# EUROPEAN PHARMACOPOEIA

## TENTH EDITION

## Volume I

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# I. PREFACE OF THE 10TH EDITION OF THE PH. EUR.

Only a few years ago, in 2014, we had occasion to commemorate the 50th year anniversary of the European Pharmacopoeia. This year, in 2019, we have another jubilee to celebrate, with the publication of the 10th edition of this fundamental reference work for the quality of medicinal products.

The world has changed considerably since the 8 founder countries started the work on the European Pharmacopoeia in 1964. Today we live in a globalised world where goods – including medicinal products, active substances and excipients – are produced and distributed all around the planet.

The European Pharmacopoeia has kept pace with this development: it is today a European single point of reference for the quality of medicines, with a global influence. The texts published within its pages provide a public, legally binding standard in the 38 member states and the European Union (EU) which are signatories to the *Convention on the elaboration of a European Pharmacopoeia*. The European Pharmacopoeia community has continued to grow in the past 3 years, with the accession of the Republic of Moldova to the Convention in 2017 and the arrival of 3 new observer states (India and Japan in 2016 and the Republic of Uzbekistan in 2018), clearly illustrating the lasting appeal and dynamism of the European Pharmacopoeia. The remarkable fact that today 6 European countries, 22 non-European countries, the Taiwan Food and Drug Administration of the Ministry of Health and Welfare (TFDA) and the World Health Organization (WHO) are observers, illustrates that the European Pharmacopoeia serves not only the health of several hundreds of millions of European citizens, but also has a worldwide impact.

The activities of the European Pharmacopoeia are coordinated by the European Directorate for the Quality of Medicines & HealthCare (EDQM). The work is carried out by 20 groups of experts and around 40 active working parties, together consisting of more than 700 members and involving experts from Europe and beyond. Since 2016, membership has also been extended to include experts from all around the world, a measure introduced to keep pace with the reality of market globalisation and which has enriched discussions within the groups.

It is my personal conviction that everyone involved in the work of the European Pharmacopoeia can be very proud that it is possible to establish and keep updated a work containing more than 2500 texts, each of which can only be adopted with the unanimous agreement of the Ph. Eur. member states. This is outstanding proof that humans can indeed overcome potential barriers and cultural differences.

The publication of the 10th Edition of the European Pharmacopoeia is the ideal time to look back on its development from the 1st to the 10th Edition.

The 1st Edition was published as 3 bound volumes between 1968 and 1976.

The 2nd Edition, published in 1980, came in loose-leaf binder format with a new fascicle issued in the middle of each year, containing all the texts adopted by the European Pharmacopoeia Commission in the previous year. This 2nd Edition culminated with the publication of fascicle 19 in 1995.

By the 3rd Edition, the European Pharmacopoeia included approximately 1200 texts and was issued for the first time in both electronic format (CD-ROM) and as a bound A4 book. This edition was published in 1996 and had a projected publication cycle of 5 years. To ensure that it remained current, a supplement was published every subsequent year and gradually compiled into a companion volume to the "main" publication.

From the 4th Edition, published in book and CD-ROM form, the publication cycle was reduced to 3 years, complemented by 3 non-cumulative supplements (2 only the year of publication of the main Edition), a schedule which continues today. In 2003 (Supplement 4.6), the European Pharmacopoeia entered a new era with the launch of the online version, giving users a choice of 3 different formats. The CD-ROM was then replaced by a DVD version in 2009 (Supplement 6.7) which in turn was replaced by a USB version in 2010 (7th Edition), itself replaced by a downloadable version in 2017 (Supplement 9.3).

The 10th Edition of the European Pharmacopoeia contains 2792 texts. We have come a long way from the original 3 bound volumes of the 1st Edition!

I have had the honour of acting as the 18th elected Chair of the European Pharmacopoeia Commission for the last 3 years. The challenges were numerous, but thanks to the willingness to collaborate, the readiness to find compromises and the enthusiasm of everyone involved, the European Pharmacopoeia continued to progress very well. To some of the advances made in this period, I would like to draw your attention:

- Following the decision of the European Pharmacopoeia Commission (COM) to elaborate **monographs on medicinal products containing chemically defined active substances**, 7 new monographs (*Deferiprone oral solution (2987)*, *Deferiprone tablets (2986)*, *Lacosamide infusion (2991)*, *Lacosamide oral solution (2990)*, *Lacosamide tablets (2989)*, *Raltegravir chewable tablets (2939)* and *Raltegravir tablets (2938)*) were adopted and published by Supplement 9.8, all elaborated under the P4 procedure for single-source products still under patent. The first monograph on a medicinal product containing a chemically-defined active substance, *Sitagliptin tablets (2927)*, had been already published as part of the 8th Edition of the European Pharmacopoeia.
- Since the last edition, the COM has achieved an important milestone in the field of **biotherapeutic products**. The P4Bio pilot phase was successfully concluded with the adoption of the monograph for *Etanercept (2895)*, further proof that the standardisation challenges stemming from the complexity and heterogeneity of biotherapeutics can be overcome and that monograph specifications are compatible with the emergence of biosimilars.

  The adoption of the monograph for *Infliximab concentrated solution (2928)* in November 2017 together with the feedback received from stakeholders during the Pharmeuropa public consultation, demonstrated that it was also feasible to set meaningful quality requirements for a complex monoclonal antibody (mAbs) with a molecular mass of approximately 150 kDa. In pursuit of this goal, the COM will carry on setting public standards for therapeutic mAbs through the development of a set of suitable general requirements and methodologies applicable to various quality attributes common to (classes/sub-classes of) mAbs (e.g. TNF-alpha product-based standards).

- The COM continued to update its **general chapters** through a programme of revisions and new elaborations. New general chapters were adopted on:
  - *Chemical imaging (5.24)*, the first of its kind to be introduced in any pharmacopoeia worldwide. This chapter provides specific recommendations on how to assess the performance of chemical imaging systems used for qualitative and quantitative investigations.
  - *Process analytical technology (5.25)* focusing on the interfacing of analytical techniques with the manufacturing process as a means of enhancing process control and improving process understanding.
  - *Scanning electron microscopy* (*2.9.52*) concentrating on scanning electron microscopy applied to pharmaceutical materials, from research to quality control.

  Revision work targeted the widely used general chapters on:
  - *Loss on drying (2.2.32)*
  - *Osmolality (2.2.35)*
  - *Infrared absorption spectrophotometry (2.2.24)*
  - *Absorption spectrophotometry, ultraviolet and visible (2.2.25)*
- The COM further fine-tuned its **implementation strategy for the ICH Q3D Guideline on elemental impurities** and adopted the revised versions of its general monographs on *Substances for pharmaceutical use (2034)* and *Pharmaceutical preparations (2619)*, as well as revised versions of the general chapters on *Elemental Impurities (5.20)* and on *Determination of elemental impurities (2.4.20)*. Numerous individual monographs have been revised within the framework of the implementation of the ICH Q3D guideline in the Ph. Eur.
- An important milestone was also achieved in the setting of quality requirements for **live biotherapeutic products** (LBPs) – which are medicinal products that contain live micro-organisms such as bacteria or yeasts - with the adoption of quality standards for LBPs for human use. These included the general monograph *Live biotherapeutic products for human use (3053)*, and 2 general chapters, *Microbial examination of live biotherapeutic products: test for enumeration of microbial contaminants* (*2.6.36*) and *Microbiological examination of live biotherapeutic products: test for specified micro-organisms* (*2.6.38*).
- A revised general monograph on ***Products of recombinant DNA technology*** *(0784)* was also adopted by the COM. This text provides general requirements for the manufacture and control of medicinal products derived from recombinant DNA technology and also includes requirements for the active substances in these products. Several parts of the text were updated, including the Production section of the monograph which has been entirely re-structured and modernised in line with the recommendations and guidelines published by the International Council for Harmonisation (ICH), the European Medicines Agency (EMA) and the World Health Organization (WHO).
- The general monograph on ***Substances for Pharmaceutical Use*** *(2034)* was revised to clarify the requirements for the bacterial endotoxin test and align them with the policy on the same topic approved by the COM at its 149th Session in June 2014. This revision goes hand-in-hand with the revision of the chapter on *Guidelines for Using the Test for Bacterial Endotoxins* (*5.1.10.*), published in European Pharmacopoeia Supplement 8.8, which also included recommendations for establishing limits, as well as information on how to evaluate the pyrogenicity of substances. The reference to the Guideline on the Limits of Genotoxic Impurities published by the EMA has also been replaced with a reference to the new Guideline on Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk (ICH M7).
- The analytical performance of chromatographic identification tests in monographs on herbal drugs and herbal drug preparations has improved since the general chapter on high-performance thin-layer chromatography of herbal drugs and herbal drug preparations (HPTLC, *2.8.25*) was introduced in Ph. Eur. The new method not only improves selectivity but also allows a more objective evaluation of the observed zones through the use of intensity markers. The equipment described ensures standardised plate preparation, and includes a system for electronic documentation of chromatograms.
- The COM has continued its efforts to critically review the number of animals required to perform scientific procedures, revising the methods used in monographs and chapters, wherever possible, to ensure that the Ph. Eur. promotes the **3Rs principles of replacing, reducing and refining the use of animals in scientific procedures**. An example of this effort is the adoption of a new chapter on the Substitution of *in vivo* methods by *in vitro* methods for the quality control of vaccines (*5.2.14*) to facilitate the transition from *in vivo* to *in vitro* methods. This chapter provides guidance on how to validate alternative *in vitro* methods in cases where a direct head-to-head comparison with an existing *in vivo* method is not possible. Following the adoption of this new chapter, the COM adopted a revised version of the general monograph on *Vaccines for human use (0153)* and on *Vaccines for veterinary use (0062)* to reduce animal testing and encourage the use of alternatives.

  In 2017, the COM endorsed the complete suppression of the test for abnormal toxicity from the Ph. Eur. As part of this exercise, the COM adopted 49 monographs revised to remove this test, 36 of which concerned vaccines for human use. The general chapter *Abnormal toxicity* (*2.6.9*) became thus obsolete and was therefore deleted from the Ph. Eur.
- The COM has also pursued its efforts to **replace the use of hazardous chemicals** whenever possible leading to the revision of a number of monographs and chapters.

This list – which is neither comprehensive nor exhaustive –illustrates the impressive dynamism of the Ph. Eur. and the willingness of the COM, together with National Pharmacopoeia Authorities and experts from member countries and beyond, to keep pace with new developments.

This period has also seen number of new working parties created or old ones reactivated to deal with emerging issues:

- A Working Party on **Pyrrolizidine Alkaloids** (PA WP) was created and tasked with the definition of a general method for testing *Pyrrolizidine alkaloids* (*2.8.26*). This decision was taken in response to a demand expressed by European regulators and following reports in some Ph. Eur. member states that herbal medicinal products, and foodstuffs, had been found to be contaminated with plants containing pyrrolizidine alkaloids.
- The Working Party on **Gene Therapy Products** has been reactivated in response to recent developments in the field. The Working Party has been tasked with the revision of general chapter *Gene transfer medicinal products for human use* (*5.14*) to take into account newly elaborated pharmacopoeial texts, such as general chapter *Raw materials of biological origin for the production of cell-based and gene therapy medicinal products* (*5.2.12*). This Working Party will also participate in the revision of transversal texts elaborated by other groups of experts

or working parties of the Ph. Eur., such as general chapter *Quantification and characterisation of residual host cell DNA* (*2.6.35*).

Furthermore, the COM initiated activities to explore new approaches for areas where existing approaches showed limitations:

- One example is the pilot phase for monographs on traditional Chinese medicine (TCM) where the possible replacement of liquid chromatographic assays by semiquantitative HPTLC is under study. The goal is to demonstrate that the same pass-fail decision for the determination of analytical markers can be obtained and that fingerprinting may lead to more meaningful characterisation of multi-component mixtures, such as herbal drugs.
- The COM also identified semiquantitative fingerprinting as a potential new approach to tackling the challenges presented by homoeopathic preparations and has reactivated important items on the work programme concerned by this issue.

Coming to the end of this preface, I would first like to thank all the members of the European Pharmacopoeia Commission for their trust and support that allowed us to make substantial progress on so many topics.

Special thanks go to the Director of the EDQM, Dr Susanne Keitel, my two vice-chairs, Prof Torbjörn Arvidsson and Dr Hilda Köszegi Szalai, the Secretary to the European Pharmacopoeia Commission, Ms Cathie Vielle, and her two deputies, Dr Emmanuelle Charton and Dr Ulrich Rose, for their excellent work and support during my time as Chair. Together, we had very open and constructive exchanges of thoughts, which have enabled us to guide the work of the European Pharmacopoeia Commission in an efficient and successful manner - and made my work a pleasure too.

Finally, I would like to express my sincere gratitude to all the chairs and experts in groups of experts and working parties, as well as the staff of the EDQM and the National Pharmacopoeia Authorities. The European Pharmacopoeia is only possible due to their omnipresent enthusiasm, hard work and collaboration.

Thanks to your dedication and efforts, the European Pharmacopoeia will continue to ensure the quality of medicines for the benefit of patients for many years to come.

Dr Tobias Gosdschan,

Chair of the European Pharmacopoeia Commission

28 February 2019

# II. INTRODUCTION

The European Pharmacopoeia is prepared under the auspices of the Council of Europe in accordance with the Convention on the Elaboration of a European Pharmacopoeia (European Treaty Series No. 50) as amended by the Protocol to the Convention (European Treaty Series No. 134), signed by the Governments of 38 member states (Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Montenegro, Netherlands, North Macedonia, Norway, Poland, Portugal, Republic of Moldova, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, United Kingdom) and the European Union. The preparation of the Pharmacopoeia is the responsibility of the European Pharmacopoeia Commission ('the Commission'), appointed in accordance with Article 5 of the above-mentioned Convention. It is composed of delegations appointed by the Contracting Parties. Each delegation consists of not more than 3 members chosen for their competence in matters within the functions of the Commission.

Observers from non-member states and international organisations are admitted to Sessions of the Commission in accordance with the Rules of Procedure. Observers are at present admitted from Albania, Algeria, Argentina, Armenia, Australia, Azerbaijan, Belarus, Brazil, Canada, China, Georgia, Guinea, India, Israel, Japan, Kazakhstan, Republic of Korea, Madagascar, Malaysia, Morocco, Russian Federation, Senegal, Singapore, South Africa, Syrian Arab Republic, Tunisia, United States of America, Uzbekistan, Taiwan Food and Drug Administration (TFDA) and the World Health Organization (WHO).

The Convention is open for signature by European countries and observer status can serve to familiarise European countries intending to become signatories with the working methods of the Commission. The Commission recognises that interactions with countries outside Europe are essential in view of the globalisation of the supply chain for pharmaceuticals. Observer status for non-European countries helps to foster these interactions by facilitating regulatory partnerships and the exchange of information and working documents as well as participation in the scientific work of the Commission. The 10th Edition of the European Pharmacopoeia contains nearly 3000 monographs and general texts. This would not have been possible without the contributions from and dedication of a network of more than 700 experts in pharmaceutical sciences from all around the world. Participation by experts and stakeholders in the European Pharmacopoeia's public standard-setting process is vital for the development of authoritative and relevant monographs.

The functions of the Commission established by Article 6 of the Convention as amended by the Protocol are:

**Article 6**

"Subject to the provisions of Article 4 of the present Convention, the functions of the Commission shall be:

(a) to determine the general principles applicable to the elaboration of the European Pharmacopoeia;

(b) to decide upon methods of analysis for that purpose;

(c) to arrange for the preparation of and to adopt monographs to be included in the European Pharmacopoeia and;

(d) to recommend the fixing of the time limits within which its decisions of a technical character relating to the European Pharmacopoeia shall be implemented within the territories of the Contracting Parties." The European Directorate for the Quality of Medicines & HealthCare (EDQM) of the Council of Europe supports the Commission in the elaboration and revision of European Pharmacopoeia texts by providing the Scientific Secretariat. In addition, it is responsible for the establishment, production, monitoring and distribution of reference standards needed when applying the monographs. The EDQM is also active in a number of other areas related to the protection of public health, for example in certifying the quality of active pharmaceutical ingredients from specific sources and in biological standardisation.

In accordance with the terms of the Convention, the Contracting Parties undertake to take the necessary measures to ensure that the monographs of the European Pharmacopoeia become the official standards applicable within their respective territories.

## PURPOSE OF THE EUROPEAN PHARMACOPOEIA

The purpose of the European Pharmacopoeia is to promote public health by the provision of recognised common standards for the quality of medicines and their components. As these standards ensure that medicines reaching the market are safe for use by patients, it is essential that they are appropriate. Their existence also facilitates the free movement of medicinal products in Europe and beyond.

European Pharmacopoeia monographs and other texts are designed to meet the needs of:

- regulatory authorities;
- those engaged in the quality control of medicinal products and their constituents;
- manufacturers of medicinal products and their individual components.

Globalisation gives rise to new challenges in terms of the quality of pharmaceutical substances and medicines. To respond to these challenges, the European Pharmacopoeia has extended its international outreach and works closely with all its stakeholders to produce quality standards appropriate for medicinal products developed in an increasingly global world.

## SEAT OF THE EUROPEAN PHARMACOPOEIA COMMISSION

The European Pharmacopoeia Commission holds its meetings in Strasbourg, the seat of the Council of Europe.

## GENERAL PRINCIPLES

General rules for interpretation of the texts of the European Pharmacopoeia are given in the General Notices. These rules are to be applied in conjunction with the information given below.

The general principles applied during elaboration of European Pharmacopoeia texts are laid down in procedures (Rules of procedure, Guide for work and Code of practice) and in Technical Guides freely available on the EDQM website. These principles are revised regularly, generally without retrospective application, so that monographs already published may not always follow the latest recommendations; however, wherever an issue with an impact on public health is identified, monographs are revised immediately.

It is recognised that general chapters are also used independently of the monographs of the Pharmacopoeia; in these circumstances users are recommended to consult the relevant technical guide, which gives extensive information on the application of many of the methods.

**General and individual monographs**. The standards of the European Pharmacopoeia take the form of general and individual monographs. General monographs provide standards that best fulfil the aims stated above and meet the needs of users. It is usually necessary to apply one or more general monographs along with any individual monograph. Where a substance or a pharmaceutical preparation is subject to the provisions of both a general monograph and an individual monograph, the two are complementary.

An individual monograph may, exceptionally, include an exemption from one or more provisions of a general monograph.

Since, for practical reasons, it is not possible to include a cross-reference to applicable or potentially applicable general monographs in each individual monograph, cross-references are not included, except where they are necessary to avoid ambiguity. A list of general monographs is included in each new edition and supplement to help users identify those required for use with an individual monograph.

**Use of animals**. In accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (CETS No. 123), elaborated under the auspices of the Council of Europe, the Commission is committed to reducing the use of animals wherever possible in pharmacopoeial testing, and encourages its stakeholders to seek alternative procedures. An animal test is included in a monograph only if it has been clearly demonstrated that it is absolutely necessary to achieve satisfactory control for pharmacopoeial purposes and no alternative is available.

**Hydrates**. When monographs refer to a hydrate form, either well-defined or not, the corresponding degree of hydration (mono-, di-, tri-, *n*-hydrate or hydrate) is indicated in the title, the chemical formula and the chemical name. For non-hydrates, 'anhydrous' is not specified in the title unless otherwise justified. When monographs cover both non-hydrates and hydrates, nothing is added to the title or the chemical name, but '$xH_2O$' is stated in the chemical formula.

**Chiral substances**. Monographs on chiral substances that describe a particular enantiomer include a test to confirm enantiomeric purity, usually by chiral liquid chromatography. A test for racemic character using optical rotation is included only if there is information on the specific optical rotation of the enantiomers that indicates that such a test would be discriminating in terms of enantiomeric purity. If other techniques, such as chiral liquid chromatography, can serve the intended purpose, they are prescribed instead of optical rotation.

**Polymorphism**. Where a substance shows polymorphism, this is usually stated under Characters. In general, no particular crystalline form is required in monographs; in rare cases, however, a monograph may cover a specific crystalline form and describe, for example, an infrared absorption spectrophotometric identification test. In these cases, the spectrum is to be recorded using the substance in the solid state without recrystallisation and the chemical reference substance described is of the required crystalline form. In addition to these exceptional cases, depending on the function of a given substance in a pharmaceutical preparation, it may be necessary for a manufacturer to ensure that a particular crystalline form is used. The information given under Characters is intended to alert users to the need to evaluate this aspect during the development of a pharmaceutical preparation. The general monograph *Substances for pharmaceutical use (2034)* and general chapter *5.9. Polymorphism* should also be consulted.

**Impurities**. Together with the general monograph *Substances for pharmaceutical use (2034)*, general chapter *5.10. Control of impurities in substances for pharmaceutical use* describes the policy for the control of impurities in individual monographs and explains how the limits in the related substances test are to be understood.

The current general policy of the Commission for substances for pharmaceutical use is to include quantitative tests for impurities in monographs. Most of the older monographs that predate this policy have been revised to introduce quantitative methods. Where a monograph does not conform to the general policy, compliance with the general monograph *Substances for pharmaceutical use (2034)* implies that the individual monograph requirements need to be supplemented by the user, using the decision tree provided in general chapter *5.10*.

Monographs on medicinal products containing chemically defined active substances describe limits for degradation products arising during manufacture and shelf-life of the medicinal product, including those synthesis impurities that are also degradation products. To this end, quantitative tests are included in the monographs.

**Elemental impurities**. The strategy for the control of elemental impurities has been aligned to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Q3D guideline and the core principles of this guideline are reproduced in general chapter *5.20. Elemental impurities*. The requirements for the control of elemental impurities, which is strongly based on risk management, are given in the general monographs on *Pharmaceutical preparations (2619)* and on *Substances for pharmaceutical use (2034)*, while to a certain extent tests for elemental impurities have been removed from individual monographs (e.g. *2.4.8. Heavy metals*).

**Residual solvents**. The requirements for residual solvents are given in the general monograph *Substances for pharmaceutical use (2034)* and in general chapter *5.4. Residual solvents*. Thus, all active substances and excipients are subjected to relevant control of residual solvents, whether or not a test is specified in the individual monograph. The requirements are aligned with the ICH Q3C guideline.

**Bacterial endotoxins**. In June 2014, the Commission approved a new policy on bacterial endotoxins in substances for pharmaceutical use. The general monograph *Substances for pharmaceutical use (2034)* states that a substance must comply with the bacterial endotoxins test (BET) if it is labelled as 'bacterial endotoxin-free', or if it is intended for use in the manufacture of parenteral preparations or preparations for irrigation without a further appropriate procedure for the removal of bacterial endotoxins. The monograph references general chapters *2.6.14. Bacterial endotoxins* and *5.1.10. Guidelines for using the test for bacterial endotoxins*. According to the general monograph on *Parenteral preparations (0520)*, pharmaceutical preparations for parenteral administration must comply with the test for bacterial endotoxins.

Monographs for substances for pharmaceutical use elaborated after the implementation of this policy do not describe a test for bacterial endotoxins, this aspect being covered by the requirements of the general monograph on *Substances for pharmaceutical use (2034*). There is an exception to this rule: the test is maintained in new monographs when, for example, a specific sample preparation must be used or a specific method applied. In such cases, no limit is given. For all monographs for substances for pharmaceutical use published before the implementation of the policy, the test for bacterial endotoxins is retained: existing limits remain in these monographs to maintain the use of well-established limits.

**Equipment, reagents**. As an aid to users, information is made available via the Knowledge database (see below) on chromatographic columns that have been found to be satisfactory during development of monographs and general methods. Information is also given on other equipment and reagents where this is considered useful. This information is given as a guide and does not imply that other columns, equipment or reagents than those specified are not suitable.

**Homoeopathic preparations**. A monograph on methods of preparation of homoeopathic stocks and potentisation, general monographs on homoeopathic preparations, mother tinctures for homoeopathic preparations and herbal drugs for homoeopathic preparations, and individual monographs on raw materials and stocks for homoeopathic preparations are included in a separate section of the European Pharmacopoeia. It is understood that when the same substance is used in both homoeopathic and other preparations, the monograph in the main body of the European Pharmacopoeia applies.

**Herbal drugs and herbal drug preparations (including traditional Chinese medicines)**. All relevant monographs are grouped together in a separate section of the European Pharmacopoeia.

**Protected species**. Monographs, notably those on herbal drugs, may cover material obtained from protected species. Inclusion of these monographs is without prejudice to the provisions for protection of these species under national and international law.

**Patents**. The description in the European Pharmacopoeia of articles subject to patent protection does not confer or imply any right to the use of such patents by any person or persons other than the proprietors of the patents concerned.

MONOGRAPHS ON PHARMACEUTICAL PREPARATIONS

The general monograph *Pharmaceutical preparations (2619)* is intended to be a reference source of standards in the European Pharmacopoeia on active substances, excipients and dosage forms which are to be applied in the manufacture/preparation of pharmaceuticals, but not a guide on how to manufacture as there is specific guidance available covering methods of manufacture and associated controls.

Harmonisation and standardisation for pharmaceutical preparations is dealt with via the drafting of general dosage form monographs setting out elements common to all preparations covered by the monograph, and via the development of standard test methods used to test medicinal products. The inclusion of these general monographs and methods in the European Pharmacopoeia provides competent authorities and manufacturers with a common basis for the preparation and evaluation of marketing authorisation applications. In addition, after a successful pilot phase, individual monographs on medicinal products containing chemically defined active substances are now elaborated on a regular basis.

Reference standards established for the assay of active substances and excipients may be suitable for use as assay standards for preparations when the conditions stated in general chapter *5.12. Reference standards* are fulfilled.

WORK PROGRAMME

The work programme (elaboration of new monographs or general chapters or revision of existing texts) is decided by the Commission at the 3 annual sessions. In general, whenever 2 member states express a wish to elaborate a monograph, the Commission adds the item to the work programme. Changes to the work programme are published on the EDQM website and in Pharmeuropa. Information is also provided to industry associations registered with the Secretariat and to manufacturers' liaison contacts and in the EDQM Knowledge database (including reasons for the revision). Interested parties are invited to contact the Secretariat for any items where they wish to be involved in the work.

CERTIFICATION PROCEDURE

The procedure for the certification of suitability to the monographs of the Pharmacopoeia allows suppliers to demonstrate that the quality of their substance is suitably controlled by the relevant monographs (see Public Health Committee (Partial Agreement) Resolution AP-CSP (07) 1 or any subsequent revision, available from the EDQM and on its website) and is an aid to the use of monographs in marketing authorisation applications where relevant, complemented by additional tests appended to the certificate. The certification procedure also applies to herbal drugs, herbal drug preparations and any substance subject to the risk of transmissible spongiform encephalopathy (TSE). Certificates of suitability are issued by the EDQM only for substances produced under a suitable quality system. Certificates are granted with respect to published monographs. Details on how this scheme is run are available from the Secretariat and on the EDQM website. A daily updated list of the certificates granted is available online on the EDQM website, including voided or suspended certificates.

PUBLICATIONS

The official version of the European Pharmacopoeia is available in English and in French. Three additional supplements are published every year.

**Implementation**. The date on which monographs are to be implemented is fixed by a Resolution of the European Committee on Pharmaceuticals and Pharmaceutical Care (Partial Agreement) of the Council of Europe, following a recommendation by the Commission. This date is usually 1 year after adoption and about 6 months after publication. Where a monograph is to be implemented at a date earlier than the next publication date of the European Pharmacopoeia or a supplement, a Resolution of the European Committee on Pharmaceuticals and Pharmaceutical Care gives the full text to be implemented. The text is also published in Pharmeuropa for information and posted on the EDQM website as part of the Resolution.

**Pharmeuropa** is the European Pharmacopoeia Forum. Texts are published for public enquiry 4 times per year (with deadlines for comments) as an aid to the elaboration of monographs and as a vehicle for information on pharmacopoeial and related matters. It is therefore extremely important that manufacturers and users of the substances provide feedback on the draft monographs. Pharmeuropa Bio & Scientific Notes, a publication indexed by bibliographic services, includes scientific papers related to the establishment of biological reference preparations and validation of biological methods within the Biological Standardisation Programme of the EDQM, and to various aspects of pharmaceutical analysis and other subjects relevant to the Pharmacopoeia. Both of these are only available online as free publications.

**Knowledge database**. The EDQM website provides access to a database containing a variety of information related to monographs and other texts and intended to facilitate their proper use. Information is provided on:

- the status (e.g. whether the text is under elaboration or a revision is ongoing, together with a brief description, if deemed appropriate);
- typical chromatograms (or other raw data) obtained for certain chromatographic separations and brand names of chromatography columns used in monograph development;
- suppliers of reagents and equipment that may be difficult to find for some users;
- revisions of the texts on a historical basis, starting with the 5th Edition;
- harmonisation status;
- other relevant topics.

**Archives (online)**. The European Pharmacopoeia Archives contain copies of the 1st to the 9th Editions in PDF format. They are available to all European Pharmacopoeia subscribers with an up-to-date subscription and a registered EPID code.

**Website**. Information on activities and many other aspects of the European Pharmacopoeia can be found on the EDQM website (www.edqm.eu).

**HelpDesk**. Many technical and other enquiries are addressed to the EDQM by users. They should be submitted via the HelpDesk on the EDQM website. The EDQM will deal with enquiries that are related to the use of monographs and other texts of the European Pharmacopoeia. The HelpDesk has a section of Frequently Asked Questions that should be consulted by users before submission of an enquiry.

**Revision programme**. Proposals to revise a text of the European Pharmacopoeia may be submitted by a delegation, by the Chair of the Commission, by the chair of a group of

experts or a working party, by a user or by the Secretariat. Requests for revision may be submitted via the national pharmacopoeia authority of a member state or, where this is not possible, to the EDQM via the HelpDesk. Proposals must be accompanied by sufficient data to justify the need for the revision. Monographs and other texts of the European Pharmacopoeia are revised as necessary following a decision of the Commission. Draft revised texts are published in Pharmeuropa.

COMBISTATS

Certain tests in monographs, particularly biological assays, require statistical analysis of the results. The EDQM has developed a computer programme, CombiStats, which can be used for statistical analysis of results of biological dilution assays. Information on the programme, with conditions of access and use, is available on the EDQM website.

INTERNATIONAL HARMONISATION

In an increasingly globalised world, the need for global quality standards has become ever more pressing. Standards are a vital instrument for marketing authorisation, market surveillance and the free movement and trade of medicines amongst regions and countries. Amongst other harmonisation initiatives, the European Pharmacopoeia is engaged in a process of harmonisation with the Japanese Pharmacopoeia and the United States Pharmacopeia, within an informal structure referred to as the Pharmacopoeial Discussion Group (PDG).

Where harmonisation of general chapters is carried out, the aim is to arrive at interchangeable methods or requirements so that demonstration of compliance using a general chapter from one of the 3 pharmacopoeias implies that the same result would be obtained using the general chapter of either of the other pharmacopoeias. To help regulatory authorities and other users recognise the interchangeability of selected harmonised general chapters, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has issued topic-specific annexes with information about a limited number of these texts in order to facilitate their implementation. More information is available from the ICH website (ich.org).

Where harmonisation of monographs is carried out, the aim is to arrive at identical requirements for all attributes of a product. Information on any non-harmonised attributes and local requirements is included in the relevant European Pharmacopoeia general chapters and monographs.

# III. EUROPEAN PHARMACOPOEIA COMMISSION

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# IV. CONTENTS OF THE 10th EDITION

The 10th Edition consists of new texts as well as all current texts from the 9th Edition, some of which have been revised or corrected.

Lists of the monographs and general chapters that, for the 10th Edition, are new, revised or corrected, or have had their titles or chapter numbers changed, are given below.

The version date (for example 01/2020 for a text that is new or revised for the 10th Edition), completed by 'corrected X.X' if a corrected version of the text has subsequently been published in Supplement X.X, and the reference number (4 digits for monographs and 5 digits for general chapters) are specified above the title of each monograph and general chapter. The version date, completed by 'corrected X.X' if appropriate, makes it possible to identify the successive versions of texts in different editions.

The volume in which the current version was first published is stated in the Knowledge database on the EDQM website.

As of the 10th Edition, all revised or corrected parts of a text are indicated by vertical lines in the margin and horizontal lines in the margin indicate where parts of a text have been deleted. Lines in the margin that were present in revised or corrected texts in the previous edition are deleted with each new edition.

Corrected texts are to be taken into account as soon as possible and not later than the end of the month following the month of publication of the volume. New and revised texts are to be taken into account not later than the implementation date.

A barcode is included at the start of each text, providing a link to further information on the text (e.g. the Knowledge database) for smartphones and tablets with a camera and a barcode reader app.

In addition to corrections made to individual texts, the following decisions and systematic modifications have been made to the texts of the European Pharmacopoeia for the 10th Edition.

- For texts not currently undergoing revision, the wording used in the calculation of percentage contents in the quantitative expression of acceptance criteria in tests for related substances has been modified to specify the full name of the substance (i.e. including counter-ions, hydrates etc.).
- Tests using diphosphorus pentoxide, predominantly tests for loss on drying, have been modified, where possible, to avoid the use of this substance due to its toxicity.
- The names and descriptions of the reagents used to describe stationary phases for gas chromatography have been revised.
- 'Tamper-proof' has been replaced by 'tamper-evident'.
- Reference to general chapter *2.8.23. Microscopic examination of herbal drugs* has been added when microscopic examination is carried out using a powder with a non-standard sieve size.
- The terms 'coarse' and 'coarsely' have been deleted from 'coarse powder (1400) (*2.9.12*)' and 'coarsely powdered herbal drug (1400) (*2.9.12*)'.
- Results are expressed in per cent instead of per cent *m/m* in the tests Dry residue of extracts (*2.8.16*) and Loss on drying of extracts (*2.8.17*).

*Individual copies of texts published in this edition will not be supplied.*

*Subscribers to the current version (book or electronic) of the European Pharmacopoeia have access to an online archive version of all obsolete editions and supplements of the European Pharmacopoeia in PDF format.*

*A list of new reagents published during the course of this edition is available under 'Useful information' in Pharmeuropa Online.*

## NEW TEXTS

*The following texts appear for the first time in the European Pharmacopoeia. They will be implemented on* ***1 January 2020*** *at the latest.*

### GENERAL CHAPTERS

2.6.35. Quantification and characterisation of residual host-cell DNA

2.9.49. Powder flow properties by shear cell methods

2.9.52. Scanning electron microscopy

3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes

5.25. Process analytical technology

### MONOGRAPHS

**Vaccines for veterinary use**

Infectious pancreatic necrosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids (3063)

**Herbal drugs and herbal drug preparations**

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**Monographs**

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# REVISED TEXTS

*The following texts have been technically revised since their last publication. They will be implemented on **1 January 2020** at the latest.*


**GENERAL CHAPTERS**

2.2.25. Absorption spectrophotometry, ultraviolet and visible
2.6.8. Pyrogens
2.6.33. Residual pertussis toxin
2.7.2. Microbiological assay of antibiotics
2.7.23. Numeration of CD34/CD45+ cells in haematopoietic products
2.7.35. Immunonephelometry for vaccine component assay
2.8.25. High-performance thin-layer chromatography of herbal drugs and herbal drug preparations
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4. Reagents
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5.8. Pharmacopoeial harmonisation
5.21. Chemometric methods applied to analytical data
5.22. Names of herbal drugs used in traditional Chinese medicine
5.24. Chemical imaging

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Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed) (1933)
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) (1934)
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed, reduced antigen(s) content) (2329)
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**Vaccines for veterinary use**

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**Herbal drugs and herbal drug preparations**

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# CORRECTED TEXTS

*The following texts have been corrected for the 10th Edition and specify 'corrected 10.0' above the title. These corrections are to be taken into account as soon as possible and not later than* ***31 August 2019*** *(the end of the month following the month of publication of the 10th Edition).*

**GENERAL CHAPTERS**

1. General notices
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2.2.29. Liquid chromatography
2.2.31. Electrophoresis
2.4.23. Sterols in fatty oils
2.4.25. Ethylene oxide and dioxan
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2.4.32. Total cholesterol in oils rich in omega-3 acids
2.5.19. *O*-Acetyl in polysaccharide vaccines
2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid
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2.9.11. Test for methanol and 2-propanol
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Sutures, sterile non-absorbable (0324)

**Herbal drugs and herbal drug preparations**

Herbal drugs: introduction (90029)
Achyranthes bidentata root (2999)
Akebia stem (2472)
Aloes dry extract, standardised (0259)
Amomum fruit (2554)
Artichoke leaf (1866)
Belladonna, prepared (0222)
Capsicum soft extract, standardised (2529)
Cassia oil (1496)
Cinnamon bark oil, Ceylon (1501)
Fennel, bitter (0824)
Fennel, sweet (0825)
Gentian tincture (1870)
Ginger (1522)
Goldenseal rhizome (1831)
Juniper oil (1832)
Long pepper (2453)
Mandarin oil (2355)
Mastic (1876)
Myrrh tincture (1877)
Nutmeg oil (1552)
Opium tincture, standardised (1841)
Oregano (1880)
Pepper (2477)
Roselle (1623)
Round amomum fruit (2555)
Sage tincture (1889)
Saw palmetto extract (2579)
Saw palmetto fruit (1848)
Star anise (1153)
Stramonium, prepared (0247)
Sweet orange oil (1811)
Thyme oil, thymol type (1374)
Turmeric rhizome (2543)

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Anacardium for homoeopathic preparations (2094)
Belladonna for homoeopathic preparations (2489)
Cocculus for homoeopathic preparations (2486)
Hydrastis canadensis for homoeopathic preparations (2500)
Ignatia for homoeopathic preparations (2513)
Magnesium phosphoricum for homoeopathic preparations (2505)
Nux-vomica for homoeopathic preparations (2514)
Staphysagria for homoeopathic preparations (2289)

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*N*-Acetyltyrosine (1384)
Alimemazine hemitartrate (2650)
Almagate (2010)
Alprazolam (1065)
Alprenolol hydrochloride (0876)
Aluminium hydroxide, hydrated, for adsorption (1664)
Aluminium stearate (1663)
Alverine citrate (2156)
Amantadine hydrochloride (0463)
Amikacin sulfate (1290)
4-Aminobenzoic acid (1687)
Amoxicillin sodium (0577)
Amphotericin B (1292)
Ampicillin sodium (0578)
Anti-T lymphocyte immunoglobulin for human use, animal (1928)
Aprotinin (0580)
Aprotinin concentrated solution (0579)
Arginine hydrochloride (0805)
Aripiprazole (2617)
Articaine hydrochloride (1688)
Atazanavir sulfate (2898)
Atropine (2056)
Atropine sulfate (0068)
Azelastine hydrochloride (1633)
Bacitracin (0465)
Bacitracin zinc (0466)
Benzalkonium chloride (0372)
Benzalkonium chloride solution (0371)
Benzyl alcohol (0256)
Benzyl benzoate (0705)
Benzylpenicillin (benzathine) tetrahydrate (0373)

Benzylpenicillin potassium (0113)
Benzylpenicillin (procaine) monohydrate (0115)
Benzylpenicillin sodium (0114)
Biperiden hydrochloride (1074)
Bismuth subsalicylate (1495)
Bleomycin sulfate (0976)
Botulinum toxin type A for injection (2113)
Botulinum toxin type B for injection (2581)
Brimonidine tartrate (2760)
Bromhexine hydrochloride (0706)
Brompheniramine maleate (0977)
Bupivacaine hydrochloride (0541)
Buserelin (1077)
Calcitonin (salmon) (0471)
Calcium folinate hydrate (0978)
Calcium glucoheptonate (1399)
Calcium levofolinate hydrate (1606)
Captopril (1079)
Carbimazole (0884)
Cefalotin sodium (0987)
Cefamandole nafate (1402)
Cefapirin sodium (1650)
Cefazolin sodium (0988)
Cefepime dihydrochloride monohydrate (2126)
Cefoperazone sodium (1404)
Cefotaxime sodium (0989)
Cefoxitin sodium (0990)
Ceftazidime pentahydrate (1405)
Ceftazidime pentahydrate with sodium carbonate for injection (2344)
Ceftriaxone sodium (0991)
Cefuroxime axetil (1300)
Cefuroxime sodium (0992)
Cetostearyl alcohol (0702)
Cetostearyl alcohol (type A), emulsifying (0801)
Cetostearyl alcohol (type B), emulsifying (0802)
Cetyl alcohol (0540)
Chloramphenicol (0071)
Chloramphenicol palmitate (0473)
Chloramphenicol sodium succinate (0709)
Chlorobutanol (0382)
Chlorobutanol hemihydrate (0383)
Chlorocresol (0384)
Chlortetracycline hydrochloride (0173)
Cholesterol (0993)
Ciclopirox (1407)
Ciclosporin (0994)
Cilastatin sodium (1408)
Cladribine (2174)
Clemastine fumarate (1190)
Clindamycin phosphate (0996)
Clioquinol (2111)
Clodronate disodium tetrahydrate (1777)
Clofibrate (0318)
Clonidine hydrochloride (0477)
Clopidogrel besilate (2790)
Clopidogrel hydrochloride (2791)
Clopidogrel hydrogen sulfate (2531)
Cloxacillin sodium (0661)
Codeine hydrochloride dihydrate (1412)
Codeine monohydrate (0076)
Codeine phosphate hemihydrate (0074)
Colestyramine (1775)
Colistimethate sodium (0319)
Colistin sulfate (0320)
Crospovidone (0892)
Cyclizine hydrochloride (1092)
Cysteine hydrochloride monohydrate (0895)
Cytarabine (0760)
Dacarbazine (1691)
Danaparoid sodium (2090)
Daunorubicin hydrochloride (0662)
Deferiprone oral solution (2987)
Deferoxamine mesilate (0896)
Demeclocycline hydrochloride (0176)
Desflurane (1666)
Desloratadine (2570)
Desmopressin (0712)
Detomidine hydrochloride for veterinary use (1414)
Dexchlorpheniramine maleate (1196)
Dibutyl phthalate (0762)
Diclofenac potassium (1508)
Diclofenac sodium (1002)
Dicloxacillin sodium (0663)
Dicycloverine hydrochloride (1197)
Diethyl phthalate (0897)
Diethylene glycol monoethyl ether (1198)
Dihydrostreptomycin sulfate for veterinary use (0485)
Dimetindene maleate (1417)
Docusate sodium (1418)
Doxorubicin hydrochloride (0714)
Doxycycline hyclate (0272)
Doxycycline monohydrate (0820)
Doxylamine hydrogen succinate (1589)
Duloxetine hydrochloride (2594)
Edrophonium chloride (2106)
Enalapril maleate (1420)
Enilconazole for veterinary use (1720)
Enrofloxacin for veterinary use (2229)
Epirubicin hydrochloride (1590)
Erythromycin lactobionate (1098)
Erythropoietin concentrated solution (1316)
Estrogens, conjugated (1512)
Ethacridine lactate monohydrate (1591)
Ethanol (96 per cent) (1317)
Ethanol, anhydrous (1318)
Ethylcellulose (0822)
Etofenamate (1513)
Eugenol (1100)
Everolimus (2918)
Felypressin (1634)
Fentanyl citrate (1103)
Fenticonazole nitrate (1211)
Filgrastim concentrated solution (2206)
Filgrastim injection (2848)
Flucloxacillin sodium (0668)
Folic acid hydrate (0067)
Foscarnet sodium hexahydrate (1520)
Fosfomycin sodium (1329)
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Gadobutrol monohydrate (2735)

Gadodiamide hydrate (2225)
Gemcitabine hydrochloride (2306)
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Glycerol (0496)
Glycerol (85 per cent) (0497)
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Glycerol monocaprylocaprate (2392)
Gonadorelin acetate (0827)
Gonadotrophin, equine serum, for veterinary use (0719)
Gramicidin (0907)
Guaiacol (1978)
Halothane (0393)
Heparins, low-molecular-mass (0828)
Heptaminol hydrochloride (1980)
Histidine hydrochloride monohydrate (0910)
Human coagulation factor VIIa (rDNA) concentrated solution (2534)
Human coagulation factor IX (rDNA) concentrated solution (2522)
Hyaluronidase (0912)
Hydroxychloroquine sulfate (2849)
Hydroxyethylcellulose (0336)
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Imipenem monohydrate (1226)
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Irinotecan hydrochloride trihydrate (2675)
Isoprenaline sulfate (0502)
Isopropyl alcohol (0970)
Isopropyl myristate (0725)
Isopropyl palmitate (0839)
Isosorbide dinitrate, diluted (1117)
Isoxsuprine hydrochloride (1119)
Kanamycin acid sulfate (0033)
Kanamycin monosulfate (0032)
Labetalol hydrochloride (0923)
Lactose (1061)
Leuprorelin (1442)
Levocabastine hydrochloride (1484)
Lincomycin hydrochloride (0583)
Lomustine (0928)
Loratadine (2124)
Lynestrenol (0558)
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Macrogol lauryl ether (1124)
Macrogol poly(vinyl alcohol) grafted copolymer (2523)
Magnesium aspartate dihydrate (1445)
Magnesium stearate (0229)
Maize starch (0344)
Meldonium dihydrate (2624)
Menadione (0507)
Mepivacaine hydrochloride (1242)
Mepyramine maleate (0278)
Meropenem trihydrate (2234)
Metacresol (2077)
Methadone hydrochloride (0408)
Methanol (1989)
Methyl salicylate (0230)
Methylcellulose (0345)
Methylene chloride (0932)
*N*-Methylpyrrolidone (1675)
Metoclopramide hydrochloride monohydrate (0674)
Mianserin hydrochloride (0846)
Minocycline hydrochloride dihydrate (1030)
Molgramostim concentrated solution (1641)
Naftidrofuryl hydrogen oxalate (1594)
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Naphazoline hydrochloride (0730)
Naphazoline nitrate (0147)
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Neostigmine bromide (0046)
Neostigmine metilsulfate (0626)
Netilmicin sulfate (1351)
Nevirapine hemihydrate (2479)
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Olsalazine sodium (1457)
Orphenadrine citrate (1759)
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Oxytetracycline dihydrate (0199)
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Phentolamine mesilate (1138)
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Piperacillin sodium (1168)
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Poly(vinyl acetate) dispersion 30 per cent (2152)
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Rabeprazole sodium (2868)
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Ranitidine hydrochloride (0946)
Remifentanil hydrochloride (2644)
Reserpine (0528)
Rifamycin sodium (0432)
Rilmenidine dihydrogen phosphate (2020)
Rivastigmine hydrogen tartrate (2630)
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Saccharin sodium (0787)
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Sertraline hydrochloride (1705)
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Sodium cetostearyl sulfate (0847)
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Sodium hyaluronate (1472)
Sodium (*S*)-lactate solution (2033)
Sodium phenylbutyrate (2183)
Sodium propionate (2041)
Sodium stearyl fumarate (1567)
Solifenacin succinate (2779)
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Somatropin concentrated solution (0950)
Somatropin for injection (0952)
Somatropin solution for injection (2370)
Spectinomycin dihydrochloride pentahydrate (1152)
Spectinomycin sulfate tetrahydrate for veterinary use (1658)
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Starch, hydroxypropyl (2165)
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Stearyl alcohol (0753)
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Sulbactam sodium (2209)
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Testosterone enantate (1048)
Testosterone isocaproate (1737)
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Thiamine hydrochloride (0303)
Thiamine nitrate (0531)
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all-*rac*-α-Tocopherol (0692)
*RRR*-α-Tocopherol (1256)
all-*rac*-α-Tocopheryl acetate (0439)
*RRR*-α-Tocopheryl acetate (1257)
α-Tocopheryl acetate concentrate (powder form) (0691)
DL-α-Tocopheryl hydrogen succinate (1258)
*RRR*-α-Tocopheryl hydrogen succinate (1259)
Tolterodine tartrate (2781)
Tosylchloramide sodium (0381)
Tributyl acetylcitrate (1770)
Tri-*n*-butyl phosphate (1682)
Triethyl citrate (1479)
Trimebutine maleate (2182)
Trimetazidine dihydrochloride (1741)
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# TEXTS WHOSE TITLE HAS CHANGED

*The titles of the following texts have been changed for the 10th Edition.*

**GENERAL CHAPTERS**

2.6.33. Residual pertussis toxin (*previously Residual pertussis toxin and irreversibility of pertussis toxoid*)

**MONOGRAPHS**

**Vaccines for veterinary use**

Enteric redmouth disease vaccine (inactivated) for rainbow trout (1950) (*previously Yersiniosis vaccine (inactivated) for salmonids*)

**Sutures for human use**

Sutures for human use: introduction (90004) (*previously Introduction*)

**Herbal drugs and herbal drug preparations**

Herbal drugs: introduction (90029) (*previously Introduction*)

**Homoeopathic preparations**

Homoeopathic preparations: introduction (90006) (*previously Introduction*)

# TEXTS WHOSE NUMBERING HAS CHANGED

*The numbering of the following texts has been changed for the 10th Edition following the modification of the structure of section 3 and the creation of chapter 3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes. Chapter 3.3 contains the following texts, which were previously in chapters 3.1. Materials used for the manufacture of containers and 3.2. Containers.*

**GENERAL CHAPTERS**

3.3.1. Materials for containers for human blood and blood components (*previously 3.1.1*)

3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components (*previously 3.1.1.1*)

3.3.3. Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components (*previously 3.1.1.2*)

3.3.4. Sterile plastic containers for human blood and blood components (*previously 3.2.3*)

3.3.5. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components (*previously 3.2.4*)

3.3.6. Sterile containers of plasticised poly(vinyl chloride) for human blood containing anticoagulant solution (*previously 3.2.5*)

3.3.7. Sets for the transfusion of blood and blood components (*previously 3.2.6*)

3.3.8. Sterile single-use plastic syringes (*previously 3.2.8*)

# 1. General notices

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# 1. GENERAL NOTICES

1.1. GENERAL STATEMENTS

The General Notices apply to all monographs and other texts of the European Pharmacopoeia.

The official texts of the European Pharmacopoeia are published in English and French. Translations in other languages may be prepared by the signatory States of the European Pharmacopoeia Convention. In case of doubt or dispute, the English and French versions are alone authoritative.

In the texts of the European Pharmacopoeia, the word 'Pharmacopoeia' without qualification means the European Pharmacopoeia. The official abbreviation Ph. Eur. may be used to indicate the European Pharmacopoeia.

The use of the title or the subtitle of a monograph implies that the article complies with the requirements of the relevant monograph. Such references to monographs in the texts of the Pharmacopoeia are shown using the monograph title and reference number in *italics*.

A preparation must comply throughout its period of validity; a distinct period of validity and/or specifications for opened or broached containers may be decided by the competent authority. The subject of any other monograph must comply throughout its period of use. The period of validity that is assigned to any given article and the time from which that period is to be calculated are decided by the competent authority in light of experimental results of stability studies.

Unless otherwise indicated in the General Notices or in the monographs, statements in monographs constitute mandatory requirements. General chapters become mandatory when referred to in a monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

The active substances, excipients, pharmaceutical preparations and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses).

**Quality systems**. The quality standards represented by monographs are valid only where the articles in question are produced within the framework of a suitable quality system. The quality system must assure that the articles consistently meet the requirements of the Pharmacopoeia.

**Alternative methods**. The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative.

**Demonstration of compliance with the Pharmacopoeia**

(1) An article is not of Pharmacopoeia quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. The manufacturer may obtain assurance that a product is of Pharmacopoeia quality on the basis of its design, together with its control strategy and data derived, for example, from validation studies of the manufacturing process.

(2) An enhanced approach to quality control could utilise process analytical technology (PAT) and/or real-time release testing (including parametric release) strategies as alternatives to end-product testing alone. Real-time release testing in circumstances deemed appropriate by the competent authority is thus not precluded by the need to comply with the Pharmacopoeia.

(3) Reduction of animal testing: the European Pharmacopoeia is dedicated to phasing out the use of animals for test purposes, in accordance with the 3Rs (Replacement, Reduction, Refinement) set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In demonstrating compliance with the Pharmacopoeia as indicated above (1), manufacturers may consider establishing additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia when animal tests are prescribed is established in such a way that animal usage is minimised as much as possible.

**Grade of materials**. Certain materials that are the subject of a pharmacopoeial monograph may exist in different grades suitable for different purposes. Unless otherwise indicated in the monograph, the requirements apply to all grades of the material. In some monographs, particularly those on excipients, a list of functionality-related characteristics that are relevant to the use of the substance may be appended to the monograph for information. Test methods for determination of one or more of these characteristics may be given, also for information.

**General monographs**. Substances and preparations that are the subject of an individual monograph are also required to comply with relevant, applicable general monographs. Cross-references to applicable general monographs are not normally given in individual monographs.

General monographs apply to all substances and preparations within the scope of the Definition section of the general monograph, except where a preamble limits the application, for example to substances and preparations that are the subject of a monograph of the Pharmacopoeia.

General monographs on dosage forms apply to all preparations of the type defined. The requirements are not necessarily comprehensive for a given specific preparation and requirements additional to those prescribed in the general monograph may be imposed by the competent authority.

General monographs and individual monographs are complementary. If the provisions of a general monograph do not apply to a particular product, this is expressly stated in the individual monograph.

**Validation of pharmacopoeial methods**. The test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. Unless otherwise stated in the monograph or general chapter, validation of the test methods by the analyst is not required.

**Implementation of pharmacopoeial methods**. When implementing a pharmacopoeial method, the user must assess whether and to what extent the suitability of the method under the actual conditions of use needs to be demonstrated according to relevant monographs, general chapters and quality systems.

**Conventional terms**. The term 'competent authority' means the national, supranational or international body or organisation vested with the authority for making decisions concerning the issue in question. It may, for example, be a national pharmacopoeia authority, a licensing authority or an official control laboratory.

The expression 'unless otherwise justified and authorised' means that the requirements have to be met, unless the

competent authority authorises a modification or an exemption where justified in a particular case.

Statements containing the word 'should' are informative or advisory.

In certain monographs or other texts, the terms 'suitable' and 'appropriate' are used to describe a reagent, micro-organism, test method etc.; if criteria for suitability are not described in the monograph, suitability is demonstrated to the satisfaction of the competent authority.

*Medicinal product*. (a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings and/or animals; or (b) any substance or combination of substances that may be used in or administered to human beings and/or animals with a view either to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

*Herbal medicinal product*. Any medicinal product, exclusively containing as active ingredients one or more herbal drugs or one or more herbal drug preparations, or one or more such herbal drugs in combination with one or more such herbal drug preparations.

*Active substance*. Any substance intended to be used in the manufacture of a medicinal product and that, when so used, becomes an active ingredient of the medicinal product. Such substances are intended to furnish a pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to affect the structure and function of the body.

*Excipient* (auxiliary substance). Any constituent of a medicinal product that is not an active substance. Adjuvants, stabilisers, antimicrobial preservatives, diluents, antioxidants, for example, are excipients.

**Interchangeable methods**. Certain general chapters contain a statement that the text in question is harmonised with the corresponding text of the Japanese Pharmacopoeia and/or the United States Pharmacopeia and that these texts are interchangeable. This implies that if a substance or preparation is found to comply with a requirement using an interchangeable method from one of these pharmacopoeias it complies with the requirements of the European Pharmacopoeia. In the event of doubt or dispute, the text of the European Pharmacopoeia is alone authoritative.

**References to regulatory documents**. Monographs and general chapters may contain references to documents issued by regulatory authorities for medicines, for example directives and notes for guidance of the European Union. These references are provided for information for users for the Pharmacopoeia. Inclusion of such a reference does not modify the status of the documents referred to, which may be mandatory or for guidance.

### 1.2. OTHER PROVISIONS APPLYING TO GENERAL CHAPTERS AND MONOGRAPHS

**Quantities**. In tests with numerical limits and assays, the quantity stated to be taken for examination is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result is calculated from this exact quantity. In tests where the limit is not numerical, but usually depends upon comparison with the behaviour of a reference substance in the same conditions, the stated quantity is taken for examination. Reagents are used in the prescribed amounts.

Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision corresponds to plus or minus 5 units after the last figure stated (for example, 0.25 g is to be interpreted as 0.245 g to 0.255 g). For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero (for example, 10.0 mL or 0.50 mL), the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; otherwise, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

It is recognised, however, that in certain cases the precision with which quantities are stated does not correspond to the number of significant figures stated in a specified numerical limit. The weighings and measurements are then carried out with a sufficiently improved accuracy.

**Apparatus and procedures**. Volumetric glassware complies with Class A requirements of the appropriate International Standard issued by the International Organisation for Standardisation.

Unless otherwise prescribed, analytical procedures are carried out at a temperature between 15 °C and 25 °C.

Unless otherwise prescribed, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base; the volumes of liquid prescribed are for use with tubes having an internal diameter of 16 mm, but tubes with a larger internal diameter may be used provided the volume of liquid used is adjusted (*2.1.5*). Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background, or if necessary against a black background. The examination is carried out in diffuse light.

Any solvent required in a test or assay in which an indicator is to be used is previously neutralised to the indicator, unless a blank test is prescribed.

**Water-bath**. The term 'water-bath' means a bath of boiling water unless water at another temperature is indicated. Other methods of heating may be substituted provided the temperature is near to but not higher than 100 °C or the indicated temperature.

**Drying and ignition to constant mass**. The terms 'dried to constant mass' and 'ignited to constant mass' mean that 2 consecutive weighings do not differ by more than 0.5 mg, the 2nd weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Where drying is prescribed using one of the expressions 'in a desiccator' or '*in vacuo*', it is carried out using the conditions described in chapter *2.2.32. Loss on drying*.

**Reagents**. The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents are described in general chapter *4*. It is assumed that reagents of analytical grade are used; for some reagents, tests to determine suitability are included in the specifications.

**Solvents**. Where the name of the solvent is not stated, the term 'solution' implies a solution in water.

Where the use of water is specified or implied in the analytical procedures described in the Pharmacopoeia or for the preparation of reagents, water complying with the requirements of the monograph *Purified water (0008)* is used, except that for many purposes the requirements for bacterial endotoxins (*Purified water in bulk*) and microbial contamination (*Purified water in containers)* are not relevant. The term 'distilled water' indicates purified water prepared by distillation.

The term 'ethanol' without qualification means anhydrous ethanol. The term 'alcohol' without qualification means ethanol (96 per cent). Other dilutions of ethanol are indicated by the term 'ethanol' or 'alcohol' followed by a statement of the percentage by volume of ethanol ($C_2H_6O$) required.

**Expression of content**. In defining content, the expression 'per cent' is used according to circumstances with one of 2 meanings:

– per cent *m/m* (percentage, mass in mass) expresses the number of grams of substance in 100 g of final product;

– per cent *V/V* (percentage, volume in volume) expresses the number of millilitres of substance in 100 mL of final product.

The expression 'parts per million' (or ppm) refers to mass in mass, unless otherwise specified.

**Temperature**. Where an analytical procedure describes temperature without a figure, the general terms used have the following meaning:

– in a deep-freeze: below – 15 °C;

– in a refrigerator: 2 °C to 8 °C;

– cold or cool: 8 °C to 15 °C;

– room temperature: 15 °C to 25 °C.

## 1.3. GENERAL CHAPTERS

**Containers**. Materials used for containers are described in general chapter *3.1*. General names used for materials, particularly plastic materials, each cover a range of products varying not only in the properties of the principal constituent but also in the additives used. The test methods and limits for materials depend on the formulation and are therefore applicable only for materials whose formulation is covered by the preamble to the specification. The use of materials with different formulations, and the test methods and limits applied to them, are subject to agreement by the competent authority.

The specifications for containers in general chapter *3.2* have been developed for general application to containers of the stated category, but in view of the wide variety of containers available and possible new developments, the publication of a specification does not exclude the use, in justified circumstances, of containers that comply with other specifications, subject to agreement by the competent authority.

Reference may be made within the monographs of the Pharmacopoeia to the definitions and specifications for containers provided in chapter *3.2. Containers*. The general monographs for pharmaceutical dosage forms may, under the heading Definition/Production, require the use of certain types of container; certain other monographs may, under the heading Storage, indicate the type of container that is recommended for use.

## 1.4. MONOGRAPHS

*TITLES*

Monograph titles are in English and French in the respective versions and there is a Latin subtitle.

*RELATIVE ATOMIC AND MOLECULAR MASSES*

The relative atomic mass ($A_r$) or the relative molecular mass ($M_r$) is shown, as and where appropriate, at the beginning of each monograph. The relative atomic and molecular masses and the molecular and graphic formulae do not constitute analytical standards for the substances described.

*CHEMICAL ABSTRACTS SERVICE (CAS) REGISTRY NUMBER*

CAS registry numbers are included for information in monographs, where applicable, to provide convenient access to useful information for users. CAS Registry Number® is a registered trademark of the American Chemical Society.

*DEFINITION*

Statements under the heading Definition constitute an official definition of the substance, preparation or other article that is the subject of the monograph.

**Limits of content**. Where limits of content are prescribed, they are those determined by the method described under Assay.

**Herbal drugs**. In monographs on herbal drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form. Where a monograph applies to the drug in several states, for example both to the whole drug and the drug in powdered form, the definition states this.

*PRODUCTION*

Statements under the heading Production draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute mandatory requirements for manufacturers, unless otherwise stated. They may relate, for example, to source materials; to the manufacturing process itself and its validation and control; to in-process testing; or to testing that is to be carried out by the manufacturer on the final article, either on selected batches or on each batch prior to release. These statements cannot necessarily be verified on a sample of the final article by an independent analyst. The competent authority may establish that the instructions have been followed, for example, by examination of data received from the manufacturer, by inspection of manufacture or by testing appropriate samples.

The absence of a Production section does not imply that attention to features such as those referred to above is not required.

**Choice of vaccine strain, Choice of vaccine composition**. The Production section of a monograph may define the characteristics of a vaccine strain or vaccine composition. Unless otherwise stated, test methods given for verification of these characteristics are provided for information as examples of suitable methods. Subject to approval by the competent authority, other test methods may be used without validation against the method shown in the monograph.

*POTENTIAL ADULTERATION*

Due to the increasing number of fraudulent activities and cases of adulteration, information may be made available to Ph. Eur. users to help detect adulterated materials (i.e. active substances, excipients, intermediate products, bulk products and finished products).

To this purpose, a method for the detection of potential adulterants and relevant limits, together with a reminder that all stages of production and sourcing are subjected to a suitable quality system, may be included in this section of monographs on substances for which an incident has occurred or that present a risk of deliberate contamination. The frequency of testing by manufacturers or by users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant) depends on a risk assessment, taking into account the level of knowledge of the whole supply chain and national requirements.

This section constitutes requirements for the whole supply chain, from manufacturers to users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant). The absence of this section does not imply that attention to features such as those referred to above is not required.

*CHARACTERS*

The statements under the heading Characters are not to be interpreted in a strict sense and are not requirements.

**Solubility**. In statements of solubility in the Characters section, the terms used have the following significance, referred to a temperature between 15 °C and 25 °C.

| Descriptive term | Approximate volume of solvent in millilitres per gram of solute | | | |
|---|---|---|---|---|
| Very soluble | less than | 1 | | |
| Freely soluble | from | 1 | to | 10 |
| Soluble | from | 10 | to | 30 |
| Sparingly soluble | from | 30 | to | 100 |
| Slightly soluble | from | 100 | to | 1000 |
| Very slightly soluble | from | 1000 | to | 10 000 |
| Practically insoluble | more than | | | 10 000 |

The term 'partly soluble' is used to describe a mixture where only some of the components dissolve. The term 'miscible' is used to describe a liquid that is miscible in all proportions with the stated solvent.

*IDENTIFICATION*

**Scope**. The tests given in the Identification section are not designed to give a full confirmation of the chemical structure or composition of the product; they are intended to give confirmation, with an acceptable degree of assurance, that the article conforms to the description on the label.

**First and second identifications**. Certain monographs have subdivisions entitled 'First identification' and 'Second identification'. The test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be used in pharmacies provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the Tests section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

**Powdered herbal drugs**. Monographs on herbal drugs may contain schematic drawings of the powdered drug. These drawings complement the description given in the relevant identification test.

*TESTS AND ASSAYS*

**Scope**. The requirements are not framed to take account of all possible impurities. It is not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated if common sense and good pharmaceutical practice require that it be absent. See also below under Impurities.

**Calculation**. Where the result of a test or assay is required to be calculated with reference to the dried or anhydrous substance or on some other specified basis, the determination of loss on drying, water content or other property is carried out by the method prescribed in the relevant test in the monograph. The words 'dried substance' or 'anhydrous substance' etc. appear in parentheses after the result. Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for the calculation of the assay content of the substance, the specific optical rotation and the specific absorbance. No further indication is given in the specific monograph.

**Limits**. The limits prescribed are based on data obtained in normal analytical practice; they take account of normal analytical errors, of acceptable variations in manufacture and compounding and of deterioration to an extent considered acceptable. No further tolerances are to be applied to the limits prescribed to determine whether the article being examined complies with the requirements of the monograph.

In determining compliance with a numerical limit, the calculated result of a test or assay is first rounded to the number of significant figures stated, unless otherwise prescribed. The limits, regardless of whether the values are expressed as percentages or as absolute values, are considered significant to the last digit shown (for example 140 indicates 3 significant figures). The last figure of the result is increased by one when the part rejected is equal to or exceeds one half-unit, whereas it is not modified when the part rejected is less than a half-unit.

**Indication of permitted limit of impurities**. The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values. For comparative tests, the approximate content of impurity tolerated, or the sum of impurities, may be indicated in brackets for information only. Acceptance or rejection is determined on the basis of compliance or non-compliance with the stated test. If the use of a reference substance for the named impurity is not prescribed, this content may be expressed as a nominal concentration of the substance used to prepare the reference solution specified in the monograph, unless otherwise described.

**Herbal drugs**. For herbal drugs, the sulfated ash, total ash, water-soluble matter, alcohol-soluble matter, water content, content of essential oil and content of active principle are calculated with reference to the drug that has not been specially dried, unless otherwise prescribed in the monograph.

**Equivalents**. Where an equivalent is given, for the purposes of the Pharmacopoeia only the figures shown are to be used in applying the requirements of the monograph.

**Culture media**. The culture media described in monographs and general chapters have been found to be satisfactory for the intended purpose. However, the components of media, particularly those of biological origin, are of variable quality, and it may be necessary for optimal performance to modulate the concentration of some ingredients, notably:

- peptones and meat or yeast extracts, with respect to their nutritive properties;
- buffering substances;
- bile salts, bile extract, deoxycholate, and colouring matter, depending on their selective properties;
- antibiotics, with respect to their activity.

*STORAGE*

The information and recommendations given under the heading Storage do not constitute a pharmacopoeial requirement but the competent authority may specify particular storage conditions that must be met.

The articles described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Where special conditions of storage are recommended, including the type of container (see section 1.3. General chapters) and limits of temperature, they are stated in the monograph.

The following expressions are used in monographs under Storage with the meaning shown.

*In an airtight container* means that the product is stored in an airtight container (*3.2*). Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

*Protected from light* means that the product is stored either in a container made of a material that absorbs actinic light sufficiently to protect the contents from change induced by such light, or in a container enclosed in an outer cover that provides such protection, or is stored in a place from which all such light is excluded.

*LABELLING*

In general, labelling of medicines is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling are not therefore comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term 'label' is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package, or a certificate of analysis accompanying the article, as decided by the competent authority.

*WARNINGS*

Materials described in monographs and reagents specified for use in the Pharmacopoeia may be injurious to health unless adequate precautions are taken. The principles of good quality control laboratory practice and the provisions of any appropriate regulations are to be observed at all times. Attention is drawn to particular hazards in certain monographs by means of a warning statement; absence of such a statement is not to be taken to mean that no hazard exists.

*IMPURITIES*

A list of all known and potential impurities that have been shown to be detected by the tests in a monograph may be given. See also chapter *5.10. Control of impurities in substances for pharmaceutical use*. The impurities are designated by a letter or letters of the alphabet. Where a letter appears to be missing, the impurity designated by this letter has been deleted from the list during monograph development prior to publication or during monograph revision.

*FUNCTIONALITY-RELATED CHARACTERISTICS OF EXCIPIENTS*

Monographs on excipients may have a section on functionality-related characteristics. The characteristics, any test methods for determination and any tolerances are not mandatory requirements; they may nevertheless be relevant for use of the excipient and are given for information (see also section 1.1. General statements).

*REFERENCE STANDARDS*

Certain monographs require the use of reference standards (chemical reference substances, herbal reference standards, biological reference preparations, reference spectra). See also chapter *5.12. Reference standards*. The European Pharmacopoeia Commission establishes the official reference standards, which are alone authoritative in case of arbitration. These reference standards are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM). Information on the available reference standards and a batch validity statement can be obtained via the EDQM website.

## 1.5. ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| $A$ | Absorbance |
| $A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}$ | Specific absorbance |
| $A_r$ | Relative atomic mass |
| $[\alpha]_D^{20}$ | Specific optical rotation |
| bp | Boiling point |
| BRP | Biological reference preparation |
| CRS | Chemical reference substance |
| $d_{20}^{20}$ | Relative density |
| λ | Wavelength |
| HRS | Herbal reference standard |
| IU | International Unit |
| M | Molarity |
| $M_r$ | Relative molecular mass |
| mp | Melting point |
| $n_D^{20}$ | Refractive index |
| Ph. Eur. U. | European Pharmacopoeia Unit |
| ppb | Parts per billion (micrograms per kilogram) |
| ppm | Parts per million (milligrams per kilogram) |
| R | Substance or solution defined under *4. Reagents* |
| $R_F$ | Retardation factor (see chapter *2.2.46*) |
| $R_{st}$ | Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by a reference substance |
| RV | Substance used as a primary standard in volumetric analysis (chapter *4.2.1*) |

**Abbreviations used in the monographs on immunoglobulins, immunosera and vaccines**

| | |
|---|---|
| CFU | Colony-forming units |
| $LD_{50}$ | The statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period |
| MLD | Minimum lethal dose |
| L+/10 dose | The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period |
| L+ dose | The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period |
| lr/100 dose | The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period |
| Lp/10 dose | The smallest quantity of toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period |
| Lo/10 dose | The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given period |
| Lf dose | The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin |

| | |
|---|---|
| $CCID_{50}$ | The statistically determined quantity of virus that may be expected to infect 50 per cent of the cell cultures to which it is added |
| $EID_{50}$ | The statistically determined quantity of virus that may be expected to infect 50 per cent of the fertilised eggs into which it is inoculated |
| $ID_{50}$ | The statistically determined quantity of a virus that may be expected to infect 50 per cent of the animals into which it is inoculated |
| $PD_{50}$ | The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to protect 50 per cent of the animals against a challenge dose of the micro-organisms or toxins against which it is active |
| $ED_{50}$ | The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to induce specific antibodies in 50 per cent of the animals for the relevant vaccine antigens |
| PFU | Pock-forming units or plaque-forming units |
| SPF | Specified-pathogen-free |

**Collections of micro-organisms**

| | |
|---|---|
| ATCC | American Type Culture Collection<br>10801 University Boulevard<br>Manassas, Virginia 20110-2209, USA |
| C.I.P. | Collection de Bactéries de l'Institut Pasteur<br>B.P. 52, 25 rue du Docteur Roux<br>75724 Paris Cedex 15, France |
| IMI | International Mycological Institute<br>Bakeham Lane<br>Surrey TW20 9TY, Great Britain |
| I.P. | Collection Nationale de Culture de Microorganismes (C.N.C.M.)<br>Institut Pasteur<br>25, rue du Docteur Roux<br>75724 Paris Cedex 15, France |
| NCIMB | National Collection of Industrial and Marine Bacteria Ltd<br>23 St Machar Drive<br>Aberdeen AB2 1RY, Great Britain |
| NCPF | National Collection of Pathogenic Fungi<br>London School of Hygiene and Tropical Medicine<br>Keppel Street<br>London WC1E 7HT, Great Britain |
| NCTC | National Collection of Type Cultures<br>Central Public Health Laboratory<br>Colindale Avenue<br>London NW9 5HT, Great Britain |
| NCYC | National Collection of Yeast Cultures<br>AFRC Food Research Institute<br>Colney Lane<br>Norwich NR4 7UA, Great Britain |
| NITE | Biological Resource Center<br>Department of Biotechnology<br>National Institute of Technology and Evaluation<br>2-5-8 Kazusakamatari, Kisarazu-shi, Chiba, 292-0818<br>Japan |
| S.S.I. | Statens Serum Institut<br>80 Amager Boulevard, Copenhagen, Denmark |

## 1.6. UNITS OF THE INTERNATIONAL SYSTEM (SI) USED IN THE PHARMACOPOEIA AND EQUIVALENCE WITH OTHER UNITS

*INTERNATIONAL SYSTEM OF UNITS (SI)*

The International System of Units comprises 2 main classes of units, namely base units and derived units[(1)]. The base units are the metre, the kilogram, the second, the ampere, the kelvin, the mole and the candela.

The derived units are formed as products of powers of the base units according to the algebraic relationships linking the corresponding quantities. Some of these derived units have special names and symbols. The derived units used in the Pharmacopoeia are shown in Table 1.6.-1.

Some important and widely used units outside the International System are shown in Table 1.6.-2.

The prefixes shown in Table 1.6.-3 are used to form the names and symbols of the decimal multiples and submultiples of SI units.

Table 1.6.-1. – *Derived units used in the European Pharmacopoeia and equivalence with other units*

| Quantity | | Unit | | | | Conversion of other units into SI units |
|---|---|---|---|---|---|---|
| Name | Symbol | Name | Symbol | Expression in SI base units | Expression in other SI units | |
| Wave number | $\nu$ | one per metre | 1/m | $m^{-1}$ | | |
| Wavelength | $\lambda$ | micrometre<br>nanometre | µm<br>nm | $10^{-6}$m<br>$10^{-9}$m | | |
| Area | $A$, $S$ | square metre | $m^2$ | $m^2$ | | |
| Volume | $V$ | cubic metre | $m^3$ | $m^3$ | | 1 mL = 1 $cm^3$ = $10^{-6}$ $m^3$ |
| Frequency | $\nu$ | hertz | Hz | $s^{-1}$ | | |
| Density | $\rho$ | kilogram per cubic metre | $kg/m^3$ | $kg \cdot m^{-3}$ | | 1 g/mL = 1 $g/cm^3$ = $10^3$ $kg \cdot m^{-3}$ |
| Velocity, speed | $v$ | metre per second | m/s | $m \cdot s^{-1}$ | | |

(1) The definitions of the units used in the International System are given in the booklet 'Le Système International d'Unités (SI)', published by the Bureau International des Poids et Mesures, Pavillon de Breteuil, F-92310 Sèvres.

| Quantity | | Unit | | | | Conversion of other units into SI units |
|---|---|---|---|---|---|---|
| **Name** | **Symbol** | **Name** | **Symbol** | **Expression in SI base units** | **Expression in other SI units** | |
| Force | *F* | newton | N | $m \cdot kg \cdot s^{-2}$ | | 1 dyne = 1 $g \cdot cm \cdot s^{-2} = 10^{-5}$ N<br>1 kp = 9.806 65 N |
| Pressure, stress | *p* | pascal | Pa | $m^{-1} \cdot kg \cdot s^{-2}$ | $N \cdot m^{-2}$ | 1 dyne/$cm^2 = 10^{-1}$ Pa = $10^{-1}$ $N \cdot m^{-2}$<br>1 atm = 101 325 Pa = 101.325 kPa<br>1 bar = $10^5$ Pa = 0.1 MPa<br>1 mm Hg = 133.322 387 Pa<br>1 Torr = 133.322 368 Pa<br>1 psi = 6.894 757 kPa |
| Dynamic viscosity | $\eta$ | pascal second | Pa·s | $m^{-1} \cdot kg \cdot s^{-1}$ | $N \cdot s \cdot m^{-2}$ | 1 P = $10^{-1}$ Pa·s = $10^{-1}$ $N \cdot s \cdot m^{-2}$<br>1 cP = 1 mPa·s |
| Kinematic viscosity | $\nu$ | square metre per second | $m^2/s$ | $m^2 \cdot s^{-1}$ | $Pa \cdot s \cdot m^3 \cdot kg^{-1}$<br>$N \cdot m \cdot s \cdot kg^{-1}$ | 1 St = 1 $cm^2 \cdot s^{-1} = 10^{-4}$ $m^2 \cdot s^{-1}$ |
| Energy | *W* | joule | J | $m^2 \cdot kg \cdot s^{-2}$ | N·m | 1 erg = 1 $cm^2 \cdot g \cdot s^{-2}$ = 1 dyne·cm = $10^{-7}$ J<br>1 cal = 4.1868 J |
| Power, radiant flux | *P* | watt | W | $m^2 \cdot kg \cdot s^{-3}$ | $N \cdot m \cdot s^{-1}$<br>$J \cdot s^{-1}$ | 1 erg/s = 1 dyne·$cm \cdot s^{-1}$ =<br>$10^{-7}$ W = $10^{-7}$ $N \cdot m \cdot s^{-1} = 10^{-7}$ $J \cdot s^{-1}$ |
| Absorbed dose (of radiant energy) | *D* | gray | Gy | $m^2 \cdot s^{-2}$ | $J \cdot kg^{-1}$ | 1 rad = $10^{-2}$ Gy |
| Electric potential difference, voltage | *U* | volt | V | $m^2 \cdot kg \cdot s^{-3} \cdot A^{-1}$ | $W \cdot A^{-1}$ | |
| Electric resistance | *R* | ohm | Ω | $m^2 \cdot kg \cdot s^{-3} \cdot A^{-2}$ | $V \cdot A^{-1}$ | |
| Electric charge | *Q* | coulomb | C | A·s | | |
| Activity referred to a radionuclide | *A* | becquerel | Bq | $s^{-1}$ | | 1 Ci = $37 \cdot 10^9$ Bq = $37 \cdot 10^9$ $s^{-1}$ |
| Concentration (of amount of substance), molar concentration | *c* | mole per cubic metre | $mol/m^3$ | $mol \cdot m^{-3}$ | | 1 mol/L = 1 M = 1 $mol/dm^3 = 10^3$ $mol \cdot m^{-3}$ |
| Mass concentration | $\rho$ | kilogram per cubic metre | $kg/m^3$ | $kg \cdot m^{-3}$ | | 1 g/L = 1 $g/dm^3$ = 1 $kg \cdot m^{-3}$ |
| Catalytic activity | *Z* | katal | kat | $mol \cdot s^{-1}$ | | |

*NOTES*

1. In the Pharmacopoeia, the Celsius temperature is used (symbol *t*). This is defined by the following equation:

$$t = T - T_0$$

where $T_0$ = 273.15 K by definition. The Celsius or centigrade temperature is expressed in degrees Celsius (symbol °C). The unit 'degree Celsius' is equal to the unit 'kelvin'.

2. The practical expressions of concentrations used in the Pharmacopoeia are defined in the General Notices.

3. The radian is the plane angle between two radii of a circle that cut off on the circumference an arc equal in length to the radius.

4. In the Pharmacopoeia, conditions of centrifugation are defined by reference to the acceleration due to gravity (*g*):

$$g = 9.806\ 65\ m \cdot s^{-2}$$

5. Certain quantities without dimensions are used in the Pharmacopoeia: relative density (*2.2.5*), absorbance (*2.2.25*), specific absorbance (*2.2.25*) and refractive index (*2.2.6*).

6. The microkatal is defined as the enzymic activity that, under defined conditions, produces the transformation (e.g. hydrolysis) of 1 micromole of the substrate per second.

Table 1.6.-2. – *Non-SI units accepted for use with the SI units*

| Quantity | Unit | | Value in SI units |
|---|---|---|---|
| | Name | Symbol | |
| Time | minute | min | 1 min = 60 s |
| | hour | h | 1 h = 60 min = 3600 s |
| | day | d | 1 d = 24 h = 86 400 s |
| Plane angle | degree | ° | 1° = (π/180) rad |
| Volume | litre | L | 1 L = 1 $dm^3$ = $10^{-3}$ $m^3$ |
| Mass | tonne | t | 1 t = $10^3$ kg |
| | dalton | Da | 1 Da = 1.660539040(20) × $10^{-27}$ kg |
| Rotational frequency | revolution per minute | r/min | 1 r/min = (1/60) $s^{-1}$ |
| Energy | electronvolt | eV | 1eV=1.602176634 × $10^{-19}$J |

Table 1.6.-3. – *Decimal multiples and sub-multiples of SI units*

| Factor | Prefix | Symbol | Factor | Prefix | Symbol |
|---|---|---|---|---|---|
| $10^{18}$ | exa | E | $10^{-1}$ | deci | d |
| $10^{15}$ | peta | P | $10^{-2}$ | centi | c |
| $10^{12}$ | tera | T | $10^{-3}$ | milli | m |
| $10^{9}$ | giga | G | $10^{-6}$ | micro | µ |
| $10^{6}$ | mega | M | $10^{-9}$ | nano | n |
| $10^{3}$ | kilo | k | $10^{-12}$ | pico | p |
| $10^{2}$ | hecto | h | $10^{-15}$ | femto | f |
| $10^{1}$ | deca | da | $10^{-18}$ | atto | a |

# 2. Methods of Analysis

# 2.1. Apparatus

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# 2.1. APPARATUS

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## 2.1.1. DROPPERS

The term 'drops' means standard drops delivered from a standard dropper as described below.

Standard droppers (Figure 2.1.1.-1) are constructed of practically colourless glass. The lower extremity has a circular orifice in a flat surface at right angles to the axis.

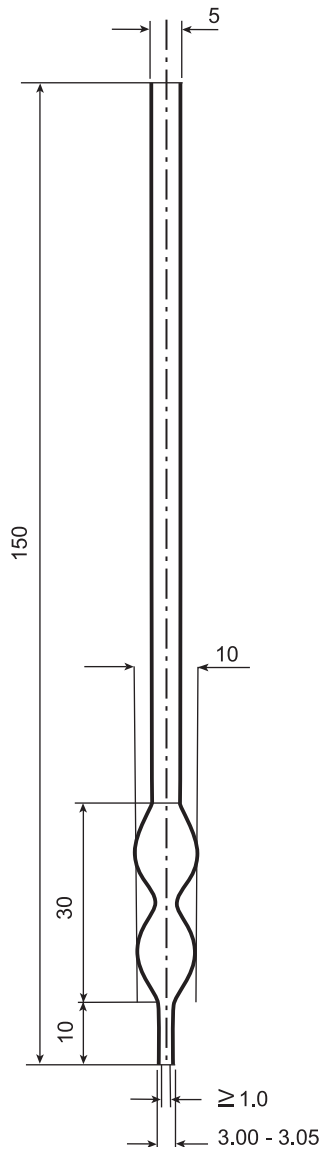


Figure 2.1.1.-1. – *Standard dropper*
*Dimensions in millimetres*

Other droppers may be used provided they comply with the following test.

20 drops of *water R* at 20 ± 1 °C flowing freely from the dropper held in the vertical position at a constant rate of 1 drop per second weighs 1000 ± 50 mg.

The dropper must be carefully cleaned before use. Carry out 3 determinations on any given dropper. No result may deviate by more than 5 per cent from the mean of the 3 determinations.

01/2008:20102

## 2.1.2. COMPARATIVE TABLE OF POROSITY OF SINTERED-GLASS FILTERS[1]

Table 2.1.2.-1

| Porosity number (Ph. Eur.)[2] | Maximum diameter of pores in micrometres | Germany | France | United Kingdom |
|---|---|---|---|---|
| 1.6 | less than 1.6 | 5f | – | – |
| – | 1 - 2.5 | 5 | – | 5 |
| 4 | 1.6 - 4 | – | – | – |
| – | 4 - 6 | – | 5 | – |
| 10 | 4 - 10 | 4f | – | 4 |
| 16 | 10 - 16 | 4 | 4 | – |
| 40 | 16 - 40 | 3 | 3 | 3 |
| – | 40 - 50 | – | – | 2 |
| 100 | 40 - 100 | 2 | 2 | – |
| – | 100 - 120 | – | – | 1 |
| 160 | 100 - 160 | 1 | 1 | – |
| – | 150 - 200 | 0 | 0 | – |
| 250 | 160 - 250 | – | – | – |
| – | 200 - 500 | – | 00 | – |

*Special Uses*

*Diameters in micrometres*

| | |
|---|---|
| < 2.5 | Bacteriological filtration |
| 4 - 10 | Ultra-fine filtration, separation of micro-organisms of large diameter |
| 10 - 40 | Analytical filtration, very fine filtration of mercury, very fine dispersion of gases |
| 40 - 100 | Fine filtration, filtration of mercury, fine dispersion of gases |
| 100 - 160 | Filtration of coarse materials, dispersion and washing of gases, support for other filter materials |
| 160 - 500 | Filtration of very coarse materials, dispersion and washing of gases. |

01/2008:20103

## 2.1.3. ULTRAVIOLET RAY LAMPS FOR ANALYTICAL PURPOSES

Mercury vapour in quartz lamps is used as the source of ultraviolet light. A suitable filter may be fitted to eliminate the visible part of the spectrum emitted by the lamp. When the Pharmacopoeia prescribes in a test the use of ultraviolet light of wavelength 254 nm or 365 nm, an instrument consisting of a mercury vapour lamp and a filter which gives an emission band with maximum intensity at about 254 nm or 365 nm is used. The lamp used should be capable of revealing without doubt a standard spot of sodium salicylate with a diameter of about 5 mm on a support of *silica gel G R*, the spot being examined while in a position normal to the radiation.

(1) The given limits are only approximate.
(2) The European Pharmacopoeia has adopted the system proposed by the International Organization for Standardization (ISO).

For this purpose apply 5 µL of a 0.4 g/L solution of *sodium salicylate R* in *alcohol R*[3] for lamps of maximum output at 254 nm and 5 µL of a 2 g/L solution in *alcohol R*[3] for lamps of maximum output at 365 nm. The distance between the lamp and the chromatographic plate under examination used in a pharmacopoeial test should never exceed the distance used to carry out the above test.

**01/2008:20104**

## 2.1.4. SIEVES

Sieves are constructed of suitable materials with square meshes. For purposes other than analytical procedures, sieves with circular meshes may be used, the internal diameters of which are 1.25 times the aperture of the square mesh of the corresponding sieve size. There must be no reaction between the material of the sieve and the substance being sifted. Degree of comminution is prescribed in the monograph using the sieve number, which is the size of the mesh in micrometres, given in parenthesis after the name of the substance (Table 2.1.4.-1).

Maximum tolerance[4] for an aperture (+ *X*): no aperture size shall exceed the nominal size by more than *X*, where:

$$X = \frac{2(w^{0.75})}{3} + 4(w^{0.25})$$

*w* = width of aperture.

Tolerance for mean aperture (± *Y*): the average aperture size shall not depart from the nominal size by more than ± *Y*, where:

$$Y = \frac{w^{0.98}}{27} + 1.6$$

Intermediary tolerance (+ *Z*): not more than 6 per cent of the total number of apertures shall have sizes between "nominal + *X*" and "nominal + *Z*", where:

$$Z = \frac{X + Y}{2}$$

Wire diameter *d*: the wire diameters given in Table 2.1.4.-1 apply to woven metal wire cloth mounted in a frame. The nominal sizes of the wire diameters may depart from these values within the limits $d_{max}$ and $d_{min}$. The limits define a permissible range of choice ± 15 per cent of the recommended nominal dimensions. The wires in a test sieve shall be of a similar diameter in warp and weft directions.

Table 2.1.4.-1 (*values in micrometers*)

| Sieve numbers (Nominal dimensions of apertures) | Tolerances for apertures | | | Wire diameters | | |
|---|---|---|---|---|---|---|
| | Maximum tolerance for an aperture | Tolerance for mean aperture | Intermediary tolerance | Recommended nominal dimensions | Admissible limits | |
| | + *X* | ± *Y* | + *Z* | *d* | $d_{max}$ | $d_{min}$ |
| 11 200 | 770 | 350 | 560 | 2500 | 2900 | 2100 |
| 8000 | 600 | 250 | 430 | 2000 | 2300 | 1700 |
| 5600 | 470 | 180 | 320 | 1600 | 1900 | 1300 |
| 4000 | 370 | 130 | 250 | 1400 | 1700 | 1200 |
| 2800 | 290 | 90 | 190 | 1120 | 1300 | 950 |
| 2000 | 230 | 70 | 150 | 900 | 1040 | 770 |
| 1400 | 180 | 50 | 110 | 710 | 820 | 600 |
| 1000 | 140 | 30 | 90 | 560 | 640 | 480 |
| 710 | 112 | 25 | 69 | 450 | 520 | 380 |
| 500 | 89 | 18 | 54 | 315 | 360 | 270 |
| 355 | 72 | 13 | 43 | 224 | 260 | 190 |
| 250 | 58 | 9.9 | 34 | 160 | 190 | 130 |
| 180 | 47 | 7.6 | 27 | 125 | 150 | 106 |
| 125 | 38 | 5.8 | 22 | 90 | 104 | 77 |
| 90 | 32 | 4.6 | 18 | 63 | 72 | 54 |
| 63 | 26 | 3.7 | 15 | 45 | 52 | 38 |
| 45 | 22 | 3.1 | 13 | 32 | 37 | 27 |
| 38 | – | – | – | 30 | 35 | 24 |

(3) The *alcohol R* used must be free from fluorescence.
(4) See the International Standard ISO 3310/1 (1975).

01/2008:20105

## 2.1.5. TUBES FOR COMPARATIVE TESTS

Tubes used for comparative tests are matched tubes of colourless glass with a uniform internal diameter. The base is transparent and flat.

A column of the liquid is examined down the vertical axis of the tube against a white background, or if necessary, against a black background. The examination is carried out in diffused light.

It is assumed that tubes with an internal diameter of 16 mm will be used. Tubes with a larger internal diameter may be used instead but the volume of liquid examined must then be increased so that the depth of liquid in the tubes is not less than where the prescribed volume of liquid and tubes 16 mm in internal diameter are used.

01/2018:20106

## 2.1.6. GAS DETECTOR TUBES

Gas detector tubes are cylindrical, sealed tubes consisting of an inert transparent material and are constructed to allow the passage of gas. They contain reagents adsorbed onto inert substrates that are suitable for the visualisation of the substance to be detected and, if necessary, they also contain preliminary layers and/or adsorbent filters to eliminate substances that interfere with the substance to be detected. The layer of indicator contains either a single reagent for the detection of a given impurity or several reagents for the detection of several substances (monolayer tube or multilayer tube).

The test is carried out by passing the required volume of the gas to be examined through the indicator tube. The length of the coloured layer or the intensity of a colour change on a graduated scale gives an indication of the impurities present.

The calibration of the detector tubes is verified according to the manufacturer's instructions.

*Operating conditions*. Examine according to the manufacturer's instructions or proceed as follows.

The gas supply is connected to a suitable pressure regulator and needle valve. Connect the flexible tubing fitted with a Y-piece to the valve and adjust the flow of gas to be examined to purge the tubing in order to obtain an appropriate flow (Figure 2.1.6.-1). Prepare the indicator tube and fit to the metering pump, following the manufacturer's instructions. Connect the open end of the indicator tube to the short leg of the tubing and operate the pump by the appropriate number of strokes to pass a suitable volume of gas to be examined through the tube. Read the value corresponding to the length of the coloured layer or the intensity of the colour on the graduated scale. If a negative result is achieved, indicator tubes can be verified with a calibration gas containing the appropriate impurity.

In view of the wide variety of available compressor oils, it is necessary to verify the reactivity of the oil detector tubes for the oil used. Information on the reactivity for various oils is given in the leaflet supplied with the tube. If the oil used is not cited in the leaflet, the tube manufacturer must verify the reactivity and if necessary provide a tube specific for this oil.

**Arsine detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for the gold salt or other appropriate indicator. The minimum value indicated is 0.25 ppm or less, with a relative standard deviation of at most 20 per cent.

**Carbon dioxide detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for hydrazine and crystal violet indicators. The minimum value indicated is 100 ppm with a relative standard deviation of at most 15 per cent.

**Carbon monoxide detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for di-iodine pentoxide, selenium dioxide and fuming sulfuric acid indicators. The minimum value indicated is 5 ppm or less, with a relative standard deviation of at most 15 per cent.

**Hydrogen sulfide detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for an appropriate lead salt indicator. The minimum value indicated is 0.2 ppm or less, with a relative standard deviation of at most 10 per cent.

**Nitrogen monoxide and nitrogen dioxide detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for an oxidising layer (Cr(VI) salt) and the diphenylbenzidine indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most 15 per cent.

**Oil detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for the sulfuric acid indicator. The minimum value indicated is 0.1 mg/m$^3$ with a relative standard deviation of at most 30 per cent.

**Phosphine detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for the gold salt or other appropriate indicator. The minimum value indicated is 0.2 ppm or less, with a relative standard deviation of at most 20 per cent.

**Sulfur dioxide detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for the iodine and starch indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most 15 per cent.

**Water vapour detector tube**. Sealed glass tube containing adsorbent filters and suitable supports for the magnesium perchlorate indicator. The minimum value indicated is 67 ppm or less, with a relative standard deviation of at most 20 per cent.

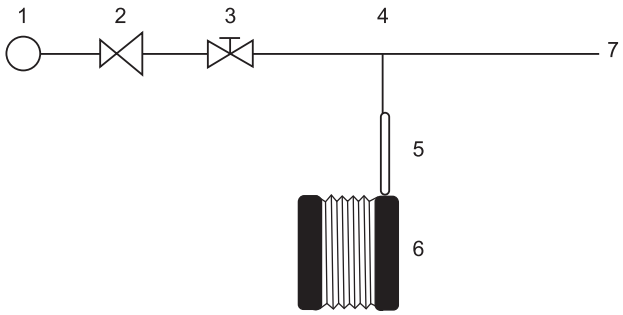


1. Gas supply
2. Pressure regulator
3. Needle valve
4. Y-piece
5. Indicator tube
6. Indicator tube pump
7. End open to atmosphere

Figure 2.1.6.-1. – *Apparatus for gas detector tubes*

# 2.2. Physical and physico-chemical methods



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# 2.2. PHYSICAL AND PHYSICO-CHEMICAL METHODS



07/2017:20201

## 2.2.1. CLARITY AND DEGREE OF OPALESCENCE OF LIQUIDS

Opalescence is the effect of light being absorbed or scattered by submicroscopic particles or optical density inhomogeneities. The absence of any particles or inhomogeneities in a solution results in a clear solution.

A liquid is considered *clear* if its clarity is the same as that of *water R* or of the solvent used, or if its opalescence is not more pronounced than that of reference suspension I (see Table 2.2.1.-1), when examined under the conditions described below.

Requirements in monographs are expressed in terms of the visual method by comparing with the defined reference suspensions (see Table 2.2.1.-1). However, instrumental methods may also be used for determining compliance with monograph requirements once the suitability of the instrument has been established as described below and calibration with reference suspensions I-IV and with *water R* or the solvent used has been performed.

VISUAL METHOD

Using identical test-tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with a reference suspension freshly prepared as described below. Ensure that the depths of the layers in the 2 test-tubes are the same (about 40 mm).

Compare the liquids in diffused daylight 5 min after preparation of the reference suspension, viewing vertically against a black background.

*System suitability*. The diffusion of light must be such that reference suspension I can readily be distinguished from *water R*, and that reference suspension II can readily be distinguished from reference suspension I (see Table 2.2.1.-1).

INSTRUMENTAL METHOD

The instrumental assessment of clarity and opalescence provides a more discriminatory test that does not depend on the visual acuity of the analyst. Numerical results are more useful for process control and quality monitoring, especially in stability studies. For example, previous numerical data on stability can be extrapolated to determine whether a given batch of a preparation will exceed shelf-life limits prior to the expiry date.

*TURBIDIMETRY AND NEPHELOMETRY*

When a suspension is viewed at right angles to the direction of the incident light, the system appears opalescent due to the scattering of light by the particles of the suspension (Tyndall effect). A certain portion of the light beam entering a turbid liquid is transmitted, another portion is absorbed and the remaining portion is scattered by the suspended particles. The light-scattering effect of suspended particles can be measured either indirectly by observation of the transmitted light (turbidimetry) or directly by measuring the scattered light (nephelometry). Turbidimetry and nephelometry are more reliable in low turbidity ranges, where there is a linear relationship between turbidity values and detector signals.

As the degree of turbidity increases, not all the particles are exposed to the incident light and the scattered or the transmitted radiation of other particles is hindered on its way to the detector.

For quantitative measurements, the construction of calibration curves is essential. Linearity must be based on at least 4 levels of concentrations. Reference suspensions must show a sufficiently stable degree of turbidity and must be produced under well-defined conditions.

*MEASUREMENTS IN RATIO MODE*

The determination of opalescence of coloured liquids is done using instruments with ratio mode, since colour provides a negative interference, attenuating both incident and scattered light and lowering the turbidity value. The effect is so great, even for moderately coloured samples, that conventional nephelometers cannot be used.

In turbidimetry or nephelometry with ratio mode, the ratio of the transmission measurement to the 90° scattered light measurement is determined. This procedure compensates for the light that is diminished by the colour of the sample. Instruments with ratio mode use as light source a tungsten lamp with spectral sensitivity at about 550 nm operating at a filament colour temperature of 2700 K. Other suitable light sources may also be used. Silicon photodiodes and photomultipliers are commonly used as detectors and record changes in light scattered or transmitted by the sample. The light scattered at 90 ± 2.5° is measured by the primary detector. Other detectors measure back and forward scatter (reflected light) as well as transmitted light. The results are obtained by calculating the ratio of the 90° scattered light measured to the sum of the components of forward scattered and transmitted light values.

The instruments used are calibrated against standards of known turbidity and are capable of automatic measurement of turbidity. The test results are obtained directly from the instrument and compared to the specifications in the individual monograph.

Alternatively, the influence of the colour of the sample may also be eliminated by using an infrared light-emitting diode (IR LED) having an emission maximum at 860 nm with a 60 nm spectral bandwidth as the light source of the instrument.

*INSTRUMENT REQUIREMENTS*

Instruments complying with the following characteristics and verified using reference suspensions as described below may be used instead of visual examination for determination of compliance with monograph requirements.

- *Measuring unit*: NTU (nephelometric turbidity units). NTU is based on the turbidity of a primary standard of formazin. FTU (formazin turbidity units) or FNU (formazin nephelometric units) are also used, and are equivalent to NTU in regions of low turbidity (up to 40 NTU). These units are used in all 3 instrumental methods (nephelometry, turbidimetry and in ratio mode).
- *Measuring range*: 0.01-1100 NTU.
- *Resolution*: 0.01 NTU within the range 0-9.99 NTU; 0.1 NTU within the range 10.0-99.9 NTU; and 1 NTU for the range > 100 NTU.
- *Accuracy*: ± (10 per cent of reading + 0.01 NTU) within the range 0-20 NTU; ± 7.5 per cent within the range 20-1100 NTU.
- *Repeatability*: ± 0.05 NTU within the range 0-20 NTU; ± 2 per cent of the reading within the range 20-1100 NTU.

Instruments with measuring range or resolution, accuracy and repeatability capabilities other than those mentioned above may be used provided they are sufficiently validated and are capable for the intended use.

*CONTROL OF INSTRUMENT PERFORMANCE*

– *Calibration*: performed with at least 4 reference suspensions of formazin covering the measuring range of interest. Reference suspensions described in this chapter or suitable reference standards calibrated against the primary reference suspensions may be used.
– *Stray light*: < 0.15 NTU within the range 0-10 NTU; < 0.5 NTU within the range 10-1100 NTU. Stray light is defined as that light that reaches the nephelometric detector without being a result of scatter from the sample. Stray light is always a positive interference and is a significant source of error in low-range turbidity measurements. Sources of stray light include: imperfections in and scratches on sample cells, internal reflections of the optical system, contamination of the optics or sample cell chamber with dust, and electronic noise. Instrument design can also affect stray light. The influence of stray light becomes negligible in ratio mode measurements.

The test methodology for the specific substance/product to be analysed must also be verified to demonstrate its analytical capability. The instrument and methodology shall be consistent with the attributes of the substance to be examined.

Measurements of standards and samples should be carried out under the same temperature conditions, preferably between 20 °C and 25 °C.

REFERENCE SUSPENSIONS

Formazin has several desirable characteristics that make it an excellent turbidity standard. It can be reproducibly prepared from assayed raw materials. The physical characteristics make it a desirable light-scatter calibration standard. The formazin polymer consists of chains of different lengths, which fold into random configurations. This results in a wide variety of particle shapes and sizes, which allows the analysis of different particle sizes and shapes that are found in real samples. Stabilised formazin suspensions that can be used to prepare stable, diluted turbidity standards are commercially available and may be used after comparison with the standards prepared as described.

All steps of the preparation of reference suspensions as described below are carried out at 25 ± 3 °C.

**Hydrazine sulfate solution**. Dissolve 1.0 g of *hydrazine sulfate R* in *water R* and dilute to 100.0 mL with the same solvent. Allow to stand for 4-6 h.

**Primary opalescent suspension (formazin suspension)**. In a 100 mL ground-glass-stoppered flask, dissolve 2.5 g of *hexamethylenetetramine R* in 25.0 mL of *water R*. Add 25.0 mL of the hydrazine sulfate solution. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be mixed thoroughly before use.

**Standard of opalescence**. Dilute 15.0 mL of the primary opalescent suspension to 1000.0 mL with *water R*. This suspension is freshly prepared and may be stored for up to 24 h.

**Reference suspensions**. Prepare the reference suspensions according to Table 2.2.1.-1. Mix and shake before use.

Table 2.2.1.-1

| | I | II | III | IV |
|---|---|---|---|---|
| Standard of opalescence | 5.0 mL | 10.0 mL | 30.0 mL | 50.0 mL |
| *Water R* | 95.0 mL | 90.0 mL | 70.0 mL | 50.0 mL |

Measurements of reference suspensions I-IV in ratio mode show a linear relationship between the concentrations and measured NTU values (see Table 2.2.1.-2).

Table 2.2.1.-2

| Formazin suspensions | Opalescent values (NTU) |
|---|---|
| Reference suspension I | 3 |
| Reference suspension II | 6 |
| Reference suspension III | 18 |
| Reference suspension IV | 30 |
| Standard of opalescence | 60 |
| Primary opalescent suspension | 4000 |

**01/2008:20202**

# 2.2.2. DEGREE OF COLORATION OF LIQUIDS

The examination of the degree of coloration of liquids in the range brown-yellow-red is carried out by one of the 2 methods below, as prescribed in the monograph.

A solution is *colourless* if it has the appearance of *water R* or the solvent or is not more intensely coloured than reference solution $B_9$.

METHOD I

Using identical tubes of colourless, transparent, neutral glass of 12 mm external diameter, compare 2.0 mL of the liquid to be examined with 2.0 mL of *water R* or of the solvent or of the reference solution (see Tables of reference solutions) prescribed in the monograph. Compare the colours in diffused daylight, viewing horizontally against a white background.

METHOD II

Using identical tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15 mm to 25 mm, compare the liquid to be examined with *water R* or the solvent or the reference solution (see Tables of reference solutions) prescribed in the monograph, the depth of the layer being 40 mm. Compare the colours in diffused daylight, viewing vertically against a white background.

REAGENTS

**Primary solutions**

*Yellow solution*. Dissolve 46 g of *ferric chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $FeCl_3,6H_2O$ per millilitre by adding the same acidic mixture. Protect the solution from light.

Titration. Place in a 250 mL conical flask fitted with a ground-glass stopper, 10.0 mL of the solution, 15 mL of *water R*, 5 mL of *hydrochloric acid R* and 4 g of *potassium iodide R*, close the flask, allow to stand in the dark for 15 min and add 100 mL of *water R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 27.03 mg of $FeCl_3,6H_2O$.

*Red solution*. Dissolve 60 g of *cobalt chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $CoCl_2,6H_2O$ per millilitre by adding the same acidic mixture.

Titration. Place in a 250 mL conical flask fitted with a ground-glass stopper, 5.0 mL of the solution, 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of a 300 g/L solution

of *sodium hydroxide R*. Boil gently for 10 min, allow to cool and add 60 mL of *dilute sulfuric acid R* and 2 g of *potassium iodide R*. Close the flask and dissolve the precipitate by shaking gently. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 23.79 mg of $CoCl_2,6H_2O$.

*Blue primary solution.* Dissolve 63 g of *copper sulfate pentahydrate R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $CuSO_4,5H_2O$ per millilitre by adding the same acidic mixture.

Titration. Place in a 250 mL conical flask fitted with a ground-glass stopper, 10.0 mL of the solution, 50 mL of *water R*, 12 mL of *dilute acetic acid R* and 3 g of *potassium iodide R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 24.97 mg of $CuSO_4,5H_2O$.

**Standard solutions**

Using the 3 primary solutions, prepare the 5 standard solutions as follows (Table 2.2.2.-1):

Table 2.2.2.-1

| Standard solution | Volume in millilitres | | | |
|---|---|---|---|---|
| | Yellow solution | Red solution | Blue solution | Hydrochloric acid (10 g/L HCl) |
| B (brown) | 3.0 | 3.0 | 2.4 | 1.6 |
| BY (brownish-yellow) | 2.4 | 1.0 | 0.4 | 6.2 |
| Y (yellow) | 2.4 | 0.6 | 0.0 | 7.0 |
| GY (greenish-yellow) | 9.6 | 0.2 | 0.2 | 0.0 |
| R (red) | 1.0 | 2.0 | 0.0 | 7.0 |

**Reference solutions for Methods I and II**

Using the 5 standard solutions, prepare the following reference solutions.

Table 2.2.2.-2. - *Reference solutions B*

| Reference solution | Volumes in millilitres | |
|---|---|---|
| | Standard solution B | Hydrochloric acid (10 g/L HCl) |
| $B_1$ | 75.0 | 25.0 |
| $B_2$ | 50.0 | 50.0 |
| $B_3$ | 37.5 | 62.5 |
| $B_4$ | 25.0 | 75.0 |
| $B_5$ | 12.5 | 87.5 |
| $B_6$ | 5.0 | 95.0 |
| $B_7$ | 2.5 | 97.5 |
| $B_8$ | 1.5 | 98.5 |
| $B_9$ | 1.0 | 99.0 |

Table 2.2.2.-3. - *Reference solutions BY*

| Reference solution | Volumes in millilitres | |
|---|---|---|
| | Standard solution BY | Hydrochloric acid (10 g/L HCl) |
| $BY_1$ | 100.0 | 0.0 |
| $BY_2$ | 75.0 | 25.0 |
| $BY_3$ | 50.0 | 50.0 |
| $BY_4$ | 25.0 | 75.0 |
| $BY_5$ | 12.5 | 87.5 |
| $BY_6$ | 5.0 | 95.0 |
| $BY_7$ | 2.5 | 97.5 |

Table 2.2.2.-4. - *Reference solutions Y*

| Reference solution | Volumes in millilitres | |
|---|---|---|
| | Standard solution Y | Hydrochloric acid (10 g/L HCl) |
| $Y_1$ | 100.0 | 0.0 |
| $Y_2$ | 75.0 | 25.0 |
| $Y_3$ | 50.0 | 50.0 |
| $Y_4$ | 25.0 | 75.0 |
| $Y_5$ | 12.5 | 87.5 |
| $Y_6$ | 5.0 | 95.0 |
| $Y_7$ | 2.5 | 97.5 |

Table 2.2.2.-5. - *Reference solutions GY*

| Reference solution | Volumes in millilitres | |
|---|---|---|
| | Standard solution GY | Hydrochloric acid (10 g/L HCl) |
| $GY_1$ | 25.0 | 75.0 |
| $GY_2$ | 15.0 | 85.0 |
| $GY_3$ | 8.5 | 91.5 |
| $GY_4$ | 5.0 | 95.0 |
| $GY_5$ | 3.0 | 97.0 |
| $GY_6$ | 1.5 | 98.5 |
| $GY_7$ | 0.75 | 99.25 |

Table 2.2.2.-6. - *Reference solutions R*

| Reference solution | Volumes in millilitres | |
|---|---|---|
| | Standard solution R | Hydrochloric acid (10 g/L HCl) |
| $R_1$ | 100.0 | 0.0 |
| $R_2$ | 75.0 | 25.0 |
| $R_3$ | 50.0 | 50.0 |
| $R_4$ | 37.5 | 62.5 |
| $R_5$ | 25.0 | 75.0 |
| $R_6$ | 12.5 | 87.5 |
| $R_7$ | 5.0 | 95.0 |

**Storage**

For Method I, the reference solutions may be stored in sealed tubes of colourless, transparent, neutral glass of 12 mm external diameter, protected from light.

For Method II, prepare the reference solutions immediately before use from the standard solutions.

07/2016:20203

# 2.2.3. POTENTIOMETRIC DETERMINATION OF pH

The pH of an aqueous solution is defined as the negative logarithm of the activity of its hydrogen ions, expressed conventionally as the hydrogen ion concentration of the solution. For practical purposes, its definition is an experimental one. The pH of a solution to be examined is related to that of a reference solution ($pH_s$) by the following equation:

$$pH = pH_s - \frac{E - E_s}{k}$$

in which $E$ is the potential, expressed in volts, of the cell containing the solution to be examined and $E_s$ is the potential, expressed in volts, of the cell containing the solution of known pH ($pH_s$), $k$ is the change in potential per unit change in pH, expressed in volts and calculated from the Nernst equation.

Table 2.2.3.-1. – *Values of k at different temperatures*

| Temperature (°C) | *k* (V) |
|---|---|
| 15 | 0.0572 |
| 20 | 0.0582 |
| 25 | 0.0592 |
| 30 | 0.0601 |
| 35 | 0.0611 |

The potentiometric determination of pH is made by measuring the potential difference between 2 appropriate electrodes immersed in the solution to be examined; 1 of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (e.g. a silver-silver chloride electrode). They are often combined as 1 compact electrode, together with a temperature probe.

*Apparatus.* The measuring apparatus is usually a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has a sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Recent pH meters are microprocessor-controlled and are operated using the manufacturer's firmware or software, according to given instructions.

*Management of electrodes.* The electrodes are stored appropriately and according to the manufacturer's recommendations (e.g. in an electrolyte solution or a suitable storage solution). Before measurement, the electrodes are visually checked. Refillable electrodes are checked for the absence of air bubbles in the glass bulb and to ensure that the inner electrolyte solution level is satisfactory. The filling orifice has to remain open during the measurement. It is also recommended that the diaphragm of the reference electrode is checked. Before first use, or if the electrode has been stored out of electrolyte solution, it is usually necessary to condition it, according to the recommendations of the manufacturer. If pH stabilisation is too slow (i.e. a long response time), or a zero point shift, reduced slope or any other difficulties in calibration are observed, the electrode will probably need to be cleaned or replaced. The cleaning is performed depending on the type of sample and as prescribed in the manufacturer's manual. Regular cleaning is recommended.

*Calibration and measurement conditions.* Unless otherwise prescribed in the monograph, all measurements are carried out at the same temperature as that used for calibration (± 2.5 °C), usually between 20 °C and 25 °C. Table 2.2.3.-2 shows the variation of pH with respect to temperature of a number of reference buffer solutions used for calibration. Follow the manufacturer's instructions for temperature correction.

The calibration consists of the determination of the slope (e.g. 95-105 per cent) and the offset of the measuring system. Most commercial pH meters offer a "self test" or "start-up test" where, for example, the slope and asymmetry potential are tested and compared to the manufacturer's specifications. The apparatus is calibrated using at least 2 buffer solutions chosen so that the expected pH value of the solution to be examined lies between the pH values of the buffer solutions. The range must be at least 2 pH units. The pH of another buffer solution of intermediate pH, read from the scale, must not differ by more than 0.05 pH units from the value corresponding to that solution.

Reference buffer solutions are preferably commercially available certified reference materials. Alternatively, buffer solutions can be prepared in-house according to Table 2.2.3.-2. These solutions must be traceable to primary standards. Calibration has to be performed on a regular basis, preferably each day of use or before each series of measurements.

Immerse the electrodes in the solution to be examined and take the reading in the same conditions as those applied for the reference buffer solutions.

If suspensions, emulsions or solutions of non-aqueous or partially non-aqueous character are measured on a system calibrated as described above, the pH reading can only be considered to be an approximation of the true value. Suitable electrodes have to be used for pH measurements of such mixtures.

Table 2.2.3.-2. – *pH of reference buffer solutions at various temperatures*

| Temperature (°C) | Potassium tetraoxalate 0.05 M | Potassium hydrogen tartrate saturated at 25 °C | Potassium dihydrogen citrate 0.05 M | Potassium hydrogen phthalate 0.05 M | Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M | Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M | Disodium tetraborate 0.01 M | Sodium carbonate 0.025 M + Sodium bicarbonate 0.025 M | Calcium hydroxide, saturated at 25°C |
|---|---|---|---|---|---|---|---|---|---|
| | $C_4H_3KO_8,2H_2O$ | $C_4H_5KO_6$ | $C_6H_7KO_7$ | $C_8H_5KO_4$ | $KH_2PO_4$+ $Na_2HPO_4$ | $KH_2PO_4$+ $Na_2HPO_4$ | $Na_2B_4O_7$, $10H_2O$ | $Na_2CO_3$+ $NaHCO_3$ | $Ca(OH)_2$ |
| 15 | 1.67 | | 3.80 | 4.00 | 6.90 | 7.45 | 9.28 | 10.12 | 12.81 |
| 20 | 1.68 | | 3.79 | 4.00 | 6.88 | 7.43 | 9.23 | 10.06 | 12.63 |
| 25 | 1.68 | 3.56 | 3.78 | 4.01 | 6.87 | 7.41 | 9.18 | 10.01 | 12.45 |
| 30 | 1.68 | 3.55 | 3.77 | 4.02 | 6.85 | 7.40 | 9.14 | 9.97 | 12.29 |

| Temperature (°C) | Potassium tetraoxalate 0.05 M | Potassium hydrogen tartrate saturated at 25 °C | Potassium dihydrogen citrate 0.05 M | Potassium hydrogen phthalate 0.05 M | Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M | Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M | Disodium tetraborate 0.01 M | Sodium carbonate 0.025 M + Sodium bicarbonate 0.025 M | Calcium hydroxide, saturated at 25°C |
|---|---|---|---|---|---|---|---|---|---|
| | $C_4H_3KO_8,2H_2O$ | $C_4H_5KO_6$ | $C_6H_7KO_7$ | $C_8H_5KO_4$ | $KH_2PO_4$+ $Na_2HPO_4$ | $KH_2PO_4$+ $Na_2HPO_4$ | $Na_2B_4O_7$, $10H_2O$ | $Na_2CO_3$+ $NaHCO_3$ | $Ca(OH)_2$ |
| 35 | 1.69 | 3.55 | 3.76 | 4.02 | 6.84 | 7.39 | 9.10 | 9.93 | 12.13 |
| $\frac{\Delta pH^{(1)}}{\Delta t}$ | + 0.001 | − 0.0014 | − 0.0022 | + 0.0012 | − 0.0028 | − 0.0028 | − 0.0082 | − 0.0096 | − 0.034 |

(1) pH variation per degree Celsius.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

**Potassium tetraoxalate 0.05 M**. Dissolve 12.61 g of $C_4H_3KO_8,2H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

**Potassium hydrogen tartrate, saturated at 25 °C**. Shake an excess of $C_4H_5KO_6$ vigorously with *carbon dioxide-free water R* at 25 °C. Filter or decant. Prepare immediately before use.

**Potassium dihydrogen citrate 0.05 M**. Dissolve 11.41 g of $C_6H_7KO_7$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Prepare immediately before use.

**Potassium hydrogen phthalate 0.05 M**. Dissolve 10.13 g of $C_8H_5KO_4$, previously dried for 1 h at 110 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

**Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M**. Dissolve 3.39 g of $KH_2PO_4$ and 3.53 g of $Na_2HPO_4$, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

**Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M**. Dissolve 1.18 g of $KH_2PO_4$ and 4.30 g of $Na_2HPO_4$, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

**Disodium tetraborate 0.01 M**. Dissolve 3.80 g of $Na_2B_4O_7,10H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

**Sodium carbonate 0.025 M + Sodium hydrogen carbonate 0.025 M**. Dissolve 2.64 g of $Na_2CO_3$ and 2.09 g of $NaHCO_3$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

**Calcium hydroxide, saturated at 25 °C**. Shake an excess of *calcium hydroxide R* with *carbon dioxide-free water R* and decant at 25 °C. Store protected from atmospheric carbon dioxide.

STORAGE OF BUFFER SOLUTIONS

Store buffer solutions in suitable chemically-resistant, airtight containers, such as type I glass bottles or plastic containers suitable for aqueous solutions.

**01/2016:20204**

## 2.2.4. APPROXIMATE pH OF SOLUTIONS

Determine the approximate pH using a *pH indicator strip R*. Alternatively, pH indicators such as those described in Table 2.2.4.-1 can be used.

Table 2.2.4.-1

| Reaction | pH | Indicator |
|---|---|---|
| **Alkaline** | > 8 | *Red litmus paper R* |
| Slightly alkaline | 8 – 10 | *Phenolphthalein solution R* |
| | | *Thymol blue solution R* |
| Strongly alkaline | > 10 | *Phenolphthalein paper R* |
| | | *Thymol blue solution R* |
| **Neutral** | 6 – 8 | *Methyl red solution R* |
| | | *Phenol red solution R* |
| **Acid** | < 6 | *Methyl red solution R* |
| | | *Bromothymol blue solution R1* |
| Slightly acid | 4 – 6 | *Methyl red solution R* |
| | | *Bromocresol green solution R* |
| Strongly acid | < 4 | *Congo red paper R* |

**01/2008:20205 corrected 10.0**

## 2.2.5. RELATIVE DENSITY

The relative density $d_{t_2}^{t_1}$ of a substance is the ratio of the mass of a certain volume of a substance at temperature $t_1$ to the mass of an equal volume of water at temperature $t_2$.

Unless otherwise indicated, the relative density $d_{20}^{20}$ is used.

Relative density is also commonly expressed as $d_4^{20}$. Density $\rho_{20}$, defined as the mass of a unit volume of the substance at 20 °C

may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre ($1\ kg{\cdot}m^{-3} = 10^{-3}\ g{\cdot}cm^{-3}$). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0.998203 \times d_{20}^{20} \text{ or } d_{20}^{20} = 1.00180 \times \rho_{20}$$

$$\rho_{20} = 0.999972 \times d_{4}^{20} \text{ or } d_{4}^{20} = 1.00003 \times \rho_{20}$$

$$d_{4}^{20} = 0.998230 \times d_{20}^{20}$$

Relative density or density is measured according to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids) or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the 3rd decimal place. When using a density meter, the buoyancy of air has no influence.

*Oscillating transducer density meter.* The apparatus consists of:

- a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;
- a means of measuring the oscillation period ($T$), which may be converted by the apparatus to give a direct reading of density, or used to calculate density using the constants $A$ and $B$ described below.

The resonant frequency ($f$) is a function of the spring constant ($c$) and the mass ($m$) of the system:

$$f^2 = \frac{1}{T^2} = \frac{c}{m} \times \frac{1}{4\pi^2}$$

Hence:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \times V}{c}\right) \times 4\pi^2$$

$M$ = mass of the tube;

$V$ = inner volume of the tube.

Introduction of 2 constants $A = c / (4\pi^2 \times V)$ and $B = M / V$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The constants $A$ and $B$ are determined by operating the instrument with the U-tube filled with 2 different samples of known density, for example, degassed *water R* and air. Control measurements are made daily using degassed *water R*. The results displayed for the control measurement using degassed *water R* shall not deviate from the reference value ($\rho_{20} = 0.998203\ g{\cdot}cm^{-3}$, $d_{20}^{20} = 1.000000$) by more than its specified error. For example, an instrument specified to $\pm 0.0001\ g{\cdot}cm^{-3}$ shall display $0.9982 \pm 0.0001\ g{\cdot}cm^{-3}$ in order to be suitable for further measurement. Otherwise a re-adjustment is necessary. Calibration with certified reference materials is carried out regularly. Measurements are made using the same procedure as for calibration. The liquid to be examined is equilibrated in a thermostat at 20 °C before introduction into the tube, if necessary, to avoid the formation of bubbles and to reduce the time required for measurement.

Factors affecting accuracy include:

- temperature uniformity throughout the tube;
- non-linearity over a range of density;
- parasitic resonant effects;
- viscosity, whereby solutions with a higher viscosity than the calibrant have a density that is apparently higher than the true value.

The effects of non-linearity and viscosity may be avoided by using calibrants that have density and viscosity close to those of the liquid to be examined (± 5 per cent for density, ± 50 per cent for viscosity). The density meter may have functions for automatic viscosity correction and for correction of errors arising from temperature changes and non-linearity.

Precision is a function of the repeatability and stability of the oscillator frequency, which is dependent on the stability of the volume, mass and spring constant of the cell.

Density meters are able to achieve measurements with an error of the order of $1 \times 10^{-3}\ g{\cdot}cm^{-3}$ to $1 \times 10^{-5}\ g{\cdot}cm^{-3}$ and a repeatability of $1 \times 10^{-4}\ g{\cdot}cm^{-3}$ to $1 \times 10^{-6}\ g{\cdot}cm^{-3}$.

**01/2008:20206**

## 2.2.6. REFRACTIVE INDEX

The refractive index of a medium with reference to air is equal to the ratio of the sine of the angle of incidence of a beam of light in air to the sine of the angle of refraction of the refracted beam in the given medium.

Unless otherwise prescribed, the refractive index is measured at 20 ± 0.5 °C, with reference to the wavelength of the D-line of sodium (λ = 589.3 nm); the symbol is then $n_D^{20}$.

Refractometers normally determine the critical angle. In such apparatus the essential part is a prism of known refractive index in contact with the liquid to be examined.

Calibrate the apparatus using certified reference materials.

When white light is used, the refractometer is provided with a compensating system. The apparatus gives readings accurate to at least the third decimal place and is provided with a means of operation at the temperature prescribed. The thermometer is graduated at intervals of 0.5 °C or less.

**07/2018:20207**

## 2.2.7. OPTICAL ROTATION

PRINCIPLE

Optical rotation (also known as optical activity) is the property displayed by chiral substances of rotating the plane of polarisation of linearly polarised light.

Optical rotation is considered to be positive (+) for dextrorotatory substances (i.e. those that rotate the plane of polarisation in a clockwise direction when viewed in the direction facing the oncoming light beam) and negative (−) for laevorotatory substances (i.e. anticlockwise rotation).

The angle of optical rotation $\alpha$ of a liquid is the angle of rotation of the plane of polarisation, expressed in degrees (°), at the wavelength of the D-line of sodium (λ = 589.3 nm) measured at 20 °C through the liquid when using a path length of 1.00 dm.

The specific optical rotation $[\alpha]_D^{20}$ of a substance in solution is calculated from the angle of optical rotation, as defined above, with reference to a path length of 1.00 dm and a concentration of the substance to be examined of 1 g/mL. The specific optical rotation of a substance in solution is always expressed with reference to a given solvent and concentration.

As some equipment may not use sodium lamps, the wavelength of measurement is given as 589 nm instead of 589.3 nm.

In certain cases specified in the monograph, the angle of optical rotation is measured at other temperatures, other wavelengths and/or in cells with a path length other than 1.00 dm.

In the conventional system adopted by the Pharmacopoeia, the specific optical rotation is expressed by its value without units; the actual units, degree millilitres per decimetre gram [(°)·ml·dm$^{-1}$·g$^{-1}$] are understood.

EQUIPMENT

The polarimeter typically consists of:

- a light source, for example a sodium discharge lamp, a light-emitting diode (LED) or another light source capable of providing radiation at the desired wavelength (589 nm unless otherwise prescribed in the monograph); if the light source is polychromatic, a means of isolating the required wavelength is necessary, e.g. an optical filter;
- a polariser and an analyser;
- a sample cell with a path length of 1.00 dm, unless otherwise specified in the monograph;
- a detection system to measure the angle of optical rotation, which must be capable of giving readings to at least the nearest 0.01°, unless otherwise specified in the monograph;
- a temperature control system that indicates the temperature with a readability of 0.1 °C; it may be embedded in the polarimeter (e.g. a Peltier system) or be an external unit (e.g. a cycle-cryostat), and must be able to maintain the temperature of the liquid to within ± 0.5 °C of that prescribed.

EQUIPMENT PERFORMANCE

The accuracy of the scale is checked near the value to be measured or over an appropriate range, usually by means of certified quartz plates. Other certified reference materials may also be suitable (e.g. sucrose solutions).

Optical rotation measurements may be used to quantify the amount of an enantiomer or the ratio of enantiomers present in a sample. For that purpose, the linearity must be checked, for example using sucrose solutions.

PROCEDURE

Determine the zero of the polarimeter and the angle of rotation of the liquid at a wavelength of 589 nm and a temperature of 20 ± 0.5 °C, unless otherwise prescribed. The zero of the polarimeter is determined with the sample cell closed.

For neat liquids, the zero is determined with an empty sample cell.

For solutions, the zero is determined with the sample cell filled with the same solvent as that used for the solution to be examined and measured at the same temperature. The sample preparation procedure is prescribed in the monograph.

Calculate the specific optical rotation at temperature $t$ and wavelength $\lambda$ using the following formulae.

For neat liquids, the density of the liquid is taken into account:

$$[\alpha]_{\lambda}^{t} = \frac{\alpha}{l \cdot \rho_t}$$

For solutions:

$$[\alpha]_{\lambda}^{t} = \frac{1000\alpha}{l \cdot c}$$

$\alpha$ = angle of rotation measured at temperature $t$ and wavelength $\lambda$, in degrees;

$l$ = path length of the polarimeter sample cell, in decimetres;

$\rho_t$ = density determined at the temperature of measurement $t$, in grams per cubic centimetre; for the purposes of the Pharmacopoeia, density is replaced by relative density (*2.2.5*);

$c$ = concentration of the solution, in grams per litre.

When the limits for optical rotation or specific optical rotation are expressed as the dried substance, the anhydrous substance or the solvent-free substance, the result must be corrected for loss on drying (*2.2.32*), water content (*2.5.12* or *2.5.32*) or content of solvent as appropriate.

**04/2018:20208**

## 2.2.8. VISCOSITY

The *dynamic* viscosity or *viscosity coefficient* $\eta$ is the tangential force per unit surface, known as *shearing stress* $\tau$ and expressed in pascals, necessary to move, parallel to the sliding plane, a layer of liquid of 1 square metre at a rate ($v$) of 1 metre per second relative to a parallel layer at a distance ($x$) of 1 metre.

The ratio d$v$/d$x$ is a speed gradient giving the *rate of shear D* expressed in reciprocal seconds (s$^{-1}$), so that $\eta = \tau/D$.

The unit of dynamic viscosity is the pascal second (Pa·s). The most commonly used submultiple is the millipascal second (mPa·s).

The *kinematic viscosity* $\nu$, expressed in square metres per second, is obtained by dividing the dynamic viscosity $\eta$ by the density $\rho$ expressed in kilograms per cubic metre, of the liquid measured at the same temperature, i.e. $\nu = \eta/\rho$. The kinematic viscosity is usually expressed in square millimetres per second.

A capillary viscometer may be used for determining the viscosity of Newtonian liquids and a rotating viscometer for determining the viscosity of Newtonian and non-Newtonian liquids. Other viscometers may be used provided that the accuracy and precision are at least as satisfactory as those obtained with the viscometers described in the related chapters.

**01/2019:20209**

## 2.2.9. CAPILLARY VISCOMETER METHOD

PRINCIPLE

The determination of viscosity is carried out using a suspended-level (Ubbelohde-type) capillary viscometer of appropriate size at a temperature of 20.0 ± 0.1 °C, unless otherwise prescribed. The time required for the level of the liquid to drop from one mark to the other is measured.

EQUIPMENT

The principal components of an Ubbelohde-type capillary viscometer(1). are shown in Figure 2.2.9.-1.

(1) The European Pharmacopoeia describes the system proposed by the International Organization for Standardization (ISO)

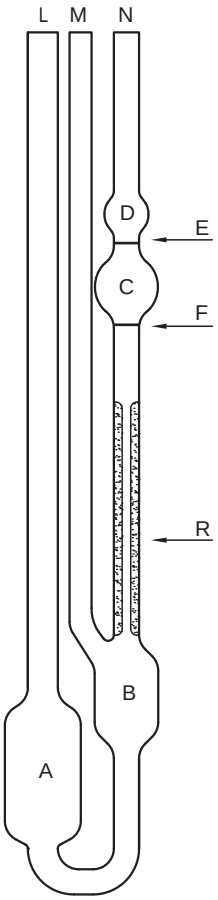


Figure 2.2.9.-1. – *Suspended-level (Ubbelohde-type) capillary viscometer*

PROCEDURE

Select a capillary viscometer of appropriate size to obtain a minimum flow time of 200 s.

**Calibration**

Capillary viscometers are calibrated at regular intervals as defined in the quality management system and dictated by the frequency of use of the equipment and the application.

Calibrate the instrument at the temperature used for the measurement by using at least 2 certified reference materials matching the viscosity range of the viscometer.

Calculate the viscometer constant ($k$) in square millimetres per second squared, using the following expression:

$$k = \frac{\eta}{\rho t}$$

$\eta$ = dynamic viscosity of the certified reference material, in millipascal seconds;

$\rho$ = density of the certified reference material, in milligrams per cubic millimetre;

$t$ = flow time for the certified reference material to drop from the upper mark to the lower mark, in seconds.

Calculate the mean of the values obtained.

**Method**

Charge the viscometer (Figure 2.2.9.-1) through tube *L* with a sufficient quantity of the liquid to be examined (previously brought to 20 °C unless otherwise prescribed) to fill bulb *A* while ensuring that the level of liquid in bulb *B* is below the exit to ventilation tube *M*. Immerse the viscometer in the upright position in a water-bath at 20.0 ± 0.1 °C (unless otherwise prescribed) and allow to stand for not less than 30 min to allow the temperature to reach equilibrium. Close tube *M* and draw the level of the liquid in tube *N* up to a level about 8 mm above mark *E*. Keep the liquid at this level by closing tube *N* and opening tube *M*. Open tube *N* and, using a stopwatch, measure the time required, to at least the nearest 1/5 of a second, for the level of the liquid to drop from mark *E* to mark *F*.

The flow time of the liquid to be examined is the mean of 3 consecutive measurements. The result is valid if the relative standard deviation of the 3 measurements is not more than 2.0 per cent.

**Calculation**

Calculate the kinematic viscosity ($\nu$) (*2.2.8*), in square millimetres per second, using the following expression:

$$\nu = kt$$

$k$ = viscometer constant, in square millimetres per second squared;

$t$ = flow time of the liquid to be examined, in seconds.

Calculate the dynamic viscosity ($\eta$) (*2.2.8*), in millipascal seconds, using the following expression:

$$\eta = k\rho t$$

$\rho$ = density of the liquid to be examined at the temperature used for the viscosity measurement, in milligrams per cubic millimetre.

The density may be obtained by multiplying the relative density of the liquid to be examined by 0.99820 (measurement at 20 °C) or 0.99704 (measurement at 25 °C).

**01/2008:20210**

## 2.2.10. VISCOSITY - ROTATING VISCOMETER METHOD

The principle of the method is to measure the force acting on a rotor (torque) when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotating viscometers are used for measuring the viscosity of Newtonian (shear-independent viscosity) or non-Newtonian liquids (shear dependent viscosity or apparent viscosity). Rotating viscometers can be divided in 2 groups, namely absolute and relative viscometers. In absolute viscometers the flow in the measuring geometry is well defined. The measurements result in absolute viscosity values, which can be compared with any other absolute values. In relative viscometers the flow in the measuring geometry is not defined. The measurements result in relative viscosity values, which cannot be compared with absolute values or other relative values if not determined by the same relative viscometer method.

Different measuring systems are available for given viscosity ranges as well as several rotational speeds.

APPARATUS

The following types of instruments are most common.

*CONCENTRIC CYLINDER VISCOMETERS (ABSOLUTE VISCOMETERS)*

In the concentric cylinder viscometer (coaxial double cylinder viscometer or simply coaxial cylinder viscometer), the viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Viscosity measurement can be performed by rotating the inner cylinder (Searle type viscometer) or the outer cylinder (Couette type viscometer), as shown in Figures 2.2.10.-1 and 2.2.10.-2, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds is given by the following formula:

$$\eta = \frac{1}{\omega}\left(\frac{M}{4\pi h}\right)\left(\frac{1}{R_i^2} - \frac{1}{R_o^2}\right) = k\frac{M}{\omega}$$

$M$ = torque in newton-metres acting on the cylinder surface,
$\omega$ = angular velocity in radians per second,
$h$ = height of immersion in metres of the inner cylinder in the liquid medium,
$R_i$ = radius in metres of the inner cylinder,
$R_o$ = radius in metres of the outer cylinder,
$k$ = constant of the apparatus, expressed in radians per cubic metre.

For non-Newtonian liquids it is indispensable to specify the shear stress ($\tau$) or the shear rate ($\gamma$) at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute viscometers), there is a proportional relationship between $M$ and $\tau$ and also between $\omega$ and $\gamma$:

$$\tau = AM \qquad \gamma = B\omega$$

where A and B are constants for the instrument and are calculated from the following expressions:

– *for concentric surface*:

$$A = \frac{1}{4\pi h}\frac{R_i^2 + R_o^2}{R_i^2 R_o^2} \qquad B = \frac{R_i^2 + R_o^2}{R_o^2 - R_i^2}$$

– *for cone-plates*:

$$A = \frac{3}{2\pi R^3} \qquad B = \frac{1}{\alpha}$$

$M$ = torque in Newton-metres acting on the cone or cylinder surface,
$\omega$ = angular velocity in radians per second,
$R_i$ = radius in metres of the inner cylinder,
$R_o$ = radius in metres of the outer cylinder,
$R$ = radius in metres of the cone,
$h$ = height of immersion in metres of the inner cylinder in the liquid medium,
$\alpha$ = angle in radians between the flat disk and the cone,
$\tau$ = shear stress in pascals (Pa),
$\gamma$ = shear rate in reciprocal seconds ($s^{-1}$).

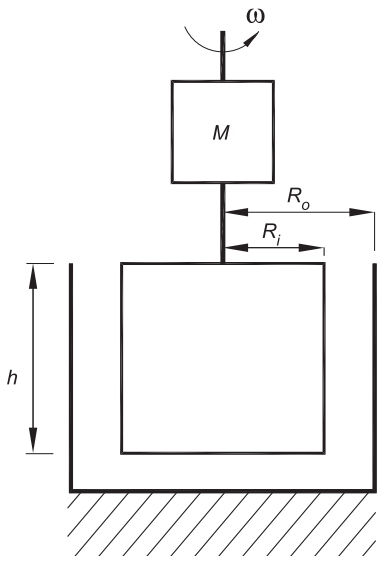


Figure 2.2.10.-1

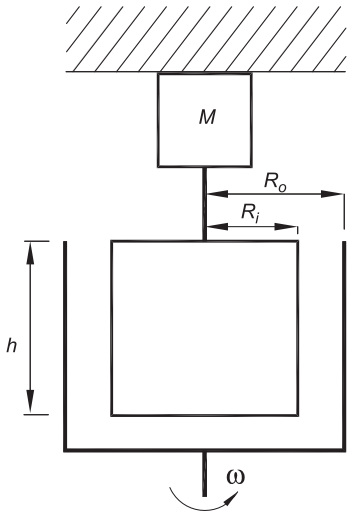


Figure 2.2.10.-2

*CONE-PLATE VISCOMETERS (ABSOLUTE VISCOMETERS)*

In the cone-plate viscometer, the liquid is introduced into the gap between a flat disc and a cone forming a define angle. Viscosity measurement can be performed by rotating the cone or the flat disc, as shown in Figures 2.2.10.-3 and 2.2.10.-4, respectively. For laminar flow, the viscosity (or apparent viscosity) $\eta$ expressed in pascal-seconds is given by the following formula:

$$\eta = \left(\frac{M}{\omega}\right)\left(\frac{3\alpha}{2\pi R^3}\right) = k\frac{M}{\omega}$$

$M$ = torque in Newton-metres acting on the flat disc or cone surface,
$\omega$ = angular velocity in radians per second,
$\alpha$ = angle in radians between the flat disc and the cone,
$R$ = radius in metres of the cone,
$k$ = constant of the apparatus, expressed in radians per cubic metre.

Constants $A$, $B$ of the apparatus (see under concentric cylinder viscometers).

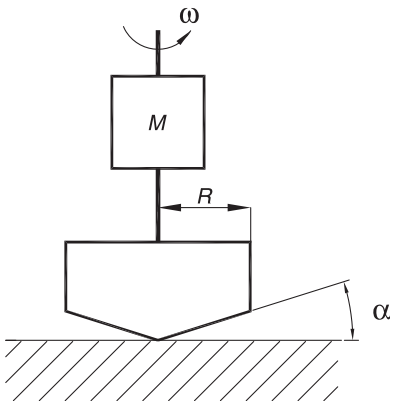


Figure 2.2.10.-3

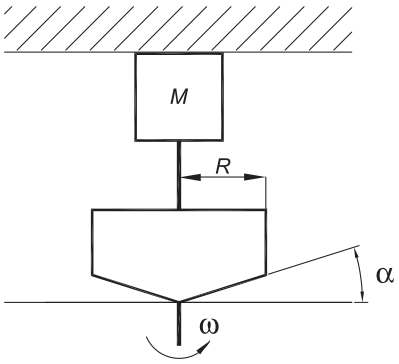


Figure 2.2.10.-4

*SPINDLE VISCOMETERS (RELATIVE VISCOMETERS)*

In the spindle viscometer, the viscosity is determined by rotating a spindle (for example, cylinder- or disc-shaped, as shown in Figures 2.2.10.-5 and 2.2.10.-6, respectively) immersed in the liquid. Relative values of viscosity (or apparent viscosity) can be directly calculated using conversion factors from the scale reading at a given rotational speed.

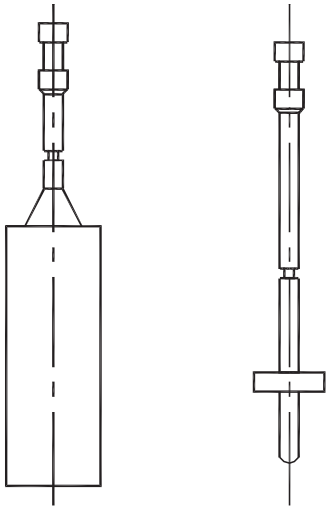

Figure 2.2.10.-5

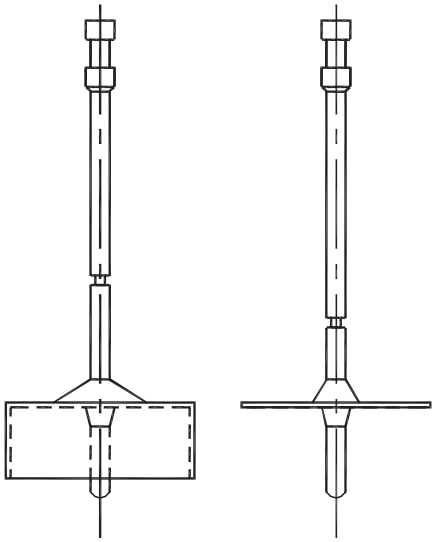

Figure 2.2.10.-6

In a general way, the constant $k$ of the apparatus may be determined at various speeds of rotation using a certified viscometer calibration liquid. The viscosity η then corresponds to the formula:

$$\eta = k\frac{M}{\omega}$$

METHOD

Measure the viscosity (or apparent viscosity) according to the instructions for the operation of the rotating viscometer. The temperature for measuring the viscosity is indicated in the monograph. For non-Newtonian systems, the monograph indicates the type of viscometer to be used and if absolute viscometers are used the angular velocity or the shear rate at which the measurement is made. If it is impossible to obtain the indicated shear rate exactly, use a shear rate slightly higher and a shear rate slightly lower and interpolate.

With relative viscometers the shear rate is not the same throughout the sample and therefore it cannot be defined. Under these conditions, the viscosity of non-Newtonian liquids determined from the previous formula has a relative character, which depends on the type of spindle and the angular velocity as well as the dimensions of the sample container (Ø = minimum 80 mm) and the depth of immersion of the spindle. The values obtained are comparable only if the method is carried out under experimental conditions that are rigorously the same.

**07/2015:20211**

## 2.2.11. DISTILLATION RANGE

The distillation range is the temperature interval, corrected for a pressure of 101.3 kPa, within which a liquid or a specified fraction of a liquid, distils under the following conditions.

*Apparatus*. The apparatus (see Figure 2.2.11.-1) consists of a distillation flask (*A*), a straight tube condenser (*B*) which fits on to the side arm of the flask and a plain-bend adaptor (*C*) attached to the end of the condenser. The lower end of the condenser may, alternatively, be bent to replace the adaptor. A thermometer is inserted in the neck of the flask so that the upper end of the bulb is 5 mm lower than the junction of the lower wall of the lateral tube. The thermometer can be read to the nearest 0.2 °C and covers a range of at least 50 °C. During the determination, the flask, including its neck, is protected from draughts by a suitable screen.

*Method*. Place in the flask (*A*) 50.0 mL of the liquid to be examined and a few pieces of porous material. Collect the distillate in a 50 mL cylinder graduated in millilitres. Cooling by circulating water is essential for liquids distilling below 150 °C. Heat the flask so that boiling is rapidly achieved and note the temperature at which the first drop of distillate falls into the cylinder. Adjust the heating to give a regular rate of distillation of 2-3 mL/min and note the temperature when the whole or the prescribed fraction of the liquid, measured at 20 °C, has distilled.

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

$t_1$ = the corrected temperature,

$t_2$ = the observed temperature, at the barometric pressure $b$,

$k$ = the correction factor taken from Table 2.2.11.-1 unless the factor is given,

$b$ = the barometric pressure, expressed in kilopascals, during the distillation.

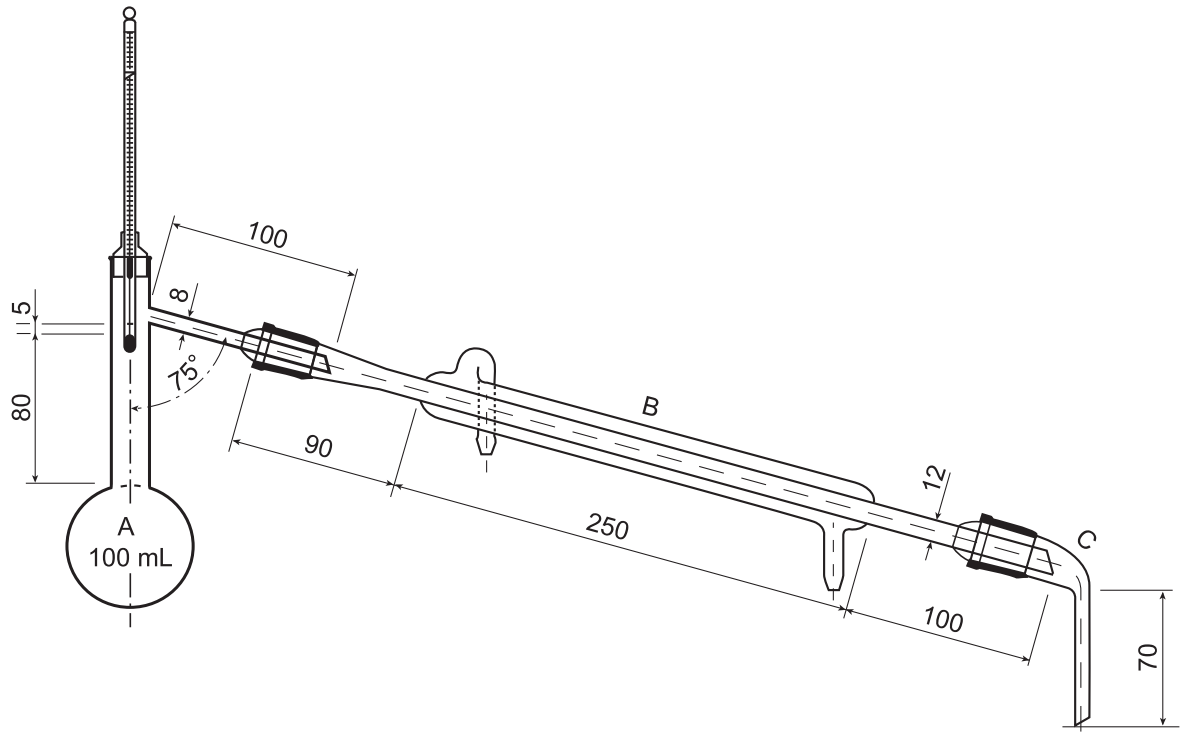


Figure 2.2.11.-1. – *Apparatus for the determination of distillation range*
*Dimensions in millimetres*

Table 2.2.11.-1. – *Temperature correction in relation to the pressure*

| Distillation temperature | Correction factor *k* |
|---|---|
| up to 100 °C | 0.30 |
| above 100 °C up to 140 °C | 0.34 |
| above 140 °C up to 190 °C | 0.38 |
| above 190 °C up to 240 °C | 0.41 |
| above 240 °C | 0.45 |

**07/2015:20212**

## 2.2.12. BOILING POINT

The boiling point is the corrected temperature at which the vapour pressure of a liquid is equal to 101.3 kPa.

*Apparatus.* The apparatus is that used for distillation range (*2.2.11*) with the exception that the thermometer is inserted in the neck of the flask so that the lower end of the bulb is level with the lower end of the neck of the distillation flask and that the flask is placed on a plate of isolating material pierced by a hole 35 mm in diameter.

*Method.* Place in the flask (*A*) 20 mL of the liquid to be examined and a few pieces of porous material. Heat the flask so that boiling is rapidly achieved and record the temperature at which liquid runs from the side-arm into the condenser.

Correct the observed temperature for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

$t_1$ = the corrected temperature,

$t_2$ = the observed temperature at barometric pressure $b$,

$k$ = the correction factor as shown in Table 2.2.11.-1 under Distillation Range,

$b$ = the barometric pressure, in kilopascals, at the time of the determination.

**01/2008:20213**

## 2.2.13. DETERMINATION OF WATER BY DISTILLATION

The apparatus (see Figure 2.2.13.-1) consists of a glass flask (*A*) connected by a tube (*D*) to a cylindrical tube (*B*) fitted with a graduated receiving tube (*E*) and reflux condenser (*C*). The receiving tube (*E*) is graduated in 0.1 mL. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

*Method.* Clean the receiving tube and the condenser of the apparatus, thoroughly rinse with water, and dry.

Introduce 200 mL of *toluene R* and about 2 mL of *water R* into the dry flask. Distil for 2 h, then allow to cool for about 30 min and read the water volume to the nearest 0.05 mL. Place in the flask a quantity of the substance, weighed with an accuracy of 1 per cent, expected to give about 2 mL to 3 mL of water. If the substance has a pasty consistency, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil, distil at the rate of about two drops per second until most of the water has distilled over, then increase the rate of distillation to about four drops per second. When the water has all distilled over, rinse the inside of the condenser tube with *toluene R*. Continue the distillation for 5 min, remove the heat, allow the receiving tube to cool to room temperature and dislodge any droplets of water which adhere to the walls of the receiving

tube. When the water and toluene have completely separated, read the volume of water and calculate the content present in the substance as millilitres per kilogram, using the formula:

$$\frac{1000(n_2 - n_1)}{m}$$

$m$ = the mass in grams of the substance to be examined,

$n_1$ = the number of millilitres of water obtained in the first distillation,

$n_2$ = the total number of millilitres of water obtained in the 2 distillations.

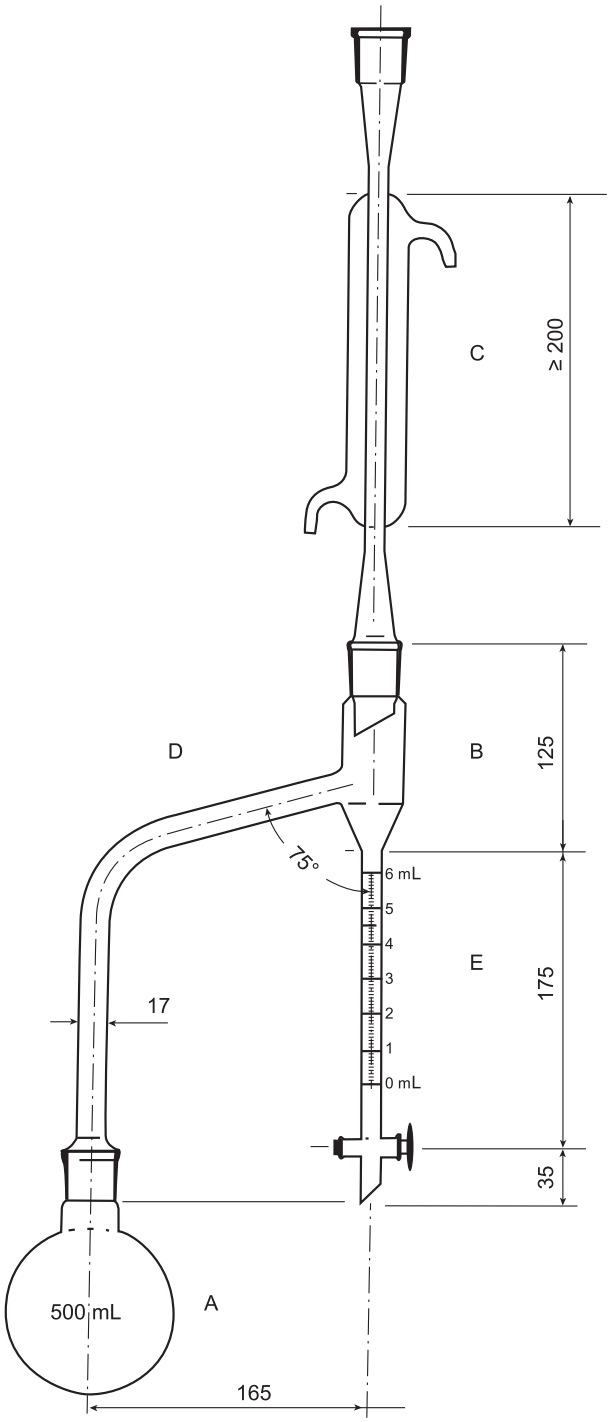


Figure 2.2.13.-1. – *Apparatus for the determination of water by distillation*

*Dimensions in millimetres*

**04/2017:20214**

# 2.2.14. MELTING POINT - CAPILLARY METHOD

The melting point determined by the capillary method is the temperature at which the last solid particle of a compact column of a substance in a tube passes into the liquid phase (i.e. clear point). The melting point determined by this method is specific to the methodology (e.g. heating rate) described in this chapter. Similarly, whenever the use of certified reference materials is required, their certified values refer to the described analytical procedure.

When prescribed in the monograph, the same apparatus and method are used for the determination of other factors, such as meniscus formation or melting range, that characterise the melting behaviour of a substance.

*Equipment.* The equipment consists of a metal heating block with 1 or more compartments for capillary tubes, or of a suitable glass vessel containing a liquid bath (e.g. water, liquid paraffin or silicone oil) and fitted with a suitable means of heating and stirring. The equipment is equipped with a temperature sensor or a suitable certified thermometer allowing readings at least to the nearest 0.1 °C.

Samples are introduced into the equipment in glass capillary tubes. The dimensions are chosen according to the manufacturer's requirements, typically with an external diameter of 1.3-1.5 mm and a wall thickness of 0.1-0.3 mm. In some equipment glass slides are used instead of capillary tubes.

The equipment is capable of heating samples at a rate of 1 °C/min or less. The accuracy of the equipment is at most ± 0.5 °C.

Detection can be performed either visually or instrumentally. In the case of instrumental detection, this is generally performed by image recording and subsequent analysis or by a photodetector that measures the transmitted or reflected light from the sample.

*Method.* The substance is previously treated as described in the monograph. Coarse crystals are to be avoided as they might lead to false results. If necessary, samples are crushed into a fine powder. Unless otherwise prescribed, dry the finely powdered substance *in vacuo* over *anhydrous silica gel R* for 24 h. Introduce a sufficient quantity into a capillary tube to give a compact column as described by the instrument manufacturer (e.g. 4-6 mm in height). Raise the temperature of the apparatus to about 5 °C below the presumed melting point. Allow the temperature to stabilise and then introduce the capillary tube into the instrument. Finally, adjust the rate of heating to about 1 °C/min unless otherwise prescribed.

In the case of instrumental detection, follow the instrument manufacturer's requirements for the determination of the melting point. For visual detection, record the temperature at which the last particle of the substance to be examined passes into the liquid phase.

Samples can be measured in parallel if the instrument allows multiple sample processing.

*System suitability.* Carry out a system suitability test before the measurements for example by choosing a suitable reference material with a melting point close to that expected for the substance to be examined.

*Qualification / Calibration of the equipment.* The qualification / calibration is carried out periodically according to the instrument manufacturer's requirements, using at least 2 certified reference materials. These are selected to cover the temperature range that is used on the equipment. Use capillary tubes with the same dimensions as those used for sample measurement.

Guidance on how to compare results obtained from certified reference materials with values from the certificates can be found on the European Reference Materials (ERM) website (Application note 1).

**01/2008:20215**

## 2.2.15. MELTING POINT - OPEN CAPILLARY METHOD

For certain substances, the following method is used to determine the melting point (also referred to as slip point and rising melting point when determined by this method).

Use glass capillary tubes open at both ends, about 80 mm long, having an external diameter of 1.4 mm to 1.5 mm and an internal diameter of 1.0 mm to 1.2 mm.

Introduce into each of 5 capillary tubes a sufficient amount of the substance, previously treated as described, to form in each tube a column about 10 mm high and allow the tubes to stand for the appropriate time and at the prescribed temperature.

Unless otherwise prescribed, substances with a waxy consistency are carefully and completely melted on a water-bath before introduction into the capillary tubes. Allow the tubes to stand at 2-8 °C for 2 h.

Attach one of the tubes to a thermometer graduated in 0.5 °C so that the substance is close to the bulb of the thermometer. Introduce the thermometer with the attached tube into a beaker so that the distance between the bottom of the beaker and the lower part of the bulb of the thermometer is 1 cm. Fill the beaker with water to a depth of 5 cm. Increase the temperature of the water gradually at a rate of 1 °C/min.

The temperature at which the substance begins to rise in the capillary tube is regarded as the melting point.

Repeat the operation with the other 4 capillary tubes and calculate the result as the mean of the 5 readings.

**07/2015:20216**

## 2.2.16. MELTING POINT - INSTANTANEOUS METHOD

The instantaneous melting point is calculated using the following expression:

$$\frac{t_1 + t_2}{2}$$

in which $t_1$ is the first temperature and $t_2$ the second temperature read under the conditions stated below.

*Apparatus.* The apparatus consists of a metal block resistant to the substance to be examined, of good heat-conducting capacity, such as brass, with a carefully polished plane upper surface. The block is uniformly heated throughout its mass by means of a micro-adjustable gas heater or an electric heating device with fine adjustment. The block has a cylindrical cavity, which is wide enough to accomodate a thermometer that is maintained in the same position during the calibration of the apparatus and the determination of the melting point of the substance to be examined. The cylindrical cavity is parallel to the upper polished surface of the block and about 3 mm from it. The apparatus is calibrated using appropriate substances of known melting point.

*Method.* Heat the block at a suitably rapid rate to a temperature about 10 °C below the presumed melting temperature, then adjust the heating rate to about 1 °C/min. At regular intervals drop a few particles of powdered and, where appropriate, dried substance, prepared as for the capillary tube method, onto the block in the vicinity of the thermometer bulb, cleaning the surface after each test. Record the temperature $t_1$ at which the substance melts instantaneously for the first time in contact with the metal. Stop the heating. During cooling drop a few particles of the substance at regular intervals on the block, cleaning the surface after each test. Record the temperature $t_2$ at which the substance ceases to melt instantaneously when it comes in contact with the metal.

*Calibration of the apparatus.* The apparatus may be calibrated using melting point reference substances such as those of the World Health Organization or other appropriate substances.

**01/2019:20217**

## 2.2.17. DROP POINT

The drop point is the temperature at which the first drop of the melting substance to be examined falls from a cup under defined conditions.

When a monograph does not specify the method to be used, apply method A. Any change from method A to method B is validated.

METHOD A

*Equipment.* The equipment (see Figure 2.2.17.-1) consists of 2 metal sheaths (*A* and *B*) screwed together. Sheath *A* is fixed to a thermometer. A metal cup is loosely fixed to the lower part of sheath *B* by means of 2 tightening bands. Fixed supports 2 mm long determine the exact position of the cup, and in addition are used to centre the thermometer. A hole pierced in the wall of sheath *B* is used to balance the pressure. The draining surface of the cup must be flat and the edges of the outflow orifice must be at right angles to it. The thermometer has the form and size shown in Figure 2.2.17.-1; it covers a range from 0 °C to 110 °C and on its scale a distance of 1 mm represents a difference of 1 °C. The thermometer bulb has a diameter of 3.5 ± 0.2 mm and a height of 6.0 ± 0.3 mm. The equipment is placed in the axis of a test-tube about 200 mm long and with an external diameter of about 40 mm. It is fixed to the test-tube by means of a laterally grooved stopper through which the thermometer passes. The opening of the cup is placed about 15 mm from the bottom of the test-tube. The whole device is immersed in a beaker with a capacity of about 1 L, filled with water. The bottom of the test-tube is placed about 25 mm from the bottom of the beaker. The water level reaches the upper part of sheath *A*. A stirrer is used to ensure that the temperature of the water remains uniform.

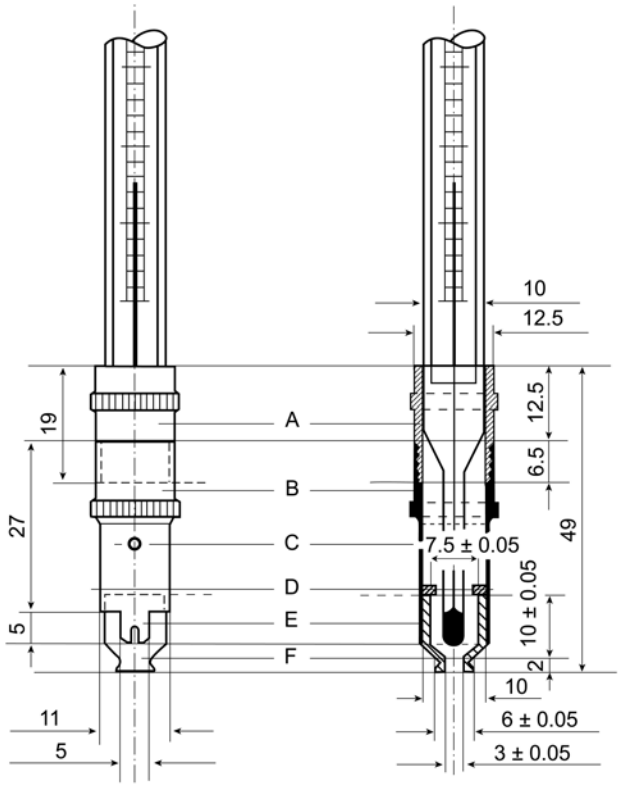


| | | |
|---|---|---|
| A. upper metal sheath | C. pressure-balancing hole | E. tightening bands |
| B. lower metal sheath | D. fixed supports | F. metal sample cup |

Figure 2.2.17.-1. – *Equipment for the determination of drop point (dimensions in millimetres)*

*Method*. Prepare the substance to be examined as described in the monograph. Fill the cup to the brim with the substance to be examined. Remove the excess substance at both ends of the cup with a spatula. When sheaths *A* and *B* have been assembled, press the cup into its housing in sheath *B* until it touches the supports. Remove with a spatula the substance pushed out by the thermometer. Place the equipment in the water-bath as described above. Heat the water-bath and, when the temperature is at about 10 °C below the presumed drop point, adjust the heating rate to about 1 °C/min. Note the temperature at the fall of the first drop. Carry out at least 3 determinations, each time with a fresh sample of the substance. The difference between the readings must not exceed 3 °C. The mean of 3 readings is the drop point of the substance.

## METHOD B - AUTOMATED METHOD

*Equipment*. The equipment (see Figure 2.2.17.-2) consists of a cartridge assembly comprising a cup holder into which the sample cup containing the sample is loosely fixed, and a collector sleeve with a horizontal light slit, which is fixed below the cup. This assembly is placed in a heating block. The block is a metal cylinder with a cylindrical hole along its vertical axis into which the cartridge assembly is placed. There is another, narrower cylindrical vertical hole in which a temperature sensor sits. This is positioned level with the sample cup. The heating block is surrounded by an electrical heating element. Below the heating block a lamp is mounted such that a beam of light shines through the light slit in the collector sleeve, and onto a photo-sensor mounted opposite. The heating element is capable of maintaining the heating block at a pre-defined temperature, and of heating at a slow and steady, pre-defined rate.

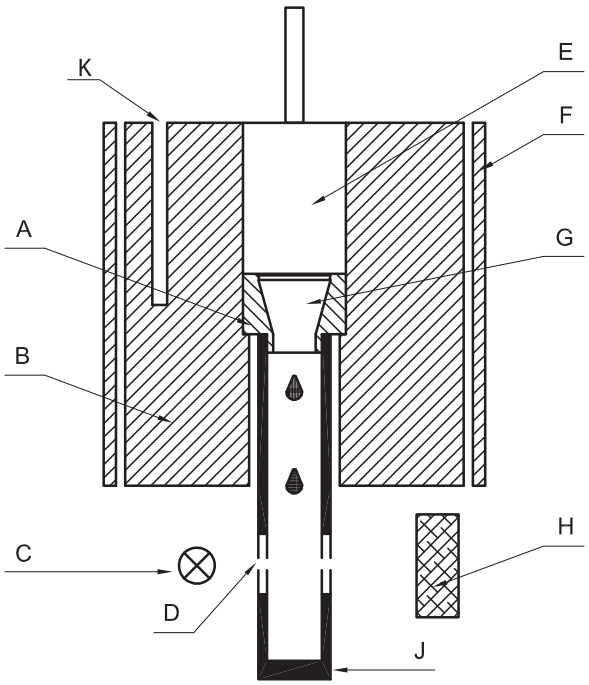


| | | | | |
|---|---|---|---|---|
| A. cup holder | C. light source | E. cartridge assembly | G. sample cup | J. collector sleeve |
| B. heating block | D. light slit | F. heating element | H. photo-sensor | K. temperature probe |

Figure 2.2.17.-2. – *Example of automated drop point equipment*

*Method*. Prepare the substance to be examined as described in the monograph, then proceed as follows or according to the manufacturer's instructions. Remove the excess substance at both ends of the cup with a spatula. Press the cup into the cup holder, and then press the collector sleeve onto the cup. Place the cartridge assembly in the heating block. Set the instrument to the initial isothermal conditions and the rate for subsequent heating as described in the monograph. Start the temperature programme. When the first drop of molten sample falls through the hole at the bottom of the sample cup, thus interrupting the light beam, a signal from the photo-sensor causes the temperature of the heating block to be recorded automatically.

*Calibration*. Use the equipment according to the manufacturer's instructions and carry out the prescribed calibrations and system performance tests at regular intervals, depending on the use of the equipment and the substances to be examined. Benzoic acid and benzophenone are usually used as certified reference materials. Other materials may be used provided they show no polymorphism. Proceed as follows or according to the manufacturer's instructions. Prepare 3 sample cups for each of 2 certified reference materials. Place the sample cups on a clean surface. Into each sample cup, introduce a small quantity of the sample and press it down with a rod (diameter about 4.5 mm). Check that the opening is completely blocked. Fill the sample cup about half full and compact the sample with a rod (diameter about 9 mm). Fill the sample cup and compact, adding more sample and compacting again if necessary, until the sample cup is completely packed.

Temperature programme for benzoic acid: start temperature = 118.0 °C; heating rate = 0.2 °C/min; end temperature = 126.0 °C. After inserting the cup at 118 °C, a waiting time of 30 s is set before heating starts.

Temperature programme for benzophenone: start temperature = 44.0 °C; heating rate = 0.2 °C/min; end temperature = 56.0 °C. After inserting the cup at 44 °C, a waiting time of 30 s is set before heating starts.

Check the 3 single results: the test is valid if the 3 results are within 0.3 °C of the mean value.

Calculate the corrected mean temperature ($T_2$) using the following expression:

$$T_1 - F$$

$T_1$ = mean drop point temperature of 3 samples, in °C;

$F$ = compensation for the difference in temperature between the sample and the point in the heating block where the temperature is measured; this will vary depending upon the design of the automatic drop point instrument and is provided by the manufacturer.

Taking into account the drop point of the certified reference material ($T_0$), the accuracy of the temperature scale is satisfactory if $|T_2 - T_0|$ is not greater than 0.3 °C.

**01/2008:20218**

## 2.2.18. FREEZING POINT

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

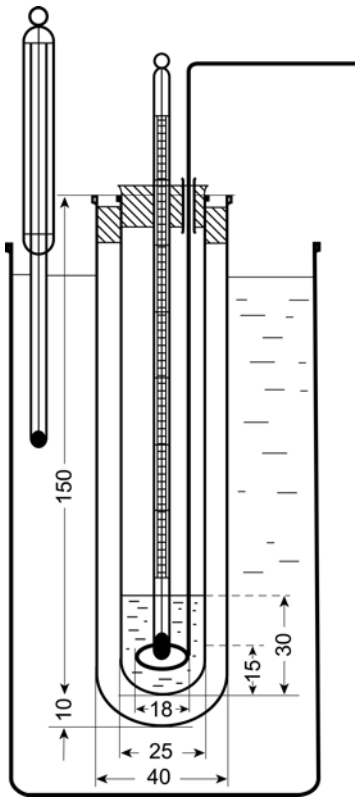


Figure 2.2.18.-1. – *Apparatus for the determination of freezing point*

*Dimensions in millimetres*

*Apparatus.* The apparatus (see Figure 2.2.18.-1) consists of a test-tube about 25 mm in diameter and 150 mm long placed inside a test-tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2 °C fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1 L beaker containing a suitable cooling liquid to within 20 mm of the top. A thermometer is supported in the cooling bath.

*Method.* Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5 °C above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride, at a temperature about 5 °C lower than the expected freezing point, insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.

**01/2016:20219**

## 2.2.19. AMPEROMETRIC TITRATION

In an amperometric titration, the end-point is determined by following the variation of the current measured between 2 electrodes (either one indicator electrode and one reference electrode or 2 indicator electrodes) immersed in the solution to be examined and maintained at a constant potential difference as a function of the quantity of titrant added.

The potential of the measuring electrode is sufficient to ensure a diffusion current for the electroactive substance.

*Apparatus.* The apparatus comprises an adjustable voltage source and a sensitive microammeter; the detection system generally consists of an indicator electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon electrode) and a reference electrode (for example, a silver-silver chloride electrode).

A three-electrode apparatus is sometimes used, consisting of an indicator electrode, a reference electrode and a polarised auxiliary electrode.

*Method.* Set the potential of the indicator electrode as prescribed and plot a graph of the initial current and the values obtained during the titration as functions of the quantity of titrant added. Add the titrant in not fewer than 3 successive quantities equal to a total of about 80 per cent of the theoretical volume corresponding to the presumed equivalence point. The 3 values must fall on a straight line. Continue adding the titrant beyond the presumed equivalence point in not fewer than 3 successive quantities. The values obtained must fall on a straight line. The point of intersection of the 2 lines represents the end-point of the titration.

For amperometric titrations with 2 indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

**01/2016:20220**

## 2.2.20. POTENTIOMETRIC TITRATION

In a potentiometric titration (volumetric titration with potentiometric end-point determination) the end-point is determined by recording the variation of the potential difference between 2 electrodes (either 1 indicator electrode and 1 reference electrode, or a combined electrode) immersed in the solution to be examined as a function of the volume of titrant added.

*Apparatus.* The apparatus used comprises a millivoltmeter. Commercial autotitrator instruments may be used and are operated in accordance with the manufacturer's instructions, using electrodes recommended for the type of titration described.

The indicator electrode to be used depends on the substance to be determined and may be a glass or metal electrode (e.g. platinum, gold or silver).

For acid-base titrations, a glass-silver-silver chloride electrode combination is generally used.

*Method.* Prepare the sample solution as described. Add the titrant in suitable aliquots paying particular attention to the rate of addition and the volume increments near the end-point. Continue the titration beyond this point to allow a clear detection of the end-point.

The end-point of the titration is reached when the maximum change in potential occurs in a plot of potential versus volume of titrant, and is expressed as the corresponding volume of titrant. Recording the first or second derivative curve can facilitate the determination of the end-point. In potentiometric titrations of weak acids or bases using non-aqueous solvents, if necessary, either carry out a blank determination or pre-neutralise the solvent mixture. Where it is impracticable to use potentiometric detection for this purpose, the solvent mixture can be pre-neutralised by titration using a suitable indicator. Some examples are given below:

| Titrant | Indicator |
|---|---|
| Perchloric acid | *Crystal violet solution R* |
| Tetrabutylammonium hydroxide | 3 g/L solution of *thymol blue R* in *methanol R* |
| Ethanolic sodium hydroxide | *Thymolphthalein solution R* |

**01/2008:20221**

## 2.2.21. FLUORIMETRY

Fluorimetry is a procedure which uses the measurement of the intensity of the fluorescent light emitted by the substance to be examined in relation to that emitted by a given standard.

*Method.* Dissolve the substance to be examined in the solvent or mixture of solvents prescribed in the monograph, transfer the solution to the cell or the tube of the fluorimeter and illuminate it with an excitant light beam of the wavelength prescribed in the monograph and as near as possible monochromatic.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence. Other types of apparatus may be used provided that the results obtained are identical.

For quantitative determinations, first introduce into the apparatus the solvent or mixture of solvents used to dissolve the substance to be examined and set the instrument to zero. Introduce the standard solution and adjust the sensitivity of the instrument so that the reading is greater than 50. If the second adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Finally introduce the solution of unknown concentration and read the result on the instrument. Calculate the concentration $c_x$ of the substance in the solution to be examined, using the formula:

$$c_x = \frac{I_x c_s}{I_s}$$

$c_x$ = concentration of the solution to be examined,

$c_s$ = concentration of the standard solution,

$I_x$ = intensity of the light emitted by the solution to be examined,

$I_s$ = intensity of the light emitted by the standard solution.

If the intensity of the fluorescence is not strictly proportional to the concentration, the measurement may be effected using a calibration curve.

In some cases, measurement can be made with reference to a fixed standard (for example a fluorescent glass or a solution of another fluorescent substance). In such cases, the concentration of the substance to be examined must be determined using a previously drawn calibration curve under the same conditions.

**01/2008:20222**

## 2.2.22. ATOMIC EMISSION SPECTROMETRY

GENERAL PRINCIPLE

Atomic emission is a process that occurs when electromagnetic radiation is emitted by excited atoms or ions. In atomic emission spectrometry the sample is subjected to temperatures high enough to cause not only dissociation into atoms, but also to cause significant amounts of collisional excitation and ionisation of the sample atoms to take place. Once the atoms and ions are in the excited states, they can decay to lower states through thermal or radiative (emission) energy transitions and electromagnetic radiation is emitted. An emission spectrum of an element contains several more lines than the corresponding absorption spectrum.

Atomic emission spectrometry is a technique for determining the concentration of an element in a sample by measuring the intensity of one of the emission lines of the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength corresponding to this emission line.

In this chapter only atomisation in flame is dealt with. The method of inductively coupled plasma-atomic emission spectrometry (ICP-AES) is described in a different general chapter.

APPARATUS

This consists essentially of:

- a sample introduction and nebulisation system;
- a flame to generate the atoms to be determined;
- a monochromator;
- a detector;
- a data-acquisition unit.

Oxygen, air and a combustible gas such as hydrogen, acetylene, propane or butane may be used in flames. The atomisation source is critical, since it must provide sufficient energy to excite and atomise the atoms. The atomic spectra emitted from flames have the advantage of being simpler than those emitted from other sources, the main limitation being that the flames are not powerful enough to cause emission for many elements allowing their determination. Acidified water is the solvent of choice for preparing test and reference solutions, although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

INTERFERENCES

Spectral interference is reduced or eliminated by choosing an appropriate emission line for measurement or by adjusting the slit for spectral band-width. Physical interference is corrected by diluting the sample solution, by matching the matrix or by using the method of standard additions. Chemical interference is reduced by using chemical modifiers or ionisation buffers.

MEMORY EFFECT

The memory effect caused by deposit of analyte in the apparatus may be limited by thoroughly rinsing between runs, diluting the solutions to be measured if possible and thus reducing their salt content, and by aspirating the solutions through as swiftly as possible.

METHOD

Use of plastic labware is recommended wherever possible.

Operate an atomic emission spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Optimise the experimental conditions (flame temperature, burner adjustment, use of an ionic buffer, concentration of solutions) for the specific element to be analysed and in respect of the sample matrix. Introduce a blank solution into the atomic generator and adjust the instrument reading to zero or to its blank value. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable reading.

It is preferable to use concentrations which fall within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

*METHOD I - DIRECT CALIBRATION*

For routine measurements 3 reference solutions of the element to be determined and a blank are prepared and examined.

Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph. Prepare not fewer than 3 reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For assay purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are between the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference solutions and to the blank solution at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

**Calculation**. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

*METHOD II - STANDARD ADDITIONS*

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if at all possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

**Calculation**. Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

*LINEARITY*

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99,
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

*ACCURACY*

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

**Recovery**. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example for trace element determination, the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

*REPEATABILITY*

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

*LIMIT OF QUANTIFICATION*

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

**01/2008:20223**

# 2.2.23. ATOMIC ABSORPTION SPECTROMETRY

GENERAL PRINCIPLE

Atomic absorption is a process that occurs when a ground state-atom absorbs electromagnetic radiation of a specific wavelength and is elevated to an excited state. The atoms in the ground state absorb energy at their resonant frequency and the electromagnetic radiation is attenuated due to resonance absorption. The energy absorption is virtually a direct function of the number of atoms present.

This chapter provides general information and defines the procedures used in element determinations by atomic absorption spectrometry, either atomisation by flame, by electrothermal vaporisation in a graphite furnace, by hydride generation or by cold vapour technique for mercury.

Atomic absorption spectrometry is a technique for determining the concentration of an element in a sample by measuring the absorption of electromagnetic radiation by the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength of one of the absorption (resonance) lines of the element concerned. The amount of radiation absorbed is, according to the Lambert-Beer law, proportional to the element concentration.

## APPARATUS

This consists essentially of:

- a source of radiation;
- a sample introduction device;
- a sample atomiser;
- a monochromator or polychromator;
- a detector;
- a data-acquisition unit.

The apparatus is usually equipped with a background correction system. Hollow-cathode lamps and electrodeless discharge lamps (EDL) are used as radiation source. The emission of such lamps consists of a spectrum showing very narrow lines with half-width of about 0.002 nm of the element being determined.

There are 3 types of sample atomisers:

- Flame technique

  A flame atomiser is composed of a nebulisation system with a pneumatic aerosol production accessory, a gas-flow regulation and a burner. Fuel-oxidant mixtures are commonly used to produce a range of temperatures from about 2000 K to 3000 K. Fuel gases include propane, hydrogen and acetylene; air and nitrous oxide are used as oxidants. The configuration of the burner is adapted to the gases used and the gas flow is adjustable. Samples are nebulised, acidified water being the solvent of choice for preparing test and reference solutions. Organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

- Electrothermal atomisation technique

  An electrothermal atomiser is generally composed of a graphite tube furnace and an electric power source. Electrothermal atomisation in a graphite tube furnace atomises the entire sample and retains the atomic vapour in the light path for an extended period. This improves the detection limit. Samples, liquid as well as solid, are introduced directly into the graphite tube furnace, which is heated in a programmed series of steps to dry the sample and remove major matrix components by pyrolysis and to then atomise all of the analyte. The furnace is cleaned using a final temperature higher than the atomisation temperature. The flow of an inert gas during the pyrolysis step in the graphite tube furnace allows a better performance of the subsequent atomisation process.

- Cold vapour and hydride technique

  The atomic vapour may also be generated outside the spectrometer. This is notably the case for the cold-vapour method for mercury or for certain hydride-forming elements such as arsenic, antimony, bismuth, selenium and tin. For mercury, atoms are generated by chemical reduction with stannous chloride or sodium borohydride and the atomic vapour is swept by a stream of an inert gas into a cold quartz cell mounted in the optical path of the instrument. Hydrides thus generated are swept by an inert gas into a heated cell in which they are dissociated into atoms.

## INTERFERENCES

Chemical, physical, ionisation and spectral interferences are encountered in atomic absorption measurements. Chemical interference is compensated by addition of matrix modifiers, of releasing agents or by using high temperature produced by a nitrous oxide-acetylene flame; the use of specific ionisation buffers (for example, lanthanum and caesium) compensates for ionisation interference; by dilution of the sample, through the method of standard additions or by matrix matching, physical interference due to high salt content or viscosity is eliminated. Spectral interference results from the overlapping of resonance lines and can be avoided by using a different resonance line. The use of Zeeman background correction also compensates for spectral interference and interferences from molecular absorption, especially when using the electrothermal atomisation technique. The use of multi-element hollow-cathode lamps may also cause spectral interference. Specific or non-specific absorption is measured in a spectral range defined by the band-width selected by the monochromator (0.2-2 nm).

## BACKGROUND CORRECTION

Scatter and background in the flame or the electrothermal atomisation technique increase the measured absorbance values. Background absorption covers a large range of wavelengths, whereas atomic absorption takes place in a very narrow wavelength range of about 0.005-0.02 nm. Background absorption can in principle be corrected by using a blank solution of exactly the same composition as the sample, but without the specific element to be determined, although this method is frequently impracticable. With the electrothermal atomisation technique the pyrolysis temperature is to be optimised to eliminate the matrix decomposition products causing background absorption. Background correction can also be made by using 2 different light sources, the hollow-cathode lamp that measures the total absorption (element + background) and a deuterium lamp with a continuum emission from which the background absorption is measured. Background is corrected by subtracting the deuterium lamp signal from the hollow-cathode lamp signal. This method is limited in the spectral range on account of the spectra emitted by a deuterium lamp from 190-400 nm. Background can also be measured by taking readings at a non-absorbing line near the resonance line and then subtracting the results from the measurement at the resonance line. Another method for the correction of background absorption is the Zeeman effect (based on the Zeeman splitting of the absorption line in a magnetic field). This is particularly useful when the background absorption shows fine structure. It permits an efficient background correction in the range of 185-900 nm.

## CHOICE OF THE OPERATING CONDITIONS

After selecting the suitable wavelength and slit width for the specific element, the need for the following has to be ascertained:

- correction for non-specific background absorption,
- chemical modifiers or ionisation buffers to be added to the sample as well as to blank and reference solutions,
- dilution of the sample to minimise, for example, physical interferences,
- details of the temperature programme, preheating, drying, pyrolysis, atomisation, post-atomisation with ramp and hold times,
- inert gas flow,
- matrix modifiers for electrothermal atomisation (furnace),
- chemical reducing reagents for measurements of mercury or other hydride-forming elements along with cold vapour cell or heating cell temperature,
- specification of furnace design (tank, L'vov platform, etc).

## METHOD

Use of plastic labware is recommended wherever possible. The preparation of the sample may require a dissolution, a digestion (mostly microwave-assisted), an ignition step or a combination thereof in order to clear up the sample matrix

and/or to remove carbon-containing material. If operating in an open system, the ignition temperature should not exceed 600 °C, due to the volatility of some metals, unless otherwise stated in the monograph.

Operate an atomic absorption spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Introduce a blank solution into the atomic generator and adjust the instrument reading so that it indicates maximum transmission. The blank value may be determined by using solvent to zero the apparatus. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a maximum absorbance reading. Rinse in order to avoid contamination and memory effects. After completing the analysis, rinse with *water R* or acidified water.

If a solid sampling technique is applied, full details of the procedure are provided in the monograph.

Ensure that the concentrations to be determined fall preferably within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

*METHOD I - DIRECT CALIBRATION*

For routine measurements 3 reference solutions and a blank solution are prepared and examined.

Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph. Prepare not fewer than 3 reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For assay purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference and blank solutions at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each of the solutions to obtain a steady reading.

**Calculation**. Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

*METHOD II - STANDARD ADDITIONS*

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

**Calculation**. Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

*LINEARITY*

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99,
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

*ACCURACY*

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

**Recovery**. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example, for trace element determination the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

*REPEATABILITY*

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

*LIMIT OF QUANTIFICATION*

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

**04/2019:20224**

## 2.2.24. ABSORPTION SPECTROPHOTOMETRY, INFRARED

PRINCIPLE

Infrared absorption spectrophotometry (also known as infrared (IR) spectroscopy) is based on the interaction of infrared radiation with matter, which affects the vibrational energy of molecules and induces intermolecular and intramolecular vibrations at specific frequencies. This results in an infrared absorption spectrum with characteristic bands that correspond to the functional groups of the molecule.

The infrared wavelength region can be further divided into 3 subregions, namely near-infrared, mid-infrared and far-infrared. These subregions have wavelength ranges that are generally accepted by convention to be 0.8-2.5 µm, 2.5-25 µm and 25-1000 µm respectively. However, in IR spectroscopy, wavenumber is more commonly used than wavelength, and can be calculated using the following equation:

$$\tilde{\nu} = \frac{1}{\lambda} \cdot 10^4$$

where $\tilde{\nu}$ is the wavenumber in reciprocal centimetres ($cm^{-1}$) and λ is the wavelength in micrometres. Thus 12 500-4000 $cm^{-1}$ is near-infrared, 4000-400 $cm^{-1}$ is mid-infrared and 400-10 $cm^{-1}$ is far-infrared.

This chapter concerns only spectroscopy in the mid-infrared region, i.e. 4000-400 $cm^{-1}$ (2.5-25 µm), which hereafter is referred to as infrared for simplicity. This region is where the fundamental molecular vibrations of functional groups appear in the spectrum as absorption bands. The region below 1500 $cm^{-1}$ is known as the 'fingerprint region', a very complex and informative part of the spectrum which characterises the molecule being investigated.

The mid-infrared region is flanked by the near-infrared region, where overtones and combinations of fundamental vibrations, mainly C-H, N-H and O-H functional groups, are detected (*2.2.40*) and the far-infrared region, where absorption bands associated with crystal lattice modes, hydrogen bonds, angle deformation vibrations of heavy atoms and molecular rotations are observed.

## APPLICATIONS

As the absorption bands in IR spectra are characteristic of the constituent functional groups of a compound, IR spectroscopy is widely used to identify substances and provide information on their structure. It can also be used for quantitative applications, which requires establishing a mathematical relationship between the intensity of the radiation absorbed by the sample and the concentration of the investigated component in the sample.

IR spectroscopy is widely used in the pharmaceutical field for chemical and physical analysis in the laboratory, and has a wide variety of applications during the manufacturing process as outlined below. IR spectroscopy thereby enables the application of Process Analytical Technology (PAT) as part of an advanced control strategy.

*Chemical analysis*:

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemicals and packaging materials;
- quality assessment of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;
- quantification of active substances in a sample matrix, determination of water and solvent content;
- quantification of impurities, e.g. in gases, inorganic materials;
- reaction monitoring, e.g. chemical synthesis.

*Physical analysis*:

- determination of solid-state properties such as polymorphism.

## LIMITATIONS

Notable limitations to the use of IR spectroscopy include the following:

- it may be necessary to use additional techniques to unambiguously identify a substance;
- pure enantiomers of a substance cannot be discriminated;
- it may not be a suitable method for trace analysis;
- sample preparation conditions (e.g. pressure, solvent) may change the crystalline form of a substance that exhibits polymorphism;
- for heterogeneous samples, the limited sampling volume may be problematic.

## MEASUREMENT MODES

IR measurements are based on passing radiation through or into a sample and measuring the attenuation of the emerging beam at various wavelengths. This corresponds to 2 main measurement modes, i.e. transmission and attenuated total reflection (ATR). However, other modes also exist for specific applications (e.g. diffuse and specular reflection).

*TRANSMISSION MODE*

This mode is based on determination of the transmittance (*T*), namely the ability of the sample to transmit IR radiation at a given wavelength (wavenumber). It is defined by the following ratio:

$$T = \frac{I}{I_0}$$

$I_0$ = intensity of incident radiation;
$I$ = intensity of transmitted radiation.

The resulting spectrum is presented in terms of transmittance (*T*) on the y-axis versus wavelength or wavenumber on the x-axis. It can also be presented in terms of absorbance (*A*) on the y-axis, which is related to transmittance (*T*) by the following equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right) = a \cdot b \cdot c$$

$a$ = molar absorption coefficient of the sample, in square centimetres per mole ($cm^2 \cdot mol^{-1}$);
$b$ = sample thickness, in centimetres;
$c$ = sample concentration, in moles per cubic centimetre ($mol \cdot cm^{-3}$).

*ATTENUATED TOTAL REFLECTION MODE*

ATR mode is based on the phenomenon of total internal reflection. The sample, with a refractive index $n_2$, is brought into close contact with a crystal (diamond, germanium, zinc selenide or any other suitable material), having a refractive index $n_1$ which is greater than $n_2$. A beam of IR light is then passed through the crystal. When the angle α between the incident beam and the sample-crystal interface exceeds a critical value $\alpha_c$, theoretically all of the radiation is reflected (total internal reflection). However, an evanescent wave is produced which slightly penetrates the sample and part of the energy is absorbed. The total reflection is attenuated, which makes it possible to generate an absorption spectrum. In practice, multiple internal reflections are often used to amplify the absorption intensity, although some accessories allow absorption measurements with a single reflection.
The penetration depth $d_p$ is usually of the order of a few micrometres and is given for a wavelength λ by the following equation:

$$d_p = \frac{\lambda / n_1}{2\pi\sqrt{sin^2\,\alpha - (n_2 / n_1)^2}}$$

where $d_p$ is the penetration depth, λ is the wavelength, α is the angle of incidence and $n_1$, $n_2$ are the refractive indices of the reflection element and the sample, respectively.

Due to the relationship between these parameters, the absorption intensity in ATR is greater at higher wavelengths (i.e. smaller wavenumbers) and slight band shifts occur compared to the corresponding transmission spectrum. It is therefore not advisable to compare ATR spectra with transmission spectra when identifying compounds.

## EQUIPMENT

The most commonly used IR spectrometers are Fourier-transform (FT-IR) spectrometers which typically consist of:

- a suitable polychromatic light source, e.g. a conducting ceramic rod;
- an interferometer;
- a sample presentation accessory, e.g. a sample holder;
- a detector;

– appropriate software for controlling the spectrometer, and for spectral evaluation and data processing.

Other spectrometers based on alternative principles may also be used if the requirements described under Control of equipment performance are fulfilled.

IR spectrometers can also be used in association with a microscope for the study of a small part of the sample or for chemical imaging.

IR spectroscopy can be coupled to other analytical techniques such as thermal analysis or chromatography.

## CONTROL OF EQUIPMENT PERFORMANCE

Accuracy of wavenumber scale and spectral resolution are critical parameters and must be verified. The tests described below can be used for the control of instrument performance and for qualification. They can also be used as system suitability tests.

These parameters are checked using suitable reference materials which are selected and presented depending on the measurement mode (e.g. transmission or ATR).

For quantitative analysis, appropriate assessment criteria for the control of absorption intensity must also be defined.

*WAVENUMBER SCALE*

The wavenumber scale is typically verified using a polystyrene film that exhibits IR absorption bands at the wavenumbers shown in Table 2.2.24.-1.

Table 2.2.24.-.1 - *Band positions and associated acceptable tolerances of the polystyrene film used to verify wavenumber accuracy*

| Band position ($cm^{-1}$) | | Tolerance ($cm^{-1}$) |
|---|---|---|
| **Transmission** | **ATR** | |
| 906.6 | 906.1 | ± 1.0 |
| 1028.3 | 1027.7 | ± 1.0 |
| 1601.2 | 1601.0 | ± 1.0 |
| 3060.0 | 3059.7 | ± 1.0 |

For measurement modes other than transmission or ATR, reference materials must be defined by the user.

*SPECTRAL RESOLUTION*

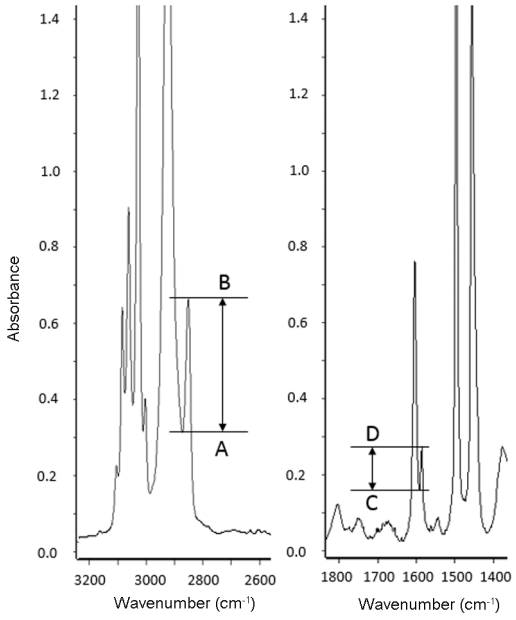


Figure 2.2.24.-1. – *Typical IR absorbance spectrum of polystyrene used to verify spectral resolution*

**Spectra recorded in transmission mode**. The spectral resolution is typically verified using a polystyrene film approximately 35 µm thick.

*Acceptance criteria (see Figure 2.2.24.-1)*: the difference between the absorbance values at the absorption minimum at 2870 $cm^{-1}$ (*A*) and the absorption maximum at 2849.5 $cm^{-1}$ (*B*) is greater than 0.33; the difference between the absorbance values at the absorption minimum at 1589 $cm^{-1}$ (*C*) and the absorption maximum at 1583 $cm^{-1}$ (*D*) is greater than 0.08.

**Spectra recorded in ATR mode**. Appropriate assessment criteria for the control of spectral resolution according to the specifications of each instrument need to be defined.

For measurement modes other than transmission or ATR, reference materials have to be defined by the user.

## PROCEDURE

*SAMPLE PREPARATION AND PRESENTATION*

Sample preparation and presentation vary according to the physical state of the sample and the measurement mode. Transmission mode is applied to transparent samples, such as neat liquids, solutions, gases or suitably prepared mulls and alkali halide discs. For liquids and gases, cells with fixed or variable pathlength and IR transparent windows can be used. For alkali-halide disks, specific sample holders are used. Reflection mode, e.g. ATR, is appropriate for the measurement of a wide range of samples in the solid and liquid state.

Some preparation modes (e.g. for discs and mulls in transmission mode or for solids in ATR mode) involve grinding and/or the application of pressure, which may induce unexpected crystal modifications.

**Transmission mode**

Prepare the substance by one of the following methods depending on the sample state (solid, liquid or gas). If sample bands in a spectrum have a minimum transmission lower than 5 per cent, this spectrum is not used for further data analysis.

*Liquids*. Examine liquids either in the form of a film between 2 plates transparent to infrared radiation or in a cell of suitable pathlength with windows that are transparent to infrared radiation.

*Liquids or solids in solution*. Prepare a solution of the substance to be examined in a suitable solvent. Choose a concentration and a pathlength that give a satisfactory spectrum. Generally, good results are obtained with concentrations of 10-100 g/L for a pathlength of 0.5-0.1 mm. The absorption due to the solvent is usually compensated by successively recording the spectra of the solvent and the sample solution and subtracting the solvent absorption bands from the spectrum of the sample solution.

*Solids dispersed in a solid (disc)*. Grind the substance to be examined taking into consideration any possible changes (e.g. crystalline form) and mix with a suitable amount of finely powdered and dried *potassium bromide R* or *potassium chloride R*, unless otherwise specified. A mixture of a few milligrams (e.g. 1-2 mg) of the substance to be examined in a few hundred milligrams (e.g. 300-400 mg) of halide is normally sufficient to give a disc of 10-15 mm diameter and a spectrum of suitable intensity. If the substance is a hydrochloride salt, it is recommended to use *potassium chloride R*. Carefully grind the mixture, spread it uniformly in a suitable die and apply a suitable pressure. A compacting force of about 800 MPa is generally sufficient to prepare a disc. For substances that are unstable under normal atmospheric conditions or are hygroscopic, the disc may be pressed under vacuum. Several factors may cause the formation of faulty discs, such as insufficient or excessive grinding, humidity or impurities in the dispersion medium. For example, any water in either the sample or the potassium bromide will cause clouding of the disc and produce a low transmission spectrum. A disc is rejected if visual examination shows a lack of uniform transparency or when, in the absence of a specific

absorption band, the transmittance is less than 60 per cent or the absorbance is more than 0.22 at about 2000 $cm^{-1}$ (5 µm) and without compensation, unless otherwise prescribed.

*Solids dispersed in a liquid (mull).* Triturate a small quantity of the substance to be examined with the minimum quantity of *liquid paraffin R* or other suitable liquid. A mixture of a few milligrams (e.g. 5-10 mg) of the substance to be examined in 1 drop of *liquid paraffin R* is generally sufficient to make an adequate mull. Compress the mull between 2 plates transparent to infrared radiation. A mull is rejected if a visual examination shows lack of uniform transparency or where the spectrum shows features such as:

- low transmission at 4000 $cm^{-1}$;
- a strongly sloping baseline between 4000 and about 2500 $cm^{-1}$;
- a ratio of relative intensities of some absorption bands that is less than expected.

*Molten solids.* If prescribed in the monograph, make a film of a molten mass and fix it on a suitable mount.

*Evaporated solution.* If prescribed in the monograph, dissolve the substance to be examined in a suitable solvent. Prepare a film by evaporating the solvent on a suitable carrier and fix it on a suitable mount.

*Gases.* Use a suitable cell transparent to infrared radiation. Evacuate the air from the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas to be examined. If necessary, adjust the pressure in the cell to atmospheric pressure using a gas transparent to infrared radiation (e.g. *nitrogen R* or *argon R*), or purge with carbon dioxide-free air. An appropriate measurement protocol must be followed to compensate for water, carbon dioxide or other atmospheric gases.

**ATR mode**

ATR is suitable for liquid and solid samples, and requires no preparation apart from simple treatments such as the grinding of large crystals and coarse material. Proceed as follows depending on the sample state (liquid or solid).

*Liquids.* Place the sample in contact with the crystal.

*Solids.* Ensure close and uniform contact between the substance to be examined and the whole crystal surface, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the crystal with the resulting solution and evaporating to dryness.

*METHODS*

Infrared spectroscopy is mostly used to identify substances, but it may also be carried out for quantitative applications. Quantitative analysis (based on the Beer-Lambert law, which relates the absorbance of a sample to its concentration) will not be described in this chapter.

The measurement is performed on an appropriately prepared sample. The data is then processed and evaluated, either to identify substances or quantify them (e.g. based on integration of IR-absorption bands).

Spectral quality may be enhanced by mathematical pretreatments. In practice, these are limited to spectral normalisation and subtraction of bands caused by carbon-dioxide and water vapour. The same pretreatments are performed on both the sample and the reference spectra.

**Identification**

Prepare the substance to be examined appropriately and record the spectra between 4000 and 650 $cm^{-1}$, unless otherwise prescribed.

Identification testing is performed by comparing the spectrum of the substance to be examined with the spectrum obtained from a Ph. Eur. chemical reference substance (CRS) or with a Ph. Eur. reference spectrum.

The spectrum of the current batch of the Ph. Eur. CRS may be recorded for immediate use or stored, for example, in a spectral library for future consultation. A stored spectrum may be used, provided traceability to the current batch of CRS is ensured.

In the case of substances that are not covered by individual monographs, a suitable reference standard may be used.

In all cases, spectra must be recorded using the same operating conditions and procedure, and especially the same measurement mode.

When comparison of the spectra recorded in the solid state show differences (see below), treat the substance to be examined and the reference substance in the same manner so that they recrystallise or are produced in the same crystalline form, or proceed as prescribed in the monograph, then record the spectra again. However, this procedure must only be done for substances where the monograph does not cover a particular form of a substance that exhibits polymorphism.

Several comparison procedures may be used, and the analyst must document and justify the method used and the specific acceptance criteria that allow a conclusion for identification. The spectra can be compared either by overlaying the spectra (in the whole spectral range or in the region of interest specified in the monograph) or by using mathematical calculations from the software. It is possible for example to perform:

- visual comparison based on band positions and relative intensities unless otherwise specified - the transmission minima (or absorption maxima) in the spectrum obtained with the substance to be examined correspond in position and relative size to those of the reference;
- calculation of the correlation coefficient between the 2 spectra - this value is calculated by the software and the identification threshold is defined by the user;
- evaluation by chemometric methods (e.g. Euclidean distance, Mahalanobis distance, classification methods); these methods involve the set-up, assessment and validation of the chemometric model by the analyst (see *5.21. Chemometric methods applied to analytical data*).

**Impurities in gases**

For the analysis of impurities, use a cell transparent to infrared radiation and of suitable optical pathlength (e.g. 1-20 m). Fill the cell as prescribed under Gases. For detection and quantification of the impurities, proceed as prescribed in the monograph.

**01/2020:20225**

# 2.2.25. ABSORPTION SPECTROPHOTOMETRY, ULTRAVIOLET AND VISIBLE

PRINCIPLE

Ultraviolet and visible (UV-Vis) spectroscopy (or spectrophotometry) is based on the ability of atoms, molecules and ions to absorb light at specific wavelengths in the ultraviolet (approximately 180-400 nm) and visible (approximately 400-800 nm) range. This absorption is associated with changes in electronic energy in the form of temporary transitions of electrons to an excited state at a higher energy orbital. As each energy level of a molecule or molecular ion also has associated vibrational and rotational sub-levels, this results in many permitted transitions, which are generally impossible to separate, thereby producing absorption bands rather than sharp lines. These bands are characteristic of the functional groups and bonds in a molecule.

UV-Vis spectroscopy measurements involve exposing a sample to light and measuring the attenuation and/or scattering of the emerging (transmitted or reflected) light either at a single wavelength or over a specified wavelength range.

## APPLICATIONS

UV-Vis spectroscopy is traditionally used for the quantitative and qualitative analysis of liquid samples, but is also suitable for solid and gaseous analytes and has other applications such as the determination of physical or chemical properties.

UV-Vis spectroscopy as described in this chapter can be applied in various ways:

- when a monograph or general chapter refers to this chapter, the requirements described in the relevant paragraphs of this chapter are mandatory;
- when used as the detection method in chromatographic systems as described in general chapter *2.2.46*, the requirements listed in the relevant paragraphs of this chapter are mandatory;
- when used as a process analytical technology (PAT) tool for PAT applications similar to the applications described in this chapter, the provisions herein apply; for other PAT applications, the principles are the same, however the criteria are established bearing in mind the intended purpose of the analysis, using a risk-based approach.

## EQUIPMENT

Spectrophotometers used for carrying out measurements in the UV-Vis region typically consist of:

- a suitable light source (such as a deuterium lamp for the UV region, a tungsten-halogen lamp for the visible region or a xenon lamp to cover the entire UV-Vis range); UV-Vis spectrophotometers often have 2 sources;
- a monochromator such as a grating system;
- other optical components, such as lenses or mirrors, that relay light through the instrument and that may also be used to generate more than one beam of light, i.e. in double-beam spectrophotometers, as opposed to single-beam spectrophotometers;
- a sample container, holder or sampling device; examples include conventional cuvettes, fibre-optic probes and immersed transmission cells (e.g. high-purity quartz or sapphire transparent to UV-Vis radiation); the choice depends on the intended application, paying particular attention to its suitability for the type of sample to be analysed;
- a single-channel (e.g. photomultiplier, photodiode) or multi-channel detector (e.g. photodiode array (PDA) or charge-coupled device (CCD));
- suitable computerised data processing and evaluation systems.

**Control of cuvettes**. For benchtop instruments, cuvettes or cells with a defined path length are used. These can be made of different materials such as quartz or glass. The tolerance for the path length of quartz and glass cuvettes is ± 0.5 per cent (e.g. ± 0.005 cm for a 1 cm cuvette). Plastic cuvettes may also be used but the tolerance interval is wider; therefore, their use must be thoroughly justified and based on a risk assessment.

The following method may be applied to check the cleanliness of optical cuvette windows and any significant differences in their thickness or parallelism: fill the cuvette with *water R* and measure its apparent absorbance against air at 240 nm for quartz cuvettes and 650 nm for glass cuvettes; rotate the cuvette 180° in its holder and measure the apparent absorbance again at the same wavelength.

When using scanning instruments, it is recommended to scan over the optical region of interest.

When using double-beam spectrophotometers, measures should be taken (e.g. matching the cuvettes) to ensure that any difference between the absorbance of the cuvettes will not have a significant impact on the analysis to be performed.

*Acceptance criteria*:

- the apparent absorbance is not greater than 0.093 for 1 cm quartz cuvettes (UV region) and 0.035 for 1 cm glass cuvettes (visible region);
- the absorbance measured after rotation (180°) does not differ by more than 0.005 from the value previously obtained.

## MEASUREMENT

**Transmission mode**. Transmission mode provides a measure of the transmittance ($T$), at a given UV-Vis wavelength, of a sample placed between the light source and the detector. Transmittance is the ratio of the intensity of the transmitted light to the intensity of the incident light and is given by the following equation:

$$T = \frac{I}{I_0}$$

$I$ = intensity of transmitted radiation;

$I_0$ = intensity of incident radiation.

A spectrum may be obtained by plotting the variation in transmittance ($T$) or absorbance ($A$) as a function of wavelength.

The absorbance is defined as the logarithm to base 10 of the reciprocal of the transmittance for monochromatic radiation. It is a dimensionless quantity expressed in absorbance units (AU), given by the following equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$

According to the Beer-Lambert law, which applies to clear diluted solutions, in the absence of interfering physico-chemical factors, the absorbance ($A$) is proportional to the path length ($l$) of the radiation through the sample, and to the concentration ($c$) of the substance in solution in accordance with the following equation:

$$A = \varepsilon c l$$

$\varepsilon$ = molar absorption coefficient, in litres per mole per centimetre;

$c$ = molar concentration of the substance in solution, in moles per litre;

$l$ = absorption path length, in centimetres.

The specific absorbance ($A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}$) of the substance is generally used in monographs and is related to absorbance ($A$) as follows:

$$A = A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}} \times c_m \times l$$

$c_m$ = mass concentration of the substance in solution, in grams per 100 millilitres.

$A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}$ represents the specific absorbance of a dissolved substance and refers to the absorbance of a 1 g/100 mL (or 1 per cent *m/V*) solution in a 1 cm cuvette or cell and is measured at a defined wavelength. The relationship between $A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}}$ and $\varepsilon$ is:

$$A_{1\ \mathrm{cm}}^{1\ \mathrm{per\ cent}} = \frac{10\varepsilon}{M_r}$$

$M_r$ = relative molecular mass.

Transmittance or absorbance measurements are generally used for liquids (dispersions and solutions), but can also be used for solids (including tablets and capsules). For measurements of solids, a suitable sample accessory is used. Liquid samples are examined using a cell or cuvette with a suitable path length (typically 0.01-1 cm) and made of a material that is transparent to UV-Vis radiation, or by using a fibre-optic probe of a suitable configuration immersed in the liquid.

**Diffuse reflection mode**. Diffuse reflection mode provides a measure of reflectance ($R$), which is given by the following equation:

$$R = \frac{I}{I_0}$$

$I$ = intensity of light reflected and/or scattered from the sample;

$I_0$ = intensity of light reflected and/or scattered from a blank or reference reflective surface.

Depending on the chemical composition and physical characteristics of the sample, the UV-Vis radiation may be absorbed as it passes through the sample. In diffuse reflection mode, it is the non-absorbed radiation which is partially reflected and/or scattered back from the sample that is measured by the detector. UV-Vis reflectance spectra are typically obtained by calculating and plotting $\log_{10}(1/R)$ as a function of the wavelength.

This measurement mode is generally selected for solids. The sample is examined either in a suitable device (e.g. a sample holder) or in direct contact with a probe. For process monitoring, the material can be analysed through a polished window interface (e.g. quartz or sapphire), or in-line using a probe. Care must be taken to ensure that the measurement conditions are as reproducible as possible from one sample to another.

**Operation of the equipment**. The factors below affect the spectral response and must always be taken into account.

Choose a measurement mode that is appropriate for the intended application and the sample type.

Define the measuring conditions taking into account the sample size and sample probe in such a way as to obtain a satisfactory signal-to-noise ratio (e.g. beam size, measurement time and number of measurements). For scanning spectrophotometers, also select the scan range, scan rate and slit-width that provide the necessary optical resolution for the intended application without losing the required signal-to-noise ratio or the linearity of the analytical method. When using spectrophotometers with array sensors, there is no need to adjust the beam size, scan range, scan rate or slit-width since the optical resolution is typically fixed and the full spectrum is always recorded.

Before an absorbance measurement is carried out, the zero position of the absorption (baseline correction) should be set or determined for the wavelengths of interest or over the appropriate range of wavelengths.

For PAT applications, when measuring moving materials or samples, ensure that there is no fouling of the sensor (e.g. no contamination or build-up of material).

Unless otherwise prescribed in the monograph, measure the absorbance using a path length of 1 cm at the prescribed wavelength. If a single value for the position of an absorption maximum or minimum is given in a monograph, the user must determine the wavelength position. The value obtained may differ by not more than ± 2 nm, unless otherwise prescribed.

Quantitative measurements relying on absorption values above 2.0 should be avoided.

**Background correction**. Select a suitable spectroscopic blank (e.g. air, blank solvent, solid material). Unless otherwise prescribed, all measurements are carried out with reference to the same solvent or the same mixture of solvents (blank).

Measure the blank and the sample within a short time-frame either in parallel in double-beam spectrophotometers or sequentially in single-beam spectrophotometers. The absorbance values of both blank and sample must be in the working range of the equipment as specified by the manufacturer.

For benchtop instruments, the absorbance of the solvent measured against air and at the prescribed wavelength must not exceed 0.4 and is preferably less than 0.2.

For chromatographic systems, the transmittance of the mobile phase may be used as the blank.

In some PAT applications, it may be impossible to remove the probe for background data collection. Various options are therefore to be considered, including the use of internal references, measurement of a blank using a second detector, etc. Only spectra measured against a blank possessing the same optical properties can be directly compared with one another.

For reflectance measurements, common reflectance blank samples include ceramics, fluoropolymers such as polytetrafluoroethylene (PTFE) and powders such as barium sulfate ($BaSO_4$) and magnesium oxide (MgO), but other suitable materials may also be used.

## MATHEMATICAL TREATMENT OF SPECTRAL DATA

In the case of single wavelength analysis used to determine the concentration of an unknown sample (e.g. as prescribed in monographs), mathematical treatment consists in determining the regression of the photometric reading (absorbance) on the concentration of the standard samples.

In the case of full range spectra, data for both diffuse reflection and transmission modes may have to be treated before a classification or calibration model can be developed. The aim can be, for example, to reduce baseline drift or to correct for scatter caused by particle size changes in solid samples. For example, first-, second- or higher-order derivative spectra can typically be used to improve resolution or sensitivity. This pretreatment may be a useful means of simplifying the data and thereby reducing the variations that may cause interference in subsequently applied mathematical models.

A wide range of treatment methods, such as scaling, smoothing, normalisation and derivatisation, can be applied either singly or in combination. More information is available in general chapter *5.21. Chemometric methods applied to analytical data*.

## CONTROL OF EQUIPMENT PERFORMANCE

Spectrophotometer performance is controlled (automatically or manually) at regular intervals as defined in the quality management system and dictated by the use of the equipment and the application. For example, equipment exposed to variations in temperature and humidity may need more frequent performance testing.

Requirements for control of equipment performance for the various measurement modes are summarised in Table 2.2.25.-1. Further such tests may be performed if appropriate.

Wavelength accuracy, absorbance accuracy and linearity are controlled using either certified reference materials such as solid filters or liquid filters in appropriate sealed cells, or solutions prepared in the laboratory as described below.

Table 2.2.25.-1. – *Minimum tests to be carried out for the control of equipment performance*

| Purpose | Method | Wavelength accuracy | Absorbance accuracy | Photometric linearity | Stray light | Resolution/spectral bandwidth |
|---|---|---|---|---|---|---|
| Quantitative or limit test | Based on measurement of the absorbance at one or more identified wavelengths (e.g. assay or impurities test) | X | X | X | X | If required in the monograph |
| Identification test | Based on wavelength of absorption maxima and minima | X | - | - | X | - |
| | Based on absorption measurement and wavelength of absorption maxima | X | X | - | X | - |
| | Based on comparison of spectrum with that of reference substance | X | X | - | - | - |

**Control of wavelength accuracy**. Control the wavelength accuracy of an appropriate number of bands in the intended spectral range using one or more reference materials; for example, use solid or liquid filters (e.g. *holmium perchlorate solution R*) to verify the position of absorption bands, or measure the emission from a light source to check emission-line position. Table 2.2.25.-2 shows examples of wavelengths used to check wavelength accuracy. When certified reference materials are used, the reference wavelength is that stated on the corresponding certificate.

Some instruments may have an automatic or inbuilt wavelength accuracy control feature.

Table 2.2.25.-2. – *Examples of wavelengths used for the control of wavelength accuracy*
*Note: the wavelength varies with the resolution of the instrument

| Material | | Wavelengths (nm)* |
|---|---|---|
| Solutions | Cerium in sulfuric acid | 201.1; 211.4; 222.6; 240.4; 253.7 |
| | Didymium in perchloric acid | 511.8; 731.6; 794.2 |
| | Holmium in perchloric acid | 241.1; 287.2; 361.3; 451.4; 485.2; 536.6; 640.5 |
| Solid filters | Didymium glass | 513.5 |
| | Holmium glass | 279.3; 360.9; 453.4; 637.5 |
| Lamps | Deuterium | 486.0; 656.1 |
| | Mercury (low pressure) | 184.9; 253.7; 312.5; 365.0; 404.7; 435.8; 546.1; 577.0; 579.1 |
| | Neon | 717.4 |
| | Xenon | 541.9; 688.2; 764.2 |

For chromatographic systems, it is also possible to control wavelength accuracy by measuring the absorbance of a 0.05 mg/mL solution of *caffeine R* in *methanol R*; the absorption maximum is obtained at 272 nm and the minimum at 244 nm.

*Acceptance criteria*

It is recommended to test at least 2 wavelengths that bracket the intended spectral range.

For benchtop instruments, the tolerance for wavelength accuracy of UV-Vis spectroscopy in cuvettes is ± 1 nm at wavelengths below 400 nm and ± 3 nm at wavelengths of 400 nm and above.

For chromatographic systems, the tolerance for wavelength accuracy is ± 2 nm for the whole UV-Vis range.

For PAT applications, a tolerance of ± 2 nm for the UV-Vis range is recommended. However, wider tolerance intervals may be needed for some PAT applications, in which case the requisite wavelength accuracy must be defined by the user depending on the intended purpose, and using a risk-based approach.

The instrument parameters (especially the entrance optics such as slit-width or optical fibre diameter) influence the resolution and must be the same as those intended for the actual measurements.

**Control of absorbance accuracy**. Control the absorbance accuracy at an appropriate number of wavelengths in the intended spectral range, using suitable solid or liquid filters to check that the absorbance measured at the test wavelength matches the certified absorbance of the filter or the absorbance value that is calculated from a certified specific absorbance. *Nicotinic acid for equipment qualification CRS* may be used.

It is recommended to test absorbance accuracy at selected wavelengths using one or more solid or liquid filters with different absorbance levels; as a minimum, values at approximately the 2 limits of the expected absorbance range should be verified.

For chromatographic systems and PAT applications, the testing of absolute absorbance accuracy may not be necessary, providing that a standard curve is measured as required.

For measurements using *nicotinic acid for equipment qualification CRS*, the certified specific absorbance is given in the corresponding leaflet.

The solution of nicotinic acid can be prepared as follows: dissolve 57.0-63.0 mg of *nicotinic acid for equipment qualification CRS* in a 0.1 M hydrochloric acid solution prepared from *hydrochloric acid R* and dilute to 200.0 mL with the same acid solution; dilute 2.0 mL of the solution to 50.0 mL with the same acid solution to obtain a final concentration of 12 mg/L. These volumes can be adjusted to obtain nicotinic acid solutions with other concentrations (up to about 40 mg/L), for the purposes of testing different absorbance levels. The absorbance is measured at 213 nm and 261 nm.

*Acceptance criteria*

The difference between the measured absorbance and the absorbance of the certified material is ± 0.010 or ± 1 per cent, whichever is greater, for each combination of wavelength and absorbance assessed (applies to absorbance values not greater than 2). Tolerances for higher absorbance values should be defined on the basis of a risk assessment.

**Control of photometric linearity**. Control the photometric linearity in the intended spectral range. In the ultraviolet range, the filters used to control absorbance accuracy may be used, as can solutions of nicotinic acid or caffeine. In the visible range, neutral glass filters may be used. Prior to performing the test, ensure that the absorbance of the standards is compatible with the intended linear range.

Solutions with increasing concentrations (e.g. 5-40 mg/L) of *nicotinic acid for equipment qualification CRS* in a 0.1 M hydrochloric acid solution prepared from *hydrochloric acid R* may be used. The absorbance is measured at 213 nm and 261 nm.

For chromatographic systems, it is also possible to check photometric linearity using 0.5-50 mg/L solutions of *caffeine R* in *water for chromatography R*. The absorbance is measured at 273 nm.

*Acceptance criterion*

The coefficient of determination ($R^2$) is not less than 0.999.

**Limit of stray light**. Stray light is determined at an appropriate wavelength using suitable solid or liquid filters or solutions prepared in-house. The instrument parameters used for the test, such as slit-width and type of light source (e.g. deuterium or tungsten lamp), must be the same as those intended for the actual measurements.

*Acceptance criterion*

The acceptance criterion depends on the filters or solutions used, for example:

- the absorbance is not less than 3.0 when using a 10 g/L solution of *sodium iodide R* at 220 nm, a 10 g/L solution of *potassium iodide R* at 250 nm or a 50 g/L solution of *sodium nitrite R* at 340 nm and 370 nm;
- the absorbance is not less than 2.0 when using a 12 g/L solution of *potassium chloride R* at 198 nm.

These values apply when using a 1 cm cell and *water R* as the compensation liquid.

**Control of resolution**. Where prescribed in a monograph, measure the resolution of the equipment either using suitable certified reference materials, or by recording the spectrum of a 0.02 per cent *V/V* solution of *toluene R* in *hexane R* or *heptane R*, with respectively *hexane R* or *heptane R* as the compensation liquid.

*Acceptance criterion*

For measurements taken with a solution prepared as described above, the minimum ratio of the absorbance at the maximum (269 nm) to that at the minimum (266 nm) is stated in the monograph.

SYSTEM SUITABILITY

System suitability tests may be required prior to sample measurement to verify critical parameters that may have an impact on the result.

These tests may cover wavelength accuracy, absorbance accuracy, stray light and photometric linearity. System functionality tests, for example those performed as part of equipment autotesting, may be considered part of the system suitability tests.

In the case of UV-Vis detection for chromatographic systems, additional system suitability tests are applicable if prescribed in the monograph and/or in general chapter *2.2.46. Chromatographic separation techniques*.

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# 2.2.26. PAPER CHROMATOGRAPHY

ASCENDING PAPER CHROMATOGRAPHY

*Apparatus*. The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting lid. In the top of the tank is a device which suspends the chromatographic paper and is capable of being lowered without opening the chamber. In the bottom of the tank is a dish to contain the mobile phase into which the paper may be lowered. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length and not less than 2.5 cm wide; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

*Method*. Place in the dish a layer 2.5 cm deep of the mobile phase prescribed in the monograph. If prescribed in the monograph, pour the stationary phase between the walls of the tank and the dish. Close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper 3 cm from one end. Using a micro pipette, apply to a spot on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper into the tank, close the lid and allow to stand for 1 h 30 min. Lower the paper into the mobile phase and allow elution to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. Protect the paper from bright light during the elution process.

DESCENDING PAPER CHROMATOGRAPHY

*Apparatus*. The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting glass lid. The lid has a central hole about 1.5 cm in diameter closed by a heavy glass plate or a stopper. In the upper part of the tank is suspended a solvent trough with a device for holding the chromatographic paper. On each side of the trough, parallel to and slightly above its upper edges, are two glass guide rods to support the paper in such a manner that no part of it is in contact with the walls of the tank. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

*Method*. Place in the bottom of the tank a layer 2.5 cm deep of the solvent prescribed in the monograph, close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper at such a distance from one end that when this end is secured in the solvent trough and the remainder of the paper is hanging freely over the guide rod, the line is a few centimetres below the guide rod and parallel with it. Using a micro-pipette, apply on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions, allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper in the tank, close the lid, and allow to stand for 1 h 30 min. Introduce into the solvent trough, through the hole in the lid, a sufficient quantity of the mobile phase, close the tank and allow elution

to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. The paper should be protected from bright light during the elution process.

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## 2.2.27. THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase).

### APPARATUS

**Plates**. The chromatography is carried out using pre-coated plates as described under *Reagents* (*4.1.1*). The particle size of the silica gel is indicated after the name of the reagent in the tests where it is used.

*Pre-treatment of the plates*. It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120 °C for 20 min.

**Chromatographic tank** with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates used and provided with a tightly fitting lid. For horizontal development the tank is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

**Micropipettes, microsyringes, calibrated disposable capillaries** or other application devices suitable for the proper application of the solutions.

**Fluorescence detection device** to measure direct fluorescence or the inhibition of fluorescence.

**Visualisation devices and reagents**. Suitable devices are used for derivatisation to transfer to the plate reagents by spraying, immersion or exposure to vapour and, where applicable, to facilitate heating for visualisation of separated components.

**Documentation**. A device may be used to provide documentation of the visualised chromatogram, for example a photograph or a computer file.

### METHOD

**Sample application**. Apply the prescribed volume of the solutions at a suitable distance from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on high-performance plates) between the centres of circular spots and 5 mm (2 mm on high-performance plates) between the edges of bands. Apply the solutions in sufficiently small portions to obtain circular spots 2-5 mm in diameter (1-2 mm on high-performance plates) or bands 10-20 mm (5-10 mm on high-performance plates) by 1-2 mm.

In a monograph, where both normal and high-performance plates may be used, the working conditions for high-performance plates are given in the brackets [ ] after those for normal plates.

**Vertical development**. Line the walls of the chromatographic tank with filter paper. Pour into the chromatographic tank a sufficient quantity of the mobile phase for the size of the tank to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the chromatographic tank, replace the lid and allow to stand at 20-25 °C for 1 h. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated tank. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the chromatographic tank, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic tank, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance, measured between the points of application and the solvent front. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

**Horizontal development**. Apply the prescribed volume of the solutions as described above. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate in the chamber after verifying that the latter is horizontal and connect the mobile phase direction device according to the manufacturer's instructions. If prescribed, develop the plate starting simultaneously at both ends. Close the chamber and maintain it at 20-25 °C. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

### VISUAL EVALUATION

**Identification**. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by comparing the colour, the size and the retardation factor ($R_F$) of both spots.

The retardation factor ($R_F$) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

*Verification of the separating power for identification*. Normally the performance given by the suitability test described in *Reagents* (*4.1.1*) is sufficient. Only in special cases an additional performance criterion is prescribed in the monograph.

**Related substances test**. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity(ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

*Verification of the separating power*. The requirements for the verification of the separating power are prescribed in the monographs concerned.

*Verification of the detecting power*. The detecting power is satisfactory if a spot or band is clearly visible in the chromatogram obtained with the most dilute reference solution.

### QUANTITATIVE MEASUREMENT

The requirements for resolution and separation are prescribed in the monographs concerned.

Substances separated by thin-layer chromatography and responding to UV-Vis irradiation can be determined directly on the plate, using appropriate instrumentation. While moving the plate or the measuring device, examine the plate by measuring the reflectance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in 3 ways: either directly by moving the plate alongside a suitable counter or vice versa (see *Radiopharmaceutical preparations (0125)*), by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter or by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail and measuring the radioactivity using a liquid scintillation counter.

**Apparatus**. The apparatus for direct measurement on the plate consists of:

- a device for exact positioning and reproducible dispensing of the amount of substances onto the plate;
- a mechanical device to move the plate or the measuring device along the *x*-axis or the *y*-axis;
- a recorder and a suitable integrator or a computer;
- *for substances responding to UV-Vis irradiation*: a photometer with a source of light, an optical device able to generate monochromatic light and a photo cell of adequate sensitivity are used for the measurement of reflectance or transmittance; if fluorescence is measured, a suitable filter is required to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass;
- *for substances containing radionuclides*: a suitable counter for radioactivity. The linearity range of the counting device is to be verified.

**Method**. Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph and, if necessary, prepare the reference solutions of the substance to be determined using the same solvent as in the test solution. Apply the same volume of each solution to the plate and develop.

*Substances responding to UV-Vis irradiation*. Prepare and apply not fewer than 3 reference solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (about 80 per cent, 100 per cent and 120 per cent). Treat with the prescribed reagent, if necessary, and record the reflectance, the transmittance or fluorescence in the chromatograms obtained with the test and reference solutions. Use the measured results for the calculation of the amount of substance in the test solution.

*Substances containing radionuclides*. Prepare and apply a test solution containing about 100 per cent of the expected value. Determine the radioactivity as a function of the path length and report the radioactivity in each resulting peak as a percentage of the total amount of radioactivity.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (*2.2.46*). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

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# 2.2.28. GAS CHROMATOGRAPHY

PRINCIPLE

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between 2 non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. It is applicable to substances or their derivatives which are volatilised under the temperatures employed.

GC is mainly based on mechanisms of adsorption or mass distribution.

EQUIPMENT

The equipment typically consists of:

- an injector;
- a chromatographic column contained in an oven;
- one or more detector(s);
- a data acquisition system.

The carrier gas flows through the column and then through the detector at a controlled rate or pressure.

The chromatography is carried out either at a constant temperature or according to a given temperature programme.

*INJECTORS*

Injection may be carried out either into a vaporisation chamber which may be equipped with a stream splitter, or directly at the head of the column using a syringe or an injection valve.

*Injections of vapour phase* may be effected by static or dynamic head-space injection systems.

*Dynamic head-space* (purge and trap) injection systems include a sparging device by which volatile substances in solution are swept into an absorbent column maintained at a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the absorbent column.

*Static head-space* injection systems include a thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow equilibration of the volatile components of the sample between the non-gaseous phase and the vapour phase. After equilibration, a predetermined amount of the head-space of the vial is flushed into the gas chromatograph.

*STATIONARY PHASES*

Stationary phases are contained in columns which may be:

- a capillary column whose stationary phase may be a solid coating the inner surface of the column (e.g. macrogol 20 000), or a liquid deposited on the inner surface (e.g. dimethylpolysiloxane); in the latter case it may be chemically bonded to the inner surface;
- a column packed with the stationary phase which may be a solid phase (e.g. alumina, silica) or an inert solid support (usually a porous polymer) impregnated or coated with a liquid.

Capillary columns, made of fused silica, are 0.1 mm to 0.53 mm in internal diameter (Ø) and at least 5 m in length. The stationary phase is a film 0.1 µm to 5.0 µm thick.

Packed columns, made of glass or metal, are usually 1 m to 3 m in length with an internal diameter (Ø) of 2 mm to 4 mm.

*MOBILE PHASES*

Retention time and peak efficiency depend on the carrier gas flow rate; retention time is directly proportional to column length and resolution is proportional to the square root of the column length.

The carrier gas flow rate is usually expressed in millilitres per minute at atmospheric pressure and at the stated temperature. Flow rate is measured at the detector outlet, either with a calibrated mechanical device or with a bubble tube, while the column is at operating temperature.

The linear velocity of the carrier gas through a column is inversely proportional to the square of the internal diameter of the column for a given flow volume.

Helium, nitrogen and hydrogen are commonly used carrier gases.

*DETECTORS*

Flame-ionisation detectors are usually employed but other detectors such as electron-capture, nitrogen-phosphorus, mass spectrometric, thermal conductivity or infrared spectrophotometric detectors may also be used.

PROCEDURE

Equilibrate the column, the injector and the detector at the temperatures and the gas flow rates/pressures specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions injected must be free from solid particles.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46 Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this general chapter.

## Static head-space gas chromatography

Static head-space gas chromatography is a technique particularly suitable for separating and determining volatile compounds present in solid or liquid samples. The method is based on the analysis of the vapour phase in equilibrium with the solid or liquid phase.

EQUIPMENT

The equipment consists of a gas chromatograph provided with a sample-introduction device that may be connected to a module that automatically controls the pressure and the temperature. If necessary, a device for eliminating solvents can be added.

The sample to be analysed is introduced into a container fitted with a suitable stopper and a valve-system which permits the passage of the carrier gas. The container is placed in a thermostatically controlled chamber at a temperature set according to the substance to be examined.

The sample is held at this temperature long enough to allow equilibration between the solid or liquid phase and the vapour phase.

The carrier gas is introduced into the container and, after the prescribed time, a suitable valve is opened so that the gas expands towards the chromatographic column taking the volatilised compounds with it.

Instead of using a chromatograph specifically equipped for the introduction of samples, it is also possible to use airtight syringes and a conventional chromatograph. Equilibration is then carried out in a separate chamber and the vapour phase is carried onto the column, while necessary precautions are taken to avoid any changes in the equilibrium.

PROCEDURE

Using the reference preparations, determine suitable instrument settings to produce an adequate response.

*DIRECT CALIBRATION*

Introduce into separate, identical containers the preparation to be examined and each of the reference preparations, as prescribed in the monograph, avoiding contact between the sampling device and the samples.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

*STANDARD ADDITIONS*

Add to a set of identical suitable containers equal volumes of the preparation to be examined. Add to all but one of the containers, suitable quantities of a reference preparation containing a known concentration of the substance to be determined so as to produce a series of preparations containing steadily increasing concentrations of the substance.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the substance to be determined in the preparation to be examined.

Alternatively, plot on a graph the mean of readings against the added quantity of the substance to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the substance to be determined in the preparation to be examined.

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# 2.2.29. LIQUID CHROMATOGRAPHY

PRINCIPLE

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between 2 non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase contained in a column.

LC is mainly based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion or stereochemical interaction.

Unless otherwise specified, all the information below is valid for both standard LC and LC using reduced particle-size columns (e.g. sub-2 µm).

The latter requires instrumentation that is able to withstand higher pressures (typically up to 100 MPa, i.e. about 15 000 psi), generates lower extra-column band broadening, provides improved gradient mixing and allows a higher sampling rate in the detection system.

EQUIPMENT

The equipment typically consists of:

- a pumping system;
- an injector;
- a chromatographic column (a column temperature controller may be used);
- 1 or more detector(s);
- a data acquisition system.

The mobile phase is supplied from 1 or more reservoirs and is pumped to the injector, then through the column, usually at a constant rate, and then through the detector(s).

*PUMPING SYSTEMS*

LC pumping systems deliver the mobile phase at a controlled flow rate. Pressure fluctuations are to be minimised, for example by passing the pressurised solvent through a pulse-dampening device. Tubing and connections are capable of withstanding the pressures developed by the pumping system. LC pumps may be fitted with a facility for 'bleeding' the system of entrapped air bubbles.

Microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

*INJECTORS*

The sample solution is introduced into the flowing mobile phase at or near the head of the column using an injection system which can operate at high pressure. Fixed-loop

and variable volume devices operated manually or by an autosampler are used. Partial filling of loops during manual injection may adversely affect injection volume precision.

*STATIONARY PHASES*

There are many types of stationary phases employed in LC, including:

- silica or alumina, commonly used in normal-phase LC (polar stationary phase and non-polar mobile phase), where the separation is based on differences in adsorption on the stationary phase and/or mass distribution between the mobile phase and the stationary phase (partition chromatography);
- a variety of chemically modified supports prepared from polymers, silica or porous graphite, used in normal-phase and reversed-phase LC (non-polar stationary phase and polar mobile phase), where the separation is based principally on partition of the molecules;
- resins or polymers with acidic or basic groups, used in ion-exchange chromatography, where separation is based on competition between the ions to be separated and those in the mobile phase;
- porous silica or polymers, used in size-exclusion chromatography (*2.2.30*), where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion;
- specially modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins etc., for the separation of enantiomers (chiral chromatography).

Most separations are based on reversed-phase LC utilising chemically modified silica as the stationary phase. The surface of the support, i.e. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Unless otherwise stated by the manufacturer, silica-based reversed-phase columns are considered to be stable in mobile phases having an apparent pH in the range 2.0 to 8.0. Columns containing porous graphite or particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase LC with unmodified silica or polar chemically modified silica (e.g. cyanopropyl or diol) as the stationary phase, with a non-polar mobile phase is applicable in certain cases.

For analytical separations, the particle size of the most commonly used stationary phases varies between 2 and 10 µm. The particles may be spherical or irregular, and of varying porosity and specific surface area. These properties contribute to the chromatographic behaviour of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g. expressed as the carbon loading, and whether the stationary phase is end-capped (i.e. part of the residual silanol groups are silylated) are additional determining factors. Tailing of peaks, particularly of basic substances, can occur when residual silanol groups are present.

In addition to porous particles, superficially porous or monolithic materials may be used.

Unless otherwise prescribed in the monograph, columns made of stainless steel of varying length and internal diameter (Ø) are used for analytical chromatography. Columns with internal diameters of less than 2 mm are often referred to as microbore columns.

The temperature of the mobile phase and the column must be kept constant during the analysis. Most separations are performed at room temperature, but some require a different temperature for optimal performance.

*MOBILE PHASES*

For normal-phase LC, low-polarity organic solvents are generally employed. The residual water content of the solvents used in the mobile phase is to be strictly controlled to obtain reproducible results.

In reversed-phase LC, aqueous mobile phases, usually with organic solvents and/or modifiers, are employed.

The components of the mobile phase are usually filtered to remove particles greater than 0.45 µm in size (or greater than 0.2 µm when the stationary phase is made of sub-2 µm particles, and when special detectors, e.g. light scattering detectors, are used). Multicomponent mobile phases are prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by mixing. Alternatively, the solvents may be delivered by individual pumps controlled by proportioning valves, by which mixing is performed according to the desired proportion. Solvents are normally degassed before pumping by sparging with helium, sonication and/or using in-line membrane/vacuum modules to avoid the creation of gas bubbles in the detector cell.

Solvents for the preparation of the mobile phase are normally free of stabilisers and, if an ultraviolet detector is employed, are transparent at the wavelength of detection. Solvents and other components employed are to be of appropriate quality. In particular, *water for chromatography R* is used for the preparation of mobile phases when water, or an aqueous solution, is 1 of the components. Any necessary adjustments of the pH are made to the aqueous component of the mobile phase and not the mixture. If buffer solutions or saline solutions are used, adequate rinsing of the system is carried out with a mixture of water and a small proportion of the organic part of the mobile phase (5 per cent *V/V*) to prevent crystallisation of salts after completion of the analysis.

Mobile phases may contain other components, for example a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase.

*DETECTORS*

Ultraviolet/visible (UV/Vis) spectrophotometers (including diode array detectors) (*2.2.25*), are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors (ECD), light scattering detectors, charged aerosol detectors (CAD), mass spectrometers (MS) (*2.2.43*), radioactivity detectors, multi-angle light scattering (MALS) detectors or other detectors may be used.

PROCEDURE

Equilibrate the column with the prescribed mobile phase and flow rate, at room temperature or at the temperature specified in the monograph, until a stable baseline is achieved. Prepare the solution(s) of the substance to be examined and the reference solution(s) required. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

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## 2.2.30. SIZE-EXCLUSION CHROMATOGRAPHY

PRINCIPLE

Size-exclusion chromatography is a liquid chromatography (*2.2.29*) technique which separates molecules in solution according to their size. With organic mobile phases, the technique is known as gel-permeation chromatography and with aqueous mobile phases it is known as gel-filtration chromatography. The sample is introduced into a column, which is filled with a gel or a porous particle packing material, and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the packing material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the total mobile phase volume $V_t$ (also known as total permeation volume). Molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the retention volume of an unretained compound $V_0$ (also known as exclusion volume or void volume). Separation according to molecular size occurs between the retention volume of an unretained compound and the total mobile phase volume, with useful separation usually occurring in the first two thirds of this range.

EQUIPMENT

The equipment consists essentially of a chromatographic column of varying length and internal diameter (Ø), if necessary temperature-controlled, packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes. The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurised systems giving faster separations.

One end of the column is usually fitted with a suitable device for applying the sample such as a flow adapter, a syringe through a septum or an injection valve and may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively, the sample may be applied directly to the drained bed surface or, where the sample is denser than the eluent, it may be layered beneath the eluent.

The mobile phase is chosen according to sample type, separation medium and method of detection. The eluent is passed through the column at a constant rate.

The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder which enables the monitoring of the relative concentrations of separated components of the sample. Ultraviolet/visible spectrophotometers (*2.2.25*), differential refractometers (RI), luminescent detectors, multi-angle light scattering (MALS) detectors and other detectors may be used. An automatic fraction collector may be attached, if necessary.

PROCEDURE

Before carrying out the separation, the packing material is treated, and the column is packed, as described in the monograph, or according to the manufacturer's instructions. Criteria for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this general chapter.

*DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURES*

Carry out the separation as stated in the monograph. If possible, monitor the elution of the components continuously and measure the corresponding peak areas. If the sample is monitored by a physico-chemical property to which all the components of interest exhibit equivalent responses (for example if they have the same specific absorbance), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components of interest. If the responses to the property used for detection of the components of interest are not equivalent, calculate the content by means of calibration curves obtained with the calibration standards prescribed in the monograph.

*DETERMINATION OF MOLECULAR MASSES*

Size-exclusion chromatography may be used to determine molecular masses by comparison with appropriate calibration standards specified in the monograph. The retention volumes of the calibration standards may be plotted against the logarithm of their molecular masses. The plot usually approximates a straight line within the exclusion and total permeation limits for the separation medium used. From the calibration curve, molecular masses may be estimated. The molecular-mass calibration is valid only for the particular macromolecular solute/solvent system used under the specified experimental conditions.

*DETERMINATION OF MOLECULAR SIZE DISTRIBUTION OF POLYMERS*

Size-exclusion chromatography may be used to determine the distribution of the molecular size of polymers. However, sample comparison may be valid only for results obtained under the same experimental conditions. The reference standards used for the calibration and the methods for determination of the distribution of molecular sizes of polymers are specified in the monograph.

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## 2.2.31. ELECTROPHORESIS[(2)]

♦1. GENERAL PRINCIPLE

Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Because of their different physico-chemical properties, different macromolecules of a mixture will migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g. free solution separation in capillary electrophoresis) and in stabilising media such as thin-layer plates, films or gels.

2. FREE OR MOVING BOUNDARY ELECTROPHORESIS

This method is mainly used for the determination of mobility, the experimental characteristics being directly measurable and reproducible. It is chiefly employed with substances of high

(2) This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. *Pharmacopoeial harmonisation*.

relative molecular mass and low diffusibility. The boundaries are initially located by a physical process such as refractometry or conductimetry. After applying a given electric field for an accurately measured time, the new boundaries and their respective positions are observed. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

3. ZONE ELECTROPHORESIS USING A SUPPORTING MEDIUM

This method requires the use of small samples only.

The nature of the support, such as paper, agar gel, cellulose acetate, starch, agarose, methacrylamide, mixed gel, introduces a number of additional factors modifying the mobility:

a) owing to channelling in the supporting medium, the apparent distance covered is less than the real distance,

b) some supporting media are not electrically neutral. As the medium is a stationary phase it may sometimes give rise to a considerable electro-endosmotic flow,

c) any heating due to the joule effect may cause some evaporation of the liquid from the supporting medium which, by capillarity, causes the solution to move from the ends towards the centre. The ionic strength therefore tends to increase gradually.

The rate of migration then depends on four main factors: the mobility of the charged particle, the electro-endosmotic flow, the evaporation flow, and the field strength. Hence it is necessary to operate under clearly defined experimental conditions and to use, wherever possible, reference substances.

An *apparatus* for electrophoresis consists of:

– a *generator supplying direct current* whose voltage can be controlled and, preferably, stabilised,

– an *electrophoresis chamber*. This is usually rectangular and made of glass or rigid plastic, with 2 separate compartments, the anodic and the cathodic, containing the electrolyte solution. In each compartment is immersed an electrode, for example of platinum or graphite. These are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the 2 compartments is kept equal to prevent siphoning.

  The electrophoresis chamber is fitted with an airtight lid which maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is preferable to cool the support.

– a *support-carrying device*:

  *Strip electrophoresis*. The supporting strip, previously wetted with the same conducting solution and dipped at each end into an electrode compartment is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte, such as a horizontal frame, inverted-V stand or a uniform surface with contact points at suitable intervals.

  *Gel electrophoresis*. The device consists essentially of a glass plate (for example, a microscope slide) over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions must be taken to avoid condensation of moisture or drying of the solid layer.

– measuring *device or means of detection*.

*Method*. Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. After the current has been switched off, remove the support from the chamber, dry and visualise.

4. POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N,N´*-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution being applied to each rod.

*Apparatus*. This consists of 2 buffer solution reservoirs made of suitable material such as poly(methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply allowing operation either at constant current or at constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

*Method*. The solutions should usually be degassed before polymerisation and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water R* to exclude air and allow to set. Gel formation usually takes about 30 min and is complete when a sharp interface appears between the gel and the water layer. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution. Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose R*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electropherogram as prescribed. ♦

5. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

5-1. *SDS-PAGE - UNIFORM PERCENTAGE GELS*

**Scope**. Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and for quantitative determinations.

**Purpose**. Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins.

Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

5-1-1. **Characteristics of polyacrylamide gels**

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerisation is usually catalysed by a free radical-generating system composed of ammonium persulfate and *N,N,N´,N´*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, i.e. by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration and the pH of the buffer, by the temperature and the field strength, and by the nature of the support material.

5-1-2. **Denaturing polyacrylamide gel electrophoresis**

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14 000 to 100 000 daltons. It is possible to extend this mass range using various techniques (e.g. gradient gels, particular buffer system). For instance, tricine-SDS gels, with tricine as the trailing ion in the electrophoresis running-buffer (instead of glycine as in the method described here), can separate very small proteins and peptides under 10 000 to 15 000 daltons.

Denaturing polyacrylamide gel electrophoresis using glycine-SDS is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes will migrate toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, can change the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

Depending on the extent of glycosylation and other post-translational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

**Reducing conditions**. Polypeptide subunits and three-dimensional structure are often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, the molecular mass of the polypeptide subunits can reasonably be calculated by linear regression (or, more accurately, by non-linear regression) with the aid of suitable molecular-mass standards.

**Non-reducing conditions**. For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. Moreover, intra-chain disulfide bonds constrain the molecular shape, usually in such a way as to reduce the Stokes radius of the molecule, thereby reducing the apparent molecular mass $M_r$. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

5-1-3. **Characteristics of discontinuous buffer system gel electrophoresis**

The most popular electrophoretic method for the characterisation of complex mixtures of proteins uses a discontinuous buffer system involving 2 contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The 2 gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anti-convective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to the focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

5-1-4. **Preparing vertical discontinuous buffer SDS polyacrylamide gels**

This section describes the preparation of gels using particular instrumentation. This does not apply to pre-cast gels. For pre-cast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and where the purity of the reagents used is not sufficient, a pre-treatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionised with a mixed-bed (anion/cation exchange) resin to remove acrylic acid and

other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

**Assembling the gel moulding cassette**. Clean the 2 glass plates (e.g. 10 cm by 8 cm in size), the polytetrafluoroethylene comb, the 2 spacers and the silicone rubber tubing (e.g. 0.6 mm in diameter by 35 cm) with mild detergent and rinse extensively with water, followed by anhydrous ethanol, and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the 2 short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with 1 finger along the long side, twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply 2 clamps on each of the 2 short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing runs along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

**Preparation of the gel**. In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the 2 gels in acrylamide-bisacrylamide, buffer and pH are different.

*Preparation of the resolving gel*. In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 2.2.31.-1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.2.31.-1, swirl and pour immediately into the gap between the 2 glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerisation.

Table 2.2.31.-1. – *Preparation of resolving gel*

| Solution components | Component volumes (mL) per gel mould volume of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 mL | 10 mL | 15 mL | 20 mL | 25 mL | 30 mL | 40 mL | 50 mL |
| **6 per cent acrylamide** | | | | | | | | |
| *Water R* | 2.6 | 5.3 | 7.9 | 10.6 | 13.2 | 15.9 | 21.2 | 26.5 |
| Acrylamide solution[1] | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 8.0 | 10.0 |
| 1.5 M Tris (pH 8.8)[2] | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS[3] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS[4] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED[5] | 0.004 | 0.008 | 0.012 | 0.016 | 0.02 | 0.024 | 0.032 | 0.04 |
| **8 per cent acrylamide** | | | | | | | | |
| *Water R* | 2.3 | 4.6 | 6.9 | 9.3 | 11.5 | 13.9 | 18.5 | 23.2 |
| Acrylamide solution[1] | 1.3 | 2.7 | 4.0 | 5.3 | 6.7 | 8.0 | 10.7 | 13.3 |
| 1.5 M Tris (pH 8.8)[2] | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS[3] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS[4] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED[5] | 0.003 | 0.006 | 0.009 | 0.012 | 0.015 | 0.018 | 0.024 | 0.03 |
| **10 per cent acrylamide** | | | | | | | | |
| *Water R* | 1.9 | 4.0 | 5.9 | 7.9 | 9.9 | 11.9 | 15.9 | 19.8 |
| Acrylamide solution[1] | 1.7 | 3.3 | 5.0 | 6.7 | 8.3 | 10.0 | 13.3 | 16.7 |
| 1.5 M Tris (pH 8.8)[2] | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS[3] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS[4] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED[5] | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |
| **12 per cent acrylamide** | | | | | | | | |
| *Water R* | 1.6 | 3.3 | 4.9 | 6.6 | 8.2 | 9.9 | 13.2 | 16.5 |
| Acrylamide solution[1] | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 | 12.0 | 16.0 | 20.0 |
| 1.5 M Tris (pH 8.8)[2] | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS[3] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS[4] | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED[5] | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

| Solution components | Component volumes (mL) per gel mould volume of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 5 mL | 10 mL | 15 mL | 20 mL | 25 mL | 30 mL | 40 mL | 50 mL |
| **14 per cent acrylamide** | | | | | | | | |
| *Water R* | 1.4 | 2.7 | 3.9 | 5.3 | 6.6 | 8.0 | 10.6 | 13.8 |
| Acrylamide solution(1) | 2.3 | 4.6 | 7.0 | 9.3 | 11.6 | 13.9 | 18.6 | 23.2 |
| 1.5 M Tris (pH 8.8)(2) | 1.2 | 2.5 | 3.6 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS(3) | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS(4) | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED(5) | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |
| **15 per cent acrylamide** | | | | | | | | |
| *Water R* | 1.1 | 2.3 | 3.4 | 4.6 | 5.7 | 6.9 | 9.2 | 11.5 |
| Acrylamide solution(1) | 2.5 | 5.0 | 7.5 | 10.0 | 12.5 | 15.0 | 20.0 | 25.0 |
| 1.5 M Tris (pH 8.8)(2) | 1.3 | 2.5 | 3.8 | 5.0 | 6.3 | 7.5 | 10.0 | 12.5 |
| 100 g/L SDS(3) | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| 100 g/L APS(4) | 0.05 | 0.1 | 0.15 | 0.2 | 0.25 | 0.3 | 0.4 | 0.5 |
| TEMED(5) | 0.002 | 0.004 | 0.006 | 0.008 | 0.01 | 0.012 | 0.016 | 0.02 |

(1) Acrylamide solution: *30 per cent acrylamide/bisacrylamide (29:1) solution R.*

(2) 1.5 M Tris (pH 8.8): *1.5 M tris-hydrochloride buffer solution pH 8.8 R.*

(3) 100 g/L SDS: a 100 g/L solution of *sodium dodecyl sulfate R.*

(4) 100 g/L APS: a 100 g/L solution of *ammonium persulfate R.* Ammonium persulfate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.

(5) TEMED: *tetramethylethylenediamine R.*

*Preparation of the stacking gel.* After polymerisation is complete (about 30 min), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2.2.31.-2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.2.31.-2. Swirl and pour immediately into the gap between the 2 glass plates of the mould directly onto the surface of the polymerised resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerise at room temperature.

**Preparation of the samples**. Unless otherwise stated in the specific monograph, the samples can be prepared as follows:

*Preparation of the samples (non-reducing conditions).* Mix equal volumes of a mixture comprising *water R* plus the preparation to be examined or the reference preparation, and *concentrated SDS-PAGE sample buffer R.*

*Preparation of the samples (reducing conditions).* Mix equal volumes of a mixture comprising *water R* plus the preparation to be examined or the reference preparation, and *concentrated SDS-PAGE sample buffer for reducing conditions R* containing 2-ME (or DTT) as the reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: keep for 5 min in a water-bath or in a block heater set at 100 °C, then cool. The temperature and time in the monograph may vary as protein cleavage may occur during the heat treatment.

Table 2.2.31.-2. – *Preparation of stacking gel*

| Solution components | Component volumes (mL) per gel mould volume of | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 mL | 2 mL | 3 mL | 4 mL | 5 mL | 6 mL | 8 mL | 10 mL |
| *Water R* | 0.68 | 1.4 | 2.1 | 2.7 | 3.4 | 4.1 | 5.5 | 6.8 |
| Acrylamide solution(1) | 0.17 | 0.33 | 0.5 | 0.67 | 0.83 | 1.0 | 1.3 | 1.7 |
| 1.0 M Tris (pH 6.8)(2) | 0.13 | 0.25 | 0.38 | 0.5 | 0.63 | 0.75 | 1.0 | 1.25 |
| 100 g/L SDS(3) | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.08 | 0.1 |
| 100 g/L APS(4) | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.08 | 0.1 |
| TEMED(5) | 0.001 | 0.002 | 0.003 | 0.004 | 0.005 | 0.006 | 0.008 | 0.01 |

(1) Acrylamide solution: *30 per cent acrylamide/bisacrylamide (29:1) solution R.*

(2) 1.0 M Tris (pH 6.8): *1 M tris-hydrochloride buffer solution pH 6.8 R.*

(3) 100 g/L SDS: a 100 g/L solution of *sodium dodecyl sulfate R.*

(4) 100 g/L APS: a 100 g/L solution of *ammonium persulfate R.* Ammonium persulfate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.

(5) TEMED: *tetramethylethylenediamine R.*

**Mounting the gel in the electrophoresis apparatus and electrophoretic separation**. After polymerisation is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE running buffer R* to remove any unpolymerised acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample carefully rinse each well with *SDS-PAGE running buffer R*. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness and electrophoresis running time and current/voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

5-2. *SDS-PAGE - GRADIENT CONCENTRATION GELS*

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols.

Gradient gels offer some advantages, as some proteins which co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis, the proteins migrate until the pore size prevents further progress and a stacking effect therefore occurs, resulting in sharper bands. As shown in Table 2.2.31.-3, gradient gels also allow separation of proteins with a wider range of molecular masses compared to a single fixed concentration gel.

The table gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular mass ranges. Note that other gradient shapes (e.g. concave) can be prepared for specific applications.

Table 2.2.31.-3

| Acrylamide (per cent) | Protein range (kDa) |
|---|---|
| 5 - 15 | 20 - 250 |
| 5 - 20 | 10 - 200 |
| 10 - 20 | 10 - 150 |
| 8 - 20 | 8 - 150 |

Gradient gels are also used for the determination of molecular mass and protein purity.

5-3. *DETECTION OF PROTEINS IN GELS*

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods and commercial kits are available. For example, fluorescent stains are visualised using a fluorescent imager and often provide a linear response over a wide range of protein concentrations, often several orders of magnitude depending on the protein. Coomassie staining has a protein detection level of approximately 1 µg to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of 1 or 2 orders of magnitude has sometimes been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining; however, the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e. the point at which the staining is deemed satisfactory. The use of dynamic ranges of reference proteins is very important as they help assess the intra-experimental sensitivity and linearity. All gel staining steps are carried out while wearing gloves, at room temperature, with gentle shaking (e.g. on an orbital shaker platform) and using any convenient container.

**Coomassie staining**. Immerse the gel in a large excess of *Coomassie staining solution R* and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *destaining solution R*. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the *destaining solution R*.

*NOTE: the acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid R, 4 volumes of methanol R and 5 volumes of water R for 1 h before it is immersed in the Coomassie staining solution R.*

**Silver staining**. Immerse the gel in a large excess of *fixing solution R* and allow to stand for 1 h. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 h or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of *water R* for 1 h. Soak the gel for 15 min in a 1 per cent *V/V* solution of *glutaraldehyde R*. Wash the gel twice for 15 min in a large excess of *water R*. Soak the gel in fresh *silver nitrate reagent R* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of *water R*. Immerse the gel for about 1 min in *developer solution R* until satisfactory staining has been obtained. Stop the development by incubation in the *blocking solution R* for 15 min. Rinse the gel with *water R*.

5-4. *RECORDING OF THE RESULTS*

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel scanning systems with data analysis software are commercially available to immediately photograph and analyse the wet gel.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of *glycerol R* for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of *glycerol R*.

Drying of stained SDS polyacrylamide gels is one of the methods used to obtain permanent documentation. This method frequently results in the cracking of gel during drying between cellulose films.

Immerse 2 sheets of porous cellulose film in *water R* and incubate for 5 min to 10 min. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of *water R* around the edges of the gel. Place

the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

5-5. *MOLECULAR-MASS DETERMINATION*

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of pre-stained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be examined.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalised migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or $R_F$. Construct a plot of the logarithm of the relative molecular masses ($M_r$) of the protein standards as a function of the $R_F$ values. Unknown molecular masses can be estimated by linear regression analysis (or more accurately by non-linear regression analysis) or interpolation from the curves of log $M_r$ against $R_F$ if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

5-6. *VALIDATION OF THE TEST*

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g. across 80 per cent of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the $R_F$. If the plot has a sigmoidal shape, then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity must also be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve to establish system suitability.

5-7. *QUANTIFICATION OF IMPURITIES*

SDS-PAGE is often used as a limit test for impurities. When impurities are quantified by normalisation to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein as described in the introduction of section 5-3, the linear range can vary but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentration.

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities may be quantified by normalisation to the main band using an integrating densitometer or by image analysis.

**07/2019:20232**



# 2.2.32. LOSS ON DRYING

PRINCIPLE

Loss on drying is the loss of mass after drying under specified conditions, calculated as a percentage (*m/m*).

Drying to constant mass means that 2 consecutive weighings do not differ by more than 0.5 mg, the 2nd weighing following an additional period of at least 30 min of drying under the conditions prescribed for the substance to be examined.

EQUIPMENT

The equipment typically consists of:

- weighing bottles that are made of suitable inert material and can easily be dried to constant mass; their diameter is large enough so that the layer of the substance to be examined does not exceed about 5 mm;
- an analytical balance by which it is possible to determine a change in mass of 0.1 mg;
- depending on the procedure to be applied, a desiccator, a vacuum cabinet, a vacuum oven or an ordinary laboratory oven; in any case, the temperature of ovens is adjustable to the specified temperature ± 2 °C; vacuum ovens in which the pressure can at least be reduced to about 2 kPa are suitable; ovens are qualified according to established quality system procedures, for example by using a suitable certified reference material (*sodium aminosalicylate dihydrate for equipment qualification CRS* may be used).

Equipment using other means of drying such as microwaves, halogen lamps, infrared lamps or mixed technologies may be used provided they are demonstrated to be fit for purpose.

PROCEDURE

It is recommended to perform the test in an environment that has minimal impact on sample measurement (e.g. humidity).

Weigh an empty weighing bottle that has been previously dried under the conditions prescribed for the substance to be examined for at least 30 min, then weigh the weighing bottle filled with the prescribed quantity of substance to be examined. Dry to constant mass or for the prescribed time. Where the drying temperature is indicated by a single value rather than a range, drying is carried out at the prescribed temperature ± 2 °C. Use one of the following procedures, unless otherwise prescribed in the monograph.

- In a desiccator: the drying is carried out over about 100 g of *molecular sieve R* at atmospheric pressure and at room temperature.
- *In vacuo*: the drying is carried out over about 100 g of *molecular sieve R* at a pressure not exceeding 2.5 kPa, at room temperature or at the temperature prescribed in the monograph.
- In an oven at a specified temperature: the drying is carried out at atmospheric pressure in an oven at the temperature prescribed in the monograph.

After drying in an oven, allow the weighing bottle and the sample to cool to room temperature in a desiccator and weigh the weighing bottle containing the dried sample.

The mass of the sample is the difference between the mass of the filled weighing bottle and the mass of the dried empty weighing bottle.

The loss on drying is the difference in the mass of the sample before and after drying, expressed as a percentage, *m/m* being implicit.

01/2009:20233

# 2.2.33. NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

## INTRODUCTION

Nuclear magnetic resonance (NMR) spectrometry is an analytical method in particular suitable for the elucidation of the chemical structure of organic molecules by means of interpretation of their NMR spectra, arising from, for example, $^{1}H$ or the X-nuclei $^{13}C$, $^{19}F$, $^{15}N$, $^{31}P$. The spectra can be used for qualitative and quantitative purposes.

Under suitable experimental conditions, the integrated NMR intensities of the signals are directly proportional to the number of nuclear spins of the molecular group responsible for the signal. These integrals can be used for quantitative analysis.

## GENERAL PRINCIPLES

Placing an ensemble of nuclei with angular momentum and a magnetic moment in a static magnetic field ($B_0$) causes the nuclei to arrange themselves in different, quantum-mechanically controlled orientations in relation to the axis of the magnetic field. These orientations are different in energy. An oscillating high-frequency magnetic field ($B_1$), perpendicular to $B_0$, will cause transitions between these orientations with net energy absorption. According to the resonance condition $\omega_0 = \gamma B_0$ ($\gamma$ = gyromagnetic ratio, $\omega_0$ = Larmor frequency), either the $B_0$ magnetic field or the frequency ($\omega_1$) of the $B_1$ field may be varied to achieve a spectrum (continuous wave (CW) method). Nowadays the $B_1$ irradiation is achieved by the use of a radiofrequency (RF) pulse (Fourier transform (FT) method). The coherent radiation emitted during the return to the initial state is observed in the form of a decay curve, called the free induction decay (FID). Subsequent Fourier transformation gives the spectrum in the frequency domain, providing information about the molecular structure. Additional radiofrequency fields may be applied during acquisition of the FID signal to suppress scalar (through-bond) interactions between nuclei (called 'decoupling'). One- and multi-dimensional techniques can be applied for qualitative and quantitative purposes, on samples in either the liquid or the solid state.

Important structural information is derived from the following spectroscopic features:

| | |
|---|---|
| resonance frequency | kind of nuclei observed |
| number of resonance signals (singlets, multiplets) | number of chemically distinct groups of nuclei |
| chemical shift δ (ppm) | chemical nature and environment of the structural group observed |
| intensity of resonance signals | relative number of resonant nuclei per chemically distinct group |
| multiplicity of coupling pattern | number of nuclei that are scalar coupled to the observed nucleus |
| coupling constant $^{n}J$ (Hz) | number of bonds in the coupling pathway, and its geometry |

Correlations of different spectral parameters (e.g. chemical shift and coupling constant, or chemical shifts of different nuclei within one molecular system) can be performed by homo- and hetero-nuclear two- and higher-dimensional methods. Information about the relaxation times $T_1$ and $T_2$, nuclear Overhauser effects (NOEs) and the kinetics of time-dependent processes are also accessible from appropriate experiments.

## APPARATUS

A high-resolution NMR spectrometer consists of at least the following parts:

- a magnet to deliver the constant magnetic field $B_0$;
- a temperature-controlled probe to contain the sample, to deliver the radiofrequency pulse and to detect radiation emitted by the sample;
- an electronic console to generate high-power radiofrequency pulses and to collect and digitise the FID signal; this unit also maintains the stability of the instrument electronics;
- a data acquisition and processing unit (computer);

and may also include:

- a continuous flow cell for coupled liquid chromatographic-NMR or flow injection analysis;
- a system for pulsed field gradient NMR.

The high magnetic field is generated by a superconducting coil in a Dewar flask filled with liquid helium. The probe typically contains the sample in a 5 mm-outer-diameter sample tube or in a flow cell, and is connected to the electronics cabinet by RF cables carrying lock, $^{1}H$-, and X-nucleus frequencies. Additional devices for tuning and matching the electronic circuits are essential, and sample temperature control is often used.

The NMR spectrometer should be demonstrated to be operating correctly. Appropriate tests to demonstrate this are, typically, measurement of linewidths at half height for defined peaks under defined acquisition conditions, signal-to-noise ratios (*S/N*) for standard mixtures, pulse power (measured as a 90° pulse width), and pulse reproducibility. All instrument manufacturers publish specifications and measurement protocols for these parameters for specific instrument/probe combinations, and compliance with these specifications should be demonstrated.

## FOURIER TRANSFORM NMR (FT-NMR)

Contemporary spectrometers generally operate according to the Fourier transform (FT) principle: after exciting the sample with a radiofrequency pulse of appropriate frequency (ν), amplitude ($B_1$) and duration ($\tau_p$) and a succeeding short dead time ($t_d$) (to enable the electronics to recover), the amplified analogue FID signal is sampled during the acquisition time ($t_{ac}$) and digitised with an analogue-to-digital converter (ADC), and the results are stored in the spectrometer memory. The receiver output is amplified prior to digitisation to maximise sensitivity without saturating the ADC. In case of observation of X-nuclei, the standard experiment includes, if necessary, broadband $^{1}H$ decoupling, i.e. irradiation of all the protons during the experiment. To increase the *S/N*, multiple FID signals may be accumulated coherently and summed. Fourier transformation of this time-domain data gives the frequency-domain spectrum.

### *PARAMETERS*

The following acquisition parameters influence the result of an FT experiment, and should be adjusted and controlled.

**Pulse width ($\tau_p$).** The excitation pulse is directed along the x-axis of the so-called rotating frame, its duration (or 'width', $\tau_p$) determines the flip angle (θ) and thus the intensity (*I*) of the resonance signal:

$$\theta = \gamma' \times B_1 \times \tau_p \quad (1)$$

$$M_y = M_o \times \sin\theta \quad (2)$$

The observed magnetisation $M_y$ is maximum at θ = 90°. The pulse duration should be short to guarantee that all signals in the spectral width (*SW*) are excited to a similar degree. The magnetisation decays due to relaxation processes.

**Dead time ($t_d$).** The dead time is the time between the end of the pulse and start of the acquisition, it is necessary for technical reasons and care should be taken as it may influence signal intensities and peak phase. Rapidly decaying signals

(giving rise to broad spectral lines) are reduced in intensity by more than slowly decaying signals (which give rise to narrow spectral lines).

**Acquisition time ($t_{ac}$)**. The acquisition time ($t_{ac}$) is related to the spectral width (i.e. the whole observed region) and the number of digital data points (*DP*) collected during signal acquisition.

$$t_{ac} = \frac{DP}{2SW} \qquad (3)$$

Maximal signal intensity and signal-to-noise ratio will be achieved if $t_{ac} \approx 1.2/(\pi\nu_{1/2})$, where $\nu_{1/2}$ is the full width at half-height (*fwhh*), but it should be set to greater than $5/(\pi\nu_{1/2})$ to minimise signal distortion.

**Repetition time ($t_r$)**. The spin-lattice relaxation ($T_1$) governs the time required for the spin system to return to equilibrium after a pulse. Relaxation can be reduced by the use of special reagents. For quantitative purposes, the repetition time used should be set relative to $T_1$ and θ to avoid saturation effects.

**Receiver gain**. The analogue signal detected by the probe is amplified prior to digitisation and storage. The amplification, or receiver gain, should be set, either automatically or manually, so that the signal does not overload the ADC, which causes signal distortion, but allows random noise generated in the probe to be digitised (i.e. is non-zero).

*OPTIMISATION OF ACQUISITION AND PROCESSING PARAMETERS FOR QUANTITATIVE PURPOSES*

Besides the acquisition parameters, signal intensity is also influenced by several processing parameters. After collecting a sufficient number of scans, the resulting FID is Fourier transformed. For reliable quantitative purposes the following parameters have to be optimised.

**Digital resolution**. The digital resolution is the frequency separation between data points. The processed signal should have at least 5 digital points above half-height of the signals to be integrated. To improve the digital resolution additional points of zero intensity may be added to the end of the experimental FID before transformation ('zero filling').

**Signal-to-noise ratio (*S/N*)**. This is the ratio between the intensities (as peak height) of a specified signal in the NMR spectrum and the random fluctuations in that signal, which is usually measured in a region of the spectrum that contains no signals from the analyte. A poor signal-to-noise ratio (*S/N*) limits the accuracy of peak integrations and quantitative analyses. An *S/N* equal to or greater than 150:1 allows peak integrations with a standard deviation of less than 1 per cent. Contemporary spectrometers have software algorithms to report the *S/N* of appropriate peaks. A sufficiently high *S/N* can be difficult to obtain when analysing dilute solutions, and especially when detecting nuclei other than $^1$H. Methods to enhance the *S/N* include:

- increasing the number of accumulated scans (*n*), as *S/N* increases with $\sqrt{n}$;
- use of exponentional multiplication on the FID signal before Fourier transformation; the exponentional multiplication factor should be in the order of the peak full width at half-height (*fwhh*);
- use of spectrometers with a higher magnetic field $B_0$, since *S/N* is proportional to $B_0^{3/2}$;
- use of digital filtering to reduce noise;
- use of probes that maximise the filling factor;
- use of cryoprobes that reduce thermal noise.

**Integration region**. The intensity of the NMR signals is obtained by a quasi-analogue signal integration either by a stepped-line plot or, more accurately, by separate line integration and digital data presentation. In liquid state, NMR signals have Lorentzian line shape. Unless otherwise specified in the monograph or when peak overlap occurs, the same integration range, expressed as a multiple of the peak *fwhh*, should be used for the monitored peak and the reference peak.

**Dynamic range**. The dynamic range of the analogue-to-digital converter (ADC) determines the minimum intensity line that can be observed or quantified when integrating 2 signals with the same linewidth in a spectrum. A 16-bit ADC allows identification of a signal of 0.003 per cent intensity relative to a strong signal completely filling the dynamic range of the ADC.

*NMR OF SAMPLES IN SOLUTION*

Most NMR experiments are performed on dilute solutions (about 1 per cent) of the analyte in an appropriate solvent, which can be spiked with a suitable standard for chemical shift calibration.

**Solvents**. The solvent should be able to dissolve the analyte without further interaction if not otherwise intended. To minimise the intense solvent signals, fully deuterated solvents (*deuterium oxide R*, *deuterated chloroform R*, *deuterated dimethyl sulfoxide R*, *deuterated acetone R*, *deuterated methanol R*, etc.) should be used. The solvent atoms give signals that are easily identified by their chemical shift and can be used to calibrate the chemical shift axis (secondary reference).

**Referencing**. The spectral feature most dependent on the chemical environment of the atom in the molecule is the chemical shift, designated as δ and reported in parts per million. The chemical shift between the resonance for an NMR active nucleus X ($\delta_{X,sample}$) is measured in parts per million as the difference between the resonance frequency of that nucleus ($\nu_{X,sample}$) and that of an internal shift reference standard ($\nu_{X,reference}$), both in hertz, divided by the basic spectrometer operating frequency ($\nu_{X,reference}$), in megahertz, at a given $B_0$:

$$\delta_{X,sample} = \frac{(\nu_{X,sample} - \nu_{X,reference})}{\nu_{X,reference}} \qquad (4)$$

By convention, the standard for exact chemical shift referencing is the $^1$H resonance of *tetramethylsilane R* (TMS), setting $\delta_{TMS}$ = 0 ppm. Formally, once the $^1$H shift scale has been referenced relative to TMS, the exact frequency of any other X resonance can be calculated and its chemical shift scale calibrated. The frequency of a (secondary) reference standard at $\delta_X$ = 0 ppm ($\nu_{X,reference}$) is calculated from the $^1$H frequency of TMS ($\nu_{H,TMS}$) and a tabulated value of the ratio ($\Xi_{X,reference}$) of the isotope-specific frequency in relation to that of $^1$H in TMS:

$$\nu_{X,reference} = \nu_{H,TMS} \times \Xi_{X,reference} \qquad (5)$$

Reference standards at $\delta_X$ = 0 ppm and corresponding $\Xi_{X,reference}$ values are shown below:

| Nucleus | Water[a] | $\Xi_{X,reference}$ | Other solvents | $\Xi_{X,reference}$ |
|---|---|---|---|---|
| $^1$H | DSS[b] | 1.00000000 | TMS | 1.00000000 |
| $^{13}$C | DSS[b] | 0.25144953 | TMS | 0.25145020 |
| $^{15}$N | $NH_3$ | 0.10132912 | $CH_3NO_2$ | 0.10136767 |
| $^{19}$F | $CF_3COOH$ | not reported | $CCl_3F$ | 0.94094011 |
| $^{31}$P | $H_3PO_4$ (85 per cent) | 0.40480742 | $(CH_3O)_3PO$ | 0.40480864 |
| [a]chemical shift depends on pH | | | | |
| [b]DSS = sodium 2,2-dimethyl-2-silapentane-5-sulfonate | | | | |

In practice, X chemical shifts are referenced directly using an appropriate standard. In $^1$H and $^{13}$C NMR, internal referencing is mainly used, where the reference is added directly to

the system under study. In $^{15}N$, $^{19}F$ and $^{31}P$ NMR, external referencing is often suitable, involving sample and reference contained separately in coaxial cylindrical sample tubes.

**Lock**. In order to prevent drifting of the spectrum over time, a stabilising procedure, called field-frequency locking, is performed. The $^{2}H$ (deuterium) signal arising from deuterated solvents is used to achieve this, unless otherwise specified in the monograph.

*QUALITATIVE ANALYSIS*

The principal use for qualitative NMR spectra is as an identity test, in which the $^{1}H$ or $^{13}C$ spectrum of a test sample is compared to the spectrum of a reference sample or, less commonly, with a published reference spectrum. Spectra of reference and test samples should be acquired using the same procedure and operational conditions. The peaks in the 2 spectra, or characteristic regions of the spectra, should correspond in position, intensity and multiplicity. In appropriate cases, mathematical comparison, such as calculation of a correlation coefficient, may be appropriate. In the absence of a reference standard, an in-house reference may be used, whose identity has been demonstrated by alternative methods, or the demonstration that the NMR spectrum is fully consistent with the reported structure for that material.

*QUANTITATIVE ANALYSIS*

Signal intensity in the basic NMR experiment is the integrated area under the signal curve measured. Only when 2 signals have equal *fwhh* and the same multiplicity may signal height serve as a measure of intensity. Under conditions of essentially complete relaxation between scans, the signal intensity ($I_A$) is a true measure of the number ($N_A$) of nuclei responsible for the respective signal:

$$I_A = K_S \times N_A \tag{6}$$

The constant $K_S$ includes fundamental constants, properties of the sample and receiver parameters, and can be omitted in cases where signal intensities are compared, giving the direct relation between the numbers of nuclei in the 2 compared structure groups A and B:

$$\frac{I_A}{I_B} = \frac{N_A}{N_B} \tag{7}$$

The numbers ($N_i$) of nuclei belonging to different structure groups of 1 molecule are small integers. The values measured are rounded to the closest integer numbers. However, the proper operation of acquisition and processing of the spectrometer is easily checked by comparing exact intensities within a spectrum of any suitable organic compound of known structure.

In addition to the fact that the intensities of signals arising from each component in a mixture are related to each other by small integer numbers, the relative molar amounts of these components can be measured by comparison of the normalised intensities of resonances from different components. The molar ratio of 2 components of a mixture is calculated according to the following equation:

$$\frac{n_A}{n_B} = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \tag{8}$$

The determination is only valid in cases where the structure of the molecules for which $I_A$ and $I_B$ are determined are known (or at least the values of $N$ for the monitored groups). Determinations are made using either an internal standard method or a peak-normalisation procedure.

**Internal standard method**. The mass ($m_A$) of an analyte (A) can be determined if a known mass ($m_B$) of a substance (B) with a known percentage content ($P_B$) is added to the solution as an intensity standard. Equation (8) can be converted to equation (9):

$$m_A = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \times \frac{M_A}{M_B} \times m_B \times \frac{P_B}{100} \tag{9}$$

Here, $M_i$ are the molecular masses.

The intensity standard has to be carefully chosen; it should be completely soluble in the solvent used for the analyte, should produce only a small number of signals, and the 'monitor group' should have a signal in an empty spectral region. A compound of high purity and with a relatively high molecular mass is recommended for this purpose.

**Normalisation procedure**. The relative proportions of components in a mixture, the degree of substitution in a structurally modified polymer, or the amount of a contaminant can be determined by comparison of the relative intensities of resonances present.

The experimental method should be validated to ensure that there is no overlap of the relevant signals. When the contaminant is of poorly defined structure or molecular mass (e.g. an emulsifier), addition of known amounts of that material to the NMR tube will allow a calibration curve to be constructed.

METHOD

**Sample handling**. Dissolve the sample in the solvent to which the appropriate reference material may have been added to calibrate chemical shift, as prescribed in the monograph. For quantitative analysis, the solutions must be free from solid particles. Some quantitative analyses may require an internal standard to be included, so that integrations of resonances from the test sample and the reference material can be compared. Appropriate references and concentrations are indicated in the specific monographs. In other quantitative analyses, the result is obtained by comparing the relative intensities of 2 or all of the resonances that arise from the test sample. After loading the sample into a tube and capping, the sample is introduced into the NMR magnet, the experimental parameters are loaded and the experiment is executed. Key experimental parameters are indicated in the monograph.

**The measurement procedure**. Equilibrate the sample in the probe, and optimise the instrument to achieve best resonance conditions and to maximise the *S/N* by tuning and matching the probe, and make adjustments to maximise magnetic field homogeneity over the sample volume (called 'shimming'). Record, or save to computer, the parameter settings. An experiment may be composed of multiple pulse-acquisition-delay sequences, and the individual FIDs are summed in the computer memory, with random noise being averaged out. When an appropriate *S/N* has been achieved, the FID is stored and the frequency-domain spectrum is generated by Fourier transformation of the summed FIDs.

NMR IN THE SOLID STATE

Samples in the solid state can be analysed using NMR spectrometers specially equipped for that purpose. Certain technical procedures make observable individual lines for individual atomic sites with a valuable extension of the applicability of NMR to inorganic materials as well.

One technique is the rapid rotation (4-30 kHz) of the powdered sample in a rotor (about 4 mm outer diameter) inclined at an angle of 54.7° (the 'magic angle') to the direction of the $B_0$ magnetic field axis. This technique is named magic angle spinning (MAS). Another effective tool is high-power decoupling and a 3rd method is the transfer of polarisation from easily excitable nuclei towards less-polarisable nuclei, i.e. cross polarisation (CP). The combination of these techniques makes available high-resolution spectra containing much information about chemical and structural details in solid glassy, amorphous, and crystalline materials of ceramic, polymeric or mineralogical origin.

If NMR is applied to a solid, full details of the procedure are provided in the monograph.

01/2016:20234

# 2.2.34. THERMAL ANALYSIS[3]

Thermal analysis is a group of techniques in which the variation of a physical property of a substance is measured as a function of temperature. The most commonly used techniques are those which measure changes in the mass or energy of a sample of a substance.

These techniques have different applications:

- determination of phase changes;
- determination of changes in chemical composition;
- determination of purity.

## THERMOGRAVIMETRY

Thermogravimetry (TG) or thermogravimetric analysis (TGA) is a technique in which the mass of a sample of a substance is recorded as a function of temperature according to a controlled temperature programme.

**Instrument**. The essential components of a thermobalance are a device for heating or cooling the substance according to a given temperature programme, a sample holder in a controlled atmosphere, an electrobalance and a means of electronic signal output to a recorder or a computer.

**Temperature calibration**. The temperature sensor close to or in contact with the sample is calibrated using the Curie temperature of a ferromagnetic substance such as nickel. In the case of an instrument capable of simultaneously conducting TG/TGA and differential thermal analysis (DTA) or differential scanning calorimetry (DSC), the same certified reference materials as those for DTA and DSC may be used, for example, indium, tin and/or zinc.

**Calibration of the electrobalance**. Place an appropriate quantity of a suitable certified reference material (e.g. *calcium oxalate monohydrate CRS*) in the sample holder and record the mass. Set the heating rate according to the manufacturer's instructions (e.g. 5 °C/min) and start the temperature increase. Record the thermogravimetric curve as a graph with temperature or time on the abscissa, increasing from left to right, and mass on the ordinate, decreasing downwards. Stop the temperature increase at about 250 °C. Measure the difference on the graph between the initial and final mass-temperature or mass-time plateaux, which corresponds to the loss of mass. The declared loss of mass for the certified reference material is stated on the label.

**Method**. Apply the same procedure to the substance to be examined using the conditions prescribed in the monograph. Calculate the loss of mass of the substance to be examined from the difference measured in the graph obtained. Express the loss of mass as $\Delta m/m$ (per cent).

If the instrument is in frequent use, carry out temperature calibration regularly. Otherwise, calibration is carried out before each measurement.

As the operating conditions are critical, the following parameters are noted for each measurement: pressure or flow rate, gas composition, mass of the sample, heating rate, temperature range and sample pre-treatment including any isothermal period.

## DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is a technique that can be used to demonstrate the energy phenomena produced during heating (or cooling) of a substance (or a mixture of substances) and to determine the changes in enthalpy and specific heat and the temperatures at which these occur. The technique is used to determine the difference in heat flow (with reference to the temperature) evolved or absorbed by the test sample compared with the reference cell, as a function of the temperature. Two types of DSC instruments are available, those using power compensation to maintain a null temperature difference between sample and reference and those that apply a constant rate of heating and detect temperature differential as a difference in heat flow between sample and reference.

**Instrument**. The instrument for power compensation DSC consists of a furnace containing a sample holder with a reference cell and a test cell. The instrument for heat flow DSC consists of a furnace containing a single cell with a sample holder for the reference crucible and the test crucible.

A temperature-programming device, thermal detector(s) and a recording system which can be connected to a computer are attached. The measurements are carried out under a controlled atmosphere.

**Calibration of the instrument**. Calibrate the instrument for temperature and enthalpy change, using suitable certified materials or reference standards.

*Temperature calibration*. It can be performed using certified reference materials having an intrinsic thermal property, such as the melting point of pure metals or organic substances, or the phase transition point of crystalline inorganic salts or oxides. Melting points of indium, tin and/or zinc are usually employed for calibration.

*Heat-quantity calibration*. For accurate estimation of the quantity of heat change (enthalpic change) of a test sample, caused by a certain physical change accompanying a temperature change, it is necessary to calibrate the instrument using suitable certified reference materials. Similar to temperature calibration, heat-quantity calibration may be performed using suitable certified reference materials showing a known definite enthalpic change caused by physical changes, such as the melting of pure metals and/or organic substances, or the phase transition of crystalline inorganic salts. The heats of fusion of indium, tin and/or zinc are usually employed for calibration.

**Operating procedure**. Weigh in a suitable crucible an appropriate quantity of the substance to be examined and place it in the sample holder. Place an empty crucible in the reference holder. Set the initial and final temperatures, and the heating rate according to the operating conditions prescribed in the monograph.

Begin the analysis and record the DSC curve with the temperature or time on the abscissa (values increasing from left to right) and the energy change on the ordinate (specify whether the change is endothermic or exothermic).

The temperature at which the phenomenon occurs (the onset temperature) corresponds to the intersection (A) of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (see Figure 2.2.34.-1). The end of the thermal phenomenon is indicated by the peak of the curve.

The enthalpy of the phenomenon is proportional to the area under the curve limited by the baseline; the proportionality factor is determined from the measurement of the heat of fusion of a known substance (e.g. indium) under the same operating conditions.

Each thermogram may be accompanied by the following data: conditions employed, record of last calibration, mass of the sample and identification (including thermal history), container, atmosphere (identity, flow rate, pressure), direction and rate of temperature change, instrument and recorder sensitivity.

(3) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8 Pharmacopoeial harmonisation*.

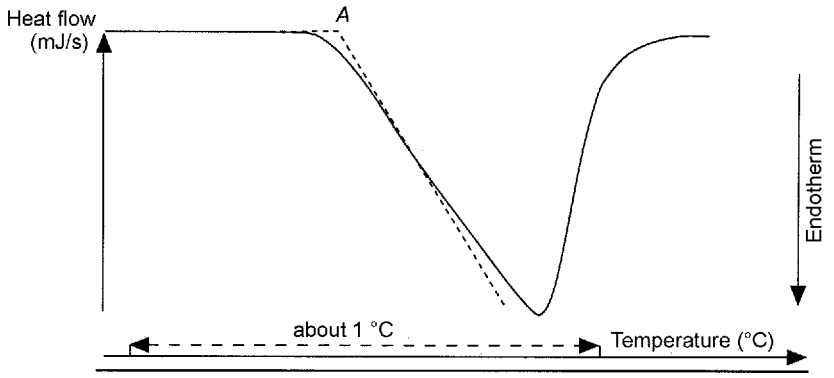


Figure 2.2.34.-1. – *Thermogram*

**Applications**

*Phase changes.* Determination of the temperature, heat capacity change and enthalpy of phase changes undergone by a substance as a function of temperature. The transitions that may be observed include those shown in Table 2.2.34.-1.

Table 2.2.34.-1.

| | |
|---|---|
| solid - solid transition: | allotropy - polymorphism<br>desolvation<br>amorphous-crystalline |
| solid - liquid transition: | melting<br>glass-transition |
| solid - gas transition: | sublimation |
| liquid - solid transition: | freezing<br>recrystallisation<br>glass-transition |
| liquid - gas transition: | evaporation |

*Changes in chemical composition.* Measurement of heat and temperatures of reaction under given experimental conditions, so that, for example, the kinetics of decomposition or desolvation can be determined.

*Application to phase diagrams.* Establishment of phase diagrams for solid mixtures. The establishment of a phase diagram may be an important step in the preformulation and optimisation of the freeze-drying process.

*Determination of purity.* The measurements of the fraction of substance melted at a given temperature and the heat of fusion by DSC enable the impurity content of a substance to be determined from a single thermal diagram, requiring the use of only a few milligrams of sample with no need for repeated accurate measurements of the true temperature.

In theory, the melting of an entirely crystalline, pure substance at constant pressure is characterised by a heat of fusion $\Delta H_f$ in an infinitely narrow range, corresponding to the melting point $T_0$. A broadening of this range is a sensitive indicator of impurities. Therefore, samples of the same substance, whose impurity contents vary by a few tenths of a per cent, give thermal diagrams that are visually distinct (see Figure 2.2.34.-2).

The determination of molar purity by DSC is based on the use of a mathematical approximation of the integrated form of the van't Hoff equation applied to the concentrations (not the activities) in a binary system [ $\ln(1 - x_2) \approx -x_2$ and $T \times T_0 \approx T_0^2$ ]. For low amounts of impurities ( $x_2 \ll 1$) and for temperatures close to the melting point $T_0$ the equation can be written as follows, in which $T$ and $x_2$ are variables:

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times x_2 \qquad (1)$$

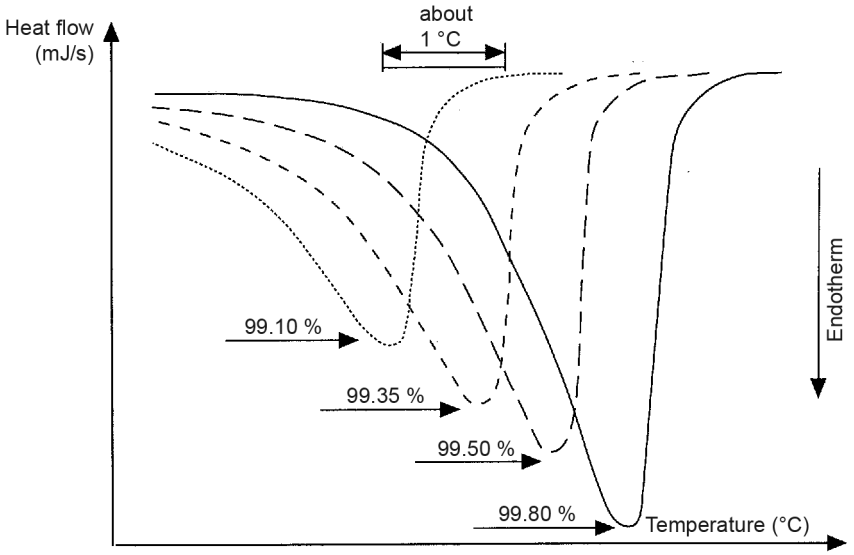


Figure 2.2.34.-2. – *Thermal diagrams according to purity*

$T$ = temperature of the sample, in kelvins;

$T_0$ = melting point of the chemically pure substance, in kelvins;

$R$ = gas constant for ideal gases, in joules·kelvin$^{-1}$·mole$^{-1}$;

$\Delta H_f$ = molar heat of fusion of the pure substance, in joules·mole$^{-1}$;

$x_2$ = mole fraction of the impurity, i.e., the number of molecules of the impurity divided by the total number of molecules in the liquid phase (or molten phase) at temperature $T$ (expressed in kelvins).

Hence, the determination of purity by DSC is limited to the detection of impurities forming a eutectic mixture with the principal compound and present at a mole fraction of typically less than 2 per cent in the substance to be examined.

This method cannot be applied to:

- amorphous substances;
- solvates or polymorphic compounds that are unstable within the experimental temperature range;
- impurities forming solid solutions with the principal substance;
- impurities that are insoluble in the liquid phase or in the melt of the principal substance.

During the heating of the substance to be examined, the impurity melts completely at the eutectic temperature. Above this temperature, the solid phase contains only the pure substance. As the temperature increases progressively from the eutectic temperature to the melting point of the pure substance, the mole fraction of the impurity in the liquid phase decreases, since the quantity of liquefied pure substance increases.

For all temperatures above the eutectic point:

$$x_2 = \frac{1}{F} \times x_2^* \qquad (2)$$

$F$ = molten fraction of the analysed sample;

$x_2^*$ = mole fraction of the impurity in the analysed sample.

When the entire sample has melted, $F = 1$ and $x_2 = x_2^*$.

If equation (2) is combined with equation (1), the following equation is obtained:

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times \frac{1}{F} \times x_2^*$$

The value of the heat of fusion of the pure substance is obtained by integrating the melting peak.

The melting point $T_0$ of the pure substance is extrapolated from the plot of temperature $T$ (expressed in kelvins) versus $1/F$. The slope α of the curve (obtained after linearisation, if necessary) corresponding to $RT_0^2 \frac{x_2^*}{\Delta H_f}$, allows $x_2^*$ to be evaluated.

The fraction $x_2^*$ multiplied by 100 gives the mole fraction in per cent for total eutectic impurities.

**07/2019:20235**

# 2.2.35. OSMOLALITY

PRINCIPLE

*GENERAL*

Osmolality is a measure of the total number of chemical entities per kilogram of solvent, and thus provides an indication of the osmotic pressure of the solution. Osmolality is dependent on the molal concentration of the solute(s) in the solution, on their dissociation and on the deviation of the solution from ideal behaviour (Raoult's law).

The unit of osmolality is the osmole per kilogram (osmol/kg), but the submultiple milliosmole per kilogram (mosmol/kg) is more commonly used.

The osmolality ($\xi_m$) of a solution containing $i$ solutes is given by the expression:

$$\xi_m = \sum \nu_i m_i \Phi_{m,i}$$

$\nu_i$ = number of entities formed by the dissociation of one molecule of the $i^{th}$ solute; if the solute is non-ionic (non-dissociating), $\nu_i$ equals 1,

$m_i$ = molality of the $i^{th}$ solute in the solution, in moles per kilogram of solvent,

$\Phi_{m,i}$ = molal osmotic coefficient, a dimensionless factor.

The molal osmotic coefficient is a measure of the deviation of the solution from ideal behaviour. For an ideal solution, osmolality equals molality ($\Phi$=1).

In the case of a real, non-ideal solution, the molal osmotic coefficient is influenced by the interactions occurring amongst the components (i.e. molecules, ions, solvent) of the solution. The more complex the composition of the solution, the harder it becomes to determine $\Phi$.

For this reason, the measurement of a colligative property such as the freezing-point depression is used as a practical means of determining osmolality by obtaining an overall measure of the contribution of the various solutes present in a solution.

*PRINCIPLE OF MEASUREMENT*

Unless otherwise prescribed, osmolality is determined by measuring the freezing-point depression ($\Delta T_f$) of a solution. The relationship between osmolality and freezing-point depression is given by the expression:

$$\Delta T_f = k_f \xi_m$$

where $k_f$ is the molal cryoscopic constant, which is solvent-dependent. For water, the value of $k_f$ is 1.86 K/osmol (i.e. adding 1 mol of a non-dissociating solute to 1 kg of water results in a decrease in freezing-point of 1.86 K).

EQUIPMENT

An osmometer for freezing-point depression measurement typically consists of:

- an appropriate sample container;
- a means of cooling the sample;
- a temperature-sensitive resistor (thermistor), with an appropriate current or potential difference measurement device that can indicate a temperature depression or give osmolality values directly;
- a means of mixing the sample and/or inducing solidification when supercooling occurs.

PROCEDURE

*CALIBRATION*

Prepare reference solutions as specified in Table 2.2.35.-1, as necessary, using dried *sodium chloride R*. Commercially available certified solutions for osmometer calibration, with osmolalities equal or similar to those listed in Table 2.2.35.-1, may be used. Calibrate the equipment according to the manufacturer's instructions using *water R* to determine the zero value and at least 2 of the reference solutions listed in Table 2.2.35.-1. Confirm the calibration using at least one additional reference solution with a known osmolality (see Table 2.2.35.-1). Select a reference solution preferably with an osmolality within ± 50 mosmol/kg of the expected value for the solution to be examined or close to the centre of the expected osmolality range of the solutions to be examined. It is recommended that the reading is within ± 4 mosmol/kg of the osmolality of the chosen reference solution.

Table 2.2.35.-1. – *Reference solutions for osmometer calibration*

| Mass of *sodium chloride R* in *water R* (g/kg) | Osmolality, $\xi_m$ (mosmol/kg) | Freezing-point depression, $\Delta T_f$ (K) |
|---|---|---|
| 3.087 | 100 | 0.186 |
| 6.260 | 200 | 0.372 |
| 9.463 | 300 | 0.558 |
| 12.684 | 400 | 0.744 |
| 15.916 | 500 | 0.930 |
| 19.147 | 600 | 1.116 |
| 22.380 | 700 | 1.302 |

*METHOD*

Rinse the sample container with the solution to be examined before each measurement. Programme the device inducing solidification to start at a defined temperature below the expected freezing-point of the solution to be examined. Introduce an appropriate volume of the solution to be examined into the sample container according to the manufacturer's instructions, and start the cooling system. The equipment indicates when equilibrium has been reached.

Perform the test under the conditions (cooling temperature and volume) used to calibrate the equipment. Depending on the type of equipment, the osmolality can be read directly or can be calculated from the measured freezing-point depression.

The test is not valid unless the measured osmolality of the solution to be examined lies within the calibrated osmolality range.

**01/2016:20236**

# 2.2.36. POTENTIOMETRIC DETERMINATION OF IONIC CONCENTRATION USING ION-SELECTIVE ELECTRODES

Ideally, the potential $E$ of an ion-selective electrode varies linearly with the logarithm of the activity $a_i$ of a given ion, as expressed by the Nernst equation:

$$E = E_0 + 2.303\frac{RT}{z_i F}\log_{10} a_i$$

$E_0$ = part of the constant potential due to the apparatus used,

R = gas constant,

$T$ = absolute temperature,

F = Faraday's number,

$z_i$ = charge number of the ion including its sign.

At a constant ionic strength, the following holds:

$$E = E_0 + \frac{k}{z_i}\log_{10} fC_i$$

$C_i$ = molar concentration of the ion,

$f$ = the activity coefficient $(a_i = fC_i)$,

$k$ = $\dfrac{RT}{F}$

If: $E_0 + \dfrac{k}{z_i}\log_{10} f = E'_0$ and $S = \dfrac{k}{z_i}$

$S$ = slope of the calibration curve of the electrode,

the following holds: $E = E'_0 + S\log_{10} C_i$

and for $-\log_{10} C_i = pC_i$: $E = E'_0 - SpC_i$.

The potentiometric determination of the ion concentration is carried out by measuring the potential difference between two suitable electrodes immersed in the solution to be examined; the indicator electrode is selective for the ion to be determined and the other is a reference electrode.

*Apparatus.* Use a voltmeter allowing measurements at least to the nearest 0.1 millivolt and whose input impedance is at least one hundred times greater than that of the electrodes used.

Ion-selective electrodes may be primary electrodes with a crystal or non-crystal membrane or with a rigid matrix (for example, glass electrodes), or electrodes with charged (positive or negative) or uncharged mobile carriers, or sensitised electrodes (enzymatic-substrate electrodes, gas-indicator electrodes). The reference electrode is generally a silver–silver chloride electrode with suitable junction liquids producing no interference.

*Procedure.* Carry out each measurement at a temperature constant to ± 0.5 °C, taking into account the variation of the slope of the electrode with temperature (see Table 2.2.36.-1). Adjust the ionic strength and possibly the pH of the solution to be analysed using the buffer reagent described in the monograph and equilibrate the electrode by immersing it in the solution to be analysed, under slow and uniform stirring, until a constant reading is obtained.

Table 2.2.36.-1. – *Values of k at different temperatures*

| Temperature (°C) | *k* |
|---|---|
| 20 | 0.0582 |
| 25 | 0.0592 |
| 30 | 0.0602 |

If the electrode system is used frequently, check regularly the repeatability and the stability of responses, and the linearity of the calibration curve or the calculation algorithm in the range of concentrations of the test solution; if not, carry out the test before each set of measurements. The response of the electrode may be regarded as linear if the slope $S$ of the calibration curve is approximately equal to $k/z_i$, per unit of $pC_i$.

METHOD I (DIRECT CALIBRATION)

Measure at least three times in succession the potential of at least three reference solutions spanning the expected concentration of the test solution. Calculate the calibration curve, or plot on a chart the mean potential E obtained against the concentration of the ion to be determined expressed as $-\log_{10} C_i$ or $pC_i$.

Prepare the test solution as prescribed in the monograph; measure the potential three times and, from the mean potential, calculate the concentration of the ion to be determined using the calibration curve.

METHOD II (MULTIPLE STANDARD ADDITIONS)

Prepare the test solution as prescribed in the monograph. Measure the potential at equilibrium $E_T$ of a volume $V_T$ of this solution of unknown concentration $C_T$ of the ion to be determined. Make at least three consecutive additions of a volume $V_S$ negligible compared to $V_T$ ($V_S \leq 0.01 V_T$) of a reference solution of a concentration $C_S$ known to be within the linear part of the calibration curve. After each addition, measure the potential and calculate the difference of potential $\Delta E$ between the measured potential and $E_T$. $\Delta E$ is related to the concentration of the ion to be determined by the equation:

$$\Delta E = S\log_{10}\left(1 + \frac{C_S V_S}{C_T V_T}\right)$$

or

$$10^{\frac{\Delta E}{S}} = 1 + \frac{C_S V_S}{C_T V_T}$$

$V_T$ = volume of the test solution,

$C_T$ = concentration of the ion to be determined in the test solution,

$V_S$ = added volume of the reference solution,

$C_S$ = concentration of the ion to be determined in the reference solution,

$S$ = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained with two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

Plot on a graph $10^{\frac{\Delta E}{S}}$ ($y$-axis) against $V_S$ ($x$-axis) and extrapolate the line obtained until it intersects the $x$-axis. At the intersection, the concentration $C_T$ of the ion to be determined in the test solution is given by the equation:

$$C_T = -\frac{C_S V_S}{V_T}$$

METHOD III (SINGLE STANDARD ADDITION)

To a volume $V_T$ of the test solution prepared as prescribed in the monograph, add a volume $V_S$ of a reference solution containing an amount of the ion to be determined known to give a response situated in the linear part of the calibration curve. Prepare a blank solution in the same conditions. Measure at least three times the potentials of the test solution and the blank solution, before and after adding the reference solution. Calculate the concentration $C_T$ of the ion to be analysed using the following equation and making the necessary corrections for the blank:

$$C_T = \frac{C_S V_S}{10^{\frac{\Delta E}{S}}(V_T + V_S) - V_T}$$

$V_T$ = volume of the test solution or the blank,

$C_T$ = concentration of the ion to be determined in the test solution,

$V_S$ = added volume of the reference solution,

$C_S$ = concentration of the ion to be determined in the reference solution,

$\Delta E$ = difference between the average potentials measured before and after adding $V_S$,

$S$ = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained from two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

**01/2018:20237**

# 2.2.37. X-RAY FLUORESCENCE SPECTROMETRY

PRINCIPLE OF THE TECHNIQUE

X-ray fluorescence (XRF) analysis is based on measurements of the X-rays emitted by the constituent atoms of a sample when it is excited by an external source of radiation. If sufficiently energetic radiation impinges on an atom of the sample material, it may eject 1 of the inner-shell electrons of that atom. The vacancy created is filled by 1 of the electrons from an outer, higher-energy shell. The energy difference between the 2 electron shells involved in the process is released in the form of fluorescent X-rays. These X-rays are characteristic since their energy is specific to each element (atom). By measuring their energy and intensity, qualitative and quantitative data about the elemental composition of the test material is obtained.

XRF spectrometry is suitable for liquid, solid and powdered materials and is widely used as a means of screening pharmaceutical ingredients and products for toxic elements or elemental impurities, for quality control and in-process testing. It is also used to identify inorganic foreign elements within falsified medicinal products. As XRF can be non-invasive, it lends itself to process analytical technology (PAT) applications, such as the analysis of an unwanted trace catalyst in an active pharmaceutical ingredient.

EQUIPMENT

An XRF spectrometer (or analyser) consists of 3 essential components:

- a source of exciting radiation (e.g. an X-ray tube, an electron beam if a scanning electron microscope is used or, more rarely, a radioisotope);
- a means for reproducible sample presentation;
- a detector.

Depending on the X-ray detection method employed, either a wavelength-dispersive (WD) or an energy-dispersive (ED) XRF spectrometer is used.

In a WD-XRF spectrometer the X-rays from the sample are directed at a crystal, which diffracts them at precise angles depending on their energy. The intensity of the diffracted X-rays is measured sequentially by a detector counter.

In an ED-XRF spectrometer the X-rays from the sample are directed at a solid-state detector, which generates an electric pulse of an amplitude proportional to the energy of each X-ray photon detected. During measurement, the spectrometer acquires an X-ray spectrum of the sample that contains complete information about its composition. An ED-XRF spectrometer can also be combined with a scanning electron microscope (SEM). Substantial advances in miniaturisation and automation have led to the development of hand-held ED-XRF spectrometers for field measurements.

Some instruments are supplied with an initial factory-set calibration that allows semi-quantitative analyses to be carried out.

MATRIX EFFECTS AND INTERFERENCE

The intensity of the characteristic X-rays of the analysed elements is not necessarily linear with concentration, owing to matrix effects. The intensity of the fluorescence measured for a given element depends not only on the concentration of that element in the sample but also on the absorption of the incident and fluorescent radiations by the matrix. The presence and concentration of other elements (analytes) in the sample, the composition of the sample matrix, and the particle size of the sample material are known to contribute to matrix effects. The presence of matrix effects must be taken into account in any calibration method utilised for quantitative determination.

At low concentrations the linearity is usually well preserved, which greatly facilitates calibration of the spectrometer. The intensity of the fluorescent radiation emitted by an element in a given matrix and at a given wavelength is then proportional to the concentration of that element and inversely proportional to the mass absorption coefficient of the matrix at that wavelength.

SAMPLE PREPARATION

It is essential that the sample is sufficiently thick that the intensities of characteristic X-rays measured are not affected by variations in sample thickness.

*Liquid samples*. Samples are analysed 'as is', provided that the solution consists of a clear, single phase and has sufficiently low volatility. A special liquid-sample holder and a commercially available support window composed of a suitable polymer film (transparent to X-rays and solvent resistant) are required. Alternatively, liquid samples can be transferred onto the surface of a disc and dried before analysis.

*Powdered samples*. Samples may be analysed 'as is' in special X-ray sample cups with bottoms made of a thin polymer film, transparent to X-rays. After transferring the powder to a sample cup, the cup is tapped gently until no further settling of the powder is observed. If necessary, more powder can be added to the cup after tapping. Another alternative for preparation of a powdered sample, which is better suited to WD-XRF analysis, is to press the sample powder into a pellet, with a binder (for example cellulose powder, wax or ethylcellulose) or without a binder. The mass of reference material and sample material must be about the same and the resulting pellets must be approximatively 5 mm thick or more.

*Solid samples*. Samples are analysed by placing them directly on the spectrometer measuring window, making sure they cover it completely. Solid samples for WD-XRF may need to be cut into a uniform shape with a flat surface for reproducible analysis, whereas samples can be measured 'as is' when using ED-XRF, provided there is adequate sample depth.

*Fusion*. The fusion bead method can be used to prepare solid and powdered samples (e.g. minerals or oxides) if the element of interest is not volatile. The sample is homogeneously mixed with a flux reagent (e.g. dilithium tetraborate) and transferred to a platinum vessel; a releasing agent and/or oxidising agent may be added if necessary, for example to prevent damage of the vessel. The mixture is heated at an appropriate temperature while swirling the vessel until a homogeneous melt is obtained. If necessary, the melt is then transferred to a flat-bottomed mould, kept in a horizontal position and cooled under conditions maintaining its property as a glass.

PROCEDURE

**Measuring conditions**. The instrument is set up and used in accordance with the manufacturer's instructions. The measurements may be carried out under vacuum, nitrogen or helium to improve the sensitivity of the method for the quantitation of light elements.

**Reference standards**. Standards required for the calibration, system suitability or control of equipment performance are prepared from certified reference materials (CRMs). Standards with a high carbon load may be more representative for pharmaceutical applications.

**Calibration**. The calibration model selected must be fit for purpose. Various calibration methods are available, including the 'fundamental parameters' approach, empirical calibration, Compton/Rayleigh normalisation and multiple linear regression (MLR).

**System suitability test**. This test must be carried out before the analysis to ensure that the performance of the measurement system is satisfactory. It may also be performed to verify the calibration of the system.

*Acceptance criteria*: the measurement system is suitable if the concentration obtained for a check material containing the element(s) of interest within the used concentration range does not differ from the actual concentration by more than 5 per cent for an assay, and 20 per cent for impurity tests; when using concentrations determined from reference methods such as atomic absorption spectrometry, the accuracy of the XRF calibration should be aligned to that of the reference method; in this case an acceptance criterion of 10 per cent for the assay can be more realistic.

**Analysis**. The samples are measured with the same parameters as used during calibration of the instrument.

CONTROL OF EQUIPMENT PERFORMANCE

*These parameters are also applicable for equipment qualification.*

Specific procedures, acceptance criteria and time intervals for characterising XRF performance depend on the instrument and its intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be obtained.

The following tests may be carried out at appropriate intervals defined according to the user's quality system procedures to ensure the adequate performance of the XRF instrument.

***x*- and *y*-axes**

It is recommended that the *x*-axis (energy or peak angle) and *y*-axis (intensity) are verified at least on installation and thereafter at appropriate intervals that are defined according to the user's quality system procedures. Consideration is to be given to peak position in ED-XRF and to peak angle in WD-XRF when verifying the *x*-axis, and to the count rate when verifying the *y*-axis.

**Detector resolution**

Calculate the resolution (total width at half-height) at the energy used during calibration of the instrument.

*Acceptance criteria*: the resolution value does not deviate by more than 20 per cent for an assay, and 25 per cent for identity and elemental impurities tests, from the value determined during calibration of the instrument.

VALIDATION REQUIREMENTS

The objective of an XRF method validation is to demonstrate that the measurement procedure is fit for purpose (assay, content uniformity, limit tests and identification tests). Where sample preparation is necessary, it is essential that test materials are spiked before any preparatory steps. For example, if a test material is to be digested, the material must be spiked at the beginning of the digestion procedure.

For the determination of impurities, the validation requirements are given in general chapter *2.4.20*. For other purposes, validation is performed according to the relevant ICH guidelines.

**01/2019:20238**

# 2.2.38. CONDUCTIVITY[4]

INTRODUCTION

This general chapter provides information on how to carry out electrical conductivity measurements (hereafter referred to as 'conductivity') of fluids, including pure liquids. It is intended for fluid applications when conductivity is used to measure, monitor or control chemical dispensing (e.g. chemical purity or ionic concentration), and other applications where the ionic character of the fluid needs to be known or controlled.

Applications include, but are not limited to, solutions that may be used in clean-in-place, chromatography detection, ionic solution preparations, end point detection, dosing, fermentation and buffer production. In some cases, conductivity measurements can be extended to pure organic

(4) This general chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

fluids such as alcohols and glycols where a weak conductivity signal exists, and the signal can be significantly increased if the organic fluids become contaminated with water or salts.

Conductivity is the measurement of the ability of a fluid to conduct electricity via its ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Conductivity is directly proportional to the concentrations of ions in the fluid according to the following equation:

$$\kappa = 1000 \sum_{i}^{all\ ions} C_i \lambda_i$$

$\kappa$ = conductivity, in siemens per centimetre;

$C_i$ = concentration of ion *i*, in moles per litre;

$\lambda_i$ = specific molar conductance of ion *i*, in siemens square centimetres per mole ($S \cdot cm^2 \cdot mol^{-1}$).

Although siemens per metre is the appropriate SI unit for conductivity, historically the unit siemens per centimetre has been selected by industry as the accepted unit.

Based on this equation, conductivity is not ion-selective because it responds to all ions. Furthermore, the specific molar conductance of each ion is different. As a result, unless the percentage composition of ions in the solution is limited and known, the precise concentrations of ionic species cannot be determined from conductivity measurements. However, for examples such as a solution of a single salt, acid or base (e.g. a caustic solution used in cleaning) the precise concentration can be directly determined. Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentrations of all ionic species (anions and cations) for diluted solutions, as described in the equation above. At higher concentrations, conductivity measurements are not perfectly linear with concentration. Conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

Another variable that influences conductivity measurements is the fluid temperature. As fluid temperature increases, the ion conductance increases, making this physico-chemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids.

The conductivity ($\kappa$) is proportional to the conductance ($G$), of a fluid between 2 electrodes:

$$\kappa = G \times \left(\frac{d}{A}\right) = G \times K$$

$\kappa$ = conductivity, in siemens per centimetre;

$G$ = conductance, in siemens;

$d$ = distance between the electrodes, in centimetres;

$A$ = area of the conducting electrodes, in square centimetres;

$K$ = cell constant, in reciprocal centimetres, which is also equal to the ratio of $d/A$.

The resistivity $\rho$, expressed in ohm centimetres, of the fluid is, by definition, the reciprocal of the conductivity:

$$\rho = \frac{1}{\kappa} = \frac{1}{G \times K} = \frac{R}{K}$$

$\rho$ = resistivity, in ohm centimetres;

$\kappa$ = conductivity, in siemens per centimetre;

$G$ = conductance, in siemens;

$R$ = resistance, in ohms, which is the reciprocal of the conductance ($G$);

$K$ = cell constant, in reciprocal centimetres.

EQUIPMENT

An electrical conductivity measurement consists of the determination of resistance of the fluid between and around the electrodes of the conductivity sensor. To achieve this measurement, the primary instrumentation is the resistance-measuring circuit and the conductivity sensor, and they are usually connected by a cable when the sensor and the user interface are separated.

The resistance measurement is made by applying an AC (alternating current, meaning the flow of electric charge periodically reverses direction) voltage (or current) to the electrodes, measuring the current (or voltage), and calculating the resistance according to Ohm's law. The alternating source is utilised to prevent polarisation (collection of ions) at the electrodes. Depending on the equipment, the frequency of the measuring system adjusts automatically according to the measuring conditions of the instrument, and there may be multiple resistance-measuring circuits embedded in the measuring system. The resistance-measurement circuit may be embedded in the transmitter or in the sensor.

The conductivity sensor consists of at least 2 electrical conductors of a fixed size and geometry, separated by an electrical insulator. The electrodes, insulator and any other wetted components are constructed of materials that are unreactive to fluids with which they may come into contact. In addition, the sensor construction must be able to withstand the environmental conditions (process or ambient temperature, pressure, cleaning applications) that it would be subjected to.

Most conductivity sensors have temperature devices such as a platinum RTD (resistance temperature device) or NTC (negative temperature coefficient) thermistor embedded inside the sensor, although external temperature measurement is possible. The purpose of the temperature measurement is for temperature compensation of the conductivity measurement.

CELL CONSTANT DETERMINATION

The purpose of the sensor's cell constant is to normalise the conductance (or resistance) measurement for the geometrical construction of the 2 electrodes.

The cell constant is determined by immersing the conductivity sensor in a solution of known conductivity. Solutions of known conductivity can be obtained by preparation of specific recipes according to national authoritative sources, or procurement of commercially available certified and traceable standard solutions. These recipes or certified solutions can range from 5 to 200 000 $\mu S \cdot cm^{-1}$ depending on the level of accuracy desired. Alternatively, the cell constant is determined by comparison to other reference conductivity measuring systems (also available as an accredited calibration service). (*NOTE*: conductivity measurements are not perfectly linear with concentration.)

The measured cell constant of the conductivity sensor must be within 5 per cent of the nominal value indicated by the sensor certificate, unless otherwise prescribed.

Modern conductivity sensors normally do not change their cell constant over their lifetime. If a change in the cell constant is detected during calibration, cleaning of the sensor according to the manufacturer's recommendations is appropriate, followed by a repeat of the calibration procedure. Sometimes 'memory effects' appear, particularly when changing from high to low concentrations if the sensor is not well flushed.

CALIBRATION OF TEMPERATURE

In addition to verifying the sensor's cell constant, the embedded temperature device (or external temperature device) must be appropriately calibrated for the application, in order to apply the temperature compensation algorithm accurately. The temperature accuracy that is required depends on the criticality of the temperature to the application. An accuracy of ± 1 °C typically suffices.

CALIBRATION OF MEASUREMENT ELECTRONICS

The measurement circuit of the system is fundamentally an AC resistance measuring device. Appropriate verification and/or calibration of the measuring circuit is required for measurement systems with signal transfer via analog cable. This is accomplished by disconnecting the measuring circuit from the sensor's electrodes, attaching traceable resistors of known value to the measuring circuit using the same measurement system cable, and verifying that the measured resistance agrees with the resistor value to an acceptable level. A typical acceptance criterion for the resistance accuracy is below 2 per cent of the reading at resistances greater than 100 Ω, and increasing to 5 per cent at lower resistances. However, it is recommended that the application criticality ultimately determines the desired accuracy.

For conductivity systems that cannot have the resistance-measuring circuit disconnected from the electrodes (e.g. measurement circuit and electrodes in 1 mutual housing), it may be difficult to directly adjust or verify the circuit accuracy, depending on the sensor design. An alternative method of verifying the measurement system integrity is a system calibration according to the procedures for cell constant determination for each measuring circuit that is intended to be used.

If verification/calibration of the sensor's cell constant, temperature device and measuring circuit are done at the same service interval, it is recommended to verify the measuring circuit first, the temperature device next and the cell constant last. Because all of these parameters are typically very stable due to modern electronics and stable sensor construction, frequent calibration (such as daily) is not usually required. Comparison to qualified reference systems is also a suitable means of calibration. Calibration is performed at appropriate intervals as defined in the quality management system.

TEMPERATURE COMPENSATION

As the conductivity of a fluid is temperature dependent, temperature compensation of the conductivity measurement is necessary unless otherwise prescribed (e.g. purified water, water for injection). An appropriate temperature compensation algorithm will ensure that changes in the conductivity measurements can be ascribed to concentration changes and not temperature changes. Conductivity measurements are normally referenced to 25 °C. A common form of linear temperature compensation uses a temperature coefficient according to the following equation:

$$\kappa_{25} = \frac{\kappa_T}{[1 + \alpha(T - 25)]}$$

$\kappa_{25}$ = conductivity compensated to 25 °C;

$\kappa_T$ = conductivity at $T$;

$\alpha$ = temperature coefficient of conductivity;

$T$ = measured temperature.

A temperature coefficient of 2.1 per cent per degree Celsius is commonly used for many salt solutions. Most salt-based solutions have temperature coefficients of conductivity ranging from 1.9 to 2.2 per cent per degree Celsius. Depending on the fluid samples, other forms of temperature compensation may be appropriate. Non-linear temperature compensation data for a variety of solutions is widely available, e.g. as described in ISO 7888 *Water Quality – Determination of electrical conductivity*. In cases of very low conductivity (below 10 $\mu S \cdot cm^{-1}$), for example, purified water for pharmaceutical use, 2 compensations need to be made. One is for the intrinsic conductivity of water, and the other is for the other ionic species in water. These compensations are normally combined and embedded in the microprocessor-controlled conductivity measurement systems. This is not supplied in all conductivity measurement technologies.

CONDUCTIVITY MEASUREMENT OF FLUIDS

For off-line batch measurements, rinse the cleaned sensor with the fluid to be measured, then perform the measurement. Ensure that the position of the sensor in the container does not impact the conductivity measurement, as the container walls can impact the measurement for some electrode designs. Record the temperature and the temperature-compensated conductivity as required.

For continuous on-line or at-line measurements, install the cleaned sensor into the pipe, tank or other containment vessel, and flush if necessary. Make sure proper installation procedures are applied to prevent bubbles or particles from collecting between the electrodes. Ensure that the position of the sensor in the pipe or tank does not impact the conductivity measurement, as the nearby surfaces can affect the measurement for some electrode designs. Record the temperature and the temperature-compensated conductivity as required.

For all batch or continuous measurements, ensure that the wetted components of the sensor are compatible with the fluid and the temperature to be measured.

**01/2008:20239**

## 2.2.39. MOLECULAR MASS DISTRIBUTION IN DEXTRANS

Examine by size-exclusion chromatography (*2.2.30*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Marker solution.* Dissolve 5 mg of *glucose R* and 2 mg of *dextran $V_0$ CRS* in 1 mL of the mobile phase.

*Calibration solutions.* Dissolve separately in 1 mL of the mobile phase 15 mg of *dextran 4 for calibration CRS*, 15 mg of *dextran 10 for calibration CRS*, 20 mg of *dextran 40 for calibration CRS*, 20 mg of *dextran 70 for calibration CRS* and 20 mg of *dextran 250 for calibration CRS*.

*System suitability solution.* Dissolve either 20 mg of *dextran 40 for performance test CRS* (for dextran 40) or 20 mg of *dextran 60/70 for performance test CRS* (for dextran 60 and dextran 70) in 1 mL of the mobile phase.

The chromatographic procedure may be carried out using:

- a column 0.3 m long and 10 mm in internal diameter, packed with *cross-linked agarose for chromatography R* or a series of columns, 0.3 m long and 10 mm in internal diameter, packed with *polyether hydroxylated gel for chromatography R*,
- as the mobile phase, at a flow rate of 0.5-1 mL/min, kept constant to ± 1 per cent per hour, a solution containing 7 g of *anhydrous sodium sulfate R* and 1 g of *chlorobutanol R* in 1 L of *water R*,
- as detector a differential refractometer,
- a 100 µL to 200 µL loop injector,

maintaining the system at a constant temperature (± 0.1 °C).

CALIBRATION OF THE CHROMATOGRAPHIC SYSTEM

Carry out replicate injections of the chosen volume of the marker solution. The chromatogram shows 2 peaks, the first of which corresponds to *dextran $V_0$ CRS* and the second of which corresponds to *glucose R*. From the elution volume of the peak corresponding to dextran $V_0$, calculate the void volume $V_0$ and from the peak corresponding to dextrose, calculate the total volume $V_t$.

Inject the chosen volume of each of the calibration solutions. Draw carefully the baseline of each of the chromatograms. Divide each chromatogram into $p$ (at least 60) equal vertical sections (corresponding to equal elution volumes). In each section $i$, corresponding to an elution volume $V_i$ measure the height ($y_i$) of the chromatogram line above the baseline and calculate the coefficient of distribution $K_i$ using the expression:

$$\frac{(V_i - V_0)}{(V_t - V_0)} \qquad (1)$$

$V_0$ = void volume of the column, determined using the peak corresponding to *dextran $V_0$ CRS* in the chromatogram obtained with the marker solution,

$V_t$ = total volume of the column, determined using the peak corresponding to glucose in the chromatogram obtained with the marker solution,

$V_i$ = elution volume of section $i$ in the chromatogram obtained with each of the calibration solutions.

Carry out the calibration using either of the following methods.

**Calibration by plotting of the curve**. For each of the dextrans for calibration calculate the coefficient of distribution $K_{max}$ corresponding to the maximum height of the chromatographic line, using expression (1). Plot on semilogarithmic paper the values of $K_{max}$ (on the $x$-axis) against the declared molecular mass at the maximum height of the chromatographic line ($M_{max}$) of each of the dextrans for calibration and glucose. Draw a first calibration curve through the points obtained, extrapolating it from the point $K_{max}$ obtained with *dextran 250 for calibration CRS* to the lowest $K$ value obtained for this CRS (Figure 2.2.39.-1). Using this first calibration curve, transform, for each chromatogram, all $K_i$ values into the corresponding molecular mass $M_i$, thus obtaining the molecular mass distribution. Calculate for each dextran for calibration the average molecular mass $M_w$ using equation (3) below. If the calculated values for $M_w$ do not differ by more than 5 per cent from those declared for each of the dextrans for calibration and the mean difference is within ± 3 per cent, the calibration curve is approved. If not, move the calibration curve along the y-axis and repeat the procedure above until the calculated and the declared values for $M_w$ do not differ by more than 5 per cent.

**Calibration by calculation of the curve**. Calculate from equations (2) and (3) below, using a suitable method[(5)], values for $b_1$, $b_2$, $b_3$, $b_4$ and $b_5$ that give values of $M_w$ within 5 per cent of the declared values of each of the dextrans for calibration and 180 ± 2 for glucose:

$$M_i = b_5 + e^{(b_4 + b_1 K_i + b_2 K_i^2 + b_3 K_i^3)} \qquad (2)$$

$$\overline{M}_w = \frac{\sum_{i=1}^{p} (y_i M_i)}{\sum_{i=1}^{p} y_i} \qquad (3)$$

$p$ = number of sections dividing the chromatograms,

$y_i$ = height of the chromatographic line above the baseline in section $i$,

$M_i$ = molecular mass in section $i$.

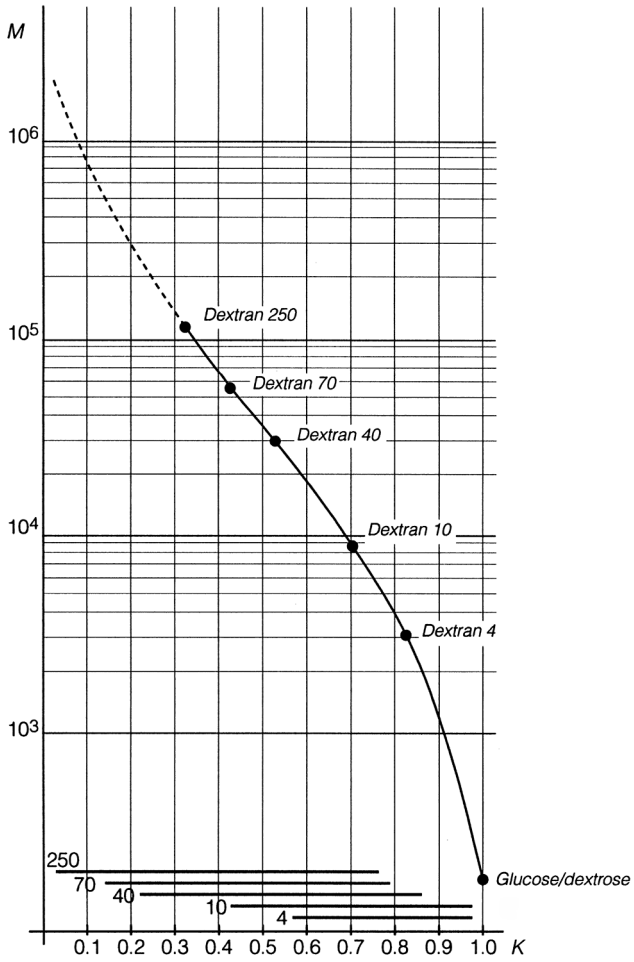


Figure 2.2.39.-1. - *Example of a calibration curve.*

*The dotted line corresponds to the part of the curve that is extrapolated. Horizontal lines at the bottom of the figure represent the width and the position of the chromatographic line obtained with each of the dextrans for calibration.*

SYSTEM SUITABILITY

Inject the chosen volume of the appropriate system suitability solution.

**Average molecular mass of dextran for performance test CRS**. Calculate the average molecular mass $M_w$ as indicated under Calibration of the chromatographic system, using either the plotted calibration curve or the values obtained above for $b_1$, $b_2$, $b_3$, $b_4$ and $b_5$. The test is not valid unless $M_w$ is:

- 41 000 to 47 000 (*dextran 40 for performance test CRS*),
- 67 000 to 75 000 (*dextran 60/70 for performance test CRS*).

(5) An iterative method such as the Gauss-Newton method modified by Hartley is suitable (see O. Hartley, Tecnometrics, 3 (1961) and G. Nilsson and K. Nilsson, J. Chromat. 101, 137 (1974)). A curve-fitting programme for microcomputers, capable of non-linear regression, may be used.

**Average molecular mass of the 10 per cent high-fraction dextran**. Calculate $M_w$ for the 10 per cent high-fraction dextran eluted through section $n$ using the equation:

$$M_w = \frac{\sum_{i=1}^{n}(y_i M_i)}{\sum_{i=1}^{n} y_i} \quad (4)$$

in which $n$ is defined by the expressions:

$$\sum_{i=1}^{n} y_i \leq 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (5)$$

$$\sum_{i=1}^{n+1} y_i > 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (6)$$

$p$ = number of sections dividing the chromatograms,

$y_i$ = height of the chromatographic line above the baseline in section $i$,

$M_i$ = molecular mass in section $i$.

The test is not valid unless $M_w$ of the 10 per cent high fraction dextran is:

– 110 000 to 130 000 (*dextran 40 for performance test CRS*),

– 190 000 to 230 000 (*dextran 60/70 for performance test CRS*).

**Average molecular mass of the 10 per cent low-fraction dextran**. Calculate $M_w$ for the 10 per cent low-fraction dextran eluted in and after section $m$ using the expression:

$$M_w = \frac{\sum_{i=m}^{p}(y_i M_i)}{\sum_{i=m}^{p} y_i} \quad (7)$$

in which $m$ is defined by the expressions:

$$\sum_{i=m}^{p} y_i \leq 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (8)$$

$$\sum_{i=m-1}^{p} y_i > 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (9)$$

$p$ = number of sections dividing the chromatograms,

$y_i$ = height of the chromatographic line above the baseline in section $i$,

$M_i$ = molecular mass in section $i$.

The test is not valid unless $M_w$ of the 10 per cent low-fraction dextran is:

– 6000 to 8500 (*dextran 40 for performance test CRS*),

– 7000 to 11 000 (*dextran 60/70 for performance test CRS*).

MOLECULAR MASS DISTRIBUTION OF THE DEXTRAN TO BE ANALYSED

Inject the chosen volume of the test solution and calculate $M_w$ of the total molecular mass distribution, $M_w$ of the 10 per cent high-fraction dextran and $M_w$ of the 10 per cent low-fraction dextran as indicated under System suitability.

01/2014:20240

# 2.2.40. NEAR-INFRARED SPECTROSCOPY

Near-infrared (NIR) spectroscopy is a technique with wide and varied applications in pharmaceutical analysis. The NIR spectral range extends from 780 nm to 2500 nm (from 12 800 $cm^{-1}$ to 4000 $cm^{-1}$). NIR spectra are dominated by C-H, N-H, O-H and S-H overtones and combinations of fundamental mid-infrared (MIR) vibrations. They contain composite chemical and physical information and in most cases this information can be extracted by suitable mathematical data treatment. NIR bands are much weaker than the fundamental MIR vibrations from which they originate. Because absorptivities in the NIR range are low, radiation can penetrate up to several millimetres into materials, including solids. Furthermore, many materials such as glass are relatively transparent in this region.

Measurements can be made directly *in situ*, in addition to standard sampling and testing procedures. NIR measurements can be performed off-line, and also at-line or in-line, and on-line for process analytical technology (PAT). Suitable chemometric methods may be required for identification. However, when the specificity criteria for a qualitative method are met, chemical identification or solid-state characterisation is possible by direct comparison of the untreated or pre-treated spectra obtained with the chemical substance being examined with a spectrum of a reference substance.

NIR spectroscopy has a wide variety of applications for chemical, physical and process analysis, for example:

*Chemical analysis*:

– identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical materials and packaging materials;

– qualification of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;

– quantification of active substances in a sample matrix, determination of chemical values such as hydroxyl value, determination of absolute water content, determination of degree of hydroxylation and control of solvent content.

*Physical analysis*:

– crystalline form and crystallinity, polymorphism, solvates, particle size;

– disintegration, hardness;

– film properties.

*Process analysis*:

– monitoring of unit operations such as synthesis, crystallisation, blending, drying, granulation and coating, for the purpose of process control;

– control and endpoint detection.

Measurements in the NIR region are influenced by many chemical and physical factors as described below; the reproducibility and relevance of results depend on control of these factors and measurements are usually valid only for a defined calibration model.

APPARATUS

All NIR measurements are based on passing light through or into a sample and measuring the attenuation of the emerging (transmitted or reflected) beam. Spectrometers for measurement in the NIR region consist of a suitable light source (such as a highly-stable quartz-tungsten lamp), a monochromator or interferometer, and a detector. Common monochromators are acousto-optic tunable filters (AOTF),

gratings or prisms. Traditionally, many NIR instruments have a single-beam design, though some process instruments use internal referencing and can therefore be dual-beam (for example in diode array instruments). Silicon, lead sulfide, and indium gallium arsenide are examples of detector materials. Conventional cuvette sample holders, fibre-optic probes, transmission dip cells, neutral borosilicate vials and spinning or traversing sample holders are a few examples of sampling devices. The selection is based on the intended application, paying particular attention to the suitability of the sampling system for the type of sample to be analysed. Suitable data processing and evaluation units (e.g. software and computer) are usually part of the system.

It is common to express the wavelength (λ) in nanometres and the wavenumber ($\nu$) in reciprocal centimetres ($cm^{-1}$), depending on the measurement technique and apparatus. Conversion between nm and $cm^{-1}$ is performed according to the following expression:

$$\nu_{cm^{-1}} = 10^7 \times \frac{1}{\lambda_{nm}}$$

MEASUREMENT METHODS

**Transmission mode**. Transmittance ($T$) is a measure of the decrease in radiation intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and the detector. The arrangement is analogous to that in many conventional spectrometers. The resulting spectrum can be presented directly in terms of transmittance ($T$) and/or absorbance ($A$) (y-axis) versus the wavelength or wavenumber (x-axis).

$$T = \frac{I}{I_0}$$

$I_0$ = intensity of incident radiation;

$I$ = intensity of transmitted radiation;

$$A = -\log_{10} T = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$

**Diffuse reflection mode**. The diffuse reflection mode gives a measure of reflectance ($R$), the ratio of the intensity of light reflected from the sample ($I$) to that reflected from a background or reference reflective surface ($I_r$). Depending on the chemical composition and physical characteristics of the sample, NIR radiation can penetrate a more or less substantial distance into the sample, where it can be absorbed by the overtones and combinations of the fundamental vibrations of the analyte species present in the sample. Non-absorbed radiation is partially reflected back from the sample to the detector. NIR reflectance spectra are typically obtained by calculating and plotting $\log_{10}(1/R)$ (y-axis) versus the wavelength or wavenumber (x-axis).

$$R = \frac{I}{I_r}$$

$I$ = intensity of light diffusively reflected from the sample;

$I_r$ = intensity of light reflected from the background or reference reflective surface;

$$A_R = \log_{10}\left(\frac{1}{R}\right) = \log_{10}\left(\frac{I_r}{I}\right)$$

**Transflection mode**. This mode is a combination of transmittance and reflectance. In the measurement of transflectance ($T^*$), a mirror or a diffuse reflectance surface is used to reflect the transmitted radiation back through the sample, thus doubling the pathlength. Non-absorbed radiation is reflected back from the sample to the detector. The resulting spectrum can be presented directly in terms of transflectance ($T^*$) and/or absorbance ($A^*$) (y-axis) versus the wavelength or wavenumber (x-axis).

$$T^* = \frac{I}{I_T}$$

$I$ = intensity of transflected radiation measured from the sample;

$I_T$ = intensity of transflected radiation of the reference material as background;

$$A^* = \log_{10}\left(\frac{1}{T^*}\right) = \log_{10}\left(\frac{I_T}{I}\right)$$

SAMPLE PREPARATION/PRESENTATION

Sample preparation and presentation may vary according to the measurement mode. The following requirements are necessary for all sampling techniques:

- optimise the measuring time and number of scans to optimise the signal-to-noise ratio;
- find the best suitable measurement mode for the intended application (transmission, diffuse reflection or transflection);
- find the best orientation of the sample (e.g. to minimise the impact of debossing on tablets);
- find the best suitable accessory (e.g. transmission cell or immersion probe);
- optimise pathlength in transmission and transflection modes;
- find a suitable spectroscopic background reference material;
- show that the background reference material is reliable over time and that the measurement of the background is reproducible and stable over time;
- when measuring moving materials or samples (for process-related measurements) it is important to obtain a representative spectrum (e.g. by adjusting the measuring time, the number of scans, co-adding individual spectra, or increasing the beam size);
- ensure there is no fouling of the sensor, for example with build-up of material or contamination;
- the measuring conditions (measuring time, beam size) in relation to the minimal sample size should be justified.

In some process analysis situations it may be impossible to remove a probe for reference background data collection; various options are therefore to be considered, including internal referencing, measurement of a background reference using a 2nd detector, etc. Only spectra measured against a background possessing the same optical properties can be directly compared with one another.

**Transmission mode**. The measurement of transmittance ($T$) is dependent on a background transmittance spectrum for its calculation. Examples of background references include air, a polymeric disc, an empty cell, a solvent blank or in special cases a reference sample. The method generally applies to liquids (diluted or undiluted), dispersions, solutions and solids (including tablets and capsules). For transmittance measurements of solids, a suitable sample accessory is used. Liquid samples are examined in a cell of suitable pathlength (typically 0.5-4 mm), transparent to NIR radiation, or alternatively by immersion of a fibre-optic probe of a suitable configuration.

**Diffuse reflection mode**. This mode generally applies to solids. The sample is examined directly, or in a suitable device (for example a sample holder), or in direct contact with a fibre-optic probe. For process monitoring, material can be analysed through a polished window interface (e.g. sapphire), or using an in-line fibre-optic probe. Care must be taken to ensure that the measuring conditions are as reproducible as

possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references include ceramic, thermoplastic resins and gold. Other suitable materials may be used.

**Transflection mode**. This mode generally applies to liquids, suspensions and clear plastic materials. A reflector is placed behind the sample so as to double the pathlength. This configuration can be adopted to share the same instrument geometry with reflectance and fibre-optic probe systems where the source and the detector are on the same side of the sample. The sample is examined through a cell with a mirror or a suitable diffusive reflector, made either of metal or of an inert substance (for example, dried titanium dioxide) not absorbing in the NIR region. Liquids can also be measured using in-line transflectance probes.

FACTORS AFFECTING SPECTRAL RESPONSE

**Environment**. The environment temperature and humidity must be taken into consideration before carrying out measurements.

**Sample presentation area**. The sample presentation area or probe end must be clean of residue prior to measurement. Similarly, the in-line or on-line interface to the sample should not have significant product or contamination build-up, which would interfere with the desired measurement.

**Sample temperature**. This parameter is important for aqueous solutions and many liquids, where a difference of a few degrees can result in measurable spectral changes which may have a significant effect on the analysis. Temperature is also an important parameter for solids and powders containing water.

**Moisture and solvent residues**. Moisture and solvent residues present in the samples will contribute significant absorption bands in the NIR region.

**Sample thickness**. Sample thickness is a known source of spectral variability and must be assessed and/or controlled, particularly for tablet and capsule analysis in transmittance mode. For the measurement of compressed powders, an infinite thickness is typically reached after 5 mm of sample depth (e.g. in a vial).

**Sample optical properties**. In solids, both surface and bulk scattering properties of samples must be taken into account. Spectra of physically, chemically or optically heterogeneous samples may require increasing the beam size, or examining multiple samples or spinning the sample to obtain a representative spectrum of the sample. Certain factors such as differing degree of compaction or particle size in powdered materials and surface finish can cause significant spectral differences.

**Solid-state forms**. Variations in solid-state forms (polymorphs, hydrates, solvates and amorphous forms) influence vibrational spectra. Hence, different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration samples have a distribution of forms relevant to the intended application.

**Age of samples**. Samples may exhibit changes in their chemical, physical or optical properties over time. Depending on the storage conditions, solid samples may either absorb or desorb water, and portions of amorphous material may crystallise. Materials used for NIR calibration should be representative of future samples and their matrix variables.

PRE-TREATMENT OF NIR SPECTRAL DATA

In many cases, and particularly for reflection mode spectra, some form of mathematical pre-treatment of the spectrum may be useful prior to the development of a classification or calibration model. The aim can be, for example, to reduce baseline variations, to reduce the impact of known variations that are interfering in the subsequent mathematical models, or to simplify data before use. In some cases spectra may also be normalised or corrected for scatter, for example using standard normal variate (SNV) transformation. Spectral pre-treatment techniques may include, for example, windowing and noise reduction and the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended because of increased spectral noise.

CONTROL OF INSTRUMENT PERFORMANCE

Use the apparatus according to the manufacturer's instructions and carry out the prescribed verification at regular intervals, according to the use of the apparatus and the application. For in-line and on-line applications, the use of alternative means of control of instrument performance must be scientifically justified. For example, utilise the standards built into the instrument or separate channels/probes to demonstrate instrument performance (pending practicality).

System suitability tests may be required prior to sample scanning, and the instrument attributes with potential impact on suitability of the final measurement (typically photometric noise and wavelength accuracy) must be tested. The frequency at which each performance test is carried out must be risk-assessed depending on the instrument type and its environment. For example, instruments placed in harsh environments with variations in temperature and humidity may need frequent performance testing. Cases where the measurement system cannot be removed such as an in-line probe or flow cell are also to be considered.

Some accessories are custom designed and therefore require adequate performance testing.

*Verification and calibration of the wavelength or wavenumber scale (except for filter apparatus)*. Verify the wavelength scale employed, generally in the region between 780 nm and 2500 nm ( 12 800 $cm^{-1}$ to 4000 $cm^{-1}$) or in the intended spectral range using one or more suitable wavelength standards which have characteristic maxima or minima within the wavelength range to be used. For example, *methylene chloride R*, *talc R*, wavelength reference lamps or a mixture of rare-earth oxides are suitable reference materials. Other suitable standards may also be used. Obtain a spectrum and measure the position of at least 3 peaks distributed over the range used. For rare-earth oxides, the National Institute of Standards and Technology (NIST) provides suitable reference materials. Fourier transform (FT) instruments have a linear frequency range, therefore wavelength certification at a single frequency is sufficient.

*Verification and calibration of photometric linearity*. The photometric linearity is demonstrated with a set of transmittance or reflectance standards with known percentage values of transmittance or reflectance. For reflectance measurements, carbon-doped polymer standards are available. Ensure that the absorbance of the materials used is relevant to the intended linear working range of the method. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. Non-linear calibration models and hence non-linear responses are acceptable with understanding demonstrated by the user.

Spectra obtained from reflectance and transmittance standards are subject to variability due to the differences between the experimental conditions under which they were factory-calibrated and those under which they are subsequently put to use. Hence, the percentage reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an 'absolute' calibration for a given instrument. As long as the standards do not change chemically or physically and the same reference background is used as was used to obtain the certified values, subsequent measurements of the same standards under

identical conditions, including precise sample positioning, give information on long-term stability of the photometric response. A tolerance of ± 2 per cent of the absorbance value is acceptable for long-term stability; this verification is only necessary if the spectra are used without pre-treatment.

Recommendations for the conditions used to control instrument performance for the various measurement modes are summarised in Table 2.2.40.-1.

Table 2.2.40.-1 – *Control of instrument performance*

| Measurement mode | Reflection | Transflection | Transmission |
|---|---|---|---|
| *Verification of wavelength scale (except for filter apparatus)* | Typical tolerances for agreement with standard values are:<br>± 1.0 nm at 780 nm (± 16 $cm^{-1}$ at 12 800 $cm^{-1}$);<br>± 1.0 nm at 1200 nm (± 8 $cm^{-1}$ at 8300 $cm^{-1}$);<br>± 1.0 nm at 1600 nm (± 6 $cm^{-1}$ at 6250 $cm^{-1}$);<br>± 1.5 nm at 2000 nm (± 4 $cm^{-1}$ at 5000 $cm^{-1}$);<br>± 1.5 nm at 2500 nm (± 2 $cm^{-1}$ at 4000 $cm^{-1}$).<br>For the reference material used, apply the tolerance for the nearest wavelength or wavenumber for each peak used. For diode array instruments, most often the pixel resolution (wavelength between pixels) can be as large as 10 nm. The pixel resolution must be adapted to match the spectral resolution. The peak-finding algorithms are critical to wavelength accuracy. Practically, ± 2 nm is appropriate for peak wavelength accuracy using such instrumentation. Alternatively, refer to manufacturer's specifications for acceptance. | | |
| Bench/mobile instrument | Measure *talc R* via a suitable medium or by fibre-optic probe. *Talc R* has characteristic peaks at 948 nm, 1391 nm and 2312 nm suitable for calibration.<br>Alternatively, other suitable standards may also be used that ensure wavelength accuracy in the region of working methodology. For example, measure an internal polystyrene standard if built-in, or measure a NIST standard or other traceable material, and assess 3 peaks across the wavelength range for calibration. | A suspension of 1.2 g of dry *titanium dioxide R* in about 4 mL of *methylene chloride R* is used directly with a cell or a probe. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 $cm^{-1}$ at 4000 $cm^{-1}$). Methylene chloride has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as a liquid transflection standard mixed with titanium dioxide or some other reflective medium. | *Methylene chloride R* may be used and has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used. |
| Process instrument | If it is not practically possible to measure a traceable reference material at the point of sample measurement, use internal material such as polystyrene, fibreglass or solvent and/or water vapour. Alternatively, adopt a second external channel/probe.<br>For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapour line, for example, the line at 7306.74 $cm^{-1}$, or 7299.45 $cm^{-1}$, or 7299.81 $cm^{-1}$ or a narrow line from a certified reference material. | | |
| *Verification of wavelength repeatability (except for filter apparatus)* | The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified. | | |
| Bench/mobile instrument | Verify the wavelength repeatability using a suitable external or internal standard. | | |
| Process instrument | Verify the wavelength repeatability using a suitable external or internal standard. | | |
| *Verification of photometric linearity and response stability*(1) | Measure 4 photometric standards across the working method absorbance range. | | |
| Bench/mobile instrument | Analyse 4 reflectance standards, for example in the range of 10-99 per cent, including 10 per cent, 20 per cent, 40 per cent and 80 per cent. In some circumstances 2 per cent may be appropriate. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. | Transflection measurements can use appropriate reflectance or transmittance standards and criteria. | Analyse 4 transmittance standards to cover the absorbance values over the working absorbance range of the modelled data. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. |

| Measurement mode | Reflection | Transflection | Transmission |
|---|---|---|---|
| Process instrument | If photometric reflectance and transmittance standards cannot be measured at the point of sample measurement, use the photometric standards built into the instrument.<br>Process instruments can use internal photometric standards for photometric linearity. Follow the manufacturer's verified tolerances in such cases. | | |

| | | |
|---|---|---|
| *Verification of photometric noise*[(1)] | Determine the photometric noise at a relevant photometric region of the spectrum using a suitable reflectance standard, for example, white reflective ceramic tiles or carbon-doped polymer standards. Follow the manufacturer's methodology and specifications. | |
| Bench/mobile instrument | Scan the reflectance low flux standard (e.g. 5 or 10 per cent, carbon-doped polymer standard) over a suitable wavelength range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. | Scan the transmittance high flux standard (e.g. 90 or 99 per cent, carbon-doped polymer standard) over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise. |
| Process instrument | As above, or if not practically possible, use the standard built into the instrument for noise testing and manufacturer specifications. | As above, or if not practically possible, use the standard built into the instrument for noise testing and manufacturer specifications. |

(1) *Verification of photometric linearity* and *Verification of photometric noise* are not required for instruments using methods to perform simple identifications which do not use the photometric absorbances as part of model strategy (for example, simple correlation with absorbing wavelengths).

QUALITATIVE ANALYSIS (IDENTIFICATION AND CHARACTERISATION)

**Establishment of a spectral reference library**. Record the spectra of a suitable number of representative samples of the substance which have known, traceable identities, and that exhibit the variation typical for the substance to be analysed (for example, solid-state form, particle size, etc.). Libraries are built using representative samples under appropriate environmental conditions. The set of spectra obtained represents the information which can be used for identification of the sample to be analysed.

The collection of spectra in the library may be represented in different ways defined by the mathematical technique used for identification. These may be:

- all individual spectra representing the substance;
- a mean spectrum of the measured batches for each chemical substance;
- if necessary, a description of the variability within the substance spectra.

The number of substances in the library depends on the specific application. All spectra in the library used have the same:

- spectral range and number of data points;
- technique of measurement;
- data pre-treatment.

If sub-groups (sub-libraries) are created, the above criteria are applied independently for each group. Sub-libraries are individually validated. Original spectral data for the preparation of the spectral library must be archived. Caution must be exercised when performing any mathematical transformation, as artefacts can be introduced or essential information (important with qualification methods) can be lost. The suitability of the algorithm used should be demonstrated by successful method validation and in all cases the rationale for the use of transform must be documented.

**Direct comparison of substance and reference spectra**. Direct comparison of representative spectra of the substance to be examined and of a reference substance for qualitative chemical or physical identification purposes may not require use of a reference spectral library where specificity permits.

**Data evaluation**. Direct comparison of the representative spectrum of the substance to be examined is made with the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification; alternatively, this can be achieved visually by overlaying spectral data if specificity is inherent. There are different techniques available, such as principal component analysis (PCA), cluster analysis, and soft independent modelling by class analogy (SIMCA). The reliability of the technique chosen for a particular application has to be validated according to the following:

**Validation of the model**. Identification methods using direct spectral comparison must be validated in accordance with identification method validation procedures.

The validation parameters for qualitative methods are robustness and specificity.

LIMIT ANALYSIS

**Relative comparison of spectra**. A calibration is not required when comparing a set of spectra for limit analysis purposes, such as the maximum or minimum absorbance at which an analyte absorbs. Also, dryer end point control may use a qualitative approach around a specific absorbing wavelength. Appropriate spectral ranges and pre-treatments (if used) must be shown to be fit for purpose.

**Specificity**. The relative discriminatory power for a limit test must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the measurement.

TREND ANALYSIS

**Relative comparison of spectra**. A calibration is not necessarily required when comparing a set of spectra for trend analysis purposes, such as the moving block approach to estimate statistical parameters such as mean, median and standard deviation. For example, blend uniformity monitoring using NIR spectroscopy has adopted such data analysis approaches. Appropriate spectral ranges and algorithms must be used for trend analyses.

**Specificity**. The relative discriminatory power for trend analysis must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the trend analysis.

QUANTITATIVE ANALYSIS

**Establishment of a spectral reference library for a calibration model**. Calibration is the process of constructing a mathematical model to relate the response from a sample scanned using an analytical instrument to the properties of the samples. Any calibration model that can be clearly defined in a mathematical expression and gives suitable results can be used. Record the spectra of a suitable number of representative samples with known or future-established values of the attribute of interest throughout the range to be measured (for example, content of water). The number of samples for calibration will depend on the complexity of the sample matrix and interferences (e.g. temperature, particle size, etc.). All samples must give quantitative results within a calibration interval as defined by the intended purpose of the method. Multiple linear regression (MLR), principal component regression (PCR) and partial least squares regression (PLS) are commonly used algorithms. For PLS or PCR calibrations, the regression coefficients and/or the loadings should be plotted and the regions of large coefficients or loadings compared with the spectrum of the analyte. Predicted residual error sum of squares (PRESS) plots (or similar) are useful to facilitate the optimising of the number of PCR or PLS factors.

**Pre-treatment of data**. Wavelength selection or excluding certain wavelength ranges may enhance the accuracy and robustness of calibration models. Wavelength compression (wavelength averaging) techniques may be applied to the data.

**Model validation parameters**. Analytical performance characteristics to be considered for demonstrating the validation of NIR methods are similar to those required for any analytical procedure. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. Validation parameters for quantitative methods are accuracy, linearity, precision (repeatability and intermediate precision), robustness and specificity.

ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters.

TRANSFER OF DATABASES

When databases are transferred to another instrument, spectral range, number of data points, spectral resolution and other parameters have to be taken into consideration. Further procedures and criteria must be applied to demonstrate that the model remains valid with the new database or new instrument.

**01/2008:20241**

# 2.2.41. CIRCULAR DICHROISM

The difference in absorbance of optically active substances within an absorption band for left and right circularly polarised light is referred to as circular dichroism.

Direct measurement gives a mean algebraic value:

$$\Delta A = A_L - A_R$$

$\Delta A$ = circular dichroic absorbance,

$A_L$ = absorbance of left circularly polarised light,

$A_R$ = absorbance of right circularly polarised light.

Circular dichroism is calculated using the equation:

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{\Delta A}{c \times l}$$

$\Delta\varepsilon$ = molar circular dichroism or molar differential dichroic absorptivity expressed in litre·mole$^{-1}$·cm$^{-1}$,

$\varepsilon_L$ = molar absorptivity (*2.2.25*) of left circularly polarised light,

$\varepsilon_R$ = molar absorptivity of right circularly polarised light,

$c$ = concentration of the test solution in mole·litre$^{-1}$,

$l$ = optical path of the cell in centimetres.

The following units may also be used to characterise circular dichroism:

*Dissymmetry factor*:

$$g = \frac{\Delta\varepsilon}{\varepsilon}$$

$\varepsilon$ = molar absorptivity (*2.2.25*).

*Molar ellipticity*:

Certain types of instruments display directly the value of ellipticity Θ, expressed in degrees. When such instruments are used, the molar ellipticity [Θ] may be calculated using the following equation:

$$[\Theta] = \frac{\Theta \times M}{c \times l \times 10}$$

[Θ] = molar ellipticity, expressed in degrees·cm$^2$·decimole$^{-1}$,

Θ = value of ellipticity given by the instrument,

$M$ = relative molecular mass of the substance to be examined,

$c$ = concentration of the solution to be examined in g/mL,

$l$ = optical path of the cell in centimetres.

Molar ellipticity is also related to molar circular dichroism by the following equation:

$$[\Theta] = 2.303\Delta\varepsilon\frac{4500}{\pi} \approx 3300\Delta\varepsilon$$

Molar ellipticity is often used in the analysis of proteins and nucleic acids. In this case, molar concentration is expressed in terms of monomeric residue, calculated using the expression:

$$\frac{\text{molecular mass}}{\text{number of amino acids}}$$

The mean relative molecular mass of the monomeric residue is 100 to 120 (generally 115) for proteins and about 330 for nucleic acids (as the sodium salt).

**Apparatus**. The light source (S) is a xenon lamp (Figure 2.2.41.-1); the light passes through a double monochromator (M) equipped with quartz prisms (P1, P2).

The linear beam from the first monochromator is split into 2 components polarised at right angles in the second monochromator. The exit slit of the monochromator eliminates the extraordinary beam.

The polarised and monochromatic light passes through a birefringent modulator (Cr): the result is alternating circularly polarised light.

The beam then passes through the sample to be examined (C) and reaches a photomultiplier (PM) followed by an amplifier circuit which produces 2 electrical signals: one is a direct current $V_c$ and the other is an alternating current at the modulation frequency $V_{ac}$ characteristic of the sample to be examined. The phase gives the sign of the circular dichroism. The ratio $V_{ac}/V_c$ is proportional to the differential absorption $\Delta A$ which created the signal. The region of wavelengths normally covered by a dichrograph is 170 nm to 800 nm.

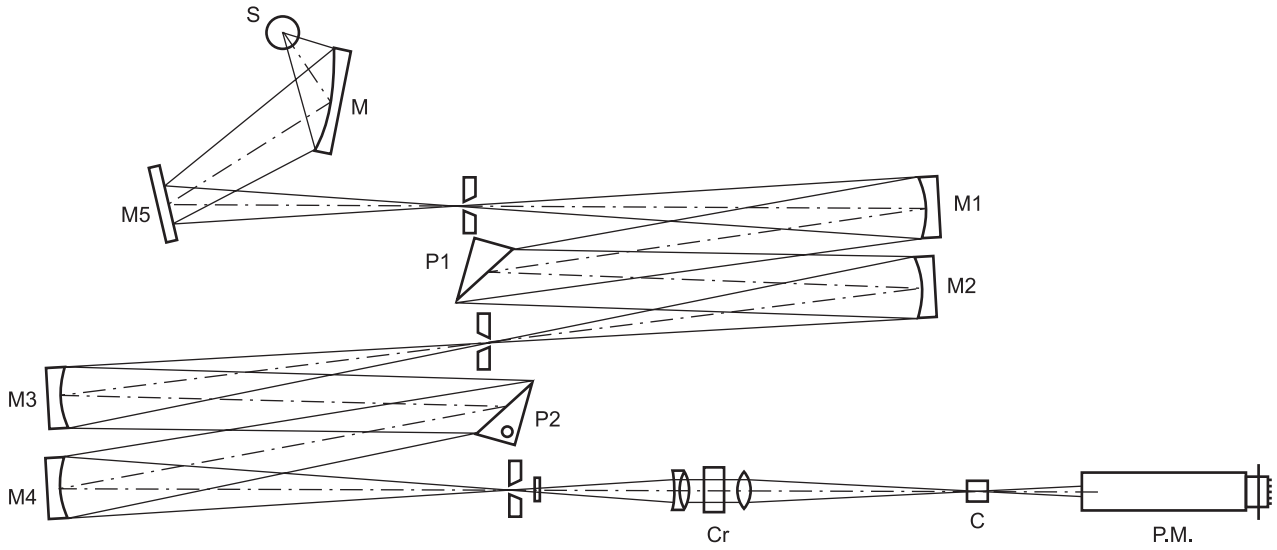


Figure 2.2.41.-1. – *Optical scheme of a dichrograph*

**Calibration of the apparatus**

*Accuracy of absorbance scale*. Dissolve 10.0 mg of *isoandrosterone R* in *dioxan R* and dilute to 10.0 mL with the same solvent. Record the circular dichroism spectrum of the solution between 280 nm and 360 nm. Measured at the maximum at 304 nm, $\Delta\varepsilon$ is + 3.3.

The solution of *(1S)-(+)-10-camphorsulfonic acid R* may also be used.

*Linearity of modulation*. Dissolve 10.0 mg of *(1S)-(+)-10-camphorsulfonic acid R* in *water R* and dilute to 10.0 mL with the same solvent. Determine the exact concentration of camphorsulfonic acid in the solution by ultraviolet spectrophotometry (*2.2.25*), taking the specific absorbance to be 1.49 at 285 nm.

Record the circular dichroism spectrum between 185 nm and 340 nm. Measured at the maximum at 290.5 nm, $\Delta\varepsilon$ is + 2.2 to + 2.5. Measured at the maximum at 192.5 nm, $\Delta\varepsilon$ is − 4.3 to − 5.

*(1S)-(+)-* or antipodal *(1R)-(−)-ammonium 10-camphorsulfonate R* can also be used.

**01/2010:20242**

# 2.2.42. DENSITY OF SOLIDS

The density of solids corresponds to their average mass per unit volume and typically is expressed in grams per cubic centimetre (g/cm$^3$) although the International Unit is the kilogram per cubic metre (1 g/cm$^3$ = 1000 kg/m$^3$).

Unlike gases and liquids whose density depends only on temperature and pressure, the density of a solid also depends on its assembly and therefore varies with the crystal structure and degree of crystallinity.

When a solid is amorphous or partially amorphous, its density may further depend upon the history of preparation, treatment and storage.

Therefore, unlike fluids, the densities of 2 chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish 3 levels of expression of density:

- the *true density*, which only includes the solid fraction of the material; in case of crystalline material, the true density is also called *crystal density*;
- the *particle density*, which also includes the volume due to intraparticulate pores;
- the *bulk density*, which further includes the interparticulate void volume formed in the powder bed.

TRUE DENSITY

The true density of a substance is the ratio of the mass to the volume of the unit cell, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is an intrinsic property of the specified crystal structure of substance, and hence should be independent of the method of determination. The true density is determined by calculation.

It is obtained using crystallographic data (volume and composition of the unit cell) from, for example, X-ray diffraction data, either on a single crystal or by refinement of the crystalline structure from X-ray powder diffraction data.

PARTICLE DENSITY

The particle density takes into account both the true density and the intraparticulate porosity (sealed and/or experimentally non-accessible open pores). Thus, particle density depends on the value of the volume determined, which in turn depends on the method of measurement. The particle density can be determined using one of the 2 following methods.

The gas *pycnometric density* is determined by measuring the volume occupied by a known mass of powder, which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer (*2.9.23*). In gas pycnometric density measurements, the volume determined excludes the volume occupied by open pores; however, it includes the volume occupied by sealed pores or pores inaccessible to the gas. Due to the high diffusivity of helium, which is the preferred choice of gas, most open pores are accessible to the gas. Therefore, the gas pycnometric density of a finely milled powder is generally not very different from the true density. Hence, this density is the best estimate of the true density of an amorphous or partially crystalline sample and is therefore widely applicable for processed pharmaceutical powder samples.

The *mercury porosimeter density* is also called *granular density*. With this method the volume determined includes the volume occupied by sealed pores or pores inaccessible to mercury; however, it includes the volume only from open pores smaller than some size limit. This pore-size limit or minimal access diameter depends on the maximal mercury intrusion pressure applied during the measurement, and under normal

operating pressures the mercury does not penetrate the finest pores accessible to helium. Various granular densities can be obtained from one sample since, for each applied mercury intrusion pressure, a density can be determined that corresponds to the pore-size limit at that pressure.

BULK AND TAPPED DENSITY

The bulk density of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed.

The bulk density of a powder is often very difficult to measure with good reproducibility since the slightest disturbance of the bed may result in a new density. Thus, it is essential in reporting bulk density to specify how the determination was made.

The bulk density and the tapped density are determined as mentioned in chapter *2.9.34. Bulk density and tapped density*.

**01/2008:20243**

# 2.2.43. MASS SPECTROMETRY

Mass spectrometry is based on the direct measurement of the ratio of the mass to the number of positive or negative elementary charges of ions (*m*/*z*) in the gas phase obtained from the substance to be analysed. This ratio is expressed in atomic mass units (1 a.m.u. = one twelfth the mass of $^{12}C$) or in daltons (1 Da = the mass of the hydrogen atom).

The ions, produced in the ion *source* of the apparatus, are accelerated and then separated by the *analyser* before reaching the *detector*. All of these operations take place in a chamber where a pumping system maintains a vacuum of $10^{-3}$ to $10^{-6}$ Pa.

The resulting spectrum shows the relative abundance of the various ionic species present as a function of *m*/*z*. The signal corresponding to an ion will be represented by several peaks corresponding to the statistical distribution of the various isotopes of that ion. This pattern is called the *isotopic profile* and (at least for small molecules) the peak representing the most abundant isotopes for each atom is called the *monoisotopic peak*.

Information obtained in mass spectrometry is essentially qualitative (determination of the molecular mass, information on the structure from the fragments observed) or quantitative (using internal or external standards) with limits of detection ranging from the picomole to the femtomole.

INTRODUCTION OF THE SAMPLE

The very first step of an analysis is the introduction of the sample into the apparatus without overly disturbing the vacuum. In a common method, called *direct liquid introduction*, the sample is placed on the end of a cylindrical rod (in a quartz crucible, on a filament or on a metal surface). This rod is introduced into the spectrometer after passing through a vacuum lock where a primary intermediate vacuum is maintained between atmospheric pressure and the secondary vacuum of the apparatus.

Other introduction systems allow the components of a mixture to be analysed as they are separated by an appropriate apparatus connected to the mass spectrometer.

**Gas chromatography/mass spectrometry**. The use of suitable columns (capillary or semi-capillary) allows the end of the column to be introduced directly into the source of the apparatus without using a separator.

**Liquid chromatography/mass spectrometry**. This combination is particularly useful for the analysis of polar compounds, which are insufficiently volatile or too heat-labile to be analysed by gas chromatography coupled with mass spectrometry. This method is complicated by the difficulty of obtaining ions in the gas phase from a liquid phase, which requires very special interfaces such as:

- *direct liquid introduction*: the mobile phase is nebulised, and the solvent is evaporated in front of the ion source of the apparatus,
- *particle-beam interface*: the mobile phase, which may flow at a rate of up to 0.6 mL/min, is nebulised in a desolvation chamber such that only the analytes, in neutral form, reach the ion source of the apparatus; this technique is used for compounds of relatively low polarity with molecular masses of less than 1000 Da,
- *moving-belt interface*: the mobile phase, which may flow at a rate of up to 1 mL/min, is applied to the surface of a moving belt; after the solvent evaporates, the components to be analysed are successively carried to the ion source of the apparatus where they are ionised; this technique is rather poorly suited to very polar or heat-labile compounds.

Other types of coupling (electrospray, thermospray, atmospheric-pressure chemical ionisation) are considered to be ionisation techniques in their own right and are described in the section on modes of ionisation.

**Supercritical fluid chromatography/mass spectrometry**. The mobile phase, usually consisting of supercritical carbon dioxide enters the gas state after passing a heated restrictor between the column and the ion source.

**Capillary electrophoresis/mass spectrometry**. The eluent is introduced into the ion source, in some cases after adding another solvent so that flow rates of the order of a few microlitres per minute can be attained. This technique is limited by the small quantities of sample introduced and the need to use volatile buffers.

MODES OF IONISATION

**Electron impact**. The sample, in the gas state, is ionised by a beam of electrons whose energy (usually 70 eV) is greater than the ionisation energy of the sample. In addition to the molecular ion $M^+$, fragments characteristic of the molecular structure are observed. This technique is limited mainly by the need to vaporise the sample. This makes it unsuited to polar, heat-labile or high molecular mass compounds. Electron impact is compatible with the coupling of gas chromatography to mass spectrometry and sometimes with the use of liquid chromatography.

**Chemical ionisation**. This type of ionisation involves a reagent gas such as methane, ammonia, nitrogen oxide, nitrogen dioxide or oxygen. The spectrum is characterised by ions of the $(M + H)^+$ or $(M - H)^-$ types, or adduct ions formed from the analyte and the gas used. Fewer fragments are produced than with electron impact. A variant of this technique is used when the substance is heat-labile: the sample, applied to a filament, is very rapidly vaporised by the Joule-Thomson effect (desorption chemical ionisation).

**Fast-atom bombardment (FAB) or fast-ion bombardment ionisation (liquid secondary-ion mass spectrometry LSIMS)**. The sample, dissolved in a viscous matrix such as glycerol, is applied to a metal surface and ionised by a beam of neutral atoms such as argon or xenon or high-kinetic-energy caesium ions. Ions of the $(M + H)^+$ or $(M - H)^-$ types or adduct ions formed from the matrix or the sample are produced. This type of ionisation, well suited to polar and heat-labile compounds, allows molecular masses of up to 10 000 Da to be obtained. The technique can be combined with liquid chromatography by adding 1 per cent to 2 per cent of glycerol to the mobile phase; however, the flow rates must be very low (a few microlitres per minute). These ionisation techniques also allow thin-layer chromatography plates to be analysed by applying a thin layer of matrix to the surface of these plates.

**Field desorption and field ionisation**. The sample is vaporised near a tungsten filament covered with microneedles (*field ionisation*) or applied to this filament (*field desorption*). A voltage of about 10 kV, applied between this filament and a counter-electrode, ionises the sample. These two techniques mainly produce molecular ions $M^+$, and $(M + H)^+$ ions and are used for low polarity and/or heat-labile compounds.

**Matrix-assisted laser desorption ionisation (MALDI)**. The sample, in a suitable matrix and deposited on a metal support, is ionised by a pulsed laser beam whose wavelength may range from UV to IR (impulses lasting from a picosecond to a few nanoseconds). This mode of ionisation plays an essential role in the analysis of very high molecular mass compounds (more than 100 000 Da) but is limited to time-of flight analysers (see below).

**Electrospray**. This mode of ionisation is carried out at atmospheric pressure. The samples, in solution, are introduced into the source through a capillary tube, the end of which has a potential of the order of 5 kV. A gas can be used to facilitate nebulisation. Desolvation of the resulting microdroplets produces singly or multiply charged ions in the gas phase. The flow rates vary from a few microlitres per minute to 1 mL/min. This technique is suited to polar compounds and to the investigation of biomolecules with molecular masses of up to 100 000 Da. It can be coupled to liquid chromatography or capillary electrophoresis.

**Atmospheric-pressure chemical ionisation (APCI)**. Ionisation is carried out at atmospheric pressure by the action of an electrode maintained at a potential of several kilovolts and placed in the path of the mobile phase, which is nebulised both by thermal effects and by the use of a stream of nitrogen. The resulting ions carry a single charge and are of the $(M + H)^+$ type in the positive mode and of the $(M - H)^-$ type in the negative mode. The high flow rates that can be used with this mode of ionisation (up to 2 mL/min) make this an ideal technique for coupling to liquid chromatography.

**Thermospray**. The sample, in the mobile phase consisting of water and organic modifiers and containing a volatile electrolyte (generally ammonium acetate) is introduced in nebulised form after having passed through a metal capillary tube at controlled temperature. Acceptable flow rates are of the order of 1 mL/min to 2 mL/min. The ions of the electrolyte ionise the compounds to be analysed. This ionisation process may be replaced or enhanced by an electrical discharge of about 800 volts, notably when the solvents are entirely organic. This technique is compatible with the use of liquid chromatography coupled with mass spectrometry.

## ANALYSERS

Differences in the performance of analysers depend mainly on two parameters:

- the range over which $m/z$ ratios can be measured, ie, the *mass range*,
- their *resolving power* characterised by the ability to separate two ions of equal intensity with $m/z$ ratios differing by $\Delta M$, and whose overlap is expressed as a given percentage of valley definition; for example, a resolving power $(M/\Delta M)$ of 1000 with 10 per cent valley definition allows the separation of $m/z$ ratios of 1000 and 1001 with the intensity returning to 10 per cent above baseline. However, the resolving power may in some cases (time-of-flight analysers, quadrupoles, ion-trap analysers) be defined as the ratio between the molecular mass and peak width at half height (50 per cent valley definition).

**Magnetic and electrostatic analysers**. The ions produced in the ion source are accelerated by a voltage $V$, and focused towards a magnetic analyser (magnetic field $B$) or an electrostatic analyser (electrostatic field $E$), depending on the configuration of the instrument. They follow a trajectory of radius $r$ according to Laplace's law:

$$\frac{m}{z} = \frac{B^2 r^2}{2V}$$

Two types of scans can be used to collect and measure the various ions produced by the ion source: a scan of $B$ holding $V$ fixed or a scan of $V$ with constant $B$. The magnetic analyser is usually followed by an electric sector that acts as a kinetic energy filter and allows the resolving power of the instrument to be increased appreciably. The maximum resolving power of such an instrument (double sector) ranges from 10 000 to 150 000 and in most cases allows the value of $m/z$ ratios to be calculated accurately enough to determine the elemental composition of the corresponding ions. For monocharged ions, the mass range is from 2000 Da to 15 000 Da. Some ions may decompose spontaneously (metastable transitions) or by colliding with a gas (collision-activated dissociation (CAD)) in field-free regions between the ion source and the detector. Examination of these decompositions is very useful for the determination of the structure as well as the characterisation of a specific compound in a mixture and involves tandem mass spectrometry. There are many such techniques depending on the region where these decompositions occur:

- *daughter-ion mode* (determination of the decomposition ions of a given parent ion): $B/E$ = constant, *MIKES (Mass-analysed Ion Kinetic Energy Spectroscopy)*,
- *parent-ion mode* (determination of all ions which by decomposition give an ion with a specific $m/z$ ratio): $B^2/E$ = constant,
- *neutral-loss mode* (detection of all the ions that lose the same fragment):

  $B/E(1 - E/E_0)^{1/2}$ = constant, where $E_0$ is the basic voltage of the electric sector.

**Quadrupoles**. The analyser consists of four parallel metal rods, which are cylindrical or hyperbolic in cross-section. They are arranged symmetrically with respect to the trajectory of the ions; the pairs diagonally opposed about the axis of symmetry of rods are connected electrically. The potentials to the two pairs of rods are opposed. They are the resultant of a constant component and an alternating component. The ions produced at the ion source are transmitted and separated by varying the voltages applied to the rods so that the ratio of continuous voltage to alternating voltage remains constant. The quadrupoles usually have a mass range of 1 a.m.u. to 2000 a.m.u., but some may range up to 4000 a.m.u. Although they have a lower resolving power than magnetic sector analysers, they nevertheless allow the monoisotopic profile of single charged ions to be obtained for the entire mass range. It is possible to obtain spectra using three quadrupoles arranged in series, $Q_1$, $Q_2$, $Q_3$ ($Q_2$ serves as a collision cell and is not really an analyser; the most commonly used collision gas is argon).

The most common types of scans are the following:

- *daughter-ion mode*: $Q_1$ selects an $m/z$ ion whose fragments obtained by collision in $Q_2$ are analysed by $Q_3$,
- *parent-ion mode*: $Q_3$ filters only a specific $m/z$ ratio, while $Q_1$ scans a given mass range. Only the ions decomposing to give the ion selected by $Q_3$ are detected,
- *neutral loss mode*: $Q_1$ and $Q_3$ scan a certain mass range but at an offset corresponding to the loss of a fragment characteristic of a product or family of compounds.

It is also possible to obtain spectra by combining quadrupole analysers with magnetic or electrostatic sector instruments; such instruments are called *hybrid mass spectrometers*.

**Ion-trap analyser**. The principle is the same as for a quadrupole, this time with the electric fields in three dimensions. This type of analyser allows product-ion spectra over several generations ($MS^n$) to be obtained.

**Ion-cyclotron resonance analysers**. Ions produced in a cell and subjected to a uniform, intense magnetic field move in circular orbits at frequencies which can be directly correlated to their *m*/*z* ratio by applying a Fourier transform algorithm. This phenomenon is called ion-cyclotron resonance. Analysers of this type consist of superconducting magnets and are capable of very high resolving power (up to 1000 000 and more) as well as $MS^n$ spectra. However, very low pressures are required (of the order of $10^{-7}$ Pa).

**Time-of-flight analysers**. The ions produced at the ion source are accelerated at a voltage *V* of 10 kV to 20 kV. They pass through the analyser, consisting of a field-free tube, 25 cm to 1.5 m long, generally called a *flight tube*. The time (*t*) for an ion to travel to the detector is proportional to the square root of the *m*/*z* ratio. Theoretically the mass range of such an analyser is infinite. In practice, it is limited by the ionisation or desorption method. Time-of-flight analysers are mainly used for high molecular mass compounds (up to several hundred thousand daltons). This technique is very sensitive (a few picomoles of product are sufficient). The accuracy of the measurements and the resolving power of such instruments may be improved considerably by using an electrostatic mirror (reflectron).

SIGNAL ACQUISITION

There are essentially three possible modes.

**Complete spectrum mode**. The entire signal obtained over a chosen mass range is recorded. The spectrum represents the relative intensity of the different ionic species present as a function of *m*/*z*. The results are essentially qualitative. The use of spectral reference libraries for more rapid identification is possible.

**Fragmentometric mode (Selected-ion monitoring)**. The acquired signal is limited to one (single-ion monitoring (SIM)) or several (multiple-ion monitoring (MIM)) ions characteristic of the substance to be analysed. The limit of detection can be considerably reduced in this mode. Quantitative or semiquantitative tests can be carried out using external or internal standards (for example, deuterated standards). Such tests cannot be carried out with time-of-flight analysers.

**Fragmentometric double mass spectrometry mode (multiple reaction monitoring (MRM))**. The unimolecular or bimolecular decomposition of a chosen precursor ion characteristic of the substance to be analysed is followed specifically. The selectivity and the highly specific nature of this mode of acquisition provide excellent sensitivity levels and make it the most appropriate for quantitative studies using suitable internal standards (for example, deuterated standards). This type of analysis can be performed only on apparatus fitted with three quadrupoles in series, ion-trap analysers or cyclotron-resonance analysers.

CALIBRATION

Calibration allows the corresponding *m*/*z* value to be attributed to the detected signal. As a general rule, this is done using a reference substance. This calibration may be external (acquisition file separate from the analysis) or internal (the reference substance(s) are mixed with the substance to be examined and appear on the same acquisition file). The number of ions or points required for reliable calibration depends on the type of analyser and on the desired accuracy of the measurement, for example, in the case of a magnetic analyser where the *m*/*z* ratio varies exponentially with the value of the magnetic field, there should be as many points as possible.

SIGNAL DETECTION AND DATA PROCESSING

Ions separated by an analyser are converted into electric signals by a detection system such as a photomultiplier or an electron multiplier. These signals are amplified before being re-converted into digital signals for data processing, allowing various functions such as calibration, reconstruction of spectra, automatic quantification, archiving, creation or use of libraries of mass spectra. The various physical parameters required for the functioning of the apparatus as a whole are controlled by computer.



**01/2008:20244**

# 2.2.44. TOTAL ORGANIC CARBON IN WATER FOR PHARMACEUTICAL USE

Total organic carbon (TOC) determination is an indirect measure of organic substances present in water for pharmaceutical use. TOC determination can also be used to monitor the performance of various operations in the preparation of medicines.

A variety of acceptable methods is available for determining TOC. Rather than prescribing a given method to be used, this general chapter describes the procedures used to qualify the chosen method and the interpretation of results in limit tests. A standard solution is analysed at suitable intervals, depending on the frequency of measurements; the solution is prepared with a substance that is expected to be easily oxidisable (for example, sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured. The suitability of the system is determined by analysis of a solution prepared with a substance expected to be oxidisable with difficulty (for example, 1,4-benzoquinone).

The various types of apparatus used to measure TOC in water for pharmaceutical use have in common the objective of completely oxidising the organic molecules in the sample water to produce carbon dioxide followed by measurement of the amount of carbon dioxide produced, the result being used to calculate the carbon concentration in the water.

The apparatus used must discriminate between organic and inorganic carbon, the latter being present as carbonate. The discrimination may be effected either by measuring the inorganic carbon and subtracting it from the total carbon, or by purging inorganic carbon from the sample before oxidisation. Purging may also entrain organic molecules, but such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

*Apparatus*. Use a calibrated instrument installed either on-line or off-line. Verify the system suitability at suitable intervals as described below. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

*TOC water*. Use highly purified water complying with the following specifications:

- conductivity: not greater than 1.0 µS·cm$^{-1}$ at 25 °C,
- total organic carbon: not greater than 0.1 mg/L.

Depending on the type of apparatus used, the content of heavy metals and copper may be critical. The manufacturer's instructions should be followed.

*Glassware preparation*. Use glassware that has been scrupulously cleaned by a method that will remove organic matter. Use *TOC water* for the final rinse of glassware.

*Standard solution*. Dissolve *sucrose R*, dried at 105 °C for 3 h in *TOC water* to obtain a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

*Test solution*. Using all due care to avoid contamination, collect water to be tested in an airtight container leaving minimal head-space. Examine the water with minimum delay to reduce contamination from the container and its closure.

*System suitability solution*. Dissolve *1,4-benzoquinone R* in *TOC water* to obtain a solution having a concentration of 0.75 mg of 1,4-benzoquinone per litre (0.50 mg of carbon per litre).

*TOC water control.* Use *TOC water* obtained at the same time as that used to prepare the standard solution and the system suitability solution.

*Control solutions.* In addition to the *TOC water control*, prepare suitable blank solutions or other solutions needed for establishing the baseline or for calibration adjustments following the manufacturer's instructions; run the appropriate blanks to zero the instrument.

*System suitability.* Run the following solutions and record the responses: *TOC water* ($r_w$); *standard solution* ($r_s$); *system suitability solution* ($r_{ss}$). Calculate the percentage response efficiency using the expression:

$$\frac{r_{ss} - r_w}{r_s - r_w} \times 100$$

The system is suitable if the response efficiency is not less than 85 per cent and not more than 115 per cent of the theoretical response.

*Procedure.* Run the test solution and record the response ($r_u$). The test solution complies with the test if $r_u$ is not greater than $r_s - r_w$.

The method can also be applied using on-line instrumentation that has been adequately calibrated and shown to have acceptable system suitability. The location of instrumentation must be chosen to ensure that the responses are representative of the water used.

**01/2008:20245**

## 2.2.45. SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical fluid chromatography (SFC) is a method of chromatographic separation in which the mobile phase is a fluid in a supercritical or a subcritical state. The stationary phase, contained in a column, consists of either finely divided solid particles, such as a silica or porous graphite, a chemically modified stationary phase, as used in liquid chromatography, or, for capillary columns, a cross-linked liquid film evenly coated on the walls of the column.

SFC is based on mechanisms of adsorption or mass distribution.

APPARATUS

The apparatus usually consists of a cooled pumping system, an injector, a chromatographic column, contained in an oven, a detector, a pressure regulator and a data acquisition device (or an integrator or a chart recorder).

**Pumping system**

Pumping systems are required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimised, e.g. by passing the pressurised solvent through a pulse-damping device. Tubing and connections are capable of withstanding the pressures developed by the pumping system.

Microprocessor controlled systems are capable of accurately delivering a mobile phase in either constant or varying conditions, according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

**Injectors**

Injection may be carried out directly at the head of the column using a valve.

**Stationary phases**

Stationary phases are contained in columns which have been described in the chapters on *Liquid chromatography* (*2.2.29*) (packed columns) and *Gas chromatography* (*2.2.28*) (capillary columns). A capillary column has a maximum internal diameter (Ø) of 100 µm.

**Mobile phases**

Usually the mobile phase is carbon-dioxide which may contain a polar modifier such as methanol, 2-propanol or acetonitrile. The composition, pressure (density), temperature and flow rate of the prescribed mobile phase may either be constant throughout the whole chromatographic procedure (isocratic, isodense, isothermic elution) or may vary according to a defined programme (gradient elution of the modifier, pressure (density), temperature or flow rate).

**Detectors**

Ultraviolet/visible (UV/Vis) spectrophotometers and flame ionisation detectors are the most commonly employed detectors. Light scattering detectors, infrared absorption spectrophotometers, thermal conductivity detectors or other special detectors may be used.

METHOD

Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (*2.2.46*). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

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## 2.2.46. CHROMATOGRAPHIC SEPARATION TECHNIQUES

Chromatographic separation techniques are multi-stage separation methods in which the components of a sample are distributed between 2 phases, one of which is stationary, while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion-exchange, etc., or may be based on differences in the physico-chemical properties of the molecules such as size, mass, volume, etc.

This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. Principles of separation, apparatus and methods are given in the following general methods:

- paper chromatography (*2.2.26*);
- thin-layer chromatography (*2.2.27*);
- gas chromatography (*2.2.28*);
- liquid chromatography (*2.2.29*);
- size-exclusion chromatography (*2.2.30*);
- supercritical fluid chromatography (*2.2.45*).

DEFINITIONS

*The system suitability and acceptance criteria in monographs have been set using parameters as defined below. With some equipment, certain parameters, such as the signal-to-noise ratio and resolution, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are equivalent to the requirements of the European Pharmacopoeia and to make any necessary corrections if this is not the case.*

**Chromatogram**

A graphical or other representation of detector response, effluent concentration or other quantity used as a measure of effluent concentration, versus time or volume. Idealised chromatograms are represented as a sequence of Gaussian peaks on a baseline (Figure 2.2.46.-1).

**Peak**

The portion of a chromatogram recording the detector response when a single component (or 2 or more unresolved components) is eluted from the column.

The peak may be defined by the peak area, or the peak height ($h$) and the peak width at half-height ($w_h$), or the peak height ($h$) and the peak width between the points of inflection ($w_i$). In Gaussian peaks (Figure 2.2.46.-1) there is the following relationship:

$$w_h = 1.18 w_i$$

**Retention time ($t_R$)**

Time required for elution of a component (Figure 2.2.46.-1, baseline scale being in minutes).

**Retention volume ($V_R$)**

Volume of the mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate ($F$) in millilitres per minute using the following equation:

$$V_R = t_R \times F$$

**Hold-up time ($t_M$)**

Time required for elution of an unretained component (Figure 2.2.46.-1, baseline scale being in minutes). In size-exclusion chromatography, the symbol $t_0$ (see below) is used.

**Hold-up volume ($V_M$)**

Volume of the mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate ($F$) in millilitres per minute using the following equation:

$$V_M = t_M \times F$$

In size-exclusion chromatography, the symbol $V_0$ (see below) is used.

**Retention factor ($k$)**

The retention factor (also known as mass distribution ratio ($D_m$) or capacity factor ($k'$)) is defined as:

$$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_C \frac{V_s}{V_M}$$

$K_C$ = distribution constant (also known as equilibrium distribution coefficient);

$V_S$ = volume of the stationary phase;

$V_M$ = volume of the mobile phase.

The retention factor of a component may be determined from the chromatogram using the following equation:

$$k = \frac{t_R - t_M}{t_M}$$

**Total mobile phase time ($t_t$)**

In size-exclusion chromatography, retention time of a component whose molecules are smaller than the smallest gel pores (Figure 2.2.46.-2).

**Total mobile phase volume ($V_t$)**

In size-exclusion chromatography, retention volume of a component whose molecules are smaller than the smallest gel pores. It may be calculated from the total mobile phase time and the flow rate ($F$) in millilitres per minute using the following equation:

$$V_t = t_t \times F$$

**Retention time of an unretained compound ($t_0$)**

In size-exclusion chromatography, retention time of a component whose molecules are larger than the largest gel pores (Figure 2.2.46.-2).

**Retention volume of an unretained compound ($V_0$)**

In size-exclusion chromatography, retention volume of a component whose molecules are larger than the largest gel pores. It may be calculated from the retention time of an unretained compound and the flow rate ($F$) in millilitres per minute using the following equation:

$$V_0 = t_0 \times F$$

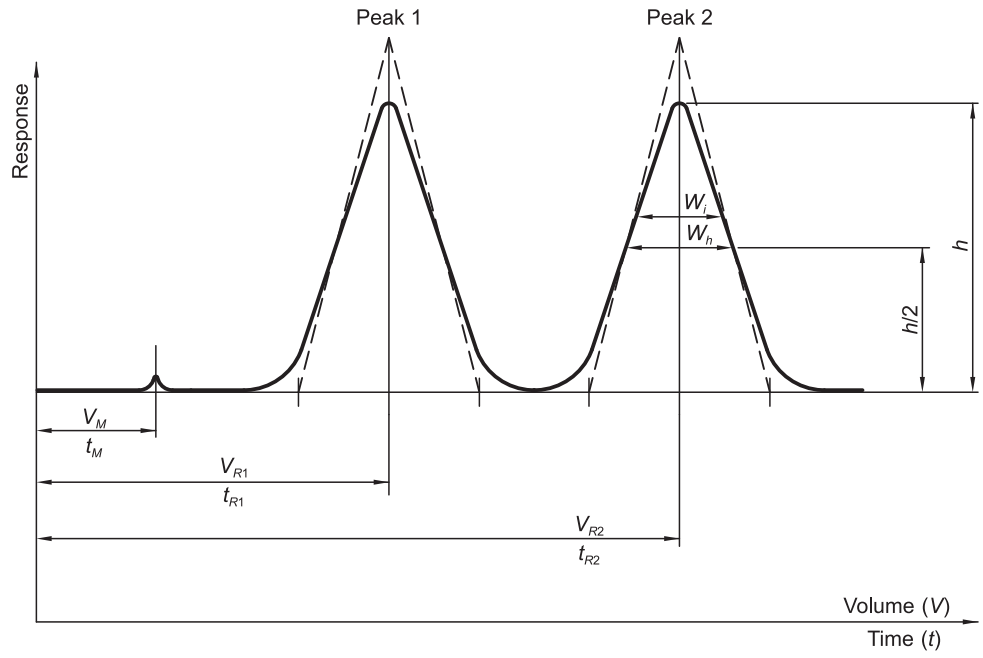


Figure 2.2.46.-1.

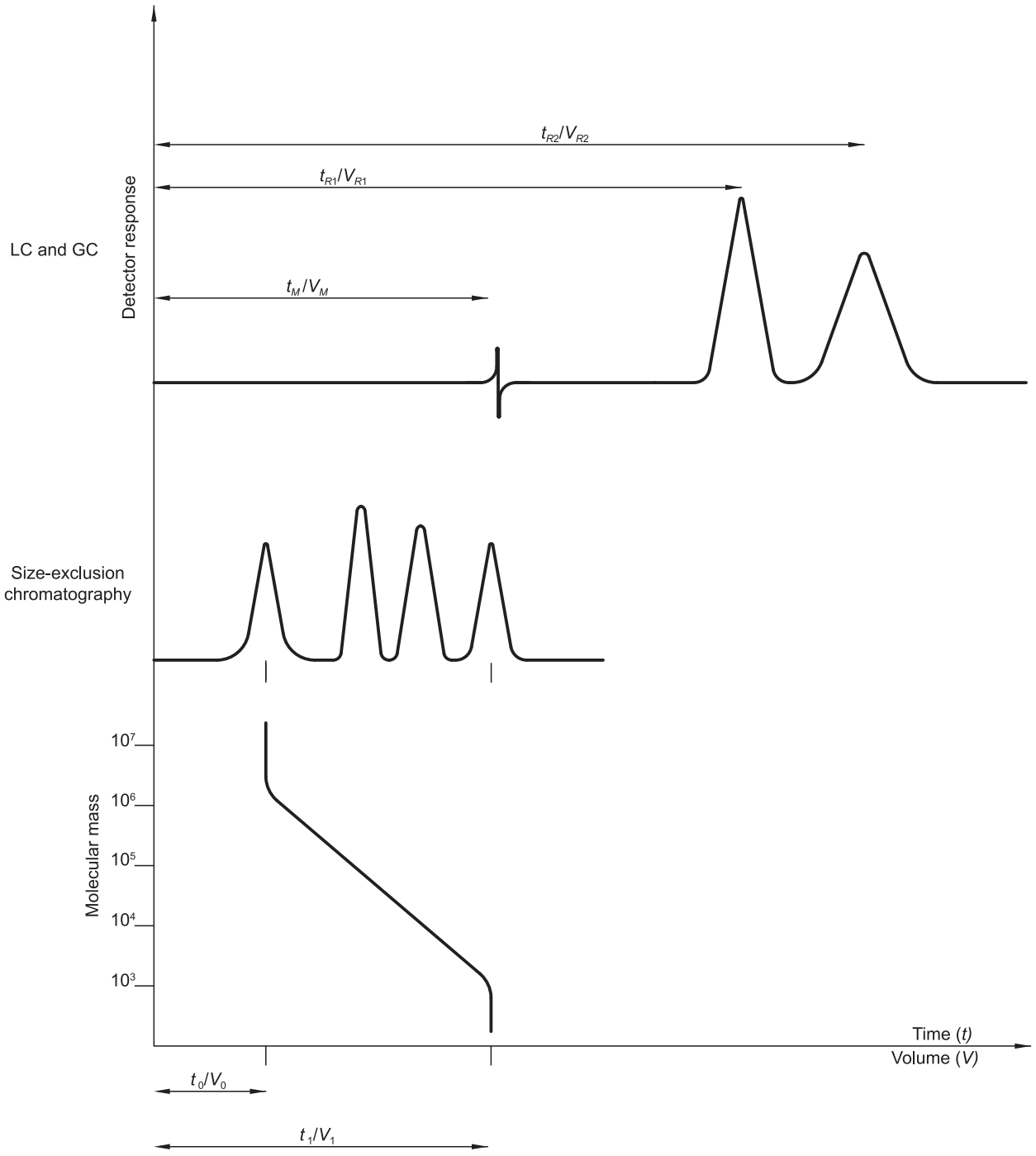


Figure 2.2.46.-2.

**Distribution constant ($K_0$)**

In size-exclusion chromatography, the elution characteristics of a component in a particular column may be given by the distribution constant (also referred to as distribution coefficient), which is calculated using the following equation:

$$K_0 = \frac{t_R - t_0}{t_t - t_0}$$

**Retardation factor ($R_F$)**

The retardation factor (also known as retention factor ($R_f$)), used in planar chromatography, is the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application (Figure 2.2.46.-3).

$$R_F = \frac{b}{a}$$

$b$ = migration distance of the component;

$a$ = migration distance of the solvent front.

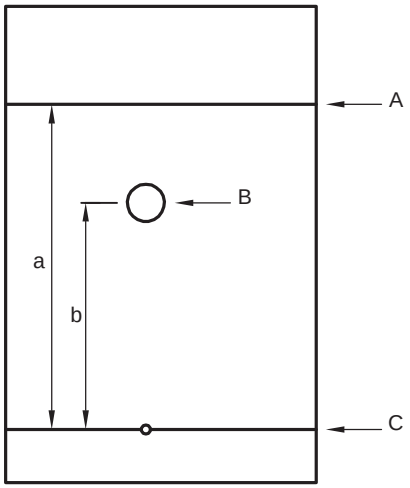


A. mobile phase front B. spot C. line of application

Figure 2.2.46.-3.

**Plate number (*N*)**

The column performance (apparent efficiency) may be calculated from data obtained under either isothermal, isocratic or isodense conditions, depending on the technique, as the plate number (also referred to as number of theoretical plates), using the following equation, the values of $t_R$ and $w_h$ being expressed in the same units:

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

$t_R$ = retention time of the peak corresponding to the component;

$w_h$ = width of the peak at half-height.

The plate number varies with the component as well as with the column, the column temperature, the mobile phase and the retention time.

**Dwell volume (*D*)**

The dwell volume (also known as gradient delay volume) is the volume between the point at which the eluents meet and the top of the column. It can be determined using the following procedure.

*Column*: replace the chromatographic column by an appropriate capillary tubing (e.g. 1 m × 0.12 mm).

*Mobile phase*:

– *mobile phase A*: *water R*;

– *mobile phase B*: 0.1 per cent *V/V* solution of *acetone R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 0 | 0 → 100 |
| 20 - 30 | 0 | 100 |

*Flow rate*: set to obtain sufficient back-pressure (e.g. 2 mL/min).

*Detection*: spectrophotometer at 265 nm.

Determine the time ($t_{0.5}$) in minutes when the absorbance has increased by 50 per cent (Figure 2.2.46.-4).

$$D = t_D \times F$$

$t_D$ = $t_{0.5} - 0.5t_G$ (in minutes);

$t_G$ = pre-defined gradient time (= 20 min);

$F$ = flow rate (in millilitres per minute).

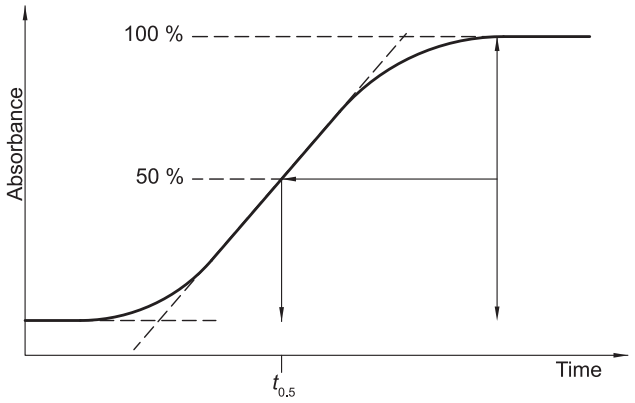


Figure 2.2.46.-4

**Symmetry factor ($A_s$)**

The symmetry factor of a peak (Figure 2.2.46.-5) is calculated using the following equation:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height;

$d$ = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

An $A_s$ value of 1.0 signifies symmetry. When $A_s > 1.0$, the peak is tailing. When $A_s < 1.0$, the peak is fronting.

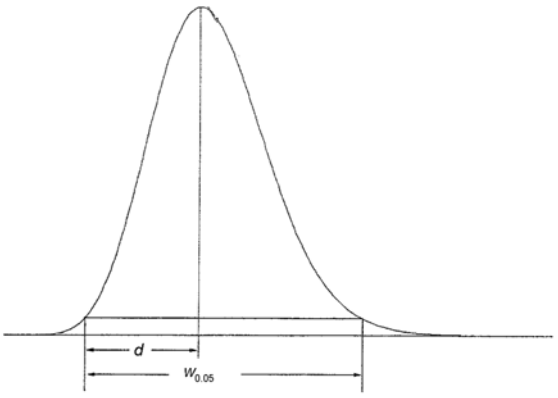


Figure 2.2.46.-5

**Resolution ($R_s$)**

The resolution between peaks of 2 components (Figure 2.2.46.-1) may be calculated using the following equation:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$t_{R2} > t_{R1}$

$t_{R1}$, $t_{R2}$ = retention times of the peaks;

$w_{h1}$, $w_{h2}$ = peak widths at half-height.

In quantitative planar chromatography, using densitometry, the migration distances are used instead of retention times and the resolution between peaks of 2 components may be calculated using the following equation:

$$R_s = \frac{1.18a(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

$R_{F1}$, $R_{F2}$ = retardation factors of the peaks;

$w_{h1}$, $w_{h2}$ = peak widths at half-height;

$a$ = migration distance of the solvent front.

**Peak-to-valley ratio (*p/v*)**

The peak-to-valley ratio may be employed as a system suitability criterion in a test for related substances when baseline separation between 2 peaks is not achieved (Figure 2.2.46.-6).

$$p / v = \frac{H_p}{H_v}$$

$H_p$ = height above the extrapolated baseline of the minor peak;

$H_v$ = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

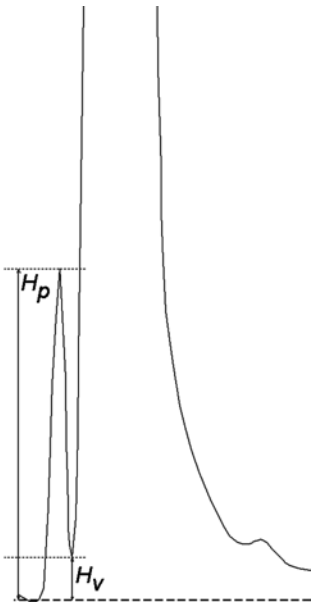


Figure 2.2.46.-6

**Relative retention (*r*)**

Relative retention is calculated as an estimate using the following equation:

$$r = \frac{t_{Ri} - t_M}{t_{Rst} - t_M}$$

$t_{Ri}$ = retention time of the peak of interest;

$t_{Rst}$ = retention time of the reference peak (usually the peak corresponding to the substance to be examined);

$t_M$ = hold-up time.

The unadjusted relative retention ($r_G$) is calculated using the following equation:

$$r_G = \frac{t_{Ri}}{t_{Rst}}$$

Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

In planar chromatography, the retardation factors $R_{Fst}$ and $R_{Fi}$ are used instead of $t_{Rst}$ and $t_{Ri}$.

**Signal-to-noise ratio (*S/N*)**

The short-term noise influences the precision of quantification. The signal-to-noise ratio is calculated using the following equation:

$$S / N = \frac{2H}{h}$$

$H$ = height of the peak (Figure 2.2.46.-7) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to at least 5 times the width at half-height;

$h$ = range of the noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

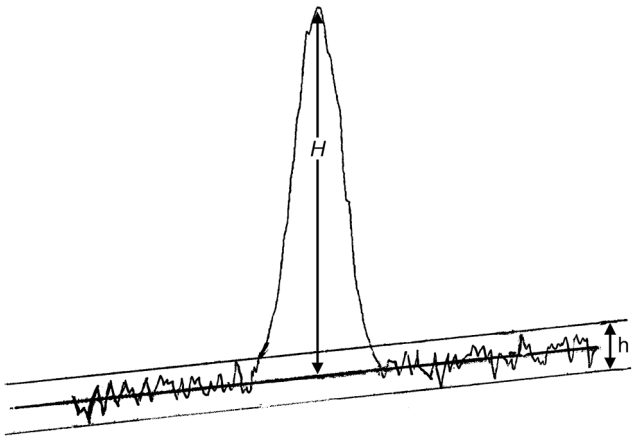


Figure 2.2.46.-7.

**System repeatability**

The repeatability of response is expressed as an estimated percentage relative standard deviation ($s_r$(%)) of a consecutive series of measurements for not fewer than 3 injections or applications of a reference solution, and is calculated using the following equation:

$$s_r(\%) = \frac{100}{\overline{y}} \sqrt{\frac{\sum (y_i - \overline{y})^2}{n - 1}}$$

$y_i$ = individual values expressed as peak area, peak height, or ratio of areas by the internal standardisation method;

$\overline{y}$ = mean of individual values;

$n$ = number of individual values.

SYSTEM SUITABILITY

The various components of the equipment employed must be qualified and be capable of achieving the performance required to conduct the test or assay.

The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. Apparent efficiency, retention factor (mass distribution ratio), resolution and symmetry factor are the parameters that are usually employed in assessing the performance of the column. Factors that may affect the chromatographic behaviour include:

- the composition, ionic strength, temperature and apparent pH of the mobile phase;
- flow rate, column dimensions, column temperature and pressure;
- stationary phase characteristics including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, specific surface area;
- reversed-phase and other surface-modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading etc.).

The following requirements and any supplementary requirements given in the individual monograph are to be fulfilled unless otherwise prescribed:

- in a related substances test or assay, for a peak in the chromatogram obtained with a reference solution used for quantification, the symmetry factor is 0.8 to 1.5, unless otherwise prescribed;
- in an assay of an active substance where the value is 100 per cent for a pure substance, the maximum permitted relative standard deviation ($s_r(\%)_{max}$) for the defined limits is calculated for a series of injections of the reference solution using the following equation:

$$s_r(\%)_{max} = \frac{KB\sqrt{n}}{t_{90\%,n-1}}$$

$K$ = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required percentage relative standard deviation after 6 injections for $B = 1.0$;

$B$ = upper limit given in the definition of the individual monograph minus 100 per cent;

$n$ = number of replicate injections of the reference solution ($3 \leq n \leq 6$);

$t_{90\%,n-1}$ = Student's $t$ at the 90 per cent probability level (double sided) with $n-1$ degrees of freedom.

Unless otherwise prescribed, the maximum permitted relative standard deviation does not exceed the appropriate value given in Table 2.2.46.-1. This requirement does not apply to tests for related substances.

Table 2.2.46.-1. – *Repeatability requirements*

| | Number of individual injections | | | |
|---|---|---|---|---|
| | 3 | 4 | 5 | 6 |
| ***B* (per cent)** | **Maximum permitted relative standard deviation** | | | |
| 2.0 | 0.41 | 0.59 | 0.73 | 0.85 |
| 2.5 | 0.52 | 0.74 | 0.92 | 1.06 |
| 3.0 | 0.62 | 0.89 | 1.10 | 1.27 |

- in a related substances test, the limit of quantification (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. Depending on various factors, such as the frequency of use of the procedure and experience with the chromatographic system, the analyst chooses an appropriate verification scheme to monitor this.

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below. Adjustment of conditions with gradient elutions is more critical than with isocratic elutions, since it may lead to shifts in peaks to a different step of the gradient, thus leading to the incorrect assignment of peaks, and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time. Changes other than those indicated require revalidation of the method. The chromatographic conditions described have been validated during the elaboration of the monograph.

The system suitability tests are included to verify that the separation required for satisfactory performance of the test or assay is achieved. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reversed-phase liquid chromatographic methods in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel), which exhibits the desired chromatographic behaviour. The Knowledge database on the EDQM website usually contains information on the column(s) used during monograph elaboration.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

**Thin-layer chromatography and paper chromatography**

*Composition of the mobile phase*: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; for a minor component at 10 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 7-13 per cent whereas a 2 per cent absolute adjustment allows a range of 8-12 per cent, the relative value therefore being the larger; for a minor component at 5 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 3.5-6.5 per cent whereas a 2 per cent absolute adjustment allows a range of 3-7 per cent, the absolute value being the larger in this case; no other component is altered by more than 10 per cent absolute.

*pH of the aqueous component of the mobile phase*: ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

*Concentration of salts in the buffer component of a mobile phase*: ± 10 per cent.

*Application volume*: 10-20 per cent of the prescribed volume if using fine particle size plates (2-10 µm).

**Liquid chromatography: isocratic elution**

*Composition of the mobile phase*: the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger (see example above); no other component is altered by more than 10 per cent absolute.

*pH of the aqueous component of the mobile phase*: ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

*Concentration of salts in the buffer component of a mobile phase*: ± 10 per cent.

*Flow rate*: ± 50 per cent; a larger adjustment is acceptable when changing the column dimensions (see the formula below).

*Column parameters*

*Stationary phase*:

- no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C18 by C8);
- *particle size*: maximum reduction of 50 per cent; no increase permitted.

*Column dimensions*:

- *length*: ± 70 per cent;
- *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

$F_1$ = flow rate indicated in the monograph, in millilitres per minute;

$F_2$ = adjusted flow rate, in millilitres per minute;

$l_1$ = length of the column indicated in the monograph, in millimetres;

$l_2$ = length of the column used, in millimetres;

$d_1$ = internal diameter of the column indicated in the monograph, in millimetres;

$d_2$ = internal diameter of the column used, in millimetres.

*Temperature*: ± 10 °C, where the operating temperature is specified, unless otherwise prescribed.

*Detector wavelength*: no adjustment permitted.

*Injection volume*: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

**Liquid chromatography: gradient elution**

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

*Composition of the mobile phase/gradient elution*: minor adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

- the system suitability requirements are fulfilled;
- the principal peak(s) elute(s) within ± 15 per cent of the indicated retention time(s);
- the final composition of the mobile phase is not weaker in elution power than the prescribed composition.

Where compliance with the system suitability requirements cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

*Dwell volume.* The configuration of the equipment employed may significantly alter the resolution, retention time and relative retentions described. Should this occur, it may be due to excessive dwell volume. Monographs preferably include an isocratic step before the start of the gradient programme so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points ($t$ min) stated in the gradient table may be replaced by adapted time points ($t_c$ min), calculated using the following equation:

$$t_c = t - \frac{(D - D_0)}{F}$$

$D$ = dwell volume, in millilitres;

$D_0$ = dwell volume used for development of the method, in millilitres;

$F$ = flow rate, in millilitres per minute.

The isocratic step introduced for this purpose may be omitted if validation data for application of the method without this step is available.

*pH of the aqueous component of the mobile phase*: no adjustment permitted.

*Concentration of salts in the buffer component of a mobile phase*: no adjustment permitted.

*Flow rate*: adjustment is acceptable when changing the column dimensions (see the formula below).

*Column parameters*

*Stationary phase*:

- no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C18 by C8);
- *particle size*: no adjustment permitted.

*Column dimensions*:

- *length*: ± 70 per cent;
- *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

$F_1$ = flow rate indicated in the monograph, in millilitres per minute;

$F_2$ = adjusted flow rate, in millilitres per minute;

$l_1$ = length of the column indicated in the monograph, in millimetres;

$l_2$ = length of the column used, in millimetres;

$d_1$ = internal diameter of the column indicated in the monograph, in millimetres;

$d_2$ = internal diameter of the column used, in millimetres.

*Temperature*: ± 5 °C, where the operating temperature is specified, unless otherwise prescribed.

*Detector wavelength*: no adjustment permitted.

*Injection volume*: may be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

**Gas chromatography**

*Column parameters*

*Stationary phase*:

- *particle size*: maximum reduction of 50 per cent; no increase permitted (packed columns);
- *film thickness*: – 50 per cent to + 100 per cent (capillary columns).

*Column dimensions*:

- *length*: ± 70 per cent;
- *internal diameter*: ± 50 per cent.

*Flow rate*: ± 50 per cent.

*Temperature*: ± 10 per cent.

*Injection volume and split volume*: may be adjusted, provided detection and repeatability are satisfactory.

**Supercritical fluid chromatography**

*Composition of the mobile phase*: for packed columns, the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; no adjustment is permitted for a capillary column system.

*Detector wavelength*: no adjustment permitted.

*Column parameters*

*Stationary phase*:

– *particle size*: maximum reduction of 50 per cent; no increase permitted (packed columns).

*Column dimensions*:

– *length*: ± 70 per cent;

– *internal diameter*:

± 25 per cent (packed columns);

± 50 per cent (capillary columns).

*Flow rate*: ± 50 per cent.

*Temperature*: ± 5 °C, where the operating temperature is specified.

*Injection volume*: may be decreased, provided detection and repeatability are satisfactory; no increase permitted.

QUANTIFICATION

Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are disregarded during quantification.

– *Detector sensitivity*. The detector sensitivity is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative detector response factor, commonly referred to as *response factor*, expresses the sensitivity of a detector for a given substance relative to a standard substance. The *correction factor* is the reciprocal of the response factor.

– *External standard method*. The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.

– *Internal standard method*. Equal amounts of a component that will be resolved from the substance to be examined (the internal standard) are introduced into the test solution and a reference solution. The internal standard is chosen such that it does not react with the substance to be examined, is stable and does not contain impurities with the same retention time as that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the reference solution.

– *Normalisation procedure*. The percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix, and those at or below the disregard limit.

– *Calibration procedure*. The relationship between the measured or evaluated signal ($y$) and the quantity (concentration, mass, etc.) of substance ($x$) is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

In tests for related substances for both the external standard method, when a dilution of the test solution is used for comparison, and the normalisation procedure, any correction factors indicated in the monograph are applied (i.e. when the response factor is outside the range 0.8-1.2).

When the related substances test prescribes the total of impurities or there is a quantitative determination of an impurity, it is important to choose an appropriate threshold setting and appropriate conditions for the integration of the peak areas. In such tests the *disregard limit*, i.e. the limit at or below which a peak is disregarded, is generally 0.05 per cent. Integration of the peak area of any impurity that is not completely separated from the principal peak is preferably performed by valley-to-valley extrapolation (tangential skim).

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**corrected 7.1**

# 2.2.47. CAPILLARY ELECTROPHORESIS(6)

GENERAL PRINCIPLES

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity $E$, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute ($\mu_{ep}$) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives). The electrophoretic velocity ($v_{ep}$) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right)$$

$q$ = effective charge of the solute,

$\eta$ = viscosity of the electrolyte solution,

$r$ = Stoke's radius of the solute,

$V$ = applied voltage,

$L$ = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility ($\mu_{eo}$) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity ($v_{eo}$) is given by the equation:

$$v_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right)$$

$\varepsilon$ = dielectric constant of the buffer,

$\zeta$ = zeta potential of the capillary surface.

The velocity of the solute ($v$) is given by:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which

(6) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time ($t$) taken by the solute to migrate the distance ($l$) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{\left(\mu_{ep} + \mu_{eo}\right) \times V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionised silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates ($N$), is given by:

$$N = \frac{\left(\mu_{ep} + \mu_{eo}\right) \times V \times l}{2 \times D \times L}$$

$D$ = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between 2 bands (expressed as the resolution, $R_s$) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}\left(\mu_{epb} - \mu_{epa}\right)}{4\left(\overline{\mu}_{ep} + \mu_{eo}\right)}$$

$\mu_{epa}$ and $\mu_{epb}$ = electrophoretic mobilities of the 2 analytes separated,

$\overline{\mu}_{ep}$ = mean electrophoretic mobility of the 2 analytes $\overline{\mu}_{ep} = \frac{1}{2}\left(\mu_{epb} + \mu_{epa}\right)$.

## APPARATUS

An apparatus for capillary electrophoresis is composed of:

- a high-voltage, controllable direct-current power supply;
- 2 buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;
- 2 electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply;
- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;
- a suitable injection system;
- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds;
- a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility;
- a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

## CAPILLARY ZONE ELECTROPHORESIS

*PRINCIPLE*

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small ($M_r$ < 2000) and large molecules (2000 < $M_r$ < 100 000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

*OPTIMISATION*

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

**Instrumental parameters**

*Voltage.* A Joule heating plot is useful in optimising the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

*Polarity.* Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

*Temperature*. The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

*Capillary*. The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

**Electrolytic solution parameters**

*Buffer type and concentration*. Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

*Buffer pH*. The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the solute from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

*Organic solvents*. Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

*Additives for chiral separations*. For the separation of optical isomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionisable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

## CAPILLARY GEL ELECTROPHORESIS

*PRINCIPLE*

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

*CHARACTERISTICS OF GELS*

2 types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels. Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary. If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used. Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility. The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

## CAPILLARY ISOELECTRIC FOCUSING

*PRINCIPLE*

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilisation.

**Loading step**. Two methods may be employed:
- loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;
- sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

**Focusing step**. When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

**Mobilisation step**. If mobilisation is required for detection, use one of the following methods.
- in the first method, mobilisation is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;
- in the second method, mobilisation is accomplished by applying positive pressure after the focusing step;
- in the third method, mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilised in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH / d$x$), the number of ampholytes having different pI values, the molecular diffusion coefficient ($D$), the intensity of the electric field ($E$) and the variation of the electrophoretic mobility of the analyte with the pH ($-\mathrm{d}\mu$ / dpH):

$$\Delta\mathrm{pI} = 3 \times \sqrt{\frac{D(\mathrm{dpH} / \mathrm{d}x)}{E(-\mathrm{d}\mu / \mathrm{dpH})}}$$

*OPTIMISATION*

The main parameters to be considered in the development of separations are:

**Voltage**. Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

**Capillary**. The electro-osmotic flow must be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

**Solutions**. The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

## MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

*PRINCIPLE*

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionised solutes is essentially chromatographic, migration of the solute and resolution can be rationalised in terms of the retention factor of the solute ($k'$), also referred to as mass distribution ratio ($D_m$), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, $k'$ is given by:

$$k' = \frac{t_R - t_0}{t_0 \times \left(1 - \frac{t_R}{t_{mc}}\right)} = K \times \frac{V_S}{V_M}$$

$t_R$ = migration time of the solute,

$t_0$ = analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol),

$t_{mc}$ = micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),

$K$ = partition coefficient of the solute,

$V_S$ = volume of the micellar phase,

$V_M$ = volume of the mobile phase.

Likewise, the resolution between 2 closely-migrating solutes ($R_s$) is given by:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k'_a \times \left(\frac{t_0}{t_{mc}}\right)}$$

$N$ = number of theoretical plates for one of the solutes,
$\alpha$ = selectivity,
$k'_a$ and $k'_b$ = retention factors for both solutes, respectively ($k'_b > k'_a$).

Similar, but not identical, equations give $k'$ and $R_s$ values for electrically charged solutes.

*OPTIMISATION*

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

**Instrumental parameters**

*Voltage.* Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

*Temperature.* Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system improves the reproducibility of the migration time for the solutes.

*Capillary.* As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

**Electrolytic solution parameters**

*Surfactant type and concentration.* The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log $k'$ of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when $k'$ approaches the value of $\sqrt{t_{mc} / t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

*Buffer pH.* Although pH does not modify the partition coefficient of non-ionised solutes, it can modify the electro-osmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electro-osmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

*Organic solvents.* To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellisation is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

*Additives for chiral separations.* For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellised achiral surfactants.

*Other additives.* Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

## QUANTIFICATION

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

- compensate for the shift in migration time from run to run, thus reducing the variation of the response,
- compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

*CALCULATIONS*

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalisation procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

## SYSTEM SUITABILITY

In order to check the behaviour of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor ($k'$) (only for micellar electrokinetic chromatography), apparent number of theoretical plates ($N$), symmetry factor ($A_s$) and resolution ($R_s$). In previous sections, the theoretical expressions for $N$ and $R_s$ have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

*APPARENT NUMBER OF THEORETICAL PLATES*

The apparent number of theoretical plates ($N$) may be calculated using the expression:

$$N = 5.54 \times \left( \frac{t_R}{w_h} \right)^2$$

$t_R$ = migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,
$w_h$ = width of the peak at half-height.

*RESOLUTION*

The resolution ($R_s$) between peaks of similar height of 2 components may be calculated using the expression:

$$R_s = \frac{1.18 \times (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

$t_{R1}$ and $t_{R2}$ = migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks,

$w_{h1}$ and $w_{h2}$ = peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley ($H_v$) between 2 partly resolved peaks in a standard preparation and the height of the smaller peak ($H_p$) and calculating the peak-to-valley ratio:

$$\frac{p}{v} = \frac{H_p}{H_v}$$

*SYMMETRY FACTOR*

The symmetry factor ($A_s$) of a peak may be calculated using the expression:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height,

$d$ = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantification) may also be useful for the determination of related substances.

*SIGNAL-TO-NOISE RATIO*

The detection limit and quantification limit correspond to signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio ($S/N$) is calculated using the expression:

$$\frac{S}{N} = \frac{2H}{h}$$

$H$ = height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height,

$h$ = range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

**04/2016:20248**

# 2.2.48. RAMAN SPECTROSCOPY

In Raman spectroscopy a sample is irradiated with an intense monochromatic light source (usually a laser). Most of the radiation is scattered from the sample at the same wavelength as that of the incident light; this process is known as Rayleigh scattering or elastic light scattering. Only $10^{-6}$ to $10^{-8}$ of the incident photons are scattered from the sample at wavelengths shifted from the original source wavelength; this scattered light is known as Raman scatter or inelastic light scatter. The differences between the incident light source wavelength and the Raman scattered wavelengths are known as the Raman shifts and are related to the molecular vibrations within the sample. The scattered light with lower energy is called Stokes scatter and with higher energy is called anti-Stokes scatter. Typically, Stokes scatter is analysed.

A Raman spectrum can be obtained from liquid, solid and even gaseous samples. Raman spectroscopy is a suitable non-invasive method for determination of solid-state properties and for chemical identification, for example, detecting changes in polymorphic form. Raman spectroscopy encompasses many methodologies, including backscattering, transmittance, resonance Raman (RR) spectroscopy, surface-enhanced Raman spectroscopy (SERS), tip-enhanced Raman spectroscopy (TERS), spatially offset Raman spectroscopy (SORS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), stimulated Raman spectroscopy (SRS) and confocal Raman (CF) spectroscopy. It also lends itself to imaging techniques.

Raman spectroscopy is complementary to infrared spectroscopy. Both techniques probe the fundamental molecular vibrations in a material. However, Raman and infrared spectroscopy have different sensitivities for different functional groups within a material. Raman spectroscopy is particularly useful in examining non-polar bonds, functional groups and vibrations that are highly symmetrical (e.g. C-C single or multiple bonds); but is less sensitive to polar bonds (e.g. C=O) and vibrations that are asymmetrical. For example, water, which strongly influences infrared spectra, exhibits weak Raman scattering and therefore has minimal interference on the resulting spectrum. Raman can therefore be used for aqueous solutions.

A major difficulty of Raman spectroscopy is that the material examined or impurities in the material may exhibit fluorescence, which can overcome the Raman signal. Fluorescence may be avoided by choosing a longer excitation wavelength, for example in the near-infrared region.

Raman spectroscopy is a rapid and non-invasive analytical method and can be performed off-line and also at-line, on-line or in-line for process analytical technology (PAT). Raman spectrometers can be situated at a great distance from the point of measurement when using long-distance optical fibres for sample interfacing. Raman spectroscopy has a wide variety of applications for chemical, physical and process analysis and can also be used for counterfeit detection and quality control, for example:

- *chemical analysis*: identification and quantification of active substances, excipients;
- *physical analysis*: identification and quantification of solid forms (e.g. polymorphs and solvates) and crystallinity;
- *process analysis*: monitoring of biological and chemical reactions, synthesis, crystallisation, granulation, mixing, drying, lyophilisation, extrusion, encapsulation and coating.

APPARATUS

There are different types of spectrometers for recording Raman spectra, the most common are benchtop dispersive instruments, including microscope-coupled Raman spectrometers, Fourier transform (FT) and hand-held Raman spectrometers.

Raman spectrometers typically consist of the following components:

- a monochromatic light source, typically a laser, with a wavelength in the ultraviolet, visible or near-infrared region; selection of laser frequency and intensity should be considered depending on the intended application and material to be examined;
- suitable optics (lens, mirrors or optical-fibre assembly) that direct the monochromatic light to and collect the Raman scattered light from the material to be examined;
- an optical device (monochromator or filter) that transmits the frequency-shifted Raman scattering and prevents the intense incident frequency of Rayleigh scattering from reaching the detector; and
- a dispersing device (grating and/or prism polychromator) combined with wavelength-selecting slits and a single or multi-channel detector (e.g. a charge-coupled device) or
- an interferometer with a detector that records the intensity of the scattered light over time, and a data-handling device (e.g. computer and appropriate software) that converts this data to the frequency or wavenumber domain by a Fourier-transform calculation.

PREPARATION OF THE SAMPLE

Raman spectra can be obtained from solids, liquids and gases either directly or in suitable containers such as glass vials, generally without prior sample preparation or dilution.

When using Raman spectroscopy, the measured sample area and volume may be small (in particular for microscope-coupled devices) and care must be taken to ensure the measurement is representative. This can be achieved by, for example, rotation of the sample, performing multiple measurements on different preparations of the sample, increasing the area of illumination by reducing the magnification (focus), by demagnification (defocusing) of the laser beam or by changing the focal length between measurements to scan at different depths.

It is not always possible to consider Raman as a non-destructive technique. The energy transmitted by the laser depends on the duration of exposure and the wavelength. It may change the physical state and may destroy the sample.

CONTROL OF INSTRUMENT PERFORMANCE

Carry out the prescribed calibrations and/or system performance tests according to the manufacturer's instructions at regular intervals, depending on the use of the apparatus. Wavenumber calibration of Fourier transform systems is maintained with an internal laser (He-Ne). For dispersive systems, emission spectra of a low pressure lamp exhibiting characteristic maxima at wavenumbers over the whole spectral range of the instrument can usually be used for calibration (e. g. neon lamps or less frequently mercury, argon, krypton or xenon lamps).

*Verification of the wavenumber scale.* Verify the wavenumber scale of the Raman shift using a suitable standard that has characteristic maxima at the wavenumbers under investigation, for example an organic substance such as polystyrene, paracetamol or cyclohexane (see Table 2.2.48.-1).

A minimum of 3 wavenumber shifts covering the working range of the instrument intended for measurements should be chosen. For dispersive Raman spectrometers that use multiple gratings for different spectral resolutions, the wavenumber scale should be verified at the same optical resolution that will occur for sample collection. All gratings used for Raman measurements should be verified for accuracy of Raman-shift.

*Verification of the response-intensity scale.* The absolute and relative intensities of the Raman bands are affected by variations in several factors including:

- polarisation of the irradiating light;
- polarisation of the Raman scattered light;
- intensity of the irradiating light;
- instrument response;
- focus and geometry at sample;
- packing density for solid samples;
- refractive index $n$ or change of $n$ ($\Delta n$) between analyte and the environment;
- the particle size and particle-size distribution;
- the scattering cross-section;
- the absorption cross-section.

Appropriate acceptance criteria will vary with the application but a day-to-day variation of ± 10 per cent or less in relative band intensities is achievable in most cases. Response calibration may involve the use of white-light standards or luminescent glass (e.g. NIST SRM 2241), usually applied by the manufacturer of Raman spectrometers.

QUALITATIVE METHODS

Since the frequency shift position is employed for identification, identical laser intensity for both the reference standard and the material to be examined may not be necessary. The material to be examined is measured in the same physical state (e.g. liquid, solid) as the reference or library material. Raman techniques offer the advantage of non-invasive measurements of the material to be examined without removal from the packaging. However, some packaging materials may alter the measurement. This is especially the case when the packaging absorbs at the laser's excitation wavelength.

*Identification using a reference standard.* Prepare the material to be examined and the reference standard by the same procedure and record the spectra under the same instrumental conditions. The maxima in the spectrum obtained with the material to be examined correspond in position and if applicable in relative intensity to those in the spectrum obtained with the reference standard. When the spectra recorded in the solid state show differences in the positions of the maxima, treat the material to be examined and the reference standard in the same manner so that they crystallise or are produced in the same solid form, or proceed as described in the monograph, then record the spectra.

*Identification using a spectral reference library.* Record the spectra of a suitable number of materials which exhibit typical variation (manufacturer, batch, particle size, impurity profile, etc.) and comply with the requirements of the monograph or established specifications. The number and selection of samples in the database depends on the specific application. A suitable mathematical transformation of the Raman spectrum may facilitate qualitative comparison of spectra (e.g. baseline correction, min-max normalisation, vector normalisation, derivatives). The selectivity of the database that makes it possible to identify a given material and distinguish it adequately from other materials in the database is to be confirmed during the validation procedure.

QUANTITATIVE METHODS

Quantitative determination requires that the reference standard and the material to be examined are measured at the same laser intensity and frequency. Ensure the material to be examined is measured in the same physical state (e.g. liquid, solid) and concentration range as the reference standard used for calibration. While Beer-Lambert's law is not valid for Raman spectroscopy, Raman intensity is directly proportional

Table 2.2.48.-1. – *Wavenumber shifts (and acceptable tolerances) of polystyrene, paracetamol and cyclohexane*

| | Wavenumber shifts[A] [$cm^{-1}$] | Tolerances | |
|---|---|---|---|
| | | Benchtop [$cm^{-1}$] | Hand-held [$cm^{-1}$] |
| Polystyrene[B] | 620.9 | ± 1.5 | ± 2.5 |
| | 1001.4 | ± 1.5 | ± 2.0 |
| | 1031.8 | ± 1.5 | ± 2.0 |
| | 1602.3 | ± 1.5 | ± 3.0 |
| | 3054.3 | ± 3.0 | NA[E] |
| Paracetamol[C] | 797.2 | ± 1.5 | ± 2.5 |
| | 857.9 | ± 1.5 | ± 2.0 |
| | 1168.5 | ± 1.5 | ± 2.0 |
| | 1236.8 | ± 1.5 | ± 2.0 |
| | 1323.9 | ± 1.5 | ± 2.5 |
| | 1648.4 | ± 1.5 | ± 3.0 |
| | 2931.1 | ± 2.0 | NA[E] |
| Cyclohexane[D] | 801.3 | ± 1.5 | ± 2.5 |
| | 1028.3 | ± 1.0 | ± 2.0 |
| | 1266.4 | ± 1.0 | ± 2.0 |
| | 1444.4 | ± 1.0 | ± 2.5 |
| | 2852.9 | ± 2.0 | ± 3.0 |

[A] *Standard guide for Raman shift standards for spectrometer calibration* (American Society for Testing and Materials ASTM E 1840).
[B] Polystyrene film (e.g. 76 µm), pellets (e.g. NIST 706a) or rod.
[C] *Paracetamol for equipment qualification CRS* (which represents monoclinic form I).
[D] *Cyclohexane R*.
[E] NA: beyond detector range.

to the concentration of the Raman scattering analytes, however for solid samples and suspensions the Raman intensity could be affected by the matrix (e.g. fluorescence and self-absorption). The Raman signal is influenced by the refractive index of the material, the particle size and the particle-size distribution (where the small particles give a relatively more intense Raman scattering than large particles), the packing density, the scattering cross-section, the absorption cross-section, etc. (see also under Verification of the response-intensity scale).

**01/2018:20249**

## 2.2.49. FALLING BALL AND AUTOMATIC ROLLING BALL VISCOMETER METHODS

The determination of dynamic viscosity of Newtonian liquids using a falling ball or rolling ball viscometer is performed at 20.0 ± 0.1 °C, unless otherwise prescribed in the monograph. The time required for a test ball to fall or roll in the liquid to be examined from one ring mark or sensor to the other is determined.

METHOD A - FALLING BALL VISCOMETER

*Apparatus.* The falling ball viscometer consists of: a glass tube enclosed in a mantle, which allows precise control of temperature; 6 balls made of glass, nickel-iron or steel with different densities and diameters. The tube is fixed in such a way that the axis is inclined by 10 ± 1° with regard to the vertical. The tube has 2 ring marks that define the distance the ball has to fall. Commercially available apparatuses are supplied with tables giving the constants, the density of the balls and the suitability of the different balls for the expected viscosity range.

*Method.* Fill the clean, dry tube of the viscometer, previously brought to 20.0 ± 0.1 °C, with the liquid to be examined, avoiding bubbles. Choose the ball that is suitable for the viscosity range of the liquid so as to obtain a falling time (run time) not less than 30 s. Place it in the tube, close the tube and maintain the solution at 20.0 ± 0.1 °C for at least 15 min. Let the ball run through the liquid between the 2 ring marks once without measurement. Let it run again and measure with a stop-watch, to at least the nearest 1/5 s, the time required for the ball to fall from the upper to the lower ring mark. Repeat the test run at least 3 times to obtain a minimum of 4 run times. The result is only valid if 2 consecutive run times do not differ by more than 1.5 per cent.

Use the mean of the run times to calculate the dynamic viscosity ($\eta$) in millipascal seconds using the formula:

$$\eta = k(\rho_1 - \rho_2) \times t$$

$k$ = constant, in square millimetres per second squared;

$\rho_1$ = density of the ball used, in grams per cubic centimetre;

$\rho_2$ = density of the liquid to be examined, in grams per cubic centimetre, obtained by multiplying its relative density ($d_{20}^{20}$) by 0.9982;

$t$ = mean of the run times of the ball, in seconds.

METHOD B – AUTOMATIC ROLLING BALL VISCOMETER

*Apparatus*. The automatic rolling ball viscometer consists of: several capillaries made of glass or other suitable materials with different diameters enclosed in a thermostatically controlled block, which allows precise control of the temperature; balls made of stainless steel (optionally coated) or other suitable materials; a motor drive that positions the capillary at an inclination angle from 10.0 ± 0.2° to 80.0 ± 0.2° with regard to the vertical. The apparatus has at least 2 sensors to measure the run time of the ball over a predefined distance. Commercially available apparatuses are supplied with tables giving the constants, the density of the balls and the suitability of the different capillaries for the expected viscosity range. Temperature control, control of the inclination angle, run-time determination, repetitions of runs and calculations of the mean and relative standard deviation are performed by the apparatus software.

*Method*. Set the instrument software to perform a measurement with at least 4 run times (2 forward/backward cycles) and a maximum relative standard deviation of 0.5 per cent. Choose the capillary, the ball and the inclination angle that are suitable for the expected viscosity range of the liquid to be examined so as to obtain a rolling time (run time) not less than 20 s over the 100 mm distance or a proportional time over other distances. If no digital density meter is connected to the viscometer, ensure that the density value of the liquid to be examined has been specified in the apparatus software. Fill the clean, dry capillary of the viscometer with the liquid to be examined, avoiding bubbles. Start the measurement immediately after filling the capillary.

The instrument calculates automatically the dynamic viscosity ($\eta$) in millipascal seconds and the kinematic viscosity ($\nu$) in square millimetres per second.

**01/2010:20254**

## 2.2.54. ISOELECTRIC FOCUSING[7]

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentrating effect is called "focusing". Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighbouring bands:

$$\Delta\mathrm{pI} = 3 \times \sqrt{\frac{D(\mathrm{dpH} / \mathrm{d}x)}{E(-\mathrm{d}\mu / \mathrm{dpH})}}$$

$D$ = diffusion coefficient of the protein,

$\frac{\mathrm{dpH}}{\mathrm{d}x}$ = pH gradient,

$E$ = intensity of the electric field, in volts per centimetre,

$-\frac{\mathrm{d}\mu}{\mathrm{dpH}}$ = variation of the solute mobility with the pH in the region close to the pI.

Since $D$ and $-\frac{\mathrm{d}\mu}{\mathrm{dpH}}$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilised pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerised within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilised pH gradients can resolve proteins differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. haemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins, the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

(7) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

APPARATUS

An apparatus for IEF consists of:

- a controllable generator for constant potential, current and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended;
- a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;
- a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

*The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.*

*PREPARATION OF THE GELS*

**Mould**. The mould (see Figure 2.2.54.-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

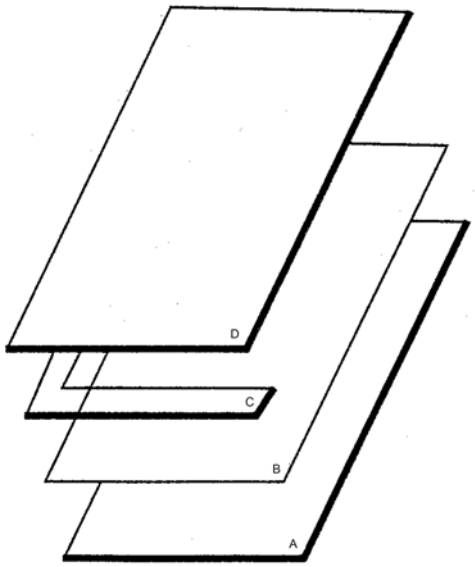


Figure 2.2.54.-1 – *Mould*

**7.5 per cent polyacrylamide gel**. Dissolve 29.1 g of *acrylamide R* and 0.9 g of *methylenebisacrylamide R* in 100 mL of *water R*. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water R*. Mix carefully and degas the solution.

**Preparation of the mould**. Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g/L solution of *ammonium persulfate R* and 0.25 volumes of *tetramethylethylenediamine R*. Immediately fill the space between the glass plates of the mould with the solution.

*METHOD*

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in *fixing solution for isoelectric focusing in polyacrylamide gel R*. Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and add 200 mL of *destaining solution R*. Incubate with shaking for 1 h. Drain the gel, add *coomassie staining solution R*. Incubate for 30 min. Destain the gel by passive diffusion with *destaining solution R* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- the use of immobilised pH gradients,
- the use of rod gels,
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,
- the inclusion of a pre-focusing step,
- the use of automated instrumentation,
- the use of agarose gels.

VALIDATION OF ISO-ELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using coloured pH markers of known isoelectric points,
- comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
- any other validation criteria as prescribed in the monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

- the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;

– the use of alternative staining methods;
– the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

**01/2010:20255**

## 2.2.55. PEPTIDE MAPPING[8]

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner. It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterise the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analysed. 4 principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

ISOLATION AND PURIFICATION

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

SELECTIVE CLEAVAGE OF PEPTIDE BONDS

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 2.2.55.-1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 2.2.55.-1. – *Examples of cleavage agents*

| Type | Agent | Specificity |
|---|---|---|
| Enzymatic | Trypsin (EC 3.4.21.4) | *C*-terminal side of Arg and Lys |
| | Chymotrypsin (EC 3.4.21.1) | *C*-terminal side of hydrophobic residues (e.g. Leu, Met, Ala, aromatics) |
| | Pepsin (EC 3.4.23.1 and 2) | Non-specific digest |
| | Lysyl endopeptidase (Lys-C endopeptidase) (EC 3.4.21.50) | *C*-terminal side of Lys |
| | Glutamyl endopeptidase (from *S. aureus* strain V8) (EC 3.4.21.19) | *C*-terminal side of Glu and Asp |
| | Peptidyl-Asp metallo-endopeptidase (endoproteinase Asp-N) | *N*-terminal side of Asp |
| | Clostripain (EC 3.4.22.8) | *C*-terminal side of Arg |
| Chemical | Cyanogen bromide | *C*-terminal side of Met |
| | 2-Nitro-5-thio-cyanobenzoic acid | *N*-terminal side of Cys |
| | *O*-Iodosobenzoic acid | *C*-terminal side of Trp and Tyr |
| | Dilute acid | Asp and Pro |
| | BNPS-skatole | Trp |

(8) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Pretreatment of sample**. Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100 000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

**Pretreatment of the cleavage agent**. Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

**Pretreatment of the protein**. Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from excipients and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography and lyophilization. Other pretreatments, such as the addition of chaotropic agents (e.g. urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the peptide map due to side reactions occurring during the digestion reaction, such as non-specific cleavage, deamidation, disulfide isomerisation, oxidation of methionine residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g. at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

**Establishment of optimal digestion conditions**. Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

*pH of the reaction milieu.* The pH of the digestion mixture is empirically determined to ensure the optimisation of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion and must not change during the course of the fragmentation reaction.

*Temperature.* A temperature between 25 °C and 37 °C is adequate for most digestions. The temperature used is intended to minimise chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4 °C, because at higher temperatures it will precipitate during digestion.

*Time.* If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 h to 30 h. The reaction is stopped by the addition of an acid which does not interfere in the map or by freezing.

*Amount of cleavage agent used.* Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e. 6-20 hours), the amount of cleavage agent is minimised to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent is added in 2 or more stages to optimise cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping, the separation step. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

## CHROMATOGRAPHIC SEPARATION

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2.2.55-2. In this section, a most widely used reversed-phase HPLC method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available, are recommended for reversed-phase HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration is also recommended.

Table 2.2.55-2. – *Techniques used for the separation of peptides*

| |
|---|
| Reversed-phase high performance liquid chromatography (HPLC) |
| Ion-exchange chromatography (IEC) |
| Hydrophobic interaction chromatography (HIC) |
| Polyacrylamide gel electrophoresis (PAGE), non-denaturating |
| Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) |
| Capillary electrophoresis (CE) |
| Paper chromatography-high voltage (PCHV) |
| High voltage-paper electrophoresis (HVPE) |

**Chromatographic column**. The selection of a chromatographic column is empirically determined for each protein. Columns with 10 nm or 30 nm pore size with silica support can give optimal separation. For smaller peptides, *octylsilyl silica gel for chromatography R* (3-10 µm) and *octadecylsilyl silica gel for chromatography R* (3-10 µm) column packings are more efficient than *butylsilyl silica gel for chromatography R* (5-10 µm).

**Solvent**. The most commonly used solvent is water with acetonitrile as the organic modifier to which not more than 0.1 per cent trifluoroacetic acid is added. If necessary, add propyl alcohol or isopropyl alcohol to solubilise the digest components, provided that the addition does not unduly increase the viscosity of the components.

**Mobile phase**. Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

**Gradient**. Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimised to provide clear resolution of 1 or 2 peaks that will become "marker" peaks for the test.

**Isocratic elution**. Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps must not be used in isocratic HPLC systems.

**Other parameters**. Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1-2.0 mL/min, and the detection of peptides is performed with a UV detector at 200-230 nm. Other methods of detection have been used (e.g. post-column derivatisation), but they are not as robust or versatile as UV detection.

**Validation**. This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint has been achieved is shown by comparison with a reference standard, which is treated in the same manner as the test protein. The use of a reference substance in parallel with the test protein is critical in the development and establishment of system suitability limits. In addition, a chromatogram is included with the reference substance for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference substance for the specified protein. The use of a digested reference substance for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterised mixture of a variant and a reference substance can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters, such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency, may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference substance for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are to be expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retentions, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (*V/V*) mixture of sample and reference substance digest. If all peaks in the sample digest and in the reference substance digest have the same relative retentions and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retentions are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and reference substance digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches are, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference substance and sample digest fragments.

The numerical comparison of the peak retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using 1 peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference substance. The possibility of auto-hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

## ANALYSIS AND IDENTIFICATION OF PEPTIDES

*This section gives guidance on the use of peptide mapping during development in support of regulatory applications.*

The use of a peptide map as a qualitative tool does not require the complete characterisation of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterisation of each of the individual peaks in the peptide map. Methods to characterise peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterisation purposes, when *N*-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase and matrix-assisted laser desorption ionisation coupled to time-of-flight analyser (MALDI-TOF) can also be used for characterisation purposes.

The use of MS for characterisation of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterisation of a protein through peptide mapping is to reconcile and account for at least 95 per cent of the theoretical composition of the protein structure.

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## 2.2.56. AMINO ACID ANALYSIS[9]

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organised as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify proteins and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyse a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatised for analysis.

### APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have post-column derivatisation capability, unless the sample is analysed using precolumn derivatisation. The detector is usually an ultraviolet/visible or fluorescence detector depending on the derivatisation method used. A recording device (e.g., integrator) is used for transforming the analogue signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

### GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

### REFERENCE MATERIAL

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analysed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

### CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analysing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analysed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

4 to 6 amino acid standard levels are analysed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nanomole of amino acid present in the standard. A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the concentration of each

(9) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nanomoles of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that correspond to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analysing many replicates (e.g., 6 analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. The amino acid composition of a standard protein (e.g., bovine serum albumin) is often analysed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits. All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilise post-column derivatisation of the amino acids are generally not affected by buffer components to the extent seen with pre-column derivatisation methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labour. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis, whose rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 h in 1 M hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of equal volumes of concentrated hydrochloric acid and nitric acid. Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500 °C for 4 h may also be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolysing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids: tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., Methods 4-11) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24 h, 48 h and 72 h) is often employed to analyse the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (e.g., serine and threonine) versus hydrolysis time, the line can be extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave

(e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins.

During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

*METHOD 1*

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

**Hydrolysis solution**. 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol.

**Procedure**

*Liquid phase hydrolysis*. Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µL of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal *in vacuo*. Samples are typically hydrolysed at 110 °C for 24 h *in vacuo* or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 h and 72 h) are investigated if there is a concern that the protein is not completely hydrolysed.

*Vapour phase hydrolysis*. This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110 °C for a 24 h hydrolysis time. Acid vapour hydrolyses the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample *in vacuo* to remove any residual acid.

*METHOD 2*

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid as the reducing acid.

**Hydrolysis solution**. 2.5 M mercaptoethanesulfonic acid solution.

**Vapour phase hydrolysis**. Dry about 1 µg to 100 µg of the protein/peptide under test in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 µL of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the hydrolysis solution. Heat the hydrolysis tube to 170-185 °C for about 12.5 min. After hydrolysis, dry the hydrolysis tube *in vacuo* for 15 min to remove the residual acid.

*METHOD 3*

Tryptophan oxidation during hydrolysis is prevented by using thioglycollic acid (TGA) as the reducing acid.

**Hydrolysis solution**. 7 M hydrochloric acid containing 1 per cent of phenol, 10 per cent of trifluoroacetic acid and 20 per cent of thioglycollic acid.

**Vapour phase hydrolysis**. Dry about 10 µg to 50 µg of the protein/peptide under test in a sample tube. Place the sample tube in a larger tube with about 200 µL of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the TGA. Heat the sample tube to 166 °C for about 15-30 min. After hydrolysis, dry the sample tube *in vacuo* for 5 min to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

*METHOD 4*

Cysteine/cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

**Oxidation solution**. Use performic acid freshly prepared by mixing 1 volume of hydrogen peroxide solution (30 per cent) and 9 volumes of anhydrous formic acid and incubating at room temperature for 1 h.

**Procedure**. Dissolve the protein/peptide sample in 20 µL of anhydrous formic acid and heat at 50 °C for 5 min; then add 100 µL of the oxidation solution. Allow the oxidation to proceed for 10-30 min. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine-sulfone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method 1 or Method 2. This technique may cause modifications to tyrosine residues in the presence of halides.

*METHOD 5*

Cysteine/cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

**Hydrolysis solution**. To 6 M hydrochloric acid containing 0.2 per cent of phenol, add sodium azide to obtain a final concentration of 2 g/L. The added phenol prevents halogenation of tyrosine.

**Liquid phase hydrolysis**. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the sodium azide present in the hydrolysis solution. This technique allows better tyrosine recovery than Method 4, but it is not quantitative for methionine. Methionine is converted to a mixture of the parent methionine and its 2 oxidative products, methionine-sulfoxide and methionine-sulfone.

*METHOD 6*

Cysteine/cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

**Hydrolysis solution**. To 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol, add dimethyl sulfoxide to obtain a final concentration of 2 per cent *V/V*.

**Vapour phase hydrolysis**. Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the DMSO present in the hydrolysis solution. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolysis of standard proteins containing 1-8 mol of cysteine per mole of protein. The response factors from protein/peptide hydrolysates are typically about 30 per cent lower than those for non-hydrolysed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

*METHOD 7*

Cysteine/cystine reduction and alkylation is accomplished by a vapour phase pyridylethylation reaction.

**Reducing solution**. Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container and mix.

**Procedure**. Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the reducing solution to the large tube, seal *in vacuo* (about 50 µm of mercury or 6.7 Pa), and heat at about 100 °C for 5 min. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 min to remove residual reagents. The pyridylethylated sample can then be acid hydrolysed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1-8 mol of cysteine per mole of protein to evaluate the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

*METHOD 8*

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

**Stock solutions**. Prepare and filter 3 solutions: 1 M Tris-hydrochloride pH 8.5 containing 4 mM disodium edetate (stock solution A), 8 M guanidine hydrochloride (stock solution B), and 10 per cent of 2-mercaptoethanol (stock solution C).

**Reducing solution**. Prepare a mixture of 1 volume of stock solution A and 3 volumes of stock solution B to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M tris-hydrochloride.

**Procedure**. Dissolve about 10 µg of the test sample in 50 µL of the reducing solution, and add about 2.5 µL of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 h at room temperature in the dark. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

*METHOD 9*

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

**Stock solutions**. Prepare as directed for Method 8.

**Carboxymethylation solution**. Prepare a 100 g/L solution of iodoacetamide in alcohol.

**Buffer solution**. Use the reducing solution, prepared as described for Method 8.

**Procedure**. Dissolve the test sample in 50 µL of the buffer solution, and add about 2.5 µL of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. Add the carboxymethylation solution in a ratio 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 min at room temperature in the dark. If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present. The reaction is stopped by adding excess of 2-mercaptoethanol. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethyl-cysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

*METHOD 10*

Cysteine/cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.

**Reducing solution**. A 10 g/L solution of dithiodiglycolic acid (or dithiodipropionic acid) in 0.2 M sodium hydroxide.

**Procedure**. Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the reducing solution. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolysed using Method 1. This method has the advantage that other amino acid residues are not derivatised by side reactions, and that the sample does not need to be desalted prior to hydrolysis.

*METHOD 11*

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

**Reducing solutions**. Prepare and filter 3 solutions: a solution of 10 mM trifluoroacetic acid (Solution A), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (Solution B), and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per millilitre (Solution C).

**Procedure**. In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of Solution A or Solution B and 2 mL of Solution C. Seal the hydrolysis tube *in vacuo*. Heat the sample at 60 °C for 4 h in the dark. The sample is then dialysed with water to remove the excess reagents. Extract the dialysed sample 3 times with equal volumes of butyl acetate, and then lyophilise. The protein can then be acid hydrolysed using previously described procedures. The α,β-diaminopropionic and α,γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatised and BTI-derivatised acid hydrolysis. The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatisation; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.

## METHODOLOGIES OF AMINO ACID ANALYSIS: GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by post-column derivatisation (e.g., with ninhydrin or *o*-phthalaldehyde). Post-column derivatisation techniques can be used with samples that contain small amounts of buffer components, (such as salts and urea) and generally require between 5 µg and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve pre-column derivatisation of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenylmethyl chloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Pre-column derivatisation techniques are very sensitive and usually require between 0.5 µg and 1.0 µg of protein sample per analysis but may be influenced by buffer

salts in the samples. Pre-column derivatisation techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Post-column derivatisation techniques are generally influenced less by performance variation of the assay than pre-column derivatisation techniques.

The following methods may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will use more than one amino acid analysis technique to exploit the advantages offered by each. In each of these methods, the analogue signal is visualised by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

*METHOD 1 - POST-COLUMN NINHYDRIN DERIVATISATION*

Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has a characteristic purple or yellow colour. Amino acids, except imino acid, give a purple colour, and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour, and show an absorption maximum at 440 nm. The post-column reaction between ninhydrin and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for the proline derivative. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

*METHOD 2 - POST-COLUMN OPA DERIVATISATION*

*o*-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is used for the post-column derivatisation in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as Method 1.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite or chloramine T allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by post-column oxidation with sodium hypochlorite or chloramine T and post-column derivatisation using OPA and a thiol compound such as *N*-acetyl-L-cysteine or 2-mercaptoethanol. The derivatisation of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite or chloramine T.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After post-column derivatisation of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatised amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of picomole level for most of the OPA-derivatised amino acids. Response linearity is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

*METHOD 3 - PRE-COLUMN PITC DERIVATISATION*

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 254 nm. Therefore, pre-column derivatisation of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyse the amino acid composition.

After the reagent is removed under vacuum, the derivatised amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after 3 days.

Separation of the PTC-amino acids on a reversed-phase HPLC with an octadecylsilyl (ODS) column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the PTC-amino acids. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

*METHOD 4 - PRE-COLUMN AQC DERIVITISATION*

Pre-column derivatisation of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase HPLC. Therefore, pre-column derivatisation of amino acids with AQC followed by reversed-phase HPLC separation with fluorimetric detection is used to analyse the amino acid composition.

Separation of the AQC-amino acids on a reversed-phase HPLC with an ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strengh. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. Excess reagent is rapidly hydrolysed ($t_{1/2}$<15 s) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 min no further derivatisation can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature. Therefore AQC-amino acids have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to range from about 40 fmol to 320 fmol for each amino acid, except for cystein. The detection limit for cystein is approximately 800 fmol. Response linearity is obtained in the range of 2.5-200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatised protein hydrolysates derived from as little as 30 ng of protein/peptide.

*METHOD 5 - PRE-COLUMN OPA DERIVATISATION*

Pre-column derivatisation of amino acids with *o*-phthalaldehyde (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as the thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reaction, make it amenable to automated derivatisation and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in Method 7 or Method 8.

Pre-column derivatisation of amino acids with OPA is followed by a reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatisation. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatised amino acids. Fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

*METHOD 6 - PRE-COLUMN DABS-Cl DERIVATISATION*

Pre-column derivatisation of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labelling of amino acids. Amino acids labelled with DABS-Cl (DABS-amino acids) are highly stable and show an absorption maximum at 436 nm.

DABS-amino acids, all naturally occurring amino acid derivatives, can be separated on an ODS column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyse the imino acids such as proline together with the amino acids at the same degree of sensitivity, DABS-Cl derivatisation method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid or methanesulfonic acid described in Method 2 under Protein hydrolysis. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI described in Method 11 under Protein hydrolysis.

The non-proteinogenic amino acid norleucine cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2-5 pmol of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10-30 ng of the dabsylated protein hydrolysate is required for each analysis.

*METHOD 7 - PRE-COLUMN FMOC-Cl DERIVATISATION*

Pre-column derivatisation of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction proceeds under mild conditions in aqueous solution and is completed in 30 s. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of 10 volumes of acetonitrile, 40 volumes of methanol and 50 volumes of acetic acid buffer to a mixture of 50 volumes of acetonitrile and 50 volumes of acetic acid buffer and 20 amino acid derivatives are separated in 20 min. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low femtomole range. A linearity range of 0.1-50 µM is obtained for most of the amino acids.

*METHOD 8 - PRE-COLUMN NBD-F DERIVATISATION*

Pre-column derivatisation of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatised with NBD-F by heating to 60 °C for 5 min.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 min. ε-Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for the pre-column OPA derivatisation method (Method 5), excluding proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis can be achieved with about 1.5 mg of protein hydrolysates in the pre-column reaction mixture.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapour phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

**Amino Acid Mole Percent**. This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular mass of the protein under investigation is unknown. This information can be used to corroborate

the identity of a protein/peptide and has other applications. Carefully identify and integrate the peaks obtained as directed for each procedure. Calculate the mole percent for each amino acid present in the test sample using the formula:

$$\frac{100r_U}{r}$$

in which $r_U$ is the peak response, in nanomoles, of the amino acid under test; and $r$ is the sum of peak responses, in nanomoles, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

**Unknown Protein Samples**. This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in micrograms, of each recovered amino acid using the formula:

$$\frac{mM_r}{1000}$$

in which $m$ is the recovered quantity, in nanomoles, of the amino acid under test; and $M_r$ is the average molecular mass for that amino acid, corrected for the mass of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analysed after appropriate correction for partially and completely destroyed amino acids. If the molecular mass of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid using the formula:

$$\frac{m}{\left(\frac{1000M}{M_{rt}}\right)}$$

in which $m$ is the recovered quantity, in nanomoles, of the amino acid under test; $M$ is the total mass, in micrograms, of the protein; and $M_{rt}$ is the molecular mass of the unknown protein.

**Known protein samples**. This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular mass and amino acid composition using the amino acid analysis data. When the composition of the protein being analysed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine) and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nanomoles, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically greater than 5 per cent variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, using the formula:

$$\frac{100m}{m_S}$$

in which $m$ is the experimentally determined quantity, in nanomoles per amino acid residue, of the amino acid under test; and $m_S$ is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

**01/2008:20257**

# 2.2.57. INDUCTIVELY COUPLED PLASMA-ATOMIC EMISSION SPECTROMETRY

GENERAL PRINCIPLE

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) is an atomic emission spectrometry method that uses an inductively coupled plasma (ICP) as the excitation source.

An ICP is a highly ionised inert gas (usually argon) with equal numbers of electrons and ions sustained by a radio-frequency (RF) field. The high temperature reached in the plasma successively desolvates, vaporises, excites - atomic emission spectrometry (AES) detection - and ionises - mass spectrometry (MS) detection - atoms from the sample. Detection limits are, generally, in the lower nanogram (ICP-MS) to microgram (ICP-AES) per litre range.

The plasma is formed by a tangential stream of support gas through a 'torch', i.e. a system consisting of 3 concentric quartz tubes. A metal coil (the load coil) surrounds the top end of the torch and is connected to a radio-frequency (RF) generator. Power (usually 700-1500 W) is applied through the coil and an oscillating magnetic field corresponding to the frequency of the generator (in most cases 27 MHz, 40 MHz) is formed. The plasma forms when the support gas is made conductive by exposing it to an electric discharge, which produces seed electrons and ions. Inside the induced magnetic field, the charged particles (electrons and ions) are forced to flow in a closed annular path. As they meet resistance to their flow, heating takes place producing additional ionisation. The process occurs almost instantaneously, and the plasma expands to its full strength and dimensions. The radio-frequency oscillation of the power applied through the coil causes radio-frequency electric and magnetic fields to be set up in the area at the top of the torch. When a spark (produced by a Tesla tube or some other seeding device) is applied to the support gas flowing through the torch, some electrons are stripped from the support gas atoms. These electrons are then caught up in the magnetic field and accelerated. Adding energy to the electrons by the use of a coil is known as inductive coupling. These high-energy electrons in turn collide with other support-gas atoms, stripping off still more electrons. The collisional ionisation of the support gas continues in a chain reaction, breaking down the gas into a physical plasma consisting of support-gas atoms, electrons and support-gas ions. The plasma is then sustained within the torch and load coil as radio-frequency energy is continually transferred to it through the inductive coupling process.

The ICP appears as an intense, very bright, plume-shaped plasma. At the base the plasma is toroidal, and this is referred to as the induction region (IR), i.e. the region in which the inductive energy transfer from the load coil to the plasma takes place. The sample is introduced through the induction region into the centre of the plasma.

## APPARATUS

The apparatus consists essentially of the following elements:

- sample-introduction system consisting of a peristaltic pump delivering the solution at constant flow rate into a nebuliser;
- radio-frequency (RF) generator;
- plasma torch;
- transfer optics focussing the image of the plasma at the entrance slit of the spectrometer; radial viewing is better for difficult matrices (alkalis, organics), whereas axial viewing gives more intensity and better detection limits in simple matrices;
- wavelength dispersive devices consisting of diffraction gratings, prisms, filters or interferometers;
- detectors converting radiant energy into electrical energy;
- data-acquisition unit.

## INTERFERENCE

Interference is anything that causes the signal from an analyte in a sample to be different from the signal for the same concentration of that analyte in a calibration solution. The well-known chemical interference that is encountered in flame atomic absorption spectrometry is usually weak in ICP-AES. In rare cases where interference occurs, it may be necessary to increase the RF power or to reduce the inner support-gas flow to eliminate it. The interference in ICP-AES can be of spectral origin or even the result of high concentrations of certain elements or matrix compounds. Physical interference (due to differences in viscosity and surface tension of the sample and calibration standards) can be minimised by dilution of the sample, matrix matching, use of internal standards or through application of the method of standard additions.

Another type of interference occasionally encountered in ICP-AES is the so-called 'easily ionised elements (EIEs) effect'. The EIEs are those elements that are ionised much more easily, for example alkaline metals and alkaline earths. In samples that contain high concentrations of EIEs (more than 0.1 per cent), suppression or enhancement of emission signals is likely to occur.

**Spectral interference**. This may be due to other lines or shifts in background intensity. These lines may correspond to argon (observed above 300 nm), OH bands due to the decomposition of water (at about 300 nm), NO bands due to the interaction of the plasma with the ambient air (between 200 nm and 300 nm), and other elements in the sample, especially those present at high concentrations. The interference falls into 4 different categories: simple background shift, sloping background shift, direct spectral overlap, and complex background shift.

**Absorption interference**. This arises when part of the emission from an analyte is absorbed before it reaches the detector. This effect is observed particularly when the concentration of a strongly emitting element is so high that the atoms or ions of that element that are in the lower energy state of transition absorb significant amounts of the radiation emitted by the relevant excited species. This effect, known as self-absorption, determines the upper end of the linear working range for a given emission line.

**Multicomponent spectral fitting**. Multiple emission-line determinations are commonly used to overcome problems with spectral interferences. A better, more accurate method for performing spectral interference corrections is to use the information obtained with advanced detector systems through multicomponent spectral fitting. This quantifies not only the interference, but also the background contribution from the matrix, thereby creating a correction formula. Multicomponent spectral fitting utilises a multiple linear-squares model based on the analysis of pure analyte, the matrix and the blank, creating an interference-corrected mathematical model. This permits the determination of the analyte emission in a complex matrix with improved detection limits and accuracy.

## PROCEDURE

### *SAMPLE PREPARATION AND SAMPLE INTRODUCTION*

The basic goal for the sample preparation is to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulfuric and phosphoric acids can contribute to background emission observed in the ICP spectra. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps used for ICP-AES usually deliver the standard and sample solutions at a rate of 1 mL/min or less.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

### *CHOICE OF OPERATING CONDITIONS*

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen:

- wavelength selection;
- support-gas flow rates (outer, intermediate and inner tubes of the torch);
- RF power;
- viewing position (radial or axial);
- pump speed;
- conditions for the detector (gain/voltage for photomultiplier tube detectors, others for array detectors);
- integration time (time set to measure the emission intensity at each wavelength).

## CONTROL OF INSTRUMENT PERFORMANCE

### System suitability

The following tests may be carried out with a multi-element control solution to ensure the adequate performance of the ICP-AES system:

- energy transfer (generator, torch, plasma); measurement of the ratio Mg II (280.270 nm)/Mg I (285.213 nm) may be used;
- sample transfer, by checking nebuliser efficiency and stability;
- resolution (optical system), by measuring peak widths at half height, for example As (189.042 nm), Mn (257.610 nm), Cu (324.754 nm) or Ba (455.403 nm);
- analytical performance, by calculating detection limits of selected elements over the wavelength range.

## VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

*LINEARITY*

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed.

If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

*ACCURACY*

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

**Recovery**. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside of the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

*REPEATABILITY*

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

*LIMIT OF QUANTIFICATION*

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

**01/2008:20258**

# 2.2.58. INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY

Inductively coupled plasma-mass spectrometry (ICP-MS) is a mass spectrometry method that uses an inductively coupled plasma (ICP) as the ionisation source. The basic principles of ICP formation are described in chapter *2.2.57* on inductively coupled plasma-atomic emission spectrometry (ICP-AES).

ICP-MS utilises the ability of the ICP to generate charged ions from the element species within a sample. These ions are then directed into a mass spectrometer, which separates them according to their mass-to-charge ratio (*m*/*z*). Most mass spectrometers have a quadrupole system or a magnetic sector. Ions are transported from the plasma through 2 cones (sampler and skimmer cones, forming the interface region) to the ion optics. The ion optics consist of an electrostatic lens, which takes ions from an area at atmospheric pressure to the mass filter at a vacuum of $10^{-8}$ Pa or less, maintained with a turbomolecular pump. After their filtration, ions of the selected mass/charge ratio are directed to a detector (channel electromultiplier, Faraday cup, dynodes), where ion currents are converted into electrical signals. The element is quantified according to the number of ions arriving and generating electrical pulses per unit time.

The sample-introduction system and data-handling techniques of an ICP-AES system are also used in ICP-MS.

APPARATUS

The apparatus consists essentially of the following elements:

- sample-introduction system, consisting of a peristaltic pump delivering the solution at constant flow rate into a nebuliser;
- radio-frequency (RF) generator;
- plasma torch;
- interface region including cones to transport ions to the ion optics;
- mass spectrometer;
- detector;
- data-acquisition unit.

INTERFERENCE

Mass interference is the major problem, for example by isobaric species that significantly overlap the mass signal of the ions of interest, especially in the central part of the mass range (for example 40-80 a.m.u.). The combination of atomic ions leads to polyatomic or molecular interferences (i.e. $^{40}Ar^{16}O$ with $^{56}Fe$ or $^{40}Ar^{40}Ar$ with $^{80}Se$). Matrix interference may also occur with some analytes. Some samples have an impact on droplet formation or on the ionisation temperature in the plasma. These phenomena may lead to the suppression of analyte signals. Physical interference is to be circumvented by using the method of internal standardisation or by standard addition. The element used as internal standard depends on the element to be measured: $^{59}Co$ and $^{115}In$, for example, can be used as internal standards.

The prime characteristic of an ICP-MS instrument is its resolution, i.e. the efficiency of separation of 2 close masses. Quadrupole instruments are, from this point of view, inferior to magnetic-sector spectrometers.

PROCEDURE

*SAMPLE PREPARATIONS AND SAMPLE INTRODUCTION*

The sample preparation usually involves a step of digestion of the matrix by a suitable method, for example in a microwave oven. Furthermore, it is important to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulfuric and phosphoric acids can contribute to background emission. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps usually deliver the standard and sample solutions at a rate of 20-1000 µL/min.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

*CHOICE OF OPERATING CONDITIONS*

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen:

- selection of cones (material of sampler and skimmer);
- support-gas flow rates (outer, intermediate and inner tubes of the torch);
- RF power;
- pump speed;
- selection of one or more isotopes of the element to be measured (mass).

ISOTOPE SELECTION

Isotope selection is made using several criteria. The most abundant isotope for a given element is selected to obtain maximum sensitivity. Furthermore, an isotope with the least interference from other species in the sample matrix and from the support gas should be selected. Information about isobaric interferences and interferences from polyatomic ions of various types, for example hydrides, oxides, chlorides, etc., is usually available in the software of ICP-MS instrument manufacturers.

CONTROL OF INSTRUMENT PERFORMANCE

**System suitability**

- Tuning of the instrument allows to monitor and adjust the measurement before running samples. ICP-MS mass accuracy is checked with a tuning solution containing several isotopes covering the whole range of masses, for example $^{9}Be$, $^{59}Co$, $^{89}Y$, $^{115}In$, $^{140}Ce$ and $^{209}Bi$.
- Sensitivity and short- and long-term stability are recorded. The instrument parameters (plasma condition, ion lenses and quadrupole parameter) are to be optimised to obtain the highest possible number of counts.
- Tuning for resolution and mass axis is to be done with a solution of Li, Y and Tl to ensure an acceptable response over a wide range of masses.
- Evaluation of the efficiency of the plasma to decompose oxides has to be performed in order to minimise these interferences. The ratio Ce/CeO and/or Ba/BaO is a good indicator, and a level less than about 3 per cent is required.
- Reduction of the formation of double-charged ions is made with Ba and Ce. The ratio of the signal for double-charged ions to the assigned element should be less than 2 per cent.
- Long-term stability is checked by running a standard first and at the end of the sample sequence, controlling whether salt deposits on the cones have reduced the signal throughout the run.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

*LINEARITY*

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed.

If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

*ACCURACY*

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

**Recovery**. For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

*REPEATABILITY*

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

*LIMIT OF QUANTIFICATION*

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

**01/2011:20259**

# 2.2.59. GLYCAN ANALYSIS OF GLYCOPROTEINS

1. INTRODUCTION

Glycan analysis is a test to analyse glycan moieties of glycoproteins. It may involve:

- whole glycoprotein analysis;
- separation and detection of protein glycoforms;
- analysis of glycopeptides obtained after enzymatic treatment of the glycoprotein;
- analysis of released glycans obtained after chemical or enzymatic treatment of the glycoprotein.

Monosaccharide analysis may complement information obtained by glycan analysis.

Glycosylation can play a predominant role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of biotherapeutics. Glycosylation, unlike transcription, is a non-template-driven enzymatic modification process that results in glycan heterogeneity. The manufacturing procedure also has an influence on glycan heterogeneity. Glycoprotein glycan analysis may therefore be an important test to identify variations in the glycosylation pattern of the glycoprotein and/or monitor the consistency of the glycosylation pattern during production.

Glycan analysis can be a comparative procedure, because the information obtained, compared to a similarly treated reference substance, confirms product consistency.

This chapter provides approaches used for glycoprotein glycan analysis and requirements for the application of methods and validation of methods.

Glycan analysis is not a single general method, but involves the application of specific procedures and the development of specific glycan maps for each unique glycoprotein. Specific procedures are therefore indicated in relevant specific monographs.

1-1. *PROTEIN GLYCOSYLATION*

There are 3 main types of enzymatic glycosylation found in proteins:

- *N*-glycosylation, which involves the addition of oligosaccharides to the nitrogen on the terminal amide group of asparagine;
- *O*-glycosylation, which involves the addition of oligosaccharides to the hydroxyl groups of serine, threonine, and/or hydroxyproline;
- *C*-glycosylation, which involves the addition of an α-mannopyranose to the C2-carbon of the indole ring of tryptophan.

Non-enzymatic additions, also known as glycation, can occur when proteins are incubated with reducing sugars.

This chapter describes analytical methods for the *N*- and *O*-linked glycosylations, which are the most commonly found in glycoprotein medicinal products.

1-2. *HETEROGENEITY OF THE PROTEIN GLYCOSYLATION*

Different levels of glycan heterogeneity can appear during the production of glycoproteins. This heterogeneity may result from variations:

- in the degree of occupancy (full, partial, unoccupied);
- in the type of glycosylation (*N*- or *O*-linked);
- in the oligosaccharide structures (extensions, branching and linkage).

This heterogeneity in glycosylation results in a set of glycoforms for one specific glycoprotein. These variations arise because, unlike transcription and translation, glycosylation is a non-template post-translational modification process. The glycosylation pattern at a given site depends on many factors including the cell-specific and/or growth-dependent availability of glycosyltransferases and exo-glycosidases found in the Golgi apparatus and endoplasmic reticulum. Protein glycosylation is also influenced by the protein structure, the production process, the host-vector expression system and the cell culture conditions.

2. GLYCAN ANALYSIS PROCEDURES

Heterogeneity in glycosylation can be assessed by 4 distinct and complementary approaches:

- analysis of the intact glycoprotein;
- analysis of glycopeptides;
- analysis of released glycans;
- monosaccharide analysis.

The present section provides methods and general requirements used for glycan analysis of glycoproteins containing *N*- and *O*-linked glycans.

Glycan analysis is usually a multistep process. There are numerous methodologies for glycan analysis. This variety is a consequence of the diversity and complexity of glycan structures, of the available technologies and detection systems, and of the wide range of approaches depending on the level of information required.

Figure 2.2.59.-1 provides an overview of glycan analysis analytical procedures that can be employed to apply the chosen approach(es). Many variations of the same techniques and conditions are available depending on the glycan structures and origin.

**Isolation and purification**. Isolation and purification may be necessary for analysis of bulk drug substances or dosage forms containing interfering excipients, and, when required, will be described in the specific monograph.

2-1. *ANALYSIS OF INTACT GLYCOPROTEIN*

Analysis of the intact glycoprotein provides information on the overall pattern of glycosylation of the glycoprotein.

This approach provides limited information when the molecule is large and contains multiple glycosylation sites.

Methods such as capillary electrophoresis (CE) (*2.2.47*) and mass spectrometry (MS) (*2.2.43*) can be used. Size-based techniques, such as size-exclusion chromatography (*2.2.30*) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*2.2.31*), may provide information on the glycosylation status of a protein. If the degree of sialylation significantly contributes to the biological activity of the glycoprotein, ion-exchange chromatography (*2.2.46*), isoelectric focusing (IEF) (*2.2.54*) or CE (*2.2.47*) may be performed to monitor sialylation. The technique must be chosen according to its suitability to provide a reliable correlation between the degree of sialylation and the bioactivity of the product.

2-2. *ANALYSIS OF GLYCOPEPTIDES*

Analysis of glycopeptides provides information on site-specific glycosylation properties, on the degree of occupancy, and on the oligosaccharide structures. It involves proteolytic digestion of the glycoprotein. Approaches to site-specific cleavage of the protein backbone are given in general chapter *2.2.55. Peptide mapping*.

After proteolysis of the glycoprotein, the following approaches can be chosen.

*Direct analysis by MS (2.2.43).* Care should be taken that the glycopeptide signal is not suppressed due to the presence of other peptides, where glycopeptides represent a minor portion of the total peptide mixture and where signal intensities are lower than those of non-glycosylated peptides.

*Separation prior to analysis by MS.* This additional step overcomes the problems raised above. Enrichment or fractionation techniques can be used either in parallel with or sequentially to direct analysis. Separation techniques such as liquid chromatography (LC) (*2.2.29*) and CE (*2.2.47*) are suitable. These techniques may be interfaced with MS to allow online MS measurements.

*Deglycosylation of the glycopeptides.* Identification of the different glycosylation sites of a glycoprotein is made possible by comparing peptide maps obtained by proteolytic digestion of the intact glycoprotein to those obtained when the glycoprotein is deglycosylated previously or following proteolytic digestion. The peptide mass gives information about the glycosylation sites and by calculating the mass difference between the intact glycopeptide and the deglycosylated glycopeptide, it is possible to obtain information about the attached glycans concerning composition and heterogeneity. Approaches to deglycosylation of the protein backbone are given in section 2-3-1. A separation step can be performed after or before deglycosylation.

2-3. *ANALYSIS OF RELEASED GLYCANS*

Analysis of released glycans provides a convenient way to obtain information on the various populations of glycans present on the protein (bi-, tri-, and tetra-antennary profile). The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatisation/labelling may be needed to allow the detection of the glycans.

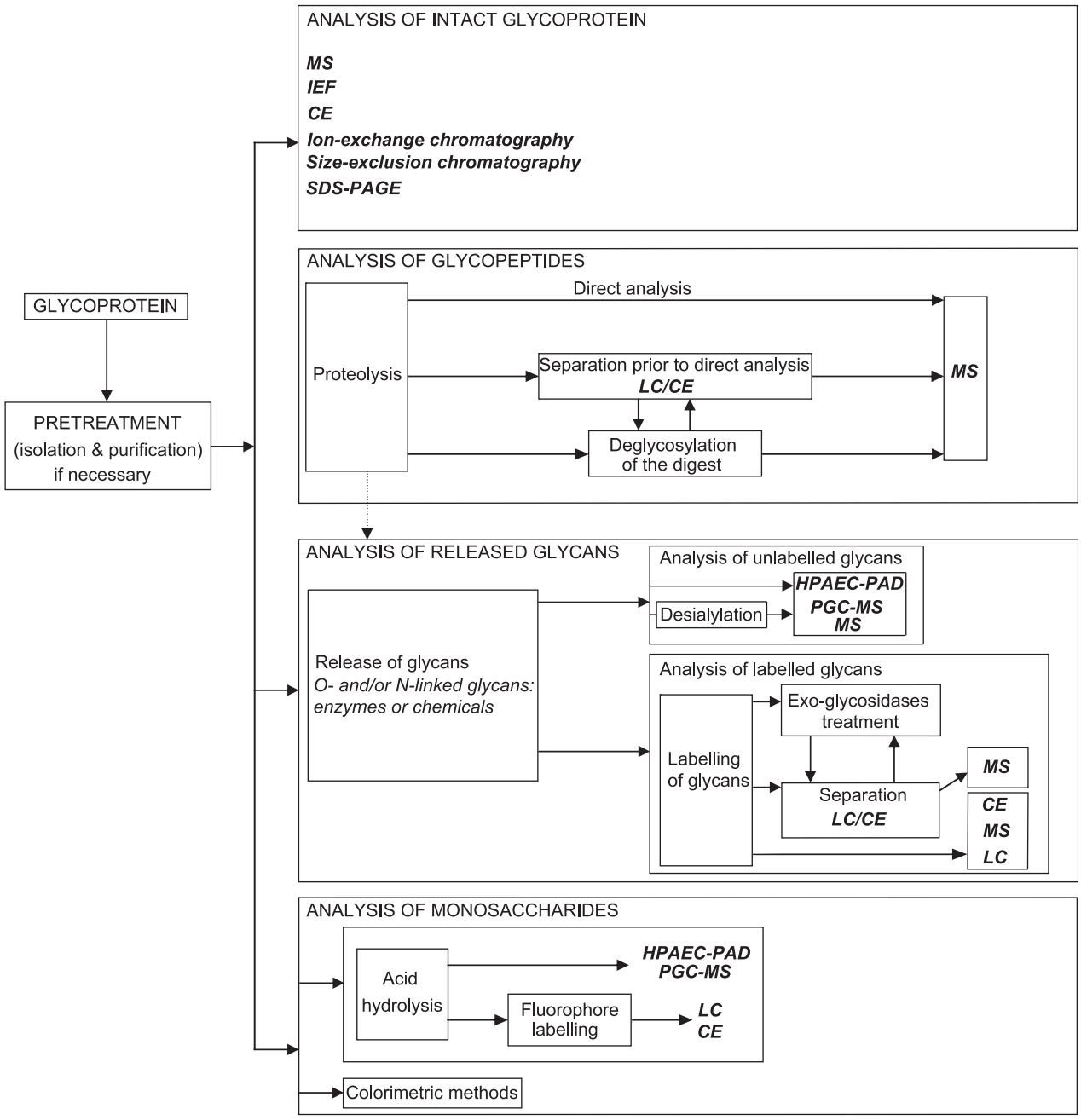


CE: Capillary electrophoresis
HPAEC-PAD: High-pH anion-exchange chromatography with pulsed amperometric detection
IEF: Isoelectric focusing
LC: Liquid chromatography
MS: Mass spectrometry
PGC: Porous graphite chromatography
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure 2.2.59.-1. – *Overview of glycan analysis procedures*

Analysis of released glycans generally involves the release and purification of glycans from the reaction mixture, followed by the labelling/derivatisation of the glycans, where needed; the glycans are then profiled (fractionation or separation).

2-3-1. **Release of glycans**

The selection of the approach used for the release of glycans will depend on the glycoprotein under test. The cleavage agent to be employed is chosen according to the type of cleavage needed and level of information required. Enzymatic or chemical cleavage may be used. Table 2.2.59.-1 gives a non-exhaustive list of enzymatic cleavage agents and their specificity.

Digestion efficiency is generally dependent on the accessibility of the glycans on the protein and hence the protein can be denatured to maximise glycosylation site exposure, unless it is desirable to distinguish between surface and buried glycans.

Chemical cleavage agents might also be used, using for example hydrazine or alkaline borohydride for β-elimination.

Table 2.2.59.-1. – *Examples of enzymatic cleavage agents*

| Agents | Specificity |
|---|---|
| ***N*-linked glycans release** | |
| **Peptide-$N^4$-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52)** | **Hydrolysis of an $N^4$-(acetyl-β-D-glucosaminyl)asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue** |
| - Peptide *N*-glycosidase F (PNGase F) | Release of *N*-glycan chain but no release of *N*-glycan chain containing (α1-3)-linked core fucose |
| - Peptide *N*-glycosidase A (PNGase A) | Release of *N*-glycan chain containing (α1-3)-linked core fucose |
| **Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96)** | **Endohydrolysis of the *N,N′*-diacetylchitobiosyl unit in high-mannose glycopeptides/glycoproteins containing the -[Man(GlcNAc)$_2$]Asn structure** |
| - Endo-β-*N*-acetylglucosaminidase F (endo F) | Release of high-mannose, hybrid and complex oligosaccharides |
| - Endo-β-*N*-acetylglucosaminidase H (endo H) | Release of high-mannose, hybrid oligosaccharides |
| ***O*-linked glycans release** | |
| **Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)*** | **Hydrolysis of terminal D-galactosyl-*N*-acetyl-α-D-galactosaminidic residues** |
| * This enzyme has limited usage because of its high substrate specificity. | |

2-3-2. **Analysis of glycans**

Released glycans can be analysed or profiled by chromatographic, electrophoretic and mass spectrometric techniques, and in general by a combination of these techniques. The choice of the method can be grouped according to the nature of the glycans and level of information required.

Analysis of glycans provides information on the various populations of glycans present on the protein (high-mannose, hybrid, complex). Information on the relative amounts of branched structures might be obtained by analysis of desialylated glycans.

A separation step may be required. It implies the use of LC (*2.2.29*) and CE (*2.2.47*) as intermediate techniques. LC (*2.2.29*) can be used preparatively with individual fractions being collected (usually labelling is required) or can be directly coupled to MS (*2.2.43*).

2-3-2-1. *Analysis of unlabelled glycans*

Native glycans can be analysed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), porous graphite chromatography (PGC) and MS (*2.2.43*).

HPAEC-PAD has high sensitivity and can also separate some linkage isomers. Response factors of the different signals are not equal for the different oligosaccharide structures. Absolute quantification of the glycan is not possible unless an oligosaccharide reference library is available. Quantification can be obtained by comparison with a well-characterised reference standard of the substance being tested, or by relating the peak area of each glycan to the total peak area of all glycans in the map.

PGC can also be used to separate native glycans because of its higher selectivity compared to the conventional non-polar columns. A PGC-electrospray-ionisation-MS approach can be applied for direct glycan analysis.

2-3-2-2. *Analysis of labelled glycans*

*Labelling of glycans*

The type of derivatisation carried out will depend on the method used to detect glycans: UV or fluorescent.

Derivatisation with fluorescent labels is the most commonly used technique for labelling glycans at their reducing end by reductive amination. One label can be attached to every single mono- and oligo-saccharide, allowing the determination of molar quantities. Table 2.2.59.-2 gives a non-exhaustive list of commonly used fluorescent labels and suitable analytical techniques.

Table 2.2.59.-2. – *Examples of fluorescent labels and suitable techniques*

| Name | Acronym | Analytical techniques |
|---|---|---|
| 2-Aminobenzoic acid | 2-AA | LC (*2.2.29*), MS (*2.2.43*) |
| 2-Aminobenzamide | 2-AB | LC (*2.2.29*), MS (*2.2.43*) |
| 2-Aminopyridine | 2-AP | LC (*2.2.29*), MS (*2.2.43*) |
| 2-Amino-9(10*H*)-acridinone | AMAC | Gel electrophoresis (*2.2.23*) |
| Trisodium 8-aminopyrene-1,3,6-trisulfonic acid | APTS | CE (*2.2.47*) |

Permethylation of glycans may also be used when MS (*2.2.43*) is used alone for detection. It is based on the methylation of the oligosaccharides.

*Analysis of labelled glycans*

Labelled glycans can be analysed by analytical techniques such as LC (*2.2.29*), CE (*2.2.47*) and MS (*2.2.43*).

According to the separation properties of the glycans, glycans can be profiled and quantified by several LC (*2.2.29*) systems using an appropriate label: reversed-phase (separation by hydrophobicity), normal-phase (separation by size), and anion-exchange (separation by charge) LC.

2-4. *MONOSACCHARIDE ANALYSIS*

Monosaccharide analysis provides information on the monosaccharide composition of a glycoprotein. Analysis of monosaccharides can be performed using either colorimetric or separation methods.

2-4-1. **Colorimetric methods**

The colorimetric methods, which are based on chemical staining, provide information on the quantity of specific classes of sugars such as sialic acids, neutral sugars and hexosamines.

2-4-2. **Separation methods**

The separation methods generate quantitative information on the overall monosaccharide composition. The methods require acid hydrolysis pre-treatment of the oligosaccharide chains of the intact glycoprotein or released glycans, prior to analysis. To release sialic acids, mild acid hydrolysis or enzymatic treatment is employed. The hydrolysis step is a significant source of variability and may require product-specific validation.

Methods for separation and quantification of monosaccharides include:

- the use of HPAEC-PAD and PGC-MS, which allow the determination of molar quantities of native monosaccharides (sialic acids, neutral sugars and alcohol sugars);
- fluorophore labelling of monosaccharides followed by separation methods such as reversed-phase or ion-exchange chromatography, or CE.

3. EVALUATION AND ANALYSIS OF DATA

Data obtained from analytical methods for the analysis of glycans can be analysed and evaluated for 3 different purposes:

- confirmation of identity of individual structures or families of structures;
- confirmation of compliance of the substance being tested with qualitative requirements;
- confirmation of compliance of the substance being tested with quantitative requirements.

Specific considerations with respect to reference standards and method development of each level of analysis are set out in sections 4 and 5 respectively.

3-1. *CONFIRMATION OF IDENTITY OF INDIVIDUAL STRUCTURES OR FAMILIES OF STRUCTURES*

The analytical target for a glycan analysis method may be an individual monosaccharide (e.g. sialic acid, fucose), a defined oligosaccharide structure (e.g. tetra-sialylated, tetra-antennary glycan) or a family of structures sharing a common analytical feature (e.g. tetra-sialylated glycans, tri-antennary glycans, glycoprotein isoforms with the same charge). Confirmation of the identity of the analytical target is an essential step in the analysis and evaluation of data, and can be achieved absolutely, by verification of molecular structure, or comparatively, by comparison with an appropriate reference standard.

3-1-1. **Absolute confirmation of identity**

Absolute confirmation of the identity of glycan structures is typically achieved during product development, and should not necessarily be the target of routine analysis. Identity of the analytical target will be assigned by reference to a known molecular property of the molecule. Such absolute identification of individual structures can require multi-step approaches using enzymatic and chemical reactions, separation techniques and online or offline detection methods, and will most commonly use the charge-to-mass ratio of a molecular ion, determined using a suitable mass spectrometric method as the final basis for structure assignment.

3-1-2. **Comparative confirmation of identity**

During routine application of the analytical method, the identity of the analytical target may be confirmed by comparison with process or system suitability reference standards. These may be generated from known, well-characterised glycoproteins, which may be of the same general class as the product being tested (e.g. fetuin for complex *N*-linked glycoproteins), or may be derived from a well-characterised batch of the product being tested, which has been established as a reference standard. The following considerations apply to comparative assignment of structural identity:

- in the case of a validated high reproducibility of the retention times, the absolute retention times can be used for correct assignment;
- alternatively, a glycan marker can be injected at the beginning and at the end of the testing sequence and checked for any drifts in the retention times; based on these reference chromatograms the glycans of the test samples can be assigned;
- in cases where no standard is available to assign all glycan peaks in the test sample, absolute or normalised retention times can be used to monitor and label unidentified glycan peaks.

3-2. *CONFIRMATION OF COMPLIANCE OF THE SUBSTANCE BEING TESTED WITH QUALITATIVE REQUIREMENTS*

At this level of evaluation, the analytical results obtained with the product being tested are evaluated to demonstrate compliance with specifications. Typically this is achieved by comparison with data obtained in parallel using a reference standard of the substance being tested. In evaluating the data it is necessary:

- to establish that the analytical result obtained using the reference standard is broadly comparable to the expected result, to verify the suitability of the system; for example, in a glycan mapping procedure, this would be achieved by comparison of the map obtained with the reference substance with a provided specimen map obtained during establishment of the reference substance, and by ensuring compliance with all stated system suitability criteria;
- to demonstrate similarity of the maps obtained with the reference substance and the test substance, using any specific compliance criteria given in the specific monograph.

3-3. *CONFIRMATION OF COMPLIANCE OF THE SUBSTANCE BEING TESTED WITH QUANTITATIVE REQUIREMENTS*

3-3-1. **Quantitative measurement of analyte levels and expression of results**

In some cases, e.g. measurement of sialic acid or other monosaccharides, data can be expressed in order to obtain a molar ratio of sialic acid to glycoprotein. Data is calculated by reference to a reference standard for sialic acid and to a validated method of protein determination. Either the internal or external standard method may be used (see general chapter *2.2.46. Chromatographic separation techniques*).

3-3-2. **Quantitative expressions of separation profile**

Profiles or distribution patterns may be expressed numerically in a number of ways, including the normalisation procedure; the percentage content of each analytical target, e.g. glycan entity, is calculated by determining the response of the glycan entity as a percentage of the total response of all the entities, excluding those due to solvents or any added reagents, and those below the disregard limit. In addition, numerical expressions such as the *Z* number, which are method- and product-specific and defined in specific monographs, can be used.

4. REFERENCE STANDARDS

Reference standards for glycan analysis serve 2 functions: the verification of the suitability of the system and the confirmation that the article under test complies with specified requirements.

The reference standards used for system suitability may be:

- a reference substance for the substance being tested;
- glycan moities liberated from a fully characterised reference standard of the substance being tested;
- well-characterised glycan moities liberated from glycoproteins (e.g. fetuin, IgG);
- glycan markers characterised for identity and purity.

The reference standard used for compliance of the glycoprotein under test is a preparation of the substances being tested. It is noted that glycan analysis procedures described in specific monographs prescribe the use of a reference standard for the substance being tested and for which the glycan analysis procedure has been validated.

5. POINTS TO CONSIDER IN METHOD DEVELOPMENT

This section provides means for measuring the overall performance of the method during development. The extent of method development and analytical validation is selected on the basis of their suitability for a specific product. Depending on the chosen approach, several steps are necessary for glycan analysis, for example:

- isolation and purification (or desalting) of the glycoprotein;
- enzymatic (or chemical) treatment of the glycoprotein to selectively release either *N*- or *O*-linked glycans from the protein backbone;
- isolation and purification of the released glycans;
- verification of released sialic acid and monosaccharide residues;
- chromophore labelling of the released glycans;
- separation of the glycans, native or fluorescence labelled;
- glycan identification and quantification (e.g. determination of the *Z* number);

– determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides.

**Protein isolation and purification**. Isolation and purification of the glycoprotein from its matrix may be necessary to remove all interfering substances (e.g. excipients, salts) and, when required, will be specified in the specific monograph. This must be performed in a reproducible manner in order to guarantee a quantitative recovery of the protein.

**Release and isolation of oligosaccharides**. The approach chosen for the release of glycans will depend on the protein under test and will be based on the types of glycosylation, i.e. *N*- or *O*-linked glycosylation. Non-compendial approaches available for the release of glycans must be optimised in order to ascertain a quantitative profiling of all glycan entities. Factors that impact cleavage efficiency, such as enzyme-to-protein concentration ratio, temperature, reaction time course, and denaturation of protein prior to digestion, must be optimised.

It is noteworthy that the enzymatic/chemical reaction must not alter the glycan composition, e.g. not destroy sialic acid residues. Where there is more than one glycosylation site, the enzymatic treatment should proportionally release all oligosaccharide moieties attached to the protein, independent of their structure and their individual position in the protein. Reproducible recovery of all glycan entities from the reaction mixture must be confirmed.

**Derivatisation of released glycans**. Derivatisation is usually carried out according to non-compendial protocols. Therefore, the reproducible derivatisation of all glycan entities must be verified. This may be achieved through optimisation of the reaction conditions such as amount of the derivatisation reagent, reaction temperature and time. The derivatisation reaction must not change the glycan composition, e.g. not destroy sialic acid residues.

**Separation, identification and system suitability**. The methods employed for glycan analysis must be capable of detecting and separating different glycan moieties to ascertain a reliable identification and quantification.

The acceptance criteria for system suitability, which also cover glycan cleavage, recovery and analysis, depend on the critical test parameters that affect the outcome of the result.

A comparison between the glycan map of the substance under test and that of a reference substance, being treated in the same conditions, is an indicator to evaluate the performance of the analytical procedure. In order to further confirm the obtained results, the analyses may be repeated with an orthogonal method. The use of a reference standard (e.g. reference substance of the product being examined, system suitability glycan marker) is essential in the establishment of system suitability parameters and validation of the analytical procedure.

Reproducibility of quantitative expression (e.g. *Z* number estimation) of glycan profiles must be verified.

**Determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides**. Where site occupancy is estimated by comparison of glycosylated and non-glycosylated peptides from an enzymatically digested glycoprotein, reproducible cleavage of both forms of the peptide must be demonstrated.

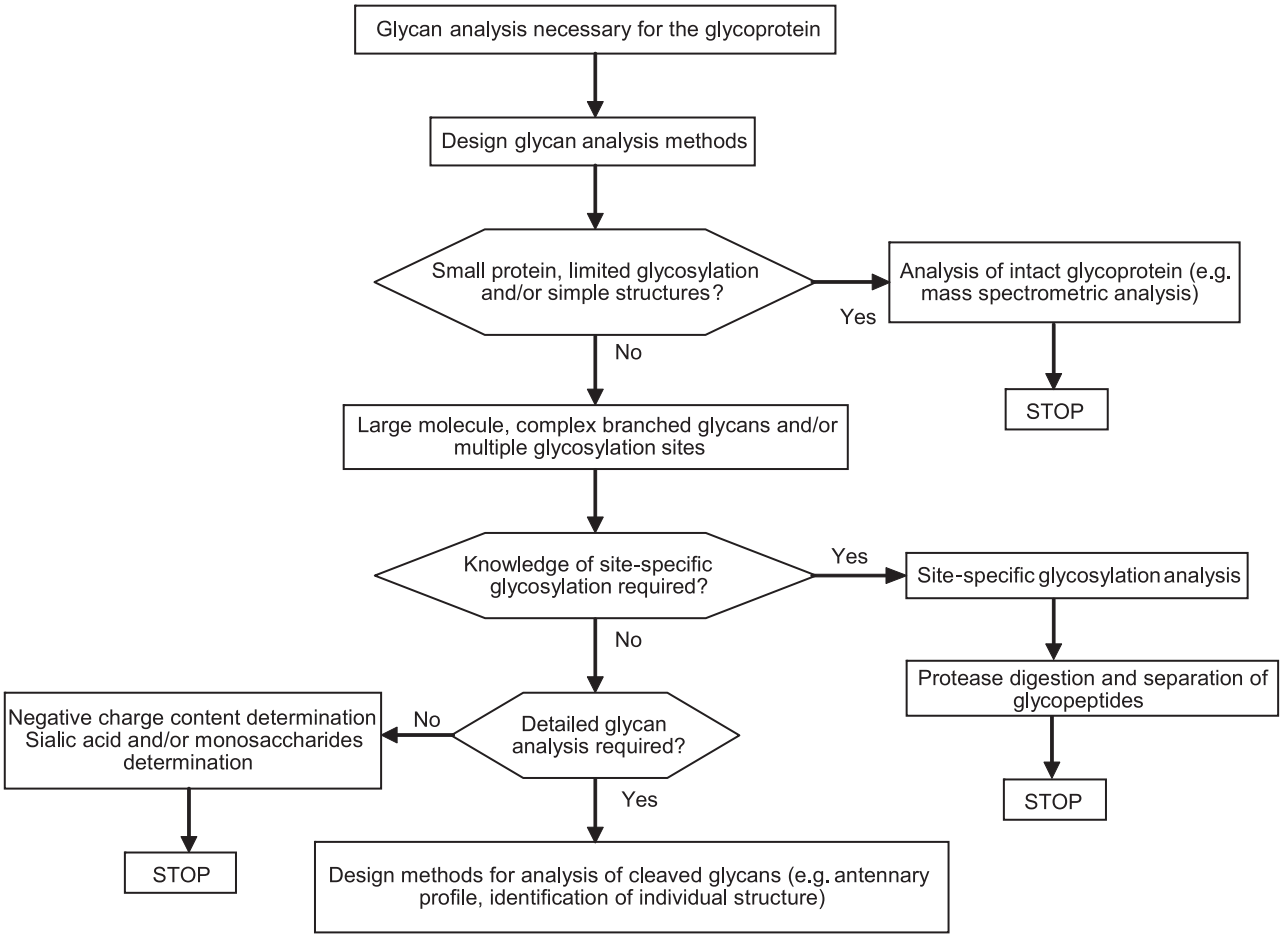


Figure 2.2.59.-2. – *Guidance on methods to be used when glycan analysis is required*

6. GLYCAN ANALYSIS DECISION-MAKING FRAMEWORK

This decision-making framework is given for information and does not constitute a mandatory part of the European Pharmacopoeia.

The choice of procedures used to analyse glycans is established according to the level of information required to ensure the quality of the glycoprotein and is set up during the development phase of the product.

Figure 2.2.59.-2 provides guidance in the choice of methods to be used when glycan analysis is required.

**01/2013:20261**

# 2.2.61. CHARACTERISATION OF CRYSTALLINE SOLIDS BY MICROCALORIMETRY AND SOLUTION CALORIMETRY

*For the purpose of this chapter, crystalline material, partially crystalline material and amorphous material are considered as solids.*

INTRODUCTION - THE CONCEPT OF CRYSTALLINITY

The perfectly ordered crystal lattice with every molecule in its expected lattice position is an ideal that is seldom, if ever, achieved. The other extreme is the amorphous state, in which a solid contains the maximum possible density of imperfections (defects of various dimensionalities), such that all long-range order is lost while only the short-range order, imposed by its nearest neighbours, remains. Real crystals lie somewhere between these 2 extremes. A crystal's position on a scale bounded by these 2 extremes is termed *crystallinity*.

All real crystals, even in the pure state, possess some lattice imperfections or defects, which increase both the energy (enthalpy under conditions of constant atmospheric pressure) and the disorder (expressed as the entropy) of the crystal lattice. A crystal with a relatively low density of imperfections is said to be highly crystalline and to possess a high crystallinity. By contrast, a particle with a relatively high density of imperfections is said to be partially amorphous and to possess a low crystallinity. In ideal terms, a totally amorphous particle corresponds to zero crystallinity. Amorphous particles may contain somewhat ordered domains that can act as nuclei for crystallisation; such so-called amorphous particles are said to possess a low-level but finite crystallinity.

The ability to detect and to quantify the amount of amorphous material within a highly crystalline substance is of great importance during the development and subsequent manufacture of a pharmaceutical preparation.

In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with varying sizes and shapes. The lower the crystallinity of a solid, the greater its enthalpy and entropy. The increase in enthalpy is never totally compensated for by the increase in entropy; therefore, the Gibbs free energy, which reflects the balance between them, actually increases. Hence, the lower the crystallinity of a material (powder), and consequently the greater its amorphous character, the greater its apparent intrinsic solubility and dissolution rate, but the lower its thermodynamic stability. Because of the great relevance of these properties, crystallinity is also an important property and requires measurement by a suitable method.

In the following chapter, the crystallinity or the content of amorphous parts of a powder are measured by calorimetric methods such as microcalorimetry or solution calorimetry, although other methods could be used (e.g. see general chapter *2.9.33. Characterisation of crystalline and partially crystalline solids by X-ray powder diffraction (XRPD)*).

Many substances are capable of crystallising in more than one type of crystal lattice, which is known as polymorphism. If water or a solvent is incorporated in the crystal lattice the crystals are termed hydrates or solvates. Because of the different crystal packing, and/or molecular conformation and lattice energy, they usually exhibit different physical properties. For simplicity, calorimetry measurements for degree of crystallinity determination discussed here assume only one solid crystalline form present in the material of interest. The theory and experimental technique can be easily expanded to polymorphic systems with proper consideration of the enthalpy differences among the polymorphs.

METHOD 1 - MICROCALORIMETRY (DETERMINATION OF AMORPHOUS CONTENT)

Most chemical, physical and biological processes are associated with the exchange of heat. Microcalorimetry is a highly sensitive technique to monitor and quantify both exothermic (heat producing) and endothermic (heat absorbing) changes associated with those processes. The technique allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure.

Thermal events producing only a fraction of a microwatt can be observed using microcalorimetry. This means that temperature differences less than $10^{-6}$ K must be detectable. Microcalorimetry typically uses the heat flow (heat leakage) principle, where the heat produced (or absorbed) in a thermally defined vessel flows away (or into) in an effort to re-establish thermal equilibrium with its surroundings. Exceptional thermal stability with its surrounding has to be achieved either by a heat sink or an electronically regulated surrounding.

Heat energy from an active sample in the reaction vessel is channelled typically through Peltier elements; they act as thermoelectric generators using the Seebeck effect. The heat energy is converted into a voltage signal proportional to the heat flow.

Results are typically presented as a measure of the thermal energy produced per unit of time (Watt) as a function of time.

*APPARATUS*

Microcalorimeters are typically designed as twin systems with a measuring vessel and a reference vessel. Vessels are typically made of glass or stainless steel. For certain applications specially designed vessels which allow the addition of a gas, a liquid or a solid material may be used.

*CALIBRATION*

The microcalorimeter is calibrated for heat flow (energy per time unit) using either calibrated external or internal electrical heat sources or a suitable standard reaction.

*SENSITIVITY*

The sensitivity of the microcalorimetric method can be assessed based on an appropriate standard sample analysed according to the corresponding method in conjunction with the determination of the instrument baseline noise.

*PROCEDURE*

Weigh in a suitable vessel an appropriate quantity of the substance to be examined. Close the vessel carefully to avoid any evaporation of solvents and place the vessel in the sample holder. If appropriate, allow the vessel to equilibrate at the temperature of the measurement before placing it in the measuring position.

Begin the analysis and record the heat flow, with the time on the abscissa and the heat flow on the ordinate (specify the direction of exothermic or endothermic heat flow).

*DETECTION AND QUANTIFICATION OF AMORPHOUS CONTENT IN POWDERS*

The amorphous state is metastable with respect to the crystalline state; recrystallisation may therefore occur. The measurement of the heat of recrystallisation enables the amorphous content to be determined by the area of the recrystallisation peak. By relating the output from the microcalorimeter for a sample to that obtained from an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1 per cent can be reached.

Recrystallisation can be initiated by subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The sample is typically placed in an ampoule which also contains a small test-tube containing an aqueous saturated salt solution, an organic solvent, or a solvent mixture.

The heat of recrystallisation is typically measured using a fixed sample mass placed in a glass or steel vessel. The test-tube containing a saturated salt solution or an organic solvent is chosen to be large enough to allow a full saturation of the atmosphere above the sample. The mass of the sample and the nature of the vapour atmosphere above the sample are chosen so that recrystallisation occurs in such a way that a distinct peak is observed, clearly separated from initial thermal events caused by introduction of the sample.

The conditions under which the transition of the amorphous phase to a thermodynamically more stable crystalline state occurs will have a significant impact on the time of recrystallisation. In particular, physical mixtures of purely amorphous and crystalline material will behave differently from a partially crystalline material. These effects should be considered when developing a method.

A typical response for the recrystallisation of a mainly amorphous material is shown in Figure 2.2.61.-1. The first part of the curve represents several concurrent processes taking place simultaneously, such as the absorption of water vapour into the amorphous parts of the powder and the generation of water vapour from the test-tube. After this initial response there is a large exothermic response caused by the recrystallisation of the amorphous material. Also included, but not seen, are the expulsion of excess water from the recrystallised parts and its condensation. Thus, the area under this exothermic recrystallisation response is proportional to the heat of recrystallisation.

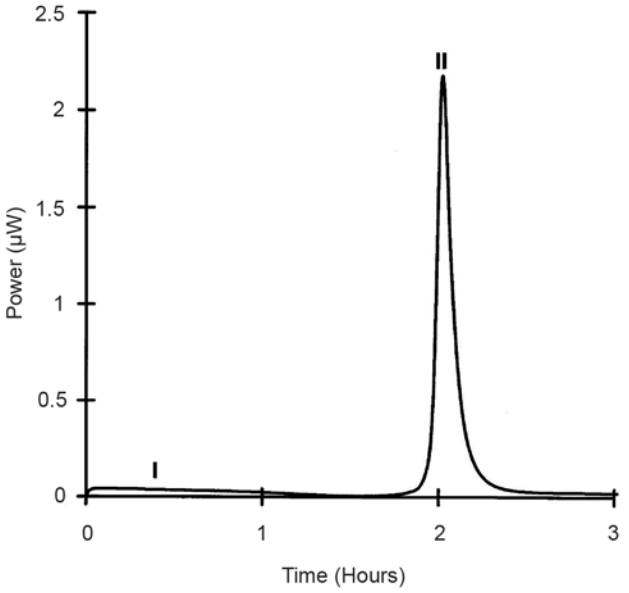


Figure 2.2.61.-1. – *Typical microcalorimetric output of power (in µW) as a function of time (in hours): amorphous collapse peak (I) and crystallisation peak (II) for mainly amorphous lactose at 25 °C and 75 per cent relative humidity*

METHOD 2 - SOLUTION CALORIMETRY (DETERMINATION OF CRYSTALLINITY)

Solution calorimetry provides a means of determining enthalpy of solution (i.e. heat of solution under constant atmospheric pressure) of a substance. Enthalpy of solution is defined as the enthalpy of the substance dissolved in the solution to a defined concentration minus the enthalpy of the original substance. The solvent for the dissolution process must be such that the mass of solid dissolves within a time frame that matches the response time of the calorimeter, as discussed below. The enthalpy of solution is proportional to the amount of solid being dissolved. This amount may be defined as 1 mol for molar enthalpy or as 1 g for specific enthalpy. If the substance possesses adequate purity (as determined by the degree of accuracy required) and if its molecular mass is known, the molar enthalpy is preferred, otherwise the specific enthalpy must be used. The enthalpy of solution is weakly dependent on both the temperature, which is usually 25.0 °C, and the final concentration of the dissolved solute.

It is usually preferred to express the crystallinity, $P_c$, of a substance on a percentage scale. This procedure requires 2 reference standards, namely a highly crystalline sample assuming 100 per cent crystallinity and having a measured enthalpy of solution of $\Delta H_c^s$, and an amorphous sample assuming 0 per cent crystallinity and having a measured enthalpy of solution of $\Delta H_a^s$. From these values and from the measured enthalpy of solution, $\Delta H_s^s$, of the solid under study, the percentage crystallinity of the solid, $P_c$, may be calculated as follows:

$$P_c(\%) = 100(\Delta H_s^s - \Delta H_a^s) / (\Delta H_c^s - \Delta H_a^s)$$

Clearly, crystallinity expressed on a percentage scale depends on 3 measured values and the enthalpies of solution may be replaced by other corresponding physical quantities that depend on crystallinity. The value of the percentage crystallinity of a sample, however, depends not only on the nature and method of preparation of the 2 reference standards, but also on the choice of the physical quantity that is measured.

The enthalpy of solution is measured either by an isoperibol (constant perimeter, i.e. jacket) solution calorimeter or by an isothermal (constant temperature) solution calorimeter. Typically, at least 3 measurements are made with each sample.

The mean of these values is then calculated. The exact requirements will depend upon the equipment capability and degree of accuracy needed.

*ISOPERIBOL SOLUTION CALORIMETRY*

In the isoperibol solution calorimeter, the heat change during the solution process causes a corresponding change in temperature of the solvent-solute system (i.e. solution). This temperature change is measured by a temperature sensor, which is wired to an electrical circuit that records an electrical signal corresponding to the temperature change. Typically, this temperature change in an electronic form is measured at precisely defined time intervals to produce temperature-time data that are collected, analysed by a computer, and then plotted. A blank run without addition of the solid solute to the solvent normally shows no discernible change in the slope of the temperature-time plot.

For isoperibol solution calorimeters, response is fairly rapid, but corrections must be made for any heat losses to or heat gains from the bath. Therefore, isoperibol solution calorimeters are more advantageous than isothermal solution calorimeters when the solution process is relatively fast. For all measurements of enthalpy of solution using isoperibol solution calorimeters, the choice of solvent is critical. The nature and mass of the solvent and the mass of sample allow the total heat change, corresponding to total dissolution of the solid, to proceed to completion within 5 min under vigorous stirring at a constant rotational speed within the range of 400-600 r/min.

The effective heat capacity of the calorimeter cell and its contents is determined for every calorimeter run. This determination is accomplished by electrical heating of the contents of the calorimeter cell. The effective heat capacity is determined according to 1 of 2 protocols: either by making 1 determination after ampoule breakage or by making 1 determination before and a 2nd determination after ampoule breakage and then averaging the 2 results. The accuracy and reliability of the electrical heating are established by the accuracy and reliability of the aforementioned chemical calibrations.

*ISOTHERMAL SOLUTION CALORIMETRY*

In the isothermal (constant temperature) solution calorimeter, the heat change during the solution process is compensated for by an equal but opposite energy change, such that the temperature of the solvent-solute system (i.e. solution) remains essentially constant. This equal but opposite energy change is measured and, when its sign is reversed, provides the enthalpy of solution. For isothermal calorimeters, response is relatively slow, but the compensation process eliminates the effects of heat losses to or heat gains from the bath. Therefore, isothermal solution calorimeters are more advantageous than isoperibol solution calorimeters when the solution process is relatively slow.

*SOLUTION CALORIMETER CALIBRATION*

To ensure the accuracy of the calorimeter, chemical calibrations must be performed on a regular basis. For an endothermic solution process, the calibration of the calorimeter is checked by measuring the heat absorbed during the dissolution of potassium chloride in distilled water at 298.15 K (25.0 °C). The established enthalpy change in this endothermic process is 235.5 J/g (17.56 kJ/mol). For an exothermic solution process, the calorimeter is checked by measuring the heat evolved during the dissolution of 5 g per litre of tromethamine [tris(hydroxymethyl)aminomethane, THAM] in a 0.1 mol/L aqueous hydrochloric acid solution at 298.15 K (25.0 °C). The established heat for the aforementioned process is – 246.0 J/g (– 29.80 kJ/mol).

*SAMPLE HANDLING*

The chemical and physical stability of solids may decrease with decreasing crystallinity. In particular, solids of low crystallinity, especially amorphous solids, tend to sorb water vapour from the atmosphere, leading to crystallisation and a corresponding gain in crystallinity. For these reasons, anhydrous samples whose crystallinity is to be determined must be stored at zero humidity or below critical humidity levels in sealed chambers containing a desiccant, preferably containing an indicator of effectiveness. If crystallinity-humidity studies are to be carried out, the sample is stored in a sealed chamber containing a saturated salt solution to provide a defined relative humidity.

**04/2019:20263**

# 2.2.63. DIRECT AMPEROMETRIC AND PULSED ELECTROCHEMICAL DETECTION

Direct amperometric detection and pulsed electrochemical detection (PED) are used to detect electroactive compounds by oxidation (or reduction) when coupled to a separation technique such as liquid chromatography (*2.2.29*). Electrochemical detection uses the redox potential of analytes and is therefore only valid for electroactive substances.

PRINCIPLE

When a constant potential is applied between 2 electrodes, the oxidation (or reduction) of an electroactive substance generates a current proportional to the amount of analyte passing the electrode surface. This analytical technique requires a supporting electrolyte, which can be added post-column if not already present in the mobile phase. Detection usually involves oxidation of the compounds of interest. The potential applied to the measuring electrode can serve as a selectivity parameter for the compounds of interest. The upper limit of the electroactivity range is defined by the oxidation curve of the mobile phase, the supporting electrolyte or the measuring electrode.

With direct amperometric detection, only a constant potential is applied. However, this method may result in the surface of the measuring electrode becoming fouled with adsorbed carbon compounds, resulting in signal variability and a decreased response. This passivation phenomenon is particularly common with sugars, thiols and phenols. The problem of electrode fouling can be resolved by the application of a series of pulses, a process known by the generic term of pulsed electrochemical detection (PED).

The most widely used PED mode is pulsed amperometric detection (PAD), which involves a 3-step series of pulses or waveform (Figure 2.2.63.-1). After applying the detection potential ($E_{det}$), a high positive potential ($E_{oxd}$) (anodic polarisation) is applied to clean the surface of the measuring electrode, followed by a negative potential ($E_{red}$) (cathodic polarisation) to restore the surface of the measuring electrode. A new cycle of the waveform can then start. Following the potential step from $E_{red}$ to $E_{det}$, background signals will originate. Although these decay quickly, a brief delay must be introduced before the analyte signal can be properly measured.

Other waveforms comprising more than 3 voltage steps have been developed.

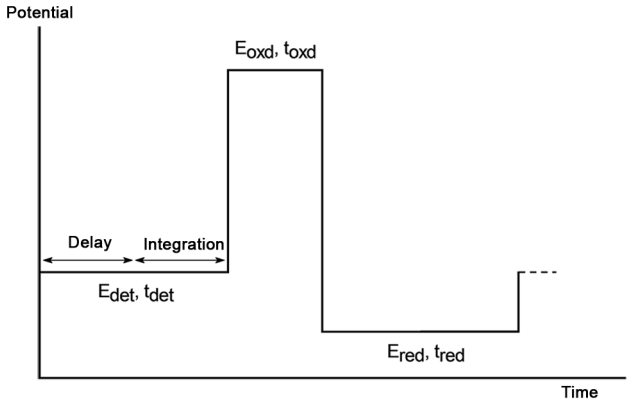


$E_{det}$: detection potential applied during a period $t_{det}$

$E_{oxd}$: oxidation potential applied during a period $t_{oxd}$

$E_{red}$: reduction potential applied during a period $t_{red}$

Figure 2.2.63.-1. – *3-Step potential-time waveform (pulse sequence)*

EQUIPMENT

An electrochemical detection system comprises 3 electrodes: a measuring electrode (gold, platinum or glassy carbon); a reference electrode (usually silver-silver chloride); and an auxiliary electrode (e.g. titanium cell body) (Figure 2.2.63.-2).

The choice of measuring electrode depends on the substance to be examined. The electrodes are coupled to a potentiostat, which controls the potentials applied to the measuring electrode without subjecting the reference electrode to a current, the auxiliary electrode functioning as a cathode whenever the measuring electrode is operating as an anode and vice versa.

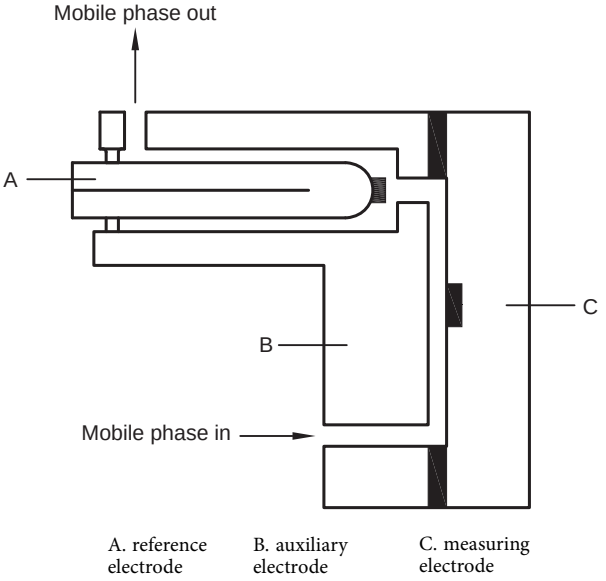


Figure 2.2.63.-2. – *Electrochemical detection system*

*EQUIPMENT PERFORMANCE*

The potentials to be applied depend on the analyte and the type of electrodes used, and the settings must therefore be adapted accordingly.

All electrodes should be in good condition and periodic replacement is recommended. When the detector is used for quantitative analysis, it is advisable to check the linear range, sensitivity and repeatability of the detector.

The quality of the reagents used is of utmost importance. It is advisable to employ reagents of the highest purity.

*ADDITIONAL INFORMATION*

*Maintenance of the electrochemical cell*

Although the application of an increased potential in PED cleans the surface of the measuring electrode effectively, it can be necessary to polish the measuring electrode mechanically from time to time when the background current increases and the sensitivity decreases. The cleaning of the measuring electrode must be carried out very carefully to avoid creating pits or scratches. It is also advisable to wipe the auxiliary electrode at the same time to remove deposited substances. The cell requires some time to stabilise after polishing.

*Sodium hydroxide solution*

Detection can be enhanced in alkaline media (at least pH 12), as is the case with aminoglycoside antibiotics. When the mobile phase is not sufficiently alkaline, this can be achieved by post-column addition of a sodium hydroxide solution to increase the pH of the column effluent. The solutions are mixed in a coil that is linked to the electrochemical cell. It is essential that the length of the mixing coil is such as to produce a homogeneous solution but with minimal peak broadening. It is important to avoid carbonates in the sodium hydroxide solution. To avoid baseline disturbances, the sodium hydroxide solution should be degassed before use, it must be added pulse-free and its flow should be constant between runs. The parts of the chromatographic system coming into contact with the sodium hydroxide-containing solutions must be alkaline resistant.

**07/2011:20264**

# 2.2.64. PEPTIDE IDENTIFICATION BY NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

This general chapter is to be used in conjunction with general chapter *2.2.33. Nuclear magnetic resonance spectrometry* in the context of peptide identification. The approach to be followed is qualitative and consists of comparing the nuclear magnetic resonance (NMR) spectrum of a test sample with that of a reference sample acquired under identical conditions.

This general chapter mainly applies to the use of proton NMR ($^1$H NMR) spectrometry, to confirm the identity of small peptide products (up to approximately 15 amino acids). It is also applicable when using $^{13}$C NMR spectrometry with some modifications. The scope is restricted to the use of one-dimensional NMR spectrometry.

GENERAL PRINCIPLES

**Equipment**. Unless otherwise specified, an apparatus with a field strength giving an operating frequency for proton NMR of at least 300 MHz.

**Spectral acquisition conditions and their optimisation**. After introduction into the magnet, the sample is allowed to come to thermal equilibrium, especially if analysis is carried out at a temperature significantly different from room temperature: monitoring the lock signal is often a valuable visual guide to the progress of this process.

The spectral width must encompass the complete spectrum of the peptide, with an empty spectral region at each side. Typically, a spectral width of 12 ppm or 16 ppm is appropriate.

The following parameters may be optimised to improve resolution of characteristic peaks: temperature and/or pH primarily, buffer and peptide concentrations. Control of sample temperature is recommended but is not mandatory; if not used, the effect of small temperature changes on the appearance of the spectrum is validated.

The number of data points collected is such as to define peaks adequately.

Solvent suppression is not recommended but, if used, the intensities of peaks close to the solvent resonance may be affected and this has to be validated when comparing spectra.

**Chemical shift referencing**. For samples in aqueous solution, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), sodium 3-(trimethylsilyl)propionate (TSP) or a deuterated analogue (TSP-$d_4$) are appropriate, and the chemical shift of the methyl signals is often set to 0 ppm. Either the reference material is added at low amounts (10-100 ppm has been found to be appropriate) to the deuterated water used to dissolve the final sample, or an easily recognised internal resonance that is consistently present (such as acetate anion) can be used as a secondary reference. In this case, a validation spectrum obtained under the same spectral conditions is used to define the chemical shift of the secondary standard.

**Sample size**. Usually a few milligrams are used. If sample sizes are variable, the effects of this variation on the appearance of the spectrum are validated.

**Sample preparation**. The test and reference samples must be comparable in terms of concentration, pH and buffer composition. Typically, samples in solution are lyophilised, and the dried samples dissolved in deuterated water or a buffer in deuterated water. It may be worthwhile to lyophilise a solution in deuterated water one or more times ('deuterium exchange') as this reduces the intensity of strong solvent signals; volatile process impurities such as ethanol will also be lost. Use of buffer for the final sample preparation can reduce aggregation and improve spectral reproducibility by reducing batch-to-batch pH variation. Some probes are intolerant to high salt concentrations, but ionic strengths up to 200 mM sodium chloride are normally tolerated. High salt concentrations tend to increase 90° pulse length.

VERIFICATION OF IDENTITY

**Determination of key spectral factors**. Use of a qualitative approach does not entail stringent requirements on spectral parameters (for example, fast pulse repetition rates can be used, as full relaxation is not required). The use of short pulse widths (for example, a 30° pulse) and fast repetition rates will have no significantly deleterious effect on spectra, and will allow faster acquisition of acceptable signal-to-noise ratios. Variation in the pulse width and acquisition time within wide limits will not affect the ability to compare spectra. The number of scans collected must give appropriate signal-to-noise ratios for low intensity resonances and therefore a minimum signal-to-noise ratio of 50:1 is recommended.

**Identification of characteristic resonances**. It is possible to compare either the complete spectrum or a portion of it. Comparison of spectra of relevant samples will highlight regions of the spectrum that are distinctive, and comparison can be constrained to these regions. It is important to define resonances from impurities, such as residual solvents, which may be essentially irrelevant to product quality and which may vary in intensity between batches.

**Spectral comparison**. See the provisions of general chapter *2.2.33*.

**01/2013:20265**

## 2.2.65. VOLTAMETRIC TITRATION

In voltametric titration the end-point of the titration is determined by following the variation of the voltage measured between 2 electrodes (either 1 indicator electrode and 1 reference electrode or 2 indicator electrodes) immersed in the solution to be examined and maintained at a constant current as a function of the quantity of titrant added.

*Apparatus*. The apparatus comprises an adjustable current source and a voltmeter; the detection system generally consists of an indicator electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon electrode) and a 2nd electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon electrode).

*Method*. Set the current to the indicator electrode as prescribed in the monograph and plot a graph of the initial voltage and the values obtained during the titration as functions of the quantity of titrant added. Add the titrant in not fewer than 3 successive quantities equal to a total of about 80 per cent of the theoretical volume corresponding to the presumed equivalence point. The 3 values must fall on a straight line. Continue adding the titrant beyond the presumed equivalence point in not fewer than 3 successive quantities. The values obtained must fall on another straight line. The point of intersection of the 2 lines represents the end-point of the titration.

Using titration systems for voltametric titration with 2 indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

**01/2014:20266**

## 2.2.66. DETECTION AND MEASUREMENT OF RADIOACTIVITY

INTRODUCTION

Within the context of the European Pharmacopoeia, the term 'radioactivity' is used both to describe the phenomenon of radioactive decay and to express the physical quantity of this phenomenon. In the monographs on radiopharmaceutical preparations, the detection and measurement of radioactivity are performed for different purposes: verification of the characters, identification, determination of radionuclidic and radiochemical purity, as well as determination of the radioactivity in a substance (assay).

Under these assumptions, the measurement can be qualitative, quantitative or both, depending whether it is directed to the identification of the radionuclide or the determination of its activity (rate of decay) or both of them.

Radioactive sources can produce various types of emissions, such as alpha particles, electrons, positrons, gamma- and X-rays, according to the radionuclidic composition.

Each radionuclide yields characteristic emissions, with specific energies and relative intensities. Such radiations can be detected as a result of their ionising properties in an ionisation chamber but without further characterisation; when they are detected and analysed using a spectrometer, an energy spectrum is obtained. A detailed spectrum analysis is typically used to identify radionuclides present in a sample. Spectrometry can also be used for quantitative determination of the radioactivity in sources made of a single radionuclide or radionuclide mixtures or of the individual radionuclides present.

A measurement of radioactivity is generally performed by counting the number of detected decay events (emissions). Therefore, the geometry of the sample during the measurement of radioactivity and the acquisition time strongly influence the result. In general, the measurement geometry must correspond to a calibrated geometry and the acquisition time must be long enough to reach sufficient counting statistics.

A measurement of radioactivity can be done in a stand-alone mode (e.g. using an ionisation chamber or a spectrometer) or in combination with a separation technique (e.g. radiochromatography) to account for relative contributions from different radioactive chemical species that may be present in a mixture.

## MEASUREMENT OF RADIOACTIVITY

A direct determination of the radioactivity of a given sample, in becquerel (Bq), may be carried out if the decay scheme of the radionuclide is known, but in practice many corrections are required to obtain accurate results. For this reason, it is possible to carry out the measurement with the aid of a primary standard source or by using measuring instruments such as an ionisation chamber or a spectrometer calibrated using suitable standards for the particular radionuclides.

A spectrometer is used when measuring the radioactivity of radionuclides in a mixture, each radionuclide being identified by its emissions and their characteristic energies.

All measurements of radioactivity must be corrected for dead-time losses and by subtracting the background signal due to radiation in the environment and to spurious signals generated in the equipment itself.

The radioactivity of a preparation is stated at a given date. If the half-life of the radionuclide is less than 70 days, the time is also indicated. This statement of the radioactive content must be made with reference to a specified time zone. The radioactivity at other times may be calculated from the exponential decay equation or from tables.

In general, a correct measurement of radioactivity requires that consideration is given to some or all of the following:

**Dead-time losses**. Due to the finite resolving time (dead time) of the detector and its associated electronic equipment, it may be necessary to correct for losses by coincidence. The resolving time of a counter is the minimum time interval required by the counter to resolve 2 single pulses. Incident radiation events at shorter intervals may not be detected or may be detected as a single event with the summed energy. These losses are sometimes referred to as 'dead-time losses'. For a counting system with a fixed dead time $\tau$ following each count, the true count rate, per second, is calculated using the following expression:

$$\frac{N_1}{1 - N_1\tau}$$

$N_1$ = the observed count rate, per second;

$\tau$ = the dead time, in seconds.

With some equipment this correction is made automatically. Corrections for losses by coincidence must be made before the correction for background radiation.

**Correction for decay during measurement**. If the time period of an individual measurement, $t_m$, is not negligibly short compared with the half-life of the radionuclide, $T_{1/2}$, the decay during this measurement time must be taken into account. For example, there is a 5 per cent cumulative loss of counts due to decay during a counting period that is 15 per cent of the half-life of the radionuclide.

After having corrected the instrument reading (count rate, ionisation current, etc.) for background signals and, if necessary, for losses due to electronic effects, the instrument reading corrected to the beginning of the individual measurement is calculated using the following expression:

$$\frac{R(\lambda t_m)}{1 - (e^{-\lambda t_m})}$$

$R$ = instrument reading before decay correction, but already corrected for background signal, etc.;

$\lambda$ = radionuclide decay constant ($\ln 2/T_{½}$);

$e$ = base of natural logarithm;

$t_m$ = measurement duration.

**Statistics of radioactivity measurement**. The results of determinations of radioactivity show variations that derive mainly from the random nature of nuclear transformations. Counting for any finite time can yield only an estimate of the true rate of nuclear transformations. A sufficient number of counts must be registered in order to compensate for variations in the number of transformations per time. In the case of measurement of radioactivity, the standard deviation of the recorded counts is the square root of the counts, so at least 10 000 counts are necessary to obtain a relative standard deviation of not more than 1 per cent.

**Linearity**. The linearity of an instrument is the range of radioactivity for a particular radionuclide over which its efficiency remains constant.

The linear range of a radioactivity measurement assembly can be determined by repeatedly counting a radioactive sample in a fixed geometry as it decays from an activity level that is above the linear range. After correction for the background signal, the natural logarithm of the count rate data is plotted against the elapsed time after the first measurement (Figure 2.2.66.-1).

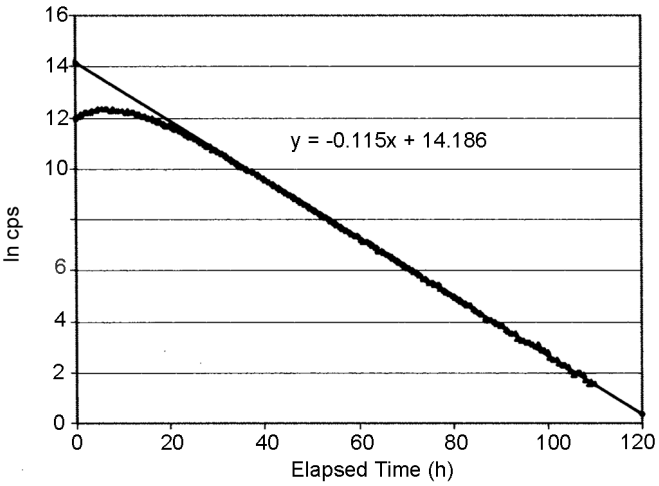


Figure 2.2.66.-1. – *Plot showing the measured and extrapolated count rate (natural logarithm of counts per second (cps)) from a technetium-99m source as a function of time, starting with a level of radioactivity above the linear range of the measuring equipment*

Linear regression analysis of the central, linear portion of the data set yields a slope which is the decay constant $\lambda$, which has a characteristic value for each radionuclide:

$$\ln \text{cps} = -\lambda t + c$$

$c$ represents the natural logarithm of the count rate at $t = 0$ of a perfectly linear instrument.

The resulting regression equation is used to calculate the theoretical count rate at each time that the actual data were recorded. Where the deviation of the measured count rate from the theoretical count rate is unacceptably high, the linear range of the measuring equipment has been exceeded.

Alternatively, a series of dilutions can be made of a radioactive solution of known radioactivity concentration. Equal volumes of each of the dilutions are then counted using standardised geometry and counter settings. The ratio of the count rate for each sample (after correction for background signals and decay) to the calculated radioactivity of the respective

sample in Bq is the counting efficiency. The range over which this ratio is constant is the useable range of the measuring equipment for the radionuclide concerned.

The limit of detection and the limit of quantification for equipment and procedures used for radioactivity measurement must be established before their routine use.

**Limit of detection**. The limit of detection (LOD) of an individual procedure is the lowest amount of radioactivity in a sample that can be detected but not necessarily quantified as an exact value. In practical terms this requires an estimate of the background signal and its standard deviation. The LOD is usually considered to be 3 times the standard deviation of the background signal.

**Limit of quantification**. The limit of quantification (LOQ) of an individual procedure is the lowest amount of radioactivity in a sample that can be quantitatively determined with suitable precision and accuracy. The LOQ is used particularly for the determination of impurities and/or degradation products. In practical terms the LOQ is usually considered to be 10 times the standard deviation of the background signal.

*MEASUREMENT OF RADIOACTIVITY USING IONISATION CHAMBERS*

**Apparatus**. Ionisation chambers (including dose calibrators) are the most common equipment for the measurement of radioactivity in the practice of radiopharmacy. It generally can measure activities from a few tens of kBq to hundreds of GBq. It usually comprises a sealed well-type ionisation chamber and built-in electronics to convert the detector signal to a measure of radioactivity.

The chamber is filled with a gas across which an electrical field is applied. When the gas is ionised by the radiation emitted by the source, the resulting ionisation current is measured and related to the radioactivity present in the ionisation chamber. The ionisation current is influenced by the applied voltage, the energy and the intensity of the radiation and the nature and pressure of the gas. The instrument settings (calibration factor) may be adjusted to keep a direct relationship between the ionisation produced by the radiation of a specific radionuclide and the radioactivity value obtained for each measurement geometry.

As an ionisation chamber measures only the current resulting from the overall ionisation produced within the chamber, it cannot discriminate between the emissions of different radionuclides.

For an accurate measurement of the radioactivity of a specific radionuclide, the measurement must be corrected for the contributions to the ionisation current caused by radionuclidic impurities present in the preparation.

The activity levels to be measured are limited by saturation considerations, the range of the amplifier and the design of the chamber itself. The linearity range of the ionisation chamber is established as described above under Linearity.

The ionisation chamber must be shielded to minimise background signals to an acceptable level.

**Method**. The sample is positioned inside the well of the ionisation chamber at a given position, using a holder. After putting the sample in the ionisation chamber, the activity reading is made once the response is stable. Measuring the sample under exactly the same geometrical conditions as the calibration source will yield the most accurate results. If necessary, dilute the preparation to be measured to the same volume as that of the calibration source.

*Calibration*. The ionisation chamber is calibrated taking into account the shape, dimensions, material of the container, volume and composition of the solution, the position within the chamber and the radionuclide being measured. Limits for uncertainty in calibration can be found in national and international regulations.

Calibrate the ionisation chamber at least once a year, by using sources of radionuclides traceable to national or international standards in the appropriate containers (vial, syringe) with regard to geometry. Establish and implement subsidiary correction factors to take account of the differing configurations of the radionuclides to be measured. Perform a linearity check of the instrument's response over the complete range of energies and activities for which the equipment is used.

For each setting and before each use (minimum once on each day of use) perform a constancy check of the ionisation chamber using standard sources of radionuclides with long half-lives to verify its calibrated state. A check with a reference source, such as caesium-137, must be performed on each day of use to verify that the ionisation chamber is still in its calibrated state.

*MEASUREMENT OF RADIOACTIVITY USING SOLID-STATE DETECTORS*

Solid-state detectors include scintillating plastic fluors and crystals, and semiconductors. Further to their application in spectrometry (see section Spectrometry), solid-state detectors can be used for the measurement of radioactivity. In particular, due to their high sensitivity, plastic and crystal scintillation detectors are used in counting low levels of radioactivity. Dead-time losses must be carefully considered with these types of detectors. Semiconductor detectors are used when a higher energy discrimination is required, for example in mixtures of radionuclides or when there are potential radionuclidic impurities with emissions of similar energy.

**Apparatus**. The equipment consists of a shielded detector comprising a plastic or crystal scintillator coupled to a photomultiplier, or a semiconductor, which are connected to an amplifier and counting electronics. The system may have an adjustable energy window, used for selecting a counting region of the radionuclide energy spectrum that may be adjusted by the operator.

Instruments have different properties of energy resolution and detection efficiency depending on the type of detector and its volume and geometry. Lower efficiency requires a longer counting time.

Samples to be measured may be placed in front of the detector or into the well of a well-type detector. Measuring chambers may be enclosed in the detector shielding and single samples may be introduced using lids or other positioning systems to ensure correct measurement geometry.

A scintillation detector can be used for dynamic radioactivity measurement when, for example, the eluate of a liquid chromatograph is directed over or through a detector, see section on Detection and measurement of radioactivity in combination with a separation technique.

**Method**. Ensure that the sample radioactivity gives a counting rate in the linearity range of the equipment. The measurement is started after any shielding is in place or the well cover is replaced and the counting time is selected to reach sufficient counts for a statistically significant value.

*Calibration*. The detector has to be calibrated by measuring its efficiency using a source of the radionuclide in question traceable to national or international standards. Calibration in terms of efficiency uses sources such as caesium-137, cobalt-60, barium-133 and others covering the desired energy range.

*MEASUREMENT OF RADIOACTIVITY USING LIQUID SCINTILLATION DETECTORS*

Liquid scintillation counting is commonly used for beta-particle emitting samples, but is also used for alpha-particle emitting samples. For the principles of the detection of radioactivity using liquid scintillation detectors see under Beta-particle spectrometry below.

*Calibration*. In order to take into account the loss of counting efficiency due to quenching, the liquid scintillation counter may make use of an external source, typically barium-133 or europium-152, which is brought close to the sample vial to release Compton electrons. The shape of the resulting spectrum is analysed automatically to compute a quench-indicating parameter. This parameter can then be related to the counting efficiency measuring sources of known activity at a determined level of quenching agent. The obtained quench curve allows the determination of the activity of an unknown sample knowing the count rate and the value of the quenching parameter.

*DETERMINATION OF HALF-LIFE*

The half-life is a characteristic of the radionuclide that may be used for its identification. The half-life is calculated by measuring the variation of radioactivity of a sample to be tested as a function of time. Perform the measurements in the linearity range of a calibrated instrument.

**Apparatus**. Half-life can be measured by using any type of quantitative radioactivity detector provided it is used within a linearity range throughout the range of activities that are present during the measurement and the geometry is not changed during the measurement.

For preparations containing a radionuclide with a short half-life and when stated in a monograph, determination of the approximate half-life contributes to the identification.

**Method**.

*Half-life*. The preparation to be examined is used as such or diluted or dried in a capsule after appropriate dilution. The radioactive sample is prepared in a manner that will avoid loss of material during handling. If it is a liquid (solution), it is contained in a closed flask or a sealed tube. If it is a residue from drying in a capsule, it is protected by a cover consisting of a sheet of adhesive cellulose acetate or of some other material.

The radioactivity of the sample must be high enough to allow measurements over a period corresponding to 3 estimated half-lives but must be, for each measurement, within the linearity range of the equipment. Correction for dead-time losses is applied if necessary.

The same source is measured in the same geometrical conditions and at intervals usually corresponding to at least half of the estimated half-life. Each value is tabulated against the time interval from the initial measurement. To avoid influence of decay during measurement, the counting time is the same for all measurements.

A graph can be drawn with time as the abscissa and the logarithm of the relative instrument reading (e.g. count rate) as the ordinate. The half-life is calculated from the slope of the best linear fit of the measured values against the time corresponding to each measurement.

*Approximate half-life*. For this purpose, not fewer than 3 measurements are made over a period of not less than 1/4 of the estimated half-life.

The sample to be examined and the instrument to be used comply with the indications given above. The data are processed in the same way as above.

SPECTROMETRY

Radionuclides can be identified by their emission spectrum. Each type of emission (i.e. alpha particles, beta particles and electrons, gamma- and X-rays) requires specific equipment to acquire an emission spectrum. Spectrometers must be calibrated in order to work properly and the following sections describe the different equipment and detail the general procedures for a reliable measurement.

*GAMMA-RAY SPECTROMETRY*

**General principles**. In gamma-ray spectrometry using a scintillation detector, absorption of gamma- and X-rays results in production of light, which is converted into an electrical pulse by a photomultiplier. In gamma-ray spectrometry using a semiconductor detector, absorption of gamma- and X-rays results in the immediate production of an electrical pulse.

In both cases the pulse amplitude is proportional to the energy of the absorbed radiation. The most common detectors for gamma- and X-ray spectrometry are thallium-activated sodium iodide (NaI(Tl)) scintillation counters and high-purity germanium (HPGe) semiconductor detectors.

A gamma-ray spectrum can be produced by collecting and analysing a sufficient number of pulses.

**Apparatus**. A gamma-ray spectrometer usually comprises a shielded measuring chamber where the sample is positioned, a detector, an electronic chain and a multichannel analyser.

The shielding of the chamber must be able to reduce the background signal to a level that allows the registration of a correct gamma-ray spectrum.

The measurement chamber has a movable cover or a drawer to allow the positioning of the sample. A sample holder may be present to ensure reproducible geometry between measurements.

The duration of measurement is related to the radioactivity of the target radionuclide and a long period of acquisition may be required to achieve the necessary counting statistics. Dead-time losses must be carefully considered with this type of detector.

The sensitivity of a NaI(Tl) detector is higher than that of a germanium detector of the same size. In general, peaks in an energy spectrum are identified with an uncertainty depending upon the full width of the peak at its half-maximum height (FWHM). The energy resolution of a solid-state scintillation detector is much poorer than that of a semiconductor detector and hence peaks obtained with a semiconductor detector are much narrower than those obtained with a scintillation detector. Figure 2.2.66.-2 shows a comparison of the spectra obtained from the same source with the 2 types of detector.

The different performances of NaI(Tl) and HPGe detectors may limit their use in some spectrometric analyses.

For the identification of the radionuclide(s) in a preparation and determination of radionuclidic purity, a risk assessment on the process of radionuclide production must assess the potential presence of other radionuclides with photon energies in the same range (± 10 per cent) as that of the radionuclide(s) present in the radiopharmaceutical.

In case radionuclidic impurities can be present that emit gamma- or X-rays with an energy in the same range as that of the photons emitted by the radionuclide in the preparation, a measured peak energy within a maximum interval of ± 2 keV or ± 2 per cent (whichever is the larger) with respect to the nominal peak energy (see *5.7. Table of physical characteristics of radionuclides)* is sufficient for peak identification.

In the case where such impurities are not expected to be present, a maximum interval of ± 10 keV or ± 6 per cent (whichever is the larger) with respect to the nominal peak energy is acceptable for peak identification.

**Method**. Ensure that the counting rate of the sample falls within the linearity range of the equipment. For liquid samples this may be achieved by appropriate dilution; for solid samples, by increasing the source-to-detector distance or by using an attenuating material. Introduce the preparation to be examined in a container into the instrument chamber and record the spectrum after closing the shielding.

Ensure that the container used for quantitative measurements is of the same shape, dimensions, volume and material as that of the calibration standard.

Ensure that the composition of the solution and the position of the container in the measuring chamber is the same for the container for the quantitative measurement as for the calibration standard.

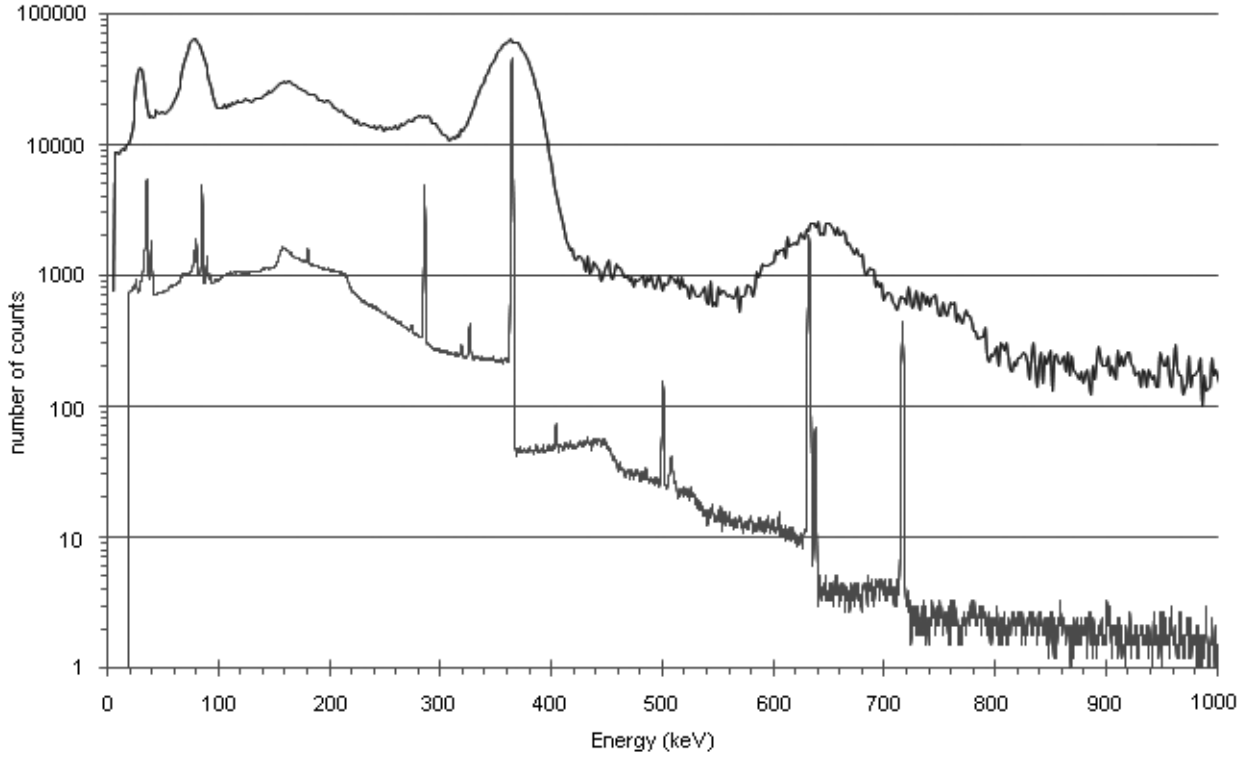


Figure 2.2.66.-2. – *Comparative pulse-height spectra recorded using a thallium-activated sodium iodide scintillator (upper curve) and a high-purity germanium semiconductor detector (lower curve). The source was gamma- and X-ray radiation from the decay of iodine-131.*

*Radionuclide identification.* Calibrate the spectrometer in relation to energy. Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

*Radionuclidic purity.* Calibrate the spectrometer in relation to efficiency and energy. Determine the LOQ and resolution of the equipment and ensure that they are in line with the limits of the radionuclides to be determined. Record the spectrum of the preparation.

Identify the radionuclides present in the preparation to be examined and determine their radioactivity with the aid of chapter *5.7. Table of physical characteristics of radionuclides*. Because the level of radionuclidic impurities, expressed as a percentage of the total radioactivity, may increase or decrease with time, the measured activity of each impurity must be recalculated to the activity during the period of validity of the preparation. The activities of all radionuclidic impurities need to be summed (taking into account the limit of quantification) and related to the total radioactivity of the preparation.

The sample is placed close to the detector or within a well-type detector. All the events within a pre-set energy range are collected and displayed on a ratemeter as counts per second or accumulated over a pre-set period of time. If there is sufficient difference in photon energies emitted by the radionuclide(s), a sodium iodide detector can be suitable, given its high sensitivity. However, if there is a need to discriminate emissions of similar energy, a HPGe detector or another semiconductor detector is needed.

*Calibration.* Calibration in relation to energy is done by using the peaks of known sources traceable to national or international standards, such as cobalt-57, caesium-137, cobalt-60 and others covering the desired energy range. A calibration in relation to efficiency can be simultaneously obtained, so that not only the energy spectrum but also the activity of the sample and the radionuclide impurities can be further determined. The calibration of efficiency can be performed with a traceable radionuclide source with energy peaks covering the desired range or with the aid of a mixed, traceable radionuclide standard with gamma-ray energies covering the desired range.

To obtain the efficiency curve, the detector response as a function of the energy has to be measured using each separate sample/detector geometry. For this reason, it is possible to carry out the measurement with the aid of a primary standard source. Primary standards may not be available for radionuclides with a short half-life, e.g. some positron emitters. When measuring, the sample will mostly have to be in a container and set at a defined position in relation to the detector. The sample/detector geometry is then defined by the position of the sample relative to the detector and the characteristics of the container and sample, e.g. shape, volume and density. Figure 2.2.66.-3 shows a typical HPGe detector efficiency curve obtained for a cylindrical container placed on top of the detector.

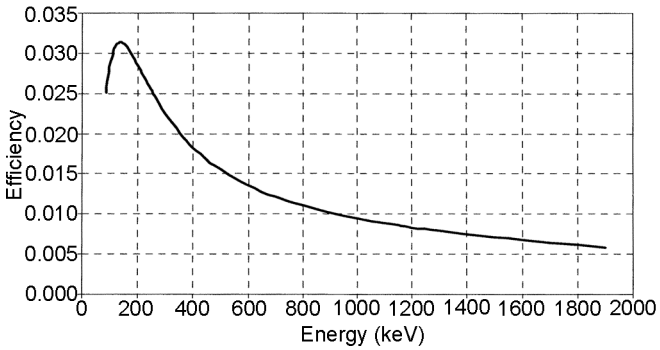


Figure 2.2.66.-3. – *Typical HPGe efficiency curve measured using a dedicated container set on top of the detector*

*BETA-PARTICLE SPECTROMETRY*

In the case of a beta-particle emitter, a beta-particle spectrometer is necessary to determine the energy distribution of the emitted beta particles. It is analogous to a gamma-ray spectrometer, but frequently uses liquid scintillators to convert the energy of the beta particles into detectable light,

which can then be analysed. Beta-particle spectrometry is mostly achieved by dissolving or suspending the sample in a liquid scintillation cocktail in transparent or translucent (glass or plastic) containers and subsequent counting of the electrical pulses generated by a photomultiplier from the emitted light. The pulse amplitude is related to the energy of the absorbed radiation. A beta-particle spectrum can be produced by collecting a sufficient number of pulses. The liquid scintillation cocktail is chosen in such a way that counting errors due to quenching, chemoluminescence, phosphorescence, etc., are minimised. Coincidence counting with 2 or more photomultipliers is also used to minimise counts from background radiation, electronics, etc.
To differentiate between alpha- and beta-particle emissions, pulse-shape discrimination is commonly used.

*Radionuclide identification.* Determination of the correspondence of the mean and/or maximum energies in the energy spectrum from the sample to the energies prescribed by a monograph is a valid identification test.

*Calibration.* A common method of energy calibration is to use an unquenched reference sample to determine the maximum energy of the beta particles emitted by the radionuclide of interest.

*ALPHA-PARTICLE SPECTROMETRY*

For the identification and assay of alpha-particle emitters, spectrometry using liquid scintillation is mostly used. The principle is explained in the previous section on beta-particle spectrometry.
For the identification and determination of radionuclidic purity of alpha-particle emitters, spectrometry using a silicon-diode semiconductor detector can be used. Using this detector, the absorption of alpha particles results in the immediate production of an electrical pulse. The movement of electron-hole pairs created by the interaction of radiation induces an electrical charge, which is amplified and measured.

The sample preparation is of crucial importance. After a chemical separation of the radionuclide of interest, the sample is electro-deposited on a stainless steel disk in the form of a very thin layer of material to minimise self-absorption. The yield of the whole procedure can be determined experimentally by adding a known amount of a tracer, which will take into account the chemical separation efficiency, the electro-deposition efficiency and the counting efficiency.

For both types of detectors, the pulse amplitude is related to the energy of the absorbed radiation. An alpha-particle spectrum can be produced by collecting a sufficient number of pulses.

*Radionuclide identification.* Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

*Calibration.* An alpha-particle spectrometer has to be calibrated in relation to energy and efficiency. This is done by using the peaks from known sources covering the desired energy range, such as americium-241 and plutonium-242. Not all alpha particles emitted by the source will produce a count in the system. The probability that an emitted alpha particle will interact with the detector material and produce a count is the efficiency of the detector, which depends on the geometry.

## DETECTION AND MEASUREMENT OF RADIOACTIVITY IN COMBINATION WITH A SEPARATION TECHNIQUE

A radioactive preparation may contain the radionuclide in different chemical forms other than the intended one. Therefore it is necessary to separate the different substances containing the radionuclide and determine the percentage of radioactivity due to the radionuclide concerned associated with the stated chemical form and the contribution to the total radioactivity due to the radionuclide concerned coming from other substances. For this purpose, instruments for the detection and measurement of radioactivity are used in combination with a physico-chemical separation technique. In principle, any method of separation may be used.

Monographs for radiopharmaceutical preparations may include the combined use of radioactivity measurement with paper chromatography (*2.2.26*), thin-layer chromatography (*2.2.27*), gas chromatography (*2.2.28*), liquid chromatography (*2.2.29*), size-exclusion chromatography (*2.2.30*) or electrophoresis (*2.2.31*).

In all cases the radioactivity of each analyte is measured after the separation has been achieved using the stated method.

Radioactivity measurement may be performed using detectors mounted in series with other detectors in analytical instruments, such as liquid chromatographs, making in-line detection of analytes, or performed off-line, i.e. after the analytical separation has been completed, by measuring the radioactivity of eluate fractions obtained after liquid chromatographic separation of equal volume or as the distribution of radioactivity on paper chromatography or thin-layer chromatography supports.

*IN-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY IN COMBINATION WITH LIQUID CHROMATOGRAPHY*

**Apparatus**. Liquid chromatography (see *2.2.29*) may be used to separate the principal radioactive substance of a radiopharmaceutical preparation from radiochemical impurities or degradation products. In-line detection is usually obtained by using a scintillation detector connected to a ratemeter and recording device. The scintillating material of the detector is selected on the basis of the emission to be detected, e.g. plastic scintillator for beta-particle emissions or scintillation crystals for gamma- and X-ray radiations. The addition of a liquid scintillation cocktail before the eluate reaches the in-line radioactivity detector may also be used in the case of beta-particle-emitting radionuclides.

The simultaneous use of a radioactivity detector and other detectors (UV, refractive index, conductimetric, etc.) connected in series may be used to identify the substance, e.g. in relation to the retention time of a known standard, to determine the amount of the substance using a suitable reference standard and to measure the radioactivity associated with such a substance. When different detectors are coupled in series, correct the experimentally obtained retention times for the delay in time between the detectors.

In liquid chromatography some radiochemical impurities, such as colloidal impurities, may be retained on the column. In such cases a separate method is required for the determination of the content of the retained radiochemical impurities and the calculation formula for the expression of the total radiochemical purity takes into account the relative amount of the retained radiochemical impurities.

One possibility to evaluate such retention problems during method validation is to evaluate the radioactivity recovery from the column by measuring the total radioactivity recovered from the chromatographic equipment with and without the column.

**Method**. The sample is diluted if necessary and then applied to the column in the prescribed volume and conditions. In this respect it is important to demonstrate the LOD and LOQ, and the linearity of the detector throughout the range of activities to be measured.

*Flow-through detector.* A portion of the tubing where the eluate containing the radioactive species is flowing is placed in front of or within the detector. Counting efficiency may be increased using a longer portion of the tube (e.g. making multiple turns in front of or within the detector); however, this will reduce the ability of the system to separate 2 closely eluting peaks of radioactivity.

When the radiochemical purity test prescribes determination of the total radiochemical impurities or there is a quantitative determination of an individual impurity, it is important to choose an appropriate threshold setting and appropriate conditions for integration of the peak areas. In such tests the disregard limit, i.e. the limit at or below which a peak is disregarded, is dependent on the method and is related to the limit of detection and limit of quantification. Thus, the threshold setting of the data collection system corresponds to at least half of the disregard limit.

Record the signal of the detectors as a function of time.

Identification of peaks in the radiometric signal (radiochromatogram) is made on the basis of the retention time of the analytes. The profile from other detectors may be used for this purpose.

Quantification of the different components of chromatogram and radiochromatogram profiles is made on the basis of peak areas. Peak areas are usually obtained by direct integration of the detector signal using commercially available software.

*OFF-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY*

Liquid chromatography (*2.2.29*). Provided the retention times of the various radiochemical species are reproducibly consistent, an alternative method of radioactivity quantification is to collect the liquid chromatography effluent in a series of timed samples (fractions) for off-line analysis for radioactivity content. The radioactivity in the fractions corresponding to the peaks can be expressed as a percentage of the total of the radioactivity in all fractions, taking into account the limit of quantification.

**Method**. The sample is applied on the column in the prescribed volume and conditions. Fractions are collected at the end of the chromatographic line.

The volume between the detector used to identify the retention time of the peaks and the collection point is measured and a delay factor is calculated on the basis of the effluent flow rate and applied to each peak to estimate the time of elution of the peak at the point of collection. The fractions are collected on the basis of a fixed time interval or at the time of appearance estimated from the delay time so that any relevant peak is collected in one or more fractions.

The radioactivity of each fraction is counted using a calibrated instrument such as a dose calibrator or a scintillation detector, taking into account the limit of quantification and the linearity.

An elution profile is obtained tabulating the counts per fraction against the elution time or volume. The activity of fractions belonging to the same peak may be summed and the relative percentage calculated to define radiochemical purity.

Thin-layer chromatography (*2.2.27*) and paper chromatography (*2.2.26*). Provided a thin-layer chromatography or a paper chromatography analytical method has been validated for the separation of components of a radioactive preparation, the number and relative intensities of the separated spots can be detected and measured using a radioactivity detector that can relate the radioactivity to a specific position on the chromatographic support.

The positions of the spots (peaks) may permit chemical identification by comparison with solutions of the same chemical substances (non-radioactive), using a suitable detection method.

**Apparatus**.

*Scanning device*. The apparatus generally comprises a radioactivity detector, such as a position-sensitive proportional counter or a collimated scintillation detector placed at a fixed distance from a scanning platform where the chromatographic support to be scanned is positioned.

The radioactivity of the sample applied to the chromatography support must result in a counting rate in the linearity range of the equipment and the sample may be diluted if necessary. The area to be scanned is positioned at the reference position so that the desired lane is aligned with the detector scanning trip. Adjust the scanning time to allow enough counting time during the run.

The detector or the platform may be moved in-plane, along the *x*-axis or the *y*-axis, so that the entire surface can be scanned during a single run.

The detector is connected to a suitable counting device, so that the radioactivity revealed can be measured quantitatively and the count rate related spatially to the surface scanned.

The radioactivity is automatically reported against the development distance and the profile describes peaks having an area proportional to the number of counts per unit of distance.

*Radioactivity counter*. In the case where a maximum of only 3 radiochemical components needs to be identified and they are fully separated, the support can be cut into equal strips, each having a size not more than half the length of the support corresponding to the difference between the retardation factors of the 2 closest spots. Each single strip is numbered starting from the origin side and counted separately. Alternatively, for well-characterised systems the support may be cut into 2 or more unequal portions, folded if necessary to approximately equal geometry before counting. An ionisation chamber or a scintillation counter can be used for this purpose, provided they are used within the instrument's linearity range and above its LOQ.

*Autoradiography*. This may also be used to acquire an image of the radioactivity distribution on the chromatographic support. In this case, the response of the system used for the acquisition of the image, such as a phosphor imager or a photographic film, must be shown to be linear with respect to the radioactivity in the chromatogram. Otherwise the system must be pre-calibrated or exposed at the same time to a series of reference radioactive sources, obtained by dilution from a calibrated standard solution, covering the expected radioactivity range that may be present on the support.

**Method**. Deposit the required amount of sample at the origin of the chromatographic support, with drying if necessary to avoid spreading of the spot. Develop the chromatogram according to the prescribed method. A carrier may be added when prescribed in a particular monograph.

In paper and thin-layer chromatography, it is preferable not to dilute the preparation to be examined but it is important to avoid depositing such a quantity of radioactivity that counting losses by coincidence (dead-time losses) occur during measurement of the radioactivity.

After development, the support is dried and the positions of the radioactive areas are detected by measurement of radioactivity over the length of the chromatogram, using a suitable collimated counter, by autoradiography, or by cutting the strips into portions and counting each portion.

Radioactivity may be measured by integration using an automatic-plotting instrument or a digital counter.

The ratios of the areas under the peaks give the ratios of the percentages of radioactivity due to the respective radiochemical substances.

When the strips are cut into portions, the ratios of the quantities of radioactivity measured give the ratio of percentages of radioactivity due to the respective radiochemical species.

*Calibration*. It is important to demonstrate the limits of detection and quantification, and the linearity of the detector throughout the range of activities to be measured and in all positions on the support of the chromatographic system. This may be done by applying samples covering a range of activities from 0.1 per cent to 100 per cent of the expected range. Prepare the samples by dilution and apply equal volumes of each, with drying if necessary. After examining the radioactivity profile using the equipment's standard settings, the peak areas are integrated for comparison with the

calculated amount of radioactivity applied to each spot. Verify that the response of the detector over the complete length and width of the detector path is the same, as the response may vary with the detector position.

The peak-resolving power is influenced by the size of the spot, the total radioactivity of the radionuclide and the detector equipment. It can be checked by applying 5 µL spots separated by distances increasing from 4 mm to 20 mm in 2 mm increments. The approximate resolution of the detection system can be determined from the radioactivity profile as the distance between the 2 spots where the baseline is only just clearly separated.

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# 2.3. IDENTIFICATION

01/2008:20301

## 2.3.1. IDENTIFICATION REACTIONS OF IONS AND FUNCTIONAL GROUPS

ACETATES

a) Heat the substance to be examined with an equal quantity of *oxalic acid R*. Acid vapours with the characteristic odour of acetic acid are liberated, showing an acid reaction (*2.2.4*).

b) Dissolve about 30 mg of the substance to be examined in 3 mL of *water R* or use 3 mL of the prescribed solution. Add successively 0.25 mL of *lanthanum nitrate solution R*, 0.1 mL of *0.05 M iodine* and 0.05 mL of *dilute ammonia R2*. Heat carefully to boiling. Within a few minutes a blue precipitate is formed or a dark blue colour develops.

ACETYL

In a test-tube about 180 mm long and 18 mm in external diameter, place about 15 mg of the substance to be examined, or the prescribed quantity, and 0.15 mL of *phosphoric acid R*. Close the tube with a stopper through which passes a small test-tube about 100 mm long and 10 mm in external diameter containing *water R* to act as a condenser. On the outside of the smaller tube, hang a drop of *lanthanum nitrate solution R*. Except for substances hydrolysable only with difficulty, place the apparatus in a water-bath for 5 min, then take out the smaller tube. Remove the drop and mix it with 0.05 mL of *0.01 M iodine* on a tile. Add at the edge 0.05 mL of *dilute ammonia R2*. After 1 min to 2 min, a blue colour develops at the junction of the two drops; the colour intensifies and persists for a short time.

For *substances hydrolysable only with difficulty* heat the mixture slowly to boiling over an open flame and then proceed as prescribed above.

ALKALOIDS

Dissolve a few milligrams of the substance to be examined, or the prescribed quantity, in 5 mL of *water R*, add *dilute hydrochloric acid R* until an acid reaction occurs (*2.2.4*), then 1 mL of *potassium iodobismuthate solution R*. An orange or orange-red precipitate is formed immediately.

ALUMINIUM

Dissolve about 15 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add about 0.5 mL of *dilute hydrochloric acid R* and about 0.5 mL of *thioacetamide reagent R*. No precipitate is formed. Add dropwise *dilute sodium hydroxide solution R*. A gelatinous white precipitate is formed which dissolves on further addition of *dilute sodium hydroxide solution R*. Gradually add *ammonium chloride solution R*. The gelatinous white precipitate is re-formed.

AMINES, PRIMARY AROMATIC

Acidify the prescribed solution with *dilute hydrochloric acid R* and add 0.2 mL of *sodium nitrite solution R*. After 1 min to 2 min, add 1 mL of *β-naphthol solution R*. An intense orange or red colour and usually a precipitate of the same colour are produced.

AMMONIUM SALTS

To the prescribed solution add 0.2 g of *magnesium oxide R*. Pass a current of air through the mixture and direct the gas that escapes just beneath the surface of a mixture of 1 mL of *0.1 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The colour of the indicator changes to yellow. On addition of 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R* a yellow precipitate is formed.

AMMONIUM SALTS AND SALTS OF VOLATILE BASES

Dissolve about 20 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 2 mL of *dilute sodium hydroxide solution R*. On heating, the solution gives off vapour that can be identified by its odour and by its alkaline reaction (*2.2.4*).

ANTIMONY

Dissolve with gentle heating about 10 mg of the substance to be examined in a solution of 0.5 g of *sodium potassium tartrate R* in 10 mL of *water R* and allow to cool: to 2 mL of this solution, or to 2 mL of the prescribed solution, add *sodium sulfide solution R* dropwise; an orange-red precipitate is formed which dissolves on addition of *dilute sodium hydroxide solution R*.

ARSENIC

Heat 5 mL of the prescribed solution on a water-bath with an equal volume of *hypophosphorous reagent R*. A brown precipitate is formed.

BARBITURATES, NON-NITROGEN SUBSTITUTED

Dissolve about 5 mg of the substance to be examined in 3 mL of *methanol R*, add 0.1 mL of a solution containing 100 g/L of *cobalt nitrate R* and 100 g/L of *calcium chloride R*. Mix and add, with shaking, 0.1 mL of *dilute sodium hydroxide solution R*. A violet-blue colour and precipitate are formed.

BENZOATES

a) To 1 mL of the prescribed solution add 0.5 mL of *ferric chloride solution R1*. A dull-yellow precipitate, soluble in *ether R*, is formed.

b) Place 0.2 g of the substance to be examined, treated if necessary as prescribed, in a test-tube. Moisten with 0.2 mL to 0.3 mL of *sulfuric acid R*. Gently warm the bottom of the tube. A white sublimate is deposited on the inner wall of the tube.

c) Dissolve 0.5 g of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.5 mL of *hydrochloric acid R*. The precipitate obtained, after crystallisation from warm *water R* and drying *in vacuo*, has a melting point (*2.2.14*) of 120 °C to 124 °C.

BISMUTH

a) To 0.5 g of the substance to be examined add 10 mL of *dilute hydrochloric acid R* or use 10 mL of the prescribed solution. Heat to boiling for 1 min. Cool and filter if necessary. To 1 mL of the solution obtained add 20 mL of *water R*. A white or slightly yellow precipitate is formed which on addition of 0.05 mL to 0.1 mL of *sodium sulfide solution R* turns brown.

b) To about 45 mg of the substance to be examined add 10 mL of *dilute nitric acid R* or use 10 mL of the prescribed solution. Boil for 1 min. Allow to cool and filter if necessary. To 5 mL of the solution obtained add 2 mL of a 100 g/L solution of *thiourea R*. A yellowish-orange colour or an orange precipitate is formed. Add 4 mL of a 25 g/L solution of *sodium fluoride R*. The solution is not decolorised within 30 min.

BROMIDES

a) Dissolve in 2 mL of *water R* a quantity of the substance to be examined equivalent to about 3 mg of bromide ($Br^-$) or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate obtained in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves with difficulty.

b) Introduce into a small test-tube a quantity of the substance to be examined equivalent to about 5 mg of bromide ($Br^-$) or the prescribed quantity. Add 0.25 mL of *water R*, about 75 mg of *lead dioxide R*, 0.25 mL of *acetic acid R* and shake gently. Dry the inside of the upper part of the test-tube with a piece of filter paper and allow to stand for 5 min. Prepare a strip of suitable filter paper of appropriate size. Impregnate it by capillarity, by dipping the tip in a drop of *decolorised fuchsin solution R* and introduce the impregnated part immediately into the tube. Starting from the tip, a violet colour appears within 10 s that is clearly distinguishable from the red colour of fuchsin, which may be visible on a small area at the top of the impregnated part of the paper strip.

CALCIUM

a) To 0.2 mL of a neutral solution containing a quantity of the substance to be examined equivalent to about 0.2 mg of calcium ($Ca^{2+}$) per millilitre or to 0.2 mL of the prescribed solution add 0.5 mL of a 2 g/L solution of *glyoxalhydroxyanil R* in *ethanol (96 per cent) R*, 0.2 mL of *dilute sodium hydroxide solution R* and 0.2 mL of *sodium carbonate solution R*. Shake with 1 mL to 2 mL of *chloroform R* and add 1 mL to 2 mL of *water R*. The chloroform layer is coloured red.

b) Dissolve about 20 mg of the substance to be examined or the prescribed quantity in 5 mL of *acetic acid R*. Add 0.5 mL of *potassium ferrocyanide solution R*. The solution remains clear. Add about 50 mg of *ammonium chloride R*. A white, crystalline precipitate is formed.

CARBONATES AND BICARBONATES

Introduce into a test-tube 0.1 g of the substance to be examined and suspend in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 3 mL of *dilute acetic acid R*. Close the tube immediately using a stopper fitted with a glass tube bent twice at right angles. The solution or the suspension becomes effervescent and gives off a colourless and odourless gas. Heat gently and collect the gas in 5 mL of *barium hydroxide solution R*. A white precipitate is formed that dissolves on addition of an excess of *hydrochloric acid R1*.

CHLORIDES

a) Dissolve in 2 mL of *water R* a quantity of the substance to be examined equivalent to about 2 mg of chloride ($Cl^-$) or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, white precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light, disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves easily with the possible exception of a few large particles which dissolve slowly.

b) Introduce into a test-tube a quantity of the substance to be examined equivalent to about 15 mg of chloride ($Cl^-$) or the prescribed quantity. Add 0.2 g of *potassium dichromate R* and 1 mL of *sulfuric acid R*. Place a filter-paper strip impregnated with 0.1 mL of *diphenylcarbazide solution R* over the opening of the test-tube. The paper turns violet-red. The impregnated paper must not come into contact with the potassium dichromate.

CITRATES

Dissolve in 5 mL of *water R* a quantity of the substance to be examined equivalent to about 50 mg of citric acid or use 5 mL of the prescribed solution. Add 0.5 mL of *sulfuric acid R* and 1 mL of *potassium permanganate solution R*. Warm until the colour of the permanganate is discharged. Add 0.5 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R* and 4 g of *sulfamic acid R*. Make alkaline with *concentrated ammonia R*, added dropwise until all the sulfamic acid has dissolved. Addition of an excess of *concentrated ammonia R* produces a violet colour, turning to violet-blue.

ESTERS

To about 30 mg of the substance to be examined or the prescribed quantity add 0.5 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* in *methanol R* and 0.5 mL of a 100 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. Heat to boiling, cool, acidify with *dilute hydrochloric acid R* and add 0.2 mL of *ferric chloride solution R1* diluted ten times. A bluish-red or red colour is produced.

IODIDES

a) Dissolve a quantity of the substance to be examined equivalent to about 4 mg of iodide ($I^-$) in 2 mL of *water R* or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, pale-yellow precipitate is formed. Centrifuge and wash with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate does not dissolve.

b) To 0.2 mL of a solution of the substance to be examined containing about 5 mg of iodide ($I^-$) per millilitre, or to 0.2 mL of the prescribed solution, add 0.5 mL of *dilute sulfuric acid R*, 0.1 mL of *potassium dichromate solution R*, 2 mL of *water R* and 2 mL of *chloroform R*. Shake for a few seconds and allow to stand. The chloroform layer is coloured violet or violet-red.

IRON

a) Dissolve a quantity of the substance to be examined equivalent to about 10 mg of iron ($Fe^{2+}$) in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *potassium ferricyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 mL of *dilute hydrochloric acid R*.

b) Dissolve a quantity of the substance to be examined equivalent to about 1 mg of iron ($Fe^{3+}$) in 30 mL of *water R*. To 3 mL of this solution or to 3 mL of the prescribed solution, add 1 mL of *dilute hydrochloric acid R* and 1 mL of *potassium thiocyanate solution R*. The solution is coloured red. Take two portions, each of 1 mL, of the mixture. To one portion add 5 mL of *isoamyl alcohol R* or 5 mL of *ether R*. Shake and allow to stand. The organic layer is coloured pink. To the other portion add 2 mL of *mercuric chloride solution R*. The red colour disappears.

c) Dissolve a quantity of the substance to be examined equivalent to not less than 1 mg of iron ($Fe^{3+}$) in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *potassium ferrocyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 mL of *dilute hydrochloric acid R*.

LACTATES

Dissolve a quantity of the substance to be examined equivalent to about 5 mg of lactic acid in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 1 mL of *bromine water R* and 0.5 mL of *dilute sulfuric acid R*. Heat on a water-bath until the colour is discharged, stirring occasionally with a glass rod. Add 4 g of *ammonium sulfate R* and mix. Add dropwise and without mixing 0.2 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R*. Still without mixing add 1 mL of *concentrated ammonia R*. Allow to stand for 30 min. A dark green ring appears at the junction of the two liquids.

LEAD

a) Dissolve 0.1 g of the substance to be examined in 1 mL of *acetic acid R* or use 1 mL of the prescribed solution. Add 2 mL of *potassium chromate solution R*. A yellow precipitate is formed that dissolves on addition of 2 mL of *strong sodium hydroxide solution R*.

b) Dissolve 50 mg of the substance to be examined in 1 mL of *acetic acid R* or use 1 mL of the prescribed solution. Add 10 mL of *water R* and 0.2 mL of *potassium iodide solution R*. A yellow precipitate is formed. Heat to boiling for 1 min to 2 min. The precipitate dissolves. Allow to cool. The precipitate is re-formed as glistening, yellow plates.

MAGNESIUM

Dissolve about 15 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 1 mL of *dilute ammonia R1*. A white precipitate is formed that dissolves on addition of 1 mL of *ammonium chloride solution R*. Add 1 mL of *disodium hydrogen phosphate solution R*. A white crystalline precipitate is formed.

MERCURY

a) Place about 0.1 mL of a solution of the substance to be examined on well-scraped copper foil. A dark-grey stain that becomes shiny on rubbing is formed. Dry the foil and heat in a test-tube. The spot disappears.

b) To the prescribed solution add *dilute sodium hydroxide solution R* until strongly alkaline (*2.2.4*). A dense yellow precipitate is formed (mercuric salts).

NITRATES

To a mixture of 0.1 mL of *nitrobenzene R* and 0.2 mL of *sulfuric acid R*, add a quantity of the powdered substance equivalent to about 1 mg of nitrate ($NO_3^-$) or the prescribed quantity. Allow to stand for 5 min. Cool in iced water and add slowly and with mixing 5 mL of *water R*, then 5 mL of *strong sodium hydroxide solution R*. Add 5 mL of *acetone R*. Shake and allow to stand. The upper layer is coloured deep violet.

PHOSPHATES (ORTHOPHOSPHATES)

a) To 5 mL of the prescribed solution, neutralised if necessary, add 5 mL of *silver nitrate solution R1*. A yellow precipitate is formed whose colour is not changed by boiling and which dissolves on addition of *ammonia R*.

b) Mix 1 mL of the prescribed solution with 2 mL of *molybdovanadic reagent R*. A yellow colour develops.

POTASSIUM

a) Dissolve 0.1 g of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 1 mL of *sodium carbonate solution R* and heat. No precipitate is formed. Add to the hot solution 0.05 mL of *sodium sulfide solution R*. No precipitate is formed. Cool in iced water and add 2 mL of a 150 g/L solution of *tartaric acid R*. Allow to stand. A white crystalline precipitate is formed.

b) Dissolve about 40 mg of the substance to be examined in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *dilute acetic acid R* and 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R*. A yellow or orange-yellow precipitate is formed immediately.

SALICYLATES

a) To 1 mL of the prescribed solution add 0.5 mL of *ferric chloride solution R1*. A violet colour is produced that persists after the addition of 0.1 mL of *acetic acid R*.

b) Dissolve 0.5 g of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.5 mL of *hydrochloric acid R*. The precipitate obtained, after recrystallisation from hot *water R* and drying *in vacuo*, has a melting point (*2.2.14*) of 156 °C to 161 °C.

SILICATES

Mix the prescribed quantity of the substance to be examined in a lead or platinum crucible by means of a copper wire with about 10 mg of *sodium fluoride R* and a few drops of *sulfuric acid R* to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of *water R* is suspended and warm gently. Within a short time a white ring is rapidly formed around the drop of water.

SILVER

Dissolve about 10 mg of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.3 mL of *hydrochloric acid R1*. A curdled, white precipitate is formed that dissolves on addition of 3 mL of *dilute ammonia R1*.

SODIUM

a) Dissolve 0.1 g of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 2 mL of a 150 g/L solution of *potassium carbonate R* and heat to boiling. No precipitate is formed. Add 4 mL of *potassium pyroantimonate solution R* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

b) Dissolve a quantity of the substance to be examined equivalent to about 2 mg of sodium ($Na^+$) in 0.5 mL of *water R* or use 0.5 mL of the prescribed solution. Add 1.5 mL of *methoxyphenylacetic reagent R* and cool in ice-water for 30 min. A voluminous, white, crystalline precipitate is formed. Place in water at 20 °C and stir for 5 min. The precipitate does not disappear. Add 1 mL of *dilute ammonia R1*. The precipitate dissolves completely. Add 1 mL of *ammonium carbonate solution R*. No precipitate is formed.

SULFATES

a) Dissolve about 45 mg of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 1 mL of *dilute hydrochloric acid R* and 1 mL of *barium chloride solution R1*. A white precipitate is formed.

b) To the suspension obtained during reaction (a), add 0.1 mL of *0.05 M iodine*. The suspension remains yellow (distinction from sulfites and dithionites), but is decolorised by adding dropwise *stannous chloride solution R* (distinction from iodates). Boil the mixture. No coloured precipitate is formed (distinction from selenates and tungstates).

TARTRATES

a) Dissolve about 15 mg of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 0.05 mL of a 10 g/L solution of *ferrous sulfate R* and 0.05 mL of *dilute hydrogen peroxide solution R*. A transient yellow colour is produced. After the colour has disappeared add *dilute sodium hydroxide solution R* dropwise. A violet or purple colour is produced.

b) To 0.1 mL of a solution of the substance to be examined containing the equivalent of about 15 mg of tartaric acid per millilitre or to 0.1 mL of the prescribed solution add 0.1 mL of a 100 g/L solution of *potassium bromide R*, 0.1 mL of a 20 g/L solution of *resorcinol R* and 3 mL of *sulfuric acid R*. Heat on a water-bath for 5 min to 10 min. A dark-blue colour develops. Allow to cool and pour the solution into *water R*. The colour changes to red.

XANTHINES

To a few milligrams of the substance to be examined or the prescribed quantity add 0.1 mL of *strong hydrogen peroxide solution R* and 0.3 mL of *dilute hydrochloric acid R*. Heat to dryness on a water-bath until a yellowish-red residue is obtained. Add 0.1 mL of *dilute ammonia R2*. The colour of the residue changes to violet-red.

ZINC

Dissolve 0.1 g of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 0.2 mL of *strong sodium hydroxide solution R*. A white precipitate is formed. Add a further 2 mL of *strong sodium hydroxide solution R*. The precipitate dissolves. Add 10 mL of *ammonium chloride solution R*. The solution remains clear. Add 0.1 mL of *sodium sulfide solution R*. A flocculent white precipitate is formed.

**01/2019:20302 corrected 9.8**

# 2.3.2. IDENTIFICATION OF FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

METHOD A

Thin-layer chromatography (*2.2.27*).

*Test solution.* Unless otherwise prescribed, dissolve about 20 mg (1 drop) of the fatty oil in 3 mL of *methylene chloride R*.

*Reference solution.* Dissolve about 20 mg (1 drop) of *maize oil R* in 3 mL of *methylene chloride R*.

*Plate*: a suitable octadecylsilyl silica gel for high-performance thin-layer chromatography as the coating substance.

*Mobile phase*:

– *mobile phase A*: *ether R*;

– *mobile phase B*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (20:40:50 *V/V/V*).

*Application*: 1 µL.

*Development*: twice over a path of 0.5 cm with mobile phase A, then twice over a path of 8 cm with mobile phase B.

*Drying*: in air.

*Detection*: spray with a 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*. Heat the plate at 120 °C for about 3 min and examine in daylight.

The chromatogram obtained typically shows spots comparable to those in Figure 2.3.2.-1.

METHOD B

Thin-layer chromatography (*2.2.27*).

*Test solution.* Unless otherwise prescribed, dissolve about 20 mg (1 drop) of the fatty oil in 3 mL of *methylene chloride R*.

*Reference solution.* Dissolve about 20 mg (1 drop) of *maize oil R* in 3 mL of *methylene chloride R*.

*Plate*: a suitable octadecylsilyl silica gel for high-performance thin-layer chromatography as the coating substance.

*Mobile phase*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (20:40:50 *V/V/V*).

*Application*: 1 µL as bands of 8 mm. A suitable automated apparatus may be used.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: treat with a 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*. Heat the plate at 120 °C for 3 min and examine in daylight.

The chromatogram obtained typically shows zones comparable to those in Figure 2.3.2.-2.

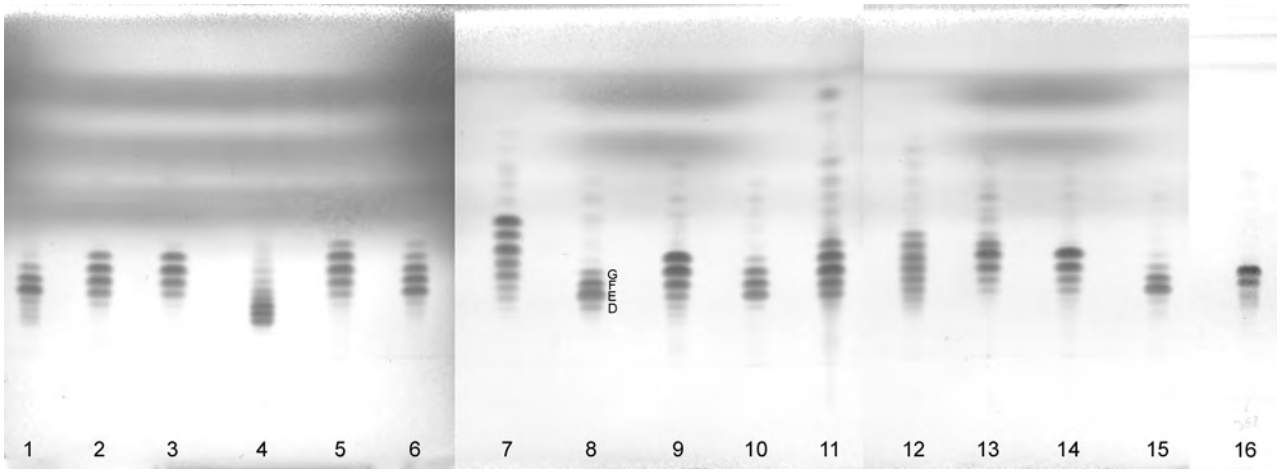


1. arachis oil
2. sesame oil
3. maize oil
4. rapeseed oil
5. soya-bean oil
6. rapeseed oil (erucic acid-free)
7. linseed oil
8. olive oil
9. sunflower oil
10. almond oil
11. wheat-germ oil
12. borage oil
13. evening primrose oil
14. safflower oil (type I)
15. safflower oil (type II)
16. hydrogenated arachis oil

Figure 2.3.2.-1. – *Chromatograms for the identification of fatty oils (method A)*

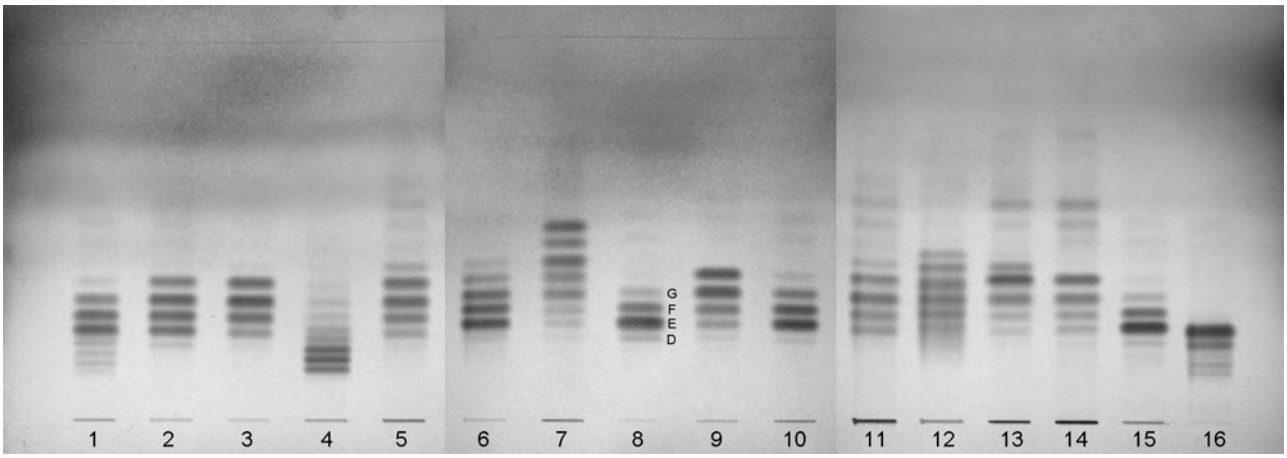


1. arachis oil
2. sesame oil
3. maize oil
4. rapeseed oil
5. soya-bean oil
6. rapeseed oil (erucic acid-free)
7. linseed oil
8. olive oil
9. sunflower oil
10. almond oil
11. wheat-germ oil
12. borage oil
13. evening primrose oil
14. safflower oil (type I)
15. safflower oil (type II)
16. hydrogenated arachis oil

Figure 2.3.2.-2. – *Chromatograms for the identification of fatty oils (method B)*

**01/2008:20303**

## 2.3.3. IDENTIFICATION OF PHENOTHIAZINES BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a closed tank containing the necessary quantity of the impregnation mixture composed of a solution containing 10 per cent *V/V* of *phenoxyethanol R* and 50 g/L of *macrogol 300 R* in *acetone R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen at least 17 cm from the lower edge of the plate, remove the plate and use immediately for chromatography. Carry out the chromatography in the same direction as the impregnation.

*Test solution.* Dissolve 20 mg of the substance to be examined in *chloroform R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of the corresponding chemical reference substance (CRS) in *chloroform R* and dilute to 10 mL with the same solvent.

Apply separately to the plate 2 µL of each solution and develop in the dark over a path of 15 cm using a mixture of 50 mL of *light petroleum R* and 1 mL of *diethylamine R* saturated with *phenoxyethanol R* (i.e. add about 3 mL to 4 mL of *phenoxyethanol R* to the above mixture of solvents to give a persistent cloudiness on shaking, decant, and use the supernatant, even if it is cloudy). After development place the plate under ultraviolet light at 365 nm and examine after a few minutes. The spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with the reference solution. Spray with a 10 per cent *V/V* solution of *sulfuric acid R* in *alcohol R*. The spot in the chromatogram obtained with the test solution is of the same colour as that in the chromatogram obtained with the reference solution and has similar stability over a period of at least 20 min.

**01/2008:20304**

## 2.3.4. ODOUR

On a watch-glass 6 cm to 8 cm in diameter, spread in a thin layer 0.5 g to 2.0 g of the substance to be examined. After 15 min, determine the odour or verify the absence of odour.

# 2.4. Limit tests



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# 2.4. LIMIT TESTS

**01/2008:20401**
**corrected 8.0**

## 2.4.1. AMMONIUM

*Unless otherwise prescribed, use method A.*

METHOD A

Introduce the prescribed solution into a test-tube or dissolve the prescribed quantity of the substance to be examined in 14 mL of *water R* in a test-tube. Make the solution alkaline if necessary by the addition of *dilute sodium hydroxide solution R*, dilute to 15 mL with *water R* and add 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. Prepare a standard by mixing 10 mL of *ammonium standard solution (1 ppm $NH_4$) R*, 5 mL of *water R* and 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. Stopper the test-tubes.

After 5 min, any yellow colour in the test solution is not more intense than that in the standard.

METHOD B

In a 25 mL jar fitted with a cap, place the prescribed quantity of the finely powdered substance to be examined and dissolve or suspend in 1 mL of *water R*. Add 0.30 g of *heavy magnesium oxide R*. Close immediately after placing a piece of *silver manganese paper R* 5 mm square, wetted with a few drops of *water R*, under the polyethylene cap. Swirl, avoiding projections of liquid, and allow to stand at 40 °C for 30 min. If the silver manganese paper shows a grey colour, it is not more intense than that of a standard prepared at the same time and in the same manner using the prescribed volume of *ammonium standard solution (1 ppm $NH_4$) R*, 1 mL of *water R* and 0.30 g of *heavy magnesium oxide R*.

**04/2018:20402**

## 2.4.2. ARSENIC

METHOD A

The apparatus (see Figure 2.4.2.-1) consists of a 100 mL conical flask closed with a ground-glass stopper through which passes a glass tube about 200 mm long and about 5 mm in internal diameter. The lower part of the tube tapers to an internal diameter of 1 mm, and about 20 mm from its tip is a lateral orifice 2-3 mm in diameter. When the tube is in position in the stopper, the lateral orifice is at least 3 mm below the lower surface of the stopper. A second glass tube of the same internal diameter is connected to the first tube. The second tube is bent twice at right angles and the free end of the tube tapers to an internal diameter of 1 mm. This end is immersed in a test-tube containing 3.0 mL of *silver diethyldithiocarbamate solution R*. Other suitable equipment may be used. Into the first tube insert 50-60 mg of *lead acetate cotton R*, loosely packed, or a small plug of cotton and a rolled piece of *lead acetate paper R* weighing 50-60 mg.

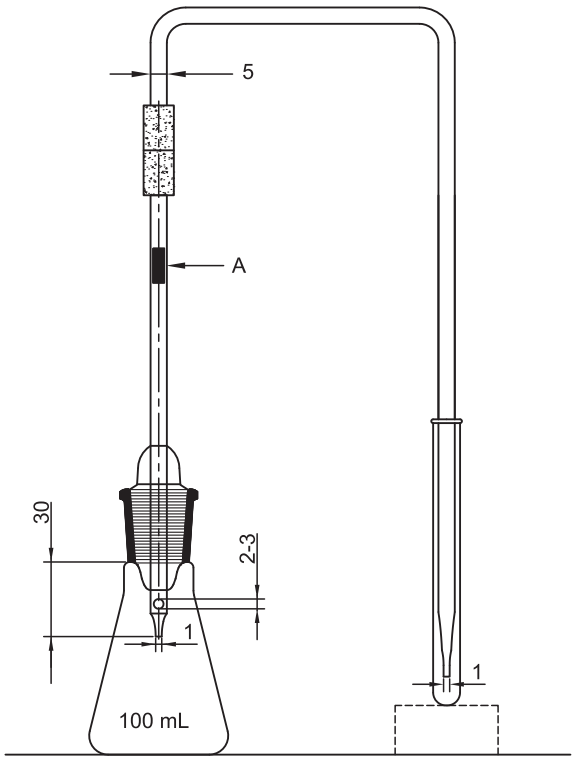


A. Lead acetate paper/cotton

Figure 2.4.2.-1. – *Apparatus for the limit test for arsenic (method A)*

*Dimensions in millimetres*

In the conical flask, dissolve the prescribed quantity of the substance to be examined in 25 mL of *water R*, or in the case of a solution adjust the prescribed volume to 25 mL with *water R*. Add 15 mL of *hydrochloric acid R*, 0.1 mL of *stannous chloride solution R* and 5 mL of *potassium iodide solution R*, allow to stand for 15 min and introduce 5 g of *activated zinc R*. Assemble the 2 parts of the apparatus immediately and immerse the flask in a water-bath at a temperature such that a uniform evolution of gas is maintained. Prepare a standard in the same manner, using 1 mL of *arsenic standard solution (1 ppm As) R*, diluted to 25 mL with *water R*. If foaming occurs, 1 mL of *2-propanol R* may be added to the flask.

After at least 2 h, the colour obtained in the test-tube with the test solution is not more intense than that obtained with the standard.

*Suitability test.* The colour obtained in the test-tube with the standard is at least as intensely coloured as 3 mL of a mixture of 3.0 mL of yellow primary solution, 0.6 mL of red primary solution and 11.40 mL of a solution of *hydrochloric acid R* (10 g/L HCl) (*2.2.2, Method I*).

METHOD B

Introduce the prescribed quantity of the substance to be examined into a test-tube containing 4 mL of *hydrochloric acid R* and about 5 mg of *potassium iodide R* and add 3 mL of *hypophosphorous reagent R*. Heat the mixture on a water-bath for 15 min, shaking occasionally. Prepare a standard in the same manner, using 0.5 mL of *arsenic standard solution (10 ppm As) R*.

After heating on the water-bath, the colour obtained in the test-tube with the test solution is not more intense than that obtained with the standard.

01/2008:20403
corrected 8.0

## 2.4.3. CALCIUM

*All solutions used for this test are prepared with distilled water R.*

To 0.2 mL of *alcoholic calcium standard solution (100 ppm Ca) R* add 1 mL of *ammonium oxalate solution R*. After 1 min add a mixture of 1 mL of *dilute acetic acid R* and 15 mL of the prescribed solution or of a solution containing the prescribed quantity of the substance to be examined, and shake. Prepare a standard in the same manner using a mixture of 10 mL of aqueous *calcium standard solution (10 ppm Ca) R*, 1 mL of *dilute acetic acid R* and 5 mL of *distilled water R*.

After 15 min, any opalescence in the test solution is not more intense than that in the standard.

01/2008:20404

## 2.4.4. CHLORIDES

To 15 mL of the prescribed solution add 1 mL of *dilute nitric acid R* and pour the mixture as a single addition into a test-tube containing 1 mL of *silver nitrate solution R2*. Prepare a standard in the same manner using 10 mL of *chloride standard solution (5 ppm Cl) R* and 5 mL of *water R*. Examine the tubes laterally against a black background.

After standing for 5 min protected from light, any opalescence in the test solution is not more intense than that in the standard.

01/2008:20405

## 2.4.5. FLUORIDES

Introduce into the inner tube of the apparatus (see Figure 2.4.5.-1) the prescribed quantity of the substance to be examined, 0.1 g of acid-washed *sand R* and 20 mL of a mixture of equal volumes of *sulfuric acid R* and *water R*. Heat the jacket containing *tetrachloroethane R* maintained at its boiling point (146 °C). Heat the steam generator and distil, collecting the distillate in a 100 mL volumetric flask containing 0.3 mL of *0.1 M sodium hydroxide* and 0.1 mL of *phenolphthalein solution R*. Maintain a constant volume (20 mL) in the tube during distillation and ensure that the distillate remains alkaline, adding *0.1 M sodium hydroxide* if necessary. Dilute the distillate to 100 mL with *water R* (test solution). Prepare a standard in the same manner by distillation, using 5 mL of *fluoride standard solution (10 ppm F) R* instead of the substance to be examined. Into two glass-stoppered cylinders introduce 20 mL of the test solution and 20 mL of the standard and 5 mL of *aminomethylalizarindiacetic acid reagent R*.

After 20 min, any blue colour in the test solution (originally red) is not more intense than that in the standard.

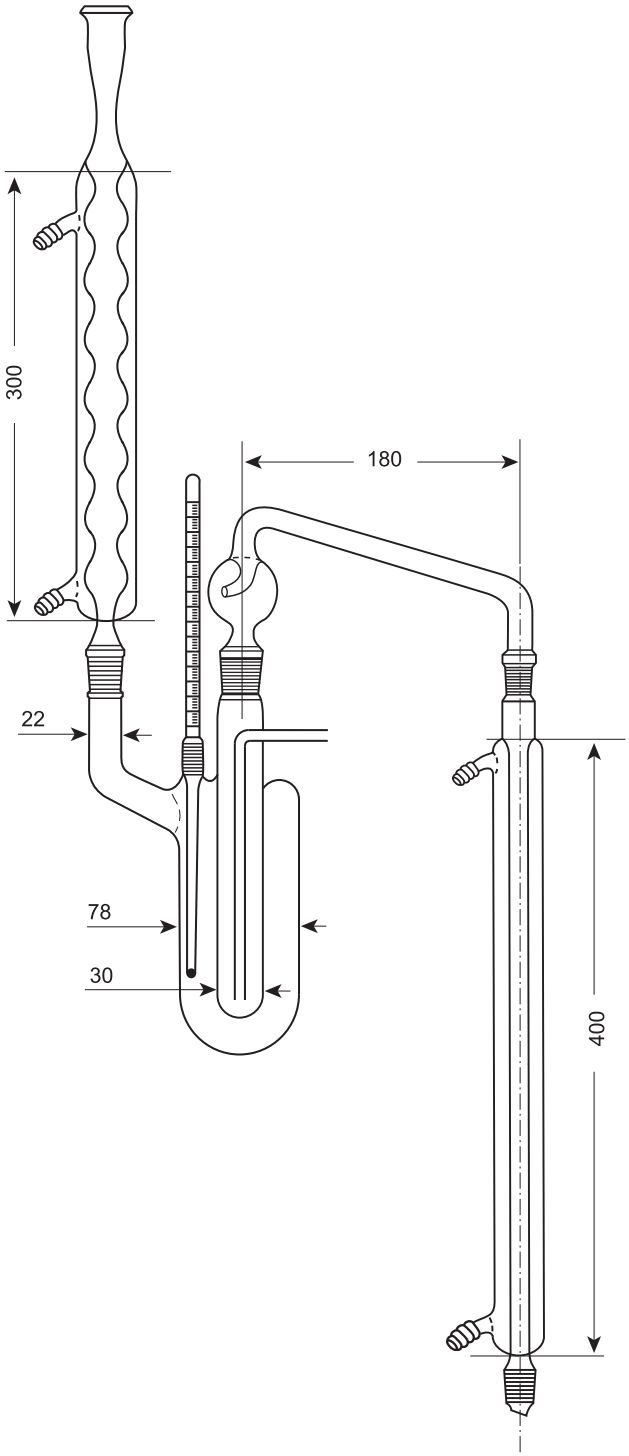


Figure 2.4.5.-1. – *Apparatus for limit test for fluorides*
*Dimensions in millimetres*

01/2008:20406

## 2.4.6. MAGNESIUM

To 10 mL of the prescribed solution add 0.1 g of *disodium tetraborate R*. Adjust the solution, if necessary, to pH 8.8 to pH 9.2 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Shake with 2 quantities, each of 5 mL, of a 1 g/L solution of *hydroxyquinoline R* in *chloroform R*, for 1 min each time. Allow to stand. Separate and discard the organic layer. To the aqueous solution add 0.4 mL of *butylamine R* and 0.1 mL of *triethanolamine R*. Adjust the

solution, if necessary, to pH 10.5 to pH 11.5. Add 4 mL of the solution of hydroxyquinoline in chloroform, shake for 1 min, allow to stand and separate. Use the lower layer for comparison. Prepare a standard in the same manner using a mixture of 1 mL of *magnesium standard solution (10 ppm Mg) R* and 9 mL of *water R*.

Any colour in the solution obtained from the substance to be examined is not more intense than that in the standard.

**01/2008:20407**

## 2.4.7. MAGNESIUM AND ALKALINE-EARTH METALS

To 200 mL of *water R* add 0.1 g of *hydroxylamine hydrochloride R*, 10 mL of *ammonium chloride buffer solution pH 10.0 R*, 1 mL of *0.1 M zinc sulfate* and about 15 mg of *mordant black 11 triturate R*. Heat to about 40 °C. Titrate with *0.01 M sodium edetate* until the violet colour changes to full blue. To the solution add the prescribed quantity of the substance to be examined dissolved in 100 mL of *water R* or use the prescribed solution. If the colour of the solution changes to violet, titrate with *0.01 M sodium edetate* until the full blue colour is again obtained.

The volume of *0.01 M sodium edetate* used in the second titration does not exceed the prescribed quantity.

**07/2010:20408**

## 2.4.8. HEAVY METALS

The methods described below require the use of *thioacetamide reagent R*. As an alternative, *sodium sulfide solution R1* (0.1 mL) is usually suitable. Since tests prescribed in monographs have been developed using *thioacetamide reagent R*, if *sodium sulfide solution R1* is used instead, it is necessary to include also for methods A, B and H a monitor solution, prepared from the quantity of the substance to be examined prescribed for the test, to which has been added the volume of lead standard solution prescribed for preparation of the reference solution. The test is invalid if the monitor solution is not at least as intense as the reference solution.

METHOD A

*Test solution.* 12 mL of the prescribed aqueous solution of the substance to be examined.

*Reference solution (standard).* A mixture of 10 mL of *lead standard solution (1 ppm Pb) R* or *lead standard solution (2 ppm Pb) R*, as prescribed, and 2 mL of the prescribed aqueous solution of the substance to be examined.

*Blank solution.* A mixture of 10 mL of *water R* and 2 mL of the prescribed aqueous solution of the substance to be examined.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

*System suitability*: the reference solution shows a slight brown colour compared to the blank solution.

*Result*: any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD B

*Test solution.* 12 mL of the prescribed solution of the substance to be examined prepared using an organic solvent containing a minimum percentage of water (for example, dioxan containing 15 per cent of water or acetone containing 15 per cent of water).

*Reference solution (standard).* A mixture of 10 mL of lead standard solution (1 or 2 ppm Pb), as prescribed, and 2 mL of the prescribed solution of the substance to be examined in an organic solvent. Prepare the lead standard solution (1 or 2 ppm Pb) by dilution of *lead standard solution (100 ppm Pb) R* with the solvent used for the substance to be examined.

*Blank solution.* A mixture of 10 mL of the solvent used for the substance to be examined and 2 mL of the prescribed solution of the substance to be examined in an organic solvent.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

*System suitability*: the reference solution shows a slight brown colour compared to the blank solution.

*Result*: any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD C

*Test solution.* Place the prescribed quantity (not more than 2 g) of the substance to be examined in a silica crucible with 4 mL of a 250 g/L solution of *magnesium sulfate R* in *dilute sulfuric acid R*. Mix using a fine glass rod. Heat cautiously. If the mixture is liquid, evaporate gently to dryness on a water-bath. Progressively heat to ignition and continue heating until an almost white or at most greyish residue is obtained. Carry out the ignition at a temperature not exceeding 800 °C. Allow to cool. Moisten the residue with a few drops of *dilute sulfuric acid R*. Evaporate, ignite again and allow to cool. The total period of ignition must not exceed 2 h. Take up the residue in 2 quantities, each of 5 mL, of *dilute hydrochloric acid R*. Add 0.1 mL of *phenolphthalein solution R*, then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 mL in excess. Filter if necessary and wash the filter. Dilute to 20 mL with *water R*.

*Reference solution (standard).* Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined. To 10 mL of the solution obtained add 2 mL of the test solution.

*Monitor solution.* Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution. To 10 mL of the solution obtained add 2 mL of the test solution.

*Blank solution.* A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

*System suitability*:

- the reference solution shows a slight brown colour compared to the blank solution,
- the monitor solution is at least as intense as the reference solution.

*Result*: any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD D

*Test solution.* In a silica crucible, mix thoroughly the prescribed quantity of the substance to be examined with 0.5 g of *magnesium oxide R1*. Ignite to dull redness until a homogeneous white or greyish-white mass is obtained. If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800 °C for about 1 h. Take up the residue in 2 quantities, each of 5 mL, of a mixture of equal volumes of *hydrochloric acid R1* and *water R*. Add 0.1 mL of *phenolphthalein solution R* and then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 mL in excess. Filter if necessary and wash the filter. Dilute to 20 mL with *water R*.

*Reference solution (standard).* Prepare as described for the test solution using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined and drying in an oven at 100-105 °C. To 10 mL of the solution obtained add 2 mL of the test solution.

*Monitor solution.* Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution and drying in an oven at 100-105 °C. To 10 mL of the solution obtained add 2 mL of the test solution.

*Blank solution.* A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

*System suitability*:

- the reference solution shows a slight brown colour compared to the blank solution,
- the monitor solution is at least as intense as the reference solution.

*Result*: any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD E

*Test solution.* Dissolve the prescribed quantity of the substance to be examined in 30 mL of *water R* or the prescribed volume.

*Reference solution (standard).* Unless otherwise prescribed, dilute the prescribed volume of *lead standard solution (1 ppm Pb) R* to the same volume as the test solution.

Prepare the filtration apparatus by adapting the barrel of a 50 mL syringe without its piston to a support containing, on the plate, a membrane filter (nominal pore size 3 µm) and above it a prefilter (Figure 2.4.8.-1).

Transfer the test solution into the syringe barrel, put the piston in place and then apply an even pressure on it until the whole of the liquid has been filtered. In opening the support and removing the prefilter, check that the membrane filter remains uncontaminated with impurities. If this is not the case replace it with another membrane filter and repeat the operation under the same conditions.

To the prefiltrate or to the prescribed volume of the prefiltrate add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately and allow to stand for 10 min and again filter as described above, but inverting the order of the filters, the liquid passing first through the membrane filter before passing through the prefilter (Figure 2.4.8.-1). The filtration must be carried out slowly and uniformly by applying moderate and constant pressure to the piston of the syringe. After complete filtration, open the support, remove the membrane filter, and dry using filter paper.

In parallel, treat the reference solution in the same manner as the test solution.

*Result*: the colour of the spot obtained with the test solution is not more intense than that obtained with the reference solution.

METHOD F

*Test solution.* Place the prescribed quantity or volume of the substance to be examined in a clean, dry, 100 mL long-necked combustion flask (a 300 mL flask may be used if the reaction

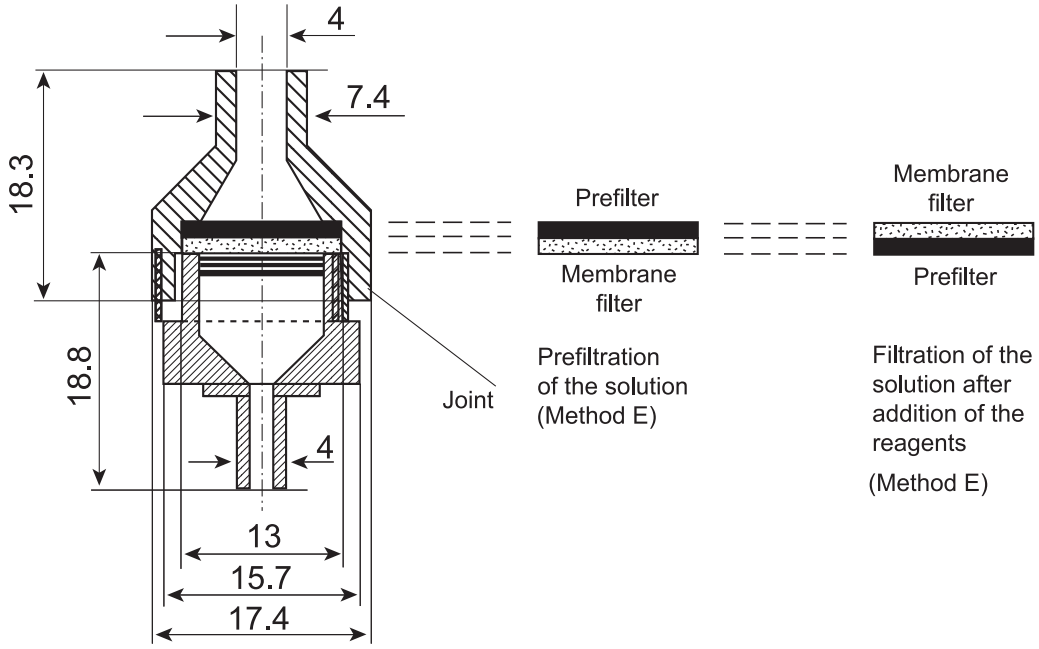


Figure 2.4.8.-1. – *Apparatus for the test for heavy metals*
*Dimensions in millimetres*

foams excessively). Clamp the flask at an angle of 45°. If the substance to be examined is a solid, add a sufficient volume of a mixture of 8 mL of *sulfuric acid R* and 10 mL of *nitric acid R* to moisten the substance thoroughly; if the substance to be examined is a liquid, add a few millilitres of a mixture of 8 mL of *sulfuric acid R* and 10 mL of *nitric acid R*. Warm gently until the reaction commences, allow the reaction to subside and add additional portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat and boil gently until the solution darkens. Cool, add 2 mL of *nitric acid R* and heat again until the solution darkens. Continue the heating, followed by the addition of *nitric acid R* until no further darkening occurs, then heat strongly until dense, white fumes are produced. Cool, cautiously add 5 mL of *water R*, boil gently until dense, white fumes are produced and continue heating to reduce to 2-3 mL. Cool, cautiously add 5 mL of *water R* and examine the colour of the solution. If the colour is yellow, cautiously add 1 mL of *strong hydrogen peroxide solution R* and again evaporate until dense, white fumes are produced and reduce to a volume of 2-3 mL. If the solution is still yellow in colour, repeat the addition of 5 mL of *water R* and 1 mL of *strong hydrogen peroxide solution R* until the solution is colourless. Cool, dilute cautiously with *water R* and rinse into a 50 mL colour comparison tube, ensuring that the total volume does not exceed 25 mL. Adjust the solution to pH 3.0-4.0, using short range pH indicator paper as external indicator, with *concentrated ammonia R1* (*dilute ammonia R1* may be used, if desired, as the specified range is approached), dilute with *water R* to 40 mL and mix. Add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Dilute to 50 mL with *water R* and mix.

*Reference solution (standard).* Prepare at the same time and in the same manner as the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R*.

*Monitor solution.* Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

*Blank solution.* Prepare as described for the test solution, omitting the substance to be examined.

Examine the solutions vertically against a white background after 2 min.

*System suitability*:

- the reference solution shows a brown colour compared to the blank solution,
- the monitor solution is at least as intense as the reference solution.

*Result*: any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD G

*CAUTION: when using high-pressure digestion vessels the safety precautions and operating instructions given by the manufacturer must be followed. The digestion cycles have to be elaborated depending on the type of microwave oven to be used (for example, energy-controlled microwave ovens, temperature-controlled microwave ovens or high-pressure ovens). The cycle must conform to the manufacturer's instructions. The digestion cycle is suitable if a clear solution is obtained.*

*Test solution.* Place the prescribed amount of the substance to be examined (not more than 0.5 g) in a suitable, clean beaker. Add successively 2.7 mL of *sulfuric acid R*, 3.3 mL of *nitric acid R* and 2.0 mL of *strong hydrogen peroxide solution R* using a magnetic stirrer. Allow the substance to react with a reagent before adding the next one. Transfer the mixture to a dry high-pressure-resistant digestion vessel (fluoropolymer or quartz glass).

*Reference solution (standard).* Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined.

*Monitor solution.* Prepare as prescribed for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

*Blank solution.* Prepare as described for the test solution, omitting the substance to be examined.

Close the vessels and place in a laboratory microwave oven. Digest using a sequence of 2 separate suitable programmes. Design the programmes in several steps in order to control the reaction, monitoring pressure, temperature or energy depending on the type of microwave oven available. After the first programme allow the digestion vessels to cool before opening. Add to each vessel 2.0 mL of *strong hydrogen peroxide solution R* and digest using the second programme. After the second programme allow the digestion vessels to cool before opening. If necessary to obtain a clear solution, repeat the addition of *strong hydrogen peroxide solution R* and the second digestion programme.

Cool, dilute cautiously with *water R* and rinse into a flask, ensuring that the total volume does not exceed 25 mL.

Using short-range pH indicator paper as external indicator, adjust the solutions to pH 3.0-4.0 with *concentrated ammonia R1* (*dilute ammonia R1* may be used as the specified range is approached). To avoid heating of the solutions use an ice-bath and a magnetic stirrer. Dilute to 40 mL with *water R* and mix. Add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Dilute to 50 mL with *water R*, mix and allow to stand for 2 min.

Filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

*System suitability*:

- the spot obtained with the reference solution shows a brown colour compared to the spot obtained with the blank solution,
- the spot obtained with the monitor solution is at least as intense as the spot obtained with the reference solution.

*Result*: the brown colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

METHOD H

*Test solution.* Dissolve the prescribed quantity of the substance to be examined in 20 mL of the solvent or solvent mixture prescribed.

*Reference solution.* Dilute the prescribed volume of *lead standard solution (10 ppm Pb) R* to 20 mL with the solvent or solvent mixture prescribed.

*Blank solution.* 20 mL of the solvent or solvent mixture prescribed.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix. *(In some cases precipitation occurs, in which case the specific monograph would describe re-dissolution in a defined volume of a given solvent.)* Add to 1.2 mL of *thioacetamide reagent R*. Mix immediately and allow to stand for 2 min. Filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Compare the spots on the filters obtained with the different solutions.

*System suitability*: the spot obtained with the reference solution shows a brownish-black colour compared to the spot obtained with the blank solution.

*Result*: the brownish-black colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

**01/2008:20409**

## 2.4.9. IRON

Dissolve the prescribed quantity of the substance to be examined in *water R* and dilute to 10 mL with the same solvent or use 10 mL of the prescribed solution. Add 2 mL of a 200 g/L solution of *citric acid monohydrate R* and 0.1 mL of *thioglycollic acid R*. Mix, make alkaline with *ammonia R* and dilute to 20 mL with *water R*. Prepare a standard in the same manner, using 10 mL of *iron standard solution (1 ppm Fe) R*.

After 5 min, any pink colour in the test solution is not more intense than that in the standard.

**01/2008:20410**

## 2.4.10. LEAD IN SUGARS

Determine the lead by atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Add 2.0 mL of a clear 10 g/L solution of *ammonium pyrrolidinedithiocarbamate R* and 10.0 mL of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

*Reference solutions.* Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *lead standard solution (10 ppm Pb) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for the test solution without the substance to be examined. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 0.5 ppm of lead, unless otherwise prescribed.

**01/2008:20411**

## 2.4.11. PHOSPHATES

To 100 mL of the solution prepared and, if necessary, neutralised as prescribed add 4 mL of *sulfomolybdic reagent R3*. Shake and add 0.1 mL of *stannous chloride solution R1*. Prepare a standard in the same manner using 2 mL of *phosphate standard solution (5 ppm $PO_4$) R* and 98 mL of *water R*. After 10 min, compare the colours using 20 mL of each solution.

Any colour in the test solution is not more intense than that in the standard.

**01/2008:20412**

## 2.4.12. POTASSIUM

To 10 mL of the prescribed solution add 2 mL of a freshly prepared 10 g/L solution of *sodium tetraphenylborate R*. Prepare a standard in the same manner using a mixture of 5 mL of *potassium standard solution (20 ppm K) R* and 5 mL of *water R*.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

**01/2008:20413 corrected 8.0**

## 2.4.13. SULFATES

*All solutions used for this test must be prepared with distilled water R.*

Add 3 mL of a 250 g/L solution of *barium chloride R* to 4.5 mL of *sulfate standard solution (10 ppm $SO_4$) R1*. Shake and allow to stand for 1 min. To 2.5 mL of this suspension add 15 mL of the prescribed solution and 0.5 mL of *acetic acid R*. Prepare a standard in the same manner using 15 mL of *sulfate standard solution (10 ppm $SO_4$) R* instead of the prescribed solution.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

**04/2010:20414**

## 2.4.14. SULFATED ASH[(1)]

Ignite a suitable crucible (for example, silica, platinum, porcelain or quartz) at 600 ± 50 °C for 30 min, allow to cool in a desiccator over silica gel or other suitable desiccant and weigh. Place the prescribed amount of the substance to be examined in the crucible and weigh. Moisten the substance to be examined with a small amount of *sulfuric acid R* (usually 1 mL) and heat gently at as low a temperature as practicable until the sample is thoroughly charred. After cooling, moisten the residue with a small amount of *sulfuric acid R* (usually 1 mL), heat gently until white fumes are no longer evolved and ignite at 600 ± 50 °C until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel or other suitable desiccant, weigh it again and calculate the percentage of residue.

If the amount of the residue so obtained exceeds the prescribed limit, repeat the moistening with *sulfuric acid R* and ignition, as previously, for 30 min periods until 2 consecutive weighings do not differ by more than 0.5 mg or until the percentage of residue complies with the prescribed limit.

The amount of substance used for the test (usually 1-2 g) is chosen so that at the prescribed limit the mass of the residue (usually about 1 mg) can be measured with sufficient accuracy.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**01/2008:20415 corrected 7.0**

## 2.4.15. NICKEL IN POLYOLS

Determine the nickel by atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Add 2.0 mL of a saturated solution of *ammonium pyrrolidinedithiocarbamate R* (about 10 g/L) and 10.0 mL of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

*Reference solutions.* Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *nickel standard solution (10 ppm Ni) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for preparation of the test solution omitting the substance to be examined. Measure the absorbance at 232.0 nm using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 1 ppm of nickel, unless otherwise prescribed.

**01/2008:20416**

## 2.4.16. TOTAL ASH

Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Unless otherwise prescribed, evenly distribute 1.00 g of the substance or the powdered herbal drug to be examined in the crucible. Dry at 100 °C to 105 °C for 1 h and ignite to constant mass in a muffle furnace at 600 °C ± 25 °C, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant mass.

**01/2008:20417**

## 2.4.17. ALUMINIUM

Place the prescribed solution in a separating funnel and shake with 2 quantities, each of 20 mL, and then with one 10 mL quantity of a 5 g/L solution of *hydroxyquinoline R* in *chloroform R*. Dilute the combined chloroform solutions to 50.0 mL with *chloroform R* (test solution).

Prepare a standard in the same manner using the prescribed reference solution.

Prepare a blank in the same manner using the prescribed blank solution.

Measure the intensity of the fluorescence (*2.2.21*) of the test solution ($I_1$), of the standard ($I_2$) and of the blank ($I_3$) using an excitant beam at 392 nm and a secondary filter with a transmission band centred on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence ($I_1 - I_3$) of the test solution is not greater than that of the standard ($I_2 - I_3$).

**01/2008:20418**

## 2.4.18. FREE FORMALDEHYDE

Use method A, unless otherwise prescribed. Method B is suitable for vaccines where sodium metabisulfite has been used to neutralise excess formaldehyde.

METHOD A

For vaccines for human use, prepare a 1 in 10 dilution of the vaccine to be examined. For bacterial toxoids for veterinary use, prepare a 1 in 25 dilution of the vaccine to be examined.

To 1 mL of the dilution, add 4 mL of *water R* and 5 mL of *acetylacetone reagent R1*. Place the tube in a water-bath at 40 °C for 40 min. Examine the tubes down their vertical axes. The solution is not more intensely coloured than a standard, prepared at the same time and in the same manner, using 1 mL of a dilution of *formaldehyde solution R* containing 20 µg of formaldehyde ($CH_2O$) per millilitre, instead of the dilution of the vaccine to be examined.

METHOD B

*Test solution.* Prepare a 1 in 200 dilution of the vaccine to be examined with *water R*. If the vaccine is an emulsion, prepare an equivalent dilution using the aqueous phase separated by a suitable procedure (see below). If one of the methods described below is used for separation of the aqueous phase, a 1 in 20 dilution of the latter is used.

*Reference solutions.* Prepare solutions containing 0.25 g/L, 0.50 g/L, 1.00 g/L and 2.00 g/L of $CH_2O$ by dilution of *formaldehyde solution R* with *water R*. Prepare a 1 in 200 dilution of each solution with *water R*.

To 0.5 mL of the test solution and of each of the reference solutions in test-tubes, add 5.0 mL of a freshly prepared 0.5 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R*. Close the tubes, shake and allow to stand for 60 min. Add 1 mL of *ferric chloride-sulfamic acid reagent R* and allow to stand for 15 min. Measure the absorbance (*2.2.25*) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine to be examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient ($r$) of the calibration curve is less than 0.97.

*Emulsions.* If the vaccine to be examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. The following procedures have been found suitable.

(a) Add 1.0 mL of the vaccine to be examined to 1.0 mL of *isopropyl myristate R* and mix. Add 1.3 mL of *1 M hydrochloric acid*, 2.0 mL of *chloroform R* and 2.7 mL of a 9 g/L solution of *sodium chloride R*. Mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*. If this procedure fails to separate the aqueous phase, add 100 g/L of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuge at 22 500 *g*.

(b) Add 1.0 mL of the vaccine to be examined to 1.0 mL of a 100 g/L solution of *sodium chloride R* and mix. Centrifuge at 1000 *g* for 15 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

(c) Add 1.0 mL of the vaccine to be examined to 2.0 mL of a 100 g/L solution of *sodium chloride R* and 3.0 mL of *chloroform R* and mix. Centrifuge at 1000 *g* for 5 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

01/2008:20419

## 2.4.19. ALKALINE IMPURITIES IN FATTY OILS

In a test-tube mix 10 mL of recently distilled *acetone R* and 0.3 mL of *water R* and add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *alcohol R*. Neutralise the solution if necessary with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*. Add 10 mL of the oil to be examined, shake and allow to stand. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

07/2018:20420
corrected 9.6

## 2.4.20. DETERMINATION OF ELEMENTAL IMPURITIES

INTRODUCTION

This chapter describes the general approach for the determination of elemental impurities in medicinal products or substances for pharmaceutical use. As the chemical composition of the considered samples and the specification limits for the element(s) of interest vary considerably, it is not possible to describe all suitable sample preparation and measurement methods. Therefore, any method that fulfils the requirements described in this chapter may be used.

The results of the analysis are acceptable only if the system suitability has been demonstrated by a suitable test. Before the initial use of a method, the analyst must ensure that the method is appropriate for the samples and instruments used. This is accomplished by applying a validation procedure to methods not described in the individual monograph or by a system suitability test for methods which are described in the monograph. Decision trees for the choice of the sample preparation and the measurement procedures are presented in Figures 2.4.20.-1 and 2.4.20.-2.

PROCEDURES

As a reference procedure is not provided for each element, matrix and concentration, the choice of procedure according to Figures 2.4.20.-1 and 2.4.20.-2, including sample preparation, detection technique and instrument parameters, is the responsibility of the user.

Use the flow chart in Figure 2.4.20.-1 to define the sample preparation method and the flow chart in Figure 2.4.20.-2 to define the measurement method. The sample preparation method should yield a sufficient quantity of sample to allow quantification of each element at the specified limit stated in the individual monograph or the general chapter.

All suitable sample preparation methods and measurement techniques (e.g. *2.2.22. Atomic emission spectrometry* (AES), *2.2.23. Atomic absorption spectrometry* (AAS), *2.2.37. X-ray fluorescence spectrometry* (XRFS), *2.2.57. Inductively coupled plasma-atomic emission spectrometry* (ICP-AES), *2.2.58. Inductively coupled plasma-mass spectrometry* (ICP-MS), *2.4.2. Arsenic*, *2.4.8. Heavy metals*, *2.4.9. Iron*, *2.4.10. Lead in sugars*, *2.4.15. Nickel in polyols*, *2.4.31. Nickel in hydrogenated vegetable oils*) can be used for the determination of elemental impurities, if the method has been verified before the initial use by a system suitability test or a validation procedure according to this chapter.

If no sample preparation and/or measurement method is described in the individual monograph, a suitable sample preparation and/or measurement method must be developed and validated (see Figures 2.4.20.-1 and 2.4.20.-2).

*SAMPLE PREPARATION*

Sample preparation is critical to the success of elemental analysis. Many techniques not using direct measurement are heavily dependent on sample transport.

If an atomisation system is used, the most conventional means by which samples are introduced into the atomisation system is by solution nebulisation. In this case, solid samples must be dissolved in order to be introduced into the atomisation system. Samples may be dissolved in any appropriate solvent. The use of aqueous or dilute nitric acid solutions is strongly recommended, due to minimal interference with these solvents compared to other solvents. Hydrochloric acid, hydrofluoric acid, perchloric acid, sulfuric acid and hydrogen peroxide, at various concentrations, can be used to dissolve the samples. The viscosity of sulfuric acid is greater than that of the other acids and is to be taken into account as it can affect the overall fluidity of the solution.

The choice of solvents also includes, but is not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

Acids, bases, and hydrogen peroxide of high purity must be used, especially when ICP-MS is employed. For aqueous solutions, use *deionised distilled water R*. Diluents must be checked for interference if they are used in an analysis. Because it is not always possible to obtain organic solvents that are free from elemental impurities, organic solvents of the highest purity possible with regard to these contaminants must be used. Specifically for ICP techniques, where samples are introduced into the plasma via solution nebulisation, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP-AES and ICP-MS analyses in cases where accuracy and precision are not sufficient. In any case, the selection of an appropriate internal standard should take into account the element(s) of interest, ionisation energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion or incineration techniques can be employed. These include hot-plate digestion, incineration and microwave-assisted digestions, using an open- or closed-vessel.

The decision regarding the type of digestion technique to be used depends on the nature of the sample being digested, as well as on the element(s) of interest and the concentration range of the elements to be quantified. Open-vessel digestion is not recommended for the analysis of volatile elements. The suitability of a digestion technique, whether open- or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile elements have not been lost during sample preparation. The digestion cycle is suitable if a clear solution is obtained.

It is important to consider the selection of the type, the material of construction, the pretreatment, and the cleaning of analytical labware used in elemental analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, care must be exercised to prevent the adsorption of elemental impurities onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of sample solutions by elemental impurities and ions present in the container can also lead to inaccurate results.

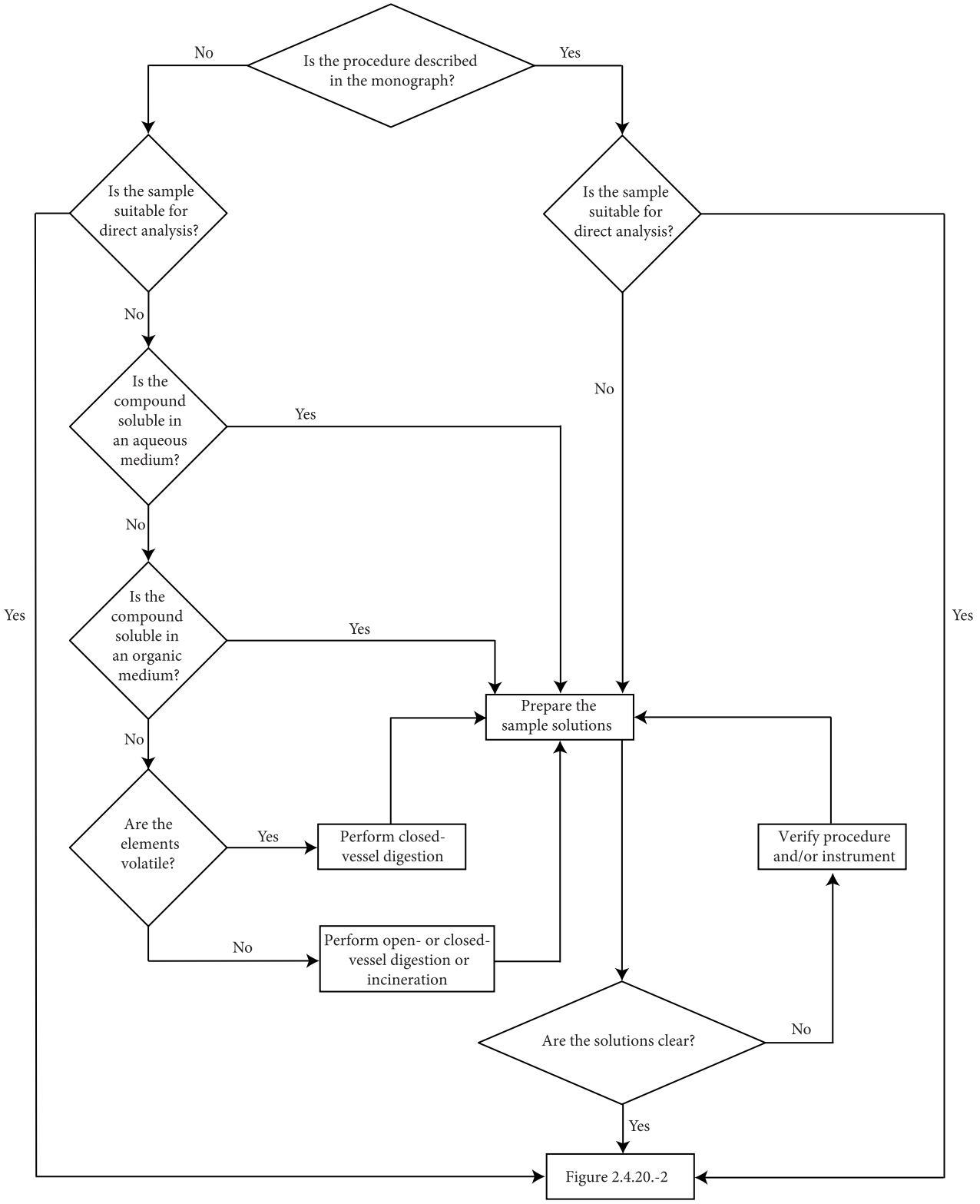


Figure 2.4.20.-1. – *Elemental impurities decision tree: sample preparation*

The use of volumetric glassware that does not comply with Class A requirements of the appropriate International Standard of the International Organization for Standardization (ISO) is acceptable if the validation or the system suitability test of the method using such glassware have experimentally demonstrated that the method is suitable for the intended purpose.

*CAUTION: when using high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.*

*MEASUREMENT*

**Method**. The choice of the techniques depends mainly on the sample matrix and the characteristics and specification limits of the element(s) of interest. Analyse according to the instructions of the manufacturer of the equipment regarding program and wavelength.

**System suitability**. A system suitability test must be carried out on the day of the analysis to ensure that the sample preparation and measurement system are appropriate.

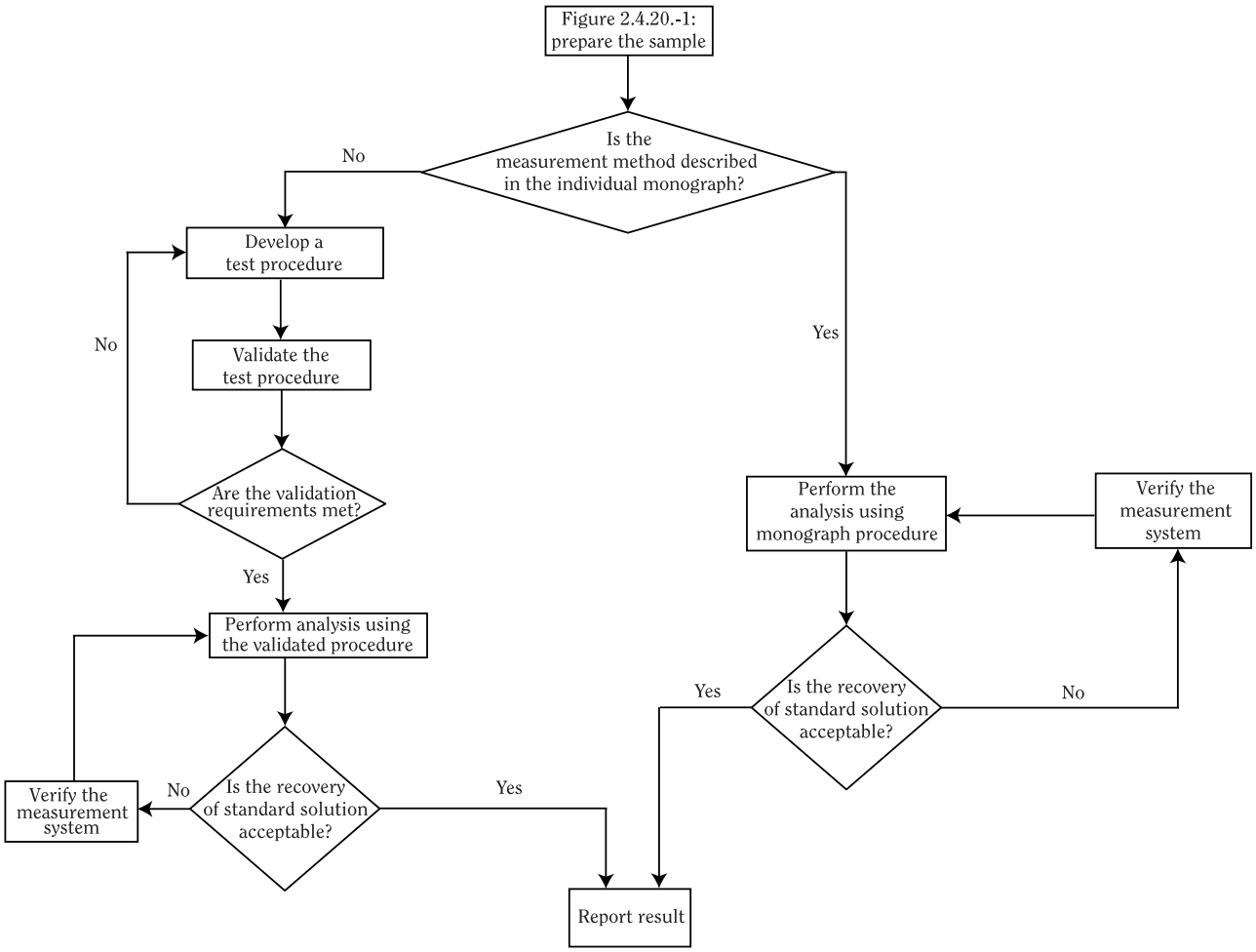


Figure 2.4.20.-2. – *Elemental impurities decision tree: measurement*

*Acceptance criterion for preparation of sample solution*: a clear solution is obtained.

*Acceptance criterion for measurement system*: the measured concentration of a standard solution of the element at a concentration within the range of the used calibration curve does not differ from the actual concentration by more than 20 per cent.

**Calculation**. The blank value of reagents must be taken into account for the calculation of the content. Upon completion of the analysis, the concentration of a given element in the sample is calculated by the software of the instrument from the concentration of the element in the test solution. If no calculation software is available or no indication for calculation is given in the general chapter corresponding to the method used, the concentration of a given element in the sample can be calculated from the concentration of the element in the solution using the following expression:

$$C = A \times \frac{V_1}{m} \times \frac{V_2}{V_3}$$

$C$ = concentration of element in the analysed sample, in micrograms per gram;

$A$ = instrument reading of the concentration of the element in the sample solution, in micrograms per millilitre;

$m$ = mass of the sample in the initial sample solution, in grams;

$V_1$ = volume of the initial sample preparation, in millilitres;

$V_2$ = total volume of any dilution performed, in millilitres;

$V_3$ = volume of initial sample preparation used in any dilution performed, in millilitres.

VALIDATION REQUIREMENTS

Some validation requirements provided below may differ from those provided in general chapters of the Ph. Eur. (e.g. *2.2.22* (AES), *2.2.23* (AAS), *2.2.57* (ICP-AES), *2.2.58* (ICP-MS)).

Before the initial use of the selected procedure, the analyst must ensure that the sample preparation and measurement method are appropriate for the element(s) of interest, sample matrix and instrument used. This is accomplished by following the validation procedure before the initial use and the system suitability test on the day of the analysis.

For elemental impurities, validation of a limit test must include specificity and limit of detection.

The following section defines the characteristics for the acceptability of a quantitative procedure. It must be demonstrated experimentally that such a procedure complies with the validation requirements, with an appropriate system suitability test using material spiked with a suitable reference material. The test materials must be spiked before any sample preparation steps. For example, if a test material is to be digested, the material must be spiked at the beginning of the digestion procedure.

*SPECIFICITY*

Specificity is the ability to ensure that the analytical procedure (sample preparation and measurement) allows a reliable determination of the element(s) of interest in the presence of components (e.g. carrier gas, impurities, matrix) that may be expected to be present.

*Acceptance criteria*: the procedure must be able to assess unequivocally each elemental impurity to be determined with this procedure in the presence of components that may be expected to be present, including other elemental impurities, matrix components and other sources of interference; specificity is demonstrated by complying with the accuracy requirement for the element(s) to be determined.

*RANGE*

*Acceptance criterion*: range is demonstrated by complying with the recovery requirement.

*ACCURACY*

Verify the accuracy using a certified reference material or by performing a test for recovery. *Elemental impurity solutions CRS* may be used.

The recovery may be determined on a sample of the substance to be examined, spiked with a known quantity of a reference standard of the element of interest (3 concentration levels in the range of 50-150 per cent of the intended specification limit, even if the original concentration of the reference standard is at the specified value), in triplicate.

*Acceptance criterion*: spike recovery is within 70 per cent and 150 per cent for the mean of 3 replicates at each concentration.

*REPEATABILITY*

*Test samples*: either 6 independent samples of the substance to be examined spiked with a suitable reference standard at the specified concentration level, or 3 concentration levels prepared in triplicate.

*Acceptance criterion*: the relative standard deviation is in both cases not more than 20 per cent.

*INTERMEDIATE PRECISION*

The effect of random events (intra-laboratory variations) on the analytical precision of the method must be established. Acceptable experiments for establishing intermediate precision include performing the repeatability analysis on different days, or with different instrumentation, or by different analysts. Only 1 of the 3 experiments is required to demonstrate intermediate precision.

*Acceptance criterion*: the relative standard deviation is not more than 25 per cent.

*LIMIT OF QUANTIFICATION*

Use the results from the accuracy study. Determine the lowest concentration meeting the acceptance criterion.

*Acceptance criterion*: the limit of quantification is below the specification limit.

*LIMIT OF DETECTION (ONLY APPLICABLE TO LIMIT TESTS)*

Determine the lowest concentration giving a signal clearly distinct from that obtained with a blank solution.

*Acceptance criterion*: the limit of detection is not more than 0.5 times the concentration of the specification limit.

**01/2008:20421**

## 2.4.21. FOREIGN OILS IN FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate a plate by placing it in a chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *liquid paraffin R* and 90 volumes of *light petroleum R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen by at least 12 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 5 min. Carry out the chromatography in the same direction as the impregnation.

*Preparation of the mixture of fatty acids.* Heat 2 g of the oil with 30 mL of *0.5 M alcoholic potassium hydroxide* under a reflux condenser for 45 min. Add 50 mL of *water R*, allow to cool, transfer to a separating funnel and extract with three quantities, each of 50 mL, of *ether R*. Discard the ether extracts, acidify the aqueous layer with *hydrochloric acid R* and extract with three quantities, each of 50 mL, of *ether R*. Combine the ether extracts and wash with three quantities, each of 10 mL, of *water R*; discard the washings, dry the ether over *anhydrous sodium sulfate R* and filter. Evaporate the ether on a water-bath. Use the residue to prepare the test solution. The fatty acids may also be obtained from the soap solution prepared during the determination of the unsaponifiable matter.

*Test solution.* Dissolve 40 mg of the mixture of fatty acids obtained from the substance to be examined in 4 mL of *chloroform R*.

*Reference solution.* Dissolve 40 mg of the mixture of fatty acids obtained from a mixture of 19 volumes of *maize oil R* and 1 volume of *rapeseed oil R* in 4 mL of *chloroform R*.

Apply to the plate 3 µL of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *water R* and 90 volumes of *glacial acetic acid R*. Dry the plate at 110 °C for 10 min. Allow to cool and, unless otherwise prescribed, place the plate in a chromatographic chamber, with a tightly fitting lid, that has previously been saturated with iodine vapour by placing *iodine R* in an evaporating dish at the bottom of the chamber. After some time brown or yellowish-brown spots become visible. Remove the plate and allow to stand for a few minutes. When the brown background colour has disappeared, spray with *starch solution R*. Blue spots appear which may become brown on drying and again become blue after spraying with *water R*. The chromatogram obtained with the test solution always shows a spot with an $R_F$ of about 0.5 (oleic acid) and a spot with an $R_F$ of about 0.65 (linoleic acid) corresponding to the spots in the chromatogram obtained with the reference solution. With some oils a spot with an $R_F$ of about 0.75 may be present (linolenic acid). By comparison with the spot in the chromatogram obtained with the reference solution, verify the absence in the chromatogram obtained with the test solution of a spot with an $R_F$ of about 0.25 (erucic acid).

**07/2016:20422**

## 2.4.22. COMPOSITION OF FATTY ACIDS BY GAS CHROMATOGRAPHY

The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil to be examined by gas chromatography (*2.2.28*).

METHOD A

*This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.*

*Test solution.* When prescribed in the monograph, dry the oil to be examined before the methylation step. Weigh 1.0 g of the oil into a 25 mL round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 mL of *anhydrous methanol R* and 0.2 mL of a 60 g/L solution of *potassium hydroxide R* in *methanol R*. Attach the

reflux condenser, pass *nitrogen R* through the mixture at a rate of about 50 mL/min, shake and heat to boiling. When the solution is clear (usually after about 10 min), continue heating for a further 5 min. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 mL of *heptane R* and transfer the rinsings to the separating funnel and shake. Add 10 mL of a 200 g/L solution of *sodium chloride R* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulfate R*. Allow to stand, then filter.

*Reference solution (a).* Prepare 0.50 g of the mixture of calibrating substances with the composition described in one of the 2.4.22 tables, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 2.4.22.-1). Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *heptane R*.

*Reference solution (c).* Prepare 0.50 g of a mixture of fatty acid methyl esters that corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance to be examined. Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

*Column*:

- *material*: fused silica, glass or quartz;
- *size*: *l* = 10-30 m, Ø = 0.2-0.8 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.1-0.5 µm) or another suitable stationary phase.

*Carrier gas*: *helium for chromatography R* or *hydrogen for chromatography R*.

*Flow rate*: 1.3 mL/min (for a column Ø = 0.32 mm).

*Split ratio*: 1:100 or less, according to the internal diameter of the column used (1:50 when Ø = 0.32 mm).

*Temperature*:

- *column*: in isothermal conditions, 160-200 °C, according to the length and type of column used (200 °C for a column 30 m long and coated with a layer of *macrogol 20 000 R*); if a linear temperature programming is necessary, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C, for example;
- *injection port*: 250 °C;
- *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability* when using the mixture of calibrating substances in Table 2.4.22.-1 or Table 2.4.22.-3:

- *resolution*: minimum 1.8 between the peaks due to methyl oleate and methyl stearate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl myristate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 30 000, calculated for the peak due to methyl stearate in the chromatogram obtained with reference solution (a).

*System suitability* when using the mixture of calibrating substances in Table 2.4.22.-2:

- *resolution*: minimum 4.0 between the peaks due to methyl caprylate and methyl decanoate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl caproate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 15 000, calculated for the peak due to methyl decanoate in the chromatogram obtained with reference solution (a).

*ASSESSMENT OF CHROMATOGRAMS*

Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

**Qualitative analysis**. Identify the peaks in the chromatogram obtained with reference solution (c) (isothermal operating conditions or linear temperature programming).

When using isothermal operating conditions, the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 2.4.22.-1, 2.4.22.-2 or 2.4.22.-3.

Table 2.4.22.-1. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

| Mixture of the following substances | Composition (per cent *m/m*) |
|---|---|
| *Methyl laurate R* | 5 |
| *Methyl myristate R* | 5 |
| *Methyl palmitate R* | 10 |
| *Methyl stearate R* | 20 |
| *Methyl arachidate R* | 40 |
| *Methyl oleate R* | 20 |

Table 2.4.22.-2. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

| Mixture of the following substances | Composition (per cent *m/m*) |
|---|---|
| *Methyl caproate R* | 10 |
| *Methyl caprylate R* | 10 |
| *Methyl decanoate R* | 20 |
| *Methyl laurate R* | 20 |
| *Methyl myristate R* | 40 |

Table 2.4.22.-3. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

| Mixture of the following substances | Composition (per cent *m/m*) |
|---|---|
| *Methyl myristate R* | 5 |
| *Methyl palmitate R* | 10 |
| *Methyl stearate R* | 15 |
| *Methyl arachidate R* | 20 |
| *Methyl oleate R* | 20 |
| *Methyl eicosenoate R* | 10 |
| *Methyl behenate R* | 10 |
| *Methyl lignocerate R* | 10 |

Measure the reduced retention time ($t'_R$) of each peak in the chromatogram obtained with reference solution (a). $t'_R$ is the retention time measured from the solvent peak and not from the time of injection. Plot the straight line:

$$\log_{10}(t'_R) = f(\text{equivalent chain length})$$

The logarithms of $t'_R$ of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as 'equivalent chain lengths'; the equivalent chain length is the length of the theoretical saturated chain that would have the same $t'_R$ as the fatty acid to be identified. For example, linoleic acid has the same $t'_R$ as the theoretical saturated fatty acid having 18.8 carbon atoms.

Identify the peaks in the chromatogram obtained with the test solution by means of the straight line and the reduced retention times. Equivalent chain lengths are given in Table 2.4.22.-4.

Table 2.4.22.-4. – *Equivalent chain lengths (this value, which is to be calculated using calibration curves, is given as an example for a column of macrogol 20 000 R)*

| Fatty acid | Equivalent chain length |
|---|---|
| Caproic acid | 6.0 |
| Caprylic acid | 8.0 |
| Capric acid | 10.0 |
| Lauric acid | 12.0 |
| Myristic acid | 14.0 |
| Palmitic acid | 16.0 |
| Palmitoleic acid | 16.3 |
| Margaric acid | 17.0 |
| Stearic acid | 18.0 |
| Oleic acid | 18.3 |
| Linoleic acid | 18.8 |
| Gamma-linolenic acid | 19.0 |
| Alpha-linolenic acid | 19.2 |
| Arachidic acid | 20.0 |
| Eicosenoic acid (gondoic acid) | 20.2 |
| Arachidonic acid | 21.2 |
| Behenic acid | 22.0 |
| Erucic acid | 22.2 |
| 12-Oxostearic acid | 22.7 |
| Ricinoleic acid | 23.9 |
| 12-Hydroxystearic acid | 23.9 |
| Lignoceric acid | 24.0 |
| Nervonic acid | 24.2 |

**Quantitative analysis**. In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except that of the solvent, is set at 100 per cent. The content of a constituent is calculated by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent *m/m*.

METHOD B

*This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.*

*Test solution.* Introduce 0.100 g of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve with 1 mL of *heptane R* and 1 mL of *dimethyl carbonate R* and mix vigorously under gentle heating (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for about 5 min. Add 3 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µL of the organic phase.

*Reference solutions and assessment of chromatograms.* Where there is no specific prescription in the individual monograph, proceed as described under Method A.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 100 |
| | 15 - 36 | 100 → 225 |
| | 36 - 61 | 225 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

METHOD C

*This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroepoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.*

*Test solution.* Dissolve 0.10 g of the substance to be examined in 2 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R* in a 25 mL conical flask and boil under a reflux condenser for 30 min. Add 2.0 mL of *boron trifluoride-methanol solution R* through the condenser and boil for 30 min. Add 4 mL of *heptane R* through the condenser and boil for 5 min. Cool and add 10.0 mL of *saturated sodium chloride solution R*, shake for about 15 s and add a quantity of *saturated sodium chloride solution R* such that the upper phase is brought into the neck of the flask. Collect 2 mL of the upper phase, wash with 3 quantities, each of 2 mL, of *water R* and dry over *anhydrous sodium sulfate R*.

*Reference solutions, chromatographic procedure and assessment of chromatograms.* Where there is no specific prescription in the individual monograph, proceed as described under Method A.

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# 2.4.23. STEROLS IN FATTY OILS

*When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B must be validated.*

METHOD A

**Separation of the sterol fraction (TLC)**

Prepare the unsaponifiable matter and then isolate the sterol fraction of the fatty oil by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R* with a 0.2 mm to 0.5 mm layer.

*Test solution (a).* In a 150 mL flask fitted with a reflux condenser, place a volume of a 2 g/L solution of *betulin R* in *methylene chloride R* containing betulin corresponding to about 10 per cent of the sterol content of the sample used for the determination (e.g. in the case of olive oil add 500 µL, in the case of other vegetable oils add 1500 µL of the betulin solution). If the monograph requires the percentage content of the individual sterols in the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness under a current of *nitrogen R*. Add 5.00 g (*m*) of the substance to be examined. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with 100 mL of *water R*. Shake the liquid carefully with 3 quantities, each of 100 mL, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with several quantities, each of 40 mL, of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 mL of *acetone R* to the residue. Carefully remove the solvent in a current of *nitrogen R*. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh. Transfer the residue to a small test tube with *methylene chloride R*. Evaporate under a stream of *nitrogen R* to a volume of about 1 mL. Depending on the unsaponifiable content of the oil, adapt the final concentration of the solution to 25-50 mg/mL.

*Test solution (b).* Treat 5.00 g of *rapeseed oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 mL of *2 M alcoholic potassium hydroxide R*".

*Test solution (c).* Treat 5.00 g of *sunflower oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 mL of *2 M alcoholic potassium hydroxide R*".

*Reference solution.* Dissolve 25 mg of *cholesterol R* and 10 mg of *betulin R* in 1 mL of *methylene chloride R*.

Use a separate plate for each test solution. Apply as a band of 10 mm, at 20 mm from the base and 10 mm from the left edge, 10 µL of the reference solution and as bands of 150 mm, at 20 mm from the base, 0.5 mL of test solutions (a), (b) or (c). Develop over a path of 17 cm using a mixture of 35 volumes of *ether R* and 65 volumes of *hexane R*. Dry the plates in a current of *nitrogen R*. Spray the plates with a 2 g/L solution of *dichlorofluorescein R* in *anhydrous ethanol R* and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows bands due to cholesterol and betulin. The chromatograms obtained with the test solutions show bands with similar $R_F$ values due to sterols. From each of the chromatograms, remove an area of coating corresponding to the area occupied by the sterol bands and additionally the area of the zones 2-3 mm above and below the visible zones corresponding to the reference solution. Place separately in three 50 mL flasks. To each flask add 15 mL of *methylene chloride R* and heat under reflux with stirring, for 15 min. Filter each solution through a sintered-glass filter (40) (*2.1.2*) or suitable filter paper and wash each filter with 3 quantities, each of 15 mL, of *methylene chloride R*. Place the combined filtrate and washings from each filter separately in 3 flasks, evaporate under a stream of *nitrogen R* to 5-10 mL. Transfer to a small test tube and evaporate to dryness under a stream of *nitrogen R*.

**Determination of the sterols (GC)**

Gas chromatography (*2.2.28*). *Carry out the operations protected from humidity and prepare the solutions immediately before use.*

*Test solution.* To the sterols separated from the substance to be examined by thin-layer chromatography add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

*Reference solution (a).* To 9 parts of the sterols separated from *rapeseed oil R* by thin-layer chromatography add 1 part of *cholesterol R*. To the mixture add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

*Reference solution (b).* To the sterols separated from *sunflower oil R* by thin-layer chromatography add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

*Column*:

- *material*: fused silica;
- *size*: *l* = 20-30 m, Ø = 0.25-0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* or *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R* or *helium for chromatography R*.

*Linear velocity*: 30-50 cm/s (hydrogen) or 20-35 cm/s (helium).

*Split ratio*: 1:50 (hydrogen) or 1:100 (helium).

*Temperature*:

- *column*: 260 °C;
- *injection port*: 280 °C;
- *detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: the chromatogram obtained with reference solution (a) shows 4 principal peaks corresponding to cholesterol, brassicasterol, campesterol and β-sitosterol and the chromatogram obtained with reference solution (b) shows 4 principal peaks corresponding to campesterol, stigmasterol, β-sitosterol and Δ7-stigmastenol. The relative retentions of the sterols with reference to β-sitosterol (main peak) are given in Table 2.4.23.-1.

Table 2.4.23.-1. – *Relative retentions of sterols with reference to β-sitosterol for 2 different columns*

| | Cyanopropyl(7)-(phenyl)(7)-(methyl)(86)polysiloxane | Phenyl(5)methyl(95)-polysiloxane |
|---|---|---|
| Cholesterol | 0.64 | 0.63 |
| Brassicasterol | 0.70 | 0.71 |
| 24-Methylenecholesterol | 0.79 | 0.80 |
| Campesterol | 0.82 | 0.81 |
| Campestanol | 0.83 | 0.82 |
| Stigmasterol | 0.87 | 0.87 |
| Δ7-Campesterol | 0.93 | 0.92 |
| Δ5,23-Stigmastadienol | 0.95 | 0.95 |
| Clerosterol | 0.96 | 0.96 |
| β-Sitosterol | 1 | 1 |
| Sitostanol | 1.01 | 1.02 |
| Δ5-Avenasterol | 1.03 | 1.03 |
| Δ5,24-Stigmastadienol | 1.09 | 1.08 |
| Δ7-Stigmastenol(1) | 1.13 | 1.12 |
| Δ7-Avenasterol | 1.18 | 1.16 |
| Betulin | 1.4 | 1.4 |

(1) This sterol may also be referred to as Δ7-stigmasterol in literature.

The peak due to the internal standard (betulin) must be clearly separated from the peaks due to the sterols to be determined.

For the chromatogram obtained with the test solution, identify the peaks and calculate the percentage content of each sterol in the sterol fraction of the substance to be examined using the following expression:

$$\frac{A}{S} \times 100$$

$A$ = area of the peak due to the component to be determined;

$S$ = sum of the areas of the peaks due to the components indicated in Table 2.4.23.-1; disregard the peak due to betulin.

If required in the monograph, calculate the content of each sterol in milligrams per 100 grams of the substance to be examined using the following expression:

$$\frac{A \times m' \times 100}{A' \times m}$$

$A$ = area of the peak due to the component to be determined;

$A'$ = area of the peak due to betulin;

$m$ = mass of the sample of the substance to be examined, in grams;

$m'$ = mass of *betulin R* added, in milligrams.

METHOD B

**Preparation of the unsaponifiable matter**

Prepare the unsaponifiable matter according to the method stated in the test for unsaponifiable matter of the monograph on the substance to be examined. Failing this, prepare the unsaponifiable matter according to the method described in chapter *2.5.7. Unsaponifiable matter*. After the final neutralisation step, evaporate the ethanol, then add 6 mL of *acetone R* and evaporate the solvent. Dry the residue at 100-105 °C. It is not necessary to dry to constant mass.

Simultaneously prepare under the same conditions the unsaponifiable matter of *sunflower oil R*. This will in particular serve to locate the sterol fraction to be collected.

**Separation of the sterol fraction (LC)**

Liquid chromatography (*2.2.29*).

*Test solution.* Take up the residue with 3 quantities, each of 4 mL, of the solvent used during the preparation of the unsaponifiable matter (generally *ether R* or *light petroleum R*) and transfer to a 15 mL tube. Evaporate to dryness under a current of *nitrogen R*. Dissolve the residue in a volume of mobile phase sufficient to obtain a solution with an approximate concentration of 40 mg/mL. Add a few drops of *2-propanol R1* to improve the solubility (3 drops are normally sufficient to ensure complete solubilisation). Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Proceed as described for the test solution with the unsaponifiable matter obtained with *sunflower oil R*.

*Precolumn*:

- *size*: *l* = 5 mm, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm) with a pore size of 6 nm.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm) with a pore size of 6 nm.

*Mobile phase*: *2-propanol R1*, *hexane R* (1:99 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Identification of the peaks due to sterols*: the sterol fraction elutes at the end of the chromatogram. Locate the fraction to be collected using the chromatogram obtained with the reference solution, which shows 2 principal peaks eluting approximately between 23 min and 32 min. Collect the fraction at the detector outlet in a 15 mL tube with a screw cap. Evaporate the solvent under a current of *nitrogen R*.

**Determination of the sterols (GC)**

Gas chromatography (*2.2.28*).

*Test solution.* Dissolve the residue of the sterol fraction obtained with the test solution in the previous LC step in 0.2 mL of *anhydrous pyridine R* and 0.2 mL of a mixture of 1 volume of *chlorotrimethylsilane R* and 99 volumes of *N,O-bis(trimethylsilyl)trifluoroacetamide R*. Stopper the tube tightly and heat at 80 °C for 20 min. Allow to cool and use the liquid phase.

*Reference solution.* Dissolve the residue of the sterol fraction obtained with the reference solution in the previous LC step in 0.2 mL of *anhydrous pyridine R* and 0.2 mL of a mixture of 1 volume of *chlorotrimethylsilane R* and 99 volumes of *N,O-bis(trimethylsilyl)trifluoroacetamide R*. Stopper the tube tightly and heat at 80 °C for 20 min. Allow to cool and use the liquid phase.

A standard of cholesterol (*cholesterol R*) may also be used, alone or as a mixture with the sterol fraction of sunflower oil. Proceed with derivatisation as described for the test solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.6 mL/min.

*Split ratio*: 1:25.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 38 | 260 |
| | 38 - 44 | 260 → 290 |
| | 44 - 49 | 290 |
| Injection port | | 290 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1-3 µL (depending on the expected amount of sterols in the substance to be examined).

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the peaks due to campesterol, stigmasterol, β-sitosterol and Δ7-stigmastenol. Identify the peaks due to the sterols in the chromatogram obtained with the test solution using the chromatogram obtained with the reference solution and the relative retentions with reference to β-sitosterol (main peak) given in Table 2.4.23.-1.

*System suitability*: reference solution:

– *resolution*: minimum 4.0 between the peaks due to campesterol and stigmasterol.

Calculate the percentage content of each sterol in the sterol fraction of the substance to be examined using the following expression:

$$\frac{A}{S} \times 100$$

*A* = area of the peak due to the component to be determined;

*S* = sum of the areas of the peaks due to the components indicated in Table 2.4.23.-1, except betulin.

**01/2017:20424**

# 2.4.24. IDENTIFICATION AND CONTROL OF RESIDUAL SOLVENTS

The test procedures described in this general method may be used:

i. for the identification of the majority of Class 1 and Class 2 residual solvents in an active substance, excipient or medicinal product when the residual solvents are unknown;

ii. as a limit test for Class 1 and Class 2 solvents when present in an active substance, excipient or medicinal product;

iii. for the quantification of Class 2 solvents when the limits are greater than 1000 ppm (0.1 per cent) or for the quantification of Class 3 solvents when required.

Class 1, Class 2 and Class 3 residual solvents are listed in general chapter *5.4. Residual solvents*.

Three diluents are described for sample preparation and the conditions to be applied for head-space injection of the gaseous sample onto the chromatographic system. Two chromatographic systems are prescribed but System A is preferred whilst System B is employed normally for confirmation of identity. The choice of sample preparation procedure depends on the solubility of the substance to be examined and in certain cases the residual solvents to be controlled.

The following residual solvents are not readily detected by the head-space injection conditions described: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, *N*-methylpyrrolidone and sulfolane. Other appropriate procedures should be employed for the control of these residual solvents.

When the test procedure is applied quantitatively to control residual solvents in a substance, then it must be validated.

PROCEDURE

Examine by gas chromatography with static head-space injection (*2.2.28*).

**Sample preparation 1**. This is intended for the control of residual solvents in water-soluble substances.

*Sample solution (1).* Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

**Sample preparation 2**. This is intended for the control of residual solvents in water-insoluble substances.

*Sample solution (2).* Dissolve 0.200 g of the substance to be examined in *dimethylformamide R* (DMF) and dilute to 20.0 mL with the same solvent.

**Sample preparation 3**. This is intended for the control of *N,N*-dimethylacetamide and/or *N,N*-dimethylformamide, when it is known or suspected that one or both of these substances are present in the substance to be examined.

*Sample solution (3).* Dissolve 0.200 g of the substance to be examined in *1,3-dimethyl-2-imidazolidinone R* (DMI) and dilute to 20.0 mL with the same solvent.

In some cases none of the above sample preparation procedures are appropriate, in which case the diluent to be used for the preparation of the sample solution and the static head-space conditions to be employed must be demonstrated to be suitable.

*Solvent solution (a).* To 1.0 mL of *Class 1 residual solvent solution CRS*, add 9 mL of *dimethyl sulfoxide R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

The reference solutions correspond to the following limits:

– benzene: 2 ppm,
– carbon tetrachloride: 4 ppm,
– 1,2-dichloroethane: 5 ppm,
– 1,1-dichloroethene: 8 ppm,
– 1,1,1-trichloroethane: 10 ppm.

*Solvent solution (b).* Dissolve appropriate quantities of the Class 2 residual solvents in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute in *water R* to give a concentration of 1/20 of the limits stated in Table 2 (see *5.4. Residual solvents*).

*Solvent solution (c).* Dissolve 1.00 g of the solvent or solvents present in the substance to be examined in *dimethyl sulfoxide R* or *water R*, if appropriate, and dilute to 100.0 mL with *water R*. Dilute to give a concentration of 1/20 of the limit(s) stated in Table 1 or 2 (see *5.4. Residual solvents*).

*Blank solution.* Prepare as described for solvent solution (c) but without the addition of solvent(s) (used to verify the absence of interfering peaks).

*Test solution.* Introduce 5.0 mL of the sample solution and 1.0 mL of the blank solution into an injection vial.

*Reference solution (a) (Class 1).* Introduce 1.0 mL of solvent solution (a) and 5.0 mL of the appropriate diluent into an injection vial.

*Reference solution ($a_1$) (Class 1).* Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (a) into an injection vial.

*Reference solution (b) (Class 2).* Introduce 1.0 mL of solvent solution (b) and 5.0 mL of the appropriate diluent into an injection vial.

*Reference solution (c).* Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (c) into an injection vial.

*Reference solution (d).* Introduce 1.0 mL of the blank solution and 5.0 mL of the appropriate diluent into an injection vial.

*Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous solution.*

The following static head-space injection conditions may be used:

| | Sample preparation procedure | | |
|---|---|---|---|
| **Operating parameters** | **1** | **2** | **3** |
| Equilibration temperature (°C) | 80 | 105 | 80 |
| Equilibration time (min) | 60 | 45 | 45 |
| Transfer-line temperature (°C) | 85 | 110 | 105 |
| Carrier gas: *Nitrogen for chromatography R* or *Helium for chromatography R* at an appropriate pressure | | | |
| Pressurisation time (s) | 30 | 30 | 30 |
| Injection volume (mL) | 1 | 1 | 1 |

The chromatographic procedure may be carried out using:

*SYSTEM A*

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with cross-linked 6 per cent polycyanopropylphenylsiloxane and 94 per cent polydimethylsiloxane (film thickness: 1.8 µm or 3 µm),
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s,
- a flame-ionisation detector (a mass spectrometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1),

maintaining the temperature of the column at 40 °C for 20 min, then raising the temperature at a rate of 10 °C per min to 240 °C and maintaining it at 240 °C for 20 min and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C, or, where there is interference from the matrix, use:

*SYSTEM B*

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with *macrogol 20 000 R* (film thickness: 0.25 µm),
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s.
- a flame-ionisation detector (a mass spectrophotometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1),

maintaining the temperature of the column at 50 °C for 20 min, then raising the temperature at a rate of 6 °C per min to 165 °C and maintaining it at 165 °C for 20 min and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C.

Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System A and record the chromatogram under such conditions that the signal-to-noise ratio for 1,1,1-trichloroethane can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.4.24.-1.

Inject 1 mL of the gaseous phase of reference solution ($a_1$) onto the column described in System A. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System A and record the chromatogram under such conditions that the resolution between acetonitrile and methylene chloride can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24.-2 and the resolution between acetonitrile and methylene chloride is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System A. If in the chromatogram obtained, there is no peak which corresponds to one of the residual solvent peaks in the chromatograms obtained with reference solution (a) or (b), then the substance to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) then System B is to be employed.

Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System B and record the chromatogram under such conditions that the signal-to-noise ratio for benzene can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.4.24.-3.

Inject 1 mL of the gaseous phase of reference solution ($a_1$) onto the column described in System B. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System B and record the chromatogram under such conditions that the resolution between acetonitrile and trichloroethene can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24.-4 and the resolution between acetonitrile and trichloroethene is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System B. If in the chromatogram obtained, there is no peak which corresponds to any of the residual solvent peaks in the chromatogram obtained with the reference solution (a) or (b), then the substance to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) and confirms the correspondence obtained when using System A, then proceed as follows.

Inject 1 mL of the gaseous phase of reference solution (c) onto the column described for System A or System B. If necessary, adjust the sensitivity of the system so that the height of the peak corresponding to the identified residual solvent(s) is at least 50 per cent of the full scale of the recorder.

Inject 1 mL of the gaseous phase of reference solution (d) onto the column. No interfering peaks should be observed.

Inject 1 mL of the gaseous phase of the test solution and 1 mL of the gaseous phase of reference solution (c) on to the column. Repeat these injections twice more.

The mean area of the peak of the residual solvent(s) in the chromatograms obtained with the test solution is not greater than half the mean area of the peak of the corresponding residual solvent(s) in the chromatograms obtained with reference solution (c). The test is not valid unless the relative standard deviation of the differences in areas between the analyte peaks obtained from 3 replicate paired injections of reference solution (c) and the test solution, is at most 15 per cent.

A flow diagram of the procedure is shown in Figure 2.4.24.-5.

When a residual solvent (Class 2 or Class 3) is present at a level of 0.1 per cent or greater then the content may be quantitatively determined by the method of standard additions.

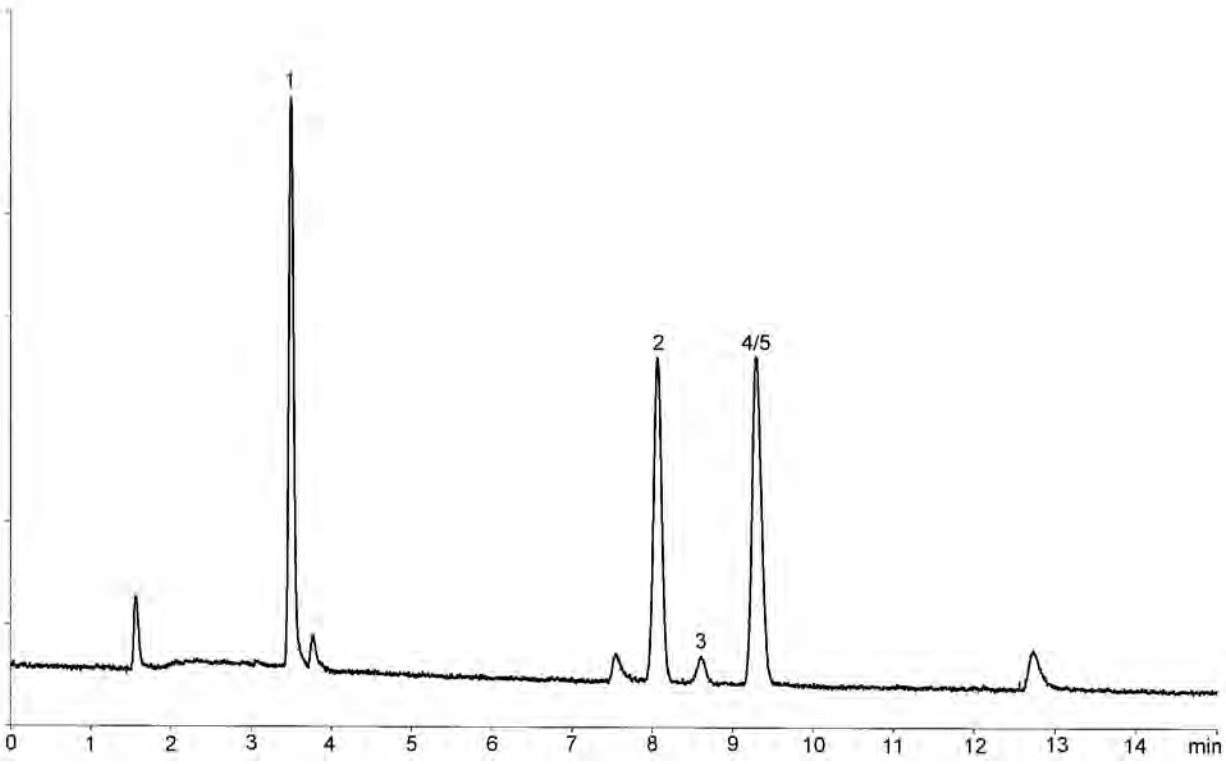


1. 1,1-dichloroethene 2. 1,1,1-trichloroethane 3. carbon tetrachloride 4. benzene 5. 1,2-dichloroethane

Figure 2.4.24.-1. – *Typical chromatogram of Class 1 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector.*

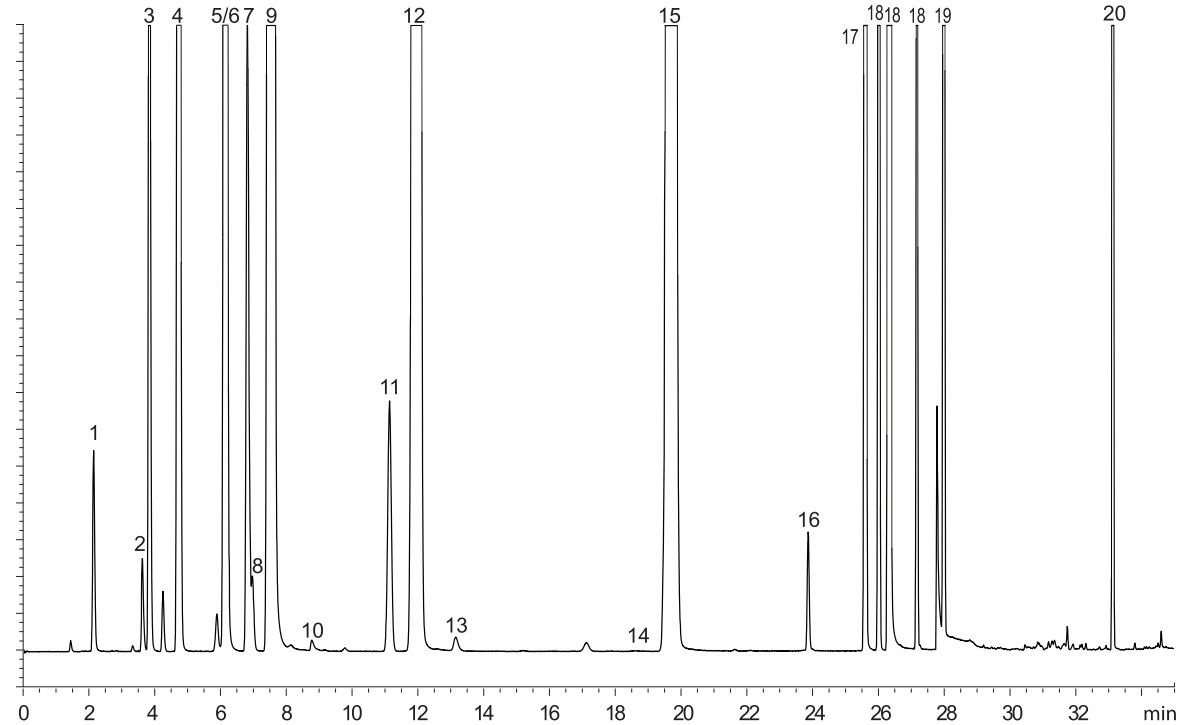


| | | | | |
|---|---|---|---|---|
| 1. methanol | 5. 1,2-dichloroethene | 9. cyclohexane | 13. 1,4-dioxane | 17. chlorobenzene |
| 2. acetonitrile | 6. nitromethane | 10. 1,2-dimethoxyethane | 14. pyridine ($t_r$ = 18.7 min) | 18. xylene *ortho, meta, para* |
| 3. dichloromethane | 7. tetrahydrofuran | 11. 1,1,2-trichloroethene | 15. toluene | 19. cumene |
| 4. hexane | 8. chloroform | 12. methylcyclohexane | 16. methylbutylketone | 20. tetralin |

Figure 2.4.24.-2. – *Chromatogram of Class 2 solvents (solvent solution (b)) using the conditions described for System A and Procedure 1. Flame-ionisation detector.*

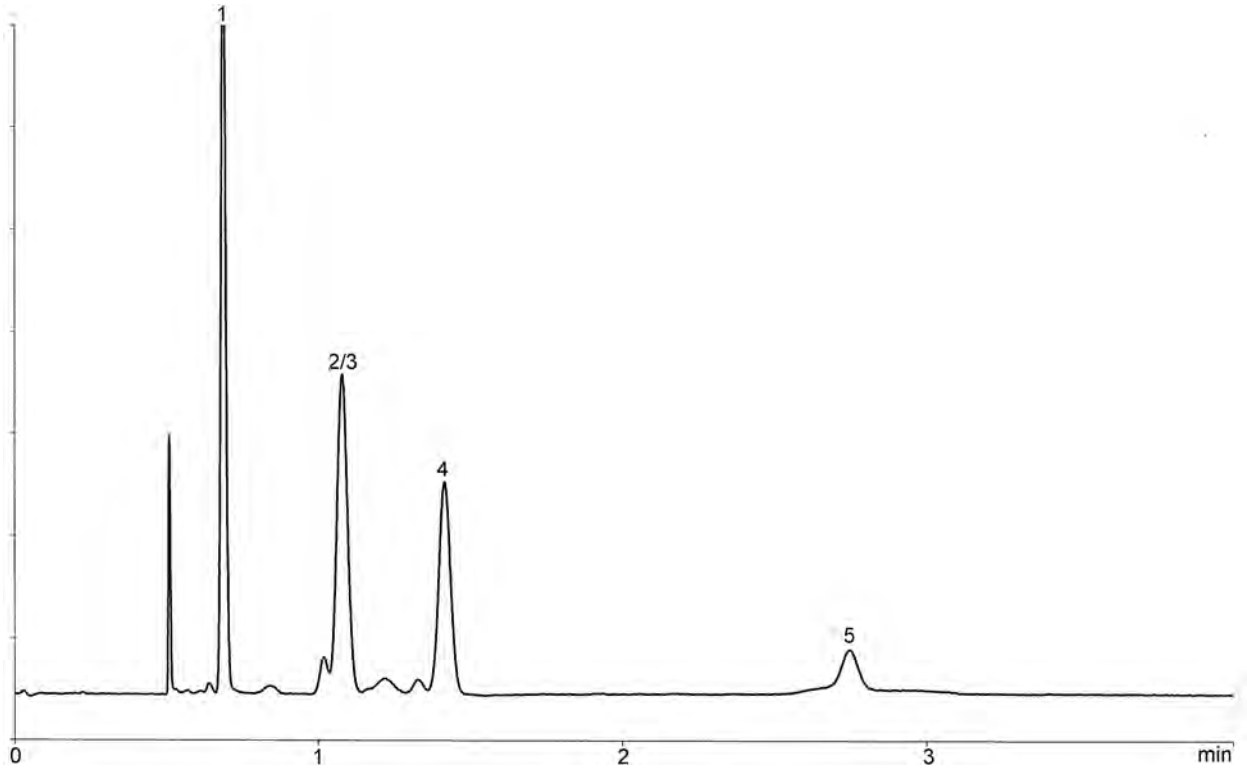


1. 1,1-dichloroethene 2. 1,1,1-trichloroethane 3. carbon tetrachloride 4. benzene 5. 1,2-dichloroethane

Figure 2.4.24.-3. – *Chromatogram of Class 1 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector.*

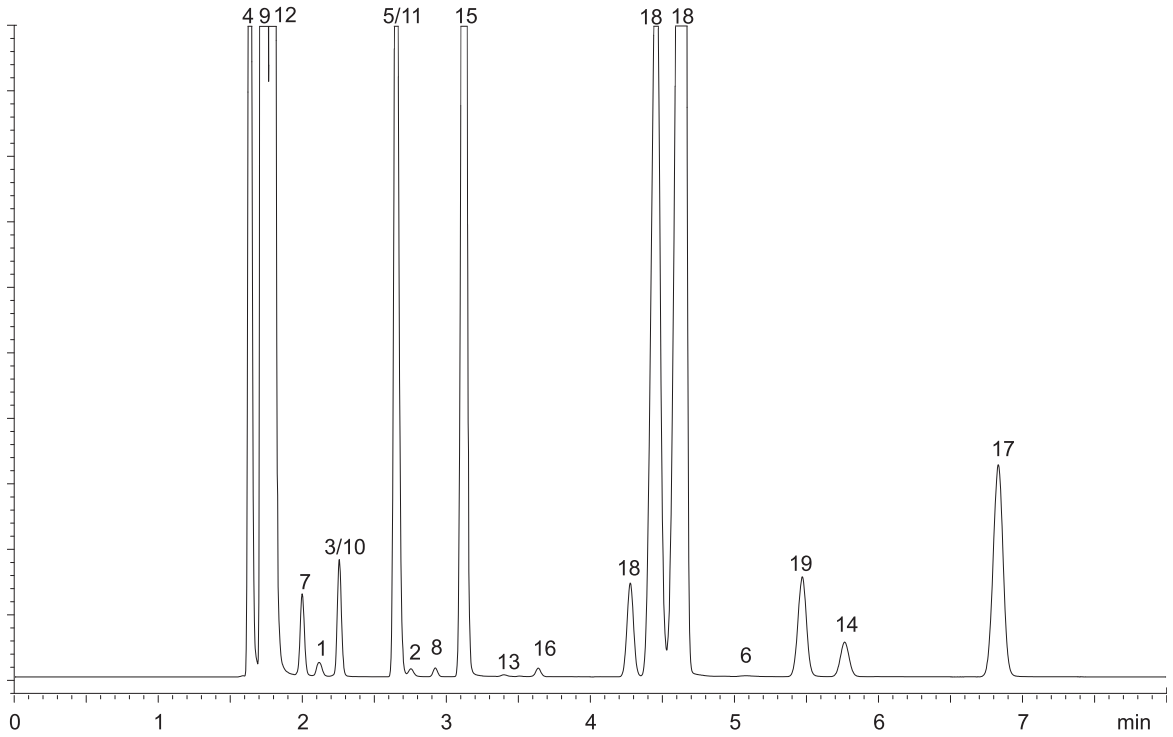


| | | | | |
|---|---|---|---|---|
| 1. methanol | 5. 1,2-dichloroethene | 9. cyclohexane | 13. 1,4-dioxane | 17. chlorobenzene |
| 2. acetonitrile | 6. nitromethane | 10. 1,2-dimethoxyethane | 14. pyridine | 18. xylene *ortho, meta, para* |
| 3. dichloromethane | 7. tetrahydrofuran | 11. 1,1,2-trichloroethene | 15. toluene | 19. cumene |
| 4. hexane | 8. chloroform | 12. methylcyclohexane | 16. methylbutylketone | 20. tetralin ($t_r$ = 27 min) |

Figure 2.4.24.-4. – *Typical chromatogram of Class 2 residual solvents (solvent solution (b)) using the conditions described for System B and Procedure 1. Flame-ionisation detector.*

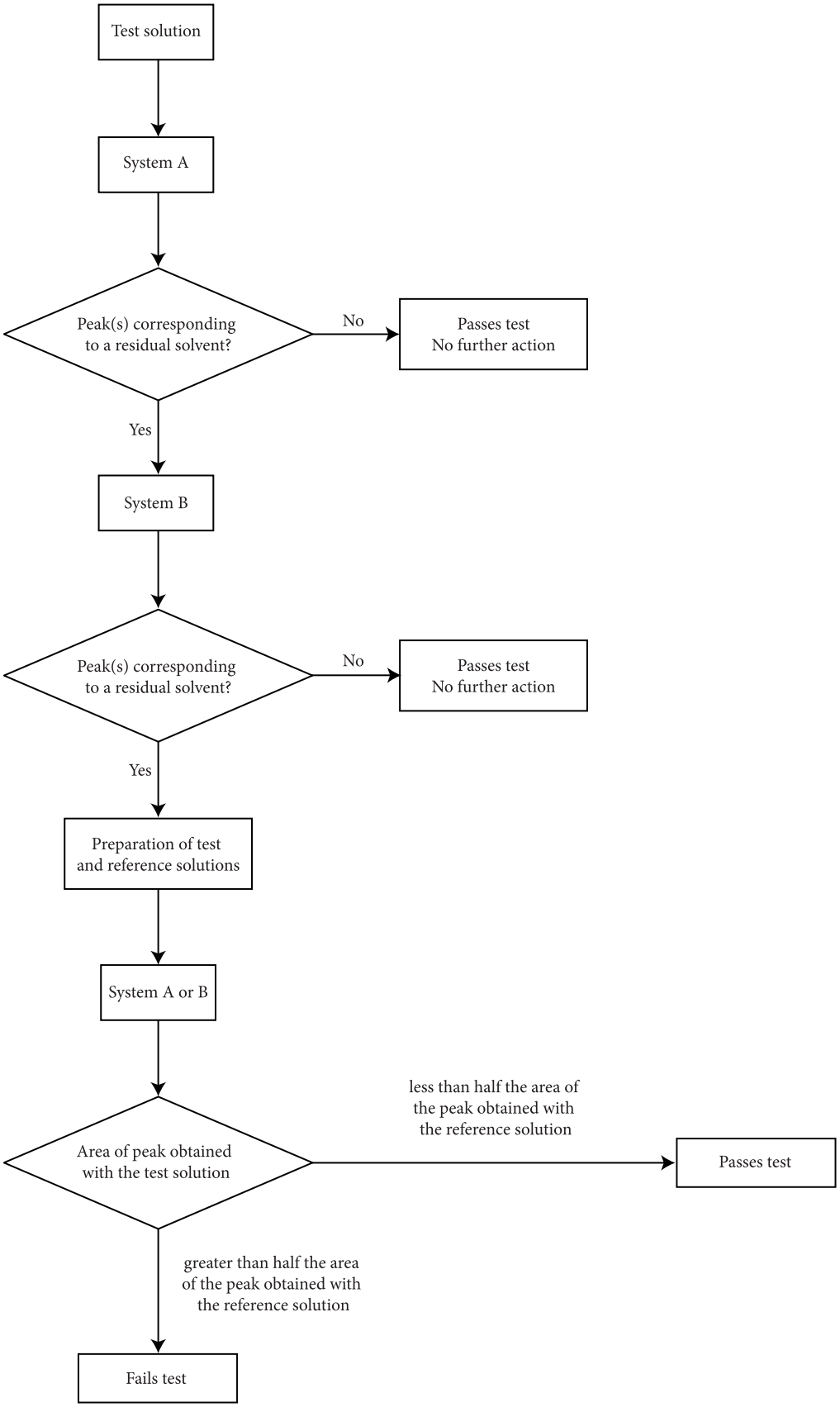


Figure 2.4.24.-5. – *Diagram relating to the identification of residual solvents and the application of limit tests*

**04/2015:20425 corrected 10.0**

# 2.4.25. ETHYLENE OXIDE AND DIOXAN

The test is intended for the determination of residual ethylene oxide and dioxan in samples soluble in water or dimethylacetamide. For substances that are insoluble or insufficiently soluble in these solvents, the preparation of the sample solution and the head-space conditions to be employed are given in the individual monograph.

Head-space gas chromatography (*2.2.28*).

A. For samples soluble in or miscible with water, the following procedure may be used.

*Test solution.* Weigh 1.00 g ($M_T$) of the substance to be examined in a 10 mL vial (other sizes may be used depending on the operating conditions) and add 1.0 mL of *water R*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

*Reference solution (a).* Weigh 1.00 g ($M_R$) of the substance to be examined into an identical 10 mL vial, add 0.50 mL of *dioxan solution R2* and 0.50 mL of *ethylene oxide solution R3*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

*Reference solution (b).* To 0.50 mL of *ethylene oxide solution R3* in a 10 mL vial add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R* and 0.10 mL of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

B. For samples soluble in or miscible with dimethylacetamide, the following procedure may be used.

*Test solution.* Weigh 1.00 g ($M_T$) of the substance to be examined in a 10 mL vial (other sizes may be used depending on the operating conditions) and add 0.20 mL of *water R* and 1.0 mL of *dimethylacetamide R*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

*Reference solution (a).* Weigh 1.00 g ($M_R$) of the substance to be examined into an identical 10 mL vial, add 0.10 mL of *dioxan solution R1*, 0.10 mL of *ethylene oxide solution R2* and 1.0 mL of *dimethylacetamide R*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

*Reference solution (b).* To 0.10 mL of *ethylene oxide solution R2* in a 10 mL vial, add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R* and 0.10 mL of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

*Column*:

- *material*: glass or fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R* or *nitrogen for chromatography R*.

*Linear velocity*: 20 cm/s.

*Split ratio*: 1:20.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 70 °C (90 °C for solutions in dimethylacetamide);
- *equilibration time*: 45 min;
- *transfer-line temperature*: 75 °C (150 °C for solutions in dimethylacetamide);
- *carrier gas*: *helium for chromatography R*;
- *pressurisation time*: 1 min;
- *injection time*: 12 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 50 |
| | 5 - 31 | 50 → 180 |
| | 31 - 32.5 | 180 → 230 |
| | 32.5 - 37.5 | 230 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: a suitable volume, for example 1.0 mL, of the gaseous phase of the test solution and of reference solutions (a) and (b). Repeat the procedure twice more.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to acetaldehyde and ethylene oxide;
- *signal-to-noise ratio*: minimum 5 for the peaks due to ethylene oxide and dioxan.

*Verification of precision*

For each pair of injections, calculate for ethylene oxide and for dioxan the difference in area between the peaks obtained with the test solution and reference solution (a). The test is not valid unless the relative standard deviation of the 3 values obtained for ethylene oxide is not greater than 15 per cent and the relative standard deviation of the 3 values obtained for dioxan is not greater than 15 per cent. If the weighings used for the test solution and reference solution (a) differ from 1.00 g by more than 0.5 per cent, the appropriate corrections must be made.

Calculate the content of ethylene oxide or dioxan in parts per million from the following expressions:

$$\frac{A_T \times C}{(A_R \times M_T) - (A_T \times M_R)}$$

$A_T$ = area of the peak due to ethylene oxide in the chromatogram obtained with the test solution;

$A_R$ = area of the peak due to ethylene oxide in the chromatogram obtained with reference solution (a);

$M_T$ = mass of the substance to be examined in the test solution, in grams;

$M_R$ = mass of the substance to be examined in reference solution (a), in grams;

$C$ = amount of ethylene oxide added to reference solution (a), in micrograms.

$$\frac{D_T \times C}{(D_R \times M_T) - (D_T \times M_R)}$$

$D_T$ = area of the peak due to dioxan in the chromatogram obtained with the test solution;

$D_R$ = area of the peak due to dioxan in the chromatogram obtained with reference solution (a);

$C$ = amount of dioxan added to reference solution (a) in micrograms.

**01/2008:20426 corrected 10.0**

## 2.4.26. *N,N*-DIMETHYLANILINE

METHOD A

Examine by gas chromatography (*2.2.28*), using *N,N-diethylaniline R* as the internal standard.

*Internal standard solution.* Dissolve 50 mg of *N,N-diethylaniline R* in 4 mL of *0.1 M hydrochloric acid* and dilute to 50 mL with *water R*. Dilute 1 mL of this solution to 100 mL with *water R*.

*Test solution.* Dissolve in a ground-glass-stoppered tube 0.50 g of the substance to be examined in 30.0 mL of *water R*. Add 1.0 mL of the internal standard solution. Adjust the solution to a temperature of 26-28 °C. Add 1.0 mL of *strong sodium hydroxide solution R* and mix until completely dissolved. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

*Reference solution.* Dissolve 50.0 mg of *N,N-dimethylaniline R* in 4.0 mL of *0.1 M hydrochloric acid* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 30.0 mL with *water R*. Add 1.0 mL of the internal standard solution and 1.0 mL of *strong sodium hydroxide solution R*. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

The chromatographic procedure may be carried out using:

- a fused-silica capillary column 25 m long and 0.32 mm in internal diameter coated with *phenyl(50)methyl(50)polysiloxane R* (film thickness 0.52 µm),
- *helium for chromatography R* as the carrier gas with a split ratio 1:20, a column head pressure of 50 kPa and a split vent of 20 mL/min,
- a flame-ionisation detector,
- a split-liner consisting of a column about 1 cm long packed with *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *methylpolysiloxane R*,

maintaining the temperature of the column at 150 °C for 5 min, then raising the temperature at a rate of 20 °C per min to 275 °C and maintaining it at 275 °C for 3 min and maintaining the temperature of the detector at 300 °C and that of the injection port at 220 °C.

The retention times are: *N,N*-dimethylaniline about 3.6 min, *N,N*-diethylaniline about 5.0 min.

Inject 1 µL of the test solution and 1 µL of the reference solution.

METHOD B

Examined by gas chromatography (*2.2.28*), using *naphthalene R* as the internal standard.

*Internal standard solution.* Dissolve 50 mg of *naphthalene R* in *cyclohexane R* and dilute to 50 mL with the same solvent. Dilute 5 mL of this solution to 100 mL with *cyclohexane R*.

*Test solution.* To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

*Reference solution.* To 50.0 mg of *N,N-dimethylaniline R* add 2 mL of *hydrochloric acid R* and 20 mL of *water R*, shake to dissolve and dilute to 50.0 mL with *water R*. Dilute 5.0 mL of this solution to 250.0 mL with *water R*. To 1.0 mL of the latter solution in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

The chromatographic procedure may be carried out using:

- a glass column 2 m long and 2 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 mL/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C and that of the injection port and of the detector at 150 °C.

Inject 1 µL of the test solution and 1 µL of the reference solution.

**07/2014:20427**

## 2.4.27. HEAVY METALS IN HERBAL DRUGS AND HERBAL DRUG PREPARATIONS

*CAUTION: when using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.*

APPARATUS

The apparatus typically consists of the following:

- as digestion flasks, polytetrafluoroethylene, perfluoroalkoxy polymer, quartz or glass flasks with a volume of 20-150 mL, fitted with an airtight closure, a valve to adjust the pressure inside the container and a polytetrafluoroethylene tube to allow release of gas;
- a system to make flasks airtight, using the same torsional force for each of them;
- a programmable microwave oven (e.g. with a magnetron frequency of 2450 MHz, with a selectable output from 0 to 1500 ± 70 W in 1 per cent increments), a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system and exhaust tubing to vent fumes;
- an atomic absorption spectrometer (*2.2.23*), an inductively coupled plasma-atomic emission spectrometer (*2.2.57*), or an inductively coupled plasma-mass spectrometer (*2.2.58*).

METHOD

Examine by atomic absorption spectrometry (AAS) (*2.2.23*), inductively coupled plasma-atomic emission spectrometry (ICP-AES) (*2.2.57*), or inductively coupled plasma-mass spectrometry (ICP-MS) (*2.2.58*).

Deviations from the experimental parameters of the sample preparation procedure and the method description below are acceptable provided that the validation requirements are met and the system suitability test is fulfilled on the day of the analysis.

**Sample preparation**

Clean all the glassware and laboratory equipment with a 10 g/L solution of *nitric acid R* before use.

*Test solution.* In a digestion flask, place the prescribed quantity of the substance to be examined (about 0.50 g of powdered herbal drug (1400) (*2.9.12*)). Add 4 mL of *heavy metal-free hydrochloric acid R* and 6 mL of *heavy metal-free nitric acid R*. Make the flask airtight.

Place the digestion flasks in the microwave oven. Carry out the digestion in 3 steps according to the following programme, used for 7 flasks each containing the test solution: 80 per cent power for 15 min, 100 per cent power for 5 min, 80 per cent power for 20 min.

At the end of the cycle, allow the flasks to cool in air or water. After cooling, open each digestion flask and introduce the clear, colourless solution obtained into a 50 mL volumetric flask. Rinse each digestion flask with 2 quantities, each of 15 mL, of *heavy metal-free dilute nitric acid R*, collect the rinsings in the volumetric flask and dilute to 50.0 mL with *water R*. Modifiers (e.g. in the case of AAS with electrothermal atomisation, 1.0 mL of a 10 g/L solution of *magnesium nitrate R* and 1.0 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R*) and stabilising agents may be used, if necessary.

*Blank solution.* Mix 4 mL of *heavy metal-free hydrochloric acid R* and 6 mL of *heavy metal-free nitric acid R* in a digestion flask. Carry out the digestion in the same manner as for the test solution.

*DETERMINATION OF ARSENIC, CADMIUM, COPPER, NICKEL AND LEAD USING AAS (2.2.23) WITH ELECTROTHERMAL ATOMISATION*

Measure the content of arsenic, cadmium, copper, nickel and lead by direct calibration (*2.2.23, Method I*) or by the standard additions method (*2.2.23, Method II*), using reference solutions of each heavy metal and the instrumental parameters recommended in Table 2.4.27.-1.

The absorbance value of the blank solution is subtracted from the value obtained with the test solution.

Table 2.4.27.-1. – *Instrumental parameters for AAS with electrothermal atomisation*

| | | As | Cd | Cu | Ni | Pb |
|---|---|---|---|---|---|---|
| Wavelength | nm | 193.7 | 228.8 | 324.8 | 232 | 283.5 |
| Slit width | nm | 0.5 | 0.5 | 0.5 | 0.2 | 0.5 |
| Lamp current | mA | 10 | 6 | 7 | 10 | 5 |
| Ignition temperature | °C | 1400 | 800 | 800 | 800 | 800 |
| Atomisation temperature | °C | 2600 | 1800 | 2300 | 2500 | 2200 |
| Gas flow rate | L/min | 3 | 3 | 3 | 3 | 3 |

*DETERMINATION OF ARSENIC AND MERCURY USING AAS (2.2.23) WITH COLD-VAPOUR OR HYDRIDE ATOMISATION*

Measure the content of arsenic and mercury by direct calibration (*2.2.23, Method I*) or by the standard additions method (*2.2.23, Method II*), using reference solutions of arsenic or mercury and an automated continuous-flow hydride vapour generation system.

The absorbance value of the blank solution is subtracted from the value obtained with the test solution.

**Arsenic**

*Sample solution.* To 19.0 mL of the test solution or of the blank solution as prescribed above, add 1 mL of a 200 g/L solution of *potassium iodide R*. Allow the test solution to stand at room temperature for about 50 min or at 70 °C for about 4 min.

*Acid reagent. Heavy metal-free hydrochloric acid R.*

*Reducing reagent.* 6 g/L solution of *sodium tetrahydroborate R* in a 5 g/L solution of *sodium hydroxide R*.

The recommended instrumental parameters in Table 2.4.27.-2 may be used.

**Mercury**

*Sample solution.* Test solution or blank solution, as prescribed above.

*Acid reagent.* 515 g/L solution of *heavy metal-free hydrochloric acid R*.

*Reducing reagent.* 10 g/L solution of *stannous chloride R* in *heavy metal-free dilute hydrochloric acid R*.

The recommended instrumental parameters in Table 2.4.27.-2 may be used.

Table 2.4.27.-2. – *Instrumental parameters for AAS with cold-vapour or hydride atomisation*

| | | As | Hg |
|---|---|---|---|
| Wavelength | nm | 193.7 | 253.7 |
| Slit width | nm | 0.2 | 0.5 |
| Lamp current | mA | 10 | 4 |
| Acid reagent flow rate | mL/min | 1.0 | 1.0 |
| Reducing reagent flow rate | mL/min | 1.0 | 1.0 |
| Sample solution flow rate | mL/min | 7.0 | 7.0 |
| Absorption cell | | Quartz (heated) | Quartz (unheated) |
| Nitrogen flow rate | L/min | 0.1 | 0.1 |

*DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL AND LEAD USING ICP-AES (2.2.57)*

Measure the content of arsenic, cadmium, copper, mercury, nickel and lead by direct calibration (*2.2.23, Method I*), using reference solutions of each heavy metal or a mixture of all measured metals, and the instrumental parameters recommended in Table 2.4.27.-3.

The emission value of the blank solution is subtracted from the value obtained with the test solution.

*DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL AND LEAD USING ICP-MS (2.2.58)*

Measure the content of arsenic, cadmium, copper, mercury, nickel and lead by direct calibration (*2.2.23, Method I*) using reference solutions of each heavy metal and the analytical isotopes and additional masses recommended in Table 2.4.27.-4.

The signal intensity of the blank solution is subtracted from the value obtained with the test solution.

SYSTEM SUITABILITY

A system suitability test must be carried out on the day of the analysis to ensure that the sample preparation and measurement system are appropriate.

Table 2.4.27.-3. – *Instrumental parameters for ICP-AES*

| | | As | Cd | Cu | Hg | Ni | Pb |
|---|---|---|---|---|---|---|---|
| Wavelength | nm | 193.696/ 197.197/ 189.042 | 214.438/ 226.502/ 228.802 | 324.754/ 327.396/ 224.700 | 184.950/ 253.652/ 435.835 | 231.604/ 231.997/ 352.454 | 220.351/ 283.306/ 168.215 |
| Ar, Monitorline | nm | 430.010 | 430.010 | 430.010 | 430.010 | 430.010 | 430.010 |
| Plasma energy | W | 1200 | 1200 | 1200 | 1200 | 1200 | 1200 |
| Peak algorithm with background correction | | yes | yes | yes | yes | yes | yes |

*Acceptance criterion for preparation of sample solution*: a clear solution is obtained.

*Acceptance criterion for measurement system*: the measured concentration of a standard solution of the metal at a concentration within the range of the used calibration curve does not differ from the actual concentration by more than 20 per cent.

Table 2.4.27.-4. – *Recommended analytical isotopes and additional masses for ICP-MS*

| Isotope | Element of Interest |
|---|---|
| 75 | Arsenic |
| 106, 108, 111, 114 | Cadmium |
| 63, 65 | Copper |
| 202 | Mercury |
| 60, 62 | Nickel |
| 206, 207, 208 | Lead |

VALIDATION REQUIREMENTS

The analytical procedures used must be validated in accordance with the relevant general methods AAS (*2.2.23*), ICP-AES (*2.2.57*) and ICP-MS (*2.2.58*). Additionally, the following criteria must be fulfilled.

*SPECIFICITY*

Specificity is the ability to ensure that the analytical procedures for sample preparation and measurement allow a reliable determination of the metal(s) in the presence of components (e.g. carrier gas, impurities, matrix) that may be expected to be present.

*Acceptance criteria*: the procedure must be able to assess unequivocally each heavy metal to be determined with this procedure in the presence of components that may be expected to be present, including other heavy metals, matrix components and other sources of interference; specificity is demonstrated by complying with the accuracy requirement for the metal(s) to be determined.

*RANGE*

The calibration range of each metal is within the linear range of the method; test solutions containing residues at a level outside the calibration range may be diluted to concentrations within the calibration range.

*Acceptance criterion*: range is demonstrated by complying with the recovery requirement.

*ACCURACY*

Verify the accuracy using a certified reference material (CRM) or by performing a test for recovery.

**Recovery**. Recovery may be determined on a sample of the substance to be examined, spiked with a known quantity of a reference standard of the metal (3 concentration levels in the range of 50-150 per cent of the intended specification limit, even if the original concentration of the reference standard is at the specified value), in triplicate.

*Acceptance criterion*: spike recovery is within 70 per cent and 150 per cent for the mean of 3 replicates at each concentration.

*REPEATABILITY*

*Test samples*: either 6 independent samples of the substance to be examined spiked with a suitable reference standard at the specified concentration level, or 3 concentration levels prepared in triplicate.

*Acceptance criterion*: the relative standard deviation is in both cases not greater than the value indicated in Table 2.4.27.-5.

*INTERMEDIATE PRECISION*

The effect of random events (intra-laboratory variations) on the analytical precision of the method must be established. Acceptable experiments for establishing intermediate precision include performing the repeatability analysis on different days, or with different instrumentation, or with different analysts. Only 1 of the 3 experiments is required to demonstrate intermediate precision.

*Acceptance criterion*: the relative standard deviation is not greater than the value indicated in Table 2.4.27.-5.

*LIMIT OF QUANTIFICATION*

Determine the lowest concentration meeting the acceptance criterion. Use the results from the accuracy study.

*Acceptance criterion*: the limit of quantification is below the specification limit.

Table 2.4.27.-5

| Concentration range of the metal (mg/kg) | Repeatability (RSD) (per cent) | Intermediate precision (RSD) (per cent) |
|---|---|---|
| 0.01 - 1 | 20 | 32 |
| > 1 | 10 | 16 |

*LIMIT OF DETECTION (ONLY APPLICABLE TO LIMIT TESTS)*

Determine the lowest concentration giving a signal clearly distinct from that obtained with a blank solution.

*Acceptance criterion*: the limit of detection is not more than 0.1 times the concentration of the specification limit.

**01/2008:20428**

# 2.4.28. 2-ETHYLHEXANOIC ACID

Examine by gas chromatography (*2.2.28*), using *3-cyclohexylpropionic acid R* as the internal standard.

*Internal standard solution.* Dissolve 100 mg of *3-cyclohexylpropionic acid R* in *cyclohexane R* and dilute to 100 mL with the same solvent.

*Test solution.* To 0.300 g of the substance to be examined, add 4.0 mL of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min with 1.0 mL of the internal standard solution. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

*Reference solution.* Dissolve 75.0 mg of *2-ethylhexanoic acid R* in the internal standard solution and dilute to 50.0 mL with the same solution. To 1.0 mL of the solution add 4.0 mL of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

The chromatographic procedure may be carried out using:

- a wide-bore fused-silica column 10 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 1.0 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 10 mL/min,
- a flame-ionisation detector,

with the following temperature programme:

| | Time (min) | Temperature (°C) | Rate (°C/min) | Comment |
|---|---|---|---|---|
| Column | 0 - 2 | 40 | – | isothermal |
| | 2 - 7.3 | 40 → 200 | 30 | linear gradient |
| | 7.3 - 10.3 | 200 | – | isothermal |
| Injection port | | 200 | | |
| Detector | | 300 | | |

Inject 1 µL of the test solution and 1 µL of the reference solution.

The test is not valid unless the resolution between the peaks due to 2-ethylhexanoic acid (first peak) and the internal standard is at least 2.0.

Calculate the percentage content of 2-ethylhexanoic acid from the expression:

$$\frac{A_T \times I_R \times m_R \times 2}{A_R \times I_T \times m_T}$$

$A_T$ = area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the test solution,

$A_R$ = area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the reference solution,

$I_T$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution,

$I_R$ = area of the peak due to the internal standard in the chromatogram obtained with the reference solution,

$m_T$ = mass of the substance to be examined in the test solution, in grams,

$m_R$ = mass of 2-ethylhexanoic acid in the reference solution, in grams.

**04/2019:20429**

# 2.4.29. COMPOSITION OF FATTY ACIDS IN OILS RICH IN OMEGA-3 ACIDS

*The assay may be used for quantitative determination of the eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total omega-3-acids content in products from fish oil containing omega-3 acids in different concentrations. The method is applicable to triglycerides or ethyl esters. The former includes fish oils/fish-liver oils and omega-3-concentrates in triglyceride form. The results are expressed as triglycerides or ethyl esters, respectively.*

EPA AND DHA

Gas chromatography (*2.2.28*). *Carry out the operations as rapidly as possible, avoiding exposure to actinic light, oxidising agents, oxidation catalysts (for example, copper and iron) and air.*

The assay is carried out on the methyl esters (after derivatisation of triglycerides - see step B below) or ethyl esters of (*all-Z*)-eicosa-5,8,11,14,17-pentaenoic acid (EPA; 20:5 n-3) and (*all-Z*)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA; 22:6 n-3) in the substance to be examined.

*Internal standard*: *methyl tricosanoate R*.

*Test solutions*: prepare all test solutions in duplicate.

**Step A**

– *Test solution (a).* Dissolve a quantity of the sample to be examined according to Table 2.4.29.-1 and 70.0 mg of the internal standard in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard. Ethyl esters are now ready for analysis. For triglycerides continue as described in step B.

Table 2.4.29.-1

| Approximate sum EPA + DHA (per cent) | Mass of sample to be examined (g) |
|---|---|
| 30 - 50 | 0.4 - 0.5 |
| 50 - 70 | 0.3 |
| 70 - 90 | 0.25 |

– *Test solution (b).* Dissolve a quantity of the sample to be examined according to Table 2.4.29.-1 in a 50 mg/mL solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Ethyl esters are now ready for analysis. For triglycerides continue as described in step B.

**Step B**

Introduce 2.0 mL of test solutions (a) and (b) obtained in step A into separate quartz tubes and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Allow to cool. Add 2 mL of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 mL of *trimethylpentane R*, cap and shake vigorously for at least 30 s. Immediately add 5 mL of a *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and shake thoroughly for at least 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer once more with 1 mL of *trimethylpentane R*. Wash the combined trimethylpentane extracts with 2 quantities, each of 1 mL, of *water R* and dry over *anhydrous sodium sulfate R*.

*Reference solutions*: prepare reference solutions ($a_1$) and ($a_2$) in duplicate; reference solution (c) only has to be prepared for triglycerides, and only if tetracos-15-enoic acid methyl ester is not clearly observed in the chromatogram obtained with test solution (a).

– *Reference solution ($a_1$).* Dissolve 70.0 mg of the internal standard and 90.0 mg of *eicosapentaenoic acid ethyl ester CRS* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard.

– *Reference solution ($a_2$).* Dissolve 60.0 mg of *docosahexaenoic acid ethyl ester CRS* and 70.0 mg of the internal standard in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard.

For ethyl ester samples, reference solutions ($a_1$) and ($a_2$) are ready for analysis. For analysis of triglycerides, continue with step B in the same manner as for test solutions (a) and (b).

– *Reference solution (b).* Dissolve 0.300 g of *methyl arachidate R*, 0.300 g of *methyl behenate R*, 0.300 g of *methyl palmitate R* and 0.300 g of *methyl stearate R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

– *Reference solution (c).* Dissolve a mixture containing 55.0 mg of *docosahexaenoic acid methyl ester R* and 5.0 mg of *tetracos-15-enoic acid methyl ester R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

*Column*:

– *material*: fused silica;

– *dimensions*: *l* = at least 25 m, Ø = 0.25 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.2 µm).

*Carrier gas*: *hydrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:200, alternatively splitless with temperature control (sample solutions need to be diluted 1/200 with a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* before injection).

If necessary, adapt the split ratio and/or sample dilution to obtain a symmetry factor of 0.8-1.5 for the peaks due to the methyl or ethyl esters of eicosapentaenoic acid and docosahexaenoic acid, while at the same time observing that for test solution (b) any peaks due to the corresponding esters of linolenic acid (C18:3; n-3), stearidonic acid (C18:4; n-3),

eicosatetraenoic acid (C20:4; n-3), heneicosapentaenoic acid (C21:5; n-3), and docosapentaenoic acid (C22:5; n-3) are clearly detectable. If both requirements are unachievable, then the clear detection of the corresponding esters mentioned above for test solution (b) takes precedence.

If necessary, adapt the split ratio and/or sample dilution to obtain a symmetry factor of 0.8-1.5 for the peaks due to the components of reference solution (b).

*Temperature*:

| | Split injection | | Splitless injection | |
|---|---|---|---|---|
| | Time (min) | Temperature (°C) | Time (min) | Temperature (°C) |
| Column | 0 - 2 | 170 | 0 - 2 | 90 |
| | 2 - 25.7 | 170 → 240 | 2 - 4.7 | 90 → 170 |
| | 25.7 - 28 | 240 | 4.7 - 28 | 170 → 240 |
| | | | 28 - 30 | 240 |
| Injection port | | 250 | | 90 - 250* |
| Detector | | 270 | | 270 |

*90 °C for on-column injection

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*:

– in the chromatogram obtained with reference solution (b), multiply the area of the peaks due to methyl palmitate, methyl stearate, methyl arachidate and methyl behenate by the corresponding response factors in Table 2.4.29.-2; normalise the corrected areas of the peaks of the fatty acid methyl esters to a sum of 100 per cent; the normalised area percentage of each fatty acid methyl ester is to be within ± 1.0 percentage units of the corresponding weight percentage;

Table 2.4.29.-2

| Fatty acid methyl ester | Theoretical response factor |
|---|---|
| Methyl palmitate | 1.049 |
| Methyl stearate | 1.029 |
| Methyl arachidate | 1.013 |
| Methyl behenate | 1.000 |

– *resolution*:
  – *ethyl esters*: minimum 1.2 between the peaks due to methyl tricosanoate and heneicosapentaenoic acid ethyl ester in the chromatogram obtained with test solution (a);
  – *triglycerides*: minimum 1.2 between the peaks due to docosahexaenoic acid methyl ester and tetracos-15-enoic acid methyl ester in the chromatogram obtained with test solution (a) or with reference solution (c);
– in the chromatogram obtained with test solution (a), the peaks due to methyl tricosanoate and any heneicosapentaenoic acid methyl ester present when compared with the chromatogram obtained with test solution (b) are clearly separated.

Calculate the percentage content of EPA and DHA using the following expression and taking into account the assigned value of the reference substances:

$$\frac{A_x \times m_1}{A_1 \times m_2} \times Rf \times C \times 100$$

$Rf$ = response factor for EPA and DHA as given by the expression:

$$\frac{A_{x,3} \times m_{x,r}}{A_{x,r} \times m_{x,3}}$$

$m_1$ = mass of the internal standard in test solution (a), in milligrams;

$m_2$ = mass of the sample to be examined in test solution (a), in milligrams;

$m_{x,3}$ = mass of the internal standard in reference solution ($a_1$) (EPA determination), or in reference solution ($a_2$) (DHA determination), in milligrams;

$m_{x,r}$ = mass of *eicosapentaenoic acid ethyl ester CRS* in reference solution ($a_1$) or *docosahexaenoic acid ethyl ester CRS* in reference solution ($a_2$), in milligrams;

$A_x$ = area of the peak due to eicosapentaenoic acid ester or docosahexaenoic acid ester in the chromatogram obtained with test solution (a);

$A_{x,r}$ = area of the peak due to eicosapentaenoic acid ester in the chromatogram obtained with reference solution ($a_1$) or docosahexaenoic acid ester in the chromatogram obtained with reference solution ($a_2$);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with test solution (a);

$A_{x,3}$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution ($a_1$) (EPA determination) or with reference solution ($a_2$) (DHA determination);

$C$ = conversion factor between ethyl ester and triglycerides:

Ethyl esters: $C = 1.00$;

Triglycerides: $C = 0.954$ for EPA;

$C = 0.957$ for DHA.

TOTAL OMEGA-3 ACIDS

From the assay for EPA and DHA, calculate the percentage content of the total omega-3 acids using the following expression and identifying the peaks from the chromatograms:

$$EPA + DHA + \frac{A_{n-3}\,(EPA + DHA)}{A_{\mathrm{EPA}} + A_{\mathrm{DHA}}}$$

$EPA$ = percentage content of EPA;

$DHA$ = percentage content of DHA;

$A_{\text{n-3}}$ = sum of the areas of the peaks due to linolenic acid (C18:3; n-3), stearidonic acid (C18:4; n-3), eicosatetraenoic acid (C20:4; n-3), heneicosapentaenoic acid (C21:5; n-3), and docosapentaenoic acid (C22:5; n-3) in the chromatogram obtained with test solution (b);

$A_{\mathrm{EPA}}$ = area of the peak due to EPA ester in the chromatogram obtained with test solution (b);

$A_{\mathrm{DHA}}$ = area of the peak due to DHA ester in the chromatogram obtained with test solution (b).

01/2008:20430

## 2.4.30. ETHYLENE GLYCOL AND DIETHYLENE GLYCOL IN ETHOXYLATED SUBSTANCES

*Ethoxylated substances may contain varied amounts of ethylene glycol and diethylene glycol, as a result of the manufacturing process. The following method may be used for the quantitative determination of these substances, in particular in the case of the following surfactants: macrogolglycerol ricinoleate, macrogolglycerol hydroxystearate, macrogol 15 hydroxystearate, nonoxinol 9 and macrogol cetostearyl ether.*

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 30.0 mg of *1,2-pentanediol R* in *acetone R* and dilute to 30.0 mL with the same solvent. Dilute 1.0 mL of this solution to 20.0 mL with *acetone R*.

*Test solution.* Dissolve 0.500 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the same solution.

*Reference solution (a).* Mix 30.0 mg of *ethylene glycol R* with *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 10.0 mL with the internal standard solution.

*Reference solution (b).* Prepare a solution of *diethylene glycol R* with a concentration corresponding to the prescribed limit and using the same solvents as for the preparation of reference solution (a).

*Column*:

– *material*: fused silica,
– *size*: *l* = 30 m, Ø = 0.53 mm,
– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 10 mL/min.

*Split ratio*: 1:3.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 40 | 80 → 200 |
| | 40 - 45 | 200 → 230 |
| | 45 - 65 | 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Relative retention* with reference to 1,2-pentanediol (retention time = about 19 min): ethylene glycol = about 0.7; diethylene glycol = about 1.3.

04/2018:20431

## 2.4.31. NICKEL IN HYDROGENATED VEGETABLE OILS

Atomic absorption spectrometry (*2.2.23, Method I*).

*The reagents magnesium nitrate R and ammonium dihydrogen phosphate R must be controlled for nickel before use. The actual nickel content is taken into account in the calculation of the nickel content of the sample.*

*Test solution.* Weigh 0.250 g (*m*) of the substance to be examined into a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), add 6.0 mL of *nickel-free nitric acid R* and 2.0 mL of *strong hydrogen peroxide solution R*. Prepare a blank solution in the same manner. Place the closed vessels in a laboratory microwave oven and digest with an appropriate programme, e.g. 1000 W for 40 min. Allow the digestion vessels to cool before opening. Add 2.0 mL of *strong hydrogen peroxide solution R* and repeat the digestion step. Allow the digestion vessels to cool before opening. Quantitatively transfer to a 25 mL flask, add 0.5 mL of a 10 g/L solution of *magnesium nitrate R* and 0.5 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R*, dilute to 25.0 mL with *water for chromatography R* and mix.

*Reference solutions.* Into 4 volumetric flasks, introduce 25 µL, 50 µL, 75 µL and 100 µL of *nickel standard solution (5 ppm Ni) R*. To each flask, add 0.5 mL of a 10 g/L solution of *magnesium nitrate R*, 0.5 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R* and 6.0 mL of *nickel-free nitric acid R* and dilute to 25.0 mL with *water for chromatography R*. Mix to obtain reference solutions containing respectively 5 ng/mL, 10 ng/mL, 15 ng/mL and 20 ng/mL (ppb) of nickel.

*Zero solution.* In a volumetric flask, introduce 1.0 mL of a 10 g/L solution of *magnesium nitrate R*, 1.0 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R* and 12.0 mL of *nickel-free nitric acid R*. Dilute to 50.0 mL with *water for chromatography R* and mix.

*Method.* Determine the absorbance of each solution at 232.0 nm using a suitable graphite furnace atomic absorption (GFAA) spectrometer equipped with a background compensation system, a pyrolytically-coated tube and a nickel hollow-cathode lamp. The optimal temperature programme may be different for each instrument, so the programme recommended by the spectrometer manufacturer may be used. The following temperature parameters for GFAA analysis are given as an example: maintain the drying temperature of the furnace at 120 °C for 35 s after a 5 s ramp, the ashing temperature at 1100 °C for 10 s after a 30 s ramp, the cooling temperature at 800 °C for 5 s after a 5 s decrease, and the atomisation temperature at 2600 °C for 7 s. Use the zero solution to set the instrument to zero. Using the calibration curve, determine the concentrations of the test solution and the blank solution from the corresponding absorptions. If necessary, dilute with the zero solution to obtain a reading within the calibrated absorbance range.

Calculate the content of Ni in micrograms per gram (ppm) using the following expression:

$$\frac{c \times f}{m \times 40}$$

$c$ = measured concentration of Ni, in nanograms per millilitre;

$f$ = dilution factor of the test solution;

$m$ = mass of the substance to be examined, in grams.

07/2016:20432 corrected 10.0

## 2.4.32. TOTAL CHOLESTEROL IN OILS RICH IN OMEGA-3 ACIDS

*This method may be used for the quantitative determination of the sum of free and esterified cholesterol in products of fish oils rich in omega-3 acids (as ethyl esters or triglycerides).*

Gas chromatography (*2.2.28*).

*Internal standard stock solution.* Dissolve 0.15 g of *(5α)-cholestane R* in *heptane R* and dilute to 50.0 mL with the same solvent. The solution may be stored in a deep-freeze for up to 6 months.

*Internal standard working solution. Prepare the solution immediately before use.* Dilute 1.0 mL of the internal standard stock solution to 10.0 mL with *heptane R*.

*Cholesterol stock solution.* Dissolve 50.0 mg of *cholesterol R* in *heptane R* and dilute to 100.0 mL with the same solvent. The solution may be stored in a deep-freeze for up to 6 months.

*Cholesterol working solution. Prepare the solution immediately before use.* Dilute 1.0 mL of the cholesterol stock solution to 10.0 mL with *heptane R*.

*Cholesterol and α-tocopherol stock solution.* Dissolve 50.0 mg of *cholesterol R* and 50.0 mg of *α-tocopherol R* in *heptane R* and dilute to 100.0 mL with the same solvent. The solution may be stored at room temperature for up to 3 months.

*Reference solution. Prepare the solution on the day of use.* Dilute 1.0 mL of the cholesterol and α-tocopherol stock solution to 100.0 mL with *heptane R*.

*Calibration solutions.* See Table 2.4.32.-1. *Prepare the solutions on the day of use.* Dilute each solution to 20.0 mL with a 10 per cent *V/V* solution of *ethyl acetate R* in *heptane R*.

For high levels of cholesterol (3.0-20.0 mg/g), use all 5 calibration solutions.

For low levels of cholesterol (0.2-3.0 mg/g), use the following calibration solutions: 0.2 mg/g, 1.0 mg/g and 3.0 mg/g.

*Test solution.* Weigh 0.100 g of the substance to be examined into a 15 mL quartz tube (for fish oils and cod-liver oils, shake the oil to be examined vigorously in a suitable container, allow to stand for 10-15 min and while maintaining the container upright, remove a sample from the middle layer of the oil for weighing). Add 1.0 mL of the internal standard working solution. Evaporate the solvent on a heating block at 50 °C under a gentle stream of *nitrogen R*. Add 0.5 mL of a 50 per cent *m/m* solution of *potassium hydroxide R* and 3.0 mL of *ethanol (96 per cent) R*. Fill the tube with *nitrogen R*, cap and homogenise. Heat on the heating block at 100 °C for 1 h. Cool for about 10 min, add 6.0 mL of *distilled water R* and homogenise. Condition a 20 mL solid phase extraction (SPE) column containing 1 g of *end-capped octadecylsilyl silica gel for chromatography R* (particles with a diameter of 55 µm and a pore size of 7 nm) with 5 mL of a 50 per cent *V/V* solution of *ethanol (96 per cent) R* in *distilled water R*. Transfer 5.0 mL of the saponified sample to the SPE column ensuring that the column does not dry out. Wash the column with 5.0 mL of a 50 per cent *V/V* solution of *ethanol (96 per cent) R* in *distilled water R*. Elute the column using 20.0 mL of a 10 per cent *V/V* solution of *ethyl acetate R* in *heptane R*. Collect the eluate and use it as the test solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Pressure*: 48 kPa (corresponding to a flow rate of about 0.6 mL/min at 200 °C and about 0.4 mL/min at 330 °C).

*Split ratio*: 1:5.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 200 |
| | 1 - 7.5 | 200 → 330 |
| | 7.5 - 10 | 330 |
| Injection port | | 250 |
| Detector | | 340 |

Prior to analysis, heat the column at 340 °C for at least 30 min using the indicated pressure.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Retention time*: (5α)-cholestane = about 7.5 min; cholesterol = about 9 min.

Plot the calibration curve. The x-axis represents the nominal concentration of cholesterol in milligrams per gram of the substance to be examined in each calibration solution. The y-axis represents the ratio of the area of the peak due to cholesterol to the area of the peak due to (5α)-cholestane in the chromatogram obtained with each calibration solution. Calculate the slope (*S*) and the intercept with the y-axis (*Y*).

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to cholesterol and α-tocopherol in the chromatogram obtained with the reference solution;
- the coefficient of determination ($r^2$) of the calibration curve is not less than 0.995.

Calculate the content of total cholesterol, expressed in milligrams of cholesterol per gram of substance to be examined, using the following expression:

$$\frac{\frac{A_1}{A_2} - Y}{m_1 \times S} \times 0.100$$

$A_1$ = area of the peak due to cholesterol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to (5α)-cholestane in the chromatogram obtained with the test solution;

$m_1$ = mass of the substance to be examined in the test solution, in grams;

$Y$ = intercept of the calibration curve with the y-axis;

$S$ = slope of the calibration curve, in grams per milligram.

Table 2.4.32.-1. – *Preparation of calibration solutions*

| Calibration solutions | | Cholesterol stock solution (mL) | Cholesterol working solution (mL) | Internal standard working solution (mL) |
|---|---|---|---|---|
| Cholesterol concentration (mg/mL) | Cholesterol concentration (mg/g)[a] | | | |
| 0.1 | 20.0 | 4.0 | | 1.0 |
| 0.05 | 10.0 | 2.0 | | 1.0 |
| 0.015 | 3.0 | 0.60 | | 1.0 |
| 0.005 | 1.0 | | 2.0 | 1.0 |
| 0.001 | 0.2 | | 0.40 | 1.0 |

(a)Based on a sample weight of 0.100 g of the substance to be examined.

# 2.5. Assays



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# 2.5. ASSAYS

01/2008:20501
corrected 8.6

## 2.5.1. ACID VALUE

The acid value $I_A$ is the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralise the free acids present in 1 g of the substance.

Dissolve 10.00 g of the substance to be examined, or the quantity prescribed, (*m* g), in 50 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *light petroleum R3*, previously neutralised with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide*, unless otherwise specified, using 0.5 mL of *phenolphthalein solution R1* as indicator. If necessary, heat to about 90 °C to dissolve the substance to be examined. When the substance to be examined has dissolved, titrate with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide* until the pink colour persists for at least 15 s (*n* mL of titrant). When heating has been applied to aid dissolution, maintain the temperature at about 90 °C during the titration.

$$I_A = \frac{5.611n}{m}$$

01/2008:20502

## 2.5.2. ESTER VALUE

The ester value $I_E$ is the number that expresses in milligrams the quantity of potassium hydroxide required to saponify the esters present in 1 g of the substance. It is calculated from the saponification value $I_S$ and the acid value $I_A$:

$$I_E = I_S - I_A$$

01/2008:20503

## 2.5.3. HYDROXYL VALUE

The hydroxyl value $I_{OH}$ is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the acid combined by acylation in 1 g of the substance.

METHOD A

Introduce the quantity of the substance to be examined shown in Table 2.5.3.-1 (*m* g) into a 150 mL acetylation flask fitted with an air condenser, unless another quantity is prescribed in the monograph. Add the quantity of *acetic anhydride solution R1* stated in Table 2.5.3.-1 and attach the air condenser.

Table 2.5.3.-1

| Presumed value $I_{OH}$ | Quantity of sample (g) | Volume of acetylating reagent (mL) |
|---|---|---|
| 10 - 100 | 2.0 | 5.0 |
| 100 - 150 | 1.5 | 5.0 |
| 150 - 200 | 1.0 | 5.0 |
| 200 - 250 | 0.75 | 5.0 |
| 250 - 300 | 0.60 or 1.20 | 5.0 or 10.0 |
| 300 - 350 | 1.0 | 10.0 |
| 350 - 700 | 0.75 | 15.0 |
| 700 - 950 | 0.5 | 15.0 |

Heat the flask in a water-bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask and allow to cool. Add 5 mL of *water R* through the upper end of the condenser. If a cloudiness appears add sufficient *pyridine R* to clear it, noting the volume added. Shake the flask and replace in the water-bath for 10 min. Withdraw the flask and allow to cool. Rinse the condenser and the walls of the flask with 5 mL of *alcohol R*, previously neutralised to *phenolphthalein solution R1*. Titrate with *0.5 M alcoholic potassium hydroxide* using 0.2 mL of *phenolphthalein solution R1* as indicator ($n_1$ mL of *0.5 M alcoholic potassium hydroxide*). Carry out a blank test under the same conditions ($n_2$ mL of *0.5 M alcoholic potassium hydroxide*).

$$I_{OH} = \frac{28.05(n_2 - n_1)}{m} + I_A$$

METHOD B

Introduce the prescribed quantity of the substance to be examined (*m* g) into a perfectly dry 5 mL conical flask fitted with a ground-glass or suitable plastic stopper and add 2.0 mL of *propionic anhydride reagent R*. Close the flask and shake gently to dissolve the substance. Allow to stand for 2 h unless otherwise prescribed. Remove the stopper and transfer the flask and its contents into a wide-mouthed 500 mL conical flask containing 25.0 mL of a 9 g/L solution of *aniline R* in *cyclohexane R* and 30 mL of *glacial acetic acid R*. Swirl the contents of the flask, allow to stand for 5 min, add 0.05 mL of *crystal violet solution R* and titrate with *0.1 M perchloric acid* until an emerald-green colour is obtained ($n_1$ mL of *0.1 M perchloric acid*). Carry out a blank test under the same conditions ($n_2$ mL of *0.1 M perchloric acid*).

$$I_{OH} = \frac{5.610(n_1 - n_2)}{m}$$

To take account of any water present, determine this (*y* per cent) by the semi-micro determination of water (*2.5.12*).

The hydroxyl value is then given by the equation:

$$I_{OH} = \text{(hydroxyl value as determined)} - 31.1y$$

01/2008:20504

## 2.5.4. IODINE VALUE

The iodine value $I_I$ is the number that expresses in grams the quantity of halogen, calculated as iodine, that can be fixed in the prescribed conditions by 100 g of the substance.

*When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.*

METHOD A

Unless otherwise prescribed, use the following quantities (Table 2.5.4.-1) for the determination.

Table 2.5.4.-1

| Presumed value $I_I$ | Quantity of sample (g) |
|---|---|
| less than 20 | 1.0 |
| 20 - 60 | 0.5 - 0.25 |
| 60 - 100 | 0.25 - 0.15 |
| more than 100 | 0.15 - 0.10 |

Introduce the prescribed quantity of the substance to be examined ($m$ g) into a 250 mL flask fitted with a ground-glass stopper and previously dried or rinsed with *glacial acetic acid R*, and dissolve it in 15 mL of *chloroform R* unless otherwise prescribed. Add very slowly 25.0 mL of *iodine bromide solution R*. Close the flask and keep it in the dark for 30 min unless otherwise prescribed, shaking frequently. Add 10 mL of a 100 g/L solution of *potassium iodide R* and 100 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, shaking vigorously until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration adding the *0.1 M sodium thiosulfate* dropwise until the colour is discharged ($n_1$ mL of *0.1 M sodium thiosulfate*). Carry out a blank test under the same conditions ($n_2$ mL of *0.1 M sodium thiosulfate*).

$$I_I = \frac{1.269\ (n_2 - n_1)}{m}$$

METHOD B

Unless otherwise prescribed, use the following quantities (Table 2.5.4.-2) for the determination.

Table 2.5.4.-2

| Presumed value $I_I$ | Mass (g) (corresponding to an excess of 150 per cent ICl) | Mass (g) (corresponding to an excess of 100 per cent ICl) | Iodine chloride solution (mL) |
|---|---|---|---|
| <3 | 10 | 10 | 25 |
| 3 | 8.4613 | 10.5760 | 25 |
| 5 | 5.0770 | 6.3460 | 25 |
| 10 | 2.5384 | 3.1730 | 20 |
| 20 | 0.8461 | 1.5865 | 20 |
| 40 | 0.6346 | 0.7935 | 20 |
| 60 | 0.4321 | 0.5288 | 20 |
| 80 | 0.3173 | 0.3966 | 20 |
| 100 | 0.2538 | 0.3173 | 20 |
| 120 | 0.2115 | 0.2644 | 20 |
| 140 | 0.1813 | 0.2266 | 20 |
| 160 | 0.1587 | 0.1983 | 20 |
| 180 | 0.1410 | 0.1762 | 20 |
| 200 | 0.1269 | 0.1586 | 20 |

The mass of the sample is such that there will be an excess of *iodine chloride solution R* of 50 per cent to 60 per cent of the amount added, i.e. 100 per cent to 150 per cent of the amount absorbed.

Introduce the prescribed quantity of the substance to be examined ($m$ g) into a 250 mL flask fitted with a ground-glass stopper and previously rinsed with *glacial acetic acid R* or dried, and dissolve it in 15 mL of a mixture of equal volumes of *cyclohexane R* and *glacial acetic acid R*, unless otherwise prescribed. If necessary, melt the substance before dissolution (melting point greater than 50 °C). Add very slowly the volume of *iodine chloride solution R* stated in Table 2.5.4.-2. Close the flask and keep it in the dark for 30 min, unless otherwise prescribed, shaking frequently. Add 10 mL of a 100 g/L solution of *potassium iodide R* and 100 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, shaking vigorously until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration adding the *0.1 M sodium thiosulfate* dropwise until the colour is discharged ($n_1$ mL of *0.1 M sodium thiosulfate*). Carry out a blank test under the same conditions ($n_2$ mL of *0.1 M sodium thiosulfate*).

$$I_I = \frac{1.269\ (n_2 - n_1)}{m}$$

**01/2016:20505**

## 2.5.5. PEROXIDE VALUE

The peroxide value $I_P$ is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance, as determined by the methods described below.

*When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.*

METHOD A

Place 5.00 g of the substance to be examined ($m$ g) in a 250 mL conical flask fitted with a ground-glass stopper. Add 30 mL of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*. Shake to dissolve the substance and add 0.5 mL of *saturated potassium iodide solution R*. Shake for exactly 1 min then add 30 mL of *water R*. Titrate with *0.01 M sodium thiosulfate*, adding the titrant slowly with continuous vigorous shaking, until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration, shaking vigorously, until the colour is discharged ($n_1$ mL of *0.01 M sodium thiosulfate*). Carry out a blank test under the same conditions ($n_2$ mL of *0.01 M sodium thiosulfate*). The volume of *0.01 M sodium thiosulfate* used in the blank titration must not exceed 0.1 mL.

$$I_P = \frac{10(n_1 - n_2)}{m}$$

METHOD B

*Carry out the operations avoiding exposure to actinic light.*

Place 50 mL of a mixture of 2 volumes of *trimethylpentane R* and 3 volumes of *glacial acetic acid R* in a conical flask and replace the stopper. Swirl the flask until the substance to be examined ($m$ g; see Table 2.5.5.-1) has dissolved. Using a suitable volumetric pipette, add 0.5 mL of *saturated potassium iodide solution R* and replace the stopper. Allow the solution to stand for 60 ± 1 s, thoroughly shaking the solution continuously, then add 30 mL of *water R*.

Table 2.5.5.-1

| Expected peroxide value $I_P$ | Mass of substance to be examined (g) |
|---|---|
| 0 to 12 | 5.00 to 2.00 |
| 12 to 20 | 2.00 to 1.20 |
| 20 to 30 | 1.20 to 0.80 |
| 30 to 50 | 0.800 to 0.500 |
| 50 to 90 | 0.500 to 0.300 |

Titrate the solution with *0.01 M sodium thiosulfate* ($V_1$ mL), adding it gradually and with constant, vigorous shaking, until the yellow iodine colour has almost disappeared. Add about 0.5 mL of *starch solution R1* and continue the titration, with

constant shaking especially near the end-point, to liberate all of the iodine from the solvent layer. Add the sodium thiosulfate solution dropwise until the blue colour just disappears.

Depending on the volume of *0.01 M sodium thiosulfate* used, it may be necessary to titrate with *0.1 M sodium thiosulfate*.

*NOTE*: there is a 15 s to 30 s delay in neutralising the starch indicator for peroxide values of 70 and greater, due to the tendency of trimethylpentane to float on the surface of the aqueous medium and the time necessary to adequately mix the solvent and the aqueous titrant, thus liberating the last traces of iodine. It is recommended to use *0.1 M sodium thiosulfate* for peroxide values greater than 150. A small amount (0.5 per cent to 1.0 per cent *m/m*) of high HLB emulsifier (for example polysorbate 60) may be added to the mixture to retard the phase separation and decrease the time lag in the liberation of iodine.

Carry out a blank determination ($V_0$ mL). If the result of the blank determination exceeds 0.1 mL of titration reagent, replace the reagents and repeat the determination.

$$I_p = \frac{1000(V_1 - V_0)c}{m}$$

c = concentration of the sodium thiosulfate solution, in moles per litre.

**01/2008:20506**

## 2.5.6. SAPONIFICATION VALUE

The saponification value $I_S$ is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids and to saponify the esters present in 1 g of the substance.

Unless otherwise prescribed, use the quantities indicated in Table 2.5.6.-1 for the determination.

Table 2.5.6.-1

| Presumed value $I_S$ | Quantity of sample (g) |
|---|---|
| <3 | 20 |
| 3 to 10 | 12 to 15 |
| 10 to 40 | 8 to 12 |
| 40 to 60 | 5 to 8 |
| 60 to 100 | 3 to 5 |
| 100 to 200 | 2.5 to 3 |
| 200 to 300 | 1 to 2 |
| 300 to 400 | 0.5 to 1 |

Introduce the prescribed quantity of the substance to be examined (*m* g) into a 250 mL borosilicate glass flask fitted with a reflux condenser. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Attach the condenser and heat under reflux for 30 min, unless otherwise prescribed. Add 1 mL of *phenolphthalein solution R1* and titrate immediately (while still hot) with *0.5 M hydrochloric acid* ($n_1$ mL of *0.5 M hydrochloric acid*). Carry out a blank test under the same conditions ($n_2$ mL of *0.5 M hydrochloric acid*).

$$I_S = \frac{28.05(n_2 - n_1)}{m}$$

**01/2008:20507**

## 2.5.7. UNSAPONIFIABLE MATTER

The term "unsaponifiable matter" is applied to the substances non-volatile at 100-105 °C obtained by extraction with an organic solvent from the substance to be examined after it has been saponified. The result is calculated as per cent *m/m*.

*Use ungreased ground-glass glassware.*

Introduce the prescribed quantity of the substance to be examined (*m* g) into a 250 mL flask fitted with a reflux condenser. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with the aid of 100 mL of *water R*. Shake the liquid carefully with 3 quantities, each of 100 mL, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with 2 quantities, each of 40 mL, of *water R* then wash successively with 40 mL of a 30 g/L solution of *potassium hydroxide R* and 40 mL of *water R*; repeat this procedure 3 times. Wash the ether phase several times, each with 40 mL of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*.

Distil off the ether with suitable precautions and add 6 mL of *acetone R* to the residue. Carefully remove the solvent in a current of air. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh (*a* g).

$$\text{Unsaponifiable matter} = \frac{100a}{m} \text{ per cent}$$

Dissolve the residue in 20 mL of *alcohol R*, previously neutralised to *phenolphthalein solution R* and titrate with *0.1 M ethanolic sodium hydroxide*. If the volume of *0.1 M ethanolic sodium hydroxide* used is greater than 0.2 mL, the separation of the layers has been incomplete; the residue weighed cannot be considered as "unsaponifiable matter". In case of doubt, the test must be repeated.

**01/2008:20508**

## 2.5.8. DETERMINATION OF PRIMARY AROMATIC AMINO-NITROGEN

Dissolve the prescribed quantity of the substance to be examined in 50 mL of *dilute hydrochloric acid R* or in another prescribed solvent and add 3 g of *potassium bromide R*. Cool in ice-water and titrate by slowly adding *0.1 M sodium nitrite* with constant stirring.

Determine the end-point electrometrically or by the use of the prescribed indicator.

01/2008:20509

## 2.5.9. DETERMINATION OF NITROGEN BY SULFURIC ACID DIGESTION

SEMI-MICRO METHOD

Place a quantity of the substance to be examined (*m* g) containing about 2 mg of nitrogen in a combustion flask, add 4 g of a powdered mixture of 100 g of *dipotassium sulfate R*, 5 g of *copper sulfate pentahydrate R* and 2.5 g of *selenium R*, and three glass beads. Wash any adhering particles from the neck into the flask with 5 mL of *sulfuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of *water R*, cool again and place in a steam-distillation apparatus. Add 30 mL of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect about 40 mL of distillate in 20.0 mL of *0.01 M hydrochloric acid* and enough *water R* to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with *0.01 M sodium hydroxide*, using *methyl red mixed solution R* as indicator ($n_1$ mL of *0.01 M sodium hydroxide*).

Repeat the test using about 50 mg of *glucose R* in place of the substance to be examined ($n_2$ mL of *0.01 M sodium hydroxide*).

$$\text{Content of nitrogen} = \frac{0.01401(n_2 - n_1)}{m} \text{ per cent}$$

01/2008:20510

## 2.5.10. OXYGEN-FLASK METHOD

Unless otherwise prescribed the combustion flask is a conical flask of at least 500 mL capacity of borosilicate glass with a ground-glass stopper fitted with a suitable carrier for the sample, for example in platinum or platinum-iridium.

Finely grind the substance to be examined, place the prescribed quantity in the centre of a piece of filter paper measuring about 30 mm by 40 mm provided with a small strip about 10 mm wide and 30 mm long. If paper impregnated with lithium carbonate is prescribed, moisten the centre of the paper with a saturated solution of *lithium carbonate R* and dry in an oven before use. Envelop the substance to be examined in the paper and place it in the sample carrier. Introduce into the flask *water R* or the prescribed solution designed to absorb the combustion products, displace the air with oxygen by means of a tube having its end just above the liquid, moisten the neck of the flask with *water R* and close with its stopper. Ignite the paper strip by suitable means with the usual precautions. Keep the flask firmly closed during the combustion. Shake the flask vigorously to completely dissolve the combustion products. Cool and after about 5 min, unless otherwise prescribed, carefully unstopper the flask. Wash the ground parts and the walls of the flask, as well as the sample carrier, with *water R*. Combine the combustion products and the washings and proceed as prescribed in the monograph.

01/2008:20511
corrected 8.0

## 2.5.11. COMPLEXOMETRIC TITRATIONS

ALUMINIUM

Introduce 20.0 mL of the prescribed solution into a 500 mL conical flask, add 25.0 mL of *0.1 M sodium edetate* and 10 mL of a mixture of equal volumes of a 155 g/L solution of *ammonium acetate R* and *dilute acetic acid R*. Boil for 2 min, then cool. Add 50 mL of *ethanol R* and 3 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *ethanol R*. Titrate the excess of sodium edetate with *0.1 M zinc sulfate* until the colour changes from greenish-blue to reddish-violet.

1 mL of *0.1 M sodium edetate* is equivalent to 2.698 mg of Al.

BISMUTH

Introduce the prescribed solution into a 500 mL conical flask. Dilute to 250 mL with *water R* and then, unless otherwise prescribed, add dropwise, with shaking, *concentrated ammonia R* until the mixture becomes cloudy. Add 0.5 mL of *nitric acid R*. Heat to about 70 °C until the cloudiness disappears completely. Add about 50 mg of *xylenol orange triturate R* and titrate with *0.1 M sodium edetate* until the colour changes from pinkish-violet to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

CALCIUM

Introduce the prescribed solution into a 500 mL conical flask, and dilute to 300 mL with *water R*. Add 6.0 mL of *strong sodium hydroxide solution R* and about 200 mg of *calconecarboxylic acid triturate R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

MAGNESIUM

Introduce the prescribed solution into a 500 mL conical flask and dilute to 300 mL with *water R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Heat to about 40 °C then titrate at this temperature with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

LEAD

Introduce the prescribed solution into a 500 mL conical flask and dilute to 200 mL with *water R*. Add about 50 mg of *xylenol orange triturate R* and *hexamethylenetetramine R* until the solution becomes violet-pink. Titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 20.72 mg of Pb.

ZINC

Introduce the prescribed solution into a 500 mL conical flask and dilute to 200 mL with *water R*. Add about 50 mg of *xylenol orange triturate R* and *hexamethylenetetramine R* until the solution becomes violet-pink. Add 2 g of *hexamethylenetetramine R* in excess. Titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 6.54 mg of Zn.

04/2018:20512

## 2.5.12. WATER: SEMI-MICRO DETERMINATION

The semi-micro determination of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in a suitable anhydrous medium in the presence of a base with sufficient buffering capacity.

APPARATUS

The apparatus consists of a titration vessel with:

- 2 identical platinum electrodes;
- tight inlets for introduction of solvent and titrant;
- an inlet for introduction of air via a desiccant;
- a sample inlet fitted with a stopper or, for liquids, a septum.

Inlet systems for introduction of dry nitrogen or for aspiration of solvents may also be fitted.

The titration is carried out according to the instrument supplier's instructions. Care is taken throughout the determination to avoid exposure of reagents and solvents to atmospheric moisture. The end-point is determined using 2 identical indicator electrodes connected to an electrical source that maintains between the electrodes either a constant current (*2.2.65. Voltametric titration*) or a constant voltage (*2.2.19. Amperometric titration*). Where direct titration is used (method A), addition of titrant causes either a decrease in voltage where constant current is maintained or an increase in current where constant voltage is maintained, until the end-point is reached. Instruments with automatic end-point detection are commonly used. Instrument qualification is carried out according to established quality system procedures, for example using a suitable certified reference material (*sodium aminosalicylate dihydrate for equipment qualification CRS* may be used).

STANDARDISATION

To the titration vessel, add *methanol R*, dried if necessary, or the solvent recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce a suitable amount of water in an appropriate form (*water R* or a certified reference material) and carry out the titration, stirring for the necessary time. The water equivalent is not less than 80 per cent of that indicated by the supplier. Standardise the titrant before the first use and at suitable intervals thereafter.

Unless otherwise prescribed, use Method A.

METHOD A

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and carry out the titration, stirring for the necessary extraction time.

METHOD B

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and in a suitable state of division. Add an accurately measured volume of the titrant, sufficient to give an excess of about 1 mL or the prescribed volume. Allow to stand protected from light for 1 min or the prescribed time, with stirring. Titrate the excess of reagent using *methanol R* or the prescribed solvent, containing an accurately known quantity of water.

SUITABILITY

The accuracy of the determination with the chosen titrant must be verified for each combination of substance, titrant and solvent to be examined. The following procedure, given as an example, is suitable for samples containing 2.5-25 mg of water.

The water content of the substance to be examined is determined using the reagent/solvent system chosen. Thereafter, in the same titration vessel, sequential known amounts of water, corresponding to about 50-100 per cent of the amount found in the substance to be examined, are added in an appropriate form (at least 5 additions) and the water content is determined after each addition. Calculate the percentage recovery ($r$) after each addition using the following expression:

$$r = 100\frac{W_2}{W_1}$$

$W_1$ = amount of water added, in milligrams;

$W_2$ = amount of water found, in milligrams.

Calculate the mean percentage recovery ($\bar{r}$). The reagent/solvent system is considered to be acceptable if $\bar{r}$ is between 97.5 per cent and 102.5 per cent.

Calculate the regression line. The $x$-axis represents the cumulative water added whereas the $y$-axis represents the sum of the initial water content determined for the substance ($M$) and the cumulative water determined after each addition. Calculate the slope ($b$), the intercept with the $y$-axis ($a$) and the intercept of the extrapolated calibration line with the $x$-axis ($d$).

Calculate the percentage errors ($e_1$ and $e_2$) using the following expressions:

$$e_1 = 100\frac{a - M}{M}$$

$$e_2 = 100\frac{|d| - M}{M}$$

$a$ = the $y$-axis intercept, in milligrams of water;

$d$ = the $x$-axis intercept, in milligrams of water;

$M$ = water content of the substance, in milligrams of water.

The reagent/solvent system is considered to be acceptable if:

- $|e_1|$ and $|e_2|$ are not greater than 2.5 per cent;
- $b$ is between 0.975 and 1.025.

01/2008:20513

## 2.5.13. ALUMINIUM IN ADSORBED VACCINES

Homogenise the preparation to be examined and transfer a suitable quantity, presumed to contain 5 mg to 6 mg of aluminium, to a 50 mL combustion flask. Add 1 mL of *sulfuric acid R*, 0.1 mL of *nitric acid R* and some glass beads. Heat the solution until thick, white fumes are evolved. If there is charring at this stage add a few more drops of *nitric acid R* and continue boiling until the colour disappears. Allow to cool for a few minutes, carefully add 10 mL of *water R* and boil until a clear solution is obtained. Allow to cool, add 0.05 mL of *methyl orange solution R* and neutralise with *strong sodium hydroxide solution R* (6.5 mL to 7 mL). If a

precipitate forms dissolve it by adding, dropwise, sufficient *dilute sulfuric acid R*. Transfer the solution to a 250 mL conical flask, rinsing the combustion flask with 25 mL of *water R*. Add 25.0 mL of *0.02 M sodium edetate*, 10 mL of *acetate buffer solution pH 4.4 R* and a few glass beads and boil gently for 3 min. Add 0.1 mL of *pyridylazonaphthol solution R* and titrate the hot solution with *0.02 M copper sulfate* until the colour changes to purplish-brown. Carry out a blank titration omitting the vaccine.

1 mL of *0.02 M sodium edetate* is equivalent to 0.5396 mg of Al.

**01/2008:20514**

## 2.5.14. CALCIUM IN ADSORBED VACCINES

*All solutions used for this test must be prepared using water R.*

Determine the calcium by atomic emission spectrometry (*2.2.22, Method I*). Homogenise the preparation to be examined. To 1.0 mL add 0.2 mL of *dilute hydrochloric acid R* and dilute to 3.0 mL with *water R*. Measure the absorbance at 620 nm.

**01/2008:20515**

## 2.5.15. PHENOL IN IMMUNOSERA AND VACCINES

Homogenise the preparation to be examined. Dilute an appropriate volume with *water R* so as to obtain a solution presumed to contain 15 µg of phenol per millilitre. Prepare a series of reference solutions with *phenol R* containing 5 µg, 10 µg, 15 µg, 20 µg and 30 µg of phenol per millilitre respectively. To 5 mL of the solution to be examined and to 5 mL of each of the reference solutions respectively, add 5 mL of *buffer solution pH 9.0 R*, 5 mL of *aminopyrazolone solution R* and 5 mL of *potassium ferricyanide solution R*. Allow to stand for 10 min and measure the intensity of colour at 546 nm.

Plot the calibration curve and calculate the phenol content of the preparation to be examined.

**01/2008:20516**

## 2.5.16. PROTEIN IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Place 1 mL of the solution in a glass tube and add 0.15 mL of a 400 g/L solution of *trichloroacetic acid R*. Shake, allow to stand for 15 min, centrifuge for 10 min at 5000 r/min and discard the supernatant. Add 0.4 mL of *0.1 M sodium hydroxide* to the centrifugation residue.

*Reference solutions.* Dissolve 0.100 g of *bovine albumin R* in 100 mL of *0.1 M sodium hydroxide* (stock solution containing 1 g of protein per litre). Dilute 1 mL of the stock solution to 20 mL with *0.1 M sodium hydroxide* (working dilution 1 : 50 mg of protein per litre). Dilute 1 mL of the stock solution to 4 mL with *0.1 M sodium hydroxide* (working dilution 2 : 250 mg of protein per litre). Place in 6 glass tubes 0.10 mL, 0.20 mL and 0.40 mL of working dilution 1 and 0.15 mL, 0.20 mL and 0.25 mL of working dilution 2. Make up the volume in each tube to 0.40 mL using *0.1 M sodium hydroxide*.

Prepare a blank using 0.40 mL of *0.1 M sodium hydroxide*.

Add 2 mL of *cupri-tartaric solution R3* to each tube, shake and allow to stand for 10 min. Add to each tube 0.2 mL of a mixture of equal volumes of *phosphomolybdotungstic reagent R* and *water R*, prepared immediately before use. Stopper the tubes, mix by inverting and allow to stand in the dark for 30 min. The blue colour is stable for 60 min. If necessary, centrifuge to obtain clear solutions.

Measure the absorbance (*2.2.25*) of each solution at 760 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbances of the 6 reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

**01/2008:20517**

## 2.5.17. NUCLEIC ACIDS IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*.

Dilute the test solution if necessary to obtain an absorbance value suitable for the instrument used. Measure the absorbance (*2.2.25*) at 260 nm using *water R* as the compensation liquid.

The absorbance of a 1 g/L solution of nucleic acid at 260 nm is 20.

**01/2008:20518**

## 2.5.18. PHOSPHORUS IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volume used in the test (1 mL) contains about 6 µg of phosphorus. Transfer 1.0 mL of the solution to a 10 mL ignition tube.

*Reference solutions.* Dissolve 0.2194 g of *potassium dihydrogen phosphate R* in 500 mL of *water R* to give a solution containing the equivalent of 0.1 mg of phosphorus per millilitre. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Introduce 0.5 mL, 1.0 mL and 2.0 mL of the dilute solution into 3 ignition tubes.

Prepare a blank solution using 2.0 mL of *water R* in an ignition tube.

To all the tubes add 0.2 mL of *sulfuric acid R* and heat in an oil bath at 120 °C for 1 h and then at 160 °C until white fumes appear (about 1 h). Add 0.1 mL of *perchloric acid R* and heat at 160 °C until the solution is decolorised (about 90 min). Cool and add to each tube 4 mL of *water R* and 4 mL of *ammonium molybdate reagent R*. Heat in a water-bath at 37 °C for 90 min and cool. Adjust the volume to 10.0 mL with *water R*. The blue colour is stable for several hours.

Measure the absorbance (*2.2.25*) of each solution at 820 nm using the blank solution as the compensation liquid. Draw a calibration curve with the absorbances of the 3 reference

solutions as a function of the quantity of phosphorus in the solutions and read from the curve the quantity of phosphorus in the test solution.

**01/2008:20519 corrected 10.0**

## 2.5.19. *O*-ACETYL IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 30 µg to 600 µg of acetylcholine chloride (*O*-acetyl). Introduce 0.3 mL, 0.5 mL and 1.0 mL in duplicate into 6 tubes (3 reaction solutions and 3 correction solutions).

*Reference solutions.* Dissolve 0.150 g of *acetylcholine chloride R* in 10 mL of *water R* (stock solution containing 15 g of acetylcholine chloride per litre). Immediately before use, dilute 1 mL of the stock solution to 50 mL with *water R* (working dilution 1: 300 µg of acetylcholine chloride per millilitre). Immediately before use, dilute 1 mL of the stock solution to 25 mL with *water R* (working dilution 2: 600 µg of acetylcholine chloride per millilitre). Introduce 0.1 mL and 0.4 mL of working dilution 1 in duplicate (reaction and correction solutions) into 4 tubes and 0.6 mL and 1.0 mL of working dilution 2 in duplicate (reaction and correction solutions) into another 4 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Add 1.0 mL of a 4 M solution of hydrochloric acid prepared from *hydrochloric acid R* to each of the correction tubes and to the blank. Add 2.0 mL of *alkaline hydroxylamine solution R* to each tube. Allow the reaction to proceed for exactly 2 min and add 1.0 mL of the 4 M solution of hydrochloric acid to each of the reaction tubes. To each tube, add 1.0 mL of a 100 g/L solution of *ferric chloride R* in a 0.1 M solution of hydrochloric acid prepared from *hydrochloric acid R*, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (*2.2.25*) of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the 4 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 3 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

**01/2008:20520**

## 2.5.20. HEXOSAMINES IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 125 µg to 500 µg of glucosamine (hexosamine). Introduce 1.0 mL of the diluted solution into a graduated tube.

*Reference solutions.* Dissolve 60 mg of *glucosamine hydrochloride R* in 100 mL of *water R* (stock solution containing 0.500 g of glucosamine per litre). Introduce 0.25 mL, 0.50 mL, 0.75 mL, and 1.0 mL of the working dilution into 4 graduated tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Add 1 mL of a solution of *hydrochloric acid R* (292 g/L) to each tube. Stopper the tubes and place in a water-bath for 1 h. Cool to room temperature. Add to each tube 0.05 mL of a 5 g/L solution of *thymolphthalein R* in *alcohol R*; add a solution of *sodium hydroxide R* (200 g/L) until a blue colour is obtained and then *1 M hydrochloric acid* until the solution is colourless. Dilute the volume in each tube to 10 mL with *water R* (neutralised hydrolysates).

In a second series of 10 mL graduated tubes, place 1 mL of each neutralised hydrolysate. Add 1 mL of acetylacetone reagent (a mixture, prepared immediately before use, of 1 volume of *acetylacetone R* and 50 volumes of a 53 g/L solution of *anhydrous sodium carbonate R*) to each tube. Stopper the tubes and place in a water-bath at 90 °C for 45 min. Cool to room temperature. Add to each tube 2.5 mL of *alcohol R* and 1.0 mL of dimethylaminobenzaldehyde solution (immediately before use dissolve 0.8 g of *dimethylaminobenzaldehyde R* in 15 mL of *alcohol R* and add 15 mL of *hydrochloric acid R*) and dilute the volume in each tube to 10 mL with *alcohol R*. Stopper the tubes, mix by inverting and allow to stand in the dark for 90 min. Measure the absorbance (*2.2.25*) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 4 reference solutions and the corresponding content of hexosamine and read from the curve the quantity of hexosamine in the test solution.

**01/2008:20521**

## 2.5.21. METHYLPENTOSES IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2 µg to 20 µg of rhamnose (methylpentoses). Introduce 0.25 mL, 0.50 mL and 1.0 mL of the diluted solution into 3 tubes.

*Reference solutions.* Dissolve 0.100 g of *rhamnose R* in 100 mL of *water R* (stock solution containing 1 g of methylpentose per litre). Immediately before use, dilute 1 mL of the stock solution to 50 mL with *water R* (working dilution: 20 mg of methylpentose per litre). Introduce 0.10 mL, 0.25 mL, 0.50 mL, 0.75 mL and 1.0 mL of the working dilution into 5 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 4.5 mL of a cooled mixture of 1 volume of *water R* and 6 volumes of *sulfuric acid R*. Warm the tubes to room temperature and place in a water-bath for a few minutes. Cool to room temperature. Add to each tube 0.10 mL of a 30 g/L solution of *cysteine hydrochloride R*, prepared immediately before use. Shake and allow to stand for 2 h.

Measure the absorbance (*2.2.25*) of each solution at 396 nm and at 430 nm using the blank as compensation liquid. For each solution, calculate the difference between the absorbance measured at 396 nm and that measured at 430 nm. Draw a calibration curve from the absorbance differences for the 5 reference solutions and the corresponding content

of methylpentose and read from the curve the quantity of methylpentose in the test solution for each volume tested. Calculate the mean of the 3 values.

**01/2008:20522**

## 2.5.22. URONIC ACIDS IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 4 µg to 40 µg of glucuronic acid (uronic acids). Introduce 0.25 mL, 0.50 mL and 1.0 mL of the diluted solution into 3 tubes.

*Reference solutions.* Dissolve 50 mg of *sodium glucuronate R* in 100 mL of *water R* (stock solution containing 0.4 g of glucuronic acid per litre). Immediately before use, dilute 5 mL of the stock solution to 50 mL with *water R* (working dilution: 40 mg of glucuronic acid per litre). Introduce 0.10 mL, 0.25 mL, 0.50 mL, 0.75 mL, and 1.0 mL of the working dilution into 5 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 5.0 mL of *borate solution R*. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature. Add 0.20 mL of a 1.25 g/L solution of *carbazole R* in *ethanol R* to each tube. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature. Measure the absorbance (*2.2.25*) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 5 reference solutions and the corresponding content of glucuronic acid and read from the curve the quantity of glucuronic acid in the test solution for each volume tested. Calculate the mean of the 3 values.

**01/2008:20523**

## 2.5.23. SIALIC ACID IN POLYSACCHARIDE VACCINES

*Test solution.* Transfer quantitatively the contents of one or several containers to a volumetric flask of a suitable volume that will give a solution with a known concentration of about 250 µg per millilitre of polysaccharide and dilute to volume with *water R*. Using a syringe, transfer 4.0 mL of this solution to a 10 mL ultrafiltration cell suitable for the passage of molecules of relative molecular mass less than 50 000. Rinse the syringe twice with *water R* and transfer the rinsings to the ultrafiltration cell. Carry out the ultrafiltration, with constant stirring, under *nitrogen R* at a pressure of about 150 kPa. Refill the cell with *water R* each time the volume of liquid in it has decreased to 1 mL and continue until 200 mL has been filtered and the remaining volume in the cell is about 2 mL. Using a syringe, transfer this residual liquid to a 10 mL volumetric flask. Wash the cell with 3 quantities, each of 2 mL, of *water R*, transfer the washings to the flask and dilute to 10.0 mL with *water R* (test solution). In each of 2 test-tubes place 2.0 mL of the test solution.

*Reference solutions.* Use the reference solutions prescribed in the monograph.

Prepare 2 series of 3 test-tubes, place in the tubes of each series 0.5 mL, 1.0 mL and 1.5 mL respectively, of the reference solution corresponding to the type of vaccine to be examined and adjust the volume in each tube to 2.0 mL with *water R*.

Prepare blank solutions using 2.0 mL of *water R* in each of 2 test-tubes.

To all the tubes add 5.0 mL of *resorcinol reagent R*. Heat at 105 °C for 15 min, cool in cold water and transfer the tubes to a bath of iced water. To each tube add 5 mL of *isoamyl alcohol R* and mix thoroughly. Place in the bath of iced water for 15 min. Centrifuge the tubes and keep them in the bath of iced water until the examination by absorption spectrophotometry. Measure the absorbance (*2.2.25*) of each supernatant solution at 580 nm and 450 nm using *isoamyl alcohol R* as the compensation liquid. For each wavelength, calculate the absorbance as the mean of the values obtained with 2 identical solutions. Subtract the mean value for the blank solution from the mean values obtained for the other solutions.

Draw a graph showing the difference between the absorbances at 580 nm and 450 nm of the reference solutions as a function of the content of *N*-acetylneuraminic acid and read from the graph the quantity of *N*-acetylneuraminic acid (sialic acid) in the test solution.

**01/2011:20524**

## 2.5.24. CARBON DIOXIDE IN GASES

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

**Description and principle of measurement**. The concentration of carbon dioxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell and it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for carbon dioxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

When carbon dioxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of carbon dioxide. The generated signal is linearised in order to obtain the carbon dioxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

**Required technical specifications**. When used for a limit test, the infrared analyser meets the following technical specifications:

- *limit of detection*: (generally defined as a signal-to-noise ratio of 2) maximum 20 per cent of the maximum admissible concentration;
- *repeatability*: maximum relative standard deviation of 10 per cent of the maximum admissible concentration, determined on 6 measurements;

– *linearity*: maximum 10 per cent of the maximum admissible concentration.

The technical specifications must be met in the presence of the other gas impurities in the sample.

**01/2011:20525**

## 2.5.25. CARBON MONOXIDE IN GASES

METHOD I

*Apparatus.* The apparatus (Figure 2.5.25.-1) consists of the following parts connected in series:

– a U-tube ($U_1$) containing *anhydrous silica gel R* impregnated with *chromium trioxide R*;

– a wash bottle ($F_1$) containing 100 mL of a 400 g/L solution of *potassium hydroxide R*;

– a U-tube ($U_2$) containing pellets of *potassium hydroxide R*;

– a U-tube ($U_3$) containing *diphosphorus pentoxide R* dispersed on previously granulated, fused pumice;

– a U-tube ($U_4$) containing 30 g of *recrystallised iodine pentoxide R* in granules, previously dried at 200 °C and kept at a temperature of 120 °C ($T$) during the test; the iodine pentoxide is packed in the tube in 1 cm columns separated by 1 cm columns of glass wool to give an effective length of 5 cm;

– a reaction tube ($F_2$) containing 2.0 mL of *potassium iodide solution R* and 0.15 mL of *starch solution R*.

*Method.* Flush the apparatus with 5.0 L of *argon R* and, if necessary, discharge the blue colour in the iodide solution by adding the smallest necessary quantity of freshly prepared *0.002 M sodium thiosulfate*. Continue flushing until not more than 0.045 mL of *0.002 M sodium thiosulfate* is required after passage of 5.0 L of *argon R*. Pass the gas to be examined from the cylinder through the apparatus, using the prescribed volume and the flow rate. Flush the last traces of liberated iodine into the reaction tube by passing through the apparatus 1.0 L of *argon R*. Titrate the liberated iodine with *0.002 M sodium thiosulfate*. Carry out a blank test, using the prescribed volume of *argon R*. The difference between the volumes of *0.002 M sodium thiosulfate* used in the titrations is not greater than the prescribed limit.

METHOD II

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

**Description and principle of measurement**. The concentration of carbon monoxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell; it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for carbon monoxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

When carbon monoxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of carbon monoxide. The generated signal is linearised in order to obtain the carbon monoxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

**Required technical specifications**. When used for a limit test, the carbon monoxide infrared analyser meets the following technical specifications:

– *limit of detection*: (generally defined as a signal-to-noise ratio of 2) maximum 20 per cent of the maximum admissible concentration;

– *repeatability*: maximum relative standard deviation of 10 per cent of the maximum admissible concentration, determined on 6 measurements;

– *linearity*: maximum 10 per cent of the maximum admissible concentration.

The technical specifications must be met in the presence of the other gas impurities in the sample.

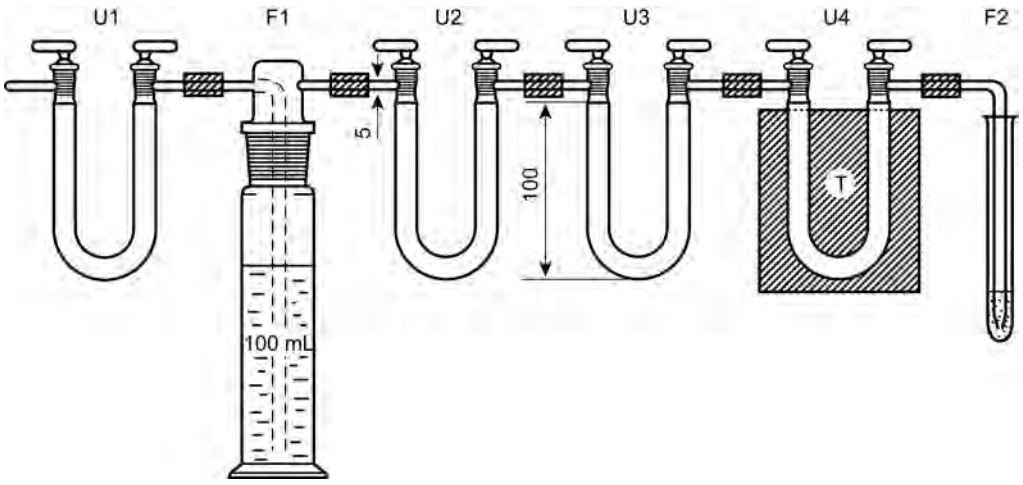


Figure 2.5.25.-1. – *Apparatus for the determination of carbon monoxide*
*Dimensions in millimetres*

01/2008:20526

## 2.5.26. NITROGEN MONOXIDE AND NITROGEN DIOXIDE IN GASES

Nitrogen monoxide and nitrogen dioxide in gases are determined using a chemiluminescence analyser (Figure 2.5.26.-1).

The apparatus consists of the following:

- a device for filtering, checking and controlling the flow of the gas to be examined,
- a converter that reduces nitrogen dioxide to nitrogen monoxide, to determine the combined content of nitrogen monoxide and nitrogen dioxide. The efficiency of the converter has to be verified prior to use,
- a controlled-flow-rate ozone generator; the ozone is produced by high-voltage electric discharges across two electrodes; the ozone generator is supplied with pure oxygen or with dehydrated ambient air and the concentration of ozone obtained must greatly exceed the maximum content of any detectable nitrogen oxides,
- a chamber in which nitrogen monoxide and ozone can react,
- a system for detecting light radiation emitted at a wavelength of 1.2 µm, consisting of a selective optical filter and a photomultiplier tube.

01/2009:20527

## 2.5.27. OXYGEN IN GASES

Oxygen in gases is determined using a paramagnetic analyser.

The principle of the method is based on the high paramagnetic sensitivity of the oxygen molecule. Oxygen exerts a strong interaction on magnetic fields, which is measured electronically, amplified and converted to a reading of oxygen concentration. The measurement of oxygen concentration is dependent upon the pressure and temperature and, if the analyser is not automatically compensated for variations in temperature and pressure, it must be calibrated immediately prior to use. As the paramagnetic effect of oxygen is linear, the instrument must have a suitable range with a readability of 0.1 per cent or better.

*Calibration of the instrument.* Make the setting in the following manner:

- set the zero by passing *nitrogen R1* through the instrument until a constant reading is obtained;
- set the scale to 100 per cent by passing *oxygen R* through the instrument at the same flow rate as for *nitrogen R1* until a constant reading is obtained.

*Assay.* Pass the gas to be examined through the instrument at a constant flow rate until a constant reading is obtained. Record the concentration of oxygen in the gas to be examined.

01/2017:20528

## 2.5.28. WATER IN GASES

Water in gases is determined using an electrolytic hygrometer, described below.

The measuring cell consists of a thin film of diphosphorus pentoxide, between 2 coiled platinum wires which act as electrodes. The water vapour in the gas to be examined is absorbed by the diphosphorus pentoxide, which is transformed to phosphoric acid, an electrical conductor. A continuous voltage applied across the electrodes produces electrolysis of the water and the regeneration of the diphosphorus pentoxide. The resulting electric current, which is proportional to the water content in the gas to be examined, is measured. This system is self-calibrating since it obeys Faraday's law.

Take a sample of the gas to be examined. Allow the gas to stabilise at room temperature. Purge the cell continuously until a stable reading is obtained. Measure the water content in the gas to be examined, making sure that the temperature is constant throughout the device used to introduce the gas into the apparatus.

The electrolytic hygrometer achieves accurate sample flows by using a mass flow controller to deliver a constant volumetric flow rate to ensure that the water content is determined accurately. The calibration of the mass flow controller is normally performed using nitrogen. When using gases other than nitrogen for calibration, consult the manufacturer's instructions for the appropriate conversion factors and ensure that the correct cell is used for the type of gas to be examined.

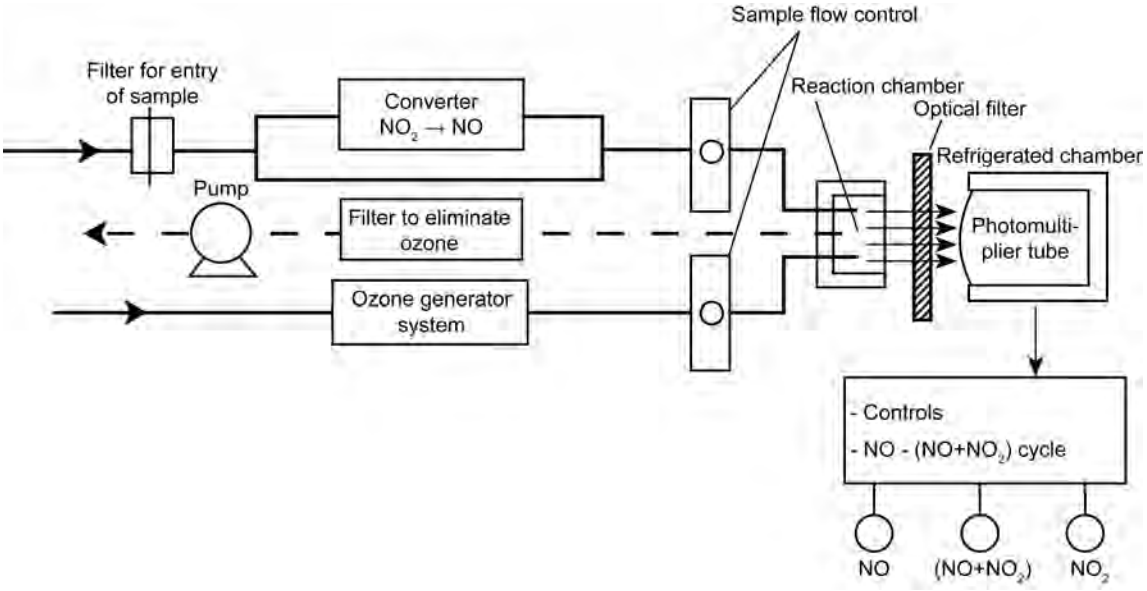


Figure 2.5.26.-1. – *Chemiluminescence analyser*

**01/2013:20529**

## 2.5.29. SULFUR DIOXIDE

Introduce 150 mL of *water R* into the flask (*A*) (see Figure 2.5.29.-1) and pass *carbon dioxide R* through the whole system for 15 min at a rate of 100 ± 5 mL/min. To 10 mL of *dilute hydrogen peroxide solution R* add 0.15 mL of a 1 g/L solution of *bromophenol blue R* in *ethanol (20 per cent V/V) R*. Add *0.1 M sodium hydroxide* until a violet-blue colour is obtained, without exceeding the end-point. Place the solution in the test-tube (*D*). Without interrupting the stream of carbon dioxide, remove the funnel (*B*) and introduce through the opening into the flask (*A*) 25.0 g (*m* g) of the substance to be examined with the aid of 100 mL of *water R*. Replace the funnel. Close the tap of the funnel and add 80 mL of *dilute hydrochloric acid R* to the funnel. Open the tap of the funnel to allow the hydrochloric acid solution to flow into the flask, making sure that no sulfur dioxide escapes into the funnel by closing the tap before the last few millilitres of hydrochloric acid solution drain out. Boil for 1 h. Open the tap of the funnel and stop the flow of carbon dioxide and also the heating and the cooling water. Transfer the contents of the test-tube with the aid of a little *water R* to a 200 mL wide-necked, conical flask. Heat on a water-bath for 15 min and allow to cool. Add 0.1 mL of a 1 g/L solution of *bromophenol blue R* in *ethanol (20 per cent V/V) R* and titrate with *0.1 M sodium hydroxide* until the colour changes from yellow to violet-blue ($V_1$ mL). Carry out a blank titration ($V_2$ mL).

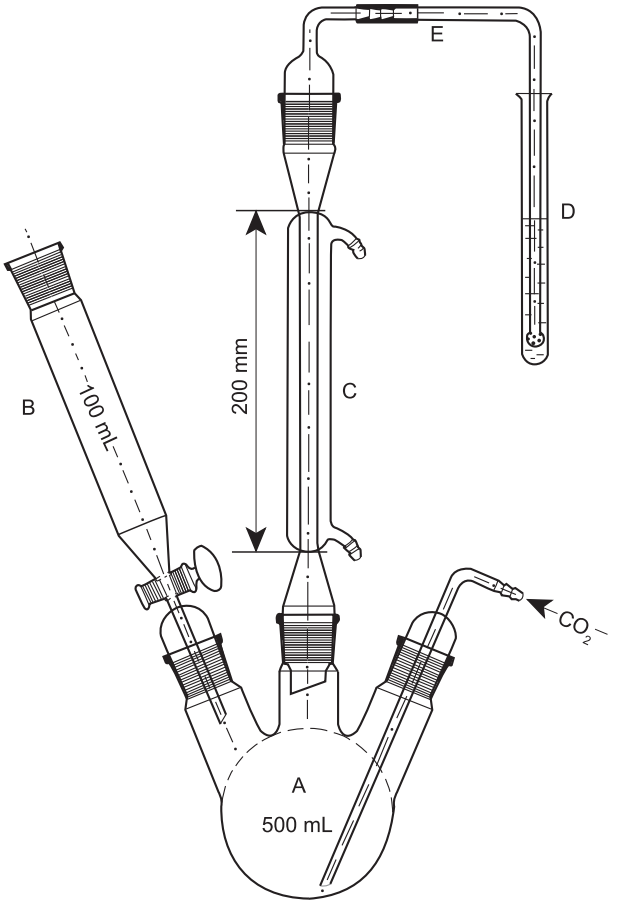


Figure 2.5.29.-1.– *Apparatus for the determination of sulfur dioxide*

Calculate the content of sulfur dioxide in parts per million using the following expression:

$$32\,030 \times (V_1 - V_2) \times \frac{n}{m}$$

$n$ = molarity of the sodium hydroxide solution used as titrant.

**01/2008:20530**

## 2.5.30. OXIDISING SUBSTANCES

Transfer 4.0 g to a glass-stoppered, 125 mL conical flask and add 50.0 mL of *water R*. Insert the stopper and swirl for 5 min. Transfer to a glass-stoppered 50 mL centrifuge tube and centrifuge. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125 mL conical flask. Add 1 mL of *glacial acetic acid R* and 0.5 g to 1.0 g of *potassium iodide R*. Insert the stopper, swirl, and allow to stand for 25 min to 30 min in the dark. Add 1 mL of *starch solution R* and titrate with *0.002 M sodium thiosulfate* until the starch-iodine colour disappears. Carry out a blank determination. Not more than 1.4 mL of *0.002 M sodium thiosulfate* is required (0.002 per cent, calculated as $H_2O_2$).

1 mL of *0.002 M sodium thiosulfate* is equivalent to 34 µg of oxidising substances, calculated as hydrogen peroxide.

**01/2008:20531**

## 2.5.31. RIBOSE IN POLYSACCHARIDE VACCINES

*Test solution.* Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2.5 µg to 25 µg of ribose. Introduce 0.20 mL and 0.40 mL of the diluted solution into tubes in triplicate.

*Reference solutions.* Dissolve 25 mg of *ribose R* in *water R* and dilute to 100.0 mL with the same solvent (stock solution containing 0.25 g/L of ribose). Immediately before use, dilute 1 mL of the stock solution to 10.0 mL with *water R* (working dilution: 25 mg/L of ribose). Introduce 0.10 mL, 0.20 mL, 0.40 mL, 0.60 mL, 0.80 mL and 1.0 mL of the working dilution into 6 tubes.

Prepare a blank using 2 mL of *water R*.

Make up the volume in each tube to 2 mL with *water R*. Shake. Add 2 mL of a 0.5 g/L solution of *ferric chloride R* in *hydrochloric acid R* to each tube. Shake. Add 0.2 mL of a 100 g/L solution of *orcinol R* in *ethanol R*. Place the tubes in a water-bath for 20 min. Cool in iced water. Measure the absorbance (*2.2.25*) of each solution at 670 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbance readings for the 6 reference solutions and the corresponding content of ribose and read from the curve the quantity of ribose in the test solution for each volume tested. Calculate the mean of the 3 values.

07/2019:20532

# 2.5.32. WATER: MICRO DETERMINATION

PRINCIPLE

The coulometric titration of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in an anhydrous medium in the presence of a base with sufficient buffering capacity. In contrast to the volumetric method described in general chapter *2.5.12. Water: semi-micro determination*, iodine is produced electrochemically in the reaction cell by oxidation of iodide. The iodine produced at the anode reacts immediately with the water and the sulfur dioxide contained in the reaction cell. The quantity of water in the substance is directly proportional to the quantity of electricity (in coulombs), corresponding to electric current (in amperes) multiplied by time (in seconds), used for iodine generation up until the titration end-point. When all of the water in the reaction cell has been consumed, the end-point is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, an amount of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the reaction cell by pre-titration, i.e. the electrolyte reagent is titrated to dryness before starting the sample analysis. Individual determinations can be carried out successively in the same reagent solution, under the following conditions:

- each component of the test mixture is compatible with the other components;
- no other reactions take place;
- the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is intended for the quantitative determination of small quantities of water (from 10 µg), however a working range of 100 µg to 10 mg of water is recommended for reproducibility reasons.

Accuracy and precision of the method are predominantly governed by the sample preparation and the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

APPARATUS

The apparatus consists of a reaction cell, electrodes and a magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in an oven and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

Instrument qualification is carried out according to established quality system procedures, for example using a suitable certified reference material. *Sodium aminosalicylate dihydrate for equipment qualification CRS* may be used when proceeding by direct or liquid sample introduction, whereas *amoxicillin trihydrate for performance verification CRS* may be used with the evaporation technique.

METHOD

Fill the compartments of the reaction cell with *electrolyte reagent for the micro determination of water R* according to the manufacturer's instructions and perform the coulometric pre-titration to a stable end-point. Introduce the prescribed quantity of the substance to be examined into the reaction cell and titrate again to a stable end-point, stirring for at least 30 s, unless otherwise indicated in the monograph. If an oven is used, the prescribed quantity of sample is introduced into the oven and heated. After evaporation of the water from the sample into the reaction cell, the titration is started. Alternatively, the evaporated moisture is immediately titrated while heating the sample in the oven to avoid loss of evaporated water already collected in the reagent solution during prolonged heating. Read the value from the instrument's output and calculate if necessary the percentage or quantity of water that is present in the substance. When appropriate to the type of sample and the sample preparation, perform a blank titration.

VERIFICATION OF ACCURACY

At appropriate intervals, such as at least at the beginning and the end of a series of sample titrations, introduce a defined quantity of water, in the same order of magnitude as the quantity of water in the sample, using a suitable certified reference material and perform the coulometric titration. The recovery is within the range of 97.5 per cent to 102.5 per cent for an addition of 1000 µg of $H_2O$ and within the range of 90.0 per cent to 110.0 per cent for the addition of 100 µg of $H_2O$.

01/2008:20533
corrected 6.0

# 2.5.33. TOTAL PROTEIN

Many of the assay methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for assay purposes. If the buffer used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid but if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may significantly reduce the content in solution. This can be prevented by preparing samples at higher concentration or by using a non-ionic detergent in the preparation.

*Test solution.* Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/mL and 2 mg/mL.

*Reference solution.* Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

**Procedure**. Keep the test solution, the reference solution and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (*2.2.25*) of the test solution and the reference solution in quartz cells at 280 nm, using the prescribed buffer as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

**Light scattering**. The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression. Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

**Calculations**. Use corrected values for the calculations. Calculate the concentration of protein in the test solution ($C_U$) from the following equation:

$$C_U = C_S(A_U / A_S)$$

where $C_S$ is the concentration of protein in the reference solution and $A_U$ and $A_S$ are the corrected absorbances of the test solution and the reference solution, respectively.

METHOD 2

This method (commonly referred to as the Lowry assay) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm. The phosphomolybdotungstic reagent reacts primarily with tyrosine residues in the protein. Colour development reaches a maximum in 20 min to 30 min at room temperature, after which there is a gradual loss of colour. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test sample may be used. Most interfering substances cause a lower colour yield; however, some detergents cause a slight increase in colour. A high salt concentration may cause a precipitate to form. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same. Where separation of interfering substances from the protein in the test sample is necessary, proceed as directed below for interfering substances prior to preparation of the test solution. The effect of interfering substances may be minimised by dilution, provided the concentration of the test protein remains sufficient for accurate measurement.

Use *distilled water R* to prepare all buffers and reagents used for this method.

*Test solution.* Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve. A suitable buffer will produce a solution of pH 10.0 to 10.5.

*Reference solutions.* Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 5 µg/mL and 100 µg/mL.

*Blank.* Use the buffer used to prepare the test solution and the reference solutions.

*Copper sulfate reagent.* Dissolve 100 mg of *copper sulfate pentahydrate R* and 0.2 g of *sodium tartrate R* in *distilled water R* and dilute to 50 mL with the same solvent. Dissolve 10 g of *anhydrous sodium carbonate R* in *distilled water R* and dilute to 50 mL with the same solvent. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Use within 24 h.

*Alkaline copper reagent.* Mix 1 volume of copper sulfate reagent, 2 volumes of a 50 g/L solution of *sodium dodecyl sulfate R* and 1 volume of a 32 g/L solution of *sodium hydroxide R*. Store at room temperature and use within 2 weeks.

*Diluted phosphomolybdotungstic reagent.* Mix 5 mL of *phosphomolybdotungstic reagent R* with 55 mL of *distilled water R*. Store in an amber bottle, at room temperature.

**Procedure**. To 1.0 mL of each reference solution, of the test solution and of the blank, add 1.0 mL of alkaline copper reagent and mix. Allow to stand for 10 min. Add 0.5 mL of the diluted phosphomolybdotungstic reagent, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution from the blank as compensation liquid.

**Calculations**. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

**Interfering substances**. In the following procedure, deoxycholate-trichloroacetic acid is added to a test sample to remove interfering substances by precipitation of proteins before determination; this technique can also be used to concentrate proteins from a dilute solution.

Add 0.1 mL of a 1.5 g/L solution of *sodium deoxycholate R* to 1 mL of a solution of the substance to be examined. Mix using a vortex mixer and allow to stand at room temperature for 10 min. Add 0.1 mL of a 720 g/L solution of *trichloroacetic acid R* and mix using a vortex mixer. Centrifuge at 3000 *g* for 30 min, decant the liquid and remove any residual liquid with a pipette. Redissolve the protein pellet in 1 mL of alkaline copper reagent.

METHOD 3

This method (commonly referred to as the Bradford assay) is based on the absorption shift from 470 nm to 595 nm observed when the acid blue 90 dye binds to protein. The acid blue 90 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the assay to different proteins. The protein used as reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water R* to prepare all buffers and reagents used for this method.

*Test solution.* Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve.

*Reference solutions.* Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg/mL and 1 mg/mL.

*Blank.* Use the buffer used to prepare the test solution and the reference solutions.

*Acid blue 90 reagent.* Dissolve 0.10 g of *acid blue 90 R* in 50 mL of *alcohol R*. Add 100 mL of *phosphoric acid R*, dilute to 1000 mL with *distilled water R* and mix. Filter the solution

and store in an amber bottle at room temperature. Slow precipitation of the dye occurs during storage. Filter the reagent before using.

**Procedure**. Add 5 mL of acid blue 90 reagent to 0.100 mL of each reference solution, of the test solution and of the blank. Mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances (*2.2.25*) of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid. Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.

**Calculations**. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 4

This method (commonly referred to as the bicinchoninic acid or BCA assay) is based on reduction of the cupric ($Cu^{2+}$) ion to cuprous ($Cu^{1+}$) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. Few substances interfere with the reaction. When interfering substances are present their effect may be minimised by dilution, provided that the concentration of the protein to be determined remains sufficient for accurate measurement. Alternatively, the protein precipitation procedure given in Method 2 may be used to remove interfering substances. Because different protein species may give different colour response intensities, the reference protein and protein to be determined must be the same.

Use *distilled water R* to prepare all buffers and reagents used for this method.

*Test solution*. Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

*Reference solutions*. Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 1200 µg/mL.

*Blank*. Use the buffer used to prepare the test solution and the reference solutions.

*BCA reagent*. Dissolve 10 g of *disodium bicinchoninate R*, 20 g of *sodium carbonate monohydrate R*, 1.6 g of *sodium tartrate R*, 4 g of *sodium hydroxide R*, and 9.5 g of *sodium hydrogen carbonate R* in *distilled water R*. Adjust, if necessary, to pH 11.25 with a solution of *sodium hydroxide R* or a solution of *sodium hydrogen carbonate R*. Dilute to 1000 mL with *distilled water R* and mix.

*Copper-BCA reagent*. Mix 1 mL of a 40 g/L solution of *copper sulfate pentahydrate R* and 50 mL of BCA reagent.

**Procedure**. Mix 0.1 mL of each reference solution, of the test solution and of the blank with 2 mL of the copper-BCA reagent. Incubate the solutions at 37 °C for 30 min, note the time and allow the mixtures to cool to room temperature. Within 60 min of the end of incubation, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution in quartz cells at 562 nm, using the blank as compensation liquid. After the solutions have cooled to room temperature, the colour intensity continues to increase gradually.

**Calculations**. The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 5

This method (commonly referred to as the biuret assay) is based on the interaction of cupric ($Cu^{2+}$) ion with protein in alkaline solution and resultant development of absorbance at 545 nm. This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimise the effects of interfering substances also can be used to determine the protein content in test samples at concentrations below 500 µg/mL.

Use *distilled water R* to prepare all buffers and reagents used for this method.

*Test solution*. Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

*Reference solutions*. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than three reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.5 mg/mL and 10 mg/mL.

*Blank*. Use a 9 g/L solution of *sodium chloride R*.

*Biuret reagent*. Dissolve 3.46 g of *copper sulfate pentahydrate R* in 10 mL of hot *distilled water R*, and allow to cool (Solution A). Dissolve 34.6 g of *sodium citrate R* and 20.0 g of *anhydrous sodium carbonate R* in 80 mL of hot *distilled water R*, and allow to cool (Solution B). Mix solutions A and B and dilute to 200 mL with *distilled water R*. Use within 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

**Procedure**. To one volume of the test solution add an equal volume of a 60 g/L solution of *sodium hydroxide R* and mix. Immediately add biuret reagent equivalent to 0.4 volumes of the test solution and mix rapidly. Allow to stand at a temperature between 15 °C and 25 °C for not less than 15 min. Within 90 min of addition of the biuret reagent, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution at the maximum at 545 nm, using the blank as compensation liquid. Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

**Calculations**. The relationship of absorbance to protein concentration is approximately linear within the indicated range of protein concentrations for the reference solutions. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the standard curve. A suitable system is one that yields a line having a correlation coefficient not less than 0.99. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

**Interfering substances**. To minimise the effect of interfering substances, the protein can be precipitated from the test sample as follows: add 0.1 volumes of a 500 g/L solution of *trichloroacetic acid R* to 1 volume of a solution of the test sample, withdraw the supernatant layer and dissolve the precipitate in a small volume of *0.5 M sodium hydroxide*. Use the solution obtained to prepare the test solution.

METHOD 6

This fluorimetric method is based on the derivatisation of the protein with *o*-phthalaldehyde, which reacts with the primary amines of the protein (*N*-terminal amino acid and the ε-amino group of lysine residues). The sensitivity of the assay can be increased by hydrolysing the protein before adding *o*-phthalaldehyde. Hydrolysis makes the α-amino group of the constituent amino acids available for reaction with the phthalaldehyde reagent. The method requires very small quantities of the protein. Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with phthalaldehyde and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this procedure may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

*Test solution*. Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions. Adjust the solution to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

*Reference solutions*. Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 200 µg/mL. Adjust the solutions to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

*Blank solution*. Use a 9 g/L solution of *sodium chloride R*.

*Borate buffer solution*. Dissolve 61.83 g of *boric acid R* in *distilled water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 mL with *distilled water R* and mix.

*Phthalaldehyde stock solution*. Dissolve 1.20 g of *phthalaldehyde R* in 1.5 mL of *methanol R*, add 100 mL of borate buffer solution and mix. Add 0.6 mL of a 300 g/L solution of *macrogol 23 lauryl ether R* and mix. Store at room temperature and use within 3 weeks.

*Phthalaldehyde reagent*. To 5 mL of phthalaldehyde stock solution add 15 µL of *2-mercaptoethanol R*. Prepare at least 30 min before use. Use within 24 h.

**Procedure**. Mix 10 µL of the test solution and of each of the reference solutions with 0.1 mL of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 mL of *0.5 M sodium hydroxide* and mix. Determine the fluorescent intensities (*2.2.21*) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

**Calculations**. The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis techniques destroy the test sample during the analysis but are not limited to protein presentation in an aqueous environment.

**Procedure A**. Proceed as prescribed for the determination of nitrogen by sulfuric acid digestion (*2.5.9*) or use commercial instrumentation for Kjeldahl nitrogen assay.

**Procedure B**. Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C), which produces nitric oxide (NO) and other oxides of nitrogen ($NO_x$) from the nitrogen present in the substance to be examined. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal-conductivity detector. Other instruments mix nitric oxide (NO) with ozone ($O_3$) to produce excited nitrogen dioxide ($NO_2$*), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

**Calculations**. The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference substance.

**01/2008:20534**

## 2.5.34. ACETIC ACID IN SYNTHETIC PEPTIDES

Examine by liquid chromatography (*2.2.29*).

*Test solution*. Prepare as described in the monograph. The concentration of peptide in the solution may be adapted, depending on the expected amount of acetic acid in the sample.

*Reference solution*. Prepare a 0.10 g/L solution of *glacial acetic acid R* in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

– as mobile phase at a flow rate of 1.2 mL/min:

*Mobile phase A*. Dilute 0.7 mL of *phosphoric acid R* to 1000 mL with *water R*; adjust the pH to 3.0 with *strong sodium hydroxide solution R*,

*Mobile phase B*. *Methanol R2*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 10 | 95 → 50 | 5 → 50 |
| 10 - 20 | 50 | 50 |
| 20 - 22 | 50 → 95 | 50 → 5 |
| 22 - 30 | 95 | 5 |

– as detector a spectrophotometer set at 210 nm.

Inject 10 µL of the reference solution and 10 µL of the test solution. In the chromatograms obtained, the peak corresponding to acetic acid has a retention time of 3-4 min. The baseline presents a steep rise after the start of the linear gradient, which corresponds to the elution of the peptide from the column. Determine the content of acetic acid in the peptide.

01/2011:20535

## 2.5.35. NITROUS OXIDE IN GASES

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

**Description and principle of measurement**. The concentration of nitrous oxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell and it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for nitrous oxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

When nitrous oxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of nitrous oxide. The generated signal is linearised in order to obtain the nitrous oxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

01/2008:20536

## 2.5.36. ANISIDINE VALUE

The anisidine value is defined as 100 times the optical density measured in a 1 cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the following method.

*Carry out the operations as rapidly as possible, avoiding exposure to actinic light.*

*Test solution (a).* Dissolve 0.500 g of the substance to be examined in *trimethylpentane R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* To 5.0 mL of test solution (a) add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

*Reference solution.* To 5.0 mL of *trimethylpentane R* add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Measure the absorbance (*2.2.25*) of test solution (a) at the maximum at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2A_1 - A_2)}{m}$$

$A_1$ = absorbance of test solution (b) at 350 nm,

$A_2$ = absorbance of test solution (a) at 350 nm,

$m$ = mass of the substance to be examined in test solution (a), in grams.

04/2011:20537 corrected 10.0

## 2.5.37. METHYL, ETHYL AND ISOPROPYL METHANESULFONATE IN METHANESULFONIC ACID

The following method has been validated for the methyl, ethyl and isopropyl esters of methanesulfonic acid at concentrations in the range of 0.5 ppm to 100 ppm.

If it is intended to be used to determine levels of methanesulfonic acid esters outside this validated range, for example in early steps of the synthesis prior to their removal, the concentration of the test solution has to be adjusted accordingly.

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution.* Dilute 7 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with *methylene chloride R*. Dilute 10 µL of the solution to 100.0 mL with *methylene chloride R*.

*Test solution.* Add 0.74 g of the substance to be examined to 10.0 mL of *water R* and extract with 10.0 mL of the internal standard solution. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulfate R*. Shake and filter.

*Reference solution (a).* Dissolve 50 mg each of *methyl methanesulfonate R* (MMS), *ethyl methanesulfonate R* (EMS) and *isopropyl methanesulfonate R* (IMS) in the internal standard solution and dilute to 50.0 mL with the same solution. Dilute 74 µL of the solution to 10.0 mL with the internal standard solution. Dilute 100 µL of this solution to 10.0 mL with the internal standard solution.

*Reference solution (b).* Dilute 3.0 mL of reference solution (a) to 10.0 mL with the internal standard solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Pulsed splitless*: 250 kPa, 0.25 min.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 1 | 55 |
| | | 1 - 9 | 55 → 135 |
| Injection port | | | 240 |
| Detector: | transfer line | | 280 |
| | source | | 230 |
| | analyser | | 150 |

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);

– mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

| Substance | *m/z* | Duration of monitoring |
|---|---|---|
| Butyl methanesulfonate (BMS) | 56 | $t_R$ between 7.0 min and 9.0 min |
| Methyl methanesulfonate (MMS) | 80 | $t_R$ between 2.0 min and 3.5 min |
| Ethyl methanesulfonate (EMS) | 79 | $t_R$ between 4.0 min and 4.7 min |
| Isopropyl methanesulfonate (IMS) | 123 | $t_R$ between 4.7 min and 5.5 min |

*Injection*: 2 µL.

*Relative retention* with reference to the internal standard (BMS) (retention time = about 7.6 min): MMS = about 0.3; EMS = about 0.5; IMS = about 0.6.

*System suitability*:

– *resolution*: minimum 3.0 between the peaks due to EMS and IMS in the chromatogram obtained with reference solution (a);
– *signal-to-noise ratio*: minimum 10 for the peaks due to MMS, EMS and IMS in the chromatogram obtained with reference solution (b).

Calculate the content of MMS, EMS or IMS in parts per million using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.148}{A_1 \times I_2 \times W_2}$$

$A_1$ = area of the peak due to MMS, EMS or IMS in the chromatogram obtained with reference solution (a);

$A_2$ = area of the peak due to MMS, EMS or IMS in the chromatogram obtained with the test solution;

$C$ = percentage content of MMS, EMS or IMS;

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a);

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$W_1$ = mass of MMS, EMS or IMS used to prepare reference solution (a), in milligrams;

$W_2$ = mass of the substance to be examined in the test solution, in milligrams;

0.148 = dilution factor.

**04/2016:20538 corrected 10.0**

## 2.5.38. METHYL, ETHYL AND ISOPROPYL METHANESULFONATE IN ACTIVE SUBSTANCES

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of methanesulfonic acid (in concentrations between 0.2 ppm and 5 ppm) in betahistine mesilate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the methanesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

*Solution A*. Dissolve with the aid of ultrasound 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* and dilute to 50.0 mL with the same solvent.

*Internal standard solution.* Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

*Blank solution.* Introduce 0.50 mL of solution A and 0.50 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Test solution.* Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.*

*Reference solution (a).* Dissolve 25.0 mg each of *methyl methanesulfonate R* (MMS), *ethyl methanesulfonate R* (EMS) and *isopropyl methanesulfonate R* (IMS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

*Reference solution (b).* Dilute 20 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Reference solution (c).* Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R.*

*The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.*

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:20.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 60 °C;
– *equilibration time*: 30 min;
– *transfer-line temperature*: 120 °C.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 1 | 40 |
| | | 1 - 10 | 40 → 130 |
| Injection port | | | 220 |
| Detector | transfer line | | 280 |
| | source | | 250 |
| | analyser | | 200 |

At the end of analysis, the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria; alternatively a suitable electron-capture detector may be used:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

| Substance | Quantitation ion (*m*/*z*) | Qualification ion (*m*/*z*) |
|---|---|---|
| Butyl iodide (BuI)* | 184 | 127 |
| Methyl iodide (MeI)* | 142 | 127 |
| Ethyl iodide (EtI)* | 156 | 127 |
| Isopropyl iodide (iPrI)* | 170 | 127 |
| * formed from BMS, MMS, EMS and IMS in the derivatisation reaction. | | |

*Injection*: 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and the blank solution.

*Relative retention* with reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl methanesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

$A_1$ = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);

$A_2$ = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;

$C$ = percentage content of each ester;

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$W_1$ = mass of each ester used to prepare reference solution (a), in milligrams;

$W_2$ = mass of the substance to be examined in the test solution, in milligrams;

0.05 = dilution factor.

**04/2013:20539 corrected 10.0**

## 2.5.39. METHANESULFONYL CHLORIDE IN METHANESULFONIC ACID

The following method has been validated for the determination of methanesulfonyl chloride in methanesulfonic acid at concentrations in the range of 0.05 ppm to 50 ppm.

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution.* Dissolve 7 µL of *butyl methanesulfonate CRS* (BMS) in *methylene chloride R* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with *methylene chloride R*.

*Test solution.* To 5 mL of *water R*, add 7.4 g of the substance to be examined and mix slowly. After cooling, add 5.0 mL of *methylene chloride R* and 100 µL of the internal standard solution and shake. Allow to separate and transfer the organic layer to a vial containing 1 g of *anhydrous sodium sulfate R*. Repeat the extraction twice with 5.0 mL of *methylene chloride R* each time, combine the organic layers and filter.

*Reference solution (a).* Dissolve 50.0 mg of *methanesulfonyl chloride R* in *methylene chloride R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methylene chloride R*. Dilute 300 µL of this solution to 10.0 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 500 µL of reference solution (a) and 100 µL of the internal standard solution to 15.0 mL with *methylene chloride R*.

*Reference solution (c).* Dilute 25 µL of reference solution (a) and 100 µL of the internal standard solution to 15.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Pulsed splitless*: 60 kPa, 0.1 min.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 4 | 40 |
| | | 4 - 8 | 40 → 200 |
| Injection port | | | 240 |
| Detector: | transfer line | | 280 |
| | source | | 230 |
| | analyser | | 150 |

At the end of analysis the temperature of the column is raised to 270 °C and maintained at this temperature for 8 min.

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

| Substance | *m*/*z* | Duration of monitoring |
|---|---|---|
| Methanesulfonyl chloride | 79 | $t_R$ between 3.3 min and 6.0 min |
| Butyl methanesulfonate (BMS) | 56 | $t_R$ between 6.0 min and 8.0 min |

*Injection*: 5 µL of the test solution, reference solutions (b) and (c), the internal standard solution and *methylene chloride R*.

*Relative retention* with reference to the internal standard (BMS) (retention time = about 7.2 min): methanesulfonyl chloride = about 0.68.

*System suitability*:

- in the chromatogram obtained with the internal standard solution, there is no peak with the same retention time as methanesulfonyl chloride;
- *resolution*: minimum 5.0 between the peaks due to methanesulfonyl chloride and BMS in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the peak due to methanesulfonyl chloride in the chromatogram obtained with reference solution (c).

Calculate the content of methanesulfonyl chloride in parts per million using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 1.5}{A_1 \times I_2 \times W_2}$$

$A_1$ = area of the peak due to methanesulfonyl chloride in the chromatogram obtained with reference solution (b);

$A_2$ = area of the peak due to methanesulfonyl chloride in the chromatogram obtained with the test solution;

$C$ = percentage content of methanesulfonyl chloride;

$I_1$ = area of the peak due to BMS in the chromatogram obtained with reference solution (b);

$I_2$ = area of the peak due to BMS in the chromatogram obtained with the test solution;

$W_1$ = mass of methanesulfonyl chloride used to prepare reference solution (a), in milligrams;

$W_2$ = mass of the sample in the test solution, in milligrams;

1.5 = dilution factor.

**04/2015:20540**
**corrected 10.0**

## 2.5.40. METHYL, ETHYL AND ISOPROPYL TOLUENESULFONATE IN ACTIVE SUBSTANCES

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of toluenesulfonic acid (in concentrations between 0.2 ppm and 5 ppm) in sultamicillin tosilate dihydrate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the toluenesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

*Solution A*. Dissolve 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* using sonication and dilute to 50.0 mL with the same solvent.

*Internal standard solution*. Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

*Blank solution*. Introduce 0.5 mL of solution A and 0.5 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Test solution*. Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.*

*Reference solution (a)*. Dissolve 25.0 mg each of *methyl toluenesulfonate R* (MTS), *ethyl toluenesulfonate R* (ETS) and *isopropyl toluenesulfonate R* (ITS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

*Reference solution (b)*. Dilute 40 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Reference solution (c)*. Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.*

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:20.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 30 min;
- *transfer-line temperature*: 120 °C.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 1 | 40 |
| | | 1 - 10 | 40 → 130 |
| Injection port | | | 220 |
| Detector | transfer line | | 280 |
| | source | | 250 |
| | analyser | | 200 |

At the end of analysis the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

| Substance | Quantitation ion (*m/z*) | Qualification ion (*m/z*) |
|---|---|---|
| Butyl iodide (BuI)* | 184 | 127 |
| Methyl iodide (MeI)* | 142 | 127 |
| Ethyl iodide (EtI)* | 156 | 127 |
| Isopropyl iodide (iPrI)* | 170 | 127 |
| * formed from BMS, MTS, ETS and ITS in the derivatisation reaction. | | |

*Injection*: 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and of the blank solution.

*Relative retention* with reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl toluenesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

$A_1$ = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);

$A_2$ = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;

$C$ = percentage content of each ester;

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$W_1$ = mass of each ester used to prepare reference solution (a), in milligrams;

$W_2$ = mass of the substance to be examined in the test solution, in milligrams;

0.05 = dilution factor.

**04/2016:20541 corrected 10.0**

## 2.5.41. METHYL, ETHYL AND ISOPROPYL BENZENESULFONATE IN ACTIVE SUBSTANCES

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of benzenesulfonic acid (in concentrations between 2.5 ppm and 40 ppm) in amlodipine besilate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the benzenesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

This method is not suitable for clopidogrel besilate as it was observed that methyl benzenesulfonate was obtained during the gas chromatographic analysis as an artefact originating from degradation.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

*Solvent mixture*: *water R, acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

*Solution A*. Dissolve with the aid of ultrasound 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* and dilute to 50.0 mL with the same solvent.

*Internal standard solution.* Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

*Blank solution.* Introduce 0.50 mL of solution A and 0.50 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Test solution.* Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.*

*Reference solution (a).* Dissolve 25.0 mg each of *methyl benzenesulfonate R* (MBS), *ethyl benzenesulfonate R* (EBS) and *isopropyl methanesulfonate R* (IMS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

*Reference solution (b).* Dilute 40 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Reference solution (c).* Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R.*

*The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.*

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:20.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 30 min;
- *transfer-line temperature*: 120 °C.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 1 | 40 |
| | | 1 - 10 | 40 → 130 |
| Injection port | | | 220 |
| Detector | transfer line | | 280 |
| | source | | 250 |
| | analyser | | 200 |

At the end of analysis, the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

| Substance | Quantitation ion (*m/z*) | Qualification ion (*m/z*) |
|---|---|---|
| Butyl iodide (BuI)* | 184 | 127 |
| Methyl iodide (MeI)* | 142 | 127 |
| Ethyl iodide (EtI)* | 156 | 127 |
| Isopropyl iodide (iPrI)* | 170 | 127 |
| * formed from BMS, MBS, EBS and IMS in the derivatisation reaction. | | |

*Injection*: 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and the blank solution.

*Relative retention* with reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl benzenesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

$A_1$ = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);

$A_2$ = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;

$C$ = percentage content of each ester;

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$W_1$ = mass of each ester used to prepare reference solution (a), in milligrams;

$W_2$ = mass of the substance to be examined in the test solution, in milligrams;

0.05 = dilution factor.

# 2.6. Biological tests



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# 2.6. BIOLOGICAL TESTS

**04/2011:20601**
**corrected 7.7**

## 2.6.1. STERILITY[(1)]

*The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test.*

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

CULTURE MEDIA AND INCUBATION TEMPERATURES

*Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.*

The following culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

**Fluid thioglycollate medium**

| | |
|---|---|
| L-Cystine | 0.5 g |
| Agar | 0.75 g |
| Sodium chloride | 2.5 g |
| Glucose monohydrate/Glucose | 5.5 g/5.0 g |
| Yeast extract (water-soluble) | 5.0 g |
| Pancreatic digest of casein | 15.0 g |
| Sodium thioglycollate or | 0.5 g |
| Thioglycollic acid | 0.3 mL |
| Resazurin sodium solution (1 g/L of resazurin sodium), freshly prepared | 1.0 mL |
| *Water R* | 1000 mL |

pH after sterilisation 7.1 ± 0.2

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the *water R* and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1 M sodium hydroxide* so that, after sterilisation, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix and place the medium in suitable vessels which provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilise using a validated process. If the medium is stored, store at a temperature between 2 °C and 25 °C in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink colour disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid thioglycollate medium is to be incubated at 30-35 °C.

For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycollate medium incubated at 20-25 °C may be used instead of soya-bean casein digest medium provided that it has been validated as described in growth promotion test.

Where prescribed or justified and authorised, the following alternative thioglycollate medium may be used. Prepare a mixture having the same composition as that of the fluid thioglycollate medium, but omitting the agar and the resazurin sodium solution, sterilise as directed above. The pH after sterilisation is 7.1 ± 0.2. Heat in a water-bath prior to use and incubate at 30-35 °C under anaerobic conditions.

**Soya-bean casein digest medium**

| | |
|---|---|
| Pancreatic digest of casein | 17.0 g |
| Papaic digest of soya-bean meal | 3.0 g |
| Sodium chloride | 5.0 g |
| Dipotassium hydrogen phosphate | 2.5 g |
| Glucose monohydrate/Glucose | 2.5 g/2.3 g |
| *Water R* | 1000 mL |

pH after sterilisation 7.3 ± 0.2

Dissolve the solids in *water R*, warming slightly to effect solution. Cool the solution to room temperature. Add *1 M sodium hydroxide*, if necessary, so that after sterilisation the solution will have a pH of 7.3 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilise using a validated process. Store at a temperature between 2 °C and 25 °C in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soya-bean casein digest medium is to be incubated at 20-25 °C.

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

**Sterility**. Incubate portions of the media for 14 days. No growth of micro-organisms occurs.

**Growth promotion test of aerobes, anaerobes and fungi**. Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of micro-organisms are indicated in Table 2.6.1.-1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Clostridium sporogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Aspergillus brasiliensis*, *Bacillus subtilis*, *Candida albicans*. Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

Table 2.6.1.-1. – *Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test*

| | |
|---|---|
| **Aerobic bacteria** | |
| *Staphylococcus aureus* | ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276 |
| *Bacillus subtilis* | ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134 |
| *Pseudomonas aeruginosa* | ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275 |
| **Anaerobic bacterium** | |
| *Clostridium sporogenes* | ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437, NBRC 14293 |
| **Fungi** | |
| *Candida albicans* | ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594 |
| *Aspergillus brasiliensis* | ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455 |

The media are suitable if a clearly visible growth of the micro-organisms occurs.

METHOD SUITABILITY TEST

Carry out a test as described below under Test for sterility of the product to be examined using exactly the same methods except for the following modifications.

**Membrane filtration**. After transferring the contents of the container or containers to be tested to the membrane add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

**Direct inoculation**. After transferring the content of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the medium.

In both cases use the same micro-organisms as those described above under Growth promotion test of aerobes, anaerobes and fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of micro-organisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test.

This method suitability test is performed:

a) when the test for sterility has to be carried out on a new product;

b) whenever there is a change in the experimental conditions of the test.

The method suitability test may be performed simultaneously with the test for sterility of the product to be examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits, that is, for filterable aqueous preparations, for alcoholic or oily preparations and for preparations miscible with or soluble in aqueous or oily solvents provided these solvents do not have an antimicrobial effect in the conditions of the test.

**Membrane filtration**. Use membrane filters having a nominal pore size not greater than 0.45 µm whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. Specially adapted filters may be needed for certain products, e.g. for antibiotics.

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilised by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

*Aqueous solutions*. If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g/L neutral solution of meat or casein peptone pH 7.1 ± 0.2 onto the membrane in the apparatus and filter. The diluent may contain suitable neutralising substances and/or appropriate inactivating substances for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary after diluting to the volume used in the method suitability test with the chosen sterile diluent but in any case using not less than the quantities of the product to be examined prescribed in Table 2.6.1.-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume of the chosen sterile diluent used in the method suitability test. Do not exceed a washing cycle of 5 times 100 mL per filter, even if during the method suitability test it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into 2 equal parts and transfer one half to each of 2 suitable media. Use the same volume of each medium as in the method suitability test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

*Soluble solids*. Use for each medium not less than the quantity prescribed in Table 2.6.1.-2 of the product dissolved in a suitable solvent such as the solvent provided with the preparation, water for injections, saline or a 1 g/L neutral solution of meat or casein peptone and proceed with the test as described above for aqueous solutions using a membrane appropriate to the chosen solvent.

*Oils and oily solutions*. Use for each medium not less than the quantity of the product prescribed in Table 2.6.1.-2. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure

Table 2.6.1.-2. – *Minimum quantity to be used for each medium*

| Quantity per container | Minimum quantity to be used for each medium unless otherwise justified and authorised |
|---|---|
| *Liquids* | |
| – less than 1 mL | The whole contents of each container |
| – 1-40 mL | Half the contents of each container but not less than 1 mL |
| – greater than 40 mL and not greater than 100 mL | 20 mL |
| – greater than 100 mL | 10 per cent of the contents of the container but not less than 20 mL |
| *Antibiotic liquids* | 1 mL |
| *Insoluble preparations, creams and ointments to be suspended or emulsified* | Use the contents of each container to provide not less than 200 mg |
| *Solids* | |
| – less than 50 mg | The whole contents of each container |
| – 50 mg or more but less than 300 mg | Half the contents of each container but not less than 50 mg |
| – 300 mg to 5 g | 150 mg |
| – greater than 5 g | 500 mg |
| *Catgut and other surgical sutures for veterinary use* | 3 sections of a strand (each 30 cm long) |

or suction gradually. Wash the membrane at least 3 times by filtering through it each time about 100 mL of a suitable sterile solution such as 1 g/L neutral meat or casein peptone containing a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L. Transfer the membrane or membranes to the culture medium or media or vice versa as described above for aqueous solutions, and incubate at the same temperatures and for the same times.

*Ointments and creams.* Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate as described above, by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described above for oils and oily solutions.

**Direct inoculation of the culture medium**. Transfer the quantity of the preparation to be examined prescribed in Table 2.6.1.-2 directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

*Oily liquids.* Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L.

*Ointments and creams.* Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as a 1 g/L neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However when fluid thioglycollate medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

*Catgut and other surgical sutures for veterinary use.* Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Open the sealed package using aseptic precautions and remove 3 sections of the strand for each culture medium. Carry out the test on 3 sections, each 30 cm long, cut off from the beginning, the centre and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

a) the data of the microbiological monitoring of the sterility testing facility show a fault;

b) a review of the testing procedure used during the test in question reveals a fault;

c) microbial growth is found in the negative controls;

d) after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid it is repeated with the same number of units as in the original test.

If no evidence of microbial growth is found in the repeat test the product examined complies with the test for sterility. If microbial growth is found in the repeat test the product examined does not comply with the test for sterility.

Table 2.6.1.-3. – *Minimum number of items to be tested*

| Number of items in the batch* | Minimum number of items to be tested for each medium, unless otherwise justified and authorised** |
|---|---|
| *Parenteral preparations* | |
| – Not more than 100 containers | 10 per cent or 4 containers, whichever is the greater |
| – More than 100 but not more than 500 containers | 10 containers |
| – More than 500 containers | 2 per cent or 20 containers (10 containers for large-volume parenterals) whichever is less |
| *Ophthalmic and other non-injectable preparations* | |
| – Not more than 200 containers | 5 per cent or 2 containers, whichever is the greater |
| – More than 200 containers | 10 containers |
| – If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral administration | |
| *Catgut and other surgical sutures for veterinary use* | 2 per cent or 5 packages whichever is the greater, up to a maximum total of 20 packages |
| *Bulk solid products* | |
| – Up to 4 containers | Each container |
| – More than 4 containers but not more than 50 containers | 20 per cent or 4 containers, whichever is the greater |
| – More than 50 containers | 2 per cent or 10 containers, whichever is the greater |
| * If the batch size is not known, use the maximum number of items prescribed. **If the contents of one container are enough to inoculate the 2 media, this column gives the number of containers needed for both the media together. | |

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC AND OTHER NON-INJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 2.6.1.-2, diluting where necessary to about 100 mL with a suitable sterile solution, such as 1 g/L neutral meat or casein peptone.

When using the technique of direct inoculation of media, use the quantities shown in Table 2.6.1.-2, unless otherwise justified and authorised. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of 2 or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in Table 2.6.1.-3.

*Guidelines on the test for sterility are given in general chapter 5.1.9.*

**01/2008:20602**

## 2.6.2. MYCOBACTERIA

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

Inoculate 0.2 mL of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 mL in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

**01/2008:20607 corrected 6.1**

## 2.6.7. MYCOPLASMAS

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

Nucleic acid amplification techniques (NAT) may be used as an alternative to one or both of the other methods after suitable validation.

CULTURE METHOD

*CHOICE OF CULTURE MEDIA*

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the micro-organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate micro-organisms in the list. When testing for mycoplasmas in the product to be examined, at least 1 of the following species will be included as a positive control:

– *Acholeplasma laidlawii* (vaccines for human and veterinary use where an antibiotic has been used during production);

- *Mycoplasma gallisepticum* (where avian material has been used during production or where the vaccine is intended for use in poultry);
- *Mycoplasma hyorhinis* (non-avian veterinary vaccines);
- *Mycoplasma orale* (vaccines for human and veterinary use);
- *Mycoplasma pneumoniae* (vaccines for human use) or other suitable species of D-glucose fermenter such as *Mycoplasma fermentans*;
- *Mycoplasma synoviae* (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone a limited number of subcultures (not more than 15), and are stored frozen or freeze-dried. After cloning, the strains are identified as being of the required species by comparison with type cultures, for example:

| | | | |
|---|---|---|---|
| *A. laidlawii* | NCTC 10116 | CIP 75.27 | ATCC 23206 |
| *M. gallisepticum* | NCTC 10115 | CIP 104967 | ATCC 19610 |
| *M. fermentans* | NCTC 10117 | CIP 105680 | ATCC 19989 |
| *M. hyorhinis* | NCTC 10130 | CIP 104968 | ATCC 17981 |
| *M. orale* | NCTC 10112 | CIP 104969 | ATCC 23714 |
| *M. pneumoniae* | NCTC 10119 | CIP 103766 | ATCC 15531 |
| *M. synoviae* | NCTC 10124 | CIP 104970 | ATCC 25204 |

*Acholeplasma laidlawii BRP*, *Mycoplasma fermentans BRP*, *Mycoplasma hyorhinis BRP*, *Mycoplasma orale BRP* and *Mycoplasma synoviae BRP* are suitable for use as low-passage reference strains.

*INCUBATION CONDITIONS*

Incubate liquid media in tightly stoppered containers at 35-38 °C. Incubate solid media in microaerophilic conditions (nitrogen containing 5-10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface) at 35-38 °C.

*NUTRITIVE PROPERTIES*

*Carry out the test for nutritive properties for each new batch of medium.* Inoculate the chosen media with the appropriate test micro-organisms; use not more than 100 CFU per 60 mm diameter plate containing 9 mL of solid medium and per 100 mL container of liquid medium; use a separate plate and container for each species of micro-organism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under Test for mycoplasmas in the product to be examined). The solid medium complies with the test if adequate growth is found for each test micro-organism (growth obtained does not differ by a factor greater than 5 from the value calculated with respect to the inoculum). The liquid medium complies with the test if growth on agar plates subcultured from the broth is found for at least 1 subculture for each test micro-organism.

*INHIBITORY SUBSTANCES*

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of mycoplasmas.

To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the product to be examined. If growth of a test micro-organism occurs more than 1 subculture sooner in the absence of the product to be examined than in its presence, or if plates directly inoculated with the product to be examined have fewer than 1/5 of the number of colonies of those inoculated without the product to be examined, inhibitory substances are present and they must be neutralised or their effect otherwise countered, for example by passage in substrates not containing inhibitors or dilution in a larger volume of medium before the test. If dilution is used, larger medium volumes may be used or the inoculum volume may be divided among several 100 mL flasks. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

*TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED*

Inoculate 10 mL of the product to be examined per 100 mL of each liquid medium. If it has been found that a significant pH change occurs upon the addition of the product to be examined, the liquid medium is restored to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the product to be examined on each plate of each solid medium. Incubate liquid media for 20-21 days. Incubate solid media for not less than 14 days, except those corresponding to the 20-21 day subculture, which are incubated for 7 days. At the same time incubate an uninoculated 100 mL portion of each liquid medium and agar plates, as a negative control. On days 2-4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between the 6$^{th}$ and 8$^{th}$ days, again between the 13$^{th}$ and 15$^{th}$ days and again between the 19$^{th}$ and 21$^{st}$ days of the test. Observe the liquid media every 2 or 3 days and if a colour change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 CFU of at least 1 test micro-organism on agar medium or into broth medium. Where the test for mycoplasmas is carried out regularly and where possible, it is recommended to use the test micro-organisms in regular rotation. The test micro-organisms used are those listed under Choice of culture media.

*INTERPRETATION OF RESULTS*

At the end of the prescribed incubation period, examine all inoculated solid media microscopically for the presence of mycoplasma colonies. The product complies with the test if growth of typical mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of mycoplasmas. If suspect colonies are observed, a suitable validated method may be used to determine whether they are due to mycoplasmas.

*The following section is published for information.*

RECOMMENDED MEDIA FOR THE CULTURE METHOD

The following media are recommended. Other media may be used, provided that their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the product to be examined.

*HAYFLICK MEDIA (RECOMMENDED FOR THE GENERAL DETECTION OF MYCOPLASMAS)*

**Liquid medium**

| | |
|---|---|
| Beef heart infusion broth (1) | 90.0 mL |
| Horse serum (unheated) | 20.0 mL |
| Yeast extract (250 g/L) | 10.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20 000 IU/mL) | 0.25 mL |
| Deoxyribonucleic acid (2 g/L solution) | 1.2 mL |

Adjust to pH 7.8.

**Solid medium**

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.

*FREY MEDIA (RECOMMENDED FOR THE DETECTION OF M. SYNOVIAE)*

**Liquid medium**

| | |
|---|---|
| Beef heart infusion broth (1) | 90.0 mL |
| Essential vitamins (2) | 0.025 mL |
| Glucose monohydrate (500 g/L solution) | 2.0 mL |
| Swine serum (inactivated at 56 °C for 30 min) | 12.0 mL |
| β-Nicotinamide adenine dinucleotide (10 g/L solution) | 1.0 mL |
| Cysteine hydrochloride (10 g/L solution) | 1.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20 000 IU/mL) | 0.25 mL |

Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to pH 7.8.

**Solid medium**

| | |
|---|---|
| Beef heart infusion broth (1) | 90.0 mL |
| Agar, purified (3) | 1.4 g |

Adjust to pH 7.8, sterilise by autoclaving then add:

| | |
|---|---|
| Essential vitamins (2) | 0.025 mL |
| Glucose monohydrate (500 g/L solution) | 2.0 mL |
| Swine serum (unheated) | 12.0 mL |
| β-Nicotinamide adenine dinucleotide (10 g/L solution) | 1.0 mL |
| Cysteine hydrochloride (10 g/L solution) | 1.0 mL |
| Phenol red (0.6 g/L solution) | 5.0 mL |
| Penicillin (20 000 IU/mL) | 0.25 mL |

*FRIIS MEDIA (RECOMMENDED FOR THE DETECTION OF NON-AVIAN MYCOPLASMAS)*

**Liquid medium**

| | |
|---|---|
| Hanks' balanced salt solution (modified) (4) | 800 mL |
| Distilled water | 67 mL |
| Brain heart infusion (5) | 135 mL |
| PPLO Broth (6) | 248 mL |
| Yeast extract (170 g/L) | 60 mL |
| Bacitracin | 250 mg |
| Meticillin | 250 mg |
| Phenol red (5 g/L) | 4.5 mL |
| Horse serum | 165 mL |
| Swine serum | 165 mL |

Adjust to pH 7.40-7.45.

**Solid medium**

| | |
|---|---|
| Hanks' balanced salt solution (modified) (4) | 200 mL |
| DEAE-dextran | 200 mg |
| Agar, purified (3) | 15.65 g |

Mix well and sterilise by autoclaving. Cool to 100 °C. Add to 1740 mL of liquid medium as described above.

(1) *Beef heart infusion broth*

| | |
|---|---|
| Beef heart (for preparation of the infusion) | 500 g |
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Distilled water | to 1000 mL |

Sterilise by autoclaving.

(2) *Essential vitamins*

| | |
|---|---|
| Biotin | 100 mg |
| Calcium pantothenate | 100 mg |
| Choline chloride | 100 mg |
| Folic acid | 100 mg |
| *i*-Inositol | 200 mg |
| Nicotinamide | 100 mg |
| Pyridoxal hydrochloride | 100 mg |
| Riboflavine | 10 mg |
| Thiamine hydrochloride | 100 mg |
| Distilled water | to 1000 mL |

(3) *Agar, purified*

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity and gel strength. It contains about:

| | |
|---|---|
| Water | 12.2 per cent |
| Ash | 1.5 per cent |
| Acid-insoluble ash | 0.2 per cent |
| Chlorine | 0 |
| Phosphate (calculated as $P_2O_5$) | 0.3 per cent |
| Total nitrogen | 0.3 per cent |
| Copper | 8 ppm |
| Iron | 170 ppm |
| Calcium | 0.28 per cent |
| Magnesium | 0.32 per cent |

(4) *Hanks' balanced salt solution (modified)*

| | |
|---|---|
| Sodium chloride | 6.4 g |
| Potassium chloride | 0.32 g |
| Magnesium sulfate heptahydrate | 0.08 g |
| Magnesium chloride hexahydrate | 0.08 g |
| Calcium chloride, anhydrous | 0.112 g |
| Disodium hydrogen phosphate dihydrate | 0.0596 g |
| Potassium dihydrogen phosphate, anhydrous | 0.048 g |
| Distilled water | to 800 mL |

(5) *Brain heart infusion*

| | |
|---|---|
| Calf-brain infusion | 200 g |
| Beef-heart infusion | 250 g |
| Proteose peptone | 10 g |
| Glucose monohydrate | 2 g |
| Sodium chloride | 5 g |
| Disodium hydrogen phosphate, anhydrous | 2.5 g |
| Distilled water | to 1000 mL |

(6) *PPLO broth*

| | |
|---|---|
| Beef-heart infusion | 50 g |
| Peptone | 10 g |
| Sodium chloride | 5 g |
| Distilled water | to 1000 mL |

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from mycoplasmas.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralised using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

*VERIFICATION OF THE SUBSTRATE*

Use Vero cells or another cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 CFU or CFU-like micro-organisms of suitable reference strains of *M. hyorhinis* and *M. orale*. The following strains have been found to be suitable:

| | | | |
|---|---|---|---|
| *M. hyorhinis* | | | ATCC 29052 |
| *M. orale* | NCTC 10112 | CIP 104969 | ATCC 23714 |

The cells are suitable if both reference strains are detected.

The indicator cells must be subcultured without an antibiotic before use in the test.

*TEST METHOD*

1. Seed the indicator cell culture at a suitable density (for example, $2 \times 10^4$ to $2 \times 10^5$ cells/mL, $4 \times 10^3$ to $2.5 \times 10^4$ cells/cm$^2$) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel and incubate at 35-38 °C.

2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50 per cent confluence after 3-5 days of incubation. Complete confluence impairs visualisation of mycoplasmas after staining and must be avoided.

3. Remove the medium and rinse the indicator cells with *phosphate buffered saline pH 7.4 R*, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of *glacial acetic acid R* and 3 volumes of *methanol R* is suitable when *bisbenzimide R* is used for staining).

4. Remove the fixing solution and wash the cells with sterile *water R*. Dry the slides completely if they are to be stained more than 1 h later (particular care is needed for staining of slides after drying owing to artefacts that may be produced).

5. Add a suitable DNA stain and allow to stand for a suitable time (*bisbenzimide working solution R* and a standing time of 10 min are suitable).

6. Remove the stain and rinse the monolayer with *water R*.

7. Mount each coverslip, where applicable (a mixture of equal volumes of *glycerol R* and *phosphate-citrate buffer solution pH 5.5 R* is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400 × magnification or greater.

8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

*INTERPRETATION OF RESULTS*

The product to be examined complies with the test if fluorescence typical of mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of mycoplasmas. The test is invalid if the negative controls show fluorescence typical of mycoplasmas.

NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT)

NAT (*2.6.21*) may be used for detection of mycoplasmas by amplification of nucleic acids extracted from a test sample with specific primers that reveal the presence of the target nucleic acid. NAT indicate the presence of a particular nucleic acid sequence and not necessarily the presence of viable mycoplasmas. A number of different techniques are available. This general chapter does not prescribe a particular method for the test. The procedure applied must be validated as described, taking account of the guidelines presented at the end of this section. Where a commercial kit is used, certain elements of the validation may be carried out by the manufacturer and information provided to the user but it must be remembered that full information on the primers may not be available and that production of the kit may be modified or discontinued.

NAT are applied where prescribed in a monograph. They may also be used instead of the culture method and the indicator cell culture method after suitable validation.

*Direct NAT* can be applied in the presence of cytotoxic material and where a rapid method is needed.

*Cell-culture enrichment followed by NAT*: the test sample and a suitable cell substrate (as described under the indicator cell-culture method) are cultured together for a suitable period; the nucleic acids are then extracted from cells and supernatant and used for detection by NAT.

*VALIDATION*

Reference standards are required at various stages during validation and for use as controls during routine application of the test. The reference standards may be mycoplasmas or nucleic acids.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

- *A. laidlawii*;
- *M. fermentans*;
- *M. hyorhinis* (where cell-culture enrichment is used, a fastidious strain such as ATCC 29052 is included);
- *M. orale*;
- *M. pneumoniae* or *M. gallisepticum*;
- *M. synoviae* (where there is use of or exposure to avian material during production);
- *Mycoplasma arginini;*
- *Spiroplasma citri* (where there is use of or exposure to insect or plant material during production).

Demonstration of specificity requires the use of a suitable range of bacterial species other than mycoplasmas. Bacterial genera with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium, Lactobacillus* and *Streptococcus*.

*Comparability studies for use of NAT as an alternative method.* For each mycoplasma test species:

- as an alternative to the culture method: the NAT test system must be shown to detect 10 CFU/mL;
- as an alternative to the indicator cell culture method: the NAT test system must be shown to detect 100 CFU/mL;

*or* an equivalent limit of detection in terms of the number of copies of mycoplasma nucleic acid in the test sample (using suitable reference standards of mycoplasma nucleic acid).

*CONTROLS*

**Internal controls**. Internal controls are necessary for routine verification of absence of inhibition. The internal control may contain the primer binding-site, or some other suitable sequence may be used. It is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

**External controls**. The external positive control contains a defined number of target-sequence copies or CFUs from 1 or more suitable species of mycoplasma chosen from those used during validation of the test conditions. 1 of the positive controls is set close to the positive cut-off point to demonstrate that the expected sensitivity is achieved. The external negative control contains no target sequence but does not necessarily represent the same matrix as the test article.

*INTERPRETATION OF RESULTS*

The primers used may also amplify non-mycoplasmal bacterial nucleic acid, leading to false positive results. Procedures are established at the time of validation for dealing with confirmation of positive results, where necessary.

*The following section is published for information.*

## Validation of nucleic acid amplification techniques (NAT) for the detection of mycoplasmas: guidelines

1. *SCOPE*

Nucleic acid amplification techniques (NAT) are either qualitative or quantitative tests for the presence of nucleic acid. For the detection of mycoplasma contamination of various samples such as vaccines and cell substrates, qualitative tests are adequate and may be considered to be limit tests. These guidelines describe methods to validate qualitative nucleic acid amplification analytical procedures for assessing mycoplasma contamination. They may also be applicable for real-time NAT used as limit tests for the control of contaminants.

The 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria).

NAT may be used as:

- a complementary test (for example, for cytotoxic viral suspensions) or for in-process control purposes;
- an alternative method to replace an official method (indicator cell culture method or culture method).

These guidelines will thus separate these 2 objectives by presenting first a guideline for the validation of the NAT themselves, and second, a guideline for a comparability study between NAT and official methods.

2. *GUIDELINE FOR MYCOPLASMA NAT VALIDATION*

3 parameters should be evaluated: specificity, detection limit and robustness.

2-1. **Specificity**. Specificity is the ability to unequivocally assess target nucleic acid in the presence of components that may be expected to be present.

The specificity of NAT is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

The ability of the NAT to detect a large panel of mycoplasma species will depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels (e.g. reference strains provided by the EDQM). Since NAT systems are usually based on a mix of primers, the theoretical analysis of primers and probes by comparison with databases is not recommended, because interpretation of the results may be quite complex and may not reflect the experimental results.

Moreover, as it is likely that the primers will detect other bacterial species, the potential cross-detection should be documented in the validation study. Bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium*, *Lactobacillus* and *Streptococcus*. However, this is not an exhaustive list and species to be tested will depend on the theoretical ability (based on primers/probes sequences) of the NAT system to detect such other species.

Based on the results from this validation of the specificity, if a gap in the specificity of the method is identified (such as detection of non-mycoplasmal bacterial nucleic acid), an appropriate strategy must be proposed in the validation study to allow interpretation of positive results on a routine basis. For example, a second test may be performed using an alternative method without this specificity gap or using an official method.

2-2. **Detection limit**. The detection limit of an individual analytical procedure is the lowest amount of target nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

For establishment of the detection limit, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in general chapter *2.6.21*) is the minimum number of target sequence copies per volume of sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of mycoplasmal genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

To determine the positive cut-off point, a dilution series of characterised and calibrated (either in CFUs or nucleic acid copies) in-house working strains or EDQM standards should be tested on different days to examine variation between test runs.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

- *A. laidlawii*;
- *M. fermentans*;
- *M. hyorhinis*;
- *M. orale*;
- *M. pneumoniae* or *M. gallisepticum*;
- *M. synoviae* (where there is use of or exposure to avian material during production);
- *M. arginini*;
- *S. citri* (where there is use of or exposure to insect or plant material during production).

For each strain, at least 3 independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory may test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a

manageable level, a preliminary test should be performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point. The concentration of mycoplasmas (CFUs or copies) that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to evaluate the variability of the analytical procedure.

2-3. **Robustness**. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

Finally, robustness of the method can be evaluated through collaborative studies.

3. *GUIDELINE FOR COMPARABILITY STUDY*

NAT may be used instead of official methods (indicator cell culture method and/or culture method). In this case a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the alternative method and official methods. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered. For the detection limit, acceptability criteria are defined as follows:

- if the alternative method is proposed to replace the culture method, the NAT system must be shown to detect 10 CFU/mL for each mycoplasma test species described in paragraph 2-2;
- if the alternative method is proposed to replace the indicator cell culture method, the NAT system must be shown to detect 100 CFU/mL for each mycoplasma test species described in paragraph 2-2.

For both cases, suitable standards calibrated for the number of nucleic acid copies and the number of CFUs may be used for establishing that these acceptability criteria are reached. The relation between CFUs and nucleic acid copies for the reference preparations should be previously established to compare the performance of the alternative NAT method with the performance of the official methods.

1 of the following 2 strategies can be used to perform this comparability study:

- perform the NAT alternative method in parallel with the official method(s) to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains;
- compare the performance of the NAT alternative method using previously obtained data from official method validation. In this case, calibration of standards used for both validations as well as their stabilities should be documented carefully.

Comparability study reports should describe all the validation elements described in section 2 (specificity, limit of detection and variability, as well as robustness) in order to assess all the advantages and/or disadvantages of the alternative NAT method compared to official methods.

**01/2020:20608**

# 2.6.8. PYROGENS

The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined.

**Selection of animals**. Use healthy, adult rabbits of either sex weighing not less than 1.5 kg, fed a complete and balanced diet not containing antibiotics, and not showing loss of body mass during the week preceding the test. A rabbit is not to be used in a pyrogen test if:

a) it has been used in a negative pyrogen test in the preceding 3 days; or

b) it has been used in the preceding 3 weeks in a pyrogen test in which the substance under examination failed to pass the test.

**Animals' quarters**. Keep the rabbits individually in a quiet area with a uniform appropriate temperature. Withhold food from the rabbits overnight and until the test is completed; withhold water during the test. Carry out the test in a quiet room where there is no risk of disturbance exciting the animals and in which the room temperature is within 3 °C of that of the rabbits' living quarters, or in which the rabbits have been kept for at least 18 h before the test.

**Materials**. Thoroughly wash all glassware, syringes and needles with water for injections and heat in a hot-air oven at 250 °C for 30 min or at 200 °C for 1 h.

**Retaining boxes**. The retaining boxes for rabbits whose temperature is being measured by an electrical device are made in such a way that the animals are retained only by loosely fitting neck-stocks; the rest of the body remains relatively free so that the rabbits may sit in a normal position. They are not restrained by straps or other similar methods that may harm the animal. The animals are put into the boxes not less than 1 h before the 1st recording of the temperature and remain in them throughout the test.

**Thermometers**. Use a thermometer or electrical device that indicates the temperature with a resolution of 0.1 °C and insert into the rectum of the rabbit to a depth of about 5 cm. The depth of insertion is constant for any 1 rabbit in any 1 test. When an electrical device is used it may be left in position throughout the test.

**Preliminary test**. After selection of the animals, 1-3 days before testing the product to be examined, treat those animals that have not been used during the previous 2 weeks by intravenous injection of 10 mL per kilogram of body mass of a pyrogen-free 9 g/L solution of *sodium chloride R* warmed to about 38.0 °C. Record the temperatures of the animals, beginning at least 90 min before injection and continuing for 3 h after the injection of the solution. Any animal showing a temperature variation greater than 0.6 °C is not used in the main test.

**Main test**. Carry out the test using a group of 3 rabbits.

*Preparation and injection of the product*. Warm the liquid to be examined to approximately 38.0 °C before the injection. The product to be examined may be dissolved in, or diluted with, a pyrogen-free 9 g/L solution of *sodium chloride R* or another prescribed liquid. Inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 min, unless otherwise prescribed in the monograph. The amount of the product to be injected varies according to the product to be examined and is prescribed in the monograph. The volume injected is not less than 0.5 mL per kilogram and not more than 10 mL per kilogram of body mass.

*Determination of the initial and maximum temperatures.* The 'initial temperature' of each rabbit is the mean of 2 temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the product to be examined. The 'maximum temperature' of each rabbit is the highest temperature recorded for that rabbit in the 3 h after the injection. Record the temperature of each rabbit at intervals of not more than 30 min, beginning at least 90 min before the injection of the product to be examined and continuing 3 h after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Rabbits showing a temperature variation greater than 0.2 °C between 2 successive readings in the determination of the initial temperature are withdrawn from the test. In any 1 test, only rabbits having initial temperatures that do not differ from one another by more than 1 °C are used. All rabbits having an initial temperature higher than 39.8 °C or less than 38.0 °C are withdrawn from the test.

**Interpretation of results**. Having carried out the test 1st on a group of 3 rabbits, repeat if necessary on further groups of 3 rabbits to a total of 4 groups, depending on the results obtained. If the summed response of the 1st group does not exceed the figure given in the 2nd column of Table 2.6.8.-1, the substance passes the test. If the summed response exceeds the figure given in the 2nd column of the table but does not exceed the figure given in the 3rd column of the table, repeat the test as indicated above. If the summed response exceeds the figure given in the 3rd column of the table, the product fails the test.

Table 2.6.8.-1

| Number of rabbits | Product passes if summed response does not exceed | Product fails if summed response exceeds |
|---|---|---|
| 3 | 1.15 °C | 2.65 °C |
| 6 | 2.80 °C | 4.30 °C |
| 9 | 4.45 °C | 5.95 °C |
| 12 | 6.60 °C | 6.60 °C |

Rabbits used in a test for pyrogens where the mean rise in the rabbits' temperature has exceeded 1.2 °C are permanently excluded.

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test (*2.6.30*).

**01/2008:20610**

## 2.6.10. HISTAMINE

Euthanise a guinea-pig weighing 250 g to 350 g that has been deprived of food for the preceding 24 h. Remove a portion of the distal small intestine 2 cm in length and empty the isolated part by rinsing carefully with solution B described below using a syringe. Attach a fine thread to each end and make a small transverse incision in the middle of the piece of intestine. Place it in an organ bath with a capacity of 10 mL to 20 mL, containing solution B maintained at a constant temperature (34 °C to 36 °C) and pass through the solution a current of a mixture of 95 parts of oxygen and 5 parts of carbon dioxide. Attach one of the threads near to the bottom of the organ bath. Attach the other thread to an isotonic myograph and record the contractions of the organ on a kymograph or other suitable means of giving a permanent record. If a lever is used, its length is such that the movements of the organ are amplified about 20 times. The tension on the intestine should be about 9.8 mN (1 g) and it should be adjusted to the sensitivity of the organ. Flush out the organ bath with solution B. Allow it to stand for 10 min. Flush 2 or 3 times more with solution B. Stimulate a series of contractions by the addition of measured volumes between 0.2 mL and 0.5 mL of a solution of *histamine dihydrochloride R* having a strength which produces reproducible submaximal responses. This dose is termed the "high dose". Flush the organ bath (preferably by overflow without emptying the bath) 3 times with solution B before each addition of histamine. The successive additions should be made at regular intervals allowing a complete relaxation between additions (about 2 min). Add equal volumes of a weaker dilution of *histamine dihydrochloride R* which produces reproducible responses approximately half as great as the "high dose". This dose is termed the "low dose". Continue the regular additions of "high" and "low" doses of histamine solution as indicated above, and alternate each addition with an equal volume of a dilution of the solution to be examined, adjusting the dilution so that the contraction of the intestine, if any, is smaller than that due to the "high dose" of histamine. Determine whether the contraction, if any, is reproducible and that the responses to the "high" and "low" doses of histamine are unchanged. Calculate the activity of the substance to be examined in terms of its equivalent in micrograms of histamine base from the dilution determined as above.

The quantity so determined does not exceed the quantity prescribed in the monograph.

If the solution to be examined does not produce a contraction, prepare a fresh solution adding a quantity of histamine corresponding to the maximum tolerated in the monograph and note whether the contractions produced by the preparation with the added histamine correspond to the amount of histamine added. If this is not the case, or if the contractions caused by the substance to be examined are not reproducible or if subsequent responses to "high" and "low" doses of histamine are diminished, the results of the tests are invalid and the test for depressor substances (*2.6.11*) must be carried out.

**Solution A**

| | |
|---|---|
| Sodium chloride | 160.0 g |
| Potassium chloride | 4.0 g |
| Calcium chloride, anhydrous | 2.0 g |
| Magnesium chloride, anhydrous | 1.0 g |
| Disodium hydrogen phosphate dodecahydrate | 0.10 g |
| *Water for injections R* | to 1000 mL |

**Solution B**

| | |
|---|---|
| Solution A | 50.0 mL |
| Atropine sulfate | 0.5 mg |
| Sodium hydrogen carbonate | 1.0 g |
| Glucose monohydrate | 0.5 g |
| *Water for injections R* | to 1000 mL |

Solution B should be freshly prepared and used within 24 h.

**01/2008:20611**

## 2.6.11. DEPRESSOR SUBSTANCES

Carry out the test on a cat weighing not less than 2 kg and anaesthetised with chloralose or with a barbiturate that allows the maintenance of uniform blood pressure. Protect

the animal from loss of body heat and maintain it so that the rectal temperature remains within physiological limits. Introduce a cannula into the trachea. Insert a cannula filled with a heparinised 9 g/L solution of sodium chloride into the common carotid artery and connect it to a device capable of giving a continuous record of the blood pressure. Insert into the femoral vein another cannula, filled with a heparinised 9 g/L solution of sodium chloride, through which can be injected the solutions of histamine and of the substance to be examined. Determine the sensitivity of the animal to histamine by injecting intravenously at regular intervals, doses of *histamine solution R* corresponding to 0.1 µg and 0.15 µg of histamine base per kilogram of body mass. Repeat the lower dose at least 3 times. Administer the second and subsequent injections not less than 1 min after the blood pressure has returned to the level it was at immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the lower dose is obtained and if the higher dose causes greater responses. Dissolve the substance to be examined in sufficient of a 9 g/L solution of sodium chloride or other prescribed solvent, to give the prescribed concentration. Inject intravenously per kilogram of body mass 1.0 mL of *histamine solution R*, followed by 2 successive injections of the prescribed amount of the solution to be examined and, finally, 1.0 mL of *histamine solution R*. The second, third and fourth injections are given not less than 1 min after the blood pressure has returned to the level it was at immediately before the preceding injection. Repeat this series of injections twice and conclude the test by giving 1.5 mL of *histamine solution R* per kilogram of body mass.

If the response to 1.5 mL of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 mL the test is invalid. The substance to be examined fails the test if the mean of the series of responses to the substance is greater than the mean of the responses to 1.0 mL of *histamine solution R* per kilogram of body mass or if any one dose of the substance causes a greater depressor response than the concluding dose of the histamine solution. The test animal must not be used in another test for depressor substances if the second criterion applies or if the response to the high dose of histamine given after the administration of the substance to be examined is less than the mean response to the low doses of histamine previously injected.

**07/2010:20612**

# 2.6.12. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: MICROBIAL ENUMERATION TESTS(2)

## 1. INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable micro-organisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

## 2. GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised. If inactivators are used for this purpose, their efficacy and their absence of toxicity for micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

## 3. ENUMERATION METHODS

Use the membrane filtration method or the plate-count methods, as prescribed. The most-probable-number (MPN) method is generally the least accurate method for microbial counts, however, for certain product groups with a very low bioburden, it may be the most appropriate method.

The choice of method is based on factors such as the nature of the product and the required limit of micro-organisms. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen must be established.

## 4. GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

### 4-1. *GENERAL CONSIDERATIONS*

The ability of the test to detect micro-organisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

### 4-2. *PREPARATION OF TEST STRAINS*

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 2.6.12.-1.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions; to suspend *A. brasiliensis* spores, 0.05 per cent of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

### 4-3. *NEGATIVE CONTROL*

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 5. A failed negative control requires an investigation.

### 4-4. *GROWTH PROMOTION OF THE MEDIA*

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

(2) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

Table 2.6.12.-1. – *Preparation and use of test micro-organisms*

| Micro-organism | Preparation of test strain | Growth promotion | | Suitability of counting method in the presence of the product | |
|---|---|---|---|---|---|
| | | Total aerobic microbial count | Total yeasts and moulds count | Total aerobic microbial count | Total yeasts and moulds count |
| *Staphylococcus aureus*<br>such as:<br>ATCC 6538<br>NCIMB 9518<br>CIP 4.83<br>NBRC 13276 | Casein soya bean digest agar or casein soya bean digest broth<br>30-35 °C<br>18-24 h | Casein soya bean digest agar and casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - | Casein soya bean digest agar/MPN casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - |
| *Pseudomonas aeruginosa*<br>such as:<br>ATCC 9027<br>NCIMB 8626<br>CIP 82.118<br>NBRC 13275 | Casein soya bean digest agar or casein soya bean digest broth<br>30-35 °C<br>18-24 h | Casein soya bean digest agar and casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - | Casein soya bean digest agar/MPN casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - |
| *Bacillus subtilis*<br>such as:<br>ATCC 6633<br>NCIMB 8054<br>CIP 52.62<br>NBRC 3134 | Casein soya bean digest agar or casein soya bean digest broth<br>30-35 °C<br>18-24 h | Casein soya bean digest agar and casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - | Casein soya bean digest agar/MPN casein soya bean digest broth<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days | - |
| *Candida albicans*<br>such as:<br>ATCC 10231<br>NCPF 3179<br>IP 48.72<br>NBRC 1594 | Sabouraud-dextrose agar or Sabouraud-dextrose broth<br>20-25 °C<br>2-3 days | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 5 days | Sabouraud-dextrose agar<br>≤ 100 CFU<br>20-25 °C<br>≤ 5 days | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 5 days<br>MPN: not applicable | Sabouraud-dextrose agar<br>≤ 100 CFU<br>20-25 °C<br>≤ 5 days |
| *Aspergillus brasiliensis*<br>such as:<br>ATCC 16404<br>IMI 149007<br>IP 1431.83<br>NBRC 9455 | Sabouraud-dextrose agar or potato-dextrose agar<br>20-25 °C<br>5-7 days, or until good sporulation is achieved | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 5 days | Sabouraud-dextrose agar<br>≤ 100 CFU<br>20-25 °C<br>≤ 5 days | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 5 days<br>MPN: not applicable | Sabouraud-dextrose agar<br>≤ 100 CFU<br>20-25 °C<br>≤ 5 days |

Inoculate portions/plates of casein soya bean digest broth and casein soya bean digest agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.12.-1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud-dextrose agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.12.-1, using a separate plate of medium for each. Incubate in the conditions described in Table 2.6.12.-1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

4-5. *SUITABILITY OF THE COUNTING METHOD IN THE PRESENCE OF PRODUCT*

4-5-1. **Preparation of the sample**. The method for sample preparation depends upon the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

*Water-soluble products.* Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

*Non-fatty products insoluble in water.* Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. A surface-active agent such as 1 g/L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

*Fatty products.* Dissolve in isopropyl myristate, sterilised by filtration or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent, heated if necessary to not more than 40 °C, or in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath. Add sufficient of the pre-warmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial tenfold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent.

*Fluids or solids in aerosol form.* Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

*Transdermal patches.* Remove the protective cover sheets ('release liners') of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a sterile porous material, for example sterile gauze, to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 min.

4-5-2. **Inoculation and dilution**. Add to the sample prepared as described above (4-5-1) and to a control (with no test material included) a sufficient volume of the microbial

suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralisation, dilution or filtration.

4-5-3. **Neutralisation/removal of antimicrobial activity**. The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-4, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

*Neutralising agents*. Neutralising agents may be used to neutralise the activity of antimicrobial agents (Table 2.6.12.-2). They may be added to the chosen diluent or the medium preferably before sterilisation. If used, their efficacy and their absence of toxicity for micro-organisms must be demonstrated by carrying out a blank with neutraliser and without product.

If no suitable neutralising method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the product is not likely to be contaminated with the given species of the micro-organism. However, it is possible that the product only inhibits some of the micro-organisms specified herein, but does not inhibit others not included amongst the test strains or for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

Table 2.6.12.-2. – *Common neutralising agents for interfering substances*

| Interfering substance | Potential neutralising method |
|---|---|
| Glutaraldehyde, mercurials | Sodium hydrogensulfite (sodium bisulfite) |
| Phenolics, alcohol, aldehydes, sorbate | Dilution |
| Aldehydes | Glycine |
| Quaternary Ammonium Compounds (QACs), parahydroxybenzoates (parabens), bis-biguanides | Lecithin |
| QACs, iodine, parabens | Polysorbate |
| Mercurials | Thioglycollate |
| Mercurials, halogens, aldehydes | Thiosulfate |
| EDTA (edetate) | $Mg^{2+}$ or $Ca^{2+}$ ions |

4-5-4. **Recovery of micro-organism in the presence of product**. For each of the micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

4-5-4-1. *Membrane filtration*. Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen such that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, one membrane filter is used.

Transfer a suitable amount of the sample prepared as described under 4-5-1 to 4-5-3 (preferably representing 1 g of the product, or less if large numbers of CFU are expected) to the membrane filter, filter immediately and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of casein soya bean digest agar. For the determination of total combined yeasts/moulds count (TYMC), transfer the membrane to the surface of Sabouraud-dextrose agar. Incubate the plates as indicated in Table 2.6.12.-1. Perform the counting.

4-5-4-2. *Plate-count methods*. Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

4-5-4-2-1. Pour-plate method

For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under 4-5-1 to 4-5-3 and 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar, both media being at not more than 45 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 2.6.12.-1, at least 2 Petri dishes are used. Incubate the plates as indicated in Table 2.6.12.-1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

4-5-4-2-2. Surface-spread method

For Petri dishes 9 cm in diameter, add 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or an incubator. For each of the micro-organisms listed in Table 2.6.12.-1, at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under 4-5-1 to 4-5-3 over the surface of the medium. Incubate and count as prescribed under 4-5-4-2-1.

4-5-4-3. *Most-probable-number (MPN) method*. The precision and accuracy of the MPN method is less than that of the membrane filtration method or the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least 3 serial tenfold dilutions of the product as described under 4-5-1 to 4-5-3. From each level of dilution, 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9-10 mL of casein soya bean digest broth. If necessary, a surface-active agent such as polysorbate 80 or an inactivator of antimicrobial agents may be added to the medium. Thus, if 3 levels of dilution are prepared, 9 tubes are inoculated.

Incubate all tubes at 30-35 °C for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or in casein soya bean digest agar, for 1-2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 2.6.12.-3.

4-6. *RESULTS AND INTERPRETATION*

When verifying the suitability of the membrane filtration method or the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in 4-5-2 in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met for one or more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

## 5. TESTING OF PRODUCTS

### 5-1. *AMOUNT USED FOR THE TEST*

Unless otherwise prescribed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches.

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g. tablet, capsule, injection) is less than or equal to 1 mg or the amount per gram or millilitre (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

Table 2.6.12.-3. – *Most-probable-number values of micro-organisms*

| Observed combinations of numbers of tubes showing growth in each set | | | MPN per gram or per millilitre of product | 95 per cent confidence limits |
|---|---|---|---|---|
| Number of grams or millilitres of product per tube | | | | |
| 0.1 | 0.01 | 0.001 | | |
| 0 | 0 | 0 | < 3 | 0-9.4 |
| 0 | 0 | 1 | 3 | 0.1-9.5 |
| 0 | 1 | 0 | 3 | 0.1-10 |
| 0 | 1 | 1 | 6.1 | 1.2-17 |
| 0 | 2 | 0 | 6.2 | 1.2-17 |
| 0 | 3 | 0 | 9.4 | 3.5-35 |
| 1 | 0 | 0 | 3.6 | 0.2-17 |
| 1 | 0 | 1 | 7.2 | 1.2-17 |
| 1 | 0 | 2 | 11 | 4-35 |
| 1 | 1 | 0 | 7.4 | 1.3-20 |
| 1 | 1 | 1 | 11 | 4-35 |
| 1 | 2 | 0 | 11 | 4-35 |
| 1 | 2 | 1 | 15 | 5-38 |
| 1 | 3 | 0 | 16 | 5-38 |
| 2 | 0 | 0 | 9.2 | 1.5-35 |
| 2 | 0 | 1 | 14 | 4-35 |
| 2 | 0 | 2 | 20 | 5-38 |
| 2 | 1 | 0 | 15 | 4-38 |
| 2 | 1 | 1 | 20 | 5-38 |
| 2 | 1 | 2 | 27 | 9-94 |
| 2 | 2 | 0 | 21 | 5-40 |
| 2 | 2 | 1 | 28 | 9-94 |
| 2 | 2 | 2 | 35 | 9-94 |
| 2 | 3 | 0 | 29 | 9-94 |
| 2 | 3 | 1 | 36 | 9-94 |
| 3 | 0 | 0 | 23 | 5-94 |
| 3 | 0 | 1 | 38 | 9-104 |
| 3 | 0 | 2 | 64 | 16-181 |
| 3 | 1 | 0 | 43 | 9-181 |
| 3 | 1 | 1 | 75 | 17-199 |
| 3 | 1 | 2 | 120 | 30-360 |
| 3 | 1 | 3 | 160 | 30-380 |
| 3 | 2 | 0 | 93 | 18-360 |
| 3 | 2 | 1 | 150 | 30-380 |
| 3 | 2 | 2 | 210 | 30-400 |
| 3 | 2 | 3 | 290 | 90-990 |
| 3 | 3 | 0 | 240 | 40-990 |
| 3 | 3 | 1 | 460 | 90-1980 |
| 3 | 3 | 2 | 1100 | 200-4000 |
| 3 | 3 | 3 | > 1100 | |

For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e. less than 1000 mL or 1000 g), the amount tested shall be 1 per cent of the batch unless a lesser amount is prescribed or justified and authorised.

For products where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

### 5-2. *EXAMINATION OF THE PRODUCT*

#### 5-2-1. **Membrane filtration**

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown suitable as described in section 4 and transfer the appropriate amount to each of 2 membrane filters and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of casein soya bean digest agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud-dextrose agar. Incubate the plate of casein soya bean digest agar at 30-35 °C for 3-5 days and the plate of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Calculate the number of CFU per gram or per millilitre of product.

When examining transdermal patches, filter 10 per cent of the volume of the preparation described under 4-5-1 separately through each of 2 sterile filter membranes. Transfer one membrane to casein soya bean digest agar for TAMC and the other membrane to Sabouraud-dextrose agar for TYMC.

#### 5-2-2. **Plate-count methods**

##### 5-2-2-1. Pour-plate method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of product.

5-2-2-2. Surface-spread method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of CFU proceed as described for the pour-plate method.

5-2-3. **Most-probable-number method**

Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes at 30-35 °C for 3-5 days. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 2.6.12.-3.

5-3. *INTERPRETATION OF THE RESULTS*

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using casein soya bean digest agar; if colonies of fungi are detected on this medium, they are counted as part of the TAMC. The total combined yeasts/mould count (TYMC) is considered to be equal to the number of CFU found using Sabouraud-dextrose agar; if colonies of bacteria are detected on this medium, they are counted as part of the TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, Sabouraud-dextrose agar containing antibiotics may be used. If the count is carried out by the MPN method the calculated value is the TAMC.

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- $10^1$ CFU: maximum acceptable count = 20;
- $10^2$ CFU: maximum acceptable count = 200;
- $10^3$ CFU: maximum acceptable count = 2000, and so forth.

The recommended solutions and media are described in general chapter *2.6.13*.

**04/2010:20613**

# 2.6.13. MICROBIOLOGICAL EXAMINATION OF NON-STERILE PRODUCTS: TEST FOR SPECIFIED MICRO-ORGANISMS(3)

1. INTRODUCTION

The tests described hereafter will allow determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2. GENERAL PROCEDURES

The preparation of samples is carried out as described in general chapter *2.6.12*.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised as described in general chapter *2.6.12*.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.12*.

3. GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect micro-organisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

3-1. *PREPARATION OF TEST STRAINS*

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

3-1-1. **Aerobic micro-organisms**. Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h. Grow the test strain for *Candida albicans* separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20-25 °C for 2-3 days.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium, such as ATCC 14028 or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;
- *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C.

3-1-2. **Clostridia**. Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03) or NBRC 14293. Grow the clostridial test strain under anaerobic conditions in reinforced medium for clostridia at 30-35 °C for 24-48 h. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *Cl. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period.

3-2. *NEGATIVE CONTROL*

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 4. A failed negative control requires an investigation.

3-3. *GROWTH PROMOTION AND INHIBITORY PROPERTIES OF THE MEDIA*

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 2.6.13.-1.

*Test for growth promoting properties, liquid media*: inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the

(3) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

Table 2.6.13.-1 – *Growth promoting, inhibitory and indicative properties of media*

| | Medium | Property | Test strains |
|---|---|---|---|
| Test for bile-tolerant gram-negative bacteria | Enterobacteria enrichment broth-Mossel | Growth promoting | *E. coli*<br>*P. aeruginosa* |
| | | Inhibitory | *S. aureus* |
| | Violet red bile glucose agar | Growth promoting + indicative | *E. coli*<br>*P. aeruginosa* |
| Test for *Escherichia coli* | MacConkey broth | Growth promoting | *E. coli* |
| | | Inhibitory | *S. aureus* |
| | MacConkey agar | Growth promoting + indicative | *E. coli* |
| Test for *Salmonella* | Rappaport Vassiliadis *Salmonella* enrichment broth | Growth promoting | *Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony |
| | | Inhibitory | *S. aureus* |
| | Xylose, lysine, deoxycholate agar | Growth promoting + indicative | *Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony |
| Test for *Pseudomonas aeruginosa* | Cetrimide agar | Growth promoting | *P. aeruginosa* |
| | | Inhibitory | *E. coli* |
| Test for *Staphylococcus aureus* | Mannitol salt agar | Growth promoting + indicative | *S. aureus* |
| | | Inhibitory | *E. coli* |
| Test for clostridia | Reinforced medium for clostridia | Growth promoting | *Cl. sporogenes* |
| | Columbia agar | Growth promoting | *Cl. sporogenes* |
| Test for *Candida albicans* | Sabouraud dextrose broth | Growth promoting | *C. albicans* |
| | Sabouraud dextrose agar | Growth promoting + indicative | *C. albicans* |

shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

*Test for growth promoting properties, solid media*: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

*Test for inhibitory properties, liquid or solid media*: inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

*Test for indicative properties*: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

3-4. *SUITABILITY OF THE TEST METHOD*

For each product to be tested, perform the sample preparation as described in the relevant paragraph in section 4. Add each test strain at the time of mixing, in the prescribed growth medium. Inoculate the test strains individually. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation.

Perform the test as described in the relevant paragraph in section 4 using the shortest incubation period prescribed.

The specified micro-organisms must be detected with the indication reactions as described in section 4.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see 4-5-3 of general chapter *2.6.12*).

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralised, then it is to be assumed that the inhibited micro-organism will not be present in the product.

4. TESTING OF PRODUCTS

4-1. *BILE-TOLERANT GRAM-NEGATIVE BACTERIA*

4-1-1. **Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h).

4-1-2. **Test for absence**. Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in 4-1-1, to inoculate enterobacteria enrichment broth-Mossel. Incubate at 30-35 °C for 24-48 h. Subculture on plates of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

The product complies with the test if there is no growth of colonies.

4-1-3. **Quantitative test**

4-1-3-1. *Selection and subculture.* Inoculate suitable quantities of enterobacteria enrichment broth-Mossel with the preparation as described under 4-1-1 and/or dilutions of it containing respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 mL, 0.01 mL and 0.001 mL) of the product to be examined. Incubate at 30-35 °C for 24-48 h. Subculture each of the cultures on a plate of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

4-1-3-2. *Interpretation.* Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 2.6.13.-2 the probable number of bacteria.

Table 2.6.13.-2 – *Interpretation of results*

| Results for each quantity of product | | | Probable number of bacteria per gram or millilitre of product |
|---|---|---|---|
| 0.1 g or 0.1 mL | 0.01 g or 0.01 mL | 0.001 g or 0.001 mL | |
| + | + | + | $> 10^3$ |
| + | + | − | $< 10^3$ and $> 10^2$ |
| + | − | − | $< 10^2$ and $> 10$ |
| − | − | − | $< 10$ |

4-2. *ESCHERICHIA COLI*

4-2-1. **Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

4-2-2. **Selection and subculture**. Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

4-2-3. **Interpretation**. Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

4-3. *SALMONELLA*

4-3-1. **Sample preparation and pre-incubation**. Prepare the product to be examined as described in general chapter *2.6.12*, and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

4-3-2. **Selection and subculture**. Transfer 0.1 mL of casein soya bean digest broth to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on plates of xylose, lysine, deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

4-3-3. **Interpretation**. The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-4. *PSEUDOMONAS AERUGINOSA*

4-4-1. **Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.

4-4-2. **Selection and subculture**. Subculture on a plate of cetrimide agar and incubate at 30-35 °C for 18-72 h.

4-4-3. **Interpretation**. Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

4-5. *STAPHYLOCOCCUS AUREUS*

4-5-1. **Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. When testing transdermal patches, filter the volume of sample corresponding to 1 patch of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.

4-5-2. **Selection and subculture**. Subculture on a plate of mannitol salt agar and incubate at 30-35 °C for 18-72 h.

4-5-3. **Interpretation**. The possible presence of *S. aureus* is indicated by the growth of yellow/white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-6. *CLOSTRIDIA*

4-6-1. **Sample preparation and heat treatment**. Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in general chapter *2.6.12*. Divide the sample into 2 portions of at least 10 mL. Heat 1 portion at 80 °C for 10 min and cool rapidly. Do not heat the other portion.

4-6-2. **Selection and subculture**. Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under 3-4) of reinforced medium for clostridia. Incubate under anaerobic conditions at 30-35 °C for 48 h. After incubation, make subcultures from each container on Columbia agar and incubate under anaerobic conditions at 30-35 °C for 48-72 h.

4-6-3. **Interpretation**. The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of clostridia. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-7. *CANDIDA ALBICANS*

4-7-1. **Sample preparation and pre-incubation**. Prepare the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL to inoculate 100 mL of Sabouraud-dextrose broth and mix. Incubate at 30-35 °C for 3-5 days.

4-7-2. **Selection and subculture**. Subculture on a plate of Sabouraud-dextrose agar and incubate at 30-35 °C for 24-48 h.

4-7-3. **Interpretation**. Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

*The following section is given for information.*

## 5. RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The following solutions and culture media have been found to be satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used provided that their suitability can be demonstrated.

*Stock buffer solution.* Place 34 g of potassium dihydrogen phosphate in a 1000 mL volumetric flask, dissolve in 500 mL of purified water, adjust to pH 7.2 ± 0.2 with sodium hydroxide, dilute to 1000.0 mL with purified water and mix. Dispense into containers and sterilise. Store at 2-8 °C.

*Phosphate buffer solution pH 7.2.* Prepare a mixture of stock buffer solution and purified water (1:800 *V/V*) and sterilise.

**Buffered sodium chloride-peptone solution pH 7.0**

| | |
|---|---|
| Potassium dihydrogen phosphate | 3.6 g |
| Disodium hydrogen phosphate dihydrate | 7.2 g, equivalent to 0.067 M phosphate |
| Sodium chloride | 4.3 g |
| Peptone (meat or casein) | 1.0 g |
| Purified water | 1000 mL |

Sterilise in an autoclave using a validated cycle.

**Casein soya bean digest broth**

| | |
|---|---|
| Pancreatic digest of casein | 17.0 g |
| Papaic digest of soya bean | 3.0 g |
| Sodium chloride | 5.0 g |
| Dipotassium hydrogen phosphate | 2.5 g |
| Glucose monohydrate | 2.5 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Casein soya bean digest agar**

| | |
|---|---|
| Pancreatic digest of casein | 15.0 g |
| Papaic digest of soya bean | 5.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Sabouraud-dextrose agar**

| | |
|---|---|
| Dextrose | 40.0 g |
| Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1) | 10.0 g |
| Agar | 15.0 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Potato dextrose agar**

| | |
|---|---|
| Infusion from potatoes | 200 g |
| Dextrose | 20.0 g |
| Agar | 15.0 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Sabouraud-dextrose broth**

| | |
|---|---|
| Dextrose | 20.0 g |
| Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1) | 10.0 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Enterobacteria enrichment broth-Mossel**

| | |
|---|---|
| Pancreatic digest of gelatin | 10.0 g |
| Glucose monohydrate | 5.0 g |
| Dehydrated ox bile | 20.0 g |
| Potassium dihydrogen phosphate | 2.0 g |
| Disodium hydrogen phosphate dihydrate | 8.0 g |
| Brilliant green | 15 mg |
| Purified water | 1000 mL |

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25 °C. Heat at 100 °C for 30 min and cool immediately.

**Violet red bile glucose agar**

| | |
|---|---|
| Yeast extract | 3.0 g |
| Pancreatic digest of gelatin | 7.0 g |
| Bile salts | 1.5 g |
| Sodium chloride | 5.0 g |
| Glucose monohydrate | 10.0 g |
| Agar | 15.0 g |
| Neutral red | 30 mg |
| Crystal violet | 2 mg |
| Purified water | 1000 mL |

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling; do not heat in an autoclave.

**MacConkey broth**

| | |
|---|---|
| Pancreatic digest of gelatin | 20.0 g |
| Lactose monohydrate | 10.0 g |
| Dehydrated ox bile | 5.0 g |
| Bromocresol purple | 10 mg |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**MacConkey agar**

| | |
|---|---|
| Pancreatic digest of gelatin | 17.0 g |
| Peptones (meat and casein) | 3.0 g |
| Lactose monohydrate | 10.0 g |
| Sodium chloride | 5.0 g |
| Bile salts | 1.5 g |
| Agar | 13.5 g |
| Neutral red | 30.0 mg |
| Crystal violet | 1 mg |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 7.1 ± 0.2 at 25 °C. Boil for 1 min with constant shaking then sterilise in an autoclave using a validated cycle.

**Rappaport Vassiliadis *Salmonella* enrichment broth**

| | |
|---|---|
| Soya peptone | 4.5 g |
| Magnesium chloride hexahydrate | 29.0 g |
| Sodium chloride | 8.0 g |
| Dipotassium phosphate | 0.4 g |
| Potassium dihydrogen phosphate | 0.6 g |
| Malachite green | 0.036 g |
| Purified water | 1000 mL |

Dissolve, warming gently. Sterilise in an autoclave using a validated cycle, at a temperature not exceeding 115 °C. The pH is to be 5.2 ± 0.2 at 25 °C after heating and autoclaving.

**Xylose, lysine, deoxycholate agar**

| | |
|---|---|
| Xylose | 3.5 g |
| L-Lysine | 5.0 g |
| Lactose monohydrate | 7.5 g |
| Sucrose | 7.5 g |
| Sodium chloride | 5.0 g |
| Yeast extract | 3.0 g |
| Phenol red | 80 mg |
| Agar | 13.5 g |
| Sodium deoxycholate | 2.5 g |
| Sodium thiosulfate | 6.8 g |
| Ferric ammonium citrate | 0.8 g |
| Purified water | 1000 mL |

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

**Cetrimide agar**

| | |
|---|---|
| Pancreatic digest of gelatin | 20.0 g |
| Magnesium chloride | 1.4 g |
| Dipotassium sulfate | 10.0 g |
| Cetrimide | 0.3 g |
| Agar | 13.6 g |
| Purified water | 1000 mL |
| Glycerol | 10.0 mL |

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.2 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Mannitol salt agar**

| | |
|---|---|
| Pancreatic digest of casein | 5.0 g |
| Peptic digest of animal tissue | 5.0 g |
| Beef extract | 1.0 g |
| D-Mannitol | 10.0 g |
| Sodium chloride | 75.0 g |
| Agar | 15.0 g |
| Phenol red | 0.025 g |
| Purified water | 1000 mL |

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.4 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Reinforced medium for clostridia**

| | |
|---|---|
| Beef extract | 10.0 g |
| Peptone | 10.0 g |
| Yeast extract | 3.0 g |
| Soluble starch | 1.0 g |
| Glucose monohydrate | 5.0 g |
| Cysteine hydrochloride | 0.5 g |
| Sodium chloride | 5.0 g |
| Sodium acetate | 3.0 g |
| Agar | 0.5 g |
| Purified water | 1000 mL |

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 6.8 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

**Columbia agar**

| | |
|---|---|
| Pancreatic digest of casein | 10.0 g |
| Meat peptic digest | 5.0 g |
| Heart pancreatic digest | 3.0 g |
| Yeast extract | 5.0 g |
| Maize starch | 1.0 g |
| Sodium chloride | 5.0 g |
| Agar, according to gelling power | 10.0-15.0 g |
| Purified water | 1000 mL |

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle. Allow to cool to 45-50 °C; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

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## 2.6.14. BACTERIAL ENDOTOXINS[4]

The test for bacterial endotoxins (BET) is used to detect or quantify endotoxins from gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

Method A. Gel-clot method: limit test

Method B. Gel-clot method: quantitative test

Method C. Turbidimetric kinetic method

Method D. Chromogenic kinetic method

Method E. Chromogenic end-point method

Method F. Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

The test is carried out in a manner that avoids endotoxin contamination.

1. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

(4) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

*NOTE: in this chapter, the term 'tube' includes all types of receptacles, for example microtitre plate wells.*

2. REAGENTS, TEST SOLUTIONS

**(1) Amoebocyte lysate**

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority.

*NOTE: amoebocyte lysate reacts with some β-glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These preparations may be used for endotoxin testing in the presence of glucans.*

**(2) Lysate solution**

Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

**(3) Water for BET (water for bacterial endotoxins test)**

*Water for injections R* or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

Endotoxin is expressed in International Units (IU). The equivalence in IU of the International Standard is stated by the World Health Organization.

*NOTE: one International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).*

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$\mathrm{MVD} = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

*Endotoxin limit*: the endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

$K$ = threshold pyrogenic dose of endotoxin per kilogram of body mass,

$M$ = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, $M$ is the maximum total dose administered in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

*Concentration of test solution*:

- mg/mL if the endotoxin limit is specified by mass (IU/mg),
- Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit),
- mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

7. GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The minimum concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity. To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under 1. Preparatory testing.

*1. PREPARATORY TESTING*

**(i) Confirmation of the labelled lysate sensitivity**

Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/mL, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 mL aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37 ± 1 °C for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

The end-point is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean end-point

concentration by calculting the mean of the logarithms of the end-point concentrations of the 4 dilution series, take the antilogarithm of this value, as indicated by the following expression:

$$\text{Geometric mean end-point concentration} = \text{antilog}\frac{\sum e}{f}$$

$\sum e$ = sum of the $\log_{10}$ end-point concentrations of the dilution series used,

$f$ = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (IU/mL). If this is not less than 0.5λ and not more than 2λ, the labelled sensitivity is confirmed and is used in the tests performed with this lysate.

**(ii) Test for interfering factors**

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors must be repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is considered valid when all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the test solution interferes with the test.

If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

*2. LIMIT TEST (METHOD A)*

**(i) Procedure**

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

| Solution | Endotoxin concentration/Solution to which endotoxin is added | Number of replicates |
|---|---|---|
| A | None/Diluted test solution | 2 |
| B | 2λ/Diluted test solution | 2 |
| C | 2λ/Water for BET | 2 |
| D | None/Water for BET | 2 |

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

**(ii) Interpretation**

The test is considered valid when both replicates of solution B and C are positive and those of solution D are negative.

When a negative result is found for both replicates of solution A, the preparation being examined complies with the test.

When a positive result is found for both replicates of solution A, the preparation being examined does not comply with the test.

When a positive result is found for one replicate of solution A and a negative result is found for the other, repeat the test. In the repeat test, the preparation being examined complies with the test if a negative result is found for both replicates of solution A. The preparation does not comply with the test if a positive result is found for one or both replicates of solution A.

However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

*3. QUANTITATIVE TEST (METHOD B)*

**(i) Procedure**

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-1

| Solution | Endotoxin concentration/Solution to which endotoxin is added | Diluent | Dilution factor | Endotoxin concentration | Number of replicates |
|---|---|---|---|---|---|
| A | None/Test solution | - | - | - | 4 |
| B | 2λ/Test solution | Test solution | 1 | 2λ | 4 |
| | | | 2 | 1λ | 4 |
| | | | 4 | 0.5λ | 4 |
| | | | 8 | 0.25λ | 4 |
| C | 2λ/Water for BET | Water for BET | 1 | 2λ | 2 |
| | | | 2 | 1λ | 2 |
| | | | 4 | 0.5λ | 2 |
| | | | 8 | 0.25λ | 2 |
| D | None/Water for BET | - | - | - | 2 |

Solution A = solution of the preparation being examined that is free of detectable endotoxins.
Solution B = test for interference.
Solution C = control of the labelled lysate sensitivity.
Solution D = negative control (water for BET).

**(ii) Calculation and interpretation**

The test is considered valid when the following 3 conditions are met:

(a) both replicates of solution D (negative control) are negative,

(b) both replicates of solution B (positive product control) are positive,

(c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate, by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the test solution is the end-point concentration of the replicates. If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, report the endotoxin concentration as less than λ (or, if a diluted sample was tested, report as less than the lowest dilution factor of the sample × λ). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the largest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor × 8 × λ).

The preparation being examined meets the requirements of the test if the endotoxin concentration in both replicates is less than that specified in the monograph.

Table 2.6.14.-3

| Solution | Endotoxin concentration/Solution to which endotoxin is added | Diluent | Dilution factor | Endotoxin concentration | Number of replicates |
|---|---|---|---|---|---|
| A | None/Test solution | Water for BET | 1 | - | 2 |
| | | | 2 | - | 2 |
| | | | 4 | - | 2 |
| | | | 8 | - | 2 |
| B | 2λ/Test solution | | 1 | 2λ | 2 |
| C | 2λ/Water for BET | Water for BET | 1 | 2λ | 2 |
| | | | 2 | 1λ | 2 |
| | | | 4 | 0.5λ | 2 |
| | | | 8 | 0.25λ | 2 |
| D | None/Water for BET | - | - | - | 2 |

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make a dilution series of 4 tubes containing the test solution at concentrations of 1, 1/2, 1/4 and 1/8, relative to the dilution used in the test for interfering factors. Other dilutions up to the MVD may be used as appropriate.

Solution B = solution A containing standard endotoxin at a concentration of 2λ (positive product control).

Solution C = a dilution series of 4 tubes of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.

Solution D = water for BET (negative control).

8. PHOTOMETRIC QUANTITATIVE TECHNIQUES (METHODS C, D, E AND F)

*1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)*

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique may be classified as being either the end-point-turbidimetric test or the kinetic-turbidimetric test.

The end-point-turbidimetric test (Method F) is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or transmission, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

*2. CHROMOGENIC TECHNIQUE (METHODS D AND E)*

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique may be classified as being either the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period.

The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

*3. PREPARATORY TESTING*

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to show that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

**(i) Assurance of criteria for the standard curve**

The test must be carried out for each lot of lysate reagent.

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 $\log_{10}$ in the kinetic methods, additional standards must be included to bracket each $\log_{10}$ increase in the range of the standard curve.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980, for the range of endotoxin concentrations set up.

**(ii) Test for interfering factors**

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-4

| Solution | Endotoxin concentration | Solution to which endotoxin is added | Number of replicates |
|---|---|---|---|
| A | None | Test solution | Not less than 2 |
| B | Middle concentration of the standard curve | Test solution | Not less than 2 |
| C | At least 3 concentrations (lowest concentration is designated λ) | Water for BET | Each concentration not less than 2 |
| D | None | Water for BET | Not less than 2 |

Solution A = test solution, that may be diluted not to exceed the MVD.

Solution B = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.

Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls).

Solution D = water for BET (negative control).

The test is considered valid when the following conditions are met:

- the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;
- the result with solution D does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A, Table 2.6.14.-4) from that in the solution containing the added endotoxin (solution B, Table 2.6.14.-4).

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution not to exceed the MVD may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

*4. TEST*

**(i) Procedure**

Follow the procedure described in 3. Preparatory testing, (ii) Test for interfering factors.

**(ii) Calculation**

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the positive control solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,

(2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent,

(3) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

**(iii) Interpretation**

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

*Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.*

**04/2016:20615**

# 2.6.15. PREKALLIKREIN ACTIVATOR

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of the International Standard, which consists of freeze-dried prekallikrein activator. The equivalence in International Units of the International Standard is stated by the World Health Organization.

REAGENTS

*Prekallikrein activator in albumin BRP* is calibrated in International Units by comparison with the International Standard.

*Buffer A*. Dissolve 6.055 g of *tris(hydroxymethyl)aminomethane R*, 1.17 g of *sodium chloride R*, 50 mg of *hexadimethrine bromide R* and 0.100 g of *sodium azide R* in *water R*. Adjust to pH 8.0 with *2 M hydrochloric acid R* and dilute to 1000 mL with *water R*.

*Buffer B*. Dissolve 6.055 g of *tris(hydroxymethyl)aminomethane R* and 8.77 g of *sodium chloride R* in *water R*. Adjust to pH 8.0 with *2 M hydrochloric acid R* and dilute to 1000 mL with *water R*.

PREPARATION OF PREKALLIKREIN SUBSTRATE

*To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.*

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or a 38 g/L solution of *sodium citrate R*) to which 1 mg/mL of *hexadimethrine bromide R* has been added. Centrifuge the mixture at 3600 *g* for 5 min. Separate the plasma and centrifuge again at 6000 *g* for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing *agarose-DEAE for ion-exchange chromatography R* that has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 mL/cm$^2$/h. Collect the eluate in fractions and record the absorbance at 280 nm (*2.2.25*). Pool the fractions containing the 1$^{st}$ protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 7 g/L of *sodium chloride R* and filter through a membrane filter (nominal pore size 0.45 µm). Freeze the filtrate in portions and store at – 25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

METHOD

The assay may be carried out using an automated enzyme analyser or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate R* or *D-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride R*), dissolved in buffer B. Record the rate of change in absorbance per minute for 2-10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Depending on the method used, $\Delta A$/min has to be corrected by subtracting the value obtained for the corresponding blank without the prekallikrein substrate. The results may be calculated using a standard curve, a parallel-line or a slope ratio assay or any other suitable statistical method. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined. During method validation, the spiking experiments must show that the sample matrix has no influence on the results. High blank values may impact assay validity and should be appropriately investigated.

**01/2018:20616**
**corrected 9.4**

# 2.6.16. TESTS FOR EXTRANEOUS AGENTS IN VIRAL VACCINES FOR HUMAN USE

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter *5.1.7. Viral safety*. This strategy includes a full package of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The list of tests summarised in Table 2.6.16.-1 must be adapted depending on the extraneous agents that have the potential to contaminate the product: for *in vitro* tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If *in vivo* tests are more relevant than *in vitro* tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilised SPF eggs for the influenza virus) the decision to maintain or to introduce such *in vivo* assays in a testing strategy must be justified by the risk assessment.

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and with the agreement of the competent authority.

In tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell cultures from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (*5.2.2*).

TEST METHODS

Relevant tests for extraneous agents to be carried out at various production stages are indicated in Table 2.6.16.-1 using the methods described below, based on the risk assessment.

Take samples at the time of harvesting, and if not tested immediately, keep at a temperature below – 40 °C.

Table 2.6.16.-1. – *Relevant tests for extraneous agents at various production stages*

| | Virus seed lots | Virus harvests | Production of culture substrates | |
|---|---|---|---|---|
| | | | Control cells | Control eggs |
| Bacterial and fungal contamination | + | + | - | - |
| Mycoplasmas | + | + | - | - |
| Spiroplasmas[1] | + | - | - | - |
| Mycobacteria | + | + | - | - |
| Test in suckling mice[2] | + | - | - | - |
| Avian viruses[3] | + | + | - | - |
| Test for extraneous agents in cell cultures[4] | + | + | + | + |
| Insect viruses[5] | + | + | - | - |
| Test on control cells (microscopic examination) | - | - | + | - |
| Haemadsorbing viruses | - | - | + | - |
| Test on control eggs (haemagglutinating agents) | - | - | - | + |
| Avian leucosis viruses[6] | - | - | + | + |

| | | | | |
|---|---|---|---|---|
| Test for specific viruses by NAT[7] | + | + | - | - |
| Test for viruses using broad molecular methods[8] | + | + | - | - |

(1) If insect cells or raw materials of plant origin are used.

(2) If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.

(3) If the virus is propagated in avian or primary avian tissues. If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.

(4) Test performed in suitable permissive cell cultures. Based on a risk assessment.

(5) If the virus is propagated in insect cells.

(6) If the virus is propagated in primary avian tissues or in eggs.

(7) Based on a risk assessment.

(8) These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority.

**Bacterial and fungal contamination**. Each virus seed lot and virus harvest complies with the test for sterility (*2.6.1*).

**Mycoplasmas** (*2.6.7*). Each virus seed lot and virus harvest complies with the test for mycoplasmas.

**Spiroplasmas**. Spiroplasmas may be introduced into virus seed lots through contamination of raw materials of plant origin or when insect cell lines are used for virus propagation. When appropriate, virus seed lots are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority.

**Mycobacteria** (*2.6.2*). A 2.7 mL sample of each virus seed lot and each virus harvest is tested for the presence of *Mycobacterium* spp. by culture methods known to be sensitive for the detection of these organisms. NAT (*2.6.21*) may be used as an alternative, provided such an assay is validated and shown to be comparable to the culture method.

**Suckling mice**. Each virus seed lot is tested in suckling mice if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package. Inoculate no fewer than 20 suckling mice, each less than 24 h old, intracerebrally with 0.01 mL and intraperitoneally with at least 0.1 mL of the virus seed lot. Observe the suckling mice daily for at least 4 weeks. Carry out an autopsy of all suckling mice that die after the first 24 h of the test or that show signs of illness, and examine for evidence of viral infection by direct macroscopical observation. The virus seed lot passes the test if no suckling mice show evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated suckling mice survive the observation period.

**Avian viruses**. Each virus seed lot propagated in avian tissues and each virus harvest propagated in primary avian tissues is tested for avian viruses if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package. Neutralise a sample equivalent to 100 human doses of vaccine or 10 mL, whichever is the greater. Using 0.5 mL per egg, inoculate a group of fertilised SPF eggs, 9-11 days old, by the allantoic route and a second group, 5-7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

**Test for extraneous agents in cell cultures**. For each virus seed lot, each virus harvest and each production cell culture (control cells or control eggs), tests for other extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate and virus strain, as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal-or plant-derived raw materials, must be taken into account when choosing suitable permissive cells.

For each virus seed lot and virus harvest, neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian and human cell cultures. If the virus is grown in simian or human cells, the neutralised virus harvest is tested on a separate culture of these cells. If the virus is grown in a mammalian cell system other than simian or human, or in avian cells, cells of that species, but from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. If the production cell culture is maintained at a temperature other than 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells used for growth of the virus. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing viruses after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

**Insect viruses**. Each virus seed lot and virus harvest propagated in insect cells is tested for insect viruses. Neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into at least 1 cell culture different from that used in production and permissible to insect viruses, and that also allows detection of human arboviruses (e.g. BHK-21). The choice of cells is approved by the competent authority and takes into account the origin of the production cells and the likely contaminants that may be detected by the chosen cells. The cells are incubated at an appropriate temperature and observed for a period of 14 days. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing virus after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

**Tests on control cells**. Where cell cultures are used for virus production, examine the control cells microscopically for the absence of any virus causing cytopathic degeneration throughout the incubation time of the inoculated production cell cultures or for no less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, pool the supernatant fluids from the control cells and examine for the presence of extraneous agents over a period of 14 days as described above for the virus seed lot and the virus harvest by inoculation of relevant cell cultures depending on the type of cells used for virus growth.

**Haemadsorbing viruses**. Where cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents in cell cultures. At 14 days or at the time of the last virus harvest, whichever is the longer, examine no fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the test for haemadsorbing viruses is not feasible, carry out a test for haemagglutinating viruses. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half

of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. No evidence of haemadsorbing agents is found.

**Tests on control eggs**. Where eggs are used for virus production, examine 0.25 mL of the allantoic fluid from each control egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 mL sample of the pooled amniotic fluids from the control eggs in 0.5 mL volumes into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

In addition, inoculate 5 mL samples of the pooled amniotic fluids from the control eggs into suitable permissive cells including human, simian and avian cells. Observe the cell cultures for 14 days at a suitable incubation temperature. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

**Avian leucosis viruses**. For each virus propagated in primary avian cell tissues or in eggs, the production cell culture (control cells or control eggs) is tested for avian leucosis viruses in accordance with general chapter *2.6.24*. When cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents prior to the test for avian leucosis viruses. At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the test for avian leucosis viruses on DF1 cells or leucosis-free chick-embryo cell cultures with amplification through 5 passages using at least 5 mL of the supernatant fluid from the control cells or at least 10 mL of a sample of the pooled amniotic fluids from the control eggs. PERT assay end-point can be used after DF1 amplification for detection of exogenous avian retroviruses (including avian leucosis virus). For specific detection of avian leucosis virus, several end-points can be used such as immunostaining, enzyme-linked immunosorbent assay (ELISA) or complement fixation for avian leucosis (COFAL). Control cells or control eggs comply with the test if there is no evidence of the presence of any avian leucosis virus.

**Tests for specific viruses by NAT**. Based on a risk assessment related to the manufacturing process, each virus seed lot and each virus harvest may be tested by NAT (*2.6.21*) for specific viruses that are not detected by conventional *in vivo* or cell culture assays.

**Test for viruses using broad molecular methods**. With the agreement of the competent authority, broad molecular methods (e.g. high-throughput sequencing) may be used either as an alternative to *in vivo* tests and specific NAT, or as a supplement/alternative to *in vitro* culture tests based on the risk assessment.

Both NAT (*2.6.21*) and broad molecular methods are carried out with or without prior amplification in suitable permissive cells. In cases of positive results with either broad molecular methods or NAT, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

01/2018:20617

# 2.6.17. TEST FOR ANTICOMPLEMENTARY ACTIVITY OF IMMUNOGLOBULIN

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 $CH_{50}$) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The haemolytic unit of complement activity ($CH_{50}$) is the amount of complement that, in the given reaction conditions, will produce the lysis of $2.5 \times 10^8$ out of a total of $5 \times 10^8$ optimally sensitised red blood cells.

*Magnesium and calcium stock solution*. Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 mL with the same solvent.

*Barbital buffer stock solution*. Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 mL of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid*. Add 12.5 mL of magnesium and calcium stock solution and dilute to 5000 mL with *water R*. Filter through a membrane filter (nominal pore size 0.22 µm). Store at 4 °C in glass containers.

*Gelatin solution*. Dissolve 12.5 g of *gelatin R* in about 800 mL of *water R* and heat to boiling in a water-bath. Cool to 20 °C and dilute to 10 L with *water R*. Filter through a membrane filter (nominal pore size 0.22 µm). Store at 4 °C. Use clear solutions only.

*Citrate solution*. Dissolve 8.0 g of *sodium citrate R*, 4.2 g of *sodium chloride R* and 20.5 g of *glucose R* in 750 mL of *water R*. Adjust to pH 6.1 using a 100 g/L solution of *citric acid monohydrate R* and dilute to 1000 mL with *water R*.

*Gelatin barbital buffer solution*. Add 4 volumes of gelatin solution to 1 volume of barbital buffer stock solution and mix. Adjust to pH 7.3, if necessary, using *1 M sodium hydroxide* or *1 M hydrochloric acid*. Maintain at 4 °C. Prepare fresh solutions daily.

*Stabilised sheep blood*. Collect 1 volume of sheep blood into 1 volume of citrate solution and mix. Store at 4 °C for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

*Haemolysin*. Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)

*Guinea-pig complement*. Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below – 70 °C.

METHOD

**Preparation of standardised 5 per cent sheep red blood cell suspension**. Separate sheep red blood cells by centrifuging an appropriate volume of stabilised sheep blood and wash the cells at least 3 times with gelatin barbital buffer solution and prepare a 5 per cent *V/V* suspension in the same solution. Measure the cell density of the suspension as follows: add 0.2 mL to 2.8 mL of *water R* and centrifuge the lysed solution for 5 min at 1000 *g*; the cell density is suitable if the absorbance (*2.2.25*) of the supernatant at 541 nm is 0.62 ± 0.01. Correct the cell density by adding gelatin barbital buffer solution according to the following equation:

$$V_f = \frac{V_i \times A}{0.62}$$

$V_f$ = final adjusted volume;

$V_i$ = the initial volume;

$A$ = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about $1 \times 10^9$ cells/mL.

**Haemolysin titration**. Prepare haemolysin dilutions as shown in Table 2.6.17.-1.

Table 2.6.17.-1

| Required dilution of haemolysin | Prepared using | | |
|---|---|---|---|
| | Gelatin barbital buffer solution | | Haemolysin |
| | Volume (mL) | Dilution (1/...) | Volume (mL) |
| 7.5 | 0.65 | undiluted | 0.1 |
| 10 | 0.90 | undiluted | 0.1 |
| 75 | 1.80 | 7.5 | 0.2 |
| 100 | 1.80 | 10 | 0.2 |
| 150 | 1.00 | 75 | 1.0 |
| 200 | 1.00 | 100 | 1.0 |
| 300 | 1.00 | 150 | 1.0 |
| 400 | 1.00 | 200 | 1.0 |
| 600 | 1.00 | 300 | 1.0 |
| 800 | 1.00 | 400 | 1.0 |
| 1200 | 1.00 | 600 | 1.0 |
| 1600 | 1.00 | 800 | 1.0 |
| 2400 | 1.00 | 1200 | 1.0 |
| 3200* | 1.00 | 1600 | 1.0 |
| 4800* | 1.00 | 2400 | 1.0 |

* discard 1.0 mL of the mixture.

Add 1.0 mL of 5 per cent sheep red blood cell suspension to each tube of the haemolysin dilution series, starting at the 1/75 dilution, and mix. Incubate at 37 °C for 30 min.

Transfer 0.2 mL of each of these incubated mixtures to new tubes and add 1.10 mL of gelatin barbital buffer solution and 0.2 mL of diluted guinea-pig complement (for example, 1/150). Perform this in duplicate.

As the unhaemolysed cell control, prepare 3 tubes with 1.4 mL of gelatin barbital buffer solution and 0.1 mL of 5 per cent sheep red blood cell suspension.

As the fully haemolysed control, prepare 3 tubes with 1.4 mL of *water R* and 0.1 mL of 5 per cent sheep red cell suspension.

Incubate all tubes at 37 °C for 60 min and centrifuge at 1000 *g* for 5 min. Measure the absorbance (*2.2.25*) of the supernatants at 541 nm and calculate the percentage degree of haemolysis in each tube using the following expression:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

$A_a$ = absorbance of tubes with haemolysin dilution;

$A_b$ = mean absorbance of the 3 tubes with full haemolysis;

$A_1$ = mean absorbance of the 3 tubes with no haemolysis.

Plot the percentage degree of haemolysis as the ordinate against the corresponding reciprocal value of the haemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the haemolysin from the graph by inspection. Select a dilution such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis. This dilution is defined as 1 minimal haemolytic unit (1 MHU) in 1.0 mL. The optimal haemolytic haemolysin dilution for preparation of sensitised sheep red blood cells contains 2 MHU/mL.

The haemolysin titration is not valid unless the maximum degree of haemolysis is 50 per cent to 70 per cent. If the maximum degree of haemolysis is not in this range, repeat the titration with more or less diluted complement solution.

**Preparation of optimised sensitised sheep red blood cells (haemolytic system)**. Prepare an appropriate volume of diluted haemolysin containing 2 MHU/mL and an equal volume of standardised 5 per cent sheep red blood cell suspension. Add the haemolysin dilution to the standardised cell suspension and mix. Incubate at 37 °C for 15 min, store at 2 °C to 8 °C and use within 6 h.

**Titration of complement**. Prepare an appropriate dilution of complement (for example 1/250) with gelatin barbital buffer solution and perform the titration in duplicate as shown in Table 2.6.17.-2.

Table 2.6.17.-2

| Tube number | Volume of diluted complement (for example 1/250) (mL) | Volume of gelatin barbital buffer solution (mL) |
|---|---|---|
| 1 | 0.1 | 1.2 |
| 2 | 0.2 | 1.1 |
| 3 | 0.3 | 1.0 |
| 4 | 0.4 | 0.9 |
| 5 | 0.5 | 0.8 |
| 6 | 0.6 | 0.7 |
| 7 | 0.7 | 0.6 |
| 8 | 0.8 | 0.5 |
| 9 | 0.9 | 0.4 |
| 10 | 1.0 | 0.3 |
| 11 | 1.1 | 0.2 |
| 12 | 1.2 | 0.1 |
| 3 tubes as cell control at 0 per cent haemolysis | – | 1.3 |
| 3 tubes at 100 per cent haemolysis | – | 1.3 mL of water |

Add 0.2 mL of sensitised sheep red blood cells to each tube, mix well and incubate at 37 °C for 60 min. Cool the tubes in an ice-bath and centrifuge at 1000 *g* for 5 min. Measure the absorbance of the supernatant at 541 nm and calculate the degree of haemolysis ($Y$) using the following expression:

$$\frac{A_c - A_1}{A_b - A_1}$$

$A_c$ = absorbance of tubes 1 to 12;

$A_b$ = mean absorbance of tubes with 100 per cent haemolysis;

$A_1$ = mean absorbance of cell controls with 0 per cent haemolysis.

Plot $Y/(1- Y)$ as the abscissa against the amount of diluted complement in millilitres as the ordinate on log–log graph paper. Fit the best line to the points and determine the

ordinate for the 50 per cent haemolytic complement dose where $Y/(1-Y) = 1.0$. Calculate the activity in haemolytic units ($CH_{50}$/mL) using the following expression:

$$\frac{C_d}{C_a \times 5}$$

$C_d$ = reciprocal value of the complement dilution;

$C_a$ = volume of diluted complement resulting in 50 per cent haemolysis, in millilitres;

5 = scaling factor to take account of the number of red blood cells.

The test is not valid unless the plot is a straight line between 15 per cent and 85 per cent haemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

**Test for anticomplementary activity**. Prepare a complement dilution having 100 $CH_{50}$/mL by diluting titrated guinea-pig complement with gelatin barbital buffer solution. Depending on the immunoglobulin to be examined and based on validation data, a pH adjustment to 7 may be necessary. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg/mL:

Table 2.6.17.-3

| | Immunoglobulin to be examined | Complement control (in duplicate) |
|---|---|---|
| Immunoglobulin (50 mg/mL) | 0.2 mL | – |
| Gelatin barbital buffer | 0.6 mL | 0.8 mL |
| Complement | 0.2 mL | 0.2 mL |

Carry out the test on the immunoglobulin to be examined and prepare ACA negative and positive controls using *human immunoglobulin for anticomplementary activity BRP*, as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of gelatin barbital buffer solution are added if the immunoglobulin concentration varies from 50 mg/mL; for example, 0.47 mL of gelatin barbital buffer solution is added to 0.33 mL of immunoglobulin containing 30 mg/mL to give 0.8 mL. Close the tubes and incubate at 37 °C for 60 min. Add 0.2 mL of each incubation mixture to 9.8 mL of gelatin barbital buffer solution to dilute the complement. Perform complement titrations on each tube as described above to determine the remaining complement activity (Table 2.6.17.-2). Calculate the anticomplementary activity of the preparation to be examined relative to the complement control considered as 100 per cent, using the following expression:

$$\frac{a-b}{a} \times 100$$

$a$ = mean complement activity ($CH_{50}$/mL) of complement control;

$b$ = complement activity ($CH_{50}$/mL) of tested sample.

The test is not valid unless:

- the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation;
- the mean complement activity of complement control ($a$) is in the range 80 $CH_{50}$/mL to 120 $CH_{50}$/mL.

**01/2008:20618**

## 2.6.18. TEST FOR NEUROVIRULENCE OF LIVE VIRUS VACCINES

For each test, use not fewer than ten monkeys that are seronegative for the virus to be tested. For each monkey, inject not more than 0.5 mL of the material to be examined into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be not less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a group of not fewer than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 h of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if: more than 20 per cent of the inoculated monkeys die from nonspecific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are euthanised show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

**07/2019:20620**

## 2.6.20. ANTI-A AND ANTI-B HAEMAGGLUTININS

METHOD A: INDIRECT METHOD

Prepare in duplicate serial dilutions of the preparation to be examined in a 9 g/L solution of *sodium chloride R*. To each dilution of 1 series add an equal volume of a 5 per cent *V/V* suspension of group $A_1$ red blood cells previously washed 3 times with the sodium chloride solution. To each dilution of the other series add an equal volume of a 5 per cent *V/V* suspension of group B red blood cells previously washed 3 times with the sodium chloride solution. Incubate the suspensions at 37 °C for 30 min then wash the cells 3 times with the sodium chloride solution. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 min. Without centrifuging, examine each suspension for agglutination under a microscope.

METHOD B: DIRECT METHOD

*MATERIALS*

*Phosphate-buffered saline (PBS)*. Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. If the solution has to be kept for several days, 0.2 g of *sodium azide R* may be added in order to avoid microbial contamination.

*PBS-BSA solution*. PBS containing 2 g/L of *bovine albumin R* (Cohn Fraction V, for ELISA). Store the solution at 2-8 °C but allow it to reach 19-25 °C before use.

*Papain solution*. Use serological-grade papain from a commercial source, the activity of which has been validated.

*Red blood cells*. Use pooled D-negative $A_1$ ($A_1$rr), D-negative B (Brr) and D-negative O (Orr) red blood cells from preferably 3 donors. When *Immunoglobulin for anti-A and anti-B antibodies limit test BRP* is used, 3 donors are to be used. $A_2$ red blood cells are not recommended as they give weaker reactions.

Wash the cells 4 times with PBS or until the supernatant is clear. Each wash consists of suspending the cells in a minimum of 2 volumes of PBS, centrifuging the cells at 1800 *g* for 5 min to pack, and discarding the supernatant. Treat the packed cells with papain solution according to the manufacturer's instructions and wash the cells 4 times with PBS.

Red blood cells may be stored for not more than 1 week in a preservative solution at 2-8 °C. A preparation of the following composition is appropriate:

| | |
|---|---|
| Trisodium citrate | 8 g/L |
| D-glucose | 20 g/L |
| Citric acid | 0.5 g/L |
| Sodium chloride | 4.2 g/L |
| Inosine | 0.938 g/L |
| Adenosine triphosphate (ATP) | 0.4 g/L |
| Chloramphenicol | 0.34 g/L |
| Neomycin sulfate | 0.1 g/L |

If using stored cells, wash the cells at least twice in PBS or until the supernatant is clear before proceeding.

*Microtitre plates*. Use V-bottomed rigid microtitre plates.

*Reference standards*. *Immunoglobulin (anti-A, anti-B antibodies test positive control) BRP* and *Immunoglobulin (anti-A, anti-B antibodies test negative control) BRP* are suitable for use as the positive control and negative control, respectively, and should be used as guides for operators establishing and performing the direct method for anti-A and anti-B haemagglutinins.

*Immunoglobulin for anti-A and anti-B antibodies limit test BRP* defines the recommended maximum limits permissible for batches of human immunoglobulin and must be used only for comparison with batches of human immunoglobulin that have higher titres than the positive control.

*METHOD*

The test described in this chapter is performed at room temperature on the positive control solutions, the negative control solutions and the test solutions at the same time and under identical conditions. Whenever necessary, a further test is performed with *Immunoglobulin for anti-A and anti-B antibodies limit test BRP*.

*Reference solutions*. Reconstitute the positive control and the negative control according to the instructions. The immunoglobulin G (IgG) concentration is 50 g/L in each of the reconstituted preparations. Make a 2-fold dilution of each reconstituted preparation with PBS-BSA solution to obtain solutions containing IgG at 25 g/L. Prepare 7 further serial 2-fold dilutions of each preparation using PBS-BSA solution to extend the dilution range to 1/256 (0.195 g/L IgG). Add 20 µL of each dilution of each preparation in triplicate to the microtitre plate.

*Test solutions*. Dilute the preparation to be examined with PBS-BSA solution to obtain a starting IgG concentration of 25 g/L. For 50 g/L preparations, this is a 2-fold dilution; adjust the dilution factor accordingly for preparations with an IgG concentration other than 50 g/L to obtain a starting concentration of 25 g/L for testing. This 25 g/L solution is assigned a nominal 2-fold dilution factor for comparison with the reference solutions, even if this does not reflect the true dilution factor used to achieve 25 g/L. Prepare 7 further serial 2-fold dilutions of the preparation using PBS-BSA solution to extend the nominal dilution range to 1/256 (0.195 g/L IgG) for comparison with the reference preparations over the same IgG concentration range. Add 20 µL of each dilution in triplicate to the microtitre plate.

For preparations with an IgG concentration lower than 25 g/L, dilute to a starting concentration corresponding to the nearest lower concentration of the reference solutions. This solution is assigned the same nominal dilution factor as the corresponding reference solution having the same IgG concentration. Proceed with the preparation of the appropriate 2-fold dilution series as described above.

Prepare 3 per cent *V/V* suspensions of papain-treated D-negative $A_1$, B and O red blood cells in PBS/BSA solution. Add 20 µL of D-negative $A_1$, B and O red blood cells respectively to the 1st, the 2nd and the 3rd dilution series of each of the preparation to be examined, the positive control and the negative control. Mix by shaking the plate on a shaker for 10 s (or until the cells are resuspended).

Centrifuge the plate at 80 *g* at room temperature for 1 min to pack the cells. Place the plate at an angle of approximately 70°. Read after 4-5 min or when the negative controls (D-negative O red blood cells and negative control solution) have streamed. A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the endpoint titre as the reciprocal of the highest dilution that gives rise to a positive result.

The positive control has nominal anti-A and anti-B titres of 32 (range 32-64 for anti-A; range 16-32 for anti-B) and the negative controls (D-negative O red blood cells and negative control solution) must not show agglutination at the starting dilution of 1 in 2. Users must validate their own test conditions, and investigate their assay conditions and reagents in the event of results being significantly different from those expected. Failure to obtain negative reactions with the negative controls may indicate that, for example, insufficient time has elapsed for the cells to stream, or that reagents have been used directly from cold storage.

If the nominal anti-A or anti-B titre of the preparation to be examined is greater than the titre of the positive control, the test preparation is to be compared with *Immunoglobulin for anti-A and anti-B antibodies limit test BRP*.

The maximum allowable titre is 64.

**07/2014:20621**

# 2.6.21. NUCLEIC ACID AMPLIFICATION TECHNIQUES

1. INTRODUCTION

Nucleic acid amplification techniques are based on 2 different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification;
2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method; in this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, 2 synthetic oligonucleotide primers of opposite polarity anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions that form as a result of specific base pairing between the primers and the complementary DNA sequence border the DNA segment to be amplified, and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:

- heat denaturation of the nucleic acid (target sequence) into 2 single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

Multiplex PCR assays use several primer pairs designed for simultaneous amplification of different targets in one reaction.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

**5.1. Prevention of contamination**

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:

- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.);
- pre-PCR (area where reagents, samples and controls are handled);
- PCR amplification (amplified material is handled in a closed system);
- post-PCR detection (the only area where the amplified material is handled in an open system).

If closed systems are used, the strict segregation of areas is not required.

**5.2. Sample preparation**

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under 7.3.2. must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

**5.3. Amplification**

PCR amplification of the target sequence is conducted under defined cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:

- the length and base composition of primer and target sequences;
- the type of DNA polymerase, buffer composition and reaction volume used for the amplification;
- the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

**5.4. Detection**

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Detection and characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, liquid chromatography). Detection and characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Detection and characterisation by chemical modification may be achieved by, for example, incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent chemiluminescent, radioisotopic or immuno-enzyme-coupled detection.

6. EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result. As soon as a measurable target threshold is defined, a quantitative test system is required.

7. QUALITY ASSURANCE

**7.1. Validation of the PCR assay system**

The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the ICH guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences are used for validation of a PCR test, when available.

*7.1.1. Determination of the positive cut-off point*

During validation of qualitative tests, the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

*7.1.2. Quantitative assay systems*

For a quantitative assay, the following parameters are determined during validation: accuracy, precision, specificity, quantitation limit, linearity, range and robustness.

**7.2. Quality control of reagents**

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, their purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

**7.3. Run controls**

*7.3.1. External controls*

In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR assay:

- positive control: this contains a defined number of target-sequence copies, the number being close to the positive cut-off value, and determined individually for each assay system and indicated as a multiple of the positive cut-off value of the assay system;
- negative control: a sample of a suitable matrix already proven to be free of the target sequences.

*7.3.2. Internal control*

Internal controls are defined nucleic acid sequences containing, unless otherwise prescribed, the primer binding sites. Internal controls must be amplified with defined efficacy, and the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA/RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

*7.3.3. Threshold control*

The threshold control for quantitative assays is a test sample with the analyte at a concentration that is defined as the threshold not to be exceeded. It contains the analyte suitably calibrated in International Units and is analysed in parallel in each run of a quantitative assay.

**7.4. External quality assessment**

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

*The following sections are published for information.*

## Validation of nucleic acid amplification techniques (NAT) for the detection of hepatitis C virus (HCV) RNA in plasma pools: guidelines

1. SCOPE

The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the *Pharmeuropa* Technical Guide for the elaboration of monographs, December 1999, Chapter III 'Validation of analytical procedures'. These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, the 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-contamination).

2. SPECIFICITY

Specificity is the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive. Suitable samples of non-reactive pools are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM).

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes (e.g. genotypes 1 and 3 in Europe) should be detected at a suitable level.

3. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to 2: either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in the general chapter *2.6.21*) is the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus BRP*, which has been calibrated against the WHO HCV International Standard, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using, for example, $\log_{10}$ dilutions of the plasma pool sample) should be carried out in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using, for example, a dilution factor of 0.5 $\log_{10}$ or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

4. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods that use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV-specific antibodies, should be tested and found positive.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least $10^2$ times the 95 per cent cut-off value or at least $10^4$ IU/mL).

5. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions, including precautions taken to prevent cross-contamination or destruction of the viral RNA, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus BRP* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard) can be considered a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

*Technical qualification*. An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. All results should be positive.

*Operator qualification*. An appropriate qualification programme should be implemented for each operator involved in the testing.

## Validation of nucleic acid amplification techniques (NAT) for the quantification of B19 virus (B19V) DNA in plasma pools: guidelines

1. SCOPE

The European Pharmacopoeia requires that plasma pools used for manufacture of certain products are tested for the presence of B19 virus (B19V) DNA with a threshold concentration that must not be exceeded. In order to comply with these requirements, quantitative NAT tests are preferred. The characteristics regarded as the most important for validation of the quantitative NAT procedure are accuracy, precision, specificity, quantitation limit, linearity and range. In addition, the robustness of the analytical procedure should be evaluated.

This guideline describes methods to validate NAT analytical procedures for assessing B19V DNA contamination of plasma pools based on the ICH guidelines. However, this document may also be used as a basis for the validation of quantitative NAT in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. precision, accuracy, range, robustness).

2. ACCURACY

Accuracy expresses the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value and the value found. The accuracy of an assay is dependent on the calibration of the assay and on the variance of the different assay steps. Though it is recommended to establish the accuracy across the specified range of the analytical procedure, the most important assessment of accuracy is in the range of the threshold concentration. In the case of B19V NAT assays for investigation of plasma pools it is recommended to assess the accuracy of the calibrated assay by assaying at least 5 concentrations (dilution factor of 0.5 $\log_{10}$) of *B19 virus DNA for NAT testing BRP* or another material, suitably calibrated in International Units against the actual WHO B19 DNA International Standard, covering the range of the currently recommended threshold concentration of 10.0 IU/µL B19V DNA (e.g. $10^5$ IU/mL, $10^{4.5}$ IU/mL, $10^4$ IU/mL, $10^{3.5}$ IU/mL and $10^3$ IU/mL), with at least 3 replicates for each dilution. Accuracy should be reported for the different concentrations in terms of percentage determined compared with the known amount of B19V DNA. It should reflect the level of technology of the respective assays, which should also be defined, for example in collaborative studies.

3. PRECISION

Precision expresses the closeness of agreement between a series of measurements, obtained from multiple sampling of the same homogenous sample. The precision is defined at 3 levels:

- repeatability expresses the precision under the same operating conditions over a short interval of time (intra-assay precision); it is assessed by using 1 assay and testing 3 replicates of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units and covering the whole quantitative range of the assay; the coefficient of variation for the individual samples is calculated (intra-assay variability);
- intermediate precision expresses the intra-laboratory variations (inter-assay precision); it is established by assaying replicates (as routinely used for the assay) of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units covering the whole quantitative range of the assay under different circumstances (e.g. different days, different analysts, different equipment, different reagents); the coefficient of variation for the individual samples is calculated;
- reproducibility expresses the precision between different laboratories (inter-laboratory precision); it is assessed by participation in quantitative collaborative studies on B19V DNA-NAT assays, e.g. under the Proficiency Testing Scheme (PTS), including the comparative analysis of the obtained quantitative results, where appropriate.

4. SPECIFICITY

Specificity expresses the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present. The specificity of NAT analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only human B19V DNA should be investigated by comparing the chosen sequences with sequences in published data banks. There should be no major homology found with sequences unrelated to B19V.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to examine the specificity of the analytical procedure, at least 20 B19V DNA-negative plasma pools should be tested and shown to be non-reactive.

*Parvovirus B19 genotypes.* The International Committee on Taxonomy of Viruses (ICTV) has classified representatives of the 3 genotypes as strains of human parvovirus B19. Genotype 1 represents prototype B19V, genotype 2 represents viral sequences like A6, and genotype 3 represents V9-like sequences. By performing sequence alignment with respective B19V genotype sequences available from nucleic acid sequence databases, primers and probes should be designed to detect and quantify consistently the different human parvovirus B19 genotypes. Reference materials should be used to check the approach chosen. Since biological reference preparations reflecting some genotypes might be difficult to obtain, respective plasmid preparations or synthetic nucleic acids may also serve as a characterised target sequence source. However, those cannot be used to validate the extraction procedure.

5. QUANTITATION LIMIT

The quantitation limit is the lowest amount of nucleic acid in a sample that can be determined quantitatively with suitable precision and accuracy. The quantitation limit of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by limiting dilution analysis. The lowest concentration of target nucleic acids that is quantitated with suitable precision and accuracy is defined.

6. LINEARITY

The linearity of an assay is its ability to obtain test results that are directly proportional to the concentration of the nucleic acid. The linearity of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples with the concentrations covering the whole quantitative range. The interval between the upper and the lower concentration of the target nucleic acid where test results are directly proportional to the concentrations is defined.

7. RANGE

The range of an assay is the interval between the upper and the lower concentration of nucleic acid in the sample for which it has been demonstrated that the procedure has a suitable level of precision, accuracy and linearity. The range of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples. The interval between the upper and the lower concentration that can be expressed with an acceptable degree of accuracy and precision is defined.

8. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. Nonetheless, the robustness of NAT can be demonstrated during the development of the method when small variations in the concentrations of reagents, for example $MgCl_2$, primers or dNTP, are tested. To demonstrate robustness, at least 20 B19V DNA-negative plasma pool samples spiked with B19V DNA at the threshold concentration should be tested and found to have acceptable quantitative values.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of plasma pools without B19V DNA or with levels below the threshold concentration

(10 samples) and plasma pools spiked with high concentrations of B19V DNA (at least $10^2$ times the threshold level, 10 samples).

9. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that may influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools by manufacturers (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions including precautions taken to prevent cross-contamination or destruction of the viral nucleic acids, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The inclusion of an appropriate threshold control (for example, plasma spiked with a B19V DNA sample suitably calibrated in International Units, such as *B19 virus DNA for NAT testing BRP*) is considered to be a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

*Technical qualification.* An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used. For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with a concentration of B19V DNA around the threshold concentration. All results should be acceptable and reflect the features of the assay as determined during the validation phase.

*Operator qualification.* An appropriate qualification programme should be implemented for each operator involved in the testing.

**01/2008:20622**

## 2.6.22. ACTIVATED COAGULATION FACTORS

Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R*. Place a series of polystyrene tubes in a water-bath at 37 °C and add to each tube 0.1 mL of *platelet-poor plasma R* and 0.1 mL of a suitable dilution of a phospholipid preparation to act as a platelet substitute. Allow to stand for 60 s. Add to each tube either 0.1 mL of 1 of the dilutions or 0.1 mL of the buffer solution (control tube). To each tube add immediately 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C, and measure, within 30 min of preparing the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 s to 350 s.

**04/2014:20624**

## 2.6.24. AVIAN VIRAL VACCINES: TESTS FOR EXTRANEOUS AGENTS IN SEED LOTS

GENERAL PROVISIONS

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (*5.2.2*).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of general chapter *5.2.4. Cell cultures for the production of veterinary vaccines*, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of test substance applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) For a freeze-dried preparation, reconstitute using a suitable liquid. Unless otherwise stated or justified, the test substance must contain a quantity of virus equivalent to at least 10 doses of vaccine in 0.1 mL of inoculum.

e) If the virus of the seed lot would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

f) Monospecific antiserum and serum of avian origin used for cell culture or any other purpose, in any of these tests, shall be free from antibodies against and free from inhibitory effects on the organisms listed hereafter under 7. Antibody specifications for sera used in extraneous agents testing.

g) Where specified in a monograph or otherwise justified, if neutralisation of the virus of the seed lot is required but difficult to achieve, the tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent.

h) Other types of tests than those indicated may be used, provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (*2.6.21*) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. TEST FOR EXTRANEOUS AGENTS USING EMBRYONATED HENS' EGGS

Use a test substance, diluted if necessary, containing a quantity of neutralised virus equivalent to at least 10 doses of vaccine in 0.2 mL of inoculum. Suitable antibiotics may be added. Inoculate the test substance into 3 groups of 10 embryonated hens' eggs as follows:

- group 1: 0.2 mL into the allantoic cavity of each 9- to 11-day-old embryonated egg;
- group 2: 0.2 mL onto the chorio-allantoic membrane of each 9- to 11-day-old embryonated egg;
- group 3: 0.2 mL into the yolk sac of each 5- to 6-day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 daily for 12 days. Discard embryos that die during the first 24 h as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 h after inoculation. Examine macroscopically for abnormalities all embryos that die more than 24 h after inoculation, or that survive the incubation period. Examine

also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos. Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The seed lot complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the seed lot and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of any extraneous agent.

2. TEST IN CHICKEN KIDNEY CELLS

Prepare 7 monolayers of chicken kidney cells, each monolayer having an area of about 25 $cm^2$. Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test substance, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of the test substance onto each of the 5 monolayers. Allow adsorption for 1 h, add culture medium and incubate the cultures for a total of at least 21 days, subculturing at 4- to 7-day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle. Inoculate 0.1 mL of pooled material onto each of 5 recently prepared monolayers of about 25 $cm^2$ each, at each passage. For the last passage, grow the cells also on a suitable substrate so as to obtain an area of about 10 $cm^2$ of cells from each of the monolayers for test A. The test is not valid if less than 80 per cent of the monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test substance. At the end of the total incubation period, carry out the following procedures.

A. Fix and stain (with Giemsa or haematoxylin and eosin) about 10 $cm^2$ of confluent cells from each of the 5 monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or other evidence of the presence of contaminating agents from the test substance.

B. Drain and wash about 25 $cm^2$ of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken erythrocytes (using at least 1 mL of suspension for each 5 $cm^2$ of cells). Incubate the cells at 4 °C for 20 min and then wash gently in phosphate buffered saline pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test substance.

C. Test individual samples of the fluids from each cell culture using chicken erythrocytes for haemagglutination attributable to the presence of a haemagglutinating agent in the test substance.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

3. TEST FOR AVIAN LEUCOSIS VIRUSES

Prepare at least 13 replicate monolayers of either DF-1 cells or primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos that are known to be genetically susceptible to subgroups A, B and J of avian leucosis viruses and that support the growth of exogenous but not endogenous avian leucosis viruses (cells from C/E strain chickens are suitable). Each replicate shall have an area of about 50 $cm^2$.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of the test substance onto each of 5 of the replicate monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 2 of the replicate monolayers with subgroup A avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 mL), 2 with subgroup B avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 mL) and 2 with subgroup J avian leucosis virus (not more than 10 $CCID_{50}$ in 0.1 mL) as positive controls. Maintain not fewer than 2 non-inoculated replicate monolayers as negative controls.

Incubate the cells for a total of at least 9 days, subculturing at 3- to 4-day intervals. Retain cells from each passage level and harvest the cells at the end of the total incubation period. Wash cells from each passage level from each replicate and resuspend the cells at $10^7$ cells per millilitre in barbital-buffered saline for subsequent testing by a Complement Fixation for Avian Leucosis (COFAL) test or in phosphate buffered saline for testing by Enzyme-Linked Immunosorbent Assay (ELISA). Then, carry out 3 cycles of freezing and thawing to release any group-specific antigen and perform a COFAL test or an ELISA test on each extract to detect group-specific avian leucosis antigen if present.

The test is not valid if group-specific antigen is detected in fewer than 5 of the 6 positive control replicate monolayers or if a positive result is obtained in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test replicate monolayers are inconclusive, then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained. If a positive result is obtained for any of the test monolayers, then the presence of avian leucosis virus in the test substance has been detected.

The seed lot complies with the test if there is no evidence of the presence of any avian leucosis virus.

4. TEST FOR AVIAN RETICULOENDOTHELIOSIS VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day old chick embryos or duck embryo fibroblasts from the tissues of 13- to 14-day-old embryos, each monolayer having an area of about 25 $cm^2$.

Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of the test substance onto each of 5 of the monolayers. Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with avian reticuloendotheliosis virus as positive controls (not more than 10 $CCID_{50}$ in 0.1 mL). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cells for a total of at least 10 days, subculturing twice at 3- to 4-day intervals. The test is not valid if fewer than 3 of the 4 positive controls or fewer than 4 of the 5 test monolayers or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the fibroblasts on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent fibroblasts from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent fibroblasts derived from each of the original 11 monolayers by immunostaining for the presence of avian reticuloendotheliosis virus. The test is not valid if avian reticuloendotheliosis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the fibroblast monolayers shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of avian reticuloendotheliosis virus.

5. TEST FOR CHICKEN ANAEMIA VIRUS

Prepare eleven 20 mL suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 $cm^2$ cell culture flasks containing about $5 \times 10^5$ cells/mL. Inoculate 0.1 mL of the test substance into each of 5 flasks. Inoculate 4 of the suspensions with 10 $CCID_{50}$ chicken anaemia virus as positive controls. Maintain not fewer than 2 non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3- to 4-day intervals. During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids becoming red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed and resuspend at about $10^6$ cells/mL and place 25 µL in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in fewer than 3 of the 4 positive controls or in any of the non-inoculated controls. If the results for more than 1 of the test suspensions are inconclusive, then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The seed lot complies with the test if there is no evidence of the presence of chicken anaemia virus.

6. TEST FOR EXTRANEOUS AGENTS USING CHICKS

Inoculate each of at least 10 chicks with the equivalent of 100 doses of vaccine by the intramuscular route and with the equivalent of 10 doses by eye-drop. Chicks that are 2 weeks of age are used in the test except that if the seed virus is pathogenic for birds of this age, older birds may be used, if required and justified. In exceptional cases, for inactivated vaccines, the virus may be neutralised by specific antiserum if the seed virus is pathogenic for birds at the age of administration. Repeat these inoculations 2 weeks later. Observe the chicks for a period of 5 weeks from the day of the first inoculation. No antimicrobial agents shall be administered to the chicks during the test period. The test is not valid if fewer than 80 per cent of the chicks survive to the end of the test period.

Collect serum from each chick at the end of the test period. Test each serum sample for antibodies against each of the agents listed below (with the exception of the virus type of the seed lot) using one of the methods indicated for testing for the agent.

Clinical signs of disease in the chicks during the test period (other than signs attributable to the virus of the seed lot) and the detection of antibodies in the chicks after inoculation (with the exception of antibodies to the virus of the seed lot), are classed as evidence of the presence of an extraneous agent in the seed lot.

It is recommended that sera from these birds is retained so that additional testing may be carried out if requirements change.

**A. Standard tests**

| Agent | Type of test |
|---|---|
| Avian adenoviruses, group 1 | SN, EIA, AGP |
| Avian encephalomyelitis virus | AGP, EIA |
| Avian infectious bronchitis virus | EIA, HI |
| Avian infectious laryngotracheitis virus | SN, EIA, IS |
| Avian leucosis viruses | SN, EIA |
| Avian nephritis virus | IS |
| Avian orthoreoviruses | IS, EIA |
| Avian reticuloendotheliosis virus | AGP, IS, EIA |
| Chicken anaemia virus | IS, EIA, SN |
| Egg drop syndrome virus | HI, EIA |
| Avian infectious bursal disease virus | Serotype 1: AGP, EIA, SN<br>Serotype 2: SN |
| Influenza A virus | AGP, EIA, HI |
| Marek's disease virus | AGP |
| Newcastle disease virus | HI, EIA |
| Turkey rhinotracheitis virus | EIA |
| *Salmonella pullorum* | Agg |

Agg: agglutination
AGP: agar gel precipitation
EIA: enzyme immunoassay (e.g. ELISA)
HI: haemagglutination inhibition
IS: immunostaining (e.g. fluorescent antibody)
SN: serum neutralisation

**B. Additional tests for turkey extraneous agents**

If the seed virus is of turkey origin or was propagated in turkey substrates, tests for antibodies against the following agents are also carried out.

| Agent | Type of test |
|---|---|
| *Chlamydia* spp. | EIA |
| Avian infectious haemorrhagic enteritis virus | AGP |
| Avian paramyxovirus 3 | HI |
| Avian infectious bursal disease virus type 2 | SN |

A test for freedom from turkey lympho-proliferative disease virus is carried out by intraperitoneal inoculation of twenty 4-week-old turkey poults. Observe the poults for 40 days. The test is not valid if more than 20 per cent of the poults die from non-specific causes. The seed lot complies with the test if sections of spleen and thymus taken from 10 poults 2 weeks after inoculation show no macroscopic or microscopic lesions (other than those attributable to the seed lot virus) and no poult dies from causes attributable to the seed lot.

**C. Additional tests for duck extraneous agents**

If the seed virus is of duck origin or was propagated in duck substrates, tests for antibodies against the following agents are also carried out.

| Agent | Type of test |
|---|---|
| *Chlamydia* spp. | EIA |
| Duck and goose parvoviruses | SN, EIA |
| Duck enteritis virus | SN |
| Duck hepatitis virus type I | SN |

The seed lot complies with the test if there is no evidence of the presence of any extraneous agent.

**D. Additional tests for goose extraneous agents**

If the seed virus is of goose origin or was prepared in goose substrates, tests for the following agents are also carried out.

| Agent | Type of test |
|---|---|
| Duck and goose parvovirus | SN, EIA |
| Duck enteritis virus | SN |
| Goose haemorrhagic polyomavirus | test in goslings shown below or another suitable test |

Inoculate subcutaneously the equivalent of at least 10 doses to each of ten 1-day-old susceptible goslings. Observe the goslings for 28 days. The test is not valid if more than

20 per cent of the goslings die from non-specific causes. The seed virus complies with the test if no gosling dies from causes attributable to the seed lot.

7. ANTIBODY SPECIFICATIONS FOR SERA USED IN EXTRANEOUS AGENTS TESTING

All batches of serum to be used in extraneous agents testing, either to neutralise the vaccine virus (seed lot or batch of finished product) or as a supplement for cell culture media, shall be shown by suitably sensitive tests to be free from antibodies against and free from inhibitory effects on the following micro-organisms - except for one type, namely, antibodies against the virus that they are supposed to neutralise.

Avian adenoviruses

Avian encephalomyelitis virus

Avian infectious bronchitis viruses

Avian infectious bursal disease virus types 1 and 2

Avian infectious haemorrhagic enteritis virus

Avian infectious laryngotracheitis virus

Avian leucosis viruses

Avian nephritis virus

Avian paramyxoviruses 1 to 9

Avian orthoreoviruses

Avian reticuloendotheliosis virus

Chicken anaemia virus

Duck enteritis virus

Duck hepatitis virus type I

Egg drop syndrome virus

Fowl pox virus

Influenza viruses

Marek's disease virus

Turkey herpesvirus

Turkey rhinotracheitis virus

Non-immune serum for addition to culture media can be assumed to be free from antibodies against any of these viruses if the agent is known not to infect the species of origin of the serum and it is not necessary to test the serum for such antibodies. Monospecific antisera for virus neutralisation can be assumed to be free from the antibodies against any of these viruses if it can be shown that the immunising antigen could not have been contaminated with antigens derived from that virus and if the virus is known not to infect the species of origin of the serum; it is not necessary to test the serum for such antibodies. It is not necessary to retest sera obtained from birds from SPF chicken flocks (*5.2.2*).

Batches of sera prepared for neutralising the vaccine virus must not be prepared from any passage level derived from the virus isolate used to prepare the master seed lot or from an isolate cultured in the same cell line.

**04/2015:20625**

## 2.6.25. AVIAN LIVE VIRUS VACCINES: TESTS FOR EXTRANEOUS AGENTS IN BATCHES OF FINISHED PRODUCT

GENERAL PROVISIONS

a) In the following tests, chickens and/or chicken material such as eggs and cell cultures shall be derived from chicken flocks free from specified pathogens (SPF) (*5.2.2*).

b) Cell cultures for the testing of extraneous agents comply with the requirements for the master cell seed of general chapter *5.2.4. Cell cultures for the production of veterinary vaccines*, with the exception of the karyotype test and the tumorigenicity test, which do not have to be carried out.

c) In tests using cell cultures, precise specifications are given for the number of replicates, monolayer surface areas and minimum survival rate of the cultures. Alternative numbers of replicates and cell surface areas are possible as well, provided that a minimum of 2 replicates are used, the total surface area and the total volume of vaccine test applied are not less than that prescribed here and the survival rate requirements are adapted accordingly.

d) In these tests, use the liquid vaccine or reconstitute a quantity of the freeze-dried preparation to be tested with the liquid stated on the label or another suitable diluent such as water for injections. Unless otherwise stated or justified, the test substance contains the equivalent of 10 doses in 0.1 mL of inoculum.

e) If the vaccine virus would interfere with the conduct and sensitivity of the test, neutralise the virus in the preparation with a monospecific antiserum.

f) Monospecific antiserum and serum of avian origin used for cell culture and any other purpose, in any of these tests, shall be free of antibodies against and free from inhibitory effects on the organisms listed under 7. Antibody specifications for sera used in extraneous agents testing (*2.6.24*).

g) Where specified in a monograph or otherwise justified, if neutralisation of the vaccine virus is required but difficult to achieve, the tests described below are adapted, as required, to provide the necessary guarantees of freedom from contamination with an extraneous agent.

If the vaccine virus cannot be completely neutralised – using monospecific antiserum – for the yolk sac inoculation test, the following tests may be performed:

- for enveloped viruses such as Newcastle disease virus: perform the test for extraneous agents using embryonated hens' eggs by the yolk sac route with prior inactivation of enveloped vaccine viruses with lipid solvents,
- for avian encephalomyelitis virus and avian nephritis viruses: perform appropriate tests to detect these viruses. For avian nephritis virus, the test in chicken kidney cells described in section 2 of general chapter *2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots* may be used.

If the vaccine virus cannot be completely neutralised – using monospecific antiserum – for the other tests below, alternatively or in addition to *in vitro* tests conducted on the batch, a test for extraneous agents may be conducted on chick sera obtained from testing the batch of vaccine, as described under 6. Test for extraneous agents using chicks in general chapter *2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots.*

h) Other types of tests than those indicated may be used, provided they are at least as sensitive as those indicated and of appropriate specificity. Nucleic acid amplification techniques (*2.6.21*) give specific detection for many agents and can be used after validation for sensitivity and specificity.

1. TEST FOR EXTRANEOUS AGENTS USING EMBRYONATED HENS' EGGS

Prepare the test vaccine, diluted if necessary, to contain neutralised virus equivalent to 10 doses of vaccine in 0.2 mL of inoculum. Suitable antibiotics may be added. Inoculate the test vaccine into 3 groups of 10 embryonated hens' eggs as follows:

- group 1: 0.2 mL into the allantoic cavity of each 9- to 11-day-old embryonated egg,

– group 2: 0.2 mL onto the chorio-allantoic membrane of each 9- to 11-day-old embryonated egg,
– group 3: 0.2 mL into the yolk sac of each 5- to 6-day-old embryonated egg.

Candle the eggs in groups 1 and 2 daily for 7 days and the eggs in group 3 for 12 days. Discard embryos that die during the first 24 h as non-specific deaths; the test is not valid unless at least 6 embryos in each group survive beyond the first 24 h after inoculation. Examine macroscopically for abnormalities all embryos which die more than 24 h after inoculation, or which survive the incubation period. Examine also the chorio-allantoic membranes of these eggs for any abnormality and test the allantoic fluids for the presence of haemagglutinating agents.

Carry out a further embryo passage. Pool separately material from live and from the dead and abnormal embryos. Inoculate each pool into 10 eggs for each route as described above, chorio-allantoic membrane material being inoculated onto chorio-allantoic membranes, allantoic fluids into the allantoic cavity and embryo material into the yolk sac. For eggs inoculated by the allantoic and chorio-allantoic routes, candle the eggs daily for 7 days, proceeding and examining the material as described above. For eggs inoculated by the yolk sac route, candle the eggs daily for 12 days, proceeding and examining the material as described above.

The batch of vaccine complies with the test if no test embryo shows macroscopic abnormalities or dies from causes attributable to the vaccine and if examination of the chorio-allantoic membranes and testing of the allantoic fluids show no evidence of the presence of extraneous agents.

As previously mentioned under General provisions (paragraph g), if neutralisation of the virus is not possible and as a consequence the yolk sac inoculation cannot be evaluated, suitable tests other than those indicated may be carried out to provide the necessary guarantees of freedom from contamination with an extraneous agent; in particular other tests to detect avian encephalomyelitis and avian nephritis viruses may be carried out. In this case, justification to use other tests must be provided, and the vaccine complies if there is no evidence of the presence of avian encephalomyelitis virus or avian nephritis virus.

2. TEST IN CHICKEN EMBRYO FIBROBLAST CELLS

Prepare 7 monolayers of primary or secondary chicken embryo fibroblasts, from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 $cm^2$. Maintain 2 monolayers as negative controls and treat these in the same way as the 5 monolayers inoculated with the test vaccine, as described below. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers. Allow adsorption for 1 h and add culture medium. Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made with pooled cells and fluids from all 5 monolayers after carrying out a freeze-thaw cycle. Inoculate 0.1 mL of pooled material onto each of 5 recently prepared monolayers of chicken embryo fibroblast cells, each monolayer having an area of about 25 $cm^2$ each as before. For the last passage, grow the cells also on a suitable substrate so as to obtain an area of about 10 $cm^2$ of cells from each of the monolayers, for test A. The test is not valid if less than 80 per cent of the test monolayers, or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of contaminating agents in the test vaccine. At the end of the total incubation period, carry out the following procedures.

A. Fix and stain (with Giemsa or haematoxylin and eosin) about 10 $cm^2$ of confluent cells from each of the 5 original monolayers. Examine the cells microscopically for any cytopathic effect, inclusion bodies, syncytial formation, or any other evidence of the presence of a contaminating agent from the test vaccine.
B. Drain and wash about 25 $cm^2$ of cells from each of the 5 monolayers. Cover these cells with a 0.5 per cent suspension of washed chicken red blood cells (using at least 1 mL of suspension for each 5 $cm^2$ of cells). Incubate the cells at 4 °C for 20 min and then wash gently in phosphate buffered saline pH 7.4. Examine the cells microscopically for haemadsorption attributable to the presence of a haemadsorbing agent in the test vaccine.
C. Test individually samples of the fluid from each cell culture using chicken red blood cells for haemagglutination attributable to the presence of a haemagglutinating agent in the test vaccine.

The test is not valid if there are any signs of extraneous agents in the negative control cultures. The batch of vaccine complies with the test if there is no evidence of the presence of any extraneous agent.

3. TEST FOR EGG DROP SYNDROME VIRUS

Prepare 11 monolayers of chicken embryo liver cells, from the tissues of 14- to 16-day-old embryos, each monolayer having an area of about 25 $cm^2$. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, add culture medium. Inoculate 4 of the monolayers with a suitable strain of egg drop syndrome virus (not more than 10 $CCID_{50}$ in 0.1 mL) to serve as positive control monolayers. Maintain 2 non-inoculated monolayers as negative control monolayers.

Incubate the cells for a total of at least 21 days, subculturing every 4-5 days. Each passage is made as follows: carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; inoculate 0.1 mL of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo liver cells, each monolayer having an area of about 25 $cm^2$ as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures at frequent intervals throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine. At the end of the total incubation period, carry out the following procedure: test separately, cell culture fluid from the test monolayers, positive control monolayers and negative control monolayers, using chicken red blood cells, for haemagglutination attributable to the presence of haemagglutinating agents.

The test is not valid if egg drop syndrome virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of egg drop syndrome virus or any other extraneous agent.

4. TEST FOR MAREK'S DISEASE VIRUS

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 $cm^2$. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain

of Marek's disease virus (not more than 10 $CCID_{50}$ in 0.1 mL) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells, prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix an appropriate quantity of each with a suspension of freshly prepared primary or secondary chick embryo fibroblasts and prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

Examine microscopically all the cell cultures frequently throughout the entire incubation period for any signs of cytopathic effect or other evidence of the presence of a contaminating agent in the test vaccine.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of Marek's disease virus. The test is not valid if Marek's disease virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of Marek's disease virus or any other extraneous agent.

5. TESTS FOR TURKEY RHINOTRACHEITIS VIRUS

A. In chicken embryo fibroblasts

*NOTE: this test can be combined with Test 2 by using the same test monolayers and negative controls, for all stages up to the final specific test for turkey rhinotracheitis virus on cells prepared from the last subculture.*

Prepare 11 monolayers of primary or secondary chick embryo fibroblasts from the tissues of 9- to 11-day-old embryos, each monolayer having an area of about 25 $cm^2$. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus as positive controls (not more than 10 $CCID_{50}$ in 0.1 mL). Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle; prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers; inoculate 0.1 mL of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of chicken embryo fibroblasts cells, each monolayer having an area of about 25 $cm^2$ as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative control monolayers survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for both of the 2 test monolayers are inconclusive then further subcultures of reserved portions of the fibroblasts shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

B. In Vero cells

Prepare 11 monolayers of Vero cells, each monolayer having an area of about 25 $cm^2$. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h, and add culture medium. Inoculate 4 of the monolayers with a suitable strain of turkey rhinotracheitis virus (not more than 10 $CCID_{50}$ in 0.1 mL) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.1 mL of the pooled material onto each of 5, 4 and 2 recently prepared monolayers of Vero cells, each monolayer having an area of about 25 $cm^2$ as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of turkey rhinotracheitis virus. The test is not valid if turkey rhinotracheitis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of turkey rhinotracheitis virus or any other extraneous agent.

6. TEST FOR CHICKEN ANAEMIA VIRUS

Prepare eleven 20 mL suspensions of the MDCC-MSBI cell line or another cell line of equivalent sensitivity in 25 $cm^2$ flasks containing about $5 \times 10^5$ cells/mL. Inoculate 0.1 mL of test vaccine into each of 5 of these flasks. Inoculate 4 other suspensions with 10 $CCID_{50}$ chicken anaemia virus as positive controls. Maintain not fewer than 2 non-inoculated suspensions. Maintain all the cell cultures for a total of at least 24 days, subculturing 8 times at 3- to 4-day intervals. During the subculturing the presence of chicken anaemia virus may be indicated by a metabolic colour change in the infected cultures, the culture fluids becoming red in comparison with the control cultures. Examine the cells microscopically for cytopathic effect. At this time or at the end of the incubation period, centrifuge the cells from each flask at low speed, resuspend at about $10^6$ cells per millilitre and place 25 µL in each of 10 wells of a multi-well slide. Examine the cells by immunostaining.

The test is not valid if chicken anaemia virus is detected in fewer than 3 of the 4 positive controls or in any of the non-inoculated controls. If the results for more than 1 of the test suspensions are inconclusive then further subcultures of reserved portions of the test suspensions shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of chicken anaemia virus.

7. TEST FOR DUCK ENTERITIS VIRUS

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare 11 monolayers of primary or secondary Muscovy duck embryo liver cells, from the tissues of 21- or 22-day-old embryos, each monolayer having an area of about 25 $cm^2$. Remove the culture medium when the cells reach confluence. Inoculate 0.1 mL of test vaccine onto each of 5 of the monolayers (test monolayers). Allow adsorption for 1 h and add culture medium. Inoculate 4 of the monolayers with a suitable strain of duck enteritis virus (not more than 10 $CCID_{50}$ in 0.1 mL) to serve as positive controls. Maintain 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: trypsinise the cells and prepare separate pools of the cells from the test monolayers, from the positive control monolayers and from the negative control monolayers. Mix a portion of each with a suspension of freshly prepared primary or secondary Muscovy duck embryo liver cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck enteritis virus. The test is not valid if duck enteritis virus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive. If the results for more than 1 of the test monolayers are inconclusive then further subcultures of reserved portions of the monolayers shall be made and tested until an unequivocal result is obtained.

The batch of vaccine complies with the test if there is no evidence of the presence of duck enteritis virus or any other extraneous agent.

8. TEST FOR DUCK AND GOOSE PARVOVIRUSES

This test is carried out for vaccines prepared on duck or goose substrates.

Prepare a suspension of sufficient primary or secondary Muscovy duck embryo fibroblasts from the tissues of 16- to 18-day-old embryos, to obtain not fewer than 11 monolayers, each having an area of about 25 $cm^2$. Inoculate 0.5 mL of test vaccine into an aliquot of cells for 5 monolayers and seed into 5 replicate containers to form 5 test monolayers. Inoculate 0.4 mL of a suitable strain of duck parvovirus (not more than 10 $CCID_{50}$ in 0.1 mL) into an aliquot of cells for 4 monolayers and seed into 4 replicate containers to form 4 positive control monolayers. Prepare 2 non-inoculated monolayers as negative controls.

Incubate the cultures for a total of at least 21 days, subculturing at 4- to 5-day intervals. Each passage is made as follows: carry out a freeze-thaw cycle. Prepare separate pools of the cells plus fluid from the test monolayers, from the positive control monolayers and from the negative control monolayers. Inoculate 0.5 mL, 0.4 mL and 0.2 mL of the pooled materials into aliquots of a fresh suspension of sufficient primary or secondary Muscovy duck embryo fibroblast cells to prepare 5, 4 and 2 monolayers, as before. The test is not valid if fewer than 4 of the 5 test monolayers or fewer than 3 of the 4 positive controls or neither of the 2 negative controls survive after any passage.

For the last subculture, grow the cells on a suitable substrate so as to obtain an area of about 10 $cm^2$ of confluent cells from each of the original 11 monolayers for the subsequent test: test about 10 $cm^2$ of confluent cells derived from each of the original 11 monolayers by immunostaining for the presence of duck or goose parvovirus. The test is not valid if duck parvovirus is detected in fewer than 3 of the 4 positive control monolayers or in any of the negative control monolayers, or if the results for both of the 2 negative control monolayers are inconclusive.

The batch of vaccine complies with the test if there is no evidence of the presence of duck (or goose) parvovirus or any other extraneous agent.

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# 2.6.26. TEST FOR ANTI-D ANTIBODIES IN HUMAN IMMUNOGLOBULIN

MATERIALS

*Phosphate-buffered saline (PBS).* Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. If the solution has to be kept for several days, 0.2 g of *sodium azide R* may be added in order to avoid microbial contamination.

*PBS-BSA solution.* PBS containing 2 g/L of *bovine albumin R* (Cohn Fraction V, for ELISA). Store the solution at 2-8 °C but allow it to reach 19-25 °C before use.

*Papain solution.* Use serological-grade papain from a commercial source, the activity of which has been validated.

*Red blood cells.* Use pooled D-positive red blood cells from not fewer than 3 donors, preferably of group $OR_2R_2$. D-positive red blood cells may also be obtained from $OR_1R_1$ or $OR_1R_2$ donors. Mixing phenotypes has not been tested and is therefore not recommended.

Use pooled D-negative red blood cells, preferably from 3 donors of group Orr. When only 1 donor of group Orr is available, D-negative red blood cells from only 1 donor may be used.

Wash the cells 4 times with PBS or until the supernatant is clear. Each wash consists of suspending the cells in a minimum of 2 volumes of PBS, centrifuging the cells at 1800 *g* for 5 min to pack, and discarding the supernatant. Treat the packed cells with papain solution according to the manufacturer's instructions and wash the cells 4 times with PBS.

Red blood cells may be stored for not more than 1 week in a preservative solution at 2-8 °C. A preparation of the following composition is appropriate:

| | |
|---|---|
| Trisodium citrate | 8 g/L |
| D-glucose | 20 g/L |
| Citric acid | 0.5 g/L |
| Sodium chloride | 4.2 g/L |
| Inosine | 0.938 g/L |
| Adenosine triphosphate (ATP) | 0.4 g/L |
| Chloramphenicol | 0.34 g/L |
| Neomycin sulfate | 0.1 g/L |

If using stored cells, wash the cells at least twice in PBS or until the supernatant is clear before proceeding.

*Microtitre plates.* Use V-bottomed rigid microtitre plates.

*Reference standards. Immunoglobulin (anti-D antibodies test) BRP* and *Immunoglobulin (anti-D antibodies test negative control) BRP* are suitable for use as the positive control and negative control, respectively.

METHOD

The test described in this chapter is performed at room temperature on the positive control solutions, the negative control solutions and the test solutions at the same time and under identical conditions.

*Reference solutions.* Reconstitute the positive control and the negative control according to the instructions. The immunoglobulin G (IgG) concentration is 50 g/L in each of the reconstituted preparations. Make a 2-fold dilution of each reconstituted preparation with PBS-BSA solution to obtain solutions containing IgG at 25 g/L. Prepare 7 further serial 2-fold dilutions of each preparation using PBS-BSA solution to extend the dilution range to 1/256 (0.195 g/L IgG). Add 20 µL of each dilution of each preparation in duplicate to the microtitre plate.

*Test solutions.* Dilute the preparation to be examined with PBS-BSA solution to obtain a starting IgG concentration of 25 g/L. For 50 g/L preparations, this is a 2-fold dilution; adjust the dilution factor accordingly for preparations with an IgG concentration other than 50 g/L to obtain a starting concentration of 25 g/L for testing. This 25 g/L solution is assigned a nominal 2-fold dilution factor for comparison with the reference solutions, even if this does not reflect the true dilution factor used to achieve 25 g/L. Prepare 7 further serial 2-fold dilutions of the preparation using PBS-BSA solution to extend the nominal dilution range to 1/256 (0.195 g/L IgG) for comparison with the reference preparations over the same IgG concentration range. Add 20 µL of each dilution in duplicate to the microtitre plate.

Prepare 3 per cent *V/V* suspensions of papain-treated D-positive (preferably $OR_2R_2$, but $OR_1R_1$ or $OR_1R_2$ may also be used) and D-negative (Orr) red blood cells in PBS-BSA solution. Add 20 µL of D-positive red blood cells to 1 dilution series of each of the preparation to be examined, the positive control and the negative control, and 20 µL of D-negative red blood cells to the other dilution series. Mix by shaking the plate on a shaker for 10 s (or until the cells are resuspended).

Centrifuge the plate at 80 *g* at room temperature for 1 min to pack the cells. Place the plate at an angle of approximately 70°. Read after 4-5 min or when the negative controls (D-negative red blood cells and negative control solution) have streamed. A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the endpoint titre as the reciprocal of the highest dilution that gives rise to a positive result.

The positive control has a nominal titre of 8 and the negative controls (D-negative red blood cells and negative control solution) must not show agglutination at the starting dilution of 1 in 2. Users must validate their own test conditions, and investigate their assay conditions and reagents in the event of results being significantly different from those expected. Failure to obtain negative reactions with the negative controls may indicate that, for example, insufficient time has elapsed for the cells to stream, or that reagents have been used directly from cold storage.

The titre of the preparation to be examined must not be greater than the titre of the positive control when both preparations are titrated from 25 g/L.

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# 2.6.27. MICROBIOLOGICAL EXAMINATION OF CELL-BASED PREPARATIONS

*This chapter does not concern the examination of human blood or blood components, which is covered by Directive 2002/98/EC of the European Parliament and of the Council of 27 January 2003 and Commission Directive 2004/33/EC of 22 March 2004 implementing Directive 2002/98/EC.*

## 1. INTRODUCTION

The approaches to microbiological examination of cell-based preparations outlined in this general chapter take into account the characteristics and limitations of these preparations, in particular their shelf-life, which may not always allow for completion of conventional microbiological examination tests before administration to the patient, as well as the amounts available for testing and sampling-related issues. These approaches may be applied when the test for sterility, described in general chapter *2.6.1. Sterility*, is required but cannot be performed for technical reasons or due to the characteristics of the specific cell-based preparation.

### 1-1. *SHELF-LIFE*

The shelf-life of cell-based preparations is dependent on the cell characteristics and on the preservation conditions. For non-cryopreserved cell-based preparations, the shelf-life usually does not exceed 3-4 days and sometimes not more than a few hours. In such cases the microbiological status of the final preparation cannot be determined, according to general chapter *2.6.1*, before the time of administration.

### 1-2. *SAMPLE COMPOSITION*

Microbial contaminants may be found either inside or on the surface of cells or other components of the cell-based preparation and may not be detected if only supernatants, such as culture or transport media, are analysed. The sample tested must be representative of all of the components of the cell-based preparation, unless otherwise justified.

### 1-3. *SAMPLE SIZE*

Due to constraints surrounding the use of a single donor or manufacturing-related capacities, the sample volume available for testing at the end of the production process may be limited. Nevertheless, with regard to the sampling error, which may lead to microbial contamination not being detected, the sample size must be sufficient to ensure suitable sensitivity and specificity of the chosen test method.

### 1-4. *RATIONALE FOR METHOD SELECTION*

Method selection must be based on the characteristics of the final preparation and the manufacturing process. To ensure safety for the intended use, the choice of method or combination of methods could be supported by a risk analysis of the potential exposure to microbiological contaminants, and the characteristics and intended use of the cell-based preparation. The media and incubation times used in these methods must be chosen taking into account the properties of the source material and the conditions during the manufacturing process that may support growth of specific micro-organisms (e.g. psychrophilic, thermophilic or fastidious bacteria or fungi). The composition of the cell-based preparation may impede certain test methods for physical reasons such as initial turbidity of the culture media after addition of the test sample.

The following approaches to microbiological examination may be applied:

– automated growth-based methods;

– a combination of preculturing and detection by alternative methods (*5.1.6*);
– direct detection by alternative methods (*5.1.6*);
– methods based on the sterility test prescribed in general chapter *2.6.1*.

## 2. GENERAL CONSIDERATIONS

### 2-1. *GENERAL PRECAUTIONS*

The test is carried out under aseptic conditions according to current regulations for potentially infective material. The precautions taken to avoid contamination are such that they do not affect any micro-organisms that are to be revealed in the test. The test is performed under working conditions that are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls. Testing shall take account of the potential for the presence of inhibitory substances in the sample that may affect the outcome of the test.

### 2-2. *HANDLING CONSTRAINTS*

#### 2-2-1. **Shelf-life**

'Negative-to-date' is understood as an intermediate reading of a test method (*2.6.1* or an automated growth-based method) that has not yet been completed. Where cell-based preparations have limiting shelf-lives, 'negative-to-date' results may be used as the readout, where justified. Based on the risk analysis of the characteristics and intended use of the cell-based preparation, results from additional microbiological in-process control may be needed at the time of use.

#### 2-2-2. **Sampling**

The test sample must be representative of all of the components of the cell-based preparation and be taken from the final preparation. Where this is not possible, surrogate testing may be performed, for example on the liquids last in contact with the cells being processed.

## 3. METHODS FOR MICROBIOLOGICAL EXAMINATION OF CELL-BASED PREPARATIONS

### 3-1. *AUTOMATED GROWTH-BASED METHOD*

#### 3-1-1. **Growth promotion test**

This section outlines the conditions for confirming the suitability of the culture media used for microbiological examination.

At least 2 suitable culture media intended for detection of fungi and aerobic and anaerobic bacteria are used. Each batch of sterile medium is tested for its growth-promoting capacities by inoculating duplicate test containers of each medium with not more than 100 CFU of each of the strains listed in Table 2.6.27.-1, and incubating for a maximum of 7 days for detection of microbial growth at the temperature defined for testing (see Table 2.6.27.-3). The test media are satisfactory if there is clear evidence of growth in all inoculated media containers within this incubation period.

Table 2.6.27.-1. – *Micro-organisms used for growth promotion test*

| **Aerobic medium** | |
|---|---|
| *Staphylococcus aureus* | for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518 |
| *Bacillus subtilis* | for example, ATCC 6633, CIP 52.62, NCIMB 8054 |
| *Pseudomonas aeruginosa* | for example, ATCC 9027, NCIMB 8626, CIP 82.118 |
| *Candida albicans* | for example, ATCC 10231, IP 48.72, NCPF 3179 |
| *Aspergillus brasiliensis* | for example, ATCC 16404, IP 1431.83, IMI 149007 |
| **Anaerobic medium** | |
| *Clostridium sporogenes* | for example, ATCC 11437, CIP 79.3, NCTC 532 or ATCC 19404 |
| *Bacteroides fragilis* | for example, ATCC 25285, CIP 77.16, NCTC 9343 |

#### 3-1-2. **Method suitability**

The test system is validated with respect to specificity (absence of false positive results), sensitivity, reproducibility and robustness. Regardless of the type of cell-based preparation, the manufacturing process, the sample volume analysed or the type of test system, the suitability of the method is to be confirmed in the presence of the specific sample composition. During the confirmation of the suitability of the method, particularly to determine sensitivity, the test is carried out using the micro-organisms listed in Table 2.6.27.-2. Sensitivity is meant as the capacity to detect 100 CFU or less. Not more than 100 CFU of the chosen micro-organisms are inoculated into the medium in the presence of the preparation to be tested, using at least 3 replicates. The microbial count in the micro-organism suspensions used for inoculation is determined by streaking an appropriate sample on agar plates. If between 1 and 100 CFU are detected for each strain within the duration of the assay, the method is valid for the intended sample composition.

It may be necessary to modify the list of micro-organisms in Table 2.6.27.-2 depending on the origin of the cells and any micro-organisms previously found or associated with the particular type of cells.

Table 2.6.27.-2. – *Micro-organisms used for method suitability*

| **Aerobic medium** | |
|---|---|
| *Aspergillus brasiliensis* | for example, ATCC 16404, IP 1431.83, IMI 149007 |
| *Bacillus subtilis* | for example, ATCC 6633, CIP 52.62, NCIMB 8054 |
| *Candida albicans* | for example, ATCC 10231, IP 48.72, NCPF 3179 |
| *Pseudomonas aeruginosa* | for example, ATCC 9027, NCIMB 8626, CIP 82.118 |
| *Staphylococcus aureus* | for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518 |
| *Streptococcus pyogenes* | for example, ATCC 19615, CIP 1042.26, NCIMB 13285 |
| *Micrococcus* sp. | for example, ATCC 700405, NCTC 7567 |
| **Anaerobic medium** | |
| *Clostridium sporogenes* | for example, ATCC 11437, CIP 79.3, NCTC 532 or ATCC 19404 |
| *Propionibacterium acnes* | for example, ATCC 11827 |

In some cases, the cell-based preparation itself can inactivate contaminating micro-organisms. Appropriate measures must be taken to ensure the suitability of any additional microbial strains used for method validation.

#### 3-1-3. **Testing of the preparation to be examined**

*Sample*. A sample that is representative of the characteristics of the cell-based preparation is tested. The sample is added to the culture medium as soon as possible. If it is necessary to store samples, the impact of the storage on potential contaminants is evaluated.

For cell-based preparations where the total volume (*V*) of the batch is between 1 mL and 1 L in a single container, the following table indicates the inoculation volume to be used.

| Total cell-based preparation volume (mL) | Total inoculum volume (divided between aerobic and anaerobic bottles) |
|---|---|
| 10 ≤ V ≤ 1000 | 1 per cent of total volume of preparation to be tested |
| 1 ≤ V < 10 | 100 µL |
| V < 1 | Not applicable |

For other volumes or multiple containers, alternative approaches should be used and have to be justified (see section 2-2-2). Enlargement of the total volume by means of dilution may be envisaged to assure complete inoculation of sample volumes of 100 µL. For preparation volumes less than 1 mL, where final sampling is not possible, surrogate testing, in-process testing or other appropriate testing should be used and has to be justified.

*Analysis.* Samples are inoculated into containers of culture medium as soon as possible and incubated for not less than 7 days. Depending on the results obtained during method suitability testing and considering relevant micro-organisms, the incubation period may be extended up to 14 days. Selection of incubation temperatures should enable detection of a broad range of micro-organisms. This is typically in the range of 30-37 °C; however, for cell-based preparations with a very short shelf-life, a growth-accelerating temperature of not less than 35 °C may be more appropriate to obtain a relevant 'negative-to-date' readout of the test. In addition, for preparations where there is a significant risk of contamination from the environment, 2 temperature ranges of, for example, 20-25 °C (for aerobic) and 30-37 °C (for anaerobic) are used, in order to cover both environmental and clinical micro-organisms.

Table 2.6.27.-3 lists possible alternative approaches for the choice of incubation temperatures. The temperature and time for incubation are based on the results of the suitability study for the specific cell-based preparation.

Table 2.6.27.-3. – *Possible temperature settings in automated culturing systems used alone or in combination with manual testing*

| | Aerobic incubation | Anaerobic incubation |
|---|---|---|
| Option 1 | 20-25 °C (automated system), if necessary 30-35 °C (automated system) | 30-35 °C (automated system) |
| Option 2 | 35-37 °C (automated system); where relevant, additional incubation at a lower temperature (manual method)* | 35-37 °C (automated system) |
| Option 3 | 30-32 °C (automated system) | 30-32 °C (automated system) |
| Option 4 | 30-32 °C (automated system) | 35-37 °C (automated system) |

* Where relevant, incubate in addition at a temperature between 20 °C and 30 °C. Incubation can be performed using commercially available microbiological media, either aerobic bottles intended for automated systems or casein soya bean digest broth.

3-1-4. **Observation and interpretation of results**

Media are examined, visually or with automated systems, at least daily and at the end of the observation period for evidence of microbial growth. If no growth is observed during or at the end of the observation period, the product is 'culture negative'. If growth is observed in a valid test, the product is 'culture positive'.

If the inoculated bottles are stored for more than 12 h before being placed into the automated culturing system, a subculture of each incubated bottle is performed to check for false negatives. This addresses cases in which, where a micro-organism is fast-growing and the conditions are optimal, micro-organisms may start to proliferate during storage. As a consequence, there may not be a significant increase in the relevant parameters at the time of testing and microbial contaminants may not be recognised by the system (false negative result).

3-2. *ALTERNATIVE METHODS*

3-2-1. **Combination of preculturing and detection by alternative methods**

The samples to be tested are incubated in both aerobic and anaerobic liquid cultivation media or equivalent solid media for a short period of time (e.g. 12-24 h depending on the sensitivity of the alternative approach used). An alternative method suitable for rapid detection of micro-organisms is then performed (e.g. nucleic acid amplification techniques (*2.6.21*), flow cytometry (*2.7.24*), bioluminescence (*5.1.6*)).

3-2-2. **Direct detection by alternative methods** (*5.1.6*)

Where a cell-based preparation has a very short shelf-life (e.g. a few hours) or where standard methods do not provide satisfactory detection of micro-organisms, non-growth-based, direct detection methods may be carried out for microbiological examination (e.g. nucleic acid amplification techniques (*2.6.21*), flow cytometry (*2.7.24*), bioluminescence (*5.1.6*)).

This approach enables a result to be obtained within a very short time, although at the expense of lower sensitivity, in comparison to growth-based methods. Depending on the approach used, both viable and non-viable micro-organisms may be detected.

3-2-3. **Method validation**

Validation is carried out according to the general recommendations of general chapter *5.1.6* and according to the recommendations specific to cell-based preparations in section 3-1-2 for automated growth-based methods. The sensitivity of these approaches must be validated considering the doubling times of potentially contaminating micro-organisms during pre-incubation.

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# 2.6.30. MONOCYTE-ACTIVATION TEST

1. INTRODUCTION

The monocyte-activation test (MAT) is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines have a role in fever pathogenesis. Consequently, the MAT will detect the presence of pyrogens in the test sample. The MAT is suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test.

Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep or non-linear dose-response curves in comparison with endotoxin dose-response curves. Preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

The following 3 methods are described in the present chapter.

Method A. Quantitative test

Method B. Semi-quantitative test

Method C. Reference lot comparison test

In addition, further useful information on practical aspects of the tests can be found in the 'Guidance notes' section at the end of this general chapter.

The test is carried out in a manner that avoids pyrogen contamination.

## 2. DEFINITIONS

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the contaminant limit can be determined. The calculation of the MVD is based on the endotoxin reference standard. Determine the MVD using the following expression:

$$\frac{CLC \times C}{LOD}$$

*CLC* = contaminant limit concentration;

*C* = concentration of test solution;

*LOD* = limit of detection.

As the LOD is not always available in advance, an estimated LOD based on historical data can be used to calculate the MVD.

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre or per units of biological activity of the preparation being examined.

The CLC is calculated using the following expression:

$$\frac{K}{M}$$

*K* = threshold pyrogenic dose per kilogram of body mass;

*M* = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, *M* is the maximum total dose administered in a single hour period.

Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. In this case, the concentration of test solution is expressed in mg/mL if the endotoxin limit is specified by mass (IU/mg), in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit), in mL/mL if the endotoxin limit is specified by volume (IU/mL).

Endotoxin equivalents are values for the contaminant concentration read off the standard endotoxin dose-response curve (Method A) or estimated by comparison with responses to standard endotoxin solutions (Method B). The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

The LOD is determined using the endotoxin standard curve. It is the concentration of endotoxin corresponding to the cut-off value. For the purpose of the test, the LOD is expressed as endotoxin equivalents per millilitre. The cut-off value is expressed in units appropriate to the read-out (e.g. for an enzyme-linked immunosorbent assay (ELISA), use the optical density).

The cut-off value may be calculated using the following expression:

$$\bar{x} + 3s$$

$\bar{x}$ = mean of the 4 replicates for the responses to the blank ($R_0$);

$s$ = standard deviation of the 4 replicates of the responses to the blank ($R_0$).

## 3. GENERAL PROCEDURE

A solution of the preparation being examined is incubated with a source of human monocytes or human monocytic cells, e.g. from human heparinised peripheral blood that is preferably not more than 4 h old, or a monocyte-containing fraction of that blood, such as human peripheral blood mononuclear cells (PBMC) isolated, e.g. by density-gradient centrifugation, or a human monocytic cell line. Human heparinised peripheral blood is usually diluted with culture medium or saline e.g. to 2-50 per cent *V/V* (final concentration). PBMC or monocytic cell lines, in culture medium and with either the donor's own plasma or AB serum, are typically used at a final cell density of 0.1-1.0 × $10^6$ cells per well, tube or other receptacle. For monocytic cell lines, heat-inactivated foetal bovine serum may be substituted for AB serum. The cell culture is carried out at 37 ± 1 °C, in an atmosphere appropriate for the culture medium, e.g. 5 per cent $CO_2$ in humidified air. The duration of the culture is sufficient to allow accumulation of the chosen read-out. The responses of the chosen read-out, e.g. a pro-inflammatory or pyrogenic cytokine, to a solution of the preparation being examined are compared with responses to standard endotoxin or to a reference lot of the preparation being examined.

## 4. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable pyrogens and which do not interfere with the test.

## 5. CELL SOURCES AND QUALIFICATION

### 5-1. *WHOLE BLOOD*

Whole blood is obtained from single donors or from pooled whole blood which are qualified according to the requirements described under sections 5-3, 5-4, 5-5 and where applicable, section 6-3.

### 5-2. *PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)*

PBMC are isolated from blood obtained from single donors or from pooled whole blood which are qualified according to the requirements described under sections 5-3, 5-4, 5-5 and where applicable, section 6-3.

### 5-3. *QUALIFICATION OF BLOOD DONORS*

Blood donors are to satisfy the following qualification criteria, together with other requirements in force that relate to consent, health and safety and ethical considerations. Blood donors are to describe themselves as being in good health, as not to be suffering from any bacterial or viral infections and to have been free from the symptoms of any such infection for a period of at least 1 week prior to the donation of blood. Blood donors are not to have taken non-steroidal anti-inflammatory drugs during the 48 h prior to donating blood and steroidal anti-inflammatory drugs during the 7 days prior to donating blood. Individuals who have been prescribed immunosuppressant or other drugs known to influence the production of the chosen readout are not to serve as blood donors. Blood donations are to be tested for infection markers according to national requirements for transfusion medicine.

### 5-4. *QUALIFICATION OF CELLS POOLED FROM A NUMBER OF DONORS*

Pools (of whole blood or blood fractions, e.g. PBMC), must consist of donations from a minimum of 4 individual donors but preferably 8 or more donors, where practicable, taking from each donation an approximately equal volume of blood, or cells from an approximately equal volume of blood. For the qualification of pooled cells proceed as follows: within 4 h of collection of blood, generate dose-response curves from the pool using standard endotoxin with at least 4 geometrically diluted endotoxin concentrations, e.g. in the

range of 0.01 IU/mL to 4 IU/mL. The dose-response curves are to meet the 2 criteria for the endotoxin standard curve described under section 6-1. If the pool is to be used for the detection of non-endotoxin contaminants, pools are to be qualified as described in section 6-3. Where cells are pooled, the averaging effect is to be considered when setting the pass/fail specification for a given product.

5-5. *QUALIFICATION OF CRYO-PRESERVED CELLS*

The cell source intended for use in a MAT, e.g. human whole blood, blood fractions, such as PBMC or monocytic cell lines, may be cryo-preserved. Pools of cryo-preserved cells are obtained by pooling before freezing, or by pooling single cryo-preserved donations immediately after thawing. Qualification of cryo-preserved blood or cells is performed immediately after thawing (and pooling if necessary). Dose-response curves for the cryo-preserved blood or cells are to comply with the 2 criteria for the endotoxin standard curve described under section 6-1. Qualification of the cryo-preserved blood or cells is to be performed according to section 6-3 if the intended use is for the detection of non-endotoxin contaminants.

5-6. *MONOCYTIC CONTINUOUS CELL LINES*

Monocytic cell lines are appropriate for the detection of bacterial endotoxins, but have limited use for the detection of non-endotoxin pyrogens.

A human monocytic cell line is cultured in order to ensure a sufficient supply for the MAT. To optimise the method, clones derived from the cell line can be used.

Cells must be maintained under aseptic conditions and regularly tested for the presence of mycoplasma contamination. Additionally, cells must be regularly checked for identity (e.g. doubling time, morphology, and function) and stability. The functional stability of a cell line is assessed by monitoring its performance in relation to the number of passages during routine testing. Criteria for functional stability are to be established and may include growth criteria, maximum signal obtained in the test, background noise and receptor expression. The receptor expression may be tested with specific ligands e.g. lipopolysaccharide (LPS) for toll-like receptor 4 (TLR4), lipoteichoic acid (LTA) for toll-like receptor 2 (TLR2), synthetic bacterial lipoprotein for TLR2-TLR1, synthetic bacterial lipoprotein for TLR2-TLR6 or flagellin.

The dose-response curves are to meet the 2 criteria for the endotoxin standard curve described under section 6-1. If the cells are to be used for the detection of non-endotoxin contaminants, they are to be qualified as described in section 6-3.

## 6. PREPARATORY TESTING

To ensure both the precision and validity of the test, preparatory tests are conducted, to assure that the criteria for the endotoxin standard curve are satisfied, that the solution does not interfere with the test, that the test detects endotoxins and non-endotoxins contaminants and that the solution does not interfere in the detection system.

A test for interfering factors is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

6-1. *ASSURANCE OF CRITERIA FOR THE ENDOTOXIN STANDARD CURVE*

Using the standard endotoxin solution, prepare at least 4 endotoxin concentrations to generate the standard curve. Perform the test using at least 4 replicates of each concentration of standard endotoxin.

The basal release of the chosen read-out (blank) in the absence of added standard endotoxin is optimised to be as low as possible (e.g. an optical density below 0.1 when using an ELISA).

There are 2 acceptance criteria for the standard curve:

- the regression of responses (appropriately transformed if necessary) on $\log_{10}$ dose shall be statistically significant ($p < 0.01$);
- the regression of responses on $\log_{10}$ dose must not deviate significantly from linearity ($p > 0.05$) (see chapter *5.3. Statistical analysis*).

6-2. *TEST FOR INTERFERING FACTORS*

To assure the validity of the test, preparatory tests are conducted to ensure that the preparation being examined does not interfere with the test. Using an appropriate diluent, dilute the preparation being examined in geometric steps, with all dilutions not exceeding the MVD. Make the same dilutions of the preparation being examined and add endotoxin at a justified concentration. Alternatively, use a diluent containing added endotoxin at a justified concentration. In both cases, this concentration is usually equal to or near the estimated middle of the endotoxin standard curve (Method A) or twice the estimated LOD (Method B). Test these dilution series in parallel in the same experiment. Use the endotoxin standard curve to calculate the concentration of endotoxin-equivalents in each solution. Calculate the mean recovery of the added endotoxin by subtracting the mean concentration of endotoxin equivalents in the solution (if any) from that in the solution containing the added endotoxin. The test solution is considered free of interfering factors if, under the conditions of the test, the measured endotoxin equivalents in the test solution to which endotoxin is added is within 50-200 per cent of the added concentration, after subtraction of any endotoxin equivalents detected in the solution without added endotoxin. When this criterion is not met, Method C is to be preferred over Methods A and B.

In Method C, the dilutions of the test and reference lots depend on the type of analysis used to make the comparison between the two. The type of analysis is to be justified and validated for each product, and is to include assay validity criteria. In an example, a solution of the preparation being examined is tested at 3 dilutions: the highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out and the 2-fold dilutions immediately below and above the chosen dilution. Since the concentration that stimulates the greatest release of the chosen read-out may be donor-dependent as well as batch-dependent, the product-specific validation is to be performed in at least 3 independent tests, each using cells from different donors. The highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out in the majority of donors, and the 2-fold dilutions immediately below and above that dilution are deemed to be validated for further testing. If undiluted test solution stimulates the greatest release of the chosen read-out, subsequent testing is to be performed using undiluted test solution and also test solution diluted in the ratios 1:2 and 1:4 before its addition to the monocytic cells. The dilution factors for these 3 solutions are designated $f_1$, $f_2$ and $f_3$.

If the pyrogen content of the product is inherently high, it may be more appropriate to carry out, for example, a parallel-line analysis on the dose-response curves for the test and reference lots. In this situation, solutions of the preparations are tested at 3 or more geometric dilutions which cover the range of the dose-response curve used for the validated analysis (see chapter *5.3. Statistical analysis*).

6-3. *METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS*

The preparatory testing is also to show that the chosen test system detects, in addition to bacterial endotoxins, non-endotoxin pro-inflammatory or pyrogenic contaminants. The suitability of the method for the particular product has to be verified. This can be achieved using historic batches found to be contaminated with non-endotoxin contaminants that caused positive responses in the rabbit pyrogens test or

adverse drug reactions in man. Where such batches are not available, the preparatory testing is to include validation of the test system using at least 2 non-endotoxin ligands for toll-like receptors, e.g. peptidoglycans, lipoteichoic acids, synthetic bacterial lipoproteins, flagellin and crude bacterial whole cell extract, at least 1 of which is to be spiked into the preparation being examined. The choice of non-endotoxin pyrogens used should reflect the most likely contaminant(s) of the preparation being examined.

6-4. *INTERFERENCE IN THE DETECTION SYSTEM*

Once the optimum dilution of the solution of the preparation being examined for further testing has been identified, this dilution is tested for interference in the detection system (e.g. ELISA) for the chosen read-out. The agreement between a dilution series of the standard for the chosen read-out, in the presence and absence of the preparation being examined, is to be within, for example ± 20 per cent of the optical density.

## 7. METHODS

### 7-1. *METHOD A: QUANTITATIVE TEST*

Method A involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test.

#### 7-1-1. **Test procedure**

Using the validated test method, prepare the solutions shown in Table 2.6.30.-1 and culture 4 replicates of each solution with the qualified cells.

Table 2.6.30.-1

| Solution | Solution | Added endotoxin (IU/mL) | Number of replicates |
|---|---|---|---|
| A | Test solution/*f* | None | 4 |
| B | Test solution/2 × *f* | None | 4 |
| C | Test solution/4 × *f* | None | 4 |
| AS | Test solution/*f* | Middle dose from endotoxin standard curve ($R_3$) | 4 |
| BS | Test solution/2 × *f* | Middle dose from endotoxin standard curve ($R_3$) | 4 |
| CS | Test solution/4 × *f* | Middle dose from endotoxin standard curve ($R_3$) | 4 |
| $R_0$ | Pyrogen-free saline or test diluent | None (negative control) | 4 |
| $R_1$-$R_4$ | Pyrogen-free saline or test diluent | 4 concentrations of standard endotoxin | 4 of each concentration |

Solution A = solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was carried out, i.e. the highest concentration (lowest dilution) for which the endotoxin recovery is within 50-200 per cent.

Solution B = 2-fold dilution of solution A, not exceeding the MVD.

Solution C = 2-fold dilution of solution B, not exceeding the MVD.

Solution AS = solution A spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve ($R_3$).

Solution BS = solution B spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve ($R_3$).

Solution CS = solution C spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve ($R_3$).

Solution $R_0$ = negative control.

Solutions $R_1$-$R_4$ = solutions of standard endotoxin at the concentrations used in the test for interfering factors.

#### 7-1-2. **Calculation and interpretation**

All data to be included in the data analysis are to relate to cells for which the 2 criteria for the endotoxin standard curve are satisfied. For each different cell source, e.g. individual donation, donor pool, or cell line, use the endotoxin standard curve $R_1$-$R_4$ to calculate the concentration of endotoxin equivalents in each of the replicates of solutions A, B and C and solutions AS, BS and CS. The recovery of endotoxin equivalents calculated from the endotoxin equivalents concentration found in solutions AS, BS and CS after subtracting the endotoxin equivalents concentration found in solutions A, B and C is within the range of 50-200 per cent. Dilutions not fulfilling the spike recovery argument are not valid and are therefore excluded from further evaluation. The preparation being examined complies with the requirements of the test for a given cell source if the mean concentrations of endotoxin equivalents measured in the replicates of solutions A, B and C, after correction for dilution and concentration, are all less than the CLC specified for the preparation being examined. One valid dilution is the minimum required for a valid test.

#### 7-1-3. **Pass/fail criteria of the preparation**

When cells from individual donors are used, the preparation being examined is required to comply with the test with the cells from each of 4 different donors. If the preparation being examined passes the test with cells from 3 of the 4 donors, the test is continued with cells from a further 4 donors, none of whom provided cells for the 1st test, and the preparation being examined is required to pass the test with cells from 7 of the 8 different donors (i.e. a maximum of 1 positive reaction in 8 donors is allowed). When the source of monocytes consists of cells pooled from a number of individual donors, the preparation being examined is required to pass the test with 1 pool of cells. Where a human monocytic cell line is used for the test, the preparation being examined is required to pass the test with 1 qualified passage of cells.

### 7-2. *METHOD B. SEMI-QUANTITATIVE TEST*

Method B involves a comparison of the preparation being examined with standard endotoxin. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test. Solution A must be chosen for the pass decision, unless otherwise justified and authorised.

#### 7-2-1. **Test procedure**

Using the validated test method, prepare the solutions shown in Table 2.6.30.-2 and culture 4 replicates of each solution with the qualified cells.

Table 2.6.30.-2

| Solution | Solution | Added endotoxin (IU/mL) | Number of replicates |
|---|---|---|---|
| A | Test solution/*f* | None | 4 |
| B | Test solution/$f_1$ | None | 4 |
| C | Test solution/$f_2$ | None | 4 |
| AS | Test solution/*f* | Standard endotoxin at 2 × estimated LOD for the test system | 4 |
| BS | Test solution/$f_1$ | Standard endotoxin at 2 × estimated LOD for the test system | 4 |

| Solution | Solution | Added endotoxin (IU/mL) | Number of replicates |
|---|---|---|---|
| CS | Test solution/$f_2$ | Standard endotoxin at 2 × estimated LOD for the test system | 4 |
| $R_0$ | Pyrogen-free saline or test diluent | None (negative control) | 4 |
| $R_1$ | Pyrogen-free saline or test diluent | Standard endotoxin at 0.5 × estimated LOD for the test system | 4 |
| $R_2$ | Pyrogen-free saline or test diluent | Standard endotoxin at 1 × estimated LOD for the test system | 4 |
| $R_3$ | Pyrogen-free saline or test diluent | Standard endotoxin at 2 × estimated LOD for the test system | 4 |
| $R_4$ | Pyrogen-free saline or test diluent | Standard endotoxin at 4 × estimated LOD for the test system | 4 |

Solution A = solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was completed.

Solution B = solution of the preparation being examined at a dilution, here designated $f_1$, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. 1:2 × MVD (i.e. 2 times less diluted than the MVD).

Solution C = solution of the preparation being examined at a dilution, here designated $f_2$, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. MVD.

Solution AS = solution A spiked with standard endotoxin at 2 × estimated LOD for the test system (as determined in preparatory testing).

Solution BS = solution B spiked with standard endotoxin at 2 × estimated LOD for the test system.

Solution CS = solution C spiked with standard endotoxin at 2 × estimated LOD for the test system.

Solution $R_0$ = negative control.

Solution $R_1$ = standard endotoxin at 0.5 × estimated LOD for the test system.

Solution $R_2$ = standard endotoxin at 1 × estimated LOD for the test system.

Solution $R_3$ = standard endotoxin at 2 × estimated LOD for the test system.

Solution $R_4$ = standard endotoxin at 4 × estimated LOD for the test system.

7-2-2. **Calculation and interpretation**

All data to be included in the data analysis are to relate to cells for which mean responses to solutions $R_0$-$R_4$ increase progressively. The mean response to $R_0$ may be equal to the mean response to $R_1$. For each different cell source, the mean response to solution $R_2$ is to be greater than a positive cut-off value. Data below this cut-off value are considered negative. If the mean response to $R_1$ or $R_2$ exceeds the cut-off value, the response to the solution chosen for the pass/fail decision must be negative (= pass). For each negative solution of the preparation being examined (A, B and C), the mean response to the corresponding spiked solution (AS, BS or CS respectively) is compared with the mean response to $R_3$ to determine the percentage spike recovery. The contaminant concentration of the preparation being examined is less than the CLC for a given cell source if the solution of the preparation being examined designated for the pass/fail-decision and the dilutions below all give negative results and the endotoxin spike recovery is within the range of 50-200 per cent.

7-2-3. **Pass/fail criteria of the preparation**

The criteria are the same as for method A (see 7-1-3).

7-3. *METHOD C: REFERENCE LOT COMPARISON TEST*

Method C involves a comparison of the preparation being examined with a validated reference lot of that preparation. The type of analysis selected to compare the two is to be justified and validated for each product and is to include assay validity criteria. The reference lot is also selected according to criteria that have been justified and authorised. The test is intended to be performed in cases where a preparation being examined shows marked interference but cannot be diluted within the MVD to overcome the interference or because it contains or is believed to contain non-endotoxin contaminants. Responses to non-endotoxin contaminants may dilute out more rapidly than responses to endotoxin, which makes it necessary to perform the test at a range of dilutions that include minimum dilution. The test procedure is described below and includes an example of a type of analysis used for the comparison of a test lot and reference lot.

7-3-1. **Test procedure**

Using the validated test method, prepare the solutions shown in Table 2.6.30.-3 and culture 4 replicates of each solution with the qualified cells.

Table 2.6.30.-3

| Solution | Solution/dilution factor | Number of replicates |
|---|---|---|
| A | Solution of reference lot/$f_1$ | 4 |
| B | Solution of reference lot/$f_2$ | 4 |
| C | Solution of reference lot/$f_3$ | 4 |
| D | Solution of preparation being examined/$f_1$ | 4 |
| E | Solution of preparation being examined/$f_2$ | 4 |
| F | Solution of preparation being examined/$f_3$ | 4 |
| G | Positive control (standard endotoxin) | 4 |
| $R_0$ | Diluent (negative control) | 4 |

Solutions A, B and C are solutions of the reference lot diluted by the dilution factors, $f_1$, $f_2$ and $f_3$, determined in the test for interfering factors.

Solutions D, E and F are solutions of the preparation being examined diluted by the dilution factors, $f_1$, $f_2$ and $f_3$, determined for the reference lot in the test for interfering factors.

Solution G is the positive test control for the viability of the cells and is a standard endotoxin concentration that gives a clear positive response.

Solution $R_0$ is the diluent used to dilute the preparation being examined and serves as the test blank.

7-3-2. **Calculation and interpretation**

All data to be included in the data analysis are to relate to cells for which solution G and at least one of solutions A, B and C give a response that is greater than the basal release of the read-out (Solution $R_0$). For each different cell source, e.g. individual donation, donor pool, or cell line, use the standard

curve for the read-out (a calibration curve in duplicate with a blank and at least 4 geometrically diluted concentrations of the standard for the chosen read-out) and calculate the mean responses of the replicates of solutions A-F. Sum the mean responses to solutions A, B and C and sum the mean responses to solutions D, E and F. Divide the sum of the mean responses to solutions D, E and F by the sum of the mean responses to solutions A, B and C. The preparation being examined complies with the test for a given cell source if the resulting value complies with a defined acceptance criterion not exceeding a justified value, e.g. 2.5.

7-3-3. **Pass/fail criteria of the preparation**

The criteria are the same as for method A (see 7-1-3).

To quantify more closely the level of contamination, Methods A, B and C may be performed using other dilutions of the solution of the preparation being examined not exceeding the MVD.

*The following section is published for information only.*

## Guidance notes

1. INTRODUCTION

The monocyte-activation test (MAT) is primarily intended to be used as a replacement for the rabbit pyrogen test. The MAT detects pyrogenic and pro-inflammatory contaminants, including endotoxins from gram-negative bacteria and 'non-endotoxin' contaminants, including pathogen-associated molecular patterns (PAMPs), derived from gram-positive and gram-negative bacteria, viruses and fungi, and product-related and process-related biological or chemical entities.

Since non-endotoxin contaminants are a physico-chemically diverse class of molecules, and usually the nature of the contaminant in a preparation being examined is unknown, the level of contamination is expressed either in endotoxin-equivalent units, derived by comparison with responses to standard endotoxin, or by comparison with a reference lot of the preparation being examined.

In the MAT, responses to standard endotoxin usually dilute out over approximatively 1 $\log_{10}$ and responses to products contaminated with non-endotoxin contaminants (alone or in combination with endotoxins) often show very steep dose-response curves, usually over only 1 or 2 dilution steps when tested for their capability to stimulate monocytes. Frequently, the largest response to such contaminated products is obtained with undiluted solutions of preparations being examined or small dilutions of the preparations being examined. For this reason test solutions of preparations being examined that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

2. METHODS

2-1. *INFORMATION REGARDING THE CHOICE OF METHODS*

Methods A, B and C, are not normally applied where a preparation being examined has the intrinsic activity of stimulating the release of the chosen read-out or where the preparation being examined is contaminated with the chosen read-out. In both cases, this fact is to be addressed by modifying and validating the chosen method accordingly. The product-specific validation of the chosen method would be expected to identify the frequency of non-responders to a particular product/contaminant(s) combination and to identify steps to address this, e.g. screening of donors, increasing the number of donors per test, and setting pass/fail criteria of appropriate stringency to maximise the likelihood of detecting contaminated batches. Method A is not appropriate if the results of different dilutions (endotoxin equivalents per millilitre) show that the dose response curve is not parallel to the standard endotoxin curve. Method B is a semi-quantitative test that can be applied when responses to dilutions of a preparation being examined are not parallel to responses to dilutions of standard endotoxin.

Method C, the reference lot comparison test, was developed to address extreme donor variability in responses to certain product/contaminant(s) combinations. In this regard, it should be noted that, while monocytes from most donors respond in a broadly similar manner to bacterial endotoxin, responses of monocytes from different donors to non-endotoxin contaminants can differ markedly, so that it is possible to identify non-responders along with low and high responders to certain product/contaminant(s) combinations.

2-2. *CALCULATION OF CONTAMINANT LIMIT CONCENTRATION*

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre or in units of biological activity of the preparation being examined. Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. The CLC is expressed in terms of endotoxin equivalents. The CLC is calculated using the following expression:

$$\frac{K}{M}$$

$K$ = threshold pyrogenic dose per kilogram of body mass;

$M$ = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, $M$ is the maximum total dose administered in a single hour period.

The CLC depends on the product and its route of administration and is stated in some monographs.

Values for $K$ are suggested in Table 2.6.30.-4.

Table 2.6.30.-4

| Route of administration | *K* |
|---|---|
| Intravenous | 5.0 IU of endotoxin per kilogram of body mass |
| Intravenous, for radiopharmaceuticals | 2.5 IU of endotoxin per kilogram of body mass |
| Intrathecal | 0.2 IU of endotoxin per kilogram of body mass |
| Parenteral formulations administered per square metre of body surface | 100 IU/m$^2$ |

2-3. *INFORMATION REGARDING CRYO-PROTECTANTS*

The influence of cryo-protectants, e.g. dimethyl sulfoxide (DMSO), and their residues in thawed cells, is to be considered: DMSO is toxic to cells in culture and, even when cells have been washed thoroughly, cryo-preservation may have altered cell properties, e.g. cell membrane permeability.

2-4. *INTERFERENCE TESTING*

Where practicable, interference testing is performed on at least 3 different lots of the preparation being examined. Preparations being examined that show marked batch-to-batch variation, that effectively renders each batch unique for the purposes of interference testing, are to be subjected to interference testing within each individual test, i.e. concomitant validation.

Interference testing is preferably performed on batches of the preparation being examined that are free of endotoxins and other pyrogenic/pro-inflammatory contaminants and, where this is not practicable, none of the batches are to be heavily contaminated. If only 1 batch is available the validation has to be performed on that batch in 3 independent tests. Precision parameters for reproducibility, e.g. ± 50 per cent, are to be fulfilled.

2-5. *CROSS-VALIDATION*

General chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*, states that an MAT should be performed on products where the presence of non-endotoxin pyrogenic substances cannot be ruled out. It is therefore recommended to perform cross-validation experiments with the MAT together with the validation experiments for the test for bacterial endotoxins (BET) using the same 3 batches. Cross-validation should be repeated on 3 batches if important process parameters have changed, as potential contamination by non-endotoxin pyrogens cannot be ruled out.

The rabbit pyrogen test (see general chapter *2.6.8. Pyrogens*) must only be performed for cross validation if none of the MAT methods (A, B or C) can be validated for a certain product.

3. REPLACEMENT OF THE RABBIT PYROGEN TEST BY THE MONOCYTE ACTIVATION TEST

As noted above, MAT is primarily intended to be used as a replacement for the rabbit pyrogen test. Monographs on pharmaceutical products intended for parenteral administration that may contain pyrogenic contaminants require either a test for bacterial endotoxins or a monocyte activation test.

As a general policy:

- in any individual monograph, when a test is required, only 1 test is included, either that for bacterial endotoxins or the MAT. Before including the MAT in a monograph, evidence is required that 1 of the 3 methods described in the MAT chapter can be applied satisfactorily to the product in question;
- the necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the MAT to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the MAT is appropriate, is to be provided.

4. VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test, or replacement of a method for detecting pro-inflammatory/pyrogenic contaminants by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

The following procedures are suggested for validating a method for the MAT other than the one indicated in the monograph:

- the procedure and the materials and reagents used in the method should be validated as described for the test concerned;
- the presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least 3 production batches.

MAT should be applied to all new products intended for parenteral administration that have to be tested for the presence of non-endotoxin pyrogens according to the requirements of the European Pharmacopoeia.

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# 2.6.31. MICROBIOLOGICAL EXAMINATION OF HERBAL MEDICINAL PRODUCTS FOR ORAL USE AND EXTRACTS USED IN THEIR PREPARATION

1. MICROBIAL ENUMERATION TESTS

**Total aerobic microbial count (TAMC)**. Perform as described in general chapter *2.6.12*.

**Total combined yeasts/moulds count (TYMC)**. Perform as described in general chapter *2.6.12*. Due to the natural high bioburden in the products covered by general chapter *5.1.8*, use of Sabouraud-dextrose agar containing antibiotics is suitable.

2. TEST FOR SPECIFIED MICRO-ORGANISMS

2-1. *INTRODUCTION*

The tests described hereafter will allow determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described.
The tests are designed primarily to determine whether a product, substance or preparation (hereinafter referred to as 'the product') complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2-2. *GENERAL PROCEDURES*

The preparation of samples is carried out as described in general chapter *2.6.12*.
If the product to be examined has antimicrobial activity, this is as far as possible removed or neutralised as described in general chapter *2.6.12*.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.12*.

2-3. *GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS*

The ability of the test to detect micro-organisms in the presence of the product to be examined must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

2-3-1. **PREPARATION OF TEST STRAINS**

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed lot.

2-3-1-1. **Aerobic micro-organisms**. Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;

– *Salmonella enterica* subsp. *enterica* serovar Typhimurium such as ATCC 14028 or, as an alternative, *S. enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;

– *Bacillus subtilis* such as ATCC 6633, NCIMB 8054, CIP 52.62 or NBRC 3134.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

2-3-2. **NEGATIVE CONTROL**

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 2-4. A failed negative control requires an investigation.

2-3-3. **GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA**

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Verify suitable properties of relevant media as described in Table 2.6.31.-1.

Table 2.6.31.-1. – *Growth-promoting, inhibitory and indicative properties of media*

| | Medium | Property | Test strains |
|---|---|---|---|
| Test for bile-tolerant gram-negative bacteria | Casein soya bean digest broth | Growth promoting | *S. aureus*<br>*P. aeruginosa*<br>*B. subtilis* |
| | Enterobacteria enrichment broth-Mossel | Growth promoting | *E. coli*<br>*P. aeruginosa* |
| | | Inhibitory | *S. aureus* |
| | Violet red bile glucose agar | Growth promoting + indicative | *E. coli*<br>*P. aeruginosa* |
| Test for *Escherichia coli* | Casein soya bean digest broth | Growth promoting | *S. aureus*<br>*P. aeruginosa*<br>*B. subtilis* |
| | MacConkey broth | Growth promoting | *E. coli* |
| | | Inhibitory | *S. aureus* |
| | MacConkey agar | Growth promoting + indicative | *E. coli* |
| Test for *Salmonella* | Buffered peptone medium | Growth promoting | *S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony |
| | Rappaport Vassiliadis *Salmonella* enrichment broth | Growth promoting | *S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony |
| | | Inhibitory | *S. aureus* |
| | Xylose, lysine, deoxycholate agar | Growth promoting + indicative | *S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony |

**Test for growth-promoting properties, liquid media**: inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Incubate the casein soya bean digest broth at 30-35 °C for not more than 3 days. Clearly visible growth of the micro-organism comparable to that obtained with a previously tested and approved batch of medium occurs.

**Test for growth-promoting properties, solid media**: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that obtained with a previously tested and approved batch of medium occurs.

**Test for inhibitory properties, liquid or solid media**: inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

**Test for indicative properties**: perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those obtained with a previously tested and approved batch of medium.

2-3-4. **SUITABILITY OF THE TEST METHOD**

For each product to be examined, perform the sample preparation as described in the relevant paragraph in section 2-4. Add each test strain at the time of mixing, in the prescribed growth medium (casein soya bean digest broth or buffered peptone medium). For the enumeration method for bile-tolerant gram-negative bacteria, inoculate *E. coli* and *P. aeruginosa* individually. For the tests for *E. coli* and *Salmonella*, inoculate the specified micro-organism individually.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see section 4-5-3 of general chapter *2.6.12*).

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralised, then it is to be assumed that the inhibited micro-organism will not be present in the product.

2-3-4-1. **Test for absence**. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation. Perform the test as described in the relevant paragraph in section 2-4 using the shortest incubation period prescribed. The specified micro-organisms must be detected with the indication reactions as described in section 2-4.

2-3-4-2. **Enumeration test**. Semi-quantitative test (probable-number method).

Use a number of micro-organisms equivalent to not more than 100 CFU per gram or millilitre of product. Perform the test as described in the relevant paragraph in section 2-4 using the shortest incubation period prescribed. The dilution corresponding to 0.1 g or 0.1 mL of product must be positive.

2-4. *TESTING OF PRODUCTS*

2-4-1. **BILE-TOLERANT GRAM-NEGATIVE BACTERIA**

2-4-1-1. **Enumeration test**. Semi-quantitative test (probable-number method).

2-4-1-1-1. *Sample preparation and pre-incubation*. Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate

the bacteria but not sufficient to encourage multiplication of the organisms (2-3 h).

2-4-1-1-2. *Selection and subculture.* Inoculate suitable quantities of enterobacteria enrichment broth-Mossel with the preparation as described above and/or, depending on the limit applied for the particular product, with 3 of the 4 dilutions of the preparation, which contain respectively 0.1 g, 0.01 g, 0.001 g and 0.0001 g (or 0.1 mL, 0.01 mL, 0.001 mL and 0.0001 mL) of the product to be examined. Incubate at 30-35 °C for 24-48 h. Subculture each of the cultures on a plate of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

2-4-1-1-3. *Interpretation.* Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result.

Determine from Table 2.6.31.-2 the probable number of bacteria.

Table 2.6.31.-2. – *Interpretation of results*

| Results for each quantity of product | | | | Probable number of bacteria per gram or millilitre of product |
|---|---|---|---|---|
| 0.1 g or 0.1 mL | 0.01 g or 0.01 mL | 0.001 g or 0.001 mL | 0.0001 g or 0.0001 mL | |
| + | + | + | + | $> 10^4$ |
| + | + | + | - | $< 10^4$ and $> 10^3$ |
| + | + | - | - | $< 10^3$ and $> 10^2$ |
| + | - | - | - | $< 10^2$ and $> 10$ |
| - | - | - | - | $< 10$ |

2-4-2. **ESCHERICHIA COLI**

2-4-2-1. **Test for absence**

2-4-2-1-1. *Sample preparation and pre-incubation.* Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described in section 2-3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

2-4-2-1-2. *Selection and subculture.* Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

2-4-2-1-3. *Interpretation.* Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

2-4-2-2. **Enumeration test**. Semi-quantitative test (probable-number method).

2-4-2-2-1. *Sample preparation and pre-incubation.* Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use the quantities corresponding respectively to 0.1 g, 0.01 g and 0.001 g (or 0.1 mL, 0.01 mL and 0.001 mL) to inoculate a suitable amount (determined as described in section 2-3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

2-4-2-2-2. *Selection and subculture.* Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

2-4-2-2-3. *Interpretation.* Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result.

Determine from Table 2.6.31.-3 the probable number of bacteria.

Table 2.6.31.-3. – *Interpretation of results*

| Results for each quantity of product | | | Probable number of bacteria per gram or millilitre of product |
|---|---|---|---|
| 0.1 g or 0.1 mL | 0.01 g or 0.01 mL | 0.001 g or 0.001 mL | |
| + | + | + | $> 10^3$ |
| + | + | - | $< 10^3$ and $> 10^2$ |
| + | - | - | $< 10^2$ and $> 10$ |
| - | - | - | $< 10$ |

2-4-3. **SALMONELLA**

2-4-3-1. **Test for absence**

2-4-3-1-1. *Sample preparation and pre-incubation.* Use 25 g or 25 mL of the product to be examined to inoculate 225 mL of buffered peptone medium and mix (e.g. homogenise in a filter bag by using a blender). Incubate at 30-35 °C for 18-24 h.

2-4-3-1-2. *Selection and subculture.* Transfer 0.1 mL of buffered peptone medium to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on plates of xylose, lysine and deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

2-4-3-1-3. *Interpretation.* The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the identification tests are negative.

*The following section is given for information.*

*RECOMMENDED SOLUTIONS AND CULTURE MEDIA*

The solutions and culture media mentioned in this chapter and described in general chapter *2.6.13* and the following buffered peptone medium have been found to be satisfactory for the purposes for which they are prescribed in this chapter. Other media may be used provided that their suitability can be demonstrated.

**Buffered peptone medium**

| | |
|---|---|
| Potassium dihydrogen phosphate | 1.5 g |
| Disodium hydrogen phosphate dodecahydrate | 9.0 g |
| Sodium chloride | 5.0 g |
| Peptone | 10.0 g |
| Purified water | 1000 mL |

Adjust the pH so that after sterilisation it is 7.0 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

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# 2.6.33. RESIDUAL PERTUSSIS TOXIN

The test for residual pertussis toxin is performed *in vitro*, using a Chinese Hamster Ovary (CHO) cell-based assay, and is intended for the assay of non-adsorbed purified pertussis components.

*Pertussis toxin BRP* is suitable as a reference pertussis toxin preparation.

The CHO cell-clustering assay (CHO assay) is based on the induction of clusters in non-confluent CHO cell cultures by active pertussis toxin. The cultures are then examined under a microscope and any clusters present are counted. The assay may be used as a quantitative test or as a limit test with the sensitivity determined within each assay using dilutions of a reference pertussis toxin preparation.

The sensitivity of the CHO assay to pertussis toxin is verified with *pertussis toxin BRP* or a suitable reference preparation calibrated in International Units using a suitable CHO assay. The assay sensitivity is defined as the lowest concentration in the reference preparation dilution series to produce a positive response, i.e. at least 10 CHO cell clusters. A suitable assay has a sensitivity of at least 5 mIU/mL.

*The following method is given as an example.*

**Reagents and equipment**

- *Pertussis toxin BRP.*
- *CHO-K1 cell line* (ATCC No. CCL-61 or ECACC No. 85051005).
- *Kaighn's modified Ham's F-12K medium.*

  Media that have a slightly different composition from that in Table 2.6.33.-1 may also be used. Proline is an essential component of the medium. Adjust to pH 7.2 ± 0.2 if necessary.

Table 2.6.33.-1. – *Kaighn's modified Ham's F-12K medium*

| | |
|---|---|
| **Amino acids** | |
| L-Alanine | 18.0 mg |
| L-Arginine hydrochloride | 0.4220 g |
| L-Asparagine monohydrate | 30.0 mg |
| L-Aspartic acid | 26.6 mg |
| L-Cysteine hydrochloride monohydrate | 70.0 mg |
| L-Glutamic acid | 29.0 mg |
| L-Glutamine | 0.2920 g |
| Glycine | 15.0 mg |
| L-Histidine hydrochloride monohydrate | 45.8 mg |
| L-Isoleucine | 7.88 mg |
| L-Leucine | 26.2 mg |
| L-Lysine hydrochloride | 73.0 mg |
| L-Methionine | 8.96 mg |
| L-Phenylalanine | 9.92 mg |
| L-Proline | 69.0 mg |
| L-Serine | 21.0 mg |
| L-Threonine | 23.0 mg |
| L-Tryptophan | 4.1 mg |
| L-Tyrosine disodium salt dihydrate | 13.5 mg |
| L-Valine | 23.0 mg |
| **Vitamins** | |
| Biotin | 70 µg |
| D-Calcium pantothenate | 0.5 mg |
| Choline chloride | 14.0 mg |
| Folic acid | 1.3 mg |
| *myo*-Inositol | 18.0 mg |
| Nicotinamide | 37 µg |
| Pyridoxine hydrochloride | 60 µg |
| Riboflavin | 40 µg |
| Thiamine hydrochloride | 0.3 mg |
| Vitamin B12 | 1.4 mg |
| **Inorganic salts** | |
| Calcium chloride, anhydrous | 0.1020 g |
| Cupric sulfate pentahydrate | 2 µg |
| Disodium hydrogen phosphate, anhydrous | 0.1155 g |
| Ferric sulfate heptahydrate | 0.8 mg |
| Magnesium chloride, anhydrous | 49.7 mg |
| Magnesium sulfate, anhydrous | 0.1920 g |
| Potassium chloride | 0.2850 g |
| Sodium bicarbonate | 2.5000 g |
| Sodium chloride | 7.5300 g |
| Zinc sulfate heptahydrate | 0.144 mg |
| **Other components** | |
| D-Glucose | 1.2600 g |
| Hypoxanthine sodium salt | 4.0 mg |
| Lipoic acid | 0.21 mg |
| Phenol red | 3.0 mg |
| Putrescine dihydrochloride | 0.32 mg |
| Sodium pyruvate | 0.2200 g |
| Thymidine | 0.7 mg |
| Water | to 1000 mL |

- *CHO cell culture medium.* Supplement Kaighn's modified Ham's F-12K medium with foetal bovine serum to obtain a final concentration of 10 per cent *V/V*. Add a sufficient quantity of 0.2 M L-glutamine solution to obtain a final concentration of 1 per cent *V/V*. An antibiotic/antimycotic solution may be added if required. Adjust to pH 7.2 ± 0.2 if necessary. Store at 5 ± 3 °C for a maximum of 3 weeks, protected from light.
- *Trypsin-EDTA solution* (trypsin 0.25 per cent).
- *Phosphate-buffered saline pH 7.4 (PBS)*, without calcium or magnesium. Dissolve 9.0 g of *sodium chloride R*, 0.144 g of *potassium dihydrogen phosphate R* and 0.795 g of *disodium hydrogen phosphate heptahydrate R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.
- *24-well flat-bottomed assay plates with lid.*
- *75 $cm^2$ tissue culture flasks.*
- *Low protein binding polypropylene microtubes.*

**CHO cell culture**. The cells are obtained from a cell bank and used between defined passage levels. CHO cells are cultured in tissue culture flasks containing 20 mL of CHO cell culture medium in a humidified incubator set at 37 °C and 5 per cent $CO_2$. The cells are passaged at a ratio of 1:5 to 1:20 as they approach confluence.

Before use, allow all reagents to reach room temperature or warm to 37 °C.

To passage the cells, remove the CHO cell culture medium. Wash the cell layer gently by rinsing briefly 2-3 times with PBS. Remove the PBS and add 2 mL of trypsin-EDTA solution to the flask, allowing it to cover the monolayer for 20 s. Remove the trypsin-EDTA solution by aspiration. Immediately observe the culture using phase-contrast microscopy. When the cells begin to contract, tap the side of the culture flask firmly to dislodge the cells. When the majority of cells are free-floating, add 10 mL of CHO cell culture medium to stop the trypsin digestion. Trypsinisation used for cell detachment must be carefully controlled to avoid over-digestion which causes cleavage of either pertussis toxin receptors or cell adherence proteins, or both.

Determine the cell concentration and viability using trypan blue exclusion or another appropriate method. The cell viability must be greater than 95 per cent to continue. Transfer a sufficient volume of the cell suspension to a fresh flask for a 1:5 to 1:20 passage ratio and add CHO cell culture medium to bring the volume to 20 mL. Incubate the cells in a humidified incubator set at 37 °C and 5 per cent $CO_2$ for at least 48 h prior to use in the CHO assay.

**Seeding density for CHO assay**. Prepare a suspension of CHO cells using the trypsinisation procedure described above. Count and dilute the cell suspension to between $4 \times 10^4$ and $8 \times 10^4$ CHO cells/mL in CHO cell culture medium. The cell concentration selected must allow clusters to be identified at the end of the stimulation period. Transfer 250 µL of the cell suspension into each well of the 24-well plates. Store the seeded plates in a humidified incubator set at 37 °C and 5 per cent $CO_2$ while the reference preparation and test sample are prepared.

**Preparation of pertussis toxin reference**. Reconstitute *pertussis toxin BRP* as stated in the leaflet accompanying the reference preparation. Prepare 7 two-fold serial dilutions in CHO cell culture medium, in such a way that the assay end-point occurs in the middle of the dilution series. The dilutions are prepared in low protein binding polypropylene microtubes. One dilution series for the reference preparation is included in each assay plate.

**Preparation of non-adsorbed purified pertussis component samples**. Dilute the test sample in CHO cell culture medium to obtain the highest concentration of the test sample which does not significantly dilute the nutrients of the medium, in order to maximise the sensitivity of the assay. Prepare a dilution series as described for the reference preparation.

**Stimulation of CHO cells**. Transfer 250 µL from each tube of the reference preparation dilution series into the assigned wells of the assay plates seeded with CHO cells. Similarly, transfer 250 µL of each dilution of the test sample into the assigned well(s). Transfer 250 µL of CHO cell culture medium into the negative control wells. Return the assay plates to a humidified incubator set at 37 °C and 5 per cent $CO_2$ for 48 h.

**Scoring and interpretation of results**. Observe the cell cultures using phase contrast microscopy at a magnification of 4× or 10×. In all wells the cell cultures must not be confluent and must have sufficient growth space to allow any clusters present to be counted. Assign a positive score when 10 or more CHO cell cluster formations are clearly evident within a single well. Assign a negative score to wells containing fewer than 10 clusters. The end-point concentration is the lowest concentration at which there is a positive score.

**Assay validity**

The test is not valid unless:

- the negative control wells show no evidence of clustering.
- wells containing the reference toxin preparation at a concentration greater than or equal to 5 mIU/mL exhibit a positive response (i.e. 10 or more CHO cell clusters).
- a clear end-point dilution is observed for the reference preparation.
- replicate values for the end-point of the reference preparation do not differ by more than one 2-fold dilution within one assay.

**Calculation**

Calculate the residual pertussis toxin activity in the test sample in International Units per millilitre, relative to the reference preparation, using the following expression:

$$\frac{GM(end-point)_S}{GM(end-point)_R} \times A_R$$

$GM(end\text{-}point)_S$ = geometric mean of the reciprocal of the end-point dilutions (last dilution at which there is a positive score) for the test sample;

$GM(end\text{-}point)_R$ = geometric mean of the reciprocal of the end-point dilutions (last dilution at which there is a positive score) for the reference preparation;

$A_R$ = activity of the reference preparation (stock solution), in International Units per millilitre.

*Example*

Series of 7 two-fold dilutions were prepared for the reference preparation and test sample. In this example, the reference stock solution has an activity of 1000 IU/mL. The scores observed at each dilution of the reference preparation and test sample, the reciprocal end-point dilutions and the geometric means of reciprocal end-point dilutions are shown in Tables 2.6.33.-2 and 2.6.33.-3.

Table 2.6.33.-2 – *Reference preparation dilutions and scores*

| **Reference preparation dilution** | **Score** | |
|---|---|---|
| | Rep. 1 | Rep. 2 |
| 1:100 000 | + | + |
| 1:200 000 | + | + |
| 1:400 000 | + | + |
| 1:800 000 | + | - |
| 1:1 600 000 | - | - |
| 1:3 200 000 | - | - |
| 1:6 400 000 | - | - |
| **End-point dilutions** | 1:800 000 | 1:400 000 |
| Reciprocal of end-point dilutions | 800 000 | 400 000 |
| ***GM(end-point)*$_R$** | 565 685 | |

Table 2.6.33.-3 – *Test sample dilutions and scores*

| Test sample dilution | Score | |
|---|---|---|
| | Rep. 1 | Rep. 2 |
| 1:100 | + | + |
| 1:200 | + | + |
| 1:400 | + | + |
| 1:800 | + | + |
| 1:1600 | + | - |
| 1:3200 | - | - |
| 1:6400 | - | - |
| **End-point dilutions** | 1:1600 | 1:800 |
| Reciprocal of end-point dilutions | 1600 | 800 |
| $GM(end\text{-}point)_s$ | 1131 | |

The residual pertussis toxin activity in the test sample can be determined as follows: (1131/565685) × 1000 = 2 IU/mL.

**04/2017:20634**

# 2.6.34. HOST-CELL PROTEIN ASSAYS

*This general chapter provides guidance for the development and validation of host-cell protein (HCP) assays used to test products obtained by recombinant DNA technology. It does not exclude the use of alternative approaches that are acceptable to the competent authority.*

INTRODUCTION

Host-cell proteins (HCPs) are process-related impurities derived from the host organism used for the production of a medicinal product by recombinant DNA technology. In order to mitigate their potential adverse effects (e.g. immunogenicity), HCP content is expected to be reduced to the lowest possible level.

HCP clearance during the purification process must be assessed and the HCP content determined using an HCP assay that has been evaluated and validated for a given product.

The HCP acceptance limit, typically expressed in nanograms of HCP per milligram of active substance (ppm), must be justified with regard to the HCP clearance capacity of the purification process and with regard to the potential impact of residual HCP on patients, taking into account the worst-case quantity of HCP that could be administered with the product.

HCPs are generally measured using an immuno-based assay containing, as reagents, the HCP antigen preparation (hereinafter 'the HCP antigens') or HCP reference standard and the corresponding polyclonal antibodies (antisera). Antisera must cover a broad spectrum of HCPs representative of the product concerned.

Sandwich-type enzyme-linked immunosorbent assays (ELISA) are the most commonly employed assays to assess quantitatively the level of HCPs. It should be noted that HCP content measured by ELISA does not represent absolute HCP mass content. The sensitivity is the result of the observed cumulative responses of many individual HCPs in comparison to the response of an HCP reference standard. The use of orthogonal analytical methods (e.g. electrophoresis, HPLC, Western blot, mass spectrometry) to characterise the various HCPs in the product is recommended to support the development and selection of the assay.

ASSAY SELECTION

Several types of assay are available, with selection taking into account several factors, including the stage of development of the product, the nature of the host cell and the protein immunogenicity, the expression mode, the manufacturing process, and prior knowledge. When selecting and developing the assay, its life cycle (e.g. reagent supply, consistency, assay validation, process change) must also be considered.

*TYPES OF ASSAY*

**Process-specific assays**

Process-specific HCP assays (also called product-specific HCP assays) are developed and validated taking into account the specificity of the production process, and using the same host organism expressing the recombinant product.

The HCP antigens are derived from a mock run of the active substance manufacturing process (or a process representative of it) up to a step capable of generating a broad spectrum of HCPs in sufficient quantities.

The antisera raised must cover a broad range of HCPs, in order to detect as many different HCPs as possible and also to accommodate process variations.

**Platform assays**

Platform assays are developed by individual manufacturers and customised for the processes and host organism used by the manufacturer for production. The same sets of reference standards and reagents may be used to monitor HCPs in several products manufactured in the same host organism, provided that upstream processes (and downstream, if relevant) are sufficiently similar for these products. The suitability of the antiserum should be evaluated as described above for process-specific assays.

**Generic assays**

Commercially available HCP test kits are commonly referred to as generic HCP assays. They are intended to work broadly across similar expression hosts. Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the HCP antigens may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest. The suitability of the antiserum should be evaluated as described above for process-specific assays.

*CRITERIA FOR ASSAY SELECTION*

In view of the potential safety issues associated with residual HCPs in the active substance, a risk assessment is performed to support the choice between a generic, a platform or a process-specific strategy, taking into account the stage of development of the product.

For early development a generic assay or a platform assay may be used. For later development phases, process-specific assays must be considered, as they are generally regarded as superior, especially when compared to generic assays. This is because process-specific assays are more likely to show immunoreactivity against representative HCPs.

Platform or generic assays may be used, provided that the assay is appropriately characterised and validated against process-specific HCPs.

PRODUCTION AND TESTING OF THE HCP ANTIGEN PREPARATION

The HCP reagents (HCP antigens and anti-HCP antibodies) are produced in such a way as to facilitate replication of the production when a replenishment for the HCP assay is needed.

The HCP antigens are used to generate the polyclonal antibody reagent for the HCP immunoassay by immunising one or more suitable animal species. In addition, they serve as the HCP reference standard in the HCP immunoassay.

As far as possible, the HCP antigens must cover the relevant HCP population expected to be derived from the manufacturing process of the protein of interest.

The HCP population must also be broad enough to cover worst-case purification scenarios and to provide robustness against potential manufacturing process changes during the life cycle of the product.

*PROCESS-SPECIFIC ASSAYS*

**Null cell line**

Development of a process-specific assay involves the selection of a null cell line that does not contain the expression gene for the product of interest and is derived from the same cell line that has been used to establish the production cell line. This null cell line may be non-transfected or mock-transfected. A mock-transfected cell line is created by transfecting the parental cell line with a blank plasmid, i.e. the plasmid used to create the production cell line, but missing the gene coding for the protein of interest.

**Mock production process**

*Upstream*

The antigens produced for process-specific assays are obtained by a mock production process that mimics the intended manufacturing process, using the null cell line and, as far as possible, the same operating conditions.

As for any mock production, the process used represents an approximation of the intended manufacturing process and leads to differences (e.g. different scale, operating parameters, product interaction). However, the impact of those differences needs to be considered carefully because they may affect the composition of the HCP population.

For example, a mock fermentation of an inclusion body manufacturing process may not deliver the desired inclusion bodies if the product is not present. Therefore, depending on the null cell line used (e.g. mock-transfected or not), the antigens may need to be isolated differently compared to the intended manufacturing process.

In some situations, operating parameters for the mock production may be adjusted to cover worst-case scenarios (e.g. to deliver antigens covering a broad spectrum of different HCP species). For example, the antigen-containing cell culture supernatant may be harvested beyond the minimum level of cell viability in order to include more cytosolic proteins, which are released by additional cell lysis.

*Downstream*

The HCP antigens derived from the upstream process are usually only minimally processed (filtration, concentration), in order to obtain a representative spectrum of HCPs. Further purification is generally not recommended as there will be a risk of losing HCP species.

However, in cases where the antigens are not representative (e.g. resulting in low coverage), mixing of mock materials from different processing steps can be considered. Enrichment may also be achieved by pooling materials from mock fermentation or purification runs using different operating conditions, or from selective purification steps (e.g. to reduce large amounts of the few immunodominant HCPs).

**Cross-contamination with the protein of interest**

The HCP antigens must be produced in a manner that avoids contamination with even minute traces of the product in order to avoid cross-reactivity with the polyclonal antibodies.

To achieve this goal, dedicated or single-use equipment is used as much as possible. Where multi-purpose equipment is used, it must be cleaned appropriately. In addition, the risk of contamination when filling or handling the antigens in the laboratory environment must also be considered.

**Characterisation and testing**

Before using the HCP antigens for immunisation, the protein content is assessed (total protein assay) and the absence of the protein of interest verified.

Comparison of the HCP population with the mock and the intended production process is performed, typically by SDS-PAGE and/or two-dimensional (2D) electrophoresis with a high sensitivity stain. The aim of this comparison is to show that the HCP antigens resulting from the mock production process contain most of the representative HCP species of the intended manufacturing process. Where necessary, complementary information may be gathered by orthogonal methods, e.g. mass spectrometry.

*PLATFORM ASSAYS*

**Null cell line**

Development of a platform assay involves a null cell line that does not contain the expression gene for the product of interest, and uses the same host species. This null cell line may be non-transfected or mock-transfected, and may be used for the production of HCP antigens for products from a given company's manufacturing platform.

**Mock production process**

*Upstream*

The HCP antigens produced for platform assays are obtained by a mock production process that mimics the platform upstream process that is used for several products, and typically uses the same media components. As for any mock production, the process used represents an approximation of the intended manufacturing process, which may impact the composition of the HCP population (see process-specific assays).

*Downstream*

As for other assays, the HCP antigens derived from the upstream process are, in general, only minimally processed (e.g. no or limited number of purification steps) to obtain a broad spectrum of HCPs, although mixing and pooling strategies may also be used to widen the spectrum of HCP species.

**Characterisation and testing**

As for process-specific assays, both the protein content and the absence of the protein of interest are tested. Comparison of the HCP population with the mock and the intended production process is performed.

*GENERIC ASSAYS*

Generic assays are commercially available and are developed by the vendor.

Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the null cell line may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest.

Nevertheless, the generic assay must be selected with consideration given to the intended manufacturing process (e.g. appropriate host cell line), and be appropriately validated for the product of interest and phase of development. As a consequence, if generic assays are used in later stages of development or during commercial manufacturing, it is recommended to validate the assay and control lot-to-lot reagent consistency using either appropriate upstream fractions from the production process or a mock preparation generated using a null cell line.

PRODUCTION AND CHARACTERISATION OF THE ANTI-HCP ANTIBODY REAGENT

*PROCESS-SPECIFIC AND PLATFORM ASSAYS*

**Immunisation**

One of the challenges of the immunisation step is to generate polyclonal antibodies that are highly specific and sensitive for each of the antigenic proteins in the complex mixture of HCPs used as an immunogen. An animal's immune response must be stimulated against both the stronger and the weaker antigens.

An animal host that yields a sufficient quantity and diversity of HCP-specific immunoglobulin G (IgG) is selected.

Where both the polyclonal capture and the polyclonal detection antibodies are from the same source, it can be assumed that they recognise different epitopes on the same HCP in the assay. Alternatively, polyclonal anti-HCP antibodies from different animal species may be used. Using several animals for a given species may reduce the impact of individual variations in immune competence and provide additional response diversity, resulting in maximised antibody coverage against the HCP antigens.

An immune response to a limited number of HCP antigens may be obtained rapidly, particularly when adjuvants are used to boost the immune response. However, in complex mixtures, differential enhancement of the immune response towards weaker antigens or those at lower concentrations may be necessary.

It usually takes several immunisations to reach a maximum immunological response and, depending on the frequency of immunisation, the process can take 3-6 months to complete.

The immune response against the HCPs for a given immunisation scheme has to be monitored by determining the antibody titre using, for example, an ELISA, and by comparing the results of 1D or 2D electrophoresis after protein staining and a Western blot, where the polyclonal anti-HCP antibodies are used as primary antibody. In practice, some minor proteins that elicit a strong immune response may not be visible in the protein-stained gel, and some poorly antigenic proteins that are detectable by protein staining may not elicit a detectable immune response. To achieve sample-dilution linearity in complex multi-analyte immunoassays, it is essential that the immune reagent simultaneously and specifically recognises as many individual analytes as possible in an assay sample and that it is present in stoichiometric excess. For this purpose, a series of sample dilutions from different process steps may be tested by ELISA using purified anti-HCP antibodies from bleedings that have shown suitable coverage by Western blot.

Finally, based on the results of the tests described above, antisera from different animals are pooled, retested and purified.

**Purification and preparation**

The HCP antibodies must be purified before an assay can be developed.

Typically, this is achieved by protein A- or protein G-chromatography and/or HCP antigen affinity chromatography. In the case of HCP antigen affinity chromatography, the antigens used for immunisation are immobilised on column chromatography media and the specific antibodies are captured by applying the antisera onto the column.

Additional purification to remove potential aggregates might be required by gel permeation chromatography.

For the ELISA, a part of the purified anti-HCP antibodies is conjugated to a detection label (e.g. biotin or horseradish peroxidase).

The purified anti-HCP antibodies and the sera must be stored at a temperature that ensures their stability.

**Characterisation and testing**

The suitability of the derived HCP assay reagent is assessed by demonstrating the coverage of the HCPs representative of the manufacturing process by the anti-HCP antibodies.

For this purpose, 2D electrophoresis of the HCP antigens is performed. The protein pattern of the immunostain is compared with the protein pattern of the total stain. The anti-HCP antibodies must recognise a broad range of HCPs over the full range of charge and molecular size. Other methods using native conditions may be considered.

*GENERIC ASSAYS*

Immunisation, purification and preparation of the anti-HCP antibody reagent are carried out by the vendor and details may not be available.

Characterisation and testing of anti-HCP antibodies are performed as for other assay types. Typically, there is limited control over lot-to-lot reagent consistency. Appropriate comparative lot testing is therefore required.

VALIDATION OF THE HCP ASSAY

The HCP ELISA is developed to detect and quantify a heterogeneous mixture of antigens at varying concentrations, and with a reagent containing antibodies that are not represented at a one-to-one ratio. The section below is intended to target the specifics in development and validation of this type of ELISA.

HCP assays such as ELISA are validated with regard to accuracy, specificity, precision, quantitation and detection limits, linearity, range and robustness.

During the life cycle of the product, a full or partial revalidation of the assay may be required, for example when implementing a manufacturing process change that may impact the suitability of the HCP reagent.

**Accuracy**

Accuracy is demonstrated by spike/recovery analysis of the HCP reference standard in a relevant background matrix (e.g. the active substance or a sample from a relevant purification step).

**Specificity**

Specificity is demonstrated by the absence of interference from the matrix background (including the active substance). For instance, data from the accuracy study can be used to assess specificity.

**Precision**

As for any other quantitative assay, repeatability, intermediate precision and reproducibility are appropriately demonstrated.

**Quantitation and detection limits**

Sensitivity is usually in the ppm range and is normally described through the quantitation limit (QL). QL is typically determined by HCP spike recovery studies in the active substance or an appropriate sample matrix, and is calculated from the minimal spike providing a response with predefined accuracy and precision from replicate analyses.

Detection limit (DL) is often not determined (optional validation parameter).

**Linearity**

The linearity of the HCP assay is demonstrated using dilution series of the HCP standard and spike/recovery experiments (accuracy study).

Additionally, due to the nature of HCP assays, the multiple HCP analytes and polyclonal anti-HCP antibodies, sample-dilution non-linearity may be observed, i.e. back-calculated results increase with increasing dilutions of samples, which in most cases is related to the excess of one or more individual HCPs in the sample when compared to the available antibodies in the HCP immunoassay. As a consequence, dilution linearity must be properly assessed for the relevant process steps by comparison of target versus measured HCP concentrations at varying sample dilutions. Dilution linearity is demonstrated if the acceptance criteria for assay variation are met for different sample dilutions. Studies demonstrating dilution linearity can be carried out either during method development or at the latest during method validation.

If a sample shows dilution non-linearity, multiple sample dilutions are prepared beyond the range where non-linear behaviour is observed.

The final HCP value is typically reported as the average HCP concentration obtained for a minimum of 2 dilutions within the linear dilution range. If justified, 1 dilution may be sufficient.

**Range**

The range of the assay is typically defined by the HCP concentrations for which a suitable level of precision, accuracy and linearity has been demonstrated.

**Robustness**

The evaluation of robustness is considered during the development phase.

CHANGE OF HCP ASSAY AND/OR REAGENT

The quantities of antigens and antibodies must be large enough to supply the HCP assay for several years. Therefore, the supply, quality and consistency of reagents must be appropriately managed throughout the life cycle of the assay.

For generic HCP assays, in order to ensure the consistency and quality of the reagents, recharacterisation or revalidation of the assay may be required for each new batch of reagent, as their quality may change from one batch to another.

For process-specific and for platform assays, there are generally 2 situations where new HCP assay reagents may be required:

- the HCP reference standard and/or antibody are depleted; antibody may then be purified from a frozen serum stock or a new immunisation is required;
- a manufacturing process change can impact the HCP composition for the purification intermediates or the final product; the assay reagent may not be properly suited to detect and quantify the modified HCPs; a manufacturing process change can therefore render the reagents unsuitable for assay use.

Newly prepared reagents must be thoroughly characterised (e.g. by 2D-SDS-PAGE/Western blot for coverage, 2D-SDS-PAGE/differential gel electrophoresis (DIGE)/identification by MS). Afterwards, the validation status of the assay using the new reagents must be assessed. It is recommended to perform these experiments side-by-side with the currently used reagents.

Table 2.6.34.-1 outlines a recommendation for reagent characterisation and assessment of immunoassay validation, as a consequence of a depletion of assay reagents or a process change.

Table 2.6.34.-1

| | Depleted reagents | | Process change |
|---|---|---|---|
| | **HCP reference standard** | **Anti-HCP antibody** | |
| Reagent characterisation | The protein concentration of the new reference standard is determined preferably using the same method as for the current reference standard to ensure that the protein concentrations are comparable.<br>Using suitable methods (e.g. 1D-/2D-PAGE, 2D-DIGE), the similarity in protein composition between the new and current HCP reference standards is assessed. | Total protein concentration of the new antibody is determined. The final assay concentration must be titrated for the new lot in order to achieve a similar standard curve as for the current lot.<br>For detection antibodies, the detection label: protein stoichiometry is controlled and ensured to be similar to the current antibody lot.<br>Immunoreactivity of the new antibody is compared qualitatively (by visual comparison) or semi-quantitatively (coverage determination) against the current lot by suitable methods (e.g. 1D or 2D Western blot). Due to the variability of the method, it is particularly advisable to perform this characterisation side-by-side with the current antibody lot. | The effects of process changes that have the potential to impact the HCP composition are analysed by suitable methods (e.g. 1D-/2D-PAGE, Western blot, HCP assay).<br>If the process change does not lead to a relevant change in HCP composition, the current HCP reagents are also suitable for the new process.<br>If the process change does lead to a relevant change in HCP composition, but the suitability of the current HCP reagents was demonstrated, the current HCP reagents are also suitable for the new process.<br>If the process change does lead to a relevant change in HCP composition, but the current HCP reagents were shown to be unsuitable for the new process, a new assay must to be developed including a mock fermentation according to the new process and a new immunisation. |
| Testing of reagents in HCP assays | The new HCP reference standard is quantitatively tested against the current reference standard for spike recovery at different concentrations covering the validated assay range.<br>Standard curves obtained with the new versus the current reference standard are assessed for similarity. | Standard curves obtained when using new versus current antibody lots are compared.<br>A bridging study is performed with testing of relevant process samples (e.g. purification steps from harvest to the final active substance). In a side-by-side experiment, new antibodies must detect HCP levels at different process steps equally or with an improved quantitation limit. | A mock run harvest from the new process is tested for spike recovery using the current HCP assay.<br>Relevant process samples (e.g. purification steps from harvest to the final active substance) from the new and the previous process are tested side-by-side. |

| | Depleted reagents | | Process change |
|---|---|---|---|
| | HCP reference standard | Anti-HCP antibody | |
| Assessment of validation status | If reagent characterisation and ELISA testing demonstrate suitability of the new HCP reference standard, the current reference standard can be replaced. No revalidation of the test method is required.<br>If the new HCP reference standard differs significantly in protein composition and/or assay performance from the current reference standard, revalidation is required. | If reagent characterisation and ELISA testing demonstrate that the new antibody is suitable, the current antibody can be replaced. No revalidation of the test method is required.<br>If the new antibody differs significantly in Western blot immunoreactivity or immunoassay sensitivity and/or assay performance compared to the current antibody, revalidation is required. | If, for the new process, the antibody shows similar or higher immunoreactivity compared to the previous process, and the HCP assay shows adequate recovery from the mock harvest of the new process and also similar or higher sensitivity for samples from the relevant process steps, then the current assay and reagents are considered suitable for the new process. No revalidation of the test method is required.<br>If reagents appear suitable to detect HCP from the new process, but the ELISA indicates significant differences in spike recovery of the mock sample or of HCP levels at relevant process steps, then revalidation is required. The process change might also impact dilution linearity of test samples from certain process steps; if these steps are essential for the HCP control strategy, revalidation or even generation of new antibody reagents might be required.<br>In case of a major change in HCP composition with the new process that leads to either a mismatch in protein composition compared to the current assay standard, reduced immunoreactivity of the antibody, or significantly decreased immunoassay sensitivity, then new assay reagents are prepared and the HCP assay is validated with the new reagents. |
| PAGE: polyacrylamide gel electrophoresis<br>DIGE: differential gel electrophoresis | | | |

**01/2020:20635**

# 2.6.35. QUANTIFICATION AND CHARACTERISATION OF RESIDUAL HOST-CELL DNA

*This general chapter describes analytical methods that may be used to measure the content and to characterise the size of residual host-cell DNA in biological products produced in cell substrates. It does not exclude the use of alternative approaches that are acceptable to the competent authority.*

## INTRODUCTION

Several sensitive analytical methods exist for the quantification of residual host-cell DNA, including real-time quantitative PCR (qPCR) (Method A) and an immunoenzymatic method (Method B).

qPCR may also be used to assess the residual host-cell DNA size distribution, as a characterisation test depending on the nature of the cell substrate (e.g. continuous cell lines) and on the amount of residual host-cell DNA.

A suitable method is selected depending on the nature of the biological product to be tested and taking into account the characteristics and limitations of each method as summarised in Table 2.6.35.-1.

Table 2.6.35.-1 - *Comparison of the characteristics of qPCR and immunoenzymatic methods*

| Characteristics | qPCR (Method A) | Immunoenzymatic method (Method B) |
|---|---|---|
| Can be used to assess DNA size distribution | Yes | No |
| Limit of quantification (*may vary depending on the matrix, method and interfering substances*) | 0.01-10 pg/mL | 2-10 pg/mL |
| Specificity (total DNA versus specific DNA) | Specific DNA | Total DNA |
| Interfering substances | Proteins | Detergents/proteins/solvents/RNA |
| Limitations | Fragments smaller than the PCR product can not be detected and quantified. | Not applicable for DNA-based products.<br>The DNA content is underestimated for fragments below 1000 base pairs (bp).<br>Detectability depends on DNA size.<br>Undetectable short DNA fragments (below 80 nucleotides) can interfere with the assay through reagent consumption.<br>Products must be free of bacterial DNA.<br>Narrow quantification range: 5-150 pg/well. |

SAMPLE PREPARATION

The concentration of residual host-cell DNA may vary depending on the type of biological product and the DNA clearance capacity of the manufacturing process.

Depending on the sample matrix, pretreatment of the sample may be necessary to ensure appropriate recovery of the residual host-cell DNA.

When analysing highly purified protein samples such as recombinant proteins or monoclonal antibodies, simple digestion with proteinase or affinity chromatography may be sufficient to recover the residual host-cell DNA. For more complex matrices such as viral vaccines and viral vectors, an additional virus lysis step may be required to release the residual host-cell DNA from the viral particles.

The immunoenzymatic method is especially sensitive to interference from proteins. This can be avoided by performing an initial pretreatment step, which may include digestion with proteinase K and sodium dodecyl sulfate (SDS). This step may suffice to recover the residual host-cell DNA. However, in other cases, the residual host-cell DNA may be bound to the sample components, and/or soluble interfering substances may be present, in which case it may be necessary to extract the residual host-cell DNA from the sample.

The DNA can be extracted using protocols which have demonstrated a satisfactory recovery rate during spiking experiments. Several suitable methods exist, including DNA precipitation or DNA-specific binding to a matrix (e.g. magnetic beads or silica columns). Commercial kits can be used to extract residual host-cell DNA samples. Some of these kits use a chaotrope (sodium iodide) and a detergent (sodium lauroylsarcosinate) to disrupt the association between the residual host-cell DNA and the sample components. The residual host-cell DNA in the sample is then recovered by co-precipitation with a carrier molecule such as glycogen in the presence of ethanol or 2-propanol. Several independent extraction procedures may be required, depending on the reproducibility of the spike recovery. Negative controls must be included in each extraction procedure. In some cases, dilution of the samples may be recommended to reduce the matrix effect. A correction factor may also be applied to take into account the recovery rate of the spike.

METHOD A – REAL-TIME QUANTITATIVE PCR (qPCR)

This method can be used to quantify a cellular DNA target sequence from a variety of samples. For the quantification of residual host-cell DNA, qPCR targeting either a stable sequence within a highly conserved host-cell region or targeting repetitive elements to enhance the sensitivity of the test can be used. When repetitive elements are targeted, it may be difficult to eliminate potential background noise due to environmental DNA (e.g. when using Alu human sequences). The specificity of the qPCR method must be established during the validation studies by demonstrating the absence of cross-reactivity with unrelated sequences.

Alternatively, digital PCR methods may be used.

**qPCR amplification**

The detection and quantification of residual host-cell DNA by qPCR may involve the use of either a non-specific fluorescent dye that intercalates with any double-stranded DNA, or sequence-specific DNA probes. The qPCR principle described in general chapter *2.6.21. Nucleic acid amplification techniques* applies.

The number of cycles required for the fluorescent measurement to exceed a threshold value (Ct or Cp) correlates to the starting amount of residual host-cell DNA in the sample.

If several extractions are performed, the resulting samples must be analysed at an appropriate dilution.

PCR negative controls are used.

A standard curve is plotted using serial dilutions of host-cell genomic DNA in order to allow residual host-cell DNA levels in biological products to be determined based on their Ct or Cp values. The use of carefully characterised representative genomic DNA extracted from the cells used for the production of the biological product is recommended for the preparation of the standard.

The same methodology is applied for residual host-cell DNA size evaluation. At least 2 sets of primers can be designed to amplify overlapping fragments of different sizes in the target sequence.

**Suitability criteria**

*Control samples*. In order to control the risk of contamination and to ensure adequate sensitivity, each PCR assay includes the following controls:

- *a negative control for qPCR* and *a negative control for extraction*, composed of a sample of a suitable matrix already proven to be free of the target sequence(s);
- *a positive control for qPCR*, which contains a defined number of target sequence copies or a defined DNA concentration which is determined individually for each assay system;
- *a control for extraction*, typically an internal control added to the test material as a defined concentration or number of target-sequence copies. In this case, the amplicons must be clearly discernible and may be detected in a separate qPCR. Alternatively, an external control consisting of test sample spiked with a well-characterised level of genomic DNA may be used.

Extraction recovery must fall within predetermined values based on the performance of the assay as demonstrated during assay validation.

*Genomic DNA standard curve*. The standard curve is linear over the chosen range.

The coefficient of determination $R^2$ associated with the standard curve must be greater or equal to 0.98. The PCR efficiency falls within pre-established limits.

The coefficient of variation for the different extracts or replicates is not higher than a predefined criterion.

**Calculation**

If several extractions are performed, each extracted sample is analysed individually. The residual host-cell DNA content is calculated from the genomic standard curve by averaging the values obtained for the different extractions or replicates. A correction factor may also be applied to take into account the recovery rate for total DNA quantification in the samples.

For the characterisation of residual host-cell DNA size, the distribution of the overlapping fragments of different sizes is calculated as the ratio of the number of copies for each amplicon size to the number of copies of the smallest amplicon size.

METHOD B – IMMUNOENZYMATIC METHOD

The immunoenzymatic method is a non-specific technique for the quantification of residual host-cell DNA (regardless of its origin). It is therefore also a total DNA assay, and consequently, it is not only critical to avoid contamination through environmental DNA, but all materials and reagents used must be DNA-free. The samples to be tested must be free of microbial contamination and all samples, controls and standards must be processed under controlled conditions until the denaturation step.

By design, the method detects single-stranded DNA.

**Principle**

This total DNA assay consists of 4 steps:

- *denaturation and formation of complexes*, where the DNA is denatured into single-stranded DNA by heating the sample. The denatured DNA is mixed with a single reagent that contains a single-stranded DNA-binding protein

conjugated to streptavidin and a monoclonal anti-DNA antibody conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but are not sequence-specific. The liquid phase, in the presence of streptavidin, facilitates the formation of a complex with the single-stranded DNA from the sample.

– *filtration*, where the complex is filtered through a biotinylated nitrocellulose membrane. The biotin in the membrane captures the complexes by binding to streptavidin. The membrane is washed to remove any unbound reagents. Non-specific binding is avoided by the use of an albumin-coated nitrocellulose membrane.

– *detection*, where the membrane is placed in a reader, which contains a urea solution that reacts with the urease in the DNA complex and produces ammonia. The associated change in pH is measured by a potentiometric sensor in µV/s and is directly proportional to the amount of DNA in the sample.

– *analysis*, where the raw data from the sample and from the standard curve are analysed using appropriate software to determine the residual host-cell DNA content in the sample.

All samples and negative controls are tested spiked and unspiked. The spike solution (1000 pg/mL) is prepared by dilution of a concentrated standard (calf thymus DNA) at 5000 pg/mL.

**Suitability criteria**

*Control samples*

– the amount of DNA in the positive control falls within the range indicated on the batch certificate provided by the supplier;

– spike recovery in the negative control is between 80 per cent and 120 per cent.

*Samples*

– when several replicates are analysed, the coefficient of variation for the different replicates is not higher than a predefined criterion;

– spike recovery is between 80 per cent and 120 per cent.

**Calculation**

The content of residual host-cell DNA is calculated in picograms per millilitre using the following expression:

$$\frac{ID \times (C - A)}{V}$$

*ID* = ratio for dilution and sampling;

*C* = raw mean value (in picograms per tube) for the test tubes containing the diluted sample;

*A* = raw mean value (in picograms per tube) for the test tubes containing the negative control;

*V* = volume in the test tube, in millilitres (usually 0.5 mL per tube).

Where necessary, this result may be corrected by the extraction recovery (e.g. mean recovery for a given product).

**04/2019:20636**

# 2.6.36. MICROBIOLOGICAL EXAMINATION OF LIVE BIOTHERAPEUTIC PRODUCTS: TESTS FOR ENUMERATION OF MICROBIAL CONTAMINANTS

## 1. INTRODUCTION

The tests described hereafter will allow enumeration of live biotherapeutic product (LBP) contaminants: mesophilic bacteria and yeasts/moulds that may grow under aerobic conditions.

The tests are designed primarily to determine whether an LBP substance or preparation complies with the established specifications for microbial contamination.

Alternative microbiological procedures (*5.1.6*), including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

## 2. GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the LBP to be tested. The precautions taken to avoid contamination must be such that they do not affect any contaminant micro-organisms that are to be revealed in the test.

If the LBP to be tested prevents the enumeration of contaminant micro-organisms, apply the decision tree shown in Figure 2.6.36.-1.

If components other than the active substance (i.e. the micro-organism) of the LBP to be tested have inhibitory activity, this is insofar as possible removed or neutralised as described in general chapter *2.6.12*. If inactivators are used for this purpose, their efficacy and their absence of toxicity for the target contaminant micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for the target contaminant micro-organisms and their compatibility with inactivators used must be demonstrated.

## 3. ENUMERATION METHODS

Use the plate-count or the most-probable-number (MPN) method. The MPN method is generally the least accurate method for microbial counts.

The choice of method is based on factors such as the nature of the LBP and the required limit of microbial contamination. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen must be established.

## 4. GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

### *4-1. GENERAL CONSIDERATIONS*

The ability of the test to detect microbial contamination in the presence of the LBP to be tested must be established.

Suitability must be confirmed if a change in testing performance, or in the LBP, which may affect the outcome of the test is introduced.

### *4-2. PREPARATION OF TEST STRAINS*

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable

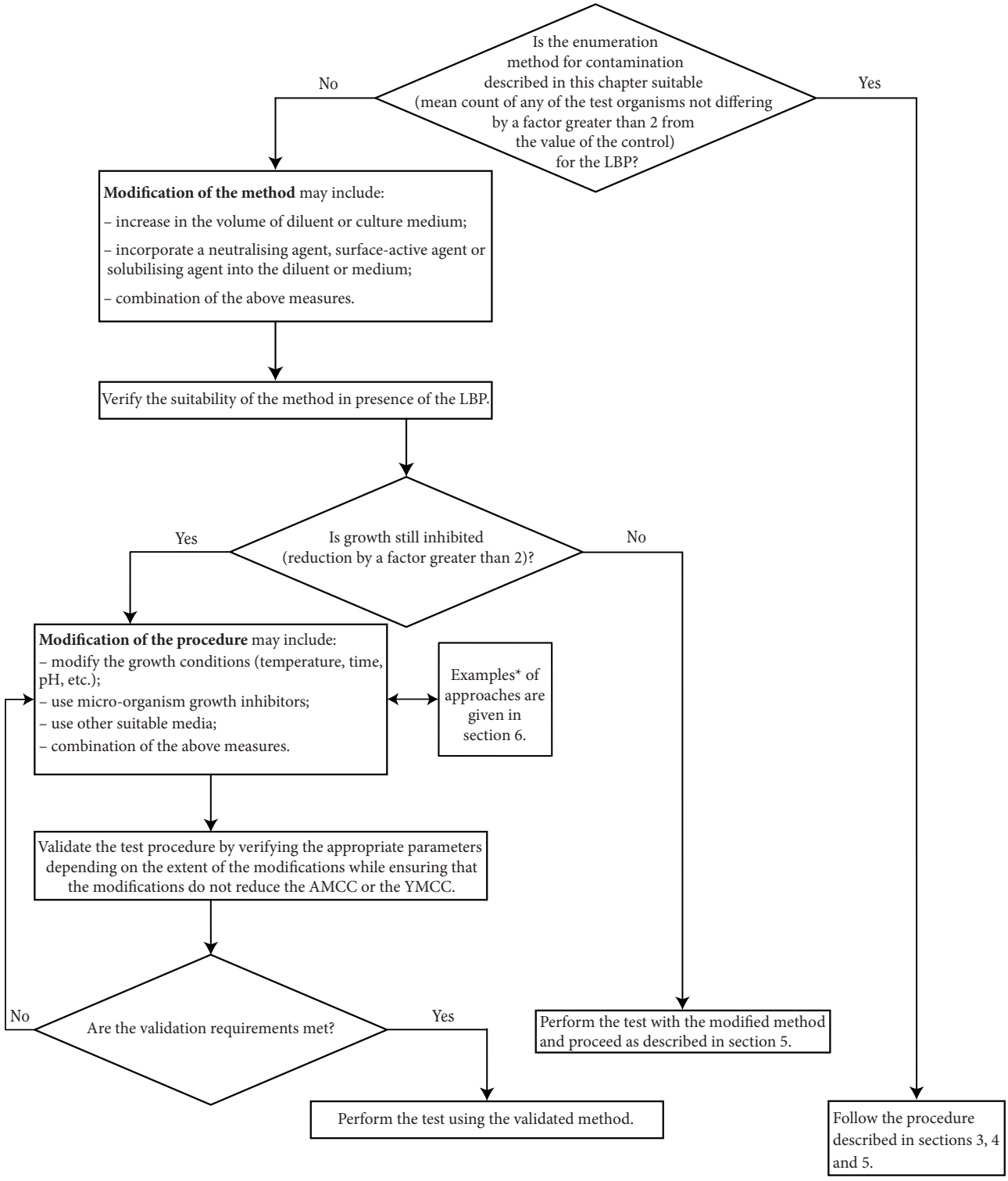


* This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

Figure 2.6.36.-1. – *Decision tree for the microbial contamination enumeration methods*

micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 2.6.36.-1.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions; to suspend *A. brasiliensis* spores, 0.05 per cent of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

*4-3. NEGATIVE CONTROL*

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the LBP as described in section 5. A failed negative control requires an investigation.

*4-4. GROWTH PROMOTION OF THE MEDIA*

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of casein soya bean digest broth and casein soya bean digest agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.36.-1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud-dextrose agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.36.-1, using a separate plate of medium for each. Incubate in the conditions described in Table 2.6.36.-1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

*4-5. SUITABILITY OF THE COUNTING METHOD IN THE PRESENCE OF THE LBP TO BE TESTED*

**4-5-1. Preparation of the sample**. The method for sample preparation depends upon the physical characteristics of the LBP to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Prepare a homogenous suspension of the LBP to be tested (usually a 1 in 10 dilution is prepared) in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. A surface-active agent such as a 1 g/L solution of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

Table 2.6.36.-1. – *Preparation and use of test micro-organisms*

| Micro-organism | Preparation of test strain | Growth promotion | | Suitability of counting method in the presence of the LBP | |
|---|---|---|---|---|---|
| | | Aerobic microbial contamination count (AMCC) | Yeasts and moulds contamination count (YMCC) | Aerobic microbial contamination count (AMCC) | Yeasts and moulds contamination count (YMCC) |
| *Staphylococcus aureus* such as: ATCC 6538 NCIMB 9518 CIP 4.83 NBRC 13276 | Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h | Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - | Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - |
| *Pseudomonas aeruginosa* such as: ATCC 9027 NCIMB 8626 CIP 82.118 NBRC 13275 | Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h | Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - | Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - |
| *Bacillus subtilis* such as: ATCC 6633 NCIMB 8054 CIP 52.62 NBRC 3134 | Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h | Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - | Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days | - |
| *Candida albicans* such as: ATCC 10231 NCPF 3179 IP 48.72 NBRC 1594 | Sabouraud- dextrose agar or Sabouraud-dextrose broth 20-25 °C 2-3 days | Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days | Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days | Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable | Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days |
| *Aspergillus brasiliensis* such as: ATCC 16404 IMI 149007 IP 1431.83 NBRC 9455 | Sabouraud- dextrose agar or potato-dextrose agar 20-25 °C 5-7 days, or until good sporulation is achieved | Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days | Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days | Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable | Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days |

**4-5-2. Inoculation and dilution**. Add to the sample prepared as described above (4-5-1) and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted LBP.

To demonstrate acceptable recovery of the test micro-organisms from the LBP, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to inhibitory activity of the LBP, further appropriate protocols must be developed (see decision tree shown in Figure 2.6.36.-1). If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the test micro-organisms may be added after neutralisation or dilution.

*Inhibitory activity*. The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-3, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), follow the decision tree shown in Figure 2.6.36.-1 and modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the

diluent or culture medium, (2) incorporation of specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

If growth is still inhibited (reduction by a factor greater than 2) continue to follow the decision tree shown in Figure 2.6.36.-1.

A new approach needs to be validated using the appropriate parameters depending on the extent of modification, while ensuring that the modifications do not reduce the AMCC or the YMCC.

**4-5-3. Recovery of the test micro-organisms in the presence of an LBP**. For each of the test micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

*4-5-3-1. Plate-count methods.* Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

4-5-3-1-1. Pour-plate method

For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under 4-5-1 and 4-5-2, and 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar, both media being at not more than 45 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 2.6.36.-1, at least 2 Petri dishes are used. Incubate the plates as indicated in Table 2.6.36.-1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

4-5-3-1-2. Surface-spread method

For Petri dishes 9 cm in diameter, add 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or an incubator. For each of the micro-organisms listed in Table 2.6.36.-1, at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under 4-5-1 and 4-5-2 over the surface of the medium. Incubate and count as prescribed under 4-5-3-1-1.

*4-5-3-2. Most-probable-number (MPN) method.* The precision and accuracy of the MPN method is less than the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of AMCC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least 3 serial tenfold dilutions of the LBP as described under 4-5-1 and 4-5-2. From each level of dilution, 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9-10 mL of casein soya bean digest broth. If necessary, a surface-active agent such as polysorbate 80 may be added to the medium. Thus, if 3 levels of dilution are prepared, 9 tubes are inoculated.

Incubate all tubes at 30-35 °C for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the LBP to be tested, subculture in the same broth, or in casein soya bean digest agar, for 1-2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per gram or millilitre of the LBP to be tested from Table 2.6.36.-2.

*4-6. RESULTS AND INTERPRETATION*

When verifying the suitability of the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in 4-5-2 in the absence of the LBP must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met, follow the decision tree shown in Figure 2.6.36.-1 and modify the procedure for the particular enumeration test to ensure the validity of the results.

5. TESTING OF LIVE BIOTHERAPEUTIC PRODUCTS

*5-1. AMOUNT USED FOR THE TEST*

Unless otherwise prescribed, use 10 g or 10 mL of the LBP to be tested taken with the precautions referred to above.

For LBP where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

Table 2.6.36.-2. – *Most-probable-number values of micro-organisms*

| Observed combinations of numbers of tubes showing growth in each set | | | MPN per gram or per millilitre of LBP | 95 per cent confidence limits |
|---|---|---|---|---|
| Number of grams or millilitres of LBP per tube | | | | |
| 0.1 | 0.01 | 0.001 | | |
| 0 | 0 | 0 | < 3 | 0 - 9.4 |
| 0 | 0 | 1 | 3 | 0.1 - 9.5 |
| 0 | 1 | 0 | 3 | 0.1 - 10 |
| 0 | 1 | 1 | 6.1 | 1.2 - 17 |
| 0 | 2 | 0 | 6.2 | 1.2 - 17 |
| 0 | 3 | 0 | 9.4 | 3.5 - 35 |
| 1 | 0 | 0 | 3.6 | 0.2 - 17 |
| 1 | 0 | 1 | 7.2 | 1.2 - 17 |
| 1 | 0 | 2 | 11 | 4 - 35 |
| 1 | 1 | 0 | 7.4 | 1.3 - 20 |
| 1 | 1 | 1 | 11 | 4 - 35 |
| 1 | 2 | 0 | 11 | 4 - 35 |
| 1 | 2 | 1 | 15 | 5 - 38 |
| 1 | 3 | 0 | 16 | 5 - 38 |
| 2 | 0 | 0 | 9.2 | 1.5 - 35 |
| 2 | 0 | 1 | 14 | 4 - 35 |
| 2 | 0 | 2 | 20 | 5 - 38 |
| 2 | 1 | 0 | 15 | 4 - 38 |
| 2 | 1 | 1 | 20 | 5 - 38 |
| 2 | 1 | 2 | 27 | 9 - 94 |
| 2 | 2 | 0 | 21 | 5 - 40 |
| 2 | 2 | 1 | 28 | 9 - 94 |
| 2 | 2 | 2 | 35 | 9 - 94 |
| 2 | 3 | 0 | 29 | 9 - 94 |
| 2 | 3 | 1 | 36 | 9 - 94 |
| 3 | 0 | 0 | 23 | 5 - 94 |
| 3 | 0 | 1 | 38 | 9 - 104 |
| 3 | 0 | 2 | 64 | 16 - 181 |
| 3 | 1 | 0 | 43 | 9 - 181 |
| 3 | 1 | 1 | 75 | 17 - 199 |
| 3 | 1 | 2 | 120 | 30 - 360 |
| 3 | 1 | 3 | 160 | 30 - 380 |

| Observed combinations of numbers of tubes showing growth in each set | | | MPN per gram or per millilitre of LBP | 95 per cent confidence limits |
|---|---|---|---|---|
| Number of grams or millilitres of LBP per tube | | | | |
| 0.1 | 0.01 | 0.001 | | |
| 3 | 2 | 0 | 93 | 18 - 360 |
| 3 | 2 | 1 | 150 | 30 - 380 |
| 3 | 2 | 2 | 210 | 30 - 400 |
| 3 | 2 | 3 | 290 | 90 - 990 |
| 3 | 3 | 0 | 240 | 40 - 990 |
| 3 | 3 | 1 | 460 | 90 - 1980 |
| 3 | 3 | 2 | 1100 | 200 - 4000 |
| 3 | 3 | 3 | > 1100 | |

*5-2. EXAMINATION OF THE LBP*

**5-2-1. Plate-count methods**

*5-2-1-1. Pour-plate method.* Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Select the plates corresponding to a given dilution and showing the highest number of contaminating colonies less than 250 for AMCC and 50 for YMCC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of LBP.

*5-2-1-2. Surface-spread method.* Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of contaminant CFU proceed as described for the pour-plate method.

**5-2-2. Most-probable-number method**. Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes at 30-35 °C for 3-5 days. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of contaminant micro-organisms per gram or millilitre of the LBP to be tested from Table 2.6.36.-2.

*5-3. INTERPRETATION OF THE RESULTS*

The aerobic microbial contamination count (AMCC) is considered to be equal to the number of contaminating CFU found using casein soya bean digest agar; if colonies of contaminating yeasts/moulds are detected on this medium, they are counted as part of the AMCC. The combined yeasts/moulds contaminants count (YMCC) is considered to be equal to the number of CFU found using Sabouraud-dextrose agar; if colonies of contaminating bacteria are detected on this medium, they may be excluded from the YMCC. When the YMCC is expected to exceed the acceptance criterion due to bacterial growth, Sabouraud-dextrose agar containing antibiotics can be used (see decision tree shown in Figure 2.6.36.-1). If the count is carried out by the MPN method the calculated value is the AMCC.

When an acceptance criterion for microbial contamination is prescribed it is interpreted as follows:

- $10^1$ CFU: maximum acceptable count = 20;
- $10^2$ CFU: maximum acceptable count = 200;
- $10^3$ CFU: maximum acceptable count = 2000.

The recommended solutions and media are described in general chapter *2.6.13*.

6. APPROACHES FOR ENUMERATION OF MICROBIAL CONTAMINANTS IN THE PRESENCE OF INHIBITION DUE TO THE LBP

This section should be understood as informative suggestion. Other procedures are possible as far as they are justified. Examples of approaches for enumeration of microbial contaminants for LBP inhibiting the microbial contamination enumeration are indicated below.

Each approach needs to be validated using the appropriate parameters depending on the extent of modification, while ensuring that the modifications do not reduce the AMCC or the YMCC.

The test strains indicated in Table 2.6.36.-1 are used. Preparation of test strains is performed as described in 4-2. Negative control is performed as described in 4-3. Growth promotion and suitability of new culture media chosen is carried out by inoculating each batch of the medium with a small number (not more than 100 CFU) of the micro-organisms. Growth obtained does not differ by a factor greater than 2 from the calculated value for a standardised inoculum counted on casein soya bean digest agar (AMCC) or Sabouraud-dextrose agar (YMCC).

*6-1. ENUMERATION OF AEROBIC MICROBIAL CONTAMINANTS*

LBP containing lactic acid bacteria may be tested for aerobic microbial contamination on sugar-free agar plates incubated aerobically at 30-35 °C for 72 h. Lactic acid bacteria grow slowly as pinpoint colonies, while contaminants can easily be detected as larger colonies and are fast growing.

Alternatively, the aerobic microbial contamination may be tested on casein soya bean digest agar plates supplemented with 5 per cent of sheep blood at 30-35 °C for 44-48 h. The addition of blood enhances growth of contaminants and formation of distinctive colony morphology that are better discriminated in the presence of lactic acid bacteria.

For LBP containing *Bacillus clausii* spores, the aerobic microbial contamination may be enumerated on sporulating agar. The medium, by promoting the sporulation of *Bacillus clausii*, inhibits the vegetative growth of the LBP micro-organism, making the detection of contaminants possible. Plates are incubated at 33-37 °C for 48 h.

LBP containing *Saccharomyces cerevisiae*, var. *boulardii* may be tested for aerobic microbial contamination on casein soya bean digest agar containing cycloheximide, a suitable inhibitor of *Saccharomyces*. Plates are incubated at 30-35 °C for 3-5 days.

For LBP for which suitable media and growth conditions to enumerate aerobic microbial contamination are not available, not only the absence of specific contaminants (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp. and bile-tolerant Gram-negative bacteria) is verified using the method described in general chapter *2.6.38*, and tests for other contaminating micro-organisms are carried out based on a risk assessment (e.g. specific environmental contaminants).

*6-2. ENUMERATION OF YEAST AND MOULD CONTAMINANTS*

For LBP containing bacteria, the test may be carried out with Sabouraud-dextrose agar containing antimicrobials (e.g. chloramphenicol) incubated at 20-25 °C for 5-7 days.

For LBP containing *Saccharomyces cerevisae*, var. *boulardii*, yeasts and moulds may be determined using several media (Sabouraud-dextrose agar supplemented with chloramphenicol and cycloheximide, Czapek-Dox agar, potato dextrose agar).

04/2019:20638

# 2.6.38. MICROBIOLOGICAL EXAMINATION OF LIVE BIOTHERAPEUTIC PRODUCTS: TESTS FOR SPECIFIED MICRO-ORGANISMS

## 1. INTRODUCTION

The tests described hereafter are intended for determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described. The tests are designed primarily to determine whether a live biotherapeutic product (LBP) complies with the established specification for microbiological quality.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

## 2. GENERAL PROCEDURES

The preparation of samples is carried out as described in general chapter *2.6.36*.

If the LBP to be tested prevents the detection of specified micro-organisms apply the decision tree shown in Figure 2.6.38.-1.

If components other than the active substance (i.e. the micro-organism) of the LBP to be tested have inhibitory activity against the target contaminant micro-organisms, this is insofar as possible removed or neutralised as described in general chapter *2.6.36*.

If surface-active agents are used for sample preparation, their absence of toxicity for the target contaminant micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.36*.

## 3. GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect the target micro-organisms in the presence of the LBP to be tested must be established. Suitability must be confirmed if a change is introduced in the testing procedure or the LBP, which may affect the outcome of the test.

### *3-1. PREPARATION OF TEST STRAINS*

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

**3-1-1. Aerobic micro-organisms**. Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h. Grow the test strain for *Candida albicans* separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20-25 °C for 2-3 days.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium, such as ATCC 14028 or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;
- *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C.

**3-1-2. Anaerobic micro-organisms**

**Clostridia**. Use a *Clostridium sporogenes* strain such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03) or NBRC 14293. Grow the clostridial test strain under anaerobic conditions in reinforced medium for Clostridia at 30-35 °C for 24-48 h. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *C. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period.

### *3-2. NEGATIVE CONTROL*

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the LBP as described in section 4. A failed negative control requires an investigation.

### *3-3. GROWTH PROMOTION AND INHIBITORY PROPERTIES OF THE MEDIA*

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 2.6.38.-1.

**3-3-1. Test for growth promoting properties, liquid media**. Inoculate a portion of the appropriate medium of the same volume to be used in the test (see sections 3-4 and 4) with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

**3-3-2. Test for growth promoting properties, solid media**. Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism is comparable to that previously obtained with a previously tested and approved batch of medium and does not differ by a factor greater than 2.

**3-3-3. Test for inhibitory properties, liquid or solid media**. Inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified

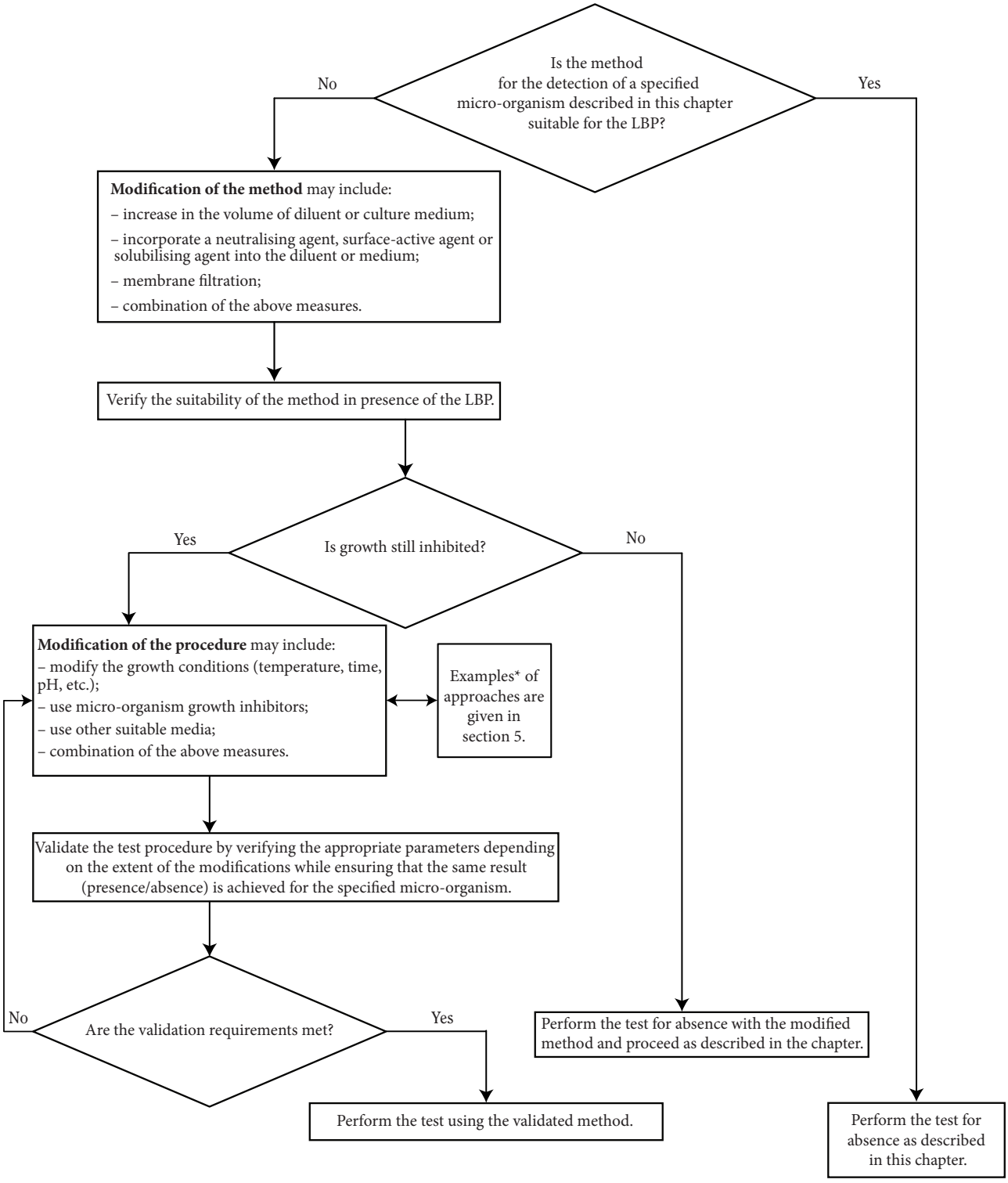


* This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

Figure 2.6.38.-1. – *Decision tree for the test for specified micro-organisms*

temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

Table 2.6.38.-1. – *Growth promoting, inhibitory and indicative properties of media*

| | Medium | Property | Test strains |
|---|---|---|---|
| Test for bile-tolerant Gram-negative bacteria | Enterobacteria enrichment broth-Mossel | Growth promoting | *E. coli*<br>*P. aeruginosa* |
| | | Inhibitory | *S. aureus* |
| | Violet red bile glucose agar | Growth promoting + indicative | *E. coli*<br>*P. aeruginosa* |
| Test for *Escherichia coli* | MacConkey broth | Growth promoting | *E. coli* |
| | | Inhibitory | *S. aureus* |
| | MacConkey agar | Growth promoting + indicative | *E. coli* |
| Test for *Salmonella* | Rappaport Vassiliadis *Salmonella* enrichment broth | Growth promoting | *Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony |
| | | Inhibitory | *S. aureus* |
| | Xylose, lysine, deoxycholate agar | Growth promoting + indicative | *Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony |
| Test for *Pseudomonas aeruginosa* | Cetrimide agar | Growth promoting | *P. aeruginosa* |
| | | Inhibitory | *E. coli* |
| Test for *Staphylococcus aureus* | Mannitol salt agar | Growth promoting + indicative | *S. aureus* |
| | | Inhibitory | *E. coli* |
| Test for Clostridia | Reinforced medium for Clostridia | Growth promoting | *C. sporogenes* |
| | Columbia agar | Growth promoting | *C. sporogenes* |
| Test for *Candida albicans* | Sabouraud-dextrose broth | Growth promoting | *C. albicans* |
| | Sabouraud-dextrose agar | Growth promoting + indicative | *C. albicans* |

**3-3-4. Test for indicative properties**. Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest validated period of time specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

*3-4. SUITABILITY OF THE TEST METHOD*

For each LBP to be tested, perform the sample preparation as described in the relevant paragraph in section 4. Inoculate the test strains individually. Add each test strain at the time of mixing, in the prescribed growth medium. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation. The inoculum should not exceed 1 per cent of the volume of the growth medium. Perform the test as described in the relevant paragraph in section 4 using for each step of the test the shortest incubation time to be used in the test.

The specified micro-organisms must be detected with the appearance and indication reactions described in section 4. If non-characteristic colonies and reactions are obtained, the method may still be suitable provided that all colony types are identified when performing the test.

Any inhibitory activity of the LBP against the target micro-organisms necessitates a modification of the test procedure (see decision tree shown in Figure 2.6.38.-1) and then the suitability for the LBP must be confirmed.

## 4. TESTING OF LIVE BIOTHERAPEUTIC PRODUCTS

*4-1. BILE-TOLERANT GRAM-NEGATIVE BACTERIA*

**4-1-1. Sample preparation and pre-incubation**. Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the micro-organisms (usually 2 h but not more than 5 h).

**4-1-2. Test for absence**. Unless otherwise prescribed, use the volume corresponding to 1 g of the LBP, as prepared in 4-1-1, to inoculate enterobacteria enrichment broth-Mossel. Incubate at 30-35 °C for 24-48 h. Subculture on violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

The LBP complies with the test if there is no growth of colonies.

*4-2. ESCHERICHIA COLI*

**4-2-1. Sample preparation and pre-enrichment**. Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

**4-2-2. Selection and subculture**. Shake the container, transfer 1 mL of the casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on MacConkey agar at 30-35 °C for 18-72 h.

**4-2-3. Interpretation**. Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The LBP complies with the test if colonies are not present or if the confirmatory identification tests are negative.

*4-3. SALMONELLA*

**4-3-1. Sample preparation and pre-enrichment**. Prepare the LBP to be tested as described in general chapter *2.6.36* and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

**4-3-2. Selection and subculture**. Transfer 0.1 mL of the casein soya bean digest broth to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on xylose, lysine, deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

**4-3-3. Interpretation**. Growth of well-developed, red colonies, with or without black centres indicates the possible presence of *Salmonella*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*4-4. PSEUDOMONAS AERUGINOSA*

**4-4-1. Sample preparation and pre-enrichment**. Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. Incubate at 30-35 °C for 18-24 h.

**4-4-2. Selection and subculture**. Subculture on cetrimide agar and incubate at 30-35 °C for 18-72 h.

**4-4-3. Interpretation**. Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The LBP complies with the test if colonies are not present or if the confirmatory identification tests are negative.

*4-5. STAPHYLOCOCCUS AUREUS*

**4-5-1. Sample preparation and pre-enrichment**. Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. Incubate at 30-35 °C for 18-24 h.

**4-5-2. Selection and subculture**. Subculture on mannitol salt agar and incubate at 30-35 °C for 18-72 h.

**4-5-3. Interpretation**. Growth of yellow/white colonies surrounded by a yellow zone indicates the possible presence of *S. aureus*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*4-6. CLOSTRIDIA*

**4-6-1. Sample preparation and heat treatment**. Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the LBP to be tested as described in general chapter *2.6.36*. Divide the sample into 2 portions of at least 10 mL. Heat 1 portion at 80 °C for 10 min and cool rapidly. Do not heat the other portion.

**4-6-2. Selection and subculture**. Use 10 mL or the quantity corresponding to 1 g or 1 mL of the LBP to be tested of both portions to inoculate suitable amounts (determined as described under 3-4) of reinforced medium for Clostridia. Incubate under anaerobic conditions at 30-35 °C for 48 h. After incubation, make subcultures from each container on Columbia agar and incubate under anaerobic conditions at 30-35 °C for 48-72 h.

**4-6-3. Interpretation**. The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction may indicate the presence of Clostridia. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

*4-7. CANDIDA ALBICANS*

**4-7-1. Sample preparation and pre-enrichment**. Prepare the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL to inoculate 100 mL of Sabouraud-dextrose broth and mix. Incubate at 30-35 °C for 3-5 days.

**4-7-2. Selection and subculture**. Subculture on Sabouraud-dextrose agar and incubate at 30-35 °C for 24-48 h.

**4-7-3. Interpretation**. Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

## 5. APPROACHES FOR TESTING SPECIFIED MICRO-ORGANISMS AND ADDITIONAL TESTING

This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

If the detection of the specified micro-organism is inhibited by the LBP, its detection is carried out under conditions that neutralise the inhibition or limit the growth of the LBP micro-organisms. The modified test for specified micro-organisms is validated using the appropriate validation parameters depending on the extent of the modifications while ensuring that the same result (presence/absence) is achieved for the specified micro-organism.

A suitable combination of antibiotics may be used to inhibit the LBP and detect the specified micro-organism. For example, for LBP samples containing *S. cerevisiae*, var. *boulardii*, pre-enrichment and selective media (Sabouraud-dextrose broth and agar) can be supplemented with chloramphenicol and cycloheximide to detect contaminant *C. albicans*.

Alternative pre-enrichment media (e.g. buffered peptone medium for *Salmonella* — see general chapter *2.6.31*), media and test/growth conditions may be used to support growth of the target micro-organisms while limiting growth of the micro-organisms of the LBP.

LBP containing *Bacillus clausii* spores are tested for contaminating *Bacillus cereus* on chromogenic selective agar media plates. Incubation time and temperature depends on the specific medium.

A proposed approach to detect *Bacillus cereus* is described below.

Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount of casein soya bean digest broth, mix and incubate at 35-37 °C for 18-24 h. Subculture on selective media (e.g. *B. cereus* Mossel agar). For each LBP to be tested, validate incubation time and temperature. The appearance of *B. cereus* colonies depends on the medium used. The LBP complies with the test if no *B. cereus* colonies are present.

For LBP containing *E. coli*, use *Enterococcus faecalis* and *Enterococcus faecium* as microbial indicators of faecal contamination to replace the test for absence of *E. coli*. Use selective or differential chromogenic media and, for each LBP to be tested, validate incubation time and temperature. The LBP complies with the test when there is no growth of *Enterococcus* spp.

A search for pathogenic *E. coli* strains may be carried out by suitable methods (e.g. molecular methods for the detection of gene *stx* or *eae*) based on risk-assessment.

## 6. RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The solutions and culture media mentioned in this chapter and described in chapter *2.6.13* have been found satisfactory for bile-tolerant Gram-negative bacteria, *E. coli*, *P. aeruginosa*, *S. aureus*, Clostridia, *Salmonella* and *C. albicans*. Other media may be used provided that their suitability can be demonstrated and the analytical method where they are used is validated with the appropriate validation parameters.

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# 2.7. BIOLOGICAL ASSAYS

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## 2.7.1. IMMUNOCHEMICAL METHODS

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen-antibody complex may be detected, and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardise the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is, a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions; it is important to ascertain that the kits are suitable for the analysis of the substance to be examined, with particular reference to selectivity and sensitivity. Guidance concerning immunoassay kits is provided by the World Health Organization, Technical Report Series 658 (1981).

METHODS IN WHICH A LABELLED ANTIGEN OR A LABELLED ANTIBODY IS USED

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radio-immunoassay". The recommendations for the measurement of radioactivity given in the monograph on *Radiopharmaceutical Preparations (0125)* are applicable to immunoassays involving radioisotopes. All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

METHODS IN WHICH AN UNLABELLED ANTIGEN OR ANTIBODY IS USED

**Immunoprecipitation methods**

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants which gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase in sensitivity can be obtained by using antigen- or antibody-coated particles (e.g. latex) as reactants.

In flocculation methods, stepwise dilutions of one of the reactants is usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium: concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are performed in tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as *simple*; when it involves related but not serologically identical reactants, the system is *complex* and where several serologically unrelated reactants are involved, the system is *multiple*.

In *simple diffusion methods*, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

*Single radial immunodiffusion* (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactant has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In *double diffusion methods*, concentration gradients are established for both reactants. Both antigen and antibody diffuse from separate sites into an initially immunologically neutral gel.

*Comparative double diffusion methods* are used for qualitatively comparing various antigens versus a suitable antibody or vice versa. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens/antibodies can be distinguished.

**Immunoelectrophoretic methods**

Immunoelectrophoresis (IE) is a qualitative technique combining 2 methods: gel electrophoresis followed by immunodiffusion.

*Crossed immunoelectrophoresis* is a modification of the IE method. It is suitable both for qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions to be determined, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out perpendicular to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

*Electroimmunoassay*, often referred to as *rocket immuno-electrophoresis* is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or vice versa. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

*Counter-immunoelectrophoresis* is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an electric field depending on the different charges. Dilutions of a standard for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

**Visualisation and characterisation of immunoprecipitation lines**

These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterisation of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

VALIDATION OF THE METHOD

**Validation criteria**

A quantitative immunochemical method is not valid unless:

1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound,

2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity,

3) The limit of quantitation is below the acceptance criteria stated in the individual monograph,

4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs,

5) The order in which the assay is performed does not give rise to systematic errors.

**Validation methods**

In order to verify these criteria, the validation design includes the following elements:

1) The assay is performed at least in triplicate,

2) The assay includes at least 3 different dilutions of the standard preparation and 3 dilutions of sample preparations of presumed activity similar to the standard preparation,

3) The assay layout is randomised,

4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared,

5) The test includes the measurement of non-specific binding of the labelled reactant,

6) For displacement immunoassay:

(a) maximum binding (zero displacement) is determined,

(b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

STATISTICAL CALCULATION

To analyse the results, response curves for test and standard may be analysed by the methods described in *5.3. Statistical Analysis of Results of Biological Assays and Tests*.

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

01/2020:20702

# 2.7.2. MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the 2 log dose-response (or transformed response) lines of the preparation to be examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated.

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by method A or method B.

A. DIFFUSION METHOD

Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example 48 °C to 50 °C for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic to be examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2-5 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 2.7.2.-1, prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than 6 solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 h. A period of diffusion prior to incubation, usually 1-4 h, at room temperature or at about 4 °C, as appropriate, may be used to minimise the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters to the nearest 0.1 mm or the areas of the circular inhibition zones to the nearest 0.01 $mm^2$ and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

B. TURBIDIMETRIC METHOD

Inoculate a suitable medium with a suspension of the chosen micro-organism having a sensitivity to the antibiotic to be examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 h.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 2.7.2.-2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number 3 consecutive doses, using corresponding doses for the reference substance and the antibiotic to be examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 mL of the solution and 9 mL of the medium). For the assay of tyrothricin add 0.1 mL of the solution to 9.9 mL of inoculated medium.

Prepare at the same time 2 control tubes without antibiotic, both containing the inoculated medium and to one of which is added immediately 0.5 mL of *formaldehyde R*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomised block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3-4 h, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the micro-organisms by adding 0.5 mL of *formaldehyde R* to each tube or by heat treatment and measure the opacity to 3 significant figures using suitable optical apparatus. Alternatively use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Table 2.7.2.-1. – *Diffusion assay*

| Antibiotic | Reference substance | Solvent to be used in preparing the stock solution | Buffer solution (pH) | Micro-organism | Medium and final pH (± 0.1 pH unit) | Incubation temperature |
|---|---|---|---|---|---|---|
| Amphotericin B | *Amphotericin B for microbiological assay CRS* | *Dimethyl sulfoxide R* | pH 10.5 (0.2 M) | *Saccharomyces cerevisiae*<br>ATCC 9763<br>IP 1432-83 | F - pH 6.1 | 35-37 °C |
| Bacitracin zinc | *Bacitracin zinc CRS* | *0.01 M hydrochloric acid* | pH 7.0 (0.05 M) | *Micrococcus luteus*<br>NCTC 7743<br>CIP 53.160<br>ATCC 10240 | A - pH 7.0 | 35-39 °C |
| Bleomycin sulfate | *Bleomycin sulfate CRS* | *Water R* | pH 6.8 (0.1 M) | *Mycobacterium smegmatis*<br>ATCC 607 | G - pH 7.0 | 35-37 °C |
| Colistimethate sodium | *Colistimethate sodium CRS* | *Water R* | pH 6.0 (0.05 M) | *Bordetella bronchiseptica*<br>NCTC 8344<br>CIP 53.157<br>ATCC 4617<br>*Escherichia coli*<br>NCIMB 8879<br>CIP 54.127<br>ATCC 10536 | B - pH 7.3<br><br>B - pH 7.3 | 35-39 °C<br><br>35-39 °C |
| Colistin sulfate | *Colistin sulfate for microbiological assay CRS* | *Water R* | pH 6.0 (0.05 M) | *Bordetella bronchiseptica*<br>NCTC 8344<br>CIP 53.157<br>ATCC 4617<br>*Escherichia coli*<br>NCIMB 8879<br>CIP 54.127<br>ATCC 10536 | B - pH 7.3<br><br>B - pH 7.3 | 35-39 °C<br><br>35-39 °C |

| Antibiotic | Reference substance | Solvent to be used in preparing the stock solution | Buffer solution (pH) | Micro-organism | Medium and final pH (± 0.1 pH unit) | Incubation temperature |
|---|---|---|---|---|---|---|
| Framycetin sulfate | *Framycetin sulfate CRS* | *Water R* | pH 8.0 (0.05 M) | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | E - pH 7.9 | 30-37 °C |
| | | | | *Bacillus pumilus* NCTC 8241 CIP 76.18 | E - pH 7.9 | 30-37 °C |
| Gentamicin sulfate | *Gentamicin sulfate CRS* | *Water R* | pH 8.0 (0.05 M) | *Bacillus pumilus* NCTC 8241 CIP 76.18 | A - pH 7.9 | 35-39 °C |
| | | | | *Staphylococcus epidermidis* NCIMB 8853 CIP 68.21 ATCC 12228 | A - pH 7.9 | 35-39 °C |
| Josamycin | *Josamycin CRS* | *Methanol R* (see the monograph) | pH 5.6 | *Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400 | A - pH 6.6 | 35-37 °C |
| Josamycin propionate | *Josamycin propionate CRS* | *Methanol R* (see the monograph) | pH 5.6 | *Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400 | A - pH 6.6 | 35-37 °C |
| Kanamycin monosulfate<br>Kanamycin acid sulfate | *Kanamycin monosulfate CRS* | *Water R* | pH 8.0 (0.05 M) | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | A - pH 7.9 | 30-37 °C |
| | | | | *Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P | A - pH 7.9 | 35-39 °C |
| Neomycin sulfate | *Neomycin sulfate for microbiological assay CRS* | *Water R* | pH 8.0 (0.05 M) | *Bacillus pumilus* NCTC 8241 CIP 76.18 | E - pH 7.9 | 30-37 °C |
| | | | | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | E - pH 7.9 | 30-37 °C |
| Netilmicin sulfate | *Netilmicin sulfate CRS* | *Water R* | pH 8.0 ± 0.1 | *Staphylococcus aureus* ATCC 6538 P CIP 53.156 | A - pH 7.9 | 32-35 °C |
| Nystatin | *Nystatin CRS* | *Dimethylformamide R* | pH 6.0 (0.05 M) containing 5 per cent *V/V* of *dimethylformamide R* | *Candida tropicalis* CIP 1433-83 NCYC 1393 | F - pH 6.0 | 30-37 °C |
| | | | | *Saccharomyces cerevisiae* NCYC 87 CIP 1432-83 ATCC 9763 | F - pH 6.0 | 30-32 °C |
| Polymyxin B sulfate | *Polymyxin B sulfate for microbiological assay CRS* | *Water R* | pH 6.0 (0.05 M) | *Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617 | B - pH 7.3 | 35-39 °C |
| Rifamycin sodium | *Rifamycin sodium CRS* | *Methanol R* | pH 7.0 (0.05 M) | *Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341 | A - pH 6.6 | 35-39 °C |
| Spiramycin | *Spiramycin CRS* | *Methanol R* | pH 8.0 (0.05 M) | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | A - pH 7.9 | 30-32 °C |

| Antibiotic | Reference substance | Solvent to be used in preparing the stock solution | Buffer solution (pH) | Micro-organism | Medium and final pH (± 0.1 pH unit) | Incubation temperature |
|---|---|---|---|---|---|---|
| Streptomycin sulfate | *Streptomycin sulfate CRS* | *Water R* | pH 8.0 (0.05 M) | *Bacillus subtilis* NCTC 8236 CIP 1.83 | A - pH 7.9 | 30-37 °C |
| | | | | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | A - pH 7.9 | 30-37 °C |
| Teicoplanin | *Teicoplanin CRS* | pH 6.0 (0.05 M) | pH 6.0 (0.05 M) | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | H - pH 7.8-8.0 | 35-37 °C |
| Tylosin for veterinary use<br>Tylosin phosphate for veterinary use<br>Tylosin tartrate for veterinary use | *Tylosin CRS* | 2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R* | A mixture of 40 volumes of *methanol R* and 60 volumes of *0.1 M phosphate buffer solution pH 8.0 R* | *Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341 | A - pH 8.0 | 32-35 °C |
| Vancomycin hydrochloride | *Vancomycin hydrochloride CRS* | *Water R* | pH 8.0 | *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633 | A - pH 8.0 | 37-39 °C |

Calculate the potency using appropriate statistical methods.

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

Table 2.7.2.-2. – *Turbidimetric assay*

| Antibiotic | Reference substance | Solvent to be used in preparing the stock solution | Buffer solution (pH) | Micro-organism | Medium and final pH (± 0.1 pH unit) | Incubation temperature |
|---|---|---|---|---|---|---|
| Colistimethate sodium | *Colistimethate sodium CRS* | *Water R* | pH 7.0 | *Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637 | C - pH 7.0 | 35-37 °C |
| Colistin sulfate | *Colistin sulfate for microbiological assay CRS* | *Water R* | pH 7.0 | *Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637 | C - pH 7.0 | 35-37 °C |
| Framycetin sulfate | *Framycetin sulfate CRS* | *Water R* | pH 8.0 | *Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| Gentamicin sulfate | *Gentamicin sulfate CRS* | *Water R* | pH 7.0 | *Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| Gramicidin | *Gramicidin CRS* | *Methanol R* | pH 7.0* | *Enterococcus hirae* CIP 58.55 ATCC 10541 *Staphylococcus aureus* ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| | *Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions, for example 0.1 mg/mL of *polysorbate 80 R* | | | | | |

| Antibiotic | Reference substance | Solvent to be used in preparing the stock solution | Buffer solution (pH) | Micro-organism | Medium and final pH (± 0.1 pH unit) | Incubation temperature |
|---|---|---|---|---|---|---|
| Josamycin | *Josamycin CRS* | *Methanol R* (see the monograph) | pH 5.6 | *Staphylococcus aureus*<br>CIP 53.156<br>ATCC 6538 P<br>NCTC 7447 | C - pH 8.0 | 35-37 °C |
| Josamycin propionate | *Josamycin propionate CRS* | *Methanol R* (see the monograph) | pH 5.6 | *Staphylococcus aureus*<br>CIP 53.156<br>ATCC 6538 P<br>NCTC 7447 | C - pH 8.0 | 35-37 °C |
| Kanamycin monosulfate<br>Kanamycin acid sulfate | *Kanamycin monosulfate CRS* | *Water R* | pH 8.0 | *Staphylococcus aureus*<br>NCTC 7447<br>CIP 53.156<br>ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| Neomycin sulfate | *Neomycin sulfate for microbiological assay CRS* | *Water R* | pH 8.0 | *Staphylococcus aureus*<br>NCTC 7447<br>CIP 53.156<br>ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| Rifamycin sodium | *Rifamycin sodium CRS* | *Methanol R* | pH 7.0 | *Escherichia coli*<br>NCIMB 8879<br>CIP 54.127<br>ATCC 10536 | C - pH 7.0 | 35-37 °C |
| Spiramycin | *Spiramycin CRS* | *Methanol R* | pH 7.0 | *Staphylococcus aureus*<br>NCTC 7447<br>CIP 53.156<br>ATCC 6538 P | C - pH 7.0 | 35-37 °C |
| Streptomycin sulfate | *Streptomycin sulfate CRS* | *Water R* | pH 8.0 | *Klebsiella pneumoniae*<br>NCTC 7427<br>CIP 53.153<br>ATCC 10031 | C - pH 7.0 | 35-37 °C |
| Tylosin for veterinary use<br>Tylosin tartrate for veterinary use | *Tylosin CRS* | 2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R* | pH 7.0 | *Staphylococcus aureus*<br>NCTC 6571<br>ATCC 9144<br>CIP 53.154 | C - pH 7.0 | 37 °C |
| Tyrothricin | *Gramicidin CRS* | *Alcohol R* | *Alcohol R* | *Enterococcus hirae*<br>ATCC 10541 | C - pH 7.0 | 37 °C |
| Vancomycin hydrochloride | *Vancomycin hydrochloride CRS* | *Water R* | pH 8.0 | *Staphylococcus aureus*<br>CIP 53.156<br>ATCC 6538 P | C - pH 7.0 | 37-39 °C |

*The following section is published for information.*

## Recommended micro-organisms

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH. The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

**Preparation of inocula**. *Bacillus cereus* var. *mycoides*; *Bacillus subtilis*; *Bacillus pumilus*. Spore suspensions of the organisms to be used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/L of *manganese sulfate R*. Using sterile *water R*, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually $10 \times 10^6$ to $100 \times 10^6$ per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 26 °C for 4-6 days, then adding, aseptically, sufficient *manganese sulfate R* to give a concentration of 0.001 g/L and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile *water R* to give a concentration of $10 \times 10^6$ to $100 \times 10^6$ spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

*Bordetella bronchiseptica*. Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile *water R* and dilute to a suitable opacity.

*Staphylococcus aureus*; *Klebsiella pneumoniae*; *Escherichia coli*; *Micrococcus luteus*; *Staphylococcus epidermidis*. Prepare as described above for *B. bronchiseptica* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

*Saccharomyces cerevisiae*; *Candida tropicalis*. Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/L solution of *sodium chloride R*. Dilute to a suitable opacity with the same solution.

**Buffer solutions**. Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 mL of *0.2 M potassium dihydrogen phosphate R* with the quantity of *0.2 M sodium hydroxide* indicated in Table 2.7.2.-3. Dilute with freshly prepared *distilled water R* to produce 200.0 mL.

Table 2.7.2.-3

| pH | *0.2 M Sodium hydroxide* (mL) |
|---|---|
| 5.8 | 3.72 |
| 6.0 | 5.70 |
| 6.2 | 8.60 |
| 6.4 | 12.60 |
| 6.6 | 17.80 |
| 6.8 | 23.65 |
| 7.0 | 29.63 |
| 7.2 | 35.00 |
| 7.4 | 39.50 |
| 7.6 | 42.80 |
| 7.8 | 45.20 |
| 8.0 | 46.80 |

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.-1 with the exception of bleomycin sulfate and amphotericin B.

For bleomycin sulfate, prepare the buffer solution pH 6.8 as follows: dissolve 6.4 g of *potassium dihydrogen phosphate R* and 18.9 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000 mL with *water R*.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, add 20 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

**Culture media**. The following media or equivalent media may be used.

Medium A

| | |
|---|---|
| Peptone | 6 g |
| Pancreatic digest of casein | 4 g |
| Beef extract | 1.5 g |
| Yeast extract | 3 g |
| Glucose monohydrate | 1 g |
| Agar | 15 g |
| Water | to 1000 mL |

Medium B

| | |
|---|---|
| Pancreatic digest of casein | 17 g |
| Papaic digest of soya bean | 3 g |
| Sodium chloride | 5 g |
| Dipotassium hydrogen phosphate | 2.5 g |
| Glucose monohydrate | 2.5 g |
| Agar | 15 g |
| Polysorbate 80 | 10 g |
| Water | to 1000 mL |

The polysorbate 80 is added to the hot solution of the other ingredients after boiling, and immediately before adjusting to volume.

Medium C

| | |
|---|---|
| Peptone | 6 g |
| Beef extract | 1.5 g |
| Yeast extract | 3 g |
| Sodium chloride | 3.5 g |
| Glucose monohydrate | 1 g |
| Dipotassium hydrogen phosphate | 3.68 g |
| Potassium dihydrogen phosphate | 1.32 g |
| Water | to 1000 mL |

Medium D

| | |
|---|---|
| Heart extract | 1.5 g |
| Yeast extract | 1.5 g |
| Peptone-casein | 5 g |
| Glucose monohydrate | 1 g |
| Sodium chloride | 3.5 g |
| Dipotassium hydrogen phosphate | 3.68 g |
| Potassium dihydrogen phosphate | 1.32 g |
| Potassium nitrate | 2 g |
| Water | to 1000 mL |

Medium E

| | |
|---|---|
| Peptone | 5 g |
| Meat extract | 3 g |
| Disodium hydrogen phosphate,12$H_2O$ | 26.9 g |
| Agar | 10 g |
| Water | to 1000 mL |

The disodium hydrogen phosphate is added as a sterile solution after sterilisation of the medium.

Medium F

| | |
|---|---|
| Peptone | 9.4 g |
| Yeast extract | 4.7 g |
| Beef extract | 2.4 g |
| Sodium chloride | 10.0 g |
| Glucose monohydrate | 10.0 g |
| Agar | 23.5 g |
| Water | to 1000 mL |

Medium G

| | |
|---|---|
| Glycerol | 10 g |
| Peptone | 10 g |
| Meat extract | 10 g |
| Sodium chloride | 3 g |
| Agar | 15 g |
| Water | to 1000 mL |

pH 7.0 ± 0.1 after sterilisation.

Medium H

| | |
|---|---|
| Peptone | 5.0 g |
| Agar | 15.0 g |
| Beef extract powder | 3.0 g |
| Water | to 1000 mL |

pH 7.8 - 8.0 adjusted with *0.1 M sodium hydroxide*.

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# 2.7.4. ASSAY OF HUMAN COAGULATION FACTOR VIII

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipid. Factor VIII activity may be measured in plasma preparations and therapeutic concentrates (plasma-derived and recombinant). The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

Quantification of factor VIII activity in plasma preparations is expressed in International Units defined by the International Standard for blood coagulation factor VIII in plasma, and *coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation. Quantification of factor VIII activity in therapeutic concentrates is expressed in International Units defined by the International Standard for blood coagulation factor VIII concentrate, and *human coagulation factor VIII concentrate BRP* is suitable for use as a reference preparation.

The chromogenic assay method consists of 2 consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme.

**Step 1**

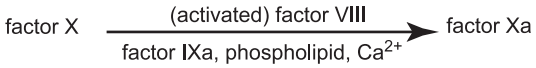


**Step 2**

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the appropriate International Standard for blood coagulation factor VIII as the standard, that the results obtained do not differ significantly.

It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa generation in order to determine the time taken to reach 50 per cent of the maximal factor Xa generation.

## REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay.

Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least 2 separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The 2nd step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between 3 and 5 amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

## ASSAY PROCEDURE

For the assay of therapeutic concentrates, add sufficient prediluent to the reference and test preparations to produce solutions containing 0.5-2.0 IU/mL. The prediluent consists of haemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma. The prediluted materials must be stable beyond the time required for the assay. Pre-dilution in haemophilia A plasma is not required for the assay of factor VIII in plasma preparations.

Prepare dilutions of the pre-diluted reference and test concentrate preparations (or the reference and test plasma preparations) using a non-chelating, appropriately buffered solution, for example, tris(hydroxymethyl)aminomethane or imidazole, containing 1 per cent of human or bovine albumin. Prepare at least 2 dilution series of at least 3 further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU/mL, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.

**Step 1**. Mix prewarmed dilutions of the factor VIII reference preparation and of the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. Allow the activation of factor X to proceed for a suitable time, terminating the reaction (step 2) when the factor Xa concentration has reached approximately 50 per cent of the maximal (plateau) level. Appropriate activation times are usually between 2 min and 5 min.

**Step 2**. Terminate the activation by addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as a 50 per cent *V/V* solution of acetic acid, or a 1 M pH 3 citrate buffer solution. Adjust the hydrolysis time to achieve a linear development of chromophore over time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

**04/2017:20705**

# 2.7.5. ASSAY OF HEPARIN

The anticoagulant activity of heparin is determined *in vitro* by its ability to accelerate the inhibition of thrombin, factor IIa (anti-IIa assay), by antithrombin. The International Unit is the activity contained in a stated amount of the International Standard for unfractionated heparin. *Heparin sodium BRP*, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as the reference preparation.

The assay of anti-factor Xa activity is carried out to determine the ratio of anti-factor Xa activity to anti-factor IIa activity.

For anti-IIa and anti-Xa assays, carry out the assay by determining the absorbance (end-point method) or the change of absorbance per minute (kinetic method).

ANTI-FACTOR IIa ACTIVITY

**Reference and test solutions**

Prepare 4 independent series of 4 dilutions each of the substance to be examined and of *heparin sodium BRP* in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*; a concentration range within 0.005 IU and 0.03 IU per millilitre is suitable. The dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

**Procedure**

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_1$, $T_2$, $T_3$, $T_4$ for each of the 4 series of dilutions of the substance to be examined and $S_1$, $S_2$, $S_3$, $S_4$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 100 µL of *antithrombin III solution R5* and 50 µL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_1$, $S_2$, $S_3$, $S_4$, $T_1$, $T_2$, $T_3$, $T_4$, $T_1$, $T_2$, $T_3$, $T_4$, $S_1$, $S_2$, $S_3$, $S_4$, allow to equilibrate at 37 °C (water-bath or heating block) for at least 1 min and add to each tube 25 µL of *human thrombin solution R2*. Incubate for exactly 1 min and add 50 µL of a chromogenic substrate specific to factor IIa at a concentration suitable for the assay (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride dissolved in *water R* to give a 1.25 mM solution).

For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (*2.2.25*) at 405 nm using a suitable reading device.

For the end-point method, stop the reaction after exactly 4 min by adding 50 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Assess whether exactly 4 min of incubation with the chromogenic substrate yields the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm using a suitable reading device.

Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (*5.3*).

ANTI-FACTOR Xa ACTIVITY

**Reference and test solutions**

Prepare 4 independent series of 4 dilutions each of the substance to be examined and of *heparin sodium BRP* in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*; a concentration range within 0.03 IU and 0.375 IU per millilitre is suitable. The dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

**Procedure**

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_1$, $T_2$, $T_3$, $T_4$ for each of the 4 series of dilutions of the substance to be examined and $S_1$, $S_2$, $S_3$, $S_4$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R6* and 50 µL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_1$, $S_2$, $S_3$, $S_4$, $T_1$, $T_2$, $T_3$, $T_4$, $T_1$, $T_2$, $T_3$, $T_4$, $S_1$, $S_2$, $S_3$, $S_4$, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *bovine factor Xa solution R2*. Incubate for exactly 2 min and add 100 µL of a chromogenic substrate specific to factor Xa at a concentration suitable for the assay (for example, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride dissolved in *water R* to give a 1 mM solution).

For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (*2.2.25*) at 405 nm using a suitable reading device.

For the end-point method, stop the reaction after exactly 4 min by adding 50 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Assess whether exactly 4 min of incubation with the chromogenic substrate yields the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm using a suitable reading device.

Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (*5.3*).

**01/2008:20706**
**corrected 6.0**

# 2.7.6. ASSAY OF DIPHTHERIA VACCINE (ADSORBED)

The potency of diphtheria vaccine is determined by administration of the vaccine to guinea-pigs followed either by challenge with diphtheria toxin (method A or B) or by determination of the titre of antibodies against diphtheria toxin or toxoid in the serum of guinea-pigs (method C). In both cases, the potency of the vaccine is calculated by comparison with a reference preparation, calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of diphtheria toxoid adsorbed on aluminium hydroxide. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

*Diphtheria vaccine (adsorbed) BRP* is suitable for use as a reference preparation.

The method chosen for the assay of diphtheria vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from diphtheria toxoid of the same origin, and with comparable levels (expressed in Lf/mL) of the same diphtheria toxoid, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccines. Any combinations containing a whole-cell pertussis component or containing haemophilus type b conjugate vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier in the same vial must always be assessed separately.

For combinations containing diphtheria and tetanus components, the serological assay (method C) can be performed with the same group of animals used for the serological assay of the tetanus vaccine (adsorbed) (*2.7.8*) when the common immunisation conditions for the diphtheria and the tetanus components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Once the analyst has sufficient experience with this method for a given vaccine, it is possible to apply a simplified model such as a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required, but does not give information on linearity, parallelism and the dose-response curve. The simplified model allows for a considerable reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- the mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation;
- the antitoxin titres or scores of run controls (positive and negative serum samples);
- the ratio of antitoxin titres or scores for the positive serum control to the serum samples corresponding to the reference vaccine.

METHOD A: INTRADERMAL CHALLENGE TEST IN GUINEA-PIGS

*SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy, white guinea-pigs from the same stock and of a size suitable for the prescribed number of challenge sites, the difference in body mass between the heaviest and the lightest animal being not greater than 100 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 5 guinea-pigs as unvaccinated controls.

*SELECTION OF THE CHALLENGE TOXIN*

Select a preparation of diphtheria toxin containing 67 to 133 lr/100 in 1 Lf and 25 000 to 50 000 minimal reacting doses for guinea-pig skin in 1 Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

*PREPARATION OF THE CHALLENGE TOXIN SOLUTION*

Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 mL. Prepare from this a further series of 5 four-fold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf in 0.2 mL.

*DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, will result in an intradermal score of approximately 3 when the animals are challenged.

*IMMUNISATION AND CHALLENGE*

Allocate the dilutions, 1 to each of the groups of guinea-pigs, and inject subcutaneously 1.0 mL of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, shave both flanks of each guinea-pig and inject 0.2 mL of each of the 6 toxin dilutions intradermally into 6 separate sites on each of the vaccinated guinea-pigs in such a way as to minimise interference between adjacent sites.

*DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN*

If necessary, inject the unvaccinated control animals with dilutions containing 80, 40, 20, 10 and $5 \times 10^{-6}$ Lf of the challenge toxin.

*READING AND INTERPRETATION OF RESULTS*

Examine all injection sites 48 h after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intra-dermal challenge score. Tabulate the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as $(\text{score})^2$ or $\arcsin((\text{score}/6)^2)$, to obtain an estimate of the relative potency for each of the test preparations by parallel-line quantitative analysis.

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the mean score obtained at the lowest dose level is less than 3 and the mean score at the highest dose level is more than 3;
- where applicable, the toxin dilution that contains $40 \times 10^{-6}$ Lf gives a positive erythema in at least 80 per cent of the control guinea-pigs and the dilution containing

$20 \times 10^{-6}$ Lf gives a positive erythema in less than 80 per cent of the guinea-pigs (if these criteria are not met a different toxin has to be selected);

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

– the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD B: LETHAL CHALLENGE TEST IN GUINEA-PIGS

*SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 4 further groups of 5 guinea-pigs as unvaccinated controls.

*SELECTION OF THE CHALLENGE TOXIN*

Select a preparation of diphtheria toxin containing not less than 100 $LD_{50}$ per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the lethal dose for every assay.

*PREPARATION OF THE CHALLENGE TOXIN SOLUTION*

Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing approximately 100 $LD_{50}$ per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 32, 1 to 100 and 1 to 320 with the same diluent.

*DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, protect approximately 50 per cent of the animals from the lethal effects of the subcutaneous injection of the quantity of diphtheria toxin prescribed for this test.

*IMMUNISATION AND CHALLENGE*

Allocate the dilutions, 1 to each of the groups of guinea-pigs, and inject subcutaneously 1.0 mL of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 mL of the challenge toxin solution (100 $LD_{50}$).

*DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN*

If necessary, allocate the challenge toxin solution and the 3 dilutions made from it, 1 to each of the 4 groups of 5 guinea-pigs, and inject subcutaneously 1.0 mL of each solution into each guinea-pig in the group to which that solution is allocated.

*READING AND INTERPRETATION OF RESULTS*

Count the number of surviving guinea-pigs 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of animals surviving in each of the groups of vaccinated guinea-pigs, using the usual statistical methods (for example, *5.3*).

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

– for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs;

– where applicable, the number of animals that die in the 4 groups of 5 injected with the challenge toxin solution and its 3 dilutions indicates that the challenge dose was approximately 100 $LD_{50}$;

– the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

– the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

*SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

*REFERENCE PREPARATION*

Use a suitable reference preparation such as *diphtheria vaccine (adsorbed) BRP* or a batch of vaccine shown to be effective in clinical studies, or a batch representative thereof, and which has been calibrated in International Units with reference to *diphtheria vaccine (adsorbed) BRP* or the International Standard for diphtheria toxoid (adsorbed).

*DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the vaccine to be examined and the reference preparation; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range of, for example, 0.5-16 IU/mL for the reference vaccine and within the range of, for example, 1:2 to 1:125 for the vaccine to be examined. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

*IMMUNISATION*

Inject subcutaneously to each guinea-pig 1.0 mL of the dilution allocated to its group.

*BLOOD SAMPLING*

35-42 days after immunisation, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

*PREPARATION OF SERUM SAMPLES*

Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

*DETERMINATION OF ANTIBODY TITRE*

Determine the relative antibody titre or score of each serum sample by a suitable immunochemical method (*2.7.1*). The methods shown below (enzyme-linked immunosorbent assay (ELISA) and Vero cell assay) have been found to be suitable.

*CALCULATION OF POTENCY*

Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the usual statistical methods (for example, *5.3*).

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

*The following section is published for information.*

## Assay of diphtheria vaccine (adsorbed): guidelines

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

*PREPARATION OF SERUM SAMPLES*

For the preparation of serum samples, the following technique has been found to be suitable. Invert the tubes containing blood samples 6 times and allow to stand at 37 °C for 2 h, then at 4 °C for 2 h. Centrifuge at room temperature at 800 *g* for 20 min. Transfer the serum to sterile tubes and store at a temperature below − 20 °C. At least a 40 per cent yield of serum is obtained by this procedure.

*DETERMINATION OF ANTIBODY TITRE*

The ELISA and Vero cell assays shown below are given as examples of immunochemical methods that have been found to be suitable for the determination of antibody titre.

**Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA)**. Dilutions of test and reference sera are made on ELISA plates coated with diphtheria toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added, followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods (for example, *5.3*).

*Reagents and equipment*

- *ELISA plates*: 96 wells, columns 1-12, rows A-H.
- *Diphtheria guinea-pig antiserum (for vaccines-human use)* (positive control serum), obtained by immunisation of guinea-pigs using *diphtheria vaccine (adsorbed) BRP*.
- *Peroxidase conjugate*. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.
- *Diphtheria toxoid*.
- *Carbonate coating buffer pH 9.6*. Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R*. Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.
- *Phosphate-buffered saline pH 7.4 (PBS)*. Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 mL of *water R*. Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water R* before use.
- *Citric acid solution*. Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust the solution to pH 4.0 with a 400 g/L solution of *sodium hydroxide R*.
- *Washing buffer*. PBS containing 0.5 g/L of *polysorbate 20 R*.
- *Diluent blocking buffer*. PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.
- *Peroxidase substrate*. Shortly before use, dissolve 10 mg of *diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R*.

*Method*

The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 12A-H are for positive control serum for assay monitoring. Wells 3-11A-H are for test samples.

Coat each well of the ELISA plates with 100 µL of diphtheria toxoid solution (0.5 Lf/mL in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with washing buffer. Block the plates by addition of 100 µL of diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing buffer. Place 100 µL of diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of negative control serum, positive control serum (from about 0.01 IU/mL) and test sera. Allocate the negative control serum to column 1, positive control serum to columns 2 and 12 and test sera to columns 3-11 and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make twofold serial dilutions from row B, down the plate to row H, by transferring 100 µL from one well to the next well. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with washing buffer. Prepare a suitable dilution (a 2000-fold dilution has been found to be suitable) of peroxidase conjugate in diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing buffer. Add 100 µL of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

**Determination of antibody titre in guinea-pig serum by Vero cell assay**. The method used relies either on metabolic inhibition (method 1) or on cytotoxicity (method 2) as the end point, and on either microscopic (cell morphology) or visual (colour) inspection of the cells.

The limit of detection is specific for each antitoxin and is usually between 0.015 IU/mL (method 1) and 0.05 IU/mL (method 2).

The endpoint is taken as the highest serum dilution protecting cells from the diphtheria toxin effect. The antitoxin activity is calculated with respect to guinea-pig or WHO reference standard, and expressed in International Units per millilitre.

*Reagents and equipment*

- *Flat-bottomed tissue culture plates*: 96 wells, columns 1-12, rows A-H.
- *75 $cm^2$ tissue culture flasks*.
- *Diphtheria toxin*.
- *Diphtheria guinea-pig antiserum (for vaccines-human use)* (positive control serum), obtained by immunisation of guinea-pigs with *diphtheria vaccine (adsorbed) BRP*.
- *Vero cells* (African Green Monkey kidney cells). Cell passages from P2 to P15 are suitable for use.

*Method 1*. The diphtheria toxin causes a cytopathogenic effect on Vero cells leading to cellular lysis. Antibodies directed against diphtheria toxin may inhibit this cytopathogenic effect. Consequently, the potency of a diphtheria vaccine may be indirectly determined with the help of this cell culture system if different serum dilutions from immunised animals are cultured with a constant toxin concentration. In the Vero

cell assay, yellow colour indicates viable cells, red colour dead cells. When only part of the cells are dead, the colour may be orange.

*Reagents and equipment*

- *Modified MEM*. Minimum Essential Medium (MEM) with Earle's Salts, without L-glutamine and sodium bicarbonate.
- *Modified medium 199*. Medium 199, with Hanks' Solution and L-glutamine, without sodium bicarbonate.
- *Foetal bovine serum*.
- *Sodium bicarbonate 7.5 per cent solution*.
- *Trypsin solution*: trypsin 2.5 per cent solution.
- *EDTA solution*: EDTA 0.02 per cent (Versene 1:5000) solution.
- *Modified D-PBS*. Dulbecco's phosphate buffered saline (D-PBS), without calcium, or magnesium.
- *L-glutamine 200mM solution*.
- *Penicillin/streptomycin solution*.
- *Primary culture medium*. To 50 mL of modified MEM add 440 mL of *water R*, 5 mL of L-glutamine 200 mM solution, and 10 mL of sodium bicarbonate 7.5 per cent solution. To 25 mL of this medium add 1.25 mL of foetal bovine serum.
- *Maintenance culture medium*. Similar to the primary culture medium except that 0.5 mL instead of 1.25 mL of foetal bovine serum is added to 20 mL of the enriched MEM medium.
- *Medium A*. To 50.0 mL of modified medium 199 add 440.0 mL of *water R*, 5.0 mL of L-glutamine 200 mM solution and 10.0 mL of sodium bicarbonate 7.5 per cent solution.
- *Medium B*. To 150.0 mL of medium A add 3.0 mL of foetal bovine serum and 0.3 mL of penicillin/streptomycin solution.
- *Medium C*. To 22.0 mL of medium A add 0.44 mL of foetal bovine serum and 0.44 mL of penicillin/streptomycin solution.

Vero cells are cultured in tissue culture flasks (for example 75 $cm^2$/250 mL) in an incubator at 36 ± 1 °C, 5 per cent $CO_2$ and 90 per cent relative humidity. Vero cells are first grown in the primary culture medium. After 2-3 days of growth, the primary culture medium is replaced by the maintenance culture medium. When a confluent monolayer is obtained, the culture supernatant is discarded and the cell layer washed gently with modified D-PBS. Add a mixture of 1 volume of trypsin solution and 1 volume of EDTA solution to the flask. Swirl the flask gently and incubate in the $CO_2$ incubator for about 3 min until the cells start to break from the monolayer. Vigorously tap the side of the flask to make the cells fall. Resuspend the cells in 5-6 mL of fresh medium C to obtain a homogeneous suspension. Prepare a cell suspension in medium C containing approximately $1 \times 10^5$ cells/mL.

Place 25 µL of medium B in each well except those of column 1. Place 25 µL of the diphtheria guinea-pig antiserum (for vaccines-human use) (positive control serum, working dilution in medium B of 0.40 IU/mL) in wells A1, A2 and A11. Place 25 µL of guinea-pig serum samples in wells B-G of columns 1, 2 and 11. Place 25 µL of negative control serum in row H of columns 1, 2 and 11. Using a multichannel micropipette, make twofold serial dilutions across the plate (from column 2 up to column 10 for rows A-G and up to column 8 for row H). Discard 25 µL from the wells in column 10 in rows A-G, and from well H8.

Reconstitute the diphtheria toxin with saline solution to give a solution of 50 IU/mL. Prepare a 50-fold dilution of this diphtheria toxin dilution in medium B to obtain a working solution of 1.0 IU/mL. Add 25 µL of this working solution to wells A12 and B12 (toxin control). Make twofold serial dilutions by tranferring 25 µL from one well to the next, from well B12 down to H12. Change the tip between each dilution. Discard 25 µL from well H12. Add 25 µL of medium B to wells B12-H12. Then, place 25 µL of the working dilution of the diphtheria toxin (1.0 IU/mL) in each well of rows A-H, from column 1-10, except in wells H9 and H10 (cells only, without serum and without toxin).

Cover the plates with lids or sealer and shake gently. Incubate the plates for at least 2 h in a humid container in a $CO_2$ incubator at 37 °C. Add 200 µL of cell suspension containing $1 \times 10^5$ cells/mL to all the wells. Cover the plates with sealer. Incubate at 37 °C for 5 days. Check for microbial contamination by microscopic examination.

Yellow wells are recorded as negative and red wells indicate dead cells and are recorded as positive. A colour between yellow and red indicates a mixture of viable and dead cells and is recorded as positive/negative. The results based on the change in colour can be confirmed by reading viable and dead cells under the microscope.

The potency of the guinea-pig antiserum samples is obtained by comparing the last well of the standard preparation showing complete neutralisation of the toxin, with the last well of the sample demonstrating the same effect. For calculations of potency, it must be remembered that the endpoint may be between a negative well and a positive/negative well.

*Method 2*: Thiazolyl blue MTT is reduced to a blue/black formazan product by the mitochondrial dehydrogenase of viable cells, and thus serves as a quantitative measure of living cells present, indicating when the toxin has been neutralised by the antitoxin. White or colourless wells indicate absence of viable cells due to insufficient antitoxin to neutralise the toxin.

*Reagents and equipment*

- *MEM* (Minimal Essential Media).
- *Newborn calf serum*.
- *Antibiotic solution* (containing 10 000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per millilitre).
- *L-glutamine 200mM solution*.
- *Trypsin-EDTA*.
- *Thiazolyl blue MTT* [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].
- *1 M HEPES buffer pH 8.1*. Dissolve 18.75 g of HEPES in 82.5 mL of *water R* and 30.0 mL of *2 M sodium hydroxide R*.
- *Glucose solution (10 per cent)*.
- *Complete culture medium*. Mix 200 mL of MEM with 10 mL of newborn calf serum, 3.0 mL of 1 M HEPES buffer pH 8.1, 2.0 mL of glucose solution (10 per cent), 2.0 mL of antibiotic solution and 2.0 mL of L-glutamine 200mM solution.
- *Phosphate-buffered saline pH 7.4 (PBS)*. Dissolve 10.0 g of *sodium chloride R*, 0.75 g of *potassium chloride R*, 1.44 g of *disodium hydrogen phosphate dodecahydrate R*, and 0.125 g of *potassium dihydrogen phosphate R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary. Autoclave at 120 °C for 15 min.
- *Thiazolyl blue MTT solution*. Dissolve 0.1 g of thiazolyl blue MTT in 20 mL of PBS. Sterilise by filtration (0.2 µm) and store in dark bottle.
- *pH adjuster solution*. Mix 40 mL of *acetic acid R* with 1.25 mL of *1 M hydrochloric acid* and 8.75 mL of *water R*.
- *Extraction buffer pH 4.7*. Dissolve 10 g of *sodium laurilsulfate R* in *water R* and add 50 mL of *dimethylformamide R*, and dilute to 100 mL with *water R*. Adjust the pH with an appropriate volume of pH adjuster solution.

Vero cells are cultured in tissue culture flasks (for example 75 $cm^2$/250 mL) in an incubator at 36 ± 1 °C, 5 per cent $CO_2$ and 90 per cent relative humidity. Vero cells are grown in the complete culture medium. After 6-7 days of growth, a confluent monolayer is obtained, the culture supernatant is discarded and the cell layer is washed 3 times with trypsin-EDTA: gently pipette out the medium, add 0.5-1 mL

of trypsin-EDTA, swirl the flask and tip the trypsin out. Do this twice, and the 3rd time, place the flask in the incubator for 5 min until the cells start to break from the monolayer. Vigorously tap the side of the flask to make the cells fall. Resuspend the cells in 6-25 mL of fresh complete culture medium to obtain a homogeneous suspension. Prepare a cell suspension in complete culture medium containing approximately $4 \times 10^5$ cells/mL.

Place 50 µL of complete culture medium in each well except those of column 1. Place 100 µL of diphtheria guinea pig antiserum (for vaccines-human use) (positive control serum, working dilution in complete culture medium of 0.12 IU/mL) in well A1 and 50 µL in well A11. Place 100 µL of guinea pig test serum samples, diluted if necessary, in wells B1-G1. Add 50 µL of the same sample to wells B11-G11 in the corresponding row. Place 100 µL of negative control serum in well H1 and 50 µL in well H11. Using a multi-channel micropipette, make twofold serial dilutions by transferring 50 µL from one well to the next working across the plate (from column 1-10 for rows A-G and from column 1-8 for row H).

Diphtheria toxin of known activity and Lf content is diluted to a suitable working stock containing at least 4 minimum cytopathic doses in complete culture medium. Add 50 µL of the diluted toxin to each well except H9 and H10 (cell control), A11-H11 (serum control) and A12-H12 (toxin control). Add 100 µL of diluted toxin to well A12 and make twofold serial dilutions by transferring 50 µL from one well to the next working down the plate (from well A12-H12). Discard 50 µL from well H12. Add 50 µL of complete medium to wells H9 and H10.

Cover the plates with a lid or sealer and leave for 1 h at room temperature to allow toxin neutralisation to occur. 50 µL of cell suspension containing approximately $4 \times 10^5$ cells/mL is added to each well. The plates are sealed and incubated at 37 °C for 6 days. Check for microbial contamination by microscopic examination. 10 µL of thyazolyl blue MTT solution is added to each well. The plates are incubated at 37 °C for a further 2-4 h. Then, the medium is removed and 100 µL of extraction buffer pH 4.7 is added to each well. The plates are incubated at 37 °C and left overnight to aid the extraction process. Once extraction and solubilisation is complete, plates are visually examined or read at 570 nm.

Blue/black wells are recorded as negative (all the cells are alive, toxin neutralisation by antitoxin) and white or colourless wells indicate dead cells (no toxin neutralisation) and are recorded as positive.

The potency of the test antitoxin is obtained by comparing the last well of the reference antitoxin preparation showing neutralisation of the toxin, with the last well of the antitoxin preparation demonstrating the same effect. The neutralising antibody titre of the sample being examined can be calculated by multiplication of the dilution factor with total number of International Units per millilitre of the reference preparation at the end point. The test is valid if all the cells in the toxin control are dead and reference antitoxin gives a neutralisation in at least the first 2 dilutions tested.

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## 2.7.7. ASSAY OF PERTUSSIS VACCINE (WHOLE CELL)

The potency of pertussis vaccine (whole cell) is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of *Bordetella pertussis*, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of dried pertussis vaccine. The equivalence in International Units of the International Standard is stated by the World Health Organization.

**Selection and distribution of the test animals**. Use in the test healthy mice less than 5 weeks old of a suitable strain from the same stock, the difference in mass between the heaviest and the lightest being not greater than 5 g. Distribute the mice in 6 groups of not fewer than 16 and 4 groups of 10. The mice must all be of the same sex or the males and females distributed equally between the groups.

**Selection of the challenge strain and preparation of the challenge suspension**. Select a suitable strain of *B. pertussis* capable of causing the death of mice within 14 days of intracerebral injection. If more than 20 per cent of the mice die within 48 h of the injection the strain is not suitable. Make one subculture from the strain and suspend the harvested *B. pertussis* in a solution containing 10 g/L of *casein R* hydrolysate and 6 g/L of *sodium chloride R* and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension. Prepare a series of dilutions in the same solution and allocate each dilution to a group of 10 mice. Inject intracerebrally into each mouse a dose (0.02 mL or 0.03 mL) of the dilution allocated to its group. After 14 days, count the number of mice surviving in each group. From the results, calculate the expected opacity of a suspension containing 100 $LD_{50}$ in each challenge dose. For the test of the vaccine to be examined make a fresh subculture from the same strain of *B. pertussis* and prepare a suspension of the harvested organisms with an opacity corresponding to about 100 $LD_{50}$ in each challenge dose. Prepare 3 dilutions of the challenge suspension.

**Determination of potency**. Prepare 3 serial dilutions of the vaccine to be examined and 3 similar dilutions of the reference preparation such that in each the intermediate dilution may be expected to protect about 50 per cent of the mice from the lethal effects of the challenge dose of *B. pertussis*. Suggested doses are 1/8, 1/40 and 1/200 of the human dose of the vaccine to be examined and 0.5 IU, 0.1 IU and 0.02 IU of the reference preparation, each dose being contained in a volume not exceeding 0.5 mL. Allocate the 6 dilutions, one to each of the groups of not fewer than 16 mice, and inject intraperitoneally into each mouse one dose of the dilution allocated to its group. After 14 - 17 days inject intracerebrally into each animal in the groups of not fewer than 16, one dose of the challenge suspension. Allocate the challenge suspension and the 3 dilutions made from it, one to each of the groups of 10 mice, and inject intracerebrally one dose of each suspension into each mouse in the group to which that suspension is allocated. Exclude from consideration any mice that die within 48 h of challenge. Count the number of mice surviving in each of the groups after 14 days. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the numbers of animals surviving in each of the groups of not fewer than 16.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and the smallest doses given to the mice;
- the number of animals that die in the 4 groups of 10 injected with the challenge suspension and its dilutions indicates that the challenge dose is approximately 100 $LD_{50}$; and
- the statistical analysis shows no deviation from linearity or parallelism.

The test may be repeated but when more than one test is performed the results of all valid tests must be combined.

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corrected 10.0

# 2.7.8. ASSAY OF TETANUS VACCINE (ADSORBED)

The potency of tetanus vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases, the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the paralysis method is not obligatory, the $LD_{50}$ method may be used. For the $LD_{50}$ method, the number of animals and the procedure are identical to those described for the paralysis method, but the end-point is the death of the animal rather than paralysis.

The International Unit is the activity contained in a stated amount of the International Standard for tetanus toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Tetanus vaccine (adsorbed) BRP* is calibrated in International Units with reference to the International Standard.

The method chosen for the assay of tetanus vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;
2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from tetanus toxoid of the same origin and with comparable levels (expressed in Lf/mL) of the same tetanus toxoid, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccines. Any combinations containing a whole-cell pertussis component or containing haemophilus type b conjugate vaccine with tetanus toxoid in the same vial must always be assessed separately.

For combinations containing diphtheria and tetanus components, the serological assay (method C) can be performed with the same group of animals used for the serological assay of the diphtheria vaccine (adsorbed) (*2.7.6*) when the common immunisation conditions for the tetanus and the diphtheria components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multiple-dilution assays, it may be possible to reduce the number of animals needed to obtain a statistically significant result by applying a simplified model such as a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required, but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model allows for a considerable reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- the mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation;
- the antitoxin titres or scores of run controls (positive and negative serum samples);
- the ratio of antitoxin titres or scores for the positive serum control to the serum samples corresponding to the reference vaccine.

## METHOD A. CHALLENGE TEST IN GUINEA-PIGS

### *SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the activity of the challenge toxin has to be determined, include 3 further groups of 5 guinea-pigs as unvaccinated controls.

### *SELECTION OF THE CHALLENGE TOXIN*

Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

### *PREPARATION OF THE CHALLENGE TOXIN SOLUTION*

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

### *DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

### *IMMUNISATION AND CHALLENGE*

Allocate the dilutions, 1 to each of the groups of guinea-pigs, and inject subcutaneously 1.0 mL of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 mL of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

### *DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN*

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of 5 guinea-pigs, and inject subcutaneously 1.0 mL of each solution into each guinea-pig in the group to which that solution is allocated. The activity and stability of the challenge toxin are determined by carrying out a suitable number of determinations of the 50 per cent paralytic dose. It is then not necessary to repeat the determination for each assay.

*READING AND INTERPRETATION OF RESULTS*

Examine the guinea-pigs twice daily. Remove and euthanise all animals showing definite signs of tetanus paralysis. Count the number of guinea-pigs without paralysis 5 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated guinea-pigs, using the usual statistical methods (for example, *5.3*).

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs;
- where applicable, the number of paralysed animals in the 3 groups of 5 injected with the dilutions of the challenge toxin solution indicates that the challenge was approximately 50 times the 50 per cent paralytic dose;
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD B. CHALLENGE TEST IN MICE

*SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use mice of the same sex or with males and females equally distributed between the groups. Distribute the mice in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 3 further groups of not fewer than 5 mice to serve as unvaccinated controls.

*SELECTION OF THE CHALLENGE TOXIN*

Select a preparation of tetanus toxin containing not less than 100 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

*PREPARATION OF THE CHALLENGE TOXIN SOLUTION*

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose in 0.5 mL. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

*DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 0.5 mL per mouse, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

*IMMUNISATION AND CHALLENGE*

Allocate the dilutions, 1 to each of the groups of mice, and inject subcutaneously 0.5 mL of each dilution into each mouse in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 0.5 mL of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

*DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN*

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of not fewer than 5 mice, and inject subcutaneously 0.5 mL of each solution into each mouse in the group to which that solution is allocated.

*READING AND INTERPRETATION OF RESULTS*

Examine the mice twice daily. Remove and euthanise all animals showing definite signs of tetanus paralysis. Count the number of mice without paralysis 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated mice, using the usual statistical methods (for example, *5.3*).

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the mice;
- where applicable, the number of paralysed animals in the 3 groups of not fewer than 5 injected with the dilutions of the challenge toxin solution, indicates that the challenge dose was approximately 50 times the 50 per cent paralytic dose;
- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

*SELECTION AND DISTRIBUTION OF THE TEST ANIMALS*

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

*REFERENCE PREPARATION*

Use a suitable reference preparation such as *tetanus vaccine (adsorbed) BRP* or a batch of vaccine shown to be effective in clinical studies, or a batch representative thereof, and which has been calibrated in International Units with reference to *tetanus vaccine (adsorbed) BRP* or the International Standard for tetanus toxoid (adsorbed).

*DILUTION OF THE TEST AND REFERENCE PREPARATIONS*

Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the vaccine to be examined and the reference preparation; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range of, for example, 0.5-16 IU/mL for each series. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

*IMMUNISATION*

Inject subcutaneously to each guinea-pig 1.0 mL of the dilution allocated to its group.

*BLOOD SAMPLING*

35-42 days after immunisation, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

*PREPARATION OF SERUM SAMPLES*

Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

*DETERMINATION OF ANTIBODY TITRE*

Determine the relative antibody titre or score of each serum sample by a suitable immunochemical method (*2.7.1*). The methods shown below (enzyme-linked immunosorbent assay (ELISA) and toxin-binding inhibition (ToBI)) have been found to be suitable.

*CALCULATION OF POTENCY*

Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the usual statistical methods (for example, *5.3*).

*REQUIREMENTS FOR A VALID ASSAY*

The test is not valid unless:

- the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

*The following section is published for information.*

## Assay of tetanus vaccine (adsorbed): guidelines

METHOD A. CHALLENGE TEST IN GUINEA-PIGS

*READING AND INTERPRETATION OF RESULTS*

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when subcutaneous injection of the challenge toxin is made mid-ventrally, directly behind the sternum with the needle pointing towards the neck of the guinea-pig. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in at least 1 of the forelimbs paralysis that can be recognised at an early stage. The tetanus grades in guinea-pigs are characterised by the following signs:

- T1: slight stiffness of 1 forelimb, but difficult to observe;
- T2: paresis of 1 forelimb which still can function;
- T3: paralysis of 1 forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis;
- T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed;
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

METHOD B. CHALLENGE TEST IN MICE

*READING AND INTERPRETATION OF RESULTS*

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in the toxin-injected hind leg paresis followed by paralysis that can be recognised at an early stage. The tetanus grades in mice are characterised by the following signs:

- T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;
- T2: paresis of the toxin-injected hind leg, which still can function for walking;
- T3: paralysis of the toxin-injected hind leg, which does not function for walking;
- T4: the toxin-injected hind leg is completely stiff with immovable toes;
- T5: tetanus seizures, continuous tonic spasm of muscles;
- D: death.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

*PREPARATION OF SERUM SAMPLES*

For the preparation of serum samples, the following technique has been found to be suitable. Invert the tubes containing blood samples 6 times and allow to stand at 37 °C for 2 h, then at 4 °C for 2 h. Centrifuge at room temperature at 800 *g* for 20 min. Transfer the serum to sterile tubes and store at a temperature below − 20 °C. At least a 40 per cent yield of serum is obtained by this procedure.

*DETERMINATION OF ANTIBODY TITRE*

The ELISA and ToBI tests shown below are given as examples of immunochemical methods that have been found to be suitable for the determination of antibody titre.

**Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA)**. Dilutions of test and reference sera are made on ELISA plates coated with tetanus toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added, followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods (for example, *5.3*).

*Reagents and equipment*

- *ELISA plates*: 96 wells, columns 1-12, rows A-H.
- *Clostridium tetani guinea-pig antiserum (for vaccines-human use) BRP* (positive control serum).
- *Peroxidase conjugate*. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.
- *Tetanus toxoid*.
- *Carbonate coating buffer pH 9.6*. Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R*. Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.
- *Phosphate-buffered saline pH 7.4 (PBS)*. Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 mL of *water R*. Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water R* before use.
- *Citric acid solution*. Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust the solution to pH 4.0 with a 400 g/L solution of *sodium hydroxide R*.
- *Washing buffer*. PBS containing 0.5 g/L of *polysorbate 20 R*.
- *Diluent block buffer*. PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.

– *Peroxidase substrate*. Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R*.

*Method*

The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 12A-H are for positive control serum for assay monitoring. Wells 3-11A-H are for test samples.

Coat each well of the ELISA plates with 100 µL of tetanus toxoid solution (0.5 Lf/mL in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with washing buffer. Block the plates by addition of 100 µL of diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing buffer. Place 100 µL of diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of negative control serum, positive control serum (from about 0.01 IU/mL) and test sera. Allocate the negative control serum to column 1, positive control serum to columns 2 and 12 and test sera to columns 3-11 and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make twofold serial dilutions from row B down the plate to row H, by transferring 100 µL from one well to the next. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with washing buffer. Prepare a suitable dilution (a 2000-fold dilution has been found to be suitable) of peroxidase conjugate in diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing buffer. Add 100 µL of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

**Determination of antibody titre in guinea-pig serum by toxin- or toxoid-binding inhibition (ToBI)**. Tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. To determine unbound toxin or toxoid, the mixtures are transferred to an ELISA plate coated with tetanus antitoxin. Peroxidase-conjugated equine anti-tetanus IgG is added followed by a peroxidase substrate. Optical density is measured and the antibody titre is calculated using the usual statistical methods (for example, *5.3*). A positive control serum and a negative control serum are included on each plate to monitor assay performance.

*Reagents and equipment*

– *Round-bottomed, rigid polystyrene microtitre plates*.

– *Flat-bottomed ELISA plates*.

– *Tetanus toxin or tetanus toxoid*.

– *Clostridium tetani guinea-pig antiserum (for vaccines-human use) BRP* (positive control serum).

– *Equine anti-tetanus IgG*.

– *Peroxidase-conjugated equine anti-tetanus IgG*.

– *Carbonate buffer pH 9.6*. Dissolve 1.5 g of *anhydrous sodium carbonate R*, 2.39 g of *sodium hydrogen carbonate R* and 0.2 g of *sodium azide R* in 1000 mL of *water R*, adjust to pH 9.6 and autoclave at 121 °C for 20 min.

– *Sodium acetate buffer pH 5.5*. Dissolve 90.2 g of *anhydrous sodium acetate R* in 900 mL of *water R*, adjust to pH 5.5 using a saturated solution of *citric acid monohydrate R* and dilute to 1000 mL with *water R*.

– *Phosphate-buffered saline pH 7.2 (PBS)*. Dissolve 135.0 g of *sodium chloride R*, 20.55 g of *disodium hydrogen phosphate dihydrate R* and 4.80 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 15 L with the same solvent. Autoclave at 100 °C for 60 min.

– *Diluent buffer*. PBS containing 5 g/L of *bovine albumin R* and 0.5 g/L of *polysorbate 80 R*.

– *Block buffer*. PBS containing 5 g/L of *bovine albumin R*.

– *Tetramethylbenzidine solution*. 6 g/L solution of *tetramethylbenzidine R* in *ethanol (96 per cent) R*. The substance dissolves within 30-40 min at room temperature.

– *Peroxidase substrate*. Mix 90 mL of *water R*, 10 mL of sodium acetate buffer pH 5.5, 1.67 mL of tetramethylbenzidine solution and 20 µL of *strong hydrogen peroxide solution R*.

– *Washing solution*. Tap water containing 0.5 g/L of *polysorbate 80 R*.

*Method*

Block the microtitre plates by placing in each well 150 µL of block buffer. Cover the plates with a lid or sealer. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing solution. Place 100 µL of PBS in each well. Place 100 µL of reference guinea-pig tetanus antitoxin in the first well of a row. Place 100 µL of undiluted test sera in the first well of the required number of rows. Using a multichannel micropipette, make twofold serial dilutions across the plate (up to column 10), by transferring 100 µL from one well to the next. Discard 100 µL from the last column so that all wells contain 100 µL. Prepare a 0.1 Lf/mL solution of tetanus toxin or toxoid using PBS as diluent. Add 40 µL of this solution to each well except those of column 12. The wells of column 11 are a positive control. Add 40 µL of PBS to the wells of column 12 (negative control). Shake the plates gently and cover them with lids. Coat the ELISA plates: immediately before use make a suitable dilution of equine anti-tetanus IgG in carbonate buffer pH 9.6 and add 100 µL to each well. Incubate the 2 series of plates overnight in a humid atmosphere at 37 °C. To avoid temperature gradient effects, do not stack more than 4 plates high. Cover the plates with lids. On the following day, wash the ELISA plates thoroughly with washing solution. Block the plates by placing in each well 125 µL of block buffer. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing solution. Transfer 100 µL of the pre-incubation mixture from the polystyrene plates to the corresponding wells of the ELISA plates, starting with column 12 and then continuing from 1 to 11. Cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 2 h. Wash the ELISA plates thoroughly with washing solution. Make a suitable dilution (a 4000-fold dilution has been found to be suitable) of the peroxidase-conjugated equine anti-tetanus IgG in diluent buffer. Add 100 µL of the dilution to each well and cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 1.5 h. Wash the ELISA plates thoroughly with washing solution. Add 100 µL of peroxidase substrate to each well. A blue colour develops. Incubate the plates at room temperature. Stop the reaction at a given time (within 10 min) by the addition of 100 µL of a 2 M solution of sulfuric acid prepared from *sulfuric acid R* to each well in the same order as the addition of substrate. The colour changes from blue to yellow. Measure the absorbance at 450 nm immediately after addition of the sulfuric acid or maintain the plates in the dark until reading.

01/2018:20709

# 2.7.9. TEST FOR Fc FUNCTION OF IMMUNOGLOBULIN

*The test for Fc function of immunoglobulin is carried out using method A or B. Method B is an adaptation of the procedure of method A for the use of microtitre plates for the measurement of complement-mediated haemolysis. Differences in the test procedures between methods A and B are addressed in the test.*

REAGENTS

*Stabilised human blood*. Collect group O human blood into ACD anticoagulant solution. Store the stabilised blood at 4 °C for not more than 3 weeks.

*Phosphate-buffered saline pH 7.2*. Dissolve 1.022 g of *anhydrous disodium hydrogen phosphate R*, 0.336 g of *anhydrous sodium dihydrogen phosphate R* and 8.766 g of *sodium chloride R* in 800 mL of *water R* and dilute to 1000 mL with the same solvent.

*Magnesium and calcium stock solution*. Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 mL with the same solvent.

*Barbital buffer stock solution*. Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 mL of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid*. Add 12.5 mL of magnesium and calcium stock solution and dilute to 5000 mL with *water R*. Store at 4 °C in transparent containers.

*Albumin barbital buffer solution*. Dissolve 0.150 g of *bovine albumin R* in 20 mL of barbital buffer stock solution and dilute to 100 mL with *water R*. Prepare immediately before use.

*Tannic acid solution*. Dissolve 10 mg of *tannic acid R* in 100 mL of phosphate-buffered saline pH 7.2. Prepare immediately before use.

*Guinea-pig complement*. Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below – 70 °C. Immediately before starting complement-initiated haemolysis, dilute to 125-200 $CH_{50}$ per millilitre with albumin barbital buffer solution and store in an ice-bath during the test.

*Rubella antigen*. Suitable rubella antigen for haemagglutination-inhibition titre (HIT). Titre > 256 HA units.

**Preparation of tanned human red blood cells**. Separate human red blood cells by centrifuging an appropriate volume of stabilised human blood, wash the cells at least 3 times with phosphate-buffered saline pH 7.2 and suspend at 2 per cent *V/V* in phosphate-buffered saline pH 7.2. Add 0.2 mL of tannic acid solution to 14.8 mL of phosphate-buffered saline pH 7.2. Mix 1 volume of the freshly prepared dilution with 1 volume of the human red blood cell suspension and incubate at 37 °C for 10 min. Collect the cells by centrifugation (800 *g* for 10 min), discard the supernatant and wash the cells once with phosphate-buffered saline pH 7.2. Resuspend the tanned cells at 1 per cent *V/V* in phosphate-buffered saline pH 7.2.

**Antigen coating of tanned human red blood cells**. Take a suitable volume ($V_s$) of tanned cells, add 0.2 mL of rubella antigen per 1.0 mL of tanned cells and incubate at 37 °C for 30 min. Collect the cells by centrifugation (800 *g* for 10 min) and discard the supernatant. Add a volume of albumin barbital buffer solution equivalent to the discarded supernatant, resuspend and collect the cells as described and repeat the washing procedure. Resuspend with albumin barbital buffer solution using a volume equivalent to 3/4 of $V_s$, thereby obtaining the initial volume ($V_i$). Mix 900 µL of albumin barbital buffer solution with 100 µL of $V_i$, which is thereby reduced to the residual volume ($V_r$), and determine the initial absorbance at 541 nm ($A$). Dilute $V_r$ by a factor equal to $A$ using albumin barbital buffer solution, thereby obtaining the final adjusted volume $V_f = V_r \times A$ of sensitised human red blood cells and adjusting $A$ to 1.0 ± 0.1 for a tenfold dilution.

**Antibody binding of antigen-coated tanned human red blood cells**. Prepare the following solutions in succession and in duplicate, using for each solution a separate half-micro cuvette (for example, disposable type) or test-tube.

(1) *Test solutions*. If necessary, adjust the immunoglobulin to be examined to pH 7.

Where method A is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 30 mg and 40 mg of immunoglobulin and adjust the volume to 900 µL with albumin barbital buffer.

Where method B is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 15 mg and 30 mg of immunoglobulin and adjust the volume to 1200 µL with albumin barbital buffer.

(2) *Reference solutions*. Prepare as for the test solutions using *human immunoglobulin for Fc function BRP*.

(3) *Complement control*. Albumin barbital buffer solution.

Where method A is performed, add to each cuvette/test-tube 100 µL of sensitised human red blood cells and mix well. Allow to stand for 15 min, add 1000 µL of albumin barbital buffer solution, collect the cells by centrifugation (1000 *g* for 10 min) of the cuvette/test-tube and remove 1900 µL of the supernatant. Replace the 1900 µL with albumin barbital buffer solution and repeat the whole of the washing procedure, finally leaving a volume of 200 µL. Test samples may be stored in sealed cuvettes/test-tubes at 4 °C for not longer than 24 h.

Where method B is performed, add to each test-tube 300 µL of sensitised human red blood cells and mix well (the final immunoglobulin concentration is in the range of 10-20 mg/mL). Allow to stand for 15 min, add 1500 µL of albumin barbital buffer solution and stir gently until homogeneous. Collect the cells by centrifugation (1000 *g* for 10 min) of the test-tube, remove the supernatant and add approximately 3 mL of albumin barbital buffer solution. Repeat this operation up to 4 times in total, leaving a final volume of 300 µL. Test samples may be stored in sealed test-tubes at 4 °C for not longer than 24 h.

**Complement-initiated haemolysis**.

To measure haemolysis where method A is performed, add 600 µL of albumin barbital buffer solution warmed to 37 °C to the test sample, resuspend the cells carefully by repeated pipetting (not fewer than 5 times) and place the cuvette in the thermostatted cuvette holder of a spectrophotometer. After 2 min, add 200 µL of diluted guinea-pig complement (125-200 $CH_{50}$/mL), mix thoroughly by pipetting twice and start immediately after the second pipetting the time-dependent recording of absorbance at 541 nm, using albumin barbital buffer solution as the compensation liquid. Stop the measurement if absorbance as a function of time has clearly passed the inflexion point.

To measure haemolysis where method B is performed, add 900 µL of albumin barbital buffer solution warmed to 37 °C to each test-tube and resuspend the cells carefully by repeated pipetting (not fewer than 5 times). The microtitre plate must be prewarmed to 37 °C before starting the test. Transfer 240 µL of each solution into 4 microtitre plate wells then incubate the microplate at 37 °C for 6 min, stirring gently every 10 s. To each microtitre plate well add 60 µL of diluted guinea-pig complement (150 $CH_{50}$/mL). Mix for 10 s and immediately start recording the absorbance at 541 nm at 37 °C, measuring every 20 s. Stop the measurement if the absorbance as a function of time has clearly passed the inflexion point.

**Evaluation**. For each cuvette/test-tube/well, determine the slope ($S$) of the haemolysis curve at the approximate inflexion point by segmenting the steepest section in suitable time

intervals (for example, $\Delta t$ = 1 min), and calculate $S$ between adjacent intersection points, expressed as $\Delta A$ per minute. The largest value for $S$ serves as $S_{exp}$. In addition, determine the absorbance at the start of measurement ($A_s$) by extrapolating the curve, which is almost linear and parallel to the time axis within the first few minutes. Correct $S_{exp}$ using the expression:

$$S' = \frac{S_{exp}}{A_s}$$

Calculate the arithmetic mean of the values of $S'$ for each preparation (test and reference solution). Calculate the index of Fc function ($I_{Fc}$) from the expression:

$$I_{Fc} = \frac{100 \times (\overline{S'} - \overline{S'}_c)}{\overline{S'}_s - \overline{S'}_c}$$

$\overline{S'}$ = arithmetic mean of the corrected slope for the preparation to be examined;

$\overline{S'}_s$ = arithmetic mean of the corrected slope for the reference preparation;

$\overline{S'}_c$ = arithmetic mean of the corrected slope for the complement control.

Calculate the index of Fc function for the preparation to be examined: the value is not less than that stated in the leaflet accompanying the reference preparation.

**01/2008:20710**

# 2.7.10. ASSAY OF HUMAN COAGULATION FACTOR VII

Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VII activity of a stated amount of the International Standard, which consists of freeze-dried plasma. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human coagulation factor VII concentrate BRP* is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of 2 consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ions, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarised in the following scheme.

**Step 1**

a) factor VII —(tissue factor, $Ca^{2+}$)→ factor VIIa

b) factor X —(factor VIIa, $Ca^{2+}$; tissue factor/phospholipid)→ factor Xa

**Step 2**

chromogenic substrate —(factor Xa)→ peptide + chromophore

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipid as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10-350 nmol/L, preferably 14-70 nmol/L. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipid component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of $Ca^{2+}$ is 15-25 mmol/L. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3-8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water R* and used at a final concentration of 0.2-2 mmol/L. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

ASSAY PROCEDURE

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water R*; use within 1 h. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU and 2.0 IU of factor VII per millilitre.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of bovine or human albumin, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicate. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU/mL.

Prepare a control solution that includes all components except factor VII.

*Prepare all dilutions in plastic tubes and use within 1 h.*

**Step 1**. Mix dilutions of the factor VII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. The

concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

**Step 2**. Terminate the activation by the addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by the addition of a suitable reagent, such as acetic acid (500 g/L $C_2H_4O_2$) or a citrate solution (1 mol/L) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

**01/2008:20711**

## 2.7.11. ASSAY OF HUMAN COAGULATION FACTOR IX

The principle of the assay is to measure the ability of a factor IX preparation to reduce the prolonged coagulation time of factor IX-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference preparation, calibrated in International Units.

The International Unit is the factor IX activity of a stated amount of the International Standard, which consists of a freeze-dried concentrate of human coagulation factor IX. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human coagulation factor IX concentrate BRP* is calibrated in International Units by comparison with the International Standard.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor IX-deficient plasma (for example *plasma substrate R2*) to produce solutions containing 0.5-2.0 IU/mL. Prepare at least 3 dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH 7.3 R*) containing 10 g/L of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or carry out the assay with incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 mL of factor IX-deficient plasma (for example *plasma substrate R2*) and 0.1 mL of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 mL of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37 °C. To each tube, add 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency using the usual statistical methods (for example, *5.3*).

**01/2008:20712**

## 2.7.12. ASSAY OF HEPARIN IN COAGULATION FACTORS

Heparin is assayed as a complex with antithrombin III (AT) via its inhibition of coagulation factor Xa (anti-Xa activity). An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralised by the heparin-AT complex and the residual factor Xa hydrolyses a specific chromogenic peptide substrate to release a chromophore. The quantity of chromophore is inversely proportional to the activity of the heparin.

*Factor Xa chromogenic substrate*. Specific chromogenic substrate for factor Xa such as: *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride. Reconstitute according to the manufacturer's instructions.

*Dilution buffer*. 6.05 g/L solution of *tris(hydroxymethyl)aminomethane R*. Adjust to pH 8.4 if necessary using *hydrochloric acid R*.

*Test solution*. Dilute the preparation to be examined with dilution buffer to obtain a solution expected to contain 0.1 IU of heparin per millilitre.

*Reference solution*. Dilute the heparin reference preparation with dilution buffer to obtain a solution containing 0.1 IU of heparin per millilitre.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Warm all solutions to 37 °C in a water-bath shortly before the test.

Distribute in a series of wells, 20 µL of normal human plasma and 20 µL of *antithrombin III solution R1*. Add to the wells a series of volumes (20 µL, 60 µL, 100 µL and 140 µL) of the test solution or the reference solution and make up the volume in each well to 200 µL using dilution buffer (0.02-0.08 IU of heparin per millilitre in the final reaction mixture).

*End-point method*. Transfer 40 µL from each well to a second series of wells, add 20 µL of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µL of a 1 mmol/L solution of factor Xa chromogenic substrate and incubate at 37 °C for 3 min. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20 per cent *V/V* solution of *glacial acetic acid R* and measure the absorbance at 405 nm (*2.2.25*). Appropriate reaction times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

*Kinetic method*. Transfer 40 µL from each well to a second series of wells, add 20 µL of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µL of a 2 mmol/L solution of factor Xa chromogenic substrate, incubate at 37 °C and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (*2.2.25*),

thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation by the usual statistical methods for a slope-ratio assay (for example, *5.3*).

**01/2008:20713**
**corrected 7.5**

## 2.7.13. ASSAY OF HUMAN ANTI-D IMMUNOGLOBULIN

METHOD A

The potency of human anti-D immunoglobulin is determined by comparing the quantity necessary to produce agglutination of D-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity contained in a stated amount of the International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

*Human anti-D immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Use pooled D-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not fewer than 4 group O $R_1R_1$ donors. To a suitable volume of the cells, previously washed 3 times with a 9 g/L solution of *sodium chloride R*, add an equal volume of *bromelains solution R*, allow to stand at 37 °C for 10 min, centrifuge, remove the supernatant and wash 3 times with a 9 g/L solution of *sodium chloride R*. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 300 g/L solution of *bovine albumin R* and 45 volumes of a 9 g/L solution of *sodium chloride R*. Stand the resulting suspension in iced water, stirring continuously.

Using a calibrated automated dilutor, prepare suitable dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 5 g/L of *bovine albumin R* and 9 g/L of *sodium chloride R*.

Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils, at 15.0 °C. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 mL/min and a 3 g/L solution of *methylcellulose 450 R* at a rate of 0.05 mL/min. Introduce the dilutions of the preparation to be examined and the reference preparation at a rate of 0.1 mL/min for 2 min, followed by the diluent solution at a rate of 0.1 mL/min for 4 min before the next dilution is introduced.

Introduce air at a rate of 0.6 mL/min. Incubate at 37 °C for 18 min and then disperse the rouleaux by introducing at a rate of 1.6 mL/min a 9 g/L solution of *sodium chloride R* containing a suitable wetting agent (for example, *polysorbate 20 R* at a final concentration of 0.2 g/L) to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 mL/min and then at 0.6 mL/min. Lyse the unagglutinated red blood cells with a solution containing 5 g/L of *octoxinol 10 R*, 0.2 g/L of *potassium ferricyanide R*, 1 g/L of *sodium hydrogen carbonate R* and 0.05 g/L of *potassium cyanide R* at a rate of 2.5 mL/min. A 10-minute delay coil is introduced to allow for conversion of the haemoglobin. Continuously record the absorbance (*2.2.25*) of the haemolysate at a wavelength between 540 nm and 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between the concentration and the resultant change in absorbance ($\Delta A$). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation to be examined.

Calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

METHOD B

The potency of human anti-D immunoglobulin is determined by competitive enzyme-linked immunoassay on erythrocyte-coated microtitre plates. The method is based on the competitive binding between a polyclonal anti-D immunoglobulin preparation and a biotinylated monoclonal anti-D antibody directed against a D-antigen-specific epitope. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

*Human anti-D immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

*MATERIALS*

Reagents not specified are of analytical grade.

*PBS (Phosphate-buffered saline).* Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R*, 0.2 g of *potassium dihydrogen phosphate R* and 0.2 g of *sodium azide R* in *water R* and dilute to 1000 mL with the same solvent.

*TBS (Tris-buffered saline).* Dissolve 8.0 g of *sodium chloride R* and 0.6 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust to pH 7.2 with *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

*Papain solution.* Prepare a solution by stirring 1 g of *papain R* at 37 °C for 30 min in 10 mL of *0.067 M phosphate buffer solution pH 5.4 R*, centrifuge at 10 000 *g* for 5 min and filter through a membrane filter (nominal pore size 0.22 µm). To activate, combine 1 mL of the filtrate with 1 mL of a 48.44 g/L solution of *L-cysteine R* and 1 mL of a 3.72 g/L solution of *sodium edetate R* and dilute to 10 mL with *0.067 M phosphate buffer solution pH 5.4 R*. Freeze in aliquots at − 20 °C or below.

*Red blood cells.* Use pooled D-positive red blood cells obtained from not fewer than 3 group O $R_2R_2$ donors. Wash the cells 4 times with PBS. Centrifuge the cells at 1800 *g* for 5 min, mix a suitable volume of prewarmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume has been found suitable) and incubate at 37 °C for 10 min. Wash the cells 4 times with PBS. Store at 4 °C in an appropriate stabiliser for up to 1 week.

*Biotinylated Brad-5.* Use according to instructions.

*Alkaline phosphatase-conjugated avidin/streptavidin reagent.* Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

*Substrate solution.* Use *para*-nitrophenyl phosphate according to instructions.

*Cell fixation buffer.* Dissolve 18.02 g of *glucose R*, 4.09 g of *sodium chloride R*, 1.24 g of *boric acid R*, 10.29 g of *sodium citrate R* and 0.74 g of *sodium edetate R* in *water R*. Adjust to pH 7.2-7.3 using *1 M sodium hydroxide* or *1 M hydrochloric acid*, and dilute to 1000 mL with *water R*. Use directly from storage at 4 °C.

*Glutaraldehyde solution.* Immediately before use, add 750 µL of a 250 g/L solution of *glutaraldehyde R* to 50 mL of cold PBS.

*Microtitre plates.* Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U- or V-bottomed polystyrene or poly(vinyl chloride) plates.

*METHOD*

Prepare a 0.1 per cent *V/V* suspension of papain-treated red blood cells in cold cell-fixation buffer. Pipette 50 µL into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 *g* for 3 min, preferably at 4 °C. Without removing the supernatant, gently add 100 µL of glutaraldehyde solution to each well and leave for 10 min.

Drain the wells by quickly inverting the plate and wash 3 times with 250-300 µL of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4 °C after draining off the PBS and adding 100 µL of cell-fixation buffer per well and sealing with plastic film. Plates can be stored at 4 °C for up to 1 month.

*Test solutions.* For freeze-dried preparations, reconstitute as stated on the label. Prepare 4 independent replicates of 5 serial 2-fold dilutions starting with 30 IU/mL in PBS containing 10 g/L of *bovine albumin R*. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

*Reference solutions.* Reconstitute the reference preparation according to instructions. Prepare 4 independent replicates of 5 serial 2-fold dilutions starting with 30 IU/mL in PBS containing 10 g/L of *bovine albumin R*.

Using U- or V-bottomed microtitre plates, add 35 µL of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 µL of biotinylated Brad-5 at 250 ng/mL.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel. Add 250 µL of PBS containing 20 g/L of *bovine albumin R* and leave at room temperature for 30 min.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 µL from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 µL of PBS containing 10 g/L of *bovine albumin R* as negative control. Seal the plate with plastic film and incubate at room temperature for 1 h.

Remove the liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 µL of TBS.

Dilute the alkaline phosphatase-conjugated avidin/streptavidin reagent in TBS containing 10 g/L of *bovine albumin R* and add 50 µL to each well. Incubate for 30 min at room temperature.

Remove the liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 µL of TBS.

Add 100 µL of substrate solution to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 50 µL of *3 M sodium hydroxide* to each of the wells.

Measure the absorbances at 405 nm and substract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

METHOD C

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference preparation is stated by the World Health Organization.

*Human anti-D immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

*MATERIALS*

Reagents not specified are of analytical grade.

*PBS.* Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *disodium hydrogen phosphate dodecahydrate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

*PBS-BSA solution.* PBS containing 10.0 g/L of *bovine albumin R*.

*Red blood cells.* Use D-positive red blood cells obtained from a group O $R_1R_1$ donor within 2 weeks of collection. Store if necessary in an appropriate stabiliser at 4 °C. Wash the cells at least twice with PBS-BSA solution and prepare a suspension containing $1 \times 10^4$ cells per microlitre but not more than $5 \times 10^4$ cells per microlitre in PBS-BSA solution.

Use D-negative red blood cells obtained from a group O rr donor and prepared similarly.

*Secondary antibody.* Use a suitable fluorescent dye-conjugated anti-IgG antibody fragment specific for human IgG or parts of it. Store and use according to the manufacturer's instructions.

*Microtitres plates.* Use flat-bottomed plates without surface treatment for enzyme immunoassays.

*METHOD*

*Test solutions.* For freeze-dried preparations, reconstitute as stated on the label. Prepare at least 3 independent replicates of at least 3 serial 1.5- or 2-fold dilutions starting with a concentration in the range of 1.2-0.15 IU/mL using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

*Reference solutions.* Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5- or 2-fold dilutions starting with a concentration in the range of 1.2-0.15 IU/mL using PBS-BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µL of the D-positive red blood cells into each well of a microtitre plate. Add 50 µL of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µL of PBS-BSA solution as negative control. Distribute 50 µL of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µL of the lowest dilution of the test preparation. To monitor spurious reactions, distribute 50 µL of the D-positive red blood cells into 4 wells of the same microtitre plate and add 50 µL of PBS-BSA solution. Seal with plastic film and incubate at 37 °C for 40 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µL of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and add 50 µL of the secondary antibody diluted with PBS-BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µL of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, resuspend the cells into 200-250 µL of PBS. Transfer the cell suspension into a tube suitable for the flow-cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate.

Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose-response curve to estimate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

**04/2019:20714**

# 2.7.14. ASSAY OF HEPATITIS A VACCINE

The assay of hepatitis A vaccine is carried out either *in vitro*, by an immunochemical determination of antigen content (method A), or *in vivo*, by comparing under given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation (method B).

METHOD A. *IN VITRO* ASSAY

The hepatitis A antigen content is determined by a suitable immunochemical method (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

*Hepatitis A vaccine (inactivated, non-adsorbed) BRP* may be used as a reference preparation.

The following method is given as an example of an immunochemical method that has been found to be suitable. Perform an ELISA assay for the determination of hepatitis A antigen content using monoclonal antibodies specific for the detection of a hepatitis A epitope that induces neutralising antibodies.

**Materials**

*ELISA microtitre plates*: 96 wells.

*Hepatitis A virus coating reagent for ELISA BRR.*

*Hepatitis A vaccine ELISA detection antibodies set BRR*, composed of *anti-hepatitis A virus primary detection antibody BRR* and *conjugated secondary detection antibody BRR.*

*Hepatitis A vaccine (inactivated, non-adsorbed) BRP.*

*Carbonate-bicarbonate coating buffer (0.05 M) pH 9.6.* Solution 1: dissolve 2.1 g of *sodium hydrogen carbonate R* in 500 mL of *water for injections R*. Solution 2: dissolve 7.16 g of *sodium carbonate R* in 500 mL of *water for injections R*. Mix 70 mL of solution 1 and about 30 mL of solution 2, adjusting the pH with solution 2 if necessary. Filter through a membrane filter (nominal pore size 0.22 µm) and prepare appropriate aliquots in sterile tubes. Store at 2-8 °C for up to 5 months.

*Blocking buffer.* Solution 1: dissolve 4.2 g of *sodium hydrogen carbonate R* in 1000 mL of *water for injections R*. Solution 2: dissolve 5.3 g of *sodium carbonate R* in 1000 mL of *water for injections R*. Mix 630 mL of solution 1 and 270 mL of solution 2, add 50 g of *sucrose R* and stir until dissolution is complete. Add 3 g of *bovine albumin R* (pour small quantities to avoid lumps) and stir until dissolution is complete, avoiding foaming. Adjust to pH 9.6 carefully with solution 2. Prepare appropriate aliquots in sterile tubes. Store at − 20 °C for up to 5 months.

*PBS buffer pH 7.5 ± 0.3.* Dissolve 0.2 g of *potassium dihydrogen phosphate R*, 0.2 g of *potassium chloride R*, 8.0 g of *sodium chloride R* and 1.15 g of *disodium hydrogen phosphate dodecahydrate R* in 1000 mL of *water for injections R*. Store at 2-8 °C for up to 1 month.

*Polysorbate 20 solution.* Dilute 5 mL of *polysorbate 20 R* to 100 mL with *water for injections R*. Store at 2-8 °C for up to 1 month.

*PBS-T buffer.* Immediately before use, add 1 mL of the polysorbate 20 solution to 1000 mL of the PBS buffer pH 7.5 ± 0.3.

*PBS-B-T buffer.* Dissolve 2.5 g of *bovine albumin R* in 400 mL of the PBS-T buffer (pour small quantities of bovine albumin to avoid lumps) and stir until dissolution is complete, avoiding foaming. Adjust to pH 7.25 with *4 M sodium hydroxide R* and dilute to 500 mL with the PBS-T buffer. Prepare appropriate aliquots in sterile tubes. Store at − 20 °C for up to 5 months.

*Chromogenic substrate.* Immediately before use, dissolve at room temperature *diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) according to the manufacturer's instructions.

*Stop solution.* Dissolve 1 g of *sodium dodecyl sulfate R* in *water for injections R* and dilute to 100 mL with the same solvent. Store at room temperature for up to 1 month, protected from light.

**Method**. Dilute an appropriate volume of *hepatitis A virus coating reagent for ELISA BRR* in an appropriate volume of the carbonate-bicarbonate coating buffer (0.05 M) pH 9.6. Transfer 100 µL of the solution to each well of the microtitre plates. Seal the plates and incubate for 3 h ± 5 min at 37 ± 2 °C in a humidified atmosphere. Store at 5 ± 3 °C overnight in a humidified atmosphere. On the following day, empty the plates by inverting them on absorbent paper and add 200 µL of the blocking buffer to each well. Incubate for 45 ± 5 min at 37 ± 2 °C in a humidified atmosphere. Empty the plates by inverting them on absorbent paper. Seal the plates and store at − 20 °C for up to 1 month.

For adsorbed vaccines, a pre-treatment or desorption step is necessary before dilution. The treatment step is considered as a pre-dilution. Prepare 2 independent dilution series in the PBS-B-T buffer for each test sample and for the reference preparation. A 2-fold serial dilution has been found suitable. Thaw an appropriate number of coated plates for the assay. Wash the plates 3 times with the PBS-T buffer. Tap the plates gently on absorbent paper to drain off the washing buffer. For the test samples and the reference preparation, transfer 100 µL of each sample (pre-dilution and serial dilutions) to separate wells of the plate following the appropriate plate layout. For blanks, transfer 100 µL of the PBS-B-T buffer per well. Seal the plates and incubate at 37 ± 2 °C in a humidified atmosphere for 1.5 h ± 5 min. Discard the incubation mix and wash the plates 3 times with the PBS-T buffer. Tap the plates gently on absorbent paper to drain off the washing buffer.

Immediately before use, dilute an appropriate volume of *anti-hepatitis A virus primary detection antibody BRR* in an appropriate volume of the PBS-B-T buffer. Transfer 100 µL of the solution to each well. Seal the plates and incubate at 37 ± 2 °C in a humidified atmosphere for 1 h ± 3 min. Discard the incubation mix and wash the plates 3 times with the PBS-T buffer. Tap the plates gently on absorbent paper to drain off the washing buffer.

Immediately before use, dilute an appropriate volume of *conjugated secondary detection antibody BRR* in an appropriate volume of the PBS-B-T buffer. Transfer 100 µL of the solution to each well. Seal the plates and incubate at 37 ± 2 °C in a humidified atmosphere for 1 h ± 3 min. Discard the incubation mix and wash the plates 3 times with the PBS-T buffer. Tap the plates gently on absorbent paper to drain off the washing buffer.

Add 100 µL of the chromogenic substrate to each well. Seal the plates and incubate at 37 ± 2 °C in a humidified atmosphere for 30 ± 2 min. Stop the reaction by adding 50 µL of the stop solution to each well. Read the absorbance immediately at 405 nm.

**Calculation**. Calculate the potency of the vaccine to be examined relative to *hepatitis A vaccine (inactivated, non-adsorbed) BRP*, using the usual statistical methods (*5.3*).

**Validity conditions**. The test is not valid unless:

- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves;
- the confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

METHOD B. *IN VIVO* ASSAY

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

**Selection and distribution of the test animals**. Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number suitable for the requirements of the assay.

**Determination of potency of the vaccine to be examined**. Using a 9 g/L solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine, prepare at least 3 dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 mL of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28-32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (*2.7.1*).

**Calculations**. Carry out the calculations by the usual statistical methods for an assay with a quantal response (*5.3*).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

**Validity conditions**. The test is not valid unless:

- for both the test and the reference vaccine, the $ED_{50}$ lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

**Potency requirement**. The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

*IN VIVO* ASSAY

**Selection and distribution of the test animals**. Use in the test healthy mice from the same stock, about 5 weeks old. The strain of mice used for this test must give a significant slope for the dose-response curve to the antigen; mice with haplotype H-$2^q$ or H-$2^d$ are suitable. Healthy guinea-pigs weighing 300 g to 350 g (about 7 weeks old) from the same stock are also suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number appropriate to the requirements of the assay.

**Determination of potency of the vaccine to be examined**. Using a 9 g/L solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least 3 dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions, 1 to each of the groups of animals, and inject intraperitoneally not more than 1.0 mL of each dilution into each animal in the group to which that dilution is allocated. One group of animals remains unvaccinated and is injected intraperitoneally with the same volume of diluent. After an appropriate time interval (for example, 4-6 weeks), anaesthetise and bleed the animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against HBsAg by a suitable immunochemical method (*2.7.1*).

**Calculations**. Calculations are carried out by the usual statistical methods for an assay with a quantal response (*5.3*).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay is determined. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

**Validity conditions**. The test is not valid unless:

- for both the test and the reference vaccine, the $ED_{50}$ lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

**Potency requirement**. The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

*IN VITRO* ASSAY

Carry out an immunochemical determination (*2.7.1*) of antigen content with acceptance criteria validated against the *in vivo* test.

Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of HBsAg have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used and a parallel-line model is used to analyse the data, which may be suitably transformed. Kits for measuring HBsAg *in vitro* are commercially available and it is possible to adapt their test procedures for use as an *in vitro* potency assay.

The acceptance criteria are approved for a given reference preparation by the competent authority in light of the validation data.

**01/2012:20715**

## 2.7.15. ASSAY OF HEPATITIS B VACCINE (rDNA)

The assay of hepatitis B vaccine (rDNA) is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content.

07/2019:20716

# 2.7.16. ASSAY OF PERTUSSIS VACCINE (ACELLULAR)

The assay of acellular pertussis vaccine measures the capacity of the vaccine to induce the formation of specific antibodies in mice or guinea-pigs. Antibody titres for each antigen are determined using a suitable immunochemical method (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

The assay results can be expressed:

- either as a ratio of the geometric mean titre (GMT) of antibodies produced following administration of the test vaccine to the GMT of antibodies produced following administration of a reference vaccine examined in parallel (relative potency assay);
- or directly as a GMT of antibodies induced by the test vaccine (geometric mean unit assay or GMU assay).

For combinations containing pertussis components together with diphtheria and tetanus components, the serological assay in guinea-pigs can be performed with the same group of animals used for the serological assay of diphtheria vaccine (adsorbed) (*2.7.6*) and of tetanus vaccine (adsorbed) (*2.7.8*) when the common immunisation conditions for all components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine. The guinea-pig model allows for a further reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Methods A and B described below are developed by testing multiple dilutions of the test vaccine and the reference vaccine or internal control (see Glossary), to determine which dilutions are suitable. Once the suitable dilutions have been confirmed for a given vaccine, it is recommended, in accordance with 3R principles (Replacement, Reduction, Refinement), to apply a simplified model such as a single dilution for both the test vaccine and the reference vaccine or internal control. Such a model enables the analyst to determine whether the immunogenicity of the test vaccine is satisfactory.

Suitable indicators to monitor the performance of the serological assay (single or multiple dilutions) are:

- the geometric mean and geometric standard deviation of antibody titres in the serum samples obtained after administration of a fixed dose of the reference vaccine or internal control;
- the antibody titres of run controls (positive control and negative serum samples).

It may be necessary to reconfirm the suitability of the selected dilution with a multiple-dilution assay, e.g. after major process changes or for investigational purposes.

## METHOD A. SEROLOGY IN MICE

*The following test model is given as an example of a multiple-dilution assay which may form the basis for the establishment of a single-dilution assay.*

**Selection and distribution of the test animals**. Use healthy mice (for example, CD1 strain from the same stock and more than 5 weeks old). Distribute the animals into not fewer than 6 equal groups; use groups containing a number of animals sufficient to meet the pre-defined criteria for variability of the antibody responses prescribed under Calculations and validity criteria. Use serial dilutions of the test vaccine and the reference vaccine or internal control and allocate each dilution to a group of mice. During validation studies a further group of mice may be used as a negative control by injecting the animals with diluent alone.

**Immunisation**. Inject intraperitoneally or subcutaneously into each mouse 0.5 mL of the dilution allocated to its group.

**Collection of serum samples**. 4-5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at − 20 °C until used for antibody determination. Avoid frequent freezing and thawing of serum samples.

**Reference antiserum**. A reference antiserum of assigned activity serves as the basis for calculation of the antibody titres in the test sera. *Bordetella pertussis mouse antiserum BRP* is suitable for use as a reference antiserum.

**Antibody determination**. Assay the individual sera for content of specific antibodies against each acellular pertussis antigen using a validated method such as the ELISA test shown below.

**ELISA test**. Microtitre plates (poly(vinyl chloride) or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating the plates with a solution of bovine serum albumin and then washed. 2-fold dilutions of sera from individual mice immunised with the test vaccine or either the reference vaccine or the internal control are prepared on the plates. Reference antiserum is included on each plate. After incubation at 22-25 °C for 1 h, the plates are washed. A suitable solution of enzyme-conjugated anti-mouse IgG antibody is added to each well and incubated at 22-25 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore that can be quantified by measurement of absorbance (*2.2.25*).

## METHOD B. SEROLOGY IN GUINEA-PIGS

*The following test model is given as an example of a multiple-dilution assay which may form the basis for the establishment of a single-dilution assay.*

**Selection and distribution of the test animals**. Use healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs into not fewer than 6 equal groups; use groups containing a number of animals sufficient to meet the pre-defined criteria for variability of the antibody responses prescribed under Calculations and validity criteria. During validation studies a further group of guinea-pigs may be used as a negative control by injecting the animals with diluent alone.

**Dilution of the test and reference preparations**. Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the test vaccine and the reference vaccine or internal control; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range found to be suitable for all the components in the test vaccine. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

**Immunisation**. Inject subcutaneously into each guinea-pig 1.0 mL of the dilution allocated to its group.

**Collection of serum samples**. 5-6 weeks after immunisation, take a blood sample from each vaccinated and negative control guinea-pig using a suitable method. Store the sera at − 20 °C until used for antibody determination. Avoid frequent freezing and thawing of serum samples.

**Reference antiserum**. An in-house guinea-pig reference antiserum of assigned activity serves as the basis for calculation of the antibody titres in the test sera.

**Antibody determination**. Assay the individual sera for content of specific antibodies against each acellular pertussis antigen using a validated method such as the ELISA test shown below.

**ELISA test**. Suitable 96-well microtitre plates are coated with the purified antigens (e.g. pertussis toxin (PT), pertactin (PRN), filamentous haemagglutinin (FHA) and/or fimbrial agglutinogens (Fim 2/3)) representing components in the combined vaccine at a concentration of 200-400 ng per well. After washing, unreacted sites are blocked by incubating the plates with a suitable blocking buffer and then washed. 2-fold dilutions of sera from individual guinea-pigs immunised with the test vaccine or either the reference vaccine or the internal control are prepared on the plates. Reference antiserum is included on each plate. After incubation at 37 °C for 1 h, the plates are washed. A suitable solution of enzyme-conjugated anti-guinea-pig IgG antibody is added to each well and incubated at 37 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore that can be quantified by measurement of absorbance (*2.2.25*).

CALCULATIONS AND VALIDITY CRITERIA

**Relative potency assay**. The antibody titres in the sera of mice or guinea-pigs immunised with the test and reference vaccines are determined for each acellular pertussis antigen using the reference antiserum. From the values obtained, the GMT ratio of the test vaccine in relation to the reference vaccine is calculated for each antigen.

The relative potency assay is not valid unless:

- the number of responder animals for the test and reference vaccines meets the pre-defined criteria;
- the GMT for the reference vaccine is within the limits of the control chart;
- the variability of the antibody responses meets the pre-defined criteria.

**GMU assay**. The antibody titres in the sera of mice or guinea-pigs immunised with the internal control and test vaccine are determined for each acellular pertussis antigen using the reference antiserum, and the GMTs are calculated for each antigen.

The GMU assay is not valid unless:

- the number of responder animals for the test vaccine and internal control meets the pre-defined criteria;
- the GMT for the internal control is within the limits of the control chart;
- the variability of the antibody responses meets the pre-defined criteria.

GLOSSARY

**Internal control for GMU assay**. A batch of vaccine shown to be representative of the current manufacturing process and whose response in mice or guinea pigs has been appropriately measured. The stability of the internal control shall be monitored and documented.

**Reference vaccine for relative potency assay**. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof. The stability of the reference vaccine shall be monitored and documented.

**Responder animals**. Immunised animals producing antibodies at a titre greater than a threshold defined during the development and validation of the method.

*The following section is published for information.*

## Assay of pertussis vaccine (acellular): guidelines

METHOD B. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

The ELISA shown below is given as an example of an immunochemical method that has been found to be suitable.

**Determination of antibody titre by ELISA method for pertussis toxin (PT), filamentous haemagglutinin (FHA), fimbrial agglutinogens (Fim 2/3) and pertactin (PRN)**. 2-fold dilutions of sera from test and reference vaccine or internal control are made on ELISA plates coated with acellular pertussis antigens (PRN, PT, FHA or Fim 2/3). A guinea-pig reference antiserum and a negative guinea-pig serum are included on each plate. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG is added, followed by a peroxidase substrate.

**Reagents and equipment:**

- *ELISA plates*: 96 wells, columns 1-12, rows A-H.
- *Reference antiserum* (guinea-pig).
- *Peroxidase conjugate*. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.
- *Bordetella pertussis antigens* (PRN, PT, FHA or Fim 2/3).
- *Carbonate coating buffer pH 9.6*. Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R*. Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.
- *Phosphate-buffered saline pH 7.4 (PBS)*. Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 mL of *water R*. Store at room temperature to prevent crystallisation. Dilute 10-fold with *water R* before use.
- *Citric acid solution*. Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust to pH 4.0 with a 400 g/L solution of *sodium hydroxide R*.
- *Washing buffer*. PBS containing 0.5 g/L of *polysorbate 20 R*.
- *Diluent block buffer*. PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.
- *Peroxidase substrate*. Shortly before use, dissolve 10 mg of *diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of the citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R*.

**Method**. The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are used for negative control serum. Wells 2-12 A-H are used for guinea-pig reference antiserum (usually in 2 positions) and individual sera from guinea-pigs immunised with the test vaccine, or either the reference vaccine or the internal control.

Coat each well of the ELISA plates with 100 µL of the appropriate antigen solution (PT, FHA and Fim 2/3 at 2 µg/mL and PRT at 4 µg/mL, in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with the washing buffer. Block the plates by addition of 150 µL of the diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with the washing buffer. Place 100 µL of the diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of individual test and reference vaccine or internal control serum samples, reference antiserum and negative control serum samples. Allocate the negative control serum to column 1, the reference antiserum to at least 2 other columns and individual test and reference vaccine or internal control

sera to the remaining columns and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make 2-fold serial dilutions from row B down the plate to row H, by transferring 100 µL from one well to the next. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with the washing buffer. Prepare a suitable dilution of the peroxidase conjugate in the diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with the washing buffer. Add 100 µL of the peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as the addition of substrate was made.

**01/2008:20717**

## 2.7.17. ASSAY OF HUMAN ANTITHROMBIN III

The antithrombin III content of the preparation to be examined is determined by comparing its ability to inactivate thrombin in the presence of an excess of heparin with the same ability of a reference preparation of human antithrombin III concentrate calibrated in International Units. Varying quantities of the preparation to be examined are mixed with a given quantity of thrombin and the remaining thrombin activity is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for human antithrombin III concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Method.* Prepare 2 independent series of 3 or 4 dilutions in the range 1/75 to 1/200 from 1 IU/mL, for both the preparation to be examined and the reference preparation, using *tris-EDTA BSA buffer solution pH 8.4 R* containing 15 IU of heparin per millilitre.

Warm 200 µL of each dilution at 37 °C for 1-2 min. Add to each dilution 200 µL of a solution of *bovine thrombin R* containing 2 IU/mL in *tris-EDTA BSA buffer solution pH 8.4 R*. Mix and maintain at 37 °C for exactly 1 min. Add 500 µL of a suitable chromogenic substrate (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide, reconstituted in *water R* to give a solution containing 4 mmol/L and further diluted to a concentration suitable for the assay using *tris-EDTA BSA buffer solution pH 8.4 R* without albumin). Immediately start measurement of the change in absorbance at 405 nm (*2.2.25*), continuing the measurement for at least 30 s. Calculate the rate of change of absorbance ($\Delta A$/min). (Alternatively, an end-point assay may be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm.)

The rate of change of absorbance ($\Delta A$/min) is inversely proportional to antithrombin III activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (*5.3*).

**07/2015:20718**

## 2.7.18. ASSAY OF HUMAN COAGULATION FACTOR II

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor II activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human blood coagulation factor II. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

*REAGENTS*

*Viper venom specific factor II activator* (ecarin). A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

*Factor IIa chromogenic substrate.* Specific chromogenic substrate for factor IIa such as: *H*-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, *H*-D-cyclohexylglycyl-α-aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate. Reconstitute according to the manufacturer's instructions.

*Dilution buffer.* Solution containing 6.06 g/L of *tris(hydroxymethyl)aminomethane R*, 17.53 g/L of *sodium chloride R* and 1 g/L of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary, using *hydrochloric acid R*.

*METHOD*

*Test solution.* Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

*Reference solution.* Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 25 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µL of dilution buffer, then 25 µL of ecarin and incubate for exactly 2 min. To each well add 25 µL of factor IIa chromogenic substrate.

Read the rate of change of absorbance (*2.2.25*) at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance ($\Delta A$/min). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate $\Delta A$/min as the slope of the line. From the $\Delta A$/min values of each individual

dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (*5.3*).

**01/2008:20719**

## 2.7.19. ASSAY OF HUMAN COAGULATION FACTOR X

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor X activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human coagulation factor X. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

REAGENTS

*Russell's viper venom specific factor X activator* (RVV). A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

*Factor Xa chromogenic substrate*. Specific chromogenic substrate for factor Xa such as: *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulfonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate. Reconstitute according to the manufacturer's instructions.

*Dilution buffer*. Solution containing 3.7 g/L of *tris(hydroxymethyl)aminomethane R*, 18.0 g/L of *sodium chloride R*, 2.1 g/L of *imidazole R*, 0.02 g/L of *hexadimethrine bromide R* and 1 g/L of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary using *hydrochloric acid R*.

METHOD

*Test solution*. Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

*Reference solution*. Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 12.5 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 µL of RVV and incubate for exactly 90 s. To each well add 150 µL of factor Xa chromogenic substrate, diluted 1 in 6 in dilution buffer. Read the rate of change of absorbance (*2.2.25*) (at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance (Δ*A*/min). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate Δ*A*/min as the slope of the line. From the Δ*A*/min values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (*5.3*).

**01/2008:20720**

## 2.7.20. *IN VIVO* ASSAY OF POLIOMYELITIS VACCINE (INACTIVATED)

The capacity of the vaccine to induce the formation of neutralising antibodies is determined *in vivo* by one of the following methods.

TEST IN CHICKS OR GUINEA-PIGS

Prepare a suitable series of not fewer than 3 dilutions of the vaccine to be examined using a suitable buffered saline solution. Distribute either guinea-pigs weighing 250-350 g or 3-week-old chicks into groups of 10, and allocate a group to each dilution of the vaccine. Inject intramuscularly into each animal 0.5 mL of the dilution intended for its group. Bleed the animals after 5-6 days and separate the sera. Examine the sera for the presence of neutralising antibodies, at a dilution of 1 in 4, to each of the human poliovirus types 1, 2 and 3. Mix 100 $CCID_{50}$ of virus with the dilution of serum and incubate at 37 °C for 4.5-6 h. Keep at 5 ± 3 °C for 12-18 h where necessary for consistency of results. Inoculate the mixtures into cell cultures for the detection of unneutralised virus and read the results up to 7 days after inoculation. For each group of animals, note the number of sera that have neutralising antibodies and calculate the dilution of the vaccine that gives an antibody response in 50 per cent of the animals. Carry out in parallel a control test using a suitable reference preparation. The vaccine complies with the test if a dilution of 1 to 100 or more produces an antibody response for each of the 3 types of virus in 50 per cent of the animals.

TEST IN RATS

A suitable *in vivo* assay method consists of intramuscular injection into the hind limb(s) of not fewer than 3 dilutions of the vaccine to be examined and a reference vaccine, using for each dilution a group of 10 specific pathogen-free rats of a suitable strain. Use of 4 dilutions is often necessary to obtain valid results for all 3 serotypes. The number of animals per group must be sufficient to obtain results that meet the validity criteria; groups of 10 rats are usually sufficient, although valid results may be obtained with fewer animals per group. If animals of different sex are used, males and females are evenly distributed between all groups. A weight range of 175-250 g has been found to be suitable. An inoculum of 0.5 mL per rat is used. The dose range is chosen such that a dose response to all 3 poliovirus types is obtained. Bleed the animals after 20-22 days. Neutralising titres against all 3 poliovirus types are measured separately using 100 $CCID_{50}$ of the Sabin strains as challenge viruses, Vero or Hep2 as indicator cells, and neutralisation conditions of 3 h at 35-37 °C followed by 18 h at 2-8 °C where necessary for consistency of results. Results are read following fixation and staining after 7 days of incubation at 35 °C. For a valid antibody assay, the titre of each challenge virus must be shown to be within the range 10 $CCID_{50}$ to 1000 $CCID_{50}$ and the neutralising antibody titre of a control serum must be within 2 twofold dilutions of the

geometric mean titre of the serum. The potency is calculated by comparison of the proportion of responders for the vaccine to be examined and the reference vaccine by the probit method or, after validation, using a parallel-line model. For the probit method it is necessary to establish a cut-off neutralising antibody titre for each poliovirus type to define a responder. Due to interlaboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values are determined for each laboratory based on a minimum series of 3 tests with the reference vaccine. The mid-point on a $\log_2$ scale of the minimum and maximum geometric mean titres of the series of 3 or more tests is used as the cut-off value. For each of the 3 poliovirus types, the potency of the vaccine is not significantly less than that of the reference preparation. The test is not valid unless:

- for both the vaccine to be examined and the reference vaccine, the $ED_{50}$ lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

*The following section is published for information.*

## Guideline on waiving of the *in vivo* assay of poliomyelitis vaccine (inactivated) and its combinations

*This guideline applies to vaccines derived from wild strains of poliovirus. The validation described should be carried out for each product before waiving of the in vivo assay, and should be repeated wherever there is a substantial change to the manufacturing process that may affect the in vitro or in vivo assays.*

The European convention on the protection of vertebrate animals used for experimental and other scientific purposes requires that tests in animals shall not be carried out if a scientifically satisfactory alternative is reasonably and practically available. The aim of this guideline is therefore to promote waiving of the *in vivo* assay wherever it can be shown for a given product that the *in vitro* assay (D-antigen determination) gives sufficient assurance of satisfactory potency for routine batch control.

For the *in vivo* assay, the test in rats is considered to be the method of choice. For vaccines that are assayed using chicks or guinea-pigs and that have an established record of production history, the *in vivo* assay may be waived if the rat assay is also applied to the batches included in the validation study described below. For vaccines not yet approved, the results of the rat assay on all final bulks should be included in all data generated for demonstration of consistency of production before waiving of the *in vivo* assay.

Once the *in vivo* assay has been waived, batches of vaccine will be released on the basis of the *in vitro* assay, and the *in vivo* assay should not be used as an alternative for the release of a batch that fails the *in vitro* assay. Repetition of the *in vitro* assay may be performed according to an authorised procedure.

PROCEDURE

The following conditions should be met before performance of the validation study:

- appropriate experience of the rat assay;
- full validation of the D-antigen assay (linearity, repeatability, intermediate precision, accuracy and limits of quantification);
- establishment of acceptance criteria for the D-antigen assay based on a suitable number of consecutive final lots;
- establishment of production consistency on recent final bulks using the currently approved *in vivo* assay; the final bulks should correspond to the final lots used to establish the acceptance criteria for the D-antigen assay and should represent different inactivated harvests of each of the 3 types of poliovirus.

The validation study should be performed on:

- a final bulk/lot that is representative of the current production method;
- 2 sub-potent batches prepared, for example, by heating normal vaccine or mixing it with heat-treated vaccine; the sub-potent batches should have expected titres of about half that of the representative final bulk/lot.

These batches are assayed using as reference standard a homologous production batch:

- by the currently approved *in vivo* assay for the vaccine;
- by the rat assay where this is not the currently approved *in vivo* assay;
- by the D-antigen assay.

Waiving of the *in vivo* assay is acceptable if the representative final bulk/lot complies with the *in vivo* and *in vitro* assays and the sub-potent batches fail to comply. If a sub-potent batch fails to comply with the D-antigen assay but complies with the *in vivo* assay, the latter may be repeated.

**01/2008:20721**

# 2.7.21. ASSAY OF HUMAN VON WILLEBRAND FACTOR

The biological functions of human von Willebrand factor are numerous. At present, its ristocetin cofactor activity and its collagen binding activity can be utilised for assays. The potency of human von Willebrand factor is determined by comparing, in given conditions, its activity with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

The International Unit is the activity of a stated amount of the International Standard, which consists of a freeze-dried human von Willebrand factor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

RISTOCETIN COFACTOR ASSAY

The ristocetin cofactor activity of von Willebrand factor is determined by measuring agglutination of a suspension of platelets in the presence of ristocetin A. The assay can be carried out for quantitative determinations by using automated instruments, or for semi-quantitative determinations by visually assessing the endpoint of agglutination in a dilution series. Quantitative assays are preferred.

*REAGENTS*

*Suspension of platelets.* Use standardised and, for example, formaldehyde- or paraformaldehyde-fixed preparations of freshly isolated and washed human platelets. The suspension may also be freeze-dried. An appropriate amount of ristocetin A is added if necessary. Some platelet reagents may already contain ristocetin A.

*Reference preparation.* The reference preparation for von Willebrand factor is the WHO International Standard for von Willebrand factor concentrate.

*METHOD*

*Semi-quantitative assay.* Prepare suitable dilutions of the preparation to be examined and of the reference preparation, using as diluent a solution containing 9 g/L of *sodium chloride R* and 10-50 g/L of *human albumin R*. Add to each

dilution an appropriate amount of the suspension of platelets and, if necessary, of ristocetin A. Mix on a glass slide by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

*Quantitative Assay*. Reconstitute the entire contents of 1 ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of the recommended diluent (for example *water R*); use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5-2.0 IU/mL. The prediluent consists of an isotonic non-chelating buffer containing, for example, 1-5 per cent of human or bovine albumin, and tris(hydroxymethyl)aminomethane or imidazole, appropriately buffered.

The test is performed in accordance with the manufacturer's instructions with at least 2 dilution series with as many dilutions as are needed to obtain a total of at least 3 different concentrations in the linear range of the assay.

Check the validity of the assay and calculate the potency of the test preparation using the usual statistical methods (for example, *5.3*).

COLLAGEN-BINDING ASSAY

Collagen-binding is determined by an enzyme-linked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

*REAGENTS*

*Collagen*. Use native equine or human fibrils of collagen type I or III. For ease of handling, collagen solutions may be used.

*Collagen diluent*. Dissolve 50 g of *glucose R* in *water R*, adjust to pH 2.7-2.9 with *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

*Phosphate-buffered saline (PBS)*. Dissolve 8.0 g of *sodium chloride R*, 1.05 g of *disodium hydrogen phosphate dihydrate R*, 0.2 g of *sodium dihydrogen phosphate R* and 0.2 g of *potassium chloride R* in *water R*. Adjust to pH 7.2 using *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

*Washing buffer*. PBS containing 1 g/L of *polysorbate 20 R*.

*Blocking reagent*. PBS containing 1 g/L of *polysorbate 20 R* and 10 g/L of *bovine albumin R*.

*Dilution buffer*. PBS containing 1 g/L of *polysorbate 20 R* and 50 g/L of *bovine albumin R*.

*Conjugate*. Rabbit anti-human von Willebrand factor serum horseradish peroxidase conjugate. Use according to the manufacturer's instructions.

*Substrate solution*. Immediately before use, dissolve a tablet of *o*-phenylenediamine dihydrochloride and a tablet of urea hydrogen peroxide in 20 mL of *water R* or use a suitable volume of hydrogen peroxide. Protect from light.

*Microtitre plates*. Flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity.

*METHOD*

*Test solutions*. Reconstitute the preparation to be examined as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

*Reference solutions*. Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen diluent to obtain a solution containing 30-75 µg/mL of collagen, mix gently to produce a uniform suspension of collagen fibrils. Pipette 100 µL into each well of the microtitre plate. Cover the plate with plastic film and incubate at 37 °C overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times. Add 250 µL of blocking reagent to each well, cover the plate with plastic film and incubate at 37 °C for 1 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µL each of the test solutions or reference solutions to the wells. Add 100 µL of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Prepare a suitable dilution of the conjugate (for example, a dilution factor of 1 to 4000) with PBS containing 5 g/L of *bovine albumin R* and add 100 µL to each well. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µL of substrate solution to each of the wells and incubate at room temperature for 20 min in the dark. Add 100 µL of *1 M hydrochloric acid* to each of the wells.

Measure the absorbance at 492 nm. Use the absorbance values to estimate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

The assay is invalid if the absorbances measured for the negative controls are greater than 0.05.

**07/2014:20722**

## 2.7.22. ASSAY OF HUMAN COAGULATION FACTOR XI

The principle of the assay is to measure the ability of a factor XI preparation to reduce the prolonged coagulation time of factor XI-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference plasma calibrated against the International Standard for blood coagulation factor XI in plasma.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. *Coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation. Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor XI-deficient plasma (for example *plasma substrate R3*) to produce solutions

containing 0.5-2.0 units/mL. Prepare at least 3 appropriate dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH 7.3 R*) containing 10 g/L of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or perform the assay with incubation tubes maintained in a water bath at 37 °C. Place in each tube 0.1 mL of factor XI-deficient plasma (for example *plasma substrate R3*) and 0.1 mL of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 mL of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37 °C. To each tube, add 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency using the usual statistical methods (for example, *5.3*).

**01/2020:20723**

# 2.7.23. NUMERATION OF CD34/CD45+ CELLS IN HAEMATOPOIETIC PRODUCTS

This chapter describes immunolabelling and analysis by flow cytometry (*2.7.24*) to determine the number of CD34/CD45+ cells contained in haematopoietic products. The determination is carried out by a single platform method using calibrated fluorospheres, after lysis of the sample red blood cells if necessary.

This method applies to all types of preparations and whole blood. However, the characteristics of this method make it particularly suitable for preparations containing very low percentages of CD34/CD45+ cells.

**Graft quality assessment by CD34/CD45+ cell enumeration**

A variety of studies have established that the 1-3 per cent of cells in the bone marrow that express the CD34 cell surface antigen are capable of reconstituting long-term, multilineage haematopoiesis after myeloablative therapy. CD34/CD45+ cells are also found in the peripheral circulation of normal individuals but are extremely rare (0.01-0.1 per cent). However, CD34/CD45+ cells may also be mobilised from marrow to the peripheral circulation in greater numbers by haematopoietic cytokines such as granulocyte colony-stimulating factor and/or chemotherapy.

The technique used for enumeration of CD34/CD45+ cells must meet the following requirements:

- high sensitivity, since haematopoietic stem cells are rare events;
- accuracy, to provide clinically relevant results;
- reproducibility, to provide clinically reliable results;
- speed, to provide real-time analysis.

**Selection of parameters**

The flow cytometry assay uses commercially available, directly conjugated fluorochrome-labelled monoclonal antibodies, routine staining and whole blood lysing procedures, and a gating strategy using light scatter and immunofluorescence analysis using a pan-CD45/CD34 monoclonal antibody combination.

It is possible to determine CD34/CD45+ cell viability by appropriate nucleic acid staining with a stain that does not cross the intact cell membrane (for example, with 7-aminoactinomycin D).

**Selection of monoclonal antibodies**

*CD34 antibodies.* Use class III CD34 antibodies that detect all glycosylation variants of the molecule (for example, clone 8G12 or 581). To detect rare events, use an antibody conjugated to the brightest fluorochrome excitable using an argon laser-based flow cytometer, for example phycoerythrin (PE).

*CD45 antibodies.* Pan-CD45 antibodies that detect all isoforms and all glycoforms of this structure are required. A CD45 antibody conjugated to fluorescein isothiocyanate (FITC) fluorochrome is generally used (for example, J33, HLe1, 2D1).

*Isotypic or isoclonic controls.* A negative control is analysed to detect any non-specific signal in the PE fluorescence region. If using an isotypic control (a monoclonal antibody to an irrelevant antigen of the same isotype as the CD34 antibody employed), the PE-conjugated isotype is combined with CD45-FITC (or PerCP). If using an isoclonic control, the unconjugated (in excess) and PE-conjugated CD34 identical monoclonal antibody is combined with conjugated CD45. Alternative combinations may be used.

**Absolute count of CD34/CD45+**

*Calibrated fluorospheres.* Depending on the technique used, the internal standard either consists of calibrated beads in suspension or is directly introduced into the associated tubes by the manufacturer.

The absolute count of the CD34/CD45+ cells per microlitre is calculated using the following expression:

$$\frac{A}{B} \times C$$

$A$ = number of CD34/CD45+ cells counted;

$B$ = number of fluorosphere singlets counted;

$C$ = known fluorosphere concentration.

**Gating strategies**

The purpose of sequential gating is to select the population of interest and simultaneously minimise interference from debris and mature cells to which antibodies can bind non-specifically. If using a commercial kit, apply the gating recommended by the manufacturer. If using an in-house assay, it is preferable to apply a currently recommended strategy. A gating strategy that uses light scattering parameters and CD34/CD45 fluorescence will aid in the accurate identification and enumeration of CD34/CD45+ cells.

**Number of events analysed**

A sufficient number of events are analysed to maintain acceptable accuracy and precision, for example not fewer than 100 CD34+ events and not fewer than 60 000 CD45+ events; the total number of cells counted may be greater if the percentage of CD34 is 0.1 per cent or less.

**Specimen collection**

Acid citrate dextrose (ACD) formula A is the anticoagulant used in apheresis procedures. This anticoagulant allows both an automated leucocyte count and flow cytometry evaluation to be performed on the same specimen. Edetic acid (EDTA) is the anticoagulant of choice for peripheral blood sampling.

**Specimen transport**

Transport conditions guarantee the physical and thermal safety of samples.

**Specimen integrity and storage**

Fresh (less than 24 h old) apheresis products, whole blood samples, umbilical cord blood specimens or bone marrow samples can be processed. Old specimens (more than 24 h old) and specimens that have been frozen and thawed are

stained with a viability dye. On receipt, the temperature within the package is verified.

TECHNIQUE

**Sample preparation**

Ensure that the concentration of leucocytes is suitable prior to staining with monoclonal antibodies. If necessary, dilute the sample with medium that is compatible with the product to be tested and the lysing system. Record the dilution factor. It is recommended to perform the test with a negative control.

**Flow cytometry analysis**

*Autostandardisation*

For analysis of cells labelled with a commercially available kit, manufacturers have developed some quality tools for setting the flow cytometer. These settings are then automatically transferred on protocol analysis of samples. Specific fluorospheres are used to set the photomultiplier tube (PMT) on target values, compensation is set and the system is checked using a control preparation.

*System settings*

- *Discriminator/threshold*: the forward angle light scatter threshold is set to exclude debris (low forward scatter) but not small lymphocytes from the light-scatter plot.
- *PMT high voltage settings*: these must be consistent with cell-surface marker analysis and established within each laboratory so that negative and positive cell populations of moderate antigen density can be distinguished; PMT voltages are reviewed and adjusted periodically according to standardised laboratory procedures.
- *Compensation*: this must be acceptable for the colour spectra overlap (for example, FITC/PE) encountered in cell-surface marker analysis; colour compensation is analysed and adjusted according to standardised laboratory procedures.
- *Flow rate*: this must be consistent with routine cell-surface marker analysis.
- *Gating regions*: the gating regions established for the CD34/CD45 samples are maintained unaltered for the analysis of the negative region.

**Calculation of absolute number of CD34/CD45+ cells**

The absolute number of CD34/CD45+ cells is calculated using the following expression:

$$n \times D \times V$$

$n$ = total number of CD34/CD45+ cells per microlitre;

$D$ = dilution factor;

$V$ = volume of the product to be tested, in microlitres.

Results are reported as both the percentage of CD34/CD45+ cells and the absolute number per microlitre. They may also be reported as the absolute number per kilogram of recipient body mass, where this is possible.

**01/2008:20724**

# 2.7.24. FLOW CYTOMETRY

Flow cytometry consists of a multiparametric analysis of optical properties of individual particles in a fluidic system.

Cells or particles in suspension are individually distributed into a linear array (stream), which flows through a detection device. Solid tissues have to be reduced to a single-cell suspension to be analysed.

The spectrum of parameters measurable by flow cytometry includes volume and morphological complexity of cells or cell-like structures, cell pigments, DNA content, RNA content, proteins, cell surface markers, intracellular markers, enzymatic activity, pH, membrane and fluidity.

It is possible to collect 2 morphological parameters plus 1 or more fluorescence signals per single cell. The multiparametric analysis allows the definition of cell populations by their phenotype.

APPARATUS

Focusing, magnifying, and choice of light source are optimised to allow the automatic detection and measurement of morphological differences and staining patterns. Flow cytofluorimetric analysis meets the following criteria:

- choice of light source depending on the parameters to be analysed;
- adjustment of instrument settings depending on the cell type to be analysed (for example, cell cultures, leucocytes, platelets, bacteria, spermatozoa, yeast) and the analysis to be performed (for example, phenotyping, cell cycle, apoptosis, cytokines, membrane fluidity, fluorescent protein).

Flow cytometry is characterised by the automated quantification of set parameters for a high number of single cells during each analysis session. For example, 100 000 particles or more (practically unlimited) are analysed one after the other, typically in about 1 min. The detection limit is as low as 100 fluorescent molecules per cell.

A flow cytometer apparatus has 5 main components:

- a fluidic system and a flow cell;
- a light source;
- a detection and Analogue to Digital Conversion (ADC) system;
- an amplification system;
- a computer provided with software for analysis of the signals.

*FLUIDIC SYSTEM AND FLOW CELL*

The single cell is exposed to the light source and detected in the flow cell. The fluidic system carries the suspended cells individually from the sample tube to the laser intercept point. To achieve this, the sample stream is drawn out to a very thin fluid thread by a sheath fluid in the flow cell (hydrodynamic focusing). The light beam is focused in an elliptical shape, by 2 confocal lenses, into the flow cell channel through which the cells pass. The flow rate must be constant during routine cell surface marker analysis and must ensure a suitable distance between the cells to allow counting.

*LIGHT SOURCES*

Commonly used light sources are:

- lamps (mercury, xenon);
- high power water-cooled lasers (argon, krypton, dye laser);
- low power air-cooled lasers (argon (488 nm), red helium-neon (633 nm), green helium-neon, helium-cadmium (UV));
- diode lasers (blue, green, red, violet).

*SIGNAL DETECTION*

When a particle passes across the light beam, it scatters some of the light in all directions. Fluorescent dyes, when added to the particle, give off their own light (fluorescence), which is also radiated in all directions. 2 types of signals may thereby be generated:

- scatter of light;
- fluorescence emission.

The instrument's light detectors collect some of this scattered and fluorescent light and produce electronic signals proportional to the amount of light collected.

**Scatter**. 2 parameters of light scattering are measured:
- the amount scattered mainly forward (forward scatter (FS))
- the amount scattered at 90° from the direction of the light beam (side scatter (SS)).

Forward scatter correlates with the cell volume while side scatter is influenced by parameters such as the shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness, and correlates with the morphological complexity of the cell, so that the higher the SS intensity, the higher the cell complexity. As a function of the morphological characteristics of cells, scatter signals will always be generated during a flow analysis; they are defined as intrinsic parameters.

**Fluorescence**. Depending on the type and number of light sources, when a cell passes through the sensing area, it will emit fluorescent light. Fluorescence signals are generated from fluorescent dyes naturally present in the cells (for example, co-enzymes, chlorophyll, seaweed pigments) and/or from fluorescent probes taken up by the cells when stained for the analysis of specific characteristics (for example, fluorescent antibodies, nucleic acid dyes, pH probes, calcium probes, fluorescent proteins). Nowadays, there is a large number and a wide range of different types of fluorescent probes available. The optical filters must be adapted to the fluorochromes used and changed if necessary. Each fluorescent probe is characterised by its excitation spectrum and its emission spectrum. They are chosen depending on the nature of the excitation source and the detection system, and according to the specific purpose of the analysis.

*SIGNAL MANAGEMENT AND ANALOGUE TO DIGITAL CONVERSION*

Scatter and fluorescence signals emitted by cells when passing across the laser beam are sorted and addressed to their detectors using optical filters. The detectors are transducers (photomultiplier tubes (PMTs)) that convert light signals radiated from the cells into voltage pulses.

The process of counting each pulse in the appropriate channel is known as Analogue to Digital Conversion (ADC). The process is finally shown as a frequency histogram.

**Amplification**. Voltage pulses need to be amplified for optimal visualisation. The amplification process accentuates the differences between cell signals, and consequently increases the resolution among cell populations of different characteristics (for example, the differentiation of viable from non-viable cells, or non-specific fluorescence from antigen-specific fluorescence after staining with a fluorescent monoclonal antibody).

There are 2 methods of amplification: linear or logarithmic; the choice between the 2 types is made for every single signal according to the morphological characteristics of the cells and the staining reagents used (for example, fluorescent monoclonal antibodies, nucleic acid dyes).

*Linear amplification*, which enhances the differences among strong pulses, is used with those parameters that generate high intensity signals, for example:
- cell scatters;
- fluorescence from nucleic acid dyes for cell cycle studies.

*Logarithmic amplification*, in contrast, is for weak pulses and parameters or analysis conditions that may generate both weak and strong pulses, for example:
- cell antigens;
- scatter from platelets, bacteria, yeast;
- fluorescence from nucleic acid dyes for apoptosis studies.

**Compensation of fluorescence signals**. Each fluorescent dye has an absorption wavelength spectrum and a higher emission wavelength spectrum. When using 2 or more fluorescent probes simultaneously for staining cells (for example, 4-antigen immunophenotyping), the fluorochromes emission spectra may overlap. As a consequence, each fluorescence detector will sense its own specific fluorescent light and a variable quantity of light emitted by the other fluorescent probes. This results in signal over-evaluation and poor separation of the cell populations.

The solution is in the use of an electronic matrix that allows the selective subtraction of the interfering signals from each fluorescence signal after detector sensing (fluorescence compensation).

Fluorescence compensation requires the use of fluorescence calibrators, preferably positive cell samples stained with the fluorochromes of interest, combined in a manner equivalent to that for the antibody used for the analysis.

*SIGNAL PLOTTING AND DISPLAY*

After amplification and compensation, the signals are plotted in 2 or 3 dimensions. Histograms show the signal intensities versus the cell counts for a given parameter. Cytograms, in which each dot represents a cell, result from the combination of 2 signal intensities (dual-parameter dot plots). The type and number of plots and signal combinations are chosen on the basis of the specimens and dyes used. When analysing acquired data, the flow cytometry software can also generate other kinds of graphs (such as overlays, surface plots, tomograms, contour plots, density plots, overlay plots). Statistical data such as mean fluorescent intensities (and their shifts in time or their dependence on cell function) can also be used.

*DATA ANALYSIS*

Different kinds of cell populations may be present inside the cell suspensions to be analysed, some of which are unwanted (such as dead cells, debris or macro-aggregates), or simply not relevant for the analysis (for example, granulocytes when studying lymphocytes). This depends on the cell sample type (whole blood, bone marrow, cell cultures, biological fluids, cell suspensions from solid tissues) and on the handling procedures (for example, staining methods, lysis, fixation, cryopreservation, thawing, paraffin-embedded tissue preparation).

As a consequence, not all the signals generated during a flow cytometry analysis belong to the cells to be studied. 2 strategies are adopted to exclude unwanted and irrelevant cell signals.

The 1st is used during data acquisition. It is a noise threshold, applied to 1 (or more) significant parameter(s), set to acquire only the cells with signal intensities higher than the pre-defined discrimination value for that parameter. Due to its characteristics of a strong signal with a low grade of interference, forward scatter is the parameter most often used as discriminator.

The 2nd, applied during data analysis, consists of the use of gating regions to restrict the analysis only to signals from those populations that satisfy given morphological and expression profile characteristics. 2 types of logical gating are commonly used. The 1st is the morphological gate. The cell populations are identified using their morphological signals (FS and SS). A region gate is drawn around the population of interest (for example, lymphocytes, viable cells) then the fluorescence plots are gated into the selected region. The 2nd is the fluorescence-based gate. The cell population of interest is identified on the basis of the expression intensity of an antigen or a dye, then a gate region is drawn around it. Afterwards the fluorescence plots are gated into the selected region.

The analysis software allows the creation of multiple gate regions, using a sequential logic order. This feature is especially useful when studying rare cell populations or for sorting purposes.

*CONTROLS*

**Internal control**. The system's optical alignment must be validated before analysis using adapted fluorospheres and the optimum fluidic stability is checked. The data obtained are reported and allow the periodical review of control values against the mean performance value. A positive control is highly desirable to prove that the test antibody is functional

and to allow the proper setting of the flow cytometer. The positive control must include samples known to be positive for the marker of interest.

**External control**. To ensure reliability in the data obtained or to check inter-laboratory reproducibility, participation in a proficiency testing study is recommended.

**07/2009:20725**

## 2.7.25. ASSAY OF HUMAN PLASMIN INHIBITOR

Human plasmin inhibitor, also called human $\alpha_2$-antiplasmin, is a plasma protein that inhibits the plasmin (a serine protease) pathway of fibrinolysis by rapidly forming a complex with free plasmin. Furthermore, upon blood coagulation, human plasmin inhibitor is cross-linked to fibrin strands by factor XIII, and interferes with binding of the proenzyme plasminogen to fibrin.

The potency of human plasmin inhibitor is estimated by comparing the ability of the preparation to be examined to inhibit the cleavage of a specific chromogenic substrate by plasmin with the same ability of a reference standard of human plasmin inhibitor. Plasmin cleavage of the chromogenic substrate yields a chromophore that can be quantified spectrophotometrically.

The individual reagents for the assay may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

REAGENTS

*Dilution buffer pH 7.5*. According to the manufacturer's instructions, a suitable buffer is used. Adjust the pH if necessary.

*Plasmin*. A preparation of human plasmin that does not contain significant amounts of other proteases is preferably used. Reconstitute and store according to the manufacturer's instructions.

*Plasmin chromogenic substrate*. A suitable specific chromogenic substrate for plasmin is used: H-D-cyclohexylalanyl-norvalyl-lysyl-*p*-nitroaniline hydrochloride (H-D-CHA-Nva-Lys-*p*NA.HCl) or L-pyroglutamyl-L-phenylalanyl-L-lysyl-*p*-nitroaniline hydrochloride (Glp-Phe-Lys-*p*NA.HCl). Reconstitute in *water R* to give a suitable concentration according to the manufacturer's instructions.

METHOD

Varying quantities of the preparation to be examined are mixed with a given quantity of plasmin and the remaining plasmin activity is determined using a suitable chromogenic substrate.

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.5 and prepare at least 2 independent series of 3 or 4 dilutions for both the preparation to be examined and the reference standard.

Mix 0.020 mL of each dilution with 0.020 mL of dilution buffer pH 7.5 and warm to 37 °C. Add 0.040 mL of a plasmin solution (test concentration in the range of 0.2 nkat/mL to 1.6 nkat/mL) previously heated to 37 °C and leave at 37 °C for 1 min. Add 0.020 mL of the chromogenic substrate solution, previously heated to 37 °C, to each mixture. Immediately start measurement of the change in absorbance at 405 nm (*2.2.25*) using a microtitre plate reader. Calculate the rate of change of absorbance (ΔA/min). Alternatively, an end-point assay might be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm.

In both cases the duration of the cleavage of the chromogenic substrate should be chosen to produce a linear increase in absorbance at 405 nm, before substrate depletion becomes significant. If the assay is performed in test tubes or cuvettes using a spectrophotometric method, the volumes of reagent solutions are changed proportionally.

Substract the optical density of the blank (prepared with dilution buffer pH 7.5) from the optical density of the preparation to be examined. Check the validity of the assay and calculate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

**01/2008:20727**

## 2.7.27. FLOCCULATION VALUE (Lf) OF DIPHTHERIA AND TETANUS TOXINS AND TOXOIDS (RAMON ASSAY)

The content of toxin or toxoid in a sample can be expressed as a flocculation value (Lf) using the Ramon assay. In this assay, antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of toxin or toxoid. At the equivalence point of toxin/toxoid and antitoxin, flocculation occurs in 1 or more tubes. The first tube in which flocculation occurs is used to determine the Lf value of the sample.

The Lf value of a toxin or toxoid is determined by the number of units of antitoxin that, when mixed with the sample, produces an optimally flocculating mixture (Ramon assay).

Practical experience has shown that the results of the calibration of antitoxins in International Units (IU), for example by comparison to international antitoxin standards, depends on the immunochemical method used. For this reason, antitoxins used for the Ramon assay must be directly calibrated against the *international biological reference reagents for diphtheria or tetanus toxoid for flocculation tests*, using the principles described below. The concentration thus determined may be indicated in Lf-equivalents per millilitre (Lf-eq./mL).

By definition, 1 Lf is the quantity of toxin or toxoid that flocculates in the shortest time with 1 Lf-eq. of specific antitoxin.

A range of volumes of the reference standard of antitoxin adjusted to a concentration of 100 Lf-eq./mL is dispensed into a series of, for example, 7 cm × 1 cm flocculation tubes. A sufficient quantity of a 9 g/L solution of *sodium chloride R* is added to each tube to give a constant total volume of, for example, 1 mL. The test sample is diluted to give an expected concentration of approximately 50 Lf/mL, and, for example, 1 mL aliquots of this dilution are dispensed into each of the tubes containing antitoxin. The tubes are properly mixed by shaking, then placed in a water-bath at a constant temperature between 30 °C and 52 °C, and observed at regular intervals for the first appearance of floccules. This may require the use of a magnifying lens and strong illumination.

The first and the second mixtures to flocculate are recorded as well as the time taken for the first flocculation to appear. 2 tubes may flocculate simultaneously.

The first tube to flocculate is the one that contains the amount of antitoxin closest in equivalence to the amount of antigen in the sample. The antitoxin content of this tube can be used to calculate the Lf value of the sample. If 2 tubes flocculate at the same time, the mean from the tubes are given as the result.

The time taken for the first tube to flocculate (Kf) is a useful indicator of the quality of the antigen. If at a given temperature and concentration of toxoid and antitoxin the Kf value is increased compared with normal, this indicates that the antigen has been damaged. The Kf value may also change with the quality of the antitoxin used.

*Example*

| Tube | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Antitoxin added (Lf-eq.) | 40 | 45 | 50 | 55 | 60 | 65 |
| Antitoxin added (mL) | 0.40 | 0.45 | 0.50 | 0.55 | 0.60 | 0.65 |
| Saline added (mL) | 0.60 | 0.55 | 0.50 | 0.45 | 0.40 | 0.35 |
| Diluted sample added | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

If in this example the first tube to flocculate is tube C then the Lf value of the diluted sample is 50 Lf/mL. However, if the first tube to flocculate is tube A or tube F this does not indicate equivalence at that level. It would be necessary to perform a repeat test using either a different dilution of test sample or selecting a different range of doses of reference antitoxin.

More precision can be obtained by making allowance for the sequence of flocculation after the first tube. Thus, in the example quoted, if the second tube to flocculate had been tube D, the final value for the diluted sample would be 52, whereas if the second tube to flocculate was tube B, the final value would be 48. The test may be performed in duplicate with slightly different dilutions of the test sample.

If there is no indication of the expected Lf value of the sample available, it is advisable to obtain a rough estimate by use of a wider range of antitoxin content in the tubes before proceeding to the final test.

*Example*

| Tube | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| Antitoxin content (Lf-eq.) | 20 | 30 | 45 | 70 | 100 | 150 |

The level of toxin or toxoid and antitoxin concentration in the test may be varied, but this will markedly affect the flocculation time, so that at very low levels the test will take too long, whilst at a high concentration the onset of flocculation may be so rapid as to make it difficult to distinguish the first and second tubes to flocculate.

**Assay of low concentrations by blend flocculation**

For very low concentrations, it is preferable to measure toxin or toxoid by the method of blend flocculation. This involves comparison of the Lf value of a known toxin or toxoid and that of a mixture of the sample with that toxin or toxoid.

When a toxin or toxoid with a known Lf value and a toxin or toxoid with an unknown Lf value are flocculated together, the mixture will flocculate as the sum of their values if they are homogeneous. If non-homogenous toxins or toxoids are mixed they will produce an aberrant pattern with 2 flocculation maxima.

**01/2008:20728**

# 2.7.28. COLONY-FORMING CELL ASSAY FOR HUMAN HAEMATOPOIETIC PROGENITOR CELLS

The haematopoietic system represents a continuum of cells whose phenotype and properties change as they progress from stem cells to differentiated cells.

Haematopoietic progenitor cells (HPCs) are capable of forming colonies or 'cell clusters' in cultures grown in semi-solid media and are said to be 'clonogenic'. The determination of the number of colony-forming cells (CFCs) in a cellular product is an indicator of the functional capacity of the progenitor cells and is a predictor of haematopoietic reconstitution. The measured number of CFCs correlates with the minimum number of progenitors present in the sample.

CELL-SURFACE MARKERS

The capacity of colony-forming cells to give rise to haematopoietic colonies *in vitro* and/or to reconstitute the haematopoietic system has been correlated with the expression of specific cell-surface antigens. The expression of the membrane antigen CD34 is an accepted marker for most of the haematopoietic progenitors and stem cells.

COLONY ASSAY SPECIFICITY

Colony-forming cells are identified with a nomenclature based on the lineages of mature cells present in the colony (for example, CFU-Mix, CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E, CFU-Meg) and are a population of progenitors able to give rise to colonies containing one or more lineages of haematopoietic cells. No or low capacity for self-renewal has been ascribed to this population of human HPCs compared with the most immature stem cells.

The amount and type of growth factors supplied during the culture modulate the type and size of colonies that will be formed.

Greater specificity on the general class of HPCs and on their relative proliferative potential is provided by the time required to differentiate *in vitro* into mature cells. The time required by post-natal colony-forming cells to give rise to a colony formed of mature cells *in vitro* is 10-14 days.

QUALITY ASSURANCE FOR A CFC ASSAY

It is paramount for the overall quality of the colony-forming cell assay to apply a strictly standardised approach.
It is therefore recommended to carry out intra- and inter-laboratory validations. The source of the materials, including reagents, growth factors and disposables, is identified.

The main factors affecting variability in the CFC assay are the number of cells plated and the identification of colonies. Up to 15 per cent intra-laboratory variability may be observed for the same test. If it is necessary to evaluate the number of colony-forming cells in a purified cell population, it is possible to use a limiting dilution approach where the number of wells positive for cell proliferation is measured with an automated system.

The other main source of variability stems from the use of undefined materials (for example, foetal bovine serum or bovine serum albumin) in the CFC assay. These products derive from pools of source materials and provide a non-specific stimulation of cellular proliferation. However, it is not uncommon to have batches with particular characteristics that selectively stimulate the proliferation of specific haematopoietic lineages.

Finally, a low level of endotoxins (less than 0.01 IU/mL or less than 0.01 IU/mg) in all the materials used for the clonogenic assay is advisable, as higher levels result first in a progressive skewing of the haematopoietic lineages expression in the cultures, and afterwards in a more general inhibition of cell proliferation and clonogenesis.

CFC CLONOGENIC ASSAY

The CFC assay is based on the capacity of progenitor cells to form a colony when plated in a semi-solid medium or in a gel in the presence of specific growth factors. Different types of semi-solid media may be used (for example, methylcellulose, collagen, agar and plasma-clot) depending on the desired readout. Commercially available media usually give more reproducible results.

*MATERIALS*

A validation is performed at least for the following critical materials.

**Growth factors**. Both multilineage (such as Kit-ligand or stem cell factor (SCF), interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF)) and lineage-specific (erythropoietin, granulocyte colony-stimulating factor (G-CSF)) growth factors are required to obtain the highest number of colonies from a cell suspension containing a mixed population of HPCs.

**Other media components**. Media may be supplemented by serum (notably by foetal bovine serum) and/or albumin.

*CELL CULTURE*

**Cells**. The sample placed in culture must be representative of the cellular product injected. Cell suspensions are required for this assay. In the case of bone marrow aspirates, such suspensions can be obtained by forcing the bone marrow through a sieve or through progressively smaller calibre needles. Repeated passages through a 21-gauge needle are usually sufficient to disperse cell clusters into a cell suspension.

*PLATING AND SCORING*

The cells diluted in the culture medium are mixed in the semi-solid medium. It is common to plate 1 mL of the mixture in an untreated sterile Petri dish (Ø 35 mm).

Because of the viscosity of the medium, the solution cannot be plated with air displacement pipettes and the use of syringes equipped with large bore (≤ 18-gauge) needles is required.

The number of cells to be plated depends on the HPC concentration in the sample to be tested. So that no colony is derived from 2 different HPCs, the number of cells plated must allow between 40 and 80 colonies per plate (Ø 35 mm) to be counted. The 'target' number of colonies per plate may be obtained either from the percentage of CD34+ (or concentration of CD34+ cells/mL) determined by flow cytometry (*2.7.24*) or from different dilutions of the cell suspension (usually 2 concentrations are tested).

The plates are incubated in aerobic conditions with a carbon dioxide concentration of 5 per cent, at 37 °C in a humid (saturated) atmosphere for 10-14 days, and the number of colonies is then scored under an inverted microscope. Care must be taken when manipulating the dishes containing the colonies as the methylcellulose-based medium is viscous but not jellified. An inclined plate will result in mixed and 'comet'-shaped colonies making the scoring likely to be incorrect.

*IDENTIFICATION OF THE COLONIES*

The size and structure of the colonies depend on the type of mature cells that are their constituents. 50 cells per colony is usually considered a minimum. The presence of haemoglobinised cells identifies progenitors of the erythroid lineage. As the amount of mature cells for each lineage largely depends on the growth factors added to the cultures, performing differentiated counts is not recommended unless otherwise prescribed.

*EXPRESSION OF THE RESULTS*

The results of CFC culture are usually expressed as the arithmetic mean of the number of colonies counted in at least 3 plates in the test. The mean number of colonies is then related to $10^4$ or $10^5$ viable nucleated cells placed in culture.

**01/2008:20729**

# 2.7.29. NUCLEATED CELL COUNT AND VIABILITY

The determination of the quality of cell suspensions requires accurate measurements of both cell concentration and percentage of viable cells. These data are essential to the decision-making process for preparing cellular products and for maintaining optimum culture conditions. The cell count may be expressed as the number of cells per volume of cell suspension and the cell viability as the number of viable cells per volume of cell suspension. The cell-count procedure may be performed manually (haemocytometer) or with an automated apparatus (for example, particle counter, flow cytometer). Other methods than that described below may be used.

CELL NUMBER

*MANUAL COUNTING*

*Description of the apparatus and test principle.* The following materials are required:

- a haemocytometer: a specialised microscope counting chamber available in different designs. It consists of a thick slide and a coverslip mounted to delimit a chamber with a specific volume for each design. The thick slide of the various haemocytometers consists of counting chambers separated by deep grooves to avoid cross-filling. The counting chamber is etched in the glass and contains a grid which is specific for each model;
- a light microscope - low power 10× to 40× magnification;
- pipettes of a suitable volume range.

The haemocytometer is used to quantify the number of cells in a given solution by calculation of the cell concentration per millilitre ($C$) using the following expression:

$$a \times 10^n \times d$$

$a$ = number of cells counted;

$d$ = dilution factor (where applicable);

$n$ = factor varying with the volume of the haemocytometer chamber.

It is possible to distinguish between mixed cell populations provided they differ in size or pigmentation (for example, leukocytes and erythrocytes).

*Preparation of the counting chamber and analysis.* Mount the coverslip (slightly moistened on the edges) on the slide. Move the coverslip back and forth over the slide, pressing slightly on the sides. Prepare a suitable dilution of the cell suspension in isotonic buffer or in haemolysis buffer.

Add an appropriate volume of the dilution to the counting chamber. The liquid is added to the border of the coverslip and is drained inside the chamber by capillarity. Carefully place the haemocytometer under the microscope and focus. Count the cells in a zone of the grid. Calculate the cell concentration in the diluted and original samples.

To increase the accuracy of the measurement, it is important to respect the following basic precautions:

- use only suitably thickened coverslips;
- wherever possible, count more than 100 cells (if necessary, count more areas);

– where cell clustering is detected (i.e. the cell suspension is not monocellular), resuspend the cells before sampling and count again;
– avoid underfilling or overflowing the chamber, otherwise the volume will no longer be accurate.

*AUTOMATED COUNTING METHODS*

*Particle counters based on conductivity variation.* Electronic particle counting devices measure the size and number of particles in a solution.

Particle counters are calibrated before use with a solution of particles of known concentration and size. To allow the counting of larger particles, tubes fitted with differently calibrated orifices are available. These apparatuses do not allow the discrimination between dead and live cells. As cell debris may also generate pulses that may cause errors, counters are also fitted with a threshold control allowing only larger particles to be counted.

The apparatus must be qualified for the counting of cellular products (in terms of linearity, accuracy, etc.).

*Particle counters based on flow cytometry* (*2.7.24*). The flow cytometer is calibrated with reference particles of known concentration and size to give an absolute cell number per volume. However, a calibrating solution is no longer necessary in instruments using 2 electrodes inserted in the sampling chamber where the fixed size of the sampling chamber and distance between the 2 electrodes allow the measurement of the content of a fixed volume. This type of instrument rarely needs to be calibrated after the initial setting.

VIABILITY

This section applies to cell staining by viability dyes and manual or automated analysis, under a light microscope or by flow cytometry, of a cell suspension in order to determine the percentage of viable cells.

Depending on the type of cells and the method used, the results may differ.

*MANUAL DYE-EXCLUSION METHOD*

*Test principle.* This test is based on the exclusion of the dye from viable cells whereas dead or damaged cells absorb the dye and are coloured. It provides information on the cytoplasmic membrane integrity but its results do not necessarily reflect cell functionality. Recently trypsinised or thawed viable cells may have leaky membranes, causing them to absorb the dye.

*Dye.* Trypan blue is the stain most commonly used to distinguish between viable and non-viable cells, but other suitable dyes such as erythrosin B or nigrosin may also be used. It is an acid dye ($M_r$ 961), an anion with 4 sulfonate groups that can easily bind to proteins; therefore the protein concentration of the preparation to be tested must be as low as possible.

*Test conditions.* Dye fixation is strongly influenced by pH, within a range of 6.6 to 7.6. Fixation is optimal at pH 7.5. The other conditions, such as the dye concentration and the staining time are validated.

*Storage conditions of the dye*: Generally a 0.4 or 0.5 per cent trypan blue solution in sterile phosphate-buffered saline is used. Store protected from light and air.

*Test preparation and analysis.* Stain the cell suspension at the required dilution (usually in phosphate-buffered saline) with, for example, a trypan blue solution having a final concentration of 0.1 to 0.2 per cent. Mix gently. Incubate for not more than 2-4 min at room temperature. Mix gently and place a suitable volume in a counting chamber. Count without delay.

Determine the percentage of viable cells from the ratio of the number of unstained cells to the total number of cells under a light microscope, considering all stained cells as dead cells. Viability (*V*) is calculated as a percentage using the following expression:

$$\frac{n}{N} \times 100$$

*n* = number of unstained (viable) cells;

*N* = total number of cells (stained and unstained).

It is essential that the incubation time be not more than 4 min as the number of stained cells may increase significantly afterwards. For a new determination, it may therefore be necessary to prepare a new test.

*AUTOMATED METHODS*

**Flow cytometry**

*Test principle.* The test is based on the ability of certain dyes to cross damaged membranes and bind to DNA by intercalating between bases so that dead cells may fluoresce and be detected by flow cytometry (*2.7.24*). Non-viable cells are evaluated and discriminated by focusing on positive staining whereas viable cells remain unstained. This analysis is generally performed with 7-aminoactinomycin D (7-AAD) or propidium iodide (PI) but other suitable dyes may also be used.

*Dye.* 7-AAD and PI are given as examples of membrane-impermeants that may be used as viability dyes.

7-AAD is an analogue of actinomycin D that contains a substituted amino group at position 7 of the chromophore. It intercalates between cytosine and guanine DNA bases. The spectral properties of 7-AAD make this molecule particularly suitable for flow-cytometry analysis. The maximum absorption of the 7-AAD/DNA complex is situated in the green spectral region and is thus suitable for an argon laser-equipped cytometer (excitation wavelength of 488 nm). The deep red fluorescence emission of the 7-AAD viability dye (635 nm to 675 nm) eases the use of the probe in combination with fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-conjugated antibodies, because in contrast to PI, the 7-AAD/DNA complex shows minimal overlap with FITC and PE.

PI binds to double-stranded DNA by intercalating between bases with little or no sequence preference and with a stoichiometry of 1 dye molecule per 4-5 DNA base pairs. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted around 30-40 nm towards the red and the fluorescence emission maximum (615 nm) is shifted around 15 nm towards the blue. Although its absorptivity is quite low, PI exhibits a sufficiently large Stokes shift to allow simultaneous detection of nucleic acids and fluorescein-labelled antibodies, provided that the suitable optical filters are used.

*Storage conditions of nucleic acid dye solution*: 5 ± 3 °C.

*Test preparation and analysis.* In the case of haematopoietic cells, the dye may be added after CD45 labelling to obtain a better separation of cells from debris and platelets with a side scatter (SS)/CD45+ gating region. The incubation conditions of the cell suspension with the dye are validated previously.

Incubation is performed at room temperature protected from light. Where necessary, lysis of red blood cells is performed using, for example, ammonium chloride. If not, add buffer alone.

Percentages of viable cells are directly given by the flow cytometer and deduced from the analysis of positive cells (dead cells) in the SS/7-AAD or SS/PI cytogram (dot plots).

Positive controls may consist of stabilised cells (dead cells) mixed with fresh viable cells at a target value.

**Digital imaging of stained cells**. Digital imaging allows the automation of dye-exclusion methods. The cell suspension and viability-dye solution are directly mixed by a machine. The system, which allows sample aspiration, reagent handling, and subsequent instrument cleaning is fully automated. Once the cellular suspension has been aspirated and mixed with the dye solution, it is pumped to the flow cell for imaging. The

stained cell suspension is aspirated through a chamber where stroboscopic light allows a camera to photograph the flowing cells. The images are digitalised and the number of dead or live cells counted by the software.

**07/2008:20730**
**corrected 7.0**

# 2.7.30. ASSAY OF HUMAN PROTEIN C

## 1. CHROMOGENIC ASSAY

Human protein C is a vitamin K-dependent plasma protein that, upon activation to activated protein C (APC), can inhibit blood coagulation through the proteolytic cleavage of factors Va and VIIIa. Human protein C activity is estimated using a two-step method: in the 1st step, human protein C in the preparation is activated by a specific activator from snake venom; in the 2nd step, APC cleaves a specific chromogenic substrate to form a chromophore that can be quantified spectrophotometrically.

**Step 1**

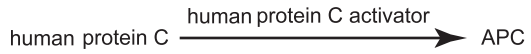


**Step 2**

The potency of human protein C is estimated by comparing the ability of the preparation to be examined to cleave a chromogenic substrate with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre plate end-point method.

### REAGENTS

*Dilution buffer pH 8.4.* Dissolve 6.055 g of *tris(hydroxymethyl)aminomethane R* and 16.84 g of *caesium chloride R* in *water R* and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

*Human protein C activator.* Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions. Dilute to 0.25 U/mL with *water R* before use in the assay.

*Activated protein C chromogenic substrate.* Specific chromogenic substrate for APC, for example L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline hydrochloride (pyroGlu-Pro-Arg-pNA.HCl). Reconstitute with *water R* to give a concentration of 4.5 mmol/L. Further dilute to 1.1 mmol/L with dilution buffer pH 8.4 before use in the assay.

### METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with *water R* to produce at least 3 separate dilutions for each preparation in the range 0.050-0.200 IU/mL, preferably in duplicate.

**Step 1**. Mix 0.025 mL of each dilution with 0.050 mL of the human protein C activator, both previously heated to 37 °C, and leave at 37 °C for exactly 10 min. For each dilution, prepare a blank in the same manner, using *water R* instead of the human protein C activator.

**Step 2**. Add 0.150 mL of diluted chromogenic substrate, previously heated to 37 °C, to each mixture and leave at 37 °C for exactly 10 min. The incubation time must be adjusted, if necessary, to ensure a linear development of chromophore with time. Terminate the reaction by adding 0.050 mL of a 50 per cent *V/V* solution of *glacial acetic acid R*.

Cleavage of the chromogenic substrate by APC causes release of the chromophore pNA, in proportion to the concentration of human protein C in the preparation. Measure the optical density at a wavelength of 405 nm. Subtract the optical density of the blank from the optical density of the test sample. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

## 2. CLOTTING ASSAY

Human protein C activity is estimated following cleavage to APC by a specific activator extracted from the venom of the viper *Agkistrodon contortrix contortrix*. The resulting APC inactivates factors Va and VIIIa, and thus prolongs the APTT (Activated Partial Thromboplastin Time) of a system in which all the coagulation factors are present, constant and in excess, except for human protein C, which is derived from the preparation being tested. Prolongation of the clotting time is proportional to the concentration of human protein C in the preparation.

The potency of human protein C is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

### REAGENTS

*Dilution buffer pH 7.4.* Isotonic non-chelating buffer.

*Human protein C-deficient plasma.* Citrated human plasma with no measurable human protein C content. Reconstitute and store according to the manufacturer's instructions.

*Human protein C activator.* Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions.

*Coagulation activator.* A suitable APTT reagent containing phospholipids and a contact activator may be used. It may be combined with the human protein C activator.

### METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.010-0.150 IU/mL, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein C-deficient plasma and 1 volume of the human protein C activator (combined with the APTT reagent where appropriate), all previously heated to 37 °C. Add 1 volume of *0.025 M calcium chloride solution R* previously heated to 37 °C, and record the clotting time.

The clotting time is proportional to the concentration of human protein C in each dilution. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

**07/2008:20731**

## 2.7.31. ASSAY OF HUMAN PROTEIN S

Human protein S is a vitamin K-dependent plasma protein that acts as a cofactor for the anticoagulant functions of activated protein C (APC). Human protein S activity may be determined by the clotting assay described below, which is sensitive to the ability of human protein S to accelerate the inactivation of factor Va by APC. In practice, the assay involves the addition of human protein S to a reagent mixture containing APC, factor Va and human protein S-deficient plasma. Prolongation of the clotting time is proportional to the concentration of human protein S in the preparation. Methods in which APC is added directly as a reagent are preferred to those in which APC is generated during the assay by the addition of a specific human protein C activator purified from snake venom. Activation of coagulation is initiated by the addition of an activating reagent such as thromboplastin or activated factor X, together with phospholipids and calcium chloride. During the assay, factor Va is generated from factor V in the human protein S-deficient plasma following the activation of coagulation. The assay procedure must ensure that human protein S is the only limiting factor.

The potency of human protein S is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein S calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein S. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

REAGENTS

*Dilution buffer pH 7.4.* Isotonic non-chelating buffer prepared as follows: dissolve 6.08 g of *tris(hydroxymethyl)aminomethane R* and 8.77 g of *sodium chloride R* in *water R* and adjust the pH if necessary; add 10 g of *bovine albumin R* or *human albumin R* and dilute to 1000.0 mL with *water R*.

*Human protein S-deficient plasma.* Citrated human plasma with no measurable human protein S content and, preferably, also free of C4b-binding protein.

*Coagulation activator.* This reagent is used to initiate coagulation in the human protein S-deficient plasma, and thereby also provides a source of activated factor V. The activator may consist of tissue factor, activated factor X, or an agent capable of directly activating factor X that may be purified from the venom of Russell's viper (*Vipera russelli*). The reagent also contains APC, phospholipids and *calcium chloride R*, or, alternatively, calcium chloride may be added separately after a timed activation period.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.020-0.100 IU/mL, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein S-deficient plasma, both previously heated to 37 °C. Add 2 volumes of the coagulation activator, previously heated to 37 °C, and record the clotting time.

Alternative procedures may use a coagulation activator without calcium chloride, and require a precisely timed activation period before the addition of calcium chloride and the measurement of clotting time.

The clotting time is proportional to the concentration of human protein S in each dilution. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

**07/2008:20732**

## 2.7.32. ASSAY OF HUMAN α-1-PROTEINASE INHIBITOR

Human α-1-proteinase inhibitor (also known as α-1-antitrypsin or α-1-antiproteinase) content is determined by comparing the ability of the preparation to be examined to inactivate the serine protease elastase (porcine pancreatic elastase or human neutrophil elastase) with the same ability of a reference standard of human α-1-proteinase inhibitor calibrated in milligrams of active (functional) α-1-proteinase inhibitor. Varying quantities of the preparation to be examined are mixed with a given quantity of elastase and the remaining elastase activity is determined using a suitable chromogenic substrate. The method described below is given as an example.

REAGENTS

*Tris-albumin buffer solution.* Dissolve 24.23 g of *trometamol R* in *water R*, adjust to pH 8.0 ± 0.3 using *hydrochloric acid R1* and dilute to 1000 mL with *water R*. To 100 mL of this solution add 0.5 mL of a 20 per cent solution of *human albumin R* or *bovine albumin R*.

Buffer solution containing human or bovine albumin must be prepared fresh on the day of its use; otherwise, it can be conserved by sterile filtration (0.2 µm) and stored at 2-8 °C for up to 2 weeks.

METHOD

Prepare 2 series of 4 or 5 dilutions in an appropriate human α-1-proteinase inhibitor concentration range, for both the preparation to be examined and the reference standard, using the tris-albumin buffer solution.

Transfer 50 µL of the reference solution dilutions into the wells of a microtitre plate and to each well, add 150 µL of a porcine pancreatic elastase solution diluted to an appropriate concentration with the tris-albumin buffer solution. Incubate for a defined period of time, 3-10 min, at room temperature. Since the activity of the solutions of the different porcine pancreatic elastases may vary, the concentration of elastase can be adjusted by evaluation of blank values containing elastase but no human α-1-proteinase inhibitor, to exhibit a suitable change of absorbance at 405 nm under the actual assay conditions.

Add to each well 100 µL of a solution of chromogenic substrate *N*-succinyl-tri-L-alanyl 4-p-nitroanilide (Suc-Ala-Ala-Ala-pNA), reconstituted in *dimethyl sulfoxide R* to give a solution containing 4.5 mg/mL, then further diluted with the tris-albumin buffer solution to a concentration of 0.45 mg/mL. Immediately start measurement of the change in absorbance (*2.2.25*) at 405 nm using a microtitre plate reader, continuing the measurement for at least 5 min. Calculate the rate of change of absorbance (ΔA/min). Alternatively, an end-point assay may be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm. If the

assay is performed in test tubes using spectrophotometers for monitoring the change in absorbance at 405 nm, the volumes of reagent solutions are changed proportionally.

The rate of change of absorbance (ΔA/min) is inversely proportional to human α-1-proteinase inhibitor activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (*5.3*).

**04/2016:20734**

## 2.7.34. ASSAY OF HUMAN C1-ESTERASE INHIBITOR

The human C1-esterase inhibitor content of the preparation to be examined is determined by comparing its ability to inhibit C1-esterase with that of a reference preparation calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human plasma-derived C1-esterase inhibitor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization. Varying quantities of the preparation to be examined are mixed with an excess of C1-esterase and the remaining C1-esterase activity is determined using a suitable chromogenic substrate.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

Reconstitute the preparation as stated on the label. Prepare an appropriate series of at least 3 dilutions, from 1 IU/mL of C1-esterase inhibitor, for both the preparation to be examined and the reference preparation, using a suitable pH 7.4 buffer solution containing 9 g/L of *sodium chloride R* and either 10 g/L of *human albumin R* or 10 g/L of *bovine albumin R*. Warm all solutions to 37 °C. Place a suitable quantity of 1 of the dilutions of the reference preparation or of the preparation to be examined in microtitre plate wells and incubate at 37 °C. To each well add a suitable quantity of C1-esterase solution and incubate at 37 °C for 5 min. Add an appropriate quantity of a suitable specific chromogenic substrate such as methoxycarbonyl-L-lysyl(ε-benzyloxycarbonyl)-glycyl-L-arginine-4-nitroanilide. Read the rate of increase of absorbance (Δ*A*/min) at 405 nm. A positive control, using the pH 7.4 buffer solution instead of the C1-esterase inhibitor, is included. For preparations that may exhibit proteolytic activity, a test is carried out on a suitable blank composed of the preparation to be examined, the pH 7.4 buffer solution and the chromogenic substrate.

Calculate the C1-esterase inhibitor content using the usual statistical methods, for example slope ratio (*5.3*).

**01/2020:20735**

## 2.7.35. IMMUNONEPHELOMETRY FOR VACCINE COMPONENT ASSAY

GENERAL PRINCIPLE

The general principle of nephelometry is described in general chapter *2.2.1. Clarity and degree of opalescence of liquids*. Immunonephelometry measures turbidity that is mainly due to immune complexes formed by the antigen-antibody reaction. Although immunonephelometry is applicable for the quantification of both antibodies and antigens, this general chapter describes the quantification of antigens as vaccine components.

APPARATUS

The nephelometer used is described in general chapter *2.2.1. Clarity and degree of opalescence of liquids*. However the measurement may be made at a non-zero angle different from 90°.

METHODS

In general, the progress of the reaction measured by light scattering can be described in 3 steps.

Initially, a baseline level of light scattering due to the reaction medium is detected. After the 1st reagent (antigen) is added, an increase in the signal is observed followed by a plateau. When the 2nd reagent (antibody) is added, a 2nd increase of the signal is observed with a 2nd plateau followed by a final increase in the intensity of the signal, which continues until a 3rd plateau is reached. The measurement zone starts from the addition of the 2nd reagent until the higher intensity of light scattering (the 3rd plateau) is reached depending on the concentrations of the component to be assayed.

The following methods can be used to quantify the immune complexes formed during this type of reaction.

*END-POINT NEPHELOMETRY*

The light scattering is measured after the immune complex has formed, i.e. after about 60 min. The value of the end-point is in this case directly proportional to the content of the component to be assayed when an excess of antibodies is used. A blank is necessary in order to subtract the value of nonspecific scattering due to the reaction mixture and to the reaction/measurement cell.

*RATE NEPHELOMETRY*

Rate nephelometry is based on the rate of immune complex formation (rate of increase in light scattering), which is proportional to the concentration of the component to be assayed.

There are 2 types of rate nephelometry.

**Fixed-time rate nephelometry**. A 1st measurement of light scattering is carried out a few seconds after the last reagent has been added. A 2nd measurement is carried out after a fixed time interval established during development of the method. The difference between the 2 values is proportional to the quantity of the component to be assayed.

**Rate nephelometry using the peak of the first derivative**. The formation of the immune complex is described by the curve of the first derivative of the variation in light scattering with time ($d\Theta_D/dt$). Two successive peaks are observed due to the addition of the 1st and then the 2nd reagent.

The height of the 3rd peak of the first derivative is proportional to the quantity of component to be assayed in the reaction medium. Successive measurements are carried out a few seconds after the 2nd reagent has been added.

In this case, the light scattered by the reaction medium, the component and the reagent does not affect the initial value of the derived signal.

OPERATING CONDITIONS

*REAGENTS AND REFERENCE STANDARDS*

The reagents, diluents and samples to be examined must be clear and free from any suspended particles. To this end, a preliminary filtration or centrifugation step may be considered.

The antisera or antibodies are selected based on their specificity, their precipitating capacity and their avidity for the antigen. Indeed, high avidity results in clearly detectable signals being generated rapidly, which thus improves the quality of the results.

In addition, it is important to define the range of antigen concentrations to be assayed with an excess of antibodies and to verify the absence of a zone effect in this range.

It is recommended to ensure that the profiles of response will be similar to that of an appropriate reference standard.

*PRE-TREATMENT OF THE SAMPLES TO BE EXAMINED*

An appropriate pre-treatment could be envisaged to eliminate an adjuvant or another interfering substance.

*ASSAY*

Ensure that all the reagents are at a suitable temperature to optimise the reaction.

To the samples diluted with a suitable medium in the reaction measurement cell, add a fixed quantity of antibodies. Carry out several determinations of the dilution being examined.

In some cases, the progress of the reaction may be improved by introducing additional substances into the reaction medium (e.g. polyethylene glycol).

The reference standards and samples must be treated in the same manner.

*PERFORMANCE OF A BLANK TEST*

To ensure that the assays are specific, only the scattering due to immune complexes must be measured. Other particles, the matrix of the sample, the reagents or the reaction measurement cell may interfere. In this case and if the end-point method is used, it is necessary to carry out a blank test to avoid taking non-specific scattering into account.

*CALIBRATION*

The calibration curve is obtained by preparing a set of dilutions of a reference standard. The concentrations are chosen in order to encompass the working range.

*METHODS OF CALCULATION*

The parallel line method (see general chapter *5.3*) or a calibration curve is used for the calculations.

VALIDITY OF THE TESTS

*VALIDITY OF THE CALIBRATION CURVES*

Only calibration curves that comply with the defined validity criteria may be used.

The following parameters are examples to be used to define validity criteria:

- minimum of light scattering units (LSU) obtained for the lowest concentration point of the calibration curve;
- coefficient of variation for the LSU measured for each point on the curve;
- coefficients of regression or of correlation of the calibration curve;
- slope and intercept of the calibration curve.

*VALIDITY OF THE RESULTS*

The following criteria are examples of validity criteria of the samples to be assayed:

- coefficient of variation or the range of values for the determinations carried out on the sample;
- assay of an in-house reference standard with trend monitoring or a control chart.

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# 2.8. METHODS IN PHARMACOGNOSY

01/2008:20801

## 2.8.1. ASH INSOLUBLE IN HYDROCHLORIC ACID

Ash insoluble in hydrochloric acid is the residue obtained after extracting the sulfated or total ash with hydrochloric acid, calculated with reference to 100 g of drug.

To the crucible containing the residue from the determination of sulfated or total ash, add 15 mL of *water R* and 10 mL of *hydrochloric acid R*, cover with a watch-glass, boil the mixture gently for 10 min and allow to cool. Filter through an ashless filter, wash the residue with hot *water R* until the filtrate is neutral, dry, ignite to dull redness, allow to cool in a desiccator and weigh. Reheat until the difference between 2 consecutive weighings is not more than 1 mg.

04/2014:20802

## 2.8.2. FOREIGN MATTER

Herbal drugs should be free from moulds, insects and other animal contamination.

Foreign matter is material consisting of any or all of the following:

1) *foreign organs*: matter coming from the source plant but not defined as the herbal drug;

2) *foreign elements*: matter not coming from the source plant and of either vegetable or mineral origin.

DRIED PLANTS

**Sampling and sample preparation**. Apply general chapter *2.8.20. Herbal drugs: sampling and sample preparation*.

**Determination of foreign matter**. Weigh 100-500 g of the sample, or the minimum quantity prescribed in the individual monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

FRESH PLANTS

Where general chapter *2.8.20. Herbal drugs: sampling and sample preparation* cannot be applied, use one of the following methods, as appropriate: use method A when the test can be carried out on the whole batch; use method B when the test cannot be carried on the whole batch.

*METHOD A*

**Sampling and sample preparation**. Carry out the test on the whole batch.

**Determination of foreign matter**. Spread the batch out in a thin layer and examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

*METHOD B*

**Sampling and sample preparation**. If it is not possible to inspect the whole batch, proceed as follows.

*Bulk sample*. Prepare the bulk sample as described in general chapter *2.8.20. Herbal drugs: sampling and sample preparation*.

*Test sample*. Use the bulk sample or, where the bulk sample is greater than 1 kg, reduce it to a mass of 500-1000 g by a suitable method which retains the representative nature of the bulk sample.

**Determination of foreign matter**. Use the sample, or the minimum quantity prescribed in the individual monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

01/2008:20803

## 2.8.3. STOMATA AND STOMATAL INDEX

STOMATA

There are several types of stomata (see Figure 2.8.3.-1), distinguished by the form and arrangement of the surrounding cells:

(1) The *anomocytic* (irregular-celled) type: the stoma is surrounded by a varying number of cells in no way differing from those of the epidermis generally,

(2) The *anisocytic* (unequal-celled) type: the stoma is usually surrounded by 3 subsidiary cells, of which one is markedly smaller than the others,

(3) The *diacytic* (cross-celled) type: the stoma is accompanied by 2 subsidiary cells, whose common wall is at right angles to the guard cells,

(4) The *paracytic* (parallel-celled) type: the stoma has on each side one or more subsidiary cells parallel to the long axis of the pore and guard cells.

STOMATAL INDEX

$$\text{Stomatal Index} = \frac{100 \times S}{E + S}$$

S = the number of stomata in a given area of leaf,

E = the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of leaf, make not fewer than 10 determinations and calculate the mean.

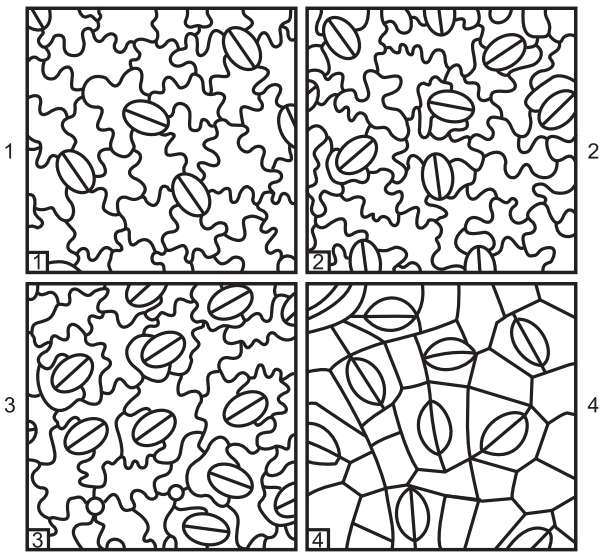


Figure 2.8.3.-1

01/2008:20804

## 2.8.4. SWELLING INDEX

The swelling index is the volume in millilitres occupied by 1 gram of a drug, including any adhering mucilage, after it has swollen in an aqueous liquid for 4 h.

In a 25 mL ground-glass stoppered cylinder graduated over a height of 125 ± 5 mm in 0.5 mL divisions, place 1.0 g of the drug, whole or of the degree of comminution prescribed in the monograph. Unless otherwise prescribed, moisten the drug with 1.0 mL of *alcohol R*, add 25 mL of *water R* and close the cylinder. Shake vigorously every 10 min for 1 h. Allow to stand for 3 h. At 90 min after the beginning of the test, release any large volumes of liquid retained in the layer of the drug and any particles of the drug floating at the surface of the liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the drug, including any adhering mucilage. Carry out 3 tests at the same time.

The swelling index is given by the mean of the 3 tests.

01/2008:20805

## 2.8.5. WATER IN ESSENTIAL OILS

Mix 10 drops of the essential oil with 1 mL of *carbon disulfide R*. The solution remains clear on standing.

01/2008:20806

## 2.8.6. FOREIGN ESTERS IN ESSENTIAL OILS

Heat 1 mL of the essential oil for 2 min on a water-bath with 3.0 mL of a freshly prepared 100 g/L solution of *potassium hydroxide R* in *alcohol R*. No crystals are formed within 30 min, even after cooling.

01/2008:20807

## 2.8.7. FATTY OILS AND RESINIFIED ESSENTIAL OILS IN ESSENTIAL OILS

Allow 1 drop of the essential oil to fall onto filter paper. The drop evaporates completely within 24 h without leaving any translucent or greasy spot.

01/2008:20808

## 2.8.8. ODOUR AND TASTE OF ESSENTIAL OILS

Mix 3 drops of the essential oil with 5 mL of *alcohol (90 per cent V/V) R* and stir in 10 g of powdered *sucrose R*. The odour and taste are similar to that of the plant or parts of the plant from which the essential oil has been obtained.

01/2008:20809
corrected 10.0

## 2.8.9. RESIDUE ON EVAPORATION OF ESSENTIAL OILS

The residue on evaporation of an essential oil is the percentage by mass of the oil which remains after evaporation on a water-bath under the conditions specified below.

*Apparatus.* The apparatus (see Figure 2.8.9.-1) consists of:
- water-bath with a cover having holes of 70 mm diameter;
- evaporating dish of heat-resistant glass which is inert to the contents;
- desiccator.

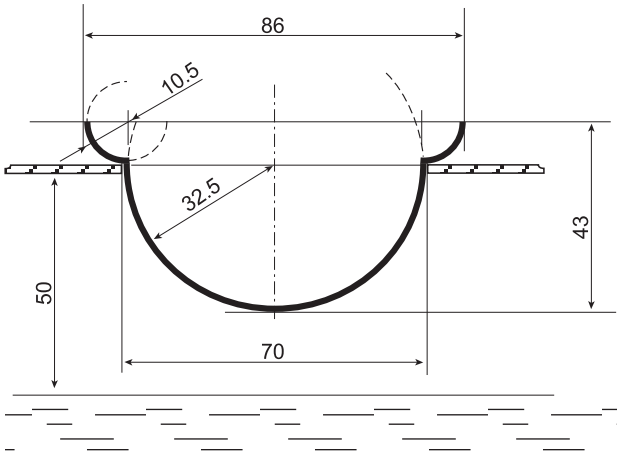


Figure 2.8.9.-1
*Dimensions in millimetres*

*Method.* Weigh the evaporating dish after having heated it on the water-bath for 1 h and cooled it in the desiccator. Weigh into the evaporating dish 5.00 g of the essential oil, unless otherwise prescribed. Evaporate the oil by heating on a water-bath in a draught-free atmosphere for the prescribed time. Allow to cool in the desiccator and weigh.

During the test, the level of water in the bath is maintained about 50 mm beneath the level of the cover.

01/2008:20810

## 2.8.10. SOLUBILITY IN ALCOHOL OF ESSENTIAL OILS

Place 1.0 mL of the essential oil in a 25 mL or 30 mL glass-stoppered cylinder. Place in a constant temperature device, maintained at a temperature of 20 ± 0.2 °C. Using a burette of at least 20 mL capacity, add the alcohol of the strength prescribed in the monograph by increments of 0.1 mL until solution is complete and then continue adding by increments of 0.5 mL to a total of 20 mL, shaking frequently and vigorously. Record the volume of alcohol added when a clear solution has been obtained and, if the solution becomes cloudy or opalescent before 20 mL of alcohol has been added, record the volume added when the cloudiness or opalescence appears and, where applicable, the volume added when the cloudiness or opalescence disappears.

If a clear solution has not been obtained when 20 mL of alcohol of the prescribed strength has been added, repeat the test using the next highest concentration of alcohol.

An essential oil is said to be "soluble in *n* volumes and more of alcohol of given strength *t*" when the clear solution in *n* volumes remains clear when compared with the undiluted oil after further addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *n* volumes of alcohol of given strength *t*, becoming cloudy when diluted" when the clear solution in *n* volumes becomes cloudy in $n_1$ volumes ($n_1$ less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *n* volumes of alcohol of given strength *t* with cloudiness between $n_1$ and $n_2$ volumes" when the clear solution in *n* volumes becomes cloudy in $n_1$ volumes ($n_1$ less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of $n_2$ volumes of alcohol and then becomes clear ($n_2$ less than 20).

An essential oil is said to be "soluble with opalescence" when the alcoholic solution shows a bluish tinge, similar to that of a standard of opalescence freshly prepared as follows: mix 0.5 mL of *silver nitrate solution R2* and 0.05 mL of *nitric acid R*; add 50 mL of a 12 mg/L solution of *sodium chloride R*; mix and allow to stand protected from light for 5 min.

**01/2008:20811**

## 2.8.11. ASSAY OF 1,8-CINEOLE IN ESSENTIAL OILS

Weigh 3.00 g of the oil, recently dried with *anhydrous sodium sulfate R*, into a dry test-tube and add 2.10 g of melted *cresol R*. Place the tube in the apparatus for the determination of freezing point (*2.2.18*) and allow to cool, stirring continuously. When crystallisation takes place there is a small rise in temperature. Note the highest temperature reached ($t_1$).

Remelt the mixture on a water-bath at a temperature that does not exceed $t_1$ by more than 5 °C and place the tube in the apparatus, maintained at a temperature 5 °C below $t_1$. When crystallisation takes place, or when the temperature of the mixture has fallen 3 °C below $t_1$, stir continuously. Note the highest temperature at which the mixture crystallises ($t_2$). Repeat the operation until 2 highest values obtained for $t_2$ do not differ by more than 0.2 °C. If supercooling occurs, induce crystallisation by adding a small crystal of the complex consisting of 3.00 g of *cineole R* and 2.10 g of melted *cresol R*. If $t_2$ is below 27.4 °C, repeat the determination after the addition of 5.10 g of the complex.

The content of cineole corresponding to the highest temperature observed ($t_2$) is given in Table 2.8.11.-1. If 5.10 g of the complex has been added, calculate the cineole content per cent *m/m* from the expression:

$$2(A - 50)$$

where $A$ is the value found in Table 2.8.11.-1.

The content of cineole, corresponding to the highest temperature observed ($t_2$), is obtained, where necessary, by interpolation.

Table 2.8.11.-1

| $t_2$ °C | cineole per cent *m/m* | $t_2$ °C | cineole per cent *m/m* | $t_2$ °C | cineole per cent *m/m* | $t_2$ °C | cineole per cent *m/m* |
|---|---|---|---|---|---|---|---|
| 24 | 45.5 | 32 | 56.0 | 40 | 67.0 | 48 | 82.0 |
| 25 | 47.0 | 33 | 57.0 | 41 | 68.5 | 49 | 84.0 |
| 26 | 48.5 | 34 | 58.5 | 42 | 70.0 | 50 | 86.0 |
| 27 | 49.5 | 35 | 60.0 | 43 | 72.5 | 51 | 88.5 |
| 28 | 50.5 | 36 | 61.0 | 44 | 74.0 | 52 | 91.0 |
| 29 | 52.0 | 37 | 62.5 | 45 | 76.0 | 53 | 93.5 |
| 30 | 53.5 | 38 | 63.5 | 46 | 78.0 | 54 | 96.0 |
| 31 | 54.5 | 39 | 65.0 | 47 | 80.0 | 55 | 99.0 |

**07/2019:20812**

## 2.8.12. ESSENTIAL OILS IN HERBAL DRUGS

PRINCIPLE

The determination of essential oils in herbal drugs is carried out by steam distillation in a special apparatus under the conditions described below. The distillate is collected in the graduated tube using the solvent prescribed in the monograph (usually *xylene R*, *trimethylpentane R* or *1,2,4-trimethylbenzene R*) to take up the essential oil; the aqueous phase is automatically returned to the distillation flask.

EQUIPMENT

The equipment typically consists of:

- a suitable round-bottomed flask with a short, ground-glass neck that has an internal diameter of about 29 mm at the wide end;
- a condenser assembly (see Figure 2.8.12.-1) that closely fits the flask, the different parts being fused into 1 piece and made of glass with a low coefficient of thermal expansion:
  - a vented stopper (*K'*) with an opening about 1 mm in diameter, and a tube (*K*), the wide end of which is made of ground-glass and has an internal diameter of 10 mm;
  - a pear-shaped swelling (*J*) with a capacity of 3 mL;
  - a tube (*JL*) graduated in 0.01 mL;
  - a bulb-shaped swelling (*L*) with a capacity of about 2 mL;
  - a 3-way tap (*M*);
  - a junction (*B*) situated at a level 20 mm higher than the uppermost graduation of tube *JL*;
- a suitable heating device, allowing fine temperature control;
- a vertical support with a horizontal ring covered with insulating material.

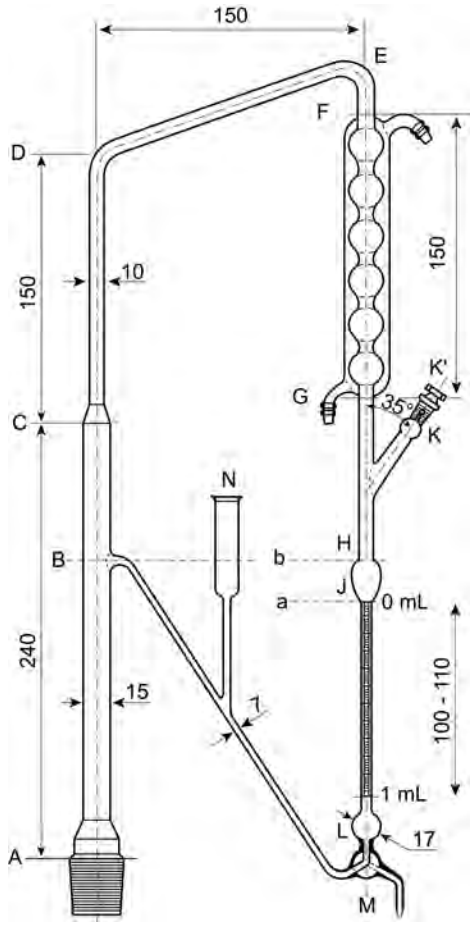


Figure 2.8.12.-1. – *Apparatus for the determination of essential oils in herbal drugs*

*Dimensions in millimetres*

PROCEDURE

Use a thoroughly cleaned apparatus. Carry out the determination according to the nature of the herbal drug to be examined. Place the prescribed volume of distillation liquid in the flask, add a few pieces of porous porcelain and attach the condenser assembly. Introduce *water R* through the filling funnel (*N*) until it reaches level *B*. Remove the stopper (*K'*) and introduce the prescribed quantity of the solvent indicated in the monograph using a pipette with its tip placed at the bottom of tube *K*. Re-insert the stopper (*K'*) and ensure that the vent is unobstructed. Heat the liquid in the flask to boiling and adjust the distillation rate to 2-3 mL/min, unless otherwise prescribed.

**Determination of the rate of distillation**. During distillation, determine the rate of distillation by lowering the level of the water by means of tap *M* until the meniscus reaches the level of the lower mark (*a*) (see Figure 2.8.12.-2). Close tap *M* and measure the time taken for the liquid to reach the upper mark (*b*). Modify the heat to obtain the target distillation rate. If the distillation rate is still not within the prescribed range, repeat the operation. If the distillation conditions are not changed, it is sufficient to determine the distillation rate at regular intervals rather than before each test.

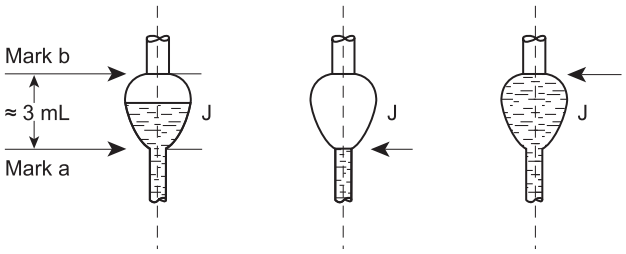


Figure 2.8.12.-2

**Determination of the solvent volume after blank distillation**. If using *xylene R* or *trimethylpentane R*, distil for 30 min. Ensure that tubes *BM* and *JM* are connected via tap *M* during distillation. Stop heating and wait at least 10 min before reading the volume of solvent in the graduated tube.

If using *1,2,4-trimethylbenzene R*, the 30 min blank distillation step is not necessary. Stop heating after adjusting the distillation rate and wait at least 10 min before reading the volume of solvent in the graduated tube.

**Determination of the essential oil in the herbal drug**. Introduce the prescribed quantity of the herbal drug into the flask and continue distillation as described above for the time and at the rate prescribed. Stop heating, read the volume of liquid collected in the graduated tube after 10 min and subtract the volume of the solvent previously noted. The difference represents the quantity of essential oil in the sample. Calculate the result in millilitres per kilogram of herbal drug.

**Recovery of the mixture of solvent and essential oil**. When the essential oil is to be used for other analytical purposes, the water-free mixture of solvent and essential oil may be recovered as follows: remove the stopper (*K'*) and introduce 0.1 mL of a 1 g/L solution of *sodium fluoresceinate R* and 0.5 mL of *water R*. Run the mixture of solvent and essential oil into the bulb-shaped swelling (*L*) by opening tap *M*, allow to stand for 5 min and lower the level of the mixture slowly until it just reaches the level of tap *M*. Open tap *M* clockwise so that the water flows out of the connecting tube (*BM*). Wash the tube with *acetone R* introduced through the filling funnel (*N*). Turn tap *M* anti-clockwise in order to recover the mixture of solvent and essential oil in an appropriate flask.

**01/2019:20813**

# 2.8.13. PESTICIDE RESIDUES

**Definition**. For the purposes of the Pharmacopoeia, a pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of herbal drugs. The item includes substances intended for use as growth-regulators, defoliants or desiccants and any substance applied to crops, either before or after harvest, to protect the commodity from deterioration during storage and transport. Pesticide residues can be present and are controlled in herbal drugs and herbal drug preparations.

**Limits**. Unless otherwise indicated in the monograph, the herbal drug to be examined at least complies with the limits indicated in Table 2.8.13.-1. The limits applying to pesticides that are not listed in Table 2.8.13.-1 and whose presence is suspected for any reason comply with the limits (levels) cross-referred to by Regulation (EC) No. 396/2005, including

annexes and successive updates. Limits for pesticides that are not listed in Table 2.8.13.-1 or in European Union texts are calculated using the following expression:

$$\frac{ADI \times M}{MDD_{HD} \times 100}$$

*ADI* = acceptable daily intake, as published by FAO-WHO, in milligrams per kilogram of body mass;

*M* = body mass in kilograms (60 kg);

$MDD_{HD}$ = daily dose of the herbal drug, in kilograms.

The limits for pesticides in herbal drug preparations are calculated using the following expressions:

If *DER* ≤ 10: $MRL_{HD} \times DER$

If *DER* > 10: $\frac{ADI \times M}{MDD_{HP} \times 100}$

$MRL_{HD}$ = maximum residue limit of the pesticide in the herbal drug as given in Table 2.8.13.-1 or in EU texts or calculated using the expression mentioned above;

*DER* = drug/extract ratio, i.e. the ratio between the quantity of herbal drug used in the manufacture of a herbal drug preparation and the quantity of herbal drug preparation obtained;

$MDD_{HP}$ = daily dose of the herbal drug preparation, in kilograms.

The competent authority may grant total or partial exemption from the test when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after the harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

Table 2.8.13.-1

| Substance | Limit (mg/kg) |
|---|---|
| Acephate | 0.1 |
| Alachlor | 0.05 |
| Aldrin and dieldrin (sum of) | 0.05 |
| Azinphos-ethyl | 0.1 |
| Azinphos-methyl | 1 |
| Bromophos-ethyl | 0.05 |
| Bromophos-methyl | 0.05 |
| Brompropylate | 3 |
| Chlordane (sum of *cis*-, *trans* - and oxychlordane) | 0.05 |
| Chlorfenvinphos | 0.5 |
| Chlorpyriphos-ethyl | 0.2 |
| Chlorpyriphos-methyl | 0.1 |
| Chlorthal-dimethyl | 0.01 |
| Cyfluthrin (sum of) | 0.1 |
| λ-Cyhalothrin | 1 |
| Cypermethrin and isomers (sum of) | 1 |
| DDT (sum of *o,p′*-DDE, *p,p′*-DDE, *o,p′*-DDT, *p,p′*-DDT, *o,p′*-TDE and *p,p′*-TDE) | 1 |
| Deltamethrin | 0.5 |
| Diazinon | 0.5 |
| Dichlofluanid | 0.1 |
| Dichlorvos | 1 |
| Dicofol | 0.5 |
| Dimethoate and omethoate (sum of) | 0.1 |
| Dithiocarbamates (expressed as CS2) | 2 |
| Endosulfan (sum of isomers and endosulfan sulfate) | 3 |
| Endrin | 0.05 |
| Ethion | 2 |
| Etrimphos | 0.05 |
| Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon) | 0.1 |
| Fenitrothion | 0.5 |
| Fenpropathrin | 0.03 |
| Fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon and fensulfothion-sulfon) | 0.05 |
| Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon-sulfon, fenthion-oxon-sulfoxid, fenthion-sulfon and fenthion-sulfoxid) | 0.05 |
| Fenvalerate | 1.5 |
| Flucytrinate | 0.05 |
| τ-Fluvalinate | 0.05 |
| Fonophos | 0.05 |
| Heptachlor (sum of heptachlor, *cis*-heptachlorepoxide and *trans*-heptachlorepoxide) | 0.05 |
| Hexachlorbenzene | 0.1 |
| Hexachlorocyclohexane (sum of isomers α-, β-, δ- and ε) | 0.3 |
| Lindan (γ-hexachlorocyclohexane) | 0.6 |
| Malathion and malaoxon (sum of) | 1 |
| Mecarbam | 0.05 |
| Methacriphos | 0.05 |
| Methamidophos | 0.05 |
| Methidathion | 0.2 |
| Methoxychlor | 0.05 |
| Mirex | 0.01 |
| Monocrotophos | 0.1 |
| Parathion-ethyl and paraoxon-ethyl (sum of) | 0.5 |
| Parathion-methyl and paraoxon-methyl (sum of) | 0.2 |
| Pendimethalin | 0.5 |
| Pentachloranisol | 0.01 |
| Permethrin and isomers (sum of) | 1 |
| Phosalone | 0.1 |
| Phosmet | 0.05 |
| Piperonyl butoxide | 3 |
| Pirimiphos-ethyl | 0.05 |
| Pirimiphos-methyl (sum of pirimiphos-methyl and *N*-desethyl-pirimiphos-methyl) | 4 |
| Procymidone | 0.1 |
| Profenophos | 0.1 |
| Prothiophos | 0.05 |

| Substance | Limit (mg/kg) |
|---|---|
| Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I and pyrethrin II) | 3 |
| Quinalphos | 0.05 |
| Quintozene (sum of quintozene, pentachloraniline and methyl penthachlorphenyl sulfide) | 1 |
| S-421 | 0.02 |
| Tecnazene | 0.05 |
| Tetradifon | 0.3 |
| Vinclozolin | 0.4 |

**Sampling of herbal drugs**. Sampling is done according to general chapter *2.8.20. Herbal drugs: sampling and sample preparation*.

**Qualitative and quantitative analysis of pesticide residues**. The analytical procedures used are validated (e.g. according to Document No. SANCO/10232/2006 or any subsequent revisions of this document). In particular, they satisfy the following criteria:

- the chosen method, especially the purification steps, is suitable for the combination pesticide residue/substance to be examined, and not susceptible to interference from co-extractives;
- natural occurrence of some constituents is considered in the interpretation of results (e.g. disulfide from crucifers);
- the concentration of test and reference solutions and the setting of the apparatus are such that the responses used for quantification of the pesticide residues are within the dynamic range of the detector; test solutions containing pesticide residues at a level outside the dynamic range, may be diluted within the calibration range, provided that the concentration of the matrix in the solution is adjusted in the case where the calibration solutions must be matrix-matched;
- between 70 per cent to 110 per cent of each pesticide is recovered;
- repeatability of the method: RSD is not greater than the values indicated in Table 2.8.13.-2;
- reproducibility of the method: RSD is not greater than the values indicated in Table 2.8.13.-2.

Table 2.8.13.-2

| Concentration range of the pesticide (mg/kg) | Repeatability (RSD) (per cent) | Reproducibility (RSD) (per cent) |
|---|---|---|
| 0.001 - 0.01 | 30 | 60 |
| > 0.01 - 0.1 | 20 | 40 |
| > 0.1 - 1 | 15 | 30 |
| > 1 | 10 | 20 |

**01/2008:20814**

## 2.8.14. TANNINS IN HERBAL DRUGS

*Carry out all the extraction and dilution operations protected from light.*

In the case of a herbal drug or a dry extract, to the stated amount of the powdered drug (180) (*2.9.12*) or the extract in a 250 mL round-bottomed flask add 150 mL of *water R*. Heat on a water-bath for 30 min. Cool under running water and transfer quantitatively to a 250 mL volumetric flask. Rinse the round-bottomed flask and collect the washings in the volumetric flask, then dilute to 250.0 mL with *water R*. Allow the solids to settle and filter the liquid through a filter paper 125 mm in diameter. Discard the first 50 mL of the filtrate.

In the case of a liquid extract or a tincture, dilute the stated amount of the liquid extract or tincture to 250.0 mL with *water R*. Filter the mixture through a filter paper 125 mm in diameter. Discard the first 50 mL of the filtrate.

*Total polyphenols*. Dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm ($A_1$), using *water R* as the compensation liquid.

*Polyphenols not adsorbed by hide powder*. To 10.0 mL of the filtrate, add 0.10 g of *hide powder CRS* and shake vigorously for 60 min. Filter and dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm ($A_2$), using *water R* as the compensation liquid.

*Standard*. Dissolve immediately before use 50.0 mg of *pyrogallol R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm ($A_3$), using *water R* as the compensation liquid.

Calculate the percentage content of tannins expressed as pyrogallol from the expression:

$$\frac{62.5(A_1 - A_2)m_2}{A_3 \times m_1}$$

$m_1$ = mass of the sample to be examined, in grams;

$m_2$ = mass of pyrogallol, in grams.

**01/2008:20815**

## 2.8.15. BITTERNESS VALUE

The bitterness value is the reciprocal of the dilution of a compound, a liquid or an extract that still has a bitter taste. It is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200 000.

*Determination of the correction factor*

A taste panel comprising at least 6 persons is recommended. The mouth must be rinsed with *water R* before tasting.

To correct for individual differences in tasting bitterness amongst the panel members it is necessary to determine a correction factor for each panel member.

*Stock solution*. Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Reference solutions*. Prepare a series of dilutions by placing in a first tube 3.6 mL of the stock solution and increasing the volume by 0.2 mL in each subsequent tube to a total of 5.8 mL; dilute the contents of each tube to 10.0 mL with *water R*.

Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 mL of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the correction factor *k* for each panel member from the expression:

$$k = \frac{n}{5.00}$$

*n* = number of millilitres of the stock solution in the dilution of lowest concentration that is judged to be bitter.

Persons who are unable to taste any bitterness when using the reference solution prepared from 5.8 mL of stock solution have to be excluded from the panel.

*Sample preparation*

If necessary, reduce the sample to a powder (710) (*2.9.12*). To 1.0 g of sample add 100 mL of boiling *water R*. Heat on a water-bath for 30 min, stirring continuously. Allow to cool and dilute to 100 mL with *water R*. Shake vigorously and filter, discarding the first 2 mL of the filtrate. The filtrate is labelled C-1 and has a dilution factor (DF) of 100.

If liquids have to be examined, 1 mL of the liquid is diluted with a suitable solvent to 100 mL and designated C-1.

*Determination of the bitterness value*

Test solutions:

10.0 mL of C-1 is diluted with *water R* to 100 mL: C-2 (DF = 1000)

10.0 mL of C-2 is diluted with *water R* to 100 mL: C-3 (DF = 10 000)

20.0 mL of C-3 is diluted with *water R* to 100 mL: C-3A (DF = 50 000)

10.0 mL of C-3 is diluted with *water R* to 100 mL: C-4 (DF = 100 000)

Starting with dilution C-4 each panel member determines the dilution which still has a bitter taste. This solution is designated D. Note the DF of solution D is Y.

Starting with solution D prepare the following sequence of dilutions:

| Solution D (mL) | 1.2 | 1.5 | 2.0 | 3.0 | 6.0 | 8.0 |
|---|---|---|---|---|---|---|
| *water R* (mL) | 8.8 | 8.5 | 8.0 | 7.0 | 4.0 | 2.0 |

Determine the number of millilitres of solution D which, when diluted to 10.0 mL with *water R*, still has a bitter taste (X).

Calculate the bitterness value for each panel member from the expression:

$$\left(\frac{Y \times k}{X \times 0.1}\right)$$

Calculate the bitterness value of the sample to be examined as the average value for all panel members.

**01/2008:20816**

## 2.8.16. DRY RESIDUE OF EXTRACTS

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, introduce rapidly 2.00 g or 2.0 mL of the extract to be examined. Evaporate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage or in grams per litre.

**01/2008:20817**

## 2.8.17. LOSS ON DRYING OF EXTRACTS

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 0.50 g of the extract to be examined, finely powdered. Dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage.

**01/2008:20818**

## 2.8.18. DETERMINATION OF AFLATOXIN $B_1$ IN HERBAL DRUGS

*CAUTION: aflatoxins are very toxic and carcinogenic. Perform manipulations in a fume cupboard whenever possible. Take particular precautions, such as use of a glove box, when toxins are in dry form because of their electrostatic properties and the tendency to disperse through the working areas. Decontamination procedures for laboratory wastes of aflatoxins were developed by the International Agency for Research on Cancer (IARC).*

Aflatoxins are naturally occurring mycotoxins produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are common and widespread in nature and are most often found when certain grains are grown under conditions of stress such as drought. The mould occurs in soil, decaying vegetation, hay, and grains undergoing microbial spoilage, and invades all types of organic substrates whenever and wherever the conditions are favourable for its growth. Favourable conditions include high moisture content and high temperature. At least 13 different types of aflatoxin are produced in nature and most of these are known to be highly toxic and carcinogenic. Aflatoxin $B_1$ is considered the most toxic. Herbal drugs that are subject to contamination by aflatoxins are tested by a validated method.

Unless otherwise indicated in the monograph, herbal drugs contain not more than 2 µg/kg of aflatoxin $B_1$. The competent authority may also require compliance with a limit of 4 µg/kg for the sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.

The method described below is cited as an example of a method that has been shown to be suitable for devil's claw root, ginger and senna pods. Its suitability for other herbal drugs must be demonstrated or another validated method used.

METHOD

Liquid chromatography (*2.2.29*).

*Aflatoxins are subject to light degradation. Carry out the determination protected from daylight by using UV-absorbing foil on windows in combination with subdued light, or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable). Protect aflatoxin-containing solutions from daylight.*

Rinse glassware before use with a 10 per cent *V/V* solution of *sulfuric acid R* and then rinse carefully with *distilled water R* until no more acid is present.

*Test solution.* Use an immunoaffinity column containing antibodies against aflatoxin $B_1$ with a capacity of not less than 100 ng of aflatoxin $B_1$ and which gives a recovery of not less than 80 per cent when a solution of 5 ng of aflatoxin $B_1$ in a mixture of 12.5 mL of *methanol R* and 87.5 mL of *water R* is passed through. Allow the immunoaffinity column to reach

room temperature. To 5.00 g of the powdered drug (500) (*2.9.12*) add 100 mL of a mixture of 30 volumes of *water R* and 70 volumes of *methanol R* and extract by sonication for 30 min. Filter through folded filter paper. Pipette 10.0 mL of the clear filtrate into a 150 mL conical flask. Add 70 mL of *water R*. Pass 40 mL through the immunoaffinity column at a flow rate of 3 mL/min (not exceeding 5 mL/min). Wash the column with 2 volumes, each of 10 mL, of *water R* at a flow rate not exceeding 5 mL/min and dry by applying a slight vacuum for 5-10 s or by passing air through the immunoaffinity column by means of a syringe for 10 s. Apply 0.5 mL of *methanol R* to the column and allow to pass through by gravity. Collect the eluate in a 5 mL volumetric flask. After 1 min, apply a 2nd portion of 0.5 mL of *methanol R*. After a further 1 min, apply a 3rd portion of 0.5 mL of *methanol R*. Collect most of the applied elution solvent by pressing air through or applying vacuum to the column. Dilute to 5 mL with *water R* and shake well. If the solution is clear it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of aflatoxin by retention.

*Aflatoxin $B_1$ primary stock solution.* Dissolve *aflatoxin $B_1$ R* in a mixture of 2 volumes of *acetonitrile R* and 98 volumes of *toluene R* to give a 10 µg/mL solution. To determine the exact concentration of aflatoxin $B_1$ in the stock solution, record the absorption curve (*2.2.25*) between 330 nm and 370 nm in quartz cells.

Calculate the aflatoxin $B_1$ mass concentration, in micrograms per millilitre, using the following expression:

$$\frac{A \times M \times 100}{\varepsilon \times l}$$

$A$ = absorbance determined at the maximum of the absorption curve;

$M$ = molar mass of aflatoxin $B_1$ (312 g/mol);

$\varepsilon$ = molar absorptivity of aflatoxin $B_1$ in the toluene-acetonitrile mixture (1930 $m^2$/mol);

$l$ = optical path length of the cell (1 cm).

*Aflatoxin $B_1$ secondary stock solution.* Prepare a secondary stock solution containing 100 ng/mL aflatoxin $B_1$ by diluting aflatoxin $B_1$ primary stock solution with a mixture of 2 volumes of *acetonitrile R* and 98 volumes of *toluene R*. Wrap the flask tightly in aluminium foil and store it below 4 °C. Before use, do not remove the aluminium foil until the contents have reached room temperature. If the solution has to be stored for a long period (for example, 1 month), weigh the flask and record the mass before and after each use of the solution.

*Aflatoxin $B_1$ standard solutions.* Place the volumes of aflatoxin secondary stock solution indicated in Table 2.8.18.-1 in separate 250 mL volumetric flasks. Pass a stream of nitrogen through at room temperature until the solvent has just evaporated. To each flask, add 75 mL of *methanol R*, allow the aflatoxin $B_1$ to dissolve and dilute to 250 mL with *water R*.

Table 2.8.18.-1. – *Aflatoxin $B_1$ standard solutions*

| Standard solution | Volume of secondary stock solution (µL) | Final concentration of standard solution (ng/mL) |
|---|---|---|
| 1 | 125 | 0.05 |
| 2 | 250 | 0.1 |
| 3 | 500 | 0.2 |
| 4 | 750 | 0.3 |
| 5 | 1000 | 0.4 |

*Calibration curve.* Prepare the calibration curve using aflatoxin $B_1$ standard solutions 1 to 5, which cover a range equivalent to 1-8 µg/kg of aflatoxin $B_1$ in the herbal drug. Check the plot for linearity. If the content of aflatoxin $B_1$ in the sample to be examined is outside of the calibration range, the test solution must be diluted to an aflatoxin content that is appropriate for the established calibration curve.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A* (for post-column derivatisation with photochemical reactor or pyridinium bromide): *acetonitrile R*, *methanol R*, *water R* (2:3:6 *V/V/V*);
- *mobile phase B* (for post-column derivatisation with electrochemically derived bromine): add 0.12 g of *potassium bromide R* and 350 µL of *dilute nitric acid R1* per litre of mobile phase A.

*Flow rate*: 1 mL/min.

*Detection*: fluorescence detector with a 360 nm excitation filter and a 420 nm cut-off emission filter, or equivalent. Recommended settings for adjustable detectors are 365 nm (excitation wavelength) and 435 nm (emission wavelength).

*Injection*: 500 µL.

*Post-column derivatisation with pyridinium hydrobromide perbromide (PBPB)*:

- pulseless pump;
- T-piece with zero dead volume;
- polytetrafluoroethylene reaction tube, $l$ = 0.45 m, Ø = 0.5 mm;
- mobile phase A;
- post-column derivatisation reagent: dissolve 50 mg of *pyridinium hydrobromide perbromide R* in 1000 mL of *water R* (store protected from light and use within 4 days);
- flow rate of the derivatisation reagent: 0.4 mL/min.

*Post-column derivatisation with photochemical reactor (PHRED)*

- reactor unit with one 254 nm low pressure mercury UV bulb (minimum 8 W);
- polished support plate;
- knitted reactor coil: polytetrafluoroethylene tubing knitted tightly around the UV bulb, $l$ = 25 m, Ø = 0.25 mm, nominal void volume 1.25 mL;
- exposure time: 2 min;
- mobile phase A.

*Post-column derivatisation with electrochemically generated bromine (KOBRA)*:

- KOBRA-cell: electrochemical cell that generates a reactive form of bromine for derivatisation of aflatoxins, resulting in enhanced fluorescence; available from various commercial suppliers;
- Derivation direct-current supply in series with the KOBRA-cell, providing a constant current of about 100 µA;
- polytetrafluoroethylene reaction tube, $l$ = 0.12 m, Ø = 0.25 mm;
- mobile phase B.

*Elution order*: aflatoxin $G_2$, aflatoxin $G_1$, aflatoxin $B_2$, aflatoxin $B_1$.

*Calculation*: calculate the calibration curve $y = ax + b$, with aflatoxin $B_1$ concentration (ng/mL) on the $x$-axis and the signal ($S$) on the $y$-axis. The aflatoxin $B_1$ concentration ($C$) in a measured solution is equal to $\frac{S-b}{a}$.

Calculate the aflatoxin $B_1$ content of the herbal drug, in nanograms per gram, using the following expression:

$$\frac{V_1 \times V_2 \times C}{m \times V_i}$$

$m$ = mass of the herbal drug taken for analysis, in grams;

$V_1$ = volume of the solvent used for extraction, in millilitres;

$V_i$ = aliquot taken for immunoaffinity clean-up, in millilitres;

$V_2$ = final volume of solution after elution from the immunoaffinity column and dilution, in millilitres;

$C$ = measured aflatoxin $B_1$ concentration of the test solution, in nanograms per millilitre.

The presence of aflatoxin $B_1$ may be confirmed by recording the chromatogram without post-column derivatisation, which leads to a large decrease (greater than 10-fold) in the response due to aflatoxin $B_1$.

01/2008:20820

# 2.8.20. HERBAL DRUGS: SAMPLING AND SAMPLE PREPARATION

In order to reduce the effect of sampling in qualitative and quantitative analysis, it is necessary to ensure that the composition of the sample used is representative of the batch of material being examined. The following procedures are the minimum considered applicable for herbal drugs. *NOTE: other procedures may be used if they can be demonstrated to produce representative batch samples.*

## BULK SAMPLE

Where external examination of containers, markings and labels of a batch indicate that it can be considered to be homogeneous, sample the number of randomly selected containers indicated below. Where a batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each sub-batch as a homogeneous batch using, as a minimum, the number of randomly selected containers indicated below.

| Number of containers in batch (*N*) | Number of containers to be sampled (*n*) |
|---|---|
| 1 - 3 | all |
| > 3 | $n^* = \sqrt{N} + 1$ |

* round *n* up to the next integer

Take one sample from each container to be sampled. The sample is taken from the upper, middle or lower section of the container, such that the samples taken are representative of different parts of the containers. In the case of large bales or bags, samples must be taken from a depth of at least 10 cm. The mass of the material taken from each container is such that the total mass of the bulk sample complies with the following values.

| Mass of herbal drug in the batch (kg) | Minimum mass of samples as a percentage of the mass of the batch of herbal drug |
|---|---|
| < 50 | 1.00* |
| 50 - 100 | 0.50 |
| > 100 - 250 | 0.25 |
| > 250 - 500 | 0.20 |
| > 500 - 1000 | 0.18 |
| > 1000 - 2500 | 0.15 |
| > 2500 - 5000 | 0.10 |
| > 5000 - 10 000 | 0.08 |
| > 10 000 - 25 000 | 0.05 |

NOTE: if the mass of the batch is greater than 25 000 kg, it is divided into sub-batches, and the procedure is applied to each sub-batch as though it were a homogeneous batch.

* subject to a minimum total mass of 125 g for the bulk sample; if this minimum requirement represents more than 10.0 per cent of the mass of herbal drug in the batch, the whole batch may be used as the sample.

Prepare the bulk sample by combining and thoroughly mixing the samples taken from each of the randomly selected containers (see Table 2.8.20.-1).

## TEST SAMPLE

Unless otherwise prescribed in the monograph, prepare the test sample as follows.

Reduce the size of the bulk sample by quartering (see Note below) or by any other method that produces a homogeneous sample, making sure that each retained portion remains representative of the whole, until the minimum retained quantity complies with the following conditions.

| Type of herbal drug | Minimum weight of test sample |
|---|---|
| Roots, rhizomes, bark, herbs | 500 g or mass of whole sample if bulk sample is less than 500 g |
| Leaves, flowers, seeds, fruits | 250 g or mass of whole sample if bulk sample is less than 250 g |
| Broken or fragmented drugs (where average mass of the pieces is less than 0.5 g) | 125 g |

*NOTE: quartering consists of placing the bulk sample, thoroughly mixed, as a level and square-shaped heap and dividing it diagonally into 4 equal parts. 2 opposite quarters are retained and carefully remixed. The process is repeated as necessary until the required minimum mass is obtained for the test sample.*

Mill the test sample in a single pass through a 1 mm screen or the screen size specified in the monograph. The use of a milling machine is recommended.

Pass the milled sample through a 1 mm standard sieve or the sieve specified in the monograph. The residue retained on the sieve must not be more than 10 per cent of the total mass of the milled sample, of which not more than 2 per cent of the total mass of the milled sample may be of a particle size greater than 1.5 mm or 1.5 times the specified particle size in the monograph. If these conditions are met, the sample and residue are to be well mixed to form the test sample for analysis.

In those cases where these requirements are not met, the test sample for analysis is composed of the 2 parts measured separately. Therefore, the quantity required for each analysis is derived by weighing proportional quantities of the powder and the residue.

*NOTE: for determination of microscopic characters, a portion of the milled test sample is re-milled through a 0.355 mm screen.*

Table 2.8.20.-1. – *Operation of the sampling procedure in order to obtain the prescribed bulk sample*

| Mass of herbal drug in container (kg) | 0.5 | | | 1 | | | 5 | | |
|---|---|---|---|---|---|---|---|---|---|
| Total mass of herbal drug in the batch (kg) | No. of containers in batch | No. of containers to be sampled | Total mass of samples (g) | No. of containers in batch | No. of containers to be sampled | Total mass of samples (g) | No. of containers in batch | No. of containers to be sampled | Total mass of samples (g) |
| 0.5 | 1 | 1 | 125 | – | – | – | – | – | – |
| 1 | 2 | 2 | 125 | 1 | 1 | 125 | – | – | – |
| 5 | 10 | 5 | 125 | 5 | 4 | 125 | 1 | 1 | 125 |
| 10 | 20 | 6 | 125 | 10 | 5 | 125 | 2 | 2 | 125 |
| 25 | – | – | – | 25 | 6 | 250 | 5 | 4 | 250 |
| 100 | – | – | – | 100 | 11 | 500 | 20 | 6 | 500 |
| 250 | – | – | – | – | – | – | 50 | 9 | 625 |
| 500 | – | – | – | – | – | – | 100 | 11 | 1000 |
| **Mass of herbal drug in container (kg)** | **25** | | | **125** | | | **500** | | |
| **Total mass of herbal drug in the batch (kg)** | **No. of containers in batch** | **No. of containers to be sampled** | **Total mass of samples (g)** | **No. of containers in batch** | **No. of containers to be sampled** | **Total mass of samples (g)** | **No. of containers in batch** | **No. of containers to be sampled** | **Total mass of samples (g)** |
| 25 | 1 | 1 | 250 | – | – | – | – | – | – |
| 100 | 4 | 3 | 500 | – | – | – | – | – | – |
| 250 | 10 | 5 | 625 | 2 | 2 | 625 | – | – | – |
| 500 | 20 | 6 | 1000 | 4 | 3 | 1000 | 1 | 1 | 1000 |
| 1000 | 40 | 8 | 1800 | 8 | 4 | 1800 | 2 | 2 | 1800 |
| 2000 | 80 | 10 | 3000 | 16 | 5 | 3000 | 4 | 3 | 3000 |
| 3000 | 120 | 12 | 3000 | 24 | 6 | 3000 | 6 | 4 | 3000 |
| 5000 | 200 | 16 | 5000 | 40 | 8 | 5000 | 10 | 5 | 5000 |
| 10 000 | 400 | 21 | 8000 | 80 | 10 | 8000 | 20 | 6 | 8000 |
| 25 000 | 800 | 30 | 12 500 | 160 | 14 | 12 500 | 40 | 8 | 12 500 |

**01/2011:20821**

# 2.8.21. TEST FOR ARISTOLOCHIC ACIDS IN HERBAL DRUGS

*CAUTION: aristolochic acids are very toxic and carcinogenic. Perform manipulations in a fume cupboard whenever possible. Take particular precautions, such as use of a glove box, when the substance is in dry form because of its electrostatic properties and the tendency to disperse through the working areas.*

Methods A and B are intended to be cross-referenced in monographs on herbal drugs that, according to chemotaxonomic knowledge, are expected to be free from aristolochic acids, but that may be subject to adulteration or substitution with plant material containing aristolochic acids. Methods A and B are intended to be used in the screening of herbal drugs for aristolochic acids at the stated limits and will usually be complemented by macroscopic and/or microscopic tests to exclude plant material containing aristolochic acids.

Method C will not be used in specific monographs but is provided as a method to confirm the presence of aristolochic acid I at levels equal to or greater than 2 ppm. It may be applied if chromatographic data suggests the presence of aristolochic acid I.

These methods are not designed for inclusion as assay methods in monographs on those drugs that produce aristolochic acids as secondary metabolites; for these, a more sensitive, validated method is required.

METHOD A: SCREENING TEST FOR ARISTOLOCHIC ACIDS

Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *anhydrous formic acid R*, *water R*, *methanol R* (1:9:40 *V/V/V*).

*Test solution.* To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 10.0 mL of the solvent mixture, sonicate for 10 min and centrifuge.

*Reference solution (a).* Disperse an amount of *aristolochia HRS* corresponding to 0.10 mg of aristolochic acid I in 20.0 mL of the solvent mixture, sonicate for 10 min and centrifuge.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 25.0 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R*, *toluene R* (3:3:30:60 *V/V/V/V*); use the upper layer.

*Application*: 20 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in a current of cold air for 5 min.

*Detection*: spray with a 100 g/L solution of *stannous chloride R* in *dilute hydrochloric acid R* until the plate is slightly wet, heat at 100 °C for 1 min and examine in ultraviolet light at 365 nm.

*System suitability*:

– the chromatogram obtained with reference solution (a) shows 2 greenish-blue zones due to aristolochic acids I and II between $R_F$ = 0.35 and $R_F$ = 0.55, which may not be completely separated;

– the chromatogram obtained with reference solution (b) shows at least 1 of these zones (corresponding to 2 ppm of aristolochic acid I).

*Results*: in the chromatogram obtained with the test solution no zone is similar in position and fluorescence to any of the zones due to aristolochic acids in the chromatogram obtained with reference solution (a).

If the chromatogram obtained with the test solution shows any zones similar in position and fluorescence to any of the zones due to aristolochic acids I and II in the chromatogram obtained with reference solution (a), apply method B.

METHOD B: LIMIT TEST FOR ARISTOLOCHIC ACID I

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Weigh 2.0 g of the powdered herbal drug (710) (*2.9.12*) into a 250 mL, brown, screw-cap bottle and add 100.0 mL of the solvent mixture. Stir for 30 min at about 300 r/min and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve the contents of a vial of *aristolochic acid I CRS* in the solvent mixture to obtain a concentration of 0.04 µg/mL of aristolochic acid I.

*Reference solution (b).* Dissolve the contents of a vial of *aristolochic acid for system suitability CRS* (containing aristolochic acids I and II) in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 2.1 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water R* (0.1:99.9 *V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R* (0.1:99.9 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 85 → 35 | 15 → 65 |
| 25 - 30 | 35 → 0 | 65 → 100 |
| 30 - 31 | 0 → 85 | 100 → 15 |

*Flow rate*: 0.3 mL/min.

*Detection*: spectrophotometer at 390 nm.

*Injection*: 25 µL.

*System suitability*:

– *resolution*: minimum 3.0 between the peaks due to aristolochic acids I and II in the chromatogram obtained with reference solution (b);
– *signal-to-noise ratio*: minimum 10 for the peak due to aristolochic acid I in the chromatogram obtained with reference solution (a).

*Limit*:

– the sample complies with the test if the chromatogram obtained with the test solution shows no peak with the same retention time as the peak due to aristolochic acid I in the chromatogram obtained with reference solution (a) (2 ppm).

METHOD C: CONFIRMATORY TEST FOR ARISTOLOCHIC ACID I

Liquid chromatography (*2.2.29*) coupled with mass spectrometry (*2.2.43*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Weigh 2.0 g of the powdered herbal drug (710) (*2.9.12*) into a 250 mL, brown, screw-cap bottle and add 100.0 mL of the solvent mixture. Sonicate for 30 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve the contents of a vial of *aristolochic acid I CRS* in the solvent mixture to obtain a concentration of 0.04 µg/mL of aristolochic acid I.

*Reference solution (b).* Prepare a solution according to the instructions supplied with *aristolochic acid I CRS* to obtain a concentration of 0.45 µg of aristolochic acid I in 10.0 mL of the test solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 2.1 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *anhydrous formic acid R*, 1 g/L solution of *ammonium acetate R* in *water R* (0.1:99.9 *V/V*);
– *mobile phase B*: *anhydrous formic acid R*, 1 g/L solution of *ammonium acetate R* in *methanol R* (0.1:99.9 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 70 → 0 | 30 → 100 |
| 15 - 16 | 0 | 100 |
| 16 - 17 | 0 → 70 | 100 → 30 |

*Flow rate*: 0.4 mL/min.

*Injection*: 20 µL; inject reference solution (a) twice, the test solution twice, reference solution (a) twice, then reference solution (b) twice.

*Detection*: mass detector as described below under A or B. Adjust the flow rate, the temperature and the detector settings so as to comply with the system suitability criterion.

A. Ion-trap mass spectrometer equipped with an electrospray ionisation (ESI) interface and $MS^n$ analyser.

Set the mass spectrometer parameters for the $MS^3$ mode as follows:

| Mode | Parent (*m/z*) | Isolation width (*m/z*) | Relative collision energy (per cent) |
|---|---|---|---|
| $MS^2$ | 359 $[M + NH_4]^+$ | 2.0 | 30 |
| $MS^3$ | 298 | 2.0 | 35 |

– full scan of product ions: from *m/z* 80 to *m/z* 370;
– product ions to be monitored: *m/z* 252, *m/z* 268 and *m/z* 281.

*System suitability*:

– *signal-to-noise ratio*: minimum 100 for the monitored product ions in the chromatogram obtained with reference solution (a);
– *matrix interference test*: the average of the 2 ratios of reference solution (b) is inside the ± 40 per cent interval of the average of the 2 ratios of reference solution (a); otherwise it is necessary to adjust the detector settings.

*Results*: evaluate the average ratios (252/268 and 281/268) of the relative intensity of the 3 product ions of aristolochic acid I in the test solution; evaluate the average of the 2 ratios of the signals at the retention time of aristolochic acid I in reference solution (a); if the average of the 2 ratios of the test solution is within the ± 40 per cent interval of the average of the 2 ratios of reference solution (a), aristolochic acid I is present in the test solution.

B. Triple-quadrupole mass spectrometer equipped with an ESI interface and $MS^n$ analyser.

Set the mass spectrometer parameters for the $MS^2$ mode as follows:

- precursor ion: *m/z* 359 $[M + NH_4]^+$;
- product ions to be monitored: *m/z* 265, *m/z* 281 and *m/z* 296.

*System suitability*:

- *signal-to-noise ratio*: minimum 100 for the monitored product ions in the chromatogram obtained with reference solution (a);
- *matrix interference test*: the average of the 2 ratios of reference solution (b) is inside the ± 40 per cent interval of the average of the 2 ratios of reference solution (a); otherwise it is necessary to adjust the detector settings.

*Results*: evaluate the average ratios (265/281 and 296/281) of the relative intensity of the 3 product ions of aristolochic acid I in the test solution; evaluate the average of the 2 ratios of the signals at the retention time of aristolochic acid I in reference solution (a); if the average of the 2 ratios of the test solution is within the ± 40 per cent interval of the average of the 2 ratios of reference solution (a), aristolochic acid I is present in the test solution.

**01/2010:20822**

## 2.8.22. DETERMINATION OF OCHRATOXIN A IN HERBAL DRUGS

*CAUTION: ochratoxin A is nephrotoxic and nephrocarcinogenic. Perform manipulations in a fume cupboard. Take particular precautions, such as use of a glove box, when toxins are in dry form because of their electrostatic properties and the tendency to disperse through the working areas. Decontamination procedures for laboratory glassware containing ochratoxin A are necessary (see appendix).*

Herbal drugs that are subject to contamination by ochratoxin A are tested by a validated method.

The method described below is cited as an example of a method that has been shown to be suitable for liquorice extract and liquorice root. Its suitability for other herbal drugs must be demonstrated or another validated method used.

METHOD

Liquid chromatography (*2.2.29*).

Use brown glassware that is free from detergent residues. If necessary rinse glassware before use with a 10 per cent *V/V* solution of *sulfuric acid R* and then rinse carefully with *distilled water R* until no more acid is present.

*Solution A*. Mix 80 volumes of *water R*, previously adjusted to pH 2.3 with *anhydrous formic acid R*, and 20 volumes of *acetonitrile R*.

*Test solution*. Use an immunoaffinity column containing antibodies against ochratoxin A with a capacity of not less than 100 ng of ochratoxin A and which gives a recovery of not less than 70 per cent. Allow the immunoaffinity column to reach room temperature.

To 2.00 g of the powdered drug (250) (*2.9.12*) add 80 mL of a 30 g/L solution of *sodium hydrogen carbonate R* and extract by sonication for 30 min (change water of ultrasonic bath after 15 min). Cool to room temperature and dilute to 100.0 mL ($V_1$) with the same solution. Centrifuge. Mix thoroughly 5.0 mL ($V_i$) of the clear supernatant with 30 mL *buffer solution pH 7.4 R* and pass the whole solution through the immunoaffinity column at a flow rate of 3 mL/min (do not exceed 5 mL/min). Wash the column first with 10 mL *buffer solution pH 7.4 R* then with 2 quantities, each of 10 mL, of *water R* at a flow rate not exceeding 5 mL/min and dry by applying a slight vacuum for 5-10 s or by passing air through the immunoaffinity column by means of a syringe for 10 s. Apply 0.5 mL of *methanol R* to the column and allow to pass through by gravity.

Collect the eluate in a 4 mL glass vial. After 30 s, apply a 2nd quantity of 0.5 mL of *methanol R* and allow to pass through the column by gravity into the same glass vial. After a further 30 s, repeat with a 3rd portion of 0.5 mL of *methanol R*. Collect any solvent retained on the column by pressing air through or applying vacuum to the column. Evaporate the combined eluates completely to dryness using a thermal block with a nitrogen blanket (40 °C). Dissolve the residue in 0.5 mL ($V_2$) of solution A. If the solution is clear it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of ochratoxin A by retention.

*Ochratoxin A primary stock solution*. Dilute 1.0 mL of *ochratoxin A solution R* to 100.0 mL with *methanol R* and shake thoroughly.

*Ochratoxin A secondary stock solution*. Dilute 10.0 mL of ochratoxin A primary stock solution to 100.0 mL with *methanol R* and shake thoroughly.

*Ochratoxin A standard solutions*. Place the volumes of ochratoxin A primary stock solution or ochratoxin A secondary stock solution indicated in Table 2.8.22.-1 into separate flasks and make up to 50.0 mL with solution A.

Table 2.8.22.-1. – *Ochratoxin A standard solutions*

| Standard solution | Volume of ochratoxin A primary stock solution (µL) | Final concentration of ochratoxin A in standard solution (ng/mL) |
|---|---|---|
| 1 | 5000 | 50 |
| 2 | 2500 | 25 |
| 3 | 1000 | 10 |
| 4 | 500 | 5 |
| 5 | 250 | 2.5 |
| **Standard solution** | **Volume of ochratoxin A secondary stock solution (µL)** | **Final concentration of ochratoxin A in standard solution (ng/mL)** |
| 6 | 500 | 0.5 |
| 7 | 100 | 0.1 |

*Calibration curve*. Prepare the calibration curve using ochratoxin A standard solutions 1 to 7, which cover a range equivalent to 0.5-250 µg/kg of ochratoxin A in the herbal drug. Check the plot for linearity. If the content of ochratoxin A in the sample to be examined is outside of the calibration range, the test solution must be diluted to an ochratoxin A content that is appropriate for the established calibration curve.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *water R* adjusted to pH 2.3 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 80 → 40 | 20 → 60 |
| 30 - 35 | 40 → 20 | 60 → 80 |
| 35 - 37 | 20 | 80 |
| 37 - 40 | 20 → 80 | 80 → 20 |

*Flow rate*: 0.8 mL/min.

*Detection*: fluorescence detector; recommended settings for adjustable detectors are 330 nm (excitation wavelength) and 460 nm (emission wavelength).

*Injection*: 20 µL.

*Calculation*: calculate the calibration curve $y = ax + b$, with ochratoxin A concentration (in nanograms per millilitre) on the x-axis and the signal ($S$) on the y-axis. The ochratoxin A concentration ($C$) in a measured solution is equal to $\frac{S-b}{a}$.

Calculate the ochratoxin A content of the herbal drug, in nanograms per gram, using the following expression:

$$\frac{V_1 \times V_2 \times C}{m \times V_i}$$

$m$ = mass of the herbal drug used to prepare the test solution, in grams;

$V_1$ = volume of dilution, in millilitres;

$V_i$ = aliquot taken for immunoaffinity clean-up, in millilitres;

$V_2$ = volume in which the residue is taken up, in millilitres;

$C$ = measured ochratoxin A concentration of the test solution, in nanograms per millilitre.

## Appendix: Decontamination procedures for laboratory glassware

Rinse glassware with *methanol R* and decontaminate by immersion in *strong sodium hypochlorite solution R* for at least 2 h, then wash thoroughly with water.

**04/2010:20823**

# 2.8.23. MICROSCOPIC EXAMINATION OF HERBAL DRUGS

The microscopic examination of herbal drugs is carried out on the powdered drug (355) (*2.9.12*) unless otherwise prescribed in the monograph.

*Chloral hydrate solution R* is the most commonly prescribed reagent. However, certain features are not visible or not easily seen after mounting in this reagent. In this case, other reagents are used, for example, a 50 per cent *V/V* solution of *glycerol R*, which makes it possible to visualise starch granules. It may also be necessary to prescribe specific reagents in a monograph, for example: *lactic reagent R* which is used to show the presence of various features, 10 per cent *V/V* alcoholic solution of *phloroglucinol R* and *hydrochloric acid R*, which are used to identify the presence of lignin in cells or tissues, *ruthenium red solution R*, which is used to show the presence of mucilage in cells or *glycerol R* used to show the presence of starch and inulin.

Examination under polarised light (between crossed nicol prisms) is used to identify starch granules (black cross phenomenon), calcium oxalate crystals (refringence) or lignified structures.

MOUNTING IN CHLORAL HYDRATE SOLUTION

Place 2-3 drops of *chloral hydrate solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling on a hot plate or a micro gas burner. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Repeat the heating until the starch granules and the water-soluble contents of the cells are no longer visible. Examine under a microscope.

Chloral hydrate tends to crystallise as long needles. To avoid this, proceed as follows: after heating, remove the cover slip; to the preparation add 1 drop of a 10 per cent *V/V* mixture of *chloral hydrate solution R* in *glycerol R*; place a clean cover slip on the preparation; examine under a microscope.

MOUNTING IN A 50 PER CENT *V/V* SOLUTION OF GLYCEROL

Place 2 drops of a 50 per cent *V/V* solution of *glycerol R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Examine under a microscope.

MOUNTING IN A 10 PER CENT *V/V* ALCOHOLIC SOLUTION OF PHLOROGLUCINOL AND HYDROCHLORIC ACID

Place a very small quantity of the powdered drug on a glass microscope slide. Add 1-2 drops of a 10 per cent *V/V* alcoholic solution of *phloroglucinol R*. Mix and allow the solvent to evaporate almost completely. Add 1-2 drops of *hydrochloric acid R* and cover the preparation with a cover slip. Examine immediately under a microscope. The red colour indicates the presence of lignin.

MOUNTING IN LACTIC REAGENT

Place 2-3 drops of *lactic reagent R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Lignified structures stain bright yellow; structures containing cellulose remain colourless. Starch granules stain more or less violet; certain secretions (e.g., essential oils, resins, oleoresins) stain orange and cork stains red.

MOUNTING IN RUTHENIUM RED SOLUTION

Place 2 drops of *ruthenium red solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. After about 1 minute, allow a drop of *distilled water R* to be taken up between the slide and the cover slip. Examine under a microscope. The mucilage stains violet red.

**07/2019:20824**

# 2.8.24. FOAM INDEX

PRINCIPLE

The foam index is determined by measuring the height of the foam produced by the equivalent of 1 g of herbal drug or herbal drug preparation under the stated test conditions.

EQUIPMENT

The equipment (see Figure 2.8.24.-1) typically consists of:

- a pipette stand;
- a 50 mL class A pipette with a rubber filler bulb;
- a 250 mL glass measuring cylinder with 2 mL graduations and an internal diameter of 38 ± 3 mm.

Mount the pipette on its stand so that the tip will be located 45 cm from the base of the cylinder placed under it. Position the cylinder so that the pipette tip is aligned with its centre. Before performing the test, fill the pipette and cylinder with

water to check that the liquid dispensed from the pipette runs into the centre of the measuring cylinder. Mark the location of the measuring cylinder.

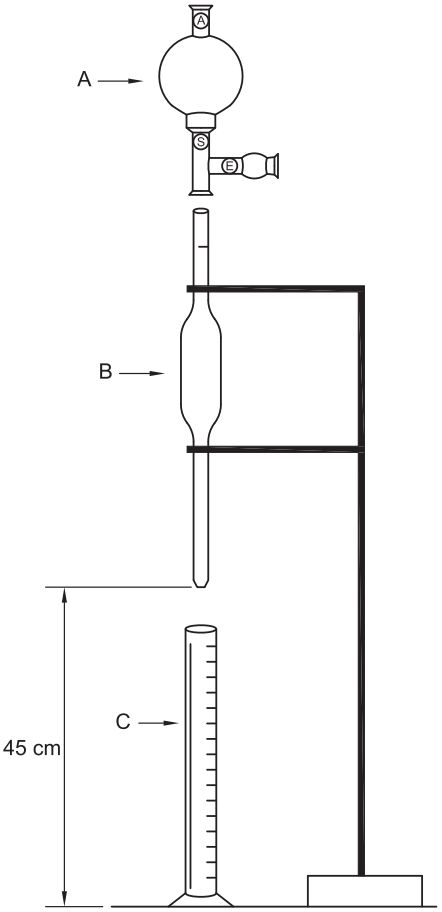


A. rubber pipette filler

B. 50 mL class A pipette

C. 250 mL glass measuring cylinder with 2 mL graduations and an internal diameter of 38 ± 3 mm

Figure 2.8.24.-1. – *Apparatus suitable for measuring the foam index*

PROCEDURE

**Preparation of the test solution**. Introduce the quantity of powdered herbal drug (355) (*2.9.12*) or herbal drug preparation prescribed in the monograph into a 250 mL beaker. Add 50 mL of *distilled water R* and allow to stand for 30 min, mixing 3-5 times with a spatula during this period to disperse the powder without producing any foam. Rinse the spatula and the internal walls of the beaker with a further 50 mL of *distilled water R* to ensure that as much of the herbal drug or herbal drug preparation as possible is contained within the liquid. Allow to stand undisturbed for 30 min. Filter through a filter paper 125 mm in diameter. Use the filtrate as the test solution.

**Method**. Mount the pipette on the stand. Take up 50.0 mL of the test solution using the pipette. Rinse the measuring cylinder with *water R* to wet its walls, then introduce the remaining 50.0 mL of test solution, taking care not to produce any foam. Position the measuring cylinder on its mark under the pipette. Open pipette filler valve E to allow the solution to run from the pipette into the measuring cylinder. Record the maximum height of the foam before it starts to collapse. Repeat the test twice, carefully rinsing the pipette and the measuring cylinder with *distilled water R* between tests.

**Results**. Determine the foam index ($I_F$) using the following equation:

$$I_F = \frac{H}{m}$$

$H$ = height of the resulting foam, in centimetres;

$m$ = mass of the herbal drug or herbal drug preparation used to prepare the test solution, in grams.

Record the average of 3 determinations as the result.

This result represents the height of the foam for a test solution with a theoretical concentration of 1 g in 100 mL.

**01/2020:20825**

# 2.8.25. HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY OF HERBAL DRUGS AND HERBAL DRUG PREPARATIONS

High-performance thin-layer chromatography (HPTLC) is used for qualitative analysis of herbal drugs and herbal drug preparations. It is a thin-layer chromatographic technique (*2.2.27*) that usually uses a glass plate coated with a uniform, porous layer (average pore size 6 nm), typically 200 µm thick, of irregular particles of silica gel between 2 µm and 10 µm in size and with an average size of 5 µm, a polymeric binder and a fluorescence indicator ($F_{254}$). The results are qualified using a system suitability test.

EQUIPMENT

The equipment used for qualitative HPTLC typically consists of:

- glass plates, as described above, usually 20 × 10 cm in size;
- devices suitable for the application of specified volumes of solutions as bands and allowing control of the dimensions and position of application;
- a device suitable for conditioning the stationary phase at the prescribed relative humidity;
- a suitable chromatographic tank (for example, a twin trough chamber);
- a device suitable for the reproducible drying of the developed plate;
- devices suitable for the application of reagents to, and heating of, the plate as part of the derivatisation procedure;
- a system suitable for the electronic documentation of chromatograms under 254 nm UV, 366 nm UV and white light.

*NOTE*: normal thin-layer chromatographic methods using glass plates or sheets coated with particles of 5-40 µm or HPTLC aluminium-backed sheets may be used, provided that the results obtained fulfil the general system suitability criteria that the bands develop perpendicular to the lower edge of the plate and the solvent front is parallel to the upper edge of the plate, and satisfy the system suitability test stated in the individual monograph.

METHOD

**Preparation of test solution**. The test solution is prescribed in the individual monograph and is usually prepared as follows.

For dry herbal drugs or dry herbal extracts, mix 0.5 g of the powdered herbal drug or 0.1 g of the dry herbal extract with 5.0 mL of *methanol R* and sonicate for 15 min; filter or centrifuge and use the filtrate or supernatant as the test solution.

For essential oils, dissolve 50 µL of the essential oil in 1.0 mL of *toluene R* and use this solution as the test solution.

**Preparation of reference solutions**. Reference solutions are prescribed in the individual monograph and are usually prepared as follows. Prepare a 1 mg/mL solution (identified as 'R' in the chromatograms associated with the monograph) of suitable reagent(s) or reference standard(s) in *methanol R* or, for essential oils, in *toluene R*. Prepare a second reference solution (diluted reference solution, identified as 'R1/4' in the chromatograms associated with the monograph) by mixing 1 volume of this solution and 3 volumes of the same solvent (in some cases a different diluted reference solution, e.g. 'R1/20', may be prescribed instead). Both solutions are used as intensity references.

**Intensity marker**. Use one or more of the substances in the reference solution and in the diluted reference solution as intensity marker(s) for the evaluation of the chromatogram.

**Preparation of system suitability test solution**. Prepare the system suitability test (SST) solution as stated in the individual monograph.

**Sample application and plate layout**. Samples are usually applied as narrow bands 8 mm in length at a distance of 8 mm from the lower edge of the plate. The centre of the first track, which is used for the system suitability test solution, is positioned 20 mm from the left edge of the plate. The minimum distance between tracks (centre to centre) is 11 mm. A maximum of 15 tracks are applied onto a standard plate. If no electronic solvent front detection device is used, the development distance is marked with a pencil close to the right or left edge of the plate.

**Conditioning of the plate**. Following sample application and unless otherwise stated in the individual monograph, expose the plate to air with a suitable relative humidity obtained using a saturated solution of *magnesium chloride R* (for example, by allowing the plate to stand in a closed chamber containing such a solution for 1 h or by using preconditioned air).

**Preparation of the tank and development of the plate**. Unless otherwise stated in the individual monograph, the chromatographic separation is performed in a saturated tank. Where a twin trough chamber is used, place a piece of filter paper in the rear trough. Load the tank with a sufficient quantity of mobile phase to wet the filter paper completely and achieve a level of 5 mm in both troughs. With the lid closed, leave the tank for 20 min for saturation. Introduce the plate in a vertical position into the front trough of the tank so that the coating layer faces the filter paper. When the mobile phase has reached the prescribed distance (usually 70 mm from the lower edge of the plate), remove the plate from the tank and dry in a vertical position in a stream of air at room temperature. Other tank configurations and development distances may be specified in an individual monograph.

*NOTE*: other tanks may be employed if the results obtained fulfil all of the system suitability criteria.

**Visualisation**. Chromatograms on the plate are visualised as stated in the individual monograph under Detection. Where derivatisation reagents are used, typically 3.5 mL of reagent solution is homogenously sprayed onto a plate of size 20 × 10 cm, or the plate is immersed into the reagent solution, typically at a speed of 5 mm/s for a dwell time of 1 s. Observation may be performed under 254 nm UV, 366 nm UV or white light prior to and/or after derivatisation. When pictures are digitally recorded, exposure time should be adjusted based on the track used for the system suitability test solution.

**System suitability test**. This test is based on the separation of 2 substances that have similar retardation factors ($R_F$ values) but that are barely separable under the specified chromatographic conditions (for example, chlorogenic acid and hyperoside in chromatographic systems used for flavonoids). The results for the test and reference solutions are only valid when the system suitability test solution satisfies the separation requirement stated in the individual monograph.

**Visual evaluation**. The chromatograms obtained with the test and reference solutions are compared with the descriptions in the results section of the individual monograph, with respect to zone position and colour, as well as intensity for the test solution. Zones of the test solution described as 'equivalent' or without an indication of intensity have intensities similar to the zone of the intensity marker in the reference solution (R). Zones described as 'intense' are visually more intense than the zone of the intensity marker in the reference solution; zones described as 'faint' are visually less intense than the zone of the intensity marker in the reference solution, but equal to or more intense than the zone of the intensity marker in the diluted reference solution (R1/4, R1/20, etc.); zones described as 'very faint' are visually less intense than the zone of the intensity marker in the diluted reference solution.

# 2.9. Pharmaceutical technical procedures



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# 2.9. PHARMACEUTICAL TECHNICAL PROCEDURES



01/2020:20901

## 2.9.1. DISINTEGRATION OF TABLETS AND CAPSULES[(1)]

This test is provided to determine whether tablets or capsules disintegrate within the prescribed time when placed in a liquid medium under the experimental conditions presented below.

For the purposes of this test, disintegration does not imply complete dissolution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the discs, if used, is a soft mass having no palpably firm core.

♦Use apparatus A for tablets and capsules that are not greater than 18 mm long. For larger tablets or capsules use apparatus B.♦

TEST A - TABLETS AND CAPSULES OF NORMAL SIZE

**Apparatus**. The apparatus consists of a basket-rack assembly, a 1 L, low-form beaker, 149 ± 11 mm in height and having an inside diameter of 106 ± 9 mm for the immersion fluid, a thermostatic arrangement for heating the fluid between 35 °C and 39 °C, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid, and descends to not less than 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

*Basket-rack assembly*. The basket-rack assembly consists of 6 open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 21.85 ± 1.15 mm and a wall 1.9 ± 0.9 mm thick; the tubes are held in a vertical position by 2 plates, each 90 ± 2 mm in diameter and 6.75 ± 1.75 mm in thickness, with 6 holes, each 24 ± 2 mm in diameter, equidistant from the centre of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 2.0 ± 0.2 mm mesh apertures and with a wire diameter of 0.615 ± 0.045 mm. The parts of the apparatus are assembled and rigidly held by means of 3 bolts passing through the 2 plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat provided the specifications for the glass tubes and the screen mesh size are maintained. The basket-rack assembly conforms to the dimensions shown in Figure 2.9.1.-1.

*Discs*. The use of discs is permitted only where specified or allowed. Each tube is provided with a cylindrical disc 9.5 ± 0.15 mm thick and 20.7 ± 0.15 mm in diameter. The disc is made of a suitable, transparent plastic material having a specific gravity of 1.18-1.20. 5 parallel 2 ± 0.1 mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are parallel to the cylindrical axis and centered 6 ± 0.2 mm from the axis on imaginary lines perpendicular to the axis and to each other, as defined in Figure 2.9.1.-1. 4 identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centres of 2 adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of 1.6 ± 0.1 mm and its bottom edges lie at a depth of 1.5 mm to 1.8 mm from the cylinder's circumference. The parallel side of the trapezoid on the top of the cylinder has a length of 9.4 ± 0.2 mm and its centre lies at a depth of 2.6 ± 0.1 mm from the cylinder's circumference. All surfaces of the disc are smooth.

If the use of discs is specified, add a disc to each tube and operate the apparatus as directed under Procedure. The discs conform to the dimensions shown in Figure 2.9.1.-1.

The use of automatic detection employing modified discs is permitted where the use of discs is specified or allowed. Such discs must comply with the requirements of density and dimension given in this chapter.

**Procedure**. Place 1 dosage unit in each of the 6 tubes of the basket and, if prescribed, add a disc. Operate the apparatus using the specified medium, maintained at 37 ± 2 °C, as the immersion fluid. At the end of the specified time, lift the basket from the fluid and observe the dosage units: all of the dosage units have disintegrated completely. If 1 or 2 dosage units fail to disintegrate, repeat the test on 12 additional dosage units. The requirements of the test are met if not less than 16 of the 18 dosage units tested have disintegrated.

♦TEST B – LARGE TABLETS AND LARGE CAPSULES

**Apparatus**. The main part of the apparatus (Figure 2.9.1.-2.) is a rigid basket-rack assembly supporting 3 cylindrical transparent tubes 77.5 ± 2.5 mm long, 33.0 mm ± 0.5 mm in internal diameter, and with a wall thickness of 2.5 ± 0.5 mm. Each tube is provided with a cylindrical disc 31.4 ± 0.13 mm in diameter and 15.3 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18-1.20. Each disc is pierced by 7 holes, each 3.15 ± 0.1 mm in diameter, 1 in the centre and the other 6 spaced equally on a circle of radius 4.2 mm from the centre of the disc. The tubes are held vertically by 2 separate and superimposed rigid plastic plates 97 mm in diameter and 9 mm thick, with 3 holes. The holes are equidistant from the centre of the plate and equally spaced. Attached to the under side of the lower plate is a piece of woven gauze made from stainless steel wire 0.63 ± 0.03 mm in diameter and having mesh apertures of 2.0 ± 0.2 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery. A metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached to a mechanical device capable of raising and lowering it smoothly at a constant frequency of between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm.

The assembly is suspended in the specified liquid medium in a suitable vessel, preferably a 1 L beaker. The volume of the liquid is such that when the assembly is in the highest position the wire mesh is at least 15 mm below the surface of the liquid, and when the assembly is in the lowest position the wire mesh is at least 25 mm above the bottom of the beaker and the upper open ends of the tubes remain above the surface of the liquid. A suitable device maintains the temperature of the liquid at 35-39 °C.

The design of the basket-rack assembly may be varied provided the specifications for the tubes and wire mesh are maintained.

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Method**. Test 6 tablets or capsules either by using 2 basket-rack assemblies in parallel or by repeating the procedure. In each of the 3 tubes, place 1 tablet or capsule and, if prescribed, add a disc; suspend the assembly in the beaker containing the specified liquid. Operate the apparatus for the prescribed period, withdraw the assembly and examine the state of the tablets or capsules. To pass the test, all 6 of the tablets or capsules must have disintegrated. ♦

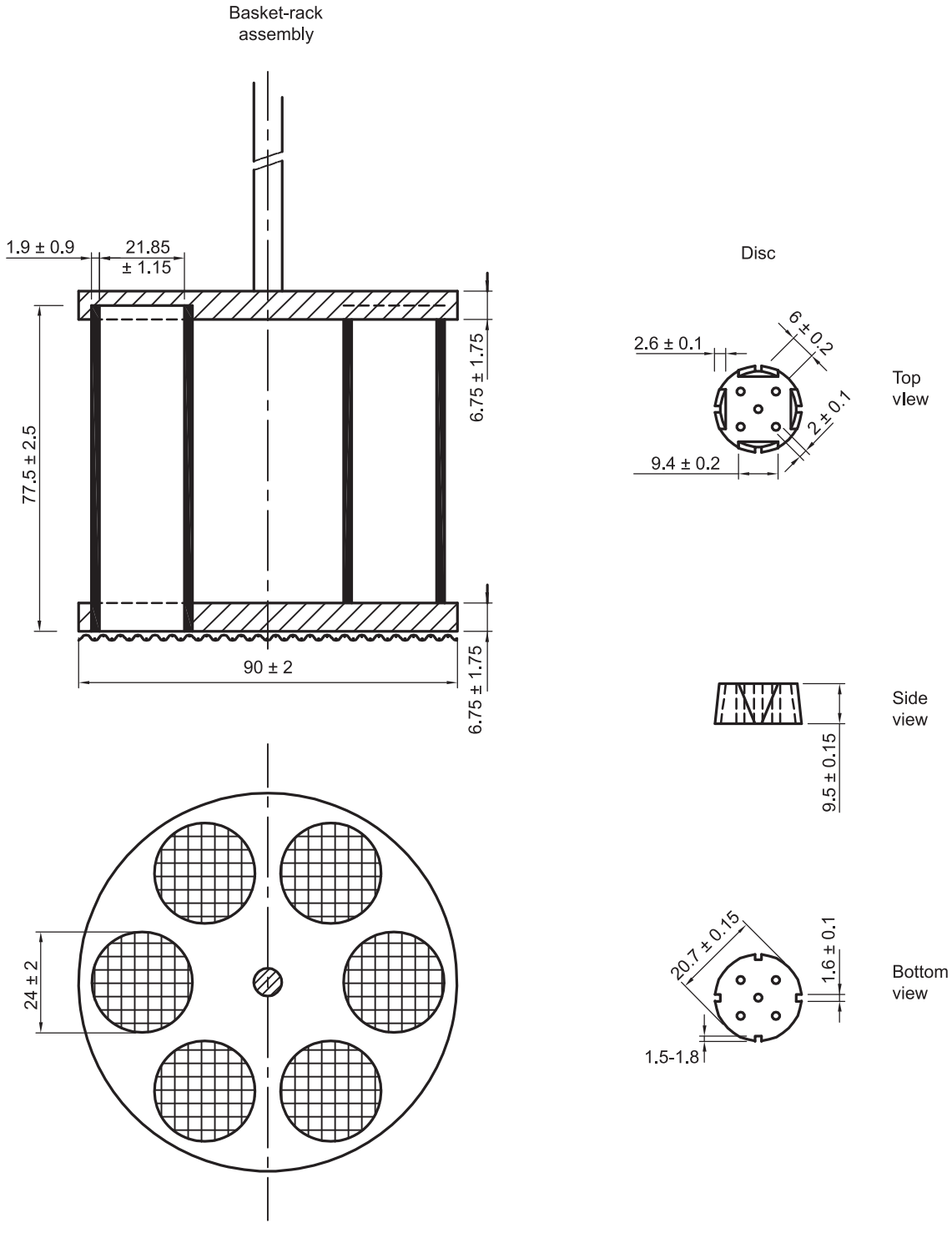


Figure 2.9.1.-1. – *Disintegration apparatus A*

*Dimensions in millimetres*

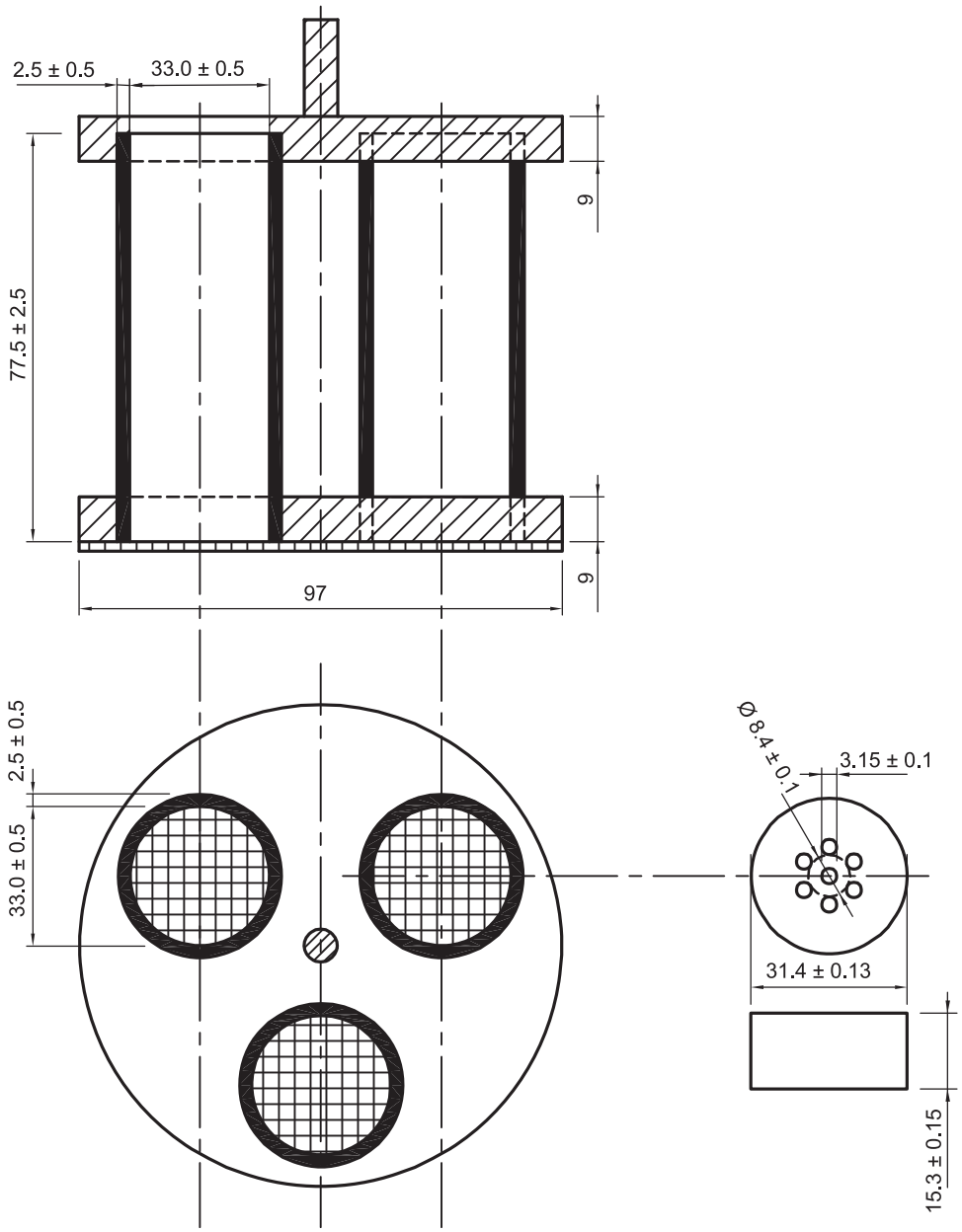


Figure 2.9.1.-2. – *Disintegration apparatus B*
*Dimensions in millimetres*

**01/2008:20902**

## 2.9.2. DISINTEGRATION OF SUPPOSITORIES AND PESSARIES

The disintegration test determines whether the suppositories or pessaries soften or disintegrate within the prescribed time when placed in a liquid medium in the experimental conditions described below.

Disintegration is considered to be achieved when:

a) dissolution is complete,

b) the components of the suppository or pessary have separated: melted fatty substances collect on the surface of the liquid, insoluble powders fall to the bottom and soluble components dissolve, depending on the type of preparation, the components may be distributed in one or more of these ways,

c) there is softening of the sample that may be accompanied by appreciable change of shape without complete separation of the components, the softening is such that the suppository or pessary no longer has a solid core offering resistance to pressure of a glass rod,

d) rupture of the gelatin shell of rectal or vaginal capsules occurs allowing release of the contents,

e) no residue remains on the perforated disc or if a residue remains, it consists only of a soft or frothy mass having no solid core offering resistance to pressure of a glass rod (vaginal tablets).

*Apparatus.* The apparatus (Figure 2.9.2.-1) consists of a sleeve of glass or suitable transparent plastic, of appropriate thickness, to the interior of which is attached by means of three hooks a metal device consisting of two perforated stainless metal discs each containing 39 holes 4 mm in diameter; the diameter of the discs is similar to that of the interior of the sleeve; the discs are about 30 mm apart. The test is carried out using three such apparatuses each containing a single sample. Each apparatus is placed in a beaker with a capacity of at least 4 L filled with water maintained at 36-37 °C, unless otherwise prescribed. The apparatuses may also be placed together in a

vessel with a capacity of at least 12 L. The beaker is fitted with a slow stirrer and a device that will hold the cylinders vertically not less than 90 mm below the surface of the water and allow them to be inverted without emerging from the water.

*Method.* Use three suppositories or pessaries. Place each one on the lower disc of a device, place the latter in the sleeve and secure. Invert the apparatuses every 10 min. Examine the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.

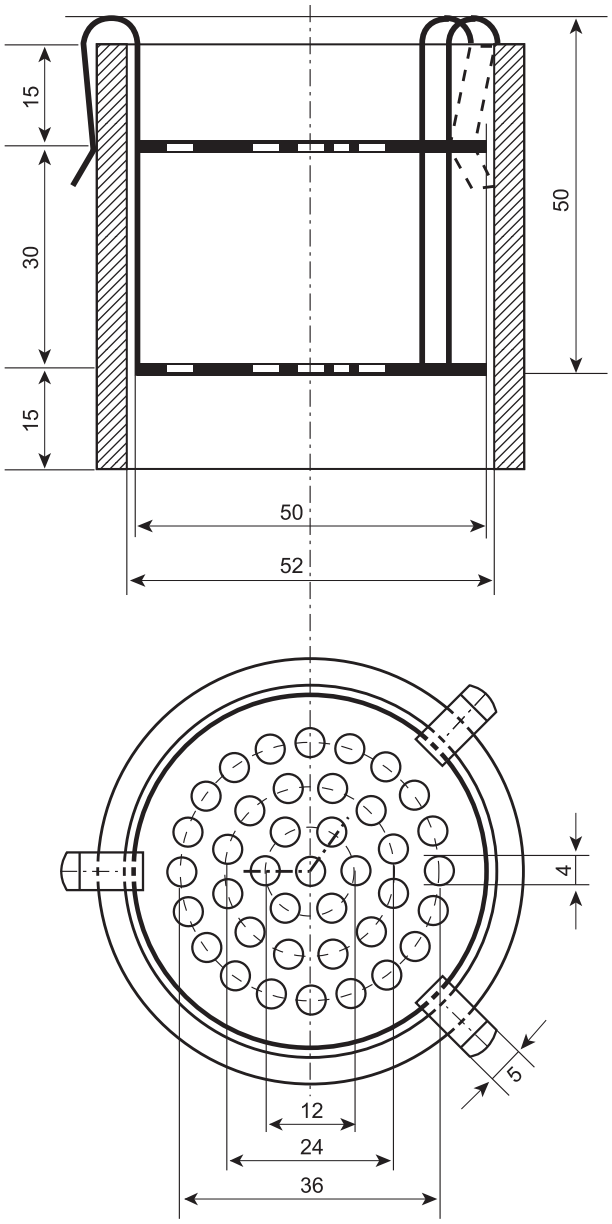


Figure 2.9.2.-1. – *Apparatus for disintegration of suppositories and pessaries*

*Dimensions in millimetres*

METHOD OF OPERATION FOR VAGINAL TABLETS

Use the apparatus described above, arranged so as to rest on the hooks (see Figure 2.9.2.-2). Place it in a beaker of suitable diameter containing water maintained at 36-37 °C with the level just below the upper perforated disc. Using a pipette, adjust the level with water at 36-37 °C until a uniform film covers the perforations of the disc. Use three vaginal tablets. Place each one on the upper plate of an apparatus and cover the latter with a glass plate to maintain appropriate conditions of humidity. Examine the state of the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.

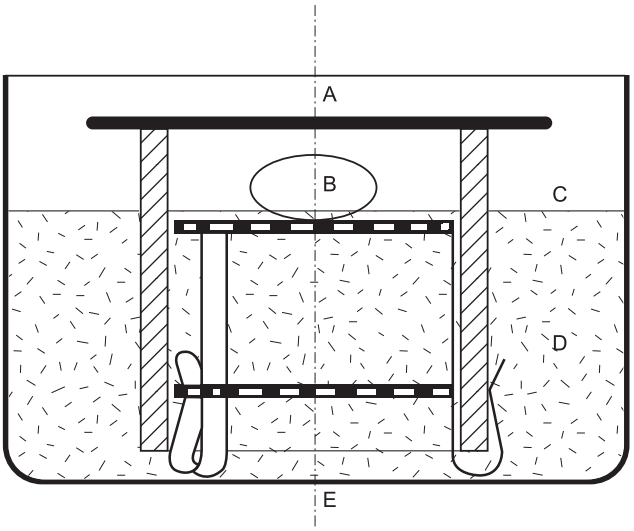


A. glass plate
B. vaginal tablet
C. water surface
D. water
E. dish, beaker

Figure 2.9.2.-2.

**01/2016:20903**

## 2.9.3. DISSOLUTION TEST FOR SOLID DOSAGE FORMS[(2)]

This test is provided to determine compliance with the dissolution requirements for solid dosage forms administered orally. In this chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified.

APPARATUS

**Apparatus 1 (Basket apparatus).** The assembly consists of the following: a vessel, which may be covered, made of glass or other inert, transparent material[(3)]; a motor; a drive shaft; and a cylindrical basket (stirring element). The vessel is partially immersed in a suitable water-bath of any convenient size or heated by a suitable device such as a heating jacket. The water-bath or heating device permits maintaining the temperature inside the vessel at 37 ± 0.5 °C during the test and keeping the dissolution medium in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. Apparatus that permits observation of the preparation and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and a capacity of 1 L. Its height is 160-210 mm and its inside diameter is 98-106 mm. Its sides are flanged at the top. A fitted cover may be used to retard evaporation[(4)]. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly and without significant wobble that could affect the results. A

(2) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*
(3) The materials must not sorb, react, or interfere with the preparation to be tested.
(4) If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of samples.

speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at a specified rate, within ± 4 per cent.

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316 or equivalent, to the specifications shown in Figure 2.9.3.-1.

A basket having a gold coating of about 2.5 µm (0.0001 inch) thick may be used. The dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm during the test.

**Apparatus 2 (Paddle apparatus)**. Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm from the vertical axis of the vessel, at any point, and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in Figure 2.9.3.-2. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test. The metallic or suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of non-reactive material, such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Figure 2.9.3.-3. Other validated sinker devices may be used.

**Apparatus 3 (Reciprocating cylinder)**. The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material) and screens that are made of suitable nonsorbing and nonreactive material, and that are designed to fit the tops and bottoms of the reciprocating cylinders; a motor and drive assembly to reciprocate the cylinders vertically inside the vessels, and if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water-bath of any convenient size that permits holding the temperature at 37 ± 0.5 °C during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be selected and maintained at the specified dip rate, within ± 5 per cent. An apparatus that permits observation of the preparations and reciprocating cylinders is preferable. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The components conform to the dimensions shown in Figure 2.9.3.-4 unless otherwise specified.

**Apparatus 4 (Flow-through cell)**. The assembly consists of a reservoir and a pump for the dissolution medium; a flow-through cell; a water-bath that maintains the dissolution medium at 37 ± 0.5 °C. Use the specified cell size.

The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range between 240 mL/h and 960 mL/h, with standard flow rates of 4 mL/min, 8 mL/min, and 16 mL/min. It must deliver a constant flow (± 5 per cent of the nominal flow rate); the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses/min. A pump without pulsation may also be used. Dissolution test procedures using the flow-through cell must be characterised with respect to rate and any pulsation.

The flow-through cell (see Figures 2.9.3.-5 and 2.9.3.-6) of transparent and inert material is mounted vertically, with a filter system that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 mm and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1 mm diameter, with 1 bead of about 5 mm positioned at the apex to protect the fluid entry tube; a tablet holder (see Figures 2.9.3.-5 and 2.9.3.-6) is available for positioning of special dosage forms. The cell is immersed in a water-bath, and the temperature is maintained at 37 ± 0.5 °C.

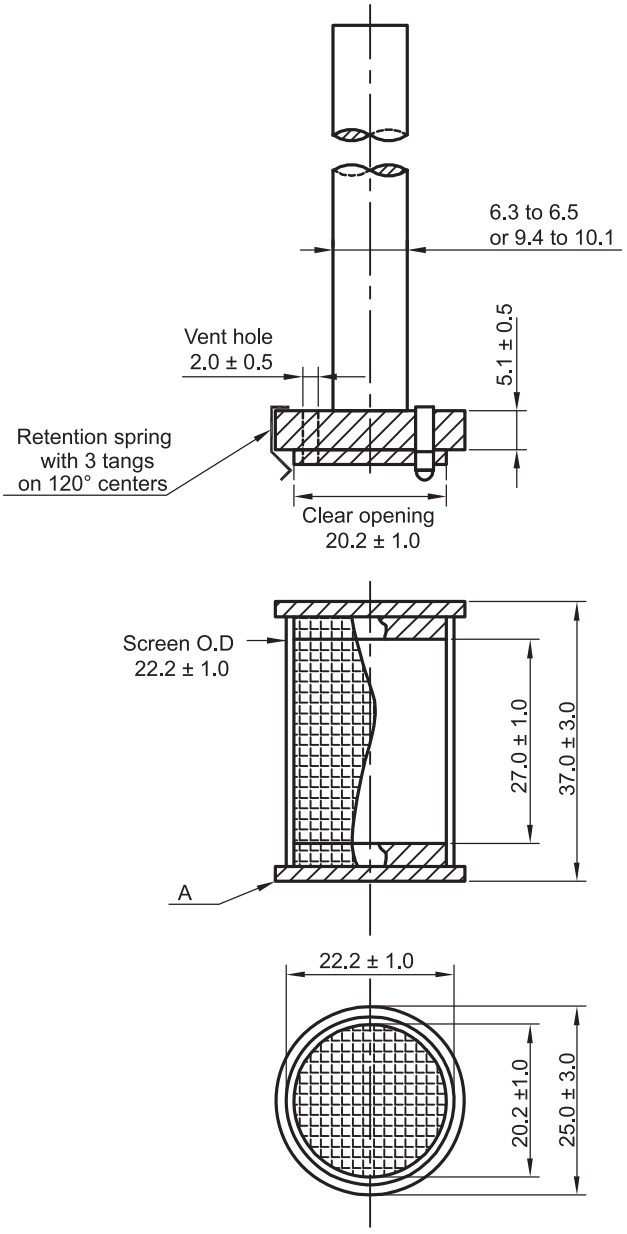


1) Screen with welded seam: 0.22-0.31 mm wire diameter with wire opening of 0.36-0.44 mm. After welding the screen may be slighty altered.

2) Maximum allowable runout at "A" is 1.0 mm when the part is rotated on center line axis with basket mounted.

Figure 2.9.3.-1. – *Apparatus 1, Basket stirring element*

*Dimensions in millimetres*

The apparatus uses a clamp mechanism and 2 O-rings for the fixation of the cell assembly. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump must not be on a level higher than the reservoir flasks.

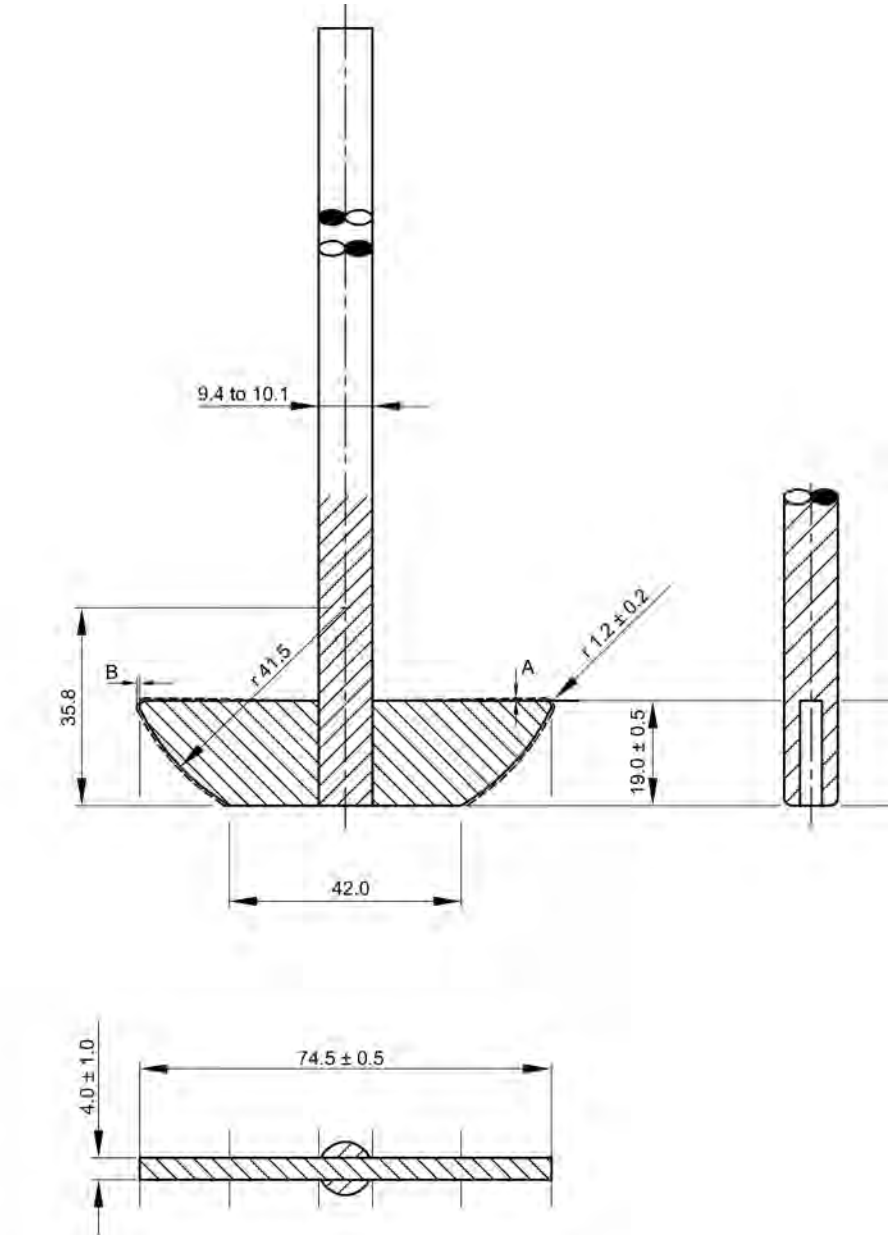


A and B dimensions do not vary more than 0.5 mm when part is rotated on center line axis. Tolerances are ± 1.0 mm unless otherwise stated.

Figure 2.9.3.-2. – *Apparatus 2, Paddle stirring element*
*Dimensions in millimetres*

Tube connections are as short as possible. Use suitably inert tubing, such as polytetrafluoroethylene, with a 1.6 mm inner diameter and inert flanged-end connections.

*Apparatus suitability.* The determination of suitability of the apparatus to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotation speed (Apparatus 1 and 2), dip rate (Apparatus 3), and flow rate of medium (Apparatus 4).

Determine the acceptable performance of the dissolution test assembly periodically.

PROCEDURE

*APPARATUS 1 AND 2*

**Conventional-release solid dosage forms**

*Procedure.* Place the stated volume of the dissolution medium (± 1 per cent) in the vessel of the specified apparatus. Assemble the apparatus, equilibrate the dissolution medium to 37 ± 0.5 °C, and remove the thermometer. ◊The test may also be carried out with the thermometer in place, provided it is shown that results equivalent to those obtained without the thermometer are obtained.◊

Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit. Operate the apparatus at the specified rate. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the dissolution medium and the top of the rotating basket or blade, not less than 1 cm from the vessel wall. Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh dissolution medium at 37 °C or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test and verify

the temperature of the medium at suitable times. Perform the analysis using a suitable assay method[5]. Repeat the test with additional dosage units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the apparatus described in this chapter, is necessary.

*Dissolution medium*. A suitable dissolution medium is used. The volume specified refers to measurements made between 20 °C and 25 °C. If the dissolution medium is a buffered solution, adjust the solution so that its pH is within 0.05 units of the specified pH. Dissolved gases can cause bubbles to form, which may change the results of the test. In such cases, dissolved gases must be removed prior to testing[6].

*Time*. Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. Samples are to be withdrawn only at the stated times, within a tolerance of ± 2 per cent.

**Prolonged-release solid dosage forms**

*Procedure*. Proceed as described for conventional-release dosage forms.

*Dissolution medium*. Proceed as described for conventional-release dosage forms.

*Time*. The test-time points, generally 3, are expressed in hours.

**Delayed-release solid dosage forms**

*Procedure*. Use Method A or Method B.

Method A

- *Acid stage*. Place 750 mL of *0.1 M hydrochloric acid* in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5 °C. Place 1 dosage unit in the apparatus, cover the vessel and operate the apparatus at the specified rate. After 2 h of operation in *0.1 M hydrochloric acid*, withdraw an aliquot of the fluid and proceed immediately as directed under Buffer stage. Perform an analysis of the aliquot using a suitable assay method.
- *Buffer stage*. Complete the operations of adding the buffer and adjusting the pH within 5 min. With the apparatus operating at the rate specified, add to the fluid in the vessel 250 mL of a 0.20 M solution of *trisodium phosphate dodecahydrate R* that has been equilibrated to 37 ± 0.5 °C. Adjust, if necessary, with *2 M hydrochloric acid R* or *2 M sodium hydroxide R* to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 min, or for the specified time. At the end of the time period, withdraw an aliquot of the fluid and perform the analysis using a suitable assay method.

Method B

- *Acid Stage*. Place 1000 mL of *0.1 M hydrochloric acid* in the vessel and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5 °C. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate. After 2 h of operation in *0.1 M hydrochloric acid*, withdraw an aliquot of the fluid, and proceed immediately as directed under Buffer stage. Perform an analysis of the aliquot using a suitable assay method.
- *Buffer stage*. For this stage of the procedure use buffer that has previously been equilibrated to a temperature of 37 ± 0.5 °C. Drain the acid from the vessel and add 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 3 volumes of *0.1 M hydrochloric acid* with 1 volume of a 0.20 M solution of *trisodium phosphate dodecahydrate R* and adjusting, if necessary, with *2 M hydrochloric acid R* or *2 M sodium hydroxide R* to a pH of 6.8 ± 0.05. This may also be accomplished by removing from the apparatus the vessel containing the acid and replacing it with another vessel, containing the buffer and transferring the dosage unit to the vessel containing the buffer. Continue to operate the apparatus for 45 min, or for the specified time. At the end of the time period, withdraw an aliquot of the fluid and perform the analysis using a suitable assay method.

*Time*. All test times stated are to be observed within a tolerance of ± 2 per cent, unless otherwise specified.

*APPARATUS 3*

**Conventional-release solid dosage forms**

*Procedure*. Place the stated volume of the dissolution medium (± 1 per cent) in each vessel of the apparatus. Assemble the apparatus, equilibrate the dissolution medium to 37 ± 0.5 °C, and remove the thermometer. Place 1 dosage unit in each of the reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9-10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the medium from a zone midway between the surface of the dissolution medium and the bottom of each vessel. Perform the analysis as directed. If necessary, repeat the test with additional dosage units.

Replace the aliquot withdrawn for analysis with equal volumes of fresh dissolution medium at 37 °C or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered with the evaporation cap for the duration of the test and verify the temperature of the medium at suitable times.

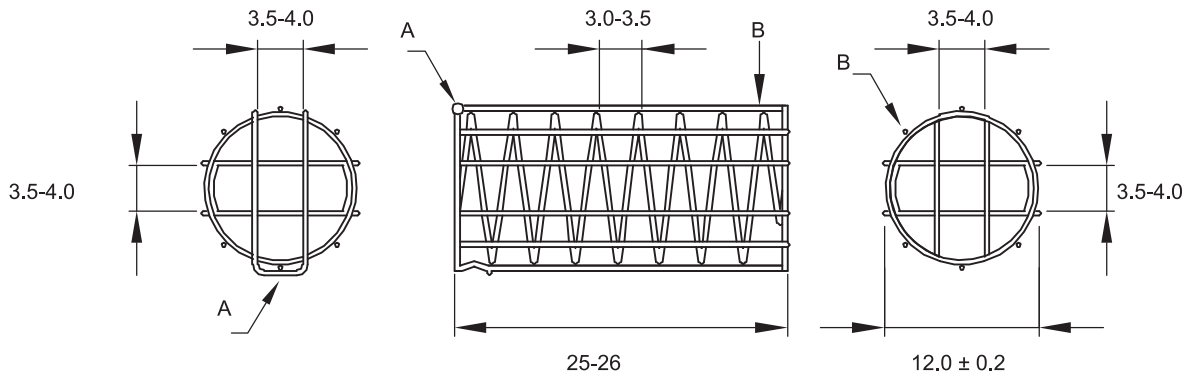


A: acid-resistant wire clasp B: acid-resistant wire support

Figure 2.9.3.-3. – *Alternative sinker*
*Dimensions in millimetres*

(5) Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active substance or contain extractable substances that would interfere with the analysis.
(6) A method of deaeration is as follows: heat the medium, while stirring gently, to about 41 °C, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other validated deaeration techniques for removal of dissolved gases may be used.

*Dissolution medium.* Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

*Time.* Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

**Prolonged-release dosage forms**

*Procedure.* Proceed as described for conventional-release dosage forms under Apparatus 3.

*Dissolution medium.* Proceed as described for prolonged-release dosage forms under Apparatus 1 and 2.

*Time.* Proceed as described for prolonged-release dosage forms under Apparatus 1 and 2.

**Delayed-release dosage forms**

*Procedure.* Proceed as described for delayed-release dosage forms, Method B, under Apparatus 1 and 2, using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media, and using the volume of medium specified (usually 300 mL).

*Time.* Proceed as directed for delayed-release dosage forms under Apparatus 1 and 2.

*APPARATUS 4*

**Conventional-release dosage forms**

*Procedure.* Place the glass beads into the cell specified. Place 1 dosage unit on top of the beads or, if specified, on a wire carrier. Assemble the filter head and fix the parts together by means of a suitable clamping device. Introduce by the pump the dissolution medium warmed to 37 ± 0.5 °C through the bottom of the cell to obtain the flow rate specified and measured with an accuracy of 5 per cent. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed. Repeat the test with additional dosage units.

*Dissolution medium.* Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

*Time.* Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

**Prolonged-release dosage forms**

*Procedure.* Proceed as described for conventional-release dosage forms under Apparatus 4.

*Dissolution medium.* Proceed as described for conventional-release dosage forms under Apparatus 4.

*Time.* Proceed as described for conventional-release dosage forms under Apparatus 4.

**Delayed-release dosage forms**

*Procedure.* Proceed as described for delayed-release dosage forms under Apparatus 1 and 2, using the specified media.

*Time.* Proceed as described for delayed-release dosage forms under Apparatus 1 and 2.

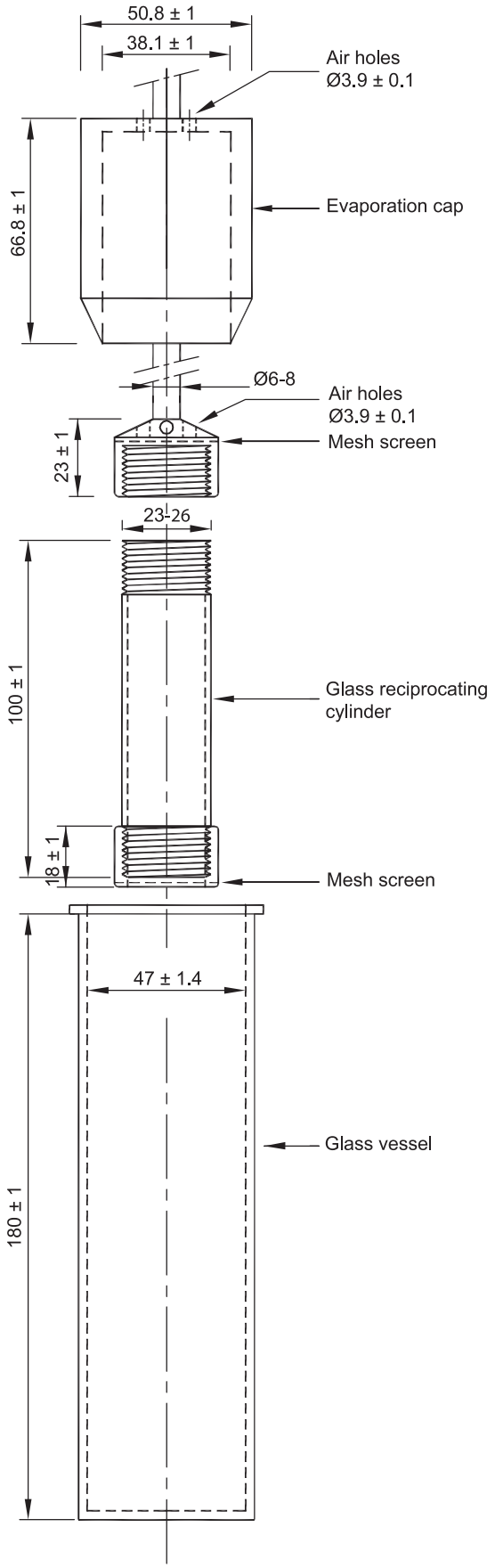


Figure 2.9.3.-4. – *Apparatus 3, glass vessel and reciprocating cylinder*
*Dimensions in millimetres*

INTERPRETATION

**Conventional-release solid dosage forms**

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units tested conform to Table 2.9.3.-1. Continue testing through the 3 levels unless the results conform at either $S_1$ or $S_2$. The quantity $Q$, is the specified amount of dissolved active substance, expressed as a percentage of the labelled content; the 5 per cent, 15 per cent, and 25 per cent values in the Table are percentages of the labelled content so that these values and $Q$ are in the same terms.

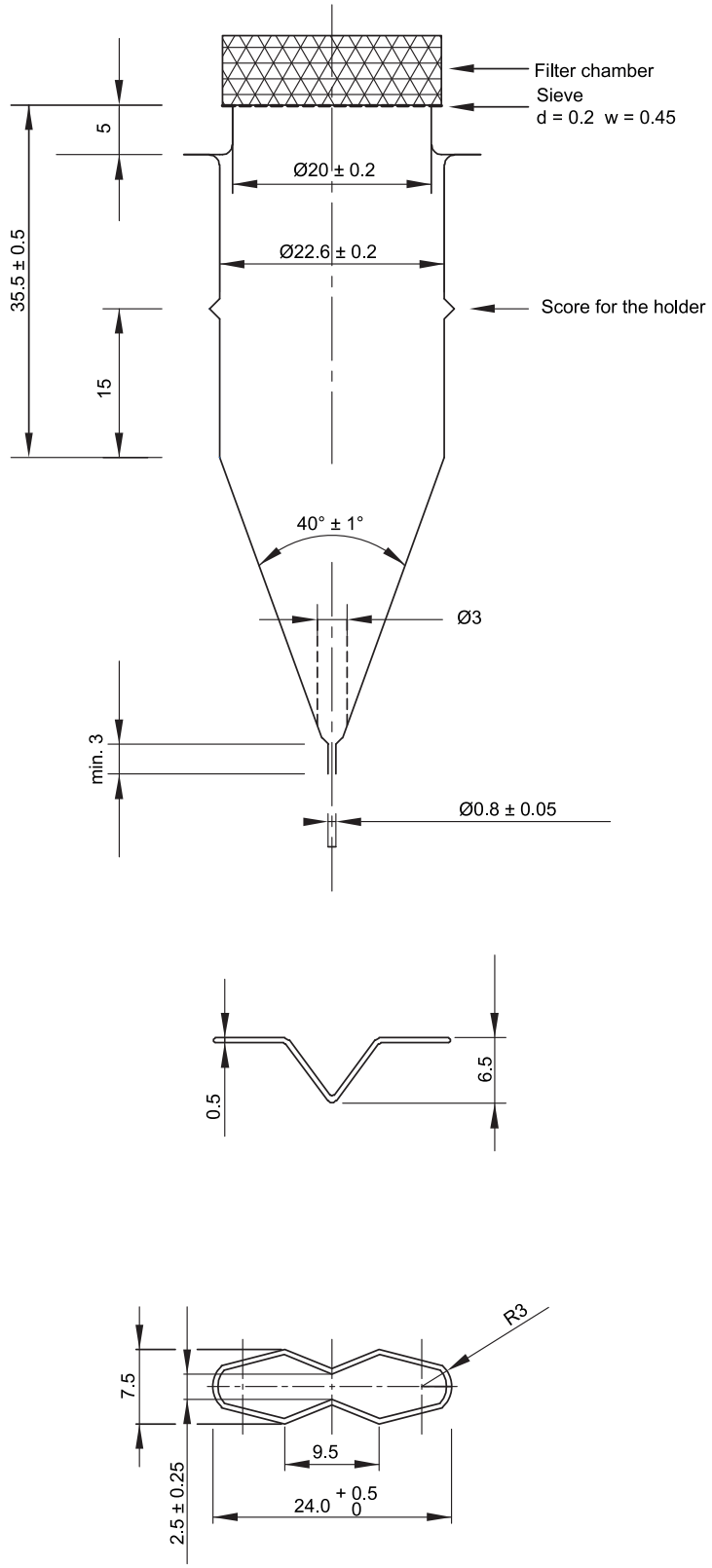


Figure 2.9.3.-5. – *Apparatus 4, large cell for tablets and capsules (top), tablet holder for the large cell (bottom)*

*Dimensions in millimetres unless otherwise specified*

Table 2.9.3.-1

| Level | Number tested | Acceptance criteria |
|---|---|---|
| $S_1$ | 6 | Each unit is not less than $Q$ + 5 per cent. |
| $S_2$ | 6 | Average of 12 units ($S_1$ + $S_2$) is equal to or greater than $Q$, and no unit is less than $Q$ − 15 per cent. |
| $S_3$ | 12 | Average of 24 units ($S_1$ + $S_2$ + $S_3$) is equal to or greater than $Q$, not more than 2 units are less than $Q$ − 15 per cent, and no is less than $Q$ − 25 per cent. |

**Prolonged-release dosage forms**

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units tested conform to Table 2.9.3.-2. Continue testing through the 3 levels unless the results conform at either $L_1$ or $L_2$. Limits on the amounts of active substance dissolved are expressed in terms of the percentage of labelled content. The limits embrace each value of $Q_i$, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified, the acceptance criteria apply individually to each range.

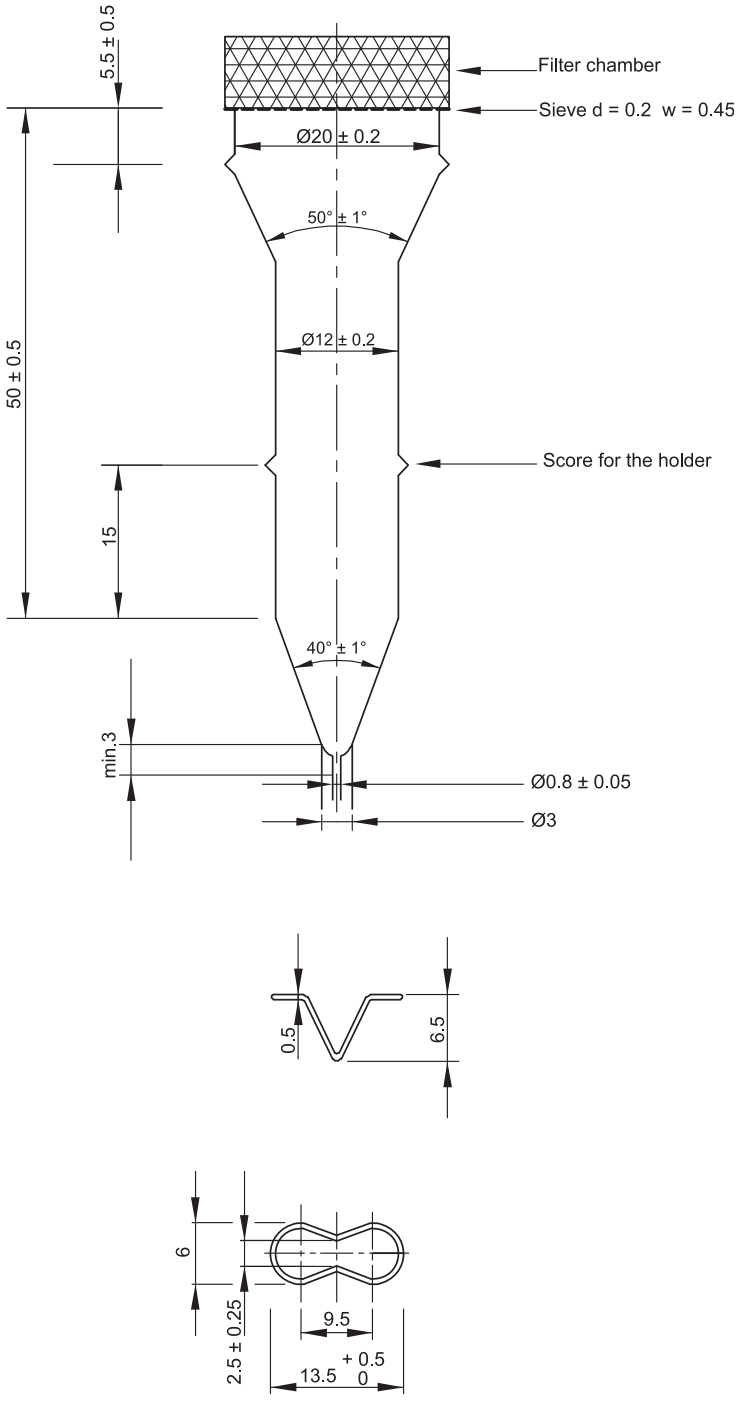


Figure 2.9.3.-6. – *Apparatus 4, small cell for tablets and capsules (top), tablet holder for the small cell (bottom)*

*Dimensions in millimetres unless otherwise specified*

Table 2.9.3.-2

| Level | Number tested | Acceptance criteria |
|---|---|---|
| $L_1$ | 6 | No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time. |
| $L_2$ | 6 | The average value of the 12 units ($L_1 + L_2$) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10 per cent of labelled content outside each of the stated ranges; and none is more than 10 per cent of labelled content below the stated amount at the final test time. |
| $L_3$ | 12 | The average value of the 24 units ($L_1 + L_2 + L_3$) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10 per cent of labelled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10 per cent of labelled content below the stated amount at the final test time; and none of the units is more than 20 per cent of labelled content outside each of the stated ranges or more than 20 per cent of labelled content below the stated amount at the final test time. |

**Delayed-release dosage forms**

*Acid stage.* Unless otherwise specified, the requirements of this portion of the test are met if the quantities, based on the percentage of the labelled content of active substance dissolved from the units tested conform to Table 2.9.3.-3. Continue testing through the 3 levels unless the results of both acid and buffer stages conform at an earlier level.

Table 2.9.3.-3

| Level | Number tested | Acceptance criteria |
|---|---|---|
| $A_1$ | 6 | No individual value exceeds 10 per cent dissolved. |
| $A_2$ | 6 | The average value of the 12 units ($A_1 + A_2$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved. |
| $A_3$ | 12 | The average value of the 24 units ($A_1 + A_2 + A_3$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved. |

*Buffer stage.* Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the units tested conform to Table 2.9.3.-4. Continue testing through the 3 levels unless the results of both stages conform at an earlier level. The value of *Q* in Table 2.9.3.-4 is 75 per cent dissolved unless otherwise specified. The quantity, *Q*, is the specified total amount of active substance dissolved in both the acid and buffer stages, expressed as a percentage of the labelled content. The 5 per cent, 15 per cent and 25 per cent values in the Table are percentages of the labelled content so that these values and *Q* are in the same terms.

Table 2.9.3.-4

| Level | Number tested | Acceptance criteria |
|---|---|---|
| $B_1$ | 6 | No unit is less than $Q$ + 5 per cent. |
| $B_2$ | 6 | The average value of the 12 units ($B_1 + B_2$) is equal to or greater than $Q$, and no unit is less than $Q$ − 15 per cent. |
| $B_3$ | 12 | The average value of the 24 units ($B_1 + B_2 + B_3$) is equal to or greater than $Q$, not more than 2 units are less than $Q$ − 15 per cent, and no unit is less than $Q$ − 25 per cent. |

*Recommendations on dissolution testing are given in general chapter 5.17.1.*

**01/2008:20904**

# 2.9.4. DISSOLUTION TEST FOR TRANSDERMAL PATCHES

This test is used to determine the dissolution rate of the active ingredients of transdermal patches.

1. DISK ASSEMBLY METHOD

**Equipment**. Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*) with the addition of a stainless steel disk assembly (SSDA) in the form of a net with an aperture of 125 µm (see Figure 2.9.4.-1).

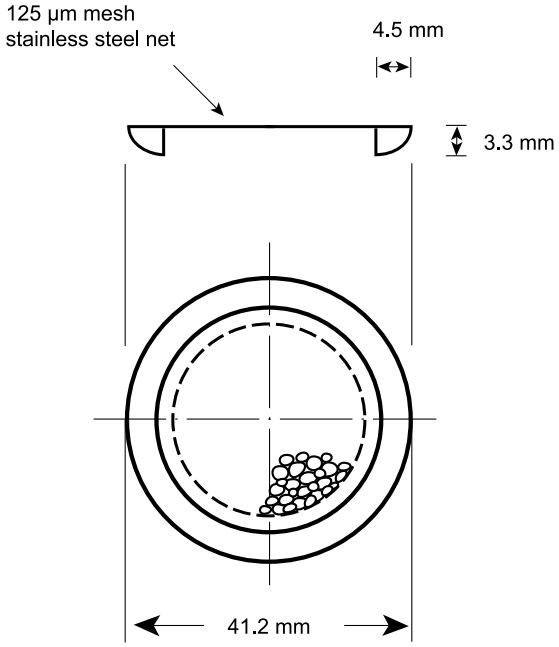


Figure 2.9.4.-1. – *Disk assembly*

The SSDA holds the system at the bottom of the vessel and is designed to minimise any dead volume between the SSDA and the bottom of the vessel. The SSDA holds the patch flat, with the release surface uppermost and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm between the bottom of the paddle blade and the surface of the SSDA is maintained during the test (see Figure 2.9.4.-2). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

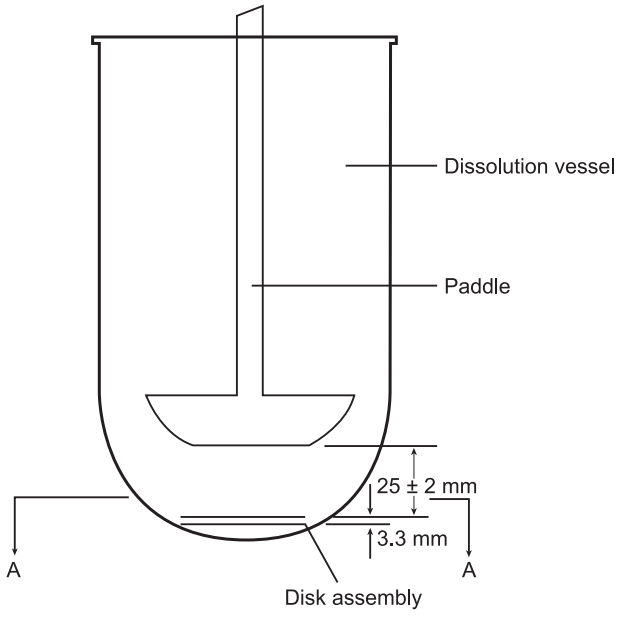


Figure 2.9.4.-2. – *Paddle and disk*

**Procedure**. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Apply the patch to the SSDA, ensuring that the release surface of the patch is as flat as possible. The patch may be attached to the SSDA by a

prescribed adhesive or by a strip of a double-sided adhesive tape. The adhesive or tape are previously tested for the absence of interference with the assay and of adsorption of the active ingredient(s). Press the patch, release surface facing up, onto the side of the SSDA made adhesive. The applied patch must not overlap the borders of the SSDA. For this purpose and provided that the preparation is homogeneous and uniformly spread on the outer covering, an appropriate and exactly measured piece of the patch may be cut and used for testing the dissolution rate. This procedure may also be necessary to achieve appropriate sink conditions. This procedure must not be applied to membrane-type patches. Place the patch mounted on the SSDA flat at the bottom of the vessel with the release surface facing upwards. Immediately rotate the paddle at 100 r/min, for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

2. CELL METHOD

**Equipment**. Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*) with the addition of the extraction cell (*cell*).

The *cell* is made of chemically inert materials and consists of a *support*, a *cover* and, if necessary, a *membrane* placed on the patch to isolate it from the medium that may modify or adversely affect the physico-chemical properties of the patch (see Figure 2.9.4.-3).

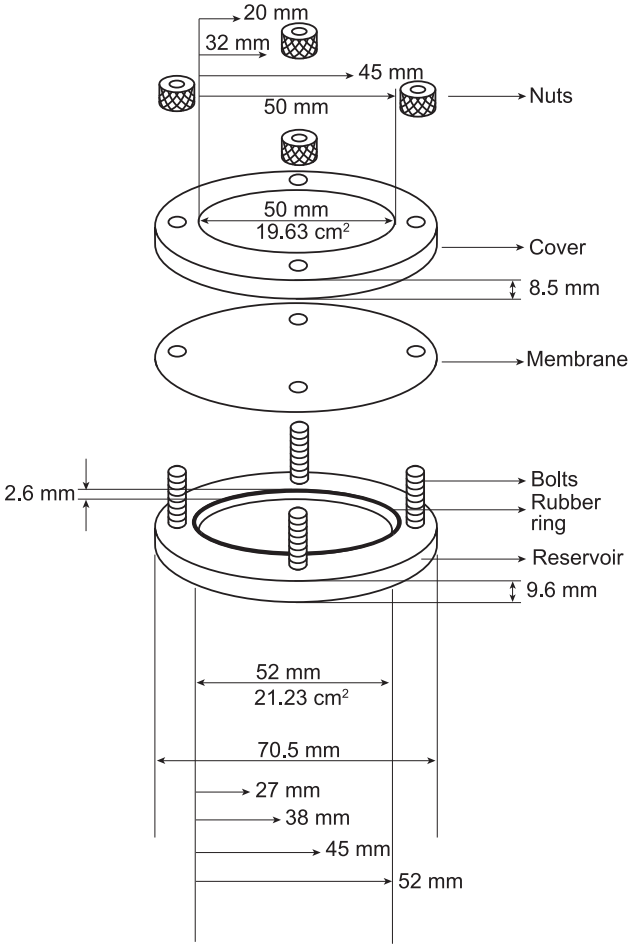


Figure 2.9.4.-3. – *Extraction cell*

*Support*. The central part of the support forms a cavity intended to hold the patch. The cavity has a depth of 2.6 mm and a diameter that is appropriate to the size of the patch to be examined. The following diameters can be used: 27 mm, 38 mm, 45 mm, 52 mm, corresponding to volumes of 1.48 mL, 2.94 mL, 4.13 mL, 5.52 mL, respectively.

*Cover*. The cover has a central opening with a diameter selected according to the size of the patch to be examined. The patch can thus be precisely centred, and its releasing surface limited. The following diameters may be used: 20 mm, 32 mm, 40 mm, 50 mm corresponding to areas of 3.14 $cm^2$, 8.03 $cm^2$, 12.56 $cm^2$, 19.63 $cm^2$, respectively. The cover is held in place by nuts screwed onto bolts projecting from the support. The cover is sealed to the support by a rubber ring set on the reservoir.

*Extraction cell*. The *cell* holds the patch flat, with the release surface uppermost and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm is maintained between the paddle blade and the surface of the patch (see Figure 2.9.4.-4). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

**Procedure**. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Precisely centre the patch in the *cell* with the releasing surface uppermost. Close the *cell*, if necessary applying a hydrophobic substance (for example, petrolatum) to the flat surfaces to ensure the seal, and ensure that the patch stays in place. Introduce the cell flat into the bottom of the vessel with the cover facing upwards. Immediately rotate the paddle, at 100 r/min for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the paddle blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

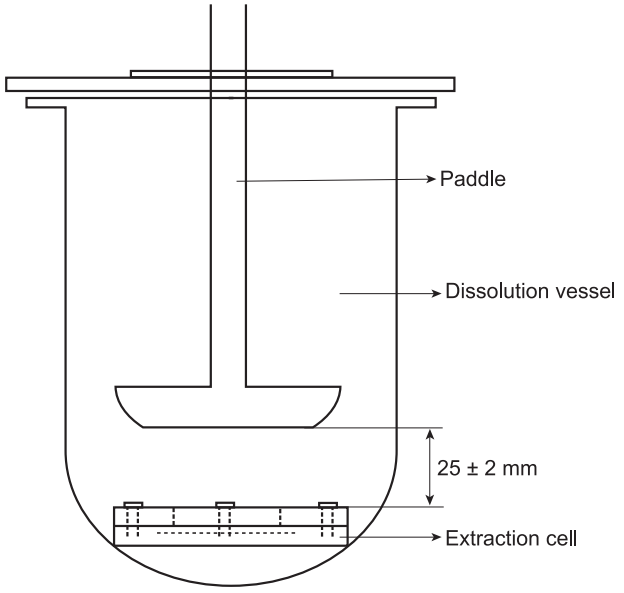


Figure 2.9.4.-4. – *Paddle over extraction cell*

3. ROTATING CYLINDER METHOD

**Equipment**. Use the assembly of the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*). Replace the paddle and shaft with a stainless steel cylinder stirring element (*cylinder*) (see Figure 2.9.4.-5). The patch is placed on the *cylinder* at the beginning of each test. The distance between the inside bottom of the vessel and the *cylinder* is maintained at 25 ± 2 mm during the test. The temperature is maintained at 32 ± 0.5 °C. The vessel is covered during the test to minimise evaporation.

**Procedure**. Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Remove the protective liner from

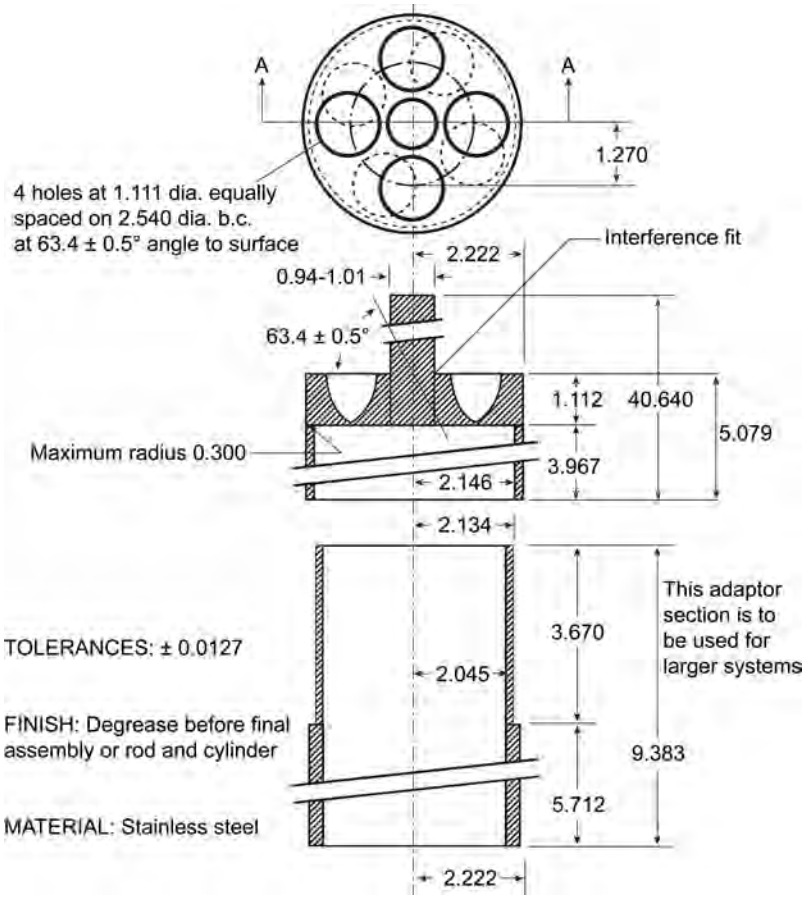


Figure 2.9.4.-5. – *Cylinder stirring element*

*Dimensions in centimetres*

the patch and place the adhesive side on a piece of suitable inert porous membrane that is at least 1 cm larger on all sides than the patch. Place the patch on a clean surface with the membrane in contact with this surface. Two systems for adhesion to the *cylinder* may be used:

– apply a suitable adhesive to the exposed membrane borders and, if necessary, to the back of the patch,

– apply a double-sided adhesive tape to the external wall of the *cylinder*.

Using gentle pressure, carefully apply the non-adhesive side of the patch to the *cylinder*, so that the release surface is in contact with the dissolution medium and the long axis of the patch fits around the circumference of the *cylinder*.

The system for adhesion used is previously tested for absence of interference with the assay and of adsorption of the active ingredient(s).

Place the *cylinder* in the apparatus, and immediately rotate the *cylinder* at 100 r/min, for example. At determined intervals, withdraw a sample of dissolution medium from a zone midway between the surface of the dissolution medium and the top of the rotating *cylinder*, and not less than 1 cm from the vessel wall.

Perform the assay on each sample as directed in the individual monograph, correcting for any volume withdrawn, as necessary. Repeat the test with additional patches.

**Interpretation**. The requirements are met if the quantity of active ingredient(s) released from the patch, expressed as the amount per surface area per time unit, is within the prescribed limits at the defined sampling times.

**01/2008:20905**

## 2.9.5. UNIFORMITY OF MASS OF SINGLE-DOSE PREPARATIONS

Weigh individually 20 units taken at random or, for single-dose preparations presented in individual containers, the contents of 20 units, and determine the average mass. Not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation shown in Table 2.9.5.-1 and none deviates by more than twice that percentage.

For capsules and powders for parenteral administration, proceed as described below.

CAPSULES

Weigh an intact capsule. Open the capsule without losing any part of the shell and remove the contents as completely as possible. For soft shell capsules, wash the shell with a suitable solvent and allow to stand until the odour of the solvent is no longer perceptible. Weigh the shell. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 capsules.

Table 2.9.5.-1

| Pharmaceutical Form | Average Mass | Percentage deviation |
|---|---|---|
| Tablets (uncoated and film-coated) | 80 mg or less | 10 |
| | More than 80 mg and less than 250 mg | 7.5 |
| | 250 mg or more | 5 |
| Capsules, granules (uncoated, single-dose) and powders (single-dose) | Less than 300 mg | 10 |
| | 300 mg or more | 7.5 |
| Powders for parenteral administration* (single-dose) | More than 40 mg | 10 |
| Suppositories and pessaries | All masses | 5 |
| Powders for eye-drops and powders for eye lotions (single-dose) | Less than 300 mg | 10 |
| | 300 mg or more | 7.5 |

* When the average mass is equal to or below 40 mg, the preparation is not submitted to the test for uniformity of mass but to the test for uniformity of content of single-dose preparations (*2.9.6*).

POWDERS FOR PARENTERAL ADMINISTRATION

Remove any paper labels from a container and wash and dry the outside. Open the container and without delay weigh the container and its contents. Empty the container as completely as possible by gentle tapping, rinse it if necessary with *water R* and then with *alcohol R* and dry at 100-105 °C for 1 h, or, if the nature of the container precludes heating at this temperature, dry at a lower temperature to constant mass. Allow to cool in a desiccator and weigh. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 containers.

**01/2017:20906**

## 2.9.6. UNIFORMITY OF CONTENT OF SINGLE-DOSE PREPARATIONS

The test for uniformity of content of single-dose preparations is based on the assay of the individual contents of active substance(s) of a number of single-dose units to determine whether the individual contents are within limits set with reference to the average content of the sample.

The test is not required for multivitamin and trace-element preparations and in other justified and authorised circumstances.

*Method.* Using a suitable analytical method, determine the individual contents of active substance(s) of 10 dosage units taken at random.

Apply the criteria of test A, test B or test C as specified in the monograph for the dosage form in question.

TEST A

The preparation complies with the test if each individual content is between 85 per cent and 115 per cent of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75 per cent to 125 per cent of the average content.

If one individual content is outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than one of the individual contents of the 30 units is outside 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

TEST B

The preparation complies with the test if not more than one individual content is outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content. The preparation fails to comply with the test if more than 3 individual contents are outside the limits of 85 per cent to 115 per cent of the average content or if one or more individual contents are outside the limits of 75 per cent to 125 per cent of the average content.

If 2 or 3 individual contents are outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than 3 individual contents of the 30 units are outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

TEST C

The preparation complies with the test if the average content of the 10 dosage units is between 90 per cent and 110 per cent of the content stated on the label and if the individual content of each dosage unit is between 75 per cent and 125 per cent of the average content.

**01/2010:20907**

## 2.9.7. FRIABILITY OF UNCOATED TABLETS(7)

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablet friability supplements other physical strength measurements, such as tablet breaking force.

Use a drum, with an internal diameter between 283-291 mm and a depth between 36-40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see Figure 2.9.7.-1.). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5-85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5-25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 r/min. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

For tablets with a unit mass equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit mass of more than 650 mg, take a sample of 10 whole tablets. The tablets are carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the targeted value, the test is repeated twice and the mean of the 3 tests determined. A

(7) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

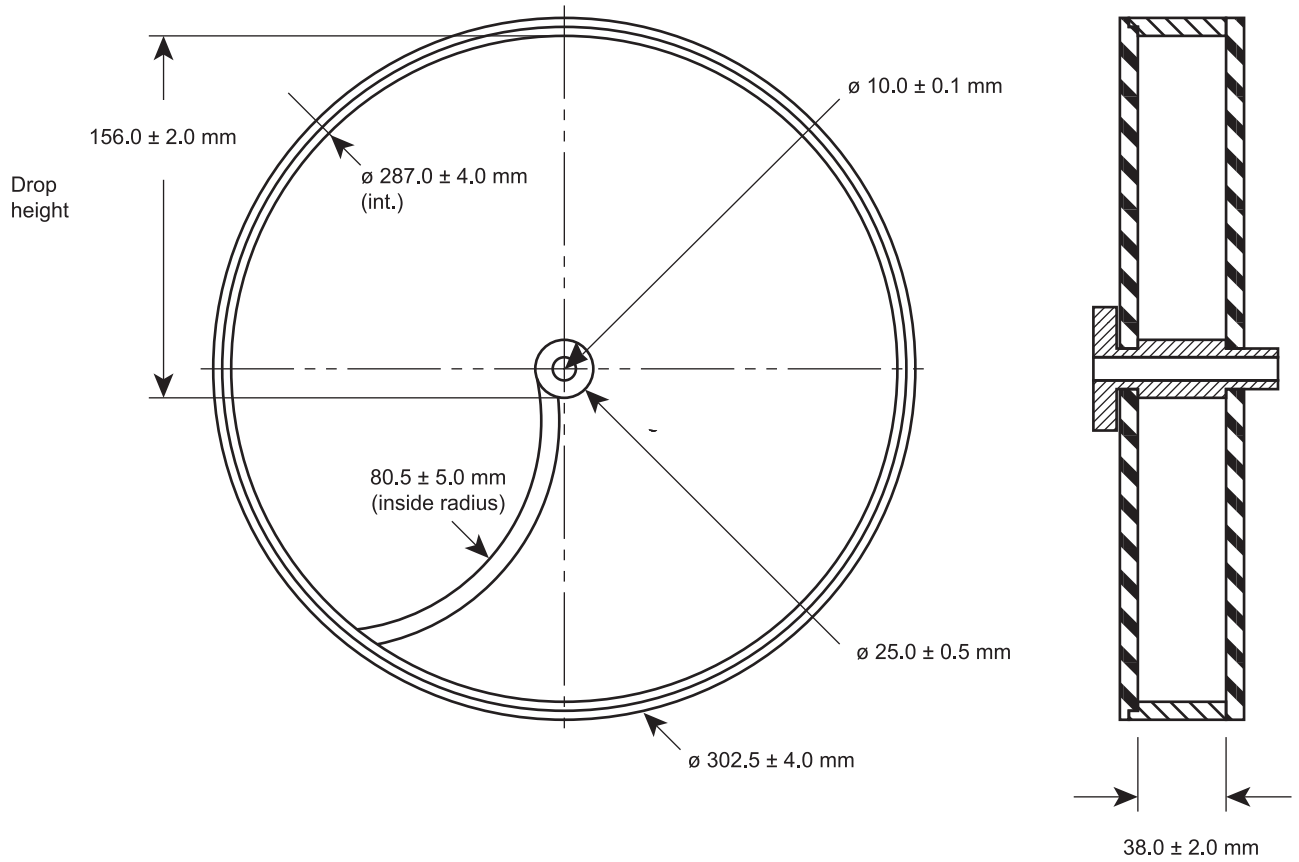


Figure 2.9.7.-1. – *Tablet friability apparatus*

maximum loss of mass (obtained from a single test or from the mean of 3 tests) not greater than 1.0 per cent is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, a humidity-controlled environment is required for testing.

A drum with dual scooping projections, or apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

**01/2008:20908**

## 2.9.8. RESISTANCE TO CRUSHING OF TABLETS

This test is intended to determine, under defined conditions, the resistance to crushing of tablets, measured by the force needed to disrupt them by crushing.

APPARATUS

The apparatus consists of 2 jaws facing each other, one of which moves towards the other. The flat surfaces of the jaws are perpendicular to the direction of movement. The crushing surfaces of the jaws are flat and larger than the zone of contact with the tablet. The apparatus is calibrated using a system with a precision of 1 newton.

OPERATING PROCEDURE

Place the tablet between the jaws, taking into account, where applicable, the shape, the break-mark and the inscription; for each measurement orient the tablet in the same way with respect to the direction of application of the force. Carry out the measurement on 10 tablets, taking care that all fragments of tablets have been removed before each determination.

*This procedure does not apply when fully automated equipment is used.*

EXPRESSION OF RESULTS

Express the results as the mean, minimum and maximum values of the forces measured, all expressed in newtons.

Indicate the type of apparatus and, where applicable, the orientation of the tablets.

**07/2008:20909**

## 2.9.9. MEASUREMENT OF CONSISTENCY BY PENETROMETRY

This test is intended to measure, under determined and validated conditions, the penetration of an object into the product to be examined in a container with a specified shape and size.

APPARATUS

The apparatus consists of a penetrometer made up of a stand and a penetrating object. A suitable apparatus is shown in Figure 2.9.9.-1.

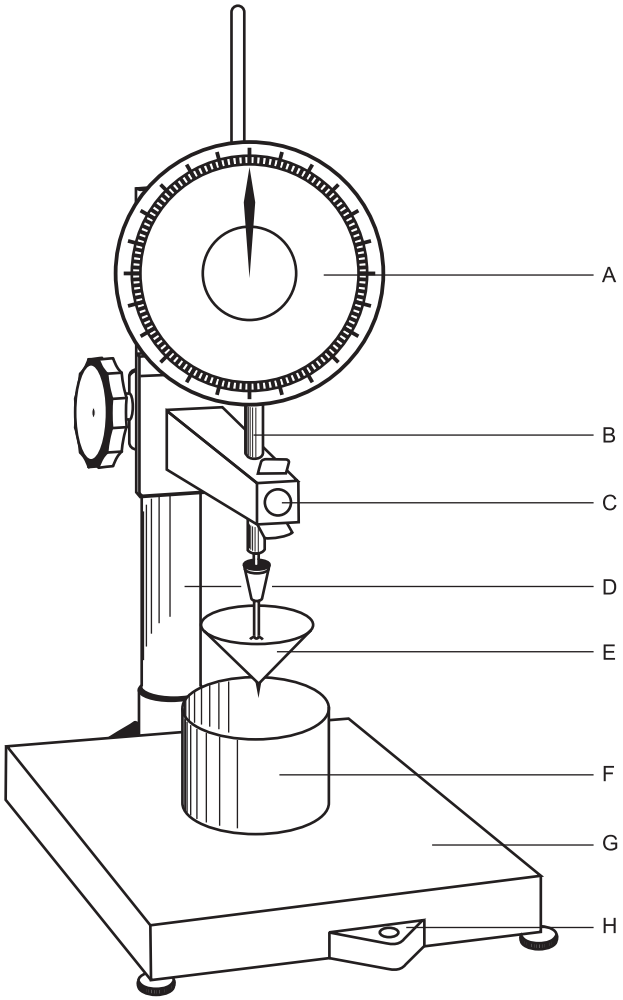


Figure 2.9.9.-1. – *Penetrometer*

A. Scale showing the depth of penetration, graduated in tenths of millimetres.
B. Vertical shaft to maintain and guide the penetrating object.
C. Device to retain and to release the penetrating object automatically and for a constant time.
D. Device to ensure that the penetrating object is vertical and that the base is horizontal.
E. Penetrating object (see Figures 2.9.9.-2 and 3).
F. Container.
G. Horizontal base.
H. Control for the horizontal base.

The stand is made up of:
- a vertical shaft to maintain and guide the penetrating object;
- a horizontal base;
- a device to ensure that the penetrating object is vertical;
- a device to check that the base is horizontal;
- a device to retain and release the penetrating object;
- a scale showing the depth of penetration, graduated in tenths of a millimetre.

The penetrating object, made of a suitable material, has a smooth surface, and is characterised by its shape, size and mass (*m*).

Suitable penetrating objects are shown in Figures 2.9.9.-2 and 2.9.9.-3.

PROCEDURE

Prepare the test samples according to one of the following procedures.

A. Carefully and completely fill 3 containers, without forming air bubbles. Level if necessary to obtain a flat surface. Store the samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed.
B. Store 3 samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed. Apply a suitable shear to the samples for 5 min. Carefully and completely fill 3 containers, without forming air bubbles, and level if necessary to obtain a flat surface.
C. Melt 3 samples and carefully and completely fill 3 containers, without forming air bubbles. Store the samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed.

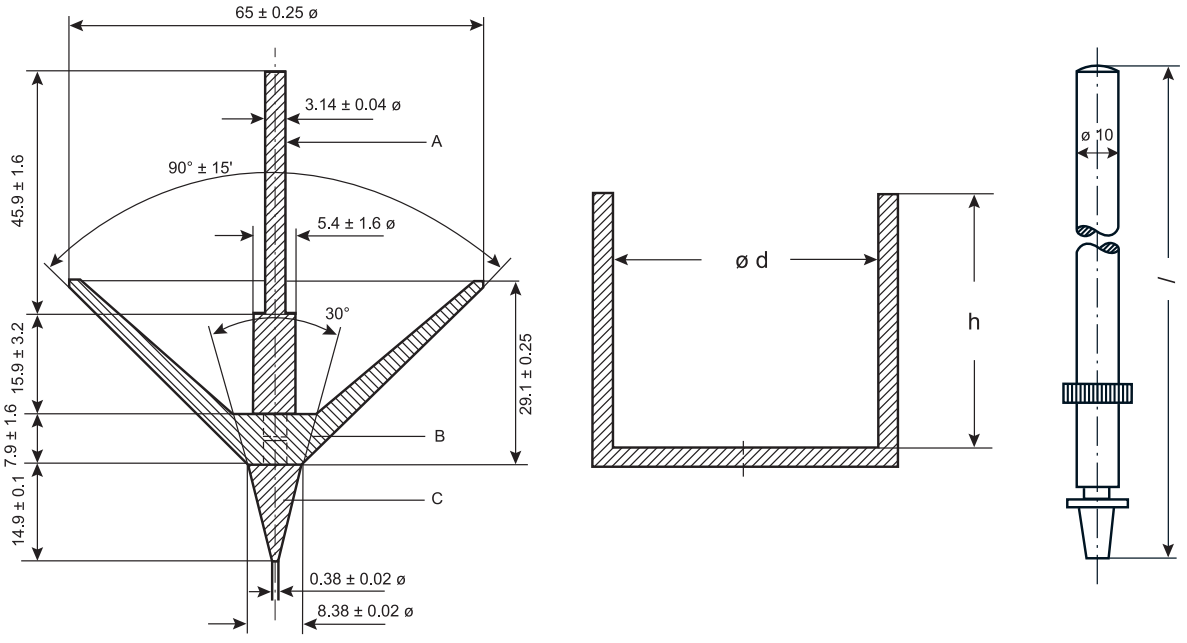


Figure 2.9.9.-2. – *Cone (m = 102.5 ± 0.05 g), suitable container (d = 102 mm or 75 mm; h ≥ 62 mm) and shaft (l = 162 mm; m = 47.5 ± 0.05 g).*
*Dimensions in millimetres*

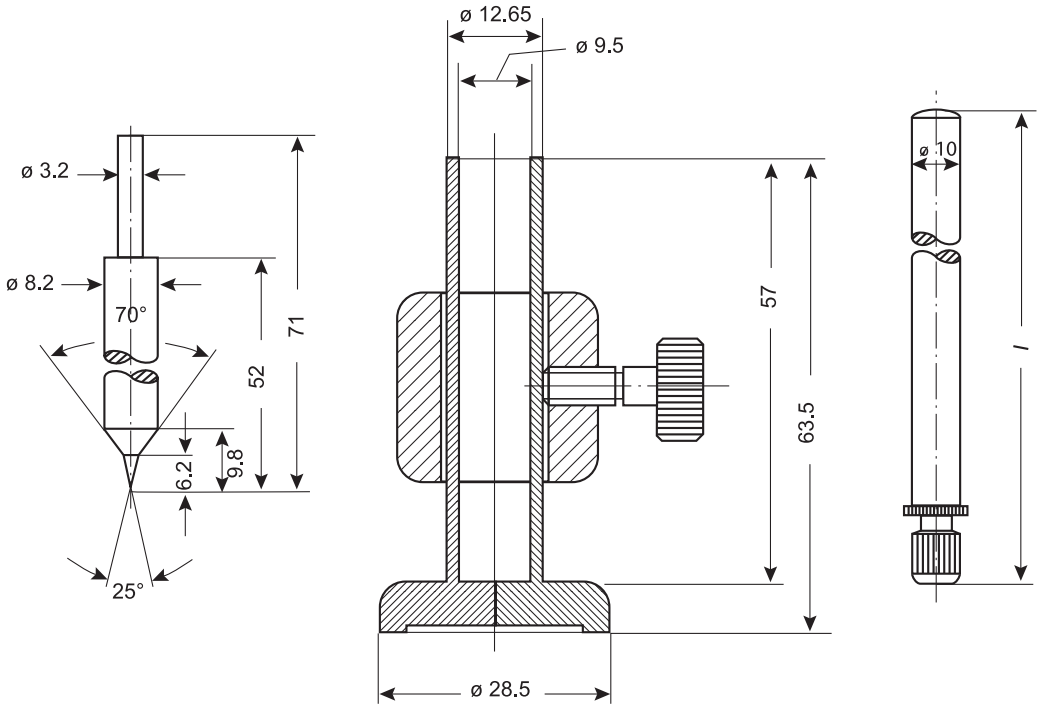


Figure 2.9.9.-3 – *Micro-cone (m = 7.0 g), suitable container and shaft (l = 116 mm; m = 16.8 g)*
*Dimensions in millimetres*

**Determination of penetration**. Place the test sample on the base of the penetrometer. Verify that its surface is perpendicular to the vertical axis of the penetrating object. Bring the temperature of the penetrating object to 25 ± 0.5 °C and then adjust its position such that its tip just touches the surface of the sample. Release the penetrating object and hold it free for 5 s. Clamp the penetrating object and measure the depth of penetration. Repeat the test with the 2 remaining containers.

EXPRESSION OF THE RESULTS

The penetration is expressed in tenths of a millimetre as the arithmetic mean of the 3 measurements. If any of the individual results differ from the mean by more than 3 per cent, repeat the test and express the results of the 6 measurements as the mean and the relative standard deviation.

**07/2019:20910**
**corrected 10.0**

## 2.9.10. ETHANOL CONTENT

*These methods are intended for the examination of liquid pharmaceutical preparations and their ingredients that contain ethanol.*

*The ethanol content of a liquid is expressed as the number of volumes of ethanol contained in 100 volumes of the liquid, the volumes being measured at 20 ± 0.1 °C. This is known as the 'percentage of ethanol by volume' (per cent V/V). The content may also be expressed in grams of ethanol per 100 g of the liquid. This is known as the 'percentage of ethanol by mass' (per cent m/m).*

METHOD A

Where preparations contain dissolved substances, the dissolved substances must be separated from the ethanol that is to be determined by distillation. Where distillation would distil volatile substances other than ethanol and water, the appropriate precautions are stated in the monograph.

The relation between the density at 20 ± 0.1 °C, the relative density (corrected to vacuum) and the ethanol content of a mixture of water and ethanol is given in the tables of the International Organisation for Legal Metrology (1972), International Recommendation No. 22.

**Apparatus**. The apparatus (see Figure 2.9.10.-1) consists of a round-bottomed flask (*A*) fitted with a distillation head (*B*) with a steam trap and attached to a vertical condenser (*C*). The latter is fitted at its lower part with a tube (*D*), which carries the distillate into the lower part of a 100 mL or 250 mL volumetric flask. The volumetric flask is immersed in a mixture of ice and water (*E*) during the distillation. A disc having a circular aperture 6 cm in diameter is placed under the flask (*A*) to reduce the risk of charring any dissolved substances.

**Method**

*Pycnometer method/oscillating transducer density meter method*. Transfer 25.0 mL of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask. Dilute with 100-150 mL of *distilled water R* and add a few pieces of pumice. Attach the distillation head and condenser. Distil and collect not less than 90 mL of distillate in a 100 mL volumetric flask. Adjust the temperature to 20 ± 0.1 °C and dilute to 100.0 mL with *distilled water R* at 20 ± 0.1 °C. Determine the relative density at 20 ± 0.1 °C using a pycnometer or an oscillating transducer density meter.

The values indicated in Table 2.9.10.-1, column 3, are multiplied by 4 to obtain the percentage of ethanol by volume (*V/V*) contained in the preparation. After calculation of the ethanol content using the table, round off the result to 1 decimal place.

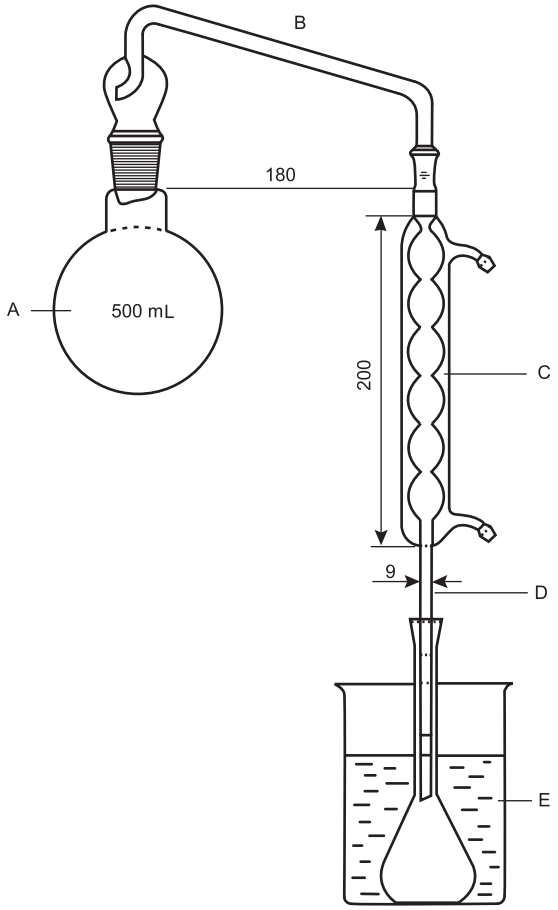


Figure 2.9.10.-1. – *Apparatus for the determination of ethanol content*

*Dimensions in millimetres*

Table 2.9.10.-1. – *Relationship between density, relative density and ethanol content*

| $\rho_{20}$ (kg·m$^{-3}$) | Relative density of the distillate measured in air $d_{20}^{20}$ | Ethanol content in per cent *V/V* at 20 °C |
|---|---|---|
| 968.0 | 0.9697 | 25.09 |
| 968.5 | 0.9702 | 24.64 |
| 969.0 | 0.9707 | 24.19 |
| 969.5 | 0.9712 | 23.74 |
| 970.0 | 0.9717 | 23.29 |
| 970.5 | 0.9722 | 22.83 |
| 971.0 | 0.9727 | 22.37 |
| 971.5 | 0.9733 | 21.91 |
| 972.0 | 0.9738 | 21.45 |
| 972.5 | 0.9743 | 20.98 |
| 973.0 | 0.9748 | 20.52 |
| 973.5 | 0.9753 | 20.05 |
| 974.0 | 0.9758 | 19.59 |
| 974.5 | 0.9763 | 19.12 |
| 975.0 | 0.9768 | 18.66 |
| 975.5 | 0.9773 | 18.19 |
| 976.0 | 0.9778 | 17.73 |
| 976.5 | 0.9783 | 17.25 |
| 977.0 | 0.9788 | 16.80 |
| 977.5 | 0.9793 | 16.34 |
| 978.0 | 0.9798 | 15.88 |
| 978.5 | 0.9803 | 15.43 |
| 979.0 | 0.9808 | 14.97 |
| 979.5 | 0.9813 | 14.52 |
| 980.0 | 0.9818 | 14.07 |
| 980.5 | 0.9823 | 13.63 |
| 981.0 | 0.9828 | 13.18 |
| 981.5 | 0.9833 | 12.74 |
| 982.0 | 0.9838 | 12.31 |
| 982.5 | 0.9843 | 11.87 |
| 983.0 | 0.9848 | 11.44 |
| 983.5 | 0.9853 | 11.02 |
| 984.0 | 0.9858 | 10.60 |
| 984.5 | 0.9863 | 10.18 |
| 985.0 | 0.9868 | 9.76 |
| 985.5 | 0.9873 | 9.35 |
| 986.0 | 0.9878 | 8.94 |
| 986.5 | 0.9883 | 8.53 |
| 987.0 | 0.9888 | 8.13 |
| 987.5 | 0.9893 | 7.73 |
| 988.0 | 0.9898 | 7.34 |
| 988.5 | 0.9903 | 6.95 |
| 989.0 | 0.9908 | 6.56 |
| 989.5 | 0.9913 | 6.17 |
| 990.0 | 0.9918 | 5.79 |
| 990.5 | 0.9923 | 5.42 |
| 991.0 | 0.9928 | 5.04 |
| 991.5 | 0.9933 | 4.67 |
| 992.0 | 0.9938 | 4.30 |
| 992.5 | 0.9943 | 3.94 |
| 993.0 | 0.9948 | 3.58 |
| 993.5 | 0.9953 | 3.22 |
| 994.0 | 0.9958 | 2.86 |
| 994.5 | 0.9963 | 2.51 |
| 995.0 | 0.9968 | 2.16 |
| 995.5 | 0.9973 | 1.82 |
| 996.0 | 0.9978 | 1.47 |
| 996.5 | 0.9983 | 1.13 |
| 997.0 | 0.9988 | 0.80 |
| 997.5 | 0.9993 | 0.46 |
| 998.0 | 0.9998 | 0.13 |

*Hydrometer method.* Transfer 50.0 mL of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask, add 200-300 mL of *distilled water R* and distil, as described above, into a volumetric flask until at least 180 mL has been collected. Adjust the temperature to 20 ± 0.1 °C and dilute to 250.0 mL with *distilled water R* at 20 ± 0.1 °C.

Transfer the distillate to a cylinder whose diameter is at least 6 mm wider than the bulb of the hydrometer. If the volume is insufficient, double the quantity of the sample and dilute the distillate to 500.0 mL with *distilled water R* at 20 ± 0.1 °C.

Multiply the strength by 5 to allow for the dilution during the determination. After calculation of the ethanol content using Table 2.9.10.-1, round off the result to 1 decimal place.

METHOD B

Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Test solution.* Dilute a volume of the preparation to be examined corresponding to 0.4 g of ethanol to 50.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*. To 2.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Reference solution (a).* Dilute 5.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 25.0 mL of the solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

*Reference solution (b).* Mix 0.5 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Reference solution (c).* Mix 1.0 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Reference solution (d).* Mix 1.5 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Reference solution (e).* Dilute 1.0 mL of *methanol R2* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (f).* Mix 1.0 mL of the internal standard solution, 2.0 mL of reference solution (a) and 2.0 mL of reference solution (e) and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Close the vials immediately with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimp cap.*

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 3 mL/min.

*Split ratio*: 1:50.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 85 °C;
- *equilibration time*: 20 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1.6 | 40 |
| | 1.6 - 9.9 | 40 → 65 |
| | 9.9 - 13.6 | 65 → 175 |
| | 13.6 - 20 | 175 |
| Injection port | | 200 |
| Detector | | 200 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL of the gaseous phase of the test solution and reference solutions (b), (c), (d) and (f), at least 3 times.

*Elution order*: methanol, ethanol, propanol.

*Relative retention* with reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; propanol = about 1.6.

*System suitability*: reference solution (f):

- *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Establish a calibration curve with the concentration of ethanol in reference solutions (b), (c), (d) and (f) as the abscissa and the mean ratio of the peak area of ethanol to the peak area of the internal standard in the corresponding chromatograms as the ordinate.

Calculate the percentage content of ethanol in the preparation to be examined.

METHOD C

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*.

*Test solution.* Dilute a volume of the preparation to be examined corresponding to 1 g of ethanol to 50.0 mL with *water R*. To 1.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of *anhydrous ethanol R* to 50.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of *methanol R2* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (c).* Mix 1.0 mL of the internal standard solution, 1.0 mL of reference solution (a) and 2.0 mL of reference solution (b) and dilute to 20.0 mL with *water R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1.6 | 40 |
| | 1.6 - 9.9 | 40 → 65 |
| | 9.9 - 13.6 | 65 → 175 |
| | 13.6 - 20 | 175 |
| Injection port | | 200 |
| Detector | | 200 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL of the test solution and reference solution (c), at least 3 times.

*Elution order*: methanol, ethanol, propanol.

*Relative retention* with reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; propanol = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the ethanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2 \times 100}{A_2 \times I_1 \times V_1}$$

$A_1$ = area of the peak due to ethanol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ethanol in the chromatogram obtained with reference solution (c);

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);

$V_1$ = volume of the preparation to be examined in the test solution, in millilitres.

**07/2019:20911 corrected 10.0**

## 2.9.11. TEST FOR METHANOL AND 2-PROPANOL

METHOD A

Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Test solution.* Mix 1.0 mL of the internal standard solution and 4.0 mL of the preparation to be examined and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Reference solution (a).* Mix 1.0 mL of *methanol R2* and 1.0 mL of *2-propanol R2* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (b).* Dilute 5.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 25.0 mL of the solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

*Reference solution (c).* Mix 1.0 mL of the internal standard solution, 2.0 mL of reference solution (a) and 2.0 mL of reference solution (b) and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

*Close the vials immediately with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimp cap.*

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3 mL/min.

*Split ratio*: 1:50.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 85 °C;
- *equilibration time*: 20 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1.6 | 40 |
| | 1.6 - 9.9 | 40 → 65 |
| | 9.9 - 13.6 | 65 → 175 |
| | 13.6 - 20 | 175 |
| Injection port | | 200 |
| Detector | | 200 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL of the gaseous phase of the test solution and reference solution (c), at least 3 times.

*Elution order*: methanol, ethanol, 2-propanol, 1-propanol.

*Relative retention* with reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; 2-propanol = about 1.2; 1-propanol = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the methanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2}{A_2 \times I_1 \times 40}$$

$A_1$ = area of the peak due to methanol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to methanol in the chromatogram obtained with reference solution (c);

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

Calculate the 2-propanol content in per cent *V/V* using the following expression:

$$\frac{A_3 \times I_2}{A_4 \times I_1 \times 40}$$

$A_3$ = area of the peak due to 2-propanol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to 2-propanol in the chromatogram obtained with reference solution (c);

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

METHOD B

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*.

*Test solution.* Mix 1.0 mL of the internal standard solution and 4.0 mL of the preparation to be examined and dilute to 20.0 mL with *water R*.

*Reference solution (a).* Mix 1.0 mL of *methanol R2* and 1.0 mL of *2-propanol R2* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of *anhydrous ethanol R* to 50.0 mL with *water R*.

*Reference solution (c).* Mix 1.0 mL of the internal standard solution, 1.0 mL of reference solution (b) and 2.0 mL of reference solution (a) and dilute to 20.0 mL with *water R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.53 mm;

– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1.6 | 40 |
| | 1.6 - 9.9 | 40 → 65 |
| | 9.9 - 13.6 | 65 → 175 |
| | 13.6 - 20 | 175 |
| Injection port | | 200 |
| Detector | | 200 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL of the test solution and reference solution (c), at least 3 times.

*Elution order*: methanol, ethanol, 2-propanol, 1-propanol.

*Relative retention* with reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; 2-propanol = about 1.2; 1-propanol = about 1.6.

*System suitability*: reference solution (c):

– *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the methanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2}{A_2 \times I_1 \times 40}$$

$A_1$ = area of the peak due to methanol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to methanol in the chromatogram obtained with reference solution (c);

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

Calculate the 2-propanol content in per cent *V/V* using the following expression:

$$\frac{A_3 \times I_2}{A_4 \times I_1 \times 40}$$

$A_3$ = area of the peak due to 2-propanol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to 2-propanol in the chromatogram obtained with reference solution (c);

$I_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$I_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).



**01/2008:20912**

## 2.9.12. SIEVE TEST

The degree of fineness of a powder may be expressed by reference to sieves that comply with the specifications for non-analytical sieves (*2.1.4*).

Where the degree of fineness of powders is determined by sieving, it is defined in relation to the sieve number(s) used either by means of the following terms or, where such terms cannot be used, by expressing the fineness of the powder as a percentage *m/m* passing the sieve(s) used.

The following terms are used in the description of powders:

**Coarse powder**. Not less than 95 per cent by mass passes through a number 1400 sieve and not more than 40 per cent by mass passes through a number 355 sieve.

**Moderately fine powder**. Not less than 95 per cent by mass passes through a number 355 sieve and not more than 40 per cent by mass passes through a number 180 sieve.

**Fine powder**. Not less than 95 per cent by mass passes through a number 180 sieve and not more than 40 per cent by mass passes through a number 125 sieve.

**Very fine powder**. Not less than 95 per cent by mass passes through a number 125 sieve and not more than 40 per cent by mass passes through a number 90 sieve.

If a single sieve number is given, not less than 97 per cent of the powder passes through the sieve of that number, unless otherwise prescribed.

Assemble the sieves and operate in a suitable manner until sifting is practically complete. Weigh the separated fractions of the powder.

**04/2017:20914**

## 2.9.14. SPECIFIC SURFACE AREA BY AIR PERMEABILITY

The test is intended for the determination of the specific surface area of dry powders expressed in square metres per gram in the sub-sieve region. The effect of molecular flow ("slip flow") which may be important when testing powders consisting of particles less than a few micrometres is not taken into account in the equation used to calculate the specific surface area.

APPARATUS

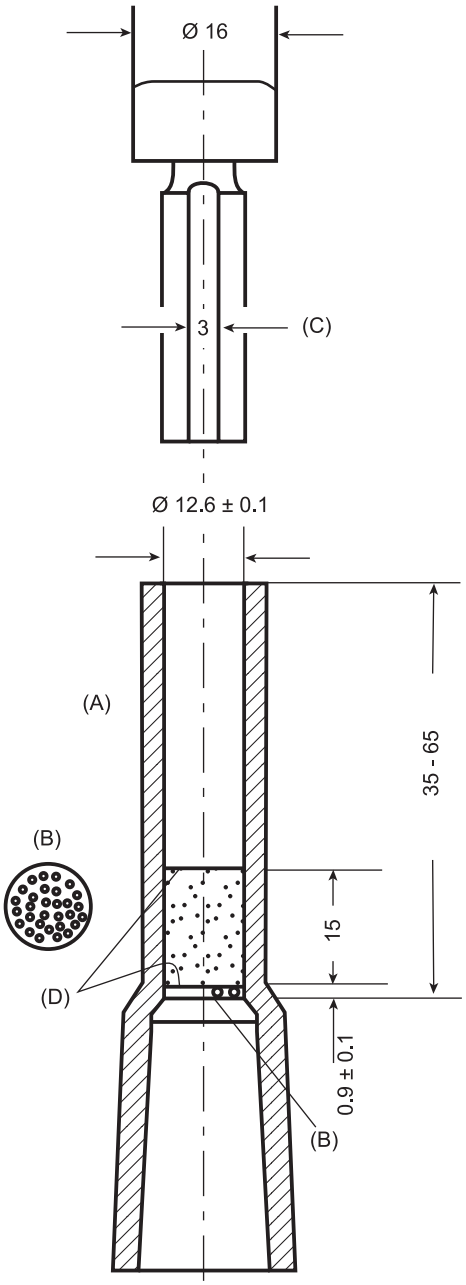


Figure 2.9.14.-1. – *Permeability cell*

*Dimensions in millimetres*

The apparatus consists of the following parts:

(a) a *permeability cell* (see Figure 2.9.14.-1), which consists of a cylinder with an inner diameter of 12.6 ± 0.1 mm (*A*), constructed of glass or non-corroding metal. The bottom of the cell forms an airtight connection (for example, via an adapter) with the manometer (Figure 2.9.14.-2). A ledge 0.5-1 mm in width is located 50 ± 15 mm from the top of the cell. It is an integral part of the cell or firmly fixed so as to be airtight. It supports a perforated metal disk (*B*), constructed of non-corroding metal. The disk has a thickness of 0.9 ± 0.1 mm and is perforated with 30 to 40 holes 1 mm in diameter evenly distributed over this area.

The plunger (*C*) is made of non-corroding metal and fits into the cell with a clearance of not more than 0.1 mm. The bottom of the plunger has sharp square edges at right angles to the principal axis. There is an air vent 3 mm long and 0.3 mm deep on one side of the plunger. The top of the plunger has a collar such that when the plunger is placed in the cell and the collar is brought into contact with the top of the cell, the distance between the bottom of the plunger and the top of the perforated disk (*B*) is 15 ± 1 mm.

The filter paper disks (*D*) have smooth edges and the same diameter as the inside of the cell.

(b) a *U-tube manometer* (*E*) (Figure 2.9.14.-2) is made of nominal 9 mm outer diameter and 7 mm inner diameter glass tubing with standard walls. The top of one arm of the manometer forms an airtight connection with the permeability cell (*F*). The manometer arm connected to the permeability cell has a line etched around the tube at 125-145 mm below the top of the side outlet and three other lines at distances of 15 mm, 70 mm and 110 mm above that line (*G*). The side outlet, 250-305 mm above the bottom of the manometer, is used to evacuate the manometer arm connected to the permeability cell. A tap is provided on the side outlet not more than 50 mm from the manometer arm.

The manometer is mounted firmly in such a manner that the arms are vertical. It is filled to the lowest mark with the liquid recommended by the manufacturer of the equipment or water.

METHOD

If prescribed, dry the powder to be examined and sift through a suitable sieve (for example no. 125) to disperse agglomerates. Calculate the mass (*M*) of the powder to be used from the following expression:

$$V \times \rho \times (1 - \varepsilon) \qquad (1)$$

$V$ = bulk volume of the compacted bed of powder,

$\rho$ = density of the substance to be examined in grams per millilitre,

$\varepsilon$ = porosity of the compacted bed of powder.

Assume first a porosity of 0.5 and introduce this value in expression (1) to calculate the mass (*M*) of the powder to be examined.

Place a filter paper disk on top of the perforated metal disk (*B*). Weigh the calculated mass (*M*) of the powder to be examined to the nearest 1 mg. Carefully transfer the powder into the cleaned, tared permeability cell and carefully tap the cell so that the surface of the powder bed is level and cover it with a second filter paper disk. Slowly compact the powder by means of the plunger, avoiding rotary movement. Maintain the pressure until the plunger is completely inserted into the permeability cell. If this is not possible, decrease the quantity of the powder used. If, on the contrary, there is not enough resistance, increase the quantity of the powder. In this case calculate the porosity again. After at least 10 s, remove the plunger.

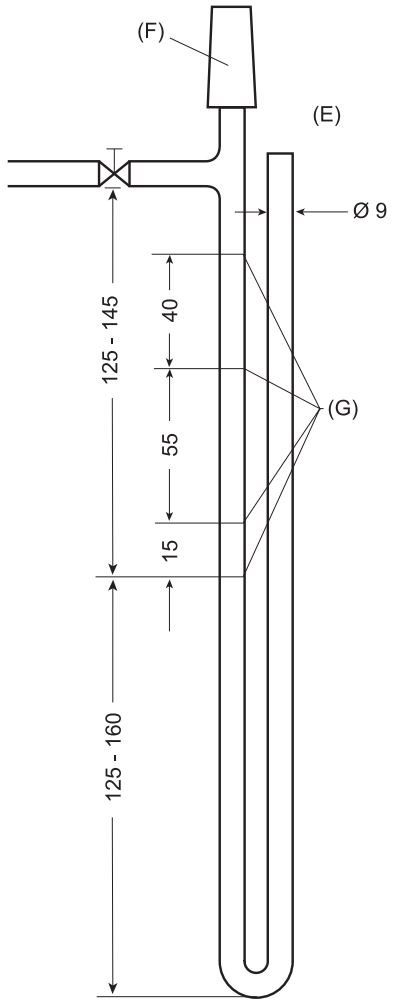


Figure 2.9.14.-2. – *Manometer*
*Dimensions in millimetres*

Attach the permeability cell to the tube of the manometer by means of an airtight connection. Evacuate the air from the manometer by means of a rubber bulb until the level of the coloured liquid is at the highest mark. Close the tap and check that the apparatus is airtight by closing the upper end of the cell, for example with a rubber stopper. Remove the stopper and, using a timer, measure the time taken for the liquid to fall from the second to the third mark.

Using the measured flow time, calculate the specific surface area (*S*), expressed in square metres per gram, from the following expression:

$$\frac{K \times \sqrt{\varepsilon^3} \times \sqrt{t}}{\rho \times (1 - \varepsilon) \times \sqrt{\eta}} \qquad (2)$$

$t$ = flow time in seconds,

$\eta$ = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1),

$K$ = apparatus constant determined according to expression (4),

$\rho$ = density of the substance to be examined in grams per millilitre,

$\varepsilon$ = porosity of the compacted bed of powder.

## CALIBRATION OF THE APPARATUS

**Bulk volume of the compacted powder bed**. It is determined by the mercury displacement method as follows:

Place two filter paper disks in the permeability cell, pressing down the edges with a rod slightly smaller than the cell diameter until the filter disks lie flat on the perforated metal disk; fill the cell with mercury, removing any air bubbles adhering to the wall of the cell and wipe away the excess to create a plane surface of mercury at the top of the cell. If the cell is made of material that will amalgamate, grease the cell and the metal disk first with a thin layer of liquid paraffin. Pour out the mercury into a tared beaker and determine the mass ($M_A$) and the temperature of the mercury.

Make a compacted bed using the reference powder and again fill the cell with mercury with a planar surface at the top of the cell. Pour out the mercury in a tared beaker and again determine the mass of the mercury ($M_B$). Calculate the bulk volume ($V$) of the compacted bed of powder from the following expression:

$$\frac{M_A - M_B}{\rho_{\mathrm{Hg}}} \qquad (3)$$

$M_A - M_B$ = difference between the determined masses of mercury in grams,

$\rho_{\mathrm{Hg}}$ = density of mercury at the determined temperature in grams per millilitre.

Repeat the procedure twice, changing the powder each time; the range of values for the calculated volume ($V$) is not greater than 0.01 mL. Use the mean value of the three determined volumes for the calculations.

**Apparatus constant (*K*)**. It is determined using a reference powder with known specific surface area and density as follows:

Calculate the required quantity of the reference powder to be used (expression (1)) using the stated density and the determined volume of the compacted powder bed (expression (3)).

Homogenise and loosen up the powder by shaking it for 2 min in a 100 mL bottle. Prepare a compacted powder bed and measure the flow time of air as previously described. Calculate the apparatus constant ($K$) from the following expression:

$$\frac{S_{sp} \times \rho \times (1 - \varepsilon) \times \sqrt{\eta}}{\sqrt{\varepsilon^3} \times \sqrt{t}} \qquad (4)$$

$S_{sp}$ = stated specific surface area of the reference powder,

$\rho$ = density of the substance to be examined in grams per millilitre,

$\varepsilon$ = porosity of the compacted bed of powder,

$t$ = flow time in seconds,

$\eta$ = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1).

The density of mercury and the viscosity of air over a range of temperatures are shown in Table 2.9.14.-1.

Table 2.9.14.-1.

| Temperature (°C) | Density of mercury (g/mL) | Viscosity of air ($\eta$) (mPa·s) | $\sqrt{\eta}$ |
|---|---|---|---|
| 16 | 13.56 | 0.01800 | 0.1342 |
| 17 | 13.56 | 0.01805 | 0.1344 |
| 18 | 13.55 | 0.01810 | 0.1345 |
| 19 | 13.55 | 0.01815 | 0.1347 |
| 20 | 13.55 | 0.01819 | 0.1349 |
| 21 | 13.54 | 0.01824 | 0.1351 |
| 22 | 13.54 | 0.01829 | 0.1353 |
| 23 | 13.54 | 0.01834 | 0.1354 |
| 24 | 13.54 | 0.01839 | 0.1356 |

01/2008:20916

# 2.9.16. FLOWABILITY

The test for flowability is intended to determine the ability of divided solids (for example, powders and granules) to flow vertically under defined conditions.

APPARATUS

According to the flow properties of the material to be tested, funnels with or without stem, with different angles and orifice diameters are used. Typical apparatuses are shown in Figures 2.9.16.-1 and 2.9.16.-2. The funnel is maintained upright by a suitable device. The assembly must be protected from vibrations.

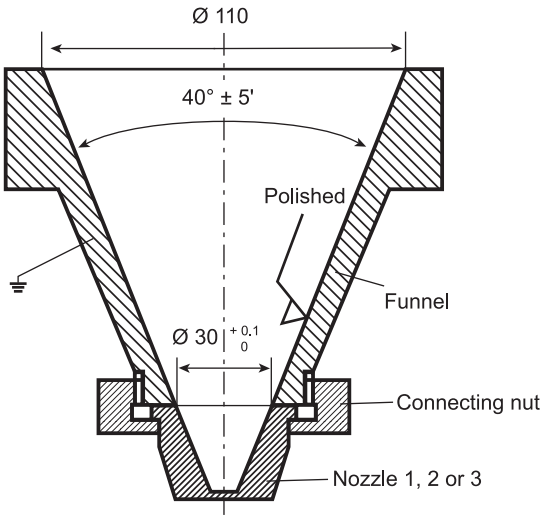


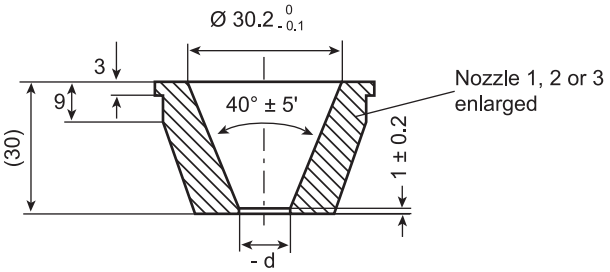


| Nozzle | Diameter (*d*) of the outflow opening (millimetres) |
|---|---|
| 1 | 10 ± 0.01 |
| 2 | 15 ± 0.01 |
| 3 | 25 ± 0.01 |

Figure 2.9.16.-1. – *Flow funnel and nozzle. Nozzle is made of stainless, acid-resistant steel (V4A,CrNi)*
*Dimensions in millimetres*

METHOD

Into a dry funnel, whose bottom opening has been blocked by suitable means, introduce without compacting a test sample weighed with 0.5 per cent accuracy. The amount of the sample depends on the apparent volume and the apparatus used. Unblock the bottom opening of the funnel and measure the time needed for the entire sample to flow out of the funnel. Carry out three determinations.

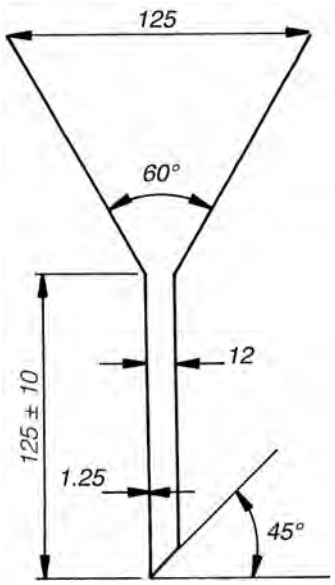


Figure 2.9.16.-2
*Dimensions in millimetres*

EXPRESSION OF RESULTS

The flowability is expressed in seconds and tenths of seconds, related to 100 g of sample.

The results depend on the storage conditions of the material to be tested.

The results can be expressed as the following:

a) the mean of the determinations, if none of the individual values deviates from the mean value by more than 10 per cent;

b) as a range, if the individual values deviate from the mean value by more than 10 per cent;

c) as a plot of the mass against the flow time;

d) as an infinite time, if the entire sample fails to flow through.

04/2010:20917

## 2.9.17. TEST FOR EXTRACTABLE VOLUME OF PARENTERAL PREPARATIONS[8]

Suspensions and emulsions are shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20-25 °C before measuring the volume.

SINGLE-DOSE CONTAINERS

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding 3 times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm in length. Expel any air bubbles from the syringe and needle, then discharge the contents of the syringe without emptying the needle into a standardised dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40 per cent of its graduated volume. Alternatively, the volume of the contents in millilitres may be calculated as the mass in grams divided by the density.

For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement provided that a separate, dry syringe assembly is used for each container. The contents of containers holding 10 mL or more may be determined by opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in case of containers examined individually, or, in case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

MULTIDOSE CONTAINERS

For injections in multidose containers labelled to yield a specific number of doses of a stated volume, select one container and proceed as directed for single-dose containers using the same number of separate syringe assemblies as the number of doses specified.

The volume is such that each syringe delivers not less than the stated dose.

CARTRIDGES AND PREFILLED SYRINGES

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in millilitres calculated as the mass in grams divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

PARENTERAL INFUSIONS

Select one container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40 per cent of the nominal volume of the cylinder. Measure the volume transferred.

The volume is not less than the nominal volume.

01/2008:20918

## 2.9.18. PREPARATIONS FOR INHALATION: AERODYNAMIC ASSESSMENT OF FINE PARTICLES

This test is used to determine the fine particle characteristics of the aerosol clouds generated by preparations for inhalation.

Unless otherwise justified and authorised, one of the following apparatus and test procedures is used.

*Stage mensuration* is performed periodically together with confirmation of other dimensions critical to the effective operation of the impactor.

*Re-entrainment (for apparatus D and E).* To ensure efficient particle capture, coat each plate with glycerol, silicone oil or similar high viscosity liquid, typically deposited from a volatile solvent. Plate coating must be part of method validation and may be omitted where justified and authorised.

*Mass balance.* The total mass of the active substance is not less than 75 per cent and not more than 125 per cent of the average delivered dose determined during testing for uniformity of delivered dose. This is not a test of the inhaler but it serves to ensure that the results are valid.

APPARATUS A - GLASS IMPINGER

The apparatus is shown in Figure 2.9.18.-1 (see also Table 2.9.18.-1).

**Procedure for nebulisers**

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump fitted with a filter (of suitable pore size) to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Introduce the liquid preparation for inhalation into the reservoir of the nebuliser. Fit the mouthpiece and connect it by means of an adapter to the device.

Switch on the pump of the apparatus and after 10 s switch on the nebuliser.

After 60 s, unless otherwise justified, switch off the nebuliser, wait for about 5 s and then switch off the pump of the apparatus. Dismantle the apparatus and wash the inner surface of the upper impingement chamber collecting the washings in a volumetric flask. Wash the inner surface of the lower impingement chamber collecting the washings in a second volumetric flask. Finally, wash the filter preceding the pump and its connections to the lower impingement chamber and combine the washings with those obtained from the lower impingement chamber. Determine the amount of active substance collected in each of the 2 flasks. Express the results for each of the 2 parts of the apparatus as a percentage of the total amount of active substance.

(8) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Procedure for pressurised inhalers**

Place the actuator adapter in position at the end of the throat so that the mouthpiece end of the actuator, when inserted to a depth of about 10 mm, lines up along the horizontal axis of the throat and the open end of the actuator, which accepts the pressurised container, is uppermost and in the same vertical plane as the rest of the apparatus.

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the lower jet-spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Prime the metering valve by shaking for 5 s and discharging once to waste; after not less than 5 s, shake and discharge again to waste. Repeat a further 3 times.

Shake for about 5 s, switch on the pump to the apparatus and locate the mouthpiece end of the actuator in the adapter, discharge once immediately. Remove the assembled inhaler from the adapter, shake for not less than 5 s, relocate the mouthpiece end of the actuator in the adapter and discharge again. Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. After the final discharge wait for not less than 5 s and then switch off the pump. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the dose stated on the label.

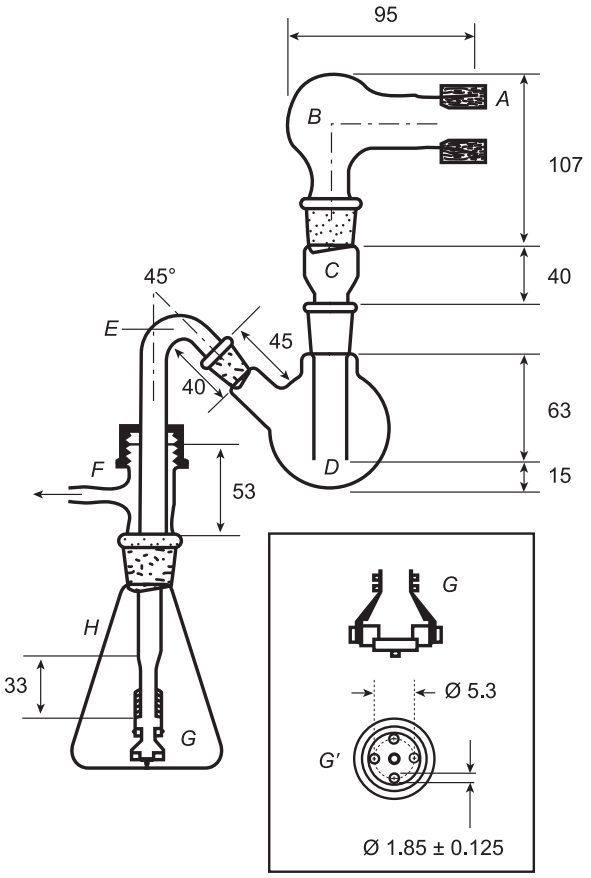


Figure 2.9.18.-1. – *Apparatus A: glass impinger*

*Dimensions in millimetres (tolerances ± 1 mm unless otherwise prescribed)*

**Procedure for powder inhalers**

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet-spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Without the inhaler in place, connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Prepare the inhaler for use and locate the mouthpiece in the apparatus by means of a suitable adapter. Switch on the pump for 5 s. Switch off the pump and remove the inhaler. Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the dose stated on the label.

Table 2.9.18.-1. – *Component specification for apparatus A in Figure 2.9.18.-1*

| Code | Item | Description | Dimensions* |
|---|---|---|---|
| A | Mouthpiece adaptor | Moulded rubber adapter for actuator mouthpiece. | |
| B | Throat | Modified round-bottomed flask: | 50 mL |
| | | – *ground-glass inlet socket* | 29/32 |
| | | – *ground-glass outlet cone* | 24/29 |
| C | Neck | Modified glass adapter: | |
| | | – *ground-glass inlet socket* | 24/29 |
| | | – *ground-glass outlet cone* | 24/29 |
| | | Lower outlet section of precision-bore glass tubing: | |
| | | – *bore diameter* | 14 |
| | | Selected bore light-wall glass tubing: | |
| | | – *external diameter* | 17 |
| D | Upper | Modified round-bottomed flask | 100 mL |
| | impingement | – *ground-glass inlet socket* | 24/29 |
| | chamber | – *ground-glass outlet cone* | 24/29 |

| Code | Item | Description | Dimensions* |
|---|---|---|---|
| E | Coupling tube | Medium-wall glass tubing: | |
| | | – *ground-glass cone* | 14/23 |
| | | Bent section and upper vertical section: | |
| | | – *external diameter* | 13 |
| | | Lower vertical section: | |
| | | – *external diameter* | 8 |
| F | Screwthread, | Plastic screw cap | 28/13 |
| | side-arm | Silicone rubber ring | 28/11 |
| | adaptor | PTFE washer | 28/11 |
| | | Glass screwthread: | |
| | | – *thread size* | 28 |
| | | Side-arm outlet to vacuum pump: | |
| | | – *minimum bore diameter* | 5 |
| G | Lower jet assembly | Modified polypropylene filter holder connected to lower vertical section of coupling tube by PTFE tubing. | see Figure 2.9.18.-1 |
| | | Acetal circular disc with the centres of four jets arranged on a projected circle of diameter 5.3 mm with an integral jet spacer peg: | 10 |
| | | – *peg diameter* | 2 |
| | | – *peg protrusion* | 2 |
| H | Lower | Conical flask | 250 mL |
| | impingement chamber | – *ground-glass inlet socket* | 24/29 |

* Dimensions in millimetres, unless otherwise stated.

## Fine particle dose and particle size distribution

APPARATUS C - MULTI-STAGE LIQUID IMPINGER

The multi-stage liquid impinger consists of impaction stages 1 (pre-separator), 2, 3 and 4 and an integral filter stage (stage 5), see Figures 2.9.18.-4/6. An impaction stage comprises an upper horizontal metal partition wall (B) through which a metal inlet jet tube (A) with its impaction plate (D) is protruding. A glass cylinder (E) with sampling port (F) forms the vertical wall of the stage, and a lower horizontal metal partition wall (G) through which the tube (H) connects to the next lower stage. The tube into stage 4 (U) ends in a multi-jet arrangement. The impaction plate (D) is secured in a metal frame (J) which is fastened by 2 wires (K) to a sleeve (L) secured on the jet tube. The horizontal face of the collection plate is perpendicular to the axis of the jet tube and centrally aligned. The upper surface of the impaction plate is slightly raised above the edge of the metal frame. A recess around the perimeter of the horizontal partition wall guides the position of the glass cylinder. The glass cylinders are sealed against the horizontal partition walls with gaskets (M) and clamped together by 6 bolts (N). The sampling ports are sealed by stoppers. The bottom-side of the lower partition wall of stage 4 has a concentrical protrusion fitted with a rubber O-ring (P) which seals against the edge of a filter placed in the filter holder. The filter holder (R) is constructed as a basin with a concentrical recess in which a perforated filter support (S) is flush-fitted. The filter holder is dimensioned for 76 mm diameter filters. The assembly of impaction stages is clamped onto the filter holder by 2 snap-locks (T). Connect an induction port (see Figure 2.9.18.-7) onto the stage 1 inlet jet tube of the impinger. A rubber O-ring on the jet tube provides an airtight connection to the induction port. A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port.

**Procedure for pressurised inhalers**

Dispense 20 mL of a solvent, capable of dissolving the active substance into each of stages 1 to 4 and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable filter capable of quantitatively collecting the active substance in stage 5 and assemble the apparatus. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler is positioned in the same orientation as intended for use. Connect a suitable vacuum pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 30 L/min (± 5 per cent). Switch off the pump.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge 1 delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active substance from the inner walls and the collection plate of each of the 4 upper stages of the apparatus into the solution in the respective stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

Table 2.9.18.-2. – *Component specification for apparatus C in Figures 2.9.18.-4/6*

| Code* | Item | Description | Dimensions** |
|---|---|---|---|
| A,H | Jet tube | Metal tube screwed onto partition wall sealed by gasket (C), polished inner surface | see Figure 2.9.18.-5 |
| B,G | Partition wall | Circular metal plate | |
| | | – *diameter* | 120 |
| | | – *thickness* | see Figure 2.9.18.-5 |
| C | Gasket | e.g. PTFE | to fit jet tube |
| D | Impaction | Porosity 0 sintered-glass disk | |
| | plate | – *diameter* | see Figure 2.9.18.-5 |

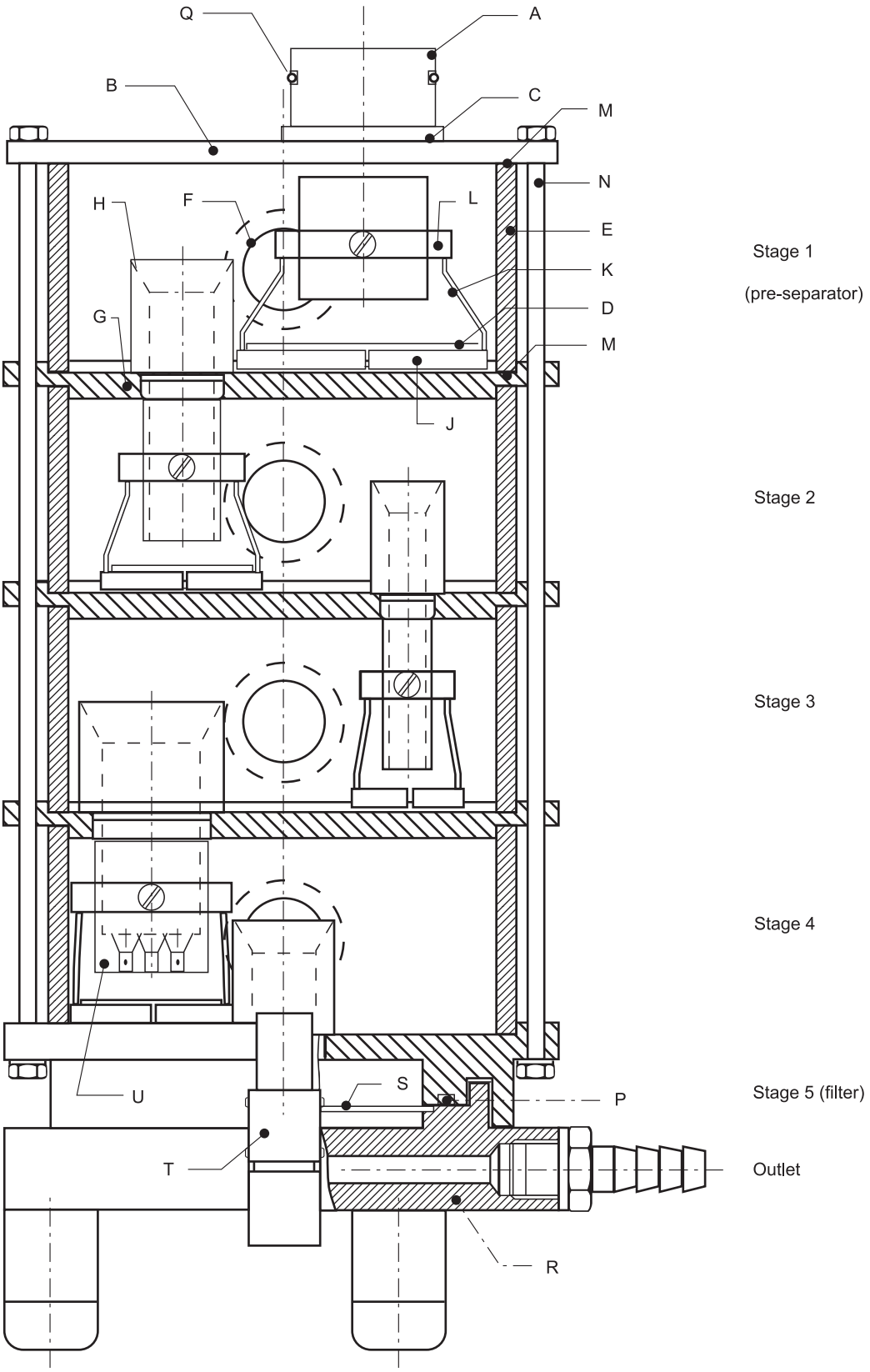


Figure 2.9.18.-4. – *Apparatus C: multi-stage liquid impinger*

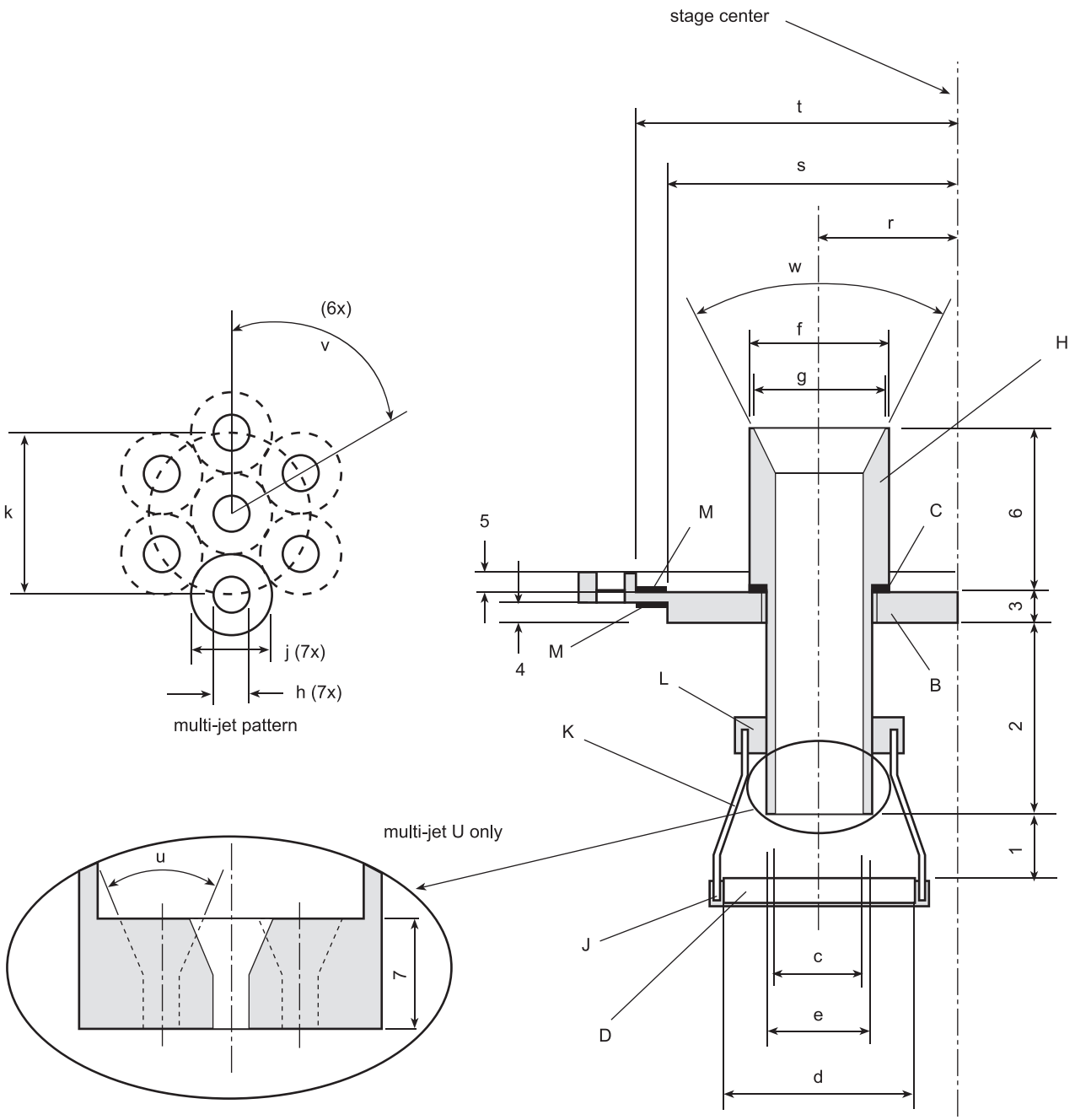


Figure 2.9.18.-5. – *Apparatus C: details of jet tube and impaction plate. Inserts show end of multi-jet tube U leading to stage 4. (Numbers and lowercase letters refer to Table 2.9.18.-3 and uppercase letters refer to Figure 2.9.18.-4).*

| Code* | Item | Description | Dimensions** |
|---|---|---|---|
| E | Glass cylinder | Plane polished cut glass tube | |
| | | – *height, including gaskets* | 46 |
| | | – *outer diameter* | 100 |
| | | – *wall thickness* | 3.5 |
| | | – *sampling port (F) diameter* | 18 |
| | | – *stopper in sampling port* | ISO 24/25 |
| J | Metal frame | L-profiled circular frame with slit | |
| | | – *inner diameter* | to fit impaction plate |
| | | – *height* | 4 |
| | | – *thickness of horizontal section* | 0.5 |
| | | – *thickness of vertical section* | 2 |

| Code* | Item | Description | Dimensions** |
|---|---|---|---|
| K | Wire | Steel wire interconnecting metal frame and sleeve (2 for each frame) | |
| | | – *diameter* | 1 |
| L | Sleeve | Metal sleeve secured on jet tube by screw | |
| | | – *inner diameter* | to fit jet tube |
| | | – *height* | 6 |
| | | – *thickness* | 5 |
| M | Gasket | e.g. silicone | to fit glass cylinder |
| N | Bolt | Metal bolt with nut (6 pairs) | |
| | | – *length* | 205 |
| | | – *diameter* | 4 |

| Code* | Item | Description | Dimensions** |
|---|---|---|---|
| P | O-ring | Rubber O-ring | |
| | | – *diameter × thickness* | 66.34 × 2.62 |
| Q | O-ring | Rubber O-ring | |
| | | – *diameter × thickness* | 29.1 × 1.6 |
| R | Filter holder | Metal housing with stand and outlet | see Figure 2.9.18.-6 |
| S | Filter support | Perforated sheet metal | |
| | | – *diameter* | 65 |
| | | – *hole diameter* | 3 |
| | | – *distance between holes (centre-points)* | 4 |
| T | Snap-locks | | |
| U | Multi-jet tube | Jet tube (H) ending in multi-jet arrangement. | see inserts Figure 2.9.18.-5 |

* Refers to Figure 2.9.18.-4.

** Measures in millimetres with tolerances according to iso 2768-m unless otherwise stated.

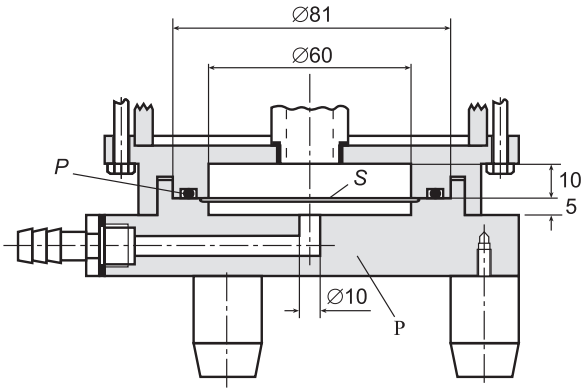


Figure 2.9.18.-6. – *Apparatus C: details of the filter stage (stage 5). Numbers refer to dimensions (Ø = diameter). Uppercase letters refer to Table 2.9.18.-2.*

*Dimensions in millimetres unless otherwise stated*

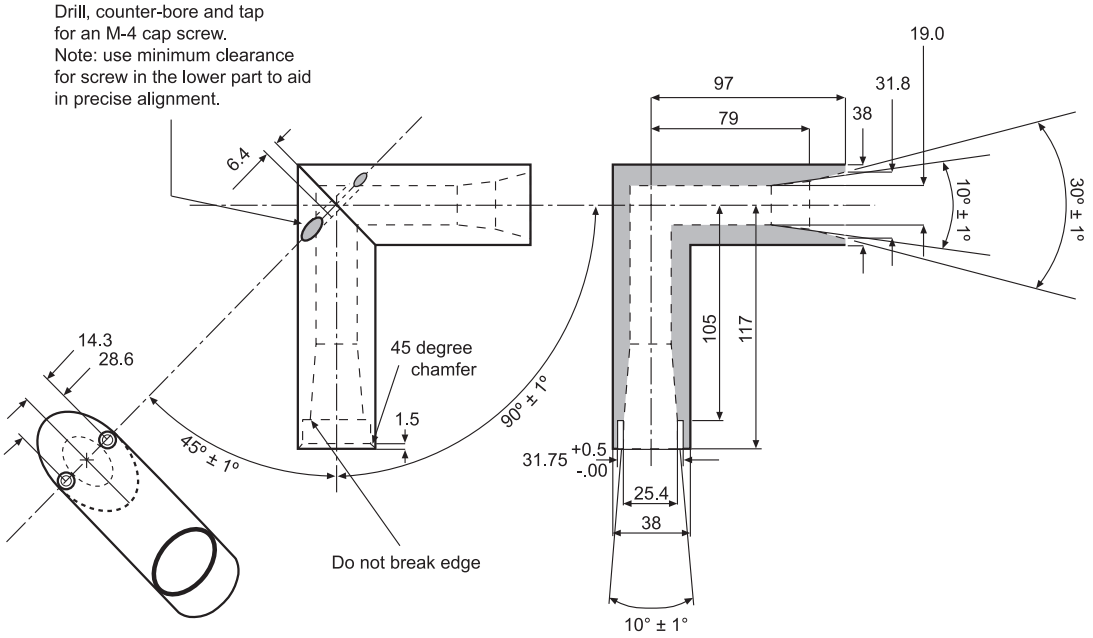


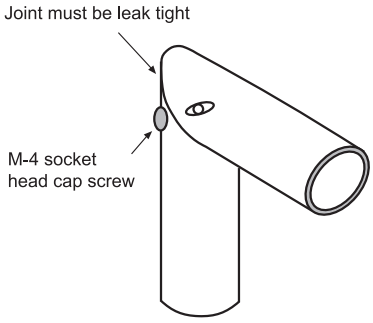


Isometric view of induction port

1. Material may be aluminium, stainless steel or other suitable material.
2. Machine from 38 mm bar stock.
3. Bore 19 mm hole through bar.
4. Cut tube to exact 45° as shown.
5. The inner bores and tapers should be smooth – surface roughness Ra approx. 0.4 µm.
6. Mill joining cads of stock to provide a liquid tight leak-free seal.
7. Set up a holding fixture for aligning the inner 19 mm bore and for drilling and tapping M4 × 0.7 threads. There must be virtually no mismatch of the inner bores in the miter joint.

Figure 2.9.18.-7. – *Induction port*

*Dimensions in millimetres unless otherwise stated*

Table 2.9.18.-3. – *Dimensions[1] of jet tube with impaction plate of apparatus C*

| Type | Code[2] | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Filter (stage 5) |
|---|---|---|---|---|---|---|
| Distance | 1 | 9.5 (-.0+.5) | 5.5 (-.0+.5) | 4.0 (-.0+.5) | 6.0 (-.0+.5) | n.a. |
| Distance | 2 | 26 | 31 | 33 | 30.5 | 0 |
| Distance | 3 | 8 | 5 | 5 | 5 | 5 |
| Distance | 4 | 3 | 3 | 3 | 3 | n.a. |
| Distance | 5 | 0 | 3 | 3 | 3 | 3 |
| Distance | 6 [3] | 20 | 25 | 25 | 25 | 25 |
| Distance | 7 | n.a. | n.a. | n.a. | 8.5 | n.a. |
| Diameter | c | 25 | 14 | 8.0 (± .1) | 21 | 14 |
| Diameter | d | 50 | 30 | 20 | 30 | n.a. |
| Diameter | e | 27.9 | 16.5 | 10.5 | 23.9 | n.a. |
| Diameter | f | 31.75 (-.0+.5) | 22 | 14 | 31 | 22 |
| Diameter | g | 25.4 | 21 | 13 | 30 | 21 |
| Diameter | h | n.a. | n.a. | n.a. | 2.70 (± .5) | n.a. |
| Diameter | j | n.a. | n.a. | n.a. | 6.3 | n.a. |
| Diameter | k | n.a. | n.a. | n.a. | 12.6 | n.a. |
| Radius[4] | r | 16 | 22 | 27 | 28.5 | 0 |
| Radius | s | 46 | 46 | 46 | 46 | n.a. |
| Radius | t | n.a. | 50 | 50 | 50 | 50 |
| Angle | w | 10° | 53° | 53° | 53° | 53° |
| Angle | u | n.a. | n.a. | n.a. | 45° | n.a. |
| Angle | v | n.a. | n.a. | n.a. | 60° | n.a. |

(1) Measures in millimetres with tolerances according to ISO 2768-m unless otherwise stated
(2) Refer to Figure 2.9.18.-5
(3) Including gasket
(4) Relative centreline of stage compartment
n.a. = not applicable

**Procedure for powder inhalers**

Place a suitable low resistance filter capable of quantitatively collecting the active substance in stage 5 and assemble the apparatus. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4. Unless otherwise defined, conduct the test at the flow rate, $Q_{out}$, used in the test for uniformity of delivered dose, drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus.

Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter ($Q_{out}$) using the ideal gas law. For a meter calibrated for the entering volumetric flow ($Q_{in}$), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

$P_0$ = atmospheric pressure,

$\Delta P$ = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, $Q_{out}$ (± 5 per cent). Switch off the pump. Ensure that critical flow occurs in the flow control valve by the following procedure.

With the inhaler in place and the test flow rate established, measure the absolute pressure on both sides of the control valve (pressure reading points P2 and P3 in Figure 2.9.18.-8). A ratio P3/P2 of less than or equal to 0.5 indicates critical flow. Switch to a more powerful pump and re-measure the test flow rate if critical flow is not indicated.

Dispense 20 mL of a solvent, capable of dissolving the active substance into each of the 4 upper stages of the apparatus and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable mouthpiece adapter in position at the end of the induction port.

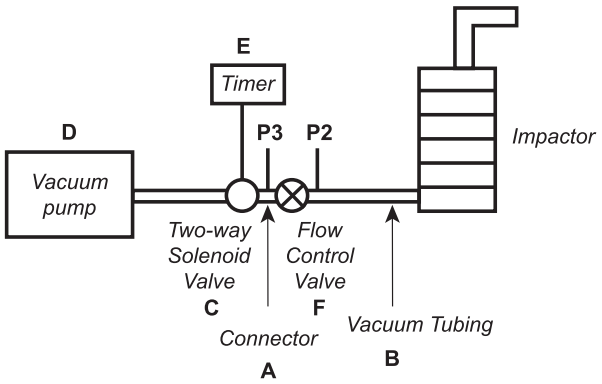


Figure 2.9.18.-8. – *Experimental set-up for testing powder inhalers*

Table 2.9.18.-4. – *Component specification for Figure 2.9.18.-8*

| Code | Item | Description |
|---|---|---|
| A | Connector | ID ≥ 8 mm, e.g., short metal coupling, with low-diameter branch to P3. |
| B | Vacuum tubing | A length of suitable tubing having an ID ≥ 8 mm and an internal volume of 25 ± 5 mL. |
| C | 2-way solenoid valve | A 2-way, 2-port solenoid valve having a minimum airflow resistance orifice with ID ≥ 8 mm and an opening time ≤ 100 ms. (e.g. type 256-A08, Bürkert GmbH, D-74653 Ingelfingen), or equivalent. |
| D | Vacuum pump | Pump must be capable of drawing the required flow rate through the assembled apparatus with the powder inhaler in the mouthpiece adapter (e.g. product type 1023, 1423 or 2565, Gast Manufacturing Inc., Benton Harbor, MI 49022), or equivalent. Connect the pump to the 2-way solenoid valve using short and/or wide (ID ≥ 10 mm) vacuum tubing and connectors to minimise pump capacity requirements. |
| E | Timer | Timer capable to drive the 2-way solenoid valve for the required duration (e.g. type G814, RS Components International, Corby, NN17 9RS, UK), or equivalent. |
| P2 P3 | Pressure measurements | Determine under steady-state flow condition with an absolute pressure transducer. |
| F | Flow control valve | Adjustable regulating valve with maximum $C_v \geq 1$, (e.g. type 8FV12LNSS, Parker Hannifin plc., Barnstaple, EX31 1NP, UK), or equivalent. |

Prepare the powder inhaler for use according to patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active substance from the inner walls and the collection plate of each of the 4 upper stages

of the apparatus into the solution in the respective stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages.

Using a suitable method of analysis, determine the amount of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

APPARATUS D - ANDERSEN CASCADE IMPACTOR

The Andersen 1 ACFM non-viable cascade impactor consists of 8 stages together with a final filter. Material of construction may be aluminium, stainless steel or other suitable material. The stages are clamped together and sealed with O-rings. Critical dimensions applied by the manufacturer of apparatus D are provided in Table 2.9.18.-5. In use, some occlusion and wear of holes will occur. In-use mensuration tolerances need to be justified. In the configuration used for pressurised inhalers (Figure 2.9.18.-9) the entry cone of the impactor is connected to an induction port (see Figure 2.9.18.-7). A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port.

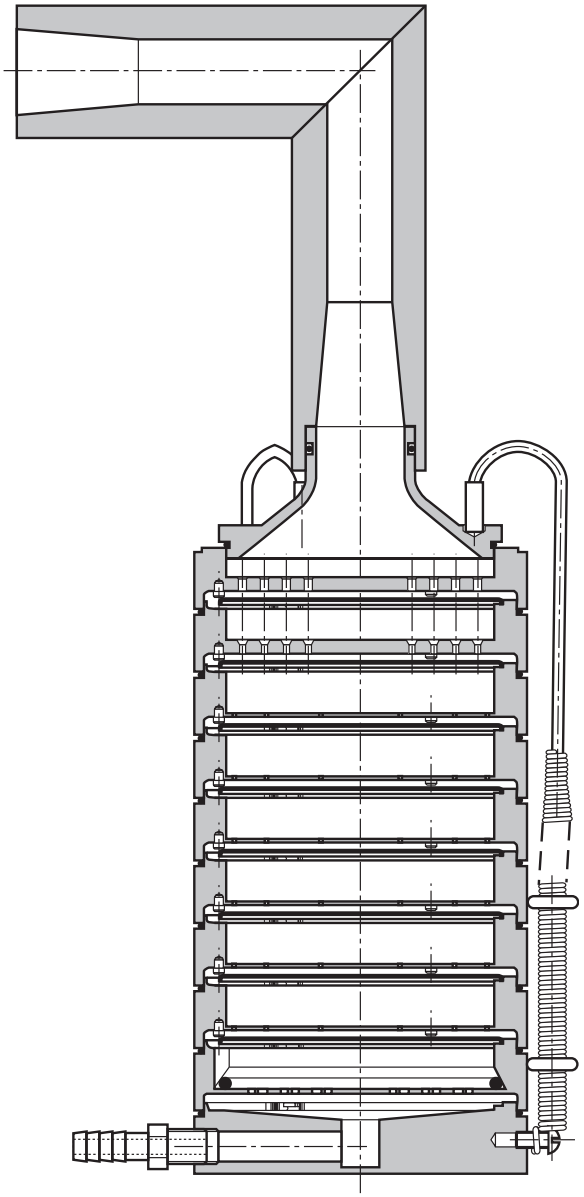

Figure 2.9.18.-9. – *Apparatus D: Andersen cascade impactor used for pressurised inhalers*

In the configuration for powder inhalers, a pre-separator is placed above the top stage to collect large masses of non-respirable powder. It is connected to the induction port as shown in Figure 2.9.18.-10. To accommodate high flow rates through the impactor, the outlet nipple, used to connect the impactor to the vacuum system is enlarged to have an internal diameter of greater than or equal to 8 mm.

Table 2.9.18.-5. – *Critical dimensions for apparatus D*

| Description | Number | Dimension (mm) |
|---|---|---|
| Stage 0 nozzle diameter | 96 | 2.55 ± 0.025 |
| Stage 1 nozzle diameter | 96 | 1.89 ± 0.025 |
| Stage 2 nozzle diameter | 400 | 0.914 ± 0.0127 |
| Stage 3 nozzle diameter | 400 | 0.711 ± 0.0127 |
| Stage 4 nozzle diameter | 400 | 0.533 ± 0.0127 |
| Stage 5 nozzle diameter | 400 | 0.343 ± 0.0127 |
| Stage 6 nozzle diameter | 400 | 0.254 ± 0.0127 |
| Stage 7 nozzle diameter | 201 | 0.254 ± 0.0127 |

**Procedure for pressurised inhalers**

Assemble the Andersen impactor with a suitable filter in place. Ensure that the system is airtight. In that respect, follow the manufacturer's instructions. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler unit is positioned in the same orientation as the intended use. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 28.3 L/min (± 5 per cent). Switch off the pump.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge one delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inverted inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. Extract the active substance from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

**Procedure for powder inhalers**

*The aerodynamic cut-off diameters of the individual stages of this apparatus are currently not well-established at flow rates other than 28.3 L/min.* Users must justify and validate the use of the impactor in the chosen conditions, when flow rates different from 28.3 L/min are selected.

Assemble the Andersen impactor with the pre-separator and a suitable filter in place and ensure that the system is airtight. Depending on the product characteristics, the pre-separator may be omitted, where justified and authorised. Stages 6 and 7 may also be omitted at high flow rates, if justified. The pre-separator may be coated in the same way as the plates

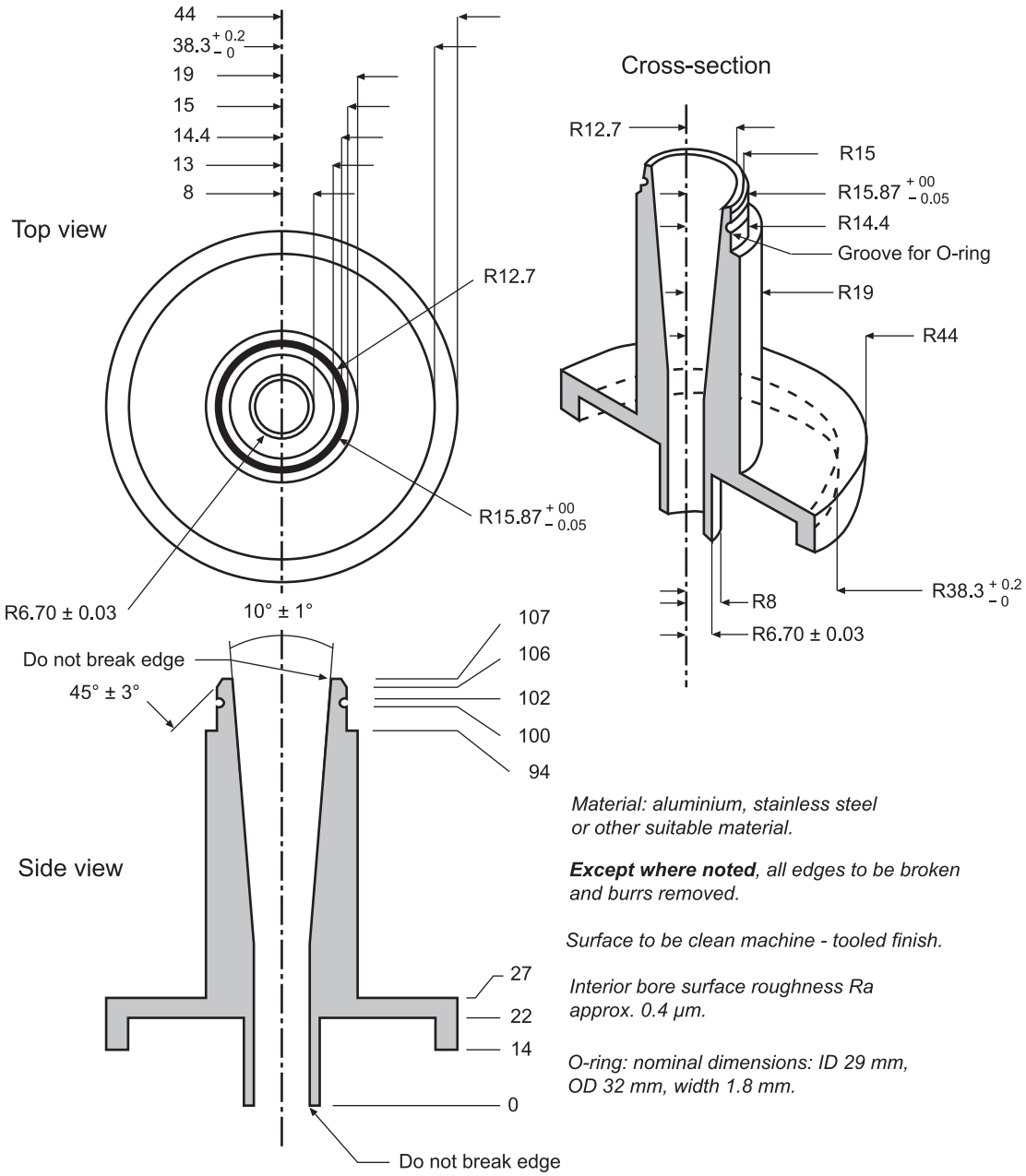


Figure 2.9.18.-10. – *Connection of the induction port to the preseparator of the Andersen cascade impactor*
*Dimensions in millimetres unless otherwise stated*

or may contain 10 mL of a suitable solvent. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4.

Unless otherwise defined, conduct the test at the flow rate, $Q_{out}$, used in the test for uniformity of delivered dose drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus.

Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter ($Q_{out}$) using the ideal gas law. For a meter calibrated for the entering volumetric flow ($Q_{in}$), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

$P_0$ = atmospheric pressure,

$\Delta P$ = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, $Q_{out}$ (± 5 per cent). Ensure that critical flow occurs in the flow control valve by the procedure described for Apparatus C. Switch off the pump.

Prepare the powder inhaler for use according to the patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

Dismantle the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the pre-separator, induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. Extract the active substance from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

APPARATUS E

Apparatus E is a cascade impactor with 7 stages and a micro-orifice collector (MOC). Over the flow rate range of 30 L/min to 100 L/min the 50 per cent-efficiency cut-off diameters ($D_{50}$ values) range between 0.24 µm to 11.7 µm, evenly spaced on a logarithmic scale. In this flow range, there are always at least 5 stages with $D_{50}$ values between 0.5 µm and 6.5 µm. The collection efficiency curves for each stage are sharp and minimise overlap between stages.

Material of construction may be aluminium, stainless steel or other suitable material.

The impactor configuration has removable impaction cups with all the cups in one plane (Figures 2.9.18.-11/14). There are 3 main sections to the impactor; the bottom frame that holds the impaction cups, the seal body that holds the jets and the lid that contains the interstage passageways (Figures 2.9.18.-11/12). Multiple nozzles are used at all but the first stage (Figure 2.9.18.-13). The flow passes through the impactor in a saw-tooth pattern.

Critical dimensions are provided in Table 2.9.18.-6.

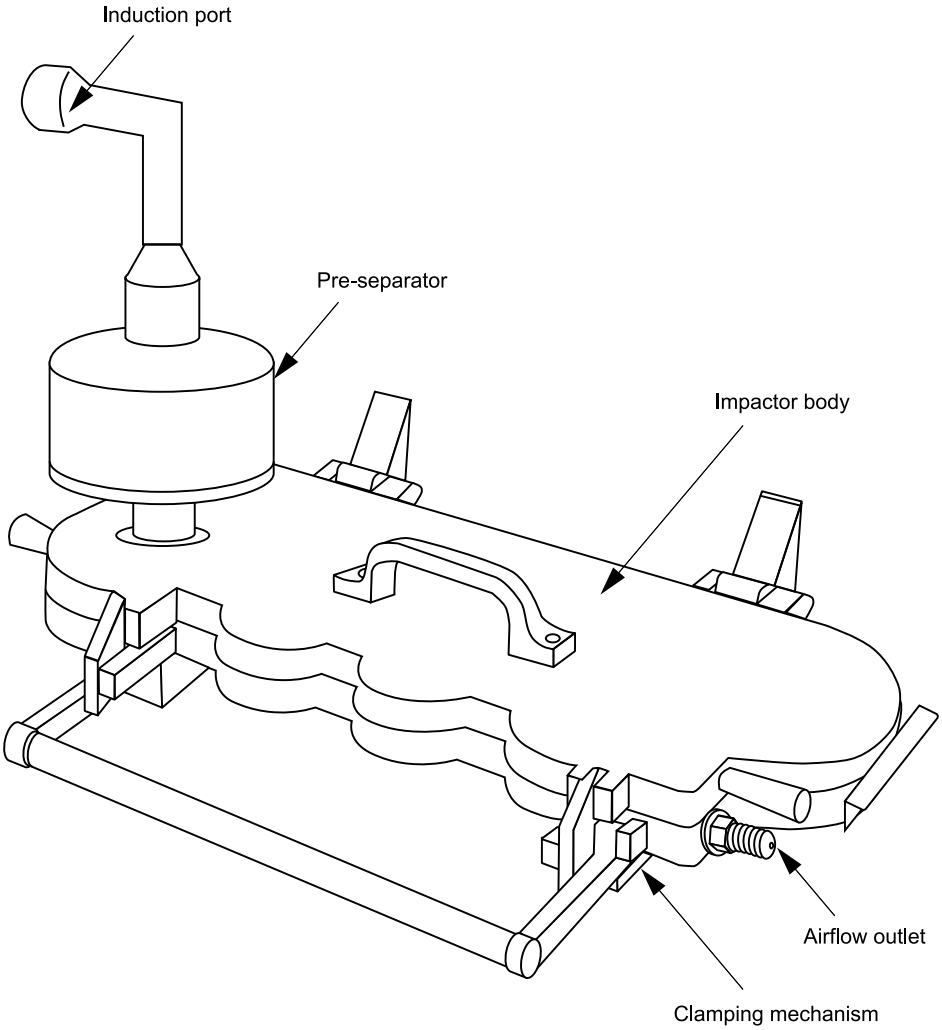


Figure 2.9.18.-11. – *Apparatus E (shown with the pre-separator in place)*

Table 2.9.18.-6. – *Critical dimensions for apparatus E*

| Description | Dimension (mm) |
|---|---|
| Pre-separator (dimension a - see Figure 2.9.18.-15) | 12.8 ± 0.05 |
| Stage 1* Nozzle diameter | 14.3 ± 0.05 |
| Stage 2* Nozzle diameter | 4.88 ± 0.04 |
| Stage 3* Nozzle diameter | 2.185 ± 0.02 |
| Stage 4* Nozzle diameter | 1.207 ± 0.01 |
| Stage 5* Nozzle diameter | 0.608 ± 0.01 |
| Stage 6* Nozzle diameter | 0.323 ± 0.01 |
| Stage 7* Nozzle diameter | 0.206 ± 0.01 |
| MOC* | approx. 0.070 |
| Cup depth (dimension b - see Figure 2.9.18.-14) | 14.625 ± 0.10 |
| Collection cup surface roughness (Ra) | 0.5 - 2 µm |
| Stage 1 nozzle to seal body distance** - dimension c | 0 ± 1.18 |
| Stage 2 nozzle to seal body distance** - dimension c | 5.236 ± 0.736 |
| Stage 3 nozzle to seal body distance** - dimension c | 8.445 ± 0.410 |
| Stage 4 nozzle to seal body distance** - dimension c | 11.379 ± 0.237 |
| Stage 5 nozzle to seal body distance** - dimension c | 13.176 ± 0.341 |
| Stage 6 nozzle to seal body distance** - dimension c | 13.999 ± 0.071 |
| Stage 7 nozzle to seal body distance** - dimension c | 14.000 ± 0.071 |
| MOC nozzle to seal body distance** - dimension c | 14.429 to 14.571 |

* See Figure 2.9.18.-13
** See Figure 2.9.18.-14

In routine operation, the seal body and lid are held together as a single assembly. The impaction cups are accessible when this assembly is opened at the end of an inhaler test. The cups are held in a support tray, so that all cups can be removed from the impactor simultaneously by lifting out the tray.

An induction port with internal dimensions (relevant to the airflow path) defined in Figure 2.9.18.-7 connects to the impactor inlet. A pre-separator can be added when required, typically with powder inhalers, and connects between the induction port and the impactor. A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port.

Apparatus E contains a terminal Micro-Orifice Collector (MOC) that for most formulations will eliminate the need for a final filter as determined by method validation. The MOC is an impactor plate with nominally 4032 holes, each approximately 70 µm in diameter. Most particles not captured on stage 7 of the impactor will be captured on the cup surface below the MOC. For impactors operated at 60 L/min, the MOC is capable of collecting 80 per cent of 0.14 µm particles. For formulations with a significant fraction of particles not captured by the MOC, there is an optional filter holder that can replace the MOC or be placed downstream of the MOC (a glass fibre filter is suitable).

**Procedure for pressurised inhalers**

Place cups into the apertures in the cup tray. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached and operate the handle to lock the impactor together so that the system is airtight.

Connect an induction port with internal dimensions defined in Figure 2.9.18.-7 to the impactor inlet. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port. When attached to the mouthpiece adapter, the inhaler is positioned in the same orientation as intended for use. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 30 L/min (± 5 per cent). Switch off the pump.

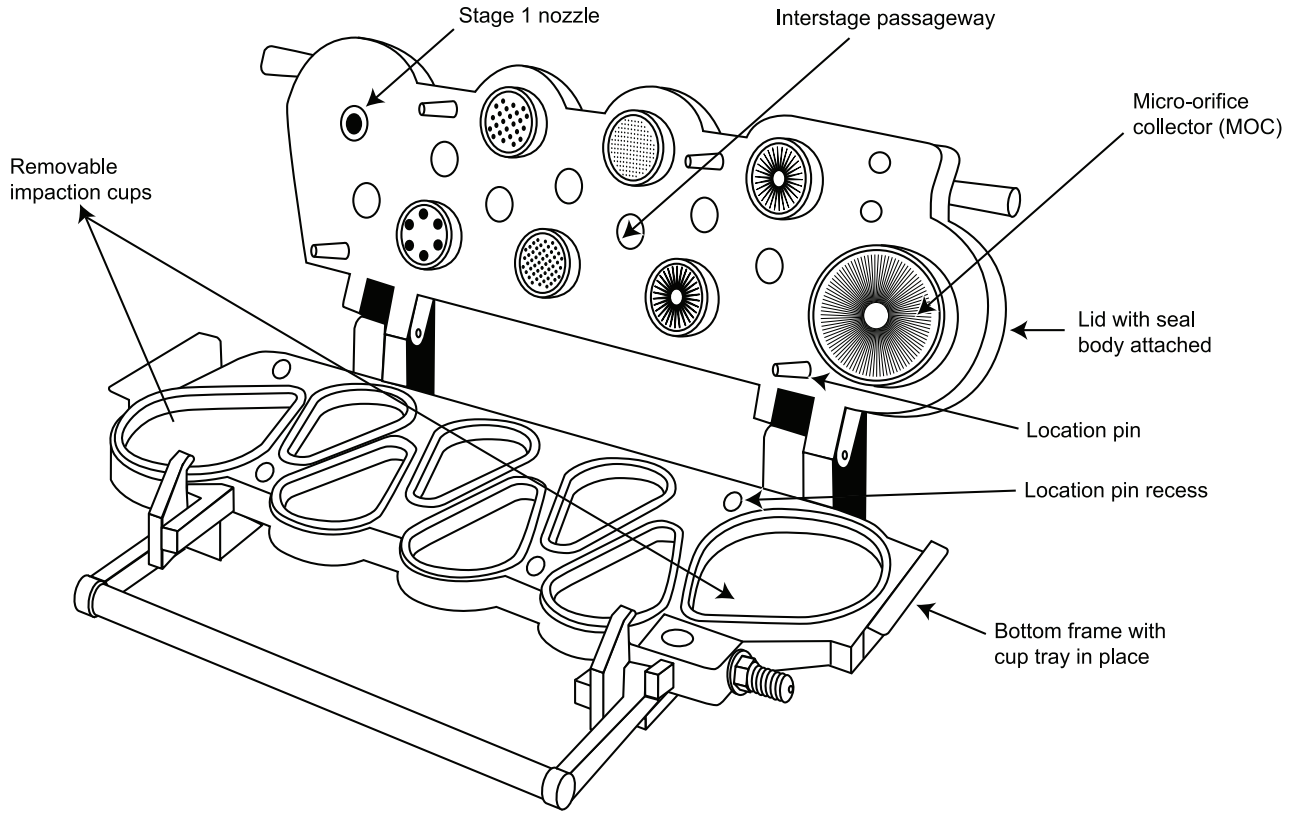


Figure 2.9.18.-12. – *Apparatus E showing component parts*

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge 1 delivery to waste. Switch on the pump to the apparatus. Prepare the inhaler for use according to the patient instructions, locate the mouthpiece end of the actuator in the adapter and discharge the inhaler into the apparatus, depressing the valve for a sufficient time to ensure a complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised, and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the apparatus and recover the active substance as follows: remove the induction port and mouthpiece adapter from the apparatus and recover the deposited active substance into an aliquot of solvent. Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active substance in each cup into an aliquot of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

**Procedure for powder inhalers**

Assemble the apparatus with the pre-separator (Figure 2.9.18.-15). Depending on the product characteristics, the pre-separator may be omitted, where justified.

Place cups into the apertures in the cup tray. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached and operate the handle to lock the impactor together so that the system is airtight.

When used, the pre-separator should be assembled as follows: assemble the pre-separator insert into the pre-separator base. Fit the pre-separator base to the impactor inlet. Add 15 mL of the solvent used for sample recovery to the central cup of the pre-separator insert. Place the pre-separator body on top of this assembly and close the 2 catches.

Connect an induction port with internal dimensions defined in Figure 2.9.18.-7 to the impactor inlet or pre-separator inlet. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the inhaler, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port. When attached to the mouthpiece adapter, the inhaler is positioned in the same orientation as intended for use. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4.

Unless otherwise prescribed, conduct the test at the flow rate, $Q_{out}$, used in the test for uniformity of delivered dose drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the

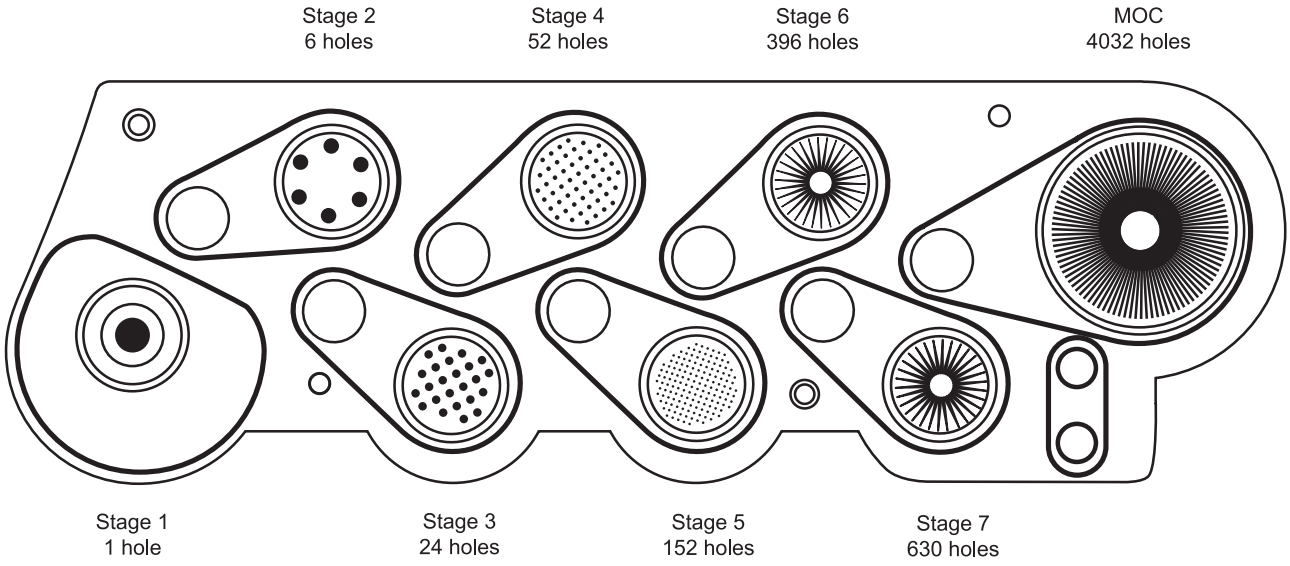


Figure 2.9.18.-13. – *Apparatus E: nozzle configuration*

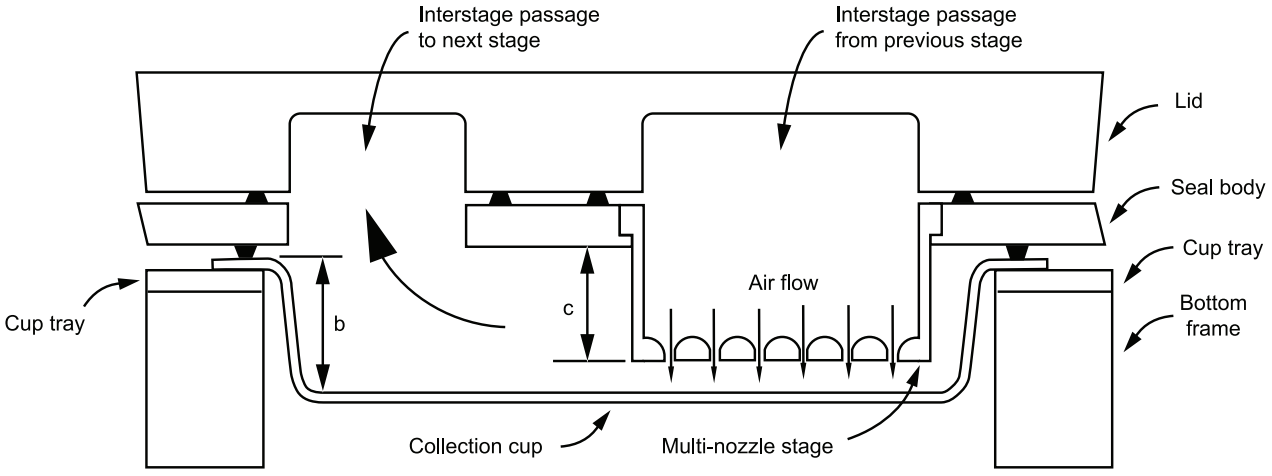


Figure 2.9.18.-14. – *Apparatus E: configuration of interstage passageways*

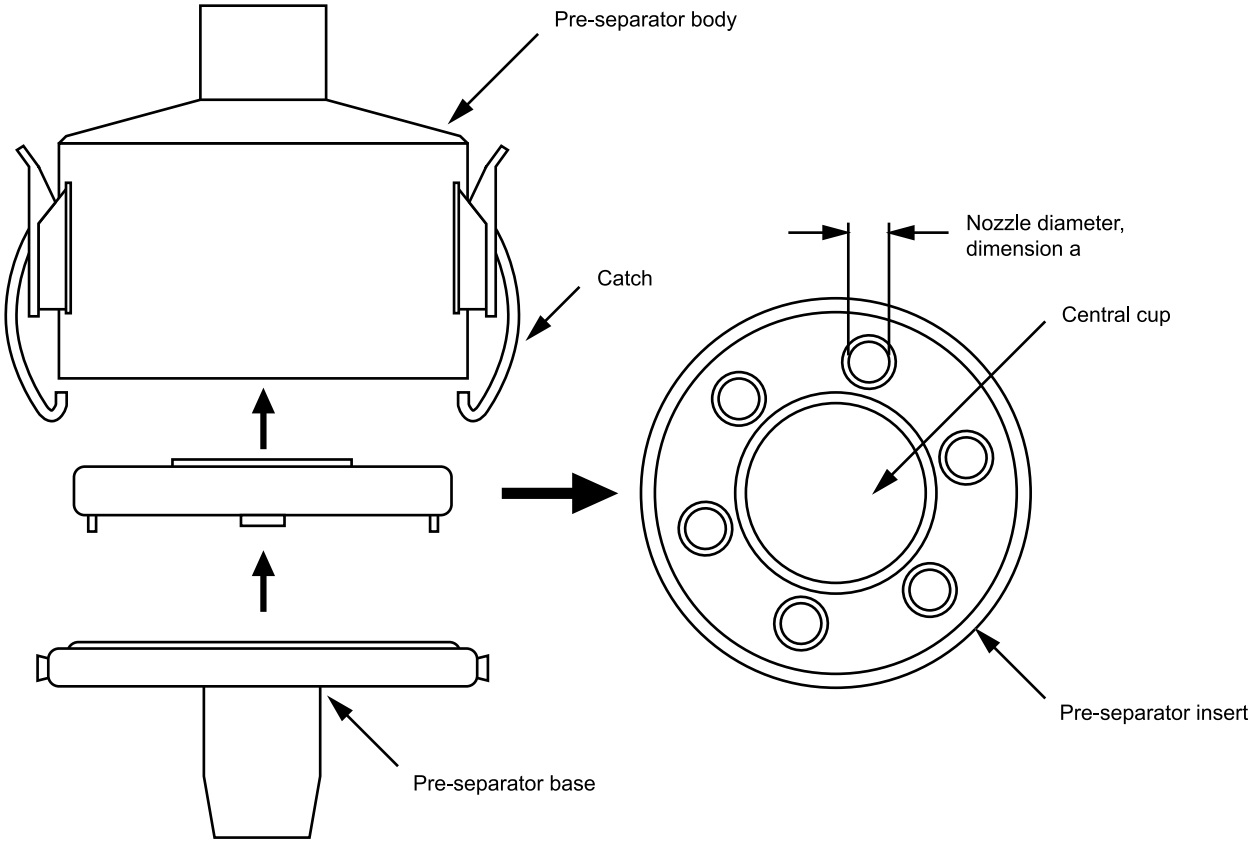


Figure 2.9.18.-15. – *Apparatus E: pre-separator configuration*

meter, or calculate the volumetric flow leaving the meter ($Q_{out}$) using the ideal gas law. For a meter calibrated for the entering volumetric flow ($Q_{in}$), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

$P_0$ = atmospheric pressure,

$\Delta P$ = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, $Q_{out}$ (± 5 per cent). Ensure that critical flow occurs in the flow control valve by the procedure described for Apparatus C. Switch off the pump.

Prepare the powder inhaler for use according to the patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, $T$ (± 5 per cent). Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

Dismantle the apparatus and recover the active substance as follows: remove the induction port and mouthpiece adapter from the pre-separator, when used, and recover the deposited active substance into an aliquot of solvent. When used, remove the pre-separator from the impactor, being careful to avoid spilling the cup liquid into the impactor. Recover the active substance from the pre-separator.

Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active substance in each cup into an aliquot of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

CALCULATIONS

From the analysis of the solutions, calculate the mass of active substance deposited on each stage per discharge and the mass of active substance per discharge deposited in the induction port, mouthpiece adapter and when used, the pre-separator.

Starting at the final collection site (filter or MOC), derive a table of cumulative mass versus cut-off diameter of the respective stage (see Tables 2.9.18.-7 for Apparatus C, 2.9.18.-8 for Apparatus D, 2.9.18.-9 for Apparatus E). Calculate by interpolation the mass of the active substance less than 5 µm. This is the Fine Particle Dose (FPD).

If necessary, and where appropriate (e.g., where there is a log-normal distribution), plot the cumulative fraction of active substance versus cut-off diameter (see Tables 2.9.18.-7/9) on log probability paper, and use this plot to determine values

Table 2.9.18.-7. – *Calculations for Apparatus C. Use* $q = \sqrt{(60 / Q)}$, *where Q is the test flow rate in litres per minute* ($Q_{out}$ *for powder inhalers)*

| Cut-off diameter (µm) | Mass of active substance deposited per discharge | Cumulative mass of active substance deposited per discharge | Cumulative fraction of active substance (per cent) |
|---|---|---|---|
| $d_4 = 1.7 \times q$ | mass from stage 5, $m_5$* | $c_4 = m_5$ | $f_4 = (c_4/c) \times 100$ |
| $d_3 = 3.1 \times q$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c) \times 100$ |
| $d_2 = 6.8 \times q$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c) \times 100$ |
| | mass from stage 2, $m_2$ | $c = c_2 + m_2$ | 100 |

* Stage 5 is the filter stage

Table 2.9.18.-8. – *Calculations for Apparatus D when used at a flow rate of 28.3 L/min*

| Cut-off diameter (µm) | Mass of active substance deposited per discharge | Cumulative mass of active substance deposited per discharge | Cumulative fraction of active substance (per cent) |
|---|---|---|---|
| $d_7 = 0.4$ | mass from stage 8, $m_8$ | $c_7 = m_8$ | $f_7 = (c_7/c) \times 100$ |
| $d_6 = 0.7$ | mass from stage 7, $m_7$ | $c_6 = c_7 + m_7$ | $f_6 = (c_6/c) \times 100$ |
| $d_5 = 1.1$ | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $f_5 = (c_5/c) \times 100$ |
| $d_4 = 2.1$ | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $f_4 = (c_4/c) \times 100$ |
| $d_3 = 3.3$ | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $f_3 = (c_3/c) \times 100$ |
| $d_2 = 4.7$ | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $f_2 = (c_2/c) \times 100$ |
| $d_1 = 5.8$ | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $f_1 = (c_1/c) \times 100$ |
| $d_0 = 9.0$ | mass from stage 1, $m_1$ | $c_0 = c_1 + m_1$ | $f_0 = (c_0/c) \times 100$ |
| | mass from stage 0, $m_0$ | $c = c_0 + m_0$ | 100 |

Table 2.9.18.-9. – *Calculations for Apparatus E. Use $q = (60/Q)^x$, where Q is the test flow rate in litres per minute, and x is listed in the table*

| Cut-off diameter (µm) | x | Mass of active substance deposited per discharge | Cumulative mass of active substance deposited per discharge | Cumulative fraction of active substance (per cent) |
|---|---|---|---|---|
| $d_7 = 0.34 \times q$ | 0.67 | mass from MOC or terminal filter, $m_8$ | $c_7 = m_8$ | $F_7 = (c_7/c) \times 100$ |
| $d_6 = 0.55 \times q$ | 0.60 | mass from stage 7, $m_7$ | $c_6 = c_7 + m_7$ | $F_6 = (c_6/c) \times 100$ |
| $d_5 = 0.94 \times q$ | 0.53 | mass from stage 6, $m_6$ | $c_5 = c_6 + m_6$ | $F_5 = (c_5/c) \times 100$ |
| $d_4 = 1.66 \times q$ | 0.47 | mass from stage 5, $m_5$ | $c_4 = c_5 + m_5$ | $F_4 = (c_4/c) \times 100$ |
| $d_3 = 2.82 \times q$ | 0.50 | mass from stage 4, $m_4$ | $c_3 = c_4 + m_4$ | $F_3 = (c_3/c) \times 100$ |
| $d_2 = 4.46 \times q$ | 0.52 | mass from stage 3, $m_3$ | $c_2 = c_3 + m_3$ | $F_2 = (c_2/c) \times 100$ |
| $d_1 = 8.06 \times q$ | 0.54 | mass from stage 2, $m_2$ | $c_1 = c_2 + m_2$ | $F_1 = (c_1/c) \times 100$ |
| | | mass from stage 1, $m_1$ | $c = c_1 + m_1$ | 100 |

for the Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) as appropriate. Appropriate computational methods may also be used.

**04/2011:20919**

# 2.9.19. PARTICULATE CONTAMINATION: SUB-VISIBLE PARTICLES[9]

Particulate contamination of injections and infusions consists of extraneous, mobile undissolved particles, other than gas bubbles, unintentionally present in the solutions.

For the determination of particulate contamination 2 procedures, Method 1 (Light Obscuration Particle Count Test) and Method 2 (Microscopic Particle Count Test), are specified hereinafter. When examining injections and infusions for sub-visible particles, Method 1 is preferably applied. However, it may be necessary to test some preparations by the light obscuration particle count test followed by the microscopic particle count test to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for sub-visible particles by one or both of these methods. When Method 1 is not applicable, e.g. in case of preparations having reduced clarity or increased viscosity, the test is carried out according to Method 2. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor may also require microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate contamination cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterise the level of particulate contamination in a large group of units.

## METHOD 1. LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage which allows an automatic determination of the size of particles and the number of particles according to size.

The apparatus is calibrated using suitable certified reference materials consisting of dispersions of spherical particles of known sizes between 10 µm and 25 µm. These standard particles are dispersed in *particle-free water R*. Care must be taken to avoid aggregation of particles during dispersion.

### General precautions

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water R*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

(9) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of 5 samples of *particle-free water R*, each of 5 mL, according to the method described below. If the number of particles of 10 µm or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware and water are suitable for the test.

**Method**

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of not less than 25 mL; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral administration are reconstituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Remove 4 portions, each of not less than 5 mL, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

**Evaluation**

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of test 1.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of test 1.B.

♦For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of test 1.B.♦

If the average number of particles exceeds the limits, test the preparation by the microscopic particle count test.

*Test 1.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL*

The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per millilitre equal to or greater than 10 µm and does not exceed 3 per millilitre equal to or greater than 25 µm.

*Test 1.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL*

The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm.

METHOD 2. MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, filter assembly for retaining particulate contamination and membrane filter for examination.

The microscope is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, 2 suitable illuminators to provide episcopic illumination in addition to oblique illumination, and is adjusted to 100 ± 10 magnifications.

The ocular micrometer is a circular diameter graticule (see Figure 2.9.19.-1.) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10 µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or international standard institution. A relative error of the linear scale of the graticule within ± 2 per cent is acceptable. The large circle is designated the graticule field of view (GFOV).

2 illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator adjustable to give reflected oblique illumination at an angle of 10-20°.

The filter assembly for retaining particulate contamination consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark grey in colour, non-gridded or gridded, and 1.0 µm or finer in nominal pore size.

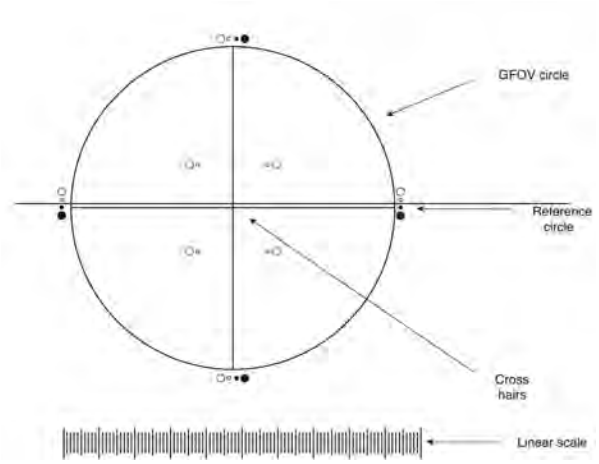


Figure 2.9.19.-1. – *Circular diameter graticule*

**General precautions**

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water R*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of a 50 mL volume of *particle-free water R* according to the method described below. If more than 20 particles 10 µm or larger in size or if more than 5 particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter and water are suitable for the test.

**Method**

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container

opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral administration are constituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several millilitres of *particle-free water R*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water R*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the filter in a Petri dish and allow the filter to air-dry with the cover slightly ajar. After the filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm. Alternatively, partial filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by transforming mentally the image of each particle into a circle and then comparing it to the 10 µm and 25 µm graticule reference circles. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the microscopic particle count test do not attempt to size or enumerate amorphous, semi-liquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discoloration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases the interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count test.

**Evaluation**

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of test 2.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of test 2.B.

♦For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of test 2.B.♦

*Test 2.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL*

The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per millilitre equal to or greater than 10 µm and does not exceed 2 per millilitre equal to or greater than 25 µm.

*Test 2.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL*

The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 µm and does not exceed 300 per container equal to or greater than 25 µm.

**01/2020:20920**

# 2.9.20. PARTICULATE CONTAMINATION : VISIBLE PARTICLES

Particulate contamination consists of mobile undissolved substances, other than gas bubbles, unintentionally present in liquid preparations.

The test is intended to provide a simple procedure for the visual assessment of the quality of liquid preparations, if applicable after reconstitution, as regards visible particles.

EQUIPMENT

The equipment (see Figure 2.9.20.-1) consists of a viewing station comprising:

- a matt black panel (A) of appropriate size held in a vertical position;
- a non-glare white panel (B) of appropriate size held in a vertical position next to the black panel;
- a non-glare white panel (C) of appropriate size held in a horizontal position next to (A) and (B);
- a lampholder (D) fitted with a suitable, shaded, white-light source and with a suitable light diffuser (e.g. a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length, or an appropriate light-emitting diode (LED) light source). The intensity of illumination at the viewing point is maintained between 2000 lux and 3750 lux, although higher values may be required for coloured glass or plastic containers and for coloured or turbid preparations.

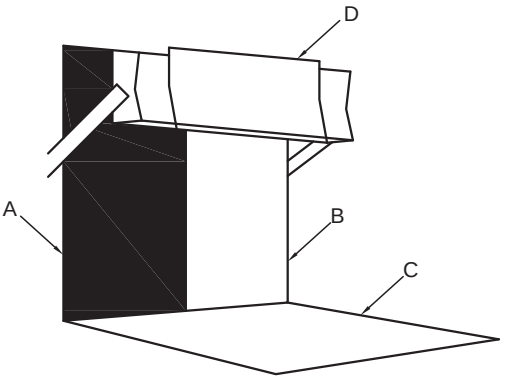


Figure 2.9.20.-1. – *Equipment for visible particles*

METHOD

Adequate visual inspection of the container and contents is necessary, which may require the removal of any adherent labels from the container. Gently swirl or invert the container, ensuring that air bubbles are not introduced, and observe without magnification for about 5 s in front of the white panel (B), although longer observation times may be required for coloured glass or plastic containers and for coloured

or turbid preparations. Where inspection in the primary container is not possible, the contents may be transferred for inspection into a sample container that is free from visible particles, taking precautions to prevent contamination during transfer. Repeat the inspection in front of the black panel (A). Record the presence of any visible particles.

**01/2008:20922**

## 2.9.22. SOFTENING TIME DETERMINATION OF LIPOPHILIC SUPPOSITORIES

The test is intended to determine, under defined conditions, the time which elapses until a suppository maintained in water softens to the extent that it no longer offers resistance when a defined weight is applied.

APPARATUS A

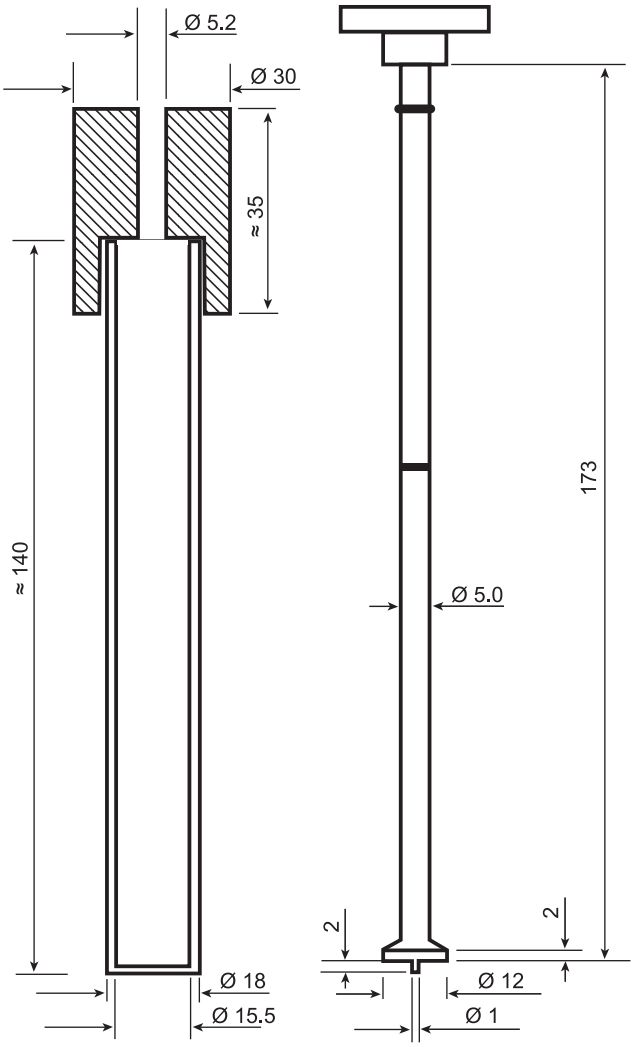


Figure 2.9.22.-1. – *Apparatus A for measuring the softening time of lipophilic suppositories*

*Dimensions in millimetres*

The apparatus (see Figure 2.9.22.-1) consists of a glass tube 15.5 mm in internal diameter with a flat bottom and a length of about 140 mm. The tube is closed by a removable plastic cover having an opening 5.2 mm in diameter. The apparatus comprises a rod 5.0 mm in diameter which becomes wider towards the lower end, reaching a diameter of 12 mm. A metal needle 2 mm in length and 1 mm in diameter is fixed on the flat underside.

The rod consists of 2 parts, a lower part made of plastic material and an upper part made of plastic material or metal with a weight disk. The upper and lower parts are either fitted together (manual version) or separate (automated version). The weight of the entire rod is 30 ± 0.4 g. The upper part of the rod carries a sliding mark ring. When the rod is introduced into the glass tube so that it touches the bottom, the mark ring is adjusted to coincide with the upper level of the plastic cover.

**Method**. Place the glass tube containing 10 mL of water in a water-bath and equilibrate at 36.5 ± 0.5 °C. Fix the glass tube vertically and immerse to a depth of at least 7 cm below the surface but without touching the bottom of the water-bath. Introduce a suppository, tip first, into the tube followed by the rod with the free gliding plastic cover into the glass tube until the metal needle touches the flat end of the suppository. Put the cover on the tube (beginning of time measurement). Note the time which elapses until the rod sinks down to the bottom of the glass tube and the mark ring reaches the upper level of the plastic cover.

APPARATUS B

The apparatus (see Figure 2.9.22.-2) consists of a water-bath (B) into which an inner tube (A) is inserted and fixed with a stopper. The inner tube is closed by a stopper at the bottom. The apparatus is fitted with a thermometer. 2 insets are available:

- a glass rod (C1) in the form of a tube sealed at both ends, carrying a rim at its lower end weighed with lead shot, which has a weight of 30 ± 0.4 g,
- a penetration inset (C2) consisting of a rod (7.5 ± 0.1 g) in a tube which has an enlargement for the suppository, both made of stainless steel.

**Method**. Pour 5 mL of water at 36.5 ± 0.5 °C into the inner tube (A), introduce a suppository with the tip downwards and onto that, place the inset (C1 or C2). Note the time which elapses between this moment and the moment when the lower, rimmed end of the glass rod (C1) or the steel rod (C2) reaches the narrowed part of the inner glass tube. Melting or dissolution is then considered as complete.

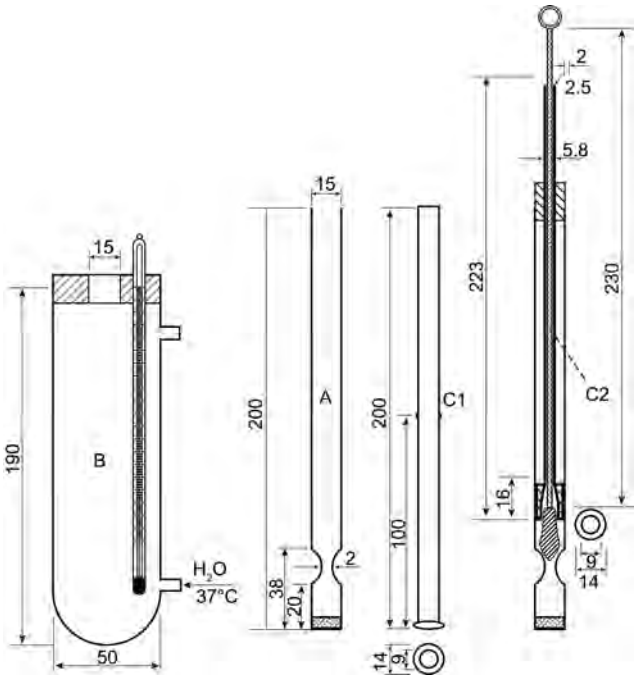


Figure 2.9.22.-2. – *Apparatus B for measuring the softening time of lipophilic suppositories*

*Dimensions in millimetres*

04/2019:20923

# 2.9.23. GAS PYCNOMETRIC DENSITY OF SOLIDS(10)

Gas pycnometric density is determined by measuring the volume occupied by a known mass of powder, which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer. In gas pycnometric density measurements, the volume determined excludes the volume occupied by open pores; however, it includes the volume occupied by sealed pores or pores inaccessible to the gas.

Usually, helium is used as a test gas due to its high diffusivity into small open pores. If gases other than helium are used, different values would be obtained, since the penetration of the gas is dependent on the size of the pore as well as the cross-sectional area of the gas molecules.

The measured density is a volume-weighted average of the densities of individual powder particles. It is called the particle density, distinct from the true density of a solid or the bulk density of a powder. The density of solids is expressed in grams per cubic centimetre (g/cm$^3$), although the International Unit is the kilogram per cubic metre (1 g/cm$^3$ = 1000 kg/m$^3$).

## APPARATUS

The apparatus (see Figure 2.9.23.-1) consists of the following:

- a sealed test cell, with empty cell volume $V_c$, connected through a valve to an expansion cell, with volume $V_r$;
- a system capable of pressurising the test cell with the measurement gas until a defined pressure ($P$) indicated by a manometer;
- the system is connected to a source of measurement gas, preferably helium, unless another gas is specified.

The gas pycnometric density measurement is performed at a temperature between 15 °C and 30 °C that does not vary by more than 2 °C during the course of measurement.

The apparatus is calibrated, which means that the volumes $V_c$ and $V_r$ are determined using a suitable calibration standard whose volume is known to the nearest 0.001 cm$^3$. The procedure described below is followed in 2 runs, firstly with an empty test cell, and secondly with the calibration standard placed in the test cell. The volumes $V_c$ and $V_r$ are calculated using the equation for the sample volume ($V_s$), taking into account that $V_s$ is zero in the first run.

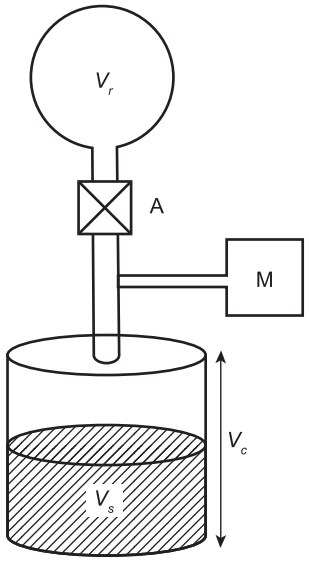


A = valve;
$V_r$ = expansion volume, in cubic centimetres;
$V_c$ = cell volume, in cubic centimetres;
$V_s$ = sample volume, in cubic centimetres;
M = manometer.

Figure 2.9.23.-1. – *Schematic diagram of a gas pycnometer*

## METHOD

Volatile contaminants in the powder are removed by degassing the powder under a constant purge of helium prior to the measurement. Occasionally, powders may have to be degassed under vacuum. Because volatiles may be evolved during the measurement, weighing of the sample is carried out after the pycnometric measurement of volume.

Weigh the test cell of the pycnometer and record the mass. Fill the test cell with a given mass of powder of the substance to be examined. Seal the test cell in the pycnometer. Record the system reference pressure ($P_r$) as indicated by the manometer while the valve that connects the expansion cell with the test cell is open. Close the valve to separate the expansion cell from the test cell. Pressurise the test cell with the gas to an initial pressure ($P_i$) and record the value obtained. Open the valve to connect the expansion cell with the test cell. Record the final pressure ($P_f$). Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume ($V_s$) agree to within 0.2 per cent. Unload the test cell and measure the final powder mass ($m$), expressed in grams. If the pycnometer differs in operation or construction from the one shown in Figure 2.9.23.-1, follow the instructions of the manufacturer of the pycnometer.

## EXPRESSION OF THE RESULTS

The sample volume ($V_s$) is given by the equation:

$$V_s = V_c - \frac{V_r}{\frac{P_i - P_r}{P_f - P_r} - 1}$$

The density ($\rho$) is given by the equation:

$$\rho = \frac{m}{V_s}$$

The sample conditioning is indicated with the results. For example, indicate whether the sample was tested as is or dried under specific conditions such as those described for loss on drying.

(10) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

**04/2012:20925** – 2 guides with bushes (Figure 2.9.25.-3).

## 2.9.25. DISSOLUTION TEST FOR MEDICATED CHEWING GUMS

PRINCIPLE

The test is used to determine the dissolution rate of active substances in medicated chewing gums. This is done by applying a mechanical kneading procedure to a piece of gum placed in a small chamber designed to simulate the process of chewing.

APPARATUS A

Chewing apparatus A (Figure 2.9.25.-1) consists of:

- 1 chewing chamber;
- 1 vertical piston;
- 2 horizontal pistons with O-rings and sealing rings.

The chewing chamber consists of 4 individual parts:

- 1 central chamber;
- 1 funnel (Figure 2.9.25.-2);

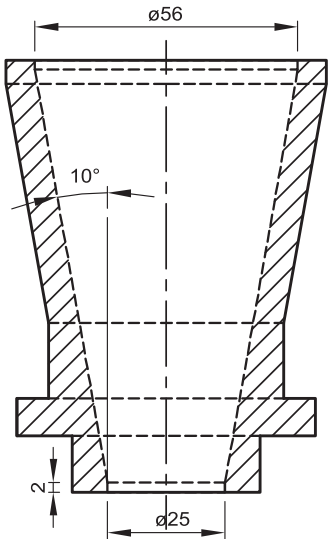


Figure 2.9.25.-2 – *Funnel*
*Dimensions in millimetres*

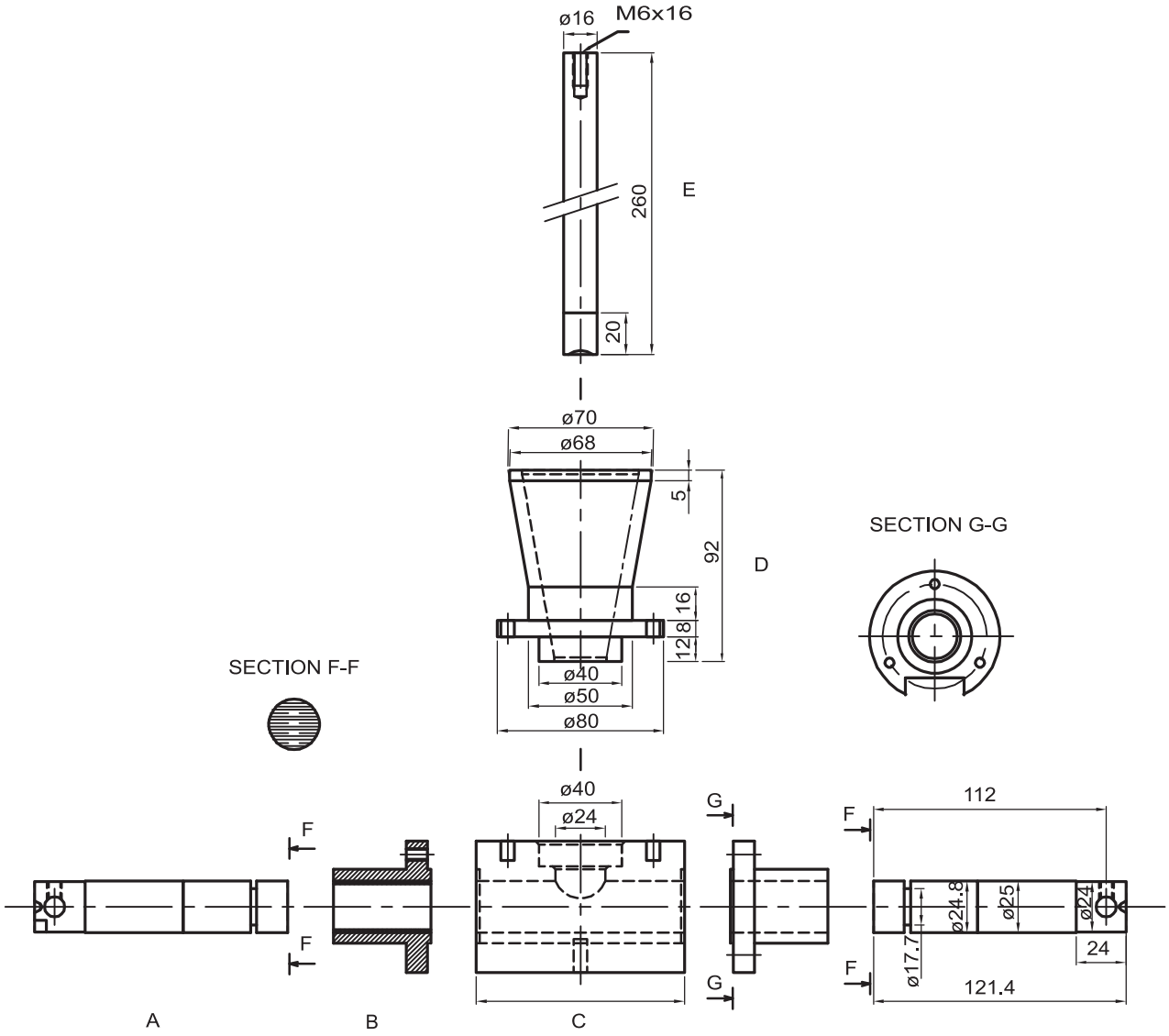


A. horizontal piston B. guide C. chewing chamber D. funnel E. vertical piston

Figure 2.9.25.-1 – *Apparatus A - Chewing chamber and pistons*
*Dimensions in millimetres*

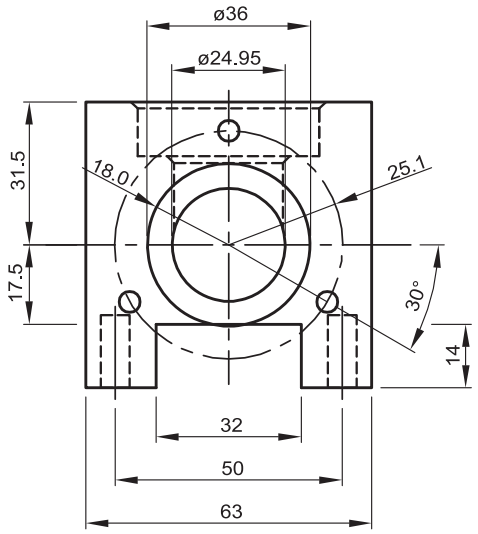


Figure 2.9.25.-3 – *Guide (section G-G)*

*Dimensions in millimetres*

The funnel and guides are mounted on the central chamber. The O-rings are incorporated in the piston recess with the sealing ring around it; the sealing rings ensure that the chamber is watertight. The horizontal pistons are placed in the chewing chamber through the guides.

The gum is artificially chewed by the horizontal pistons, and the vertical piston ensures that the gum stays in the right place between chews.

Machine speed is controlled to ensure a constant cycle. One cycle (chew) is defined as follows: the horizontal pistons start from their outermost position, move to their innermost position then return to their outermost position. Within one cycle, the vertical piston moves from its lowest position to its uppermost position and back to its lowest position.

Each horizontal piston has a stroke of 25.0 mm. The maximum distance between these 2 pistons is 50 mm. The minimum distance between the 2 horizontal pistons is 0.1 mm to 1.0 mm. The vertical piston has a stroke of 22.0 mm.

Horizontal piston movement is controlled so that the 2 pistons are at their innermost position at the same time. Vertical piston movement is controlled so that it does not conflict with the movement of the horizontal pistons.

If necessary, the machine can be constructed so that the horizontal pistons rotate around their own axes in opposite direction to each other by the end of the chew in order to obtain maximum chewing.

All parts of the apparatus that may come into contact with the preparation or the dissolution medium are chemically inert and do not adsorb, react with or interfere with the sample.

APPARATUS B

Chewing apparatus B (Figure 2.9.25.-4) consists of:

– 1 test cell (Figure 2.9.25.-5 or 2.9.25.-6);

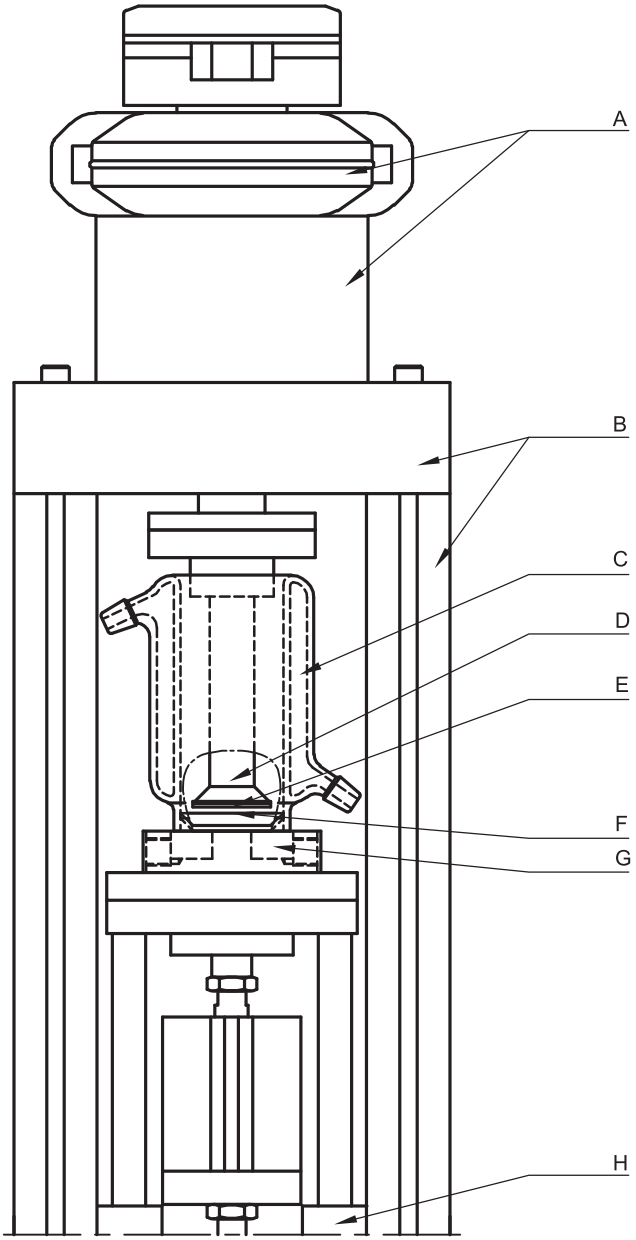


| | |
|---|---|
| A. revolving device for the upper chewing surface | E. upper chewing surface |
| B. stand | F. lower chewing surface |
| C. test cell | G. base chamber |
| D. axle | H. device for up-and-down chewing motion |

Figure 2.9.25.-4 – *Apparatus B*

– 1 vertical axle with upper chewing surface (Figures 2.9.25.-7 and 2.9.25.-8);
– 1 base chamber with lower chewing surface (Figures 2.9.25.-9 and 2.9.25.-10);
– 1 device for up-and-down chewing motion;
– 1 revolving device for the vertical axle.

The gum is artificially chewed by the lower and upper chewing surfaces. Machine speed is controlled to ensure a constant cycle. The distance between the lower and upper chewing surfaces may be set up to 5 mm. The turning angle of the revolving device is about 20°.

The test cells may also be equipped with 1 or 2 glass sampling tubes, coming through the thermostatic double wall. These tubes also make it possible to have an external sink, which may be necessary to achieve sink conditions for sparingly soluble substances.

The gum is usually sandwiched between 2 circular plastic nets to prevent disintegration.

Nets made from nylon (PA6) with an aperture of 1.4 mm and a wire diameter of 0.405 mm may be used.

All parts of the apparatus that may come into contact with the preparation or the dissolution medium are chemically inert and do not adsorb, react with or interfere with the sample.

PROCEDURE

For each determination, the following information is needed:

- apparatus used (type A or type B);
- composition, volume and temperature of the dissolution medium;
- number of chews per minute;
- time and sampling method;
- whether the analysis is performed on the gum residue or on the dissolution medium;
- method of analysis.

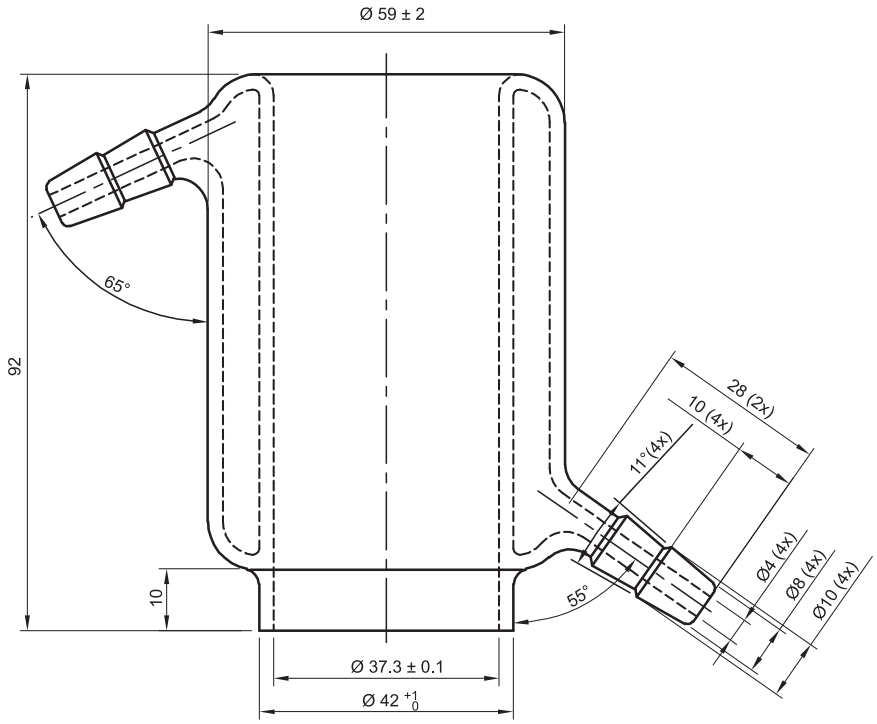


Figure 2.9.25.-5 – *Test cell*
*Dimensions in millimetres*

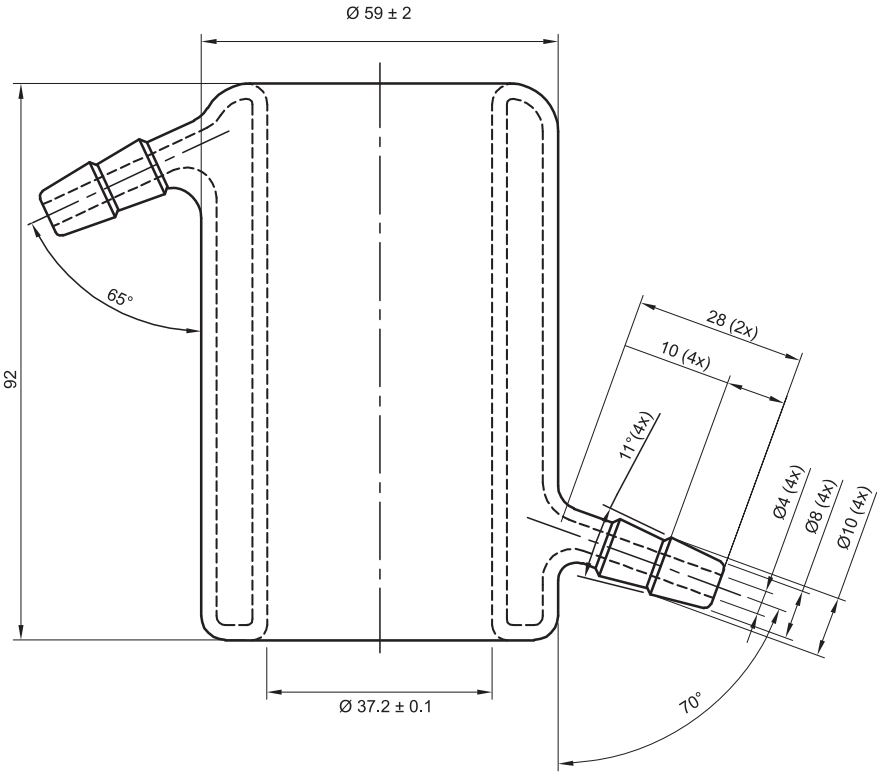


Figure 2.9.25.-6 – *Test cell (straight)*
*Dimensions in millimetres*

Place the prescribed volume of dissolution medium in the chewing chamber, usually 20 mL of *phosphate buffer solution pH 6.0 R2*. Maintain the medium temperature at 37 ± 0.5 °C using an electrical device with external control (apparatus A) or with a thermostat (apparatus B). Set the machine speed at the prescribed number of chews per minute (typically up to 60). Accurately weigh a portion of gum or the whole gum, put it into the chewing chamber and start the machine.

SAMPLING AND EVALUATION

Stop the apparatus at the prescribed time. Remove the gum residue and take a sample of the dissolution medium. Determine the content of active substance(s) by a suitable method. Medium replacement may be made after each sampling procedure; compensation by calculation of medium volume change or sample dilution is needed. Alternatively, determine the content of active substance(s) remaining in the gum residue. Carry out the test successively on 6 medicated chewing gums.

The quantity of active substance(s) dissolved in a specified time is expressed as a percentage of the content stated on the label.

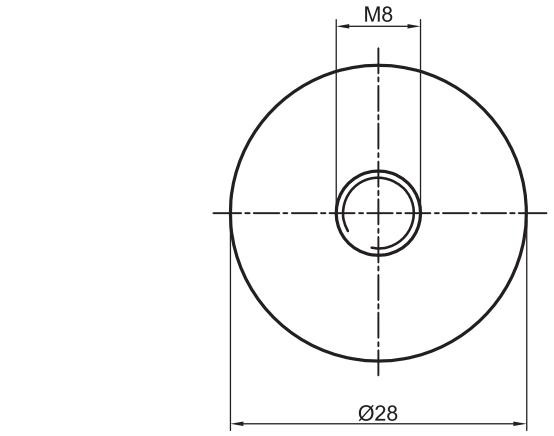


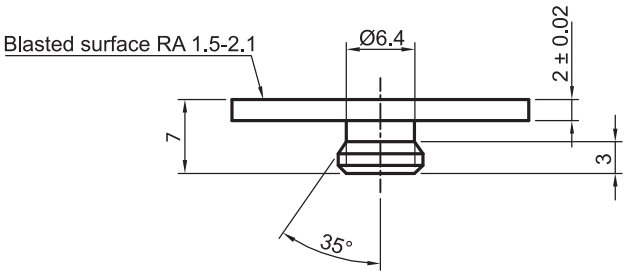


Figure 2.9.25.-8 – *Upper chewing surface*
*Dimensions in millimetres*

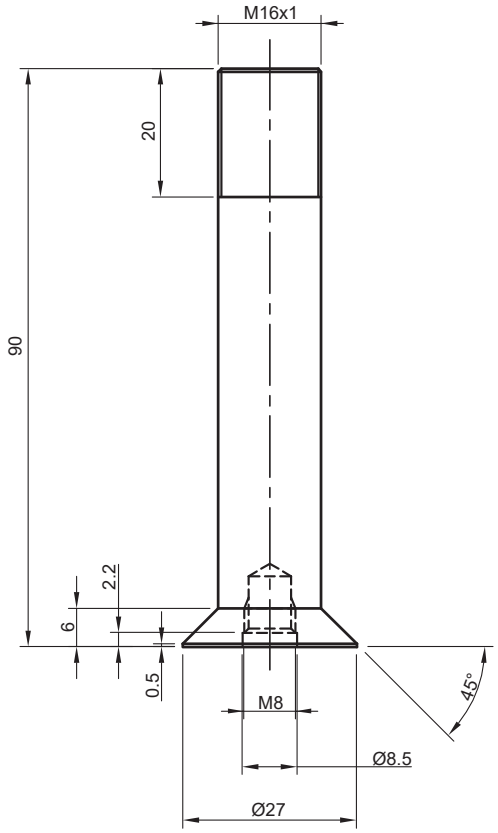


Figure 2.9.25.-7 – *Axle*

*Dimensions in millimetres*

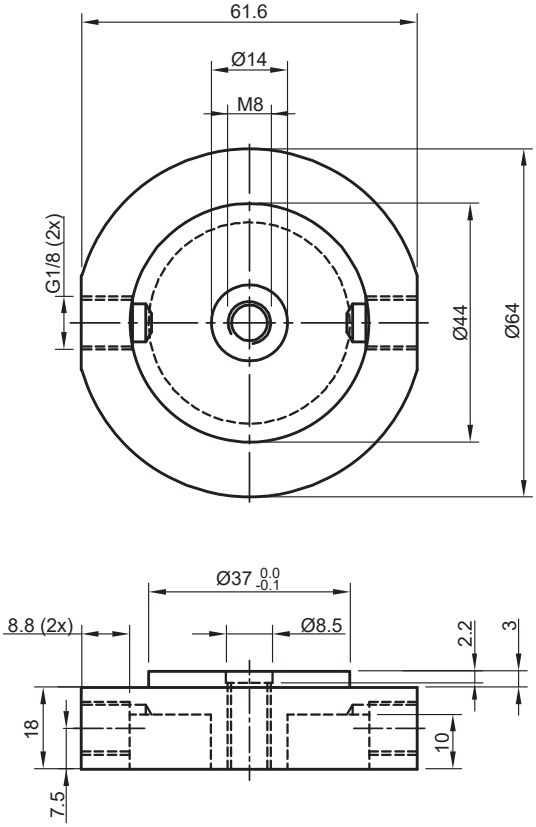


Figure 2.9.25.-9 – *Base chamber*
*Dimensions in millimetres*

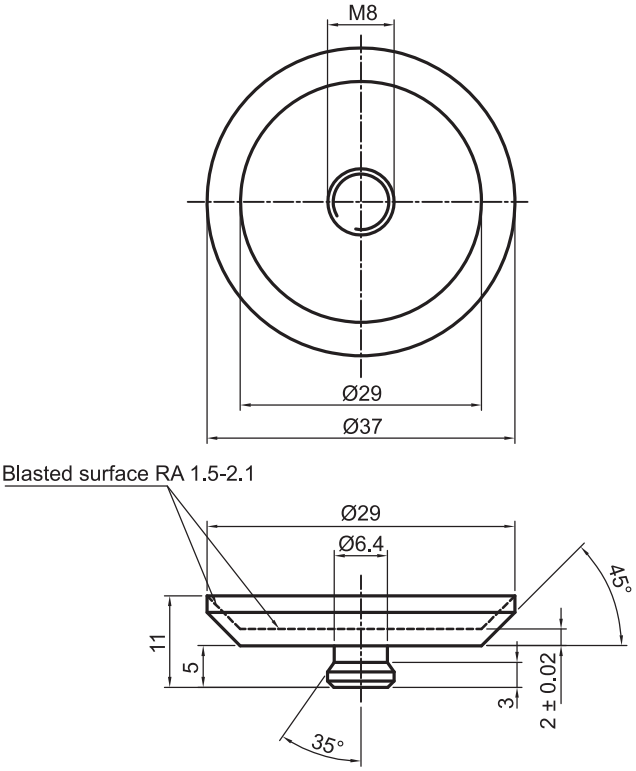


Figure 2.9.25.-10 – *Lower chewing surface*

*Dimensions in millimetres*

**01/2010:20926**

## 2.9.26. SPECIFIC SURFACE AREA BY GAS ADSORPTION[(11)]

INTRODUCTION

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen. The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

*MULTI-POINT MEASUREMENT*

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$\frac{1}{\left[V_a\left(\frac{P_o}{P}-1\right)\right]} = \frac{C-1}{V_mC}\times\frac{P}{P_o}+\frac{1}{V_mC} \qquad (1)$$

$P$ = partial vapour pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in pascals,

$P_o$ = saturated pressure of adsorbate gas, in pascals,

$V_a$ = volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure ($1.013 \times 10^5$ Pa)], in millilitres,

$V_m$ = volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in millilitres,

$C$ = dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of $V_a$ is measured at each of not less than 3 values of $P/P_o$.

Then the BET value

$$\frac{1}{\left[V_a\left(\frac{P_o}{P}-1\right)\right]}$$

is plotted against $P/P_o$ according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, $r$, of the linear regression is not less than 0.9975; that is, $r^2$ is not less than 0.995. From the resulting linear plot, the slope, which is equal to $(C-1)/V_mC$, and the intercept, which is equal to $1/V_mC$, are evaluated by linear regression analysis. From these values, $V_m$ is calculated as $1/(slope + intercept)$, while $C$ is calculated as $(slope/intercept) + 1$. From the value of $V_m$ so determined, the specific surface area, $S$, in $m^2 \cdot g^{-1}$, is calculated by the equation:

$$S = \frac{V_mNa}{m \times 22400} \qquad (2)$$

$N$ = Avogadro constant ($6.022 \times 10^{23}$ $mol^{-1}$),

$a$ = effective cross-sectional area of one adsorbate molecule, in square metres (0.162 $nm^2$ for nitrogen and 0.195 $nm^2$ for krypton),

$m$ = mass of test powder, in grams,

22400 = volume occupied by 1 mole of the adsorbate gas at STP allowing for minor departures from the ideal, in millilitres.

A minimum of 3 data points is required. Additional measurements may be carried out, especially when non-linearity is obtained at a $P/P_o$ value close to 0.3. Because non-linearity is often obtained at a $P/P_o$ value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

*SINGLE-POINT MEASUREMENT*

Normally, at least 3 measurements of $V_a$ each at different values of $P/P_o$ are required for the determination of specific surface area by the dynamic flow gas adsorption technique (*Method I*) or by volumetric gas adsorption (*Method II*). However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of $V_a$ measured at a single value of $P/P_o$ such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating $V_m$:

$$V_m = V_a\left(1-\frac{P}{P_o}\right) \qquad (3)$$

The specific surface area is then calculated from the value of $V_m$ by equation (2) given above.

(11) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant $C$ is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multiple-point method for the series of powder samples. Close similarity between the single-point values and multiple-point values suggests that $1/C$ approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant $C$ is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multiple-point method to evaluate $C$ for one of the samples of the series from the BET plot, from which $C$ is calculated as (1 + *slope/intercept*). Then $V_m$ is calculated from the single value of $V_a$ measured at a single value of $P/P_o$ by the equation:

$$V_m = V_a\left(\frac{P_o}{P} - 1\right)\left[\frac{1}{C} + \frac{C-1}{C} \times \left(\frac{P}{P_o}\right)\right] \qquad (4)$$

The specific surface area is calculated from $V_m$ by equation (2) given above.

EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique (*Method I*) and the volumetric gas adsorption technique (*Method II*).

*SAMPLE PREPARATION*

**Outgassing**

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapours that may have become physically adsorbed onto the surface after manufacture and during treatment, handling and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapours. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

*Conditions.* The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure and time should be chosen so that the original surface of the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum, by purging the sample in a flowing stream of a non-reactive, dry gas, or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

**Adsorbate**

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area (< 0.2 $m^2 \cdot g^{-1}$) the proportion adsorbed is low. In such cases the use of krypton at liquid nitrogen temperature is preferred because the low vapour pressure exerted by this gas greatly reduces error. The use of larger sample quantities where feasible (equivalent to 1 $m^2$ or greater total surface area using nitrogen) may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

**Quantity of sample**

Accurately weigh a quantity of the test powder such that the total surface of the sample is at least 1 $m^2$ when the adsorbate is nitrogen and 0.5 $m^2$ when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

*MEASUREMENTS*

Because the amount of gas adsorbed under a given pressure tends to increase on decreasing the temperature, adsorption measurements are usually made at a low temperature. Measurement is performed at 77.4 K, the boiling point of liquid nitrogen.

**Method I: the dynamic flow method**

*Principle*

In the dynamic flow method (see Figure 2.9.26.-1), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

A minimum of 3 mixtures of the appropriate adsorbate gas with helium are required within the $P/P_o$ range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of 3 data points within the recommended range of 0.05 to 0.30 for $P/P_o$ is to be determined.

*Procedure*

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample, again through the thermal conductivity cell and then to a recording potentiometer.

Immerse the sample cell in liquid nitrogen, then the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

Remove from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Since this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, inject a known quantity of adsorbate into the system, sufficient to give a peak of similar magnitude to the desorption peak and obtain the proportion of gas volume per unit peak area.

Use a nitrogen/helium mixture for a single-point determination and several such mixtures or premixing 2 streams of gas for a multiple-point determination.

Calculation is essentially the same as for the volumetric method.

**Method II: the volumetric method**

*Principle*

In the volumetric method (see Figure 2.9.26.-2), the recommended adsorbate gas is nitrogen which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, $P$, of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Since only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

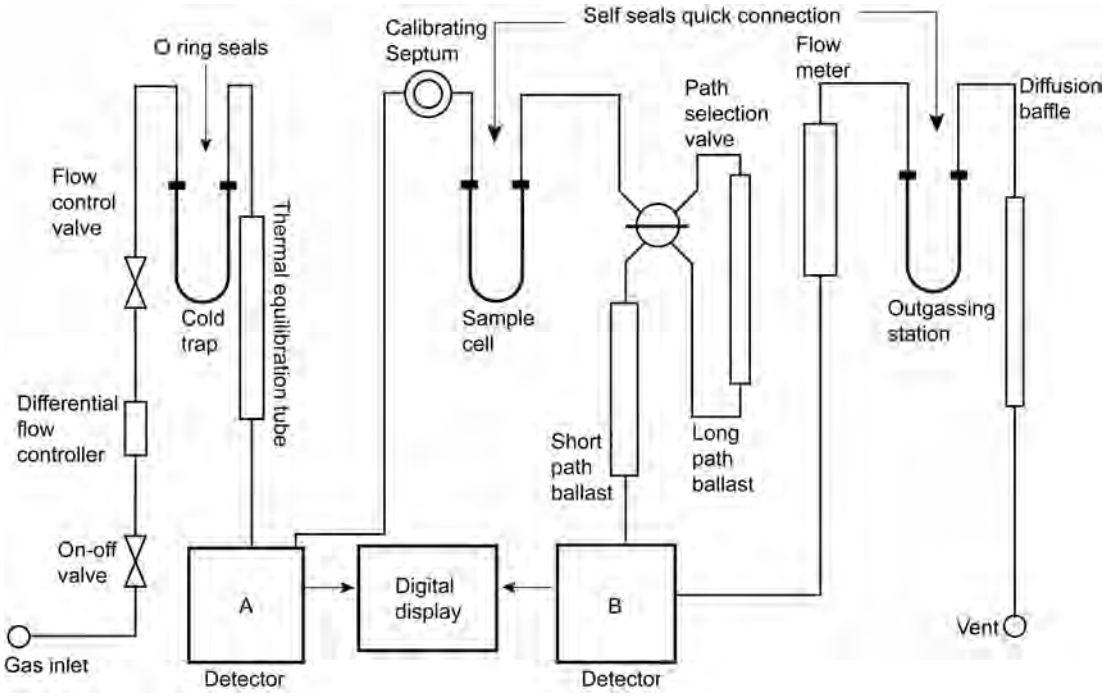


Figure 2.9.26.-1. – *Schematic diagram of the dynamic flow method apparatus*

*Procedure*

Admit a small amount of dry nitrogen into the sample tube to prevent contamination of the clean surface, remove the sample tube, insert the stopper, and weigh it. Calculate the weight of the sample. Attach the sample tube to the volumetric apparatus. Cautiously evacuate the sample down to the specified pressure (e.g. between 2 Pa and 10 Pa). Alternatively, some instruments operate by evacuating to a defined rate of pressure change (e.g. less than 13 Pa/30 s) and holding for a defined period of time before commencing the next step.

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a non-adsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements, that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed, $V_a$. For multipoint measurements, repeat the measurement of $V_a$ at successively higher $P/P_o$ values. When nitrogen is used as the adsorbate gas, $P/P_o$ values of 0.10, 0.20, and 0.30 are often suitable.

REFERENCE MATERIALS

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area, such as α-alumina, which should have a specific surface area similar to that of the sample to be examined.

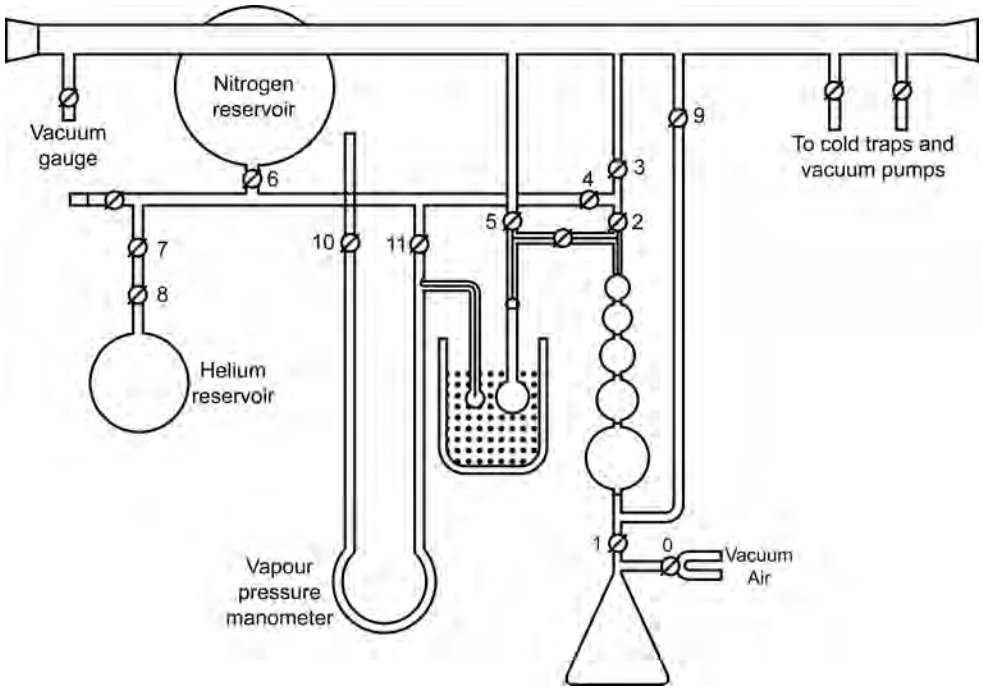


Figure 2.9.26.-2. – *Schematic diagram of the volumetric method apparatus*

01/2008:20927

## 2.9.27. UNIFORMITY OF MASS OF DELIVERED DOSES FROM MULTIDOSE CONTAINERS

*The following test is intended for oral dosage forms such as granules, powders for oral use and liquids for oral use, which are supplied in multidose containers provided at manufacture with a measuring device.*

Weigh individually 20 doses taken at random from one or more containers with the measuring device provided and determine the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 per cent and none deviates by more than 20 per cent.

01/2008:20929

## 2.9.29. INTRINSIC DISSOLUTION

The test is intended to determine the intrinsic dissolution rate of pure solid substances following compaction. It is carried out under specified experimental conditions such that a practical measure of the intrinsic dissolution rate is obtained.

The intrinsic dissolution rate is a theoretical value referring to pure solid substances having null porosity, but, practically, intrinsic dissolution rate is determined on substances having a minimal porosity.

PRINCIPLE

The intrinsic dissolution rate is defined as the dissolution rate of pure substances following compaction under the condition of constant surface area. Its assessment is useful in the characterisation of active substances and excipients.

The dissolution rate of pure substances can be affected by all the solid state properties such as crystal habit, crystallinity, amorphism, polymorphism, pseudo-polymorphism, particle size and specific surface area. In addition, it can also be influenced by extrinsic factors (test conditions), such as hydrodynamics, temperature, viscosity, pH, buffer strength and ionic strength of the dissolution medium.

The assessment of intrinsic dissolution rate of a solid substance involves the preparation of a compact. Assurance of appropriate compaction properties of the powder to be tested is needed prior to performing the test.

The intrinsic dissolution rate is determined by exposing a constant area of the compacted substance to an appropriate dissolution medium, while maintaining constant stirring rate, temperature, ionic strength and pH.

The intrinsic dissolution rate is expressed in terms of dissolved mass of substance per time per exposed area, typically in milligrams per minute per square centimetre ($mg \cdot min^{-1} \cdot cm^{-2}$).

APPARATUS

A typical apparatus consists of a punch and die fabricated out of hardened steel. The base of the die has 3 threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compact. The die has a 0.1-1.0 cm diameter cavity into which a measured amount of the powder to be tested is placed. The punch is then inserted in the die cavity and the material is compressed, generally using a benchtop hydraulic press. A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test. A compact is formed in the cavity with a single face of defined area exposed on the bottom of the die (Figure 2.9.29.-1). The bottom of the die cavity is threaded so that at least 50-75 per cent of the compact can dissolve without falling out of the die. The top of the die has a threaded shoulder that allows it to be attached to a holder. The holder is mounted on a laboratory stirring device, and the entire die, with the compact still in place, is immersed in the dissolution medium and rotated by the stirring device.

PROCEDURE

Weigh the material onto a piece of weighing paper. Attach the surface plate to the underside of the die, and secure it with the 3 provided screws. Transfer the sample of powder tested into the die cavity. Place the punch into the chamber, and secure the metal plate on the top of the assembly. Compress the powder using a hydraulic press by applying a suitable pressure for a sufficient dwell time to ensure a stable compact with minimal porosity; the disintegration of the compact has to be prevented as far as possible, since it would cause an increase in surface area and hence in dissolution rate. Detach the surface plate, and screw the die with punch still in place into the holder. Tighten securely. Remove all loose powder from the surface of the die by blowing compressed air or nitrogen across the surface of the compact.

Slide the die-holder assembly into the dissolution test chuck and tighten. Position the shaft in the spindle so that when the test head is lowered, the exposed surface of the compact will be 3.8 cm from the bottom of the vessel. The disc assembly is aligned to minimise wobble and air bubbles are not allowed to form as this could decrease the compact surface in contact with the dissolution medium. If possible, sink conditions are maintained throughout the test. However, in order to obtain detectable concentrations of solute, the use of a relatively small volume of medium may be necessary as a consequence of the limited surface available for dissolution.

Warm the dissolution medium to the temperature chosen for the test. Lower the test head into position before rotation. Care should be taken to ensure that air bubbles are excluded from the surface of the compact as this could decrease the compact surface in contact with the dissolution medium. Operate the apparatus immediately at the speed of rotation chosen for the test.

Collect samples at fixed time intervals and assay them by means of an analytical method of suitable sensitivity and accuracy.

ASSESSMENT OF THE RESULTS

The data for the cumulative amount dissolved at each time point are corrected for sampling losses. To calculate the intrinsic dissolution rate, plot the cumulative amount of sample dissolved per unit area of the compact against time. The cumulative amount dissolved per unit area is given by the cumulative amount dissolved at each time point divided by the surface area exposed. Linear regression is then performed on the normalised experimental data relevant to an appropriate time interval preceding the possible disintegration of the compact. The intrinsic dissolution rate of the substance tested, expressed in milligrams per minute per square centimetre, is determined from the slope of the regression line. The result for intrinsic dissolution rate must be accompanied by a statement of the precise conditions of compact preparation and test method (dissolution medium, volume of medium used, stirring rate, temperature etc.).

*NOTE*: when necessary and justified, an apparatus with a different configuration may be used, such as a die holder that holds the compact in a fixed vertical position, with agitation provided by a paddle positioned at a defined distance from the surface of the compact.

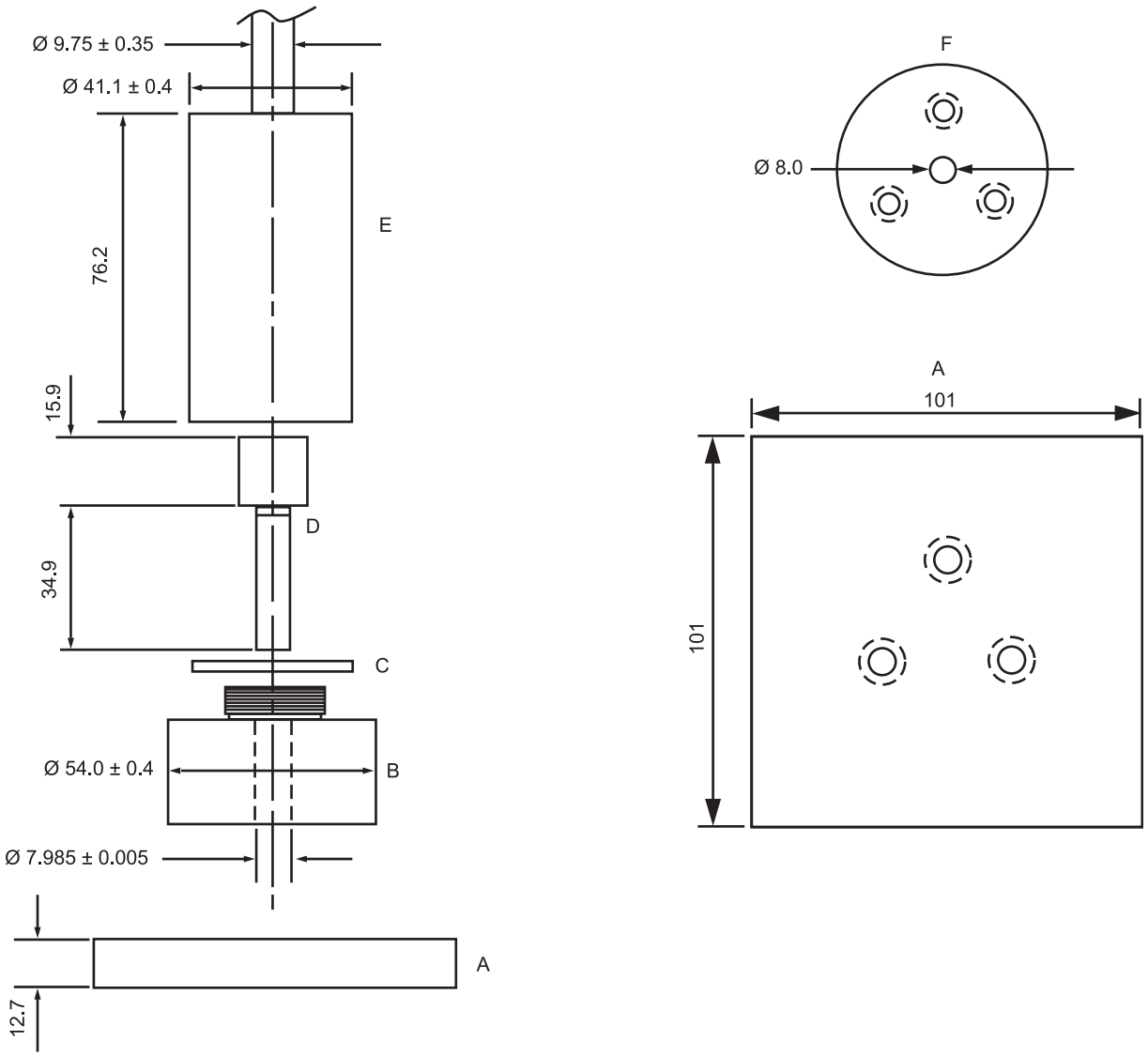


Figure 2.9.29.-1. – *Typical apparatus used to obtain the compact for the determination of the intrinsic dissolution Dimensions in millimetres*

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## 2.9.31. PARTICLE SIZE ANALYSIS BY LASER LIGHT DIFFRACTION(12)

The method is based on the ISO standards 13320-1(1999) and 9276-1(1998).

### INTRODUCTION

The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider angular range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e. by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For non-spherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g. sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems, for example, powders, sprays, aerosols, suspensions,

(12) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

emulsions, and gas bubbles in liquids, through analysis of their angular light-scattering patterns. It does not address specific requirements of particle size measurement of specific products.

## PRINCIPLE

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

## INSTRUMENT

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity or direct bright light.

An example of a set-up of a laser light diffraction instrument is given in Figure 2.9.31.-1. Other equipment may be used.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size distribution and associated data analysis and reporting.

The particles can enter the laser beam in 2 positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance. In so-called reversed Fourier optics the particles enter behind the collecting lens and thus, in a converging beam. The advantage of the conventional set-up is that a reasonable path length for the sample is allowed within the working distance of the lens. The second set-up allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

## DEVELOPMENT OF THE METHOD

The measurement of particle size by laser diffraction can give reproducible data, even in the sub-micron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g. dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately 0.1 µm to 3 mm. Because of recent advances in lens and equipment design, newer instruments are capable of exceeding this range routinely. With the validation report the user demonstrates the applicability of the method for its intended use.

**Sampling**. The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.

**Evaluation of the dispersion procedure**. Inspect the sample to be analysed, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method it is highly advisable to check that comminution of the particles does not occur, and conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change significantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g. crystallisation, milling) of the material has changed, the applicability of the method must be verified (e.g. by microscopic comparison).

Sprays, aerosols and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilisers) and/or mechanical forces (e.g. agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilisation of the dispersion. For these liquid dispersions, a recirculating system is most commonly used, consisting of an optical

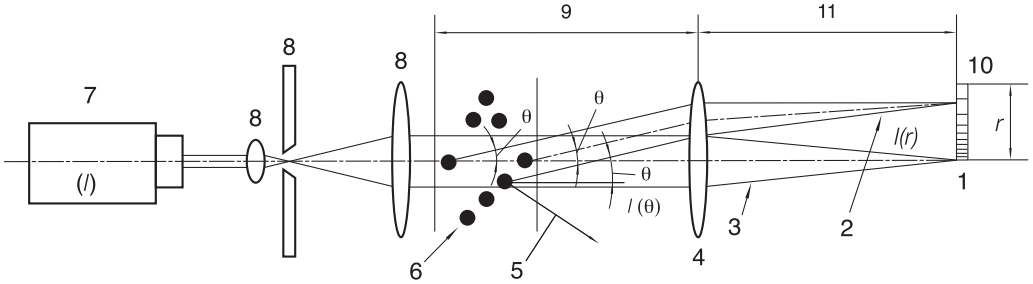


1. Obscuration detector
2. Scattered beam
3. Direct beam
4. Fourier lens
5. Scattered light not collected by lens (4)
6. Particle ensemble
7. Light source laser
8. Beam processing unit
9. Working distance of lens (4)
10. Multi-element detector
11. Focal distance of lens (4)

Figure 2.9.31.-1. – *Example of a set-up of a laser light diffraction instrument*

measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing. Non-recirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers, which apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol, which is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free flowing, coarser particles or granules the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving and the mass and percentage of removed material are reported. However, after pre-sieving, note that the sample is no longer representative, unless otherwise proven.

**Optimisation of the liquid dispersion**. Liquids, surfactants, and dispersing aids used to disperse powders must:

- be transparent at the laser wavelength and practically free from air bubbles or particles;
- have a refractive index that differs from that of the test material;
- be non-solvent of the test material (pure liquid or pre-filtered, saturated solution);
- not alter the size of the test materials (e.g. by solubility, solubility enhancement, or recrystallisation effects);
- favour easy formation and stability of the dispersion;
- be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.);
- possess a suitable viscosity to facilitate recirculation, stirring and filtration.

Surfactants and/or dispersing aids are often used to wet the particles and to stabilise the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH respectively can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

**Optimisation of the gas dispersion**. For sprays and dry powder dispersions, a compressed gas free from oil, water and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

**Determination of the concentration range**. In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropriate concentration range for any typical sample of material. (Note: in different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g. obscuration, optical concentration, proportional number of total mass).

**Determination of the measuring time**. The time of measurement, the reading time of the detector and the acquisition frequency are determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

**Selection of an appropriate optical model**. Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, since small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about 0.01-0.1 i) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g. shape, surface roughness and porosity), bear upon the final result.

**Validation**. Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate between different components in a sample, nor is it possible to discriminate agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly on the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled, robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as $s_{rel} \leq 10$ per cent [$n = 6$] for any central value of the distribution (e.g. for $x_{50}$). Values at the sides of the distribution (e.g. $x_{10}$ and $x_{90}$) are oriented towards less stringent acceptance criteria such as $s_{rel} \leq 15$ per cent [$n = 6$]. Below 10 µm, these values must be doubled. Robustness may be tested during the selection and optimisation of the dispersion media and forces.

The change of the dispersing energy may be monitored by the change in the particle-size distribution.

MEASUREMENT

**Precautions**. The instructions given in the instrument manual are followed:

- never look into the direct path of the laser beam or its reflections;
- earth all instrument components to prevent ignition of solvents or dust explosions;
- check the instrument set-up (e.g. warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight);
- in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent air-flow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

**Measurement of the light scattering of dispersed sample(s)**. After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to substract them later from the data obtained with the sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation. The magnitude of the signal from each detector element depends upon the detection area, the light intensity and the quantum efficiency. The co-ordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (a blank measurement) indicates the proportion of scattered light and hence the particle concentration.

**Conversion of scattering pattern into particle-size distribution**. This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of deviations between measured and calculated scattering patterns (e.g. least squares), some constraints (e.g. non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

**Replicates**. The number of replicate measurements (with individual sample preparations) to be performed depends on the required measurement precision. It is recommended to set this number in a substance-specific method.

REPORTING OF RESULTS

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol $x$ is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. $Q3(x)$ denotes the volume fraction undersize at the particle size $x$. In a graphical representation, $x$ is plotted on the abscissa and the dependent variable Q3 on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10 per cent, 50 per cent, and 90 per cent (denoted as $x_{10}$, $x_{50}$, and $x_{90}$ respectively) are frequently used. $x_{50}$ is also known as the median particle size. It is recognised that the symbol $d$ is also widely used to designate the particle size, thus the symbol $x$ may be replaced by $d$.

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. As the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

CONTROL OF THE INSTRUMENT PERFORMANCE

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

**Calibration**. Laser diffraction systems, although assuming idealised properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument is considered to meet the requirements if the mean value of $x_{50}$ from at least 3 independent measurements does not deviate by more than 3 per cent from the certified range of values of the certified reference material. The mean values for $x_{10}$ and $x_{90}$ must not deviate by more than 5 per cent from the certified range of values. Below 10 µm, these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, non-spherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analyses performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a significant bias. The reason for this bias is that the different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same non-spherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

**Qualification of the system**. In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the $x_{50}$ value does not deviate by more than 10 per cent from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g. $x_{10}$ and $x_{90}$), then these values must not deviate by more than 15 per cent from the certified range of values. Below 10 µm, these values must be doubled.

*NOTE: for calibration of the instrument, stricter requirements are laid down in the paragraph Calibration.*

**07/2008:20932**

# 2.9.32. POROSITY AND PORE-SIZE DISTRIBUTION OF SOLIDS BY MERCURY POROSIMETRY

## INTRODUCTION

In general, different types of pores may be pictured as apertures, channels or cavities within a solid body, or as space (i.e. interstices or voids) between solid particles in a bed, compact or aggregate. Porosity is a term that is often used to indicate the porous nature of solid material, and is more precisely defined as the ratio of the volume of accessible pores and voids to the total volume occupied by a given amount of the solid. In addition to the accessible pores, a solid may contain closed pores, which are isolated from the external surface and into which fluids are not able to penetrate. The characterisation of closed pores, i.e. cavities with no access to an external surface, is not covered in this chapter.

Porous materials may take the form of fine or coarse powders, compacts, extrudates, sheets or monoliths. Their characterisation usually involves the determination of the total pore volume or porosity as well as the pore-size distribution.

It is well established that the performance of a porous solid (e.g. its strength, reactivity, permeability or adsorbent power) is dependent upon its pore structure. Many different methods have been developed for the characterisation of pore structure. In view of the complexity of most porous solids, it is not surprising to find that the results obtained are not always in agreement and that no single technique can be relied upon to provide a complete picture of the pore structure. The choice of the most appropriate method depends on the application of the porous solid, its chemical and physical nature and the range of pore-size.

This chapter provides guidance for measurement of porosity and pore-size distribution by mercury porosimetry. It is a comparative test, usually destructive, in which the volume of mercury penetrating a pore or void is determined as a function of an applied hydrostatic pressure, which can be related to a pore diameter. Other information such as pore shape and inter-connectivity, the internal and external surface area, powder granulometry, bulk and tapped density could also be inferred from volume-pressure curves; however, these aspects of the technique do not fall under the scope of this chapter.

Practical considerations presently limit the maximum applied absolute pressure reached by some equipment to about 400 MPa, corresponding to a minimum equivalent pore diameter of approximately 0.003 µm. The maximum diameter will be limited for samples having a significant depth due to the difference in hydrostatic head of mercury from the top to the bottom of the sample. For most purposes this limit may be regarded as 400 µm.

Inter-particle and intra-particle porosity can be determined, but the method does not distinguish between these porosities where they co-exist.

The method is suitable for the study of most porous materials. Samples that amalgamate with mercury, such as certain metals, may be unsuitable for this technique or may require a preliminary passivation. Other materials may deform or compact under the applied pressure. In some cases it may be possible to apply sample-compressibility corrections and useful comparative data may still be obtained.

Mercury porosimetry is considered to be comparative, as for most porous media a theory is not available to allow an absolute calculation of results of pore-size distribution. Therefore this technique is mainly recommended for development studies.

Mercury is toxic. Appropriate precautions must be observed to safeguard the health of the operator and others working in the area. Waste material must also be disposed of in a suitable manner, according to local regulations.

## PRINCIPLE

The technique is based on the measurement of the mercury volume intruded into a porous solid as a function of the applied pressure. The measurement includes only those pores into which mercury can penetrate at the pressure applied.

A non-wetting liquid penetrates into a porous system only under pressure. The pressure to be applied is in inverse proportion to the inner diameter of the pore aperture. In the case of cylindrical pores, the correlation between pore diameter and pressure is given by the Washburn equation:

$$d_p = -\frac{4 \cdot \sigma}{p} \cos\theta$$

$d_p$ = pore diameter, in metres;

$\sigma$ = surface tension, in newtons per metre;

$\theta$ = contact angle of mercury on the sample, in degrees;

$p$ = applied pressure, in pascals.

## APPARATUS

The sample holder, referred to as penetrometer or dilatometer, has a calibrated capillary tube, through which the sample can be evacuated and through which mercury can enter. The capillary tube is attached to a wider tube in which the test sample is placed. The change in the volume of mercury intruded is usually measured by the change in capacitance between the mercury column in the capillary tube and a metal sleeve around the outside of the capillary tube. If precise measurements are required the expected total void and pore volume of the sample should be between 20 per cent and 90 per cent of the internal volume of the capillary tube. Since different materials exhibit a wide range of open porosities, a number of penetrometers with different capillary tube diameters and sample volumes may be required. A typical set-up for a mercury porosimeter instrument is given in Figure 2.9.32.-1. The porosimeter may have separate ports for high- and low-pressure operation, or the low-pressure measurement may be carried out on a separate unit.

The pressure range is typically 4-300 kPa for low-pressure operation and above 300 kPa for high-pressure operation, depending on the design of the particular apparatus and on the intended use.

METHOD

**Sample preparation**

The sample is pre-treated to remove adsorbed material that can obscure its accessible porosity, for example by heating and/or evacuation, or by flowing inert gas. It may be possible to passivate the surface of wettable or amalgam-forming solids, for example by producing a thin layer of oxide, or by coating with stearate.

The sample of the pre-treated solid is weighed and transferred to the penetrometer. The pore system of the sample is then degassed in a vacuum to a maximum residual pressure of 7 Pa.

**Filling the penetrometer with mercury**

The mercury used is of analytical quality. Overlay the sample with mercury under vacuum. The vacuum is required to ensure the transfer of mercury from the reservoir to the penetrometer. In a filled penetrometer the filling pressure comprises the applied pressure plus the pressure contribution created by the head of mercury contacting the sample. A typical filling pressure would be about 4 kPa. The hydrostatic pressure of the mercury over the sample may be minimised by filling the penetrometer in the horizontal position.

**Low-pressure measurement**

Admit air or nitrogen in a controlled manner to increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The concomitant change in the length of the mercury column in the capillary tube is recorded. When the maximum required pressure has been reached, return to atmospheric pressure.

**High-pressure measurement**

After measurement at low pressure, the penetrometer filled with mercury is transferred to the high-pressure port or unit of the instrument and overlaid with hydraulic fluid. Mercury is intruded into the pore system via the hydraulic fluid. Increase the pressure in the system to the maximum pressure reached in the low-pressure measurement and record the intrusion volume at this pressure, since subsequent intrusion volumes are calculated from this initial volume. Increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The fall in the mercury column is measured up to the maximum required pressure. If required the pressure may be decreased either in stages or continuously at a slow rate to determine the mercury extrusion curve.

Corrections are made to take account of changes in the volume of the mercury, the penetrometer and other components of the volume detector system under elevated pressure. The extent of the corrections may be determined by means of blank measurements under the same conditions.

An experimentally determined volume-pressure curve is shown in Figure 2.9.32.-2.

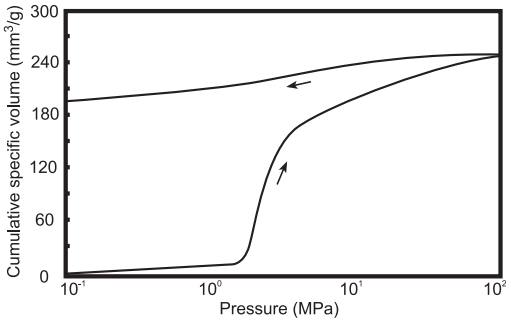


Figure 2.9.32.-2. – *Volume-pressure curve as semilogarithmic plot*

REPORTING OF RESULTS

The pressure readings can be converted to pore diameters by means of the Washburn equation or by another model.

The surface tension of mercury (σ) depends not only on the temperature, but also, in the case of markedly curved surfaces areas, on the radius of curvature. In general, values between 0.41 $N \cdot m^{-1}$ and 0.52 $N \cdot m^{-1}$ are measured at room temperature. If the value is not known, $\sigma = 0.48\ N \cdot m^{-1}$ can be used.

The contact angle of mercury (θ) in most cases is more than 90°. It may be determined using a contact angle instrument. If the value is not known, θ = 130° can be used. The values of contact angle and surface tension and the model used in the calculation are reported.

Visualisation of the data can be done with several types of graphs. Frequently, in a graphical representation the pore diameter is plotted on the abscissa and the intruded volume per sample mass on the ordinate to give the pore-size

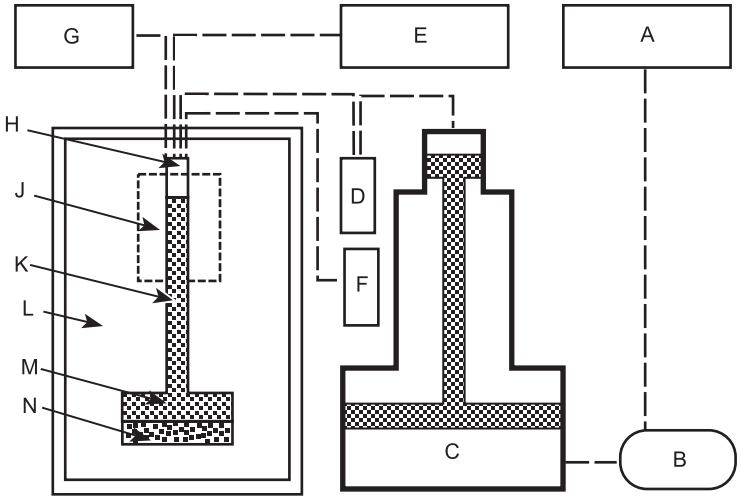


| | | | |
|---|---|---|---|
| A. Low-pressure hydraulic fluid reservoir | E. High-pressure hydraulic fluid reservoir | J. Penetration volume indicator | N. Sample |
| B. Hydraulic pump | F. Vacuum pump with gauge | K. Capillary tube | |
| C. Pressure multiplier | G. Mercury reservoir | L. High-pressure chamber | |
| D. Pressure transducer | H. Oil | M. Mercury | |

Figure 2.9.32.-1. – *Example of the set-up of a mercury porosimeter instrument*

distribution. It is appropriate here to choose a logarithmic scale for the abscissa (see Figure 2.9.32.-3). The spaces between the particles of the solid sample are included as pores in the calculation. If the pores differ in size from the voids, the latter can be separated by choosing the appropriate pore-size range.

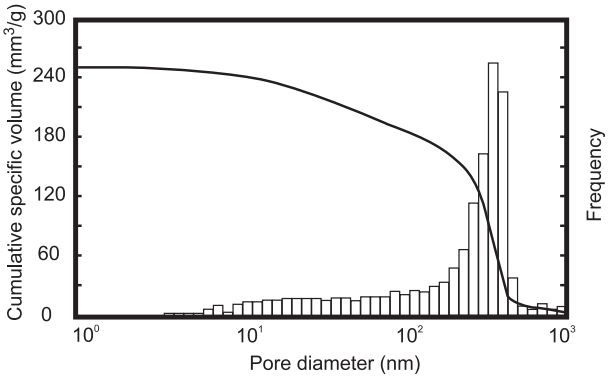


Figure 2.9.32.-3. – *Pore-size distribution as semilogarithmic plots of the cumulative and the normalised density distribution*

Extrusion curves may not be used for calculating the pore-size distribution (for hysteresis, see Figure 2.9.32.-2), because an intruded part of the mercury always remains in the pore system. The retention ratio may however be useful for the qualitative characterisation of pores that are only accessible via narrow openings ('ink-bottle pores').

Most common characteristic values, such as the total intruded specific volume and the mean and median pore diameters, are calculated from the pore-size distribution. Moreover, sufficient information must be documented about the sample, the sample preparation, the evacuation conditions and the instrument used.

CONTROL OF INSTRUMENT PERFORMANCE

As mercury porosimetry is considered to be used as a comparative test, no details are given in this chapter. However, it is recommended that a stable comparison material is tested on a regular basis to monitor instrument calibration and performance.

**04/2019:20933**

# 2.9.33. CHARACTERISATION OF CRYSTALLINE AND PARTIALLY CRYSTALLINE SOLIDS BY X-RAY POWDER DIFFRACTION (XRPD)[13]

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern.

Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially 3 types of information can be derived from a powder diffraction pattern: angular position of diffraction lines (depending on geometry and size of the unit cell); intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample); and diffraction line profiles (depending on instrumental resolution, crystallite size, strain and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (for example, identification of crystalline phases) and quantitative phase analysis of crystalline materials. An estimate of the amorphous and crystalline fractions[14] can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually non-destructive in nature (specimen preparation is usually limited to grinding to ensure a randomly oriented sample). XRPD investigations can also be carried out under *in situ* conditions on specimens exposed to non-ambient conditions, such as low or high temperature and humidity.

PRINCIPLE

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on the atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between 2 diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (see Figure 2.9.33.-1):

$$2d_{hkl}\sin\theta_{hkl} = n\lambda$$

The wavelength λ of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or $d_{hkl}$ (also called 'd-spacings'). $\theta_{hkl}$ is the angle between the incident ray and the family of lattice planes, and $\sin\theta_{hkl}$ is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices {*hkl*}. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings *a*, *b* and *c* and the angles between them, α, β, and γ.

The interplanar spacing for a specified set of parallel *hkl* planes is denoted by $d_{hkl}$. Each such family of planes may show higher orders of diffraction where the *d* values for the related families of planes n*h*, n*k*, n*l* are diminished by the factor 1/n (n being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, $\theta_{hkl}$, associated with it (for a specific wavelength λ).

A powder specimen is assumed to be polycrystalline so that at any angle $\theta_{hkl}$ there are always crystallites in an orientation allowing diffraction according to Bragg's law[15]. For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as 'lines', 'reflections' or 'Bragg reflections') are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles on the perfection and extent of the crystal lattice. Under these conditions the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarisation factor, multiplicity and Lorentz factor.

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area and shape (characterised by, for example, peak width or asymmetry, analytical function, empirical representation). An example of the type of powder patterns obtained for 5 different solid phases of a substance are shown in Figure 2.9.33.-2.

(13) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.
(14) There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances such as: determination of crystal structures, refinement of crystal structures, determination of crystallographic purity of crystalline phases, characterisation of crystallographic texture, etc. These applications are not described in this chapter.
(15) An 'ideal' powder for diffraction experiments consists of a large number of small, randomly oriented spherical crystallites (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.

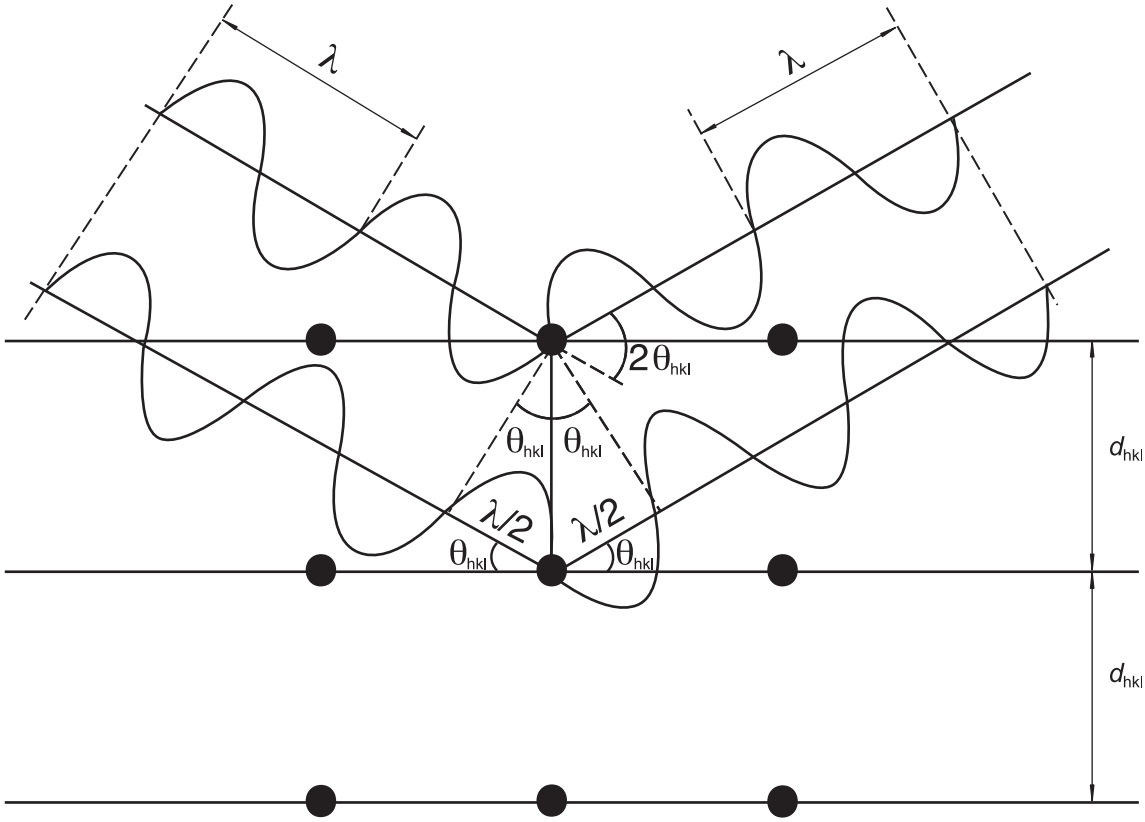


Figure 2.9.33.-1. – *Diffraction of X-rays by a crystal according to Bragg's law*

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more-or-less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background, for instance the sample holder, diffuse scattering from air and equipment, other instrumental parameters such as detector noise, general radiation from the X-ray tube, etc. The peak-to-background ratio can be increased by minimising background and by choosing prolonged exposure times.

INSTRUMENT

**Instrument set-up**. X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises 5 main parts: an X-ray source; incident beam optics, which may perform monochromatisation, filtering, collimation and/or focusing of the beam; a goniometer; diffraction beam optics, which may perform monochromatisation, filtering, collimation and focusing or parallelising of the beam; and a detector. Data-collection and data-processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required. The simplest instruments used to measure XRPD patterns are powder cameras. The replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing but parafocusing, such as in the Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used and is therefore briefly described here.

A given instrument may provide a horizontal or vertical θ/2θ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane). The basic geometric arrangement is represented in Figure 2.9.33.-3. The divergent beam of radiation from the X-ray tube (the so-called 'primary beam') passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For θ/2θ scans the goniometer rotates the specimen about the same axis as that of the detector, but at half the rotational speed, in a θ/2θ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5-2 mm thickness can also be used for small sample amounts.

**X-ray radiation**. In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends

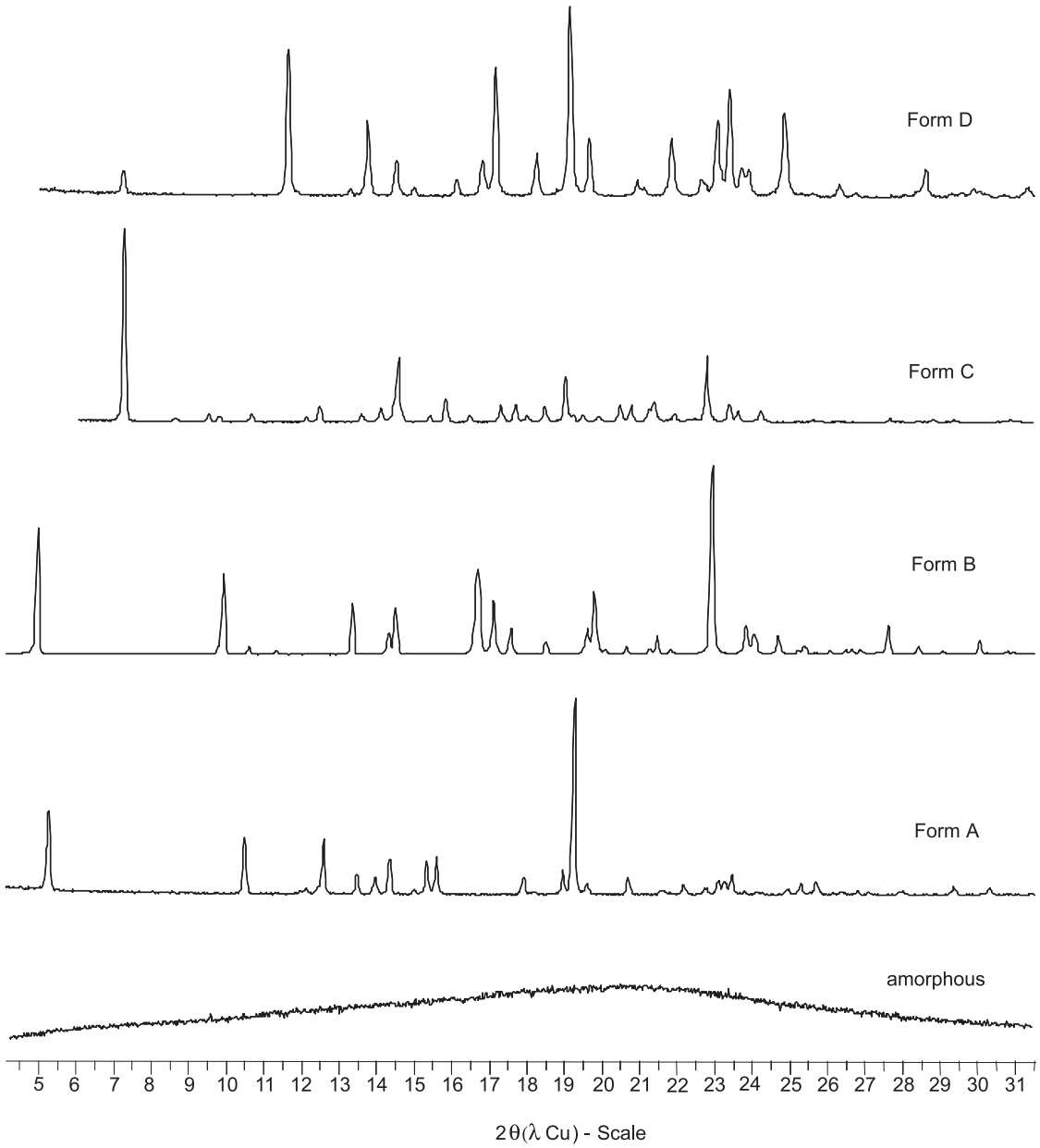


Figure 2.9.33.-2. – *X-ray powder diffraction patterns collected for 5 different solid phases of a substance (the intensities are normalised)*

on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources utilised for X-ray diffraction are vacuum tubes utilising copper, molybdenum, iron, cobalt or chromium as anodes; copper, molybdenum or cobalt X-rays are employed most commonly for organic substances (the use of cobalt anodes can be especially preferred to separate distinct X-ray lines). The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen. The wavelengths used in powder diffraction generally correspond to the $K_\alpha$ radiation from the anode. Consequently, it is advantageous to make the X-ray beam 'monochromatic' by eliminating all the other components of the emission spectrum. This can be partly obtained using $K_\beta$ filters, i.e. metal filters selected as having an absorption edge between the $K_\alpha$ and $K_\beta$ wavelengths emitted by the tube.

Such a filter is usually inserted between the X-ray tube and the specimen. Another, more-and-more-commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a 'monochromator'). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e. $K_\alpha$ and $K_\beta$) at different angles, so that only one of them may be selected to enter into the detector. It is even possible to separate $K_{\alpha1}$ and $K_{\alpha2}$ radiations by using a specialised monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating $K_\alpha$ and $K_\beta$ wavelengths is by using curved X-rays mirrors that can simultaneously monochromate and focus or parallelise the X-ray beam.

*RADIATION PROTECTION. Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions are taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.*

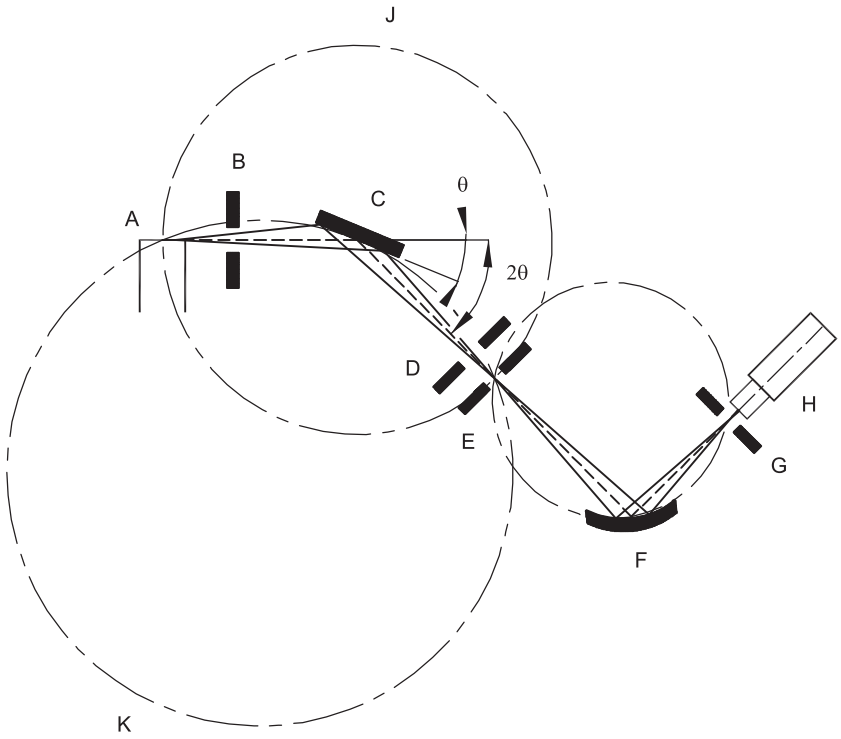


| | | | | |
|---|---|---|---|---|
| A. X-ray tube | C. sample | E. receiving slit | G. detector receiving slit | J. diffractometer circle |
| B. divergence slit | D. anti-diffusion slit | F. monochromator | H. detector | K. focusing circle |

Figure 2.9.33.-3. – *Geometric arrangement of the Bragg-Brentano parafocusing geometry*

## SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and mounting of the specimen in a suitable holder are critical steps in many analytical methods, and are particularly so for XRPD analysis, since they can greatly affect the quality of the data to be collected[16]. The main sources of error due to specimen preparation and mounting are briefly discussed here for instruments in Bragg-Brentano parafocusing geometry.

### *SPECIMEN PREPARATION*

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or plate-like crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections, so that some are more intense and others are less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimise preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 µm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 µm) may cause line broadening and significant changes to the sample itself such as:

- specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.);
- reduced degree of crystallinity;
- solid-state transition to another polymorph;
- chemical decomposition;
- introduction of internal stress;
- solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the non-ground specimen with that corresponding to a specimen of smaller particle size (e.g. a milled specimen). If the XRPD pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

### *SPECIMEN MOUNTING*

**Effect of specimen displacement**. A specimen surface that is offset by $D$ with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D{\cdot}\cos\theta$ shifts[17] in $2\theta$ positions (typically of the order of 0.01° in $2\theta$ at low angles ($\cos\theta \simeq 1$) for a displacement $D$ = 15 µm) and asymmetric broadening of the profile towards low $2\theta$ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This is by far the largest source of errors in data collected on well-aligned diffractometers.

**Effect of specimen thickness and transparency**. When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of 'infinite thickness'. To minimise the transparency effect, it is advisable to use a non-diffracting substrate (zero background holder), for example a plate of single crystalline silicon cut parallel to the 510 lattice planes[18]. One advantage of the transmission mode is that problems with sample height and specimen transparency are less important. The use of an appropriate

(16) Similarly, changes in the specimen can occur during data collection in the case of a non-equilibrium specimen (temperature, humidity).
(17) Note that a goniometer zero alignment shift would result in constant shift on all observed $2\theta$-line positions, in other words, the whole diffraction pattern is in this case translated by an offset of Z° in $2\theta$.
(18) In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.

internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

CONTROL OF THE INSTRUMENT PERFORMANCE

Goniometers and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to minimise adequately systematic errors, while optimising the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought whilst performing the alignment procedure. There are many different configurations and each supplier's equipment requires specific alignment procedures.

The overall diffractometer performance must be tested and monitored periodically using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its XRPD pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterised single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its 2θ diffraction angles or d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based either on a more-or-less extended 2θ-range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalised intensities ($I_{norm}$), a so-called ($d$, $I_{norm}$)-list extracted from the pattern, is the crystallographic fingerprint of the material, and can be compared to ($d$, $I_{norm}$)-lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu $K_\alpha$ radiation, it is appropriate to record the diffraction pattern in a 2θ-range from as near 0° as possible to at least 40°. The agreement in the 2θ-diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognised to have varying unit cell dimensions and as such shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ-positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g. inorganic salts), it may be necessary to extend the 2θ-region scanned to well beyond 40°. It is generally sufficient to scan past the 10 strongest reflections identified in single phase XRPD database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

- non-crystallised or amorphous substances;
- the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10 per cent *m/m*);
- pronounced preferred orientation effects;
- the phase has not been filed in the database used;
- formation of solid solutions;
- presence of disordered structures that alter the unit cell;
- the specimen comprises too many phases;
- presence of lattice deformations;
- structural similarity of different phases;

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of 2 or more known phases, of which not more than 1 is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can, in many cases, be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines[(19)], or on the full pattern. These integrated intensities, peak heights or full-pattern data points are compared to the corresponding values of reference materials. These reference materials shall be single-phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require in particular homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects. In favourable cases, amounts of crystalline phases as small as 10 per cent may be determined in solid matrices.

*POLYMORPHIC SAMPLES*

For a sample composed of 2 polymorphic phases *a* and *b*, the following expression may be used to quantify the fraction $F_a$ of phase *a*:

$$F_a = \frac{1}{1 + K(I_b / I_a)}$$

The fraction is derived by measuring the intensity ratio between the 2 phases, knowing the value of the constant *K*. *K* is the ratio of the absolute intensities of the 2 pure polymorphic phases $I_{oa}/I_{ob}$. Its value can be determined by measuring standard samples.

*METHODS USING A STANDARD*

The most commonly used methods for quantitative analysis are:

- the 'external standard method';
- the 'internal standard method';
- the 'spiking method' (often also called the 'standard addition method').

The 'external standard method' is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material with crystallite size and X-ray absorption coefficient comparable to those of the components of the sample, and with a diffraction pattern that does not overlap at all that of the sample to be analysed, can be used. A known quantity of this reference material is added to the sample to be analysed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the 'internal standard method', requires a precise measurement of diffraction intensities.

In the 'spiking method' (or 'standard addition method'), some of the pure phase *a* is added to the mixture containing the unknown concentration of *a*. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative *x* intercept is the concentration of the phase *a* in the original sample.

(19) If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley or least squares methods can be used.

ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:

- if the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances as described above; the amorphous fraction is then deduced indirectly by subtraction;
- if the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase ('the degree of crystallinity') can be estimated by measuring 3 areas of the diffractogram:

  $A$ = total area of the peaks arising from diffraction from the crystalline fraction of the sample;

  $B$ = total area below area $A$;

  $C$ = background area (due to air scattering, fluorescence, equipment, etc).

When these areas have been measured, the degree of crystallinity can be roughly estimated using the following formula:

$$\% \text{ crystallinity} = 100A / (A + B - C)$$

It is noteworthy that this method does not yield absolute degree-of-crystallinity values and hence is generally used for comparative purposes only.

More sophisticated methods are also available, such as the Ruland method.

SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low and the scattering properties are normally very low.

For any given crystalline form of a substance, knowledge of the crystal structure allows the calculation of the corresponding XRPD pattern, thereby providing a 'preferred-orientation-free' reference XRPD pattern, which may be used for phase identification.

**04/2019:20934**

# 2.9.34. BULK DENSITY AND TAPPED DENSITY OF POWDERS[20]

## Bulk density

The bulk density of a powder is the ratio of the mass of an untapped powder sample to its volume, including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per millilitre despite the International Unit being kilogram per cubic metre (1 g/mL = 1000 kg/m$^3$), because the measurements are made using cylinders. It may also be expressed in grams per cubic centimetre.

The bulking properties of a powder are dependent upon the preparation, treatment and storage of the sample, i.e. how it has been handled. The particles can be packed to have a range of bulk densities and, moreover, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made.

The bulk density of a powder is determined either by measuring the volume of a known mass of powder sample, which may have been passed through a sieve, in a graduated cylinder (Method 1), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (Method 2) or has been introduced into a measuring vessel (Method 3).

*Methods 1 and 3 are favoured.*

METHOD 1: MEASUREMENT IN A GRADUATED CYLINDER

*Procedure.* Pass a quantity of powder sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry, graduated, 250 mL cylinder (readable to 2 mL), gently introduce, without compacting, approximately 100 g ($m$) of the test sample weighed with 0.1 per cent accuracy. If necessary, carefully level the powder without compacting, and read the unsettled apparent volume ($V_0$) to the nearest graduated unit. Calculate the bulk density in grams per millilitre using the formula $m/V_0$. Generally, replicate determinations are desirable for the determination of this property.

If the powder density is too low or too high, such that the test sample has an untapped apparent volume of more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. In this case, a different amount of powder is selected as the test sample, such that its untapped apparent volume is between 150 mL and 250 mL (apparent volume greater than or equal to 60 per cent of the total volume of the cylinder); the mass of the test sample is specified in the expression of results.

For test samples having an apparent volume between 50 mL and 100 mL, a 100 mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

METHOD 2: MEASUREMENT IN A VOLUMETER

*Apparatus.* The apparatus (Figure 2.9.34.-1) consists of a top funnel fitted with a 1.0 mm sieve, mounted over a baffle box containing 4 glass baffles over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an internal diameter of 30.00 ± 2.00 mm) or cubical (16.39 ± 0.20 mL volume with internal dimensions of 25.400 ± 0.076 mm).

(20) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

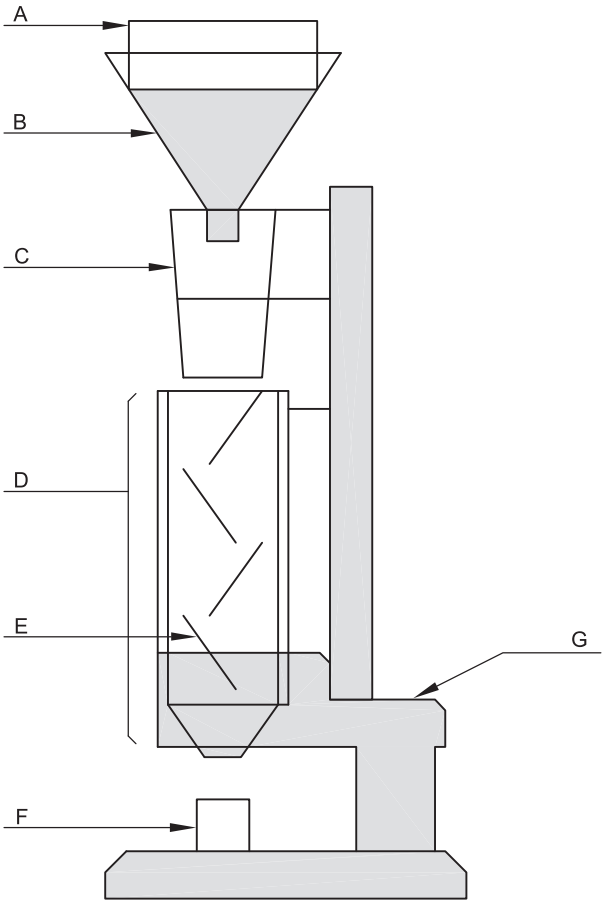


A. 1.0 mm sieve
B. powder funnel
C. loading funnel
D. baffle box
E. glass baffle
F. cup
G. stand

Figure 2.9.34.-1. – *Volumeter*

*Procedure.* Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 $cm^3$ of powder with the cubical cup and 35 $cm^3$ of powder with the cylindrical cup. Carefully, scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the side of the cup and determine the mass ($M$) of the powder to the nearest 0.1 per cent. Calculate the bulk density in grams per millilitre using the formula $M/V_0$ (where $V_0$ is the volume of the cup) and record the average of 3 determinations using 3 different powder samples.

METHOD 3: MEASUREMENT IN A VESSEL

*Apparatus.* The apparatus consists of a 100 mL cylindrical vessel of stainless steel with dimensions as specified in Figure 2.9.34.-2.

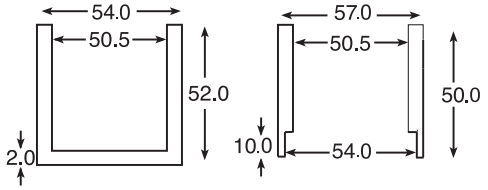


Figure 2.9.34.-2. – *Measuring vessel (left) and cap (right) Dimensions in millimetres*

*Procedure.* Pass a quantity of powder sufficient to complete the test through a 1.0 mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described under Method 2. Determine the mass ($M_0$) of the powder to the nearest 0.1 per cent by subtracting the previously determined mass of the empty measuring vessel. Calculate the bulk density in grams per millilitre using the formula $M_0/100$ and record the average of 3 determinations using 3 different powder samples.

## Tapped density

The tapped density is an increased bulk density attained after mechanically tapping a receptacle containing the powder sample.

The tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing the powder sample. After observing the initial powder volume or mass, the measuring cylinder or vessel is mechanically tapped, and volume or mass readings are taken until little further volume or mass change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop, under its own mass, a specified distance by one of 3 methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimise any possible separation of the mass during tapping down.

METHOD 1

*Apparatus.* The apparatus (Figure 2.9.34.-3) consists of the following:

– a 250 mL graduated cylinder (readable to 2 mL) with a mass of 220 ± 44 g;

– a settling apparatus capable of producing, per minute, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.

*Procedure.* Proceed as described above for the determination of the bulk volume ($V_0$). Secure the cylinder in the support. Carry out 10, 500 and 1250 taps on the same powder sample and read the corresponding volumes $V_{10}$, $V_{500}$ and $V_{1250}$ to the nearest graduated unit. If the difference between $V_{500}$ and $V_{1250}$ is less than or equal to 2 mL, $V_{1250}$ is the tapped volume. If the difference between $V_{500}$ and $V_{1250}$ exceeds 2 mL, repeat in increments of, for example, 1250 taps, until the difference between successive measurements is less than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate the tapped density in grams per millilitre using the formula $m/V_f$ (where $V_f$ is the final tapped volume). Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results.

If it is not possible to use a 100 g test sample, use a reduced amount and a suitable 100 mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a support weighing 240 ± 12 g. If the difference between $V_{500}$ and $V_{1250}$ is less than or equal to 1 mL, $V_{1250}$ is the tapped volume. If the difference between $V_{500}$ and $V_{1250}$ exceeds 1 mL, repeat in increments of, for example, 1250 taps, until the difference between successive measurements is less than or equal to 1 mL. The modified test conditions are specified in the expression of the results.

METHOD 2

*Procedure.* Proceed as directed under Method 1 except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per minute.

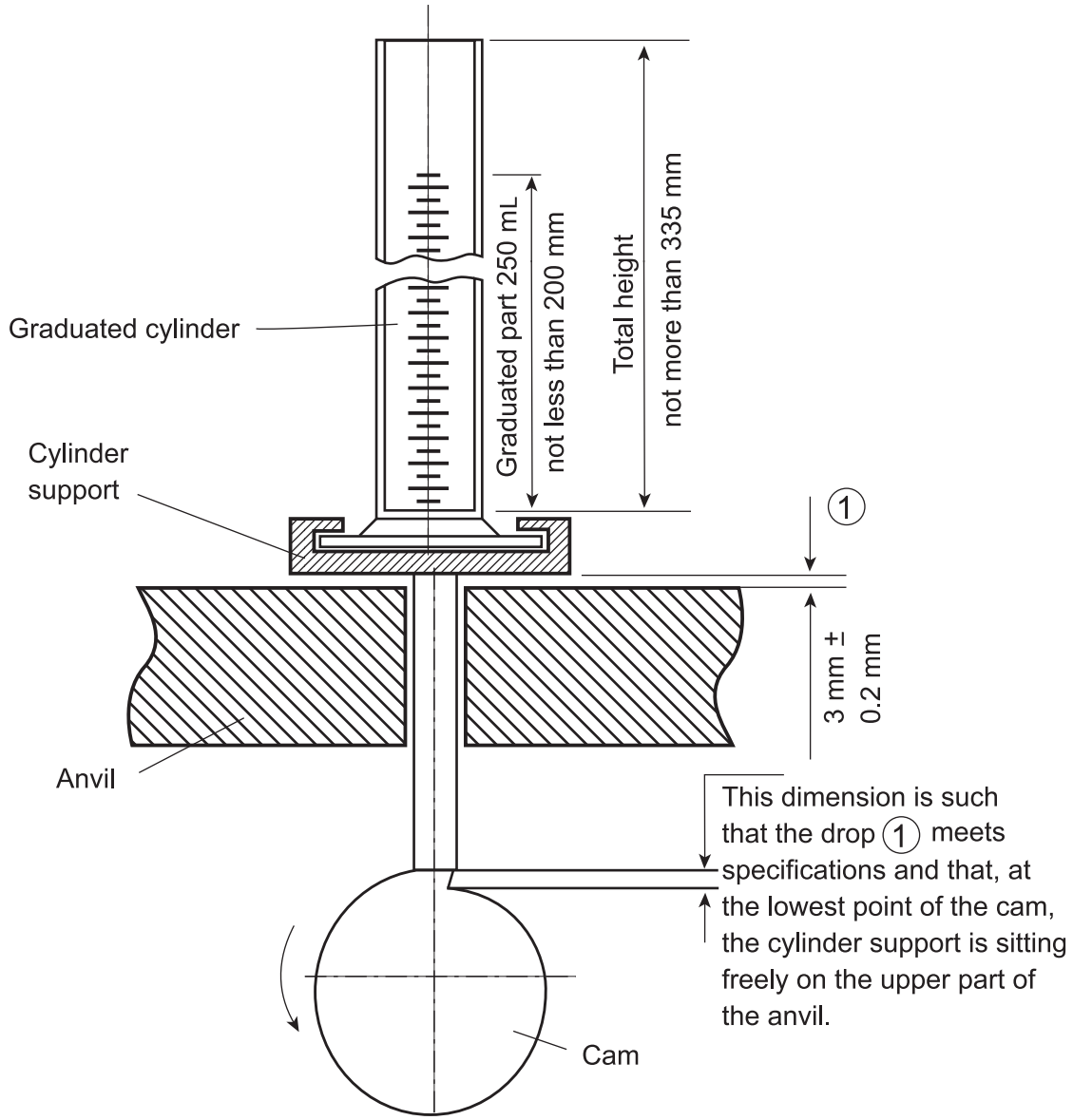


Figure 2.9.34.-3. – *Settling device for powder samples*
*Dimensions in millimetres*

METHOD 3

*Procedure*. Proceed as described under Method 3 for measuring the bulk density, using the measuring vessel equipped with the cap shown in Figure 2.9.34.-2. The measuring vessel with the cap is lifted 50-60 times per minute by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap and carefully scrape excess powder from the top of the measuring vessel as described under Method 3 for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the 2 masses obtained after 200 and 400 taps exceeds 2 per cent, repeat the test using 200 additional taps until the difference between successive measurements is less than 2 per cent. Calculate the tapped density in grams per millilitre using the formula $M_f/100$ (where $M_f$ is the mass of powder in the measuring vessel). Record the average of 3 determinations using 3 different powder samples. The test conditions, including tapping height, are specified in the expression of the results.

## Measures of powder compressibility

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the compressibility index or the Hausner ratio.

The compressibility index and Hausner ratio are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For more-poorly flowing materials, there are frequently greater interparticulate interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the compressibility index and the Hausner ratio.

Compressibility index:

$$\frac{100\left(V_0 - V_f\right)}{V_0}$$

$V_0$ = unsettled apparent volume;

$V_f$ = final tapped volume.

Hausner Ratio:

$$\frac{V_0}{V_f}$$

Depending on the material, the compressibility index can be determined using $V_{10}$ instead of $V_0$. If $V_{10}$ is used, it is clearly stated with the results.

**04/2019:20935**

## 2.9.35. POWDER FINENESS[21]

Particle-size distribution is estimated by analytical sieving (*2.9.38*) or by application of other suitable methods where appropriate. A simple descriptive classification of powder fineness is provided in this chapter. For practical reasons, sieves are commonly used to measure powder fineness. Sieving is most suitable where a majority of the particles are larger than about 75 µm, although it can be used for some powders having smaller particle sizes where the method can be validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles.

Where the cumulative distribution has been determined by analytical sieving or by application of other methods, particle size may be characterised in the following manner:

$x_{90}$ = particle size corresponding to 90 per cent of the cumulative undersize distribution;

$x_{50}$ = median particle size (i.e. 50 per cent of the particles are smaller and 50 per cent of the particles are larger);

$x_{10}$ = particle size corresponding to 10 per cent of the cumulative undersize distribution.

It is recognised that the symbol $d$ is also widely used to designate these values. Therefore, the symbols $d_{90}$, $d_{50}$, $d_{10}$ may be used.

The following parameters may be defined based on the cumulative distribution.

$Q_r(x)$ = cumulative distribution of particles with a dimension less than or equal to $x$ where the subscript $r$ reflects the distribution type.

| *r* | Distribution type |
|---|---|
| 0 | Number |
| 1 | Length |
| 2 | Area |
| 3 | Volume |

Therefore, by definition:

$Q_r(x) = 0.90$ when $x = x_{90}$

$Q_r(x) = 0.50$ when $x = x_{50}$

$Q_r(x) = 0.10$ when $x = x_{10}$

An alternative but less informative method of classifying powder fineness is by use of the descriptive terms in Table 2.9.35.-1.

Table 2.9.35.-1.

| Classification of powders by fineness | | |
|---|---|---|
| **Descriptive term** | **$x_{50}$ (µm)** | **Cumulative distribution by volume basis, $Q_3(x)$** |
| Coarse | > 355 | $Q_3(355) < 0.50$ |
| Moderately fine | 180 - 355 | $Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$ |
| Fine | 125 - 180 | $Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$ |
| Very fine | ≤ 125 | $Q_3(125) \geq 0.50$ |

**01/2010:20936**

## 2.9.36. POWDER FLOW[22]

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterising powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterise powder flow.

The purpose of this chapter is to review the methods for characterising powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterise the flow properties of pharmaceutical powders, this chapter proposes the standardisation of test methods that may be valuable during pharmaceutical development.

4 commonly reported methods for testing powder flow are:

- angle of repose,
- compressibility index or Hausner ratio,
- flow rate through an orifice,
- shear cell.

In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardising the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardisation of the methods. In general, any method of measuring powder flow must be practical, useful, reproducible and sensitive, and must yield meaningful results. It bears repeating that no simple powder flow method will adequately or completely characterise the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardised test methods to characterise the various aspects of powder flow as needed by the pharmaceutical scientist.

ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterise the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction, or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise due to segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the method continues

(21) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*
(22) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods, described briefly below.

**Basic methods for angle of repose**

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified based on 2 important experimental variables:

- the height of the 'funnel' through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms;
- the base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

**Variations in angle of repose methods**

Variations of the above methods have also been used to some extent in the pharmaceutical literature:

- *drained angle of repose*: this is determined by allowing an excess quantity of material positioned above a fixed diameter base to 'drain' from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose;
- *dynamic angle of repose*: this is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

**General scale of flowability for angle of repose**

While there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr[23], which is shown in Table 2.9.36.-1. There are examples in the literature of formulations with an angle of repose in the range of 40-50 degrees that manufactured satisfactorily. When the angle of repose exceeds 50 degrees, the flow is rarely acceptable for manufacturing purposes.

Table 2.9.36.-1. – *Flow properties and corresponding angles of repose*[23]

| Flow property | Angle of repose (degrees) |
|---|---|
| Excellent | 25-30 |
| Good | 31-35 |
| Fair (aid not needed) | 36-40 |
| Passable (may hang up) | 41-45 |
| Poor (must agitate, vibrate) | 46-55 |
| Very poor | 56-65 |
| Very, very poor | > 66 |

**Experimental considerations for angle of repose**

Angle of repose is not an intrinsic property of the powder, that is to say, it is very much dependent upon the method used to form the cone of powder. On this subject, the existing literature raises these important considerations:

- the peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimised;
- the nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a 'common base', which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

**Recommended procedure for angle of repose**

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base must be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care must be taken to prevent vibration as the funnel is moved. The funnel height is maintained at approximately 2-4 cm from the top of the powder pile as it is being formed in order to minimise the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan(\alpha) = \frac{\text{height}}{0.5 \times \text{base}}$$

COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials, because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and tapped volume of a powder.

**Basic methods for compressibility index and Hausner ratio**

While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure the unsettled apparent volume, ($V_0$), and the final tapped volume, ($V_f$), of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\textit{Compressibility Index} = 100 \times \frac{V_0 - V_f}{V_0}$$

$$\textit{Hausner Ratio} = \frac{V_0}{V_f}$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values of bulk density ($\rho_{bulk}$) and tapped density ($\rho_{tapped}$) as follows:

$$\textit{Compressibility Index} = 100 \times \frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}$$

$$\textit{Hausner Ratio} = \frac{\rho_{tapped}}{\rho_{bulk}}$$

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in Table 2.9.36.-2.

(23) Carr RL. Evaluating flow properties of solids. *Chem. Eng* 1965; 72:163-168.

Table 2.9.36.-2. – *Scale of flowability*[23]

| Compressibility index (per cent) | Flow character | Hausner ratio |
|---|---|---|
| 1-10 | Excellent | 1.00-1.11 |
| 11-15 | Good | 1.12-1.18 |
| 16-20 | Fair | 1.19-1.25 |
| 21-25 | Passable | 1.26-1.34 |
| 26-31 | Poor | 1.35-1.45 |
| 32-37 | Very poor | 1.46-1.59 |
| > 38 | Very, very poor | > 1.60 |

**Experimental considerations for the compressibility index and Hausner ratio**

Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the unsettled apparent volume, $V_0$, of the final tapped volume, $V_f$, of the bulk density, $\rho_{bulk}$, and of the tapped density, $\rho_{tapped}$:

- the diameter of the cylinder used,
- the number of times the powder is tapped to achieve the tapped density,
- the mass of material used in the test,
- rotation of the sample during tapping.

**Recommended procedure for compressibility index and Hausner ratio**

Use a 250 mL volumetric cylinder with a test sample mass of 100 g. Smaller amounts and volumes may be used, but variations in the method must be described with the results. An average of 3 determinations is recommended.

## FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously, since pulsating flow patterns have been observed even for free-flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

**Basic methods for flow through an orifice**

There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on 3 important experimental variables:

- the type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment;
- the size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate;
- the method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 s to the nearest tenth of a gram).

**Variations in methods for flow through an orifice**

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favour of high-density materials. Since die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container, however, this appears to complicate interpretation of results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

**General scale of flowability for flow through an orifice**

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

**Experimental considerations for flow through an orifice**

Flow rate through an orifice is not an intrinsic property of the powder. It is very much dependent upon the methodology used. The existing literature points out several important considerations affecting these methods:

- the diameter and shape of the orifice,
- the type of container material (metal, glass, plastic),
- the diameter and height of the powder bed.

**Recommended procedure for flow through an orifice**

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the 'head' of powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. It is advisable to use a cylinder as the container, because the walls of the container must have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder, rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than twice the diameter of the column. The orifice must be circular and the cylinder must be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- diameter of the opening greater than 6 times the diameter of the particles,
- diameter of the cylinder greater than twice the diameter of the opening.

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, thus, selection of an appropriate construction material is important.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum flexibility and better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

## SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as

the flow factor and other flowability indices. Because of the ability to control experimental parameters more precisely, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. These methods have been successfully used to determine critical hopper and bin parameters.

**Basic methods for shear cell**

One type of shear cell is the cylindrical shear cell which is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly because material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterising powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology generally is rather time-consuming and requires significant amounts of material and a well-trained operator.

**Recommendations for shear cell**

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterise powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterisation using shear cell methodology include a complete description of equipment and methodology used.

**01/2010:20937**

## 2.9.37. OPTICAL MICROSCOPY *(24)*

Optical microscopy for particle characterisation can generally be applied to particles of 1 µm and greater. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterisation of larger particles. Various alternative techniques are available for particle characterisation outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterising particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

**Apparatus**. Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterisation of the smallest particles to be classified in the test sample. The greatest numerical aperture of the objective is sought for each magnification range. Polarising filters may be used in conjunction with suitable analysers and retardation plates. Colour filters of relatively narrow spectral transmission are used with achromatic objectives, and are preferable with apochromats; they are required for appropriate colour rendition in photomicrography. Condensers, corrected at least for spherical aberration are used in the microscope substage and with the lamp. The numerical aperture of the substage condenser matches that of the objective under the conditions of use; this is affected by the actual aperture of the condenser diaphragm and the presence of immersion oils.

**Adjustment**. The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements is done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

**Illumination**. A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Köhler illumination is preferred. With coloured particles, choose the colour of the filters so as to control the contrast and detail of the image.

**Visual characterisation**. The magnification and numerical aperture must be sufficiently high to allow adequate resolution of the images of the particles to be characterised. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimised if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale must be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterise materials having a wide particle size distribution.

**Photographic characterisation**. If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing must be identical for photographs of both the test sample and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

**Preparation of the mount**. The mounting medium will vary according to the physical properties of the test sample. Sufficient, but not excessive, contrast between the sample and the mounting medium is required to ensure adequate detail of the sample edge. The particles must rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care must be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

**Crystallinity characterisation**. The crystallinity of a material may be characterised to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the sample in mineral oil on a clean glass slide. Examine the mixture using a polarising microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

**Limit test of particle size by microscopy**. Weigh a suitable quantity of the powder to be examined (for example, 10-100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles

(24) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

**Particle size characterisation**. The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterised must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterisation of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined in Figure 2.9.37.-1.

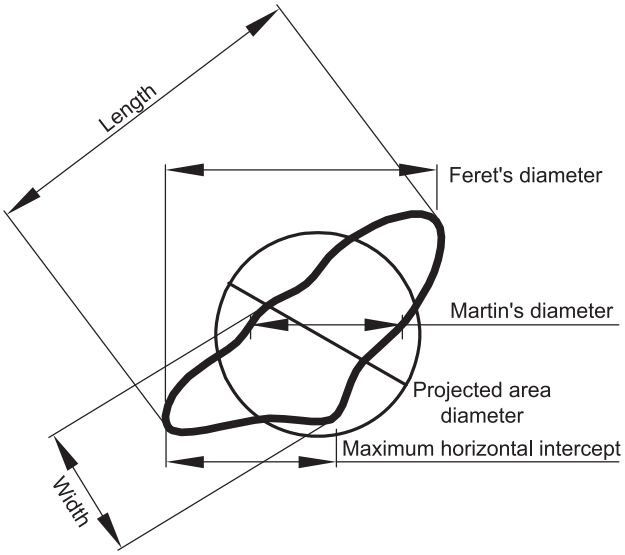


Figure 2.9.37.-1. – *Commonly used measurements of particle size*

- *Feret's diameter*: the distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale,
- *Martin's diameter*: the diameter of the particle at the point that divides a randomly oriented particle into 2 equal projected areas,
- *projected area diameter*: the diameter of a circle that has the same projected area as the particle,
- *length*: the longest dimension from edge to edge of a particle oriented parallel to the ocular scale,
- *width*: the longest dimension of the particle measured at right angles to the length.

**Particle shape characterisation**. For irregularly shaped particles, characterisation of particle size must also include information on particle shape. The homogeneity of the powder must be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see Figure 2.9.37.-2).

- *acicular*: slender, needle-like particle of similar width and thickness,
- *columnar*: long, thin particle with a width and thickness that are greater than those of an acicular particle,
- *flake*: thin, flat particle of similar length and width,
- *plate*: flat particle of similar length and width but with greater thickness than a flake particle,
- *lath*: long, thin, blade-like particle,
- *equant*: particle of similar length, width, and thickness; both cubical and spherical particles are included.

**General observations**. A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semi-solid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

- *lamellar*: stacked plates,
- *aggregate*: mass of adhered particles,
- *agglomerate*: fused or cemented particles,
- *conglomerate*: mixture of 2 or more types of particles,
- *spherulite*: radial cluster,
- *drusy*: particle covered with tiny particles.

Particle condition may be described by the following terms:

- *edges*: angular, rounded, smooth, sharp, fractured,
- *optical*: color (using proper color balancing filters), transparent, translucent, opaque,
- *defects*: occlusions, inclusions.

Surface characteristics may be described as:

- *cracked*: partial split, break, or fissure,
- *smooth*: free of irregularities, roughness, or projections,
- *porous*: having openings or passageways,
- *rough*: bumpy, uneven, not smooth,
- *pitted*: small indentations.

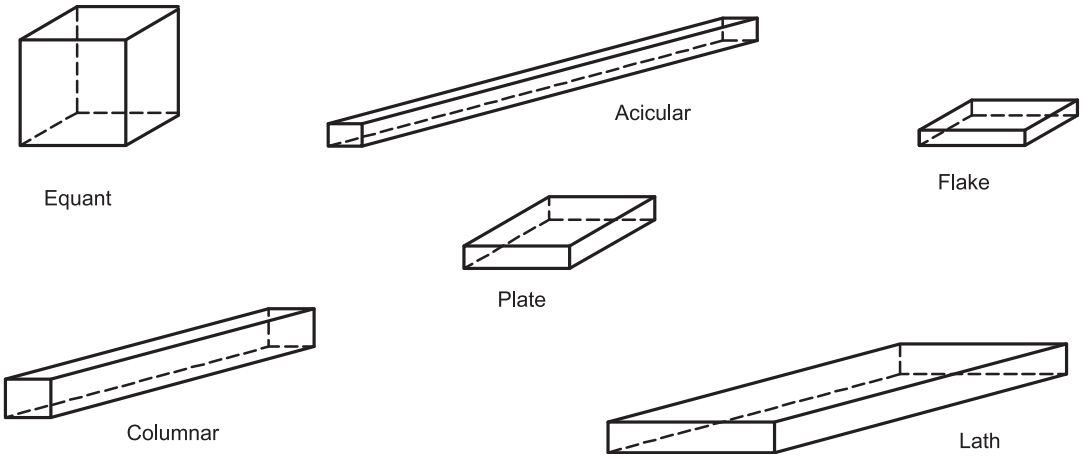


Figure 2.9.37.-2. – *Commonly used descriptions of particle shape*

01/2010:20938

# 2.9.38. PARTICLE-SIZE DISTRIBUTION ESTIMATION BY ANALYTICAL SIEVING[25]

Sieving is one of the oldest methods of classifying powders and granules by particle-size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e. breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 µm. For smaller particles, their light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials other means of agitation such as air-jet sieving or sonic-sifter sieving may be more appropriate. Nevertheless, sieving can sometimes be used for some powders or granules having median particle sizes smaller than 75 µm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of the test sieves) and the difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle-size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on 1 or 2 sieves.

Estimate the particle-size distribution as described under Dry sieving method, unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e. material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 µm), serious consideration must be given to the use of an alternative particle-sizing method.

Sieving is carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out must be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material must be detailed in the individual monograph.

**Principles of analytical sieving**. Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is joined to the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve. The nest of sieves is subjected to a standardised period of agitation, and then the mass of material retained on each sieve is accurately determined. The test gives the mass percentage of powder in each sieve size range.

This sieving process for estimating the particle-size distribution of a single pharmaceutical powder is generally intended for use where at least 80 per cent of the particles are larger than 75 µm. The size parameter involved in determining particle-size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

## TEST SIEVES

Test sieves suitable for pharmacopoeial tests conform to the current edition of *ISO 3310-1: Test sieves – Technical requirements and testing - Part 1: Test sieves of metal wire cloth* (see Table 2.9.38.-1). Unless otherwise specified in the monograph, use those ISO sieves listed as principal sizes in Table 2.9.38.-1 that are recommended in the particular region.

Table 2.9.38.-1.

| ISO Nominal Aperture | | | US Sieve No. | Recommended USP Sieves (µm) | European Sieve No. | Japanese Sieve No. |
|---|---|---|---|---|---|---|
| Principal sizes | Supplementary sizes | | | | | |
| R 20/3 | R 20 | R 40/3 | | | | |
| 11.20 mm | 11.20 mm | 11.20 mm | | | 11 200 | |
| | 10.00 mm | | | | | |
| | | 9.50 mm | | | | |
| | 9.00 mm | | | | | |
| 8.00 mm | 8.00 mm | 8.00 mm | | | | |
| | 7.10 mm | | | | | |
| | | 6.70 mm | | | | |
| | 6.30 mm | | | | | |
| 5.60 mm | 5.60 mm | 5.60 mm | | | 5600 | 3.5 |
| | 5.00 mm | | | | | |
| | | 4.75 mm | | | | 4 |
| | 4.50 mm | | | | | |
| 4.00 mm | 4.00 mm | 4.00 mm | 5 | 4000 | 4000 | 4.7 |
| | 3.55 mm | | | | | |
| | | 3.35 mm | 6 | | | 5.5 |
| | 3.15 mm | | | | | |
| 2.80 mm | 2.80 mm | 2.80 mm | 7 | 2800 | 2800 | 6.5 |
| | 2.50 mm | | | | | |
| | | 2.36 mm | 8 | | | 7.5 |
| | 2.24 mm | | | | | |
| 2.00 mm | 2.00 mm | 2.00 mm | 10 | 2000 | 2000 | 8.6 |
| | 1.80 mm | | | | | |
| | | 1.70 mm | 12 | | | 10 |
| | 1.60 mm | | | | | |
| 1.40 mm | 1.40 mm | 1.40 mm | 14 | 1400 | 1400 | 12 |
| | 1.25 mm | | | | | |
| | | 1.18 mm | 16 | | | 14 |

(25) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

| ISO Nominal Aperture | | | US Sieve No. | Recommended USP Sieves (µm) | European Sieve No. | Japanese Sieve No. |
|---|---|---|---|---|---|---|
| Principal sizes | Supplementary sizes | | | | | |
| R 20/3 | R 20 | R 40/3 | | | | |
| | 1.12 mm | | | | | |
| 1.00 mm | 1.00 mm | 1.00 mm | 18 | 1000 | 1000 | 16 |
| | 900 µm | | | | | |
| | | 850 µm | 20 | | | 18 |
| | 800 µm | | | | | |
| 710 µm | 710 µm | 710 µm | 25 | 710 | 710 | 22 |
| | 630 µm | | | | | |
| | | 600 µm | 30 | | | 26 |
| | 560 µm | | | | | |
| 500 µm | 500 µm | 500 µm | 35 | 500 | 500 | 30 |
| | 450 µm | | | | | |
| | | 425 µm | 40 | | | 36 |
| | 400 µm | | | | | |
| 355 µm | 355 µm | 355 µm | 45 | 355 | 355 | 42 |
| | 315 µm | | | | | |
| | | 300 µm | 50 | | | 50 |
| | 280 µm | | | | | |
| 250 µm | 250 µm | 250 µm | 60 | 250 | 250 | 60 |
| | 224 µm | | | | | |
| | | 212 µm | 70 | | | 70 |
| | 200 µm | | | | | |
| 180 µm | 180 µm | 180 µm | 80 | 180 | 180 | 83 |
| | 160 µm | | | | | |
| | | 150 µm | 100 | | | 100 |
| | 140 µm | | | | | |
| 125 µm | 125 µm | 125 µm | 120 | 125 | 125 | 119 |
| | 112 µm | | | | | |
| | | 106 µm | 140 | | | 140 |
| | 100 µm | | | | | |
| 90 µm | 90 µm | 90 µm | 170 | 90 | 90 | 166 |
| | 80 µm | | | | | |
| | | 75 µm | 200 | | | 200 |
| | 71 µm | | | | | |
| 63 µm | 63 µm | 63 µm | 230 | 63 | 63 | 235 |
| | 56 µm | | | | | |
| | | 53 µm | 270 | | | 282 |
| | 50 µm | | | | | |
| 45 µm | 45 µm | 45 µm | 325 | 45 | 45 | 330 |
| | 40 µm | | | | | |
| | | 38 µm | | | 38 | 391 |

Sieves are selected to cover the entire range of particle sizes present in the test sample. A nest of sieves having a $\sqrt{2}$ progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometres or millimetres in denoting test sieve openings.

Test sieves are made from stainless steel or, less preferably, from brass or another suitable non-reactive wire.

Calibration and recalibration of test sieves is in accordance with the current edition of ISO 3310-1. Sieves are carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212-850 µm, standard glass spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

*Cleaning test sieves*. Ideally, test sieves are cleaned using only a low-pressure air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

**Test sample**. If the test sample mass is not given in the monograph for a particular material, use a test sample having a mass of 25-100 g, depending on the bulk density of the material, for test sieves having a 200 mm diameter. For 76 mm sieves, the amount of material that can be accommodated is approximately 1/7 that which can be accommodated by a 200 mm sieve. Determine the most appropriate mass for a given material by test sieving accurately weighed samples of different masses, such as 25 g, 50 g and 100 g, for the same time period on a mechanical shaker (note: if the test results are similar for the 25 g and 50 g samples, but the 100 g sample shows a lower percentage through the finest sieve, the 100 g sample size is too large). Where only a sample of 10-25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g. down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly iso-diametrical shape, sample masses below 5 g for a 200 mm screen may be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve-analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to absorbing or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging does not influence the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 per cent (*m/m*) level to minimise this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

**Agitation methods**. Several different sieve and powder-agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitudes of the forces acting on the individual particles under test. Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), since changes in the agitation conditions will give different results for the sieve analysis and endpoint determination, and may be sufficiently different to give a failing result under some circumstances.

**Endpoint determination**. The test sieving analysis is complete when the mass on any of the test sieves does not change by more than 5 per cent or 0.1 g (10 per cent in the case of 76 mm

sieves) of the previous mass on that sieve. If less than 5 per cent of the total sample mass is present on a given sieve, the endpoint for that sieve is increased to a mass change of not more than 20 per cent of the previous mass on that sieve.

If more than 50 per cent of the total sample mass is found on any one sieve, unless this is indicated in the monograph, the test is repeated, but with the addition to the sieve nest of a more coarse sieve intermediate between that carrying the excessive mass and the next coarsest sieve in the original nest, i.e. addition of the ISO series sieve omitted from the nest of sieves.

SIEVING METHODS

**Mechanical agitation (Dry sieving method)**. Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test sample on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 min, then carefully remove each sieve from the nest without loss of material. Reweigh each sieve, and determine the mass of material on each one. Determine the mass of material in the collecting pan in a similar manner. Re-assemble the nest of sieves, and agitate for 5 min. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see Endpoint determination under Test sieves). Upon completion of the analysis, reconcile the masses of material. Total loss must not exceed 5 per cent of the mass of the original test sample.

Repeat the analysis with a fresh sample, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle-size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle-size analysis method must be used.

**Air-entrainment methods (Air-jet and sonic-sifter sieving)**. Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air-jet sieving. It uses the same general sieving methodology as that described under Dry sieving method, but with a standardised air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle-size distribution. Air-jet sieving often includes the use of finer test sieves than used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic-sifter method, a nest of sieves is used, and the test sample is carried in a vertically oscillating column of air that lifts the sample and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g when sonic sifting is employed.

The air-jet sieving and sonic-sifter sieving methods may be useful for powders or granules when the mechanical sieving techniques are incapable of giving a meaningful analysis.

These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e. below 75 µm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

INTERPRETATION

The raw data must include the mass of the test sample, the total sieving time, the precise sieving methodology, and the set values for any variable parameters, in addition to the masses retained on the individual sieves and in the pan.

It may be convenient to convert the raw data into a cumulative mass distribution, and if it is desired to express the distribution in terms of a cumulative mass undersize, the range of sieves used must include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

**04/2019:20939**

# 2.9.39. WATER-SOLID INTERACTIONS: DETERMINATION OF SORPTION-DESORPTION ISOTHERMS AND OF WATER ACTIVITY(26)

INTRODUCTION

Pharmaceutical solids as raw materials or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallisation, lyophilisation, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapour or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the "solid-state", crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e. eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognise that there are many situations where a certain level of water in a solid is required for proper performance, e.g. powder compaction. It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage and use.

Some of the more critical pieces of required information concerning water-solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;
- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapour can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

(26) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

## PHYSICAL STATES OF SORBED WATER

Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterised by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, or lose their crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling or dissolution. Such behaviour is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g. by lyophilisation, or after milling. The introduction of defects into highly crystalline solids will also produce this behaviour. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of 2 states, "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature, $T_g$.

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticiser and reduce the value of $T_g$. Since the rheological properties of "fluid" and "glassy" states are quite different, i.e. the "fluid" state exhibits much less viscosity as one goes increasingly above the glass transition temperature, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Since amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a "fluid" state. This is the basis of "cake collapse" often observed during the lyophilisation process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e. to dissolve in their own sorbed water, at relative humidities, $RH_i$, in excess of the relative humidity of a saturated solution of the solid, $RH_0$. Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as $RH_i$ is greater than $RH_0$.

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heats of sorption, freezing point, nuclear magnetic resonance, dielectric properties and diffusion. Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water-solid interactions. Recognising that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of $T_g$. Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g. $10^{13}$ Pa·s. As the amount of water sorbed increases and $T_g$ decreases, approaching ambient temperatures, the glassy state approaches that of a "fluid" state and water mobility along with the mobility of the solid itself increases significantly. At high *RH*, the degree of water plasticisation of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticiser.

**Rates of water uptake**. The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapour can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapour diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

## DETERMINATION OF SORPTION-DESORPTION ISOTHERMS

**Principle**. The tendency to take up water vapour is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e. equilibrium. Relative humidity, *RH*, is defined by the following expression:

$$\frac{P_c}{P_0} \times 100$$

$P_c$ = pressure of water vapour in the system;

$P_0$ = saturation pressure of water vapour under the same conditions.

The ratio $P_c/P_0$ is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption-desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption-desorption hysteresis.

**Methods**. Samples may be stored in chambers at various relative humidities (Figure 2.9.39.-1). The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing.

Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored. Data points for the determination of the sorption isotherm (e.g. from 0 per cent to approximately 95 per cent *RH*, non condensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g. deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of the relative humidity generation. The required relative humidities can be generated, e.g. by accurately mixing dry and saturated vapour gas with flow controllers. The electrostatic behaviour of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions or deliquescence points of certified salts over an adequate range), must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques. In some cases, direct analysis of water content by different methods such as determination of the boiling point, determination of water by distillation, loss on drying or gas chromatography may be advantageous. In the case of adsorption, to improve sensitivity, one can increase the specific surface area of the sample by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity. For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, one must be aware of any adverse effects this might have on the solid such as dehydration, chemical degradation or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

**Report and interpretation of the data**. Sorption data are usually reported as a graph of the apparent mass change in per cent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph. The measurement method must be traceable with the data.

Adsorption-desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the

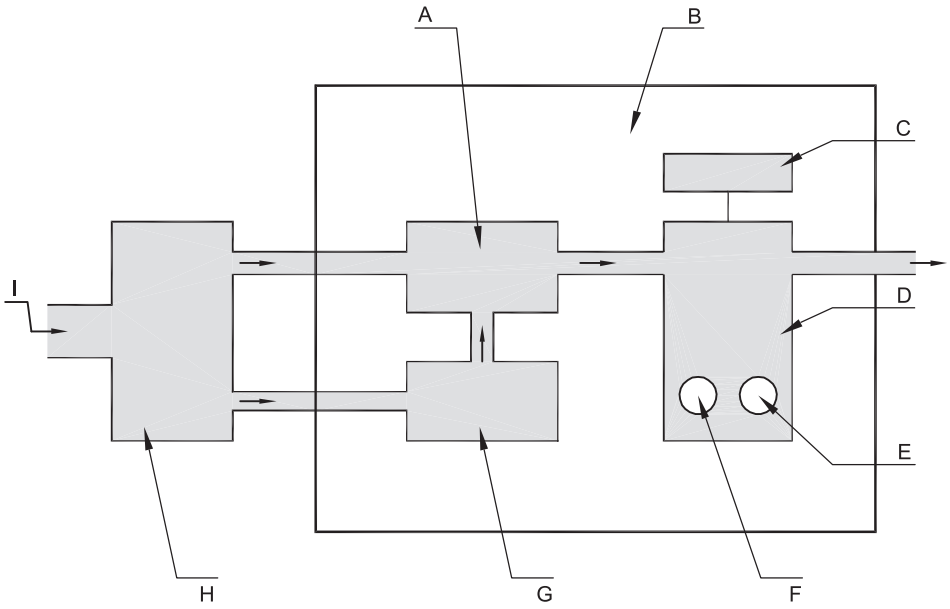


A. Humidity controller
B. Temperature controlled chamber
C. Balance module
D. Humidity regulated module
E. Reference
F. Sample
G. Vapour humidifier
H. Flow control module
I. Dry gas

Figure 2.9.39.-1. – *Example of an apparatus for the determination of the water sorption (other designs are possible)*

sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapour. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapour adsorption-desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they "fill" (adsorption) and "empty" (desorption) under different equilibrium conditions. For non-porous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapour-solid interaction due to a change in the equilibrium state of the solid, e.g. conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption-desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, since one is usually dealing with a polymer plasticised into its "fluid" state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapour adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5 per cent *m/m* uptake of water could hardly cover the bare surface of 100 $m^2/g$, while for 1.0 $m^2/g$ this amounts to 100 times more surface coverage. In the case of pharmaceutical solids which have a specific surface area in the range of 0.01 $m^2/g$ to 10 $m^2/g$, what appears to be low water content could represent a significant amount of water for the available surface. Since the "dry surface area" is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

## DETERMINATION OF THE WATER ACTIVITY

**Principle**. Water activity, $A_w$, is the ratio of vapour pressure of water in the product ($P$) to saturation pressure of water vapour ($P_0$) at the same temperature. It is numerically equal to 1/100 of the relative humidity (*RH*) generated by the product in a closed system. *RH* can be calculated from direct measurements of partial vapour pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the *RH* to which they are exposed. Ignoring activity coefficients, the relationship between $A_w$ and equilibrium relative humidity (*ERH*) are represented by the following equations:

$$A_w = \frac{P}{P_0}$$

$$ERH \text{ (per cent)} = A_w \times 100$$

**Method**. The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key component. Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturisation and robustness are a precondition. The $A_w$ measurement may be conducted using the dew point/chilled mirror method[27]. A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror, which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the *ERH* is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, qualified, and calibrated when used to make water activity determinations. These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25 °C such as those listed in Table 2.9.39.-1.

Table 2.9.39.-1. – *Standard saturated salt solutions*

| Saturated salts solutions at 25 °C | *ERH* (per cent) | $A_w$ |
|---|---|---|
| Potassium sulfate ($K_2SO_4$) | 97.3 | 0.973 |
| Barium chloride ($BaCl_2$) | 90.2 | 0.902 |
| Sodium chloride (NaCl) | 75.3 | 0.753 |
| Magnesium nitrate ($Mg(NO_3)_2$) | 52.9 | 0.529 |
| Magnesium chloride ($MgCl_2$) | 32.8 | 0.328 |
| Lithium chloride (LiCl) | 11.2 | 0.112 |

(27) AOAC International Official Method 978.18.

04/2017:20940

# 2.9.40. UNIFORMITY OF DOSAGE UNITS[28]

To ensure the consistency of dosage units, each unit in a batch should have an active substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of an active substance in each dosage unit. ◊Unless otherwise stated,◊ the uniformity of dosage units specification is not intended to apply to solutions, suspensions, emulsions or gels in single-dose containers intended for local action following cutaneous administration. ◊The test for content uniformity is not required for multivitamin, single-vitamin and trace-element preparations.◊

The term 'uniformity of dosage unit' is defined as the degree of uniformity in the amount of the active substance among dosage units. Therefore, the requirements of this chapter apply to each active substance being comprised in dosage units containing 1 or more active substances, unless otherwise specified elsewere in this Pharmacopoeia.

The uniformity of dosage units can be demonstrated by either of 2 methods: content uniformity or mass variation (see Table 2.9.40.-1).

The test for content uniformity of preparations presented in dosage units is based on the assay of the individual contents of active substance(s) of a number of dosage units to determine whether the individual contents are within the limits set. The content uniformity method may be applied in all cases.

The test for mass variation is applicable for the following dosage forms:

(1) solutions enclosed in single-dose containers and in soft capsules;

(2) solids (including powders, granules and sterile solids) that are packaged in single-dose containers and contain no added active or inactive substances;

(3) solids (including sterile solids) that are packaged in single-dose containers, with or without added active or inactive substances, that have been prepared from true solutions and freeze-dried in the final containers and are labelled to indicate this method of preparation;

(4) hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of an active substance comprising 25 per cent or more, by mass, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other active substances present in lesser proportions is demonstrated by meeting content uniformity requirements.

The test for content uniformity is required for all dosage forms not meeting the above conditions for the mass variation test. ♦ Alternatively, products that do not meet the 25 mg/25 per cent threshold limit may be tested for uniformity of dosage units by mass variation instead of the content uniformity test on the following condition: the concentration Relative Standard Deviation (RSD) of the active substance in the final dosage units is not more than 2 per cent, based on process validation data and development data, and if there has been regulatory approval of such a change. The concentration RSD is the RSD of the concentration per dosage unit (*m/m* or *m/V*), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit mass. See the RSD formula in Table 2.9.40.-2. ♦

Table 2.9.40.-1. – *Application of Content Uniformity (CU) and Mass Variation (MV) test for dosage forms*

| Dosage forms | Type | Sub-Type | Dose and ratio of active substance | |
|---|---|---|---|---|
| | | | ≥ 25 mg and ≥ 25 per cent | < 25 mg or < 25 per cent |
| Tablets | uncoated | | MV | CU |
| | coated | film-coated | MV | CU |
| | | others | CU | CU |
| Capsules | hard | | MV | CU |
| | soft | suspensions, emulsions, gels | CU | CU |
| | | solutions | MV | MV |
| Solids in single-dose containers | single component | | MV | MV |
| | multiple components | solution freeze-dried in final container | MV | MV |
| | | others | CU | CU |
| Solutions enclosed in single-dose containers | | | MV | MV |
| Others: dosage forms not addressed by the other categories in this table including but not limited to suppositories, transdermal patches and semi-solid preparations applied cutaneously and intended for systemic distribution of the active substance | | | CU | CU |

(28) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

Table 2.9.40.-2.

| Variable | Definition | Conditions | Value |
|---|---|---|---|
| $\overline{X}$ | Mean of individual contents ($x_1$, $x_2$,..., $x_n$), expressed as a percentage of the label claim | | |
| $x_1$, $x_2$,..., $x_n$ | Individual contents of the dosage units tested, expressed as a percentage of the label claim | | |
| $n$ | Sample size (number of dosage units in a sample) | | |
| $k$ | Acceptability constant | If $n$ = 10, then | 2.4 |
| | | If $n$ = 30, then | 2.0 |
| $s$ | Sample standard deviation | | $\left[\frac{\sum_{i=1}^{n}(x_i - \overline{X})^2}{n-1}\right]^{1/2}$ |
| $RSD$ | Relative standard deviation | | $\frac{100s}{\overline{X}}$ |
| $M$ (case 1)<br>To be applied when $T \leq 101.5$ | Reference value | If 98.5 per cent ≤ $\overline{X}$ ≤ 101.5 per cent, then | $M = \overline{X}$<br>($AV = ks$) |
| | | If $\overline{X}$ < 98.5 per cent, then | $M$ = 98.5 per cent<br>($AV$ = 98.5 − $\overline{X}$ + $ks$) |
| | | If $\overline{X}$ > 101.5 per cent, then | $M$ = 101.5 per cent<br>($AV = \overline{X}$ − 101.5 + $ks$) |
| $M$ (case 2)<br>To be applied when $T > 101.5$ | Reference value | If 98.5 per cent ≤ $\overline{X} \leq T$, then | $M = \overline{X}$<br>($AV = ks$) |
| | | If $\overline{X}$ < 98.5 per cent, then | $M$ = 98.5 per cent<br>($AV$ = 98.5 − $\overline{X}$ + $ks$) |
| | | If $\overline{X} > T$, then | $M = T$ per cent<br>($AV = \overline{X} - T + ks$) |
| Acceptance value ($AV$) | | | General formula:<br>$\lvert M - \overline{X} \rvert + ks$<br>Calculations are specified above for the different cases. |
| $L1$ | Maximum allowed acceptance value | | $L1$ = 15.0 unless otherwise specified |
| $L2$ | Maximum allowed range for deviation of each dosage unit tested from the calculated value of $M$ | On the low side, no dosage unit result can be less than 0.75 $M$ while on the high side, no dosage unit result can be greater than 1.25 $M$ (This is based on $L2$ value of 25.0) | $L2$ = 25.0 unless otherwise specified |
| $T$ | Target content per dosage unit at time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, $T$ is equal to 100 per cent or $T$ is the manufacturer's approved target content per dosage unit | | |

CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated. Where different procedures are used for assay of the preparation and for the content uniformity test, it may be necessary to establish a correction factor to be applied to the results of the latter.

**Solid dosage forms**. Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see Table 2.9.40.-2).

**Liquid or semi-solid dosage forms**. Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use. Express the results as delivered dose. Calculate the acceptance value (see Table 2.9.40.-2).

**Calculation of Acceptance Value**

Calculate the Acceptance Value ($AV$) using the formula:

$$\lvert M - \overline{X} \rvert + ks$$

for which the terms are as defined in Table 2.9.40.-2.

MASS VARIATION

Carry out an assay for the active substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result $A$, expressed as percentage of label claim (see Calculation of Acceptance Value). Assume that the concentration (mass of active substance per mass of dosage unit) is uniform. Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated.

**Uncoated or film-coated tablets**. Accurately weigh 10 tablets individually. Calculate the active substance content, expressed as percentage of label claim, of each tablet from the mass of the individual tablets and the result of the assay. Calculate the acceptance value.

**Hard capsules**. Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net mass of its contents by subtracting the mass of the shell from the respective gross mass. Calculate the active substance content in each capsule from the mass of product removed from the individual capsules and the result of the assay. Calculate the acceptance value.

**Soft capsules**. Accurately weigh 10 intact capsules individually to obtain their gross masses, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the active substance content in each capsule from the mass of product removed from the individual capsules and the result of the assay. Calculate the acceptance value.

**Solid dosage forms other than tablets and capsules**. Proceed as directed for hard capsules, treating each unit as described therein. Calculate the acceptance value.

**Liquid ◊or semi-solid◊ dosage forms**. Accurately weigh the amount of liquid or semi-solid that is removed from each of 10 individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the active substance content in each container from the mass of product removed from the individual containers and the result of the assay. Calculate the acceptance value.

**Calculation of Acceptance Value**. Calculate the acceptance value ($AV$) as shown in content uniformity, except that the individual contents of the units are replaced with the individual estimated contents defined below.

$x_1, x_2, ..., x_n$ = individual estimated contents of the dosage units tested;

where

$$x_i = w_i \times \frac{A}{\overline{W}}$$

$w_1, w_2, ..., w_n$ = individual masses of the dosage units tested;

$A$ = content of active substance (percentage of label claim) obtained using an appropriate analytical method (assay);

$\overline{W}$ = mean of individual masses ($w_1, w_2, ..., w_n$).

CRITERIA

Apply the following criteria, unless otherwise specified.

**Solid, semi-solid and liquid dosage forms**. The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to $L1$ per cent. If the acceptance value is greater than $L1$ per cent, test the next 20 dosage units and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to $L1$ per cent and no individual content of the dosage unit is less than $(1 - L2 \times 0.01)M$ or more than $(1 + L2 \times 0.01)M$ in calculation of acceptance value under content uniformity or under mass variation. Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

**04/2012:20941**

# 2.9.41. FRIABILITY OF GRANULES AND SPHEROIDS

*This chapter describes 2 methods for determination of the friability of granules and spheroids, which may be used during development studies. It is recognised, however, that many methods with equal suitability may be used.*

This test is intended to determine, under defined conditions, the friability of granules and spheroids. Friability is defined as a reduction in the mass of the granules or spheroids or in the formation of fragments of granules or spheroids, occurring when the granules or spheroids are subjected to mechanical strain during handling (tumbling, vibration, fluidisation, etc.). Examples of changes are abrasion, breakage or deformation of granules or spheroids.

METHOD A

**Apparatus (fluidised-bed apparatus)**. The apparatus (see Figure 2.9.41.-1) consists of a glass cylinder (A) with a conical lower part. The cylinder is provided with a sieve lid (B) having an aperture size of 500 µm or any other suitable sieve. The conical end is connected to a U-shaped glass tube (C) that can be disconnected from the cylinder for removal of the granules or spheroids. The U-tube is attached to a T-coupling (D). One inlet of the T-coupling is joined by a silicone tube to a manometer for regulating the compressed-air flow (use compressed air complying with the test for water in the monograph *Medicinal air (1238)*), the other one is connected via a silicone tube to a by-pass flowmeter (E) (0.10-1.00 $m^3 \cdot h^{-1}$).

**Procedure**. *The following procedure is usually suitable.* Remove the fine particles by sieving (sieve having an aperture size of 710 µm or any other suitable sieve). Introduce about 8.0 g ($m_1$) of granules or spheroids into the cylinder (A). Close the apparatus with the sieve lid (B). Adjust the flow rate of the compressed air to 0.45 $m^3 \cdot h^{-1}$. After 15 min, remove the granules or spheroids from the apparatus by disconnecting the U-tube and weigh again ($m_2$). Test 3 samples and calculate the mean value. It is recommended to spray the inside of the apparatus with an antistatic agent every 3 determinations in order to prevent electrostatic charging.

*Loss on drying.* Dry in an oven at 105 °C, unless otherwise prescribed. Alternatively, other drying conditions as described in general chapter *2.2.32* may be used.

**Calculation**

$$F = \frac{m_1(100 - T_1) - m_2(100 - T_2)}{m_1} \times 100$$

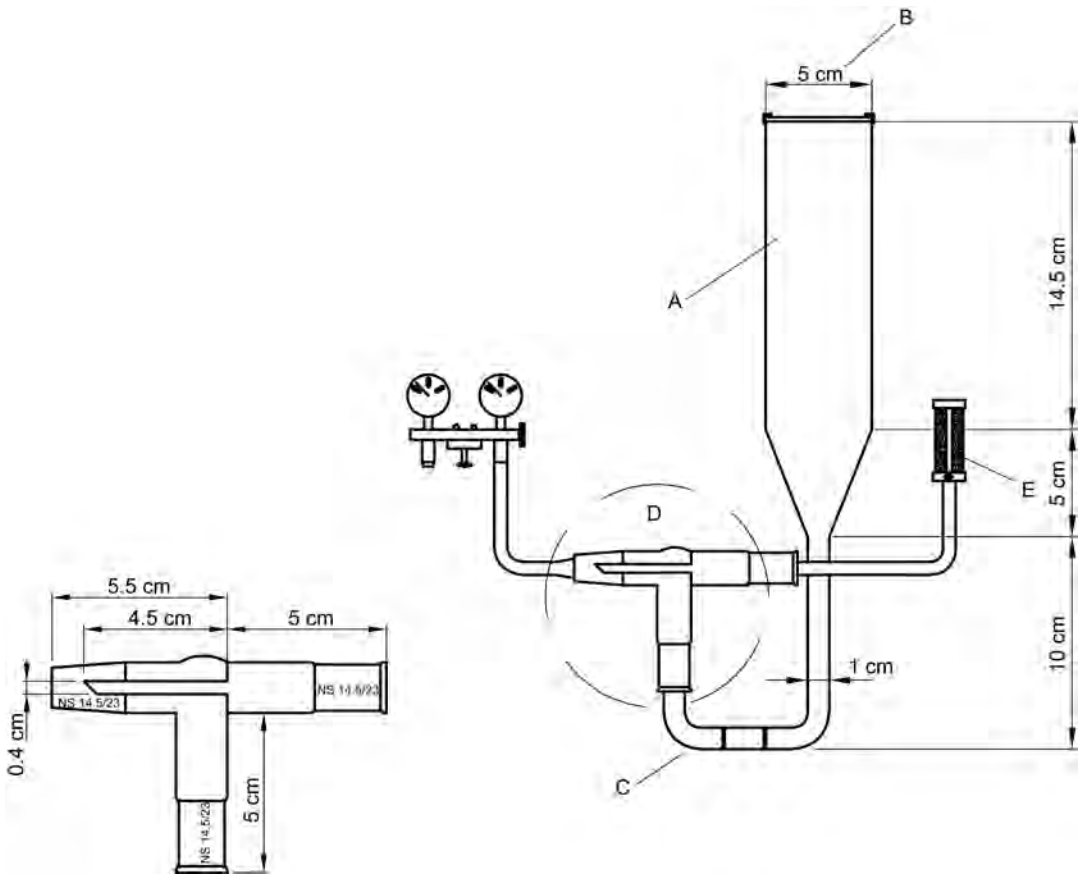


Figure 2.9.41.-1. – *Fluidised-bed apparatus*

$F$ = friability;

$T_1$ = percentage loss on drying before the test (mean of 2 determinations);

$T_2$ = percentage loss on drying after the test (mean of 2 determinations);

$m_1$ = mass of the granules or spheroids before the test, in grams;

$m_2$ = mass of the granules or spheroids after the test, in grams.

METHOD B

**Apparatus (oscillating apparatus)**. The apparatus (see Figure 2.9.41.-2) consists of a glass container, containing the granules or spheroids to be examined, which is subjected to horizontal oscillations. The frequency and duration of the oscillations can be varied continuously. The frequency can be adjusted, using a scale, to a value in the range 0-400 oscillations/min. The duration can be set to a value in the range 0-9999 s.

**Procedure**. *The following procedure is usually suitable.* Remove the fine particles by sieving (sieve having an aperture size of 355 µm or any other suitable sieve). In the glass container, weigh about 10.00 g ($m_1$) of the granules or spheroids. Install the container in the apparatus. Shake for 240 s at the highest frequency for hard granules or spheroids, or for 120 s at a lower frequency (e.g. 140 oscillations/min) for soft granules or spheroids. Sieve (355 µm, or the same sieve as used previously) and weigh the granules or spheroids again ($m_2$). Test 3 samples and calculate the mean value.

*Loss on drying.* Dry in an oven at 105 °C, unless otherwise prescribed. Alternatively, other drying conditions as described in general chapter *2.2.32* may be used.

**Calculation**

$$F = \frac{m_1(100 - T_1) - m_2(100 - T_2)}{m_1} \times 100$$

$F$ = friability;

$T_1$ = percentage loss on drying before the test (mean of 2 determinations);

$T_2$ = percentage loss on drying after the test (mean of 2 determinations);

$m_1$ = mass of the granules or spheroids before the test, in grams;

$m_2$ = mass of the granules or spheroids after the test, in grams.

**01/2008:20942**

## 2.9.42. DISSOLUTION TEST FOR LIPOPHILIC SOLID DOSAGE FORMS

APPARATUS

The apparatus (see Figure 2.9.42.-1) consists of:

- A reservoir for the dissolution medium.
- A pump that forces the dissolution medium upwards through the flow-through cell.
- A flow-through cell shown in Figure 2.9.42.-2 specifically intended for lipophilic solid dosage forms such as suppositories and soft capsules. It consists of 3 transparent parts which fit into each other. The lower part (1) is made up of 2 adjacent chambers connected to an overflow device.

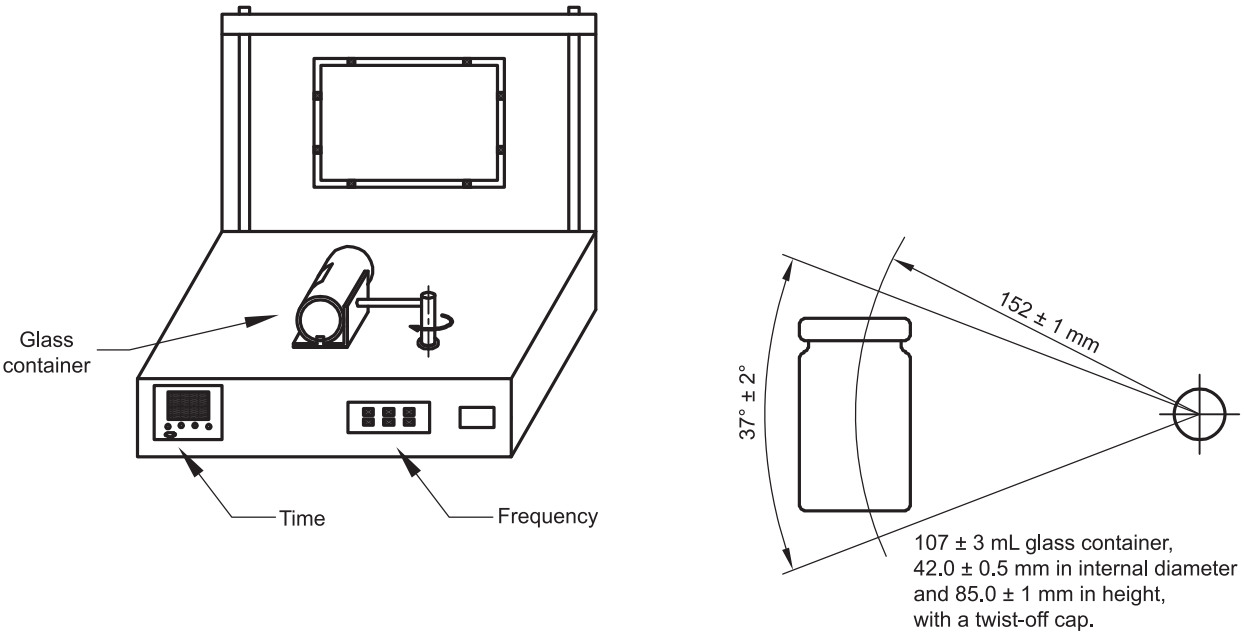


Figure 2.9.41.-2. – *Oscillating apparatus*

The dissolution medium passes through chamber A and is subjected to an upwards flow. The flow in chamber B is downwards directed to a small-size bore exit which leads upwards to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients which float on the dissolution medium. A metal grill serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fibre or cellulose filters.

– A water-bath that will maintain the dissolution medium at 37 ± 0.5 °C.

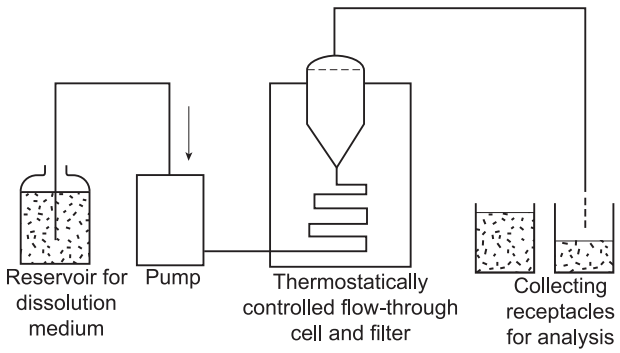


Figure 2.9.42.-1. – *Flow-through apparatus*

**Dissolution medium**. If the dissolution medium is buffered, adjust its pH to within ± 0.05 units of the prescribed value. Remove any dissolved gases from the dissolution medium before the test since they can cause the formation of bubbles that significantly affect the results.

METHOD

Place 1 unit of the preparation to be examined in chamber A. Close the cell with the prepared filter assembly. At the beginning of the test, chamber A requires air removal via a small orifice connected to the filter assembly. Heat the dissolution medium to an appropriate temperature taking the melting point of the preparation into consideration. Using a suitable pump, introduce the warmed dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate (± 5 per cent). When the dissolution medium reaches the overflow, air starts to escape through the capillary and chamber B fills with the dissolution medium. The preparation spreads through the dissolution medium according to its physico-chemical properties.

In justified and authorised cases, representative fractions of large volume suppositories may be tested.

SAMPLING AND EVALUATION

Samples are always collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the active substance from the solution and does not contain substances extractable by the dissolution medium that would interfere with the prescribed analytical method. Proceed with analysis of the filtrate as prescribed.

The quantity of the active substance dissolved in a specified time is expressed as a percentage of the content stated on the label.

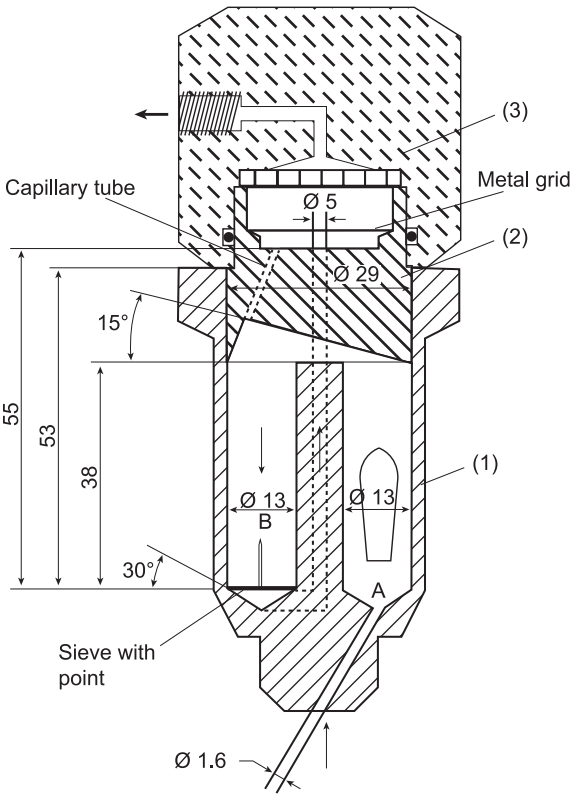


Figure 2.9.42.-2. – *Flow-through cell*

*Dimensions in millimetres*

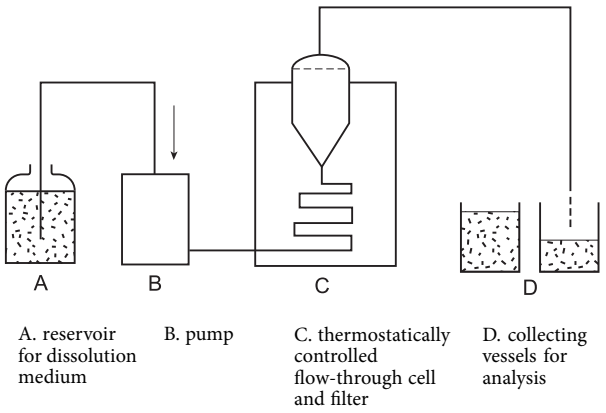


Figure 2.9.43.-1. – *Flow-through apparatus*

**01/2008:20943 corrected 6.1**

## 2.9.43. APPARENT DISSOLUTION

This method is mainly used to determine the apparent dissolution rate of pure solid substances. It may also be used for the determination of the apparent dissolution rate of active substances in preparations presented as powders or granules.

APPARATUS

All parts of the apparatus that may come into contact with the sample or the dissolution medium are chemically inert and do not adsorb, react with, or interfere with the test sample. No part of the assembly or its environment contributes significant motion, agitation or vibration beyond that resulting from the flow-through system.

Apparatus that permits observation of the sample is preferable.

The apparatus (see Figure 2.9.43.-1) consists of:

- a reservoir for the dissolution medium;
- a pump that forces the dissolution medium upwards through the flow-through cell;
- a flow-through cell, preferably of transparent material, mounted vertically with a filter system preventing escape of undissolved particles;
- a water-bath that will maintain the dissolution medium at the chosen temperature (generally 37 ± 0.5 °C).

The flow-through cell shown in Figure 2.9.43.-2 consists of 3 parts that fit into each other. The lower part supports a system of grids and filters on which the powder is placed. The middle part, which fits onto the lower part, contains an insert that sieves the sample when the dissolution medium flows through the cell. This insert is made up of 2 parts: a conical sieve that is placed on the sample and a clip placed midway down the middle part to hold the sieve in place when the dissolution medium passes through. A 2nd filtration assembly (grid and filter) is placed on top of the middle part before fitting the upper part through which the dissolution medium flows out of the cell.

DISSOLUTION MEDIUM

If the dissolution medium is buffered, adjust its pH to within ± 0.05 units. Remove any dissolved gases from the dissolution medium before the test, since they can cause the formation of bubbles, which significantly affect the results.

METHOD

Place a bead of 5 ± 0.5 mm diameter at the bottom of the cone of the lower part followed by glass beads of suitable size, preferably of 1 ± 0.1 mm diameter. Place a sieve (with 0.2 mm apertures), a suitable filter and a 2nd sieve on top of the lower part. Fit the middle part onto the lower part. Weigh the assembly. Place the sample on the filtration assembly and weigh the sample in the cell. Place the sieve of the insert, cone upwards, on the sample, and position the clip midway down the middle part. Place a sieve (with 0.2 mm apertures) and a suitable filter on top of the middle part. Fit the upper part. Heat the dissolution medium to the chosen temperature. Using a suitable pump, introduce the dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate ± 5 per cent.

SAMPLING

Samples of dissolution medium are collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Immediately filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the substances from the solution and does not contain substances extractable by the dissolution medium that would interfere with the prescribed analytical method. Proceed with the analysis of the filtrate as prescribed.

ASSESSMENT OF THE RESULTS

When the test is performed for batch release purposes, an adequate number of replicates is carried out.

The results are expressed as:

- the amount of dissolved substance by time unit (if the dissolution is linear);
- the dissolution time of the whole sample and at appropriate intermediate stages.

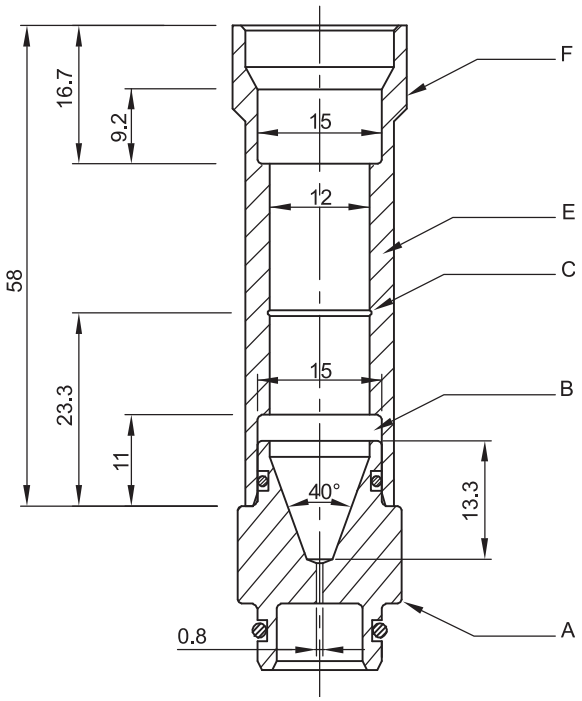


A. lower part C. clip E. middle part
B. sieve D. insert F. upper part

Figure 2.9.43.-2. – *Flow-through cell*
*Dimensions in millimetres*

**01/2012:20944**

## 2.9.44. PREPARATIONS FOR NEBULISATION: CHARACTERISATION

Products used for nebulisation and intended for pulmonary delivery are characterised using the following tests:

- Active substance delivery rate and total active substance delivered;
- Aerodynamic assessment of nebulised aerosols.

These tests standardise the approach given to the assessment of the dose that would be delivered to a patient but are not intended to provide assessment of the nebuliser device itself, which is described in the European standard EN 13544-1:2007+A1:2009, Respiratory therapy equipment - Part 1: Nebulizing systems and their components.

The mass- rather than the number-weighted size distribution is more appropriate to evaluate product performance. Indeed, active substance mass as a function of aerodynamic diameter is more indicative of therapeutic effect within the respiratory tract.

### ACTIVE SUBSTANCE DELIVERY RATE AND TOTAL ACTIVE SUBSTANCE DELIVERED

These tests are performed to assess the rate of delivery to the patient and the total active substance delivered to the patient, using standardised conditions of volumetric flow rate. It is essential that breath-enhanced and breath-actuated nebulisers be evaluated by a breathing simulator, as the output of these types of device is highly dependent on inhalation flow rate. The methodology below describes the use of a standard breathing pattern defined for adults. Should a particular product for nebulisation only be indicated for paediatric (i.e. neonate, infant or child) use, then paediatric breathing pattern(s) must be used. Breathing patterns are used, rather than continuous flow rates, to provide a more appropriate measure of the mass of active substance that would be delivered to patients.

Active substance delivery rate and total active substance delivered are appropriate characteristics because they allow the mass delivered to be characterised in a standard way regardless of the nebuliser used. Accordingly, the test methodology described below allows that the mass of active substance delivered in the 1st period (typically 1 min) is measured (consequently giving an assessment of active substance delivery rate) as well as capturing the total mass of active substance delivered.

*APPARATUS*

**Breathing simulator**. A commercially available breathing simulator, which is able to generate the breathing profiles specified in Table 2.9.44.-1, is used for the test. The breathing profile indicated for adults is used unless the medicinal product is specifically intended for use in paediatrics, where alternate patterns should be used, as indicated in Table 2.9.44.-1.

Table 2.9.44.-1. – *Breathing simulator specifications*

| Item | Specification | | | |
|---|---|---|---|---|
| | Adult | Neonate | Infant | Child |
| Tidal volume | 500 mL | 25 mL | 50 mL | 155 mL |
| Frequency | 15 cycles/min | 40 cycles/min | 30 cycles/min | 25 cycles/min |
| Waveform | sinusoidal | sinusoidal | sinusoidal | sinusoidal |
| Inhalation/exhalation ratio | 1:1 | 1:3 | 1:3 | 1:2 |

**Filter system**. A suitably validated low-resistance filter, capable of quantitatively collecting the aerosol and enabling recovery of the active substance with an appropriate solvent, is used for the test. The dead volume of the filter casing does not exceed 10 per cent of the tidal volume used in the breath simulation.

*METHOD*

Attach the filter (contained in the filter holder) (A) to the breath simulator (B) according to Figure 2.9.44.-1. Fill the nebuliser (C) with the volume of the medicinal product as specified in the patient instructions. Attach the mouthpiece of the nebuliser to the inhalation filter using a mouthpiece adapter if required, ensuring that connections are airtight. Make sure the nebuliser is positioned in the same orientation as intended for use; this may require tilting the breathing simulator and filter holder. Set the breathing simulator to generate the specified breathing pattern.

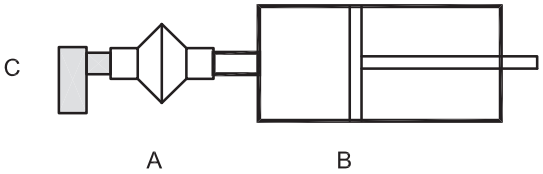


A. inhalation filter and filter holder B. breathing simulator C. nebuliser

Figure 2.9.44.-1. – *Experimental set-up for breathing simulator testing*

Start the breathing simulator then, at the beginning of an inhalation cycle, start the nebuliser. Operate the nebuliser for a defined initial time period. The time chosen, usually 60 ± 1 s, must allow sufficient active substance deposition on the inhalation filter to allow quantitative analysis. If the quantity of active substance deposited on the inhalation filter in 60 s is insufficient for this analysis, the length of the time interval for aerosol collection can be increased. If the filter is soaked with the preparation, this time can be decreased. At the end of this initial period, stop the nebuliser.

Place a fresh filter and filter holder in position and continue until nebulisation ceases. Interrupt nebulisation and exchange filters if necessary, to avoid filter saturation.

*RESULTS*

Using a suitable method of analysis, determine the mass of active substance collected on the filters and filter holders during each time interval. Determine the active substance delivery rate by dividing the mass of active substance collected on the first inhalation filter by the time interval used for collection. Determine the total mass of active substance delivered by summing the mass of active substance collected on all inhalation filters and filter holders.

AERODYNAMIC ASSESSMENT OF NEBULISED AEROSOLS

Nebulised products need to be size-characterised at flow rates lower than the range that is normally used for powder inhalers and metered-dose inhalers. A flow rate of 15 L/min is recommended in the European standard because this value represents a good approximation to the mid-inhalation flow rate achievable by a tidally breathing healthy adult (500 mL tidal volume).

Although low-angle laser light scattering instruments (laser diffractometers) can provide rapid size-distribution measurements of nebuliser-generated aerosols, these techniques do not detect the active substance; rather they measure the size distribution of the droplets irrespective of their content. This may not be a problem with homogeneous solutions, but can result in significant error if the product to be nebulised is a suspension, or if droplet evaporation is significant as can be the case with certain nebuliser types. Cascade impactors enable the aerosol to be characterised unambiguously in terms of the mass of active substance as a function of aerodynamic diameter. Laser diffraction may be used if validated against a cascade impaction method.

Apparatus E (see below under Apparatus), a cascade impactor, has been calibrated at 15 L/min specifically to meet the recommendation of the European standard, and is therefore used for this test. Determining mass balance in the same way as for powder inhalers and metered-dose inhalers is not straightforward, in that the dose is being captured as a continuous output, and hence is not included. As part of method development, recovery experiments must be performed to validate the method.

It is also recognised that the control of evaporation of droplets produced by nebulisers may be critical to avoid bias in the droplet size assessment process. Evaporation can be minimised by cooling the impactor to a temperature of about 5 °C, typically achieved by cooling the impactor in a refrigerator for about 90 min. Typically, at least after each day of use, the apparatus must be fully cleaned, including the inter-stage passageways, in view of the greater risk of corrosion caused by the condensation/accumulation of saline-containing droplets on inter-stage metalwork associated with cooling the impactor. It is recommended to dry all surfaces of the apparatus after each test, for example with compressed air. Note: the micro-orifice collector (MOC) should not be dried with compressed air.

*APPARATUS*

A detailed description of Apparatus E and the induction port is contained in general chapter *2.9.18*, and includes details of critical dimensions and the qualification process for the impactor (stage mensuration).

A back-up filter in addition to the micro-orifice collector (MOC) must be used to ensure quantitative recovery of active substance from the nebulised aerosol at the specified flow rate of 15 L/min. The filter is located below the MOC (internal filter option) or a filter in holder, external to the impactor, is used to capture any fine droplets that pass beyond the last size fractionating stage.

A pre-separator is not used for testing nebuliser-generated aerosols.

*METHOD VALIDATION*

**Impactor stage overloading**. During method development and validation, it is important to confirm that the volume of liquid sampled from the nebuliser does not overload the impactor. Visual inspection of the collection surfaces on stages collecting most of the droplets may reveal streaking if overloading has occurred. This phenomenon is usually also associated with an increase in mass of active substance collected on the final stage and back-up filter. Reducing the sampling period ($T_0$) is the most effective way to avoid overloading in any given system, balancing overloading with analytical sensitivity.

**Re-entrainment**. Droplet bounce and re-entrainment are less likely with nebuliser-produced droplets than with solid particles from inhalers and for that reason coating would not normally be required.

*METHOD*

Pre-cool the assembled impactor and induction port in a refrigerator (set at about 5 °C) for not less than 90 min and start the determination within about 5 min of removal of the impactor from the refrigerator. Other methods that maintain the impactor at a constant temperature (for example, use of a cooling cabinet) can also be employed when validated.

Set up the nebuliser with a supply of driving gas (usually air or oxygen), or use a compressor, at the pressure and flow rate specified by the manufacturer of the nebuliser. Take precautions to ensure that the gas supply line does not become detached from the nebuliser when under pressure. Fill the nebuliser with the volume of the medicinal product as specified in the patient instructions.

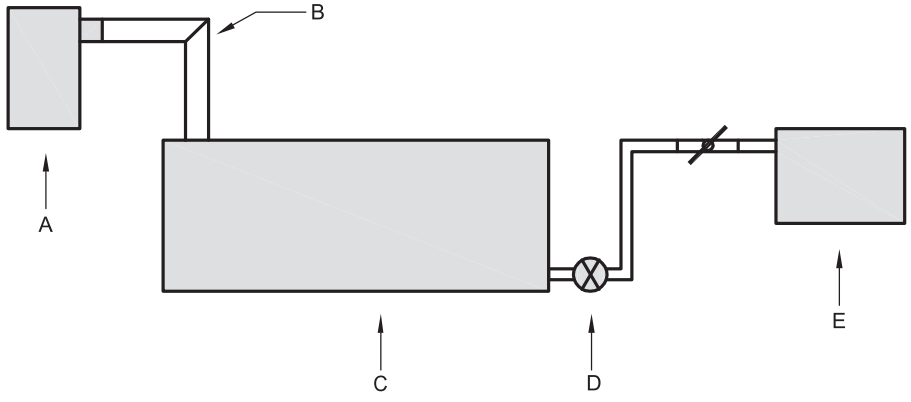


A. nebuliser B. induction port C. impactor (apparatus E) D. flow control valve E. vacuum source

Figure 2.9.44.-2. – *Apparatus E for measuring the size distribution of preparations for nebulisation*

Remove the impactor from the refrigerator. Attach the induction port to the impactor, and connect the outlet of the impactor/external filter to a vacuum source that is capable of drawing air through the system at 15 L/min as specified in Figure 2.9.44.-2. Turn on the flow through the impactor.

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve located between the impactor and the vacuum source to achieve a steady flow through the system at 15 L/min (± 5 per cent). Remove the flow meter.

Make sure the nebuliser is positioned in the same orientation as intended for use then attach the mouthpiece of the nebuliser to the induction port, using a mouthpiece adapter if required, ensuring that connections are airtight. Switch on the flow/compressor for the nebuliser. Sample for a predetermined time ($T_0$). Once determined, this time ($T_0$) must be defined and used in the analytical method for a particular medicinal product to ensure that mass fraction data can be compared. At the end of the sampling period, switch off the driving gas flow/compressor to the nebuliser, remove the nebuliser from the induction port and switch off the flow from the vacuum source to the impactor.

Dismantle the impactor and, using a suitable method of analysis, determine the mass of active substance collected in the induction port, on each stage and on the back-up filter/external filter as described for Apparatus E in general chapter *2.9.18*. Add the mass of active substance collected in the MOC to that deposited on the back-up filter/external filter and treat as a single sample for the purpose of subsequent calculations.

Calculate the mass fraction ($F_{m,comp}$) of the active substance deposited on each component of the impactor, commencing with the induction port and proceeding in order through the impactor, using the following expression:

$$F_{m,comp} = \frac{m_{comp}}{M}$$

$m_{comp}$ = mass associated with the component under evaluation;

$M$ = total mass collected by the system.

Present $F_{m,comp}$ in order of location within the measurement equipment, beginning at the induction port and ending with the back-up filter of the impactor (see Figure 2.9.44.-3). Where appropriate, $F_{m,comp}$ for adjacent stages of the impactor may be combined in order to report the mass fraction collected on a group of stages as a single value.

Determine the cumulative mass-weighted particle-size distribution of the aerosol size-fractionated by the impactor in accordance with the procedure given in general chapter *2.9.18*. Starting at the filter, derive a cumulative mass versus effective cut-off diameter of the respective stages (see Table 2.9.44.-2 for the appropriate cut-off diameters at 15 L/min). Plot the cumulative fraction of active substance versus cut-off diameter in a suitable format, for example logarithmic or log-probability format. Where appropriate, determine by interpolation the fraction either below a given size or between an upper and a lower size limit.

Table 2.9.44.-2. – *Cut-off sizes for Apparatus E at 15 L/min*

| Stage | Cut-off diameter (µm) |
|---|---|
| 1 | 14.1 |
| 2 | 8.61 |
| 3 | 5.39 |
| 4 | 3.30 |
| 5 | 2.08 |
| 6 | 1.36 |
| 7 | 0.98 |

If necessary, and where appropriate, determine values for the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD), as appropriate.

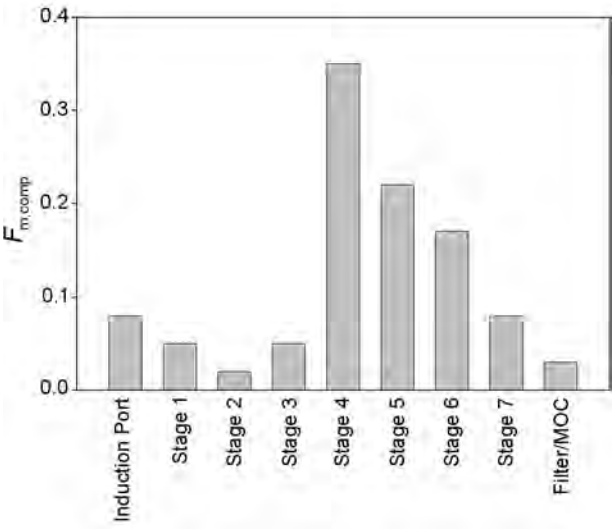


Figure 2.9.44.-3. – *Example of mass fraction of droplets presented in terms of location within the sampling system*

**07/2009:20945**

# 2.9.45. WETTABILITY OF POROUS SOLIDS INCLUDING POWDERS

INTRODUCTION

The wettability of solid surfaces is commonly characterised by direct or indirect contact angle measurements. The contact angle (θ) between a liquid and a solid is the angle naturally formed when a drop of a liquid is placed on a solid surface. This is depicted in Figure 2.9.45.-1. For a given liquid, wettable solids show a low contact angle and non-wettable solids show a contact angle of 90° or more.

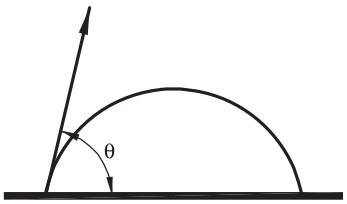


Figure 2.9.45.-1. – *Contact angle (θ) of a sessile drop observed on a non-porous surface*

2 methods for the determination of wettability are described below. The methods are capable of measuring the wettability of porous solids like powders or granules. Both methods express the wettability by a contact angle measurement between the porous solid and a given liquid.

The sessile drop method is based on direct measurement of a contact angle of a sessile drop on a compacted powder disc.

With the Washburn method the contact angle is indirectly measured. The method is based on the capillary effect of the powder pores. The effect (mass gain) is recorded by special electronic balances starting the moment when the powder sample touches the surface of a liquid, preferably not dissolving or poorly dissolving the sample. The measurement has very little or no effect on the state of the powder.

Any pre-treatment of the sample to be examined is disadvantageous, since the properties may be significantly altered. For example, the compaction of a powder as a disc may decrease the surface free energy when the crystalline

state of the powder is changed (e.g. metastable forms), or may increase surface free energy by creating crystal defects (disadvantage of the sessile drop method since compacted powder discs are tested).

The methods are usually applied to examine the following parameters:

- batch-to-batch consistency of samples in terms of wettability;
- effect of liquid viscosity on wettability;
- effect of surface tension of a liquid on wettability;
- alteration of surface properties of samples.

SESSILE DROP METHOD

This method may be used to characterise directly the wettability of coatings and compacted formulations such as tablets. Moreover, it is sometimes possible to use the sessile drop instrument in a dynamic measurement (dynamic contact angle measurement, Figure 2.9.45.-2) of porous solid/liquid systems where the contact angle decreases. By taking several contact angle measurements as a function of time, the rate of spreading accompanied by penetration of a liquid droplet into a slightly porous solid may be studied.

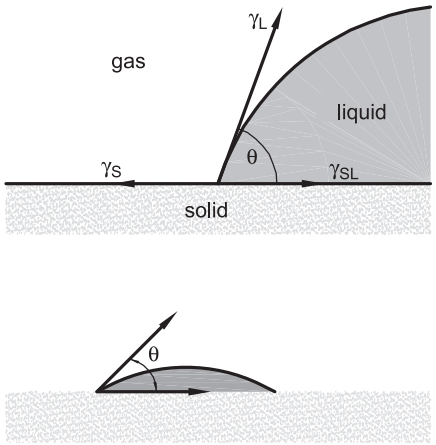


Figure 2.9.45.-2. – *Sessile drop determination with visual inspection of the droplet*

Under equilibrium conditions the contact angle of a sessile drop depends on 3 interrelated surface tensions and is determined using Young's equation (see Figure 2.9.45.-2, 1st part):

$$\gamma_S = \gamma_{SL} + \gamma_L \cos\theta$$

$\gamma_S$ = surface tension of the solid with air;

$\gamma_{SL}$ = interfacial tension of the solid with the liquid;

$\gamma_L$ = surface tension of the liquid with air.

*PROCEDURE*

Since powders are unable to form a completely flat surface, the powder is usually compacted as a disc in an attempt to make the surface smoother. A liquid drop with a given volume is placed on the disc (see Figure 2.9.45.-2) allowing direct measurement of the contact angle using a goniometer fitted with an eyepiece protractor, or by geometric construction on a photomicrograph. Other physical and mathematical procedures of data analysis may also be appropriate. The drop volume may influence the result. Several determinations of the contact angle (θ) (n = 6) are usually carried out and the average is calculated.

WASHBURN METHOD

The Washburn method is able to measure the contact angle of porous solids with a contact angle in the range of 0-90°.

The tested material is the combination of the sample, the holder and the filter system. Therefore, an estimation or determination of the true value is not possible and only apparent values of the contact angle can be determined. However, the contact angle of the sample is the functional property on which the result is significantly dependent. The outcome of the test is a ranking order listing the wettability of different substances or formulations characterised by an apparent contact angle.

*PRINCIPLE*

If a porous solid is brought into contact with a liquid, such that the solid is not submerged in the liquid, but rather is just touching the liquid surface, then the rise of liquid into the pores of the solid due to capillary action will be governed by the following equations:

$$m^2 = \frac{t}{A} \quad (1)$$

$m$ = mass of liquid sucked into the solid;

$t$ = time elapsed since the solid and the liquid were brought into contact;

$A$ = constant, dependent on the properties of the liquid and the solid to be examined, calculated using the following equation:

$$A = \frac{\eta}{c \times \rho^2 \times \gamma \times \cos\theta} \quad (2)$$

$\eta$ = viscosity of the liquid;

$\rho$ = density of the liquid;

$\gamma$ = surface tension of the liquid;

$\theta$ = contact angle between the solid and the liquid;

$c$ = material constant, dependent on the porous texture of the solid.

Equations (1) and (2) lead to equation (3):

$$\cos\theta = \frac{m^2}{t} \times \frac{\eta}{c \times \rho^2 \times \gamma} \quad (3)$$

In setting up a Washburn determination, a liquid with known density (ρ), viscosity (η), and surface tension (γ) is used. Under these conditions, when the mass of liquid rising into the porous solid is monitored as a function of time (such that capillary penetration rate ($\frac{m^2}{t}$) is the experimental data), 2 unknowns remain according to equation (3): the contact angle (θ) of the liquid on the solid, and the solid material constant ($c$).

**Determination of the material constant (*c*).** The material constant for a porous solid is determined by the following equation, considering cylindrical pores:

$$\frac{\pi^2 \times r^5 \times N^2}{2} \quad (4)$$

$r$ = average capillary radius within the porous solid;

$N$ = number of capillaries per volumetric unit.

If a Washburn determination is performed with a liquid considered to have a contact angle of 0° (cos 0° = 1) on the solid, then the solid material constant ($c$) is the only remaining unknown in equation (3) and can thus be determined. *n*-Heptane is the liquid of choice for determining material constants because of its low surface tension (20.14 $mN \cdot m^{-1}$ at 25 °C). *n*-Hexane may also be used (18.43 $mN \cdot m^{-1}$ at 25 °C) but is more volatile. If the powder dissolves too

quickly in these liquids, hexamethyldisiloxane may be used instead (15.9 $mN \cdot m^{-1}$ at 25 °C). Replicate determinations are performed (n = 6) and the average value calculated.

Once the material constant (*c*) has been determined for the solid to be examined, a sample of the solid can be tested for wettability by another liquid. The material constant determined by the *n*-heptane test is used in the Washburn equation, in combination with the capillary penetration rate ($\frac{m^2}{t}$) data obtained while testing the substance to be examined in the prescribed liquid. This allows calculation of the contact angle.

NOTE: if a series of liquids (at least 2 liquids in addition to the liquid used to determine the material constant) is tested against a given solid then the resultant contact angle data can be used to calculate the surface energy of the porous solid.

*APPARATUS*

Figure 2.9.45.-3 shows the principal components of the apparatus. The main device is an electronic balance with a suitable processor ensuring a suitable resolution in force measurement and a suitable resolution in lifting up the immersion liquid towards the sample.

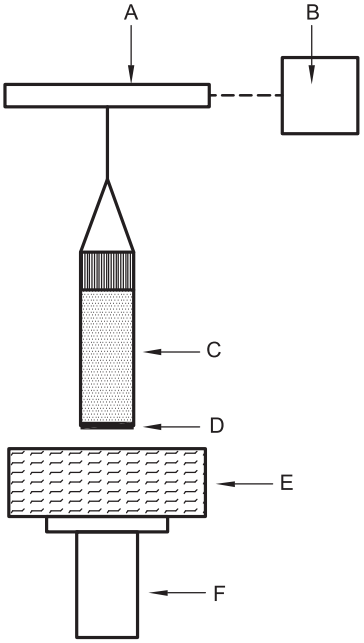


A. electronic balance C. sample holder E. immersion liquid

B. computer D. filter F. lift

Figure 2.9.45.-3. – *Apparatus for contact angle measurement by the Washburn method*

Table 2.9.45.-1 indicates parameters of the electronic balance that are generally considered suitable.

Table 2.9.45.-1. – *Technical parameters of the electronic balance*

| | Lift | Mass measurement |
|---|---|---|
| Range | > 110 mm | 0 - 210 g |
| Resolution | 0.1 µm | 10 µg |
| Speed | 0.099 - 500 mm/min | - |

**Sample holders**. The sample holder may be a small glass cylinder with a sintered-glass filter at one end.

Powder material holders (see Figure 2.9.45-4) may also be made of aluminium; they are less fragile than those made of glass and have small holes in the bottom that render them easier to clean than a sintered-glass filter. The cover for the cell is equipped with 2 screw threads. One connects it with the sample chamber while the other allows the user to guide a piston down onto the sample itself and compact it. The apparatus is similar to an automatic tensiometer, except for the sample holder.

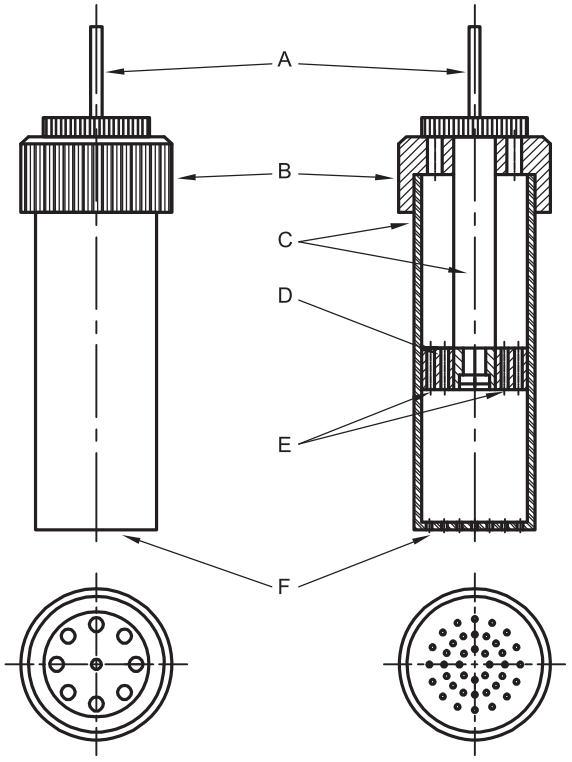


A. fixing C. thread E. capillary holes

B. cover D. plunger F. capillary holes

Figure 2.9.45.-4. – *Example of sample holder with plunger for compaction of a powder*

*PROCEDURE*

**Filling of the sample holder**. Place a disc of filter paper in the bottom of the aluminium or glass sample holder. This prevents powder from leaking out of the bottom of the cell. The filter does not have to be made of paper, but it must be a material that is easily wetted by the liquid to be tested. A black-band filter (used for reverse osmosis) is recommended because of its high porosity and minimum flow resistance.

Place a known amount of powder into the cell. The reproducibility of material constants and contact angles will depend on the ability to weigh out the same amount of powder for each test when a sufficient and adjusted amount of powder is compacted in a uniform way (i.e. tapping/compaction of the powder).

For most powders, a correct amount is in the range of a few grams, typically filling about 2/3 of the capacity of the holder. Place a second piece of filter paper on top of the powder in the cell. This will prevent powder from rising through the holes in the piston during the compaction process and/or during the determination.

**Tapping/compaction of the powder**. A bulk powder bed is very porous and thus very sensitive to small influences that can easily alter the porosity and consequently the *c*-constant. Therefore a tapped powder may be advantageous and will show more reproducible results. The appropriate number of taps must first be evaluated: 50-100 taps are usually appropriate.

If the aluminium sample holder is used then it may be mounted in the cylinder of a stamp volumeter, which can run the evaluated number of taps.

If tapping is not appropriate, the powder bed is compacted by screwing the piston of the aluminium sample holder applying a specified pressure.

A further possibility is centrifugation under defined conditions. Where applicable, a compacted disc of the powder sample may also be mounted on the electronic balance. A sample holder is omitted in this case.

After connecting to the balance, the sample holder is positioned with the porous solid just above the surface of the liquid (see Figure 2.9.45.-3), using the lift.

The liquid is raised further until it just touches the bottom of the porous sample. Mass-versus-time data is then collected as liquid penetrates into the solid. Data can be presented in either graphical or tabular format. The apparatus may perform the whole determination automatically.

*CRITICAL PARAMETERS*

The following points must be considered.

*Sample properties*:

- water content of the sample;
- crystalline or solid-state properties of the sample (polymorphic form, type of solvate).

*Sample preparation*:

- homogeneity of any powder blend to be examined;
- particle-size distribution; before testing it is sometimes advisable to sieve the sample (e.g. using a 250 µm sieve);
- the optimal compaction parameters (amount of sample, number of taps or piston mass) must be determined;
- the compaction state of the different powder samples must be uniform;
- the sample holder or, if used, the glass frit must be carefully cleaned;
- uniformity of the results is improved by using a sample holder made of aluminium.

*Immersion liquid*:

- specifications of the immersion liquid must be indicated.

**04/2013:20947**
**corrected 8.1**

## 2.9.47. DEMONSTRATION OF UNIFORMITY OF DOSAGE UNITS USING LARGE SAMPLE SIZES

*The procedure is intended for, but not limited to, the evaluation of medicinal products that are manufactured using process analytical technology (PAT) methodology.*

Table 2.9.47.-1. – *Acceptability constant (k) and acceptable number of dosage units with a content outside (1 ± L2 × 0.01)M (= c2) for a given sample size n*

| n (≥) | k | c2 | n (≥) | k | c2 | n (≥) | k | c2 | n (≥) | k | c2 | n (≥) | k | c2 | n (≥) | k | c2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **100** | 2.15 | | **804** | 2.26 | 7 | **2480** | 2.29 | 23 | **4366** | 2.30 | 41 | **6252** | 2.31 | 59 | **8243** | 2.31 | 78 |
| **105** | 2.16 | | **905** | 2.27 | | **2585** | 2.29 | 24 | **4471** | 2.30 | 42 | **6357** | 2.31 | 60 | **8347** | 2.31 | 79 |
| **120** | 2.17 | 0 | **908** | 2.27 | 8 | **2690** | 2.29 | 25 | **4576** | 2.30 | 43 | **6462** | 2.31 | 61 | **8452** | 2.31 | 80 |
| **139** | 2.18 | | **1013** | 2.27 | 9 | **2794** | 2.29 | 26 | **4680** | 2.30 | 44 | **6566** | 2.31 | 62 | **8557** | 2.31 | 81 |
| **161** | 2.19 | | **1118** | 2.27 | 10 | **2899** | 2.29 | 27 | **4785** | 2.30 | 45 | **6671** | 2.31 | 63 | **8662** | 2.31 | 82 |
| **176** | 2.19 | | **1223** | 2.27 | 11 | **3004** | 2.29 | 28 | **4890** | 2.30 | 46 | **6776** | 2.31 | 64 | **8767** | 2.31 | 83 |
| **189** | 2.20 | | **1276** | 2.28 | | **3109** | 2.29 | 29 | **4995** | 2.30 | 47 | **6881** | 2.31 | 65 | **8871** | 2.31 | 84 |
| **224** | 2.21 | 1 | **1328** | 2.28 | 12 | **3171** | 2.30 | | **5099** | 2.30 | 48 | **6985** | 2.31 | 66 | **8976** | 2.31 | 85 |
| **270** | 2.22 | | **1432** | 2.28 | 13 | **3213** | 2.30 | 30 | **5204** | 2.30 | 49 | **7090** | 2.31 | 67 | **9081** | 2.31 | 86 |
| **280** | 2.22 | | **1537** | 2.28 | 14 | **3318** | 2.30 | 31 | **5309** | 2.30 | 50 | **7195** | 2.31 | 68 | **9186** | 2.31 | 87 |
| **328** | 2.23 | 2 | **1642** | 2.28 | 15 | **3423** | 2.30 | 32 | **5414** | 2.30 | 51 | **7300** | 2.31 | 69 | **9290** | 2.31 | 88 |
| **385** | 2.23 | | **1747** | 2.28 | 16 | **3528** | 2.30 | 33 | **5519** | 2.30 | 52 | **7404** | 2.31 | 70 | **9395** | 2.31 | 89 |
| **407** | 2.24 | 3 | **1851** | 2.28 | 17 | **3633** | 2.30 | 34 | **5623** | 2.30 | 53 | **7509** | 2.31 | 71 | **9500** | 2.31 | 90 |
| **490** | 2.24 | | **1918** | 2.29 | | **3737** | 2.30 | 35 | **5728** | 2.30 | 54 | **7614** | 2.31 | 72 | **9605** | 2.31 | 91 |
| **516** | 2.25 | 4 | **1956** | 2.29 | 18 | **3842** | 2.30 | 36 | **5833** | 2.30 | 55 | **7719** | 2.31 | 73 | **9710** | 2.31 | 92 |
| **594** | 2.25 | | **2061** | 2.29 | 19 | **3947** | 2.30 | 37 | **5938** | 2.30 | 56 | **7824** | 2.31 | 74 | **9814** | 2.31 | 93 |
| **672** | 2.26 | 5 | **2166** | 2.29 | 20 | **4052** | 2.30 | 38 | **6042** | 2.30 | 57 | **7928** | 2.31 | 75 | **9919** | 2.31 | 94 |
| **699** | 2.26 | 6 | **2270** | 2.29 | 21 | **4156** | 2.30 | 39 | **6136** | 2.31 | | **8033** | 2.31 | 76 | | | |
| | | | **2375** | 2.29 | 22 | **4261** | 2.30 | 40 | **6147** | 2.31 | 58 | **8138** | 2.31 | 77 | | | |

Table 2.9.47.-2. – *Acceptable number of individual dosage units with a content outside (1 ± L1 × 0.01)T (= c1) and (1 ± L2 × 0.01)T (= c2) respectively, for a given sample size n*

| n (≥) | c1 | c2 |
|---|---|---|
| 100 | 3 | 0 |
| 123 | 4 | |
| 159 | 5 | |
| 176 | 5 | 1 |
| 196 | 6 | |
| 234 | 7 | |
| 273 | 8 | |
| 280 | 8 | 2 |
| 313 | 9 | |
| 353 | 10 | |
| 385 | 10 | 3 |
| 394 | 11 | |
| 434 | 12 | |
| 476 | 13 | |
| 490 | 13 | 4 |
| 517 | 14 | |
| 559 | 15 | |
| 594 | 15 | 5 |
| 601 | 16 | |
| 644 | 17 | |
| 686 | 18 | |
| 699 | 18 | 6 |
| 729 | 19 | |
| 772 | 20 | |
| 804 | 20 | 7 |
| 815 | 21 | |
| 858 | 22 | |
| 902 | 23 | |
| 908 | 23 | 8 |
| 945 | 24 | |
| 989 | 25 | |
| 1013 | 25 | 9 |
| 1033 | 26 | |
| 1077 | 27 | |
| 1118 | 27 | 10 |
| 1121 | 28 | |
| 1165 | 29 | |
| 1209 | 30 | |
| 1223 | 30 | 11 |
| 1253 | 31 | |
| 1298 | 32 | |
| 1328 | 32 | 12 |
| 1342 | 33 | |
| 1387 | 34 | |
| 1432 | 35 | 13 |
| 1476 | 36 | |
| 1521 | 37 | |
| 1537 | 37 | 14 |
| 1566 | 38 | |
| 1611 | 39 | |
| 1642 | 39 | 15 |
| 1656 | 40 | |
| 1701 | 41 | |
| 1746 | 42 | |
| 1747 | 42 | 16 |
| 1791 | 43 | |
| 1836 | 44 | |
| 1851 | 44 | 17 |
| 1882 | 45 | |
| 1927 | 46 | |
| 1956 | 46 | 18 |
| 1972 | 47 | |
| 2018 | 48 | |
| 2061 | 48 | 19 |
| 2063 | 49 | |
| 2109 | 50 | |
| 2154 | 51 | |
| 2166 | 51 | 20 |
| 2200 | 52 | |
| 2246 | 53 | |
| 2270 | 53 | 21 |
| 2291 | 54 | |
| 2337 | 55 | |
| 2375 | 55 | 22 |
| 2383 | 56 | |
| 2429 | 57 | |
| 2475 | 58 | |
| 2480 | 58 | 23 |
| 2520 | 59 | |
| 2566 | 60 | |
| 2585 | 60 | 24 |
| 2612 | 61 | |
| 2658 | 62 | |
| 2690 | 62 | 25 |
| 2704 | 63 | |
| 2750 | 64 | |
| 2794 | 64 | 26 |
| 2796 | 65 | |
| 2843 | 66 | |
| 2889 | 67 | |
| 2899 | 67 | 27 |
| 2935 | 68 | |
| 2981 | 69 | |
| 3004 | 69 | 28 |
| 3027 | 70 | |
| 3073 | 71 | |
| 3109 | 71 | 29 |
| 3120 | 72 | |
| 3166 | 73 | |
| 3212 | 74 | |
| 3213 | 74 | 30 |
| 3259 | 75 | |
| 3305 | 76 | |
| 3318 | 76 | 31 |
| 3351 | 77 | |
| 3398 | 78 | |
| 3423 | 78 | 32 |
| 3444 | 79 | |
| 3491 | 80 | |
| 3528 | 80 | 33 |
| 3537 | 81 | |
| 3584 | 82 | |
| 3630 | 83 | |
| 3633 | 83 | 34 |
| 3677 | 84 | |
| 3723 | 85 | |
| 3737 | 85 | 35 |
| 3770 | 86 | |
| 3817 | 87 | |
| 3842 | 87 | 36 |
| 3863 | 88 | |
| 3910 | 89 | |
| 3947 | 89 | 37 |
| 3956 | 90 | |
| 4003 | 91 | |
| 4050 | 92 | |
| 4052 | 92 | 38 |
| 4097 | 93 | |
| 4143 | 94 | |
| 4156 | 94 | 39 |
| 4190 | 95 | |
| 4237 | 96 | |
| 4261 | 96 | 40 |
| 4284 | 97 | |
| 4330 | 98 | |
| 4366 | 98 | 41 |
| 4377 | 99 | |
| 4424 | 100 | |
| 4471 | 101 | 42 |
| 4518 | 102 | |
| 4565 | 103 | |
| 4576 | 103 | 43 |
| 4612 | 104 | |
| 4658 | 105 | |
| 4680 | 105 | 44 |
| 4705 | 106 | |
| 4752 | 107 | |
| 4785 | 107 | 45 |
| 4799 | 108 | |
| 4846 | 109 | |
| 4890 | 109 | 46 |
| 4893 | 110 | |
| 4940 | 111 | |
| 4987 | 112 | |
| 4995 | 112 | 47 |
| 5034 | 113 | |
| 5081 | 114 | |
| 5099 | 114 | 48 |
| 5128 | 115 | |
| 5175 | 116 | |
| 5204 | 116 | 49 |
| 5222 | 117 | |
| 5269 | 118 | |
| 5309 | 118 | 50 |
| 5317 | 119 | |
| 5364 | 120 | |
| 5411 | 121 | |
| 5414 | 121 | 51 |
| 5458 | 122 | |
| 5505 | 123 | |
| 5519 | 123 | 52 |
| 5552 | 124 | |
| 5599 | 125 | |
| 5623 | 125 | 53 |
| 5647 | 126 | |
| 5694 | 127 | |
| 5728 | 127 | 54 |
| 5741 | 128 | |
| 5788 | 129 | |
| 5833 | 129 | 55 |
| 5835 | 130 | |
| 5883 | 131 | |
| 5930 | 132 | |
| 5938 | 132 | 56 |
| 5977 | 133 | |
| 6024 | 134 | |
| 6042 | 134 | 57 |
| 6072 | 135 | |
| 6119 | 136 | |
| 6147 | 136 | 58 |
| 6166 | 137 | |
| 6214 | 138 | |
| 6252 | 138 | 59 |
| 6261 | 139 | |
| 6308 | 140 | |
| 6355 | 141 | |
| 6357 | 141 | 60 |
| 6403 | 142 | |
| 6450 | 143 | |
| 6462 | 143 | 61 |
| 6498 | 144 | |
| 6545 | 145 | |
| 6566 | 145 | 62 |
| 6592 | 146 | |
| 6640 | 147 | |
| 6671 | 147 | 63 |
| 6687 | 148 | |
| 6734 | 149 | |
| 6776 | 149 | 64 |
| 6782 | 150 | |
| 6829 | 151 | |
| 6877 | 152 | |
| 6881 | 152 | 65 |
| 6924 | 153 | |
| 6972 | 154 | |
| 6985 | 154 | 66 |
| 7019 | 155 | |
| 7067 | 156 | |
| 7090 | 156 | 67 |
| 7114 | 157 | |
| 7161 | 158 | |
| 7195 | 158 | 68 |
| 7209 | 159 | |
| 7256 | 160 | |
| 7300 | 160 | 69 |
| 7304 | 161 | |
| 7351 | 162 | |
| 7399 | 163 | |
| 7404 | 163 | 70 |
| 7447 | 164 | |
| 7494 | 165 | |
| 7509 | 165 | 71 |
| 7542 | 166 | |
| 7589 | 167 | |
| 7614 | 167 | 72 |
| 7637 | 168 | |
| 7684 | 169 | |
| 7719 | 169 | 73 |
| 7732 | 170 | |
| 7779 | 171 | |
| 7824 | 171 | 74 |
| 7827 | 172 | |
| 7875 | 173 | |
| 7922 | 174 | |
| 7928 | 174 | 75 |
| 7970 | 175 | |
| 8017 | 176 | |
| 8033 | 176 | 76 |
| 8065 | 177 | |
| 8113 | 178 | |
| 8138 | 178 | 77 |
| 8160 | 179 | |
| 8208 | 180 | |
| 8243 | 180 | 78 |
| 8256 | 181 | |
| 8303 | 182 | |
| 8347 | 182 | 79 |
| 8351 | 183 | |
| 8399 | 184 | |
| 8446 | 185 | |
| 8452 | 185 | 80 |
| 8494 | 186 | |
| 8542 | 187 | |
| 8557 | 187 | 81 |
| 8589 | 188 | |
| 8637 | 189 | |
| 8662 | 189 | 82 |
| 8685 | 190 | |
| 8732 | 191 | |
| 8767 | 191 | 83 |
| 8780 | 192 | |
| 8828 | 193 | |
| 8871 | 193 | 84 |
| 8875 | 194 | |
| 8923 | 195 | |
| 8971 | 196 | |
| 8976 | 196 | 85 |
| 9019 | 197 | |
| 9066 | 198 | |
| 9081 | 198 | 86 |
| 9114 | 199 | |
| 9162 | 200 | |
| 9186 | 200 | 87 |
| 9210 | 201 | |
| 9257 | 202 | |
| 9290 | 202 | 88 |
| 9305 | 203 | |
| 9353 | 204 | |
| 9395 | 204 | 89 |
| 9401 | 205 | |
| 9449 | 206 | |
| 9496 | 207 | |
| 9500 | 207 | 90 |
| 9544 | 208 | |
| 9592 | 209 | |
| 9605 | 209 | 91 |
| 9640 | 210 | |
| 9688 | 211 | |
| 9710 | 211 | 92 |
| 9735 | 212 | |
| 9783 | 213 | |
| 9814 | 213 | 93 |
| 9831 | 214 | |
| 9879 | 215 | |
| 9919 | 215 | 94 |
| 9927 | 216 | |
| 9975 | 217 | |
| 10023 | 218 | |
| 10070 | 219 | |

Compliance with general chapter *2.9.40. Uniformity of dosage units* can be demonstrated by the following procedure, when large samples (sample size $n \geq 100$) are evaluated.

Application of this chapter does not constitute a mandatory requirement. It presents 2 alternative tests (Alternative 1 and Alternative 2). Fulfilling the requirements of either of the 2 alternatives is considered as evidence that the medicinal product tested complies with general chapter *2.9.40*. The 2 alternatives are considered equivalent in their demonstration of compliance with general chapter *2.9.40*.

ALTERNATIVE 1 (PARAMETRIC)

Select not fewer than 100 units according to a predefined sampling plan.

The consistency of dosage units is evaluated by content uniformity or mass variation as prescribed in Table 2.9.40.-1. Calculate the acceptance value (*AV*) using the following expression:

$$|M - \overline{X}| + ks$$

for which the terms are defined in Table 2.9.40.-2, but using the sample size-dependent value for *k* defined in Table 2.9.47.-1.

*CRITERIA*

Apply the following criteria, unless otherwise specified.

The requirements for dosage form uniformity are met if:

1. the acceptance value (*AV*) is less than or equal to $L1$; and
2. in the calculation of acceptance value (*AV*) under content uniformity or under mass variation, the number of individual dosage units outside $(1 \pm L2 \times 0.01)M$ is less than or equal to $c2$ as defined for a given sample size *n* in Table 2.9.47.-1.

Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

Table 2.9.47.-1. is to be interpreted as follows:

- for a sample size of $n = 400$, enter the table at $n \geq 385$: $k = 2.23$ and $c2 = 3$;
- for a sample size of $n = 450$, enter the table at $n \geq 407$: $k = 2.24$ and $c2 = 3$;
- for a sample size of $n = 500$, enter the table at $n \geq 490$: $k = 2.24$ and $c2 = 4$.

ALTERNATIVE 2 (NON-PARAMETRIC)

Select not fewer than 100 units according to a predefined sampling plan.

The consistency of dosage units is evaluated by content uniformity or mass variation as prescribed in Table 2.9.40.-1. Assay individually or weigh the units and calculate individual contents as prescribed in general chapter *2.9.40*. Count the number of individual dosage units with a content outside $(1 \pm L1 \times 0.01)T$ and the number of individual dosage units with a content outside $(1 \pm L2 \times 0.01)T$. Evaluate if the values are within the limits defined in Table 2.9.47.-2.

*CRITERIA*

Apply the following criteria, unless otherwise specified.

The requirements for dosage form uniformity are met if:

1. the number of individual dosage units outside $(1 \pm L1 \times 0.01)T$ is less than or equal to $c1$; and
2. the number of individual dosage units outside $(1 \pm L2 \times 0.01)T$ is less than or equal to $c2$.

$c1$ and $c2$ for a given sample size *n* are defined in Table 2.9.47.-2. Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

Table 2.9.47.-2 is to be interpreted as follows:

- for a sample size of $n = 400$, enter the table at $n \geq 394$: $c1 = 11$ and $c2 = 3$;
- for a sample size of $n = 450$, enter the table at $n \geq 434$: $c1 = 12$ and $c2 = 3$;
- for a sample size of $n = 500$, enter the table at $n \geq 490$: $c1 = 13$ and $c2 = 4$.

01/2020:20949



# 2.9.49. POWDER FLOW PROPERTIES BY SHEAR CELL METHODS

The methodology in this general chapter is based on standard test methods ASTM D6773-08 and ASTM D6128-14.

Powder flow properties play an important role in the design of formulations, processes and pharmaceutical production equipment, and an approach commonly used to characterise these properties is to submit the powder to shear tests, which are experiments designed to determine the flow properties of the powder by applying different states of stress and strain to it. Using these tests, a wide variety of parameters describing the flow properties of a powder can be studied, including the yield locus, the angle of internal friction, the compressive strength, the tensile strength, and a variety of derived parameters such as the flowability ratio. Because of the ability to control experimental parameters precisely, flow properties can also be determined as a function of consolidation load, time, and environmental conditions such as temperature and humidity.

Many suitable shear cell configurations and test methods are available; the most widely used are those based on the Jenike-type shear cell or on ring shear cells such as the Schulze-type ring shear tester.

PRINCIPLE

*UNIAXIAL COMPRESSION TEST*

The uniaxial compression test illustrates the concept of flowability. As shown in Figure 2.9.49.-1, a hollow cylinder (cross-sectional area A, internal wall assumed as frictionless) is filled with a powder. The powder is loaded by the consolidation stress ($\sigma_1$) in the vertical direction. The more the volume of the powder can be reduced, the more compressible the powder is. In addition to the increase in bulk density ($\rho_b$) from the consolidation stress, an increase in the strength of the powder is also observed. Hence, the powder is both consolidated and compressed through the effect of the consolidation stress.

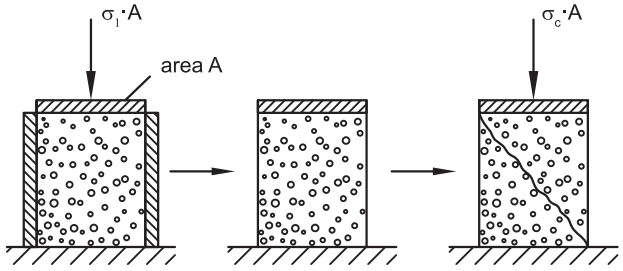


Figure 2.9.49.-1. – *Uniaxial compression test*

After consolidation, the powder is relieved of the consolidation stress ($\sigma_1$) and the hollow cylinder is removed. The consolidated cylindrical powder sample is subsequently loaded with an increasing vertical compressive stress and, at a certain stress, the sample fails along the shear plane and the powder starts to flow. The compressive strength ($\sigma_c$), or unconfined yield strength, is defined as the stress causing failure. Since the powder fails only at a sufficiently large vertical stress, there is a specific yield limit for the powder.

The yield limit of a powder is dependent on its stress history, i.e. its previous consolidation. The greater the consolidation stress ($\sigma_1$) the greater the bulk density ($\rho_b$) and compressive strength ($\sigma_c$). Thus, uniaxial compression tests conducted at different consolidation stresses ($\sigma_1$) yield different pairs of values ($\sigma_1$, $\sigma_c$) and ($\sigma_1$, $\rho_b$). Plotting these pairs of values as points in a ($\sigma_1$, $\sigma_c$) diagram and a ($\sigma_1$, $\rho_b$) diagram, respectively, and drawing in each diagram a curve through these points, usually results in curves similar to those shown for product A

in Figure 2.9.49.-2, where bulk density ($\rho_b$) and compressive strength ($\sigma_c$) typically increase with consolidation stress ($\sigma_1$). Very rarely a progressive slope similar to that of the left part of curve B is observed. The graph of $\sigma_c$ versus $\sigma_1$ is called the flow function.

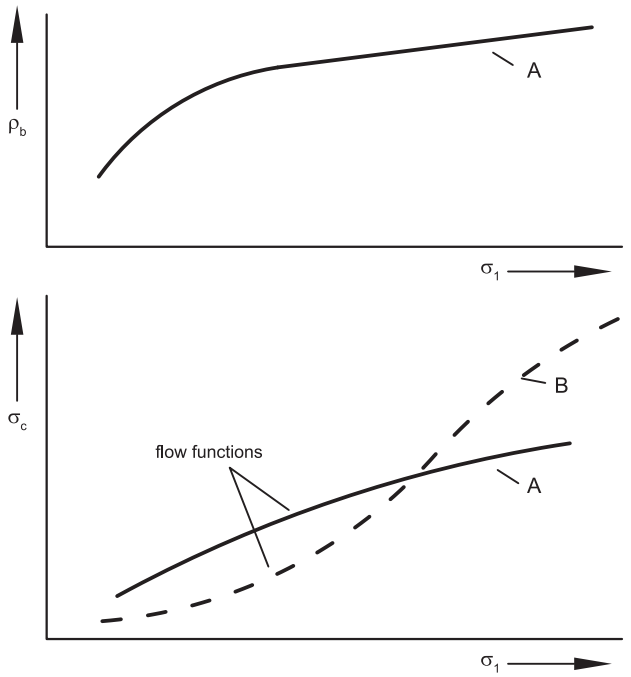


Figure 2.9.49.-2. – *Bulk density ($\rho_b$) and compressive strength ($\sigma_c$) as a function of consolidation stress ($\sigma_1$)*

*NUMERICAL CHARACTERISATION OF FLOWABILITY*

The flow function can be used to characterise the flow behaviour of a powder in terms of the flowability ratio ($ff_c$), which is given by:

$$ff_c = \sigma_1 / \sigma_c$$

The larger the $ff_c$, the better a powder flows. The $ff_c$ value can be used to classify the flow behaviour as follows:

| | |
|---|---|
| $ff_c < 1$ | not flowing |
| $1 < ff_c < 2$ | very cohesive |
| $2 < ff_c < 4$ | cohesive |
| $4 < ff_c < 10$ | easy-flowing |
| $10 < ff_c$ | free-flowing |

Figure 2.9.49.-3 shows the flow function A taken from the ($\sigma_1$, $\sigma_c$) diagram in Figure 2.9.49.-2. Additionally, the boundaries of the ranges of the classifications listed above are shown as straight lines, each representing a constant value of the flowability ratio ($ff_c$). This diagram clearly shows that the flowability ratio ($ff_c$) of a specific powder is dependent on the consolidation stress ($\sigma_1$) applied.

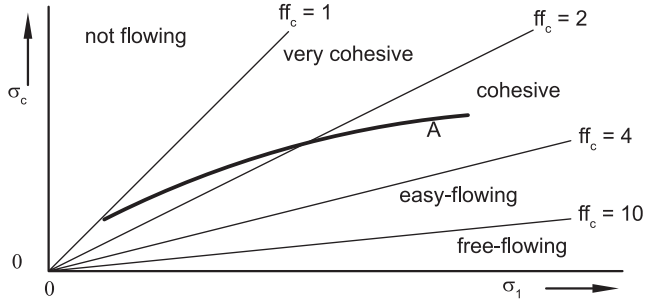


Figure 2.9.49.-3. – *Flow function and lines of constant flowability ratio*

Furthermore, the flow function may change depending on the consolidation time, for example, at the same $\sigma_1$ level, higher $\sigma_c$ values may be obtained with longer consolidation times; this is known as the time consolidation effect. Possible mechanisms are solid or liquid bridges, solid crystallisation, viscoelastic or viscoplastic deformation, or chemical reactions at the particle contacts.

*YIELD LIMIT AND MOHR STRESS CIRCLE*

Assuming that the force of gravity and wall friction effects can be disregarded, the uniaxial compression test can be represented as shown in Figure 2.9.49.-4, in a ($\sigma$, $\tau$) diagram where $\sigma$ is the normal stress and $\tau$ is the shear stress. In such a diagram, all pairs of values ($\sigma$, $\tau$) form a circle representing the stresses in the powder; this is called a 'Mohr stress circle'. The Mohr stress circle is centred on the sigma axis. The two intersect points with the sigma axis are called the minor and major principal stresses. Figure 2.9.49.-4 shows the Mohr stress circles corresponding to the uniaxial compression test and a possible yield limit of the sample (the real course of the yield limit cannot be determined with only the uniaxial compression test).

Mohr stress circle A describes the stresses in the powder sample at consolidation. Since no shear stress is applied, $\sigma_1$ corresponds to the normal stress or vertical stress ($\sigma_v$) and $\sigma_2$ corresponds to the horizontal stress ($\sigma_h$).

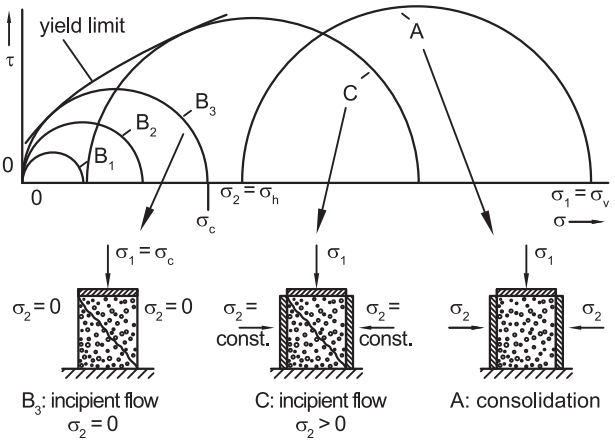


Figure 2.9.49.-4. – *Measurement of compressive strength and corresponding ($\sigma$, $\tau$) diagram*

In the second part of the test shown in Figure 2.9.49.-1, the sample is loaded with increasing vertical stress after it has been relieved of the consolidation stress and the hollow cylinder has been removed. As the vertical load increases, the stress states at different load steps are represented by stress circles with increasing diameter (stress circles $B_1$, $B_2$, $B_3$ in Figure 2.9.49.-4). The minor principal stress, i.e. the horizontal stress, is equal to zero for all stress circles since the lateral surface of the sample is uncovered and not loaded.

Mohr stress circle $B_3$ represents the stresses in the powder sample at failure of the sample. Since the load corresponding to this Mohr stress circle causes incipient flow of the sample, Mohr stress circle $B_3$ must be tangential to the yield limit in the ($\sigma$, $\tau$) diagram.

If, during the second part of the experiment shown in Figure 2.9.49.-1 (i.e. the measurement of compressive strength), a constant horizontal stress $\sigma_h > 0$ were also applied to the sample in addition to the vertical stress ($\sigma_v$), stress circles that indicate failure of the sample and are tangential to the yield limit (e.g. stress circle C in Figure 2.9.49.-4) would likewise be found. Thus the yield limit is the envelope of all stress circles that indicate failure of a powder sample.

## MEASUREMENT WITH A SHEAR TESTER

### *PRINCIPLE*

The test sample must be representative of the powder with respect to particle-size distribution, moisture, temperature and other properties that may have an influence on the flow behaviour. The test sample is filled into a shear cell of circular or annular cross-section, depending on the type of tester used. In order to achieve a homogenous and representative powder bed, filling should be carried out uniformly in small horizontal layers using a spoon or spatula, without applying force to the surface of the material, until the cell is slightly overfilled. Excess powder is then removed by scraping off with a blade until the powder is flush with the top of the shear cell. The shear cell is then completed with a lid placed on top of the sample. The latter step may vary with the type of apparatus and is therefore carried out according to the manufacturer's instructions to achieve a homogenous and representative powder bed.

In general, a shear test measures the yield limit of a consolidated powder bed. This yield limit is also called the yield locus. It depends to a certain extent on the apparatus and experimental conditions. Typically the yield locus is measured immediately after consolidation of the powder bed. If however the yield locus is measured after a certain consolidation time, then it is called the time yield locus. When running a shear test, a normal stress (σ) is applied vertically to the powder in the shear cell. A shear deformation of the sample is then induced by moving the lid of the shear cell relative to the bottom of the shear cell in a horizontal direction with constant velocity (*V*) (see Figure 2.9.49.-5). This results in a horizontal shear stress (τ), which develops due to the friction between the particles in the shearing plane. The lid of the shear cell is allowed to move vertically in order to adjust to changes in the sample's bulk density.

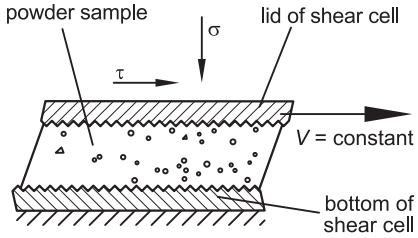


Figure 2.9.49.-5. – *Principle of shear deformation in a shear cell*

When a point of a yield locus is measured, as in the uniaxial compression test, 2 steps are necessary: first the powder sample is consolidated in a preshear step, and then a point of the yield locus is measured in a shear or shear-to-failure step.

### *PRESHEAR STEP*

For preshear, the powder sample is loaded in the vertical direction under a well-defined normal stress ($\sigma = \sigma_{pre}$), and is then sheared. Preshear is stopped when steady-state flow, characterised by constant shear stress ($\tau_{pre}$), is achieved. The pair of values of normal stress and shear stress at steady-state flow ($\sigma_{pre}$, $\tau_{pre}$) is plotted in a normal stress - shear stress diagram ((σ, τ) diagram, Figure 2.9.49.-6, right). Point ($\sigma_{pre}$, $\tau_{pre}$) is called the preshear point.

### *SHEAR-TO-FAILURE STEP*

After the powder has been consolidated by the preshear procedure, the shear stress (τ) is reduced to zero by reversing the relative motion of the lid with respect to the bottom of the shear cell.

For the next step of the test procedure, the so-called shear or shear-to-failure step, the normal stress acting on the sample is decreased to a value $\sigma_{sh}$, which is less than the normal stress at preshear ($\sigma_{pre}$).

If the consolidated sample is sheared under the normal stress $\sigma_{sh} < \sigma_{pre}$, it will start to flow when a sufficiently large shear stress ($\tau_{sh}$) is attained. At that point particles start to move against each other. The material will start to dilate, i.e. decrease in bulk density and shear resistance, and thus shear stress will decrease. The maximum shear stress ($\tau_{sh}$) characterises incipient flow. The corresponding pair of values ($\sigma_{sh}$, $\tau_{sh}$) is a point of the yield locus in the (σ, τ) diagram (Figure 2.9.49.-6, right). Such a point is called a shear point or a point of incipient flow.

In order to measure the course of the yield locus, several of the tests described above must be performed, where the samples must first be consolidated at the same normal stress ($\sigma_{pre}$, preshear). Then the samples are sheared to failure under different normal stresses $\sigma_{sh} < \sigma_{pre}$. The yield locus follows a curve plotted through all measured shear points (Figure 2.9.49.-6, right). In general, at least 3 shear points should be measured.

With the Jenike-type shear cell a new sample has to be prepared for each shear point (indicated as 'next test' in Figure 2.9.49.-6). However, with ring shear testers a complete yield locus is usually measured with 1 sample. In addition, with the Schulze-type ring shear tester the bulk density is measured during the test, depending on the consolidation stress applied.

## DATA EVALUATION

Parameters that describe the flow properties can be determined from the yield locus as shown in Figure 2.9.49.-7.

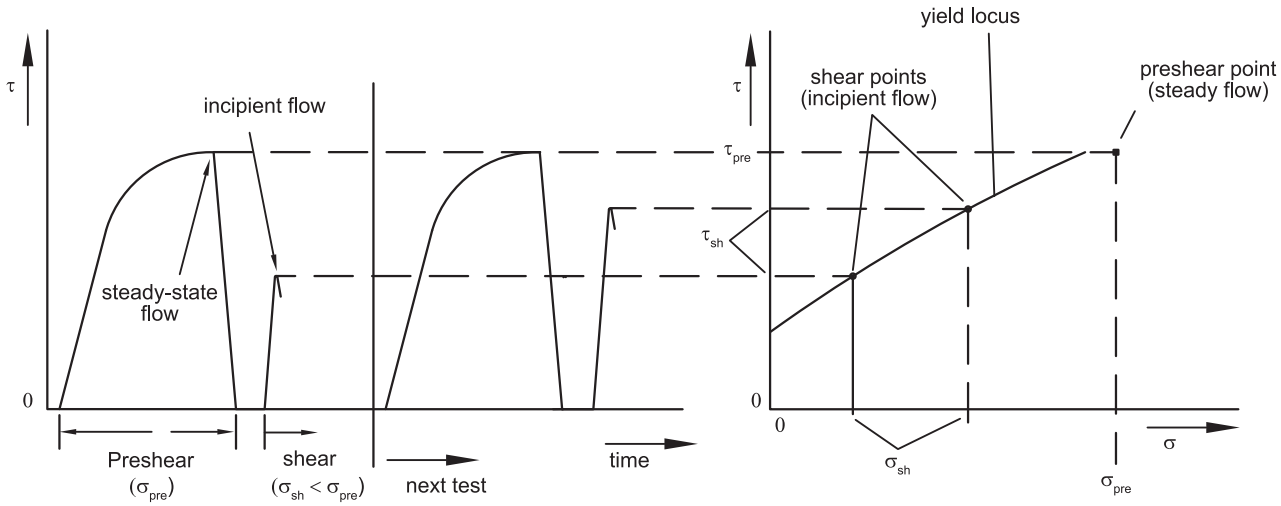


Figure 2.9.49.-6. – *Plot of shear stress versus time (left) and corresponding yield locus (right)*

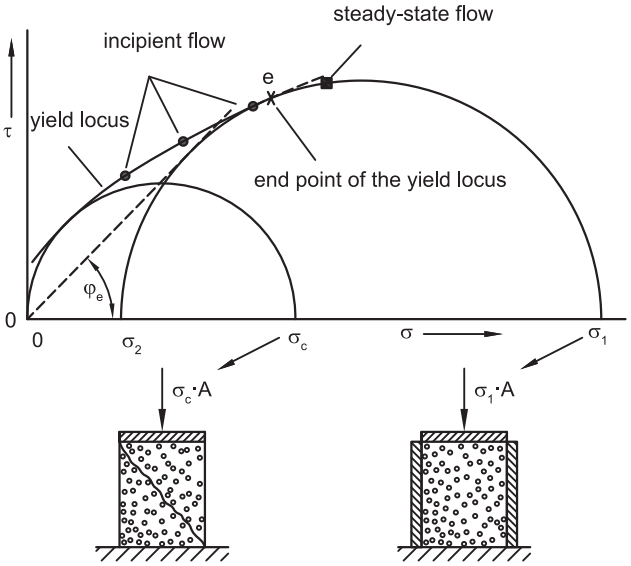


Figure 2.9.49.-7. – *Yield locus, Mohr stress circles and analogy with uniaxial compression test*

The consolidation stress ($\sigma_1$) is equal to the major principal stress of the Mohr stress circle, which is tangential to the yield locus, and intersects at the point of steady-state flow ($\sigma_{pre}$, $\tau_{pre}$). This stress circle represents the stress distribution in the sample at the end of the consolidation procedure (stresses at steady-state flow). It corresponds to the stress circle at the end of consolidation in the uniaxial compression test.

The compressive strength ($\sigma_c$) is determined from the stress circle that is tangential to the yield locus and runs through the origin (minor principal stress $\sigma_2 = 0$). This stress circle represents a similar stress state to the one that prevails in the second step of the uniaxial compression test.

A straight line through the origin of the ($\sigma$, $\tau$) diagram, tangent to the greater Mohr circle, is the effective yield locus, shown as a broken line in Figure 2.9.49.-7. It encloses the $\sigma$-axis with the effective angle of internal friction ($\varphi_e$). Because the largest Mohr stress circle indicates a state of steady-state flow, the angle $\varphi_e$ can be regarded as a measure of the internal friction at steady-state flow.

Further flow properties can be determined from the yield locus as illustrated in Figure 2.9.49.-8.

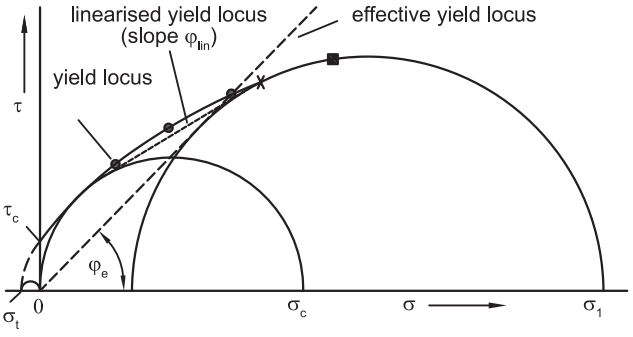


Figure 2.9.49.-8. – *Yield locus and characteristic values for flow properties*

For many applications the yield locus is linearised as the tangent to both Mohr circles. The linearised yield locus is characterised by its slope angle $\varphi_{lin}$.

The cohesion ($\tau_c$) is the value of the shear stress where the yield locus intersects with the $\tau$-axis, i.e. at normal stress $\sigma = 0$. The uniaxial tensile strength ($\sigma_t$) is the normal stress at the left end of the yield locus at shear stress $\tau = 0$. As it is difficult to measure the yield locus at small and negative stress levels, cohesion may be obtained by extrapolating the yield locus to the intersection with the $\tau$-axis. Due to the pronounced non-linearity of the yield locus at low stresses, the cohesion obtained in this way is less accurate than the compressive strength, and it is hardly possible to determine tensile strength by extrapolation.

If several yield loci are measured at different stress levels, i.e. with different normal stresses at preshear ($\sigma_{pre}$), each yield locus represents another state of consolidation and another bulk density. The above-mentioned flow properties, namely compressive strength, effective angle of internal friction or slope angle of the linearised yield locus, can be indicated as functions of the consolidation stress ($\sigma_1$), similar to Figure 2.9.49.-2 where bulk density and compressive strength are plotted versus consolidation stress.

**01/2020:20952**

# 2.9.52. SCANNING ELECTRON MICROSCOPY

*This general chapter focuses on scanning electron microscopy applied to pharmaceutical materials, from research to quality control.*

Scanning electron microscopy (SEM) is a powerful imaging technique that is superior to and complements light microscopy in terms of resolution, magnification and depth of field.

In addition, by exploiting the different interactions between the electrons and the specimen, it provides topographical and compositional information and can thus also be used as an analytical tool. When it is combined with elemental X-ray microanalysis, in particular, chemical analysis can be performed (see general chapter *2.2.37. X-ray fluorescence spectrometry*).

SEM is a well-established technique and, increasingly, it is being applied to examine and characterise a wide range of pharmaceutical materials in solid form but also, with the use of specialised specimen preparation methods, in semi-solid and liquid form.

## PRINCIPLE

To produce magnified images, a scanning electron microscope uses a finely-focused beam of accelerated electrons instead of light; the specimen is scanned (rastered) in a rectangular pattern with the electron beam. This technique exploits the interactions between the electron beam and the specimen using different types of detectors. Due to the nature of these interactions, a variety of signals are produced and these can be used to provide characteristic information about the specimen at and from just below its surface.

### *INTERACTION WITH THE SPECIMEN*

As the surface of the specimen is scanned by the electron beam, these electrons (also known as primary electrons) interact with the specimen and can penetrate to a depth of up to a few tens of micrometres. Electrons are scattered and absorbed within a teardrop-shaped volume just below the surface of the specimen, known as the interaction volume (Figure 2.9.52-1). The depth to which the electron beam penetrates is directly proportional to the beam voltage (a high-energy beam will penetrate deeper than a low-energy beam) and inversely proportional to the average atomic number of the constituent elements of the specimen (the beam will penetrate much deeper into a specimen rich in light elements than into one rich in heavy elements).

**Types of emitted signals**

Figure 2.9.52-1 illustrates the sources of the principal emitted signals, which are described as follows:

- *secondary electrons* are low-energy electrons that are ejected from just below the specimen surface as a result of the inelastic scattering of the incident electrons;
- *backscattered electrons* are high-energy electrons that result from the elastic scattering of incident electrons deeper in the interaction volume; as a consequence, backscattered electron images typically have a lower spatial resolution than secondary electron images and show less surface detail;
- characteristic *X-rays* are produced when the incident electron beam interacts with the elements in the specimen;
- *cathodoluminescence* is the emission of photons in the visible spectrum when atoms and molecules deep within the interaction volume return to their ground state after being excited by the electron beam.

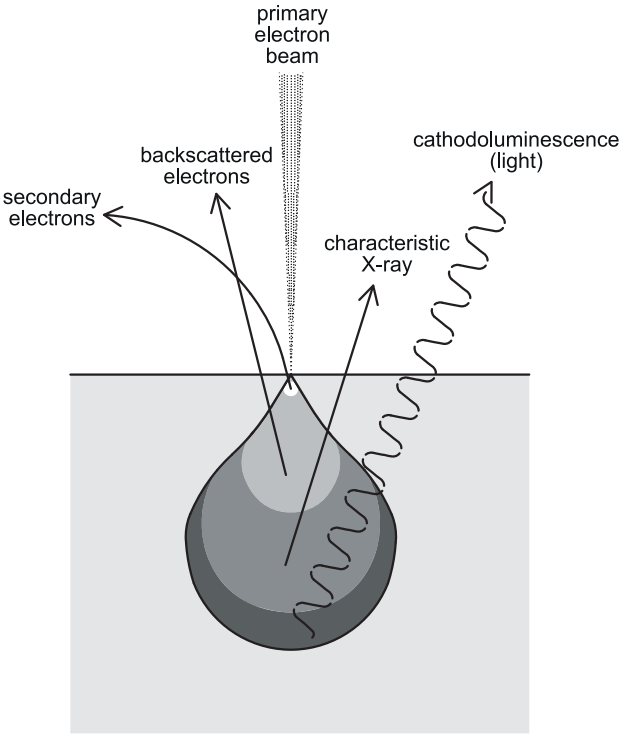


Figure 2.9.52-1. – *Schematic diagram showing the signals generated from a teardrop-shaped interaction volume*

*APPLICATIONS*

SEM is used to support many pharmaceutical processes during the formulation and analytical development, manufacturing and quality control of medicinal products. Its applications include:

- physical and chemical analysis of samples;
- comparison of raw materials from different suppliers;
- support for the development of particle sizing methods;
- particle size analysis (using image analysis);
- characterisation of the surface roughness of tablet film coatings;
- investigation of complaints;
- analysis and identification of particulate matter contamination;
- trouble-shooting of manufacturing problems;
- examination of falsified products.

SEM is used to examine a wide range of materials, such as active pharmaceutical ingredients (APIs), excipients, powder blends, solid dosage forms, medical devices, primary and secondary packaging materials and contaminants. The examination of SEM images permits the qualitative and quantitative assessment of the homogeneity and consistency of powders as raw materials or after processing (e.g. compressed into tablets), with respect to the shapes, sizes and size distributions of particles in powders and of the texture, porosity and shapes of the crystals. This information can be correlated with dissolution behaviour, bioavailability and the crystallinity of the components in solid dosage forms. SEM has particular value in supporting the development and optimisation of manufacturing processes for most solid dosage forms, such as tablets, powder mixtures for oral suspensions, granules, powders for inhalation, spray- or freeze-dried powders, and powders for injections.

*SPECIFICITIES OF THE TECHNIQUE*

SEM offers many distinct advantages over conventional optical light microscopy because specimens can be examined at higher magnifications (250 000 × compared to 1000 ×, for example) with greater lateral resolution (3 nm or better compared to about 200 nm) and superior depths of field. However, the interaction between the electrons and the specimen could induce a strong charging effect due to the accumulation of electrical charge on its surface. Specific preparation procedures may thus be required for non-conducting specimens so that the accumulated charges can be dissipated.

Therefore, the ultimate performance of an electron microscope depends upon a number of factors, with the nature of the specimen having a major influence on the quality of the final image.

EQUIPMENT

Figure 2.9.52-2 is a schematic diagram of an electron microscope. It typically consists of:

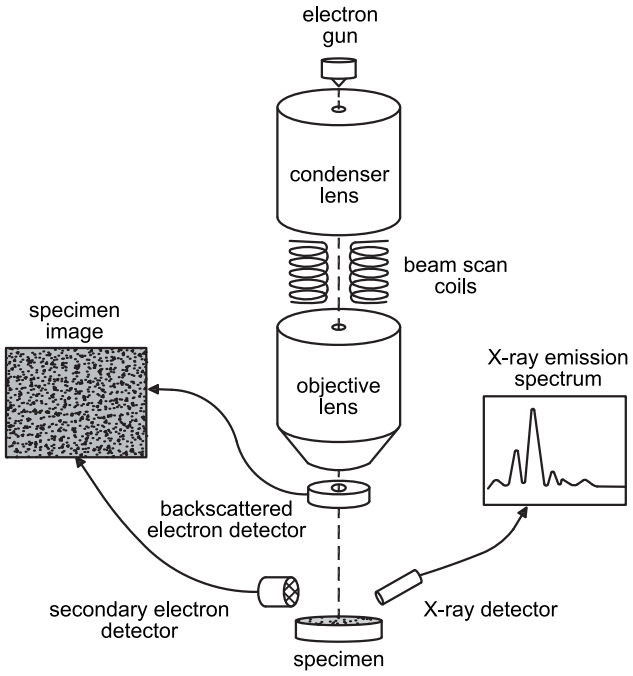


Figure 2.9.52-2. – *Schematic diagram of a scanning electron microscope*

- an electron gun that is housed at the top of an electron-optical column (electron column) and emits a beam of electrons; the electrons are accelerated down the column under the influence of a high voltage that is typically variable from 100 V to 30 kV;

- an electron column, which contains various components used to control and focus the electron beam:
  - a condenser lens system that controls the diameter and energy of the electron beam;
  - scan coils that are used to move the beam in a raster-fashion across a rectangular area on the specimen;
  - an objective lens that focuses the beam to a fine point and directs it towards the specimen;
- a specimen chamber positioned at the bottom of the electron column; it contains the specimen and an array of detectors that collect the emitted signals;
- a system of pumps that maintains the electron gun chamber and electron column under high vacuum to preserve the reliability and stability of the electron source.

*VACUUM IN THE SPECIMEN CHAMBER*

Conventional SEM is performed with the electron column and specimen chamber maintained at a high vacuum so that conductive specimens can be examined. However, recent developments have enabled the examination of non-conductive specimens and hydrated materials either in a humid or wet atmosphere using environmental SEM (ESEM), or in a dry partial vacuum using variable pressure SEM (VPSEM) at pressures ranging from 10 Pa to $10^3$ Pa. A differential vacuum pumping system keeps the gun chamber at a high vacuum relative to the low vacuum (sometimes close to atmospheric pressure) in the specimen chamber. An advantage of using ESEM or VPSEM is that the microscope can also be operated in high vacuum mode.

*ELECTRON EMITTERS*

There are 2 main types of electron sources:

- *thermionic emission*. A widely-used thermionic source consists of a filament, usually a tungsten wire, heated by passing an electrical current directly through it. An alternative is a lanthanum hexaboride ($LaB_6$) emitter, which consists of an indirectly heated single crystal; it has a much greater electron yield and is much brighter than a tungsten emitter.
- *field emission (FE)*. Electrons are emitted from the ultra-sharp tip of a fine-pointed tungsten wire immersed within an intense electrostatic field. Used in high-resolution electron microscopes, FE emitters give an intensely bright, small-diameter and low-current electron beam. They are very stable and have an operating life of many thousands of hours. FE-SEM offers significant advantages, because it produces high-resolution images at low accelerating voltages (down to a few hundreds of volts), with increased signal-to-noise ratios (giving low-noise, high-quality images) and with a superior depth of field.

*DETECTORS*

A cluster of different detectors (see Figure 2.9.52-2) are positioned at optimised distances close to the specimen and collect the variety of signals emitted during interaction with the electron beam.

**Secondary electron detectors**

Most high-vacuum electron microscopes are fitted with an Everhart-Thornley (E-T) detector positioned to one side of the specimen. E-T detectors are sensitive to the low-energy secondary electrons that originate from a shallow depth (typically less than 50 nm) below the surface of a specimen.

The E-T detector has a positively biased wire grid in front of it to attract electrons towards a scintillator which converts the secondary electrons into flashes of light that are directed into a photomultiplier. Secondary electrons can follow curved paths from areas on specimens that may not be in the direct line of sight of the detector. As a consequence, secondary electron images tend to have high contrast and show considerable surface details that emphasise topographical features and variations in surface roughness across specimens.

In addition to secondary electrons, E-T detectors are also sensitive to backscattered electrons emitted from areas on the specimen that happen to be in the direct line of sight of the detector. As a consequence, secondary electron images include contributions from both secondary electrons and backscattered electrons and it is this combination that produces the three-dimensional effect that is a distinctive characteristic of secondary electron images.

An E-T detector cannot be used in ESEM or VPSEM because secondary electrons emitted from a specimen are scattered and absorbed by the gas molecules in the chamber. To observe secondary electron images in ESEM or VPSEM, a detector that is sensitive to the small amount of light that is emitted from the ionised gas molecules surrounding the specimen (a process called gas luminescence) in the specimen chamber is used. It is this cloud of positive ions around the specimen that also neutralises any negative charge accumulation.

**Backscattered electron detectors**

Backscattered electrons have higher energies than secondary electrons and are emitted over a broad angular range. Consequently, the detector is positioned close to and above the specimen. Most high-vacuum and low-vacuum electron microscopes are fitted with a dedicated backscattered electron detector that uses either a scintillator or solid-state diodes.

Scintillator detectors work in the same way as E-T secondary electron detectors by detecting electrons as flashes of light, but have no biased grid to attract electrons towards them. Solid-state backscattered electron detectors consist of an annulus having 4 or 5 separate photodiodes arranged as an array of segments. The diodes can be turned on or off in different combinations to allow topographic imaging, compositional imaging or both together.

The emission of backscattered electrons increases with the average atomic number of the various components (pure or composite) of the specimen. This behaviour can be exploited to examine the spatial distribution and homogeneity of components containing light and heavy atoms in mixtures such as powder blends, compressed tablets and specimens contaminated with foreign matter. Backscattered electron compositional images do not reveal which elements are present in a specimen but they do show where high atomic number materials are relative to low atomic number materials. Materials consisting mainly of heavy atoms (e.g. iron, bromine) appear brighter than those having lighter atoms (e.g. carbon, nitrogen, oxygen, aluminium). To determine which elements are actually present, elemental X-ray microanalysis is used.

Unlike secondary electron detectors, backscattered electron detectors are not greatly affected by electrical charging at the specimen surface. Therefore, non-conducting specimens can be imaged in high-vacuum mode and in low-vacuum mode using a backscattered electron detector.

Both scintillator and solid-state backscattered electron detectors are also very sensitive to visible light and can therefore also function as cathodoluminescence detectors.

**Cathodoluminescence detectors**

The weak-intensity light emitted from cathodoluminescent materials is collected by a retractable paraboloidal mirror and directed into a light-sensitive scintillator detector (not shown in Figure 2.9.52-2). The mirror is inserted just above the specimen and is retracted when not in use. The electron beam passes through a small hole in the mirror so that it strikes the specimen to induce the emission of visible light.

In its simplest form, the detector does not discriminate between different wavelengths of light and greyscale images are produced with cathodoluminescent areas on a specimen being shown as bright. More complex detectors have the capability to select different wavelengths of light being emitted to produce monochromatic images tuned to a single wavelength that represents a specific material. Cathodoluminescence

spectral analysis is achieved by passing polychromatic light emitted from the specimen into a spectrometer where it is dispersed to produce a visible light spectrum.

Spectral analysis of the emitted light is not widely used but has applications in the examination and characterisation of some organic and inorganic materials. It can be used to acquire diagnostic information about the chemistry and structure of single materials and spectral imaging can be used for analysing mixtures of materials.

**Elemental X-ray microanalysis detectors**

An X-ray analyser fitted to the specimen chamber of an electron microscope enables the rapid and non-destructive elemental analysis of materials being examined. 2 main detector types are available and these detect either the dispersed energies (energy-dispersive X-ray microanalysis, EDX) or the dispersed wavelengths (wavelength-dispersive X-ray microanalysis, WDX) of the characteristic X-rays emitted from specimens (for more details, see general chapter *2.2.37*).

PROCEDURE

*SPECIMEN PREPARATION / PRESENTATION*

Prior to preparing a specimen for examination by SEM, the purpose of the examination must be considered because this can affect the way the sample is treated.

In addition, the electrical conductivity of the specimen must be considered to minimise charge accumulation as the electron beam scans across the specimen. Numerous methods have been developed to prepare specimens to maintain integrity whilst minimising artefacts. The preparation of most pharmaceutical samples is simple and quick and does not require specialised equipment or complex processes.

Specimen holders, called stubs, are used to support powders, single particles, tablets, freeze-dried cakes, capsules and beads. Rapid-curing, vacuum compatible glue or electrically conductive silver or carbon paints are ideal for holding large objects that are up to a few millimetres in diameter. Fine powders and small objects can be attached to stubs on double-sided adhesive tape or on sticky tabs. Large samples may have to be reduced in size to fit onto a stub or into the specimen chamber and tablets can be broken open to expose their cores before being attached securely to a stub.

Powders can be simply sprinkled or poured onto a stub that has had a thin layer of adhesive applied to it and excess loose powder is then tapped off or blown off with a gentle stream of inert compressed gas. A rapid and simple method for powders is to attach double-sided adhesive tape onto a stub and dip it gently into the powder so that a thin layer sticks to the tape, and excess powder is then blown off (taking care not to inhale the airborne powder).

When preparing powder samples, cross-contamination must be avoided because the presence in the specimen of just a single particle from another material can lead to an incorrect interpretation of an image or chemical analysis. For this reason, it is not good practice to disperse powder particles onto a specimen stub using a brush, because the brush must be decontaminated or discarded after a single use. For microanalysis, the use of inert/plastic tools (needles, spatulas, tweezers, etc.) is preferred for sample manipulation since contaminant particles could potentially be released from metal tools.

*SPECIMEN COATING*

Most pharmaceutical materials are readily examined without the need for prolonged and complicated preparation techniques. However, such materials tend to be electrically non-conductive and so specimens that are examined using high-vacuum SEM need to be coated with an ultra-thin layer of conductive material. Without a conductive coating, specimens will acquire a net negative charge as the electron beam scans across them; this causes the specimen to glow brightly and, when it discharges, disturbing flashes and bright streaks will be seen in the image. Suitable conductive materials for coating include metals, such as platinum and gold, and these can be applied to rough and smooth specimen surfaces using a plasma sputter coater. If carbon is to be used, it needs to be evaporated in a very high vacuum because it cannot be sputtered. In addition to reducing charging effects, a conductive coating will increase the emission of secondary electrons to give brighter images and will also transfer localised electron beam-induced heat away from the specimen. Some powders consisting of very small particles (such as colloidal silica) may need to be sputter coated 3 or 4 times to prevent charging because the coating material initially fails to form a continuous conductive layer.

Some beam-sensitive specimens, such as semi-solids or wet materials, may have to be cooled or frozen using a cooling stage to minimise degradation or evaporation. With respect to this, FE-SEM allows the surfaces of labile specimens to be examined with an electron beam that has a very low energy (e.g. 500 volts or less), and this can help to minimise or even eliminate beam-induced degradation.

Specimens to be analysed by elemental X-ray microanalysis are typically not coated with metal because the coat would add extra peaks to the X-ray spectrum and this could interfere with the analysis or even obscure minor amounts of some elements, thereby precluding the possibility of quantitative determinations of unknowns. Most elemental analysers are capable of detecting light elements, so even a thin carbon coat could hinder the interpretation of the spectrum. To minimise or exclude potential problems and artefacts associated with specimen coating, non-conductive materials can be examined uncoated using ESEM or VPSEM. Software correction for any coating may need to be applied.

*OPERATION*

The electron microscope is operated with a beam-accelerating voltage that is appropriate to produce images that reveal the information of interest about a specimen. For example, a low voltage (e.g. less than 5 kV) can be used to image surface details and a higher voltage (e.g. greater than 10 kV) increases the resolving power and can be used for the analysis of a wide range of elements. The most useful accelerating voltage range is about 2 kV to 20 kV because most elements of interest can be ionized by electrons with energies in this range.

Adjustment of the objective lens focuses the beam on the specimen over a wide range of working distances to accommodate large and small specimens. The depth of field can also be increased by selecting smaller beam apertures to enable the full depth of thick specimens to be in acceptable focus simultaneously.

When SEM is used to examine specimens in a high vacuum, very high magnifications in excess of 10 000 × can be achieved to resolve fine detail down to about 3 nm, depending upon the specimen. This is possible because the electron beam can be focused without being scattered by gas molecules. For ESEM or VPSEM, a consequence of gas or water vapour being present in the specimen chamber is that the electron beam suffers some lateral scattering (called skirting). This scattering can inhibit high-resolution imaging because the beam cannot be focused as finely as it would be in a high vacuum. By selecting a shorter working distance and optimising the gas pressure, this adverse effect can be minimised.

Lateral spatial resolutions of 1 nm or better become possible (depending upon the specimen being examined) when using FE-SEM. To achieve this high resolving power, a beam-accelerating voltage in excess of about 15 kV may be required, but a high beam voltage could damage beam-sensitive materials. For most organic solids, the electron beam will penetrate many micrometres into the specimen and surface detail will be lost due to the scattering and absorption of the emitted electrons. In order to reveal the surface details of specimens, the electron microscope must be operated at a much lower voltage, and this is at the expense of resolution.

Most pharmaceutical specimens studied by SEM are likely to be examined at a magnification less than 10 000 × and so a high resolving power is typically not needed.

*COMBINATION OF SEM AND X-RAY MICROANALYSIS*

This combination enables the identification of the elemental compositions of specimens and elemental mapping to visualise the distributions of elements, for example in tablets and blends.

Element maps are easily and quickly generated to visualise the spatial distribution of the elements present in a specimen at low and high magnifications. Cross-sections cut or fractured through tablet cores and powders that have been lightly pressed flat can be mapped to reveal the relationships between their components. This technique is useful to compare the differences between samples to indicate why some perform better than others. It is important to have surfaces that are not rough because emitted X-rays follow straight paths and so may not be detected in areas (effectively in shadows) that are not in direct line of sight of the X-ray detector. This artefact can be reduced by tilting the specimen towards the detector.

X-ray microanalysis is also used for the investigation of particulate matter. Black spots in tablets, for example, are often caused by particles of metal or rubber debris, caused by the wearing of tablet presses, that have subsequently been incorporated into the tablet as a visible defect. SEM-EDX is an ideal technique to identify them because it is non-destructive and can often be used to distinguish between different metals so that the source and root cause can be identified. Also, particles in vials and syringes of solutions for injection can be examined and analysed after they have been isolated by filtration or removed using a micropipette.

EQUIPMENT PERFORMANCE

SEM user requirements, in addition to manufacturer's recommendations, provide a basis for the system performance evaluation. For size measurements, accuracy of the displayed scale bar represents a critical parameter that should be checked at both low and high magnifications using certified calibration test specimens (available from SEM accessory suppliers). As accuracy of scale can be influenced by instrumental parameters, such as accelerating voltage and working distance, or by the specimen itself, it should be checked under different operating conditions to allow for instrument variations using, for example, a bracketing concept. Accuracy of scale should be checked in orthogonal directions and the typical accuracy of the displayed scale should be 10 per cent or better. If justified, other acceptance criteria, such as image resolution, might be applied.

For elemental analysis by EDX, the accuracy and reproducibility of the energy scale over a specific energy range (e.g. 0-5 keV or 0-20 keV) represents a parameter that should be checked and calibrated according to the manufacturer's instrument operating instructions using suitable reference materials (see general chapter *2.2.37*). These single element materials are used because they have multiple X-ray emission peaks at well-defined energies and intensities across the spectral range being calibrated. The intensities of the emitted X-rays can be influenced by other instrumental parameters, such as accelerating voltage or the working distance, and should be checked for relevant instrumental parameters using, for example, a bracketing concept. If justified, other acceptance criteria (such as detector resolution) might be used.

# 3. Materials for containers and containers

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**01/2013:30100**

## 3.1. MATERIALS USED FOR THE MANUFACTURE OF CONTAINERS

The materials described in this chapter are used for the manufacture of containers for pharmaceutical use. Their use may also be considered for the manufacture of part or all of objects used for medico-surgical purposes.

Materials and polymers other than those described in the Pharmacopoeia may be used subject to approval in each case by the competent authority responsible for the licensing for sale of the preparation in the container.

**Transmissible spongiform encephalopathies** (*5.2.8*). A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out, and suitable measures are taken to minimise any such risk.

**04/2018:30103**

## 3.1.3. POLYOLEFINS

DEFINITION

Polyolefins are obtained by polymerisation of ethylene or propylene or by copolymerisation of these substances with not more than 25 per cent of higher homologues ($C_4$ to $C_{10}$) or of carboxylic acids or of esters. Certain materials may be mixtures of polyolefins.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polyolefins may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*S*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.3 per cent;
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;
- dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
- 4,4′,4″-(2,4,6-trimethylbenzene-1,3,5-triyltrismethylene)tris[2,6-bis(1,1-dimethylethyl)phenol] (plastic additive 10): maximum 0.3 per cent;
- 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14): maximum 0.3 per cent;
- didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
- dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
- tris[2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12): maximum 0.3 per cent;
- plastic additive 18: maximum 0.1 per cent;
- copolymer of dimethyl succinate and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)ethanol (plastic additive 22): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite: maximum 0.5 per cent;
- alkanamides: maximum 0.5 per cent;
- alkenamides: maximum 0.5 per cent;
- sodium silico-aluminate: maximum 0.5 per cent;
- silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
- sodium benzoate: maximum 0.5 per cent;
- fatty acid esters or salts: maximum 0.5 per cent;
- trisodium phosphate: maximum 0.5 per cent;
- liquid paraffin: maximum 0.5 per cent;
- zinc oxide: maximum 0.5 per cent;
- talc: maximum 0.5 per cent;
- magnesium oxide: maximum 0.2 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- titanium dioxide: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

*Appearance*: powder, beads, granules or, after transformation, sheets of varying thickness or containers.

*Solubility*: practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

They soften at temperatures between 65 °C and 165 °C. They burn with a blue flame.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

**Solution S2**. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser, for 90 min with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

**Solution S3**. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h with constant stirring. Allow to cool and decant the solution.

**Appearance of solution**. Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*): maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

**Reducing substances**. To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 3.0 mL.

**Extractable aluminium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

**Extractable titanium** *(not for materials opacified with titanium dioxide)*: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

**Extractable zinc**: maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

**Extractable heavy metals** (*2.4.8*): maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS

*These tests are to be carried out, in whole or in part, only if required by the stated composition or the use of the material.*

**Phenolic antioxidants**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *tetrahydrofuran R* (50:50 *V/V*).

*Test solution S21.* Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

*Test solution S22.* Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of a mixture of equal volumes of *acetonitrile R* and a 10 g/L solution of *tert-butylhydroperoxide R* in *tetrahydrofuran R*. Close the flask and allow to stand for 1 h. Prepare a blank solution using the blank of solution S2.

*Of the following reference solutions, prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.*

*Reference solution (a).* Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (f).* Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (g).* Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (h).* Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (i).* Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (j). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (k).* Dissolve 20.0 mg of *plastic additive 18 CRS* in 10.0 mL of a mixture of equal volumes of *acetonitrile R* and a 10 g/L solution of *tert-butylhydroperoxide R* in *tetrahydrofuran R*. Allow to stand in a closed container for 1 h. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R* (30:70 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either the reference solution (d) or (e) or reference solutions (d) and (e).

*Run time*: 30 min.

*System suitability*:
- *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

*Limits*:
- *plastic additive 07*: maximum 0.125 per cent;
- *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:
- plastic additive 09;
- plastic additive 10;
- plastic additive 11;
- plastic additive 12;
- plastic additive 13;

proceed as described above with the following modifications.

*Mobile phase*: *water for chromatography R*, *tetrahydrofuran R*, *acetonitrile R* (10:30:60 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

*System suitability*:
- *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

*Limits*:
- *plastic additive 09*: maximum 0.3 per cent;
- *plastic additive 10*: maximum 0.3 per cent;
- *plastic additive 11*: maximum 0.3 per cent;
- *plastic additive 12*: maximum 0.3 per cent;
- *plastic additive 13*: maximum 0.3 per cent.

C. If the substance to be examined contains plastic additive 18, proceed as described for plastic additive 07 and/or plastic additive 08 with the following modifications.

*Mobile phase*: *tetrahydrofuran R*, *acetonitrile R* (20:80 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL of test solution S22, the corresponding blank solution and reference solution (k).

*Identification of peaks*: use the chromatogram supplied with *plastic additive 18 CRS* and the chromatogram obtained with reference solution (k) to identify the peaks due to plastic additive 18.

*Retention time* of the 2 principal peaks due to plastic additive 18: about 3.3 min and about 6.6 min.

The chromatogram obtained with test solution S22 shows 2 principal peaks due to plastic additive 18. The sum of the areas of these peaks is not less than 50 per cent of the sum of the areas of all the peaks due to plastic additive 18.

*System suitability*:
- *resolution*: minimum 6.0 between the 2 principal peaks in the chromatogram obtained with reference solution (k).

The chromatogram obtained with test solution S22 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage content*:
- sum up the areas of all the peaks due to plastic additive 18 eluting between 2.0 min and 9.5 min; use the concentration of *plastic additive 18 CRS* in reference solution (k).

*Limit*:
- *sum of the areas of the peaks due to plastic additive 18*: maximum 0.1 per cent; disregard any peak with an area less than 0.3 per cent of the total area.

**Non-phenolic antioxidants**. Thin-layer chromatography (*2.2.27*).

*Test solution S23.* Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

*Reference solution (l).* Dissolve 60 mg of *plastic additive 14 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (m).* Dissolve 60 mg of *plastic additive 15 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (n).* Dissolve 60 mg of *plastic additive 16 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (o).* Dissolve 60 mg of *plastic additive 17 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (p).* Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase A*: *hexane R*.

*Mobile phase B*: *methylene chloride R*.

*Application*: 20 µL of test solution S23, reference solution (p) and the reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

*Development A*: over a path of 18 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 17 cm with mobile phase B.

*Drying B*: in air.

*Detection*: examine in ultraviolet light at 254 nm; spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

*System suitability*: reference solution (p):

– the chromatogram shows 2 clearly separated spots.

*Limit*: any spots in the chromatogram obtained with test solution S23 are not more intense than the spots in the corresponding positions in the chromatograms obtained with the reference solutions.

**Plastic additive 22**. Liquid chromatography (*2.2.29*).

*Test solution*. Evaporate 25 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 mL of *toluene R* and 10 mL of a 10 g/L solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *anhydrous ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

*Reference solution*. Dissolve 30 mg of *plastic additive 22 CRS* in 50 mL of *toluene R*. Add 1 mL of this solution to 25 mL of blank solution S2 and evaporate to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 mL of *toluene R* and 10 mL of a 10 g/L solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *anhydrous ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *anhydrous ethanol R*, *hexane R* (11:89 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 227 nm.

*Injection*: 20 µL.

*Run time*: 10 min.

*System suitability*:

– *resolution*: minimum 7 between the peaks due to the 'diol' component and to the diluent of the reference solution.

*Limit*: the area of the peak due to the 'diol' component from plastic additive 22 in the chromatogram obtained with the test solution is less than the corresponding peak in the chromatogram obtained with the reference solution.

**Amides and stearates**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Use test solution S23 described in the test for non-phenolic antioxidants.

*Reference solution (q)*. Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in 10 mL of *methylene chloride R*.

*Reference solution (r)*. Dissolve 40 mg of oleamide (*plastic additive 20 CRS*) in 20 mL of *methylene chloride R*.

*Reference solution (s)*. Dissolve 40 mg of erucamide (*plastic additive 21 CRS*) in 20 mL of *methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (25:75 *V/V*).

*Application*: 10 µL of test solution S23 and reference solution (q).

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

*Limit*: any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S23 is similar in position ($R_F$ = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (q).

B. *Mobile phase A*: *hexane R*.

*Mobile phase B*: *methanol R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL of the test solution S23 and the reference solutions (r) and (s).

*Development A*: over a path of 13 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 10 cm with mobile phase B.

*Drying B*: in air.

*Detection*: spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R*. Heat in an oven at 120 °C until spots appear.

*Limits*: any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S23 are similar in position ($R_F$ = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (r) and (s).

**07/2017:30104**

# 3.1.4. POLYETHYLENE WITHOUT ADDITIVES FOR CONTAINERS FOR PARENTERAL PREPARATIONS AND FOR OPHTHALMIC PREPARATIONS

DEFINITION

Polyethylene without additives is obtained by polymerisation of ethylene under high pressure in the presence of oxygen or free-radical-forming initiators as catalyst.

CHARACTERS

*Appearance*: beads, granules, powder or, after transformation, translucent sheets of varying thickness or containers.

*Solubility*: practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures beginning at 65 °C.

*Relative density*: 0.910 to 0.937.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

*Absorption maxima at some of the following wavenumbers (tolerance: ± 5 $cm^{-1}$)*: at 2915 $cm^{-1}$, 2848 $cm^{-1}$, 1471 $cm^{-1}$, 1465 $cm^{-1}$, 729 $cm^{-1}$ and 719 $cm^{-1}$.

The spectrum obtained is identical to that obtained with the material selected for the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample').

B. Additives (see Tests).

TESTS

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and heat under a reflux condenser for 5 h. Allow to cool and decant. Keep part of the solution for the test for appearance of solution. Filter the rest through a sintered glass filter (16) (*2.1.2*). Use within 4 h of preparation.

**Solution S2**. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser with constant stirring for 90 min. Allow to cool to 60 °C and add, with contant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

**Solution S3**. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

**Appearance of solution**. Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*): maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

**Reducing substances**. To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add l g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

**Additives**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5 mL of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

*Reference solution*. Dissolve 20 mg of *plastic additive 15 CRS* and 20 mg of *plastic additive 08 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase A*: *hexane R*.

*Mobile phase B*: *methanol R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL.

*Development A*: over a path of 13 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 10 cm with mobile phase B.

*Drying B*: in air.

*Detection*: spray with a 40 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 120 °C until the spots appear in the chromatogram obtained with the reference solution.

*System suitability*: reference solution:

– the chromatogram shows 2 separated spots.

*Limit*: no spot appears in the chromatogram obtained with the test solution, except for a spot which may be at the solvent front from the first development and which corresponds to oligomers. Disregard any spots corresponding to those obtained in the chromatogram with the blank solution.

**Extractable heavy metals** (*2.4.8*): maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20 mL with *water R*. 12 mL of solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

**Sulfated ash** (*2.4.14*): maximum 0.02 per cent, determined on 5.0 g.

**04/2018:30105**

## 3.1.5. POLYETHYLENE WITH ADDITIVES FOR CONTAINERS FOR PARENTERAL PREPARATIONS AND FOR OPHTHALMIC PREPARATIONS

DEFINITION

Polyethylene with additives is obtained by polymerisation of ethylene under pressure in the presence of a catalyst or by copolymerisation of ethylene with not more than 25 per cent of higher alkene homologues ($C_3$ to $C_{10}$).

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polyethylene may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

– butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
– pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
– 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*S*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
– octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.3 per cent;
– ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;
– dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
– 4,4′,4″-(2,4,6-trimethylbenzene-1,3,5-triyltrismethylene)tris[2,6-bis(1,1-dimethylethyl)phenol] (plastic additive 10): maximum 0.3 per cent;
– 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14): maximum 0.3 per cent;
– didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
– dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
– tris [2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite: maximum 0.5 per cent;
- alkanamides: maximum 0.5 per cent;
- alkenamides: maximum 0.5 per cent;
- sodium silico-aluminate: maximum 0.5 per cent;
- silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
- sodium benzoate: maximum 0.5 per cent;
- fatty acid esters or salts: maximum 0.5 per cent;
- trisodium phosphate: maximum 0.5 per cent;
- liquid paraffin: maximum 0.5 per cent;
- zinc oxide: maximum 0.5 per cent;
- magnesium oxide: maximum 0.2 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- titanium dioxide only for materials for containers for ophthalmic use: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

*Appearance*: powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers.

*Solubility*: practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures between 70 °C and 140 °C.

Relative density: 0.890 to 0.965.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

*Absorption maxima at some of the following wavenumbers* (*tolerance:* ± *5 cm*$^{-1}$): at 2915 cm$^{-1}$, 2848 cm$^{-1}$, 1471 cm$^{-1}$, 1465 cm$^{-1}$, 729 cm$^{-1}$ and 719 cm$^{-1}$.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

**Solution S2**. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser for 90 min with constant stirring. Allow to cool to 60 °C and add with continued stirring 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

**Solution S3**. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h with constant stirring. Allow to cool and decant the solution.

**Appearance of solution**. Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*): maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

**Reducing substances**. To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

**Extractable aluminium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

**Extractable chromium**: maximum 0.05 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

*Wavelength*: use the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

**Extractable titanium** *(not for materials opacified with titanium dioxide)*: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

**Extractable vanadium**: maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *vanadium standard solution (1 g/L V) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

*Wavelength*: use the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

**Extractable zinc**: maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

**Extractable zirconium**: maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Use solution S3.

*Reference solutions.* Prepare the reference solutions using *zirconium standard solution (1 g/L Zr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

*Wavelength*: use the emission of zirconium at 343.82 nm, the spectral background being taken as 343.92 nm.

Verify the absence of zirconium in the hydrochloric acid used.

**Extractable heavy metals** (*2.4.8*): maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material opacified with titanium dioxide.

SUPPLEMENTARY TESTS

*These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.*

**Phenolic antioxidants**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *tetrahydrofuran R* (50:50 *V/V*).

*Test solution S21.* Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

*Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.*

*Reference solution (a).* Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (f).* Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (g).* Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (h).* Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (i).* Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (j). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R* (30:70 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either reference solution (d) or (e), or reference solutions (d) and (e).

*Run time*: 30 min.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

*Limits*:

- *plastic additive 07*: maximum 0.125 per cent;
- *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:

- plastic additive 09;
- plastic additive 10;
- plastic additive 11;
- plastic additive 12;
- plastic additive 13;

proceed as described above with the following modifications.

*Mobile phase*: *water for chromatography R*, *tetrahydrofuran R*, *acetonitrile R* (10:30:60 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

*Limits*:

– *plastic additive 09*: maximum 0.3 per cent;

– *plastic additive 10*: maximum 0.3 per cent;

– *plastic additive 11*: maximum 0.3 per cent;

– *plastic additive 12*: maximum 0.3 per cent;

– *plastic additive 13*: maximum 0.3 per cent.

**Non-phenolic antioxidants**. Thin-layer chromatography (*2.2.27*).

*Test solution S22*. Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

*Reference solution (k)*. Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (l)*. Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (m)*. Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (n)*. Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (o)*. Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase A*: *hexane R*.

*Mobile phase B*: *methylene chloride R*.

*Application*: 20 µL of test solution S22, reference solution (o) and reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

*Development A*: over a path of 18 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 17 cm with mobile phase B.

*Drying B*: in air.

*Detection*: examine in ultraviolet light at 254 nm, spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

*System suitability*: reference solution (o):

– the chromatogram shows 2 clearly separated spots.

*Limits*: any spots in the chromatogram obtained with test solution S22 are not more intense than the spots in the same positions in the chromatograms obtained with the reference solutions.

**Amides and stearates**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Use test solution S22 described in the test for non-phenolic antioxidants.

*Reference solution (p)*. Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (q)*. Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (r)*. Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plates*: *TLC silica gel $F_{254}$ plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (25:75 *V/V*).

*Application*: 10 µL of test solution S22 and reference solution (p).

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

*Limit*: any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S22 is similar in position ($R_F$ = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (p).

B. *Mobile phase A*: *hexane R*.

*Mobile phase B*: *methanol R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL of test solution S22 and reference solutions (q) and (r).

*Development A*: over a path of 13 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 10 cm with mobile phase B.

*Drying B*: in air.

*Detection*: spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R* and heat in an oven at 120 °C until spots appear.

*Limits*: any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with the test solution S22 are similar in position ($R_F$ = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

**04/2018:30106**

# 3.1.6. POLYPROPYLENE FOR CONTAINERS AND CLOSURES FOR PARENTERAL PREPARATIONS AND OPHTHALMIC PREPARATIONS

DEFINITION

Polypropylene consists of the homopolymer of propylene or of a copolymer of propylene with not more than 25 per cent of ethylene or of a mixture (alloy) of polypropylene with not more than 25 per cent of polyethylene. It may contain additives.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polypropylene may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, (plastic additive 11): maximum 0.3 per cent;
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;
- dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
- 2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10): maximum 0.3 per cent;
- 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxa-phosphinane] (plastic additive 14): maximum 0.3 per cent;
- didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
- dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
- tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite: maximum 0.5 per cent;
- alkanamides: maximum 0.5 per cent;
- alkenamides: maximum 0.5 per cent;
- sodium silico-aluminate: maximum 0.5 per cent;
- silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
- sodium benzoate: maximum 0.5 per cent;
- fatty acid esters or salts: maximum 0.5 per cent;
- trisodium phosphate: maximum 0.5 per cent;
- liquid paraffin: maximum 0.5 per cent;
- zinc oxide: maximum 0.5 per cent;
- talc: maximum 0.5 per cent;
- magnesium oxide: maximum 0.2 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- titanium dioxide, only for materials for containers for ophthalmic use: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

*Appearance*: powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers.

*Solubility*: practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures beginning at about 120 °C.

IDENTIFICATION

*If necessary, cut the samples of material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

*Absorption maxima at some of the following wavenumbers (tolerance: ± 5 $cm^{-1}$)*: at 1375 $cm^{-1}$, 1170 $cm^{-1}$, 995 $cm^{-1}$ and 973 $cm^{-1}$.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

*If necessary, cut the samples of material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

**Solution S2**. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser for 90 min, with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

**Solution S3**. Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

**Appearance of solution**. Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*): maximum 0.2, determined between wavelengths of 220 nm to 340 nm on solution S1.

**Reducing substances**. To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

**Extractable aluminium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

**Extractable chromium**: maximum 0.05 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

*Wavelength*: use the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

**Extractable titanium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

**Extractable vanadium**: maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *vanadium standard solution (1 g/L V) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

*Wavelength*: use the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

**Extractable zinc**: maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Use solution S3.

*Reference solutions*. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

**Extractable heavy metals** (*2.4.8*): maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS

*These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.*

**Phenolic antioxidants**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *tetrahydrofuran R* (50:50 *V/V*).

*Test solution S21*. Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

*Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.*

*Reference solution (a)*. Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (e)*. Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (f)*. Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (g)*. Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (h)*. Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (i)*. Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (j). Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R* (30:70 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either reference solution (d) or (e), or reference solutions (d) and (e).

*Run time*: 30 min.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

*Limits*:

– *plastic additive 07*: maximum 0.125 per cent;

– *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:

– plastic additive 09;

– plastic additive 10;

– plastic additive 11;

– plastic additive 12;

– plastic additive 13;

proceed as described above with the following modifications.

*Mobile phase*: *water for chromatography R*, *tetrahydrofuran R*, *acetonitrile R* (10:30:60 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Injection*: 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

*Calculation of percentage contents*:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

*Limits*:

– *plastic additive 09*: maximum 0.3 per cent;

– *plastic additive 10*: maximum 0.3 per cent;

– *plastic additive 11*: maximum 0.3 per cent;

– *plastic additive 12*: maximum 0.3 per cent;

– *plastic additive 13*: maximum 0.3 per cent.

**Non-phenolic antioxidants**. Thin-layer chromatography (*2.2.27*).

*Test solution S22*. Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

*Reference solution (k)*. Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (l)*. Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (m)*. Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (n)*. Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

*Reference solution (o)*. Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase A*: *hexane R*.

*Mobile phase B*: *methylene chloride R*.

*Application*: 20 µL of test solution S22, reference solution (o) and reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

*Development A*: over a path of 18 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 17 cm with mobile phase B.

*Drying B*: in air.

*Detection*: examine in ultraviolet light at 254 nm; spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

*System suitability*: reference solution (o):

– the chromatogram shows 2 clearly separated spots.

*Limits*: any spots in the chromatogram obtained with test solution S22 are not more intense than the spots in the same positions in the chromatograms obtained with the reference solutions.

**Amides and stearates**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Use test solution S22 described in the test for non-phenolic antioxidants.

*Reference solution (p)*. Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (q)*. Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (r)*. Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (25:75 *V/V*).

*Application*: 10 µL of solution S22 and reference solution (p).

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

*Limit*: any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S22 is similar in position ($R_F$ = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (p).

B. *Mobile phase A*: *hexane R*.

*Mobile phase B*: *methanol R, methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL of solution S22 and reference solutions (q) and (r).

*Development A*: over a path of 13 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 10 cm with mobile phase B.

*Drying B*: in air.

*Detection*: spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R*; heat in an oven at 120 °C until spots appear.

*Limits*: any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S22 are similar in position ($R_F$ = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

**07/2017:30107**

# 3.1.7. POLY(ETHYLENE - VINYL ACETATE) FOR CONTAINERS AND TUBING FOR TOTAL PARENTERAL NUTRITION PREPARATIONS

DEFINITION

Poly(ethylene - vinyl acetate), complying with the following requirements, is suitable for the manufacture of containers and tubing for total parenteral nutrition preparations. It is obtained by copolymerisation of mixtures of ethylene and vinyl acetate.

*Content of vinyl acetate*:

- *material used for containers*: a defined quantity of not more than 25 per cent;
- *material used for tubing*: a defined quantity of not more than 30 per cent.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Poly(ethylene - vinyl acetate) may contain not more than 3 of the following antioxidants:

- butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.2 per cent;
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.2 per cent;
- tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12): maximum 0.2 per cent;
- 2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10): maximum 0.2 per cent.

It may also contain:

- oleamide (plastic additive 20): maximum 0.5 per cent;
- erucamide (plastic additive 21): maximum 0.5 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- calcium carbonate or potassium hydroxide: maximum 0.5 per cent;
- colloidal silica: maximum 0.2 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

*Appearance*: beads, granules or, after transformation, translucent sheets or tubing of varying thickness or samples of finished objects.

*Solubility*: practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in methanol and in hexane, which dissolves, however, low molecular mass polymers.

It burns with a blue flame.

The temperature at which the substance softens changes with the vinyl acetate content: from about 100 °C for contents of a few per cent to about 70 °C for contents of 30 per cent.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

*Absorption maxima (tolerance: ± 5 $cm^{-1}$)*: at 2920 - 2850 $cm^{-1}$, 1740 $cm^{-1}$, 1240 $cm^{-1}$, 1020 $cm^{-1}$, 720 $cm^{-1}$ and 610 $cm^{-1}$.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

TESTS

*If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser for 90 min, with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents.

**Solution S2**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

**Appearance of solution**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*): maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S2.

**Reducing substances**. To 20 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

**Amides and stearates**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Evaporate 100 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

*Reference solution (a)*. Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in 10 mL of *methylene chloride R*.

*Reference solution (b)*. Dissolve 40 mg of *plastic additive 20 CRS* in 20 mL of *methylene chloride R*.

*Reference solution (c)*. Dissolve 40 mg of *plastic additive 21 CRS* in 20 mL of *methylene chloride R*.

*Plates*: *TLC silica gel $F_{254}$ plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (25:75 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

*Limit*: any spot corresponding to plastic additive 19 in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a).

B. *Mobile phase A*: *hexane R*.

*Mobile phase B*: *methanol R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL.

*Development A*: over a path of 13 cm with mobile phase A.

*Drying A*: in air.

*Development B*: over a path of 10 cm with mobile phase B.

*Drying B*: in air.

*Detection*: spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R* and heat at 120 °C until spots appear.

*Limit*: any spots corresponding to plastic additive 21 or plastic additive 20 in the chromatogram obtained with the test solution are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively.

**Phenolic antioxidants**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *tetrahydrofuran R* (50:50 *V/V*).

*Test solution (a)*. Evaporate 50 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture.

*Test solution (b)*. Evaporate 50 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of *methylene chloride R*.

*Reference solution (a)*. Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07), 40.0 mg of *plastic additive 10 CRS*, 40.0 mg of *plastic additive 09 CRS* and 40.0 mg of *plastic additive 11 CRS* in 10 mL of the solvent mixture. Dilute 2 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 40.0 mg of *plastic additive 11 CRS* and 40.0 mg of *plastic additive 12 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of the solution to 50.0 mL with *methylene chloride R*.

*Column*:

- *size*: *l* = 0.25 m; Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *tetrahydrofuran R*, *acetonitrile R* (10:30:60 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10;
- *number of theoretical plates*: minimum 2500, calculated for the peak due to plastic additive 07.

*Limits*:

- the chromatogram obtained with test solution (a) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (a) with a retention time greater than 2 min;
- the areas of the peaks in the chromatogram obtained with test solution (a) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (a), except for the last peak eluted in the chromatogram obtained with reference solution (a).

If the chromatogram obtained with test solution (a) shows a peak with the same retention time as the last antioxidant eluted from reference solution (a), carry out the test as described with the following modifications.

*Mobile phase*: *water R*, *2-propanol R*, *methanol R* (5:45:50 *V/V/V*).

*Injection*: 20 µL of test solution (b) and reference solution (b).

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12.

*Limits*:

- the chromatogram obtained with test solution (b) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (b) with a retention time greater than 3 min;
- the areas of the peaks in the chromatogram obtained with test solution (b) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (b).

**Sulfated ash** (*2.4.14*): maximum 1.2 per cent, determined on 5.0 g.

ASSAY

Introduce 0.250 g to 1.000 g of the substance to be examined, according to the vinyl acetate content of the copolymer to be examined, into a 300 mL conical flask with a ground-glass neck containing a magnetic stirrer. Add 40 mL of *xylene R*. Boil under a reflux condenser with stirring for 4 h. Stirring continuously, allow to cool until precipitation begins before slowly adding 25.0 mL of *alcoholic potassium hydroxide solution R1*. Boil again under a reflux condenser with stirring for 3 h. Allow to cool with continued stirring, rinse the condenser with 50 mL of *water R* and add 30.0 mL of *0.05 M sulfuric acid* to the flask. Transfer the contents of the flask into

a 400 mL beaker; rinse the flask with 2 quantities, each of 50 mL, of a 200 g/L solution of *anhydrous sodium sulfate R* and 3 quantities, each of 20 mL, of *water R* and add all the rinsings to the beaker containing the initial solution. Titrate the excess sulfuric acid with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.05 M sulfuric acid* is equivalent to 8.609 mg of vinyl acetate.

**01/2008:30108**



## 3.1.8. SILICONE OIL USED AS A LUBRICANT

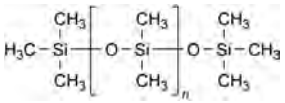

DEFINITION

Silicone oil used as a lubricant is a poly(dimethylsiloxane) obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. Different grades exist which are characterised by a number indicating the nominal viscosity placed after the name.

Silicone oils used as lubricants have a degree of polymerisation ($n$ = 400 to 1200) such that their kinematic viscosities are nominally between 1000 $mm^2 \cdot s^{-1}$ and 30 000 $mm^2 \cdot s^{-1}$.

CHARACTERS

*Appearance*: clear, colourless liquid of various viscosities.

*Solubility*: practically insoluble in water and in methanol, very slightly soluble in anhydrous ethanol, miscible with ethyl acetate, with methyl ethyl ketone and with toluene.

IDENTIFICATION

A. Kinematic viscosity at 25 °C (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *silicone oil CRS*.

The region of the spectrum from 850-750 $cm^{-1}$ is not taken into account since it may show slight differences depending on the degree of polymerisation.

C. Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

D. In a platinum crucible, prepare the sulfated ash (*2.4.14*) using 50 mg. The white powder obtained gives the reaction of silicates (*2.3.1*).

TESTS

**Acidity**. To 2.0 g add 25 mL of a mixture of equal volumes of *anhydrous ethanol R* and *ether R*, previously neutralised to 0.2 mL of *bromothymol blue solution R1*, and shake. Not more than 0.15 mL of *0.01 M sodium hydroxide* is required to change the colour of the solution to blue.

**Viscosity** (*2.2.10*). Determine the dynamic viscosity at 25 °C. Calculate the kinematic viscosity taking the relative density to be 0.97. The kinematic viscosity is within the range 95 per cent to 105 per cent of the nominal viscosity stated on the label.

**Mineral oils**. Place 2 mL in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

**Phenylated compounds**. The refractive index (*2.2.6*) is not greater than 1.410.

**Heavy metals**: maximum 5 ppm.

*Solvent mixture*: *dilute ammonia R2*, 2 g/L solution of *hydroxylamine hydrochloride R* (1:9 *V/V*).

Mix 1.0 g with *methylene chloride R* and dilute to 20 mL with the same solvent. Add 1.0 mL of a freshly prepared 0.02 g/L solution of *dithizone R* in *methylene chloride R*, 0.5 mL of *water R* and 0.5 mL of the solvent mixture. At the same time, prepare the reference solution as follows: to 20 mL of *methylene chloride R* add 1.0 mL of a freshly prepared 0.02 g/L solution of *dithizone R* in *methylene chloride R*, 0.5 mL of *lead standard solution (10 ppm Pb) R* and 0.5 mL of the solvent mixture. Immediately shake each solution vigorously for 1 min. Any red colour in the test solution is not more intense than that in the reference solution.

**Volatile matter**: maximum 2.0 per cent, determined on 2.00 g by heating in an oven at 150 °C for 24 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

LABELLING

The label states:

- the nominal viscosity by a number placed after the name of the product;
- that the contents are to be used as a lubricant.

**01/2008:30109**

## 3.1.9. SILICONE ELASTOMER FOR CLOSURES AND TUBING

DEFINITION

Silicone elastomer complying with the following requirements is suitable for the manufacture of closures and tubing.

Silicone elastomer is obtained by cross-linking a linear polysiloxane constructed mainly of dimethylsiloxy units with small quantities of methylvinylsiloxy groups; the chain ends are blocked by trimethylsiloxy or dimethylvinylsiloxy groups.

The general formula of the polysiloxane is:

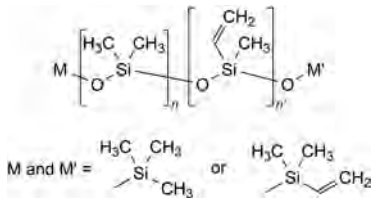

The cross-linking is carried out in the hot state:

- either with:
  - 2,4-dichlorobenzoyl peroxide for extruded products; or
  - 2,4-dichlorobenzoyl peroxide or dicumyl peroxide or *OO*-(1,1-dimethylethyl) *O*-isopropyl monoperoxycarbonate or 2,5-bis[(1,1-dimethylethyl)dioxy]-2,5-dimethylhexane for moulded products;
- or by hydrosilylation by means of polysiloxane with -SiH groups using platinum as a catalyst.

In all cases, appropriate additives are used such as silica and sometimes small quantities of organosilicon additives (α,ω-dihydroxypolydimethylsiloxane).

CHARACTERS

*Appearance*: transparent or translucent material.

*Solubility*: practically insoluble in organic solvents, some of which, for example cyclohexane, hexane and methylene chloride, cause a reversible swelling of the material.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*) by the multiple reflection method for solids.

*Comparison*: *silicone elastomer CRS*.

B. Heat 1.0 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

C. 50 mg of the residue of combustion gives the reaction of silicates (*2.3.1*).

TESTS

*If necessary, cut the material into pieces of maximum dimension on a side of not greater than 1 cm.*

**Solution S**. Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant the solution.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Acidity or alkalinity**. To 100 mL of solution S add 0.15 mL of *bromothymol blue solution R1*. Not more than 2.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To a further 100 mL of solution S, add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

**Relative density** (*2.2.5*): 1.05 to 1.25, determined using a density bottle with *anhydrous ethanol R* as the immersion liquid.

**Reducing substances**. To 20 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Allow to stand for 15 min. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R* instead of solution S. The difference between the titration volumes is not more than 1.0 mL.

**Substances soluble in hexane**: maximum 3 per cent.

Evaporate 25 mL of the solution obtained in the test for phenylated compounds in a glass evaporating dish on a water-bath and dry in an oven at 100-105 °C for 1 h. The residue weighs not more than 15 mg.

**Phenylated compounds**. Place 2.0 g in a borosilicate-glass flask with a ground-glass neck and add 100 mL of *hexane R*. Boil under a reflux condenser for 4 h. Cool, then filter rapidly through a sintered-glass filter (16) (*2.1.2*). Collect the filtrate and close the container immediately to avoid evaporation. At wavelengths from 250 nm to 340 nm, the absorbance (*2.2.25*) is not greater than 0.4.

**Mineral oils**. Place 2 g in a 100 mL conical flask containing 30 mL of a mixture of 5 volumes of *ammonia R* and 95 volumes of *pyridine R*. Allow to stand for 2 h, shaking frequently. Decant the pyridine solution and examine in ultraviolet light at 365 nm. The fluorescence is not greater than that of a solution containing 1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

**Volatile matter**: maximum 0.5 per cent for silicone elastomer prepared using peroxides; maximum 2.0 per cent for silicone elastomer prepared using platinum.

Weigh 10.0 g of the substance previously stored for 48 h in a desiccator over *anhydrous calcium chloride R*. Heat in an oven at 200 °C for 4 h, allow to cool in a desiccator and weigh again.

*Silicone elastomer prepared using peroxides complies with the following additional test*:

**Residual peroxides**: maximum 0.08 per cent calculated as dichlorobenzoyl peroxide.

Place 5 g in a borosilicate-glass flask, add 150 mL of *methylene chloride R* and close the flask. Stir with a mechanical stirrer for 16 h. Filter rapidly, collecting the filtrate in a flask with a ground-glass neck. Replace the air in the container with *oxygen-free nitrogen R*, introduce 1 mL of a 200 g/L solution of *sodium iodide R* in *anhydrous acetic acid R*, close the flask, shake thoroughly and allow to stand protected from light for 30 min. Add 50 mL of *water R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not greater than 2.0 mL.

*Silicone elastomer prepared using platinum complies with the following additional test*:

**Platinum**: maximum 30 ppm.

In a quartz crucible, ignite 1.0 g of the material to be examined, raising the temperature gradually until a white residue is obtained. Transfer the residue to a graphite crucible. To the quartz crucible add 10 mL of a freshly prepared mixture of 1 volume of *nitric acid R* and 3 volumes of *hydrochloric acid R*, heat on a water-bath for 1-2 min and transfer to the graphite crucible. Add 5 mg of *potassium chloride R* and 5 mL of *hydrofluoric acid R* and evaporate to dryness on a water-bath. Add 5 mL of *hydrofluoric acid R* and evaporate to dryness again; repeat this operation twice. Dissolve the residue in 5 mL of *1 M hydrochloric acid*, warming on a water-bath. Allow to cool and add the solution to 1 mL of a 250 g/L solution of *stannous chloride R* in *1 M hydrochloric acid*, rinse the graphite crucible with a few millilitres of *1 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

Prepare simultaneously the reference solution as follows: to 1 mL of a 250 g/L solution of *stannous chloride R* in *1 M hydrochloric acid*, add 1.0 mL of *platinum standard solution (30 ppm Pt) R* and dilute to 10.0 mL with *1 M hydrochloric acid*.

The colour of the test solution is not more intense than that of the standard.

LABELLING

The label states whether the material was prepared using peroxides or platinum.

**04/2015:30110**

## 3.1.10. MATERIALS BASED ON NON-PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR NON-INJECTABLE, AQUEOUS SOLUTIONS

DEFINITION

Materials based on non-plasticised poly(vinyl chloride) that comply with the following specifications are suitable for the manufacture of containers for non-injectable aqueous solutions. They may also be used for solid forms for oral administration and in some cases, subject to special studies on the compatibility of the container with its contents, these materials may be suitable for the preparation of containers for suppositories. They consist of 1 or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

The chlorine content expressed as poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION

Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm. The manufacturing process is validated to demonstrate that the product complies with the following test.

**Vinyl chloride**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

*Test solution.* Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

*Vinyl chloride primary solution. Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject these 25 mL of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

*Vinyl chloride standard solution*: vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

*Reference solutions.* Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the head space.

*Limit*:

- *vinyl chloride*: maximum 1 ppm.

**Additives**

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. These additives are chosen from the following list, which specifies for each substance the maximum allowable content:

- epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6: maximum 8 per cent;
- calcium salt or zinc salts of aliphatic fatty acids with more than 7 carbon atoms: maximum 1.5 per cent or maximum 1.5 per cent of their mixture;
- liquid paraffin: maximum 1.5 per cent;
- waxes: maximum 1.5 per cent;
- hydrogenated oils or esters of aliphatic fatty acids: maximum 2 per cent;
- macrogol esters: maximum 1.5 per cent;
- sorbitol: maximum 1.5 per cent;
- 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite: maximum 1 per cent.

They may contain one of the following groups of stabilisers (where isooctyl is, for example, 2-ethylhexyl):

- tin as di(isooctyl) 2,2′-[(dioctylstannylene)bis(thio)]-diacetate containing about 27 per cent of tri(isooctyl) 2,2′,2″-[(monooctylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
- tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2′-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2′,2″-[(monomethylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
- 1-phenyleicosane-1,3-dione (benzoylstearoylmethane) or 2-(4-dodecylphenyl)indole or didodecyl 1,4-dihydropyridine-2,6-dimethyl-3,5-dicarboxylate: maximum 1 per cent or 1 per cent of a mixture of 2 of these.

Colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority. The material may be opacified with titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

*Appearance*: powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects.

*Solubility*: practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, insoluble in anhydrous ethanol.

They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation.* Dissolve residue A (see Tests: solution S2) in 5 mL of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

*Absorption maxima* (tolerance ± 5 $cm^{-1}$): at 2910 $cm^{-1}$, 1425 $cm^{-1}$, 1330 $cm^{-1}$, 1252 $cm^{-1}$, 958 $cm^{-1}$ and 690 $cm^{-1}$.

The spectrum obtained is identical to that of the material selected for the type sample.

TESTS

*If necessary, cut the samples of the material to be examined into pieces with a maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

**Solution S2**. Dissolve 5.0 g of the material to be examined in 80 mL of *tetrahydrofuran R* and dilute to 100 mL with the same solvent. Filter if necessary (the solution may remain opalescent). To 20 mL of the solution add, dropwise and with gentle shaking, 70 mL of *ethanol (96 per cent) R*. Cool in ice for 1 h. Filter or centrifuge (residue A). Wash residue A with *ethanol (96 per cent) R*, add the washings to the filtrate or the centrifugation liquid and dilute to 100 mL with *ethanol (96 per cent) R*.

**Solution S3**. Place 5 g of the material to be examined in a borosilicate-glass flask with a ground-glass neck. Add 100 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

**Appearance of solution S1**. Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Absorbance of solution S1** (*2.2.25*). Evaporate 100 mL of solution S1 to dryness. Dissolve the residue in 5 mL of *hexane R*. Filter if necessary through a filter previously rinsed with *hexane R*. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.25.

**Absorbance of solution S2** (*2.2.25*): maximum 0.2 for tin-stabilised materials or 0.4 for other materials determined between wavelengths of 250 nm and 330 nm on solution S2.

**Extractable barium**: maximum 2 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solution.* A solution containing 0.1 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Examined at 455.40 nm, the emission of the test solution is not greater than that of the reference solution.

**Extractable cadmium**: maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S3.

*Reference solution.* A solution containing 0.03 ppm of cadmium prepared by diluting *cadmium standard solution (0.1 per cent Cd) R* with *0.1 M hydrochloric acid*.

Verify the absence of cadmium in the hydrochloric acid used.

Examined at 228.8 nm, the absorbance of the test solution is not greater than that of the reference solution.

**Tin-stabilised materials**: maximum 0.25 per cent of Sn.

*Tin stock solution.* Dilute 81 mg of *plastic additive 23 CRS* to 100.0 mL with *tetrahydrofuran R*.

*Tin standard solution.* Dilute 20 mL of the tin stock solution to 100.0 mL with *ethanol (96 per cent) R*.

To 0.10 mL of solution S2 in a test tube add 0.05 mL of *1 M hydrochloric acid*, 0.5 mL of *potassium iodide solution R* and 5 mL of *ethanol (96 per cent) R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 mL of the tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution. The greenish-blue colour of dithizone solution turns pink in the presence of tin.

**Non-tin stabilised materials**: maximum 25 ppm of Sn.

To 5 mL of solution S2 in a test tube add 0.05 mL of *1 M hydrochloric acid* and 0.5 mL of *potassium iodide solution R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. If the solution obtained is not colourless, add the sodium sulfite solution in 0.05 mL fractions. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.05 mL of the tin standard solution (see test above).

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution.

**Extractable heavy metals** (*2.4.8*): maximum 20 ppm.

12 mL of solution S3 complies with test A. Prepare the reference solution using 10 mL of *lead standard solution (1 ppm Pb) R*.

**Extractable zinc**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S3 diluted 10-fold with *water R*.

*Reference solution.* A solution containing 0.50 ppm of zinc prepared by dilution of *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g; maximum 4.0 per cent when the materials are opacified with titanium dioxide.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

**04/2015:30111**

## 3.1.11. MATERIALS BASED ON NON-PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR SOLID DOSAGE FORMS FOR ORAL ADMINISTRATION

DEFINITION

Materials based on non-plasticised poly(vinyl chloride) for containers for solid dosage forms for oral administration are suitable for the manufacture of sheets or containers, and consist of 1 or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

The chlorine content expressed as poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION

Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm. The manufacturing process is validated to demonstrate that the product complies with the following test for vinyl chloride.

**Vinyl chloride**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution*. Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

*Test solution*. Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

*Vinyl chloride primary solution. Prepare in a fume cupboard*. Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the 25 mL of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

*Vinyl chloride standard solution*: vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

*Reference solutions*. Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the head space.

*Limit*:

- *vinyl chloride*: maximum 1 ppm.

**Additives**

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. These additives are chosen from the following list, which specifies for each substance the maximum allowable content:

- epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for tin-stabilised materials: maximum 2 per cent;
- epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for non-tin-stabilised materials: maximum 3 per cent;
- calcium, magnesium or zinc salts of aliphatic fatty acids with more than 7 carbon atoms: maximum 1.5 per cent or maximum 1.5 per cent of their mixture;
- waxes: maximum 4 per cent;
- liquid paraffin: maximum 1.5 per cent;
- hydrogenated oils or esters of aliphatic fatty acids: maximum 2 per cent;
- the percentage sum of the 3 lubricants above: maximum 4 per cent;
- macrogol esters: maximum 1.5 per cent;
- sorbitol: maximum 1.5 per cent;
- 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite: maximum 1 per cent;
- calcium carbonate: maximum 1 per cent;
- silica: maximum 1 per cent.

They may contain one of the following groups of stabilisers (where isooctyl is, for example, 2-ethylhexyl):

- tin as di(isooctyl) 2,2′-[(dioctylstannylene)bis(thio)]-diacetate containing about 27 per cent of tri(isooctyl) 2,2′2″-[(monooctylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
- tin as tri(isooctyl) 2,2′2″-[(monooctylstannylidyne)-tris(thio)]triacetate: maximum 0.25 per cent;
- tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2′-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2′,2″-[(monomethylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
- 1-phenyleicosane-1,3-dione (benzoylstearoylmethane): maximum 1 per cent.

Colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority. The material may be opacified with titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

*Appearance*: powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects.

*Solubility*: practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, practically insoluble in anhydrous ethanol.

They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*. Dissolve residue A (see Tests: solution S2) in 5 mL of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

*Absorption maxima* (tolerance ± 5 $cm^{-1}$): at 2910 $cm^{-1}$, 1425 $cm^{-1}$, 1330 $cm^{-1}$, 1252 $cm^{-1}$, 958 $cm^{-1}$ and 690 $cm^{-1}$.

The spectrum obtained is identical to that of the material selected for the type sample.

TESTS

*If necessary, cut the samples of the material to be examined into pieces with a maximum dimension on a side of not greater than 1 cm.*

**Solution S1**. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

**Solution S2**. Dissolve 5.0 g of the material to be examined in 80 mL of *tetrahydrofuran R* and dilute to 100 mL with the same solvent. Filter if necessary (the solution may remain opalescent). To 20 mL of the solution add, dropwise and with gentle shaking, 70 mL of *ethanol (96 per cent) R*. Cool in ice

for 1 h. Filter or centrifuge (residue A). Wash residue A with *ethanol (96 per cent) R*, add the washings to the filtrate or the centrifugation liquid and dilute to 100 mL with *ethanol (96 per cent) R*.

**Solution S3**. Place 5 g of the material to be examined in a borosilicate-glass flask with a ground-glass neck. Add 100 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

**Appearance of solution S1**. Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Absorbance of solution S1** (*2.2.25*). Evaporate 100 mL of solution S1 to dryness. Dissolve the residue in 5 mL of *hexane R*. Filter if necessary through a filter previously rinsed with *hexane R*. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.3.

**Absorbance of solution S2** (*2.2.25*): maximum 1.0, determined between wavelengths of 250 nm and 330 nm on solution S2 for material that does not contain 1-phenyleicosane-1,3-dione; maximum 0.4, determined between wavelengths of 250 nm and 330 nm on a 10-fold dilution of solution S2 in *ethanol (96 per cent) R* for material that contains 1-phenyleicosane-1,3-dione.

**Tin-stabilised materials**: maximum 0.25 per cent of Sn.

*Tin stock solution*. Dilute 81 mg of *plastic additive 23 CRS* to 100.0 mL with *tetrahydrofuran R*.

*Tin standard solution*. Dilute 20 mL of the tin stock solution to 100.0 mL with *ethanol (96 per cent) R*.

To 0.10 mL of solution S2 in a test-tube add 0.05 mL of *1 M hydrochloric acid*, 0.5 mL of *potassium iodide solution R* and 5 mL of *ethanol (96 per cent) R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 mL of the tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution. The greenish-blue colour of dithizone solution turns pink in the presence of tin.

**Non-tin-stabilised materials**: maximum 25 ppm of Sn.

To 5 mL of solution S2 in a test-tube add 0.05 mL of *1 M hydrochloric acid* and 0.5 mL of *potassium iodide solution R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. If the solution obtained is not colourless, add the sodium sulfite solution in 0.05 mL fractions. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.05 mL of the tin standard solution (see test above).

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution.

**Extractable heavy metals** (*2.4.8*): maximum 20 ppm.

12 mL of solution S3 complies with test A. Prepare the reference solution using 10 mL of *lead standard solution (1 ppm Pb) R*.

**Extractable zinc**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Solution S3 diluted 10-fold with *water R*.

*Reference solution*. A solution containing 0.50 ppm of zinc prepared by dilution of *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g; maximum 4.0 per cent when the materials are opacified with titanium dioxide.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

**01/2020:30113**

# 3.1.13. PLASTIC ADDITIVES

## DEFINITION

Plastic additives are chemical substances that are intentionally added to plastic materials to achieve a physical or chemical effect during processing of the plastic or in the final material or container. They may consist of a single chemical substance, a polymeric substance or a defined mixture of different components. Additives are intended to be present in the final material or container.

Substances present that have not been added intentionally are considered to be impurities and include reaction and degradation products, which may be limited by a suitable specification.

## GENERAL REQUIREMENTS

Each plastic additive and, if likely to be present in the final material or container, its impurities and reaction and degradation products, must be toxicologically qualified, taking into account the maximum potential exposure as a result of leaching into the contents of the container and product contamination.

Acceptance criteria must be specified for the identity, physico-chemical characteristics, impurities and the assay for each component.

A plastic material contains no more than the strict minimum of additives, at the lowest effective concentration for the intended use and to ensure the stability and quality of the final material or container.

Additives specified for plastic materials described in the European Pharmacopoeia are stated below. Plastic additives other than those described in the Pharmacopoeia may be used subject to agreement on a case-by-case basis by the competent authority.

## LIST

*NOTE. The nomenclature according to IUPAC rules is given first. The synonym in bold corresponds to the name given in the texts of Chapter 3. The synonym according to Chemical Abstracts (CA) Index rules is also given.*

**plastic additive 01**. $C_{24}H_{38}O_4$. [117-81-7].

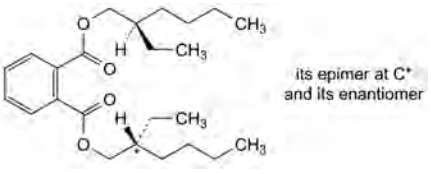


bis[(2*RS*)-2-ethylhexyl] benzene-1,2-dicarboxylate

synonyms: – **di(2-ethylhexyl) phthalate,**

– 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester,

– DEHP.

**plastic additive 02**. $C_{16}H_{30}O_4Zn$. [136-53-8].

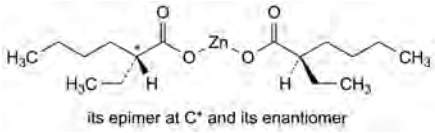


zinc bis[(2*RS*)-2-ethylhexanoate]

synonyms: – **zinc octanoate,**

– hexanoic acid, 2-ethyl, zinc salt (2:1),

– zinc 2-ethylcaproate.

**plastic additive 03**. [05518-18-3]/[00110-30-5].

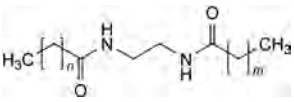

*N,N′*-(ethane-1,2-diyl)dialcanamide (with *n* and *m* = 14 or 16)

synonyms: – ***N,N′*-diacylethylenediamines,**

– *N,N′*-diacylethylenediamine (in this context acyl means in particular palmitoyl and stearoyl).

**plastic additive 04**. [8013-07-8].

epoxidised soya oil

**plastic additive 05**. [8016-11-3].

epoxidised linseed oil

**plastic additive 06**. [57455-37-5](TSCA)/[101357-30-6] (EINECS)/Pigment blue 29 (CI 77007)

ultramarine blue

**plastic additive 07**. $C_{15}H_{24}O$. [128-37-0]

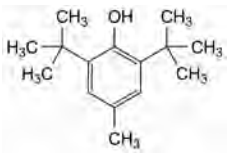

2,6-di-*tert*-butyl-4-methylphenol

synonyms: – **butylhydroxytoluene,**

– phenol, 2,6-bis(1,1-dimethylethyl)-4 methyl-

– 2,6-bis(1,1-dimethylethyl)-4-methylphenol.

**plastic additive 08**. $C_{50}H_{66}O_8$. [32509-66-3].

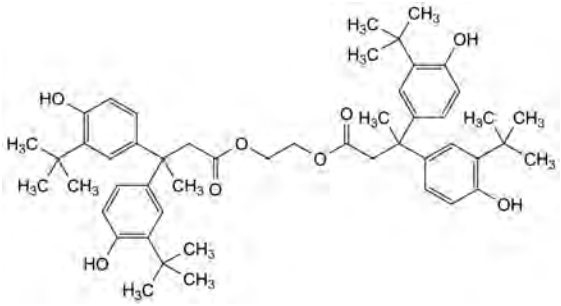

ethane-1,2-diyl bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)-butanoate]

synonyms: – **ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate],**

– benzenepropanoic acid, 3-(1,1-dimethylethyl)-β-[3-(1,1-dimethylethyl)-4-hydroxyphenyl]-4-hydroxy-β-methyl-, 1,1′-(1,2-ethanediyl) ester,

– ethylene bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)butyrate].

**plastic additive 09**. $C_{73}H_{108}O_{12}$. [6683-19-8].

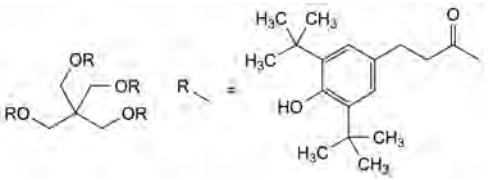

2,2-bis[[[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoyl]-oxy]methyl]propan-1,3-diyl bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate]

synonyms: – **pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate],**

– benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, 1,1′-[2,2-bis[[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-1-oxopropoxy]methyl]-1,3-propanediyl] ester,

– 2,2-bis[[[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoyl]oxy]methyl]propane-1,3-diyl 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoate,

– 2,2-bis(hydroxymethyl)propane-1,3-diol tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-propionate].

**plastic additive 10**. $C_{54}H_{78}O_3$. [1709-70-2].

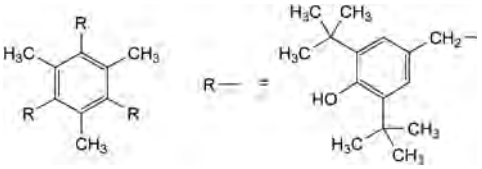

4,4′,4″-[(2,4,6-trimethylbenzene-1,3,5-triyl)tris(methylene)]-tris(2,6-di-*tert*-butylphenol)

synonyms: – **2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol,**

– phenol, 4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)tris(methylene)]tris[2,6-bis(1,1-dimethylethyl)-,

– 1,3,5-tris[3,5-di-*tert*-butyl-4-hydroxybenzyl]-2,4,6-trimethylbenzene.

**plastic additive 11**. $C_{35}H_{62}O_3$. [2082-79-3].

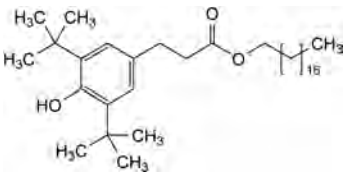

octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate

synonyms: – **octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate,**

– benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester.

**plastic additive 12**. $C_{42}H_{63}O_3P$. [31570-04-4].

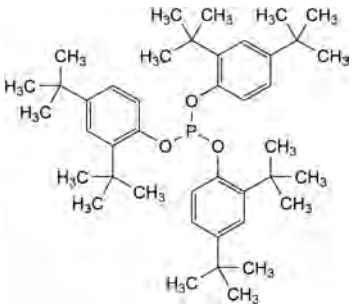

tris(2,4-di-*tert*-butylphenyl) phosphite

synonyms: – **tris(2,4-di-*tert*-butylphenyl) phosphite,**

– phenol, 2,4-bis(1,1-dimethylethyl)-, 1,1′,1″-phosphite,

– 2,4-bis(1,1-dimethylethyl)phenyl, phosphite.

**plastic additive 13**. $C_{48}H_{69}N_3O_6$. [27676-62-6].

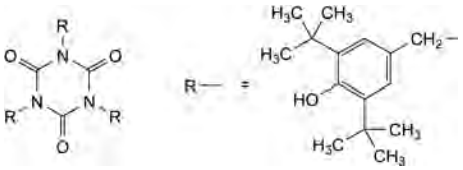

1,3,5-tris[(3,5-di-*tert*-butyl-4-hydroxyphenyl)methyl]-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione

synonyms: – **1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione,**

– 1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione, 1,3,5-tris[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methyl]-.

**plastic additive 14**. $C_{41}H_{82}O_6P_2$. [3806-34-6].

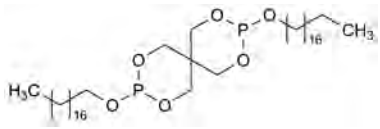

3,9-bis(octadecyloxy)-2,4,8,10-tetraoxa-3,9-diphospha-spiro[5.5]undecane

synonyms: – **2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane],**

– 2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5]undecane, 3,9-bis(octadecyloxy)-.

**plastic additive 15**. $C_{36}H_{74}S_2$. [2500-88-1].

(octadecyldisulfanyl)octadecane

synonyms: – **dioctadecyl disulfide,**

– octadecane, 1,1′-dithio-.

**plastic additive 16**. $C_{30}H_{58}O_4S$. [123-28-4].

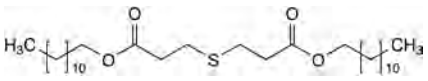

didodecyl 3,3′-sulfanediyldipropanoate

synonyms: – **didodecyl 3,3′-thiodipropionate,**

– propanoic acid, 3,3′-thiobis-, 1,1′-didodecyl ester,

– lauryl thiodipropionate.

**plastic additive 17**. $C_{42}H_{82}O_4S$. [693-36-7].

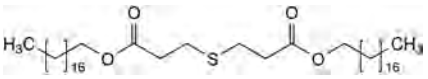

dioctadecyl 3,3′-sulfanediyldipropanoate

synonyms: – **dioctadecyl 3,3′-thiodipropionate,**

– propanoic acid, 3,3′-thiobis-, 1,1′-dioctadecyl ester,

– stearyl thiodipropionate.

**plastic additive 18**. [119345-01-6].

mixture of 7 products corresponding to reaction product of di-*tert*-butyl phosphonite with phosphorous trichloride, reaction products with 1,1′-biphenyl and 2,4-di-*tert*-butylphenol:

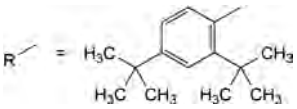

*component I*

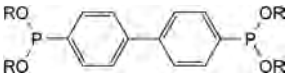

tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-4,4′-diyl)bis(phosphonite)

*component II*

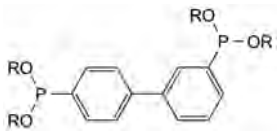

tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-3,4′-diyl)bis(phosphonite)

*component III*

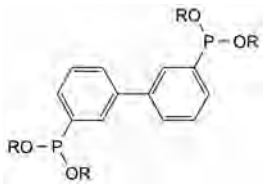

tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-3,3′-diyl)bis(phosphonite)

*component IV*

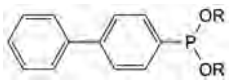

bis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-4-yl)phosphonite

*component V*

tris(2,4-di-*tert*-butylphenyl) phosphite

*component VI*

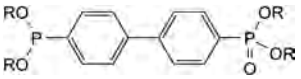

bis(2,4-di-*tert*-butylphenyl) 4′-[bis(2,4-di-*tert*-butylphenoxy)phosphanyl]([1,1′-biphenyl]-4-yl)phosphonate

*component VII*

R-OH: 2,4-di-*tert*-butylphenol

**plastic additive 19**. $C_{18}H_{36}O_2$. [57-11-4].

octadecanoic acid

synonyms: – **stearic acid.**

**plastic additive 20**. $C_{18}H_{35}NO$. [301-02-0].

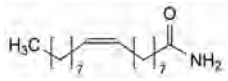

(9*Z*)-octadec-9-enamide

synonyms: – **oleamide,**

– 9-octadecenamide, (9*Z*)-,

– 9-*cis*-oleamide.

**plastic additive 21**. $C_{22}H_{43}NO$. [112-84-5].

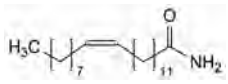

(13*Z*)-docos-13-enamide

synonyms: – **erucamide,**

– 13-docosenamide, (13*Z*)-,

– 13-*cis*-docosenamide.

**plastic additive 22**. [65447-77-0].

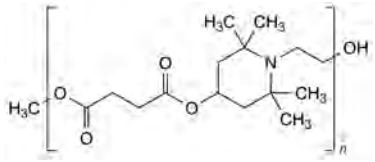

copolymer of dimethyl butanedioate and 1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidin-4-ol

synonyms: – **copolymer of dimethyl succinate and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)ethanol,**

– butanedioic acid, 1,4-dimethyl ester, polymer with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidineethanol.

**plastic additive 23**.

mixture of component I and about 27 per cent of component II

*component I* [26401-97-8]

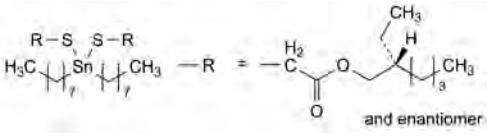


bis[(2*RS*)-2-ethylhexyl][(dioctylstannanetriyl)bis-(sulfanediyl)]diacetate

synonyms: – **di(isooctyl) 2,2′-[(dioctylstannylene)-bis(thio)]diacetate,**

– acetic acid, 2,2′-[(dioctylstannylene)-bis(thio)]bis-, 1,1′-diisooctyl ester.

*component II* [26401-86-5]

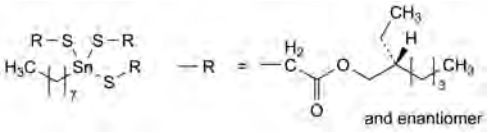


tris[(2*RS*)-2-ethylhexyl][(octylstannanetriyl)tris-(sulfanediyl)]triacetate

synonyms: – **tri(isooctyl) 2,2′,2″-[(monooctylstannylidyne)tris(thio)]triacetate,**

– acetic acid, 2,2′,2″-[(octylstannylidyne)-tris(thio)]tris-, 1,1′,1″-triisooctyl ester.

**plastic additive 24**. $C_{26}H_{48}O_4$. [166412-78-8].

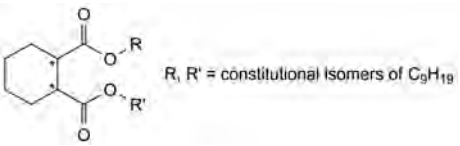


mixture of constitutional isomers of diisononyl (1*Ξ*,2*Ξ*)-cyclohexane-1,2-dicarboxylate.

synonyms: – **cyclohexane 1,2-dicarboxylic acid, diisononyl ester,**

– 1,2-cyclohexanedicarboxylic acid, 1,2-diisononyl ester.

**plastic additive 25** $C_{28}H_{50}O_8$. [82469-79-2].

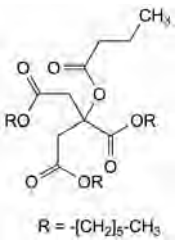


trihexyl 2-(butanoyloxy)propane-1,2,3-tricarboxylate

synonyms: – **butyryl tri-*n*-hexyl citrate,**

– 1,2,3-propanetricarboxylic acid, 2-(1-oxobutoxy)-, 1,2,3-trihexyl ester.

**plastic additive 26**. $C_{33}H_{54}O_6$. [3319-31-1].

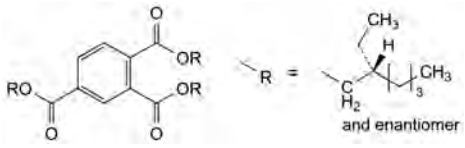


tris[(2*RS*)-2-ethylhexyl] benzene-1,2,4-tricarboxylate

synonyms: – **tris(2-ethylhexyl) trimellitate,**

– 1,2,4-benzentricarboxylic acid, 1,2,4-tris (2-ethylhexyl] estertricarboxylate.

**plastic additive 27**. $C_{24}H_{38}O_4$. [6422-86-2].

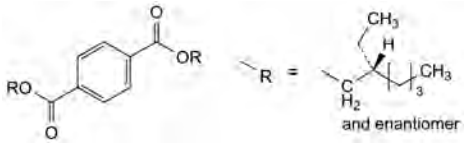


bis[(2*RS*)-2-ethylhexyl] benzene-1,4-dicarboxylate

synonyms: – **bis(2-ethylhexyl) terephthalate,**

– 1,4-benzenedicarboxylic acid,

– 1,4-bis(2-ethylhexyl) ester.

**01/2019:30114 corrected 10.0**

# 3.1.14. MATERIALS BASED ON PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR AQUEOUS SOLUTIONS FOR INTRAVENOUS INFUSION

DEFINITION

Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion are defined by the nature and the proportions of the substances used in their manufacture.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

**Vinyl chloride**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution*. Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

*Test solution*. Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

*Vinyl chloride primary solution. Prepare in a fume cupboard*. Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

*Vinyl chloride standard solution*: vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

*Reference solutions*. Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain, respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the head-space.

*Limit*:

- *vinyl chloride*: maximum 1 ppm.

**Additives**

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:

- di(2-ethylhexyl) phthalate (plastic additive 01): maximum 40 per cent;
- zinc octanoate (zinc 2-ethylhexanoate) (plastic additive 02): maximum 1 per cent;
- calcium stearate or zinc stearate: maximum 1 per cent or 1 per cent of a mixture of the two;
- *N,N′*-diacylethylenediamines (plastic additive 03): maximum 1 per cent;
- maximum 10 per cent of one of the following epoxidised oils or 10 per cent of a mixture of the two:
  - epoxidised soya oil (plastic additive 04) of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6;
  - epoxidised linseed oil (plastic additive 05) of which the oxiran oxygen content is not greater than 10 per cent and the iodine value is not greater than 7.

When colouring materials are added, ultramarine blue (plastic additive 06) is used. Other colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Colourless, slightly blue or pale yellow powder, beads, granules or, after transformation, translucent sheets of varying thickness, with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

To 2.0 g of the material to be examined add 200 mL of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue (residue B) and the solution (solution A) by filtration.

Evaporate solution A to dryness under reduced pressure in a water-bath at 30 °C. Dissolve residue in 10 mL of *toluene R* (solution A1). Dissolve residue B in 60 mL of *ethylene chloride R*, heating on a water-bath under a reflux condenser. Filter. Add the obtained solution dropwise and with vigorous shaking to 600 mL of *heptane R* heated almost to boiling. Separate by hot filtration the coagulum (coagulum B1) and the organic solution. Allow the latter to cool; separate the precipitate that forms (precipitate B2) and filter through a tared sintered-glass filter (40) (*2.1.2*).

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve coagulum B1 in 30 mL of *tetrahydrofuran R* and add, in small volumes with shaking, 40 mL of *anhydrous ethanol R*; separate the precipitate (precipitate B3) by filtration and dry *in vacuo* at a temperature not exceeding 50 °C over *diphosphorus pentoxide R*; dissolve a few milligrams of precipitate B3 in 1 mL of *tetrahydrofuran R*, place a few drops of the solution obtained on a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

*Comparison*: *poly(vinyl chloride) CRS*.

B. Plastic additive 01 (see Tests).

TESTS

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

**Solution S1**. Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of strong hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

**Solution S2**. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

**Appearance of solution S2**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*). Evaporate 100.0 mL of solution S2 to dryness. Dissolve the residue in 5.0 mL of *hexane R*. From 250 nm to 310 nm the absorbance is not greater than 0.25.

**Reducing substances**. *Carry out the test within 4 h of preparation of solution S2.* To 20.0 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R*. The difference between the 2 titration volumes is not greater than 2.0 mL.

**Primary aromatic amines**: maximum 20 ppm.

To 2.5 mL of solution A1 obtained during the identification, add 6 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*. Shake vigorously and discard the upper layer. To the aqueous layer add 0.4 mL of a freshly prepared 10 g/L solution of *sodium nitrite R*. Mix and allow to stand for 1 min. Add 0.8 mL of a 25 g/L solution of *ammonium sulfamate R*, allow to stand for 1 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. After 30 min, any colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner, replacing the aqueous layer with a mixture of 1 mL of a 0.01 g/L solution of *naphthylamine R* in *0.1 M hydrochloric acid*, 5 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*.

**Plastic additive 01**. Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution S3*: 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

*Internal standard solution S4*: 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

*Test solution*. Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solution may be stored at 4 °C for up to 2 weeks.

*Stock solution*. Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with the internal standard solution S4.

*Reference solutions (a1)-(a5)*. Dilute the stock solution with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 100 |
| | 0 - 3.3 | 100 → 200 |
| | 3.3 - 20 | 200 → 250 |
| | 20 - 22.5 | 250 |
| | 22.5 - 23 | 250 → 270 |
| | 23 - 25 | 270 |
| | 25 - 25.6 | 270 → 320 |
| | 25.6 - 30.6 | 320 |
| Injection port | | 300 |

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] |
|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 |
| DnOP (internal standard) | 149 | 279 | 167 |

*Injection*: 1 µL.

*Relative retention* with reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] | Ion ratio 2/1 (%) | Ion ratio 3/1 (%) |
|---|---|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 | 50 | 30 |
| DnOP (internal standard) | 149 | 279 | 167 | / | / |

*System suitability*:

- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to plastic additive 01, determined on 6 injections of a reference solution situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to plastic additive 01 to that due to the internal standard, determined on 6 injections of a reference solution situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additive 01 in the material to be examined.

*Limit*:

- *plastic additive 01*: maximum 40 per cent.

**Plastic additive 03**. Wash precipitate B2 obtained during the identification and contained in the tared sintered-glass filter (40) (*2.1.2*) with *anhydrous ethanol R*. Dry to constant mass over *diphosphorus pentoxide R* and weigh the filter. The residue weighs not more than 20 mg.

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: the residue obtained above. When the amount of residue is insufficient to prepare a disc, record the spectrum of the residue placed between 2 plates transparent to infrared radiation or examine by attenuated total reflectance (ATR).

*Comparison*: *plastic additive 03 CRS*.

**Plastic additives 04 and 05**. Thin-layer chromatography (*2.2.27*).

*Reference solutions*. Prepare 10 mg/mL solutions of *plastic additive 04 CRS* and *plastic additive 05 CRS*, respectively, in *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *toluene R*.

*Application*: 0.5 mL of solution A1 obtained during the identification, as a band 30 mm by 3 mm, and 5 µL of each reference solution.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour for 5 min.

Examine the chromatogram and locate the zone corresponding to plastic additives 04 and 05 ($R_F$ = 0). Remove the area of silica gel corresponding to this zone. Similarly remove a corresponding area of silica gel as a blank reference. Separately shake both samples for 15 min with 40 mL of *methanol R*. Filter, rinse with 2 quantities, each of 10 mL, of *methanol R*, add the rinsings to the filtrate and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

**Barium**: maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid*.

*Reference solution*. A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

**Cadmium**: maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Evaporate 10 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid solution.

*Reference solutions*. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

**Calcium**: maximum 0.07 per cent.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Use the test solution prepared for the determination of barium.

*Reference solution*. A solution containing 50.0 ppm of calcium prepared by dilution of *calcium standard solution (400 ppm Ca) R* with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of calcium at 315.89 nm, the spectral background being taken at 315.60 nm.

Verify the absence of calcium in the hydrochloric acid used.

**Tin**: maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Dilute solution S1 10-fold with *water R* immediately before use.

*Reference solution*. Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

*Wavelength*: use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the hydrochloric acid used.

**Zinc**: maximum 0.2 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dilute solution S1 100-fold with *0.1 M hydrochloric acid*.

*Reference solutions*. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

**Heavy metals** (*2.4.8*): maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

**Water extractable substances**: maximum 0.3 per cent.

Evaporate 50.0 mL of solution S2 to dryness on a water-bath and dry in an oven at 100-105 °C to constant mass. Carry out a blank test with 50.0 mL of *water R*. The residue weighs not more than 7.5 mg taking into account the blank test.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

**01/2008:30115**
**corrected 7.5**

# 3.1.15. POLYETHYLENE TEREPHTHALATE FOR CONTAINERS FOR PREPARATIONS NOT FOR PARENTERAL USE

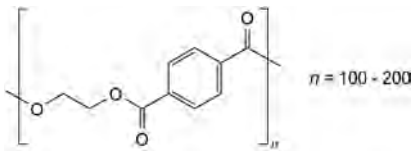


DEFINITION

Polyethylene terephthalate is obtained from the polymerisation of terephthalic acid or dimethyl terephthalate with ethylene glycol. Isophthalic acid, dimethyl isophthalate, 1,4-bis(hydroxymethyl)cyclohexane (cyclohexane-1,4-dimethanol) or diethylene glycol may be used in the polymerisation. It may contain not more than 0.5 per cent of silica or silicates and colouring matter approved by the competent authority.

PRODUCTION

The manufacturing process is validated to demonstrate that the residual acetaldehyde content is not greater than 10 ppm in the granules.

CHARACTERS

*Appearance*: clear or opaque granules.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It is hydrolysed by strong bases.

IDENTIFICATION

A. Place 0.10 g of the material to be examined into a borosilicate glass flask with a ground-glass neck. Add 25 mL of a 200 g/L solution of *potassium hydroxide R* in a 50 per cent *V/V* solution of *anhydrous ethanol R*. Reflux for 30 min. Allow to cool and dilute to 100 mL with *water R*. Filter if necessary. Dilute 1.0 mL of the filtrate to 100 mL with *water R*. Examined between 210 nm and 330 nm (*2.2.25*), the solution shows an absorption maximum at 240 nm.

B. Dissolve 0.05 g of the material to be examined in 2 mL of *1,1,1,3,3,3-hexafluoropropan-2-ol R*. Apply to a glass plate on a water-bath in a fume cupboard several drops of the solution to produce a film of about 15 mm by 15 mm. Allow the solvent to evaporate and remove the film using a stream of water and a scraper. Dry in an oven at 100-105 °C for 1-2 h. Examine the film by infrared absorption spectrophotometry (*2.2.24*). The spectrum of the material to be examined shows maxima in particular at 1725 $cm^{-1}$, 1410 $cm^{-1}$, 1265 $cm^{-1}$, 1120 $cm^{-1}$, 1100 $cm^{-1}$, 1020 $cm^{-1}$, 875 $cm^{-1}$, 725 $cm^{-1}$. The spectrum obtained, in addition, is identical to that of the material selected for the type sample.

TESTS

*If necessary, cut out samples for testing to a maximum size of 1 cm per side.*

**Solution S1**. Place 10.0 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 200 mL of *water R* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S1 within 4 h of its preparation.*

**Solution S2**. Place 10 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 100 mL of *ethanol (96 per cent) R* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S2 within 4 h of its preparation.*

**Solution S3**. Place 20 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 50 mL of *0.1 M hydrochloric acid* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S3 within 4 h of its preparation.*

**Solution S4**. Place 20 g of the material to be examined into a borosilicate glass flask with a ground-glass neck. Add 50 mL of *0.01 M sodium hydroxide* and heat at 50 °C for 5 h. Allow to cool and decant. *Use solution S4 within 4 h of its preparation.*

**Appearance of solution S1**. Solution S1 is clear (*2.2.1*).

**Appearance of solution S2**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. The solution turns yellow. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To another 50 mL of solution S1 add 0.2 mL of *methyl orange solution R*. The solution turns yellow. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator to orange.

**Absorbance of solution S1** (*2.2.25*): maximum 0.20 between 220 nm and 340 nm. In addition, for coloured polyethylene terephthalate: maximum 0.05 between 400 nm to 800 nm.

**Absorbance of solution S2** (*2.2.25*): maximum 0.05 between 400 nm and 800 nm.

**Reducing substances**. Add 2 mL of *0.5 M sulfuric acid* and 20.0 mL of *0.002 M potassium permanganate* to 20.0 mL of solution S1. Boil for 3 min. Cool immediately to ambient temperature. Add 1 g of *potassium iodide R*, 0.25 mL of *starch solution R* as indicator and titrate with *0.01 M sodium thiosulfate*. Perform a blank titration using 20.0 mL of *water R*. The difference in volume used in the 2 titrations is not greater than 0.5 mL.

**Substances soluble in dioxan**: maximum 3 per cent.

Place 2 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 20 mL of *dioxan R* and heat under reflux for 2 h. Evaporate 10 mL of the solution to dryness on a water-bath and then dry the residue at 100-105 °C. The residue weighs a maximum of 30 mg.

**Extractable aluminium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 396.15 nm, the spectral background being taken at 396.25 nm.

Verify the absence of aluminium in the *0.1 M hydrochloric acid* used.

**Extractable antimony**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S4.

*Reference solutions.* Prepare the reference solutions using *antimony standard solution (100 ppm Sb) R*, diluting with *0.01 M sodium hydroxide*.

*Wavelength*: 231.15 nm or 217.58 nm, the spectral background being taken at 231.05 nm.

**Extractable barium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *barium standard solution (50 ppm Ba) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the *0.1 M hydrochloric acid* used.

**Extractable cobalt**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *cobalt standard solution (100 ppm Co) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 228.62 nm, the spectral background being taken at 228.50 nm.

Verify the absence of cobalt in the *0.1 M hydrochloric acid* used.

**Extractable germanium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S4.

*Reference solutions.* Prepare the reference solutions using *germanium standard solution (100 ppm Ge) R*, diluting with *0.01 M sodium hydroxide*.

*Wavelength*: 206.87 nm or 265.12 nm, the spectral background being taken at 206.75 nm.

**Extractable manganese**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *manganese standard solution (100 ppm Mn) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 257.61 nm, the spectral background being taken at 257.50 nm.

Verify the absence of manganese in the *0.1 M hydrochloric acid* used.

**Extractable titanium**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 323.45 nm or 334.94 nm, the spectral background being taken at 323.35 nm.

Verify the absence of titanium in the 0.1M hydrochloric acid used.

**Extractable zinc**: maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Solution S3.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Wavelength*: 213.86 nm, the spectral background being taken at 213.75 nm.

Verify the absence of zinc in the *0.1 M hydrochloric acid* used.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent determined on 1.0 g.

# 3.2. Containers

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**01/2008:30200 corrected 10.0**

# 3.2. CONTAINERS

A container for pharmaceutical use is an article that contains or is intended to contain a product and is, or may be, in direct contact with it. The closure is a part of the container.

The container (see General Notices section 1.3) is so designed that the contents may be removed in a manner appropriate to the intended use of the preparation. It provides a varying degree of protection depending on the nature of the product and the hazards of the environment, and minimises the loss of constituents. The container does not interact physically or chemically with the contents in a way that alters their quality beyond the limits tolerated by official requirements.

*Single-dose container*. A single-dose container holds a quantity of the preparation intended for total or partial use on 1 occasion only.

*Multidose container*. A multidose container holds a quantity of the preparation suitable for 2 or more doses.

*Well-closed container*. A well-closed container protects the contents from contamination with extraneous solids and liquids and from loss of contents under ordinary conditions of handling, storage and transport.

*Airtight container*. An airtight container is impermeable to solids, liquids and gases under ordinary conditions of handling, storage and transport. If the container is intended to be opened on more than 1 occasion, it must be so designed that it remains airtight after re-closure.

*Sealed container*. A sealed container is a container closed by fusion of the material of the container.

*Tamper-evident container*. A tamper-evident container is a closed container fitted with a device that reveals irreversibly whether the container has been opened.

*Child-proof container*. A container that is fitted with a closure that prevents opening by children.

**01/2019:30201**

## 3.2.1. GLASS CONTAINERS FOR PHARMACEUTICAL USE

Glass containers for pharmaceutical use are glass articles intended to come into direct contact with pharmaceutical preparations.

*Colourless glass* is highly transparent in the visible spectrum.

*Coloured glass* is obtained by the addition of small amounts of metal oxides, chosen according to the desired spectral absorbance.

*Neutral glass* is a borosilicate glass containing significant amounts of boric oxide, aluminium oxide, alkali metal oxides and/or alkaline earth oxides in the glass network. Due to its composition, neutral glass has a high hydrolytic resistance and a high thermal shock resistance.

*Soda-lime-silica glass* is a silica glass containing alkali metal oxides, mainly sodium oxide, and alkaline earth oxides, mainly calcium oxide, in the glass network. Due to its composition, soda-lime-silica glass has only a moderate hydrolytic resistance.

The hydrolytic stability of glass containers for pharmaceutical use is expressed by the resistance to the release of soluble mineral substances into water under the prescribed conditions of contact between the inner surface of the container or glass grains and water. The hydrolytic resistance is evaluated by titrating released alkali reacting ions. According to their hydrolytic resistance, glass containers are classified as follows:

- type I glass containers: neutral glass, with a high hydrolytic resistance due to the chemical composition of the glass itself;
- type II glass containers: usually of soda-lime-silica glass with a high hydrolytic resistance resulting from suitable treatment of the inner surface;
- type III glass containers: usually of soda-lime-silica glass with only moderate hydrolytic resistance.

The following italicised statements constitute general recommendations concerning the type of glass container that may be used for different types of pharmaceutical preparations. The manufacturer of a pharmaceutical product is responsible for ensuring the suitability of the chosen container.

*Type I glass containers are suitable for most preparations whether or not for parenteral administration.*

*Type II glass containers are suitable for most acidic and neutral, aqueous preparations whether or not for parenteral administration.*

*Type III glass containers are in general suitable for non-aqueous preparations for parenteral administration, for powders for parenteral administration (except for freeze-dried preparations) and for preparations not for parenteral administration.*

Glass containers with a hydrolytic resistance higher than that recommended above for a particular type of preparation may generally also be used.

The container chosen for a given preparation shall be such that the glass material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity. In justified cases, further detailed information may be necessary to assess the impact on chronic use and for vulnerable patient groups.

Preparations for parenteral administration are normally presented in colourless glass, but coloured glass may be used for substances known to be light-sensitive. Colourless or coloured glass is used for the other pharmaceutical preparations. It is recommended that all glass containers for liquid preparations and for powders for parenteral administration permit the visual inspection of the contents.

The inner surface of glass containers may be specially treated to improve hydrolytic resistance, to confer water-repellancy, etc. The outer surface may also be treated, for example to reduce friction and to improve resistance to abrasion. The outer treatment is such that it does not contaminate the inner surface of the container.

Except for type I glass containers, glass containers for pharmaceutical preparations are not to be re-used. Containers for human blood and blood components must not be re-used.

PRODUCTION

When glass containers for pharmaceutical use are manufactured under stressed conditions (e.g. temperature-time profile) and/or are placed in contact with particularly aggressive pharmaceutical preparations, they may undergo delamination, i.e the separation of the inner glass surface into thin layers called lamellae or flakes. Glass delamination may be the result of a chemical attack that occurs according to well-known glass corrosion mechanisms, such as dissolution by hydrolysis and ion exchange (leaching) as a function of the pH. The process of interaction between the glass surface and the pharmaceutical preparation requires incubation time, and flaking may only become visible a number of months after filling.

Several risk factors are known to increase the propensity of a glass to delaminate. The chemical composition of the pharmaceutical preparation, the presence of buffers like citrate or phosphate, which are known to corrode glass, and

the ionic strength of the liquid medium may all strongly favour delamination. The manufacturing process of the container, chemical treatments of the inner surface, and terminal sterilisation and processing at the pharmaceutical filling lines are other important risk factors to be considered. It is recommended that the user of the container assesses the compatibility of the glass container and the pharmaceutical preparation on a case-by-case basis, considering for example the dosage form, properties of the formulation and glass quality.

The propensity to delamination of glass containers from different sources can be assessed and ranked by exposing the container to accelerated degradation testing, carried out at specified temperatures for a short time and using the solutions associated with the actual pharmaceutical preparation as extractants. The presence of particles in the extraction solution, the occurrence of phase separation on the inner surface, and the steep increase of silica concentration in the extraction solution are all indicators of a potential propensity for delamination. Accelerated degradation testing can be used as a predictive tool to select the most appropriate container for the intended preparation, but the full compatibility of the active substance with the glass leachate can only be assessed by a stability test under normal conditions of use.

## TESTS

Glass containers for pharmaceutical use comply with the relevant test or tests for hydrolytic resistance. When glass containers have non-glass components, the tests apply only to the glass part of the container.

To define the quality of glass containers according to the intended use, one or more of the following tests are necessary.

Tests for hydrolytic resistance are carried out to define the type of glass (I, II or III) and to control its hydrolytic resistance.

In addition, containers for aqueous parenteral preparations are tested for arsenic release and coloured glass containers are tested for spectral transmission.

### *HYDROLYTIC RESISTANCE*

Table 3.2.1.-1. – *Types of glass*

| Type of container | Test to be performed |
|---|---|
| Type I and type II glass containers (to distinguish from type III glass containers) | Test A (surface test) |
| Type I glass containers (to distinguish from type II and type III glass containers) | Test B (glass grains test) or test C (etching test) |
| Type I and type II glass containers (if there are doubts whether the high hydrolytic resistance is due to the chemical composition or to the surface treatment) | Tests A and B, or tests A and C |

The test is carried out by titration of the extraction solutions obtained under the conditions described for tests A, B and C. Test C is performed if there are uncertainties whether the container is type I or type II.

#### EQUIPMENT

- An autoclave or steam steriliser capable of withstanding a pressure of $2.5 \times 10^5$ N/m$^2$ (equivalent to 0.25 MPa = 2.5 bar) or more and capable of carrying out the heating cycle described under Autoclaving process. Preferably it is equipped with a constant-pressure regulator or other suitable means in order to maintain the temperature at 121 ± 1 °C. The autoclave vessel is equipped with a heating device, a thermometer integrated in the autoclave, a pressure gauge, a vent cock (for manually operated autoclaves only) and a tray of sufficient capacity to accommodate, above the water level, the number of containers needed to carry out the test. The autoclave has the possibility to connect a calibrated resistance thermometer or a calibrated thermocouple from the inner chamber to an external measuring device to allow a temperature measurement independent from the autoclave system.

  *The autoclave vessel and all ancillary equipment must be washed thoroughly with water R before use.*
- A calibrated resistance thermometer or calibrated thermocouple connected to a suitable temperature measuring device.
- Burettes with a suitable capacity.
- One-mark volumetric flasks, with a capacity of 1000 mL.
- Pipettes and beakers.
- Conical flasks with capacities of 100 mL and 250 mL.
- A water-bath.
- Metal foil (e.g. aluminium, stainless steel).

Flasks and beakers must already have been used for the test or have been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h before being used.

#### DETERMINATION OF THE FILLING VOLUME

The filling volume is the volume of water to be introduced into the container for the purpose of the test. For vials and bottles the filling volume is 90 per cent of the brimful capacity. For ampoules it is the volume up to the height of the shoulder.

**Vials and bottles**. Select, at random, 6 containers from the sample lot, or 3 if their capacity exceeds 100 mL, and remove any debris or dust. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface and fill them with *water R* until about the rim edge, avoiding overflow and introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of the water expressed to 2 decimal places for containers having a nominal volume less than or equal to 30 mL, and expressed to 1 decimal place for containers having a nominal volume greater than 30 mL. Calculate the mean value of the brimful capacity in millilitres and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

**Ampoules**. Place at least 6 dry ampoules on a flat, horizontal surface and fill them with *water R* from a burette, until the water reaches point A, where the body of the ampoule declines to the shoulder (see Figure 3.2.1.-1). Read the capacities (expressed to 2 decimal places) and calculate the mean value. This volume, expressed to 1 decimal place, is the filling volume for the particular ampoule lot. The filling volume may also be determined by weighing.

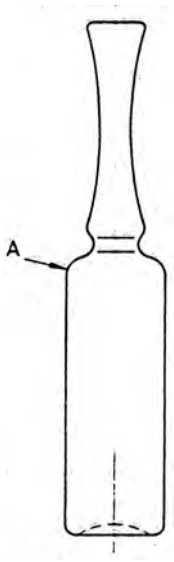


Figure 3.2.1.-1. – *Filling volume of ampoules (up to point A)*

**Syringes and cartridges**. Select 6 syringes or cartridges. Close the small opening (mouth of cartridges and needle and/or Luer cone of syringes) using an inert material (e.g. a

tip cap) or any other suitable means to prevent water leakage. Determine the mean brimful volume in accordance with the procedure described under Vials and bottles and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

**TEST A. HYDROLYTIC RESISTANCE OF THE INNER SURFACES OF GLASS CONTAINERS (SURFACE TEST)**

The determination is carried out on unused containers. The volumes of the test liquid necessary are indicated in Table 3.2.1.-2.

Table 3.2.1.-2. – *Volume of test liquid and number of titrations*

| Filling volume (mL) | Volume of test liquid for 1 titration (mL) | Number of titrations |
|---|---|---|
| Up to 3 | 25.0 | 1 |
| Above 3 and up to 30 | 50.0 | 2 |
| Above 30 and up to 100 | 100.0 | 2 |
| Above 100 | 100.0 | 3 |

**Cleaning**. Remove any debris or dust. Shortly before the test, fill each container to the brim with *water R* and allow to stand, filled with water, for 20 ± 5 min. Empty the containers, carefully rinse twice with *water R* and once with *water R1* and allow to drain.

Closed ampoules are not rinsed before testing. Closed ampoules may be warmed on a water-bath or in an oven at about 40 °C for approximately 2 min before opening to avoid underpressure when opening.

**Filling**. Fill the containers with *water R1* up to the filling volume.

Loosely cap each container with an inert material, for example with inverted beakers of such a size that the bottoms of the beakers fit snugly down on the rims of the sample. Ampoules and vials capped with clean aluminium foil are further examples. Place syringes and cartridges in a beaker and cover the beaker with clean aluminium foil.

Containers of a volume of 2 mL or less, in which the water is not sufficiently retained during the autoclaving process, may be closed in a suitable way, e.g. with a stopper or plug of inert material, such as silicone, and fixed using a plunger or a stable fixing or clamping device.

Place the samples, gathered in groups in glass dishes or in beakers or other suitable holders, on the rack in the autoclave containing *water R* at room temperature. Ensure that they are held above the level of the water in the autoclave.

**Autoclaving process**

*Reference thermal cycle*

The autoclave is run in such a way that the temperature in the containers to be tested follows a thermal cycle with the following characteristics: temperature raised from room temperature to 100 °C within 20-30 min; temperature maintained at 100 ± 1 °C for 10 ± 1 min; temperature in the containers raised from 100 °C to 121 °C within 20-22 min; temperature maintained at 121 ± 1 °C for 60 ± 1 min; temperature cooled to 100 °C within 40-44 min.

*Autoclave calibration*

Before being used for the first time, the autoclave and the temperature measuring system are calibrated to ensure that the autoclave settings are suitable to guarantee that the temperature inside the containers is 121 ± 1 °C.

*NOTE: significant differences may be observed between the temperature measured in the autoclave chamber and inside the containers.*

Take a set of containers of intermediate capacity (10 mL for instance) and fill them with *water R1*. Select a sufficient number of containers to fill completely the tray within the autoclave chamber. Insert the end of the calibrated resistance thermometer or calibrated thermocouple into one of the containers through a hole in the closure having approximately the same diameter as the probe and connect it to the external measuring device. If the container is too small to insert a thermocouple, place the thermocouple in a similar container of suitable size filled with *water R1*. Close the autoclave door or lid securely and run the autoclave to achieve the target thermal cycle in the containers. Where a manual autoclave is run, leave the vent cock open. Heat the autoclave at a regular rate so that steam issues vigorously from the vent cock after 20-30 min, and maintain a vigorous evolution of steam for a further 10 min.

Close the vent cock, follow the temperature increase on the calibrated thermocouple measuring device by comparison with readings taken from the autoclave thermometer and adjust the autoclave settings accordingly in order to match the target thermal cycle. Keep the temperature ramp as smooth as possible.

Using the calibrated thermocouple measuring device, ensure that deviations from the holding temperature of 121 ± 1 °C are within the tolerance. When cooling down, vent to prevent the formation of a vacuum. For safety reasons (boiling retardation) do not open the autoclave before the water in the containers has reached a temperature of 95 °C. Remove the hot samples from the autoclave and cool cautiously to room temperature within 30 min.

Record the autoclave settings used to carry out the thermal cycle and use these settings for routine autoclave runs.

At regular intervals verify the validation of the calibration. Establish a re-calibration plan based on quality assurance criteria, recalibrate as appropriate and keep records.

*Routine autoclave runs*

Use the autoclave settings established during the calibration stage and follow the same thermal cycle described above. Container sets of different capacity can be tested during the same run. Keep the glass load very similar in size and mass to the load used during the calibration stage. The use of the calibrated thermocouple is no longer necessary provided the calibration is proved to be valid over a defined time span.

At the end of the cycle, remove the hot samples from the autoclave and cool them cautiously to room temperature within 30 min.

*NOTE: depending on the type or size of the autoclave the heat transfer and thus the resulting thermal cycle in the containers may vary with the total load of the autoclave. It may therefore be necessary to adjust the autoclave load.*

**Method**. Carry out the titration within 1 h of removal of the containers from the autoclave. Combine the liquids obtained from the containers and mix. Introduce the prescribed volume (Table 3.2.1.-2) into a conical flask (test solution). Place the same volume of *water R1* into a 2nd similar flask as a blank. Add to each flask 0.05 mL of *methyl red solution R* for each 25 mL of liquid. Titrate the blank with *0.01 M hydrochloric acid*. Titrate the test solution with the same acid until the colour of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test solution and express the results in millilitres of *0.01 M hydrochloric acid* per 100 mL. Express titration values of less than 1.0 mL to 2 decimal places and titration values of more than or equal to 1.0 mL to 1 decimal place.

**Limits**. The results, or the average of the results if more than 1 titration is performed, is not greater than the values stated in Table 3.2.1.-3.

Table 3.2.1.-3. – *Limit values in the test for surface hydrolytic resistance*

| | Maximum volume of 0.01 M HCl per 100 mL of test solution (mL) | |
|---|---|---|
| **Filling volume (mL)** | **Types I and II glass containers** | **Type III glass containers** |
| Up to 0.5 | 3.0 | 30.0 |
| Above 0.5 and up to 1 | 2.0 | 20.0 |
| Above 1 and up to 2 | 1.8 | 17.6 |
| Above 2 and up to 3 | 1.6 | 16.1 |
| Above 3 and up to 5 | 1.3 | 13.2 |
| Above 5 and up to 10 | 1.0 | 10.2 |
| Above 10 and up to 20 | 0.80 | 8.1 |
| Above 20 and up to 50 | 0.60 | 6.1 |
| Above 50 and up to 100 | 0.50 | 4.8 |
| Above 100 and up to 200 | 0.40 | 3.8 |
| Above 200 and up to 500 | 0.30 | 2.9 |
| Above 500 | 0.20 | 2.2 |

**TEST B. HYDROLYTIC RESISTANCE OF GLASS GRAINS (GLASS GRAINS TEST)**

*Check that the articles as received have been annealed to a commercially acceptable quality.*

The test may be performed on the canes used for the manufacture of tubing glass containers or on the containers.

**Equipment**

- a mortar, pestle (see Figure 3.2.1.-2) and hammer made of tempered, magnetic steel;
- as an alternative to the mortar, pestle and hammer, a ball mill can be used; the ball mill is made of agate, zirconia or stainless steel with a volume of 250 mL; 2 balls with a diameter of 40 mm or 3 balls with a diameter of 30 mm are suitable;
- a set of 3 square-mesh sieves of stainless steel, mounted on frames of the same material and consisting of the following:
  - (a) sieve no. 710;
  - (b) sieve no. 425;
  - (c) sieve no. 300;
- a mechanical sieve-shaker or a sieving machine may be used to sieve the grains;
- a permanent magnet;
- metal foil (e.g. aluminium, stainless steel);
- a hot-air oven, capable of maintaining a temperature of 140 ± 5 °C;
- a balance, capable of weighing up to 500 g with an accuracy of 0.005 g;
- a desiccator;
- an ultrasonic bath.

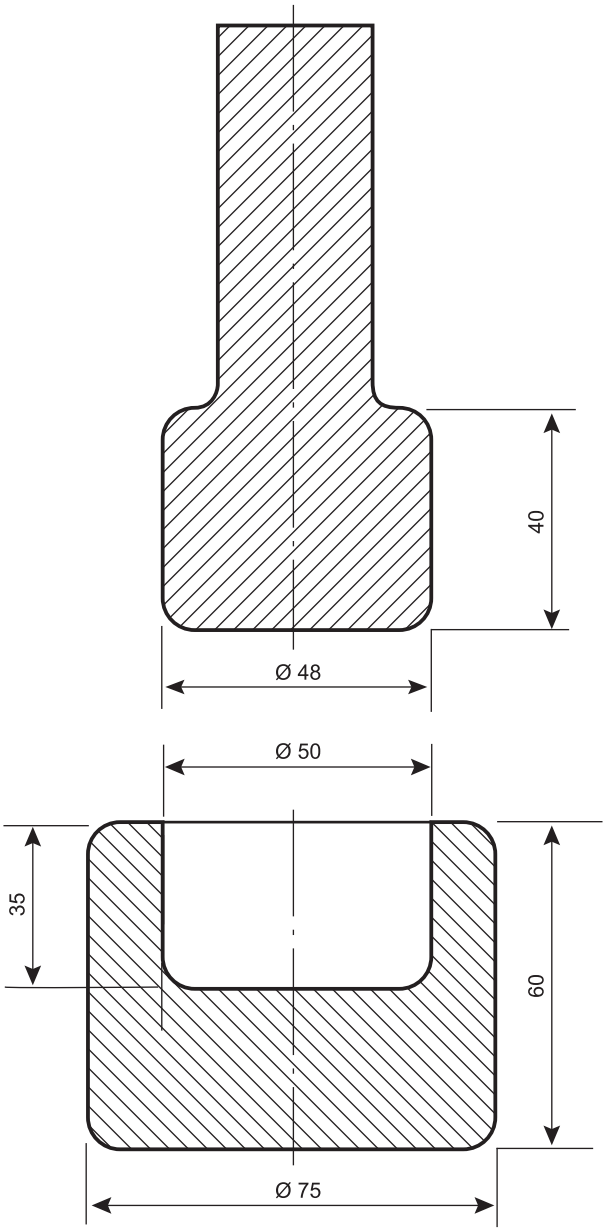


Figure 3.2.1.-2. – *Mortar and pestle apparatus for glass grains method (dimensions in millimetres)*

**Method**. Rinse the containers to be tested with *water R* and dry in the oven. Wrap at least 3 of the glass articles in clean paper and crush to produce 2 samples of about 100 g each, in pieces not more than 30 mm across.

Where a mortar, pestle and hammer are used, place in the mortar 30-40 g of the pieces 10-30 mm across taken from 1 of the samples, insert the pestle and strike it heavily, once only, with the hammer. Transfer the contents of the mortar to sieve (a), the coarsest of the set. Repeat the operation until all fragments have been transferred to the sieve. Shake the set of sieves for a short time by hand and remove the glass that remains on sieves (a) and (b). Submit these portions to further fracture, repeating the operation until about 10 g of glass remains on sieve (a). Reject this portion and the portion that passes through sieve (c). Reassemble the set of sieves and shake for 5 min. Transfer to a weighing bottle those glass grains that pass through sieve (b) and are retained on sieve (c).

Where a ball mill is used, place in the ball mill beaker about 50 g of the pieces 10-30 mm across taken from 1 of the samples, add the balls and crush thin-walled glass (wall thickness up to 1.5 mm) for up to 2 min and thick-walled glass (wall thickness greater than 1.5 mm) for up to 5 min. Transfer the grains to

sieve (a), sieve for about 30 s and collect the grains retained on sieve (c). Transfer the glass from sieves (a) and (b) into the ball mill and crush and sieve again as indicated above. Combine the grains retained on sieve (c).

Repeat the crushing and sieving procedure with the other glass sample and thus 2 samples of grains, each of which shall be in excess of 10 g, are obtained. Spread each sample on a piece of clean glazed paper and remove any iron particles by passing the magnet over them. Transfer each sample into a beaker for cleaning. Add to the grains in each beaker 30 mL of *acetone R* and scour the grains by suitable means, such as a rubber- or plastic-coated glass rod. After scouring the grains, allow to settle and decant as much acetone as possible. Add another 30 mL of *acetone R*, swirl, allow to settle and decant again, and add 30 mL of *acetone R*.

Fill the bath of the ultrasonic vessel with water at room temperature, then place the beaker in the rack and immerse it until the level of the acetone is at the level of the water; apply the ultrasound for 1 min. Swirl the beaker, allow to settle and decant the acetone as completely as possible, add 30 mL of *acetone R* and repeat the ultrasonic cleaning operation. If a fine turbidity persists, repeat the ultrasonic cleaning and acetone washing until the solution remains clear. Swirl and decant the acetone then dry the grains, first by putting the beaker on a warm plate to remove excess acetone and then by heating at 140 °C for 20 min in the drying oven. Transfer the dried grains from each beaker into separate weighing bottles, insert the stoppers and cool in the desiccator. Weigh 10.00 g of the cleaned and dried grains into 2 separate conical flasks. Add 50 mL of *water R1* into each by means of a pipette (test solutions). Pipette 50 mL of *water R1* into a 3rd conical flask as a blank. Distribute the grains evenly over the flat bases of the flasks by gentle shaking. Close the flasks with neutral glass dishes or aluminium foil rinsed with *water R*, or with inverted beakers so that the inner surface of the beakers fit snugly down onto the top rims of the flasks. Place all 3 flasks in the rack in the autoclave containing the water at room temperature, and ensure that they are held above the level of the water in the vessel. Carry out the autoclaving procedure in a similar manner to that described under test A, but maintain the temperature of 121 ± 1 °C only for 30 ± 1 min. Do not open the autoclave until it has cooled to 95 °C. Remove the hot samples from the autoclave and cool the flasks in running tap water as soon as possible, avoiding thermal shock. To each of the 3 flasks add 0.05 mL of *methyl red solution R*. Titrate the blank solution immediately with *0.02 M hydrochloric acid* then titrate the test solutions with the same acid until the colour matches that obtained with the blank solution. Subtract the titration volume for the blank solution from that for the test solution.

*NOTE: where necessary to obtain a sharp end-point, the clear solution is to be decanted into a separate 250 mL flask. Rinse the grains with 3 quantities, each of 15 mL, of water R1 by swirling and add the washings to the main solution. Add 0.05 mL of methyl red solution R. Titrate and calculate as described below. In this case also add 45 mL of water R1 and 0.05 mL of methyl red solution R to the blank solution.*

Calculate the mean value of the results in millilitres of *0.02 M hydrochloric acid* per gram of the sample and if required its equivalent in alkali extracted, calculated as micrograms of sodium oxide per gram of glass grains.

1 mL of *0.02 M hydrochloric acid* is equivalent to 620 µg of sodium oxide.

Repeat the test if the highest and lowest observed values differ by more than 20 per cent.

**Limits**. Type I glass containers require not more than 0.1 mL of *0.02 M hydrochloric acid* per gram of glass, type II and type III glass containers require not more than 0.85 mL of *0.02 M hydrochloric acid* per gram of glass.

#### TEST C. TO DETERMINE WHETHER THE CONTAINERS HAVE BEEN SURFACE-TREATED (ETCHING TEST)

If there are uncertainties whether a container has been surface-treated, and/or to distinguish between type I and type II glass containers, test C is used in addition to test A. Alternatively, tests A and B may be used. Test C may be carried out either on unused samples or on samples previously used in test A.

**Vials and bottles**. The volumes of test liquid required are shown in Table 3.2.1.-2.

Rinse the containers twice with *water R*, fill to the brimful point with a mixture of 1 volume of *hydrofluoric acid R* and 9 volumes of *hydrochloric acid R* and allow to stand for 10 min. Empty the containers and rinse carefully 5 times with *water R*. Immediately before the test, rinse once again with *water R*. Submit the containers thus prepared to the same autoclaving and determination procedure as described in Test A for surface hydrolytic resistance. If the results are considerably higher than those obtained from the original surfaces (by about a factor of 5 to 10), the samples have been surface-treated.

**Ampoules, cartridges and syringes**

*NOTE: ampoules, cartridges and syringes made from glass tubing are not normally subjected to internal surface treatment because their high chemical resistance is dependent upon the chemical composition of the glass as a material.*

Apply the test method as described above for vials and bottles. If the ampoules are not surface-treated, the new values are slightly lower than those obtained in previous tests.

**Distinction between type I and type II glass containers**

The results obtained in Test C are compared to those obtained in Test A. The interpretation of the result is shown in Table 3.2.1.-4.

Table 3.2.1.-4. – *Distinction between type I and type II glass containers*

| Type I | Type II |
|---|---|
| The values are closely similar to those found in the test for surface hydrolytic resistance for type I glass containers. | The values greatly exceed those found in the test for surface hydrolytic resistance and are similar to but not larger than those for type III glass containers. |

*ARSENIC*

*The test applies to glass containers for aqueous parenteral preparations.*

Hydride generation atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use the extraction solution obtained from containers of types I and II, after autoclaving at 121 °C for 1 h as described under Test A for surface hydrolytic resistance. Transfer 10.0 mL to a 100 mL volumetric flask. Add 10 mL of *hydrochloric acid R* and 5 mL of a 200 g/L solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *arsenic standard solution (1 ppm As) R*. Add 10 mL of *hydrochloric acid R* and 5 mL of a 200 g/L solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 mL with *water R*. The concentration range of the reference solutions is typically 0.005-0.015 ppm of As.

*Acid reservoir. Hydrochloric acid R.*

*Reducing reservoir. Sodium tetrahydroborate reducing solution R.*

Use a hydride generation device to introduce the test solution into the cuvette of the spectrometer. Establish and standardise instrumental operating conditions according to the manufacturer's instructions, optimise the uptake rate of the peristaltic pump, then connect it to the acid reservoir, the reducing reservoir and the test solution.

*Source*: hollow-cathode lamp.

*Wavelength*: 193.7 nm.

*Atomisation device*: air-acetylene flame.

*Limit*: maximum 0.1 ppm of As.

*SPECTRAL TRANSMISSION FOR COLOURED GLASS CONTAINERS*

**Equipment**. A UV-Vis spectrophotometer, equipped with a photodiode detector or equipped with a photomultiplier tube coupled with an integrating sphere.

**Preparation of the specimen**. Break the glass container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded-diamond wheel. Select sections representative of the wall thickness and trim them as suitable for mounting in a spectrophotometer. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion with opaque paper or tape, provided that the length of the specimen is greater than that of the slit. Before placing in the holder, wash, dry and wipe the specimen with a lens tissue. Mount the specimen with the aid of wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks.

**Method**. Place the specimen in the spectrophotometer with its cylindrical axis parallel to the slit and in such a way that the light beam is perpendicular to the surface of the section and that the losses due to reflection are at a minimum. Measure the transmission of the specimen with reference to air in the spectral region of 290-450 nm, continuously or at intervals of 20 nm.

**Limits**. The observed spectral transmission for coloured glass containers for preparations that are not for parenteral administration does not exceed 10 per cent at any wavelength in the range of 290-450 nm, irrespective of the type and the capacity of the glass container. The observed spectral transmission in coloured glass containers for parenteral preparations does not exceed the limits given in Table 3.2.1.-5.

Table 3.2.1.-5. – *Limits of spectral transmission for coloured glass containers for parenteral preparations*

| | Maximum percentage of spectral transmission at any wavelength between 290 nm and 450 nm | |
|---|---|---|
| **Nominal volume (mL)** | **Flame-sealed containers** | **Containers with closures** |
| Up to 1 | 50 | 25 |
| Above 1 and up to 2 | 45 | 20 |
| Above 2 and up to 5 | 40 | 15 |
| Above 5 and up to 10 | 35 | 13 |
| Above 10 and up to 20 | 30 | 12 |
| Above 20 | 25 | 10 |

## Annex – test for surface hydrolytic resistance – determination by flame spectrometry

*The surface hydrolytic resistance of glass of types I and II may be determined by analysis of the leaching solution by flame spectrometry. A number of elements that, when present as oxides in glass, contribute to the alkalinity of the solution, are determined and used to express an alkali equivalent. The spectrometric method has the advantage of allowing the use of a much smaller sample of extract so that it can be applied to small individual containers. This enables an evaluation of the uniformity of the containers in a given batch where this is critical. The results of this measurement are not equivalent to those of titrimetry and the 2 methods cannot be considered interchangeable. A correlation between the 2 is dependent on the type of glass and the size and shape of the container. The titrimetric method is the reference method of the Pharmacopoeia; the spectrometric method may be used in justified and authorised cases.*

*A method suitable for this type of analysis is shown below.*

The determination is carried out on unused containers. The number of containers to be examined is indicated in Table 3.2.1.-6.

Table 3.2.1.-6. – *Number of containers to be examined for the spectrometric method*

| Filling volume (mL) | Number of containers to be measured separately | Additional containers for preliminary measurements |
|---|---|---|
| Up to 2 | 20 | 2 |
| Above 2 and up to 5 | 15 | 2 |
| Above 5 and up to 30 | 10 | 2 |
| Above 30 and up to 100 | 5 | 1 |
| Above 100 | 3 | 1 |

Instructions on determination of the filling volume, cleaning of the containers, filling and heating are given above under Hydrolytic resistance and Test A.

*SOLUTIONS*

*Spectrochemical buffer solution.* Dissolve 80 g of *caesium chloride R* in about 300 mL of *water R1*, add 10mL of *6 M hydrochloric acid R*, dilute to 1.0 L with *water R1* and mix.

*Stock solutions*:

- sodium oxide, $c(Na_2O)$ = 1 mg/mL;
- potassium oxide, $c(K_2O)$ = 1 mg/mL;
- calcium oxide, $c(CaO)$ = 1 mg/mL.

Commercially available stock solutions may also be used.

*Standard solutions.* Prepare standard solutions by diluting the stock solutions with *water R1* to obtain concentrations suitable for establishing the reference solutions in an appropriate manner, e.g. with concentrations of 20 µg/mL of sodium oxide, potassium oxide and calcium oxide, respectively. Commercially available standard solutions may also be used.

*Reference solutions.* Prepare the reference solutions for establishing the calibration graph (set of calibration solutions) by diluting suitable concentrated standard solutions with *water R1*, so that the normal working ranges of the specific elements are covered, taking into account the instrument used for the measurement. Typical concentration ranges of the reference solutions are:

- for determination by atomic emission spectrometry of sodium oxide and potassium oxide: up to 10 µg/mL;
- for determination by atomic absorption spectrometry of sodium oxide and potassium oxide: up to 3 µg/mL;
- for determination by atomic absorption spectrometry of calcium oxide: up to 7 µg/mL.

Use reference solutions containing 5 per cent *V/V* of the spectrochemical buffer solution.

*METHOD*

Carry out preliminary measurements of the potassium oxide and calcium oxide concentrations on one of the extraction solutions. If, for one container type, the concentration of potassium oxide is less than 0.2 µg/mL and the concentration of calcium oxide is less than 0.1 µg/mL, the remaining extraction solutions of this container type need not be analysed for these ions. Aspirate the extraction solution from each sample directly into the flame of the atomic absorption or atomic emission instrument and determine the approximate concentrations of sodium oxide (and potassium oxide and calcium oxide, if present) by reference to calibration graphs produced from the reference solutions of suitable concentration.

*FINAL ANALYSIS*

If dilution is unnecessary, add to each container a volume of the spectrochemical buffer solution equivalent to 5 per cent of the filling volume, mix well and determine sodium oxide, calcium oxide and potassium oxide, by reference to calibration graphs. For the determination of the calcium oxide concentration by flame spectrometry, a nitrous oxide/acetylene flame is used.

If dilution is necessary, determine sodium oxide, calcium oxide and potassium oxide, if present, following the procedures as described above. The solutions shall contain 5 per cent *V/V* of the spectrochemical buffer solution. Concentration values less than 1.0 µg/mL are expressed to 2 decimal places, values greater than or equal to 1.0 µg/mL to 1 decimal place. Correct the result for the buffer addition and for any dilution.

*DETERMINATION*

Determine the mean value of the concentration of individual oxides found in the samples tested, in micrograms of the oxide per millilitre of the extraction solution, and calculate the sum of the individual oxides, expressed as micrograms of sodium oxide per millilitre of the extraction solution, using the following mass conversion factors:

- 1 µg of potassium oxide corresponds to 0.658 µg of sodium oxide;
- 1 µg of calcium oxide corresponds to 1.105 µg of sodium oxide.

**Limits**. The mean value is not greater than the value given in Table 3.2.1.-7.

Table 3.2.1.-7. – *Limit values in the test for surface hydrolytic resistance by flame spectrometry, for type I and type II glass containers*

| Filling volume (mL) | Limit values for the concentration of oxides, expressed as sodium oxide (µg/mL) |
|---|---|
| Up to 0.5 | 7.50 |
| Above 0.5 and up to 1 | 5.00 |
| Above 1 and up to 2 | 4.50 |
| Above 2 and up to 3 | 4.10 |
| Above 3 and up to 5 | 3.20 |
| Above 5 and up to 10 | 2.50 |
| Above 10 and up to 20 | 2.00 |
| Above 20 and up to 50 | 1.50 |
| Above 50 and up to 100 | 1.20 |
| Above 100 and up to 200 | 1.00 |
| Above 200 and up to 500 | 0.75 |
| Above 500 | 0.50 |

**04/2015:30202**

## 3.2.2. PLASTIC CONTAINERS AND CLOSURES FOR PHARMACEUTICAL USE

A plastic container for pharmaceutical use is a plastic article which contains or is intended to contain a pharmaceutical product and is, or may be, in direct contact with it. The closure is a part of the container.

Plastic containers and closures for pharmaceutical use are made of materials in which may be included certain additives; these materials do not include in their composition any substance that can be extracted by the contents in such quantities as to alter the efficacy or the stability of the product or to present a risk of toxicity.

The most commonly used polymers are polyethylene (with and without additives), polypropylene, poly(vinyl chloride), poly(ethylene terephthalate) and poly(ethylene-vinyl acetate).

The nature and amount of the additives are determined by the type of the polymer, the process used to convert the polymer into the container and the intended purpose of the container. Additives may consist of antioxidants, stabilisers, plasticisers, lubricants, colouring matter and impact modifiers. Antistatic agents and mould-release agents may be used only for containers for preparations for oral use or for external use for which they are authorised. Acceptable additives are indicated in the type specification for each material described in the Pharmacopoeia. Other additives may be used provided they are approved in each case by the competent authority responsible for the licensing for sale of the preparation.

For selection of a suitable plastic container, it is necessary to know the full manufacturing formula of the plastic, including all materials added during formation of the container so that the potential hazards can be assessed. In justified cases, further detailed information may be necessary to assess the impact on chronic use and for vulnerable patient groups. The plastic container chosen for any particular preparation should be such that:

- the ingredients of the preparation in contact with the plastic material are not significantly adsorbed on its surface and do not significantly migrate into or through the plastic,
- the plastic material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity.

Using material or materials selected to satisfy these criteria, a number of identical type samples of the container are made by a well-defined procedure and submitted to practical testing in conditions that reproduce those of the intended use, including, where appropriate, sterilisation. In order to confirm the compatibility of the container and the contents and to ensure that there are no changes detrimental to the quality of the preparation, various tests are carried out such as verification of the absence of changes in physical characteristics, assessment of any loss or gain through permeation, detection of pH changes, assessment of changes caused by light, chemical tests and, where appropriate, biological tests.

The method of manufacture is such as to ensure reproducibility for subsequent bulk manufacture and the conditions of manufacture are chosen so as to preclude the possibility of contamination with other plastic materials or their ingredients. The manufacturer of the product must ensure that containers made in production are similar in every respect to the type samples.

For the results of the testing on type samples to remain valid, it is important that:

- there is no change in the composition of the material as defined for the type samples,
- there is no change in the manufacturing process as defined for the type samples, especially as regards the temperatures to which the plastic material is exposed during conversion or subsequent procedures such as sterilisation,
- scrap material is not used.

Recycling of excess material of well-defined nature and proportions may be permitted after appropriate validation.

Subject to satisfactory testing for compatibility of each different combination of container and contents, the materials described in the Pharmacopoeia are recognised as being suitable for the specific purposes indicated, as defined above.

**01/2008:90003 corrected 6.0**

# 3.2.2.1. PLASTIC CONTAINERS FOR AQUEOUS SOLUTIONS FOR INFUSION

DEFINITION

Plastic containers for aqueous solutions for infusion are manufactured from one or more polymers, if necessary with additives. The containers described in this section are not necessarily suitable for emulsions. The polymers most commonly used are polyethylene, polypropylene and poly(vinyl chloride). The specifications of this text are to be read in conjunction with section *3.2.2. Plastic containers and closures for pharmaceutical use*.

The containers may be bags or bottles. They have a site suitable for the attachment of an infusion set designed to ensure a secure connection. They may have a site that allows an injection to be made at the time of use. They usually have a part that allows them to be suspended and which will withstand the tension occurring during use. The containers must withstand the sterilisation conditions to which they will be submitted. The design of the container and the method of sterilisation chosen are such that all parts of the containers that may be in contact with the infusion are sterilised. The containers are impermeable to micro-organisms after closure. The containers are such that after filling they are resistant to damage from accidental freezing which may occur during transport of the final preparation. The containers are and remain sufficiently transparent to allow the appearance of the contents to be examined at any time, unless otherwise justified and authorised.

The empty containers display no defects that may lead to leakage and the filled and closed containers show no leakage.

For satisfactory storage of some preparations, the container has to be enclosed in a protective envelope. The initial evaluation of storage has then to be carried out using the container enclosed in the envelope.

TESTS

**Solution S**. *Use solution S within 4 h of preparation*. Fill a container to its nominal capacity with *water R* and close it, if possible using the usual means of closure; otherwise close using a sheet of pure aluminium. Heat in an autoclave so that a temperature of 121 ± 2 °C is reached within 20 min to 30 min and maintain at this temperature for 30 min. If heating at 121 °C leads to deterioration of the container, heat at 100 °C for 2 h.

*Blank*. Prepare a blank by heating *water R* in a borosilicate-glass flask closed by a sheet of pure aluminium at the temperature and for the time used for the preparation of solution S.

**Appearance of solution S**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To a volume of solution S corresponding to 4 per cent of the nominal capacity of the container add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 mL of *0.01 M hydrochloric acid* and 0.1 mL of *methyl red solution R*. The solution is orange-red or red.

**Absorbance** (*2.2.25*). Measure the absorbance of solution S from 230 nm to 360 nm, using the blank (see solution S) as the compensation liquid. At these wavelengths, the absorbance is not greater than 0.20.

**Reducing substances**. To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil for 3 min. Cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a titration using 20.0 mL of the blank. The difference between the titration volumes is not greater than 1.5 mL.

**Transparency**. Fill a container previously used for the preparation of solution S with a volume equal to the nominal capacity of the primary opalescent suspension (*2.2.1*) diluted 1 in 200 for a container made from polyethylene or polypropylene and 1 in 400 for other containers. The cloudiness of the suspension is perceptible when viewed through the container and compared with a similar container filled with *water R*.

LABELLING

The label accompanying a batch of empty containers includes a statement of:

- the name and address of the manufacturer,
- a batch number which enables the history of the container and of the plastic material of which it is manufactured to be traced.

**07/2018:30209**

# 3.2.9. RUBBER CLOSURES FOR CONTAINERS FOR AQUEOUS PARENTERAL PREPARATIONS, FOR POWDERS AND FOR FREEZE-DRIED POWDERS

Rubber closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders are elastomers made of materials obtained by vulcanisation (cross-linking) of macromolecular organic substances, using appropriate additives. They cover all types of rubber closures including stoppers for vials, sealing disks and plunger stoppers for cartridges, as well as rubber tip caps, needle shields and plunger stoppers for syringes. The elastomers are polymers, obtained by chemical synthesis, or are polymers of natural origin. The choice of the principal components and of the various additives (for example, vulcanisers, accelerators, stabilisers, pigments) depends on the properties required for the finished article. The specifications apply to rubber closures made from rubber of 1 kind only, to coated closures, to bi-layer seals and to lubricated closures. Coated closures consist of a bulk of rubber, bearing on its surface or part of its surface a layer of a different polymer. Bi-layer seals are composed of 2 different layers of rubber, 1 of which exhibits a higher level of chemical purity and is intended for contact with a pharmaceutical preparation; the other layer exhibits a higher level of elasticity and is intended to improve self-sealing and fragmentation resistance of the seal. Lubricated rubber closures are closures treated with silicone oil (*3.1.8*) or other lubricants, for example materials chemically or mechanically bonded to the closures.

If closures are lubricated they comply in lubricated state with the requirements as defined in this general chapter. The specifications do not apply to closures made from silicone elastomer (which are dealt with in general chapter *3.1.9. Silicone elastomer for closures and tubing*).

Rubber closures may be classified in 2 types: type I closures meet the strictest requirements and are preferred; type II closures have mechanical properties suitable for special uses (for example, multiple piercing) and cannot meet requirements as severe as for type I closures because of their chemical composition.

The closures chosen for use with a particular preparation are such that:

- the components of the preparation in contact with the closures are not adsorbed onto the surface of the closures and do not migrate into or through the closures to an extent sufficient to affect the preparation adversely;
- the closures do not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity;
- the closures are compatible with the preparation for which they are used throughout its period of validity.

The manufacturer of the preparation must obtain from the supplier an assurance that the composition of the closure does not vary and that it is identical to that of the closure used during compatibility testing. If the supplier informs the manufacturer of the preparation that changes have been made to the composition, a risk assessment should be applied to determine the need to repeat the compatibility testing, totally or partly, depending on the nature of the changes.

The closures are washed and may be sterilised before use.

## CHARACTERS

Rubber closures are elastic. They are translucent or opaque and have no characteristic colour, the latter depending on the additives used. They are practically insoluble in tetrahydrofuran, in which, however, a considerable reversible swelling may occur. They are homogeneous and practically free from flash and adventitious materials (for example, fibres, foreign particles, waste rubber).

## IDENTIFICATION

*Identification of the type of rubber used for the closures is not within the scope of this specification. The identification tests given below distinguish between closures made from rubber and those made from silicone elastomer and plastic materials but do not differentiate all types of rubber. Other identity tests may be carried out with the aim of detecting differences in a batch compared with the closures used for compatibility testing. One or more of the following analytical methods may be applied for this purpose: determination of relative density, determination of sulfated ash, determination of sulfur content, thin-layer chromatography carried out on an extract, ultraviolet absorption spectrophotometry of an extract, infrared absorption spectrophotometry of a pyrolysate or attenuated total reflectance (ATR).*

A. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflectance (ATR).

   If necessary, cut the sample along an appropriate axis and examine the cut surface. For coated, bi-layer and lubricated closures, perform the test for each different part of the closure. This is not required for silicone oil used as lubricant. Identification of silicone oil can be performed prior to it being used.

   *Comparison*: type sample.

   If direct ATR measurement on the surface is not feasible (mainly rubber closures filled with carbon black), heat an appropriate amount of rubber in a heat-resistant test-tube over an open flame to dry the sample and continue heating until pyrolysate vapours are condensed near the top edge of the test-tube. Examine the pyrolysate of the sample by ATR and compare the spectrum with that obtained with the pyrolysate of the type sample.

B. Total ash (*2.4.16*).

   If the sample has not been subjected to steam sterilisation, drying at 100-105 °C can be omitted. Determine the percentage content of total ash in the sample to be examined and compare with the percentage content of total ash in the type sample ($A_0$). The total ash content falls within the following ranges depending on the total ash content of the type sample, or, if not available, in the range defined as the target for the specific rubber type.

| Total ash in the type sample, $A_0$ (per cent) | Limit for total ash in the sample (per cent) |
|---|---|
| $A_0 \leq 5.0$ | $(A_0 - 0.75)$ to $(A_0 + 0.75)$ |
| $5.0 < A_0 \leq 10$ | $(A_0 - 1.0)$ to $(A_0 + 1.0)$ |
| $A_0 > 10$ | $(A_0 - 2.0)$ to $(A_0 + 2.0)$ |

   In addition to the use of platinum and silica crucibles described in general chapter *2.4.16*, porcelain crucibles may be used. The sample may be ignited using a microwave oven instead of a muffle furnace.

## TESTS

**Solution S**. Place a number of uncut closures with a total surface area of about 100 $cm^2$ in a wide-necked flask (type I glass, *3.2.1*), add 200 mL of *water R* and weigh. Cover the mouth of the flask with a borosilicate-glass beaker. Heat in an autoclave so that a temperature of 121 ± 2 °C is reached within 20-30 min and maintain at this temperature for 30 min. Immerse the temperature probe for the autoclave programme-control in water in a container comparable to that used for the sample. Cool to room temperature over about 30 min. Make up to the original mass with *water R*. Shake and decant the solution immediately. Shake solution S before each test. If using a tightly closed flask (type I glass, *3.2.1*) with an inert closure instead of a wide-necked flask covered with a borosilicate-glass beaker, it is not necessary to make up to the original mass.

*Blank solution*. Prepare a blank solution in the same manner using 200 mL of *water R*.

**Appearance of solution S**. Solution S is not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*). For type I closures, solution S is not more opalescent than reference suspension II (*2.2.1*) and for type II closures, solution S is not more opalescent than reference suspension III. In case of nephelometric determination, the limit for type I closures is 6 NTU and the limit for type II closures is 18 NTU.

**Acidity or alkalinity**. To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Carry out a titration using 20.0 mL of the blank (see solution S). Not more than 0.3 mL of *0.01 M sodium hydroxide* or 0.8 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to blue or yellow, respectively. If after adding the indicator the solution is green, it is neutral and no titration is needed.

**Absorbance**. *Carry out the test within 5 h of preparation of solution S.* Filter solution S through a membrane filter (nominal pore size 0.45 µm), rejecting the first few millilitres of filtrate. Measure the absorbance (*2.2.25*) of the filtrate at wavelengths from 220-360 nm using the blank (see solution S) as compensation liquid: absorbance within the 220-360 nm range does not exceed 0.2 for type I closures or 4.0 for type II closures. If necessary, dilute the filtrate before measurement of the absorbance and correct the result for the dilution.

**Reducing substances**. *Carry out the test within 4 h of preparation of solution S.* To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil for 3 min. Cool. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a titration using 20.0 mL of the blank (see solution S). The difference between the titration volumes is not greater than 3.0 mL for type I closures and 7.0 mL for type II closures.

**Ammonium** (*2.4.1, Method A*): maximum 2 ppm.

Dilute 5 mL of solution S to 14 mL with *water R*.

**Extractable zinc**: maximum 5 µg of extractable Zn per millilitre of solution S.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S. If results are outside the calibration range, dilute 10.0 mL of solution S to an appropriate volume with *0.1 M hydrochloric acid*.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R* diluted with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

**Extractable heavy metals** (*2.4.8*): maximum 2 ppm.

Solution S complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

**Residue on evaporation**. Evaporate 50.0 mL of solution S to dryness on a water-bath and dry at 100-105 °C. The residue weighs not more than 2.0 mg for type I closures and not more than 4.0 mg for type II closures.

**Volatile sulfides**. Place closures, cut if necessary, with a total surface area of 20 ± 2 $cm^2$ in a 100 mL conical flask and add 50 mL of a 20 g/L solution of *citric acid monohydrate R*. Place a piece of *lead acetate paper R* over the mouth of the flask and maintain the paper in position by placing over it an inverted weighing bottle. Heat in an autoclave at 121 ± 2 °C for 30 min. Any black stain on the paper is not more intense than that of a standard, treated in the same manner, prepared by mixing 50 mL of a 20 g/L solution of *citric acid monohydrate R* and 5.0 mL of a freshly prepared 0.0308 g/L solution of *sodium sulfide R*.

The tests for penetrability, fragmentation and self-sealing are performed on whole closures.

*For the tests for penetrability, fragmentation and self-sealing, treat non-sterilised closures as described for the preparation of solution S and allow to dry.* To perform these 3 tests, use for each closure a new, lubricated, long-bevel(1) (bevel angle 12 ± 2°) hypodermic needle with an external diameter of 0.8 mm and pierce the closures with the needle perpendicular to the surface without rotating the needle.

**Penetrability**. For closures intended to be pierced by a hypodermic needle, carry out the following test. Fill 10 suitable vials to the nominal volume with *water R*, fit the closures to be examined and secure with a cap. The force required for piercing, determined with an accuracy of ± 0.25 N, is not greater than 10 N for each closure.

**Fragmentation**. For closures intended to be pierced by a hypodermic needle, carry out the following test. If the closures are to be used for aqueous preparations, introduce in 12 clean vials a volume of *water R* corresponding to the nominal volume minus 4 mL, close the vials with the closures to be examined, secure with a cap and allow to stand for 16 h. If the closures are to be used with dry preparations, close 12 clean vials with the closures to be examined. Using a needle fitted to a clean syringe, inject into the vial 1 mL of *water R* and remove 1 mL of air; carry out this operation 4 times for each closure, piercing the closure each time at a different site. Use a new needle for each closure and check that the needle is not blunted during the test. Pass the liquid in the vials through a filter with a pore size of 0.5 µm. Count the fragments of rubber visible to the naked eye. The total number of fragments does not exceed 5. This limit is based on the assumption that fragments with a diameter equal to or greater than 50 µm are visible to the naked eye; in cases of doubt or dispute, the fragments are examined with a microscope to verify their nature and size.

**Self-sealing test**. For closures intended to be used with multidose containers, carry out the following test. Fill 10 vials matching the design of the stopper to the nominal volume with *water R*, fit the closures to be examined, secure with a cap and crimp tightly. Pierce each closure 10 times, piercing the closure each time at a different site. Immerse the vials upright in a 1 g/L solution of *methylene blue R* and reduce the external pressure by 27 kPa for 10 min. Restore atmospheric pressure and leave the vials immersed for 30 min. Rinse the outside of the vials. None of the vials contains any trace of coloured solution.

(1) See ISO 7864, *Sterile hypodermic needles for single use.*

# 3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes



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01/2020:30301

# 3.3.1. MATERIALS FOR CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

*This general chapter is published for information.*

*NOTE: for materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion, see text 3.1.14.*

Plastic containers for the collection, storage, processing and administration of blood and its components may be manufactured from one or more polymers, if necessary with certain additives.

If all or part of the container consists of a material described in a text of the Pharmacopoeia, the quality of the material is controlled by the methods indicated in that text. (See *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components*).

In normal conditions of use the materials and containers made from such materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood or blood components.

01/2020:30302

# 3.3.2. MATERIALS BASED ON PLASTICISED POLY(VINYL CHLORIDE) FOR CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

*This general chapter is published for information.*

DEFINITION

Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components are defined by the nature and the proportions of the substances used in their manufacture.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

**Vinyl chloride**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

*Test solution.* Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

*Vinyl chloride primary solution. Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

*Vinyl chloride standard solution*: vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

*Reference solutions.* Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain, respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL.

*Limit*:

- *vinyl chloride*: maximum 1 ppm.

**Additives**

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:

- di(2-ethylhexyl)phthalate (plastic additive 01): maximum 40 per cent;
- zinc octanoate (zinc 2-ethylhexanoate) (plastic additive 02): maximum 1 per cent;
- calcium stearate or zinc stearate: maximum 1 per cent or 1 per cent of a mixture of the two;
- *N,N′*-diacylethylenediamines (plastic additive 03): maximum 1 per cent;
- one of the following epoxidised oils: maximum 10 per cent or 10 per cent of a mixture of the two:
  - epoxidised soya oil (plastic additive 04), of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6;
  - epoxidised linseed oil (plastic additive 05), of which the oxiran oxygen content is not greater than 10 per cent and the iodine value is not greater than 7;
- cyclohexane 1,2-dicarboxylic acid, diisononyl ester (plastic additive 24): maximum 45 per cent;
- butyryl tri-*n*-hexyl citrate (plastic additive 25): maximum 45 per cent;
- tris(2-ethylhexyl) trimellitate (plastic additive 26): maximum 45 per cent;

– bis(2-ethylhexyl) terephthalate (plastic additive 27): maximum 45 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

Blood and blood components have different requirements, for example with respect to gas exchange, storage temperature and the mechanical properties of the containers. In addition, the stability and the quality of blood or blood components stored in containers can be influenced by the plasticisers/additives present in the materials used in the composition of the containers. To ensure the stability of blood and blood components during their manufacture and storage, the materials from which the containers are composed must be carefully selected according to the intended use.

CHARACTERS

Almost colourless or pale yellow powder, beads, granules or, after transformation, translucent sheets of varying thickness, with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

To 2.0 g of the material to be examined add 200 mL of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue (residue B) and the solution (solution A) by filtration.

Evaporate solution A to dryness under reduced pressure in a water-bath at 30 °C. Dissolve the residue in 10 mL of *toluene R* (solution A1). Dissolve residue B in 60 mL of *ethylene chloride R*, heating on a water-bath under a reflux condenser. Filter. Add the solution obtained dropwise and with vigorous shaking to 600 mL of *heptane R* heated almost to boiling. Separate the coagulum (coagulum B1) and the organic solution by hot filtration. Allow the latter to cool; separate the precipitate that forms (precipitate B2) and filter through a tared sintered-glass filter (40) (*2.1.2*).

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve coagulum B1 in 30 mL of *tetrahydrofuran R* and add, in small volumes with shaking, 40 mL of *anhydrous ethanol R*; separate the precipitate (precipitate B3) by filtration and dry *in vacuo* at a temperature not exceeding 50 °C over *diphosphorus pentoxide R*; dissolve a few milligrams of precipitate B3 in 1 mL of *tetrahydrofuran R*, place a few drops of the solution obtained on a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

*Comparison*: *poly(vinyl chloride) CRS*.

B. Plastic additives 01, 24, 25, 26 and 27 (see Tests).

TESTS

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

**Solution S1**. Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

**Solution S2**. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool, decant the solution and dilute to 500 mL with *water R*.

**Appearance of solution S2**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

**Absorbance** (*2.2.25*). Evaporate 100.0 mL of solution S2 to dryness. Dissolve the residue in 5.0 mL of *hexane R*. From 250 nm to 310 nm the absorbance is not greater than 0.25.

**Reducing substances**. *Carry out the test within 4 h of preparation of solution S2.* To 20.0 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R*. The difference between the 2 titration volumes is not more than 2.0 mL.

**Primary aromatic amines**: maximum 20 ppm.

To 2.5 mL of solution A1 obtained during the identification, add 6 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*. Shake vigorously and discard the upper layer. To the aqueous layer add 0.4 mL of a freshly prepared 10 g/L solution of *sodium nitrite R*. Mix and allow to stand for 1 min. Add 0.8 mL of a 25 g/L solution of *ammonium sulfamate R*, allow to stand for 1 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. After 30 min, any colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner, replacing the aqueous layer with a mixture of 1 mL of a 0.01 g/L solution of *naphthylamine R* in *0.1 M hydrochloric acid*, 5 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*.

**Plastic additives 01, 24, 25, 26 and 27**. Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution S3*: 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

*Internal standard solution S4*: 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

*Test solution.* Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

*Stock solution (a).* Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (b).* Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (c).* Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (d).* Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (e).* Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Reference solutions (a1)-(a5).* Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

*Reference solutions (b1)-(b5).* Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS*.

*Reference solutions (c1)-(c5).* Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS*.

*Reference solutions (d1)-(d5).* Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS*.

*Reference solutions (e1)-(e5).* Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3.3 | 100 → 200 |
| | 3.3 - 20 | 200 → 250 |
| | 20 - 22.5 | 250 |
| | 22.5 - 23 | 250 → 270 |
| | 23 - 25 | 270 |
| | 25 - 25.6 | 270 → 320 |
| | 25.6 - 30.6 | 320 |
| Injection port | | 300 |

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] |
|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 |
| Plastic additive 24 | 155 | 127 | 299 |
| Plastic additive 25 | 71 | 213 | 315 |
| Plastic additive 26 | 305 | 193 | 323 |
| Plastic additive 27 | 261 | 149 | 167 |
| DnOP (internal standard) | 149 | 279 | 167 |

*Injection*: 1 µL.

*Relative retention* with reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1.09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] | Ion ratio 2/1 (%) | Ion ratio 3/1 (%) |
|---|---|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 | 50 | 30 |
| Plastic additive 24 | 155 | 127 | 299 | 30 | 13 |
| Plastic additive 25 | 71 | 213 | 315 | 45 | 20 |
| Plastic additive 26 | 305 | 193 | 323 | 55 | 20 |
| Plastic additive 27 | 261 | 149 | 167 | 130 | 85 |
| DnOP (internal standard) | 149 | 279 | 167 | / | / |

*System suitability*:

- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

*Limits*:

- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

**Plastic additive 03**. Wash precipitate B2 obtained during the identification and contained in the tared sintered-glass filter (40) (*2.1.2*) with *anhydrous ethanol R*. Dry to constant mass over *diphosphorus pentoxide R* and weigh the filter. The residue weighs not more than 20 mg.

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: the residue obtained above. When the amount of residue is insufficient to prepare a disc, record the spectrum of the residue placed between 2 plates transparent to infrared radiation or examine by attenuated total reflectance (ATR).

*Comparison*: *plastic additive 03 CRS*.

**Plastic additives 04 and 05**. Thin-layer chromatography (*2.2.27*).

*Reference solutions.* Prepare 10 mg/mL solutions of *plastic additive 04 CRS* and *plastic additive 05 CRS*, respectively, in *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *toluene R*.

*Application*: 0.5 mL of solution A1 obtained during the identification, as a band 30 mm by 3 mm, and 5 µL of each reference solution.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour for 5 min.

Examine the chromatogram and locate the zone corresponding to plastic additives 04 and 05 ($R_F$ = 0). Remove the area of silica gel corresponding to this zone. Similarly remove a

corresponding area of silica gel as a blank reference. Separately shake both samples for 15 min with 40 mL of *methanol R*. Filter, rinse with 2 quantities, each of 10 mL, of *methanol R*, add the rinsings to the filtrate and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

**Barium**: maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid*.

*Reference solution.* A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

**Cadmium**: maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Evaporate 10 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

**Calcium**: maximum 0.07 per cent.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Use the test solution prepared for the determination of barium.

*Reference solution.* A solution containing 50.0 ppm of calcium prepared by dilution of *calcium standard solution (400 ppm Ca) R* with *0.1 M hydrochloric acid*.

*Wavelength*: use the emission of calcium at 315.89 nm, the spectral background being taken at 315.60 nm.

Verify the absence of calcium in the hydrochloric acid used.

**Tin**: maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Dilute solution S1 10-fold with *water R* immediately before use.

*Reference solution.* Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

*Wavelength*: use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulfuric acid used.

**Zinc**: maximum 0.2 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute solution S1 100-fold with *0.1 M hydrochloric acid*.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

**Heavy metals** (*2.4.8*): maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

**Water extractable substances**: maximum 0.3 per cent.

Evaporate 50 mL of solution S2 to dryness on a water-bath and dry in an oven at 100-105 °C to constant mass. Carry out a blank test with 50.0 mL of *water R*. The residue weighs not more than 7.5 mg taking into account the blank test.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

*Additional tests for sterile plastic containers for human blood and blood components are described in chapter 3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes and relevant subsections.*

*An additional test for the absorbance of an anticoagulant solution is described in general chapter 3.3.6. Sterile containers of plasticised poly(vinyl chloride) for human blood containing anticoagulant solution.*

**01/2020:30303**

# 3.3.3. MATERIALS BASED ON PLASTICISED POLY(VINYL CHLORIDE) FOR TUBING USED IN SETS FOR THE TRANSFUSION OF BLOOD AND BLOOD COMPONENTS

*This general chapter is published for information.*

DEFINITION

Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components are defined by the nature and the proportions of the substances used in their manufacture.

Connecting ports are also to be considered as tubing.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

**Vinyl chloride**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

*Test solution*. Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

*Vinyl chloride primary solution. Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

*Vinyl chloride standard solution*: vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

*Reference solutions*. Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL.

*Limit*:

- *vinyl chloride*: maximum 1 ppm.

**Additives**

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:

- di(2-ethylhexyl)phthalate (plastic additive 01): maximum 40 per cent;
- cyclohexane 1,2-dicarboxylic acid, diisononyl ester (plastic additive 24): maximum 45 per cent;
- butyryl tri-*n*-hexyl citrate (plastic additive 25): maximum 45 per cent;
- tris(2-ethylhexyl) trimellitate (plastic additive 26): maximum 45 per cent;
- bis(2-ethylhexyl) terephthalate (plastic additive 27): maximum 45 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

Blood and blood components have different requirements, for example with respect to gas exchange, storage temperature and the mechanical properties of the tubing. In addition, the stability and the quality of blood or blood components during transfusion can be influenced by the plasticisers/additives present in the materials used in the composition of the tubing. To ensure the stability of blood and blood components during transfusion, the materials from which the tubing is composed must be carefully selected according to the intended use.

CHARACTERS

Almost colourless or pale-yellow material in the form of powder, beads, granules or, after transformation, tubes with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*. To 0.5 g of the material to be examined add 30 mL of *tetrahydrofuran R*. Heat with stirring on a water-bath in a fume cupboard for 10 min; the material dissolves completely. Add *methanol R* dropwise with stirring; a granular precipitate is formed. Filter the precipitate and dry at 60 °C. Examine the precipitate by infrared absorption spectrophotometry (*2.2.24*). Dissolve 50 mg in 2 mL of *tetrahydrofuran R* and pour on a glass slide. Dry in an oven at 80 °C, remove the film and fix on a suitable mount. Examine by infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *poly(vinyl chloride) CRS*.

B. Plastic additives 01, 24, 25, 26 and 27 (see Tests).

TESTS

*If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.*

**Solution S1**. Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

**Solution S2**. Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool. Decant the solution and dilute to 500 mL with *water R*.

**Appearance of solution S2**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Plastic additives 01, 24, 25, 26 and 27**. Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution S3*: 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

*Internal standard solution S4*: 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

*Test solution*. Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

*Stock solution (a).* Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (b).* Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (c).* Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (d).* Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (e).* Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Reference solutions (a1)-(a5).* Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS.*

*Reference solutions (b1)-(b5).* Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS.*

*Reference solutions (c1)-(c5).* Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS.*

*Reference solutions (d1)-(d5).* Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS.*

*Reference solutions (e1)-(e5).* Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS.*

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3.3 | 100 → 200 |
| | 3.3 - 20 | 200 → 250 |
| | 20 - 22.5 | 250 |
| | 22.5 - 23 | 250 → 270 |
| | 23 - 25 | 270 |
| | 25 - 25.6 | 270 → 320 |
| | 25.6 - 30.6 | 320 |
| Injection port | | 300 |

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] |
|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 |
| Plastic additive 24 | 155 | 127 | 299 |
| Plastic additive 25 | 71 | 213 | 315 |
| Plastic additive 26 | 305 | 193 | 323 |
| Plastic additive 27 | 261 | 149 | 167 |
| DnOP (internal standard) | 149 | 279 | 167 |

*Injection*: 1 µL.

*Relative retention* with reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1,09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] | Ion ratio 2/1 (%) | Ion ratio 3/1 (%) |
|---|---|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 | 50 | 30 |
| Plastic additive 24 | 155 | 127 | 299 | 30 | 13 |
| Plastic additive 25 | 71 | 213 | 315 | 45 | 20 |
| Plastic additive 26 | 305 | 193 | 323 | 55 | 20 |
| Plastic additive 27 | 261 | 149 | 167 | 130 | 85 |
| DnOP (internal standard) | 149 | 279 | 167 | / | / |

*System suitability*:

- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

*Limits*:

- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

**Barium**: maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid.*

*Reference solution.* A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid.*

*Wavelength*: use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

**Cadmium**: maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Evaporate 10.0 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

**Tin**: maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Dilute solution S1 10-fold with *water R* immediately before use.

*Reference solution.* Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

*Wavelength*: use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulfuric acid used.

**Heavy metals** (*2.4.8*): maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

ASSAY

To 0.500 g of the material to be examined add 30 mL of *tetrahydrofuran R* and heat with stirring on a water-bath in a fume cupboard for 10 min. The material dissolves completely. Add 60 mL of *methanol R* dropwise with stirring. A granular precipitate of poly(vinyl chloride) is formed. Allow to stand for a few minutes. Continue addition of *methanol R* until no further precipitation is observed. Transfer to a sintered-glass filter (40) (*2.1.2*), using 3 small quantities of *methanol R* to aid transfer and to wash the precipitate. Dry the filter and the precipitate to constant mass at 60 °C and weigh.

*Additional tests for sterilised sets are described in general chapter 3.3.7. Sets for the transfusion of blood and blood components.*

**01/2020:30304**

## 3.3.4. STERILE PLASTIC CONTAINERS FOR HUMAN BLOOD AND BLOOD COMPONENTS

Plastic containers for the collection, storage, processing and administration of blood and its components are manufactured from one or more polymers, if necessary with additives. The composition and the conditions of manufacture of the containers are registered by the appropriate competent authorities in accordance with the relevant national legislation and international agreements.

When the composition of the materials of the different parts of the containers corresponds to the appropriate specifications, their quality is controlled by the methods indicated in those specifications (see *3.1. Materials used for the manufacture of containers* and subsections and *3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes* and subsections).

Materials other than those described in the Pharmacopoeia may be used provided that their composition is authorised by the competent authority and that the containers manufactured from them comply with the requirements prescribed in this general chapter.

In normal conditions of use the materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood.

The containers may contain anticoagulant solutions, depending on their intended use, and are supplied sterile.

Each container is fitted with attachments suitable for the intended use. The container may be in the form of a single unit or the collecting container may be connected by one or more tubes to one or more secondary containers to allow separation of the blood components to be effected within a closed system.

The outlets are of a shape and size allowing for adequate connection of the container with the blood-giving equipment. The protective coverings on the blood-taking needle and on the appendages must be such as to ensure the maintenance of sterility. They must be easily removable but must be tamper-evident.

The capacity of the containers is related to the nominal capacity prescribed by the national authorities and to the appropriate volume of anticoagulant solution. The nominal capacity is the volume of blood to be collected in the container. The containers are of a shape such that when filled they may be centrifuged.

The containers are fitted with a suitable device for suspending or fixing which does not hinder the collection, storage, processing or administration of the blood.

The containers are enclosed in sealed, protective envelopes.

CHARACTERS

The container is sufficiently transparent to allow adequate visual examination of its contents before and after the taking of the blood and is sufficiently flexible to offer minimum resistance during filling and emptying under normal conditions of use. The container contains not more than 5 mL of air.

TESTS

**Solution $S_1$.** Fill the container with 100 mL of a sterile, pyrogen-free 9 g/L solution of *sodium chloride R*. Close the container and heat it in an autoclave so that the contents are maintained at 110 °C for 30 min.

If the container to be examined contains an anticoagulant solution, first empty it, rinse the container with 250 mL of *water for injections R* at 20 ± 1 °C and discard the rinsings.

**Solution $S_2$.** Introduce into the container a volume of *water for injections R* corresponding to the intended volume of anticoagulant solution. Close the container and heat it in an autoclave so that the contents are maintained at 110 °C for 30 min. After cooling, add sufficient *water for injections R* to fill the container to its nominal capacity.

If the container to be examined contains an anticoagulant solution, first empty it and rinse it as indicated above.

**Resistance to centrifugation**. Introduce into the container a volume of *water R*, acidified by the addition of 1 mL of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Envelop the container with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue solution R1* or other suitable indicator and then dried. Centrifuge at 5000 *g* for 10 min. No leakage perceptible on the indicator paper and no permanent distortion occur.

**Resistance to stretch**. Introduce into the container a volume of *water R*, acidified by the addition of 1 mL of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Suspend the container by the suspending device at the opposite end from the blood-taking tube and apply along the axis of this tube an immediate force of 20 N (2.05 kgf). Maintain the traction for 5 s. Repeat the test with the force applied to each of the parts for filling and emptying. No break and no deterioration occur.

**Leakage**. Place the container which has been submitted to the stretch test between two plates covered with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue solution R1* or other suitable indicator and then dried. Progressively apply force to the plates to press the container so that its internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) reaches 67 kPa within 1 min. Maintain the pressure for 10 min. No signs of leakage are detectable on the indicator paper or at any point of attachment (seals, joints, etc.).

**Vapour permeability**. For a container containing an anticoagulant solution, fill with a volume of a 9 g/L solution of *sodium chloride R* equal to the volume of blood for which the container is intended.

For an empty container, fill with the same mixture of anticoagulant solution and sodium chloride solution. Close the container, weigh it and store it at 5 ± 1 °C in an atmosphere with a relative humidity of (50 ± 5) per cent for 21 days. At the end of this period the loss in mass is not greater than 1 per cent.

**Emptying under pressure**. Fill the container with a volume of *water R* at 5 ± 1 °C equal to the nominal capacity. Attach a transfusion set without an intravenous cannula to one of the connectors. Compress the container so as to maintain throughout the emptying an internal pressure (i.e the difference between the applied pressure and atmospheric pressure) of 40 kPa. The container empties in less than 2 min.

**Speed of filling**. Attach the container by means of the blood-taking tube fitted with the needle to a reservoir containing a suitable solution having a viscosity equal to that of blood, such as a 335 g/L solution of *sucrose R* at 37 °C. Maintain the internal pressure of the reservoir (i.e. the difference between the applied pressure and atmospheric pressure) at 9.3 kPa with the base of the reservoir and the upper part of the container at the same level. The volume of liquid which flows into the container in 8 min is not less than the nominal capacity of the container.

**Resistance to temperature variations**. Place the container in a suitable chamber having an initial temperature of 20-23 °C. Cool it rapidly in a deep-freeze to – 80 °C and maintain it at this temperature for 24 h. Raise the temperature to 50 °C and maintain for 12 h. Allow to cool to room temperature. The container complies with the tests for resistance to centrifugation, resistance to stretch, leakage, vapour permeability emptying under pressure and speed of filling prescribed above.

**Transparency**. Fill the empty container with a volume equal to its nominal capacity of the primary opalescent suspension (*2.2.1*) diluted so as to have an absorbance (*2.2.25*) at 640 nm of 0.37 to 0.43 (dilution factor about 1 in 16). The cloudiness of the suspension must be perceptible when viewed through the bag, as compared with a similar container filled with *water R*.

**Extractable matter**. Tests are carried out by methods designed to simulate as far as possible the conditions of contact between the container and its contents which occur in conditions of use.

The conditions of contact and the tests to be carried out on the eluates are prescribed, according to the nature of the constituent materials, in the particular requirements for each type of container.

**Haemolytic effects in buffered systems**

*Stock buffer solution*. Dissolve 90.0 g of *sodium chloride R*, 34.6 g of *disodium hydrogen phosphate dodecahydrate R* and 2.43 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

*Buffer solution* $A_0$. To 30.0 mL of stock buffer solution add 10.0 mL of *water R*.

*Buffer solution* $B_0$. To 30.0 mL of stock buffer solution add 20.0 mL of *water R*.

*Buffer solution* $C_0$. To 15.0 mL of stock buffer solution add 85.0 mL of *water R*.

Introduce 1.4 mL of solution $S_2$ into each of three centrifuge tubes. To tube I add 0.1 mL of buffer solution $A_0$, to tube II add 0.1 mL of buffer solution $B_0$ and to tube III add 0.1 mL of buffer solution $C_0$. To each tube add 0.02 mL of fresh, heparinised human blood, mix well and warm on a water-bath at 30 ± 1 °C for 40 min. Use blood collected less than 3 h previously or blood collected into an anticoagulant citrate-phosphate-dextrose solution (CPD) less than 24 h previously.

Prepare three solutions containing, respectively:

3.0 mL of buffer solution $A_0$ and 12.0 mL of *water R* (solution $A_1$),

4.0 mL of buffer solution $B_0$ and 11.0 mL of *water R* (solution $B_1$),

4.75 mL of buffer solution $B_0$ and 10.25 mL of *water R* (solution $C_1$).

To tubes I, II and III add, respectively, 1.5 mL of solution $A_1$, 1.5 mL of solution $B_1$ and 1.5 mL of solution $C_1$. At the same time and in the same manner, prepare three other tubes, replacing solution $S_2$ by *water R*. Centrifuge simultaneously the tubes to be examined and the control tubes at exactly 2500 *g* in the same horizontal centrifuge for 5 min. After centrifuging, measure the absorbances (*2.2.25*) of the liquids at 540 nm using the stock buffer solution as compensation liquid. Calculate the haemolytic value as a percentage from the expression:

$$\frac{A_{exp}}{A_{100}} \times 100$$

$A_{100}$ = absorbance of tube III;

$A_{exp}$ = absorbance of tube I or II or of the corresponding control tubes.

The solution in tube I gives a haemolytic value not greater than 10 per cent and the haemolytic value of the solution in tube II does not differ by more than 10 per cent from that of the corresponding control tube.

**Sterility** (*2.6.1*). The containers comply with the test for sterility. Introduce aseptically into the container 100 mL of a sterile 9 g/L solution of sodium chloride and shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents of the container through a membrane filter and place the membrane in the appropriate culture medium, as prescribed in the test for sterility.

**Pyrogens** (*2.6.8*). Solution $S_1$ complies with the test for pyrogens. Inject 10 mL of the solution per kilogram of the rabbit's mass.

PACKAGING

The containers are packed in protective envelopes.

On removal from its protective envelope the container shows no leakage and no growth of micro-organisms. The protective envelope is sufficiently robust to withstand normal handling.

The protective envelope is sealed in such a manner that it cannot be opened and re-closed without leaving visible traces that the seal has been broken.

LABELLING

The labelling complies with the relevant national legislation and international agreements. The label states:

- the name and address of the manufacturer;
- a batch number which enables the history of the container and of the plastic material of which it is manufactured to be traced.

A part of the label is reserved for:

- the statement of the blood group, the reference number and all other information required by national legislation or international agreements, and an empty space is provided for the insertion of supplementary labelling.

The label of the *protective envelope* or the *label* on the container, visible through the envelope, states:

- the expiry date;
- that, once withdrawn from its protective envelope, the container must be used within 10 days.

The ink or other substance used to print the labels or the writing must not diffuse into the plastic material of the container and must remain legible up to the time of use.

**01/2020:30305**

# 3.3.5. EMPTY STERILE CONTAINERS OF PLASTICISED POLY(VINYL CHLORIDE) FOR HUMAN BLOOD AND BLOOD COMPONENTS

*This general chapter is published for information.*

DEFINITION

Unless otherwise authorised as described in general chapter *3.3.4. Sterile plastic containers for human blood and blood components*, the nature and composition of the material from which the containers are made comply with the requirements in general chapter *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components*.

TESTS

They comply with the tests prescribed in general chapter *3.3.4. Sterile plastic containers for human blood and blood components* and with the following tests.

**Reference solution**. Heat *water R* in a borosilicate-glass flask in an autoclave at 110 °C for 30 min.

**Acidity or alkalinity**. To a volume of solution $S_2$ (see *3.3.4*) corresponding to 4 per cent of the nominal value of the container add 0.1 mL of *phenolphthalein solution R*. The solution remains colourless. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 mL of *0.01 M hydrochloric acid* and 0.1 mL of *methyl red solution R*. The solution is orange-red or red.

**Absorbance** (*2.2.25*): maximum 0.30, determined between wavelengths of 230 nm and 250 nm on solution $S_2$ (see *3.3.4*); maximum 0.10, determined between wavelengths of 251 nm and 360 nm on solution $S_2$. Use the reference solution as the compensation liquid.

**Reducing substances**. Immediately after preparation of solution $S_2$ (see *3.3.4*), transfer to a borosilicate-glass flask a volume corresponding to 8 per cent of the nominal value of the container. At the same time, prepare a blank using an equal volume of the freshly prepared reference solution in another borosilicate-glass flask. To each solution add 20.0 mL of *0.002 M potassium permanganate* and 1 mL of *dilute sulfuric acid R*. Allow to stand protected from light for 15 min. To each solution add 0.1 g of *potassium iodide R*. Allow to stand protected from light for 5 min and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. The difference between the 2 titrations is not greater than 2.0 mL.

**Plastic additives 01, 24, 25, 26 and 27**. Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

*Internal standard solution S3*: 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

*Internal standard solution S4*. 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

*Test solution*. Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

*Stock solution (a)*. Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (b)*. Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (c)*. Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (d)*. Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Stock solution (e)*. Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

*Reference solutions (a1)-(a5)*. Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

*Reference solutions (b1)-(b5)*. Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS*.

*Reference solutions (c1)-(c5)*. Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS*.

*Reference solutions (d1)-(d5)*. Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS*.

*Reference solutions (e1)-(e5)*. Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3.3 | 100 → 200 |
| | 3.3 - 20 | 200 → 250 |
| | 20 - 22.5 | 250 |
| | 22.5 - 23 | 250 → 270 |
| | 23 - 25 | 270 |
| | 25 - 25.6 | 270 → 320 |
| | 25.6 - 30.6 | 320 |
| Injection port | | 300 |

*Detection*: mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] |
|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 |
| Plastic additive 24 | 155 | 127 | 299 |
| Plastic additive 25 | 71 | 213 | 315 |
| Plastic additive 26 | 305 | 193 | 323 |
| Plastic additive 27 | 261 | 149 | 167 |
| DnOP (internal standard) | 149 | 279 | 167 |

*Injection*: 1 µL.

*Relative retention* with reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1.09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

| Substance | Ion 1 [*m/z*] | Ion 2 [*m/z*] | Ion 3 [*m/z*] | Ion ratio 2/1 (%) | Ion ratio 3/1 (%) |
|---|---|---|---|---|---|
| Plastic additive 01 | 149 | 167 | 279 | 50 | 30 |
| Plastic additive 24 | 155 | 127 | 299 | 30 | 13 |
| Plastic additive 25 | 71 | 213 | 315 | 45 | 20 |
| Plastic additive 26 | 305 | 193 | 323 | 55 | 20 |
| Plastic additive 27 | 261 | 149 | 167 | 130 | 85 |
| DnOP (internal standard) | 149 | 279 | 167 | / | / |

*System suitability*:

- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

*Limits*:

- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

**Chlorides** (*2.4.4*): maximum 0.4 ppm, determined with solution $S_2$ (see *3.3.4*).

Prepare the standard using a mixture of 1.2 mL of *chloride standard solution (5 ppm Cl) R* and 13.8 mL of *water R*.

**Residue on evaporation**. Evaporate to dryness 100 mL of solution $S_2$ (see *3.3.4*) in a borosilicate-glass beaker of appropriate capacity, previously heated to 105 °C. Evaporate to dryness in the same conditions 100 mL of the reference solution (blank test). Dry to constant mass at 100-105 °C. The residue from solution $S_2$ weighs a maximum of 3 mg, taking into account the blank test.

STORAGE

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components*.

LABELLING

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components*.

**01/2020:30306**

# 3.3.6. STERILE CONTAINERS OF PLASTICISED POLY(VINYL CHLORIDE) FOR HUMAN BLOOD CONTAINING ANTICOAGULANT SOLUTION

*This general chapter is published for information.*

DEFINITION

Sterile plastic containers containing an anticoagulant solution complying with the monograph *Anticoagulant and preservative solutions for human blood (0209)* are used for the collection, storage and administration of blood. Before filling they comply with the description and characters given in general chapter *3.3.5. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components*.

Unless otherwise authorised as described in general chapter *3.3.4. Sterile plastic containers for human blood and blood components*, the nature and composition of the material from which the containers are made comply with the requirements prescribed in general chapter *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components*.

TESTS

They comply with the tests prescribed in general chapter *3.3.4. Sterile plastic containers for human blood and blood components* and with the following tests.

**Volume of anticoagulant solution**. Empty the container, collecting the anticoagulant solution in a graduated cylinder. The volume does not differ by more than ± 10 per cent from the stated volume.

**Absorbance** (*2.2.25*). Measure the absorbance of the anticoagulant solution removed from the container between 250 nm and 350 nm, using as the compensation liquid an anticoagulant solution of the same composition that has not been in contact with a plastic material. The absorbance at the maximum at 280 nm is not greater than 0.5.

**Plastic additives 01, 24, 25, 26 and 27**. Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Carefully remove the anticoagulant solution by means of the flexible transfer tube. Using a funnel fitted to the tube, completely fill the container with *water R*, leave in contact for 1 min while squeezing the container gently, then empty completely. Repeat the rinsing. The container, emptied and rinsed in this manner, complies with the test for plastic additives 01, 24, 25, 26 and 27 prescribed in general chapter *3.3.5. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components*.

STORAGE

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components*.

LABELLING

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components*.

**01/2020:30307**

## 3.3.7. SETS FOR THE TRANSFUSION OF BLOOD AND BLOOD COMPONENTS

*This general chapter is published for information.*

DEFINITION

Sets for the transfusion of blood and blood components consist principally of plastic tubing to which are fitted the parts necessary to enable the set to be used for transfusion in the appropriate manner. Sets include a closure-piercing device, a blood filter, a drip chamber, a flow regulator, a Luer connector and, usually, a site that allows an injection to be made at the time of use. When the sets are to be used with containers requiring an air filter, this may be incorporated in the closure-piercing device or a separate air-inlet device may be used. The chamber enclosing the blood filter, the drip chamber and the main tubing are transparent. The materials chosen and the design of the set are such as to ensure absence of haemolytic effects. The sets comply with current standards regarding dimensions and performance.

All parts of the set that may be in contact with blood and blood components are sterile and pyrogen-free. Each set is presented in an individual package that maintains the sterility of the contents. The sets are not to be re-sterilised or re-used.

Sets for the transfusion of blood and blood components are manufactured within the framework of a suitable quality system and in accordance with any relevant national regulations.

TESTS

*Carry out the tests on sterilised sets.*

**Solution S**. Make a closed circulation system from 3 sets and a 300 mL borosilicate-glass vessel. Fit to the vessel a suitable thermostat device that maintains the temperature of the liquid in the vessel at 37 ± 1 °C. Circulate 250 mL of *water R* through the system in the direction used for transfusion for 2 h at a rate of 1 L/h (for example using a peristaltic pump applied to as short a piece of suitable silicone elastomer tubing as possible). Collect the whole of the solution and allow to cool.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 25 mL of solution S add 0.15 mL of *BRP indicator solution R*. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 25 mL of solution S add 0.2 mL of *methyl orange solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator.

**Absorbance** (*2.2.25*): maximum 0.30, determined between wavelengths of 230 nm and 250 nm on solution S; maximum 0.15, determined between wavelengths of 251 nm and 360 nm on solution S.

**Reducing substances**. *Carry out the test within 4 h of preparation of solution S.* To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20.0 mL of *water R*. The difference between the 2 titration volumes is not greater than 2.0 mL.

**Ethylene oxide**. Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Carry out the test on each plastic part of the set for transfusion and prepare the reference solutions immediately before use.*

*Test sample.* For hard plastic materials (e.g. cyclo-olefin polymers and copolymers), crush the sample into fine particles. For soft plastic materials (e.g. silicone or plasticised poly(vinyl chloride)), cut the sample into pieces not greater than 0.5 $cm^2$ in size. In both cases, weigh 0.10-3.00 g of the sample, depending on the amount of ethylene oxide residue expected in the plastic part tested, into a 20 mL injection vial and close the vial. For low ethylene-oxide-releasing plastic materials (e.g. cyclo-olefin polymers and copolymers), heat the vial in an oven at 120 °C for at least 15 h (pre-thermal extraction step) before the 1st injection.

*Reference solution (a).* Dilute 1.0 mL of *ethylene oxide stock solution R1* to 50.0 mL with *anhydrous ethanol R* (1000 µg of ethylene oxide per millilitre). *If the solution is prepared using commercial ethylene oxide standard from a previously opened container, it should be noted that some ethylene oxide may have been lost owing to its high volatility.*

*Reference solution (b).* Dilute 10 mL of reference solution (a) to 20 mL with *anhydrous ethanol R* (500 µg of ethylene oxide per millilitre).

*Reference solution (c).* Dilute 8.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (400 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (c) into an injection vial and close immediately.

*Reference solution (d).* Dilute 6.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (300 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (d) into an injection vial and close immediately.

*Reference solution (e).* Dilute 4.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (200 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (e) into an injection vial and close immediately.

*Reference solution (f).* Dilute 2.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (100 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (f) into an injection vial and close immediately.

*Reference solution (g).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (50 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (g) into an injection vial and close immediately.

*Reference solution (h).* Dilute 0.5 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (25 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (h) into an injection vial and close immediately.

*Reference solution (i).* Dilute 100 mg of *acetaldehyde R* to 100 mL with *anhydrous ethanol R* (1000 µg of *acetaldehyde R* per millilitre).

*Reference solution (j).* Transfer 10 µL of reference solution (i) and 20 µL of reference solution (b) into an injection vial, mix and close immediately.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *porous silica for chromatography R* (film thickness 4 µm).

A particle trap may be used to prevent particles that are dislodged from the column from damaging the detector.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:50.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 80 °C for plasticised poly(vinyl chloride); 120 °C for cyclo-olefin polymers and copolymers, and polyurethane; 160 °C for silicone;
- *equilibration time*: 60 min;
- *transfer-line temperature*: 130 °C;
- *pressurisation time*: 0.5 min;
- *injection time*: 3 min;
- *shaking mode*: high agitation.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 2 | 100 |
| | | 2 - 8.25 | 100 → 225 |
| | | 8.25 - 13.25 | 225 |
| Injection port | | | 160 |
| Detector: | transfer line | | 260 |
| | source | | 230 |
| | analyser | | 150 |

*Detection*: mass spectrometer; the following settings have been found to be suitable:

- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *acquisition system*: single-ion monitoring (SIM) for ethylene oxide quantification and complete spectrum mode (*m/z* = 10-350) for ethylene oxide identification;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows: *m/z* = 44 as ethylene oxide quantitation ion; *m/z* = 29 and *m/z* =15 as ethylene oxide qualification ions.

*Injection*: 1 mL of the test sample and reference solutions (c), (d), (e), (f), (g), (h) and (j).

After injection of the test sample, remove the cap from the vial in a fume cupboard and purge the vial with dry nitrogen for 30 s. Close the vial with the cap and a new septum and repeat the heating and injection to exhaustion. Exhaustion is achieved when the amount of ethylene oxide extracted is less than 10 per cent of the 1st extraction or when no analytically significant increase in the cumulative residue levels is detected.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained with reference solution (j);
- *signal-to-noise ratio*: minimum 10 for the peak due to ethylene oxide in the chromatogram obtained with reference solution (h).

Verify the absence of peaks interfering with the peak due to ethylene oxide by carrying out the test on an unsterilised sample.

*Calculation of content.* Establish a calibration curve with the mass of ethylene oxide in each reference solution as the abscissa and the corresponding peak areas as the ordinate.

For sets for transfusion, the total amount of ethylene oxide is calculated using the following formula:

$$EO = \sum_{i=1}^{n} (mi \times Ci) / M$$

EO = total amount of ethylene oxide, in ppm;

*mi* = mass of each plastic part of the set for transfusion, in grams;

*Ci* = cumulative amount of ethylene oxide determined in the corresponding plastic part, in micrograms per gram;

*M* = mass of the set for transfusion, in grams.

*Limit*: if the label states that ethylene oxide has been used for sterilisation:

- *total (sum of amounts of ethylene oxide quantified in each plastic part of the set for transfusion)*: maximum 10 ppm.

**Extraneous particles**. Fill the set via the normal inlet with a 0.1 g/L solution of *sodium laurilsulfate R*, previously filtered through a sintered-glass filter (16) (*2.1.2*) and heated to 37 °C. Collect the liquid via the normal outlet. When examined under suitable conditions of visibility, the liquid is clear and practically free from visible particles and filaments (it is assumed that particles and filaments with a diameter equal to or greater than 50 µm are visible to the naked eye).

**Flow rate**. Pass through a complete set with the flow regulator fully open 50 mL of a solution having a viscosity of 3 mPa·s (3 cP) (for example a 33 g/L solution of *macrogol 4000 R* at 20 °C) under a static head of 1 m. The time required for passage of 50 mL of the solution is not greater than 90 s.

**Resistance to pressure**. Make tight the extremities of the set and any air-inlet device. Connect the set to a compressed air outlet fitted with a pressure regulator. Immerse the set in a tank of water at 20-23 °C. Apply progressively an excess pressure of 100 kPa and maintain for 1 min. No air bubble escapes from the set.

**Transparency**. Use as reference suspension the primary opalescent suspension (*2.2.1*) diluted 8-fold for sets having tubing with an external diameter less than 5 mm and diluted 16-fold for sets having tubing with an external diameter of 5 mm or greater. Circulate the reference suspension through the set and compare with a set from the same batch filled with *water R*. The opalescence and presence of bubbles are discernible.

**Residue on evaporation**. Evaporate 50.0 mL of solution S to dryness on a water-bath and dry to constant mass in an oven at 100-105 °C. Carry out a blank test using 50.0 mL of *water R*. The residue obtained with solution S weighs not more than 1.5 mg, taking into account the blank test.

**Sterility** (*2.6.1*). The sets comply with the test for sterility. If the sets are stated to be sterile only internally, pass 50 mL of buffered sodium chloride-peptone solution pH 7.0 (*2.6.12*) through the set and use the solution to carry out the test by the membrane filtration method.

If the sets are stated to be sterile both internally and externally, open the package with the necessary aseptic precautions and:

- for the direct inoculation method, place the set or its components in a suitable container containing a sufficient quantity of the culture medium to ensure complete immersion;
- for the membrane filtration method, place the set or its components in a suitable container containing a sufficient quantity of buffered sodium chloride-peptone solution pH 7.0 (*2.6.12*) to allow total rinsing for 10 min.

**Pyrogens** (*2.6.8*). Connect together 5 sets and pass through the assembly, at a flow rate not exceeding 10 mL/min, 250 mL of a sterile, pyrogen-free, 9 g/L solution of *sodium chloride R*. Collect the solution aseptically in a pyrogen-free container. The solution complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the solution.

LABELLING

The label states, where applicable, that the set has been sterilised using ethylene oxide.

**01/2020:30308**

## 3.3.8. STERILE SINGLE-USE PLASTIC SYRINGES

DEFINITION

Sterile single-use plastic syringes are medical devices intended for immediate use for the administration of injectable preparations. They are supplied sterile and bacterial endotoxin-free and are not to be re-sterilised or re-used. They consist of a syringe barrel and a piston that may have an elastomer sealing ring; they may be fitted with a needle that may be non-detachable. Each syringe is presented with individual protection for maintaining sterility.

The barrel of the syringe is sufficiently transparent to permit dosages to be read without difficulty and allow air bubbles and foreign particles to be discerned.

The plastics and elastomer materials of which the barrel and piston are made comply with appropriate specifications or with requirements of the competent authority. The most commonly used materials are polypropylene and polyethylene. The syringes comply with current standards regarding dimensions and performance.

Silicone oil (*3.1.8*) may be applied to the internal wall of the barrel, in which case there remains no excess capable of contaminating the contents at the time of use. The inks, glues and adhesives for the marking on the syringe or on the package and, where necessary, the assembly of the syringe and its package, do not migrate across the walls.

TESTS

**Solution S**. Prepare the solution in a manner that avoids contamination by foreign particles. Using a sufficient number of syringes to produce 50 mL of solution, fill the syringes to their nominal volume with *water R* and maintain at 37 °C for 24 h. Combine the contents of the syringes in a suitable borosilicate-glass container.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*) and is practically free from foreign solid particles.

**Acidity or alkalinity**. To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.3 mL of *0.01 M sodium hydroxide* or *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Absorbance** (*2.2.25*): maximum 0.40, determined between wavelengths of 220 nm and 360 nm on solution S.

**Ethylene oxide**. Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the reference solutions immediately before use.*

*Test sample.* Crush the sample into fine particles. Weigh 0.10-3.00 g of the sample, depending on the amount of ethylene oxide residue expected, into a 20 mL injection vial and close the vial. For low ethylene-oxide-releasing plastic materials (e.g. cyclo-olefin polymers and copolymers), heat the vial in an oven at 120 °C for at least 15 h (pre-thermal extraction step) before the 1st injection.

*Reference solution (a).* Dilute 1.0 mL of *ethylene oxide stock solution R1* to 50.0 mL with *anhydrous ethanol R* (1000 µg of ethylene oxide per millilitre). *If the solution is prepared using commercial ethylene oxide standard from a previously opened container, it should be noted that some ethylene oxide may have been lost owing to its high volatility.*

*Reference solution (b).* Dilute 10 mL of reference solution (a) to 20 mL with *anhydrous ethanol R* (500 µg of ethylene oxide per millilitre).

*Reference solution (c).* Dilute 8.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (400 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (c) into an injection vial and close immediately.

*Reference solution (d).* Dilute 6.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (300 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (d) into an injection vial and close immediately.

*Reference solution (e).* Dilute 4.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (200 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (e) into an injection vial and close immediately.

*Reference solution (f).* Dilute 2.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (100 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (f) into an injection vial and close immediately.

*Reference solution (g).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (50 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (g) into an injection vial and close immediately.

*Reference solution (h).* Dilute 0.5 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (25 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (h) into an injection vial and close immediately.

*Reference solution (i).* Dilute 100 mg of *acetaldehyde R* to 100 mL with *anhydrous ethanol R* (1000 µg of acetaldehyde per millilitre).

*Reference solution (j).* Transfer 10 µL of reference solution (i) and 20 µL of reference solution (b) into an injection vial, mix and close immediately.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *porous silica for chromatography R* (film thickness 4 µm).

A particle trap may be used to prevent particles that are dislodged from the column from damaging the detector.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:50.

*Static head-space conditions that may be used*:
- *equilibration temperature*: 80 °C for plasticised poly(vinyl chloride); 120 °C for cyclo-olefin polymers and copolymers, and polyurethane; 160 °C for silicone;
- *equilibration time*: 60 min;
- *transfer-line temperature*: 130 °C;
- *pressurisation time*: 0.5 min;
- *injection time*: 3 min;
- *shaking mode*: high agitation.

*Temperature*:

| | | Time (min) | Temperature (°C) |
|---|---|---|---|
| Column | | 0 - 2 | 100 |
| | | 2 - 8.25 | 100 → 225 |
| | | 8.25 - 13.25 | 225 |
| Injection port | | | 160 |
| Detector: | transfer line | | 260 |
| | source | | 230 |
| | analyser | | 150 |

*Detection*: mass spectrometer; the following settings have been found to be suitable:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *acquisition system*: single-ion monitoring (SIM) for ethylene oxide quantification and complete spectrum mode ($m/z$ = 10-350) for ethylene oxide identification;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows: $m/z$ = 44 as ethylene oxide quantitation ion; $m/z$ = 29 and $m/z$ =15 as ethylene oxide qualification ions.

*Injection*: 1 mL of the test sample and reference solutions (c), (d), (e), (f), (g), (h) and (j).

After injection of the test sample, remove the cap from the vial in a fume cupboard, and purge the vial with dry nitrogen for 30 s. Close the vial with the cap and a new septum and repeat the heating and injection to exhaustion. Exhaustion is achieved when the amount of ethylene oxide extracted is less than 10 per cent of the 1st extraction or when no analytically significant increase in the cumulative residue levels is detected.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained with reference solution (j);
- *signal-to-noise ratio*: minimum 10 for the peak due to ethylene oxide in the chromatogram obtained with reference solution (h).

Verify the absence of peaks interfering with the peak due to ethylene oxide by carrying out the test on an unsterilised sample.

*Calculation of content*. Establish a calibration curve with the mass of ethylene oxide in each reference solution as the abscissa and the corresponding peak areas as the ordinate.

*Limit*: if the label states that ethylene oxide has been used for sterilisation:
- *ethylene oxide*: maximum 10 ppm.

**Silicone oil**. Calculate the internal surface area of a syringe in square centimetres using the following expression:

$$2\sqrt{V \cdot \pi \cdot h}$$

$V$ = nominal volume of the syringe, in cubic centimetres;

$h$ = height of the graduation, in centimetres.

Take a sufficient number of syringes to give an internal surface area of 100 $cm^2$ to 200 $cm^2$. Aspirate into each syringe a volume of *methylene chloride R* equal to half the nominal volume and make up to the nominal volume with air. Rinse the internal surface corresponding to the nominal volume with the solvent by inverting the syringe 10 times in succession with the needle fitting closed by a finger covered by a plastic film inert to methylene chloride. Expel the extracts into a tared dish and repeat the operation. Evaporate the combined extracts to dryness on a water-bath. Dry at 100-105 °C for 1 h. The residue weighs not more than 0.25 mg per square centimetre of internal surface area.

Examine the residue by infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *silicone oil CRS*.

**Reducing substances**. To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20.0 mL of *water R*. The difference between the 2 titration volumes is not greater than 3.0 mL.

**Transparency**. Fill a syringe with *water R* (blank) and fill another with a 10-fold dilution of primary opalescent suspension (*2.2.1*). Use primary opalescent suspension that has been allowed to stand at 20 ± 2 °C for 24 h before use. Compare with the naked eye in diffused light against a dark background. The opalescence of the suspension is detectable when compared with the blank.

**Sterility** (*2.6.1*). *Syringes stated to be sterile comply with the test for sterility carried out as follows.* Using aseptic technique, open the package, withdraw the syringe, separate the components and place each in a suitable container containing sufficient culture media to cover the part completely. Use both the recommended media (*2.6.1*).

*Syringes stated to be sterile only internally comply with the test for sterility carried out as follows.* Use 50 mL of inoculation medium for each test syringe. Using aseptic technique, remove the needle protector and submerge the needle in the culture medium. Flush the syringe 5 times by withdrawing the plunger to its fullest extent.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mL, determined on 10 syringes filled with an extraction volume of 40 mL of water for BET, unless otherwise justified and authorised.

LABELLING

The label states, where applicable, that the syringe has been sterilised using ethylene oxide.

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**04/2013:40000**

# 4. REAGENTS

*Additional information for reagents that can only be fully identified by a trademark or whose availability is limited may be found in the Knowledge database on the EDQM website. This information is given only to make it easier to obtain such reagents and this does not suggest in any way that the mentioned suppliers are especially recommended or certified by the European Pharmacopoeia Commission or the Council of Europe. It is therefore acceptable to use reagents from another source provided that they comply with the standards of the Pharmacopoeia.*

**07/2018:40100**

## 4.1. REAGENTS, STANDARD SOLUTIONS, BUFFER SOLUTIONS

Where the name of a substance or a solution is followed by the letter R (the whole in italics), this indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicines.

Within the description of each reagent there is a 7-digit reference code in italics (for example, *1002501*). This number, which will remain unchanged for a given reagent during subsequent revisions of the list, is used for identification purposes by the Secretariat, and users of the Pharmacopoeia may also find it useful, for example in the management of reagent stocks. The description may also include a CAS number (Chemical Abstract Service Registry Number) recognisable by its typical format, for example 9002-93-1.

Some of the reagents included in the list are toxic and are to be handled in conformity with good quality control laboratory practice.

Reagents in aqueous solution are prepared using *water R*. For liquid chromatography, *water for chromatography R* is used for the preparation of mobile phases when water, or an aqueous solution, is one of the components. Where a reagent solution is described using an expression such as 'hydrochloric acid (10 g/L HCl)', the solution is prepared by an appropriate dilution with *water R* of a more concentrated reagent solution specified in this chapter. Reagent solutions used in the limit tests for barium, calcium and sulfates are prepared using *distilled water R*. Where the name of the solvent is not stated, an aqueous solution is intended.

The reagents and reagent solutions are to be stored in well-closed containers. The labelling should comply with the relevant national legislation and international agreements.

**01/2020:40101**

## 4.1.1. REAGENTS

**Acacia.** *1000100.*

See *Acacia (0307)*.

**Acacia solution.** *1000101.*

Dissolve 100 g of *acacia R* in 1000 mL of *water R*. Stir with a mechanical stirrer for 2 h. Centrifuge at about 2000 *g* for 30 min to obtain a clear solution.

*Storage*: in polyethylene containers of about 250 mL capacity at a temperature of 0 °C to − 20 °C.

**Acebutolol hydrochloride.** *1148900.* [34381-68-5].

See *Acebutolol hydrochloride (0871)*.

**Acetal.** $C_6H_{14}O_2$. ($M_r$ 118.2). *1112300.* [105-57-7].
Acetaldehyde diethyl acetal. 1,1-Diethoxyethane.

Clear, colourless, volatile liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.824.

$n_D^{20}$: about 1.382.

bp: about 103 °C.

**Acetaldehyde.** $C_2H_4O$. ($M_r$ 44.1). *1000200.* [75-07-0].
Ethanal.

Clear, colourless flammable liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.788.

$n_D^{20}$: about 1.332.

bp: about 21 °C.

**Acetaldehyde ammonia trimer trihydrate.**
$C_6H_{15}N_3,3H_2O$. ($M_r$ 183.3). *1133500.* [58052-80-5].
2,4,6-Trimethylhexahydro-1,3,5-triazine trihydrate.

*Content*: minimum 95.0 per cent.
Colourless or white or pale yellow crystals or powder.

mp: 95 °C to 97 °C.

*Assay*. Dissolve 0.900 g in *water R* and dilute to 50.0 mL with the same solvent. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M hydrochloric acid* is equivalent to 61.08 mg of $C_6H_{15}N_3,3H_2O$.

**Acetic acid, anhydrous.** $C_2H_4O_2$. ($M_r$ 60.1). *1000300.* [64-19-7].

*Content*: minimum 99.6 per cent *m/m* of $C_2H_4O_2$.

Colourless liquid or white or almost white, shining, fern-like crystals, miscible with or very soluble in water, in ethanol (96 per cent), in glycerol (85 per cent), and in most fatty and essential oils.

$d_{20}^{20}$: 1.052 to 1.053.

bp: 117 °C to 119 °C.

A 100 g/L solution is strongly acid (*2.2.4*).

A 5 g/L solution neutralised with *dilute ammonia R2* gives reaction (b) of acetates (*2.3.1*).

*Freezing point* (*2.2.18*): minimum 15.8 °C.

*Water* (*2.5.12*): maximum 0.4 per cent. If the water content is more than 0.4 per cent it may be adjusted by adding the calculated amount of *acetic anhydride R*.

*Storage*: protected from light.

**Acetic acid, glacial.** $C_2H_4O_2$. ($M_r$ 60.1). *1000400.* [64-19-7].

See *Acetic acid, glacial (0590)*.

**Acetic acid.** *1000401.*

*Content*: 290 g/L to 310 g/L of $C_2H_4O_2$ ($M_r$ 60.1).

Dilute 30 g of *glacial acetic acid R* to 100 mL with *water R*.

**Acetic acid, dilute.** *1000402.*

*Content*: 115 g/L to 125 g/L of $C_2H_4O_2$ ($M_r$ 60.1).

Dilute 12 g of *glacial acetic acid R* to 100 mL with *water R*.

**Acetic acid, dilute R1.** *1000403.*

*Content*: 57.5 g/L to 62.5 g/L ($M_r$ 60.1).

Dilute 6 g of *glacial acetic acid R* to 100 mL with *water R*.

**Acetic anhydride.** $C_4H_6O_3$. ($M_r$ 102.1). *1000500.* [108-24-7].

*Content*: minimum 97.0 per cent *m/m* of $C_4H_6O_3$.

Clear, colourless liquid.

bp: 136 °C to 142 °C.

*Assay.* Dissolve 2.00 g in 50.0 mL of *1 M sodium hydroxide* in a ground-glass-stoppered flask and boil under a reflux condenser for 1 h. Titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* required for 1 g ($n_1$). Dissolve 2.00 g in 20 mL of *cyclohexane R* in a ground-glass-stoppered flask, cool in ice and add a cold mixture of 10 mL of *aniline R* and 20 mL of *cyclohexane R*. Boil the mixture under a reflux condenser for 1 h, add 50.0 mL of *1 M sodium hydroxide* and shake vigorously. Titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* required for 1 g ($n_2$). Calculate the percentage of $C_4H_6O_3$ from the following expression:

$$10.2(n_1 - n_2)$$

**Acetic anhydride solution R1.** *1000501.*

Dissolve 25.0 mL of *acetic anhydride R* in *anhydrous pyridine R* and dilute to 100.0 mL with the same solvent.

*Storage*: protected from light and air.

**Acetic anhydride - sulfuric acid solution.** *1000502.*

Carefully mix 5 mL of *acetic anhydride R* with 5 mL of *sulfuric acid R*. Add dropwise and with cooling to 50 mL of *anhydrous ethanol R*.

Prepare immediately before use.

**Acetone.** *1000600.* [67-64-1].

See *Acetone (0872)*.

**Acetonitrile.** $C_2H_3N$. ($M_r$ 41.05). *1000700.* [75-05-8]. Methyl cyanide. Ethanenitrile.

Clear, colourless liquid, miscible with water, with acetone and with methanol.

$d_{20}^{20}$: about 0.78.

$n_D^{20}$: about 1.344.

A 100 g/L solution is neutral to litmus paper.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 80 °C and 82 °C.

*Acetonitrile used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 0.01 from 255 nm to 420 nm, determined using *water R* as compensation liquid.

**Acetonitrile for chromatography.** *1000701.*

See *Acetonitrile R*.

*Acetonitrile used in chromatography complies with the following additional tests.*

*Absorbance* (*2.2.25*): maximum 0.01 at 240 nm and higher wavelengths, determined using *water R* as compensation liquid.

*Content* (*2.2.28*): minimum 99.8 per cent.

**Acetonitrile R1.** *1000702.*

Complies with the requirements prescribed for *acetonitrile R* and with the following additional requirements.

*Content*: minimum 99.9 per cent.

*Absorbance* (*2.2.25*): maximum 0.10, determined at 200 nm using *water R* as the compensation liquid.

**Acetoxyvalerenic acid.** $C_{17}H_{24}O_4$. ($M_r$ 292.4). *1165800.* [81397-67-3]. (2*E*)-3-[(1*RS*,4*S*,7*R*,7a*R*)-1-(Acetyloxy)-3,7-dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl]-2-methylprop-2-enoic acid.

Colourless or pale yellow viscous oil.

*Absorbance* (*2.2.25*). A solution in *methanol R* shows an absorption maximum at about 216 nm.

**Acetylacetamide.** $C_4H_7NO_2$. ($M_r$ 101.1). *1102600.* [5977-14-0]. 3-Oxobutanamide.

mp: 53 °C to 56 °C.

**Acetylacetone.** $C_5H_8O_2$. ($M_r$ 100.1). *1000900.* [123-54-6]. 2,4-Pentanedione.

Colourless or slightly yellow, easily flammable liquid, freely soluble in water, miscible with acetone, with ethanol (96 per cent) and with glacial acetic acid.

$n_D^{20}$: 1.452 to 1.453.

bp: 138 °C to 140 °C.

**Acetylacetone reagent R1.** *1000901.*

To 100 mL of *ammonium acetate solution R* add 0.2 mL of *acetylacetone R*.

**Acetylacetone reagent R2.** *1000902.*

Dissolve 0.2 mL of *acetylacetone R*, 3 mL of *glacial acetic acid R* and 25 g of *ammonium acetate R* in *water R* and dilute to 100 mL with the same solvent.

***N*-Acetyl-ε-caprolactam.** $C_8H_{13}NO_2$. ($M_r$ 155.2). *1102700.* [1888-91-1]. *N*-Acetylhexane-6-lactam.

Colourless liquid, miscible with anhydrous ethanol.

$d_{20}^{20}$: about 1.100.

$n_D^{20}$: about 1.489.

bp: about 135 °C.

**Acetyl chloride.** $C_2H_3ClO$. ($M_r$ 78.5). *1000800.* [75-36-5].

Clear, colourless liquid, flammable, decomposes in contact with water and with ethanol (96 per cent), miscible with ethylene chloride.

$d_{20}^{20}$: about 1.10.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 49 °C and 53 °C.

**Acetylcholine chloride.** $C_7H_{16}ClNO_2$. ($M_r$ 181.7). *1001000.* [60-31-1].

Crystalline powder, very soluble in cold water and in ethanol (96 per cent). It decomposes in hot water and in alkalis.

*Storage*: at − 20 °C.

**Acetylene.** $C_2H_2$. ($M_r$ 26.04). *1199800.* [74-86-2]. Ethyne.

*Content*: minimum 99.0 per cent *V/V*.

**Acetyleugenol.** $C_{12}H_{14}O_3$. ($M_r$ 206.2). *1100700.* [93-28-7]. 2-Methoxy-4-(2-propenyl)phenylacetate.

Yellow coloured, oily liquid, practically insoluble in water, freely soluble in ethanol (96 per cent).

$n_D^{20}$: about 1.521.

bp: 281 °C to 282 °C.

*Acetyleugenol used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

*Test solution.* The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

***N*-Acetylglucosamine.** $C_8H_{15}NO_6$. ($M_r$ 221.2). *1133600.* [7512-17-6]. 2-(Acetylamino)-2-deoxy-D-glucopyranose.

mp: about 202 °C.

**Acetyl-11-keto-β-boswellic acid.** $C_{32}H_{48}O_5$. ($M_r$ 512.7). *1167700.* [67416-61-9]. 3α-(Acetyloxy)-11-oxours-12-en-24-oic acid. (4β)-3α-(Acetyloxy)-11-oxours-12-en-23-oic acid.

White or almost white powder, insoluble in water, soluble in acetone, in anhydrous ethanol and in methanol.

mp: 271 °C to 274 °C.

*Acetyl-11-keto-β-boswellic acid used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph on *Indian frankincense (2310)*.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

***N*-Acetylneuraminic acid.** $C_{11}H_{19}NO_9$. ($M_r$ 309.3). *1001100.* [131-48-6]. *O*-Sialic acid.

White or almost white acicular crystals, soluble in water and in methanol, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

$[\alpha]_D^{20}$: about − 36, determined on a 10 g/L solution.

mp: about 186 °C, with decomposition.

***N*-Acetyltryptophan.** $C_{13}H_{14}N_2O_3$. ($M_r$ 246.3). *1102800.* [1218-34-4]. 2-Acetylamino-3-(indol-3-yl)propanoic acid.

White or almost white powder or colourless crystals, slightly soluble in water. It dissolves in dilute solutions of alkali hydroxides.

mp: about 205 °C.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Tryptophan (1272)*.

*Test solution*. Dissolve 10.0 mg in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Acetyltyrosine ethyl ester.** $C_{13}H_{17}NO_4,H_2O$. ($M_r$ 269.3). *1001200.* [36546-50-6]. *N*-Acetyl-L-tyrosine ethyl ester monohydrate. Ethyl (*S*)-2-acetamido-3-(4-hydroxyphenyl)propionate monohydrate.

White or almost white, crystalline powder suitable for the assay of chymotrypsin.

$[\alpha]_D^{20}$: + 21 to + 25, determined on a 10 g/L solution in *ethanol (96 per cent) R*.

$A_{1\,cm}^{1\%}$: 60 to 68, determined at 278 nm in *ethanol (96 per cent) R*.

**Acetyltyrosine ethyl ester, 0.2 M.** *1001201.*

Dissolve 0.54 g of *acetyltyrosine ethyl ester R* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Acid blue 83.** $C_{45}H_{44}N_3NaO_7S_2$. ($M_r$ 826). *1012200.* [6104-59-2].

Colour Index No. 42660.
Brilliant blue. Coomassie brilliant blue R 250.

Brown powder insoluble in cold water, slightly soluble in boiling water and in anhydrous ethanol, soluble in sulfuric acid, glacial acetic acid and in dilute solutions of alkali hydroxides.

**Acid blue 90.** $C_{47}H_{48}N_3NaO_7S_2$. ($M_r$ 854). *1001300.* [6104-58-1].

Colour Index No. 42655.
Sodium [4-[[4-[(4-ethoxyphenyl)amino]phenyl][[4-(ethyl)(3-sulfonatobenzyl)amino]phenyl]methylene]cyclo-hexa-2,5-dien-1-ylidene](ethyl)-(3-sulfonatobenzyl)ammonium.

A dark brown powder, with a violet sheen and some particles having a metallic lustre, soluble in water and in anhydrous ethanol.

$A_{1\,cm}^{1\%}$: greater than 500, determined at 577 nm in a 0.01 g/L solution in buffer solution pH 7.0 and calculated with reference to the dried substance.

*Loss on drying* (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Acid blue 92.** $C_{26}H_{16}N_3Na_3O_{10}S_3$. ($M_r$ 696). *1001400.* [3861-73-2].

Colour Index No. 13390.
Coomassie blue. Anazolene sodium. Trisodium 8-hydroxy-4′-(phenylamino)azonaphthalene-3,5′,6-trisulfonate.

Dark blue crystals, soluble in water, in acetone and in ethylene glycol monoethylether, slightly soluble in ethanol (96 per cent).

**Acid blue 92 solution.** *1001401.*

Dissolve 0.5 g of *acid blue 92 R* in a mixture of 10 mL of *glacial acetic acid R*, 45 mL of *ethanol (96 per cent) R* and 45 mL of *water R*.

**Acid blue 93.** $C_{37}H_{27}N_3Na_2O_9S_3$. ($M_r$ 800). *1134200.* [28983-56-4].

Colour Index No. 42780.
Methyl blue. Poirrier blue.

Mixture of triphenylrosaniline di- and trisulfonate and of triphenylpararosaniline.

Dark blue powder.

*Colour change*: pH 9.4 to pH 14.0.

**Acid blue 93 solution.** *1134201.*

Dissolve 0.2 g of *acid blue 93 R* in *water R* and dilute to 100 mL with the same solvent.

**α1-Acid-glycoprotein silica gel for chiral separation.** *1148700.*

A very finely divided silica gel for chromatography consisting of spherical particles coated with α1-acid glycoprotein.

**Acrylamide.** $C_3H_5NO$. ($M_r$ 71.1). *1001500.* [79-06-1]. Propenamide.

Colourless or white flakes or a white or almost white, crystalline powder, very soluble in water and in methanol, freely soluble in anhydrous ethanol.

mp: about 84 °C.

**30 per cent acrylamide/bisacrylamide (29:1) solution.** *1001501.*

Prepare a solution containing 290 g of *acrylamide R* and 10 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

**30 per cent acrylamide/bisacrylamide (36.5:1) solution.** *1001502.*

Prepare a solution containing 292 g of *acrylamide R* and 8 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

**Acrylic acid.** $C_3H_4O_2$. ($M_r$ 72.1). *1133700.* [79-10-7]. Prop-2-enoic acid. Vinylformic acid.

*Content*: minimum 99 per cent.

It is stabilised with 0.02 per cent of hydroquinone monomethyl ether.

Corrosive liquid, miscible with water and ethanol (96 per cent). It polymerises readily in the presence of oxygen.

$d_{20}^{20}$: about 1.05.

$n_D^{20}$: about 1.421.

bp: about 141 °C.

mp: 12 °C to 15 °C.

**Actein.** $C_{37}H_{56}O_{11}$. ($M_r$ 677). *1181500.* [18642-44-9]. (23*R*,24*R*,25*S*,26*S*)-3β-(β-D-Xylopyranosyloxy)-16β,23:23,26:24,25-triepoxy-26-hydroxy-9,19-cyclolanostan-12β-yl acetate.

**Acteoside.** $C_{29}H_{36}O_{15}$. ($M_r$ 624.6). *1145100.* [61276-17-3]. 2-(3,4-Dihydroxyphenyl)ethyl 3-*O*-(6-deoxy-α-L-mannopyranosyl)-4-*O*-[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-β-D-glucopyranoside. Verbascoside.

Light yellowish powder, freely soluble in water and in methanol.

mp: about 140 °C, with decomposition.

**Adamantane.** $C_{10}H_{16}$. ($M_r$ 136.2). *1181600.* [281-23-2]. Tricyclo[3.3.1.1$^{3,7}$]decane.

mp: about 270 °C.

**Adenine.** *1172800.* [73-24-5].

See *Adenine (0800)*.

**Adenosine.** $C_{10}H_{13}N_5O_4$. ($M_r$ 267.2). *1001600.* [58-61-7]. 6-Amino-9-β-D-ribofuranosyl-9*H*-purine.

White or almost white, crystalline powder, slightly soluble in water, practically insoluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of acids.

mp: about 234 °C.

**Adipic acid.** $C_6H_{10}O_4$. ($M_r$ 146.1). *1095600.* [124-04-9].

Prisms, freely soluble in methanol, soluble in acetone, practically insoluble in light petroleum.

mp: about 152 °C.

**Adrenaline.** $C_9H_{13}NO_3$. ($M_r$ 183.2). *1155000.* [51-43-4]. (1*R*)-1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanol. 4-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol.

White or almost white powder, gradually becoming brown on exposure to light and air, very slightly soluble in water and in ethanol (96 per cent), insoluble in acetone. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

mp: about 215 °C.

**Adrenalone hydrochloride.** $C_9H_{12}ClNO_3$. ($M_r$ 217.7). *1155100.* [62-13-5]. 1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanone hydrochloride. 3′,4′-Dihydroxy-2-(methylamino)acetophenone hydrochloride.

Pale yellow crystals, freely soluble in water, soluble in ethanol (96 per cent).

mp: about 244 °C.

**Aescin.** *1001700.* [6805-41-0].

A mixture of related saponins obtained from the seeds of *Aesculus hippocastanum* L.

Fine, almost white or slightly reddish or yellowish, amorphous powder.

*Chromatography.* Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of *aescin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: the upper layer of a mixture of 10 volumes of *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *butanol R.*

*Application*: 20 µL of the test solution as bands of 20 mm by 3 mm.

*Development*: over a path of 12 cm.

*Drying*: at 100-105 °C.

*Detection*: spray with about 10 mL of *anisaldehyde solution R* for a plate 200 mm square and heat again at 100-105 °C.

*Results*: the chromatogram shows a principal band with an $R_F$ of about 0.4.

**Aflatoxin $B_1$.** $C_{17}H_{12}O_6$. ($M_r$ 312.3). *1166000.* [1162-65-8]. (6a*R*,9a*S*)-4-Methoxy-2,3,6a,9a-tetrahydrocyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][1]benzopyran-1,11-dione.

White or faint yellow crystals.

**Agarose/cross-linked polyacrylamide.** *1002200.*

Agarose trapped within a cross-linked polyacrylamide network; it is used for the separation of globular proteins with relative molecular masses of $2 \times 10^4$ to $35 \times 10^4$.

**Agarose-DEAE for ion-exchange chromatography.** *1002100.* [57407-08-6].

Cross-linked agarose substituted with diethylaminoethyl groups, presented as beads.

**Agarose for chromatography.** *1001800.* [9012-36-6].

Swollen beads 60-140 µm in diameter presented as a 4 per cent suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of $6 \times 10^4$ to $20 \times 10^6$ and of polysaccharides with relative molecular masses of $3 \times 10^3$ to $5 \times 10^6$.

**Agarose for chromatography, cross-linked.** *1001900.* [61970-08-9].

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads 60-140 µm in diameter and is presented as a 4 per cent suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of $6 \times 10^4$ to $20 \times 10^6$ and of polysaccharides with relative molecular masses of $3 \times 10^3$ to $5 \times 10^6$.

**Agarose for chromatography, cross-linked R1.** *1001901.* [65099-79-8].

Prepared for agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads 60-140 µm in diameter and is presented as a 4 per cent suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of $7 \times 10^4$ to $40 \times 10^6$ and of polysaccharides with relative molecular masses of $1 \times 10^5$ to $2 \times 10^7$.

**Agarose for electrophoresis.** *1002000.* [9012-36-6].

A neutral, linear polysaccharide, the main component of which is derived from agar.

White or almost white powder, practically insoluble in cold water, very slightly soluble in hot water.

**Agnuside.** $C_{22}H_{26}O_{11}$. ($M_r$ 466.4). *1162000.* [11027-63-7]. (1*RS*,4a*SR*,5*RS*,7a*RS*)-5-Hydroxy-7-[[(4-hydroxybenzoyl)oxy]methyl]-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-1-yl β-D-glucopyranoside.

White or almost white crystals.

**Air, hydrocarbon-free.** *1188700.*

Complies with the requirements prescribed for the monograph *Medicinal air (1238)* with the following additional requirement.

*Hydrocarbons*: maximum 5 ppm *V/V*, calculated as $CH_4$.

**Alanine.** *1102900.* [56-41-7].

See *Alanine (0752)*.

**β-Alanine.** *1004500.* [107-95-9].

See *3-aminopropionic acid R*.

**Albumin, bovine.** *1002300.* [9048-46-8].

Bovine serum albumin containing about 96 per cent of protein.

White to light brownish-yellow powder.

*Water* (*2.5.12*): maximum 3.0 per cent, determined on 0.800 g.

**Albumin, bovine R1.** *1183500.* [9048-46-8].

Bovine serum albumin containing about 96 per cent of protein.

White or light brownish-yellow powder.

**Albumin, human.** *1133800.*

Human serum albumin containing not less than 96 per cent of albumin.

**Albumin solution, human.** *1002400.* [9048-46-8].

See *Human albumin solution (0255)*.

**Albumin solution, human R1.** *1002401.*

Dilute *human albumin solution R* with a 9 g/L solution of *sodium chloride R* to a concentration of 1 g/L of protein. Adjust the pH to 3.5-4.5 with *glacial acetic acid R*.

**Alcohol.** *1002500.* [64-17-5].

See *Ethanol (96 per cent) R*.

**Alcohol (*x* per cent *V/V*).** *1002502.*

See *Ethanol (x per cent V/V) R*.

**Alcohol, aldehyde-free.** *1002501.*

Mix 1200 mL of *ethanol (96 per cent) R* with 5 mL of a 400 g/L solution of *silver nitrate R* and 10 mL of a cooled 500 g/L solution of *potassium hydroxide R*. Shake, allow to stand for a few days and filter. Distil the filtrate immediately before use.

**Aldehyde dehydrogenase.** *1103000.*

Enzyme obtained from baker's yeast which oxidises acetaldehyde to acetic acid in the presence of nicotinamide-adenine dinucleotide, potassium salts and thiols, at pH 8.0.

**Aldehyde dehydrogenase solution.** *1103001.*

Dissolve in *water R* a quantity of *aldehyde dehydrogenase R*, equivalent to 70 units and dilute to 10 mL with the same solvent. This solution is stable for 8 h at 4 °C.

**Aldrin.** $C_{12}H_8Cl_6$. ($M_r$ 364.9). *1123100.* [309-00-2].

bp: about 145 °C.

mp: about 104 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Aleuritic acid.** $C_{16}H_{32}O_5$. ($M_r$ 304.4). *1095700.* [533-87-9]. (9*RS*,10*SR*)-9,10,16-Trihydroxyhexadecanoic acid.

White or almost white powder, greasy to the touch, soluble in methanol.

mp: about 101 °C.

**Alizarin S.** $C_{14}H_7NaO_7S,H_2O$. ($M_r$ 360.3). *1002600.* [130-22-3].

Schultz No. 1145.

Colour Index No. 58005.
Sodium 1,2-dihydroxyanthraquinone-3-sulfonate monohydrate. Sodium 3,4-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate monohydrate.

Orange-yellow powder, freely soluble in water and in ethanol (96 per cent).

**Alizarin S solution.** *1002601.*

A 1 g/L solution of *alizarin S R*.

*Test for sensitivity.* If alizarin S solution is used for the standardisation of *0.05 M barium perchlorate*, it shows a colour change from yellow to orange-red when it is tested according to the standardisation of *0.05 M barium perchlorate*.

*Colour change*: pH 3.7 (yellow) to pH 5.2 (violet).

**Aloe emodin.** $C_{15}H_{10}O_5$. ($M_r$ 270.2). *1188800.* [481-72-1].

1,8-Dihydroxy-3-(hydroxymethyl)anthracene-9,10-dione.

1,8-Dihydroxy-3-(hydroxymethyl)anthraquinone.

**Alovudine.** $C_{10}H_{13}FN_2O_4$. ($M_r$ 244.2). *1185400.* [25526-93-6]. 1-[(2*R*,4*S*,5*R*)-4-Fluoro-5-(hydroxymethyl)tetrahydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione. Fluorodeoxythymidine. 3′-Deoxy-3′-fluorothymidine.

*Content*: minimum 95 per cent.

Colourless crystals.

**Aluminium.** Al. ($A_r$ 26.98). *1118200.* [7429-90-5].

White or almost white, malleable, flexible, bluish metal, available as bars, sheets, powder, strips or wire. In moist air an oxide film forms which protects the metal from corrosion.

Analytical grade.

**Aluminium chloride.** $AlCl_3,6H_2O$. ($M_r$ 241.4). *1002700.* [7784-13-6]. Aluminium chloride hexahydrate.

*Content*: minimum 98.0 per cent of $AlCl_3,6H_2O$.

White or slightly yellowish, crystalline powder, hygroscopic, freely soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Aluminium chloride reagent.** *1002702.*

Dissolve 2.0 g of *aluminium chloride R* in 100 mL of a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

**Aluminium chloride solution.** *1002701.*

Dissolve 65.0 g of *aluminium chloride R* in *water R* and dilute to 100 mL with the same solvent. Add 0.5 g of *activated charcoal R*, stir for 10 min, filter and add to the filtrate, with continuous stirring, sufficient of a 10 g/L solution of *sodium hydroxide R* (about 60 mL) to adjust the pH to about 1.5.

**Aluminium nitrate.** $Al(NO_3)_3,9H_2O$. ($M_r$ 375.1). *1002800.* [7784-27-2]. Aluminium nitrate nonahydrate.

Crystals, deliquescent, very soluble in water and ethanol (96 per cent), very slightly soluble in acetone.

*Storage*: in an airtight container.

**Aluminium oxide, anhydrous.** *1002900.* [1344-28-1].

Aluminium oxide, consisting of γ-$Al_2O_3$, dehydrated and activated by heat treatment.

*Particle size*: 75 µm to 150 µm.

**Aluminium oxide, basic.** *1118300.*

A basic grade of *anhydrous aluminium oxide R* suitable for column chromatography.

*pH* (*2.2.3*). Shake 1 g with 10 mL of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 9 to 10.

**Aluminium oxide for chromatography, deactivated.** *1188900.*

Aluminium oxide suitably deactivated for the separation and detection of traces of polar hydrocarbons, with porous layer open tubular (PLOT) design.

**Aluminium oxide, neutral.** *1118400.*

See *Aluminium oxide, hydrated (0311)*.

**Aluminium potassium sulfate.** *1003000.* [7784-24-9].

See *Alum (0006)*.

**Aluminium test strip.** *1199900.*

Commercially available test strip for the determination of aluminium in aqueous solvents at a level below 5 ppm.

**Americium-243 spiking solution.** *1167500.*

Contains 50 Bq/L $^{243}Am$ and a 134 mg/L solution of *lanthanum chloride heptahydrate R* in a 103 g/L solution of *hydrochloric acid R*.

**Amido black 10B.** $C_{22}H_{14}N_6Na_2O_9S_2$. ($M_r$ 617). *1003100.* [1064-48-8].

Schultz No. 299.

Colour Index No. 20470.

Disodium 5-amino-4-hydroxy-6-[(4-nitrophenyl)azo]-3-(phenylazo)naphthalene-2,7-disulfonate.

Dark-brown to black powder, sparingly soluble in water, soluble in ethanol (96 per cent).

**Amido black 10B solution.** *1003101.*

A 5 g/L solution of *amido black 10B R* in a mixture of 10 volumes of *acetic acid R* and 90 volumes of *methanol R*.

**Aminoazobenzene.** $C_{12}H_{11}N_3$. ($M_r$ 197.2). *1003200.* [60-09-3].

Colour Index No. 11000.
4-(Phenylazo)aniline.

Brownish-yellow needles with a bluish tinge, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 128 °C.

**2-Aminobenzoic acid.** $C_7H_7NO_2$. ($M_r$ 137.1). *1003400.* [118-92-3]. Anthranilic acid.

A white or pale-yellow, crystalline powder, sparingly soluble in cold water, freely soluble in hot water, in ethanol (96 per cent) and in glycerol. Solutions in ethanol (96 per cent) or in ether and, particularly, in glycerol show a violet fluorescence.

mp: about 145 °C.

**3-Aminobenzoic acid.** $C_7H_7NO_2$. ($M_r$ 137.1). *1147400.* [99-05-8].

White or almost white crystals. An aqueous solution turns brown on standing in air.

mp: about 174 °C.

*Storage*: in an airtight container, protected from light.

**4-Aminobenzoic acid.** $C_7H_7NO_2$. ($M_r$ 137.1). *1003300.* [150-13-0].

White or almost white, crystalline powder, slightly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in light petroleum.

mp: about 187 °C.

*Chromatography.* Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Procaine hydrochloride (0050)*; the chromatogram shows only one principal spot.

*Storage*: protected from light.

**4-Aminobenzoic acid solution.** *1003301.*

Dissolve 1 g of *4-aminobenzoic acid R* in a mixture of 18 mL of *anhydrous acetic acid R*, 20 mL of *water R* and 1 mL of *phosphoric acid R*. Immediately before use, mix 2 volumes of the solution with 3 volumes of *acetone R*.

***N*-(4-Aminobenzoyl)-L-glutamic acid.** $C_{12}H_{14}N_2O_5$. ($M_r$ 266.3). *1141700.* [4271-30-1]. ABGA. (2*S*)-2-[(4-Aminobenzoyl)amino]pentanedioic acid.

White or almost white, crystalline powder.

mp: about 175 °C, with decomposition.

**4-Aminobutanoic acid.** $C_4H_9NO_2$. ($M_r$ 103.1). *1123200.* [56-12-2]. γ-Aminobutyric acid. GABA.

Leaflets from methanol and ether, needles from water and ethanol (96 per cent). Freely soluble in water, practically insoluble or slightly soluble in other solvents.

mp: about 202 °C (decreases on rapid heating).

**Aminobutanol.** $C_4H_{11}NO$. ($M_r$ 89.1). *1003500.* [5856-63-3]. 2-Aminobutanol.

Oily liquid, miscible with water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.94.

$n_D^{20}$: about 1.453.

bp: about 180 °C.

**Aminochlorobenzophenone.** $C_{13}H_{10}ClNO$. ($M_r$ 231.7). *1003600.* [719-59-5]. 2-Amino-5-chlorobenzophenone.

Yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

mp: about 97 °C.

*Content*: minimum 95.0 per cent.

*Storage*: protected from light.

**4-Aminofolic acid.** $C_{19}H_{20}N_8O_5$. ($M_r$ 440.4). *1163700.* [54-62-6]. (2*S*)-2-[[4-[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]amino]pentanedioic acid. *N*-[4-[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzoyl]-L-glutamic acid. Aminopterine.

Yellowish powder.

mp: about 230 °C.

**6-Aminohexanoic acid.** $C_6H_{13}NO_2$. ($M_r$ 131.2). *1103100.* [60-32-2].

Colourless crystals, freely soluble in water, sparingly soluble in methanol, practically insoluble in anhydrous ethanol.

mp: about 205 °C.

**Aminohippuric acid.** $C_9H_{10}N_2O_3$. ($M_r$ 194.2). *1003700.* [61-78-9]. (4-Aminobenzamido)acetic acid.

White or almost white powder, sparingly soluble in water, soluble in ethanol (96 per cent).

mp: about 200 °C.

**Aminohippuric acid reagent.** *1003701.*

Dissolve 3 g of *phthalic acid R* and 0.3 g of *aminohippuric acid R* in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent.

**Aminohydroxynaphthalenesulfonic acid.** $C_{10}H_9NO_4S$. ($M_r$ 239.3). *1112400.* [116-63-2]. 4-Amino-3-hydroxynaphthalene-1-sulfonic acid.

White or grey needles, turning pink on exposure to light, especially when moist, practically insoluble in water and in ethanol (96 per cent), soluble in solutions of alkali hydroxides and in hot solutions of sodium metabisulfite.

*Storage*: protected from light.

**Aminohydroxynaphthalenesulfonic acid solution.** *1112401.*

Mix 5.0 g of *anhydrous sodium sulfite R* with 94.3 g of *sodium hydrogensulfite R* and 0.7 g of *aminohydroxynaphthalenesulfonic acid R*. Dissolve 1.5 g of the mixture in *water R* and dilute to 10.0 mL with the same solvent. Prepare the solution daily.

***cis*-Aminoindanol.** $C_9H_{11}NO$. ($M_r$ 149.2). *1168300.* [126456-43-7]. (1*S*,2*R*)-1-Amino-2,3-dihydro-1*H*-inden-2-ol. (−)-*cis*-1-Aminoindan-2-ol.

*Content*: minimum 98.0 per cent (sum of enantiomers, determined by gas chromatography).

$[\alpha]_D^{20}$: − 69 to − 59, determined on a 2 g/L solution in *chloroform R*.

mp: 118 °C to 122 °C.

**Aminomethylalizarindiacetic acid.** $C_{19}H_{15}NO_8$,$2H_2O$. ($M_r$ 421.4). *1003900.* [3952-78-1]. 2,2′-[(3,4-dihydroxyanthraquinon-3-yl)methylenenitrilo]diacetic acid dihydrate. Alizarin complexone dihydrate.

Fine, pale brownish-yellow or orange-brown powder, practically insoluble in water, soluble in solutions of alkali hydroxides.

mp: about 185 °C.

*Loss on drying* (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g.

**Aminomethylalizarindiacetic acid reagent.** *1003901.*

*Solution A*. Dissolve 0.36 g of *cerous nitrate R* in *water R* and dilute to 50 mL with the same solvent.

*Solution B*. Suspend 0.7 g of *aminomethylalizarindiacetic acid R* in 50 mL of *water R*. Dissolve with the aid of about 0.25 mL of *concentrated ammonia R*, add 0.25 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

*Solution C*. Dissolve 6 g of *sodium acetate R* in 50 mL of *water R*, add 11.5 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

To 33 mL of *acetone R* add 6.8 mL of solution C, 1.0 mL of solution B and 1.0 mL of solution A and dilute to 50 mL with *water R*.

*Test for sensitivity.* To 1.0 mL of *fluoride standard solution (10 ppm F) R* add 19.0 mL of *water R* and 5.0 mL of the aminomethylalizarindiacetic acid reagent. After 20 min, the solution assumes a blue colour.

*Storage*: use within 5 days.

**Aminomethylalizarindiacetic acid solution.** *1003902.*

Dissolve 0.192 g of *aminomethylalizarindiacetic acid R* in 6 mL of freshly prepared *1 M sodium hydroxide*. Add 750 mL of *water R*, 25 mL of *succinate buffer solution pH 4.6 R* and, dropwise, *0.5 M hydrochloric acid* until the colour changes from violet-red to yellow (pH 4.5 to 5). Add 100 mL of *acetone R* and dilute to 1000 mL with *water R*.

**4-Aminomethylbenzoic acid.** $C_8H_9NO_2$. ($M_r$ 151.2). *1167800.* [56-91-7].

**Aminonitrobenzophenone.** $C_{13}H_{10}N_2O_3$. ($M_r$ 242.2). *1004000.* [1775-95-7]. 2-Amino-5-nitrobenzophenone.

Yellow, crystalline powder, practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methanol.

mp: about 160 °C.

$A_{1\,cm}^{1\%}$: 690 to 720, determined at 233 nm using a 0.01 g/L solution in *methanol R*.

**6-Aminopenicillanic acid.** $C_8H_{12}N_2O_3S$. ($M_r$ 216.3). *1162100.* [551-16-6]. (2*S*,5*R*,6*R*)-6-Amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

*Appearance*: white or almost white powder.

mp: about 205 °C, with decomposition.

**Aminophenazone.** $C_{13}H_{17}N_3O$. (231.3). *1133900.* [58-15-1]. 4-(Dimethylamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one.

White or almost white, crystalline powder or colourless crystals, soluble in water, freely soluble in ethanol (96 per cent).

mp: about 108 °C.

**2-Aminophenol.** $C_6H_7NO$. ($M_r$ 109.1). *1147500.* [95-55-6].

Pale yellowish-brown crystals which rapidly become brown, sparingly soluble in water, soluble in ethanol (96 per cent).

mp: about 172 °C.

*Storage*: in an airtight container, protected from light.

**3-Aminophenol.** $C_6H_7NO$. ($M_r$ 109.1). *1147600.* [591-27-5].

Pale yellowish-brown crystals, sparingly soluble in water.

mp: about 122 °C.

**4-Aminophenol.** $C_6H_7NO$. ($M_r$ 109.1). *1004300.* [123-30-8].

*Content*: minimum 95 per cent.

White or slightly coloured, crystalline powder, becoming coloured on exposure to air and light, sparingly soluble in water, soluble in anhydrous ethanol.

mp: about 186 °C, with decomposition.

*Storage*: protected from light.

**Aminopolyether.** $C_{18}H_{36}N_2O_6$. ($M_r$ 376.5). *1112500.* [23978-09-8]. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8,8,8]hexacosane.

mp: 70 °C to 73 °C.

**3-Aminopropanol.** $C_3H_9NO$. ($M_r$ 75.1). *1004400.* [156-87-6]. 3-Aminopropan-1-ol. Propanolamine.

Clear, colourless, viscous liquid.

$d_{20}^{20}$: about 0.99.

$n_D^{20}$: about 1.461.

mp: about 11 °C.

**3-Aminopropionic acid.** $C_3H_7NO_2$. ($M_r$ 89.1). *1004500.* [107-95-9]. β-Alanine.

*Content*: minimum 99 per cent.

White or almost white, crystalline powder, freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: about 200 °C, with decomposition.

**Aminopyrazolone.** $C_{11}H_{13}N_3O$. ($M_r$ 203.2). *1004600.* [83-07-8]. 4-Amino-2,3-dimethyl-1-phenylpyrazolin-5-one.

Light-yellow needles or powder, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 108 °C.

**Aminopyrazolone solution.** *1004601.*

A 1 g/L solution of *aminopyrazolone R* in *buffer solution pH 9.0 R*.

**3-Aminosalicylic acid.** $C_7H_7NO_3$. ($M_r$ 153.1). *1183600.* [570-23-0]. 3-Amino-2-hydroxybenzoic acid.

mp: about 240 °C.

Slightly soluble in water.

**4-Aminosalicylic acid.** $C_7H_7NO_3$. ($M_r$ 153.1). *1183700.* [65-49-6]. 4-Amino-2-hydroxybenzoic acid.

White or almost white, bulky powder, slightly soluble in water, soluble in ethanol (96 per cent), in dilute nitric acid and in sodium hydroxide. It darkens on exposure to air and light.

mp: 135 °C to 145 °C.

*Storage*: at a temperature not exceeding 30 °C, in an airtight container, protected from light.

**Ammonia, concentrated.** *1004700.*

See *Concentrated ammonia solution (0877)*.

**Ammonia.** *1004701.*

*Content*: 170 g/L to 180 g/L of $NH_3$ ($M_r$ 17.03).

Dilute 67 g of *concentrated ammonia R* to 100 mL with *water R*.

$d_{20}^{20}$: 0.931 to 0.934.

When used in the test for iron, *ammonia R* complies with the following additional requirement. Evaporate 5 mL of ammonia to dryness on a water-bath, add 10 mL of *water R*, 2 mL of a 200 g/L solution of *citric acid monohydrate R* and 0.1 mL of *thioglycollic acid R*. Make alkaline by adding *ammonia R* and dilute to 20 mL with *water R*. No pink colour develops.

*Storage*: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

**Ammonia, dilute R1.** *1004702.*

*Content*: 100 g/L to 104 g/L of $NH_3$ ($M_r$ 17.03).

Dilute 41 g of *concentrated ammonia R* to 100 mL with *water R*.

**Ammonia, dilute R2.** *1004703.*

*Content*: 33 g/L to 35 g/L of $NH_3$ ($M_r$ 17.03).

Dilute 14 g of *concentrated ammonia R* to 100 mL with *water R*.

**Ammonia, dilute R3.** *1004704.*

*Content*: 1.6 g/L to 1.8 g/L of $NH_3$ ($M_r$ 17.03).

Dilute 0.7 g of *concentrated ammonia R* to 100 mL with *water R*.

**Ammonia, dilute R4.** *1004706.*

*Content*: 8.4 g/L to 8.6 g/L of $NH_3$ ($M_r$ 17.03).

Dilute 3.5 g of *concentrated ammonia R* to 100 mL with *water R*.

**Ammonia, lead-free.** *1004705.*

Complies with the requirements prescribed for *dilute ammonia R1* with the following additional test: to 20 mL of lead-free ammonia, add 1 mL of *lead-free potassium cyanide solution R*, dilute to 50 mL with *water R* and add 0.10 mL of *sodium sulfide solution R*. The solution is not more intensely coloured than a reference solution prepared without sodium sulfide.

**Ammonia, concentrated R1.** *1004800.*

*Content*: minimum 30.0 per cent *m/m* of $NH_3$ ($M_r$ 17.03).

A clear, colourless liquid.

$d_{20}^{20}$: less than 0.892.

*Assay.* Weigh accurately a ground-glass-stoppered flask containing 50.0 mL of *1 M hydrochloric acid*. Introduce 2 mL of *concentrated ammonia R1* and weigh again. Titrate the solution with *1 M sodium hydroxide*, using 0.5 mL of *methyl red mixed solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of $NH_3$.

*Storage*: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

**Ammonium acetate.** $C_2H_7NO_2$. ($M_r$ 77.1). *1004900.* [631-61-8].

Colourless crystals, very deliquescent, very soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Ammonium acetate solution.** *1004901.*

Dissolve 150 g of *ammonium acetate R* in *water R*. Add 3 mL of *glacial acetic acid R* and dilute to 1000 mL with *water R*.

*Storage*: use within 1 week.

**Ammonium and cerium nitrate.** $(NH_4)_2Ce(NO_3)_6$. ($M_r$ 548.2). *1005000.* [16774-21-3].

Orange-yellow, crystalline powder, or orange transparent crystals, soluble in water.

**Ammonium and cerium sulfate.** $(NH_4)_4Ce(SO_4)_4,2H_2O$. ($M_r$ 633). *1005100.* [10378-47-9].

Orange-yellow, crystalline powder or crystals, slowly soluble in water.

**(1*R*)-(–)-Ammonium 10-camphorsulfonate.** $C_{10}H_{19}NO_4S$. ($M_r$ 249.3). *1103200.*

*Content*: minimum 97.0 per cent of (1*R*)-(–)-ammonium 10-camphorsulfonate.

$[\alpha]_D^{20}$: – 18 ± 2, determined on a 50 g/L solution.

**Ammonium carbamate.** $CH_6N_2O_2$. ($M_r$ 78.1). *1168400.* [1111-78-0]. Carbamic acid ammonium salt.

**Ammonium carbonate.** *1005200.* [506-87-6]. A mixture of varying proportions of ammonium hydrogen carbonate ($NH_4HCO_3$, $M_r$ 79.1) and ammonium carbamate ($NH_2COONH_4$, $M_r$ 78.1).

White or almost white translucent mass, slowly soluble in about 4 parts of water. It is decomposed by boiling water. Ammonium carbonate liberates not less than 30 per cent *m/m* of $NH_3$ ($M_r$ 17.03).

*Assay.* Dissolve 2.00 g in 25 mL of *water R*. Slowly add 50.0 mL of *1 M hydrochloric acid*, titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl orange solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of $NH_3$.

*Storage*: at a temperature below 20 °C.

**Ammonium carbonate solution.** *1005201.*

A 158 g/L solution of *ammonium carbonate R*.

**Ammonium carbonate solution R1.** *1005202.*

Dissolve 20 g of *ammonium carbonate R* in 20 mL of *dilute ammonia R1* and dilute to 100 mL with *water R*.

**Ammonium chloride.** *1005300.* [12125-02-9].

See *Ammonium chloride (0007)*.

**Ammonium chloride solution.** *1005301.*

A 107 g/L solution of *ammonium chloride R*.

**Ammonium citrate.** $C_6H_{14}N_2O_7$. ($M_r$ 226.2). *1103300.* [3012-65-5]. Diammonium hydrogen citrate.

White or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

*pH* (*2.2.3*): about 4.3 for a 22.6 g/L solution.

**Ammonium dihydrogen phosphate.** $(NH_4)H_2PO_4$. ($M_r$ 115.0). *1005400.* [7722-76-1]. Monobasic ammonium phosphate.

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

*pH* (*2.2.3*): about 4.2 for a 23 g/L solution.

**Ammonium formate.** $CH_5NO_2$. ($M_r$ 63.1). *1112600.* [540-69-2].

Deliquescent crystals or granules, very soluble in water, soluble in ethanol (96 per cent).

mp: 119 °C to 121 °C.

*Storage*: in an airtight container.

**Ammonium hexafluorogermanate(IV).** $(NH_4)_2GeF_6$. ($M_r$ 222.7). *1134000.* [16962-47-3].

White or almost white crystals, freely soluble in water.

**Ammonium hydrogen carbonate.** $NH_4HCO_3$. ($M_r$ 79.1). *1005500.* [1066-33-7].

*Content*: minimum 99 per cent.

**Ammonium molybdate.** $(NH_4)_6Mo_7O_{24},4H_2O$. ($M_r$ 1236). *1005700.* [12054-85-2].

Colourless or slightly yellow or greenish crystals, soluble in water, practically insoluble in ethanol (96 per cent).

**Ammonium molybdate reagent.** *1005701.*

Mix, in the given order, 1 volume of a 25 g/L solution of *ammonium molybdate R*, 1 volume of a 100 g/L solution of *ascorbic acid R* and 1 volume of *sulfuric acid R* (294.5 g/L $H_2SO_4$). Add 2 volumes of *water R*.

*Storage*: use within 1 day.

**Ammonium molybdate reagent R1.** *1005706.*

Mix 10 mL of a 60 g/L solution of *disodium arsenate R*, 50 mL of *ammonium molybdate solution R*, 90 mL of *dilute sulfuric acid R* and dilute to 200 mL in *water R*.

*Storage*: in amber flasks at 37 °C for 24 h.

**Ammonium molybdate reagent R2.** *1005708.*

Dissolve 50 g of *ammonium molybdate R* in 600 mL of *water R*. To 250 mL of cold *water R* add 150 mL of *sulfuric acid R* and cool. Mix the 2 solutions together. *Storage*: use within 1 day.

**Ammonium molybdate solution.** *1005702.*

A 100 g/L solution of *ammonium molybdate R*.

**Ammonium molybdate solution R2.** *1005703.*

Dissolve 5.0 g of *ammonium molybdate R* with heating in 30 mL of *water R*. Cool, adjust the pH to 7.0 with *dilute ammonia R2* and dilute to 50 mL with *water R*.

**Ammonium molybdate solution R3.** *1005704.*

*Solution A*. Dissolve 5 g of *ammonium molybdate R* in 20 mL of *water R* with heating.

*Solution B*. Mix 150 mL of *ethanol (96 per cent) R* with 150 mL of *water R*. Add with cooling 100 mL of *sulfuric acid R*.

Immediately before use add 80 volumes of solution B to 20 volumes of solution A.

**Ammonium molybdate solution R4.** *1005705.*

Dissolve 1.0 g of *ammonium molybdate R* in *water R* and dilute to 40 mL with the same solvent. Add 3 mL of *hydrochloric acid R* and 5 mL of *perchloric acid R* and dilute to 100 mL with *acetone R*.

*Storage*: protected from light; use within 1 month.

**Ammonium molybdate solution R5.** *1005707.*

Dissolve 1.0 g of *ammonium molybdate R* in 40.0 mL of a 15 per cent *V/V* solution of *sulfuric acid R*. Prepare the solution daily.

**Ammonium molybdate solution R6.** *1005709.*

Slowly add 10 mL of *sulfuric acid R* to about 40 mL of *water R*. Mix and allow to cool. Dilute to 100 mL with *water R* and mix. Add 2.5 g of *ammonium molybdate R* and 1 g of *cerium sulfate R*, and shake for 15 min to dissolve.

**Ammonium nitrate.** $NH_4NO_3$. ($M_r$ 80.0). *1005800.* [6484-52-2].

White or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Ammonium nitrate R1.** *1005801.*

Complies with the requirements prescribed for *ammonium nitrate R* with the following additional requirements.

*Acidity.* The solution of the substance is slightly acid (*2.2.4*).

*Chlorides* (*2.4.4*): maximum 100 ppm, determined on 0.50 g.

*Sulfates* (*2.4.13*): maximum 150 ppm, determined on 1.0 g.

*Sulfated ash* (*2.4.14*): maximum 0.05 per cent, determined on 1.0 g.

**Ammonium oxalate.** $C_2H_8N_2O_4,H_2O$. ($M_r$ 142.1). *1005900.* [6009-70-7].

Colourless crystals, soluble in water.

**Ammonium oxalate solution.** *1005901.*

A 40 g/L solution of *ammonium oxalate R*.

**Ammonium persulfate.** $(NH_4)_2S_2O_8$. ($M_r$ 228.2). *1006000.* [7727-54-0].

White or almost white, crystalline powder or granular crystals, freely soluble in water.

**Ammonium phosphate.** $(NH_4)_2HPO_4$. ($M_r$ 132.1). *1006100.* [7783-28-0]. Diammonium hydrogen phosphate.

White or almost white crystals or granules, hygroscopic, very soluble in water, practically insoluble in ethanol (96 per cent).

*pH* (*2.2.3*): about 8 for a 200 g/L solution.

*Storage*: in an airtight container.

**Ammonium pyrrolidinedithiocarbamate.** $C_5H_{12}N_2S_2$. ($M_r$ 164.3). *1006200.* [5108-96-3]. Ammonium 1-pyrrolidinyl-dithioformate.

White or pale yellow, crystalline powder, sparingly soluble in water, very slightly soluble in ethanol (96 per cent).

*Storage*: in a bottle containing a piece of ammonium carbonate in a muslin bag.

**Ammonium reineckate.** $NH_4[Cr(NCS)_4(NH_3)_2],H_2O$. ($M_r$ 354.4). *1006300.* [13573-16-5]. Ammonium diamine-tetrakis(isothiocyanato)chromate(III) monohydrate.

Red powder or crystals, sparingly soluble in cold water, soluble in hot water and in ethanol (96 per cent).

**Ammonium reineckate solution.** *1006301.*

A 10 g/L solution of *ammonium reineckate R*. Prepare immediately before use.

**Ammonium sulfamate.** $NH_2SO_3NH_4$. ($M_r$ 114.1). *1006400.* [7773-06-0].

White or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 130 °C.

*Storage*: in an airtight container.

**Ammonium sulfate.** $(NH_4)_2SO_4$. ($M_r$ 132.1). *1006500.* [7783-20-2].

Colourless crystals or white or almost white granules, very soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

*pH* (*2.2.3*): 4.5 to 6.0 for a 50 g/L solution in *carbon dioxide-free water R*.

*Sulfated ash* (*2.4.14*): maximum 0.1 per cent.

**Ammonium sulfide solution.** *1123300.*

Saturate 120 mL of *dilute ammonia R1* with *hydrogen sulfide R* and add 80 mL of *dilute ammonia R1*. Prepare immediately before use.

**Ammonium thiocyanate.** $NH_4SCN$. ($M_r$ 76.1). *1006700.* [1762-95-4].

Colourless crystals, deliquescent, very soluble in water, soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Ammonium thiocyanate solution.** *1006701.*

A 76 g/L solution of *ammonium thiocyanate R*.

**Ammonium vanadate.** $NH_4VO_3$. ($M_r$ 117.0). *1006800.* [7803-55-6]. Ammonium trioxovanadate(V).

White or slightly yellowish, crystalline powder, slightly soluble in water, soluble in *dilute ammonia R1*.

**Ammonium vanadate solution.** *1006801.*

Dissolve 1.2 g of *ammonium vanadate R* in 95 mL of *water R* and dilute to 100 mL with *sulfuric acid R*.

**Amoxicillin trihydrate.** *1103400.*

See *Amoxicillin trihydrate (0260)*.

**α-Amylase.** *1100800.* 1,4-α-D-glucane-glucanohydrolase (EC *3.2.1.1*).

White or light brown powder.

**α-Amylase solution.** *1100801.*

A solution of *α-amylase R* with an activity of 800 FAU/g.

**β-Amyrin.** $C_{30}H_{50}O$. ($M_r$ 426.7). *1141800.* [559-70-6]. Olean-12-en-3β-ol.

White or almost white powder.

mp: 187 °C to 190 °C.

**Andrographolide.** $C_{20}H_{30}O_5$. ($M_r$ 350.4). *1198100.* [5508-58-7]. (3*E*,4*S*)-3-[2-[(1*R*,4a*S*,5*R*,6*R*,8a*S*)-6-Hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl]ethylidene]-4-hydroxydihydrofuran-2(3*H*)-one.

**Anethole.** $C_{10}H_{12}O$. ($M_r$ 148.2). *1006900.* [4180-23-8]. 1-Methoxy-4-(propen-1-yl)benzene.

White or almost white, crystalline mass up to 20 °C to 21 °C, liquid above 23 °C, practically insoluble in water, freely soluble in anhydrous ethanol, soluble in ethyl acetate and in light petroleum.

$n_D^{25}$: about 1.56.

bp: about 230 °C.

*Anethole used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

*Test solution*. The substance to be examined.

*Content*: minimum 99.0 per cent of *trans*-anethole (retention time: about 41 min), calculated by the normalisation procedure.

**Anhydrous colloidal silica.** *1202000.* [7631-86-9].

See *Anhydrous colloidal silica (0434)*.

**Aniline.** $C_6H_7N$. ($M_r$ 93.1). *1007100.* [62-53-3]. Benzeneamine.

Colourless or slightly yellowish liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 1.02.

bp: 183 °C to 186 °C.

*Storage*: protected from light.

**Aniline hydrochloride.** $C_6H_8ClN$. ($M_r$ 129.6). *1147700.* [142-04-1]. Benzenamine hydrochloride.

Crystals.
It darkens on exposure to air and light.

mp: about 198 °C.

*Storage*: protected from light.

*Content*: minimum 97.0 per cent.

**Anion-exchange resin.** *1007200.*

Resin in chlorinated form containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$] attached to a polymer lattice consisting of polystyrene cross-linked with 2 per cent of divinylbenzene. It is available as spherical beads.

Wash the resin with *1 M sodium hydroxide* on a sintered-glass filter (40) (*2.1.2*) until the washings are free from chloride, then wash with *water R* until the washings are neutral. Suspend in freshly prepared *ammonium-free water R* and protect from atmospheric carbon dioxide.

**Anion-exchange resin R1.** *1123400.*

Resin containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$] attached to a lattice consisting of methacrylate.

**Anion-exchange resin R2.** *1141900.*

Conjugate of homogeneous 10 µm hydrophilic polyether particles, and a quaternary ammonium salt, providing a matrix suitable for strong anion-exchange chromatography of proteins.

**Anion-exchange resin R3.** *1180900.*

Resin with quaternary ammonium groups attached to a lattice of ethylvinylbenzene crosslinked with 55 per cent of divinylbenzene.

**Anion-exchange resin for chromatography, strongly basic.** *1112700.*

Resin with quaternary amine groups attached to a lattice of latex cross linked with divinylbenzene.

**Anion-exchange resin for chromatography, strongly basic R1.** *1187400.*

Non-porous resin agglomerated with a 100 nm alkyl quaternary ammonium functionalised latex.

**Anion-exchange resin for chromatography, strongly basic R2.** *1203000.*

Non-porous resin agglomerated with a 43 nm quaternary amine functionalised latex, cross-linked with ethylvinylbenzene/divinylbenzene.

**Anion-exchange resin, strongly basic.** *1026600.*

Gel-type resin in hydroxide form containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$, type 1] attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene.

Brown transparent beads.

*Particle size*: 0.2 mm to 1.0 mm.

*Moisture content*: about 50 per cent.

*Total exchange capacity*: minimum 1.2 meq/mL.

**Anion-exchange resin, weak.** *1146700.*

Resin with diethylaminoethyl groups attached to a lattice consisting of poly(methyl methacrylate).

**Anisaldehyde.** $C_8H_8O_2$. ($M_r$ 136.1). *1007300.* [123-11-5]. 4-Methoxybenzaldehyde.

Oily liquid, very slightly soluble in water, miscible with ethanol (96 per cent).

bp: about 248 °C.

*Anisaldehyde used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

*Test solution*. The substance to be examined.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Anisaldehyde solution.** *1007301.*

Mix in the following order, 0.5 mL of *anisaldehyde R*, 10 mL of *glacial acetic acid R*, 85 mL of *methanol R* and 5 mL of *sulfuric acid R*.

**Anisaldehyde solution R1.** *1007302.*

To 10 mL of *anisaldehyde R* add 90 mL of *ethanol (96 per cent) R*, mix, add 10 mL of *sulfuric acid R* and mix again.

**Anise ketone.** $C_{10}H_{12}O_2$. ($M_r$ 164.2). *1174700.* [122-84-9]. 1-(4-Methoxyphenyl)propan-2-one.

***p*-Anisidine.** $C_7H_9NO$. ($M_r$ 123.2). *1103500.* [104-94-9]. 4-Methoxyaniline.

White or almost white crystals, sparingly soluble in water, soluble in anhydrous ethanol.

*Content*: minimum 97.0 per cent.

*Caution: skin irritant, sensitiser.*

*Storage*: protected from light, at 0 °C to 4 °C.

On storage, *p*-anisidine tends to darken as a result of oxidation. A discoloured reagent can be reduced and decolorised in the following way: dissolve 20 g of *p-anisidine R* in 500 mL of *water R* at 75 °C. Add 1 g of *sodium sulfite heptahydrate R* and 10 g of *activated charcoal R* and stir for 5 min. Filter, cool the

filtrate to about 0 °C and allow to stand at this temperature for at least 4 h. Filter, wash the crystals with a small quantity of *water R* at about 0 °C and dry the crystals *in vacuo* (*2.2.32*).

**Anthracene.** $C_{14}H_{10}$. ($M_r$ 178.2). *1007400.* [120-12-7].
White or almost white, crystalline powder, practically insoluble in water, slightly soluble in chloroform.
mp: about 218 °C.

**Anthrone.** $C_{14}H_{10}O$. ($M_r$ 194.2). *1007500.* [90-44-8].
9(10*H*)-Anthracenone.
Pale yellow, crystalline powder.
mp: about 155 °C.

**Antimony potassium tartrate.** $C_8H_4K_2O_{12}Sb_2,3H_2O$. ($M_r$ 668). *1007600.* [28300-74-5]. Dipotassium di[tartrato(4-)*O*$^1$,*O*$^2$,*O*$^3$,*O*$^4$]bis[antimonate(III)] trihydrate.
White or almost white, granular powder or colourless, transparent crystals, soluble in water and in glycerol, freely soluble in boiling water, practically insoluble in ethanol (96 per cent). The aqueous solution is slightly acid.

**Antimony trichloride.** $SbCl_3$. ($M_r$ 228.1). *1007700.* [10025-91-9].
Colourless crystals or a transparent crystalline mass, hygroscopic, freely soluble in anhydrous ethanol. Antimony trichloride is hydrolysed by water.
*Storage*: in an airtight container, protected from moisture.

**Antimony trichloride solution.** *1007701.*
Rapidly wash 30 g of *antimony trichloride R* with two quantities, each of 15 mL, of *ethanol-free chloroform R*, drain off the washings, and dissolve the washed crystals immediately in 100 mL of *ethanol-free chloroform R*, warming slightly.
*Storage*: over a few grams of *anhydrous sodium sulfate R*.

**Antithrombin III.** *1007800.* [90170-80-2].
Antithrombin III is purified from human plasma by heparin agarose chromatography and should have a specific activity of at least 6 IU/mg.

**Antithrombin III solution R1.** *1007801.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 1 IU/mL.

**Antithrombin III solution R2.** *1007802.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 0.5 IU/mL.

**Antithrombin III solution R3.** *1007803.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.3 IU/mL with *phosphate buffer solution pH 6.5 R*.

**Antithrombin III solution R4.** *1007804.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.1 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R*.

**Antithrombin III solution R5.** *1007805.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.125 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

**Antithrombin III solution R6.** *1007806.*
Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 1.0 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

**Apigenin.** $C_{15}H_{10}O_5$. ($M_r$ 270.2). *1095800.* [520-36-5]. 4′,5,7-Trihydroxyflavone.
Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).
mp: about 310 °C, with decomposition.
*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Roman chamomile flower (0380)*: apply 10 µL of a 0.25 g/L solution in *methanol R*; the chromatogram shows in the upper third a principal zone of yellowish-green fluorescence.

**Apigenin 7-glucoside.** $C_{21}H_{20}O_{10}$. ($M_r$ 432.4). *1095900.* [578-74-5]. Apigetrin. 7-(β-D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.
Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).
mp: 198 °C to 201 °C.
*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Roman chamomile flower (0380)*: apply 10 µL of a 0.25 g/L solution in *methanol R*; the chromatogram shows in the middle third a principal zone of yellowish fluorescence.
*Apigenin-7-glucoside used in liquid chromatography complies with the following additional test.*
*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Matricaria flower (0404)*.
*Test solution*. Dissolve 10.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent.
*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Aprotinin.** *1007900.* [9087-70-1].
See *Aprotinin (0580)*.

**Arabinose.** $C_5H_{10}O_5$. ($M_r$ 150.1). *1008000.* [87-72-9]. (3*R*,4*S*,5*S*)-Tetrahydro-2*H*-pyran-2,3,4,5-tetrol. L-Arabinopyranose. L-(+)-Arabinose.
White or almost white, crystalline powder, freely soluble in water.
$[\alpha]_D^{20}$: + 103 to + 105, determined on a 50 g/L solution in *water R* containing about 0.05 per cent of $NH_3$.

**Arachidyl alcohol.** $C_{20}H_{42}O$. ($M_r$ 298.5). *1156300.* [629-96-9]. 1-Eicosanol.
mp: about 65 °C.
*Content*: minimum 96 per cent of $C_{20}H_{42}O$.

**Arbutin.** $C_{12}H_{16}O_7$. ($M_r$ 272.3). *1008100.* [497-76-7]. Arbutoside. 4-Hydroxyphenyl-β-D-glucopyranoside.
Fine, white or almost white, shiny needles, freely soluble in water, very soluble in hot water, soluble in ethanol (96 per cent).
*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Bearberry leaf (1054)*; the chromatogram shows only one principal spot.

**Arginine.** *1103600.* [74-79-3].
See *Arginine (0806)*.

**Argon.** Ar. ($A_r$ 39.95). *1008200.* [7440-37-1].
*Content*: minimum 99.995 per cent *V/V*.
*Carbon monoxide* (*2.5.25, Method I*): maximum 0.6 ppm *V/V*; after passage of 10 L of *argon R* at a flow rate of 4 L/h, not more than 0.05 mL of *0.002 M sodium thiosulfate* is required for the titration.

**Argon R1.** Ar. ($A_r$ 39.95). *1176000.* [7440-37-1].
*Content*: minimum 99.99990 per cent *V/V*.

**Argon for chromatography.** Ar. ($A_r$ 39.95). *1166200.* [7440-37-1].
*Content*: minimum 99.95 per cent *V/V*.

**Aromadendrene.** $C_{15}H_{24}$. ($M_r$ 204.4 ). *1139100.* [489-39-4]. (1*R*,2*S*,4*R*,8*R*,11*R*)-3,3,11-Trimethyl-7-methylenetricyclo-[6.3.0.0$^{2,4}$]undecane.

Clear, almost colourless liquid.

$d_4^{20}$: about 0.911.

$n_D^{20}$: about 1.497.

$[\alpha]_D^{20}$: about + 12.

bp: about 263 °C.

*Aromadendrene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph on *Tea tree oil (1837)*.

*Content*: minimum 92 per cent, calculated by the normalisation procedure.

**Arsenazo III.** $C_{22}H_{18}As_2N_4O_{14}S_2$. ($M_r$ 776). *1198200.* [1668-00-4]. 3,6-Bis[(2-arsonophenyl)diazenyl]-4,5-dihydroxynaphthalene-2,7-disulfonic acid.

Brown powder.

**Arsenious trioxide.** $As_2O_3$. ($M_r$ 197.8). *1008300.* [1327-53-3]. Arsenious anhydride. Diarsenic trioxide.

Crystalline powder or a white or almost white mass, slightly soluble in water, soluble in boiling water.

**Ascorbic acid.** *1008400.* [50-81-7].

See *Ascorbic acid (0253)*.

**Ascorbic acid solution.** *1008401.*

Dissolve 50 mg in 0.5 mL of *water R* and dilute to 50 mL with *dimethylformamide R*.

**Asiaticoside.** $C_{48}H_{78}O_{19}$. ($M_r$ 959). *1123500.* [16830-15-2]. *O*-6-Deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl 2α,3β,23-trihydroxy-4α-urs-12-en-28-oate.

White or almost white powder, hygroscopic, soluble in methanol, slightly soluble in anhydrous ethanol, insoluble in acetonitrile.

mp: about 232 °C, with decomposition.

*Water* (*2.5.12*): 6.0 per cent.

*Asiaticoside used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Centella (1498)*.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

*Storage*: protected from humidity.

**Asparagine.** $C_4H_8N_2O_3$. ($M_r$ 132.12). *1200000.* [70-47-3].

**Aspartic acid.** *1134100.* [56-84-8].

See *Aspartic acid (0797)*.

**D-Aspartic acid.** $C_4H_7NO_4$. ($M_r$ 133.1). *1200100.* [1783-96-6].

**L-Aspartyl-L-phenylalanine.** $C_{13}H_{16}N_2O_5$. ($M_r$ 280.3). *1008500.* [13433-09-5]. (*S*)-3-Amino-*N*-[(*S*)-1-carboxy-2-phenylethyl]-succinamic acid.

White or almost white powder.

mp: about 210 °C, with decomposition.

**Astragaloside IV.** $C_{41}H_{68}O_{14}$. ($M_r$ 785). *1178200.* [84687-43-4]. (20*R*,24*S*)-20,24-Epoxy-16β,25-dihydroxy-3β-(β-D-xylopyranosyloxy)-9,19-cyclolanostan-6α-yl β-D-glucopyranoside.

**Atropine sulfate.** *1159000.* [5908-99-6].

See *Atropine sulfate (0068)*.

**Aucubin.** $C_{15}H_{22}O_9$. ($M_r$ 346.3 ). *1145200.* [479-98-1]. [1*S*,4a*R*,5*S*,7a*S*)-5-Hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-1-yl β-D-glucopyranoside.

Crystals, soluble in water, in ethanol (96 per cent) and in methanol, practically insoluble in light petroleum.

$[\alpha]_D^{20}$: about − 163.

mp: about 181 °C.

**Azomethine H.** $C_{17}H_{12}NNaO_8S_2$. ($M_r$ 445.4). *1008700.* [5941-07-1]. Sodium hydrogeno-4-hydroxy-5-(2-hydroxybenzylideneamino)-2,7-naphthalenedisulfonate.

**Azomethine H solution.** *1008701.*

Dissolve 0.45 g of *azomethine H R* and 1 g of *ascorbic acid R* with gentle heating in *water R* and dilute to 100 mL with the same solvent.

**Baicalin.** $C_{21}H_{18}O_{11}$. ($M_r$ 446.4). *1179200.* [21967-41-9]. 5,6-Dihydroxy-4-oxo-2-phenyl-4*H*-1-benzopyran-7-yl-β-D-glucopyranosiduronic acid.

**Barbaloin.** $C_{21}H_{22}O_9,H_2O$. ($M_r$ 436.4). *1008800.* [1415-73-2]. Aloin. 1,8-Dihydroxy-3-hydroxymethyl-10-β-D-glucopyranosyl-10*H*-anthracen-9-one.

Yellow to dark-yellow, crystalline powder, or yellow needles, darkening on exposure to air and light, sparingly soluble in water and in ethanol (96 per cent), soluble in acetone, in ammonia and in solutions of alkali hydroxides.

$A_{1\,cm}^{1\%}$: about 192 at 269 nm, about 226 at 296.5 nm, about 259 at 354 nm, determined on a solution in *methanol R* and calculated with reference to the anhydrous substance.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Frangula bark (0025)*; the chromatogram shows only one principal spot.

**Barbital.** *1008900.* [57-44-3].

See *Barbital (0170)*.

**Barbital sodium.** $C_8H_{11}N_2NaO_3$. ($M_r$ 206.2). *1009000.* [144-02-5]. Sodium derivative of 5,5-diethyl-1*H*,3*H*,5*H*-pyrimidine-2,4,6-trione.

*Content*: minimum 98.0 per cent.

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Barbituric acid.** $C_4H_4N_2O_3$. ($M_r$ 128.1). *1009100.* [67-52-7]. 1*H*,3*H*,5*H*-Pyrimidine-2,4,6-trione.

White or almost white powder, slightly soluble in water, freely soluble in boiling water and in dilute acids.

mp: about 253 °C.

**Barium acetate.** $C_4H_6BaO_4$. ($M_r$ 255.4). *1162700.* [543-80-6]. Barium diacetate.

White or almost white powder, soluble in water.

$d_{20}^{20}$: 2.47.

**Barium carbonate.** $BaCO_3$. ($M_r$ 197.3). *1009200.* [513-77-9].

White or almost white powder or friable masses, practically insoluble in water.

**Barium chloride.** $BaCl_2,2H_2O$. ($M_r$ 244.3). *1009300.* [10326-27-9]. Barium dichloride.

Colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Barium chloride solution R1.** *1009301.*

A 61 g/L solution of *barium chloride R*.

**Barium chloride solution R2.** *1009302.*

A 36.5 g/L solution of *barium chloride R*.

**Barium hydroxide.** $Ba(OH)_2,8H_2O$. ($M_r$ 315.5). *1009400.* [12230-71-6]. Barium dihydroxide.

Colourless crystals, soluble in water.

**Barium hydroxide solution.** *1009401.*

A 47.3 g/L solution of *barium hydroxide R*.

**Barium nitrate.** $Ba(NO_3)_2$. ($M_r$ 261.3). *1163800.* [10022-31-8].

Crystals or crystalline powder, freely soluble in water, very slightly soluble in ethanol (96 per cent) and in acetone.

mp: about 590 °C.

**Barium sulfate.** *1009500.* [7727-43-7].

See *Barium sulfate (0010)*.

**Benzalacetone.** $C_{10}H_{10}O$. ($M_r$ 146.2). *1168500.* [122-57-6]. (3*E*)-4-phenylbut-3-en-2-one.

White or pale yellow mass.

*Content*: minimum 98.0 per cent.

bp: about 261 °C.

mp: about 39 °C.

**Benzaldehyde.** $C_7H_6O$. ($M_r$ 106.1). *1009600.* [100-52-7].

Colourless or slightly yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 1.05.

$n_D^{20}$: about 1.545.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 177 °C and 180 °C.

*Storage*: protected from light.

**Benzene.** $C_6H_6$. ($M_r$ 78.1). *1009800.* [71-43-2].

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: about 80 °C.

Where benzene is used to prepare a reference solution, for safety reasons, the pure reagent may be replaced by a commercially available reference material containing a certified amount of benzene.

**Benzene-1,2,4-triol.** $C_6H_6O_3$. ($M_r$ 126.1). *1177500.* [533-73-3]. Hydroxyhydroquinone. Hydroxyquinol.

Freely soluble in water, in ethanol (96 per cent) and in ethyl acetate.

mp: about 140 °C.

**Benzethonium chloride.** $C_{27}H_{42}ClNO_2,H_2O$. ($M_r$ 466.1). *1009900.* [121-54-0]. Benzyldimethyl[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]ammonium chloride monohydrate.

Fine, white or almost white powder or colourless crystals, soluble in water and in ethanol (96 per cent).

mp: about 163 °C.

*Storage*: protected from light.

**Benzidine.** $C_{12}H_{12}N_2$. ($M_r$ 184.2). *1145300.* [92-87-5]. Biphenyl-4,4′-diamine.

*Content*: minimum 95 per cent.

White or slightly yellowish or reddish powder, darkening on exposure to air and light.

mp: about 120 °C.

*Storage*: protected from light.

**Benzil.** $C_{14}H_{10}O_2$. ($M_r$ 210.2). *1117800.* [134-81-6]. Diphenylethanedione.

Yellow, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent), ethyl acetate and toluene.

mp: 95 °C.

**Benzocaine.** $C_9H_{11}NO_2$. ($M_r$ 165.2). *1123600.* [94-09-7].

See *Benzocaine (0011)*.

**Benzohydrazide.** $C_7H_8N_2O$. ($M_r$ 136.2). *1194400.* [613-94-5]. Benzoyldiazane.

**Benzoic acid.** *1010100.* [65-85-0].

See *Benzoic acid (0066)*.

**Benzoin.** $C_{14}H_{12}O_2$. ($M_r$ 212.3). *1010200.* [579-44-2]. 2-Hydroxy-1,2-diphenylethanone.

Slightly yellowish crystals, very slightly soluble in water, freely soluble in acetone, soluble in hot ethanol (96 per cent).

mp: about 137 °C.

**Benzophenone.** $C_{13}H_{10}O$. ($M_r$ 182.2). *1010300.* [119-61-9]. Diphenylmethanone.

Prismatic crystals, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 48 °C.

**1,4-Benzoquinone.** $C_6H_4O_2$. ($M_r$ 108.1). *1118500.* [106-51-4]. Cyclohexa-2,5-diene-1,4-dione.

*Content*: minimum 98.0 per cent.

**Benzoylarginine ethyl ester hydrochloride.** $C_{15}H_{23}ClN_4O_3$. ($M_r$ 342.8). *1010500.* [2645-08-1]. *N*-Benzoyl-L-arginine ethyl ester hydrochloride. Ethyl (*S*)-2-benzamido-5-guanidinovalerate hydrochloride.

White or almost white, crystalline powder, very soluble in water and in anhydrous ethanol.

$[\alpha]_D^{20}$: − 15 to − 18, determined on a 10 g/L solution.

mp: about 129 °C.

$A_{1\,cm}^{1\%}$: 310 to 340, determined at 227 nm using a 0.01 g/L solution.

**Benzoyl chloride.** $C_7H_5ClO$. ($M_r$ 140.6). *1010400.* [98-88-4].

Colourless, lachrymatory liquid, decomposed by water and by ethanol (96 per cent).

$d_{20}^{20}$: about 1.21.

bp: about 197 °C.

***N*-Benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate.** $C_{35}H_{42}N_8O_8$. ($M_r$ 703). *1010600.*

**3-Benzoylpropionic acid.** $C_{10}H_{10}O_3$. ($M_r$ 178.2). *1171000.* [2051-95-8]. 4-Oxo-4-phenylbutanoic acid.

mp: about 118 °C.

**2-Benzoylpyridine.** $C_{12}H_9NO$. ($M_r$ 183.2). *1134300.* [91-02-1]. Phenyl(pyridin-2-yl)methanone.

Colourless crystals, soluble in ethanol (96 per cent).

mp: about 43 °C.

**Benzyl alcohol.** *1010700.* [100-51-6].

See *Benzyl alcohol (0256)*.

**Benzyl benzoate.** *1010800.* [120-51-4].

See *Benzyl benzoate (0705)*.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Peru balsam (0754)*: apply 20 µL of a 0.3 per cent *V/V* solution in *ethyl acetate R*; after spraying and heating, the chromatogram shows a principal band with an $R_F$ of about 0.8.

**Benzyl cinnamate.** $C_{16}H_{14}O_2$. ($M_r$ 238.3). *1010900.* [103-41-3]. Benzyl 3-phenylprop-2-enoate.

Colourless or yellowish crystals, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 39 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Peru balsam (0754)*: apply 20 µL of a 3 g/L solution in *ethyl acetate R*; after spraying and heating, the chromatogram shows a principal band with an $R_F$ of about 0.6.

**Benzyl cyanide.** $C_8H_7N$. ($M_r$ 117.2). *1171100.* [140-29-4]. Phenylacetonitrile.

*Content*: minimum 95.0 per cent.

Clear, colourless or light yellow liquid.

$n_D^{20}$: about 1.523.

bp: about 233 °C.

**Benzyl ether.** $C_{14}H_{14}O$. ($M_r$ 198.3). *1140900.* [103-50-4]. Dibenzyl ether.

Clear, colourless liquid, practically insoluble in water, miscible with acetone and with anhydrous ethanol.

$d_{20}^{20}$: about 1.043.

$n_D^{20}$: about 1.562.

bp: about 296 °C, with decomposition.

**Benzylpenicillin sodium.** *1011000.* [69-57-8].

See *Benzylpenicillin sodium (0114)*.

**2-Benzylpyridine.** $C_{12}H_{11}N$. ($M_r$ 169.2). *1112900.* [101-82-6].

*Content*: minimum 98.0 per cent.

Yellow liquid.

mp: 13 °C to 16 °C.

**4-Benzylpyridine.** $C_{12}H_{11}N$. ($M_r$ 169.2). *1181200.* [2116-65-6].

*Content*: minimum 98.0 per cent.

Yellow liquid.

mp: 72 °C to 78 °C.

**Benzyltrimethylammonium chloride.** $C_{10}H_{16}ClN$. ($M_r$ 185.7). *1155700.* [56-93-9]. *N*,*N*,*N*-Trimethylphenylmethanaminium chloride. *N*,*N*,*N*-Trimethylbenzenemethanaminium chloride.

White or almost white powder, soluble in water.

mp: about 230 °C, with decomposition.

**Berberine chloride.** $C_{20}H_{18}ClNO_4,2H_2O$. ($M_r$ 407.8). *1153400.* [5956-60-5]. 9,10-Dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium chloride.

Yellow crystals, slightly soluble in water, practically insoluble in ethanol (96 per cent).

mp: 204 °C to 206 °C.

*Berberine chloride used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Goldenseal rhizome (1831)*.

*Content*: minimum 95 per cent, calculated by the normalisation procedure.

**Bergapten.** $C_{12}H_8O_4$. ($M_r$ 216.2). *1103700.* [484-20-8]. 5-Methoxypsoralen.

Colourless crystals, practically insoluble in water, sparingly soluble in ethanol (96 per cent) and slightly soluble in glacial acetic acid.

mp: about 188 °C.

**Betulin.** $C_{30}H_{50}O_2$. ($M_r$ 442.7). *1011100.* [473-98-3]. Lup-20(39)-ene-3β,28-diol.

White or almost white, crystalline powder.

mp: 248 °C to 251 °C.

**Bibenzyl.** $C_{14}H_{14}$. ($M_r$ 182.3). *1011200.* [103-29-7]. 1,2-Diphenylethane.

White or almost white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in ethanol (96 per cent).

mp: 50 °C to 53 °C.

**Biphenyl.** $C_{12}H_{10}$. ($M_r$ 154.2). *1168600.* [92-52-4].

mp: 68 °C to 70 °C.

**(–)-α-Bisabolol.** $C_{15}H_{26}O$. ($M_r$ 222.4). *1128800.* [23089-26-1]. (2*S*)-6-Methyl-2-[(1*S*)-4-methylcyclohex-3-enyl]hept-5-en-2-ol. Levomenol.

Colourless, viscous liquid with a slight, characteristic odour, practically insoluble in water, freely soluble in ethanol (96 per cent), in methanol, in toluene, in fatty oils and in essential oils.

$d_{20}^{20}$: 0.925 to 0.935.

$n_D^{20}$: 1.492 to 1.500.

$[\alpha]_D^{20}$: – 54.5 to – 58.0, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

*(– )-α-Bisabolol used for gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Matricaria oil (1836)*.

*Test solution*. A 4 g/L solution in *cyclohexane R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Bisbenzimide.** $C_{25}H_{27}Cl_3N_6O,5H_2O$. ($M_r$ 624). *1103800.* [23491-44-3]. 4-[5-[5-(4-Methylpiperazin-1-yl)benzimidazol-2-yl]benzimidazol-2-yl]phenol trihydrochloride pentahydrate.

**Bisbenzimide stock solution.** *1103801.*

Dissolve 5 mg of *bisbenzimide R* in *water R* and dilute to 100 mL with the same solvent.

*Storage*: in the dark.

**Bisbenzimide working solution.** *1103802.*

Immediately before use, dilute 100 µL of *bisbenzimide stock solution R* to 100 mL with *phosphate buffered saline pH 7.4 R*.

**Bis(diphenylmethyl) ether.** $C_{26}H_{22}O$. ($M_r$ 350.5). *1203100.* [574-42-5].

[Oxybis(methanetriyl)]tetrakisbenzene. 1,1′,1″,1‴-(Oxymethylidyne)tetrakisbenzene.

**Bismuth nitrate pentahydrate.** $Bi(NO_3)_3,5H_2O$. ($M_r$ 485.1). *1165600.* [10035-06-0].

mp: about 30 °C.

**Bismuth subnitrate.** $4BiNO_3(OH)_2,BiO(OH)$. ($M_r$ 1462). *1011500.* [1304-85-4].

White or almost white powder, practically insoluble in water.

**Bismuth subnitrate R1.** *1011501.*

*Content*: 71.5 per cent to 74.0 per cent of bismuth (Bi), and 14.5 per cent to 16.5 per cent of nitrate, calculated as nitrogen pentoxide ($N_2O_5$).

**Bismuth subnitrate solution.** *1011502.*

Dissolve 5 g of *bismuth subnitrate R1* in a mixture of 8.4 mL of *nitric acid R* and 50 mL of *water R* and dilute to 250 mL with *water R*. Filter if necessary.

*Acidity*. To 10 mL add 0.05 mL of *methyl orange solution R*. 5.0 mL to 6.25 mL of *1 M sodium hydroxide* is required to change the colour of the indicator.

**Bis-tris propane.** $C_{11}H_{26}N_2O_6$. ($M_r$ 282.3). *1185500.* [64431-96-5]. 2,2′-(Propane-1,3-diyldiimino)bis[2-(hydroxymethyl)-1,3-propanediol.

*Content*: minimum 99.0 per cent.

**Biuret.** $C_2H_5N_3O_2$. ($M_r$ 103.1). *1011600.* [108-19-0].
White or almost white crystals, hygroscopic, soluble in water, sparingly soluble in ethanol (96 per cent).
mp: 188 °C to 190 °C, with decomposition.
*Storage*: in an airtight container.

**Biuret reagent.** *1011601.*
Dissolve 1.5 g of *copper sulfate pentahydrate R* and 6.0 g of *sodium potassium tartrate R* in 500 mL of *water R*. Add 300 mL of a carbonate-free 100 g/L solution of *sodium hydroxide R*, dilute to 1000 mL with the same solution and mix.

**Blocking solution.** *1122400.*
A 10 per cent *V/V* solution of *acetic acid R*.

**Blue dextran 2000.** *1011700.* [9049-32-5].
Prepared from dextran having an average relative molecular mass of $2 \times 10^6$ by introduction of a polycyclic chromophore that colours the substance blue. The degree of substitution is 0.017.
It is freeze-dried and dissolves rapidly and completely in water and aqueous saline solutions.
*Absorbance* (*2.2.25*). A 1 g/L solution in a *phosphate buffer solution pH 7.0 R* shows an absorption maximum at 280 nm.

**Boldine.** $C_{19}H_{21}NO_4$. ($M_r$ 327.3). *1118800.* [476-70-0].
1,10-Dimethoxy-6aα-aporphine-2,9-diol.
White or almost white crystalline powder, very slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of acids.
$[\alpha]_D^{25}$: about + 127, determined on a 1 g/L solution in *anhydrous ethanol R*.
mp: about 163 °C.

**Boric acid.** *1011800.* [10043-35-3].
See *Boric acid (0001)*.

**Boric acid solution, saturated, cold.** *1011801.*
To 3 g of *boric acid R* add 50 mL of *water R* and shake for 10 min. Place the solution for 2 h in the refrigerator.

**Borneol.** $C_{10}H_{18}O$. ($M_r$ 154.3). *1011900.* [507-70-0].
*endo*-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol.
Colourless crystals, readily sublimes, practically insoluble in water, freely soluble in ethanol (96 per cent) and in light petroleum.
mp: about 208 °C.
*Chromatography*. Thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. Apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 mL for a plate 200 mm square, and heat at 100-105 °C for 10 min. The chromatogram obtained shows only one principal spot.

**Bornyl acetate.** $C_{12}H_{20}O_2$. ($M_r$ 196.3). *1012000.* [5655-61-8].
*endo*-1,7,7-Trimethylbicyclo[2.2.1]hept-2-yl acetate.
Colourless crystals or a colourless liquid, very slightly soluble in water, soluble in ethanol (96 per cent).
mp: about 28 °C.
*Chromatography*. Thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. Apply to the plate 10 µL of a 2 g/L solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 mL for a plate 200 mm square, and heat at 100-105 °C for 10 min. The chromatogram obtained shows only one principal spot.

**Boron trichloride.** $BCl_3$. ($M_r$ 117.2). *1112000.* [10294-34-5].
Colourless gas. Reacts violently with water. Available as solutions in suitable solvents (2-chloroethanol, methylene chloride, hexane, heptane, methanol).
$n_D^{20}$: about 1.420.
bp: about 12.6 °C.
*Caution: toxic and corrosive.*

**Boron trichloride-methanol solution.** *1112001.*
A 12 per cent *m/m* solution of *boron trichloride R* in *methanol R*.
*Storage*: protected from light at − 20 °C, preferably in sealed tubes.

**Boron trifluoride.** $BF_3$. ($M_r$ 67.8). *1012100.* [7637-07-2].
Colourless gas.

**Boron trifluoride-methanol solution.** *1012101.*
A 140 g/L solution of *boron trifluoride R* in *methanol R*.

**Brilliant blue.** *1012200.* [6104-59-2].
See *acid blue 83 R*.

**Bromelains.** *1012300.* [37189-34-7].
Concentrate of proteolytic enzymes derived from *Ananas comosus* Merr.
Dull-yellow powder.
*Activity*. 1 g liberates about 1.2 g of amino-nitrogen from a solution of *gelatin R* in 20 min at 45 °C and pH 4.5.

**Bromelains solution.** *1012301.*
A 10 g/L solution of *bromelains R* in a mixture of 1 volume of *phosphate buffer solution pH 5.5 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*.

**Bromine.** $Br_2$. ($M_r$ 159.8). *1012400.* [7726-95-6].
Brownish-red fuming liquid, slightly soluble in water, soluble in ethanol (96 per cent).
$d_{20}^{20}$: about 3.1.

**Bromine solution.** *1012401.*
Dissolve 30 g of *bromine R* and 30 g of *potassium bromide R* in *water R* and dilute to 100 mL with the same solvent.

**Bromine water.** *1012402.*
Shake 3 mL of *bromine R* with 100 mL of *water R* to saturation.
*Storage*: over an excess of *bromine R*, protected from light.

**Bromine water R1.** *1012403.*
Shake 0.5 mL of *bromine R* with 100 mL of *water R*.
*Storage*: protected from light; use within 1 week.

**Bromocresol green.** $C_{21}H_{14}Br_4O_5S$. ($M_r$ 698). *1012600.* [76-60-8]. 3′,3″,5′,5″-Tetrabromo-*m*-cresol-sulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromo-3-methylphenol)-*S*,*S*-dioxide.
Brownish-white powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Bromocresol green-methyl red solution.** *1012602.*
Dissolve 0.15 g of *bromocresol green R* and 0.1 g of *methyl red R* in 180 mL of *anhydrous ethanol R* and dilute to 200 mL with *water R*.

**Bromocresol green solution.** *1012601.*
Dissolve 50 mg of *bromocresol green R* in 0.72 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.
*Test for sensitivity*. To 0.2 mL of the bromocresol green solution add 100 mL of *carbon dioxide-free water R*. The solution is blue. Not more than 0.2 mL of *0.02 M hydrochloric acid* is required to change the colour to green.
*Colour change*: pH 3.6 (yellow) to pH 5.2 (blue).

**Bromocresol purple.** $C_{21}H_{16}Br_2O_5S$. ($M_r$ 540.2). *1012700.* [115-40-2]. 3′,3″-Dibromo-*o*-cresolsulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-bromo-6-methylphenol)-*S*,*S*-dioxide.

Pinkish powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Bromocresol purple solution.** *1012701.*

Dissolve 50 mg of *bromocresol purple R* in 0.92 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.2 mL of the bromocresol purple solution add 100 mL of *carbon dioxide-free water R* and 0.05 mL of *0.02 M sodium hydroxide*. The solution is bluish-violet. Not more than 0.2 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

*Colour change*: pH 5.2 (yellow) to pH 6.8 (bluish-violet).

**5-Bromo-2′-deoxyuridine.** $C_9H_{11}BrN_2O_5$. ($M_r$ 307.1). *1012500.* [59-14-3]. 5-Bromo-1-(2-deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione.

mp: about 194 °C.

*Chromatography.* Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram shows only one principal spot.

**Bromomethoxynaphthalene.** $C_{11}H_9BrO$. ($M_r$ 237.1). *1159100.* [5111-65-9]. 2-Bromo-6-methoxynaphthalene.

mp: about 109 °C.

**Bromophenol blue.** $C_{19}H_{10}Br_4O_5S$. ($M_r$ 670). *1012800.* [115-39-9]. 3′,3″,5′,5″-Tetrabromophenolsulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromophenol) *S*,*S*-dioxide.

Light orange-yellow powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent), freely soluble in solutions of alkali hydroxides.

**Bromophenol blue solution.** *1012801.*

Dissolve 0.1 g of *bromophenol blue R* in 1.5 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.05 mL of the bromophenol blue solution add 20 mL of *carbon dioxide-free water R* and 0.05 mL of *0.1 M hydrochloric acid*. The solution is yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour to bluish-violet.

*Colour change*: pH 2.8 (yellow) to pH 4.4 (bluish-violet).

**Bromophenol blue solution R1.** *1012802.*

Dissolve 50 mg of *bromophenol blue R* with gentle heating in 3.73 mL of *0.02 M sodium hydroxide* and dilute to 100 mL with *water R*.

**Bromophenol blue solution R2.** *1012803.*

Dissolve with heating 0.2 g of *bromophenol blue R* in 3 mL of *0.1 M sodium hydroxide* and 10 mL of *ethanol (96 per cent) R*. After solution is effected, allow to cool and dilute to 100 mL with *ethanol (96 per cent) R*.

**Bromophos.** $C_8H_8BrCl_2O_3PS$. ($M_r$ 366.0). *1123700.* [2104-96-3].

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Bromophos-ethyl.** $C_{10}H_{12}BrCl_2O_3PS$. ($M_r$ 394.0). *1123800.* [4824-78-6].

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Bromothymol blue.** $C_{27}H_{28}Br_2O_5S$. ($M_r$ 624). *1012900.* [76-59-5]. 3′,3″-Dibromothymolsulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-bromo-6-isopropyl-3-methylphenol) *S*,*S*-dioxide.

Reddish-pink or brownish powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Bromothymol blue solution R1.** *1012901.*

Dissolve 50 mg of *bromothymol blue R* in a mixture of 4 mL of *0.02 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.3 mL of *bromothymol blue solution R1* add 100 mL of *carbon dioxide-free water R*. The solution is yellow. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to blue.

*Colour change*: pH 5.8 (yellow) to pH 7.4 (blue).

**Bromothymol blue solution R2.** *1012902.*

A 10 g/L solution of *bromothymol blue R* in *dimethylformamide R*.

**Bromothymol blue solution R3.** *1012903.*

Warm 0.1 g of *bromothymol blue R* with 3.2 mL of *0.05 M sodium hydroxide* and 5 mL of *ethanol (90 per cent V/V) R*. After solution is effected, dilute to 250 mL with *ethanol (90 per cent V/V) R*.

**Bromothymol blue solution R4.** *1012904.*

Dissolve 100 mg of *bromothymol blue R* in a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and dilute to 100 mL with the same mixture of solvents. Filter if necessary.

**BRP indicator solution.** *1013000.*

Dissolve 0.1 g of *bromothymol blue R*, 20 mg of *methyl red R* and 0.2 g of *phenolphthalein R* in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent. Filter.

**Brucine.** $C_{23}H_{26}N_2O_4$. ($M_r$ 394.5). *1013100.* [357-57-3]. 2,3-Dimethoxystrychnidin-10-one. 2,3-Dimethoxystrychnine.

Colourless crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 178 °C.

**Butanal.** $C_4H_8O$. ($M_r$ 72.1). *1134400.* [123-72-8]. Butyraldehyde.

$d_{20}^{20}$: 0.806.

$n_D^{20}$: 1.380.

bp: 75 °C.

***i*-Butane.** $C_4H_{10}$. ($M_r$ 58.12). *1189000.* [75-28-5]. Isobutane. 2-Methylpropane.

*Content*: minimum 99.0 per cent *V/V*.

***n*-Butane.** $C_4H_{10}$. ($M_r$ 58.12). *1189100.* [106-97-8]. Butane.

*Content*: minimum 99.0 per cent *V/V*.

**Butane-1,4-diol.** $HO(CH_2)_4OH$. ($M_r$ 90.12). *1174800.* [110-63-4].

**Butanol.** $C_4H_{10}O$. ($M_r$ 74.1). *1013200.* [71-36-3]. Butan-1-ol.

Clear, colourless liquid, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.81.

bp: 116 °C to 119 °C.

**2-Butanol R1.** $C_4H_{10}O$. ($M_r$ 74.1). *1013301.* [78-92-2]. Butan-2-ol. *sec*-Butyl alcohol.

*Content*: minimum 99.0 per cent.

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.81.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 99 °C and 100 °C.

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Isopropyl alcohol (0970)*.

**Butyl acetate.** $C_6H_{12}O_2$. ($M_r$ 116.2). *1013400.* [123-86-4].

Clear, colourless liquid, flammable, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.88.

$n_D^{20}$: about 1.395.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 123 °C and 126 °C.

**Butyl acetate R1.** *1013401.*

*Content*: minimum 99.5 per cent, determined by gas chromatography.

Clear, colourless liquid, flammable, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.883.

$n_D^{20}$: about 1.395.

*Butanol*: maximum 0.2 per cent, determined by gas chromatography.

*n-Butyl formate*: maximum 0.1 per cent, determined by gas chromatography.

*n-Butyl propionate*: maximum 0.1 per cent, determined by gas chromatography.

*Water*: maximum 0.1 per cent.

**Butylamine.** $C_4H_{11}N$. ($M_r$ 73.1). *1013600.* [109-73-9]. Butan-1-amine.

Distil and use within one month.

Colourless liquid, miscible with water, with ethanol (96 per cent).

$n_D^{20}$: about 1.401.

bp: about 78 °C.

***tert*-Butylamine.** *1100900.* [75-64-9].

See *1,1-dimethylethylamine R*.

**4-(Butylamino)benzoic acid.** $C_{11}H_{15}NO_2$. ($M_r$ 193.2). *1206700.* [4740-24-3].

White or almost white powder.

*Content*: 96.5 per cent to 103.5 per cent.

**Butylated hydroxytoluene.** *1013800.* [128-37-0].

See *Butylhydroxytoluene R*.

**Butylboronic acid.** $C_4H_{11}BO_2$. ($M_r$ 101.9). *1013700.* [4426-47-5].

*Content*: minimum 98 per cent.

mp: 90 °C to 92 °C.

***tert*-Butylhydroperoxide.** $C_4H_{10}O_2$. ($M_r$ 90.1). *1118000.* [75-91-2]. 1,1-Dimethylethylhydroperoxide.

Flammable liquid, soluble in organic solvents.

$d_{20}^{20}$: 0.898.

$n_D^{20}$: 1.401.

bp: 35 °C.

**Butyl 4-hydroxybenzoate.** *1103900.* [94-26-8].

See *Butyl parahydroxybenzoate R*.

**Butylhydroxytoluene.** *1013800.* [128-37-0].

See *Butylhydroxytoluene (0581)*.

**Butyl methacrylate.** $C_8H_{14}O_2$. ($M_r$ 142.2). *1145400.* [97-88-1]. Butyl 2-methylpropenoate.

Clear, colourless solution.

$d_4^{20}$: about 0.894.

$n_D^{20}$: about 1.424.

bp: about 163 °C.

***tert*-Butyl methyl ether.** *1013900.* [1634-04-4].

See *1,1-dimethylethyl methyl ether R*.

**2-Butyloctanol.** $C_{12}H_{26}O$. ($M_r$ 186.3). *1206100.* [3913-02-8]. (2Ξ)-2-Butyloctan-1-ol.

**Butyl parahydroxybenzoate.** *1103900.* [94-26-8].

See *Butyl parahydroxybenzoate (0881)*.

**Butyric acid.** $C_4H_8O_2$. ($M_r$ 88.1). *1014000.* [107-92-6]. Butanoic acid.

*Content*: minimum 99.0 per cent.

Oily liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.96.

$n_D^{20}$: about 1.398.

bp: about 163 °C.

**Butyrolactone.** $C_4H_6O_2$. ($M_r$ 86.1). *1104000.* [96-48-0]. Dihydro-2(3*H*)-furanone. γ-Butyrolactone.

Oily liquid, miscible with water, soluble in methanol.

$n_D^{25}$: about 1.435.

bp: about 204 °C.

**Cadmium.** Cd. ($A_r$ 112.4). *1014100.* [7440-43-9].

Silvery-white, lustrous metal, practically insoluble in water, freely soluble in nitric acid and in hot hydrochloric acid.

**Cadmium nitrate tetrahydrate.** $Cd(NO_3)_2,4H_2O$. ($M_r$ 308.5). *1174900.* [10022-68-1].

Hygroscopic orthorhombic crystals, very soluble in water, soluble in acetone and in ethanol (96 per cent).

mp: about 59.5 °C.

**Caesium chloride.** CsCl. ($M_r$ 168.4). *1014200.* [7647-17-8].

White or almost white powder, very soluble in water, freely soluble in methanol, practically insoluble in acetone.

**Caffeic acid.** $C_9H_8O_4$. ($M_r$ 180.2). *1014300.* [331-39-5]. (*E*)-3-(3,4-Dihydroxyphenyl)propenoic acid.

White or almost white crystals or plates, freely soluble in hot water and in ethanol (96 per cent), sparingly soluble in cold water.

*Absorbance* (*2.2.25*). A freshly prepared solution at pH 7.6 shows 2 absorption maxima at about 288 nm and about 313 nm.

**Caffeine.** *1014400.* [58-08-2].

See *Caffeine (0267)*.

**Calcium acetate.** $C_4H_6CaO_4$. ($M_r$ 158.2). *1191600.* [62-54-4]. Calcium diacetate. See *Calcium acetate (2128)*.

**Calcium carbonate.** *1014500.* [471-34-1].

See *Calcium carbonate (0014)*.

**Calcium carbonate R1.** *1014501.*

Complies with the requirements prescribed for *calcium carbonate R* with the following additional requirement.

*Chlorides* (*2.4.4*): maximum 50 ppm.

**Calcium chloride.** *1014600.* [10035-04-8].

See *Calcium chloride (0015)*.

**Calcium chloride solution.** *1014601.*

A 73.5 g/L solution of *calcium chloride R*.

**Calcium chloride solution, 0.01 M.** *1014602.*

Dissolve 0.147 g of *calcium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

**Calcium chloride solution, 0.02 M.** *1014603.*

Dissolve 2.94 g of *calcium chloride R* in 900 mL of *water R*, adjust to pH 6.0 to 6.2 and dilute to 1000.0 mL with *water R*.

*Storage*: at 2 °C to 8 °C.

**Calcium chloride solution, 0.025 M.** *1014604.*

Dissolve 0.368 g of *calcium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

**Calcium chloride R1.** $CaCl_2,4H_2O$. ($M_r$ 183.1). *1014700.* Calcium chloride tetrahydrate.

*Iron*: maximum 0.05 ppm.

**Calcium chloride, anhydrous.** $CaCl_2$. ($M_r$ 111.0). *1014800.* [10043-52-4].

*Content*: minimum 98.0 per cent (dried substance).

White or almost white granules, deliquescent, very soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

*Loss on drying* (*2.2.32*): maximum 5.0 per cent, determined by drying in an oven at 200 °C.

*Storage*: in an airtight container, protected from moisture.

**Calcium hydroxide.** $Ca(OH)_2$. ($M_r$ 74.1). *1015000.* [1305-62-0]. Calcium dihydroxide.

White or almost white powder, almost completely soluble in 600 parts of water.

**Calcium hydroxide solution.** *1015001.*

A freshly prepared saturated solution.

**Calcium lactate pentahydrate.** *1015100.* [41372-22-9].

See *Calcium lactate pentahydrate (0468)*.

**Calcium phosphate monobasic monohydrate.** $CaH_4O_8P_2,H_2O$. ($M_r$ 252.1). *1157200.* [10031-30-8]. Calcium tetrahydrogen bisphosphate monohydrate. Phosphoric acid calcium salt (2:1) monohydrate.

White or almost white, crystalline powder, soluble in water.

**Calcium sulfate.** $CaSO_4,{}^1/_2H_2O$. ($M_r$ 145.1). *1015200.* [10034-76-1]. Calcium sulfate hemihydrate.

White or almost white powder, soluble in about 1500 parts of water, practically insoluble in ethanol (96 per cent). When mixed with half its mass of water it rapidly solidifies to a hard and porous mass.

**Calcium sulfate solution.** *1015201.*

Shake 5 g of *calcium sulfate R* with 100 mL of *water R* for 1 h and filter.

**Calconecarboxylic acid.** $C_{21}H_{14}N_2O_7S$. ($M_r$ 438.4). *1015300.* [3737-95-9]. 2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)naphthalene-3-carboxylic acid.

Brownish-black powder, slightly soluble in water, very slightly soluble in acetone and in ethanol (96 per cent), sparingly soluble in dilute solutions of sodium hydroxide.

**Calconecarboxylic acid triturate.** *1015301.*

Mix 1 part of *calconecarboxylic acid R* with 99 parts of *sodium chloride R*.

*Test for sensitivity.* Dissolve 50 mg of calconecarboxylic acid triturate in a mixture of 2 mL of *strong sodium hydroxide solution R* and 100 mL of *water R*. The solution is blue but becomes violet on addition of 1 mL of a 10 g/L solution of *magnesium sulfate R* and 0.1 mL of a 1.5 g/L solution of *calcium chloride R* and turns pure blue on addition of 0.15 mL of *0.01 M sodium edetate*.

**Camphene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1139200.* [79-92-5]. 2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane.

*Camphene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Rosemary Oil (1846)*.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

**Camphor.** *1113000.* [76-22-2].

See *Camphor, racemic (0655)*.

*Camphor used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

*Test solution.* A 10 g/L solution of the substance to be examined in *hexane R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**(1*S*)-(+)-10-Camphorsulfonic acid.** $C_{10}H_{16}O_4S$. ($M_r$ 232.3). *1104100.* [3144-16-9]. (1*S*,4*R*)-(+)-2-Oxo-10-bornenesulfonic acid. [(1*S*)-7,7-Dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl]methanesulfonic acid. Reychler's acid.

Prismatic crystals, hygroscopic, soluble in water.

*Content*: minimum 99.0 per cent of (1*S*)-(+)-10-camphorsulfonic acid.

$[\alpha]_D^{20}$: + 20 ± 1, determined on a 43 g/L solution.

mp: about 194 °C, with decomposition.

$\Delta A$ (*2.2.41*): $10.2 \times 10^3$ determined at 290.5 nm on a 1.0 g/L solution.

**Capric acid.** $C_{10}H_{20}O_2$. ($M_r$ 172.3). *1142000.* [334-48-5]. Decanoic acid.

Crystalline solid, very slightly soluble in water, soluble in anhydrous ethanol.

bp: about 270 °C.

mp: about 31.4 °C.

*Capric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Capric alcohol.** *1024700.*

See *Decanol R*.

**Caproic acid.** $C_6H_{12}O_2$. ($M_r$ 116.2). *1142100.* [142-62-1]. Hexanoic acid.

Oily liquid, sparingly soluble in water.

$d_4^{20}$: about 0.926.

$n_D^{20}$: about 1.417.

bp: about 205 °C.

*Caproic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**ε-Caprolactam.** $C_6H_{11}NO$. ($M_r$ 113.2). *1104200.* [105-60-2]. Hexane-6-lactam.

Hygroscopic flakes, freely soluble in water, in anhydrous ethanol and in methanol.

mp: about 70 °C.

**Caprylic acid.** $C_8H_{16}O_2$. ($M_r$ 144.2). *1142200.* [124-07-2]. Octanoic acid.

Slightly yellow, oily liquid.

$d_4^{20}$: about 0.910.

$n_D^{20}$: about 1.428.

bp: about 239.7 °C.

mp: about 16.7 °C.

*Caprylic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Capsaicin.** $C_{18}H_{27}NO_3$. ($M_r$ 305.4). *1147900.* [404-86-4]. (*E*)-*N*-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide.

White or almost white, crystalline powder, practically insoluble in water, freely soluble in anhydrous ethanol.

mp: about 65 °C.

*Capsaicin used in the assay in Capsicum (1859) complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Capsicum (1859)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Carbazole.** $C_{12}H_9N$. ($M_r$ 167.2). *1015400.* [86-74-8]. Dibenzopyrrole.

Crystals, practically insoluble in water, freely soluble in acetone, slightly soluble in anhydrous ethanol.

mp: about 245 °C.

**Carbomer.** *1015500.* [9007-20-9].

A cross-linked polymer of acrylic acid; it contains a large proportion (56 per cent to 68 per cent) of carboxylic acid ($CO_2H$) groups after drying at 80 °C for 1 h. Average relative molecular mass about $3 \times 10^6$.

*pH* (*2.2.3*): about 3 for a 10 g/L suspension.

**Carbon dioxide.** *1015600.* [124-38-9].

See *Carbon dioxide (0375)*.

**Carbon dioxide R1.** $CO_2$. ($M_r$ 44.01). *1015700.* [124-38-9].

*Content*: minimum 99.995 per cent *V/V*.

*Carbon monoxide*: less than 5 ppm.

*Oxygen:* less than 25 ppm.

*Nitric oxide:* less than 1 ppm.

**Carbon dioxide R2.** $CO_2$. ($M_r$ 44.01). *1134500.* [124-38-9].

*Content*: minimum 99 per cent *V/V*.

**Carbon disulfide.** $CS_2$. ($M_r$ 76.1). *1015800.* [75-15-0].

Colourless or yellowish, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

$d_{20}^{20}$: about 1.26.

bp: 46 °C to 47 °C.

**Carbon for chromatography, graphitised.** *1015900.*

Carbon chains having a length greater than $C_9$.

*Particle size*: 400 µm to 850 µm.

*Relative density*: 0.72.

*Surface area*: 10 $m^2$/g.

Do not use at a temperature higher than 400 °C.

**Carbon for chromatography, graphitised R1.** *1153500.*

Porous spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms.

*Particle size*: 5 µm to 7 µm.

*Pore volume*: 0.7 $cm^3$/g.

**Carbon monoxide.** CO. ($M_r$ 28.01). *1016000.* [630-08-0].

*Content*: minimum 99.97 per cent *V/V*.

**Carbon monoxide R1.** CO. ($M_r$ 28.01). *1134600.* [630-08-0].

*Content*: minimum 99 per cent *V/V*.

**Carbon tetrachloride.** $CCl_4$. ($M_r$ 153.8). *1016100.* [56-23-5]. Tetrachloromethane.

Clear, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 1.595 to 1.598.

bp: 76 °C to 77 °C.

**Carbophenothion.** $C_{11}H_{16}ClO_2PS_3$. ($M_r$ 342.9). *1016200.* [786-19-6]. *O,O*-Diethyl *S*-[[(4-chlorophenyl)thio]methyl]-phosphorodithioate.

Yellowish liquid, practically insoluble in water, miscible with organic solvents.

$d_4^{25}$: about 1.27.

For the monograph *Wool Fat (0134)*, a suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Car-3-ene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1124000.* [498-15-7]. 3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene. 4,7,7-Trimethyl-3-norcarene.

Liquid with a pungent odour, slightly soluble in water, soluble in organic solvents.

$d_{20}^{20}$: about 0.864.

$n_D^{20}$: 1.473 to 1.474.

$[\alpha]_D^{20}$: + 15 to + 17.

bp: 170 °C to 172 °C.

*Car-3-ene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Nutmeg oil (1552)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Carminic acid.** $C_{22}H_{20}O_{13}$. ($M_r$ 492.4). *1156700.* [1260-17-9]. 7-α-D-Glucopyranosyl-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid.

Dark red powder, very slightly soluble in water, soluble in dimethyl sulfoxide, very slightly soluble in ethanol (96 per cent).

**Carob bean gum.** *1104500.*

The ground endosperm of the fruit kernels of *Ceratonia siliqua* L. Taub.

White or almost white powder containing 70 per cent to 80 per cent of a water-soluble gum consisting mainly of galactomannoglycone.

**Carvacrol.** $C_{10}H_{14}O$. ($M_r$ 150.2). *1016400.* [499-75-2]. 5-Isopropyl-2-methylphenol.

Brownish liquid, practically insoluble in water, very soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.975.

$n_D^{20}$: about 1.523.

bp: about 237 °C.

*Carvacrol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. Dissolve 0.1 g in about 10 mL of *acetone R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Carveol.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1160400.* [99-48-9]. *p*-Mentha-1(6),8-dien-2-ol. 2-Methyl-5-(1-methylethenyl)cyclohex-2-enol.

The substance contains a variable content of *trans*- and *cis*-carveol.

*Carveol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the test for chromatographic profile in the monograph *Caraway oil (1817)*.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Carvone.** $C_{10}H_{14}O$. ($M_r$ 150.2). *1016500.* [2244-16-8]. (+)-*p*-Mentha-6,8-dien-2-one. (5*S*)-2-Methyl-5-(1-methylethenyl)-cyclohex-2-enone.

Liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.965

$n_D^{20}$: about 1.500.

$[\alpha]_D^{20}$: about + 61.

bp: about 230 °C.

*Carvone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)* using the substance to be examined as the test solution.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Carvone R1.** *1016501.*

Complies with the requirements prescribed for *carvone R* with the following additional requirement.

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the test for chiral purity in the monograph *Caraway oil (1817)*.

*Content*: minimum 98 per cent.

**(−)-Carvone.** $C_{10}H_{14}O$. ($M_r$ 150.2). *1160500.* [6485-40-1]. (−)-*p*-Mentha-1(6),8-dien-2-one. (5*R*)-2-Methyl-5-(1-methylethenyl)cyclohex-2-enone.

Liquid.

$d_{20}^{20}$: about 0.965.

$n_D^{20}$: about 1.4988.

$[\alpha]_D^{20}$: about − 62.

bp: about 230 °C.

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the test for chiral purity in the monograph *Caraway oil (1817)*.

*Content*: minimum 99 per cent.

**β-Caryophyllene.** $C_{15}H_{24}$. ($M_r$ 204.4). *1101000.* [87-44-5]. (*E*)-(1*R*,9*S*)-4,11,11-Trimethyl-8-methylene-bicyclo[7.2.0]undec-4-ene.

Oily liquid, practically insoluble in water, miscible with ethanol (96 per cent).

*β-Caryophyllene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

*Test solution*. The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**Caryophyllene oxide.** $C_{15}H_{24}O$. ($M_r$ 220.4). *1149000.* [1139-30-6]. (-)-β-Caryophyllene epoxide. (1*R*,4*R*,6*R*,10*S*)-4,12,12-Trimethyl-9-methylene-5-oxatricyclo[8.2.0.0$^{4,6}$]dodecane.

Colourless, fine crystals with lumps.

mp: 62 °C to 63 °C.

*Caryophyllene oxide used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Turpentine oil, Pinus pinaster type (1627)*.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Casein.** *1016600.* [9000-71-9].

Mixture of related phosphoproteins obtained from milk.

White or almost white, amorphous powder or granules, very slightly soluble in water and in non-polar organic solvents. It dissolves in concentrated hydrochloric acid giving a pale-violet solution. It forms salts with acids and bases. Its isoelectric point is at about pH 4.7. Alkaline solutions are laevorotatory.

**Casticin.** $C_{19}H_{18}O_8$. ($M_r$ 374.3). *1162200.* [479-91-4]. 5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxy-4*H*-1-benzopyran-4-one.

Yellow crystals.

**Catalpol.** $C_{15}H_{22}O_{10}$. ($M_r$ 362.3). *1142300.* [2415-24-9]. (1a*S*,1b*S*,2*S*,5a*R*,6*S*,6a*S*)-6-Hydroxy-1a-(hydroxymethyl)-1a,1b,2,5a,6,6a-hexahydrooxireno[4,5]cyclopenta[1,2-*c*]pyran-2-yl β-D-glucopyranoside.

mp: 203 °C to 205 °C.

**Catechin.** $C_{15}H_{14}O_6,xH_2O$. ($M_r$ 290.3 for the anhydrous substance). *1119000.* [154-23-4]. (+)-(2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol. Catechol. Cianidanol. Cyanidol.

**Cathine hydrochloride.** $C_9H_{14}ClNO$. ($M_r$ 187.7). *1206800.* [2153-98-2]. (1*S*,2*S*)-2-Amino-1-phenylpropan-1-ol hydrochloride. Norpseudoephedrine hydrochloride.

White or almost white solid.

*Content*: minimum 95.0 per cent.

**Cation-exchange resin.** *1016700.*

A resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as spherical beads.

**Cation-exchange resin R1.** *1121900.*

A resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 4 per cent of divinylbenzene. It is available as spherical beads.

**Cation-exchange resin R2.** *1195400.*

Resin containing strongly acidic propylenesulfonic acid groups.

**Cation-exchange resin (calcium form), strong.** *1104600.*

Resin in calcium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene.

**Cation-exchange resin (sodium form), strong.** *1176100.*

Resin in sodium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

**Cation-exchange resin, strong.** *1156800.*

Strong cation-exchange resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

**Cation-exchange resin, weak.** *1203200.*

Weak cation-exchange resin in protonated form with carboxylate functional groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

**Cellulose for chromatography.** *1016800.* [9004-34-6].

Fine, white or almost white, homogeneous powder with an average particle size less than 30 µm.

*Preparation of a thin layer.* Suspend 15 g in 100 mL of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

**Cellulose for chromatography R1.** *1016900.*

Microcrystalline cellulose.

*Preparation of a thin layer.* Suspend 25 g in 90 mL of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

**Cellulose for chromatography $F_{254}$.** *1017000.*

Microcrystalline cellulose $F_{254}$. A fine, white or almost white, homogeneous powder with an average particle size less than 30 µm, containing a fluorescent indicator having an optimal intensity at 254 nm.

*Preparation of a thin layer.* Suspend 25 g in 100 mL of *water R* and homogenise using an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

**Cerium sulfate.** $Ce(SO_4)_2,4H_2O$. ($M_r$ 404.3). *1017300.* [10294-42-5]. Cerium(IV) sulfate tetrahydrate. Ceric sulfate.

Yellow or orange-yellow, crystalline powder or crystals, very slightly soluble in water, slowly soluble in dilute acids.

**Cerous nitrate.** $Ce(NO_3)_3,6H_2O$. ($M_r$ 434.3). *1017400.* [10294-41-4]. Cerium trinitrate hexahydrate.

Colourless or pale yellow, crystalline powder, freely soluble in water and in ethanol (96 per cent).

**Cetostearyl alcohol.** *1017500.* [67762-27-0].

See *Cetostearyl alcohol (0702)*.

**Cetrimide.** *1017600.* [8044-71-1].

See *Cetrimide (0378)*.

**Cetyl alcohol.** $C_{16}H_{34}O$. ($M_r$ 242.4). *1160600.* [36653-82-4]. Hexadecan-1-ol.

*Content*: minimum 95.0 per cent.

mp: about 48 °C.

**Cetylpyridinium chloride monohydrate.** $C_{21}H_{38}ClN,H_2O$. ($M_r$ 358.0). *1162800.* [6004-24-6]. 1-Hexadecylpyridinium chloride monohydrate.

White or almost white powder, freely soluble in water and in ethanol (96 per cent).

mp: 80 °C to 83 °C.

**Cetyltrimethylammonium bromide.** $C_{19}H_{42}BrN$. ($M_r$ 364.5). *1017700.* [57-09-0]. Cetrimonium bromide. *N*-Hexadecyl-*N,N,N*-trimethylammonium bromide.

White or almost white, crystalline powder, soluble in water, freely soluble in ethanol (96 per cent).

mp: about 240 °C.

**Chamazulene.** $C_{14}H_{16}$. ($M_r$ 184.3). *1148000.* [529-05-5]. 7-Ethyl-1,4-dimethylazulene.

Blue liquid, very slightly soluble in water, soluble in ethanol (96 per cent), miscible with fatty oils, with essential oils and with liquid paraffin, soluble with discolouration in phosphoric acid (85 per cent *m/m*) and sulfuric acid (50 per cent *V/V*).

*Appearance of solution.* 50 mg is soluble in 2.5 mL of *hexane R*. The blue solution is clear in a thin-layer obtained by tilting the test-tube.

*Chamazulene used for gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Matricaria oil (1836)*.

*Test solution*: a 4 g/L solution in *cyclohexane R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Charcoal, activated.** *1017800.* [64365-11-3].

See *Activated charcoal (0313)*.

**Chloral hydrate.** *1017900.* [302-17-0].

See *Choral hydrate (0265)*.

**Chloral hydrate solution.** *1017901.*

A solution of 80 g in 20 mL of *water R*.

**Chloramine.** *1018000.* [7080-50-4].

See *Tosylchloramide sodium (0381)*.

**Chloramine solution.** *1018001.*

A 20 g/L solution of *chloramine R*. Prepare immediately before use.

**Chloramine solution R1.** *1018002.*

A 0.1 g/L solution of *chloramine R*. Prepare immediately before use.

**Chloramine solution R2.** *1018003.*

A 0.2 g/L solution of *chloramine R*. Prepare immediately before use.

**Chlordane.** $C_{10}H_6Cl_8$. ($M_r$ 409.8). *1124100.* [12789-03-6].

bp: about 175 °C.

mp: about 106 °C.

A suitable certified reference solution of technical grade (10 ng/µL in iso-octane) may be used.

**Chlordiazepoxide.** *1113200.* [58-25-3].

See *Chlordiazepoxide (0656)*.

**Chlorfenvinphos.** $C_{12}H_{14}Cl_3O_4P$. ($M_r$ 359.6). *1124200.* [470-90-6].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Chloroacetanilide.** $C_8H_8ClNO$. ($M_r$ 169.6). *1018100.* [539-03-7]. 4′-Chloroacetanilide.

*Content*: minimum 95 per cent.

Crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 178 °C.

**Chloroacetic acid.** $C_2H_3ClO_2$. ($M_r$ 94.5). *1018200.* [79-11-8].

Colourless or white or almost white crystals, deliquescent, very soluble in water, soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Chloroaniline.** $C_6H_6ClN$. ($M_r$ 127.6). *1018300.* [106-47-8]. 4-Chloroaniline.

Crystals, soluble in hot water, freely soluble in ethanol (96 per cent).

mp: about 71 °C.

**4-Chlorobenzenesulfonamide.** $C_6H_6ClNO_2S$. ($M_r$ 191.6). *1097400.* [98-64-6].

White or almost white powder.

mp: about 145 °C.

**2-Chlorobenzoic acid.** $C_7H_5ClO_2$. ($M_r$ 156.6). *1139300.* [118-91-2].

Soluble in water, slightly soluble in anhydrous ethanol.

bp: about 285 °C.

mp: about 140 °C.

**Chlorobutanol.** *1018400.* [57-15-8].

See *Chlorobutanol (0382)*.

**2-Chloro-2-deoxy-D-glucose.** $C_6H_{11}ClO_5$. ($M_r$ 198.6). *1134700.* [14685-79-1].

White or almost white crystalline, very hygroscopic powder, soluble in water and in dimethyl sulfoxide, practically insoluble in ethanol (96 per cent).

**2-Chloroethanol.** $C_2H_5ClO$. ($M_r$ 80.5). *1097500.* [107-07-3].

Colourless liquid, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 1.197.

$n_D^{20}$: about 1.442.

bp: about 130 °C.

mp: about – 89 °C.

**2-Chloroethanol solution.** *1097501.*

Dissolve 125 mg of *2-chloroethanol R* in *2-propanol R* and dilute to 50 mL with the same solvent. Dilute 5 mL of the solution to 50 mL with *2-propanol R*.

**Chloroethylamine hydrochloride.** $C_2H_7Cl_2N$. ($M_r$ 116.0). *1124300.* [870-24-6]. 2-Chloroethanamine hydrochloride.

mp: about 145 °C.

**(2-Chloroethyl)diethylamine hydrochloride.** $C_6H_{15}Cl_2N$. ($M_r$ 172.1). *1018500.* [869-24-9].

White or almost white, crystalline powder, very soluble in water and in methanol, freely soluble in methylene chloride, practically insoluble in hexane.

mp: about 211 °C.

**Chloroform.** $CHCl_3$. ($M_r$ 119.4). *1018600.* [67-66-3]. Trichloromethane.

Clear, colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 1.475 to 1.481.

bp: about 60 °C.

*Ethanol*: 0.4 per cent *m/m* to 1.0 per cent *m/m*.

**Chloroform, acidified.** *1018601.*

To 100 mL of *chloroform R* add 10 mL of *hydrochloric acid R*. Shake, allow to stand and separate the 2 layers.

**Chloroform, ethanol-free.** *1018602.*

Shake 200 mL of *chloroform R* with four quantities, each of 100 mL, of *water R*. Dry over 20 g of *anhydrous sodium sulfate R* for 24 h. Distil the filtrate over 10 g of *anhydrous sodium sulfate R*. Discard the first 20 mL of distillate. Prepare immediately before use.

**Chlorogenic acid.** $C_{16}H_{18}O_9$. ($M_r$ 354.3). *1104700.* [327-97-9]. (1*S*,3*R*,4*R*,5*R*)-3-[(3,4-Dihydroxycinnamoyl)oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid.

White or almost white, crystalline powder or needles, freely soluble in boiling water, in acetone and in ethanol (96 per cent).

$[\alpha]_D^{26}$: about – 35.2.

mp: about 208 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed on Identification A in the monograph *Belladonna leaf dry extract, standardised (1294)*; the chromatogram shows only one principal zone.

*Chlorogenic acid used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Artichoke Leaf (1866)*.

*Content*: minimum 97.0 per cent.

**3-Chloro-2-methylaniline.** $C_7H_8ClN$. ($M_r$ 141.6). *1139400.* [87-60-5]. 6-Chloro-2-toluidine.

Not miscible with water, slightly soluble in anhydrous ethanol.

$d_{20}^{20}$: about 1.171.

$n_D^{20}$: about 1.587.

bp: about 115 °C.

mp: about 2 °C.

**2-Chloro-*N*-(2,6-dimethylphenyl)acetamide.** $C_{10}H_{12}ClNO$. ($M_r$ 197.7). *1168700.* [1131-01-7].

**2-Chloronicotinic acid.** $C_6H_4ClNO_2$. ($M_r$ 157.6). *1157300.* [2942-59-8]. 2-Chloropyridine-3-carboxylic acid.

White or almost white powder.

mp: about 177 °C.

*Content*: minimum 95 per cent.

**2-Chloro-4-nitroaniline.** $C_6H_5ClN_2O_2$. ($M_r$ 172.6). *1018800.* [121-87-9].

Yellow, crystalline powder, freely soluble in methanol.

mp: about 107 °C.

*Storage*: protected from light.

**2-Chloro-5-nitrobenzoic acid.** $C_7H_4ClNO_4$. ($M_r$ 201.6). *1183800.* [2516-96-3].

mp: 165 °C to 168 °C.

**Chlorophenol.** $C_6H_5ClO$. ($M_r$ 128.6). *1018900.* [106-48-9]. 4-Chlorophenol.

Colourless or almost colourless crystals, slightly soluble in water, very soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

mp: about 42 °C.

**2-[2-(4-Chlorophenyl)acetyl]benzoic acid.** $C_{15}H_{11}ClO_3$. ($M_r$ 274.7). *1194500.* [53242-76-5].

**Chloroplatinic acid.** $H_2Cl_6Pt,6H_2O$. ($M_r$ 517.9). *1019000.* [18497-13-7]. Hydrogen hexachloroplatinate(IV) hexahydrate.

*Content*: minimum 37.0 per cent *m/m* of platinum ($A_r$ 195.1).

Brownish-red crystals or a crystalline mass, very soluble in water, soluble in ethanol (96 per cent).

*Assay*. Ignite 0.200 g to constant mass at 900 ± 50 °C and weigh the residue (platinum).

*Storage*: protected from light.

**3-Chloropropane-1,2-diol.** $C_3H_7ClO_2$. ($M_r$ 110.5). *1097600.* [96-24-2].

Colourless liquid, soluble in water and ethanol (96 per cent).

$d_{20}^{20}$: about 1.322.

$n_D^{20}$: about 1.480.

bp: about 213 °C.

**5-Chloroquinolin-8-ol.** $C_9H_6ClNO$. ($M_r$ 179.6). *1156900.* [130-16-5]. 5-Chlorooxine.

Sparingly soluble in cold dilute hydrochloric acid.

mp: about 123 °C.

*Content*: minimum 95.0 per cent.

**4-Chlororesorcinol.** $C_6H_5ClO_2$. ($M_r$ 144.6). *1177700.* [95-88-5]. 4-Chlorobenzene-1,3-diol. 1,3-Dihydroxy-4-chlorobenzene.

mp: 106 °C to 108 °C.

**5-Chlorosalicylic acid.** $C_7H_5ClO_3$. ($M_r$ 172.6). *1019100.* [321-14-2].

White or almost white, crystalline powder, soluble in methanol.

mp: about 173 °C.

**Chlorothiazide.** $C_7H_6ClN_3O_4S_2$. ($M_r$ 295.7). *1112100.* [58-94-6]. 6-Chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

*Content*: minimum 98.0 per cent.

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

**Chlorotrimethylsilane.** $C_3H_9ClSi$. ($M_r$ 108.6). *1019300.* [75-77-4].

Clear, colourless liquid, fuming in air.

$d_{20}^{20}$: about 0.86.

$n_D^{20}$: about 1.388.

bp: about 57 °C.

**Chlorpyriphos.** $C_9H_{11}Cl_3NO_3PS$. ($M_r$ 350.6). *1124400.* [2921-88-2].

bp: about 200 °C.

mp: 42 °C to 44 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Chlorpyriphos-methyl.** $C_7H_7Cl_3NO_3PS$. ($M_r$ 322.5). *1124500.* [5598-13-0].

mp: 45 °C to 47 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Chlortetracycline hydrochloride.** *1145500.*

See *Chlortetracycline hydrochloride (0173)*.

**(5α)-Cholestane.** $C_{27}H_{48}$. ($M_r$ 372.7). *1167900.* [481-21-0].

Slightly soluble in anhydrous ethanol.

mp: about 81 °C.

**Cholesterol.** *1019400.* [57-88-5].

See *Cholesterol (0993)*.

**Choline chloride.** $C_5H_{14}ClNO$. ($M_r$ 139.6). *1019500.* [67-48-1]. (2-Hydroxyethyl)trimethylammonium chloride.

Deliquescent crystals, very soluble in water and in ethanol (96 per cent).

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Suxamethonium chloride (0248)*: apply 5 µL of a 0.2 g/L solution in *methanol R*; the chromatogram shows one principal spot.

*Storage*: in an airtight container.

**Chondroitinase ABC.** *1162900.*

Pectin lyase-like enzyme secreted by *Flavobacterium heparinum*. Available in vials containing 5-10 units. It cleaves both glucuronate-containing disaccharides, e.g. chondroitin sulfate, and iduronate-containing disaccharides, e.g. dermatan sulfate.

**Chondroitinase AC.** *1163000.*

Pectin lyase-like enzyme secreted by *Flavobacterium heparinum*. Available in vials containing 5-10 units. It cleaves only glucuronate-containing disaccharides, e.g. chondroitin sulfate.

**Chromazurol S.** $C_{23}H_{13}Cl_2Na_3O_9S$. ($M_r$ 605). *1019600.* [1667-99-8].

Schultz No. 841.

Colour Index No. 43825.

Trisodium 5-[(3-carboxylato-5-methyl-4-oxocyclohexa-2,5-dien-1-ylidene)(2,6-dichloro-3-sulfonatophenyl)methyl]-2-hydroxy-3-methylbenzoate.

Brownish-black powder, soluble in water, slightly soluble in ethanol (96 per cent).

**Chromic potassium sulfate.** $CrK(SO_4)_2,12H_2O$. ($M_r$ 499.4). *1019800.* [7788-99-0]. Chrome alum.

Large, violet-red or black crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

**Chromium(III) acetylacetonate.** $C_{15}H_{21}CrO_6$. ($M_r$ 349.3). *1172900.* [21679-31-2]. (*OC*-6-11)-Tris(2,4-pentanedionato-κ*O*,κ*O*′)chromium.

**Chromium(III) trichloride hexahydrate.** $[Cr(H_2O)_4Cl_2]Cl,2H_2O$. ($M_r$ 266.5). *1104800.* [10060-12-5].

Dark green crystalline powder, hygroscopic.

*Storage*: protected from humidity and oxidising agents.

**Chromium trioxide.** $CrO_3$. ($M_r$ 100.0). *1019900.* [1333-82-0].

Dark brownish-red needles or granules, deliquescent, very soluble in water.

*Storage*: in an airtight glass container.

**Chromogenic substrate R1.** *1020000.*

Dissolve *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to 0.0005 M before use.

**Chromogenic substrate R2.** *1020100.*

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute before use in titrating in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to give a 0.0005 M solution.

**Chromogenic substrate R3.** *1149100.*

Dissolve D-valyl-leucyl-lysyl-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution.

**Chromogenic substrate R4.** *1163100.*

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.008 M solution. Dilute to 0.0025 M with *phosphate buffer solution pH 8.5 R* before use.

**Chromogenic substrate R5.** *1163200.*

Dissolve *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride in *water R* to give a 0.003 M solution.

**Chromotrope II B.** $C_{16}H_9N_3Na_2O_{10}S_2$. ($M_r$ 513.4). *1020200.* [548-80-1].

Schultz No. 67.

Colour Index No. 16575.

Disodium 4,5-dihydroxy-3-(4-nitrophenylazo)naphthalene-2,7-disulfonate.

Reddish-brown powder, soluble in water giving a yellowish-red colour, practically insoluble in ethanol (96 per cent).

**Chromotrope II B solution.** *1020201.*

A 0.05 g/L solution of *chromotrope II B R* in *sulfuric acid R*.

**Chromotropic acid, sodium salt.** $C_{10}H_6Na_2O_8S_2,2H_2O$. ($M_r$ 400.3). *1020300.* [5808-22-0].

Schultz No. 1136.

Disodium 4,5-dihydroxynaphthalene-2,7-disulfonate dihydrate. Disodium 1,8-dihydroxynaphthalene-3,6-disulfonate dihydrate.

A yellowish-white powder, soluble in water, practically insoluble in ethanol (96 per cent).

**Chromotropic acid, sodium salt solution.** *1020301.*

Dissolve 0.60 g of *chromotropic acid, sodium salt R* in about 80 mL of *water R* and dilute to 100 mL with the same solvent. Use this solution within 24 h.

**Chromotropic acid-sulfuric acid solution.** *1020302.*

Dissolve 5 mg of *chromotropic acid, sodium salt R* in 10 mL of a mixture of 9 mL of *sulfuric acid R* and 4 mL of *water R*.

**Chrysanthemin.** $C_{21}H_{21}ClO_{11}$. ($M_r$ 485.8). *1134800.* [7084-24-4]. Cyanidin 3-*O*-glucoside chloride. Kuromanin chloride. 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucopyranosyl)oxy-5,7-dihydroxy-1-benzopyrylium chloride.

Reddish-brown crystalline powder, soluble in water and in ethanol (96 per cent).

*Absorbance* (*2.2.25*). A 0.01 g/L solution in a mixture of 1 volume of *hydrochloric acid R* and 999 volumes of *methanol R* shows an absorption maximum at 528 nm.

**α-Chymotrypsin for peptide mapping.** *1142400.*

α-Chymotrypsin of high purity, treated to eliminate tryptic activity.

**Cimifugin.** $C_{16}H_{18}O_6$. ($M_r$ 306.3). *1181700.* [37921-38-3]. (2*S*)-7-(Hydroxymethyl)-2-(1-hydroxy-1-methylethyl)-4-methoxy-2,3-dihydro-5*H*-furo[3,2-*g*][1]benzopyran-5-one.

**Cinchonidine.** $C_{19}H_{22}N_2O$. ($M_r$ 294.4). *1020400.* [485-71-2]. (*R*)-(Quinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

White or almost white, crystalline powder, very slightly soluble in water and in light petroleum, soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: − 105 to − 110, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

mp: about 208 °C, with decomposition.

*Storage*: protected from light.

**Cinchonine.** $C_{19}H_{22}N_2O$. ($M_r$ 294.4). *1020500.* [118-10-5]. (*S*)-(Quinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in ethanol (96 per cent) and in methanol.

$[\alpha]_D^{20}$: + 225 to + 230, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

mp: about 263 °C.

*Storage*: protected from light.

**Cineole.** $C_{10}H_{18}O$. ($M_r$ 154.3). *1020600.* [470-82-6]. 1,8-Cineole. Eucalyptol. 1,8-Epoxy-*p*-menthane.

Colourless liquid, practically insoluble in water, miscible with anhydrous ethanol.

$d_{20}^{20}$: 0.922 to 0.927.

$n_D^{20}$: 1.456 to 1.459.

*Freezing point* (*2.2.18*): 0 °C to 1 °C.

*Distillation range* (*2.2.11*): 174 °C to 177 °C.

*Phenol.* Shake 1 g with 20 mL of *water R*. Allow to separate and add to 10 mL of the aqueous layer 0.1 mL of *ferric chloride solution R1*. No violet colour develops.

*Turpentine oil.* Dissolve 1 g in 5 mL of *ethanol (90 per cent V/V) R*. Add dropwise freshly prepared *bromine water R*. Not more than 0.5 mL is required to give a yellow colour lasting for 30 min.

*Residue on evaporation*: maximum 0.05 per cent.

To 10.0 mL add 25 mL of *water R*, evaporate on a water-bath and dry the residue to constant mass at 100-105 °C.

*Cineole used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution.* The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**1,4-Cineole.** $C_{10}H_{18}O$. ($M_r$ 154.3). *1142500.* [470-67-7]. 1-Methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane. 1-Isopropyl-4-methyl-7-oxabicyclo[2.2.1]heptane.

Colourless liquid.

$d_4^{20}$: about 0.900.

$n_D^{20}$: about 1.445.

bp: about 173 °C.

**Cinnamamide.** $C_9H_9NO$. ($M_r$ 147.2). *1154800.* [621-79-4]. (*E*)-3-Phenylprop-2-enamide.

White or almost white powder.

mp: about 149 °C.

***trans*-Cinnamic acid.** $C_9H_8O_2$. ($M_r$ 148.2). *1159200.* [140-10-3]. *trans*-3-Phenylacrylic acid. (2*E*)-3-Phenylprop-2-enoic acid.

Colourless crystals, very slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: 133 °C.

**Cinnamic aldehyde.** $C_9H_8O$. ($M_r$ 132.2). *1020700.* [104-55-2]. 3-Phenylpropenal.

Yellowish or greenish-yellow, oily liquid, slightly soluble in water, very soluble in ethanol (96 per cent).

$n_D^{20}$: about 1.620.

*Storage*: protected from light.

***trans*-Cinnamic aldehyde.** $C_9H_8O$. ($M_r$ 132.2). *1124600.* [14371-10-9]. *(E)*-3-Phenylprop-2-enal.

*trans-Cinnamic aldehyde used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Cinnamyl acetate.** $C_{11}H_{12}O_2$. ($M_r$ 176.2). *1124700.* [103-54-8]. 3-Phenylprop-2-en-1-yl acetate.

$n_D^{20}$: about 1.542.

bp: about 262 °C.

*Cinnamyl acetate used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Citral.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1020800.* [5392-40-5]. Mixture of (2*E*)- and (2*Z*)-3,7-Dimethylocta-2,6-dienal.

Light yellow liquid, practically insoluble in water, miscible with ethanol (96 per cent) and with propylene glycol.

*Chromatography.* Thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance: apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

*Citral used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

*Content* of citral (neral + geranial): minimum 95.0 per cent, calculated by the normalisation procedure.

**Citrated rabbit plasma.** *1020900.*

Collect blood by intracardiac puncture from a rabbit kept fasting for 12 h, using a plastic syringe with a No. 1 needle containing a suitable volume of 38 g/L solution of *sodium citrate R* so that the final volume ratio of citrate solution to blood is 1 : 9. Separate the plasma by centrifugation at 1500 *g* to 1800 *g* at 15 °C to 20 °C for 30 min.

*Storage*: at 0 °C to 6 °C; use within 4 h of collection.

**Citric acid, anhydrous.** *1021200.* [77-92-9].

See *Citric acid (0455)*.

**Citric acid monohydrate.** *1021000.* [5949-29-1].

See *Citric acid monohydrate (0456)*.
*When used in the test for iron, it complies with the following additional requirement.*

Dissolve 0.5 g in 10 mL of *water R*, add 0.1 mL of *thioglycollic acid R*, mix and make alkaline with *ammonia R*. Dilute to 20 mL with *water R*. No pink colour appears in the solution.

**Citronellal.** $C_{10}H_{18}O$. ($M_r$ 154.3). *1113300.* [106-23-0]. 3,7-Dimethyl-6-octenal.

Very slightly soluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: 0.848 to 0.856.

$n_D^{20}$: about 1.446.

*Citronellal used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Citronellol.** $C_{10}H_{20}O$. ($M_r$ 156.3). *1134900.* [106-22-9]. 3,7-Dimethyloct-6-en-1-ol.

Clear, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 0.857.

$n_D^{20}$: 1.456.

bp: 220 °C to 222 °C.

*Citronellol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

*Storage*: in an airtight container, protected from light.

**Citronellyl acetate.** $C_{12}H_{22}O_2$. ($M_r$ 198.3). *1135000.* [150-84-5]. 3,7-Dimethyl-6-octen-1-yl acetate.

$d_{20}^{20}$: 0.890.

$n_D^{20}$: 1.443.

bp: 229 °C.

*Citronellyl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

*Storage*: in an airtight container, protected from light.

**Citropten.** $C_{11}H_{10}O_4$. ($M_r$ 206.2). *1021300.* [487-06-9]. Limettin. 5,7-Dimethoxy-2*H*-1-benzopyran-2-one.

Needle-shaped crystals, practically insoluble in water and in light petroleum, freely soluble in acetone and in ethanol (96 per cent).

mp: about 145 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance: apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained shows only one principal spot.

**Clobetasol propionate.** $C_{25}H_{32}ClFO_5$. ($M_r$ 467.0). *1097700.* [25122-46-7]. 21-Chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17-propionate.

White or almost white crystalline powder, insoluble in water, soluble in ethanol (96 per cent) and in acetone.

$[\alpha]_D^{20}$: about + 104 (in dioxan).

mp: about 196 °C.

**Coagulation factor V solution.** *1021400.*

Coagulation factor V solution may be prepared by the following method or by any other method which excludes factor VIII.

Prepare the factor V reagent from fresh oxalated bovine plasma, by fractionation at 4 °C with a saturated solution of *ammonium sulfate R* prepared at 4 °C. Separate the fraction which precipitates between 38 per cent and 50 per cent of saturation, which contains factor V without significant contamination with factor VIII. Remove the ammonium sulfate by dialysis and dilute the solution with a 9 g/L solution of *sodium chloride R* to give a solution containing between 10 per cent and 20 per cent of the quantity of factor V present in fresh human normal plasma.

*Assay of factor V*. Prepare two dilutions of the preparation of factor V in *imidazole buffer solution pH 7.3 R* containing 1 volume of the preparation in 10 volumes and in 20 volumes of the buffer solution respectively. Test each dilution as follows: mix 0.1 mL of *plasma substrate deficient in factor V R*, 0.1 mL of the solution to be examined, 0.1 mL of *thromboplastin R* and 0.1 mL of a 3.5 g/L solution of *calcium chloride R* and measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the first indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time (in duplicate) of four dilutions of human normal plasma in *imidazole buffer solution pH 7.3 R*, containing respectively, 1 volume in 10 (equivalent to 100 per cent of factor V), 1 volume in 50 (20 per cent), 1 volume in 100 (10 per cent), and 1 volume in 1000 (1 per cent). Using two-way logarithmic paper plot the average coagulation times for each dilution of human plasma against the equivalent percentage of factor V and read the percentage of factor V for the two dilutions of the factor V solution by interpolation. The mean of the two results gives the percentage of factor V in the solution to be examined.

*Storage*: in the frozen state at a temperature not higher than − 20 °C.

**Cobalt chloride.** $CoCl_2,6H_2O$. ($M_r$ 237.9). *1021600.* [7791-13-1].

Red, crystalline powder or deep-red crystals, very soluble in water, soluble in ethanol (96 per cent).

**Cobalt nitrate.** $Co(NO_3)_2,6H_2O$. ($M_r$ 291.0). *1021700.* [10026-22-9].

Small garnet-red crystals, very soluble in water.

**Codeine.** *1021800.* [6059-47-8].

See *Codeine monohydrate (0076)*.

**Codeine phosphate.** *1021900.* [52-28-8].

See *Codeine phosphate hemihydrate (0074)*.

**Congo red.** $C_{32}H_{22}N_6Na_2O_6S_2$. ($M_r$ 697). *1022000.* [573-58-0].

Schultz No. 360.

Colour Index No. 22120.
Disodium (biphenyl-4,4′-diyl-bis-2,2′-azo)bis(1-amino-naphthalene-4-sulfonate).

Brownish-red powder, soluble in water.

**Congo red paper.** *1022002.*

Immerse strips of filter paper for a few minutes in *congo red solution R*. Allow to dry.

**Congo red solution.** *1022001.*

Dissolve 0.1 g of *congo red R* in a mixture of 20 mL of *ethanol (96 per cent) R* and *water R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.2 mL of the congo red solution add 100 mL of *carbon dioxide-free water R* and 0.3 mL of *0.1 M hydrochloric acid*. The solution is blue. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour to pink.

Colour change: pH 3.0 (blue) to pH 5.0 (pink).

**Coomassie blue.** *1001400.* [3861-73-2].

See *acid blue 92 R*.

**Coomassie blue solution.** *1001401.*

See *acid blue 92 solution R*.

**Coomassie staining solution.** *1012201.*

A 1.25 g/L solution of *acid blue 83 R* in a mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*. Filter.

**Coomassie staining solution R1.** *1173000.*

Dissolve 0.275 g of *acid blue 83 R* in 200 mL of *methanol R*. Stir until complete dissolution of the crystals (for about 2 h). Add 750 mL of *water R* and 50 mL of *glacial acetic acid R*. Stir overnight (for at least 16 h); filter.

**Copper.** Cu. ($A_r$ 63.55). *1022100.* [7440-50-8].

Cleaned foil, turnings, wire or powder of the pure metal of electrolytic grade.

**Copper acetate.** $C_4H_6CuO_4,H_2O$. ($M_r$ 199.7). *1022200.* [6046-93-1].

Blue-green crystals or powder, freely soluble in boiling water, soluble in water and in ethanol (96 per cent), slightly soluble in glycerol (85 per cent).

**Copper edetate solution.** *1022300.*

To 2 mL of a 20 g/L solution of *copper acetate R* add 2 mL of *0.1 M sodium edetate* and dilute to 50 mL with *water R*.

**Copper nitrate.** $Cu(NO_3)_2,3H_2O$. ($M_r$ 241.6). *1022400.* [10031-43-3]. Copper dinitrate trihydrate.

Dark blue crystals, hygroscopic, very soluble in water giving a strongly acid reaction, freely soluble in ethanol (96 per cent) and in dilute nitric acid.

*Storage*: in an airtight container.

**Copper sulfate, anhydrous.** $CuSO_4$. ($M_r$ 159.6). *1199000.* [7758-98-7].

Greenish-grey powder, hygroscopic, freely soluble in water, slightly soluble in methanol and practically insoluble in ethanol (96 per cent).

**Copper sulfate solution R1.** *1199001.*

To 600 mL of *water R* slowly add 80 mL of *phosphoric acid R*. Dissolve with stirring 100 g of *anhydrous copper sulfate R* and dilute to 1 L with *water R*.

**Copper sulfate pentahydrate.** $CuSO_4,5H_2O$. ($M_r$ 249.7). *1022500.* [7758-99-8].

Blue powder or deep-blue crystals, slowly efflorescent, very soluble in water, slightly soluble in ethanol (96 per cent).

**Copper sulfate solution.** *1022501.*

A 125 g/L solution of *copper sulfate pentahydrate R*.

**Copper tetrammine, ammoniacal solution of.** *1022600.*

Dissolve 34.5 g of *copper sulfate pentahydrate R* in 100 mL of *water R* and, whilst stirring, add dropwise *concentrated ammonia R* until the precipitate which forms dissolves completely. Keeping the temperature below 20 °C, add dropwise with continuous shaking 30 mL of *strong sodium hydroxide solution R*. Filter through a sintered-glass filter (40) (*2.1.2*), wash with *water R* until the filtrate is clear and take up the precipitate with 200 mL of *concentrated ammonia R*. Filter through a sintered-glass filter (*2.1.2*) and repeat the filtration to reduce the residue to a minimum.

**Cortisone.** $C_{21}H_{28}O_5$. ($M_r$ 360.4). *1175000.* [53-06-5].

*Content*: minimum 95.0 per cent.

mp: 223-228 °C.

**Cortisone acetate.** *1097800.* [50-04-4].

See *Cortisone acetate (0321)*.

**Corydaline.** $C_{22}H_{27}NO_4$. ($M_r$ 369.4). *1204400.* [518-69-4]. (13*S*,13a*R*)-5,8,13,13a-Tetrahydro-2,3,9,10-tetramethoxy-13-methyl-6*H*-dibenzo[*a*,*g*]quinolizine.

**Costunolide.** $C_{15}H_{20}O_2$. ($M_r$ 232.3). *1194600.* [553-21-9]. (3a*S*,6*E*,10*E*,11a*R*)-6,10-Dimethyl-3-methylene-3a,4,5,8,9,11a-hexahydrocyclodeca[*b*]furan-2(3*H*)-one.

**Coumaphos.** $C_{14}H_{16}ClO_5PS$. ($M_r$ 362.8). *1124800.* [56-72-4].

mp: 91 °C to 92 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

***o*-Coumaric acid.** $C_9H_8O_3$. ($M_r$ 164.2). *1157400.* [614-60-8]. (*E*)-2-Hydroxycinnamic acid. (2*E*)-3-(2-Hydroxyphenyl)prop-2-enoic acid.

White or almost white powder.

mp: about 217 °C.

**Coumarin.** $C_9H_6O_2$. ($M_r$ 146.1). *1124900.* [91-64-5]. 2*H*-Chromen-2-one. 2*H*-1-Benzopyran-2-one.

Colourless, crystalline powder or orthorhombic or rectangular crystals, very soluble in boiling water, soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides.

mp: 68 °C to 70 °C.

*Coumarin used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Cresol.** $C_7H_8O$. ($M_r$ 108.1). *1022700.* [95-48-7]. *o*-Cresol. 2-Methylphenol.

Crystals or a super-cooled liquid becoming dark on exposure to light and air, miscible with anhydrous ethanol, soluble in about 50 parts of water and soluble in solutions of alkali hydroxides.

$d_{20}^{20}$: about 1.05.

$n_D^{20}$: 1.540 to 1.550.

bp: about 190 °C.

*Freezing point* (*2.2.18*): minimum 30.5 °C.

*Residue on evaporation*: maximum 0.1 per cent *m/m*, determined by evaporating on a water-bath and drying in an oven at 100-105 °C.

*Storage*: protected from light, moisture and oxygen.

Distil before use.

**_m_-Cresol.** *1177100.* [108-39-4].

See *metacresol (2077)*.

**_p_-Cresol.** $C_7H_8O$. ($M_r$ 108.1). *1153100.* [106-44-5]. 4-Methylphenol.

Colourless or white or almost white crystals or crystalline mass.

$d_{20}^{20}$: about 1.02.

bp: about 202 °C.

**_m_-Cresol purple.** $C_{21}H_{18}O_5S$. ($M_r$ 382.44). *1121700.* [2303-01-7]. *m*-Cresolsulfonphthalein.

Olive-green, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent), in glacial acetic acid and in methanol.

**_m_-Cresol purple solution.** *1121701.*

Dissolve 0.1 g of *m-cresol purple R* in 13 mL of *0.01 M sodium hydroxide*, dilute to 100 mL with *water R* and mix.

*Colour change*: pH 1.2 (red) to pH 2.8 (yellow); pH 7.4 (yellow) to pH 9.0 (purple).

**Cresol red.** $C_{21}H_{18}O_5S$. ($M_r$ 382.4). *1022800.* [1733-12-6]. Cresolsulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis-(2-methylphenol) *S*,*S*-dioxide.

A reddish-brown crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Cresol red solution.** *1022801.*

Dissolve 0.1 g of *cresol red R* in a mixture of 2.65 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* A mixture of 0.1 mL of the cresol red solution and 100 mL of *carbon dioxide-free water R* to which 0.15 mL of *0.02 M sodium hydroxide* has been added is purple-red. Not more than 0.15 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

*Colour change*: pH 7.0 (yellow) to pH 8.6 (red).

**Crown-ether silica gel for chiral separation.** *1192400.*

A very finely divided silica gel for chromatography coated with the following chiral crown ether:

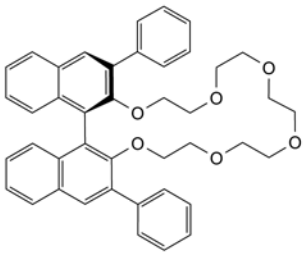

($R_a$)-6,23-Diphenyl-8,9,11,12,14,15,17,18,20,21-decahydrodinaphtho[2,1-*q*:1′,2′-*s*][1,4,7,10,13,16]-hexaoxacycloicosine.

**Crystal violet.** $C_{25}H_{30}ClN_3$. ($M_r$ 408.0). *1022900.* [548-62-9].

Schultz No. 78.

Colour Index No. 42555.
Hexamethyl-pararosanilinium chloride.

Dark-green powder or crystals, soluble in water and in ethanol (96 per cent).

**Crystal violet solution.** *1022901.*

Dissolve 0.5 g of *crystal violet R* in *anhydrous acetic acid R* and dilute to 100 mL with the same solvent.

*Test for sensitivity.* To 50 mL of *anhydrous acetic acid R* add 0.1 mL of the crystal violet solution. On addition of 0.1 mL of *0.1 M perchloric acid* the bluish-purple solution turns bluish-green.

**Cupric chloride.** $CuCl_2,2H_2O$. ($M_r$ 170.5). *1023000.* [10125-13-0]. Cupric chloride dihydrate.

Greenish-blue powder or crystals, deliquescent in moist air, efflorescent in dry air, freely soluble in water, in ethanol (96 per cent) and in methanol, sparingly soluble in acetone.

*Storage*: in an airtight container.

**Cupri-citric solution.** *1023100.*

Dissolve 25 g of *copper sulfate pentahydrate R*, 50 g of *citric acid monohydrate R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

**Cupri-citric solution R1.** *1023200.*

Dissolve 25 g of *copper sulfate pentahydrate R*, 50 g of *citric acid monohydrate R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

Adjust the solution so that it complies with the following requirements.

a) To 25.0 mL add 3 g of *potassium iodide R*. Add 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R* with precaution and in small quantities. Titrate with *0.1 M sodium thiosulfate* using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

24.5 mL to 25.5 mL of *0.1 M sodium thiosulfate* is used in the titration.

b) Dilute 10.0 mL to 100.0 mL with *water R* and mix. To 10.0 mL of the solution, add 25.0 mL of *0.1 M hydrochloric acid* and heat for 1 h on a water-bath. Cool, adjust with *water R* to the initial volume and titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

5.7 mL to 6.3 mL of *0.1 M sodium hydroxide* is used in the titration.

c) Dilute 10.0 mL to 100.0 mL with *water R* and mix. Titrate 10.0 mL of the solution with *0.1 M hydrochloric acid*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

6.0 mL to 7.5 mL of *0.1 M hydrochloric acid* is used in the titration.

**Cupriethylenediamine hydroxide solution.** *3008700.* [14552-35-3].

The molar ratio of ethylenediamine to copper is 2.00 ± 0.04.

This solution is commercially available.

**Cupri-tartaric solution.** *1023300.*

*Solution A*. Dissolve 34.6 g of *copper sulfate pentahydrate R* in *water R* and dilute to 500 mL with the same solvent.

*Solution B*. Dissolve 173 g of *sodium potassium tartrate R* and 50 g of *sodium hydroxide R* in 400 mL of *water R*. Heat to boiling, allow to cool and dilute to 500 mL with *carbon dioxide-free water R*.

Mix equal volumes of the 2 solutions immediately before use.

**Cupri-tartaric solution R2.** *1023302.*

Add 1 mL of a solution containing 5 g/L of *copper sulfate pentahydrate R* and 10 g/L of *potassium tartrate R* to 50 mL of *sodium carbonate solution R1*. Prepare immediately before use.

**Cupri-tartaric solution R3.** *1023303.*

Prepare a solution containing 10 g/L of *copper sulfate pentahydrate R* and 20 g/L of *sodium tartrate R*. To 1.0 mL of the solution add 50 mL of *sodium carbonate solution R2*. Prepare immediately before use.

**Cupri-tartaric solution R4.** *1023304.*

*Solution A*. 150 g/L *copper sulfate pentahydrate R*.

*Solution B*. Dissolve 2.5 g of *anhydrous sodium carbonate R*, 2.5 g of *sodium potassium tartrate R*, 2.0 g of *sodium hydrogen carbonate R*, and 20.0 g of *anhydrous sodium sulfate R* in *water R* and dilute to 100 mL with the same solvent.

Mix 1 part of solution A with 25 parts of solution B immediately before use.

**Curcumin.** $C_{21}H_{20}O_6$. ($M_r$ 368.4). *1023500.* [458-37-7]. 1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione.

Orange-brown, crystalline powder, practically insoluble in water, soluble in glacial acetic acid.

mp: about 183 °C.

**Curcuminoids.** *1183900.*

A mixture of curcumin ($C_{21}H_{20}O_6$; $M_r$ 368.4), demethoxycurcumin ($C_{20}H_{18}O_5$; $M_r$ 338.4) and bis-demethoxycurcumin ($C_{19}H_{16}O_4$; $M_r$ 308.3).

**Cyanoacetic acid.** $C_3H_3NO_2$. ($M_r$ 85.1). *1097900.* [372-09-8].

White or yellowish-white, hygroscopic crystals, very soluble in water.

*Storage*: in an airtight container.

**Cyanocobalamin.** *1023600.* [68-19-9].

See *Cyanocobalamin (0547)*.

**Cyanogen bromide solution.** *1023700.* [506-68-3].

Add dropwise, with cooling *0.1 M ammonium thiocyanate* to *bromine water R* until the yellow colour disappears. Prepare immediately before use.

**Cyanoguanidine.** $C_2H_4N_4$. ($M_r$ 84.1). *1023800.* [461-58-5]. Dicyandiamide. 1-Cyanoguanidine.

White or almost white, crystalline powder, sparingly soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: about 210 °C.

**Cyanopropyl(3)phenyl(3)methyl(94)polysiloxane.** *1114800.*

Polysiloxane substituted with 3 per cent of cyanopropyl groups, 3 per cent of phenyl groups and 94 per cent of methyl groups.

**Cyanopropyl(7)phenyl(7)methyl(86)polysiloxane.** *1109200.*

Polysiloxane substituted with 7 per cent of cyanopropyl groups, 7 per cent of phenyl groups and 86 per cent of methyl groups.

**Cyanopropyl(25)phenyl(25)methyl(50)polysiloxane.** *1066500.*

Polysiloxane substituted with 25 per cent of cyanopropyl groups, 25 per cent of phenyl groups and 50 per cent of methyl groups.

**Cyanopropylpolysiloxane.** *1066700.*

Polysiloxane substituted with 100 per cent of cyanopropyl groups.

**Cyasterone.** $C_{29}H_{44}O_8$. ($M_r$ 520.7). *1204500.* [17086-76-9]. (2β,3β,5β,22*R*,24*S*,24$^1$*R*,25*S*)-24$^1$,26-Epoxy-2,3,14,20,22-pentahydroxystigmast-7-ene-6,26-dione.

**α-Cyclodextrin.** $C_{36}H_{60}O_{30}$. ($M_r$ 972). *1176200.* [10016-20-3]. Cyclohexakis-(1→4)-(α-D-glucopyranosyl). Cyclomaltohexaose. Alfadex.

**β-Cyclodextrin.** *1184000.* [7585-39-9].

See *Betadex (1070)*.

**β-Cyclodextrin for chiral chromatography, modified.** *1154600.*

30 per cent of 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin dissolved in polysiloxane substituted with 15 per cent of phenyl groups and 85 per cent of methyl groups.

**β-Cyclodextrin for chiral chromatography, modified R1.** *1160700.*

30 per cent of 2,3-di-*O*-acetyl-6-*O*-*tert*-butylsilyl-β-cyclodextrin dissolved in polysiloxane substituted with 15 per cent of phenyl groups and 85 per cent of methyl groups.

**Cyclohexane.** $C_6H_{12}$. ($M_r$ 84.2). *1023900.* [110-82-7].

Clear, colourless, flammable liquid, practically insoluble in water, miscible with organic solvents.

$d_{20}^{20}$: about 0.78.

bp: about 80.5 °C.

*Cyclohexane used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 0.35 at 220 nm, 0.16 at 235 nm, 0.05 at 240 nm, 0.01 at 250 nm, determined using *water R* as compensation liquid.

**Cyclohexane R1.** *1023901.*

Complies with the requirements prescribed for *cyclohexane R* with the following additional requirement.

The fluorescence, measured at 460 nm, under illumination with an excitant light beam at 365 nm, is not more intense than that of a solution containing 0.002 ppm of *quinine R* in *dilute sulfuric acid R1*.

**Cyclohexylamine.** $C_6H_{13}N$. ($M_r$ 99.2). *1024000.* [108-91-8]. Cyclohexanamine.

Colourless liquid, soluble in water, miscible with usual organic solvents.

$n_D^{20}$: about 1.460.

bp: 134 °C to 135 °C.

**Cyclohexylenedinitrilotetra-acetic acid.** $C_{14}H_{22}N_2O_8,H_2O$. ($M_r$ 364.4). *1024100. trans*-Cyclohexylene-1,2-dinitrilo-*N,N,N',N'*-tetra-acetic acid.

White or almost white, crystalline powder.

mp: about 204 °C.

**Cyclohexylmethanol.** $C_7H_{14}O$. ($M_r$ 114.2). *1135200.* [100-49-2]. Cyclohexylcarbinol.

Liquid with a slight odour of camphor, soluble in ethanol (96 per cent).

$n_D^{25}$: about 1.464.

bp: about 185 °C.

**3-Cyclohexylpropionic acid.** $C_9H_{16}O_2$. ($M_r$ 156.2). *1119200.* [701-97-3].

Clear liquid.

$d_{20}^{20}$: about 0.998.

$n_D^{20}$: about 1.4648.

bp: about 130 °C.

**Cyhalothrin.** $C_{23}H_{19}ClF_3NO_3$. ($M_r$ 449.9). *1125000.* [91465-08-6].

bp: 187 °C to 190 °C.

mp: about 49 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p*-Cymene.** $C_{10}H_{14}$. ($M_r$ 134.2). *1113400.* [99-87-6]. 1-Isopropyl-4-methylbenzene.

Colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.858.

$n_D^{20}$: about 1.4895.

bp: 175 °C to 178 °C.

*p-Cymene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. The substance to be examined.

*Content*: minimum 96.0 per cent, calculated by the normalisation procedure.

**Cynarin.** $C_{25}H_{24}O_{12}$. ($M_r$ 516.4). *1159300.* [30964-13-7]. (1α,3α,4α,5β)-1,3-Bis[[3-(3,4-Dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-4,5-dihydroxycyclohexanecarboxylic acid.

White or almost white amorphous mass, odourless.

**Cypermethrin.** $C_{22}H_{19}Cl_2NO_3$. ($M_r$ 416.3). *1125100.* [52315-07-8].

bp: 170 °C to 195 °C.

mp: 60 °C to 80 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**L-Cysteine.** $C_3H_7NO_2S$. ($M_r$ 121.1). *1024200.* [52-90-4].

Powder, freely soluble in water, in ethanol (96 per cent) and in acetic acid, practically insoluble in acetone.

**Cysteine hydrochloride.** *1024300.* [7048-04-6].

See *Cysteine hydrochloride monohydrate (0895)*.

**L-Cystine.** $C_6H_{12}N_2O_4S_2$. ($M_r$ 240.3). *1024400.* [56-89-3].

White or almost white, crystalline powder, practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

$[\alpha]_D^{20}$: − 218 to − 224, determined in *1 M hydrochloric acid*.

mp: 250 °C, with decomposition.

**Cytosine.** $C_4H_5N_3O$. ($M_r$ 111.1). *1160800.* [71-30-7].

*Content*: minimum 95.0 per cent.

**Daidzein.** $C_{15}H_{10}O_4$. ($M_r$ 254.2). *1178400.* [486-66-8]. 7-Hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

**Daidzin.** $C_{21}H_{20}O_9$. ($M_r$ 416.4). *1178300.* [552-66-9]. Daidzein-7-*O*-glucoside. 7-(β-D-Glucopyranosyloxy)-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

**Dantron.** $C_{14}H_8O_4$. ($M_r$ 240.2). *1024500.* [117-10-2]. 1,8-Dihydroxyanthraquinone. 1,8-Dihydroxyanthracene-9,10-dione.

Crystalline orange powder, practically insoluble in water, slightly soluble in ethanol (96 per cent), soluble in solutions of alkali hydroxides.

mp: about 195 °C.

***o,p′*-DDD.** $C_{14}H_{10}Cl_4$. ($M_r$ 320.0). *1125200.* [53-19-0]. 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p′*-DDD.** $C_{14}H_{10}Cl_4$. ($M_r$ 320.0). *1125300.* [72-54-8]. 1,1-Bis(4-chlorophenyl)-2,2-dichloroethane.

bp: about 193 °C.

mp: about 109 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***o,p′*-DDE.** $C_{14}H_8Cl_4$. ($M_r$ 318.0). *1125400.* [3424-82-6]. 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p′*-DDE.** $C_{14}H_8Cl_4$. ($M_r$ 318.0). *1125500.* [72-55-9]. 1,1-Bis(4-chlorophenyl)-2,2-dichloroethylene.

bp: 316 °C to 317 °C.

mp: 88 °C to 89 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***o,p′*-DDT.** $C_{14}H_9Cl_5$. ($M_r$ 354.5). *1125600.* [789-02-6]. 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p′*-DDT.** $C_{14}H_9Cl_5$. ($M_r$ 354.5). *1125700.* [50-29-3]. 1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane.

bp: about 260 °C.

mp: 108 °C to 109 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Decanal.** $C_{10}H_{20}O$. ($M_r$ 156.3). *1149200.* [112-31-2]. Decyl aldehyde.

Oily, colourless liquid, practically insoluble in water.

*Decanal used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Sweet orange oil (1811)*.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Decane.** $C_{10}H_{22}$. ($M_r$ 142.3). *1024600.* [124-18-5].

Colourless liquid, practically insoluble in water.

$n_D^{20}$: about 1.411.

bp: about 174 °C.

**Decanol.** $C_{10}H_{22}O$. ($M_r$ 158.3). *1024700.* [112-30-1]. Decan-1-ol.

Viscous liquid, solidifying at about 6 °C, practically insoluble in water, soluble in ethanol (96 per cent).

$n_D^{20}$: about 1.436.

bp: about 230 °C.

**Dehydrocostus lactone.** $C_{15}H_{18}O_2$. ($M_r$ 230.3). *1194700.* [477-43-0]. (3a*S*,6a*R*,9a*R*,9b*S*)-3,6,9-Trismethylenedecahydroazuleno[4,5-*b*]furan-2(3*H*)-one.

**Deltamethrin.** $C_{22}H_{19}Br_2NO_3$. ($M_r$ 505.2). *1125800.* [52918-63-5].

bp: about 300 °C.

mp: about 98 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Demeclocycline hydrochloride.** *1145600.*

See *Demeclocycline hydrochloride (0176)*.

**Demethylflumazenil.** $C_{14}H_{12}FN_3O_3$. ($M_r$ 289.3). *1149300.* [79089-72-8]. Ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate.

Colourless needles, soluble in dimethyl sulfoxide and in hot methanol.

mp: about 288 °C.

**14-Deoxy-11,12-didehydroandrographolide.** $C_{20}H_{28}O_4$. ($M_r$ 332.4). *1198300.* [42895-58-9]. 3-[(1*E*)-2-[(1*R*,4a*S*,5*R*,6*R*,8a*R*)-6-Hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl]ethenyl]furan-2(5*H*)-one.

**2-Deoxy-D-ribose.** $C_5H_{10}O_4$. ($M_r$ 134.1). *1163900.* [533-67-5]. Thyminose. 2-Deoxy-D-*erythro*-pentose.

**2′-Deoxyuridine.** $C_9H_{12}N_2O_5$. ($M_r$ 228.2). *1024800.* [951-78-0]. 1-(2-Deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione.

mp: about 165 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram shows only one principal spot.

**4-Deoxypyridoxine hydrochloride.** $C_8H_{12}NO_2Cl$. ($M_r$ 189.6). *1175500.* [148-51-6]. 5-(Hydroxymethyl)-2,4-dimethylpyridin-3-ol.

**Desmethylmisonidazole.** $C_6H_9N_3O_4$. ($M_r$ 187.2). *1185600.* [13551-92-3]. (2*RS*)-3-(2-Nitro-1*H*-imidazol-1-yl)propane-1,2-diol.

*Content*: minimum 95 per cent.

Yellow powder.

**Destaining solution.** *1012202.*

A mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*.

**Deuterated acetic acid.** $C_2{}^2H_4O_2$. ($M_r$ 64.1). *1101100.* [1186-52-3]. Tetradeuteroacetic acid. Acetic-$d_3$ acid-*d*.

Degree of deuteration: minimum 99.7 per cent.

$d_{20}^{20}$: about 1.12.

$n_D^{20}$: about 1.368.

bp: about 115 °C.

mp: about 16 °C.

**Deuterated acetone.** $C_3{}^2H_6O$. ($M_r$ 64.1). *1024900.* [666-52-4]. Acetone-$d_6$. ($^2H_6$)-Acetone.

Degree of deuteration: minimum 99.5 per cent.

Clear, colourless liquid, miscible with water, with dimethylformamide, with anhydrous ethanol and with methanol.

$d_{20}^{20}$: about 0.87.

$n_D^{20}$: about 1.357.

bp: about 55 °C.

*Water and deuterium oxide.* Not more than 0.1 per cent.

**Deuterated acetonitrile.** $C_2{}^2H_3N$. ($M_r$ 44.1). *1173100.* [2206-26-0].

Degree of deuteration: minimum 99.8 per cent.

Clear, colourless liquid, miscible with water, with acetone and with methanol.

$d_{20}^{20}$: about 0.78.

$n_D^{20}$: about 1.344.

**Deuterated chloroform.** $C^2HCl_3$. ($M_r$ 120.4). *1025000.* [865-49-6]. ($^2H$)-Chloroform. Chloroform-*d*.

Degree of deuteration: minimum 99.7 per cent.

Clear, colourless liquid, practically insoluble in water, miscible with acetone and with ethanol (96 per cent). It may be stabilised over silver foil.

$d_{20}^{20}$: about 1.51.

$n_D^{20}$: about 1.445.

bp: about 60 °C.

*Water and deuterium oxide*: maximum 0.05 per cent.

**Deuterated dimethyl sulfoxide.** $C_2{}^2H_6OS$. ($M_r$ 84.2). *1025100.* [2206-27-1]. ($^2H_6$)-Dimethyl sulfoxide. Dimethyl sulfoxide-$d_6$.

Degree of deuteration: minimum 99.8 per cent.

Very hygroscopic liquid, practically colourless, viscous, soluble in water, in acetone and in anhydrous ethanol.

$d_{20}^{20}$: about 1.18.

mp: about 20 °C.

*Water and deuterium oxide*: maximum 0.1 per cent.

*Storage*: in an airtight container.

**Deuterated methanol.** $C^2H_4O$. ($M_r$ 36.1). *1025200.* [811-98-3]. ($^2H$)-Methanol. Methanol-*d*.

Degree of deuteration: minimum 99.8 per cent.

Clear, colourless liquid miscible with water, with ethanol (96 per cent) and with methylene chloride.

$d_{20}^{20}$: about 0.888.

$n_D^{20}$: about 1.326.

bp: 65.4 °C.

**Deuterated sodium trimethylsilylpropionate.** $C_6H_9{}^2H_4NaO_2Si$. ($M_r$ 172.3). *1179100.* [24493-21-8]. Sodium 3-(trimethylsilyl)(2,2,3,3-$^2H_4$)propionate. TSP-$d_4$.

*Degree of deuteration*: minimum 98 per cent.

White or almost white powder.

**Deuterium chloride.** $^2HCl$. ($M_r$ 37.47). *1178800.* [7698-05-7]. Deuterated hydrochloric acid.

Gas.

*Degree of deuteration*: minimum 99 per cent.

*Caution*: *toxic*.

**Deuterium chloride solution.** *1178801.*

Dilute 1 mL of *deuterium chloride R* (38 per cent *m*/*m*) with 5 mL of *deuterium oxide R*.

**Deuterium oxide.** $^2H_2O$. ($M_r$ 20.03). *1025300.* [7789-20-0]. Deuterated water.

Degree of deuteration: minimum 99.7 per cent.

$d_{20}^{20}$: about 1.11.

$n_D^{20}$: about 1.328.

bp: about 101 °C.

**Deuterium oxide R1.** $^2H_2O$. ($M_r$ 20.03). *1025301.* [7789-20-0]. Deuterated water.

Degree of deuteration: minimum 99.95 per cent.

**Developer solution.** *1122500.*

Dilute 2.5 mL of a 20 g/L solution of *citric acid monohydrate R* and 0.27 mL of *formaldehyde R* to 500.0 mL with *water R*.

**Dextran for chromatography, cross-linked R2.** *1025500.*

Bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of $15 \times 10^2$ to $30 \times 10^3$. When dry, the beads have a diameter of 20-80 µm.

**Dextran for chromatography, cross-linked R3.** *1025600.*

Bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of $4 \times 10^3$ to $15 \times 10^4$. When dry, the beads have a diameter of 40-120 µm.

**Dextrose.** *1025700.* [50-99-7].

See *glucose R*.

**3,3′-Diaminobenzidine tetrahydrochloride.** $C_{12}H_{18}Cl_4N_4$, $2H_2O$. ($M_r$ 396.1). *1098000.* [7411-49-6]. 3,3′,4,4′-Biphenyl-tetramine.

Almost white or slightly pink powder, soluble in water.

mp: about 280 °C, with decomposition.

**1,2-Diamino-4,5-methylenedioxybenzene dihydrochloride.** $C_7H_{10}Cl_2N_2O_2$. ($M_r$ 225.1). *1202100.* [81864-15-5]. 2*H*-1,3-Benzodioxole-5,6-diamine dihydrochloride.

*Content*: minimum 99 per cent (HPLC).

**Diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate).** $C_{18}H_{24}N_6O_6S_4$. ($M_r$ 548.7). *1153000.* [30931-67-0]. ABTS. Diammonium 2,2′-(diazanediylidene)bis[3-ethyl-2,3-dihydrobenzothiazole-6-sulfonate].

Chromogenic substrate suitable for use in ELISA procedures.

Green tablets, freely soluble in water.

pH (*2.2.3*): 4.2 to 5.8 for a 0.1 g/L solution.

**Diatomaceous earth.** *1025900.* [91053-39-3].

White or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in ethanol (96 per cent).

The substance may be identified by microscopic examination with a magnification of × 500.

**Diatomaceous earth for gas chromatography.** *1026000.*

White or almost white, fine granular powder, practically insoluble in water and in ethanol (96 per cent), made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms. The substance may be identified by microscopic examination with a magnification of × 500. The substance is acid-washed, then water-washed until neutral.

**Diatomaceous earth for gas chromatography, silanised.** *1026300.*

*Diatomaceous earth for gas chromatography R* silanised with dimethyldichlorosilane or other suitable silanising agents.

**Diazinon.** $C_{12}H_{21}N_2O_3PS$. ($M_r$ 304.3). *1125900.* [333-41-5].

bp: about 306 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Diazobenzenesulfonic acid solution R1.** *1026500.*

Dissolve 0.9 g of *sulfanilic acid R* in a mixture of 30 mL of *dilute hydrochloric acid R* and 70 mL of *water R*. To 3 mL of the solution add 3 mL of a 50 g/L solution of *sodium nitrite R*. Cool in an ice-bath for 5 min, add 12 mL of the sodium nitrite solution and cool again. Dilute to 100 mL with *water R* and keep the reagent in an ice-bath. Prepare extemporaneously but allow to stand for 15 min before use.

**Dibromomethane.** $CH_2Br_2$. ($M_r$ 173.8). *1195500.* [74-95-3].

Colourless liquid, slightly soluble in water.

bp: about 96 °C.

**Dibutylamine.** $C_8H_{19}N$. ($M_r$ 129.3). *1126000.* [111-92-2]. *N*-Butylbutan-1-amine.

Colourless liquid.

$n_D^{20}$: about 1.417.

bp: about 159 °C.

**Dibutylammonium phosphate for ion-pairing.** *1168800.*

A colourless solution of 10 per cent to 15 per cent *V/V* of di-*n*-butylamine and 12 per cent to 17 per cent *V/V* of phosphoric acid in water, suitable for ion-pairing in liquid chromatography.

**Dibutyl ether.** $C_8H_{18}O$. ($M_r$ 130.2). *1026700.* [142-96-1].

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

$d_{20}^{20}$: about 0.77.

$n_D^{20}$: about 1.399.

*Do not distil if the dibutyl ether does not comply with the test for peroxides.*

*Peroxides.* Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

**Dibutyl phthalate.** $C_{16}H_{22}O_4$. ($M_r$ 278.3). *1026800.* [84-74-2]. Dibutyl benzene-1,2-dicarboxylate.

Clear, colourless or faintly coloured, oily liquid, very slightly soluble in water, miscible with acetone and with ethanol (96 per cent).

$d_{20}^{20}$: 1.043 to 1.048.

$n_D^{20}$: 1.490 to 1.495.

**Dicarboxidine hydrochloride.** $C_{20}H_{26}Cl_2N_2O_6$. ($M_r$ 461.3). *1026900.* [56455-90-4]. 4,4′-[(4,4′-Diaminobiphenyl-3,3′-diyl)dioxy]dibutanoic acid dihydrochloride.

**Dichlofenthion.** $C_{10}H_{13}Cl_2O_3PS$. ($M_r$ 315.2). *1126100.* [97-17-6].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Dichloroacetic acid.** $C_2H_2Cl_2O_2$. ($M_r$ 128.9). *1027000.* [79-43-6].

Colourless liquid, miscible with water and ethanol (96 per cent).

$d_{20}^{20}$: about 1.566.

$n_D^{20}$: about 1.466.

bp: about 193 °C.

**Dichloroacetic acid solution.** *1027001.*

Dilute 67 mL of *dichloroacetic acid R* to 300 mL with *water R* and neutralise to *blue litmus paper R* using *ammonia R*. Cool, add 33 mL of *dichloroacetic acid R* and dilute to 600 mL with *water R*.

**3,5-Dichloroaniline.** $C_6H_5Cl_2N$. ($M_r$ 162.0). *1177800.* [626-43-7]. 3,5-dichlorophenylamine.

mp: 46 °C to 52 °C.

**Dichlorobenzene.** $C_6H_4Cl_2$. ($M_r$ 147.0). *1027100.* [95-50-1]. 1,2-Dichlorobenzene.

Colourless, oily liquid, practically insoluble in water, soluble in anhydrous ethanol.

$d_{20}^{20}$: about 1.31.

bp: about 180 °C.

**2,4-Dichlorobenzoic acid.** $C_7H_4Cl_2O_2$. ($M_r$ 191.0). *1185700.* [50-84-0].

Faintly beige powder.

mp: about 160 °C.

**2,3-Dichloro-5,6-dicyanobenzoquinone.** $C_8Cl_2N_2O_2$. ($M_r$ 227.0). *1153600.* [84-58-2]. 4,5-Dichloro-3,6-dioxocyclohexa-1,4-diene-1,2-dicarbonitrile.

Yellow or orange crystals, soluble in dioxan and in acetic acid, slightly soluble in methylene chloride. It decomposes in water.

mp: about 214 °C.

*Storage*: at a temperature of 2 °C to 8 °C.

**(*S*)-3,5-Dichloro-2,6-dihydroxy-*N*-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide.** $C_{14}H_{19}BrCl_2N_2O_3$. ($M_r$ 414.1). *1142600.* [113310-88-6].

White or almost white, crystalline powder.

$[\alpha]_D^{22}$: + 11.4, determined on a 15.0 g/L solution in *anhydrous ethanol R*.

mp: about 212 °C.

**Dichlorofluorescein.** $C_{20}H_{10}Cl_2O_5$. ($M_r$ 401.2). *1027200.* [76-54-0]. 2,7-Dichlorofluorescein. 2-(2,7-Dichloro-6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid.

Yellowish-brown or yellow-orange powder, slightly soluble in water, freely soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides giving a solution showing a yellowish-green fluorescence.

**2,6-Dichlorophenol.** $C_6H_4Cl_2O$. ($M_r$ 163.0). *1177600.* [87-65-0].

mp: 64 °C to 66 °C.

**Dichlorophenolindophenol, sodium salt.** $C_{12}H_6Cl_2NNaO_2,2H_2O$. ($M_r$ 326.1). *1027300.* [620-45-1]. The sodium derivative of 2,6-dichloro-*N*-(4-hydroxyphenyl)-1,4-benzoquinone monoimine dihydrate.

Dark-green powder, freely soluble in water and in anhydrous ethanol. The aqueous solution is dark blue; when acidified it becomes pink.

**Dichlorophenolindophenol standard solution.** *1027301.*

Dissolve 50.0 mg of *dichlorophenolindophenol, sodium salt R* in 100.0 mL of *water R* and filter.

*Assay*. Dissolve 20.0 mg of *ascorbic acid R* in 10 mL of a freshly prepared 200 g/L solution of *metaphosphoric acid R* and dilute to 250.0 mL with *water R*. Titrate 5.0 mL rapidly with the dichloro-phenolindophenol standard solution, added from a microburette graduated in 0.01 mL, until the pink colour persists for 10 s, the titration occupying not more than 2 min. Dilute the dichlorophenolindophenol solution with *water R* to make 1 mL of the solution equivalent to 0.1 mg of ascorbic acid ($C_6H_8O_6$).

*Storage*: use within 3 days.

Standardise immediately before use.

**5,7-Dichloroquinolin-8-ol.** $C_9H_5Cl_2NO$. ($M_r$ 214.1). *1157000.* [773-76-2]. 5,7-Dichlorooxine.

Yellow, crystalline powder, soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 179 °C.

*Content*: minimum 95.0 per cent.

**Dichloroquinonechlorimide.** $C_6H_2Cl_3NO$. ($M_r$ 210.4). *1027400.* [101-38-2]. 2,6-Dichloro-*N*-chloro-1,4-benzoquinone mono-imine.

Pale yellow or greenish-yellow crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute alkaline solutions.

mp: about 66 °C.

**Dichlorvos.** $C_4H_7Cl_2O_4P$. ($M_r$ 221). *1101200.* [62-73-7]. 2,2-Dichlorovinyl dimethyl phosphate.

Colourless or brownish-yellow liquid, soluble in water, miscible with most organic solvents.

$n_D^{25}$: about 1.452.

**Dicyclohexyl.** $C_{12}H_{22}$. ($M_r$ 166.3). *1135300.* [92-51-3]. Bicyclohexyl.

$d_{20}^{20}$: about 0.864.

bp: about 227 °C.

mp: about 4 °C.

**Dicyclohexylamine.** $C_{12}H_{23}N$. ($M_r$ 181.3). *1027500.* [101-83-7]. *N,N*-Dicyclohexylamine.

Colourless liquid, sparingly soluble in water, miscible with the usual organic solvents.

$n_D^{20}$: about 1.484.

bp: about 256 °C.

*Freezing point* (*2.2.18*): 0 °C to 1 °C.

**Dicyclohexylurea.** $C_{13}H_{24}N_2O$. ($M_r$ 224.4). *1027600.* [2387-23-7]. 1,3-Dicyclohexylurea.

White or almost white, crystalline powder.

mp: about 232 °C.

**Didocosahexaenoin.** $C_{47}H_{68}O_5$. ($M_r$ 713.0). *1142700.* [88315-12-2]. Diglyceride of docosahexaenoic acid (C22:6). Glycerol didocosahexaenoate. (*all-Z*)-Docosahexaenoic acid, diester with propane-1,2,3-triol.

**Didodecyl 3,3′-thiodipropionate.** $C_{30}H_{58}O_4S$. ($M_r$ 514.8). *1027700.* [123-28-4].

White or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in light petroleum, slightly soluble in ethanol (96 per cent).

mp: about 39 °C.

**Dieldrin.** $C_{12}H_8Cl_6O$. ($M_r$ 380.9). *1126200.* [60-57-1].

bp: about 385 °C.

mp: about 176 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Diethanolamine.** $C_4H_{11}NO_2$. ($M_r$ 105.1). *1027800.* [111-42-2]. 2,2′-Iminobisethanol.

Viscous, clear, slightly yellow liquid or deliquescent crystals melting at about 28 °C, very soluble in water, in acetone and in methanol.

$d_{20}^{20}$: about 1.09.

*pH* (*2.2.3*): 10.0 to 11.5 for a 50 g/L solution.

*Diethanolamine used in the test for alkaline phosphatase complies with the following additional test.*

*Ethanolamine.* Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 1.00 g of *3-aminopropanol R* in *acetone R* and dilute to 10.0 mL with the same solvent.

*Test solution (a).* Dissolve 5.00 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dissolve 5.00 g of the substance to be examined in *acetone R*, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with the same solvent.

*Reference solutions.* Dissolve 0.50 g of *ethanolamine R* in *acetone R* and dilute to 10.0 mL with the same solvent. To 0.5 mL, 1.0 mL and 2.0 mL of this solution, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *acetone R*.

*Column*:

- *size*: *l* = 1 m, Ø = 4 mm;
- *stationary phase*: *diphenylphenylene oxide polymer R* (180-250 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 40 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 → 3 | 125 |
| | 3 → 17.6 | 125 → 300 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame-ionisation.

*Injection*: 1.0 µL.

*Limit*:

- *ethanolamine*: maximum 1.0 per cent.

**Diethoxytetrahydrofuran.** $C_8H_{16}O_3$. ($M_r$ 160.2). *1027900.* [3320-90-9]. 2,5-Diethoxytetrahydrofuran. A mixture of the *cis* and *trans* isomers.

Clear, colourless or slightly yellowish liquid, practically insoluble in water, soluble in ethanol (96 per cent) and in most other organic solvents.

$d_{20}^{20}$: about 0.98.

$n_D^{20}$: about 1.418.

**Diethylamine.** $C_4H_{11}N$. ($M_r$ 73.1). *1028000.* [109-89-7].

Clear, colourless, flammable liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.71.

bp: about 55 °C.

**Diethylamine R1.** $C_4H_{11}N$. ($M_r$ 73.1). *1028001.* [109-89-7]. *N*-Ethylethanamine.

*Content*: minimum 99.5 per cent.

Clear, colourless, flammable liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.71.

bp: about 55 °C.

**Diethylaminoethyldextran.** *1028200.*

Anion-exchange resin presented as the hydrochloride.

Powder forming gels with water.

***N,N*-Diethylaniline.** $C_{10}H_{15}N$. ($M_r$ 149.2). *1028400.* [91-66-7].

$d_{20}^{20}$: about 0.938.

bp: about 217 °C.

mp: about – 38 °C.

**Diethylene glycol.** $C_4H_{10}O_3$. ($M_r$ 106.1). *1028300.* [111-46-6]. 2,2′-Oxydiethanol.

*Content*: minimum 99.5 per cent *m/m*.

Clear, colourless liquid, hygroscopic, miscible with water, with acetone and with ethanol (96 per cent).

$d_{20}^{20}$: about 1.118.

$n_D^{20}$: about 1.447.

bp: 244 °C to 246 °C.

*Storage*: in an airtight container.

***N,N*-Diethylethane-1,2-diamine.** $C_6H_{16}N_2$. ($M_r$ 116.2). *1028500.* [100-36-7]. *N,N*-Diethylethylenediamine.

*Content*: minimum 98.0 per cent.

Slightly oily liquid, colourless or slightly yellow, strong odour of ammonia, irritant to the skin, eyes and mucous membranes.

$d_{20}^{20}$: 0.827.

bp: 145 °C to 147 °C.

*Water* (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Di(2-ethylhexyl) phthalate.** $C_{24}H_{38}O_4$. ($M_r$ 390.5). *1028100.* Di(2-ethylhexyl) benzene-1,2-dicarboxylate.

Colourless, oily liquid, practically insoluble in water, soluble in organic solvents.

$d_{20}^{20}$: about 0.98.

$n_D^{20}$: about 1.486.

*Viscosity* (*2.2.9*): about 80 mPa·s.

**Diethylphenylenediamine sulfate.** $C_{10}H_{18}N_2O_4S$. ($M_r$ 262.3). *1028600.* [6283-63-2]. *N,N*'-Diethyl-*p*-phenylenediamine sulfate. *N,N*'-Diethylbenzene-1,4-diamine sulfate.

White or slightly yellow powder, soluble in water.

mp: about 185 °C, with decomposition.

*Storage*: protected from light.

**Diethylphenylenediamine sulfate solution.** *1028601.*

To 250 mL of *water R* add 2 mL of *sulfuric acid R* and 25 mL of *0.02 M sodium edetate*. Dissolve in this solution 1.1 g of *diethylphenylenediamine sulfate R* and dilute to 1000 mL with *water R*.

Do not use if the solution is not colourless.

*Storage*: protected from light and heat for 1 month.

**Diethyl sulfone.** $C_4H_{10}O_2S$. ($M_r$ 122.2). *1203300.* [597-35-3]. 1-(Ethylsulfonyl)ethane. 1-(Ethanesulfonyl)ethane.

*Content*: minimum 97 per cent.

Crystalline powder.

mp: about 73 °C.

**Diflubenzuron.** $C_{14}H_9ClF_2N_2O_2$. ($M_r$ 310.7). *1180000.* [35367-38-5]. 1-(4-Chlorophenyl)-3-(2,6-difluorobenzoyl)urea.

Colourless or white or almost white crystals, practically insoluble in water, freely soluble in dimethyl sulfoxide, slightly soluble in acetone.

mp: 230 to 232 °C.

**Digitonin.** $C_{56}H_{92}O_{29}$. ($M_r$ 1229). *1028700.* [11024-24-1]. 3β-[*O*-β-D-Glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyloxy]-(25*R*)-5α-spirostan-2α,15β-diol.

Crystals, practically insoluble in water, sparingly soluble in anhydrous ethanol, slightly soluble in ethanol (96 per cent).

**Digitoxin.** *1028800.* [71-63-6].

See *Digitoxin (0078)*.

**Diglycine.** $C_4H_8N_2O_3$. ($M_r$ 132.1). *1191700.* [556-50-3]. 2-[(2-Aminoacetyl)amino]acetic acid. Glycylglycine.

**Digoxin.** *1203400.*

See *Digoxin (0079)*.

**Dihydrocapsaicin.** $C_{18}H_{29}NO_3$. ($M_r$ 307.4). *1148100.* [19408-84-5]. *N*-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnonanamide.

White or almost white, crystalline powder, practically insoluble in cold water, freely soluble in anhydrous ethanol.

**10,11-Dihydrocarbamazepine.** $C_{15}H_{14}N_2O$. ($M_r$ 238.3). *1028900.* [3564-73-6]. 10,11-Dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide.

mp: 205 °C to 210 °C.

**Dihydrocarvone.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1160900.* [7764-50-3]. *p*-Menth-8-en-2-one. 2-Methyl-5-(1-methylethenyl)cyclohexanone.

*Dihydrocarvone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the test for chromatographic profile in the monograph *Caraway oil (1817)*.

*Content* calculated by the normalisation procedure:

- *major component (trans-dihydrocarvone)*: minimum 70 per cent;
- *sum of cis- and trans-dihydrocarvone*: minimum 98 per cent.

**2,5-Dihydroxybenzoic acid.** $C_7H_6O_4$. ($M_r$ 154.1). *1148200.* [490-79-9]. Gentisic acid.

Light yellow crystals.

mp: about 200 °C.

**5,7-Dihydroxy-4-methylcoumarin.** $C_{10}H_8O_4$. ($M_r$ 192.2). *1149400.* [2107-76-8]. 5,7-Dihydroxy-4-methyl-2*H*-1-benzopyran-2-one.

Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: 295 °C to 303 °C.

**Dihydroxynaphthalene.** *1029000.* [132-86-5].

See *1,3-dihydroxynaphthalene R*.

**1,3-Dihydroxynaphthalene.** $C_{10}H_8O_2$. ($M_r$ 160.2). *1029000.* [132-86-5]. Naphthalene-1,3-diol.

Crystalline, generally brownish-violet powder, freely soluble in water and in ethanol (96 per cent).

mp: about 125 °C.

**2,7-Dihydroxynaphthalene.** $C_{10}H_8O_2$. ($M_r$ 160.2). *1029100.* [582-17-2]. Naphthalene-2,7-diol.

Needles, soluble in water and in ethanol (96 per cent).

mp: about 190 °C.

**2,7-Dihydroxynaphthalene solution.** *1029101.*

Dissolve 10 mg of *2,7-dihydroxynaphthalene R* in 100 mL of *sulfuric acid R* and allow to stand until decolorised.

*Storage*: use within 2 days.

**5,7-Diiodoquinolin-8-ol.** $C_9H_5I_2NO$. ($M_r$ 397.0). *1157100.* [83-73-8]. 5,7-Diiodooxine.

Yellowish-brown powder, sparingly soluble in acetone and in ethanol (96 per cent).

*Content*: minimum 95.0 per cent.

**Di-isobutyl ketone.** $C_9H_{18}O$. ($M_r$ 142.2). *1029200.* [108-83-8].

Clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

$n_D^{20}$: about 1.414

bp: about 168 °C.

**Di-isopropyl ether.** $C_6H_{14}O$. ($M_r$ 102.2). *1029300.* [108-20-3].

Clear, colourless liquid, very slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 0.723 to 0.728.

bp: 67 °C to 69 °C.

*Do not distil if the di-isopropyl ether does not comply with the test for peroxides.*

*Peroxides.* Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

*Storage*: protected from light.

***N,N*-Diisopropylethylamine.** $C_8H_{19}N$. ($M_r$ 129.2). *1204600.* [7087-68-5]. *N*-Ethyl-*N*-(propan-2-yl)propan-2-amine. *N*-Ethyldiisopropylamine.

Clear, colourless or light yellow liquid.

bp: 127 °C.

***N,N′*-Diisopropylethylenediamine.** $C_8H_{20}N_2$. ($M_r$ 144.3). *1140600.* [4013-94-9]. *N,N′*-Bis(1-methylethyl)-1,2-ethanediamine.

Colourless or yellowish, corrosive, flammable, hygroscopic liquid.

$d_{20}^{20}$: about 0.798.

$n_D^{20}$: about 1.429.

bp: about 170 °C.

**4,4′-Dimethoxybenzophenone.** $C_{15}H_{14}O_3$. ($M_r$ 242.3). *1126300.* [90-96-0]. Bis(4-methoxyphenyl)methanone.

White or almost white powder, practically insoluble in water and slightly soluble in ethanol (96 per cent).

mp: about 142 °C.

**3,4-Dimethoxy-L-phenylalanine.** $C_{11}H_{15}NO_4$. ($M_r$ 225.2). *1191800.* [32161-30-1]. (2*S*)-2-Amino-3-(3,4-dimethoxyphenyl)propanoic acid.

*Content*: minimum 95 per cent.

White or almost white powder.

**Dimethoxypropane.** $C_5H_{12}O_2$. ($M_r$ 104.1). *1105200.* [77-76-9]. 2,2-Dimethoxypropane.

Colourless liquid, decomposing on exposure to moist air or water.

$d_{20}^{20}$: about 0.847.

$n_D^{20}$: about 1.378.

bp: about 83 °C.

**Dimethylacetamide.** $C_4H_9NO$. ($M_r$ 87.1). *1029700.* [127-19-5]. *N,N*-Dimethylacetamide.

*Content*: minimum 99.5 per cent.

Colourless liquid, miscible with water and with many organic solvents.

$d_{20}^{20}$: about 0.94.

$n_D^{20}$: about 1.437.

bp: about 165 °C.

**Dimethylamine.** $C_2H_7N$. ($M_r$ 45.08). *1168900.* [124-40-3]. *N*-Methylmethanamine.

Colourless, flammable gas.

bp: about 7 °C.

mp: about − 92.2 °C.

**Dimethylamine solution.** *1168901.*

A 400 g/L solution of *dimethylamine R*.

Clear, colourless solution.

Density: about 0.89.

bp: about 54 °C.

mp: about − 37 °C.

**Dimethylaminobenzaldehyde.** $C_9H_{11}NO$. ($M_r$ 149.2). *1029800.* [100-10-7]. 4-Dimethylaminobenzaldehyde.

White or yellowish-white crystals, soluble in ethanol (96 per cent) and in dilute acids.

mp: about 74 °C.

**Dimethylaminobenzaldehyde solution R1.** *1029801.*

Dissolve 0.2 g of *dimethylaminobenzaldehyde R* in 20 mL of *ethanol (96 per cent) R* and add 0.5 mL of *hydrochloric acid R*. Shake the solution with *activated charcoal R* and filter. The colour of the reagent is less intense than that of *iodine solution R3*. Prepare immediately before use.

**Dimethylaminobenzaldehyde solution R2.** *1029802.*

Dissolve 0.2 g of *dimethylaminobenzaldehyde R*, without heating, in a mixture of 4.5 mL of *water R* and 5.5 mL of *hydrochloric acid R*. Prepare immediately before use.

**Dimethylaminobenzaldehyde solution R6.** *1029803.*

Dissolve 0.125 g of *dimethylaminobenzaldehyde R* in a cooled mixture of 35 mL of *water R* and 65 mL of *sulfuric acid R*. Add 0.1 mL of a 50 g/L solution of *ferric chloride R*. Before use allow to stand for 24 h, protected from light.

*Storage*: when stored at room temperature, use within 1 week; when stored in a refrigerator use within several months.

**Dimethylaminobenzaldehyde solution R7.** *1029804.*

Dissolve 1.0 g of *dimethylaminobenzaldehyde R* in 50 mL of *hydrochloric acid R* and add 50 mL of *ethanol (96 per cent) R*.

*Storage*: protected from light; use within 4 weeks.

**Dimethylaminobenzaldehyde solution R8.** *1029805.*

Dissolve 0.25 g of *dimethylaminobenzaldehyde R* in a mixture of 5 g of *phosphoric acid R*, 45 g of *water R* and 50 g of *anhydrous acetic acid R*. Prepare immediately before use.

**Dimethylaminobenzaldehyde solution R9.** *1029806.*

Dissolve 1.0 g of *dimethylaminobenzaldehyde R* in 3.5 mL of perchloric acid (600 g/L $HClO_4$) and slowly add 6.5 mL of *2-propanol R*. Prepare immediately before use.

**4-Dimethylaminocinnamaldehyde.** $C_{11}H_{13}NO$. ($M_r$ 175.2). *1029900.* [6203-18-5]. 3-(4-Dimethylaminophenyl)prop-2-enal.

Orange or orange-brown crystals or powder. Sensitive to light.

mp: about 138 °C.

**4-Dimethylaminocinnamaldehyde solution.** *1029901.*

Dissolve 2 g of *4-dimethylaminocinnamaldehyde R* in a mixture of 100 mL of *hydrochloric acid R1* and 100 mL of *anhydrous ethanol R*. Dilute the solution to four times its volume with *anhydrous ethanol R* immediately before use.

**Dimethylaminoethanol.** $C_4H_{11}NO$. ($M_r$ 89.1). *1195600.* [108-01-0]. 2-(Dimethylamino)ethan-1-ol.

Colourless or slightly yellow liquid, miscible with water.

bp: about 135 °C.

**2-(Dimethylamino)ethyl methacrylate.** $C_8H_{15}NO_2$. ($M_r$ 157.2). *1147200.* [2867-47-2]. 2-(Dimethylamino)ethyl 2-methylpropenoate.

$d_4^{20}$: about 0.930.

bp: about 187 °C.

**Dimethylaminonaphthalenesulfonyl chloride.** $C_{12}H_{12}ClNO_2S$. ($M_r$ 269.8). *1030000.* [605-65-2]. 5-Dimethyl-amino-1-naphthalenesulfonyl chloride.

Yellow, crystalline powder, slightly soluble in water, soluble in methanol.

mp: about 70 °C.

**3-Dimethylaminophenol.** $C_8H_{11}NO$. ($M_r$ 137.2). *1156500.* [99-07-0]. 3-(Dimethylamino)phenol.

Grey powder, slightly soluble in water.

mp: about 80 °C.

**2-(Dimethylamino)thioacetamide hydrochloride.** $C_4H_{11}ClN_2S$. ($M_r$ 154.7). *1181800.* [27366-72-9].

**Dimethylaniline.** *1030100.* [121-69-7].

See *N,N-Dimethylaniline R*.

***N,N*-Dimethylaniline.** $C_8H_{11}N$. ($M_r$ 121.2). *1030100.* [121-69-7].

Clear, oily liquid, almost colourless when freshly distilled, darkening on storage to reddish-brown, practically insoluble in water, freely soluble in ethanol (96 per cent).

$n_D^{20}$: about 1.558.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 192 °C and 194 °C.

**2,3-Dimethylaniline.** $C_8H_{11}N$. ($M_r$ 121.2). *1105300.* [87-59-2]. 2,3-Xylidine.

Yellowish liquid, sparingly soluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: 0.993 to 0.995.

$n_D^{20}$: about 1.569.

bp: about 224 °C.

**2,6-Dimethylaniline.** $C_8H_{11}N$. ($M_r$ 121.2). *1030200.* [87-62-7]. 2,6-Xylidine.

Colourless liquid, sparingly soluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.98.

**2,6-Dimethylaniline hydrochloride.** $C_8H_{12}ClN$. ($M_r$ 157.6). *1169000.* [21436-98-6]. 2,6-Dimethylbenzenamide hydrochloride. 2,6-Xylidine hydrochloride.

*Content*: minimum 98.0 per cent.

**2,4-Dimethyl-6-*tert*-butylphenol.** $C_{12}H_{18}O$. ($M_r$ 178.3). *1126500.* [1879-09-0].

**Dimethyl carbonate.** $C_3H_6O_3$. ($M_r$ 90.1). *1119300.* [616-38-6]. Carbonic acid dimethyl ester.

Liquid, insoluble in water, miscible with ethanol (96 per cent).

$d_4^{17}$: 1.065.

$n_D^{20}$: 1.368.

bp: about 90 °C.

**Dimethyl-β-cyclodextrin.** $C_{56}H_{98}O_{35}$. ($M_r$ 1331). *1169100.* [51166-71-3]. Heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose. Cycloheptakis-(1→4)-(2,6-di-*O*-methyl-α-D-glucopyranosyl). $2^A,2^B,2^C,2^D,2^E,2^F,2^G,6^A,6^B,6^C,6^D,6^E,6^F,6^G$-Tetradeca-*O*-methyl-β-cyclodextrin.

White or almost white powder.

**Dimethyldecylamine.** $C_{12}H_{27}N$. ($M_r$ 185.4). *1113500.* [1120-24-7]. *N,N*-dimethyldecylamine.

*Content*: minimum 98.0 per cent *m/m*.

bp: about 234 °C.

**1,1-Dimethylethylamine.** $C_4H_{11}N$. ($M_r$ 73.1). *1100900.* [75-64-9]. 2-Amino-2-methylpropane. *tert*-Butylamine.

Liquid, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.694.

$n_D^{20}$: about 1.378.

bp: about 46 °C.

**1,1-Dimethylethyl methyl ether.** $C_5H_{12}O$. ($M_r$ 88.1). *1013900.* [1634-04-4]. 2-Methoxy-2-methylpropane. *tert*-Butyl methyl ether.

Colourless, clear, flammable liquid.

$n_D^{20}$: about 1.376.

*Absorbance* (*2.2.25*): maximum 0.30 at 240 nm, 0.10 at 255 nm, 0.01 at 280 nm, determined using *water R* as compensation liquid.

**1,1-Dimethylethyl methyl ether R1.** $C_5H_{12}O$. ($M_r$ 88.1). *1126400.* [1634-04-4]. 2-Methoxy-2-methylpropane. *tert*-Butyl methyl ether.

*Content*: minimum 99.5 per cent.

$d_{20}^{20}$: about 0.741.

$n_D^{20}$: about 1.369.

bp: about 55 °C.

**Dimethylformamide.** $C_3H_7NO$. ($M_r$ 73.1). *1030300.* [68-12-2].

Clear, colourless neutral liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: 0.949 to 0.952.

bp: about 153 °C.

*Water* (*2.5.12*): maximum 0.1 per cent.

**Dimethylformamide diethylacetal.** $C_7H_{17}NO_2$. ($M_r$ 147.2). *1113600.* [1188-33-6]. *N,N*-Dimethylformamide diethylacetal.

$n_D^{20}$: about 1.40.

bp: 128 °C to 130 °C.

***N,N*-Dimethylformamide dimethylacetal.** $C_5H_{13}NO_2$. ($M_r$ 119.2). *1140700.* [4637-24-5]. 1,1-Dimethoxytrimethylamine.

Clear, colourless liquid.

$d_{20}^{20}$: about 0.896.

$n_D^{20}$: about 1.396.

bp: about 103 °C.

**Dimethylglyoxime.** $C_4H_8N_2O_2$. ($M_r$ 116.1). *1030400.* [95-45-4]. 2,3-Butanedione dioxime.

White or almost white, crystalline powder or colourless crystals, practically insoluble in cold water, very slightly soluble in boiling water, soluble in ethanol (96 per cent).

mp: about 240 °C, with decomposition.

*Sulfated ash* (*2.4.14*): maximum 0.05 per cent.

**1,3-Dimethyl-2-imidazolidinone.** $C_5H_{10}N_2O$. ($M_r$ 114.2). *1135400.* [80-73-9]. *N,N′*-Dimethylethylene urea. 1,3-Dimethyl-2-imidazolidone.

$n_D^{20}$: 1.4720.

bp: about 224 °C.

***N,N*-Dimethyloctylamine.** $C_{10}H_{23}N$. ($M_r$ 157.3). *1030500.* [7378-99-6]. Octyldimethylamine.

Colourless liquid.

$d_{20}^{20}$: about 0.765.

$n_D^{20}$: about 1.424.

bp: about 195 °C.

**2,5-Dimethylphenol.** $C_8H_{10}O$. ($M_r$ 122.2). *1162300.* [95-87-4]. *p*-Xylenol.

White or almost white crystals.

**2,6-Dimethylphenol.** $C_8H_{10}O$. ($M_r$ 122.2). *1030600.* [576-26-1].

Colourless needles, slightly soluble in water, very soluble in ethanol (96 per cent).

bp: about 203 °C.

mp: 46 °C to 48 °C.

**3,4-Dimethylphenol.** $C_8H_{10}O$. ($M_r$ 122.2). *1098100.* [95-65-8].

White or almost white crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

bp: about 226 °C.

mp: 25 °C to 27 °C.

***N,N*-Dimethyl-L-phenylalanine.** $C_{11}H_{15}NO_2$. ($M_r$ 193.2). *1164000.* [17469-89-5]. (2*S*)-2-(Dimethylamino)-3-phenylpropanoic acid.

mp: about 226 °C.

**Dimethylpiperazine.** $C_6H_{14}N_2$. ($M_r$ 114.2). *1030700.* [106-58-1]. 1,4-Dimethylpiperazine.

A colourless liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.85.

$n_D^{20}$: about 1.446.

bp: about 131 °C.

**Dimethylstearamide.** $C_{20}H_{41}NO$. ($M_r$ 311.6). *1030800.* *N,N*-Dimethylstearamide.

White or almost white solid mass, soluble in many organic solvents, including acetone.

mp: about 51 °C.

**Dimethylstearylamide.** *1030800.*

See *dimethylstearamide R*.

**Dimethyl sulfone.** $C_2H_6O_2S$. ($M_r$ 94.1). *1030900.* [67-71-0].

White or almost white, crystalline powder, freely soluble in water, soluble in acetone and ethanol (96 per cent).

mp: 108 °C to 110 °C.

**Dimethyl sulfoxide.** *1029500.* [67-68-5].

See *Dimethyl sulfoxide (0763)*.

*Dimethyl sulfoxide used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 1.00 at 262 nm, 0.46 at 270 nm, 0.16 at 290 nm, 0.01 at 340 nm and higher wavelengths, determined using *water R* as compensation liquid.

**Dimethyl sulfoxide R1.** *1029501.*

*Content*: minimum 99.7 per cent, determined by gas chromatography.

**Dimethyl sulfoxide R2.** *1029502.*

*Content*: minimum 99.9 per cent, determined by gas chromatography.

*Residue on evaporation*: maximum 0.0005 per cent.

*Water (2.5.32)*: maximum 0.005 per cent.

**Dimeticone.** *1105400.* [9006-65-9].

See *Dimeticone (0138)*.

**Dimidium bromide.** $C_{20}H_{18}BrN_3$. ($M_r$ 380.3). *1031100.* [518-67-2]. 3,8-Diamino-5-methyl-6-phenylphenanthridinium bromide.

Dark-red crystals, slightly soluble in water at 20 °C, sparingly soluble in water at 60 °C and in ethanol (96 per cent).

**Dimidium bromide-sulfan blue mixed solution.** *1031101.*

Dissolve separately 0.5 g of *dimidium bromide R* and 0.25 g of *sulfan blue R* in 30 mL of a hot mixture of 1 volume of *anhydrous ethanol R* and 9 volumes of *water R*, stir, mix the two solutions, and dilute to 250 mL with the same mixture of solvents. Mix 20 mL of this solution with 20 mL of a 14.0 per cent *V/V* solution of *sulfuric acid R* previously diluted with about 250 mL of *water R* and dilute to 500 mL with *water R*.

*Storage*: protected from light.

**Dinitrobenzene.** $C_6H_4N_2O_4$. ($M_r$ 168.1). *1031200.* [99-65-0]. 1,3-Dinitrobenzene.

Yellowish crystalline powder or crystals, practically insoluble in water, slightly soluble in ethanol (96 per cent).

mp: about 90 °C.

**Dinitrobenzene solution.** *1031201.*

A 10 g/L solution of *dinitrobenzene R* in *ethanol (96 per cent) R*.

**Dinitrobenzoic acid.** $C_7H_4N_2O_6$. ($M_r$ 212.1). *1031300.* [99-34-3]. 3,5-Dinitrobenzoic acid.

Almost colourless crystals, slightly soluble in water, very soluble in ethanol (96 per cent).

mp: about 206 °C.

**Dinitrobenzoic acid solution.** *1031301.*

A 20 g/L solution of *dinitrobenzoic acid R* in *ethanol (96 per cent) R*.

**Dinitrobenzoyl chloride.** $C_7H_3ClN_2O_5$. ($M_r$ 230.6). *1031400.* [99-33-2]. 3,5-Dinitrobenzoyl chloride.

Translucent, yellow or greenish-yellow powder or yellowish crystals, soluble in acetone and in toluene.

mp: about 68 °C.

*Suitability test.* To 1 mL of *anhydrous ethanol R* and 0.1 g of *dinitrobenzoyl chloride R* add 0.05 mL of *dilute sulfuric acid R* and boil under a reflux condenser for 30 min. After evaporation on a water-bath add 5 mL of *heptane R* to the residue and heat to boiling. Filter the hot solution. Wash the crystals formed on cooling to room temperature with a small quantity of *heptane R* and dry in a desiccator. The crystals melt (*2.2.14*) at 94 °C to 95 °C.

**Dinitrophenylhydrazine.** $C_6H_6N_4O_4$. ($M_r$ 198.1). *1031500.* [119-26-6]. 2,4-Dinitrophenylhydrazine.

Reddish-orange crystals, very slightly soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 203 °C (instantaneous method).

**Dinitrophenylhydrazine-aceto-hydrochloric solution.** *1031501.*

Dissolve 0.2 g of *dinitrophenylhydrazine R* in 20 mL of *methanol R* and add 80 mL of a mixture of equal volumes of *acetic acid R* and *hydrochloric acid R1*. Prepare immediately before use.

**Dinitrophenylhydrazine-hydrochloric solution.** *1031502.*

Dissolve by heating 0.50 g of *dinitrophenylhydrazine R* in *dilute hydrochloric acid R* and dilute to 100 mL with the same solvent. Allow to cool and filter. Prepare immediately before use.

**Dinitrophenylhydrazine-sulfuric acid solution.** *1031503.*

Dissolve 1.5 g of *dinitrophenylhydrazine R* in 50 mL of a 20 per cent *V/V* solution of *sulfuric acid R*. Prepare immediately before use.

**Dinonyl phthalate.** $C_{26}H_{42}O_4$. ($M_r$ 418.6). *1031600.* [28553-12-0].

Colourless to pale yellow, viscous liquid.

$d_{20}^{20}$: 0.97 to 0.98.

$n_D^{20}$: 1.482 to 1.489.

*Acidity*. Shake 5.0 g with 25 mL of *water R* for 1 min. Allow to stand, filter the separated aqueous layer and add 0.1 mL of *phenolphthalein solution R*. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour of the solution (0.05 per cent, calculated as phthalic acid).

*Water* (*2.5.12*): maximum 0.1 per cent.

**Dioctadecyl disulfide.** $C_{36}H_{74}S_2$. ($M_r$ 571.1). *1031700.* [2500-88-1].

White or almost white powder, practically insoluble in water.

mp: 53 °C to 58 °C.

**2,2′-Di(octadecyloxy)-5,5′-spirobi(1,3,2-dioxaphosphorinane).** $C_{41}H_{82}O_6P_2$. ($M_r$ 733). *1031800.*

White or almost white, waxy solid, practically insoluble in water, soluble in hydrocarbons.

mp: 40 °C to 70 °C.

**Dioctadecyl 3,3′-thiodipropionate.** $C_{42}H_{82}O_4S$. ($M_r$ 683). *1031900.* [693-36-7].

White or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, in ethanol (96 per cent) and in light petroleum.

mp: 58 °C to 67 °C.

**Di-*n*-octyl phthalate.** $C_{24}H_{38}O_4$. ($M_r$ 390.6). *1203500.* [117-84-0]. Dioctyl benzene-1,2-dicarboxylate.

Colourless viscous liquid, insoluble in water.

*Density*: about 0.98 g/mL (20 °C).

**Dioxan.** $C_4H_8O_2$. ($M_r$ 88.1). *1032000.* [123-91-1]. 1,4-Dioxane.

Clear, colourless liquid, miscible with water and with most organic solvents.

$d_{20}^{20}$: about 1.03.

*Freezing point* (*2.2.18*): minimum 11.0 °C.

*Water* (*2.5.12*): maximum 0.5 per cent.

*Do not distil if the dioxan does not comply with the test for peroxides.*

*Peroxides*. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

*Dioxan used for liquid scintillation is of a suitable analytical grade.*

**Dioxan solution.** *1032002.*

Dilute 50.0 mL of *dioxan stock solution R* to 100.0 mL with *water R*. (0.5 mg/mL of dioxan).

**Dioxan solution R1.** *1032003.*

Dilute 10.0 mL of *dioxan solution R* to 50.0 mL with *water R*. (0.1 mg/mL of dioxan).

**Dioxan solution R2.** *1032004.*

Dilute 2.0 mL of *dioxan solution R* to 50.0 mL with *water R* (0.02 mg/mL of dioxan).

**Dioxan stock solution.** *1032001.*

Dissolve 1.00 g of *dioxan R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with *water R* (1.0 mg/mL).

**Diphenylamine.** $C_{12}H_{11}N$. ($M_r$ 169.2). *1032100.* [122-39-4].

White or almost white crystals, slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 55 °C.

*Storage*: protected from light.

**Diphenylamine solution.** *1032101.*

A 1 g/L solution of *diphenylamine R* in *sulfuric acid R*.

*Storage*: protected from light.

**Diphenylamine solution R1.** *1032102.*

A 10 g/L solution of *diphenylamine R* in *sulfuric acid R*. The solution is colourless.

**Diphenylamine solution R2.** *1032103.*

Dissolve 1 g of *diphenylamine R* in 100 mL of *glacial acetic acid R* and add 2.75 mL of *sulfuric acid R*. Use immediately.

**Diphenylanthracene.** $C_{26}H_{18}$. ($M_r$ 330.4). *1032200.* [1499-10-1]. 9,10-Diphenylanthracene.

Yellowish or yellow, crystalline powder, practically insoluble in water.

mp: about 248 °C.

**Diphenylbenzidine.** $C_{24}H_{20}N_2$. ($M_r$ 336.4). *1032300.* [531-91-9]. *N,N'*-Diphenylbenzidine. *N,N'*-Diphenylbiphenyl-4,4′-diamine.

White or faintly grey, crystalline powder, practically insoluble in water, slightly soluble in acetone and in ethanol (96 per cent).

mp: about 248 °C.

*Nitrates*. Dissolve 8 mg in a cooled mixture of 5 mL of *water R* and 45 mL of *nitrogen-free sulfuric acid R*. The solution is colourless or very pale blue.

*Sulfated ash* (*2.4.14*): maximum 0.1 per cent.

*Storage*: protected from light.

**Diphenylboric acid aminoethyl ester.** $C_{14}H_{16}BNO$. ($M_r$ 225.1). *1032400.* [524-95-8].

White or slightly yellow, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 193 °C.

**Diphenylcarbazide.** $C_{13}H_{14}N_4O$. ($M_r$ 242.3). *1032500.* [140-22-7]. 1,5-Diphenylcarbonodihydrazide.

White or almost white, crystalline powder which gradually becomes pink on exposure to air, very slightly soluble in water, soluble in acetone, in ethanol (96 per cent) and in glacial acetic acid.

mp: about 170 °C.

*Sulfated ash* (*2.4.14*): maximum 0.1 per cent.

*Storage*: protected from light.

**Diphenylcarbazide solution.** *1032501.*

Dissolve 0.2 g of *diphenylcarbazide R* in 10 mL of *glacial acetic acid R* and dilute to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

**Diphenylcarbazone.** $C_{13}H_{12}N_4O$. ($M_r$ 240.3). *1032600.* [538-62-5]. 1,5-Diphenylcarbazone.

Orange-yellow, crystalline powder, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 157 °C, with decomposition.

**Diphenylcarbazone mercuric reagent.** *1032601.*

*Solution A*. Dissolve 0.1 g of *diphenylcarbazone R* in *anhydrous ethanol R* and dilute to 50 mL with the same solvent.

*Solution B*. Dissolve 1 g of *mercuric chloride R* in *anhydrous ethanol R* and dilute to 50 mL with the same solvent.

Mix equal volumes of the two solutions.

**2,2-Diphenylglycine.** $C_{14}H_{13}NO_2$. ($M_r$ 227.26). *1174300.* [3060-50-2]. Amino(diphenyl)acetic acid.

**1,2-Diphenylhydrazine.** $C_{12}H_{12}N_2$. ($M_r$ 184.3). *1140800.* [122-66-7]. Hydrazobenzene. 1,2-Diphenyldiazane.

Orange powder.

mp: about 125 °C.

**Diphenylmethanol.** $C_{13}H_{12}O$. ($M_r$ 184.2). *1145700.* [91-01-0]. Benzhydrol.

White or almost white, crystalline powder.

mp: about 66 °C.

**Diphenyloxazole.** $C_{15}H_{11}NO$. ($M_r$ 221.3). *1032700.* [92-71-7]. 2,5-Diphenyloxazole.

White or almost white powder, practically insoluble in water, soluble in methanol, sparingly soluble in dioxan and in glacial acetic acid.

mp: about 70 °C.

$A_{1\ cm}^{1\%}$: about 1260 determined at 305 nm in *methanol R*.

*Diphenyloxazole used for liquid scintillation is of a suitable analytical grade.*

**Diphenylphenylene oxide polymer.** *1032800.* 2,6-Diphenyl-*p*-phenylene oxide polymer.

White or almost white, porous beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

**Diphosphorus pentoxide.** $P_2O_5$. ($M_r$ 141.9). *1032900.* [1314-56-3]. Phosphorus pentoxide. Phosphoric anhydride.

White or almost white powder, amorphous, deliquescent. It is hydrated by water with the evolution of heat.

*Storage*: in an airtight container.

**Dipotassium hydrogen phosphate.** $K_2HPO_4$. ($M_r$ 174.2). *1033000.* [7758-11-4].

White or almost white, crystalline powder, hygroscopic, very soluble in water, slightly soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Dipotassium hydrogen phosphate trihydrate.** $K_2HPO_4,3H_2O$. ($M_r$ 228.2). *1157600.* [16788-57-1].

Colourless or white or almost white powder or crystals, freely soluble in water.

**Dipotassium sulfate.** $K_2SO_4$. ($M_r$ 174.3). *1033100.* [7778-80-5].

Colourless crystals, soluble in water.

**2,2′-Dipyridylamine.** $C_{10}H_9N_3$. ($M_r$ 171.2). *1157700.* [1202-34-2]. *N*-(Pyridin-2-yl)pyridin-2-amine.

mp: about 95 °C.

**Disodium arsenate.** $Na_2HAsO_4,7H_2O$. ($M_r$ 312.0). *1102500.* [10048-95-0]. Disodium hydrogen arsenate heptahydrate. Dibasic sodium arsenate.

Crystals, efflorescent in warm air, freely soluble in water, soluble in glycerol, slightly soluble in ethanol (96 per cent). The aqueous solution is alcaline to litmus.

$d_{20}^{20}$: about 1.87.

mp: about 57 °C when rapidly heated.

**Disodium bicinchoninate.** $C_{20}H_{10}N_2Na_2O_4$. ($M_r$ 388.3). *1126600.* [979-88-4]. Disodium 2,2′-biquinoline-4-4′-dicarboxylate.

**Disodium hydrogen citrate.** $C_6H_6Na_2O_7,1^1/_2H_2O$. ($M_r$ 263.1). *1033200.* [144-33-2]. Sodium acid citrate. Disodium hydrogen 2-hydroxypropane-1,2,3-tricarboxylate sesquihydrate.

White or almost white powder, soluble in less than 2 parts of water, practically insoluble in ethanol (96 per cent).

**Disodium hydrogen phosphate, anhydrous.** $Na_2HPO_4$. ($M_r$ 142.0). *1033400.* [7558-79-4].

**Disodium hydrogen phosphate dihydrate.** *1033500.* [10028-24-7].

See *Disodium phosphate dihydrate (0602)*.

**Disodium hydrogen phosphate dodecahydrate.** *1033300.* [10039-32-4].

See *Disodium phosphate dodecahydrate (0118)*.

**Disodium hydrogen phosphate solution.** *1033301.*

A 90 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.

**Disodium hydrogen phosphate heptahydrate.** $Na_2HPO_4,7H_2O$. ($M_r$ 268.1). *1206900.* [7782-85-6].

**Disodium tetraborate.** *1033600.* [1303-96-4].

See *Borax (0013)*.

**Borate solution.** *1033601.*

Dissolve 9.55 g of *disodium tetraborate R* in *sulfuric acid R*, heating on a water-bath, and dilute to 1 L with the same acid.

**Ditalimphos.** $C_{12}H_{14}NO_4PS$. ($M_r$ 299.3). *1126700.* [5131-24-8]. *O*,*O*-Diethyl (1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)phosphonothioate.

Very slightly soluble in water, in ethyl acetate and in anhydrous ethanol.

A suitable certified reference solution may be used.

**5,5′-Dithiobis(2-nitrobenzoic acid).** $C_{14}H_8N_2O_8S_2$. ($M_r$ 396.4). *1097300.* [69-78-3]. 3-Carboxy-4-nitrophenyldisulfide. Ellman's reagent. DTNB.

Yellow powder sparingly soluble in ethanol (96 per cent).

mp: about 242 °C.

**Dithioerythritol.** $C_4H_{10}O_2S_2$. ($M_r$ 154.3). *1187500.* [6892-68-8].

(2*R*,3*S*)-1,4-Disulfanylbutane-2,3-diol. DTE.

mp: about 83 °C.

**Dithiol.** $C_7H_8S_2$. ($M_r$ 156.3). *1033800.* [496-74-2]. Toluene-3,4-dithiol. 4-Methylbenzene-1,2-dithiol.

White or almost white crystals, hygroscopic, soluble in methanol and in solutions of alkali hydroxides.

mp: about 30 °C.

*Storage*: in an airtight container.

**Dithiol reagent.** *1033801.*

To 1 g of *dithiol R* add 2 mL of *thioglycollic acid R* and dilute to 250 mL with a 20 g/L solution of *sodium hydroxide R*. Prepare immediately before use.

**Dithiothreitol.** $C_4H_{10}O_2S_2$. ($M_r$ 154.2). *1098200.* [27565-41-9]. *threo*-1,4-Dimercaptobutane-2,3-diol.

Slightly hygroscopic needles, freely soluble in water, in acetone and in anhydrous ethanol.

*Storage*: in an airtight container.

**Dithizone.** $C_{13}H_{12}N_4S$. ($M_r$ 256.3). *1033900.* [60-10-6]. 1,5-Diphenylthiocarbazone.

A bluish-black, brownish-black or black powder, practically insoluble in water, soluble in ethanol (96 per cent).

*Storage*: protected from light.

**Dithizone solution.** *1033901.*

A 0.5 g/L solution of *dithizone R* in *chloroform R*. Prepare immediately before use.

**Dithizone solution R2.** *1033903.*

Dissolve 40.0 mg of *dithizone R* in *chloroform R* and dilute to 1000.0 mL with the same solvent. Dilute 30.0 mL of the solution to 100.0 mL with *chloroform R*.

*Assay*. Dissolve a quantity of *mercuric chloride R* equivalent to 0.1354 g of $HgCl_2$ in a mixture of equal volumes of *dilute sulfuric acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 2.0 mL of this solution to 100.0 mL with a mixture of equal volumes of *dilute sulfuric acid R* and *water R*. (This solution contains 20 ppm of Hg). Transfer 1.0 mL of the solution to a separating funnel and add 50 mL of *dilute sulfuric acid R*, 140 mL of *water R* and 10 mL of a 200 g/L solution of *hydroxylamine hydrochloride R*. Titrate with the dithizone solution; after each addition, shake the mixture twenty times and towards the end of the titration allow to separate and discard the chloroform layer. Titrate until a bluish-green colour is obtained. Calculate the equivalent in micrograms of mercury per millilitre of the dithizone solution from the expression $20/V$, where $V$ is the volume in millilitres of the dithizone solution used in the titration.

**Dithizone R1.** $C_{13}H_{12}N_4S$. ($M_r$ 256.3). *1105500.* [60-10-6]. 1,5-Diphenylthiocarbazone.

*Content*: minimum 98.0 per cent.

Bluish-black, brownish-black or black powder, practically insoluble in water, soluble in ethanol (96 per cent).

*Storage*: protected from light.

**Divanadium pentoxide.** $V_2O_5$. ($M_r$ 181.9). *1034000.* [1314-62-1]. Vanadic anhydride.

*Content*: minimum 98.5 per cent.

Yellow-brown or rust-brown powder, slightly soluble in water, soluble in strong mineral acids and in solutions of alkali hydroxides with formation of salts.

*Appearance of solution.* Heat 1 g for 30 min with 10 mL of *sulfuric acid R*. Allow to cool and dilute to 10 mL with the same acid. The solution is clear (*2.2.1*).

*Sensitivity to hydrogen peroxide.* Dilute 1.0 mL of the solution prepared for the test for appearance of solution cautiously to 50.0 mL with *water R*. To 0.5 mL of the solution add 0.1 mL of a solution of hydrogen peroxide (0.1 g/L of $H_2O_2$) prepared from *dilute hydrogen peroxide solution R*. The solution has a distinct orange colour compared with a blank prepared from 0.5 mL of the solution to be examined and 0.1 mL of *water R*. After the addition of 0.4 mL of a solution of hydrogen peroxide (0.1 g/L of $H_2O_2$) prepared from *dilute hydrogen peroxide solution R*, the orange solution becomes orange-yellow.

*Loss on ignition*: maximum 1.0 per cent, determined on 1.00 g at 700 ± 50 °C.

*Assay.* Dissolve 0.200 g with heating in 20 mL of a 70 per cent *m/m* solution of *sulfuric acid R*. Add 100 mL of *water R* and *0.02 M potassium permanganate* until a reddish colour is obtained. Decolorise the excess of potassium permanganate by the addition of a 30 g/L solution of *sodium nitrite R*. Add 5 g of *urea R* and 80 mL of a 70 per cent *m/m* solution of *sulfuric acid R*. Cool. Using 0.1 mL of *ferroin R* as indicator, titrate the solution immediately with *0.1 M ferrous sulfate* until a greenish-red colour is obtained.

1 mL of *0.1 M ferrous sulfate* is equivalent to 9.095 mg of $V_2O_5$.

**Divanadium pentoxide solution in sulfuric acid.** *1034001.*

Dissolve 0.2 g of *divanadium pentoxide R* in 4 mL of *sulfuric acid R* and dilute to 100 mL with *water R*.

**Docosahexaenoic acid methyl ester.** $C_{23}H_{34}O_2$. ($M_r$ 342.5). *1142800.* [301-01-9]. DHA methyl ester. Cervonic acid methyl ester. (all-*Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid methyl ester.

*Content*: minimum 90.0 per cent, determined by gas chromatography.

**Docusate sodium.** *1034100.* [577-11-7].

See *Docusate sodium (1418)*.

**Dodecyltrimethylammonium bromide.** $C_{15}H_{34}BrN$. ($M_r$ 308.4). *1135500.* [1119-94-4]. *N,N,N*-Trimethyldodecan-1-aminium bromide.

White or almost white crystals.

mp: about 246 °C.

**D-Dopa.** $C_9H_{11}NO_4$. ($M_r$ 197.2). *1164100.* [5796-17-8]. (2*R*)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid. 3-Hydroxy-D-tyrosine. 3,4-Dihydroxy-D-phenylalanine.

$[\alpha]_D^{20}$: + 9.5 to + 11.5, determined on a 10 g/L solution in *1 M hydrochloric acid*.

mp: about 277 °C.

**Dotriacontane.** $C_{32}H_{66}$. ($M_r$ 450.9). *1034200.* [544-85-4]. *n*-Dotriacontane.

White or almost white plates, practically insoluble in water, sparingly soluble in hexane.

mp: about 69 °C.

*Impurities.* Not more than 0.1 per cent of impurities with the same $t_R$ value as α-tocopherol acetate, determined by the gas chromatographic method prescribed in the monograph *α-Tocopherol acetate (0439)*.

**Doxycycline.** *1145800.*

See *Doxycycline monohydrate (0820)*.

**β-Ecdysterone.** $C_{27}H_{44}O_7$. ($M_r$ 480.6). *1204700.* [5289-74-7]. (2β,3β,5β,22*R*)-2,3,14,20,22,25-Hexahydroxycholest-7-en-6-one.

**Echinacoside.** $C_{35}H_{46}O_{20}$. ($M_r$ 786.5). *1159400.* [82854-37-3]. β-(3′,4′-Dihydroxyphenyl)-ethyl-*O*-α-L-rhamnopyranosyl (1→3)-*O*-β-D-[β-D-glucopyranosyl(1→6)]-(4-*O*-caffeoyl)-glucopyranoside.

Pale yellow powder, odourless.

**Edotreotide.** $C_{65}H_{92}N_{14}O_{18}S_2$. ($M_r$ 1422). *1182400.* [204318-14-9]. *N*-[[4,7,10-Tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl]-D-phenylalanyl-L-cysteinyl-

L-tyrosyl-D-tryptophyl-L-lysyl-L-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide. DOTATOC. DOTA-[Tyr$^3$]-octreotide.

White or almost white powder.

*Content*: minimum 95.0 per cent.

**Electrolyte reagent for the micro determination of water.** *1113700.*

Commercially available anhydrous reagent or a combination of anhydrous reagents for the coulometric titration of water, containing suitable organic bases, sulfur dioxide and iodide dissolved in a suitable solvent.

**Elementary standard solution for atomic spectrometry (1.000 g/L).** *5004000.*

This solution is prepared, generally in acid conditions, from the element or a salt of the element whose minimum content is not less than 99.0 per cent. The quantity per litre of solution is greater than 0.995 g throughout the guaranteed period, as long as the vial has not been opened. The starting material (element or salt) and the characteristics of the final solvent (nature and acidity, etc.) are mentioned on the label.

**Emodin.** $C_{15}H_{10}O_5$. ($M_r$ 270.2). *1034400.* [518-82-1].
1,3,8-Trihydroxy-6-methylanthraquinone.

Orange-red needles, practically insoluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Rhubarb (0291)*; the chromatogram shows only one principal spot.

**Endoprotease LysC.** *1173200.*

Microbial extracellular proteolytic enzyme secreted by *Achromobacter lyticus*. A lyophilised powder, free of salts.

**α-Endosulfan.** $C_9H_6Cl_6O_3S$. ($M_r$ 406.9). *1126800.* [959-98-8].

bp: about 200 °C.

mp: about 108 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**β-Endosulfan.** $C_9H_6Cl_6O_3S$. ($M_r$ 406.9). *1126900.* [33213-65-9].

bp: about 390 °C.

mp: about 207 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Endrin.** $C_{12}H_8Cl_6O$. ($M_r$ 380.9). *1127000.* [72-20-8].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**(−)-Epicatechin.** $C_{15}H_{14}O_6$. ($M_r$ 290.3). *1201300.* [490-46-0].
(2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3,5,7-triol.

**(−)-Epigallocatechin-3-*O*-gallate.** $C_{22}H_{18}O_{11}$. ($M_r$ 458.4). *1201400.* [989-51-5]. (2*R*,3*R*)-5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate.

**Epilactose.** $C_{12}H_{22}O_{11}$. ($M_r$ 342.3). *1189200.* [20869-27-6].
4-*O*-β-D-Galactopyranosyl-D-mannopyranose.

*Content*: minimum 98 per cent.

**Erucamide.** $C_{22}H_{43}NO$. ($M_r$ 337.6). *1034500.* [112-84-5].
(*Z*)-Docos-13-enoamide.

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

mp: about 70 °C.

**Erythritol.** *1113800.* [149-32-6].

See *Erythritol (1803)*.

**Esculetin.** $C_9H_6O_4$. ($M_r$ 178.1). *1185800.* [305-01-1].
6,7-Dihydroxy-2*H*-1-benzopyran-2-one. Aesculetin.

**Esculin.** $C_{15}H_{16}O_9,1^1/_2H_2O$. ($M_r$ 367.3). *1119400.* [531-75-9].
6-(β-D-Glucopyranosyloxy)-7-hydroxy-2*H*-chromen-2-one.

White or almost white powder or colourless crystals, sparingly soluble in water and in ethanol (96 per cent), freely soluble in hot water and in hot ethanol (96 per cent).

*Chromatography* (*2.2.27*). Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Eleutherococcus (1419)*; the chromatogram shows only one principal spot.

**Estradiol.** $C_{18}H_{24}O_2$. ($M_r$ 272.4). *1135600.* [50-28-2].
Estra-1,3,5(10)-triene-3,17β-diol. β-Estradiol.

Prisms stable in air, practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in acetone and in dioxan, sparingly soluble in vegetable oils.

mp: 173 °C to 179 °C.

**17α-Estradiol.** $C_{18}H_{24}O_2$. ($M_r$ 272.4). *1034600.* [57-91-0].

White or almost white, crystalline powder or colourless crystals.

mp: 220 °C to 223 °C.

**Estragole.** $C_{10}H_{12}O$. ($M_r$ 148.2). *1034700.* [140-67-0].
1-Methoxy-4-prop-2-enylbenzene.

Liquid, miscible with ethanol (96 per cent).

$n_D^{20}$: about 1.52.

bp: about 216 °C.

*Estragole used in gas chromatography complies with the following test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

*Test solution*. The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Ethane.** $C_2H_6$. ($M_r$ 30.07). *1189300.* [74-84-0].

*Content*: minimum 99.0 per cent *V/V*.

**Ethanol.** *1034800.* [64-17-5].

See *Ethanol, anhydrous R*.

**Ethanol, anhydrous.** *1034800.* [64-17-5].

See *Ethanol, anhydrous (1318)*.

**Ethanol R1.** *1034801.*

Complies with the requirements prescribed for the monograph *Ethanol, anhydrous (1318)* with the following additional requirement.

*Methanol*. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Reference solution*. Dilute 0.50 mL of *anhydrous methanol R* to 100.0 mL with the substance to be examined. Dilute 1.0 mL of this solution to 100.0 mL with the substance to be examined.

*Column*:

– *material*: glass;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (75-100 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 130 °C;
– *injection port*: 150 °C;
– *detector*: 200 °C.

*Detection*: flame-ionisation.

*Injection*: 1 µL of the test solution and 1 µL of the reference solution, alternately, three times.

After each chromatography, heat the column to 230 °C for 8 min. Integrate the methanol peak. Calculate the percentage methanol content from the following expression:

$$\frac{a \times b}{c - b}$$

$a$ = percentage *V/V* content of methanol in the reference solution,

$b$ = area of the methanol peak in the chromatogram obtained with the test solution,

$c$ = area of the methanol peak in the chromatogram obtained with the reference solution.

*Limit*:

– *methanol*: maximum 0.005 per cent *V/V*.

**Ethanol (96 per cent).** *1002500.* [64-17-5].

See *Ethanol (96 per cent) (1317)*.

**Ethanol (*x* per cent *V/V*).** *1002502.*

Mix appropriate volumes of *water R* and *ethanol (96 per cent) R*, allowing for the effects of warming and volume contraction inherent to the preparation of such a mixture, to obtain a solution whose final content of ethanol corresponds to the value of *x*.

**Ethanolamine.** $C_2H_7NO$. ($M_r$ 61.1). *1034900.* [141-43-5]. 2-Aminoethanol.

Clear, colourless, viscous, hygroscopic liquid, miscible with water and with methanol.

$d_{20}^{20}$: about 1.014.

$n_D^{20}$: about 1.454.

mp: about 11 °C.

*Storage*: in an airtight container.

**Ether.** $C_4H_{10}O$. ($M_r$ 74.1). *1035000.* [60-29-7].

Clear, colourless, volatile and very mobile liquid, very flammable, hygroscopic, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 0.713 to 0.715.

bp: 34 °C to 35 °C.

*Do not distil if the ether does not comply with the test for peroxides.*

*Peroxides.* Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

The name and concentration of any added stabilisers are stated on the label.

*Storage*: in an airtight container, protected from light, at a temperature not exceeding 15 °C.

**Ether, peroxide-free.** *1035100.*

See *Anaesthetic ether (0367)*.

**Ethion.** $C_9H_{22}O_4P_2S_4$. ($M_r$ 384.5). *1127100.* [563-12-2].

mp: – 24 °C to – 25 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Ethoxychrysoidine hydrochloride.** $C_{14}H_{17}ClN_4O$. ($M_r$ 292.8). *1035200.* [2313-87-3]. 4-[(4-Ethoxyphenyl)diazenyl]phenylene-1,3-diamine hydrochloride.

Reddish powder, soluble in ethanol (96 per cent).

**Ethoxychrysoidine solution.** *1035201.*

A 1 g/L solution of *ethoxychrysoidine hydrochloride R* in *ethanol (96 per cent) R*.

*Test for sensitivity.* To a mixture of 5 mL of *dilute hydrochloric acid R* and 0.05 mL of the ethoxy-chrysoidine solution add 0.05 mL of *0.0167 M bromide-bromate*. The colour changes from red to light yellow within 2 min.

**Ethyl acetate.** $C_4H_8O_2$. ($M_r$ 88.1). *1035300.* [141-78-6].

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 0.901 to 0.904.

bp: 76 °C to 78 °C.

**Ethyl acetate, treated.** *1035301.*

Disperse 200 g of *sulfamic acid R* in *ethyl acetate R* and make up to 1000 mL with the same solvent. Stir the suspension obtained for three days and filter through a filter paper.

*Storage*: use within 1 month.

**Ethyl acrylate.** $C_5H_8O_2$. ($M_r$ 100.1). *1035400.* [140-88-5]. Ethyl prop-2-enoate.

Colourless liquid.

$d_{20}^{20}$: about 0.924.

$n_D^{20}$: about 1.406.

bp: about 99 °C.

mp: about – 71 °C.

**4-[(Ethylamino)methyl]pyridine.** $C_8H_{12}N_2$. ($M_r$ 136.2). *1101300.* [33403-97-3].

Pale yellow liquid.

$d_{20}^{20}$: about 0.98.

$n_D^{20}$: about 1.516.

bp: about 98 °C.

**Ethylbenzene.** $C_8H_{10}$. ($M_r$ 106.2). *1035800.* [100-41-4].

*Content*: minimum 99.5 per cent *m/m*, determined by gas chromatography.

Clear, colourless liquid, practically insoluble in water, soluble in acetone, and in ethanol (96 per cent).

$d_{20}^{20}$: about 0.87.

$n_D^{20}$: about 1.496.

bp: about 135 °C.

**Ethyl benzenesulfonate.** $C_8H_{10}O_3S$. ($M_r$ 186.2). *1194800.* [515-46-8].

*Content*: minimum 97.0 per cent.

Colourless or slightly yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

*Density*: about 1.22 g/mL (25 °C).

**Ethyl benzoate.** $C_9H_{10}O_2$. ($M_r$ 150.2). *1135700.* [93-89-0].

A clear, colourless, refractive liquid, practically insoluble in water, miscible with ethanol (96 per cent) and with light petroleum.

$d_4^{25}$: about 1.050.

$n_D^{20}$: about 1.506.

bp: 211 °C to 213 °C.

**Ethyl 5-bromovalerate.** $C_7H_{13}BrO_2$. ($M_r$ 209.1). *1142900.* [14660-52-7]. Ethyl 5-bromopentanoate.

Clear, colourless liquid.

$d_{20}^{20}$: about 1.321.

bp: 104 °C to 109 °C.

**Ethyl clorazepate.** $C_{18}H_{15}ClN_2O_3$. ($M_r$ 342.8). *1204800.* [5606-55-3]. Ethyl (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine-3-carboxylate.

**Ethyl cyanoacetate.** $C_5H_7NO_2$. ($M_r$ 113.1). *1035500.* [105-56-6].

Colourless or pale yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

bp: 205 °C to 209 °C, with decomposition.

**Ethylene chloride.** $C_2H_4Cl_2$. ($M_r$ 99.0). *1036000.* [107-06-2]. 1,2-Dichloroethane.

Clear, colourless liquid, soluble in about 120 parts of water and in 2 parts of ethanol (96 per cent).

$d_{20}^{20}$: about 1.25.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 82 °C and 84 °C.

**Ethylenediamine.** $C_2H_8N_2$. ($M_r$ 60.1). *1036500.* [107-15-3]. Ethane-1,2-diamine.

Clear, colourless, fuming liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

bp: about 116 °C.

**Ethylene bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate].** *1035900.* [32509-66-3].

See *ethylene bis[3,3-di(3-(1,1-dimethylethyl)-4-hydroxyphenyl)butyrate] R*.

**Ethylene bis[3,3-di(3-(1,1-dimethylethyl)-4-hydroxyphenyl)butyrate].** $C_{50}H_{66}O_8$. ($M_r$ 795). *1035900.* [32509-66-3]. Ethylene bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate].

Crystalline powder, practically insoluble in water and in light petroleum, very soluble in acetone and in methanol.

mp: about 165 °C.

**(Ethylenedinitrilo)tetra-acetic acid.** $C_{10}H_{16}N_2O_8$. ($M_r$ 292.2). *1105800.* [60-00-4]. *N,N'*-1,2-Ethanediylbis[*N*-(carboxymethyl)glycine]. Edetic acid.

White or almost white crystalline powder, very slightly soluble in water.

mp: about 250 °C, with decomposition.

**Ethylene glycol.** $C_2H_6O_2$. ($M_r$ 62.1). *1036100.* [107-21-1]. Ethane-1,2-diol.

*Content*: minimum 99.0 per cent.

Colourless, slightly viscous liquid, hygroscopic, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: 1.113 to 1.115.

$n_D^{20}$: about 1.432.

bp: about 198 °C.

mp: about − 12 °C.

*Acidity*. To 10 mL add 20 mL of *water R* and 1 mL of *phenolphthalein solution R*. Not more than 0.15 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator to pink.

*Water* (*2.5.12*): maximum 0.2 per cent

**Ethylene glycol monododecyl ether.** $C_{14}H_{30}O_2$. ($M_r$ 230.4). *1191900.* [4536-30-5]. 2-(Dodecyloxy)ethan-1-ol.

Colourless or faintly green liquid.

**Ethylene glycol monoethyl ether.** $C_4H_{10}O_2$. ($M_r$ 90.1). *1036200.* [110-80-5]. 2-Ethoxyethanol.

*Content*: minimum 99.0 per cent.

Clear, colourless liquid, miscible with water, with acetone and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.93.

$n_D^{25}$: about 1.406.

bp: about 135 °C.

**Ethylene glycol monomethyl ether.** $C_3H_8O_2$. ($M_r$ 76.1). *1036300.* [109-86-4]. 2-Methoxyethanol.

*Content*: minimum 99.0 per cent.

Clear, colourless liquid, miscible with water, with acetone and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.97.

$n_D^{20}$: about 1.403.

bp: about 125 °C.

**Ethylene oxide.** $C_2H_4O$. ($M_r$ 44.05). *1036400.* [75-21-8]. Oxirane.

Colourless, flammable gas, very soluble in water and in anhydrous ethanol.

*Liquefaction point*: about 12 °C.

**Ethylene oxide solution.** *1036402.*

Weigh a quantity of cool *ethylene oxide stock solution R* equivalent to 2.5 mg of ethylene oxide into a cool flask and dilute to 50.0 g with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 mL with *macrogol 200 R1* (5 µg of ethylene oxide per gram of solution). *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

**Ethylene oxide solution R1.** *1036403.*

Dilute 1.0 mL of cooled *ethylene oxide stock solution R* (check the exact volume by weighing) to 50.0 mL with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 mL with *macrogol 200 R1*. Calculate the exact amount of ethylene oxide in parts per million from the volume determined by weighing and taking the relative density of *macrogol 200 R1* as 1.127. *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

**Ethylene oxide solution R2.** *1036404.*

Weigh 1.00 g of cold *ethylene oxide stock solution R* (equivalent to 2.5 mg of ethylene oxide) into a cold flask containing 40.0 g of cold *macrogol 200 R1*. Mix and determine the exact mass and dilute to a calculated mass to obtain a solution containing 50 µg of ethylene oxide per gram of solution. Weigh 10.00 g into a flask containing about 30 mL of *water R*, mix and dilute to 50.0 mL with *water R* (10 µg/mL of ethylene oxide). *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

**Ethylene oxide solution R3.** *1036405.*

Dilute 10.0 mL of *ethylene oxide solution R2* to 50.0 mL with *water R* (2 µg/mL of ethylene oxide). *Prepare immediately before use.*

**Ethylene oxide solution R4.** *1036407.*

Dilute 1.0 mL of *ethylene oxide stock solution R1* to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

**Ethylene oxide stock solution.** *1036401.*

*All operations carried out in the preparation of these solutions must be conducted in a fume cupboard. The operator must protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask.*

*Store all solutions in an airtight container in a refrigerator at 4 °C to 8 °C. Carry out all determinations three times.*

Into a dry, clean test-tube, cooled in a mixture of 1 part of *sodium chloride R* and 3 parts of crushed ice, introduce a slow current of *ethylene oxide R* gas, allowing condensation onto the inner wall of the test-tube. Using a glass syringe, previously cooled to − 10 °C, inject about 300 µL (corresponding to about 0.25 g) of liquid *ethylene oxide R* into 50 mL of *macrogol 200 R1*. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption ($M_{eo}$). Dilute to 100.0 mL with *macrogol 200 R1*. Mix well before use.

*Assay*. To 10 mL of a 500 g/L suspension of *magnesium chloride R* in *anhydrous ethanol R* add 20.0 mL of *0.1 M alcoholic hydrochloric acid R* in a flask. Stopper and shake to obtain a saturated solution and allow to stand overnight to equilibrate. Weigh 5.00 g of ethylene oxide stock solution (2.5 g/L) into the flask and allow to stand for 30 min. Titrate with *0.1 M alcoholic potassium hydroxide R* determining the end-point potentiometrically (*2.2.20*).

Carry out a blank titration, replacing the substance to be examined with the same quantity of *macrogol 200 R1*.

Ethylene oxide content in milligrams per gram is given by:

$$\frac{(V_0 - V_1) \times f \times 4.404}{m}$$

$V_0$, $V_1$ = volumes of *0.1 M alcoholic potassium hydroxide* used respectively for the blank titration and the assay,

$f$ = factor of the alcoholic potassium hydroxide solution,

$m$ = mass of the sample taken, in grams.

**Ethylene oxide stock solution R1.** *1036406.*

A 50 g/L solution of *ethylene oxide R* in *methanol R*.

Either use a commercially available reagent or prepare the solution corresponding to the aforementioned composition.

**Ethylene oxide stock solution R2.** *1036408.*

A 50 g/L solution of *ethylene oxide R* in *methylene chloride R*.

Either use a commercially available reagent or prepare the solution corresponding to the aforementioned composition.

**Ethyl formate.** $C_3H_6O_2$. ($M_r$ 74.1). *1035600.* [109-94-4]. Ethyl methanoate.

Clear, colourless, flammable liquid, freely soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.919.

$n_D^{20}$: about 1.36.

bp: about 54 °C.

**2-Ethylhexane-1,3-diol.** $C_8H_{18}O_2$. ($M_r$ 146.2). *1105900.* [94-96-2].

Slightly oily liquid, soluble in anhydrous ethanol, 2-propanol, propylene glycol and castor oil.

$d_{20}^{20}$: about 0.942.

$n_D^{20}$: about 1.451.

bp: about 244 °C.

**2-Ethylhexanoic acid.** $C_8H_{16}O_2$. ($M_r$ 144.2). *1036600.* [149-57-5].

Colourless liquid.

$d_{20}^{20}$: about 0.91.

$n_D^{20}$: about 1.425.

*Related substances*. Gas chromatography (*2.2.28*).

*Injection*: 1 µL of the test solution.

*Test solution*: suspend 0.2 g of the 2-ethylhexanoic acid in 5 mL of *water R*, add 3 mL of *dilute hydrochloric acid R* and 5 mL of *hexane R*, shake for 1 min, allow the layers to separate and use the upper layer. Carry out the chromatographic procedure as prescribed in the test for 2-ethylhexanoic acid in the monograph on *Amoxicillin sodium (0577)*.

*Limit*: the sum of the area of any peaks, apart from the principal peak and the peak due to the solvent, is not greater than 2.5 per cent of the area of the principal peak.

**Ethyl 4-hydroxybenzoate.** *1035700.* [120-47-8].

See *Ethyl parahydroxybenzoate R*.

***N*-Ethylmaleimide.** $C_6H_7NO_2$. ($M_r$ 125.1). *1036700.* [128-53-0]. 1-Ethyl-1*H*-pyrrole-2,5-dione.

Colourless crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: 41 °C to 45 °C.

*Storage*: at a temperature of 2 °C to 8 °C.

**Ethyl methanesulfonate.** $C_3H_8O_3S$. ($M_r$ 124.2). *1179300.* [62-50-0].

Clear, colourless liquid.

*Content*: minimum 99.0 per cent.

*Density*: about 1.206 g/cm$^3$ (20 °C).

$n_D^{20}$: about 1.418.

bp: about 213 °C.

**Ethyl methyl ketone.** *1054100.* [78-93-3].

See *methyl ethyl ketone R*.

**2-Ethyl-2-methylsuccinic acid.** $C_7H_{12}O_4$. ($M_r$ 160.2). *1036800.* [631-31-2]. 2-Ethyl-2-methylbutanedioic acid.

mp: 104 °C to 107 °C.

**Ethyl parahydroxybenzoate.** *1035700.* [120-47-8].

See *Ethyl parahydroxybenzoate (0900)*.

**2-Ethylpyridine.** $C_7H_9N$. ($M_r$ 107.2). *1133400.* [100-71-0].

Colourless or brownish liquid.

$d_{20}^{20}$: about 0.939.

$n_D^{20}$: about 1.496.

bp: about 149 °C.

**Ethyl toluenesulfonate.** $C_9H_{12}O_3S$. ($M_r$ 200.3). *1191000.* [80-40-0]. Ethyl 4-methylbenzenesulfonate. Ethyl tosilate.

*Content*: minimum 97.0 per cent.

*Density*: about 1.17 g/mL (25 °C).

bp: about 160 °C.

mp: about 33 °C.

**Ethylvinylbenzene-divinylbenzene copolymer.** *1036900.*

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of bead. The size range of the beads is specified after the name of the reagent in the tests where it is used.

**Eugenol.** $C_{10}H_{12}O_2$. ($M_r$ 164.2). *1037000.* [97-53-0]. 4-Allyl-2-methoxyphenol.

Colourless or pale yellow, oily liquid, darkening on exposure to air and light and becoming more viscous, practically insoluble in water, miscible with ethanol (96 per cent) and with fatty and essential oils.

$d_{20}^{20}$: about 1.07.

bp: about 250 °C.

*Eugenol used in gas chromatography complies with the following additional test*.

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

*Test solution*. The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

*Storage*: protected from light.

**Euglobulins, bovine.** *1037100.*

Use fresh bovine blood collected into an anticoagulant solution (for example, sodium citrate solution). Discard any haemolysed blood. Centrifuge at 1500-1800 *g* at 15-20 °C to obtain a supernatant plasma poor in platelets.

To 1 L of bovine plasma add 75 g of *barium sulfate R* and shake for 30 min. Centrifuge at not less than 1500-1800 *g* at 15-20 °C and draw off the clear supernatant. Add 10 mL of a 0.2 mg/mL solution of *aprotinin R* and shake to ensure mixing. In a container with a minimum capacity of 30 L in a chamber at 4 °C introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10-15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 mL of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L *sodium citrate R* and adjust to pH 7.2-7.4 by adding a 10 g/L solution of *sodium hydroxide R*. Filter through a sintered glass filter (*2.1.2*); to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of the chloride-citrate solution described above and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of bovine plasma.

*Test for suitability.* For this test, prepare the solutions using *phosphate buffer solution pH 7.4 R* containing 30 g/L of *bovine albumin R*.

Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.2 mL of a solution of a reference preparation of urokinase containing 100 IU/mL and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL. Add rapidly 0.5 mL of a solution containing 10 mg of bovine euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of bovine euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

*Storage*: protected from moisture at 4 °C; use within 1 year.

**Euglobulins, human.** *1037200.*

For the preparation, use fresh human blood collected into an anticoagulant solution (for example sodium citrate solution) or human blood for transfusion that has been collected in plastic blood bags and which has just reached its expiry date. Discard any haemolysed blood. Centrifuge at 1500-1800 *g* at 15 °C to obtain a supernatant plasma poor in platelets. Iso-group plasmas may be mixed.

To 1 L of the plasma add 75 g of *barium sulfate R* and shake for 30 min. Centrifuge at not less than 15 000 *g* at 15 °C and draw off the clear supernatant. Add 10 mL of a solution of *aprotinin R* containing 0.2 mg/mL and shake to ensure mixing. In a container with a minimum capacity of 30 L in a chamber at 4 °C introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add while stirring the supernatant obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10-15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 mL of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, and adjust the pH to 7.2-7.4 by adding a 10 g/L solution of *sodium hydroxide R*. Filter through a sintered-glass filter (*2.1.2*); to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of the chloride-citrate solution described above and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of human plasma.

*Test for suitability.* For this test, prepare the solutions using *phosphate buffer solution pH 7.2 R* containing 30 g/L of *bovine albumin R*. Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.1 mL of a solution of a reference preparation of streptokinase containing 10 IU of streptokinase activity per millilitre and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL. Add rapidly 1 mL of a solution containing 10 mg of human euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of human euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

*Storage*: in an airtight container at 4 °C; use within 1 year.

**Evodiamine.** $C_{19}H_{17}N_3O$. ($M_r$ 303.4). *1199400.* [518-17-2]. (13b*S*)-14-Methyl-8,13,13b,14-tetrahydro-indolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one.

**Extraction resin.** *1204900.*

Solid phase extraction resin containing 2,2′-oxybis(*N*,*N*-dioctylacetamide) (*N*,*N*,*N*′,*N*′-tetra-*n*-octyldiglycolamide).

**Factor VII-deficient plasma.** *1185900.*

Plasma that is deficient in factor VII.

**Factor Xa, bovine, coagulation.** *1037300.* [9002-05-5].

An enzyme which converts prothrombin to thrombin. The semi-purified preparation is obtained from liquid bovine plasma and it may be prepared by activation of the zymogen factor X with a suitable activator such as Russell's viper venom.

*Storage*: freeze-dried preparation at − 20 °C and frozen solution at a temperature lower than − 20 °C.

**Factor Xa solution, bovine.** *1037301.*

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Any change in the absorbance of the solution, measured at 405 nm (*2.2.25*) against *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* and from which the blank absorbance has been substracted, is not more than 0.20 per minute.

**Factor Xa solution, bovine R1.** *1037302.*

Reconstitute as directed by the manufacturer and dilute to 1.4 nkat/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R*.

**Factor Xa solution, bovine R2.** *1037303.*

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* to obtain a solution that gives an absorbance between 0.65 and 1.25 at 405 nm when determining the blank amidolytic activity according to general chapter *2.7.5* using the end-point method.

**Fargesin.** $C_{21}H_{22}O_6$. ($M_r$ 370.4). *1200200.* [31008-19-2].

5-[(3*SR*,3a*RS*,6*RS*,6a*RS*)-6-(3,4-Dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-*c*]furan-3-yl]-1,3-benzodioxole.

***(E,E)*-Farnesol.** $C_{15}H_{26}O$. ($M_r$ 222.4). *1161000.* [106-28-5]. *trans,trans*-Farnesol. (2*E*,6*E*)-3,7,11-Trimethyldodeca-2,6,10-trien-1-ol.

**Fast blue B salt.** $C_{14}H_{12}Cl_2N_4O_2$. ($M_r$ 339.2). *1037400.* [84633-94-3].

Schultz No. 490.

Colour Index No. 37235.
3,3′-Dimethoxy(biphenyl)-4,4′-bisdiazonium dichloride.

Dark green powder, soluble in water. It is stabilised by addition of zinc chloride.

*Storage*: in an airtight container, at a temperature between 2 °C and 8 °C.

**Fast blue B salt solution.** *1037401.*

Dissolve 140 mg of *fast blue B salt R* in 10 mL of *water R* and mix with 50 mL of *methylene chloride R* and 140 mL of *methanol R*.

*Storage*: protected from light at a temperature of 4 °C; use within 1 week.

**Fast red B salt.** $C_{17}H_{13}N_3O_9S_2$. ($M_r$ 467.4). *1037500.* [49735-71-9].

Schultz No. 155.

Colour Index No. 37125.
2-Methoxy-4-nitrobenzenediazonium hydrogen naphthalene-1,5-disulfonate.

Orange-yellow powder, soluble in water, slightly soluble in ethanol (96 per cent).

*Storage*: in an airtight container, protected from light, at 2 °C to 8 °C.

**Fenchlorphos.** $C_8H_8Cl_3O_3PS$. ($M_r$ 321.5). *1127200.* [299-84-3].

mp: about 35 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Fenchone.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1037600.* [7787-20-4].
(1*R*)-1,3,3-Trimethylbicyclo[2.2.1]heptan-2-one.

Oily liquid, miscible with ethanol (96 per cent), practically insoluble in water.

$n_D^{20}$: about 1.46.

$bp_{15mm}$: 192 °C to 194 °C.

*Fenchone used in gas chromatography complies with the following test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter fennel (0824)*.

*Test solution*. The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Fenvalerate.** $C_{25}H_{22}ClNO_3$. ($M_r$ 419.9). *1127300.* [51630-58-1].

bp: about 300 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Ferric ammonium sulfate.** $FeNH_4(SO_4)_2,12H_2O$. ($M_r$ 482.2). *1037700.* [7783-83-7]. Ammonium iron disulfate dodecahydrate.

Pale-violet crystals, efflorescent, very soluble in water, practically insoluble in ethanol (96 per cent).

**Ferric ammonium sulfate solution R2.** *1037702.*

A 100 g/L solution of *ferric ammonium sulfate R*. If necessary filter before use.

**Ferric ammonium sulfate solution R5.** *1037704.*

Shake 30.0 g of *ferric ammonium sulfate R* with 40 mL of *nitric acid R* and dilute to 100 mL with *water R*. If the solution is turbid, centrifuge or filter it.

*Storage*: protected from light.

**Ferric ammonium sulfate solution R6.** *1037705.*

Dissolve 20 g of *ferric ammonium sulfate R* in 75 mL of *water R*, add 10 mL of a 2.8 per cent *V/V* solution of *sulfuric acid R* and dilute to 100 mL with *water R*.

**Ferric chloride.** $FeCl_3,6H_2O$. ($M_r$ 270.3). *1037800.* [10025-77-1]. Iron trichloride hexahydrate.

Yellowish-orange or brownish crystalline masses, deliquescent, very soluble in water, soluble in ethanol (96 per cent). On exposure to light, ferric chloride and its solutions are partly reduced.

*Storage*: in an airtight container.

**Ferric chloride solution R1.** *1037801.*

A 105 g/L solution of *ferric chloride R*.

**Ferric chloride solution R2.** *1037802.*

A 13 g/L solution of *ferric chloride R*.

**Ferric chloride solution R3.** *1037803.*

Dissolve 2.0 g of *ferric chloride R* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

**Ferric chloride-ferricyanide-arsenite reagent.** *1037805.*

Immediately before use mix 10 mL of a 27 g/L solution of *ferric chloride R* in *dilute hydrochloric acid R*, 7 mL of *potassium ferricyanide solution R*, 3 mL of *water R* and 10 mL of *sodium arsenite solution R*.

**Ferric chloride-sulfamic acid reagent.** *1037804.*

A solution containing 10 g/L of *ferric chloride R* and 16 g/L of *sulfamic acid R*.

**Ferric nitrate.** $Fe(NO_3)_3,9H_2O$. ($M_r$ 404). *1106100.* [7782-61-8].

*Content*: minimum 99.0 per cent *m/m* of $Fe(NO_3)_3,9H_2O$.

Light-purple crystals or crystalline mass, very soluble in water.

*Free acid*: not more than 0.3 per cent (as $HNO_3$).

**Ferric sulfate.** $Fe_2(SO_4)_3,xH_2O$. *1037900.* [15244-10-7]. Iron(III) trisulfate hydrated.

Yellowish-white powder, very hygroscopic, decomposes in air, slightly soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container, protected from light.

**Ferric sulfate solution.** *1037901.*

Dissolve 50 g of *ferric sulfate R* in an excess of *water R*, add 200 mL of *sulfuric acid R* and dilute to 1000 mL with *water R*.

**Ferric sulfate pentahydrate.** $Fe_2(SO_4)_3,5H_2O$. ($M_r$ 489.9). *1153700.* [142906-29-4].

White or yellowish powder.

**Ferrocyphene.** $C_{26}H_{16}FeN_6$. ($M_r$ 468.3). *1038000.* [14768-11-7]. Dicyanobis(1,10-phenanthroline)iron(II).

Violet-bronze, crystalline powder, practically insoluble in water and in ethanol (96 per cent).

*Storage*: protected from light and moisture.

**Ferroin.** *1038100.* [14634-91-4].

Dissolve 0.7 g of *ferrous sulfate R* and 1.76 g of *phenanthroline hydrochloride R* in 70 mL of *water R* and dilute to 100 mL with the same solvent.

*Test for sensitivity.* To 50 mL of *dilute sulfuric acid R* add 0.1 mL of *ferroin R*. After the addition of 0.1 mL of *0.1 M ammonium and cerium nitrate* the colour changes from red to light blue.

**Ferrous ammonium sulfate.** $Fe(NH_4)_2(SO_4)_2,6H_2O$. ($M_r$ 392.2). *1038200.* [7783-85-9]. Diammonium iron disulfate hexahydrate.

Pale bluish-green crystals or granules, freely soluble in water, practically insoluble in ethanol (96 per cent).

*Storage*: protected from light.

**Ferrous sulfate.** *1038300.* [7782-63-0].

See *Ferrous sulfate heptahydrate (0083)*.

**Ferrous sulfate solution R2.** *1038301.*

Dissolve 0.45 g of *ferrous sulfate R* in 50 mL of *0.1 M hydrochloric acid* and dilute to 100 mL with *carbon dioxide-free water R*. Prepare immediately before use.

**Ferulic acid.** $C_{10}H_{10}O_4$. ($M_r$ 194.2). *1149500.* [1135-24-6]. 4-Hydroxy-3-methoxycinnamic acid. 3-(4-Hydroxy-3-methoxyphenyl)propenoic acid.

Faint yellow powder, freely soluble in methanol.

mp: 172.9 °C to 173.9 °C.

*Ferulic acid used in the assay of eleutherosides in Eleutherococcus (1419) complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Eleutherococcus (1419)*.

*Content*: minimum 99 per cent, calculated by the normalisation procedure.

**Fibrin blue.** *1101400.*

Mix 1.5 g of fibrin with 30 mL of a 5 g/L solution of *indigo carmine R* in 1 per cent *V/V dilute hydrochloric acid R*. Heat the mixture to 80 °C and maintain at this temperature whilst stirring for about 30 min. Allow to cool. Filter. Wash extensively by resuspension in 1 per cent *V/V dilute hydrochloric acid R* and mixing for about 30 min; filter. Repeat the washing operation three times. Dry at 50 °C. Grind.

**Fibrin congo red.** *1038400.*

Take 1.5 g of fibrin and leave overnight in 50 mL of a 20 g/L solution of *congo red R* in *ethanol (90 per cent V/V) R*. Filter, rinse the fibrin with *water R* and store under *ether R*.

**Fibrinogen.** *1038500.* [9001-32-5].

See *Human fibrinogen, freeze-dried (0024)*.

**Fixing solution.** *1122600.*

To 250 mL of *methanol R*, add 0.27 mL of *formaldehyde R* and dilute to 500.0 mL with *water R*.

**Fixing solution for isoelectric focusing in polyacrylamide gel.** *1138700.*

A solution containing 35 g of *sulfosalicylic acid R* and 100 g of *trichloroacetic acid R* per litre of *water R*.

**Flufenamic acid.** $C_{14}H_{10}F_3NO_2$. ($M_r$ 281.2). *1106200.* [530-78-9]. 2-[[3-(Trifluoromethyl)phenyl]amino]benzoic acid.

Pale yellow, crystalline powder or needles, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: 132 °C to 135 °C.

**Flumazenil.** *1149600.* [78755-81-4].

See *Flumazenil (1326)*.

**Flunitrazepam.** *1153800.* [1622-62-4].

See *Flunitrazepam (0717)*.

**Fluorene.** $C_{13}H_{10}$. ($M_r$ 166.2). *1127400.* [86-73-7]. Diphenylenemethane.

White or almost white crystals, freely soluble in anhydrous acetic acid, soluble in hot ethanol (96 per cent).

mp: 113 °C to 115 °C.

**(9-Fluorenyl)methyl chloroformate.** $C_{15}H_{11}ClO_2$. ($M_r$ 258.7). *1180100.* [28920-43-6]. Fluoren-9-ylmethyl chloromethanoate.

mp: about 63 °C.

**Fluorescamine.** $C_{17}H_{10}O_4$. ($M_r$ 278.3). *1135800.* [38183-12-9]. 4-Phenylspiro[furan-2(3*H*),1'(3'*H*)-isobenzofuran]-3,3'-dione.

mp: 154 °C to 155 °C.

**Fluorescein.** $C_{20}H_{12}O_5$. ($M_r$ 332.3). *1106300.* [2321-07-5]. 3',6'-Dihydroxyspiro[isobenzofurane-1(3*H*),9'-[9*H*]xanthen]-3-one.

Orange-red powder, practically insoluble in water, soluble in warm ethanol (96 per cent), soluble in alkaline solutions. In solution, fluorescein displays a green fluorescence.

mp: about 315 °C.

**Fluorescein-conjugated rabies antiserum.** *1038700.*

Immunoglobulin fraction with a high rabies antibody titre, prepared from the sera of suitable animals that have been immunised with inactivated rabies virus; the immunoglobulin is conjugated with fluorescein isothiocyanate.

**Fluorocholine chloride.** $C_5H_{13}ClFNO$. ($M_r$ 157.6). *1195700.* [459424-38-5]. *N*-(Fluoromethyl)-2-hydroxy-*N*,*N*-dimethylethan-1-aminium chloride.

Colourless, hygroscopic crystals.

mp: about 184 °C.

**2-Fluoro-2-deoxy-D-glucose.** $C_6H_{11}FO_5$. ($M_r$ 182.2). *1113900.* [86783-82-6].

White or almost white crystalline powder.

mp: 174 °C to 176 °C.

**2-Fluoro-2-deoxy-D-mannose.** $C_6H_{11}FO_5$. ($M_r$ 182.1). *1172100.* [38440-79-8].

Colourless semi-solid.

**Fluorodinitrobenzene.** $C_6H_3FN_2O_4$. ($M_r$ 186.1). *1038800.* [70-34-8]. 1-Fluoro-2,4-dinitrobenzene.

Pale yellow liquid or crystals, soluble in propylene glycol.

mp: about 29 °C.

*Content*: minimum 99.0 per cent, determined by gas chromatography.

**1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide.** $C_9H_9FN_4O_5$. ($M_r$ 272.2). *1194900.* [95713-52-3]. $N^{\alpha}$-(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide. Marfey's reagent. FDAA.

Yellow or orange powder.

mp: about 228 °C.

*Enantiomeric purity*: minimum 99.5 per cent.

**DL-6-Fluorodopa hydrochloride.** $C_9H_{11}ClFNO_4$. ($M_r$ 251.6). *1169200.* (2*RS*)-2-Amino-3-(2-fluoro-4,5-dihydroxyphenyl)propanoic acid hydrochloride. 2-Fluoro-5-hydroxy-DL-tyrosine hydrochloride.

White or almost white powder.

**Fluoroethyl(2-hydroxyethyl)dimethylammonium chloride.** $C_6H_{15}ClFNO$. ($M_r$ 171.6). *1195800.* [479407-08-4]. *N*-(2-Fluoroethyl)-2-hydroxy-*N*,*N*-dimethylethan-1-aminium chloride.

Slightly yellow powder.

**Fluoroethyl-D-tyrosine hydrochloride.** $C_{11}H_{15}FNO_3Cl$. ($M_r$ 263.7). *1192000.* (2*R*)-2-Amino-3-[4-(2-fluoroethoxy)phenyl]propanoic acid hydrochloride.

*Content*: minimum 95 per cent.

Colourless or almost colourless crystals.

**Fluoroethyl-L-tyrosine hydrochloride.** $C_{11}H_{15}FNO_3Cl$. ($M_r$ 263.7). *1192100.* (2*S*)-2-Amino-3-[4-(2-fluoroethoxy)phenyl]propanoic acid hydrochloride.

*Content*: minimum 95 per cent.

Colourless or almost colourless crystals.

**6-Fluorolevodopa hydrochloride.** $C_9H_{11}ClFNO_4$. ($M_r$ 251.6). *1169300.* [144334-59-8]. (2*S*)-2-Amino-3-(2-fluoro-4,5-dihydroxyphenyl)propanoic acid hydrochloride. 2-Fluoro-5-hydroxy-L-tyrosine hydrochloride.

Colourless or almost colourless solid, soluble in water.

**Fluoromisonidazole.** $C_6H_8FN_3O_3$. ($M_r$ 189.1). *1186000.* [13551-89-8]. (2*RS*)-1-Fluoro-3-(2-nitro-1*H*-imidazol-1-yl)propan-2-ol. FMISO.

*Content*: minimum 95 per cent.

Yellow crystals.

**1-Fluoro-2-nitro-4-(trifluoromethyl)benzene.** $C_7H_3F_4NO_2$. ($M_r$ 209.1). *1038900.* [367-86-2].

mp: about 197 °C.

**Folic acid.** *1039000.* [75708-92-8].

See *Folic acid hydrate (0067)*.

**Formaldehyde.** *1039100.* [50-00-0].

See *Formaldehyde solution R*.

**Formaldehyde solution.** *1039101.*

See *Formaldehyde solution (35 per cent) (0826)*.

**Formaldehyde solution R1.** *1039102.*

Complies with the requirements prescribed in the monograph *Formaldehyde solution (35 per cent) (0826)* with the following modification.

*Content*: 36.5 per cent *m/m* to 38.0 per cent *m/m* of formaldehyde ($CH_2O$; $M_r$ 30.03).

**Formamide.** $CH_3NO$. ($M_r$ 45.0). *1039200.* [75-12-7].

Clear, colourless, oily liquid, hygroscopic, miscible with water and with ethanol (96 per cent). It is hydrolysed by water.

$d_{20}^{20}$: about 1.134.

bp: about 210 °C.

*Content*: minimum 99.5 per cent.

*Storage*: in an airtight container.

**Formamide R1.** *1039202.*

Complies with the requirements prescribed for *formamide R* with the following additional requirement.

*Water* (*2.5.12*): maximum 0.1 per cent determined with an equal volume of *anhydrous methanol R*.

**Formamide, treated.** *1039201.*

Disperse 1.0 g of *sulfamic acid R* in 20.0 mL of *formamide R* containing 5 per cent *V/V* of *water R*.

**Formic acid, anhydrous.** $CH_2O_2$. ($M_r$ 46.03). *1039300.* [64-18-6].

*Content*: minimum 98.0 per cent *m/m*.

Colourless liquid, corrosive, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 1.22.

*Assay.* Weigh accurately a conical flask containing 10 mL of *water R*, quickly add about 1 mL of the acid and weigh again. Add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 46.03 mg of $CH_2O_2$.

**Fructose.** *1106400.* [57-48-7].

See *Fructose (0188)*.

**Fuchsin, basic.** *1039400.* [632-99-5].

A mixture of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$; $M_r$ 337.9; Colour Index No. 42510; Schultz No. 780) and *para*-rosaniline hydrochloride ($C_{19}H_{18}ClN_3$; $M_r$ 323.8; Colour Index No. 42500; Schultz No. 779).

If necessary, purify in the following manner. Dissolve 1 g in 250 mL of *dilute hydrochloric acid R*. Allow to stand for 2 h at room temperature, filter and neutralise with *dilute sodium hydroxide solution R* and add 1 mL to 2 mL in excess. Filter the precipitate through a sintered-glass filter (40) (*2.1.2*) and wash with *water R*. Dissolve the precipitate in 70 mL of *methanol R*, previously heated to boiling, and add 300 mL of *water R* at 80 °C. Allow to cool to room temperature, filter and dry the crystals *in vacuo*.

Crystals with a greenish-bronze sheen, soluble in water and in ethanol (96 per cent).

*Storage*: protected from light.

**Fuchsin solution, decolorised.** *1039401.*

Dissolve 0.1 g of *basic fuchsin R* in 60 mL of *water R*. Add a solution containing 1 g of *anhydrous sodium sulfite R* or 2 g of *sodium sulfite heptahydrate R* in 10 mL of *water R*. Slowly and with continuous shaking add 2 mL of *hydrochloric acid R*. Dilute to 100 mL with *water R*. Allow to stand protected from light for at least 12 h, decolorise with *activated charcoal R* and filter. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolorise again by adding *activated charcoal R*.

*Test for sensitivity.* To 1.0 mL add 1.0 mL of *water R* and 0.1 mL of *aldehyde-free alcohol R*. Add 0.2 mL of a solution containing 0.1 g/L of formaldehyde ($CH_2O$, $M_r$ 30.03). A pale-pink colour develops within 5 min.

*Storage*: protected from light.

**Fuchsin solution, decolorised R1.** *1039402.*

To 1 g of *basic fuchsin R* add 100 mL of *water R*. Heat to 50 °C and allow to cool with occasional shaking. Allow to stand for 48 h, shake and filter. To 4 mL of the filtrate add 6 mL of *hydrochloric acid R*, mix and dilute to 100 mL with *water R*. Allow to stand for at least 1 h before use.

**Fucose.** $C_6H_{12}O_5$. ($M_r$ 164.2). *1039500.* [6696-41-9]. 6-Deoxy-L-galactose.

White or almost white powder, soluble in water and in ethanol (96 per cent).

$[\alpha]_D^{20}$: about − 76, determined on a 90 g/L solution 24 h after dissolution.

mp: about 140 °C.

**Fumaric acid.** $C_4H_4O_4$. ($M_r$ 116.1). *1153200.* [110-17-8]. (*E*)-Butenedioic acid.

White or almost white crystals, slightly soluble in water, soluble in ethanol (96 per cent), slightly soluble in acetone.

mp: about 300 °C.

**Furfural.** $C_5H_4O_2$. ($M_r$ 96.1). *1039600.* [98-01-1]. 2-Furaldehyde. 2-Furanecarbaldehyde.

Clear, colourless to brownish-yellow, oily liquid, miscible in 11 parts of water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 1.155 to 1.161.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 159 °C and 163 °C.

*Storage*: in a dark place.

**Gadolinium chloride hexahydrate.** $GdCl_3,6H_2O$. ($M_r$ 371.7). *1198400.* [13450-84-5]. Gadolinium trichloride hexahydrate.

*Content*: minimum 99.9 per cent.

**Gadolinium sulfate octahydrate.** $Gd_2(SO_4)_3,8H_2O$. ($M_r$ 747). *1195300.* [13450-87-8].

Colourless, crystalline powder.

**Galactose.** $C_6H_{12}O_6$. ($M_r$ 180.2). *1039700.* [59-23-4]. D-(+)-Galactose.

White or almost white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 79 to + 81, determined on a 100 g/L solution in *water R* containing about 0.05 per cent of $NH_3$.

**1,6-Galactosylgalactose.** $C_{12}H_{22}O_{11}$. ($M_r$ 342.3). *1195900.* [5077-31-6]. 6-*O*-β-D-Galactopyranosyl-D-galactopyranose.

White or almost white powder.

**Galacturonic acid.** $C_6H_{10}O_7$. ($M_r$ 194.1). *1196000.* [685-73-4]. D-(+)-galacturonic acid. (2*S*,3*R*,4*S*,5*R*)-2,3,4,5-Tetrahydroxy-6-oxo-hexanoic acid.

$[\alpha]_D^{20}$: about + 53°, determined on a 100 g/L solution.

**Gallic acid.** $C_7H_6O_5,H_2O$. ($M_r$ 188.1). *1039800.* [5995-86-8]. 3,4,5-Trihydroxybenzoic acid monohydrate.

Crystalline powder or long needles, colourless or slightly yellow, soluble in water, freely soluble in hot water, in ethanol (96 per cent) and in glycerol.

It loses its water of crystallisation at 120 °C.

mp: about 260 °C, with decomposition.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Bearberry leaf (1054)*; the chromatogram shows only one principal spot.

**Gallium ($^{68}$Ga) chloride solution.** $^{68}GaCl_3$. ($M_r$ 174.3). *1182500.*

Solution containing gallium-68 in the form of gallium chloride in *dilute hydrochloric acid R*.

*Content*: 90 per cent to 110 per cent of the declared gallium-68 radioactivity at the date and time stated on the label.

**Gastric juice, artificial.** *1039900.*

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*. Add 80 mL of *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

**Gastrodin.** $C_{13}H_{18}O_7$. ($M_r$ 286.3). *1203600.* [62499-27-8]. 4-(Hydroxymethyl)phenyl α-D-glucopyranoside. (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(Hydroxymethyl)-6-[4-(hydroxymethyl)-phenoxy]oxane-3,4,5-triol.

**GC concentrical column.** *1135100.*

A commercially available system consisting of 2 concentrically arranged tubes. The outer tube is packed with molecular sieves and the inner tube is packed with a porous polymer mixture. The main application is the separation of gases.

**Gelatin.** *1040000.* [9000-70-8].

See *Gelatin (0330)*.

**Gelatin, hydrolysed.** *1040100.*

Dissolve 50 g of *gelatin R* in 1000 mL of *water R*. Autoclave in saturated steam at 121 °C for 90 min and freeze dry.

**Geniposide.** $C_{17}H_{24}O_{10}$. ($M_r$ 388.4). *1196800.* [24512-63-8]. Methyl (1*S*,4a*S*,7a*S*)-1-(β-D-glucopyranosyloxy)-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-4-carboxylate.

**Geraniol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1135900.* [106-24-1]. (*E*)-3,7-Dimethylocta-2,6-dien-1-ol.

Oily liquid, slight odour of rose, practically insoluble in water, miscible with ethanol (96 per cent).

*Geraniol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

*Content*: minimum 98.5 per cent, calculated by the normalisation procedure.

*Storage*: in an airtight container, protected from light

**Geranyl acetate.** $C_{12}H_{20}O_2$. ($M_r$ 196.3). *1106500.* [105-87-3]. (*E*)-3,7-Dimethylocta-2,6-dien-1-yl acetate.

Colourless or slightly yellow liquid, slight odour of rose and lavender.

*Geranyl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution.* The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Ginsenoside Rb1.** $C_{54}H_{92}O_{23},3H_2O$. ($M_r$ 1163). *1127500.* [41753-43-9]. (20*S*)-3β-Di-D-glucopyranosyl-20-di-D-glucopyranosylprotopanaxadiol. (20*S*)-3β-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-20-[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-5α-dammar-24-en-12β-ol. (20*S*)-3β-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-20-[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-4,4,8,14-tetramethyl-18-nor-5α-cholest-24-en-12β-ol.

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 11.3 determined on a 10 g/L solution in *methanol R*.

mp: about 199 °C.

*Water (2.5.12)*: maximum 6.8 per cent.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginseng (1523)*.

*Test solution.* Dissolve 3.0 mg, accurately weighed, of *ginsenoside Rb1* in 10 mL of *methanol R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Ginsenoside Re.** $C_{48}H_{82}O_{18}$. ($M_r$ 947.2). *1157800.* [52286-59-6]. (3β,6α,12β)-20-(β-D-Glucopyranosyloxy)-3,12-dihydroxydammar-24-en-6-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside.

Colourless solid, soluble in water, in ethanol (96 per cent) and in methanol.

**Ginsenoside Rf.** $C_{42}H_{72}O_{14},2H_2O$. ($M_r$ 837). *1127700.* [52286-58-5]. (20*S*)-6-*O*-[β-D-Glucopyranosyl-(1→2)-β-D-glycopyranoside]-dammar-24-ene-3β,6α,12β,20-tetrol.

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 12.8 determined on a 10 g/L solution in *methanol R*.

mp: about 198 °C.

**Ginsenoside Rg1.** $C_{42}H_{72}O_{14},2H_2O$. ($M_r$ 837). *1127600.* [22427-39-0]. (20*S*)-6β-D-Glucopyranosyl-D-glucopyranosylprotopanaxatriol. (20*S*)-6α,20-Bis(β-D-glucopyranosyloxy)-5α-dammar-24-ene-3β,12β-diol. (20*S*)-6α,20-Bis(β-D-glucopyranosyloxy)-4,4,8,14-tetramethyl-18-nor-5α-cholest-24-ene-3β,12β-diol.

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 31.2 determined on a 10 g/L solution in *methanol R*.

mp: 188 °C to 191 °C.

*Water (2.5.12)*: maximum 4.8 per cent.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginseng (1523)*.

*Test solution.* Dissolve 3.0 mg, accurately weighed, of *ginsenoside Rg1* in 10 mL of *methanol R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Ginsenoside Rg2.** $C_{42}H_{72}O_{13}$. ($M_r$ 785). *1182600.* [52286-74-5]. 3β,12β,20-Trihydroxydammar-24-en-6α-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside.

**Ginsenoside Ro.** $C_{48}H_{76}O_{19}$. ($M_r$ 957). *1205000.* [34367-04-9]. (3β)-28-(β-D-Glucopyranosyloxy)-28-oxoolean-12-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid.

**Gitoxin.** $C_{41}H_{64}O_{14}$. ($M_r$ 781). *1040200.* [4562-36-1]. Glycoside of *Digitalis purpurea* L. 3β-(*O*-2,6-Dideoxy-β-d-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-d-

*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-d-*ribo*-hexopyranosyloxy)-14,16β-dihydroxy-5β,14β-card-20(22)-enolide.

A white or almost white, crystalline powder, practically insoluble in water and in most common organic solvents, soluble in pyridine.

$[\alpha]_D^{20}$: + 20 to + 24, determined on a 5 g/L solution in a mixture of equal volumes of *chloroform R* and *methanol R*.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Digitalis leaf (0117)*; the chromatogram shows only one principal spot.

**Glucosamine hydrochloride.** $C_6H_{14}ClNO_5$. ($M_r$ 215.6). *1040300.* [66-84-2]. D-Glucosamine hydrochloride.

Crystals, soluble in water.

$[\alpha]_D^{20}$: + 100, decreasing to + 47.5 after 30 min, determined on a 100 g/L solution.

**Glucose.** *1025700.* [50-99-7].

See *Glucose (0177)*.

**D-Glucuronic acid.** $C_6H_{10}O_7$. ($M_r$ 194.1). *1119700.* [6556-12-3].

*Content*: minimum 96.0 per cent, calculated with reference to the substance dried *in vacuo* (*2.2.32*).

Soluble in water and in ethanol (96 per cent).

Shows mutarotation: $[\alpha]_D^{24}$: + 11.7 → + 36.3.

*Assay*. Dissolve 0.150 g in 50 mL of *anhydrous methanol R* while stirring under nitrogen. Titrate with *0.1 M tetrabutylammonium hydroxide*, protecting the solution from atmospheric carbon dioxide throughout solubilisation and titration. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 19.41 mg of $C_6H_{10}O_7$.

**Glutamic acid.** *1040400.* [56-86-0].

See *Glutamic acid (0750)*.

**L-Glutamine.** $C_5H_{10}N_2O_3$. ($M_r$ 146.2). *1203700.* [56-85-9]. (*S*)-2,5-Diamino-5-oxopentanoic acid.

White crystalline powder.

mp: about 185 °C, with decomposition.

**Glutamyl endopeptidase for peptide mapping.** *1173300.* [137010-42-5]. Endoproteinase Glu-C of high purity from *Staphylococcus aureus* strain V8 (EC 3.4.21.19).

**L-γ-Glutamyl-L-cysteine.** $C_8H_{14}N_2O_5S$. ($M_r$ 250.3). *1157900.* [636-58-8].

**Glutaraldehyde.** $C_5H_8O_2$. ($M_r$ 100.1). *1098300.* [111-30-8].

Oily liquid, soluble in water.

$n_D^{25}$: about 1.434.

bp: about 188 °C.

**Glutaric acid.** $C_5H_8O_4$. ($M_r$ 132.1). *1149700.* [110-94-1]. Pentanedioic acid.

White or almost white, crystalline powder.

**L-Glutathione, oxidised.** $C_{20}H_{32}N_6O_{12}S_2$. ($M_r$ 612.6). *1158000.* [27025-41-8]. Bis(L-γ-glutamyl-L-cysteinylglycine) disulfide.

**Glycerol.** *1040500.* [56-81-5].

See *Glycerol (0496)*.

**Glycerol R1.** *1040501.*

Complies with the requirements prescribed for the monograph *Glycerol (0496)* and free from diethylene glycol when examined as prescribed in the test for impurity A and related substances in that monograph.

**Glycerol (85 per cent).** *1040600.*

See *Glycerol (85 per cent) (0497)*.

**Glycerol (85 per cent) R1.** *1040601.*

Complies with the requirements prescribed for the monograph *Glycerol 85 per cent (0497)* and free from diethylene glycol when examined as prescribed in the test for impurity A and related substances in that monograph.

**Glycerol 1-decanoate.** $C_{13}H_{26}O_4$. ($M_r$ 246.3). *1169400.* [2277-23-8]. (2*RS*)-2,3-Dihydroxypropyl decanoate. α-Monocaprin. 1-Monodecanoyl-*rac*-glycerol.

*Content*: about 99 per cent.

**Glycerol 1-octanoate.** $C_{11}H_{22}O_4$. ($M_r$ 218.3). *1169500.* [502-54-5]. (2*RS*)-2,3-Dihydroxypropyl octanoate. α-Monocaprylin. 1-Monooctanoyl-*rac*-glycerol.

*Content*: about 99 per cent.

**Glycidol.** $C_3H_6O_2$. ($M_r$ 74.1). *1127800.* [556-52-5].

Slightly viscous liquid, miscible with water.

$d_4^{20}$: about 1.115.

$n_D^{20}$: about 1.432.

**Glycine.** *1040700.* [56-40-6].

See *Glycine (0614)*.

**Glycine anhydride.** $C_4H_6N_2O_2$. ($M_r$ 114.1). *1192200.* [106-57-0]. Piperazine-2,5-dione (2,5-DKP).

**Glycolic acid.** $C_2H_4O_3$. ($M_r$ 76.0). *1040800.* [79-14-1]. 2-Hydroxyacetic acid.

Crystals, soluble in water, in acetone, in ethanol (96 per cent) and in methanol.

mp: about 80 °C.

**Glycyrrhetic acid.** $C_{30}H_{46}O_4$. ($M_r$ 470.7). *1040900.* [471-53-4]. Glycyrrhetinic acid. 12,13-Didehydro-3β-hydroxy-11-oxo-olean-30-oic acid.

A mixture of α- and β-glycyrrhetic acids in which the β-isomer is predominant.

White or yellowish-brown powder, practically insoluble in water, soluble in anhydrous ethanol and in glacial acetic acid.

$[\alpha]_D^{20}$: + 145 to + 155, determined on a 10.0 g/L solution in *anhydrous ethanol R*.

*Chromatography*. Thin-layer chromatography (*2.2.27*) using *silica gel* $GF_{254}$ *R* as the coating substance; prepare the slurry using a 0.25 per cent *V/V* solution of *phosphoric acid R*. Apply to the plate 5 µL of a 5 g/L solution of the glycyrrhetic acid in a mixture of equal volumes of *chloroform R* and *methanol R*. Develop over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *chloroform R*. Examine the chromatogram in ultraviolet light at 254 nm. The chromatogram shows a dark spot ($R_F$ about 0.3) corresponding to β-glycyrrhetic acid and a smaller spot ($R_F$ about 0.5) corresponding to α-glycyrrhetic acid. Spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min. Both spots are coloured bluish-violet. Between them a smaller bluish-violet spot may be present.

**18α-Glycyrrhetinic acid.** $C_{30}H_{46}O_4$. ($M_r$ 470.7). *1127900.* [1449-05-4]. (20β)-3β-Hydroxy-11-oxo-18α-olean-12-en-29-oic acid.

White or almost white powder, practically insoluble in water, soluble in anhydrous ethanol, sparingly soluble in methylene chloride.

**Glyoxalhydroxyanil.** $C_{14}H_{12}N_2O_2$. ($M_r$ 240.3). *1041000.* [1149-16-2]. Glyoxal bis(2-hydroxyanil).

White or almost white crystals, soluble in hot ethanol (96 per cent).

mp: about 200 °C.

**Glyoxal solution.** *1098400.* [107-22-2].

Contains about 40 per cent ( *m/m*) glyoxal.

*Assay.* In a ground-glass stoppered flask place 1.000 g of glyoxal solution, 20 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* and 50 mL of *water R*. Allow to stand for 30 min and add 1 mL of *methyl red mixed solution R* and titrate with *1 M sodium hydroxide* until the colour changes from red to green. Carry out a blank titration.

1 mL of *1 M sodium hydroxide* is equivalent to 29.02 mg of glyoxal ($C_2H_2O_2$).

**Gonadotrophin, chorionic.** *1041100.* [9002-61-3].

See *Chorionic gonadotrophin (0498)*.

**Gonadotrophin, serum.** *1041200.*

See *Equine serum gonadotrophin for veterinary use (0719)*.

**Gramine.** $C_{11}H_{14}N_2$. ($M_r$ 174.2). *1189400.* [87-52-5]. 1-(1*H*-Indol-3-yl)-*N*,*N*-dimethylmethanamine.

Flakes, practically insoluble in water, soluble in ethanol (96 per cent), slightly soluble in acetone.

mp: 132 °C to 134 °C.

**Guaiacol.** $C_7H_8O_2$. ($M_r$ 124.1). *1148300.* [90-05-1]. 2-Methoxyphenol. 1-Hydroxy-2-methoxybenzene.

Crystalline mass or colourless or yellowish liquid, hygroscopic, slightly soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

bp: about 205 °C.

mp: about 28 °C.

**Guaiacum resin.** *1041400.*

Resin obtained from the heartwood of *Guaiacum officinale* L. and *Guaiacum sanctum* L.

Reddish-brown or greenish-brown, hard, glassy fragments; fracture shiny.

**Guaiazulene.** $C_{15}H_{18}$. ($M_r$ 198.3). *1041500.* [489-84-9]. 1,4-Dimethyl-7-isopropylazulene.

Dark-blue crystals or blue liquid, very slightly soluble in water, miscible with fatty and essential oils and with liquid paraffin, sparingly soluble in ethanol (96 per cent), soluble in 500 g/L sulfuric acid and 80 per cent *m/m* phosphoric acid, giving a colourless solution.

mp: about 30 °C.

*Storage*: protected from light and air.

**Guanidine hydrochloride.** $CH_5N_3HCl$. ($M_r$ 95.5). *1098500.* [50-01-1].

Crystalline powder, freely soluble in water and in ethanol (96 per cent).

**Guanine.** $C_5H_5N_5O$. ($M_r$ 151.1). *1041600.* [73-40-5]. 2-Amino-1,7-dihydro-6*H*-purin-6-one.

Amorphous white or almost white powder, practically insoluble in water, slightly soluble in ethanol (96 per cent). It dissolves in ammonia and in dilute solutions of alkali hydroxides.

**Haemoglobin.** *1041700.* [9008-02-0].

*Nitrogen*: 15 per cent to 16 per cent.

*Iron*: 0.2 per cent to 0.3 per cent.

*Loss on drying* (*2.2.32*): maximum 2 per cent.

*Sulfated ash* (*2.4.14*): maximum 1.5 per cent.

**Haemoglobin solution.** *1041701.*

Transfer 2 g of *haemoglobin R* to a 250 mL beaker and add 75 mL of *dilute hydrochloric acid R2*. Stir until solution is complete. Adjust the pH to 1.6 ± 0.1 using *1 M hydrochloric acid*. Transfer to a 100 mL flask with the aid of *dilute hydrochloric acid R2*. Add 25 mg of *thiomersal R*. Prepare daily, store at 5 ± 3 °C and readjust to pH 1.6 before use.

*Storage*: at 2 °C to 8 °C.

**Hamamelitannin.** $C_{20}H_{20}O_{14}$. ($M_r$ 484.4). *1192700.* [469-32-9]. (2*R*,3*R*,4*R*)-2-Formyl-2,3,4-trihydroxypentane-1,5-diyl bis(3,4,5-trihydroxybenzoate). 2-*C*-[(Galloyloxy)methyl]-D-ribose 5-gallate.

**Harpagoside.** $C_{24}H_{30}O_{11}$. ($M_r$ 494.5). *1098600.*

White or almost white, crystalline powder, very hygroscopic, soluble in water and in ethanol (96 per cent).

mp: 117 °C to 121 °C.

*Storage*: in an airtight container.

**Hederacoside C.** $C_{59}H_{96}O_{26}$. ($M_r$ 1221). *1158100.* [14216-03-6]. *O*-6-Deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl (4*R*)-3β-[[2-*O*(-6-deoxy-α-L-mannopyranosyl)-α-L-arabinopyranosyl]oxy]-23-hydroxyolean-12-en-28-oate.

Colourless crystals or white or almost white powder.

mp: about 220 °C.

*Hederacoside C used in liquid chromatography complies with the following additional test.*

*Assay.* Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ivy leaf (2148)*.

*Test solution.* Dissolve 5.0 mg of hederacoside C in 5.0 mL of *methanol R*.

*Content*: minimum 95 per cent, calculated by the normalisation procedure.

**Hederagenin.** $C_{30}H_{48}O_4$. ($M_r$ 472.7). *1184100.* [465-99-6]. Astrantiagenin E. Caulosapogenin. 3β,23-Dihydroxy-4α-olean-12-en-28-oic acid.

**α-Hederin.** $C_{41}H_{66}O_{12}$. ($M_r$ 751.0). *1158200.* [27013-91-8]. (+)-(4*R*)-3β-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-α-L-arabinopyranosyl]oxy]-23-hydroxyolean-12-en-28-oic acid.

White or almost white powder.

mp: about 256 °C.

**Helium for chromatography.** He. ($A_r$ 4.003). *1041800.* [7440-59-7].

*Content*: minimum 99.995 per cent *V/V* of He.

**Heparin.** *1041900.* [9041-08-1].

See *Heparin sodium (0333)*.

**Heparinase I.** *1187600.* [9025-39-2]. Heparin lyase (EC *4.2.2.7*).

Enzyme from *Flavobacterium heparinum* that performs eliminative cleavage of polysaccharides containing (1→4)-linked D-glucuronate or L-iduronate residues and (1→4)-α-linked 2-sulfoamino-2-deoxy-6-sulfo-D-glucose residues to give oligosaccharides with terminal 4-deoxy-α-D-gluc-4-enuronosyl groups at their non-reducing ends.

**Heparinase II.** *1187700.* [149371-12-0].

Enzyme from *Flavobacterium heparinum* that depolymerises sulfated polysaccharide chains containing 1→4 linkages between hexosamines and uronic acid residues (both iduronic and glucuronic acid residues). The reaction yields oligosaccharide products (mainly disaccharides) containing unsaturated uronic acids.

**Heparinase III.** *1187800.* [37290-86-1]. Heparin-sulfate lyase (EC *4.2.2.8*).

Enzyme from *Flavobacterium heparinum* that depolymerises selectively sulfated polysaccharide chains containing 1→4 linkages between hexosamines and glucuronic acid residues to give oligosaccharide products (mainly disaccharides) containing unsaturated uronic acids.

**HEPES.** $C_8H_{18}N_2O_4S$. ($M_r$ 238.3). *1106800.* [7365-45-9]. 2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethanesulfonic acid.

White or almost white powder.

mp: about 236 °C, with decomposition

**Heptachlor.** $C_{10}H_5Cl_7$. ($M_r$ 373.3). *1128000.* [76-44-8].

bp: about 135 °C.

mp: about 95 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Heptachlor epoxide.** $C_{10}H_5Cl_7O$. ($M_r$ 389.3). *1128100.* [1024-57-3].

bp: about 200 °C.

mp: about 160 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Heptafluorobutyric acid.** $C_4HF_7O_2$. ($M_r$ 214.0). *1162400.* [375-22-4]. HFBA.

Clear, colourless liquid. Corrosive.

$d_{20}^{20}$: about 1.645.

$n_D^{20}$: about 1.300.

bp: about 120 °C.

*Content*: minimum 99.5 per cent.

**Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butanamide.** $C_8H_{12}F_7NOSi$. ($M_r$ 299.3). *1139500.* [53296-64-3]. 2,2,3,3,4,4,4-Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)-butyramide.

Clear, colourless liquid, flammable.

$n_D^{20}$: about 1.351.

bp: about 148 °C.

**Heptane.** $C_7H_{16}$. ($M_r$ 100.2). *1042000.* [142-82-5].

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

$d_{20}^{20}$: 0.683 to 0.686.

$n_D^{20}$: 1.387 to 1.388.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 97 °C and 98 °C.

**Hesperidin.** $C_{28}H_{34}O_{15}$. ($M_r$ 611). *1139000.* [520-26-3]. (*S*)-7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one.

Hygroscopic powder, slightly soluble in water and in methanol.

mp: 258 °C to 262 °C.

**Hexachlorobenzene.** $C_6Cl_6$. ($M_r$ 284.8). *1128200.* [118-74-1].

bp: about 332 °C.

mp: about 230 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**α-Hexachlorocyclohexane.** $C_6H_6Cl_6$. ($M_r$ 290.8). *1128300.* [319-84-6].

bp: about 288 °C.

mp: about 158 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**β-Hexachlorocyclohexane.** $C_6H_6Cl_6$. ($M_r$ 290.8). *1128400.* [319-85-7].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**δ-Hexachlorocyclohexane.** $C_6H_6Cl_6$. ($M_r$ 290.8). *1128500.* [319-86-8].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Hexacosane.** $C_{26}H_{54}$. ($M_r$ 366.7). *1042200.* [630-01-3].

Colourless or white or almost white flakes.

mp: about 57 °C.

**Hexadimethrine bromide.** $(C_{13}H_{30}Br_2N_2)_n$. *1042300.* [28728-55-4]. 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide. Poly(1,1,5,5-tetramethyl-1,5-azonia-undecamethylene dibromide).

White or almost white, amorphous powder, hygroscopic, soluble in water.

*Storage*: in an airtight container.

**2,2′,2″,6,6′,6″-Hexa(1,1-dimethylethyl)-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol.** $C_{54}H_{78}O_3$. ($M_r$ 775). *1042100.* 2,2′,2″,6,6′,6″-Hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol.

Crystalline powder, practically insoluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 244 °C.

**1,1,1,3,3,3-Hexafluoropropan-2-ol.** $C_3H_2F_6O$. ($M_r$ 168.0). *1136000.* [920-66-1].

*Content*: minimum 99.0 per cent, determined by gas chromatography.

Clear, colourless liquid, miscible with water and with anhydrous ethanol.

$d_{20}^{20}$: about 1.596.

bp: about 59 °C.

**Hexamethyldisilazane.** $C_6H_{19}NSi_2$. ($M_r$ 161.4). *1042400.* [999-97-3].

Clear, colourless liquid.

$d_{20}^{20}$: about 0.78.

$n_D^{20}$: about 1.408.

bp: about 125 °C.

*Storage*: in an airtight container.

**Hexamethylenetetramine.** $C_6H_{12}N_4$. ($M_r$ 140.2). *1042500.* [100-97-0]. Hexamine. 1,3,5,7-Tetraazatricyclo[3.3.1.1$^{3,7}$]-decane.

Colourless, crystalline powder, very soluble in water.

**Hexane.** $C_6H_{14}$. ($M_r$ 86.2). *1042600.* [110-54-3].

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

$d_{20}^{20}$: 0.659 to 0.663.

$n_D^{20}$: 1.375 to 1.376.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 67 °C and 69 °C.

*Hexane used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 0.01 from 260 nm to 420 nm, determined using *water R* as compensation liquid.

**Hexylamine.** $C_6H_{15}N$. ($M_r$ 101.2). *1042700.* [111-26-2]. Hexan-1-amine.

Colourless liquid, slightly soluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.766.

$n_D^{20}$: about 1.418.

bp: 127 °C to 131 °C.

**Hibifolin.** $C_{21}H_{18}O_{14}$. ($M_r$ 494.4). *1207000.* [55366-56-8]. 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4*H*-1-benzopyran-8-yl β-D-glucopyranosiduronic acid. Gossypetin 8-*O*-glucuronide. Gossypetin 8-*O*-β-D-glucuropyranoside.

*Storage*: protected from light, at a temperature not exceeding 8 °C, in a dry place.

**Histamine dihydrochloride.** *1042800.* [56-92-8].

See *Histamine dihydrochloride (0143)*.

**Histamine solution.** *1042901.*

A 9 g/L solution of *sodium chloride R* containing 0.1 µg per millilitre of histamine base (as the phosphate or dihydrochloride).

**Histidine.** *1187900.* [71-00-1]. (2*S*)-2-Amino-3-(1*H*-imidazol-4-yl)propanoic acid.

**Histidine monohydrochloride.** $C_6H_{10}ClN_3O_2,H_2O$. ($M_r$ 209.6). *1043000.* [123333-71-1]. (*RS*)-2-Amino-3-(imidazol-4-yl)propionic acid hydrochloride monohydrate.

Crystalline powder or colourless crystals, soluble in water.

mp: about 250 °C, with decomposition.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Histamine dihydrochloride (0143)*; the chromatogram shows only one principal spot.

**Holmium oxide.** $Ho_2O_3$. ($M_r$ 377.9). *1043100.* [12055-62-8]. Diholmium trioxide.

Yellowish powder, practically insoluble in water.

**Holmium perchlorate solution.** *1043101.*

A 40 g/L solution of *holmium oxide R* in a solution of *perchloric acid R* containing 141 g/L of $HClO_4$.

**DL-Homocysteine.** $C_4H_9NO_2S$. ($M_r$ 135.2). *1136100.* [454-29-5]. (2*RS*)-2-Amino-4-sulfanylbutanoic acid.

White or almost white, crystalline powder.

mp: about 232 °C.

**L-Homocysteine thiolactone hydrochloride.** $C_4H_8ClNOS$. ($M_r$ 153.6). *1136200.* [31828-68-9]. (3*S*)-3-Aminodihydrothiophen-2(3*H*)-one hydrochloride.

White or almost white, crystalline powder.

mp: about 202 °C.

**Homoorientin.** $C_{21}H_{20}O_{11}$. ($M_r$ 448.4). *1189500.* [4261-42-1]. 2-(3,4-Dihydroxyphenyl)-6-β-D-glucopyranosyl-5,7-dihydroxy-4*H*-1-benzopyran-4-one. Isoorientin. Luteolin-6-*C*-glucoside.

**Honokiol.** $C_{18}H_{18}O_2$. ($M_r$ 266.3). *1182700.* [35354-74-6]. 3′,5-Di(prop-2-enyl)biphenyl-2,4′-diol. 3′,5-Diallyl-2,4′-dihydroxybiphenyl. 3′,5-Di-2-propenyl-[1,1′-biphenyl]-2,4′-diol.

**Human tissue factor solution.** *1186100.*

Solution containing human tissue factor, which may be produced by recombinant DNA technology, combined with phospholipids and calcium buffers. Suitable stabilisers may be added.

**Hyaluronidase diluent.** *1043300.*

Mix 100 mL of *phosphate buffer solution pH 6.4 R* with 100 mL of *water R*. Dissolve 0.140 g of *hydrolysed gelatin R* in the solution at 37 °C.

*Storage*: use within 2 h.

**Hydrastine hydrochloride.** $C_{21}H_{22}ClNO_6$. ($M_r$ 419.9). *1154000.* [5936-28-7]. (3*S*)-6,7-Dimethoxy-3-[(5*R*)-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one hydrochloride.

White or almost white powder, hygroscopic, very soluble in water and in ethanol (96 per cent).

$[\alpha]_D^{17}$: about + 127.

mp: about 116 °C.

*Hydrastine hydrochloride used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Goldenseal rhizome (1831)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Hydrazine.** $H_4N_2$. ($M_r$ 32.05). *1136300.* [302-01-2]. Diazane.

Slightly oily liquid, colourless, with a strong odour of ammonia, miscible with water. Dilute solutions in water are commercially available.

$n_D^{20}$: about 1.470.

bp: about 113 °C.

mp: about 1.5 °C.

*Caution: toxic and corrosive.*

**Hydrazine sulfate.** $H_6N_2O_4S$. ($M_r$ 130.1). *1043400.* [10034-93-2].

Colourless crystals, sparingly soluble in cold water, soluble in hot water (50 °C) and freely soluble in boiling water, practically insoluble in ethanol (96 per cent).

*Content*: minimum 99 per cent.

**Hydriodic acid.** HI. ($M_r$ 127.9). *1098900.* [10034-85-2].

Prepare by distilling hydriodic acid over red phosphorus, passing *carbon dioxide R* or *nitrogen R* through the apparatus during the distillation. Use the colourless or almost colourless, constant-boiling mixture (55 per cent to 58 per cent of HI) distilling between 126 °C and 127 °C.

Place the acid in small, amber, glass-stoppered bottles previously flushed with *carbon dioxide R* or *nitrogen R*, seal with paraffin.

*Storage*: in a dark place.

**Hydrobromic acid, 30 per cent.** *1098700.* [10035-10-6].

A 30 per cent solution of hydrobromic acid in *glacial acetic acid R*.

Degas with caution the contents before opening.

**Hydrobromic acid, dilute.** *1098701.*

Place 5.0 mL of *30 per cent hydrobromic acid R* in amber vials equipped with polyethylene stoppers. Seal under *argon R* and store in the dark. Add 5.0 mL of *glacial acetic acid R* immediately before use. Shake.

*Storage*: in the dark.

**Hydrobromic acid, 47 per cent.** *1118900.*

A 47 per cent *m/m* solution of hydrobromic acid.

**Hydrobromic acid, dilute R1.** *1118901.*

Contains 7,9 g/L of HBr.

Dissolve 16.81 g of *47 per cent hydrobromic acid R* in *water R* and dilute to 1000 mL with the same solvent.

**Hydrochloric acid.** *1043500.* [7647-01-0].

See *Concentrated hydrochloric acid (0002)*.

**0.1 M Hydrochloric acid, alcoholic.** *3008800.*

Dilute 9.0 mL of *hydrochloric acid R* to 1000.0 mL with *aldehyde-free alcohol R*.

**2 M Hydrochloric acid.** *3001700.*

Dilute 206.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

**3 M Hydrochloric acid.** *3001600.*

Dilute 309.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

**6 M Hydrochloric acid.** *3001500.*

Dilute 618.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

**Hydrochloric acid R1.** *1043501.*

Contains 250 g/L of HCl.

Dilute 70 g of *hydrochloric acid R* to 100 mL with *water R*.

**Hydrochloric acid, brominated.** *1043507.*

To 1 mL of *bromine solution R* add 100 mL of *hydrochloric acid R*.

**Hydrochloric acid, dilute.** *1043503.*

Contains 73 g/L of HCl.

Dilute 20 g of *hydrochloric acid R* to 100 mL with *water R*.

**Hydrochloric acid, dilute, heavy metal-free.** *1043509.*

Complies with the requirements prescribed for *dilute hydrochloric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.003 ppm.

Cu: 0.003 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.004 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

**Hydrochloric acid, dilute R1.** *1043504.*

Contains 0.37 g/L of HCl.

Dilute 1.0 mL of *dilute hydrochloric acid R* to 200.0 mL with *water R*.

**Hydrochloric acid, dilute R2.** *1043505.*

Dilute 30 mL of *1 M hydrochloric acid* to 1000 mL with *water R*; adjust to pH 1.6 ± 0.1.

**Hydrochloric acid, dilute R3.** *1203800.*

Contains 3.7 g/L of HCl.

Dilute 10.0 mL of *dilute hydrochloric acid R* to 200.0 mL with *water R*.

**Hydrochloric acid, ethanolic.** *1043506.*

Dilute 5.0 mL of *1 M hydrochloric acid* to 500.0 mL with *ethanol (96 per cent) R*.

**Hydrochloric acid, heavy metal-free.** *1043510.*

Complies with the requirements prescribed for *hydrochloric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.003 ppm.

Cu: 0.003 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.004 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

**Hydrochloric acid, lead-free.** *1043508.*

Complies with the requirements prescribed for *hydrochloric acid R* with the following additional requirement.

*Lead*: maximum 20 ppb.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. In a quartz crucible evaporate 200 g of the acid to be examined almost to dryness. Take up the residue in 5 mL of nitric acid prepared by sub-boiling distillation of *nitric acid R* and evaporate to dryness. Take up the residue in 5 mL of nitric acid prepared by sub-boiling distillation of *nitric acid R*.

*Reference solutions*. Prepare the reference solutions using *lead standard solution (0.1 ppm Pb) R* diluted with nitric acid prepared by sub-boiling distillation of *nitric acid R*.

*Wavelength*: 220.35 nm.

**Hydrochloric acid, methanolic.** *1043511.*

Dilute 4.0 mL of *hydrochloric acid R* to 1000.0 mL with *methanol R2*.

**Hydrocortisone acetate.** *1098800.* [50-03-3].

See *Hydrocortisone acetate (0334)*.

**Hydrofluoric acid.** HF. ($M_r$ 20.01). *1043600.* [7664-39-3].

*Content*: minimum 40.0 per cent *m/m*.

Clear, colourless liquid.

*Loss on ignition*: not more than 0.05 per cent *m/m*; evaporate the hydrofluoric acid in a platinum crucible and gently ignite the residue to constant mass.

*Assay*. Weigh accurately a glass-stoppered flask containing 50.0 mL of *1 M sodium hydroxide*. Introduce 2 g of the hydrofluoric acid and weigh again. Titrate the solution with *0.5 M sulfuric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 20.01 mg of HF.

*Storage*: in a polyethylene container.

**Hydrogen for chromatography.** $H_2$. ($M_r$ 2.016). *1043700.* [1333-74-0].

*Content*: minimum 99.95 per cent *V/V*.

**Hydrogen peroxide solution, dilute.** *1043800.* [7722-84-1].

See *Hydrogen peroxide solution (3 per cent) (0395)*.

**Hydrogen peroxide solution, strong.** *1043900.* [7722-84-1].

See *Hydrogen peroxide solution (30 per cent) (0396)*.

**Hydrogen sulfide.** $H_2S$. ($M_r$ 34.08). *1044000.* [7783-06-4].

Gas, slightly soluble in water.

**Hydrogen sulfide solution.** *1136400.*

A recently prepared solution of *hydrogen sulfide R* in *water R*. The saturated solution contains about 0.4 per cent to 0.5 per cent of $H_2S$ at 20 °C.

**Hydrogen sulfide R1.** $H_2S$. ($M_r$ 34.08). *1106600.* [7783-06-4].

*Content*: minimum 99.7 per cent *V/V*.

**Hydroquinone.** $C_6H_6O_2$. ($M_r$ 110.1). *1044100.* [123-31-9].

Benzene-1,4-diol.

Fine, colourless or white or almost white needles, darkening on exposure to air and light, soluble in water and in ethanol (96 per cent).

mp: about 173 °C.

*Storage*: protected from light and air.

**Hydroquinone solution.** *1044101.*

Dissolve 0.5 g of *hydroquinone R* in *water R*, add 20 µL of *sulfuric acid R* and dilute to 50 mL with *water R*.

**4′-Hydroxyacetophenone.** $C_8H_8O_2$. ($M_r$ 136.2). *1196900.* [99-93-4]. 1-(4-Hydroxyphenyl)ethan-1-one.

**2-Hydroxybenzimidazole.** $C_7H_6N_2O$. ($M_r$ 134.1). *1169600.* [615-16-7]. 1*H*-benzimidazol-2-ol.

**4-Hydroxybenzohydrazide.** $C_7H_8N_2O_2$. ($M_r$ 152.2). *1145900.* [5351-23-5]. *p*-Hydroxybenzohydrazide.

**4-Hydroxybenzoic acid.** $C_7H_6O_3$. ($M_r$ 138.1). *1106700.* [99-96-7].

Crystals, slightly soluble in water, very soluble in ethanol (96 per cent), soluble in acetone.

mp: 214 °C to 215 °C.

**4-Hydroxycoumarin.** $C_9H_6O_3$. ($M_r$ 162.2). *1169700.* [1076-38-6]. 4-Hydroxy-2*H*-1-benzopyran-2-one.

White or almost white powder, freely soluble in methanol.

*Content*: minimum 98.0 per cent.

**6-Hydroxydopa.** $C_9H_{11}NO_5$. ($M_r$ 213.2). *1169800.* [21373-30-8]. (2*RS*)-2-Amino-3-(2,4,5-trihydroxyphenyl)-propanoic acid. 2,5-Dihydroxy-DL-tyrosine.

mp: about 257 °C.

**4-Hydroxyisophthalic acid.** $C_8H_6O_5$. ($M_r$ 182.1). *1106900.* [636-46-4]. 4-Hydroxybenzene-1,3-dicarboxylic acid.

Needles or platelets, very slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 314 °C, with decomposition.

**Hydroxylamine hydrochloride.** $NH_4ClO$. ($M_r$ 69.5). *1044300.* [5470-11-1].

White or almost white, crystalline powder, very soluble in water, soluble in ethanol (96 per cent).

**Hydroxylamine hydrochloride solution R2.** *1044304.*

Dissolve 2.5 g of *hydroxylamine hydrochloride R* in 4.5 mL of hot *water R* and add 40 mL of *ethanol (96 per cent) R* and 0.4 mL of *bromophenol blue solution R2*. Add *0.5 M alcoholic potassium hydroxide* until a greenish-yellow colour is obtained. Dilute to 50.0 mL with *ethanol (96 per cent) R*.

**Hydroxylamine solution, alcoholic.** *1044301.*

Dissolve 3.5 g of *hydroxylamine hydrochloride R* in 95 mL of *ethanol (60 per cent V/V) R*, add 0.5 mL of a 2 g/L solution of *methyl orange R* in *ethanol (60 per cent V/V) R* and sufficient *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* to give a pure yellow colour. Dilute to 100 mL with *ethanol (60 per cent V/V) R*.

**Hydroxylamine solution, alkaline.** *1044302.*

Immediately before use, mix equal volumes of a 139 g/L solution of *hydroxylamine hydrochloride R* and a 150 g/L solution of *sodium hydroxide R*.

**Hydroxylamine solution, alkaline R1.** *1044303.*

*Solution A*. Dissolve 12.5 g of *hydroxylamine hydrochloride R* in *methanol R* and dilute to 100 mL with the same solvent.

*Solution B*. Dissolve 12.5 g of *sodium hydroxide R* in *methanol R* and dilute to 100 mL with the same solvent.

Mix equal volumes of solution A and solution B immediately before use.

**Hydroxymethylfurfural.** $C_6H_6O_3$. ($M_r$ 126.1). *1044400.* [67-47-0]. 5-Hydroxymethylfurfural.

Acicular crystals, freely soluble in water, in acetone and in ethanol (96 per cent).

mp: about 32 °C.

**Hydroxynaphthol blue, sodium salt.** $C_{20}H_{11}N_2Na_3O_{11}S_3$. ($M_r$ 620). *1044500.* [63451-35-4]. Trisodium 2,2′-dihydroxy-1,1′-azonaphthalene-3′,4,6′-trisulfonate.

**2-Hydroxypropylbetadex for chromatography.** *1146000.* Betacyclodextrin modified by the bonding of (*R*) or (*RS*) propylene oxide groups on the hydroxyl groups.

**Hydroxypropyl-β-cyclodextrin.** *1128600.* [94035-02-6].

See *Hydroxypropylbetadex (1804)*.

pH (*2.2.3*): 5.0 to 7.5 for a 20 g/L solution.

**Hydroxyquinoline.** $C_9H_7NO$. ($M_r$ 145.2). *1044600.* [148-24-3]. 8-Hydroxyquinoline. Quinolin-8-ol.

White or slightly yellowish, crystalline powder, slightly soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in dilute mineral acids.

mp: about 75 °C.

*Sulfated ash* (*2.4.14*): maximum 0.05 per cent.

**12-Hydroxystearic acid.** $C_{18}H_{36}O_3$. ($M_r$ 300.5). *1099000.* [106-14-9]. 12-Hydroxyoctadecanoic acid.

White or almost white powder.

mp: 71 °C to 74 °C.

**5-Hydroxyuracil.** $C_4H_4N_2O_3$. ($M_r$ 128.1). *1044700.* [496-76-4]. Isobarbituric acid. Pyrimidine-2,4,5-triol.

White or almost white, crystalline powder.

mp: about 310 °C, with decomposition.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Fluorouracil (0611)*; the chromatogram shows a principal spot with an $R_F$ of about 0.3.

*Storage*: in an airtight container.

**Hyoscine hydrobromide.** *1044800.* [6533-68-2].

See *Hyoscine hydrobromide (0106)*.

**Hyoscyamine sulfate.** *1044900.* [620-61-1].

See *Hyoscyamine sulfate (0501)*.

**Hypericin.** $C_{30}H_{16}O_8$. ($M_r$ 504.4). *1149800.* [548-04-9]. 1,3,4,6,8,13-Hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-*opqra*]perylene-7,14-dione.

*Content*: minimum 85 per cent.

**Hyperoside.** $C_{21}H_{20}O_{12}$. ($M_r$ 464.4). *1045000.* 2-(3,4-Dihydroxyphenyl)-3-β-D-galactopyranosyloxy-5,7-dihydroxychromen-4-one.

Faint yellow needles, soluble in methanol.

*Absorbance* (*2.2.25*). A solution in *methanol R* shows 2 absorption maxima at about 257 nm and at about 359 nm.

**Hypophosphorous reagent.** *1045200.*

Dissolve with the aid of gentle heat, 10 g of *sodium hypophosphite R* in 20 mL of *water R* and dilute to 100 mL with *hydrochloric acid R*. Allow to settle and decant or filter through glass wool.

**Hypoxanthine.** $C_5H_4N_4O$. ($M_r$ 136.1). *1045300.* [68-94-0]. 1*H*-Purin-6-one.

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in dilute acids and in dilute alkali hydroxide solutions, decomposes without melting at about 150 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Mercaptopurine (0096)*; the chromatogram shows only one principal spot.

**Ibuprofen.** *1197000.* [15687-27-1].

See *Ibuprofen (0721)*.

**Imidazole.** $C_3H_4N_2$. ($M_r$ 68.1). *1045400.* [288-32-4].

White or almost white, crystalline powder, soluble in water and in ethanol (96 per cent).

mp: about 90 °C.

**Iminodiacetic acid.** $C_4H_7NO_4$. ($M_r$ 133.1). *1192300.* [142-73-4]. 2,2′-Iminodiacetic acid.

**Iminodibenzyl.** $C_{14}H_{13}N$. ($M_r$ 195.3). *1045500.* [494-19-9]. 10,11-Dihydrodibenz[*b,f*]azepine.

Pale yellow, crystalline powder, practically insoluble in water, freely soluble in acetone.

mp: about 106 °C.

**Imipramine hydrochloride.** *1207100.* [113-52-0].

See *Imipramine hydrochloride (0029)*.

**Imperatorin.** $C_{16}H_{14}O_4$. ($M_r$ 270.3). *1180200.* [482-44-0]. 9-[(3-Methylbut-2-enyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one.

**2-Indanamine hydrochloride.** $C_9H_{12}ClN$. ($M_r$ 169.7). *1175800.* [2338-18-3]. 2-Aminoindane hydrochloride.

2,3-Dihydro-1*H*-inden-2-amine hydrochloride.

**Indigo.** $C_{16}H_{10}N_2O_2$. ($M_r$ 262.3). *1192800.* [482-89-3]. Indigotin. 1,1′,3,3′-Tetrahydro-2-2′-bi(indolylidene)-3,3′-dione.

**Indigo carmine.** $C_{16}H_8N_2Na_2O_8S_2$. ($M_r$ 466.3). *1045600.* [860-22-0].

Schultz No. 1309.

Colour Index No. 73015.
3,3′-Dioxo-2,2′-bisindolylidene-5,5′-disulfonate disodium. E 132.

It usually contains sodium chloride.

Blue or violet-blue powder or blue granules with a coppery lustre, sparingly soluble in water, practically insoluble in ethanol (96 per cent). It is precipitated from an aqueous solution by sodium chloride.

**Indigo carmine solution.** *1045601.*

To a mixture of 10 mL of *hydrochloric acid R* and 990 mL of 200 g/L *nitrogen-free sulfuric acid R* add 0.2 g of *indigo carmine R*.

*The solution complies with the following test*: add 10 mL to a solution of 1.0 mg of *potassium nitrate R* in 10 mL of *water R*, rapidly add 20 mL of *nitrogen-free sulfuric acid R* and heat to boiling. The blue colour is discharged within 1 min.

**Indigo carmine solution R1.** *1045602.*

Dissolve 4 g of *indigo carmine R* in about 900 mL of *water R* added in several portions. Add 2 mL of *sulfuric acid R* and dilute to 1000 mL with *water R*.

*Assay*. Place in a 100 mL conical flask with a wide neck 10.0 mL of *nitrate standard solution (100 ppm $NO_3$) R*, 10 mL of *water R*, 0.05 mL of the *indigo carmine solution R1*, and then in a single addition, but with caution, 30 mL of *sulfuric acid R*. Titrate the solution immediately, using the *indigo carmine solution R1*, until a stable blue colour is obtained.

The number of millilitres used, *n*, is equivalent to 1 mg of $NO_3$.

**Indirubin.** $C_{16}H_{10}N_2O_2$. ($M_r$ 262.3). *1192900.* [479-41-4].

1,1′,2′,3-Tetrahydro-2,3′-bi(indolylidene)-2′,3-dione.

**Indometacin.** *1101500.* [53-86-1].

See *Indometacin (0092)*.

**Inosine.** $C_{10}H_{12}N_4O_5$. ($M_r$ 268.2). *1169900.* [58-63-9]. 9-β-D-Ribofuranosylhypoxanthine. 9-β-D-Ribofuranosyl-1,9-dihydro-6*H*-purin-6-one.

mp: 222 °C to 226 °C.

***myo*-Inositol.** *1161100.*

See *myo-Inositol (1805)*.

**Iodine.** *1045800.* [7553-56-2].

See *Iodine (0031)*.

**Iodine solution R1.** *1045801.*

To 10.0 mL of *0.05 M iodine* add 0.6 g of *potassium iodide R* and dilute to 100.0 mL with *water R*. Prepare immediately before use.

**Iodine solution R2.** *1045802.*

To 10.0 mL of *0.05 M iodine* add 0.6 g of *potassium iodide R* and dilute to 1000.0 mL with *water R*. Prepare immediately before use.

**Iodine solution R3.** *1045803.*

Dilute 2.0 mL of *iodine solution R1* to 100.0 mL with *water R*. Prepare immediately before use.

**Iodine solution R4.** *1045806.*

Dissolve 14 g of *iodine R* in 100 mL of a 400 g/L solution of *potassium iodide R*, add 1 mL of *dilute hydrochloric acid R* and dilute to 1000 mL with *water R*.

*Storage*: protected from light.

**Iodine solution R5.** *1045807.*

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent (0.05 M solution).

**Iodine solution, alcoholic.** *1045804.*

A 10 g/L solution of *iodine R* in *ethanol (96 per cent) R*.

*Storage*: protected from light.

**Iodine solution, chloroformic.** *1045805.*

A 5 g/L solution of *iodine R* in *chloroform R*.

*Storage*: protected from light.

**Iodine-123 and ruthenium-106 spiking solution.** *1166700.*

*Prepare immediately before use*. Mix 3.5 mL of an 18.5 kBq/mL solution of ruthenium-106 in the form of ruthenium trichloride in a mixture of equal volumes of *glacial acetic acid R* and *water R* with 200 µL of a 75 kBq/mL solution of iodine-123 in the form of sodium iodide in *water R*.

**Iodine bromide.** IBr. ($M_r$ 206.8). *1045900.* [7789-33-5].

Bluish-black or brownish-black crystals, freely soluble in water, in ethanol (96 per cent) and in glacial acetic acid.

bp: about 116 °C.

mp: about 40 °C.

*Storage*: protected from light.

**Iodine bromide solution.** *1045901.*

Dissolve 20 g of *iodine bromide R* in *glacial acetic acid R* and dilute to 1000 mL with the same solvent.

*Storage*: protected from light.

**Iodine chloride.** ICl. ($M_r$ 162.4). *1143000.* [7790-99-0].

Black crystals, soluble in water, in acetic acid and in ethanol (96 per cent).

bp: about 97.4 °C.

**Iodine chloride solution.** *1143001.*

Dissolve 1.4 g of *iodine chloride R* in *glacial acetic acid R* and dilute to 100 mL with the same acid.

*Storage*: protected from light.

**Iodine pentoxide, recrystallised.** $I_2O_5$. ($M_r$ 333.8). *1046000.* [12029-98-0]. Di-iodine pentoxide. Iodic anhydride.

*Content*: minimum 99.5 per cent.

White or almost white, crystalline powder, or white or greyish-white granules, hygroscopic, very soluble in water forming $HIO_3$.

*Stability on heating.* Dissolve 2 g, previously heated for 1 h at 200 °C, in 50 mL of *water R*. A colourless solution is obtained.

*Assay.* Dissolve 0.100 g in 50 mL of *water R*, add 3 g of *potassium iodide R* and 10 mL of *dilute hydrochloric acid R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.782 mg of $I_2O_5$.

*Storage*: in an airtight container, protected from light.

**Iodoacetamide.** $C_2H_4INO$. ($M_r$ 185.0). *1186200.* [144-48-9]. 2-Iodoacetamide.

Slightly yellow, crystalline powder, soluble in water.

mp: about 92 °C.

**Iodoacetic acid.** $C_2H_3IO_2$. ($M_r$ 185.9). *1107000.* [64-69-7].

Colourless or white or almost white crystals, soluble in water and in ethanol (96 per cent).

mp: 82 °C to 83 °C.

**2-Iodobenzoic acid.** $C_7H_5IO_2$. ($M_r$ 248.0). *1046100.* [88-67-5].

White or slightly yellow, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 160 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*), using cellulose for chromatography $f_{254}$ R as the coating substance: apply to the plate 20 µL of a solution of the 2-iodobenzoic acid, prepared by dissolving 40 mg in 4 mL of *0.1 M sodium hydroxide* and diluting to 10 mL with *water R*. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of *water R*, 40 volumes of *glacial acetic acid R* and 40 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

**3-Iodobenzylammonium chloride.** $C_7H_9ClIN$. ($M_r$ 269.5). *1168000.* [3718-88-5]. 1-(3-Iodophenyl)methanamine hydrochloride. 1-(3-Iodophenyl)methanaminium chloride. *m*-Iodobenzylamine hydrochloride.

White or almost white crystals.

mp: 188 °C to 190 °C.

**Iodoethane.** $C_2H_5I$. ($M_r$ 156.0). *1099100.* [75-03-6].

*Content*: minimum 99 per cent.

Colourless or slightly yellowish liquid, darkening on exposure to air and light, miscible with ethanol (96 per cent) and most organic solvents.

$d_{20}^{20}$: about 1.95.

$n_D^{20}$: about 1.513.

bp: about 72 °C.

*Storage*: in an airtight container, protected from light.

**2-Iodohippuric acid.** $C_9H_8INO_3,2H_2O$. ($M_r$ 341.1). *1046200.* [147-58-0]. 2-(2-Iodobenzamido)acetic acid.

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 170 °C.

*Water* (*2.5.12*): 9 per cent to 13 per cent, determined on 1.000 g.

*Chromatography*. Thin-layer chromatography (*2.2.27*), using *cellulose for chromatography $F_{254}$ R* as the coating substance: apply to the plate 20 µL of a solution of the 2-iodohippuric acid, prepared by dissolving 40 mg in 4 mL of *0.1 M sodium hydroxide* and diluting to 10 mL with *water R*. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of *water R*, 40 volumes of *glacial acetic acid R* and 40 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

**Iodoplatinate reagent.** *1046300.*

To 3 mL of a 100 g/L solution of *chloroplatinic acid R* add 97 mL of *water R* and 100 mL of a 60 g/L solution of *potassium iodide R*.

*Storage*: protected from light.

**Iodoplatinate reagent R1.** *1172200.*

Mix 2.5 mL of a 50 g/L solution of *chloroplatinic acid R*, 22.5 mL of a 100 g/L solution of *potassium iodide R* and 50 mL of *water R*.

*Storage*: protected from light, at a temperature of 2-8 °C.

**Iodosulfurous reagent.** *1046400.*

The apparatus, which must be kept closed and dry during the preparation, consists of a 3000 mL to 4000 mL round-bottomed flask with three inlets for a stirrer and a thermometer and fitted with a drying tube. To 700 mL of *anhydrous pyridine R* and 700 mL of *ethylene glycol monomethyl ether R* add, with constant stirring, 220 g of finely powdered *iodine R*, previously dried over *diphosphorus pentoxide R*. Continue stirring until the iodine has completely dissolved (about 30 min). Cool to − 10 °C, and add quickly, still stirring, 190 g of *sulfur dioxide R*. Do not allow the temperature to exceed 30 °C. Cool.

*Assay*. Add about 20 mL of *anhydrous methanol R* to a titration vessel and titrate to the end-point with the iodosulfurous reagent (*2.5.12*). Introduce in an appropriate form a suitable amount of *water R*, accurately weighed, and repeat the determination of water. Calculate the water equivalent in milligrams per millilitre of iodosulfurous reagent.

The minimum water equivalent is 3.5 mg of water per millilitre of reagent.

Work protected from humidity. Standardise immediately before use.

*Storage*: in a dry container.

**5-Iodouracil.** $C_4H_3IN_2O_2$. ($M_r$ 238.0). *1046500.* [696-07-1]. 5-Iodo-1*H*,3*H*-pyrimidine-2,4-dione.

mp: about 276 °C, with decomposition.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram obtained shows only one principal spot.

**Ion-exclusion resin for chromatography.** *1131000.*

A resin with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

**Ion-exchange resin, strongly acidic.** *1085400.*

Resin in protonated form with sulfonic acid groups attached to a lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as spherical beads; unless otherwise prescribed, the particle size is 0.3 mm to 1.2 mm.

*Capacity*. 4.5 mmol to 5 mmol per gram, with a water content of 50 per cent to 60 per cent.

*Preparation of a column.* Unless otherwise prescribed, use a tube with a fused-in sintered glass disc having a length of 400 mm, an internal diameter of 20 mm and a filling height of about 200 mm. Introduce the resin, mixing it with *water R* and pouring the slurry into the tube, ensuring that no air bubbles are trapped between the particles. When in use, the liquid must not be allowed to fall below the surface of the resin. If the resin is in its protonated form, wash with *water R* until 50 mL requires not more than 0.05 mL of *0.1 M sodium hydroxide* for neutralisation, using 0.1 mL of *methyl orange solution R* as indicator.

If the resin is in its sodium form or if it requires regeneration, pass about 100 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R* slowly through the column and then wash with *water R* as described above.

**Irisflorentin.** $C_{20}H_{18}O_8$. ($M_r$ 386.4). *1186300.* [41743-73-1]. 9-Methoxy-7-(3,4,5-trimethoxyphenyl)-8*H*-1,3-dioxolo-[4,5-*g*][1]benzopyran-8-one.

**Iron.** Fe. ($A_r$ 55.85). *1046600.* [7439-89-6].

Grey powder or wire, soluble in dilute mineral acids.

**Iron salicylate solution.** *1046700.*

Dissolve 0.1 g of *ferric ammonium sulfate R* in a mixture of 2 mL of *dilute sulfuric acid R* and 48 mL of *water R* and dilute to 100 mL with *water R*. Add 50 mL of a 11.5 g/L solution of *sodium salicylate R*, 10 mL of *dilute acetic acid R*, 80 mL of a 136 g/L solution of *sodium acetate R* and dilute to 500 mL with *water R*. The solution should be recently prepared.

*Storage*: in an airtight container, protected from light.

**Isatin.** $C_8H_5NO_2$. ($M_r$ 147.1). *1046800.* [91-56-5]. Indoline-2,3-dione.

Small, yellowish-red crystals, slightly soluble in water, soluble in hot water and in ethanol (96 per cent), soluble in solutions of alkali hydroxides giving a violet colour becoming yellow on standing.

mp: about 200 °C, with partial sublimation.

*Sulfated ash* (*2.4.14*): maximum 0.2 per cent.

**Isatin reagent.** *1046801.*

Dissolve 6 mg of *ferric sulfate R* in 8 mL of *water R* and add cautiously 50 mL of *sulfuric acid R*. Add 6 mg of *isatin R* and stir until dissolved.

The reagent should be pale yellow, but not orange or red.

**Isoamyl alcohol.** $C_5H_{12}O$. ($M_r$ 88.1). *1046900.* [123-51-3]. 3-Methylbutan-1-ol.

Colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

bp: about 130 °C.

**Isoamyl benzoate.** $C_{12}H_{16}O_2$. ($M_r$ 192.3). *1164200.* [94-46-2]. Isopentyl benzoate. 3-Methylbutyl benzoate.

$n_D^{20}$: about 1.494.

bp: about 261 °C.

Colourless or pale yellow liquid.

**Isoandrosterone.** $C_{19}H_{30}O_2$. ($M_r$ 290.4). *1107100.* [481-29-8]. Epiandrosterone. 3β-Hydroxy-5α-androstan-17-one.

White or almost white powder, practically insoluble in water, soluble in organic solvents.

$[\alpha]_D^{20}$: + 88, determined on 20 g/L solution in *methanol R*.

mp: 172 °C to 174 °C.

$\Delta A$ (*2.2.41*): $14.24 \times 10^3$, determined at 304 nm on a 1.25 g/L solution.

***N*-Isobutyldodecatetraenamide.** $C_{16}H_{25}NO$. ($M_r$ 247.4). *1159500.* [866602-52-0]. (2*E*,4*E*,8*Z*,10*EZ*)-*N*-2-(Methylpropyl)dodeca-2,4,8,10-tetraenamide.

White or almost white or non-coloured crystals.

mp: about 70 °C.

***N*-Isobutyldodecatetraenamide solution.** *1159501.*

A solution of *N-isobutyldodecatetraenamide R*, exactly weighed, in *methanol R* at a concentration of about 10 mg/mL.

**Isodrin.** $C_{12}H_8Cl_6$. ($M_r$ 364.9). *1128700.* [465-73-6]. 1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-*endo*,*endo*-1,4:5,8-dimethanonaphthalene.

Practically insoluble in water, soluble in common organic solvents such as acetone.

A suitable certified reference solution may be used.

**Isoeugenol.** $C_{10}H_{12}O_2$. ($M_r$ 164.2). *1206200.* [97-54-1]. 2-Methoxy-4-[(1Ξ)-prop-1-en-1-yl]phenol.

**Isoleucine.** *1185000.* [73-32-5].

See *Isoleucine (0770)*.

**Isomalt.** $C_{12}H_{24}O_{11}$. ($M_r$ 344.3). *1164300.* [64519-82-0].

Mixture of 6-*O*-α-D-glucopyranosyl-D-glucitol and of 1-*O*-α-D-glucopyranosyl-D-mannitol.

White or almost white powder or granules, freely soluble in water.

**Isomaltitol.** $C_{12}H_{24}O_{11}$. ($M_r$ 344.3). *1161200.* [534-73-6]. 6-*O*-α-D-Glucopyranosyl-D-glucitol.

White or almost white powder, freely soluble in water.

**Isomenthol.** $C_{10}H_{20}O$. ($M_r$ 156.3). *1047000.* [23283-97-8]. (+)-*Isomenthol:* (1*S*,2*R*,5*R*)-2-isopropyl-5-methylcyclohexanol. (±)-*Isomenthol:* a mixture of equal parts of (1*S*,2*R*,5*R*)- and (1*R*,2*S*,5*S*)-2-isopropyl-5-methylcyclohexanol.

Colourless crystals, practically insoluble in water, very soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: (+)-*Isomenthol*: about + 24, determined on a 100 g/L solution in *ethanol (96 per cent) R*.

bp: (+)-*Isomenthol*: about 218 °C. (±)-*Isomenthol*: about 218 °C.

mp: (+)-*Isomenthol*: about 80 °C. (±)-*Isomenthol*: about 53 °C.

**(+)-Isomenthone.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1047100.* (1*R*)-*cis*-*p*-Menthan-3-one. (1*R*)-*cis*-2-Isopropyl-5-methylcyclohexanone.

Contains variable amounts of menthone. A colourless liquid, very slightly soluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.904.

$n_D^{20}$: about 1.453.

$[\alpha]_D^{20}$: about + 93.2.

*Isomenthone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. The substance to be examined.

*Content*: minimum 80.0 per cent, calculated by the normalisation procedure.

**Isomethyleugenol.** $C_{11}H_{14}O_2$. ($M_r$ 178.2). *1181900.* [93-16-3]. 1,2-Dimethoxy-4-prop-1-enylbenzene.

Isomethyleugenol used in gas chromatography complies with the following additional test.

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Niaouli oil, cineole type (2468)*.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

**Isonicotinamide.** $C_6H_6N_2O$. ($M_r$ 122.1). *1193000.* [1453-82-3]. 4-Pyridinecarboxamide. Pyridine-4-carboxamide.

White or almost white, crystalline powder, soluble in water.

**Isonicotinic acid.** $C_6H_5NO_2$. ($M_r$ 123.1). *1202200.* [55-22-1]. Pyridine-4-carboxylic acid.

Creamish-white powder, sparingly soluble in water.

mp: about 311 °C.

**Isopropylamine.** $C_3H_9N$. ($M_r$ 59.1). *1119800.* [75-31-0]. Propan-2-amine.

Colourless, highly volatile, flammable liquid.

$n_D^{20}$: about 1.374.

bp: 32 °C to 34 °C.

**Isopropyl iodide.** $C_3H_7I$. ($M_r$ 170.0). *1166600.* [75-30-9]. 2-Iodopropane.

*Content*: minimum 99 per cent.

**Isopropyl methanesulfonate.** $C_4H_{10}O_3S$. ($M_r$ 138.2). *1179400.* [926-06-7]. 1-methylethyl methanesulfonate.

Clear, colourless liquid.

*Content*: minimum 99.0 per cent.

*Density*: about 1.129 g/cm³ (20 °C).

$n_D^{20}$: 1.418-1.421.

bp: about 82 °C at 6 mm Hg.

**Isopropyl myristate.** *1047200.* [110-27-0].

See *Isopropyl myristate (0725)*.

**4-Isopropylphenol.** $C_9H_{12}O$. ($M_r$ 136.2). *1047300.* [99-89-8].

*Content*: minimum 98 per cent.

bp: about 212 °C.

mp: 59 °C to 61 °C.

**Isopropyl toluenesulfonate.** $C_{10}H_{14}O_3S$. ($M_r$ 214.3). *1191100.* [2307-69-9]. 1-Methylethyl 4-methylbenzenesulfonate. Propan-2-yl 4-methylbenzenesulfonate. Isopropyl tosilate.

*Content*: minimum 97.0 per cent.

Clear liquid.

mp: about 20 °C.

**Isopulegol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1139600.* [89-79-2]. (−)-Isopulegol. (1*R*,2*S*,5*R*)-2-Isopropenyl-5-methylcyclohexanol.

$d_4^{20}$: about 0.911.

$n_D^{20}$: about 1.472.

bp: about 91 °C.

*Isopulegol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Mint oil, partly dementholised (1838)*.

*Content*: minimum 99 per cent, calculated by the normalisation procedure.

**Isoquercitrin.** $C_{21}H_{20}O_{12}$. ($M_r$ 464.4). *1201600.* [482-35-9]. 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

**Isoquercitroside.** $C_{21}H_{20}O_{12}$. ($M_r$ 464.4). *1136500.* [21637-25-2]. 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucofuranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

**Isorhamnetin-3-*O*-neohesperidoside.** $C_{28}H_{32}O_{16}$. ($M_r$ 625). *1205100.* [55033-90-4]. 3-[6-Deoxy-α-L-mannopyranosyl-(1→2)-β-D-glucopyranosyloxy]-5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one.

**Isorhynchophylline.** $C_{22}H_{28}N_2O_4$. ($M_r$ 384.5). *1197100.* [6859-01-4]. Methyl (16*E*)-17-methoxy-2-oxo-16,17-didehydro-20α-corynoxan-16-carboxylate. Methyl (16*E*)-16-(methoxymethylidene)-2-oxo-20α-corynoxan-17-oate.

**Isosilibinin.** $C_{25}H_{22}O_{10}$. ($M_r$ 482.4). *1149900.* [72581-71-6]. 3,5,7-Trihydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one.

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

**Kaempferol.** $C_{15}H_{10}O_6$. ($M_r$ 286.2). *1197200.* [520-18-3]. 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

**Kaolin, light.** *1047400.* [1332-58-7].

A purified native hydrated aluminium silicate. It contains a suitable dispersing agent.

Light, white or almost white powder free from gritty particles, unctuous to the touch, practically insoluble in water and in mineral acids.

*Coarse particles*: maximum 0.5 per cent.

Place 5.0 g in a ground-glass-stoppered cylinder about 160 mm long and 35 mm in diameter and add 60 mL of a 10 g/L solution of *sodium pyrophosphate R*. Shake vigorously and allow to stand for 5 min. Using a pipette, remove 50 mL of the liquid from a point about 5 cm below the surface. To the remaining liquid add 50 mL of *water R*, shake, allow to stand for 5 min and remove 50 mL as before. Repeat the operations until a total of 400 mL has been removed. Transfer the remaining suspension to an evaporating dish. Evaporate to dryness on a water-bath and dry the residue to constant mass at 100-105 °C. The residue weighs not more than 25 mg.

*Fine particles.* Disperse 5.0 g in 250 mL of *water R* by shaking vigorously for 2 min. Immediately pour into a glass cylinder 50 mm in diameter and, using a pipette, transfer 20 mL to a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100-105 °C. Allow the remainder of the suspension to stand at 20 °C for 4 h and, using a pipette with its tip exactly 5 cm below the surface, withdraw a further 20 mL without disturbing the sediment, place in a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100-105 °C. The mass of the second residue is not less than 70 per cent of that of the first residue.

**11-Keto-β-boswellic acid.** $C_{30}H_{46}O_4$. ($M_r$ 470.7). *1167600.* [17019-92-0]. 3α-Hydroxy-11-oxours-12-en-24-oic acid. (4β)-3α-Hydroxy-11-oxours-12-en-23-oic acid.

White or almost white powder, insoluble in water, soluble in acetone, in anhydrous ethanol and in methanol.

mp: 195 °C to 197 °C.

*11-Keto-β-boswellic acid used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Indian frankincense (2310)*.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

**Kieselguhr for chromatography.** *1047500.*

White or yellowish-white, light powder, practically insoluble in water, in dilute acids and in organic solvents.

*Filtration rate.* Use a chromatography column 0.25 m long and 10 mm in internal diameter with a sintered-glass (100) plate and two marks at 0.10 m and 0.20 m above the plate. Place sufficient of the substance to be examined in the column to reach the first mark and fill to the second mark with *water R*. When the first drops begin to flow from the column, fill to the second mark again with *water R* and measure the time required for the first 5 mL to flow from the column. The flow rate is not less than 1 mL/min.

*Appearance of the eluate.* The eluate obtained in the test for filtration rate is colourless (*2.2.2, Method I*).

*Acidity or alkalinity.* To 1.00 g add 10 mL of *water R*, shake vigorously and allow to stand for 5 min. Filter the suspension on a filter previously washed with hot *water R* until the washings are neutral. To 2.0 mL of the filtrate add 0.05 mL of *methyl red solution R*; the solution is yellow. To 2.0 mL of the filtrate add 0.05 mL of *phenolphthalein solution R1*; the solution is at most slightly pink.

*Water-soluble substances.* Place 10.0 g in a chromatography column 0.25 m long and 10 mm in internal diameter and elute with *water R*. Collect the first 20 mL of eluate, evaporate to dryness and dry the residue at 100 °C to 105 °C. The residue weighs not more than 10 mg.

*Iron* (*2.4.9*): maximum 200 ppm.

To 0.50 g add 10 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R*, shake vigorously, allow to stand for 5 min and filter. 1.0 mL of the filtrate complies with the test for iron.

*Loss on ignition*: maximum 0.5 per cent. During heating to red heat (600 ± 50 °C) the substance does not become brown or black.

**Kieselguhr G.** *1047600.*

Consists of kieselguhr treated with hydrochloric acid and calcined, to which is added about 15 per cent of calcium sulfate hemihydrate.

A fine greyish-white powder; the grey colour becomes more pronounced on triturating with water. The average particle size is 10-40 µm.

*Calcium sulfate content.* Determine by the method prescribed for *silica gel G R*.

*pH* (*2.2.3*). Shake 1 g with 10 mL of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 7 to 8.

*Chromatographic separation.* Thin-layer chromatography (*2.2.27*). Prepare plates using a slurry of the kieselguhr G with a 2.7 g/L solution of *sodium acetate R*. Apply 5 µL of a solution containing 0.1 g/L of lactose, sucrose, glucose and fructose in *pyridine R*. Develop over a path of 14 cm using a mixture of 12 volumes of *water R*, 23 volumes of *2-propanol R* and 65 volumes of *ethyl acetate R*. The migration time of the solvent is about 40 min. Dry, spray onto the plate about 10 mL of *anisaldehyde solution R* and heat for 5-10 min at 100-105 °C. The chromatogram shows four well-defined spots without tailing and well separated from each other.

**Lactic acid.** *1047800.* [50-21-5].

See *Lactic acid (0458)*.

**Lactic reagent.** *1047801.*

*Solution A.* To 60 mL of *lactic acid R* add 45 mL of previously filtered *lactic acid R* saturated without heating with *Sudan red G R*; as lactic acid saturates slowly without heating, an excess of colorant is always necessary.

*Solution B.* Prepare 10 mL of a saturated solution of *aniline R*. Filter.

*Solution C.* Dissolve 75 mg of *potassium iodide R* in water and dilute to 70 mL with the same solvent. Add 10 mL of *ethanol (96 per cent) R* and 0.1 g of *iodine R*. Shake.

Mix solutions A and B. Add solution C.

**Lactobionic acid.** $C_{12}H_{22}O_{12}$. ($M_r$ 358.3). *1101600.* [96-82-2].

White or almost white, crystalline powder, freely soluble in water, practically insoluble in ethanol (96 per cent).

mp: about 115 °C.

**β-Lactose.** $C_{12}H_{22}O_{11}$. ($M_r$ 342.3). *1150100.* [5965-66-2]. β-D-Lactose.

White or slightly yellowish powder.

*Content*: minimum 99 per cent.

*α-D-Lactose*: not greater than 35 per cent.

*Assay.* Gas chromatography (*2.2.28*): use the normalisation procedure.

*Column*:

- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 32.5 | 20 → 280 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: an appropriate derivatised sample.

**Lactose monohydrate.** *1047900.* [5989-81-1].

See *Lactose monohydrate (0187)*.

**α-Lactose monohydrate.** $C_{12}H_{22}O_{11},H_2O$. ($M_r$ 360.3). *1150000.* [5989-81-1]. α-D-Lactose monohydrate.

White or almost white powder.

*Content*: minimum 97 per cent.

*β-D-Lactose*: less than 3 per cent.

*Assay.* Gas chromatography (*2.2.28*): use the normalisation procedure.

*Column*:

- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12.5 | 230 → 280 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: an appropriate derivatised sample.

**Lactulose.** *1189600.* [4618-18-2].

See *Lactulose (1230)*.

**Lanatoside C.** $C_{49}H_{76}O_{20}$. ($M_r$ 985). *1163300.* [17575-22-3]. 3β-[(β-D-Glucopyranosyl-(1→4)-3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide.

Long, flat prisms obtained after recrystallisation in ethanol (96 per cent), freely soluble in pyridine and in dioxan.

**Lanthanum chloride heptahydrate.** $LaCl_3,7H_2O$. ($M_r$ 371.4). *1167200.*

White or almost white powder or colourless crystals, freely soluble in water.

**Lanthanum chloride solution.** *1114001.*

To 58.65 g of *lanthanum trioxide R* slowly add 100 mL of *hydrochloric acid R*. Heat to boiling. Allow to cool and dilute to 1000.0 mL with *water R*.

**Lanthanum nitrate.** $La(NO_3)_3,6H_2O$. ($M_r$ 433.0). *1048000.* [10277-43-7]. Lanthanum trinitrate hexahydrate.

Colourless crystals, deliquescent, freely soluble in water.

*Storage*: in an airtight container.

**Lanthanum nitrate solution.** *1048001.*

A 50 g/L solution of *lanthanum nitrate R*.

**Lanthanum trioxide.** $La_2O_3$. ($M_r$ 325.8). *1114000.* [1312-81-8].

An almost white, amorphous powder, practically insoluble in *water R*. It dissolves in dilute solutions of mineral acids and absorbs atmospheric carbon dioxide.

*Calcium*: maximum 5 ppm.

**Lauric acid.** $C_{12}H_{24}O_2$. ($M_r$ 200.3). *1143100.* [143-07-7]. Dodecanoic acid.

White or almost white, crystalline powder, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 44 °C.

*Lauric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Lauryl alcohol.** $C_{12}H_{26}O$. ($M_r$ 186.3). *1119900.* [112-53-8]. Dodecan-1-ol.

$d_{20}^{20}$: about 0.820.

mp: 24 °C to 27 °C.

*Content*: minimum 98.0 per cent, determined by gas chromatography.

**Lavandulol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1114100.* [498-16-8]. (*R*)-5-Methyl-2-(1-methylethenyl)-4-hexen-1-ol.

Oily liquid with a characteristic odour.

*Lavandulol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

*Test solution.* The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**Lavandulyl acetate.** $C_{12}H_{20}O_2$. ($M_r$ 196.3). *1114200.* [25905-14-0]. 2-Isopropenyl-5-methylhex-4-en-1-yl acetate.

Colourless liquid with a characteristic odour.

*Lavandulyl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

*Test solution.* The substance to be examined.

*Content*: minimum 93.0 per cent, calculated by the normalisation procedure.

**Lead acetate.** $C_4H_6O_4Pb,3H_2O$. ($M_r$ 379.3). *1048100.* [6080-56-4]. Lead di-acetate.

Colourless crystals, efflorescent, freely soluble in water, soluble in ethanol (96 per cent).

**Lead acetate cotton.** *1048101.*

Immerse absorbent cotton in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. Drain off the excess of liquid, without squeezing the cotton, by placing it on several layers of filter paper. Allow to dry in air.

*Storage*: in an airtight container.

**Lead acetate paper.** *1048102.*

Immerse filter paper weighing about 80 g/m$^2$ in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. After drying, cut the paper into strips 15 mm by 40 mm.

**Lead acetate solution.** *1048103.*

A 95 g/L solution of *lead acetate R* in *carbon dioxide-free water R*.

**Lead dioxide.** $PbO_2$. ($M_r$ 239.2). *1048200.* [1309-60-0].

Dark brown powder, evolving oxygen when heated, practically insoluble in water, soluble in hydrochloric acid with evolution of chlorine, soluble in dilute nitric acid in the presence of hydrogen peroxide, oxalic acid or other reducing agents, soluble in hot, concentrated alkali hydroxide solutions.

**Lead nitrate.** $Pb(NO_3)_2$. ($M_r$ 331.2). *1048300.* [10099-74-8]. Lead dinitrate.

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

**Lead nitrate solution.** *1048301.*

A 33 g/L solution of *lead nitrate R*.

**Lead subacetate solution.** *1048400.* [1335-32-6]. Basic lead acetate solution.

*Content*: 16.7 per cent *m/m* to 17.4 per cent *m/m* of Pb ($A_r$ 207.2) in a form corresponding approximately to the formula $C_8H_{14}O_{10}Pb_3$.

Dissolve 40.0 g of *lead acetate R* in 90 mL of *carbon dioxide-free water R*. Adjust the pH to 7.5 with *strong sodium hydroxide solution R*. Centrifuge and use the clear colourless supernatant solution.

The solution remains clear when stored in a well-closed container.

**Leiocarposide.** $C_{27}H_{34}O_{16}$. ($M_r$ 614.5). *1150200.* [71953-77-0]. 2-(β-D-Glucopyranosyloxy)benzyl 3-(β-D-glucopyranosyloxy)-6-hydroxy-2-methoxybenzoate. 2-[[[3-(β-D-Glucopyranosyloxy)-6-hydroxy-2-methoxybenzoyl]oxy]methyl]phenyl-β-D-glucopyranoside.

White or almost white powder, soluble in water, freely soluble in methanol, slightly soluble in ethanol (96 per cent).

mp: 190 °C to 193 °C.

**Lemon oil.** *1101700.*

See *Lemon oil (0620)*.

**Leucine.** *1048500.* [61-90-5].

See *Leucine (0771)*.

**Levodopa.** *1170000.* [59-92-7].

See *Levodopa (0038)*.

**(*Z*)-Ligustilide.** $C_{12}H_{14}O_2$. ($M_r$ 190.2). *1180300.* [81944-09-4]. (3*Z*)-3-Butylidene-1,3,4,5-tetrahydroisobenzofuran-1-one.

**Limonene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1048600.* [5989-27-5]. D-Limonene. (+)-*p*-Mentha-1,8-diene. (*R*)-4-Isopropenyl-1-methylcyclohex-1-ene.

Colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.84.

$n_D^{20}$: 1.471 to 1.474.

$[\alpha]_D^{20}$: about + 124.

bp: 175 °C to 177 °C.

*Limonene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution.* The substance to be examined.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Linalol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1048700.* [78-70-6]. (*RS*)-3,7-Dimethylocta-1,6-dien-3-ol.

Mixture of two stereoisomers (licareol and coriandrol).

Liquid, practically insoluble in water.

$d_{20}^{20}$: about 0.860.

$n_D^{20}$: about 1.462.

bp: about 200 °C.

*Linalol used in gas chromatography complies with the following test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

*Test solution.* The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Linalyl acetate.** $C_{12}H_{20}O_2$. ($M_r$ 196.3). *1107200.* [115-95-7]. (*RS*)-1,5-Dimethyl-1-vinylhex-4-enyl acetate.

Colourless or slightly yellow liquid with a strong odour of bergamot and lavender.

$d_{25}^{25}$: 0.895 to 0.912.

$n_D^{20}$: 1.448 to 1.451.

bp: about 215 °C.

*Linalyl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Lindane.** $C_6H_6Cl_6$. ($M_r$ 290.8). *1128900.* [58-89-9]. γ-Hexachlorocyclohexane.

For the monograph *Wool fat (0134)*, a suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Linoleic acid.** $C_{18}H_{32}O_2$. ($M_r$ 280.5). *1143200.* [60-33-3]. (9*Z*,12*Z*)-Octadeca-9,12-dienoic acid.

Colourless, oily liquid.

$d_4^{20}$: about 0.903.

$n_D^{20}$: about 1.470.

*Linoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Linolenic acid.** $C_{18}H_{30}O_2$. ($M_r$ 278.4). *1143300.* [463-40-1]. (9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid. α-Linolenic acid.

Colourless liquid, practically insoluble in water, soluble in organic solvents.

$d_4^{20}$: about 0.915.

$n_D^{20}$: about 1.480.

*Linolenic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Linolenyl alcohol.** $C_{18}H_{32}O$. ($M_r$ 264.4). *1156200.* [24149-05-1]. (9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trien-1-ol. α-Linolenyl alcohol.

*Content*: minimum 96 per cent.

**Linoleyl alcohol.** $C_{18}H_{34}O$. ($M_r$ 266.5). *1155900.* [506-43-4]. (9*Z*,12*Z*)-Octadeca-9,12-dien-1-ol.

*Relative density*: 0.830.

*Content*: minimum 85 per cent.

**Linsidomine hydrochloride.** $C_6H_{11}ClN_4O_2$. ($M_r$ 206.6). *1171200.* [16142-27-1]. 3-(Morpholin-4-yl)sydnonimine hydrochloride. 3-(Morpholin-4-yl)-1,2,3-oxadiazol-3-ium-5-aminide hydrochloride.

White or almost white powder.

**Liquid scintillation cocktail.** *1167300.*

Commercially available solution for the determination of radioactivity by liquid scintillation counting. It contains one or more fluorescent agents and mostly one or more emulsifying agents in a suitable organic solvent or mixture of organic solvents.

**Liquid scintillation cocktail R1.** *1176800.*

To 1000 mL of *dioxan R*, add 0.3 g of *methylphenyloxazolylbenzene R*, 7 g of *diphenyloxazole R* and 100 g of *naphthalene R*.

**Lithium.** Li. ($A_r$ 6.94). *1048800.* [7439-93-2].

A soft metal whose freshly cut surface is silvery-grey. It rapidly tarnishes in contact with air. It reacts violently with water, yielding hydrogen and giving a solution of lithium hydroxide; soluble in methanol, yielding hydrogen and a solution of lithium methoxide; practically insoluble in light petroleum.

*Storage*: under light petroleum or liquid paraffin.

**Lithium carbonate.** $Li_2CO_3$. ($M_r$ 73.9). *1048900.* [554-13-2]. Dilithium carbonate.

White or almost white, light powder, sparingly soluble in water, very slightly soluble in ethanol (96 per cent). A saturated solution at 20 °C contains about 13 g/L of $Li_2CO_3$.

**Lithium chloride.** LiCl. ($M_r$ 42.39). *1049000.* [7447-41-8].

Crystalline powder or granules or cubic crystals, deliquescent, freely soluble in water, soluble in acetone and in ethanol (96 per cent). Aqueous solutions are neutral or slightly alkaline.

*Storage*: in an airtight container.

**Lithium hydroxide.** $LiOH,H_2O$. ($M_r$ 41.96). *1049100.* [1310-66-3]. Lithium hydroxide monohydrate.

White or almost white, granular powder, strongly alkaline, it rapidly absorbs water and carbon dioxide, soluble in water, sparingly soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Lithium metaborate, anhydrous.** $LiBO_2$. ($M_r$ 49.75). *1120000.* [13453-69-5].

**Lithium sulfate.** $Li_2SO_4,H_2O$. ($M_r$ 128.0). *1049200.* [10102-25-7]. Dilithium sulfate monohydrate.

Colourless crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

**Lithium trifluoromethanesulfonate.** $CF_3LiO_3S$. ($M_r$ 156.0). *1173400.* [33454-82-9].

**Litmus.** *1049300.* [1393-92-6].

Schultz No. 1386.

Indigo-blue fragments prepared from various species of Rocella, Lecanora or other lichens, soluble in water, practically insoluble in ethanol (96 per cent).

*Colour change*: pH 5 (red) to pH 8 (blue).

**Litmus paper, blue.** *1049301.*

Boil 10 parts of coarsely powdered *litmus R* for 1 h with 100 parts of *ethanol (96 per cent) R*. Decant the alcohol and add to the residue a mixture of 45 parts of *ethanol (96 per cent) R* and 55 parts of *water R*. After 2 days decant the clear liquid. Impregnate strips of filter paper with the solution and allow to dry.

*Test for sensitivity.* Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 mL of *0.02 M hydrochloric acid* and 90 mL of *water R*. On shaking the paper turns red within 45 s.

**Litmus paper, red.** *1049302.*

To the blue litmus extract, add *dilute hydrochloric acid R* dropwise until the blue colour becomes red. Impregnate strips of filter paper with the solution and allow to dry.

*Test for sensitivity.* Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 mL of *0.02 M sodium hydroxide* and 90 mL of *water R*. On shaking the paper turns blue within 45 s.

**Loganin.** $C_{17}H_{26}O_{10}$. ($M_r$ 390.4). *1136700.* [18524-94-2]. Methyl (1*S*,4a*S*,6*S*,7*R*,7a*S*)-1-(β-D-glucopyranosyloxy)-6-hydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[*c*]pyran-4-carboxylate.

mp: 220 °C to 221 °C.

**Longifolene.** $C_{15}H_{24}$. ($M_r$ 204.4). *1150300.* [475-20-7]. (1*S*,3a*R*,4*S*,8a*S*)-4,8,8-Trimethyl-9-methylenedecahydro-1,4-methanoazulene.

Oily, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_4^{18}$: 0.9319.

$n_D^{20}$: 1.5050.

$[\alpha]_D^{20}$: + 42.7.

bp: 254 °C to 256 °C.

*Longifolene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Turpentine oil, Pinus pinaster type (1627)*.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Low-vapour-pressure hydrocarbons (type L).** *1049400.*

Unctuous mass, soluble in benzene and in toluene.

**Lumiflavine.** $C_{13}H_{12}N_4O_2$. ($M_r$ 256.3). *1141000.* [1088-56-8]. 7,8,10-Trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione.

Yellow powder or orange crystals, very slightly soluble in water, freely soluble in methylene chloride.

**Luteolin.** $C_{15}H_{10}O_6$. ($M_r$ 286.2). *1198500.* [491-70-3]. 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one.

**Luteolin-7-glucoside.** $C_{21}H_{20}O_{11}$. ($M_r$ 448.4). *1163400.* [5373-11-5]. 2-(3,4-Dihydroxyphenyl)-7-(β-D-glucopyranosyloxy)-5-hydroxy-4*H*-1-benzopyran-4-one.

Yellow powder.

*Absorbance* (*2.2.25*). A solution in *methanol R* shows absorption maxima at 255 nm, 267 nm and 350 nm.

mp: about 247 °C.

**Lutetium chloride hexahydrate.** $LuCl_3,6H_2O$. ($M_r$ 389.4). *1199600.* [15230-79-2].

White to yellow, crystalline powder, freely soluble in water.

**Lysyl endopeptidase.** *1188000.* [78642-25-8].

*Achromobacter* endoproteinase I. Lysyl bond specific proteinase (EC 3.4.21.50).

It belongs to the serine endopeptidase family. Initially isolated from *Achromobacter lyticus*. Enzymes with similar specificity are produced by *Lysobacter enzymogenes* (endoproteinase Lys-C) and *Pseudomonas aeruginosa* (Ps-1). It cleaves peptide bonds at the carboxy-terminal of both lysine residues and *S*-aminoethylcysteine residues with a high degree of specificity. 1 amidase unit (U) is the amount of enzyme that will produce 1 micromole of *p*-nitroaniline from *N*-benzoyl-DL-lysine-*p*-nitroaniline per minute at 30 °C at pH 9.5.

**Macrogol 23 lauryl ether.** *1129000.*

See *Macrogol lauryl ether (1124)*, the number of moles of ethylene oxide reacted per mole of lauryl alcohol being 23 (nominal value).

**Macrogol 200.** *1099200.* [25322-68-3]. Polyethyleneglycol 200.

Clear, colourless or almost colourless viscous liquid, very soluble in acetone and in anhydrous ethanol, practically insoluble in fatty oils.

$d_{20}^{20}$: about 1.127.

$n_D^{20}$: about 1.450.

**Macrogol 200 R1.** *1099201.*

Introduce 500 mL of *macrogol 200 R* into a 1000 mL round bottom flask. Using a rotation evaporator remove any volatile components applying for 6 h a temperature of 60 °C and a vacuum with a pressure of 1.5-2.5 kPa.

**Macrogol 300.** *1067100.* [25322-68-3]. Polyethyleneglycol 300.

See *Macrogols (1444)*.

**Macrogol 400.** *1067200.* [25322-68-3]. Polyethyleneglycol 400.

See *Macrogols (1444)*.

**Macrogol 600.** *1189700.* [25322-68-3]. Polyethyleneglycol 600.

See *Macrogols (1444)*.

**Macrogol 1000.** *1067300.* [25322-68-3]. Polyethyleneglycol 1000.

See *Macrogols (1444)*.

**Macrogol 1500.** *1067400.* [25322-68-3]. Polyethyleneglycol 1500.

See *Macrogols (1444)*.

**Macrogol 4000.** *1198000.* [25322-68-3]. Polyethyleneglycol 4000.

See *Macrogols (1444)*.

**Macrogol 6000.** *1189800.* [25322-68-3]. Polyethyleneglycol 6000.

White or almost white solid with a waxy or paraffin-like appearance, very soluble in water and in methylene chloride, practically insoluble in ethanol (96 per cent), in fatty oils and in mineral oils.

**Macrogol 20 000.** *1067600.* Polyethyleneglycol 20 000.

See *Macrogols (1444)*.

**Macrogol 20 000 2-nitroterephthalate.** *1067601.*

Polyethyleneglycol 20 000 with embedded 2-nitroterephthalate groups.

**Macrogol, base-deactivated.** *1170300.*

Base-deactivated polyethyleneglycol.

**Macrogol cetostearyl ether.** *1196100.*

See *Macrogol cetostearyl ether (1123)*.

**Macrogol, polar-deactivated.** *1179000.*

Polar-deactivated polyethyleneglycol.

**Magnesium.** Mg. ($A_r$ 24.30). *1049500.* [7439-95-4].

Silver-white ribbon, turnings or wire, or a grey powder.

**Magnesium acetate.** $C_4H_6MgO_4,4H_2O$. ($M_r$ 214.5). *1049600.* [16674-78-5]. Magnesium diacetate tetrahydrate.

Colourless crystals, deliquescent, freely soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Magnesium chloride.** *1049700.* [7791-18-6].

See *Magnesium chloride hexahydrate (0402)*.

**Magnesium nitrate.** $Mg(NO_3)_2,6H_2O$. ($M_r$ 256.4). *1049800.* [13446-18-9]. Magnesium nitrate hexahydrate.

Colourless, clear crystals, deliquescent, very soluble in water, freely soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Magnesium nitrate solution.** *1049801.*

Dissolve 17.3 g of *magnesium nitrate R* in 5 mL of *water R* warming gently and add 80 mL of *ethanol (96 per cent) R*. Cool and dilute to 100.0 mL with the same solvent.

**Magnesium oxide.** *1049900.* [1309-48-4].

See *Light magnesium oxide (0040)*.

**Magnesium oxide R1.** *1049901.*

Complies with the requirements prescribed for *magnesium oxide R* with the following modifications.

*Arsenic* (*2.4.2, Method A*): maximum 2 ppm.

Dissolve 0.5 g in a mixture of 5 mL of *water R* and 5 mL of *hydrochloric acid R1*.

*Heavy metals* (*2.4.8*): maximum 10 ppm.

Dissolve 1.0 g in a mixture of 3 mL of *water R* and 7 mL of *hydrochloric acid R1*. Add 0.05 mL of *phenolphthalein solution R* and *concentrated ammonia R* until a pink colour is obtained. Neutralise the excess of ammonia by the addition of *glacial acetic acid R*. Add 0.5 mL in excess and dilute to 20 mL with *water R*. Filter, if necessary. 12 mL of the solution complies with test A. Prepare the reference solution using a mixture of 5 mL of *lead standard solution (1 ppm Pb) R* and 5 mL of *water R*.

*Iron* (*2.4.9*): maximum 50 ppm.

Dissolve 0.2 g in 6 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*.

**Magnesium oxide, heavy.** *1050000.* [1309-48-4].

See *Heavy magnesium oxide (0041)*.

**Magnesium silicate for pesticide residue analysis.** *1129100.* [1343-88-0].

Magnesium silicate for chromatography (60-100 mesh).

**Magnesium sulfate.** *1050200.* [10034-99-8].

See *Magnesium sulfate heptahydrate (0044)*.

**Magnolin.** $C_{23}H_{28}O_7$. ($M_r$ 416.5). *1200300.* [31008-18-1].

(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-Dimethoxyphenyl)-6-(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-*c*]furan.

**Magnolol.** $C_{18}H_{18}O_2$. ($M_r$ 266.3). *1182800.* [528-43-8]. 5,5′-Di(prop-2-enyl)biphenyl-2,2′-diol. 5,5′-Diallyl-2,2′-dihydroxybiphenyl. 5,5′-Di-2-propenyl-[1,1′-biphenyl]-2,2′-diol.

**Maize oil.** *1050400.*

See *Maize oil, refined (1342)*.

**Makisterone A.** $C_{28}H_{46}O_7$. ($M_r$ 494.7). *1207200.* [20137-14-8]. (22*R*)-2β,3β,14,20,22,25-Hexahydroxy-5β-ergost-7-en-6-one.

**Malachite green.** $C_{23}H_{25}ClN_2$. ($M_r$ 364.9). *1050500.* [123333-61-9].

Schultz No. 754.

Colour Index No. 42000.
[4-[[4-(Dimethylamino)phenyl]phenylmethylene]cyclohexa-2,5-dien-1-ylidene]dimethylammonium chloride.

Green crystals with a metallic lustre, very soluble in water giving a bluish-green solution, soluble in ethanol (96 per cent) and in methanol.

Absorbance (*2.2.25*). A 0.01 g/L solution in *ethanol (96 per cent) R* shows an absorption maximum at 617 nm.

**Malachite green solution.** *1050501.*

A 5 g/L solution of *malachite green R* in *anhydrous acetic acid R*.

**Malathion.** $C_{10}H_{19}O_6PS_2$. ($M_r$ 330.3). *1129200.* [121-75-5].

bp: about 156 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Maleic acid.** *1050600.* [110-16-7].

See *Maleic acid (0365)*.

**Maleic anhydride.** $C_4H_2O_3$. ($M_r$ 98.1). *1050700.* [108-31-6]. Butenedioic anhydride. 2,5-Furandione.

White or almost white crystals, soluble in water forming maleic acid, very soluble in acetone and in ethyl acetate, freely soluble in toluene, soluble in ethanol (96 per cent) with ester formation, very slightly soluble in light petroleum.

mp: about 52 °C.

Any residue insoluble in toluene does not exceed 5 per cent (maleic acid).

**Maleic anhydride solution.** *1050701.*

Dissolve 5 g of *maleic anhydride R* in *toluene R* and dilute to 100 mL with the same solvent. Use within one month. If the solution becomes turbid, filter.

**Malic acid.** *1200400.* [6915-15-7].

See *Malic acid (2080)*.

**Maltitol.** *1136800.* [585-88-6].

See *Maltitol (1235)*.

**Maltol.** $C_6H_6O_3$. ($M_r$ 126.1). *1202300.* [118-71-8]. 3-Hydroxy-2-methyl-4*H*-pyran-4-one.

White or almost white crystalline powder, soluble in hot water.

mp: 161 °C to 162 °C.

**Maltose monohydrate.** $C_{12}H_{22}O_{11},H_2O$. ($M_r$ 360.3). *1193100.* [6363-53-7]. 4-*O*-α-D-glucopyranosyl-D-glucopyranose monohydrate.

**Maltotriose.** $C_{18}H_{32}O_{16}$. ($M_r$ 504.4). *1176300.* [1109-28-0]. α-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucose.

White or almost white, crystalline powder, very soluble in water.

mp: about 134 °C.

**Mandelic acid.** $C_8H_8O_3$. ($M_r$ 152.1). *1171300.* [90-64-2]. 2-Hydroxy-2-phenylacetic acid.

White crystalline flakes, soluble in water.

mp: 118 to 121 °C.

**Manganese sulfate.** $MnSO_4,H_2O$. ($M_r$ 169.0). *1050900.* [10034-96-5]. Manganese sulfate monohydrate.

Pale-pink, crystalline powder or crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

*Loss on ignition*: 10.0 per cent to 12.0 per cent, determined on 1.000 g at 500 ± 50 °C.

**Mannitol.** *1051000.* [69-65-8].

See *Mannitol (0559)*.

**Mannose.** $C_6H_{12}O_6$. ($M_r$ 180.2). *1051100.* [3458-28-4]. D-(+)-Mannose.

white or almost white, crystalline powder or small crystals, very soluble in water, slightly soluble in anhydrous ethanol.

$[\alpha]_D^{20}$: + 13.7 + 14.7, determined on a 200 g/L solution in *water R* containing about 0.05 per cent of $NH_3$.

mp: about 132 °C, with decomposition.

**Marrubiin.** $C_{20}H_{28}O_4$. ($M_r$ 332.4). *1158300.* [465-92-9]. (2a*S*,5a*S*,6*R*,7*R*,8a*R*,8b*R*)-6-[2-(Furan-3-yl)ethyl]-6-hydroxy-2a,5a,7-trimethyldecahydro-2*H*-naphtho[1,8-*bc*]furan-2-one.

Colourless, microcrystalline powder.

*Marrubiin used in liquid chromatography complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *White horehound (1835)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Meclozine dihydrochloride.** *1051200.* [1104-22-9].

See *Meclozine dihydrochloride (0622)*.

**Medronic acid.** *1193200.* [1984-15-2].

See *Medronic acid for radiopharmaceutical preparations (2350)*.

**Melamine.** $C_3H_6N_6$. ($M_r$ 126.1). *1051300.* [108-78-1]. 1,3,5-Triazine-2,4,6-triamine.

A white or almost white, amorphous powder, very slightly soluble in water and in ethanol (96 per cent).

**Menadione.** *1051400.* [58-27-5].

See *Menadione (0507)*.

**Menthofuran.** $C_{10}H_{14}O$. ($M_r$ 150.2). *1051500.* [17957-94-7]. 3,9-Epoxy-*p*-mentha-3,8-diene. 3,6-Dimethyl-4,5,6,7-tetrahydro-benzofuran.

Slightly bluish liquid, very slightly soluble in water, soluble in ethanol (96 per cent).

$d_{15}^{20}$: about 0.965.

$n_D^{20}$: about 1.480.

$[\alpha]_D^{20}$: about + 93.

bp: 196 °C.

*Menthofuran used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. The substance to be examined.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

**Menthol.** *1051600.* [2216-51-5].

See *Levomenthol (0619)* and *Racemic menthol (0623)*.

*Menthol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the related substances test included in the monograph *Racemic menthol (0623)*.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Menthone.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1051700.* [14073-97-3]. (2*S*,5*R*)-2-Isopropyl-5-methylcyclohexanone. (–)-*trans*-*p*-Menthan-3-one.

Contains variable amounts of isomenthone.

Colourless liquid, very slightly soluble in water, very soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.897.

$n_D^{20}$: about 1.450.

*Menthone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**Menthyl acetate.** $C_{12}H_{22}O_2$. ($M_r$ 198.3). *1051800.* [2623-23-6]. (1*R*,2*S*,5*R*)-5-Methyl-2-(propan-2-yl)cyclohexyl acetate.

Colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.92.

$n_D^{20}$: about 1.447.

bp: about 228 °C.

*Menthyl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. The substance to be examined.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

**2-Mercaptobenzimidazole.** $C_7H_6N_2S$. ($M_r$ 150.2). *1170100.* [583-39-1]. 1*H*-benzimidazole-2-thiol.

mp: about 302 °C.

**2-Mercaptoethanol.** $C_2H_6OS$. ($M_r$ 78.1). *1099300.* [60-24-2].

Liquid, miscible with water.

$d_{20}^{20}$: about 1.116.

bp: about 157 °C.

**Mercaptopurine.** *1051900.* [6112-76-1].

See *Mercaptopurine (0096)*.

**Mercuric acetate.** $C_4H_6HgO_4$. ($M_r$ 318.7). *1052000.* [1600-27-7]. Mercury diacetate.

White or almost white crystals, freely soluble in water, soluble in ethanol (96 per cent).

**Mercuric acetate solution.** *1052001.*

Dissolve 3.19 g of *mercuric acetate R* in *anhydrous acetic acid R* and dilute to 100 mL with the same acid. If necessary, neutralise the solution with *0.1 M perchloric acid* using 0.05 mL of *crystal violet solution R* as indicator.

**Mercuric chloride.** *1052200.* [7487-94-7].

See *Mercuric chloride (0120)*.

**Mercuric chloride solution.** *1052201.*

A 54 g/L solution of *mercuric chloride R*.

**Mercuric iodide.** $HgI_2$. ($M_r$ 454.4). *1052300.* [7774-29-0]. Mercury di-iodide.

Dense, scarlet, crystalline powder, slightly soluble in water, sparingly soluble in acetone and in ethanol (96 per cent), soluble in an excess of *potassium iodide solution R*.

*Storage*: protected from light.

**Mercuric nitrate.** $Hg(NO_3)_2,H_2O$. ($M_r$ 342.6). *1052400.* [7783-34-8]. Mercury dinitrate monohydrate.

Colourless or slightly coloured crystals, hygroscopic, soluble in water in the presence of a small quantity of nitric acid.

*Storage*: in an airtight container, protected from light.

**Mercuric oxide.** HgO. ($M_r$ 216.6). *1052500.* [21908-53-2]. Yellow mercuric oxide. Mercury oxide.

A yellow to orange-yellow powder, practically insoluble in water and in ethanol (96 per cent).

*Storage*: protected from light.

**Mercuric sulfate solution.** *1052600.* [7783-35-9].

Dissolve 1 g of *mercuric oxide R* in a mixture of 20 mL of *water R* and 4 mL of *sulfuric acid R*.

**Mercuric thiocyanate.** $Hg(SCN)_2$. ($M_r$ 316.7). *1052700.* [592-85-8]. Mercury di(thiocyanate).

White or almost white, crystalline powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent), soluble in solutions of sodium chloride.

**Mercuric thiocyanate solution.** *1052701.*

Dissolve 0.3 g of *mercuric thiocyanate R* in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

*Storage*: use within 1 week.

**Mesityl oxide.** $C_6H_{10}O$. ($M_r$ 98.1). *1120100.* [141-79-7]. 4-Methylpent-3-en-2-one.

Colourless, oily liquid, soluble in 30 parts of water, miscible with most organic solvents.

$d_{20}^{20}$: about 0.858.

bp: 129 °C to 130 °C.

**Metanil yellow.** $C_{18}H_{14}N_3NaO_3S$. ($M_r$ 375.4). *1052900.* [587-98-4].

Schultz No. 169.

Colour Index No. 13065.
Sodium 3-[4-(phenylamino)phenylazo]benzenesulfonate.

A brownish-yellow powder, soluble in water and in ethanol (96 per cent).

**Metanil yellow solution.** *1052901.*

A 1 g/L solution of *metanil yellow R* in *methanol R*.

*Test for sensitivity.* To 50 mL of *anhydrous acetic acid R* add 0.1 mL of the metanil yellow solution. Add 0.05 mL of *0.1 M perchloric acid*; the colour changes from pinkish-red to violet.

*Colour change*: pH 1.2 (red) to pH 2.3 (orange-yellow).

**Metaphosphoric acid.** $(HPO_3)_x$. *1053000.* [37267-86-0].

Glassy lumps or sticks containing a proportion of sodium metaphosphate, hygroscopic, very soluble in water.

*Nitrates.* Boil 1.0 g with 10 mL of *water R*, cool, add 1 mL of *indigo carmine solution R*, 10 mL of *nitrogen-free sulfuric acid R* and heat to boiling. The blue colour is not entirely discharged.

*Reducing substances*: maximum 0.01 per cent, calculated as $H_3PO_3$.

Dissolve 35.0 g in 50 mL of *water R*. Add 5 mL of a 200 g/L solution of *sulfuric acid R*, 50 mg of *potassium bromide R* and 5.0 mL of *0.02 M potassium bromate* and heat on a water-bath for 30 min. Allow to cool and add 0.5 g of *potassium iodide R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator. Carry out a blank test.

1 mL of *0.02 M potassium bromate* is equivalent to 4.10 mg of $H_3PO_3$.

*Storage*: in an airtight container.

**Methacrylic acid.** $C_4H_6O_2$. ($M_r$ 86.1). *1101800.* [79-41-4].
2-Methylprop-2-enoic acid.

Colourless liquid.

$n_D^{20}$: about 1.431.

bp: about 160 °C.

mp: about 16 °C.

**Methane.** $CH_4$. ($M_r$ 16). *1166300.* [74-82-8].

*Content*: minimum 99.0 per cent *V/V*.

**Methane R1.** $CH_4$. ($M_r$ 16). *1176400.* [74-82-8].

*Content*: minimum 99.995 per cent *V/V*.

**Methanesulfonic acid.** $CH_4O_3S$. ($M_r$ 96.1). *1053100.* [75-75-2].

Clear, colourless liquid, solidifying at about 20 °C, miscible with water, slightly soluble in toluene, practically insoluble in hexane.

$d_{20}^{20}$: about 1.48.

$n_D^{20}$: about 1.430.

**Methanesulfonyl chloride.** $CH_3ClO_2S$. ($M_r$ 114.6). *1181300.* [124-63-0].

Clear, colourless or slightly yellow liquid.

*Content*: minimum 99.0 per cent.

*Density*: 1.48 g/cm$^3$.

$n_D^{20}$: about 1.452.

bp: about 161 °C.

**Methanol.** $CH_4O$. ($M_r$ 32.04). *1053200.* [67-56-1].

Clear, colourless, flammable liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: 0.791 to 0.793.

bp: 64 °C to 65 °C.

**Methanol R1.** *1053201.*

Complies with the requirements prescribed for *methanol R* with the following additional requirement.

*Absorbance* (*2.2.25*): maximum 0.70 at 210 nm, 0.30 at 220 nm, 0.13 at 230 nm, 0.02 at 250 nm, 0.01 at 260 nm and higher wavelengths, determined using *water R* as compensation liquid.

**Methanol R2.** *1053202.*

Complies with the requirements prescribed for *methanol R* and the following additional requirements.

*Content*: minimum 99.8 per cent.

*Absorbance (2.2.25)*: maximum 0.17, determined at 225 nm using *water R* as the compensation liquid.

**Methanol, hydrochloric.** *1053203.*

Dilute 1.0 mL of *hydrochloric acid R1* to 100.0 mL with *methanol R*.

**Methanol, aldehyde-free.** *1053300.*

Dissolve 25 g of *iodine R* in 1 L of *methanol R* and pour the solution, with constant stirring, into 400 mL of *1 M sodium hydroxide*. Add 150 mL of *water R* and allow to stand for 16 h. Filter. Boil under a reflux condenser until the odour of iodoform disappears. Distil the solution by fractional distillation.

*Aldehydes and ketones*: maximum 0.001 per cent.

**Methanol, anhydrous.** *1053400.* [67-56-1].

Treat 1000 mL of *methanol R* with 5 g of *magnesium R*. If necessary initiate the reaction by adding 0.1 mL of *mercuric chloride solution R*. When the evolution of gas has ceased, distil the liquid and collect the distillate in a dry container protected from moisture.

*Water* (*2.5.12*): maximum 0.3 g/L.

**DL-Methionine.** *1129400.* [59-51-8].

See *DL-Methionine (0624)*.

**L-Methionine.** *1053500.* [63-68-3].

See *Methionine (1027)*.

**L-Methionine sulfoxide.** $C_5H_{11}NO_3S$. ($M_r$ 165.2). *1193300.* [3226-65-1]. (2*S*)-2-Amino-4-[(*RS*)-methylsulfinyl]butanoic acid.

**(*RS*)-Methotrexate.** $C_{20}H_{22}N_8O_5$. *1120200.* [60388-53-6].
(*RS*)-2-[4-[[(2,4-diaminopteridin-6-yl)methyl]-methylamino]benzoylamino]pentanedioic acid.

*Content*: minimum 96.0 per cent.

mp: about 195 °C.

**Methoxychlor.** $C_{16}H_{15}Cl_3O_2$. ($M_r$ 345.7). *1129300.* [72-43-5].
1,1-(2,2,2-Trichloroethylidene)-bis(4-methoxybenzene).

Practically insoluble in water, freely soluble in most organic solvents.

bp: about 346 °C.

mp: 78 °C to 86 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

***trans*-2-Methoxycinnamaldehyde.** $C_{10}H_{10}O_2$. ($M_r$ 162.2). *1129500.* [60125-24-8].

mp: 44 °C to 46 °C.

*trans-2-Methoxycinnamaldehyde used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

*Content*: minimum 96.0 per cent, calculated by the normalisation procedure.

**(1*RS*)-1-(6-Methoxynaphthalen-2-yl)ethanol.** $C_{13}H_{14}O_2$. ($M_r$ 202.3). *1159600.* [77301-42-9]. 6-Methoxy-α-methyl-2-naphthalenemethanol.

White or almost white powder.

mp: about 113 °C.

**1-(6-Methoxynaphthalen-2-yl)ethanone.** $C_{13}H_{12}O_2$. ($M_r$ 200.2). *1159700.* [3900-45-6]. 6′-Methoxy-2′-acetonaphthone.

White or almost white powder.

mp: about 108 °C.

**6-Methoxy-2-naphthoic acid.** $C_{12}H_{10}O_3$. ($M_r$ 202.2). *1184200.* [2471-70-7]. 6-Methoxynaphthalene-2-carboxylic acid.

White or almost white, crystalline powder.

mp: 201 °C to 206 °C.

**Methoxyphenylacetic acid.** $C_9H_{10}O_3$. ($M_r$ 166.2). *1053600.* [7021-09-2]. (*RS*)-2-Methoxy-2-phenylacetic acid.

White, crystalline powder or white or almost white crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 70 °C.

**Methoxyphenylacetic reagent.** *1053601.*

Dissolve 2.7 g of *methoxyphenylacetic acid R* in 6 mL of *tetramethylammonium hydroxide solution R* and add 20 mL of *anhydrous ethanol R*.

*Storage*: in a polyethylene container.

**3-Methoxy-L-tyrosine.** $C_{10}H_{13}NO_4H_2O$. ($M_r$ 229.2). *1164400.* [200630-46-2].

Off-white or yellow powder.

**Methyl acetate.** $C_3H_6O_2$. ($M_r$ 74.1). *1053700.* [79-20-9].

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.933.

$n_D^{20}$: about 1.361.

bp: 56 °C to 58 °C.

**Methyl 4-acetylbenzoate.** $C_{10}H_{10}O_3$. ($M_r$ 178.2). *1154100.* [3609-53-8].

mp: about 94 °C.

**Methyl 4-acetylbenzoate reagent.** *1154101.*

Dissolve 0.25 g of *methyl 4-acetylbenzoate R* in a mixture of 5 mL of *sulfuric acid R* and 85 mL of cooled *methanol R*.

**Methyl acrylate.** $C_4H_6O_2$. ($M_r$ 86.1). *1199200.* [96-33-3]. Methyl prop-2-enoate.

Clear, colourless liquid.

bp: about 80 °C.

**Methylal.** $C_3H_8O_2$. ($M_r$ 76.1). *1173500.* [109-87-5]. Dimethoxymethane. Dioxapentane. Formaldehyde dimethyl acetal. Methylene dimethyl ether.

Clear, colourless, volatile, flammable liquid, soluble in water and miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.860.

$n_D^{20}$: about 1.354.

bp: about 41 °C.

*Methylal used in gas chromatography complies with the following additional test.*

*Content*: minimum 99.5 per cent, determined by gas chromatography.

**Methylamine hydrochloride.** $CH_6ClN$. ($M_r$ 67.5). *1198600.* [593-51-1]. Methanamine hydrochloride.

White or almost white powder.

*Content*: minimum 98.0 per cent.

**Methyl 4-aminobenzoate.** $C_8H_9NO_2$. ($M_r$ 151.2). *1175600.* [619-45-4].

mp: 110 °C to 113 °C.

**4-Methylaminophenol sulfate.** $C_{14}H_{20}N_2O_6S$. ($M_r$ 344.4). *1053800.* [55-55-0].

Colourless crystals, very soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 260 °C.

**3-(Methylamino)-1-phenylpropan-1-ol.** $C_{10}H_{15}NO$. ($M_r$ 165.2). *1186400.* [42142-52-9].

White or almost white powder.

mp: 59 °C to 64 °C.

**Methyl anthranilate.** $C_8H_9NO_2$. ($M_r$ 151.2). *1107300.* [134-20-3]. Methyl 2-aminobenzoate.

Colourless crystals or a colourless or yellowish liquid, soluble in water, freely soluble in ethanol (96 per cent).

mp: 24 °C to 25 °C.

*Methyl anthranilate used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution.* The substance to be examined.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Methyl arachidate.** $C_{21}H_{42}O_2$. ($M_r$ 326.6). *1053900.* [1120-28-1]. Methyl eicosanoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 46 °C.

**Methyl behenate.** $C_{23}H_{46}O_2$. ($M_r$ 354.6). *1107500.* [929-77-1]. Methyl docosanoate.

mp: 54 °C to 55 °C.

**Methyl benzenesulfonate.** $C_7H_8O_3S$. ($M_r$ 172.2). *1159800.* [80-18-2].

*Content*: minimum 98.0 per cent.

Clear, colourless liquid.

bp: about 148 °C.

**Methyl benzoate.** $C_8H_8O_2$. ($M_r$ 136.2). *1164500.* [93-58-3]. Benzoic acid, methyl ester.

Colourless liquid.

$d_4^{20}$: 1.088.

bp: about 200 °C.

**Methylbenzothiazolone hydrazone hydrochloride.** $C_8H_{10}ClN_3S,H_2O$. ($M_r$ 233.7). *1055300.* [38894-11-0]. 3-Methylbenzothiazol-2(3*H*)-one hydrazone hydrochloride monohydrate.

Almost white or yellowish, crystalline powder.

mp: about 270 °C.

*Suitability for determination of aldehydes.* To 2 mL of *aldehyde-free methanol R* add 60 µL of a 1 g/L solution of *propionaldehyde R* in *aldehyde-free methanol R* and 5 mL of a 4 g/L solution of methylbenzothiazolone hydrazone hydrochloride. Mix. Allow to stand for 30 min. Prepare a blank omitting the propionaldehyde solution. Add 25.0 mL of a 2 g/L solution of *ferric chloride R* to the test solution and to the blank, dilute to 100.0 mL with *acetone R* and mix. The absorbance (*2.2.25*) of the test solution, measured at 660 nm using the blank as compensation liquid, is not less than 0.62.

**(*R*)-(+)-α-Methylbenzyl isocyanate.** $C_9H_9NO$. ($M_r$ 147.2). *1171400.* [33375-06-3]. (+)-(*R*)-α-Methylbenzyl isocyanate. (+)-[(1*R*)-1-Isocyanatoethyl]benzene. (+)-(1*R*)-1-Phenylethyl isocyanate.

*Content*: minimum 99.0 per cent.

Colourless liquid.

$d_{20}^{20}$: about 1.045.

$n_D^{20}$: about 1.513.

bp: 55 °C to 56 °C at 2.5 mm Hg.

*Enantiomeric purity*: minimum 99.5.

*Storage*: at a temperature of 2 °C to 8 °C.

**(*S*)-(−)-α-Methylbenzyl isocyanate.** $C_9H_9NO$. ($M_r$ 147.2). *1170200.* [14649-03-7]. (−)-(*S*)-α-Methylbenzyl isocyanate. (−)-[(1*S*)-1-Isocyanatoethyl]benzene. (−)-(1*S*)-1-Phenylethyl isocyanate.

*Content*: minimum 99.0 per cent.

Colourless liquid.

$d_{20}^{20}$: about 1.045.

$n_D^{20}$: about 1.514.

bp: 55 °C to 56 °C at 2.5 mm Hg.

*Enantiomeric purity*: minimum 99.5 per cent.

*Storage*: at a temperature of 2 °C to 8 °C.

*NOTE: do not use the reagent if it is coloured.*

**2-Methylbutane.** $C_5H_{12}$. ($M_r$ 72.2). *1099500.* [78-78-4]. Isopentane.

*Content*: minimum 99.5 per cent of $C_5H_{12}$.

Very flammable colourless liquid.

$d_{20}^{20}$: about 0.621.

$n_D^{20}$: about 1.354.

bp: about 29 °C.

*Water (2.5.12)*: maximum 0.02 per cent.

*Residue on evaporation*: maximum 0.0003 per cent.

*Absorbance* (*2.2.25*): maximum 0.30 at 210 nm, 0.07 at 220 nm, 0.01 at 240 nm and higher wavelengths, determined using *water R* as compensation liquid.

**2-Methylbut-2-ene.** $C_5H_{10}$. ($M_r$ 70.1). *1055400.* [513-35-9].

Very flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: 37.5 °C to 38.5 °C.

**Methyl 4-(butylamino)benzoate.** $C_{12}H_{17}NO_2$. ($M_r$ 207.3). *1207300.* [71839-12-8].

White or almost white solid.

*Content*: minimum 99.9 per cent.

**Methyl caprate.** *1054000.*

See *Methyl decanoate R*.

**Methyl caproate.** $C_7H_{14}O_2$. ($M_r$ 130.2). *1120300.* [106-70-7]. Methyl hexanoate.

$d_{20}^{20}$: about 0.885.

$n_D^{20}$: about 1.405.

bp: 150 °C to 151 °C.

**Methyl caprylate.** $C_9H_{18}O_2$. ($M_r$ 158.2). *1120400.* [111-11-5]. Methyl octanoate.

$d_{20}^{20}$: about 0.876.

$n_D^{20}$: about 1.417.

bp: 193 °C to 194 °C.

**Methylcellulose 450.** *1055500.* [9004-67-5].

See *Methylcellulose (0345)*.

Nominal viscosity: 450 mPa·s.

**Methyl cinnamate.** $C_{10}H_{10}O_2$. ($M_r$ 162.2). *1099400.* [103-26-4].

Colourless crystals practically insoluble in water, soluble in ethanol (96 per cent).

$n_D^{20}$: about 1.56.

bp: about 260 °C.

mp: 34 °C to 36 °C.

**Methylcyclohexane.** $C_7H_{14}$. ($M_r$ 98.2). *1189900.* [108-87-2].

**Methyl decanoate.** $C_{11}H_{22}O_2$. ($M_r$ 186.3). *1054000.* [110-42-9].

*Content*: minimum 99.0 per cent.

Clear, colourless or yellow liquid, soluble in light petroleum.

$d_{20}^{20}$: 0.871 to 0.876.

$n_D^{20}$: 1.425 to 1.426.

*Foreign substances.* Gas chromatography (*2.2.28*), injecting equal volumes of each of the following:

A 0.02 g/L solution of the substance to be examined in *carbon disulfide R* (solution A), a 2 g/L solution of the substance to be examined in *carbon disulfide R* (solution B), and *carbon disulfide R* (solution C). Carry out the chromatographic procedure under the conditions of the test for butylated hydroxytoluene prescribed in the monograph *Wool fat (0134)*. The total area of any peaks, apart from the solvent peak and the principal peak, in the chromatogram obtained with solution B is less than the area of the principal peak in the chromatogram obtained with solution A.

**Methyldopa, racemic.** $C_{10}H_{13}NO_4$,1½$H_2O$. ($M_r$ 238.2). *1175100.*

Mixture of equal volumes of (2*S*)- and (2*R*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acids.

**3-*O*-Methyldopamine hydrochloride.** $C_9H_{14}ClNO_2$. ($M_r$ 203.7). *1055600.* [1477-68-5]. 4-(2-Aminoethyl)-2-methoxyphenol hydrochloride.

mp: 213 °C to 215 °C.

**4-*O*-Methyldopamine hydrochloride.** $C_9H_{14}ClNO_2$. ($M_r$ 203.7). *1055700.* [645-33-0]. 5-(2-Aminoethyl)-2-methoxyphenol hydrochloride.

mp: 207 °C to 208 °C.

**Methylenebisacrylamide.** $C_7H_{10}N_2O_2$. ($M_r$ 154.2). *1056000.* [110-26-9]. *N,N´*-Methylenebispropenamide.

Fine, white or almost white powder, slightly soluble in water, soluble in ethanol (96 per cent).

mp: 300 °C, with decomposition.

**Methylene blue.** $C_{16}H_{18}ClN_3S$,*x*$H_2O$. ($M_r$ 319.9 for the anhydrous substance). *1055800.* [122965-43-9].

Schultz No. 1038.

Colour Index No. 52015.
3,7-Dimethylaminophenothiazin-5-ium chloride.
It occurs in different hydrated forms and may contain up to 22 per cent of water.

Dark-green or bronze, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent).

**Methylene blue solution.** *1055801.*

Dissolve 3 mg of *methylene blue R*, 1.2 g of *sulfuric acid R* and 5.0 g of *anhydrous sodium sulfate R* in 100 mL of *water R*.

**Methylene chloride.** $CH_2Cl_2$. ($M_r$ 84.9). *1055900.* [75-09-2]. Dichloromethane.

Colourless liquid, sparingly soluble in water, miscible with ethanol (96 per cent).

bp: 39 °C to 42 °C.

Methylene chloride used in fluorimetry complies with the following additional test.

*Fluorescence.* Under irradiation at 365 nm, the fluorescence (*2.2.21*) measured at 460 nm in a 1 cm cell is not more intense than that of a solution containing 0.002 ppm of *quinine R* in *0.5 M sulfuric acid* measured in the same conditions.

**Methylene chloride, acidified.** *1055901.*

To 100 mL of *methylene chloride R* add 10 mL of *hydrochloric acid R*, shake, allow to stand and separate the two layers. Use the lower layer.

**Methyl eicosenoate.** $C_{21}H_{40}O_2$. ($M_r$ 324.5). *1120500.* [2390-09-2]. Methyl (11*Z*)-eicos-11-enoate.

**Methyl erucate.** $C_{23}H_{44}O_2$. ($M_r$ 352.6). *1146100.* [1120-34-9]. Methyl (13*Z*)-docos-13-enoate.

$d_{20}^{20}$: about 0.871.

$n_D^{20}$: about 1.456.

**3-*O*-Methylestrone.** $C_{19}H_{24}O_2$. ($M_r$ 284.4). *1137000.* [1624-62-0]. 3-Methoxy-1,3,5(10)-estratrien-17-one.

White to yellowish-white powder.

$[\alpha]_D^{20}$: about + 157.

mp: about 173 °C.

**Methyl ethyl ketone.** $C_4H_8O$. ($M_r$ 72.1). *1054100.* [78-93-3]. Ethyl methyl ketone. 2-Butanone.

Clear, colourless, flammable liquid, very soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.81.

bp: 79 °C to 80 °C.

**Methyleugenol.** $C_{11}H_{14}O_2$. ($M_r$ 178.2). *1182000.* [93-15-2]. 1,2-Dimethoxy-4-prop-2-enylbenzene.

Methyleugenol used in gas chromatography complies with the following additional test.

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Niaouli oil, cineole type (2468)*.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

**Methyl 4-hydroxybenzoate.** *1055000.* [99-76-3].

See *Methyl parahydroxybenzoate R*.

**1-Methylimidazole.** $C_4H_6N_2$. ($M_r$ 82.1). *1139700.* [616-47-7]. 1-Methyl-1*H*-imidazole.

Colourless or slightly yellowish liquid.

$n_D^{20}$: about 1.495.

bp: 195 °C to 197 °C.

*Storage*: in an airtight container, protected from light.

**1-Methylimidazole R1.** *1139701.*

Complies with the requirements prescribed for *1-methylimidazole R* with the following additional requirement.

*Content*: minimum 95.0 per cent.

**2-Methylimidazole.** $C_4H_6N_2$. ($M_r$ 82.1). *1143400.* [693-98-1].

White or almost white, crystalline powder.

mp: about 145 °C.

**Methyl iodide.** $CH_3I$. ($M_r$ 141.9). *1166400.* [74-88-4]. Iodomethane.

*Content*: minimum 99.0 per cent.

**Methyl isobutyl ketone.** $C_6H_{12}O$. ($M_r$ 100.2). *1054300.* [108-10-1]. 4-Methyl-2-pentanone.

Clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

$d_{20}^{20}$: about 0.80.

bp: about 115 °C.

*Distillation range* (*2.2.11*). Distil 100 mL. The range of temperature of distillation from 1 mL to 95 mL of distillate does not exceed 4.0 °C.

*Residue on evaporation*: maximum 0.01 per cent, determined by evaporating on a water-bath and drying at 100-105 °C.

**Methyl isobutyl ketone R1.** *1054301.*

Shake 50 mL of freshly distilled *methyl isobutyl ketone R* with 0.5 mL of *hydrochloric acid R1* for 1 min. Allow the phases to separate and discard the lower phase. Prepare immediately before use.

**Methyl isobutyl ketone R3.** *1054302.*

Complies with the requirements for *methyl isobutyl ketone R* and with the following limits.

*Cr*: maximum 0.02 ppm.

*Cu*: maximum 0.02 ppm.

*Pb*: maximum 0.1 ppm.

*Ni*: maximum 0.02 ppm.

*Sn*: maximum 0.1 ppm.

**Methyl isobutyl ketone, water-saturated.** *1054303.*

Shake *methyl isobutyl ketone R* with *water R* prior to use.

**Methyl laurate.** $C_{13}H_{26}O_2$. ($M_r$ 214.4). *1054400.* [111-82-0]. Methyl dodecanoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

Colourless or yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

$d_{20}^{20}$: about 0.87.

$n_D^{20}$: about 1.431.

mp: about 5 °C.

**Methyl lignocerate.** $C_{25}H_{50}O_2$. ($M_r$ 382.7). *1120600.* [2442-49-1]. Methyl tetracosanoate.

Flakes.

mp: about 58 °C.

**Methyl linoleate.** $C_{19}H_{34}O_2$. ($M_r$ 294.5). *1120700.* [112-63-0]. Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate.

$d_{20}^{20}$: about 0.888.

$n_D^{20}$: about 1.466.

bp: 207 °C to 208 °C.

**Methyl linolenate.** $C_{19}H_{32}O_2$. ($M_r$ 292.5). *1120800.* [301-00-8]. Methyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate. Methyl α-linolenate.

$d_{20}^{20}$: about 0.901.

$n_D^{20}$: about 1.471.

bp: about 207 °C.

**Methyl γ-linolenate.** $C_{19}H_{32}O_2$. ($M_r$ 292.5). *1158400.* [16326-32-2]. Methyl (6*Z*,9*Z*,12*Z*)-octadeca-6,9,12-trienoate.

*Content*: minimum 99.0 per cent, determined by gas chromatography.

**Methyl margarate.** $C_{18}H_{36}O_2$. ($M_r$ 284.5). *1120900.* [1731-92-6]. Methyl heptadecanoate.

White or almost white powder.

mp: 32 °C to 34 °C.

*Methyl margarate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Methyl methacrylate.** $C_5H_8O_2$. ($M_r$ 100.1). *1054500.* [80-62-6]. Methyl 2-methylprop-2-enoate.

Colourless liquid.

$n_D^{20}$: about 1.414.

bp: about 100 °C.

mp: about − 48 °C.

It contains a suitable stabilising reagent.

**Methyl methanesulfonate.** $C_2H_6O_3S$. ($M_r$ 110.1). *1179500.* [66-27-3].

Clear, colourless or slightly yellow liquid.

*Content*: minimum 99.0 per cent.

*Density*: about 1.3 g/cm$^3$ (25 °C).

$n_D^{20}$: about 1.414.

bp: about 202 °C.

**Methyl 2-methoxybenzoate.** $C_9H_{10}O_3$. ($M_r$ 166.2). *1206300.* [606-45-1].

Colourless liquid.

**Methyl 4-methoxybenzoate.** $C_9H_{10}O_3$. ($M_r$ 166.2). *1206400.* [121-98-2].

White or almost white powder.

**Methyl *N*-methylanthranilate.** $C_9H_{11}NO_2$. ($M_r$ 165.2). *1164600.* [85-91-6]. Methyl 2-(methylamino)benzoate.

Pale yellow liquid.

$d_4^{20}$: about 1.128.

$n_D^{20}$: about 1.579.

bp: 255 °C to 258 °C.

*Methyl N-methylanthranilate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Mandarin oil (2355)*.

*Test solution*. The substance to be examined.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Methyl myristate.** $C_{15}H_{30}O_2$. ($M_r$ 242.4). *1054600.* [124-10-7]. Methyl tetradecanoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

$d_{20}^{20}$: about 0.87.

$n_D^{20}$: about 1.437.

mp: about 20 °C.

**Methyl nervonate.** *1144800.* [2733-88-2].

See *Tetracos-15-enoic acid methyl ester R*.

**Methyl oleate.** $C_{19}H_{36}O_2$. ($M_r$ 296.4). *1054700.* [112-62-9]. Methyl (9*Z*)-octadec-9-enoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

$d_{20}^{20}$: about 0.88.

$n_D^{20}$: about 1.452.

**Methylophiopogonanone A.** $C_{19}H_{18}O_6$. ($M_r$ 342.3). *1206500.* [74805-92-8]. (3*R*)-3-[(1,3-Benzodioxol-5-yl)methyl]-2,3-dihydro-5,7-dihydroxy-6,8-dimethyl-4*H*-1-benzopyran-4-one.

**Methyl orange.** $C_{14}H_{14}N_3NaO_3S$. ($M_r$ 327.3). *1054800.* [547-58-0].

Schultz No. 176.

Colour Index No. 13025.

Sodium 4′-(dimethylamino)azobenzene-4-sulfonate.

Orange-yellow, crystalline powder, slightly soluble in water, practically insoluble in ethanol (96 per cent).

**Methyl orange mixed solution.** *1054801.*

Dissolve 20 mg of *methyl orange R* and 0.1 g of *bromocresol green R* in 1 mL of *0.2 M sodium hydroxide* and dilute to 100 mL with *water R*.

*Colour change*: pH 3.0 (orange) to pH 4.4 (olive-green).

**Methyl orange solution.** *1054802.*

Dissolve 0.1 g of *methyl orange R* in 80 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

*Test for sensitivity.* A mixture of 0.1 mL of the methyl orange solution and 100 mL of *carbon dioxide-free water R* is yellow. Not more than 0.1 mL of *1 M hydrochloric acid* is required to change the colour to red.

*Colour change*: pH 3.0 (red) to pH 4.4 (yellow).

**Methyl palmitate.** $C_{17}H_{34}O_2$. ($M_r$ 270.5). *1054900.* [112-39-0]. Methyl hexadecanoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 30 °C.

**Methyl palmitoleate.** $C_{17}H_{32}O_2$. ($M_r$ 268.4). *1121000.* [1120-25-8]. Methyl (9*Z*)-hexadec-9-enoate.

$d_{20}^{20}$: about 0.876.

$n_D^{20}$: about 1.451.

**Methyl parahydroxybenzoate.** *1055000.* [99-76-3].

See *Methyl parahydroxybenzoate (0409)*.

**Methyl pelargonate.** $C_{10}H_{20}O_2$. ($M_r$ 172.3). *1143500.* [1731-84-6]. Methyl nonanoate.

Clear, colourless liquid.

$d_4^{20}$: about 0.873.

$n_D^{20}$: about 1.422.

bp: 91 °C to 92 °C.

*Methyl pelargonate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**2-Methylpentane.** $C_6H_{14}$. ($M_r$ 86.2). *1180400.* [107-83-5].

Isohexane.

$d_{20}^{20}$: about 0.653.

bp: about 60.0 °C.

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

**3-Methylpentan-2-one.** $C_6H_{12}O$. ($M_r$ 100.2). *1141100.* [565-61-7].

Colourless, flammable liquid.

$d_{20}^{20}$: about 0.815.

$n_D^{20}$: about 1.400.

bp: about 118 °C

**4-Methylpentan-2-ol.** $C_6H_{14}O$. ($M_r$ 102.2). *1114300.* [108-11-2].

Clear, colourless, volatile liquid.

$d_4^{20}$: about 0.802.

$n_D^{20}$: about 1.411.

bp: about 132 °C.

**Methylphenyloxazolylbenzene.** $C_{26}H_{20}N_2O_2$. ($M_r$ 392.5). *1056200.* [3073-87-8]. 1,4-Bis[2-(4-methyl-5-phenyl)-oxazolyl]benzene.

Fine, greenish-yellow powder with a blue fluorescence or small crystals, soluble in ethanol (96 per cent), sparingly soluble in xylene.

mp: about 233 °C.

*Methylphenyloxazolylbenzene used for liquid scintillation is of a suitable analytical grade.*

**1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine.** $C_{12}H_{15}N$. ($M_r$ 173.3). *1137100.* [28289-54-5]. MPTP.

White or almost white, crystalline powder, slightly soluble in water.

mp: about 41 °C.

**Methylpiperazine.** $C_5H_{12}N_2$. ($M_r$ 100.2). *1056300.* [109-01-3]. 1-Methylpiperazine.

Colourless liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.90.

$n_D^{20}$: about 1.466.

bp: about 138 °C.

**4-(4-Methylpiperidin-1-yl)pyridine.** $C_{11}H_{16}N_2$. ($M_r$ 176.3). *1114400.* [80965-30-6].

Clear liquid.

$n_D^{20}$: about 1.565.

**Methylpolysiloxane.** *1066800.*

Polysiloxane substituted with 100 per cent of methyl groups.

**Methylprednisolone.** $C_{22}H_{30}O_5$. ($M_r$ 374.5). *1193400.* [83-43-2]. 11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione.

White or almost white, crystalline powder.

**2-Methylpropanol.** $C_4H_{10}O$. ($M_r$ 74.1). *1056400.* [78-83-1]. Isobutyl alcohol. 2-Methylpropan-1-ol.

Clear colourless liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.80.

$n_D^{15}$: 1.397 to 1.399.

bp: about 107 °C.

*Distillation range* (*2.2.11*). Not less than 96 per cent distils between 107 °C and 109 °C.

**2-Methyl-2-propanol.** $C_4H_{10}O$. ($M_r$ 74.1). *1056500.* [75-65-0]. 1,1-Dimethyl ethyl alcohol. *tert*-Butyl alcohol.

Clear, colourless liquid or crystalline mass, soluble in water, miscible with ethanol (96 per cent).

*Freezing point* (*2.2.18*): about 25 °C.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 81 °C and 83 °C.

**(15*R*)-15-Methylprostaglandin $F_{2\alpha}$.** $C_{21}H_{36}O_5$. ($M_r$ 368.5). *1159900.* [35864-81-4]. (5*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-Dihydroxy-2-[(1*E*)-(3*R*)-3-hydroxy-3-methyloct-1-enyl]cyclopentyl]hept-5-enoic acid.

Available as a 10 g/L solution in *methyl acetate R*.

*Storage*: at a temperature below − 15 °C.

**5-Methylpyridin-2-amine.** $C_6H_8N_2$. ($M_r$ 108.1). *1193500.* [1603-41-4]. 6-Amino-3-picoline.

White or yellow crystals or crystalline powder.

mp: about 76 °C.

**5-Methylpyridin-2(1*H*)-one.** $C_6H_7NO$. ($M_r$ 109.1). *1193600.* [1003-68-5].

White or almost white powder, soluble in anhydrous ethanol and in methanol.

mp: about 181 °C.

*Storage*: at a temperature of 2 °C to 8 °C.

***N*-Methylpyrrolidine.** $C_5H_{11}N$. ($M_r$ 85.2). *1164700.* [120-94-5].

*Content*: minimum 97.0 per cent.

bp: about 80 °C.

***N*-Methylpyrrolidone.** $C_5H_9NO$. ($M_r$ 99.1). *1164800.* [872-50-4]. 1-Methylpyrrolidin-2-one.

$d_{20}^{20}$: about 1.028.

bp: about 202 °C.

mp: about − 24 °C.

**Methyl red.** $C_{15}H_{15}N_3O_2$. ($M_r$ 269.3). *1055100.* [493-52-7].

Schultz No. 250.

Colour Index No. 13020.

2-(4-Dimethylamino-phenylazo)benzoic acid.

Dark-red powder or violet crystals, practically insoluble in water, soluble in ethanol (96 per cent).

**Methyl red mixed solution.** *1055101.*

Dissolve 0.1 g of *methyl red R* and 50 mg of *methylene blue R* in 100 mL of *ethanol (96 per cent) R*.

*Colour change*: pH 5.2 (red-violet) to pH 5.6 (green).

**Methyl red solution.** *1055102.*

Dissolve 50 mg of *methyl red R* in a mixture of 1.86 mL of *0.1 M sodium hydroxide* and 50 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.1 mL of the methyl red solution add 100 mL of *carbon dioxide-free water R* and 0.05 mL of *0.02 M hydrochloric acid*. The solution is red. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to yellow.

*Colour change*: pH 4.4 (red) to pH 6.0 (yellow).

**Methyl salicylate.** *1146200.* [119-36-8].

See *Methyl salicylate (0230)*

**Methyl stearate.** $C_{19}H_{38}O_2$. ($M_r$ 298.5). *1055200.* [112-61-8]. Methyl octadecanoate.

*Content*: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 38 °C.

**Methylthymol blue.** $C_{37}H_{40}N_2Na_4O_{13}S$. ($M_r$ 845). *1158500.* [1945-77-3]. Tetrasodium 2,2′,2″,2‴-[3*H*-2,1-benzoxathiol-3-ylidenebis[[6-hydroxy-2-methyl-5-(1-methylethyl)-3,1-phenylene]methylenenitrilo]]tetraacetate *S*,*S*-dioxide.

Produces a blue colour with calcium in alkaline solution.

**Methylthymol blue mixture.** *1158501.*

A mixture of 1 part of *methylthymol blue R* and 100 parts of *potassium nitrate R*.

**Methyl toluenesulfonate.** $C_8H_{10}O_3S$. ($M_r$ 186.2). *1191200.* [80-48-8]. Methyl 4-methylbenzenesulfonate. Methyl tosilate.

*Content*: minimum 97.0 per cent.

*Density*: about 1.234 g/mL (25 °C).

bp: about 292 °C.

mp: 25 °C to 28 °C.

***N*-Methyl-*m*-toluidine.** $C_8H_{11}N$. ($M_r$ 121.2). *1175200.* [696-44-6]. *N*,3-Dimethylaniline. *N*,3-Dimethylbenzenamine. Methyl-*m*-tolylamine.

*Content*: minimum 97 per cent.

**Methyl tricosanoate.** $C_{24}H_{48}O_2$. ($M_r$ 368.6). *1111500.* [2433-97-8]. Tricosanoic acid methyl ester.

*Content*: minimum 99.0 per cent.

White or almost white crystals, practically insoluble in water, soluble in hexane.

mp: 55 °C to 56 °C.

**Methyl tridecanoate.** $C_{14}H_{28}O_2$. ($M_r$ 228.4). *1121100.* [1731-88-0].

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

$d_{20}^{20}$: about 0.86.

$n_D^{20}$: about 1.441.

mp: about 6 °C.

**Methyl 3,4,5-trimethoxybenzoate.** $C_{11}H_{14}O_5$. ($M_r$ 226.23). *1177200.* [1916-07-0].

***N*-Methyltrimethylsilyl-trifluoroacetamide.** $C_6H_{12}F_3NOSi$. ($M_r$ 199.3). *1129600.* [24589-78-4]. 2,2,2-Trifluoro-*N*-methyl-*N*-(trimethylsilyl)acetamide.

$n_D^{20}$: about 1.380.

bp: 130 °C to 132 °C.

**Minocycline hydrochloride.** *1146300.*

See *Minocycline hydrochloride (1030)*.

**Molecular sieve.** *1056600.*

Molecular sieve composed of sodium aluminosilicate. It is available as beads or powder with a pore size of 0.4 nm.

When reused, it is recommended that the molecular sieve be regenerated according to the manufacturer's instructions.

**Molecular sieve for chromatography.** *1129700.*

Molecular sieve composed of sodium aluminosilicate. The pore size is indicated after the name of the reagent in the tests where it is used. If necessary, the particle size is also indicated.

**Molybdovanadic reagent.** *1056700.*

In a 150 mL beaker, mix 4 g of finely powdered *ammonium molybdate R* and 0.1 g of finely powdered *ammonium vanadate R*. Add 70 mL of *water R* and grind the particles using a glass rod. A clear solution is obtained within a few minutes. Add 20 mL of *nitric acid R* and dilute to 100 mL with *water R*.

**Monodocosahexaenoin.** $C_{25}H_{38}O_4$. ($M_r$ 402.6). *1143600.* [124516-13-8]. Monoglyceride of docosahexaenoic acid (C22:6). Glycerol monodocosahexaenoate. (*all-Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid, monoester with propane-1,2,3-triol.

**Mordant black 11.** $C_{20}H_{12}N_3NaO_7S$. ($M_r$ 461.4). *1056800.* [1787-61-7].

Schultz No. 241.

Colour Index No. 14645.

Sodium 2-hydroxy-1-[(1-hydroxynaphth-2-yl)azo]-6-nitronaph-thalene-4-sulfonate. Eriochrome black.

Brownish-black powder, soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container, protected from light.

**Mordant black 11 triturate.** *1056801.*

Mix 1 g of *mordant black 11 R* with 99 g of *sodium chloride R*.

*Test for sensitivity.* Dissolve 50 mg in 100 mL of *water R*. The solution is brownish-violet. On addition of 0.3 mL of *dilute ammonia R1* the solution turns blue. On the subsequent addition of 0.1 mL of a 10 g/L solution of *magnesium sulfate R*, it turns violet.

*Storage*: in an airtight container, protected from light.

**Mordant black 11 triturate R1.** *1056802.*

Mix 1.0 g of *mordant black 11 R*, 0.4 g of *methyl orange R* and 100 g of *sodium chloride R*.

**Morphine hydrochloride.** *1056900.*

See *Morphine hydrochloride (0097)*.

**Morpholine.** $C_4H_9NO$. ($M_r$ 87.1). *1057000.* [110-91-8]. Tetrahydro-1,4-oxazine.

Colourless, hygroscopic liquid, flammable, soluble in water and in ethanol (96 per cent).

$d_{20}^{20}$: about 1.01.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 126 °C and 130 °C.

*Storage*: in an airtight container.

**Morpholine for chromatography.** *1057001.*

Complies with the requirements prescribed for *morpholine R* with the following additional requirement.

*Content*: minimum 99.5 per cent.

**2-[*N*-Morpholino]ethanesulfonic acid.** $C_6H_{13}NO_4S$. ($M_r$ 195.2). *1186500.* [4432-31-9]. 2-(Morpholin-4-yl)sulfonic acid. MES.

White or almost white, crystalline powder, soluble in water.

mp: about 300 °C.

**Murexide.** $C_8H_8N_6O_6,H_2O$. ($M_r$ 302.2). *1137200.* 5,5′-Nitrilobis(pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione) monoammonium salt.

Brownish-red crystalline powder, sparingly soluble in cold water, soluble in hot water, practically insoluble in ethanol (96 per cent), soluble in solutions of potassium hydroxide or sodium hydroxide giving a blue colour.

**Myosmine.** $C_9H_{10}N_2$. ($M_r$ 146.2). *1121200.* [532-12-7]. 3-(4,5-Dihydro-3*H*-pyrrol-2-yl)pyridine.

Colourless crystals.

mp: about 45 °C.

**β-Myrcene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1114500.* [123-35-3]. 7-Methyl-3-methylenocta-1,6-diene.

Oily liquid with a pleasant odour, practically insoluble in water, miscible with ethanol (96 per cent), soluble in glacial acetic acid. It dissolves in solutions of alkali hydroxides.

$d_4^{20}$: about 0.794.

$n_D^{20}$: about 1.470.

*β-Myrcene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution.* The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**Myristic acid.** $C_{14}H_{28}O_2$. ($M_r$ 228.4). *1143700.* [544-63-8]. Tetradecanoic acid.

Colourless or white or almost white flakes.

mp: about 58.5 °C.

*Myristic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Myristicine.** $C_{11}H_{12}O_3$. ($M_r$ 192.2). *1099600.* [607-91-0]. 5-Allyl-1-methoxy-2,3-methylenedioxybenzene. 4-Methoxy-6-(prop-2-enyl)-1,3-benzodioxole.

Oily colourless liquid, practically insoluble in water, slightly soluble in anhydrous ethanol, miscible with toluene and with xylene.

$d_{20}^{20}$: about 1.144.

$n_D^{20}$: about 1.540.

bp: 276 °C to 277 °C.

mp: about 173 °C.

*Chromatography.* Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Star anise (1153)*; the chromatogram shows only one principal spot.

*Myristicine used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Nutmeg oil (1552)*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

*Storage*: protected from light.

**Myristyl alcohol.** $C_{14}H_{30}O$. ($M_r$ 214.4). *1121300.* [112-72-1]. Tetradecan-1-ol.

$d_{20}^{20}$: about 0.823.

mp: 38 °C to 40 °C.

**Myrtillin.** $C_{21}H_{21}ClO_{12}$. ($M_r$ 500.8). *1172300.* [6906-38-3]. Delphinidin 3-*O*-glucoside chloride.

**Naphthalene.** $C_{10}H_8$. ($M_r$ 128.2). *1057100.* [91-20-3].

White or almost white crystals, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 80 °C.

*Naphthalene used for liquid scintillation is of a suitable analytical grade.*

**2,3-Naphthalenediamine.** $C_{10}H_{10}N_2$. ($M_r$ 158.2). *1199700.* [771-97-1]. Naphthalene-2,3-diamine. 2,3-Diaminonaphthalene.

Brownish-yellow crystalline powder, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: 195 °C to 198 °C.

**Naphtharson.** $C_{16}H_{11}AsN_2Na_2O_{10}S_2$. ($M_r$ 576.3). *1121400.* [3688-92-4]. Thorin. Disodium 4-[(2-arsonophenyl)azo]-3-hydroxynaphthalene-2,7-disulfonate.

Red powder, soluble in water.

**Naphtharson solution.** *1121401.*

A 0.58 g/L solution of *naphtharson R*.

*Test for sensitivity.* To 50 mL of *ethanol (96 per cent) R*, add 20 mL of *water R*, 1 mL of *dilute sulfuric acid R1* and 1 mL of the naphtharson solution. Titrate with *0.025 M barium perchlorate*; the colour changes from orange-yellow to orange-pink.

*Storage*: protected from light; use within 1 week.

**Naphtharson solution R1.** *1121402.*

A 1 g/L solution in *deionised distilled water R*.

*Test for sensitivity.* To 50 mL of *ethanol (96 per cent) R*, add 20 mL of *water R*, 1 mL of *dilute sulfuric acid R1* and 1 mL of *naphtharson solution R1*. Titrate with *0.025 M barium perchlorate*; the colour changes from orange-yellow to orange-pink.

*Storage*: protected from light; use within 1 week.

**α-Naphthol.** $C_{10}H_8O$. ($M_r$ 144.2). *1057300.* [90-15-3]. 1-Naphthol.

White or almost white, crystalline powder or colourless or white or almost white crystals, darkening on exposure to light, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 95 °C.

*Storage*: protected from light.

**α-Naphthol solution.** *1057301.*

Dissolve 0.10 g of *α-naphthol R* in 3 mL of a 150 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*. Prepare immediately before use.

**β-Naphthol.** $C_{10}H_8O$. ($M_r$ 144.2). *1057400.* [135-19-3]. 2-Naphthol.

White or slightly pink plates or crystals, very slightly soluble in water, very soluble in ethanol (96 per cent).

mp: about 122 °C.

*Storage*: protected from light.

**β-Naphthol solution.** *1057401.*

Dissolve 5 g of freshly recrystallised *β-naphthol R* in 40 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*. Prepare immediately before use.

**β-Naphthol solution R1.** *1057402.*

Dissolve 3.0 mg of *β-naphthol R* in 50 mL of *sulfuric acid R* and dilute to 100.0 mL with the same acid. Use the recently prepared solution.

**Naphtholbenzein.** $C_{27}H_{18}O_2$. ($M_r$ 374.4). *1057600.* [145-50-6]. α-Naphtholbenzein. 4-[(4-Hydroxynaphthalen-1-yl)(phenyl)methylidene] naphthalen-1(4*H*)-one.

Brownish-red powder or shiny brownish-black crystals, practically insoluble in water, soluble in ethanol (96 per cent) and in glacial acetic acid.

**Naphtholbenzein solution.** *1057601.*

A 2 g/L solution of *naphtholbenzein R* in *anhydrous acetic acid R*.

*Test for sensitivity.* To 50 mL of *glacial acetic acid R* add 0.25 mL of the naphtholbenzein solution. The solution is brownish-yellow. Not more than 0.05 mL of *0.1 M perchloric acid* is required to change the colour to green.

**Naphthol yellow.** $C_{10}H_5N_2NaO_5$. ($M_r$ 256.2). *1136600.* 2,4-Dinitro-1-naphthol, sodium salt.

Orange-yellow powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Naphthol yellow S.** $C_{10}H_4N_2Na_2O_8S$. ($M_r$ 358.2). *1143800.* [846-70-8].

Colour Index No. 10316. 8-Hydroxy-5,7-dinitro-2-naphthalenesulfonic acid disodium salt. Disodium 5,7-dinitro-8-oxidonaphthalene-2-sulfonate.

Yellow or orange-yellow powder, freely soluble in water.

**1-Naphthylacetic acid.** $C_{12}H_{10}O_2$. ($M_r$ 186.2). *1148400.* [86-87-3]. (Naphthalen-1-yl)acetic acid.

White or yellow crystalline powder, very slightly soluble in water, freely soluble in acetone.

mp: about 135 °C.

**Naphthylamine.** $C_{10}H_9N$. ($M_r$ 143.2). *1057700.* [134-32-7]. 1-Naphthylamine.

White or almost white, crystalline powder, turning pink on exposure to light and air, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 51 °C.

*Storage*: protected from light.

**Naphthylethylenediamine dihydrochloride.** $C_{12}H_{16}Cl_2N_2$. ($M_r$ 259.2). *1057800.* [1465-25-4]. *N*-(1-Naphthyl)ethylene-diamine dihydrochloride.

It may contain methanol of crystallisation.

White or yellowish-white powder, soluble in water, slightly soluble in ethanol (96 per cent).

**Naphthylethylenediamine dihydrochloride solution.** *1057801.*

Dissolve 0.1 g of *naphthylethylenediamine dihydrochloride R* in *water R* and dilute to 100 mL with the same solvent. Prepare immediately before use.

**Naringin.** $C_{27}H_{32}O_{14}$. ($M_r$ 580.5). *1137300.* [10236-47-2]. 7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one.

White or almost white crystalline powder, slightly soluble in water, soluble in methanol and in dimethylformamide.

mp: about 171 °C.

*Absorbance* (*2.2.25*). Naringin dissolved in a 5 g/L solution of *dimethylformamide R* in *methanol R* shows an absorption maximum at 283 nm.

**Neohesperidin.** $C_{28}H_{34}O_{15}$. ($M_r$ 610.6). *1182200.* [13241-33-3]. Hesperetin-7-neohesperidoside. (2*S*)-7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one.

***trans*-Nerolidol.** $C_{15}H_{26}O$. ($M_r$ 222.4). *1107900.* [40716-66-3]. 3,7,11-Trimethyldodeca-1,6,10-trien-3-ol.

Slightly yellow liquid, slight odour of lily and lily of the valley, practically insoluble in water and in glycerol, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.876.

$n_D^{20}$: about 1.479.

$bp_{12}$: 145 °C to 146 °C.

*trans-Nerolidol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**Neryl acetate.** $C_{12}H_{20}O_2$. ($M_r$ 196.3). *1108000.* [141-12-8]. (*Z*)-3,7-Dimethylocta-2,6-dienyl acetate.

Colourless, oily liquid.

$d_{20}^{20}$: about 0.907.

$n_D^{20}$: about 1.460.

$bp_{25}$: 134 °C.

*Neryl acetate used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 93.0 per cent, calculated by the normalisation procedure.

**Nickel-aluminium alloy.** *1058100.*

Contains 48 per cent to 52 per cent of aluminium (Al; $A_r$ 26.98) and 48 per cent to 52 per cent of nickel (Ni; $A_r$ 58.70).

Before use, reduce to a fine powder (180) (*2.9.12*).

It is practically insoluble in water and soluble in mineral acids.

**Nickel-aluminium alloy (halogen-free).** *1118100.*

Contains 48 per cent to 52 per cent of aluminium (Al; $A_r$ 26.98) and 48 per cent to 52 per cent of nickel (Ni; $A_r$ 58.71).

Fine, grey powder, practically insoluble in water, soluble in mineral acids with formation of salts.
*Chlorides*: maximum 10 ppm.

Dissolve 0.400 g in 40 mL of a mixture of 67 volumes of *sulfuric acid R* and 33 volumes of *dilute nitric acid R*. Evaporate the solution nearly to dryness, dissolve the residue in *water R* and dilute to 20.0 mL with the same solvent. To one half-aliquot of the solution, add 1.0 mL of *0.1 M silver nitrate*. Filter after 15 min and add 0.2 mL of sodium chloride solution (containing 10 µg of chlorides per millilitre) to the filtrate. After 5 min the solution is more opalescent than a mixture of the second half-aliquot of the solution with 1.0 mL of *0.1 M silver nitrate*.

**Nickel chloride.** $NiCl_2$. ($M_r$ 129.6). *1057900.* [7718-54-9]. Nickel chloride, anhydrous.

Yellow, crystalline powder, very soluble in water, soluble in ethanol (96 per cent). It sublimes in the absence of air and readily absorbs ammonia. The aqueous solution is acid.

**Nickel nitrate hexahydrate.** $Ni(NO_3)_2,6H_2O$. ($M_r$ 290.8). *1175300.* [13478-00-7].

**Nickel sulfate.** $NiSO_4,7H_2O$. ($M_r$ 280.9). *1058000.* [10101-98-1]. Nickel sulfate heptahydrate.

Green, crystalline powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Nicotinamide-adenine dinucleotide.** $C_{21}H_{27}N_7O_{14}P_2$. ($M_r$ 663). *1108100.* [-84-9]. $NAD^+$.

White or almost white powder, very hygroscopic, freely soluble in water.

**Nicotinamide-adenine dinucleotide solution.** *1108101.*

Dissolve 40 mg of *nicotinamide-adenine dinucleotide R* in *water R* and dilute to 10 mL with the same solvent. Prepare immediately before use.

**Nicotinic acid.** *1158600.* [59-67-6].

See *Nicotinic acid (0459)*.

**Nicotinoyl hydrazide.** $C_6H_7N_3O$. ($M_r$ 137.1). *1202400.* [553-53-7]. Pyridine-3-carbohydrazide.

White or almost white powder or crystalline powder, soluble in water.

mp: about 160 °C.

**Nile blue A.** $C_{20}H_{21}N_3O_5S$. ($M_r$ 415.5). *1058200.* [3625-57-8].

Schultz No. 1029.

Colour Index No. 51180.
5-Amino-9-(diethylamino)benzo[*a*]phenoxazinylium hydrogen sulfate.

Green, crystalline powder with a bronze lustre, sparingly soluble in ethanol (96 per cent), in glacial acetic acid and in pyridine.

*Absorbance* (*2.2.25*). A 0.005 g/L solution in *ethanol (50 per cent V/V) R* shows an absorption maximum at 640 nm.

**Nile blue A solution.** *1058201.*

A 10 g/L solution of *Nile blue A R* in *anhydrous acetic acid R*.

*Test for sensitivity.* To 50 mL of *anhydrous acetic acid R* add 0.25 mL of the Nile blue A solution. The solution is blue. On the addition of 0.1 mL of *0.1 M perchloric acid*, the colour changes to blue-green.

*Colour change*: pH 9.0 (blue) to pH 13.0 (red).

**Ninhydrin.** $C_9H_4O_3,H_2O$. ($M_r$ 178.1). *1058300.* [485-47-2]. 1,2,3-Indanetrione monohydrate.

White or very pale yellow, crystalline powder, soluble in water and in ethanol (96 per cent).

*Storage*: protected from light.

**Ninhydrin and stannous chloride reagent.** *1058301.*

Dissolve 0.2 g of *ninhydrin R* in 4 mL of hot *water R*, add 5 mL of a 1.6 g/L solution of *stannous chloride R*, allow to stand for 30 min, then filter and store at a temperature of 2 °C to 8 °C. Immediately before use dilute 2.5 mL of the solution with 5 mL of *water R* and 45 mL of *2-propanol R*.

**Ninhydrin solution.** *1058303.*

A 2 g/L solution of *Ninhydrin R* in a mixture of 5 volumes of *dilute acetic acid R* and 95 volumes of *butanol R*.

**Ninhydrin solution R1.** *1058304.*

Dissolve 1.0 g of *ninhydrin R* in 50 mL of *ethanol (96 per cent) R* and add 10 mL of *glacial acetic acid R*.

**Ninhydrin solution R2.** *1058305.*

Dissolve 3 g of *ninhydrin R* in 100 mL of a 45.5 g/L solution of *sodium metabisulfite R*.

**Ninhydrin solution R3.** *1058306.*

A 4 g/L solution in a mixture of 5 volumes of *anhydrous acetic acid R* and 95 volumes of *butanol R*.

**Ninhydrin solution R4.** *1058307.*

A 3 g/L solution of *ninhydrin R* in a mixture of 5 volumes of *glacial acetic acid R* and 95 volumes of *2-propanol R*.

**Nitrazepam.** *1143900.* [146-22-5].

See *Nitrazepam (0415)*.

**Nitric acid.** $HNO_3$. ($M_r$ 63.0). *1058400.* [7697-37-2].

*Content*: 63.0 per cent *m/m* to 70.0 per cent *m/m*.

Clear, colourless or almost colourless liquid, miscible with water.

$d_{20}^{20}$: 1.384 to 1.416.

A 10 g/L solution is strongly acid and gives the reaction of nitrates (*2.3.1*).

*Appearance.* Nitric acid is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

*Chlorides* (*2.4.4*): maximum 0.5 ppm.

To 5 g add 10 mL of *water R* and 0.3 mL of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared in the same manner using 13 mL of *water R*, 0.5 mL of *nitric acid R*, 0.5 mL of *chloride standard solution (5 ppm Cl) R* and 0.3 mL of *silver nitrate solution R2*.

*Sulfates* (*2.4.13*): maximum 2 ppm.

Evaporate 10 g to dryness with 0.2 g of *sodium carbonate R*. Dissolve the residue in 15 mL of *distilled water R*. Prepare the standard using a mixture of 2 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 13 mL of *distilled water R*.

*Arsenic* (*2.4.2, Method A*): maximum 0.02 ppm.

Gently heat 50 g with 0.5 mL of *sulfuric acid R* until white fumes begin to evolve. To the residue add 1 mL of a 100 g/L solution of *hydroxylamine hydrochloride R* and dilute to 2 mL with *water R*. Prepare the standard using 1.0 mL of *arsenic standard solution (1 ppm As) R*.

*Iron* (*2.4.9*): maximum 1 ppm.

Dissolve the residue from the determination of sulfated ash in 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Dilute 5 mL of this solution to 10 mL with *water R*.

*Heavy metals* (*2.4.8*): maximum 2 ppm.

Dilute 10 mL of the solution prepared for the limit test for iron to 20 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

*Sulfated ash*: maximum 0.001 per cent.

Carefully evaporate 100 g to dryness. Moisten the residue with a few drops of *sulfuric acid R* and heat to dull red.

*Assay.* To 1.50 g add about 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 63.0 mg of $HNO_3$.

*Storage*: protected from light.

**Nitric acid, cadmium- and lead-free.** *1058401.*

Complies with the requirements prescribed for *nitric acid R* and with the following additional test.

*Test solution.* To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R* heating slightly, and dilute to 50.0 mL with the same solvent.

*Cadmium*: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene or air-propane flame.

*Lead*: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm or 217.0 nm.

*Atomisation device*: air-acetylene flame.

**Nitric acid, dilute.** *1058402.*

Contains about 125 g/L of $HNO_3$ ($M_r$ 63.0).

Dilute 20 g of *nitric acid R* to 100 mL with *water R*.

**Nitric acid, dilute R1.** *1058407.*

Dilute 40 g of *nitric acid R* to 100 mL with *water R*.

**Nitric acid, dilute R2.** *1058409.*

Dilute 30 g of *nitric acid R* to 100 mL with *water R*.

**Nitric acid, dilute, heavy metal-free.** *1058410.*

Complies with the requirements prescribed for *dilute nitric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.
Cd: 0.005 ppm.
Cu: 0.001 ppm.
Fe: 0.02 ppm.
Hg: 0.002 ppm.
Ni: 0.005 ppm.
Pb: 0.001 ppm.
Zn: 0.01 ppm.

**Nitric acid, heavy metal-free.** *1058404.*

Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.
Cd: 0.005 ppm.
Cu: 0.001 ppm.
Fe: 0.02 ppm.
Hg: 0.002 ppm.

Ni: 0.005 ppm.

Pb: 0.001 ppm.

Zn: 0.01 ppm.

**Nitric acid, lead-free.** *1058403.*

Complies with the requirements prescribed for *Nitric acid R* with the following additional test.

*Lead*: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R*, heating slightly, and dilute to 50.0 mL with the same solvent.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm or 217.0 nm.

*Atomisation device*: air-acetylene flame.

**Nitric acid, lead-free R1.** *1058405.*

*Nitric acid R* containing not more than 1 µg/kg of lead.

**Nitric acid, lead-free, dilute.** *1058406.*

Dilute 5 g of *lead-free nitric acid R1* to 100 mL with *deionised distilled water R*.

**Nitric acid, nickel-free.** *1058408.*

Complies with the requirements prescribed for *nitric acid R* with the following additional requirement.

*Nickel*: maximum 0.005 ppm.

**Nitric acid, fuming.** *1058500.* [7697-37-2].

Clear, slightly yellowish liquid, fuming on contact with air.

$d_{20}^{20}$: about 1.5.

**Nitrilotriacetic acid.** $C_6H_9NO_6$. ($M_r$ 191.1). *1137400.* [139-13-9].

White or almost white crystalline powder, practically insoluble in water and in most organic solvents.

mp: about 240 °C, with decomposition.

**Nitroaniline.** $C_6H_6N_2O_2$. ($M_r$ 138.1). *1058600.* [100-01-6]. 4-Nitroaniline.

Bright yellow, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in ethanol (96 per cent), forms water-soluble salts with strong mineral acids.

mp: about 147 °C.

**Nitrobenzaldehyde.** $C_7H_5NO_3$. ($M_r$ 151.1). *1058700.* [552-89-6]. 2-Nitrobenzaldehyde.

Yellow needles, slightly soluble in water, freely soluble in ethanol (96 per cent), volatile in steam.

mp: about 42 °C.

**Nitrobenzaldehyde paper.** *1058701.*

Dissolve 0.2 g of *nitrobenzaldehyde R* in 10 mL of a 200 g/L solution of *sodium hydroxide R*. Use the solution within 1 h. Immerse the lower half of a slow filter paper strip 10 cm long and 0.8-1 cm wide. Absorb the excess reagent between two sheets of filter paper. Use within a few minutes of preparation.

**Nitrobenzaldehyde solution.** *1058702.*

Add 0.12 g of powdered *nitrobenzaldehyde R* to 10 mL of *dilute sodium hydroxide solution R*; allow to stand for 10 min shaking frequently and filter. Prepare immediately before use.

**4-Nitrobenzaldehyde.** $C_7H_5NO_3$. ($M_r$ 151.1). *1198700.* [555-16-8].

**Nitrobenzene.** $C_6H_5NO_2$. ($M_r$ 123.1). *1058800.* [98-95-3].

Colourless or very slightly yellow liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: about 211 °C.

*Dinitrobenzene.* To 0.1 mL add 5 mL of *acetone R*, 5 mL of *water R* and 5 mL of *strong sodium hydroxide solution R*. Shake and allow to stand. The upper layer is almost colourless.

**4-Nitrobenzoic acid.** $C_7H_5NO_4$. ($M_r$ 167.1). *1144000.* [62-23-7].

Yellow crystals.

mp: about 240 °C.

**Nitrobenzoyl chloride.** $C_7H_4ClNO_3$. ($M_r$ 185.6). *1058900.* [122-04-3]. 4-Nitrobenzoyl chloride.

Yellow crystals or a crystalline mass, decomposing in moist air, completely soluble in sodium hydroxide solution giving a yellowish-orange colour.

mp: about 72 °C.

**Nitrobenzyl chloride.** $C_7H_6ClNO_2$. ($M_r$ 171.6). *1059000.* [100-14-1]. 4-Nitrobenzyl chloride.

Pale-yellow crystals, lachrymatory, practically insoluble in water, very soluble in ethanol (96 per cent).

**4-(4-Nitrobenzyl)pyridine.** $C_{12}H_{10}N_2O_2$. ($M_r$ 214.2). *1101900.* [1083-48-3].

Yellow powder.

mp: about 70 °C.

**Nitroethane.** $C_2H_5NO_2$. ($M_r$ 75.1). *1059200.* [79-24-3].

Clear, oily, colourless liquid.

bp: about 114 °C.

**Nitrofurantoin.** *1099700.* [67-20-9].

See *Nitrofurantoin (0101)*.

**Nitrogen.** $N_2$. ($M_r$ 28.01). *1059300.* [7727-37-9].

Nitrogen, washed and dried.

**Nitrogen gas mixture.** *1136900.*

*Nitrogen R* containing 1 per cent *V/V* of each of the following gases: *carbon dioxide R2*, *carbon monoxide R1* and *oxygen R1*.

**Nitrogen, oxygen-free.** *1059600.*

*Nitrogen R* which has been freed from oxygen by passing it through *alkaline pyrogallol solution R*.

**Nitrogen R1.** $N_2$. ($M_r$ 28.01). *1059400.* [7727-37-9].

*Content*: minimum 99.999 per cent *V/V*.

*Carbon monoxide*: less than 5 ppm.

*Oxygen*: less than 5 ppm.

**Nitrogen dioxide.** $NO_2$. ($M_r$ 46.01). *1179600.* [10102-44-0].

*Content*: minimum 98.0 per cent *V/V*.

**Nitrogen for chromatography.** $N_2$. ($M_r$ 28.01). *1059500.* [7727-37-9].

*Content*: minimum 99.95 per cent *V/V*.

**Nitrogen monoxide.** NO. ($M_r$ 30.01). *1108300.*

*Content*: minimum 98.0 per cent *V/V*.

**Nitromethane.** $CH_3NO_2$. ($M_r$ 61.0). *1059700.* [75-52-5].

Clear, colourless, oily liquid, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 1.132 to 1.134.

$n_D^{20}$: 1.381 to 1.383.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 100 °C and 103 °C.

**Nitro-molybdovanadic reagent.** *1060100.*

*Solution A*. Dissolve 10 g of *ammonium molybdate R* in *water R*, add 1 mL of *ammonia R* and dilute to 100 mL with *water R*.

*Solution B*. Dissolve 2.5 g of *ammonium vanadate R* in hot *water R*, add 14 mL of *nitric acid R* and dilute to 500 mL with *water R*.

To 96 mL of *nitric acid R* add 100 mL of solution A and 100 mL of solution B and dilute to 500 mL with *water R*.

**4-Nitrophenol.** $C_6H_5NO_3$. ($M_r$ 139.1). *1146400.* [100-02-7]. *p*-Nitrophenol.

*Content*: minimum 95 per cent.

Colourless or slightly yellow powder, sparingly soluble in water and in methanol.

mp: about 114 °C.

**3-Nitrosalicylic acid.** $C_7H_5NO_5$. ($M_r$ 183.1). *1184300.* [85-38-1]. 2-Hydroxy-3-nitrobenzoic acid.

Yellowish crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: 142 °C to 147 °C.

***N*-Nitrosodiethanolamine.** $C_4H_{10}N_2O_3$. ($M_r$ 134.1). *1129800.* [1116-54-7]. 2,2′-(Nitrosoimino)diethanol.

Yellow liquid, miscible with anhydrous ethanol.

$n_D^{20}$: about 1.485.

bp: about 125 °C.

***N*-Nitrosodiisopropanolamine.** $C_6H_{14}N_2O_3$. ($M_r$ 162.2). *1176500.* [53609-64-6]. 1,1′-(Nitrosoimino)bispropan-2-ol.

bp: 122-124 °C.

**Nitrosodipropylamine.** $C_6H_{14}N_2O$. ($M_r$ 130.2). *1099900.* [621-64-7]. Dipropylnitrosamine.

Liquid, soluble in anhydrous ethanol and in strong acids.

$d_{20}^{20}$: about 0.915.

bp: about 78 °C.

Appropriate grade for chemiluminescence determination.

**Nitrosodipropylamine solution.** *1099901.*

Inject 78.62 g of *anhydrous ethanol R* through the septum of a vial containing *nitrosodipropylamine R*. Dilute 1/100 in *anhydrous ethanol R* and place 0.5 mL aliquots in crimp-sealed vials.

*Storage*: in the dark at 5 °C.

**Nitrotetrazolium blue.** $C_{40}H_{30}Cl_2N_{10}O_6$. ($M_r$ 818). *1060000.* [298-83-9]. 3,3′-(3,3′-Dimethoxy-4,4′-diphenylene)di[2-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium] dichloride. *p*-Nitro-tetrazolium blue.

Crystals, soluble in methanol, giving a clear, yellow solution.

mp: about 189 °C, with decomposition.

**Nitrous oxide.** $N_2O$. ($M_r$ 44.01). *1108500.*

*Content*: minimum 99.99 per cent *V/V*.

*Nitrogen monoxide*: less than 1 ppm.

*Carbon monoxide*: less than 1 ppm.

**Nonivamide.** $C_{17}H_{27}NO_3$. ($M_r$ 293.4). *1148500.* [2444-46-4]. *N*-[(4-Hydroxy-3-methoxyphenyl)methyl]nonanamide.

White or almost white, crystalline powder, practically insoluble in cold water, freely soluble in anhydrous ethanol.

*Nonivamide used in the test for nonivamide in the monograph Capsicum (1859) complies with the following additional test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Capsicum (1859)*.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Nonylamine.** $C_9H_{21}N$. ($M_r$ 143.3). *1139800.* [112-20-9]. Nonan-1-amine. 1-Aminononane.

Corrosive, colourless, clear liquid.

$d_4^{20}$: about 0.788.

$n_D^{20}$: about 1.433.

**Nordazepam.** $C_{15}H_{11}ClN_2O$. ($M_r$ 270.7). *1060200.* [1088-11-5]. 7-Chloro-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one.

White or pale yellow, crystalline powder, practically insoluble in water, slightly soluble in ethanol (96 per cent).

mp: about 216 °C.

**DL-Norleucine.** $C_6H_{13}NO_2$. ($M_r$ 131.2). *1060300.* [616-06-8]. (*RS*)-2-Aminohexanoic acid.

Shiny crystals, sparingly soluble in water and in ethanol (96 per cent), soluble in acids.

**Noscapine hydrochloride.** *1060500.* [912-60-7].

See *Noscapine hydrochloride (0515)*.

**Ochratoxin A solution.** *1175700.*

50 µg/mL solution of (2*S*)-2-([[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-2-benzopyran-7-yl]carbonyl]amino)-3-phenylpropanoic acid (ochratoxin A) in a mixture of 1 volume of *acetic acid R* and 99 volumes of *benzene R*.

**Octadecyl [3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-propionate].** $C_{35}H_{62}O_3$. ($M_r$ 530.9). *1060600.* [2082-79-3]. Octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate.

White or slightly yellowish, crystalline powder, practically insoluble in water, very soluble in acetone and in hexane, slightly soluble in methanol.

mp: 49 °C to 55 °C.

**Octanal.** $C_8H_{16}O$. ($M_r$ 128.2). *1150400.* [124-13-0]. Octyl aldehyde.

Oily, colourless liquid. Practically insoluble in water.

*Octanal used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Sweet orange oil (1811)*.

*Content*: minimum 99 per cent, calculated by the normalisation procedure.

**Octane.** $C_8H_{18}$. ($M_r$ 114.2). *1166500.* [111-65-9]. *n*-Octane.

*Content*: minimum 99 per cent.

**Octanol.** $C_8H_{18}O$. ($M_r$ 130.2). *1060700.* [111-87-5]. Octan-1-ol. Caprylic alcohol.

Colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.828.

bp: about 195 °C.

**3-Octanone.** $C_8H_{16}O$. ($M_r$ 128.2). *1114600.* [106-68-3]. Octan-3-one. Ethylpentylketone.

Colourless liquid with a characteristic odour.

$d_{20}^{20}$: about 0.822.

$n_D^{20}$: about 1.415.

bp: about 167 °C.

*3-Octanone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

*Test solution.* The substance to be examined.

*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Octoxinol 10.** $C_{34}H_{62}O_{11}$ (average). ($M_r$ 647). *1060800.* [9002-93-1]. α-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-ω-hydroxypoly-(oxyethylene).

Clear, pale-yellow, viscous liquid, miscible with water, with acetone and with ethanol (96 per cent), soluble in toluene.

*Storage*: in an airtight container.

**Octreotide acetate.** $C_{49}H_{66}N_{10}O_{10}S_2,xC_2H_4O_2$. *1182900.* [79517-01-4]. (Acetate-free peptide: $M_r$ 1019. [83150-76-9]). D-Phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide acetate. It contains a variable quantity of acetic acid.

White or almost white powder, freely soluble in water and acetic acid.

*Content*: minimum 96.0 per cent.

**Octylamine.** $C_8H_{19}N$. ($M_r$ 129.2). *1150500.* [111-86-4]. Octan-1-amine.

Colourless liquid.

$d_{20}^{20}$: about 0.782.

bp: 175 °C to 179 °C.

**Oleamide.** $C_{18}H_{35}NO$. ($M_r$ 281.5). *1060900.* (9*Z*)-Octadec-9-enoamide.

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

mp: about 80 °C.

**Oleanolic acid.** $C_{30}H_{48}O_3$. ($M_r$ 456.7). *1183000.* [508-02-1]. 3β-Hydroxyolean-12-en-28-oic acid. Astrantiagenin C.

**Oleic acid.** $C_{18}H_{34}O_2$. ($M_r$ 282.5). *1144100.* [112-80-1]. (9*Z*)-Octadec-9-enoic acid.

Clear, colourless liquid, practically insoluble in water.

$d_4^{20}$: about 0.891.

$n_D^{20}$: about 1.459.

mp: 13 °C to 14 °C.

*Oleic acid used in the assay of total fatty acids in the monograph Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Oleuropein.** $C_{25}H_{32}O_{13}$. ($M_r$ 540.5). *1152900.* [32619-42-4]. 2-(3,4-Dihydroxyphenyl)ethyl[(2*S*,3*E*,4*S*)-3-ethylidene-2-(β-D-glucopyranosyloxy)-5-(methoxycarbonyl)-3,4-dihydro-2*H*-pyran-4-yl]acetate.

Powder, soluble in methanol.

*Oleuropein used in Olive leaf (1878) complies with the following test.*

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Olive leaf (1878)*.

*Content*: minimum 80 per cent, calculated by the normalisation procedure.

**Oleyl alcohol.** $C_{18}H_{36}O$. ($M_r$ 268.5). *1156000.* [143-28-2]. (9*Z*)-Octadec-9-en-1-ol.

bp: about 207 °C.

$n_D^{20}$: 1.460.

*Content*: minimum 85 per cent.

**Olive oil.** *1061000.* [8001-25-0].

See *Olive oil, virgin (0518)*.

**Orcinol.** $C_7H_8O_2,H_2O$. ($M_r$ 142.2). *1108700.* [6153-39-5]. 5-Methylbenzene-1,3-diol monohydrate.

Crystalline powder, sensitive to light.

bp: about 290 °C.

mp: 58 °C to 61 °C.

**Organosilica polymer, amorphous, octadecylsilyl.** *1144200.*

Synthetic, spherical hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by trifunctionally bonded octadecylsilyl groups.

**Organosilica polymer, amorphous, polar-embedded octadecylsilyl, end-capped.** *1150600.*

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Organosilica polymer, amorphous, propyl-2-phenylsilyl, end-capped.** *1178100.*

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of propyl-2-phenylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Organosilica polymer compatible with 100 per cent aqueous mobile phases, octadecylsilyl, solid core, end-capped.** *1201700.*

Silica gel with spherical silica particles containing a solid non-porous silica core surrounded by a thin outer organosilica polymer coating with octadecylsilyl groups, suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Organosilica polymer for chromatography, amorphous, octadecylsilyl, end-capped.** *1164900.*

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Organosilica polymer, multi-layered, octadecylsilyl, end-capped.** *1202500.*

Synthetic, spherical hybrid particles, multi-layered, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Osthole.** $C_{15}H_{16}O_3$. ($M_r$ 244.3). *1180500.* [484-12-8]. 7-Methoxy-8-(3-methylbut-2-enyl)-2*H*-1-benzopyran-2-one. 7-Methoxy-8-isopentenylcoumarin.

**Oxalic acid.** $C_2H_2O_4,2H_2O$. ($M_r$ 126.1). *1061400.* [6153-56-6]. Ethanedioic acid dihydrate.

White or almost white crystals, soluble in water, freely soluble in ethanol (96 per cent).

**Oxalic acid and sulfuric acid solution.** *1061401.*

A 50 g/L solution of *oxalic acid R* in a cooled mixture of equal volumes of *sulfuric acid R* and *water R*.

**Oxazepam.** *1144300.* [604-75-1].

See *Oxazepam (0778)*.

**Ox brain, acetone-dried.** *1061300.*

Cut into small pieces a fresh ox brain previously freed from vascular and connective tissue. Place in *acetone R* for preliminary dehydration. Complete the dehydration by pounding in a mortar 30 g of this material with successive quantities, each of 75 mL, of *acetone R* until a dry powder is obtained after filtration. Dry at 37 °C for 2 h or until the odour of acetone is no longer present.

**2,2′-Oxybis(*N*,*N*-dimethylethylamine).** $C_8H_{20}N_2O$. ($M_r$ 160.3). *1141200.* [3033-62-3]. Bis(2-dimethylaminoethyl) ether.

Colourless, corrosive liquid.

$d_{20}^{20}$: about 0.85.

$n_D^{20}$: about 1.430.

**Oxygen.** $O_2$. ($M_r$ 32.00). *1108800.*

*Content*: minimum 99.99 per cent *V/V*.

*Nitrogen and argon*: less than 100 ppm.

*Carbon dioxide*: less than 10 ppm.

*Carbon monoxide*: less than 5 ppm.

**Oxygen R1.** $O_2$. ($M_r$ 32.00). *1137600.*

*Content*: minimum 99 per cent *V/V*.

**Oxytetracycline hydrochloride.** *1146500.*

See *Oxytetracycline hydrochloride (0198)*.

**Paeoniflorin.** $C_{23}H_{28}O_{11}$. ($M_r$ 480.5). *1197300.* [23180-57-6]. [(1*R*,2*S*,3*R*,5*R*,6*R*,8*S*)-3-(β-D-Glucopyranosyloxy)6-hydroxy-8-methyl-9,10-dioxatetracyclo[4.3.1.$0^{2,5}$.$0^{3,8}$]decan-2-yl]methyl benzoate.

**Paeonol.** $C_9H_{10}O_3$. ($M_r$ 166.2). *1197400.* [552-41-0]. 1-(2-Hydroxy-4-methoxyphenyl)ethan-1-one. 2′-Hydroxy-4′-methoxyacetophenone.

**Palladium.** Pd. ($A_r$ 106.4). *1114700.* [7440-05-3].

Grey white metal, soluble in hydrochloric acid.

**Palladium chloride.** $PdCl_2$. ($M_r$ 177.3). *1061500.* [7647-10-1].

Red crystals.

mp: 678 °C to 680 °C.

**Palladium chloride solution.** *1061501.*

Dissolve 1 g of *palladium chloride R* in 10 mL of warm *hydrochloric acid R*. Dilute the solution to 250 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Dilute this solution immediately before use with 2 volumes of *water R*.

**Palmatine.** $C_{21}H_{22}NO_4^+$. ($M_r$ 352.4). *1198800.* [3486-67-7]. 2,3,9,10-Tetramethoxy-5,6-dihydro-7λ⁵-isoquinolino-[3,2-*a*]isoquinolin-7-ylium. 7,8,13,13a-Tetradehydro-2,3,9,10-tetramethoxyberbinium.

**Palmitic acid.** $C_{16}H_{32}O_2$. ($M_r$ 256.4). *1061600.* [57-10-3]. Hexadecanoic acid.

White or almost white, crystalline scales, practically insoluble in water, freely soluble in hot ethanol (96 per cent).

mp: about 63 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Chloramphenicol palmitate (0473)*; the chromatogram shows only one principal spot.

*Palmitic acid used in the assay of total fatty acids in the monograph Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Palmitoleic acid.** $C_{16}H_{30}O_2$. ($M_r$ 254.4). *1144400.* [373-49-9]. (9*Z*)-Hexadec-9-enoic acid.

Clear, colourless liquid.

bp: about 162 °C.

*Palmitoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Palmityl alcohol.** $C_{16}H_{34}O$. ($M_r$ 242.4). *1156100.* [36653-82-4]. Hexadecan-1-ol. Cetyl alcohol.

mp: about 48 °C.

*Content*: minimum 96 per cent.

**Pancreas powder.** *1061700.*

See *Pancreas powder (0350)*.

**Papain.** *1150700.* [9001-73-4].

A proteolytic enzyme obtained from the latex of the green fruit and leaves of *Carica papaya* L.

**Papaverine hydrochloride.** *1061800.* [61-25-6].

See *Papaverine hydrochloride (0102)*.

**Paper chromatography performance test solutions.** *1150800.*

*Test solution (A). Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283).*

*Test solution (B)*. In a closed vial mix 100 µL of a 5 g/L solution of *stannous chloride R* in *0.05 M hydrochloric acid* and 100 MBq to 200 MBq of *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* in a volume not exceeding 2 mL.

**Paper for chromatography.** *1150900.*

Pure cellulose grade thin paper with a smooth surface and a thickness of about 0.2 mm.

*Chromatographic separation*. To 2 strips of *paper for chromatography R* apply separately 2-5 µL of test solution (a) and test solution (b) of *paper chromatography performance test solutions R*. Develop over a pathlength of 3/4 of the paper height, using a mixture of equal volumes of *methanol R* and *water R*. Allow to dry and determine the distribution of radioactivity using a suitable detector. The paper is not satisfactory, unless the chromatogram obtained with test solution (a) shows a single radioactivity spot with an $R_F$ value in the range 0.8-1.0 and the chromatogram obtained with test solution (b) shows a single radioactivity spot at the application point ($R_F$ value in the range 0.0-0.1).

**Paracetamol.** *1061900.* [103-90-2].

See *Paracetamol (0049)*.

**Paracetamol, 4-aminophenol-free.** *1061901.*

Recrystallise *paracetamol R* from *water R* and dry *in vacuo* at 70 °C; repeat the procedure until the product complies with the following test: dissolve 5 g of the dried substance in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same mixture of solvents. Add 1 mL of a freshly prepared solution containing 10 g/L of *sodium nitroprusside R* and 10 g/L of *anhydrous sodium carbonate R*, mix and allow to stand for 30 min protected from light. No blue or green colour is produced.

**Paraffin, liquid.** *1062000.* [8042-47-5].

See *Liquid paraffin (0239)*.

**Paraffin, white soft.** *1062100.*

A semi-liquid mixture of hydrocarbons obtained from petroleum and bleached, practically insoluble in water and in ethanol (96 per cent), soluble in *light petroleum R1*, the solution sometimes showing a slight opalescence.

**Paraldehyde.** *1151000.* [123-63-7].

See *Paraldehyde (0351)*.

**Pararosaniline hydrochloride.** $C_{19}H_{18}ClN_3$. ($M_r$ 323.8). *1062200.* [569-61-9].

Schultz No. 779.

Colour Index No. 42500.

4-[Bis(4-aminophenyl)methylene]cyclohexa-2,5-dieniminium chloride.

Bluish-red, crystalline powder, slightly soluble in water, soluble in anhydrous ethanol. Solutions in water and anhydrous ethanol are deep-red; solutions in sulfuric acid and in hydrochloric acid are yellow.

mp: about 270 °C, with decomposition.

**Decolorised pararosaniline solution.** *1062201.*

To 0.1 g of *pararosaniline hydrochloride R* in a ground-glass-stoppered flask add 60 mL of *water R* and a solution of 1.0 g of *anhydrous sodium sulfite R* or 2.0 g of *sodium sulfite heptahydrate R* or 0.75 g of *sodium metabisulfite R* in 10 mL of *water R*. Slowly and with stirring add 6 mL of *dilute hydrochloric acid R*, stopper the flask and continue stirring until dissolution is complete. Dilute to 100 mL with *water R*. Allow to stand for 12 h before use.

*Storage*: protected from light.

**Parthenolide.** $C_{15}H_{20}O_3$. ($M_r$ 248.3). *1129900.* [20554-84-1]. (4*E*)-(1a*R*,7a*S*,10a*S*,10b*S*)-1a,5-Dimethyl-8-methylene-2,3,6,7,7a,8,10a,10b-octahydro-oxireno[9,10]cyclodeca[1,2-*b*]furan-9(1a*H*)-one. (*E*)-(5*S*,6*S*)-4,5-Epoxygermacra-1(10),11(13)-dieno-12(6)-lactone.

White or almost white, crystalline powder, very slightly soluble in water, very soluble in methylene chloride, soluble in methanol.

$[\alpha]_D^{22}$: – 71.4, determined on a 2.2 g/L solution in *methylene chloride R*.

mp: 115 °C to 116 °C.

*Absorbance* (*2.2.25*). A 0.01 g/L solution in *ethanol (96 per cent) R* shows an absorption maximum at 214 nm.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Feverfew (1516)*, at the concentration of the reference solution.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

**L-Penicillamine coated silica gel for chiral separations.** *1200500.*

A very finely divided silica gel for chromatography coated with L-penicillamine.

**Penicillinase solution.** *1062300.*

Dissolve 10 g of casein hydrolysate, 2.72 g of *potassium dihydrogen phosphate R* and 5.88 g of *sodium citrate R* in 200 mL of *water R*, adjust to pH 7.2 with a 200 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*. Dissolve 0.41 g of *magnesium sulfate R* in 5 mL of *water R* and add 1 mL of a 1.6 g/L solution of *ferrous ammonium sulfate R* and sufficient *water R* to produce 10 mL. Sterilise both solutions by heating in an autoclave, cool, mix, distribute in shallow layers in conical flasks and inoculate with *Bacillus cereus* (NCTC 9946). Allow the flasks to stand at 18 °C to 37 °C until growth is apparent and then maintain at 35 °C to 37 °C for 16 h, shaking constantly to ensure maximum aeration. Centrifuge and sterilise the supernatant by filtration through a membrane filter. 1.0 mL of penicillinase solution contains not less than 0.4 microkatals (corresponding to the hydrolysis of not less than 500 mg of benzylpenicillin to benzylpenicilloic acid per hour) at 30 °C and pH 7, provided that the concentration of benzylpenicillin does not fall below the level necessary for enzyme saturation.

The Michaelis constant for benzylpenicillin of the penicillinase in penicillinase solution is approximately 12 µg/mL.

*Sterility* (*2.6.1*). It complies with the test for sterility.

*Storage*: at a temperature between 0 °C and 2 °C for 2 to 3 days. When freeze-dried and kept in sealed ampoules, it may be stored for several months.

**Pentaerythrityl tetrakis[3-(3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl)propionate].** $C_{73}H_{108}O_{12}$. ($M_r$ 1178). *1062400.* [6683-19-8]. Pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propionate]. 2,2′-Bis(hydroxymethyl)propane-1,3-diol tetrakis[3-[3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl]]propionate.

White or slightly yellow, crystalline powder, practically insoluble in water, very soluble in acetone, soluble in methanol, slightly soluble in hexane.

mp: 110 °C to 125 °C.

α-form: 120 °C to 125 °C.

β-form: 110 °C to 115 °C.

**Pentafluoropropanoic acid.** $C_3HF_5O_2$. ($M_r$ 164.0). *1151100.* [422-64-0].

Clear, colourless liquid.

$d_{20}^{20}$: about 1.561.

$n_D^{20}$: about 1.284.

bp: about 97 °C.

**Pentafluoropropionic anhydride.** $C_6F_{10}O_3$. ($M_r$ 310.0). *1177300.* [356-42-3]. Pentafluoropropanoic anhydride.

**Pentane.** $C_5H_{12}$. ($M_r$ 72.2). *1062500.* [109-66-0].

Clear, colourless, flammable liquid, very slightly soluble in water, miscible with acetone and with anhydrous ethanol.

$d_{20}^{20}$: about 0.63.

$n_D^{20}$: about 1.359.

bp: about 36 °C.

*Pentane used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 0.70 at 200 nm, 0.30 at 210 nm, 0.07 at 220 nm, 0.03 at 230 nm, 0.01 at 240 nm, determined using *water R* as compensation liquid.

**1,2-Pentanediol.** $C_5H_{12}O_2$. ($M_r$ 104.2). *1155800.* [5343-92-0]. (2*RS*)-Pentane-1,2-diol.

$d_4^{20}$: about 0.971.

$n_D^{20}$: about 1.439.

bp: about 201 °C.

**Pentanol.** $C_5H_{12}O$. ($M_r$ 88.1). *1062600.* [71-41-0]. Pentan-1-ol.

Colourless liquid, sparingly soluble in water, miscible with ethanol (96 per cent).

$n_D^{20}$: about 1.410.

bp: about 137 °C.

**3-Pentanone.** $C_5H_{10}O$. ($M_r$ 86.13). *1173600.* [96-22-0]. Pentan-3-one. Diethyl ketone.

***tert*-Pentyl alcohol.** $C_5H_{12}O$. ($M_r$ 88.1). *1062700.* [75-85-4]. *tert*-Amyl alcohol. 2-Methyl-2-butanol.

Volatile, flammable liquid, freely soluble in water, miscible with ethanol (96 per cent) and with glycerol.

$d_{20}^{20}$: about 0.81.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 100 °C and 104 °C.

*Storage*: protected from light.

**Pentetic acid.** $C_{14}H_{23}N_3O_{10}$. ($M_r$ 393.3). *1183100.* [67-43-6]. [[(Carboxymethyl)imino]bis(ethylenenitrilo)]tetraacetic acid. White or almost white powder, slightly soluble in water.

mp: 219 °C to 220 °C, with decomposition.

**Pepsin powder.** *1062800.* [9001-75-6].

See *Pepsin powder (0682)*.

**Peptide *N*-glycosidase F.** *1186600.* [83534-39-8].

Peptide-$N^4$-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52). PNGase F.

**Perchloric acid.** $HClO_4$. ($M_r$ 100.5). *1062900.* [7601-90-3].

*Content*: 70.0 per cent *m/m* to 73.0 per cent *m/m*.

Clear, colourless liquid, miscible with water.

$d_{20}^{20}$: about 1.7.

*Assay.* To 2.50 g add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 100.5 mg of $HClO_4$.

**Perchloric acid solution.** *1062901.*

Dilute 8.5 mL of *perchloric acid R* to 100 mL with *water R*.

**Perfluoroheptanoic acid.** $C_7HF_{13}O_2$. ($M_r$ 364.1). *1207400.* [375-85-9]. Tridecafluoroheptanoic acid.

**Periodic acetic acid solution.** *1063000.*

Dissolve 0.446 g of *sodium periodate R* in 2.5 mL of a 25 per cent *V/V* solution of *sulfuric acid R*. Dilute to 100.0 mL with *glacial acetic acid R*.

**Periodic acid.** $H_5IO_6$. ($M_r$ 227.9). *1108900.* [10450-60-9].

Crystals, freely soluble in water and soluble in ethanol (96 per cent).

mp: about 122 °C.

**Permethrin.** $C_{21}H_{20}Cl_2O_3$. ($M_r$ 391.3). *1130000.* [52645-53-1].

mp: 34 °C to 35 °C.

A suitable certified reference solution (10 ng/μL in cyclohexane) may be used.

**Peroxide test strips.** *1147800.*

Use commercial test strips with a suitable scale in the range from 0 ppm to 25 ppm peroxide.

**Perylene.** $C_{20}H_{12}$. ($M_r$ 252.3). *1130100.* [198-55-0]. Dibenz[*de*,*kl*]anthracene.

Orange powder.

mp: about 279 °C.

**Petroleum, light.** *1063100.* [8032-32-4]. Petroleum ether 50-70 °C.

Clear, colourless, flammable liquid without fluorescence, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: 0.661 to 0.664.

*Distillation range* (*2.2.11*): 50 °C to 70 °C.

**Petroleum, light R1.** *1063101.* Petroleum ether 40-60 °C.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

$d_{20}^{20}$: 0.630 to 0.656.

*Distillation range* (*2.2.11*): 40 °C to 60 °C. It does not become cloudy at 0 °C.

**Petroleum, light R2.** *1063102.* Petroleum ether 30-40 °C.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

$d_{20}^{20}$: 0.620 to 0.630.

*Distillation range* (*2.2.11*): 30 °C to 40 °C. It does not become cloudy at 0 °C.

**Petroleum, light R3.** *1063103.* Petroleum ether 100-120 °C.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

$d_{20}^{20}$: about 0.720.

*Distillation range* (*2.2.11*): 100 °C to 120 °C.

*Water* (*2.5.12*): maximum 0.03 per cent.

**Petroleum, light R4.** *1063104.* Petroleum ether 80-100 °C.

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

$d_{20}^{20}$: about 0.70.

*Distillation range* (*2.2.11*): 80 °C to 100 °C.

**pH indicator strip.** *1178900.*

Paper strip, or plastic strip containing multiple segments of different dye-impregnated papers, allowing visual determination of pH in the prescribed range, by comparison with the corresponding master chart.

**α-Phellandrene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1130400.* [4221-98-1]. (*R*)-5-Isopropyl-2-methyl-cyclohexa-1,3-diene. (–)-*p*-Mentha-1,5-diene.

$n_D^{20}$: about 1.471.

bp: 171 °C to 174 °C.

*α-Phellandrene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Eucalyptus oil (0390)*.

*Test solution.* The substance to be examined.

*Content*: 95.0 per cent, calculated by the normalisation procedure.

**Phenanthrene.** $C_{14}H_{10}$. ($M_r$ 178.2). *1063200.* [85-01-8].

White or almost white crystals, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: about 100 °C.

**Phenanthroline hydrochloride.** $C_{12}H_9ClN_2,H_2O$. ($M_r$ 234.7). *1063300.* [18851-33-7]. 1,10-Phenanthroline hydrochloride monohydrate.

White or almost white, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent).

mp: about 215 °C, with decomposition.

**Phenazone.** *1063400.* [60-80-0].

See *Phenazone (0421)*.

**Phenol.** *1063500.* [108-95-2].

See *Phenol (0631)*.

**Phenolphthalein.** $C_{20}H_{14}O_4$. ($M_r$ 318.3). *1063700.* [77-09-8]. 3,3-Bis(4-hydroxyphenyl)-3*H*-isobenzofuran-1-one.

White or yellowish-white powder, practically insoluble in water, soluble in ethanol (96 per cent).

**Phenolphthalein paper.** *1063704.*

Immerse strips of filter paper for a few minutes in *phenolphthalein solution R*. Allow to dry.

**Phenolphthalein solution.** *1063702.*

Dissolve 0.1 g of *phenolphthalein R* in 80 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.1 mL of the phenolphthalein solution add 100 mL of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.2 mL of *0.02 M sodium hydroxide* is required to change the colour to pink.

*Colour change*: pH 8.2 (colourless) to pH 10.0 (red).

**Phenolphthalein solution R1.** *1063703.*

A 10 g/L solution of *phenolphthalein R* in *ethanol (96 per cent) R*.

**Phenol red.** *1063600.* [143-74-8].

Bright red or dark red, crystalline powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent).

**Phenol red solution.** *1063601.*

Dissolve 0.1 g of *phenol red R* in a mixture of 2.82 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* Add 0.1 mL of the phenol red solution to 100 mL of *carbon dioxide-free water R*. The solution is yellow. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to reddish-violet.

*Colour change*: pH 6.8 (yellow) to pH 8.4 (reddish-violet).

**Phenol red solution R2.** *1063603.*

*Solution A*. Dissolve 33 mg of *phenol red R* in 1.5 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*.

*Solution B*. Dissolve 25 mg of *ammonium sulfate R* in 235 mL of *water R*; add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Add 25 mL of solution A to solution B. If necessary, adjust the pH of the mixture to 4.7.

**Phenol red solution R3.** *1063604.*

*Solution A*. Dissolve 33 mg of *phenol red R* in 1.5 mL of *dilute sodium hydroxide solution R* and dilute to 50 mL with *water R*.

*Solution B*. Dissolve 50 mg of *ammonium sulfate R* in 235 mL of *water R*; add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Add 25 mL of solution A to solution B; if necessary, adjust the pH of the mixture to 4.7.

**Phenoxyacetic acid.** $C_8H_8O_3$. ($M_r$ 152.1). *1063800.* [122-59-8]. 2-Phenoxyethanoic acid.

Almost white crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent), and in glacial acetic acid.

mp: about 98 °C.

*Chromatography.* Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Phenoxymethylpenicillin (0148)*; the chromatogram shows only one principal spot.

**2-Phenoxyaniline.** $C_{12}H_{11}NO$. ($M_r$ 185.2). *1165500.* [2688-84-8]. 2-Phenoxybenzenamine. 2-Aminophenyl phenyl ether.

**Phenoxyethanol.** $C_8H_{10}O_2$. ($M_r$ 138.2). *1064000.* [122-99-6]. 2-Phenoxyethanol.

Clear, colourless, oily liquid, slightly soluble in water, freely soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 1.11.

$n_D^{20}$: about 1.537.

*Freezing point* (*2.2.18*): minimum 12 °C.

**Phenylacetic acid.** $C_8H_8O_2$. ($M_r$ 136.2). *1160000.* [103-82-2].

White or almost white powder, soluble in water.

bp: about 265 °C.

mp: about 75 °C.

**Phenylalanine.** *1064100.* [63-91-2].

See *Phenylalanine (0782)*.

***p*-Phenylenediamine dihydrochloride.** $C_6H_{10}Cl_2N_2$. ($M_r$ 181.1). *1064200.* [615-28-1]. 1,4-Diaminobenzene dihydrochloride.

Crystalline powder or white or slightly coloured crystals, turning reddish on exposure to air, freely soluble in water, slightly soluble in ethanol (96 per cent).

**α-Phenylglycine.** $C_8H_9NO_2$. ($M_r$ 151.2). *1064300.* [2835-06-5]. (*RS*)-2-Amino-2-phenylacetic acid.

**D-Phenylglycine.** $C_8H_9NO_2$. ($M_r$ 151.2). *1144500.* [875-74-1]. (2*R*)-2-Amino-2-phenylacetic acid.

*Content*: minimum 99 per cent.

White or almost white, crystalline powder.

**Phenylhydrazine.** $C_6H_8N_2$. ($M_r$ 108.1). *1190800.* [100-63-0].

White or almost white, crystalline powder, becoming yellow or dark red on exposure to air, melting at room temperature giving an oily liquid, miscible with anhydrous ethanol, sparingly soluble in water.

bp: about 244 °C, with decomposition.

mp: about 20 °C.

**Phenylhydrazine hydrochloride.** $C_6H_9ClN_2$. ($M_r$ 144.6). *1064500.* [59-88-1].

White or almost white, crystalline powder, becoming brown on exposure to air, soluble in water and in ethanol (96 per cent).

mp: about 245 °C, with decomposition.

*Storage*: protected from light.

**Phenylhydrazine hydrochloride solution.** *1064501.*

Dissolve 0.9 g of *phenylhydrazine hydrochloride R* in 50 mL of *water R*. Decolorise with *activated charcoal R* and filter. To the filtrate add 30 mL of *hydrochloric acid R* and dilute to 250 mL with *water R*.

**Phenylhydrazine-sulfuric acid solution.** *1064502.*

Dissolve 65 mg of *phenylhydrazine hydrochloride R*, previously recrystallised from *ethanol (85 per cent V/V) R*, in a mixture of 80 volumes of *water R* and 170 volumes of *sulfuric acid R* and dilute to 100 mL with the same mixture of solvents. Prepare immediately before use.

**Phenyl isothiocyanate.** $C_7H_5NS$. ($M_r$ 135.2). *1121500.* [103-72-0].

Liquid, insoluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 1.13.

$n_D^{20}$: about 1.65.

bp: about 221 °C.

mp: about − 21 °C.

Use a grade suitable for protein sequencing.

**Phenyl(5)methyl(95)polysiloxane.** *1066900.*

Polysiloxane substituted with 5 per cent of phenyl groups and 95 per cent of methyl groups.

**Phenyl(5)methyl(95)polysiloxane, base-deactivated.** *1176600.*

Base-deactivated polysiloxane substituted with 5 per cent of phenyl groups and 95 per cent of methyl groups.

**Phenyl(50)methyl(50)polysiloxane.** *1067900.*

Polysiloxane substituted with 50 per cent of phenyl groups and 50 per cent of methyl groups.

**1-Phenylpiperazine.** $C_{10}H_{14}N_2$. ($M_r$ 162.2). *1130500.* [92-54-6].

Slightly viscous, yellow liquid, not miscible with water.

$d_4^{20}$: about 1.07.

$n_D^{20}$: about 1.588.

**1-Phenylpropan-2-ol.** $C_9H_{12}O$. ($M_r$ 136.2). *1205200.* [698-87-3]. (2*RS*)-1-Phenylpropan-2-ol.
mp: 65 °C to 67 °C.

**1-Phenyl-1,2,3,4-tetrahydroisoquinoline.** $C_{15}H_{15}N$. ($M_r$ 209.3). *1193700.* [22990-19-8].

**Phloroglucide.** $C_{12}H_{10}O_5$. ($M_r$ 234.2). *1177400.* [491-45-2]. 2,3′,4,5′,6-Biphenylpentol.
White or almost white powder, hygroscopic, light sensitive. Slowly discolours on exposure to light.

**Phloroglucinol.** $C_6H_6O_3,2H_2O$. ($M_r$ 162.1). *1064600.* [6099-90-7]. Benzene-1,3,5-triol.
White or yellowish crystals, slightly soluble in water, soluble in ethanol (96 per cent).
mp: about 223 °C (instantaneous method).

**Phloroglucinol solution.** *1064601.*
To 1 mL of a 100 g/L solution of *phloroglucinol R* in *ethanol (96 per cent) R*, add 9 mL of *hydrochloric acid R*.
*Storage*: protected from light.

**Phosalone.** $C_{12}H_{15}ClNO_4PS_2$. ($M_r$ 367.8). *1130200.* [2310-17-0].
mp: 45 °C to 48 °C
A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Phosphomolybdic acid.** $12MoO_3,H_3PO_4,xH_2O$. *1064900.* [51429-74-4].
Orange-yellow, fine crystals, freely soluble in water, soluble in ethanol (96 per cent).

**Phosphomolybdic acid solution.** *1064901.*
Dissolve 4 g of *phosphomolybdic acid R* in *water R* and dilute to 40 mL with the same solvent. Add cautiously and with cooling 60 mL of *sulfuric acid R*. Prepare immediately before use.

**Phosphomolybdotungstic reagent.** *1065000.*
Dissolve 100 g of *sodium tungstate R* and 25 g of *sodium molybdate R* in 700 mL of *water R*. Add 100 mL of *hydrochloric acid R* and 50 mL of *phosphoric acid R*. Heat the mixture under a reflux condenser in a glass apparatus for 10 h. Add 150 g of *lithium sulfate R*, 50 mL of *water R* and a few drops of *bromine R*. Boil to remove the excess of bromine (15 min), allow to cool, dilute to 1000 mL with *water R* and filter. The reagent should be yellow in colour. If it acquires a greenish tint, it is unsatisfactory for use but may be regenerated by boiling with a few drops of *bromine R*. Care must be taken to remove the excess of bromine by boiling.
*Storage*: at 2 °C to 8 °C.

**Phosphomolybdotungstic reagent, dilute.** *1065001.*
To 1 volume of *phosphomolybdotungstic reagent R* add 2 volumes of *water R*.

**Phosphoric acid.** *1065100.* [7664-38-2].
See *Concentrated phosphoric acid (0004)*.

**Phosphoric acid, dilute.** *1065101.*
See *Dilute phosphoric acid (0005)*.

**Phosphoric acid, dilute R1.** *1065102.*
Dilute 93 mL of *dilute phosphoric acid R* to 1000 mL with *water R*.

**Phosphorous acid.** $H_3PO_3$. ($M_r$ 82.0). *1130600.* [13598-36-2].
White or almost white, very hygroscopic and deliquescent crystalline mass; slowly oxidised by oxygen (air) to $H_3PO_4$.
Unstable, orthorhombic crystals, soluble in water, in ethanol (96 per cent) and in a mixture of 3 volumes of ether and 1 volume of ethanol (96 per cent).
$d_4^{21}$: 1.651.
mp: about 73 °C.

**Phosphotungstic acid solution.** *1065200.*
Heat under a reflux condenser for 3 h, 10 g of *sodium tungstate R* with 8 mL of *phosphoric acid R* and 75 mL of *water R*. Allow to cool and dilute to 100 mL with *water R*.

**Phthalaldehyde.** $C_8H_6O_2$. ($M_r$ 134.1). *1065300.* [643-79-8]. Benzene-1,2-dicarboxaldehyde.
Yellow, crystalline powder.
mp: about 55 °C.
*Storage*: protected from light and air.

**Phthalaldehyde reagent.** *1065301.*
Dissolve 2.47 g of *boric acid R* in 75 mL of *water R*, adjust to pH 10.4 using a 450 g/L solution of *potassium hydroxide R* and dilute to 100 mL with *water R*. Dissolve 1.0 g of *phthalaldehyde R* in 5 mL of *methanol R*, add 95 mL of the boric acid solution and 2 mL of *thioglycollic acid R* and adjust to pH 10.4 with a 450 g/L solution of *potassium hydroxide R*.
*Storage*: protected from light; use within 3 days.

**Phthalazine.** $C_8H_6N_2$. ($M_r$ 130.1). *1065400.* [253-52-1].
Pale yellow crystals, freely soluble in water, soluble in anhydrous ethanol, in ethyl acetate and in methanol.
mp: 89 °C to 92 °C.

**Phthalein purple.** $C_{32}H_{32}N_2O_{12},xH_2O$. ($M_r$ 637, anhydrous substance). *1065500.* [2411-89-4]. Metalphthalein. 2,2′,2″,2‴-[*o*-Cresolphthalein-3′,3″-bis(methylenenitrilo)]tetra-acetic acid. (1,3-Dihydro-3-oxo-isobenzofuran-1-ylidene)bis[(6-hydroxy-5-methyl-3,1-phenylene)bis(methyleneimino)diacetic acid].
Yellowish-white or brownish powder, practically insoluble in water, soluble in ethanol (96 per cent). The product may be found in commerce in the form of the sodium salt: a yellowish-white to pink powder, soluble in water, practically insoluble in ethanol (96 per cent).
*Test for sensitivity.* Dissolve 10 mg in 1 mL of *concentrated ammonia R* and dilute to 100 mL with *water R*. To 5 mL of the solution add 95 mL of *water R*, 4 mL of *concentrated ammonia R*, 50 mL of *ethanol (96 per cent) R* and 0.1 mL of *0.1 M barium chloride*. The solution is blue-violet. Add 0.15 mL of *0.1 M sodium edetate*. The solution becomes colourless.

**Phthalic acid.** $C_8H_6O_4$. ($M_r$ 166.1). *1065600.* [88-99-3]. Benzene-1,2-dicarboxylic acid.
White or almost white, crystalline powder, soluble in hot water and in ethanol (96 per cent).

**Phthalic anhydride.** $C_8H_4O_3$. ($M_r$ 148.1). *1065700.* [85-44-9]. Isobenzofuran-1,3-dione.
*Content*: minimum 99.0 per cent.
White or almost white flakes.
mp: 130 °C to 132 °C.
*Assay.* Dissolve 2.000 g in 100 mL of *water R* and boil under a reflux condenser for 30 min. Cool and titrate with *1 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.
1 mL of *1 M sodium hydroxide* is equivalent to 74.05 mg of $C_8H_4O_3$.

**Phthalic anhydride solution.** *1065701.*
Dissolve 42 g of *phthalic anhydride R* in 300 mL of *anhydrous pyridine R*. Allow to stand for 16 h.
*Storage*: protected from light; use within 1 week.

**Picein.** $C_{14}H_{18}O_7$. ($M_r$ 298.3). *1130700.* [530-14-3]. 1-[4-(β-D-Glucopyranosyloxy)phenyl]ethanone. *p*-(Acetylphenyl)-β-D-glucopyranoside.
mp: 194 °C to 195 °C.

**Picric acid.** $C_6H_3N_3O_7$. ($M_r$ 229.1). *1065800.* [88-89-1]. 2,4,6-Trinitrophenol.

Yellow prisms or plates, soluble in water and in ethanol (96 per cent).

*Storage*: moistened with *water R*.

**Picric acid solution.** *1065801.*

A 10 g/L solution of *picric acid R*.

**Picric acid solution R1.** *1065802.*

Prepare 100 mL of a saturated solution of *picric acid R* and add 0.25 mL of *strong sodium hydroxide solution R*.

**Picrotin.** $C_{15}H_{18}O_7$. ($M_r$ 310.3). *1188100.* [21416-53-5]. (1*R*,3*R*,5*S*,8*S*,9*R*,12*S*,13*R*,14*S*)-1-hydroxy-14-(2-hydroxypropan-2-yl)-13-methyl-4,7,10-trioxapentacyclo[6.4.1.1.$^{9,12}$.0$^{3,5}$.0$^{5,13}$]tetradecane-6,11-dione.

White or colourless crystalline powder or crystals, soluble in boiling water and in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: 248 °C to 250 °C.

**Picrotoxinin.** $C_{15}H_{16}O_6$. ($M_r$ 292.2). *1188200.* [17617-45-7]. (1*R*,3*R*,5*S*,8*S*,9*R*,12*S*,13*R*,14*R*)-1-hydroxy-13-methyl-14-(prop-1-en-2-yl)-4,7,10-trioxapentacyclo[6.4.1.1$^{9,12}$.0$^{3,5}$.0$^{5,13}$]tetradecane-6,11-dione.

White or colourless crystalline powder or crystals, soluble in methylene chloride, in ethanol (96 per cent) and in alkaline solutions.

mp: 207 to 210 °C.

**α-Pinene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1130800.* [7785-70-8]. (1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene.

Liquid not miscible with water.

$d_{20}^{20}$: about 0.859.

$n_D^{20}$: about 1.466.

bp: 154 °C to 156 °C.

*α-Pinene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**β-Pinene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1109000.* [127-91-3]. 6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane.

Colourless, oily liquid, odour reminiscent of turpentine, practically insoluble in water, miscible with ethanol (96 per cent).

*β-Pinene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 95.0 per cent.

**1,4-Piperazinediethanesulfonic acid.** $C_8H_{18}N_2O_6S_2$. ($M_r$ 302.4). *1186700.* [5625-37-6]. Piperazine-1,4-bis(2-ethanesulfonic acid). 2,2′-(Piperazine-1,4-diyl)bis(ethanesulfonic acid). Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid). PIPES.

*Content*: minimum 99 per cent.

White, crystalline powder.

**Piperazine hydrate.** *1065900.* [142-63-2].

See *Piperazine hydrate (0425)*.

**Piperidine.** $C_5H_{11}N$. ($M_r$ 85.2). *1066000.* [110-89-4]. Hexahydropyridine.

Colourless to slightly yellow, alkaline liquid, miscible with water, with ethanol (96 per cent) and with light petroleum.

bp: about 106 °C.

**Piperine.** $C_{17}H_{19}NO_3$. ($M_r$ 285.3). *1183200.* [94-62-2]. (2*E*,4*E*)-1-(Piperidin-1-yl)-5-(1,3-benzodioxol-5-yl)penta-2,4-dien-1-one. 1-Piperoyl-piperidine. 1-[(2*E*,4*E*)-5-(3,4-Methylenedioxyphenyl)-1-oxo-2,4-pentadienyl]piperidine.

**Piperitone.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1151200.* [89-81-6]. 6-Isopropyl-3-methyl-cyclohex-2-en-1-one.

**Pirimiphos-ethyl.** $C_{13}H_{24}N_3O_3PS$. ($M_r$ 333.4). *1130300.* [23505-41-1].

mp: 15 °C to 18 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Plasma, platelet-poor.** *1066100.*

Withdraw 45 mL of human blood into a 50 mL plastic syringe containing 5 mL of a sterile 38 g/L solution of *sodium citrate R*. Without delay, centrifuge at 1500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the supernatant plasma using a plastic syringe and without delay centrifuge at 3500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the liquid and freeze it rapidly in suitable amounts in plastic tubes at or below − 40 °C. Use plastic or silicone-treated equipment.

**Plasma substrate.** *1066200.*

Separate the plasma from human or bovine blood collected into one-ninth its volume of a 38 g/L solution of *sodium citrate R*, or into two-sevenths its volume of a solution containing 20 g/L of *disodium hydrogen citrate R* and 25 g/L of *glucose R*. With the former, prepare the substrate on the day of collection of the blood. With the latter, prepare within two days of collection of the blood.

*Storage*: at − 20 °C.

**Plasma substrate R1.** *1066201.*

*Use water-repellent equipment (made from materials such as suitable plastics or suitably silicone-treated glass) for taking and handling blood.*

Collect a suitable volume of blood from each of at least five sheep; a 285 mL volume of blood collected into 15 mL of anticoagulant solution is suitable but smaller volumes may be collected, taking the blood, either from a live animal or at the time of slaughter, using a needle attached to a suitable cannula which is long enough to reach the bottom of the collecting vessel. Discarding the first few millilitres and collecting only free-flowing blood, collect the blood in a sufficient quantity of an anticoagulant solution containing 8.7 g of *sodium citrate R* and 4 mg of *aprotinin R* per 100 mL of *water R* to give a final ratio of blood to anticoagulant solution of 19 to 1. During and immediately after collection, swirl the flask gently to ensure mixing but do not allow frothing to occur. When collection is complete, close the flask and cool to 10-15 °C. When cold, pool the contents of all the flasks with the exception of any that show obvious haemolysis or clots and keep the pooled blood at 10-15 °C.

As soon as possible and within 4 h of collection, centrifuge the pooled blood at 1000-2000 *g* at 10-15 °C for 30 min. Separate the supernatant and centrifuge it at 5000 *g* for 30 min. (Faster centrifugation, for example 20 000 *g* for 30 min, may be used if necessary to clarify the plasma, but filtration procedures should not be used.) Separate the supernatant and, without delay, mix thoroughly and distribute the plasma substrate into small stoppered containers in portions sufficient for a complete heparin assay (for example 10 mL to 30 mL). Without delay, rapidly

cool to a temperature below – 70 °C (for example by immersing the containers into liquid nitrogen) and store at a temperature below – 30 °C.

The plasma is suitable for use as plasma substrate in the assay for heparin if, under the conditions of the assay, it gives a clotting time appropriate to the method of detection used and if it provides reproducible, steep log dose-response curves.

When required for use, thaw a portion of the plasma substrate in a water-bath at 37 °C, gently swirling until thawing is complete; once thawed it should be kept at 10-20 °C and used without delay. The thawed plasma substrate may be lightly centrifuged if necessary; filtration procedures should not be used.

**Plasma substrate R2.** *1066202.*

Prepare from human blood containing less than 1 per cent of the normal amount of factor IX. Collect the blood into one-ninth its volume of a 38 g/L solution of *sodium citrate R*.

*Storage*: in small amounts in plastic tubes at a temperature of – 30 °C or lower.

**Plasma substrate R3.** *1066203.*

Prepare from human blood containing less than 1 per cent of the normal amount of factor XI. Collect the blood into one-ninth its volume of a 38 g/L solution of *sodium citrate R*.

*Storage*: in small amounts in plastic tubes at a temperature of – 30 °C or lower.

**Plasma substrate deficient in factor V.** *1066300.*

Use preferably a plasma which is congenitally deficient, or prepare it as follows: separate the plasma from human blood collected into one tenth of its volume of a 13.4 g/L solution of *sodium oxalate R*. Incubate at 37 °C for 24 h to 36 h. The coagulation time determined by the method prescribed for *coagulation factor V solution R* should be 70 s to 100 s. If the coagulation time is less than 70 s, incubate again for 12 h to 24 h.

*Storage*: in small quantities at a temperature of – 20 °C or lower.

**Plasminogen, human.** *1109100.* [9001-91-6].

A substance present in blood that may be activated to plasmin, an enzyme that lyses fibrin in blood clots.

**Plutonium-242 spiking solution.** *1167400.*

Contains 50 Bq/L $^{242}Pu$ and a 134 mg/L solution of *lanthanum chloride heptahydrate R* in a 284 g/L solution of *nitric acid R*.

**Poloxamer 188.** *1186800.*

See *Poloxamers (1464)*.

**Polyamine grafted poly(vinyl alcohol) copolymer.** *1188300.*

Copolymer beads of poly(vinyl alcohol) to which polyamine is covalently bonded. The size range of the beads is specified after the name of the reagent in the tests where it is used.

**Polydatin.** $C_{20}H_{22}O_8$. ($M_r$ 390.4). *1197500.* [65914-17-2].

3-Hydroxy-5-[2-(4-hydroxyphenyl)eth-1-en-1-yl]phenyl β-D-glucopyranoside. Resveratrol-3-β-mono-D-glucoside.

**Polyether hydroxylated gel for chromatography.** *1067000.*

Gel with a small particle size having a hydrophilic surface with hydroxyl groups. It has an exclusion limit for dextran of relative molecular mass $2 \times 10^5$ to $2.5 \times 10^6$.

**Polyethyleneglycol adipate.** $(C_8H_{12}O_4)_n$. ($M_r$ $(172.2)_n$). *1067700.*

White or almost white, wax-like mass, practically insoluble in water.

mp: about 43 °C.

**Polyethyleneglycol succinate.** $(C_6H_8O_4)_n$. ($M_r$ $(144.1)_n$). *1067800.*

White or almost white, crystalline powder, practically insoluble in water.

mp: about 102 °C.

**Polymethacrylate gel.** *1181100.*

A methacrylate-based size-exclusion stationary phase for water-soluble samples.

**Polymethacrylate gel, hydroxylated.** *1151300.*

Stationary phase for size-exclusion chromatography.

Gel based on hydroxylated methacrylic acid polymer.

**Polyorganosiloxane for oxygen-containing compounds.** *1200600.*

Combination of suitable polyorganosiloxanes with high affinity for oxygen-containing compounds.

**Polyoxyethylated castor oil.** *1068200.*

Light yellow liquid. It becomes clear above 26 °C.

**Polysorbate 20.** *1068300.* [9005-64-5].

See *Polysorbate 20 (0426)*.

**Polysorbate 65.** *1196200.* [9005-71-4].

**Polysorbate 80.** *1068400.* [9005-65-6].

See *Polysorbate 80 (0428)*.

**Polystyrene 900-1000.** *1112200.* [9003-53-6].

Organic standard used for calibration in gas chromatography.

$M_w$: about 950.

$M_w/M_n$: 1.10.

**Potassium acetate.** *1175900.* [127-08-2].

See *Potassium acetate (1139)*.

**Potassium bicarbonate.** *1069900.* [298-14-6].

See *Potassium hydrogen carbonate R*.

**Potassium bicarbonate solution, saturated methanolic.** *1069901.*

See *potassium hydrogen carbonate solution, saturated methanolic R*.

**Potassium bromate.** $KBrO_3$. ($M_r$ 167.0). *1068700.* [7758-01-2].

White or almost white granular powder or crystals, soluble in water, slightly soluble in ethanol (96 per cent).

**Potassium bromide.** *1068800.* [7758-02-3].

See *Potassium bromide (0184)*.

*Potassium bromide used for infrared absorption spectrophotometry (2.2.24) also complies with the following additional test.*

A disc 2 mm thick prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 $cm^{-1}$ to 620 $cm^{-1}$. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 $cm^{-1}$ and 1630 $cm^{-1}$.

**Potassium carbonate.** $K_2CO_3$. ($M_r$ 138.2). *1068900.* [584-08-7]. Dipotassium carbonate.

White or almost white, granular powder, hygroscopic, very soluble in water, practically insoluble in anhydrous ethanol.

*Storage*: in an airtight container.

**Potassium chlorate.** $KClO_3$. ($M_r$ 122.6). *1069000.* [3811-04-9].

A white or almost white powder, granules or crystals, soluble in water.

**Potassium chloride.** *1069100.* [7447-40-7].

See *Potassium chloride (0185)*.

*Potassium chloride used for infrared absorption spectrophotometry (2.2.24) also complies with the following additional test.*

A disc 2 mm thick, prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 $cm^{-1}$ to 620 $cm^{-1}$. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 $cm^{-1}$ and 1630 $cm^{-1}$.

**Potassium chloride, 0.1 M.** *1069101.*

A solution of *potassium chloride R* containing the equivalent of 7.46 g of KCl in 1000.0 mL.

**Potassium chromate.** $K_2CrO_4$. ($M_r$ 194.2). *1069200.* [7789-00-6]. Dipotassium chromate.

Yellow crystals, freely soluble in water.

**Potassium chromate solution.** *1069201.*

A 50 g/L solution of *potassium chromate R*.

**Potassium citrate.** *1069300.* [6100-05-6].

See *Potassium citrate (0400)*.

**Potassium cyanide.** KCN. ($M_r$ 65.1). *1069400.* [151-50-8].

White or almost white, crystalline powder or white or almost white mass or granules, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Potassium cyanide solution.** *1069401.*

A 100 g/L solution of *potassium cyanide R*.

**Potassium cyanide solution, lead-free.** *1069402.*

Dissolve 10 g of *potassium cyanide R* in 90 mL of *water R*, add 2 mL of *strong hydrogen peroxide solution R* diluted 1 to 5. Allow to stand for 24 h, dilute to 100 mL with *water R* and filter.

The solution complies with the following test: take 10 mL of the solution, add 10 mL of *water R* and 10 mL of *hydrogen sulfide solution R*. No colour is evolved even after addition of 5 mL of *dilute hydrochloric acid R*.

**Potassium dichromate.** $K_2Cr_2O_7$. ($M_r$ 294.2). *1069500.* [7778-50-9]. Dipotassium dichromate.

Potassium dichromate used for the calibration of spectrophotometers (*2.2.25*) contains not less than 99.9 per cent of $K_2Cr_2O_7$, calculated with reference to the substance dried at 130 °C.

Orange-red crystals, soluble in water, practically insoluble in ethanol (96 per cent).

*Assay.* Dissolve 1.000 g in *water R* and dilute to 250.0 mL with the same solvent. To 50.0 mL of this solution add a freshly prepared solution of 4 g of *potassium iodide R*, 2 g of *sodium hydrogen carbonate R* and 6 mL of *hydrochloric acid R* in 100 mL of *water R* in a 500 mL flask. Stopper the flask and allow to stand protected from light for 5 min. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *iodide-free starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 4.903 mg of $K_2Cr_2O_7$.

**Potassium dichromate solution.** *1069501.*

A 106 g/L solution of *potassium dichromate R*.

**Potassium dichromate solution R1.** *1069502.*

A 5 g/L solution of *potassium dichromate R*.

**Potassium dihydrogen phosphate.** *1069600.* [7778-77-0].

See *Potassium dihydrogen phosphate (0920)*.

**Potassium dihydrogen phosphate, 0.2 M.** *1069601.*

A solution of *potassium dihydrogen phosphate R* containing the equivalent of 27.22 g of $KH_2PO_4$ in 1000.0 mL.

**Potassium ferricyanide.** $K_3[Fe(CN)_6]$. ($M_r$ 329.3). *1069700.* [13746-66-2]. Potassium hexacyanoferrate(III).

Red crystals, freely soluble in water.

**Potassium ferricyanide solution.** *1069701.*

Wash 5 g of *potassium ferricyanide R* with a little *water R*, dissolve and dilute to 100 mL with *water R*. Prepare immediately before use.

**Potassium ferriperiodate solution.** *1070801.*

Dissolve 1 g of *potassium periodate R* in 5 mL of a freshly prepared 120 g/L solution of *potassium hydroxide R*. Add 20 mL of *water R* and 1.5 mL of *ferric chloride solution R1*. Dilute to 50 mL with a freshly prepared 120 g/L solution of *potassium hydroxide R*.

**Potassium ferrocyanide.** $K_4[Fe(CN)_6],3H_2O$. ($M_r$ 422.4). *1069800.* [14459-95-1]. Potassium hexacyanoferrate(II).

Transparent yellow crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

**Potassium ferrocyanide solution.** *1069801.*

A 53 g/L solution of *potassium ferrocyanide R*.

**Potassium fluoride.** KF. ($M_r$ 58.1). *1137800.* [7789-23-3].

Colourless crystals or white or almost white crystalline powder, deliquescent, soluble in water, practically insoluble in ethanol (96 per cent).

**Potassium hydrogen carbonate.** $KHCO_3$. ($M_r$ 100.1). *1069900.* [298-14-6]. Potassium bicarbonate.

Transparent, colourless crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

**Potassium hydrogen carbonate solution, saturated methanolic.** *1069901.*

Dissolve 0.1 g of *potassium hydrogen carbonate R* in 0.4 mL of *water R*, heating on water-bath. Add 25 mL of *methanol R* and swirl, keeping the solution on the water-bath until dissolution is complete. Use a freshly prepared solution.

**Potassium hydrogen phthalate.** $C_8H_5KO_4$. ($M_r$ 204.2). *1070000.* [877-24-7]. Potassium hydrogen benzene-1,2-dicarboxylate.

White or almost white crystals, soluble in water, slightly soluble in ethanol (96 per cent).

**Potassium hydrogen phthalate, 0.2 M.** *1070001.*

A solution of *potassium hydrogen phthalate R* containing the equivalent of 40.84 g of $C_8H_5KO_4$ in 1000.0 mL.

**Potassium hydrogen sulfate.** $KHSO_4$. ($M_r$ 136.2). *1070100.* [7646-93-7].

Colourless, transparent, hygroscopic crystals, freely soluble in water giving a strongly acid solution.

*Storage*: in an airtight container.

**Potassium hydrogen tartrate.** $C_4H_5KO_6$. ($M_r$ 188.2). *1070200.* [868-14-4]. Potassium hydrogen (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate.

White or almost white, crystalline powder or colourless, slightly opaque crystals, slightly soluble in water, soluble in boiling water, practically insoluble in ethanol (96 per cent).

**Potassium hydroxide.** *1070300.* [1310-58-3].

See *Potassium hydroxide (0840)*.

**Potassium hydroxide, alcoholic, 2 M.** *1070301.*

Dissolve 12 g of *potassium hydroxide R* in 10 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

**Potassium hydroxide in alcohol (10 per cent *V/V*), 0.5 M.** *1070302.*

Dissolve 28 g of *potassium hydroxide R* in 100 mL of *ethanol (96 per cent) R* and dilute to 1000 mL with *water R*.

**Potassium hydroxide solution, alcoholic.** *1070303.*

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100 mL with *aldehyde-free alcohol R*. Decant the clear solution. The solution should be almost colourless.

**Potassium hydroxide solution, alcoholic R1.** *1070304.*

Dissolve 6.6 g of *potassium hydroxide R* in 50 mL of *water R* and dilute to 1000 mL with *anhydrous ethanol R*.

**Potassium iodate.** $KIO_3$. ($M_r$ 214.0). *1070400.* [7758-05-6].

White or almost white, crystalline powder, soluble in water.

**Potassium iodide.** *1070500.* [7681-11-0].

See *Potassium iodide (0186)*.

**Potassium iodide and starch solution.** *1070501.*

Dissolve 0.75 g of *potassium iodide R* in 100 mL of *water R*. Heat to boiling and add whilst stirring a solution of 0.5 g of *soluble starch R* in 35 mL of *water R*. Boil for 2 min and allow to cool.

*Test for sensitivity.* A mixture of 15 mL of the potassium iodide and starch solution, 0.05 mL of *glacial acetic acid R* and 0.3 mL of *iodine solution R2* is blue.

**Potassium iodide solution.** *1070502.*

A 166 g/L solution of *potassium iodide R*.

**Potassium iodide solution, iodinated.** *1070503.*

Dissolve 2 g of *iodine R* and 4 g of *potassium iodide R* in 10 mL of *water R*. When solution is complete dilute to 100 mL with *water R*.

**Potassium iodide solution, iodinated R1.** *1070505.*

Dissolve 500 mg of *iodine R* and 1.5 g of *potassium iodide R* in *water R* and dilute to 25 mL with the same solvent.

**Potassium iodide solution, saturated.** *1070504.*

A saturated solution of *potassium iodide R* in *carbon dioxide-free water R*. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Test by adding to 0.5 mL of the saturated potassium iodide solution 30 mL of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*, as well as 0.1 mL of *starch solution R*. Any blue colour formed should be discharged by the addition of 0.05 mL of *0.1 M sodium thiosulfate*.

*Storage*: protected from light.

**Potassium iodobismuthate solution.** *1070600.*

To 0.85 g of *bismuth subnitrate R* add 40 mL of *water R*, 10 mL of *glacial acetic acid R* and 20 mL of a 400 g/L solution of *potassium iodide R*.

**Potassium iodobismuthate solution, dilute.** *1070603.*

Dissolve 100 g of *tartaric acid R* in 500 mL of *water R* and add 50 mL of *potassium iodobismuthate solution R1*.

*Storage*: protected from light.

**Potassium iodobismuthate solution R1.** *1070601.*

Dissolve 100 g of *tartaric acid R* in 400 mL of *water R* and add 8.5 g of *bismuth subnitrate R*. Shake for 1 h, add 200 mL of a 400 g/L solution of *potassium iodide R* and shake well. Allow to stand for 24 h and filter.

*Storage*: protected from light.

**Potassium iodobismuthate solution R2.** *1070602.*

*Stock solution.* Suspend 1.7 g of *bismuth subnitrate R* and 20 g of *tartaric acid R* in 40 mL of *water R*. To the suspension add 40 mL of a 400 g/L solution of *potassium iodide R* and stir for 1 h. Filter. The solution may be kept for several days in brown bottles.

*Spray solution.* Mix immediately before use 5 mL of the stock solution with 15 mL of *water R*.

**Potassium iodobismuthate solution R3.** *1070604.*

Dissolve 0.17 g of *bismuth subnitrate R* in a mixture of 2 mL of *glacial acetic acid R* and 18 mL of *water R*. Add 4 g of *potassium iodide R*, 1 g of *iodine R* and dilute to 100 mL with *dilute sulfuric acid R*.

**Potassium iodobismuthate solution R4.** *1070605.*

Dissolve 1.7 g of *bismuth subnitrate R* in 20 mL of *glacial acetic acid R*. Add 80 mL of *distilled water R*, 100 mL of a 400 g/L solution of *potassium iodide R*, 200 mL of *glacial acetic acid R* and dilute to 1000 mL with *distilled water R*. Mix 2 volumes of this solution with 1 volume of a 200 g/L solution of *barium chloride R*.

**Potassium iodobismuthate solution R5.** *1070606.*

To 0.85 g of *bismuth subnitrate R* add 10 mL of *glacial acetic acid R* and gently heat until completely dissolved. Add 40 mL of *water R* and allow to cool. To 5 mL of this solution, add 5 mL of a 400 g/L solution of *potassium iodide R*, 20 mL of *glacial acetic acid R* and 70 mL of *water R*.

**Potassium nitrate.** $KNO_3$. ($M_r$ 101.1). *1070700.* [7757-79-1].

Colourless crystals, very soluble in water.

**Potassium periodate.** $KIO_4$. ($M_r$ 230.0). *1070800.* [7790-21-8].

White or almost white, crystalline powder or colourless crystals, soluble in water.

**Potassium permanganate.** *1070900.* [7722-64-7].

See *Potassium permanganate (0121)*.

**Potassium permanganate and phosphoric acid solution.** *1070901.*

Dissolve 3 g of *potassium permanganate R* in a mixture of 15 mL of *phosphoric acid R* and 70 mL of *water R*. Dilute to 100 mL with *water R*.

**Potassium permanganate solution.** *1070902.*

A 30 g/L solution of *potassium permanganate R*.

**Potassium perrhenate.** $KReO_4$. ($M_r$ 289.3). *1071000.* [10466-65-6].

White or almost white, crystalline powder, soluble in water, slightly soluble in ethanol (96 per cent), in methanol and in propylene glycol.

**Potassium persulfate.** $K_2S_2O_8$. ($M_r$ 270.3). *1071100.* [7727-21-1]. Dipotassium peroxodisulfate.

Colourless crystals or white or almost white, crystalline powder, sparingly soluble in water, practically insoluble in ethanol (96 per cent). Aqueous solutions decompose at room temperature and more rapidly on warming.

**Potassium plumbite solution.** *1071200.*

Dissolve 1.7 g of *lead acetate R*, 3.4 g of *potassium citrate R* and 50 g of *potassium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

**Potassium pyroantimonate.** $KSb(OH)_6$. ($M_r$ 262.9). *1071300.* [12208-13-8]. Potassium hexahydroxoantimoniate.

White or almost white, crystals or crystalline powder, sparingly soluble in water.

**Potassium pyroantimonate solution.** *1071301.*

Dissolve 2 g of *potassium pyroantimonate R* in 95 mL of hot *water R*. Cool quickly and add a solution containing 2.5 g of *potassium hydroxide R* in 50 mL of *water R* and 1 mL of *dilute sodium hydroxide solution R*. Allow to stand for 24 h, filter and dilute to 150 mL with *water R*.

**Potassium pyroantimonate solution R1.** *1071302.*

Dissolve 2.0 g of *potassium pyroantimonate R* in 100 mL of hot *water R*. Boil for about 5 min, cool quickly and add 10 mL of a 150 g/L solution of *potassium hydroxide R*. Allow to stand for 24 h and filter.

**Potassium 4-sulfobenzoate.** $C_7H_5KO_5S$. ($M_r$ 240.3). *1190000.* [5399-63-3]. 4-Sulfobenzoic acid potassium salt. Potassium 4-carboxybenzenesulfonate.

White, crystalline powder.

**Potassium tartrate.** $C_4H_4K_2O_6,{}^1/_2H_2O$. ($M_r$ 235.3). *1071400.* [921-53-9]. Dipotassium (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate hemihydrate.

White or almost white, granular powder or crystals, very soluble in water, very slightly soluble in ethanol (96 per cent).

**Potassium tetraiodomercurate solution.** *1071500.*

Dissolve 1.35 g of *mercuric chloride R* in 50 mL of *water R*. Add 5 g of *potassium iodide R* and dilute to 100 mL with *water R*.

**Potassium tetraiodomercurate solution, alkaline.** *1071600.*

Dissolve 11 g of *potassium iodide R* and 15 g of *mercuric iodide R* in *water R* and dilute to 100 mL with the same solvent. Immediately before use, mix 1 volume of this solution with an equal volume of a 250 g/L solution of *sodium hydroxide R*.

**Potassium tetroxalate.** $C_4H_3KO_8,2H_2O$. ($M_r$ 254.2). *1071700.* [6100-20-5].

White or almost white, crystalline powder, sparingly soluble in water, soluble in boiling water, slightly soluble in ethanol (96 per cent).

**Potassium thiocyanate.** KSCN. ($M_r$ 97.2). *1071800.* [333-20-0].

Colourless crystals, deliquescent, very soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Potassium thiocyanate solution.** *1071801.*

A 97 g/L solution of *potassium thiocyanate R*.

**Povidone.** *1068500.* [9003-39-8].

See *Povidone (0685)*.

**Procaine hydrochloride.** *1109400.*

See *Procaine hydrochloride (0050)*.

**Proline.** *1152200.* [147-85-3].

See *Proline (0785)*.

**Propane.** $C_3H_8$. ($M_r$ 44.10). *1190100.* [74-98-6].

*Content*: minimum 99.0 per cent *V/V*.

**Propane-1,3-diol.** $C_3H_8O_2$. ($M_r$ 76.1). *1185100.* [504-63-2]. 1,3-Dihydroxypropane.

Colourless, viscous liquid.

bp: about 214 °C.

mp: about − 27 °C.

**Propanol.** $C_3H_8O$. ($M_r$ 60.1). *1072000.* [71-23-8]. Propan-1-ol.

Clear colourless liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.802 to 0.806.

bp: about 97.2 °C.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 96 °C and 99 °C.

**Propanol R1.** *1184400.* [71-23-8].

See *Propanol (2036)*.

**2-Propanol.** $C_3H_8O$. ($M_r$ 60.1). *1072100.* [67-63-0]. Propan-2-ol. Isopropyl alcohol.

Clear, colourless, flammable liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.785.

bp: 81 °C to 83 °C.

**2-Propanol R1.** *1072101.*

Complies with the requirements prescribed for *2-propanol R* with the following additional requirements.

$n_D^{20}$: about 1.378.

*Water* (*2.5.12*): maximum 0.05 per cent, determined on 10 g.

*Absorbance* (*2.2.25*): maximum 0.60 at 210 nm, 0.26 at 220 nm, 0.13 at 230 nm, 0.02 at 250 nm, 0.01 at 260 nm, determined using *water R* as compensation liquid.

**2-Propanol R2.** *1184900.* [67-63-0].

See *Isopropyl alcohol (0970)*.

**Propetamphos.** $C_{10}H_{20}NO_4PS$. ($M_r$ 281.3). *1130900.* [31218-83-4].

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

**Propidium iodide.** $C_{27}H_{34}I_2N_4$. ($M_r$ 668.4). *1154200.* [25535-16-4]. 3,8-Diamino-5-[3(diethylmethylammonio)-propyl]-6-phenylphenanthridinium diiodide.

Dark red solid.

**Propionaldehyde.** $C_3H_6O$. ($M_r$ 58.1). *1072300.* [123-38-6]. Propanal.

Liquid freely soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.81.

$n_D^{20}$: about 1.365.

bp: about 49 °C.

mp: about − 81 °C.

**Propionic acid.** $C_3H_6O_2$. ($M_r$ 74.1). *1072400.* [79-09-4].

Oily liquid, soluble in ethanol (96 per cent), miscible with water.

$d_{20}^{20}$: about 0.993.

$n_D^{20}$: about 1.387.

bp: about 141 °C.

mp: about − 21 °C.

**Propionic anhydride.** $C_6H_{10}O_3$. ($M_r$ 130.1). *1072500.* [123-62-6].

Clear, colourless liquid, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 1.01.

bp: about 167 °C.

**Propionic anhydride reagent.** *1072501.*

Dissolve 1 g of *toluenesulfonic acid R* in 30 mL of *glacial acetic acid R*, add 5 mL of *propionic anhydride R* and allow to stand for at least 15 min before use.

*Storage*: use within 24 h.

**Propyl acetate.** $C_5H_{10}O_2$. ($M_r$ 102.1). *1072600.* [109-60-4].

$d_{20}^{20}$: about 0.888.

bp: about 102 °C.

mp: about − 95 °C.

**Propyl parahydroxybenzoate.** *1072700.* [94-13-3].

See *Propyl parahydroxybenzoate (0431)*.

**D-Prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride.** $C_{26}H_{36}Cl_2N_8O_5$. ($M_r$ 612). *1072800.*

**Propylene glycol.** *1072900.* [57-55-6].
See *Propylene glycol (0430)*.

**Propylene oxide.** $C_3H_6O$. ($M_r$ 58.1). *1121800.* [75-56-9].
Colourless liquid, miscible with ethanol (96 per cent).

**Protamine sulfate.** *1073000.* [53597-25-4 (salmine) 9007-31-2 (clupeine)].
See *Protamine sulfate (0569)*.

**Protopine hydrochloride.** $C_{20}H_{20}ClNO_5$. ($M_r$ 389.8). *1163500.* [6164-47-2].
5-Methyl-4,6,7,14-tetrahydrobis[1,3]benzodioxolo[4,5-*c*:5′,6′-*g*]azecin-13(5*H*)-one hydrochloride.

**Pteroic acid.** $C_{14}H_{12}N_6O_3$. ($M_r$ 312.3). *1144600.* [119-24-4]. 4-[[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoic acid.
Crystals, soluble in solutions of alkali hydroxides.

**Puerarin.** $C_{21}H_{20}O_9$. ($M_r$ 416.4). *1180600.* [3681-99-0]. 7,4′-Dihydroxy-8-*C*-glucosyliso-haloprone. 8-β-D-Glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one.

**Pulegone.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1073100.* [89-82-7]. (*R*)-2-Isopropylidene-5-methylcyclohexanone. (+)-*p*-Menth-4-en-3-one.
Oily, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).
$d_{15}^{20}$: about 0.936.
$n_D^{20}$: 1.485 to 1.489.
bp: 222 °C to 224 °C.
*Pulegone used in gas chromatography complies with the following additional test.*
*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.
*Test solution*. The substance to be examined.
*Content*: minimum 98.0 per cent, calculated by the normalisation procedure.

**Pullulanase.** *1190200.* [9075-68-7]. Pullulan-6-glucanohydrolase obtained from *Klebsiella pneumoniae*.
*Content*: minimum 30 units/mg of protein.
One unit represents the enzymatic activity required to produce 1.0 µmol of maltotriose from pullulan per minute at pH 5.0 at 30 °C.
DETERMINATION OF PULLULANASE ACTIVITY
*Substrate*. Dissolve 0.250 g of pullulan in 20.0 mL of *water R*, adding pullulan to the water.
*Buffer solution A*. 21 g/L solution of *citric acid monohydrate R* adjusted to pH 5.0 with a 27 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.
*Buffer solution B*. Prepare a 136 g/L solution of *sodium acetate R* adjusted to pH 6.0 with *dilute acetic acid R*. Dilute 1 mL of this solution to 100 mL with *water R*.
*Somogyi reagent*. To 28 g of *anhydrous disodium hydrogen phosphate R* and 40 g of *sodium potassium tartrate R* add about 700 mL of *water R*. Add 100 mL of a 42 g/L solution of *sodium hydroxide R* and mix. Add 80 mL of a 100 g/L solution of *copper sulfate pentahydrate R*. Heat until complete dissolution. Add 180 g of *anhydrous sodium sulfate R* and adjust the volume to 1 L with *water R*. Allow to stand at room temperature for 1 or 2 days to let insoluble matter precipitate. Filter the solution and keep the filtrate in a brown-glass bottle with a ground-glass stopper.
*Nelson reagent*. Dissolve 50 g of *ammonium molybdate R* in 900 mL of *water R*. Add 42 g of *sulfuric acid R* and mix. Dissolve 6 g of *disodium arsenate R* in 50 mL of *water R*. Add the latter solution to the 1st solution, and allow to stand in a brown-glass bottle with a ground-glass stopper at 37 °C for 1 or 2 days.
*Glucose standard solution*. Dry *glucose R* at a pressure less than 6 kPa at 60 °C for 5 h, and calculate the water content. Transfer 10.00 g of dried glucose to a volumetric flask, dissolve with *water R*, dilute to 1.0 L with the same solvent, and mix. Transfer 10.0 mL of this solution to a volumetric flask and dilute to 1.0 L with *water R*. Each millilitre contains 100 µg of glucose.
*Pullulanase diluent*. Dilute *pullulanase R* with buffer solution B to prepare a solution with an enzyme activity of about 0.2 units/mL. The measurement range is between 0.1 and 0.4 units/mL. Record the dilution factor (*D*). This diluent is used as a diluted enzyme solution.
*Procedure*. Transfer 4.0 mL of substrate to a test tube and add 0.5 mL of buffer solution A, mix, and incubate at 30 °C. Add 0.5 mL of pullulanase diluent and mix thoroughly. After 30 s, transfer 1.0 mL of this solution to a test tube labelled "pullulan test solution 1", add 2.0 mL of Somogyi reagent, and mix. After 30.5 min, transfer 1.0 mL of the mixture of substrate and pullulanase diluent to a second test tube labelled "pullulan test solution 2", add 2.0 mL of Somogyi reagent, and mix. In a third test tube labelled "standard blank", mix 2.0 mL of Somogyi reagent and 1.0 mL of *water R*. In a fourth test tube labelled "glucose standard solution", mix 2.0 mL of Somogyi reagent and 1.0 mL of glucose standard solution, and add 1.0 mL of *water R*. Incubate the fourth test tube in a water-bath for exactly 10 min. Remove the tube and allow it to cool under running water. Add 2.0 mL of Nelson reagent, mix well, and allow the solution to stand for at least 15 min. Add 5.0 mL of *water R* to each of the 4 test tubes and mix thoroughly. Determine the absorbance at 520 nm of the standard blank ($A_{blank}$), the glucose standard solution ($A_{Std}$), pullulan test solution 1 ($A_0$) and pullulan test solution 2 ($A_{30}$), using *water R* as the blank. One unit is defined as the enzymatic activity required to produce 1 µmol of maltotriose (measured as glucose) from pullulan per minute. Calculate the pullulanase activity, *P*, in units/mL, using the following expression:

$$[(A_{30} - A_0) / (A_{Std} - A_{blank})] \times 0.185 \times D$$

MEASUREMENT OF PROTEIN CONTENT (MEASURED AS ALBUMINOID CONTENT) FOR THE CALCULATION OF SPECIFIC ACTIVITY
*Reagent A*. Prepare a solution having known concentrations of about 4 g/L of *sodium hydroxide R* and about 21 g/L of *anhydrous sodium carbonate R*.
*Reagent B*. Transfer 0.5 g of *copper sulfate pentahydrate R* and 1.0 g of *sodium citrate R* to a volumetric flask, dissolve in and dilute with *water R* to 100.0 mL, and mix.
*Lowry solution*. Mix 50 volumes of reagent A and 1 volume of reagent B.
*Diluted Folin-Ciocalteu's phenol reagent (for albuminoid quantification)*. Prepare a two-fold dilution of the commercially available 2 N Folin-Ciocalteu's phenol reagent or prepare a solution by making an appropriate dilution of *phosphomolybdotungstic reagent R*.
*Bovine albumin standard stock solution*. Transfer 50.0 mg of *bovine albumin R* to a volumetric flask, dissolve in and dilute with *water R* to 500.0 mL, and mix. It contains 100 µg/mL of bovine albumin.
*Standard solutions*. Using appropriate dilutions of bovine albumin standard stock solution in *water R*, prepare 5 standard solutions having concentrations equally spaced between 5 µg/mL and 100 µg/mL of bovine albumin.
*Test solution*. Dilute *pullulanase R* with buffer solution B in order to obtain a solution having a concentration of 60-70 µg/mL of albuminoid. Water may be used as diluent. Record the dilution factor, $D_f$.

*Procedure.* Introduce into separate tubes 0.3 mL of each of the standard solutions, the test solution and *water R*. Add 3.0 mL of Lowry solution to each tube and mix. Incubate at room temperature for 10 min. Add 0.3 mL of diluted Folin-Ciocalteu's phenol reagent to each tube, mix immediately, and allow to stand at room temperature for 60 min. Determine the absorbances of the standard solutions and the test solution at the wavelength of maximum absorbance, about 750 nm, using *water R* as the blank.

*Calculation.* The relationship of absorbance to protein concentration is non-linear; however, if the standard curve concentration range is sufficiently small, it will approach linearity. Using linear regression method, plot the absorbances of the standard solutions versus the protein (bovine albumin) concentrations, in µg/mL. Using the plot, determine the concentration of protein (albuminoid content), $C_{albuminoid}$, in µg/mL, in the test solution. Calculate the albuminoid concentration, in mg/mL, in *pullulanase R* using the following expression:

$$C_{protein} = (C_{albuminoid} \times D_f) / 1000$$

Calculate the specific activity, in units/mg, of pullulanase using the formula:

$$P / C_{protein}$$

$P$ = pullulanase activity in units/mL.

**Putrescine.** $C_4H_{12}N_2$. ($M_r$ 88.15). *1137900.* [110-60-1]. 1,4-Butanediamine. Tetramethylenediamine.

Colourless oily liquid, very soluble in water. Strong piperidine-like odour.

bp: about 159 °C.

mp: about 23 °C.

**Pyrazine-2-carbonitrile.** $C_5H_3N_3$. ($M_r$ 105.1). *1183300.* [19847-12-2]. 2-Cyanopyrazine.
Clear, pale yellow liquid.

*Content*: minimum 99 per cent.

**Pyridin-2-amine.** $C_5H_6N_2$. ($M_r$ 94.1). *1073400.* [504-29-0]. 2-Aminopyridine.

Large crystals soluble in water and in ethanol (96 per cent).

bp: about 210 °C.

mp: about 58 °C.

**Pyridine.** $C_5H_5N$. ($M_r$ 79.1). *1073200.* [110-86-1].

Clear, colourless liquid, hygroscopic, miscible with water and with ethanol (96 per cent).

bp: about 115 °C.

*Storage*: in an airtight container.

**Pyridine, anhydrous.** *1073300.*

Dry *pyridine R* over *anhydrous sodium carbonate R*. Filter and distil.

*Water* (*2.5.12*): maximum 0.01 per cent *m/m*.

**Pyridine-4-carbonitrile.** $C_6H_4N_2$. ($M_r$ 104.1). *1190300.* [100-48-1]. 4-Cyanopyridine.

White or almost white crystalline powder.

bp: 194 °C to 196 °C.

mp: 76 °C to 79 °C.

**Pyridinium hydrobromide perbromide.** $C_5H_6Br_3N$. ($M_r$ 319.8). *1166100.* [39416-48-3]. Pyridinium tribromide(1-).

Red crystals.

**Pyridylazonaphthol.** $C_{15}H_{11}N_3O$. ($M_r$ 249.3). *1073500.* [85-85-8]. 1-(2-Pyridylazo)-2-naphthol.

Brick-red powder, practically insoluble in water, soluble in ethanol (96 per cent), in methanol and in hot dilute alkali solutions.

mp: about 138 °C.

**Pyridylazonaphthol solution.** *1073501.*

A 1 g/L solution of *pyridylazonaphthol R* in *anhydrous ethanol R*.

*Test for sensitivity.* To 50 mL of *water R* add 10 mL of *acetate buffer solution pH 4.4 R*, 0.10 mL of *0.02 M sodium edetate* and 0.25 mL of the pyridylazonaphthol solution. After addition of 0.15 mL of a 5 g/L solution of *copper sulfate pentahydrate R*, the colour changes from light yellow to violet.

**4-(2-Pyridylazo)resorcinol monosodium salt.** $C_{11}H_8N_3NaO_2$, $H_2O$. ($M_r$ 255.2). *1131500.* [16593-81-0].

Orange crystalline powder.

**Pyrocatechol.** $C_6H_6O_2$. ($M_r$ 110.1). *1073600.* [120-80-9]. Benzene-1,2-diol.

Colourless or slightly yellow crystals, soluble in water, in acetone and in ethanol (96 per cent).

mp: about 102 °C.

*Storage*: protected from light.

**Pyrogallol.** $C_6H_6O_3$. ($M_r$ 126.1). *1073700.* [87-66-1]. Benzene-1,2,3-triol.

White or almost white crystals, becoming brownish on exposure to air and light, very soluble in water and in ethanol (96 per cent), slightly soluble in carbon disulfide. On exposure to air, aqueous solutions, and more rapidly alkaline solutions, become brown owing to the absorption of oxygen.

mp: about 131 °C.

*Storage*: protected from light.

**Pyrogallol solution, alkaline.** *1073701.*

Dissolve 0.5 g of *pyrogallol R* in 2 mL of *carbon dioxide-free water R*. Dissolve 12 g of *potassium hydroxide R* in 8 mL of *carbon dioxide-free water R*. Mix the two solutions immediately before use.

**Pyrrolidine.** $C_4H_9N$. ($M_r$ 71.1). *1165000.* [123-75-1].

*Content*: minimum 99 per cent.

bp: 87 °C to 88 °C.

**2-Pyrrolidone.** $C_4H_7NO$. ($M_r$ 85.1). *1138000.* [616-45-5]. Pyrrolidin-2-one.

Liquid above 25 °C, miscible with water, with anhydrous ethanol and with ethyl acetate.

$d_4^{25}$: 1.116.

*Water* (*2.5.12*): maximum 0.2 per cent determined on 2.00 g.

*Assay.* Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 1.0 g in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *material*: glass;
- *size*: $l$ = 30 m; Ø = 0.53 mm;
- *stationary phase*: *macrogol 20 000 R* (1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: adjusted so that the retention time of 2-pyrrolidone is about 10 min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 80 |
| | 1 - 12 | 80 → 190 |
| | 12 - 32 | 190 |
| Injection port | | 200 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution.

*Content*: minimum 98.0 per cent.

**Pyruvic acid.** $C_3H_4O_3$. ($M_r$ 88.1). *1109300.* [127-17-3]. 2-Oxopropanoic acid.

Yellowish liquid, miscible with water and with anhydrous ethanol.

$d_{20}^{20}$: about 1.267.

$n_D^{20}$: about 1.413.

bp: about 165 °C.

**Quercetin dihydrate.** $C_{15}H_{10}O_7,2H_2O$. ($M_r$ 338.2). *1138100.* 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one.

Yellow crystals or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

*Water* (*2.5.12*): maximum 12.0 per cent, determined on 0.100 g.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginkgo leaf (1828)*.

*Content*:minimum 90 per cent (anhydrous substance) calculated by the normalisation procedure.

*Storage*: protected from light.

**Quercitrin.** $C_{21}H_{20}O_{11}$. ($M_r$ 448.4). *1138200.* [522-12-3]. Quercetin 3-L-rhamnopyranoside. 3-[(6-Deoxy-α-L-mannopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one. Quercitroside.

Yellow crystals, practically insoluble in cold water, soluble in ethanol (96 per cent).

mp: 176 °C to 179 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Goldenrod (1892)*: apply 20 µL of the solution; after spraying, the chromatogram shows a yellowish-brown fluorescent zone with an $R_F$ of about 0.6.

*Storage*: at a temperature of 2 °C to 8 °C.

**Quillaia saponins, purified.** *1184500.*

A mixture of related saponins obtained from the bark of *Quillaja saponaria* Molina *s.l.*

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Quillaia bark (1843)*: apply 5 µL of the solution; after treating with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 120 °C for 5 min and examine in daylight; the chromatogram shows 3 principal zones in the upper part of the middle third.

**Quinaldine red.** $C_{21}H_{23}IN_2$. ($M_r$ 430.3). *1073800.* [117-92-0]. 2-[2-[4-(Dimethylamino)phenyl]ethenyl]-1-ethylquinolinium iodide.

Dark bluish-black powder, sparingly soluble in water, freely soluble in ethanol (96 per cent).

**Quinaldine red solution.** *1073801.*

Dissolve 0.1 g of *quinaldine red R* in *methanol R* and dilute to 100 mL with the same solvent.

*Colour change*: pH 1.4 (colourless) to pH 3.2 (red).

**Quinhydrone.** $C_{12}H_{10}O_4$. ($M_r$ 218.2). *1073900.* [106-34-3]. Equimolecular compound of 1,4-benzoquinone and hydroquinone.

Dark green, lustrous crystals or a crystalline powder, slightly soluble in water, sparingly soluble in hot water, soluble in ethanol (96 per cent) and in concentrated ammonia.

mp: about 170 °C.

**Quinidine.** $C_{20}H_{24}N_2O_2$. ($M_r$ 324.4). *1074000.* [56-54-2]. (*S*)-(6-Methoxyquinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

White or almost white crystals, very slightly soluble in water, sparingly soluble in ethanol (96 per cent), slightly soluble in methanol.

$[\alpha]_D^{20}$: about + 260, determined on a 10 g/L solution in *anhydrous ethanol R*.

mp: about 172 °C.

*Storage*: protected from light.

**Quinidine sulfate.** *1109500.* [6591-63-5].

See *Quinidine sulfate (0017)*.

**Quinine.** $C_{20}H_{24}N_2O_2$. ($M_r$ 324.4). *1074100.* [130-95-0]. (*R*)-(6-Methoxyquinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol.

White or almost white, microcrystalline powder, very slightly soluble in water, slightly soluble in boiling water, very soluble in anhydrous ethanol.

$[\alpha]_D^{20}$: about − 167, determined on a 10 g/L solution in *anhydrous ethanol R*.

mp: about 175 °C.

*Storage*: protected from light.

**Quinine hydrochloride.** *1074200.* [6119-47-7].

See *Quinine hydrochloride (0018)*.

**Quinine sulfate.** *1074300.* [6119-70-6].

See *Quinine sulfate (0019)*.

**3-Quinuclidinol.** $C_7H_{13}NO$. ($M_r$ 127.2). *1193800.* [1619-34-7]. (3*R*)-1-Azabicyclo[2.2.2]octan-3-ol.

*Content*: minimum 99 per cent.

Light yellow powder.

**Rabbit erythrocyte suspension.** *1074500.*

Prepare a 1.6 per cent *V/V* suspension of rabbit erythrocytes as follows: defibrinate 15 mL of freshly drawn rabbit blood by shaking with glass beads, centrifuge at 2000 *g* for 10 min and wash the erythrocytes with three quantities, each of 30 mL, of a 9 g/L solution of *sodium chloride R*. Dilute 1.6 mL of the suspension of erythrocytes to 100 mL with a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*.

**Raclopride tartrate.** $C_{19}H_{26}Cl_2N_2O_9$. ($M_r$ 497.3). *1144700.* [98185-20-7]. Raclopride L-tartrate.

White or almost white solid, sensitive to light, soluble in water.

$[\alpha]_D^{25}$: + 0.3, determined on a 3 g/L solution.

mp: about 141 °C.

**Raffinose pentahydrate.** $C_{18}H_{32}O_{16},5H_2O$. ($M_r$ 594.5). *1201800.* [17629-30-0]. β-D-Fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside pentahydrate.

*Content*: minimum 98.0 per cent.

Crystalline powder.

mp: about 80 °C.

**Raltegravir potassium.** $C_{20}H_{20}FKN_6O_5$. *1202600.* [871038-72-1].

See *Raltegravir potassium (2887)*.

**Rapeseed oil.** *1074600.*

See *Rapeseed oil, refined (1369)*.

**Reducing mixture.** *1074700.*

Grind the substances added in the following order to obtain a homogeneous mixture: 20 mg of *potassium bromide R*, 0.5 g of *hydrazine sulfate R* and 5 g of *sodium chloride R*.

**Reichstein's substance S.** $C_{21}H_{30}O_4$. ($M_r$ 346.5). *1175400.* [152-58-9].

*Content*: minimum 95.0 per cent.

mp: about 208 °C.

**Resin for hydrophobic interaction chromatography.** *1202700.*

Non-porous resin consisting of spherical polymethacrylate particles bonded with butyl groups.

*pH limits of use*: 2 to 12.

**Resin for reversed-phase ion chromatography.** *1131100.*

A neutral, macroporous, high specific surface area with a non-polar character resin consisting of polymer lattice of polystyrene cross-linked with divinylbenzene.

**Resin, weak cationic.** *1096000.*

See *weak cationic resin R*.

**Resorcinol.** *1074800.* [108-46-3].

See *Resorcinol (0290)*.

**Resorcinol reagent.** *1074801.*

To 80 mL of *hydrochloric acid R1* add 10 mL of a 20 g/L solution of *resorcinol R* and 0.25 mL of a 25 g/L solution of *copper sulfate pentahydrate R* and dilute to 100.0 mL with *water R*. Prepare the solution at least 4 h before use.

*Storage*: at 2 °C to 8 °C for 1 week.

**Resveratrol.** $C_{14}H_{12}O_3$. ($M_r$ 228.2). *1186900.* [501-36-0]. 3,4′,5-Stilbenetriol. 5-[(*E*)-2-(4-Hydroxyphenyl)ethenyl]benzene-1,3-diol.

**Rhamnose.** $C_6H_{12}O_5,H_2O$. ($M_r$ 182.2). *1074900.* [6155-35-7]. (2*R*,3*R*,4*R*,5*R*,6*S*)-6-Methyltetrahydro-2*H*-pyran-2,3,4,5-tetrol monohydrate. 6-Deoxy-α-L-mannopyranose monohydrate. α-L-Rhamnopyranose monohydrate. L-(+)-Rhamnose monohydrate.

White or almost white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 7.8 to + 8.3, determined on a 50 g/L solution in *water R* containing about 0.05 per cent of $NH_3$.

**Rhaponticin.** $C_{21}H_{24}O_9$. ($M_r$ 420.4). *1075000.* [155-58-8]. 3-Hydroxy-5-[2-(3-hydroxy-4-methoxyphenyl)ethenyl]phenyl β-D-glucopyranoside.

Yellowish-grey, crystalline powder, soluble in ethanol (96 per cent) and in methanol.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Rhubarb (0291)*; the chromatogram shows only one principal spot.

**Rhein.** $C_{15}H_8O_6$. ($M_r$ 284.2). *1197700.* [478-43-3]. 4,5-Dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid. 1,8-Dihydroxy-3-carboxyanthraquinone.

**Rhodamine 6 G.** $C_{28}H_{31}ClN_2O_3$. ($M_r$ 479.0). *1153300.* [989-38-8].

Colour Index No. 45160.

9-[2-(Ethoxycarbonyl)phenyl]-3,6-bis(ethylamino)-2,7-dimethylxanthenylium chloride.

Brownish-red powder.

**Rhodamine B.** $C_{28}H_{31}ClN_2O_3$. ($M_r$ 479.0). *1075100.* [81-88-9].

Schultz No. 864.

Colour Index No. 45170.

[9-(2-Carboxyphen-yl)-6-(diethylamino)-3*H*-xanthen-3-ylidene]diethylammonium chloride.

Green crystals or reddish-violet powder, very soluble in water and in ethanol (96 per cent).

**Rhynchophylline.** $C_{22}H_{28}N_2O_4$. ($M_r$ 384.5). *1197800.* [76-66-4]. Methyl (16*E*)-17-methoxy-2-oxo-16,17-didehydro-7β,20α-corynoxan-16-carboxylate. Methyl (16*E*)-16-(methoxymethylidene)-2-oxo-7β,20α-corynoxan-17-oate.

**Ribose.** $C_5H_{10}O_5$. ($M_r$ 150.1). *1109600.* [50-69-1]. D-Ribose.

Soluble in water, slightly soluble in ethanol (96 per cent).

mp: 88 °C to 92 °C.

**Ricinoleic acid.** $C_{18}H_{34}O_3$. ($M_r$ 298.5). *1100100.* [141-22-0]. (9*Z*,12*R*)-12-Hydroxyoctadec-9-enoic acid. 12-Hydroxyoleic acid.

Yellow or yellowish-brown viscous liquid, consisting of a mixture of fatty acids obtained by the hydrolysis of castor oil, practically insoluble in water, very soluble in anhydrous ethanol.

$d_{20}^{20}$: about 0.942.

$n_D^{20}$: about 1.472.

mp: about 285 °C, with decomposition.

**Rosmarinic acid.** $C_{18}H_{16}O_8$. ($M_r$ 360.3). *1138300.* [20283-92-5].

mp: 170 °C to 174 °C.

**Rutecarpine.** $C_{18}H_{13}N_3O$. ($M_r$ 287.3). *1199500.* [84-26-4]. 8,13-Dihydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one.

**Ruthenium red.** $[(NH_3)_5RuORu(NH_3)_4ORu(NH_3)_5]Cl_6,4H_2O$. ($M_r$ 858). *1075200.* [11103-72-3].

Brownish-red powder, soluble in water.

**Ruthenium red solution.** *1075201.*

A 0.8 g/L solution of *ruthenium red R* in *lead acetate solution R*.

**Rutin.** *1075300.* [250249-75-3].

See *Rutoside trihydrate R*.

**Rutoside trihydrate.** *1075300.* [250249-75-3].

See *Rutoside trihydrate (1795)*.

**Sabinene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1109700.* [3387-41-5]. Thuj-4(10)-ene. 4-Methylene-1-isopropylbicyclo[3.1.0]hexane.

A colourless, oily liquid.

*Sabinene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

*Test solution*. The substance to be examined.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Saccharin sodium.** *1131400.* [128-44-9].

See *Saccharin sodium (0787)*.

**Safrole.** $C_{10}H_{10}O_2$. ($M_r$ 162.2). *1131200.* [94-59-7]. 5-(Prop-2-enyl)-1,3-benzodioxole. 4-Allyl-1,2-(methylenedioxy)benzene.

Colourless or slightly yellow, oily liquid, with the odour of sassafras, insoluble in water, very soluble in ethanol (96 per cent), miscible with hexane.

$d_{20}^{20}$: 1.095 to 1.096.

$n_D^{20}$: 1.537 to 1.538.

bp: 232 °C to 234 °C.

*Freezing point*: about 11 °C.

*Safrole used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cinnamon bark oil, Ceylon (1501)*.

*Content*: minimum 96.0 per cent, calculated by the normalisation procedure.

**Saikosaponin A.** $C_{42}H_{68}O_{13}$. ($M_r$ 781). *1201900.* [20736-09-8]. 13,28-Epoxy-16β,23-dihydroxy-4α-olean-11-en-3β-yl 6-deoxy-3-*O*-β-D-glucopyranosyl-β-D-galactopyranoside.

**Saikosaponin D.** $C_{42}H_{68}O_{13}$. ($M_r$ 781). *1201200.* [20874-52-6]. 13,28-Epoxy-16α,23-dihydroxy-4α-olean-11-en-3β-yl 6-deoxy-3-*O*-β-D-glucopyranosyl-β-D-galactopyranoside.

**Salicin.** $C_{13}H_{18}O_7$. ($M_r$ 286.3). *1131300.* [138-52-3]. 2-(Hydroxymethyl)phenyl-β-D-glucopyranoside. Salicoside.

$[\alpha]_D^{20}$: − 62.5 ± 2.

mp: 199 °C to 201 °C.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Willow bark (1583)* at the concentration of the reference solution.

*Content*: minimum 99.0 per cent, calculated by the normalisation procedure.

**Salicylaldehyde.** $C_7H_6O_2$. ($M_r$ 122.1). *1075400.* [90-02-8]. 2-Hydroxybenzaldehyde.

Clear, colourless, oily liquid.

$d_{20}^{20}$: about 1.167.

$n_D^{20}$: about 1.574.

bp: about 196 °C.

mp: about − 7 °C.

**Salicylaldehyde azine.** $C_{14}H_{12}N_2O_2$. ($M_r$ 240.3). *1075500.* [959-36-4]. 2,2′-Azinodimethyldiphenol.

Dissolve 0.30 g of *hydrazine sulfate R* in 5 mL of *water R*, add 1 mL of *glacial acetic acid R* and 2 mL of a freshly prepared 20 per cent *V/V* solution of *salicylaldehyde R* in *2-propanol R*. Mix, allow to stand until a yellow precipate is formed. Shake with two quantities, each of 15 mL, of *methylene chloride R*. Combine the organic layers and dry over *anhydrous sodium sulfate R*. Decant or filter the solution and evaporate to dryness. Recrystallise from a mixture of 40 volumes of *methanol R* and 60 volumes of *toluene R* with cooling. Dry the crystals *in vacuo*.

mp: about 213 °C.

*Chromatography*. Thin-layer chromatography (*2.2.27*) as prescribed in the test for hydrazine in the monograph *Povidone (0685)*; the chromatogram shows only one principal spot.

**Salicylic acid.** *1075600.* [69-72-7].

See *Salicylic acid (0366)*.

**Salvianolic acid B.** $C_{36}H_{30}O_{16}$. ($M_r$ 719). *1184600.* [121521-90-2]. (2*R*)-2-[[(2*E*)-3-[(2*S*,3*S*)-3-[[(1*R*)-1-Carboxy-2-(3,4-dihydroxyphenyl)ethoxy]carbonyl]-2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrobenzofuran-4-yl]prop-2-enoyl]oxy]-3-(3,4-dihydroxyphenyl)propanoic acid.

**Sand.** *1075800.*

White or slightly greyish grains of silica with a particle size between 150 μm and 300 μm.

**Sarafloxacin hydrochloride.** $C_{20}H_{18}ClF_2N_3O_3$. ($M_r$ 421.8). *1181400.* [91296-87-6]. 6-Fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid hydrochloride.

**Schisandrin.** $C_{24}H_{32}O_7$. ($M_r$ 432.5). *1173800.* [7432-28-2]. Schisandrol A. Wuweizichun A. (6*S*,7*S*,12a*R*$_a$)-5,6,7,8-Tetrahydro-1,2,3,10,11,12-hexamethoxy-6,7-dimethyldibenzo[*a,c*]cyclooctan-6-ol.

White or almost white, crystalline powder.

Schisandrin used in the assay in the monograph *Schisandra fruit (2428)* complies with the following additional test.

*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Schisandra fruit (2428)*.

*Content*: minimum 95 per cent, calculated by the normalisation procedure.

*Storage*: in an airtight container, at − 20 °C or below.

**γ-Schisandrin.** $C_{23}H_{28}O_6$. ($M_r$ 400.5). *1173900.* [61281-37-6]. Schisandrin B. Wuweizisu B. *rac*-(6*R*,7*S*,13a*R*$_a$)-1,2,3,13-Tetramethoxy-6,7-dimethyl-5,6,7,8-tetrahydrobenzo[3,4]cycloocta[1,2-*f*][1,3]benzodioxole.

White or almost white, crystalline powder.

*Storage*: in an airtight container, at − 20 °C or below.

**Sclareol.** $C_{20}H_{36}O_2$. ($M_r$ 308.5). *1139900.* [515-03-7]. (1*R*,2*R*,4a*S*,8a*S*)-1-[(3*R*)-3-Hydroxy-3-methylpent-4-enyl]-2,5,5,8a-tetramethyldecahydronaphthalen-2-ol.

Odourless crystals.

$[\alpha]_D^{20}$: 6.7, determined with a solution in anhydrous ethanol.

$bp_{19\ mm}$: 218 °C to 220 °C.

mp: 96 °C to 98 °C.

*Sclareol used in the chromatographic profile test in the monograph Clary sage oil (1850) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clary sage oil (1850)*.

*Content*: minimum 97 per cent, calculated by the normalisation procedure.

**Scopoletin.** $C_{10}H_8O_4$. ($M_r$ 192.2). *1158700.* [92-61-5]. 7-Hydroxy-6-methoxy-2*H*-1-benzopyran-2-one. 7-Hydroxy-6-methoxycoumarin.

Faintly beige, fine crystals.

mp: 202 °C to 208 °C.

**SDS-PAGE running buffer.** *1114900.*

Dissolve 151.4 g of *tris(hydroxymethyl)aminomethane R*, 721.0 g of *glycine R* and 50.0 g of *sodium laurilsulfate R* in *water R* and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with *water R* and mix. Measure the pH (*2.2.3*) of the diluted solution. The pH is between 8.1 and 8.8.

**SDS-PAGE sample buffer (concentrated).** *1115000.*

Dissolve 1.89 g of *tris(hydroxymethyl)aminomethane R*, 5.0 g of *sodium laurilsulfate R* and 50 mg of *bromophenol blue R* in *water R*. Add 25.0 mL of *glycerol R* and dilute to 100 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R*, and dilute to 125 mL with *water R*.

**SDS-PAGE sample buffer for reducing conditions (concentrated).** *1122100.*

Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 200 mL with *water R*. Add 25.0 mL of *2-mercaptoethanol R*. Adjust to pH 6.8 with *hydrochloric acid R*, and dilute to 250.0 mL with *water R*.

Alternatively, dithiothreitol may be used as reducing agent instead of 2-mercaptoethanol. In this case prepare the sample buffer as follows: dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 200 mL with *water R*. Adjust

to pH 6.8 with *hydrochloric acid R*, and dilute to 250.0 mL with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 100 mM.

**Selenious acid.** $H_2SeO_3$. ($M_r$ 129.0). *1100200.* [7783-00-8].
Deliquescent crystals, freely soluble in water.
*Storage*: in an airtight container.

**Selenium.** Se. ($A_r$ 79.0). *1075900.* [7782-49-2].
Brown-red or black powder or granules, practically insoluble in water and in ethanol (96 per cent), soluble in nitric acid.
mp: about 220 °C.

**Sennoside B.** $C_{42}H_{38}O_{20}$. ($M_r$ 863). *1190400.* [128-57-4].
(9*R*,9′*S*)-5,5′-Bis(β-D-glucopyranosyloxy)-4,4′-dihydroxy-10,10′-dioxo-9,9′,10,10′-tetrahydro-9,9′-bianthracene-2,2′-dicarboxylic acid.
Pale yellow crystals, practically insoluble in water, very slightly soluble in ethanol (96 per cent), soluble in dilute solutions of alkali hydroxides.
mp: 180 °C to 186 °C.

**Serine.** *1076000.* [56-45-1].
See *Serine (0788)*.

**Sialic acid.** *1001100.* [131-48-6].
See *N-acetylneuraminic acid R*.

**Silibinin.** $C_{25}H_{22}O_{10}$. ($M_r$ 482.4). *1151400.* [22888-70-6].
Silybin. (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4*H*-1-benzopyran-4-one.
White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.
*Silibinin used in the assay of Milk thistle fruit (1860) complies with the following additional test.*
*Assay*. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Milk thistle fruit (1860)*.
*Test solution*. Dissolve 5.0 mg of silibinin, dried *in vacuo*, in *methanol R* and dilute to 50.0 mL with the same solvent.
Silibinin A and silibinin B content: minimum 95.0 per cent, calculated by the normalisation procedure.

**Silica for chromatography, porous.** *1207800.*
Porous silica with porous layer open tubular (PLOT) design.

**Silica gel π-acceptor/π-donor for chiral separations.** *1160100.*
A very finely divided silica gel for chromatography consisting of spherical particles to which 1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenantrene has been covalently bound, showing both π-electron acceptor and π-electron donor characteristics.

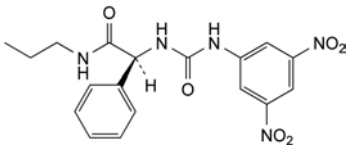


**Silica gel AGP for chiral chromatography.** *1148700.*
See *α1-Acid-glycoprotein silica gel for chiral separation R*.

**Silica gel, anhydrous.** *1076100.* [112926-00-8].
Partly dehydrated polymerised, amorphous silicic acid, absorbing at 20 °C about 30 per cent of its mass of water. Practically insoluble in water, partly soluble in solutions of sodium hydroxide. It contains a suitable indicator for detection of the humidity status, for which the colour change from the hydrated to anhydrous form is given on the label.

**Silica gel BC for chiral chromatography.** *1161300.*
A very finely divided silica gel for chromatography (5 µm) coated with β-cyclodextrin. Higher selectivity may be obtained when cyclodextrin has been derivatized with propylene oxide.

**Silica gel for chiral chromatography, urea type.** *1181000.*
A very finely divided silica gel (5 µm) coated with the following derivative:

**Silica gel for chiral separation, amylose derivative of.** *1171700.*
Substituted amylose coated on a very finely divided silica gel for chromatography.

**Silica gel for chiral separation, cellulose derivative of.** *1110300.*
Substituted cellulose coated on a very finely divided silica gel for chromatography.

**Silica gel for chiral separation, human albumin coated.** *1138500.*
A very finely divided silica gel, chemically modified at the surface by the bonding of human albumin.

**Silica gel for chiral separation, protein derivative of.** *1196300.*
A very finely divided silica gel for chromatography consisting of spherical particles coated with a protein derivative.

**Silica gel for chiral separation, vancomycin-bonded.** *1205300.*
High-purity silica gel chemically modified by the bonding of vancomycin through multiple covalent linkages.

**Silica gel for chromatography.** *1076900.*
A very finely divided silica gel.

**Silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases.** *1160200.*
A very finely divided silica gel with bonded alkyl groups suitable for use with highly aqueous mobile phases.

**Silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases, end-capped.** *1176900.*
A very finely divided silica gel with bonded alkyl groups suitable for use with highly aqueous mobile phases. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, alkylsilyl, solid core, end-capped.** *1194300.*
Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with alkylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, amidoalkylsilyl.** *1205400.*
A very finely divided silica gel, chemically modified at the surface by the bonding of amidoalkylsilyl groups.

**Silica gel for chromatography, amidohexadecylsilyl.** *1170400.*
A very finely divided silica gel with a fine particle size, chemically modified at the surface by the bonding of amidohexadecylsilyl groups.

**Silica gel for chromatography, amidohexadecylsilyl, end-capped.** *1201100.*
A very finely divided silica gel, chemically modified at the surface by the bonding of amidohexadecylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, aminopropylmethylsilyl.** *1102400.*
Silica gel with a fine particle size, chemically modified by bonding aminopropylmethylsilyl groups on the surface.

**Silica gel for chromatography, aminopropylsilyl.** *1077000.*

Silica gel with a fine particle size, chemically modified by bonding aminopropylsilyl groups on the surface.

**Silica gel for chromatography, aminopropylsilyl R1.** *1077001.*

Silica gel with a particle size of about 55 µm, chemically modified by bonding aminopropylsilyl groups on the surface.

**Silica gel for chromatography, amylose derivative of.** *1109800.*

A very finely divided (10 µm) silica gel, chemically modified at the surface by the bonding of an amylose derivative.

**Silica gel for chromatography, butylsilyl.** *1076200.*

A very finely divided silica gel, chemically modified at the surface by the bonding of butylsilyl groups.

**Silica gel for chromatography, butylsilyl, end-capped.** *1170500.*

A very finely divided silica, chemically modified at the surface by the bonding of butylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography compatible with 100 per cent aqueous mobile phases, octadecylsilyl.** *1203900.*

A very finely divided silica gel with bonded octadecylsilyl groups suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases.

**Silica gel for chromatography compatible with 100 per cent aqueous mobile phases, octadecylsilyl, end-capped.** *1188400.*

A very finely divided silica gel with bonded octadecylsilyl groups suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography compatible with highly aqueous mobile phases, octadecylsilyl diol, end-capped.** *1207500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups and end-capping. Free diol groups are also present. For use with highly aqueous mobile phases.

**Silica gel for chromatography, cyanopropylsilyl, end-capped, base-deactivated.** *1194200.*

A very finely divided silica gel, pre-treated before the bonding of cyanopropylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, cyanosilyl.** *1109900.*

A very finely divided silica gel chemically modified at the surface by the bonding of cyanosilyl groups.

**Silica gel for chromatography, cyanosilyl, end-capped.** *1195000.*

A very finely divided silica gel chemically modified at the surface by the bonding of cyanosilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, di-isobutyloctadecylsilyl.** *1140000.*

A very finely divided silica gel chemically modified at the surface by the bonding of di-isobutyloctadecylsilyl groups.

**Silica gel for chromatography, diisopropylcyanosilyl.** *1168100.*

A very finely divided silica gel chemically modified at the surface by the bonding of diisopropylcyanosilyl groups.

**Silica gel for chromatography, 4-dimethylaminobenzylcarbamidesilyl.** *1204000.*

A very finely divided silica gel, chemically modified at the surface by the bonding of 4-dimethylaminobenzylcarbamide groups.

**Silica gel for chromatography, dimethyloctadecylsilyl.** *1115100.*

A very finely divided silica gel, chemically modified at the surface by the bonding of dimethyloctadecylsilyl groups.

*Specific surface area*: 300 $m^2$/g.

**Silica gel for chromatography, diol.** *1110000.*

Spherical silica particles to which dihydroxypropyl groups are bonded. Pore size 10 nm.

**Silica gel for chromatography, dodecylsilyl, end-capped.** *1179700.*

A very finely divided silica gel, chemically modified at the surface by the introduction of dodecylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, hexadecylamidylsilyl.** *1162500.*

A very finely divided (5 µm) silica gel, chemically modified at the surface by the introduction of hexadecylcarboxamidopropyldimethylsilyl groups.

**Silica gel for chromatography, hexadecylamidylsilyl, end-capped.** *1172400.*

A very finely divided (5 µm) silica gel, chemically modified at the surface by the introduction of hexadecylcarboxamidopropyldimethylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, hexylsilyl.** *1077100.*

A very finely divided silica gel, chemically modified at the surface by the bonding of hexylsilyl groups.

**Silica gel for chromatography, hexylsilyl, end-capped.** *1174400.*

A very finely divided silica gel, chemically modified at the surface by the bonding of hexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, human albumin coated.** *1138500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of human albumin.

**Silica gel for chromatography (hybrid material), octadecylsilyl, ethylene-bridged, charged surface, end-capped.** *1202800.*

Synthetic, spherical ethylene-bridged hybrid particles with a charged surface, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography (hybrid material), octadecylsilyl, ethylene-bridged, end-capped.** *1190500.*

Synthetic, spherical, ethylene-bridged hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To

minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography (hybrid material), phenylhexylsilyl, ethylene-bridged, charged surface, end-capped.** *1204100.*

Synthetic, spherical ethylene-bridged hybrid particles with a charged surface, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of phenylhexylsilyl groups. To minimise any interaction with basic compounds they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography (hybrid material), phenylsilyl, ethylene-bridged, end-capped.** *1200700.*

Synthetic, spherical, ethylene-bridged hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of phenylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography (hybrid material), polar-embedded, octadecylsilyl, ethylene-bridged, end-capped.** *1200800.*

Synthetic, spherical, ethylene-bridged hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, hydrophilic.** *1077200.*

A very finely divided silica gel whose surface has been modified to provide hydrophilic characteristics.

**Silica gel for chromatography, nitrile.** *1077300.*

A very finely divided silica gel, chemically modified at the surface by the bonding of cyanopropylsilyl groups.

**Silica gel for chromatography, nitrile R1.** *1077400.*

A very finely divided silica gel consisting of porous, spherical particles with chemically bonded nitrile groups.

**Silica gel for chromatography, nitrile R2.** *1119500.*

Ultrapure silica gel, chemically modified at the surface by the introduction of cyanopropylsilyl groups. Less than 20 ppm of metals.

**Silica gel for chromatography, nitrile, end-capped.** *1174500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of cyanopropylsilyl groups. To minimise any interaction with basic components it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, 4-nitrophenylcarbamidesilyl.** *1185200.*

A very finely divided silica gel, chemically modified at the surface by bonding of 4-nitrophenylcarbamide groups.

**Silica gel for chromatography, octadecanoylaminopropylsilyl.** *1115200.*

A very finely divided silica gel, chemically modified at the surface by the bonding of aminopropylsilyl groups which are acylated with octadecanoyl groups.

**Silica gel for chromatography, octadecylphenylsilyl, end-capped.** *1199300.*

A very finely divided silica gel, chemically modified at the surface by bonding of octadecylphenylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl.** *1077500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups.

**Silica gel for chromatography, octadecylsilyl R1.** *1110100.*

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. Less than 20 ppm of metals.

**Silica gel for chromatography, octadecylsilyl R2.** *1115300.*

A very finely divided (15 nm pore size) ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups (20 per cent carbon load), optimised for the analysis of polycyclic aromatic hydrocarbons.

**Silica gel for chromatography, octadecylsilyl, base-deactivated.** *1077600.*

A very finely divided silica gel, pretreated before the bonding of octadecylsilyl groups by careful washing and hydrolysing most of the superficial siloxane bridges to minimise the interaction with basic components.

**Silica gel for chromatography, octadecylsilyl, cross-linked, end-capped.** *1204200.*

A very finely divided silica gel, chemically modified at the surface by the cross-linking and bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, end-capped.** *1115400.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, end-capped R1.** *1115401.*

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, end-capped, base-deactivated.** *1108600.*

A very finely divided silica gel, pre-treated before the bonding of octadecylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To further minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, extra-dense bonded, end-capped.** *1188500.*

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, for separation of polycyclic aromatic hydrocarbons.** *1202900.*

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups, optimised for the analysis of polycyclic aromatic hydrocarbons.

**Silica gel for chromatography, octadecylsilyl, monolithic, end-capped.** *1154500.*

Monolithic rods of highly porous (greater than 80 per cent) metal-free silica with a bimodal pore structure, modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, polar-embedded, encapsulated.** *1206600.*

Silica gel chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, it is carefully encapsulated to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, polar end-capped.** *1205500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully polar end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, solid core.** *1205600.* Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octadecylsilyl groups.

**Silica gel for chromatography, octadecylsilyl, solid core, end-capped.** *1193900.*

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, with embedded polar groups, end-capped.** *1177900.*

A very finely divided silica gel. The particles are based on a mixture of silica chemically modified at the surface by the bonding of octadecylsilyl groups and silica chemically modified with a reagent providing a surface with chains having embedded polar groups. Furthermore, the packing material is end-capped.

**Silica gel for chromatography, octadecylsilyl, with extended pH range, end-capped.** *1196700.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups resistant to bases up to pH 11. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octadecylsilyl, with polar incorporated groups, end-capped.** *1165100.*

A very finely divided silica gel. The particles are based on silica, chemically modified with a reagent providing a surface with chains having polar incorporated groups and terminating octadecyl groups. Furthermore, the packing material is end-capped.

**Silica gel for chromatography, octylsilyl.** *1077700.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl groups.

**Silica gel for chromatography, octylsilyl R1.** *1077701.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl and methyl groups (double bonded phase).

**Silica gel for chromatography, octylsilyl R2.** *1077702.*

Ultrapure very finely divided (10 nm pore size) silica gel, chemically modified at the surface by the bonding of octylsilyl groups (19 per cent carbon load). Less than 20 ppm of metals.

**Silica gel for chromatography, octylsilyl R3.** *1155200.*

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octylsilyl groups and sterically protected with branched hydrocarbons at the silanes.

**Silica gel for chromatography, octylsilyl, base-deactivated.** *1131600.*

A very finely divided silica gel, pretreated before the bonding of octylsilyl groups by careful washing and hydrolysing most of the superficial siloxane bridges to minimise the interaction with basic components.

**Silica gel for chromatography, octylsilyl, end-capped.** *1119600.*

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octylsilyl, end-capped, base-deactivated.** *1148800.*

A very finely divided silica gel, pre-treated before the bonding of octylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octylsilyl, extra-dense bonded, end-capped.** *1200900.*

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, octylsilyl, with polar incorporated groups, end-capped.** *1152600.*

A very finely divided silica gel. The particles are based on silica, chemically modified with a reagent providing a surface with chains having polar incorporated groups and terminating octyl groups. Furthermore, the packing material is end-capped.

**Silica gel for chromatography, oxypropionitrilsilyl.** *1184700.*

A very finely divided silica gel chemically modified at the surface by the bonding of oxypropionitrilsilyl groups.

**Silica gel for chromatography, palmitamidopropylsilyl, end-capped.** *1161900.*

A very finely divided silica gel, chemically modified at the surface by the bonding of palmitamidopropyl groups and end-capped with acetamidopropyl groups.

**Silica gel for chromatography, pentafluorophenylpropylsilyl, solid core, end-capped.** *1207600.*

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with pentafluorophenylpropylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, phenylhexylsilyl.** *1153900.*

A very finely divided silica gel, chemically modified at the surface by the bonding of phenylhexyl groups.

**Silica gel for chromatography, phenylhexylsilyl, end-capped.** *1170600.*

A very finely divided silica gel, chemically modified at the surface by the bonding of phenylhexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, phenylhexylsilyl, solid core, end-capped.** *1198900.*

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with phenylhexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, phenylsilyl.** *1110200.*

A very finely divided silica gel, chemically modified at the surface by the bonding of phenyl groups.

**Silica gel for chromatography, phenylsilyl, end-capped.** *1154900.*

A very finely divided silica gel, chemically modified at the surface by the bonding of phenyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, phenylsilyl, end-capped, base-deactivated.** *1197900.*

A very finely divided silica gel pre-treated before the bonding of phenylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges, chemically modified at the surface by bonding of phenyl groups. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, phenylsilyl, extra-dense bonded, end-capped.** *1207700.*

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of phenylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

**Silica gel for chromatography, propoxybenzene, end-capped.** *1174600.*

A very finely divided silica gel, chemically modified at the surface by the bonding of propoxybenzene groups.

**Silica gel for chromatography, propylsilyl.** *1170700.*

A very finely divided silica gel, chemically modified at the surface by the bonding of propylsilyl groups.

**Silica gel for chromatography, strong-anion-exchange.** *1077800.*

A very finely divided silica gel, chemically modified at the surface by the bonding of quaternary ammonium groups.

pH limit of use: 2 to 8.

**Silica gel for chromatography, strong cation-exchange.** *1161400.*

A very finely divided silica gel, chemically modified at the surface by the bonding of sulfonic acid groups.

**Silica gel for chromatography, trimethylsilyl.** *1115500.*

A very finely divided silica gel, chemically modified at the surface by the bonding of trimethylsilyl groups.

**Silica gel for size-exclusion chromatography.** *1077900.*

A very finely divided silica gel (10 µm) with a very hydrophilic surface. The average diameter of the pores is about 30 nm. It is compatible with aqueous solutions between pH 2 and 8 and with organic solvents. It is suitable for the separation of proteins with relative molecular masses of $1 \times 10^3$ to $3 \times 10^5$.

**Silica gel G.** *1076300.* [112926-00-8].

Contains about 13 per cent of calcium sulfate hemihydrate. The particle size is about 15 µm.

*Calcium sulfate content.* Place 0.25 g in a ground-glass stoppered flask, add 3 mL of *dilute hydrochloric acid R* and 100 mL of *water R* and shake vigorously for 30 min. Filter through a sintered-glass filter (*2.1.2*) and wash the residue. Carry out on the combined filtrate and washings the complexometric assay of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 14.51 mg of $CaSO_4,{}^1/_2H_2O$.

*pH* (*2.2.3*). Shake 1 g for 5 min with 10 mL of *carbon dioxide-free water R*. The pH of the suspension is about 7.

**Silica gel $GF_{254}$.** *1076400.* [112926-00-8].

Contains about 13 per cent of calcium sulfate hemihydrate and about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm. The particle size is about 15 µm.

*Calcium sulfate content.* Determine by the method prescribed for *silica gel G R*.

*pH.* Complies with the test prescribed for *silica gel G R*.

*Fluorescence.* Thin-layer chromatography (*2.2.27*) using *silica gel $GF_{254}$ R* as the coating substance. Apply separately to the plate at ten points increasing volumes from 1 µL to 10 µL of a 1 g/L solution of *benzoic acid R* in a mixture of 10 volumes of *anhydrous formic acid R* and 90 volumes of *2-propanol R*. Develop over a path of 10 cm with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for quantities of 2 µg and greater.

**Silica gel H.** *1076500.* [112926-00-8].

The particle size is of about 15 µm.

*pH (2.2.3).* Complies with the test prescribed for *silica gel G R*.

**Silica gel H, silanised.** *1076600.*

*Preparation of a thin layer.* See *silanised silica gel $HF_{254}$ R*

*Chromatographic separation.* Complies with the test prescribed for *silanised silica gel $HF_{254}$ R*.

**Silica gel $HF_{254}$.** *1076700.*

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm. The particle size is about 15 µm.

*pH.* Complies with the test prescribed for *silica gel G R*.

*Fluorescence.* Complies with the test prescribed for *silica gel $GF_{254}$ R*.

**Silica gel $HF_{254}$, silanised.** *1076800.*

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm.

*Preparation of a thin layer.* Vigorously shake 30 g for 2 min with 60 mL of a mixture of 1 volume of *methanol R* and 2 volumes of *water R*. Coat carefully cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air and then heat in an oven at 100 °C to 105 °C for 30 min.

*Chromatographic separation.* Introduce 0.1 g each of *methyl laurate R*, *methyl myristate R*, *methyl palmitate R* and *methyl stearate R* into a 250 mL conical flask. Add 40 mL of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 mL of *water R*, acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantities, each of 10 mL of *chloroform R*. Dry the combined chloroform extracts over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 mL of *chloroform R*. Examine by thin-layer chromatography (*2.2.27*), using silanised silica gel $HF_{254}$ as the coating substance. Apply to the plate at each of three separate points 10 µL of the chloroformic solution. Develop over a path of 14 cm with a mixture of 10 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *dioxan R*. Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible.

Treat the plate with ammonia vapour until the background is white. The chromatograms show four clearly separated, well-defined spots.

**Silicotungstic acid.** $H_4SiW_{12}O_{40},xH_2O$. *1078000.* [11130-20-4].

White or yellowish-white crystals, deliquescent, very soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Silicristin.** $C_{25}H_{22}O_{10}$. ($M_r$ 482.4). *1151500.* [33889-69-9]. (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*S*)-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1-benzofuran-5-yl]chroman-4-one.

White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

**Silidianin.** $C_{25}H_{22}O_{10}$. ($M_r$ 482.4). *1151600.* [29782-68-1]. (3*R*,3a*R*,6*R*,7a*R*,8*R*)-7a-Hydroxy-8-(4-hydroxy-3-methoxyphenyl)-4-[(2*R*, 3*R*)-3,5,7-trihydroxy-4-oxochroman-2-yl]-2,3,3a,7a-tetrahydro-3,6-methano-1-benzofuran-7(6a*H*)-one.

White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

**Silver diethyldithiocarbamate.** $C_5H_{10}AgNS_2$. ($M_r$ 256.1). *1110400.* [1470-61-7]. Silver diethylcarbamodithioate.

Pale-yellow or greyish-yellow powder, practically insoluble in water, soluble in pyridine.

Storage below 8 °C is recommended.

It may be prepared as follows. Dissolve 1.7 g of *silver nitrate R* in 100 mL of *water R*. Separately dissolve 2.3 g of *sodium diethyldithiocarbamate R* in 100 mL of *water R*. Cool both solutions to 10 °C, then mix, and while stirring, collect the yellow precipitate on a sintered-glass filter (16) (*2.1.2*) and wash with 200 mL of cold *water R*. Dry the precipitate *in vacuo* for 10 h (*2.2.32*).

**Silver diethyldithiocarbamate solution.** *1110401.*

*Prepare the solution immediately before use.* Dissolve 0.100 g of *silver diethyldithiocarbamate R* in *pyridine R* and dilute to 20.0 mL with the same solvent.

*Suitability test.* The solution is clear (*2.2.1*). The absorbance (*2.2.25*) of the solution is maximum 0.20 at 450 nm, maximum 0.01 at 510 nm and maximum 0.010 at 538 nm.

**Silver manganese paper.** *1078200.*

Immerse strips of slow filter paper into a solution containing 8.5 g/L of *manganese sulfate R* and 8.5 g/L of *silver nitrate R*. Maintain for a few minutes and allow to dry over an appropriate desiccant, protected from acid and alkaline vapours.

**Silver nitrate.** *1078300.* [7761-88-8].

See *Silver nitrate (0009)*.

**Silver nitrate reagent.** *1078305.*

*Prepare immediately before use.* To a mixture of 3 mL of *concentrated ammonia R* and 40 mL of *1 M sodium hydroxide*, add 8 mL of a 200 g/L solution of *silver nitrate R*, dropwise, with stirring. Dilute to 200 mL with *water R*.

**Silver nitrate solution R1.** *1078301.*

A 42.5 g/L solution of *silver nitrate R*.

*Storage*: protected from light.

**Silver nitrate solution R2.** *1078302.*

A 17 g/L solution of *silver nitrate R*.

*Storage*: protected from light.

**Silver nitrate solution, ammoniacal.** *1078303.*

Dissolve 2.5 g of *silver nitrate R* in 80 mL of *water R* and add *dilute ammonia R1* dropwise until the precipitate has dissolved. Dilute to 100 mL with *water R*. Prepare immediately before use.

**Silver nitrate solution in pyridine.** *1078304.*

An 85 g/L solution of *silver nitrate R* in *pyridine R*.

*Storage*: protected from light.

**Silver oxide.** $Ag_2O$. ($M_r$ 231.7). *1078400.* [20667-12-3]. Disilver oxide.

Brownish-black powder, practically insoluble in water and in ethanol (96 per cent), freely soluble in dilute nitric acid and in ammonia.

*Storage*: protected from light.

**Silver sulfate.** $Ag_2SO_4$. ($M_r$ 311.8). *1201000.* [10294-26-5].

*Content*: minimum 99.0 per cent.

White or light grey powder, slightly soluble in water.

mp: about 652 °C.

*Storage*: protected from light.

**Sinensetin.** $C_{20}H_{20}O_7$. ($M_r$ 372.4). *1110500.* [2306-27-6]. 3′,4′,5,6,7-Pentamethoxyflavone.

White or almost white, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 177 °C.

*Absorbance* (*2.2.25*). A solution in *methanol R* shows 3 absorption maxima, at 243 nm, 268 nm and 330 nm.

*Assay.* Liquid chromatography (*2.2.29*) as prescribed in the monograph *Java tea (1229)*.

*Content*: minimum 95 per cent, calculated by the normalisation procedure.

**Sinomenine.** $C_{19}H_{23}NO_4$. ($M_r$ 329.4). *1183400.* [115-53-7]. 7,8-Didehydro-4-hydroxy-3,7-dimethoxy-17-methyl-9α,13α,14α-morphinan-6-one. Cucoline.

**Sirolimus.** $C_{51}H_{79}NO_{13}$. ($M_r$ 914). *1205700.* [53123-88-9]. Rapamycin.

mp: 183 °C to 185 °C.

**Sitostanol.** $C_{29}H_{52}O$. ($M_r$ 416.7). *1140100.* [19466-47-8]. Dihydro-β-sitosterol.

*Content*: minimum 95.0 per cent.

**β-Sitosterol.** $C_{29}H_{50}O$. ($M_r$ 414.7). *1140200.* [83-46-5]. Stigmast-5-en-3β-ol. 22,23-Dihydrostigmasterol.

White or almost white powder, practically insoluble in water, sparingly soluble in tetrahydrofuran.

*Content*: minimum 75.0 per cent *m/m* (dried substance).

*Assay.* Gas chromatography (*2.2.28*), as prescribed in the monograph *Phytosterol (1911)*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in *tetrahydrofuran R* and dilute to 10.0 mL with the same solvent. Introduce 100 µL of this solution into a suitable 3 mL flask and evaporate to dryness under *nitrogen R*. To the residue add 100 µL of a freshly prepared mixture of 50 µL of *1-methylimidazole R* and 1.0 mL of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat at 100 °C for 15 min. Allow to cool.

*Injection*: 1 µL of the test solution.

**Sodium.** Na. ($A_r$ 22.99). *1078500.* [7440-23-5].

A metal whose freshly cut surface is bright silver-grey. It rapidly tarnishes in contact with air and is oxidised completely to sodium hydroxide and converted to sodium carbonate. It reacts violently with water, yielding hydrogen and a solution

of sodium hydroxide; soluble in anhydrous methanol, yielding hydrogen and a solution of sodium methoxide; practically insoluble in light petroleum.

*Storage*: under light petroleum or liquid paraffin.

**Sodium acetate.** *1078600.* [6131-90-4].

See *Sodium acetate trihydrate (0411)*.

**Sodium acetate, anhydrous.** $C_2H_3NaO_2$. ($M_r$ 82.0). *1078700.* [127-09-3].

Colourless crystals or granules, very soluble in water, sparingly soluble in ethanol (96 per cent).

*Loss on drying* (*2.2.32*). Not more than 2.0 per cent, determined by drying in an oven at 105 °C.

**Sodium arsenite.** $NaAsO_2$. ($M_r$ 129.9). *1165900.* [7784-46-5]. Sodium metaarsenite.

**Sodium arsenite solution.** *1165901.*

Dissolve 5.0 g of *sodium arsenite R* in 30 mL of *1 M sodium hydroxide*. Cool to 0 °C and add, while stirring, 65 mL of *dilute hydrochloric acid R*.

**Sodium ascorbate solution.** *1078800.* [134-03-2].

Dissolve 3.5 g of *ascorbic acid R* in 20 mL of *1 M sodium hydroxide*. Prepare immediately before use.

**Sodium azide.** $NaN_3$. ($M_r$ 65.0). *1078900.* [26628-22-8].

White or almost white, crystalline powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Sodium benzenesulfonate.** $C_6H_5SO_3Na$. ($M_r$ 180.16). *1196600.* [515-42-4].

White crystalline powder, soluble in water.

**Sodium bicarbonate.** *1081300.* [144-55-8].

See *sodium hydrogen carbonate R*.

**Sodium bismuthate.** $NaBiO_3$. ($M_r$ 280.0). *1079000.* [12232-99-4].

*Content*: minimum 85.0 per cent.

Yellow or yellowish-brown powder, slowly decomposing when moist or at a high temperature, practically insoluble in cold water.

*Assay*. Suspend 0.200 g in 10 mL of a 200 g/L solution of *potassium iodide R* and add 20 mL of *dilute sulfuric acid R*. Using 1 mL of *starch solution R* as indicator, titrate with *0.1 M sodium thiosulfate* until an orange colour is obtained.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 14.00 mg of $NaBiO_3$.

**Sodium bromide.** *1154300.* [7647-15-6].

See *Sodium bromide (0190)*.

**Sodium butanesulfonate.** $C_4H_9NaO_3S$. ($M_r$ 160.2). *1115600.* [2386-54-1].

White or almost white, crystalline powder, soluble in water.

mp: greater than 300 °C.

**Sodium calcium edetate.** *1174000.* [62-33-9].

See *sodium calcium edetate (0231)*.

**Sodium carbonate.** *1079200.* [6132-02-1].

See *Sodium carbonate decahydrate (0191)*.

**Sodium carbonate, anhydrous.** $Na_2CO_3$. ($M_r$ 106.0). *1079300.* [497-19-8]. Disodium carbonate.

White or almost white powder, hygroscopic, freely soluble in water.

When heated to about 300 °C it loses not more than 1 per cent of its mass.

*Storage*: in an airtight container.

**Sodium carbonate solution.** *1079301.*

A 106 g/L solution of *anhydrous sodium carbonate R*.

**Sodium carbonate solution R1.** *1079302.*

A 20 g/L solution of *anhydrous sodium carbonate R* in *0.1 M sodium hydroxide*.

**Sodium carbonate solution R2.** *1079303.*

A 40 g/L solution of *anhydrous sodium carbonate R* in *0.2 M sodium hydroxide*.

**Sodium carbonate monohydrate.** *1131700.* [5968-11-6].

See *Sodium carbonate monohydrate (0192)*.

**Sodium cetostearyl sulfate.** *1079400.*

See *Sodium cetostearyl sulfate (0847)*.

**Sodium chloride.** *1079500.* [7647-14-5].

See *Sodium chloride (0193)*.

**Sodium chloride solution.** *1079502.*

A 20 per cent *m/m* solution of *sodium chloride R*.

**Sodium chloride solution, saturated.** *1079503.*

Mix 1 part of *sodium chloride R* with 2 parts of *water R*, shake from time to time and allow to stand. Before use, decant the solution from any undissolved substance and filter, if necessary.

**Sodium citrate.** *1079600.* [6132-04-3].

See *Sodium citrate (0412)*.

**Sodium cobaltinitrite.** $Na_3[Co(NO_2)_6]$. ($M_r$ 403.9). *1079700.* [13600-98-1]. Trisodium hexanitrocobaltate(III).

Orange-yellow powder, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Sodium cobaltinitrite solution.** *1079701.*

A 100 g/L solution of *sodium cobaltinitrite R*. Prepare immediately before use.

**Sodium decanesulfonate.** $C_{10}H_{21}NaO_3S$. ($M_r$ 244.3). *1079800.* [13419-61-9].

Crystalline powder or flakes, white or almost white, freely soluble in water, soluble in methanol.

**Sodium decyl sulfate.** $C_{10}H_{21}NaO_4S$. ($M_r$ 260.3). *1138600.* [142-87-0].

*Content*: minimum 95.0 per cent.

White or almost white powder, freely soluble in water.

**Sodium deoxycholate.** $C_{24}H_{39}NaO_4$. ($M_r$ 414.6). *1131800.* [302-95-4]. Sodium 3α,12α-dihydroxy-5β-cholan-24-oate.

**Sodium deoxyribonucleate.** (About 85 per cent has a relative molecular mass of $2 \times 10^7$ or greater). *1079900.* [73049-39-5].

White or almost white, fibrous preparation obtained from calf thymus.

*Test for suitability.* Dissolve 10 mg in *imidazole buffer solution pH 6.5 R* and dilute to 10.0 mL with the same buffer solution (solution A). Dilute 2.0 mL of solution A to 50.0 mL with *imidazole buffer solution pH 6.5 R*. The absorbance (*2.2.25*) of the solution, measured at 260 nm, is 0.4 to 0.8.

To 0.5 mL of solution A add 0.5 mL of *imidazole buffer solution pH 6.5 R* and 3 mL of perchloric acid (25 g/L $HClO_4$). A precipitate is formed. Centrifuge. The absorbance of the supernatant, measured at 260 nm using a mixture of 1 mL of *imidazole buffer solution pH 6.5 R* and 3 mL of perchloric acid (25 g/L $HClO_4$) as compensation liquid, is not greater than 0.3.

In each of two tubes, place 0.5 mL of solution A and 0.5 mL of a solution of a reference preparation of streptodornase containing 10 IU/mL in *imidazole buffer solution pH 6.5 R*. To one tube add immediately 3 mL of perchloric acid (25 g/L $HClO_4$). A precipitate is formed. Centrifuge and collect supernatant A. Heat the other tube at 37 °C for 15 min and add 3 mL of perchloric acid (25 g/L $HClO_4$). Centrifuge and collect supernatant B. The absorbance of supernatant B, measured at 260 nm with reference to supernatant A is not less than 0.15.

**Sodium diethyldithiocarbamate.** $C_5H_{10}NNaS_2,3H_2O$. ($M_r$ 225.3). *1080000.* [20624-25-3].
White or almost white or colourless crystals, freely soluble in water, soluble in ethanol (96 per cent). The aqueous solution is colourless.

**Sodium dihydrogen phosphate.** *1080100.* [13472-35-0].
See *Sodium dihydrogen phosphate dihydrate (0194)*.

**Sodium dihydrogen phosphate, anhydrous.** $NaH_2PO_4$. ($M_r$ 120.0). *1080200.* [7558-80-7].
White or almost white powder, hygroscopic.
*Storage*: in an airtight container.

**Sodium dihydrogen phosphate monohydrate.** $NaH_2PO4,H_2O$. ($M_r$ 138.0). *1080300.* [10049-21-5].
White or almost white, slightly deliquescent crystals or granules, freely soluble in water, practically insoluble in ethanol (96 per cent).
*Storage*: in an airtight container.

**Sodium dioctyl sulfosuccinate.** $C_{20}H_{37}NaO_7S$. ($M_r$ 444.6). *1170800.* [577-11-7]. Sodium 1,4-bis[(2-ethylhexyl)oxy]-1,4-dioxobutane-2-sulfonate. 1,4-Bis(2-ethylhexyl) sulfobutanedioate sodium salt.
White or almost white, waxy solid.

**Sodium dithionite.** $Na_2S_2O_4$. ($M_r$ 174.1). *1080400.* [7775-14-6].
White or greyish-white, crystalline powder, oxidises in air, very soluble in water, slightly soluble in ethanol (96 per cent).
*Storage*: in an airtight container.

**Sodium dodecyl sulfate.** *1080500.* [151-21-3].
See *Sodium laurilsulfate (0098)*.
*Content*: minimum 99.0 per cent.

**Sodium edetate.** *1080600.* [6381-92-6].
See *Disodium edetate (0232)*.

**Sodium fluoresceinate.** $C_{20}H_{10}Na_2O_5$. ($M_r$ 376.3). *1080700.* [518-47-8].
Schultz No. 880.
Colour Index No. 45350.
Fluorescein sodium. Disodium 2-(3-oxo-6-oxido-3H-xanthen-9-yl)benzoate.
Orange-red powder, freely soluble in water. Aqueous solutions display an intense yellowish-green fluorescence.

**Sodium fluoride.** *1080800.* [7681-49-4].
See *Sodium fluoride (0514)*.

**Sodium formate.** $CHNaO_2$. ($M_r$ 68.0). *1122200.* [141-53-7]. Sodium methanoate.
White or almost white, crystalline powder or deliquescent granules, soluble in water and in glycerol, slightly soluble in ethanol (96 per cent).
mp: about 253 °C.

**Sodium glucuronate.** $C_6H_9NaO_7,H_2O$. ($M_r$ 234.1). *1080900.* Sodium D-glucuronate monohydrate.
$[\alpha]_D^{20}$: about + 21.5, determined on a 20 g/L solution.

**Sodium glycocholate.** $C_{26}H_{42}NNaO_6,2H_2O$. ($M_r$ 523.6). *1155500.* [207300-80-9]. Sodium [(3,7,12-trihydroxy-5-cholan-24-oyl)amino]acetate dihydrate. *N*-[(3,5,7,12)-3,7,12-Trihydroxy-24-oxocholan-24-yl]glycine monosodium salt dihydrate.
*Content*: minimum 97 per cent of $C_{26}H_{42}NNaO_6,2H_2O$.

**Sodium heptanesulfonate.** $C_7H_{15}NaO_3S$. ($M_r$ 202.3). *1081000.* [22767-50-6].
White or almost white, crystalline mass, freely soluble in water, soluble in methanol.

**Sodium heptanesulfonate monohydrate.** $C_7H_{15}NaO_3S,H_2O$. ($M_r$ 220.3). *1081100.*
*Content*: minimum 96 per cent (anhydrous substance).
White or almost white, crystalline powder, soluble in water, very slightly soluble in anhydrous ethanol.
*Water* (*2.5.12*): maximum 8 per cent, determined on 0.300 g.
*Assay.* Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).
1 mL of *0.1 M perchloric acid* is equivalent to 20.22 mg of $C_7H_{15}NaO_3S$.

**Sodium hexanesulfonate.** $C_6H_{13}NaO_3S$. ($M_r$ 188.2). *1081200.* [2832-45-3].
White or almost white powder, freely soluble in water.

**Sodium hexanesulfonate monohydrate.** $C_6H_{13}NaO_3S,H_2O$. ($M_r$ 206.2). *1161500.* [207300-91-2].
White or almost white powder, soluble in water.

**Sodium hexanesulfonate monohydrate for ion-pair chromatography.** $C_6H_{13}NaO_3S,H_2O$. ($M_r$ 206.2). *1182300.* [207300-91-2].
*Content*: minimum 99.0 per cent.

**Sodium hydrogen carbonate.** *1081300.* [144-55-8].
See *Sodium hydrogen carbonate (0195)*.

**Sodium hydrogen carbonate solution.** *1081301.*
A 42 g/L solution of *sodium hydrogen carbonate R*.

**Sodium hydrogen sulfate.** $NaHSO_4$. ($M_r$ 120.1). *1131900.* [7681-38-1]. Sodium bisulfate.
Freely soluble in water, very soluble in boiling water. It decomposes in ethanol (96 per cent) into sodium sulfate and free sulfuric acid.
mp: about 315 °C.

**Sodium hydrogensulfite.** $NaHO_3S$. ($M_r$ 104.1). *1115700.* [7631-90-5].
White or almost white, crystalline powder, freely soluble in water, sparingly soluble in ethanol (96 per cent).
On exposure to air, some sulfur dioxide is lost and the substance is gradually oxidated to sulfate.

**Sodium hydroxide.** *1081400.* [1310-73-2].
See *Sodium hydroxide (0677)*.

**2 M Sodium hydroxide.** *3009800.*
Dissolve 84 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

**4 M Sodium hydroxide.** *1081407.*
Dissolve 168 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1.0 L with the same solvent.

**Sodium hydroxide solution.** *1081401.*
Dissolve 20.0 g of *sodium hydroxide R* in *water R* and dilute to 100.0 mL with the same solvent. Verify the concentration by titration with *1 M hydrochloric acid*, using *methyl orange solution R* as indicator, and adjust if necessary to 200 g/L.

**Sodium hydroxide solution, carbonate-free.** *1081406.*
Dissolve *sodium hydroxide R* in *carbon dioxide-free water R* to give a concentration of 500 g/L and allow to stand. Decant the clear supernatant, taking precautions to avoid the introduction of carbon dioxide.

**Sodium hydroxide solution, dilute.** *1081402.*
Dissolve 8.5 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

**Sodium hydroxide solution, methanolic.** *1081403.*
Dissolve 40 mg of *sodium hydroxide R* in 50 mL of *water R*. Cool and add 50 mL of *methanol R*.

**Sodium hydroxide solution, methanolic R1.** *1081405.*

Dissolve 200 mg of *sodium hydroxide R* in 50 mL of *water R*. Cool and add 50 mL of *methanol R*.

**Sodium hydroxide solution, strong.** *1081404.*

Dissolve 42 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

**Sodium 2-hydroxybutyrate.** $C_4H_7NaO_3$. ($M_r$ 126.1). *1158800.* [19054-57-0]. Sodium (2*RS*)-2-hydroxybutanoate.

**Sodium hypobromite solution.** *1081500.*

In a bath of iced water mix 20 mL of *strong sodium hydroxide solution R* and 500 mL of *water R*, add 5 mL of *bromine solution R* and stir gently until solution is complete. Prepare immediately before use.

**Sodium hypochlorite solution, strong.** *1081600.*

*Content*: 25 g/L to 30 g/L of active chlorine.

Yellowish liquid with an alkaline reaction.

*Assay.* Introduce into a flask, successively, 50 mL of *water R*, 1 g of *potassium iodide R* and 12.5 mL of *dilute acetic acid R*. Dilute 10.0 mL of the substance to be examined to 100.0 mL with *water R*. Introduce 10.0 mL of this solution into the flask and titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 3.546 mg of active chlorine.

*Storage*: protected from light.

**Sodium hypophosphite.** $NaH_2PO_2,H_2O$. ($M_r$ 106.0). *1081700.* [10039-56-2]. Sodium phosphinate monohydrate.

White or almost white, crystalline powder or colourless crystals, hygroscopic, freely soluble in water, soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Sodium iodide.** *1081800.* [7681-82-5].

See *Sodium iodide (0196)*.

**Sodium laurilsulfate.** *1081900.* [151-21-3].

See *Sodium laurilsulfate (0098)*.

**Sodium lauryl sulfate.** *1081900.* [151-21-3].

See *Sodium laurilsulfate R*.

**Sodium laurylsulfonate for chromatography.** $C_{12}H_{25}NaO_3S$. ($M_r$ 272.4). *1132000.* [2386-53-0].

White or almost white powder or crystals, freely soluble in water.

Absorbance $A_{1\,cm}^{5\%}$ (*2.2.25*), determined in *water R*: about 0.05 at 210 nm; about 0.03 at 220 nm; about 0.02 at 230 nm; about 0.02 at 500 nm.

**Sodium metabisulfite.** *1082000.* [7681-57-4].

See *Sodium metabisulfite (0849)*.

**Sodium methanesulfonate.** $CH_3SO_3Na$. ($M_r$ 118.1). *1082100.* [2386-57-4].

White or almost white, crystalline powder, hygroscopic.

*Storage*: in an airtight container.

**Sodium molybdate.** $Na_2MoO_4,2H_2O$. ($M_r$ 242.0). *1082200.* [10102-40-6]. Disodium molybdate dihydrate.

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

**Sodium naphthoquinonesulfonate.** $C_{10}H_5NaO_5S$. ($M_r$ 260.2). *1082300.* [521-24-4]. Sodium 1,2-naphthoquinone-4-sulfonate.

Yellow or orange-yellow, crystalline powder, freely soluble in water, practically insoluble in ethanol (96 per cent).

**Sodium nitrate.** $NaNO_3$. ($M_r$ 85.0). *1082400.* [7631-99-4].

White or almost white powder or granules or colourless, transparent crystals, deliquescent in moist air, freely soluble in water, slightly soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Sodium nitrite.** $NaNO_2$. ($M_r$ 69.0). *1082500.* [7632-00-0].

*Content*: minimum 97.0 per cent.

White or almost white, granular powder or a slightly yellow, crystalline powder, freely soluble in water.

*Assay.* Dissolve 0.100 g in 50 mL of *water R*. Add 50.0 mL of *0.02 M potassium permanganate* and 15 mL of *dilute sulfuric acid R*. Add 3 g of *potassium iodide R*. Titrate with *0.1 M sodium thiosulfate*, using 1.0 mL of *starch solution R* added towards the end of the titration as indicator.

1 mL of *0.02 M potassium permanganate* is equivalent to 3.450 mg of $NaNO_2$.

**Sodium nitrite solution.** *1082501.*

A 100 g/L solution of *sodium nitrite R*. Prepare immediately before use.

**Sodium nitroprusside.** $Na_2[Fe(CN)_5(NO)],2H_2O$. ($M_r$ 298.0). *1082600.* [13755-38-9]. Sodium pentacyano-nitrosylferrate(III) dihydrate.

Reddish-brown powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

**Sodium octanesulfonate.** $C_8H_{17}NaO_3S$. ($M_r$ 216.3). *1082700.* [5324-84-5].

*Content*: minimum 98.0 per cent.

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

*Absorbance* (*2.2.25*): maximum 0.10, determined at 200 nm and maximum 0.01, determined at 250 nm using a 54 g/L solution.

**Sodium octanesulfonate monohydrate.** $C_8H_{17}NaO_3S,H_2O$. ($M_r$ 234.3). *1176700.* [207596-29-0].

White or almost white powder.

**Sodium octyl sulfate.** $C_8H_{17}NaO_4S$. ($M_r$ 232.3). *1082800.* [142-31-4].

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

**Sodium oxalate.** $C_2Na_2O_4$. ($M_r$ 134.0). *1082900.* [62-76-0].

White or almost white, crystalline powder, soluble in water, practically insoluble in ethanol (96 per cent).

**Sodium oxidronate.** $CH_4Na_2O_7P_2$. ($M_r$ 236.0). *1194000.* [14255-61-9]. Sodium hydroxymethylenediphosphonate.

White or almost white powder or colourless crystals, very soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

**Sodium pentanesulfonate.** $C_5H_{11}NaO_3S$. ($M_r$ 174.2). *1083000.* [22767-49-3].

White or almost white, crystalline solid, soluble in water.

**Sodium pentanesulfonate monohydrate.** $C_5H_{11}NaO_3S,H_2O$. ($M_r$ 192.2). *1132100.* [207605-40-1].

White or almost white crystalline solid, soluble in water.

**Sodium pentanesulfonate monohydrate R1.** $C_5H_{11}NaO_3S,H_2O$. ($M_r$ 192.2). *1172500.* [207605-40-1].

*Content*: minimum 99 per cent of $C_5H_{11}NaO_3S,H_2O$.

**Sodium perchlorate.** $NaClO_4,H_2O$. ($M_r$ 140.5). *1083100.* [7791-07-3].

*Content*: minimum 99.0 per cent of $NaClO_4,H_2O$.

White or almost white, deliquescent crystals, very soluble in water.

*Storage*: in a well-closed container.

**Sodium periodate.** $NaIO_4$. ($M_r$ 213.9). *1083200.* [7790-28-5]. Sodium metaperiodate.

*Content*: minimum 99.0 per cent.

White or almost white, crystalline powder or crystals, soluble in water and in mineral acids.

**Sodium periodate solution.** *1083201.*

Dissolve 1.07 g of *sodium periodate R* in *water R*, add 5 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*. Use a freshly prepared solution.

**Sodium phosphite pentahydrate.** $Na_2HPO_3,5H_2O$. ($M_r$ 216.0). *1132200.* [13517-23-2].

White or almost white, crystalline powder, hygroscopic, freely soluble in water.

*Storage*: in an airtight container.

**Sodium picrate solution, alkaline.** *1083300.*

Mix 20 mL of *picric acid solution R* and 10 mL of a 50 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*.

*Storage*: use within 2 days.

**Sodium potassium tartrate.** $C_4H_4KNaO_6,4H_2O$. ($M_r$ 282.2). *1083500.* [6381-59-5].

Colourless, prismatic crystals, very soluble in water.

**Sodium 1-propanesulfonate.** $C_3H_9SO_4Na$. ($M_r$ 164.2). *1197600.* [304672-01-3]. Sodium propane-1-sulfonate monohydrate.

mp: about 250 °C.

**Sodium pyrophosphate.** $Na_4P_2O_7,10H_2O$. ($M_r$ 446.1). *1083600.* [13472-36-1]. Tetrasodium diphosphate decahydrate.

Colourless, slightly efflorescent crystals, freely soluble in water.

**Sodium pyruvate.** $C_3H_3NaO_3$. ($M_r$ 110.0). *1204300.* [113-24-6]. 2-Oxopropanoic acid sodium salt.

White or faint yellow powder, soluble in water (100 mg/mL).

mp: greater than 300 °C.

**Sodium rhodizonate.** $C_6Na_2O_6$. ($M_r$ 214.0). *1122300.* [523-21-7]. [(3,4,5,6-Tetraoxocyclohex-1-en-1,2-ylene)dioxy]disodium.

Violet crystals, soluble in water with an orange-yellow colour.

Solutions are unstable and must be prepared on the day of use.

**Sodium salicylate.** *1083700.* [54-21-7].

See *Sodium salicylate (0413)*.

**Sodium stearyl fumarate.** $C_{22}H_{39}NaO_4$. *1195100.* [4070-80-8].

See *Sodium stearyl fumarate (1567)*.

**Sodium sulfate, anhydrous.** *1083800.* [7757-82-6].

Ignite at 600 °C to 700 °C anhydrous sodium sulfate complying with the requirements prescribed in the monograph on *Anhydrous sodium sulfate (0099)*.

*Loss on drying* (*2.2.32*): maximum 0.5 per cent, determined by drying in an oven at 130 °C.

**Sodium sulfate, anhydrous R1.** *1083801.*

Complies with the requirements prescribed for *anhydrous sodium sulfate R* with the following maximum contents.

Cl: 20 ppm.

Pb: 10 ppm.

As: 3 ppm.

Ca: 50 ppm.

Fe: 10 ppm.

Mg: 10 ppm.

**Sodium sulfate decahydrate.** $Na_2SO_4,10H_2O$. ($M_r$ 322.2). *1132300.* [7727-73-3].

See *Sodium sulfate decahydrate (0100)*.

**Sodium sulfide.** $Na_2S,9H_2O$. ($M_r$ 240.2). *1083900.* [1313-84-4]. Disodium sulfide nonahydrate.

Colourless, rapidly yellowing crystals, deliquescent, very soluble in water.

*Storage*: in an airtight container.

**Sodium sulfide solution.** *1083901.*

Dissolve 12 g of *sodium sulfide R* with heating in 45 mL of a mixture of 10 volumes of *water R* and 29 volumes of *glycerol (85 per cent) R*, allow to cool and dilute to 100 mL with the same mixture of solvents.

The solution should be colourless.

**Sodium sulfide solution R1.** *1083902.*

Prepare by one of the following methods.

– Dissolve 5 g of *sodium sulfide R* in a mixture of 10 mL of *water R* and 30 mL of *glycerol R*.

– Dissolve 5 g of *sodium hydroxide R* in a mixture of 30 mL of *water R* and 90 mL of *glycerol R*. Divide the solution into 2 equal portions. Saturate 1 portion with *hydrogen sulfide R*, with cooling. Mix the 2 portions.

*Storage*: in a well-filled container, protected from light; use within 3 months.

**Sodium sulfite, anhydrous.** *1084100.* [7757-83-7].

See *Sodium sulfite (0775)*.

**Sodium sulfite heptahydrate.** *1084000.* [10102-15-5].

See *Sodium sulfite heptahydrate (0776)*.

**Sodium tartrate.** $C_4H_4Na_2O_6,2H_2O$. ($M_r$ 230.1). *1084200.* [6106-24-7]. Disodium (2*R*,3*R*)-2,3-dihydroxybutanedioate dihydrate.

White or almost white crystals or granules, very soluble in water, practically insoluble in ethanol (96 per cent).

**Sodium taurodeoxycholate.** $C_{26}H_{44}NNaO_6S,H_2O$. ($M_r$ 539.7). *1155600.* [110026-03-4]. Sodium 2-[(3,12-dihydroxy-5-cholan-24-oyl)amino]ethanesulfonate monohydrate. 2-[[(3,5,12)-3,12-Dihydroxy-24-oxocholan-24-yl]amino]ethanesulfonic acid monosodium salt monohydrate.

*Content*: minimum 94 per cent of $C_{26}H_{44}NNaO_6S,H_2O$.

**Sodium tetrahydroborate.** $NaBH_4$. ($M_r$ 37.8). *1146900.* [16940-66-2]. Sodium borohydride.

Colourless, hygroscopic crystals, freely soluble in water, soluble in anhydrous ethanol, decomposing at higher temperature or in the presence of acids or certain metal salts forming borax and hydrogen.

*Storage*: in an airtight container.

**Sodium tetrahydroborate reducing solution.** *1146901.*

Introduce about 100 mL of *water R* into a 500 mL volumetric flask containing a stirring bar. Add 5.0 g of *sodium hydroxide R* in pellets and 2.5 g of *sodium tetrahydroborate R*. Stir until complete dissolution, dilute to 500.0 mL with *water R* and mix. Prepare immediately before use.

**Sodium tetraphenylborate.** $NaB(C_6H_5)_4$. ($M_r$ 342.2). *1084400.* [143-66-8].

White or slightly yellowish, bulky powder, freely soluble in water and in acetone.

**Sodium tetraphenylborate solution.** *1084401.*

Filter before use if necessary.

A 10 g/L solution of *sodium tetraphenylborate R*.

*Storage*: use within 1 week.

**Sodium thioglycollate.** $C_2H_3NaO_2S$. ($M_r$ 114.1). *1084500.* [367-51-1]. Sodium mercaptoacetate.

White or almost white, granular powder or crystals, hygroscopic, freely soluble in water and in methanol, slightly soluble in ethanol (96 per cent).

*Storage*: in an airtight container.

**Sodium thiosulfate.** *1084600.* [10102-17-7].

See *Sodium thiosulfate (0414)*.

**Sodium thiosulfate, anhydrous.** $Na_2S_2O_3$. ($M_r$ 158.1). *1180700.* [7772-98-7]. Disodium thiosulfate.

*Content*: minimum 98.0 per cent.

**Sodium tungstate.** $Na_2WO_4,2H_2O$. ($M_r$ 329.9). *1084700.* [10213-10-2]. Disodium tungstate dihydrate.

White or almost white, crystalline powder or colourless crystals, freely soluble in water forming a clear solution, practically insoluble in ethanol (96 per cent).

**Sorbitol.** *1084800.* [50-70-4].

See *Sorbitol (0435)*.

**Soya bean lecithin.** *1196400.* [8030-76-0].

**Soya-bean oil, refined.** *1201500.*

See *Soya-bean oil, refined (1473)*.

**Sphingomyelin from egg yolk.** *1199100.* [85187-10-6].

(2*R*,3*S*,4*E*)-2-(Acylamino)-3-hydroxyoctadec-4-en-1-yl 2-(trimethylazaniumyl)ethyl phosphate.

**Squalane.** $C_{30}H_{62}$. ($M_r$ 422.8). *1084900.* [111-01-3]. (6*Ξ*,10*Ξ*,15*Ξ*,19*Ξ*)-2,6,10,15,19,23-Hexamethyltetracosane. Perhydrosqualene.

Colourless, oily liquid, freely soluble in fatty oils, slightly soluble in acetone, in ethanol (96 per cent), in glacial acetic acid and in methanol.

$d_{20}^{20}$: 0.811 to 0.813.

$n_D^{20}$: 1.451 to 1.453.

**Stannous chloride.** $SnCl_2,2H_2O$. ($M_r$ 225.6). *1085000.* [10025-69-1]. Tin dichloride dihydrate.

*Content*: minimum 97.0 per cent of $SnCl_2,2H_2O$.

Colourless crystals, very soluble in water, freely soluble in ethanol (96 per cent), in glacial acetic acid and in dilute and concentrated hydrochloric acid.

*Assay.* Dissolve 0.500 g in 15 mL of *hydrochloric acid R* in a ground-glass-stoppered flask. Add 10 mL of *water R* and 5 mL of *chloroform R*. Titrate rapidly with *0.05 M potassium iodate* until the chloroform layer is colourless.

1 mL of *0.05 M potassium iodate* is equivalent to 22.56 mg of $SnCl_2,2H_2O$.

**Stannous chloride solution.** *1085001.*

Heat 20 g of *tin R* with 85 mL of *hydrochloric acid R* until no more hydrogen is released. Allow to cool.

*Storage*: over an excess of *tin R*, protected from air.

**Stannous chloride solution R1.** *1085002.*

Immediately before use, dilute 1 volume of *stannous chloride solution R* with 10 volumes of *dilute hydrochloric acid R*.

**Stannous chloride solution R2.** *1085003.*

To 8 g of *stannous chloride R* add 100 mL of a 20 per cent *V/V* solution of *hydrochloric acid R*. Shake until dissolved, heating, if necessary, on a water-bath at 50 °C. Pass a current of *nitrogen R* for 15 min. Prepare immediately before use.

**Stanolone.** $C_{19}H_{30}O_2$. ($M_r$ 290.4). *1154400.* [521-18-6]. 17β-Hydroxy-5α-androstan-3-one.

White or almost white powder.

mp: about 180 °C.

**Standard solution for the micro determination of water.** *1147300.*

Commercially available standard solution for the coulometric titration of water, containing a certified content of water in a suitable solvent.

***Staphylococcus aureus* strain V8 protease, type XVII-B.** *1115800.* [66676-43-5].

Microbial extracellular proteolytic enzyme. A lyophilised powder containing 500 units to 1000 units per milligram of solid.

**Starch, soluble.** *1085100.* [9005-84-9].

White or almost white powder.

**Starch iodate paper.** *1085101.*

Immerse strips of filter paper in 100 mL of *iodide-free starch solution R* containing 0.1 g of *potassium iodate R*. Drain and allow to dry protected from light.

**Starch iodide paper.** *1085106.*

Immerse strips of filter paper in 100 mL of *potassium iodide and starch solution R*. Drain and allow to dry protected from light.

*Test for sensitivity.* Mix 0.05 mL of *0.1 M sodium nitrite* with 4 mL of *hydrochloric acid R* and dilute to 100 mL with *water R*. Apply one drop of the solution to starch iodide paper; a blue spot appears.

**Starch solution.** *1085103.*

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R* and whilst stirring pour the mixture into 100 mL of boiling *water R* containing 10 mg of *mercuric iodide R*.

*NOTE*: commercially available reagents may be used; including mercury-free solutions or those containing alternative preservatives.

Carry out the test for sensitivity each time the reagent is used.

*Test for sensitivity.* To a mixture of 1 mL of the starch solution and 20 mL of *water R*, add about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1*. The solution is blue.

**Starch solution, iodide-free.** *1085104.*

Prepare the solution as prescribed for *starch solution R* omitting the mercuric iodide. Prepare immediately before use.

**Starch solution R1.** *1085105.*

Mix 1 g of *soluble starch R* and a small amount of cold *water R*. Add this mixture, while stirring, to 200 mL of boiling *water R*. Add 0.25 g of *salicylic acid R* and boil for 3 min. Immediately remove from the heat and cool.

*Storage*: if long storage is required, the solution shall be stored at 4 °C to 10 °C. A fresh starch solution shall be prepared when the end-point of the titration from blue to colourless fails to be sharp. If stored under refrigeration, the starch solution is stable for about 2 to 3 weeks.

*Test for sensitivity.* A mixture of 2 mL of *starch solution R1*, 20 mL of *water R*, about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1* is blue.

**Starch solution R2.** *1085107.*

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R* and whilst stirring pour the mixture into 100 mL of boiling *water R*. Use a freshly prepared solution.

*Test for sensitivity*. To a mixture of 1 mL of the starch solution and 20 mL of *water R*, add about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1*. The solution is blue.

**Stavudine.** *1187000.* [3056-17-5].

See *Stavudine (2130)*.

**Stearic acid.** $C_{18}H_{36}O_2$. ($M_r$ 284.5). *1085200.* [57-11-4].
Octadecanoic acid.

White or almost white powder or flakes, greasy to the touch, practically insoluble in water, soluble in hot ethanol (96 per cent).

mp: about 70 °C.

*Stearic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

*Content*: minimum 98 per cent, calculated by the normalisation procedure.

**Stearyl alcohol.** $C_{18}H_{38}O$. ($M_r$ 270.5). *1156400.* [112-92-5].
Octadecan-1-ol.

mp: about 60 °C.

*Content*: minimum 95 per cent.

**Stigmasterol.** $C_{29}H_{48}O$. ($M_r$ 412.7). *1141400.* [83-48-7].
(22*E*)-Stigmasta-5,22-dien-3β-ol. (22*E*)-24-Ethylcholesta-5,22-dien-3β-ol.

White or almost white powder, insoluble in water.

mp: about 170 °C.

$[\alpha]_D^{22}$: about – 51, determined with a 20 g/L solution in *chloroform R*.

**Streptomycin sulfate.** *1085300.* [3810-74-0].

See *Streptomycin sulfate (0053)*.

**Strongly acidic ion-exchange resin.** *1085400.*

See *ion-exchange resin, strongly acidic R*.

**Strontium carbonate.** $SrCO_3$. ($M_r$ 147.6). *1122700.* [1633-05-2].

White or almost white, crystalline powder.

*Content*: minimum 99.5 per cent.

**Strontium chloride hexahydrate.** $SrCl_2,6H_2O$. ($M_r$ 266.6). *1167000.* [10025-70-4].

White or almost white crystals, very soluble in water.

mp: about 115 °C (loss of water) and 872 °C.

**Strontium selective extraction resin.** *1167100.*

Commercially available resin prepared by loading a suspension of 4,4′(5′)-di-*tert*-butylcyclohexano-18-crown-6 (crown ether) in octanol onto an inert chromatographic support. The bed density of this resin is approximately 0.35 g/mL.

**Strontium-85 spiking solution.** *1166800.*

Dilute *strontium-85 standard solution R* to a radioactivity concentration of approximately 10 kBq/mL with a 0.27 g/L solution of *strontium chloride hexahydrate R* in a 1.03 g/L solution of *hydrochloric acid R*.

**Strontium-85 standard solution.** *1166900.*

A solution of strontium-85 in the form of $Sr^{2+}$ ions in a 51.5 g/L solution of *hydrochloric acid R*.

**Strychnine.** $C_{21}H_{22}N_2O_2$. ($M_r$ 334.4). *1190600.* [57-24-9].
(4a*R*,4b*R*,5a*S*,8a*R*,13a*S*,15a*S*)-2,4a,4b,5,5a,7,8,13a,15,15a-Decahydro-4,6-methano-6*H*-indolo[3,2,1-*ij*]oxepino[2,3,4-*de*]pyrrolo[2,3-*h*]quinolin-14-one. Strychnidin-10-one.

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 285 °C.

**Styrene.** $C_8H_8$. ($M_r$ 104.2). *1151700.* [100-42-5].
Ethenylbenzene.

bp: about 145 °C.

Colourless, oily liquid, very slightly soluble in water.

**Styrene-divinylbenzene copolymer.** *1085500.*

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

**Succinic acid.** $C_4H_6O_4$. ($M_r$ 118.1). *1085600.* [110-15-6].
Butanedioic acid.

White or almost white, crystalline powder or colourless crystals, soluble in water and in ethanol (96 per cent).

mp: 184 °C to 187 °C.

**Sucrose.** *1085700.* [57-50-1].

See *Sucrose (0204)*.

**Sudan orange.** $C_{16}H_{12}N_2O$. ($M_r$ 248.3). *1110700.* [842-07-9].
Colour Index No. 12055.
1-(Phenylazo)naphthalen-2-ol. Sudan I.

Orange-red powder, practically insoluble in water, soluble in methylene chloride.

mp: about 131 °C.

**Sudan red G.** $C_{17}H_{14}N_2O_2$. ($M_r$ 278.3). *1085800.*
Schultz No. 149.

Colour Index No. 12150.
Solvent Red 1. 1-[(2-Methoxyphenyl)azo]naphtalen-2-ol.

Reddish-brown powder, practically insoluble in water.

*Chromatography*. Thin-layer chromatography (*2.2.27*) using *silica gel G R* as the coating substance: apply 10 µL of a 0.1 g/L solution in *methylene chloride R* and develop over a path of 10 cm with the same solvent; the chromatogram shows only one principal spot.

**Sulfanilamide.** $C_6H_8N_2O_2S$. ($M_r$ 172.2). *1086100.* [63-74-1].
4-Aminobenzenesulfonamide.

White or almost white powder, slightly soluble in water, freely soluble in boiling water, in acetone, in dilute acids and in solutions of the alkali hydroxides, sparingly soluble in ethanol (96 per cent) and in light petroleum.

mp: about 165 °C.

**Sulfathiazole.** $C_9H_9N_3O_2S_2$. ($M_r$ 255.3). *1086300.* [72-14-0].
4-Amino-*N*-(thiazol-2-yl)benzenesulfonamide.

White or yellowish-white powder or crystals, very slightly soluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in solutions of alkali hydroxides and carbonates.

mp: about 200 °C.

**Sulfamic acid.** $H_3NO_3S$. ($M_r$ 97.1). *1085900.* [5329-14-6].

White or almost white crystalline powder or crystals, freely soluble in water, sparingly soluble in acetone, in ethanol (96 per cent) and in methanol.

mp: about 205 °C, with decomposition.

**Sulfan blue.** $C_{27}H_{31}N_2NaO_6S_2$. ($M_r$ 566.6). *1086000.* [129-17-9].

Schultz No. 769.

Colour Index No. 42045.
Acid Blue 1. Patent Blue VF. Disulfine blue. Blue VS.
Sodium [[[(4-diethylamino)phenyl](2,4-disulfonatophenyl)-methylene]cyclohexa-2,5-dien-1-ylidene]diethylammonium.

Violet powder, soluble in water. Dilute solutions are blue and turn yellow on the addition of concentrated hydrochloric acid.

**Sulfanilic acid.** $C_6H_7NO_3S$. ($M_r$ 173.2). *1086200.* [121-57-3]. 4-Aminobenzenesulfonic acid.

Colourless crystals, sparingly soluble in water, practically insoluble in ethanol (96 per cent).

**Sulfanilic acid solution.** *1086203.*

Dissolve 0.33 g of *sulfanilic acid R* in 75 mL of *water R* heating gently if necessary and dilute to 100 mL with *glacial acetic acid R*.

**Sulfanilic acid solution R1.** *1086201.*

Dissolve 0.5 g of *sulfanilic acid R* in a mixture of 75 mL of *dilute acetic acid R* and 75 mL of *water R*.

**Sulfanilic acid solution, diazotised.** *1086202.*

Dissolve, with warming, 0.9 g of *sulfanilic acid R* in 9 mL of *hydrochloric acid R*, and dilute to 100 mL with *water R*. Cool 10 mL of this solution in iced water and add 10 mL of an ice-cold 45 g/L solution of *sodium nitrite R*. Allow to stand at 0 °C for 15 min (if stored at this temperature, the solution is stable for 3 days) and immediately before use add 20 mL of a 100 g/L solution of *sodium carbonate R*.

**Sulfomolybdic reagent R2.** *1086400.*

Dissolve about 50 mg of *ammonium molybdate R* in 10 mL of *sulfuric acid R*.

**Sulfomolybdic reagent R3.** *1086500.*

Dissolve with heating 2.5 g of *ammonium molybdate R* in 20 mL of *water R*. Dilute 28 mL of *sulfuric acid R* in 50 mL of *water R*, then cool. Mix the two solutions and dilute to 100 mL with *water R*.

*Storage*: in a polyethylene container.

**Sulfosalicylic acid.** $C_7H_6O_6S,2H_2O$. ($M_r$ 254.2). *1086600.* [5965-83-3]. 2-Hydroxy-5-sulfobenzoic acid.

White or almost white, crystalline powder or crystals, very soluble in water and in ethanol (96 per cent).

mp: about 109 °C.

**Sulfur.** *1110800.* [7704-34-9].

See *Sulfur for external use (0953)*.

**Sulfur dioxide.** $SO_2$. ($M_r$ 64.1). *1086700.* [7446-09-5]. Sulfurous anhydride.

A colourless gas. When compressed it is a colourless liquid.

**Sulfur dioxide R1.** $SO_2$. ($M_r$ 64.1). *1110900.* [7446-09-5].

*Content*: minimum 99.9 per cent *V/V*.

**Sulfuric acid.** $H_2SO_4$. ($M_r$ 98.1). *1086800.* [7664-93-9].

*Content*: 95.0 per cent *m/m* to 97.0 per cent *m/m*.

Colourless, caustic liquid with an oily consistency, highly hygroscopic, miscible with water and with ethanol (96 per cent) producing intense heat.

$d_{20}^{20}$: 1.834 to 1.837.

A 10 g/L solution is strongly acid and gives the reactions of sulfates (*2.3.1*).

*Appearance.* It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

*Oxidisable substances.* Pour 20 g cautiously, with cooling, into 40 mL of *water R*. Add 0.5 mL of *0.002 M potassium permanganate*. The violet colour persists for at least 5 min.

*Chlorides*: maximum 0.5 ppm.

Pour 10 g, carefully and while cooling, into 10 mL of *water R* and after cooling dilute to 20 mL with the same solvent. Add 0.5 mL of *silver nitrate solution R2*. Allow to stand for 2 min protected from bright light. The solution is not more opalescent than a standard prepared at the same time using a mixture of 1 mL of *chloride standard solution (5 ppm Cl) R*, 19 mL of *water R* and 0.5 mL of *silver nitrate solution R2*.

*Nitrates*: maximum 0.5 ppm.

Pour 50 g or 27.2 mL, carefully and while cooling, into 15 mL of *water R*. Add 0.2 mL of a freshly prepared 50 g/L solution of *brucine R* in *glacial acetic acid R*. After 5 min any colour is less intense than that of a reference mixture prepared in the same manner and containing 12.5 mL of *water R*, 50 g of *nitrogen-free sulfuric acid R*, 2.5 mL of *nitrate standard solution (10 ppm $NO_3$) R* and 0.2 mL of a 50 g/L solution of *brucine R* in *glacial acetic acid R*.

*Ammonium*: maximum 2 ppm.

Pour 2.5 g, carefully and while cooling, into *water R* and dilute to 20 mL with the same solvent. Cool, and add dropwise 10 mL of a 200 g/L solution of *sodium hydroxide R*, followed by 1 mL of *alkaline potassium tetraiodomercurate solution R*. The colour of the solution is less intense than that of a mixture of 5 mL of *ammonium standard solution (1 ppm $NH_4$) R*, 15 mL of *water R*, 10 mL of a 200 g/L solution of *sodium hydroxide R* and 1 mL of *alkaline potassium tetraiodomercurate solution R*.

*Arsenic* (*2.4.2, Method A*): maximum 0.02 ppm.

To 50 g add 3 mL of *nitric acid R* and evaporate carefully until the volume is reduced to about 10 mL. Cool, add to the residue 20 mL of *water R* and concentrate to 5 mL. Prepare the standard using 1.0 mL of *arsenic standard solution (1 ppm As) R*.

*Iron* (*2.4.9*): maximum 1 ppm.

Dissolve the residue on ignition with slight heating in 1 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*. Dilute 5 mL of this solution to 10 mL with *water R*.

*Heavy metals* (*2.4.8*): maximum 2 ppm.

Dilute 10 mL of the solution obtained in the test for iron to 20 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

*Residue on ignition*: maximum 0.001 per cent, determined on 100 g by evaporating cautiously in a small crucible over a naked flame and igniting the residue to redness.

*Assay.* Weigh accurately a ground-glass-stoppered flask containing 30 mL of *water R*, introduce 0.8 mL of the sulfuric acid, cool and weigh again. Titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.04 mg of $H_2SO_4$.

*Storage*: in a ground-glass-stoppered container made of glass or other inert material.

**5 M Sulfuric acid.** *1086809.*

Dilute 28 mL of *sulfuric acid R* to 100 mL with *water R*.

**Sulfuric acid, alcoholic, 2.5 M.** *1086801.*

Carefully and with constant cooling, stir 14 mL of *sulfuric acid R* into 60 mL of *anhydrous ethanol R*. Allow to cool and dilute to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

**Sulfuric acid, alcoholic, 0.25 M.** *1086802.*

Dilute 10 mL of *2.5 M alcoholic sulfuric acid R* to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

**Sulfuric acid, alcoholic solution of.** *1086803.*

Carefully and with constant cooling, stir 20 mL of *sulfuric acid R* into 60 mL of *ethanol (96 per cent) R*. Allow to cool and dilute to 100 mL with *ethanol (96 per cent) R*. Prepare immediately before use.

**Sulfuric acid, dilute.** *1086804.*

Contains 98 g/L of $H_2SO_4$.

Add 5.5 mL of *sulfuric acid R* to 60 mL of *water R*, allow to cool and dilute to 100 mL with the same solvent.

*Assay.* Into a ground-glass-stoppered flask containing 30 mL of *water R*, introduce 10.0 mL of the dilute sulfuric acid. Titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.04 mg of $H_2SO_4$.

**Sulfuric acid, dilute R1.** *1086810.*

Contains 4.9 g/L of $H_2SO_4$.

Prepared from *sulfuric acid R*.

**Sulfuric acid-formaldehyde reagent.** *1086805.*

Mix 2 mL of *formaldehyde solution R* with 100 mL of *sulfuric acid R*.

**Sulfuric acid, heavy metal-free.** *1086807.*

Complies with the requirements prescribed for *sulfuric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.002 ppm.

Cu: 0.001 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.002 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

**Sulfuric acid, nitrogen-free.** *1086806.*

Complies with the requirements prescribed for *sulfuric acid R* with the following additional test.

*Nitrates.* To 5 mL of *water R* add carefully 45 mL of the sulfuric acid, allow to cool to 40 °C and add 8 mg of *diphenylbenzidine R*. The solution is colourless or very pale blue.

**Sulfuric acid, nitrogen-free R1.** *1086808.*

Complies with the requirements prescribed for *nitrogen-free sulfuric acid R*.

*Content*: 95.0 per cent *m/m* to 95.5 per cent *m/m*.

**Sulfuric acid R1.** $H_2SO_4$. ($M_r$ 98.1). *1190900.* [7664-93-9].

*Content*: 75 per cent *V/V*.

**Sunflower oil.** *1086900.*

See *Sunflower oil, refined (1371)*.

**Swertiamarin.** $C_{16}H_{22}O_{10}$. ($M_r$ 374.3). *1163600.* [17388-39-5]. Swertiamaroside. (4*R*,5*R*,6*S*)-5-Ethenyl-6-(β-D-glucopyranosyloxy)-4a-hydroxy-4,4a,5,6-tetrahydro-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one.

**Tagatose.** $C_6H_{12}O_6$. ($M_r$ 180.16). *1111000.* [87-81-0]. D-*lyxo*-Hexulose.

White or almost white powder.

$[\alpha]_D^{20}$: – 2.3 determined on a 21.9 g/L solution.

mp: 134 °C to 135 °C.

**Talc.** *1087000.* [14807-96-6].

See *Talc (0438)*.

**Tannic acid.** *1087100.* [1401-55-4].

Yellowish or light-brown, glistening scales or amorphous powder, very soluble in water, freely soluble in ethanol (96 per cent), soluble in acetone.

*Storage*: protected from light.

**Tanshinone $II_A$.** $C_{19}H_{18}O_3$. ($M_r$ 294.3). *1184800.* [568-72-9].

1,6,6-Trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-*b*]furan-10,11-dione.

**Tartaric acid.** *1087200.* [87-69-4].

See *Tartaric acid (0460)*.

**Taxifolin.** $C_{15}H_{12}O_7$. ($M_r$ 304.3). *1151800.* [480-18-2]. (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4*H*-1-benzopyran-4-one.

White or almost white powder, slightly soluble in anhydrous ethanol.

*Absorbance* (*2.2.25*). A solution in *anhydrous ethanol R* shows an absorption maximum at 290 nm.

**Tecnazene.** $C_6HCl_4NO_2$. ($M_r$ 260.9). *1132400.* [117-18-0].

bp: about 304 °C.

mp: 99 °C to 100 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***trans*-Terpin.** $C_{10}H_{20}O_2$. ($M_r$ 172.3). *1205800.* [565-50-4]. (1*r*,4*r*)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexan-1-ol. *p*-Menthane-1,8-diol.

mp: about 116 °C.

**α-Terpinene.** $C_{10}H_{16}$. ( $M_r$ 136.2). *1140300.* [99-86-5]. 1-Isopropyl-4-methylcyclohexa-1,3-diene.

Clear, almost colourless liquid.

$d_4^{20}$: about 0.837.

$n_D^{20}$: about 1.478.

bp: about 174 °C.

*α-Terpinene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Tea tree oil (1837)*.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

**γ-Terpinene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1115900.* [99-85-4]. 1-Isopropyl-4-methylcyclohexa-1,4-diene.

Oily liquid.

*γ-Terpinene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution.* The substance to be examined.

*Content*: minimum 93.0 per cent, calculated by the normalisation procedure.

**Terpinen-4-ol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1116000.* [562-74-3]. 4-Methyl-1-(1-methylethyl)cyclohex-3-en-1-ol. *p*-Menth-1-en-4-ol.

Oily, colourless liquid.

*Terpinen-4-ol used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

*Test solution.* The substance to be examined.

*Content*: minimum 90.0 per cent, calculated by the normalisation procedure.

**α-Terpineol.** $C_{10}H_{18}O$. ($M_r$ 154.2). *1087300.* [98-55-5]. (*RS*)-2-(4-Methylcyclohex-3-enyl)-2-propanol.

Colourless crystals, practically insoluble in water, soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.935.

$n_D^{20}$: about 1.483.

$[\alpha]_D^{20}$: about 92.5.

mp: about 35 °C.

It may contain 1 to 3 per cent of β-terpineol.

*α-Terpineol used in gas chromatography complies with the following test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

*Test solution.* A 100 g/L solution in *hexane R*.

*Content*: minimum 97.0 per cent, calculated by the normalisation procedure.

**Terpinolene.** $C_{10}H_{16}$. ($M_r$ 136.2). *1140400.* [586-62-9]. *p*-Mentha-1,4(8)-diene. 4-Isopropylidene-1-methylcyclohexene.

Clear, almost colourless liquid.

$d_4^{20}$: about 0.863.

$n_D^{20}$: about 1.488.

bp: about 184 °C.

*Terpinolene used in gas chromatography complies with the following additional test.*

*Assay.* Gas chromatography (*2.2.28*) as prescribed in the monograph *Tea tree oil (1837)*.

*Content*: minimum 90 per cent, calculated by the normalisation procedure.

**Testosterone.** *1116100.* [58-22-0].

See *Testosterone (1373)*.

**Testosterone propionate.** *1087400.* [57-85-2].

See *Testosterone propionate (0297)*.

**1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose.** $C_{14}H_{20}O_{10}$. ($M_r$ 348.3). *1172600.* [13100-46-4].

White or almost white powder, soluble in water with gentle heating.

$[\alpha]_D^{20}$: + 11, determined on a 6 g/L solution in *chloroform R*.

mp: 126 °C to 128 °C.

**1,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranose.** $C_{14}H_{20}O_{10}$. ($M_r$ 348.3). *1174100.* [18968-05-3].

Colourless or white powder or crystals.

mp: 160 °C to 161 °C.

$[\alpha]_D^{20}$: − 68, determined on a 7 g/L solution in *methylene chloride R*.

**Tetrabutylammonium bromide.** $C_{16}H_{36}BrN$. ($M_r$ 322.4). *1087500.* [1643-19-2].

White or almost white crystals.

mp: 102 °C to 104 °C.

**Tetrabutylammonium dihydrogen phosphate.** $C_{16}H_{38}NO_4P$. ($M_r$ 339.5). *1087600.* [5574-97-0].

White or almost white powder, hygroscopic.

*pH* (*2.2.3*): about 7.5 for a 170 g/L solution.

*Absorbance* (*2.2.25*): about 0.10 determined at 210 nm using a 170 g/L solution.

*Storage*: in an airtight container.

**Tetrabutylammonium dihydrogen phosphate solution.** *1087601.*

A 1.0 M solution of *tetrabutylammonium dihydrogen phosphate R*. This solution is commercially available.

**Tetrabutylammonium hydrogen sulfate.** $C_{16}H_{37}NO_4S$. ($M_r$ 339.5). *1087700.* [32503-27-8].

Crystalline powder or colourless crystals, freely soluble in water and in methanol.

mp: 169 °C to 173 °C.

*Absorbance* (*2.2.25*): maximum 0.05, determined between 240 nm and 300 nm using a 50 g/L solution.

**Tetrabutylammonium hydrogen sulfate R1.** *1087701.*

Complies with the requirements prescribed for *tetrabutylammonium hydrogen sulfate R* with the following additional requirement.

*Absorbance* (*2.2.25*): maximum 0.02, determined between 215 nm and 300 nm using a 50 g/L solution.

**Tetrabutylammonium hydroxide.** $C_{16}H_{37}NO,30H_2O$. ($M_r$ 800). *1087800.* [147741-30-8].

*Content*: minimum 98.0 per cent of $C_{16}H_{37}NO,30H_2O$.

White or almost white crystals, soluble in water.

*Assay.* Dissolve 1.000 g in 100 mL of *water R*. Titrate immediately with *0.1 M hydrochloric acid* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M hydrochloric acid* is equivalent to 80.0 mg of $C_{16}H_{37}NO,30H_2O$.

**Tetrabutylammonium hydroxide solution (104 g/L).** *1087801.*

A solution containing 104 g/L of $C_{16}H_{37}NO$ ($M_r$ 259.5), prepared by dilution of a suitable reagent grade.

**Tetrabutylammonium hydroxide solution (400 g/L).** *1087802.*

A solution containing 400 g/L of $C_{16}H_{37}NO$ ($M_r$ 259.5) of a suitable grade.

**Tetrabutylammonium iodide.** $C_{16}H_{36}IN$. ($M_r$ 369.4). *1087900.* [311-28-4].

*Content*: minimum 98.0 per cent.

White or slightly coloured, crystalline powder or crystals, soluble in ethanol (96 per cent).

*Sulfated ash* (*2.4.14*): maximum 0.02 per cent.

*Assay.* Dissolve 1.200 g in 30 mL of *water R*. Add 50.0 mL of *0.1 M silver nitrate* and 5 mL of *dilute nitric acid R*. Titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate*, using 2 mL of *ferric ammonium sulfate solution R2* as indicator.

1 mL of *0.1 M silver nitrate* is equivalent to 36.94 mg of $C_{16}H_{36}IN$.

**Tetrachloroethane.** $C_2H_2Cl_4$. ($M_r$ 167.9). *1088000.* [79-34-5]. 1,1,2,2-Tetrachloroethane.

Clear, colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 1.59.

$n_D^{20}$: about 1.495.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 145 °C and 147 °C.

**Tetrachlorvinphos.** $C_{10}H_9Cl_4O_4P$. ($M_r$ 366.0). *1132500.* [22248-79-9].

mp: about 95 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Tetracos-15-enoic acid methyl ester.** $C_{25}H_{48}O_2$. ($M_r$ 380.7). *1144800.* [2733-88-2]. 15-Tetracosaenoic acid methyl ester. Methyl tetracos-15-enoate. Nervonic acid methyl ester.

*Content*: minimum 99.0 per cent, determined by gas chromatography.

Liquid.

**Tetracycline hydrochloride.** *1147000.*

See *Tetracycline hydrochloride (0210)*.

**Tetradecane.** $C_{14}H_{30}$. ($M_r$ 198.4). *1088200.* [629-59-4]. *n*-Tetradecane.

*Content*: minimum 99.5 per cent *m/m*.

A colourless liquid.

$d_{20}^{20}$: about 0.76.

$n_D^{20}$: about 1.429.

bp: about 252 °C.

mp: about − 5 °C.

**Tetradecylammonium bromide.** $C_{40}H_{84}BrN$. ($M_r$ 659). *1088300.* [14937-42-9]. Tetrakis(decyl)ammonium bromide.
White or slightly coloured, crystalline powder or crystals.
mp: 88 °C to 89 °C.

**Tetraethylammonium hydrogen sulfate.** $C_8H_{21}NO_4S$. ($M_r$ 227.3). *1116200.* [16873-13-5].
Hygroscopic powder.
mp: about 245 °C.

**Tetraethylammonium hydroxide solution.** $C_8H_{21}NO$. ($M_r$ 147.3). *1100300.* [77-98-5].
A 200 g/L solution.
Colourless liquid, strongly alkaline.
$d_{20}^{20}$: about 1.01.
$n_D^{20}$: about 1.372.
*HPLC grade.*

**Tetraethylene pentamine.** $C_8H_{23}N_5$. ($M_r$ 189.3). *1102000.* [112-57-2]. 3,6,9-Triazaundecan-1,11-diamine.
Colourless liquid, soluble in acetone.
$n_D^{20}$: about1.506.
*Storage*: protected from humidity and heat.

**Tetraheptylammonium bromide.** $C_{28}H_{60}BrN$. ($M_r$ 490.7). *1088400.* [4368-51-8].
White or slightly coloured, crystalline powder or crystals.
mp: 89 °C to 91 °C.

**Tetrahexylammonium bromide.** $C_{24}H_{52}BrN$. ($M_r$ 434.6). *1152500.* [4328-13-6]. *N,N,N*-Trihexylhexan-1-aminium bromide.
White or almost white, crystalline powder, hygroscopic.
mp: about 100 °C.

**Tetrahexylammonium hydrogen sulfate.** $C_{24}H_{53}NO_4S$. ($M_r$ 451.8). *1116300.* [32503-34-7]. *N,N,N*-Trihexylhexan-1-aminium hydrogen sulfate.
White or almost white crystals.
mp: 100 °C to 102 °C.

**Tetrahydrofuran.** $C_4H_8O$. ($M_r$ 72.1). *1088500.* [109-99-9]. Tetramethylene oxide.
Clear, colourless, flammable liquid, miscible with water, with ethanol (96 per cent).
$d_{20}^{20}$: about 0.89.
*Do not distil if the tetrahydrofuran does not comply with the test for peroxides.*
*Peroxides.* Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.
*Tetrahydrofuran used in spectrophotometry complies with the following additional test.*
*Absorbance* (*2.2.25*): maximum 0.70 at 255 nm, 0.10 at 270 nm, 0.01 at 310 nm, determined using *water R* as compensation liquid.

**Tetrahydrofuran for chromatography.** *1147100.*
Complies with the requirements prescribed for *tetrahydrofuran R* with the following additional requirements:
$d_4^{20}$ = 0.8892.
bp: about 66 °C.
*Content*: minimum 99.8 per cent of $C_4H_8O$.

**Tetrahydropalmatine.** $C_{21}H_{25}NO_4$. ($M_r$ 355.4). *1205900.* [2934-97-6]. (13a*RS*)-5,8,13,13a-Tetrahydro-2,3,9,10-tetramethoxy-6*H*-dibenzo[*a*,*g*]quinolizine.

**α-Tetralone.** $C_{10}H_{10}O$. ($M_r$ 146.2). *1171800.* [529-34-0]. 1-Oxotetraline. 3,4-Dihydronaphthalen-1(2*H*)-one.
bp: about 115 °C.
mp: about 5 °C.

**Tetramethylammonium bromide.** $C_4H_{12}BrN$. ($M_r$ 154.1). *1156600.* [64-20-0]. *N,N,N*-Trimethylmethanaminium bromide.
White or slightly yellow crystals, freely soluble in water.
mp: about 285 °C, with decomposition.

**Tetramethylammonium chloride.** $C_4H_{12}ClN$. ($M_r$ 109.6). *1100400.* [75-57-0].
Colourless crystals, soluble in water and in ethanol (96 per cent).
mp: about 300 °C, with decomposition.

**Tetramethylammonium hydrogen sulfate.** $C_4H_{13}NO_4S$. ($M_r$ 171.2). *1116400.* [80526-82-5].
Hygroscopic powder.
mp: about 295 °C.

**Tetramethylammonium hydroxide.** $C_4H_{13}NO,5H_2O$. ($M_r$ 181.2). *1122800.* [10424-65-4]. Tetramethylammonium hydroxide pentahydrate.
Suitable grade for HPLC.

**Tetramethylammonium hydroxide solution.** *1088600.* [75-59-2].
*Content*: minimum 10.0 per cent *m/m* of $C_4H_{13}NO$. ($M_r$ 91.2).
Clear, colourless or very pale yellow liquid, miscible with water and with ethanol (96 per cent).
*Assay.* To 1.000 g add 50 mL of *water R* and titrate with *0.05 M sulfuric acid*, using 0.1 mL of *methyl red solution R* as indicator.
1 mL of *0.05 M sulfuric acid* is equivalent to 9.12 mg of $C_4H_{13}NO$.

**Tetramethylammonium hydroxide solution, dilute.** *1088601.*
Dilute 10 mL of *tetramethylammonium hydroxide solution R* to 100 mL with *aldehyde-free alcohol R*. Prepare immediately before use.

**Tetramethylbenzidine.** $C_{16}H_{20}N_2$. ($M_r$ 240.3). *1132600.* [54827-17-7]. 3,3′,5,5′-Tetramethylbiphenyl-4,4′-diamine.
Powder, practically insoluble in water, very soluble in methanol.
mp: about 169 °C.

**1,1,3,3-Tetramethylbutylamine.** $C_8H_{19}N$. ($M_r$ 129.3). *1141500.* [107-45-9]. 2-Amino-2,4,4-trimethylpentane.
Clear, colourless liquid.
$d_{20}^{20}$: about 0.805.
$n_D^{20}$: about 1.424.
bp: about 140 °C.

**Tetramethyldiaminodiphenylmethane.** $C_{17}H_{22}N_2$. ($M_r$ 254.4). *1088700.* [101-61-1]. 4,4′-Methylenebis-(*N,N*-dimethylaniline).
White or bluish-white crystals or leaflets, practically insoluble in water, slightly soluble in ethanol (96 per cent), soluble in mineral acids.
mp: about 90 °C.

**Tetramethyldiaminodiphenylmethane reagent.** *1088701.*
*Solution A.* Dissolve 2.5 g of *tetramethyldiaminodiphenylmethane R* in 10 mL of *glacial acetic acid R* and add 50 mL of *water R*.
*Solution B.* Dissolve 5 g of *potassium iodide R* in 100 mL of *water R*.

*Solution C.* Dissolve 0.30 g of *ninhydrin R* in 10 mL of *glacial acetic acid R* and add 90 mL of *water R*.

Mix solution A, solution B and 1.5 mL of solution C.

**Tetramethylethylenediamine.** $C_6H_{16}N_2$. ($M_r$ 116.2). *1088800.* [110-18-9]. *N,N,N',N'*-Tetramethylethylenediamine.

Colourless liquid, miscible with water and with ethanol (96 per cent).

$d_{20}^{20}$: about 0.78.

$n_D^{20}$: about 1.418.

bp: about 121 °C.

**Tetramethylsilane.** $C_4H_{12}Si$. ($M_r$ 88.2). *1088900.* [75-76-3]. TMS.

Clear, colourless liquid, very slightly soluble in water, soluble in acetone and in ethanol (96 per cent).

$d_{20}^{20}$: about 0.64.

$n_D^{20}$: about 1.358.

bp: about 26 °C.

*Tetramethylsilane used in nuclear magnetic resonance spectrometry complies with the following additional test.*

In the nuclear magnetic resonance spectrum of an approximately 10 per cent *V/V* solution of the tetramethylsilane in *deuterated chloroform R*, the intensity of any foreign signal, excluding those due to spinning side bands and to chloroform, is not greater than the intensity of the C-13 satellite signals located at a distance of 59.1 Hz on each side of the principal signal of tetramethylsilane.

**Tetrandrine.** $C_{38}H_{42}N_2O_6$. ($M_r$ 623). *1178500.* [518-34-3].

**Tetrapropylammonium chloride.** $C_{12}H_{28}ClN$. ($M_r$ 221.8). *1151900.* [5810-42-4].

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 241 °C.

**Tetrapropylammonium hydrogen sulfate.** $C_{12}H_{29}NO_4S$. ($M_r$ 283.4). *1191300.* [56211-70-2]. *N,N,N*-Tripropylpropan-1-aminium hydrogen sulfate.

White or almost white, crystalline, hygroscopic powder.

**Tetrazolium blue.** $C_{40}H_{32}Cl_2N_8O_2$. ($M_r$ 728). *1089000.* [1871-22-3]. 3,3′-(3,3′-Dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis[2,5-diphenyl-2*H*-tetrazolium] dichloride.

Yellow crystals, slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol, practically insoluble in acetone.

mp: about 245 °C, with decomposition.

**Tetrazolium bromide.** $C_{18}H_{16}BrN_5S$. ($M_r$ 414.3). *1152700.* [298-93-1]. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. MTT.

**Tetrazolium salt.** $C_{20}H_{17}N_5O_6S_2$. ($M_r$ 487.5). *1174200.* [138169-43-4]. 5-(3-Carboxymethoxyphenyl)-3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt. MTS.

**Thallous sulfate.** $Tl_2SO_4$. ($M_r$ 504.8). *1089100.* [7446-18-6]. Dithallium sulfate.

White or almost white, rhomboid prisms, slightly soluble in water, practically insoluble in ethanol (96 per cent).

**Thebaine.** $C_{19}H_{21}NO_3$. ($M_r$ 311.4). *1089200.* [115-37-7]. (5*R*,9*R*,13*S*)-4,5-Epoxy-3,6-dimethoxy-9a-methylmorphina-6,8-diene.

White or pale yellow, crystalline powder, very slightly soluble in water, soluble in hot anhydrous ethanol and in toluene.

mp: about 193 °C.

*Chromatography* (*2.2.27*). Thin-layer chromatography (*2.2.27*) as prescribed in identification test B in the monograph *Raw opium (0777)*: apply to the plate as a band (20 mm × 3 mm) 20 µL of a 0.5 g/L solution; the chromatogram shows an orange-red or red principal band with an $R_F$ of about 0.5.

**Theobromine.** *1138800.* [83-67-0].

See *Theobromine (0298)*.

**Theophylline.** *1089300.* [58-55-9].

See *Theophylline (0299)*.

**Thiamazole.** $C_4H_6N_2S$. ($M_r$ 114.2). *1089400.* [60-56-0]. Methimazole. 1-Methyl-1*H*-imidazole-2-thiol.

White or almost white, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 145 °C.

**2-(2-Thienyl)acetic acid.** $C_6H_6O_2S$. ($M_r$ 142.1). *1089500.* [1918-77-0].

Brown powder.

mp: about 65 °C.

**Thioacetamide.** $C_2H_5NS$. ($M_r$ 75.1). *1089600.* [62-55-5].

Crystalline powder or colourless crystals, freely soluble in water and in ethanol (96 per cent).

mp: about 113 °C.

**Thioacetamide reagent.** *1089601.*

To 0.2 mL of *thioacetamide solution R* add 1 mL of a mixture of 5 mL of *water R*, 15 mL of *1 M sodium hydroxide* and 20 mL of *glycerol (85 per cent) R*. Heat in a water-bath for 20 s. Prepare immediately before use.

**Thioacetamide solution.** *1089602.*

A 40 g/L solution of *thioacetamide R*.

**Thiobarbituric acid.** $C_4H_4N_2O_2S$. ($M_r$ 144.2). *1111200.* [504-17-6]. 4,6-Dihydroxy-2-sulfanylpyrimidine.

**Thiodiethylene glycol.** $C_4H_{10}O_2S$. ($M_r$ 122.2). *1122900.* [111-48-8]. Di(2-hydroxyethyl) sulfide.

Colourless or yellow, viscous liquid.

*Content*: minimum 99.0 per cent.

$d_{20}^{20}$: about 1.18.

**Thioglycollic acid.** $C_2H_4O_2S$. ($M_r$ 92.1). *1089700.* [68-11-1]. 2-Mercaptoacetic acid.

Colourless liquid, miscible with water, soluble in ethanol (96 per cent).

**Thiomalic acid.** $C_4H_6O_4S$. ($M_r$ 150.2). *1161600.* [70-49-5]. (2*RS*)-2-Sulfanylbutanedioic acid.

mp: 150 °C to 152 °C.

**Thiomersal.** $C_9H_9HgNaO_2S$. ($M_r$ 404.8). *1089800.* [54-64-8]. Sodium mercurothiolate. Sodium 2-[(ethylmercurio)thio]benzoate.

Light, yellowish-white, crystalline powder, very soluble in water, freely soluble in ethanol (96 per cent).

**Thiourea.** $CH_4N_2S$. ($M_r$ 76.1). *1089900.* [62-56-6].

White or almost white, crystalline powder or crystals, soluble in water and in ethanol (96 per cent).

mp: about 178 °C.

**Threonine.** *1090000.* [72-19-5].

See *Threonine (1049)*.

**Thrombin, bovine.** *1090200.* [9002-04-4].

A preparation of the enzyme, obtained from bovine plasma, that converts fibrinogen into fibrin.

A yellowish-white powder.

*Storage*: at a temperature below 0 °C.

**Thrombin, human.** *1090100.* [9002-04-4].

Dried human thrombin. A preparation of the enzyme which converts human fibrinogen into fibrin. It is obtained from liquid human plasma and may be prepared by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength and temperature.

Yellowish-white powder, freely soluble in a 9 g/L solution of sodium chloride forming a cloudy, pale yellow solution.

*Storage*: in a sealed, sterile container under nitrogen, protected from light, at a temperature below 25 °C.

**Thrombin solution, human.** *1090101.*

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

**Thrombin solution, human R1.** *1090102.*

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 2.5 IU/mL with *phosphate buffer solution pH 6.5 R*.

**Thrombin solution, human R2.** *1090103.*

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*.

**Thromboplastin.** *1090300.*

A preparation containing the membrane glycoprotein tissue factor and phospholipid, either purified from animal brain (usually rabbit) or human placenta or manufactured using recombinant DNA technology with added phospholipid. The preparation is formulated for routine use in the prothrombin time test and may contain calcium.

**Thujone.** $C_{10}H_{16}O$. ($M_r$ 152.2). *1116500.* [76231-76-0].
4-Methyl-1-(1-methylethyl)bicyclo[3.1.0]hexan-3-one.

Colourless or almost colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent) and in many other organic solvents.

**Thymidine.** $C_{10}H_{14}N_2O_5$. ($M_r$ 242.2). *1158900.* 1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione.

Needles, soluble in water, in hot ethanol (96 per cent) and in glacial acetic acid.

**Thymine.** $C_5H_6N_2O_2$. ($M_r$ 126.1). *1090400.* [65-71-4].
5-Methylpyrimidine-2,4(1*H*,3*H*)-dione.

Short needles or plates, slightly soluble in cold water, soluble in hot water. It dissolves in dilute solution of alkali hydroxides.

**Thymol.** *1090500.* [89-83-8]. See *Thymol (0791)*.

*Thymol used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

*Test solution*. Dissolve 0.1 g in about 10 mL of *acetone R*.

*Content*: minimum 95.0 per cent, calculated by the normalisation procedure.

**Thymol blue.** $C_{27}H_{30}O_5S$. ($M_r$ 466.6). *1090600.* [76-61-9].
Thymolsulfonphthalein. 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-isopropyl-5-methylphenol) *S*,*S*-dioxide.

Brownish-green or greenish-blue, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Thymol blue solution.** *1090601.*

Dissolve 0.1 g of *thymol blue R* in a mixture of 2.15 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

*Test for sensitivity.* To 0.1 mL of the thymol blue solution add 100 mL of *carbon dioxide-free water R* and 0.2 mL of *0.02 M sodium hydroxide*. The solution is blue. Not more than 0.15 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

*Colour change*: pH 1.2 (red) to pH 2.8 (yellow); pH 8.0 (olive-green) to pH 9.6 (blue).

**Thymolphthalein.** $C_{28}H_{30}O_4$. ($M_r$ 430.5). *1090700.* [125-20-2]. 3,3-Bis(4-hydroxy-5-isopropyl-2-methylphenyl)-3*H*-isobenzo-furan-1-one.

White or yellowish-white powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

**Thymolphthalein solution.** *1090701.*

A 1 g/L solution of *thymolphthalein R* in *ethanol (96 per cent) R*.

*Test for sensitivity.* To 0.2 mL of the thymolphthalein solution add 100 mL of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.05 mL of *0.1 M sodium hydroxide* is required to change the colour to blue.

*Colour change*: pH 9.3 (colourless) to pH 10.5 (blue).

**Tin.** Sn. ($A_r$ 118.7). *1090800.* [7440-31-5].

Silvery-white granules, soluble in hydrochloric acid with release of hydrogen.

*Arsenic* (*2.4.2, Method A*): maximum 10 ppm, determined on 0.1 g.

**Tin test kit, semi-quantitative.** *1194100.*

Commercially available set of reagents consisting of tin test strips and a reagent mixture for the determination of tin in aqueous solutions, in a range of 10-200 µg/mL.

**Titan yellow.** $C_{28}H_{19}N_5Na_2O_6S_4$. ($M_r$ 696). *1090900.* [1829-00-1].

Schultz No. 280.

Colour Index No. 19540.
Thiazol yellow. Disodium 2,2′-[(1-triazene-1,3-diyl)di-4,1-phenylene]bis-[6-methylbenzothiazole-7-sulfonate].

A yellowish-brown powder, freely soluble in water and in ethanol (96 per cent).

**Titan yellow paper.** *1090901.*

Immerse strips of filter paper in *titan yellow solution R* and leave for a few minutes. Allow to dry at room temperature.

**Titan yellow solution.** *1090902.*

A 0.5 g/L solution of *titan yellow R*.

*Test for sensitivity.* To 0.1 mL of the titan yellow solution add 10 mL of *water R*, 0.2 mL of *magnesium standard solution (10 ppm Mg) R* and 1.0 mL of *1 M sodium hydroxide*. A distinct pink colour is visible by comparison with a reference solution prepared in a similar manner omitting the magnesium.

**Titanium.** Ti. ($A_r$ 47.88). *1091000.* [7440-32-6].

*Content*: minimum 99 per cent.

Metal powder, fine wire (diameter not more than 0.5 mm), sponge.

mp: about 1668 °C.

*Density*: about 4.507 g/cm$^3$.

**Titanium dioxide.** *1117900.* [13463-67-7].

See *Titanium dioxide (0150)*.

**Titanium trichloride.** $TiCl_3$. ($M_r$ 154.3). *1091200.* [7705-07-9]. Titanium(III) chloride.

Reddish-violet crystals, deliquescent, soluble in water and in ethanol (96 per cent).

mp: about 440 °C.

*Storage*: in an airtight container.

**Titanium trichloride solution.** *1091201.*

$d_{20}^{20}$: about 1.19.

A 150 g/L solution of *titanium trichloride R* in hydrochloric acid (100 g/L HCl).

**Titanium trichloride-sulfuric acid reagent.** *1091202.*

Carefully mix 20 mL of *titanium trichloride solution R* with 13 mL of *sulfuric acid R*. Add sufficient *strong hydrogen peroxide solution R* to give a yellow colour. Heat until white fumes are evolved. Allow to cool. Dilute with *water R* and repeat the evaporation and addition of *water R* until a colourless solution is obtained. Dilute to 100 mL with *water R*.

**TLC aluminium oxide G plate.** *1165200.*

Support of metal, glass or plastic, coated with a layer of aluminium oxide (particle size 5-40 µm) containing about 10 per cent of calcium sulfate hemihydrate as a binder.

**TLC cellulose plate.** *1191400.*

Support of glass, metal or plastic, coated with a layer of cellulose.

**TLC octadecylsilyl silica gel plate.** *1148600.*

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel. The plate may contain an organic binder.

**TLC octadecylsilyl silica gel $F_{254}$ plate.** *1146600.*

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel.

It contains a fluorescent indicator having a maximum absorbance in ultraviolet light at 254 nm.

**TLC performance test solution.** *1116600.*

Prepare a mixture of 1.0 mL of each of the following solutions and dilute to 10.0 mL with *acetone R*: a 0.5 g/L solution of *Sudan red G R* in *toluene R*, a 0.5 g/L solution of *methyl orange R* in *ethanol R* prepared immediately before use, a 0.5 g/L solution of *bromocresol green R* in *acetone R* and a 0.25 g/L solution of *methyl red R* in *acetone R*.

**TLC silica gel plate.** *1116700.*

Support of glass, metal or plastic, coated with a layer of silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

*Chromatographic separation*. Apply to the plate an appropriate volume (10 µL for a normal TLC plate and 1 µL to 2 µL for a fine particle size plate) of *TLC performance test solution R*. Develop over a pathlength two-thirds of the plate height, using a mixture of 20 volumes of *methanol R* and 80 volumes of *toluene R*. The plate is not satisfactory, unless the chromatogram shows four clearly separated spots, the spot of bromocresol green with an $R_F$ value less than 0.15, the spot of methyl orange with an $R_F$ value in the range of 0.1 to 0.25, the spot of methyl red with an $R_F$ value in the range of 0.35 to 0.55 and the spot of Sudan red G with an $R_F$ value in the range of 0.75 to 0.98.

**TLC silica gel $F_{254}$ plate.** *1116800.*

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

*Fluorescence suppression*. Apply separately to the plate at five points increasing volumes (1 µL to 10 µL for normal TLC plates and 0.2 µL to 2 µL for fine particle size plates) of a 1 g/L solution of *benzoic acid R* in a mixture of 15 volumes of *anhydrous ethanol R* and 85 volumes of *cyclohexane R*. Develop over a pathlength half of the plate height with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. For normal TLC plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 2 µg and greater. For fine particle size plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 0.2 µg and greater.

**TLC silica gel $F_{254}$, silanised plate.** *1117200.*

It complies with the requirements prescribed for *TLC silanised silica gel plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

**TLC silica gel G plate.** *1116900.*

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains calcium sulfate hemihydrate as binder.

**TLC silica gel $GF_{254}$ plate.** *1117000.*

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modifications.

It contains calcium sulfate hemihydrate as binder and a fluorescent indicator having a maximum absorbance at 254 nm.

*Fluorescence suppression*. Complies with the test prescribed for *TLC silica gel $F_{254}$ plate R*.

**TLC silica gel plate for aminopolyether test.** *1172700.*

Immerse a *TLC silica gel plate R* in *iodoplatinate reagent R1* for 5-10 s. Dry at room temperature for 12 h, protected from light.

*Storage*: protected from light, in an open container; use within 30 days after preparation.

**TLC silica gel plate for chiral separations, octadecylsilyl.** *1137700.*

Support of glass, metal or plastic, coated with a layer of octadecylsilyl silica gel, impregnated with $Cu^{2+}$ ions and enantiomerically pure hydroxyproline. The plate may contain an organic binder.

**TLC silica gel, silanised plate.** *1117100.*

Support of glass, metal or plastic, coated with a layer of silanised silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

*Chromatographic separation*. Introduce 0.1 g each of *methyl laurate R*, *methyl myristate R*, *methyl palmitate R* and *methyl stearate R* into a 250 mL conical flask. Add 40 mL of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 mL of

*water R*, acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantitites each of 10 mL of *methylene chloride R*. Dry the combined methylene chloride extracts over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 mL of *methylene chloride R*. Examine by thin-layer chromatography (*2.2.27*), using *TLC silanised silica gel plate R*. Apply an appropriate quantity (about 10 µL for normal TLC plates and about 1 µL to 2 µL for fine particle size plates) of the methylene chloride solution at each of three separate points. Develop over a pathlength two-thirds of the plate height with a mixture of 10 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *dioxan R*. Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until the background is white. The chromatograms show four clearly separated, well-defined spots.

**α-Tocopherol.** *1152300.* [10191-41-0].
See *all-rac-α-Tocopherol (0692)*.

**α-Tocopheryl acetate.** *1152400.* [7695-91-2].
See *all-rac-α-Tocopheryl acetate (0439)*.

***o*-Tolidine.** $C_{14}H_{16}N_2$. ($M_r$ 212.3). *1123000.* [119-93-7]. 3,3′-Dimethylbenzidine.
*Content*: minimum 97.0 per cent.
Light brownish, crystalline power.
mp: about 130 °C.

***o*-Tolidine solution.** *1123001.*
Dissolve 0.16 g of *o-tolidine R* in 30.0 mL of *glacial acetic acid R*, add 1.0 g of *potassium iodide R* and dilute to 500.0 mL with *water R*.

**Toluene.** $C_7H_8$. ($M_r$ 92.1). *1091300.* [108-88-3]. Methylbenzene.
Clear, colourless, flammable liquid, very slightly soluble in water, miscible with ethanol (96 per cent).
$d_{20}^{20}$: 0.865 to 0.870.
bp: about 110 °C.

**Toluene, sulfur-free.** *1091301.*
Complies with the requirements prescribed for *toluene R* with the following additional requirements.
*Sulfur compounds.* To 10 mL add 1 mL of *anhydrous ethanol R* and 3 mL of *potassium plumbite solution R* and boil under a reflux condenser for 15 min. Allow to stand for 5 min. No darkening is produced in the aqueous layer.
*Thiophen-related substances.* Shake 2 mL with 5 mL of *isatin reagent R* for 5 min and allow to stand for 15 min. No blue colour is produced in the lower layer.

**Toluenesulfonamide.** $C_7H_9NO_2S$. ($M_r$ 171.2). *1091500.* [70-55-3]. 4-Methylbenzenesulfonamide. *p*-Toluenesulfonamide.
*Content*: minimum 99.0 per cent.
White or almost white, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.
mp: about 136 °C.

***o*-Toluenesulfonamide.** $C_7H_9NO_2S$. ($M_r$ 171.2). *1091400.* [88-19-7]. 2-Methylbenzenesulfonamide.
White or almost white, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.
mp: about 156 °C.

***p*-Toluenesulfonamide.** *1091500.* [70-55-3].
See *toluenesulfonamide R*.

**Toluenesulfonic acid.** $C_7H_8O_3S,H_2O$. ($M_r$ 190.2). *1091600.* [6192-52-5]. 4-Methylbenzenesulfonic acid.
*Content*: minimum 87.0 per cent of $C_7H_8O_3S$.
White or almost white, crystalline powder or crystals, freely soluble in water, soluble in ethanol (96 per cent).

**Toluenesulfonylurea.** $C_8H_{10}N_2O_3S$. ($M_r$ 214.2). *1177000.* [1694-06-0]. 4-Methylbenzenesulfonylurea. *p*-Toluenesulfonylurea. (4-Methylphenyl)sulfonylurea.
White or almost white, crystalline powder.
mp: 196 to 198 °C.

***o*-Toluidine.** $C_7H_9N$. ($M_r$ 107.2). *1091700.* [95-53-4]. 2-Methylaniline.
Pale-yellow liquid becoming reddish-brown on exposure to air and light, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute acids.
$d_{20}^{20}$: about 1.01.
$n_D^{20}$: about 1.569.
bp: about 200 °C.
*Storage*: in an airtight container, protected from light.

***o*-Toluidine hydrochloride.** $C_7H_{10}ClN$. ($M_r$ 143.6). *1117300.* [636-21-5]. 2-Methylaniline hydrochloride. 2-Methylbenzenamine hydrochloride.
*Content*: minimum 98.0 per cent.
mp: 215 °C to 217 °C.

***p*-Toluidine.** $C_7H_9N$. ($M_r$ 107.2). *1091800.* [106-49-0]. 4-Methylaniline.
Lustrous plates or flakes, slightly soluble in water, freely soluble in acetone and in ethanol (96 per cent).
mp: about 44 °C.

**Toluidine blue.** $C_{15}H_{16}ClN_3S$. ($M_r$ 305.8). *1091900.* [92-31-9].
Schultz No. 1041.
Colour Index No. 52040.
Toluidine Blue O. 3-Amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride.
Dark-green powder, soluble in water, slightly soluble in ethanol (96 per cent).

**Tosylarginine methyl ester hydrochloride.** $C_{14}H_{23}ClN_4O_4S$. ($M_r$ 378.9). *1092000.* [1784-03-8]. *N*-Tosyl-L-arginine methyl ester hydrochloride. Methyl (*S*)-5-guanidino-2-(4-methylbenzenesulfonamido)valerate hydrochloride.
$[\alpha]_D^{20}$: − 12 to − 16, determined on a 40 g/L solution.
mp: about 145 °C.

**Tosylarginine methyl ester hydrochloride solution.** *1092001.*
To 98.5 mg of *tosylarginine methyl ester hydrochloride R* add 5 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and shake to dissolve. Add 2.5 mL of *methyl red mixed solution R* and dilute to 25.0 mL with *water R*.

**Tosyl-lysyl-chloromethane hydrochloride.** $C_{14}H_{22}Cl_2N_2O_3S$. ($M_r$ 369.3). *1092100.* [4238-41-9]. *N*-Tosyl-L-lysyl-chloromethane hydrochloride. (3*S*)-7-Amino-1-chloro-3-(4-methylbenzenesulfonamido)heptan-2-one hydrochloride.
$[\alpha]_D^{20}$: − 7 to − 9, determined on a 20 g/L solution.
mp: about 155 °C, with decomposition.
$A_{1\,cm}^{1\%}$: 310 to 340, determined at 230 nm in *water R*.

**Tosylphenylalanylchloromethane.** $C_{17}H_{18}ClNO_3S$. ($M_r$ 351.9). *1092200.* [402-71-1]. *N*-Tosyl-L-phenylalanylchloromethane.

$[\alpha]_D^{20}$: – 85 to – 89, determined on a 10 g/L solution in *ethanol (96 per cent) R*.

mp: about 105 °C.

$A_{1\,cm}^{1\%}$: 290 to 320, determined at 228.5 nm in *ethanol (96 per cent) R*.

**Toxaphene.** *1132800.* [8001-35-2].

A mixture of polychloro derivatives.

mp: 65 °C to 90 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

**Tragacanth.** *1092300.* [9000-65-1].

See *Tragacanth (0532)*.

**Triacetin.** $C_9H_{14}O_6$. ($M_r$ 218.2). *1092400.* [102-76-1]. Propane-1,2,3-triyl triacetate. Glycerol triacetate.

Almost clear, colourless to yellowish liquid, soluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 1.16.

$n_D^{20}$: about 1.43.

bp: about 260 °C.

**Triamcinolone.** $C_{21}H_{27}FO_6$. ($M_r$ 394.4). *1111300.* [124-94-7]. 9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione.

A crystalline powder.

mp: 262 °C to 263 °C.

**Triamcinolone acetonide.** *1133100.* [76-25-5].

See *Triamcinolone acetonide (0533)*.

**Tribromophenol.** $C_6H_3Br_3O$. ($M_r$ 330.8). *1165300.* [118-79-6]. 2,4,6-Tribromophenol.

**Tributyl citrate.** $C_{18}H_{32}O_7$. ($M_r$ 360.4). *1152800.* [77-94-1]. Tributyl 2-hydroxypropane-1,2,3-tricarboxylate.

$d_4^{20}$: about 1.043.

$n_D^{20}$: about 1.445.

**Tributyl phosphate.** $C_{12}H_{27}O_4P$. ($M_r$ 266.3). *1179900.* [126-73-8]. Tributoxyphosphine oxide. Tributoxyphosphane oxide.

Colourless liquid, slightly soluble in water, soluble in the usual organic solvents.

$d_{25}^{25}$: about 0.976.

$n_D^{25}$: about 1.422.

bp: about 289 °C, with decomposition.

**Tributylphosphine.** $C_{12}H_{27}P$. ($M_r$ 202.3). *1187100.* [998-40-3].

Clear, colourless liquid.

bp: about 240 °C.

mp: about – 60 °C.

**Trichloroacetic acid.** $C_2HCl_3O_2$. ($M_r$ 163.4). *1092500.* [76-03-9].

Colourless crystals or a crystalline mass, very deliquescent, very soluble in water and in ethanol (96 per cent).

*Storage*: in an airtight container.

**Trichloroacetic acid solution.** *1092501.*

Dissolve 40.0 g of *trichloroacetic acid R* in *water R* and dilute to 1000.0 mL with the same solvent. Verify the concentration by titration with *0.1 M sodium hydroxide* and adjust if necessary to 40 ± 1 g/L.

**1,1,1-Trichloroethane.** $C_2H_3Cl_3$. ($M_r$ 133.4). *1092600.* [71-55-6]. Methylchloroform.

Non-flammable liquid, practically insoluble in water, soluble in acetone and in methanol.

$d_{20}^{20}$: about 1.34.

$n_D^{20}$: about 1.438.

bp: about 74 °C.

**Trichloroethylene.** $C_2HCl_3$. ($M_r$ 131.4). *1102100.* [79-01-6].

Colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 1.46.

$n_D^{20}$: about 1.477.

**Trichlorotrifluoroethane.** $C_2Cl_3F_3$. ($M_r$ 187.4). *1092700.* [76-13-1]. 1,1,2-Trichloro-1,2,2-trifluoroethane.

Colourless, volatile liquid, practically insoluble in water, miscible with acetone.

$d_{20}^{20}$: about 1.58.

*Distillation range* (*2.2.11*). Not less than 98 per cent distils between 47 °C and 48 °C.

**Tricine.** $C_6H_{13}NO_5$. ($M_r$ 179.2). *1138900.* [5704-04-1]. *N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Use electrophoresis-grade reagent.

mp: about 183 °C.

**Tricosane.** $C_{23}H_{48}$. ($M_r$ 324.6). *1092800.* [638-67-5].

White or almost white crystals, practically insoluble in water, soluble in hexane.

mp: about 48 °C.

**Tridecyl alcohol.** $C_{13}H_{28}O$. ($M_r$ 200.4). *1192500.* [112-70-9]. Tridecanol.

**Tridocosahexaenoin.** $C_{69}H_{98}O_6$. ($M_r$ 1023.5). *1144900.* [124596-98-1]. Triglyceride of docosahexaenoic acid (C22:6). Glycerol tridocosahexaenoate. Propane-1,2,3-triyl tri-(*all-Z*)-docosa-4,7,10,13,16,19-hexaenoate.

The reagent from Nu-Chek Prep, Inc. has been found suitable.

**Triethanolamine.** *1092900.* [102-71-6].

See *Trolamine (1577)*.

**Triethylamine.** $C_6H_{15}N$. ($M_r$ 101.2). *1093000.* [121-44-8]. *N*,*N*-Diethylethanamine.

Colourless liquid, slightly soluble in water at a temperature below 18.7 °C, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.727.

$n_D^{20}$: about 1.401.

bp: about 90 °C.

**Triethylamine R1.** $C_6H_{15}N$. ($M_r$ 101.2). *1093001.* [121-44-8]. *N*,*N*-Diethylethanamine.

Complies with the requirements prescribed for *triethylamine R* with the following additional requirements.

*Content*: minimum 99.5 per cent, determined by gas chromatography.

*Water*: maximum 0.1 per cent.

Use freshly distilled or from a freshly opened container.

**Triethylamine R2.** $C_6H_{15}N$. ($M_r$ 101.2). *1093002.* [121-44-8]. *N*,*N*-Diethylethanamine.

Complies with the requirements prescribed for *triethylamine R* and with the following additional requirements.

*Content*: minimum 99.5 per cent, determined by gas chromatography.

*Water*: maximum 0.2 per cent.

It is suitable for gradient elution in liquid chromatography.

*Use freshly distilled or from a freshly opened container.*

**Triethylenediamine.** $C_6H_{12}N_2$. ($M_r$ 112.2). *1093100.* 1,4-Diazabicyclo[*2.2.2*]octane.

Crystals, very hygroscopic, sublimes readily at room temperature, freely soluble in water, in acetone and in anhydrous ethanol.

bp: about 174 °C.

mp: about 158 °C.

*Storage*: in an airtight container.

**Triethyl phosphonoformate.** $C_7H_{15}O_5P$. ($M_r$ 210.2). *1132900.* [1474-78-8]. Ethyl (diethoxyphosphoryl)formate.

Colourless liquid.

$bp_{12\ mm}$: about 135 °C.

**Triflumuron.** $C_{15}H_{10}ClF_3N_2O_3$. ($M_r$ 358.7). *1180800.* [64628-44-0]. 1-(2-Chlorobenzoyl)-3-(4-triflumoromethoxyphenyl)urea.

White or almost white crystalline powder, practically insoluble in water, sparingly soluble in acetone and in methylene chloride.

**Trifluoroacetic acid.** $C_2HF_3O_2$. ($M_r$ 114.0). *1093200.* [76-05-1].

*Content*: minimum 99 per cent.

Liquid, miscible with acetone and with ethanol (96 per cent).

$d_{20}^{20}$: about 1.53.

bp: about 72 °C.

Use a grade suitable for protein sequencing.

*Storage*: in an airtight container.

**Trifluoroacetic anhydride.** $C_4F_6O_3$. ($M_r$ 210.0). *1093300.* [407-25-0].

Colourless liquid.

$d_{20}^{20}$: about 1.5.

**3-Trifluoromethylaniline.** $C_7H_6F_3N$. ($M_r$ 161.1). *1171900.* [98-16-8]. 3-(Trifluoromethyl)aniline. α,α,α-Trifluoro-*m*-toluidine. 3-(Trifluoromethyl)benzenamide.

Colourless liquid.

*Density*: 1.30 g/cm³ (20 °C).

**4-Trifluoromethylphenol.** $C_7H_5F_3O$. ($M_r$ 162.1). *1161700.* [402-45-9].

White or light yellow, crystalline solid or powder.

mp: about 46 °C.

**Trifluoropropylmethylpolysiloxane.** *1171600.*

Polysiloxane substituted with trifluoropropyl groups and methyl groups.

**Triglycine.** $C_6H_{11}N_3O_4$. ($M_r$ 189.2). *1192600.* [556-33-2]. 2-[[2-[(2-Aminoacetyl)amino]acetyl]amino]acetic acid. Glycylglycylglycine.

**Trigonelline hydrochloride.** $C_7H_8ClNO_2$. ($M_r$ 173.6). *1117400.* [6138-41-6]. 3-Carboxy-1-methylpyridinium chloride. Nicotinic acid *N*-methylbetaine hydrochloride.

Crystalline powder, very soluble in water, soluble in ethanol (96 per cent).

mp: about 258 °C.

**1,2,4-Trimethylbenzene.** $C_9H_{12}$. ($M_r$ 120.2). *1188600.* [95-63-6]. Pseudocumene.

**Trimethylpentane.** $C_8H_{18}$. ($M_r$ 114.2). *1093400.* [540-84-1]. Iso-octane. 2,2,4-Trimethylpentane.

Colourless, flammable liquid, practically insoluble in water, soluble in anhydrous ethanol.

$d_{20}^{20}$: 0.691 to 0.696.

$n_D^{20}$: 1.391 to 1.393.

*Distillation range* (*2.2.11*). Not less than 95 per cent distils between 98 °C and 100 °C.

*Trimethylpentane used in spectrophotometry complies with the following additional test.*

*Absorbance* (*2.2.25*): maximum 0.01 from 250 nm to 420 nm, determined using *water R* as compensation liquid.

**Trimethylpentane R1.** *1093401.*

Complies with the requirements prescribed for *trimethylpentane R* with the following modification.

*Absorbance (2.2.25).* Not more than 0.07 from 220 nm to 360 nm, determined using *water R* as the compensation liquid.

**Trimethylpentane for chromatography.** *1093402.*

Complies with the requirements prescribed for *trimethylpentane R* with the following additional requirement.

*Residue on evaporation*: maximum 2 mg/L.

***N,O*-bis(Trimethylsilyl)acetamide.** $C_8H_{21}NOSi_2$. ($M_r$ 203.4). *1093600.* [10416-59-8].

Colourless liquid.

$d_{20}^{20}$: about 0.83.

***N*-Trimethylsilylimidazole.** $C_6H_{12}N_2Si$. ($M_r$ 140.3). *1100500.* [18156-74-6]. 1-Trimethylsilylimidazole.

Colourless, hygroscopic liquid.

$d_{20}^{20}$: about 0.96.

$n_D^{20}$: about 1.48.

*Storage*: in an airtight container.

***N,O*-bis(Trimethylsilyl)trifluoroacetamide.** $C_8H_{18}F_3NOSi_2$. ($M_r$ 257.4). *1133200.* [25561-30-2]. BSTFA.

Colourless liquid.

$d_{20}^{20}$: about 0.97.

$n_D^{20}$: about 1.38.

$bp_{12mm}$: about 40 °C

**Trimethylsulfonium hydroxide.** $C_3H_{10}OS$. ($M_r$ 94.2). *1145000.* [17287-03-5].

$d_4^{20}$: about 0.81.

**Trimethyltin chloride.** $C_3H_9ClSn$. ($M_r$ 199.3). *1170900.* [1066-45-1]. Chlorotrimethylstannane.

**2,4,6-Trinitrobenzene sulfonic acid.** $C_6H_3N_3O_9S,3H_2O$. ($M_r$ 347.2). *1117500.* [2508-19-2].

White or almost white, crystalline powder, soluble in water.

mp: 190 °C to 195 °C.

**Triolein.** $C_{57}H_{104}O_6$. ($M_r$ 885.4). *1168200.* [122-32-7]. Propane-1,2,3-triyl tris[(9*Z*)-octadec-9-enoate]. *sn*-Glyceryl trioleate. Glycerol trioleate. Oleyl triglyceride.

*Content*: minimum 99.0 per cent.

**Triphenylmethanol.** $C_{19}H_{16}O$. ($M_r$ 260.3). *1093700.* [76-84-6]. Triphenylcarbinol.

Colourless crystals, practically insoluble in water, freely soluble in ethanol (96 per cent).

**Triphenyltetrazolium chloride.** $C_{19}H_{15}ClN_4$. ($M_r$ 334.8). *1093800.* [298-96-4]. 2,3,5-Triphenyl-2*H*-tetrazol-3-ium chloride.

Pale or dull-yellow powder, soluble in water, in acetone and in ethanol (96 per cent).

mp: about 240 °C, with decomposition.

*Storage*: protected from light.

**Triscyanoethoxypropane.** $C_{12}H_{17}N_3O_3$. ($M_r$ 251.3). *1093900.* 1,2,3-Tris(2-cyanoethoxy)propane.

Viscous, brown-yellow liquid, soluble in methanol. Used as a stationary phase in gas chromatography.

$d_{20}^{20}$: about 1.11.

*Viscosity* (*2.2.9*): about 172 mPa·s.

**1,3,5-Tris[3,5-di(1,1-dimethylethyl)-4-hydroxybenzyl]-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione.** $C_{48}H_{69}O_6N_3$. ($M_r$ 784.1). *1094000.* [27676-62-6].

White or almost white, crystalline powder.

mp: 218 °C to 222 °C.

**Tris[2,4-di(1,1-dimethylethyl)phenyl] phosphite.** $C_{42}H_{63}O_3P$. ($M_r$ 647). *1094100.* [31570-04-4].

White or almost white powder.

mp: 182 °C to 186 °C.

**Tris(hydroxymethyl)aminomethane.** *1094200.* [77-86-1].

See *Trometamol (1053)*.

**Tris(hydroxymethyl)aminomethane solution.** *1094201.*

A solution containing the equivalent of 24.22 g of $C_4H_{11}NO_3$ in 1000.0 mL.

**Tris(hydroxymethyl)aminomethane solution R1.** *1094202.*

Dissolve 60.6 mg of *tris(hydroxymethyl)aminomethane R* and 0.234 g of *sodium chloride R* in *water R* and dilute to 100 mL with the same solvent.

*Storage*: at 2 °C to 8 °C; use within 3 days.

**Tripotassium phosphate trihydrate.** $K_3PO_4,3H_2O$. ($M_r$ 266.3). *1155300.* [22763-03-7].

White or almost white crystalline powder, freely soluble in water.

**Trisodium phosphate dodecahydrate.** $Na_3PO_4,12H_2O$. ($M_r$ 380.1). *1094300.* [10101-89-0].

Colourless or white or almost white crystals, freely soluble in water.

**Trometamol.** *1094200.* [77-86-1].

See *Tris(hydroxymethyl)aminomethane R*.

**Tropic acid.** $C_9H_{10}O_3$. ($M_r$ 166.17). *1172000.* [529-64-6]. (2*RS*)-3-Hydroxy-2-phenylpropanoic acid.

**Troxerutin.** $C_{33}H_{42}O_{19}$. ($M_r$ 743). *1160300.* [7085-55-4]. Trihydroxyethylrutin. 3′,4′,7-Tris[*O*-(2-hydroxyethyl)]rutin. 2-[3,4-Bis(2-hydroxyethoxy)phenyl]-3-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-7-(2-hydroxyethoxy)-4*H*-1-benzopyran-4-one.

mp: 168 °C to 176 °C.

**Trypsin.** *1094500.* [9002-07-7].

A proteolytic enzyme obtained by activation of trypsinogen extracted from the pancreas of beef (*Bos taurus* L.).

White or almost white, crystalline or amorphous powder, sparingly soluble in water.

**Trypsin for peptide mapping.** *1094600.* [9002-07-7].

Trypsin of high purity treated to eliminate chymotryptic activity.

**Tryptophan.** $C_{11}H_{12}N_2O_2$. ($M_r$ 204.2). *1094700.* [73-22-3].

White or yellowish-white, crystalline powder or colourless crystals, slightly soluble in water, very slightly soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: about − 30, determined on a 10 g/L solution.

**Typhaneoside.** $C_{34}H_{42}O_{20}$. ($M_r$ 771). *1206000.* [104472-68-6]. 3-[6-Deoxy-α-L-mannopyranosyl-(1→2)-[6-deoxy-α-L-mannopyranosyl-(1→6)]-β-D-glucopyranosyloxy]-5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one.

**Tyramine.** $C_8H_{11}NO$. ($M_r$ 137.2). *1117600.* [51-67-2]. 4-(2-Aminoethyl)phenol.

Crystals, sparingly soluble in water, soluble in boiling anhydrous ethanol.

mp: 164 °C to 165 °C.

**Tyrosine.** $C_9H_{11}NO_3$. ($M_r$ 181.2). *1094800.* [60-18-4]. 2-Amino-3-(4-hydroxyphenyl)propionic acid.

White or almost white, crystalline powder, or colourless or white or almost white crystals, slightly soluble in water, practically insoluble in acetone and in anhydrous ethanol, soluble in dilute hydrochloric acid and in solutions of alkali hydroxides.

**Umbelliferone.** $C_9H_6O_3$. ($M_r$ 162.1). *1137500.* [93-35-6]. 7-Hydroxycoumarin. 7-Hydroxy-2*H*-1-benzopyran-2-one.

Needles from water.

mp: 225 °C to 228 °C.

**Undecanoic acid.** $C_{11}H_{22}O_2$. ($M_r$ 186.29). *1195200.* [112-37-8]. Hendecanoic acid. Undecylic acid.

mp: about 30 °C.

*Content*: minimum 97.0 per cent of $C_{11}H_{22}O_2$.

**Uracil.** $C_4H_4N_2O_2$. ($M_r$ 112.1). *1161800.* [66-22-8].

*Content*: minimum 95.0 per cent.

**Urea.** *1095000.* [57-13-6].

See *Urea (0743)*.

**Uridine.** $C_9H_{12}N_2O_6$. ($M_r$ 244.2). *1095100.* [58-96-8]. 1-β-D-Ribofuranosyluracil.

White or almost white, crystalline powder, soluble in water.

mp: about 165 °C.

**Ursolic acid.** $C_{30}H_{48}O_3$. ($M_r$ 456.7). *1141600.* [77-52-1]. 3β-Hydroxyurs-12-en-28-oic acid.

White or almost white powder, practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

$[\alpha]_D^{21}$: about 67.50, determined on a 10 g/L solution in a 56.1 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*.

mp: 285 °C to 288 °C.

**Valencene.** $C_{15}H_{24}$. ($M_r$ 204.4). *1152100.* [4630-07-3]. 4β*H*,5α-Eremophila-1(10),11-diene. (1*R*,7*R*,8a*S*)-1,8a-Dimethyl-7-(1-methylethenyl)-1,2,3,5,6,7,8,8a-octahydronaphthalene.

Oily, colourless or pale yellow liquid, with a characteristic odour, practically insoluble in water, soluble in ethanol (96 per cent).

$d_4^{20}$: about 0.918.

$n_D^{20}$: about 1.508.

bp: about 123 °C.

*Valencene used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Sweet orange oil (1811)*.

*Content*: minimum 80 per cent, calculated by the normalisation procedure.

**Valerenic acid.** $C_{15}H_{22}O_2$. ($M_r$ 234.3). *1165700.* [3569-10-6]. (2*E*)-3-[(4*S*,7*R*,7a*R*)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl]-2-methylprop-2-enoic acid.

mp: 134 °C to 138 °C.

**Valeric acid.** $C_5H_{10}O_2$. ($M_r$ 102.1). *1095200.* [109-52-4]. Pentanoic acid.

Colourless liquid, soluble in water, freely soluble in ethanol (96 per cent).

$d_{20}^{20}$: about 0.94.

$n_D^{20}$: about 1.409.

bp: about 186 °C.

**Valine.** *1185300.* [72-18-4].

See *Valine (0796)*.

**Vanillin.** *1095300.* [121-33-5].

See *Vanillin (0747)*.

**Vanillin reagent.** *1095301.*

Carefully add, dropwise, 2 mL of *sulfuric acid R* to 100 mL of a 10 g/L solution of *vanillin R* in *ethanol (96 per cent) R*.

*Storage*: use within 48 h.

**Vanillin solution, phosphoric.** *1095302.*

Dissolve 1.0 g of *vanillin R* in 25 mL of *ethanol (96 per cent) R*. Add 25 mL of *water R* and 35 mL of *phosphoric acid R*.

**Veratrole.** $C_8H_{10}O_2$. ($M_r$ 138.2). *1165400.* [91-16-7]. 1,2-Dimethoxybenzene.

$d_4^{20}$: 1.085.

$n_D^{20}$: 1.534.

bp: about 206 °C.

mp: about 22 °C.

**Verbenone.** $C_{10}H_{14}O$. ($M_r$ 150.2). *1140500.* [1196-01-6]. (1*S*,5*S*)-4,6,6-Trimethylbicyclo[3.1.1]hept-3-en-2-one.

Oil with a characteristic odour, practically insoluble in water, miscible with organic solvents.

$d_{20}^{20}$: about 0.978.

$n_D^{18}$: about 1.49.

$[\alpha]_D^{18}$: about + 249.6.

bp: 227 °C to 228 °C.

mp: about 6.5 °C.

*Verbenone used in gas chromatography complies with the following additional test.*

*Assay*. Gas chromatography (*2.2.28*) as prescribed in the monograph *Rosemary oil (1846)*.

*Content*: minimum 99 per cent, calculated by the normalisation procedure.

**Vinyl acetate.** $C_4H_6O_2$. ($M_r$ 86,10). *1111800.* [108-05-4]. Ethenyl acetate.

$d_{20}^{20}$: about 0.930.

bp: about 72 °C.

**Vinyl chloride.** $C_2H_3Cl$. ($M_r$ 62.5). *1095400.* [75-01-4].

Colourless gas, slightly soluble in organic solvents.

**Vinyl(1)phenyl(5)methyl(94)polysiloxane.** *1100000.*

Polysiloxane substituted with 1 per cent of vinyl groups, 5 per cent of phenyl groups and 94 per cent of methyl groups.

**Vinyl polymer for chromatography, amino alkyl.** *1191500.*

Spherical particles (5 µm) of a vinyl alcohol copolymer chemically modified by bonding of amino alkyl groups.

**Vinyl polymer for chromatography, octadecyl.** *1155400.*

Spherical particles (5 µm) of a vinyl alcohol copolymer chemically modified by bonding of octadecyl groups on the hydroxyl groups.

**Vinyl polymer for chromatography, octadecylsilyl.** *1121600.*

Spherical particles (5 µm) of a vinyl alcohol copolymer bonded to an octadecylsilane. Carbon content of 17 per cent.

**2-Vinylpyridine.** $C_7H_7N$. ($M_r$ 105.1). *1102200.* [100-69-6].

Yellow liquid, miscible in water.

$d_{20}^{20}$: about 0.97.

$n_D^{20}$: about 1.549.

**4-Vinylpyridine.** $C_7H_7N$. ($M_r$ 105.1). *1187200.* [100-43-6]. 4-Ethenylpyridine.

Clear, deep yellowish-brown liquid.

bp: 58-61 °C.

**1-Vinylpyrrolidin-2-one.** $C_6H_9NO$. ($M_r$ 111.1). *1111900.* [88-12-0]. 1-Ethenylpyrrolidin-2-one.

*Content*: minimum 99.0 per cent.

Clear colourless liquid.

*Water* (*2.5.12*): maximum 0.1 per cent, determined on 2.5 g. Use as the solvent, a mixture of 50 mL of *anhydrous methanol R* and 10 mL of *butyrolactone R*.

*Assay*. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Column*:

- *material*: fused-silica;
- *size*: *l* = 30 m, Ø = 0.5 mm;
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 80 |
| | 1 - 12 | 80 → 190 |
| | 12 - 27 | 190 |
| Injection port | | 190 |

*Detection*: flame-ionisation.

*Injection*: 0.3 µL of the substance to be examined.

Adjust the flow rate of the carrier gas so that the retention time of the peak corresponding to 1-vinylpyrrolidin-2-one is about 17 min.

**Vitexin.** $C_{21}H_{20}O_{10}$. ($M_r$ 432.4). *1133300.* [3681-93-4]. Apigenin 8-glucoside.

Yellow powder.

*Storage*: in an airtight container, protected from light.

**Water.** *1095500.* [7732-18-5].

See *Purified water (0008)*.

**Water R1.** *1095509.*

Prepared from *distilled water R* by multiple distillation. Remove carbon dioxide by boiling for at least 15 min before use in a boiling flask of fused silica or borosilicate glass and cool. Any other suitable method may be used. The boiling flask has been already used for the test or has been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h prior to first use. When tested immediately before use, *water R1* is neutral to *methyl red solution R*, i.e. it shall produce an orange-red (not a violet-red or yellow) colour corresponding to pH 5.5 ± 0.1 when 0.05 mL of *methyl red solution R* is added to 50 mL of the water to be examined.

*Conductivity*: maximum 1 $\mu S \cdot cm^{-1}$, determined at 25 °C by an in-line conductivity meter (see *Purified water (0008)*).

**Water, ammonium-free.** *1095501.*

To 100 mL of *water R* add 0.1 mL of *sulfuric acid R*. Distil using the apparatus described for the determination of *Distillation range* (*2.2.11*). Reject the first 10 mL and collect the following 50 mL.

**Water, carbon dioxide-free.** *1095502.*

*Water R* which has been boiled for a few minutes and protected from the atmosphere during cooling and storage or deionised *water R* with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C.

**Water, distilled.** *1095504.*

*Water R* prepared by distillation.

**Water, distilled, deionised.** *1095508.*

Deionised *water R* prepared by distillation with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C.

**Water for chromatography.** *1095503.*

Deionised water with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C, prepared by distillation, ion exchange, reverse osmosis or any other suitable method, using water that complies with the regulations on water intended for human consumption, as laid down by the competent authority.

Its quality is such that no significant interfering peaks or loss of sensitivity are observed when used in chromatography. Isocratic elution with UV detection at low wavelengths (i.e. less than 230 nm), with evaporative detectors (e.g. light scattering detector, particle counter detector, charged aerosol detector) or mass detectors, or gradient elution, may require the use of water with a total organic carbon content of maximum 5 ppb.

**Water for injections.** *1095505.*

See *Water for injections (0169)*.

**Water, nitrate-free.** *1095506.*

To 100 mL of *water R* add a few milligrams of *potassium permanganate R* and of *barium hydroxide R*. Distil using the apparatus described for the determination of *Distillation range* (*2.2.11*). Reject the first 10 mL and collect the following 50 mL.

**Water, particle-free.** *1095507.*

Filter *water R* through a membrane with a pore size of 0.22 µm.

**Weak cationic resin.** *1096000.*

Polymethacrylic resin, slightly acid, with carboxyl groups present in a protonated form.

*Particle size*: 75 µm to 160 µm.

*pH limits of use*: 5 to 14.

*Maximum temperature of use*: 120 °C.

**Wedelolactone.** $C_{16}H_{10}O_7$. ($M_r$ 314.3). *1187300.* [524-12-9]. 1,8,9-Trihydroxy-3-methoxy-6*H*-benzofuro[3,2-*c*][1]benzopyran-6-one.

**White beeswax.** *1196500.*

See *White beeswax (0069)*.

**Xanthydrol.** $C_{13}H_{10}O_2$. ($M_r$ 198.2). *1096100.* [90-46-0]. 9-Xanthenol.

*Content*: minimum 90.0 per cent.

White or pale-yellow powder, very slightly soluble in water, soluble in ethanol (96 per cent) and in glacial acetic acid. It is also available as a methanolic solution containing 90 g/L to 110 g/L of xanthydrol.

mp: about 123 °C.

*Assay.* In a 250 mL flask dissolve 0.300 g in 3 mL of *methanol R* or use 3.0 mL of solution. Add 50 mL of *glacial acetic acid R* and, dropwise with shaking, 25 mL of a 20 g/L solution of *urea R*. Allow to stand for 12 h, collect the precipitate on a sintered-glass filter (16) (*2.1.2*), wash with 20 mL of *ethanol (96 per cent) R*, dry in an oven at 100 °C to 105 °C and weigh.

1 g of precipitate is equivalent to 0.9429 g of xanthydrol.

*Storage*: protected from light. If a methanolic solution is used, store in small sealed ampoules and filter before use if necessary.

**Xanthydrol R1.** *1096101.*

Complies with the requirements prescribed for *xanthydrol R* with the following requirement.

*Content*: minimum 98.0 per cent of $C_{13}H_{10}O_2$.

**Xanthydrol solution.** *1096102.*

To 0.1 mL of a 100 g/L solution of *xanthydrol R* in *methanol R* add 100 mL of *anhydrous acetic acid R* and 1 mL of *hydrochloric acid R*. Allow to stand for 24 h before using.

**Xylene.** $C_8H_{10}$. ($M_r$ 106.2). *1096200.* [1330-20-7].

Mixture of isomers. Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.867.

$n_D^{20}$: about 1.497.

bp: about 138 °C.

***m*-Xylene.** $C_8H_{10}$. ($M_r$ 106.2). *1117700.* [108-38-3]. 1,3-Dimethylbenzene.

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.884.

$n_D^{20}$: about 1.497.

bp: about 139 °C.

mp: about − 47 °C.

***o*-Xylene.** $C_8H_{10}$. ($M_r$ 106.2). *1100600.* [95-47-6]. 1,2-Dimethylbenzene.

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

$d_{20}^{20}$: about 0.881.

$n_D^{20}$: about 1.505.

bp: about 144 °C.

mp: about − 25 °C.

**Xylenol orange.** $C_{31}H_{28}N_2Na_4O_{13}S$. ($M_r$ 761). *1096300.* [3618-43-7]. Tetrasodium 3,3′-(3*H*-2,1-benzoxathiol-3-ylidene)bis[(6-hydroxy-5-methyl-3,1-phenylene)methyleneiminobisacetate] *S,S*-dioxide.

Reddish-brown crystalline powder, soluble in water.

**Xylenol orange solution.** *1096302.*

Dissolve 50.8 mg of *xylenol orange R* in *water R* and dilute to 100.0 mL with the same solvent.

**Xylenol orange triturate.** *1096301.*

Triturate 1 part of *xylenol orange R* with 99 parts of *potassium nitrate R*.

*Test for sensitivity.* To 50 mL of *water R* add 1 mL of *dilute acetic acid R*, 50 mg of the xylenol orange triturate and 0.05 mL of *lead nitrate solution R*. Add

*hexamethylenetetramine R* until the colour changes from yellow to violet-red. After addition of 0.1 mL of *0.1 M sodium edetate* the colour changes to yellow.

**Xylitol.** $C_5H_{12}O_5$. ($M_r$ 152.1). *1190700.* [87-99-0].
White or almost white, crystalline powder or crystals.
*Content*: minimum 96.0 per cent.

**Xylose.** *1096400.* [58-86-6].
See *Xylose (1278)*.

**Zinc.** Zn. ($A_r$ 65.4). *1096500.* [7440-66-6].
*Content*: minimum 99.5 per cent.
Silver-white cylinders, granules, pellets or filings with a blue sheen.
*Arsenic* (*2.4.2, Method A*): maximum 0.2 ppm.
Dissolve 5.0 g in a mixture of the 15 mL of *hydrochloric acid R* and 25 mL of *water R* prescribed.

**Zinc, activated.** *1096501.*
Place the zinc cylinders or pellets to be activated in a conical flask and add a sufficient quantity of a 50 ppm solution of *chloroplatinic acid R* to cover the metal. Allow the metal to remain in contact with the solution for 10 min, wash, drain and dry immediately.
*Arsenic* (*2.4.2, Method A*). To 5 g of the activated zinc add 15 mL of *hydrochloric acid R*, 25 mL of *water R*, 0.1 mL of *stannous chloride solution R* and 5 mL of *potassium iodide solution R*. No colour is produced during the test.
*Activity*. The requirements of the suitability test for arsenic (*2.4.2, Method A*) are met.

**Zinc acetate.** $(C_2H_3O_2)_2Zn,2H_2O$. ($M_r$ 219.5). *1102300.* [5970-45-6]. Zinc acetate dihydrate.
Bright white or almost white crystals, slightly efflorescent, freely soluble in water, soluble in ethanol (96 per cent). It loses its crystallisation water at 100 °C.
$d_{20}^{20}$: about 1.735.
mp: about 237 °C.

**Zinc acetate solution.** *1102301.*
Mix 600 mL of *water R* with 150 mL of *glacial acetic acid R*, 54.9 g of *zinc acetate R* and stir to dissolve. Continue stirring while adding 150 mL of *concentrated ammonia R*. Cool to room temperature and adjust with *ammonia R* to pH 6.4. Dilute the mixture to 1 L with *water R*.

**Zinc chloride.** *1096600.* [7646-85-7].
See *Zinc chloride (0110)*.

**Zinc chloride-formic acid solution.** *1096601.*
Dissolve 20 g of *zinc chloride R* in 80 g of an 850 g/L solution of *anhydrous formic acid R*.

**Zinc chloride solution, iodinated.** *1096602.*
Dissolve 20 g of *zinc chloride R* and 6.5 g of *potassium iodide R* in 10.5 mL of *water R*. Add 0.5 g of *iodine R* and shake for 15 min. Filter if necessary.
*Storage*: protected from light.

**Zinc iodide and starch solution.** *1096502.*
To a solution of 2 g of *zinc chloride R* in 10 mL of *water R* add 0.4 g of *soluble starch R* and heat until the starch has dissolved. After cooling to room temperature add 1.0 mL of a colourless solution containing 0.10 g *zinc R* as filings and 0.2 g of *iodine R* in *water R*. Dilute the solution to 100 mL with *water R* and filter.
*Storage*: protected from light.
*Test for sensitivity*. Dilute 0.05 mL of *sodium nitrite solution R* to 50 mL with *water R*. To 5 mL of this solution add 0.1 mL of *dilute sulfuric acid R* and 0.05 mL of the zinc iodide and starch solution and mix. The solution becomes blue.

**Zinc oxide.** *1096700.* [1314-13-2].
See *Zinc oxide (0252)*.

**Zinc powder.** Zn. ($A_r$ 65.4). *1096800.* [7440-66-6].
*Content*: minimum 90.0 per cent.
Very fine, grey powder, soluble in *dilute hydrochloric acid R*.

**Zinc sulfate.** *1097000.* [7446-20-0].
See *Zinc sulfate (0111)*.

**Zirconyl nitrate.** A basic salt corresponding approximately to the formula $ZrO(NO_3)_2,2H_2O$. *1097200.* [14985-18-3].
A white or almost white powder or crystals, hygroscopic, soluble in water. The aqueous solution is a clear or at most slightly opalescent liquid.
*Storage*: in an airtight container.

**Zirconyl nitrate solution.** *1097201.*
A 1 g/L solution in a mixture of 40 mL of *water R* and 60 mL of *hydrochloric acid R*.

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## 4.1.2. STANDARD SOLUTIONS FOR LIMIT TESTS

**Acetaldehyde standard solution (100 ppm $C_2H_4O$).** *5000100.*
Dissolve 1.0 g of *acetaldehyde R* in *2-propanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 500.0 mL with *2-propanol R*. Prepare immediately before use.

**Acetaldehyde standard solution (100 ppm $C_2H_4O$) R1.** *5000101.*
Dissolve 1.0 g of *acetaldehyde R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 500.0 mL with *water R*. Prepare immediately before use.

**Aluminium standard solution (200 ppm Al).** *5000200.*
Dissolve in *water R* a quantity of *aluminium potassium sulfate R* equivalent to 0.352 g of $AlK(SO_4)_2,12H_2O$. Add 10 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*.

**Aluminium standard solution (100 ppm Al).** *5000203.*
Immediately before use, dilute with *water R* to 10 times its volume a solution containing 8.947 g of *aluminium chloride R* in 1000.0 mL of *water R*.

**Aluminium standard solution (10 ppm Al).** *5000201.*
Immediately before use, dilute with *water R* to 100 times its volume in a solution containing *aluminium nitrate R* equivalent to 1.39 g of $Al(NO_3)_3,9H_2O$ in 100.0 mL.

**Aluminium standard solution (5 ppm Al).** *5006600.*
Immediately before use, dilute with *water R* to 100 times its volume in a solution containing *aluminium nitrate R* equivalent to 0.695 g of $Al(NO_3)_3,9H_2O$ in 100.0 mL. Alternatively, use a commercially available standard solution containing a known amount of aluminium (5 ppm Al).

**Aluminium standard solution (2 ppm Al).** *5000202.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *aluminium potassium sulfate R* equivalent to 0.352 g of $AlK(SO_4)_2,12H_2O$ and 10 mL of *dilute sulfuric acid R* in 100.0 mL.

**Ammonium standard solution (100 ppm $NH_4$).** *5000300.*
Immediately before use, dilute to 25 mL with *water R* 10 mL of a solution containing *ammonium chloride R* equivalent to 0.741 g of $NH_4Cl$ in 1000 mL.

**Ammonium standard solution (3 ppm $NH_4$).** *5006100.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ammonium chloride R* equivalent to 0.889 g of $NH_4Cl$ in 1000.0 mL.

**Ammonium standard solution (2.5 ppm $NH_4$).** *5000301.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ammonium chloride R* equivalent to 0.741 g of $NH_4Cl$ in 1000.0 mL.

**Ammonium standard solution (1 ppm $NH_4$).** *5000302.*
Immediately before use, dilute *ammonium standard solution (2.5 ppm $NH_4$) R* to 2.5 times its volume with *water R*.

**Antimony standard solution (100 ppm Sb).** *5000401.*
Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $C_8H_4K_2O_{12}Sb_2,3H_2O$ in 500 mL of *1 M hydrochloric acid* and dilute the clear solution to 1000 mL with *water R*.

**Antimony standard solution (1 ppm Sb).** *5000400.*
Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $C_8H_4K_2O_{12}Sb_2,3H_2O$ in 20 mL of *hydrochloric acid R1* and dilute the clear solution to 100.0 mL with *water R*. To 10.0 mL of this solution add 200 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*. To 100.0 mL of this solution add 300 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*. Prepare the dilute solutions immediately before use.

**Arsenic standard solution (10 ppm As).** *5000500.*
Immediately before use, dilute with *water R* to 100 times its volume a solution prepared by dissolving *arsenious trioxide R* equivalent to 0.330 g of $As_2O_3$ in 5 mL of *dilute sodium hydroxide solution R* and diluting to 250.0 mL with *water R*.

**Arsenic standard solution (1 ppm As).** *5000501.*
Immediately before use, dilute *arsenic standard solution (10 ppm As) R* to 10 times its volume with *water R*.

**Barium standard solution (0.1 per cent Ba).** *5000601.*
Dissolve *barium chloride R* equivalent to 0.178 g of $BaCl_2,2H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Barium standard solution (50 ppm Ba).** *5000600.*
Immediately before use, dilute with *distilled water R* to 20 times its volume a solution in *distilled water R* containing *barium chloride R* equivalent to 0.178 g of $BaCl_2,2H_2O$ in 100.0 mL.

**Barium standard solution (2 ppm Ba).** *5005600.*
Immediately before use, dilute *barium standard solution (50 ppm Ba) R* to 25 times its volume with *distilled water R*.

**Bismuth standard solution (100 ppm Bi).** *5005300.*
Dissolve *bismuth subnitrate R* equivalent to 0.500 g of Bi in 50 mL of *nitric acid R* and dilute to 500.0 mL with *water R*. Dilute the solution to 10 times its volume with *dilute nitric acid R* immediately before use.

**Cadmium standard solution (0.1 per cent Cd).** *5000700.*
Dissolve *cadmium R* equivalent to 0.100 g of Cd in the smallest necessary amount of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 mL with a 1 per cent *V/V* solution of *hydrochloric acid R*.

**Cadmium standard solution (10 ppm Cd) .** *5000701.*
Immediately before use, dilute *cadmium standard solution (0.1 per cent Cd) R* to 100 times its volume with a 1 per cent *V/V* solution of *hydrochloric acid R*.

**Calcium standard solution (400 ppm Ca).** *5000800.*
Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 1.000 g of $CaCO_3$ and 23 mL of *1 M hydrochloric acid* in 100.0 mL.

**Calcium standard solution (100 ppm Ca).** *5000801.*
Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ and 3 mL of *acetic acid R* in 250.0 mL.

**Calcium standard solution (100 ppm Ca) R1.** *5000804.*
Immediately before use, dilute with *water R* to 10 times its volume a solution containing *anhydrous calcium chloride R* equivalent to 2.769 g of $CaCl_2$ in 1000.0 mL of *dilute hydrochloric acid R*.

**Calcium standard solution (100 ppm Ca), alcoholic.** *5000802.*
Immediately before use, dilute with *ethanol (96 per cent) R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 2.50 g of $CaCO_3$ and 12 mL of *acetic acid R* in 1000.0 mL.

**Calcium standard solution (10 ppm Ca).** *5000803.*
Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ and 3 mL of *acetic acid R* in 250.0 mL.

**Chloride standard solution (50 ppm Cl).** *5004100.*
Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 mL.

**Chloride standard solution (8 ppm Cl).** *5000900.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 1.32 g of NaCl in 1000.0 mL.

**Chloride standard solution (5 ppm Cl).** *5000901.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 mL.

**Chromium liposoluble standard solution (1000 ppm Cr).** *5004600.*
A chromium (metal) organic compound in an oil.

**Chromium standard solution (0.1 per cent Cr).** *5001002.*
Dissolve *potassium dichromate R* equivalent to 2.83 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

**Chromium standard solution (100 ppm Cr).** *5001000.*
Dissolve *potassium dichromate R* equivalent to 0.283 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

**Chromium standard solution (0.1 ppm Cr).** *5001001.*
Immediately before use, dilute *chromium standard solution (100 ppm Cr) R* to 1000 times its volume with *water R*.

**Cobalt standard solution (100 ppm Co).** *5004300.*
Dissolve *cobalt nitrate R* equivalent to 0.494 g of $Co(NO_3)_2,6H_2O$ in 500 mL of *1 M nitric acid* and dilute the clear solution to 1000 mL with *water R*.

**Copper liposoluble standard solution (1000 ppm Cu).** *5004700.*
A copper (metal) organic compound in an oil.

**Copper standard solution (0.1 per cent Cu).** *5001100.*
Dissolve *copper sulfate pentahydrate R* equivalent to 0.393 g of $CuSO_4,5H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

**Copper standard solution (0.1 per cent Cu) for ICP.** *5006300.*

A copper standard solution (1000 mg/L) suitable for inductively coupled plasma (ICP) applications and traceable to national or international standards.

**Copper standard solution (10 ppm Cu).** *5001101.*

Immediately before use, dilute *copper standard solution (0.1 per cent Cu) R* to 100 times its volume with *water R*.

**Copper standard solution (0.1 ppm Cu).** *5001102.*

Immediately before use, dilute *copper standard solution (10 ppm Cu) R* to 100 times its volume with *water R*.

**Ferrocyanide standard solution (100 ppm $Fe(CN)_6$).** *5001200.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium ferrocyanide R* equivalent to 0.20 g of $K_4Fe(CN)_6,3H_2O$ in 100.0 mL.

**Ferricyanide standard solution (50 ppm $Fe(CN)_6$).** *5001300.*

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium ferricyanide R* equivalent to 0.78 g of $K_3Fe(CN)_6$ in 100.0 mL.

**Fluoride standard solution (10 ppm F).** *5001400.*

Dissolve in *water R sodium fluoride R* previously dried at 300 °C for 12 h, equivalent to 0.442 g of NaF, and dilute to 1000.0 mL with the same solvent (1 mL = 0.2 mg F). Store in a polyethylene container. Immediately before use, dilute the solution to 20 times its volume with *water R*.

**Fluoride standard solution (1 ppm F).** *5001401.*

Immediately before use, dilute *fluoride standard solution (10 ppm F) R* to 10 times its volume with *water R*.

**Formaldehyde standard solution (5 ppm $CH_2O$).** *5001500.*

Immediately before use, dilute with *water R* to 200 times its volume a solution containing 1.0 g of $CH_2O$ per litre prepared from *formaldehyde solution R*.

**Germanium standard solution (100 ppm Ge).** *5004400.*

Dissolve *ammonium hexafluorogermanate(IV) R* equivalent to 0.307 g of $(NH_4)_2GeF_6$ in a 0.01 per cent *V/V* solution of *hydrofluoric acid R*. Dilute the clear solution to 1000 mL with *water R*.

**Glyoxal standard solution (20 ppm $C_2H_2O_2$).** *5003700.*

In a 100 mL graduated flask weigh a quantity of *glyoxal solution R* corresponding to 0.200 g of $C_2H_2O_2$ and make up to volume with *anhydrous ethanol R*. Immediately before use dilute the solution to 100 times its volume with the same solvent.

**Glyoxal standard solution (2 ppm $C_2H_2O_2$).** *5003701.*

Immediately before use, dilute *glyoxal standard solution (20 ppm $C_2H_2O_2$) R* to 10 times its volume with *anhydrous ethanol R*.

**Hydrogen peroxide standard solution (2 ppm $H_2O_2$).** *5005200.*

Dilute 10.0 mL of *dilute hydrogen peroxide solution R* to 300.0 mL with *water R*. Dilute 2.0 mL of this solution to 1000.0 mL with *water R*. Prepare immediately before use.

**Iodide standard solution (10 ppm I).** *5003800.*

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium iodide R* equivalent to 0.131 g of KI in 100.0 mL.

**Iron standard solution (0.1 per cent Fe).** *5001605.*

Dissolve 0.100 g of Fe in the smallest amount necessary of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 mL with *water R*.

**Iron standard solution (250 ppm Fe).** *5001606.*

Immediately before use, dilute with *water R* to 40 times its volume a solution containing 4.840 g of *ferric chloride R* in a 150 g/L solution of *hydrochloric acid R* diluted to 100.0 mL.

**Iron standard solution (20 ppm Fe).** *5001600.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *ferric ammonium sulfate R* equivalent to 0.863 g of $FeNH_4(SO_4)_2,12H_2O$ and 25 mL of *dilute sulfuric acid R* in 500.0 mL.

**Iron standard solution (10 ppm Fe).** *5001601.*

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ferrous ammonium sulfate R* equivalent to 7.022 g of $Fe(NH_4)_2(SO_4)_2,6H_2O$ and 25 mL of *dilute sulfuric acid R* in 1000.0 mL.

**Iron standard solution (8 ppm Fe).** *5001602.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 80 mg of *iron R* and 50 mL of *hydrochloric acid R* (220 g/L HCl) in 1000.0 mL.

**Iron standard solution (2 ppm Fe).** *5001603.*

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 10 times its volume with *water R*.

**Iron standard solution (1 ppm Fe).** *5001604.*

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 20 times its volume with *water R*.

**Lead liposoluble standard solution (1000 ppm Pb).** *5004800.*

A lead (metal) organic compound in an oil.

**Lead standard solution (0.1 per cent Pb).** *5001700.*

Dissolve *lead nitrate R* equivalent to 0.400 g of $Pb(NO_3)_2$ in *water R* and dilute to 250.0 mL with the same solvent.

**Lead standard solution (100 ppm Pb).** *5001701.*

Immediately before use, dilute *lead standard solution (0.1 per cent Pb) R* to 10 times its volume with *water R*.

**Lead standard solution (10 ppm Pb).** *5001702.*

Immediately before use, dilute *lead standard solution (100 ppm Pb) R* to 10 times its volume with *water R*.

**Lead standard solution (10 ppm Pb) R1.** *5001706.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 0.160 g of *lead nitrate R* in 100 mL of *water R*, to which is added 1 mL of *lead-free nitric acid R* and dilute to 1000.0 mL.

**Lead standard solution (2 ppm Pb).** *5001703.*

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 5 times its volume with *water R*.

**Lead standard solution (1 ppm Pb).** *5001704.*

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 10 times its volume with *water R*.

**Lead standard solution (0.25 ppm Pb).** *5006000.*

Immediately before use, dilute *lead standard solution (1 ppm Pb) R* to 4 times its volume with *water R*.

**Lead standard solution (0.1 ppm Pb).** *5001705.*

Immediately before use, dilute *lead standard solution (1 ppm Pb) R* to 10 times its volume with *water R*.

**Lutetium standard solution (20 ppm Lu).** *5006500.*

Immediately before use, dissolve 0.445 g of *lutetium chloride hexahydrate R* in a mixture of equal volumes of *heavy metal-free nitric acid R* and *water R*, and dilute to 100.0 mL with the same mixture of solvents.

Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

**Magnesium standard solution (0.1 per cent Mg).** *5001803.*
Dissolve *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4,7H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Magnesium standard solution (1000 ppm Mg).** *5006200.*
Dissolve 5.275 g of *magnesium nitrate R* in 16 mL of *dilute nitric acid R* and dilute to 500.0 mL with *water R*.
*Standardisation*: carry out the determination of magnesium by complexometry (*2.5.11*).

**Magnesium standard solution (100 ppm Mg).** *5001800.*
Immediately before use, dilute with *water R* to 10 times its volume a solution containing *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4,7H_2O$ in 100.0 mL.

**Magnesium standard solution (10 ppm Mg).** *5001801.*
Immediately before use, dilute *magnesium standard solution (100 ppm Mg) R* to 10 times its volume with *water R*.

**Magnesium standard solution (10 ppm Mg) R1.** *5001802.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing 8.365 g of *magnesium chloride R* in 1000.0 mL of *dilute hydrochloric acid R*.

**Manganese standard solution (1000 ppm Mn).** *5005800.*
Dissolve *manganese sulfate R* equivalent to 3.08 g of $MnSO_4,H_2O$ in 500 mL of *1 M nitric acid* and dilute the solution to 1000 mL with *water R*.

**Manganese standard solution (100 ppm Mn).** *5004500.*
Dissolve *manganese sulfate R* equivalent to 0.308 g of $MnSO_4,H_2O$ in 500 mL of *1 M nitric acid* and dilute the clear solution to 1000 mL with *water R*.

**Mercury standard solution (1000 ppm Hg).** *5001900.*
Dissolve *mercuric chloride R* equivalent to 1.354 g of $HgCl_2$ in 50 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R*.

**Mercury standard solution (10 ppm Hg).** *5001901.*
Immediately before use, dilute with water to 100 times its volume a solution containing *mercuric chloride R* equivalent to 0.338 g of $HgCl_2$ in 250.0 mL.

**Nickel liposoluble standard solution (1000 ppm Ni).** *5004900.*
A nickel (metal) organic compound in an oil.

**Nickel standard solution (10 ppm Ni).** *5002000.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *nickel sulfate R* equivalent to 4.78 g of $NiSO_4,7H_2O$ in 1000.0 mL.

**Nickel standard solution (5 ppm Ni).** *5005900.*
Immediately before use dilute *nickel standard solution (10 ppm Ni) R* to twice its volume with *water for chromatography R*.

**Nickel standard solution (0.2 ppm Ni).** *5002002.*
Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 50 times its volume with *water R*.

**Nickel standard solution (0.1 ppm Ni).** *5002001.*
Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 100 times its volume with *water R*.

**Nitrate standard solution (100 ppm $NO_3$).** *5002100.*
Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium nitrate R* equivalent to 0.815 g of $KNO_3$ in 500.0 mL.

**Nitrate standard solution (10 ppm $NO_3$).** *5002101.*
Immediately before use, dilute *nitrate standard solution (100 ppm $NO_3$) R* to 10 times its volume with *water R*.

**Nitrate standard solution (2 ppm $NO_3$).** *5002102.*
Immediately before use, dilute *nitrate standard solution (10 ppm $NO_3$) R* to 5 times its volume with *water R*.

**Palladium standard solution (500 ppm Pd).** *5003600.*
Dissolve 50.0 mg of *palladium R* in 9 mL of *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

**Palladium standard solution (20 ppm Pd).** *5003602.*
Dissolve 0.333 g of *palladium chloride R* in 2 mL of warm *hydrochloric acid R*. Dilute the solution to 1000.0 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Immediately before use dilute to 10 times its volume with *water R*.

**Palladium standard solution (0.5 ppm Pd).** *5003601.*
Dilute 1 mL of *palladium standard solution (500 ppm Pd) R* to 1000 mL with a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R*.

**Phosphate standard solution (200 ppm $PO_4$).** *5004200.*
Dissolve *potassium dihydrogen phosphate R* equivalent to 0.286 g of $KH_2PO_4$ in *water R* and dilute to 1000.0 mL with the same solvent.

**Phosphate standard solution (5 ppm $PO_4$).** *5002200.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium dihydrogen phosphate R* equivalent to 0.716 g of $KH_2PO_4$ in 1000.0 mL.

**Platinum standard solution (30 ppm Pt).** *5002300.*
Immediately before use, dilute with *1 M hydrochloric acid* to 10 times its volume a solution containing 80 mg of *chloroplatinic acid R* in 100.0 mL of *1 M hydrochloric acid*.

**Potassium standard solution (0.2 per cent K).** *5002402.*
Dissolve *dipotassium sulfate R* equivalent to 0.446 g of $K_2SO_4$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Potassium standard solution (600 ppm K).** *5005100.*
Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulfate R* equivalent to 2.676 g of $K_2SO_4$ in 100.0 mL.

**Potassium standard solution (100 ppm K).** *5002400.*
Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulfate R* equivalent to 0.446 g of $K_2SO_4$ in 100.0 mL.

**Potassium standard solution (20 ppm K).** *5002401.*
Immediately before use, dilute *potassium standard solution (100 ppm K) R* to 5 times its volume with *water R*.

**Scandium standard solution (0.1 per cent Sc) for ICP.** *5006400.*
A scandium standard solution (1000 mg/L) suitable for inductively coupled plasma (ICP) applications and traceable to national or international standards.

**Selenium standard solution (100 ppm Se).** *5002500.*
Dissolve 0.100 g of *selenium R* in 2 mL of *nitric acid R*. Evaporate to dryness. Take up the residue in 2 mL of *water R* and evaporate to dryness; carry out three times. Dissolve the residue in 50 mL of *dilute hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

**Selenium standard solution (1 ppm Se).** *5002501.*
Immediately before use, dilute with *water R* to 40 times its volume a solution containing *selenious acid R* equivalent to 6.54 mg of $H_2SeO_3$ in 100.0 mL.

**Silver standard solution (5 ppm Ag).** *5002600.*
Immediately before use, dilute with *water R* to 100 times its volume a solution containing *silver nitrate R* equivalent to 0.790 g of $AgNO_3$ in 1000.0 mL.

**Sodium standard solution (1000 ppm Na).** *5005700.*

Dissolve a quantity of *anhydrous sodium carbonate R* equivalent to 2.305 g of $Na_2CO_3$ in a mixture of 25 mL of *water R* and 25 mL of *nitric acid R* and dilute to 1000.0 mL with *water R*.

**Sodium standard solution (200 ppm Na).** *5002700.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.509 g of NaCl in 100.0 mL.

**Sodium standard solution (50 ppm Na).** *5002701.*

Dilute the *sodium standard solution (200 ppm Na) R* to four times its volume with *water R*.

**Strontium standard solution (1.0 per cent Sr).** *5003900.*

Cover with *water R*, *strontium carbonate R* equivalent to 1.6849 g of $SrCO_3$. Cautiously add *hydrochloric acid R* until all the solid has dissolved and there is no sign of further effervescence. Dilute to 100.0 mL with *water R*.

**Sulfate standard solution (100 ppm $SO_4$).** *5002802.*

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *dipotassium sulfate R* equivalent to 0.181 g of $K_2SO_4$ in 100.0 mL.

**Sulfate standard solution (10 ppm $SO_4$).** *5002800.*

Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *dipotassium sulfate R* equivalent to 0.181 g of $K_2SO_4$ in 100.0 mL.

**Sulfate standard solution (10 ppm $SO_4$) R1.** *5002801.*

Immediately before use, dilute with *ethanol (30 per cent V/V) R* to 100 times its volume a solution containing *dipotassium sulfate R* equivalent to 0.181 g of $K_2SO_4$ in 100.0 mL of *ethanol (30 per cent V/V) R*.

**Sulfite standard solution (80 ppm $SO_2$).** *5005500.*

Dissolve 3.150 g of *anhydrous sodium sulfite R* in freshly prepared *distilled water R* and dilute to 100.0 mL with the same solvent. Dilute 0.5 mL to 100.0 mL with freshly prepared *distilled water R*.

**Sulfite standard solution (1.5 ppm $SO_2$).** *5002900.*

Dissolve *sodium metabisulfite R* equivalent to 0.152 g of $Na_2S_2O_5$ in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*. To 3.0 mL of the resulting solution, add 4.0 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

**Thallium standard solution (10 ppm Tl).** *5003000.*

Dissolve *thallous sulfate R* equivalent to 0.1235 g of $Tl_2SO_4$ in a 9 g/L solution of *sodium chloride R* and dilute to 1000.0 mL with the same solution. Dilute 10.0 mL of the solution to 100.0 mL with the 9 g/L solution of *sodium chloride R*.

**Tin liposoluble standard solution (1000 ppm Sn).** *5005000.*

A tin (metal) organic compound in an oil.

**Tin standard solution (5 ppm Sn).** *5003100.*

Dissolve *tin R* equivalent to 0.500 g of Sn in a mixture of 5 mL of *water R* and 25 mL of *hydrochloric acid R* and dilute to 1000.0 mL with *water R*. Dilute the solution to 100 times its volume with a 2.5 per cent *V/V* solution of *hydrochloric acid R* immediately before use.

**Tin standard solution (0.1 ppm Sn).** *5003101.*

Immediately before use, dilute *tin standard solution (5 ppm Sn) R* to 50 times its volume with *water R*.

**Titanium standard solution (100 ppm Ti).** *5003200.*

Dissolve 100.0 mg of *titanium R* in 100 mL of *hydrochloric acid R* diluted to 150 mL with *water R*, heating if necessary. Allow to cool and dilute to 1000 mL with *water R*.

**Vanadium standard solution (1 g/L V).** *5003300.*

Dissolve in *water R ammonium vanadate R* equivalent to 0.230 g of $NH_4VO_3$ and dilute to 100.0 mL with the same solvent.

**Zinc standard solution (5 mg/mL Zn).** *5003400.*

Dissolve 3.15 g of *zinc oxide R* in 15 mL of *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

**Zinc standard solution (100 ppm Zn).** *5003401.*

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *zinc sulfate R* equivalent to 0.440 g of $ZnSO_4,7H_2O$ and 1 mL of *acetic acid R* in 100.0 mL.

**Zinc standard solution (10 ppm Zn).** *5003402.*

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 10 times its volume with *water R*.

**Zinc standard solution (5 ppm Zn).** *5003403.*

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 20 times its volume with *water R*.

**Zirconium standard solution (1 g/L Zr).** *5003500.*

Dissolve *zirconyl nitrate R* equivalent to 0.293 g of $ZrO(NO_3)_2,2H_2O$ in a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

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## 4.1.3. BUFFER SOLUTIONS

**Buffered acetone solution.** *4000100.*

Dissolve 8.15 g of *sodium acetate R* and 42 g of *sodium chloride R* in *water R*, add 68 mL of *0.1 M hydrochloric acid* and 150 mL of *acetone R* and dilute to 500 mL with *water R*.

**Buffer solution pH 2.0.** *4000200.*

Dissolve 6.57 g of *potassium chloride R* in *water R* and add 119.0 mL of *0.1 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

**0.125 M Phosphate buffer solution pH 2.0.** *4015600.*

Dissolve 17.0 g of *potassium dihydrogen phosphate R* and 17.8 g of *anhydrous disodium hydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent. If necessary adjust the pH with *phosphoric acid R*.

**Phosphate buffer solution pH 2.0.** *4007900.*

Dissolve 8.95 g of *disodium hydrogen phosphate dodecahydrate R* and 3.40 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary adjust the pH with *phosphoric acid R*.

**Sulfate buffer solution pH 2.0.** *4008900.*

Dissolve 132.1 g of *ammonium sulfate R* in *water R* and dilute to 500.0 mL with the same solvent (Solution A). Carefully and with constant cooling stir 14 mL of *sulfuric acid R* into about 400 mL of *water R*; allow to cool and dilute to 500.0 mL with *water R* (Solution B). Mix equal volumes of solutions A and B. Adjust the pH if necessary.

**Buffer solution pH 2.2.** *4010500.*

Mix 6.7 mL of *phosphoric acid R* with 55.0 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R*.

**Buffer solution pH 2.5.** *4000300.*

Dissolve 100 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*; adjust to pH 2.5 with *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

**Buffer solution pH 2.5 R1.** *4000400.*

To 4.9 g of *dilute phosphoric acid R* add 250 mL of *water R*. Adjust the pH with *dilute sodium hydroxide solution R* and dilute to 500.0 mL with *water R*.

**0.2 M Phosphate buffer solution pH 2.5.** *4014100.*

Dissolve 27.2 g of *potassium dihydrogen phosphate R* in about 900 mL of *water R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1.0 L with *water R*.

**Phosphate buffer solution pH 2.8.** *4010600.*

Dissolve 7.8 g of *sodium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 2.8 with *phosphoric acid R* and dilute to 1000 mL with the same solvent.

**Buffer solution pH 3.0.** *4008000.*

Dissolve 21.0 g of *citric acid monohydrate R* in 200 mL of *1 M sodium hydroxide* and dilute to 1000 mL with *water R*. Dilute 40.3 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

**0.25 M Citrate buffer solution pH 3.0.** *4012600.*

Dissolve 5.3 g of *citric acid monohydrate R* in 80 mL of *water R*. Adjust the pH with *1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

**0.1 M Phosphate buffer solution pH 3.0.** *4011500.*

Dissolve 12.0 g of *anhydrous sodium dihydrogen phosphate R* in *water R*, adjust the pH with *dilute phosphoric acid R1* and dilute to 1000 mL with *water R*.

**Phosphate buffer solution pH 3.0.** *4000500.*

Mix 0.7 mL of *phosphoric acid R* with 100 mL of *water R*. Dilute to 900 mL with the same solvent. Adjust to pH 3.0 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water R*.

**Phosphate buffer solution pH 3.0 R1.** *4010000.*

Dissolve 3.40 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*. Adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000.0 mL with *water R*.

**Phosphate buffer solution pH 3.2.** *4008100.*

To 900 mL of a 4 g/L solution of *sodium dihydrogen phosphate R*, add 100 mL of a 2.5 g/L solution of *phosphoric acid R*. Adjust the pH if necessary.

**Phosphate buffer solution pH 3.2 R1.** *4008500.*

Adjust a 35.8 g/L solution of *disodium hydrogen phosphate dodecahydrate R* to pH 3.2 with *dilute phosphoric acid R*. Dilute 100.0 mL of the solution to 2000.0 mL with *water R*.

**Phosphate buffer solution pH 3.25.** *4014900.*

Dissolve about 1.36 g of *potassium dihydrogen phosphate R* in 1000 mL of *water R* and adjust to pH 3.25 ± 0.05 with *dilute phosphoric acid R*. Filter through a membrane filter (nominal pore size 0.45 µm or finer).

**Phosphate buffer solution pH 3.4.** *4015800.*

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH with *phosphoric acid R*.

**Buffer solution pH 3.5.** *4000600.*

Dissolve 25.0 g of *ammonium acetate R* in 25 mL of *water R* and add 38.0 mL of *hydrochloric acid R1*. Adjust the pH if necessary with *dilute hydrochloric acid R* or *dilute ammonia R1*. Dilute to 100.0 mL with *water R*.

**Phosphate buffer solution pH 3.5.** *4000700.*

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH with *phosphoric acid R*.

**Buffer solution pH 3.6.** *4000800.*

To 250.0 mL of *0.2 M potassium hydrogen phthalate R* add 11.94 mL of *0.2 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

**Buffer solution pH 3.7.** *4000900.*

To 15.0 mL of *acetic acid R* add 60 mL of *ethanol (96 per cent) R* and 20 mL of *water R*. Adjust to pH 3.7 by the addition of *ammonia R*. Dilute to 100.0 mL with *water R*.

**Buffered copper sulfate solution pH 4.0.** *4001000.*

Dissolve 0.25 g of *copper sulfate pentahydrate R* and 4.5 g of *ammonium acetate R* in *dilute acetic acid R* and dilute to 100.0 mL with the same solvent.

**0.1 M Sodium acetate buffer solution pH 4.0.** *4013800.*

Dissolve 822 mg of *sodium acetate R* in 100 mL of *water R* (solution A). Dilute 1.44 mL of *glacial acetic acid R* in 250 mL of *water R* (solution B). Titrate 100 mL of solution B using about 20 mL of solution A.

**Acetate buffer solution pH 4.4.** *4001100.*

Dissolve 136 g of *sodium acetate R* and 77 g of *ammonium acetate R* in *water R* and dilute to 1000.0 mL with the same solvent; add 250.0 mL of *glacial acetic acid R* and mix.

**Phthalate buffer solution pH 4.4.** *4001200.*

Dissolve 2.042 g of *potassium hydrogen phthalate R* in 50 mL of *water R*, add 7.5 mL of *0.2 M sodium hydroxide* and dilute to 200.0 mL with *water R*.

**Acetate buffer solution pH 4.5.** *4012500.*

Dissolve 77.1 g of *ammonium acetate R* in *water R*. Add 70 mL of *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

**0.5 M Ammonium acetate buffer solution pH 4.5.** *4014200.*

Mix 14.3 mL of *glacial acetic acid R* and 470 mL of *water R* and adjust to pH 4.5 with *concentrated ammonia R*. Dilute to 500.0 mL with *water R*.

**0.05 M Phosphate buffer solution pH 4.5.** *4009000.*

Dissolve 6.80 g of *potassium dihydrogen phosphate R* in 1000.0 mL of *water R*. The pH of the solution is 4.5.

**Sodium acetate buffer solution pH 4.5.** *4010100.*

Dissolve 63 g of *anhydrous sodium acetate R* in *water R*, add 90 mL *acetic acid R* and adjust to pH 4.5, and dilute to 1000 mL with *water R*.

**Acetate buffer solution pH 4.6.** *4001400.*

Dissolve 5.4 g of *sodium acetate R* in 50 mL of *water R*, add 2.4 g of *glacial acetic acid R* and dilute to 100.0 mL with *water R*. Adjust the pH if necessary.

**Succinate buffer solution pH 4.6.** *4001500.*

Disssolve 11.8 g of *succinic acid R* in a mixture of 600 mL of *water R* and 82 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

**Acetate buffer solution pH 4.7.** *4001600.*

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of this solution with 250 mL of *dilute acetic acid R*. Shake twice with a freshly prepared, filtered, 0.1 g/L solution of *dithizone R* in *chloroform R*. Shake with *carbon tetrachloride R* until the extract is colourless. Filter the aqueous layer to remove traces of carbon tetrachloride.

**Acetate buffer solution pH 4.7 R1.** *4013600.*

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of this solution with 250 mL of *dilute acetic acid R*.

**Acetate buffer solution pH 5.0.** *4009100.*

To 120 mL of a 6 g/L solution of *glacial acetic acid R* add 100 mL of *0.1 M potassium hydroxide* and about 250 mL of *water R*. Mix. Adjust the pH to 5.0 with a 6 g/L solution of *acetic acid R* or with *0.1 M potassium hydroxide* and dilute to 1000.0 mL with *water R*.

**Citrate buffer solution pH 5.0.** *4010700.*

Prepare a solution containing 20.1 g/L of *citric acid monohydrate R* and 8.0 g/L of *sodium hydroxide R*. Adjust the pH with *dilute hydrochloric acid R*.

**0.2 M Deuterated sodium phosphate buffer solution pH 5.0.** *4013900.*

Dissolve 2.76 g of *sodium dihydrogen phosphate monohydrate R* in 90 mL of *deuterium oxide R*, adjust the pH with a deuterated solution of *phosphoric acid R* or a deuterated 1 M solution of *sodium hydroxide R*, dilute to 100 mL with *deuterium oxide R* and mix.

**Phosphate buffer solution pH 5.0.** *4011300.*

Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*. Adjust the pH with a 1 M potassium hydroxide solution prepared from *potassium hydroxide R* and dilute to 1000 mL with *water R*.

**Sodium acetate buffer solution pH 5.0.** *4015500.*

Dissolve 50.0 g of *sodium acetate R* in 10.0 mL of *glacial acetic acid R* and add *water R*. Adjust to pH 5.0 with a 4.2 g/L solution of *sodium hydroxide R* or with *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

**Buffer solution pH 5.2.** *4001700.*

Dissolve 1.02 g of *potassium hydrogen phthalate R* in 30.0 mL of *0.1 M sodium hydroxide*. Dilute to 100.0 mL with *water R*.

**0.067 M Phosphate buffer solution pH 5.4.** *4012000.*

Mix appropriate volumes of a 23.99 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with a 9.12 g/L solution of *sodium dihydrogen phosphate monohydrate R* to obtain pH 5.4.

**Acetate-edetate buffer solution pH 5.5.** *4001900.*

Dissolve 250 g of *ammonium acetate R* and 15 g *sodium edetate R* in 400 mL of *water R* and add 125 mL of *glacial acetic acid R*.

**Buffer solution pH 5.5.** *4001800.*

Dissolve 54.4 g of *sodium acetate R* in 50 mL of *water R*, heating to 35 °C if necessary. After cooling, slowly add 10 mL of *anhydrous acetic acid R*. Shake and dilute to 100.0 mL with *water R*.

**Phosphate buffer solution pH 5.5.** *4002000.*

Dissolve 13.61 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution A). Dissolve 35.81 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution B). Mix 96.4 mL of solution A and 3.6 mL of solution B.

**Phosphate-citrate buffer solution pH 5.5.** *4008700.*

Mix 56.85 mL of a 28.4 g/L solution of *anhydrous disodium hydrogen phosphate R* and 43.15 mL of a 21 g/L solution of *citric acid monohydrate R*.

**Phosphate buffer solution pH 5.6.** *4011200.*

Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution A). Dissolve 1.161 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution B). Mix 94.4 mL of solution A and 5.6 mL of solution B. If necessary, adjust to pH 5.6 using solution A or solution B.

**Phosphate buffer solution pH 5.8.** *4002100.*

Dissolve 1.19 g of *disodium hydrogen phosphate dihydrate R* and 8.25 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

**Acetate buffer solution pH 6.0.** *4002200.*

Dissolve 100 g of *ammonium acetate R* in 300 mL of *water R*, add 4.1 mL of *glacial acetic acid R*, adjust the pH if necessary using *ammonia R* or *acetic acid R* and dilute to 500.0 mL with *water R*.

**Diethylammonium phosphate buffer solution pH 6.0.** *4002300.*

Dilute 68 mL of *phosphoric acid R* to 500 mL with *water R*. To 25 mL of this solution add 450 mL of *water R* and 6 mL of *diethylamine R*, adjust to pH 6 ± 0.05, if necessary, using *diethylamine R* or *phosphoric acid R* and dilute to 500.0 mL with *water R*.

**1 M Morpholinoethanesulfonate buffer solution pH 6.0.** *4015900.*

Dissolve 48.8 g of *2-[N-morpholino]ethanesulfonic acid R* in 160 mL of *water R* and add 25 mL of *2 M sodium hydroxide R*. Adjust to pH 6.0 with *2 M sodium hydroxide R*. Dilute to almost 250 mL with *water R*. Adjust the pH, if necessary, with *2 M sodium hydroxide R* and dilute to 250.0 mL with *water R*.

**Phosphate buffer solution pH 6.0.** *4002400.*

Mix 63.2 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and 36.8 mL of a 21 g/L solution of *citric acid monohydrate R*.

**Phosphate buffer solution pH 6.0 R1.** *4002500.*

Dissolve 6.8 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with *water R*. Adjust the pH with *strong sodium hydroxide solution R*.

**Phosphate buffer solution pH 6.0 R2.** *4002600.*

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 28.5 mL of *0.2 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

**Phosphate buffer solution pH 6.4.** *4002800.*

Dissolve 2.5 g of *disodium hydrogen phosphate dodecahydrate R*, 2.5 g of *sodium dihydrogen phosphate R* and 8.2 g of *sodium chloride R* in 950 mL of *water R*. Adjust the pH of the solution to 6.4 with *1 M sodium hydroxide* or *1 M hydrochloric acid*, if necessary. Dilute to 1000.0 mL with *water R*.

**0.5 M Phthalate buffer solution pH 6.4.** *4009200.*

Dissolve 100 g of *potassium hydrogen phthalate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary, using *strong sodium hydroxide solution R*.

**Buffer solution pH 6.5.** *4002900.*

Dissolve 60.5 g of *disodium hydrogen phosphate dodecahydrate R* and 46 g of *potassium dihydrogen phosphate R* in *water R*. Add 100 mL of *0.02 M sodium edetate* and 20 mg of *mercuric chloride R* and dilute to 1000.0 mL with *water R*.

**Imidazole buffer solution pH 6.5.** *4003000.*

Dissolve 6.81 g of *imidazole R*, 1.23 g of *magnesium sulfate R* and 0.73 g of *calcium sulfate R* in 752 mL of *0.1 M hydrochloric acid*. Adjust the pH if necessary and dilute to 1000.0 mL with *water R*.

**0.1 M phosphate buffer solution pH 6.5.** *4010800.*

Dissolve 13.80 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of *distilled water R*. Adjust the pH using a 400 g/L solution of *sodium hydroxide R*. Dilute to 1000 mL with *distilled water R*.

**Phosphate buffer solution pH 6.5.** *4012800.*

Dissolve 2.75 g of *sodium dihydrogen phosphate R* and 4.5 g of *sodium chloride R* in 500 mL of *water R*. Adjust the pH with *phosphate buffer solution pH 8.5 R*.

**Buffer solution pH 6.6.** *4003100.*

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 89.0 mL of *0.2 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*.

**0.1 M Phosphate buffer solution pH 6.7.** *4014300.*

Dissolve 15.6 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent. Dissolve 17.8 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1.0 L with the same solvent. Mix the solutions, check the pH and if necessary adjust to pH 6.7.

**Phosphate buffered saline pH 6.8.** *4003200.*

Dissolve 1.0 g of *potassium dihydrogen phosphate R*, 2.0 g of *dipotassium hydrogen phosphate R* and 8.5 g of *sodium chloride R* in 900 mL of *water R*, adjust the pH if necessary and dilute to 1000.0 mL with the same solvent.

**Phosphate buffer solution pH 6.8.** *4003300.*

Mix 77.3 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 22.7 mL of a 21 g/L solution of *citric acid monohydrate R*.

**Phosphate buffer solution pH 6.8 R1.** *4003400.*

To 51.0 mL of a 27.2 g/L solution of *potassium dihydrogen phosphate R* add 49.0 mL of a 71.6 g/L solution of *disodium hydrogen phosphate dodecahydrate R*. Adjust the pH if necessary.

*Storage*: at 2 °C to 8 °C.

**1 M Tris-hydrochloride buffer solution pH 6.8.** *4009300.*

Dissolve 60.6 g of *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

**Buffer solution pH 7.0.** *4003500.*

To 1000 mL of a solution containing 18 g/L of *disodium hydrogen phosphate dodecahydrate R* and 23 g/L of *sodium chloride R* add sufficient (about 280 mL) of a solution containing 7.8 g/L of *sodium dihydrogen phosphate R* and 23 g/L of *sodium chloride R* to adjust the pH. Dissolve in the solution sufficient *sodium azide R* to give a 0.2 g/L solution.

**Maleate buffer solution pH 7.0.** *4003600.*

Dissolve 10.0 g of *sodium chloride R*, 6.06 g of *tris(hydroxymethyl)aminomethane R* and 4.90 g of *maleic anhydride R* in 900 mL of *water R*. Adjust the pH using a 170 g/L solution of *sodium hydroxide R*. Dilute to 1000.0 mL with *water R*.

*Storage*: at 2 °C to 8 °C; use within 3 days.

**0.025 M Phosphate buffer solution pH 7.0.** *4009400.*

Mix 1 volume of *0.063 M phosphate buffer solution pH 7.0 R* with 1.5 volumes of *water R*.

**0.03 M Phosphate buffer solution pH 7.0.** *4010300.*

Dissolve 5.2 g of *dipotassium hydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust the solution to pH 7.0 ± 0.1 using *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

**0.05 M Phosphate buffer solution pH 7.0.** *4012400.*

Mix 34 mL of *water R* and 100 mL of *0.067 M phosphate buffer solution pH 7.0 R*.

**0.063 M Phosphate buffer solution pH 7.0.** *4009500.*

Dissolve 5.18 g of *anhydrous disodium hydrogen phosphate R* and 3.65 g of *sodium dihydrogen phosphate monohydrate R* in 950 mL of *water R* and adjust the pH with *phosphoric acid R*; dilute to 1000.0 mL with *water R*.

**0.067 M Phosphate buffer solution pH 7.0.** *4003800.*

Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution A). Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 100.0 mL with the same solvent (solution B). Mix 38.9 mL of solution A and 61.1 mL of solution B. Adjust the pH if necessary.

**0.1 M Phosphate buffer solution pH 7.0.** *4008200.*

Dissolve 1.361 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent. Adjust the pH using a 35 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.

**Phosphate buffer solution pH 7.0.** *4003700.*

Mix 82.4 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 17.6 mL of a 21 g/L solution of *citric acid monohydrate R*.

**Phosphate buffer solution pH 7.0 R1.** *4003900.*

Mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and 148.2 mL of a 8 g/L solution of *sodium hydroxide R*, adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

**Phosphate buffer solution pH 7.0 R2.** *4004000.*

Mix 50.0 mL of a 136 g/L solution of *potassium dihydrogen phosphate R* with 29.5 mL of *1 M sodium hydroxide* and dilute to 100.0 mL with *water R*. Adjust the pH to 7.0 ± 0.1.

**Phosphate buffer solution pH 7.0 R3.** *4008600.*

Dissolve 5 g of *potassium dihydrogen phosphate R* and 11 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*. Adjust to pH 7.0 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*. Dilute to 1000 mL with *water R* and mix.

**Phosphate buffer solution pH 7.0 R4.** *4010200.*

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 18.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 500 mL with the same solvent.

**Phosphate buffer solution pH 7.0 R5.** *4011400.*

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* in 800 mL of *water R*. Adjust the pH using a 30 per cent *m/m* solution of *phosphoric acid R* and dilute to 1000 mL with *water R*.

**Phosphate buffer solution pH 7.0 R6.** *4015300.*

Dissolve 3.56 g of *disodium hydrogen phosphate dihydrate R* in 950 mL of *water for chromatography R*. Adjust the pH with *phosphoric acid R* and dilute to 1.0 L with *water for chromatography R*.

**Phosphate buffer solution pH 7.0 R7.** *4015700.*

Dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.0 with *dilute phosphoric acid R* and dilute to 1.0 L with *water R*.

**Potassium phosphate buffer solution pH 7.0.** *4014700.*

Dissolve 10 mg of *bovine albumin R* and 68 mg of *potassium dihydrogen phosphate R* in 30 mL of *water R*. If necessary, adjust to pH 7.0 with *potassium hydroxide R*. Dilute to 50 mL with *water R* and filter.

**Sodium/calcium acetate buffer solution pH 7.0.** *4014800.*

Dissolve 10 mg of *bovine albumin R* and 32 mg of *calcium acetate R* in 60 mL of *water R*. Add 580 µL of *glacial acetic acid R* and adjust to pH 7.0 with *2 M sodium hydroxide R*. Dilute to 100 mL with *water R* and filter.

**Tetrabutylammonium buffer solution pH 7.0.** *4010900.*

Dissolve 6.16 g of *ammonium acetate R* in a mixture of 15 mL of *tetrabutylammonium hydroxide solution (400 g/L) R* and 185 mL of *water R*. Adjust the pH with *nitric acid R*.

**Buffered salt solution pH 7.2.** *4004300.*

Dissolve in *water R* 8.0 g of *sodium chloride R*, 0.2 g of *potassium chloride R*, 0.1 g of *anhydrous calcium chloride R*, 0.1 g of *magnesium chloride R*, 3.18 g of *disodium hydrogen phosphate dodecahydrate R* and 0.2 g of *potassium dihydrogen phosphate R* and dilute to 1000.0 mL with *water R*.

**Buffer solution pH 7.2.** *4004100.*

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 175.0 mL of *0.2 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*. Adjust the pH if necessary.

**Phosphate-albumin buffered saline pH 7.2.** *4004400.*

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R*, 7.6 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use adjust the pH using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R*.

**Phosphate-albumin buffered saline pH 7.2 R1.** *4009600.*

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R*, 7.6 g of *sodium chloride R* and 1 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use adjust the pH using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R*.

**Phosphate buffer solution pH 7.2.** *4004200.*

Mix 87.0 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 13.0 mL of a 21 g/L solution of *citric acid monohydrate R*.

**Imidazole buffer solution pH 7.3.** *4004500.*

Dissolve 3.4 g of *imidazole R* and 5.8 g of *sodium chloride R* in *water R*, add 18.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*. Adjust the pH if necessary.

**Barbital buffer solution pH 7.4.** *4004700.*

Mix 50 mL of a solution in *water R* containing 19.44 g/L of *sodium acetate R* and 29.46 g/L of *barbital sodium R* with 50.5 mL of *0.1 M hydrochloric acid*, add 20 mL of an 85 g/L of *sodium chloride R* and dilute to 250 mL with *water R*.

**Buffer solution pH 7.4.** *4004600.*

Dissolve 0.6 g of *potassium dihydrogen phosphate R*, 6.4 g of *disodium hydrogen phosphate dodecahydrate R* and 5.85 g of *sodium chloride R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

**Phosphate buffered saline pH 7.4.** *4005000.*

Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R*, 0.19 g of *potassium dihydrogen phosphate R* and 8.0 g of *sodium chloride R* in water. Dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

**Phosphate buffer solution pH 7.4.** *4004800.*

Add 250.0 mL of *0.2 M potassium dihydrogen phosphate R* to 393.4 mL of *0.1 M sodium hydroxide*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 7.4.** *4012100.*

Dissolve 30.3 g of *tris(hydroxymethyl)aminomethane R* in approximately 200 mL of *water R*. Add 183 mL of *1 M hydrochloric acid*. Dilute to 500.0 mL with *water R*. *Note: the pH is 7.7-7.8 at room temperature and 7.4 at 37 °C. This solution is stable for several months at 4 °C.*

**Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4.** *4004900.*

Dissolve 6.08 g of *tris(hydroxymethyl)aminomethane R*, 8.77 g of *sodium chloride R* in 500 mL of *distilled water R*. Add 10.0 g of *bovine albumin R*. Adjust the pH using *hydrochloric acid R*. Dilute to 1000.0 mL with *distilled water R*.

**Tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1.** *4012200.*

Dissolve 0.1 g of *bovine albumin R* in a mixture containing 2 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 7.4 R* and 50 mL of a 5.84 mg/mL solution of *sodium chloride R*. Dilute to 100.0 mL with *water R*.

**Tris-sodium acetate buffer solution pH 7.4.** *4012900.*

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*. Adjust to pH 7.4 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

**Tris-sodium acetate-sodium chloride buffer solution pH 7.4.** *4013000.*

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R* and 14.6 g of *sodium chloride R* in 900 mL of *water R*. Add 0.50 g of *bovine albumin R*. Adjust to pH 7.4 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

**Borate buffer solution pH 7.5.** *4005200.*

Dissolve 2.5 g of *sodium chloride R*, 2.85 g of *disodium tetraborate R* and 10.5 g of *boric acid R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

*Storage*: at 2 °C to 8 °C.

**Buffer (HEPES) solution pH 7.5.** *4009700.*

Dissolve 2.38 g of *HEPES R* in about 90 mL of *water R*. Adjust the pH to 7.5 with *sodium hydroxide solution R*. Dilute to 100 mL with *water R*.

**0.05 M Phosphate buffer solution pH 7.5.** *4014400.*

Dissolve 0.89 g of *disodium hydrogen phosphate dihydrate R* in about 80 mL of *water R*. Adjust to pH 7.5 with an 8.5 per cent *V/V* solution of *phosphoric acid R* and dilute to 100.0 mL with *water R*.

**0.2 M Phosphate buffer solution pH 7.5.** *4005400.*

Dissolve 27.22 g of *potassium dihydrogen phosphate R* in 930 mL of *water R*, adjust to pH 7.5 with a 300 g/L solution of *potassium hydroxide R* and dilute to 1000.0 mL with *water R*.

**0.33 M Phosphate buffer solution pH 7.5.** *4005300.*

Dissolve 119.31 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution A). Dissolve 45.36 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution B). Mix 85 mL of solution A and 15 mL of solution B. Adjust the pH if necessary.

**0.25 M Sodium phosphate buffer solution pH 7.5.** *4016100.*

Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 70 mL of *water R*, adjust to pH 7.5 with a 300 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*.

**0.05 M Tris-hydrochloride buffer solution pH 7.5.** *4005600.*

Dissolve 6.057 g of *tris(hydroxymethyl)aminomethane R* in *water R* and adjust the pH with *hydrochloric acid R*. Dilute to 1000.0 mL with *water R*.

**0.1 M Tris-hydrochloride buffer solution pH 7.5.** *4016200.*

Dissolve 3.03 g of *tris(hydroxymethyl)aminomethane R* in 200 mL of *water R*, adjust to pH 7.5 with *hydrochloric acid R* and dilute to 250 mL with *water R*.

**1 M Tris-hydrochloride buffer solution pH 7.5.** *4014500.*

Dissolve 12.11 g of *tris(hydroxymethyl)aminomethane R* in 90 mL of *water R*, adjust to pH 7.5 with *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 7.5.** *4005500.*

Dissolve 7.27 g of *tris(hydroxymethyl)aminomethane R* and 5.27 g of *sodium chloride R* in *water R*, and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R1.** *4016400.*

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 900 mL of *water R* and add 10 mL of *0.01 M calcium chloride solution R*. Adjust the pH if necessary with *sodium hydroxide solution R* or *hydrochloric acid R*, and dilute to 1000.0 mL with *water R*.

**Sodium citrate buffer solution pH 7.8 (0.034 M sodium citrate, 0.101 M sodium chloride).** *4009800.*

Dissolve 10.0 g of *sodium citrate R* and 5.90 g of *sodium chloride R* in 900 mL of *water R*. Adjust the pH by addition of *hydrochloric acid R* and dilute to 1000 mL with *water R*.

**0.0015 M Borate buffer solution pH 8.0.** *4006000.*

Dissolve 0.572 g of *disodium tetraborate R* and 2.94 g of *calcium chloride R* in 800 mL of *water R*. Adjust the pH with *1 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

**Buffer solution pH 8.0.** *4005900.*

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R* add 46.8 mL of *0.2 M sodium hydroxide*. Dilute to 200.0 mL with *water R*.

**Buffer solution pH 8.0 R1.** *4010400.*

Dissolve 20 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*. Adjust the pH with *phosphoric acid R*. Dilute to 1000 mL with *water R*.

**0.02 M Phosphate buffer solution pH 8.0.** *4006100.*

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R* add 46.8 mL of *0.2 M sodium hydroxide*. Dilute to 500.0 mL with *water R*.

**0.1 M Phosphate buffer solution pH 8.0.** *4008400.*

Dissolve 0.523 g of *potassium dihydrogen phosphate R* and 16.73 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

**1 M Phosphate buffer solution pH 8.0.** *4007800.*

Dissolve 136.1 g of *potassium dihydrogen phosphate R* in *water R*, adjust the pH with *1 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*.

**0.02 M Sodium phosphate buffer solution pH 8.0.** *4013700.*

Dissolve 0.31 g of *sodium dihydrogen phosphate R* in 70 mL of *water R* and adjust to pH 8.0 with *1 M sodium hydroxide*, then dilute to 100 mL with *water R*.

**1 M Tris-hydrochloride buffer solution pH 8.0.** *4012700.*

Dissolve 121.1 g of *tris(hydroxymethyl)aminomethane R* and 1.47 g of *calcium chloride R* in 900 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

**Tris-hydrochloride buffer solution pH 8.0.** *4012300.*

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* and 29.4 mg of *calcium chloride R* in *water R*. Adjust the pH with *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

**Tris-sodium acetate buffer solution pH 8.0.** *4013100.*

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*. Adjust to pH 8.0 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

**Tris-sodium acetate-sodium chloride buffer solution pH 8.0.** *4013200.*

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R* and 14.6 g of *sodium chloride R* in 900 mL of *water R*. Add 0.50 g of *bovine albumin R*. Adjust to pH 8.0 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 8.1.** *4006200.*

Dissolve 0.294 g of *calcium chloride R* in 40 mL of *tris(hydroxymethyl)aminomethane solution R* and adjust the pH with *1 M hydrochloric acid*. Dilute to 100.0 mL with *water R*.

**Guanidine-tris(hydroxymethyl)aminomethane buffer solution pH 8.3.** *4016300.*

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 87.5 mL of a 764 g/L solution of *guanidine hydrochloride R*. Adjust to pH 8.3 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

**Tris-glycine buffer solution pH 8.3.** *4006300.*

Dissolve 6.0 g of *tris(hydroxymethyl)aminomethane R* and 28.8 g of *glycine R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 1 volume to 10 volumes with *water R* immediately before use.

**Tris-hydrochloride buffer solution pH 8.3.** *4011800.*

Dissolve 9.0 g of *tris(hydroxymethyl)aminomethane R* in 2.9 L of *water R*. Adjust the pH with *1 M hydrochloric acid*. Adjust the volume to 3 L with *water R*.

**Barbital buffer solution pH 8.4.** *4006400.*

Dissolve 8.25 g of *barbital sodium R* in *water R* and dilute to 1000.0 mL with the same solvent.

**Tris-EDTA BSA buffer solution pH 8.4.** *4006500.*

Dissolve 6.1 g of *tris(hydroxymethyl)aminomethane R*, 2.8 g of *sodium edetate R*, 10.2 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R*, adjust to pH 8.4 using *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

**Tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4.** *4006600.*

Dissolve 5.12 g of *sodium chloride R*, 3.03 g of *tris(hydroxymethyl)aminomethane R* and 1.40 g of *sodium edetate R* in 250 mL of *distilled water R*. Adjust the pH to 8.4 using *hydrochloric acid R*. Dilute to 500.0 mL with *distilled water R*.

**Tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1.** *4015100.*

Dissolve 10.20 g of *sodium chloride R*, 6.10 g of *tris(hydroxymethyl)aminomethane R*, 2.80 g of *sodium edetate R* and 1.00 g of *macrogol 6000 R* or 2.00 g of *bovine albumin R* or of *human albumin R* in 800 mL of *water R*. Adjust to pH 8.4 with *hydrochloric acid R* and dilute to 1.0 L with *water R*.

**Guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.5.** *4014600.*

Dissolve 1.0 g of *sodium edetate R*, 12.1 g of *tris(hydroxymethyl)aminomethane R* and 57.0 g of *guanidine hydrochloride R* in 35 mL of *water R*. Adjust to pH 8.5 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

**Phosphate buffer solution pH 8.5.** *4013300.*

Dissolve 3.5 g of *dipotassium hydrogen phosphate R* and 4.5 g of *sodium chloride R* in 500 mL of *water R*. Adjust the pH with a mixture of equal volumes of *dilute phosphoric acid R* and *water R*.

**Tris acetate buffer solution pH 8.5.** *4006700.*

Dissolve 0.294 g of *calcium chloride R* and 12.11 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust the pH with *acetic acid R*. Dilute to 1000.0 mL with *water R*.

**Barbital buffer solution pH 8.6 R1.** *4006900.*

Dissolve in *water R* 1.38 g of *barbital R*, 8.76 g of *barbital sodium R* and 0.38 g of *calcium lactate pentahydrate R* and dilute to 1000.0 mL with the same solvent.

**Guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.6.** *4016500.*

Dissolve 0.018 g of *sodium edetate R*, 2.2 g of *tris(hydroxymethyl)aminomethane R* and 28.7 g of *guanidine hydrochloride R* in 20 mL of *water R*. Adjust to pH 8.6 with *acetic acid R* and dilute to 50 mL with *water R*.

**1.5 M Tris-hydrochloride buffer solution pH 8.8.** *4009900.*

Dissolve 90.8 g of *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

**3 M Tris-hydrochloride buffer solution pH 8.8.** *4015000.*

Dissolve 363.3 g of *tris(hydroxymethyl)aminomethane R* in 500 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 1 L with *water R*.

**Buffer (phosphate) solution pH 9.0.** *4008300.*

Dissolve 1.74 g of *potassium dihydrogen phosphate R* in 80 mL of *water R*, adjust the pH with a 1 M potassium hydroxide solution prepared from *potassium hydroxide R* and dilute to 100.0 mL with *water R*.

**Buffer solution pH 9.0.** *4007000.*

Dissolve 6.18 g of *boric acid R* in *0.1 M potassium chloride R* and dilute to 1000.0 mL with the same solvent. Mix 1000.0 mL of this solution and 420.0 mL of *0.1 M sodium hydroxide*.

**Buffer solution pH 9.0 R1.** *4007100.*

Dissolve 6.20 g of *boric acid R* in 500 mL of *water R* and adjust the pH with *1 M sodium hydroxide* (about 41.5 mL). Dilute to 1000.0 mL with *water R*.

**0.05 M Tris-hydrochloride buffer solution pH 9.0.** *4013500.*

Dissolve 0.605 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust the pH with *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 9.0.** *4015200.*

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 950 mL of *water for chromatography R*. Adjust to pH 9.0 with *acetic acid R* and dilute to 1000.0 mL with *water for chromatography R*.

**Tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R1.** *4016600.*

Dissolve 12.1 g of *tris(hydroxymethyl)aminomethane R* in 950 mL of *water R*. Adjust to pH 9.0 with *acetic acid R* and dilute to 1000.0 mL with *water R*.

**Ammonium chloride buffer solution pH 9.5.** *4007200.*

Dissolve 33.5 g of *ammonium chloride R* in 150 mL of *water R*, add 42.0 mL of *concentrated ammonia R* and dilute to 250.0 mL with *water R*.

*Storage*: in a polyethylene container.

**Ammonium chloride buffer solution pH 10.0.** *4007300.*

Dissolve 5.4 g of *ammonium chloride R* in 20 mL of *water R*, add 35.0 mL of *ammonia R* and dilute to 100.0 mL with *water R*.

**Borate buffer solution pH 10.0.** *4016000.*

Introduce 12.4 g of *boric acid R* into a 500.0 mL volumetric flask. Add 300 mL of *water R* to suspend the boric acid. Add 100 mL of a 56 g/L solution of *potassium hydroxide R* and mix to dissolve the boric acid. Adjust to pH 10.0 by slowly adding a 56 g/L solution of *potassium hydroxide R* (about 60 mL is usually needed). Mix. Dilute almost to volume with *water R*. If necessary, adjust the pH with *boric acid R* or with a 56 g/L solution of *potassium hydroxide R*. Dilute to 500.0 mL with *water R*.

**Diethanolamine buffer solution pH 10.0.** *4007500.*

Dissolve 96.4 g of *diethanolamine R* in *water R* and dilute to 400 mL with the same solvent. Add 0.5 mL of an 186 g/L solution of *magnesium chloride R* and adjust the pH with *1 M hydrochloric acid*. Dilute to 500.0 mL with *water R*.

**0.1 M Ammonium carbonate buffer solution pH 10.3.** *4011900.*

Dissolve 7.91 g of *ammonium carbonate R* in 800 mL of *water R*. Adjust the pH with *dilute sodium hydroxide solution R*. Dilute to 1000.0 mL with *water R*.

**Ammonium chloride buffer solution pH 10.4.** *4011000.*

Dissolve 70 g of *ammonium chloride R* in 200 mL of *water R*, add 330 mL of *concentrated ammonia R* and dilute to 1000.0 mL with *water R*. If necessary, adjust to pH 10.4 with *ammonia R*.

**Borate buffer solution pH 10.4.** *4011100.*

Dissolve 24.64 g of *boric acid R* in 900 mL of *distilled water R*. Adjust the pH using a 400 g/L solution of *sodium hydroxide R*. Dilute to 1000 mL with *distilled water R*.

**Ammonium chloride buffer solution pH 10.7.** *4013400.*

Dissolve 67.5 g of *ammonium chloride R* in *water R*, add 570 mL of *concentrated ammonia R* and dilute to 1000.0 mL with *water R*.

**Buffer solution pH 10.9.** *4007600.*

Dissolve 6.75 g of *ammonium chloride R* in *ammonia R* and dilute to 100.0 mL with the same solvent.

**Total-ionic-strength-adjustment buffer.** *4007700.*

Dissolve 58.5 g of *sodium chloride R*, 57.0 mL of *glacial acetic acid R*, 61.5 g of *sodium acetate R* and 5.0 g of *cyclohexylenedinitrilotetra-acetic acid R* in *water R* and dilute to 500.0 mL with the same solvent. Adjust to pH 5.0 to 5.5 with a 335 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *distilled water R*.

**Total-ionic-strength-adjustment buffer R1.** *4008800.*

Dissolve 210 g of *citric acid monohydrate R* in 400 mL of *distilled water R*. Adjust to pH 7.0 with *concentrated ammonia R*. Dilute to 1000.0 mL with *distilled water R* (solution A). Dissolve 132 g of *ammonium phosphate R* in *distilled water R* and dilute to 1000.0 mL with the same solvent (solution B). To a suspension of 292 g of (ethylenedinitrilo)tetra-*acetic acid R* in about 500 mL of *distilled water R*, add about 200 mL of *concentrated ammonia R* to dissolve. Adjust the pH to 6 to 7 with *concentrated ammonia R*. Dilute to 1000.0 mL with *distilled water R* (solution C). Mix equal volumes of solution A, B, and C and adjust to pH 7.5 with *concentrated ammonia R*.

**Buffer solution pH 11.** *4014000.*

Dissolve 6.21 g of *boric acid R*, 4.00 g of *sodium hydroxide R* and 3.70 g of *potassium chloride R* in 500 mL of *water R* and dilute to 1000 mL with the same solvent.

**0.1 M Phosphate buffer solution pH 11.3.** *4015400.*

Dissolve 17.4 g of *dipotassium hydrogen phosphate R* in about 950 mL of *water R*, adjust to pH 11.3 using a 100 g/L solution of *potassium hydroxide R* and dilute to 1.0 L with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

# 4.2. VOLUMETRIC ANALYSIS

**07/2017:40201**

## 4.2.1. PRIMARY STANDARDS FOR VOLUMETRIC SOLUTIONS

Primary standards for volumetric solutions are indicated by the suffix RV. Primary standards of suitable quality may be obtained from commercial sources or prepared by the following methods.

For primary standards from commercial sources, a pre-treatment step may be necessary. Follow the supplier's instructions.

A secondary standard may be used provided its traceability to a primary standard has been demonstrated.

**Arsenious trioxide.** $As_2O_3$. ($M_r$ 197.8). *2000100.* [1327-53-3]. Arsenic(III) oxide.

Sublime *arsenious trioxide R* in a suitable apparatus.

*Storage*: over *anhydrous silica gel R*.

**Benzoic acid.** $C_7H_6O_2$. ($M_r$ 122.1). *2000200.* [65-85-0].

Sublime *benzoic acid R* in a suitable apparatus.

**Ferrous ethylenediammonium sulfate.** $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$. ($M_r$ 382.1). *2000900.* [113193-60-5]. Ethylenediammonium iron(II) disulfate tetrahydrate. Ethylenediammonium tetraaquabis(sulfato)iron(II).

*Content*: minimum 99.5 per cent.

**Potassium bromate.** $KBrO_3$. ($M_r$ 167.0). *2000300.* [7758-01-2].

Crystallise *potassium bromate R* from boiling *water R*. Collect the crystals and dry to constant mass at 180 °C.

**Potassium hydrogen phthalate.** $C_8H_5KO_4$. ($M_r$ 204.2). *2000400.* [877-24-7]. Potassium 2-carboxybenzoate.

Recrystallise *potassium hydrogen phthalate R* from boiling *water R*, collect the crystals at a temperature above 35 °C and dry to constant mass at 110 °C.

**Sodium chloride.** NaCl. ($M_r$ 58.44). *2000600.* [7647-14-5].

To 1 volume of the *saturated sodium chloride solution R* add 2 volumes of *hydrochloric acid R*. Collect the crystals formed and wash with *hydrochloric acid R1*. Remove the hydrochloric acid by heating on a water-bath and dry the crystals to constant mass at 300 °C.

**Sulfanilic acid.** $C_6H_7NO_3S$. ($M_r$ 173.2). *2000700.* [121-57-3]. 4-Aminobenzenesulfonic acid.

Recrystallise *sulfanilic acid R* from boiling *water R*. Filter and dry to constant mass at 100-105 °C.

**Trometamol.** $C_4H_{11}NO_3$. ($M_r$ 121.1). *2001000.* [77-86-1]. 2-Amino-2-(hydroxymethyl)propane-1,3-diol. Tris(hydroxymethyl)aminomethane.

*Content*: minimum 99.5 per cent.

**Zinc.** Zn. ($M_r$ 65.4). *2000800.* [7440-66-6].

*Content*: minimum 99.9 per cent.

**07/2019:40202**

## 4.2.2. VOLUMETRIC SOLUTIONS

Volumetric solutions are prepared according to the usual chemical analytical methods. The accuracy of the apparatus used is verified to ensure that it is appropriate for the intended use.

The concentration of volumetric solutions is indicated in terms of molarity. Molarity expresses, as the number of moles, the amount of substance dissolved in 1 L of solution. A solution which contains *x* moles of substance per litre is said to be *x* M.

Volumetric solutions do not differ from the prescribed strength by more than 10 per cent. The molarity of the volumetric solutions is determined by an appropriate number of titrations. The repeatability does not exceed 0.2 per cent (relative standard deviation).

Volumetric solutions are standardised by the methods described below. When a volumetric solution is to be used in an assay in which the end-point is determined by an electrochemical process (for example, amperometry or potentiometry) the solution is standardised by the same method. The composition of the medium in which a volumetric solution is standardised should be the same as that in which it is to be used.

Solutions more dilute than those described below are either prepared by adapting the quantities stated or by dilution, with *carbon dioxide-free water R* (unless otherwise prescribed), of a more concentrated solution that has been previously standardised. In the first case, the correction factor is determined on the volumetric solution to be used in the monograph. In the latter case, the correction factor of the dilute solution is the same as that of the standardised solution from which it was prepared.

Commercially available volumetric solutions traceable to a primary standard may be used provided their titre is determined or verified prior to first use.

Titres of volumetric solutions are verified at appropriate intervals that are defined in the quality system procedures.

**0.1 M Ammonium and cerium nitrate.** *3000100.*

Shake for 2 min a solution containing 56 mL of *sulfuric acid R* and 54.82 g of *ammonium and cerium nitrate R*, then add 5 successive quantities, each of 100 mL, of *water R*, shaking after each addition. Dilute the clear solution to 1000.0 mL with *water R*. Standardise the solution after 10 days.

*Standardisation.* Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L $H_2SO_4$). Titrate with the ammonium and cerium nitrate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

*Storage*: protected from light.

**0.1 M Ammonium and cerium sulfate.** *3000300.*

Dissolve 65.0 g of *ammonium and cerium sulfate R* in a mixture of 500 mL of *water R* and 30 mL of *sulfuric acid R*. Allow to cool and dilute to 1000.0 mL with *water R*.

*Standardisation.* Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L $H_2SO_4$). Titrate with the ammonium and cerium sulfate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium sulfate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

*Dilution.* Use a diluted solution of *sulfuric acid R* (59 g/L $H_2SO_4$) while cooling the solution.

**0.1 M Ammonium thiocyanate.** *3000500.*

Dissolve 7.612 g of *ammonium thiocyanate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 20.0 mL of *0.1 M silver nitrate* add 25 mL of *water R*, 2 mL of *dilute nitric acid R* and 2 mL of *ferric ammonium sulfate solution R2*. Titrate with the ammonium thiocyanate solution until a reddish-yellow colour is obtained.

**0.1 M Barium chloride.** *3000600.*

Dissolve 24.4 g of *barium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 10.0 mL of the barium chloride solution add 60 mL of *water R*, 3 mL of *concentrated ammonia R* and 0.5-1 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*. When the solution begins to decolorise, add 50 mL of *ethanol (96 per cent) R* and continue the titration until the blue-violet colour disappears.

**0.05 M Barium perchlorate.** *3000700.*

Dissolve 15.8 g of *barium hydroxide R* in a mixture of 7.5 mL of *perchloric acid R* and 75 mL of *water R*, adjust the solution to pH 3 by adding *perchloric acid R* and filter if necessary. Add 150 mL of *ethanol (96 per cent) R* and dilute to 250 mL with *water R*. Dilute to 1000.0 mL with *buffer solution pH 3.7 R*.

*Standardisation.* To 5.0 mL of *0.05 M sulfuric acid* add 5 mL of *water R*, 50 mL of *buffer solution pH 3.7 R* and 0.5 mL of *alizarin S solution R*. Titrate with the barium perchlorate solution until an orange-red colour appears. Standardise immediately before use.

*Dilution.* Use *buffer solution pH 3.7 R*.

**0.005 M Barium perchlorate.** *3010200.*

Dilute 10.0 mL of *0.05 M barium perchlorate* to 100.0 mL with a buffer solution prepared as follows: to 15.0 mL of *acetic acid R* add 60.0 mL of *2-propanol R*. Adjust to pH 3.7 with *ammonia R* and dilute to 100.0 mL with *water R*.

**0.004 M Benzethonium chloride.** *3000900.*

Dissolve in *water R* 1.792 g of *benzethonium chloride R*, previously dried to constant mass at 100-105 °C, and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 0.350 g of the dried substance in 35 mL of a mixture of 30 volumes of *anhydrous acetic acid R* and 70 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, using 0.05 mL of *crystal violet solution R* as indicator. Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 44.81 mg of $C_{27}H_{42}ClNO_2$.

**0.01 M Bismuth nitrate.** *3010000.*

Dissolve 4.86 g of *bismuth nitrate pentahydrate R* in 60 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R*.

*Standardisation.* To 25.0 mL of the bismuth nitrate solution, add 50 mL of *water R* and titrate with *0.01 M sodium edetate* using 0.05 mL of a 1 g/L solution of *xylenol orange R* as indicator.

**0.0167 M Bromide-bromate.** *3001000.*

Dissolve 2.7835 g of *potassium bromate RV* and 13 g of *potassium bromide R* in *water R* and dilute to 1000.0 mL with the same solvent.

**0.1 M Cerium sulfate.** *3001100.*

Dissolve 40.4 g of *cerium sulfate R* in a mixture of 500 mL of *water R* and 50 mL of *sulfuric acid R*. Allow to cool and dilute to 1000.0 mL with *water R*.

*Standardisation.* Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L $H_2SO_4$). Titrate with the cerium sulfate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M cerium sulfate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

**0.02 M Copper sulfate.** *3001200.*

Dissolve 5.0 g of *copper sulfate pentahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 20.0 mL of the copper sulfate solution add 2 g of *sodium acetate R* and 0.1 mL of *pyridylazonaphthol solution R*. Titrate with *0.02 M sodium edetate* until the colour changes from violet-blue to bright green. Titrate slowly towards the end of the titration.

**0.1 M Ferric ammonium sulfate.** *3001300.*

Dissolve 50.0 g of *ferric ammonium sulfate R* in a mixture of 6 mL of *sulfuric acid R* and 300 mL of *water R* and dilute to 1000.0 mL with *water R*.

*Standardisation.* To 10.0 mL of the ferric ammonium sulfate solution add 35 mL of *water R*, 3 mL of *hydrochloric acid R* and 1 g of *potassium iodide R*. Allow to stand for 10 min. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*) or using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 48.22 mg of $FeNH_4(SO_4)_2,12H_2O$.

**0.1 M Ferrous sulfate.** *3001400.*

Dissolve 27.80 g of *ferrous sulfate R* in 500 mL of *dilute sulfuric acid R* and dilute to 1000.0 mL with *water R*.

*Standardisation.* To 25.0 mL of the ferrous sulfate solution add 3 mL of *phosphoric acid R* and titrate immediately with *0.02 M potassium permanganate*. Standardise immediately before use.

**1 M Hydrochloric acid.** *3001800.*

Dilute 103.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

*Standardisation.* Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*. Titrate with the hydrochloric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until a yellowish-red colour is obtained.

1 mL of *1 M hydrochloric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

**0.1 M Hydrochloric acid.** *3002100.*

Dilute 100.0 mL of *1 M hydrochloric acid* to 1000.0 mL with *carbon dioxide-free water R*.

*Standardisation.* Carry out the titration described for *1 M hydrochloric acid* using 95 mg of *trometamol RV* dissolved in 50 mL of *water R*.

1 mL of *0.1 M hydrochloric acid* is equivalent to 12.11 mg of $C_4H_{11}NO_3$.

**0.5 M Iodine.** *3009400.*

Dissolve 127 g of *iodine R* and 200 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 2.0 mL of the iodine solution add 1 mL of *dilute acetic acid R* and 50 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, using *starch solution R* as indicator.

*Storage*: protected from light.

**0.05 M Iodine.** *3002700.*

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 10.0 mL of the iodine solution add 1 mL of *dilute acetic acid R* and 40 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*) or using *starch solution R* as indicator.

*Storage*: protected from light.

**0.01 M Iodine.** *3002900.*

Add 0.3 g of *potassium iodide R* to 20.0 mL of *0.05 M iodine* and dilute to 100.0 mL with *water R*.

**0.1 M Lanthanum nitrate.** *3010100.*

Dissolve 43.30 g of *lanthanum nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 20.0 mL of the lanthanum nitrate solution, add 15 mL of *water R* and 25 mL of *0.1 M sodium edetate*. Add about 50 mg of *xylenol orange triturate R* and about 2 g of *hexamethylenetetramine R*. Titrate with *0.1 M zinc sulfate* until the colour changes from yellow to violet-pink.

1 mL of *0.1 M sodium edetate* is equivalent to 43.30 mg of $La(NO_3)_3,6H_2O$.

**0.1 M Lead nitrate.** *3003100.*

Dissolve 33 g of *lead nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Take 20.0 mL of the lead nitrate solution and carry out the determination of lead by complexometry (*2.5.11*).

**0.1 M Lithium methoxide.** *3003300.*

Dissolve 0.694 g of *lithium R* in 150 mL of *anhydrous methanol R* and dilute to 1000.0 mL with *toluene R*.

*Standardisation.* To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the lithium methoxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*. Stir to effect solution and titrate with the lithium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the lithium methoxide solution. Standardise immediately before use.

1 mL of *0.1 M lithium methoxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**0.1 M Magnesium chloride.** *3003400.*

Dissolve 20.33 g of *magnesium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Carry out the determination of magnesium by complexometry (*2.5.11*).

**1 M Nitric acid.** *3003600.*

Dilute 96.6 g of *nitric acid R* to 1000.0 mL with *water R*.

*Standardisation.* Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*. Titrate with the nitric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until a reddish-yellow colour is obtained.

1 mL of *1 M nitric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

**0.1 M Perchloric acid.** *3003900.*

Place 8.5 mL of *perchloric acid R* in a volumetric flask containing about 900 mL of *glacial acetic acid R* and mix. Add 30 mL of *acetic anhydride R*, dilute to 1000.0 mL with *glacial acetic acid R*, mix and allow to stand for 24 h. Determine the water content (*2.5.12*) without addition of methanol and, if necessary, adjust the water content to 0.1-0.2 per cent by adding either *acetic anhydride R* or *water R*. Allow to stand for 24 h.

*Standardisation.* Dissolve 0.170 g of *potassium hydrogen phthalate RV* in 50 mL of *anhydrous acetic acid R*, warming gently if necessary. Allow to cool protected from air, and titrate with the perchloric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.05 mL of *crystal violet solution R* as indicator. Note the temperature of the perchloric acid solution at the time of the titration. If the temperature at which an assay is carried out is different from that at which the *0.1 M perchloric acid* has been standardised, the volume used in the assay becomes:

$$V_c = V[1 + (t_1 - t_2)0.0011]$$

$t_1$ = temperature during standardisation,

$t_2$ = temperature during the assay,

$V_c$ = corrected volume,

$V$ = observed volume.

1 mL of *0.1 M perchloric acid* is equivalent to 20.42 mg of $C_8H_5KO_4$.

*Dilution.* Use *anhydrous acetic acid R*.

**0.033 M Potassium bromate.** *3004200.*

Dissolve 5.5670 g of *potassium bromate RV* in *water R* and dilute to 1000.0 mL with the same solvent.

**0.1 M Potassium hydrogen phthalate.** *3004700.*

In a conical flask containing about 800 mL of *anhydrous acetic acid R*, dissolve 20.42 g of *potassium hydrogen phthalate RV*. Heat on a water-bath until completely dissolved, protected from humidity. Cool to 20 °C and dilute to 1000.0 mL with *anhydrous acetic acid R*.

**0.1 M Potassium hydroxide.** *3004800.*

Dissolve 6 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 0.150 g of *potassium hydrogen phthalate RV* in 50 mL of *water R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M potassium hydroxide* is equivalent to 20.42 mg of $C_8H_5KO_4$.

**0.5 M Potassium hydroxide in alcohol (60 per cent *V/V*).** *3004900.*

Dissolve 3 g of *potassium hydroxide R* in *aldehyde-free alcohol R* (60 per cent *V/V*) and dilute to 100.0 mL with the same solvent.

*Standardisation.* Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* is equivalent to 61.06 mg of $C_7H_6O_2$.

**0.5 M Potassium hydroxide, alcoholic.** *3005000.*

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100.0 mL with *aldehyde-free alcohol R*.

*Standardisation.* Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.06 mg of $C_7H_6O_2$.

*Dilution.* Use *aldehyde-free alcohol R*.

**0.05 M Potassium iodate.** *3005200.*

Dissolve 10.70 g of *potassium iodate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 3.0 mL of the potassium iodate solution add 40.0 mL of *water R*, 1 g of *potassium iodide R* and 5 mL of *dilute sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*) or using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 3.567 mg of $KIO_3$.

**0.001 M Potassium iodide.** *3009200.*

Dilute 10.0 mL of *potassium iodide solution R* to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 500.0 mL with *water R*.

**0.02 M Potassium permanganate.** *3005300.*

Dissolve 3.2 g of *potassium permanganate R* in *water R* and dilute to 1000.0 mL with the same solvent. Heat the solution for 1 h on a water-bath, allow to cool and filter through a sintered-glass filter (*2.1.2*).

*Standardisation.* Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L $H_2SO_4$). Titrate with the potassium permanganate solution, determining the end-point potentiometrically (*2.2.20*) or by the colour of the solution changing to pink. Standardise immediately before use.

1 mL of *0.02 M potassium permanganate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

*Storage*: protected from light.

**0.1 M Silver nitrate.** *3005600.*

Dissolve 17.0 g of *silver nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 50 mg of *sodium chloride RV* in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with the silver nitrate solution, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

*Storage*: protected from light.

**0.1 M Sodium arsenite.** *3005800.*

Dissolve *arsenious trioxide RV* equivalent to 4.946 g of $As_2O_3$ in a mixture of 20 mL of *strong sodium hydroxide solution R* and 20 mL of *water R*, dilute to 400 mL with *water R* and add *dilute hydrochloric acid R* until the solution is neutral to *blue litmus paper R*. Dissolve 2 g of *sodium hydrogen carbonate R* in the solution and dilute to 500.0 mL with *water R*.

**0.1 M Sodium edetate.** *3005900.*

Dissolve 37.5 g of *sodium edetate R* in 500 mL of *water R*, add 100 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

*Standardisation.* Dissolve 0.120 g of *zinc RV* in 4 mL of *hydrochloric acid R1*. Add *dilute sodium hydroxide solution R* until the solution is weakly acid and carry out the assay of zinc by complexometry (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 6.538 mg of Zn.

*Storage*: in a polyethylene container.

**1 M Sodium hydroxide.** *3006300.*

Dissolve 42 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 1.50 g of *potassium hydrogen phthalate RV* in 50 mL of *water R*. Titrate with the sodium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 204.2 mg of $C_8H_5KO_4$.

If sodium hydroxide free from carbonate is prescribed, prepare it as follows. Dissolve *sodium hydroxide R* in *water R* to give a concentration of 400-600 g/L and allow to stand. Decant the clear supernatant, taking precautions to avoid the introduction of carbon dioxide, and dilute with *carbon dioxide-free water R* to the required molarity. The solution complies with the following test. Titrate 20.0 mL of hydrochloric acid of the same molarity with the solution of sodium hydroxide, using 0.1 mL of *phenolphthalein solution R* as indicator. At the end-point add just sufficient of the acid to discharge the pink colour and concentrate the solution to 20 mL by boiling. During boiling add just sufficient acid to discharge the pink colour, which should not reappear after prolonged boiling. The volume of acid used does not exceed 0.1 mL.

**0.1 M Sodium hydroxide.** *3006600.*

Dilute 100.0 mL of *1 M sodium hydroxide* to 1000.0 mL with *carbon dioxide-free water R*.

*Standardisation.* Carry out the titration described for *1 M sodium hydroxide* using 0.150 g of *potassium hydrogen phthalate RV* in 50 mL of *water R*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.42 mg of $C_8H_5KO_4$.

*Standardisation (for use in the assay of halide salts of organic bases).* Dissolve 0.100 g of *benzoic acid RV* in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out the titration (*2.2.20*), using the sodium hydroxide solution. Note the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**0.1 M Sodium hydroxide, ethanolic.** *3007000.*

To 250 mL of *anhydrous ethanol R* add 3.3 g of *strong sodium hydroxide solution R*.

*Standardisation.* Dissolve 0.100 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with the ethanolic sodium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.2 mL of *thymolphthalein solution R* as indicator. Standardise immediately before use.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**0.1 M Sodium methoxide.** *3007100.*

Cool 175 mL of *anhydrous methanol R* in iced *water R* and add, in small portions, about 2.5 g of freshly cut *sodium R*. When the metal has dissolved, dilute to 1000.0 mL with *toluene R*.

*Standardisation.* To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R*, and titrate with the sodium methoxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*. Stir until dissolution and titrate with the sodium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the sodium methoxide solution. Standardise immediately before use.

1 mL of *0.1 M sodium methoxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**0.1 M Sodium nitrite.** *3007200.*

Dissolve 7.5 g of *sodium nitrite R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 0.150 g of *sulfanilic acid RV* in 50 mL of *dilute hydrochloric acid R* and carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using the sodium nitrite solution and determining the end-point electrometrically. Standardise immediately before use.

1 mL of *0.1 M sodium nitrite* is equivalent to 17.32 mg of $C_6H_7NO_3S$.

**0.1 M Sodium periodate.** *3009500.*

Dissolve 21.4 g of *sodium periodate R* in about 500 mL of *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* In a stoppered flask, introduce 5.0 mL of the sodium periodate solution and add 100 mL of *water R*. Add 10 mL of *potassium iodide solution R* and 5 mL of *hydrochloric acid R1*, close, shake and allow to stand for 2 min. Titrate with *0.1 M sodium thiosulfate* until the yellow colour almost disappears. Determine the end-point potentiometrically (*2.2.20*) or add 2 mL of *starch solution R* and titrate slowly until the colour is completely discharged.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.674 mg of $NaIO_4$ or 0.125 mL of *0.1 M sodium periodate*.

**0.1 M Sodium thiosulfate.** *3007300.*

Dissolve 25 g of *sodium thiosulfate R* and 0.2 g of *sodium carbonate R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 10.0 mL of *0.033 M potassium bromate*, add 40 mL of *water R*, 10 mL of *potassium iodide solution R* and 5 mL of *hydrochloric acid R1*. Titrate with the sodium thiosulfate solution, using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.783 mg of $KBrO_3$ or 0.5 mL of *0.033 M potassium bromate*.

**0.5 M Sulfuric acid.** *3007800.*

Dissolve 28 mL of *sulfuric acid R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*. Titrate with the sulfuric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until the solution turns reddish-yellow.

1 mL of *0.5 M sulfuric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

**0.1 M Tetrabutylammonium hydroxide.** *3008300.*

Dissolve 40 g of *tetrabutylammonium iodide R* in 90 mL of *anhydrous methanol R*, add 20 g of finely powdered *silver oxide R* and shake vigorously for 1 h. Centrifuge a few millilitres of the mixture and test the supernatant for iodides. If a positive reaction is obtained, add an additional 2 g of *silver oxide R* and shake for a further 30 min. Repeat this procedure until the liquid is free from iodides, filter the mixture through a fine sintered-glass filter (*2.1.2*) and rinse the reaction vessel and filter with three quantities, each of 50 mL, of *toluene R*. Add the washings to the filtrate and dilute to 1000.0 mL with *toluene R*. Pass dry carbon dioxide-free nitrogen through the solution for 5 min.

*Standardisation.* To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the tetrabutylammonium hydroxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*. Stir to effect solution, and titrate with the tetrabutylammonium hydroxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the tetrabutylammonium hydroxide solution. Standardise immediately before use.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**0.1 M Tetrabutylammonium hydroxide in 2-propanol.** *3008400.*

Prepare as described for *0.1 M tetrabutylammonium hydroxide* using *2-propanol R* instead of *toluene R* and standardise as described.

**0.05 M Zinc chloride.** *3008500.*

Dissolve 6.82 g of *zinc chloride R*, weighed with appropriate precautions, in *water R*. If necessary, add dropwise *dilute hydrochloric acid R* until the opalescence disappears. Dilute to 1000.0 mL with *water R*.

*Standardisation.* To 20.0 mL of the zinc chloride solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.5.11*).

**0.1 M Zinc sulfate.** *3008600.*

Dissolve 29 g of *zinc sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Standardisation.* To 20.0 mL of the zinc sulfate solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.5.11*).

# 5. General texts

# 5.1. General texts on microbiology

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# 5.1. GENERAL TEXTS ON MICROBIOLOGY

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## 5.1.1. METHODS OF PREPARATION OF STERILE PRODUCTS

GENERAL INTRODUCTION

Sterility is the absence of viable micro-organisms, as defined by a sterility assurance level equal to or less than $10^{-6}$. Sterility is a critical quality attribute for a wide variety of human and veterinary preparations, including but not restricted to:

- preparations required to be sterile due to their route of administration, such as parenteral, ophthalmic and intramammary preparations, and some inhalation, irrigation and intrauterine preparations;
- preparations applied to severely injured skin, such as semi-solid preparations for cutaneous application.

The achievement of sterility for any one item in a population of items submitted to a sterilisation process can neither be guaranteed nor demonstrated. It is essential to study the effect of the chosen sterilisation procedure on the product (including its final container) to ensure its effectiveness and the integrity of the product, and to validate the procedure before it is applied in practice. Failure to follow meticulously a validated process introduces the risk of a non-sterile and/or deteriorated product.

Sterile products are prepared under appropriate conditions and are packed in suitable containers. It is recommended that the choice of container permits application of the optimum sterilisation process for the product. The container and closure system are required to maintain the sterility of the product throughout its shelf life.

Sterilisation process conditions are chosen to achieve the highest level of sterility assurance compatible with the drug product and, wherever possible, a process in which the product is sterilised in its final container (terminal sterilisation) is chosen. When a fully validated terminal sterilisation method by steam (moist heat), dry heat or ionising sterilisation is used, parametric release (i.e. the release of a batch of sterilised items based on process data rather than submission of a sample of the items to sterility testing) may be carried out, subject to the approval of the competent authority. If terminal sterilisation is not possible, aseptic assembly or filtration through a bacterial retentive filter is used. Wherever possible, an appropriate additional treatment (e.g. heating) of the product in its final container is applied to further ensure the sterility assurance level.

Requirements for the use of biological indicators for validation of sterilisation processes are given in general chapter *5.1.2*.

The present general chapter provides guidance on conditions, validation and control of sterilisation processes. The methods described here apply mainly to the inactivation or removal of bacteria, yeasts and moulds. For biological products of animal or human origin, or in cases where such material has been used in the production process, it is necessary to demonstrate during validation that the process is capable of the removal or inactivation of any relevant viral contamination. Further guidance is provided in general chapter *5.1.7. Viral safety*.

The efficacy of a sterilisation process is dependent on its nature, the processing conditions (e.g. time, temperature, moisture), the pre-sterilisation microbial contamination and the formulation of the product. The inactivation of micro-organisms by physical or chemical means follows an exponential law and hence there is always a non-zero probability that a micro-organism may survive the sterilisation process.

**Sterility assurance level (SAL)**

In the methods described, reference is made to a sterility assurance level (SAL) where appropriate. The SAL for a given sterilisation process is expressed as the probability of micro-organisms surviving in a product item after exposure to the process. An SAL of $10^{-6}$, for example, denotes a probability of not more than 1 non-sterile item in $1 \times 10^{6}$ sterilised items of the final product. The SAL of a process for a given product is established by appropriate validation studies. Microbial contamination may be described by the number, type and resistance of any micro-organisms present. Microbiological monitoring and setting of suitable limits is therefore essential for all components of sterile preparations. Steps designed to reduce microbial contamination, such as filtration prior to sterilisation, will contribute significantly to sterility assurance. The composition of a product can affect the behaviour of micro-organisms present in the product, which in turn can affect the efficacy of the sterilisation process. The water activity ($A_w$), the pH and the presence of compounds with antimicrobial activity are examples of factors that can influence the resistance of any micro-organisms present. The water activity or the product formulation (including the presence of nutrients) can affect the number of micro-organisms, which in turn can affect the efficacy of the membrane-filtration process.

METHODS AND CONDITIONS OF STERILISATION

Sterilisation may be carried out by one of the methods described hereafter. Modifications to, or combinations of, these methods may be used, provided that the chosen procedure is validated with respect both to its effectiveness and to the integrity of the product including its container. For all sterilisation methods, the critical parameters of the procedure are monitored in order to confirm that any previously determined requirements or conditions are respected throughout the batch during the entire sterilisation process. This applies in all cases, including those where the reference conditions are used. Guidance concerning validation of a steam sterilisation process using the $F_0$ concept is described in general chapter *5.1.5*. Biological indicators of sterilisation are used to develop and validate sterilisation processes and also to monitor gas sterilisation processes. Guidance on the use of biological indicators is provided in general chapter *5.1.2*.

Precautions shall be taken to prevent contamination of the sterilised articles after the sterilisation phase.

*STEAM STERILISATION*

**Principle**

Steam sterilisation is achieved by heat transfer during condensation of water from a saturated vapour phase on the surfaces of the sterilised items. Where items (open or wrapped) are sterilised in direct contact with steam, the hydrating effect of the condensate adds to the sterilising effect. For direct steam exposure, it is essential that the items are fully penetrated by saturated steam, i.e. free of air and other non-condensable gases. Where items are sterilised in closed-containers, the chamber of the steriliser serves as a steam jacket. Condensation on the surface of the containers still serves as a highly effective mechanism for energy transfer, but has no additional sterilising effect on its own. In closed-container sterilisation, the sterilising effect is determined by the conditions reached within the closed containers, where sterilisation must be achieved in the product itself and in the head-space.

**Equipment**

Steam sterilisation is performed in autoclaves, i.e. pressure vessels designed to admit or generate steam continuously and to remove condensate from the chamber to maintain the pressure and temperature at controlled levels.

For equipment used to perform direct steam exposure cycles, the supply of saturated steam, free of non-condensable gases, is assured. In autoclaves intended for the sterilisation of closed containers, steam-air mixtures or a superheated water spray can be used to achieve heat transfer. Suitable autoclaves are qualified to achieve homogeneous conditions within the chamber and the load. The principles of operation are appropriate for the items to be sterilised and the loading configuration. The suitability of the equipment for the items to be sterilised and its performance in the chosen cycle is demonstrated in autoclave performance qualification studies. Temperature profiles in the slowest-to-heat items are recorded.

Suitable autoclaves are equipped with temperature and pressure sensors of appropriate sensitivity that are placed in relevant positions to ensure effective process control. Chamber temperature and pressure profiles are recorded for each cycle. There is at least 1 independent thermal probe that controls the load temperature at the slowest-to-heat position or in the slowest-to-heat closed container of the load.

Cooling water sprayed into the chamber at the end of a sterilisation process for closed containers is of sufficient quality not to impact negatively the sterility of the sterilised items.

**Sterilisation cycle**

Suitable sterilisation cycles are chosen to be compatible with the items to be sterilised and the loading configuration. Where air is displaced from the chamber by gravity, the items to be autoclaved are designed to allow the removal of air and are arranged within the autoclave to prevent the formation of inaccessible air pockets. Where air is removed by vacuum cycles followed by steam pulses, it is assured that the items are not affected by the evacuation process. For pressure-sensitive products in closed containers, saturated steam sterilisation may not be possible. Steam-air mixtures may be applied to the chamber in order to balance pressure conditions inside the closed containers. Steam penetration is assured by choosing suitable cycles to remove air from porous loads or hollow bodies. Steam penetration is verified during cycle development by, for example, the use of physical/chemical indicators, while the biological effectiveness of the cycle is verified by the use of biological indicators (*5.1.2*). Appropriate loading patterns are specified.

**Cycle effectiveness**

The reference cycle for steam sterilisation is 15 min at 121 °C in saturated steam determined in the coldest position of the chamber. Product- and load-specific cycles, e.g. applying another combination of time and temperature, may be adopted based on cycle development and validation. The minimum temperature acceptable for a steam sterilisation process is 110 °C. The minimum $F_0$, calculated in the slowest-to-heat position of the load is not less than 8 min. The calculation of sterilisation effectiveness by the $F_0$ concept is performed according to general chapter *5.1.5*.

Calculated effectiveness from physical parameters ($F_{phys}$) is correlated with biological effectiveness ($F_{bio}$). $F_{bio}$ expresses the lethality, in minutes, provided by the process in terms of destruction of the biological indicators used. $F_{bio}$ is calculated by the following equation:

$$F_{bio} = D_{121}\left(\log_{10} N_0 - \log_{10} N\right)$$

$D_{121}$ is the $D$-value of the biological indicator at an exposure temperature of 121 °C, $N_0$ is the number of viable micro-organisms in the biological indicator before exposure, and $N$ is the number of viable micro-organisms in the biological indicator after exposure.

In cycle validation, the relevant positions in the load that are the most difficult to sterilise are determined and adequate biological effectiveness is verified by exposure of biological indicators (*5.1.2*) in these positions or products, whichever is relevant. Protection of spores from the sterilising effect (e.g. by physical occlusion of steam or by the protective properties of the product) are suitably addressed. The $F_{bio}$ determined for the most-difficult-to-sterilise position is used to define the parameters necessary to achieve reliably the required SAL equal to or less than $10^{-6}$ for the chosen cycle.

**Routine control**

Autoclave cycles are monitored by physical determination of chamber pressure and temperature profiles, at a minimum, in the coldest position of the chamber. For each cycle, pressure, time and temperature are recorded and, if possible, $F_0$ is calculated and recorded.

*DRY HEAT STERILISATION*

**Principle**

Dry heat sterilisation is a terminal sterilisation method based on the transfer of heat to the articles to be sterilised. Heat may be transferred by means of convection, radiation or direct transfer.

**Equipment**

Dry heat sterilisation is carried out in an oven with forced air circulation or using other equipment specifically designed for this purpose, e.g. a tunnel.

**Sterilisation cycle**

The steriliser is loaded in such a way that the specified or required temperature is achieved throughout the load. Knowledge of the temperature within the steriliser during the sterilisation cycle is obtained by means of temperature-sensing elements suitably placed in or on representative items situated in the coolest part (as previously established) of the loaded steriliser. The time and temperature throughout each cycle is suitably recorded.

**Cycle effectiveness**

The reference conditions for this method of sterilisation are a minimum of 160 °C for at least 2 h. Other combinations of time and temperature may be used if it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operated within the established tolerances. The procedures and precautions employed are such as to achieve an SAL equal to or less than $10^{-6}$. Dry heat sterilisation processes are validated using a combination of temperature mapping and biological indicator studies (*5.1.2*).

Dry heat at temperatures greater than 220 °C, for a validated time, is frequently used for depyrogenation of glassware. In this case, demonstration of a 3 $\log_{10}$ reduction in heat-resistant endotoxin can be used as validation criteria and biological indicators will not be needed.

**Routine control**

Dry heat sterilisation cycles are monitored by determination of temperature profiles, at a minimum, in the coldest position of the chamber. Time and temperature are recorded for each cycle.

*IONISING RADIATION STERILISATION*

**Principle**

Sterilisation by irradiation is achieved by exposure of the product to ionising radiation in the form of either gamma rays from a suitable isotopic source (such as cobalt 60), a beam of electrons energised by a suitable electron accelerator, or X-rays resulting from bombarding a suitable target with energised electrons. Ionising radiation may be used for the terminal sterilisation of finished dosage forms, the microbial inactivation of tissues and cells, or the sterilisation of materials or containers to be employed in aseptic processing. Low-energy electrons may be used for the surface sterilisation of materials upon entry to isolators used in the preparation of sterile products.

**Cycle effectiveness**

For this method of sterilisation, the reference absorbed dose is 25 kGy. Other doses may be used if, during validation of

the sterilising dose, it has been satisfactorily demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to achieve an SAL equal to or less than $10^{-6}$. Biological indicators may be required for the development and validation of the sterilisation of tissues and cell products. They may also be required for products with a potential to prevent spore inactivation.

**Routine control**

During the sterilisation process, the sterilisation dose delivered is monitored using a dosimetry system, measurements from which are traceable to national standards.

*GAS STERILISATION (VAPOR PHASE STERILISATION)*

**Principle**

Gas sterilisation of surfaces may be used for the sterilisation of primary packaging materials, equipment and some pharmaceuticals.

It is essential that penetration by gas and moisture into the material to be sterilised is ensured, and that it is followed by a process whereby the gas is eliminated under conditions that have been previously established as sufficient to ensure that any residues of gas or related transformation by-products are below concentrations that could give rise to toxic effects during product use.

**Sterilising agents**

There are 2 main categories of gaseous sterilising agents as distinguished by their antimicrobial action: alkylating agents and oxidising agents.

*Alkylating agents.* Alkylating agents are highly reactive compounds and interact with many components, such as amino, sulfhydryl and hydroxyl groups in proteins and purine bases in nucleic acids.

Ethylene oxide is an alkylating agent that is associated with cytotoxic, carcinogenic and mutagenic effects.

*Oxidising agents.* Oxidising agents are highly reactive, toxic compounds. Such compounds currently used as sterilising agents include hydrogen peroxide and peracetic acid.

**Development and validation of sterilisation processes**

Gas sterilisation is performed by exposure of the product to the sterilising agent in a leak-proof chamber under specified conditions.

A typical gas sterilisation process consists of 3 phases: (pre)conditioning, sterilisation and aeration. The parameters necessary for these phases to produce the required SAL are established during process development. A combination of physical and biological methods is used to determine the optimum sterilisation conditions. The cycle shall not compromise the functionality of either product or the container.

**Sterilisation cycle**

Specialised equipment may be required for the monitoring of temperature, humidity and gas concentration during both validation and routine operation.

**Cycle effectiveness**

Validation of microbiological performance shall confirm the effectiveness of the defined process for the product/load combination in the steriliser. The lethality of the cycle may be determined by using an appropriate approach: after time-graded exposures, the rate of inactivation (*D*-value) of the test organisms can be established by construction of a survivor curve or by using a fraction-negative method.

Biological indicators shall be shown to be, at a minimum, as resistant to the sterilising agent as the microbiological contaminants of the product to be sterilised. They shall be placed within the product at locations where sterilising conditions are most difficult to achieve.

The effectiveness of the process is dependent on a number of parameters, including gas concentration, temperature, humidity, exposure time, load configuration and characteristics of the product and its packaging materials. The effect on the process effectiveness of any change in one or more of these parameters shall be investigated.

**Routine control**

The relevant cycle process parameters (including the results of the biological indicator test) are recorded.

*MEMBRANE FILTRATION*

**Principle**

Membrane filtration is used for reduction of viable and non-viable particles in gases and fluid products that are not amenable to sterilisation by heat or irradiation. In contrast to other sterilisation methods, the principle of membrane filtration is not inactivation but removal of microorganisms from the product. Removal is achieved by a combination of sieving and surface interaction.

**Equipment**

Membrane filters are available as flat stock (discs) in appropriate holders or as cartridges. Pore size ratings are based on the correlation between microbial retention and diffusion characteristics or bubble-point measurement. Many factors contribute to the effectiveness of the filtration process, e.g. shape, pore size, structure, surface properties, the structure and arrangement of the filter unit, interaction of the filter matrix with the product, applied pressure, flow and duration of the process. Filter characteristics have to be determined in a product-specific validation. Suitable integrity test procedures (e.g. diffusive flow measurement, bubble-point determination or water-intrusion testing) are employed, as recommended by filter manufacturers. Chemical and physical compatibility of the membranes with the product to be filtered and the conditions of the filtration process are demonstrated in development studies. The filter size is suitable for the volume of the product to be filtered and the bioburden.

For sterilisation of process gases, an appropriate frequency for physical integrity testing is established.

**Filtration effectiveness**

Microbial challenge tests with a suitable model system shall demonstrate the effectiveness of the filtration process. Where testing with the product is not possible (e.g. due to the antimicrobial properties of the product), a fluid that is representative of the product shall be used, or the test conditions are modified.

It is recommended that the filtration process is carried out as close as possible to the filling point.

**Sterilisation of membrane filters**

Membrane filters may be sterilised off-line or in-line. If sterilisation is off-line, steam penetration is verified and the filter is suitably protected against contamination. The sterilised filter is aseptically assembled in the production line by means of a validated procedure. For in-line sterilisation, steam penetration throughout the filtration equipment is assured and the pressure difference across the membrane is controlled to prevent damage to the membrane itself.

**Filtration process**

Sterilisation by membrane filtration is performed by passage of the product through a microporous membrane with a nominal pore size not greater than 0.22 µm.

The pre-sterilisation microbial contamination is determined for each batch of product and process parameters are applied as established and validated in the development of the filtration process.

Where multiple bioburden-reduction filters are used to increase the efficacy of the filtration process, the filter closest to the filling point in the final container is characterised as the sterilising filter.

The sterility and integrity of the equipment downstream from the point of filtration, the qualified environmental conditions and the validated aseptic procedures applied in the handling of the filtered product all contribute to preventing recontamination of the product. This is addressed in the section on aseptic assembly.

**Routine control**

Filtration processes are monitored by physical and microbiological determination of parameters established during validation studies. These parameters include the following: pre-sterilisation microbial contamination, pre-filtration integrity test results, duration of filtration, volume filtered, differential pressure and post-filtration integrity test results.

ASEPTIC ASSEMBLY

**Principle**

The objective of aseptic assembly is to maintain the sterility of a product that is assembled from components, each of which has been sterilised by one of the above methods. This is achieved by using conditions and facilities designed to prevent microbial contamination.

Aseptic processing may include aseptic filling of products into container/closure systems, freeze-drying under aseptic conditions, aseptic blending of formulations followed by aseptic filling, and aseptic packaging.

**Development and validation of aseptic assembly**

In order to maintain the sterility of the components and the product during assembly, careful attention needs to be given to the following:

- environment;
- personnel;
- critical surfaces;
- container/closure sterilisation and transfer procedures;
- the maximum holding period of the product before filling into the final container.

Process validation includes appropriate checks on all of the above and also regular checks on the process, which are carried out by means of process simulation tests using microbial growth media that are then incubated and examined for microbial contamination (media fill tests). In addition, a suitable sample of each batch of any product that is aseptically processed is tested for sterility (*2.6.1*).

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# 5.1.2. BIOLOGICAL INDICATORS AND RELATED MICROBIAL PREPARATIONS USED IN THE MANUFACTURE OF STERILE PRODUCTS

1. INTRODUCTION

The use of biological indicators in this general chapter is intended to cover the sterilisation of finished products and relevant related sterilisation processes i.e. sterilisation processes for items coming into direct contact with the final sterilised product. Other uses of biological indicators to validate the sterilisation of other non-terminal units is outside the scope of this general chapter.

Biological indicators are test systems containing viable micro-organisms (usually spores of bacteria) that provide a defined challenge to verify the required effectiveness of a specified sterilisation process.

Biological indicators are intended for the development and validation of the sterilisation processes and not for routine monitoring unless otherwise stated in this general chapter.

The validity of the sterilisation process and the validity of the biological indicators can be assured by the use of reduced sterilisation process conditions, whereby a small proportion of the micro-organisms within the biological indicator will be shown to survive. However, when the validated sterilisation process is used, there will be no surviving viable micro-organisms (see section 3-1-2).

Bacterial spores are resistant forms of life, they can be produced and standardised, and may be stored for long periods of time under appropriate conditions.

Commercially available biological indicators typically contain a standardised population of spores of a suitable bacterium. In cases where no suitable commercial biological indicators are available to characterise the sterilising effect in the product or at a position difficult to penetrate by the sterilant, custom-made biological indicators may be used. Such biological indicators can be prepared by inoculating a standardised spore suspension onto or into the item or product to be sterilised, such action may change the characteristics of the biological indicator.

A suspension of vegetative bacterial cells is used to validate the bacterial retention capability of sterilising grade filters when applied as a sterilisation step in an aseptic production process.

2. BIOLOGICAL INDICATORS FOR STERILISATION PROCESSES

In addition to the physical sterilisation parameters, the effectiveness of a sterilisation process as described in general chapter *5.1.1* is dependent on a large number of variables, which may include, but is not necessarily restricted to, the number and resistance of contaminating micro-organisms, penetration of the sterilant, time, temperature, concentration, pH, moisture content, and the chemical composition of the product or item being sterilised.

To validate a sterilisation process, physical conditions are chosen that are expected to sterilise the items in the load to achieve a sterility assurance level (SAL) equal to or less than $10^{-6}$ as described in general chapter *5.1.1*. In a physical validation process it is demonstrated that these conditions are delivered homogeneously to all parts and positions of the load. It is the aim of biological validation to demonstrate the correlation between the predicted effect of the physical conditions applied during the process and the observed biological effect on biological indicators. Using process parameters that have been demonstrated to deliver the required biological effect will ensure sterility of the resulting product in routine processing.

The selection of the type of biological indicator used will depend on:

- the nature of the sterilising agent (e.g. heat, gas or radiation);
- the expected effectiveness of the treatment (e.g. the $F_{phys}$ calculated from the process parameters);
- the process conditions (e.g. temperature, time, relative humidity, gas concentration, radiation dose);
- the characteristics of the pharmaceutical product or item (e.g. product in final container, packaging material, utensils such as tubes or pumps) to be sterilised.

In the development of a sterilisation process, the load and the product should be assessed to determine the most difficult position to sterilise (e.g. cold spots, vial-stopper interface, difficult to penetrate areas). When choosing the optimum biological challenge to a sterilisation process, the conditions in the most difficult position to sterilise in the load and the product should be simulated as closely as possible.

Spores inoculated into a product or onto surfaces are known to react differently to sterilising conditions as compared to biological indicator units. In these cases, commercially available biological indicator units may not be suitable to test sterilisation effectiveness and an inoculated test product/item prepared from a well-characterised spore suspension may be a better model to evaluate the effectiveness of the sterilisation cycle.

2-1. *DESCRIPTION OF BIOLOGICAL INDICATORS FOR STERILISATION PROCESSES*

Depending on the process to be characterised, a suitable biological challenge may consist of biological indicators presented as test micro-organism suspensions, inoculated carriers, or self-contained biological indicators. The user must establish a high level of confidence in the manufacturer's compliance to quality standards for the biological indicator (e.g. by means of auditing) in order to rely on the characteristics stated by the manufacturer (see section 2-2). Alternatively, the labelled characteristics of biological indicators shall be verified by the user or by an independent, contract laboratory that is formally approved by the user. For custom-made biological indicators (see section 2-1-4), the characteristics shall be verified by the user or by a contract laboratory.

2-1-1. **Inoculated carriers**

Inoculated carriers consist of a defined population of bacterial spores inoculated into or onto a suitable carrier, and in most cases, in a protective envelope. The type of carrier (and the envelope if used) may influence the resistance of the bacterial spores and must be compatible with the chosen sterilisation process (e.g. strips of filter paper in glassine envelopes are frequently used for steam and ethylene oxide, while metal discs packaged in non-woven fibre envelopes are used for hydrogen peroxide vapour). After exposure to the sterilisation process, the carrier is aseptically handled according to the manufacturer's instructions, transferred to a suitable culture medium and incubated for a sufficient period of time at the appropriate temperature.

2-1-2. **Self-contained biological indicators**

A self-contained biological indicator may be, for example:

- a system consisting of an inoculated carrier and a container (e.g. ampoule) with a nutrient medium suitable for the test micro-organism used; the system is designed in such a way that the sterilising agent comes into contact with the inoculated carrier (e.g. through a tortuous path or a filter) while the growth-promoting properties of the nutrient medium are not adversely affected by the sterilisation process. After sterilisation, the carrier is brought into contact with the nutrient medium by simple manipulation. This type of biological indicator system may be used to characterise moist heat sterilisation processes including assurance of the penetration of steam into the system;
- a container (e.g. ampoule) of a population of the test micro-organism in an appropriate nutrient medium. After sterilisation, the container is incubated without further manipulation. This type of biological indicator is sensitive only to an exposure time and temperature and may be used primarily to monitor sterilisation of aqueous fluids. In order to facilitate detection of growth, the medium may contain an indicator (e.g. a pH indicator).

Self-contained biological indicators might not be suitable for the validation of certain sterilisation processes.

2-1-3. **Characterised spore suspensions**

Characterised spore suspensions consist of a defined population of bacterial spores, prepared from a clearly characterised and suitably maintained strain of a spore-forming bacterial species (e.g. of the genera *Bacillus* or *Clostridium*) in a stable suspension.

2-1-4. **Custom-made biological indicators**

Custom-made biological indicators are test items (e.g. rubber stoppers), or products, inoculated with a suitable test micro-organism, usually from a characterised spore suspension but also from spore suspensions prepared from isolates from environmental monitoring or other microbiological testing using a well-defined procedure designed to give satisfactory sporulation. The *D*-value (time for 90 per cent reduction of micro-organisms under the stated conditions) and, when appropriate, the *z*-value (see section 3-1-1) of the spore suspension must be determined. Also the *D*-value and *z*-value (if appropriate) of the spores of the inoculated test items/ products must be determined as this may be different from the spores in suspension.

After exposure to the sterilisation cycle, the custom-made biological indicator is enumerated or tested for the presence/absence of surviving test micro-organisms using a validated, appropriate microbiological technique.

2-2. *QUALITY REQUIREMENTS FOR BIOLOGICAL INDICATORS*

The following are required to be known by the user per delivery of each batch:

- genus and species of the micro-organism (including the type culture collection number where applicable);
- unique reference (e.g. batch number);
- logarithm of the viable spore count expressed to 1 decimal place in scientific notation;
- recovery method used;
- type of carrier;
- type of packaging (e.g. envelope);
- composition of the recovery medium, if needed (e.g. in case of self-contained biological indicators);
- type of indicator (e.g. pH indicator) for growth, if relevant;
- type of sterilisation process(es) and the conditions for which the biological indicator has been characterised;
- resistance (*D*-value) per batch of finished biological indicator against the specified sterilisation processes throughout the labelled shelf-life; the *D*-value should be stated in applicable units (e.g. time or dose) and expressed to 1 decimal place, together with a 95 per cent confidence interval if feasible;
- method (inactivation kinetics or fraction negative method) used to determine the resistance (*D*-value); parameters to verify e.g. exposure conditions, number of replicates tested, medium and incubation conditions used for recovery after exposure, etc.;
- the *z*-value (where relevant) for the biological indicator stated in temperature units expressed to 1 decimal place in scientific notation, including the range of temperatures used to determine the *z*-value;
- the storage conditions and the expiry date.

2-2-1. **User requirement specification (URS)**

The particular sterilisation process (moist heat, dry heat, gas, or ionising radiation) is considered as the basis for the choice of the biological indicator. This choice includes the selection of the test micro-organism, the type of biological indicator (inoculated carrier, self-contained, or custom-made), the *D*-value, and the initial spore count. Moreover, the resistance of the test strain is suitable for the particular sterilisation method and is great compared to the resistance of micro-organisms potentially contaminating the product.

2-2-2. **Quality control**

Quality control for biological indicators consists of testing for purity, identity and estimation of the number of viable cells. The biological indicator should be compliant with the URS. Users employing biological indicators outside of the manufacturer's labelled recommendations should thoroughly

characterise the biological indicators for the particular sterilisation process.

*Purity*

Examination of the micro-organisms on a suitable culture medium incubated under appropriate conditions shall not show any evidence of contamination.

*Identification*

Colony morphology and homogeneity of the population are verified, as appropriate.

*Viable count*

The viable count is performed according to the manufacturer's instructions or by any other validated method.

2-2-3. **Suitability for purpose**

The user shall ensure that the biological indicator is inactivated to the expected survival rate by the particular range of sterilisation conditions used.

## 3. BIOLOGICAL INDICATORS FOR HEAT STERILISATION

### 3-1. *PARAMETERS OF BIOLOGICAL INDICATORS FOR HEAT STERILISATION*

3-1-1. ***z*-Value**

Sterilisation processes can be operated at temperatures lower than the standard 121 °C (for longer exposure times) or at higher temperatures (for shorter exposure times). The *z*-value (the temperature difference that leads to a 10-fold change in the *D*-value of the biological indicator) is used to compare the efficacy of 2 cycles operated at different temperatures. For a *z*-value determination, the *D*-value must be determined at 3 or more temperatures. The intended process temperature should be within the range of the 3 temperatures. The $\log_{10}$ of the *D*-value is plotted against the temperature in degrees Celsius. The *z*-value is equal to the negative reciprocal of the slope of the best-fit linear curve as determined by $\log_{10}$-linear regression analysis.

3-1-2. **Establishment of validation cycle**

The characteristics of the sterilisation process (e.g. time-temperature combination, level of sterility assurance or $F_0$ required) are the basis for the choice of the biological indicator (type of biological indicator, test micro-organism, and initial viable count).

Inactivation of micro-organisms under sterilising conditions can be described by lethality kinetics and statistical probabilities. For a number of biological indicator units with an initial population of $N_0$ micro-organisms per unit and a given *D*-value, the exposure time in minutes where all units are expected to carry survivors (average of 100 surviving spores per unit) is calculated by equation (1).

$$t_s = D \times \left(\log_{10} N_0 - 2\right) \qquad (1)$$

$t_s$ = survival time

The exposure time in minutes where all units are expected to be inactivated (average of $10^{-4}$ surviving spores per unit) is calculated by equation (2).

$$t_k = D \times \left(\log_{10} N_0 + 4\right) \qquad (2)$$

$t_k$ = kill time

The objective of a validation study is to demonstrate that the sterilisation effectiveness anticipated from the physical process parameters is equivalent to the biological sterilisation effectiveness. As part of that objective, the exposure time during validation $t_{vl}$, shall not exceed $t_k$. If a too high $t_{vl}$ is chosen, even a relatively large increase in the *D*-value would still result in biological indicator units with no surviving micro-organisms. In this case, the suboptimal sterilising conditions would not be detected. It is considered reasonable to choose a $t_{vl}$ not higher than required to expect 1 in 1000 biological indicator units having surviving micro-organisms. However, too short a $t_{vl}$ shall not be chosen. If a $t_{vl}$ is chosen such that 50 per cent of the biological indicator units have surviving micro-organisms, changes in the sterilising conditions (e.g. time, temperature) could still result in 100 per cent of the biological indicator units having surviving micro-organisms, and the test would be meaningless. For these reasons, a $t_{vl}$ is chosen such that a theoretical survival rate between $10^{-1}$ and $10^{-3}$ is expected, thus:

$$D \times \left(\log_{10} N_0 + 1\right) \leq t_{vl} \leq D \times \left(\log_{10} N_0 + 3\right) \qquad (3)$$

In general, biological indicators are subjected to the intended sterilisation process. However, for highly effective sterilisation processes, the calculated effectiveness of the cycle may be such that the $t_k$ is exceeded by a wide margin. In such instances, biological validation is carried out with reduced sterilisation cycles. Such reduced cycles may be shorter in time (e.g. half cycle) or be performed at a lower temperature. In the latter case the *z*-value for the test micro-organism under the actual sterilising conditions shall be known. A reduced cycle is chosen such that the temperature is not more than 1 *z*-value below the reference sterilisation process temperature. Biological indicators of an appropriate resistance for that cycle show an expected micro-organism survival rate within a window between the lower $t_{vl}$ and the $t_k$ (see equation (3)). A decision not to perform this test must be justified.

Depending on the *D*-value of the test micro-organism and the $t_{vl}$ chosen, biological indicators having surviving micro-organisms can be expected with a low frequency (not more than 1 in 10). If it can be demonstrated that the frequency of biological indicators having surviving micro-organisms is within the expected range and is not due to inappropriate sterilising conditions, the process can be accepted.

Following a full sterilisation cycle, all biological indicators in a validation study must be inactivated, thereby proving at least a $10^6$ reduction in micro-organisms. It can then be concluded, from the resistance of the spore preparation used, that the process has delivered sufficient lethality to achieve the required sterility assurance level.

### 3-2. *BIOLOGICAL INDICATORS FOR MOIST HEAT STERILISATION*

3-2-1. **Test micro-organisms**

*Geobacillus stearothermophilus* is the most widely accepted biological indicator micro-organism for moist heat sterilisation processes. Reported $D_{121\,°C}$-values for its spores are in the range of 1.5 min to about 4.5 min, depending on sporulation conditions, the carrier material on which the spores are inoculated, the primary package surrounding the inoculated carrier, and the environment during sterilisation. Strains ATCC 7953, NCTC 10007, CIP 52.81, NCIMB 8157 and ATCC 12980 (equivalent to NRRL B-4419) have been found to be suitable. Other strains may be used, provided equivalent performance has been demonstrated. It is recognised that a $10^5$ or $10^6$ population of *Geobacillus stearothermophilus* may not be suitable for sterilisation processes delivering an $F_0$ between 8 and 15, therefore a lower spore number (i.e. $10^3$ or $10^4$) or a different test micro-organism may be used. Where a test micro-organism other than *Geobacillus stearothermophilus* (e.g. *Bacillus subtilis* ATCC 35021) is used, the resistance of the test micro-organism is evaluated to ensure its suitability for the process.

### 3-3. *BIOLOGICAL INDICATORS FOR DRY HEAT STERILISATION*

The reference conditions are stated in general chapter *5.1.1*. Heat transfer is less effective with dry heat than with steam, and temperature distribution in dry heat sterilisers is less homogeneous compared to steam sterilisers.

For example, biological indicators available for dry heat sterilisation have $D_{160\,°C}$-values within a range of 1 to 5 min. When exposed to the reference cycle of 2 h at 160 °C, a biological indicator with a $D_{160\,°C}$-value of 2.5 min would be inactivated by 48 $\log_{10}$ scales. For dry-heat sterilisation processes, *z*-values of about 20 °C are typically assumed in calculations of equivalence of cycle effectiveness ($F_H$-calculations). $F_H$ is the equivalent time in minutes at a temperature of 160 °C delivered by the sterilisation process to the product in its final container. For a biological indicator with a $D_{160\,°C}$-value of 5 min, the $D_{150\,°C}$-value would be about 16 min, and inactivation in the reference cycle would be 7.5 $\log_{10}$ scales. The use of a sterilisation process at a temperature reduced from the target temperature by 10 °C would give an expected 1 in 30 biological indicator units having surviving micro-organisms.

3-3-1. **Test micro-organisms**

Spores of *Bacillus atrophaeus* (e.g. ATCC 9372, NCIMB 8058, NRRL B-4418, or CIP 77.18) have been found to be suitable for use as biological indicators for dry heat sterilisation processes performed at temperatures between 160 °C and 180 °C. Where a test micro-organism other than *Bacillus atrophaeus* is used, to ensure its suitability, the resistance of the test micro-organism for the sterilisation process is evaluated as described in section 3-1-2.

4. BIOLOGICAL INDICATORS FOR GAS STERILISATION

The use of biological indicators is necessary for the development, validation and monitoring of all gaseous sterilisation processes. Gas sterilisation is a multi-factorial process: gas concentration, humidity, temperature, time, surface characteristics interact in a complex manner. A number of gas sterilisation processes are currently used, including ethylene oxide, hydrogen peroxide and peracetic acid or combinations of the latter.

Gas surface disinfection is widely used for medical devices, isolators, chambers, etc. Use for such purposes is outside the scope of the European Pharmacopoeia but the use of biological indicators as described in this general chapter may assist in the validation of such disinfection processes.

4-1. *TEST MICRO-ORGANISMS*

4-1-1. **Ethylene oxide sterilisation**

The use of spores of *Bacillus atrophaeus* (e.g. ATCC 9372, NCIMB 8058, NRRL B-4418, or CIP 77.18), or other strains of micro-organism having demonstrated equivalent performance, is recommended for ethylene oxide sterilisation. The number of viable spores is greater than or equal to $10^6$ per carrier. Test micro-organisms shall have *D*-values relevant to the process to be validated. These biological indicators are used routinely during each sterilisation cycle thus allowing the effectiveness of the process to be checked.

4-1-2. **Other processes**

It is the responsibility of the user to define the sterilisation cycle and the suitability of any biological indicator used. *Geobacillus stearothermophilus* has been found suitable for vaporised hydrogen peroxide processes.

5. BIOLOGICAL INDICATORS FOR IONISING RADIATION STERILISATION

Unless otherwise indicated, biological indicators are not generally considered necessary for validation of the sterilising dose for radiation sterilisation. The use of biological indicators may however be required for the development and validation of ionising radiation sterilisation e.g. of tissues, cell preparations or other specific cases (e.g. products with a potential for spore protection).

5-1. *TEST MICRO-ORGANISMS*

Spores of *Bacillus pumilus* (e.g. ATCC 27142, NCTC 10327, NCIMB 10692 or CIP 77.25) or other strains of micro-organisms having demonstrated equivalent or better performance are recommended.

6. MICROBIAL PREPARATIONS FOR STERILISATION GRADE FILTRATION

As stated in general chapter *5.1.1*, certain products that cannot be sterilised in their final container may be sterilised by a filtration process. In contrast to the biological indicators discussed in the previous sections, which assess kill-based sterilisation, the biological challenge assesses the retention of micro-organisms by the filters.

To validate the sterilisation process, it must be demonstrated that the filtration process (usually in a scaled-down model) is capable of completely retaining a microbial challenge of at least $10^7$ CFU per square centimetre of effective filter surface using a suitable test micro-organism. This test should mimic the actual filtration process as closely as possible. Where feasible, the test is carried out in the product using the specified filtration conditions. If this is not possible, e.g. due to the antimicrobial properties of the product, a medium as similar as possible to the product must be used in the test.

6-1. *TEST MICRO-ORGANISMS*

For processes using a filtration system with a nominal pore size not greater than 0.22 µm, a suspension of *Brevundimonas diminuta* (ATCC 19146, NCIMB 11091 or CIP 103020) is recommended. The *Brevundimonas diminuta* suspension must be prepared in order to achieve predominantly single cells of the smallest possible size. Other micro-organisms, for example natural flora isolated from the product or process in question, may be used if presenting a stronger challenge to the sterile filtration system than *Brevundimonas diminuta*. For filtration systems with a nominal pore size of 0.1 µm or less, a suspension of *Acholeplasma laidlawii* (ATCC 23206) may be used.

**01/2011:50103**

## 5.1.3. EFFICACY OF ANTIMICROBIAL PRESERVATION

If a pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present a hazard to the patient from infection and spoilage of the preparation. Antimicrobial preservatives must not be used as a substitute for good manufacturing practice.

The efficacy of an antimicrobial preservative may be enhanced or diminished by the active constituent of the preparation or by the formulation in which it is incorporated or by the container and closure used. The antimicrobial activity of the preparation in its final container is investigated over the period of validity to ensure that such activity has not been impaired by storage. Such investigations may be carried out on samples removed from the final container immediately prior to testing.

During development of a pharmaceutical preparation, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage and use of the preparation.

The efficacy of the antimicrobial activity may be demonstrated by the test described below. The test is not intended to be used for routine control purposes.

TEST FOR EFFICACY OF ANTIMICROBIAL PRESERVATION

The test consists of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples so removed.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or no increase, as appropriate, in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The acceptance criteria, in terms of decrease in the number of micro-organisms with time, vary for different types of preparations according to the degree of protection intended (see Tables 5.1.3.-1/2/3).

*Test micro-organisms*

| | |
|---|---|
| *Pseudomonas aeruginosa* | ATCC 9027; NCIMB 8626; CIP 82.118. |
| *Staphylococcus aureus* | ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83. |
| *Candida albicans* | ATCC 10231; NCPF 3179; IP 48.72. |
| *Aspergillus brasiliensis* | ATCC 16404; IMI 149007; IP 1431.83. |

Single-strain challenges are used and the designated micro-organisms are supplemented, where appropriate, by other strains or species that may represent likely contaminants to the preparation. It is recommended, for example, that *Escherichia coli* (ATCC 8739; NCIMB 8545; CIP 53.126) is used for all oral preparations and *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92) for oral preparations containing a high concentration of sugar.

**Preparation of inoculum**

Preparatory to the test, inoculate the surface of casein soya bean digest agar (*2.6.12*) for bacteria or Sabouraud-dextrose agar without the addition of antibiotics (*2.6.12*) for fungi, with the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of *C. albicans* at 20-25 °C for 48 h, and the culture of *A. brasiliensis* at 20-25 °C for 1 week or until good sporulation is obtained. Subcultures may be needed after revival before the micro-organism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, use a sterile suspending fluid, containing 9 g/L of *sodium chloride R*, for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about $10^8$ micro-organisms per millilitre. To harvest the *A. brasiliensis* culture, use a sterile suspending fluid containing 9 g/L of *sodium chloride R* and 0.5 g/L of *polysorbate 80 R* and adjust the spore count to about $10^8$ per millilitre by adding the same solution.

Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per millilitre in each suspension by plate count or membrane filtration (*2.6.12*). This value serves to determine the inoculum and the baseline to use in the test. The suspensions shall be used immediately.

METHOD

To count the viable micro-organisms in the inoculated products, use the agar medium used for the initial cultivation of the respective micro-organisms.

Inoculate a series of containers of the product to be examined, each with a suspension of one of the test organisms to give an inoculum of $10^5$ to $10^6$ micro-organisms per millilitre or per gram of the preparation. The volume of the suspension of inoculum does not exceed 1 per cent of the volume of the product. Mix thoroughly to ensure homogeneous distribution.

Maintain the inoculated product at 20-25 °C, protected from light. Remove a suitable sample from each container, typically 1 mL or 1 g, at zero hour and at appropriate intervals according to the type of the product and determine the number of viable micro-organisms by plate count or membrane filtration (*2.6.12*). Ensure that any residual antimicrobial activity of the product is eliminated by dilution, by filtration or by the use of a specific inactivator. When dilution procedures are used, due allowance is made for the reduced sensitivity in the recovery of small numbers of viable micro-organisms. When a specific inactivator is used, the ability of the system to support the growth of the test organisms is confirmed by the use of appropriate controls.

The procedure is validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

ACCEPTANCE CRITERIA

The criteria for evaluation of antimicrobial activity are given in Tables 5.1.3.-1/2/3 in terms of the $\log_{10}$ reduction in the number of viable micro-organisms against the value obtained for the inoculum.

Table 5.1.3.-1. - *Parenteral preparations, eye preparations, intrauterine preparations and intramammary preparations*

| | | $\log_{10}$ reduction | | | | |
|---|---|---|---|---|---|---|
| | | **6 h** | **24 h** | **7 d** | **14 d** | **28 d** |
| Bacteria | A | 2 | 3 | - | - | NR |
| | B | - | 1 | 3 | - | NI |
| Fungi | A | - | - | 2 | - | NI |
| | B | - | - | - | 1 | NI |

NR: no recovery.

NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-2. - *Ear preparations, nasal preparations, preparations for cutaneous application and preparations for inhalation*

| | | $\log_{10}$ reduction | | | |
|---|---|---|---|---|---|
| | | **2 d** | **7 d** | **14 d** | **28 d** |
| Bacteria | A | 2 | 3 | - | NI |
| | B | - | - | 3 | NI |
| Fungi | A | - | - | 2 | NI |
| | B | - | - | 1 | NI |

NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-3. - *Oral preparations, oromucosal preparations and rectal preparations*

| | $\log_{10}$ reduction | |
|---|---|---|
| | **14 d** | **28 d** |
| Bacteria | 3 | NI |
| Fungi | 1 | NI |

NI: no increase in number of viable micro-organisms compared to the previous reading.

The above criteria express the recommended efficacy to be achieved.

**04/2019:50104**

# 5.1.4. MICROBIOLOGICAL QUALITY OF NON-STERILE PHARMACEUTICAL PREPARATIONS AND SUBSTANCES FOR PHARMACEUTICAL USE[(1)]

◊*This chapter does not apply to products containing viable micro-organisms as active ingredients.*◊

The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers therefore have to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage and distribution of pharmaceutical preparations.

Microbial examination of non-sterile products is performed according to the methods given in general chapters *2.6.12* and *2.6.13*. Acceptance criteria for non-sterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC) are given in Tables 5.1.4.-1 and 5.1.4.-2. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- $10^1$ CFU: maximum acceptable count = 20;
- $10^2$ CFU: maximum acceptable count = 200;
- $10^3$ CFU: maximum acceptable count = 2000, and so forth.

Table 5.1.4.-1 includes a list of specified micro-organisms for which acceptance criteria are set. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the micro-organisms listed in Table 5.1.4.-1, the significance of other micro-organisms recovered is evaluated in terms of:

- use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);
- nature of the product: its ability to support growth, the presence of adequate antimicrobial preservation;
- method of application;
- intended recipient: risk may differ for neonates, infants, the debilitated;
- use of immunosuppressive agents, corticosteroids;
- presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialised training in microbiology and the interpretation of microbiological data. For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the desired quality.

Table 5.1.4.-1. – *Acceptance criteria for microbiological quality of non-sterile dosage forms*

| Route of administration | TAMC (CFU/g or CFU/mL) | TYMC (CFU/g or CFU/mL) | Specified micro-organisms |
|---|---|---|---|
| Non-aqueous preparations for oral use | $10^3$ | $10^2$ | Absence of *Escherichia coli* (1 g or 1 mL) |
| Aqueous preparations for oral use | $10^2$ | $10^1$ | Absence of *Escherichia coli* (1 g or 1 mL) |
| Rectal use | $10^3$ | $10^2$ | - |
| Oromucosal use<br>Gingival use<br>Cutaneous use<br>Nasal use<br>Auricular use | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL)<br>Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) |
| Vaginal use | $10^2$ | $10^1$ | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)<br>Absence of *Staphylococcus aureus* (1 g or 1 mL)<br>Absence of *Candida albicans* (1 g or 1 mL) |
| Transdermal patches (limits for one patch including adhesive layer and backing) | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 patch)<br>Absence of *Pseudomonas aeruginosa* (1 patch) |
| Inhalation use (special requirements apply to liquid preparations for nebulisation) | $10^2$ | $10^1$ | Absence of *Staphylococcus aureus* (1 g or 1 mL)<br>Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)<br>Absence of bile-tolerant gram-negative bacteria (1 g or 1 mL) |
| ♦Special Ph. Eur. provision for oral dosage forms containing raw materials of natural (animal, vegetal or mineral) origin for which antimicrobial pretreatment is not feasible and for which the competent authority accepts TAMC of the raw material exceeding $10^3$ CFU/g or CFU/mL. | $10^4$ | $10^2$ | Not more than $10^2$ CFU of bile-tolerant gram-negative bacteria (1 g or 1 mL)<br>Absence of *Salmonella* (10 g or 10 mL)<br>Absence of *Escherichia coli* (1 g or 1 mL)<br>Absence of *Staphylococcus aureus* (1 g or 1 mL)♦ |
| ♦Special Ph. Eur. provision for premixes for medicated feeding stuffs for veterinary use using excipients of plant origin for which antimicrobial treatment is not feasible. | $10^5$ | $10^4$ | Not more than $10^4$ CFU of bile-tolerant gram-negative bacteria (1 g or 1 mL)<br>Absence of *Escherichia coli* (1 g or 1 mL)<br>Absence of *Salmonella* (25 g or 25 mL)♦ |

(1) This chapter has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

Table 5.1.4.-2. – *Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use*

| | TAMC (CFU/g or CFU/mL) | TYMC (CFU/g or CFU/mL) |
|---|---|---|
| Substances for pharmaceutical use | $10^3$ | $10^2$ |

♦ *Recommended acceptance criteria for microbiological quality of herbal medicinal products for oral use and extracts used in their preparation are given in general chapter 5.1.8.* ♦

**01/2009:50105**

# 5.1.5. APPLICATION OF THE $F_0$ CONCEPT TO STEAM STERILISATION OF AQUEOUS PREPARATIONS

*The following chapter is published for information.*

The $F_0$ value of a saturated steam sterilisation process is the lethality expressed in terms of the equivalent time in minutes at a temperature of 121 °C delivered by the process to the product in its final container with reference to micro-organisms possessing a theoretical $Z$-value of 10.

The total $F_0$ of a process takes account of the heating up and cooling down phases of the cycle and can be calculated by integration of lethal rates with respect to time at discrete temperature intervals.

When a steam sterilisation cycle is chosen on the basis of the $F_0$ concept, great care must be taken to ensure that an adequate assurance of sterility is consistently achieved. In addition to validating the process, it may also be necessary to perform continuous, rigorous microbiological monitoring during routine production to demonstrate that the microbiological parameters are within the established tolerances so as to give an SAL of $10^{-6}$ or better.

In connection with sterilisation by steam, the $Z$-value relates the heat resistance of a micro-organism to changes in temperature. The $Z$-value is the change in temperature required to alter the $D$-value by a factor of 10.

The $D$-value (or decimal reduction value) is the value of a parameter of sterilisation (duration or absorbed dose) required to reduce the number of viable organisms to 10 per cent of the original number. It is only of significance under precisely defined experimental conditions.

The following mathematical relationships apply:

$$F_0 = D_{121}\left(\log_{10}N_0 - \log_{10}N\right) = D_{121}\log_{10}IF$$

$D_{121}$ = $D$-value of the reference spores (*5.1.2*) at 121 °C;

$N_0$ = initial number of viable micro-organisms;

$N$ = final number of viable micro-organisms;

$IF$ = inactivation factor.

$$Z = \frac{T_2 - T_1}{\log_{10}D_1 - \log_{10}D_2}$$

$D_1$ = $D$-value of the micro-organism at temperature $T_1$;

$D_2$ = $D$-value of the micro-organism at temperature $T_2$.

$$IF = \frac{N_0}{N} = 10^{t/D}$$

$t$ = exposure time;

$D$ = $D$-value of micro-organism in the exposure conditions.

**07/2017:50106 corrected 10.0**

# 5.1.6. ALTERNATIVE METHODS FOR CONTROL OF MICROBIOLOGICAL QUALITY

*The following chapter is published for information.*

## 1. GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

The microbiological methods described in the European Pharmacopoeia have been used for over a century and these methods for detecting, enumerating and identifying micro-organisms still serve microbiologists well. Over the years, these methods have been invaluable for the production of microbiologically safe pharmaceutical products. However, these microbiological methods are slow, and in the case of sterility tests, results are not available before an incubation period of 14 days. Consequently, the results from these methods seldom enable proactive corrective action to be taken.

Alternative methods for the control of microbiological quality have shown potential for real-time or near real-time results with the possibility of earlier corrective action. These new methods, if validated and adapted for routine use, can also offer significant improvements in the quality of testing.

Alternative methods may be used for in-process samples of pharmaceutical products, particularly for the application of Process Analytical Technology (PAT), for environmental monitoring and for industrial utilities (e.g. production and distribution of water, steam etc.), thereby contributing to the quality control of these products.

In this chapter, alternative microbiological methods for pharmaceutical application are described. For each method, the basic principle is stated and the advantages and disadvantages of the method are discussed along with any critical aspects to be considered. Potential uses that may be envisaged based on the principles of the method concerned are given, but it is not intended to suggest that such applications have been realised or that the list provided is exhaustive.

It is not the intention of this chapter to recommend one method over another, nor is it the intention to provide an exclusive or exhaustive list of alternative methods that can be used for pharmaceutical microbiological control. The information herein may be used, however, in the process of choosing an alternative microbiological method as a supplement or as an alternative to pharmacopoeial microbiological methods and to give guidance on validation of the chosen method. If a suitable method is described in the Pharmacopoeia, this method is the reference method. In this rapidly developing field, other methods are likely to appear and the guidance offered herein may be equally applicable in these cases.

There are 3 major types of determination specific to microbiological tests:

- qualitative tests for the presence or absence of micro-organisms;
- quantitative tests for enumeration of micro-organisms;
- identification tests.

### 1-1. *QUALITATIVE TESTS FOR THE PRESENCE OR ABSENCE OF MICRO-ORGANISMS*

In conventional microbiological analysis, this type of test is characterised by the use of turbidity or other growth-related changes in a culture medium as evidence of the presence of

viable micro-organisms in the test sample. The most common example of this test is the test for sterility (*2.6.1*). Other examples include those tests designed to evaluate the presence or absence of a particular type of viable micro-organism in a sample. The conventional sterility test may be replaced by, for example, tests based on bioluminescence or solid phase cytometry, gas detection or autofluorescence. Nucleic acid amplification techniques (NAT) (*2.6.21*) may also be used for the detection of mycoplasmas (*2.6.7*).

1-2. *QUANTITATIVE TESTS FOR ENUMERATION OF MICRO-ORGANISMS*

Membrane filtration and plate count methods are conventional methods used to estimate the number of viable micro-organisms present in a sample. The Most Probable Number (MPN) method is another example of such methods and was developed as a means of estimating the number of viable micro-organisms present in a sample not amenable to direct plating. Examples of alternative methods for enumeration include autofluorescence, flow cytometry, direct epifluorescent filter technique (DEFT) and solid phase cytometry.

1-3. *IDENTIFICATION TESTS*

Biochemical and morphological characterisation of an unknown micro-organism is the classical approach to identification. Recently developed methods have streamlined and automated aspects of this identification, especially in the areas of data handling, analysis and storage. Several alternative approaches that have been integrated into these methods include biochemical reactions, carbon substrate utilisation, characterisation of fatty acid composition, mass spectroscopy and Raman spectroscopy, restriction endonuclease banding patterns and the use of genome sequencing methods such as 16S rRNA gene sequence analysis for prokaryotes.

Traditional biochemical and phenotypic techniques have been shown to be less accurate and precise than genotypic methods.

Pure cultures are required for a precise identification and such cultures must be fresh and cultivated in appropriate media.

Databases are part of the systems and are included in the primary validation. As identification methods depend on the use of databases, the extent of coverage of the database with respect to the range of micro-organisms of interest must be taken into account during validation. Appropriate software allows customisation of the database, thereby allowing the user to add micro-organisms not previously included. This possibility must be considered during the validation.

## 2. GENERAL PRINCIPLES OF ALTERNATIVE METHODS

Alternative microbiological methods employ direct and indirect methods of detection; in some instances amplification of the signal is achieved by enrichment methods. In recognition of these differences, and for convenience within this chapter, alternative methods for the control of microbiological quality are divided into 3 categories:

- growth-based methods, where a detectable signal is usually achieved by a period of culture;
- direct measurement, where individual cells are differentiated and/or imaged;
- cell component analysis, where the expression of specific cell components offers an indirect measure of microbial presence and identification of micro-organisms.

In some instances, these distinctions are artificial, but enable a working classification to be created.

2-1. *GROWTH-BASED METHODS*

2-1-1. **General critical aspects of methods based on early detection of growth**

Such methods are critically dependent on microbial growth in order to provide an indication of the presence and/or number of micro-organisms. For the typically low levels of microbial contamination seen in pharmaceutical products, detection may take 24 h or longer. Increased sensitivity can be achieved with filtered products. In this case, after filtration, the membrane filter is incubated in or on the medium and the result is expressed as presence or absence in the quantity corresponding to the filtered volume. These systems, if they use an incubation step in liquid media, do not offer quantitative information, but a presence/absence determination in the quantity analysed. Analysis of more than one sample quantity may offer a semi-quantitative estimation (limit test). The major benefit of early detection methods compared to classical methods is often the capacity to simultaneously process a large number of samples and the potential to obtain a result in a shorter time.

The methods described below can be used for quantitative, semi-quantitative or qualitative analyses. They are also non-destructive, therefore subsequent identification of the micro-organism is possible.

2-1-2. **Electrochemical methods**

*Principles of measurement.* Micro-organisms multiplying and metabolising in appropriate growth media produce highly charged ionic metabolites from weakly charged organic nutrients leading to the modification of electrical properties in such media. These changes in impedance (measured by conductance or capacitance) are monitored with electrodes included in the culture vessels and in contact with the culture medium. The measurable end-point is the time taken to detect a predetermined impedance change; for particular types of micro-organisms, the detection time is inversely proportional to the initial inoculum size. For yeasts and moulds, which only produce small changes in electrical impedance, an indirect measurement of conductance can be used. Direct measurement of capacitance can also be carried out.

*Critical aspects.* There is no direct relationship between the original microbial level and the detectable end-point.

*Potential uses.* Microbiological assay of antibiotics, efficacy of antimicrobial preservation and presence/absence testing.

2-1-3. **Measurement of consumption or production of gas**

*Principles of measurement.* Appropriate growth media is utilised by actively multiplying and metabolising micro-organisms, leading to the production of metabolites or the elimination of specific nutrients. These methods detect microbial growth either by changes in the electrical properties of a sensor in response to a change in gas composition or by colorimetric changes of a sensor in response to physico-chemical changes in the growth medium in contact with that sensor. The systems are based on non-destructive techniques which enable subsequent identification or strain typing of the micro-organisms. Bacteria and/or fungi may be grown in closed containers and continuous monitoring can be performed using automated instruments that measure gas evolution (e.g. $CO_2$) or consumption (e.g. $O_2$) as surrogate markers of microbial growth. Furthermore, the production of metabolites or elimination of nutrients can lead to changes in pH or redox potential. All of these changes can be measured either directly or indirectly as changes in colorimetric markers in the growth medium.

*Critical aspects.* There is no direct relationship between the original microbial level and the detectable end-point. The incubation temperature, the physiological state and type of micro-organism, the initial load and the algorithm for data processing can significantly affect the results or the time to detection.

*Potential uses.* Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill or container closure integrity testing).

2-1-4. **Bioluminescence**

*Principles of measurement.* Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP first needs to be released from the micro-organisms using an appropriate extractant, followed by an assay using

the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The signal-to-noise ratio can be increased by addition of ADP and converting this ADP into released ATP.

Qualitative method: micro-organisms are cultivated in liquid medium. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) (e.g. bioluminescence in a tube or a well of a microtitre plate). The RLU obtained from the sample is compared with a pre-determined threshold value. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value.

Quantitative method: micro-organisms are captured on a membrane and cultivated by incubation on agar medium. Using a charge coupled device (CCD) camera, the ATP released from microcolonies can be detected by light emission and a quantitative determination is possible.

*Critical aspects.* If the sample has a high level of bacterial contamination, the detection is rapid. For low levels of contamination, it is necessary to increase the number of micro-organisms using an incubation step in culture media (liquid or solid). The yield of ATP varies from one micro-organism to another and can depend on several factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age. Additional factors such as turbidity, sample colour or product matrix effects can also influence bioluminescence measurements. Extraction of ATP is generally a destructive process which should be considered with respect to any subsequent need for identification of detected micro-organisms.

*Potential uses.* Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill), total aerobic microbial count (TAMC), environmental and water monitoring, testing for efficacy of antimicrobial preservation.

2-1-5. **Turbidimetry**

*Principles of measurement.* Microbial growth leads to detectable changes in medium opacity, which can be accurately quantified by optical density measurements at a specified wavelength. In its simplest form, such measurements are performed using a standard spectrophotometer, generally over a wavelength range of 420-615 nm. Alternative automated systems employ microtitre plate readers offering a continuous readout with early detection of optical density change.

*Critical aspects.* Attempts have been made to extrapolate the initial microbial contamination from the time to detection, but this is limited to healthy micro-organisms with reproducible growth characteristics.

*Potential uses.* By means of calibration graphs, determination of the inoculum size of microbial suspensions for use in pharmacopoeial tests. In automated mode, microbiological assay of antibiotics and testing for efficacy of antimicrobial preservation.

2-1-6. **Growth detection using selective and/or indicative media**

*Principles of measurement.* The ability to detect the presence of specific enzymes using suitable chromogenic substrates has led to the development of a large number of methods for the identification of micro-organisms employing either manual or automated techniques. The incorporation of such substrates into a selective or non-selective primary isolation medium can eliminate the need for further subculture and biochemical testing for the identification of certain micro-organisms. Consequently, chromogenic liquid or solid culture media are designed to reveal specific enzymatic activities for detection and differentiation of micro-organisms. In these particular media, defined substrates are introduced into the formulation and are metabolised by the specific cell enzyme of a given bacterium or fungus during growth. These substrates, which are linked to coloured indicators, are chosen according to the diagnostic enzymatic activity sought. Furthermore, chromogenic broth can be used for early or improved detection of contamination (e.g. in media fill or broth-based detection methods).

The use of innovative media presents several advantages, namely improved discrimination of colonies in a mixed culture, ease of use and ease of interpretation. In addition, response times are shorter as the growth and identification of the micro-organism are simultaneous.

*Critical aspects.* Validation of the media must be undertaken carefully to ensure a combination of specificity, selectivity and robustness. The quality of the signal is based not only on the careful choice of the enzymes or indicators used as the basis of detection (as these enzymes may be present in different micro-organism genera), but also on the physico-chemical characteristics of the medium, e.g. pH.

*Potential uses.* Detection of specified micro-organisms and qualitative testing (e.g. media fill and container closure integrity testing) and quantitative testing (e.g. water testing).

2-2. *DIRECT MEASUREMENT*

2-2-1. **Solid phase cytometry**

*Principles of measurement.* Micro-organisms are stained for viability by exposure to a conjugated, initially non-fluorogenic, fluorophore. An intact cellular membrane is required to retain and accumulate the fluorophore within the cytoplasm. Inside metabolically-active microbial cells, the conjugate is enzymatically cleaved and the fluorescent derivative is released intracellularly. Micro-organisms are collected on a membrane filter either before or after viability staining.

Membrane surfaces retaining vital-stained cells are then scanned by a laser beam and epifluorescent excitation allows the detection of single, viable fluorescent micro-organisms. Appropriate software allows differentiation of viable micro-organisms from autofluorescent particles. The high sensitivity and rapidity of the method permit detection of microbial contaminants within a few hours. Total cell counts (viable and non-viable) can be obtained using fluorescent staining.

*Critical aspects.* Metabolically active, fastidious and viable non-culturable micro-organisms can all be detected. This may result in reappraisal of the microbial limits established for the samples under evaluation. Spores require initiation of germination to enable detection. Single cell detection may be achievable, but identification of isolates might not be possible. False positives may occur due to autofluorescent particles that can be difficult to differentiate from micro-organisms. Signal discrimination and enhancement can be aided by microcolony growth.

*Potential uses.* Rapid and sensitive method for the non-specific evaluation of microbial contamination.

2-2-2. **Flow cytometry**

*Principles of measurement.* Fluorophore-labelled micro-organisms can be detected in suspension as they pass through a flow cytometer. Viable micro-organisms can be differentiated from non-viable particles by use of a viability-indicating fluorophore (see 2-2-1). The cell suspension stream is dispersed into a narrow channel and exposed to a laser which excites the fluorophore. Micro-organisms and particles are then counted in different channels depending on whether or not they contain a fluorescent cell.

*Critical aspects.* Direct flow cytometry may be applied to the microbiological analysis of both filterable and non-filterable materials, and after possible enrichment in the case of the low contamination levels. It gives near real-time detection, but is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it is often necessary to add an incubation step in culture media, in which case the method becomes a combination of a growth-based

method and a direct detection method. Particle size and number may have a significant effect on performance, and samples may require serial dilution. With the exception of filterability, similar considerations to those in solid phase cytometry apply. Clumping of bacteria can be a problem (e.g. *Staphylococcus aureus*).

*Potential uses.* In contrast to solid phase cytometry, this method offers the potential to detect and enumerate microbial contamination in materials containing particulate matter and if the material cannot be filtered. If a pre-incubation step is needed, the method becomes a qualitative determination.

2-2-3. **Direct epifluorescent filtration technique (DEFT)**

*Principles of measurement.* This technique may be considered a forerunner of solid phase cytometry. Micro-organisms, concentrated by filtration of the sample, are stained with a fluorescent dye (formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI)), that can be detected by epifluorescent illumination. Fluorescent vital staining techniques, as employed in solid phase cytometry (see 2-2-1), are amenable to DEFT, and fluorescent redox dyes such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be used to highlight respiring cells. Coupled with microscopy, the method allows rapid detection of micro-organisms with an absolute sensitivity that is dependent on the volume of product filtered and the number of fields of view examined. Semi-automated auto-focusing systems coupled to image analysis have served to improve the utility of this method. A modification of the principle involves sampling using an adhesive sheet (which permits collection of cells from surfaces), subsequent staining on the sheet itself, followed by direct observation using an epifluorescence microscope.

*Critical aspects.* The distribution of micro-organisms on the membrane affects method robustness. The intensity of fluorescence can be influenced by the staining process and the metabolic status of the micro-organisms. Fluorescence is not necessarily an indicator of viability. A brief period of culture on the filter surface prior to staining allows microcolony formation; these microcolonies stain readily, can be easily enumerated and are demonstrable evidence of viability.

*Potential uses.* DEFT is generally limited to low viscosity fluids, although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Monitoring of microbial contamination has been successfully applied to aqueous pharmaceuticals.

2-2-4. **Autofluorescence**

*Principles of measurement.* The presence of endogenous autofluorescent molecules and metabolites (e.g. NADPH, flavoproteins) within micro-organisms allows the early detection and quantitative enumeration of microcolonies or single cells. For direct measurements, the laser-induced autofluorescence of a single micro-organism is captured by a detector, while for growth-based systems, automated sequential imaging of the membrane surface on agar medium over the incubation period is employed and image overlay allows differentiation of growing microcolonies from fluorescent particulates. The emitted light is detected by a CCD camera. Non-destructive detection allows identification of contaminants at the end of the incubation period.

*Critical aspects.* For a non-growth based measurement, viable, but non-culturable, micro-organisms might be detected. It may be difficult to distinguish between culturable micro-organisms, viable but non-culturable micro-organisms and/or other particles.

*Potential uses.* Environmental monitoring, filterable in-process samples, water testing and product release for both sterile and non-sterile applications.

2-3. *CELL COMPONENT ANALYSIS*

2-3-1. **Phenotypic techniques**

2-3-1-1. *Immunological methods*

*Principles of measurement.* Antibody-antigen reactions can be employed to detect unique cellular determinants of specific micro-organisms. These reactions can be linked to agglutination phenomena and colorimetric or fluorimetric end-points, which offer both quantitative and qualitative detection. Enzyme-linked immunosorbent assays (ELISA) offer simple solid-phase methodologies.

*Critical aspects.* Immunological detection methods depend on the unique expression of specific identifiers, but do not necessarily demonstrate the presence of viable micro-organisms.

*Potential uses.* Detection and identification of specified micro-organisms.

2-3-1-2. *Fatty acid profiles*

*Principles of measurement.* The fatty acid composition of micro-organisms is stable, well conserved and shows a high degree of homogeneity within different taxonomic groups. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted, and the occurrence and amount of the resulting fatty acid methyl esters are measured using high-resolution gas chromatography. The fatty acid composition of an unknown isolate is compared with a database of known isolates for a possible match and identification.

*Critical aspects.* The use of fatty acid profiles for microbial identification requires a high degree of standardisation. It is critical for the fatty acid composition of microbial cells that isolates are grown using standardised media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must also be employed, with frequent runs of calibration standards and known isolates being very important.

*Potential uses.* Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-3. *Fourier transform infrared (FTIR) spectroscopy*

*Principles of measurement.* A Fourier transformation of the infrared spectrum of whole micro-organisms gives a stable, recognisable pattern typical of the taxonomic groups of micro-organisms. The analysis of the FTIR pattern can be performed with commercially available instruments. The isolate is grown on a standard medium and harvested. Cell mass is transferred to a carrier, and the infrared spectrum is recorded. The Fourier transformation is calculated and the pattern is compared with a database of known isolates for a possible match and identification.

*Critical aspects.* The use of FTIR patterns for microbial identification requires a high degree of standardisation. It is critical for the FTIR pattern of microbial cells that isolates are grown using standardised media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed, and particular attention must be paid to this in the validation process.

*Potential uses.* Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-4. *Mass spectrometry*

*Principles of measurement.* Ionised particles released by exposing microbial isolates to a laser in a vacuum can be analysed by mass spectrometry, providing characteristic spectra. Similarly, intact microbial cells, when subject to intense ionisation under matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, release a distinctive pattern of charged species. Such spectra can be compared with known profiles.

*Critical aspects.* The isolates must be cultured under standardised conditions prior to analysis.

*Potential uses.* Identification or characterisation of environmental and product microbial contaminants (for contaminant tracing and detection of specified micro-organisms).

2-3-1-5. *Biochemical assays based on physiological reactions*

*Principles of measurement.* Systems capable of performing biochemical assays based on physiological reactions are used for the identification of micro-organisms. In the presence of a pure colony, the five basic steps for these assays are preparation, inoculation, incubation, readings and interpretation. These steps are usually preceded by a description of the colony morphology, a differentiation test (e.g. Gram stain), a description of the cellular morphology and/or other early biochemical differentiation tests (e.g. oxidase, catalase, coagulase) in order to determine the appropriate testing protocol.

The Gram stain is often a key characteristic upon which further testing is based. Alternatives to the traditional staining method include the potassium hydroxide (KOH) string test, the aminopeptidase test, a fluorescent staining method and a limulus amoebocyte lysate (LAL) based assay. Test kits are available for the latter 3 methods. The fluorescent staining method requires a fluorescence microscope or a flow cytometer.

Microbial cell suspensions are tested using biochemical (assimilation or susceptibility) test kits (plates or strips). Anaerobic and aerobic micro-organisms develop characteristic reactions to selected biochemical substances. They are also known to utilise specific carbon, nitrogen, phosphorus and sulfur sources or to be inhibited by a specific concentration of an antimicrobial agent. The results are based on measurable changes (e.g. turbidity, chromogenic or fluorogenic reaction) due to the growth or inhibition of the micro-organism under investigation. Comparison of the metabolic and/or antimicrobial resistance profile with a database allows for identification of the culture. These methods can be performed manually or by semi- or fully automated instruments. Complementary tests can be performed in cases of poor discrimination. Subcultures can help in cases of indeterminate results.

*Critical aspects.* A fresh physiological culture is required. The performance of the system is also dependent on the selected phenotypic parameters, which must be stable, significant and in sufficient number.

*Potential uses.* Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-2. **Genotypic techniques**

Identification and detection of micro-organisms as well as characterisation of strains belonging to the same species may be achieved by direct detection of nucleotide target sequences that are unique for a particular microbial species or microbial group, and are targets of the genotypic (DNA or RNA-based) detection techniques. These detection techniques may be separated into 3 broad categories: direct hybridisation, nucleic acid amplification and genetic fingerprinting.

2-3-2-1. *Direct hybridisation*

*General principles of measurement.* DNA probes are short, labelled, single-strand segments of DNA that hybridise with a complementary region of microbial DNA or RNA. The probe or target DNA is usually labelled with either radioactive, fluorescent or chromogenic molecules in order to provide a hybridisation signal. Hybridisation assays include fluorescence *in situ* hybridisation (FISH) and microarray-based techniques.

*General critical aspects.* Hybridisation generally requires a large amount of the target DNA for analysis, which may result in lower detection sensitivity. The availability of suitable probes may be limited.

*Potential uses.* Due to the high specificity of the sequence-based hybridisation reaction, this method may be used for both detection and identification of micro-organisms.

2-3-2-2. *Nucleic acid amplification techniques (NAT)*

*General principles of measurement.* NAT relies on the reiteration of the DNA polymerisation process, leading to an exponential increase of a specific nucleic acid fragment. The polymerase chain reaction (PCR) is the most widely used method for target DNA amplification. In this cyclic process, a specific DNA fragment is copied by a thermostable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers, previously designed to flank the target sequence and to hybridise with it (see also general chapter *2.6.21*). After PCR, the amplified nucleic acid targets can be analysed using several methods of post-amplification analysis: fragment size analysis in gel electrophoresis, DNA sequencing or specific detection by hybridisation with a fluorescent-labelled probe. Real-time PCR eliminates the need for further post-amplification processing and offers the additional advantage that the likelihood of cross-contamination is minimised. An important advantage of real-time PCR is the ability to quantify the starting amount of the DNA target sequence in the original sample, in contrast to conventional PCR techniques, which are based on end-point detection. Since the amount of PCR product detected at the beginning of the exponential phase of the amplification reaction correlates with the initial starting amount of the DNA target, modern real-time PCR techniques have been developed to measure this exponential phase of the reaction. Automated real-time PCR systems are commercially available. For identification of species, either species-specific probes or primers can be used.

RNA can also be amplified by both conventional and real-time PCR after transcription into cDNA using a reverse transcriptase enzyme. This technique is known as reverse transcriptase PCR (RT-PCR) and it enables detection and identification of RNA viruses or viable organisms. Alternatively, specific RNA-based amplification techniques, for example nucleic acid sequence-based amplification or transcription-mediated amplification, are available. Both techniques produce RNA amplicons, in contrast to PCR which only produces DNA amplicons, even when starting from an RNA target.

*Types of target to be amplified.* Regardless of the type of NAT used, the specificity of the test is determined by the target DNA sequence under evaluation. For identification/characterisation purposes, the 16S or 23S ribosomal RNA genes may be used as targets. The 16S rRNA gene is an evolutionary-conserved gene present in all bacterial species, and is a broad range target as it is a universal marker for bacterial detection. The 23S rRNA gene is not widely used as a single target, but the 16S-23S rRNA transcribed intergenic spacer regions can be employed to distinguish between certain closely related species and/or to identify subtypes. Alternative broad-range targets include the *groEL* and *tuf* genes. Apart from broad-range targets, species-specific sequences can be used as targets for micro-organism identification. Depending on the species, either specific surface antigens, virulence factors or genes which code toxins may be amplified to detect and identify micro-organisms.

*General critical aspects*:

- the target and the primers chosen must be specific for a particular micro-organism or group of micro-organisms;
- the sensitivity of the methods is highly dependent on the efficiency of the lysis protocol and how successfully the DNA targets can be purified and concentrated in the sample;

- the presence of inhibitors of the enzymatic process results in false negative reactions;
- the procedures are prone to cross-contamination from background DNA resulting in false positive results.

Depending on the aim, a choice must be made between amplification of either a DNA or an RNA target, as this target choice affects the correlation with viability. DNA targets are generally more widely used for identification purposes, but the use of DNA as a marker has the disadvantage that dead micro-organisms can also be detected. As mRNA is rapidly degraded in dead cells, it is considered a marker for viability. Furthermore, mRNA is the obligatory target for the identification of RNA viruses.

*Critical aspects of (semi-) quantitative detection by real-time PCR*. Quantification of the target requires generation of appropriate standards and the use of standardised procedures.

*Critical aspects of RT-PCR*. RNA is less stable compared to DNA, so it requires more attention during processing. Depending on the quality of the RNA isolation, the efficiency of the cDNA synthesis can vary. RT-PCR can be used to specifically detect RNA if DNA contamination of the RNA sample is low.

*Critical aspects of using the 16S or 23S rRNA gene as a target for species identification*. 16S rRNA gene sequencing is a valuable method for identification of bacteria provided that appropriate universal primers from databases are selected. Its discriminatory power depends on the variability and the length of the 16S rRNA gene within a certain species. Regarding the use of assays targeting the 16S-23S rRNA intergenic spacer regions, the choice of appropriate species-specific primers/probes is of critical importance due to the potential polymorphism of such regions.

*Potential uses of NAT*. Due to the high sensitivity and specificity of amplification techniques, they are suitable for both detection and identification of micro-organisms. Real-time PCR is needed for quantitative or semi-quantitative analysis of the target. Besides quantitative determinations, the real-time PCR technique allows simultaneous detection of multiple targets in a single sample, as long as appropriate primers and probes that allow for multiplexing are employed. The sequencing of different genes (e.g.16S rDNA, 23S rDNA, rpoB, Gyr) is best applied to the identification of micro-organisms.

2-3-2-3. *Genetic fingerprinting*

*Principles of measurement*. Genetic fingerprinting is the identification of a strain on the basis of its DNA profile (or RNA for RNA viruses). Individual DNA profiles may be different due to genetic diversity between strains of the same species, and the aim of the fingerprinting methods is to discriminate between these strains. The classical genetic fingerprinting technique characterises micro-organisms using restriction fragments of chromosomal DNA from bacterial and fungal genomes.

Different strains from the same species may exhibit different patterns and these differences are referred to as restriction fragment length polymorphisms (RFLPs). As cutting the chromosomal DNA with restriction enzymes generates too many fragment bands to be efficiently and accurately compared, several modifications of the conventional RFLP-based method have been developed. Examples of the kind of technologies used are ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Several other fingerprinting methods use PCR to selectively amplify defined subsets of DNA restriction fragments from the entire genome, for example random amplified polymorphic DNA (RAPD) and variable number tandem repeats (VNTR).

*Critical aspects*. All fingerprinting techniques require that the micro-organism is present as a pure culture. Depending on the method, a preliminary enrichment cultivation step may be necessary if a defined quantity or a specific DNA preparation is required for the test, e.g. AFLP and PFGE. The discriminatory power, the reproducibility, the expertise needed and the labour-burden vary among techniques. The major criticism of conventional RFLP analysis is the complexity of the banding patterns. The discriminatory power of ribotyping (based on patterns of rRNA genes) is less than that of PFGE (based on patterns of the whole genomic DNA) or some PCR-based methods, but it has the advantage that it can be a highly automated system. Although PFGE is one of the most highly discriminatory fingerprinting methods, it is time-consuming and technically demanding in the laboratory as it is not automated. It also requires the use of standardised protocols. AFLP has high reproducibility, but requires technical expertise and the interpretation of results may need automated computer analysis. The reproducibility of RAPD may be poor, so it must be performed in a standardised way.

*Potential uses*. Genetic fingerprinting methods are mainly used for strain discrimination (characterisation below species level). They are a powerful tool for investigating and tracing the source and the spread of microbial contamination.

## 3. VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

### 3-1. *INTRODUCTION*

Validation, whilst subject to a variety of context-specific definitions, can be generally defined as a method to establish documented evidence that a process will consistently achieve its intended goal. Therefore, to validate an alternative microbiological method, it is essential to understand and define what the procedure is intended to achieve.
Typically, pharmaceutical microbiological methods use specific characteristics of micro-organisms as indicators or detection principles in order to determine microbiological quality. The information generally sought is presence/absence, number, viability and/or identity of micro-organisms in a given product or environment. Any given method will usually provide an indirect and conditional measure of microbiological quality. For example, the total number and viability of micro-organisms can be indicated by the number of colonies appearing under a certain set of conditions of sample preparation, cultivation and incubation; reproduction in classical microbiology is hence taken as the general indicator for viability. There are other parameters, however, that can be used as a viability measure, such as the level of ATP or the accumulation or metabolism of substrates in living cells. The results from different viability-indicating methods may not always be identical; micro-organisms may not be able to reproduce on a given medium, but may still accumulate and metabolise a substrate. Conversely, micro-organisms may be unable, at a given state of damage, to accumulate a substrate, but may still be able to recover and reproduce.

Similar considerations arise with the multiplicity of methods used for identification of micro-organisms. Therefore, while characterisation of the pattern of metabolic activity is frequently used for species identification, alternative methods also exist. Again, the outcomes obtained may not be fully consistent for the different identification methods, as one answer may be appropriate for the construction of a correct phylogenetic correlation tree, while another may be more useful in the context of pathogenicity or other property of the differentiated micro-organisms.

### 3-2. *VALIDATION PROCESS*

Two levels of validation must be envisaged for the application of alternative microbiological methods, namely primary validation and validation for the intended use. The supplier of the alternative technology typically performs primary validation of a method, whereas validation for the intended use, which is a verification of the suitability or applicability of the method in a given situation, must be seen as the responsibility of the user.

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified.

3-2-1. **Description of the technique**

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the supplier. Through primary validation, the method must be fully detailed with respect to the conditions required for application, the materials and equipment needed and the expected signal. The user shall critically review the available information.

3-2-2. **Risk-benefit analysis**

For validation of specific alternative microbiological methods, it is critical that the purpose of the quality assurance procedure is precisely outlined, as this defines the type and depth of information needed. The information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis.

The risk level in adopting an alternative method varies depending on the technology considered, the methodology it replaces, the nature of the measurements taken (qualitative, quantitative or identification), the particular product or process attribute being evaluated, the location of the measurement in the manufacturing process chain and various other factors.

Risk analysis tools may be utilised in order to determine which alternative method is to be implemented, to assist in the justification of its implementation or to better understand the impact of implementation on production and/or product quality. An alternative method can be justified for use if the information obtained gives a scientifically sound measure of microbiological quality, and if the limitations of the method are not more severe than those of the pharmacopoeial method.

3-2-3. **Primary validation**

The supplier, using a panel of test micro-organisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

- prerequisite treatment of sample or micro-organisms;
- type of response;
- specificity;
- detection limit;
- quantitation limit;
- range;
- linearity;
- accuracy and precision;
- robustness of the method in a model system.

3-2-4. **Validation for the intended use**

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

- user requirement specification (URS);
- design qualification (DQ);
- installation qualification (IQ);
- operational qualification (OQ);
- performance qualification (PQ).

The supplier and user have different tasks to perform with regard to the validation and implementation of an alternative method. These tasks are summarised in Table 5.1.6.-1.

Table 5.1.6.-1 – *Tasks to be undertaken during the validation process*

| Activity | Normally carried out by | |
|---|---|---|
| | Supplier | User |
| Primary validation | + | -(1) |
| URS (instrument, application) | - | + |
| Description of the technique | + | -(2) |
| Risk benefit analysis | -(3) | + |
| Design qualification (DQ) | - | + |
| Installation qualification (IQ) | -(4) | + |
| Operational qualification (OQ) | -(4) | + |
| Performance qualification (PQ): | | |
| - verification of primary validation data given by the supplier; | - | + |
| - verification for the intended use (e.g. sterility testing, TAMC/TYMC, …); | - | + |
| - method suitability test | - | + |

(1) The user performs primary validation if they employ the alternative method for a use other than that defined by the supplier.

(2) The user shall critically review information provided by the supplier.

(3) As part of commercialisation, the supplier may list advantages of the alternative method over pharmacopoeial techniques.

(4) IQ/OQ for complex equipment, IQ/OQ is often outsourced to supplier.

3-2-4-1. *User requirement specification (URS)*

The URS describes the functions that the method must be capable of performing and will form the basis of the method selection process. It is an essential document, as acceptance testing will be based on the requirements detailed therein. It is important to consider data management capabilities at this stage, particularly within a regulatory context. The URS shall at least address the following items:

- application of the instrument:
  - the type of analysis to be performed (e.g. quantitative, semi-quantitative, qualitative or identification).
- detection limit or quantitation limit (sensitivity):
  - the detection limit may be linked to time to detection (TTD);
  - the required level of sensitivity, which will depend on the current specification, the dilution regime and the test sample size for the existing test method under replacement.
- specificity:
  - the ability of the alternative test method to selectively detect the micro-organisms or classes of micro-organisms; this should be based on historical data generated from the pharmacopoeial test method and complemented by information from the supplier of the alternative method;
  - the ability to detect only the required viable micro-organisms;
  - for identification methods, the extent of coverage of the database with respect to the range of micro-organisms of interest.
- number and type of samples:
  - the nature of samples to be tested and the manufacturing output per batch or work-shift.

- time to detection (TTD) or time to result (TTR):
  - the TTD or TTR is an important attribute for alternative microbiological methods; for monitoring purposes, a relatively short TTD (e.g. a few hours) allows corrective actions to be taken at an early stage; for quality control purposes, a short TTD may be less critical.
- data management capabilities:
  - the new instrumentation may need to have laboratory information management system (LIMS) interface capability and external server compatibility, and the data management tools should be defined; evidence of software validation and functional testing reports will be required to support each part of the software and firmware functions.

3-2-4-2. *Design qualification (DQ)*

The DQ provides documented evidence that the design of any associated equipment is suitable for correct performance of the method. Most alternative method systems are based on commercial off-the-shelf equipment. The DQ is most suitably performed, therefore, by the instrument developer/manufacturer. Nevertheless, the user shall verify that the equipment meets the specifications laid down in the URS for the intended application.

3-2-4-3. *Installation qualification (IQ)*

The IQ provides documented evidence that the equipment has been provided and installed in accordance with its specifications.

3-2-4-4. *Operational qualification (OQ)*

The OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures.

3-2-4-5. *Performance qualification (PQ)*

The PQ provides documented evidence that the method, with the equipment installed and operated according to operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method. This is typically done with a panel of micro-organisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing micro-organisms). This assures that the conditions employed by the user laboratory make it possible to satisfy the criteria described by the supplier of the method in the model system used for the primary validation.

*Verification of primary validation data given by the supplier* (see 3-2-3). The method is verified using the panel of test micro-organisms given by the corresponding pharmacopoeial chapter. The alternative method must be applied according to the specified procedure of the supplier, without the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in the model system used by the supplier.

*Verification for the intended use (e.g. sterility testing, total aerobic microbial count (TAMC)/total combined yeasts/moulds count (TYMC), etc).* The following points, where applicable, should be addressed:

- compatibility of the response with the sample preparation that the user normally performs for product testing (method suitability testing);
- limit and range of detection of the method with regard to sample size and sample availability;
- specificity of the response with regard to the influence of the product ingredients;
- linearity of the response with regard to the types of samples to be analysed;
- accuracy and precision of the response with regard to the types of samples to be analysed.

Acceptance criteria for the method will need to be defined as a function of the application and the validation data.

3-3. *TYPES OF MICROBIOLOGICAL TESTS*

Validation of a microbiological method is the process whereby it is experimentally established by the user that the performance characteristics of the method meet the requirements of the intended application. As microbiological tests have 3 basic applications (qualitative, quantitative and identification), 3 separate sets of validation criteria are required. These criteria are described below and summarised in Table 5.1.6.-2.

Table 5.1.6.-2 – *Validation criteria for qualitative, quantitative and identification tests*

| Criterion | Qualitative test | Quantitative test | Identification test |
|---|---|---|---|
| Accuracy | +(1) | + | + |
| Precision | - | + | - |
| Specificity | + | + | + |
| Detection limit | + | -(2) | - |
| Quantitation limit | - | + | - |
| Linearity | - | + | - |
| Range | - | + | - |
| Robustness | + | + | + |
| Suitability testing | + | + | - |
| Equivalence testing | + | + | - |

(1) Performing an accuracy test of the alternate method with respect to the pharmacopoeial method can be used instead of the validation of the limit of detection test.

(2) May be needed in some cases.

3-3-1. **Validation of alternative qualitative tests for the presence or absence of micro-organisms**

3-3-1-1. *Specificity*

The specificity of an alternative qualitative method is its ability to detect only the required micro-organisms, i.e. does not generate false positive results. This can be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For qualitative methods that rely on growth to demonstrate presence or absence of micro-organisms, specificity is adequately addressed by demonstrating the growth promotion properties of the media. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-1-2. *Detection Limit*

The detection limit of an alternative qualitative method is the lowest number of micro-organisms in a sample that can be detected under the stated analytical conditions. A microbiological limit test determines the presence or absence of micro-organisms in a defined quantity of the sample under test. Due to the nature of microbiological tests, the detection limit reflects the number of micro-organisms present in the original sample before any dilution or incubation steps. The detection limit of the alternative method must not be a number greater than that of the pharmacopoeial method.

It is essential that the detection limit is determined using a sufficient number of replicates and a number of independent determinations.

3-3-1-3. *Robustness*

The robustness of an alternative qualitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, any effect on robustness must be evaluated.

Robustness of a qualitative method is judged by its ability to detect the test micro-organisms after deliberate variations to the method parameters.

3-3-1-4. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's detection capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method in routine use will need to be defined as a function of the application and the validation data.

3-3-1-5. *Equivalence testing*

Equivalence testing of 2 qualitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment. The alternative method must enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-2. **Validation of alternative quantitative tests for enumeration of micro-organisms**

3-3-2-1. *Accuracy*

The accuracy of an alternative quantitative method is the closeness of the test results obtained by the alternative method to those obtained by the pharmacopoeial method. Accuracy must be demonstrated across the practical range of the test. It is usually expressed as the percentage recovery of micro-organisms by the alternative method compared to the percentage recovery using the pharmacopoeial method, taking into account statistical analysis.

Accuracy may be shown by preparing and testing a suspension of micro-organisms at the upper end of the test range and serially diluting to the lower end of the test range. For example, if the alternative method is meant to replace the pharmacopoeial plate count method for viable counts, then a reasonable range might be $10^0$-$10^6$ CFU/mL. If instead, it is a replacement for the MPN method, a much narrower range may be used. At least 1 suspension for each test micro-organism dilution must be analysed.

The alternative method should be shown to recover at least as many micro-organisms as the pharmacopoeial method using appropriate statistical analysis.

The protocol used to check the linearity of the method (see 3-3-2-5) may also be used to check the accuracy. The suspensions of micro-organisms prepared for the alternative method are counted at the same time using the pharmacopoeial method.

3-3-2-2. *Precision*

The precision of an alternative quantitative method is the degree of agreement between individual test results when the procedure is applied repeatedly to multiple samplings of homogeneous suspensions of micro-organisms under the prescribed conditions. Precision should be split into repeatability and intermediate precision under normal or routine operating conditions. Repeatability (also referred to as within-run variability) refers to the use of the microbiological method with the same sample (replicate) in the same laboratory over a short period of time with the same analyst and the same equipment. It gives the minimum variability of the method. Intermediate precision (includes run-to-run variability and within-run variability) refers to the use of the microbiological method applied to different sample preparations of the product under test in the same laboratory with different analysts, equipment and/or on different days. It gives the maximum variability of the method. The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). At least 1 suspension in the middle of the test range is analysed. The number of replicates is chosen so that the entire test can be carried out during the same working session, i.e. under the same operating conditions and without any change in the suspension of micro-organisms. For intermediate precision, other working sessions are then carried out under conditions of maximum variability (different reagents, operators and/or days, etc.). The variance in the results observed in each of the working sessions is calculated. If the variances are homogeneous, the variance of the repeatability can be calculated. The inter-group variance of the results is also calculated and the resultant variance of the intermediate precision is given as the sum of the variance of the repeatability and the inter-group variance. The coefficient of variation is then calculated. Alternative methods must demonstrate precision comparable to that of the pharmacopoeial methods.

3-3-2-3. *Specificity*

The specificity of an alternative quantitative method is its ability to quantify only the required micro-organisms, i.e. does not generate false positive results. This may be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-2-4. *Quantitation limit*

The quantitation limit of an alternative quantitative method is the lowest number of CFUs in a sample which can be quantitatively determined with suitable precision and accuracy. It is essential that the quantitation limit is determined from a number of replicates. The results of the linearity and accuracy studies can also be used. In this case, the lowest concentration in the linear range is considered to be the quantitation limit of the method. The quantitation limit of the alternative method must not be greater than that of the pharmacopoeial method.

3-3-2-5. *Linearity*

The linearity of an alternative quantitative method is its ability (within a given range) to produce results that are proportional to the concentration of micro-organisms present in the sample. The linearity must be determined over a reasonable range (e.g. $10^0$-$10^6$ CFU/mL) so as to correspond to the purpose of the alternative method. One approach would be to select different concentrations of each test micro-organism and test several replicates. For each concentration, an appropriate number of replicates is chosen to confirm linearity. The number of replicates is chosen so that the entire test can be carried out during the same working session. After checking the homogeneity of the variances of the results obtained for each concentration, the regression line is calculated. Linearity is demonstrated if the estimated slope is significant and if the test for deviation from linearity is non-significant (see general chapter *5.3*).

3-3-2-6. *Range*

The range of an alternative quantitative method is the interval between the upper and lower levels of micro-organisms as determined from the related studies of precision, accuracy and linearity using the specified method; it is dependent on the intended application.

3-3-2-7. *Robustness*

The robustness of an alternative quantitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness must be evaluated. Robustness of an alternative quantitative method is judged by its ability to accurately enumerate the test micro-organisms after deliberate variations to the method parameters.

3-3-2-8. *Suitability testing*

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's enumeration capacity or microbial recovery. Specific points to be addressed are:

- the ability of the test to detect micro-organisms in the presence of the sample matrix;
- verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method are defined as a function of the application and of the validation data.

3-3-2-9. *Equivalence testing*

Equivalence testing of 2 quantitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment.

If the result of the alternative method can be expressed as the number of CFUs per weight or per volume, statistical analysis of the results shall demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

If the result of the alternative method cannot be expressed as the number of CFUs, equivalence testing is performed using suitable parameters, followed by statistical analysis to demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-3. **Validation of alternative identification tests**

There is a large body of evidence that different methods vary considerably in their ability to identify micro-organisms. It must be accepted that a method of identification needs to be internally consistent, but may differ from others in its identification of micro-organisms.

3-3-3-1. *Accuracy*

The accuracy of an alternative identification method is its ability to identify the desired micro-organism to the required taxonomic level. It must be demonstrated using well-characterised reference micro-organisms, e.g. type strains. Accuracy of the identification method is usually expressed as the number of correct identifications divided by the total number of identifications.

3-3-3-2. *Specificity*

The specificity of an alternative identification method is its ability to discriminate micro-organisms actually present from interfering factors that cause false identification results. Such factors include chemical substances and mixtures of micro-organisms, which cause the test to identify micro-organisms not actually present in the sample material (e.g. the presence of mixtures of DNA material from 2 micro-organisms in a sequencing test leading to the false identification of a third micro-organism).

3-3-3-3. *Robustness*

The robustness of an alternative identification method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness have to be evaluated. Robustness of an identification method is judged by its ability to correctly identify the test micro-organisms after deliberate variations to the method parameters.

**01/2008:50107**

# 5.1.7. VIRAL SAFETY

This chapter provides general requirements concerning the viral safety of medicinal products whose manufacture has involved the use of materials of human or animal origin. Since viral safety is a complex issue, it is important that a risk assessment is carried out. Requirements to be applied to a specific medicinal product are decided by the competent authority.

Where the risk of viral contamination exists, complementary measures are used as appropriate to assure the viral safety of medicinal products, based on:

- selection of source materials and testing for viral contaminants;
- testing the capacity of the production process to remove and/or inactivate viruses;
- testing for viral contamination at appropriate stages of production.

Where appropriate, one or more validated procedures for removal or inactivation of viruses are applied.

Further detailed recommendations on viral safety, including validation studies, are provided, in particular, by the *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95)* of the Committee for Proprietary Medicinal Products, and the *ICH guideline Q5A: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* (including any subsequent revisions of these documents).

Requirements concerning immunological products for veterinary use are dealt with in the monographs *Vaccines for veterinary use (0062)* and *Immunosera for veterinary use (0030)* and related general chapters.

**Risk assessment**

A risk assessment with respect to viral safety is carried out where materials of human or animal origin are used as ingredients of medicinal products or in the manufacture of active substances, excipients or medicinal products.

The principle of the risk assessment is to consider various factors that may influence the potential level of infectious particles in the medicinal product and factors related to the use of the medicinal product that determine or influence the viral risk to the recipients.

The risk assessment takes into consideration relevant factors, for example:

- the species of origin;
- the organ, tissue, fluid of origin;

- the potential contaminants in view of the origin of the raw material and the history of the donor(s), preferably including epidemiological data;
- the potential contaminants from the manufacturing process (for example, from risk materials used during manufacture);
- the infectivity and pathogenicity of the potential contaminants for the intended recipients of the medicinal product, taking account of the route of administration of the medicinal product;
- the amount of material used to produce a dose of medicinal product;
- controls carried out on the donor(s), on the raw material, during production and on the final product;
- the manufacturing process of the product and its capacity to remove and/or inactivate viruses.

The risk assessment can be based mainly on the manufacturing conditions if these include rigorous inactivation steps (for example, for gelatin etc., and products terminally sterilised by steam or dry heat as described in the general texts on sterility (*5.1*)).



04/2019:50108

# 5.1.8. MICROBIOLOGICAL QUALITY OF HERBAL MEDICINAL PRODUCTS FOR ORAL USE AND EXTRACTS USED IN THEIR PREPARATION

*This general chapter presents recommended acceptance criteria for the microbiological quality of both herbal medicinal products for oral use and the extracts that are used in their preparation.*

Microbial examination of non-sterile products is performed according to the methods given in general chapters *2.6.12*, *2.6.13* and *2.6.31*. Acceptance criteria based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC) are given below.

Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

A list of specified micro-organisms for which acceptance criteria are set can be found below. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials, the manufacturing process and the intended use.

Medicinal products containing live yeasts (live biotherapeutic products) are not within the scope of this general chapter.

## HERBAL MEDICINAL PRODUCTS

**A. Herbal medicinal products containing herbal drugs, with or without excipients, intended for the preparation of infusions and decoctions using boiling water (for example herbal teas, with or without added flavourings)**

| | |
|---|---|
| TAMC (*2.6.12*) | Acceptance criterion: $10^7$ CFU/g<br>Maximum acceptable count: 50 000 000 CFU/g |
| TYMC (*2.6.12*) | Acceptance criterion: $10^5$ CFU/g<br>Maximum acceptable count: 500 000 CFU/g |
| *Escherichia coli* (*2.6.31*) | Acceptance criterion: $10^3$ CFU/g |
| *Salmonella* (*2.6.31*) | Absence (25 g) |

**B. Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where the method of processing (for example, extraction) or, where appropriate, in the case of herbal drugs, of pre-treatment reduces the levels of organisms to below those stated for this category**

| | |
|---|---|
| TAMC (*2.6.12*) | Acceptance criterion: $10^4$ CFU/g or CFU/mL<br>Maximum acceptable count: 50 000 CFU/g or CFU/mL |
| TYMC (*2.6.12*) | Acceptance criterion: $10^2$ CFU/g or CFU/mL<br>Maximum acceptable count: 500 CFU/g or CFU/mL |
| Bile-tolerant gram-negative bacteria (*2.6.31*) | Acceptance criterion: $10^2$ CFU/g or CFU/mL |
| *Escherichia coli* (*2.6.31*) | Absence (1 g or 1 mL) |
| *Salmonella* (*2.6.31*) | Absence (25 g or 25 mL) |

**C. Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where it can be demonstrated that the method of processing (for example, extraction with low-strength ethanol or water that is not boiling, or low-temperature concentration) or, in the case of herbal drugs, of pre-treatment, would not reduce the level of organisms sufficiently to reach the criteria required under B**

| | |
|---|---|
| TAMC (*2.6.12*) | Acceptance criterion: $10^5$ CFU/g or CFU/mL<br>Maximum acceptable count: 500 000 CFU/g or CFU/mL |
| TYMC (*2.6.12*) | Acceptance criterion: $10^4$ CFU/g or CFU/mL<br>Maximum acceptable count: 50 000 CFU/g or CFU/mL |
| Bile-tolerant gram-negative bacteria (*2.6.31*) | Acceptance criterion: $10^4$ CFU/g or CFU/mL |
| *Escherichia coli* (*2.6.31*) | Absence (1 g or 1 mL) |
| *Salmonella* (*2.6.31*) | Absence (25 g or 25 mL) |

## EXTRACTS

Extracts should fulfill the acceptance criteria for category B herbal medicinal products. However, where it can be demonstrated that the method of processing would not reduce the level of micro-organisms sufficiently to reach the category B criteria, the extracts shall meet the requirements for category C herbal medicinal products.

The recommended acceptance criteria apply to extracts that are to be incorporated into herbal medicinal products for oral use. More-stringent acceptance criteria may be required for extracts that are to be incorporated into pharmaceutical preparations to be administered by other routes in order to satisfy the acceptance criteria for the intended route of administration (*5.1.4*).

*It is recognised that for some herbal medicinal products and extracts used in their preparation the criteria given above for TAMC, TYMC and bile-tolerant gram-negative bacteria cannot be met because of the typical level of microbial contamination. Less-stringent acceptance criteria may be applied on the basis of a risk assessment that takes account of qualitative and quantitative characterisation of the microbial contamination and the intended use of the herbal medicinal product or extract.*

*If it has been shown that none of the prescribed tests for a herbal medicinal product or extract will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.*

01/2009:50109

## 5.1.9. GUIDELINES FOR USING THE TEST FOR STERILITY

The purpose of the test for sterility (*2.6.1*), as that of all pharmacopoeial tests, is to provide an independent control analyst with the means of verifying that a particular material meets the requirements of the European Pharmacopoeia. A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or alternatives to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

Aseptic conditions for performance of the test can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean room, or an isolator

GUIDANCE TO MANUFACTURERS

The level of assurance provided by a satisfactory result of a test for sterility (the absence of contaminated units in the sample) as applied to the quality of the batch is a function of the homogeneity of the batch, the conditions of manufacture and the efficiency of the adopted sampling plan. Hence for the purpose of this text a batch is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units contained therein.

In the case of terminally sterilised products, physical proofs, biologically based and automatically documented, showing correct treatment throughout the batch during sterilisation are of greater assurance than the sterility test. The circumstances in which parametric release may be considered appropriate are described under *5.1.1. Methods of preparation of sterile products*. The method of media-fill runs may be used to evaluate the process of aseptic production. Apart from that, the sterility test is the only analytical method available for products prepared under aseptic conditions and furthermore it is, in all cases, the only analytical method available to the authorities who have to examine a specimen of a product for sterility.

The probability of detecting micro-organisms by the test for sterility increases with their number present in the sample tested and varies according to the readiness of growth of micro-organism present. The probability of detecting very low levels of contamination even when it is homogenous throughout the batch is very low. The interpretation of the results of the test for sterility rests on the assumption that the contents of every container in the batch, had they been tested, would have given the same result. Since it is manifest that every container cannot be tested, an appropriate sampling plan should be adopted. In the case of aseptic production, it is recommended to include samples filled at the beginning and at the end of the batch and after significant intervention.

OBSERVATION AND INTERPRETATION OF RESULTS

Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that 2 isolates are from the same source. More sensitive tests, for example molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

07/2016:50110

## 5.1.10. GUIDELINES FOR USING THE TEST FOR BACTERIAL ENDOTOXINS

1. INTRODUCTION

Endotoxins from gram-negative bacteria are the most common cause of toxic reactions resulting from contamination of pharmaceutical products with pyrogens; their common pyrogenic activity is much higher than that of other known pyrogenic substances. These endotoxins are lipopolysaccharides. Although there are a small number of pyrogens that possess a different structure, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. The monocyte-activation test (*2.6.30*) is a suitable method to use to rule out the presence of non-endotoxin pyrogens in substances or products.

The presence of endotoxins in a substance or product may be masked by factors interfering with the reaction between the endotoxins, the test reagents and the amoebocyte lysate. Also, the ability to detect endotoxins may be affected by storage conditions or storage time. Hence, the analyst who wishes to implement a test for bacterial endotoxins or to replace the pyrogen test by a test for bacterial endotoxins has to demonstrate that a valid test can be carried out on the substance or product concerned; this may entail a procedure for removing interference.

As indicated in general chapter *2.6.14. Bacterial endotoxins*, information must be available on the following 2 aspects before a test on a sample can be regarded as valid.

- The suitability of the material to be used for the test has to be established. The absence of endotoxins in the water for BET (water for bacterial endotoxins test) and in the other reagents and consumables must be assured and the sensitivity of the amoebocyte lysate must be checked to confirm the sensitivity declared by the manufacturer.
- As the substance or product to be examined may interfere with the test, the sensitivity of the amoebocyte lysate is determined in the presence and in the absence of the substance or product to be examined. There must be no difference between the 2 sensitivity values.

General chapter *2.6.14. Bacterial endotoxins* indicates methods for removing interfering factors; in the case of interference, another test must be carried out after such a method has been applied to check whether the interference has indeed been neutralised or removed.

This general chapter explains the reasons for the requirements in the test for bacterial endotoxins, then deals with reading and interpretation of the results.

Replacement of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test, or by other methods such as the monocyte-activation test or a test using recombinant factor C reagent as a replacement for the amoebocyte lysate, constitutes the use of an alternative method of analysis and hence requires demonstration that the method is appropriate for the given substance or product

and gives a result consistent with that obtained with the prescribed method as described in the General Notices (see also section 12).

The prescribed method for bacterial endotoxins may be stated in the monograph on a given substance or product. The use of a method other than the method prescribed in the monograph is considered as the use of an alternative method. Where no method is stated, any of methods A to F of general chapter *2.6.14. Bacterial endotoxins* can be used.

## 2. METHOD AND ACCEPTANCE CRITERIA

### 2-1. *METHODS AND PRECAUTIONS TO BE TAKEN*

The addition of endotoxins to amoebocyte lysate may result in turbidity, precipitation or gelation (gel-clot); initially only the gel-clot method was used in the Pharmacopoeia as an evaluation criterion in the test for bacterial endotoxins. The advantage was the simplicity of basing the decision to pass or fail the substance or product to be examined on the absence or presence of a gel-clot, visible with the naked eye. The quantitative methods C, D, E and F were developed later: they require more instrumentation, but they are easier to automate for the regular testing of large numbers of samples of the same substance or product.

Endotoxins may be adsorbed onto the surface of tubes or pipettes made from certain plastics or types of glass. Interference may appear due to the release of substances from plastic materials. Hence, the materials used must be checked.

### 2-2. *ENDOTOXIN LIMIT CONCENTRATION*

The decision to use the test for bacterial endotoxins as a limit test implies firstly that an endotoxin limit concentration must be defined for the substance or product to be examined, and secondly that the objective of the test is to know whether the endotoxin concentration in the sample to be examined is below or above this limit. The quantitative methods C, D, E and F make it possible to determine the endotoxin concentration in the sample to be examined, but for compliance with the Pharmacopoeia and in routine quality control the final question is whether or not this concentration exceeds a defined limit.

The dose of the substance or product to be examined must be taken into account in setting the endotoxin limit concentration: the limit is set so as to ensure that, as long as the endotoxin concentration in the substance or product remains below this limit, even the maximal dose administered by the intended route per hour does not contain sufficient endotoxin to cause a toxic reaction.

When the endotoxin concentration in the substance or product exactly equals the endotoxin limit concentration, gelation will occur, as is the case when the endotoxin concentration is much higher, and the substance or product will fail the test, because the all-or-none character of the test makes it impossible to differentiate between a concentration exactly equal to the endotoxin limit concentration and one that is higher. It is only when no gelation occurs that the analyst may conclude that the endotoxin concentration is below the endotoxin limit.

For substances or products in the solid state, this endotoxin limit concentration per mass unit or per International Unit (IU) of substance or product has to be converted into a concentration of endotoxin per millilitre of solution to be examined, as the test can only be carried out on a solution. The case of substances or products that already exist in the liquid state (such as infusion fluids) is discussed below.

### 2-3. *CALCULATION OF THE ENDOTOXIN LIMIT*

The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

$K$ = threshold pyrogenic dose of endotoxin per kilogram of body mass;

$M$ = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, $M$ is the maximum total dose administered per hour.

The endotoxin limit depends on the product and its route of administration and may be stated in the monograph. Values for $K$ are suggested in Table 5.1.10.-1.

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

Table 5.1.10.-1

| Route of administration | *K* |
|---|---|
| Intravenous | 5.0 IU of endotoxin per kilogram of body mass |
| Intravenous for radiopharmaceuticals | 2.5 IU of endotoxin per kilogram of body mass |
| Intrathecal | 0.2 IU of endotoxin per kilogram of body mass |
| Parenteral formulations administered per square metre of body surface | 100 IU/m² |

### 2-4. *CONSIDERATIONS WHEN ESTABLISHING AN ENDOTOXIN LIMIT FOR A SPECIFIC SUBSTANCE OR PRODUCT*

The endotoxin limit for a substance or product is established with consideration of the following aspects.

**Calculated endotoxin limit**. The endotoxin limit is calculated as described in section 2-3. This represents a safety limit not to be exceeded if the product is to be administered to humans.

**Limit prescribed in an individual substance monograph**. The limit stated in an individual substance monograph frequently reflects what is achievable in a controlled production environment. The limit prescribed in a monograph can therefore be lower than the calculated endotoxin limit. However a manufacturer may specify a limit that is more stringent than that stated in the monograph.

**Process capability**. The capability of the process to reduce or remove bacterial endotoxins during manufacture might result in lower endotoxin limits for specific processes.

**Additional safety requirements**. Precautions are taken in consideration of patient population (such as paediatric use, malnourished or cachectic patients, etc.), specific local requirements (e.g. countries might wish to operate with a lower average body weight of 60 kg instead of 70 kg frequently employed in Europe) or any additional safety margins requested by the competent authority.

**Formulation of the product**. The limit must take into consideration any theoretical bacterial endotoxin load introduced by any other components used for reconstitution and/or dilution of the product (e.g. water for injections) or introduced by starting materials and/or raw materials.

### 2-5. *MAXIMUM VALID DILUTION*

Which dilution of the substance or product is to be used in the test to obtain maximal assurance that a negative result means that the endotoxin concentration of the substance or product is less than the endotoxin limit and that a positive result means that the lysate detected an endotoxin concentration equal to or greater than the endotoxin limit? This dilution depends on the endotoxin limit and on the sensitivity of the lysate; it is called the maximum valid dilution (MVD) and its value may be calculated using the following expression:

$$\frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

*Concentration of test solution*:

- in mg/mL if the endotoxin limit is specified by mass (IU/mg);
- in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);
- in mL/mL if the endotoxin limit is specified by volume (IU/mL).

$\lambda$ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

When the value of the MVD is not a whole number, a convenient whole number smaller than the MVD may be used for routine purposes (which means preparing a solution of the substance or product that is less diluted than the MVD indicates). In this case, a negative result indicates that the endotoxin concentration of the substance or product lies below the limit value. However, when the endotoxin concentration of the substance or product in such a test is less than the endotoxin limit but high enough to make the reaction with the lysate result in a clot, the test may be positive under these conditions. Hence, when a test with this 'convenient' dilution factor is positive, the substance or product is diluted to the MVD and the test is repeated. In any case of doubt or dispute, the MVD must be used.

This stresses the importance of the confirmation of the sensitivity of the lysate.

*Example*

A 50 mg/mL solution of phenytoin sodium (intended for intravenous injection) has to be tested. Determine the MVD, given the following variables:

$M$ = maximum human dose = 15 mg per kilogram of body mass;

$c$ = 50 mg/mL;

$K$ = 5 IU of endotoxin per kilogram of body mass;

$\lambda$ = 0.4 IU of endotoxin per millilitre.

$$\text{MVD} = \frac{5 \times 50}{15} \times \frac{1}{0.4} = 41.67$$

For routine tests on this product, it may be expedient to dilute 1 mL of the solution to be examined to 20 mL (MVD/2 rounded to the next lower whole number). However, if this test result is positive the analyst will have to dilute 1 mL to 41.67 mL and repeat the test. A dilution to 41.67 mL is also necessary when the test is performed to settle a dispute.

## 3. RISK ASSESSMENT

As stated in section 1 of this general chapter, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. To rule out the presence of non-endotoxin pyrogens in substances or products, the use of the monocyte-activation test (*2.6.30*) is recommended at release or during development of the production process; if any changes are made to the production process that could influence the quality of the product regarding pyrogenicity, the monocyte-activation test is repeated. Examples of such changes include the use of different raw materials, a different production site and different process parameters.

The decision to use the test for bacterial endotoxins as the sole pyrogenicity test is to be made after careful evaluation of the risk of the substance or product containing non-endotoxin pyrogens. The risk assessment is made with consideration given to any factor that could result in the inclusion of pyrogens not detected by the test for bacterial endotoxins. The items below constitute a non-exhaustive list of factors to be considered in the risk assessment.

**Production process** (chemical synthesis, fermentation, biotechnological method). For products of fermentation, the expression system is to be considered (prokaryotic, eukaryotic) and, for a prokaryotic expression system, whether gram-positive or gram-negative bacteria are used. Also, the culture media components are examined with consideration given to their origin (synthetic, animal, plant).

**Bioburden**. The potential presence of gram-positive bacteria and fungi as contaminants of the active substance, excipients or starting materials and raw materials used in the production of the medicinal product, and the origin of the raw materials (synthetic, animal, plant) have to be taken into consideration. The quality of the water plays an important role on the overall evaluation.

**Capability of the downstream process**. It must be verified whether bacterial endotoxin removal steps are part of the downstream process.

**Safety**. The target population and the route of administration (e.g. intravenous, intrathecal) have to be taken into account in the risk assessment.

**Stability of the detectable endotoxins**. It has to be considered that the ability to detect endotoxins can be affected by interaction with certain components, storage conditions or storage time, temperature and handling of the test sample. Procedures that demonstrate stability of the detectable endotoxin content have to be established for storing, handling and mixing of samples.

## 4. REFERENCE MATERIAL

*Endotoxin standard BRP* is intended for use as the reference preparation. It has been assayed against the WHO International Standard for Endotoxin and its potency is expressed in International Units of endotoxin per vial. The International Unit of endotoxin is defined as the specific activity of a defined mass of the International Standard.

For routine purposes, another preparation of endotoxin may be used, provided it has been assayed against the International Standard for Endotoxin or the BRP and its potency is expressed in International Units of endotoxin.

*NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (E.U.).*

## 5. WATER FOR BET

Water for BET is sterile water that is free of detectable levels of endotoxin. Usually it is commercially available and certified.

General chapter *2.6.14. Bacterial endotoxins* indicates that methods other than triple distillation may be used to prepare water for BET. Reverse osmosis has been used with good results; some analysts may prefer to distil the water more than 3 times. Whatever method is used, the resultant product must be free of detectable bacterial endotoxins.

## 6. pH OF THE MIXTURE

In the test for bacterial endotoxins, optimum gel-clot occurs for a mixture at pH 6.0-8.0. However, the addition of the lysate to the sample may result in a lowering of the pH.

## 7. VALIDATION OF THE LYSATE

It is important to follow the manufacturer's instructions for the preparation of the solutions of the lysate.

The positive end-point dilution factors in gel-clot methods A and B are converted to logarithms. The reason is that if the frequency distribution of these logarithmic values is plotted, it usually approaches a normal distribution curve much more closely than the frequency distribution of the dilution factors themselves; in fact it is so similar that it is acceptable to use the normal frequency distribution as a mathematical model and to calculate confidence limits with Student's *t*-test.

## 8. PRELIMINARY TEST FOR INTERFERING FACTORS

Some substances or products cannot be tested directly for the presence of bacterial endotoxins because they are not miscible with the reagents, they cannot be adjusted to pH 6.0-8.0 or they inhibit or activate enzymatic reaction (such as β-D-glucans).

Therefore a preliminary test is required to check for the presence of interfering factors; when these are found the analyst must demonstrate that the procedure to remove them has been effective and that by applying this procedure, any bacterial endotoxins present have not been removed.

The object of the preliminary test is to test the null hypothesis that the sensitivity of the lysate in the presence of the substance or product to be examined does not differ significantly from the sensitivity of the lysate in the absence of the product. A simple criterion is used in methods A and B: the null hypothesis is accepted when the sensitivity of the lysate in the presence of the product is at least 0.5 times and not more than twice the sensitivity of the lysate by itself.

The test for interfering factors in gel-clot methods A and B requires the use of a sample of the substance or product in which no endotoxins are detectable. This presents a theoretical problem when an entirely new product has to be tested. Hence, a different approach was designed for quantitative methods C, D, E and F.

Note that methods D and E, which use a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

## 9. REMOVAL OF INTERFERING FACTORS

The procedures to remove interfering factors must not increase or decrease (for example, by adsorption) the amount of endotoxin in the substance or product to be examined. The correct way of checking this is to apply the procedures to a spiked sample of the substance or product to be examined, that is, a sample to which a known amount of endotoxin has been added, and then to measure the recovery of the endotoxin after the removal process has been conducted.

**Methods C and D**. If the nature of the product to be examined results in an interference that cannot be removed by classical methods (e.g. dilution or centrifugation), it may be possible to determine the standard curve in the same type of substance or product freed from endotoxins by appropriate treatment or by dilution of the substance or product. The endotoxins test is then carried out by comparison with this standard curve.

Ultrafiltration with cellulose triacetate asymmetric membrane filters has been found to be suitable in most cases. The filters must be properly validated, because under some circumstances cellulose derivatives (β-D-glucans) can cause false positive results.

Another option to remove interfering factors is a 2-step procedure in which 1) endotoxin within the interfering sample is fixed on a solid phase, and 2) after removal of the interfering substance (e.g. by washing) the endotoxin is detected unimpaired under suitable testing conditions.

## 10. THE PURPOSE OF THE CONTROLS

The purpose of the control made up with water for BET and the reference preparation of endotoxin at twice the concentration of the labelled lysate sensitivity is to verify the activity of the lysate at the time and under the conditions of the test (for method A and B). The purpose of the negative control is to verify the absence of a detectable concentration of endotoxin in the water for BET.

The positive control, which contains the product to be examined at the concentration used in the test, is intended to show the absence of inhibiting factors at the time and under the conditions of the test.

## 11. READING AND INTERPRETATION OF RESULTS

Minute amounts of bacterial endotoxin in the water for BET, or in any other reagent or material to which the lysate is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lysate. However, they may raise the amount of bacterial endotoxin in the solution containing the substance or product to be examined to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lysate available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a 'false positive result' cannot be ruled out completely.

## 12. REPLACEMENT OF METHODS PRESCRIBED IN MONOGRAPHS

### 12-1. *REPLACEMENT BY ANOTHER PH. EUR. METHOD*

As stated in the General Notices, the test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. The methods described in general chapters *2.6.14. Bacterial endotoxins* and *2.6.30. Monocyte-activation test* therefore do not have to be re-validated *per se*, other than in consideration of their use for a specific substance or product in a specific analytical environment.

The procedure and the materials and reagents used in the method must be validated as described for the test concerned.

The absence of interfering factors (and, if necessary, the procedure for removing them) is verified on samples of at least 3 production batches.

The necessary information is sought from manufacturers; companies are invited to provide any validation data that they have concerning the applicability of the replacement test to the substances and products of interest; such data includes details of sample preparation and of any procedures necessary to eliminate interfering factors.

As stated in general chapter *2.6.30. Monocyte-activation test*, the monocyte-activation test is primarily intended as a replacement of the rabbit pyrogen test. Guidelines on which methods to use (A, B or C) and on how to validate the monocyte-activation test are described in general chapter *2.6.30. Monocyte-activation test*.

### 12-2. *REPLACEMENT BY AN ALTERNATIVE METHOD NOT DESCRIBED IN THE PH. EUR.*

The use of alternative reagents such as recombinant factor C as a replacement to the amoebocyte lysate eliminates the use of a reagent extracted from live animals.

Replacement of a rabbit pyrogen test or a bacterial endotoxin test prescribed in a monograph by a test using recombinant factor C reagent or any other reagent as a replacement of the amoebocyte lysate is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

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# 5.1.11. DETERMINATION OF BACTERICIDAL, FUNGICIDAL OR YEASTICIDAL ACTIVITY OF ANTISEPTIC MEDICINAL PRODUCTS

*This general chapter describes a test that can be used for the determination of antimicrobial activity in antiseptic medicinal products that are miscible with water and intended for administration by direct contact with the skin or mucous membranes. The extent of testing is dependent on the declared antimicrobial activity of the product.*

*The test determines whether a product exhibits bactericidal, fungicidal or yeasticidal activity and complies with an established specification for such activity.*

*This test cannot replace or confirm the assessment of the clinical efficacy of such preparations.*

## 1. PRINCIPLE

Antimicrobial activity is determined by adding test suspensions of micro-organisms (bacteria, fungi or yeasts, separately) to the sample antiseptic product. The mixture is maintained at 33 ± 1 °C for contact times of 5 min for bactericidal activity and 15 min for fungicidal or yeasticidal activity. Additional contact times may be chosen, according to the stated use of the antiseptic medicinal product. At the end of the contact time, an aliquot is taken and the antimicrobial activity in this aliquot is immediately stopped by a validated method. 2 methods are available: dilution-neutralisation and membrane filtration.

The procedure is validated to verify its ability to demonstrate the required reduction in the count of viable micro-organisms by the use of appropriate controls.

## 2. TEST MICRO-ORGANISMS AND GROWTH CONDITIONS

Prepare standardised stable suspensions of test strains as stated in section 2-1. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the microbial test strains separately as described in Table 5.1.11.-1.

Table 5.1.11.-1. – *Test micro-organisms and growth conditions*

| | |
|---|---|
| **Strains for bactericidal activity testing** | |
| *Staphylococcus aureus* | such as ATCC 6538, NCIMB 9518, CIP 4.83, NBRC 13276 |
| *Enterococcus hirae* | such as ATCC 10541, NCIMB 8192, CIP 58.55, DSM 3320 |
| *Escherichia coli* | such as NCIMB 10083, CIP 54.117, NCTC 10538, DSM 11250 |
| *Pseudomonas aeruginosa* | such as ATCC 15442, NCIMB 8626, CIP 103467, NBRC 13275 |
| **Bacterial growth conditions** | |
| Casein soya bean digest agar or casein soya bean digest broth<br>- for preparation of test strains: 30-35 °C, 18-24 h and subculture at least twice<br>- for testing of the product and validation of the test: 30-35 °C, ≤ 3 days | number of CFU in test suspension: 1-5 × $10^8$ CFU/mL |
| **Strain for yeasticidal activity testing** | |
| *Staphylococcus aureus* | such as ATCC 6538, NCIMB 9518, CIP 4.83, NBRC 13276 |
| *Candida albicans* | such as ATCC 10231, NCPF 3179, CIP 48.72, NBRC 1594 |
| **Yeast growth conditions** | |
| Sabouraud-dextrose agar or Sabouraud-dextrose broth<br>- for preparation of test strains: 20-25 °C, 2-3 days and subculture at least twice<br>- for testing of the product and validation of the test: 20-25 °C, ≤ 5 days | number of CFU in test suspension: 1-5 × $10^7$ CFU/mL |
| **Strains for fungicidal activity testing** | |
| *Candida albicans* | such as ATCC 10231, NCPF 3179, CIP 48.72, NBRC 1594 |
| *Aspergillus brasiliensis* | such as ATCC 16404, IMI 149007, CIP 1431.83, NBRC 9455 |
| **Fungal growth conditions** | |
| Sabouraud-dextrose agar or Sabouraud-dextrose broth<br>- for preparation of test suspension of *C. albicans*: 20-25 °C, 2-3 days<br>- for preparation of test suspension of *A. brasiliensis* spores: 20-25 °C, at least 5 days until good sporulation<br>- for testing of the product and validation of the test with *C. albicans* and *A. brasiliensis*: 20-25 °C, ≤ 5 days | number of CFU in test suspension: 1-5 × $10^7$ CFU/mL |

The recommended solutions and media are described in general chapter *2.6.13*. Purified water is used. All reagents are sterile prior to use.

The test for bactericidal, fungicidal or yeasticidal activity is performed with the designated strains as described in Table 5.1.11.-1. In addition to these micro-organisms, it may be necessary to add other bacterial or fungal strains that represent the indications of the antiseptic medicinal product tested.

Single-strain challenges are used. The counts are performed in duplicate and the arithmetic mean of the results is calculated and expressed in CFU/mL.

### 2-1. *PREPARATION OF TEST SUSPENSION*

For harvesting the micro-organisms use a sufficient volume of a 9 g/L solution of *sodium chloride R* (for bacteria and *C. albicans*) or a solution containing 9 g/L of *sodium chloride R* and 0.5 g/L of *polysorbate 80 R* (for *A. brasiliensis*), to obtain a test suspension with the number of CFU described in Table 5.1.11.-1. Use the suspension within 2 h or within 24 h if stored at 2-8 °C.

### 2-2. *PREPARATION OF ANTISEPTIC PRODUCT TEST SOLUTION*

The concentration of the antiseptic product test solution shall be, if possible, 1.25 times the in-use test concentration because it is diluted to 80 per cent during the test and the method validation.

### 2-3. *NEUTRALISING AGENTS*

Neutralising agents are used to neutralise the antimicrobial activity of the antiseptic product. The common neutralising agents are listed in Table 2.6.12.-2 of general chapter *2.6.12. Microbiological examination of non-sterile products: microbial enumeration tests*. The neutralisation time is not less than 10 s and not more than 60 s.

## 3. METHODS

Prior to testing, equilibrate the temperature of all reagents to 33 ± 1 °C.

3-1. *DILUTION-NEUTRALISATION METHOD*

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the test suspension and maintain at 33 ± 1 °C for 2 min. Add 8.0 mL of the antiseptic product test solution and maintain at 33 ± 1 °C for the chosen contact time. Then, take a 1.0 mL sample of the test mixture and transfer into a tube containing 1.0 mL of *water R* and 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL of the neutralised test mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-1-1. **Suitability of the test/controls**

For all methods, prepare a validation suspension containing 100-1000 CFU of the test micro-organisms per millilitre.

3-1-1-1. *Experimental conditions control*

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the validation suspension and maintain at 33 ± 1 °C for 2 min. Add 8.0 mL of *water R* and maintain at 33 ± 1 °C for the chosen contact time. Take 1.0 mL of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-2. *Neutralising agent control*

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the validation suspension and 8.0 mL of the neutralising agent used in the test and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-3. *Dilution-neutralisation method control*

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of a 9 g/L solution of *sodium chloride R* and 8.0 mL of the product test solution and maintain at 33 ± 1 °C for the chosen contact time. Transfer 1.0 mL of this mixture into a tube containing 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Then add 1.0 mL of the validation suspension and mix. After 30 min, take a sample of 1.0 mL of the mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2. *MEMBRANE FILTRATION METHOD*

Proceed as described in section 3-1, carrying out immediately the filtration step in place of the neutralisation step. Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen such that the microbe-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, a single membrane filter is used. Appropriately dilute 0.1 mL of the test solution and immediately filter the total volume, then rinse the membrane filter with an appropriate volume of the diluent. Perform the test in duplicate. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-2-1. **Verification of the selected experimental conditions and of the membrane filtration method**

3-2-1-1. *Experimental conditions control*

Proceed as described in section 3-1-1-1, except at the end of the contact time, take the sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter immediately and then transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2-1-2. *Membrane filtration method control*

Proceed as described in section 3-1-1-3, except at the end of the chosen contact time, take that sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter and rinse as described in section 3-2, then cover the membranes with rinsing liquid and add a sample of the validation suspension. Filter again and transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

4. ACCEPTANCE CRITERIA

Unless otherwise justified and authorised, the preparation has a:

- bactericidal activity if the defined number of CFU is reduced by at least 5 $\log_{10}$;
- fungicidal activity if the defined number of CFU is reduced by at least 4 $\log_{10}$;
- yeasticidal activity if the defined number of CFU is reduced by at least 4 $\log_{10}$.

# 5.2. General texts on biological products

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# 5.2. GENERAL TEXTS ON BIOLOGICAL PRODUCTS

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corrected 6.0

## 5.2.1. TERMINOLOGY USED IN MONOGRAPHS ON BIOLOGICAL PRODUCTS

For some items, alternative terms commonly used in connection with veterinary vaccines are shown in parenthesis.

**Seed-lot system**. A seed-lot system is a system according to which successive batches of a product are derived from the same master seed lot. For routine production, a working seed lot may be prepared from the master seed lot. The origin and the passage history of the master seed lot and the working seed lot are recorded.

**Master seed lot**. A culture of a micro-organism distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability and to prevent contamination. A master seed lot in liquid form is usually stored at or below − 70 °C. A freeze-dried master seed lot is stored at a temperature known to ensure stability.

**Working seed lot**. A culture of a micro-organism derived from the master seed lot and intended for use in production. Working seed lots are distributed into containers and stored as described above for master seed lots.

**Cell-bank system (Cell-seed system)**. A system whereby successive final lots (batches) of a product are manufactured by culture in cells derived from the same master cell bank (master cell seed). A number of containers from the master cell bank (master cell seed) are used to prepare a working cell bank (working cell seed). The cell-bank system (cell-seed system) is validated for the highest passage level achieved during routine production.

**Master cell bank (Master cell seed)**. A culture of cells distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination. A master cell bank (master cell seed) is usually stored at − 70 °C or lower.

**Working cell bank (Working cell seed)**. A culture of cells derived from the master cell bank (master cell seed) and intended for use in the preparation of production cell cultures. The working cell bank (working cell seed) is distributed into containers, processed and stored as described for the master cell bank (master cell seed).

**Primary cell cultures**. Cultures of cells obtained by trypsination of a suitable tissue or organ. The cells are essentially identical to those of the tissue of origin and are no more than 5 *in vitro* passages from the initial preparation from the animal tissue.

**Cell lines**. Cultures of cells that have a high capacity for multiplication *in vitro*. In diploid cell lines, the cells have essentially the same characteristics as those of the tissue of origin. In continuous cell lines, the cells are able to multiply indefinitely in culture and may be obtained from healthy or tumoral tissue. Some continuous cell lines have oncogenic potential under certain conditions.

**Production cell culture**. A culture of cells intended for use in production; it may be derived from one or more containers of the working cell bank (working cell seed) or it may be a primary cell culture.

**Control cells**. A quantity of cells set aside, at the time of virus inoculation, as uninfected cell cultures. The uninfected cells are incubated under similar conditions to those used for the production cell cultures.

**Single harvest**. Material derived on one or more occasions from a single production cell culture inoculated with the same working seed lot or a suspension derived from the working seed lot, incubated, and harvested in a single production run.

**Monovalent pooled harvest**. Pooled material containing a single strain or type of micro-organism or antigen and derived from a number of eggs, cell culture containers etc. that are processed at the same time.

**Final bulk vaccine**. Material that has undergone all the steps of production except for the final filling. It consists of one or more monovalent pooled harvests, from cultures of one or more species or types of micro-organism, after clarification, dilution or addition of any adjuvant or other auxiliary substance. It is treated to ensure its homogeneity and is used for filling the containers of one or more final lots (batches).

**Final lot (Batch)**. A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk vaccine, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final lot (batch). Where a final bulk vaccine is filled and/or freeze-dried on several separate sessions, there results a related set of final lots (batches) that are usually identified by the use of a common part in the distinctive number or code; these related final lots (batches) are sometimes referred to as sub-batches, sub-lots or filling lots.

**Combined vaccine**. A multicomponent preparation formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use.

07/2010:50202

## 5.2.2. CHICKEN FLOCKS FREE FROM SPECIFIED PATHOGENS FOR THE PRODUCTION AND QUALITY CONTROL OF VACCINES

Where specified, chickens, embryos or cell cultures used for the production or quality control of vaccines are derived from eggs produced by chicken flocks free from specified pathogens (SPF). The SPF status of a flock is ensured by means of the system described below. The list of micro-organisms given is based on current knowledge and will be updated as necessary.

A flock is defined as a group of birds sharing a common environment and having their own caretakers who have no contact with non-SPF flocks. Once a flock is defined, no non-SPF birds are added to it.

Each flock is housed so as to minimise the risk of contamination. The facility in which the flock is housed must not be sited near to any non-SPF flocks of birds with the exception of flocks that are in the process of being established as SPF flocks and that are housed in facilities and conditions appropriate to SPF flocks. The SPF flock is housed within

an isolator or in a building with filtered air under positive pressure. Appropriate measures are taken to prevent entry of rodents, wild birds, insects and unauthorised personnel.

Personnel authorised to enter the facility must have no contact with other birds or with agents potentially capable of infecting the flock. It is advisable for personnel to shower and change clothing or to wear protective clothing before entering the controlled facility.

Wherever possible, items taken into the facility are sterilised. In particular it is recommended that the feed is suitably treated to avoid introduction of undesirable micro-organisms and that water is at least of potable quality, for example from a chlorinated supply. No medication is administered to birds within the flock that might interfere with detection of any disease.

A permanent record is kept of the general health of the flock and any abnormality is investigated. Factors to be monitored include morbidity, mortality, general physical condition, feed consumption, daily egg production and egg quality, fertility and hatchability. Records are maintained for a period of at least 5 years. Details of any deviation from normal in these performance parameters or detection of any infection are notified to the users of the eggs as soon as practicable.

The tests or combination of tests described below must have suitable specificity and sensitivity with respect to relevant serotypes of the viruses. Samples for testing are taken at random.

A positive result for chicken anaemia virus (CAV) does not necessarily exclude use of material derived from the flock, but live vaccines for use in birds less than 7 days old shall be produced using material from CAV-negative flocks. Inactivated vaccines for use in birds less than 7 days old may be produced using material from flocks that have not been shown to be free from CAV, provided it has been demonstrated that the inactivation process inactivates CAV.

## ESTABLISHMENT OF AN SPF FLOCK

A designated SPF flock is derived from chickens shown to be free from vertically-transmissible agents listed in Table 5.2.2-1. This is achieved by testing of 2 generations prior to the designated SPF flock. A general scheme for the procedure to be followed in establishing and maintaining an SPF flock is shown diagrammatically in Table 5.2.2.-2. In order to establish a new SPF flock, a series of tests must be conducted on 3 generations of birds. All birds in the 1st generation must be tested at least once before the age of 20 weeks for freedom from avian leucosis group-antigen and tested by an enzyme immunoassay (EIA) or by virus neutralisation (VN) for freedom of antibodies to avian leucosis virus subtypes A, B and J. All birds must also be tested for freedom from antibodies to the vertically-transmissible agents listed in Table 5.2.2-1. From the age of 8 weeks the flock is tested for freedom from *Salmonella*. Clinical examination is carried out on the flock from 8 weeks of age and the birds must not exhibit any signs of infectious disease. The test methods to be used for these tests are given in the table and further guidance is also given in the section below on routine testing of designated SPF flocks. From 20 weeks of age, the flock is tested as described under Routine testing of designated SPF flocks. All stages of this testing regime are also applied to the subsequent 2 generations, except the testing of every bird before lay for vertically-transmissible agents. All test results must indicate freedom from pathogens in all 3 generations for the flock consisting of the 3rd generation to be designated as SPF.

SPF embryos derived from another designated SPF flock contained within a separate facility on the same site may be introduced. From 8 weeks of age, these replacement birds are regarded as a flock and are tested in accordance with test procedures described above.

## INITIAL TESTING REQUIREMENTS FOR SUBSEQUENT GENERATIONS DERIVED FROM A DESIGNATED SPF FLOCK

Where a replacement flock is derived exclusively from a fully established SPF flock the new generation is tested prior to being designated as SPF. In addition to the tests for *Salmonella* and monitoring of the general health and performance of the flock, further specific testing from the age of 8 weeks is required. Tests are performed on two 5 per cent samples of the flock (minimum 10, maximum 200 birds) taken with an interval of at least 4 weeks between the ages of 12-16 weeks and 16-20 weeks.

All samples are collected and tested individually. Blood samples for antibody tests and suitable samples for testing for leucosis antigen are collected. The test methods to be used are as described under Routine testing of designated SPF flocks. Only when all tests have confirmed the absence of infection may the new generation be designated as SPF.

## ROUTINE TESTING OF DESIGNATED SPF FLOCKS

*General examination and necropsy*. Clinical examination is carried out at least once per week throughout the life of the flock in order to verify that the birds are free from fowl-pox virus and signs of any other infection. In the event of mortality exceeding 0.2 per cent per week, necropsy is performed on all available carcasses to verify that there is no sign of infection. Where appropriate, histopathological and/or microbiological/virological studies are performed to confirm diagnosis. Specific examination for tuberculosis lesions is carried out and histological samples from any suspected lesions are specifically stained to verify freedom from *Mycobacterium avium*. Caecal contents of all available carcasses are examined microbiologically for the presence of *Salmonella* spp. using the techniques described below. Where appropriate, caecal samples from up to 5 birds may be pooled.

*Cultural testing for Salmonella spp*. Cultural testing for *Salmonella* spp. is performed either by testing samples of droppings or cloacal swabs or by testing of drag swabs. Where droppings or cloacal swabs are tested, a total of 60 samples within each 4-week period is tested throughout the entire life of the flock. Tests may be performed on pools of up to 10 samples. Where drag swabs are tested, a minimum of 2 drag swabs are tested during each 4-week period throughout the entire life of the flock. Detection of *Salmonella* spp. in these samples is performed by pre-enrichment of the samples followed by culture using *Salmonella*-selective media.

*Tests for avian leucosis antigen*. Prior to the commencement of laying, cloacal swabs or blood samples (using buffy coat cultivation) are tested for the presence of group-specific leucosis antigen. A total of 5 per cent (minimum 10, maximum 200) of the flock is sampled during each 4-week period. During lay, albumen samples from 5 per cent (minimum 10, maximum 200) of the eggs are tested in each 4-week period. Tests are performed by EIA for group-specific antigen using methods that are capable of detecting antigen from subgroups A, B and J.

*Test for antibodies to other agents*. Tests for antibodies to all agents listed in Table 5.2.2.-1 are performed throughout the laying period of the flock. In each 4-week period, samples are taken from 5 per cent (minimum 10, maximum 200) of the flock. It is recommended that 1.25 per cent of the flock is sampled each week since some test methods for some agents must be conducted on a weekly basis. Table 5.2.2.-1 classifies the agents into those that spread rapidly through the flock and those that spread slowly or may not infect the entire flock. For those agents listed as slowly spreading, each sample is tested individually. For those agents listed as rapidly spreading, at least 20 per cent of the samples collected in each 4-week period are tested individually or, where serum neutralisation

or ELISA tests are employed, all of the samples may be tested individually or by preparing pools of 5 samples, collected at the same time.

Suitable methods to be used for detection of the agents are shown in Table 5.2.2.-1. Subject to agreement by the competent authority, other test methods may be used provided they are shown to be at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2.-1

| Agent | Test to be used** | Vertical transmission | Rapid/slow spread |
|---|---|---|---|
| Avian adenoviruses, group 1 | AGP, EIA | yes | slow |
| Avian encephalomyelitis virus | AGP, EIA | yes | rapid |
| Avian infectious bronchitis virus | HI, EIA | no | rapid |
| Avian infectious laryngotracheitis virus | VN, EIA | no | slow |
| Avian leucosis viruses | EIA for virus, VN, EIA for antibody | yes | slow |
| Avian nephritis virus | IS | no | slow |
| Avian orthoreoviruses | IS, EIA | yes | slow |
| Avian reticuloendotheliosis virus | AGP, IS, EIA | yes | slow |
| Chicken anaemia virus | IS, EIA, VN | yes | slow |
| Egg drop syndrome virus | HI, EIA | yes | slow |
| Infectious bursal disease virus | Serotype 1: AGP, EIA, VN<br>Serotype 2: VN | no | rapid |
| Influenza A virus | AGP, EIA, HI | no | rapid |
| Marek's disease virus | AGP | no | rapid |
| Newcastle disease virus | HI, EIA | no | rapid |
| Turkey rhinotracheitis virus | EIA | no | slow |
| *Mycoplasma gallisepticum* | Agg and HI to confirm a positive test, EIA, HI | yes | slow |
| *Mycoplasma synoviae* | Agg and HI to confirm a positive test, EIA, HI | yes | rapid |
| *Salmonella pullorum* | Agg | yes | slow |

Agg: agglutination
AGP: agar gel precipitation; the technique is suitable where testing is carried out weekly
EIA: enzyme immunoassay
HI: haemagglutination inhibition
IS: immunostaining
VN: virus neutralisation

**Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2-2. – *Schematic description of the establishment and maintenance of SPF flocks*

| | |
|---|---|
| NEW STOCK | Establish freedom from vertically-transmissible agents |
| | Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age |
| | Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age |
| | Carry out routine testing for specified agents from 20 weeks of age |
| 2nd GENERATION | Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age |
| | Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age |
| | Carry out routine testing for specified agents from 20 weeks of age |
| 3rd GENERATION | Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age |
| | Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age |
| DESIGNATE FLOCK AS SPF IF ALL TESTS ARE SATISFACTORY | |
| 3rd GENERATION | Carry out routine testing for specified agents from 20 weeks of age |
| | Carry out post-lay testing for vertically-transmissible agents |
| SUBSEQUENT GENERATIONS | Test two 5 per cent samples for avian leucosis antigen and for antibodies against specified agents between 12 and 20 weeks of age |
| | Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age |
| | Carry out routine testing for specified agents from 20 weeks of age |
| | Carry out post-lay testing for vertically-transmissible agents |

TESTS TO BE CONDUCTED AT THE END OF THE LAYING PERIOD

Following the last egg collection, final testing to confirm the absence of vertically-transmissible agents indicated in Table 5.2.2.-1 is performed. After the last egg collection, a minimum of 5 per cent of the flock (minimum 10, maximum 200) is retained for at least 4 weeks. Blood samples are collected from every bird in the group during the 4-week period with at least 1.25 per cent of the birds (25 per cent of the sample) being bled not earlier than 4 weeks after the final egg collection. Serum samples are tested for vertically-transmissible agents (as defined by Table 5.2.2.-1) using the methods indicated. Where sampling is performed on a weekly basis, at least 1.25 per cent of the birds (25 per cent of the sample) are tested each week during this period. Alternatively, within 4 weeks of the final egg collection blood and/or other suitable sample materials are collected from at least 5 per cent of the flock and tested for the presence of vertically-transmissible agents using validated nucleic acid amplification techniques (*2.6.21*).

ACTION TO BE TAKEN IN THE EVENT OF DETECTION OF A SPECIFIED AGENT

If evidence is found of contamination of the flock by an agent listed as slowly spreading in Table 5.2.2.-1, all materials derived from the flock during the 4-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Similarly, if evidence is found of contamination of the flock by an agent listed as rapidly spreading in Table 5.2.2.-1, all materials derived from the flock during the 2-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Any product manufactured with such materials, and for which the use of SPF materials is required, is considered unsatisfactory and must be discarded; any quality control tests conducted using the materials are invalid.

Producers must notify users of all eggs of the evidence of contamination as soon as possible following the outbreak.

Any flock in which an outbreak of any specified agent is confirmed may not be redesignated as an SPF flock. Any progeny derived from that flock during or after the 4-week period prior to the last negative sample being collected may not be designated as SPF.

**01/2018:50203**

# 5.2.3. CELL SUBSTRATES FOR THE PRODUCTION OF VACCINES FOR HUMAN USE

This general chapter deals with diploid cell lines and continuous cell lines used as cell substrates for the production of vaccines for human use; additional issues specifically related to vaccines prepared by recombinant DNA technology are covered by the monograph *Products of recombinant DNA technology (0784)*. The testing to be carried out at the various stages (cell seed, master cell bank (MCB), working cell bank (WCB), end of productions cells (EOPC) or extended cell bank (ECB) corresponding to cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

**Diploid cell lines**. A diploid cell line has a high but finite capacity for multiplication *in vitro*.

**Continuous cell lines**. A continuous cell line has the capacity to multiply indefinitely *in vitro*; the cells often have differences in karyotype compared to the original cells; they may be obtained from healthy or tumour tissue either from mammals or from insects.

There are perceived theoretical risks associated with the use of continuous cell lines, especially if their tumorigenic potential has been demonstrated experimentally. These risks are linked to the potential biological activity of the residual host-cell DNA present in the vaccine. The residual host-cell DNA may be associated with an infectivity risk if the genome of a DNA virus or a provirus is present in the cellular DNA (either integrated or extra chromosomal). In addition, there is a potential risk of oncogenicity if the cell substrate is tumorigenic.

For vaccines produced in continuous cell lines, whether tumorigenic or not, risk assessment and risk mitigation must be performed to evaluate the suitability of the cell substrate, to define the acceptable criteria for residual host-cell DNA in the final product and to evaluate the consistency of host-cell proteins.

**Cell-bank system**. Production of vaccines in diploid or continuous cell lines is based on a cell-bank system. The *in vitro* age of the cells is counted from the MCB. Each WCB is prepared from one or more containers of the MCB. The use, identity and inventory control of the containers is carefully documented.

**Media and substances of human or animal origin**. The composition of media used for isolation and all subsequent culture is recorded in detail, and if substances of human or animal origin are used they must be free from extraneous agents (*2.6.16*) and must comply with general chapter *5.1.7. Viral safety*.

If human albumin is used, it complies with the monograph *Human albumin solution (0255)*.

If bovine serum is used, it complies with the monograph *Bovine serum (2262)*.

Unless of recombinant origin, trypsin used for the preparation of cell cultures is tested by suitable methods and shown to be sterile and free from mycoplasmas and viruses.

**Cell seed**. The data used to assess the suitability of the cell seed comprises information, where available, on source, history and characterisation.

*Source of the cell seed.* For human cell lines, the following information concerning the donor is recorded: ethnic and geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

For animal cell lines, the following information concerning the source of the cells is recorded: species; strain; breeding conditions; geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

Cells of neural origin, such as neuroblastoma and P12 cell lines are not used for vaccine production since they may contain substances that concentrate agents of spongiform encephalopathies.

*History of the cell seed.* The following information is recorded: the method used to isolate the cell seed; culture methods; any other procedures used to establish the MCB, notably any that might expose the cells to extraneous agents.

Full information may not be available on the media ingredients used in the past for cultivation of cells, for example on the source of substances of animal origin; where justified and authorised, cell banks already established using such media may be used for vaccine production.

*Characterisation of the cell seed.* The following properties are investigated:

(1) the identity of the cells, using methods such as isoenzyme analysis, *in vitro* immunochemical assays, nucleic acid fingerprinting and nucleic acid amplification techniques (NAT);

(2) the growth characteristics of the cells and their morphological properties (optical and electron microscopy);

(3) for diploid cell lines, karyotype;

(4) for diploid cell lines, the *in vitro* life span in terms of population doubling level.

**Cell substrate stability**. Suitable viability of the cell line in the intended storage conditions must be demonstrated. For a given product to be prepared in the cell line, it is necessary to demonstrate that consistent production can be obtained with cells at passage and/or population doubling levels at the beginning and end of the intended period of use.

**Infectious extraneous agents**. For cell lines for vaccine production, the testing for infectious extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal or plant derived raw materials must be taken into account in the choice of suitable permissive cells. One such strategy is given in Table 5.2.3.-1, but alternative strategies could focus on more extensive testing at the MCB or WCB level. In any case, any strategy must be justified and lead to the same level of safety as outlined in Table 5.2.3-1. New, sensitive molecular techniques with broad detection capabilities are available, including massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. These methods may be used either as an alternative to *in vivo* or specific NAT tests or as a supplement/alternative to *in vitro* culture tests, in agreement with the competent authority. The capacity of the process to remove/inactivate specific viruses must take into account the origin and culture history of the cell line and adventitious viruses that are known to persistently infect the species of origin, for example, simian virus 40 in rhesus monkeys, Flock house virus in insect cells or viruses that may inadvertently be introduced during production processes or through the use of raw materials of animal or plant origin. For cell lines of insect origin, tests for specific viruses relevant to the species of origin of the insect cells and for arboviruses (arthropod-borne viruses) are carried out. The panel of viruses tested is chosen according to the current state of scientific knowledge. For cell lines shown to express endogenous retroviral particles (e.g. rodent cells), the test for reverse transcriptase is not needed because it is expected to be positive, and thus infectivity tests must be performed to determine whether these endogenous retroviral particles are infectious or not.

Cell lines that show the presence of infectious retroviruses are not acceptable for production of vaccines, unless otherwise justified and authorised.



Table 5.2.3.-1. – *Testing of cell lines*

| Test | Cell seed | Master cell bank (MCB) | Working cell bank (WCB) | EOPC/ECB (Cells at or beyond the maximum population doubling level used for production) |
|---|---|---|---|---|
| | | 1. IDENTITY AND PURITY | | |
| Morphology | + | + | + | + |
| Identification | + | + | + | + |
| Karyotype (diploid cell lines) | + | + | +(1) | +(1) |
| Life span (diploid cell lines) | – | + | + | – |
| | | 2. EXTRANEOUS AGENTS | | |
| Bacterial and fungal contamination | – | + | + | – |
| Mycobacteria | – | +(2) | +(2) | – |
| Mycoplasmas | – | + | + | – |
| Spiroplasmas(3) | – | + | + | – |
| Electron microscopy | – | +(4) | – | +(4) |
| Tests for extraneous agents in cell cultures (with viable cells or equivalent cell lysate) | – | + | + | + |
| Tests in suckling mice and eggs | – | – | +(5) | +(5) |
| Test for specific viruses by NAT | – | +(6) | +(6) | +(6) |
| Test for viruses using broad molecular methods | +(7) | +(7) | +(7) | +(7) |
| Retroviruses | – | +(4) | – | +(4) |
| | | 3. TUMORIGENICITY | | |
| Tumorigenicity | +(8, 9) | – | – | +(8) |

(1) The diploid character is established for each WCB but using cells at or beyond the maximum population doubling level used for production.

(2) If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species.

(3) If insect cells or raw materials of plant origin are used.

(4) Testing is carried out for the MCB, but using cells at or beyond the maximum population doubling level used for production.

(5) Testing is carried out for each WCB, but using cells at or beyond the maximum population doubling level used for production.

(6) Specific tests for possible contaminants (e.g. viruses) defined according to a risk assessment based on the origin of the cells and on the potential extraneous agents inadvertently introduced during production processes or through the use of animal or plant derived raw materials. The appropriate testing stages should be selected based on the risk assessment.

| Test | Cell seed | Master cell bank (MCB) | Working cell bank (WCB) | EOPC/ECB (Cells at or beyond the maximum population doubling level used for production) |
|---|---|---|---|---|

(7) These methods may be used either as alternative to *in vivo* tests and specific NAT or as supplement or alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority. The appropriate testing stages should be selected based on the risk assessment.

(8) The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and they do not need to be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic, for example CHO and BHK-21.

(9) Testing is carried out on the cell seed, but using cells at or beyond the maximum population doubling level used for production.

**Tumorigenicity**. Tumorigenicity is defined as the potential of a given cell line to induce a tumour after injection of intact live cells into immunodeficient/immunosuppressed animals (usually rodents). The tumorigenicity test is carried out using cells at or beyond the maximum population doubling level that will be used for vaccine production.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and further testing is not necessary. Known tumorigenic cell lines (e.g. CHO) do not need to be documented further.

When a previously uncharacterised cell line is tumorigenic, an oncogenicity study must be performed using purified DNA from the cell line and/or cell line lysate to demonstrate the absence of oncogenic components. The results are used as part of the risk analysis performed to support the use of the cell line for vaccine production. The determination of the $TPD_{50}$ (tumour-producing dose in 50 per cent of animals) and the capacity to form metastases are characteristic properties that must be determined as part of the risk analysis.

Despite the difficulty in demonstrating a perfect and conclusive correlation with a tumorigenic phenotype, additional *in vitro* characterisation tests may be performed to document other cell substrate properties, such as the ability to grow in soft agar gels, the ability to induce invasive cell growth in muscle and/or the ability of the cell substrate to induce transformation of 3T3 cells.

**Residual host-cell DNA**. For each particular vaccine produced on continuous cell lines, residual host-cell DNA content must be tested and an acceptable upper limit, based on a risk assessment, must be established in the final product taking into consideration:

(1) the nature of the cell substrate (non-tumorigenic, level of tumorigenicity) and its origin (human/non-human);

(2) the presence in the production process of any steps to inactivate the potential biological activity (oncogenicity, infectivity) of the residual host-cell DNA (e.g. chemical agents such as betapropiolactone and/or DNase treatment);

(3) the capacity of the process to reduce the amount and size of the contaminating residual host-cell DNA;

(4) the intended use of the vaccine (e.g. route of administration);

(5) the method used to measure the residual host-cell DNA.

In general, a purification process for parenteral vaccines is able to reduce residual host-cell DNA in final products to less than 10 ng per dose, but the acceptance limits must be approved by the competent authority.

Once validation studies (e.g. spiking studies using an adequate size distribution of DNA) have been performed and the reproducibility of the production process in reducing residual host-cell DNA to the level expected has been demonstrated, residual host-cell DNA testing may be omitted after agreement from the competent authority.

**Chromosomal characterisation**. Diploid cell lines shall be shown to be diploid. More extensive characterisation of a diploid cell line by karyotype analysis is required if the removal of intact cells during post-harvest processing has not been validated. Samples from 4 passage levels evenly spaced over the life span of the cell line are examined. A minimum of 200 cells in metaphase are examined for exact chromosome count and for the frequency of hyperploidy, hypoploidy, polyploidy, breaks and structural abnormalities.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being diploid and well characterised; where they are not genetically modified, further characterisation is not necessary.

TEST METHODS FOR CELL CULTURES

**Morphology**. The morphology of the cells is adequately described and documented.

**Identification**. Nucleic acid fingerprint analysis and a relevant selection of the following are used to establish the identity of the cells:

(1) biochemical characteristics (isoenzyme analysis);

(2) immunological characteristics (histocompatibility antigens, *in vitro* immunochemical assays);

(3) cytogenetic markers;

(4) NAT.

**Contaminating cells**. The nucleic acid fingerprint analysis carried out for identification also serves to demonstrate freedom from contaminating cells.

**Bacterial and fungal contamination**. The MCB and each WCB comply with the test for sterility (*2.6.1*), carried out using, for each medium, 10 mL of supernatant from cell cultures. Carry out the test on 1 per cent of the containers, with a minimum of 2 containers.

**Mycobacteria**. If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species, the MCB and each WCB comply with the test for mycobacteria (*2.6.2*). NAT (*2.6.21*) may be used as an alternative to this culture method provided such an assay is validated and shown to be comparable to the culture method.

**Mycoplasmas** (*2.6.7*). The MCB and each WCB comply with the test for mycoplasmas. Use one or more containers for the test.

**Spiroplasmas**. Spiroplasmas may be introduced into cell substrates through contamination of raw materials of plant origin or when insect cell lines are used. When appropriate, the MCB and each WCB are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority. Use one or more containers for the test.

**Electron microscopy**. The MCB is examined by electron microscopy for the presence of extraneous agents. Cell lines are maintained at the temperature routinely used for production and taken at or beyond the maximum population doubling level used for production. In addition, insect cell lines are maintained at temperatures above and below those routinely used for production and may also be subjected to other treatments such as exposure to chemical stressors. For insect cell lines the maintenance temperatures and treatments used are agreed with the competent authority, along with the number of sectioned cells to be examined.

**Test for extraneous agents in cell cultures**. For mammalian cells, viable cells (at least $10^7$ cells) or the equivalent cell lysate, in their culture supernatant, are either co-cultivated (for viable cells) or inoculated (for cell lysate) onto monolayer cultures of:

(1) human diploid cells;

(2) continuous simian kidney cells; and

(3) for cell substrates other than human or simian, cells of that species, from a separate batch.

For insect cell lines, cell lysates are inoculated onto monolayer cultures of other cell systems, including human, simian and, in addition, at least 1 cell line that is different from that used in production, is permissible to insect viruses and allows detection of human arboviruses (e.g. BHK-21).

The resulting co-cultivated cell culture (for viable cells) or inoculated cell cultures (for cell lysate) are observed for evidence of viruses by cytopathic effect for at least 2 weeks. If the cell line is known to be capable of supporting the growth of human or simian cytomegalovirus, the human diploid cultures are observed for at least 4 weeks. The extended 4-week cell culture of human diploid cells, for the purpose of detecting human or simian cytomegalovirus, can be replaced by the use of NAT (*2.6.21*). In cases where it is difficult to keep the cell cultures healthy for the additional 2 weeks, it may be necessary to introduce fresh medium or to subculture after 2 weeks onto fresh cultures in order to be able to detect viral agents. At the end of the observation period, carry out tests on the cell culture supernatants for haemagglutinating viruses, or on the viable cells for haemadsorbing viruses using guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Analyse half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. The test for haemagglutinating viruses is not valid for arboviruses.

The test is not valid unless at least 80 per cent of the cell cultures remain viable. The cell substrate complies with the test if no evidence of any extraneous agent is found.

**Retroviruses**

If the cell line is not known to produce retroviral particles, examine for the presence of retroviruses using:

(1) product-enhanced reverse transcriptase (PERT) assay (*2.6.21*) carried out for cell bank supernatants using cells at or beyond the maximum population doubling level that will be used for production;

(2) transmission electron microscopy.

If tests (1) and/or (2) give a positive result, infectivity assays are carried out on permissible human cells with a PERT assay end-point on the supernatant.

If the cell line is shown to produce retroviral particles (e.g. rodent cell lines), examine for the presence of retroviruses using:

- transmission electron microscopy;
- infectivity assays carried out on permissible human cells and on relevant additional cells (e.g. *Mus dunni* cells or SC-1 cells for CHO cell substrate) with a PERT assay end-point on the supernatant, except when the amplification cells are positive for reverse transcriptase, in which case the readout is performed using plaque assay or a fluorescent focus assay.

Since the sensitivity of PERT assays is very high, interpretation of a positive signal may be equivocal and a decision on the acceptability of a cell substrate is based on all available data.

**Tests in suckling mice**. The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate.

Inject $10^7$ viable cells or the equivalent cell lysate, in their culture supernatant into 2 litters of suckling mice less than 24 h old, comprising not fewer than 10 animals;

Inject at least 0.1 mL intraperitoneally and 0.01 mL intracerebrally.

Observe the suckling mice for at least 4 weeks. Investigate suckling mice that become sick or show any abnormality to establish the cause of illness. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the suckling mice in each group remain healthy and survive to the end of the observation period.

**Tests in eggs (only required for avian cell substrates)**. The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate. Inject an inoculum of $10^6$ viable cells or the equivalent cell lysate, in their culture supernatant, into the allantoic cavity of ten 9- to 11-day-old SPF embryonated hens' eggs (*5.2.2*) and into the yolk sac of ten 5- to 7-day-old SPF embryonated hens' eggs. Incubate for not less than 5 days. Test the allantoic fluids for the presence of haemagglutinins using mammalian and avian red blood cells; carry out the test at 5 ± 3 °C and 20-25 °C and read the results after 30-60 min. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the embryos remain healthy and survive to the end of the observation period.

**Tests for specific viruses**. The list of specific viruses to be tested is defined based on a viral contamination risk assessment in accordance with the principles detailed in general chapter *5.1.7. Viral Safety*, and takes into account (but is not limited to) the origin of the cells and the potential sources of viral contamination (e.g. raw material of animal or plant origin). NAT tests (*2.6.21*) are carried out with or without prior amplification in cells. For cell lines of rodent origin, NAT (*2.6.21*) or antibody production tests in mice, rats or hamsters are used to detect species-specific viruses.

**Tests for viruses using broad molecular methods**. In agreement with the competent authority, broad molecular methods (e.g. High Throughput Sequencing) may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement or alternative to *in vitro* culture tests based on the risk assessment.

For both NAT (*2.6.21*) and broad molecular methods, the stage at which testing is to be conducted (e.g. MCB, WCB, EOPC/ECB) is also based on the risk assessment and depends on the steps where viral contaminants may be introduced. In case of positive results with either broad molecular methods or NAT tests, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

**Tests for tumorigenicity *in vivo***. The test establishes a comparison between the continuous cell line and a suitable positive control cell line as reference (for example, HeLa or Hep2 cells).

Animal systems that have been shown to be suitable for this test include:

(1) athymic mice (Nu/Nu genotype);

(2) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin;

(3) thymectomised and irradiated mice that have been reconstituted ($T^-$, $B^+$) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the positive control cells are injected into separate groups of 10 animals each. In both cases, the inoculum for each animal is $10^7$ cells suspended in a volume of 0.2 mL, and the injection may be given by the intramuscular or the subcutaneous route. Newborn animals are treated with 0.1 mL of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of growing animals to the extent that the subsequent inoculum of $10^7$ positive control cells

regularly produces tumours and metastases. Severely affected animals showing evident, progressively growing tumours are euthanised before the end of the test to avoid unnecessary suffering.

At the end of the observation period all animals, including the positive control group, are euthanised and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other organs (for example, lymph nodes, lungs, kidneys and liver).

In all test systems, the animals are observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed are measured in 2 perpendicular directions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules that begin to regress during the period of observation are euthanised before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules are observed for 1-2 weeks. Among those without nodule formation, half are observed for 3 weeks and half for 12 weeks before they are euthanised and processed for histological examination. A necropsy is performed on each animal and includes examination for gross evidence of tumour formation at the site of injection and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of injection are examined histologically. In addition, since some cell lines may give rise to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals are examined histologically.

The test is invalid if fewer than 9 of the 10 animals injected with the reference positive-control cells show progressively growing tumours.

For a new tumorigenic cell line, in order to document the level of tumorigenicity, a dose range of cell substrate (e.g. dose of cells in the range of $10^5$, $10^6$ and $10^7$) is injected in different groups of 10 animals. The number of animals showing progressively growing nodules within the animal groups is monitored to calculate the $TPD_{50}$.

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## 5.2.4. CELL CULTURES FOR THE PRODUCTION OF VETERINARY VACCINES

Cell cultures for the production of vaccines for veterinary use comply with the requirements of this section. It may also be necessary that cell cultures used for testing of vaccines for veterinary use also comply with some or all of these requirements.

For most mammalian viruses, propagation in cell lines is possible and the use of primary cells is then not acceptable.

Permanently infected cells used for production of veterinary vaccines comply with the appropriate requirements described below. The cells shall be shown to be infected only with the agent stated.

CELL LINES

Cell lines are normally handled according to a cell-seed system. Each master cell seed is assigned a specific code for identification purposes. The master cell seed is stored in aliquots at – 70 °C or lower. Production of vaccine is not normally undertaken on cells more than twenty passages from the master cell seed. Where suspension cultures are used, an increase in cell numbers equivalent to approximately three population doublings is considered equivalent to one passage. If cells beyond twenty passage levels are to be used for production, it shall be demonstrated, by validation or further testing, that the production cell cultures are essentially similar to the master cell seed with regard to their biological characteristics and purity and that the use of such cells has no deleterious effect on vaccine production.

The history of the cell line shall be known and recorded in detail (for example, origin, number of passages and media used for multiplication, storage conditions).

The method of storing and using the cells, including details of how it is ensured that the maximum number of passages permitted is not exceeded during product manufacture, are recorded. A sufficient quantity of the master cell seed and each working cell seed are kept for analytical purposes.

The tests described below are carried out (as prescribed in Table 5.2.4.-1) on a culture of the master cell seed and the working cell seed or on cell cultures from the working cell seed at the highest passage level used for production and derived from a homogeneous sample demonstrated to be representative.

**Characteristics of culture**. The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is provided especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

**Karyotype**. A chromosomal examination is made of not fewer than fifty cells undergoing mitosis in the master cell seed and at a passage level at least as high as that to be used in production. Any chromosomal marker present in the master cell seed must also be found in the high passage cells and the modal number of chromosomes in these cells must not be more than 15 per cent higher than of cells of the master cell seed. The karyotypes must be identical. If the modal number exceeds the level stated, if the chromosomal markers are not found in the working cell seed at the highest level used for production or if the karyotype differs, the cell line shall not be used for manufacture.

Table 5.2.4.-1. – *Cell culture stage at which tests are carried out*

| | Master cell seed | Working cell seed | Cell from working cell seed at highest passage level |
|---|---|---|---|
| General microscopy | + | + | + |
| Bacteria and fungi | + | + | – |
| Mycoplasmas | + | + | – |
| Viruses | + | + | – |
| Retroviruses | + | – | + |
| Identification of species | + | – | + |
| Karyotype | + | – | + |
| Tumorigenicity | + | – | – |

**Identification of the species**. It shall be shown, by one validated method, that the master cell seed and the cells from the working cell seed at the highest passage level used for production come from the species of origin specified. When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

**Bacterial and fungal contamination**. The cells comply with the test for sterility (*2.6.1*). The sample of cells to be examined consists of not less than the number of cells in a monolayer with an area of 70 $cm^2$ or, for cells grown in suspension, an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Mycoplasmas** (*2.6.7*). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Absence of contaminating viruses**. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below, are carried out.

The monolayers tested shall have an area of at least 70 $cm^2$, and shall be prepared and maintained using medium and additives, and grown under similar conditions to those used for the preparation of the vaccine. The monolayers are maintained in culture for a total of at least 28 days. Subcultures are made at 7-day intervals, unless the cells do not survive for this length of time, when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers, are produced for the final subculture to carry out the tests specified below.

The monolayers are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immuno-fluorescence and other suitable tests as indicated below.

**Detection of cytopathic viruses**. Two monolayers of at least 6 $cm^2$ each are stained with an appropriate cytological stain. The entire area of each stained monolayer is examined for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality which might be attributable to a contaminant.

**Detection of haemadsorbent viruses**. Monolayers totalling at least 70 $cm^2$ are washed several times with an appropriate buffer and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times cells are examined for the presence of haemadsorption.

**Detection of specified viruses**. Tests are carried out for freedom from contaminants specific for the species of origin of the cell line and for the species for which the product is intended. Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to suitable tests, for example using fluorescein-conjugated antibodies or similar reagents.

**Tests in other cell cultures**. Monolayers totalling at least 140 $cm^2$ are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Inoculate aliquots onto the following cells at any time up to 70 per cent confluency:

- primary cells of the source species;
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended;
- cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze-thawed extracts are prepared as above and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. The cultures are examined regularly for the presence of any cytopathic changes indicative of living organisms.

At the end of this period of 14 days, the inoculated cells are subjected to the following checks:

- freedom from cytopathic and haemadsorbent organisms, using the methods specified in the relevant paragraphs above,
- absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on Detection of Specified Viruses.

**Retroviruses**. A validated *in vitro* test is carried out to detect the presence of retroviruses in cell lines. If the presence of retrovirus is known or established by testing such as an endpoint product-enhanced reverse transcriptase (PERT) assay (*2.6.21*), then infectivity assays should be carried out. A PERT assay may be suitable to detect infective retrovirus after passage on permissive cells.

Since the sensitivity of PERT assays is very high, interpretation of a positive signal may be equivocal.

Cell seeds that show the presence of infectious retroviruses are not acceptable for the production of vaccines. In exceptional cases of positive or equivocal result in the infectivity assay, it may be justified and authorised to use such cells. Such a justification is based on a risk assessment including all available data and any down stream processing steps until the final product stage. The results of the risk assessment must demonstrate that the risk associated with the presence of infectious retroviruses is negligible in the final product.

**Tumorigenicity**. The risk of a cell line for the target species must be evaluated and, if necessary, tests are carried out.

PRIMARY CELLS

For most mammalian vaccines, the use of primary cells is not acceptable for the manufacture of vaccines since cell lines can be used. If there is no alternative to the use of primary cells, the cells are obtained from a herd or flock free from specified pathogens, with complete protection from introduction of diseases (for example, disease barriers, filters on air inlets, suitable quarantine before introduction of animals). Chicken flocks comply with the requirements prescribed in general chapter *5.2.2. Chicken Flocks Free from Specified Pathogens for the Production and Quality Control of Vaccines*. For all other species, the herd or flock is shown to be free from relevant specified pathogens. All the breeding stock in the herd or flock intended to be used to produce primary cells for vaccine manufacture is subject to a suitable monitoring procedure including regular serological checks carried out at least twice a year and two supplementary serological examinations performed in 15 per cent of the breeding stock in the herd between the two checks mentioned above.

Wherever possible, particularly for mammalian cells, a seed-lot system is used with, for example, a master cell seed formed after less than five passages, the working cell seed being no more than five passages from the initial preparation of the cell suspension from the animal tissues.

Each master cell seed, working cell seed and cells of the highest passage of primary cells are checked in accordance with Table 5.2.4.-2 and the procedure described below. The sample tested shall cover all the sources of cells used for the manufacture of the batch. No batches of vaccine manufactured using the cells may be released if any one of the checks performed produces unsatisfactory results.

Table 5.2.4.-2. – *Cell culture stage at which tests are carried out*

| | Master cell seed | Working cell seed | Highest passage level |
|---|---|---|---|
| General microscopy | + | + | + |
| Bacteria and fungi | + | + | − |
| Mycoplasmas | + | + | − |
| Viruses | + | + | − |
| Retroviruses | + | + | − |
| Identification of species | + | − | − |

**Characteristics of cultures**. The appearance of cell monolayers, before and after histological staining, is described. Information, if possible numerical data, is recorded, especially on the speed and rate of growth. Similarly, the presence or absence of contact inhibition, polynucleated cells and any other cellular abnormalities are specified.

**Identification of species**. It shall be demonstrated by one validated test that the master cell seed comes from the specified species of origin.

When a fluorescence test is carried out and the corresponding serum to the species of origin of cells is used and shows that all the tested cells are fluorescent, it is not necessary to carry out other tests with reagents able to detect contamination by cells of other species.

**Bacterial and fungal sterility**. The cells comply with the test for sterility (*2.6.1*). The sample of cells to be examined consists of not less than the number of cells in a monolayer with an area of 70 $cm^2$ or for cells grown in suspension an approximately equivalent number of cells. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Mycoplasmas** (*2.6.7*). The cells comply with the test for mycoplasmas. The cells are maintained in culture for at least 15 days without antibiotics before carrying out the test.

**Absence of contaminating viruses**. The cells must not be contaminated by viruses; suitably sensitive tests, including those prescribed below are carried out.

The monolayers tested shall be at least 70 $cm^2$, and shall be prepared and maintained in culture using the same medium and additives, and under similar conditions to those used for the preparation of the vaccine.

The monolayers are maintained in culture for a total of at least 28 days or for the longest period possible if culture for 28 days is impossible. Subcultures are made at 7-day intervals, unless the cells do not survive for this length of time when the subcultures are made on the latest day possible. Sufficient cells, in suitable containers are produced for the final subculture to carry out the tests specified below.

The monolayers are examined regularly throughout the incubation period for the possible presence of cytopathic effects and at the end of the observation period for cytopathic effects, haemadsorbent viruses and specific viruses by immunofluorescence and other suitable tests as indicated below.

**Detection of cytopathic viruses**. Two monolayers of at least 6 $cm^2$ each are stained with an appropriate cytological stain. Examine the entire area of each stained monolayer for any inclusion bodies, abnormal numbers of giant cells or any other lesion indicative of a cellular abnormality that might be attributable to a contaminant.

**Detection of haemadsorbent viruses**. Monolayers totalling at least 70 $cm^2$ are washed several times with a suitable buffer solution and a sufficient volume of a suspension of suitable red blood cells added to cover the surface of the monolayer evenly. After different incubation times, examine cells for the presence of haemadsorption.

**Detection of specified viruses**. Tests are be carried out for freedom of contaminants specific for the species of origin of the cells and for the species for which the product is intended.

Sufficient cells on suitable supports are prepared to carry out tests for the agents specified. Suitable positive controls are included in each test. The cells are subjected to suitable tests using fluorescein-conjugated antibodies or similar reagents.

**Tests in other cell cultures**. Monolayers totalling at least 140 $cm^2$ are required. The cells are frozen and thawed at least three times and then centrifuged to remove cellular debris. Aliquots are inoculated onto the following cells at any time up to 70 per cent confluency:

- primary cells of the source species;
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended;
- cells sensitive to pestiviruses.

The inoculated cells are maintained in culture for at least 7 days, after which freeze-thawed extracts are prepared as above, and inoculated onto sufficient fresh cultures of the same cell types to allow for the testing as described below. The cells are incubated for at least a further 7 days. All cultures are regularly examined for the presence of any cytopathic changes indicative of living organisms.

At the end of this period of 14 days, the inoculated cells are subjected to the following checks:

- freedom from cytopathic and haemadsorbent organisms is demonstrated using the methods specified in the relevant paragraphs above;
- relevant substrates are tested for the absence of pestiviruses and other specific contaminants by immunofluorescence or other validated methods as indicated in the paragraph above on Detection of Specified Viruses.

**Retroviruses**. A validated *in vitro* test is carried out to detect the presence of retroviruses in primary cells. If the presence of retrovirus is known or established by testing such as an endpoint product-enhanced reverse transcriptase (PERT) assay (*2.6.21*), then infectivity assays should be carried out. A PERT assay may be suitable to detect infective retrovirus after passage on permissive cells.

Since the sensitivity of PERT assays is very high, interpretation of a positive signal may be equivocal.

Primary cells that show the presence of infectious retroviruses are not acceptable for the production of vaccines. In exceptional cases of positive or equivocal result in the infectivity assay, it may be justified and authorised to use such cells. Such a justification is based on a risk assessment including all available data and any down stream processing steps until the final product stage. The results of the risk assessment must demonstrate that the risk associated with the presence of infectious retroviruses is negligible in the final product.

**07/2009:50205**

# 5.2.5. SUBSTANCES OF ANIMAL ORIGIN FOR THE PRODUCTION OF IMMUNOLOGICAL VETERINARY MEDICINAL PRODUCTS

1. SCOPE

Substances of animal origin (for example serum, trypsin and serum albumin) may be used during the manufacture of immunological veterinary medicinal products.

The requirements set out in this chapter apply to substances of animal origin produced on a batch basis, for use at all stages of manufacture, for example in culture media or as added constituents of products during blending. These requirements are not intended for the control of seed materials or substrates of animal origin that are covered by requirements in other pharmacopoeial texts such as the monograph *Vaccines for veterinary use (0062)* and chapter *5.2.4. Cell cultures for the production of veterinary vaccines*.

2. GENERAL PRINCIPLES AND REQUIREMENTS

*Substances of animal origin comply with the requirements of the European Pharmacopoeia (where a relevant monograph exists).*

Restrictions are placed on the use of substances of animal origin because of safety concerns associated with pathogens that may be present in them and epidemiological and/or regulatory concerns associated with the presence of particular antigens (either live or inactivated).

General principles:

- it is recommended to minimise, wherever practicable, the use of substances of animal origin;

– unless otherwise justified, the use of substances of animal origin as constituents in the formulation of medicinal products is not acceptable except where such substances are subject to a treatment validated for the inactivation of live extraneous agents.

General requirements:

– any batch of substance (after inactivation and/or processing, if relevant) found to contain or suspected of containing any living extraneous agent shall be discarded or used only in exceptional and justified circumstances; to be accepted for use, further processing must be applied that will ensure elimination and/or inactivation of the extraneous agent, and it shall then be demonstrated that the elimination and/or inactivation has been satisfactory;

– any batch of substance that, as concluded from the risk assessment, may induce an unacceptable detectable immune response in the target species as a consequence of contamination with inactivated extraneous agents, must not be used for the manufacture of that particular immunological veterinary medicinal product.

## 3. RISK MANAGEMENT

No single measure or combination of measures can guarantee the safety of the use of substances of animal origin, but they can reduce the risk from such use. It is therefore necessary for the manufacturer of immunological veterinary medicinal products to take account of this when choosing a substance of animal origin to use in manufacture, and to conduct a risk assessment, taking into account the origin of the substance and the manufacturing steps applied to it.

In addition, risk management procedures must be applied. Any residual risk must be evaluated in relation to the potential benefits derived from the use of the substance for the manufacture of the immunological veterinary medicinal product.

### 3-1. *RISK ASSESSMENT*

The risk assessment must take account of the animal diseases occurring in the country of origin of the animals used as a source of the substance, the potential infectious diseases occurring in the source species and the likely infectivity in the source organ or tissue. From this information, as part of the risk assessment, a list can be prepared of the extraneous agents that may be present in the substance.

The risk of contamination of the substance and the resultant immunological veterinary medicinal product with living extraneous agents needs to be assessed. The risk of contamination of the substance and the resultant immunological veterinary medicinal product with inactivated extraneous agents may also need to be taken into account. This would be the case if, for example, the contaminant was one from which a European country is officially free and/or is the subject of a specific disease control program in a European country and where the presence of the inactivated agent could lead to the stimulation of a detectable immune response in recipient animals.

As part of the risk assessment, the presence in the substance of antibodies that can interfere with the detection and/or inactivation of living extraneous agents must also be taken into account.

The risk assessment may need to be repeated and the risk management steps described below re-evaluated and revised in order to take account of changes:

– in the incidence of diseases occurring in the country or countries of origin of animals used as the source for the substance, including emerging diseases (new pathogens);

– in the incidence of diseases and of disease control measures applied in the European countries in which immunological veterinary medicinal products manufactured with the substance are used.

### 3-2. *RISK CONTROL*

For each of the potential extraneous agents identified by the risk assessment, and taking into account the proposed use of the substance, the risk must be controlled by the use of one or a combination of the followings measures:

– placing restrictions on the source of the material and auditing this;

– using validated inactivation procedures;

– demonstrating the ability of a production step to remove or inactivate extraneous agents;

– testing for extraneous agents.

## 4. CONTROL MEASURES

### 4-1. *SOURCE*

All substances of animal origin used in the manufacture (including blending) of immunological veterinary medicinal products must be from a known and documented source (including species of origin and country of origin of source animals and tissues).

### 4-2. *PREPARATION*

Substances of animal origin are prepared from a homogeneous bulk designated with a batch number. A batch may contain substances derived from as many animals as desired but once defined and given a batch number, the batch is not added to or contaminated in any way.

The production method used to prepare the substance of animal origin from the raw material may contribute to the removal and/or inactivation of extraneous agents (see section 4-3).

### 4-3. *INACTIVATION AND/OR OTHER PROCESSING STEPS FOR REMOVAL OF EXTRANEOUS AGENTS*

The inactivation procedure and/or other processing steps chosen shall have been validated and shown to be capable of reducing the titre of potential extraneous agents described below in the substance concerned by a factor of at least $10^6$.

If this reduction in titre cannot be shown experimentally, a maximum pre-treatment titre of the extraneous agent must be set, taking into account the reduction in titre afforded by the inactivation/processing step and including a safety margin factor of 100; each batch of substance must be tested to determine the pre-treatment starting titre and confirm it is no greater than the specified limit, unless proper risk assessment, based on valid and suitable data, shows that titres will always be at least 100-fold below the titre that can effectively be inactivated.

The validation of the procedure(s) is conducted with a suitable representative range of viruses covering different types and sizes (enveloped and non-enveloped, DNA and RNA, single- and double-stranded, temperature- and pH-resistant), including test viruses with different degrees of resistance, taking into account the type of procedure(s) to be applied and the viruses that may be present in the material. The evidence for the efficacy of the procedure may take the form of references to published literature and/or experimental data generated by the manufacturer, but must be relevant to the conditions that will be present during the production and inactivation/processing of the substance.

For inactivated immunological veterinary medicinal products, the method used for inactivation of the active ingredient may also be validated for inactivation of possible contaminants from substances of animal origin used in the manufacture of this active ingredient.

### 4-4. *TESTS*

Depending on the outcome of the risk assessment and the validation data available for any procedure applied, tests for extraneous agents may be conducted on each batch before and/or after the application of an inactivation/processing step. For examination of the substance for freedom from extraneous agents, any solids are dissolved or suspended in a

suitable medium to provide a suitable preparation for testing. A sufficient quantity of the preparation is tested to give a suitably sensitive test, as established in the validation studies. As well as tests for living extraneous agents, tests may need to be conducted for the presence of inactivated extraneous agents, depending on the risks identified.

**Freedom from living extraneous viruses**. A sample from each batch of the substance is tested for extraneous viruses by general and specific tests. These tests are validated with respect to sensitivity and specificity for detection of a suitable range of potential extraneous viruses. Suitably sensitive cell cultures are used for the tests for extraneous viruses, including primary cells from the same species as the substance to be examined.

*General test*. The inoculated cell cultures are observed regularly for 21 days for cytopathic effects. At the end of each 7-day period, a proportion of the original cultures is fixed, stained and examined for cytopathic effects, and a proportion is tested for haemadsorbing agents.

*Specific tests*. A proportion of the cells available at the end of the general test is tested for specific viruses. The specific viruses to be tested for are potential extraneous viruses that are identified through the risk assessment and that would not be detected by the general test. A test for pestiviruses is conducted if the source species is susceptible to these.

**Bacteria and fungi**. Before use, substances are tested for sterility (*2.6.1*), or sterilised to inactivate any bacterial or fungal contaminants.

**Mycoplasma**. Before use, substances are tested for freedom from mycoplasma (*2.6.7*), or sterilised to inactivate any mycoplasmal contaminants.

**04/2013:50206**

## 5.2.6. EVALUATION OF SAFETY OF VETERINARY VACCINES AND IMMUNOSERA

The term 'product' means either a vaccine or an immunoserum throughout the text.

During development, safety tests are carried out in the target species to show the risks from use of the product.

*Immune status for tests on vaccines*. The immune status of animals to be used for the safety test is specified in the specific monograph. For most monographs, 1 of the 3 following categories is specified:

1) the animals must be free from antibodies against the virus/bacterium/toxin etc. contained in the vaccine;

2) the animals are preferably free from antibodies against the virus/bacterium/toxin etc. contained in the vaccine, but animals with a low level of antibody may be used as long as the animals have not been vaccinated and the administration of the vaccine does not cause an anamnestic response;

3) the animals must not have been vaccinated against the disease that the vaccine is intended to prevent.

As a general rule, category 1 is specified for live vaccines. For other vaccines, category 2 is usually specified, but where most animals available for use in tests would comply with category 1, this may be specified for inactivated vaccines also. Category 3 is specified for some inactivated vaccines where determination of antibodies prior to testing is unnecessary or impractical. For poultry vaccines, as a general rule the use of specified-pathogen-free (SPF) birds is specified.

For avian vaccines, the safety test is generally carried out using SPF chickens (*5.2.2*), except that for vaccines not recommended for use in chickens it is carried out using birds of one of the species for which the vaccine is recommended, the birds being free from antibodies against the disease agent for which the vaccine is intended to provide protection.

*Vaccines*. In laboratory tests, 'dose' means that quantity of the product to be recommended for use and containing the maximum titre or potency likely to be contained in production batches. Live vaccines are prepared only from strains of organisms that have been shown to be safe. For live vaccines, use a batch or batches of vaccine containing virus/bacteria at the least attenuated passage level that will be present in a batch of vaccine.

For combined vaccines, the safety shall be demonstrated; for live components of combined vaccines, compliance with the special requirements for live vaccines stated below shall be demonstrated separately for each vaccine strain.

For inactivated vaccines, safety tests carried out on the combined vaccine may be regarded as sufficient to demonstrate the safety of the individual components.

*Immunosera*. In the tests, 'dose' means the maximum quantity of the product to be recommended for use and containing the maximum potency and maximum total protein likely to be contained in production batches. In addition, if appropriate, the dose tested also contains maximum quantities of immunoglobulin or gammaglobulin.

The tests described below, modified or supplemented by tests described in the Production section of a monograph, may be carried out as part of the tests necessary during development to demonstrate the safety of the product.

1. LABORATORY TESTS

1-1. *SAFETY OF THE ADMINISTRATION OF 1 DOSE*

For each of the recommended routes of administration, administer 1 dose of product to animals of each species and category for which use of the product is to be recommended. This must include animals of the youngest recommended age and pregnant animals, if appropriate.

For vaccines intended for use in mammals, in general 8 animals per group are used unless otherwise justified or specified in a specific monograph.

For fish vaccines administered by immersion, bathe the fish for twice the recommended time using a bath at twice the recommended concentration.

For vaccines intended for use in fish, in general 50 fish per group are used unless otherwise justified or specified in a specific monograph.

For vaccines intended for use in birds older than 3 weeks, in general 8 birds per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in birds younger than 3 weeks, in general 10 birds per group are used unless otherwise justified or specified in a specific monograph.

The animals are observed and examined at least daily for signs of abnormal local and systemic reactions. Where appropriate, these studies shall include detailed post-mortem macroscopic and microscopic examinations of the injection site. Other objective criteria are recorded, such as body temperature (for mammals) and performance measurements. The body temperatures are recorded on at least the day before and at the time of administration of the product, 4 h later and on the following 4 days. The animals are observed and examined at least daily until reactions may no longer be expected but, in all cases, the observation and examination period extends at least until 14 days after administration.

Unless otherwise prescribed in a specific monograph or, in the absence of a specific monograph, unless otherwise justified and authorised, the vaccine complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine.

1-2. *SAFETY OF 1 ADMINISTRATION OF AN OVERDOSE*

Overdose testing is required only for live vaccines. An overdose of the product is administered by each recommended route of administration to animals of the categories of the target species that are expected to be the most sensitive, such as animals of the youngest age. If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If 1 route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. The overdose normally consists of 10 doses of a live vaccine. For freeze-dried live vaccines, the 10 doses shall be reconstituted in a suitable volume of diluent for the test. For vaccines intended for use in mammals, in general 8 animals per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in fish, in general 50 fish per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in birds older than 3 weeks, in general 8 birds per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in birds younger than 3 weeks, in general 10 birds per group are used unless otherwise justified or specified in a specific monograph. The animals are observed and examined at least daily for signs of local and systemic reactions. Other objective criteria are recorded, such as body temperature (for mammals) and performance measurements. The animals are observed and examined for at least 14 days after administration.

Unless otherwise prescribed in a specific monograph or, in the absence of a specific monograph, unless otherwise justified and authorised, the vaccine complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine.

1-3. *SAFETY OF THE REPEATED ADMINISTRATION OF 1 DOSE*

Repeated administration of 1 dose may be required to reveal any adverse effects induced by such administration. These tests are particularly important where the product, notably an immunoserum, may be administered on several occasions over a relatively short period of time. These tests are carried out on the most sensitive categories of the target species, using each recommended route of administration. If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If 1 route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study. The number of administrations must be not less than the maximum number recommended; for vaccines, this shall take account of the number of administrations for primary vaccination and the 1st re-vaccination; for immunosera, it shall take account of the number of administrations required for treatment. The interval between administrations shall be suitable (e.g. period of risk or required for treatment) and appropriate to the recommendations of use. Although, for convenience, as far as vaccines are concerned, a shorter interval may be used in the study than that recommended in the field, an interval of at least 14 days must be allowed between administrations for the development of any hypersensitivity reaction. For immunosera, however, administration shall follow the recommended schedule. For vaccines intended for use in mammals, in general 8 animals per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in fish, in general 50 fish per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in birds older than 3 weeks, in general 8 birds per group are used unless otherwise justified or specified in a specific monograph. For vaccines intended for use in birds younger than 3 weeks, in general 10 birds per group are used unless otherwise justified or specified in a specific monograph. The animals are observed and examined at least daily for at least 14 days after the last administration for signs of systemic and local reactions. Other objective criteria are recorded, such as body temperature and performance measurements.

Unless otherwise prescribed in a specific monograph or, in the absence of a specific monograph, unless otherwise justified and authorised, the product complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the product.

1-4. *EXAMINATION OF REPRODUCTIVE PERFORMANCE*

When the vaccine is recommended for use or may be used in pregnant animals or laying birds, carry out a test for safety in this category of animals. If the reproductive safety studies are not performed, an exclusion statement appears on the label, unless a scientific justification for absence of risk is provided. Examination of reproductive performance must also be considered when data suggest that the starting material from which the product is derived may be a risk factor. Where appropriate, reproductive performance of males and females and harmful effects on the progeny, including teratogenic or abortifacient effects, are investigated by each of the recommended routes of administration. If multiple routes and methods of administration are specified for the product concerned, administration by all routes is recommended. If 1 route of administration has been shown to cause the most severe effects, this single route may be selected as the only one for use in the study.

For vaccines intended for use in mammals, in general 8 animals per group are used unless otherwise justified or specified in a specific monograph. Vaccines recommended for use or that may be used in pregnant animals, are tested in each of the specific periods of gestation recommended for use on the label. An exclusion statement will be required for those gestation periods not tested.

The observation period is extended to parturition, to examine any harmful effects during gestation or on progeny, unless otherwise justified or specified in a specific monograph.

The following protocol is given as an example of an appropriate test for vaccines.

*Safety in pregnant animals.* Use not fewer than 8 animals per group, at the recommended stage of gestation or at a range of stages of gestation according to the recommended schedule. Not fewer than 8 animals are used for each stage of pregnancy (i.e. 24 animals for 3 trimesters of pregnancy in cattle). Administer to each animal a recommended dose of the vaccine. If the recommended schedule requires a 2nd dose, administer another dose after an interval of at least 14 days. Unless otherwise prescribed in a specific monograph, observe the animals at least daily until 1 day after parturition. Unless otherwise prescribed in a specific monograph, or, in the absence of a specific monograph, unless otherwise justified and authorised, the vaccine complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine, and if no adverse effects on the pregnancy or the offspring are noted.

1-5. *RESIDUES*

In the case of live vaccines for well-established zoonotic diseases, the determination of residual vaccine organisms at the injection site may be required, in addition to the studies of dissemination described below.

1-6. *ADVERSE EFFECTS ON IMMUNOLOGICAL FUNCTIONS*

Where the product might adversely affect the immune response of the animal to which the product is administered or of its progeny, suitable tests on the immunological functions are carried out.

1-7. *ADVERSE EFFECTS FROM INTERACTIONS*

Studies are undertaken to show a lack of adverse effect on the safety of the product when simultaneous administration is recommended or where administration of the product is recommended as part of a schedule of administration of products within a short period of time.

1-8. *SPECIAL REQUIREMENTS FOR LIVE VACCINES*

The following laboratory tests must also be carried out with live vaccines.

For the following tests except for the test for increase in virulence (section 1-8-3), use the vaccine strain at the least attenuated passage level that will be present between the master seed lot and a batch of vaccine.

1-8-1. **Spread of the vaccine strain**. Spread of the vaccine strain from vaccinated to unvaccinated target animals is investigated using the recommended route of administration most likely to result in spread. Moreover, it may be necessary to investigate the safety of spread to non-target species that could be highly susceptible to a live vaccine strain. An assessment must be made of how many animal-to-animal passages are likely to be sustainable under normal circumstances together with an assessment of the likely consequences.

1-8-2. **Dissemination in vaccinated animal**. Faeces, urine, milk, eggs, and oral, nasal and other secretions shall be tested for the presence of the organism as appropriate. Moreover, studies may be required of the dissemination of the vaccine strain in the body, with particular attention being paid to the predilection sites for replication of the organism. In the case of live vaccines for well-established zoonotic diseases for food-producing animals, these studies are obligatory and shall particularly take into account the persistence of the strain at the injection site.

1-8-3. **Increase in virulence**. Unless otherwise prescribed in a specific monograph or, in the absence of a specific monograph, unless otherwise justified and authorised, the following applies. This test is carried out using the master seed lot. If the quantity of the master seed lot sufficient for performing the test is not available, the lowest passage material used for the production that is available in sufficient quantity may be used. At the time of inoculation, the animals in all groups are of an age suitable for recovery of the strain. Serial passages are carried out in target animals using 5 groups of animals, unless there is justification to carry out more passages or unless the strain disappears from the test animal sooner. *In vitro* propagation may not be used to expand the passage inoculum.

The passages are carried out using animals most appropriate to the potential risk being assessed.

The initial administration is carried out using the recommended route of administration most likely to lead to reversion to virulence, using an initial inoculum containing the maximum release titre. After this, not fewer than 4 further serial passages through animals of the target species are undertaken. The passages are undertaken by the route of administration most likely to lead to reversion to virulence. If the properties of the strain allow sequential passage via natural spreading, this method may be used, otherwise passage as described in each specific monograph is carried out and the micro-organisms that have been recovered at the final passage are tested for increase in virulence. For the first 4 groups, a minimum of 2 animals is used for mammalian vaccines, and a minimum of 5 birds is used for avian vaccines. The last group consist of a minimum of 8 mammals or 10 birds. At each passage, the presence of living vaccine-derived micro-organisms in the material used for passage is demonstrated. Care must be taken to avoid contamination by micro-organisms from previous passages. When the micro-organism is not recovered from any intermediate *in vivo* passage, repeat the passage in 10 animals using *in vivo* passaged material from the last passage in which the micro-organism was recovered. The micro-organism recovered is used as the inoculum for the next passage. If the target micro-organism is not recovered, the experiment is considered to be completed with the conclusion that the target micro-organism does not show an increase in virulence.

General clinical observations are made during the study. Animals in the last group are observed for 21 days unless otherwise justified. These observations include all relevant parameters typical for the disease that could indicate increase in virulence. Compare the clinical signs and other relevant parameters with those observed in the animals used in the test for safety of the administration of 1 dose (section 1-1). If the last group of animals shows no evidence of an increase in virulence, further testing is not required. Otherwise, material used for the 1st passage and the micro-organisms recovered at the final passage level are used in a separate experiment using at least 8 animals per group for mammal vaccines and at least 10 birds per group for avian vaccines, to compare directly the clinical signs and other relevant parameters. This study is carried out using the route of administration that was used for previous passages. An alternative route of administration may be used if justified.

Unless otherwise justified and authorised, the product complies with the test if no animal dies or shows signs attributable to the vaccine strain and no indication of increased virulence is observed in the animals of the last group.

1-8-4. **Biological properties of the vaccine strain**. Other tests may be necessary to determine as precisely as possible the intrinsic biological properties of the vaccine strain (for example, neurotropism). For vector vaccines, evaluation is made of the risk of changing the tropism or virulence of the strain and where necessary specific tests are carried out. Such tests are systematically carried out where the product of a foreign gene is incorporated into the strain as a structural protein.

1-8-5. **Recombination or genomic reassortment of strain.** The probability of recombination or genomic reassortment with field or other strains shall be considered.

2. FIELD STUDIES

Results from laboratory studies shall normally be supplemented with supportive data from field studies. Provided that laboratory tests have adequately assessed the safety and efficacy of a product under experimental conditions using vaccines of maximum and minimum titre or potency respectively, a single batch of product may be used to assess both safety and efficacy under field conditions. In these cases, a typical routine batch of intermediate titre or potency may be used.

For food-producing mammals, the studies include measurement of the body temperatures of a sufficient number of animals, before and after administration of the product; for other mammals, such measurements are carried out if the laboratory studies indicate that there might be a problem. The size and persistence of any local reaction and the proportion of animals showing local or systemic reactions are recorded. Performance measurements are made, where appropriate.

Performance measures for broilers include weekly mortality, feed conversion ratios, age at slaughter and weight, down grading and rejects at the processing plant. For vaccines for use in laying birds or in birds that may be maintained to lay, the effect of the vaccine on laying performance and hatchability is investigated, as appropriate.

3. ECOTOXICITY

An assessment is made of the potential harmful effects of the product for the environment and any necessary precautionary measures to reduce such risks are identified. The likely degree of exposure of the environment to the product is assessed, taking into account: the target species and mode of administration; excretion of the product; and disposal of unused product. If these factors indicate that there will be

significant exposure of the environment to the product, the potential ecotoxicity is evaluated, taking into account the properties of the product.

**04/2008:50207**

## 5.2.7. EVALUATION OF EFFICACY OF VETERINARY VACCINES AND IMMUNOSERA

The term 'product' means either a vaccine or an immunoserum throughout the text.

During development of the product, tests are carried out to demonstrate that the product is efficacious when administered by each of the recommended routes and methods of administration and using the recommended schedule to animals of each species and category for which use of the product is to be recommended. The type of efficacy testing to be carried out varies considerably depending on the particular type of product.

As part of tests carried out during development to establish efficacy, the tests described in the Production section of a monograph may be carried out; the following must be taken into account.

The dose to be used is that quantity of the product to be recommended for use and containing the minimum titre or potency expected at the end of the period of validity.

For live vaccines, use vaccine containing virus/bacteria at the most attenuated passage level that will be present in a batch of vaccine.

For immunosera, if appropriate, the dose tested also contains minimum quantities of immunoglobulin or gammaglobulin and/or total protein.

The efficacy evidence must support all the claims being made. For example, claims for protection against respiratory disease must be supported at least by evidence of protection from clinical signs of respiratory disease. Where it is claimed that there is protection from infection this must be demonstrated using re-isolation techniques. If more than one claim is made, supporting evidence for each claim is required.

*Vaccines.* The influence of passively acquired and maternally derived antibodies on the efficacy of a vaccine is adequately evaluated. Any claims, stated or implied, regarding onset and duration of protection shall be supported by data from trials.

Claims related to duration of immunity are supported by evidence of protection. The test model described under Immunogenicity and/or Potency is not necessarily used to support claims regarding the duration of immunity afforded by a vaccine.

The efficacy of each of the components of multivalent and combined vaccines shall be demonstrated using the combined vaccine.

*Immunosera.* Particular attention must be paid to providing supporting data for the efficacy of the regime that is to be recommended. For example, if it is recommended that the immunoserum needs only to be administered once to achieve a prophylactic or therapeutic effect then this must be demonstrated. Any claims, stated or implied, regarding onset and duration of protection or therapeutic effect must be supported by data from trials. For example, the duration of the protection afforded by a prophylactic dose of an antiserum must be studied so that appropriate guidance for the user can be given on the label.

Studies of immunological compatibility are undertaken when simultaneous administration is recommended or where it is a part of a usual administration schedule. Wherever a product is recommended as part of an administration scheme, the priming or booster effect or the contribution of the product to the efficacy of the scheme as a whole is demonstrated.

LABORATORY TESTS

In principle, demonstration of efficacy is undertaken under well-controlled laboratory conditions by challenge of the target animal under the recommended conditions of use.

In so far as possible, the conditions under which the challenge is carried out shall mimic the natural conditions for infection, for example with regard to the amount of challenge organism and the route of administration of the challenge.

*Vaccines.* Unless otherwise justified, challenge is carried out using a strain different from the one used in the production of the vaccine.

If possible, the immune mechanism (cell-mediated/humoral, local/general, classes of immunoglobulin) that is initiated after the administration of the vaccine to target animals shall be determined.

*Immunosera.* Data are provided from measurements of the antibody levels achieved in the target species after administration of the product, as recommended. Where suitable published data exist, references are provided to relevant published literature on protective antibody levels and challenge studies are avoided.

Where challenges are required, these can be given before or after administration of the product, in accordance with the indications and specific claims to be made.

FIELD TRIALS

In general, results from laboratory tests are supplemented with data from field trials, carried out, unless otherwise justified, with untreated control animals. Provided that laboratory tests have adequately assessed the safety and efficacy of a product under experimental conditions using vaccines of maximum and minimum titre or potency respectively, a single batch of product could be used to assess both safety and efficacy under field conditions. In these cases, a typical routine batch of intermediate titre or potency may be used. Where laboratory trials cannot be supportive of efficacy, the performance of field trials alone may be acceptable.

**07/2011:50208**

## 5.2.8. MINIMISING THE RISK OF TRANSMITTING ANIMAL SPONGIFORM ENCEPHALOPATHY AGENTS VIA HUMAN AND VETERINARY MEDICINAL PRODUCTS

*This chapter is identical with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products – Revision 3, (EMA/410/01 rev. 3).*

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## 1. INTRODUCTION

### 1-1. *SCIENTIFIC BACKGROUND*

Transmissible Spongiform Encephalopathies (TSEs) are chronic degenerative nervous diseases characterised by the accumulation of an abnormal isoform of a cellular glycoprotein (known as PrP or prion protein). The abnormal isoform of PrP ($PrP^{TSE}$) differs from normal PrP (PrPc) in being highly resistant to protease and heat denaturation treatments. $PrP^{TSE}$ is considered to be the infective agent responsible for transmitting TSE disease.

TSE diseases in animals include:

- bovine spongiform encephalopathy (BSE) in cattle,
- scrapie in sheep and goats,
- chronic wasting disease (CWD) in cervids (deer and elk),
- transmissible mink encephalopathy (TME) in farmed mink,
- feline spongiform encephalopathy (FSE) in felids (specifically domestic cats and captive large cats), and
- spongiform encephalopathy of exotic ungulates in zoos.

In humans, spongiform encephalopathies include different forms of Creutzfeldt-Jakob Disease (CJD), Kuru, Gerstmann-Sträussler-Scheinker Syndrome (GSS), and Fatal Familial Insomnia (FFI).

Iatrogenic transmission of spongiform encephalopathies has been reported. In sheep, scrapie has been accidentally transmitted by the use of Louping Ill vaccine prepared from pooled, formaldehyde treated ovine brain and spleen in which material from scrapie-infected sheep had been inadvertently incorporated. Also, transmission of scrapie to sheep and goats occurred following use of a formol-inactivated vaccine against contagious agalactia, prepared with brain and mammary gland homogenates of sheep infected with *Mycoplasma agalactiae*. In man, cases of transmission of CJD have been reported which have been attributed to the parenteral administration of growth hormone and gonadotropin derived from human cadaveric pituitary glands. Cases of CJD have also been attributed to the use of contaminated instruments in brain surgery and with the transplantation of human dura mater and cornea.

Interspecies TSE transmission is restricted by a number of natural barriers, transmissibility being affected by the species of origin, the prion strain, dose, route of exposure and, in some species, the host allele of the PRNP gene. Species barriers can be crossed under appropriate conditions.

BSE was first diagnosed in the United Kingdom in 1986 and a large number of cattle and individual herds have been affected. It is clear that BSE is a food borne disease associated with feed (e.g. meat and bone meal) derived from TSE affected animals. Other countries have experienced cases of BSE, either in animals imported from the United Kingdom or in indigenous animals. There is convincing evidence to show that the variant form of CJD (vCJD) is caused by the agent which is responsible for BSE in cattle. Therefore, a cautious approach continues to be warranted if biological materials from species naturally affected by TSE diseases, especially bovine species, are used for the manufacture of medicinal products.

In the course of active surveillance programs, two previously unrecognized forms of atypical BSE (BSE-L, also named BASE, and BSE-H) have been identified in rare sporadic cases from Europe, North America, and Japan. The 'L' and 'H' identify the higher and lower electrophoretic positions of their protease-resistant $PrP^{TSE}$ isoforms. It is noteworthy that atypical cases have been found in countries that did not experience classical BSE so far, like Sweden, or in which only few classical BSE cases have been found like Canada or USA. The atypical BSE agent has been experimentally transmitted to transgenic mice expressing the human prion protein and to a cynomolgus monkey.

Scrapie occurs worldwide and has been reported in most European countries. It has the highest incidence in Cyprus. While humans have been exposed to naturally occurring scrapie for over 250 years, there is no epidemiological evidence directly linking scrapie to spongiform encephalopathies in humans(1). However, there remains a theoretical and currently unquantifiable risk that some BSE-contaminated protein supplement may have been fed to sheep. Further, it should also be assumed that any BSE agent introduced into the small ruminant population via contaminated feed is likely to be recycled and amplified(2).

There is interest in infecting cells with TSE agents to develop assays and for basic scientific reasons. Some success has been reported, usually but not always with neural cell lines. The conditions needed to infect a cell are not well understood and the process is difficult requiring particular combinations of agent and cell. It is not considered appropriate to make specific recommendations in terms of cell substrates to be used for production of biological/biotechnology-derived substances. Nevertheless, the possibility of infection of cell lines with TSE agents should be taken into account in risk assessments.

### 1-2. *REGULATORY COMPLIANCE*

**Risk assessment**. Since the use of animal-derived materials is unavoidable for the production of some medicinal products and that complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination. Consequently, the basis for regulatory compliance should be based on a risk assessment, taking into consideration all pertinent factors as identified in this chapter (see below).

**Legal basis**. The note for guidance is published by the European Commission following

- Annex I, part I, module 3, section 3.2: *Content: basic principles and requirements*, point (9) of Directive 2001/83/EC of the European Parliament and

(1) This is currently being assessed by EFSA and ECDC. For updated information, please refer to the following link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2009-0221

(2) In January 2005, after confirmation of BSE in a goat in France, additional legislative measures were taken related to monitoring and an increased testing of small ruminants. The increased surveillance did not identify additional cases of BSE in sheep and goats in the EU.

of the Council of 6 November 2001 on the Community code relating to medicinal products for human use[3], as amended, and

– Annex I, Title I, part 2, section C *Production and control of starting material* of Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products[4], as amended.

These directives require that applicants for marketing authorisation for human and veterinary medicinal products must demonstrate that medicinal products are manufactured in accordance with the latest version of this note for guidance published in the *Official Journal of the European Union*. This is a continuing obligation after the marketing authorisation has been granted.

By definition, the principle of Specified Risk Materials as defined in Regulation (EC) No 999/2001 of the European Parliament and of the Council[5] does not apply to medicinal products. However, Regulation (EC) No 1774/2002 of the European Parliament and of the Council[6], which applies since 1st May 2003, lays down health rules concerning animal by-products not intended for human consumption. As a general rule, and unless properly justified, all animal by-products used as starting materials in the manufacture of medicinal products should be 'Category 3 (i.e. safe) materials or equivalent', as defined in Regulation (EC) No 1774/2002. Justification for the use of substances derived from other, high infectivity materials must follow an appropriate benefit/risk evaluation (see further below).

The note for guidance should be read in conjunction with the various EU legal instruments including Commission decisions progressively implemented since 1991. Where appropriate, references to these decisions are given in the text. Position statements and explanatory notes made by the Committee for Medicinal Products for Human Use (CHMP) and Committee for Medicinal Products for Veterinary Use (CVMP) are still applicable for the purpose of regulatory compliance unless otherwise superseded by the note for guidance.

The general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies* of the European Pharmacopoeia refers to this chapter, which is identical with the note for guidance. The monograph forms the basis for issuing Certificates of Suitability as a procedure for demonstrating TSE compliance for substances and materials used in the manufacture of human and veterinary medicinal products.

**Clarification of note for guidance**. As the scientific understanding of TSEs, especially the pathogenesis of the diseases, is evolving, from time to time CHMP and its Biologics Working Party in collaboration with CVMP and its Immunologicals Working Party may be required in the future to develop supplementary guidance in the form of position statements or explanatory notes for the purpose of clarifying the note for guidance. The supplementary guidance shall be published by the Commission and on the website of the European Medicines Agency and taken into consideration accordingly in the scope of the certification of the European Directorate for the Quality of Medicines & HealthCare (EDQM).

2. SCOPE

*TSE-RELEVANT ANIMAL SPECIES*

Cattle, sheep, goats and animals that are naturally susceptible to infection with transmissible spongiform encephalopathy agents or susceptible to infection through the oral route other than humans[7] and non-human primates are defined as "TSE-relevant animal species"[8].

*MATERIALS*

This chapter is concerned with materials derived from "TSE-relevant animal species" that are used for the preparation of:

– active substances,
– excipients and adjuvants, and
– raw and starting materials and reagents used in production (e.g. bovine serum albumin, enzymes, culture media including those used to prepare working cell banks, or new master cell banks for medicinal products which are subject to a new marketing authorisation).

This chapter is also applicable to materials that come into direct contact with the equipment used in manufacture of the medicinal product or that come in contact with the medicinal product and therefore have the potential for contamination.

Materials used in the qualification of plant and equipment, such as culture media used in media fill experiments to validate the aseptic filling process, shall be considered in compliance with this chapter provided that the constituent or constituents are derived from tissues with no detectable infectivity (category IC tissues), where the risk of cross-contamination with potentially infective tissues has been considered (see section 3-3) and where the materials are sourced from countries with negligible BSE risk or controlled BSE risk (Categories A and B, respectively – see section 3-2). Such information shall be provided in the dossier for a marketing authorisation and verified during routine inspection for compliance with Good Manufacturing Practice (GMP).

Other materials such as cleaning agents, softeners and lubricants that come into contact with the medicinal product during its routine manufacture or in the finishing stage or in the primary packaging are considered in compliance with this chapter if they are tallow derivatives prepared using the rigorous physicochemical processes as described in section 6.

*SEED LOTS, CELL BANKS AND ROUTINE FERMENTATION/PRODUCTION*[9]

For the purpose of regulatory compliance, master seeds or master cell banks in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products) shall be covered by the note for guidance.

Master seeds and master cell banks,

– for vaccine antigens,
– for a biotechnology-derived medicinal product as described in the Annex to Regulation (EC) No 726/2004 of the European Parliament and of the Council[10], and
– for other medicinal products using seed lots or cell banking systems in their manufacture,

that have already been approved for the manufacture of a constituent of an authorised medicinal product shall be considered in compliance with the note for guidance even if they are incorporated in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products).

(3) OJ L 311, 28.11.2001, p. 67.
(4) OJ L 311, 28.11.2001, p. 1.
(5) OJ L 147, 31.5.2001, p. 1.
(6) OJ L 273, 10.10.2002, p. 1. Regulation (EC) 1774/2002 has been repealed by Regulation (EC) 1069/2009 that will apply from 4 March 2011 (OJ L 300, 14.11.2009, p. 1).
(7) Regulatory guidance and position papers have been issued by the Committee for Medicinal Products for Human Use and its Biologics Working Party on human tissue derived medicinal products in relation to CJD and vCJD. Such guidance can be found on http://www.ema.europa.eu
(8) Pigs and birds, which are animal species of particular interest for the production of medicinal products, are not naturally susceptible to infection via the oral route. Therefore they are not TSE-relevant animal species within the meaning of this chapter. Also dogs, rabbits and fish are non TSE-relevant animal species within the meaning of this chapter.
(9) See also: Position paper on the assessment of the risk of transmission of animal spongiform encephalopathy agents by master seed materials used in the production of veterinary vaccines (EMEA/CVMP/019/01-February 2001 adopted by the Committee for Medicinal Products for Veterinary Use (CVMP) in July 2001, (OJ C 286, 12.10.2001, p. 12)).
(10) OJ L 136, 30.4.2004, p. 1.

Master cell banks and master seeds established before 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products), but not yet approved as a constituent of an authorised medicinal product shall demonstrate that they fulfil the requirements of the note for guidance. If, for some raw or starting materials or reagents used for the establishment of these cell banks or seeds, full documentary evidence is no longer available, the applicant should present a risk assessment as described in Section 4 of the note for guidance.

Established working seeds or cell banks used in the manufacture of medicinal products authorised before 1 July 2000 (human medicines) or 1 October 2000 (veterinary medicines), which have been subjected to a properly conducted risk assessment by a Competent Authority of the Member States or the European Medicines Agency and declared to be acceptable, shall also be considered compliant.

However, where materials derived from the "TSE-relevant animal species" are used in fermentation/routine production processes or in the establishment of working seeds and working cell banks, the applicant must demonstrate that they fulfil the requirements of the note for guidance.



## 3. GENERAL CONSIDERATIONS

### 3-1. *SCIENTIFIC PRINCIPLES FOR MINIMISING RISK*

When manufacturers have a choice, the use of materials from "non TSE-relevant animal species" or non-animal origin is preferred. The rationale for using materials derived from "TSE-relevant animal species" instead of materials from "non-TSE-relevant species" or of non-animal origin should be given. If materials from "TSE-relevant animal species" have to be used, consideration should be given to all the necessary measures to minimise the risk of transmission of TSE.

Readily applicable diagnostic tests for TSE infectivity *in vivo* are not yet available. Diagnosis is based on post-mortem confirmation of characteristic brain lesions by histopathology and/or detection of $PrP^{TSE}$ by Western blot or immunoassay. The demonstration of infectivity by the inoculation of suspect tissue into target species or laboratory animals is also used for confirmation. However, due to the long incubation periods of all TSEs, results of *in vivo* tests are available only after months or years.

Several immunochemical tests have been developed for the detection of $PrP^{TSE}$ in post-mortem samples and some are now considered to be extremely sensitive. However, their ability to detect an infected animal depends on the timing of sample collection in relation to timing of exposure, the type of tissue collected and infectious dose acquired, together with consequential timing of onset of clinical disease. There is currently insufficient information on how this might be affected by strain variations.

Although screening of source animals by *in vitro* tests may prevent the use of animals at late stages of incubation of the disease and may provide information about the epidemiological status of a given country or region, none of the tests are considered suitable to unambiguously confirm the negative status of an animal.

Minimising the risks of transmission of TSE is based upon three complementary parameters:

- the source animals and their geographical origin,
- nature of animal material used in manufacture and any procedures in place to avoid cross-contamination with higher risk materials,
- production process(es) including the quality assurance system in place to ensure product consistency and traceability.

### 3-2. *ANIMAL SOURCE*

The source materials used for the production of materials for the manufacture of medicinal products shall be derived from animals fit for human consumption following ante- and post-mortem inspection in accordance with EU or equivalent (third country) conditions, except for materials derived from live animals, which should be found healthy after clinical examination.

#### 3-2-1. **Geographical sourcing**

##### 3-2-1-1. *Bovine materials*

The World Organisation for Animal Health (OIE)[(11)] lays down the criteria for the assessment of the status of countries in the chapter of the International Animal Health Code on bovine spongiform encephalopathy. Countries or regions are classified as follows:

A. countries or regions with a negligible BSE risk;

B. countries or regions with a controlled BSE risk;

C. countries or regions with an undetermined BSE risk.

As stipulated in Commission Regulation (EC) No 999/2001, as amended[(12)], the classification of countries or regions thereof according to their BSE risk, based on the rules laid down by OIE, is legally binding in the EU since 1 July 2007. Commission Decision 2007/453/EC[(13)] as amended, provides the classification of countries or regions according to their BSE risk.

Previously, the European Commission Scientific Steering Committee (SSC)[(14)] had established a temporary system for classifying the countries according to their geographical BSE risk (GBR)[(15)].

For the purposes of this chapter the BSE classification based on the OIE rules should be used. If a country, which was previously classified in accordance to the SSC GBR criteria, has not been classified yet according to the OIE rules, the GBR classification can be used until OIE classification has taken place, provided that there is no evidence of significant change in its BSE risk[(16)].

Where there is a choice, animals should be sourced from countries with the lowest possible BSE risk (negligible BSE risk countries (Category A)) unless the use of material from countries with a higher BSE risk is justified. Some of the materials identified in Section 6, "Specific Conditions" can be sourced from countries with controlled BSE risk (Category B)

(11) http://www.oie.int/eng/Status/BSE/en_BSE_free.htm
(12) Regulation (EC) No 722/2007 (OJ L 164, 26.6.2007, p. 7)
(13) OJ L 172, 30.6.2007, p. 84
(14) The Scientific Steering Committee established by Commission Decision 97/404/EC (OJ L 169, 27.6.1997, p. 85) shall assist the Commission to obtain the best scientific advice available on matters relating to consumer health. Since May 2003, its tasks have been taken over by the European Food Safety Authority (EFSA): http://www.efsa.europa.eu
(15) The European Scientific Steering Committee classification for geographical BSE risk (GBR) gives an indication of the level of likelihood of the presence of one or more cattle clinically or pre-clinically infected with BSE in a given country or region. A definition of the four categories is provided in the following Table.

| GBR level | Presence of one or more cattle clinically or pre-clinically infected with BSE in a geographical region/country |
|---|---|
| I | Highly unlikely |
| II | Unlikely but not excluded |
| III | Likely but not confirmed or confirmed at a lower level |
| IV | Confirmed at a higher level (≥ 100 cases/1 Million adult cattle per year) |

Reports of the GBR assessment of the countries are available on the SSC website (http://ec.europa.eu/food/fs/sc/ssc/outcome_en.html)
(16) Experts consider that the GBR classification system is stable enough, so that it can continue to be used, during the interim period, for the demonstration of compliance with this chapter.

and, in some cases, from countries with undetermined BSE risk (Category C), provided that the controls and requirements as specified in the relevant sections below are applied. Apart from these exceptions, animals must not be sourced from countries with undetermined BSE risk (Category C), and justifications for the use of animals from countries with undetermined BSE risk (Category C) must always be provided.

*3-2-1-2. Sheep and goats (small ruminants)*

Naturally occurring clinical scrapie cases have been reported in a number of countries worldwide. As BSE in sheep and goats could possibly be mistaken for scrapie, as a precautionary measure, sourcing of materials derived from small ruminants shall take into account the prevalence of both BSE and scrapie in the country and the tissues from which the materials are derived.

The principles related to "BSE negligible risk (closed) bovine herds" (see section 3-2-2) could equally be applied in the context of small ruminants in order to develop a framework to define the TSE status of a flock of small ruminants. For sheep, because of the concern over the possibility of BSE in sheep, the use of a genotype(s) showing resistance to BSE/scrapie infection could be considered in establishing TSE free flocks[17]. However, the possibility that genotypes resistant to scrapie could be susceptible to BSE (experimental oral exposure) or atypical scrapie (natural cases) should also be taken into account. Goats have not been studied sufficiently with regard to a genotype specific sensitivity.

Material of small ruminant origin should preferably be sourced from countries with a long history of absence of scrapie. Justification shall be required if the material is sourced from some other origin.

3-2-2. **BSE negligible risk (closed) bovine herds**. The safest sourcing is from countries or regions with a negligible risk (Category A countries). Other countries may have or have had cases of BSE at some point in time and the practical concept of "Negligible risk (closed) bovine herds" has been developed by the SSC and endorsed by the CHMP and CVMP. Criteria for establishing and maintaining a "BSE negligible risk (closed) bovine herd" can be found in the SSC opinion of 22-23 July 1999[18].

For the time being it is not possible to quantify the reduction of the geographical BSE risk for cattle from BSE 'negligible risk (closed) bovine herds'. However, it is expected that this risk reduction is substantial. Therefore, sourcing from such closed bovine herds shall be considered in the risk assessment in conjunction with the OIE classification of the country.

3-3. *ANIMAL PARTS, BODY FLUIDS AND SECRETIONS AS STARTING MATERIAL*

In a TSE infected animal, different organs and secretions have different levels of infectivity. If materials from 'TSE-relevant animal species' have to be used, consideration should be given to use materials of the lowest category of risk. The tables given in the Annex of this chapter[19] summarise current data about the distribution of infectivity and $PrP^{TSE}$ in cattle with BSE, and in sheep and goats with scrapie[20].

The information in the tables is based exclusively upon observations of naturally occurring disease or primary experimental infection by the oral route (in cattle) but does not include data on models using strains of TSE that have been adapted to experimental animals, because passaged strain phenotypes can differ significantly and unpredictably from those of naturally occurring disease. Because immunohistochemical and/or Western blot detection of misfolded host protein ($PrP^{TSE}$) have proven to be a surrogate marker of infectivity, $PrP^{TSE}$ testing results have been presented in parallel with bioassay data. Tissues are grouped into three major infectivity categories, irrespective of the stage of disease:

| | |
|---|---|
| **Category IA** | **High-infectivity tissues**<br><br>central nervous system (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS |
| **Category IB** | **Lower-infectivity tissues**<br><br>peripheral tissues that have tested positive for infectivity and/or $PrP^{TSE}$ in at least one form of TSE |
| **Category IC** | **Tissues with no detectable infectivity**<br><br>tissues that have been examined for infectivity, without any infectivity detected, and/or $PrP^{TSE}$, with negative results |

Category IA tissues and substances derived from them shall not be used in the manufacture of medicinal products, unless justified (see Section 5).

Although the category of lower risk tissues (category IB tissues) almost certainly includes some (e.g. blood) with a lower risk than others (e.g. lymphoreticular tissues), the data about infectivity levels in these tissues are too limited to subdivide the category into different levels of risk. It is also evident that the placement of a given tissue in one or another category can be disease and species specific, and subject to revision as new data emerge.

For the risk assessment (see section 4), manufacturers and/or marketing authorisation holders/applicants shall take into account the tissue classification tables in the Annex to this chapter.

The categories in the tables are only indicative and it is important to note the following points.

- In certain situations there could be **cross-contamination** of tissues of different categories of infectivity. The potential risk will be influenced by the circumstances in which tissues were removed, especially by contact of tissues with lower-infectivity tissues or no detectable infectivity (categories IB and IC tissues) with high-infectivity tissues (category IA tissues). Thus, cross-contamination of some tissues may be increased if infected animals are slaughtered by brain stunning (penetrative or non penetrative) or if the brain and/or spinal cord is sawed. The risk of cross-contamination will be decreased if body fluids are collected with minimal damage to tissue and cellular components are removed, and if foetal blood is collected without contamination from other maternal or foetal tissues including placenta, amniotic and allantoic fluids. For certain tissues, it is very difficult or impossible to prevent cross-contamination with category IA tissues (e.g. skull). This has to be considered in the risk assessment.
- For certain classes of substances the **stunning/slaughtering techniques** used may be important in determining the potential risk[21] because of the likelihood of disseminating the brain particles into the peripheral organs, particularly to the lungs. Stunning/slaughtering techniques should be described as well as the procedures to remove high infectivity tissues. The procedures to collect the animal

(17) Opinion of the Scientific Panel on Biological Hazards on 'the breeding programme for TSE resistance in sheep': http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620775678.htm

(18) SSC Scientific Opinion on the conditions related to "BSE Negligible Risk (Closed) Bovine Herds" adopted at the meeting of 22-23 July 1999. http://ec.europa.eu/food/fs/sc/ssc/out56_en.html

(19) The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloddproducts/tablestissueinfectivity.pdf

(20) A Scientific opinion on BSE/TSE infectivity in small ruminant tissues is currently being reviewed by EFSA (Question No EFSA-Q-2010-052). For updated information please follow this link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2010-0041

(21) SSC opinion on stunning methods and BSE risk (The risk of dissemination of brain particles into the blood and carcass when applying certain stunning methods), adopted at the meeting of 10-11 January 2002. http://ec.europa.eu/food/fs/sc/ssc/out245_en.pdf. Report of the EFSA Working group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-122, adopted on 21 October 2004, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620777397.htm

tissues/organs to be used and the measures in place to avoid cross-contamination with a higher risk material must also be described in detail.

- The risk of contamination of tissues and organs with BSE-infectivity potentially harboured in central nervous material as a consequence of the stunning method used for cattle slaughtering depends on the following factors:
  - the amount of BSE-infectivity in the brain of the slaughtered animal,
  - the extent of brain damage,
  - the dissemination of brain particles in the animal body.

  These factors must be considered in conjunction with the OIE/GBR classification of the source animals, the age of the animals in the case of cattle and the *post-mortem* testing of the cattle using a validated method.

  The underlying principles indicated above would be equally applicable to sheep and goats.

The risk posed by cross-contamination will be dependent on several complementary factors including:

- measures adopted to avoid contamination during collection of tissues (see above),
- level of contamination (amount of the contaminating tissue),
- amount and type of materials collected at the same time.

Manufacturers or the marketing authorisation holders/applicants should take into account the risk with respect to cross-contamination.

3-4. *AGE OF ANIMALS*

As the TSE infectivity accumulates in bovine animals over an incubation period of several years, it is prudent to source from young animals.

Presence of infectious material has essentially been reported in the central nervous system and related tissues, as well as in the lymphoreticular system, depending on the TSE agent (BSE in cattle or scrapie in sheep and goat). The exact time course of infectivity in the respective body parts and tissues, from the date of infection, is not known in both species and, as such, it is difficult to give clear guidance on the age above which the various tissues may be infected and should not be collected. The initial recommendation to collect tissues in the youngest age is still valid. In addition, it is noteworthy that the age criteria depend also on the geographical origin. Age is a more important parameter for materials from countries where the risk is higher (Category B and C countries), than from countries with a negligible BSE risk (Category A countries).

3-5. *MANUFACTURING PROCESS*

The assessment of the overall TSE risk reduction of a medicinal product shall take into account the control measures instituted with respect to:

- sourcing of the raw/starting materials, and
- the manufacturing process.

Controlled sourcing is a very important criterion in achieving acceptable safety of the product, due to the documented resistance of TSE agents to most inactivation procedures.

A quality assurance system, such as ISO 9000 certification, HACCP[22] or GMP, must be put in place for monitoring the production process and for batch delineation (i.e. definition of batch, separation of batches, cleaning between batches). Procedures shall be put in place to ensure traceability as well as self-auditing and to auditing suppliers of raw/starting materials.

Certain production procedures may contribute considerably to the reduction of the risk of TSE contamination, e.g. procedures used in the manufacture of tallow derivatives (see section 6). As such rigorous processing cannot be applied to many products, processes involving physical removal, such as precipitation and filtration to remove prion-rich material, are likely to be more appropriate than chemical treatments. A description of the manufacturing process, including in-process controls applied, shall be presented and the steps that might contribute to reduction or elimination of TSE contamination should be discussed. Whenever different manufacturing sites are involved, the steps performed at each site shall be clearly identified. The measures in place in order to ensure traceability of every production batch to the source material should be described.

**Cleaning process**. Cleaning of process equipment may be difficult to validate for the elimination of TSE agents. It is reported that after exposure to high titre preparations of TSE agent, detectable infectivity can remain bound to the surface of stainless steel. The removal of all adsorbed protein by the use of 1 M sodium hydroxide or chlorine releasing disinfectants (e.g. 20 000 ppm chlorine for 1 h) have been considered acceptable approaches where equipment that cannot be replaced has been exposed to potentially contaminated material. Milder treatments with limited concentrations of alkali or stabilized bleach, when properly formulated with detergents and used at specified temperatures, have been shown to exhibit similar efficiency for removing prions as did classical NaOH or chlorine treatments. A system based on vaporised hydrogen peroxide also appeared to be efficient for inactivating TSE agents. These new treatments are more compatible with delicate materials and may be suitable for practical use[23].

If risk materials are used in the manufacture of a product, cleaning procedures, including control measures, shall be put in place in order to minimise the risk of cross-contamination between production batches. This is especially important if materials from different risk categories are handled in the same plant with the same equipment. In the case of using category IA materials in the manufacture of a product, dedicated equipment shall be used, unless otherwise justified.

Further research is needed to develop and validate new decontamination procedures to lower the risk of cross-contamination for material and devices which are not compatible with WHO-recommended procedures.

**Removal/Inactivation validation**. Validation studies of removal/inactivation procedures for TSEs can be difficult to interpret. It is necessary to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent (*in vitro* or *in vivo* assay). Further research is needed to develop an understanding of the most appropriate "spike preparation" for validation studies. Therefore, validation studies are currently not generally required. However, if claims are made for the safety of the product with respect to TSEs based on the ability of manufacturing processes to remove or inactivate TSE agents, they must be substantiated by appropriate investigational studies[24].

In addition to appropriate sourcing, manufacturers are encouraged to continue their investigations into removal and inactivation methods to identify steps/processes that would have benefit in assuring the removal or inactivation of TSE agents. In any event, a production process wherever possible shall be designed taking account of available information on methods which are thought to inactivate or remove TSE agents.

(22) Hazard Analysis Critical Control Point.
(23) WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006) http://www.who.int/bloodproducts/tse/WHO%20TSE%20Guidelines%20FINAL-22%20JuneupdatedNL.pdf
(24) Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03

For certain types of products (see section 6-3 Bovine blood and blood derivatives), where validated removal/inactivation is not readily applicable, process evaluation might be required. This should be based on the starting material and any published data on TSE risk.

4. RISK ASSESSMENT OF MATERIALS OR SUBSTANCES USED IN THE MANUFACTURE AND PREPARATION OF A MEDICINAL PRODUCT IN THE CONTEXT OF REGULATORY COMPLIANCE

The assessment of the risk associated with TSE needs careful consideration of all of the parameters as outlined in section 3-1 (Scientific Principles for Minimising Risk).

As indicated in the introduction to this chapter, regulatory compliance is based on a favourable outcome from a risk assessment. The risk assessments, conducted by the manufacturers and/or the marketing authorisation holders or applicants for the different materials or substances from "TSE-relevant animal species" used in the manufacture of a medicinal product shall show that all TSE risk factors have been taken into account and, where possible, risk has been minimised by application of the principles described in this chapter. TSE Certificates of suitability issued by the EDQM may be used by the marketing authorisation holders or applicants as the basis of the risk assessments.

An overall risk assessment for the medicinal product, conducted by the marketing authorisation holders or applicants, shall take into account the risk assessments for all the different materials from "TSE-relevant animal species" and, where appropriate, TSE reduction or inactivation by the manufacturing steps of the active substance and/or finished product.

The final determination of regulatory compliance rests with the competent authority.

It is incumbent upon the manufacturers and/or the marketing authorisation holders or applicants for both human and veterinary medicinal products to select and justify the control measures for a given "TSE-relevant animal species" derivative, taking into account the latest scientific and technical progress.

5. BENEFIT/RISK EVALUATION

In addition to the parameters as mentioned in sections 3 (that may be covered by a TSE Certificate of Suitability issued by the EDQM) and 4, the acceptability of a particular medicinal product containing materials derived from a "TSE-relevant animal species", or which as a result of manufacture could contain these materials, shall take into account the following factors:

- route of administration of the medicinal product,
- quantity of animal material used in the medicinal product,
- maximum therapeutic dosage (daily dose and duration of treatment),
- intended use of the medicinal product and its clinical benefit,
- presence of a species barrier.

High-infectivity tissues (category IA tissues) and substances derived thereof shall not be used in manufacture of medicinal products, their starting materials and intermediate products (including active substances, excipients and reagents), unless justified. A justification why no other materials can be used shall be provided. In these exceptional and justified circumstances, the use of high-infectivity tissues could be envisaged for the manufacture of active substances, when, after performing the risk assessment as described in Section 4 of this chapter, and taking into account the intended clinical use, a positive benefit/risk assessment can be presented by the marketing authorisation applicant. Substances from category IA materials, if their use is justified, must be produced from animals of countries with negligible BSE risk (Category A).

6. SPECIFIC CONSIDERATIONS

The following materials prepared from "TSE-relevant animal species" are considered in compliance with this chapter provided that they meet at least the conditions specified below. The relevant information or a certificate of suitability granted by the EDQM shall be provided by the marketing authorisation applicant/holder.

6-1. *COLLAGEN*

Collagen is a fibrous protein component of mammalian connective tissue.

For collagen, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

- For collagen produced from bones, the conditions specified for gelatin are applicable (see below). Lower inactivation capacity is expected from the collagen manufacturing process than from that of gelatin. Therefore, sourcing becomes a more critical aspect to consider.
- Collagen produced from tissues such as hides, skins, tendons and sinews do not usually present a measurable TSE risk provided that contamination with potentially infected materials, for example spillage of blood and/or central nervous tissues, is avoided during procurement. Therefore, hides represent a safer raw material for human implants derived from collagen. However, cross-contamination with brain material released during the slaughtering process that may have dried on the surface of hides would be difficult to eliminate. This is another aspect to consider in the evaluation of safety of this source material.

The collagen manufacturing process can have some steps in common with the manufacture of gelatin such as alkaline and sodium sulphate treatment, calcium hydroxide and sodium hydroxide treatments or enzyme treatment. However, even these common steps can differ in duration and pH condition which can result in significant differences in their inactivation capacity. Manufacturers should at least conduct a process evaluation based on the similarities of the collagen processing steps, as compared to known inactivation steps in the manufacture of gelatin, in order to support the safety of the product. In addition to processing, differences also exist in the final use of the material and, consequently, in their risk assessment, while gelatin is widely used for oral administration, many collagen applications are in the form of surgical implants. This aspect should also be considered in the final risk assessment.

6-2. *GELATIN*

Gelatin is a natural, soluble protein, gelling or non-gelling, obtained by the partial hydrolysis of collagen produced from bones, hides and skins of animals.

For gelatin, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following[(25)].

**The source material used**

Gelatin used in medicinal products can be manufactured from bones or hides.

*Hides as the starting material.* On the basis of current knowledge, hides used for gelatin production represent a safer source material as compared to bones. However, it is

(25) Based on the Opinion of the Scientific Panel on Biological Hazards of the European Food Safety Authority on the 'Quantitative assessment of the human BSE risk posed by gelatine with respect to residual BSE risk'. The EFSA Journal, 312, (1-28). http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620776107.htm
The requirements for source material selection and manufacture are appropriate for oral or parenteral gelatin for use in human and veterinary medicinal products.

highly recommended that measures should be put in place to avoid cross-contamination with potentially infected materials during procurement.

*Bones as the starting material.* Where bones are used to manufacture gelatin, the quality of the starting materials needs to be controlled as an additional parameter to ensure the safety of the final product. Therefore, the following should be applied.

1. Skulls and spinal cord shall be removed from the collected bones (raw/starting material) independent of the age or the country of origin of the cattle.

2. Vertebrae shall be removed from the raw/starting materials from cattle over 30 months from countries with a controlled or an undetermined BSE risk (Categories B or C).

3. Gelatin for parenteral use should only be manufactured from bones coming from countries with a negligible or a controlled BSE risk (Category A and B, respectively). Gelatin for oral use can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Category A, B and C, respectively).

4. Gelatin shall be manufactured using one of the manufacturing methods described below.

**Manufacturing methods**

*Hides.* No specific measures with regard to the processing conditions are required for gelatin produced from hides provided that control measures are put in place to avoid cross-contamination both during the procurement of the hides and during the manufacturing process.

*Bones.* Where bones are used as the starting material, the mode of manufacture will be the second parameter that will ensure the safety of gelatin.

- Gelatin can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Categories A, B or C) sourced in accordance with the conditions described in section 6-2 under The source material used, using the acid, alkaline or heat/pressure manufacturing process.
- The manufacturing process shall be taken into consideration when performing the risk assessment as described in Section 4 of this chapter. Both the acid and the alkaline manufacturing methods have shown similar overall inactivation/removal of TSE infectivity in the gelatin validation experiments. Studies have shown that an additional alkaline treatment (pH 13, 2 h) of the bones/ossein further increases the TSE inactivation/removal capacity of the manufacturing process. Other processing steps such as filtration, ion-exchange chromatography and UHT sterilisation also contributes to the safety of gelatin.
- For a typical alkaline manufacturing process, bones are finely crushed, degreased with hot water and demineralised with dilute hydrochloric acid (at a minimum of 4 per cent and pH < 1.5) over a period of at least 2 days to produce the ossein. This is followed by an alkaline treatment with saturated lime solution (pH at least 12.5) for a period of at least 20 days.
- Bovine bones may also be treated by an acid process. The liming step is then replaced by an acid pre-treatment where the ossein is treated at pH < 3.5 for a minimum of 10 hours.
- A "flash" heat treatment (sterilisation) step at 138 °C minimum for 4 s at least is applied to both acid and alkaline manufacturing process.
- In the heat/pressure process, the dried degreased crushed bones are autoclaved with saturated steam at a pressure greater than 3 bar and a minimum temperature of 133 °C, for at least 20 min, followed by extraction of the protein with hot water.

The finishing steps are similar for the alkaline, acid and heat/pressure process and include extraction of the gelatine, washing, filtration and concentration.

6-3. *BOVINE BLOOD AND BLOOD DERIVATIVES*

Foetal bovine serum is commonly used in cell cultures. Foetal bovine serum should be obtained from foetuses harvested in abattoirs from healthy dams fit for human consumption and the womb should be completely removed and the foetal blood harvested in dedicated space or area by cardiac puncture into a closed collection system using aseptic technique.

Newborn calf serum is obtained from calves under 20 days old and calf serum from animals under the age of 12 months. In the case of donor bovine serum, given that it may be derived from animals less than 36 months old, the TSE negative status of the donor herd shall be well defined and documented. In all cases, serum shall be collected according to specified protocols by personnel trained in these procedures to avoid cross-contamination with higher risk tissues.

For bovine blood and blood derivatives, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

**Traceability**

Traceability to the slaughterhouse must be assured for each batch of serum or plasma. Slaughterhouses must have available lists of farms from which the animals are originated. If serum is produced from living animals, records must be available for each serum batch which assures the traceability to the farms.

**Geographical origin**

Whilst tissue infectivity of BSE in cattle is more restricted than scrapie, as a precautionary measure bovine blood should be sourced from Category A countries. Bovine blood from Category B countries is also acceptable provided that there is no risk for cross-contamination of blood with brain material from the slaughter of animals over 21 months[(26)] of age.

**Stunning methods**

If it is sampled from slaughtered animals, the method of slaughter is of importance to assure the safety of the material. It has been demonstrated that stunning by captive bolt stunner with or without pithing as well as by pneumatic stunner, especially if it injects air, can destroy the brain and disseminate brain material into the blood stream. Non-penetrative stunning is no more considered as an alternative to penetrative stunning because contamination of blood with brain material has been demonstrated[(27)]. Negligible risk can be expected from electro-narcosis[(28)], but this even does not provide strict safety because, when unsuccessful, animals may have to be additionally stunned. The stunning methods must therefore be described for the bovine blood collection process.

Whenever a risk of cross-contamination of blood with brain cannot be avoided at routine slaughtering in countries with a controlled BSE risk (Category B), safety measures such as restriction of the age of the cattle and/or reduction of infectious agents during manufacture have to be applied.

**Age**

For countries with a controlled BSE risk (Category B), a precautionary age limit of 21 months shall apply for bovine blood or blood derivatives where no significant reduction of

(26) Opinion of the Scientific Panel on Biological Hazards on the assessment of the age limit in cattle for the removal of certain Specified Risk Materials (SRM). Question No EFSA-Q-2004-146, adopted on 28 April 2005

(27) The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloodproducts/tablestissueinfectivity.pdf

(28) Report of the EFSA Working Group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-112, adopted on 21 October 2004, http://www.efsa.europa.eu/en/sciencebiohaz/biohaz_opinions/opinion_annexes/733.html

TSE agents can be assumed from manufacture. An age limit of 30 months is considered sufficient for blood derivatives where significant reduction of TSE agents can be demonstrated as described below.

**Reduction of TSE agents during manufacture**

For blood derivatives, the capacity of the manufacturing process to reduce/eliminate TSE agents should be estimated from investigational studies. The estimation may be based on published data or in house data whenever it can be shown that such data is relevant to the specific manufacturing process. If it cannot be concluded that the reduction capacity is comparable, it is recommended that manufacturers undertake product-specific investigational studies. Investigations using biochemical assay may be sufficient if there is scientific evidence that this assay correlates with infectivity data. General guidance for investigational studies on reduction of TSE agents has been outlined[(29)]. Brain-derived spike preparations are appropriate for studies investigating the risk from brain-contaminated blood.

6-4. *TALLOW DERIVATIVES*

Tallow is fat obtained from tissues including subcutaneous, abdominal and inter-muscular areas and bones. Tallow used as the starting material for the manufacture of tallow derivatives shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Tallow derivatives, such as glycerol and fatty acids, manufactured from tallow by rigorous processes are thought unlikely to be infectious and they have been the subject of specific consideration by CHMP and CVMP. For this reason, such materials manufactured under the conditions at least as rigorous as those given below shall be considered in compliance for this chapter, irrespective of the geographical origin and the nature of the tissues from which tallow derivatives are derived. Examples of rigorous processes are:

- trans-esterification or hydrolysis at not less than 200 °C for not less than 20 min under pressure (glycerol, fatty acids and fatty acid esters production),
- saponification with 12 M NaOH (glycerol and soap production):
  - batch process: at not less than 95 °C for not less than 3 h,
  - continuous process: at not less than 140 °C, under pressure for not less than 8 min, or equivalent,
- distillation at 200 °C.

Tallow derivatives manufactured according to these conditions are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

Tallow derivatives produced using other conditions must demonstrate compliance with this chapter.

6-5. *ANIMAL CHARCOAL*

Animal charcoal is prepared by carbonisation of animal tissues, such as bones, using temperatures higher than 800 °C. Unless otherwise justified, the starting material for the manufacture of animal charcoal shall be Category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. Irrespective of the geographical origin and the nature of the tissue, for the purpose of regulatory compliance, animal charcoal shall be considered in compliance with this chapter.

Charcoal manufactured according to these conditions is unlikely to present any TSE risk and shall therefore be considered compliant with this chapter. Charcoal produced using other conditions must demonstrate compliance with this chapter.

6-6. *MILK AND MILK DERIVATIVES*

In the light of the current scientific knowledge and irrespective of the geographical origin, bovine milk is unlikely to present any risk of TSE contamination[(30)].

Certain materials, including lactose, are extracted from whey, the spent liquid from cheese production following coagulation. Coagulation can involve the use of calf rennet, an extract from abomasum, or rennet derived from other ruminants. The CHMP/CVMP have performed a risk assessment for lactose and other whey derivatives produced using calf rennet and concluded that the TSE risk is negligible if the calf rennet is produced in accordance with the process described in the risk assessment report[(31)]. The conclusion was endorsed by the SSC[(32)] which has also performed an assessment of the TSE risk of rennet in general[(33)].

Table 5.2.8.-1. – *Concept for acceptance of bovine blood/sera and derivatives*

| Product | Foetal bovine serum | Donor calf serum | Adult bovine donor serum | Calf serum | Adult bovine serum / plasma | Adult bovine serum / plasma / serum derivative | Adult bovine serum derivative | Adult bovine serum derivative |
|---|---|---|---|---|---|---|---|---|
| Geographical origin of cattle | Cat. A and B | Cat. A and B | Cat. A and B[1] | Cat. A and B | Cat. A | Cat. B | Cat. A | Cat. B |
| Age of cattle | unborn | < 1 year | < 36 months | < 1 year | No limit | < 21 months[2] | No limit | < 30 months |
| Slaughtering/cross-contamination of blood with CNS material | No risk of cross-contamination | | | Risk of cross-contamination | | | | |
| Demonstration of Prion reduction during manufacture | No | | | No | | | | Yes[3] |

1. When sourced in Category B countries, cattle should be from well-defined and documented herds.

2. A higher age may be allowed if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).

3. Demonstration of prion reduction may not be required if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).

(29) Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03.
(30) For milk and milk derivatives from small ruminants, please see EFSA opinion on Question No EFSA-Q-2008-310, adopted on 22 October 2008, http://www.efsa.europa.eu/en/scdocs/scdoc/849.htm
(31) Committee for Medicinal Products for Human Use and its Biologics Working Party conducted a risk and regulatory assessment of lactose prepared using calf rennet. The risk assessment included the source of the animals, the excision of the abomasums and the availability of well-defined quality assurance procedures. The quality of any milk replacers used as feed for the animals from which abomasums are obtained is particularly important. The report can be found on http://www.ema.europa.eu/pdfs/human/press/pus/057102.pdf
(32) Provisional statement on the safety of calf-derived rennet for the manufacture of lactose, adopted by the SSC at its meeting of 4-5 April 2002 (http://ec.europa.eu/food/fs/sc/ssc/out255_en.pdf)
(33) The SSC issued an opinion on the safety of animal rennet in regard to risks from animal TSE and BSE in particular, adopted at its meeting of 16 May 2002 (http://ec.europa.eu/food/fs/sc/ssc/out265_en.pdf)

Bovine milk derivatives manufactured according to the conditions described below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- The milk is sourced from healthy animals in the same conditions as milk collected for human consumption, and
- no other ruminant materials, with the exception of calf rennet, are used in the preparation of such derivatives (e.g. pancreatic enzyme digests of casein).

Milk derivatives produced using other processes or rennet derived from other ruminant species must demonstrate compliance with this chapter.

6-7. *WOOL DERIVATIVES*

Derivatives of wool and hair of ruminants, such as lanolin and wool alcohols derived from hair shall be considered in compliance with this chapter, provided the wool and hair are sourced from live animals.

Wool derivatives produced from wool which is sourced from slaughtered animals declared "fit for human consumption" and the manufacturing process in relation to pH, temperature and duration of treatment meets at least one of the stipulated processing conditions listed below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

- Treatment at pH ≥ 13 (initial; corresponding to a NaOH concentration of at least 0.1 M NaOH) at 60 °C for at least 1 h. This occurs normally during the reflux stage of the organic-alkaline treatment.
- Molecular distillation at ≥ 220 °C under reduced pressure.

Wool derivatives produced using other conditions must demonstrate compliance with this chapter.

6-8. *AMINO ACIDS*

Amino acids can be obtained by hydrolysis of materials from various sources.

Unless otherwise justified, the starting material for the manufacture of amino acids shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Amino acids prepared using the following processing conditions are unlikely to present any TSE risk and shall be considered compliant with this chapter:

- amino acids produced from hides and skins by a process which involves exposure of the material to a pH of 1 to 2, followed by a pH of > 11, followed by heat treatment at 140 °C for 30 min at 3 bar,
- the resulting amino acids or peptides must be filtered after production, and
- analysis is performed using a validated and sensitive method to control any residual intact macromolecules, with an appropriate limit set.

Amino acids prepared using other conditions must demonstrate compliance with this chapter.

*6-9 PEPTONES*

Peptones are partial hydrolysates of protein, achieved by enzymic or acid digestion. They are used in microbiological culture media to support the nutritional requirements of micro-organisms, which might be used as seed stocks or in industrial scale fermentations for the production of human and veterinary medicinal products, including vaccines. There is considerable interest in the use of vegetable protein as an alternative to animal sourced protein. However:

- where gelatin is used as the protein source material, reference is made to section 6-2 Gelatin, of this chapter,
- where casein is used as the protein source material, reference is made to section 6-6 Milk and milk derivatives, of this chapter,
- where tissue of TSE-relevant animal species is the protein source material, the tissue must be sourced from animals fit for consumption (see section 3-2 Source animals, of this chapter) with a maximum age of 30 months old for cattle from countries with a controlled BSE risk (Category B). The age of animals is of minimal concern for animals from countries with a negligible BSE risk (Category A).

## Annex: major categories of infectivity

The tables below are taken from the *WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010)*.

Data entries are shown as follows:

| | | |
|---|---|---|
| + | = | Presence of infectivity or $PrP^{TSE}$ |
| − | = | Absence of detectable infectivity or $PrP^{TSE}$ |
| NT | = | Not tested |
| NA | = | Not applicable |
| ? | = | Uncertain interpretation |
| () | = | Limited or preliminary data |
| [] | = | Infectivity or $PrP^{TSE}$ data based exclusively on bioassays in transgenic (Tg) mice over-expressing the PrP-encoding gene or $PRP^{TSE}$ amplification methods |

Category IA: High-infectivity tissues

| Tissue | Cattle BSE | | Sheep and goats Scrapie | | Elk and deer CWD | |
|---|---|---|---|---|---|---|
| | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ |
| Brain | + | + | + | + | + | + |
| Spinal cord | + | + | + | + | NT | + |
| Retina | + | NT | NT | + | NT | + |
| Optic nerve[2] | + | NT | NT | + | NT | + |
| Spinal ganglia | + | + | + | + | NT | + |
| Trigeminal ganglia | + | + | NT | + | NT | - |
| Pituitary gland[3] | − | NT | + | + | NT | + |
| Dura mater[3] | NT | NT | NT | NT | NT | NT |

Category IB: Lower-infectivity tissues

| Tissue | Cattle BSE | | Sheep and goats Scrapie | | Elk and deer CWD | |
|---|---|---|---|---|---|---|
| | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ |
| **Peripheral nervous system** | | | | | | |
| Peripheral nerves | [+] | + | + | + | NT | + |
| Autonomic ganglia[4] | NT | + | NT | + | NT | + |
| **Lymphoreticular tissues** | | | | | | |
| Spleen | − | − | + | + | NT | + |
| Lymph nodes | − | − | + | + | NT | + |
| Tonsil | + | − | + | + | NT | + |
| Nictitating membrane | + | − | [+] | + | NT | + |
| Thymus | − | NT | + | + | NT | − |
| **Alimentary tract[5]** | | | | | | |
| Oesophagus | − | NT | [+] | + | NT | + |
| Fore-stomach[6] (ruminants only) | − | NT | [+] | + | NT | + |
| Stomach/abomasum | − | NT | [+] | + | NT | + |
| Duodenum | − | − | [+] | + | NT | + |
| Jejunum[7] | − | + | [+] | + | NT | NT |
| Ileum[7] | + | + | + | + | NT | + |
| Appendix | NA | NA | NA | NA | NA | NA |
| Colon/caecum[7] | − | − | + | + | NT | + |
| Rectum | NT | NT | NT | + | NT | + |
| **Reproductive tissues** | | | | | | |
| Placenta[8] | − | NT | + | + | NT | − |
| Ovary[3] | − | NT | − | − | NT | − |
| Uterus[3] | − | NT | − | − | NT | − |
| **Other tissues** | | | | | | |
| Mammary gland/udder[9] | − | NT | − | + | NT | NT |
| Skin[3, 10] | − | NT | − | + | [+] | [+] |
| Adipose tissue | − | NT | NT | NT | [+] | NT |
| Heart/pericardium | − | NT | − | NT | NT | + |
| Lung | − | NT | − | − | NT | + |
| Liver[3] | − | NT | + | − | NT | − |
| Kidney[3, 11] | − | − | [+] | + | NT | + |
| Adrenal | [+] | + | + | − | NT | + |
| Pancreas[3] | − | NT | + | NT | NT | + |
| Bone marrow[12] | [+] | NT | + | NT | NT | − |
| Skeletal muscle[13] | [+] | NT | [+] | + | [+] | − |
| Tongue[14] | − | NT | [+] | + | NT | − |
| Blood vessels | − | NT | NT | + | NT | − |
| Nasal mucosa[15] | − | NT | + | + | NT | + |
| Salivary gland | − | NT | + | NT | − | − |
| Cornea[16] | NT | NT | NT | NT | NT | NT |
| **Body fluids, secretion and excretions** | | | | | | |
| CSF | − | NT | + | − | NT | NT |
| Blood[17] | − | ? | + | ? | + | ? |
| Saliva | NT | NT | − | NT | + | [−] |

| Tissue | Cattle BSE | | Sheep and goats Scrapie | | Elk and deer CWD | |
|---|---|---|---|---|---|---|
| | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ |
| Milk[18] | − | − | + | [+] | NT | NT |
| Urine[19] | − | NT | − | − | −[+] | [+] |
| Feces[19] | − | NT | − | NT | −[+] | NT |

Category IC: Tissues with no detectable infectivity

| Tissue | Cattle BSE | | Sheep and goats Scrapie | | Elk and deer CWD | |
|---|---|---|---|---|---|---|
| | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ | Infectivity[1] | $PrP^{TSE}$ |
| **Reproductive tissues** | | | | | | |
| Testis | − | NT | − | − | NT | − |
| Prostate/Epididymis/Seminal vesicle | − | NT | − | − | NT | − |
| Semen | − | NT | − | − | NT | NT |
| Placenta fluids | − | NT | NT | NT | NT | NT |
| Foetus[20] | − | NT | − | − | NT | (–) |
| Embryos[20] | − | NT | ? | NT | NT | NT |
| **Musculo-skeletal tissues** | | | | | | |
| Bone | − | NT | NT | NT | NT | NT |
| Tendon | − | NT | NT | NT | NT | NT |
| **Other tissues** | | | | | | |
| Gingival tissues | NT | NT | NT | NT | NT | NT |
| Dental pulp | NT | NT | NT | NT | NT | NT |
| Trachea | − | NT | NT | NT | NT | − |
| Thyroid gland | NT | NT | − | NT | NT | − |
| **Body fluids, secretions and excretions** | | | | | | |
| Colostrum[21] | (–) | − | (?) | NT | NT | NT |
| Cord blood[21] | − | NT | NT | NT | NT | NT |
| Sweat | NT | NT | NT | NT | NT | NT |
| Tears | NT | NT | NT | NT | NT | NT |
| Nasal mucus | NT | NT | NT | NT | NT | NT |
| Bile | NT | NT | NT | NT | NT | NT |

1. Infectivity bioassays of human tissues have been conducted in either primates or mice (or both), bioassays of cattle tissues have been conducted in either cattle or mice (or both), and most bioassays of sheep and/or goat tissues have been conducted only in mice. In regard to sheep and goats not all results are consistent for both species, for example, two goats (but no sheep) have contracted BSE naturally [Eurosurveillance, 2005, Jeffrey et al., 2006]. Similarly, most of the results described for CWD were derived from studies in deer, and findings may not be identical in elk or other cervids.

2. In experimental models of TSE, the optic nerve has been shown to be a route of neuroinvasion, and contains high titres of infectivity.

3. No experimental data about infectivity in pituitary gland or dura mater in humans with all forms of human TSE have been reported, but cadaveric dura mater patches, and growth hormone derived from cadaveric pituitaries have transmitted disease to hundreds of people and therefore must be included in the category of high-risk tissues. $PrP^{TSE}$ was detected by immunoblot in the dura mater of a vCJD patient who died in the US after an unusually long incubation period (see also Table IB for other positive tissues: skin, kidney, liver, pancreas, ovary and uterus) [Notari et al., 2010]. It must be mentioned that earlier studies of numerous cases examined in the UK reported all of these tissues to be negative [Ironside et al., 2002, Head et al., 2004].

4. In cattle, $PrP^{TSE}$ is reported to be inconsistently present in the enteric plexus in the distal ileum, but immunohistochemical examination of tissues from a single 'fallen stock' case of BSE in Japan suggested (albeit equivocally) involvement of myenteric plexuses throughout the small and large intestine [Kimura and Haritani, 2008].

5. In vCJD, $PrP^{TSE}$ is limited to gut-associated lymphoid and nervous tissue (mucosa, muscle, and serosa are negative).

6. Ruminant fore stomachs (reticulum, rumen, and omasum) are widely consumed, as is the true stomach (abomasum). The abomasum of cattle (and sometimes sheep) is also a source of rennet.

7. When a large BSE oral dose was used to infect cattle experimentally, infectivity was detected in the jejunum and the ileo-caecum junction in Tg mice overexpressing PrP [courtesy of Dr M Groschup]. $PrP^{TSE}$ was detected at low incidence in lymphoid tissue of ileum [Terry et al., 2003] and has been detected at an even lower frequency in jejunal lymphoid tissue of cattle similarly infected by the oral route [EFSA, 2009].

8. A single report of transmission of sporadic CJD infectivity from human placenta has never been confirmed and is considered improbable.

9. $PrP^{TSE}$ has been detected in scrapie-infected sheep with chronic mastitis, but not from infected sheep without mastitis [Ligios et al., 2005].

10. Studies in hamsters orally infected with scrapie revealed that $PrP^{TSE}$ deposition in skin was primarily located within small nerve fibres. Also, apical skin 'velvet' from the antlers of CWD-infected deer is reported to contain $PrP^{TSE}$ and infectivity [Angers et al., 2009].

11. $PrP^{TSE}$ detected by immunocytochemistry in the renal pelvis of scrapie-infected sheep [Siso et al., 2006], and in lymphoid follicles within connective tissue adjacent to the renal pelvis in CWD-infected mule deer [Fox et al., 2006].

12. A single positive marrow in multiple transmission attempts from cattle orally dosed with BSE-infected brain [Wells et al., 1999, Wells et al., 2005, Sohn et al., 2009].

13. Muscle homogenates have not transmitted disease to primates from humans with sporadic CJD, or to cattle from cattle with BSE. However, intra-cerebral inoculation of a semitendinosus muscle homogenate (including nervous and lymphatic elements) from a single cow with clinical BSE has transmitted disease to transgenic mice that overexpress PrP at a rate indicative of trace levels of infectivity [Buschmann and Groschup, 2005]. Also, recent published and unpublished studies have reported the presence of $PrP^{TSE}$ in skeletal muscle in experimental rodent models of scrapie and vCJD [Beekes et al., 2005], in experimental and natural scrapie infections of sheep and goats [Andreoletti et al., 2004], in sheep orally dosed with BSE [Andreoletti, unpublished data], and in humans with sporadic, iatrogenic, and variant forms of CJD [Glatzel et al., 2003, Kovacs et al., 2004, Peden et al., 2006]. Bioassays of muscle in transgenic mice expressing cervid PrP have documented infectivity in CWD-infected mule deer [Angers et al., 2006], and experiments are underway to determine whether detectable $PrP^{TSE}$ in other forms of TSE is also associated with infectivity.

14. In cattle, bioassay of infectivity in the tongue was negative, but the presence of infectivity in palatine tonsil has raised concern about possible infectivity in lingual tonsillar tissue at the base of the tongue that may not be removed at slaughter [Wells et al., 2005, EFSA, 2008]. In sheep naturally infected with scrapie, 7 of 10 animals had detectable $PrP^{TSE}$ in the tongue [Casalone et al., 2005, Corona et al., 2006].

15. Limited chiefly to regions involved in olfactory sensory reception.

16. Because only one case of iatrogenic CJD has been certainly attributed to a corneal transplant among hundreds of thousands of recipients (one additional case is considered probable, and another case only possible), cornea has been categorized as a lower-risk tissue, other anterior chamber tissues (lens, aqueous humour, iris, conjunctiva) have been tested with a negative result both in vCJD and other human TSEs, and there is no epidemiological evidence that they have been associated with iatrogenic disease transmission.

17. A wealth of data from studies of blood infectivity in experimental rodent models of TSE have been extended by recent studies documenting infectivity in the blood of sheep with naturally occurring scrapie and in sheep transfused with blood from BSE-infected cattle [Houston et al., 2008], of deer with naturally occurring CWD [Mathiason et al., 2006], and (from epidemiological observations) in the red cell fraction (which includes significant amounts of both plasma and leukocytes) of four blood donors in the pre-clinical phase of vCJD infections [reviewed in Brown, 2006, Hewitt et al., 2006]. Plasma Factor VIII administration has also been potentially implicated in a subclinical case of vCJD in a haemophilia patient [Peden et al., 2010]. Blood has not been shown to transmit disease from humans with any form of 'classical' TSE [Dorsey et al., 2009], or from cattle with BSE (including fetal calf blood). A number of laboratories using new, highly sensitive methods to detect $PrP^{TSE}$ are reporting success in a variety of animal and human TSEs. However, several have experienced difficulty obtaining reproducible results in plasma, and it is not yet clear that positive results imply a potential for disease transmissibility, either because of false positives, or of 'true' positives that are due to sub-transmissible concentrations of $PrP^{TSE}$. Because of these considerations (and the fact that no data are yet available on blinded testing of specimens from naturally infected humans or animals) the expert group felt that it was still too early to evaluate the validity of these tests with sufficient confidence to permit either a negative or positive conclusion.

18. Evidence that infectivity is not present in milk from BSE-infected bovines includes temporo-spatial epidemiologic observations failing to detect maternal transmission to calves suckled for long periods, clinical observations of over a hundred calves suckled by infected cows that have not developed BSE, and experimental observations that milk from infected cows reared to an age exceeding the minimum incubation period has not transmitted disease when administered intra-cerebrally or orally to mice [Middleton and Barlow, 1993, Taylor et al., 1995]. Also, $PrP^{TSE}$ has not been detected in milk from cattle incubating BSE following experimental oral challenge [SEAC, 2005]. However, low levels (µg to ng/L) of normal PrP have been detected in milk from both animals and humans [Franscini et al., 2006]. $PrP^{TSE}$ has been detected in the mammary glands of scrapie-infected sheep with chronic mastitis [Ligios et al., 2005], and very recently it has been reported that milk (which in some cases also contained colostrum) from scrapie-infected sheep transmitted disease to healthy animals [Konold et al., 2008, Lacroux et al., 2008].

19. A mixed inoculum of urine and faeces from naturally infected CWD deer did not transmit disease during an 18-month observation period after inoculation of healthy deer with a heterozygous (96 G/S) PRNP genotype [Mathiason et al., 2006]. However, recent bioassays in Tg mice have transmitted disease from both urine [Haley et al., 2009] and faeces [Tamgüney et al., 2009]. In addition, mice with lymphocytic nephritis that were experimentally infected with scrapie shed both $PrP^{TSE}$ and infectivity in urine, when bioassayed in Tg mice [Seegeret al., 2005]. Very low levels of infectivity have also been detected in the urine (and histologically normal kidneys) of hamsters experimentally infected with scrapie [Gregori and Rohwer, 2007, Gonzalez-Romero et al., 2008]. Finally, in an experimental scrapie-hamster model, oral dosing resulted in infectious faeces when bioassayed in Tg mice over-expressing PrP [Safar et al., 2008].

20. Embryos from BSE-affected cattle have not transmitted disease to mice, but no infectivity measurements have been made on fetal calf tissues other than blood (negative mouse bioassay) [Fraser and Foster, 1994]. Calves born of dams that received embryos from BSE- affected cattle have survived for observations periods of up to seven years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy or $PrP^{TSE}$ [Wrathall et al., 2002].

21. Early reports of transmission of sporadic CJD infectivity from human cord blood and colostrum have never been confirmed and are considered improbable. A bioassay from a cow with BSE in transgenic mice over-expressing bovine PrP gave a negative result [Buschmann and Groschup, 2005], and $PrP^{TSE}$ has not been detected in colostrum from cattle incubating BSE following experimental oral challenge [SEAC, 2005].

**04/2013:50209**

## 5.2.9. EVALUATION OF SAFETY OF EACH BATCH OF IMMUNOSERA FOR VETERINARY USE

**Definition of abnormal reactions**. During development studies, the type and degree of reactions expected after administration of the immunoserum are defined in the light of safety testing. This definition of normal or abnormal local and systemic reactions is then used as part of the operation procedure for the batch safety test to evaluate acceptable and unacceptable reactions.

**Amount to be administered in the test**. In the tests, 'dose' means the quantity of the immunoserum to be recommended for use and containing the titre or potency within the limits specified for production batches. The amount to be administered in the test is usually defined in a number of doses.

**Route of administration**. The immunoserum is administered by a recommended route. In principle, preference should be given to the application route with the higher possibility to detect reactions.

**Target animal species and category of animals**. Use animals of the most sensitive species and of the minimum age recommended for administration of the immunoserum, unless otherwise justified and authorised.

**Animal numbers**. The number of animals to be used for the test is prescribed in the general monograph *Immunosera for veterinary use (0030)*.

**Identification of animals**. Unless otherwise justified and authorised, all animals are marked in a suitable way to ensure individual documentation of data for the whole observation period.

**Observation period**. Where objective criteria such as body temperature are to be recorded as described below, the animals are examined and observed for at least 3 days prior to administration of the immunoserum. After administration of the immunoserum, the animals are observed and examined at least once every day for a period of at least 14 days for signs of local and systemic reactions. On the day of administration of the immunoserum, at least 1 additional inspection is necessary after 4 h or at intervals as specified in the monograph. Where there is a 2nd administration of the immunoserum the period usually ends 14 days after the 2nd administration.

**Local and systemic reactions**. Animals showing severe abnormal local or systemic reactions are euthanised. All dead animals undergo a post-mortem with macroscopic examination. Additional microscopic and microbiological investigations may be indicated.

The animals are observed and examined for signs of local and systemic reactions. Where it is known to be a useful indicator, other criteria are recorded, such as body temperature, body mass, other performance measurements and food intake.

*Local reactions*. As far as appropriate and possible, the size and persistence of any local reaction (including incidence of painful reactions) and the proportion of animals showing local reactions are recorded.

*Systemic reactions*. Body temperature and, if appropriate, body mass are documented as general indicators of systemic effects of administration of the immunoserum. In addition, all clinical signs are recorded.

*Body temperature*. For mammals, the studies include measurement of body temperature during the observation period. The body temperatures are recorded beginning at least 3 days before administration of the immunoserum, at the time of administration, 4 h after and at suitable intervals. The body temperature before administration of the immunoserum has to be within the physiological range. At least for immunosera where a significant increase in body temperature may be expected or where an increase in body temperature is specified in an individual monograph, it is recommended to use the mean temperature of the days before administration of the immunoserum (e.g. day – 3 to day 0) as the baseline temperature to have clear guidance for evaluation of the test.

*Body mass and food intake*. Where it is known to be a reliable and useful indicator of safety, for example in young growing animals, the body mass is measured and documented shortly before administration of the immunoserum and during the observation period. The food intake is monitored and documented as an indicator of the effect of administering the immunoserum. In most cases, it will be sufficient to record the daily ration has been consumed or partly or wholly rejected but, in some cases it may be necessary to record the actual weight of food consumed, if this is a relevant indicator of the safety of the immunoserum.

*Clinical signs*. All expected and unexpected clinical signs of a general nature are recorded, including changes in health status and behaviour changes.

*Score sheets*. The score sheets are prepared for each immunoserum in the light of expected signs. All parameters and data are recorded in score sheets. The score sheets contain general parameters but are also adapted for each kind of immunoserum to list clinical signs that might be more evident for a given immunoserum.

**Criteria for repeating the test**. If an abnormal sign occurs, the responsible veterinarian determines, based on post-mortem examination if necessary, whether this was due to the immunoserum or not. If it is not clear what caused the abnormal sign or where an animal is withdrawn for reasons unrelated to the immunoserum, the test may be repeated. If in the 2nd test there is the same abnormal sign as in the 1st test, the immunoserum does not comply with the test. Any treatment administered to an animal during the observation period is recorded. If the treatment may interfere with the test, the test is invalid.

**01/2015:50211**

# 5.2.11. CARRIER PROTEINS FOR THE PRODUCTION OF CONJUGATED POLYSACCHARIDE VACCINES FOR HUMAN USE

*The use of alternative carrier proteins, production methods and tests are acceptable provided they have been authorised by the competent authority.*

Bacterial polysaccharides are not able to induce a T-cell-dependent B-cell immune response, which is needed to obtain an immunological memory response, and are generally poorly immunogenic in children under 2 years of age. The limitations are overcome by conjugating polysaccharides to carrier proteins. Carrier proteins are highly immunogenic and, when conjugated to bacterial polysaccharides, increase the capacity of polysaccharides to induce a protective response in infants.

Carrier proteins currently used in polysaccharide vaccines for human use are toxoids, non-toxic mutated toxins, surface or outer membrane proteins extracted from micro-organisms. Micro-organisms used for the production of the protein may be of genetically modified origin.

The production method used for a carrier protein shall have been shown to yield consistently batches suitable for conjugation of the carrier protein to a polysaccharide antigen.

Appropriate acceptance criteria for low bioburden before conjugation with the polysaccharide may be established. It is a prerequisite that the carrier protein is filtered through a bacteria-retentive filter prior to storage and that adequate measures are in place to avoid contamination and growth of micro-organisms during storage.

The production of carrier proteins is based on a seed-lot system. The seed lots are shown to be free from contamination using suitable methods of appropriate sensitivity. The culture may be inactivated and the carrier protein is purified by a suitable method.

The protein is characterised by one or more suitable method(s) (such as SDS-PAGE, isoelectric focusing, HPLC, size-exclusion chromatography with multiple-angle laser light scattering detection (MALLS), amino-acid analysis, amino-acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry) and its purity is verified by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that where applicable, the product is free from specific toxins. If purification steps are present, the reduction of selected process-related impurities and residuals is monitored to establish consistency of the purification process. In the case of recombinant carrier proteins, tests for at least the following impurities are also carried out:

- residual host-cell proteins, including proteins derived from the expression vector;
- residual cellular DNA.

Only a carrier protein that complies with the following tests may be used in the preparation of the conjugate.

**Identification**. The carrier protein is identified using a suitable method.

**pH** (*2.2.3*). Where applicable, the pH of the carrier protein prior to conjugation is monitored and is within the limits approved for the particular product.

**Protein content** (*2.5.16*). The content of the carrier protein is determined using a suitable method and is within the limits approved by the competent authority.

**Bacterial endotoxins**(*2.6.14*). The content is within the limits approved for the particular product.

In addition, the following requirements apply for the carrier proteins listed below.

**Diphtheria toxoid**. It is produced as described in the monograph *Diphtheria vaccine (adsorbed) (0443)* and complies with the requirements prescribed therein for bulk purified toxoid, except that the test for sterility (*2.6.1*) is not required.

**Tetanus toxoid**. It is produced as described in the monograph *Tetanus vaccine (adsorbed) (0452)* and complies with the requirements prescribed therein for bulk purified toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen and that the test for sterility (*2.6.1*) is not required.

**CRM 197 diphtheria protein**. It may be prepared from the growth of genetically modified (C7/β197) or non-genetically modified (mCRM) *Corynebacterium diphtheriae* or prepared by recombinant DNA technology in organisms such as *Escherichia coli*. The culture supernatant may be concentrated by ultrafiltration and purified by successive precipitation, filtration, and chromatography steps. When it is produced on the same premises as diphtheria toxin, CRM 197 diphtheria protein should be distinguished from the active toxin by a suitable method. The purity is not less than 90 per cent of diphtheria protein.

**OMP (*Neisseria meningitidis* group B outer membrane protein complex)**. *Neisseria meningitidis* group B outer membrane protein complex is extracted from bacterial cell cultures with a buffer containing a detergent. Cell debris is first removed. The membrane protein complex may be concentrated and purified by successive filtration and additional suitable purification steps. The lipopolysaccharide content does not exceed 8 per cent. The relative quantity of the major OMP is set and approved by the competent authority. The OMP complex complies with the test for pyrogens (*2.6.8*): inject into each rabbit 0.25 µg of OMP per kilogram of body mass.

**Recombinant protein D**. Protein D is a surface protein of nontypable *Haemophilus influenzae*. It is produced using a specific strain of *E. coli* carrying a plasmid containing the coding sequence for protein D. In order to express protein D, the modified strain is grown in a suitable liquid medium. At the end of cultivation, a purification step is carried out. The purity of the product is not less than 95 per cent of protein D.

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**corrected 10.0**

# 5.2.12. RAW MATERIALS OF BIOLOGICAL ORIGIN FOR THE PRODUCTION OF CELL-BASED AND GENE THERAPY MEDICINAL PRODUCTS

*This general chapter is published for information.*

*It contains sections on the quality requirements of raw materials used for the production of cell-based and gene therapy medicinal products for human use. The provisions of the chapter do not exclude the use of different production and control methods. It is the responsibility of the manufacturer of a raw material to qualify (prove to be suitable for the intended use) the raw material in accordance with the requirements given in this general chapter.*

*However, it is ultimately the responsibility of the user of a raw material to ensure it is of suitable quality for the specific use.*

*The quality of the raw materials may be considered according to the stage of development of the cell-based or gene therapy medicinal product, thereby acknowledging the inherent evolution of the quality profile of the product during its pharmaceutical and clinical development. Nevertheless, patient safety needs to be ensured in early phase clinical development. The aim is to have an appropriate qualification strategy for the raw materials when used for the production of cell-based/gene therapy medicinal products. It should be noted that changes in raw materials during the lifecycle of the cell-based/gene therapy medicinal product may affect the quality of the medicinal product and thus require additional studies to demonstrate comparability.*

*The impact of the raw material on the quality, safety and efficacy of the cell-based/gene therapy medicinal product is evaluated using a risk-based approach. Raw materials are used in order to consistently yield an active substance or medicinal product of a specified quality in terms of, for example, biological activity, purity/impurity profile, the risk of adventitious agents (bacteria, viruses, etc.) and stability.*

*From a risk perspective, the use of raw materials free from human or animal substances is preferred.*

*The biological nature of a raw material used for the production of cell-based/gene therapy medicinal products places special requirements on its quality. Examples of the critical quality attributes specific to each class of raw material are given in this general chapter.*

## 1. SCOPE

This general chapter applies to raw materials of biological origin used for the production of cell-based/gene therapy medicinal products. The raw materials used in the manufacture of active substances are not intended to form part of the active substance. The raw materials can be extracted from various biological sources or produced by recombinant DNA technology.

This general chapter applies to the following classes of raw materials:

- sera and serum replacements;
- proteins produced by recombinant DNA technology such as growth factors, cytokines, hormones, enzymes and monoclonal antibodies;
- proteins extracted from biological material such as enzymes and polyclonal antibodies;
- vectors.

The principles of this general chapter may also be applied to other classes of biological raw materials where appropriate.

Medical devices, plastics and chemically synthesised raw materials, such as basal media (purely composed of chemicals), synthetic peptides or synthetic polynucleotides, are not within the scope of this general chapter.

## 2. RISK ASSESSMENT

Evaluation of the impact of the raw material on the quality, safety and efficacy of cell-based/gene therapy medicinal products must be performed by the user of the raw material. No single measure or combination of measures can guarantee the quality, functionality and safety of a raw material for its intended use. Therefore, a risk assessment must consider the biological origin and traceability of the raw material, the production steps applied to it and the ability of the drug product manufacturing process to control or remove the raw material from the final medicinal product.

Any risk factor must be evaluated in relation to the clinical benefit/risk of the cell-based or gene therapy medicinal product. When evaluating the risk posed by the raw material to the final medicinal product, the exposure of a patient to residual amounts of raw material with potential harmful effects (e.g. adverse immune reactions) should be considered in relation to the clinical benefit/risk of the cell-based or gene therapy medicinal product.

## 3. GENERAL REQUIREMENTS

### 3-1. *ORIGIN*

The origin of the raw material and if relevant any biological substances used for the production of the raw material must be known. Special attention must be paid to risks related to the sourcing (including pooling) of the substances used for the production of the raw material. Depending on the source of the raw material and the substances used in its production, raw materials can be divided into 3 categories:

1) raw materials of human or animal origin;
2) raw materials produced using substances of human or animal origin;
3) raw materials free from substances of human or animal origin.

Traceability of all raw materials is required, with particular attention to those materials with an inherent safety concern i.e. those of human or animal origin.

Due to the inherent risk of transmitting adventitious agents, it is recommended to minimise, wherever possible, the use of raw materials of human or animal origin. If such raw materials are required for the production of cell-based/gene therapy medicinal products, appropriate measures are taken to minimise the risks of transmitting adventitious agents such as viruses, prions, bacteria and protozoa.

For human blood and tissue-derived materials, only carefully evaluated donors who have been adequately tested for infectious transmissible agents may be used. These materials comply with appropriate EU and/or national legislation applicable to transplantation and transfusion. Traceability measures enable each donation to be followed from the donation to the raw material and to the final product, and vice-versa.

When raw materials of animal origin are used, these animals fulfil specific health requirements and should be fit for human consumption and reared under controlled conditions, when applicable. If the origin of the animals is not fully traceable (e.g. animals collected from the wild), information on their geographic location at the time of sourcing should be considered.

When vectors or proteins produced by recombinant DNA technology are used as raw materials, traceability to the master cell bank/virus seed lot is required.

For all raw materials of human or animal origin, or raw materials produced using substances of human or animal origin, a viral risk assessment is performed according to the requirements of general chapter *5.1.7. Viral safety*. The extent of viral safety testing is dependent on the results of the initial risk assessment. In addition, a risk assessment with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risks as described in general chapter *5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products*.

### 3-2. *PRODUCTION*

All raw materials are produced within a suitable quality management system and production facilities.
Suitable in-process controls are in place to ensure that the production process is under control and consistently produces raw materials of defined quality.

Quality attributes for raw materials include identity, purity and biological activity where applicable, and they are to be demonstrated using appropriate, qualified control methods. Relevant specifications in terms of identity, purity/impurity profile and assays are to be established.

The production process is optimised to consistently minimise and/or remove adventitious agents and harmful impurities, whilst retaining the quality of the raw material. This can be achieved using one or a combination of the following measures:

- using validated inactivation/removal procedures such as gamma sterilisation or low pH during chromatography, where possible;
- demonstrating the ability of a production process to minimise, remove or inactivate adventitious agents or harmful impurities;
- testing for adventitious agents or harmful impurities.

A raw material is sterile and produced under aseptic conditions and/or subject to terminal sterilisation, unless otherwise justified. If the raw material is not sterile, the level of microbial contamination must be known.

Additives, such as stabilisers, may be added to the raw material. In cases where antibiotics and stabilisers of biological origin are used in the production of the raw material, their presence is justified and careful consideration is given to their selection, use, quality and concentration in the raw material, as well as their impact on the actual raw material itself.

### 3-3. *GENERAL QUALITY REQUIREMENTS*

Raw materials must meet pre-defined quality requirements for identity, purity and biological activity. In order to ensure the function of the raw material, it is subject to testing using appropriately qualified methods. The identity test must reflect the uniqueness of the raw material and distinguish it from other related or similar substances. Impurities include both process-related substances (e.g. in the case of recombinant proteins: host-cell-derived proteins (HCP), host-cell-derived DNA and vector-derived DNA (residual DNA), other biological or chemical substances) and product-related substances (e.g. aggregates and degradation products). The content of a raw material may be expressed either in absolute or relative terms. The assay for determination of biological activity may be used to establish the content.

#### 3-3-1. IDENTIFICATION

The identity tests are specific for the particular raw material and address the molecular structure/composition or other relevant physico-chemical, biological or immunochemical properties. Methods used in the determination of biological activity and purity may also serve to identify the raw material. Identification may be carried out by comparison with a defined reference material or a representative batch of the raw material.

3-3-2. **TESTS**

Tests that may be applicable to raw materials include the following (see also the sections below for specific raw materials):

**Appearance**. Liquid or reconstituted freeze-dried raw materials comply with the limits defined for the particular raw material with regard to degree of opalescence (*2.2.1*) and degree of coloration (*2.2.2*).

**Solubility**. Freeze-dried raw materials dissolve completely in the prescribed volume of reconstituting liquid within a specified time, at a specified temperature, as defined for the particular raw material.

**Osmolality** (*2.2.35*): within the limits defined for the particular raw material.

**pH** (*2.2.3*): within the limits defined for the particular raw material.

**Elemental impurities**: within the limits defined for the particular raw material.

**Total protein** (*2.5.33*): within the limits defined for the particular raw material.

**Related substances**. The content of product-related substances is within the limits defined for the particular raw material.

**Microbiological control**. Depending on the raw material concerned, it complies with the test for sterility (*2.6.1*) or the microbial contamination is determined (*2.6.12*).

**Viral contaminants**. Depending on the raw material concerned, relevant virus contamination is determined.

**Bacterial endotoxins** (*2.6.14*): less than the limit defined for the particular raw material.

**Mycoplasmas** (*2.6.7*). Raw materials are free from mycoplasmas.

**Stabiliser**. Where applicable, it complies with the limits defined for the particular raw material.

**Water** (*2.5.12*). Freeze-dried raw materials comply with the limits defined for the particular raw material.

3-3-3. **ASSAY**

**Content**. The content (e.g. protein content)/composition of the raw material is determined by an appropriate qualified method.

**Biological activity**. Where relevant, the biological activity is determined by a suitable assay. Where relevant (e.g. for enzymes), the biological activity is expressed per milligram of total protein (specific activity).

3-3-4. **REFERENCE MATERIAL OR REFERENCE BATCH**

An appropriate reference material or a representative batch of the raw material is used to perform the above-mentioned identification, tests and assay. Where available, the use of established reference standards, such as European Pharmacopoeia reference standards or WHO International Standards, is recommended.

3-4. *STORAGE*

The shelf life and storage conditions are defined.

3-5. *LABELLING*

The label states the expiry date, conditions for storage and use and any code that may be required for traceability including the biological origin of the raw material.

## 4. SERA AND SERUM REPLACEMENTS

4-1. *DEFINITION*

Sera from human or animal sources and serum replacements (including platelet lysates and other undefined growth additives, conditioned media, blood and other cellular components) are used as growth additives for cell culture. Sera and serum replacements used to promote cellular growth are typically complex biological mixtures, whose exact composition is not always possible to define. Due to this complex nature, special attention is given to verifying the consistency and performance of every batch.

**Bovine serum**. If bovine serum is used, it complies with the monograph *Bovine Serum (2262)*.

**Human serum and platelet lysates**. Human serum and platelet lysates used as raw materials for the production of cell-based/gene therapy medicinal products are human blood-derived materials, which can originate from the recipient (autologous) or from another individual (allogeneic).

**Conditioned media**. Conditioned media, isolated and purified from cultured cell supernatant, may also be used to enhance cell proliferation due to various growth factors and cytokines secreted by the cells into the medium.

**Other growth additives with undefined composition**. Cell and/or tissue lysates may be used as growth additives.

**Composite media**. Composite media contain growth additives such as bovine serum, growth factors etc. The principles described in this section of the general chapter apply to individual ingredients of biological origin and/or biologically active ingredients of the composite media.

4-2. *PRODUCTION*

Due to potential differences in quality between batches of serum, cell or tissue lysate, suitable measures are implemented to verify the consistency of each batch before using them as raw materials for the production of cell-based/gene therapy medicinal products.

Because of the inherent risk of transmitting infectious agents from pooled plasma, pooled sera, or other derivatives from pooled allogeneic human blood or plasma, consideration is given to limit the number of donations which are pooled, unless sufficient methods for inactivation/removal of viruses are applied during production, where applicable.

For conditioned media, a cell bank system is preferred. The removal of the cells from the media must be ensured and potential impurities originating from these cells determined if possible.

4-3. *IDENTIFICATION*

It is recognised that the exact qualitative composition of sera and serum replacements may be difficult to determine. However, the approximate protein composition in both cases may be determined by, for example, protein electrophoresis. Where relevant, tests for total protein content or any chemical additives are performed. For human serum, the electrophoretic pattern corresponds to that of an appropriate serum reference batch. Alternatively, identity may be determined by comparison of albumin content with an appropriate serum reference batch. For serum replacements, the electrophoretic pattern or the use of markers secreted by cells/platelets may be used. Human origin is determined by a suitable immunochemical method (*2.7.1*), unless otherwise justified.

4-4. *TESTS*

See section 3-3-2.

**Haemoglobin**: where relevant, within the limits defined for the particular raw material.

**Cell-derived impurities**: where relevant, within the limits defined for the particular raw material.

**Specific tests for viral contaminants**. For bovine serum, the tests for viral contaminants specified in the monograph *Bovine serum (2262)* apply. For human serum, the tests for viral safety specified in the monograph *Human plasma for fractionation (0853)* apply.

4-5. *ASSAY*

The serum or serum replacement must show cell growth promoting properties that are within the limits defined for the particular raw material. More than one type of assay may be necessary to show suitability for the intended use.

5. PROTEINS PRODUCED BY RECOMBINANT DNA TECHNOLOGY

5-1. *DEFINITION*

Proteins and peptides produced by recombinant DNA technology, which are used as raw materials, include growth factors, cytokines, hormones, enzymes and monoclonal antibodies.

*Growth factors, cytokines and hormones*. They are substances typically used for stimulation or inactivation, growth promotion or differentiation of cells in cell culture systems.

*Other proteins*. Enzymes (e.g. collagenases), as raw materials, may be used for extraction of active substances from tissues and/or fluids. Other proteins (e.g. fibronectin) may be used as culture supports or media components.

*Monoclonal antibodies*. Used as raw materials, they include immunoglobulins and fragments of an immunoglobulin with defined specificity. Antibodies can either be conjugated (chemically modified) or non-conjugated. Typical chemical modifications include fluorescent labelling and conjugation to magnetic beads. Antibodies, as raw materials, may be used for selection, activation/stimulation, isolation or purification of cells in cell culture.

5-2. *PRODUCTION*

Production of proteins using recombinant DNA technology is based on a well-characterised host-vector system, using a master cell bank and, if applicable, a working cell bank derived from the master cell bank. The expressed protein is extracted and purified using a variety of techniques, such as extraction, precipitation, centrifugation, concentration, filtration and/or chromatography.

During protein production using recombinant DNA technology, process-related impurities including residual host-cell or vector DNA and host-cell proteins must be reduced to acceptable levels. Particular attention must also be given to product-related impurities.

5-3. *IDENTIFICATION*

Identity is established by appropriate, qualified methods, such as electrophoresis (*2.2.31*), peptide mapping (*2.2.55*), isoelectric focusing (*2.2.54*) or liquid chromatography (*2.2.29*). For antibodies, identification is based on immunoglobulin class, isotype and/or specificity. In addition to the above-mentioned methods, immunochemical methods (*2.7.1*) and determination of activity are also considered suitable for identification.

5-4. *TESTS*

See section 3-3-2.

**Host-cell-derived proteins and residual host-cell or vector DNA**. Where relevant for the particular raw material, the content of residual host-cell or vector DNA and/or protein is determined using a suitable method unless the production process has been qualified to demonstrate suitable clearance. The content is within the limits defined for the particular raw material.

**Related proteins**. Related proteins (e.g. polyclonal antibodies with undefined specificities, glycoforms, degradation and oxidation products, oligomers and aggregates) are determined using liquid chromatography, electrophoretic or immunological methods and are within the limits defined for the particular raw material.

5-5. *ASSAY*

**Content**. The protein content is determined by an appropriate qualified method, for example by liquid chromatography (*2.2.29*) or UV spectrophotometry (*2.2.25*).

**Biological activity**. The biological activity of a recombinant protein is determined using, for example, cell proliferation, cell differentiation or an enzyme assay. Several acceptable bioassays may exist for a particular protein. For antibodies, cell-based immunoassays and assays based on ligand-binding and affinity may be used.

Where relevant, the biological activity is expressed per milligram of total protein (specific activity).

6. PROTEINS EXTRACTED FROM BIOLOGICAL MATERIAL

6-1. *DEFINITION*

Proteins extracted from biological material and used as raw materials include enzymes (e.g. porcine - derived trypsin and endonucleases), polyclonal antibodies, other proteins of biological origin (e.g. albumin and transferrin) and peptides of biological origin. They may be of human, animal, plant or microbiological origin.

Proteins extracted from biological material are used in a wide range of applications such as growth promotion, differentiation or purification of cultured cells and extraction of active substances from tissues and/or fluids.

6-2. *PRODUCTION*

Proteins are extracted from the blood or tissue of animals or humans, or from plant or microbiological sources using mechanical and/or chemical techniques. They are then subjected to further purification processes using a variety of techniques such as centrifugation, filtration, chromatography and concentration.

Polyclonal antibodies are produced by immunisation with a specific antigen, followed by purification. Antibody purification involves selective enrichment or specific isolation of antibodies from serum based on physico-chemical fractionation, class-specific affinity and/or antigen-specific affinity.

During production of these proteins, process-related impurities, such as blood components, tissue fragments or contaminating proteins, must be reduced to acceptable levels. Particular attention is given to product-related impurities.

6-3. *IDENTIFICATION*

Identity is established by appropriate, qualified methods, such as electrophoresis (*2.2.31*), isoelectric focusing (*2.2.54*) peptide mapping (*2.2.55*), liquid chromatography (*2.2.29*) and immunochemical methods (*2.7.1*).

6-4. *TESTS*

See section 3-3-2.

**Process-related impurities**. Substances derived from the starting material (e.g. blood components, tissue fragments or contaminating proteins) are determined using suitable methods and are within the limits defined for the particular raw material.

**Related proteins**. Related proteins (e.g. antibodies with undefined specificity, degradation and oxidation products, oligomers and aggregates) are determined using suitable methods and are within the limits defined for the particular raw material.

6-5. *ASSAY*

**Content**. The protein content is determined using an appropriate qualified method, for example by liquid chromatography (*2.2.29*) or UV spectrophotometry (*2.2.25*).

**Biological activity**. Where relevant, the biological activity of a protein is determined using, for example, enzyme assays, immunoassays or assays based on cell proliferation/differentiation. For trypsin, the assay may be performed as described in the monograph *Trypsin (0694)*.

Where relevant, the biological activity is expressed per milligram of total protein (specific activity).

7. VECTORS

Vectors that may be used as raw materials in the production of cell-based and gene therapy medicinal products include DNA vectors (e.g. plasmids, transposon vectors) as well as

viral vectors and bacteria (e.g. modified *Lactococcus* species). Vectors are usually considered as starting materials, thus not under the scope of this general chapter. In cases where vectors are not considered as starting materials, such as vectors used as helper plasmids or helper viruses, the principles of this general chapter and the principles of production and quality control as outlined in general chapter *5.14.Gene transfer medicinal products for human use* are to be followed.

**01/2017:50213**

## 5.2.13. HEALTHY CHICKEN FLOCKS FOR THE PRODUCTION OF INACTIVATED VACCINES FOR VETERINARY USE

The risk involved with the use of eggs from healthy chicken flocks has to be assessed in accordance with general chapter *5.2.5. Substances of animal origin for the production of immunological veterinary medicinal products*.

Where eggs from healthy chicken flocks are used for the production of inactivated vaccines for veterinary use, the health status of the flock is ensured by means of the system described below.

A flock is defined as a group of chickens sharing a common environment, that have no contact with other poultry flocks of lower health status.

A designated healthy chicken flock is derived from embryonated eggs obtained from healthy flocks or breeder flocks that are of the same health status.

Once the layer flock is defined, no chickens of lower health status may be added. Appropriate measures are taken to prevent or significantly reduce entry of rodents, wild birds or insects and to prevent entry of unauthorised personnel.

Personnel authorised to enter the facility in which the flock is housed must have no contact with birds of lower health status or with agents potentially capable of infecting the flock. It is advisable for personnel to shower and change clothing, or to wear protective clothing before entering the controlled facility.

Wherever possible, it is recommended that the feed is of a quality that reduces the introduction of undesirable micro-organisms as much as possible and that water is at least of potable quality. The flocks may be vaccinated. Live vaccination prior to and during the egg collection period should be avoided where possible, otherwise the associated risks should be carefully considered and the vaccination justified in accordance with general chapter *5.2.5. Substances of animal origin for the production of immunological veterinary medicinal products*.

A permanent record of the general health of the flock is kept and any abnormality is investigated. Factors to be monitored include medical treatments (especially vaccinations), morbidity, mortality, general physical condition, feed consumption, daily egg production and egg quality. Records are maintained for a period of at least 5 years. Details of any deviation from normal in these performance parameters are notified to the users of the eggs as soon as possible and within no more than 14 days.

The flocks must be free from *Mycoplasma gallisepticum*, *M. synoviae* and from salmonella which is of public health significance or pathogenic for chickens. The flocks are tested before lay and at regular intervals during lay and at the end of egg collection.

The tests or combination of tests must have suitable specificity and sensitivity. Samples are taken for testing from at least 4 different locations, and from 5 animals per thousand, with a minimum of 20 samples.

ROUTINE TESTING OF HEALTHY FLOCKS

Clinical examination is carried out at least once per week throughout the life of the flock in order to verify that the birds are free from signs of any infection. In the event of mortality due to unknown causes during lay, a necropsy is performed on a representative number of available carcasses. Where appropriate, histopathological, microbiological and virological studies are performed to confirm diagnosis.

ACTION TO BE TAKEN IN THE EVENT OF DETECTION OF INFECTIOUS DISEASES

If evidence is found of contamination of the flock, all materials derived from the flock during the 4-week period immediately preceding the date on which the positive diagnosis was made may present a risk of contamination. Any product manufactured with such materials should undergo a risk assessment to determine whether or not they can be used further in vaccine production.

Producers must notify users of all eggs of the evidence of contamination within 14 days of the outbreak.

Any flock in which a clinical outbreak or a positive test is confirmed for *M. gallisepticum*, *M. synoviae* or for salmonella which is of public health significance or pathogenic for chickens, cannot be redesignated as a healthy flock.

**01/2018:50214**

## 5.2.14. SUBSTITUTION OF *IN VIVO* METHOD(S) BY *IN VITRO* METHOD(S) FOR THE QUALITY CONTROL OF VACCINES

PURPOSE

The purpose of this general chapter is to provide guidance to facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo* methods, in cases where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more *in vitro* methods. This general chapter will not discuss the details of assay validation as such, since those principles are described elsewhere.

The general chapter applies primarily to vaccines for human or veterinary use, however the principles described may also apply to other biologicals such as sera.

CONTEXT

The test methods used for routine quality control of vaccines are intended to monitor production consistency and to ensure comparability of the quality attributes between commercial batches and those batches originally found to be safe and efficacious in clinical studies or, for veterinary vaccines, in the target species.

While the *in vivo* potency and safety assays described within Ph. Eur. vaccine monographs have historically played a central role in safeguarding the quality of vaccines, the inherent variability of *in vivo* assays can make them less suitable than appropriately designed *in vitro* assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential continually to challenge the scientific value and relevance of these *in vivo* test methods. When *in vivo* tests are found to be of limited or no value, it is imperative to eliminate them, given the ethical considerations and the obligations under the relevant conventions. In addition, there is a substantial effort to develop *in vitro* methods (including immunological, molecular and physico-chemical tests) to replace the animal tests. In several cases this has led to the successful introduction

of new *in vitro* methods in vaccine monographs. The use of appropriate *in vitro* methods not only reduces the use of animals while maintaining or improving the scientific relevance of the assays involved, but also substantially reduces assay variability and the time and resources required, and enhances the predictability of the release of safe and effective vaccine lots for use.

In addition to the benefits resulting from the substitution of appropriate *in vitro* methods for existing *in vivo* methods, under the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, the Ph. Eur. Commission has committed to the reduction of animal usage wherever possible in pharmacopoeial testing. Under the convention, those associated with the work of the European Pharmacopoeia are encouraged to develop and/or implement *in vitro* procedures, and the General Notices support the introduction of alternatives to *in vivo* methods described in Ph. Eur. monographs.



## GENERAL CONSIDERATIONS

One of the consequences associated with the inherent variability of *in vivo* assays is the problem this poses with their replacement by the more-consistent *in vitro* methods, which typically requires one-to-one assay comparison. This can be a challenge in some cases as repeated efforts through multicentre international collaborative studies can fail due to the variability inherent in the *in vivo* methods. Another consideration is that many of the legacy *in vivo* safety and potency assays for vaccines were generally shown to be fit for purpose and have historically proven their value in ensuring the efficacy and safety of vaccines. However, this was in an era when validation requirements, such as ICH Q2 (R1) or VICH GL2 guideline, were not in place, making a formal one-to-one comparison challenging or even impossible in some cases. Since precision, reproducibility, limits of detection and quantification were not established for the *in vivo* method, the comparability of one method to another becomes difficult to evaluate. It should also be noted that, because Ph. Eur. methods are considered validated under the General Notices, it is not only impractical and excessively costly now to undertake a retrospective ICH/VICH validation of these methods, but it would also be unethical given the above-mentioned convention on animal use in pharmacopoeial testing.

When considering the transition from an *in vivo*-based to an *in vitro*-based quality control assay system, it is important to understand what *in vivo* assays can and cannot offer. Although properly established *in vivo* potency assays in laboratory animals have the potential to measure complex functional responses for demonstrating proof of concept, these do not necessarily predict the actual responses in the target population. In addition, *in vitro* bioassays have the potential to mimic specific elements of complex *in vivo* responses with generally lower variability and higher sensitivity.

Another key consideration is that when an *in vivo* test for a given product is to be replaced with an *in vitro* test, the quality attribute(s) of the product will likely be assessed differently. Examples include: the determination of antigen content or a functional response (e.g. virus or toxin neutralisation) in an *in vitro* bioassay instead of *in vivo* potency; molecular consistency instead of *in vivo* neurovirulence or attenuated phenotype; absence of the extraneous agent genomes using molecular methods instead of absence of micro-organisms through *in vivo* testing; and demonstration of toxin binding and enzyme activity instead of *in vivo* specific toxicity. As a consequence, a demonstration of agreement between the 2 methods is generally not scientifically justified and should not always be expected. Even where pass/fail results from the 2 test procedures are in agreement, the correlation between 2 quantitative methods across the assay range may still be low. Regardless, the *in vitro* method(s) or testing strategy must provide at least the same confidence that the key quality attributes, which are necessary to ensure the consistency of a product's safety and effectiveness, are adequately controlled.

While the focus of this general chapter is on the replacement of existing methods for approved products, it is important to consider the use of *in vitro* methods for quality control during product development and to understand that the use of *in vivo* assays is not mandatory.

## ALTERNATIVE APPROACHES FOR THE SUBSTITUTION OF *IN VIVO* METHODS

The primary focus for the implementation of any proposed *in vitro* methods within a quality control system should be the scientific relevance of *in vitro* assays for control of the relevant quality attributes. Additionally, any *in vitro* methods will have to meet the current validation requirements.

In the Ph. Eur., *in vivo* assays for vaccines are typically replaced by *in vitro* assays following multicentre collaborative studies, but this should not be a prerequisite for *in vivo* assay replacement initiatives for individual products. Additionally, while it may be desirable to have assays that are widely applicable to a class of products, this should not be a requirement.

As explained in the guidance below, in some cases an existing method may need to be substituted by more than 1 *in vitro* test, in order to characterise the key qualitative and quantitative attributes measured by the existing test.

### *POTENCY TESTS*

When it is not possible to show agreement between the *in vitro* and *in vivo* methods due to low discriminating power and/or high variability of the *in vivo* assay, the following approach can be used. It is assumed that the product under consideration has a well-established safety and efficacy profile, with consistent manufacturing.

The *in vitro* test(s) should be able to detect differences that are relevant to the control of the production process as justified scientifically. This should be supported by data demonstrating the capability of the proposed assay(s) to control key quality attributes of the vaccine and maintain the link between the quality of the batches to be released and those batches found to be safe and efficacious through clinical studies or routine use. With the setting of appropriate specifications, the consistency of manufacturing with the *in vitro* method(s) will be maintained.

The design of an assay/assay system for vaccine quality control needs to reflect both antigen content and functionality. If a single method is used, it should preferably measure the content and integrity of the antigen by targeting epitope(s) relevant to the protection offered by the vaccine. An example of this would be a monoclonal antibody or monoclonal antibodies against an epitope or epitopes as the main target for generating neutralising antibodies. The epitope or epitopes should preferably be conformational in order to have a stability-indicating assay (as is the case for rabies vaccine). In some cases, a single *in vitro* method may not adequately reflect the content and functionality. This can be remedied through the use of multiple assays, as is the case with conjugate polysaccharide vaccines, where molecular size, conjugate integrity, and total and free polysaccharides are examples of relevant measures.

To establish quantitative measurements with an *in vitro* method, samples that differ in the magnitude of the response will be needed. In most cases, samples that are below the minimum approved potency specification with the *in vivo* method will not be available because production consistency is generally well maintained, and potency between batches does not differ significantly and/or the precision of the *in vivo* assay is such that it cannot discriminate between batches unless the difference is very large. Therefore, initial assay evaluation should be performed with samples at different concentrations, which could be followed by testing of samples

subjected to different types of stress conditions to assess further the stability-indicating potential of the new method. The inability to demonstrate agreement between an *in vitro* and an *in vivo* method does not necessarily mean that the *in vitro* method is not suitable/relevant. In many cases, an *in vitro* method will detect changes in the product profile that would not be detected by the *in vivo* method. In such cases, the *in vitro* method may be considered superior for monitoring the consistency of production and may be more relevant to assess the impact of manufacturing changes.

*SAFETY TESTS*

**Specific toxicity**

An *in vitro* method for detection of residual toxic components should be specific and at least as sensitive as the existing *in vivo* method. Where possible, a fully functional *in vitro* system should be used (e.g. toxin-sensitive cell line). Where no functional *in vitro* system is available, an *in vitro* testing strategy could be based on the detection/measurement of more than 1 parameter, sequentially where relevant, that together reflect the mode of action for the toxic components in question. Examples include the use of assays with immunological and biochemical steps to detect receptor binding and enzyme activity. In most cases, where an *in vivo* assay is to be replaced there will be data available on the sensitivity of that model for detection of the toxin in question. Therefore, new *in vitro* methods can be characterised to demonstrate that they are sufficiently sensitive using spiking experiments and referring to historic data for the *in vivo* assay. Such assays, in conjunction with the appropriate time and temperature conditions, could also be used to demonstrate the absence of reversion of a specific toxoid.

**Molecular consistency by deep sequencing versus the neurovirulence test**

An *in vitro* genotypic method to assess the molecular consistency of a viral vaccine has the potential to replace an existing *in vivo* neurovirulence test. A prerequisite for any *in vitro* genotypic method is an in-depth knowledge of the molecular markers responsible for the attenuation of the live viral vaccine (as is the case for oral poliovirus vaccine, for example). In such a case, monitoring the consistency of the vaccine lots would be achieved by confirming the presence of the required molecular attenuation markers and percentage of mutants with methods such as deep sequencing.

**Detection of viral extraneous agents by novel molecular methods**

Detection of viral extraneous agents in cell banks, seed lots and cell culture harvests is currently conducted using a panel of *in vivo* and *in vitro* methods at different stages of the manufacturing process. Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high-throughput sequencing methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. The use of these new molecular methods has highlighted gaps in the existing testing strategy by identifying previously undetected viral contaminants in final product, the cell banks from which it was produced and intermediate manufacturing stages. These new molecular methods (e.g. deep sequencing or high-throughput sequencing) detect genomes while the existing *in vivo* methods are based on observations of the effects viruses have on experimental animals. The implementation of such new molecular methods as substitutes for *in vivo* methods requires a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. For this purpose, an appropriate panel of representative, well-characterised model viruses should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the *in vivo* methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the *in vivo* methods. This last element is particularly complex since these new molecular methods do not detect the same characteristic of the viral contaminant (genome for molecular methods versus infectious virus for *in vivo* methods) and also since no or limited validation data exist for the *in vivo* methods. It should also be emphasised that the outcome of the new molecular methods is not the final result since the detection of a genome or fragments of a genome does not necessarily indicate the presence of an infectious virus.

# 5.3. Statistical analysis of results of biological assays and tests

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01/2020:50300

# 5.3. STATISTICAL ANALYSIS OF RESULTS OF BIOLOGICAL ASSAYS AND TESTS

## 1. INTRODUCTION

This chapter provides guidance for the design of bioassays prescribed in the European Pharmacopoeia (Ph. Eur.) and for analysis of their results. It is intended for use by those whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the results of these assays, often without the help and advice of a statistician. The methods of calculation described in this annex are not mandatory for the bioassays which themselves constitute a mandatory part of the Ph. Eur. Alternative methods can be used and may be accepted by the competent authorities, provided that they are supported by relevant data and justified during the assay validation process. A wide range of computer software is available and may be useful depending on the facilities available to, and the expertise of, the analyst.

Professional advice should be obtained in situations where: a comprehensive treatment of design and analysis suitable for research or development of new products is required; the restrictions imposed on the assay design by this chapter are not satisfied, for example particular laboratory constraints may require customized assay designs, or equal numbers of equally spaced doses may not be suitable; analysis is required for extended non-linear dose-response curves, for example as may be encountered in immunoassays. An outline of extended dose-response curve analysis for one widely used model is nevertheless included in Section 3.4 and a simple example is given in Section 5.4.

### 1.1. GENERAL DESIGN AND PRECISION

Biological methods are described for the assay of certain substances and preparations whose potency cannot be adequately assured by chemical or physical analysis. The principle applied wherever possible throughout these assays is that of comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the *Unit*, of the standard preparation. It is an essential condition of such methods of biological assay that the tests on the standard preparation and on the substance to be examined be carried out at the same time and under identical conditions.

For certain assays (determination of virus titre for example) the potency of the test sample is not expressed relative to a standard. This type of assay is dealt with in Section 4.5.

Any estimate of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay, even when the official method of assay is used. Methods for the design of assays and the calculation of their errors are, therefore, described below. In every case, before a statistical method is adopted, a preliminary test is to be carried out with an appropriate number of assays, in order to ascertain the applicability of this method.

The confidence interval for the potency gives an indication of the precision with which the potency has been estimated in the assay. It is calculated with due regard to the experimental design and the sample size. The 95 per cent confidence interval is usually chosen in biological assays. Mathematical statistical methods are used to calculate these limits so as to warrant the statement that there is a 95 per cent probability that these limits include the true potency. Whether this precision is acceptable to the European Pharmacopoeia depends on the requirements set in the monograph for the preparation concerned.

The terms "mean" and "standard deviation" are used here as defined in most current textbooks of biometry.

The terms "stated potency" or "labelled potency", "assigned potency", "assumed potency", "potency ratio" and "estimated potency" are used in this section to indicate the following concepts:

- "stated potency" or "labelled potency": in the case of a formulated product a nominal value assigned from knowledge of the potency of the bulk material; in the case of bulk material the potency estimated by the manufacturer;
- "assigned potency": the potency of the standard preparation;
- "assumed potency": the provisionally assigned potency of a preparation to be examined which forms the basis of calculating the doses that would be equipotent with the doses to be used of the standard preparation;
- "potency ratio" of an unknown preparation; the ratio of equipotent doses of the standard preparation and the unknown preparation under the conditions of the assay;
- "estimated potency": the potency calculated from assay data.

Section 9 (Glossary of symbols) is a tabulation of the more important uses of symbols throughout this annex. Where the text refers to a symbol not shown in this section or uses a symbol to denote a different concept, this is defined in that part of the text.

## 2. RANDOMISATION AND INDEPENDENCE OF INDIVIDUAL TREATMENTS

The allocation of the different treatments to different experimental units (animals, tubes, etc.) should be made by some strictly random process. Any other choice of experimental conditions that is not deliberately allowed for in the experimental design should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered. In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be obtained from computers by using the built-in randomisation function. The analyst must check whether a different series of numbers is produced every time the function is started.

The preparations allocated to each experimental unit should be as independent as possible. Within each experimental group, the dilutions allocated to each treatment are not normally divisions of the same dose, but should be prepared individually. Without this precaution, the variability inherent in the preparation will not be fully represented in the experimental error variance. The result will be an under-estimation of the residual error leading to:

1) an unjustified increase in the stringency of the test for the analysis of variance (see Sections 3.2.3 and 3.2.4),

2) an under-estimation of the true confidence limits for the test, which, as shown in Section 3.2.5, are calculated from the estimate of $s^2$, the residual error mean square.

## 3. ASSAYS DEPENDING UPON QUANTITATIVE RESPONSES

### 3.1. STATISTICAL MODELS

#### *3.1.1. GENERAL PRINCIPLES*

The bioassays included in the Ph. Eur. have been conceived as "dilution assays", which means that the unknown preparation to be assayed is supposed to contain the same active principle as the standard preparation, but in a different ratio of active and inert components. In such a case the

unknown preparation may in theory be derived from the standard preparation by dilution with inert components. To check whether any particular assay may be regarded as a dilution assay, it is necessary to compare the dose-response relationships of the standard and unknown preparations. If these dose-response relationships differ significantly, then the theoretical dilution assay model is not valid. Significant differences in the dose-response relationships for the standard and unknown preparations may suggest that one of the preparations contains, in addition to the active principle, other components which are not inert but which influence the measured responses.

To make the effect of dilution in the theoretical model apparent, it is useful to transform the dose-response relationship to a linear function on the widest possible range of doses. 2 statistical models are of interest as models for the bioassays prescribed: the parallel-line model and the slope-ratio model.

The application of either is dependent on the fulfilment of the following conditions:

1) the different treatments have been randomly assigned to the experimental units,

2) the responses to each treatment are normally distributed,

3) the standard deviations of the responses within each treatment group of both standard and unknown preparations do not differ significantly from one another.

When an assay is being developed for use, the analyst has to determine that the data collected from many assays meet these theoretical conditions.

- Condition 1 can be fulfilled by an efficient use of Section 2.
- Condition 2 is an assumption which in practice is almost always fulfilled. Minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are included. In case of doubt, a test for deviations from normality (e.g. the Shapiro-Wilk[(1)] test) may be performed.
- Condition 3 can be checked with a test for homogeneity of variances (e.g. Bartlett's[(2)] test, Cochran's[(3)] test). Inspection of graphical representations of the data can also be very instructive for this purpose (see examples in Section 5).

When conditions 2 and/or 3 are not met, a transformation of the responses may bring a better fulfilment of these conditions. Examples are $\ln y$, $\sqrt{y}$, $y^2$.

- Logarithmic transformation of the responses $y$ to $\ln y$ can be useful when the homogeneity of variances is not satisfactory. It can also improve the normality if the distribution is skewed to the right.
- The transformation of $y$ to $\sqrt{y}$ is useful when the observations follow a Poisson distribution i.e. when they are obtained by counting.
- The square transformation of $y$ to $y^2$ can be useful if, for example, the dose is more likely to be proportional to the area of an inhibition zone rather than the measured diameter of that zone.

For some assays depending on quantitative responses, such as immunoassays or cell-based *in vitro* assays, a large number of doses is used. These doses give responses that completely span the possible response range and produce an extended non-linear dose-response curve. Such curves are typical for all bioassays, but for many assays the use of a large number of doses is not ethical (for example, *in vivo* assays) or practical, and the aims of the assay may be achieved with a limited number of doses. It is therefore customary to restrict doses to that part of the dose-response range which is linear under suitable transformation, so that the methods of Sections 3.2 or 3.3 apply. However, in some cases analysis of extended dose-response curves may be desirable. An outline of one model which may be used for such analysis is given in Section 3.4 and a simple example is shown in Section 5.4.

There is another category of assays in which the response cannot be measured in each experimental unit, but in which only the fraction of units responding to each treatment can be counted. This category is dealt with in Section 4.

*3.1.2. ROUTINE ASSAYS*

When an assay is in routine use, it is seldom possible to check systematically for conditions 1 to 3, because the limited number of observations per assay is likely to influence the sensitivity of the statistical tests. Fortunately, statisticians have shown that, in symmetrical balanced assays, small deviations from homogeneity of variance and normality do not seriously affect the assay results. The applicability of the statistical model needs to be questioned only if a series of assays shows doubtful validity. It may then be necessary to perform a new series of preliminary investigations as discussed in Section 3.1.1.

Two other necessary conditions depend on the statistical model to be used:

- for the parallel-line model:

  4A) the relationship between the logarithm of the dose and the response can be represented by a straight line over the range of doses used,

  5A) for any unknown preparation in the assay the straight line is parallel to that for the standard.

- for the slope-ratio model:

  4B) the relationship between the dose and the response can be represented by a straight line for each preparation in the assay over the range of doses used,

  5B) for any unknown preparation in the assay the straight line intersects the $y$-axis (at zero dose) at the same point as the straight line of the standard preparation (i.e. the response functions of all preparations in the assay must have the same intercept as the response function of the standard).

Conditions 4A and 4B can be verified only in assays in which at least 3 dilutions of each preparation have been tested. The use of an assay with only 1 or 2 dilutions may be justified when experience has shown that linearity and parallelism or equal intercept are regularly fulfilled.

After having collected the results of an assay, and before calculating the relative potency of each test sample, an analysis of variance is performed, in order to check whether conditions 4A and 5A (or 4B and 5B) are fulfilled. For this, the total sum of squares is subdivided into a certain number of sum of squares corresponding to each condition which has to be fulfilled. The remaining sum of squares represents the residual experimental error to which the absence or existence of the relevant sources of variation can be compared by a series of $F$-ratios.

When validity is established, the potency of each unknown relative to the standard may be calculated and expressed as a potency ratio or converted to some unit relevant to the preparation under test e.g. an International Unit. Confidence limits may also be estimated from each set of assay data.

Assays based on the parallel-line model are discussed in Section 3.2 and those based on the slope-ratio model in Section 3.3.

If any of the 5 conditions (1, 2, 3, 4A, 5A or 1, 2, 3, 4B, 5B) are not fulfilled, the methods of calculation described here are invalid and an investigation of the assay technique should be made.

(1) Wilk, M.B. and Shapiro, S.S. (1968). The joint assessment of normality of several independent samples, *Technometrics* 10, 825-839.
(2) Bartlett, M.S. (1937). Properties of sufficiency and statistical tests, *Proc. Roy. Soc. London*, Series A 160, 280-282.
(3) Cochran, W.G. (1951). Testing a linear relation among variances, *Biometrics* 7, 17-32.

The analyst should not adopt another transformation unless it is shown that non-fulfilment of the requirements is not incidental but is due to a systematic change in the experimental conditions. In this case, testing as described in Section 3.1.1 should be repeated before a new transformation is adopted for the routine assays.

Excess numbers of invalid assays due to non-parallelism or non-linearity, in a routine assay carried out to compare similar materials, are likely to reflect assay designs with inadequate replication. This inadequacy commonly results from incomplete recognition of all sources of variability affecting the assay, which can result in underestimation of the residual error leading to large *F*-ratios.

It is not always feasible to take account of all possible sources of variation within one single assay (e.g. day-to-day variation). In such a case, the confidence intervals from repeated assays on the same sample may not satisfactorily overlap, and care should be exercised in the interpretation of the individual confidence intervals. In order to obtain a more reliable estimate of the confidence interval it may be necessary to perform several independent assays and to combine these into one single potency estimate and confidence interval (see Section 6).

For the purpose of quality control of routine assays it is recommended to keep record of the estimates of the slope of regression and of the estimate of the residual error in control charts.

– An exceptionally high residual error may indicate some technical problem. This should be investigated and, if it can be made evident that something went wrong during the assay procedure, the assay should be repeated. An unusually high residual error may also indicate the presence of an occasional outlying or aberrant observation. A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. If an aberrant value is discovered after the responses have been recorded, but can then be traced to assay irregularities, omission may be justified. The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias. In general, the rejection of observations solely because a test for outliers is significant, is discouraged.

– An exceptionally low residual error may once in a while occur and cause the *F*-ratios to exceed the critical values. In such a case it may be justified to replace the residual error estimated from the individual assay, by an average residual error based on historical data recorded in the control charts.

*3.1.3. CALCULATIONS AND RESTRICTIONS*

According to general principles of good design the following 3 restrictions are normally imposed on the assay design. They have advantages both for ease of computation and for precision.

a) Each preparation in the assay must be tested with the same number of dilutions.

b) In the parallel-line model, the ratio of adjacent doses must be constant for all treatments in the assay; in the slope-ratio model, the interval between adjacent doses must be constant for all treatments in the assay.

c) There must be an equal number of experimental units to each treatment.

If a design is used which meets these restrictions, the calculations are simple. The formulae are given in Sections 3.2 and 3.3. It is recommended to use software which has been developed for this special purpose. There are several programs in existence which can easily deal with all assay-designs described in the monographs. Not all programs may use the same formulae and algorithms, but they should all lead to the same results.

Assay designs not meeting the above mentioned restrictions may be both possible and correct, but the necessary formulae are too complicated to describe in this text. A brief description of methods for calculation is given in Section 7.1. These methods can also be used for the restricted designs, in which case they are equivalent with the simple formulae.

The formulae for the restricted designs given in this text may be used, for example, to create *ad hoc* programs in a spreadsheet. The examples in Section 5 can be used to clarify the statistics and to check whether such a program gives correct results.

## 3.2. THE PARALLEL-LINE MODEL

*3.2.1. INTRODUCTION*

The parallel-line model is illustrated in Figure 3.2.1.-I. The logarithm of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated ln(dose)-response relationship for the standard and the unknown.

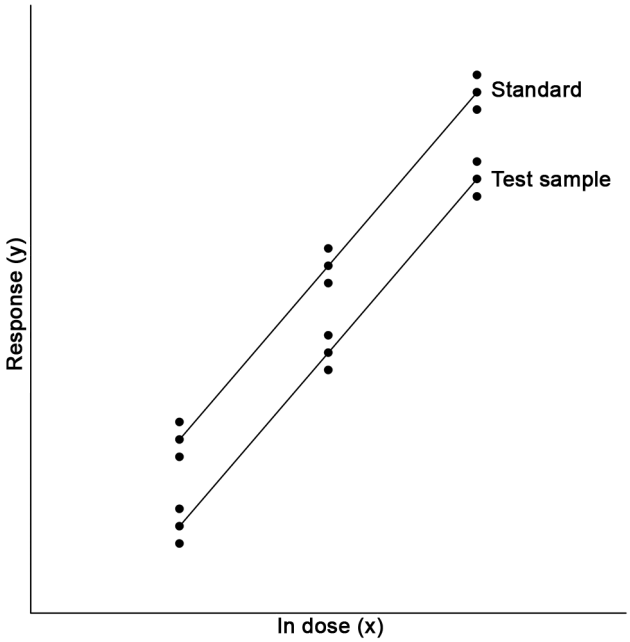


Figure 3.2.1.-I. – *The parallel-line model for a 3 + 3 assay*

*Note*: the natural logarithm (ln or $\log_e$) is used throughout this text. Wherever the term "antilogarithm" is used, the quantity $e^x$ is meant. However, the Briggs or "common" logarithm (log or $\log_{10}$) can equally well be used. In this case the corresponding antilogarithm is $10^x$.

For a satisfactory assay the assumed potency of the test sample must be close to the true potency. On the basis of this assumed potency and the assigned potency of the standard, equipotent dilutions (if feasible) are prepared, i.e. corresponding doses of standard and unknown are expected to give the same response. If no information on the assumed potency is available, preliminary assays are carried out over a wide range of doses to determine the range where the curve is linear.

The more nearly correct the assumed potency of the unknown, the closer the 2 lines will be together, for they should give equal responses at equal doses. The horizontal distance between the lines represents the "true" potency of the unknown, relative to its assumed potency. The greater the distance between the 2 lines, the poorer the assumed potency of the unknown. If the line of the unknown is situated to the right of the standard, the assumed potency was overestimated, and the calculations will indicate an estimated potency lower than the assumed potency. Similarly, if the line of the unknown is situated to the

left of the standard, the assumed potency was underestimated, and the calculations will indicate an estimated potency higher than the assumed potency.

*3.2.2. ASSAY DESIGN*

The following considerations will be useful in optimising the precision of the assay design:

1) the ratio between the slope and the residual error should be as large as possible,

2) the range of doses should be as large as possible,

3) the lines should be as close together as possible, i.e. the assumed potency should be a good estimate of the true potency.

The allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways.

**3.2.2.1. Completely randomised design**

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognisable sub-groups, the allocation of the units to the different treatments should be made randomly.

If units in sub-groups such as physical positions or experimental days are likely to be more homogeneous than the totality of the units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful distribution of the units over these restrictions permits irrelevant sources of variation to be eliminated.

**3.2.2.2. Randomised block design**

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment be applied an equal number of times in every block (litter or Petri dish) and is suitable only when the block is large enough to accommodate all treatments once. This is illustrated in Section 5.1.3. It is also possible to use a randomised design with repetitions. The treatments should be allocated randomly within each block. An algorithm to obtain random permutations is given in Section 8.5.

**3.2.2.3. Latin square design**

This design is appropriate when the response may be affected by two different sources of variation each of which can assume $k$ different levels or positions. For example, in a plate assay of an antibiotic the treatments may be arranged in a $k \times k$ array on a large plate, each treatment occurring once in each row and each column. The design is suitable when the number of rows, the number of columns and the number of treatments are equal. Responses are recorded in a square format known as a Latin square. Variations due to differences in response among the $k$ rows and among the $k$ columns may be segregated, thus reducing the error. An example of a Latin square design is given in Section 5.1.2. An algorithm to obtain Latin squares is given in Section 8.6. More complex designs in which one or more treatments are replicated within the Latin square may be useful in some circumstances. The simplified formulae given in this Chapter are not appropriate for such designs, and professional advice should be obtained.

**3.2.2.4. Cross-over design**

This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only 2 treatments to each block. For example, a block may be a single unit that can be tested on 2 occasions. The design is intended to increase precision by eliminating the effects of differences between units while balancing the effect of any difference between general levels of response at the 2 occasions. If 2 doses of a standard and of an unknown preparation are tested, this is known as a twin cross-over test.

The experiment is divided into 2 parts separated by a suitable time interval. Units are divided into 4 groups and each group receives 1 of the 4 treatments in the first part of the test. Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other. The arrangement of doses is shown in Table 3.2.2.-I. An example can be found in Section 5.1.5.

Table 3.2.2.-I. – *Arrangement of doses in cross-over design*

| Group of units | Time I | Time II |
|---|---|---|
| 1 | $S_1$ | $T_2$ |
| 2 | $S_2$ | $T_1$ |
| 3 | $T_1$ | $S_2$ |
| 4 | $T_2$ | $S_1$ |

*3.2.3. ANALYSIS OF VARIANCE*

This section gives formulae that are required to carry out the analysis of variance and will be more easily understood by reference to the worked examples in Section 5.1. Reference should also be made to the glossary of symbols (Section 9).

The formulae are appropriate for symmetrical assays where one or more preparations to be examined ($T$, $U$, etc.) are compared with a standard preparation ($S$). It is stressed that the formulae can only be used if the doses are equally spaced, if equal numbers of treatments per preparation are applied, and each treatment is applied an equal number of times. It should not be attempted to use the formulae in any other situation.

Apart from some adjustments to the error term, the basic analysis of data derived from an assay is the same for completely randomised, randomised block and Latin square designs. The formulae for cross-over tests do not entirely fit this scheme and these are incorporated into Example 5.1.5.

Having considered the points discussed in Section 3.1 and transformed the responses, if necessary, the values should be averaged over each treatment and each preparation, as shown in Table 3.2.3.-I. The linear contrasts, which relate to the slopes of the ln(dose)-response lines, should also be formed. 3 additional formulae, which are necessary for the construction of the analysis of variance, are shown in Table 3.2.3.-II.

The total variation in response caused by the different treatments is now partitioned as shown in Table 3.2.3.-III the sums of squares being derived from the values obtained in Tables 3.2.3.-I and 3.2.3.-II. The sum of squares due to non-linearity can only be calculated if at least 3 doses per preparation are included in the assay.

The residual error of the assay is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.2.3.-IV). In this table $\bar{y}$ represents the mean of all responses recorded in the assay. It should be noted that for a Latin square the number of replicate responses ($n$) is equal to the number of rows, columns or treatments ($dh$).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to the residual error ($s^2$) and the significance of these values (known as $F$-ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

Table 3.2.3.-I. – *Formulae for parallel-line assays with d doses of each preparation*

| | **Standard (*S*)** | **1st Test sample (*T*)** | **2nd Test sample (*U*, etc.)** |
|---|---|---|---|
| **Mean response lowest dose** | $S_1$ | $T_1$ | $U_1$ |
| **Mean response 2nd dose** | $S_2$ | $T_2$ | $U_2$ |
| **...** | ... | ... | ... |
| **Mean response highest dose** | $S_d$ | $T_d$ | $U_d$ |
| **Total preparation** | $P_S = S_1 + S_2 + ... + S_d$ | $P_T = T_1 + T_2 + ... + T_d$ | $P_U$ = ...etc. |
| **Linear contrast** | $L_S = 1S_1 + 2S_2 + ... + dS_d - \frac{1}{2}(d+1)P_S$ | $L_T = 1T_1 + 2T_2 + ... + dT_d - \frac{1}{2}(d+1)P_T$ | $L_U$ = ...etc. |

Table 3.2.3.-II. – *Additional formulae for the construction of the analysis of variance*

| | | |
|---|---|---|
| $H_P = \frac{n}{d}$ | $H_L = \frac{12n}{d^3 - d}$ | $K = \frac{n(P_S + P_T + ...)^2}{hd}$ |

Table 3.2.3.-III. – *Formulae to calculate the sum of squares and degrees of freedom*

| **Source of variation** | **Degrees of freedom (*f*)** | **Sum of squares** |
|---|---|---|
| **Preparations** | $h - 1$ | $SS_{\text{prep}} = H_P(P_S^2 + P_T^2 + ...) - K$ |
| **Linear regression** | 1 | $SS_{\text{reg}} = \frac{1}{h}H_L(L_S + L_T + ...)^2$ |
| **Non-parallelism** | $h - 1$ | $SS_{\text{par}} = H_L(L_S^2 + L_T^2 + ...) - SS_{\text{reg}}$ |
| **Non-linearity*** | $h(d - 2)$ | $SS_{\text{lin}} = SS_{\text{treat}} - SS_{\text{prep}} - SS_{\text{reg}} - SS_{\text{par}}$ |
| **Treatments** | $hd - 1$ | $SS_{\text{treat}} = n(S_1^2 + ... + S_d^2 + T_1^2 + ... + T_d^2 + ...) - K$ |
| * Not calculated for 2-dose assays | | |

Table 3.2.3.-IV. – *Estimation of the residual error*

| **Source of variation** | | **Degrees of freedom** | **Sum of squares** |
|---|---|---|---|
| **Blocks (rows)*** | | $n - 1$ | $SS_{\text{block}} = hd(R_1^2 + ... + R_n^2) - K$ |
| **Columns**** | | $n - 1$ | $SS_{\text{col}} = hd(C_1^2 + ... + C_n^2) - K$ |
| **Residual error***** | **Completely randomised** | $hd(n - 1)$ | $SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}}$ |
| | **Randomised block** | $(hd - 1)(n - 1)$ | $SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}}$ |
| | **Latin square** | $(hd - 2)(n - 1)$ | $SS_{\text{res}} = SS_{\text{tot}} - SS_{\text{treat}} - SS_{\text{block}} - SS_{\text{col}}$ |
| **Total** | | $nhd - 1$ | $SS_{\text{tot}} = \sum(y - \bar{y})^2$ |

For Latin square designs, these formulae are only applicable if $n = hd$
* Not calculated for completely randomised designs
** Only calculated for Latin square designs
*** Depends on the type of design

*3.2.4. TESTS OF VALIDITY*

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows.

1) The linear regression term is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95 per cent confidence limits.

2) The term for non-parallelism is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5A, Section 3.1, is satisfied.

3) The term for non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4A, Section 3.1, is satisfied.

A significant deviation from parallelism in a multiple assay may be due to the inclusion in the assay-design of a preparation to be examined that gives an ln(dose)-response line with a slope different from those for the other preparations. Instead of declaring the whole assay invalid, it may then be decided to eliminate all data relating to that preparation and to restart the analysis from the beginning.

When statistical validity is established, potencies and confidence limits may be estimated by the methods described in the next section.

*3.2.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS*

If $I$ is the ln of the ratio between adjacent doses of any preparation, the common slope ($b$) for assays with $d$ doses of each preparation is obtained from:

$$b = \frac{H_L(L_S + L_T + ...)}{Inh} \qquad (3.2.5.\text{-}1)$$

and the logarithm of the potency ratio of a test preparation, for example $T$, is:

$$M_T' = \frac{P_T - P_S}{db} \qquad (3.2.5.\text{-}2)$$

The calculated potency is an estimate of the "true potency" of each unknown. Confidence limits may be calculated as the antilogarithms of:

$$CM_T' \pm \sqrt{(C-1)(CM_T'^2 + 2V)} \qquad (3.2.5.\text{-}3)$$

$$\text{where } C = \frac{SS_{\text{reg}}}{SS_{\text{reg}} - s^2t^2} \quad \text{and } V = \frac{SS_{\text{reg}}}{b^2dn}$$

The value of $t$ may be obtained from Table 8.2 for $p = 0.05$ and degrees of freedom equal to the number of the degrees of freedom of the residual error. The estimated potency ($R_T$) and associated confidence limits are obtained by multiplying the values obtained by $A_T$ after antilogarithms have been taken. If the stock solutions are not exactly equipotent on the basis of assigned and assumed potencies, a correction factor is necessary (See Examples 5.1.2 and 5.1.3).

*3.2.6. MISSING VALUES*

In a balanced assay, an accident totally unconnected with the applied treatments may lead to the loss of one or more responses, for example because an animal dies. If it is considered that the accident is in no way connected with the composition of the preparation administered, the exact calculations can still be performed but the formulae are necessarily more complicated and can only be given within the framework of general linear models (see Section 7.1). However, there exists an approximate method which keeps the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom for the total sum of squares and for the residual error by the number of missing values and using one of the formulae below for the missing values. It should be borne in mind that this is only an approximate method, and that the exact method is to be preferred.

If more than one observation is missing, the same formulae can be used. The procedure is to make a rough guess at all the missing values except one, and to use the proper formula for this one, using all the remaining values including the rough guesses. Fill in the calculated value. Continue by similarly calculating a value for the first rough guess. After calculating all the missing values in this way the whole cycle is repeated from the beginning, each calculation using the most recent guessed or calculated value for every response to which the formula is being applied. This continues until 2 consecutive cycles give the same values; convergence is usually rapid.

Provided that the number of values replaced is small relative to the total number of observations in the full experiment (say less than 5 per cent), the approximation implied in this replacement and reduction of degrees of freedom by the number of missing values so replaced is usually fairly satisfactory. The analysis should be interpreted with great care however, especially if there is a preponderance of missing values in one treatment or block, and a biometrician should be consulted if any unusual features are encountered. Replacing missing values in a test without replication is a particularly delicate operation.

*Completely randomised design*

In a completely randomised assay the missing value can be replaced by the arithmetic mean of the other responses to the same treatment.

*Randomised block design*

The missing value is obtained using the equation:

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)} \qquad (3.2.6.\text{-}1)$$

where $B'$ is the sum of the responses in the block containing the missing value, $T'$ the corresponding treatment total and $G'$ is the sum of all responses recorded in the assay.

*Latin square design*

The missing value $y'$ is obtained from:

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)} \qquad (3.2.6.\text{-}2)$$

where $B'$ and $C'$ are the sums of the responses in the row and column containing the missing value. In this case $k = n$.

*Cross-over design*

If an accident leading to loss of values occurs in a cross-over design, a book on statistics should be consulted (e.g. D.J. Finney, see Section 10), because the appropriate formulae depend upon the particular treatment combinations.

## 3.3. THE SLOPE-RATIO MODEL

*3.3.1. INTRODUCTION*

This model is suitable, for example, for some microbiological assays when the independent variable is the concentration of an essential growth factor below the optimal concentration of the medium. The slope-ratio model is illustrated in Figure 3.3.1.-I.

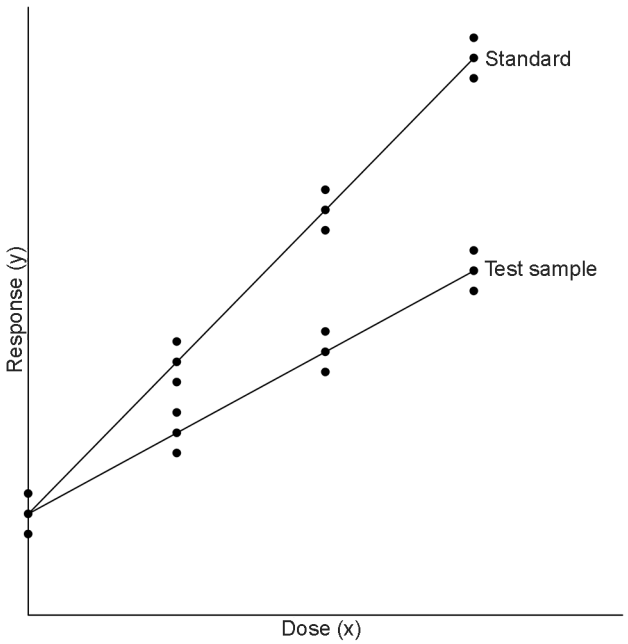


Figure 3.3.1.-I. – *The slope-ratio model for a 2 × 3 + 1 assay*

The doses are represented on the horizontal axis with zero concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated dose-response relationship for the standard and the unknown under the assumption that they intersect each other at zero-dose. Unlike the parallel-line model, the doses are not transformed to logarithms.

Just as in the case of an assay based on the parallel-line model, it is important that the assumed potency is close to the true potency, and to prepare equipotent dilutions of the test preparations and the standard (if feasible). The more nearly correct the assumed potency, the closer the 2 lines will be together. The ratio of the slopes represents the "true" potency of the unknown, relative to its assumed potency. If the slope of the unknown preparation is steeper than that of the standard, the potency was underestimated and the calculations will indicate an estimated potency higher than the assumed potency. Similarly, if the slope of the unknown is less steep than that of the standard, the potency was overestimated and the calculations will result in an estimated potency lower than the assumed potency.

In setting up an experiment, all responses should be examined for the fulfilment of the conditions 1, 2 and 3 in Section 3.1. The analysis of variance to be performed in routine is described in Section 3.3.3 so that compliance with conditions 4B and 5B of Section 3.1 may be examined.

*3.3.2. ASSAY DESIGN*

The use of the statistical analysis presented below imposes the following restrictions on the assay:

a) the standard and the test preparations must be tested with the same number of equally spaced dilutions,

b) an extra group of experimental units receiving no treatment may be tested (the blanks),

c) there must be an equal number of experimental units to each treatment.

As already remarked in Section 3.1.3, assay designs not meeting these restrictions may be both possible and correct, but the simple statistical analyses presented here are no longer applicable and either expert advice should be sought or suitable software should be used.

A design with 2 doses per preparation and 1 blank, the "common zero ($2h$ + 1)-design", is usually preferred, since it gives the highest precision combined with the possibility to check validity within the constraints mentioned above. However, a linear relationship cannot always be assumed to be valid down to zero-dose. With a slight loss of precision a design without blanks may be adopted. In this case 3 doses per preparation, the "common zero ($3h$)-design", are preferred to 2 doses per preparation. The doses are thus given as follows:

1) the standard is given in a high dose, near to but not exceeding the highest dose giving a mean response on the straight portion of the dose-response line,

2) the other doses are uniformly spaced between the highest dose and zero dose,

3) the test preparations are given in corresponding doses based on the assumed potency of the material.

A completely randomised, a randomised block or a Latin square design may be used, such as described in Section 3.2.2. The use of any of these designs necessitates an adjustment to the error sum of squares as described for assays based on the parallel-line model. The analysis of an assay of one or more test preparations against a standard is described below.

*3.3.3. ANALYSIS OF VARIANCE*

**3.3.3.1. The (*hd* + 1)-design**

The responses are verified as described in Section 3.1 and, if necessary, transformed. The responses are then averaged over each treatment and each preparation as shown in Table 3.3.3.1.-I. Additionally, the mean response for blanks ($B$) is calculated.

The sums of squares in the analysis of variance are calculated as shown in Tables 3.3.3.1.-I to 3.3.3.1.-III. The sum of squares due to non-linearity can only be calculated if at least 3 doses of each preparation have been included in the assay. The residual error is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.3.3.1.-IV).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to the residual error ($s^2$) and the significance of these values (known as $F$-ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

Table 3.3.3.1.-I. – *Formulae for slope-ratio assays with d doses of each preparation and a blank*

| | **Standard (*S*)** | **1st Test sample (*T*)** | **2nd Test sample (*U*, etc.)** |
|---|---|---|---|
| **Mean response lowest dose** | $S_1$ | $T_1$ | $U_1$ |
| **Mean response 2nd dose** | $S_2$ | $T_2$ | $U_2$ |
| **…** | … | … | … |
| **Mean response highest dose** | $S_d$ | $T_d$ | $U_d$ |
| **Total preparation** | $P_S = S_1 + S_2 + ... + S_d$ | $P_T = T_1 + T_2 + ... + T_d$ | $P_U = ...$ |
| **Linear product** | $L_S = 1S_1 + 2S_2 + ... + dS_d$ | $L_T = 1T_1 + 2T_2 + ... + dT_d$ | $L_U = ...$ |
| **Intercept value** | $a_S = (4d+2)P_S - 6L_S$ | $a_T = (4d+2)P_T - 6L_T$ | $a_U = ...$ |
| **Slope value** | $b_S = 2L_S - (d+1)P_S$ | $b_T = 2L_T - (d+1)P_T$ | $b_U = ...$ |
| **Treatment value** | $G_S = S_1^2 + ... + S_d^2$ | $G_T = T_1^2 + ... + T_d^2$ | $G_U = ...$ |
| **Non-linearity*** | $J_S = G_S - \frac{P_S^2}{d} - \frac{3b_S^2}{d^3 - d}$ | $J_T = G_T - \frac{P_T^2}{d} - \frac{3b_T^2}{d^3 - d}$ | $J_U = ...$ |
| * Not calculated for 2-dose assays | | | |

Table 3.3.3.1.-II. – *Additional formulae for the construction of the analysis of variance*

| | | | |
|---|---|---|---|
| $H_B = \frac{nhd^2 - nhd}{hd^2 - hd + 4d + 2}$ | $H_I = \frac{n}{4d^3 - 2d^2 - 2d}$ | $a = \frac{a_S + a_T + ...}{h(d^2 - d)}$ | $K = \frac{n(B + P_S + P_T + ...)^2}{hd + 1}$ |

Table 3.3.3.1.-III. – *Formulae to calculate the sum of squares and degrees of freedom*

| Source of variation | Degrees of freedom ($f$) | Sum of squares |
|---|---|---|
| **Regression** | $h$ | $SS_{reg} = SS_{treat} - SS_{blank} - SS_{int} - SS_{lin}$ |
| **Blanks** | 1 | $SS_{blank} = H_B(B - a)^2$ |
| **Intersection** | $h - 1$ | $SS_{int} = H_I\left((a_S^2 + a_T^2 + ...) - h(d^2 - d)^2 a^2\right)$ |
| **Non-linearity*** | $h(d - 2)$ | $SS_{lin} = n(J_S + J_T + ...)$ |
| **Treatments** | $hd$ | $SS_{treat} = n(B^2 + G_S + G_T + ...) - K$ |

* Not calculated for 2-dose assays

Table 3.3.3.1.-IV. – *Estimation of the residual error*

| Source of variation | | Degrees of freedom | Sum of squares |
|---|---|---|---|
| **Blocks (rows)*** | | $n - 1$ | $SS_{block} = hd(R_1^2 + ... + R_n^2) - K$ |
| **Columns**** | | $n - 1$ | $SS_{col} = hd(C_1^2 + ... + C_n^2) - K$ |
| **Residual error***** | **Completely randomised** | $(hd + 1)(n - 1)$ | $SS_{res} = SS_{tot} - SS_{treat}$ |
| | **Randomised block** | $hd(n - 1)$ | $SS_{res} = SS_{tot} - SS_{treat} - SS_{block}$ |
| | **Latin square** | $(hd - 1)(n - 1)$ | $SS_{res} = SS_{tot} - SS_{treat} - SS_{block} - SS_{col}$ |
| **Total** | | $nhd + n - 1$ | $SS_{tot} = \sum(y - \overline{y})^2$ |

For Latin square designs, these formulae are only applicable if $n = hd$
* Not calculated for completely randomised designs
** Only calculated for Latin square designs
*** Depends on the type of design

### 3.3.3.2. The (*hd*)-design

The formulae are basically the same as those for the ($hd$ + 1)-design, but there are some slight differences.

- $B$ is discarded from all formulae.
- $K = \dfrac{n(P_S + P_T + ...)^2}{hd}$
- $SS_{blank}$ is removed from the analysis of variance.
- The number of degrees of freedom for treatments becomes $hd - 1$.
- The number of degrees of freedom of the residual error and the total variance is calculated as described for the parallel-line model (see Table 3.2.3.-IV).

Validity of the assay, potency and confidence interval are found as described in Sections 3.3.4 and 3.3.5.

*3.3.4. TESTS OF VALIDITY*

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows:

1) the variation due to blanks in ($hd$ + 1)-designs is not significant, i.e. the calculated probability is not smaller than 0.05. This indicates that the responses of the blanks do not significantly differ from the common intercept and the linear relationship is valid down to zero dose;

2) the variation due to intersection is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5B, Section 3.1 is satisfied;

3) in assays including at least 3 doses per preparation, the variation due to non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4B, Section 3.1 is satisfied.

A significant variation due to blanks indicates that the hypothesis of linearity is not valid near zero dose. If this is likely to be systematic rather than incidental for the type of assay, the ($hd$-design) is more appropriate. Any response to blanks should then be disregarded.

When these tests indicate that the assay is valid, the potency is calculated with its confidence limits as described in Section 3.3.5.

*3.3.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS*

### 3.3.5.1. The (*hd* + 1)-design

The common intersection $a'$ of the preparations can be calculated from:

$$a' = \frac{(2d + 1)B + (2d - 3)ha}{h(2d - 3) + 2d + 1} \qquad (3.3.5.1.\text{-}1)$$

The slope of the standard, and similarly for each of the other preparations, is calculated from:

$$b_S' = \frac{6L_S - 3d(d + 1)a'}{2d^3 + 3d^2 + d} \qquad (3.3.5.1.\text{-}2)$$

The potency ratio of each of the test preparations can now be calculated from:

$$R_T' = \frac{b_T'}{b_S'} \qquad (3.3.5.1.\text{-}3)$$

which has to be multiplied by $A_T$, the assumed potency of the test preparation, in order to find the estimated potency $R_T$. If the step between adjacent doses was not identical for the standard and the test preparation, the potency has to be multiplied by $I_S/I_T$. Note that, unlike the parallel-line analysis, no antilogarithms are calculated.

The confidence interval for $R_T'$ is calculated from:

$$CR'_T - K' \pm \sqrt{(C - 1)(CR'^2_T + 1) + K'(K' - 2CR'_T)} \qquad (3.3.5.1.\text{-}4)$$

$$\text{where } C = \frac{b'^2_S}{b'^2_S - s^2 t^2 V_1} \text{ and } K' = (C - 1)V_2$$

$V_1$ and $V_2$ are related to the variance and covariance of the numerator and denominator of $R_T$. They can be obtained from:

$$V_1 = \frac{6}{n(2d+1)}\left(\frac{1}{d(d+1)} + \frac{3}{2(2d+1)+hd(d-1)}\right) \qquad (3.3.5.1.\text{-}5)$$

$$V_2 = \frac{3d(d+1)}{(3d+1)(d+2)+hd(d-1)} \qquad (3.3.5.1.\text{-}6)$$

The confidence limits are multiplied by $A_T$, and if necessary by $I_S/I_T$.

**3.3.5.2. The (*hd*)-design**

The formulae are the same as for the ($hd$ + 1)-design, with the following modifications:

$$a' = a \qquad (3.3.5.2.\text{-}1)$$

$$V_1 = \frac{6}{nd(2d+1)}\left(\frac{1}{d+1} + \frac{3}{h(d-1)}\right) \qquad (3.3.5.2.\text{-}2)$$

$$V_2 = \frac{3(d+1)}{3(d+1)+h(d-1)} \qquad (3.3.5.2.\text{-}3)$$

3.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

This model is suitable, for example, for some immunoassays when analysis is required of extended sigmoid dose-response curves. This model is illustrated in Figure 3.4.-I.

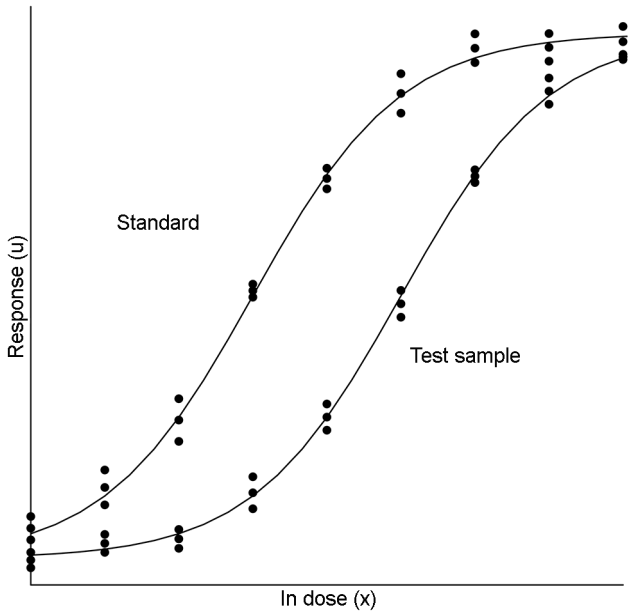


Figure 3.4.-I. – *The four-parameter logistic curve model*

The logarithms of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 curves are the calculated ln(dose)-response relationship for the standard and the test preparation.

The general shape of the curves can usually be described by a logistic function but other shapes are also possible. Each curve can be characterised by 4 parameters: The upper asymptote (α), the lower asymptote (δ), the slope-factor (β), and the horizontal location (γ). This model is therefore often referred to as a four-parameter model. A mathematical representation of the ln(dose)-response curve is:

$$u = \delta + \frac{\alpha - \delta}{1 + e^{-\beta(x-\gamma)}}$$

For a valid assay it is necessary that the curves of the standard and the test preparations have the same slope-factor, and the same maximum and minimum response level at the extreme parts. Only the horizontal location (γ) of the curves may be different. The horizontal distance between the curves is related to the "true" potency of the unknown. If the assay is used routinely, it may be sufficient to test the condition of equal upper and lower response levels when the assay is developed, and then to retest this condition directly only at suitable intervals or when there are changes in materials or assay conditions.

The maximum-likelihood estimates of the parameters and their confidence intervals can be obtained with suitable computer programs. These computer programs may include some statistical tests reflecting validity. For example, if the maximum likelihood estimation shows significant deviations from the fitted model under the assumed conditions of equal upper and lower asymptotes and slopes, then one or all of these conditions may not be satisfied.

The logistic model raises a number of statistical problems which may require different solutions for different types of assays, and no simple summary is possible. A wide variety of possible approaches is described in the relevant literature. Professional advice is therefore recommended for this type of analysis. A simple example is nevertheless included in Section 5.4 to illustrate a "possible" way to analyse the data presented. A short discussion of alternative approaches and other statistical considerations is given in Section 7.5.

If professional advice or suitable software is not available, alternative approaches are possible: 1) if "reasonable" estimates of the upper limit (α) and lower limit (δ) are available, select for all preparations the doses with mean of the responses ($u$) falling between approximately 20 per cent and 80 per cent of the limits, transform responses of the selected doses to:

$$y = \ln\left(\frac{u-\delta}{\alpha-u}\right)$$

and use the parallel line model (Section 3.2) for the analysis; 2) select a range of doses for which the responses ($u$) or suitably transformed responses, for example ln $u$, are approximately linear when plotted against ln(dose); the parallel line model (Section 3.2) may then be used for analysis.

## 4. ASSAYS DEPENDING UPON QUANTAL RESPONSES

4.1. INTRODUCTION

In certain assays it is impossible or excessively laborious to measure the effect on each experimental unit on a quantitative scale. Instead, an effect such as death or hypoglycaemic symptoms may be observed as either occurring or not occurring in each unit, and the result depends on the number of units in which it occurs. Such assays are called quantal or all-or-none.

The situation is very similar to that described for quantitative assays in Section 3.1, but in place of $n$ separate responses to each treatment a single value is recorded, i.e. the fraction of units in each treatment group showing a response. When these fractions are plotted against the logarithms of the doses the resulting curve will tend to be sigmoid (S-shaped) rather than linear. A mathematical function that represents this sigmoid curvature is used to estimate the dose-response curve. The most commonly used function is the cumulative normal distribution function. This function has some theoretical merit, and is perhaps the best choice if the response is a reflection of the tolerance of the units. If the response is more likely to depend upon a process of growth, the logistic distribution model is preferred, although the difference in outcome between the 2 models is usually very small.

The maximum likelihood estimators of the slope and location of the curves can be found only by applying an iterative procedure. There are many procedures which lead to the

same outcome, but they differ in efficiency due to the speed of convergence. One of the most rapid methods is direct optimisation of the maximum-likelihood function (see Section 7.1), which can easily be performed with computer programs having a built-in procedure for this purpose. Unfortunately, most of these procedures do not yield an estimate of the confidence interval, and the technique to obtain it is too complicated to describe here. The technique described below is not the most rapid, but has been chosen for its simplicity compared to the alternatives. It can be used for assays in which one or more test preparations are compared to a standard. Furthermore, the following conditions must be fulfilled:

1) the relationship between the logarithm of the dose and the response can be represented by a cumulative normal distribution curve,

2) the curves for the standard and the test preparation are parallel, i.e. they are identically shaped and may only differ in their horizontal location,

3) in theory, there is no natural response to extremely low doses and no natural non-response to extremely high doses.



## 4.2. THE PROBIT METHOD

The sigmoid curve can be made linear by replacing each response, i.e. the fraction of positive responses per group, by the corresponding value of the cumulative standard normal distribution. This value, often referred to as "normit", ranges theoretically from $-\infty$ to $+\infty$. In the past it was proposed to add 5 to each normit to obtain "probits". This facilitated the hand-performed calculations because negative values were avoided. With the arrival of computers the need to add 5 to the normits has disappeared. The term "normit method" would therefore be better for the method described below. However, since the term "probit analysis" is so widely spread, the term will, for historical reasons, be maintained in this text.

Once the responses have been linearised, it should be possible to apply the parallel-line analysis as described in Section 3.2. Unfortunately, the validity condition of homogeneity of variance for each dose is not fulfilled. The variance is minimal at normit = 0 and increases for positive and negative values of the normit. It is therefore necessary to give more weight to responses in the middle part of the curve, and less weight to the more extreme parts of the curve. This method, the analysis of variance, and the estimation of the potency and confidence interval are described below.

### *4.2.1. TABULATION OF THE RESULTS*

Table 4.2.1.-I is used to enter the data into the columns indicated by numbers:

(1) the dose of the standard or the test preparation,

(2) the number $n$ of units submitted to that treatment,

(3) the number of units $r$ giving a positive response to the treatment,

(4) the logarithm $x$ of the dose,

(5) the fraction $p = r/n$ of positive responses per group.

The first cycle starts here.

(6) column $Y$ is filled with zeros at the first iteration,

(7) the corresponding value $\Phi = \Phi(Y)$ of the cumulative standard normal distribution function (see also Table 8.4).

The columns (8) to (10) are calculated with the following formulae:

$$Z = \frac{e^{-Y^2/2}}{\sqrt{2\pi}} \qquad (4.2.1.\text{-}1)$$

$$y = Y + \frac{p - \Phi}{Z} \qquad (4.2.1.\text{-}2)$$

$$w = \frac{nZ^2}{\Phi - \Phi^2} \qquad (4.2.1.\text{-}3)$$

The columns (11) to (15) can easily be calculated from columns (4), (9) and (10) as $wx$, $wy$, $wx^2$, $wy^2$ and $wxy$ respectively, and the sum (Σ) of each of the columns (10) to (15) is calculated separately for each of the preparations.

The sums calculated in Table 4.2.1.-I are transferred to columns (1) to (6) of Table 4.2.1.-II and 6 additional columns (7) to (12) are calculated as follows:

$$S_{xx} = \sum wx^2 - \frac{\left(\sum wx\right)^2}{\sum w} \qquad (4.2.1.\text{-}4)$$

$$S_{xy} = \sum wxy - \frac{\left(\sum wx\right)\left(\sum wy\right)}{\sum w} \qquad (4.2.1.\text{-}5)$$

Table 4.2.1.-I. – *First working table*

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) | (13) | (14) | (15) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | dose | $n$ | $r$ | $x$ | $p$ | $Y$ | $\Phi$ | $Z$ | $y$ | $w$ | $wx$ | $wy$ | $wx^2$ | $wy^2$ | $wxy$ |
| $S$ | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | | | | | | | | | | Σ = | Σ = | Σ = | Σ = | Σ = | Σ = |
| $T$ | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| | | | | | | | | | | Σ = | Σ = | Σ = | Σ = | Σ = | Σ = |
| etc. | | | | | | | | | | | | | | | |

Table 4.2.1.-II. – *Second working table*

| | (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) | (12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $\Sigma w$ | $\Sigma wx$ | $\Sigma wy$ | $\Sigma wx^2$ | $\Sigma wy^2$ | $\Sigma wxy$ | $S_{xx}$ | $S_{xy}$ | $S_{yy}$ | $\bar{x}$ | $\bar{y}$ | $a$ |
| $S$ | . | . | . | . | . | . | . | . | . | . | . | . |
| $T$ | . | . | . | . | . | . | . | . | . | . | . | . |
| etc. | . | . | . | . | . | . | . | . | . | . | . | . |
| | | | | | | | Σ = | Σ = | | | | |

(9) $$S_{yy} = \sum wy^2 - \frac{(\sum wy)^2}{\sum w} \qquad (4.2.1.\text{-}6)$$

(10) $$\overline{x} = \frac{\sum wx}{\sum w} \qquad (4.2.1.\text{-}7)$$

(11) $$\overline{y} = \frac{\sum wy}{\sum w} \qquad (4.2.1.\text{-}8)$$

The common slope $b$ can now be obtained as:

$$b = \frac{\sum S_{xy}}{\sum S_{xx}} \qquad (4.2.1.\text{-}9)$$

and the intercept $a$ of the standard, and similarly for the test preparations is obtained as:

(12) $$a = \overline{y} - b\overline{x} \qquad (4.2.1.\text{-}10)$$

Column (6) of the first working table can now be replaced by $Y = a + bx$ and the cycle is repeated until the difference between 2 cycles has become small (e.g. the maximum difference of $Y$ between 2 consecutive cycles is smaller than $10^{-8}$).

*4.2.2. TESTS OF VALIDITY*

Before calculating the potencies and confidence intervals, validity of the assay must be assessed. If at least 3 doses for each preparation have been included, the deviations from linearity can be measured as follows: add a 13th column to Table 4.2.1.-II and fill it with:

$$S_{yy} - \frac{S_{xy}^2}{S_{xx}} \qquad (4.2.2.\text{-}1)$$

The column total is a measure of deviations from linearity and is approximately $\chi^2$ distributed with degrees of freedom equal to $N - 2h$. Significance of this value may be assessed with the aid of Table 8.3 or a suitable sub-routine in a computer program. If the value is significant at the 0.05 probability level, the assay must probably be rejected (see Section 4.2.4).

When the above test gives no indication of significant deviations from linear regression, the deviations from parallelism are tested at the 0.05 significance level with:

$$\chi^2 = \sum \frac{S_{xy}^2}{S_{xx}} - \frac{\left(\sum S_{xy}\right)^2}{\sum S_{xx}} \qquad (4.2.2.\text{-}2)$$

with $h - 1$ degrees of freedom.

*4.2.3. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS*

When there are no indications for a significant departure from parallelism and linearity the ln(potency ratio) $M'_T$ is calculated as:

$$M'_T = \frac{a_T - a_S}{b} \qquad (4.2.3.\text{-}1)$$

and the antilogarithm is taken. Now let $t = 1.96$ and $s = 1$. Confidence limits are calculated as the antilogarithms of:

$$CM'_T - (C-1)(\overline{x}_S - \overline{x}_T) \pm \sqrt{(C-1)\left(V\sum S_{xx} + C(M'_T - \overline{x}_S + \overline{x}_T)^2\right)} \qquad (4.2.3.\text{-}2)$$

where $C = \dfrac{b^2 \sum S_{xx}}{b^2 \sum S_{xx} - s^2 t^2}$ and $V = \dfrac{1}{\sum\limits_S w} + \dfrac{1}{\sum\limits_T w}$

*4.2.4. INVALID ASSAYS*

If the test for deviations from linearity described in Section 4.2.2 is significant, the assay should normally be rejected. If there are reasons to retain the assay, the formulae are slightly modified. $t$ becomes the $t$-value ($p = 0.05$) with the same number of degrees of freedom as used in the check for linearity and $s^2$ becomes the $\chi^2$ value divided by the same number of degrees of freedom (and thus typically is greater than 1).

The test for parallelism is also slightly modified. The $\chi^2$ value for non-parallelism is divided by its number of degrees of freedom. The resulting value is divided by $s^2$ calculated above to obtain an $F$-ratio with $h$ - 1 and $N$ - $2h$ degrees of freedom, which is evaluated in the usual way at the 0.05 significance level.

4.3. THE LOGIT METHOD

As indicated in Section 4.1 the logit method may sometimes be more appropriate. The name of the method is derived from the logit function which is the inverse of the logistic distribution. The procedure is similar to that described for the probit method with the following modifications in the formulae for $\Phi$ and $Z$.

$$\Phi = \frac{1}{1 + e^{-Y}} \qquad (4.3.\text{-}1)$$

$$Z = \frac{e^{-Y}}{(1 + e^{-Y})^2} \qquad (4.3.\text{-}2)$$

4.4. OTHER SHAPES OF THE CURVE

The probit and logit method are almost always adequate for the analysis of quantal responses called for in the European Pharmacopoeia. However, if it can be made evident that the ln(dose)-response curve has another shape than the 2 curves described above, another curve $\Phi$ may be adopted. $Z$ is then taken to be the first derivative of $\Phi$.

For example, if it can be shown that the curve is not symmetric, the Gompertz distribution may be appropriate (the gompit method) in which case $\Phi = 1 - e^{-e^Y}$ and $Z = e^{Y - e^Y}$.

4.5. THE MEDIAN EFFECTIVE DOSE

In some types of assay it is desirable to determine a median effective dose which is the dose that produces a response in 50 per cent of the units. The probit method can be used to determine this median effective dose ($ED_{50}$), but since there is no need to express this dose relative to a standard, the formulae are slightly different.

*Note*: a standard can optionally be included in order to validate the assay. Usually the assay is considered valid if the calculated $ED_{50}$ of the standard is close enough to the assigned $ED_{50}$. What "close enough" in this context means depends on the requirements specified in the monograph.

The tabulation of the responses to the test samples, and optionally a standard, is as described in Section 4.2.1. The test for linearity is as described in Section 4.2.2. A test for parallelism is not necessary for this type of assay. The $ED_{50}$ of test sample $T$, and similarly for the other samples, is obtained as described in Section 4.2.3, with the following modifications in formulae 4.2.3.-1 and 4.2.3.-2:

$$M'_T = \frac{-a_T}{b} \qquad (4.5.\text{-}1)$$

$$CM'_T - (C-1)\overline{x}_T \pm \sqrt{(C-1)\left(V\sum S_{xx} + C(M'_T - \overline{x}_T)^2\right)} \qquad (4.5.\text{-}2)$$

where $V = \dfrac{1}{\sum\limits_T w}$ and $C$ is left unchanged

**5. EXAMPLES**

This section consists of worked examples illustrating the application of the formulae. The examples have been selected primarily to illustrate the statistical method of calculation. They are not intended to reflect the most suitable method

5. General texts

of assay, if alternatives are permitted in the individual monographs. To increase their value as program checks, more decimal places are given than would usually be necessary. It should also be noted that other, but equivalent methods of calculation exist. These methods should lead to exactly the same final results as those given in the examples.

## 5.1. PARALLEL-LINE MODEL

### *5.1.1. TWO-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN*

*An assay of corticotrophin by subcutaneous injection in rats*

The standard preparation is administered at 0.25 and 1.0 units per 100 g of body mass. Two preparations to be examined are both assumed to have a potency of 1 unit per milligram and they are administered in the same quantities as the standard. The individual responses and means per treatment are given in Table 5.1.1.-I. A graphical presentation (Figure 5.1.1.-I) gives no rise to doubt the homogeneity of variance and normality of the data, but suggests problems with parallelism for preparation *U*.

Table 5.1.1.-I. – *Response metameter y: mass of ascorbic acid (mg) per 100 g of adrenal gland*

| | **Standard *S*** | | **Preparation *T*** | | **Preparation *U*** | |
|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $T_1$ | $T_2$ | $U_1$ | $U_2$ |
| | 300 | 289 | 310 | 230 | 250 | 236 |
| | 310 | 221 | 290 | 210 | 268 | 213 |
| | 330 | 267 | 360 | 280 | 273 | 283 |
| | 290 | 236 | 341 | 261 | 240 | 269 |
| | 364 | 250 | 321 | 241 | 307 | 251 |
| | 328 | 231 | 370 | 290 | 270 | 294 |
| | 390 | 229 | 303 | 223 | 317 | 223 |
| | 360 | 269 | 334 | 254 | 312 | 250 |
| | 342 | 233 | 295 | 216 | 320 | 216 |
| | 306 | 259 | 315 | 235 | 265 | 265 |
| Mean | 332.0 | 248.4 | 323.9 | 244.0 | 282.2 | 250.0 |

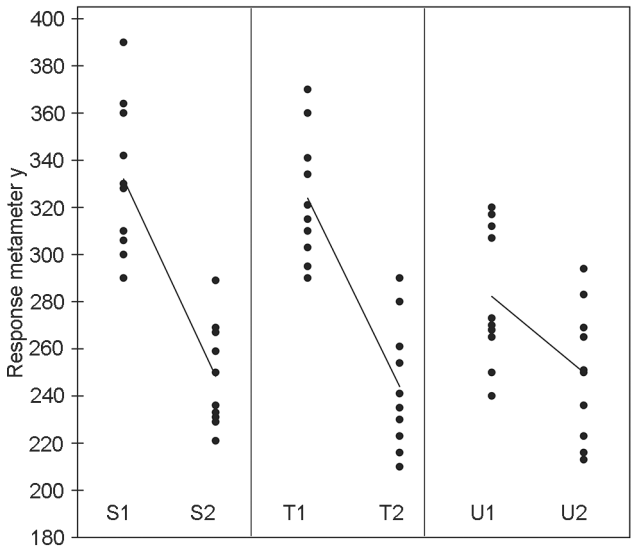


Figure 5.1.1.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

$P_S = 580.4$ $L_S = -41.8$

$P_T = 567.9$ $L_T = -39.95$

$P_U = 532.2$ $L_U = -16.1$

$H_P = \frac{10}{2} = 5$ $H_L = \frac{120}{6} = 20$

The analysis of variance can now be completed with the formulae in Tables 3.2.3-III and 3.2.3.-IV. This is shown in Table 5.1.1.-II.

Table 5.1.1.-II. – *Analysis of variance*

| **Source of variation** | **Degrees of freedom** | **Sum of squares** | **Mean square** | ***F*-ratio** | **Probability** |
|---|---|---|---|---|---|
| Preparations | 2 | 6256.6 | 3128.3 | | |
| Regression | 1 | 63 830.8 | 63 830.8 | 83.38 | 0.000 |
| Non-parallelism | 2 | 8218.2 | 4109.1 | 5.37 | 0.007 |
| Treatments | 5 | 78 305.7 | | | |
| Residual error | 54 | 41 340.9 | 765.57 | | |
| Total | 59 | 119 646.6 | | | |

The analysis confirms a highly significant linear regression. Departure from parallelism, however, is also significant ($p = 0.0075$) which was to be expected from the graphical observation that preparation *U* is not parallel to the standard. This preparation is therefore rejected and the analysis repeated using only preparation *T* and the standard (Table 5.1.1.-III).

Table 5.1.1.-III. – *Analysis of variance without sample U*

| **Source of variation** | **Degrees of freedom** | **Sum of squares** | **Mean square** | ***F*-ratio** | **Probability** |
|---|---|---|---|---|---|
| Preparations | 1 | 390.6 | 390.6 | | |
| Regression | 1 | 66 830.6 | 66 830.6 | 90.5 | 0.000 |
| Non-parallelism | 1 | 34.2 | 34.2 | 0.05 | 0.831 |
| Treatments | 3 | 67 255.5 | | | |
| Residual error | 36 | 26 587.3 | 738.54 | | |
| Total | 39 | 93 842.8 | | | |

The analysis without preparation *U* results in compliance with the requirements with respect to both regression and parallelism and so the potency can be calculated. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{20(-41.8 - 39.95)}{\ln 4 \times 10 \times 2} = -58.970$$

– the ln(potency ratio) is:

$$M_T' = \frac{567.9 - 580.4}{2 \times (-58.970)} = 0.1060$$

$$C = \frac{66\,830.6}{66\,830.6 - 738.54 \times 2.028^2} = 1.0476$$

$$V = \frac{66\,830.6}{(-58.970)^2 \times 2 \times 10} = 0.9609$$

– and ln(confidence limits) are:

$$1.0476 \times 0.1060 \pm \sqrt{0.0476 \times (1.0476 \times 0.1060^2 + 2 \times 0.9609)}$$
$$= 0.1110 \pm 0.3034$$

By taking the antilogarithms we find a potency ratio of 1.11 with 95 per cent confidence limits from 0.82-1.51.

Multiplying by the assumed potency of preparation *T* yields a potency of 1.11 units/mg with 95 per cent confidence limits from 0.82 to 1.51 units/mg.

### *5.1.2. THREE-DOSE LATIN SQUARE DESIGN*

*Antibiotic agar diffusion assay using a rectangular tray*

The standard has an assigned potency of 4855 IU/mg. The test preparation has an assumed potency of 5600 IU/mg. For the stock solutions 25.2 mg of the standard is dissolved in 24.5 mL of solvent and 21.4 mg of the test preparation is dissolved in 23.95 mL of solvent. The final solutions are prepared by first diluting both stock solutions to 1/20 and further using a dilution ratio of 1.5.

A Latin square is generated with the method described in Section 8.6 (see Table 5.1.2.-I). The responses of this routine assay are shown in Table 5.1.2.-II (inhibition zones in mm × 10). The treatment mean values are shown in Table 5.1.2.-III. A graphical representation of the data (see Figure 5.1.2.-I) gives no rise to doubt the normality or homogeneity of variance of the data.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

$P_S = 529.667$ $L_S = 35.833$

$P_T = 526.333$ $L_T = 39.333$

$H_P = \frac{6}{3} = 2$ $H_L = \frac{72}{24} = 3$

The analysis of variance can now be completed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. The result is shown in Table 5.1.2.-IV.

The analysis shows significant differences between the rows. This indicates the increased precision achieved by using a Latin square design rather than a completely randomised design. A highly significant regression and no significant departure of the individual regression lines from parallelism and linearity confirms that the assay is satisfactory for potency calculations.

Table 5.1.2.-I. – *Distribution of treatments over the plate*

| | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| 1 | $S_1$ | $T_1$ | $T_2$ | $S_3$ | $S_2$ | $T_3$ |
| 2 | $T_1$ | $T_3$ | $S_1$ | $S_2$ | $T_2$ | $S_3$ |
| 3 | $T_2$ | $S_3$ | $S_2$ | $S_1$ | $T_3$ | $T_1$ |
| 4 | $S_3$ | $S_2$ | $T_3$ | $T_1$ | $S_1$ | $T_2$ |
| 5 | $S_2$ | $T_2$ | $S_3$ | $T_3$ | $T_1$ | $S_1$ |
| 6 | $T_3$ | $S_1$ | $T_1$ | $T_2$ | $S_3$ | $S_2$ |

Table 5.1.2.-II. – *Measured inhibition zones in mm × 10*

| | 1 | 2 | 3 | 4 | 5 | 6 | Row mean |
|---|---|---|---|---|---|---|---|
| 1 | 161 | 160 | 178 | 187 | 171 | 194 | 175.2 = $R_1$ |
| 2 | 151 | 192 | 150 | 172 | 170 | 192 | 171.2 = $R_2$ |
| 3 | 162 | 195 | 174 | 161 | 193 | 151 | 172.7 = $R_3$ |
| 4 | 194 | 184 | 199 | 160 | 163 | 171 | 178.5 = $R_4$ |
| 5 | 176 | 181 | 201 | 202 | 154 | 151 | 177.5 = $R_5$ |
| 6 | 193 | 166 | 161 | 186 | 198 | 182 | 181.0 = $R_6$ |
| Col. Mean = | 172.8 = $C_1$ | 179.7 = $C_2$ | 177.2 = $C_3$ | 178.0 = $C_4$ | 174.8 = $C_5$ | 173.5 = $C_6$ | |

Table 5.1.2.-III. – *Means of the treatments*

| | **Standard *S*** | | | **Preparation *T*** | | |
|---|---|---|---|---|---|---|
| | $S_1$ | $S_2$ | $S_3$ | $T_1$ | $T_2$ | $T_3$ |
| Mean | 158.67 | 176.50 | 194.50 | 156.17 | 174.67 | 195.50 |

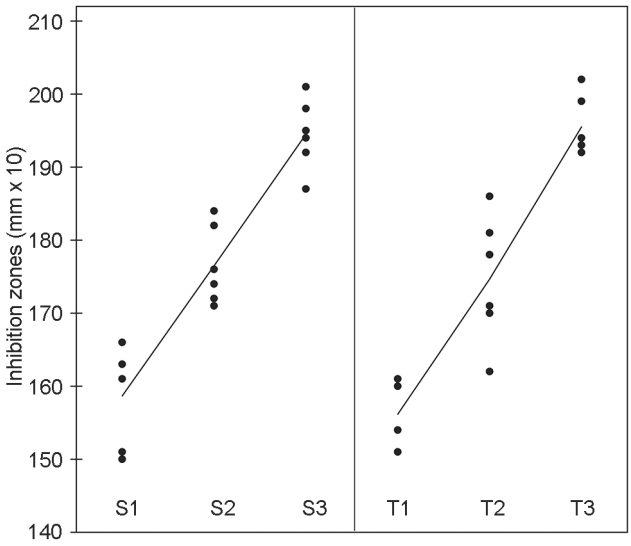


Figure 5.1.2.-I.

Table 5.1.2.-IV. – *Analysis of variance*

| **Source of variation** | **Degrees of freedom** | **Sum of squares** | **Mean square** | ***F*-ratio** | **Probability** |
|---|---|---|---|---|---|
| Preparations | 1 | 11.1111 | 11.1111 | | |
| Regression | 1 | 8475.0417 | 8475.0417 | 408.1 | 0.000 |
| Non-parallelism | 1 | 18.3750 | 18.3750 | 0.885 | 0.358 |
| Non-linearity | 2 | 5.4722 | 2.7361 | 0.132 | 0.877 |
| Treatments | 5 | 8510 | | | |
| Rows | 5 | 412 | 82.40 | 3.968 | 0.012 |
| Columns | 5 | 218.6667 | 43.73 | 2.106 | 0.107 |
| Residual error | 20 | 415.3333 | 20.7667 | | |
| Total | 35 | 9556 | | | |

The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{3 \times (35.833 + 39.333)}{\ln(1.5) \times 6 \times 2} = 46.346$$

– the ln(potency ratio) is:

$$M'_T = \frac{526.333 - 529.667}{3 \times 46.346} = -0.023974$$

$$C = \frac{8475.0417}{8475.0417 - 20.7667 \times 2.086^2} = 1.0108$$

$$V = \frac{8475.0417}{46.346^2 \times 3 \times 6} = 0.2192$$

– and ln(confidence limits) are:

$$1.0108 \times (-0.0240) \pm \sqrt{0.0108 \times (1.0108 \times (-0.0240)^2 + 2 \times 0.2192)}$$
$$= -0.02423 \pm 0.06878$$

The potency ratio is found by taking the antilogarithms, resulting in 0.9763 with 95 per cent confidence limits from 0.9112-1.0456.

A correction factor of:

$$\frac{4855 \times 25.2 / 24.5}{5600 \times 21.4 / 23.95} = 0.99799$$

is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 5600 IU/mg yields a potency of 5456 IU/mg with 95 per cent confidence limits from 5092 to 5843 IU/mg.

*5.1.3. FOUR-DOSE RANDOMISED BLOCK DESIGN*

*Antibiotic turbidimetric assay*

This assay is designed to assign a potency in international units per vial. The standard has an assigned potency of 670 IU/mg. The test preparation has an assumed potency of 20 000 IU/vial. On the basis of this information the stock solutions are prepared as follows. 16.7 mg of the standard is dissolved in 25 mL solvent and the contents of one vial of the test preparation are dissolved in 40 mL solvent. The final solutions are prepared by first diluting to 1/40 and further using a dilution ratio of 1.5. The tubes are placed in a water-bath in a randomised block arrangement (see Section 8.5). The responses are listed in Table 5.1.3.-I.

Inspection of Figure 5.1.3.-I gives no rise to doubt the validity of the assumptions of normality and homogeneity of variance of the data. The standard deviation of $S_3$ is somewhat high but is no reason for concern.

Table 5.1.3.-I. – *Absorbances of the suspensions (× 1000)*

| | Standard $S$ | | | | Preparation $T$ | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Block | $S_1$ | $S_2$ | $S_3$ | $S_4$ | $T_1$ | $T_2$ | $T_3$ | $T_4$ | Mean |
| 1 | 252 | 207 | 168 | 113 | 242 | 206 | 146 | 115 | 181.1 |
| 2 | 249 | 201 | 187 | 107 | 236 | 197 | 153 | 102 | 179.0 |
| 3 | 247 | 193 | 162 | 111 | 246 | 197 | 148 | 104 | 176.0 |
| 4 | 250 | 207 | 155 | 108 | 231 | 191 | 159 | 106 | 175.9 |
| 5 | 235 | 207 | 140 | 98 | 232 | 186 | 146 | 95 | 167.4 |
| Mean | 246.6 | 203.0 | 162.4 | 107.4 | 237.4 | 195.4 | 150.4 | 104.4 | |

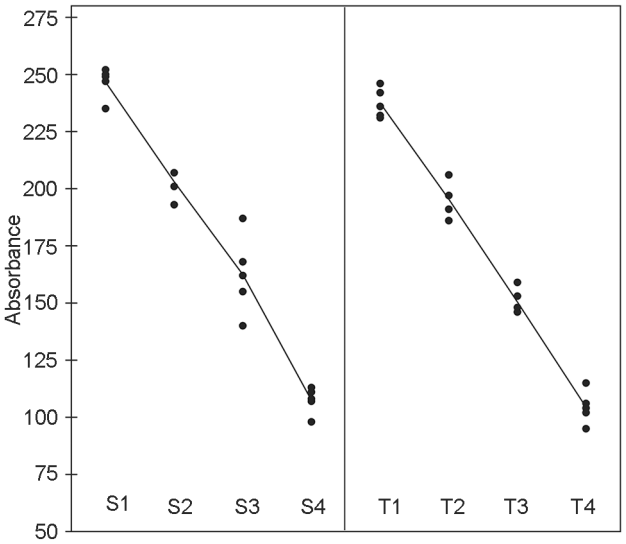


Figure 5.1.3.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

$P_S = 719.4$ $L_S = -229.1$

$P_T = 687.6$ $L_T = -222$

$H_P = \frac{5}{4} = 1.25$ $H_L = \frac{60}{60} = 1$

The analysis of variance is constructed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. The result is shown in Table 5.1.3.-II.

Table 5.1.3.-II. – *Analysis of variance*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | $F$-ratio | Probability |
|---|---|---|---|---|---|
| Preparations | 1 | 632.025 | 632.025 | | |
| Regression | 1 | 101 745.6 | 101 745.6 | 1887.1 | 0.000 |
| Non-parallelism | 1 | 25.205 | 25.205 | 0.467 | 0.500 |
| Non-linearity | 4 | 259.14 | 64.785 | 1.202 | 0.332 |
| Treatments | 7 | 102 662 | | | |
| Blocks | 4 | 876.75 | 219.188 | 4.065 | 0.010 |
| Residual error | 28 | 1509.65 | 53.916 | | |
| Total | 39 | 105 048.4 | | | |

A significant difference is found between the blocks. This indicates the increased precision achieved by using a randomised block design. A highly significant regression and no significant departure from parallelism and linearity confirms that the assay is satisfactory for potency calculations. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{1 \times (-229.1 - 222)}{\ln(1.5) \times 5 \times 2} = -111.255$$

– the ln(potency ratio) is:

$$M_T' = \frac{687.6 - 719.4}{4 \times (-111.255)} = 0.071457$$

$$C = \frac{101\,745.6}{101\,745.6 - 53.916 \times 2.048^2} = 1.00223$$

$$V = \frac{101\,745.6}{(-111.255)^2 \times 4 \times 5} = 0.4110$$

– and ln(confidence limits) are:

$$1.00223 \times 0.0715 \pm$$

$$\sqrt{0.00223 \times (1.00223 \times 0.0715^2 + 2 \times 0.4110)}$$

$$= 0.07162 \pm 0.04293$$

The potency ratio is found by taking the antilogarithms, resulting in 1.0741 with 95 per cent confidence limits from 1.0291 to 1.1214. A correction factor of:

$$\frac{670 \times 16.7 / 25}{20\,000 \times 1 / 40} = 0.89512$$

is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 20 000 IU/vial yields a potency of 19 228 IU/vial with 95 per cent confidence limits from 18 423-20 075 IU/vial.

*5.1.4. FIVE-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN*

*An in-vitro assay of three hepatitis B vaccines against a standard*

Three independent two-fold dilution series of 5 dilutions were prepared from each of the vaccines. After some additional steps in the assay procedure, absorbances were measured. They are shown in Table 5.1.4.-I.

Table 5.1.4.-I. – *Optical densities*

| Dilution | Standard *S* | | | Preparation *T* | | |
|---|---|---|---|---|---|---|
| 1:16 000 | 0.043 | 0.045 | 0.051 | 0.097 | 0.097 | 0.094 |
| 1:8000 | 0.093 | 0.099 | 0.082 | 0.167 | 0.157 | 0.178 |
| 1:4000 | 0.159 | 0.154 | 0.166 | 0.327 | 0.355 | 0.345 |
| 1:2000 | 0.283 | 0.295 | 0.362 | 0.501 | 0.665 | 0.576 |
| 1:1000 | 0.514 | 0.531 | 0.545 | 1.140 | 1.386 | 1.051 |

| Dilution | Preparation *U* | | | Preparation *V* | | |
|---|---|---|---|---|---|---|
| 1:16 000 | 0.086 | 0.071 | 0.073 | 0.082 | 0.082 | 0.086 |
| 1:8000 | 0.127 | 0.146 | 0.133 | 0.145 | 0.144 | 0.173 |
| 1:4000 | 0.277 | 0.268 | 0.269 | 0.318 | 0.306 | 0.316 |
| 1:2000 | 0.586 | 0.489 | 0.546 | 0.552 | 0.551 | 0.624 |
| 1:1000 | 0.957 | 0.866 | 1.045 | 1.037 | 1.039 | 1.068 |

The logarithms of the optical densities are known to have a linear relationship with the logarithms of the doses. The mean responses of the ln-transformed optical densities are listed in Table 5.1.4.-II. No unusual features are discovered in a graphical presentation of the data (Figure 5.1.4.-I).

Table 5.1.4.-II. – *Means of the ln-transformed absorbances*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| $S_1$ | − 3.075 | $T_1$ | − 2.344 | $U_1$ | − 2.572 | $V_1$ | − 2.485 |
| $S_2$ | − 2.396 | $T_2$ | − 1.789 | $U_2$ | − 2.002 | $V_2$ | − 1.874 |
| $S_3$ | − 1.835 | $T_3$ | − 1.073 | $U_3$ | − 1.305 | $V_3$ | − 1.161 |
| $S_4$ | − 1.166 | $T_4$ | − 0.550 | $U_4$ | − 0.618 | $V_4$ | − 0.554 |
| $S_5$ | − 0.635 | $T_5$ | 0.169 | $U_5$ | − 0.048 | $V_5$ | 0.047 |

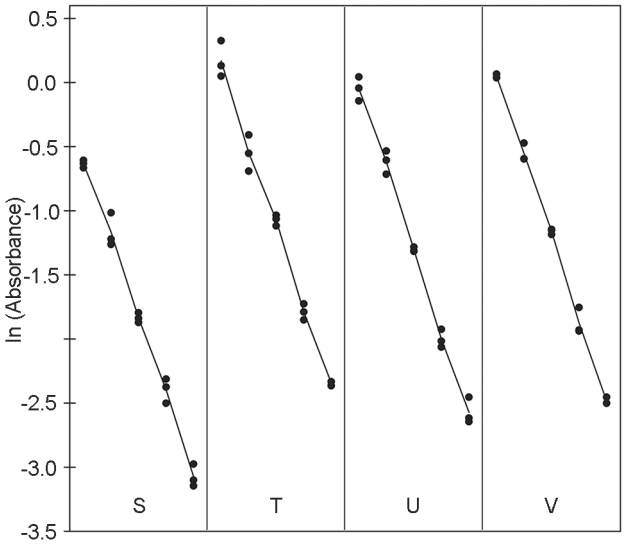


Figure 5.1.4.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II give:

$P_S = -9.108$ $L_S = 6.109$

$P_T = -5.586$ $L_T = 6.264$

$P_U = -6.544$ $L_U = 6.431$

$P_V = -6.027$ $L_V = 6.384$

$H_P = \frac{3}{5} = 0.6$ $H_L = \frac{36}{120} = 0.3$

The analysis of variance is completed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. This is shown in Table 5.1.4.-III.

Table 5.1.4.-III. – *Analysis of variance*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F*-ratio | Probability |
|---|---|---|---|---|---|
| Preparations | 3 | 4.475 | 1.492 | | |
| Regression | 1 | 47.58 | 47.58 | 7126 | 0.000 |
| Non-parallelism | 3 | 0.0187 | 0.006 | 0.933 | 0.434 |
| Non-linearity | 12 | 0.0742 | 0.006 | 0.926 | 0.531 |
| Treatments | 19 | 52.152 | | | |
| Residual error | 40 | 0.267 | 0.0067 | | |
| Total | 59 | 52.42 | | | |

A highly significant regression and a non-significant departure from parallelism and linearity confirm that the potencies can be safely calculated. The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{0.3 \times (6.109 + 6.264 + 6.431 + 6.384)}{\ln 2 \times 3 \times 4} = 0.90848$$

– the ln(potency ratio) for preparation *T* is:

$$M'_T = \frac{-5.586 - (-9.108)}{5 \times 0.90848} = 0.7752$$

$$C = \frac{47.58}{47.58 - 0.0067 \times 2.021^2} = 1.00057$$

$$V = \frac{47.58}{0.9085^2 \times 5 \times 3} = 3.8436$$

– and ln(confidence limits) for preparation *T* are:

$$1.00057 \times 0.7752 \pm \sqrt{0.00057 \times (1.00057 \times 0.7752^2 + 2 \times 3.8436)}$$

$$= 0.7756 \pm 0.0689$$

By taking the antilogarithms a potency ratio of 2.171 is found with 95 per cent confidence limits from 2.027 to 2.327. All samples have an assigned potency of 20 µg protein/mL and so a potency of 43.4 µg protein/mL is found for test preparation *T* with 95 per cent confidence limits from 40.5-46.5 µg protein/mL.

The same procedure is followed to estimate the potency and confidence interval of the other test preparations. The results are listed in Table 5.1.4.-IV.

Table 5.1.4.-IV. – *Final potency estimates and 95 per cent confidence intervals of the test vaccines (in µg protein/mL)*

| | Lower limit | Estimate | Upper limit |
|---|---|---|---|
| **Vaccine *T*** | 40.5 | 43.4 | 46.5 |
| **Vaccine *U*** | 32.9 | 35.2 | 37.6 |
| **Vaccine *V*** | 36.8 | 39.4 | 42.2 |

### *5.1.5. TWIN CROSS-OVER DESIGN*

*Assay of insulin by subcutaneous injection in rabbits*

The standard preparation was administered at 1 unit and 2 units per millilitre. Equivalent doses of the unknown preparation were used based on an assumed potency of 40 units per millilitre. The rabbits received subcutaneously 0.5 mL of the appropriate solutions according to the design in Table 5.1.5.-I and responses obtained are shown in Table 5.1.5.-II and Figure 5.1.5.-I. The large variance illustrates the variation between rabbits and the need to employ a cross-over design.

Table 5.1.5.-I. – *Arrangements of treatments*

| | Group of rabbits | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| Day 1 | $S_1$ | $S_2$ | $T_1$ | $T_2$ |
| Day 2 | $T_2$ | $T_1$ | $S_2$ | $S_1$ |

Table 5.1.5.-II. – *Response y: sum of blood glucose readings (mg/100 mL) at 1 h and 2.5 h*

| | Group 1 | | Group 2 | | Group 3 | | Group 4 | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $T_2$ | $S_2$ | $T_1$ | $T_1$ | $S_2$ | $T_2$ | $S_1$ |
| | 112 | 104 | 65 | 72 | 105 | 91 | 118 | 144 |
| | 126 | 112 | 116 | 160 | 83 | 67 | 119 | 149 |
| | 62 | 58 | 73 | 72 | 125 | 67 | 42 | 51 |
| | 86 | 63 | 47 | 93 | 56 | 45 | 64 | 107 |
| | 52 | 53 | 88 | 113 | 92 | 84 | 93 | 117 |
| | 110 | 113 | 63 | 71 | 101 | 56 | 73 | 128 |
| | 116 | 91 | 50 | 65 | 66 | 55 | 39 | 87 |
| | 101 | 68 | 55 | 100 | 91 | 68 | 31 | 71 |
| Mean | 95.6 | 82.8 | 69.6 | 93.3 | 89.9 | 66.6 | 72.4 | 106.8 |

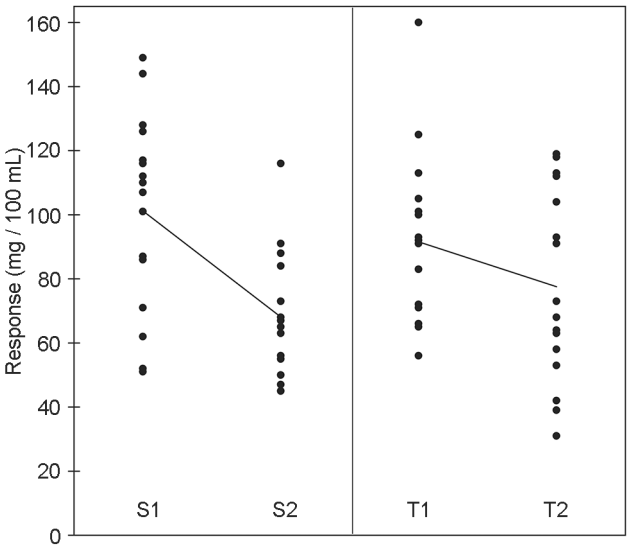


Figure 5.1.5.-I.

The analysis of variance is more complicated for this assay than for the other designs given because the component of the sum of squares due to parallelism is not independent of the component due to rabbit differences. Testing of the parallelism of the regression lines involves a second error-mean-square term obtained by subtracting the parallelism component and 2 "interaction" components from the component due to rabbit differences.

Three "interaction" components are present in the analysis of variance due to replication within each group:

days × preparation; days × regression; days × parallelism.

These terms indicate the tendency for the components (preparations, regression and parallelism) to vary from day to day. The corresponding *F*-ratios thus provide checks on these aspects of assay validity. If the values of *F* obtained are significantly high, care should be exercised in interpreting the results of the assay and, if possible, the assay should be repeated.

The analysis of variance is constructed by applying the formulae given in Tables 3.2.3.-I to 3.2.3.-III separately for both days and for the pooled set of data. The formulae in Tables 3.2.3.-I and 3.2.3.-II give:

Day 1: $P_S = 165.25$ $L_S = -13$

$P_T = 162.25$ $L_T = -8.75$

$H_P = \frac{8}{2} = 4$ $H_L = \frac{96}{6} = 16$

Day 2: $P_S = 173.38$ $L_S = -20.06$

$P_T = 176.00$ $L_T = -5.25$

$H_P = \frac{8}{2} = 4$ $H_L = \frac{96}{6} = 16$

Pooled: $P_S = 169.31$ $L_S = -16.53$

$P_T = 169.13$ $L_T = -7.00$

$H_P = \frac{16}{2} = 8$ $H_L = \frac{192}{6} = 32$

and with the formulae in Table 3.2.3.-III this leads to:

| Day 1 | Day 2 | Pooled |
|---|---|---|
| $SS_{prep} = 18.000$ | $SS_{prep} = 13.781$ | $SS_{prep} = 0.141$ |
| $SS_{reg} = 3784.5$ | $SS_{reg} = 5125.8$ | $SS_{reg} = 8859.5$ |
| $SS_{par} = 144.5$ | $SS_{par} = 1755.3$ | $SS_{par} = 1453.5$ |

The interaction terms are found as Day 1 + Day 2 - Pooled.

$$SS_{\text{days}\times\text{prep}} = 31.64$$

$$SS_{\text{days}\times\text{reg}} = 50.77$$

$$SS_{\text{days}\times\text{par}} = 446.27$$

In addition the sum of squares due to day-to-day variation is calculated as:

$$SS_{\text{days}} = \frac{1}{2}N(D_1^2 + D_2^2) - K = 478.52$$

and the sum of squares due to blocks (the variation between rabbits) as:

$$SS_{\text{block}} = 2\sum B_i^2 - K = 39\,794.7$$

where $B_i$ is the mean response per rabbit.

The analysis of variance can now be completed as shown in Table 5.1.5.-III.

Table 5.1.5.-III. – *Analysis of variance*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F*-ratio | Probability |
|---|---|---|---|---|---|
| Non-parallelism | 1 | 1453.5 | 1453.5 | 1.064 | 0.311 |
| Days × Prep. | 1 | 31.6 | 31.6 | 0.023 | 0.880 |
| Days × Regr. | 1 | 50.8 | 50.8 | 0.037 | 0.849 |
| Residual error between rabbits | 28 | 38 258.8 | 1366.4 | | |
| Rabbits | 31 | 39 794.7 | 1283.7 | | |
| Preparations | 1 | 0.14 | 0.14 | 0.001 | 0.975 |
| Regression | 1 | 8859.5 | 8859.5 | 64.532 | 0.000 |
| Days | 1 | 478.5 | 478.5 | 3.485 | 0.072 |
| Days × non-par. | 1 | 446.3 | 446.3 | 3.251 | 0.082 |
| Residual error within rabbits | 28 | 3844.1 | 137.3 | | |
| Total | 63 | 53 423.2 | | | |

The analysis of variance confirms that the data fulfil the necessary conditions for a satisfactory assay: a highly significant regression, no significant departures from parallelism, and none of the three interaction components is significant.

The formulae in Section 3.2.5 give:

– for the common slope:

$$b = \frac{32 \times (-16.53 - 7)}{\ln 2 \times 16 \times 2} = -33.95$$

– the ln(potency ratio) is:

$$M'_T = \frac{169.13 - 169.31}{2 \times (-33.95)} = 0.00276$$

$$C = \frac{8859.5}{8859.5 - 137.3 \times 2.048^2} = 1.0695$$

$$V = \frac{8859.5}{(-33.95)^2 \times 2 \times 16} = 0.2402$$

– and ln(confidence limits) are:

$$1.0695 \times 0.00276 \pm \sqrt{0.0695 \times (1.0695 \times 0.00276^2 + 2 \times 0.2402)}$$
$$= 0.00295 \pm 0.18279$$

By taking the antilogarithms a potency ratio of 1.003 with 95 per cent confidence limits from 0.835 to 1.204 is found. Multiplying by $A_T$ = 40 yields a potency of 40.1 units per millilitre with 95 per cent confidence limits from 33.4-48.2 units per millilitre.

## 5.2. SLOPE-RATIO MODEL

### *5.2.1. A COMPLETELY RANDOMISED (0,3,3)-DESIGN*

*An assay of factor VIII*

A laboratory carries out a chromogenic assay of factor VIII activity in concentrates. The laboratory has no experience with the type of assay but is trying to make it operational. Three equivalent dilutions are prepared of both the standard and the test preparation. In addition a blank is prepared, although a linear dose-response relationship is not expected for low doses. 8 replicates of each dilution are prepared, which is more than would be done in a routine assay.

A graphical presentation of the data shows clearly that the dose-response relationship is indeed not linear at low doses. The responses to blanks will therefore not be used in the calculations (further assays are of course needed to justify this decision). The formulae in Tables 3.3.3.1.-I and 3.3.3.1.-II yield:

| | | | |
|---|---|---|---|
| $P_S$ | = 0.6524 | $P_T$ | = 0.5651 |
| $L_S$ | = 1.4693 | $L_T$ | = 1.2656 |
| $a_S$ | = 0.318 | $a_T$ | = 0.318 |
| $b_S$ | = 0.329 | $b_T$ | = 0.271 |
| $G_S$ | = 0.1554 | $G_T$ | = 0.1156 |
| $J_S$ | = $4.17 \cdot 10^{-8}$ | $J_T$ | = $2.84 \cdot 10^{-6}$ |

and

$H_I$ = 0.09524 $a'$ = 0.05298 $K$ = 1.9764

and the analysis of variance is completed with the formulae in Tables 3.3.3.1.-III and 3.3.3.1.-IV.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 1.469 - 36 \times 0.0530}{84} = 0.0822$$

Slope of test sample:

$$b'_T = \frac{6 \times 1.266 - 36 \times 0.0530}{84} = 0.0677$$

Formula 3.3.5.1.-3 gives:

$$R = \frac{0.0677}{0.0822} = 0.823$$

$$C = \frac{0.0822^2}{0.0822^2 - 3.86 \cdot 10^{-6} \times 2.018^2 \times 0.0357} = 1.000083$$

$$K' = 0.000083 \times 0.75 = 0.000062$$

and the 95 per cent confidence limits are:

$$0.823 \pm \sqrt{0.000083 \times 1.678 + 0.000062 \times (-1.646)}$$
$$= 0.823 \pm 0.006$$

The potency ratio is thus estimated as 0.823 with 95 per cent confidence limits from 0.817 to 0.829.

Table 5.2.1.-I. – *Absorbances*

| | **Blank** | **Standard *S* (in IU/mL)** | | | **Preparation *T* (in IU/mL)** | | |
|---|---|---|---|---|---|---|---|
| Conc. | *B* | $S_1$ 0.01 | $S_2$ 0.02 | $S_3$ 0.03 | $T_1$ 0.01 | $T_2$ 0.02 | $T_3$ 0.03 |
| | 0.022 | 0.133 | 0.215 | 0.299 | 0.120 | 0.188 | 0.254 |
| | 0.024 | 0.133 | 0.215 | 0.299 | 0.119 | 0.188 | 0.253 |
| | 0.024 | 0.131 | 0.216 | 0.299 | 0.118 | 0.190 | 0.255 |
| | 0.026 | 0.136 | 0.218 | 0.297 | 0.120 | 0.190 | 0.258 |
| | 0.023 | 0.137 | 0.220 | 0.297 | 0.120 | 0.190 | 0.257 |
| | 0.022 | 0.136 | 0.220 | 0.305 | 0.121 | 0.191 | 0.257 |
| | 0.022 | 0.138 | 0.219 | 0.299 | 0.121 | 0.191 | 0.255 |
| | 0.023 | 0.137 | 0.218 | 0.302 | 0.121 | 0.190 | 0.254 |
| Mean | 0.0235 | 0.1351 | 0.2176 | 0.2996 | 0.1200 | 0.1898 | 0.2554 |

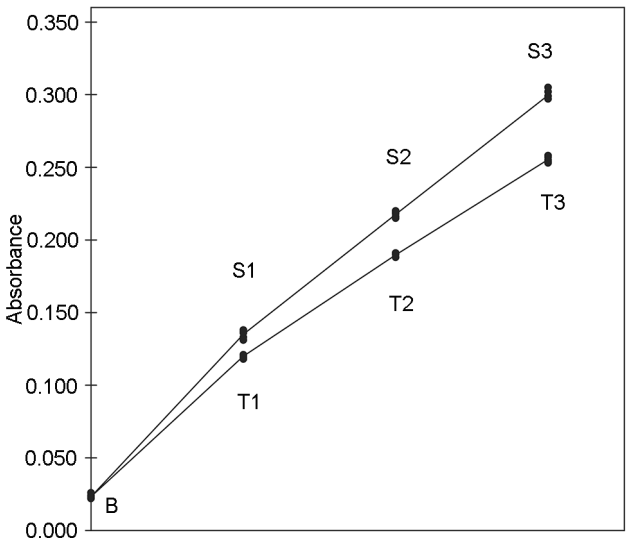


Figure 5.2.1.-I.

Table 5.2.1.-II. – *Analysis of variance*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F*-ratio | Probability |
|---|---|---|---|---|---|
| Regression | 2 | 0.1917 | 0.0958 | 24 850 | 0.000 |
| Intersection | 1 | $3 \cdot 10^{-9}$ | $3 \cdot 10^{-9}$ | $7 \cdot 10^{-4}$ | 0.978 |
| Non-linearity | 2 | $2 \cdot 10^{-5}$ | $1 \cdot 10^{-5}$ | 2.984 | 0.061 |
| Treatments | 5 | 0.1917 | | | |
| Residual error | 42 | $1.62 \cdot 10^{-4}$ | $3.86 \cdot 10^{-6}$ | | |
| Total | 47 | 0.1919 | | | |

*5.2.2. A COMPLETELY RANDOMISED (0,4,4,4)-DESIGN*

*An in-vitro assay of influenza vaccines*

The haemagglutinin antigen (HA) content of 2 influenza vaccines is determined by single radial immunodiffusion. Both have a labelled potency of 15 µg HA per dose, which is equivalent with a content of 30 µg HA/mL. The standard has an assigned content of 39 µg HA/mL.

Standard and test vaccines are applied in 4 duplicate concentrations which are prepared on the basis of the assigned and the labelled contents. When the equilibrium between the external and the internal reactant is established, the zone of the annulus precipitation area is measured. The results are shown in Table 5.2.2.-I.

Table 5.2.2.-I. – *Zone of precipitation area ($mm^2$)*

| Conc. | Standard *S* | | Preparation *T* | | Preparation *U* | |
|---|---|---|---|---|---|---|
| (µg/mL) | I | II | I | II | I | II |
| 7.5 | 18.0 | 18.0 | 15.1 | 16.8 | 15.4 | 15.7 |
| 15.0 | 22.8 | 24.5 | 23.1 | 24.2 | 20.2 | 18.6 |
| 22.5 | 30.4 | 30.4 | 28.9 | 27.4 | 24.2 | 23.1 |
| 30.0 | 35.7 | 36.6 | 34.4 | 37.8 | 27.4 | 27.0 |

A graphical presentation of the data shows no unusual features (see Figure 5.2.2.-I). The formulae in Tables 3.3.3.1.-I and 3.3.3.1.-II yield:

$P_S = 108.2$ $P_T = 103.85$ $P_U = 85.8$

$L_S = 301.1$ $L_T = 292.1$ $L_U = 234.1$

$a_S = 141.0$ $a_T = 116.7$ $a_U = 139.8$

$b_S = 61.2$ $b_T = 64.95$ $b_U = 39.2$

$G_S = 3114.3$ $G_T = 2909.4$ $G_U = 1917.3$

$J_S = 0.223$ $J_T = 2.227$ $J_U = 0.083$

and

$H_I = 0.0093$ $a' = 11.04$ $K = 14\,785.8$

and the analysis of variance is completed with the formulae in Tables 3.3.3.1.-III and 3.3.3.1.-IV. This is shown in Table 5.2.2.-II.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 301.1 - 60 \times 11.04}{180} = 6.356$$

Slope of *T* is:

$$b'_T = \frac{6 \times 292.1 - 60 \times 11.04}{180} = 6.056$$

Slope of *U* is:

$$b'_U = \frac{6 \times 234.1 - 60 \times 11.04}{180} = 4.123$$

This leads to a potency ratio of 6.056/6.356 = 0.953 for vaccine *T* and 4.123/6.356 = 0.649 for vaccine *U*.

$$C = \frac{6.356^2}{6.356^2 - 1.068 \times 2.179^2 \times 0.0444} = 1.0056$$

$$K' = 0.0056 \times 0.625 = 0.0035$$

And the confidence limits are found with formula 3.3.5.1.-4.

For vaccine *T*:

$$0.955 \pm \sqrt{0.0056 \times 1.913 + 0.0035 \times (-1.913)} = 0.955 \pm 0.063$$

For vaccine *U*:

$$0.649 \pm \sqrt{0.0056 \times 1.423 + 0.0035 \times (-1.301)} = 0.649 \pm 0.058$$

The HA content in µg/dose can be found by multiplying the potency ratios and confidence limits by the assumed content of 15 µg/dose. The results are given in Table 5.2.2.-III.

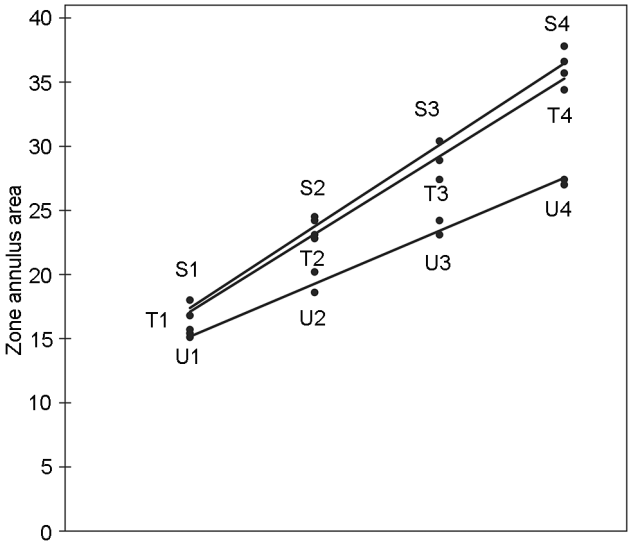


Figure 5.2.2.-I.

Table 5.2.2.-II. – *Analysis of variance*

| Source of variation | Degrees of freedom | Sum of squares | Mean square | *F*-ratio | Probability |
|---|---|---|---|---|---|
| Regression | 3 | 1087.7 | 362.6 | 339.5 | 0.000 |
| Intersection | 2 | 3.474 | 1.737 | 1.626 | 0.237 |
| Non-linearity | 6 | 5.066 | 0.844 | 0.791 | 0.594 |
| Treatments | 11 | 1096.2 | | | |
| Residual error | 12 | 12.815 | 1.068 | | |
| Total | 23 | 1109.0 | | | |

Table 5.2.2.-III. – *Estimates of HA content* (µg/dose)

| | Lower limit | Estimate | Upper limit |
|---|---|---|---|
| **Vaccine *T*** | 13.4 | 14.3 | 15.3 |
| **Vaccine *U*** | 8.9 | 9.7 | 10.6 |

## 5.3. QUANTAL RESPONSES

*5.3.1. PROBIT ANALYSIS OF A TEST PREPARATION AGAINST A REFERENCE*

*An in-vivo assay of a diphtheria vaccine*

A diphtheria vaccine (assumed potency 140 IU/vial) is assayed against a standard (assigned potency 132 IU/vial). On the basis of this information, equivalent doses are prepared and randomly administered to groups of guinea-pigs. After a

given period, the animals are challenged with diphtheria toxin and the number of surviving animals recorded as shown in Table 5.3.1.-I.

Table 5.3.1.-I. – *Raw data from a diphtheria assay in guinea-pigs*

| **Standard (*S*) Assigned potency 132 IU/vial** | | | **Test preparation (*T*) Assumed potency 140 IU/vial** | | |
|---|---|---|---|---|---|
| dose (IU/mL) | chal-lenged | protected | dose (I.U./mL) | chal-lenged | protected |
| 1.0 | 12 | 0 | 1.0 | 11 | 0 |
| 1.6 | 12 | 3 | 1.6 | 12 | 4 |
| 2.5 | 12 | 6 | 2.5 | 11 | 8 |
| 4.0 | 11 | 10 | 4.0 | 11 | 10 |

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.1.-II shows the first cycle of this procedure.

The sums of the last 6 columns are then calculated per preparation and transferred to the second working table (see Table 5.3.1.-III). The results in the other columns are found with formulae 4.2.1.-4 to 4.2.1.-10. This yields a common slope *b* of 1.655.

The values for *Y* in the first working table are now replaced by $a + bx$ and a second cycle is carried out (see Table 5.3.1.-IV).

The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.1.-V.

Linearity is tested as described in Section 4.2.2. The $\chi^2$-value with 4 degrees of freedom is 0.851 + 1.070 = 1.921 representing a *p*-value of 0.750 which is not significant.

Since there are no significant deviations from linearity, the test for parallelism can be carried out as described in the same section. The $\chi^2$-value with 1 degree of freedom is:

$$(16.71 + 17.27) - \frac{14.15^2}{5.89} = 0.001$$

representing a *p*-value of 0.974 which is not significant.

The ln(potency ratio) can now be estimated as described in Section 4.2.3.

$$M'_T = \frac{-1.721 - (-2.050)}{2.401} = 0.137$$

Further:

$$C = \frac{2.401^2 \times 5.893}{2.401^2 \times 5.893 - 1^2 \times 1.960^2} = 1.127$$

$$V = \frac{1}{18.37} + \frac{1}{17.96} = 0.110$$

Table 5.3.1.-II. – *First working table in the first cycle*

| **Vaccine** | **Dose** | ***n*** | ***r*** | ***x*** | ***p*** | ***Y*** | **Φ** | ***Z*** | ***y*** | ***w*** | ***wx*** | ***wy*** | ***wx²*** | ***wy²*** | ***wxy*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S* | 1.0 | 12 | 0 | 0.000 | 0.000 | 0 | 0.5 | 0.399 | − 1.253 | 7.64 | 0.00 | − 9.57 | 0.00 | 12.00 | 0.00 |
| | 1.6 | 12 | 3 | 0.470 | 0.250 | 0 | 0.5 | 0.399 | − 0.627 | 7.64 | 3.59 | − 4.79 | 1.69 | 3.00 | − 2.25 |
| | 2.5 | 12 | 6 | 0.916 | 0.500 | 0 | 0.5 | 0.399 | 0.000 | 7.64 | 7.00 | 0.00 | 6.41 | 0.00 | 0.00 |
| | 4.0 | 11 | 10 | 1.386 | 0.909 | 0 | 0.5 | 0.399 | 1.025 | 7.00 | 9.71 | 7.18 | 13.46 | 7.36 | 9.95 |
| *T* | 1.0 | 11 | 0 | 0.000 | 0.000 | 0 | 0.5 | 0.399 | − 1.253 | 7.00 | 0.00 | − 8.78 | 0.00 | 11.00 | 0.00 |
| | 1.6 | 12 | 4 | 0.470 | 0.333 | 0 | 0.5 | 0.399 | − 0.418 | 7.64 | 3.59 | − 3.19 | 1.69 | 1.33 | − 1.50 |
| | 2.5 | 11 | 8 | 0.916 | 0.727 | 0 | 0.5 | 0.399 | 0.570 | 7.00 | 6.42 | 3.99 | 5.88 | 2.27 | 3.66 |
| | 4.0 | 11 | 10 | 1.386 | 0.909 | 0 | 0.5 | 0.399 | 1.025 | 7.00 | 9.71 | 7.18 | 13.46 | 7.36 | 9.95 |

Table 5.3.1.-III. – *Second working table in the first cycle*

| **Vaccine** | **Σ*w*** | **Σ*wx*** | **Σ*wy*** | **Σ*wx²*** | **Σ*wy²*** | **Σ*wxy*** | $S_{xx}$ | $S_{xy}$ | $S_{yy}$ | $\bar{x}$ | $\bar{y}$ | ***a*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S* | 29.92 | 20.30 | − 7.18 | 21.56 | 22.36 | 7.70 | 7.79 | 12.58 | 20.64 | 0.68 | − 0.24 | − 1.36 |
| *T* | 28.65 | 19.72 | − 0.80 | 21.03 | 21.97 | 12.11 | 7.46 | 12.66 | 21.95 | 0.69 | − 0.03 | − 1.17 |

Table 5.3.1.-IV. – *First working table in the second cycle*

| **Vaccine** | **Dose** | ***n*** | ***r*** | ***x*** | ***p*** | ***Y*** | **Φ** | ***Z*** | ***y*** | ***w*** | ***wx*** | ***wy*** | ***wx²*** | ***wy²*** | ***wxy*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S* | 1.0 | 12 | 0 | 0.000 | 0.000 | − 1.36 | 0.086 | 0.158 | − 1.911 | 3.77 | 0.00 | − 7.21 | 0.00 | 13.79 | 0.00 |
| | 1.6 | 12 | 3 | 0.470 | 0.250 | − 0.58 | 0.279 | 0.336 | − 0.672 | 6.74 | 3.17 | − 4.53 | 1.49 | 3.04 | − 2.13 |
| | 2.5 | 12 | 6 | 0.916 | 0.500 | 0.15 | 0.561 | 0.394 | − 0.001 | 7.57 | 6.94 | − 0.01 | 6.36 | 0.00 | − 0.01 |
| | 4.0 | 11 | 10 | 1.386 | 0.909 | 0.93 | 0.824 | 0.258 | 1.260 | 5.07 | 7.03 | 6.39 | 9.75 | 8.05 | 8.86 |
| *T* | 1.0 | 11 | 0 | 0.000 | 0.000 | − 1.17 | 0.122 | 0.202 | − 1.769 | 4.20 | 0.00 | − 7.43 | 0.00 | 13.14 | 0.00 |
| | 1.6 | 12 | 4 | 0.470 | 0.333 | − 0.39 | 0.349 | 0.370 | − 0.430 | 7.23 | 3.40 | − 3.11 | 1.60 | 1.34 | − 1.46 |
| | 2.5 | 11 | 8 | 0.916 | 0.727 | 0.35 | 0.637 | 0.375 | 0.591 | 6.70 | 6.14 | 3.96 | 5.62 | 2.34 | 3.63 |
| | 4.0 | 11 | 10 | 1.386 | 0.909 | 1.13 | 0.870 | 0.211 | 1.311 | 4.35 | 6.03 | 5.70 | 8.36 | 7.48 | 7.90 |

Table 5.3.1.-V. – *Second working table after sufficient cycles*

| **Vaccine** | **Σ*w*** | **Σ*wx*** | **Σ*wy*** | **Σ*wx²*** | **Σ*wy²*** | **Σ*wxy*** | $S_{xx}$ | $S_{xy}$ | $S_{yy}$ | $\bar{x}$ | $\bar{y}$ | ***a*** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *S* | 18.37 | 14.80 | − 2.14 | 14.85 | 17.81 | 5.28 | 2.93 | 7.00 | 17.56 | 0.81 | − 0.12 | − 2.05 |
| *T* | 17.96 | 12.64 | − 0.55 | 11.86 | 18.35 | 6.76 | 2.96 | 7.15 | 18.34 | 0.70 | − 0.03 | − 1.72 |

So ln confidence limits are:

$$0.155 - 0.013 \pm \sqrt{0.127(0.649 + 1.127 \times 0.036^2)} = 0.142 \pm 0.288$$

The potency and confidence limits can now be found by taking the antilogarithms and multiplying these by the assumed potency of 140 IU/vial. This yields an estimate of 160.6 IU/vial with 95 per cent confidence limits from 121.0-215.2 IU/vial.

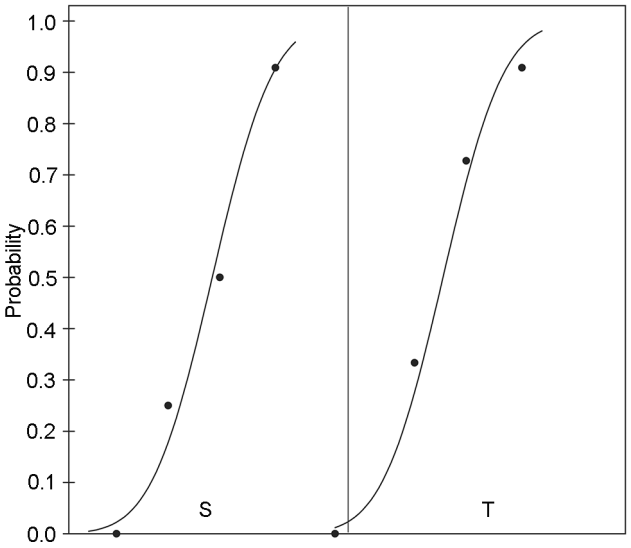


Figure 5.3.1.-I.

*5.3.2. LOGIT ANALYSIS AND OTHER TYPES OF ANALYSES OF A TEST PREPARATION AGAINST A REFERENCE*

Results will be given for the situation where the logit method and other "classical" methods of this family are applied to the data in Section 5.3.1. This should be regarded as an exercise rather than an alternative to the probit method in this specific case. Another shape of the curve may be adopted only if this is supported by experimental or theoretical evidence. See Table 5.3.2.-I.

Table 5.3.2.-I. – *Results by using alternative curves*

| | **Logit** | **Gompit** | **Angle*** |
|---|---|---|---|
| Φ | $\frac{1}{1+e^{-Y}}$ | $1-e^{-e^{Y}}$ | $\frac{1}{2}\sin Y+\frac{1}{2}$ |
| Z | $\frac{e^{-Y}}{(1+e^{-Y})^2}$ | $e^{Y-e^{Y}}$ | $\frac{1}{2}\cos Y$ |
| slope b | 4.101 | 2.590 | 1.717 |
| $\chi^2$ lin | 2.15 | 3.56 | 1.50 |
| $\chi^2$ par | 0.0066 | 0.168 | 0.0010 |
| Potency | 162.9 | 158.3 | 155.8 |
| Lower limit | 121.1 | 118.7 | 122.6 |
| Upper limit | 221.1 | 213.3 | 200.7 |

* $\begin{cases} \text{If } Y - \frac{1}{2}\pi \text{ then } \Phi = 0 \text{ and } Z = 0 \\ \text{If } Y\frac{1}{2}\pi \text{ then } \Phi = 1 \text{ and } Z = 0 \end{cases}$

*5.3.3. THE $ED_{50}$ DETERMINATION OF A SUBSTANCE USING THE PROBIT METHOD*

*An in-vitro assay of oral poliomyelitis vaccine*

In an $ED_{50}$ assay of oral poliomyelitis vaccine with 10 different dilutions in 8 replicates of 50 µL on an ELISA-plate, results were obtained as shown in Table 5.3.3.-I.

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.3.-II shows the first cycle of this procedure.

Table 5.3.3.-I. – *Dilutions ($10^x$ µL of the undiluted vaccine)*

| −3.5 | −4.0 | −4.5 | −5.0 | −5.5 | −6.0 | −6.5 | −7.0 | −7.5 | −8.0 |
|---|---|---|---|---|---|---|---|---|---|
| + | + | + | + | − | − | − | − | − | − |
| + | + | + | + | − | − | − | − | − | − |
| + | + | − | − | − | − | − | − | − | − |
| + | + | + | + | − | − | − | − | − | − |
| + | + | + | − | − | − | − | − | − | − |
| + | + | + | + | + | − | − | − | − | − |
| + | + | + | + | + | − | + | − | − | − |
| + | + | + | + | − | + | − | − | − | − |

The sums of the last 6 columns are calculated and transferred to the second working table (see Table 5.3.3.-III). The results in the other columns are found with formulae 4.2.1.-4 to 4.2.1.-10. This yields a common slope *b* of – 0.295.

The values for *Y* in the first working table are now replaced by $a + bx$ and a second cycle is carried out. The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.3.-IV.

Linearity is tested as described in Section 4.2.2. The $\chi^2$-value with 8 degrees of freedom is 2.711 representing a *p*-value of 0.951 which is not significant.

The potency ratio can now be estimated as described in Section 4.5.

$$M'_T = \frac{-(-7.931)}{-0.646} = -12.273$$

Further:

$$C = \frac{(-0.646)^2 \times 55.883}{(-0.646)^2 \times 55.883 - 1^2 \times 1.960^2} = 1.197$$

$$V = \frac{1}{19.39} = 0.052$$

So ln confidence limits are:

$$-14.692 - (-2.420) \pm \sqrt{0.197 \times (2.882 + 1.197 \times 0.009^2)}$$

$$= -12.272 \pm 0.754$$

This estimate is still expressed in terms of the ln(dilutions). In order to obtain estimates expressed in $\ln(ED_{50})$/mL the values are transformed to:

$$-M'_T + \ln\left(\frac{1000}{50}\right)$$

Since it has become common use to express the potency of this type of vaccine in terms of $\log_{10}(ED_{50})$/mL, the results have to be divided by ln(10). The potency is thus estimated as 6.63 $\log_{10}(ED_{50})$/mL with 95 per cent confidence limits from 6.30 to 6.96 $\log_{10}(ED_{50})$/mL.

Table 5.3.3.-II. – *First working table in the first cycle*

| Vaccine | Dose | *n* | *r* | *x* | *p* | *Y* | Φ | *Z* | *y* | *w* | *wx* | *wy* | $wx^2$ | $wy^2$ | *wxy* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T* | $10^{-3.5}$ | 8 | 0 | − 8.06 | 0.000 | 0.00 | 0.5 | 0.399 | − 1.253 | 5.09 | − 41.04 | − 6.38 | 330.8 | 8.00 | 51.4 |
| | $10^{-4.0}$ | 8 | 0 | − 9.21 | 0.000 | 0.00 | 0.5 | 0.399 | − 1.253 | 5.09 | − 46.91 | − 6.38 | 432.0 | 8.00 | 58.8 |
| | $10^{-4.5}$ | 8 | 1 | − 10.36 | 0.125 | 0.00 | 0.5 | 0.399 | − 0.940 | 5.09 | − 52.77 | − 4.79 | 546.8 | 4.50 | 49.6 |
| | $10^{-5.0}$ | 8 | 2 | − 11.51 | 0.250 | 0.00 | 0.5 | 0.399 | − 0.627 | 5.09 | − 58.63 | − 3.19 | 675.1 | 2.00 | 36.7 |
| | $10^{-5.5}$ | 8 | 6 | − 12.66 | 0.750 | 0.00 | 0.5 | 0.399 | 0.627 | 5.09 | − 64.50 | 3.19 | 816.8 | 2.00 | − 40.4 |
| | $10^{-6.0}$ | 8 | 7 | − 13.82 | 0.875 | 0.00 | 0.5 | 0.399 | 0.940 | 5.09 | − 70.36 | 4.79 | 972.1 | 4.50 | − 66.1 |
| | $10^{-6.5}$ | 8 | 7 | − 14.97 | 0.875 | 0.00 | 0.5 | 0.399 | 0.940 | 5.09 | − 76.23 | 4.79 | 1140.8 | 4.50 | − 71.7 |
| | $10^{-7.0}$ | 8 | 8 | − 16.12 | 1.000 | 0.00 | 0.5 | 0.399 | 1.253 | 5.09 | − 82.09 | 6.38 | 1323.1 | 8.00 | − 102.9 |
| | $10^{-7.5}$ | 8 | 8 | − 17.27 | 1.000 | 0.00 | 0.5 | 0.399 | 1.253 | 5.09 | − 87.95 | 6.38 | 1518.9 | 8.00 | − 110.2 |
| | $10^{-8.0}$ | 8 | 8 | − 18.42 | 1.000 | 0.00 | 0.5 | 0.399 | 1.253 | 5.09 | − 93.82 | 6.38 | 1728.2 | 8.00 | − 117.6 |

Table 5.3.3.-III. – *Second working table in the first cycle*

| Vaccine | $\Sigma w$ | $\Sigma wx$ | $\Sigma wy$ | $\Sigma wx^2$ | $\Sigma wy^2$ | $\Sigma wxy$ | $S_{xx}$ | $S_{xy}$ | $S_{yy}$ | $\bar{x}$ | $\bar{y}$ | *a* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T* | 50.93 | − 674.3 | 11.17 | 9484.6 | 57.50 | − 312.32 | 556.92 | − 164.43 | 55.05 | − 13.24 | 0.219 | − 3.690 |

Table 5.3.3.-IV. – *Second working table after sufficient cycles*

| Vaccine | $\Sigma w$ | $\Sigma wx$ | $\Sigma wy$ | $\Sigma wx^2$ | $\Sigma wy^2$ | $\Sigma wxy$ | $S_{xx}$ | $S_{xy}$ | $S_{yy}$ | $\bar{x}$ | $\bar{y}$ | *a* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T* | 19.39 | − 238.2 | 0.11 | 2981.1 | 26.05 | − 37.45 | 55.88 | − 36.11 | 26.05 | − 12.28 | 0.006 | − 7.931 |

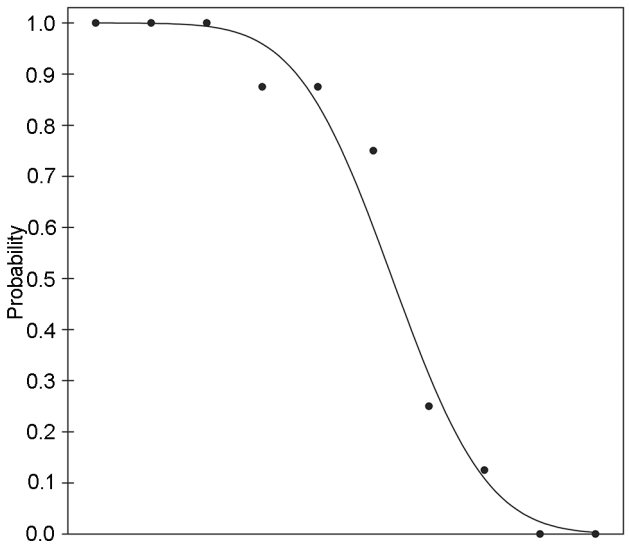


Figure 5.3.3.-I.

## 5.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

### *5.4.1. FOUR-PARAMETER LOGISTIC CURVE ANALYSIS*

*A serological assay of tetanus sera*

As already stated in Section 3.4, this example is intended to illustrate a "possible" way to analyse the data presented, but not necessarily to reflect the "only" or the "most appropriate" way. Many other approaches can be found in the literature, but in most cases they should not yield dramatically different outcomes. A short discussion of alternative approaches and other statistical considerations is given in Section 7.5.

A guinea-pig antiserum is assayed against a standard serum (0.4 IU/mL) using an enzyme-linked immunosorbent assay technique (ELISA). 10 two-fold dilutions of each serum were applied on a 96-well ELISA plate. Each dilution was applied twice. The observed responses are listed in Table 5.4.1.-I.

Table 5.4.1.-I. – *Observed responses*

| Standard *S* | | | Preparation to be examined *T* | | |
|---|---|---|---|---|---|
| Dil. | Obs. 1 | Obs. 2 | Dil. | Obs. 1 | Obs. 2 |
| 1/10 | 2.912 | 2.917 | 1/10 | 3.017 | 2.987 |
| 1/20 | 2.579 | 2.654 | 1/20 | 2.801 | 2.808 |
| 1/40 | 2.130 | 2.212 | 1/40 | 2.401 | 2.450 |
| 1/80 | 1.651 | 1.638 | 1/80 | 1.918 | 1.963 |
| 1/160 | 1.073 | 0.973 | 1/160 | 1.364 | 1.299 |
| 1/320 | 0.585 | 0.666 | 1/320 | 0.861 | 0.854 |
| 1/640 | 0.463 | 0.356 | 1/640 | 0.497 | 0.496 |
| 1/1280 | 0.266 | 0.234 | 1/1280 | 0.340 | 0.344 |
| 1/2560 | 0.228 | 0.197 | 1/2560 | 0.242 | 0.217 |
| 1/5120 | 0.176 | 0.215 | 1/5120 | 0.178 | 0.125 |

For this example, it will be assumed that the laboratory has validated conditions 1 to 3 in Section 3.1.1 when the assay was being developed for routine use. In addition, the laboratory has validated that the upper limit and lower limit of the samples can be assumed to be equal.

No unusual features are discovered in a graphical representation. A least squares method of a suitable computer program is used to fit the parameters of the logistic function, assuming that the residual error terms are independent and identically distributed normal random variables. In this case, 3 parameters (α, β and δ) are needed to describe the common slope-factor and the common lower and upper asymptotes. 2 additional parameters ($\gamma_S$ and $\gamma_T$) are needed to describe the horizontal location of the 2 curves.

The following estimates of the parameters are returned by the program:

$\alpha = 3.196$ $\qquad$ $\gamma_S = -4.307$

$\beta = 1.125$ $\qquad$ $\gamma_T = -4.684$

$\delta = 0.145$

In addition, the estimated residual variance ($s^2$) is returned as 0.001429 with 20 degrees of freedom (within-treatments variation).

In order to obtain confidence limits, and also to check for parallelism and linearity, the observed responses ($u$) are linearised and submitted to a weighted parallel-line analysis by the program. This procedure is very similar to that described in Section 4.2 for probit analysis with the following modifications:

$$Y = \beta(x - \gamma) \qquad y = Y + \frac{\left(\frac{u-\delta}{\alpha-\delta}\right) - \Phi}{Z}$$

$$\Phi = \frac{1}{1+e^{-Y}} \qquad w = \frac{Z^2(\alpha-\delta)^2}{s^2}$$

$$Z = \frac{e^{-Y}}{(1+e^{-Y})^2}$$

The resulting weighted analysis of variance of the transformed responses ($y$) using weights ($w$) is shown in Table 5.4.1.-II.

Table 5.4.1.-II – *Weighted analysis of variance*

| Source of variation | Degrees of freedom | $\chi^2$ | Probability |
|---|---|---|---|
| Preparations | 1 | 0.529653 | 0.467 |
| Regression | 1 | 6599.51 | 0.000 |
| Non-parallelism | 1 | 0.0458738 | 0.830 |
| Non-linearity | 16 | 8.89337 | 0.918 |
| Treatments | 19 | 6608.98 | 0.000 |
| Residual error | 20 | 20.0000 | |
| Total | 39 | 6628.98 | |

There are no significant deviations from parallelism and linearity and thus the assay is satisfactory for potency calculations. If the condition of equal upper and lower asymptotes is not fulfilled, significant deviations from linearity and/or parallelism are likely to occur because the tests for linearity and parallelism reflect the goodness of fit of the complete four-parameter model. The residual error in the analysis of variance is always equal to 1 as a result of the transformation. However, a heterogeneity factor (analogous to that for the probit model) can be computed.

The relative potency of the test preparation can be obtained as the antilogarithm of $\gamma_S - \gamma_T$. Multiplying by the assigned potency of the standard yields an estimate of $1.459 \times 0.4 = 0.584$ IU/mL. Formula 4.2.3.-2 gives 95 per cent confidence limits from 0.557-0.612 IU/mL.

## 6. COMBINATION OF ASSAY RESULTS

### 6.1. INTRODUCTION

Replication of independent assays and combination of their results is often needed to fulfil the requirements of the European Pharmacopoeia. The question then arises as to whether it is appropriate to combine the results of such assays and if so in what way.

Two assays may be regarded as mutually independent when the execution of either does not affect the probabilities of the possible outcomes of the other. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined, the sensitivity of the biological indicator) in 1 assay must be independent of the corresponding random errors in the other one. Assays on successive days using the original and retained dilutions of the standard therefore are not independent assays.

There are several methods for combining the results of independent assays, the most theoretically acceptable being the most difficult to apply. Three simple, approximate methods are described below; others may be used provided the necessary conditions are fulfilled.

Before potencies from assays based on the parallel-line or probit model are combined they must be expressed in logarithms; potencies derived from assays based on the slope-ratio model are used as such. As the former models are more common than those based on the slope-ratio model, the symbol $M$ denoting ln potency is used in the formulae in this section; by reading $R$ (slope-ratio) for $M$, the analyst may use the same formulae for potencies derived from assays based on the slope-ratio model. All estimates of potency must be corrected for the potency assigned to the reference preparation before they are combined.

### 6.2. COMBINATION OF INDEPENDENT ASSAY RESULTS

This method can be used provided the following conditions are fulfilled:

1) the potency estimates are derived from independent assays;

2) for each assay $C$ is close to 1 (say less than 1.1);

3) the number of degrees of freedom of the individual residual errors is not smaller than 6, but preferably larger than 15.

When these conditions are not fulfilled, an independent combination of the results cannot be applied. The method described in Section 6.3 may then be used to obtain the best estimate of the mean potency to be adopted in further assays as an assumed potency.

#### *6.2.1. HOMOGENEITY OF POTENCY ESTIMATES*

It is assumed that the results of each of the $n'$ assays have been analysed to give $n'$ values of $M$ with associated confidence limits. For each assay the logarithmic confidence interval $L$ is obtained by subtracting the lower limit from the upper. A weight $W$ for each value of $M$ is calculated from equation 6.2.1.-1, where $t$ has the same value as that used in the calculation of confidence limits.

$$W = \frac{4t^2}{L^2} \qquad (6.2.1.\text{-}1)$$

By squaring the deviation of each value of $M$ from the weighted mean, multiplying by the appropriate weight and summing over all assays, a statistic is obtained which is approximately distributed as $\chi^2$ (see Table 8.3) and which may be used to test the homogeneity of a set of ln potency estimates:

$$\chi^2 \approx \sum_{n'} W(M - \overline{M})^2 \text{ where } \overline{M} = \frac{\sum WM}{\sum W} \qquad (6.2.1.\text{-}2)$$

If the calculated $\chi^2$ is smaller than the tabulated value corresponding to $(n' - 1)$ degrees of freedom the potencies are homogeneous and the mean potency and limits obtained in Section 6.2.2 will be meaningful.

If the calculated value of this statistic is greater than the tabulated value, the potencies are heterogeneous. This means that the variation between individual estimates of $M$ is greater than would have been predicted from the estimates of the confidence limits, i.e. that there is a significant variability between the assays. Under these circumstances the equations in Section 6.2.2 are no longer applicable. Instead, the formulae in Section 6.2.3 may be used.

#### *6.2.2. WEIGHTED COMBINATION (BASED ON THE INTRA-ASSAY VARIATION)*

The products $WM$ are formed for each assay and their sum divided by the total weight for all assays to give the logarithm of the weighted mean potency.

$$\overline{M} = \frac{\sum WM}{\sum W} \qquad (6.2.2.\text{-}1)$$

The standard error of the ln (mean potency) is taken to be the square root of the reciprocal of the total weight:

$$s_{\overline{M}} = \sqrt{\frac{1}{\sum W}} \qquad (6.2.2.\text{-}2)$$

and approximate confidence limits are obtained from the antilogarithms of the value given by:

$$\overline{M} \pm t \times s_{\overline{M}} \qquad (6.2.2.\text{-}3)$$

where the number of degrees of freedom of $t$ equals the sum of the number of degrees of freedom for the error mean squares in the individual assays.

*6.2.3. SEMI-WEIGHTED COMBINATION (BASED ON THE INTRA- AND INTER-ASSAY VARIATION)*

When results of several repeated assays are combined, the $\chi^2$-value may be significant. The observed variation is then considered to have two components:

– the intra-assay variation:

$$s_M^2 = 1 / W$$

– the inter-assay variation:

$$s_m^2 = \frac{\sum (M - \overline{M})^2}{n' - 1} - \frac{\sum s_M^2}{n'}$$

where $\overline{M}$ is the unweighted mean. The intra-assay variation varies from assay to assay whereas the inter-assay variation is common to all $M$.

For each $M$ a weighting coefficient is then calculated as:

$$W' = \frac{1}{s_M^2 + s_m^2}$$

which replaces $W$ in Section 6.2.2. where $t$ is taken to be approximately 2.

Note: if $s_m^2$ is negative, then it is replaced by 0.

## 6.3. UNWEIGHTED COMBINATION OF ASSAY RESULTS

The method described below may be used when the conditions described in Section 6.2 are not fulfilled.

To combine the $n'$ estimates of $M$ from $n'$ assays, the mean is calculated and an estimate of its standard error is obtained by calculating:

$$s_{\overline{M}}^2 = \frac{\sum (M - \overline{M})^2}{n'(n' - 1)} \qquad (6.3.\text{-}1)$$

and the limits are:

$$\overline{M} \pm t s_{\overline{M}} \qquad (6.3.\text{-}2)$$

where $t$ has $(n' - 1)$ degrees of freedom. The number $n'$ of estimates of $M$ is usually small, and hence the value of $t$ is quite large.

## 6.4. EXAMPLE OF A WEIGHTED MEAN POTENCY WITH CONFIDENCE LIMITS

Table 6.4.-I lists 6 independent potency estimates of the same preparation together with their 95 per cent confidence limits and the number of degrees of freedom of their error variances. Conditions 1, 2 and 3 in Section 6.2. are met. The ln potencies and the weights are calculated as described in Section 6.2.

Table 6.4.-I. – *Potency estimates and confidence intervals of 6 independent assays*

| Potency estimate (IU/vial) | Lower limit (IU/vial) | Upper limit (IU/vial) | Degrees of freedom | ln potency $M$ | Weight $W$ |
|---|---|---|---|---|---|
| 18 367 | 17 755 | 19 002 | 20 | 9.8183 | 3777.7 |
| 18 003 | 17 415 | 18 610 | 20 | 9.7983 | 3951.5 |
| 18 064 | 17 319 | 18 838 | 20 | 9.8017 | 2462.5 |
| 17 832 | 17 253 | 18 429 | 20 | 9.7887 | 4003.0 |
| 18 635 | 17 959 | 19 339 | 20 | 9.8328 | 3175.6 |
| 18 269 | 17 722 | 18 834 | 20 | 9.8130 | 4699.5 |

Homogeneity of potency estimates is assessed with formula 6.2.1.-2 which gives a $\chi^2$ of 4.42 with 5 degrees of freedom. This is not significant ($p = 0.49$) and thus, a weighted mean potency is calculated with formula 6.2.2.-1. which yields 9.8085.

Formula 6.2.2.-2 gives a standard error of 0.00673 and approximate 95 per cent confidence limits of 9.7951 and 9.8218 are calculated with formula 6.2.2.-3 where $t$ has 120 degrees of freedom.

By taking the antilogarithms a potency of 18 187 IU/vial is found with 95 per cent confidence limits from 17 946-18 431 IU/vial.

## 7. BEYOND THIS ANNEX

It is impossible to give a comprehensive treatise of statistical methods in a pharmacopoeial text. However, the methods described in this annex should suffice for most pharmacopoeial purposes. This section tries to give a more abstract survey of alternative or more general methods that have been developed. The interested reader is encouraged to further explore the existing literature in this area. The use of more specialised statistical methods should, in any case, be left to qualified personnel.

## 7.1. GENERAL LINEAR MODELS

The methods given in this annex can be described in terms of general linear models (or generalised linear models to include the probit and logit methods). The principle is to define a linear structure matrix $\boldsymbol{X}$ (or design matrix) in which each row represents an observation and each column a linear effect (preparation, block, column, dose). For example: the Latin square design in example 5.1.2 would involve a matrix with 36 rows and 13 columns. 1 column for each of the preparations, 1 column for the doses, 5 columns for each block except the first, and 5 columns for each row except the first. All columns, except the one for doses, are filled with 0 or 1 depending on whether or not the observation relates to the effect. A vector $Y$ is filled with the (transformed) observations. The effects are estimated with the formula $(\boldsymbol{X}^T\boldsymbol{X})^{-1}\boldsymbol{X}^T Y$ from which the potency estimate $m$ can easily be derived as a ratio of relevant effects. Confidence intervals are calculated from Fieller's theorem:

$$m_L, m_U = \frac{\left[m - \frac{g v_{12}}{v_{22}} \pm \frac{ts}{b}\sqrt{v_{11} - 2m v_{12} + m^2 v_{22} - g\left(v_{11} - \frac{v_{12}^2}{v_{22}}\right)}\right]}{(1 - g)}$$

where $g = \dfrac{t^2 s^2 v_{22}}{b^2}$

and $v_{11}$, $v_{22}$, $v_{12}$ represent the variance multipliers for the numerator, the denominator and their covariance multiplier respectively. These are taken directly from $(\boldsymbol{X}^T\boldsymbol{X})^{-1}$ or indirectly by noting that:

5. General texts

$$Var(a_1 - a_2) = Var(a_1) + Var(a_2) - 2Cov(a_1, a_2)$$

and $$Cov(a_1 - a_2, b) = Cov(a_1, b) - Cov(a_2, b)$$

A full analysis of variance in which all components are partitioned is slightly more complicated as it involves a renewed definition of $\boldsymbol{X}$ with more columns to relax the assumptions of parallelism and linearity, after which the linear hypotheses can be tested. For assays depending upon quantal responses the linear effects (intercepts $a_S$, $a_T$ etc. and the common slope $b$ are found by maximising the sum over treatments of $n\ln \Phi(a_i + bx) + (n - r)\ln(1 - \Phi(a_i + bx))$ where $x$ is the ln(dose), $\Phi$ denotes the shape of the distribution and $i \in \{S, T, ...\}$.

### 7.2. HETEROGENEITY OF VARIANCE

Heterogeneity of variance cannot always be solved by simply transforming the responses. A possible way to cope with this problem is to perform a weighted linear regression. In order to obtain an unbiased estimate, the weight of the observations is taken to be proportional to the reciprocal of the error variances. Since the true error variance is not always known, an iterative reweighted linear procedure may be followed. However, the calculation of the confidence interval involves new problems.

### 7.3. OUTLIERS AND ROBUST METHODS

The method of least squares described in this annex has the disadvantage of being very sensitive to outliers. A clear outlier may completely corrupt the calculations. This problem is often remedied by discarding the outlying result from the dataset. This policy can lead to arbitrary rejection of data and is not always without danger. It is not easy to give a general guideline on how to decide whether or not a specific observation is an outlier and it is for this reason that many robust methods have been developed. These methods are less sensitive to outliers because they give less weight to observations that are far away from the predicted value. New problems usually arise in computing confidence intervals or defining a satisfactory function to be minimised.

### 7.4. CORRELATED ERRORS

Absolute randomisation is not always feasible or very undesirable from a practical point of view. Thus, subsequent doses within a dilution series often exhibit correlated errors leading to confidence limits that are far too narrow. Some methods have been developed that take account of this autocorrelation effect.

### 7.5. EXTENDED NON-LINEAR DOSE-RESPONSE CURVES

Analysis of extended non-linear dose-response curves raises a number of statistical questions which require consideration, and for which professional advice is recommended. Some of these are indicated below.

1) An example using the four-parameter logistic function has been shown. However, models based on functions giving other sigmoid curves may also be used. Models incorporating additional asymmetry parameters have been suggested.

2) Heterogeneity of variance is common when responses cover a wide range. If the analysis ignores the heterogeneity, interpretation of results may not be correct and estimates may be biased. Use of the reciprocal of the error variances as weights is unlikely to be reliable with limited numbers of replicates. It may be appropriate to estimate a function which relates variance to mean response.

3) The statistical curve-fitting procedures may give different estimates depending on assumptions made about the homogeneity of the variance and on the range of responses used.

4) In principle, equality of upper and lower response limits for the different preparations included in an assay can be directly tested in each assay. However, interpretation of the results of these tests may not be straightforward. The tests for linearity and parallelism given by the simplified method of analysis (Example 5.4.1) indirectly incorporate tests for equality and accuracy of upper and lower limits.

5) Many assays include "controls" which are intended to identify the upper and/or lower response limits. However, these values may not be consistent with the statistically fitted upper and lower response limits based on the extended dose-response curve.

6) The simplified method of analysis given in Example 5.4.1 provides approximate confidence intervals. Other methods may also be used, for example intervals based on lack-of-fit of the completely specified model. For typical assay data, with responses covering the complete range for each preparation tested, all methods give similar results.

### 7.6. NON-PARALLELISM OF DOSE-RESPONSE CURVES

Similarity of dose-response relationships is a fundamental criterion for assessing whether an assay may be regarded as a dilution assay and hence whether the estimation of relative potency is valid (see Section 3.1.1).

This criterion is frequently met by showing that dose-response curves for standard and test samples do not deviate significantly from parallelism. Underestimation of the residual error can lead to excess rejection of assays due to significant deviations from parallelism and/or linearity. This is often an artefact of inappropriate assay design or analysis. Minor modifications to assay designs might in many cases substantially improve the estimation of the residual error. Analysis allowing for the actual level of replication may also improve the situation, e.g. by accounting for multiple applications of the same dilution or correlation of errors between serial dilutions. If estimation of the relevant residual error is not feasible for individual assays, for example because it is impractical to create independent doses and/or replicates, it might be possible to obtain a more correct estimate of the residual error during the assay validation process. In such a case it may be justified to replace the residual error, estimated from the individual assay, by an average (pooled) residual error, determined during the validation process and agreed by the competent authorities.

Where not specified by relevant procedures, curve similarity may be assessed by another approach described as "equivalence testing", whereby, during the validation process, a range of the allowable difference between slopes or other measures of curve similarity, an equivalence acceptance criterion, is determined. If supported by appropriate documentation and agreed by the competent authorities, meeting the equivalence acceptance criteria may be used to satisfy the curve similarity requirement.

There may be cases where the assay system is sufficiently precise to detect genuine non-parallelism. If there is true non-parallelism this needs to be recognised and a suitable solution adopted. A solution might, for example, require a suitable standard that is similar in composition to, and therefore parallel to, the test samples. If the assay system is responding in a non-specific manner to extraneous components of the standard or test samples, then a more specific assay system that does not respond to the irrelevant components may be the solution. No simple, generally applicable statistical solution exists to overcome these fundamental problems. The appropriate action has to be decided on a case-by-case basis with the help of statistical expertise.

## 8. TABLES AND GENERATING PROCEDURES

The tables in this section list the critical values for the most frequently occurring numbers of degrees of freedom. If a critical value is not listed, reference should be made to more extensive tables. Many computer programs include statistical functions and their use is recommended instead of the tables in this section. Alternatively, the generating procedures given

below each table can be used to compute the probability corresponding to a given statistic and number of degrees of freedom.

8.1. THE *F*-DISTRIBUTION

If an observed value is higher than the value in Table 8.1.-I, it is considered to be significant (upper lines, $p = 0.05$) or highly significant (lower lines, $p = 0.01$). $df1$ is the number of degrees of freedom of the numerator and $df2$ is the number of degrees of freedom of the denominator.

*Generating procedure.* Let `F` be the *F*-ratio and `df1` and `df2` as described above. Let `pi` = $\pi$ = 3.14159265358979... The procedure in Table 8.1.-II will then generate the *p*-value.

Table 8.1.-I – *Critical values of the F-distribution*

| **df1 →** | **1** | **2** | **3** | **4** | **5** | **6** | **8** | **10** | **12** | **15** | **20** | **∞** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **df2 ↓** | | | | | | | | | | | | |
| 10 | 4.965 | 4.103 | 3.708 | 3.478 | 3.326 | 3.217 | 3.072 | 2.978 | 2.913 | 2.845 | 2.774 | 2.538 |
| | 10.044 | 7.559 | 6.552 | 5.994 | 5.636 | 5.386 | 5.057 | 4.849 | 4.706 | 4.558 | 4.405 | 3.909 |
| 12 | 4.747 | 3.885 | 3.490 | 3.259 | 3.106 | 2.996 | 2.849 | 2.753 | 2.687 | 2.617 | 2.544 | 2.296 |
| | 9.330 | 6.927 | 5.953 | 5.412 | 5.064 | 4.821 | 4.499 | 4.296 | 4.155 | 4.010 | 3.858 | 3.361 |
| 15 | 4.543 | 3.682 | 3.287 | 3.056 | 2.901 | 2.790 | 2.641 | 2.544 | 2.475 | 2.403 | 2.328 | 2.066 |
| | 8.683 | 6.359 | 5.417 | 4.893 | 4.556 | 4.318 | 4.004 | 3.805 | 3.666 | 3.522 | 3.372 | 2.868 |
| 20 | 4.351 | 3.493 | 3.098 | 2.866 | 2.711 | 2.599 | 2.447 | 2.348 | 2.278 | 2.203 | 2.124 | 1.843 |
| | 8.096 | 5.849 | 4.938 | 4.431 | 4.103 | 3.871 | 3.564 | 3.368 | 3.231 | 3.088 | 2.938 | 2.421 |
| 25 | 4.242 | 3.385 | 2.991 | 2.759 | 2.603 | 2.490 | 2.337 | 2.236 | 2.165 | 2.089 | 2.007 | 1.711 |
| | 7.770 | 5.568 | 4.675 | 4.177 | 3.855 | 3.627 | 3.324 | 3.129 | 2.993 | 2.850 | 2.699 | 2.169 |
| 30 | 4.171 | 3.316 | 2.922 | 2.690 | 2.534 | 2.421 | 2.266 | 2.165 | 2.092 | 2.015 | 1.932 | 1.622 |
| | 7.562 | 5.390 | 4.510 | 4.018 | 3.699 | 3.473 | 3.173 | 2.979 | 2.843 | 2.700 | 2.549 | 2.006 |
| 50 | 4.034 | 3.183 | 2.790 | 2.557 | 2.400 | 2.286 | 2.130 | 2.026 | 1.952 | 1.871 | 1.784 | 1.438 |
| | 7.171 | 5.057 | 4.199 | 3.720 | 3.408 | 3.186 | 2.890 | 2.698 | 2.563 | 2.419 | 2.265 | 1.683 |
| ∞ | 3.841 | 2.996 | 2.605 | 2.372 | 2.214 | 2.099 | 1.938 | 1.831 | 1.752 | 1.666 | 1.571 | 1.000 |
| | 6.635 | 4.605 | 3.782 | 3.319 | 3.017 | 2.802 | 2.511 | 2.321 | 2.185 | 2.039 | 1.878 | 1.000 |

Table 8.1.-II – *Generating procedure for the F-distribution*

| **If df1 is even** | **If df1 is odd and df2 is even** | **If df1 and df2 are odd** |
|---|---|---|
| `x=df1/(df1+df2/F)` | `x=df2/(df2+df1*F)` | `x=atn(sqr(df1*F/df2))` |
| `s=1` | `s=1` | `cs=cos(x)` |
| `t=1` | `t=1` | `sn=sin(x)` |
| `for i=2 to(df1-2) step 2` | `for i=2 to(df2-2) step 2` | `x=x/2` |
| `t=t*x*(df2+i-2)/i` | `t=t*x*(df1+i-2)/i` | `s=0` |
| `s=s+t` | `s=s+t` | `t=sn*cs/2` |
| `next i` | `next i` | `v=0` |
| `p=s*(1-x)^(df2/2)` | `p=1-s*(1-x)^(df1/2)` | `w=1` |
| | | `for i=2 to(df2-1) step 2` |
| | | `s=s+t` |
| | | `t=t*i/(i+1)*cs*cs` |
| | | `next i` |
| | | `for i=1 to(df1-2) step 2` |
| | | `v=v+w` |
| | | `w=w*(df2+i)/(i+2)*sn*sn` |
| | | `next i` |
| | | `p=1+(t*df2*v-x-s)/pi*4` |

8.2. THE *t*-DISTRIBUTION

If an observed value is higher than the value in Table 8.2.-I, it is considered to be significant ($p = 0.05$) or highly significant ($p = 0.01$).

Table 8.2.-I – *Critical values of the t-distribution*

| df | $p = 0.05$ | $p = 0.01$ | df | $p = 0.05$ | $p = 0.01$ |
|---|---|---|---|---|---|
| 1 | 12.706 | 63.656 | 22 | 2.074 | 2.819 |
| 2 | 4.303 | 9.925 | 24 | 2.064 | 2.797 |
| 3 | 3.182 | 5.841 | 26 | 2.056 | 2.779 |
| 4 | 2.776 | 4.604 | 28 | 2.048 | 2.763 |
| 5 | 2.571 | 4.032 | 30 | 2.042 | 2.750 |
| 6 | 2.447 | 3.707 | 35 | 2.030 | 2.724 |
| 7 | 2.365 | 3.499 | 40 | 2.021 | 2.704 |
| 8 | 2.306 | 3.355 | 45 | 2.014 | 2.690 |
| 9 | 2.262 | 3.250 | 50 | 2.009 | 2.678 |
| 10 | 2.228 | 3.169 | 60 | 2.000 | 2.660 |
| 12 | 2.179 | 3.055 | 70 | 1.994 | 2.648 |
| 14 | 2.145 | 2.977 | 80 | 1.990 | 2.639 |
| 16 | 2.120 | 2.921 | 90 | 1.987 | 2.632 |
| 18 | 2.101 | 2.878 | 100 | 1.984 | 2.626 |
| 20 | 2.086 | 2.845 | ∞ | 1.960 | 2.576 |

*Generating procedures.* The *p*-value for a given *t* with df degrees of freedom can be found with the procedures in Section 8.1 where `F` = $t^2$, `df1` = 1 and `df2` = df.

The *t*-value ($p = 0.05$) for a given number of degrees of freedom df can be found with the procedure in Table 8.2.-II, which should be accurate up to 6 decimal places.

Table 8.2.-II – *Generating procedure for the t-distribution*

```
t = 1.959964+
    2.37228/df+
    2.82202/df^2+
    2.56449/df^3+
    1.51956/df^4+
    1.02579/df^5+
    0.44210/df^7
```

8.3. THE $\chi^2$-DISTRIBUTION

Table 8.3.-I – *Critical values of the $\chi^2$-distribution*

| df | $p = 0.05$ | $p = 0.01$ | df | $p = 0.05$ | $p = 0.01$ |
|---|---|---|---|---|---|
| 1 | 3.841 | 6.635 | 11 | 19.675 | 24.725 |
| 2 | 5.991 | 9.210 | 12 | 21.026 | 26.217 |
| 3 | 7.815 | 11.345 | 13 | 22.362 | 27.688 |
| 4 | 9.488 | 13.277 | 14 | 23.685 | 29.141 |
| 5 | 11.070 | 15.086 | 15 | 24.996 | 30.578 |
| 6 | 12.592 | 16.812 | 16 | 26.296 | 32.000 |
| 7 | 14.067 | 18.475 | 20 | 31.410 | 37.566 |
| 8 | 15.507 | 20.090 | 25 | 37.652 | 44.314 |
| 9 | 16.919 | 21.666 | 30 | 43.773 | 50.892 |
| 10 | 18.307 | 23.209 | 40 | 55.758 | 63.691 |

If an observed value is higher than the value in Table 8.3.-I, it is considered to be significant ($p = 0.05$) or highly significant ($p = 0.01$).

*Generating procedure.* Let `X2` be the $\chi^2$-value and `df` as described above. The procedure in Table 8.3.-II will then generate the *p*-value.

Table 8.3.-II – *Generating procedure for the $\chi^2$-distribution*

| If df is even | If df is odd |
|---|---|
| `s=0` | `x=sqr(x2)` |
| `t=exp(-x2/2)` | `s=0` |
| `for i=2 to df step 2` | `t=x*exp(-x2/2)/sqr(pi/2)` |
| `s=s+t` | `for i=3 to df step 2` |
| `t=t*x2/i` | `s=s+t` |
| `next i` | `t=t*x2/i` |
| `p=1-s` | `next i` |
| | `p=1-s-2*phi(x)` |

In this procedure `phi` is the cumulative standard normal distribution function Φ (see Section 8.4).

8.4. THE Φ-DISTRIBUTION (THE CUMULATIVE STANDARD NORMAL DISTRIBUTION)

The Φ-value for negative *x* is found from Table 8.4.-I as $1 - \Phi(-x)$.

Table 8.4.-I – *Values of the Φ-distribution*

| x | Φ | x | Φ | x | Φ |
|---|---|---|---|---|---|
| 0.00 | 0.500 | 1.00 | 0.841 | 2.00 | 0.977 |
| 0.05 | 0.520 | 1.05 | 0.853 | 2.05 | 0.980 |
| 0.10 | 0.540 | 1.10 | 0.864 | 2.10 | 0.982 |
| 0.15 | 0.560 | 1.15 | 0.875 | 2.15 | 0.984 |
| 0.20 | 0.579 | 1.20 | 0.885 | 2.20 | 0.986 |
| 0.25 | 0.599 | 1.25 | 0.894 | 2.25 | 0.988 |
| 0.30 | 0.618 | 1.30 | 0.903 | 2.30 | 0.989 |
| 0.35 | 0.637 | 1.35 | 0.911 | 2.35 | 0.991 |
| 0.40 | 0.655 | 1.40 | 0.919 | 2.40 | 0.992 |
| 0.45 | 0.674 | 1.45 | 0.926 | 2.45 | 0.993 |
| 0.50 | 0.691 | 1.50 | 0.933 | 2.50 | 0.994 |
| 0.55 | 0.709 | 1.55 | 0.939 | 2.55 | 0.995 |
| 0.60 | 0.726 | 1.60 | 0.945 | 2.60 | 0.995 |
| 0.65 | 0.742 | 1.65 | 0.951 | 2.65 | 0.996 |
| 0.70 | 0.758 | 1.70 | 0.955 | 2.70 | 0.997 |
| 0.75 | 0.773 | 1.75 | 0.960 | 2.75 | 0.997 |
| 0.80 | 0.788 | 1.80 | 0.964 | 2.80 | 0.997 |
| 0.85 | 0.802 | 1.85 | 0.968 | 2.85 | 0.998 |
| 0.90 | 0.816 | 1.90 | 0.971 | 2.90 | 0.998 |
| 0.95 | 0.829 | 1.95 | 0.974 | 2.95 | 0.998 |

*Generating procedure*: Let `x` be the *x*-value. The procedure in Table 8.4.-II will generate the corresponding Φ-value if $0 \leq x \leq 8.15$. If *x* is greater than 8.15 the Φ-value can be set to 1. If *x* is negative, the formula given above can be used.

This procedure assumes that the computer can represent about 15 decimal places. If less digits or more digits can be represented, the procedure needs some trivial modifications.

Table 8.4.-II – *Generating procedure for the Φ-distribution*

```
s=0
t=x
i=1
repeat
s=s+t
i=i+2
t=t*x*x/i
until t<1E-16
phi=0.5+s*exp(-x*x/2)/sqr(2*pi)
```

## 8.5. RANDOM PERMUTATIONS

Random permutations are needed in randomised block designs. The following algorithm shows how the built-in random generator of a computer can be used to create random permutations of $N$ treatments.

Step 1. Write the $N$ possible treatments down in a row.

Step 2. Obtain a random integer $r$ such that $1 \leq r \leq N$.

Step 3. Exchange the $r$-th treatment with the $N$-th treatment in the row.

Step 4. Let $N = N - 1$ and repeat steps 2 to 4 until $N = 1$.

An example with 6 treatments will illustrate this algorithm.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1. | $N = 6$ | $S_1$ | $S_2$ | $S_3$ | $T_1$ | $T_2$ | $T_3$ |
| 2. | $r = 2$ | | → | | | | ← |
| 3. | | $S_1$ | $T_3$ | $S_3$ | $T_1$ | $T_2$ | $S_2$ |
| 4. | $N = 5$ | | | | | | |
| 2. | $r = 4$ | | | | → | ← | |
| 3. | | $S_1$ | $T_3$ | $S_3$ | $T_2$ | $T_1$ | $S_2$ |
| 4. | $N = 4$ | | | | | | |
| 2. | $r = 4$ | | | | ↓ | | |
| 3. | | $S_1$ | $T_3$ | $S_3$ | $T_2$ | $T_1$ | $S_2$ |
| 4. | $N = 3$ | | | | | | |
| 2. | $r = 1$ | → | | ← | | | |
| 3. | | $S_3$ | $T_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ |
| 4. | $N = 2$ | | | | | | |
| 2. | $r = 1$ | → | ← | | | | |
| 3. | | $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ |
| 4. | $N = 1$ | | | | | | |

## 8.6. LATIN SQUARES

The following example shows how 3 independent permutations can be used to obtain a Latin square.

1) Generate a random permutation of the $N$ possible treatments (see Section 8.5):

| | | | | | |
|---|---|---|---|---|---|
| $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ |

2) A simple Latin square can now be constructed by "rotating" this permutation to the right. This can be done as follows. Write the permutation found in step 1 down on the first row. The second row consists of the same permutation, but with all treatments shifted to the right. The rightmost treatment is put on the empty place at the left. This is repeated for all the rows until all the treatments appear once in each column:

| | | | | | |
|---|---|---|---|---|---|
| $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ |
| $S_2$ | $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ |
| $T_1$ | $S_2$ | $T_3$ | $S_3$ | $S_1$ | $T_2$ |
| $T_2$ | $T_1$ | $S_2$ | $T_3$ | $S_3$ | $S_1$ |
| $S_1$ | $T_2$ | $T_1$ | $S_2$ | $T_3$ | $S_3$ |
| $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ | $T_3$ |

3) Generate 2 independent random permutations of the figures 1 to $N$:

– one for the rows:

| | | | | | |
|---|---|---|---|---|---|
| **2** | **3** | **6** | **1** | **4** | **5** |

– and one for the columns:

| | | | | | |
|---|---|---|---|---|---|
| **3** | **4** | **6** | **2** | **5** | **1** |

4) The Latin square can now be found by sorting the rows and columns of the simple Latin square according to the 2 permutations for the rows and columns:

| | 3 | 4 | 6 | 2 | 5 | 1 |
|---|---|---|---|---|---|---|
| 2 | $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ |
| 3 | $S_2$ | $T_3$ | $S_3$ | $S_1$ | $T_2$ | $T_1$ |
| 6 | $T_1$ | $S_2$ | $T_3$ | $S_3$ | $S_1$ | $T_2$ |
| 1 | $T_2$ | $T_1$ | $S_2$ | $T_3$ | $S_3$ | $S_1$ |
| 4 | $S_1$ | $T_2$ | $T_1$ | $S_2$ | $T_3$ | $S_3$ |
| 5 | $S_3$ | $S_1$ | $T_2$ | $T_1$ | $S_2$ | $T_3$ |
| | | | ↓ | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| 1 | $S_1$ | $T_3$ | $T_2$ | $T_1$ | $S_3$ | $S_2$ |
| 2 | $S_2$ | $T_2$ | $T_3$ | $S_3$ | $T_1$ | $S_1$ |
| 3 | $T_1$ | $S_1$ | $S_2$ | $T_3$ | $T_2$ | $S_3$ |
| 4 | $S_3$ | $S_2$ | $S_1$ | $T_2$ | $T_3$ | $T_1$ |
| 5 | $T_3$ | $T_1$ | $S_3$ | $S_1$ | $S_2$ | $T_2$ |
| 6 | $T_2$ | $S_3$ | $T_1$ | $S_2$ | $S_1$ | $T_3$ |

## 9. GLOSSARY OF SYMBOLS

| Symbol | Definition |
|---|---|
| $a$ | Intersection of linear regression of responses on dose or ln(dose) |
| $b$ | Slope of linear regression of responses on dose or on ln(dose) |
| $d$ | Number of dose levels for each preparation (excluding the blank in slope-ratio assays) |
| $e$ | Base of natural logarithms (= 2.71828182845905...) |
| $g$ | Statistic used in Fieller's theorem: $g = \frac{C-1}{C}$ |
| $h$ | Number of preparations in an assay, including the standard preparation |

| Symbol | Definition |
|---|---|
| $m$ | Potency estimate obtained as a ratio of effects in general linear models |
| $n$ | Number of replicates for each treatment |
| $p$ | Probability of a given statistic being larger than the observed value. Also used as the ratio $r/n$ in probit analysis |
| $r$ | The number of responding units per treatment group in assays depending upon quantal responses |
| $s$ | Estimate of standard deviation ($= \sqrt{s^2}$) |
| $s^2$ | Estimate of residual variance given by error mean square in analysis of variance |
| $t$ | Student's statistic (Table 8.2.) |
| $u$ | Observed response in four-parameter analysis |
| $v_{11}, v_{12}, v_{22}$ | (Co)variance multipliers for numerator and denominator of ratio $m$ in Fieller's theorem |
| $w$ | Weighting coefficient |
| $x$ | The ln(dose) |
| $y$ | Individual response or transformed response |
| $A$ | Assumed potencies of test preparations when making up doses |
| $B$ | Mean response to blanks in slope-ratio assays |
| $C$ | Statistic used in the calculation of confidence intervals: $C = \frac{1}{1-g}$ |
| $C_1, \ldots, C_n$ | Mean response to each column of a Latin square design |
| $D_1, D_2$ | Mean response on time 1 or time 2 in the twin cross-over design |
| $F$ | Ratio of 2 independent estimates of variance following an $F$-distribution (Table 8.1.) |
| $G_S, G_T, \ldots$ | Treatment values used in the analysis of variance for slope-ratio assays |
| $H_P, H_L$ | Multipliers used in the analysis of variance for parallel-line assays |
| $H_B, H_I$ | Multipliers used in the analysis of variance for slope-ratio assays |
| $I$ | In parallel-line assays, the ln of the ratio between adjacent doses. In slope-ratio assays, the interval between adjacent doses |
| $J_S, J_T, \ldots$ | Linearity values used in the analysis of variance for slope-ratio assays |
| $K$ | Correction term used in the calculation of sums of squares in the analysis of variance |
| $L$ | Width of confidence interval in logarithms |
| $L_S, L_T, \ldots$ | Linear contrasts of standard and test preparations |
| $M$ | ln potency |
| $M'$ | ln potency ratio of a given test preparation |
| $N$ | Total number of treatments in the assay ($= dh$) |
| $P_S, P_T, \ldots$ | Sum of standard and test preparations |
| $R$ | Estimated potency of a given test preparation |
| $R'$ | Potency ratio of a given test preparation |
| $R_1, \ldots, R_n$ | Mean response in each of rows 1 to $n$ of a Latin square design, or in each block of a randomised block design |
| $S$ | Standard preparation |
| $S_1, \ldots, S_d$ | Mean response to the lowest dose 1 up to the highest dose $d$ of the standard preparation $S$ |
| $SS$ | Sum of squares due to a given source of variation |
| $T, U, V, \ldots$ | Test preparations |
| $T_1, \ldots, T_d$ | Mean response to the lowest dose 1 up to the highest dose d of test preparation $T$ |
| $V$ | Variance coefficient in the calculation of confidence limits |
| $W$ | Weighting factor in combination of assay results |
| $\boldsymbol{X}$ | Linear structure or design matrix used in general linear models |
| $Y$ | Vector representing the (transformed) responses in general linear models |
| $Z$ | The first derivative of $\Phi$ |
| $\alpha$ | Upper asymptote of the ln(dose)-response curve in four-parameter analysis |
| $\beta$ | Slope-factor of the ln(dose)-response curve in four-parameter analysis |
| $\gamma$ | The ln(dose) giving 50 per cent response in the four-parameter analysis |
| $\delta$ | Lower asymptote of the ln(dose)-response curve in four-parameter analysis |
| $\pi$ | 3.141592653589793238... |
| $\Phi$ | Cumulative standard normal distribution function (Table 8.4.) |
| $\chi^2$ | Chi-square statistic (Table 8.3.) |

**10. LITERATURE**

*This section lists some recommended literature for further study.*

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# 5.4. Residual solvents

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# 5.4. RESIDUAL SOLVENTS

## LIMITING RESIDUAL SOLVENT LEVELS IN ACTIVE SUBSTANCES, EXCIPIENTS AND MEDICINAL PRODUCTS

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has adopted Impurities Guidelines for Residual Solvents which prescribes limits for the content of solvents which may remain in active substances, excipients and medicinal products after processing. This guideline, the text of which is reproduced below, excludes existing marketed products. The European Pharmacopoeia is, however, applying the same principles enshrined in the guideline to existing active substances, excipients and medicinal products whether or not they are the subject of a monograph of the Pharmacopoeia. All substances and products are to be tested for the content of solvents likely to be present in a substance or product.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs since the solvents employed may vary from one manufacturer to another and the requirements of this general chapter are applied via the general monograph on *Substances for Pharmaceutical Use (2034)*. The competent authority is to be informed of the solvents employed during the production process. This information is also given in the dossier submitted for a certificate of suitability of the monographs of the European Pharmacopoeia and is mentioned on the certificate.

Where only Class 3 solvents are used, a test for loss on drying may be applied or a specific determination of the solvent may be made. If for a Class 3 solvent a justified and authorised limit higher than 0.5 per cent is applied, a specific determination of the solvent is required.

When Class 1 residual solvents or Class 2 residual solvents (or Class 3 residual solvents which exceed the 0.5 per cent) are used, the methodology described in the general method (*2.4.24*) is to be applied wherever possible. Otherwise an appropriate validated method is to be employed.

When a quantitative determination of a residual solvent is carried out, the result is taken into account for the calculation of the content of the substance except where a test for drying is carried out.

## IMPURITIES: GUIDELINES FOR RESIDUAL SOLVENTS (CHMP/ICH/82260/2006)

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2. SCOPE OF THE GUIDELINE

3. GENERAL PRINCIPLES

*3.1. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT*

*3.2. METHODS FOR ESTABLISHING EXPOSURE LIMITS*

*3.3. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS*

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GLOSSARY

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE

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*A2.1. ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS*

*A2.2. RESIDUAL SOLVENTS IN PHARMACEUTICALS*

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

### 1. INTRODUCTION

The objective of this guideline is to recommend acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient. The guideline recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

Residual solvents in pharmaceuticals are defined here as organic volatile chemicals that are used or produced in the manufacture of active substances or excipients, or in the preparation of medicinal products. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of active substance may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. This guideline does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements. Medicinal products should contain no higher levels of residual solvents than can be supported by safety data. Some solvents that are known to cause unacceptable toxicities (Class 1, Table 1) should be avoided in the production of active substances, excipients, or medicinal products unless their use can be strongly justified in a risk-benefit assessment. Some solvents associated with less severe toxicity (Class 2, Table 2) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, Table 3) should be used where practical. The complete list of solvents included in this guideline is given in Appendix 1.

The lists are not exhaustive and other solvents can be used and later added to the lists. Recommended limits of Class 1 and 2 solvents or classification of solvents may change as new safety data becomes available. Supporting safety data in a marketing application for a new medicinal product containing a new solvent may be based on concepts in this guideline or the concept of qualification of impurities as expressed in the guideline for active substances (*Q3A*, Impurities in New Active Substances) or medicinal products (*Q3B*, Impurities in New Medicinal Products), or all three guidelines.

## 2. SCOPE OF THE GUIDELINE

Residual solvents in active substances, excipients, and in medicinal products are within the scope of this guideline. Therefore, testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. It is only necessary to test for solvents that are used or produced in the manufacture or purification of active substances, excipients, or medicinal product. Although manufacturers may choose to test the medicinal product, a cumulative method may be used to calculate the residual solvent levels in the medicinal product from the levels in the ingredients used to produce the medicinal product. If the calculation results in a level equal to or below that recommended in this guideline, no testing of the medicinal product for residual solvents need be considered. If however, the calculated level is above the recommended level, the medicinal product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. Medicinal product should also be tested if a solvent is used during its manufacture.

This guideline does not apply to potential new active substances, excipients, or medicinal products used during the clinical research stages of development, nor does it apply to existing marketed medicinal products.

The guideline applies to all dosage forms and routes of administration. Higher levels of residual solvents may be acceptable in certain cases such as short term (30 days or less) or topical application. Justification for these levels should be made on a case by case basis.

See Appendix 2 for additional background information related to residual solvents.

## 3. GENERAL PRINCIPLES

### *3.1. CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT*

The term "tolerable daily intake" (TDI) is used by the International Program on Chemical Safety (IPCS) to describe exposure limits of toxic chemicals and "acceptable daily intake" (ADI) is used by the World Health Organization (WHO) and other national and international health authorities and institutes. The new term "permitted daily exposure" (PDE) is defined in the present guideline as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADI's of the same substance.

Residual solvents assessed in this guideline are listed in Appendix 1 by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

**Class 1 solvents: solvents to be avoided**

Known human carcinogens, strongly suspected human carcinogens, and environmental hazards.

**Class 2 solvents: solvents to be limited**

Non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity.

Solvents suspected of other significant but reversible toxicities.

**Class 3 solvents: solvents with low toxic potential**

Solvents with low toxic potential to man; no health-based exposure limit is needed. Class 3 solvents have PDEs of 50 mg or more per day.

### *3.2. METHODS FOR ESTABLISHING EXPOSURE LIMITS*

The method used to establish permitted daily exposures for residual solvents is presented in Appendix 3. Summaries of the toxicity data that were used to establish limits are published in *Pharmeuropa*, Vol. 9, No. 1, Supplement April 1997.

### *3.3. OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS*

Two options are available when setting limits for Class 2 solvents.

**Option 1**: the concentration limits in parts per million stated in Table 2 can be used. They were calculated using equation (1) below by assuming a product mass of 10 g administered daily.

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{dose}} \qquad (1)$$

Here, PDE is given in terms of mg/day and dose is given in g/day.

These limits are considered acceptable for all substances, excipients, or products. Therefore this option may be applied if the daily dose is not known or fixed. If all excipients and active substances in a formulation meet the limits given in Option 1, then these components may be used in any proportion. No further calculation is necessary provided the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day should be considered under Option 2.

**Option 2**: it is not considered necessary for each component of the medicinal product to comply with the limits given in Option 1. The PDE in terms of mg/day as stated in Table 2 can be used with the known maximum daily dose and equation (1) above to determine the concentration of residual solvent allowed in a medicinal product. Such limits are considered acceptable provided that is has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, reasonable variation in the manufacturing process, and the limits should reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the medicinal product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the use of Option 1 and Option 2 applied to acetonitrile in a medicinal product. The permitted daily exposure to acetonitrile is 4.1 mg per day; thus, the Option 1 limit is 410 ppm. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile are given in the following table.

| Component | Amount in formulation | Acetonitrile content | Daily exposure |
|---|---|---|---|
| Active substance | 0.3 g | 800 ppm | 0.24 mg |
| Excipient 1 | 0.9 g | 400 ppm | 0.36 mg |
| Excipient 2 | 3.8 g | 800 ppm | 3.04 mg |
| Medicinal product | 5.0 g | 728 ppm | 3.64 mg |

Excipient 1 meets the Option 1 limit, but the active substance, excipient 2, and medicinal product do not meet the Option 1 limit. Nevertheless, the product meets the Option 2 limit of 4.1 mg per day and thus conforms to the recommendations in this guideline.

Consider another example using acetonitrile as residual solvent. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile is given in the following table.

| Component | Amount in formulation | Acetonitrile content | Daily exposure |
|---|---|---|---|
| Active substance | 0.3 g | 800 ppm | 0.24 mg |
| Excipient 1 | 0.9 g | 2000 ppm | 1.80 mg |
| Excipient 2 | 3.8 g | 800 ppm | 3.04 mg |
| Medicinal product | 5.0 g | 1016 ppm | 5.08 mg |

In this example, the product meets neither the Option 1 nor the Option 2 limit according to this summation. The manufacturer could test the medicinal product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the medicinal product should take other steps to reduce the amount of acetonitrile in the medicinal product. If all of these steps fail to reduce the level of residual solvent, in exceptional cases the manufacturer could provide a summary of efforts made to reduce the solvent level to meet the guideline value, and provide a risk-benefit analysis to support allowing the product to be utilised containing residual solvent at a higher level.

*3.4. ANALYTICAL PROCEDURES*

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Any harmonised procedures for determining levels of residual solvents as described in the pharmacopoeias should be used, if feasible. Otherwise, manufacturers would be free to select the most appropriate validated analytical procedure for a particular application. If only Class 3 solvents are present, a non-specific method such as loss on drying may be used.

Validation of methods for residual solvents should conform to ICH guidelines "Text on Validation of Analytical Procedures" and "Extension of the ICH Text on Validation of Analytical Procedures".

*3.5. REPORTING LEVELS OF RESIDUAL SOLVENTS*

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in excipients or active substances in order to meet the criteria of this guideline. The following statements are given as acceptable examples of the information that could be provided from a supplier of excipients or active substances to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

– only Class 3 solvents are likely to be present. Loss on drying is less than 0.5 per cent;

– only Class 2 solvents X, Y, ... are likely to be present. All are below the Option 1 limit;

(Here the supplier would name the Class 2 solvents represented by X, Y, ...)

– only Class 2 solvents X, Y, ... and Class 3 solvents are likely to be present. Residual Class 2 solvents are below the Option 1 limit and residual Class 3 solvents are below 0.5 per cent.

If Class 1 solvents are likely to be present, they should be identified and quantified. "Likely to be present" refers to the solvent used in the final manufacturing step and to solvents that are used in earlier manufacturing steps and not removed consistently by a validated process.

If solvents of Class 2 or Class 3 are present at greater than their Option 1 limits or 0.5 per cent, respectively, they should be identified and quantified.

## 4. LIMITS OF RESIDUAL SOLVENTS

*4.1. SOLVENTS TO BE AVOIDED*

Solvents in Class 1 should not be employed in the manufacture of active substances, excipients, and medicinal products because of their unacceptable toxicity or their deleterious environmental effect. However, if their use is unavoidable in order to produce a medicinal product with a significant therapeutic advance, then their levels should be restricted as shown in Table 1, unless otherwise justified. 1,1,1-Trichloroethane is included in Table 1 because it is an environmental hazard. The stated limit of 1500 ppm is based on a review of the safety data.

Table 1. – *Class 1 solvents in pharmaceutical products (solvents that should be avoided)*

| Solvent | Concentration limit (ppm) | Concern |
|---|---|---|
| Benzene | 2 | Carcinogen |
| Carbon tetrachloride | 4 | Toxic and environmental hazard |
| 1,2-Dichloroethane | 5 | Toxic |
| 1,1-Dichloroethene | 8 | Toxic |
| 1,1,1-Trichloroethane | 1500 | Environmental hazard |

*4.2. SOLVENTS TO BE LIMITED*

Solvents in Table 2 should be limited in pharmaceutical products because of their inherent toxicity. PDEs are given to the nearest 0.1 mg/day, and concentrations are given to the nearest 10 ppm. The stated values do not reflect the necessary analytical precision of determination. Precision should be determined as part of the validation of the method.

Table 2. – *Class 2 solvents in pharmaceutical products*

| Solvent | PDE (mg/day) | Concentration limit (ppm) |
|---|---|---|
| Acetonitrile | 4.1 | 410 |
| Chlorobenzene | 3.6 | 360 |
| Chloroform | 0.6 | 60 |
| Cumene | 0.7 | 70 |
| Cyclohexane | 38.8 | 3880 |
| 1,2-Dichloroethene | 18.7 | 1870 |
| Dichloromethane | 6.0 | 600 |
| 1,2-Dimethoxyethane | 1.0 | 100 |
| *N,N*-Dimethylacetamide | 10.9 | 1090 |
| *N,N*-Dimethylformamide | 8.8 | 880 |
| 1,4-Dioxane | 3.8 | 380 |
| 2-Ethoxyethanol | 1.6 | 160 |
| Ethyleneglycol | 6.2 | 620 |
| Formamide | 2.2 | 220 |
| Hexane | 2.9 | 290 |
| Methanol | 30.0 | 3000 |
| 2-Methoxyethanol | 0.5 | 50 |
| Methylbutylketone | 0.5 | 50 |
| Methylcyclohexane | 11.8 | 1180 |
| Methylisobutylketone | 45.0 | 4500 |
| *N*-Methylpyrrolidone | 5.3 | 530 |
| Nitromethane | 0.5 | 50 |
| Pyridine | 2.0 | 200 |
| Sulfolane | 1.6 | 160 |

| Solvent | PDE (mg/day) | Concentration limit (ppm) |
|---|---|---|
| Tetrahydrofuran | 7.2 | 720 |
| Tetralin | 1.0 | 100 |
| Toluene | 8.9 | 890 |
| 1,1,2-Trichloroethene | 0.8 | 80 |
| Xylene* | 21.7 | 2170 |

*usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene.

*4.3. SOLVENTS WITH LOW TOXIC POTENTIAL*

Solvents in Class 3 (shown in Table 3) may be regarded as less toxic and of lower risk to human health. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies. It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5000 ppm or 0.5 per cent under Option l) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice.

Table 3. – *Class 3 solvents which should be limited by GMP or other quality-based requirements*

| | |
|---|---|
| Acetic acid | Heptane |
| Acetone | Isobutyl acetate |
| Anisole | Isopropyl acetate |
| 1-Butanol | Methyl acetate |
| 2-Butanol | 3-Methyl-1-butanol |
| Butyl acetate | Methylethylketone |
| *tert*-Butylmethyl ether | 2-Methyl-1-propanol |
| Dimethyl sulfoxide | Pentane |
| Ethanol | 1-Pentanol |
| Ethyl acetate | 1-Propanol |
| Ethyl ether | 2-Propanol |
| Ethyl formate | Propyl acetate |
| Formic acid | Triethylamine |

*4.4. SOLVENTS FOR WHICH NO ADEQUATE TOXICOLOGICAL DATA WAS FOUND*

The following solvents (Table 4) may also be of interest to manufacturers of excipients, active substances, or medicinal products. However, no adequate toxicological data on which to base a PDE was found. Manufacturers should supply justification for residual levels of these solvents in pharmaceutical products.

Table 4. – *Solvents for which no adequate toxicological data was found*

| | |
|---|---|
| 1,1-Diethoxypropane | Methylisopropylketone |
| 1,1-Dimethoxymethane | Methyltetrahydrofuran |
| 2,2-Dimethoxypropane | Petroleum ether |
| Isooctane | Trichloroacetic acid |
| Isopropyl ether | Trifluoroacetic acid |

GLOSSARY

*Genotoxic carcinogens*: carcinogens which produce cancer by affecting genes or chromosomes.

*LOEL:* abbreviation for *lowest-observed effect level.*

*Lowest-observed effect level*: the lowest dose of substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in the exposed humans or animals.

*Modifying factor*: a factor determined by professional judgement of a toxicologist and applied to bioassay data to relate that data safely to humans.

*Neurotoxicity*: the ability of a substance to cause adverse effects on the nervous system.

*NOEL*: abbreviation for *no-observed-effect level.*

*No-observed-effect level*: the highest dose of substance at which there are no biologically significant increases in frequency or severity of any effects in the exposed humans or animals.

*PDE*: abbreviation for *permitted daily exposure.*

*Permitted daily exposure*: the maximum acceptable intake per day of residual solvent in pharmaceutical products.

*Reversible toxicity*: the occurrence of harmful effects that are caused by a substance and which disappear after exposure to the substance ends.

*Strongly suspected human carcinogen*: a substance for which there is no epidemiological evidence of carcinogenesis but there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.

*Teratogenicity*: the occurrence of structural malformations in a developing foetus when a substance is administered during pregnancy.

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE

| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| Acetic acid | Ethanoic acid | $CH_3COOH$ | Class 3 |
| Acetone | 2-Propanone<br>Propan-2-one | $CH_3COCH_3$ | Class 3 |
| Acetonitrile | | $CH_3CN$ | Class 2 |
| Anisole | Methoxybenzene | 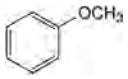 | Class 3 |
| Benzene | Benzol | | Class 1 |
| 1-Butanol | *n*-Butyl alcohol<br>Butan-1-ol | $CH_3[CH_2]_3OH$ | Class 3 |
| 2-Butanol | *sec*-Butyl alcohol<br>Butan-2-ol | $CH_3CH_2CH(OH)CH_3$ | Class 3 |
| Butyl acetate | Acetic acid butyl ester | $CH_3COO[CH_2]_3CH_3$ | Class 3 |

| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| *tert*-Butylmethyl ether | 2-Methoxy-2-methylpropane | $(CH_3)_3COCH_3$ | Class 3 |
| Carbon tetrachloride | Tetrachloromethane | $CCl_4$ | Class 1 |
| Chlorobenzene | | 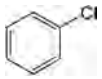 | Class 2 |
| Chloroform | Trichloromethane | $CHCl_3$ | Class 2 |
| Cumene | Isopropylbenzene<br>(1-Methylethyl)benzene | 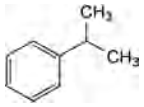 | Class 2 |
| Cyclohexane | Hexamethylene | | Class 2 |
| 1,2-Dichloroethane | *sym*-Dichloroethane<br>Ethylene dichloride<br>Ethylene chloride | $CH_2ClCH_2Cl$ | Class 1 |
| 1,1-Dichloroethene | 1,1-Dichloroethylene<br>Vinylidene chloride | $H_2C=CCl_2$ | Class 1 |
| 1,2-Dichloroethene | 1,2-Dichloroethylene<br>Acetylene dichloride | $ClHC=CHCl$ | Class 2 |
| Dichloromethane | Methylene chloride | $CH_2Cl_2$ | Class 2 |
| 1,2-Dimethoxyethane | Ethyleneglycol dimethyl ether<br>Monoglyme<br>Dimethyl cellosolve | $H_3COCH_2CH_2OCH_3$ | Class 2 |
| *N*,*N*-Dimethylacetamide | DMA | $CH_3CON(CH_3)_2$ | Class 2 |
| *N*,*N*-Dimethylformamide | DMF | $HCON(CH_3)_2$ | Class 2 |
| Dimethyl sulfoxide | Methylsulfinylmethane<br>Methyl sulfoxide<br>DMSO | $(CH_3)_2SO$ | Class 3 |
| 1,4-Dioxane | *p*-Dioxane<br>[1,4]Dioxane | | Class 2 |
| Ethanol | Ethyl alcohol | $CH_3CH_2OH$ | Class 3 |
| 2-Ethoxyethanol | Cellosolve | $CH_3CH_2OCH_2CH_2OH$ | Class 2 |
| Ethyl acetate | Acetic acid ethyl ester | $CH_3COOCH_2CH_3$ | Class 3 |
| Ethyleneglycol | 1,2-Dihydroxyethane<br>1,2-Ethanediol | $HOCH_2CH_2OH$ | Class 2 |
| Ethyl ether | Diethyl ether<br>Ethoxyethane<br>1,1′-Oxybisethane | $CH_3CH_2OCH_2CH_3$ | Class 3 |
| Ethyl formate | Formic acid ethyl ester | $HCOOCH_2CH_3$ | Class 3 |
| Formamide | Methanamide | $HCONH_2$ | Class 2 |
| Formic acid | | $HCOOH$ | Class 3 |
| Heptane | *n*-Heptane | $CH_3[CH_2]_5CH_3$ | Class 3 |
| Hexane | *n*-Hexane | $CH_3[CH_2]_4CH_3$ | Class 2 |
| Isobutyl acetate | Acetic acid isobutyl ester | $CH_3COOCH_2CH(CH_3)_2$ | Class 3 |
| Isopropyl acetate | Acetic acid isopropyl ester | $CH_3COOCH(CH_3)_2$ | Class 3 |
| Methanol | Methyl alcohol | $CH_3OH$ | Class 2 |
| 2-Methoxyethanol | Methyl cellosolve | $CH_3OCH_2CH_2OH$ | Class 2 |
| Methyl acetate | Acetic acid methyl ester | $CH_3COOCH_3$ | Class 3 |
| 3-Methyl-1-butanol | Isoamyl alcohol<br>Isopentyl alcohol<br>3-Methylbutan-1-ol | $(CH_3)_2CHCH_2CH_2OH$ | Class 3 |
| Methylbutylketone | 2-Hexanone<br>Hexan-2-one | $CH_3[CH_2]_3COCH_3$ | Class 2 |

| Solvent | Other Names | Structure | Class |
|---|---|---|---|
| Methylcyclohexane | Cyclohexylmethane | 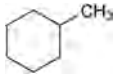 | Class 2 |
| Methylethylketone | 2-Butanone<br>MEK<br>Butan-2-one | $CH_3CH_2COCH_3$ | Class 3 |
| Methylisobutylketone | 4-Methylpentan-2-one<br>4-Methyl-2-pentanone<br>MIBK | $CH_3COCH_2CH(CH_3)_2$ | Class 2 |
| 2-Methyl-1-propanol | Isobutyl alcohol<br>2-Methylpropan-1-ol | $(CH_3)_2CHCH_2OH$ | Class 3 |
| *N*-Methylpyrrolidone | 1-Methylpyrrolidin-2-one<br>1-Methyl-2-pyrrolidinone | | Class 2 |
| Nitromethane | | $CH_3NO_2$ | Class 2 |
| Pentane | *n*-Pentane | $CH_3[CH_2]_3CH_3$ | Class 3 |
| 1-Pentanol | Amyl alcohol<br>Pentan-1-ol<br>Pentyl alcohol | $CH_3[CH_2]_3CH_2OH$ | Class 3 |
| 1-Propanol | Propan-1-ol<br>Propyl alcohol | $CH_3CH_2CH_2OH$ | Class 3 |
| 2-Propanol | Propan-2-ol<br>Isopropyl alcohol | $(CH_3)_2CHOH$ | Class 3 |
| Propyl acetate | Acetic acid propyl ester | $CH_3COOCH_2CH_2CH_3$ | Class 3 |
| Pyridine | | | Class 2 |
| Sulfonane | Tetrahydrothiophene 1,1-dioxide | | Class 2 |
| Tetrahydrofuran | Tetramethylene oxide<br>Oxacyclopentane | | Class 2 |
| Tetralin | 1,2,3,4-Tetrahydronaphthalene | 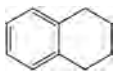 | Class 2 |
| Toluene | Methylbenzene | 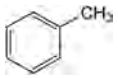 | Class 2 |
| 1,1,1-Trichloroethane | Methylchloroform | $CH_3CCl_3$ | Class 1 |
| 1,1,2-Trichloroethene | Trichloroethene | $HClC{=}CCl_2$ | Class 2 |
| Triethylamine | *N*,*N*-Diethylethanamine | $N(CH_2CH_3)_3$ | Class 3 |
| Xylene* | Dimethybenzene<br>Xylol | 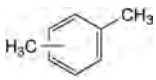 | Class 2 |

*usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene.

## APPENDIX 2. ADDITIONAL BACKGROUND

### *A2.1. ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS*

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in Environmental Health Criteria (EHC) monographs and the Integrated Risk Information System (IRIS). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (USEPA) and the United States Food and Drug Administration (USFDA) include the determination of acceptable exposure levels. The goal is protection of human health and maintenance of environmental integrity against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The methods involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter term study data can be used with modification of the approach such as use of larger safety factors. The approach described therein relates primarily to long-term or life-time exposure of the general population in the ambient environment, i.e. ambient air, food, drinking water and other media.

*A2.2. RESIDUAL SOLVENTS IN PHARMACEUTICALS*

Exposure limits in this guideline are established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, some specific assumptions about residual solvents to be used in the synthesis and formulation of pharmaceutical products should be taken into account in establishing exposure limits. They are:

1) Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.

2) The assumption of life-time patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.

3) Residual solvents are unavoidable components in pharmaceutical production and will often be a part of medicinal products.

4) Residual solvents should not exceed recommended levels except in exceptional circumstances.

5) Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described for example, by OECD, EPA, and the FDA Red Book.

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment (Gaylor, D. W. and Kodell, R. L. Linear Interpolation algorithm for low dose assessment of toxic substance. J. Environ. Pathology, 4, 305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 solvents could be determined with the use of a large safety factor (i.e., 10 000 to 100 000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these solvents should be by state-of-the-art analytical techniques.

Acceptable exposure levels in this guideline for Class 2 solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (*Pharmacopeial Forum*, Nov-Dec 1989), and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (*Environmental Health Criteria 170*, WHO, 1994). These methods are similar to those used by the USEPA (IRIS) and the USFDA (*Red Book*) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values tabulated in Section 4 of this document.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE} = \frac{\text{NOEL} \times \text{Weight Adjustment}}{\text{F1} \times \text{F2} \times \text{F3} \times \text{F4} \times \text{F5}}$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of "uncertainty factors" used in Environmental Health Criteria (*Environmental Health Criteria* 170, World Health Organization, Geneva, 1994), and "modifying factors" or "safety factors" in *Pharmacopoeial Forum*. The assumption of 100 per cent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

F1 = a factor to account for extrapolation between species:

- F1 = 2 for extrapolation from dogs to humans;
- F1 = 2.5 for extrapolation from rabbits to humans;
- F1 = 3 for extrapolation from monkeys to humans;
- F1 = 5 for extrapolation from rats to humans;
- F1 = 10 for extrapolation from other animals to humans;
- F1 = 12 for extrapolation from mice to humans.

F1 takes into account the comparative surface area: body weight ratios for the species concerned and for man. Surface area ($S$) is calculated as:

$$S = km^{0.67}$$

in which $m$ = body mass, and the constant $k$ has been taken to be 10. The body weight used in the equation are those shown below in Table A3.-1.

Table A3.-1. – *Values used in the calculations in this document*

| | |
|---|---|
| Rat body weight | 425 g |
| Pregnant rat body weight | 330 g |
| Mouse body weight | 28 g |
| Pregnant mouse body weight | 30 g |
| Guinea-pig body weight | 500 g |
| Rhesus monkey body weight | 2.5 kg |
| Rabbit body weight (pregnant or not) | 4 kg |
| Beagle dog body weight | 11.5 kg |
| Rat respiratory volume | 290 L/day |
| Mouse respiratory volume | 43 L/day |
| Rabbit respiratory volume | 1440 L/day |
| Guinea-pig respiratory volume | 430 L/day |
| Human respiratory volume | 28800 L/day |
| Dog respiratory volume | 9000 L/day |
| Monkey respiratory volume | 1150 L/day |
| Mouse water consumption | 5 mL/day |
| Rat water consumption | 30 mL/day |
| Rat food consumption | 30 g/day |

F2 = a factor of 10 to account for variability between individuals.

A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this guideline.

F3 = a variable factor to account for toxicity studies of short-term exposure:

F3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs and monkeys);

F3 = 1 for reproductive studies in which the whole period of organogenesis is covered;

F3 = 2 for a 6 month study in rodents, or a 3.5 year study in non-rodents;

F3 = 5 for a 3 month study in rodents, or a 2 year study in non-rodents;

F3 = 10 for studies of a shorter duration.

In all cases, the higher factor has been used for study durations between the time points, e.g. a factor of 2 for a 9 month rodent study.

F4 = a factor that may be applied in cases of severe toxicity, e.g. non-genotoxic carcinogenicity, neurotoxicity or teratogenicity.

In studies of reproductive toxicity, the following factors are used:

F4 = 1 for foetal toxicity associated with maternal toxicity;

F4 = 5 for foetal toxicity without maternal toxicity;

F4 = 5 for a teratogenic effect with maternal toxicity;

F4 = 10 for a teratogenic effect without maternal toxicity.

F5 = a variable factor that may be applied if the no-effect level was not established.

When only a LOEL is available, a factor of up to 10 can be used depending on the severity of the toxicity.

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognised that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for paediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider the toxicity study of acetonitrile in mice that is summarised in *Pharmeuropa*, Vol. 9. No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg $kg^{-1}$ $day^{-1}$. The PDE for acetonitrile in this study is calculated as follows:

$$\text{PDE} = \frac{50.7 \text{mg kg}^{-1} \text{day}^{-1} \times 50 \text{ kg}}{12 \times 10 \times 5 \times 1 \times 1} = 4.22 \text{ mg day}^{-1}$$

In this example,

F1 = 12 to account for the extrapolation from mice to humans;

F2 = 10 to account for differences between individual humans;

F3 = 5 because the duration of the study was only 13 weeks;

F4 = 1 because no severe toxicity was encountered;

F5 = 1 because the no-effect level was determined.

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or $mg/m^3$. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight 153.84) summarised in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6} \text{ atm} \times 153\,840 \text{ mg mol}^{-1}}{0.082 \text{ L atm K}^{-1}\text{mol}^{-1} \times 298 \text{ K}}$$

$$= \frac{46.15 \text{ mg}}{24.45 \text{ L}} = 1.89 \text{ mg / L}$$

The relationship 1000 L = 1 $m^3$ is used to convert to $mg/m^3$.

# 5.5. Alcoholimetric tables

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# 5.5. ALCOHOLIMETRIC TABLES

The general formula agreed by the Council of the European Communities in its Directive of 27 July 1976 on alcoholimetry served as the basis for establishing the following tables.

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 0.0 | 0.0 | 998.20 |
| 0.1 | 0.08 | 998.05 |
| 0.2 | 0.16 | 997.90 |
| 0.3 | 0.24 | 997.75 |
| 0.4 | 0.32 | 997.59 |
| 0.5 | 0.40 | 997.44 |
| 0.6 | 0.47 | 997.29 |
| 0.7 | 0.55 | 997.14 |
| 0.8 | 0.63 | 996.99 |
| 0.9 | 0.71 | 996.85 |
| 1.0 | 0.79 | 996.70 |
| 1.1 | 0.87 | 996.55 |
| 1.2 | 0.95 | 996.40 |
| 1.3 | 1.03 | 996.25 |
| 1.4 | 1.11 | 996.11 |
| 1.5 | 1.19 | 995.96 |
| 1.6 | 1.27 | 995.81 |
| 1.7 | 1.35 | 995.67 |
| 1.8 | 1.43 | 995.52 |
| 1.9 | 1.51 | 995.38 |
| 2.0 | 1.59 | 995.23 |
| 2.1 | 1.67 | 995.09 |
| 2.2 | 1.75 | 994.94 |
| 2.3 | 1.82 | 994.80 |
| 2.4 | 1.90 | 994.66 |
| 2.5 | 1.98 | 994.51 |
| 2.6 | 2.06 | 994.37 |
| 2.7 | 2.14 | 994.23 |
| 2.8 | 2.22 | 994.09 |
| 2.9 | 2.30 | 993.95 |
| 3.0 | 2.38 | 993.81 |
| 3.1 | 2.46 | 993.66 |
| 3.2 | 2.54 | 993.52 |
| 3.3 | 2.62 | 993.38 |
| 3.4 | 2.70 | 993.24 |
| 3.5 | 2.78 | 993.11 |
| 3.6 | 2.86 | 992.97 |
| 3.7 | 2.94 | 992.83 |
| 3.8 | 3.02 | 992.69 |
| 3.9 | 3.10 | 992.55 |
| 4.0 | 3.18 | 992.41 |
| 4.1 | 3.26 | 992.28 |
| 4.2 | 3.34 | 992.14 |
| 4.3 | 3.42 | 992.00 |
| 4.4 | 3.50 | 991.87 |
| 4.5 | 3.58 | 991.73 |
| 4.6 | 3.66 | 991.59 |
| 4.7 | 3.74 | 991.46 |
| 4.8 | 3.82 | 991.32 |
| 4.9 | 3.90 | 991.19 |
| 5.0 | 3.98 | 991.06 |
| 5.1 | 4.06 | 990.92 |
| 5.2 | 4.14 | 990.79 |
| 5.3 | 4.22 | 990.65 |
| 5.4 | 4.30 | 990.52 |
| 5.5 | 4.38 | 990.39 |
| 5.6 | 4.46 | 990.26 |
| 5.7 | 4.54 | 990.12 |
| 5.8 | 4.62 | 989.99 |
| 5.9 | 4.70 | 989.86 |
| 6.0 | 4.78 | 989.73 |
| 6.1 | 4.86 | 989.60 |
| 6.2 | 4.95 | 989.47 |
| 6.3 | 5.03 | 989.34 |
| 6.4 | 5.11 | 989.21 |
| 6.5 | 5.19 | 989.08 |
| 6.6 | 5.27 | 988.95 |
| 6.7 | 5.35 | 988.82 |
| 6.8 | 5.43 | 988.69 |
| 6.9 | 5.51 | 988.56 |
| 7.0 | 5.59 | 988.43 |
| 7.1 | 5.67 | 988.30 |
| 7.2 | 5.75 | 988.18 |
| 7.3 | 5.83 | 988.05 |
| 7.4 | 5.91 | 987.92 |
| 7.5 | 5.99 | 987.79 |
| 7.6 | 6.07 | 987.67 |
| 7.7 | 6.15 | 987.54 |
| 7.8 | 6.23 | 987.42 |
| 7.9 | 6.32 | 987.29 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| | | |
| 8.0 | 6.40 | 987.16 |
| 8.1 | 6.48 | 987.04 |
| 8.2 | 6.56 | 986.91 |
| 8.3 | 6.64 | 986.79 |
| 8.4 | 6.72 | 986.66 |
| 8.5 | 6.80 | 986.54 |
| 8.6 | 6.88 | 986.42 |
| 8.7 | 6.96 | 986.29 |
| 8.8 | 7.04 | 986.17 |
| 8.9 | 7.12 | 986.05 |
| | | |
| 9.0 | 7.20 | 985.92 |
| 9.1 | 7.29 | 985.80 |
| 9.2 | 7.37 | 985.68 |
| 9.3 | 7.45 | 985.56 |
| 9.4 | 7.53 | 985.44 |
| 9.5 | 7.61 | 985.31 |
| 9.6 | 7.69 | 985.19 |
| 9.7 | 7.77 | 985.07 |
| 9.8 | 7.85 | 984.95 |
| 9.9 | 7.93 | 984.83 |
| | | |
| 10.0 | 8.01 | 984.71 |
| 10.1 | 8.10 | 984.59 |
| 10.2 | 8.18 | 984.47 |
| 10.3 | 8.26 | 984.35 |
| 10.4 | 8.34 | 984.23 |
| 10.5 | 8.42 | 984.11 |
| 10.6 | 8.50 | 983.99 |
| 10.7 | 8.58 | 983.88 |
| 10.8 | 8.66 | 983.76 |
| 10.9 | 8.75 | 983.64 |
| | | |
| 11.0 | 8.83 | 983.52 |
| 11.1 | 8.91 | 983.40 |
| 11.2 | 8.99 | 983.29 |
| 11.3 | 9.07 | 983.17 |
| 11.4 | 9.15 | 983.05 |
| 11.5 | 9.23 | 982.94 |
| 11.6 | 9.32 | 982.82 |
| 11.7 | 9.40 | 982.70 |
| 11.8 | 9.48 | 982.59 |
| 11.9 | 9.56 | 982.47 |
| | | |
| 12.0 | 9.64 | 982.35 |
| 12.1 | 9.72 | 982.24 |
| 12.2 | 9.80 | 982.12 |
| 12.3 | 9.89 | 982.01 |
| 12.4 | 9.97 | 981.89 |
| 12.5 | 10.05 | 981.78 |
| 12.6 | 10.13 | 981.67 |
| 12.7 | 10.21 | 981.55 |
| 12.8 | 10.29 | 981.44 |
| 12.9 | 10.37 | 981.32 |
| | | |
| 13.0 | 10.46 | 981.21 |
| 13.1 | 10.54 | 981.10 |
| 13.2 | 10.62 | 980.98 |
| 13.3 | 10.70 | 980.87 |
| 13.4 | 10.78 | 980.76 |
| 13.5 | 10.87 | 980.64 |
| 13.6 | 10.95 | 980.53 |
| 13.7 | 11.03 | 980.42 |
| 13.8 | 11.11 | 980.31 |
| 13.9 | 11.19 | 980.19 |
| | | |
| 14.0 | 11.27 | 980.08 |
| 14.1 | 11.36 | 979.97 |
| 14.2 | 11.44 | 979.86 |
| 14.3 | 11.52 | 979.75 |
| 14.4 | 11.60 | 979.64 |
| 14.5 | 11.68 | 979.52 |
| 14.6 | 11.77 | 979.41 |
| 14.7 | 11.85 | 979.30 |
| 14.8 | 11.93 | 979.19 |
| 14.9 | 12.01 | 979.08 |
| | | |
| 15.0 | 12.09 | 978.97 |
| 15.1 | 12.17 | 978.86 |
| 15.2 | 12.26 | 978.75 |
| 15.3 | 12.34 | 978.64 |
| 15.4 | 12.42 | 978.53 |
| 15.5 | 12.50 | 978.42 |
| 15.6 | 12.59 | 978.31 |
| 15.7 | 12.67 | 978.20 |
| 15.8 | 12.75 | 978.09 |
| 15.9 | 12.83 | 977.98 |
| | | |
| 16.0 | 12.91 | 977.87 |
| 16.1 | 13.00 | 977.76 |
| 16.2 | 13.08 | 977.65 |
| 16.3 | 13.16 | 977.55 |
| 16.4 | 13.24 | 977.44 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 16.5 | 13.32 | 977.33 |
| 16.6 | 13.41 | 977.22 |
| 16.7 | 13.49 | 977.11 |
| 16.8 | 13.57 | 977.00 |
| 16.9 | 13.65 | 976.89 |
| | | |
| 17.0 | 13.74 | 976.79 |
| 17.1 | 13.82 | 976.68 |
| 17.2 | 13.90 | 976.57 |
| 17.3 | 13.98 | 976.46 |
| 17.4 | 14.07 | 976.35 |
| 17.5 | 14.15 | 976.25 |
| 17.6 | 14.23 | 976.14 |
| 17.7 | 14.31 | 976.03 |
| 17.8 | 14.40 | 975.92 |
| 17.9 | 14.48 | 975.81 |
| | | |
| 18.0 | 14.56 | 975.71 |
| 18.1 | 14.64 | 975.60 |
| 18.2 | 14.73 | 975.49 |
| 18.3 | 14.81 | 975.38 |
| 18.4 | 14.89 | 975.28 |
| 18.5 | 14.97 | 975.17 |
| 18.6 | 15.06 | 975.06 |
| 18.7 | 15.14 | 974.95 |
| 18.8 | 15.22 | 974.85 |
| 18.9 | 15.30 | 974.74 |
| | | |
| 19.0 | 15.39 | 974.63 |
| 19.1 | 15.47 | 974.52 |
| 19.2 | 15.55 | 974.42 |
| 19.3 | 15.63 | 974.31 |
| 19.4 | 15.72 | 974.20 |
| 19.5 | 15.80 | 974.09 |
| 19.6 | 15.88 | 973.99 |
| 19.7 | 15.97 | 973.88 |
| 19.8 | 16.05 | 973.77 |
| 19.9 | 16.13 | 973.66 |
| | | |
| 20.0 | 16.21 | 973.56 |
| 20.1 | 16.30 | 973.45 |
| 20.2 | 16.38 | 973.34 |
| 20.3 | 16.46 | 973.24 |
| 20.4 | 16.55 | 973.13 |
| 20.5 | 16.63 | 973.02 |
| 20.6 | 16.71 | 972.91 |
| 20.7 | 16.79 | 972.80 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 20.8 | 16.88 | 972.70 |
| 20.9 | 16.96 | 972.59 |
| | | |
| 21.0 | 17.04 | 972.48 |
| 21.1 | 17.13 | 972.37 |
| 21.2 | 17.21 | 972.27 |
| 21.3 | 17.29 | 972.16 |
| 21.4 | 17.38 | 972.05 |
| 21.5 | 17.46 | 971.94 |
| 21.6 | 17.54 | 971.83 |
| 21.7 | 17.62 | 971.73 |
| 21.8 | 17.71 | 971.62 |
| 21.9 | 17.79 | 971.51 |
| | | |
| 22.0 | 17.87 | 971.40 |
| 22.1 | 17.96 | 971.29 |
| 22.2 | 18.04 | 971.18 |
| 22.3 | 18.12 | 971.08 |
| 22.4 | 18.21 | 970.97 |
| 22.5 | 18.29 | 970.86 |
| 22.6 | 18.37 | 970.75 |
| 22.7 | 18.46 | 970.64 |
| 22.8 | 18.54 | 970.53 |
| 22.9 | 18.62 | 970.42 |
| | | |
| 23.0 | 18.71 | 970.31 |
| 23.1 | 18.79 | 970.20 |
| 23.2 | 18.87 | 970.09 |
| 23.3 | 18.96 | 969.98 |
| 23.4 | 19.04 | 969.87 |
| 23.5 | 19.13 | 969.76 |
| 23.6 | 19.21 | 969.65 |
| 23.7 | 19.29 | 969.54 |
| 23.8 | 19.38 | 969.43 |
| 23.9 | 19.46 | 969.32 |
| | | |
| 24.0 | 19.54 | 969.21 |
| 24.1 | 19.63 | 969.10 |
| 24.2 | 19.71 | 968.99 |
| 24.3 | 19.79 | 968.88 |
| 24.4 | 19.88 | 968.77 |
| 24.5 | 19.96 | 968.66 |
| 24.6 | 20.05 | 968.55 |
| 24.7 | 20.13 | 968.43 |
| 24.8 | 20.21 | 968.32 |
| 24.9 | 20.30 | 968.21 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) | % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|---|---|---|
| 25.0 | 20.38 | 968.10 | 29.3 | 24.01 | 963.07 |
| 25.1 | 20.47 | 967.99 | 29.4 | 24.10 | 962.95 |
| 25.2 | 20.55 | 967.87 | 29.5 | 24.18 | 962.83 |
| 25.3 | 20.63 | 967.76 | 29.6 | 24.27 | 962.71 |
| 25.4 | 20.72 | 967.65 | 29.7 | 24.35 | 962.58 |
| 25.5 | 20.80 | 967.53 | 29.8 | 24.44 | 962.46 |
| 25.6 | 20.88 | 967.42 | 29.9 | 24.52 | 962.33 |
| 25.7 | 20.97 | 967.31 | | | |
| 25.8 | 21.05 | 967.19 | 30.0 | 24.61 | 962.21 |
| 25.9 | 21.14 | 967.08 | 30.1 | 24.69 | 962.09 |
| | | | 30.2 | 24.78 | 961.96 |
| 26.0 | 21.22 | 966.97 | 30.3 | 24.86 | 961.84 |
| 26.1 | 21.31 | 966.85 | 30.4 | 24.95 | 961.71 |
| 26.2 | 21.39 | 966.74 | 30.5 | 25.03 | 961.59 |
| 26.3 | 21.47 | 966.62 | 30.6 | 25.12 | 961.46 |
| 26.4 | 21.56 | 966.51 | 30.7 | 25.20 | 961.33 |
| 26.5 | 21.64 | 966.39 | 30.8 | 25.29 | 961.21 |
| 26.6 | 21.73 | 966.28 | 30.9 | 25.38 | 961.08 |
| 26.7 | 21.81 | 966.16 | | | |
| 26.8 | 21.90 | 966.05 | 31.0 | 25.46 | 960.95 |
| 26.9 | 21.98 | 965.93 | 31.1 | 25.55 | 960.82 |
| | | | 31.2 | 25.63 | 960.70 |
| 27.0 | 22.06 | 965.81 | 31.3 | 25.72 | 960.57 |
| 27.1 | 22.15 | 965.70 | 31.4 | 25.80 | 960.44 |
| 27.2 | 22.23 | 965.58 | 31.5 | 25.89 | 960.31 |
| 27.3 | 22.32 | 965.46 | 31.6 | 25.97 | 960.18 |
| 27.4 | 22.40 | 965.35 | 31.7 | 26.06 | 960.05 |
| 27.5 | 22.49 | 965.23 | 31.8 | 26.15 | 959.92 |
| 27.6 | 22.57 | 965.11 | 31.9 | 26.23 | 959.79 |
| 27.7 | 22.65 | 964.99 | | | |
| 27.8 | 22.74 | 964.88 | 32.0 | 26.32 | 959.66 |
| 27.9 | 22.82 | 964.76 | 32.1 | 26.40 | 959.53 |
| | | | 32.2 | 26.49 | 959.40 |
| 28.0 | 22.91 | 964.64 | 32.3 | 26.57 | 959.27 |
| 28.1 | 22.99 | 964.52 | 32.4 | 26.66 | 959.14 |
| 28.2 | 23.08 | 964.40 | 32.5 | 26.75 | 959.01 |
| 28.3 | 23.16 | 964.28 | 32.6 | 26.83 | 958.87 |
| 28.4 | 23.25 | 964.16 | 32.7 | 26.92 | 958.74 |
| 28.5 | 23.33 | 964.04 | 32.8 | 27.00 | 958.61 |
| 28.6 | 23.42 | 963.92 | 32.9 | 27.09 | 958.47 |
| 28.7 | 23.50 | 963.80 | | | |
| 28.8 | 23.59 | 963.68 | 33.0 | 27.18 | 958.34 |
| 28.9 | 23.67 | 963.56 | 33.1 | 27.26 | 958.20 |
| | | | 33.2 | 27.35 | 958.07 |
| 29.0 | 23.76 | 963.44 | 33.3 | 27.44 | 957.94 |
| 29.1 | 23.84 | 963.32 | 33.4 | 27.52 | 957.80 |
| 29.2 | 23.93 | 963.20 | 33.5 | 27.61 | 957.66 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 33.6 | 27.69 | 957.53 |
| 33.7 | 27.78 | 957.39 |
| 33.8 | 27.87 | 957.26 |
| 33.9 | 27.95 | 957.12 |
| | | |
| 34.0 | 28.04 | 956.98 |
| 34.1 | 28.13 | 956.84 |
| 34.2 | 28.21 | 956.70 |
| 34.3 | 28.30 | 956.57 |
| 34.4 | 28.39 | 956.43 |
| 34.5 | 28.47 | 956.29 |
| 34.6 | 28.56 | 956.15 |
| 34.7 | 28.65 | 956.01 |
| 34.8 | 28.73 | 955.87 |
| 34.9 | 28.82 | 955.73 |
| | | |
| 35.0 | 28.91 | 955.59 |
| 35.1 | 28.99 | 955.45 |
| 35.2 | 29.08 | 955.30 |
| 35.3 | 29.17 | 955.16 |
| 35.4 | 29.26 | 955.02 |
| 35.5 | 29.34 | 954.88 |
| 35.6 | 29.43 | 954.73 |
| 35.7 | 29.52 | 954.59 |
| 35.8 | 29.60 | 954.44 |
| 35.9 | 29.69 | 954.30 |
| | | |
| 36.0 | 29.78 | 954.15 |
| 36.1 | 29.87 | 954.01 |
| 36.2 | 29.95 | 953.86 |
| 36.3 | 30.04 | 953.72 |
| 36.4 | 30.13 | 953.57 |
| 36.5 | 30.21 | 953.42 |
| 36.6 | 30.30 | 953.28 |
| 36.7 | 30.39 | 953.13 |
| 36.8 | 30.48 | 952.98 |
| 36.9 | 30.56 | 952.83 |
| | | |
| 37.0 | 30.65 | 952.69 |
| 37.1 | 30.74 | 952.54 |
| 37.2 | 30.83 | 952.39 |
| 37.3 | 30.92 | 952.24 |
| 37.4 | 31.00 | 952.09 |
| 37.5 | 31.09 | 951.94 |
| 37.6 | 31.18 | 951.79 |
| 37.7 | 31.27 | 951.63 |
| 37.8 | 31.35 | 951.48 |
| 37.9 | 31.44 | 951.33 |
| | | |
| 38.0 | 31.53 | 951.18 |
| 38.1 | 31.62 | 951.02 |
| 38.2 | 31.71 | 950.87 |
| 38.3 | 31.79 | 950.72 |
| 38.4 | 31.88 | 950.56 |
| 38.5 | 31.97 | 950.41 |
| 38.6 | 32.06 | 950.25 |
| 38.7 | 32.15 | 950.10 |
| 38.8 | 32.24 | 949.94 |
| 38.9 | 32.32 | 949.79 |
| | | |
| 39.0 | 32.41 | 949.63 |
| 39.1 | 32.50 | 949.47 |
| 39.2 | 32.59 | 949.32 |
| 39.3 | 32.68 | 949.16 |
| 39.4 | 32.77 | 949.00 |
| 39.5 | 32.86 | 948.84 |
| 39.6 | 32.94 | 948.68 |
| 39.7 | 33.03 | 948.52 |
| 39.8 | 33.12 | 948.37 |
| 39.9 | 33.21 | 948.21 |
| | | |
| 40.0 | 33.30 | 948.05 |
| 40.1 | 33.39 | 947.88 |
| 40.2 | 33.48 | 947.72 |
| 40.3 | 33.57 | 947.56 |
| 40.4 | 33.66 | 947.40 |
| 40.5 | 33.74 | 947.24 |
| 40.6 | 33.83 | 947.08 |
| 40.7 | 33.92 | 946.91 |
| 40.8 | 34.01 | 946.75 |
| 40.9 | 34.10 | 946.58 |
| | | |
| 41.0 | 34.19 | 946.42 |
| 41.1 | 34.28 | 946.26 |
| 41.2 | 34.37 | 946.09 |
| 41.3 | 34.46 | 945.93 |
| 41.4 | 34.55 | 945.76 |
| 41.5 | 34.64 | 945.59 |
| 41.6 | 34.73 | 945.43 |
| 41.7 | 34.82 | 945.26 |
| 41.8 | 34.91 | 945.09 |
| 41.9 | 35.00 | 944.93 |
| | | |
| 42.0 | 35.09 | 944.76 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) | % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|---|---|---|
| 42.1 | 35.18 | 944.59 | 46.4 | 39.08 | 937.00 |
| 42.2 | 35.27 | 944.42 | 46.5 | 39.18 | 936.81 |
| 42.3 | 35.36 | 944.25 | 46.6 | 39.27 | 936.63 |
| 42.4 | 35.45 | 944.08 | 46.7 | 39.36 | 936.44 |
| 42.5 | 35.54 | 943.91 | 46.8 | 39.45 | 936.26 |
| 42.6 | 35.63 | 943.74 | 46.9 | 39.54 | 936.07 |
| 42.7 | 35.72 | 943.57 | | | |
| 42.8 | 35.81 | 943.40 | 47.0 | 39.64 | 935.88 |
| 42.9 | 35.90 | 943.23 | 47.1 | 39.73 | 935.70 |
| | | | 47.2 | 39.82 | 935.51 |
| 43.0 | 35.99 | 943.06 | 47.3 | 39.91 | 935.32 |
| 43.1 | 36.08 | 942.88 | 47.4 | 40.00 | 935.14 |
| 43.2 | 36.17 | 942.71 | 47.5 | 40.10 | 934.95 |
| 43.3 | 36.26 | 942.54 | 47.6 | 40.19 | 934.76 |
| 43.4 | 36.35 | 942.37 | 47.7 | 40.28 | 934.57 |
| 43.5 | 36.44 | 942.19 | 47.8 | 40.37 | 934.38 |
| 43.6 | 36.53 | 942.02 | 47.9 | 40.47 | 934.19 |
| 43.7 | 36.62 | 941.84 | | | |
| 43.8 | 36.71 | 941.67 | 48.0 | 40.56 | 934.00 |
| 43.9 | 36.80 | 941.49 | 48.1 | 40.65 | 933.81 |
| | | | 48.2 | 40.75 | 933.62 |
| 44.0 | 36.89 | 941.32 | 48.3 | 40.84 | 933.43 |
| 44.1 | 36.98 | 941.14 | 48.4 | 40.93 | 933.24 |
| 44.2 | 37.07 | 940.97 | 48.5 | 41.02 | 933.05 |
| 44.3 | 37.16 | 940.79 | 48.6 | 41.12 | 932.86 |
| 44.4 | 37.25 | 940.61 | 48.7 | 41.21 | 932.67 |
| 44.5 | 37.35 | 940.43 | 48.8 | 41.30 | 932.47 |
| 44.6 | 37.44 | 940.26 | 48.9 | 41.40 | 932.28 |
| 44.7 | 37.53 | 940.08 | | | |
| 44.8 | 37.62 | 939.90 | 49.0 | 41.49 | 932.09 |
| 44.9 | 37.71 | 939.72 | 49.1 | 41.58 | 931.90 |
| | | | 49.2 | 41.68 | 931.70 |
| 45.0 | 37.80 | 939.54 | 49.3 | 41.77 | 931.51 |
| 45.1 | 37.89 | 939.36 | 49.4 | 41.86 | 931.31 |
| 45.2 | 37.98 | 939.18 | 49.5 | 41.96 | 931.12 |
| 45.3 | 38.08 | 939.00 | 49.6 | 42.05 | 930.92 |
| 45.4 | 38.17 | 938.82 | 49.7 | 42.14 | 930.73 |
| 45.5 | 38.26 | 938.64 | 49.8 | 42.24 | 930.53 |
| 45.6 | 38.35 | 938.46 | 49.9 | 42.33 | 930.34 |
| 45.7 | 38.44 | 938.28 | | | |
| 45.8 | 38.53 | 938.10 | 50.0 | 42.43 | 930.14 |
| 45.9 | 38.62 | 937.91 | 50.1 | 42.52 | 929.95 |
| | | | 50.2 | 42.61 | 929.75 |
| 46.0 | 38.72 | 937.73 | 50.3 | 42.71 | 929.55 |
| 46.1 | 38.81 | 937.55 | 50.4 | 42.80 | 929.35 |
| 46.2 | 38.90 | 937.36 | 50.5 | 42.90 | 929.16 |
| 46.3 | 38.99 | 937.18 | 50.6 | 42.99 | 928.96 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 50.7 | 43.08 | 928.76 |
| 50.8 | 43.18 | 928.56 |
| 50.9 | 43.27 | 928.36 |
| | | |
| 51.0 | 43.37 | 928.16 |
| 51.1 | 43.46 | 927.96 |
| 51.2 | 43.56 | 927.77 |
| 51.3 | 43.65 | 927.57 |
| 51.4 | 43.74 | 927.36 |
| 51.5 | 43.84 | 927.16 |
| 51.6 | 43.93 | 926.96 |
| 51.7 | 44.03 | 926.76 |
| 51.8 | 44.12 | 926.56 |
| 51.9 | 44.22 | 926.36 |
| | | |
| 52.0 | 44.31 | 926.16 |
| 52.1 | 44.41 | 925.95 |
| 52.2 | 44.50 | 925.75 |
| 52.3 | 44.60 | 925.55 |
| 52.4 | 44.69 | 925.35 |
| 52.5 | 44.79 | 925.14 |
| 52.6 | 44.88 | 924.94 |
| 52.7 | 44.98 | 924.73 |
| 52.8 | 45.07 | 924.53 |
| 52.9 | 45.17 | 924.32 |
| | | |
| 53.0 | 45.26 | 924.12 |
| 53.1 | 45.36 | 923.91 |
| 53.2 | 45.46 | 923.71 |
| 53.3 | 45.55 | 923.50 |
| 53.4 | 45.65 | 923.30 |
| 53.5 | 45.74 | 923.09 |
| 53.6 | 45.84 | 922.88 |
| 53.7 | 45.93 | 922.68 |
| 53.8 | 46.03 | 922.47 |
| 53.9 | 46.13 | 922.26 |
| | | |
| 54.0 | 46.22 | 922.06 |
| 54.1 | 46.32 | 921.85 |
| 54.2 | 46.41 | 921.64 |
| 54.3 | 46.51 | 921.43 |
| 54.4 | 46.61 | 921.22 |
| 54.5 | 46.70 | 921.01 |
| 54.6 | 46.80 | 920.80 |
| 54.7 | 46.90 | 920.59 |
| 54.8 | 46.99 | 920.38 |
| 54.9 | 47.09 | 920.17 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| | | |
| 55.0 | 47.18 | 919.96 |
| 55.1 | 47.28 | 919.75 |
| 55.2 | 47.38 | 919.54 |
| 55.3 | 47.47 | 919.33 |
| 55.4 | 47.57 | 919.12 |
| 55.5 | 47.67 | 918.91 |
| 55.6 | 47.77 | 918.69 |
| 55.7 | 47.86 | 918.48 |
| 55.8 | 47.96 | 918.27 |
| 55.9 | 48.06 | 918.06 |
| | | |
| 56.0 | 48.15 | 917.84 |
| 56.1 | 48.25 | 917.63 |
| 56.2 | 48.35 | 917.42 |
| 56.3 | 48.45 | 917.20 |
| 56.4 | 48.54 | 916.99 |
| 56.5 | 48.64 | 916.77 |
| 56.6 | 48.74 | 916.56 |
| 56.7 | 48.84 | 916.35 |
| 56.8 | 48.93 | 916.13 |
| 56.9 | 49.03 | 915.91 |
| | | |
| 57.0 | 49.13 | 915.70 |
| 57.1 | 49.23 | 915.48 |
| 57.2 | 49.32 | 915.27 |
| 57.3 | 49.42 | 915.05 |
| 57.4 | 49.52 | 914.83 |
| 57.5 | 49.62 | 914.62 |
| 57.6 | 49.72 | 914.40 |
| 57.7 | 49.81 | 914.18 |
| 57.8 | 49.91 | 913.97 |
| 57.9 | 50.01 | 913.75 |
| | | |
| 58.0 | 50.11 | 913.53 |
| 58.1 | 50.21 | 913.31 |
| 58.2 | 50.31 | 913.09 |
| 58.3 | 50.40 | 912.87 |
| 58.4 | 50.50 | 912.65 |
| 58.5 | 50.60 | 912.43 |
| 58.6 | 50.70 | 912.22 |
| 58.7 | 50.80 | 912.00 |
| 58.8 | 50.90 | 911.78 |
| 58.9 | 51.00 | 911.55 |
| | | |
| 59.0 | 51.10 | 911.33 |
| 59.1 | 51.19 | 911.11 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) | % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|---|---|---|
| 59.2 | 51.29 | 910.89 | 63.5 | 55.61 | 901.15 |
| 59.3 | 51.39 | 910.67 | 63.6 | 55.72 | 900.92 |
| 59.4 | 51.49 | 910.45 | 63.7 | 55.82 | 900.69 |
| 59.5 | 51.59 | 910.23 | 63.8 | 55.92 | 900.46 |
| 59.6 | 51.69 | 910.01 | 63.9 | 56.02 | 900.23 |
| 59.7 | 51.79 | 909.78 | | | |
| 59.8 | 51.89 | 909.56 | 64.0 | 56.12 | 899.99 |
| 59.9 | 51.99 | 909.34 | 64.1 | 56.23 | 899.76 |
| | | | 64.2 | 56.33 | 899.53 |
| 60.0 | 52.09 | 909.11 | 64.3 | 56.43 | 899.29 |
| 60.1 | 52.19 | 908.89 | 64.4 | 56.53 | 899.06 |
| 60.2 | 52.29 | 908.67 | 64.5 | 56.64 | 898.83 |
| 60.3 | 52.39 | 908.44 | 64.6 | 56.74 | 898.59 |
| 60.4 | 52.49 | 908.22 | 64.7 | 56.84 | 898.36 |
| 60.5 | 52.59 | 908.00 | 64.8 | 56.94 | 898.12 |
| 60.6 | 52.69 | 907.77 | 64.9 | 57.05 | 897.89 |
| 60.7 | 52.79 | 907.55 | | | |
| 60.8 | 52.89 | 907.32 | 65.0 | 57.15 | 897.65 |
| 60.9 | 52.99 | 907.10 | 65.1 | 57.25 | 897.42 |
| | | | 65.2 | 57.36 | 897.18 |
| 61.0 | 53.09 | 906.87 | 65.3 | 57.46 | 896.94 |
| 61.1 | 53.19 | 906.64 | 65.4 | 57.56 | 896.71 |
| 61.2 | 53.29 | 906.42 | 65.5 | 57.67 | 896.47 |
| 61.3 | 53.39 | 906.19 | 65.6 | 57.77 | 896.23 |
| 61.4 | 53.49 | 905.97 | 65.7 | 57.87 | 896.00 |
| 61.5 | 53.59 | 905.74 | 65.8 | 57.98 | 895.76 |
| 61.6 | 53.69 | 905.51 | 65.9 | 58.08 | 895.52 |
| 61.7 | 53.79 | 905.29 | | | |
| 61.8 | 53.89 | 905.06 | 66.0 | 58.18 | 895.28 |
| 61.9 | 53.99 | 904.83 | 66.1 | 58.29 | 895.05 |
| | | | 66.2 | 58.39 | 894.81 |
| 62.0 | 54.09 | 904.60 | 66.3 | 58.49 | 894.57 |
| 62.1 | 54.19 | 904.37 | 66.4 | 58.60 | 894.33 |
| 62.2 | 54.30 | 904.15 | 66.5 | 58.70 | 894.09 |
| 62.3 | 54.40 | 903.92 | 66.6 | 58.81 | 893.85 |
| 62.4 | 54.50 | 903.69 | 66.7 | 58.91 | 893.61 |
| 62.5 | 54.60 | 903.46 | 66.8 | 59.01 | 893.37 |
| 62.6 | 54.70 | 903.23 | 66.9 | 59.12 | 893.13 |
| 62.7 | 54.80 | 903.00 | | | |
| 62.8 | 54.90 | 902.77 | 67.0 | 59.22 | 892.89 |
| 62.9 | 55.00 | 902.54 | 67.1 | 59.33 | 892.65 |
| | | | 67.2 | 59.43 | 892.41 |
| 63.0 | 55.11 | 902.31 | 67.3 | 59.54 | 892.17 |
| 63.1 | 55.21 | 902.08 | 67.4 | 59.64 | 891.93 |
| 63.2 | 55.31 | 901.85 | 67.5 | 59.74 | 891.69 |
| 63.3 | 55.41 | 901.62 | 67.6 | 59.85 | 891.45 |
| 63.4 | 55.51 | 901.39 | 67.7 | 59.95 | 891.20 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 67.8 | 60.06 | 890.96 |
| 67.9 | 60.16 | 890.72 |
| | | |
| 68.0 | 60.27 | 890.48 |
| 68.1 | 60.37 | 890.23 |
| 68.2 | 60.48 | 889.99 |
| 68.3 | 60.58 | 889.75 |
| 68.4 | 60.69 | 889.50 |
| 68.5 | 60.80 | 889.26 |
| 68.6 | 60.90 | 889.01 |
| 68.7 | 61.01 | 888.77 |
| 68.8 | 61.11 | 888.52 |
| 68.9 | 61.22 | 888.28 |
| | | |
| 69.0 | 61.32 | 888.03 |
| 69.1 | 61.43 | 887.79 |
| 69.2 | 61.54 | 887.54 |
| 69.3 | 61.64 | 887.29 |
| 69.4 | 61.75 | 887.05 |
| 69.5 | 61.85 | 886.80 |
| 69.6 | 61.96 | 886.55 |
| 69.7 | 62.07 | 886.31 |
| 69.8 | 62.17 | 886.06 |
| 69.9 | 62.28 | 885.81 |
| | | |
| 70.0 | 62.39 | 885.56 |
| 70.1 | 62.49 | 885.31 |
| 70.2 | 62.60 | 885.06 |
| 70.3 | 62.71 | 884.82 |
| 70.4 | 62.81 | 884.57 |
| 70.5 | 62.92 | 884.32 |
| 70.6 | 63.03 | 884.07 |
| 70.7 | 63.13 | 883.82 |
| 70.8 | 63.24 | 883.57 |
| 70.9 | 63.35 | 883.32 |
| | | |
| 71.0 | 63.46 | 883.06 |
| 71.1 | 63.56 | 882.81 |
| 71.2 | 63.67 | 882.56 |
| 71.3 | 63.78 | 882.31 |
| 71.4 | 63.89 | 882.06 |
| 71.5 | 63.99 | 881.81 |
| 71.6 | 64.10 | 881.55 |
| 71.7 | 64.21 | 881.30 |
| 71.8 | 64.32 | 881.05 |
| 71.9 | 64.43 | 880.79 |
| | | |
| 72.0 | 64.53 | 880.54 |
| 72.1 | 64.64 | 880.29 |
| 72.2 | 64.75 | 880.03 |
| 72.3 | 64.86 | 879.78 |
| 72.4 | 64.97 | 879.52 |
| 72.5 | 65.08 | 879.27 |
| 72.6 | 65.19 | 879.01 |
| 72.7 | 65.29 | 878.75 |
| 72.8 | 65.40 | 878.50 |
| 72.9 | 65.51 | 878.24 |
| | | |
| 73.0 | 65.62 | 877.99 |
| 73.1 | 65.73 | 877.73 |
| 73.2 | 65.84 | 877.47 |
| 73.3 | 65.95 | 877.21 |
| 73.4 | 66.06 | 876.96 |
| 73.5 | 66.17 | 876.70 |
| 73.6 | 66.28 | 876.44 |
| 73.7 | 66.39 | 876.18 |
| 73.8 | 66.50 | 875.92 |
| 73.9 | 66.61 | 875.66 |
| | | |
| 74.0 | 66.72 | 875.40 |
| 74.1 | 66.83 | 875.14 |
| 74.2 | 66.94 | 874.88 |
| 74.3 | 67.05 | 874.62 |
| 74.4 | 67.16 | 874.36 |
| 74.5 | 67.27 | 874.10 |
| 74.6 | 67.38 | 873.84 |
| 74.7 | 67.49 | 873.58 |
| 74.8 | 67.60 | 873.32 |
| 74.9 | 67.71 | 873.06 |
| | | |
| 75.0 | 67.82 | 872.79 |
| 75.1 | 67.93 | 872.53 |
| 75.2 | 68.04 | 872.27 |
| 75.3 | 68.15 | 872.00 |
| 75.4 | 68.26 | 871.74 |
| 75.5 | 68.38 | 871.48 |
| 75.6 | 68.49 | 871.21 |
| 75.7 | 68.60 | 870.95 |
| 75.8 | 68.71 | 870.68 |
| 75.9 | 68.82 | 870.42 |
| | | |
| 76.0 | 68.93 | 870.15 |
| 76.1 | 69.04 | 869.89 |
| 76.2 | 69.16 | 869.62 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 76.3 | 69.27 | 869.35 |
| 76.4 | 69.38 | 869.09 |
| 76.5 | 69.49 | 868.82 |
| 76.6 | 69.61 | 868.55 |
| 76.7 | 69.72 | 868.28 |
| 76.8 | 69.83 | 868.02 |
| 76.9 | 69.94 | 867.75 |
| | | |
| 77.0 | 70.06 | 867.48 |
| 77.1 | 70.17 | 867.21 |
| 77.2 | 70.28 | 866.94 |
| 77.3 | 70.39 | 866.67 |
| 77.4 | 70.51 | 866.40 |
| 77.5 | 70.62 | 866.13 |
| 77.6 | 70.73 | 865.86 |
| 77.7 | 70.85 | 865.59 |
| 77.8 | 70.96 | 865.32 |
| 77.9 | 71.07 | 865.05 |
| | | |
| 78.0 | 71.19 | 864.78 |
| 78.1 | 71.30 | 864.50 |
| 78.2 | 71.41 | 864.23 |
| 78.3 | 71.53 | 863.96 |
| 78.4 | 71.64 | 863.69 |
| 78.5 | 71.76 | 863.41 |
| 78.6 | 71.87 | 863.14 |
| 78.7 | 71.98 | 862.86 |
| 78.8 | 72.10 | 862.59 |
| 78.9 | 72.21 | 862.31 |
| | | |
| 79.0 | 72.33 | 862.04 |
| 79.1 | 72.44 | 861.76 |
| 79.2 | 72.56 | 861.49 |
| 79.3 | 72.67 | 861.21 |
| 79.4 | 72.79 | 860.94 |
| 79.5 | 72.90 | 860.66 |
| 79.6 | 73.02 | 860.38 |
| 79.7 | 73.13 | 860.10 |
| 79.8 | 73.25 | 859.83 |
| 79.9 | 73.36 | 859.55 |
| | | |
| 80.0 | 73.48 | 859.27 |
| 80.1 | 73.60 | 858.99 |
| 80.2 | 73.71 | 858.71 |
| 80.3 | 73.83 | 858.43 |
| 80.4 | 73.94 | 858.15 |
| 80.5 | 74.06 | 857.87 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 80.6 | 74.18 | 857.59 |
| 80.7 | 74.29 | 857.31 |
| 80.8 | 74.41 | 857.03 |
| 80.9 | 74.53 | 856.75 |
| | | |
| 81.0 | 74.64 | 856.46 |
| 81.1 | 74.76 | 856.18 |
| 81.2 | 74.88 | 855.90 |
| 81.3 | 74.99 | 855.62 |
| 81.4 | 75.11 | 855.33 |
| 81.5 | 75.23 | 855.05 |
| 81.6 | 75.34 | 854.76 |
| 81.7 | 75.46 | 854.48 |
| 81.8 | 75.58 | 854.19 |
| 81.9 | 75.70 | 853.91 |
| | | |
| 82.0 | 75.82 | 853.62 |
| 82.1 | 75.93 | 853.34 |
| 82.2 | 76.05 | 853.05 |
| 82.3 | 76.17 | 852.76 |
| 82.4 | 76.29 | 852.48 |
| 82.5 | 76.41 | 852.19 |
| 82.6 | 76.52 | 851.90 |
| 82.7 | 76.64 | 851.61 |
| 82.8 | 76.76 | 851.32 |
| 82.9 | 76.88 | 851.03 |
| | | |
| 83.0 | 77.00 | 850.74 |
| 83.1 | 77.12 | 850.45 |
| 83.2 | 77.24 | 850.16 |
| 83.3 | 77.36 | 849.87 |
| 83.4 | 77.48 | 849.58 |
| 83.5 | 77.60 | 849.29 |
| 83.6 | 77.72 | 848.99 |
| 83.7 | 77.84 | 848.70 |
| 83.8 | 77.96 | 848.41 |
| 83.9 | 78.08 | 848.11 |
| | | |
| 84.0 | 78.20 | 847.82 |
| 84.1 | 78.32 | 847.53 |
| 84.2 | 78.44 | 847.23 |
| 84.3 | 78.56 | 846.93 |
| 84.4 | 78.68 | 846.64 |
| 84.5 | 78.80 | 846.34 |
| 84.6 | 78.92 | 846.05 |
| 84.7 | 79.04 | 845.75 |
| 84.8 | 79.16 | 845.45 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 84.9 | 79.28 | 845.15 |
| | | |
| 85.0 | 79.40 | 844.85 |
| 85.1 | 79.53 | 844.55 |
| 85.2 | 79.65 | 844.25 |
| 85.3 | 79.77 | 843.95 |
| 85.4 | 79.89 | 843.65 |
| 85.5 | 80.01 | 843.35 |
| 85.6 | 80.14 | 843.05 |
| 85.7 | 80.26 | 842.75 |
| 85.8 | 80.38 | 842.44 |
| 85.9 | 80.50 | 842.14 |
| | | |
| 86.0 | 80.63 | 841.84 |
| 86.1 | 80.75 | 841.53 |
| 86.2 | 80.87 | 841.23 |
| 86.3 | 81.00 | 840.92 |
| 86.4 | 81.12 | 840.62 |
| 86.5 | 81.24 | 840.31 |
| 86.6 | 81.37 | 840.00 |
| 86.7 | 81.49 | 839.70 |
| 86.8 | 81.61 | 839.39 |
| 86.9 | 81.74 | 839.08 |
| | | |
| 87.0 | 81.86 | 838.77 |
| 87.1 | 81.99 | 838.46 |
| 87.2 | 82.11 | 838.15 |
| 87.3 | 82.24 | 837.84 |
| 87.4 | 82.36 | 837.52 |
| 87.5 | 82.49 | 837.21 |
| 87.6 | 82.61 | 836.90 |
| 87.7 | 82.74 | 836.59 |
| 87.8 | 82.86 | 836.27 |
| 87.9 | 82.99 | 835.96 |
| | | |
| 88.0 | 83.11 | 835.64 |
| 88.1 | 83.24 | 835.32 |
| 88.2 | 83.37 | 835.01 |
| 88.3 | 83.49 | 834.69 |
| 88.4 | 83.62 | 834.37 |
| 88.5 | 83.74 | 834.05 |
| 88.6 | 83.87 | 833.73 |
| 88.7 | 84.00 | 833.41 |
| 88.8 | 84.13 | 833.09 |
| 88.9 | 84.25 | 832.77 |
| | | |
| 89.0 | 84.38 | 832.45 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|
| 89.1 | 84.51 | 832.12 |
| 89.2 | 84.64 | 831.80 |
| 89.3 | 84.76 | 831.48 |
| 89.4 | 84.89 | 831.15 |
| 89.5 | 85.02 | 830.82 |
| 89.6 | 85.15 | 830.50 |
| 89.7 | 85.28 | 830.17 |
| 89.8 | 85.41 | 829.84 |
| 89.9 | 85.54 | 829.51 |
| | | |
| 90.0 | 85.66 | 829.18 |
| 90.1 | 85.79 | 828.85 |
| 90.2 | 85.92 | 828.52 |
| 90.3 | 86.05 | 828.19 |
| 90.4 | 86.18 | 827.85 |
| 90.5 | 86.31 | 827.52 |
| 90.6 | 86.44 | 827.18 |
| 90.7 | 86.57 | 826.85 |
| 90.8 | 86.71 | 826.51 |
| 90.9 | 86.84 | 826.17 |
| | | |
| 91.0 | 86.97 | 825.83 |
| 91.1 | 87.10 | 825.49 |
| 91.2 | 87.23 | 825.15 |
| 91.3 | 87.36 | 824.81 |
| 91.4 | 87.49 | 824.47 |
| 91.5 | 87.63 | 824.13 |
| 91.6 | 87.76 | 823.78 |
| 91.7 | 87.89 | 823.44 |
| 91.8 | 88.02 | 823.09 |
| 91.9 | 88.16 | 822.74 |
| | | |
| 92.0 | 88.29 | 822.39 |
| 92.1 | 88.42 | 822.04 |
| 92.2 | 88.56 | 821.69 |
| 92.3 | 88.69 | 821.34 |
| 92.4 | 88.83 | 820.99 |
| 92.5 | 88.96 | 820.63 |
| 92.6 | 89.10 | 820.28 |
| 92.7 | 89.23 | 819.92 |
| 92.8 | 89.37 | 819.57 |
| 92.9 | 89.50 | 819.21 |
| | | |
| 93.0 | 89.64 | 818.85 |
| 93.1 | 89.77 | 818.49 |
| 93.2 | 89.91 | 818.12 |
| 93.3 | 90.05 | 817.76 |

| % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) | % *V/V* | % *m/m* | $\rho_{20}$ (kg/m$^3$) |
|---|---|---|---|---|---|
| 93.4 | 90.18 | 817.40 | 96.8 | 95.01 | 804.12 |
| 93.5 | 90.32 | 817.03 | 96.9 | 95.16 | 803.70 |
| 93.6 | 90.46 | 816.66 | | | |
| 93.7 | 90.59 | 816.30 | 97.0 | 95.31 | 803.27 |
| 93.8 | 90.73 | 815.93 | 97.1 | 95.45 | 802.85 |
| 93.9 | 90.87 | 815.55 | 97.2 | 95.60 | 802.42 |
| | | | 97.3 | 95.75 | 801.99 |
| 94.0 | 91.01 | 815.18 | 97.4 | 95.90 | 801.55 |
| 94.1 | 91.15 | 814.81 | 97.5 | 96.05 | 801.12 |
| 94.2 | 91.29 | 814.43 | 97.6 | 96.21 | 800.68 |
| 94.3 | 91.43 | 814.06 | 97.7 | 96.36 | 800.24 |
| 94.4 | 91.56 | 813.68 | 97.8 | 96.51 | 799.80 |
| 94.5 | 91.70 | 813.30 | 97.9 | 96.66 | 799.35 |
| 94.6 | 91.84 | 812.92 | | | |
| 94.7 | 91.98 | 812.54 | 98.0 | 96.81 | 798.90 |
| 94.8 | 92.13 | 812.15 | 98.1 | 96.97 | 798.45 |
| 94.9 | 92.27 | 811.77 | 98.2 | 97.12 | 798.00 |
| | | | 98.3 | 97.28 | 797.54 |
| 95.0 | 92.41 | 811.38 | 98.4 | 97.43 | 797.08 |
| 95.1 | 92.55 | 810.99 | 98.5 | 97.59 | 796.62 |
| 95.2 | 92.69 | 810.60 | 98.6 | 97.74 | 796.15 |
| 95.3 | 92.83 | 810.21 | 98.7 | 97.90 | 795.68 |
| 95.4 | 92.98 | 809.82 | 98.8 | 98.06 | 795.21 |
| 95.5 | 93.12 | 809.42 | 98.9 | 98.22 | 794.73 |
| 95.6 | 93.26 | 809.02 | | | |
| 95.7 | 93.41 | 808.63 | 99.0 | 98.38 | 794.25 |
| 95.8 | 93.55 | 808.23 | 99.1 | 98.53 | 793.77 |
| 95.9 | 93.69 | 807.82 | 99.2 | 98.69 | 793.28 |
| | | | 99.3 | 98.86 | 792.79 |
| 96.0 | 93.84 | 807.42 | 99.4 | 99.02 | 792.30 |
| 96.1 | 93.98 | 807.01 | 99.5 | 99.18 | 791.80 |
| 96.2 | 94.13 | 806.61 | 99.6 | 99.34 | 791.29 |
| 96.3 | 94.27 | 806.20 | 99.7 | 99.50 | 790.79 |
| 96.4 | 94.42 | 805.78 | 99.8 | 99.67 | 790.28 |
| 96.5 | 94.57 | 805.37 | 99.9 | 99.83 | 789.76 |
| 96.6 | 94.71 | 804.96 | | | |
| 96.7 | 94.86 | 804.54 | 100.0 | 100.0 | 789.24 |

# 5.6. Assay of interferons

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# 5.6. ASSAY OF INTERFERONS

*The following chapter is published for information.*

## 1. INTRODUCTION

Monographs on human interferons generally contain a bioassay based on the inhibitory activity of the interferon on the cytopathic action of a virus on a cell line in culture. In most cases, however, the virus, cell line and the assay details are not specified, in order to allow the appropriate flexibility, where the monograph covers more than one sub-class of interferon.

The present text is intended to provide outline information for the analyst on how to design, optimise and validate such an assay once an appropriate combination of cell line and cytopathic virus has been identified. A detailed procedure for a particular cytopathic antiviral assay is described as an example of a suitable method, together with information on other virus-cell line combinations and guidance on how to adapt and validate the procedure for these other combinations.

## 2. ANTIVIRAL (CYTOPATHIC EFFECT REDUCTION) ASSAYS

The antiviral assay of human interferons is based on the induction of a cellular response in human cells, which prevents or reduces the cytopathic effect of an infectious virus. The potency of interferon is estimated by comparing its protective effect against a viral cytopathic effect with the same effect of the appropriate reference preparation calibrated in International Units.

## 3. INTERFERON ASSAY USING Hep2c CELLS AND INFECTIOUS ENCEPHALOMYOCARDITIS VIRUS

The antiviral assay of human interferons described is of the cytopathic effect reduction type. It uses human Hep2c cells infected by encephalomyocarditis virus (EMCV) to measure the potencies of different human interferon test preparations. This assay has been used in three World Health Organization (WHO) international collaborative studies of candidate International Standards for human interferon alpha, human interferon beta and human interferon gamma and has repeatedly been demonstrated to be sensitive, reliable and reproducible for potency estimations of the different types of human interferon.

For the culture of mammalian cells, all procedures are carried out using standard operating procedures for the maintenance of such cell lines in culture. Volumes of reagents are indicated for cell cultures carried out in 75 $cm^2$ flasks. Other types of containers (flasks or plates) may be used but volumes must be adapted accordingly.

### *3.1. MAINTENANCE AND PREPARATION OF Hep2c CELLS*

Hep2c cells are maintained and passaged in culture medium A.

Cells are stored as frozen stocks using standard operating procedures. Growing cells may be maintained in culture up to a permitted passage number of 30, after which new cultures are established from frozen stocks.

At the beginning of the assay procedure, harvest the cells from the flasks showing 90 per cent confluent monolayers using the trypsin-treatment procedure described below.

- Remove the culture medium from the flasks.
- To each flask, add 5 mL of trypsin solution heated at 37 °C (a trypsin stock solution contains 4 mg/mL of *trypsin R* and 4 mg/mL of *sodium edetate R*; immediately before use, dilute 50 times with phosphate buffered saline). Swirl the capped flask to wash the cell monolayer. Remove the excess of trypsin solution.
- Incubate the flasks for 5 min to 10 min at 37 °C. Microscopically or visually observe the cells for signs of detachment. When viewed microscopically, the cells appear rounded up or detached and free-floating. Shake the flask vigorously to detach all the cells, add approximately 5 mL of culture medium A. Shake vigorously to yield a suspension of single cells.
- To prepare the cell suspensions for the assay procedure, carefully disperse the cells by pipetting up and down to disrupt cell aggregates, count the cells and resuspend at a concentration of $6 \times 10^5$ cells/mL.

### *3.2. PROPAGATION OF ENCEPHALOMYOCARDITIS VIRUS*

Encephalomyocarditis virus is propagated in mouse L-929 cells in order to produce a stock of progeny virus. L-929 cells are maintained by trypsin treatment and passage as described for Hep2c cells (*NOTE: it may be necessary to substitute neonatal calf serum with foetal bovine serum if the cells show poor growth*).

Take several flasks containing confluent cultures of L-929 cells. Pour off the medium from the flasks. Inoculate with 2 mL of the EMCV suspension appropriately diluted in culture medium B so that it contains approximately $2.5 \times 10^8$ plaque forming units (PFU) per millilitre. Each flask will contain $4\text{-}6 \times 10^7$ L-929 cells and therefore the multiplicity of infection (m.o.i.) will be approximately 10 PFU/cell. Carefully swirl the virus suspension over the entire cell monolayer and return the flasks to the incubator for approximately 1 h. Maintain the medium at pH 7.4 to 7.8.

After adsorption of the EMCV, add approximately 40 mL of culture medium B to each flask and return the flasks to the incubator at 37 °C for about 30 h. Maintain the medium at pH 7.4 to 7.8 to obtain a maximum virus yield. Remove the culture fluid and store at approximately 4 °C.

Place the flasks at − 20 °C to freeze the cell monolayer. Then thaw to room temperature. Add approximately 5 mL of culture medium and shake the flask to disrupt the cell walls. Transfer the contents of each flask to the container of culture fluid. Transfer the culture fluid containing the EMCV to 50 mL plastic centrifuge tubes and centrifuge at approximately 500 *g* for about 10 min to remove cell debris. Dispense the clarified culture fluid into glass screw-capped bottles, in quantities of 20 mL, 10 mL, 5 mL, 1 mL, 0.5 mL or 0.2 mL, as appropriate. Store at − 70 °C. Larger volumes can be thawed, dispensed into smaller quantities and re-frozen when required. The EMCV stock will retain its original titre if stored permanently at approximately − 70 °C, but repeated freeze-thaw cycles or storage at higher temperatures, e.g. at approximately − 20 °C, results in progressive loss of titre.

### *3.3. ASSAY PROCEDURE*

#### 3.3.1. Determination of the dose-response range

*Preparation of the solutions*

Dilute the appropriate standard for interferon (for example a specific WHO sub-type interferon standard) in culture medium A, in 10-fold dose increments, to give doses covering the range of 1000 - 0.001 IU/mL. Carry out the assay procedure in 96-well microtitre plates. To each well add 100 µL of culture medium A. Add approximately 100 µL of each dilution of the reference preparation to each well except for those intended for virus controls. Using a multichannel pipette set at 100 µL, mix the contents of the wells.

*Dispensing of the cell suspension*

Pour the cell suspension of Hep2c cells, which has been adjusted to contain approximately $6 \times 10^5$ cells/mL of culture medium A, into a plastic Petri-dish. Dispense the cell suspension from the Petri-dish into each well of the microtitre plates, using a multichannel pipette set at 100 µL.

Incubate the plates for about 24 h in an incubator set at 37 °C and 5 per cent $CO_2$.

*Viral infection*

At this stage, using an inverted microscope, check that the monolayers of Hep2c cells are confluent, that they show a relatively even distribution of cells, that they have correct morphology and that they are healthy.

Remove most of the culture medium from the wells by inverting the plate and shaking it and blotting on a paper towel (proceed in an identical way when discarding fluids from micro-titre plates as described later). Dilute the EMCV stock with fresh culture medium A to a titre of approximately $3 \times 10^7$ PFU/mL (*NOTE: each plate requires approximately 20 mL of diluted virus, plus 5 per cent to 10 per cent of extra volume*). Dispense the diluted suspension from a 9 cm sterile Petri-dish using a multichannel pipette set at 200 µL to all wells including virus controls, but excluding cell controls. Add approximately 200 µL of culture medium A without virus to each of the cell control wells.

Return the plates to the incubator set at 37 °C and 5 per cent $CO_2$ for approximately 24 h.

*Staining*

Examine the plates microscopically to check that the EMCV has caused a cytopathic effect (c.p.e.) in the virus controls. The time interval for maximum c.p.e. may vary from one assay to the next because of inherent variation of Hep2c cells to virus challenge over a given period of continuous cultivation.

Remove most of the culture medium from the wells by discarding into an appropriate decontaminating solution (sodium hypochlorite is suitable). Dispense *phosphate buffered saline pH 7.4 R* into each well. Discard the *phosphate buffered saline pH 7.4 R* into a decontaminating solution. Dispense into each well 150 µL of staining solution. Stain the cells for approximately 30 min at room temperature. Discard the staining solution into a decontaminating solution. Dispense approximately 150 µL of fixing solution. Fix for 10 min at room temperature. Discard the fixing solution into a decontaminating solution and wash the cell monolayers by immersing the assay plates in a plastic box containing running water. Discard the water and superficially dry the plates with paper towels. Dry the assay plates at 20 °C to 37 °C until all moisture has evaporated.

Add 150 µL of *0.1 M sodium hydroxide* to each well. Elute the stain by gentle agitation of the plates or by hitting them against the palm of the hand. Make sure that the stain is evenly distributed in all wells before making spectrophotometric readings.

Read the absorbance at 610 nm to 620 nm, using a microtitre plate reader, taking as a blank a well or a column of wells containing no cells and approximately 150 µL of *0.1 M sodium hydroxide*.

Estimate the concentrations of interferon standard that give the maximum and minimum reduction of cytopathic effect. This is the dose response corresponding to the working range of the assay.

**3.3.2. Assay procedure**

Carry out the assay as described above, using:

- as test solutions, the substance to be examined, diluted in two-fold increments with culture medium A to give nominal concentrations covering the working range of the assay,
- as reference solutions, the appropriate standard for interferon (for example, a specific WHO sub-type interferon) in culture medium A, diluted in two-fold increments to give nominal concentrations covering the working range of the assay.

**3.3.3. Data analysis**

Results of the cytopathic effect reduction assay generally fit a sigmoidal dose-response curve, when the interferon concentration (the log of the reciprocal of the interferon dilution) is plotted versus stain absorbance.

Plot the interferon concentration (log reciprocal of dilution) versus the stain absorbance for the interferon reference preparation and for the interferon test solutions. Using the linear portion of the curve, calculate the concentration of interferon in the sample by comparing the responses for test and reference solutions, using the usual statistical methods for a parallel line assay.

## 4. VALIDATION OF OTHER PROCEDURES

*4.1. CHOICE OF CELL LINE AND VIRUS*

A number of other combinations of cell line and virus have been used in anti-viral assays for interferons. For example, EMCV has been used in combination with the A549 epithelial lung carcinoma cell line, Semliki Forest virus or Sindbis virus have been used with human fibroblasts, and vesicular stomatitis virus has been used with either human diploid fibroblasts, the human amnion WISH cell line or the Madin-Darby bovine kidney cell line. In each case the choice of the cell line/virus combination is usually based on that which gives the most sensitive response to the interferon preparation to be assayed, and gives parallel responses when comparing the test preparation and interferon standard.

*4.2. CHOICE OF RESPONSE*

The staining procedure described above measures remaining viable cells. A number of other responses have been used, including methyl violet or crystal violet staining, or the thiazolyl blue (MTT) conversion procedure. In each case, the method is selected on the basis of producing a suitably linear and sensitive relationship between response colour and viable cell count.

*4.3. STATISTICAL VALIDATION*

As with all parallel line bioassays, the assay must satisfy the usual statistical criteria of linearity of response, parallelism and variance.

*4.4. VALIDATION OF ASSAY LAYOUT*

As with all microtitre plate assay procedures, attention must be given to validating the assay layout. In particular, bias due to non-random pipetting order or plate edge effects must be investigated and eliminated, by randomising the assay layout, or by avoiding the use of edge wells.

## REAGENTS AND CULTURE MEDIA

*Culture medium A (10 per cent neonatal calf serum)*

| | |
|---|---|
| RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/mL; streptomycin 10 ng/mL) | 450 mL |
| L-Glutamine, 200 mM, sterile | 5 mL |
| Neonatal calf serum | 50 mL |

*Culture medium B (2 per cent foetal bovine serum)*

| | |
|---|---|
| RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/mL; streptomycin 10 ng/mL) | 490 mL |
| L-Glutamine, 200 mM, sterile | 5 mL |
| Foetal bovine serum | 10 mL |

*Staining solution*

| | |
|---|---|
| Naphthalene black | 0.5 g |
| Acetic acid, glacial | 90 mL |
| Sodium acetate, anhydrous | 8.2 g |
| Water | to 1000 mL |

*Fixing solution*

| | |
|---|---|
| Formaldehyde, 40 per cent | 100 mL |
| Acetic acid, glacial | 90 mL |
| Sodium acetate, anhydrous | 8.2 g |
| Water | to 1000 mL |

# 5.7. Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia

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**01/2008:50700 corrected 9.2**

# 5.7. TABLE OF PHYSICAL CHARACTERISTICS OF RADIONUCLIDES MENTIONED IN THE EUROPEAN PHARMACOPOEIA

The following table is given to complete the general monograph *Radiopharmaceutical preparations (0125)*.

The values are obtained from the database of the National Nuclear Data Center (NNDC) at Brookhaven National Laboratory, Upton. N.Y., USA, directly accessible at: www.nndc.bnl.gov/nndc/nudat/radform.html

In case another source of information is preferred (more recent values), this source is explicitly mentioned.

The uncertainty of the half-lives are given in parentheses. In principle the digits in parentheses are the standard uncertainty of the corresponding last digits of the indicated numerical value ('Guide to the Expression of Uncertainty in Measurement', International Organization for Standardization (ISO), 1993, ISBN 92-67-10188-9 or any subsequent revisions of this document).

The following abbreviations are used:

$e_A$ = Auger electrons,

ce = conversion electrons,

$\beta^-$ = electrons,

$\beta^+$ = positrons,

$\gamma$ = gamma rays,

X = X-rays.

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 disintegrations) | Type | Energy (MeV) | Emission probability (per 100 disintegrations) |
| Tritium ($^3$H) | 12.33 (6) years | $\beta^-$ | 0.006 (I) (max: 0.019) | 100 | | | |
| Carbon-11 ($^{11}$C) | 20.385 (20) min | $\beta^+$ | 0.386 (I) (max: 0.960) | 99.8 | $\gamma$ | 0.511 | 199.5 (II) |
| Nitrogen-13 ($^{13}$N) | 9.965 (4) min | $\beta^+$ | 0.492 (I) (max: 1.198) | 99.8 | $\gamma$ | 0.511 | 199.6 (II) |
| Oxygen-15 ($^{15}$O) | 122.24 (16) s | $\beta^+$ | 0.735 (I) (max: 1.732) | 99.9 | $\gamma$ | 0.511 | 199.8 (II) |
| Fluorine-18 ($^{18}$F) | 109.77 (5) min | $\beta^+$ | 0.250 (I) (max: 0.633) | 96.7 | $\gamma$ | 0.511 | 193.5 (II) |
| Phosphorus-32 ($^{32}$P) | 14.26 (4) days | $\beta^-$ | 0.695 (I) (max: 1.71) | 100 | | | |
| Phosphorus-33 ($^{33}$P) | 25.34 (12) days | $\beta^-$ | 0.076 (I) (max: 0.249) | 100 | | | |
| Sulfur-35 ($^{35}$S) | 87.51 (12) days | $\beta^-$ | 0.049 (I) (max: 0.167) | 100 | | | |
| Chromium-51 ($^{51}$Cr) | 27.7025 (24) days | $e_A$ | 0.004 | 67 | X | 0.005 | 22.3 |
| | | | | | $\gamma$ | 0.320 | 9.9 |
| Cobalt-56 ($^{56}$Co) | 77.27 (3) days | $e_A$ | 0.006 | 47 | X | 0.006-0.007 | 25 |
| | | $\beta^+$ | 0.179 (I) | 0.9 | $\gamma$ | 0.511 | 38.0 (II) |
| | | | 0.631 (I) | 18.1 | | 0.847 | 100.0 |
| | | | | | | 1.038 | 14.1 |
| | | | | | | 1.175 | 2.2 |
| | | | | | | 1.238 | 66.1 |
| | | | | | | 1.360 | 4.3 |
| | | | | | | 1.771 | 15.5 |
| | | | | | | 2.015 | 3.0 |
| | | | | | | 2.035 | 7.8 |
| | | | | | | 2.598 | 17.0 |
| | | | | | | 3.202 | 3.1 |
| | | | | | | 3.253 | 7.6 |
| Cobalt-57 ($^{57}$Co) | 271.79 (9) days | $e_A$+ce | 0.006-0.007 | 177.4 | X | 0.006-0.007 | 57 |
| | | ce | 0.014 | 7.4 | $\gamma$ | 0.014 | 9.2 |
| | | | 0.115 | 1.8 | | 0.122 | 85.6 |
| | | | 0.129 | 1.3 | | 0.136 | 10.7 |
| | | | | | | 0.692 | 0.15 |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) |
| Cobalt-58 ($^{58}$Co) | 70.86 (7) days | $e_A$ | 0.006 | 49.4 | X | 0.006-0.007 | 26.3 |
| | | $\beta^+$ | 0.201 (I) | 14.9 | γ | 0.511 | 29.9 (II) |
| | | | | | | 0.811 | 99.4 |
| | | | | | | 0.864 | 0.7 |
| | | | | | | 1.675 | 0.5 |
| Cobalt-60 ($^{60}$Co) | 5.2714 (5) years | $\beta^-$ | 0.096 (I) (max: 0.318) | 99.9 | γ | 1.173 | 100.0 |
| | | | | | | 1.333 | 100.0 |
| Gallium-66 ($^{66}$Ga) | 9.49 (7) hours | $e_A$ | 0.008 | 21 | X | 0.009-0.010 | 19.1 |
| | | $\beta^+$ | 0.157 (I) | 1 | γ | 0.511 | 112 (II) |
| | | | 0.331 (I) | 0.7 | | 0.834 | 5.9 |
| | | | 0.397 (I) | 3.8 | | 1.039 | 37 |
| | | | 0.782 (I) | 0.3 | | 1.333 | 1.2 |
| | | | 1.90 (I) | 50 | | 1.919 | 2.1 |
| | | | | | | 2.190 | 5.6 |
| | | | | | | 2.423 | 1.9 |
| | | | | | | 2.752 | 23.4 |
| | | | | | | 3.229 | 1.5 |
| | | | | | | 3.381 | 1.5 |
| | | | | | | 3.792 | 1.1 |
| | | | | | | 4.086 | 1.3 |
| | | | | | | 4.295 | 4.1 |
| | | | | | | 4.807 | 1.8 |
| Gallium-67 ($^{67}$Ga) | 3.2612 (6) days | $e_A$ | 0.008 | 62 | X | 0.008-0.010 | 57 |
| | | ce | 0.082-0.084 | 30.4 | γ | 0.091-0.093 | 42.4 |
| | | | 0.090-0.092 | 3.6 | | 0.185 | 21.2 |
| | | | 0.175 | 0.3 | | 0.209 | 2.4 |
| | | | | | | 0.300 | 16.8 |
| | | | | | | 0.394 | 4.7 |
| | | | | | | 0.888 | 0.15 |
| Germanium-68 ($^{68}$Ge) in equilibrium with Gallium-68 ($^{68}$Ga) | 270.82 (27) days ($^{68}$Ga: 67.629 (24) min) | $e_A$ | 0.008 | 42.4 | X | 0.009-0.010 | 44.1 |
| | | $\beta^+$ | 0.353 (I) | 1.2 | γ | 0.511 | 178.3 |
| | | | 0.836 (I) | 88.0 | | 1.077 | 3.0 |
| Gallium-68 ($^{68}$Ga) | 67.629 (24) min | $e_A$ | 0.008 | 5.1 | X | 0.009-0.010 | 4.7 |
| | | $\beta^+$ | 0.353 (I) | 1.2 | γ | 0.511 | 178.3 |
| | | | 0.836 (I) | 88.0 | | 1.077 | 3.0 |
| Krypton-81m ($^{81m}$Kr) | 13.10 (3) s | ce | 0.176 | 26.4 | X | 0.012-0.014 | 17.0 |
| | | | 0.189 | 4.6 | | | |
| | | | | | γ | 0.190 | 67.6 |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 disintegrations) | Type | Energy (MeV) | Emission probability (per 100 disintegrations) |
| Rubidium-81 ($^{81}$Rb) in equilibrium with Krypton-81m ($^{81m}$Kr) | 4.576 (5) hours<br>($^{81m}$Kr: 13.10 (3) s) | $e_A$<br>ce<br><br><br>$\beta^+$<br><br><br> | 0.011<br>0.176<br>0.188<br><br>0.253 (I)<br>0.447 (I) | 31.3<br>25.0<br>4.3<br><br>1.8<br>25.0 | X<br>γ<br><br><br><br><br> | 0.013-0.014<br>0.190<br>0.446<br>0.457<br>0.510<br>0.511<br>0.538 | 57.2<br>64<br>23.2<br>3.0<br>5.3<br>54.2<br>2.2 |
| Strontium-89 ($^{89}$Sr) in equilibrium with Yttrium-89m ($^{89m}$Y) | 50.53 (7) days<br>($^{89m}$Y: 16.06 (4) s) | $\beta^-$ | 0.583 (I) (max: 1.492) | 99.99 | γ | 0.909 | 0.01 |
| Strontium-90 ($^{90}$Sr) in equilibrium with Yttrium-90 ($^{90}$Y) | 28.74 (4) years<br>($^{90}$Y: 64.10 (8) hours) | $\beta^-$ | 0.196 (I) (max: 0.546) | 100 | | | |
| Yttrium-90 ($^{90}$Y) | 64.10 (8) hours | $\beta^-$ | 0.934 (I) (max: 2.280) | 100 | | | |
| Molybdene-99 ($^{99}$Mo) in equilibrium with Technetium-99m ($^{99m}$Tc) | 65.94 (1) hours<br>($^{99m}$Tc: 6.01 (1) hours) | $\beta^-$ | 0.133 (I)<br>0.290 (I)<br>0.443 (I) | 16.4<br>1.1<br>82.4 | X<br><br>γ<br><br><br><br><br> | 0.018-0.021<br><br>0.041<br>0.141<br>0.181<br>0.366<br>0.740<br>0.778 | 3.6<br><br>1.1<br>4.5<br>6<br>1.2<br>12.1<br>4.3 |
| Technetium-99m ($^{99m}$Tc) | 6.01 (1) hours | ce<br><br>$e_A$<br><br>ce<br> | 0.002<br><br>0.015<br><br>0.120<br>0.137-0.140 | 74<br><br>2.1<br><br>9.4<br>1.3 | X<br><br>γ<br><br><br> | 0.018-0.021<br><br>0.141<br><br><br> | 7.3<br><br>89.1<br><br><br> |
| Technetium-99 ($^{99}$Tc) | 2.11 × $10^5$ years | $\beta^-$ | 0.085 (I) (max: 0.294) | 100 | | | |
| Ruthenium-103 ($^{103}$Ru) in equilibrium with Rhodium-103m ($^{103m}$Rh) | 39.26 (2) days<br>($^{103m}$Rh: 56.114 (20) min) | $e_A$+ce<br><br>ce<br><br>$\beta^-$<br> | 0.017<br><br>0.030-0.039<br><br>0.031 (I)<br>0.064 (I) | 12<br><br>88.3<br><br>6.6<br>92.2 | X<br><br>γ<br><br><br> | 0.020-0.023<br><br>0.497<br>0.610<br><br> | 9.0<br><br>91<br>5.8<br><br> |
| Indium-110 ($^{110}$In) | 4.9 (1) hours | $e_A$ | 0.019 | 13.4 | X<br><br>γ<br><br><br><br> | 0.023-0.026<br><br>0.642<br>0.658<br>0.885<br>0.938<br>0.997 | 70.5<br><br>25.9<br>98.3<br>92.9<br>68.4<br>10.5 |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) |
| Indium-110m ($^{110m}$In) | 69.1 (5) min | $e_A$ | 0.019 | 5.3 | X | 0.023-0.026 | 27.8 |
| | | $\beta^+$ | 1.015 (I) | 61 | γ | 0.511 | 123.4 (II) |
| | | | | | | 0.658 | 97.8 |
| | | | | | | 2.129 | 2.1 |
| Indium-111 ($^{111}$In) | 2.8047 (5) days | $e_A$ | 0.019 | 15.6 | X | 0.003 | 6.9 |
| | | | | | | 0.023-0.026 | 82.3 |
| | | ce | 0.145 | 7.8 | | | |
| | | | 0.167-0.171 | 1.3 | γ | 0.171 | 90.2 |
| | | | 0.219 | 4.9 | | 0.245 | 94.0 |
| | | | 0.241-0.245 | 1.0 | | | |
| Indium-114m ($^{114m}$In) in equilibrium with Indium-114 ($^{114}$In) | 49.51 (1) days | ce | 0.162 | 40 | X | 0.023-0.027 | 36.3 |
| | | | 0.186-0.190 | 40 | | | |
| | | | | | γ | 0.190 | 15.6 |
| | | $\beta^-$ | 0.777 (I) (max: 1.985) | 95 | | 0.558 | 3.2 |
| | ($^{114}$In: 71.9 (1) s) | | | | | 0.725 | 3.2 |
| Tellurium-121m ($^{121m}$Te) in equilibrium with Tellure-121 ($^{121}$Te) | 154.0 (7) days | $e_A$ | 0.003 | 88.0 | X | 0.026-0.031 | 50.5 |
| | | | 0.022-0.023 | 7.4 | | | |
| | | | | | γ | 0.212 | 81.4 |
| | | ce | 0.050 | 33.2 | | 1.102 | 2.5 |
| | ($^{121}$Te: 19.16 (5) days) | | 0.077 | 40.0 | | | |
| | | | 0.180 | 6.1 | | | |
| Tellurium-121 ($^{121}$Te) | 19.16 (5) days | $e_A$ | 0.022 | 11.6 | X | 0.026-0.030 | 75.6 |
| | | | | | γ | 0.470 | 1.4 |
| | | | | | | 0.508 | 17.7 |
| | | | | | | 0.573 | 80.3 |
| Iodine-123 ($^{123}$I) | 13.27 (8) hours | $e_A$ | 0.023 | 12.3 | X | 0.004 | 9.3 |
| | | | | | | 0.027-0.031 | 86.6 |
| | | ce | 0.127 | 13.6 | | | |
| | | | 0.154 | 1.8 | γ | 0.159 | 83.3 |
| | | | 0.158 | 0.4 | | 0.346 | 0.1 |
| | | | | | | 0.440 | 0.4 |
| | | | | | | 0.505 | 0.3 |
| | | | | | | 0.529 | 1.4 |
| | | | | | | 0.538 | 0.4 |
| Iodine-125 ($^{125}$I) | 59.402 (14) days | $e_A$+ce | 0.004 | 80 | X | 0.004 | 15.5 |
| | | | 0.023-0.035 | 33 | | 0.027 | 114 |
| | | | | | | 0.031 | 26 |
| | | | | | γ | 0.035 | 6.7 |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) |
| Iodine-126 ($^{126}$I) | 13.11 (5) days | $e_A$ | 0.023 | 6 | X | 0.027-0.031 | 42.2 |
| | | ce | 0.354 | 0.5 | γ | 0.388 | 34 |
| | | | 0.634 | 0.1 | | 0.491 | 2.9 |
| | | | | | | 0.511 | 2.3 (II) |
| | | $\beta^-$ | 0.109 (I) | 3.6 | | 0.666 | 33 |
| | | | 0.290 (I) | 32.1 | | 0.754 | 4.2 |
| | | | 0.459 (I) | 8.0 | | 0.880 | 0.8 |
| | | | | | | 1.420 | 0.3 |
| | | $\beta^+$ | 0.530 (I) | 1 | | | |
| Iodine-131 ($^{131}$I) | 8.02070 (11) days | ce | 0.46 | 3.5 | X | 0.029-0.030 | 3.9 |
| | | | 0.330 | 1.6 | | | |
| | | | | | γ | 0.080 | 2.6 |
| | | $\beta^-$ | 0.069 (I) | 2.1 | | 0.284 | 6.1 |
| | | | 0.097 (I) | 7.3 | | 0.365 | 81.7 |
| | | | 0.192 (I) | 89.9 | | 0.637 | 7.2 |
| | | | | | | 0.723 | 1.8 |
| Xenon-131m ($^{131m}$Xe) | 11.84 (7) days | $e_A$ | 0.025 | 6.8 | X | 0.004 | 8.3 |
| | | | | | | 0.030 | 44.0 |
| | | ce | 0.129 | 61 | | 0.034 | 10.2 |
| | | | 0.159 | 28.5 | | | |
| | | | 0.163 | 8.3 | γ | 0.164 | 2.0 |
| Iodine-133 ($^{133}$I) (decays to radioactive Xenon-133) | 20.8 (1) hours | $\beta^-$ | 0.140 (I) | 3.8 | γ | 0.530 | 87 |
| | | | 0.162 (I) | 3.2 | | 0.875 | 4.5 |
| | | | 0.299 (I) | 4.2 | | 1.298 | 2.4 |
| | | | 0.441 (I) | 83 | | | |
| Xenon-133 ($^{133}$Xe) | 5.243 (1) days | $e_A$ | 0.026 | 5.8 | X | 0.004 | 6.3 |
| | | | | | | 0.031 | 40.3 |
| | | ce | 0.045 | 55.1 | | 0.035 | 9.4 |
| | | | 0.075-0.080 | 9.9 | | | |
| | | | | | γ | 0.080 | 38.3 |
| | | $\beta^-$ | 0.101 (I) | 99.0 | | | |
| Xenon-133m ($^{133m}$Xe) (decays to radioactive Xenon-133) | 2.19 (1) days | $e_A$ | 0.025 | 7 | X | 0.004 | 7.8 |
| | | | | | | 0.030 | 45.9 |
| | | ce | 0.199 | 64.0 | | 0.034 | 10.6 |
| | | | 0.228 | 20.7 | | | |
| | | | 0.232 | 4.6 | γ | 0.233 | 10.0 |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) | Type | Energy (MeV) | Emission probability (per 100 dis-integrations) |
| Iodine-135 ($^{135}$I) (decays to radioactive Xenon-135) | 6.57 (2) hours | $\beta^-$ | 0.140 (I) | 7.4 | γ | 0.527 | 13.8 |
| | | | 0.237 (I) | 8 | | 0.547 | 7.2 |
| | | | 0.307 (I) | 8.8 | | 0.837 | 6.7 |
| | | | 0.352 (I) | 21.9 | | 1.039 | 8.0 |
| | | | 0.399 (I) | 8 | | 1.132 | 22.7 |
| | | | 0.444 (I) | 7.5 | | 1.260 | 28.9 |
| | | | 0.529 (I) | 23.8 | | 1.458 | 8.7 |
| | | | | | | 1.678 | 9.6 |
| | | | | | | 1.791 | 7.8 |
| Xenon-135 ($^{135}$Xe) | 9.14 (2) hours | ce | 0.214 | 5.5 | X | 0.031-0.035 | 5.0 |
| | | $\beta^-$ | 0.171 | 3.1 | γ | 0.250 | 90.2 |
| | | | 0.308 | 96.0 | | 0.608 | 2.9 |
| Caesium-137 ($^{137}$Cs) in equilibrium with Barium-137m ($^{137m}$Ba) | 30.04 (3) years | $e_A$ | 0.026 | 0.8 | X | 0.005 | 1 |
| | | | | | | 0.032-0.036 | 7 |
| | | ce | 0.624 | 8.0 | | | |
| | | | 0.656 | 1.4 | γ | 0.662 | 85.1 |
| | | $\beta^-$ | 0.174 (I) | 94.4 | | | |
| | ($^{137m}$Ba: 2.552 (1) min) | | 0.416 (I) | 5.6 | | | |
| Ytterbium-175 ($^{175}$Yb) | 4.185 (1) days | $e_A$ | 0.00602 | 6.34 | X | 0.0530-0.541 | 5.90 |
| | | ce | 0.05049 | 7.42 | | | |
| | | | 0.1029 | 1.76 | γ | 0.1138 | 3.87 |
| | | $\beta^-$ | 0.0190 | 20.4 | | 0.2825 | 6.13 |
| | | | 0.1024 | 6.7 | | 0.3963 | 13.2 |
| | | | 0.1399 | 72.9 | | | |
| Lutetium-177m ($^{177m}$Lu) | 160.44 (6) days | $e_A$ | 0.00618 | 129.8 | X | 0.0079 | 45.6 |
| | | ce | 0.04001 | 33.6 | | 0.0546-0.0649 | 115.2 |
| | | | 0.04760 | 18.2 | γ | 0.2285 | 37.1 |
| | | | 0.06315 | 23.9 | | 0.2818 | 14.2 |
| | | | 0.08793 | 16.1 | | 0.3277 | 18.1 |
| | | | 0.1017 | 23.9 | | 0.3785 | 29.9 |
| | | $\beta^-$ | 0.04082(I) | 78.6 | | 0.4137 | 17.5 |
| | | | | | | 0.4185 | 21.3 |
| Lutetium-177 ($^{177}$Lu) | 6.647 (4) days | $e_A$ | 4.3-11.2 | 8.8 | X | 0.007-0.011 | 3.2 |
| | | | | | | 0.054-0.056 | 4.4 |
| | | $\beta^-$ | 0.0477 | 11.6 | γ | 0.1129 | 6.2 |
| | | | 0.1117 | 9.1 | | 0.2084 | 10.4 |
| | | | 0.1494 | 79.3 | | | |

| Radionuclide | Half-life | Electronic emission | | | Photon emission | | |
|---|---|---|---|---|---|---|---|
| | | Type | Energy (MeV) | Emission probability (per 100 disintegrations) | Type | Energy (MeV) | Emission probability (per 100 disintegrations) |
| Thallium-200 ($^{200}$Tl) | 26.1 (1) hours | ce | 0.285 | 3.4 | X | 0.010 | 32.0 |
| | | | 0.353 | 1.4 | | 0.069-0.071 | 63.3 |
| | | | | | | 0.08 | 17.5 |
| | | $\beta^+$ | 0.495 (I) | 0.3 | | | |
| | | | | | γ | 0.368 | 87.2 |
| | | | | | | 0.579 | 13.8 |
| | | | | | | 0.828 | 10.8 |
| | | | | | | 1.206 | 29.9 |
| | | | | | | 1.226 | 3.4 |
| | | | | | | 1.274 | 3.3 |
| | | | | | | 1.363 | 3.4 |
| | | | | | | 1.515 | 4.0 |
| Lead-201 ($^{201}$Pb) (decays to radioactive Thallium-201) | 9.33 (3) hours | $e_A$ | 0.055 | 3 | X | 0.070-0.073 | 69 |
| | | | | | | 0.083 | 19 |
| | | ce | 0.246 | 8.5 | | | |
| | | | 0.276 | 2 | γ | 0.331 | 79 |
| | | | 0.316 | 2.3 | | 0.361 | 9.9 |
| | | | | | | 0.406 | 2.0 |
| | | | | | | 0.585 | 3.6 |
| | | | | | | 0.692 | 4.3 |
| | | | | | | 0.767 | 3.2 |
| | | | | | | 0.826 | 2.4 |
| | | | | | | 0.908 | 5.7 |
| | | | | | | 0.946 | 7.9 |
| | | | | | | 1.099 | 1.8 |
| | | | | | | 1.277 | 1.6 |
| Thallium-201 ($^{201}$Tl) | 72.912 (17) hours | ce | 0.016-0.017 | 17.7 | X | 0.010 | 46.0 |
| | | | 0.027-0.029 | 4.1 | | 0.069-0.071 | 73.7 |
| | | | 0.052 | 7.2 | | 0.080 | 20.4 |
| | | | 0.084 | 15.4 | | | |
| | | | 0.153 | 2.6 | γ | 0.135 | 2.6 |
| | | | | | | 0,167 | 10.0 |
| Thallium-202 ($^{202}$Tl) | 12.23 (2) days | $e_A$ | 0.054 | 2.8 | X | 0.010 | 31.0 |
| | | | | | | 0.069-0.071 | 61.6 |
| | | ce | 0.357 | 2.4 | | 0.080 | 17.1 |
| | | | | | γ | 0.440 | 91.4 |
| Lead-203 ($^{203}$Pb) | 51.873 (9) hours | $e_A$ | 0.055 | 3.0 | X | 0.010 | 37.0 |
| | | | | | | 0.071-0.073 | 69.6 |
| | | ce | 0.194 | 13.3 | | 0.083 | 19.4 |
| | | | | | γ | 0.279 | 80.8 |
| | | | | | | 0.401 | 3.4 |

(I) Mean energy of the β spectrum.
(II) Maximum emission probability corresponding to a total annihilation in the source per 100 disintegrations.

# 5.8. Pharmacopoeial harmonisation

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## 5.8. PHARMACOPOEIAL HARMONISATION

*This general chapter is included for guidance of users.*

The chapter does not affect in any way the status of the monographs and general chapters as the authoritative reference in case of doubt or dispute, where compliance with the European Pharmacopoeia is required.

The European Pharmacopoeia Commission recognises the utility of working with other pharmacopoeial bodies to develop harmonised monographs and general chapters. Such harmonisation is fully compatible with the declared aims of the Commission and has benefits of different kinds, notably the simplification and rationalisation of quality control methods and licensing procedures. Such harmonisation also enhances the benefits of the work of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), since some of the guidelines developed depend on pharmacopoeial general chapters for their application.

Work on harmonisation is carried out by a well-defined but informal process in the Pharmacopoeial Discussion Group (PDG), in which the European Pharmacopoeia, the Japanese Pharmacopoeia and the United States Pharmacopeia are associated. Pharmacopoeial harmonisation has the following 2 aims:

- for general chapters, the aim is to arrive at interchangeable methods or requirements so that demonstration of compliance using a general chapter from one of the 3 pharmacopoeias implies that the same result would be obtained using the general chapter of either of the other pharmacopoeias; when a formal declaration of interchangeability has been recommended by ICH, the corresponding information is available on the ICH website;
- for monographs, the aim is to arrive at identical requirements for all attributes of a substance; for most substances it can be extremely difficult to achieve complete harmonisation, for example because of differences in legal status and interpretation; it has therefore appeared worthwhile to the PDG to approve and publish monographs in which as many attributes as possible are harmonised.

Any non-harmonised attributes/provisions and any local requirements (i.e. attributes/provisions that are present only in the Ph. Eur. text) are indicated in the relevant Ph. Eur. general chapters and monographs: the non-harmonised attributes/provisions are placed between black diamonds (♦ ♦), while the local requirements are placed between white diamonds (◊◊).

The non-mandatory Functionality-related characteristics section is specific to the Ph. Eur.; it is not subject to pharmacopoeial harmonisation and is therefore not placed between black or white diamonds.

Harmonisation is not achieved until the text becomes official in all 3 pharmacopoeias.

*It remains the ultimate responsibility of the user to verify the current content of the texts in force in the respective pharmacopoeias.*

The 3 pharmacopoeias have undertaken not to make unilateral changes to harmonised monographs and general chapters but rather to apply the agreed revision procedure whereby all partners adopt a revision simultaneously.

# 5.9. Polymorphism

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# 5.9. POLYMORPHISM

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulfur), the term allotropy is used instead of polymorphism.

The term pseudopolymorphism is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include compounds where the solvent is trapped in the matrix in variable proportions. However the term pseudopolymorphism is ambiguous because of its use in different circumstances. It is therefore preferable to use only the terms "solvates" and "hydrates".

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, occurrence of solvates, allotropy or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At normal temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. The change from one phase to another is a univariate equilibrium, so that at a given pressure this state is characterised by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

Different crystalline forms or solvates may be produced by varying the crystallisation conditions (temperature, pressure, solvent, concentration, rate of crystallisation, seeding of the crystallisation medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism:

- X-ray diffraction of powders (*2.9.33*),
- X-ray diffraction of single crystals,
- thermal analysis (*2.2.34*) (differential scanning calorimetry, thermogravimetry, thermomicroscopy),
- microcalorimetry,
- moisture absorption analysis,
- optical and electronic microscopy,
- solid-state nuclear magnetic resonance,
- infrared absorption spectrophotometry (*2.2.24*),
- Raman spectroscopy (*2.2.48*),
- measurement of solubility and intrinsic dissolution rate,
- density measurement.

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise solvates are less soluble in their solvent than unsolvated forms.

# 5.10. Control of impurities in substances for pharmaceutical use

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# 5.10. CONTROL OF IMPURITIES IN SUBSTANCES FOR PHARMACEUTICAL USE

**Preamble**

The monographs of the European Pharmacopoeia on substances for pharmaceutical use are designed to ensure acceptable quality for users. The role of the Pharmacopoeia in public health protection requires that adequate control of impurities be provided by monographs. The quality required is based on scientific, technical and regulatory considerations.

Requirements concerning impurities are given in specific monographs and in the general monograph *Substances for pharmaceutical use (2034)*. Specific monographs and the general monograph are complementary: specific monographs prescribe acceptance criteria for impurities whereas the general monograph deals with the need for qualification, identification and reporting of any organic impurities that occur in *active substances*.

The thresholds for reporting, identification and qualification contained in the general monograph *Substances for pharmaceutical use (2034)* apply to all related substances. However, if a monograph does not contain a related substances test based on a quantitative method, any new impurities occurring above a threshold may be overlooked since the test is not capable to detect those impurities.

The provisions of the Related substances section of the general monograph *Substances for pharmaceutical use (2034)*, notably those concerning thresholds, do not apply to excipients; also excluded from the provisions of this section are: biological and biotechnological products; oligonucleotides; radiopharmaceuticals; fermentation products and semi-synthetic products derived therefrom; herbal products and crude products of animal and plant origin. Although the thresholds stated in the general monograph do not apply, the general concepts of reporting, identification (wherever possible) and qualification of impurities are equally valid for these classes.

**Basis for the elaboration of monographs of the European Pharmacopoeia**

European Pharmacopoeia monographs are elaborated on substances that are present in medicinal products that have been authorised by the competent authorities of Parties to the *European Pharmacopoeia Convention*. Consequently, these monographs do not necessarily cover all sources of substances for pharmaceutical use on the world market.

Organic and inorganic impurities present in those substances that have been evaluated by the competent authorities are qualified with respect to safety at the maximum authorised content (at the maximum daily dose) unless new safety data that become available following evaluation justify lower limits.

European Pharmacopoeia monographs on substances for pharmaceutical use are elaborated by groups of experts and working parties collaborating with national pharmacopoeia authorities, the competent authorities for marketing authorisation, national control laboratories and the European Pharmacopoeia laboratory; they are also assisted by the producers of the substances and/or the pharmaceutical manufacturers that use these substances.

**Control of impurities in substances for pharmaceutical use**

The quality with respect to impurities is controlled by a set of tests within a monograph. These tests are intended to cover organic and inorganic impurities that are relevant in view of the sources of active substances in authorised medicinal products.

Control of residual solvents is provided by the general monograph *Substances for pharmaceutical use (2034)* and general chapter *5.4. Residual solvents*. The certificate of suitability of a monograph of the European Pharmacopoeia for a given source of a substance indicates the residual solvents that are controlled together with the specified acceptance criteria and the validated control method where this differs from those described in general chapter *2.4.24. Identification and control of residual solvents*.

Monographs on organic chemicals usually have a test entitled "Related substances" that covers relevant organic impurities. This test may be supplemented by specific tests where the general test does not control a given impurity or where there are particular reasons (for example, safety reasons) for requiring special control.

Where a monograph has no Related substances (or equivalent) test but only specific tests, the user of a substance must nevertheless ensure that there is suitable control of organic impurities; those occurring above the identification threshold are to be identified (wherever possible) and, unless justified, those occurring above the qualification threshold are to be qualified (see also under Recommendations to users of monographs of active substances).

Where the monograph covers substances with different impurity profiles, it may have a single related substances test to cover all impurities mentioned in the Impurities section or several tests may be necessary to give control of all known profiles. Compliance may be established by carrying out only the tests relevant to the known impurity profile for the source of the substance.

Instructions for control of impurities may be included in the Production section of a monograph, for example where the only analytical method appropriate for the control of a given impurity is to be performed by the manufacturer since the method is too technically complex for general use or cannot be applied to the final drug substance and/or where validation of the production process (including the purification step) will give sufficient control.

**Impurities section in monographs on active substances**

The Impurities section in a monograph includes impurities (chemical structure and name wherever possible), which are usually organic, that are known to be detected by the tests prescribed in the monograph. It is based on information available at the time of elaboration or revision of the monograph and is not necessarily exhaustive. The section includes specified impurities and, where so indicated, other detectable impurities.

*Specified impurities* have an acceptance criterion not greater than that authorised by the competent authorities.

*Other detectable impurities* are potential impurities with a defined structure but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are given in the Impurities section for information.

Where an impurity other than a specified impurity is found in an active substance it is the responsibility of the user of the substance to check whether it has to be identified/qualified, depending on its content, nature, maximum daily dose and relevant identification/qualification threshold, in accordance with the general monograph on *Substances for pharmaceutical use (2034)*, Related substances section.

It should be noted that specific thresholds are applied to substances exclusively for veterinary use.

**Interpretation of the test for related substances in the monographs on active substances**

A specific monograph on a substance for pharmaceutical use is to be read and interpreted in conjunction with the general monograph on *Substances for pharmaceutical use (2034)*.

Where a general acceptance criterion for impurities ("any other impurity", "other impurities", "any impurity") equivalent to a nominal content greater than the applicable identification threshold (see the general monograph on *Substances for pharmaceutical use (2034)*) is prescribed, this is valid only for specified impurities mentioned in the Impurities section. The need for identification (wherever possible), reporting, specification and qualification of other impurities that occur must be considered according to the requirements of the general monograph. It is the responsibility of the user of the substance to determine the validity of the acceptance criteria for impurities not mentioned in the Impurities section and for those indicated as other detectable impurities.

Acceptance criteria for the related substances test are presented in different ways in existing monographs; the decision tree (Figure 5.10.-1) may be used as an aid in the interpretation of general acceptance criteria and their relation with the Impurities section of the monograph.

General acceptance criteria for "other" impurities are expressed in various ways in the monographs: "any other impurity", "other impurities", "any impurity", "any spot", "any band", etc. The general acceptance criteria may apply to certain specified impurities only or to unspecified impurities and certain specified impurities, depending on the nature of the active substance and the applicable identification threshold. Pending editorial adaptation of already published monographs using unequivocal terminology, the decision tree (Figure 5.10.-1) may be used to determine the acceptance criterion to be applied.

**Recommendations to users of monographs of active substances**

Monographs give a specification for suitable quality of substances with impurity profiles corresponding to those taken into account during elaboration and/or revision of the monograph. It is the responsibility of the user of the substance to check that the monograph provides adequate control of impurities for a substance for pharmaceutical use from a given source, notably by using the procedure for certification of suitability of the monographs of the European Pharmacopoeia.

A monograph with a related substances test based on a quantitative method (such as liquid chromatography, gas chromatography and capillary electrophoresis) provides adequate control of impurities for a substance from a given source if impurities present in amounts above the applicable identification threshold are specified impurities mentioned in the Impurities section.

If the substance contains impurities other than those mentioned in the Impurities section, it has to be verified that these impurities are detectable by the method described

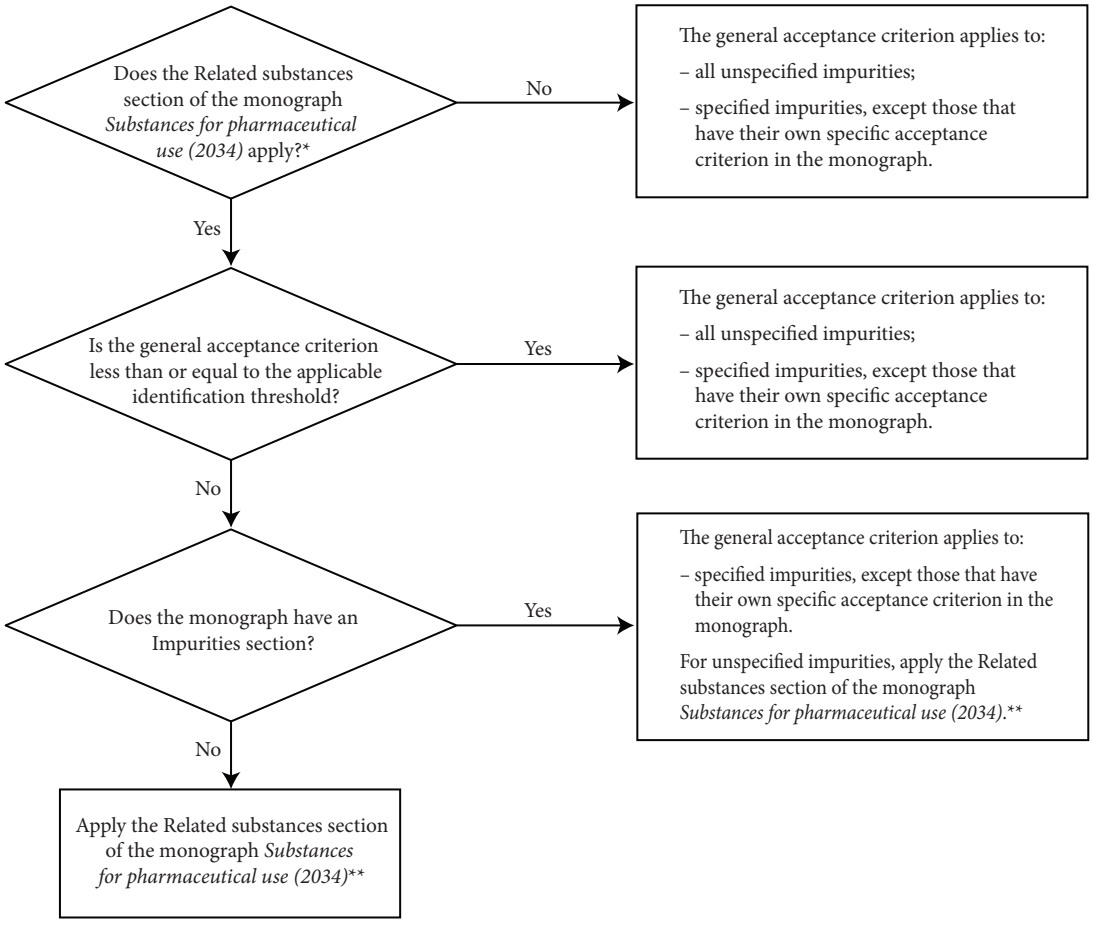


* The requirements of this section apply to active substances with the exception of: biological and biotechnological products; oligonucleotides; radiopharmaceuticals; products of fermentation and semi-synthetic products derived therefrom; crude products of animal or plant origin; herbal products.

** To apply the Related substances section of the monograph *Substances for pharmaceutical use (2034)*:
– an individual acceptance criterion must be defined for any impurity that may be present above the identification threshold;
– any impurity with an acceptance criterion above the identification threshold must wherever possible be identified;
– any impurity with an acceptance criterion above the qualification threshold must be qualified.

Figure 5.10.-1. – *Decision tree for interpretation of general acceptance criteria for 'other' impurities in monographs*

in the monograph, otherwise a new method must be developed and revision of the monograph must be requested. Depending on the contents found and the limits proposed, the identification and/or the qualification of these impurities must be considered.

Where a single related substances test covers different impurity profiles, only impurities for the known profile from a single source need to be reported in the certificate of analysis unless the marketing authorisation holder uses active substances with different impurity profiles.

**Identification of impurities (peak assignment)**

Where a monograph has an individual limit for an impurity, it is often necessary to define means of identification, for example using a reference substance, a representative chromatogram or relative retention. The user of the substance may find it necessary to identify impurities other than those for which the monograph provides a means of identification, for example to check the suitability of the specification for a given impurity profile by comparison with the Impurities section. The European Pharmacopoeia does not provide reference substances, representative chromatograms or information on relative retentions for this purpose, unless prescribed in the monograph. Users will therefore have to apply the available scientific techniques for identification.

**New impurities/Specified impurities above the specified limit**

Where a new manufacturing process or change in an established process leads to the occurrence of a new impurity, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding identification and qualification and to verify the suitability of the monograph for control of the impurity. A certificate of suitability is a means for confirming for a substance from a given source that the new impurity is adequately controlled or the certificate contains a method for control with a defined acceptance criterion. In the latter case revision of the monograph will be initiated.

Where a new manufacturing process or change in an established process leads to the occurrence of a specified impurity above the specified limit, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding qualification.

**Expression of acceptance criteria**

The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values.

**Chromatographic methods**

General chapter *2.2.46. Chromatographic separation techniques* deals with various aspects of impurities control.

Information is available via the EDQM website on commercial names for columns and other reagents and equipment found suitable during monograph development, where this is considered useful.

GLOSSARY

**Disregard limit**: in chromatographic tests, the nominal content at or below which peaks/signals are not taken into account for calculating a sum of impurities. The numerical values for the disregard limit and the reporting threshold are usually the same.

**Identification threshold**: a limit above which an impurity is to be identified.

**Identified impurity**: an impurity for which structural characterisation has been achieved.

**Impurity**: any component of a substance for pharmaceutical use that is not the chemical entity defined as the substance.

**Nominal concentration**: concentration calculated on the basis of the concentration of the prescribed reference and taking account of the prescribed correction factor.

**Other detectable impurities**: potential impurities with a defined structure that are known to be detected by the tests in a monograph but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are unspecified impurities and are thus limited by a general acceptance criterion.

**Potential impurity**: an impurity that theoretically can arise during manufacture or storage. It may or may not actually appear in the substance. Where a potential impurity is known to be detected by the tests in a monograph but not known to be normally present in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention, it will be included in the Impurities section under *Other detectable impurities* for information.

**Qualification**: the process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified.

**Qualification threshold**: a limit above which an impurity is to be qualified.

**Related substances**: title used in monographs for general tests for organic impurities.

**Reporting threshold**: a limit above which an impurity is to be reported. Synonym: reporting level.

**Specified impurity**: an impurity that is individually listed and limited with a specific acceptance criterion in a monograph. A specified impurity can be either identified or unidentified.

**Unidentified impurity**: an impurity for which a structural characterisation has not been achieved and that is defined solely by qualitative analytical properties (for example, relative retention).

**Unspecified impurity**: an impurity that is limited by a general acceptance criterion and not individually listed with its own specific acceptance criterion.

# 5.11. Characters section in monographs

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# 5.11. CHARACTERS SECTION IN MONOGRAPHS

The General Notices indicate that the statements included in the Characters section are not to be interpreted in a strict sense and are not requirements. For information of users, the methods recommended to authors of monographs as the basis for statements concerning hygroscopicity, crystallinity and solubility are given below.

HYGROSCOPICITY

This method is to be carried out on a substance that complies with the test for loss on drying or water content of the monograph. The method gives an indication of the degree of hygroscopicity rather than a true determination.

Use a glass weighing vessel 50 mm in external diameter and 15 mm high. Weigh the vessel and stopper ($m_1$). Place the amount of substance prescribed for the test for loss on drying or water in the vessel and weigh ($m_2$). Place the unstoppered vessel in a desiccator at 25 °C containing a saturated solution of ammonium chloride or ammonium sulfate or place it in a climatic cabinet set at 25 ± 1 °C and 80 ± 2 per cent relative humidity. Allow to stand for 24 h. Stopper the weighing vessel and weigh ($m_3$).

Calculate the percentage increase in mass using the expression:

$$\frac{m_3 - m_2}{m_2 - m_1} \times 100$$

The result is interpreted as follows:

- *deliquescent*: sufficient water is absorbed to form a liquid,
- *very hygroscopic*: increase in mass is equal to or greater than 15 per cent,
- *hygroscopic*: increase in mass is less than 15 per cent and equal to or greater than 2 per cent,
- *slightly hygroscopic*: increase in mass is less than 2 per cent and equal to or greater than 0.2 per cent.

CRYSTALLINITY

This method is employed to establish the crystalline or amorphous nature of a substance.

Mount a few particles of the substance to be examined in mineral oil on a clean glass slide. Examine under a polarising microscope. Crystalline particles exhibit birefringence and extinction positions when the microscope stage is revolved.

SOLUBILITY

For this test a maximum of 111 mg of substance (for each solvent) and a maximum of 30 mL of each solvent are necessary.

**Dissolving procedure**

Shake vigorously for 1 min and place in a constant temperature device, maintained at a temperature of 25.0 ± 0.5 °C for 15 min. If the substance is not completely dissolved, repeat the shaking for 1 min and place the tube in the constant temperature device for 15 min.

**Method**

Weigh 100 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube (16 mm in internal diameter and 160 mm long), add 0.1 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very soluble*.

If the substance is not completely dissolved, add 0.9 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *freely soluble*.

If the substance is not completely dissolved, add 2.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *soluble*.

If the substance is not completely dissolved, add 7.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *sparingly soluble*.

If the substance is not completely dissolved, weigh 10 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *slightly soluble*.

If the substance is not completely dissolved, weigh 1 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very slightly soluble*.

# 5.12. Reference standards

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# 5.12. REFERENCE STANDARDS

*This chapter is published for information.*

## 1. INTRODUCTION

'Reference standard' is used in this chapter as a general term covering reference substances, reference preparations and reference spectra.

Reference standards are frequently necessary to achieve adequate quality control of medicinal products and their components.

Reference standards are established using suitable procedures and their continued suitability for use is monitored according to a predefined programme. Where a reference standard is needed, it is an integral part of the pharmacopoeial monograph or the manufacturer's specification. Where a European Pharmacopoeia reference standard is referred to in a monograph or general chapter, it represents the official standard that is alone authoritative in case of doubt or dispute.

## 2. TERMINOLOGY

*Primary standard.* A standard designated or widely acknowledged as having the highest metrological qualities and whose property value is accepted without reference to other standards of the same property or quantity, within a specified context. This definition does not cover international standards.

*International standard.* A primary standard provided to enable the results of biological or immunological assay procedures to be expressed in the same way throughout the world. The value assignment is in International Units (IU) or another suitable unit. The unitage is normally assigned to a first international standard by the World Health Organization (WHO) in an arbitrary manner based on an international interlaboratory study. Activities in International Units are assigned to replacement international standards, where appropriate, by comparing them with a previous standard.

*Secondary standard.* A standard whose property value is assigned by comparison with a primary standard of the same property or quantity.

*European Pharmacopoeia reference standard.* A reference standard established under the aegis of and adopted by the European Pharmacopoeia Commission.

*European Pharmacopoeia chemical reference substance (CRS).* A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. CRSs are in general primary standards, except for those (notably antibiotics) that are calibrated in International Units. The latter are secondary standards traceable to the international standard.

*European Pharmacopoeia herbal reference standard (HRS).* A herbal drug preparation (usually an extract) or a herbal drug intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. Unless otherwise specified, HRSs are designated as primary reference standards for their intended use.

*European Pharmacopoeia biological reference preparation (BRP).* A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. BRPs are either secondary standards calibrated in International Units or primary standards, which may be used to define a European Pharmacopoeia Unit (Ph. Eur. U.). Other assigned contents may also be used, for example, virus titre or number of bacteria.

*Reference material (RM).* A material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in the measurement process.

*Certified reference material (CRM).* A reference material characterised by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that states the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

## 3. USE OF EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS

European Pharmacopoeia reference standards are employed in the identification, purity testing and assay of articles subject to a European Pharmacopoeia monograph or general chapter. European Pharmacopoeia reference standards are shown to be suitable for their intended purpose; they are not necessarily suitable for other purposes. If a European Pharmacopoeia reference standard is to be used for any purpose other than that for which it has been established, its suitability for the new use has to be fully demonstrated and when applicable, to be described in the marketing authorisation application. Any value assigned to a reference standard is valid for the intended use and not necessarily for other uses.

A European Pharmacopoeia reference standard with an assigned content/potency for use in the assay of a substance for pharmaceutical use (see general monograph *Substances for pharmaceutical use (2034)*) may be suitable to determine the content/potency of that substance in a pharmaceutical preparation provided all of the following conditions are fulfilled:

- the chromatographic assay method described in the active substance monograph is employed;
- the applicability of the method to the particular pharmaceutical preparation (absence of interference) is verified by the user;
- any pre-treatment of the sample (e.g. extraction, filtration) is validated for the particular pharmaceutical preparation.

It is the policy of the European Pharmacopoeia to supply reference standards in adequate quantities for immediate use (i.e. the quantity needed to perform the test(s) described in the monograph or general chapter) after opening of the container. Use in other conditions is the responsibility of the analyst. If an unopened container is stored in the recommended conditions, it remains suitable for use as long as it is of the current batch. Information on current batch numbers is provided in the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS). Storage of reconstituted or diluted solutions of reference standards is not recommended unless suitability has been demonstrated by the user.

*Secondary standards.* A secondary standard is usually established to reduce the use of the primary standard and may be used for routine quality control purposes. A secondary standard shall exhibit the same property or properties as the primary standard to which it is traceable. It shall therefore be used for the same purpose as the primary standard.

International standards are usually available in relatively limited quantities and are intended to be used for the characterisation and calibration of secondary standards; these secondary standards may then be used as working standards.

## 4. ESTABLISHMENT OF REFERENCE STANDARDS

### *4-1. PRIMARY STANDARDS*

A substance or preparation to be established as a primary standard is characterised by a variety of analytical techniques chosen to demonstrate its suitability for use.

For reference standards used for the control of medicinal products and their components, relevant parts of the following test programme are usually applied.

Test programme:

- Characterisation of the substance (structural elucidation) by appropriate chemical attributes such as structural formula, empirical formula, molecular mass or composition. A number of techniques may be used including:
  - nuclear magnetic resonance spectrometry;
  - mass spectrometry;
  - infrared spectrophotometry;
  - elemental analysis.
- Determination of purity:
  - determination of the content of related substances by an appropriate separation technique and/or spectrometric method, where applicable;
  - quantitative determination of water;
  - determination of the content of residual solvents;
  - determination of loss on drying, which may in certain circumstances replace the determinations of water and residual solvents;
  - determination of inorganic impurities (e.g. sulfated ash, atomic absorption spectrometry, inductively coupled plasma spectrometry, X-ray fluorescence spectrometry); the results are usually not used to determine an assigned content, except where they would have an appreciable impact upon it;
  - determination of purity by an independent method (e.g. quantitative nuclear magnetic resonance spectrometry, differential scanning calorimetry or titration where appropriate; the results of these determinations are usually used to support and confirm the results obtained from separation techniques; they are not used in the calculation of the assigned content).

For biologicals, guidance is given in the WHO recommendations for the preparation, characterisation and establishment of international and other biological reference standards (WHO Technical Report Series).

*4-2. EUROPEAN PHARMACOPOEIA CHEMICAL REFERENCE SUBSTANCES*

The extent of testing and the number of laboratories involved in the establishment of a CRS depend on the use of the CRS and are tailored to ensure fitness for purpose.

Where an interlaboratory study is carried out during establishment, a protocol is provided for each participant and only valid results derived according to the protocol are used to determine an assigned content or otherwise confirm suitability.

Relevant parts of the following programme are typically applied.

**4-2-1. Identification**. In general, the candidate batch is shown to comply with the relevant requirements of the monograph; full structural elucidation is carried out for the first batch.

**4-2-2. Related substances test**. A CRS corresponding to an impurity is characterised for identity and purity. Where a CRS is used to determine the content of a given impurity, the preferred minimum content is 95.0 per cent; where this is achieved the assigned content of the CRS is not given and it is considered to be 100.0 per cent; this approximation is acceptable since there will be no appreciable effect on the determination of impurities. When this minimum content cannot be obtained, an assigned content is given to the CRS.

CRSs used to determine the content of a given impurity are normally in the same acid, base or salt form as the substance that is the subject of the corresponding monograph. Where this is not the case, unless otherwise justified, a corresponding stoichiometric conversion factor is applied.

If an impurity is not available in a sufficient quantity to establish a CRS, a number of other options exist:

- preparation of a CRS that contains a mixture of the compound(s) and the impurity or impurities;
- preparation of a CRS containing a mixture of specified impurities.

Where such a mixture is also used to determine the content of a given impurity, the content of the impurity in the CRS is determined by appropriate separation methods and a content is assigned to the reference standard.

**4-2-3. Assay**

*4-2-3-1. Chemical assay*. When a CRS is to be used for quantitative determination of a substance for pharmaceutical use (assay standard), the extent of testing is greater than when a CRS is used for other purposes. Unless the substance is of high purity, several collaborating laboratories are usually involved in testing. The results obtained are used to assign a content. It is particularly important to quantify the impurities if a selective assay is employed. In such a case, it is best to characterise the candidate substance by additional analytical procedures that are scientifically justified, including, where possible, independent methods and methods based on different principles.

For a European Pharmacopoeia chemical reference substance established for assay purposes, the assigned content is usually calculated from the values obtained from the analyses performed for the determination of impurities (organic, inorganic, water and solvents) by applying the principle of mass balance; other suitable methods may also be used. When possible, the assigned content is confirmed by comparing with the result obtained by an independent method.

If a CRS is required for a non-chromatographic assay method (e.g. colorimetry or ultraviolet spectrophotometry), the relative reactivity or relative absorbance of the impurities present in a substance must be checked to ensure that they are not markedly different from those of the substance.

Unless otherwise stated, an assigned content is given for the substance or preparation as presented in the container ('as is'), and the contents are not to be dried before use. For assay standards prepared by lyophilisation, the content of the pure substance is indicated in milligrams or International Units per vial.

*4-2-3-2. Microbiological assay*. The potency is expressed in International Units or in European Pharmacopoeia Units if no international standard exists. The assigned potency together with the confidence limits are calculated from statistically valid results of an interlaboratory study, according to the usual statistical procedures (*5.3*).

*4-2-3-3. Assay of components of herbal drugs and herbal drug preparations*. Reference standards used in monographs on herbal drugs vary in the extent of testing depending on the type of reference standard.

An active component or marker constituent used as a CRS is usually characterised and evaluated for identity and purity; a value for content is assigned irrespective of the purity.

**4-2-4. Establishment report**. A report containing the results of the establishment study as well as information concerning the use of the CRS is prepared by EDQM, approved by the relevant group of experts and adopted by the European Pharmacopoeia Commission. The report for an assay standard indicates the content assigned to the substance with the rationale for this assignment. The estimated uncertainty of the assigned content is calculated, and where it is less than a predefined value, which is considered to be negligible in relation to the acceptance criteria for the assay, then the study is accepted. Otherwise, the study may be repeated, in whole or in part, or the limits defined for the pharmaceutical substance may be widened. The uncertainty of the assigned content is usually not given as part of the information provided with

the CRS, since the method performance and the uncertainty of the content assigned to the CRS are taken into account when setting the limit(s) in a monograph.

*4-3. EUROPEAN PHARMACOPOEIA HERBAL REFERENCE STANDARDS*

A HRS is used when the available quantity of pure constituent to be assayed is considered either insufficient or non-sustainable. HRSs may also be established for purposes other than assays, notably for use in tests for adulteration or for system suitability. A HRS is characterised by a variety of analytical techniques chosen to demonstrate its suitability for the intended use; relevant parts of the following test programme may be applied.

Test programme:

- macroscopy;
- microscopy;
- thin-layer chromatography;
- gas chromatography;
- liquid chromatography;
- quantitative determination of water;
- content of residual solvents;
- loss on drying;
- foreign matter;
- assay of constituents (e.g. constituents with known therapeutic activity, active markers, analytical markers) relevant to the intended use of the reference standard.

The extent of testing and the number of laboratories involved in the establishment of a HRS depend on its intended use.

For a European Pharmacopoeia herbal reference standard used for assay purposes, the assigned content is usually established by an interlaboratory study, using the assay method specified in the individual monograph in which the reference standard is intended to be used, comparing against a suitable pure sample of the constituent or constituents for which the content is to be assigned.

**Establishment report**. The establishment report for the HRS is prepared in the same manner as CRSs (see section 4-2-4).

*4-4. EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS AND CHEMICAL REFERENCE SUBSTANCES FOR BIOLOGICALS*

Most BRPs and some CRSs used for the testing of biological substances and preparations are established through the Biological Standardisation Programme, under the aegis of the Council of Europe and the European Commission. These reference standards are usually secondary standards calibrated against the corresponding WHO international standard. Where no international standard is available, they are primary standards with an assigned content/potency in European Pharmacopoeia Units or another suitable unit. They are established through interlaboratory studies where participating laboratories test the candidate material(s) and valid data are used to assign the official potency/content. Some of these studies are jointly organised with other organisations to establish a common material or batch of material as standard. In these cases, although the material constituting the European Pharmacopoeia reference standard may be identical to the international standard and its use may be validated through the same interlaboratory study, it is considered as a secondary standard for use as a working standard.

The study reports are endorsed by the study participants and approved by the relevant European Pharmacopoeia group of experts, where applicable, and by the Steering Committee of the Biological Standardisation Programme. The study results are then submitted to the European Pharmacopoeia Commission. Reference standards/materials established through the Biological Standardisation Programme are officially adopted by the European Pharmacopoeia Commission. The establishment reports are published in *Pharmeuropa Bio* & *Scientific Notes* (http://pharmeuropa.edqm.eu/PharmeuropaBioSN).

*4-5. SECONDARY STANDARDS*

A secondary standard should exhibit the same property or properties as the primary standard, relevant for the test(s) for which it is established. The extent of testing may not be as comprehensive as is required for the establishment of a primary standard. The secondary standard is established by comparison with the primary standard to which it is traceable. An official primary standard is used wherever possible for establishment of secondary standards.

## 5. MANUFACTURING, LABELLING, STORAGE AND DISTRIBUTION OF EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS

*5-1. MANUFACTURING*

All operations are carried out according to the relevant best practices to ensure the traceability and integrity of the reference standard. The manufacturing record includes information regarding filling, labelling and storage. Reference standards are dispensed into containers under appropriate filling and closure conditions to ensure the integrity of the reference standard. The containers employed may be multi-use or single-use, but the latter is preferred to minimise the risk of decomposition, contamination or water uptake.

*5-2. LABELLING*

The label bears the name of the reference standard, the name and address of the supplier, the batch number and unit quantity (quantity per vial/ampoule).

An accompanying leaflet, considered as part of the labelling, is normally provided.

If used as an assay standard, the following information is also given:

- the assigned percentage content;
- or, the content in milligrams or millilitres of the chemical entity in the container;
- or, the assigned potency (for biological assays or microbiological assays) in units either per milligram, per millilitre or per vial/ampoule.

For European Pharmacopoeia reference standards, no re-test or expiry date is given since the re-test programme (see section 6) monitors continued fitness for use. A batch validity statement (BVS) for each European Pharmacopoeia reference standard is available from the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS).

*5-3. STORAGE AND DISTRIBUTION*

Reference standards are to be stored and distributed in conditions suitable to ensure optimal stability.

Most European Pharmacopoeia reference standards are stored in temperature-controlled rooms at 5 ± 3 °C. However, a number of reference standards are stored at − 20 ± 5 °C and some (e.g. live virus preparations) are stored at − 80 ± 10 °C or at − 196 °C to − 170 °C under liquid nitrogen.

Appropriate packaging is used to minimise the risk of damage during transport, to keep the reference standard at the appropriate temperature when necessary and to comply with the current transport regulations.

Reference standards that are stored at 5 ± 3 °C are normally transported without cooling when short-term excursions from the long-term storage temperature are not deleterious to the reference standard. Some of them may nevertheless be sent at + 5 °C, packed with cold packs in cases where an increase in temperature is detrimental to their stability. Reference standards stored at − 20 °C are packed with cold packs or on dry ice (solid carbon dioxide) and dispatched by express courier or air freight. Reference standards stored at − 80 °C or stored under liquid nitrogen are packed on solid carbon dioxide.

Delivery conditions, dispatch and storage temperatures are available in the Reference Standards Catalogue, the Terms of Supply and the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS).

6. RE-TEST PROGRAMME OF EUROPEAN PHARMACOPOEIA STANDARDS

A system is established and implemented to ensure the continued fitness-for-use of the European Pharmacopoeia reference standards. Normally, a re-test programme is applied, taking account of the known physico-chemical properties and stability data for the reference standard. Reference standards are periodically tested for stability during storage. A monitoring programme is applied that is designed to detect at an early stage any sign of degradation using appropriate analytical techniques. The methods employed are typically chosen from amongst those performed during establishment of the reference standard so that baseline data is available.

The periodicity and extent of re-testing reference standards depends on a number of factors including:

- stability;
- container and closure system;
- storage conditions;
- hygroscopicity;
- physical form;
- intended use;
- presentation (single use/multiple use).

The re-test period may be lengthened with the support of sufficient data. The maximum permitted variation from the assigned content should be pre-defined, and if exceeded, the batch should be re-established or replaced.

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# 5.14. GENE TRANSFER MEDICINAL PRODUCTS FOR HUMAN USE

*This general chapter is published for information.*

*This general chapter contains a series of texts on gene transfer medicinal products for human use. The texts provide a framework of requirements applicable to the production and control of these products. For a specific medicinal product, application of these requirements and the need for any further texts is decided by the competent authority. The texts are designed to be applicable to approved products; the need for application of part or all of the texts to products used during the different phases of clinical trials is decided by the competent authority. The provisions of the chapter do not exclude the use of alternative production and control methods that are acceptable to the competent authority.*

*Further detailed recommendations on gene transfer medicinal products for human use are provided by the Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99) and the Guideline on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03) of the Committee for Medicinal Products for Human Use (including any subsequent revisions of these documents).*

DEFINITION

For the purposes of this general chapter, gene transfer medicinal product (GTMP) shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either *in vivo* or *ex vivo*, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid) to human/animal cells, and its subsequent expression *in vivo*. The gene transfer involves an expression system known as a vector, which can be of viral as well as non-viral origin. The vector can also be included in a human or animal cell.

*Recombinant vectors, such as viral vectors and plasmids.* Recombinant vectors are either injected directly into the patient's body (*in vivo* gene transfer) or transferred into host cells before administration of these genetically modified cells to the patient (*ex vivo* gene transfer). Viral vectors are derived from various viruses (for example, adenoviruses, poxviruses, retroviruses, lentiviruses, adeno-associated-viruses, herpesviruses). These vectors can be replicative, non-replicative or conditionally replicative. Plasmid vectors include nucleic acids in a simple formulation (for example, naked DNA) or complexed to various molecules (synthetic vectors such as lipids or polymers). Genetic material transferred by GTMPs consists of nucleotide sequences, which may notably encode gene products, antisense transcripts or ribozymes. Chemically synthesised oligonucleotides are not within the scope of this general chapter. After transfer, the genetic material may remain either cytoplasmic or episomal, or may be integrated into the host cell genome, depending on the integrating or non-integrating status of the vector.

*Genetically modified cells.* Genetically modified eukaryotic or bacterial cells are modified by vectors to express a product of interest.

PRODUCTION

**Substances used in production**. The raw materials used during the manufacturing process, including viral seed lot and cell bank establishment, where applicable, are qualified. Unless otherwise justified, all substances used are produced within a recognised quality management system using suitable production facilities. Suitable specifications are established to control notably their identity, potency (where applicable), purity and safety in terms of microbiological quality and bacterial endotoxin contamination. The quality of water used complies with the relevant corresponding monographs (*Purified water (0008)*, *Water for injections (0169)*). Where bovine serum is used, it complies with the monograph *Bovine serum (2262)*. The use of antibiotics is avoided wherever possible during production.

**Viral safety**. The requirements of chapter *5.1.7* apply.

**Transmissible spongiform encephalopathies** (*5.2.8*). A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risk.

## Recombinant vectors

PRODUCTION

*GENERAL PROVISIONS*

For viral vectors, production is based on a cell bank system and a virus seed-lot system, wherever possible.

For plasmid vectors, production is based on a bacterial cell bank system.

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages or subcultures from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

*SUBSTRATE FOR VECTOR PROPAGATION*

The substrates used comply with relevant requirements of the European Pharmacopoeia (*5.2.2*, *5.2.3*, and the section Bacterial cells used for the manufacture of plasmid vectors for human use).

*CHARACTERISATION OF THE VECTOR*

Historical records of vector construction are documented, including the origin of the vector and its subsequent manipulation, notably deleted or modified regions.

The vector is characterised using suitable and validated methods.

The genetic stability of the vector at or beyond the maximum passage level or the maximum number of cell doublings of the cell line used for production is assessed by suitable methods.

*PROPAGATION AND HARVEST*

All processing of the cell banks and subsequent cell cultures is done in an area where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The purity of the harvest is verified by suitable tests as defined in the corresponding specific sections.

*PURIFIED HARVEST*

The bulk of active substance is defined as a lot of purified recombinant vectors (viral vectors, or naked or complexed plasmids).

*FINAL LOT*

Unless otherwise justified and authorised, formulation and distribution of the final bulk is carried out under aseptic conditions using sterile containers (*3.2*).

The stability of the final lot is assessed using stability protocols including the duration, storage conditions, number of lots to be tested, test schedule and assays to be performed.

ASSAYS AND TESTS

The GTMPs comply with assays and tests described in the corresponding specific sections.

## Genetically modified cells

For cells to be modified with a recombinant vector, the data related to the recombinant vector are documented above, under Recombinant vectors.

PRODUCTION

*CELL SUBSTRATE*

For xenogeneic cell lines, including bacterial cells, a cell bank system comprising a master cell bank and working cell banks is established.

For autologous and allogeneic cells, a cell banking system comprising a master cell bank and working cell banks is established wherever possible.

*TRANSFECTION / TRANSDUCTION*

Cells are transfected or transduced using a recombinant vector (plasmid or viral vector) qualified as described under Recombinant vectors; the process is validated. They are handled under aseptic conditions in an area where no other cells or vectors are handled at the same time. All reagents used during cell manipulation steps are fully qualified. Antibiotics are avoided unless otherwise justified and authorised. Transfection or transduction is carried out under aseptic conditions.

*FINAL LOT*

In the case of frozen storage, the viability of genetically modified cells is assessed before freezing and after thawing.

If the cells are not used within a short period, stability is determined by verifying cell viability and expression of the genetic insert.

In the case of genetically modified cells encapsulated before implantation in man, any encapsulating component used is considered as part of the final product, and is therefore quality-controlled and fully characterised (for example, physical integrity, selective permeability, sterility).

ASSAYS AND TESTS

Controls of xenogeneic, allogeneic or autologous cells include the following:

- identity, counting and viability of cells;
- overall integrity, functionality, copies per cell, transfer and expression efficiency of the genetic insert;
- microbiological controls (*2.6.1* or *2.6.27*), endotoxin content, mycoplasma contamination (*2.6.7*), adventitious virus contamination and, where applicable, replicative vector generation.

The competent authority may approve a reduced testing programme where necessary because of limited availability of cells. Where necessary because of time constraints, the product may be released for use before the completion of certain tests.

## PLASMID VECTORS FOR HUMAN USE

DEFINITION

Plasmid vectors for human use are double-stranded circular forms of bacterial DNA that carry a gene of interest or a nucleotide sequence encoding antisense sequences or ribozymes and its expression cassette; they are amplified in bacteria extrachromosomally. They are used to transfer genetic material into human somatic cells *in vivo* or to genetically modify autologous, allogeneic, xenogeneic or bacterial cells before administration to humans. Plasmid vectors may be presented as naked DNA or may be formulated with synthetic delivery systems such as lipids (lipoplexes), polymers (polyplexes) and/or peptide ligands that facilitate transfer across the cell membrane and delivery to the cell, or that target delivery via specific receptors.

Plasmids formulated with synthetic delivery systems are not within the scope of this section.

PRODUCTION

*PLASMID CONSTRUCTION*

A typical plasmid vector is composed of:

- the plasmid vector backbone that contains multiple restriction endonuclease recognition sites for insertion of the genetic insert and the bacterial elements necessary for plasmid production, such as selectable genetic markers for the identification of cells that carry the recombinant vector;
- the required regulatory genetic elements to facilitate expression of the genetic insert;
- the genetic insert;
- a polyadenylation signal.

A complete description of the plasmid DNA, including its nucleotide sequence, is established with the identification, source, means of isolation and nucleotide sequence of the genetic insert. The source and function of component parts of the plasmid, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

*GENERAL PROVISIONS*

**Cell banks**. Production of plasmid vectors is based on a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs), which comply with the section Bacterial cells used for the manufacture of plasmid vectors for human use. The raw materials used during the manufacturing process, including cell bank establishment, are qualified.

**Selection techniques**. Unless otherwise justified and authorised, antibiotic-resistance genes used as selectable genetic markers, particularly for clinically useful antibiotics, are not included in the vector construct. Other selection techniques for the recombinant plasmid are preferred.

**Reference standards**. A suitable batch of the formulated plasmid, preferably one that has been clinically evaluated, is fully characterised and retained for use as a reference standard as necessary in routine control tests.

*PROPAGATION AND HARVEST*

Plasmid DNA is transferred to host strain bacterial cells and a single clone of transformed bacteria is expanded to create the MCB. The WCB is then derived from the MCB. The EOPCs are obtained from the WCB by fermentation in production conditions.

Plasmid DNA is isolated from harvested cells using an extraction step and is purified to obtain the bulk product.

Unless otherwise justified and authorised, caesium chloride-ethidium bromide density gradients are not used for production.

*PURIFIED PLASMID*

The production process is optimised to remove impurities consistently while retaining product activity. The requirement to test for a particular impurity depends on the following:

- the demonstrated capability of the manufacture and purification processes to remove or inactivate the impurity through process validation, using specific quantification methods;
- the potential toxicity associated with the impurity;
- the potential decrease of the efficacy of the genetic insert product associated with the impurity.

If selective resistance to specific antibiotics has been used for selection, data from validation studies of purification procedures are required to demonstrate the clearance capability for residual antibiotics.

Relevant in-process controls are performed to ensure that the process is continuously under control, for example, amount and form of plasmid after the extraction steps and amount of endotoxins after the extraction steps.

Only a batch of purified plasmid that complies with the following requirements may be used.

**Identity and integrity of the purified plasmid**. Identity and integrity of the purified plasmid are established by suitable methods such as sequencing or nucleic acid amplification techniques (NAT) (*2.6.21*); restriction enzyme analysis may be used where it is sufficient to detect potential critical modifications in the plasmid and confirm the plasmid identity.

**Plasmid DNA**. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

**DNA forms**. Plasmid DNA is characterised in terms of the proportions of supercoiled, multimeric, relaxed monomer and linear forms, using suitable analytical methods, examples of which are given below. For quantification of supercoiled forms, anion-exchange high performance liquid chromatography (HPLC) or capillary electrophoresis may be used. Capillary electrophoresis is also suitable for the quantification of other forms.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Residual RNA**. The content of residual RNA is determined, unless the process has been validated to demonstrate suitable clearance. Reverse-phase HPLC (RP-HPLC) may be used, or quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (*2.6.21*) when a lower limit of detection is required.

**Residual host-cell protein**. The concentration of residual host-cell protein is determined using standard protein assays (*2.5.33*), SDS-PAGE followed by silver staining, or specific immuno-assays such as western blot or ELISA, unless the process has been validated to demonstrate suitable clearance.

**Microbiological control**. Depending on the preparation concerned, it complies with the test for sterility (*2.6.1*) or the bioburden is determined (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

*FINAL BULK*

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

The plasmid vector is identified by restriction enzyme analysis or by sequencing. The test for biological activity also serves to identify the product.

TESTS

Tests carried out on the final lot include the following.

**Appearance**.

**pH** (*2.2.3*): within the limits approved for the particular preparation.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Residual moisture** (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

**DNA forms**. The percentage of the specific monomeric supercoiled form is determined as described for the purified plasmid.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

**Plasmid DNA**: not less than the quantity stated on the label, determined, for example, by 1 of the following methods.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

**Biological activity**. Wherever possible, biological activity is assessed through *in vitro* or *in vivo* bioassays. A well-defined, representative reference standard is required as a positive control for the assay. Bioassays employed to assay plasmid vectors generally involve transfection of a relevant cell line *in vitro*, followed by some functional measure of the expressed genetic insert. Such functional assays provide information about the activity of the product encoded by the genetic insert instead of the expression level of the genetic insert itself.

It may be necessary to supplement the bioassay with western-blot and ELISA assays to assess the integrity and quantity of the expressed product.

LABELLING

The label states:

- the plasmid DNA concentration;
- the recommended human dose;
- for freeze-dried preparations:
  - the name and volume of the liquid to be added;
  - the time within which the product is to be used after reconstitution.

## BACTERIAL CELLS USED FOR THE MANUFACTURE OF PLASMID VECTORS FOR HUMAN USE

Production of plasmid vectors for human use is based on the use of a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs). A bacterial cell bank for the manufacture of plasmid vectors is a collection of vials containing bacterial cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single clone of a transformed host strain. The MCB has a known, documented history; it is preferably derived from a qualified repository source. The

WCB is produced by expanding one or more vials of the MCB. Methods and reagents used to produce the bank and storage conditions are documented.

MCBs and WCBs are qualified by testing an aliquot of the banked material or testing a subculture of the cell bank.

The following table indicates the tests required at each stage of production.

| Assay | Host strain | MCB | WCB | EOPCs* |
|---|---|---|---|---|
| **Identity and purity** | | | | |
| Viability | + | + | + | + |
| Bacterial strain characterisation | + | + | – | + |
| Genotyping / phenotyping | + | + | – | + |
| Presence of the plasmid | | | | |
| – Sequence of the DNA plasmid | – | + | – | + |
| – Copy number | – | + | + | + |
| – Restriction map | – | + | + | + |
| – Percentage of cells retaining the plasmid | – | + | + | + |
| **Adventitious agents** | | | | |
| Purity by plating | + | + | + | + |
| Presence of bacteriophage | + | + | – | + |

* EOPCs are cells with a passage number at least equivalent to that used for production. The analysis has to be done once to validate each new WCB, except for purity, which has to be tested for each fermentation.

IDENTITY AND PURITY TESTING

**Viability**. The number of viable cells is determined by plating a diluted aliquot of bacterial cells on an appropriate medium and counting individual colonies.

**Biochemical and physiological bacterial strain characterisation**. Depending on the bacterial strain used for production, relevant biochemical and physiological characterisation is performed to confirm cell identity at the species level.

**Genotyping / phenotyping**. The genotype of bacterial cells is verified by determination of the suitable specific phenotypic markers or by appropriate genetic analysis.

**Presence of the plasmid**

*Sequencing*. The whole nucleotide sequence of the plasmid is verified.

*Copy number*. The plasmid DNA is isolated and purified from a known number of bacteria and the copy number determined by a suitable method such as quantitative PCR (*2.6.21*).

*Restriction map*. Restriction endonuclease digestion is performed with sufficient resolution to verify that the structure of the plasmid is unaltered in bacterial cells.

*Percentage of cells retaining the plasmid*. Bacterial elements present in the plasmid, such as selectable genetic markers, are used to define the percentage of bacteria retaining the plasmid.

ADVENTITIOUS AGENTS AND ENDOGENOUS VIRUSES

**Purity by plating**. Bacterial cells are streaked out onto suitable media and incubated in the required conditions in order to detect potential bacterial contaminants. In order to test for inhibition of the growth of contaminating organisms, additional tests in the presence of a definite amount of relevant positive control bacteria are carried out. A suitable number of colonies is examined; no contamination is detected.

**Presence of bacteriophage**. Bacterial cells are plated and incubated in a medium allowing proliferation of bacteriophages, to test for bacteriophage presence. The test is validated by the use of a reference bacteriophage strain and permissive cells as positive controls. A suitable number of colonies is examined; no contamination is detected.

# ADENOVIRUS VECTORS FOR HUMAN USE

DEFINITION

Adenovirus vectors for human use are freeze-dried or liquid preparations of recombinant adenoviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

*VECTOR CONSTRUCTION*

There are different approaches for the design and construction of an adenovirus vector. The purpose of clinical use determines which approach is optimal. A method is chosen that minimises the risk of generating replication-competent adenovirus vectors or that effectively eliminates helper viruses that might be used during production.

*VECTOR PRODUCTION*

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

*SUBSTRATE FOR VECTOR PROPAGATION*

The vector is propagated in continuous cell lines (*5.2.3*) based on a cell bank system. The occurrence of replication-competent adenoviruses may be significant when large regions of homology exist between the viral genome and the genome of the complementation cells. This occurrence may be minimised by minimising the homology between both genomes. The use of cells with no sequence homology with the vector is recommended for production.

*VECTOR SEED LOT*

Production of the vector is based on a seed-lot system.

The strain of adenovirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

**Identification**. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Genetic and phenotypic characterisation**. The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.

– A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.

**Vector concentration**. The titre of infectious vector or the concentration of vector particles in the master seed lot and each working seed lot are determined.

**Extraneous agents** (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents.

**Replication-competent adenoviruses**. Replication-competent adenoviruses are generated by homologous recombination between the recombinant viral DNA and the adenovirus sequences integrated into the genome of the complementation cells.

Detection of replication-competent adenoviruses is performed by a suitable method approved by the competent authority. It is generally performed by an infectivity assay on sensitive detector cell lines, which are not able to complement for the genes deleted from the vector. Other indicators of viral replication may be used as appropriate.

When replication-competent adenoviruses are not supposed to be present in the test sample, considering vector construction and cell lines used, at least 2, but preferably 3 or 4 successive passages are performed on the detector cell line, where applicable. Detection of a cytopathic effect at the end of the passages reveals the presence of replication-competent adenoviruses in the preparation. Positive controls are included in each assay to monitor its sensitivity.

When replication-competent adenoviruses are expected to be present in the test sample, plaque-assays or limit dilution assays on a detector cell line may be performed.

*PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Vector concentration**. The titre of infectious vector and the concentration of vector particles in single harvests are determined.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents.

**Control cells**. Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

*PURIFIED HARVEST*

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate the satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Genomic integrity**. Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

**Vector concentration**. The titre of infectious vector and the concentration of vector particles in purified harvests are determined.

**Residual host-cell protein**. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative polymerase chain reaction (PCR) is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Residual reagents**. Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

**Residual antibiotics**. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

*FINAL BULK*

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent adenoviruses have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

**Osmolality** (*2.2.35*): within the limits approved for the particular preparation.

**pH** (*2.2.3*): within the limits approved for the particular preparation.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Residual moisture** (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

**Bovine serum albumin**: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

**Replication-competent adenovirus concentration**: within the limits approved for the particular preparation.

**Vector aggregates**. Vector aggregates are determined by suitable methods (for example, light scattering).

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

**Thermal stability**. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

**Vector particle concentration**. Physical titration is performed by a suitable technique (for example, liquid chromatography, absorbance measurement or NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

The vector particle concentration of the preparation to be examined is not less than the concentration stated on the label.

**Infectious vector titre**. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

**Ratio of vector particle concentration to infectious vector titre**: within the limits approved for the particular preparation.

**Expression of the genetic insert product**. The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

**Biological activity**. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the content of active substance;
- the recommended human dose, expressed in vector particle concentration;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

## POXVIRUS VECTORS FOR HUMAN USE

DEFINITION

Poxvirus vectors for human use are freeze-dried or liquid preparations of recombinant poxviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

*VECTOR CONSTRUCTION*

The general design of a poxvirus vector is currently as follows: the genetic insert is inserted downstream of a poxvirus promoter. This expression cassette is inserted into the poxvirus genome in such a manner that it interrupts a viral gene non-essential for replication or is positioned between 2 virus open reading frames.

In most strategies used so far for the construction of the vector, the expression cassette is first inserted within the target site of a virus DNA fragment cloned into a bacterial plasmid. The plasmid is then introduced into host cells, cultured *in vitro*, which are simultaneously infected with the parental poxvirus. DNA recombination occurs within the infected cells, between homologous sequences in the viral genome and viral sequences in the plasmid so as to transfer the genetic insert into the targeted site of the viral genome. The correct targeting of the inserted DNA is checked by restriction-enzyme mapping, NAT (*2.6.21*) and sequencing. Successive plaque-cloning steps are performed to purify the recombinant poxvirus from the mixture of parental and recombinant poxviruses. A variety of methods (for example, foreign marker genes, DNA hybridisation, immunological detection, phenotypic changes in the virus) are employed to facilitate recognition and/or selection of the recombinant poxvirus from the background of parental virus. Where foreign marker genes have been transiently employed, they are removed by appropriate methods from the final recombinant poxvirus.

An alternative strategy for creating poxvirus vectors begins with the *in vitro* construction of a full-length virus genome harbouring the expression cassette within a chosen target site. This recombinant genome is then introduced into host cells simultaneously infected with a helper poxvirus that is unable to multiply. The helper virus may be a poxvirus of the same species whose ability to multiply has been inactivated, or another poxvirus species that does not multiply in the host cells.

The construction of non-replicative poxvirus vectors relies on specific host cell lines or primary cells that are naturally permissive, or on host cell lines that have been modified to express an essential poxvirus gene. These cells fulfill the general requirements for the production of medicinal products (*5.2.3*) and do not allow the generation of replicative vectors.

*VECTOR PRODUCTION*

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

*SUBSTRATE FOR VECTOR PROPAGATION*

The vector is propagated under aseptic conditions in human diploid cells (*5.2.3*), in continuous cell lines (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*). When the vector is propagated in a continuous cell line or in human diploid cells, a cell-bank system is established.

*VECTOR SEED LOT*

Production of the vector is based on a seed-lot system.

The strain of poxvirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

**Identification**. The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

**Genetic and phenotypic characterisation**. The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.

- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
- A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.
- The host range is verified by determining the replication properties of the vector and comparing them with that of the parental virus, at a passage level comparable to a production batch.

**Infectious vector titre**. The titre of infectious vector in the master seed lot and each working seed lot is determined.

**Extraneous agents** (*2.6.16*). The master seed lot and each working seed lot comply with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

*PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell culture is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

**Infectious vector titre**. The titre of infectious vector in single harvests is determined.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

**Control cells**. If human diploid cells or a continuous cell line are used for production, the control cells comply with a test for identification (*5.2.3*). They comply with the tests for extraneous agents (*2.6.16*).

*PURIFIED HARVEST*

Processing is carried out under aseptic conditions. Several single harvests may be pooled before the purification process. The harvest is first clarified to remove cells and then, where applicable, purified by validated methods.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

**Genomic integrity**. Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

**Infectious vector titre**. The titre of infectious vector in purified harvests is determined.

**Ratio of infectious vector titre to total protein concentration**. The total protein concentration is determined by a suitable method (*2.5.33*). The ratio between infectious vector titre and total protein concentration is calculated.

**Residual host-cell protein**. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Residual reagents**. Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

**Residual antibiotics**. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

*FINAL BULK*

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for bovine serum albumin (when bovine serum is used to manufacture the vector) has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

TESTS

**Osmolality** (*2.2.35*): within the limits approved for the particular preparation.

**pH** (*2.2.3*): within the limits approved for the particular preparation.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Residual moisture** (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

**Bovine serum albumin**: not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

**Thermal stability**. Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

**Infectious vector titre**. Titrate at least 3 vials of the preparation to be examined by inoculation into cell cultures. Titrate a vial of an appropriate vector reference standard to validate each assay.

The vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:

- the confidence interval ($P$ = 0.95) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

**Expression of the genetic insert product**. The expression of the genetic insert product(s) is determined, wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

**Biological activity**. Unless otherwise justifed and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

# RETROVIRIDAE-DERIVED VECTORS FOR HUMAN USE

DEFINITION

*Retroviridae*-derived vectors for human use are liquid or freeze-dried preparations of recombinant retroviruses, lentiviruses or spumaviruses, genetically modified to render them replication-incompetent, which are used to transfer genetic material to human somatic cells *in vivo* or *ex vivo*. This section applies to non-replicative vectors.

PRODUCTION

*VECTOR CONSTRUCTION*

A typical vector is composed of:

- the minimal genome from parental viruses containing the structural genetic elements shown to be indispensable for vector production;
- the required regulatory genetic elements for expression of the genetic insert (for example, long terminal repeats (LTRs));
- the genetic insert.

The vector construction is designed to prevent the generation of replication-competent viruses.

*VECTOR PRODUCTION*

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the packaging or producer cells shall have undergone no more cell doublings from the master cell bank (MCB) than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy. The genetic and phenotypic stability of the packaging or producer cells at or beyond the maximum number of cell doublings used for production is assessed by suitable methods.

Vectors are produced in continuous cell lines (*5.2.3*) using a cell-bank system. Production may involve either stably or transiently transfected cells.

**DEFINITIONS**

*Packaging cells*: a source cell line stably transfected with plasmids containing the viral genes necessary for production of empty vector particles: *gag*, *pol*, *env*.

*Producer cells*: contain the viral genes and expression cassette necessary for vector production.

- In stable production systems, the producer cells are generated by stable transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.
- In transient production systems, the producer cells are generated at the time of manufacture by simultaneous transfection of the source cell line with both the viral genes and the transgene expression plasmid, or by transient transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.

**PRODUCTION INTERMEDIATES**

*Packaging cells*

**Copy number**. The genomic DNA is isolated and purified from a known number of cells and the *gag*, *pol* and *env* genes copy number is determined by a suitable method such as quantitative PCR (*2.6.21*).

**Sequence integrity of the viral genes**. Complete nucleotide sequencing of the inserted viral genes and their regulatory elements is performed.

**Genetic stability**. Genetic stability of the packaging cells is verified at or beyond the maximum number of cell doublings used for production.

*Plasmids*

Production of the vector requires the use of plasmid intermediates. For each plasmid DNA used during production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The MCB complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the vector.

**Identification**. Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

**Genomic integrity**. Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the viral genes, the genetic insert and their respective regulation elements.

**Plasmid DNA**. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*Producer cells used in a stable production system*

**Copy number**. The copy number of the integrated viral genes and expression cassette is determined by a suitable method.

**Genetic stability**. Genetic stability of the producer cells at or beyond the maximum number of cell doublings used for production is confirmed.

**Sequence integrity of the viral genes and expression cassette**. Complete nucleotide sequencing of the inserted viral genes, the expression cassette and their respective regulation elements (for example, LTRs, promoters, psi sequence, polyadenylation signal) is performed.

**Replication-competent viruses**. The detection of replication-competent viruses is performed by suitable methods. Detection may be based on a co-cultivation for several cell doublings of the producer cells with a permissive cell line, followed by detection (either by observation of a cytopathic or haemadsorbing effect on indicator cells like PG4 S+L-, by detection using indicator cell lines by NAT (*2.6.21*) or by marker-rescue assay). Positive controls are included in each assay to monitor its sensitivity. No replication competent viruses are found.

*PRODUCTION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media must be qualified. It is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used.

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Vector concentration**. The titre of infectious vector and/or the concentration of vector particles in single harvests is determined.

**Extraneous agents**. Each single harvest complies with the tests for extraneous agents (*2.6.16*).

**Control cells**. Where a transient production process is used, control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

*PURIFIED HARVEST*

Several single harvests may be pooled before purification. Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Genomic integrity**. Genomic integrity of the vector is verified by a suitable method.

**Vector concentration**. The infectious particle titre is determined by a suitable method, for example infection of permissive cells followed by quantitative NAT (for example, quantitative PCR), Southern blot or protein expression. For lentivirus vectors, the physical titre is measured, for example by ELISA (p24).

**Replication-competent viruses**. Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), by detection of a viral antigen (for example, p24 by ELISA) or by marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

**Residual host-cell protein**. The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Residual reagents**. Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

**Residual antibiotics**. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

**Residual plasmids**. Where a transient production process is used, the concentration of residual contaminating plasmids must be quantified.

*FINAL BULK*

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent viruses have been carried out with satisfactory results on the purified harvest, they may be omitted on the final lot.

IDENTIFICATION

*Retroviridae*-derived vectors are identified by NAT (*2.6.21*), immunochemical methods (*2.7.1*) or restriction enzyme analysis.

TESTS

**Osmolality** (*2.2.35*): within the limits approved for the particular preparation.

**pH** (*2.2.3*): within the limits approved for the particular preparation.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Residual moisture** (*2.5.12*): within the limits approved for the particular freeze-dried preparation.

**Bovine serum albumin**: where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

**Replication-competent viruses**. Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), detection of a viral antigen (for example, p24 by ELISA) or marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

**Vector-particle concentration**. Physical titration is performed by a suitable technique (for example, immunochemical methods (*2.7.1*) or NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

**Infectious vector titre**. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

**Ratio of vector-particle concentration to infectious vector titre**: within the limits approved for the particular product, where applicable.

**Expression of the genetic insert product**. The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

**Biological activity**. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

# ADENO-ASSOCIATED-VIRUS VECTORS FOR HUMAN USE

DEFINITION

Adeno-associated-virus (AAV) vectors for human use are freeze-dried or liquid preparations of recombinant AAV (rAAV), genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

*VECTOR CONSTRUCTION*

rAAV vectors are developed by replacement of the *rep* and *cap* genes with the genetic insert of interest. The inverted terminal repeat (ITR) sequences are retained in the rAAV vector since these are the only AAV sequences absolutely required in *cis* to function as the origin of replication. The *rep* and *cap* genes are required in *trans* and function for replication and packaging respectively. In summary, the rAAV vector contains the ITRs and the genetic insert.

Wild-type AAV normally replicate only in the presence of helper functions, provided by a coinfecting adenovirus or herpes virus. Therefore, there are different approaches to the manufacture of an AAV vector. The manufacturing strategy chosen is designed to minimise the risk for the generation of replication-competent AAV vectors and effectively eliminate helper viruses that might be used during production.

*VECTOR PRODUCTION*

The production method shall have been shown to yield a vector of consistent quality and stability.

To produce AAV vectors, several strategies are currently used, for example:

- transient co-transfection of a cell line with plasmids containing the ITRs and the genetic insert, *rep* and *cap* genes and helper functions;
- infection with a replication-deficient helper virus of a producer cell line harbouring *rep* and *cap* genes, the ITRs and the genetic insert;
- infection of a permissive cell line with 1 or several production viruses encoding *rep* and/or *cap* and/or the genetic insert and the ITRs, and that may or may not provide helper functions (helper viruses and baculoviruses, respectively).

Depending on the strategy used to produce AAV vectors, different production intermediates are required (plasmids, viruses used for production, packaging cells).

The occurrence of replication-competent AAV may be significant when regions of homology exist between the genomes of the production intermediates and the rAAV vector. This occurrence may be minimised by reducing the homology between these genomes to a minimum. The use of production intermediates with no sequence homology is recommended for production.

The genetic and phenotypic stability of the vector at or beyond the maximum number of passage levels used for production is assessed by suitable methods.

**PRODUCTION INTERMEDIATES**

Viruses used for production and the rAAV vector are produced in continuous cell lines (*5.2.3*) using a seed lot and a cell-bank system.

*Packaging and producer cells*

**Copy number**. The genomic DNA is isolated and purified from a known number of cells and the copy number of the inserted viral genes and of the expression cassette is determined by a suitable method such as quantitative PCR (*2.6.21*).

**Sequence integrity of the viral genes and expression cassette**. Complete nucleotide sequencing of the inserted viral genes, of their regulatory elements and where applicable, of the expression cassette is performed.

**Genetic stability**. Genetic stability of the cells is verified at or beyond the maximum number of cell doublings used for production.

**Wild-type AAV**. The absence of wild-type AAV is verified using NAT (*2.6.21*).

*Plasmids*

Production of the AAV vector by transient co-transfection requires the use of plasmid intermediates. For each plasmid DNA used during production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The master cell bank complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the AAV vector.

**Identification**. Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

**Genomic integrity**. Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the region corresponding to *rep*, *cap* and the expression cassette.

**Plasmid DNA**. The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

**Residual host-cell DNA**. The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*Viruses used for production*

Their production is based on a seed lot and a cell-bank system or, where applicable (for example, for baculoviruses), on a transient system. The strain of virus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. The nucleotide sequence of the viruses is documented.

Only a virus used for production that complies with the following requirements may be used.

**Identification**. Viruses used for production are identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Genomic integrity**. Genomic integrity of the virus used for production is verified by suitable methods such as restriction enzyme analysis. Where viruses are modified to express *rep* or *cap* genes or the expression cassette, genomic integrity is assessed by sequencing or by quantitative PCR of these regions.

**Genetic stability**. Where a stable production system is used, genetic stability is verified at or beyond the maximum number of cell doublings used for production.

**Virus titration**. The infectious titre is determined by a suitable assay.

**Wild-type AAV**. Where applicable, the absence of wild-type AAV in helper virus seed lots is verified using NAT (*2.6.21*).

**Replication-competent viruses**. Detection of replication-competent viruses is performed by suitable methods. No replication-competent viruses are found.

**Extraneous agents** (*2.6.16*). It complies with the test for extraneous agents. In addition, detection of potential contamination with specific insect viruses is required where applicable.

*PRODUCTION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable classified space with appropriate containment level where no other cells, viruses or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production, and at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Vector concentration**. The titre of infectious vector and the concentration of vector particles in single harvests are determined.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents.

**Control cells**. Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*) and specific insect viruses, where insect cell lines are used for production.

*PURIFIED HARVEST*

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

**Identification**. The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

**Genetic characterisation**. The following tests are carried out.

- The entire genome of the vector is sequenced for a suitable number of production runs at the level of the purified harvest or final bulk and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Genomic integrity is checked on the vector DNA. PCR analysis may be used.

**Vector concentration**. The titre of infectious vector and the concentration of vector particles are determined.

**Residual viruses used for production**. Residual viruses used for production are assessed by plaque assays or tissue culture infective dose 50 ($TCID_{50}$) on permissive cell lines or by quantitative PCR, according to the production system used.

**Residual proteins**. The concentrations of residual host-cell and/or viral proteins are determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

**Residual DNA**. The content of residual producer-cell DNA and of residual DNA from intermediates such as plasmids and production viruses where applicable, is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

**Residual reagents**. Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

**Residual antibiotics**. Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general

method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

*FINAL BULK*

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector), replication-competent AAV and residual viruses used for production have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

**Osmolality** (*2.2.35*): within the limits approved for the particular preparation.

**pH** (*2.2.3*): within the limits approved for the particular preparation.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Residual moisture** (*2.5.12*): within the limits approved for the particular freeze-dried product.

**Bovine serum albumin**: where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

**Replication-competent AAV concentration**: within the limits approved by the competent authority.

Detection of replication-competent AAV is performed by a replication assay on a permissive cell line previously infected with a helper virus and analysis of the replicative forms by Southern blot on low-molecular-weight DNA, or by detection of the *rep* gene by quantitative PCR.

**Vector aggregates**. Vector aggregates are determined by suitable methods (for example, light scattering).

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the particular preparation.

ASSAY

**Vector-particle concentration**. Vector-particle concentration is determined using a suitable method such as quantitative PCR by comparison with a standard curve obtained using the recombinant AAV plasmid or an AAV reference standard. This concentration is within the limits approved for the particular product.

**Infectious vector titre**. Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum amount stated on the label.

The assay is invalid if:

- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

**Ratio of vector-particle concentration to infectious vector titre**: within the limits approved for the particular product.

**Expression of the genetic insert product**. The expression of the genetic insert product is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

**Biological activity**. Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

- the content of active substance;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

# 5.15. Functionality-related characteristics of excipients

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# 5.15. FUNCTIONALITY-RELATED CHARACTERISTICS OF EXCIPIENTS

*This chapter and the Functionality-related characteristics (FRCs) sections in individual monographs are not mandatory and are published for information and guidance.*

## PREAMBLE

Excipients that have previously been evaluated for safety are used in the formulation of pharmaceutical preparations to bring functionality to the formulation. The intended function of an excipient is to guarantee the required physico-chemical and biopharmaceutical properties of the pharmaceutical preparation.

The functionality of an excipient is determined by its physical and chemical attributes and, in some cases, also by its content of by-products or of additives used to improve the intended functionality. In addition, the functionality may depend on complex interactions between the constituents of the formulation and stresses related to the process. Excipient functionality can therefore be evaluated only in the context of a particular formulation and manufacturing process, frequently by the use of a number of analytical methods.

Pharmaceutical development should be science-based and ensure a quality medicinal product. It is defined in the current ICH guideline Q8 Pharmaceutical Development. Its purpose is to provide justification for the dosage form selected, the qualitative and quantitative composition of the medicinal product, the manufacturing process, the critical quality attributes (CQAs) of the constituents and medicinal product, and the critical process parameters (CPPs). The 'quality by design' (QbD) concept described in the ICH Q8 guideline requires a comprehensive understanding of the chemical and physical nature of the individual active substance(s) and excipients, and of the way their attributes interact in the formulation and with the manufacturing process.

Pharmaceutical development includes identification of which excipient quality attributes are critical.

A CQA is a physical, chemical, biological or microbiological property or characteristic that must be within appropriate limits to ensure the desired medicinal product quality.

Monographs of the European Pharmacopoeia on excipients are designed to ensure acceptable quality for users. Information on the characters of the excipient, and requirements concerning identity, chemical and microbiological purity and physical characteristics associated with the chemical structure, such as optical rotation, are given in individual monographs and in the general monograph *Substances for pharmaceutical use (2034)*.

Certain excipient attributes, such as the particle size of an excipient intended for a solid dosage form or the molecular mass of a polymeric material used as a viscosity-increasing agent, may however relate to functionality in a more general sense. These attributes are called functionality-related characteristics (FRCs). When the pharmaceutical development work of a specific product has identified the existence of one or more critical FRCs for an excipient, they are considered to be CQAs for that excipient and should be controlled accordingly.

Knowledge of FRCs may facilitate the application of process analytical technology (PAT).

FRCs are included in excipient monographs to aid excipient manufacturers and users in establishing specifications based on standard analytical methods. They provide a common language to support the supply of excipients with specified properties. FRCs may be stated (in the certificate of analysis, for example) by the excipient manufacturer with a reference to the Pharmacopoeia monograph, thus indicating the method used to test a particular characteristic. The FRCs section in individual monographs contains FRCs that are known to have an impact on the functionality of the excipient for the stated uses. The uses and the FRCs listed are not exhaustive due to the multiple uses of many excipients and the development of new uses.

## REGULATORY GUIDANCE

According to current regulatory guidelines, for example the ICH Q8 guideline, the marketing authorisation application should discuss the excipients chosen, their concentration, and the attributes that can influence the medicinal product quality or manufacturability relative to the respective function of each excipient. The ability of excipients to provide their intended functionality and to perform throughout the intended medicinal product shelf life should also be demonstrated. The information on excipient performance can be used as appropriate to justify the choice and quality attributes of the excipient.

During pharmaceutical development, the attributes that are critical to the manufacturing process and quality of the medicinal product are identified. Having identified the critical attributes of the excipients, preferably by a risk-based approach, pharmaceutical development may establish the acceptable range of the critical material attributes including both the physical and the chemical property variation. The FRCs concerned may not be properties controlled by the excipient manufacturer and could therefore be variable. The design of a robust manufacturing process for the medicinal product that limits the effect of the normal excipient variability is preferable.

Evaluation of the physical and chemical grades and, where appropriate, the setting of a specification for the critical attributes and thus the critical FRCs, is part of the pharmaceutical development irrespective of the non-mandatory character of FRCs. This evaluation should be seen in light of regulatory guidance on pharmaceutical development and the appropriate acceptance criteria should be based on an understanding of the extent to which the variation of the critical FRCs can have an impact on the quality of the medicinal drug product. An acceptable range of FRCs may be established within the limits of the design space. The design space may be described as a space in which the quality attributes of the constituents and the process parameters may vary without modification of the quality of the medicinal product.

## PHYSICAL GRADES

Excipients that are particulate solids can be available in a variety of physical grades, for example with regard to particle-size distribution, which is usually controlled by the excipient supplier. However, FRCs for these excipients may concern a wide range of properties, resulting from solid-state properties and properties of the particulate solid, which may not be controlled by the excipient supplier.

Properties of particulate solids include for example particle-size distribution, specific surface area, bulk density, flowability, wettability and water sorption. Depending on the size range, the particle-size distribution can be determined by sieve analysis (see general chapter *2.9.38. Particle-size distribution estimation by analytical sieving*) or instrumental methods, for example *2.9.31. Particle size analysis by laser light diffraction*. The method described in general chapter *2.9.26. Specific surface area by gas adsorption* is based on the Brunauer-Emmett-Teller (BET) technique. Methods to characterise flowability and bulk density of powders are described in general chapters *2.9.36. Powder flow* and *2.9.34. Bulk density and tapped density*. Solid-state properties may have an impact on the wettability (see general chapter *2.9.45. Wettability of porous*

*solids including powders*) and water-solid interactions (see general chapter *2.9.39. Water-solid interactions: determination of sorption-desorption isotherms and of water activity)* of particulate solids.

Examples of solid-state properties to be considered in the development of solid dosage forms include polymorphism, pseudopolymorphism, crystallinity and density. Techniques to study them are given in general chapters *5.9. Polymorphism*, *5.16. Crystallinity* and *2.2.42. Density of solids*.

## CHEMICAL GRADES

Excipients that are available in different chemical grades are of natural, semi-synthetic or synthetic origin. Individual monographs usually control the chemical composition of excipients that are composed of a mixture of related compounds, for example the composition of fatty acids in vegetable oils or surfactants. There are, however, individual monographs in the Pharmacopoeia each describing a class of polymeric materials that may vary in their composition with regard to the structure of homopolymers, block polymers and copolymers, the degree of polymerisation, the molecular mass and mass distribution, the degree of substitution and in some cases even different substituents on the polymer backbone. This variation may, however, have a profound effect on the functionality of the excipient and should be subject to investigations during the pharmaceutical development, preferably to establish the acceptable range of each attribute being critical to the manufacturing process and quality of the medicinal product.

## FUNCTIONALITY-RELATED CHARACTERISTICS SECTION IN MONOGRAPHS

Monographs on excipients may have a section entitled 'Functionality-related characteristics'. This section is included for information for the user and is not a mandatory part of the monograph. The section gives a statement of characteristics that are known to be relevant for certain uses of the excipient. The use for which the characteristic is relevant is stated. For other uses, the characteristic may be irrelevant. For this reason, the section should not be seen simply as a supplement to the monograph. It is the responsibility of the manufacturer of the medicinal product to decide how the information on FRCs will be applied in the manufacturing process in light of the use of the excipient and data from pharmaceutical development.

The information on the FRCs may be given in different ways:

- name of the FRC;
- name of the FRC and a recommended method for its determination, referring wherever possible to a general chapter of the Pharmacopoeia;
- name of the FRC with a recommended method for its determination and typical values, which may be in the form of tolerances from the nominal value.

A given characteristic may be the subject of a mandatory requirement in the monograph. If it is relevant for certain uses, it is also referred to in the FRCs section as a relevant characteristic that the manufacturer of the medicinal product may choose to specify for the grade used of a particular pharmaceutical preparation.

The section on FRCs is intended to reflect current knowledge related to the major uses of an excipient. In view of the multiple uses of some excipients and the continuous development of new uses, the section may not be complete. In addition, the methods cited for the determination of a particular characteristic are given as recommendations for methods that are known to be satisfactory for the purpose, and the use of other methods is not excluded.

## PHARMACOPOEIAL HARMONISATION

A number of excipient monographs are subject to pharmacopoeial harmonisation among the European, Japanese and United States pharmacopoeias (see general chapter *5.8. Pharmacopoeial harmonisation*). Introduction of the FRCs section in the monographs of the European Pharmacopoeia means that the presentation of harmonised monographs differs. Tests for physical and chemical attributes regarded as both quality-related and functionality-related in the European Pharmacopoeia are, in the 2 other pharmacopoeias, included only in the body of the monograph. The different format has no implications on the specification of excipient characteristics for the manufacturer of the medicinal product. Current regulatory guidance recommends the identification and specification of only such critical attributes that impact the manufacturing process and the quality of the medicinal product. The different legal environments of the 3 pharmacopoeias allow for different formats of the monographs without affecting the pharmacopoeial harmonisation status.

## GLOSSARY

**Critical characteristic or critical quality attribute (CQA)**: a physical, chemical, biological or microbiological property or characteristic that must be within appropriate limits or an appropriate range or distribution to ensure the desired product quality.

**Critical process parameter (CPP)**: a process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.

**Design space**: the multidimensional combination and interaction of input variables (e.g. material attributes) and process parameters that have been demonstrated to provide assurance of quality.

**Functionality-related characteristic (FRC)**: a controllable physical or chemical characteristic of an excipient that is shown to impact on its functionality.

**Process analytical technology (PAT)**: a system for designing, analysing and controlling manufacturing through timely measurements (i.e. during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality.

**Quality by design (QbD)**: a systematic approach to development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management.

# 5.16. Crystallinity

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# 5.16. CRYSTALLINITY

*This chapter provides general information on crystallinity and refers to the various techniques described in the European Pharmacopoeia that are used for its determination.*

INTRODUCTION - THE CONCEPT OF CRYSTALLINITY

Most organic and inorganic compounds of pharmaceutical relevance exist as a solid material, which can be characterised by a structure located between a perfectly ordered crystal and an amorphous material.

Real crystals lie somewhere between an ideal crystal state and the amorphous state. The position of a crystal on a scale bounded by these 2 extremes is termed its crystallinity.

A perfectly ordered crystal is an ideal state that is seldom, if ever, achieved. The structural units of a crystal, termed unit cells, are repeated regularly and indefinitely in 3 dimensions in space. The unit cell has a definite orientation and shape defined by the translational vectors *a, b* and *c*, and the angles α, β and γ, and hence has a definite volume, *V*, that contains the atoms and molecules necessary for forming the crystal. A crystalline system is defined by 3 long-range order symmetry operators (translational, orientational and conformational); the various mesophases (liquid crystals, crystals and plastic crystals) have 1 or 2 of the long-range symmetry operators and the ideal amorphous state is defined by the absence of all 3 operators.

Each crystal can be classified as a member of one of 7 possible crystal systems that are defined by the relationships between the individual dimensions *a, b* and *c* and between the individual angles α, β and γ of the unit cell. The structure of a given crystal may be classified according to one of the 7 crystal systems, to one of the 14 Bravais lattices and to one of the 230 space groups. All the 230 possible space groups, their symmetries and the symmetries of their diffraction patterns are compiled in the International Tables for Crystallography.

Many substances are capable of crystallising in more than one type of crystal lattice, which is known as polymorphism. The occurrence of polymorphism is a common phenomenon among organic molecules, giving rise to different physico-chemical properties. Crystalline polymorphs have the same chemical composition but different internal crystal structures and, therefore, possess different physico-chemical properties. The different crystal structures in polymorphs are due to different atomic packing arrangements and/or different conformations of the molecules (see chapter *5.9. Polymorphism*).

The other extreme of a crystal state is the ideal or true amorphous state, where all long-range order is lost. For most organic systems certain short-range order remains, but this is not expected to extend over distances much larger than nearest neighbour (NN) or next nearest neighbour (NNN) interactions, which are typically less than 2-2.5 nm for small organic molecules.

Amorphous material is characterised by the absence of distinct reflections in the X-ray powder diffraction (XRPD) pattern (*2.9.33*).

The crystallinity of a real powder can be considered by 2 models of crystallinity. In the 1-state model all particles will be of the same crystallinity whereas in the 2-state model each particle can be either crystalline or amorphous, such that the actual crystallinity of the powder is the weighted average of these 2 extreme crystallinities. Such a powder is obtained when pure crystalline and amorphous phases are physically mixed. In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with different sizes and shapes.

The extent of disorder in a crystalline solid can affect many physico-chemical properties of substances for pharmaceutical use. Because of the great relevance of these properties, it is important to be able to assess the extent of disorder or the crystallinity of a solid by a suitable quantitative method.

METHODS FOR MONITORING AND DETERMINING CRYSTALLINITY

Various methods are available for determining the crystallinity of a solid. Many techniques cannot detect or quantify these properties independently; for this reason, it is useful to combine several of the methods described below. Such methods often do not give accurate results and limits of quantitation are usually much greater than those for chemical impurities. In addition, certain assumptions have to be made about the relationship between standards used for calibration, which are typically mixtures of crystalline and amorphous particles (2-state model), and the samples to be analysed that are likely to have at least a small component of material exhibiting 1-state model behaviour. Finally, the lack of well-defined standards for 100 per cent crystalline or 100 per cent amorphous material complicates the validation of such methods. As explained above, it is obvious that different amorphous or non-crystalline phases exist and even co-exist in a solid powder. These different non-crystalline forms of a solid can give different responses depending on the techniques used for determining the degree of crystallinity.

**X-ray powder diffraction** (*2.9.33*). XRPD is still the most commonly used method for determining the degree of crystallinity, although this method suffers from some limitations due to peak broadening, amorphous halo and preferred orientation, which make interpretation and quantitation difficult.

XRPD alone is often insufficient to distinguish between the different non-crystalline phases. The X-ray diffraction pattern of a purely amorphous and nanocrystalline phase is characteristic of a broad diffuse halo. In-depth analysis of the X-ray diffraction patterns will show that the diffuse halo in the pattern of nanocrystalline material shows some correlation to the pattern of the parent crystalline phase, while in the case of a pure amorphous phase such a correlation does not exist. Additional techniques may be required to establish the true nature of X-ray amorphous materials.

**Thermal analysis**. Thermal analysis (*2.2.34*) of crystalline materials exhibits a melting transition that is often accompanied by decomposition or evaporation of solvents. In the case of true amorphous materials, thermal analysis reveals a glass transition, whereas only a melt would be expected for a nanocrystalline material.

**Microcalorimetry** (*2.2.61*). It is a highly sensitive technique which allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure. Amorphous parts of a substance can recrystallise by subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The measurement of the heat of recrystallisation enables the amorphous content to be determined from the enthalpy of recrystallisation. By relating the output from the microcalorimeter for a sample to that obtained for an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1 per cent can be reached.

**Solution calorimetry** (*2.2.61*). Solution calorimetry provides a means of determining enthalpy of solution for a solid substance. The crystallinity of the solid sample to be examined is given by the enthalpy of solution of the solid sample ($\Delta H_x^s$) minus the enthalpy of solution of the chosen reference

standard of the same substance ($\Delta H_r^s$) when determined under the same conditions. Because the reference standard is usually chosen for its perceived high crystallinity, its enthalpy of solution is usually algebraically greater (more endothermic or less exothermic) than that of the solid sample to be examined in the same solvent. Consequently, the crystallinity determined is a negative quantity with the SI units kJ/mol or J/g (J/kg is avoided because of its unwieldiness and potential for error). The preference for a negative value with respect to a highly crystalline reference standard recognises the fact that most samples have a lower crystallinity than this reference standard.

**Near-infrared (NIR) spectroscopy**. Near-infrared (NIR) spectroscopy (*2.2.40*) is another technique used to measure the degree of crystallinity, and has also been proven to be useful in studies of polymorphism. The NIR spectrum of a sample contains both physical and chemical information. Being non-invasive, non-destructive and operable at room temperature, the method is a valuable tool to assess changes in the amorphous and crystalline state.

**Infrared absorption spectrophotometry and Raman spectroscopy**. Infrared absorption spectrophotometry (*2.2.24*) and Raman spectroscopy (*2.2.48*) are other techniques used to measure the degree of crystallinity, and have also been proven to be useful in studies of polymorphism. The IR spectrum and Raman spectrum of a sample contain both physical and chemical information.

**Solid-state NMR**. Solid-state nuclear magnetic resonance spectrometry (ss NMR) (*2.2.33*) can be used to provide information about polymorphism and related relative molecular conformations. However, some caution has to be exercised in the interpretation of results, since it is not always simple to distinguish between samples that comprise a mixture of different physical forms (2-state model) and those that comprise crystals having disorder with exchange that is slow on the NMR timescale. Similarly, samples that contain defects arising from different molecular conformations or slightly different packing arrangements (1-state model) may show additional signals in the spectra. Solid-state NMR may be quite sensitive to this, even if lattice parameters are hardly affected and, consequently, little or no change is observed by XRPD. It is evident that the crystallinity of substances for pharmaceutical use can be complex, and both crystalline defects and amorphous material may co-exist.

**Optical microscopy**. A method to detect whether or not particles are crystalline is to use a polarising microscope (*2.9.37*), where particles show birefringence and extinction positions when the microscope stage is revolved.

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# 5.17. RECOMMENDATIONS ON METHODS FOR DOSAGE FORMS TESTING

07/2010:51701

## 5.17.1. RECOMMENDATIONS ON DISSOLUTION TESTING

*This general chapter is non-mandatory; it provides information on dissolution testing, on recommended dissolution media and on the expression of dissolution specifications for oral dosage forms (see general chapter 2.9.3. Dissolution test for solid dosage forms). This information represents generally accepted parameters used in the field of dissolution.*

In the determination of the dissolution rate of the active substance(s) of a solid dosage form, the following are to be specified:

- the apparatus to be used, and in cases where the flow-through apparatus is specified, which flow-through cell is to be used;
- the composition, the volume and the temperature of the dissolution medium;
- the rotation speed or the flow rate of the dissolution medium;
- the time, the method and the amount for sampling of the test solution or the conditions for continuous monitoring;
- the method of analysis;
- the acceptance criteria.

The choice of apparatus to be used depends on the physico-chemical characteristics of the dosage form. When a large quantity of dissolution medium is required to ensure sink conditions, or when a change of pH is necessary, the flow-through apparatus may be preferred.

EXPERIMENTAL TESTING CONDITIONS

The use of the basket and the paddle apparatus and the reciprocating cylinder apparatus is generally based on the principle of operating under sink conditions, i.e. in such a manner that the material already in solution does not exert a significant modifying effect on the rate of dissolution of the remainder. Sink conditions normally occur in a volume of dissolution medium that is at least 3-10 times the saturation volume.

In general, an aqueous medium is used. The composition of the medium is chosen on the basis of the physico-chemical characteristics of the active substance(s) and excipient(s) within the range of conditions to which the dosage form is likely to be exposed after its administration. This applies in particular to the pH and the ionic strength of the dissolution medium.

The pH of the dissolution medium is usually set between pH 1 and pH 8. In justified cases, a higher pH may be needed. For the lower pH values in the acidic range, *0.1 M hydrochloric acid* is normally used. Recommended dissolution media are described hereafter.

Water is recommended as a dissolution medium only when it is proven that the pH variations do not have an influence on the dissolution characteristics.

In specific cases, and subject to approval by the competent authority, dissolution media may contain enzymes, surfactants, further inorganic substances and organic substances. For the testing of preparations containing poorly aqueous-soluble active substances, modification of the medium may be necessary. In such circumstances, a low concentration of surfactant is recommended; it is recommended to avoid the use of organic solvents.

Gases dissolved in the dissolution medium can affect the results of the dissolution test. This is true in particular for the flow-through apparatus, where de-aeration of the medium is necessary to avoid the formation of gas bubbles in the flow-through cell. A suitable method of de-aeration is as follows: heat the medium while stirring gently to about 41 °C, immediately filter under vacuum using a filter with a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other de-aeration techniques for removal of dissolved gases may be used.

Using the paddle or basket apparatus, the volume of dissolution medium is normally 500-1000 mL. A stirring speed of between 50 r/min and 100 r/min is normally chosen; it must not exceed 150 r/min.

For the flow-through apparatus, the liquid flow rate is normally set between 4 mL/min and 50 mL/min.

RECOMMENDED DISSOLUTION MEDIA

The following dissolution media may be used.

Table 5.17.1.-1. – *Examples of dissolution media*

| pH | Dissolution media |
|---|---|
| pH 1.0 | HCl |
| pH 1.2 | NaCl, HCl |
| pH 1.5 | NaCl, HCl |
| pH 4.5 | Phosphate or acetate buffer |
| pH 5.5 and pH 5.8 | Phosphate or acetate buffer |
| pH 6.8 | Phosphate buffer |
| pH 7.2 and pH 7.5 | Phosphate buffer |

The composition and preparation of these various media are indicated below.

**Hydrochloric acid media**

- *0.2 M hydrochloric acid.*
- *0.2 M sodium chloride.* Dissolve 11.69 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

For preparing media with the pH values indicated in Table 5.17.1.-2, mix 250.0 mL of 0.2 M sodium chloride and the specified volume of *0.2 M hydrochloric acid*, and dilute to 1000.0 mL with *water R*.

Table 5.17.1.-2. – *Hydrochloric acid media*

| pH | HCl (mL) |
|---|---|
| 1.2 | 425.0 |
| 1.3 | 336.0 |
| 1.4 | 266.0 |
| 1.5 | 207.0 |
| 1.6 | 162.0 |
| 1.7 | 130.0 |
| 1.8 | 102.0 |
| 1.9 | 81.0 |
| 2.0 | 65.0 |
| 2.1 | 51.0 |
| 2.2 | 39.0 |

The hydrochloric acid media may also be prepared by replacing sodium chloride with potassium chloride.

**Acetate buffer solutions**

- *2 M acetic acid.* Dilute 120.0 g of *glacial acetic acid R* to 1000.0 mL with *water R*.

- *Acetate buffer solution pH 4.5.* Dissolve 2.99 g of *sodium acetate R* in *water R*. Add 14.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.
- *Acetate buffer solution pH 5.5.* Dissolve 5.98 g of *sodium acetate R* in *water R*. Add 3.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.
- *Acetate buffer solution pH 5.8.* Dissolve 6.23 g of *sodium acetate R* in *water R*. Add 2.1 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

**Phosphate buffer solutions**

For preparing buffers with the pH values indicated in Table 5.17.1.-3, mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and the specified volume of *0.2 M sodium hydroxide*, and dilute to 1000.0 mL with *water R*.

Table 5.17.1.-3. – *Phosphate buffer solutions*

| | | | | | | |
|---|---|---|---|---|---|---|
| **pH** | 5.8 | 6.0 | 6.2 | 6.4 | 6.6 | 6.8 |
| **NaOH (mL)** | 18.0 | 28.0 | 40.5 | 58.0 | 82.0 | 112.0 |
| **pH** | 7.0 | 7.2 | 7.4 | 7.6 | 7.8 | 8.0 |
| **NaOH (mL)** | 145.5 | 173.5 | 195.5 | 212.0 | 222.5 | 230.5 |

**Other phosphate buffer solutions**

- *Phosphate buffer solution pH 4.5.* Dissolve 13.61 g of *potassium dihydrogen phosphate R* in 750 mL of *water R*. Adjust the pH if necessary with *0.1 M sodium hydroxide* or with *0.1 M hydrochloric acid.* Dilute to 1000.0 mL with *water R*.
- *Phosphate buffer solution pH 5.5 R.*
- *Phosphate buffer solution pH 6.8 R1.*
- *Buffer solution pH 7.2 R.*
- *0.33 M phosphate buffer solution pH 7.5 R.*

**Simulated intestinal fluid pH 6.8**

Mix 77.0 mL of *0.2 M sodium hydroxide*, 250.0 mL of a solution containing 6.8 g of *potassium dihydrogen phosphate R*, and 500 mL of *water R*. Add 10.0 g of *pancreas powder R*, mix and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

**Simulated gastric fluid**

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*, add 80 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*. If required, pepsin powder may be omitted.

**Increasing pH**

For a test involving increasing pH, one of the following sequences may be used:

| **Time (h)** | 0 - 1 | 1 - 2 | 2 - 3 | 3 - 4 | 4 - 5 | 5 - 6 | 6 - 7 | 7 |
|---|---|---|---|---|---|---|---|---|
| **pH** | 1.0 | | | | | | | |
| **pH** | 1.2 | 6.8 | | | | | | |
| **pH** | 1.2 | 2.5 | 4.5 | | 7.0 | | 7.5 | |
| **pH** | 1.5 | 4.5 | | | | 7.2 | | |

To achieve this pH variation, it is possible either:

- to substitute one buffer solution for another (whole substitution);
- to remove only half of the medium each time (half change method) and replace it with a buffer solution of higher pH: the initial pH is 1.2 and the second solution is phosphate buffer solution pH 7.5; or,
- to an initial solution at pH 1.5, to add a dose of a powder mixture containing *tris(hydroxymethyl)aminomethane R* and *anhydrous sodium acetate R* to obtain pH 4.5 and a second dose to obtain pH 7.2, as described below:
  - *hydrochloric acid pH 1.5*: dissolve 2 g of *sodium chloride R* in *water R*, add 31.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*;
  - *buffer solution pH 4.5*: mix 2.28 g of *tris(hydroxymethyl)aminomethane R* with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the hydrochloric acid pH 1.5 described above;
  - *buffer solution pH 7.2*: mix 2.28 g of *tris(hydroxymethyl)aminomethane R* with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the buffer solution pH 4.5 described above.

The flow-through cell may be used for the continuous change of pH.

## QUALIFICATION AND VALIDATION

Due to the nature of the test method, quality by design is an important qualification aspect for *in vitro* dissolution test equipment. Any irregularities such as vibration or undesired agitation by mechanical imperfections are to be avoided.

Qualification of the dissolution test equipment has to consider the dimensions and tolerances of the apparatus. Critical test parameters, such as temperature and volume of dissolution medium, rotation speed or liquid flow rate, sampling probes and procedures, have to be monitored periodically during the periods of use.

The performance of the dissolution test equipment may be monitored by testing a reference product that is sensitive to hydrodynamic conditions. Such tests may be performed periodically or continuously for comparative reasons with other laboratories.

During testing, critical inspection and observation are required. This approach is especially important to explain any outlying results.

Validation of automated systems, whether concerning the sampling and analytical part or the dissolution media preparation and test performance, has to consider accuracy, precision, and the avoidance of contamination by any dilutions, transfers, cleaning and sample or solvent preparation procedures.

## EXPRESSION OF DISSOLUTION SPECIFICATIONS FOR ORAL DOSAGE FORMS

The dissolution specification is expressed in terms of the quantity ($Q$) of active substance dissolved in a specified time, expressed as a percentage of the content stated on the product label.

**Conventional-release dosage forms**

In most cases, when tested under reasonable and justified test conditions, the acceptance criteria at level $S_1$ are that at least 80 per cent of the active substance is released within a specified time, typically 45 min or less. This corresponds to a $Q$ value of 75 per cent, since, as referred to in Table 2.9.3.-1, for level $S_1$ the individual value of each of the 6 units tested is not less than $Q$ + 5 per cent, i.e. not less than 80 per cent.

Typically, a single-point acceptance criterion is sufficient to demonstrate that the majority of the active substance has been released, although in certain circumstances it may be necessary to test at additional time point(s), in order to demonstrate adequate dissolution.

**Prolonged-release dosage forms**

The dissolution test acceptance criteria for prolonged-release dosage forms is normally expected to consist of 3 or more points. The 1st specification point is intended to prevent unintended rapid release of the active substance ('dose dumping'). It is therefore set after a testing period corresponding to a dissolved amount typically of 20 per cent to 30 per cent. The 2nd specification point defines the dissolution pattern and so is set at around 50 per cent release. The final specification point is intended to ensure almost complete release, which is generally understood as more than 80 per cent release.

**Delayed-release dosage forms**

A delayed-release dosage form may release the active substance(s) fractionally or totally according to the formulation design when tested in different dissolution media, e.g. in increasing pH conditions. Dissolution specifications therefore have to be decided on a case-by-case basis.

Gastro-resistant dosage forms require at least 2 specification points in a sequential test and 2 different specifications in a parallel test. In a sequential test, the 1st specification point represents an upper limit and is set after 1 h or 2 h in acidic medium, and the 2nd after a pre-set time period of testing in an adequate buffer solution (preferably pH 6.8).

In most cases the acceptance criteria at level $B_1$ are that at least 80 per cent of the active substance is released. This corresponds to a $Q$ value of 75 per cent, since, as referred to in Table 2.9.3.-4, for level $B_1$ the individual value of each of the 6 units tested is not less than $Q$ + 5 per cent, i.e. not less than 80 per cent.

# 5.19. Extemporaneous preparation of radiopharmaceuticals

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**04/2016:51900**

# 5.19. EXTEMPORANEOUS PREPARATION OF RADIOPHARMACEUTICALS

*This general chapter is published for information.*

## 1. SCOPE AND DEFINITION

Many radiopharmaceuticals are prepared on-site on a regular basis, typically as doses for a few patients based on specific clinical needs (extemporaneously prepared radiopharmaceuticals, EPRs). Whereas the manufacture of radiopharmaceuticals and investigational medicinal products is well covered by existing regulations, this general chapter only covers EPRs, which are also to be considered in the light of any national competent authority requirements. Radiopharmaceuticals are pharmaceutical preparations, and the provisions and terminology of the general monograph *Pharmaceutical preparations (2619)* apply.

EPRs are either prepared in accordance with a medical prescription for an individual patient, or in accordance with a pharmacopoeial monograph, and intended to be supplied directly to patients. The radiopharmaceuticals concerned are used within their specified expiration time, and include both kit-based preparations (from licensed and unlicensed kits) and unlicensed preparations containing radionuclides for positron emission tomography (PET), single photon emission computed tomography (SPECT) or for therapeutic applications.

For the purpose of this general chapter, the preparation of radiopharmaceuticals is considered as a process involving some or all of the following steps: purchase of materials and products, production of radionuclides for radiolabelling, radiolabelling, chemical modification and/or purification, formulation, dispensing of the pharmaceutical form, sterilisation, analytical control, packaging, labelling and release. Drawing patient doses for immediate application (e.g. from a multidose vial) is considered as part of clinical practice, and not part of the preparation of radiopharmaceuticals.

EPRs require an appropriate framework to ensure the desired quality, hereinafter referred to as the quality system. The extent of the quality system is driven by the risks for the patient concerned, such as microbial contamination, failure of chemical reactions and its consequences, malfunctioning of equipment involved in the preparation process and inappropriate storage conditions. Risk assessment is employed to determine the level of risk and the required level of quality assurance to achieve appropriate product quality and to assure radiation safety. Examples of quality systems can be found in the following guidance documents or any subsequent revisions: *Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S): Guide to good practices for the preparation of medicinal products in healthcare establishments (PE 010); EudraLex Volume 4, EU Guidelines to Good Manufacturing Practice, Medicinal Products for Human and Veterinary Use; European Association of Nuclear Medicine (EANM) guideline: Guidance on current good radiopharmacy practice (cGRPP) for the small-scale preparation of radiopharmaceuticals*; and national guidelines from the competent authority. Guidance on risk assessment can be found in, for example, *ICH Guideline Q9 Quality Risk Management*.

Particular attention is to be paid to:

- qualified personnel with appropriate training;
- adequate premises;
- qualified and suitable equipment for production and analysis;
- validated procedures for all critical production and testing steps;
- environmental monitoring;
- appropriate documentation;
- procurement of materials and services used in the preparation;
- analytical methods / quality control.

All steps in the preparation of radiopharmaceuticals are designed to meet the radiation safety requirements for the personnel involved and the environment, thereby complying with national or international regulations. This includes appropriate shielding, and measures to avoid and monitor radioactive contamination.

## 2. PREMISES AND EQUIPMENT

The relevant premises and equipment are designed, built, maintained, cleaned and sanitised so that they protect product quality, ensure a low level of particulates and microbial contamination and protect staff and the environment from the effects of radiation.

Radiopharmacies may characteristically prepare a wide variety of radiopharmaceuticals, often in the same session and place. The premises, equipment and workflow are arranged in order to minimise the risk of cross-contamination and mix-up. Facilities and equipment are designed and controlled to reflect the specific risk of all preparations concerned, taking into account the potential for microbial contamination of the preparation. Additional considerations are taken into account when handling biological material.

Detailed knowledge of process parameters, workflow, environmental conditions and microbial aspects of the preparation helps to avoid possible chemical, radiochemical, radionuclidic and microbial contamination. In the particular case of blood cell radiolabelling, movement of operators within the laboratory between the area for blood cell labelling and the area for the preparation of other radiopharmaceuticals is prevented by the appropriate design and layout of the premises. Any biological hazardous material is stored and handled separately from other substances for pharmaceutical use, pharmaceutical preparations or starting materials.

Measurement of radioactivity is carried out as described in general chapter *2.2.66. Detection and measurement of radioactivity*. Measurement equipment is properly shielded, particularly when high levels of radioactivity are handled in adjacent areas. A system to ensure proper performance of this equipment, including daily checks and periodic calibration, is implemented. All deviations, such as changes to the range of linearity, calibration for energy and efficiency, and unexpected changes in background readings are investigated.

## 3. PREPARATION PROCESS

In the case of a licensed product used as a part of the preparation process, it is the responsibility of the marketing authorisation holder to ensure that the licensed product complies with the requirements of its marketing authorisation. The radiopharmacy preparing licensed radiopharmaceuticals according to the instructions for use carries the responsibility for the quality of the preparation and the handling of these radiopharmaceuticals at its site.

If the instructions for use of a licensed radiopharmaceutical are not strictly followed or if one or more components used for the preparation do not have a marketing authorisation, risk assessment (including rationales and pharmaceutical equivalence, if applicable) is undertaken and documented. It is the responsibility of the radiopharmacy to demonstrate that the quality of the final preparation is suitable for the intended use.

In general for EPRs, the bioburden of starting materials is an important factor in maintaining a low bacterial endotoxin content and achieving a high sterility assurance level in subsequent operations. Opened or partially used

packages of starting materials intended for subsequent use are properly indicated (labelled) and stored under restricted access conditions. Shelf-life periods are defined for opened, unopened and dissolved starting materials, especially in light of the microbiological background in the specific working conditions. The use of single-use packages is recommended. A shelf-life for starting material sets is defined with consideration given to the degradation of ingredients, microbial contamination and the stability of packaging materials, taking into account the permeability of plastic and elastomeric packaging. Shelf-life is indicated and justified by stability studies reflecting the mode of use.

Monitoring of the environment and personnel during the extemporaneous preparation of radiopharmaceuticals is essential in defining the quality of the final preparation, irrespective of the origin of material used in the preparation. Recommendations for the frequency of monitoring can be found in guidance documents such as PIC/S Guide PE 010 or any subsequent revisions. Deviations from the recommended frequency are made based on risk assessment and justified. When a sterile preparation is to be obtained and terminal sterilising filtration is not possible, all starting materials are sterile. Components of the equipment that come into direct contact with the preparation during the preparation process are sterile and disposable or re-used only after a validated cleaning and sterilisation procedure has been carried out.

3-1. *PRODUCTION OF RADIONUCLIDES*

The procedure for production of the radionuclide describes major parameters, such as:

- target material;
- nuclear reaction;
- construction of the holder for the target material;
- maintenance of the holder for the target material and the transfer lines;
- irradiation data, such as beam energy and intensity;
- typical radionuclidic contaminants for the adopted conditions (excitation function);
- separation/purification process of the desired radionuclide;

and evaluates all effects on the efficiency of the production in terms of quality and quantity of the radionuclide produced.

Radionuclide precursors and radiolabelled molecules comply with the requirements of the general monograph *Radiopharmaceutical preparations (0125)* and any individual monographs where available.

3-2. *CHEMICAL PRECURSORS*

Chemical precursors are usually obtained by chemical synthesis. They can be combined or pre-loaded with other substances in the form of pre-prepared sets for radiolabelling procedures and/or used as starting materials in cassettes or kits.

Chemical precursors, either in isolated form or in the form of starting material sets, have an acceptable, low degree of microbial contamination, irrespective of whether the final product is terminally sterilised or sterilised by filtration. Sterilisation is to be considered if there is a risk that the chemical precursors support microbial growth.

Quality requirements for chemical precursors are stated in the respective individual monographs. Where no monograph is available, the general monograph *Substances for pharmaceutical use (2034)* applies and a programme to test the quality is implemented. However, it is to be noted that certain provisions of the general monograph *Substances for pharmaceutical use (2034)* are not applicable to radiopharmaceutical preparations or chemical precursors. These provisions are covered by the general monograph *Radiopharmaceutical preparations (0125)*.

3-3. *RADIOLABELLING*

The radiolabelling step is the reaction of the radionuclide with a chemical precursor. Biological materials such as proteins or cells can also be substrates for direct radiolabelling.

The radiolabelling includes the mixing of starting materials in controlled conditions (i.e. temperature or pressure). After radiolabelling, subsequent steps may be involved to remove protecting groups or to couple the radiolabelled compound to another molecule, which may be an organic moiety or a more complex structure such as a peptide or an antibody.

Risks to radiolabelling efficiency, quality, safety and efficacy of the radiopharmaceutical, associated with the chemical and physical composition of the kit, the components or the starting materials, are evaluated and documented. Chemical and physical stability and risks of microbial contamination are examined closely.

The source and quality of starting materials (e.g. metal contaminants), the quantitative and qualitative composition (e.g. concentration, pH, sterility, osmolarity, viscosity, solubility, stability) and the operating conditions (e.g. use of inert gas, temperature, pressure) are considered when developing the synthesis. Special attention is also paid to possible side products of the synthesis. Automation and/or use of cassettes are possible ways of improving the reliability of synthesis processes, reducing the risk of microbial contamination and increasing radiation safety.

Before introducing a new synthesis in clinical application, the synthesis process is validated by suitable controls during the preparation (in-process control) and extensive quality control of the final preparation using at least 3 batches. Once the process is validated, the routine controls that need to be performed before patient administration are considered based on a risk assessment, taking into account different factors such as chemical complexity, factors affecting the efficacy of the product, and radiation dose concerns for the patient, for example through the control of radiochemical and radionuclidic impurities.

3-3-1. **Radiopharmaceuticals not requiring a purification step**

This type of synthesis is characterised by combining a radionuclide with a mixture of starting materials. This addition is followed by a near-quantitative reaction of the radionuclide with the chemical precursor, so that the extemporaneous preparation process does not require a purification step. Open methods for radiosynthesis are to be avoided due to the elevated risk of microbial contamination. All components are co-injected with the resulting radiopharmaceutical active ingredient. The required risk assessment focuses on the chemical, radiochemical and microbiological quality of all starting materials, including the radionuclide. In case of multiple additions, the risk assessment also focuses on the conditions of addition and reaction of the different starting materials, and especially the reaction container.

3-3-2. **Radiopharmaceuticals requiring purification**

This type of synthesis is characterised by a single addition of a radionuclide solution to a mixture of starting materials or by multiple additions of different starting materials, which then requires subsequent purification (see also section 3-5). An efficient purification of the desired radioactive compound from the reaction mixture is necessary in order to ensure low levels of radionuclidic, chemical and/or radiochemical impurities. Physico-chemical and chemical separation of intermediates or the final product is essential to yield a radiopharmaceutical that meets the desired quality specifications. If possible, the preparation process, including the critical separation steps, is monitored with suitable detectors, and controls are performed with regard to radiation safety. The required risk assessment focuses on the same points as in section 3-3-1, as well as on the conditions of purification, especially the efficiency of separation and the effect of chromatographic media on the

subsequent microbiological quality of the product (endotoxin content).

3-3-3. **Cell radiolabelling**

Cross-contamination, cross-infection, mix-up of blood and blood components, and integrity and/or viability of the cells after radiolabelling are all specific points of attention for the risk assessment of cell radiolabelling. This type of radiolabelling is considered more extensively in section 3-14.

3-4. *AUTOMATED SYSTEMS*

Some of the steps described above can be subject to automation. An automated module (synthesiser) usually consists of a combination of power supplies, actuators, pumps, heaters and sensors that are used in combination with an interconnected network of containers, reactors, tubing, syringes, solid phase cartridges and/or preparative liquid chromatography systems. The automated module can be a commercial piece of equipment or can be custom made. It is common for different radiopharmaceuticals to be made on the same automated module.

Within the synthesis process, the automated module controls process parameters in such a way that a solution of a radiopharmaceutical is produced. The containers and the purification system used with the automated module can be single-use ('radiopharmaceutical cassette') or used in multiple production runs.

When using consecutive production runs, the risk of cross-contamination is considered. Appropriate measures are taken to prevent cross-contamination by using dedicated components or equipment or by assessing the effectiveness of the cleaning procedure.

The containers and purification systems (e.g. the column of a preparative liquid chromatography system) are considered part of the synthesiser.

The electronic components of the synthesiser are resistant to high radiation levels.

Components of the automated module that come into contact with the starting materials, solvents and/or the radiopharmaceutical are chemically inert. Special care is taken with components that may degrade under the influence of radiation and that come into contact with the starting materials, solvents and/or the radiopharmaceutical, as they may release impurities over time.

Automated modules may also control formulation and dispensing of the radiopharmaceutical, usually by using volume- or weight-measuring devices and radioactivity detectors in order to measure and dispense the correct quantities. For dispensing, single-use tubing systems are used. The measuring system is calibrated.

For an automated synthesis and/or dispensing module, 2 levels of qualification/validation are required. The automated module itself is qualified by the supplier and/or the user. After this qualification, the extemporaneous preparation/dispensing process is validated.

The synthesis process on the synthesiser is usually controlled by software and is validated. The user of the automated system has the list of the sequence steps used in the synthesis and a history of changes made to them. The software is under access control, and any changes to it are controlled and documented. Guidance on the use of computerised systems can be found, for example, in EudraLex Volume 4, Annex 11.

Manual interventions or adjustments of parameters (e.g. manual operation of valves) are documented and investigated as a process deviation if outside the validated ranges. The version of the software used for a production is recorded as a batch parameter. When changes are made to the software, the old version of the software is archived for the same period as the documentation of the batches made with that version.

Automated systems may involve the use of radiopharmaceutical cassettes and other disposable devices. Cassettes are used with a set of starting materials (such as precursors, solvents, catalysts, etc.), which may be contained in the cassette (prefilled cassette) or provided separately (empty cassette).

Cassettes can be made by commercial manufacturers or assembled in-house. The requirements apply to both, and the related information is directed towards the users of the cassette to help them establish their user requirements.

All materials in the system that come into contact with reagents or product exhibit suitable stability during storage and use. The compatibility of the materials (e.g. plastics) with the chemical process is assessed and documented. Glass components are at least type I (see general chapter *3.2.1. Glass containers for pharmaceutical use*).

Before administration of a preparation produced with the aid of cassettes, it is validated that the combination of the cassette and the automated system consistently produces the radiopharmaceutical of the desired quality.

The quality of the chemicals used complies with the requirements mentioned in section 3-2.

The cassette is able to synthesise the radiopharmaceutical to the agreed specification during the entire shelf-life of the cassette.

In order to maintain a low bacterial endotoxin content and achieve a high sterility assurance level for the radiopharmaceutical prepared with the use of a cassette, the cassette has a low initial bioburden.

The suitability of the manufacturing process is assured and the user confirms the final product quality by appropriate analytical tests.

The user of the automated system has the necessary information on the chemicals and reaction processes applied within the system in order to evaluate potential deviations that may occur during the production of the radiopharmaceuticals. In the case of suboptimal reaction or system malfunction, yields might be lower and/or additional impurities may occur. Sufficient information about potential system malfunctions is made available to the user in order to set up appropriate release specifications.

3-5. *PURIFICATION*

Separation of the product is often required, particularly when organic chemical reactions are carried out. Since the purification step ensures the final quality of the radiopharmaceutical, separation efficiency has to be carefully evaluated in terms of final radiochemical, radionuclidic and chemical purity. Special attention is paid to residual solvents (see general chapter *5.4. Residual solvents*). All purification procedures are validated.

A microbial contamination risk exists when using chromatographic media, especially in the case of multiple-use liquid chromatography columns. Risk assessment focuses on cleaning/conditioning procedures and conditions of storage of chromatographic media. Bioburden and bacterial endotoxin content are maintained below suitable limits to allow sterilisation in case of parenteral dosage forms.

The radiolabelling process for biological materials such as blood cells are developed in such a way that the purification step, typically centrifugation, guarantees a reproducible quality of product.

3-6. *FORMULATION*

After purification of the labelled compound, the radiolabelled molecule is formulated into a suitable form for administration to patients.

The source and quality of excipients and additives are documented.

When an in-house starting material set is used, the use of components with no microbial contamination (or an acceptably low level) is recommended, irrespective of whether the final product is terminally sterilised or sterile-filtered.

In case different types of radiopharmaceuticals from kits are to be prepared in the same period, separate vials of diluent are used to prevent cross-contamination.

Most radiopharmaceuticals are intended for parenteral administration. In this respect, pH, osmolarity, viscosity, ionic strength and solubility are appropriately addressed when radiopharmaceuticals and in-house starting material sets are developed.

3-7. *DISPENSING*

Dispensing is the process of aliquoting formulation solution into final product dosage forms, subject to release before medical administration (see section 3-12). It includes preparation of a batch consisting of one or more final product vials or syringes. In order to keep the bioburden as low as possible, components used in the dispensing process are sterile. If unavailable, components are sterilised by a validated process. If components are reused, it is ensured by a validated cleaning procedure that no cross-contamination from one product to another can take place.

3-8. *STERILISATION*

Radiopharmaceuticals for parenteral administration are sterile. Terminal sterilisation provides the highest level of assurance that a product will be sterile. In most cases, only sterilising filtration steps can be performed, but in others, no sterilisation is possible (e.g. when autologous cells are radiolabelled). These are to be considered as aseptic preparations. The methods of sterilisation that can be used are described in general chapter *5.1.1. Methods of preparation of sterile products*.

Aseptic manipulations take place in a grade A environment (class A zone). The grade of the surrounding environment will depend on the containment system used, the risk of contamination for the preparation, the shelf-life of the preparation and the number of units prepared during a preparation run. With respect to air cleanliness, a grade C surrounding area for open workstations, or a grade D surrounding area for isolators is typically acceptable.

The complexity of operation and the shelf-life determine the measures that need to be taken to ensure a sterile product, for example:

- for simple operations in a closed system requiring little handling (e.g. preparing radiopharmaceuticals from licensed kits and generators), suitable control of the immediate surrounding area of a workstation may provide the appropriate level of air cleanliness when additional measures (e.g. gowning flow) are in place; a risk assessment is crucial in this respect;
- for complex operations (e.g. open-vial preparation or vial filling after sterile filtration, aseptic preparation, labelling of autologous cells), additional measures may be required in the immediate surrounding area of an open workstation to ensure a sterile product.

Closed procedures for dispensing are used whenever possible as an alternative to open-vial filling, especially for very small batches or individual patient preparations. The dispensing set (sterilising filter, needles, tubes and vials) that is used in closed aseptic dispensing operations is sterile. This can be achieved by sterilisation of the dispensing set, or by using sterile components. These sterile components are assembled and connected in a grade A air-supply area located in a grade C area with respect to air cleanliness. The process of closed aseptic dispensing can be performed in an area that is at least grade C with respect to air cleanliness.

Monitoring of critical grade A air-supply areas and the background environment for particulates and microbial contamination is carried out on a regular basis. When sterilising filtration is used to sterilise the preparation, the filter is tested for integrity before administration of the preparation to the patient. Filter-integrity testing for each type of preparation, for example by bubble-point determination, is validated.

Preparations that contain a radionuclide with a half-life shorter than 10 min are exempt from filter integrity testing before release of the product.

Where the administration is performed directly from the equipment to the patient, the filter used is suitable for direct human use.

Compatibility of the filter membrane and housing with the product solution is verified experimentally using the supplier specifications. In some cases it is not possible to find acceptable certified filters for certain applications (e.g. for hydrophobic radiopharmaceuticals). In these cases, filters need to be tested for bacterial endotoxin content, efficiency and product recovery.

3-9. *ANALYTICAL CONTROL*

All analytical systems are qualified and all methods validated according to the recognised standards (e.g. *ICH Guideline Q2 Validation of Analytical Procedures: Text and Methodology*). Wherever possible, quality control testing is carried out by a person other than the person who prepared the radiopharmaceutical.

3-9-1. **Starting materials**

Starting materials used for extemporaneous preparation comply with the general monograph *Substances for pharmaceutical use (2034)* and with their individual monographs where available.

For starting materials that are not present in the radiopharmaceutical (e.g. reagents removed by purification, catalysts, solvents, cartridges), specifications are verified by evaluation of the certificate of analysis provided by the manufacturer, completed if necessary by specific tests. If testing is not possible from a technical point of view, for example when using commercially available prefilled cassettes, testing can be omitted provided that the omission is backed up by a risk assessment. Specifications are adapted to the level of chemical and microbiological purity needed to ensure a suitable quality for the intended purpose in the radiosynthesis.

The identity of excipients included in the final formulation is verified by a suitable analytical method, unless they are licensed. Specifications are adapted to the level of purity required to ensure a quality suitable for a component of a pharmaceutical for injection, especially bioburden and bacterial endotoxin content.

Some radionuclides cannot be systematically evaluated by analysis before use in the radiosynthesis process. The suitability for the intended purpose is established each time a new batch of the target material is used or a modification of the radionuclide preparation process takes place.

For chemical precursors, (i) identity is verified by a suitable analytical method, (ii) suitability for radiosynthesis is verified by performing a complete radiosynthesis with a final radiopharmaceutical product complying with all its specifications (test synthesis), and (iii) specifications are verified by evaluation of the certificate of analysis provided by the manufacturer, completed if necessary by specific tests.

3-9-2. **Radiopharmaceuticals**

Extemporaneously prepared radiopharmaceuticals comply with the general monograph *Radiopharmaceutical preparations (0125)* and with individual monographs where available. Moreover, other applicable general monographs and general texts also apply, especially *Pharmaceutical preparations (2619)*, *5.1.1. Methods of preparation of sterile products* and *5.4. Residual solvents*.

Where no individual monograph or authorised summary of product characteristics exists, specifications and corresponding test methods need to be established for each

radiopharmaceutical. Table 5.19.-1 provides examples for determining the suitable analytical parameters and methods. Details on the measurement of radioactivity in test methods can be found in general chapter *2.2.66. Detection and measurement of radioactivity*. For each scheduled test, it is stated whether the result has to be available before release for use of the radiopharmaceutical. When a test is delayed until after release for use, this must be justified and a maximum period of delay for performing the test is established.

Table 5.19.-1. – *Examples of analytical parameters and methods for release of an extemporaneously prepared radiopharmaceutical*

| Test or parameter | Equipment and/or method |
|---|---|
| Characters, appearance | Visual inspection |
| Identity of radionuclide | Half-life determination; alpha spectrometry; beta spectrometry; gamma-ray spectrometry |
| Radiochemical identity | Liquid chromatography, thin-layer chromatography |
| Radiochemical purity | Liquid chromatography, thin-layer chromatography |
| Chemical purity | Liquid chromatography, thin-layer chromatography |
| Radionuclidic purity | Half-life determination; gamma-ray spectrometry, alpha spectrometry, beta spectrometry |
| Residual solvents | Gas chromatography |
| Pharmaceutical or physiological parameters | pH, osmolality |
| Microbiological contamination | Bacterial endotoxins, sterility |
| Radioactivity content, concentration | Ionisation chamber |
| Specific radioactivity | Liquid chromatography, ionisation chamber |
| Enantiomeric purity | Chiral chromatography |

3-10. *PACKAGING*

The primary packaging material is compatible with the preparation.

3-11. *LABELLING*

Where a radiopharmaceutical is prepared and used on the same site, the labelling of the primary packaging of the radiopharmaceutical indicates the identity and ensures traceability. It complies with the relevant national and European legislation.

The label typically states:

- the name of the preparation/active substance and/or its reference;
- an unequivocal reference to the preparation (batch number or date of the EPR);
- where applicable, a serial number for the dispensed unit (where several units are dispensed);
- the international symbol for radiation (trefoil).

Where applicable, the shielding labelling contains a reference to the patient (identification number or name).

For liquid and gaseous preparations, the total radioactivity in the container or the radioactive concentration per millilitre at a stated date and measurement time, and the volume of liquid in the container are stated on the shielding labelling.

For solid preparations (such as capsules), the total radioactivity at a stated date and, if necessary, measurement time are stated.

The labelling can be adapted in certain cases where, due to the extremely short half-life (i.e. less than 10 min) of the product, the preparation is used before all of the information is available.

In addition, the label on the shielding or on the outer package states:

- where applicable, the name of any excipients;
- the name of the manufacturer (site where the preparation was made);
- the route of administration;
- the period of validity or the expiry date;
- where applicable, any special storage conditions.

3-12. *RELEASE*

The decision to release a radiopharmaceutical as suitable for administration is dependent on the conformance of the analytical results to the specifications, and to the process data related to its preparation, especially in-process controls and monitoring (e.g. particle, microbial, environmental). However, due to the short-lived nature of radiopharmaceuticals, not all quality parameters of the preparation can be known at the time of release for administration. The list of analytical tests to be performed before release for administration is established according to section 3-9-2. Release for administration follows a written procedure indicating all relevant data (preparation, quality control, assessment of deviations, etc.) required. This procedure is based on a risk assessment. Retrospective examination of analytical results is acceptable in cases where the test results can technically not be obtained before administration of the radiopharmaceutical and is based on a risk assessment. The review and the release of the preparation before administration by the responsible person is confirmed in writing in the batch documentation.

A written procedure also describes the actions to be taken by the responsible person in case unsatisfactory test results are obtained after the preparation has been released (recall or provision of information to users of the preparation, depending on the time of discovery).

The final review and final release of the preparation by the responsible person is confirmed in writing in the batch documentation.

3-13. *RETENTION SAMPLES*

In the case of preparations without a marketing authorisation, retention samples are kept for a period of 1 month from the time all testing is completed or 1 month after expiry of the preparation, whichever is the longer. In the case of single vial dispensing, retention samples may not be available; this is carefully considered in any risk assessment. Where technically possible, the same approach is applied to chemical precursors and starting materials. No retention samples are needed for preparations of radiolabelled blood cells.

3-14. *PREPARATION OF RADIOLABELLED BLOOD CELLS*

During cell manipulation and radiolabelling, it is necessary to maintain both cell viability and sterility. Operator protection is of paramount importance, and operator exposure to biological and radiation hazards is avoided.

3-14-1. **Collection of blood cells and cellular components for radiolabelling and reinjection into the original donor/patient**

Blood cells and cellular components are collected in such a way as to preserve their function (use of a wide-bore needle, use of a syringe pre-coated with an appropriate anticoagulant, avoiding excessive centrifugation). The containers are suitably labelled with the patient's information in order to prevent mix-up. Quality requirements for all substances used in the separation of the cells are stated in the respective individual monographs, if available. The general monograph *Substances for pharmaceutical use (2034)* applies, whether or not an individual monograph is available. Further precautions may

be necessary where the use of heterologous cells is required, as provided in respective regulations.

A centrifuge, constructed to ensure containment in case of spills and/or breakage (with closed buckets), is required for blood-cell component separations. The equipment used in the labelling of cells is only used for one procedure (one patient) at a time, and single-use utensils are the preferred choice. The processing of samples from different patients is separated by a suitable period of time, and includes a cleaning/disinfection process for utensils and equipment that ensures the destruction of blood-borne pathogens and viruses.

3-14-2. **Radiolabelling of the cells**

Precautions are taken to prevent cross-contamination, cross-infection or mix-up of blood, and the introduction of microbial contamination. Radiolabelling conditions do not impair the integrity and/or viability of the cells. As terminal sterilisation is not possible, radiolabelling of cells is considered as an aseptic preparation (see section 3-8).

3-14-3. **Quality control**

Identification, calculation of the labelling yield and absence of aggregation or clumping of cells is assessed to verify the suitability of radiolabelled cells before release and reinjection/administration. At regular time intervals, testing for cell viability/integrity is performed.

Validation of the preparation of radiolabelled blood cells includes testing of cell viability, morphology or function depending on the cell type. Any changes to the procedure for preparation of radiolabelled cells is validated.



## GLOSSARY

**Automated module for synthesis and/or dispensing**. Electromechanical device controlled by software to automatically perform a sequence of operations needed for radiolabelling, purification, formulation, dispensing and/or sterilisation of a radiopharmaceutical.

**Cassette**. Single use production hardware consisting of a pre-assembled network of containers, valves and syringes, including or not the starting material set, intended to be mounted on a synthesis module in order to prepare an EPR.

**Closed method for radiosynthesis**. Method where a solution is never directly exposed to the environment during the radiosynthesis process, but is contained within the synthesis system (e.g. a cassette).

**Closed vial dispensing**. Method of dispensing, in which the solution to be filled after sterile filtration is not in direct contact with the environment and no aseptic connections are made in the system after the sterilising filter during the dispensing process.

**Open method for radiosynthesis or dispensing (open vial filling)**. Method where at some point during the process, a solution is directly exposed to the environment (*NOTE*: for dispensing, contact with the environment through a sterilising air filter is not considered a direct exposure).

**Responsible person**. Person designated as responsible for the release of a radiopharmaceutical, who meets the requirements provided by the national legislation.

**Starting material**. Substance used for the preparation of a medicinal product. Generators, chemicals for synthesis, ion-exchange resins, packing components and consumables are considered as starting materials.

**Starting material set**. Set of reagents, solvents and precursors in their usable forms for the EPR. Usable forms are mostly either a weighed amount of a solid or a volumetric sample of a suitable solution. Solids and liquids are often dispensed in closed vials for storage before use. Starting material sets may be available commercially or be prepared on-site from commercial or locally synthesised chemicals and packaging materials.

# 5.20. Metal catalyst or metal reagent residues

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# 5.20. ELEMENTAL IMPURITIES

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has adopted the ICH Q3D guideline for elemental impurities in medicinal products.

The European Pharmacopoeia (Ph. Eur.) applies this guideline to medicinal products (referred to hereunder and in the ICH Q3D guideline as 'drug products'), with the exception of products for veterinary use, unlicensed preparations and products excluded from the scope of the guideline. The guideline prescribes a permitted daily exposure (PDE) according to the route of administration for elements of toxicological concern which may be present in medicinal products. The guideline also describes some acceptable approaches to establishing concentrations of elemental impurities in medicinal products or components that would ensure that the PDEs for these impurities are not exceeded.

Parts of the introduction and the scope of the guideline are reproduced hereunder, and the full text is available from the ICH website.

*The drug products containing purified proteins and polypeptides (including proteins and polypeptides produced from recombinant or non-recombinant origins), their derivatives, and products of which they are components (e.g., conjugates) are within the scope of the guideline, as are drug products containing synthetically produced polypeptides, polynucleotides, and oligosaccharides.*

*The guideline does not apply to herbal products, radiopharmaceuticals, vaccines, cell metabolites, DNA products, allergenic extracts, cells, whole blood, cellular blood components or blood derivatives including plasma and plasma derivatives, dialysate solutions not intended for systemic circulation, and elements that are intentionally included in the drug product for therapeutic benefit. The guideline does not apply to products based on genes (gene therapy), cells (cell therapy) and tissue (tissue engineering). In some regions, these products are known as advanced therapy medicinal products.*

*The guideline does not apply to drug products used during clinical research stages of development. As the commercial process is developed, the principles contained in the guideline can be useful in evaluating elemental impurities that may be present in a new drug product.*

*Elemental impurities in drug products may arise from several sources; they may be residual catalysts that were added intentionally in synthesis or they may be present as impurities (e.g., through interactions with processing equipment or container/closure systems or by being present in components of the drug product). Because elemental impurities do not provide any therapeutic benefit to the patient, their levels in the drug product should be controlled within acceptable limits.*

*An applicant is not expected to tighten the limits based on process capability, provided that the elemental impurities in drug products do not exceed the PDEs. The PDEs established in the guideline are considered to be protective of public health for all patient populations. In some cases, lower levels of elemental impurities may be warranted when levels below toxicity thresholds have been shown to have an impact on other quality attributes of the drug product (e.g., element catalysed degradation of drug substances).*

# 5.21. Chemometric methods applied to analytical data

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# 5.21. CHEMOMETRIC METHODS APPLIED TO ANALYTICAL DATA

*The following chapter is published for information only. It is an introduction to the use of chemometric techniques for processing analytical data sets. The objective is to provide indications on good chemometric practice and requirements.*

1. GENERAL ASPECTS

1-1. *INTRODUCTION*

1-1-1. **Scope of the chapter**

This chapter is an introduction to the use of chemometric techniques for the processing of analytical data sets, which is an area of interest for research, quality control and manufacturing in the pharmaceutical industry. The objective is to provide information on the requirements for good chemometric practice and to also present a selection of established chemometric methods, but not an exhaustive review of these techniques, as refinements and innovations are constantly being introduced. The principles of the proposed methods will be briefly described along with their critical aspects and limitations. Mathematical details and algorithms are mostly omitted and a glossary is provided at the end of the chapter.

1-1-2. **Definition**

The actual definition of chemometrics is "the chemical discipline that uses mathematical and statistical methods, (a) to design or select optimal measurement procedures and experiments, and (b) to provide maximum chemical information by analysing chemical data".

From a more general point of view, chemometrics is not limited to chemical data and can contribute greatly to system understanding by analysing data, when limited knowledge and theory do not sufficiently explain observations and behaviour. Chemometric methods consist mainly of multivariate data-driven modelling techniques that result in empirical mathematical models that are subsequently used for the indirect prediction of properties of interest.

1-1-3. **Background**

Applications of chemometrics can be qualitative or quantitative, and it can help the analyst to structure the data set and to recognise hidden variable relationships within the system. However, it should be stressed that although such data-driven methods may be powerful, they would not replace a verified or established theory if available.

Chemometric methods have revolutionised near-infrared spectroscopy (NIR) and such techniques are now integral components of process analytical technology (PAT) and quality by design (QbD) for use in improved process monitoring and quality control in a variety of fields. Chemometric methods can be found throughout the scientific and technological community, with a principal but non-exclusive focus on life and health sciences such as agriculture, food, pharmacy, chemistry, biochemistry and genomics, but also other industries such as petrochemicals, textiles, sensors and cosmetics, with the potential to expand even further into other domains.

The associated mathematical principles have been understood since the early twentieth century, but chemometrics came of age with the development of digital technology and the related progress in the elaboration of mathematical algorithms. Many techniques and methods are based on geometric data representations, transformations and modelling. Later, mathematical and theoretical developments were also consolidated.

1-1-4. **Introducing chemometrics**

In chemometrics, a property of interest is evaluated solely in terms of the information contained in the measured samples. Algorithms are applied directly to the data set, and information of interest is extracted with models (modelling or calibration step). Chemometrics is associated with multivariate data analysis, which usually depends less on assumptions about the distribution of the data than many other statistical methods since it rarely involves hypothesis testing. During modelling the most sensitive changes in properties of interest can be amplified, while the less relevant changes in disturbing factors, whatever their origin, i.e. physical, chemical, experimental or instrumental variation, are minimised to the level of noise.

A model in chemometrics is a prediction method and not a formal or simplified representation of a phenomenon from physics, chemistry, etc. The ability of a model to predict properties has to be assessed with regard to its performance. The best model or calibration will provide the best estimations of properties of interest. A useful model is one that can be trusted and used for decision-making, for example. Adoption of a model in decision-making must be based on acceptable, reliable, and well-understood assessment procedures.

In univariate analysis, identified variables in a system are analysed individually. However, in reality, systems tend to be more complex, where interactions and combination effects occur between sample variables and cannot be separated. Multivariate data analysis handles many variables simultaneously and the relationship within or between data sets (typically matrices) has to be rearranged to reveal the relevant information. In multivariate methods, the original data is often combined linearly to account as much as possible for the explainable part of the data and ideally, only noise will remain unmodelled. The model, when properly validated, can be used in place of costly and time-consuming measurements in order to predict new values.

Generally, projection techniques such as principal components analysis (PCA), principal components regression (PCR) or partial least squares regression (PLS) are recommended. However, the approach will be different depending on whether the data has been generated using experimental design (i.e. designed data) or has been collected at random from a given population (i.e. non-designed data). With designed data matrices, the variables are orthogonal by construction and traditional multilinear statistical methods are therefore well suited to describing the data within. However, in non-designed data matrices, the variables are seldom orthogonal, but are more or less collinear, which favours the use of multivariate data analysis.

1-1-5. **Qualitative and quantitative data analysis**

Qualitative data analysis can be divided into exploration, an unsupervised analysis where data from a new system is to be analysed, and classification, a supervised analysis where class-labels are predicted.

*Unsupervised analysis*

In exploratory data analysis, multivariate tools are used to gather an overview of the data in order to build hypotheses, select suitable analytical methods and sampling schemes, and to determine how multivariate analysis of current and future data of similar type can be performed. When the first exploratory treatment is finalised, classification can be subsequently carried out in the form of a secondary treatment, where samples are organised into specific groups or classes.

*Supervised analysis*

Classification is the process of determining whether or not samples belong to the same class as those used to build the model. If an unknown sample fits a particular model well, it is said to be a member of that class. Many analytical tasks fall into this category, e.g. materials may be sorted according to quality, physical grade and so on. Identity testing is a special

situation where unknown samples are compared with suitable reference materials, either by direct comparison or indirect estimation, e.g. using a chemometric model.

Quantitative data analysis, on the other hand, mainly consists of calibration, followed by direct application to new and unknown samples. Calibration consists of predicting the mathematical relationship between the property to be evaluated (e.g. concentration) and the variables measured.

1-2. *GOOD CHEMOMETRIC PRACTICE*

The following notation will be used in the chapter:

| | |
|---|---|
| $X$, $Y$ | data sets |
| $X$ | independent variable |
| $Y$ | dependent variable |
| $\boldsymbol{X}$, $\boldsymbol{Y}$ | matrices |
| $\boldsymbol{x}$, $\boldsymbol{y}$ | vectors |
| $x$, $y$ | scalar values |
| $i$, $j$ | indices, points |
| $x_i$ | $i^{th}$ value of vector $\boldsymbol{x}$ |
| $x_{i,j}$ | $i^{th}$ and $j^{th}$ value of matrix $\boldsymbol{X}$ |
| $\boldsymbol{X}^T$ | transpose of matrix $\boldsymbol{X}$ |
| $\boldsymbol{X}^{-1}$ | inverse (if it exists) of matrix $\boldsymbol{X}$ |
| $\hat{X}$ | estimate of matrix $\boldsymbol{X}$ |
| $\lvert\boldsymbol{X}\rvert$ | determinant of (square) matrix $\boldsymbol{X}$ |
| $\lvert\lvert\boldsymbol{x}\rvert\rvert$ | norm of vector $\boldsymbol{x}$ |
| $b$ | regression equation coefficient |
| $e$ | residuals of $X$ |
| $f$ | residuals of $Y$ |

1-2-1. **Figures of merits for regression**

In quantitative analysis, building a regression model involves fitting a mathematical relationship to the corresponding independent data ($X$) and dependent data ($Y$). The independent data may represent a collection of signals, i.e. responses from a number of calibration samples, while the dependent data may correspond to the values of an attribute, i.e. the property of interest in the calibration samples. It is advisable to test the regression model with internal and external test sets. The internal test set consists of samples that are used to build the model (or achieve calibration) by applying resampling within the calibration data and samples that are initially left out of the calibration in order to validate the model. Use of the internal test set is part of model optimisation and model selection. The external independent test set represents data that normally is available after the model has been fixed, thus the external test set challenges the model and tests its robustness for the analysis of future data.

1-2-1-1. *Root mean square error of prediction*

The link between $X$ and $Y$ is explored through a common set of samples (calibration set) from which both $x$ and $y$-values have been collected and are clearly known. For a second set of samples (validation set) the predicted $y$-values are then compared to the reference $y$-values, resulting in a prediction residual that can be used to compute a validation residual variance, i.e. a measure of the uncertainty of future predictions, which is referred to as root mean square error of prediction (*RMSEP*). This value estimates the average uncertainty that can be expected when predicting $y$-values for new samples. Since no assumptions concerning statistical error distribution are made during modelling, prediction error cannot be used to report a valuable statistical interval for the predicted values. Nevertheless, *RMSEP* is a good error estimate in cases where both calibration and validation sample sets are representative of future samples.

A confidence interval for predicted $y$-values would be $\pm\, n \times RMSEP$, with $n$ fixed by the operator. A common choice is $n = 2$. This choice should be dependent on the requirements of the specific analytical method.

Chemometric models can end up with better precision than the reference methods used to acquire calibration and testing data. This is typically observed for water content determinations by NIR and PLS where semi-micro determination titration (*2.5.12*) is the reference method.

1-2-1-2. *Standard error of calibration and coefficient of determination*

Figures of merit can be calculated to help assess how well the calibration fits the data. Two examples of such statistical expressions are the standard error of calibration (*SEC*) and the coefficient of determination ($R^2$).

*SEC* has the same units as the dependent variables and reflects the degree of modelling error, but cannot be used to estimate future prediction errors. It is an indication of whether the calculation using the calibration equation will be sufficiently accurate for its intended purpose. In practice *SEC* has to be compared with the error of the reference method (*SEL, Standard Error of Laboratory*, see Glossary). Usually *SEC* is larger than *SEL*, in particular if modelling does not account for all interferences in the samples or if other physical phenomena are present.

The coefficient of determination ($R^2$) is a dimensionless measure of how well the calibration fits the data. $R^2$ can have values between 0 and 1. A value close to 0 indicates that the calibration fails to relate the data to the reference values and as the coefficient of determination increases, the $X$-data becomes an increasingly more accurate predictor of the reference values. Where there is more than 1 independent variable, adjusted $R^2$ should be used rather than $R^2$, since the number of independent variables in the model inflates the latter even if the fraction of variance explained by the model is not increased.

1-2-2. **Implementation steps**

The implementation of chemometric methods varies case by case depending on the specific requirements of the system to be analysed. The following generic approach can be followed when analysing non-designed data sets:

- in formulating the study problem, define the precise objective of data collection and the expected analysis results;
- investigate the origin and availability of the data. The data set should cover the variation of the explored variable(s) or attribute(s);
- if the available data does not cover the expected variation, prepare and measure samples that fill the gap;
- variable selection: sometimes selecting the right variables can give more robustness and also enhance model accuracy;
- raw data may have to be transformed and mathematical pre-treatments performed;
- elaborate the model through calibration and validation;
- challenge the model and check its performance on new samples or data;
- validate the method according to current pharmaceutical usage and requirements.

1-2-3. **Data considerations**

1-2-3-1. *Sample quality*

Careful sample selection increases the likelihood of extracting useful information from the analytical data. Whenever it is possible to actively adjust selected variables or parameters according to an experimental design, the quality of the results is increased. Experimental design (also referred to as design of experiments, DoE) can be used to introduce

systematic and controlled changes between samples, not only for analytes, but also for interferences. When modelling, common considerations include the determination of which variables are necessary to adequately describe the samples, which samples are similar to each other and whether the data set contains related sub-groups.

1-2-3-2. *Data tables, geometrical representations*

Sample responses result in a group of numerical values relating to signal intensities (*X*-data), i.e. the independent variables. However, it should be recognised that these variables are not necessarily linearly independent (i.e. orthogonal) according to mathematical definitions. These values are best represented in data tables and by convention each sample is associated with a specific row of data. A collection of such rows constitutes a matrix, where the columns are the variables. Samples can then be associated with certain features reflecting their characteristics, i.e. the value of a physical or chemical property or attribute and these data are usually referred to as the *Y*-data, i.e. the dependent variables. It is possible to add this column of values to the sample response matrix, thereby combining both the response and the attribute of each sample.

When *n* objects are described by *m* variables the data table corresponds to an *n*×*m* matrix. Each of the *m* variables represents a vector containing *n* data values corresponding to the objects. Each object therefore appears as a point in an *m* dimensional space described by its *m* coordinate values (1 value for each variable in the *m* axes).

1-2-3-3. *First assessment of data*

Before performing multivariate data analysis, the quality of the sample response can be optionally assessed using statistical tools. Graphical tools are recommended for the 1st visual assessment of the data, e.g. histograms and/or boxplots for variables for evaluation of the data distribution, and scatter plots for detection of correlations. Descriptive statistics are useful for obtaining a rapid evaluation of each variable, taken separately, before starting multivariate analysis. For example, mean, standard deviation, variance, median, minimum, maximum and lower/upper quartile can be used to assess the data and detect out-of-range values and outliers, abnormal spread or asymmetry. These statistics reveal anomalies in a data table and indicate whether a transformation might be useful or not. Two-way statistics, e.g. correlation, show how variations in 2 variables may correlate in a data table. Verification of these statistics is also useful when reducing the size of the data table, as they help in avoiding redundancies.

1-2-3-4. *Outliers*

An outlier is a sample that is not well described by the model. Outliers can be *X* or *Y* in origin. They reflect unexpected interference in the original data or measurement error. The predicted data that is very different from the expected value calls into question the suitability of the modelling procedure and the range spanned by the original data. In prediction mode, outliers can be caused by changes in the interaction between the instrument and samples or if samples are outside the model's scope. If this new source of variability is confirmed and is relevant, the corresponding data constitutes a valuable source of information. Investigation is recommended to decide whether the existing calibration requires strengthening (updating) or whether the outliers should be ignored as uncritical or unrelated to the process (i.e. operator error).

In the case of classification an outlier test should be performed on each class separately.

1-2-3-5. *Data error*

Types of data error include random error in the reference values of the attributes, random error in the collected response data and systematic error in the relationship between the two. Sources of calibration error are problem specific, for example, reference method errors and errors due to either sample non-homogeneity or the presence of non-representative samples in the calibration set. Model selection during calibration usually accounts for only a fraction of the variance or error attributable to the modelled analytical technique. However, it is difficult to assess if this error is more significant than the reference method error or vice versa.

1-2-3-6. *Pre-processing and variable selection*

The raw data may not be optimal for analysis and are generally pre-processed before performing chemometric calculations to improve the extraction of physical and chemical information.

Interferences, for example background effects, baseline shifts and measurements in different conditions, can impede the extraction of information when using multivariate methods. It is therefore important to minimise the noise introduced by such effects by carrying out pre-processing operations.

A wide range of transformations (scaling, smoothing, normalisation, derivatives, etc.) can be applied to *X*-data as well as *Y*-data for pre-processing prior to multivariate data analysis in order to enhance the modelling. The main purpose of these transformations is focussing the data analysis on the pertinent variability within the data set. For example, pre-processing may involve mean centering of variables so that the mean does not influence the model and thus reduce the model rank.

The selection of the pre-processing is mostly driven by parameters such as type of data, instrument or sample, the purpose of the model and user experience. Pre-processing methods can be combined, for example standard normal variate (SNV) with 1st derivative, as an empirical choice.

1-2-4. **Maintenance of chemometric models**

Chemometric methods should be reassessed regularly to demonstrate a consistent level of acceptable performance. In addition to this periodical task, an assessment should be carried out for critical parameters when changes are made to application conditions of the chemometric model (process, sample sources, measurement conditions, analytical equipment, software, etc.).

The aim of maintaining chemometric models up-to-date is to provide applications that are reliable over a longer period of use. The extent of the validation required, including the choice of the necessary parameters, should be based on risk analysis, taking into account the analytical method used and the chemometric model.

1-3. *ASSESSMENT AND VALIDATION OF CHEMOMETRIC METHODS*

1-3-1. **Introduction**

Current use of the term 'validation' refers to the regulatory context as applied to analytical methods, but the term is also used to characterise a typical computation step in chemometrics. Assessment of a chemometric model consists of evaluating the performance of the selected model in order to design the best model possible with a given set of data and prerequisites. Provided sufficient data are available, a distribution into 3 subsets should always be considered: 1) a learning set to elaborate models, 2) a validation set to select the best model, i.e. the model that enables the best predictions to be made, 3) an independent test set to estimate objectively the performance of the selected final model. Introducing a 3rd set for objective model performance evaluation is necessary to estimate the model error, among other performance indicators. An outline is given below on how to perform a typical assessment of a chemometric model, starting with the initial validation, followed by an independent test validation and finally association/correlation with regulatory requirements.

1-3-2. **Assessment of chemometric models**

1-3-2-1. *Validation during modelling*

Typically, algorithms are iterative and perform self-optimisation during modelling through an on-going evaluation of performance criteria and figures of merit. This step is called validation. The performance criteria are specific to the chemometric technique used and to the

nature of the analytical data, as well as the purpose of the overall method which includes both the analytical side and the chemometric model. The objective of the validation is to evaluate the model and provide help to select the best performing model. Selected samples are either specifically assigned for this purpose or are selected dynamically through reselection/re-use of data from a previous data set (sometimes called resampling – for clarification, see Glossary). A typical example of data reselection is cross-validation with specific segments, for example 'leave-one-out' cross-validation when samples are only a few, or 'leave-subset-out' cross-validation (Figure 5.21.-1). Another type of resampling is bootstrapping.

1-3-2-2. *Assessment of the model*

Once the model matches the optimisation requirements, fitness for purpose is assessed. Independent samples not used for modelling or model optimisation are introduced at this stage as an independent test-set in order to evaluate the performance of the model. Ideally, when sufficient data are available, the sample set can be split into 3 subsets comprising 1 learning set for model computation, 1 validation set for optimisation of the model, and 1 test set for evaluation of the prediction ability, i.e. whether the model is fit for purpose. The 3 subsets are treated independently and their separation should be performed in such a way that model computation is not biased. The aim is to obtain a representative distribution of the samples within the 3 subsets with regard to their properties and expected values.

1-3-2-3. *Size and partitioning of data sets*

The size of the data set needed for building the calibration is dependent on the number of analytes and interfering properties that needs to be handled in the model. The size of the learning data set for calibration usually needs to be larger when the interfering variations are acquired randomly than when all major interferences are known and they can be varied according to a statistical experimental design. The lowest possible number of samples needed to cover the calibration range can be estimated from the corresponding design. The size of the independent test set should be in the order of 20-40 per cent of the samples used for the calibration model. However, when representative samples are abundant, the larger the test data set (above 40 per cent), the more reliably the prediction performance can be estimated. It is common practice to mix learning and model validation sets and as a result, the definitive assessment of the model relies on the separate test set.

1-3-3. **Validation according to the regulatory framework**

Validation principles and considerations are described in established international guidelines and apply to the validation of analytical methods. However, due to the special nature of data treatment and evaluation, as carried out in most chemometric methods, additional aspects have to be taken into account when validating analytical procedures. In this context, validation comprises both the assessment of the analytical method performance and the evaluation of the model. In some special cases, it might only be necessary to validate the chemometric model (see section 1-2-4.).

1-3-3-1. *Qualitative models*

For validation of qualitative models, the most critical parameters are specificity and robustness. When not applicable, scientific justification is required.

*Specificity*

During validation it has to be shown that the model possesses sufficient discriminatory capability. Therefore, a suitable set of materials that pose a risk of mix-up must be defined and justified. If, in addition to chemical identification, other parameters (such as polymorphism, particle size, moisture content, etc.) are relevant, a justification for these parameters should also be included. The selection of materials to be included when validating specificity should be based on logistic considerations (e.g. materials handled close to the process under review, especially those with similar appearance), chemical considerations (e.g. materials with similar structure) and also physical considerations where relevant (e.g. materials with different physical properties). After definition of this set of materials, the discriminatory ability of the chemometric method to reject them must be proven. Therefore, for each material a representative set of samples covering typical variance within the material has to be analysed and evaluated. If the specificity of the chemometric model is insufficient, the parameters of the model should be optimised accordingly and the method revalidated.

Whenever new elements that may potentially affect identification are introduced, e.g. new materials that are handled at the same site and represent a risk of mix-up, a revalidation of specificity should be carried out. This revalidation can be limited to the new element and does not necessarily need to encompass the complete set of elements, whose constituents may not all be affected by the change.

If properties of materials change over time (e.g. batches of materials with lower or higher particle size, lower or higher moisture content etc.) and these changes become relevant, they should also be included as part of the validation. This can be achieved for example, by an amendment to the validation protocol and does not necessarily require a complete revalidation of the chemometric model.

To assess specificity, the number of false-positive and false-negative errors can be evaluated by classification of the test set.

*Robustness*

For validation of robustness, a comprehensive set of critical parameters (e.g. process parameters such as temperature, humidity, instrumental performance of the analytical equipment) should be considered. The reliability of the analytical method should be challenged by variation of these parameters. It can be advantageous to use experimental design (DoE) to evaluate the method.

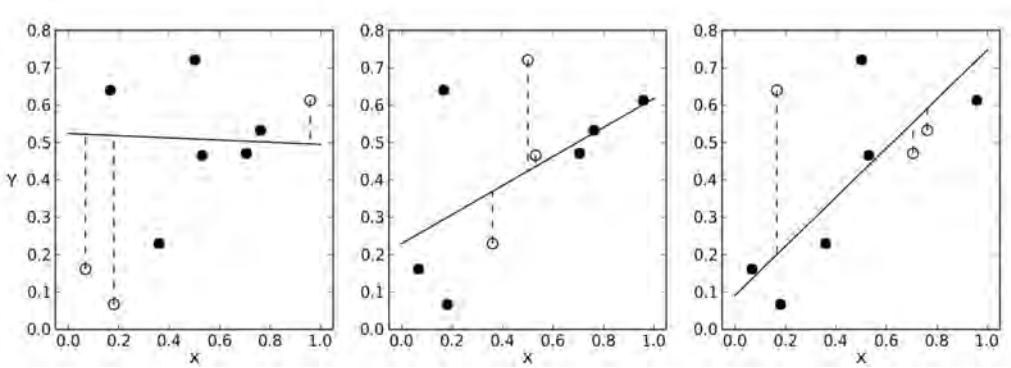


Figure 5.21.-1. – *Cross-validation with leave subset of 3 out applied to linear regression. Regression model data = ●. Subset used for test = ○. The errors of fit (interrupted lines) are collected to form the cumulated cross-validation error.*

To assess robustness the number of correct classifications, correct rejections, false-positive and false-negative errors can be evaluated by classification of samples under robustness conditions.

1-3-3-2. *Quantitative models*

The following parameters should be addressed unless otherwise justified: specificity, linearity, range, accuracy, precision and robustness.

*Specificity*

It is important to detect that the sample that is quantified is not an outlier with respect to the calibration space. This can be done using the correlation coefficient between the sample and the calibration mean, as well as Hotelling $T^2$ among others.

*Linearity*

Linearity should be validated by correlating results from the chemometric model with those from an analytical reference method. It should cover the entire range of the method and should involve a specifically selected set of samples that is not part of the calibration set. For orientation purposes, a 'leave-subset-out' cross-validation based on the calibration set may be sufficient, but should not replace assessment using an independent test set. Linearity can be evaluated through the correlation coefficient, slope and intercept of predicted values versus reference values.

*Range*

The range of analyte reference values defines the range of the chemometric model, and its lower limit determines the limits of detection and quantification of the analytical method. Controls must be in place to ensure that results outside this range are recognised as such and identified. Within the range of the model, acceptance criteria for accuracy and precision have to be fulfilled.

*Accuracy*

The accuracy of the chemometric model can be determined by comparison of analytical results obtained from the chemometric model with those obtained using a reference method. The evaluation of accuracy should be carried out over the defined range of the chemometric model using an independent test set. It may also be helpful to assess the accuracy of the model using a 'leave-subset-out' cross-validation, although, this should not replace assessment using an independent test set. Accuracy can be evaluated through the slope and intercept of predicted values versus reference values.

*Precision*

The precision of the analytical method should be validated by assessing the standard deviation of the measurements performed through the chemometric model. Precision covers repeatability (replicate measurements of the same sample by the same person on the same day) and intermediate precision (replicate measurements of the same sample by another person on different days). Precision should be assessed at different analyte values covering the range of the chemometric model, or at least at a target value.

*Robustness*

For validation of robustness, the same principles as described for qualitative methods apply. Extra care should be taken to investigate the effects of any parameters relevant for robustness on the accuracy and precision of the chemometric model. It can be an advantage to evaluate these parameters using experimental design.

The chemometric model can also be investigated using challenge samples, which may be samples with analyte concentrations outside the range of the method or samples of different identity. During the validation, it must be shown that these samples are clearly recognised as outliers.

## 2. CHEMOMETRIC TECHNIQUES

A non-exhaustive selection of chemometric methods are discussed below. A map of the selected methods is given in Figure 5.21.-2.

### 2-1. *PRINCIPAL COMPONENTS ANALYSIS*

#### 2-1-1. **Introduction**

The complexity of large data sets or tables makes human interpretation difficult without supplementary methods to aid in the process. Principal components analysis (PCA) is a projection method used to visualise the main variation in the data. PCA can show in what respect 1 sample differs from another, which variables contribute most to this difference and whether these variables contribute in the same way and are correlated or are independent of each other. It also reveals sample set patterns or groupings within the data set. In addition, PCA can be used to estimate the amount of useful information contained in the data table, as opposed to noise or meaningless variations.

#### 2-1-2. **Principle**

PCA is a linear data projection method that compresses data by decomposing it to so-called latent variables. The procedure yields columns of orthogonal vectors (scores), and rows of orthonormal vectors (loadings). The principal components (PCs), or latent variables, are a linear combination of the original variable axes. Individual latent variables can be interpreted via their connection to the original variables. In essence, the same data is shown but in a new coordinate system. The relationships between samples are revealed by their projections (scores) on the PCs. Similar samples group together in respect to PCs. The distance between samples is a measure of similarity/dissimilarity.

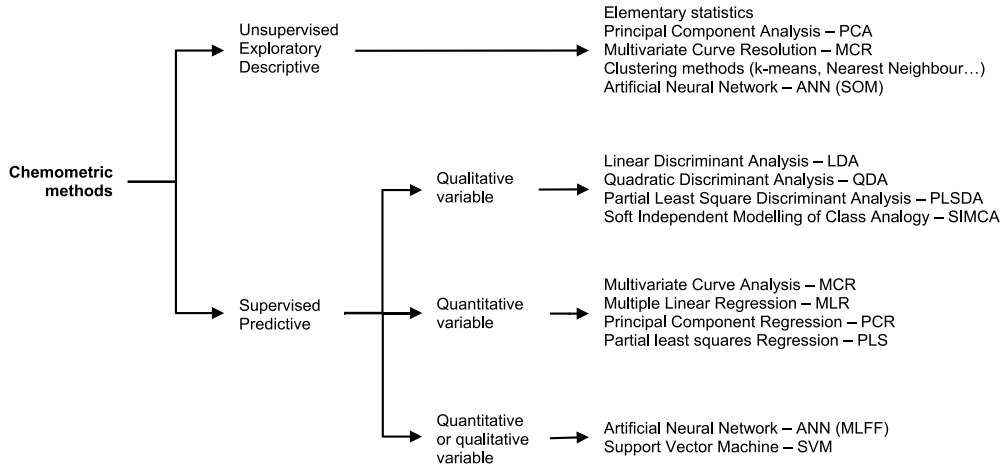


Figure 5.21.-2. – *Map of chemometric methods discussed in the chapter*

The original data table is transformed into a new, rearranged matrix whose structure reveals the relationships between rows and columns that may be hidden in the original matrix (Figure 5.21.-3). The new structure constitutes the explained part of the original data. The procedure models the original data down to a residual error, which is considered the unexplained part of the data and is minimised during the decomposition step.

The underlying idea is to replace a complex data table with a simpler counterpart version having fewer dimensions, but still fitting the original data closely enough to be considered a good approximation (Figure 5.21.-4). Extraction of information from a data table consists of exploring variations between samples, i.e. finding out what makes a sample different from or similar to another. Two samples can be described as similar if they have similar values for most variables. From a geometric perspective, the combination of measurements for 1 sample defines a point in a multidimensional space with as many dimensions as there are variables. In the case of close coordinates the 2 points are located in the same area or volume. With PCA, the number of dimensions can be reduced while keeping similar samples close to each other and dissimilar samples further apart in the same way as in the multidimensional space, but compressed into an alternate lower dimensional coordinate system.

The principle of PCA is to find the directions in the data space that describe the largest variation of the data set, i.e. where the data points are furthest apart. Each direction is a linear combination of the initial variables that contribute most to the actual variation between samples. By construction, principal components (PCs) are orthogonal to each other and are also ranked so that each carries more information than any of those that follow. Priority is therefore given to the interpretation of these PCs, starting with the 1st, which incorporates the greatest variation and thereby constitutes an alternative less complex system that is more suitable for interpreting the data structure. Normally, only the 1st PCs contain pertinent information, with later PCs being more likely to describe noise. In practice, a specific criterion is used to ensure that noise is not mistaken for information and this criterion should be used in conjunction with a method such as cross-validation or evaluation of loadings in order to determine the number of PCs to be used for the analysis. The relationships between samples can then be subsequently viewed in 1 or a series of score plots. Residuals $\hat{\boldsymbol{E}}$ keep the variation that is not included in the model, as a measure of how well samples or variables fit that model. If all PCs were retained, there would be no approximation at all and the gain in simplicity would consist only of ordering the variation of the PCs themselves by size. Deciding on the number of components to retain in a PCA model is a compromise between simplicity, robustness and goodness of fit/performance.

2-1-3. **Assessment of model**

Total explained variance $R^2$ is a measure of how much of the original variation in the data is described by the model. It expresses the proportion of structure found in the data by the model. Total residual and explained variances show how well the model fits the data. Models with small total residual variance (close to 0 per cent) or large total explained variance (close to 100 per cent) can explain most of the variation in the data. With simple models consisting of only a few components, residual variance falls to 0; otherwise, it usually means that the data contains a large amount of noise. Alternatively, it can also mean that the data structure is too complex to be explained using only a few components. Variables with small residual variance and large explained variance for a particular component are well defined by the model. Variables with large residual variance for all or the 1st components have a small or moderate relationship with other variables. If some variables have much larger residual variance than others for all or the 1st components, they may be excluded in a new calculation and this may produce a model that is more suitable for its purpose. Independent test set variance is determined by testing the model using data that was not used in the actual building of the model itself.

2-1-4. **Critical aspects**

PCA catches the main variation within a data set. Thus comparatively smaller variations may not be distinguished.

2-1-5. **Potential use**

PCA is an unsupervised method, making it a useful tool for exploratory data analysis. It can be used for visualisation, data compression, checking groups and trends in the data, detecting outliers, etc.

For exploratory data analysis, PCA modelling can be applied to the entire data table once. However, for a more detailed overview of where a new variation occurs, evolving factor analysis (EFA) can be used and, in this case, PCA is applied in an expanding or fixed window, where it is possible to identify, for example, the manifestation of a new component from a series of consecutive samples.

PCA also forms the basis for classification techniques such as SIMCA and regression methods such as PCR. The property of PCA to capture the largest variations in the 1st principal components allows subsequent regression to be based on fewer latent variables. Examples of utilising components as independent data in regression are PCR, MCR, and ANN.

PCA is used in multivariate statistical process control (MSPC) to combine all available data into a single trace and to apply a signature for each unit operation or even an entire manufacturing process based on, for example, Hotelling $T^2$ statistics, PCA model residuals or individual scores. In addition to univariate control charts, 1 significant advantage with PCA is that it can be used to detect multivariate outliers, i.e. process conditions or process output that has a different correlation structure than the one present in the previously modelled data.

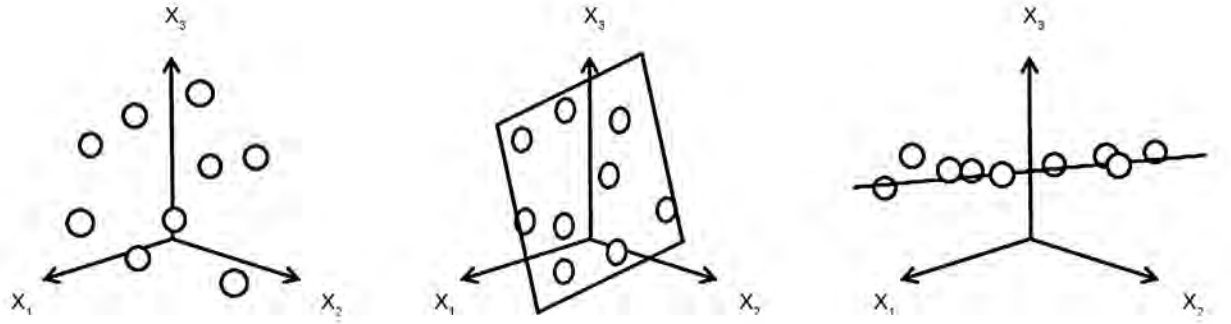


Figure 5.21.-3. – *Geometrical representation of 3 different X-data sets. On the left, objects are plotted in the multivariate space, and the following examples reveal a hidden structure, i.e. a plane and a line respectively*

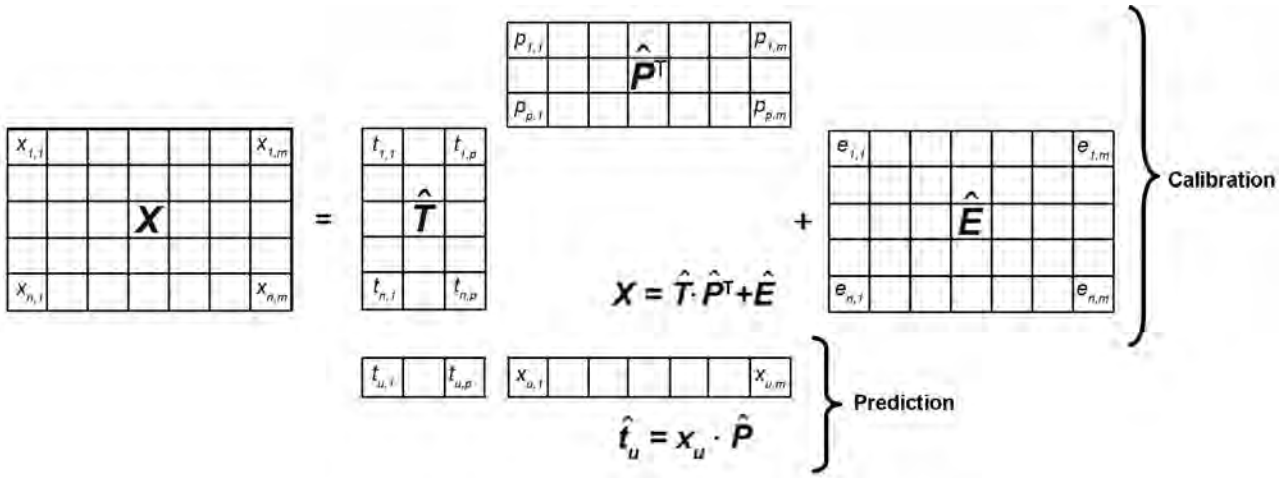


$\boldsymbol{X}$ = original data matrix of $n$ rows and $m$ columns
$\hat{\boldsymbol{T}}$ = score matrix with $n$ rows and $p$ columns
$\hat{\boldsymbol{P}}^T$ = loadings matrix with $p$ rows and $m$ columns
$\hat{\boldsymbol{E}}$ = residual matrix (same size as matrix $\boldsymbol{X}$)
$m$ = number of data points (variables)
$n$ = number of measurements (samples)
$p$ = number of factors
$\boldsymbol{x}_u$ = data of unknown sample
$\hat{\boldsymbol{t}}_u$ = score values for unknown sample

Figure 5.21.-4. – *Decomposition of the* ***X****-matrix for principal components analysis (PCA)*

2-2. *MEASURES BETWEEN OBJECTS*

*The primary use of the following algorithms is to measure the degree of similarity between an object and a group or the centre of the data.*

2-2-1. **Similarity measures**

Calculation of the correlation is the simplest statistical tool used to compare data and to determine the degree of similarity, provided the data sets have the same dimension, e.g. spectral data. It is a measure of the linear association between a pair of vectors. A correlation score between -1 and +1 is calculated for the match, based on the system below, where a perfect match (mirror image) would have a score of +1 and 2 lines that are complete opposites would have a score of -1 (Figure 5.21.-5).

Correlation is used to compare data sets in any of the following ways:

- comparison of 2 selected samples;
- comparison of 1 or more selected samples represented by vectors with a reference data library (from a group or class).

The reference data can be the average of a group of typical characteristics.

Correlation $r$ between 2 mean centred vectors $\boldsymbol{x}$ and $\boldsymbol{y}$ of the same dimension can be calculated using the following equation:

$$r = \frac{\Sigma_i x_i y_i}{\sqrt{\Sigma_i x_i^2 \Sigma_i y_i^2}}$$

2-2-2. **Distance measures**

In the object space, a collection of objects will be seen as points that are more or less close to each other and will gather into groups or clusters. Measuring the distance between points will express the degree of similarity between objects. In the same way, measuring the distance of a point to the centre of a group will give information about the group membership of this object. The following algorithms are given to illustrate the way objects can be compared.

2-2-2-1. *Euclidean distance*

The Euclidean distance $ed_{i,j}$ between 2 points $i$ and $j$ can be calculated as:

$$ed_{i,j} = \sqrt{\sum_{k=1}^{m} (x_{i,k} - x_{j,k})^2}$$

Similarly, the Euclidean distance $ed_{i,c}$ between the point $i$ and the centre $c$ of the data can be calculated as the square root of the sum of the squared differences of the coordinates of point $i$ to the mean value of the $x$-coordinates for each of the $m$ axes, which can be expressed by the following matrix notation:

$$ed_{i,c} = \sqrt{(x_i - \overline{x})(x_i - \overline{x})^T}$$

where $x_i$ denotes the $m$ values of coordinates describing the point $i$ and $\overline{x}$ denotes the mean coordinates calculated for the $m$ variables. The superscript T indicates that the 2nd term of the equation is transposed.

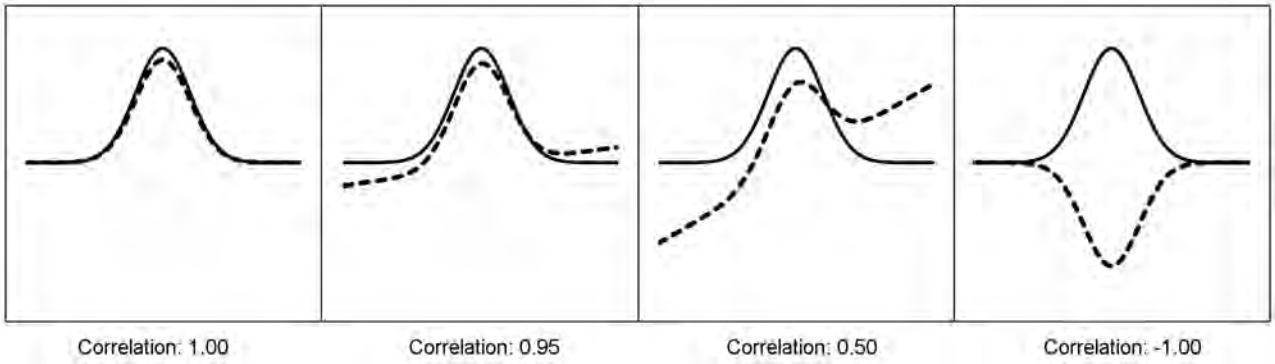


Figure 5.21.-5. – *Examples of correlation scores illustrated by matching of the shapes*

2-2-2-2. *Mahalanobis distance*

The Mahalanobis distance (*md*) takes into account the correlation between variables by using the inverse of the variance-covariance matrix.

The variance-covariance matrix $\boldsymbol{C}_x$ is calculated using the following equation:

$$\boldsymbol{C}_x = (1/(n-1))\boldsymbol{X}_c^{\mathrm{T}}\boldsymbol{X}_c$$

where $\boldsymbol{X}_c$ is the $n \times m$ data matrix centred over the mean of each column. Thus $\boldsymbol{C}_x$ is a square matrix that contains the variance of each variable over its diagonal and the covariance between variables on both sides of the diagonal. The Mahalanobis distance of point *i* to the centre *c* of the data is given by the following equation:

$$md_{i,c} = \sqrt{(x_i - \bar{x})\boldsymbol{C}_x{}^{-1}(x_i - \bar{x})^{\mathrm{T}}}$$

The $\boldsymbol{C}_x^{-1}$ matrix is the inverse of the variance-covariance matrix and $\boldsymbol{C}_x\boldsymbol{C}_x^{-1} = \boldsymbol{I}$, where $\boldsymbol{I}$ is the identity matrix. The number of variables or principal components involved in calculating the distance is designated *p*, and *n* is the number of objects in the group or in the data set. Under the assumption that the data is normally distributed, the random variable $(n\text{-}1)^2/n \times md^2$ is beta distributed with degrees of freedom $u = p/2$ and $v = (n\text{-}p\text{-}1)/2$. Thus, if for a point $x_i$ this expression exceeds the (1 - α)-quantile of the beta distribution then the point can be classified as an outlier with the significance level α (i.e. α is the probability of the type-I error classifying the point as an outlier although it is not).

In the same way, the leverage effect (*h*) of a data point located at the extremity of the *X*-space on the regression parameters of a multivariate model can be calculated using the following equation:

$$h = \frac{1}{n} + \frac{(md_{i,c})^2}{(n-1)}$$

Data points with high leverage have a large influence on the model.

2-2-2-3. *Critical aspects*

Euclidean distances only express the similarities or differences between data points when the variables are strictly uncorrelated. If correlations between variables exist they contain at least partially the same information and the dimensionality of data space is in fact smaller than the number of variables. Mahalanobis distances allow for the correction of correlations but their calculation supposes the variance-covariance matrix to be invertible. In some instances where there is high collinearity in the data set, this matrix is singular and cannot be inverted. This is especially the case with spectroscopic data where the high resolution of spectrometers introduces redundancy by essentially describing the same signal through measurements at several consecutive wavelengths. Another constraint in variance-covariance matrix inversion is that the number of variables has to be smaller than the number of objects ($n>m$).

Distances can be computed in the PC space, thus providing the benefits of reduced dimensionality, orthogonality between PCs and also PC ordering. As the 1st PCs carry the maximum amount of information, data reduction without loss of information can be achieved by eliminating the later insignificant PCs.

If a sufficient number of PCs are used to closely model the data, the Euclidean distance of data points to the centre of the data set will be identical when calculated from PC scores and from the coordinates on the original variables. This can be understood by considering that PCA calculation does not transform data but only extracts latent variables to describe the data space without distorting it. The same applies when using Mahalanobis distances, where the values are identical whether the original data space or that of the PCs is used. The only difference is the simplification of the calculation of Mahalanobis distances. Due to the orthogonality between PCs, Mahalanobis distances can be computed as the Euclidean distances calculated over the range of the normalised scores using the multiplication factor $\sqrt{n-1}$.

2-2-3. **Linear and quadratic discriminant analysis**

2-2-3-1. *Principle*

In Linear Discriminant Analysis and Quadratic Discriminant Analysis (LDA, QDA), the assignment of a test object $x_i$ to one of *K* predefined groups (or classes) identified in the data set is determined by the classification score, which is given in the following equation for LDA:

$$cf(x_{i,K}) = (x_i - \bar{x}_K)^{\mathrm{T}}\boldsymbol{C}^{-1}(x_i - \bar{x}_K) + \ln|\boldsymbol{C}| - 2\ln(\pi_K)$$

where $\pi_K$ is the prior probability of group *K* and is equal to the number of objects contained in group *K* divided by the total number of objects in the training set. $\boldsymbol{C}$ is the variance-covariance matrix and $|\boldsymbol{C}|$ is its determinant.

2-2-3-2. *Critical aspects*

LDA assumes that the variance-covariance matrix for all classes is identical, while QDA estimates a variance-covariance matrix for each class. Hence in QDA far more parameters need to be estimated which should only be done if sufficient data are available.

2-2-3-3. *Potential use*

It can be used in the case of straightforward classification schemes.

2-3. *SOFT INDEPENDENT MODELLING OF CLASS ANALOGY*

2-3-1. **Introduction**

Soft independent modelling of class analogy (SIMCA) is a method for supervised classification of data. The method requires a training set, which consists of samples with known attributes that have been pre-assigned to different classes. SIMCA classes can be overlapping and share common elements. Therefore, a sample can belong to 1, multiple or none of the classes.

2-3-2. **Principle**

PCA models are 1st established for individual classes. The samples of the training set have to be analysed by PCA (see the section on PCA) and for each class a distinct principal components model is generated. The number of relevant principal components can be adjusted for each class of objects separately. According to this procedure the data sets of each class can be reduced to the relevant principal components models.

New objects are then classified based on the individual PCA models. A new object is projected into each of these models and assigned to a certain class when its residual distance from this model is below the limit for this class (Figure 5.21.-6). Distances of objects to the respective classes can be calculated by procedures such as either Euclidean or Mahalanobis distance. Consequently an object may belong to either 1 or multiple classes if the corresponding distances are within the required threshold. If the distance of an object to all of the SIMCA classes is above the threshold, then it will be classified as an outlier.

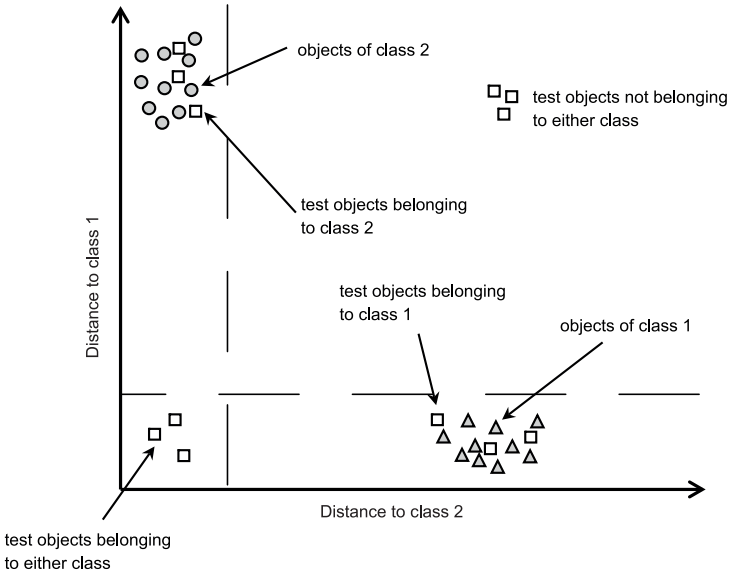


Figure 5.21.-6. – *Plot representing the 4 possible classifications of test objects in a two-class SIMCA analysis (□ = unknown sample to classify, Δ = class 1 sample, ○ = class 2 sample)*

2-3-3. **Critical aspects**

Since SIMCA is mainly based on PCA principles, the validation of the method should follow that of PCA. In addition to this, the overlap of different classes must also be taken into account. For example, a molecule can have several chemical groups that appear in its spectroscopic profile. Thus, grouping such data into chemical subgroups results in overlap since separation is not possible.

2-3-4. **Potential use**

SIMCA is often used for the classification of analytical data from techniques such as near-infrared (NIR) or mass spectroscopy, and other analytical techniques such as chromatography and chemical imaging. SIMCA is more suitable than PCA for discriminating between classes that are difficult to separate.

2-4. *CLUSTERING*

2-4-1. **Introduction**

A cluster consists of a group of objects or data points similar to each other. Clustering tools can be used to visualise how data points 'self-organise' into distinct groups or to highlight the degree of similarity between data objects. Data points from a particular cluster share some common characteristics that differentiate them from those gathered in other clusters. Clusters are characterised using 3 main properties; size, shape and distance to the nearest cluster. Clustering is an unsupervised method of data analysis and is used either for explanatory or confirmatory analysis. It differs from discriminant analysis, which is a supervised classification technique, where an unlabelled object is assigned to a group of pre-classified objects.

2-4-2. **Principle**

Numerous data clustering approaches are available and are typically classed as either hierarchical or non-hierarchical. Hierarchical clustering leads to the classical dendrogram graphical representation of the data, whereas non-hierarchical clustering finds clusters without imposing a hierarchical structure. Numerous algorithms are described in the literature, where data is partitioned either in a specific way or by optimising a particular clustering criterion. This simple and exclusive distinction is incomplete since mixed algorithms have similarities to both approaches. Hierarchical clustering recursively finds clusters either in agglomerative (bottom-up) or divisive (top-down) mode to form a tree shaped structure. Agglomerative mode starts with defining each data point as its own cluster and then merging similar clusters in pairs before repeating this step until the complete data set is classified (Figure 5.21.-7). Divisive mode starts by considering the entire data set as a single cluster, which is then recursively divided until only clusters containing a unique data point are obtained. Algorithms differ in the way they calculate the similarity between clusters. Complete link and single link algorithms calculate the distance between all pairs of objects that belong to different clusters in order to evaluate the similarity between them. In the single link method, this distance corresponds to the minimum distance separating 2 objects originating from 2 different clusters whereas in complete link algorithm this distance corresponds to the largest distance between 2 objects from 2 different clusters. Ward's algorithm, also called the minimum variance algorithm, calculates the similarity between clusters by means of decreasing cluster variance when the 2 most similar clusters are merged.

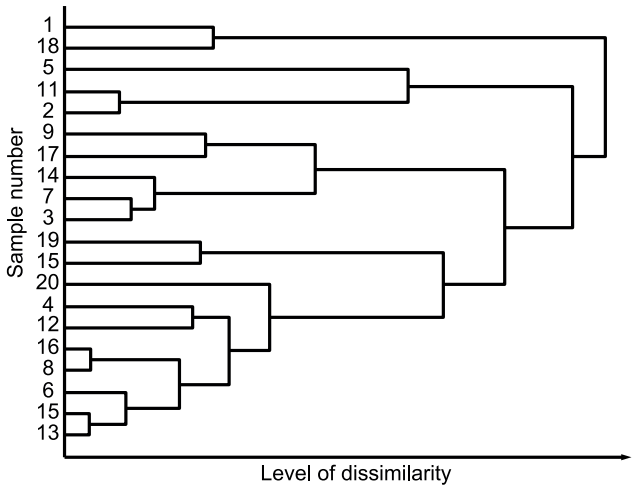


Figure 5.21.-7. – *Dendrogram for agglomerative hierarchical clustering until clusters containing a unique data point are obtained*

Non-hierarchical clustering cannot be described and categorised as easily as hierarchical clustering. Different algorithms exist, which give rise to different classification schemes. An overview of the different categories of algorithms is given below ranging from simple distance based methods such as the minimum spanning tree and the nearest neighbour algorithms, to more sophisticated methods such as the *K*-means algorithm (often cited as a classical partition

method), the expectation-maximisation algorithm (for 'model-based' methods) and DBSCAN for 'density-based' algorithms and, also, the 'grid-based' methods which are exemplified by the statistical information grid (STING) algorithm.

Minimum spanning tree clustering, such as Kruskal's algorithm, is similar to the graph theory algorithm as all the data points are first of all connected by drawing a line between the closest points. When all data points are linked, the lines of largest length are broken, leaving clusters of closely connected points. For nearest neighbour clustering, an iterative procedure is used to assign a data point to a cluster when the distance between this point and its immediate neighbour (that belongs to a cluster) is below a pre-defined threshold value.

The *K*-means algorithm is one of the most popular and as with partition algorithms, the number of clusters must be chosen *a priori*, together with the initial position of the cluster centres. A squared error criterion measures the sum of the squared distance between each object and the centroid of its corresponding cluster. The *K*-means algorithm starts with a random initial partition and progresses by reassigning objects to clusters until the desired criteria reach a minimum. Some variants of the *K*-means algorithm allow the splitting or merging of clusters in order to find the optimum number of clusters, even when starting from an arbitrary initial clustering. Model-based clustering attempts to find the best fit for the data using a preconceived model. An example of this is the EM or expectation-maximisation algorithm, which assigns each object to a particular cluster according to the probability of membership for that object. In the EM algorithm, the probability function is a multivariate Gaussian distribution and that is iteratively adjusted to data by use of the maximum-likelihood estimation. The EM algorithm is considered as an extension of the *K*-means algorithm since the residual sum of squares used for *K*-means convergence is similar to the maximum-likelihood criterion.

Density-based (DB) clustering, such as the DBSCAN algorithm, assimilates clusters to regions of high density separated by regions of low or no density. The neighbourhood of each object is examined to determine the number of other objects that fit within a specified radius and a cluster is defined when a sufficient number of objects inhabit this neighbourhood.

Grid-based algorithms, such as STING, divide the data space into a finite number of cells. The distribution of objects within each cell is then computed in terms of mean, variance, minimum, maximum and type of distribution. There are several levels of cells, providing different levels of resolution and each cell of a particular level corresponds to the union of 4 child cells from the lower level.

2-4-3. **Critical aspects**

Algorithms are sensitive to the starting conditions used to initialise the clustering of data. For example, *K*-means needs a pre-set number of clusters and the resultant partitioning will vary according to the chosen number of clusters. The metrics used in distance calculation will also influence data clustering. For Euclidean distances, the *K*-means algorithm will define spherical clusters whereas they could be ellipsoidal when using Mahalanobis distances. The cluster shape can be modified by data pre-treatments prior to cluster analysis. DB algorithms can deal with arbitrarily shaped clusters, but their weakness is their limitation in handling high-dimensional data, where objects are sparsely distributed among dimensions.

When an object is considered to belong to a cluster with a certain probability, algorithms such as density based clustering, allow a soft or fuzzy clustering. In this case, the border region of 2 adjacent clusters can house some objects belonging to both clusters.

2-4-4. **Potential use**

Clustering is an exploratory method of analysis that helps in the understanding of data structure by grouping objects that share the same characteristics and in addition, hierarchical clustering allows for classification within data objects. Clustering is used in a vast variety of fields, in particular for information retrieval from large databases. For the latter, the term 'data mining' is frequently used, where the objective is to extract hidden and unexploited information from a large volume of raw data in search of associations, trends and relationships between variables.

2-5. *MULTIVARIATE CURVE RESOLUTION*

2-5-1. **Introduction**

Multivariate curve resolution (MCR) is related to principal components analysis (PCA) but, where PCA looks for directions that represent maximum variance and are mutually orthogonal, MCR strives to find contribution profiles (i.e. MCR scores) and pure component profiles (i.e. MCR loadings). MCR is also known as self-modelling curve resolution (SMCR) or end-member extraction. When optimising MCR parameters the alternating least squares (ALS) algorithm is commonly used.

2-5-2. **Principle**

MCR-ALS estimates the contribution profiles $\boldsymbol{C}$ and the pure component profiles $\boldsymbol{S}$ from the data matrix $\boldsymbol{X}$, i.e. $\boldsymbol{X} = \boldsymbol{C}\cdot\boldsymbol{S}^{T} + \boldsymbol{E}$ just as in classical least squares (CLS). The difference between CLS and ALS is that ALS is an iterative procedure that can incorporate information that is known about the physico-chemical system studied and use this information to constrain the components/factors. For example, neither contribution nor absorbance can be negative by definition. This fact can be used to extract pure component profiles and contributions from a well-behaved data set. There are also other types of constraints that may be used, such as equality, unimodality, closure and mass balance.

It is often possible to obtain an accurate estimation of the pure component spectra or the contribution profiles and these estimates can then be used as initial values in the constrained ALS optimisation. New estimates of the profile matrix $\boldsymbol{S}$ and of the contribution profile $\boldsymbol{C}$ are obtained during each iteration. In addition, the physical and chemical knowledge of the system can be used to verify the result, and the resolved pure component contribution profiles should be explainable using existing knowledge. If the MCR results do not match the known system information, then other constraints may be needed.

2-5-3. **Critical aspects**

Selection of the correct number of components for the ALS calculations is important for a robust solution and a good estimate can be obtained using for example, evolving factor analysis (EFA) or fixed-size moving window EFA. Furthermore, the constraints can be set as either 'hard' or 'soft', where hard constraints are strictly enforced while soft constraints leave room for deviations from the restricted value. Generally, due to inherent ambiguities in the solution obtained, the MCR scores will need to be translated into, for example, the concentration of the active pharmaceutical ingredient, using a simple linear regression step. This means that the actual content must be known for at least 1 sample. When variations of 2 or more chemical entities are in some way correlated, rank deficiency occurs, for example 1 entity is formed while the other is consumed, or 2 entities are consumed at the same rate to yield a third. As a result, the variation of the individual substance is essentially masked and in such cases, simultaneous analysis of data from independent experiments using varied conditions or combined measurements from 2 measurement techniques generally results in better strategies than analysing the experiments separately one by one.

2-5-4. **Potential use**

MCR can be applied when the analytical method produces multivariate data for which the response is either linear or can be linearised. This has the advantage that only 1 standard is needed per analyte, which is particularly beneficial when the

measurements are at least partly selective between analytes. When linearity and selectivity is an issue, more standards per analyte may be required for calibration. When there is no pure analytical response for an analyte, it is also possible to estimate starting vectors by applying PCA to analyte mixtures together alongside varimax rotation of the PCA coordinate system. ALS implementations of MCR may also allow analyte profiles that are freely varied by the algorithm, which can then be used to model a profile that is difficult to estimate separately, for example a baseline.

2-6. *MULTIPLE LINEAR REGRESSION*

2-6-1. **Introduction**

Multiple Linear Regression (MLR) is a classical multivariate method that uses a combined set of **x**-vectors (***X***-data matrix) in linear combinations that are fitted as closely as possible to the corresponding single ***y***-vector.

MLR extends linear regression to more than 1 selected variable in order to perform a calibration using least squares fit.

2-6-2. **Principle**

In MLR, a direct least squares regression is performed between the ***X***- and the ***Y***-data. For the sake of simplicity, the regression of only 1 column vector **y** will be addressed here, but the method can be readily extended to a ***Y***-matrix, as is common when MLR is applied to data from experimental design (DoE), with multiple responses. In this case, single independent MLR models for each **y**-variable can be applied to the same ***X***-matrix.

The following MLR model equation is an extension of the normal univariate straight line equation; it may also contain cross and square terms:

$$y = b_0 + b_1x_1 + b_2x_2 + ... + b_kx_k + f$$

This can be compressed into the convenient matrix form:

$$\boldsymbol{y} = \boldsymbol{Xb} + \boldsymbol{f}$$

The objective is to find the vector of regression coefficients ***b*** that best minimises the error term ***f***. This is where the least squares criterion is applied to the squared error terms, i.e. to find ***b***-values so that ***y***-residuals ***f*** are minimised. MLR estimates the model coefficients using the following equation:

$$\boldsymbol{b} = (\boldsymbol{X}^T\boldsymbol{X})^{-1}\boldsymbol{X}^T\boldsymbol{y}$$

This operation involves the matrix inversion of the matrix $(\boldsymbol{X}^T\boldsymbol{X})^{-1}$. If any of the ***X***-variables show any collinearity with each other i.e. if the variables are not linearly independent, then the MLR solution will not be robust or a solution may not even be possible.

2-6-3. **Critical aspects**

MLR requires independent variables in order to adequately explain the data set, but as pharmaceutical samples are comprised of a complex matrix in which components interact to various degrees, the selection of appropriate variables is not straightforward. For example, in ultra-violet spectroscopy, observed absorbance values are linked because they may describe related behaviours in the spectroscopic data set. When observing the spectra of mixtures, collinearity is commonly found among the wavelengths, and consequently, MLR will struggle to perform a usable linear calibration.

The ability to vary the ***x***-variables independently of each other is a crucial requirement when using variables as predictors with this method. This is why in DoE the initial design matrix is generated in such a way as to establish this independence (i.e. orthogonality) from the start. MLR has the following constraints and characteristics:

- the number of ***X***-variables must be smaller than the number of samples ($n>m$), otherwise the matrix cannot be inverted;
- in case of collinearity among ***X***-variables, the *b*-coefficients are not reliable and the model may be unstable;
- MLR tends to over-fit.

To avoid overfitting MLR is often used with variable selection. The selection of the optimal number of ***X***-variables can be based on their residual variance, but also on the prediction error.

2-6-4. **Potential use**

MLR is typically suited to simple matrices/data sets, where there is a high degree of specificity and full rank. As matrices become more complex, more suitable methods such as PLS may be required to provide more accurate and/or robust calibration. In these cases, MLR may be used as a screening technique prior to the application of more advanced calibration methodologies.

2-7. *PRINCIPAL COMPONENTS REGRESSION*

2-7-1. **Introduction**

Principal components regression (PCR) is an expansion of principal components analysis (PCA) for use in quantitative applications. It is a two-step procedure whereby the calibration matrix ***X*** is first of all transformed by PCA into the scores and loadings matrices $\hat{T}$ and $\hat{P}$ respectively. In the following step, the score matrix for the principal components is used as the input for an MLR model to establish the relationship between the ***X***- and the ***Y***-data.

2-7-2. **Principle**

As in PCA, the calibration matrix is decomposed into scores and loadings matrices in such a way as to minimise the residual matrix $\hat{E}$ that ideally consists only of random errors, i.e. noise. For quantitative calibration, an additional matrix ***Y*** with the reference analytical data of the calibration samples is necessary. As the concentration information is contained in the orthogonal score vectors of the $\hat{T}$-matrix it can be optimally correlated by multiple linear regression using the actual concentrations in the ***Y***-matrix via the matrix $\hat{Q}$ (Figure 5.21.-8), while minimising the entries in the residual matrix $\hat{F}$.

2-7-3. **Critical aspects**

A crucial point in the development of a model is the selection of the optimal number of principal components. In this respect, the plot of the number of principal components versus the residual ***Y***-variance is an extremely useful diagnostic tool when defining the optimal number of PCs, i.e. when the minimum of the residual ***Y***-variance observed during model assessment has been reached. In most cases, additional PCs beyond this point do not improve the prediction performance but the calibration model falls into overfitting.

Despite its value as an important tool when dealing with collinear ***X***-data, the weakness of PCR lies in its independent decomposition of the ***X*** and ***Y*** matrices. This approach may take into account variations of the ***X***-data that are not necessarily relevant for an optimal regression with the ***Y***-data. Also, ***Y***-correlated information may even get lost in higher order principal components that are neglected in the above-mentioned selection process of the optimal number of PCs.

A stepwise principal component selection (e.g. selection of PC2 instead of PC1) may be useful to improve the performance of the calibration model.

2-7-4. **Potential use**

PCR is a multivariate technique with many diagnostic tools for the optimisation of the quantitative calibration models and the detection of erroneous measurements. In spectroscopy for example, PCR provides stable solutions when dealing with the calibration data of either complete spectra or large spectral regions. However, it generally requires more principal components than PLS and in view of the limitations and disadvantages discussed above, PLS regression has become the preferred alternative for quantitative modelling of spectroscopic data.

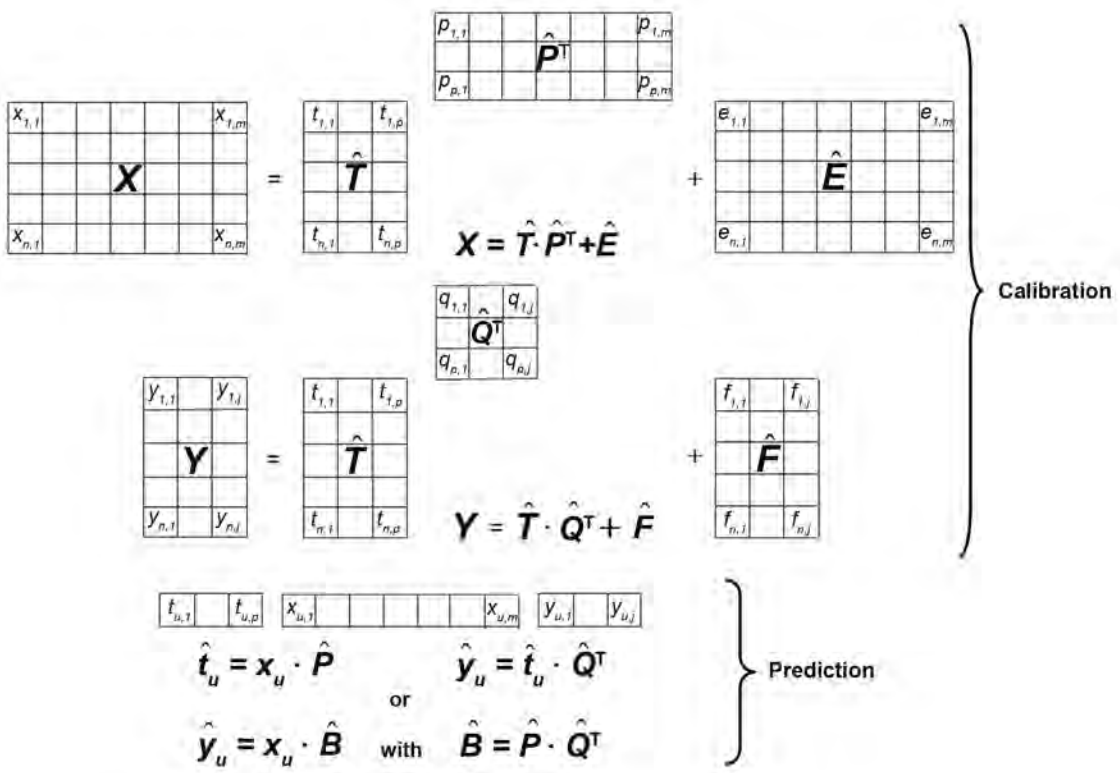


| | | | | | |
|---|---|---|---|---|---|
| $\boldsymbol{X}$ | = | original data matrix of $n$ rows and $m$ columns | $m$ | = | number of data points (variables) |
| $\hat{\boldsymbol{T}}$ | = | scores matrix with $n$ rows and $p$ columns | $n$ | = | number of measurements (samples) |
| $\hat{\boldsymbol{P}}^{\mathrm{T}}$ | = | loadings matrix with $p$ rows and $m$ columns | $j$ | = | number of property values per sample |
| $\hat{\boldsymbol{E}}$ | = | residual matrix (same size as $\boldsymbol{X}$-matrix) | $p$ | = | number of principal components (factors) |
| $\boldsymbol{Y}$ | = | property matrix of $n$ rows and $j$ columns | $\boldsymbol{x}_u$ | = | data of unknown sample |
| $\hat{\boldsymbol{Q}}^{\mathrm{T}}$ | = | correlation matrix of $p$ rows and $j$ columns | $\hat{\boldsymbol{y}}_u$ | = | predicted property values of unkown sample |
| $\hat{\boldsymbol{F}}$ | = | residual matrix (same size as $\boldsymbol{Y}$-matrix) | $\hat{\boldsymbol{t}}_u$ | = | score values for unknown sample |
| $\hat{\boldsymbol{B}}$ | = | matrix of regression coefficients | | | |

Figure 5.21.-8. – *Decomposition of the matrices for principal components regression (PCR)*

2-8. *PARTIAL LEAST SQUARES REGRESSION*

2-8-1. **Introduction**

Partial least squares regression (PLSR, generally known as PLS and alternatively named projection on latent structures) has developed into the most popular algorithm for multivariate regression.

PLS relates 2 data sets (***X*** and ***Y***) irrespective of collinearity. PLS finds latent variables from ***X*** and ***Y*** data blocks simultaneously, while maximising the covariance structure between these blocks. In a simple approximation PLS can be viewed as 2 simultaneous PCA analyses applied to the ***X*** and ***Y***-data in such a way that the structure of the ***Y***-data is used for the search of the principal components in the ***X***-data. The amount of variance modelled, i.e. the explained part of the data, is maximised for each component. The non-explained part of the data set is made up of residuals, which function as a measure of the modelling quality.

2-8-2. **Principle**

The major difference between PCR and PLS regression is that the latter is based on the simultaneous decomposition of the ***X*** and ***Y***-matrices for the derivation of the components (preferably denoted as PLS factors, factors, or latent-variables). Consequently, for the important factors, the information that describes a maximum variation in ***X***, while correlating as much as possible with ***Y***, is collected. This is precisely the information that is most relevant for the prediction of the ***Y***-values of unknown samples. In practice PLS can be applied to either 1 ***Y***-variable only (PLS1), or to the simultaneous calibration of several ***Y***-variables (PLS2 model).

As the detailed PLS algorithms are beyond the scope of this chapter, a simplified overview is instead given (Figure 5.21.-9). Arrows have been included between the $\hat{T}$ and $\hat{U}$ scores matrices in order to symbolise the interaction of their elements in the process of this iteration. While the ***Y***-matrix is decomposed into the loadings and scores matrices $\hat{Q}$ and $\hat{U}$ respectively, the decomposition of the ***X***-matrix produces not only the loadings and scores matrices $\hat{P}$ and $\hat{T}$, but also a loading weights matrix $\hat{W}$, which represents the relationship between the ***X*** and ***Y***-data.

To connect the ***Y***-matrix with the ***X***-matrix decomposition for the first estimation of the $\hat{T}$ score values, the ***Y***-data are used as a guide for the decomposition of the ***X***-matrix. By interchanging the score values of the $\hat{U}$ and $\hat{T}$ matrices, an interdependent modelling of the ***X*** and ***Y*** data is achieved, thereby reducing the influence of large ***X***-variations that do not correlate with ***Y***. Furthermore, simpler calibration models with fewer PLS-factors can also be developed where, as is the case for PCR, residual variances are used during validation to determine the optimal number of factors that model useful information and consequently, avoid overfitting.

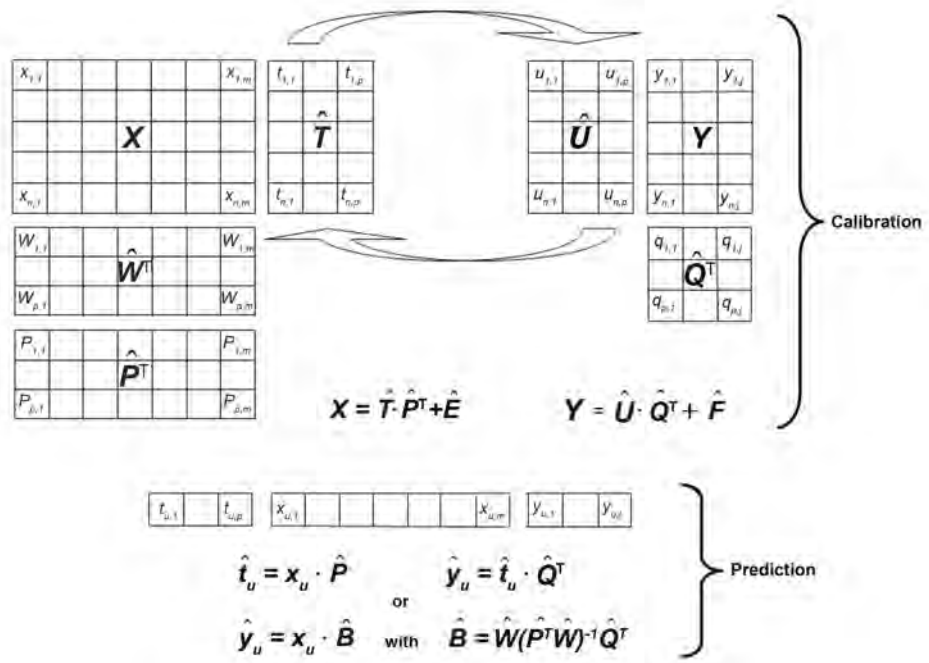


| | | | | | |
|---|---|---|---|---|---|
| $\boldsymbol{X}$ | = | original data matrix of $n$ rows and $m$ columns | $\hat{\boldsymbol{B}}$ | = | matrix of regression coefficients |
| $\boldsymbol{Y}$ | = | property matrix of $n$ rows and $j$ columns | $m$ | = | number of data points (variables) |
| $\hat{\boldsymbol{T}}$ | = | scores matrix with $n$ rows and $p$ columns | $n$ | = | number of measurements (samples) |
| $\hat{\boldsymbol{P}}^{\mathrm{T}}$ | = | loadings matrix with $p$ rows and $m$ columns | $j$ | = | number of property values per sample |
| $\hat{\boldsymbol{W}}^{\mathrm{T}}$ | = | loading weights matrix with $p$ rows and $m$ columns | $p$ | = | number of factors |
| $\hat{\boldsymbol{E}}$ | = | residual matrix (same size as $\boldsymbol{X}$-matrix) | $\boldsymbol{x}_u$ | = | data of unknown sample |
| $\hat{\boldsymbol{Q}}^{\mathrm{T}}$ | = | loadings matrix of $\boldsymbol{Y}$-data | $\hat{\boldsymbol{y}}_u$ | = | predicted property values of unknown sample |
| $\hat{\boldsymbol{U}}$ | = | scores matrix of $\boldsymbol{Y}$-data | $\hat{\boldsymbol{t}}_u$ | = | score values for unknown sample |
| $\hat{\boldsymbol{F}}$ | = | residual matrix (same size as $\boldsymbol{Y}$-matrix) | | | |

Figure 5.21.-9. – *Decomposition of the data matrices for PLS regression*

2-8-3. **Critical aspects**

A critical step in PLS is the selection of the number of factors. Selecting too few factors will inadequately explain variability in the training data set, while too many factors will cause overfitting and instability in the resulting calibration (Figure 5.21.-10). The optimal number of factors is estimated during validation of the calibration. Figure 5.21.-10 shows the changes in the calibration error (A) of a model and 2 cases of prediction errors (B, C) according to the number of factors used in the model. The calibration error decreases continuously as the number of factors increases. In case B prediction error reveals that no minimum can be observed; however, a minimum is observed in case C. In the absence of a minimum, the number of components can be chosen based on where no significant decreasing of error is observed.

As far as the decision between PLS1 or PLS2 models is concerned PLS1 modelling is chosen if there is only 1 ***Y***-variable of interest. In cases where there is more than 1 ***Y***-variable of interest, either one PLS2 model or individual PLS1 models for each ***Y***-variable can be calculated. In general, PLS2 is the preferred approach for screening purposes and in cases of highly correlated ***Y***-variables of interest; otherwise separate PLS1 models for the different ***Y***-variables will yield more satisfactory prediction results.

2-8-4. **Potential use**

PLS has emerged as a preferable alternative to PCR for quantitative calibration because it incorporates the intervention of the ***Y***-data structure for the decomposition of the calibration ***X***-matrix. Consequently, information from the most important factors is collected and is capable of describing maximal variation in the ***X***-data, while also correlating as closely as possible with the ***Y***-data. In general, this yields simpler models with fewer factors compared to PCR and also provides superior interpretation possibilities and visualisation diagnostics for the optimisation of the calibration performance. In addition PLS can handle presence of noise in both ***X***- and ***Y***-data.

PLS discriminant analysis (PLS-DA) is a special case of PLS where ***X***-matrix is regressed into a dummy ***Y***-matrix consisting of ones and zeroes. Ones and zeroes indicate the class to which samples are belonging or not. PLS-DA is used as a semi-quantitative method in, for example, chemical imaging estimating the pixel components.

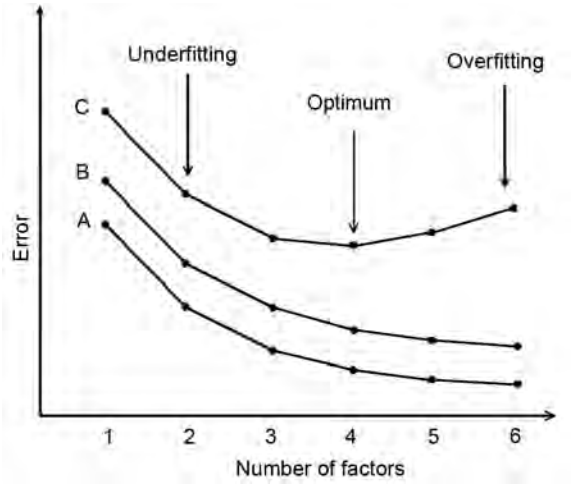


Figure 5.21.-10. – *Effects of adding factors to a model; the calibration becomes more accurate i.e. the residual error diminishes, while the predictive performance of the model could deteriorate as for prediction C. The optimum number of factors reflects a compromise.*



2-9. *SUPPORT VECTOR MACHINES*

2-9-1. **Introduction**

To achieve classification, multivariate techniques reduce the dimensionality and complexity of the data set. Kernel methods project data into higher dimensional feature spaces.

2-9-2. **Principle**

Support vector machines (SVMs) project *X*-data of the training set into a feature space of usually much higher dimension than the original data space. In the feature space a hyperplane (also called decision plane) is computed that separates individual points of known group membership (Figure 5.21.-11). The best discriminating separation is achieved by maximising the margin between groups. The margin is defined by 2 parallel hyperplanes at an equal distance from the decision plane. The optimum position of the decision plane is obtained if the margin is maximal. Points in the feature space that define the margin are called support vectors.

For each training point the distance to the decision plane is computed. In the case of a two-class separation for example, the sign of the distance gives the group membership and the value corresponds to the certainty of classification. During modelling the distance between the training points and the hyperplane contributes to the weight attributed to the point. Very distant points will have a lesser weight, and to avoid overfitting, distances smaller than a trade-off parameter will not be considered.

For non-separable object groups overlapping is allowed to a certain extent. So-called slack variables are added to objects, with value 0 if correctly classified and a positive value otherwise. The optimal hyperplane is found by maximising the margin at the same time allowing for a minimum number of training points to be misclassified (Figure 5.21.-12). The proportion of misclassified points becomes a control parameter during margin maximisation.

In practice SVM computation is extremely complex and would be infeasible without simplifying of the optimisation problem. To project *X*-data into the feature space the original data is expanded by a set of basis functions. Selecting particular basis functions makes it possible to reformulate the whole optimisation procedure. Only products of the expanded variables remain part of the optimisation procedure and they can be advantageously replaced by a Kernel function.

2-9-3. **Critical aspects**

Numerous algorithms and different types of software can be used to compute SVMs, which may lead to differing results. Optimisation will vary depending on the algorithm used. Control criteria may differ, thus leading either to divergence during the iterations, or to unstable computations sensitive to redundant and uninformative data.

During SVM computation training points that are well inside their class boundaries have little or even no effect on the position of the decision plane. The latter focusses mainly on points that are difficult to separate and not on objects that are clearly distinct. Thus, SVMs are sensitive to redundant values and atypical points like outliers, for example. As a consequence it may be pertinent to select or screen out specific variables prior to performing SVM. The data should be normalised and standardised to avoid having input data of different scales, which may lead to poor conditions for the boundary optimisation.

The best performing model must be adequately validated and test data that is completely untouched during iterations is required for this purpose. It should be ensured that this data is well balanced in the sense that both easy and difficult samples are equally represented in the training and validation sets.

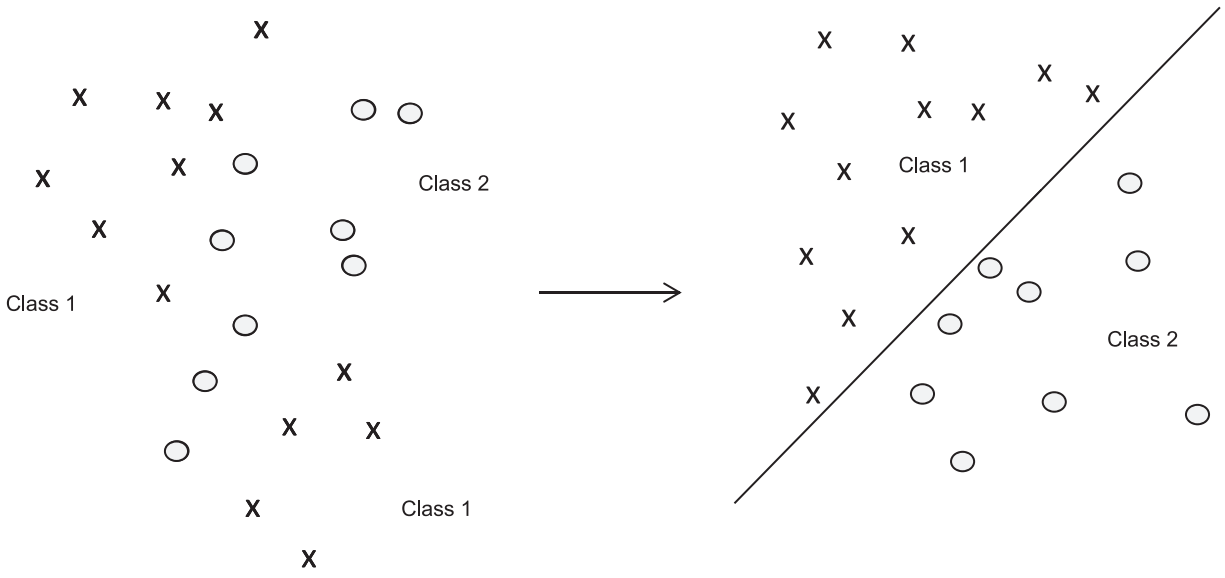


Figure 5.21.-11. – *The object space, where separation of the 2 classes is not possible, is mapped into a feature space where separation is possible*

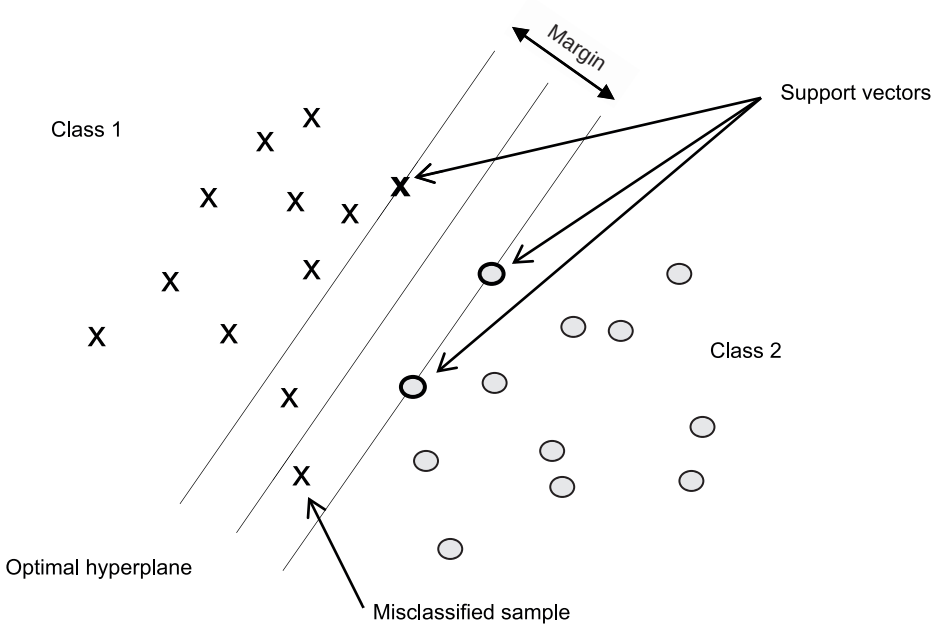


Figure 5.21.-12. – *In the feature space, the separation of classes 1 and 2 was achieved with toleration of certain misclassified samples*

2-9-4. **Potential use**

SVMs are mainly used for binary supervised classification. They can be generalised to multiclass classification or extended to regression problems, though these applications are not considered within the scope of this chapter. Objects that are difficult to classify rather than those that are clearly distinct drive the optimisation process in SVMs. SVMs can be used for the separation of classes of objects, but not for the identification of these objects. They operate well on large data sets that are obtained, for example, by NIR spectroscopy, magnetic resonance, chemical imaging or process data mining, where PCA and related methods fail. Their strength mainly lies in separation of samples featuring highly correlated signals, i.e. polymorphs, excipients, tracing of adulterated substances, counterfeits etc.

2-10. *ARTIFICIAL NEURAL NETWORKS*

2-10-1. **Introduction**

Artificial neural networks (ANNs) are general computational tools, whose initial development was inspired by the need for further understanding of biological neural networks and which have since been widely used in various areas that require data processing with computers or machines. The methods for building ANN models and their subsequent applications can be dramatically different depending on the architecture of the neural networks themselves. In the field of chemometrics, ANNs are generally used for multivariate calibration and unsupervised classification, which is achieved by using multi-layer feed-forward (MLFF) neural networks, or self-organising maps (SOM) respectively. As a multivariate calibration tool, ANNs are more generally associated with the mapping of non-linear relationships.

2-10-2. **Principle**

2-10-2-1. *General*

The basic data processing element in an artificial neural network is the artificial neuron, which can be understood as a mathematical function that uses the sum of a weighted vector and a bias as the input. The vector is the 'input' of the neuron and is obtained either directly from a sample in the data set or calculated from previous neurons. The user chooses the form of the function (called transfer function). The weights and bias are the coefficients of the ANN model and are determined through a learning process using known examples. An ANN often contains many neurons arranged in layers, where the neurons in each layer are arranged in parallel. They are connected to neurons in the preceding layer from which they receive inputs and also to neurons in the following layer where the outputs are sent (Figure 5.21.-13). The output of 1 neuron is therefore used as the input for neurons in the following layer. The input layer is a special layer that receives data directly from the user and sends this information directly to the next layer without applying a transfer function. The output layer is similar in that its output is also directly used as the model output without any additional processing. The unlimited possibilities when connecting different numbers and layers of neurons, is often called an ANN architecture, and provides the potential for ANNs to meet any complicated data modelling requirements.

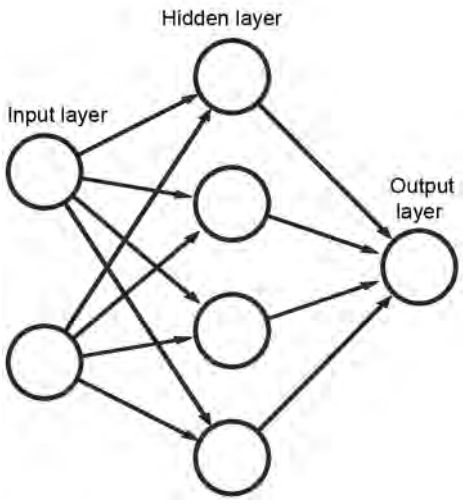


Figure 5.21.-13. - *Typical arrangements of neuron layers and their inter-connections*

2-10-2-2. *Multi-layer feed-forward artificial neural network*

A multi-layer feed-forward network (MLFF ANN) contains an input layer, an output layer and 1 or more layers of neurons in-between called hidden layers. Even though there is no limit on how many hidden layers may be included, an MLFF ANN with only 1 hidden layer is sufficiently capable of handling most multivariate calibration tasks in chemometrics. In an MLFF ANN, each neuron is fully connected to all the neurons

in the neighbouring layers. A hyperbolic tangent sigmoid transfer function is usually used in MLFF ANN, but other transfer functions, including linear functions, can also be used.

The initial weights and biases can be set as small random numbers, but can also be initialised using other algorithms. The most popular training algorithm for determining the final weights and biases is the back-propagation (BP) algorithm or its related variants. In the BP algorithm, the prediction error, calculated as the difference between the ANN output and the actual value, is propagated backward to calculate the changes needed to adjust the weights and biases in order to minimise the prediction error.

An MLFF ANN must be optimised in order to achieve acceptable performance. This often involves a number of considerations including the number of layers, the number of neurons in each layer, transfer functions for each layer or neuron, initialisation of weights, learning rate, etc.

2-10-2-3. *Self-organising map*

The aim of the self-organising map (SOM) is to create a map where observations that are close to each other have more similar properties than more distant observations. The neurons in the output layer are usually arranged in a two-dimensional map, where each neuron can be represented as a square or a hexagon. SOMs are trained using competitive learning that is different from the above described method using BP. The final trained SOM is represented as a two-dimensional map of the observations.

2-10-3. **Critical aspects**

The 2 most common pitfalls of using ANNs are over-training and under-training. Over-training means that an ANN model can predict the training set very well but ultimately fails to make good predictions. Under-training means that the ANN training ended too soon and therefore the resultant ANN model underperforms when making predictions. Both of these pitfalls should be avoided when using ANNs for calibration. A representative data set with a proper size, i.e. more observations or samples than variables, is required before a good ANN model can be trained. Generally, since the models are non-linear, more observations are needed than for a comparable data set subjected to linear modelling. As for other multivariate calibration methods, the input may need pre-processing to balance the relative influence of variables. One advantage of pre-processing is the reduction in the number of degrees of freedom of input to the ANN, for example by compression of the *X*-data to scores by PCA and then using the resulting scores for the observations as input.

2-10-4. **Potential use**

The advantage of MLFF ANN in multivariate calibration lies in its ability to model non-linear relationships. Since the neurons are fully connected, all the interactions between variables are automatically considered. It has been proven that a MLFF ANN with sufficient hidden neurons can map any complicated relationship between the inputs and outputs.

SOMs can be used to visualise high-dimensional data while preserving the topology in the original data. They are based on unsupervised learning, and are mainly useful as tools to explore features in data sets where no prior knowledge of the patterns and relationships of the samples exists.

The ANNs often have a large number of coefficients (weights and biases) that give the ANN the potential to model any complicated relationships in the data set but as a result, can also make the interpretation of the coefficients more difficult. However, when linear modelling methods are not flexible enough to provide the required prediction or classification accuracy, ANNs may be a good alternative.

3. GLOSSARY

**β-distribution**: continuous probability distribution with density function

$$f(x;u,v) = \frac{1}{B(u,v)} x^{u-1}(1-x)^{v-1}$$

where $u > 0$, $v > 0$ are shape parameters (degrees of freedom) and $B$ is the beta function,

$$B(u,v) = \int_0^1 t^{u-1}(1-t)^{v-1}dt$$

The $\gamma$-quantile of the $B(u,v)$-distribution is denoted by $\beta_{u,v;\gamma}$ and it is the value $q$ such that the value of the distribution function $F$ is $\gamma$:

$$F(x;u,v) = \int_0^q f(x;u,v)dx = \gamma$$

**Bootstrapping**: a number of sample sets of size $n$ that is produced from an original sample set of the same size $n$ by means of a random selection of samples with replacement.

**Centring**: a data set is mean centred by calculating the mean value of each variable and subtracting the variable mean values from each column of variables, in order to make the comparison and interpretation of the data easier.

**Collinear/non-collinear**: a family of vectors is collinear if at least 1 of the vectors can be represented as a linear combination of the other vectors. Hence a family of vectors is non-collinear if none of the vectors can be represented as a linear combination of the others.

**Component (or factor, latent variable)**: in chemometrics: underlying, non-observed, non-measured, hypothetical variable that contributes to the variance of a collection of measured variables. The variables are linear combinations of the factors and these factors are assumed to be uncorrelated with each other.

**Data mining**: process of exploration, extraction and modelling of large collections of data in order to discover *a priori* unknown relationships or patterns.

**Dependent variable**: also a response, regressand: a variable that is related by a formal (explicit) or empirical mathematical relationship to 1 or more other variables (typically the *Y*-data).

**Empirical model**: a data-driven model established without assuming an explicit mathematical relationship, or without a description of the behaviour of a system based on accepted laws of physics.

**Exploratory data analysis**: the process for uncovering unexpected or latent patterns in order to build future hypotheses.

**Factor**: see component.

**Hotelling $T^2$ statistics**: multivariate version of the t-statistic. In general, this statistic can be used to test if the mean vector of a multivariate data set has a certain value or to compare the means of the variables. The $T^2$ statistic is also used for detection of outliers in multivariate data sets. A multivariate statistical test using the Hotelling $T^2$ statistic can be done. A confidence ellipse can be included in score plots to reveal points outside the ellipse as potential outliers.

**Independent variable**: input variable on which other variables are dependent through a mathematical relationship (typically the *X*-data).

**Indirect prediction**: process for estimating the value of a response on the basis of a multivariate model and observed data.

**Interference**: effect of substances, physical phenomena or instrument artefacts, separate from the target analyte, that can be measured by the chosen analytical method. Then there is a

risk of confusion between the analyte and interference if the interference is not varied independently or at least randomly in relation to the analyte.

**Latent variable**: see component.

**Leave-one-out**: in a 'leave-one-out' procedure only 1 sample at a time is removed from the data set in order to create a new data set.

**Leave-subset-out**: in a 'leave-subset-out' procedure a subset of samples is removed from the data set in order to create a new data set.

**Leverage**: a measure of how extreme a data point or a variable is compared to the majority. Points or variables with a high leverage are likely to have a large influence on the model.

**Loadings**: loadings are estimated when information carried by several variables is focussed onto a few components. Each variable has a loading alongside each model component. The loadings show how well a variable is taken into account by the model components.

**Orthogonal**: 2 vectors are orthogonal if their scalar product is 0.

**Orthonormal vectors**: orthogonal and normalised (unit-length) vectors.

**Outlier**: for a numerical data set, it relates to a value statistically different from the rest. Also refers to the sample associated with that value. Specific statistical testing for outliers may be used.

**Overfitting**: for a model, overfitting is a tendency to describe too much of the variation in the data, so that in addition to the consistent underlying structure, some noise or non-informative variation is also taken into account and unreliable predictions will be obtained.

**Property**: see variable.

**Resampling**: the process of impartial rearrangement and sub-sampling of the original data set. This occurs during optimisation/validation procedures that repeatedly calculate a property and the error associated with it. Typical examples are cross-validation and bootstrapping, which create successive evaluation data sets by repeated sub-sampling.

**Reselection**: reuse of samples (see resampling).

**Residuals**: a measure of the variation that is not taken into account by the model or a deviation between predicted and reference values.

**Root mean square error of prediction**: a function of the predictive residual sum of squares to estimate the accuracy:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)^2}{n}}$$

where $\hat{y}_i$ is the predicted response for the $i^{th}$ sample of the test data set and $y_i$ the observed response of the $i^{th}$ sample, and $n$ is the number of samples.

**Sample**: object, observation, or individual from which data values are collected.

**Sample attribute**: qualitative or quantitative property of the sample.

**Sample selection**: the process of drawing a subset or a collection from a population in order to estimate the properties of the population.

**Scores or factor score coefficients**: coordinates of the samples in the new coordinate system defined by the principal components. Scores represent how samples are related to each other's given the measurement variables.

**Score (normalised)**: $j^{th}$ score value $t_{i,j}$ of the $i^{th}$ sample divided by the norm of the scores matrix:

$$\left|t_{i,j}\right| = \frac{t_{i,j}}{\sqrt{\sum_{j=1}^{p} t_{i,j}^2}}$$

where $p$ is the number of parameters in the model.

**Standard error of calibration**: a function of the predictive residual sum of squares to estimate the accuracy considering the number of parameters:

$$SEC = \sqrt{\frac{\sum_{i=1}^{n}\left(y_i - \hat{y}_i\right)^2}{n-p}}$$

where $n$ is the number of samples of the learning set, $p$ the number of parameters in the model to be estimated by using the sample data, $\hat{y}_i$ the $i^{th}$ fitted value in the calibration, and $y_i$ the $i^{th}$ reference value. In multiple regression with $m$ variables $p = m + 1$ (1 coefficient for each of the $m$ variables and 1 intercept).

**Standard error of laboratory**: concerns to the intermediate precision or reproducibility, whichever is applicable.

**Supervised**: refers to modelling data labelled by classes or values.

**Unsupervised (non-supervised)**: refers to exploring data without prior assumptions.

**Underfitting**: the reverse of overfitting.

**Variable**: property of a sample that can be assessed (attribute, descriptor, feature, property, characteristics).

**Varimax rotation**: orthogonal analytical rotation of factors that maximises the variance of squared factor loadings, thereby increasing the large factor loadings and large eigenvalues and decreasing the small ones in each factor.

4. ABBREVIATIONS

| | |
|---|---|
| ALS | alternating least squares |
| ANN | artificial neural network |
| BP | back-propagation |
| CLS | classical least squares |
| DB | density-based |
| DBSCAN | density-based spatial clustering of applications with noise |
| DoE | design of experiments |
| EFA | evolving factor analysis |
| EM | expectation maximisation |
| LDA | linear discriminant analysis |
| MCR | multivariate curve resolution |
| MLFF | multi-layer feed-forward |
| MLR | multiple linear regression |
| MSPC | multivariate statistical process control |
| NIR | near-infrared |
| PAT | process analytical technology |
| PC | principle component |

| | | | |
|---|---|---|---|
| PCA | principal components analysis | SEC | standard error of calibration |
| PCR | principal components regression | SEL | standard error of laboratory |
| PLS | partial least squares regression | SIMCA | soft independent modelling of class analogy |
| PLS-DA | partial least squares discriminant analysis | SMCR | self-modelling curve resolution |
| PLSR | partial least squares regression | SNV | standard normal variate |
| QbD | quality by design | SOM | self-organising map |
| QDA | quadratic discriminant analysis | STING | statistical information grid |
| RMSEP | root mean square error of prediction | SVM | support vector machine |

# 5.22. Names of herbal drugs used in traditional Chinese medicine

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# 5.22. NAMES OF HERBAL DRUGS USED IN TRADITIONAL CHINESE MEDICINE

**01/2020:52200** For transparency, this general chapter provides cross-references to the Chinese names in pinyin and in sinograms for herbal drugs used in traditional Chinese medicine for which a monograph is published in the Ph. Eur.

However, the titles in English, French and Latin are the only official names; the samples of herbal drugs must be labelled with at least one official title.

*This general chapter is published for information.*

| Monograph number | Latin title | English title | Pinyin | Sinogram |
|---|---|---|---|---|
| *2827* | Abelmoschi corolla | Abelmoschi corolla | *huangshukuihua* | 黄蜀葵花 |
| *2432* | Acanthopanacis gracilistyli cortex | Acanthopanax bark | *wujiapi* | 五加皮 |
| *2999* | Achyranthis bidentatae radix | Achyranthes bidentata root | *niuxi* | 牛膝 |
| *2472* | Akebiae caulis | Akebia stem | *mutong* | 木通 |
| *2554* | Amomi fructus | Amomum fruit | *sharen* | 砂仁 |
| *2555* | Amomi fructus rotundus | Round amomum fruit | *doukou* | 豆蔻 |
| *2712* | Andrographidis herba | Andrographis herb | *chuanxinlian* | 穿心莲 |
| *2661* | Anemarrhenae asphodeloides rhizoma | Anemarrhena asphodeloides rhizome | *zhimu* | 知母 |
| *2556* | Angelicae dahuricae radix | Angelica dahurica root | *baizhi* | 白芷 |
| *2557* | Angelicae pubescentis radix | Angelica pubescens root | *duhuo* | 独活 |
| *2558* | Angelicae sinensis radix | Angelica sinensis root | *danggui* | 当归 |
| *2435* | Astragali mongholici radix | Astragalus mongholicus root | *huangqi* | 黄芪 |
| *2559* | Atractylodis lanceae rhizoma | Atractylodes lancea rhizome | *cangzhu* | 苍术 |
| *2560* | Atractylodis macrocephalae rhizoma | Atractylodes rhizome, largehead | *baizhu* | 白术 |
| *1797* | Aucklandiae radix | Aucklandia root | *muxiang* | 木香 |
| *2561* | Belamcandae chinensis rhizoma | Belamcanda chinensis rhizome | *shegan* | 射干 |
| *2384* | Bistortae rhizoma | Bistort rhizome | *quanshen* | 拳参 |
| *2562* | Bupleuri radix | Bupleurum root | *chaihu* | 柴胡 |
| *2386* | Carthami flos | Safflower flower | *honghua* | 红花 |
| *2430* | Citri reticulatae epicarpium et mesocarpium | Mandarin epicarp and mesocarp | *chenpi* | 陈皮 |
| *2463* | Clematidis armandii caulis | Clematis armandii stem | *chuanmutong* | 川木通 |
| *2714* | Codonopsidis radix | Codonopsis root | *dangshen* | 党参 |
| *2454* | Coicis semen | Coix seed | *yiyiren* | 薏苡仁 |
| *2715* | Coptidis rhizoma | Chinese goldthread rhizome | *huang lian* | 黄连 |
| *2976* | Corydalis rhizoma | Corydalis rhizome | *yan husuo* | 延胡索 |
| *2890* | Dioscoreae nipponicae rhizoma | Dioscorea nipponica rhizome | *chuanshanlong* | 穿山龙 |
| *2473* | Dioscoreae oppositifoliae rhizoma | Dioscorea oppositifolia rhizome | *shanyao* | 山药 |
| *2563* | Drynariae rhizoma | Drynaria rhizome | *gusuibu* | 骨碎补 |
| *2564* | Ecliptae herba | Eclipta herb | *mohanlian* | 墨旱莲 |
| *2451* | Ephedrae herba | Ephedra herb | *mahuang* | 麻黄 |
| *2412* | Eucommiae cortex | Eucommia bark | *duzhong* | 杜仲 |
| *2718* | Evodiae fructus | Evodia fruit | *wuzhuyu* | 吴茱萸 |
| *2452* | Fraxini rhynchophyllae cortex | Fraxinus rhynchophylla bark | *qinpi* | 秦皮 |
| *2565* | Gardeniae fructus | Cape jasmine fruit | *zhizi* | 栀子 |
| *2721* | Gastrodiae rhizoma | Gastrodia rhizome | *tianma* | 天麻 |
| *2722* | Houttuyniae herba | Houttuynia herb | *yuxingcao* | 鱼腥草 |
| *2566* | Isatidis radix | Isatis root | *banlangen* | 板蓝根 |
| *2634* | Ligustici chuanxiong rhizoma | Szechwan lovage rhizome | *chuanxiong* | 川芎 |
| *2431* | Ligustici radix et rhizoma | Ligusticum root and rhizome | *gaoben* | 藁本 |
| *2612* | Lycii fructus | Barbary wolfberry fruit | *gouqizi* | 枸杞子 |

| Monograph number | Latin title | English title | Pinyin | Sinogram |
|---|---|---|---|---|
| *2723* | Lycopi herba | Lycopus lucidus herb | *zelan* | 泽兰 |
| *2742* | Magnoliae biondii flos immaturus | Magnolia biondii flower bud | *xinyi* | 辛夷 |
| *2567* | Magnoliae officinalis cortex | Magnolia officinalis bark | *houpo* | 厚朴 |
| *2568* | Magnoliae officinalis flos | Magnolia officinalis flower | *houpohua* | 厚朴花 |
| *2474* | Moutan cortex | Moutan bark | *mudanpi* | 牡丹皮 |
| *2383* | Notoginseng radix | Notoginseng root | *sanqi* | 三七 |
| *3000* | Ophiopogonis radix | Dwarf lilyturf tuber | *maidong* | 麦冬 |
| *2424* | Paeoniae radix alba | Peony root, white | *baishao* | 白芍 |
| *2425* | Paeoniae radix rubra | Peony root, red | *chishao* | 赤芍 |
| *2727* | Persicariae tinctoriae folium | Indigo plant leaf | *liaodaqingye* | 蓼大青叶 |
| *2477* | Piperis fructus | Pepper | *hujiao* | 胡椒 |
| *2453* | Piperis longi fructus | Long pepper | *bibo* | 荜茇 |
| *2660* | Platycodonis radix | Platycodon root | *jiegeng* | 桔梗 |
| *2724* | Polygoni cuspidati rhizoma et radix | Polygonum cuspidatum rhizome and root | *huzhang* | 虎杖 |
| *2433* | Polygoni multiflori radix | Fleeceflower root | *heshouwu* | 何首乌 |
| *2726* | Polygoni orientalis fructus | Polygonum orientale fruit | *shuihonghuazhi* | 水红花子 |
| *2475* | Poria | Poria | *fuling* | 茯苓 |
| *2439* | Prunellae spica | Common selfheal fruit-spike | *xiakucao* | 夏枯草 |
| *2434* | Puerariae lobatae radix | Kudzuvine root | *gegen (yege)* | 葛根 (野葛) |
| *2483* | Puerariae thomsonii radix | Thomson kudzuvine root | *fenge* | 粉葛 |
| *2663* | Salviae miltiorrhizae radix et rhizoma | Salvia miltiorrhiza root and rhizome | *danshen* | 丹参 |
| *2385* | Sanguisorbae radix | Sanguisorba root | *diyu* | 地榆 |
| *2428* | Schisandrae chinensis fructus | Schisandra fruit | *wuweizi* (*bei wuweizi*) | 五味子 (北五味子) |
| *2438* | Scutellariae baicalensis radix | Baical skullcap root | *huangqin* | 黄芩 |
| *2450* | Sinomenii caulis | Orientvine stem | *qingfengteng* | 青风藤 |
| *2440* | Sophorae flavescentis radix | Lightyellow sophora root | *kushen* | 苦参 |
| *2639* | Sophorae japonicae flos | Sophora flower | *huaihua* | 槐花 |
| *2427* | Sophorae japonicae flos immaturus | Sophora flower-bud | *huaimi* | 槐米 |
| *2478* | Stephaniae tetrandrae radix | Fourstamen stephania root | *fenfangji* (*hanfangji*) | 粉防己 (汉防己) |
| *2937* | Typhae pollis | Typhae pollen | *puhuang* | 蒲黄 |
| *2729* | Uncariae rhynchophyllae ramulus cum uncis | Uncaria stem with hooks | *gou teng* | 钩藤 |
| *2656* | Zanthoxyli bungeani pericarpium | Zanthoxylum bungeanum pericarp | *huajiao* | 花椒 |

# 5.23. Monographs on herbal drug extracts (information chapter)

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**04/2019:52300**

# 5.23. MONOGRAPHS ON HERBAL DRUG EXTRACTS (INFORMATION CHAPTER)

**Basis for elaboration of monographs on herbal drug extracts**

European Pharmacopoeia monographs on herbal drug extracts are elaborated on the basis of extracts present in medicinal products that have been authorised and/or registered by the competent authorities of Parties to the Convention on the Elaboration of a European Pharmacopoeia. However, these monographs do not necessarily cover all extracts which may be available on the market.

European Pharmacopoeia monographs on herbal drug extracts are elaborated by groups of experts and working parties in collaboration with national pharmacopoeia authorities, the competent authorities for marketing authorisation, Official Medicines Control Laboratories (OMCLs) and the EDQM laboratory; they are also assisted by the producers of herbal drug extracts and/or the pharmaceutical manufacturers that use these extracts.

During the elaboration of an extract monograph, the group of experts works on the basis of a number of extracts from the specified herbal drug that are incorporated into authorised and/or registered medicinal products originating from different sources. These extracts may have been produced using different extraction solvents and/or extraction processes and may include different types and contents of excipients (added for technological purposes during production of the extract).

Where the results from the analysis of the extracts indicate that all of the extracts are in compliance with all of the quality parameters, the monograph is intended to apply to all types of production of that specific herbal extract (for example, *Devil's claw dry extract (1871)*, where differences in the extraction solvent used, as stated in the Production section of the monograph, and any differences between the extracts in terms of their production process have no significant effect on the quality parameters).

Where there is a definable difference in the quality parameters between the extracts due to one or more aspects of the production process, the monograph is presented as a family monograph in which there will be a qualitative or quantitative difference applicable to one or more analytical parameters (for example, *Boldo leaf dry extract (1816)*, where differences in the extraction solvents used, as stated in the Production section of the monograph, necessitates defining a lower minimum content of assayed constituents in the aqueous extract in comparison with the minimum content in the hydroalcoholic extract; all other quality parameters are identical).

Where there is a significant difference in the quality parameters between the extracts due to one or more aspects of the production process, more than one extract monograph is elaborated (for example, *Valerian dry aqueous extract (2400)* and *Valerian dry hydroalcoholic extract (1898)*, where differences in the extraction solvents used and the processing method, as stated in the Production section of the monographs, necessitates defining different minimum values for the assayed constituents and different chromatographic profiles for the aqueous and the hydroalcoholic extracts).

**Types of extract**

The general monograph *Herbal drug extracts (0765)* distinguishes different types of extracts. This classification is based on the principles applied by European competent authorities during the assessment of extracts in applications for marketing authorisation/registration of medicinal products. These principles are summarised in Table 5.23.-1.

This classification of extracts implies that, for each of these types of extract, distinct principles of production and of defining or adjusting the content of assayed constituents are required by the general monograph *Herbal drug extracts (0765)*.

*Genuine (native) extract*. The concept of the genuine (native) drug extract ratio ($DER_{genuine}$) (see the Glossary in the monograph *Herbal drug extracts (0765)*) was originally devised for application to dry extracts where, after extraction of the herbal drug, all solvent is removed leaving only the extracted dry matter from the herbal drug, that is, the genuine (native) extract. However, this extracted dry matter often requires additives (inert excipients as processing aids) to produce a technologically suitable extract. Such extracts made by different manufacturers from a given herbal drug may have been produced using different solvents and processing methods and contain different amounts of excipients relative to the quantity of extracted dry matter. In order to permit comparison of such extracts, the declaration of the drug extract ratio (*DER*) on the basis of the genuine (native) extract was introduced. Thus, for those dry extracts which can be produced without excipients, the $DER_{total}$ and the $DER_{genuine}$ are identical, but where excipients are required, the $DER_{total}$ and the $DER_{genuine}$ are different. It was then thought necessary to apply this concept to soft extracts and liquid extraction preparations where solvent and, in some cases, other substances are present as an integral part of the extract. For these extracts, the concept of basing the $DER_{genuine}$ on extracted dry matter was abandoned and the extract in its entirety (including solvents, processing aids, etc.) is considered to be the genuine (native) extract. Therefore, for these extracts, the

Table 5.23.-1. – *Classification and principles of production of extracts*

| **Information available during assessment as regards pharmacological/therapeutic relevance of constituents** | **Extract type** | **Extract concept** | | **Extract adjustment** |
|---|---|---|---|---|
| | | ***Quantitative parameters*** | | |
| | | **Constituent to be analysed** | **Quantity of genuine (native) extract that is included in finished products** | |
| Constituents with known therapeutic activity | Standardised | Constituents with known therapeutic activity<br>*Constant* | *Variable* | 1) By addition of inert excipients (dry extracts) or solvents (liquid extraction preparations or soft extracts)<br>2) By blending batches |
| Constituents which are generally accepted to contribute to the therapeutic activity | Quantified | Active marker<br>*Range* | *Constant* | By blending batches |
| Constituents chosen solely for analytical purposes irrespective of any pharmacological or therapeutic activity which they may be reported to possess | Other | Analytical marker<br>*Variable* | *Constant* | None |

$DER_{total}$ and the $DER_{genuine}$ are identical. For the declaration of the active substance in the finished product, only the $DER_{genuine}$ is to be used.

**Constituents for assay**

For the purposes of quality control, monographs of the European Pharmacopoeia generally include an assay. The choice of the constituent(s) to be assayed is linked, wherever possible, to the regulatory process and is based upon the following criteria:

- where constituents with known therapeutic activity are present, these are selected for assay.

  *Constituents with known therapeutic activity* are chemically defined substances or groups of substances which are generally accepted to contribute substantially to the therapeutic activity of a herbal drug, a herbal drug preparation or a herbal medicinal product.
- where constituents with known therapeutic activity are absent, marker constituents are selected for assay.

  *Active markers* are constituents or groups of constituents which are generally accepted to contribute to the therapeutic activity of an extract.

  *Analytical markers* are constituents or groups of constituents that serve solely for analytical purposes, irrespective of any pharmacological or therapeutic activity which they may be reported to possess.

**Use of analytical markers in 'other' extracts**

The assay method described in an individual monograph:

- is usually derived from the method used to assay the corresponding herbal drug;
- is considered appropriate to determine the content of the chosen analytical marker;
- serves as a general analytical platform for determining the content of the marker in the individual extracts covered by the monograph for the purpose of quality control. The method may also be suitable for stability studies;
- may also be suitable as a basis for establishing assay methods to quantify the content of an extract in a finished medicinal product.

The minimum content of the analytical marker when using the assay method:

- is established by assaying extracts used in authorised and registered medicinal products;
- is given based on the final extract (genuine (native) extract with, where present, excipients);
- and thus:
  - represents the minimum value found in the extracts used for establishing the monograph after confirmation or modification following comments received from users, producers or national authorities during the public enquiry;
  - is related to the minimum value as stated in the corresponding herbal drug monograph;
  - reflects the range of yields of that particular marker from the extraction process as well as the stability of that marker during manufacturing;
  - is dependent on the amount of excipients added for technological reasons.

Since the monograph may encompass a broad range of extracts, the minimum value given for the analytical marker is not regarded as a stand-alone quality criterion (as compared to other minimum values given in the Pharmacopoeia). However, it gives an indication of the minimum values that can be expected when producing an extract from a herbal drug compliant with its monograph.

Based on the information given above, manufacturers may be requested by competent authorities to complement this information with a minimum value for their own extract depending on their individual manufacturing process and excipients added.

*NOTE: information on the characteristics of the extracts (for example, strength of extraction solvent, percentage of genuine extract, etc.) analysed during the elaboration of an individual extract monograph will be made available to users of the European Pharmacopoeia in the Knowledge database on the EDQM website.*

# 5.24. Chemical imaging

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# 5.24. CHEMICAL IMAGING

## 1. SCOPE

Chemical imaging (CI) combines spatially resolved sensing technologies with data analysis techniques to characterise a sample in chemical and physical terms, using information primarily obtained from its surface. Chemical imaging is particularly suited to the analysis of solid, semi-solid and liquid samples with regard to material properties, including component identity (active pharmaceutical ingredients and excipients), domain size and distribution, polymorphism, and particle morphology. Thus, imaging can be applied to assess identity, quality and quantity of active ingredients, intermediates, and excipients in bulk or solid dosage forms, biological samples, packaging and devices. Imaging is used to explore sample homogeneity, detect physical sample defects (e.g. cracks in cores or coatings), and identify foreign particles or contaminants. It also facilitates process understanding and root cause determination. Lastly, it is a tool to evaluate falsified or counterfeit medicinal products.

This general chapter's primary focus is on chemical imaging systems (CIS) based on surface analysis performed with vibrational spectroscopy, e.g. mid-infrared spectroscopy, near-infrared spectroscopy and Raman spectroscopy. However, it also applies to other techniques that supply images.

It offers specific recommendations to assess the performance of chemical imaging systems for the qualitative and quantitative exploitation of images. Where chemical imaging systems are primarily intended for investigative purposes, the performance requirements for infrared absorption spectrophotometry (*2.2.24*), near-infrared spectroscopy (*2.2.40*) and Raman spectroscopy (*2.2.48*) need not be applied. Instead, individual criteria have to be established using a risk-based approach.

## 2. ASPECTS OF CHEMICAL IMAGING

### 2-1. *DEFINITION*

Chemical imaging of pharmaceutical samples is a method that consists of a collection of responses to the illumination at multiple wavelengths of positions spatially distributed across the sample surface. For a given surface location (pixel), a set of responses will be associated with the impinging wavelengths. When *x* and *y* positions are varied successively over the range of wavelengths and responses are collected at each location, an image of a wide surface of the sample is constructed.
Imaging systems resolve spatial information primarily at the sample surface. Chemical and morphological sample characteristics or features are collated into an image made up of contributions from multiple domains distributed on the surface of the sample. As a result each mapped sample point (pixel) contains a wealth of information, and the recorded signal reflects chemical and physical properties such as ingredient identity, concentration, crystallinity, orientation, domain size and particle size.

### 2-2. *IMAGING MODES*

Chemical images may be acquired in broadband, multispectral or hyperspectral modes. An example of the broadband mode is the camera, where filters are used to weight the relative importance of different wavelengths for the 3 colour variables red, green, and blue (RGB). Multispectral imaging involves sampling spectral bands that may be spread unevenly, not unlike a combination of different detectors in different frequency ranges.
Hyperspectral imaging (HS imaging, HSI) is a two-dimensional (2D) visualisation technique that records a full range spectrum for each pixel. In practice, HSI expands spectroscopic single point analysis of samples into 2D projections of a slice of finite depth for each measured area of the sample. Hyperspectral sensors take advantage of numerous contiguous channels to uncover features that usually cannot be resolved using the average spectra obtained from a single measurement in classical spectroscopy. Images may be produced with a spectral quality similar to that of conventionally obtained spectra. However, this comes at the cost of longer measurement times, larger amounts of data, and complex computation. The spectral quality is sometimes lowered to increase the image throughput. Sample domains may include complex spectral patterns embedded in the individual pixel data that cannot be identified solely by visual inspection, and appropriate image analysis is required to extract analytical information from such a multitude of measurements. Computational and numerical methods are essential for processing, extracting and analysing content in hyperspectral images.

### 2-3. *DATACUBE*

In hyperspectral imaging each pixel is associated with one spectrum. Measurements are spread over both dimensions of the sample surface. At each measurement, a column vector of dimensions equal to the number of recorded spectral data points is associated. As a result, data is packaged into a three-dimensional array called a datacube or hypercube. The datacube is a signal intensity data set made up of the pixel *x-y* coordinates and the corresponding series of responses (e.g. absorbance or transmittance) to the scanned spectrum (see Figure 5.24.-1).
Not unlike classical spectroscopy, which gathers chemical and physical information about the single area under examination, imaging captures spectral and spatial characteristics of surface areas. Spectral and spatial analysis of image pixels can be iterative, or combined in no particular order.

### 2-4. *HIGHER DIMENSIONAL CHEMICAL IMAGING*

Spatially resolved three-dimensional imaging can be seen as extending spatial resolution along a third direction (*z*) that complements *x-y* imaging. It is the process of stacking a collection of images covering a depth into the sample which is larger compared to the analysis depth. This can be obtained indirectly (invasive, destructive process), by sequentially collecting images of appropriately prepared solid samples, e.g. by slicing the surface stepwise with a microtome. Meanwhile there are techniques which enable direct (non-invasive, non-destructive process) 3D imaging of solids using *x-y-z* spatially resolved spectroscopy. These include tomography methods such as optical coherence tomography (OCT), far-infrared (FIR)/terahertz (THz) and time-domain THz spectroscopy, confocal Raman, and others, for example, X-ray tomography or nuclear magnetic resonance imaging (MRI). Ultimately 4D and higher dimensional imaging would mean simultaneous spatial (*x*, *y*, and *z*) and spectroscopically resolved mapping. Although higher dimensional imaging is not within the remit of the current general chapter, similar recommendations may initially apply.

### 2-5. *APPLICATIONS*

Visual display of the distribution of sample surface features complements classical analytical methods by facilitating rapid and non-destructive comparison between samples.
CI can be used to analyse small to large surface areas of a sample. Imaging comes into its own as soon as constituents and morphological characteristics differ from one position to the next. Thus, the technique is particularly suited to exploring samples that are heterogeneous with regard to chemical content and physical morphology. Imaging captures the distribution of selected components and features, i.e. attributes of various parts of a sample. For example, images can display the location of features that directly impact product performance. They can show whether a component is evenly distributed on a relevant scale. Therefore, it is important to choose CI methods depending on the needs with regard to spatial resolution.

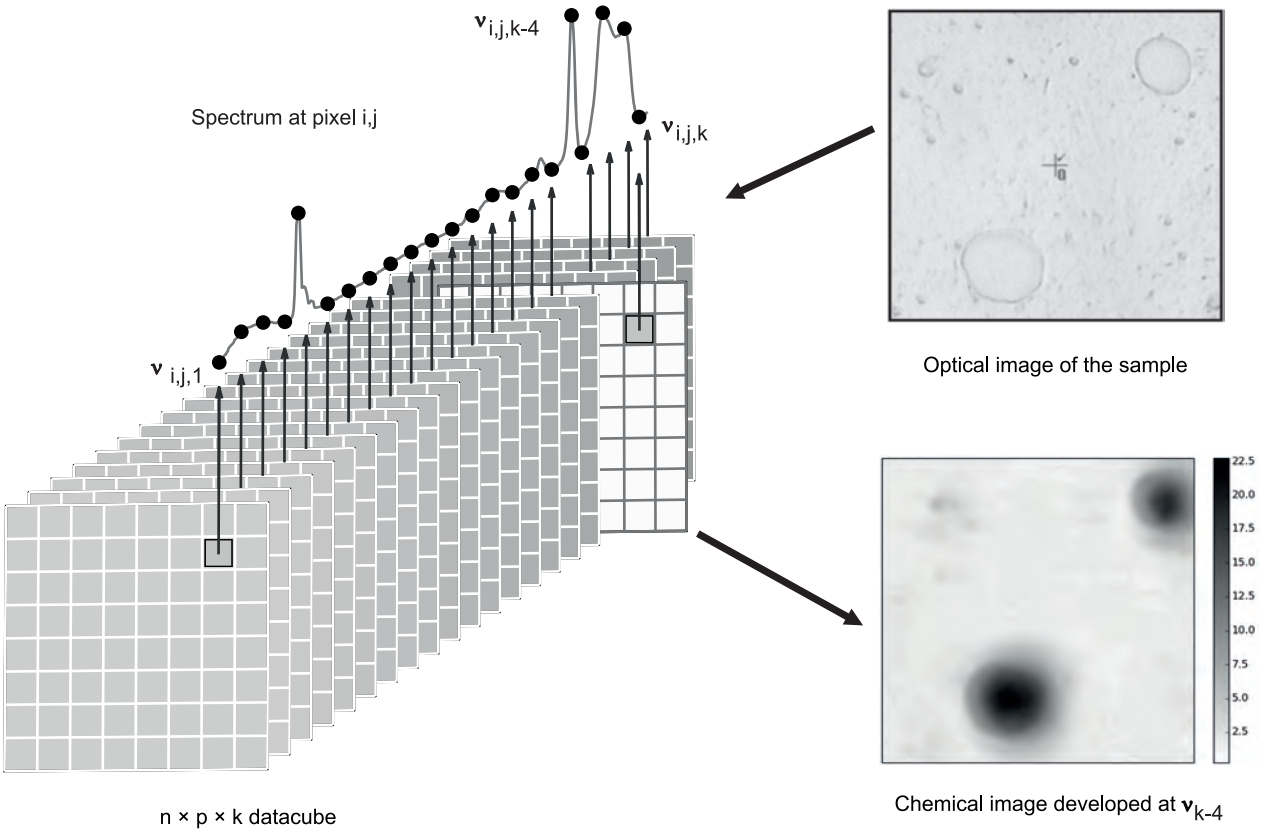


Figure 5.24.-1. – *Example of a n × p × k datacube obtained by recording spectra of k wavenumbers at n × p pixels of the sample; a grey-scale image was developed at wavenumber* $\nu_{k-4}$

The main applications of chemical imaging focus on solid-state property analysis, determination of chemical or physical features, contaminant identification, anti-counterfeit, and chemical identification. For example, the measurement of thickness and uniformity of coating on tablets, and the characterisation of surface properties, such as component mapping, determination of adhesion force and deformation depth, can be considered. Moreover, particle characterisation can be performed based on measurements of size, agglomeration and morphology, determination of roughness of the surface, and detection of broken particles and foreign particles. Analysis of particles can also be performed in liquids.

Spatial distribution of different polymorphic forms can be analysed with CI techniques and the investigation of polymorphic transitions and multiphasic materials (e.g. solid dispersions) can also be considered. The characterisation of nano- and micro-crystalline materials can be performed to monitor structural changes under stress conditions and over time, and to evaluate defects in crystals, for example, resulting from milling and micronisation of the material. The chemical characterisation of samples based on chemical images is mainly performed to identify and characterise the distribution and abundance of individual components in a mixture based on characteristic spectral features (see Figure 5.24.-2). This analysis can be performed to determine molecular but also atomic elemental species present on the surface. The kinetics and mechanism of active substance dissolution and release can be modelled, and, as another example, the drug concentration gradient between the solid/solution interface and the bulk solution can be determined.

2-6. *CHEMICAL IMAGING SYSTEMS*

The selection of a specific imaging instrument or technique depends on the particular analytical application intended. A chemical imaging system is characterised by setup, spatial and spectral resolutions, magnification, type and size of sample, sample preparation and presentation, moving or resting sample, acquisition time, number of measurements, data analysis algorithms, software, etc.

Numerous techniques enable the production of hyperspectral images. A brief description of techniques relying on vibrational spectroscopy is given below, along with their potential uses and limitations.

*Mid-infrared (MIR)*

MIR spectroscopy is based on the interaction of light with the sample, for the study of intra-molecular vibrations of a material. The electromagnetic spectrum range usually spans the region of 4000-400 $cm^{-1}$ (2.5-25 µm). MIR imaging may be used for the characterisation of chemical species in a mixture of ingredients. Measurements are often carried out using attenuated total reflectance (ATR) microscopy where the sample is in contact with an IR-transparent crystal of higher refractive index than the sample. The measurement may be difficult for samples with excessive moisture due to interference with water bands.

*Near-infrared (NIR)*

NIR spectroscopy detects molecular vibrations originating from C-H, N-H, O-H and S-H overtones, and combinations of fundamental mid-infrared vibrations. The electromagnetic spectral range usually extends from 12500-4000 $cm^{-1}$ (0.8-2.5 µm). The measurement is contactless, usually in diffuse reflectance mode, and delivers physical and chemical information.

*Far-infrared (FIR) and terahertz (THz)*

In the FIR range, electromagnetic radiation typically spans from 400 to approximately 10 $cm^{-1}$ (25-1000 µm). This allows spectra to cover inter-molecular and lattice vibration modes. The selectivity of the technique to the hydrogen bonding networks makes possible the identification and characterisation of solid state forms, e.g. polymorphic forms and degree of crystallinity.

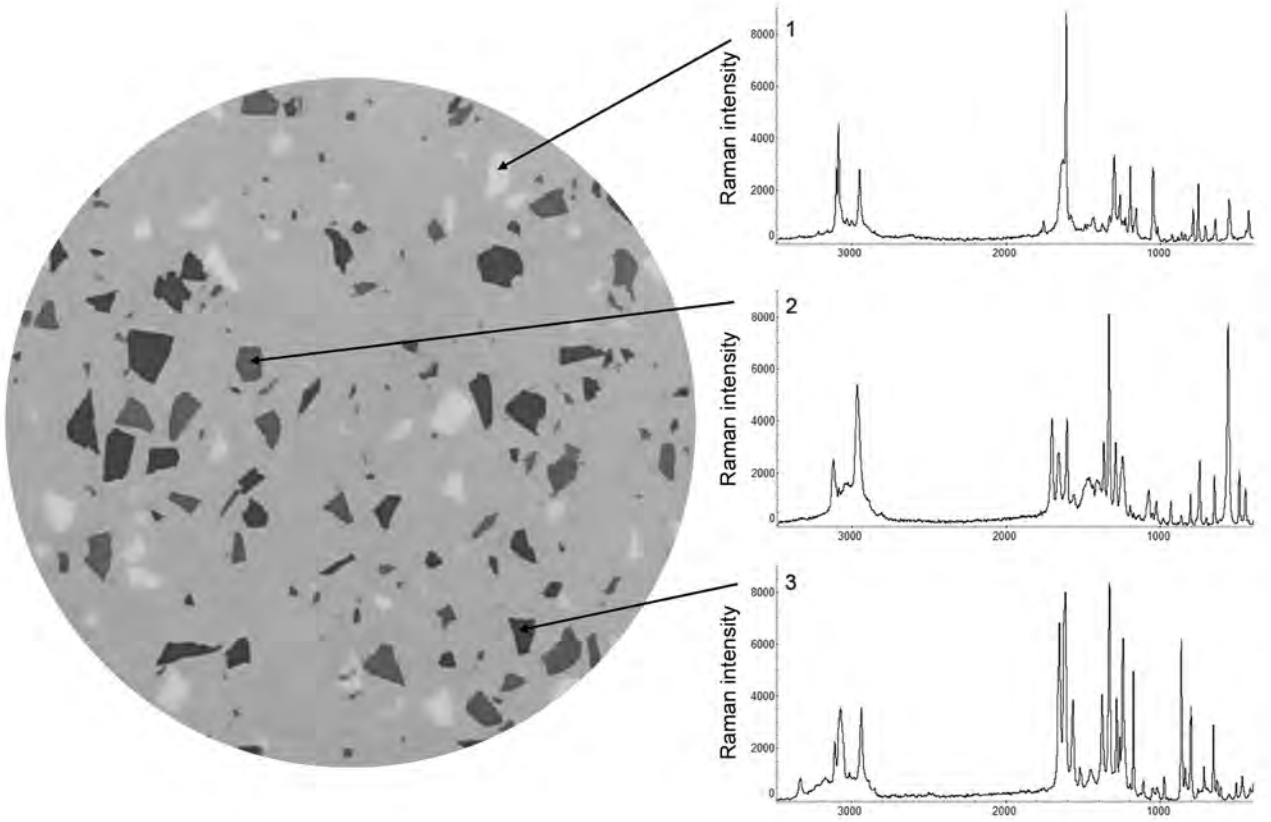


1. acetylsalicylic acid 2. caffeine 3. paracetamol

Figure 5.24.-2. – *Example of characterisation of ingredients with Raman imaging*

The radiation can pass through a wide variety of non-conducting materials, but cannot pass through conducting materials such as metal and water, and penetrates deep into the sample.

*Raman*

Raman spectroscopy detects frequency shifts originating from the inelastic part of the light scattered by a sample previously irradiated with an intense monochromatic light source (usually a laser). Raman spectra contain numerous narrow bands which makes possible the identification of chemical substances. As a result, Raman imaging gives information on chemical species in a sample (including polymorphs).

Water, air and glass are weak Raman scatterers and therefore the analysis of aqueous samples may be performed in atmospheric conditions or in sealed vials. However, the measured signal may be disturbed by fluorescence.

2-7. *ACQUISITION MODES*

Either spatial sequencing or wavelength sequencing techniques are required to produce an image. The 3 modes used to record datacubes are point mapping, line mapping and global imaging. Point mapping (point scanning) is the simplest and typically follows a rectangular grid pattern. A spectrum is recorded at each point on the grid with the sample stage being moved to the next neighbouring location. Scanning is computer-controlled along both spatial axes. In line mapping (linear scanning), the sample is illuminated along a line and the image signal is dispersed along one spatial axis onto the detector. The sample is moved along the other spatial axis to capture the next neighbouring line until full mapping is eventually achieved. Here the speed and flexibility of the experimental setup enable on-line application of continuous CI. Global imaging (focal plane scanning) is performed when all image points of a sample are imaged simultaneously onto the detector array. This can be done on a per wavelength basis with filters or tuneable filters.

2-8. *RESOLUTION*

Pixels contain a wealth of contributions from chemical and physical features occurring at the surface and near sub-surface of the sample. The performance of an imaging system is directly related to the spatial and spectral resolutions that can be achieved. Linear line mapping systems are more sensitive to spatial interference, whereas spectral resolution is more critical with focal plane acquisition.

Depending on the imaging technique, spectral resolution may be affected for example by laser wavelength, grating, detector and spectrometer focal length. Spectral resolution has an impact on chemical feature extraction because it influences the performance of qualitative and quantitative image analysis, e.g. identity of ingredients.

Spatial resolution is the smallest distance between two consecutive points which can be distinguished. It has an impact on image processing as information may appear as unresolved. For example, the domain size of components of interest should be consistent with the spatial resolution achieved by the system. The spatial resolution obtainable is limited by the instrument characteristics, for example spot size of the laser, diffraction limits, detector size, magnification, numerical aperture, etc. The spatial resolution obtained by a specific CI system is also affected by the diffusion of radiation across the sample surface. Scattering occurring below the surface is likely to distort the image obtained and limit the spatial resolution.

2-9. *REPRESENTATIVENESS OF SAMPLE SURFACE*

Depending on the instrument setup, the topology of the sample surface may impact imaging performance.

The sample surface investigated has to be representative for the intended purpose of analysis. Depending on the latter, the impact of sample homogeneity has to be evaluated. For example, with a design based on reflectance, due to the limitation of penetration depth and spatial resolution, a true measure of morphology or distribution of particle shapes in

the sample is only estimated. Thus, results obtained from surface image analysis may not be representative of the whole sample because it may not be homogeneous.

One approach to overcome this limitation would be to measure a number of cross-sections of the sample to improve estimations for the whole sample. Another approach would be to use an alternative optical design where penetration depth can be increased, e.g. confocal or spatially resolved offset measurements to capture information near the surface, tomography or transmission measurements to collect whole matrix information.

## 3. ELEMENTS OF A CHEMICAL IMAGING PROCESS

Proceeding with imaging encompasses numerous steps such as:

- sample preparation;
- control of instrument components and system performance;
- calibration of instrument and system suitability test;
- measurement, image processing, display and storage;
- image analysis and computation of numerical results.

### 3-1. *SAMPLE*

#### 3-1-1. **Sample preparation**

Sample preparation has to be in accordance with the imaging technique used. *In situ* measurements with probes can be performed without sample preparation. On the other hand, setups for Raman scattering for example proceed by non-contact focusing, meaning that the sample surface may need to be prepared to obtain a reasonably flat surface. If the instrumentation is unable to compensate for topographical variation, sample surfaces may be mechanically modified, for example by flattening a concave tablet surface. Focusing adjustments by automatic refocusing during mapping compensates for slightly uneven surfaces. Sample surface preparation has also to be considered with ATR-IR imaging for which contact between optics and sample is required.

#### 3-1-2. **Sample presentation**

Appropriate sample presentation is dictated by instrument setup. A particular setup will be more or less adapted to a specific sample type and analytical task. The sample is positioned so that it is optimally imaged. The setup is optimised to reduce specular reflection as much as possible. Angles and distances between probe (or beam), sample and detector match the setup requirements.

### 3-2. *CONTROL OF INSTRUMENT PERFORMANCE*

Evaluating both instrument performance and image analysis methods is essential to avoid misinterpretation or artefacts. Parameters include spectral as well as spatial components. The instrument is to be used according to the manufacturer's instructions.

Instrument performance verification consists of periodic performance qualification as well as system suitability tests. The intervals depend on the use of the instrument and its application. The system suitability tests are carried out before measurements to check if the CI system is operating properly for the intended application.

The parameters to be assessed and the acceptance criteria applied during performance qualification and system suitability tests have to be justified and depend on the CI technique and the purpose of the analysis. Parameters which might be verified are described below.

#### 3-2-1. **CIS component adjustment**

In a schematic setup, a typical CIS would be made up of components including source of radiation, optical devices, sample holder, detector, and software.

The system and its individual components comply with all expected requirements.

##### 3-2-1-1. *Sources, optics and detectors*

Source intensity is monitored as part of the setup verification. In particular, the source intensity should be verified before starting calibration routines or a new series of sample measurements. Optical path, confocality, wavelength accuracy and energy throughput at any *x-y* position (pixel) match the specifications.

Alignment of optics, sample and detectors comply with measurement requirements in terms of distances, angles and polarisation. This alignment may shift with the temperature. In particular, illumination of the sample or regions of interest has to be as homogeneous as possible and reproducible.

Parasitic or adverse effects, for example scattering, background, noise, bad pixels, cosmic rays and fluorescent lights in the laboratory, and side effects such as sample fluorescence have to be controlled. Stray light, ghost lines, and ghost images can be caused by reflections from imperfect surface elements. They constitute parasitic light that has to be dealt with.

##### 3-2-1-2. *Multi-wavelength and multispectral systems*

Multi-wavelength systems should be tested at wavelengths spread over the wavelength scale using the peaks of the selected reference standard with good signal-to-noise properties.

Multispectral systems are verified for all signal sources involved.

##### 3-2-1-3. *Mapping*

When images from more than one instrument are going to be aligned, it is important to have verified *x-y* scales.

##### 3-2-1-4. *Magnification*

In the case where the CIS permits different magnifications, the optical or electronic magnification level needs to be optimised. If magnification is inadequate for resolving relevant features, the value of imaging may be diminished; whereas if magnification is set higher than required, the field of view is reduced.

#### 3-2-2. **Calibration**

The purpose of calibration is to transform the recorded signal into data suitable for analysis, interpretation, or comparison with reference data.

Basic instrument calibration of imaging systems consists of *x*- and *y*-axis calibration in both wavelengths and intensity.

##### 3-2-2-1. *Spectral axis*

Optimal wavenumber accuracy is critical and should be achieved at the same level as for a standard spectrometer. Spectral axis calibration assigns wavelength and intensity values to every mapped pixel of the sample surface. To determine these relationships, well-characterised light sources or reference materials can be used. Calibration should be carried out using referenced and certified/normalised internal or external standards provided either by the supplier or a third party. Technology and environmental conditions are other critical points.

##### 3-2-2-2. *Spatial axes*

Spatial axis calibration has an impact on the actual field of view of the CIS and the sampled surface area, i.e. the pixel location. Calibration corrects deformations on the spatial axis such as those caused by the optics. For example, the pixel position may be accompanied by variation of the spectral resolution from the centre towards the detector boundaries. In addition, mixing of signals from adjacent pixels can occur. Both errors change the appearance of the pixel spectra and may reduce the accuracy of subsequent data analysis. If it is not possible to deduce the position of the imaged pixels on the detector surface from the pattern on the target surface, a special test target can be used or developed for the application.

Spatial resolution in both *x* and *y* directions is also limited, for example, by image size and step size of the moving table or the conveyor belt.

3-2-2-3. *Method*

Appropriate data processing and modelling are needed to deliver information about the property that correlates with the observed features in images. Calibration related to the analytical objective requires, for example, identifying and estimating a characteristic identity or morphological features of the sample.

3-3. *IMAGE PROCESSING*

Processing means that the image is treated for display and subsequent feature estimations. Imaging requires steps to improve the image for analysis, e.g. to control brightness and contrast, reduce noise, sharpen edges and borders, remove unwanted features, etc. This improvement allows features to be recognised i.e. separated and discriminated from the background.

Chemical images are generally processed by chemometric modelling for qualitative or quantitative purposes (see general chapter *5.21. Chemometric methods applied to analytical data*).

3-3-1. **Spectral selection**

Image contrast is built upon specific signatures of the components at the sample surface and derived from spectroscopic data such as peak intensity, peak position, baseline variations (scattering differences), or values calculated with multivariate analysis.

3-3-2. **Image pre-processing**

Data analysis methods for imaging data sets usually begin with the same steps as for single point spectroscopy. Pre-processing is utilised to separate chemical and physical effects, unless an analysis is based on the physical effects (e.g. scattering differences seen as baseline variations). The collected data is typically affected by noise or instrumental distortions and pre-processing is often applied to improve image quality. There may also be a need to remove irrelevant sources of variation such as cosmic ray spikes or stripes due to detector configuration. Common pre-processing operations for spectra are: baseline correction, derivatives, smoothing, wavelet-based filtering, standard normal variate (SNV), multiplicative scatter correction (MSC), normalisation, truncation and Fourier-transform. It has to be verified that pre-processing does not cause artefacts.

3-4. *IMAGE ANALYSIS*

Pixel arrangements in an image of the sample represent a map of the distribution of chemical or physical properties or features. Depending on the objective of the analysis, data can be explored for spectral features, spatial features, and a combination of both. Examination of the datacube can be performed in a direct way, as for conventional spectroscopy (e.g. by analysing height of peaks or area ratios), or by techniques including image processing, multivariate data analysis, etc.

3-4-1. **Visual exploration**

A review by the user of an image displayed on screen may deliver a first impression before beginning enhancement, exploration and evaluation. Images can be analysed for features at the surface of the sample, such as location, distribution, size and shape of domains, constituent identification, etc. Standard image processing tools, such as morphological filtering and particle statistics, may be applied directly for samples that are significantly heterogeneous.

For a chemical image, both spectral and spatial analyses can be equally important and there are different steps of chemical image analysis. Spectral and spatial analyses are often iterative by nature. In practice, the methods used depend upon whether it is the spectral information or the spatial information that is of more interest. The goal is to reduce data dimensionality and extract as many features as possible. Unsupervised multivariate analysis such as principal component analysis (PCA) may be applied prior to feature extraction to describe the information contained in the image. A preliminary segmentation of PCA images may be used to facilitate identification of regions of interest in the image for further processing. If the ingredients of a mixture are known and available for reference measurements, the corresponding data is used to reduce the number of spectral layers in the hypercube down to a few chemically or physically meaningful layers by using supervised regression methods, e.g. multivariate curve resolution-alternating least squares (MCR-ALS), independent component analysis (ICA), and partial least squares regression (PLS).

3-4-2. **Feature extraction**

The extraction of chemical or morphological features of samples involves supervised techniques to perform dimensional reduction. It concentrates further image processing on characteristics that are meaningful for the analysis. For example, a selected feature could be the quantity of a given substance or a solid-state form of interest. If the sample is comprised of components whose spectral features are well separated, it is possible to proceed directly with a univariate method based on a single wavelength per feature, thus utilising only a small fraction of available data. However, in many cases spectral overlap is significant and the univariate approach is insufficient. The extraction of features can then be improved by including a greater proportion of the spectral information and applying a multivariate approach, such as clustering, classification, PLS discriminant analysis (PLS-DA), linear discriminant analysis (LDA), etc.

3-4-3. **Quantification of ingredients**

There are several ways to measure ingredient variation using regression methods. A quantitative model can be computed by using average spectra or binning of regions of interest at the surface of the sample. The known bulk concentration may be used when the ingredient present varies in responses for the analytical method used. This can be performed by peak integration at a single wavelength or alternative chemometric methods.

When individual pixel spectra become increasingly mixed, chemometric tools, typically multivariate data analysis, can be deployed with similar data analytical strategies as those for single-point spectroscopy.

For example, it is possible to estimate relative component abundance by using pure component spectra as the starting point. The reference spectra used to create the library are collected from separate pure component samples. These spectra can be measured with a non-imaging spectrometer, or as separate images of pure components. The inherent variation in pure component spectra when acquired across a large number of pixels provides additional statistical robustness. The reference spectra and the unknown sample can also be measured simultaneously in the same field of view to create one single data set. If pure component reference spectra can be derived directly from an isolated area of the imaged sample, there is no need to acquire additional reference spectra for calibration. A multivariate model can be developed either based on external reference spectra or based on spectra from isolated areas of the respective image.

Both with simultaneously and separately acquired pure component spectra, it is important to verify that they are representative for the present analysis by for example regression residuals.

3-4-4. **Spatial analysis of image features**

Spatial analysis of the images can be performed with regard to distributions of shapes, sizes and locations of domains, etc. Analysis of shape and distribution relies on a variety of numerical methods and algorithms.

Images display the samples by setting a threshold value and converting data into a set of colours or shades according to a monochrome or pseudo-colour intensity scale to underline the presence or proportion of the analytes of interest at each pixel. Alternatively, the distribution of several analytes can be combined into colour images. Further analysis can

be performed, such as counting pixels, estimating the size distributions of particles of the same chemical origin, and segmentation which is a process to divide the image between regions that correspond to surface areas of interest.

3-4-5. **Measuring dimensions**

Images can be used for example to determine area, dimensions, perimeter, aspect ratio and roundness.

3-4-6. **Statistics**

Pixel statistics can be performed to facilitate further image analysis. Application of methods such as variance analysis and box-plot for domain size, shape, distribution, and distances between domains, enables estimation of the variability within an image. These methods deliver a condensed description of relations between domains in the images with simplification of the image analysis. Property estimations can also be derived from these values.

# 5.25. Process analytical technology

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# 5.25. PROCESS ANALYTICAL TECHNOLOGY

*This general chapter focuses on the interfacing of analytical techniques with the manufacturing process as a means of enhancing process control and improving process understanding. It should be noted that it is not within the scope of the chapter to give specific instructions for all possible process analytical technology sensors; instead, it offers a general approach to the integration of analytical techniques in the process environment together with aspects to be taken into consideration for the application of process analytical technology.*

## 1. INTRODUCTION

Process analytical technology (PAT) can be defined as a system for designing, analysing and controlling manufacturing processes through timely measurements (i.e. during processing) of critical quality attributes (CQA) of raw and in-process materials and critical performance characteristics of processes in order to ensure the quality of the final product. It is important to note that the term 'analytical' in PAT is used in a broad sense to include chemical, physical and microbiological measurements conducted in an integrated manner and combined with data analysis. The goal of PAT is control of the manufacturing process and enhanced process understanding, guided by risk management. Interfacing manufacturing processes with analytical techniques is therefore essential in PAT, as it facilitates process development in accordance with quality by design (QbD) principles, enables real-time release testing (RTRT) and supports continuous manufacturing processes.

Time delays between obtaining a sample for testing, analysis of that sample and any consequent outcomes must be taken into consideration when applying PAT. When the analytical results are used on a continuous basis to monitor and control a process, it is important to minimise such delays. This can be achieved most effectively with sensor-based continuous measurement systems directly interfacing with the process stream during a specific unit operation. The sensors interfaced with the process continuously measure the process conditions and material characteristics within the process environment (*in situ*) and send the measured data (e.g. a spectrum) to an operating system where it is recorded and analysed, and where any necessary adjustments to processing conditions can be determined on a continuous basis. These *in situ* measurements can generate very large volumes of data representative of the process.

## 2. INTERFACING MODES

The interfacing of analytical techniques with the manufacturing process is central to the application of PAT. Results generated by analytical equipment interfaced with a process can be used for monitoring purposes and to ensure that the process is stable, for example via automated feedback or feedforward control loops.

The terms 'off-line', 'at-line', 'on-line' and 'in-line' describe interfacing modes (see Figure 5.25.-1). Measurements using on-line and in-line systems generally support rapid and automated process adjustments since they move the analytical techniques into the process stream. In contrast, off-line and at-line measurements involve transferring the samples away from the process stream to the analytical equipment.

**Off-line measurements**

These correspond to conventional analytical testing in which the sample is removed from the manufacturing process environment and tested in a laboratory, typically located away from the production environment. This transfer of samples away from the process stream can result in a significant time lag and generally does not permit immediate process adjustments. However, off-line measurements can nonetheless be useful for PAT purposes if relevant analytical data can be obtained within a time frame that is compatible with process dynamics.

**At-line measurements**

With at-line measurements, the sample is also removed (manually or automatically) from the process stream for testing, but the testing equipment is usually located within the production environment, i.e. in close physical proximity to the process stream, and testing can therefore take place with minimal delay. This results in a shorter time frame for obtaining analytical data, and also differentiates at-line measurements from off-line measurements. It is therefore possible to make process adjustments based on the results of at-line measurements.

**On-line measurements**

On-line measurements typically involve sensor-based measurements made under real-time conditions by diverting a portion of the material from the process stream directly into a measuring device and the results are available after a minimal time delay.

Depending on the nature of the test, e.g. whether or not it is detrimental to the product, the diverted portion may be returned to the process stream, otherwise it is discarded.

**In-line measurements**

In-line measurements are taken within the process stream by placing measuring devices, typically sensors, directly in contact with or into the process stream. No portions of the material are therefore removed from the process stream.

In-line measurements must also be non-detrimental to the product.

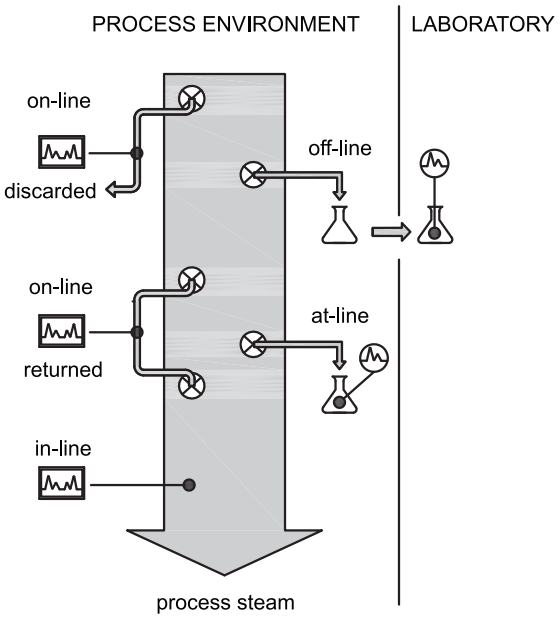


Figure 5.25.-1. – *Interfacing modes*

## 3. COMPARISON OF INTERFACING MODES

Both off-line and at-line measurements are based on analysis of discrete samples removed from a process stream or bulk material. These samples are considered representative of the material in the process stream at the time they are removed. Frequent at-line measurements also provide data supporting the application of PAT, as they are carried out within a short time frame and in the immediate vicinity of the process stream.

Neither in-line nor on-line measurements involve sampling in the conventional sense. The tested portion might not be separated from the process stream, and is usually smaller than a conventional sample.

Scale of scrutiny must be considered and will determine the frequency and duration of measurements. Furthermore, measurements can be influenced by physical attributes interfering with the acquisition properties of the measuring system. When measuring solids or suspensions, for example using spectroscopic methods, consideration must be given to the surface and bulk scattering properties of samples, and movement of the material. Influencing factors such as particle size, surface roughness and solid density can cause significant spectral differences, which must be taken into account when a method is designed and used in order to ensure that the process and material are described adequately.

In-line and on-line modes offer the advantage of fast data acquisition, allowing high measurement frequency, and thus enabling rapid continuous monitoring as well as immediate action and control of the process.

In some cases, PAT measurements can be used qualitatively to build models such as process trajectories or process signatures which can be used to characterise process variability and highlight unusual process behaviours. These models are not necessarily directly linked to a critical in-process quality attribute or performance characteristic. It is therefore recommended to demonstrate the causal relationship between the PAT measurements, the model and the relevant quality attributes or performance characteristics when using such models for process control purposes.

General validation principles also apply to in-line or on-line measurements although some validation procedures may be different to those for conventional quality control methods.

Analytical validation procedures for at-line and off-line measurements assume homogenous and authentic samples, which are crucial for the assessment of precision. However, this requirement is rarely fulfilled for on-line or in-line methods which are often used to measure processes in which the material rapidly changes and/or moves during the measurement, for example a fast-drying process or reaction monitoring. Comparison of results from on-line/in-line measurements with results from a reference test procedure would normally be needed.

## 4. STATISTICAL PROCESS CONTROL

Statistical process control (SPC) comprises a set of data analysis methods applied in order to monitor and control a process based on analysis of process variability (e.g. of CQAs). For PAT purposes, the process can be monitored using, for example, real-time data collected by process analysers.

Based on this data, SPC can be used to make process adjustments, when necessary, to maintain or attain a desired state and to ensure that the process remains under control. This includes, for example, trend analysis of quality attributes or process variables. SPC can also be used to measure process variability and process capability (i.e. the ability of a process to produce a product that complies with requirements), with a view to enhancing process understanding, thereby improving lifecycle management.

Rather than using univariate SPC methods to monitor several individual variables, it is often preferable to use multivariate statistical process control (MSPC) to analyse several variables simultaneously, taking into account potential correlations.

## 5. EUROPEAN PHARMACOPOEIA TEXTS SUPPORTING THE APPLICATION OF PAT

Typically, the analytical techniques described in the Ph. Eur. include qualification criteria designed for off-line analytical systems. These criteria are not always relevant or practical in a PAT setting, for instance when the equipment is specifically designed for on-line or in-line measurements. For this reason, certain general chapters (including *2.2.40. Near-infrared spectroscopy*, *2.2.48. Raman spectroscopy* and *2.9.47. Demonstration of uniformity of dosage units using large sample sizes*) have been revised or specifically elaborated to support and promote the use of the techniques in conjunction with PAT.

**General Notices**

Section 1.1, under Demonstration of compliance with the Pharmacopoeia, states that a substance can be demonstrated to be of pharmacopoeial quality on the basis of product design, together with the control strategy applied and data derived, for example, from validation studies of the manufacturing process.

The section also states that an enhanced approach to quality control could utilise PAT and/or real-time release testing strategies (including parametric release) as alternatives to end-product testing alone.

**Absorption spectrophotometry, infrared**

General chapter *2.2.24* has a wide variety of applications in the manufacturing process that enable PAT, such as reaction monitoring in chemical synthesis. Wavenumber shifts and acceptable tolerances for the reference material polystyrene are described for both transmission and ATR measurement modes.

**Absorption spectrophotometry, ultraviolet and visible**

General chapter *2.2.25* covers the use of PAT in the UV-Vis range using modern detectors such as photodiode arrays (PDA) or charge-coupled devices (CCD). Both transmission and diffuse reflection measurement modes are possible with off-line, at-line, on-line and in-line measurements. The table with reference wavelengths for the control of wavelength accuracy includes wavelengths in the UV range down to 180 nm.

**X-ray fluorescence spectrometry**

General chapter *2.2.37* includes references to modern equipment and the current applications of the XRF technique.

Substantial advances in miniaturisation and automation have led to the development of hand-held energy dispersive XRF (ED-XRF) spectrometers for rapid field measurements. XRF is potentially non-destructive, although it may cause sample instability. Hence, XRF lends itself to applications of PAT such as the analysis of unwanted trace catalysts in active pharmaceutical ingredients (API).

**Near-infrared spectroscopy**

General chapter *2.2.40* takes into account the increasing use of NIR spectroscopy for process monitoring and control, and the development of modern NIR spectrometers. NIR measurements can be performed off-line, but the technique also lends itself to at-line, on-line and in-line testing.

The 'Sample preparation/presentation' section includes moving materials or samples, and the use of fibre-optic probe systems in different measurement modes (i.e. transmission, diffuse reflection and transflection). The use of internal referencing for process analysis purposes is permitted when it is impossible to remove a probe for reference background data collection. A detailed table for the control of instrument performance depending on measurement mode and instrument used (benchtop, mobile or process instrument) is also provided. The chapter includes a section on qualitative analysis for identification and characterisation of a substance in addition to sections on limit analysis (e.g. for dryer end-point control) and trend analysis (for monitoring blend uniformity).

**Raman spectroscopy**

General chapter *2.2.48* includes Raman technologies that have potential uses for the application of PAT, including hand-held instruments.

Wavenumber shifts and acceptable tolerances for the reference materials cyclohexane, paracetamol and polystyrene (values derived from an inter-laboratory study) are described for both benchtop and hand-held Raman systems.

**Demonstration of uniformity of dosage units using large sample sizes**

General chapter *2.9.47* allows the determination of dosage unit uniformity in a PAT environment where the sample size is markedly greater than 30 units.

Compliance with the acceptance criteria of general chapter *2.9.47* is considered as evidence that the batch would also comply with general chapter *2.9.40. Uniformity of dosage units* if tested using a small sample size (e.g. 30-unit). Therefore, general chapter *2.9.47* does not constitute stand-alone acceptance criteria.

**Alternative methods for control of microbiological quality**

General chapter *5.1.6* describes alternative methods that might be used for the application of PAT in order to contribute to real-time or near-real-time microbiological quality control (e.g. test for microbiological examination of non-sterile products or for sterility based on laser-induced fluorescence) of in-process samples, active substances, medicinal products or excipients (particularly water). The chapter also provides guidance on how to validate these alternative methods.

**Chemometric methods applied to analytical data**

Chemometrics (general chapter *5.21*) has proven to be well suited for PAT. The investigation of large data tables and treatment of intricate signals, i.e. data collections with a hidden structure, of the type accumulated during measurements in PAT setups, requires alternative analytical tools to those used in a one-variable-at-a-time approach.

**Chemical imaging**

Chemical imaging (CI) (general chapter *5.24*) can be used to support PAT applications. CI measures spatial distribution and contributes to the understanding of the properties of materials such as finished products, pharmaceutical intermediates, excipients, APIs and starting materials.

The uses of CI include, for example, detection of defects at the sample surface such as cracks in tablet coatings and identification of foreign particles. It is a versatile tool used in process development and improvement, root cause analysis (e.g. for out-of-specification results), and may also be used to enhance process understanding.

# General monographs

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**01/2019:1063**

# ALLERGEN PRODUCTS

## Producta allergenica

*This monograph does not apply to: chemicals that are used solely for diagnosis of contact dermatitis; chemically synthesised products; allergens derived by recombinant DNA technology. It does not necessarily apply to allergen products for veterinary use.*

### DEFINITION

Allergen products are pharmaceutical preparations derived from extracts of naturally occurring source materials containing allergens, which are substances that lead to and/or provoke allergic reactions. The allergenic components are most often of a proteinaceous nature. Allergen products are intended for *in vivo* diagnosis or treatment of allergic diseases attributed to these allergens.

Allergen products are available as finished products, and as finished products used on a named-patient basis. Allergen products are generally presented as parenteral preparations, eye preparations, preparations for inhalation, preparations for oral use, sublingual preparations or preparations for skin tests.

For *in vivo* diagnostic use, allergen products are usually prepared as unmodified extracts in a 50 per cent *V/V* solution of glycerol for skin testing. For intradermal diagnosis or for provocation tests by nasal, ocular or bronchial administration, suitable dilutions of allergen products may be prepared by dilution of aqueous or glycerinated extracts, or by reconstitution of unmodified freeze-dried extracts.

For *specific immunotherapy*, allergen products may be either unmodified extracts or extracts modified chemically and/or by adsorption onto different carriers (for example, aluminium hydroxide, calcium phosphate or tyrosine).

### PRODUCTION

Where allergen products or source materials are manufactured using materials of human or animal origin, the requirements of general chapter *5.1.7. Viral safety* apply.

*SOURCE MATERIALS*

Source materials are obtained from qualified suppliers. The source materials comply with the requirements of the appropriate individual monographs (where a relevant monograph exists) and the statements in this section are intended to be read in conjunction with the individual monographs.

Source materials for the preparation of allergen products are products of animal or vegetable origin, mostly pollens, moulds, mites, animal epithelia and outgrowths, and Hymenoptera venoms. Other source materials include certain insects and foods.

The source materials are defined, where possible, by their origin, nature, method of collection or production and pre-treatment. Control methods and acceptance criteria relating to identity and purity are established. The acceptance criteria must ensure the consistency of the allergenic source material from a qualitative and quantitative point of view. The source materials are stored under controlled conditions justified by stability data.

The collection or production, as well as the handling of the source materials, are such that consistent composition is ensured from batch to batch.

When applicable, pesticides, heavy metals and residual solvents are limited according to the principles defined in general chapters *2.8.13. Pesticide residues*, *2.4.27. Heavy metals in herbal drugs and herbal drug preparations* and *2.4.24. Identification and control of residual solvents*, respectively.

Microbial contamination of the source material may be unavoidable and should be monitored according to a justified sampling plan; if a determination of microbial contamination is not applicable, this must be justified.

The scientific name (species, variety, strain etc.) of the source material is indicated and the part used is stated, if applicable.

Foods must be of a quality suitable for human consumption. The origin of the food stuff as well as its processing stage is stated.

*MANUFACTURING PROCESS*

Allergen products are generally obtained by extraction, and may be purified, from the source materials using appropriate methods shown to preserve the allergenic properties of the components. Allergens for which there are not enough patients to determine the total allergenic activity *in vivo* or *in vitro*, the extraction ratio indicating the relative proportions (*m/V*) of allergenic source materials and solvents is a minimum requirement. Allergen products presented as parenteral preparations, eye preparations, preparations for inhalation and preparations for skin testing are manufactured under aseptic conditions.

In the manufacture, packaging, storage and distribution of allergen products intended for administration by other routes, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

All allergen preparations are manufactured under conditions designed to minimise exogenous and endogenous enzymatic degradation.

Any purification procedure is designed to minimise the content of any potential irritant low-molecular-mass components and non-allergenic components.

Allergen products may contain suitable antimicrobial preservatives. The nature and concentration of the antimicrobial preservatives have to be justified and their efficacy complies with chapter *5.1.3. Efficacy of antimicrobial preservation*.

The manufacturing process comprises various stages:

- source material;
- active substance: it is generally a modified or an unmodified allergen extract; where applicable it is stored under conditions ensuring its stability, for example freeze-dried;
- finished product.

All other stages of the manufacturing process are considered as intermediates.

*IN-HOUSE REFERENCE PREPARATION*

An appropriate representative preparation is selected as the in-house reference preparation (IHRP), characterised and used to verify batch-to-batch consistency. The IHRP is stored in suitably sized aliquots under conditions ensuring its stability, for example freeze-dried.

**Characterisation of the in-house reference preparation**. *The extent of characterisation of the IHRP depends on the source material, knowledge of the allergenic components and availability of suitable reagents, as well as the intended use. The characterised IHRP is used as the reference in the batch control of active substances and intermediates and, if possible, in the batch control of finished products.*

The IHRP is characterised by the protein content determination and a protein profile using appropriate methods (such as isoelectric focusing, sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoelectrophoresis,

capillary electrophoresis, chromatographic techniques and mass spectrometry).

Allergenic components may be detected by appropriate methods (for example, immunoblotting or crossed radio-immunoelectrophoresis). Characterisation of the allergenic components may include identification of relevant allergens based on serological or other techniques using pooled or individual sera from allergic patients, or allergen-specific polyclonal or monoclonal antibodies.

Determination of the content of relevant allergens is performed wherever possible. This determination may be made using individual allergen-specific reference standards, when available. The choice of the relevant allergen components subjected to the determination must be justified. Individual allergens are identified and named according to internationally established nomenclature wherever possible.

The biological potency of the first IHRP is determined in patients by *in vivo* techniques such as skin testing, and expressed in units of biological activity, except when not enough patients are available. In this case, the potency of the first IHRP is determined by an *in vitro* method. Subsequently, the biological activity of future IHRPs is demonstrated by *in vitro* methods by comparison with the results obtained with the first IHRP. The *in vitro* potency may be measured by a suitable immunoassay (for example, an assay based on the inhibition of the binding capacity of specific immunoglobulin E antibodies).



## IDENTIFICATION

The tests for identification are performed as late as possible in the manufacturing process. In the case of products used on a named-patient basis, the control is performed on the active substance and/or at the intermediate stage between the active substance and the finished product.

Identity is confirmed by comparison with the IHRP using protein profiling by appropriate methods (for example, isoelectric focusing, sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunoelectrophoresis, immunoblotting, liquid chromatography or mass spectrometry).

In exceptional cases, if no IHRP is available, a representative batch may be used to confirm identity.

Identity may also be confirmed by comparison with individual allergen-specific reference standards, when available.

## TESTS

The tests are performed as late as possible in the manufacturing process. In the case of products used on a named-patient basis, the control is performed on the active substance and/or at the intermediate stage between the active substance and the finished product.

Various biochemical and immunological tests have been developed in order to characterise allergens qualitatively and quantitatively. In those cases where such methods cannot be applied, particularly for the determination of allergenic activity and allergen and/or protein profile, justification must be provided.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*): maximum 5 per cent for freeze-dried products. In the case of oral lyophilisates, the water content may be higher than 5 per cent, where justified and authorised.

**Sterility** (*2.6.1*). Allergen products presented as parenteral preparations, eye preparations, preparations for inhalation or preparations for skin testing comply with the test for sterility.

**Microbial contamination**. For non-sterile allergen products, recommendations are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

**Protein content** (*2.5.33*): 80 per cent to 120 per cent of the stated content, unless otherwise justified and authorised. If the biological potency can be determined then the test for protein content is performed as a batch-to-batch consistency test and the protein content is within 50 per cent to 150 per cent of the stated content. When the finished product contains proteinaceous excipients, the test for protein content is performed as late as possible during production before addition of the proteinaceous excipient.

**Protein profile**. The protein profile determined by suitable methods corresponds to that of the IHRP. The presence of relevant allergen components is verified, where possible. The choice of relevant allergen components to be tested for must be justified.

*Various additional tests, some with increasing selectivity, depending on the allergen product concerned can be applied, but in any case for allergen products intended for therapeutic use, a validated test measuring the potency (total allergenic activity, determination of individual allergens or any other justified tests) must be applied.*

**Aluminium** (*2.5.13*): 80 per cent to 120 per cent of the stated amount but in any case not more than 1.25 mg per human dose unless otherwise justified and authorised, when aluminium hydroxide or aluminium phosphate is used as adsorbent.

**Calcium** (*2.5.14*): 80 per cent to 120 per cent of the stated amount when calcium phosphate is used as adsorbent.

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific human or animal antibodies.

**Total allergenic activity**: 50 per cent to 150 per cent of the stated amount as assayed by inhibition of the binding capacity of specific immunoglobulin E antibodies or a suitable equivalent *in vitro* method.

**Individual allergens**: 50 per cent to 200 per cent of the stated amount of each relevant allergen component, determined by a suitable method.

## STORAGE

Adsorbed allergen products are not to be frozen, unless otherwise justified and authorised.

## LABELLING

The label states:

- the name of the allergen product;
- the biological potency and/or the protein content and/or the extraction concentration;
- the route of administration and the intended use;
- the storage conditions;
- where applicable, the name and amount of added antimicrobial preservative;
- where applicable, for freeze-dried preparations:
  - the name, composition and volume of the reconstituting liquid to be added;
  - the period of time within which the preparation is to be used after reconstitution;
- where applicable, that the preparation is sterile;
- where applicable, the name and amount of adsorbent.

**07/2016:2902**
**corrected 10.0**

# CHEMICAL PRECURSORS FOR RADIOPHARMACEUTICAL PREPARATIONS

## Praecursores chimici ad radiopharmaceutica

DEFINITION AND SCOPE

Chemical precursors for radiopharmaceutical preparations, hereinafter referred to as 'chemical precursors', are non-radioactive substances obtained by chemical synthesis for combination with a radionuclide.

Where a chemical precursor not described in an individual monograph of the European Pharmacopoeia is used in a radiopharmaceutical preparation prepared for the special needs of individual patients, the need for compliance with this general monograph is decided in the light of a risk assessment.

This risk assessment takes account of:

- the quality of the chemical precursor and the information available for quality evaluation;
- any further processing after radiolabelling (which may or may not include purification before administration to the patient);
- the amount used to prepare a patient dose (e.g. diagnostic use versus therapeutic use) and the frequency of administration to the patient.

Where chemical precursors are manufactured using substances of human or animal origin, the requirements of chapter *5.1.7. Viral safety* apply.

Where chemical precursors are obtained from animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge, where applicable, they also comply with the requirements of the general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*.

PRODUCTION

Chemical precursors are prepared by procedures designed to ensure a consistent quality and provide compounds that comply with the requirements of the individual monograph or approved specifications. Chemical precursors must be produced within the framework of a suitable quality system.

CHARACTERS

The statements under the heading Characters (e.g. statements about the solubility or a melting point) are not to be interpreted in a strict sense and are not requirements. They are given for information.

IDENTIFICATION

Appropriate analytical methods, such as nuclear magnetic resonance spectrometry, infrared absorption spectrophotometry, mass spectrometry and chromatographic methods are used to provide confirmation of the identity of the chemical precursor.

TESTS

**Related substances**. Unless otherwise prescribed or justified and authorised, organic impurities in chemical precursors and inorganic impurities present in inorganic chemical precursors are to be reported, identified and controlled as follows:

| | |
|---|---|
| Reporting threshold | 0.2 per cent |
| Identification threshold | 2.0 per cent |
| Total unspecified impurities | maximum 3.0 per cent |

Specific thresholds may be applied for impurities known to be unusually potent or to produce toxic or unacceptable pharmacological effects.

If the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance.

**Residual solvents**. Residual solvents are limited according to the principles defined in chapter *5.4* using general method *2.4.24* or another suitable method.

Class 1 solvents must not be employed in the final step of the manufacturing process of chemical precursors. If the use of a Class 1 solvent in an earlier step in the production process is unavoidable, the limits stated in chapter *5.4*, Table 1 apply.

Based on the permitted daily exposure (PDE), Class 2 and Class 3 solvents are limited to 0.5 per cent.

For Class 2 and Class 3 solvents, a test for loss on drying or a specific determination of the solvent may be carried out. If for a Class 2 or a Class 3 solvent, a justified and authorised limit greater than 0.5 per cent is prescribed, a specific determination of the solvent is required.

**Metal catalysts or metal reagent residues**. If the production processes of chemical precursors are known or suspected to lead to the presence of metal residues due to the use of a specific metal catalyst or metal containing reagent, the chemical precursors comply with the limit of 0.01 per cent for each of the following metals: Pt, Pd, Ir, Rh, Ru, Os, Mo, Ni, Cr, V, Pb, Hg, Cd and Tl, unless stricter limits are stated in an individual monograph.

The methodology described in general chapter *2.4.20. Determination of elemental impurities* is to be applied wherever possible.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU per gram for bulk material or maximum $10^2$ CFU per container for chemical precursors packed in single and multidose containers (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU per gram for bulk material or maximum $10^1$ CFU per container for chemical precursors packed in single-dose and multidose containers (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*). Unless otherwise justified and authorised, bacterial endotoxins are limited to a maximum 100 IU per gram for bulk material or maximum 10 IU per container for chemical precursors packed in single-dose and multidose containers.

ASSAY

Unless otherwise justified and authorised, the content of chemical precursors for radiopharmaceutical preparations is determined using a suitable method.

STORAGE

Store chemical precursors in an airtight container and in conditions that ensure adequate stability.

LABELLING

The labelling of chemical precursors complies with the relevant national and European legislation where relevant information is available. The label indicates the intended use as a chemical precursor for radiopharmaceutical preparations.

The accompanying information may recommend testing the substance in one or more production runs before its use for the manufacture of radiopharmaceutical preparations to ensure that, under specified production conditions, the substance yields a radiopharmaceutical preparation in the desired quantity and of the quality specified.

**01/2008:2098**

# ESSENTIAL OILS

## Aetherolea

*The statements in this monograph are intended to be read in conjunction with individual monographs on essential oils in the European Pharmacopoeia. Application of the monograph to other essential oils may be decided by the competent authority.*

DEFINITION

Odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating. Essential oils are usually separated from the aqueous phase by a physical process that does not significantly affect their composition.

Essential oils may be subjected to a suitable subsequent treatment. Thus an essential oil may be commercially known as being deterpenated, desesquiterpenated, rectified or 'x'-free.

- A *deterpenated essential oil* is an essential oil from which monoterpene hydrocarbons have been removed, partially or totally.
- A *deterpenated and desesquiterpenated essential oil* is an essential oil from which mono- and sesquiterpene hydrocarbons have been removed, partially or totally.
- A *rectified essential oil* is an essential oil that has been subjected to fractional distillation to remove certain constituents or modify the content.
- An *'x'-free essential oil* is an essential oil that has been subjected to partial or complete removal of one or more constituents.

PRODUCTION

Depending on the monograph, the plant raw material may be fresh, wilted, dried, whole, broken or ground.

*Steam distillation.* The essential oil is produced by the passage of steam through the plant raw material in a suitable apparatus. The steam may be introduced from an external source or generated by boiling water below the raw material or by boiling water in which the raw material is immersed. The steam and oil vapours are condensed. The water and essential oil are separated by decantation.

*Dry distillation.* The essential oil is produced by high-temperature heating of stems or barks in a suitable apparatus without the addition of water or steam.

*Mechanical process.* The essential oil, usually known as 'cold-pressed', is produced by a mechanical process without any heating. It is mainly applied to *Citrus* fruit and involves expression of the oil from the pericarp and subsequent separation by physical means.

In certain cases, a suitable antioxidant may be added to the essential oil.

CHARACTERS

The appearance and the odour of the essential oil is determined.

IDENTIFICATION

Essential oils are identified by their gas chromatographic profile, or failing this, by any other test that may be required (for example, a test by thin-layer chromatography).

TESTS

*GENERAL TESTS*

The essential oil complies with the prescribed limits for the following tests.

**Relative density** (*2.2.5*).

**Refractive index** (*2.2.6*).

**Optical rotation** (*2.2.7*).

**Fatty oils and resinified essential oils** (*2.8.7*).

*SUPPLEMENTARY TESTS*

If necessary, the essential oil complies with the prescribed limits for the following tests.

**Freezing point** (*2.2.18*).

**Acid value** (*2.5.1*).

**Peroxide value** (*2.5.5*).

**Foreign esters** (*2.8.6*).

**Residue on evaporation** (*2.8.9*).

**Water** (*2.8.5*).

**Solubility in alcohol** (*2.8.10*).

**Falsification**. If appropriate, a test for one or more falsifications may be carried out by thin-layer chromatography (*2.2.27*), by gas chromatography (*2.2.28*) using a chiral column if necessary, or by any other suitable method.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

In addition to the system suitability test given in the specific monograph, it is necessary to check the suitability of the chromatographic system using the following test, which is to be carried out periodically within the framework of performance qualification.

The chromatogram shown in Figure 2098.-1 is given as an example.

*Reference solution*: *essential oil CRS*. If necessary, the reference solution can be diluted with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:500. The split ratio/injection volume can be adjusted in order to fit the specific equipment used, provided that the column load stays the same.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 70 |
| | 15 - 100 | 70 → 240 |
| | 100 - 105 | 240 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of components*: use the chromatogram supplied with *essential oil CRS*.

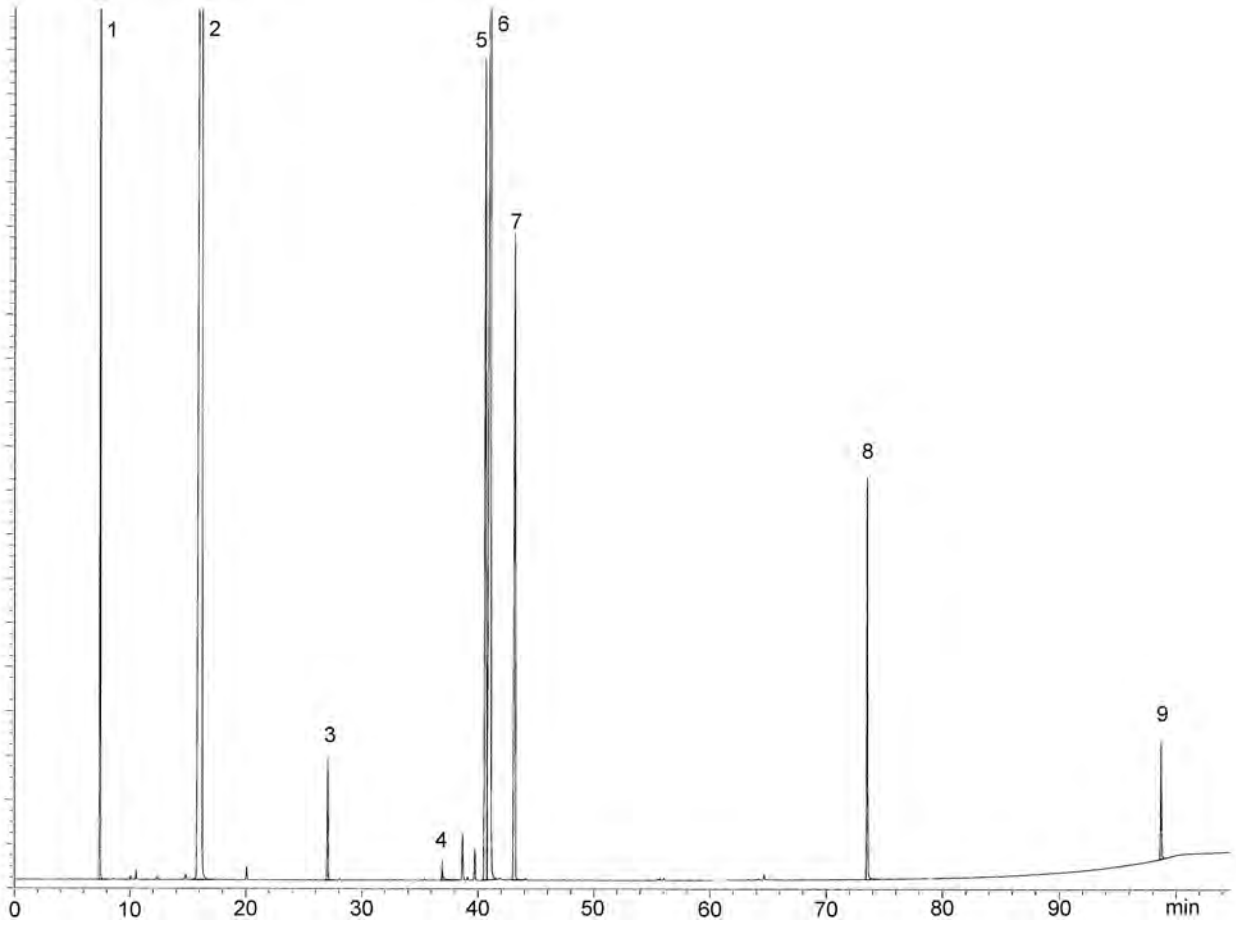


1. α-pinene
2. cineole
3. hexanol
4. decanal
5. linalol
6. linalyl acetate
7. β-caryophyllene
8. eugenol
9. benzyl salicylate

Figure 2098.-1. – *Chromatogram for the test for chromatographic profile of essential oils*

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to linalol and linalyl acetate;
- *signal-to-noise ratio*: minimum 100 for the peak due to decanal;
- *limits*: the percentage content of each of the 9 components is within the limits stated on the leaflet provided with *essential oil CRS*.

STORAGE

In a well-filled, airtight container, protected from light.

LABELLING

The label states:

- the scientific name of the plant raw material used;
- where applicable, the type and/or the chemotype of the essential oil;
- where applicable, the method of production;
- where applicable, the name and concentration of any added antioxidant;
- where applicable, additional processing steps that are not specified under Definition.

**04/2019:0765**

# HERBAL DRUG EXTRACTS

## Plantarum medicinalium extracta

DEFINITION

Herbal drug extracts are liquid (liquid extraction preparations), semi-solid (soft extracts and oleoresins) or solid (dry extracts) preparations obtained from *Herbal drugs (1433)* using suitable solvents.

An extract is essentially defined by the quality of the herbal drug, by its production process (extraction solvent(s), method of processing, etc.) and by its specifications.

European Pharmacopoeia monographs for extracts cover the genuine (native) extract and, where present, excipients.

Different types of extract may be distinguished.

**Standardised extracts** are adjusted to a defined content of one or more constituents with known therapeutic activity. This is achieved by adjustment of the extract with inert excipients or by blending batches of the extract.

**Quantified extracts** are adjusted to one or more active markers, the content of which is controlled within a limited, specified range. Adjustments are made by blending batches of the extract.

**Other extracts** are not adjusted to a particular content of constituents. For control purposes, one or more constituents are used as analytical markers. The minimum content for these analytical markers is given in an individual monograph.

PRODUCTION

Herbal drugs, solvents and other materials used for the preparation of extracts are of suitable quality and where applicable comply with the requirements of any relevant monograph in the European Pharmacopoeia. Where justified, herbal drugs used for the production of extracts may exceed the limits for heavy metals specified in the monograph *Herbal drugs (1433)* provided that the resulting extract satisfies the requirements for heavy metals (see Tests).

Different batches of the herbal drug which are compliant with the relevant monograph, or in the absence of an individual monograph with other suitable specifications, may be combined prior to extraction, for example for the purpose of achieving the quantity of herbal drug required for the production process or, in the case of standardised and quantified extracts, to achieve a certain range of content for one or more constituents in the herbal drug to be extracted. The herbal drug may also undergo a preliminary treatment, for example, grinding, inactivation of enzymes or defatting. In addition, unwanted constituents (e.g. toxic constituents) or unwanted matter (e.g. insoluble matter) may be removed at a suitable stage in the production process.

Where solvents are recovered from the production process, such recovered or recycled solvents may be used, provided that the recovery procedures are controlled and monitored to ensure that solvents meet appropriate standards before re-use or admixture with other approved materials. Water used for the production of extracts complies with the requirements of the monograph *Water for preparation of extracts (2249)*.

Where applicable, miscella (extraction liquors) are concentrated to the intended consistency using suitable methods, usually under reduced pressure and at a temperature at which deterioration of the constituents is reduced to a minimum. Essential oils that have been separated during processing may be restored to the extracts at an appropriate stage in the production process. Suitable excipients may be added at various stages of the production process for technological reasons (for example, as part of the drying process or to improve the homogeneity or consistency of an extract). For standardised extracts, suitable inert excipients may also be added to adjust one or more constituents to a defined content. For quantified extracts and 'other' extracts, the addition of inert excipients to adjust the content of assayed constituents is not permitted. Excipients are included for technological reasons only, and the manufacturer must declare the content of such excipients as a fixed percentage. In some applications, an excipient may be added in a narrow percentage range (e.g. silicon dioxide between 0.1-0.5 per cent, to improve flowability of the extract). The proposed range must be justified by the manufacturer. Suitable stabilisers, antioxidants and antimicrobial preservatives may be added to extracts where justified and authorised.

Extraction with a given solvent leads to a typical content of selected constituents in the extracted dry matter; during production of standardised and quantified extracts, purification procedures may be applied that increase the content of these selected constituents with respect to the expected values; such extracts are referred to as 'refined'.

IDENTIFICATION

Extracts are identified using suitable methods.

TESTS

Where applicable, as a result of analysis of the herbal drug used for production and in view of the production process, tests for microbiological quality (*5.1.4* or *5.1.8*), heavy metals (*2.4.27*), aflatoxins (*2.8.18*), ochratoxin A (*2.8.22*) and pesticide residues (*2.8.13*) in the extracts may be necessary. Where a test for heavy metals is carried out, the same limits for heavy metals as those given in the monograph *Herbal drugs (1433)* are applicable to extracts unless otherwise stated in an individual extract monograph or unless otherwise justified and authorised.

ASSAY

Extracts are assayed by a suitable method, unless otherwise justified.

**Standardised extracts**. The Definition section of an individual monograph on a standardised extract states the content of the assayed constituents as either a defined single content or within a defined range of content.

*Defined single content*. For example, in the monograph *Ipecacuanha liquid extract, standardised (1875)*, the content of assayed constituents is stated as 1.80 per cent to 2.20 per cent. In this case, the declaration is based on a defined single content of 2.0 per cent with a tolerance of ± 10 per cent. The acceptable tolerance is usually within the range ± 5 per cent to ± 10 per cent taking into account the nature of the extract and the method of assay.

*Defined range of content*. For example, in the monograph *Frangula bark dry extract, standardised (1214)*, the content of assayed constituents is stated as 15.0 per cent to 30.0 per cent. In this case, it is intended that an extract will consistently be produced to a defined single content selected from within the defined range taking into account an acceptable tolerance. Where there is an individual monograph in the pharmacopoeia for a standardised extract with a defined range of content, the acceptable tolerance will be stated in the individual monograph (for example, for *Frangula bark dry extract, standardised (1214)*, the acceptable tolerance is stated as ± 10 per cent relative to the declared content).

**Quantified extracts**. The content of assayed constituents must be within the values given in the Definition section of an individual monograph.

**Other extracts**. The content of assayed constituents must not be lower than the minimum value given in the Definition section of an individual monograph. Where justified and authorised, this does not preclude the selection of alternative constituents as a basis for assay using a corresponding validated analytical method, which may be more appropriate to the physical and/or chemical properties of the medicinal product into which the extract is to be incorporated. Where alternative constituents are selected for assay, a suitable minimum value for such constituents must be established.

LABELLING

The label states:

- the herbal drug used;
- where applicable, that fresh herbal drug has been used;
- the form of the extract (for example, liquid, tincture, soft, oleoresin or dry);
- where applicable, that the extract is standardised or quantified;
- for standardised extracts, the defined content of constituents with known therapeutic activity;
- for quantified extracts, the specified range of content of active markers;
- where applicable, that the extract is 'refined';
- the first solvent or solvents used for extraction (for example, ethanol 60 per cent *V/V*);
- the name and amount of any excipients present in the extract (for example, diluents, stabilisers, antimicrobial preservatives, antioxidants);
- for quantified extracts and 'other' extracts, the ratio of the quantity of herbal drug to the quantity of genuine (native) extract ($DER_{genuine}$) expressed on a mass/mass basis for

soft extracts, oleoresins and dry extracts, and on either a mass/mass or a mass/volume basis for liquid extraction preparations;
- where applicable, the percentage of dry residue;
- the storage conditions.

## Liquid extraction preparations - Praeparationes fluidae ab extractione

Liquid extraction preparations are liquid preparations consisting of a diverse range of products which are described by their extraction solvents, methods of production and drug solvent ratios or drug extract ratios. Included in this range are products obtained using ethanol, water, glycerol, propylene glycol and fatty oils as extraction solvents. Liquid (fluid) extracts and tinctures belong to this category and are described below.

### LIQUID (FLUID) EXTRACTS – EXTRACTA FLUIDA

*DEFINITION*

Quantified liquid (fluid) extracts and 'other' liquid (fluid) extracts are liquid extraction preparations of which, in general, 1 part by mass or volume is equivalent to 1 part by mass of the dried herbal drug.

Standardised liquid (fluid) extracts are only defined by their content of constituents with known therapeutic activity.

*PRODUCTION*

Liquid extracts are prepared using ethanol of a suitable concentration and/or water together with, where necessary, other substances (e.g. glycerol or ammonia solution) to extract the herbal drug, or by dissolving a soft or dry extract of the herbal drug (which has been produced using the same extraction solvent as would be used to prepare the liquid extract by direct extraction) in either ethanol of the required concentration or water.

Where the liquid extract contains ethanol, it is tested for 2-propanol (*2.9.11*), with a maximum of 0.05 per cent *V/V*, unless assurance of compliance with this limit is provided by a detailed knowledge of the ethanol supply chain and the extract manufacturing process.

Except for standardised liquid extracts, liquid extracts produced from soft or dry extracts do not contain any excipients other than those that would be present in the liquid extract prepared by direct extraction. However, exceptions may be justified in certain cases such as when the soft extract used to produce the liquid extract contains stabilisers, antioxidants or antimicrobial preservatives that have been added to ensure its stability.

Liquid extracts are adjusted, if necessary, so that they satisfy the requirements for content of solvent. Liquid extracts may be filtered, if necessary.

A slight sediment may form on standing.

*TESTS*

**Relative density** (*2.2.5*). Where applicable, the liquid extract complies with the limits prescribed.

**Ethanol** (*2.9.10*). For ethanolic liquid extracts, carry out the determination of ethanol content. The ethanol content complies with the limits prescribed.

**Methanol** (*2.9.11*): maximum 0.05 per cent *V/V* for ethanolic liquid extracts, unless otherwise prescribed or justified and authorised.

**Dry residue** (*2.8.16*). Where applicable, the liquid extract complies with the limits prescribed.

*STORAGE*

Protected from light.

*LABELLING*

The label states in addition to the requirements listed above, the ethanol content in per cent *V/V*, where applicable.

### TINCTURES – TINCTURAE

*DEFINITION*

Quantified tinctures and 'other' tinctures are liquid extraction preparations that are obtained using either 1 part by mass of herbal drug and 10 parts by mass or volume of extraction solvent, or 1 part by mass of herbal drug and 5 parts by mass or volume of extraction solvent. Alternatively, they may be obtained using either 1 part by mass of herbal drug and sufficient extraction solvent to produce 10 parts by mass or volume of tincture or 1 part by mass of herbal drug and sufficient extraction solvent to produce 5 parts by mass or volume of tincture. Other ratios of herbal drug to extraction solvent may be used.

Standardised tinctures are only defined by their content of constituents with known therapeutic activity.

*PRODUCTION*

Tinctures are usually prepared by either maceration or percolation, using ethanol of a suitable concentration to extract the herbal drug, or by dissolving a soft or dry extract of the herbal drug (which has been produced using the same extraction solvent as would be used to prepare the tincture by direct extraction) in ethanol of the required concentration. The tincture is tested for 2-propanol (*2.9.11*), with a maximum of 0.05 per cent *V/V*, unless assurance of compliance with this limit is provided by a detailed knowledge of the ethanol supply chain and the tincture manufacturing process.

Except for standardised tinctures, tinctures produced from soft or dry extracts do not contain any excipients other than those that would be present in the tincture prepared by direct extraction. However, exceptions may be justified in certain cases such as when the soft extract used to produce the tincture contains stabilisers, antioxidants or antimicrobial preservatives that have been added to ensure its stability.

Tinctures are adjusted, if necessary so that they satisfy the requirements for content of solvent. Tinctures may be filtered if necessary.

Tinctures are usually clear. A slight sediment may form on standing.

*TESTS*

**Relative density** (*2.2.5*). Where applicable, the tincture complies with the limits prescribed.

**Ethanol** (*2.9.10*). The ethanol content complies with the limits prescribed.

**Methanol** (*2.9.11*): maximum 0.05 per cent *V/V*, unless otherwise prescribed or justified and authorised.

**Dry residue** (*2.8.16*). Where applicable, the tincture complies with the limits prescribed.

*STORAGE*

Protected from light.

*LABELLING*

The label states, in addition to the requirements listed above, the ethanol content in per cent *V/V*.

## Soft extracts – extracta spissa

### DEFINITION

Soft extracts are semi-solid preparations obtained by evaporation or partial evaporation of the solvent used for production.

### TESTS

**Dry residue** (*2.8.16*). The soft extract complies with the limits prescribed.

**Solvents**. Residual solvents are controlled as described in chapter *5.4*, unless otherwise prescribed or justified and authorised.

STORAGE

In an airtight container, protected from light.

## Oleoresins – oleoresina

DEFINITION

Oleoresins are semi-solid extracts composed of a resin in solution in an essential and/or fatty oil and are obtained by evaporation of the solvent(s) used for their production.

This monograph applies to oleoresins produced by extraction and not to natural oleoresins.

TESTS

**Water** (*2.2.13*). The oleoresin complies with the limits prescribed.

**Solvents**. Residual solvents are controlled as described in chapter *5.4*, unless otherwise prescribed or justified and authorised.

STORAGE

In an airtight container, protected from light.

## Dry extracts – extracta sicca

DEFINITION

Dry extracts are solid preparations obtained by evaporation of the solvent used for their production.

Dry extracts usually have a loss on drying of not greater than 5 per cent *m/m*. Where justified and authorised, a loss on drying with a different limit or a test for water may be prescribed.

TESTS

**Loss on drying** (*2.8.17*). Where applicable, the dry extract complies with the limits prescribed.

**Water** (*2.5.12*). Where a test for loss on drying is not applicable, the dry extract complies with the limits prescribed.

**Solvents**. Residual solvents are controlled as described in chapter *5.4*, unless otherwise prescribed or justified and authorised.

STORAGE

In an airtight container, protected from light.

## Glossary - glossa

**Constituents with known therapeutic activity**. Chemically defined substances or groups of substances which are generally accepted to contribute substantially to the therapeutic activity of a herbal drug, a herbal drug preparation or a herbal medicinal product.

**Drug extract ratio (*DER*)**. The ratio between the quantity of herbal drug used in the manufacture of an extract and the quantity of extract obtained. The number (given as the actual range) written before the colon is the relative quantity of the herbal drug; the number written after the colon is the relative quantity of the extract obtained. Two *DERs* can be differentiated:

- **Genuine (native) drug extract ratio (*$DER_{genuine}$*)**. The ratio between the quantity of herbal drug used in the manufacture of an extract and the quantity of genuine (native) extract obtained.
- **Total drug extract ratio (*$DER_{total}$*)**. The ratio between the quantity of herbal drug used in the manufacture of an extract and the quantity of whole extract (including excipients) obtained.

For example, $DER_{genuine}$ 2.5-4.5:1 means that between 2.5 and 4.5 parts of herbal drug are required to produce 1 part of genuine (native) extract. Where processing aids are added to the genuine (native) extract to produce, for example, a dry extract, the $DER_{total}$ and the $DER_{genuine}$ will have different values; where a dry extract is produced without the need for any processing aids, the $DER_{total}$ and the $DER_{genuine}$ will be identical. Oleoresins are usually produced without the need to include processing aids, therefore the $DER_{total}$ and the $DER_{genuine}$ are usually identical. For soft extracts and liquid extraction preparations, where the genuine (native) extract does not exist without excipients and/or processing aids (e.g. usually 20-30 per cent of water in soft extracts, ethanolic extraction solvent in tinctures), the $DER_{total}$ and the $DER_{genuine}$ are identical.

**Drug solvent ratio (*DSR*)**. The ratio between the quantity of herbal drug, expressed in mass, used in the manufacture of an extract and the quantity of the first extraction solvent, expressed in mass or volume.

**Extraction solvents**. Solvents which are used for the extraction process.

**Genuine (native) herbal drug extract**. Refers to the extract without excipients, even if for technological reasons the genuine extract is not available. However, for soft extracts and liquid extraction preparations the genuine extract may contain variable amounts of (extraction) solvent.

**Markers**. Chemically defined constituents or groups of constituents of a herbal drug, a herbal drug preparation or a herbal medicinal product which are of interest for control purposes independent of whether they have any therapeutic activity. Markers serve to calculate the quantity of herbal drug(s) or herbal drug preparation(s) in the herbal medicinal product if the marker has been quantitatively determined in the herbal drug or herbal drug preparation.

There are 2 categories of markers:

- *active markers* are constituents or groups of constituents which are generally accepted to contribute to the therapeutic activity;
- *analytical markers* are constituents or groups of constituents that serve solely for analytical purposes, irrespective of any pharmacological or therapeutic activity which they may be reported to possess.

**Miscella (extraction liquor)**. Liquid obtained from the extraction process.

**Production of tinctures by maceration**. A process whereby, unless otherwise prescribed, the herbal drug to be extracted is reduced to pieces of suitable size, mixed thoroughly with the prescribed extraction solvent and allowed to stand in a closed container for an appropriate time, with agitation where required. The residue is separated from the extraction solvent and, if necessary, pressed out. If the residue is pressed, the 2 liquids are combined.

**Production of tinctures by percolation**. A process whereby, unless otherwise prescribed, the herbal drug to be extracted is reduced to pieces of suitable size and mixed thoroughly with a portion of the prescribed extraction solvent and allowed to stand for an appropriate time. The mixture is transferred to a percolator and more extraction solvent is added until the herbal drug is covered with a layer of extraction solvent. The percolate is allowed to flow slowly from the base of the percolator while extraction solvent is slowly added to the top of the percolator, ensuring that the herbal drug to be extracted is constantly covered with extraction solvent, until all the extraction solvent has been added. Percolation continues until the percolate is recovered. If the residue is pressed, the 2 liquids are combined.

**07/2010:1434**

# HERBAL DRUG PREPARATIONS

## Plantae medicinales praeparatae

DEFINITION

Herbal drug preparations are homogeneous products obtained by subjecting herbal drugs to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation.

Herbal drug preparations include, for example, extracts, essential oils, expressed juices, processed exudates, and herbal drugs that have been subjected to size reduction for specific applications, for example herbal drugs cut for herbal teas or powdered for encapsulation.

Herbal teas comply with the monograph *Herbal teas (1435)*.

*NOTE*: the term *comminuted* used in European Community legislation on herbal medicinal products describes a herbal drug that has been either cut or powdered.

The term *herbal drug preparation* is synonymous with the term *herbal preparation* used in European Community legislation on herbal medicinal products.

**07/2017:1433**

# HERBAL DRUGS

## Plantae medicinales

DEFINITION

Herbal drugs are mainly whole, fragmented or broken plants or parts of plants in an unprocessed state, usually in dried form but sometimes fresh. In this general monograph, the word 'plant' is used in the broader sense to also include algae, fungi and lichens. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs. Herbal drugs are precisely defined by the botanical scientific name according to the binominal system (genus, species, variety and author).

*Whole* describes a herbal drug that has not been reduced in size and is presented, dried or undried, as harvested; for example: dog rose, bitter fennel or sweet fennel, Roman chamomile flower.

*Fragmented* describes a herbal drug that has been reduced in size after harvesting to permit ease of handling, drying and/or packaging; for example: cinchona bark, rhubarb, passion flower.

*Broken* describes a herbal drug in which the more-fragile parts of the plant have broken during drying, packaging or transportation; for example: belladonna leaf, matricaria flower, hop strobile.

*Cut* describes a herbal drug that has been reduced in size, other than by powdering, to the extent that the macroscopic description in the monograph of the herbal drug can no longer be applied. When a herbal drug is cut for a specific purpose that results in the cut herbal drug being homogeneous, for example when cut for herbal teas, it is a herbal drug preparation. Certain cut herbal drugs processed in this way may be the subject of an individual monograph.

A herbal drug that complies with its monograph and is subsequently cut for extraction shall comply in its cut form, except for its macroscopic description, with the monograph for that herbal drug, unless otherwise justified.

The term *herbal drug* is synonymous with the term *herbal substance* used in European Community legislation on herbal medicinal products.

DRIED HERBAL DRUGS

*PRODUCTION*

Dried herbal drugs are obtained from cultivated or wild plants. Suitable collection, cultivation, harvesting, drying, fragmentation and storage conditions are essential to guarantee their quality.

Dried herbal drugs are, as far as possible, free from impurities such as soil, dust, dirt and other contaminants such as fungal, insect and other animal contaminations. They are not rotten.

If a decontaminating treatment has been used, it is necessary to demonstrate that the constituents of the herbal drug are not affected and that no harmful residues remain. The use of ethylene oxide is prohibited for the decontamination of herbal drugs.

*IDENTIFICATION*

Dried herbal drugs are identified using their macroscopic and microscopic descriptions and any further tests that may be required (for example, thin-layer chromatography).

*TESTS*

**Foreign matter** (*2.8.2*). Carry out a test for foreign matter, unless otherwise prescribed or justified and authorised. The content of foreign matter is not more than 2 per cent *m/m*, unless otherwise prescribed or justified and authorised. An appropriate specific test may apply to dried herbal drugs liable to be adulterated. It may not be possible to perform the test for foreign matter on a dried herbal drug that is cut, as described under Definition, for either a specific purpose or for extraction. Under these circumstances the cut material is presumed to comply with the test for foreign matter providing that the dried herbal drug prior to cutting was compliant with this test.

**Loss on drying** (*2.2.32*). Carry out a test for loss on drying, unless otherwise prescribed or justified and authorised.

**Water** (*2.2.13*). A determination of water may be carried out instead of a test for loss on drying for dried herbal drugs with a high essential-oil content.

**Pesticides** (*2.8.13*). Dried herbal drugs comply with the requirements for pesticide residues. The requirements take into account the nature of the plant, where necessary the preparation in which the plant might be used, and where available the knowledge of the complete treatment record of the batch of the plant.

**Heavy metals** (*2.4.27*). Unless otherwise stated in an individual monograph or unless otherwise justified and authorised:

- *cadmium*: maximum 1.0 ppm;
- *lead*: maximum 5.0 ppm;
- *mercury*: maximum 0.1 ppm.

Where necessary, limits for other heavy metals may be required.

*Where necessary, dried herbal drugs comply with other tests, such as the following, for example.*

**Total ash** (*2.4.16*).

**Ash insoluble in hydrochloric acid** (*2.8.1*).

**Extractable matter**.

**Swelling index** (*2.8.4*).

**Bitterness value** (*2.8.15*).

**Aflatoxin $B_1$** (*2.8.18*). Where necessary, limits for aflatoxins may be required.

**Ochratoxin A** (*2.8.22*). Where necessary, a limit for ochratoxin A may be required.

**Radioactive contamination**. In some specific circumstances, the risk of radioactive contamination is to be considered.

**Microbial contamination**. Where a dried herbal drug is used whole, cut or powdered as an ingredient in a medicinal product, the microbial contamination is controlled (*5.1.8. Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation* or *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* (e.g. for cutaneous use)).

*ASSAY*

Unless otherwise prescribed or justified and authorised, dried herbal drugs are assayed by an appropriate method.

*STORAGE*

Protected from light.

*Where used for the production of essential oils, some of the tests prescribed for dried herbal drugs may no longer be necessary. This is considered on a case-by-case basis depending on the provenance of the dried herbal drug and the production process. The need for testing (e.g. for potential contaminants) and the stage of testing (herbal drug/essential oil) is to be considered.*

FRESH HERBAL DRUGS

A fresh herbal drug is one that is intended to be processed into a herbal drug preparation (e.g. essential oil, juice, tincture) within a relatively short period of time after harvesting. Under these circumstances, the extensive analysis prescribed for dried herbal drugs is not appropriate and the following analytical requirements, based on the provenance of the fresh herbal drug, are considered suitable, provided that processing to the herbal drug preparation takes place within a validated time period after harvesting.

(1) For a fresh herbal drug that has been cultivated from seeds, cuttings, etc., whose origin and traceability can be demonstrated, and where the complete history of the herbal drug from planting to harvesting is documented:

- macroscopic identification of the plant and plant parts to be processed;
- compliance with a suitable limit test for foreign matter.

(2) For a cultivated fresh herbal drug where the information on life cycle from seed to harvesting, as described under (1), is incomplete, the same analytical requirements as described under (1) apply, as well as any additional tests that may be necessary depending on the information available on the herbal drug to be processed and any potential or known quality issues.

(3) For a fresh herbal drug that is wild-crafted, the analytical requirements should be assessed on a case-by-case basis and will depend on the ease of identification or characterisation of the herbal drug/herbal drug preparation and potential adulterants, and the method of processing or type of herbal drug preparation to be manufactured. For example, in the case of essential oils, the majority of the oil composition is determined when it is analysed, whereas for a juice or a tincture, such analytical capabilities are limited. For the processing of plant parts, a wild-crafted fresh flower, easily identifiable by its distinctive appearance and colour, would be expected to require fewer analytical control parameters than a wild-crafted fresh root with few, if any, visually distinctive features. Analytical requirements as described under (1) may be acceptable when fully justified.

For fresh herbal drugs, where the extensive analysis described for dried herbal drugs is not feasible prior to processing into the herbal drug preparation, appropriate tests (e.g. for contaminants) are performed on a suitable retained sample of the fresh herbal drug or on the herbal drug preparation.

Fresh herbal drugs may be frozen for storage purposes. Defined processes for freezing and thawing are required to ensure the quality of the herbal drug. Appropriate testing of the herbal drug, justified on the basis of the freezing and manufacturing processes, is put in place and may include testing prior to freezing and at the time of use.

When handling and processing fresh herbal drugs, it is necessary to ensure, by visual inspection or other suitable means, the absence of unwanted fermentation, the presence of which may alter the quality of the herbal drug preparation, including possible mycotoxin production.

**01/2013:1435**

# HERBAL TEAS

## Plantae ad ptisanam

DEFINITION

Herbal teas consist exclusively of one or more herbal drugs intended for oral aqueous preparations by means of decoction, infusion or maceration. The preparation is prepared immediately before use.

Herbal teas are usually supplied in bulk form or in bags for single use.

The herbal drugs used comply with the appropriate individual European Pharmacopoeia monographs or in their absence with the general monograph *Herbal drugs (1433)*.

IDENTIFICATION

The identity of herbal drugs present in herbal teas is checked by suitable methods such as botanical examinations and/or chromatographic profiles.

TESTS

Recommendations on the microbiological quality of herbal teas (*5.1.8*) take into account the prescribed preparation method (use of boiling or non-boiling water).

The proportion of herbal drugs present in herbal teas is checked by appropriate methods.

Herbal teas in bags comply with the following test:

**Uniformity of mass**. Determine the individual and the average mass of the contents of 20 randomly chosen units as follows: weigh a single full bag of herbal tea, open it without losing any fragments. Empty it completely using a brush. Weigh the empty bag and calculate the mass of the contents by subtraction. Repeat the operation on the 19 remaining bags and calculate the average mass of the contents of the 20 units. Unless otherwise justified, not more than 2 of the 20 individual masses deviate from the average mass by more than the percentage deviation shown in the table below and none deviates by more than twice that percentage.

| Average mass | Percentage deviation |
|---|---|
| less than 1.5 g | 15 per cent |
| 1.5 g to 2.0 g included | 10 per cent |
| more than 2.0 g | 7.5 per cent |

STORAGE

Protected from light.

**01/2013:2620**

# HERBAL TEAS, INSTANT

## Praeparationes celeres ad ptisanam

DEFINITION

Instant herbal teas consist of 1 or more herbal drug preparations (primarily extracts with or without added essential oils), and are intended for the preparation of an oral solution immediately before use.

Instant herbal teas may also contain, in addition to herbal drug preparations, suitable excipients such as maltodextrin and added flavourings.

Instant herbal teas are presented as a powder or granules and are usually supplied in bulk form or in sachets.

The herbal drug preparations used comply with the appropriate individual European Pharmacopoeia monographs or, in the absence of such individual monographs, with the general monograph *Herbal drug preparations (1434)* and with other appropriate general monographs, for example *Herbal drug extracts (0765)* or *Essential oils (2098)*.

IDENTIFICATION

The identity of herbal drug preparations present in instant herbal teas is checked by suitable methods.

TESTS

General chapter *5.1.8* contains recommendations on the microbiological quality of extract-containing herbal medicinal products such as instant herbal teas.

The proportion of herbal drug preparations present in instant herbal teas is checked by suitable methods.

Instant herbal teas in sachets comply with the following test.

**Uniformity of mass**. Determine the individual and the average mass of the contents of 20 randomly chosen units as follows: weigh a single full sachet of instant herbal tea, open it without losing any fragments. Empty it completely using a brush. Weigh the empty sachet and calculate the mass of the contents by subtraction. Repeat the operation on the 19 remaining sachets and calculate the average mass of the contents of the 20 units. Unless otherwise justified, not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation shown in the table below and none deviates by more than twice that percentage.

| Average mass | Percentage deviation |
|---|---|
| less than 1.5 g | 15 per cent |
| 1.5 g to 2.0 g included | 10 per cent |
| more than 2.0 g | 7.5 per cent |

STORAGE

Protected from light.

**01/2019:0084**
**corrected 10.0**

# IMMUNOSERA FOR HUMAN USE, ANIMAL

## Immunosera ex animale ad usum humanum

DEFINITION

Animal immunosera for human use are liquid or freeze-dried preparations containing purified immunoglobulins or immunoglobulin fragments obtained from serum or plasma of immunised animals of different species.

The immunoglobulins or immunoglobulin fragments have the power of specifically neutralising or binding to the antigen used for immunisation. The antigens include microbial or other toxins, human antigens, suspensions of bacterial and viral antigens and venoms of snakes, scorpions and spiders. The preparation is intended for intravenous or intramuscular administration, after dilution where applicable.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently immunosera of acceptable safety, potency in man and stability.

Any reagent of biological origin used in the production of immunosera shall be free of contamination with bacteria, fungi and viruses. The general requirements of chapter *5.1.7. Viral safety* apply to the manufacture of animal immunosera for human use, in conjunction with the more specific requirements relating to viral safety in this monograph. The method of preparation includes a step or steps that have been shown to remove or inactivate known agents of infection.

Methods used for production are validated, effective, reproducible and do not impair the biological activity of the product.

*Reference preparation*. A batch shown to be suitable in clinical trials, or a batch representative thereof, is used as the reference preparation for the tests for high molecular mass proteins and purity.

*ANIMALS*

The animals used are of a species approved by the competent authority, are healthy and are exclusively reserved for production of immunoserum. They are tested and shown to be free from a defined list of infectious agents. The introduction of animals into a closed herd follows specified procedures, including definition of quarantine measures. Where appropriate, additional specific agents are considered depending on the geographical localisation of the establishment used for the breeding and production of the animals. The feed originates from a controlled source and no animal proteins are added. The suppliers of animals are certified by the competent authority.

If the animals are treated with antibiotics, a suitable withdrawal period is allowed before collection of blood or plasma. The animals are not treated with penicillin antibiotics. If a live vaccine is administered, a suitable waiting period is imposed between vaccination and collection of serum or plasma for immunoserum production.

*IMMUNISATION*

The antigens used are identified and characterised, where appropriate; where relevant, they are shown to be free from extraneous infectious agents. They are identified by their names and a batch number; information on the source and preparation are recorded.

The selected animals are isolated for at least 1 week before being immunised according to a defined schedule, with booster injections at suitable intervals. Adjuvants may be used.

Animals are kept under general health surveillance and specific antibody production is controlled at each cycle of immunisation.

Animals are thoroughly examined before collection of blood or plasma. If an animal shows any pathological lesion not related to the immunisation process, it is not used, nor are any other of the animals in the group concerned, unless it is evident that their use will not impair the safety of the product.

*COLLECTION OF BLOOD OR PLASMA*

Collection of blood is made by venepuncture or plasmapheresis. The puncture area is shaved, cleaned and disinfected. The animals may be anaesthetised under conditions that do not influence the quality of the product. Unless otherwise prescribed, an antimicrobial preservative may be added. The blood or plasma is collected in such a manner as to maintain sterility of the product. The blood or plasma collection is conducted at a site separate from the area where the animals are kept or bred and the area where the immunoserum is purified. If the serum or plasma is stored before further processing, precautions are taken to avoid microbial contamination.

Several single plasma or serum samples may be pooled before purification. The single or pooled samples are tested before purification for the following tests.

**Tests for contaminating viruses**. If an antimicrobial preservative is added, it must be neutralised before carrying out the tests, or the tests are carried out on a sample taken before addition of the antimicrobial preservative. Each pool is tested for contaminating viruses by suitable *in vitro* tests.

Each pool is tested for viruses by inoculation to cell cultures capable of detecting a wide range of viruses relevant for the particular product.

**Potency**. Carry out a biological assay as indicated in the monograph and express the result in International Units per millilitre, where applicable. A validated *in vitro* method may also be used.

**Protein content**. Dilute the product to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2 mL of this solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying by 6.25. The protein content is within approved limits.

*PURIFICATION AND VIRAL INACTIVATION*

The immunoglobulins are concentrated and purified by fractional precipitation, chromatography, immunoadsorption or by other chemical or physical methods. They may be processed further by enzyme treatment. The methods are selected and validated to avoid contamination at all steps of processing and to avoid formation of protein aggregates that affect the immunobiological characteristics of the product. For products intended to consist of immunoglobulin fragments, the methods are validated to guarantee total fragmentation. The methods of purification used are such that they do not generate additional components that compromise the quality and the safety of the product.

Unless otherwise justified and authorised, validated procedures are applied for removal and/or inactivation of viruses. The procedures are selected to avoid the formation of polymers or aggregates and, unless the product is intended to consist of Fab′ fragments, to minimise the splitting of F(ab′)2 into Fab′ fragments.

After purification and treatment for removal and/or inactivation of viruses, a stabiliser may be added to the intermediate product, which may be stored for a period defined in light of stability data.

Only an intermediate product that complies with the following requirements may be used in the preparation of the final bulk.

**Purity**. Examine by non-reducing polyacrylamide gel electrophoresis (*2.2.31*), by comparison with the reference preparation. The bands are compared in intensity and no additional bands are found.

*FINAL BULK*

The final bulk is prepared from a single intermediate product or from a pool of intermediate products obtained from animals of the same species. Intermediate products with different specificities may be pooled.

An antimicrobial preservative and a stabiliser may be added. If an antimicrobial preservative has been added to the blood or plasma, the same substance is used as the antimicrobial preservative in the final bulk.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable physico-chemical method. It contains not less than 85 per cent and not more than 115 per cent of the amount stated on the label.

**Sterility** (*2.6.1*). It complies with the test for sterility.

*FINAL LOT*

The final bulk of immunoserum is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that complies with the requirements prescribed below under Identification, Tests and Assay may be released for use. Provided that the tests for osmolality, protein content, molecular-size distribution, antimicrobial preservative, stabiliser, purity, foreign proteins and albumin and the assay have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

The identity is established by immunological tests and, where necessary, by determination of biological activity. The assay may also serve for identification.

CHARACTERS

Immunosera are clear to opalescent and colourless to very faintly yellow liquids. They are free from turbidity. Freeze-dried products are white or slightly yellow powders or solid friable masses. After reconstitution they show the same characteristics as liquid preparations.

TESTS

**Solubility**. To a container of the preparation to be examined, add the volume of the liquid for reconstitution stated on the label. The preparation dissolves completely within the time stated on the label.

**Extractable volume** (*2.9.17*). It complies with the requirement for extractable volume.

**pH** (*2.2.3*). The pH is within the limits approved for the particular product.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg after dilution, where applicable.

**Protein content**: 90 per cent to 110 per cent of the amount stated on the label, and, unless otherwise justified and authorised, not more than 100 g/L.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2 mL of this solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying by 6.25.

**Molecular-size distribution**. Examine by liquid chromatography (*2.2.29* or *2.2.30*). It complies with the specification approved for the particular product.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Phenol** (*2.5.15*): maximum 2.5 g/L for preparations containing phenol.

**Stabiliser**. Determine the amount of stabiliser by a suitable physico-chemical method. The preparation contains not less than 80 per cent and not more than 120 per cent of the quantity stated on the label.

**Purity**. Examine by non-reducing polyacrylamide gel electrophoresis (*2.2.31*), by comparison with the reference preparation. No additional bands are found for the preparation to be examined.

**Foreign proteins**. When examined by precipitation tests with specific antisera, only protein from the declared animal species is shown to be present, unless otherwise prescribed, for example where material of human origin is used during production.

**Albumin**. Unless otherwise prescribed in the monograph, when examined electrophoretically, the content of albumin is not greater than the limit approved for the particular product and, in any case, is not greater than 3 per cent.

**Water** (*2.5.12*): maximum 3 per cent.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Pyrogens** (*2.6.8*). Unless otherwise justified and authorised, it complies with the test for pyrogens. Unless otherwise prescribed, inject 1 mL per kilogram of the rabbit's body mass.

ASSAY

Carry out a biological assay as indicated in the monograph and express the result in International Units per millilitre, where appropriate. A validated *in vitro* method may also be used.

STORAGE

Protected from light, at the temperature stated on the label. Do not allow liquid preparations to freeze.

*Expiry date*. The expiry date is calculated from the beginning of the assay.

LABELLING

The label states:

- the number of International Units per millilitre, where applicable;
- the amount of protein per container;
- for freeze-dried preparations:
  - the name and volume of the reconstituting liquid to be added;
  - that the immunoserum is to be used immediately after reconstitution;
  - the time required for complete dissolution;
- the route of administration;
- the storage conditions;
- the expiry date, except for containers of less than 1 mL which are individually packed; the expiry date may be omitted from the label on the container, provided it is shown on the package and the label on the package states that the container must be kept in the package until required for use;
- the animal species of origin;
- the name and amount of any antimicrobial preservative, any stabiliser and any other excipient.

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# IMMUNOSERA FOR VETERINARY USE

## Immunosera ad usum veterinarium

DEFINITION

Immunosera for veterinary use are preparations containing immunoglobulins, purified immunoglobulins or immunoglobulin fragments obtained from serum or plasma of immunised animals. They may be preparations of crude polyclonal antisera or purified preparations.

The immunoglobulins or immunoglobulin fragments have the power of specifically neutralising the antigen used for immunisation. The antigens include microbial or other toxins, bacterial and viral antigens, venoms of snakes and hormones. The preparation is intended for parenteral administration to provide passive immunity.

PRODUCTION

*GENERAL PROVISIONS*

Immunosera are obtained from the serum or plasma of healthy animals immunised by administration of one or more suitable antigens.

The production method shall have been shown to yield consistently batches of immunosera of acceptable safety (*5.2.6*) and efficacy (*5.2.7*).

*DONOR ANIMALS*

The animals used are exclusively reserved for production of immunoserum. They are maintained under conditions protecting them from the introduction of disease, as far as possible. The donor animals, and any animals in contact with them, are tested and shown to be free from a defined list of infectious agents and re-tested at suitable intervals. The list of agents for testing includes not only those agents that are relevant to the donor animal, but also those that are relevant to the recipient target species for the product. Where the donor animals have not been demonstrated to be free from a relevant pathogen, a justification must be provided and a validated inactivation or purification procedure must be included in the manufacturing procedure. The feed originates from a controlled source. Where the donor animals are chickens, use chickens from a flock free from specified pathogens (*5.2.2*). Where applicable for the species used, measures are taken to avoid contamination with agents of transmissible spongiform encephalopathies.

As far as possible, animals being introduced into the herd are from a known source and have a known breeding and rearing history. The introduction of animals into the herd follows specified procedures, including defined quarantine measures. During the quarantine period the animals are observed and tested to establish that they are free from the list of agents relevant for the donor animals. It may be necessary to test the

animals in quarantine for freedom from additional agents, depending on their known breeding and rearing history or any lack of information on their source.

Any routine or therapeutic medicinal treatment administered to the animals in quarantine or thereafter must be recorded.

*IMMUNISING ANTIGEN*

The principles described in the Production section of *Vaccines for veterinary use (0062)* are applied to the production of the immunogen. The antigen used is identified and characterised. The starting materials used for antigen preparation must be controlled to minimise the risk of contamination with extraneous agents. The antigen may be blended with a suitable adjuvant. The immunogen is produced on a batch basis. The batches must be prepared and tested in such a manner that assures that each batch will be equally safe and free from extraneous agents and will produce a satisfactory, consistent immune response.

*IMMUNISATION*

The donor animals are immunised according to a defined schedule. For each animal, the details of the dose of immunising antigen, route of administration and dates of administration are recorded. Animals are kept under general health surveillance and the development of specific antibodies are monitored at appropriate stages of the immunisation process.

*COLLECTION OF BLOOD OR PLASMA*

Animals are thoroughly examined before each collection. Only healthy animals may be used as a donor animal. Collection of blood is made by venepuncture or plasmapheresis. The puncture area is shaved, cleaned and disinfected. The method of collection and the volume to be collected on each occasion are specified. The blood or plasma is collected in such a manner as to maintain sterility of the product. If the serum or plasma is stored before further processing, precautions are taken to avoid microbial contamination.

The blood or plasma collection is conducted at a site separate from the area where the animals are kept or bred and the area where the immunoserum is further processed.

Clear criteria are established for determining the time between immunisation and first collection of blood or plasma as well as the time between subsequent collections and the length of time over which collections are made. The criteria applied must take into account the effect of the collections on the health and welfare of the animal as well as the effect on the consistency of production of batches of the finished product, over time.

The rate of clearance of any residues that may arise from the immunising antigen or medication given needs to be taken into account. In the case of the risk of residues from chemical substances, consideration could be given to the inclusion of a withdrawal period for the finished product. If the immunising agent consists of a live organism, the time between immunisation and collection may need to take into account the time required for the donor to eliminate the immunogen, particularly if any residual live organisms might be harmful to the recipient.

*PREPARATION OF THE FINISHED PRODUCT*

Several single plasma or serum collections from one or more animals may be pooled to form a bulk for preparation of a batch. The number of collections that may be used to produce a bulk and the size of the bulk are defined. Where pooling is not undertaken, the production procedure must be very carefully controlled to ensure that the consistency of the product is satisfactory.

The active substance is subjected to a purification and/or inactivation procedure unless omission of such a step has been justifed and agreed with the competent authority.

The procedure applied must have been validated and be shown not to adversely impair the biological activity of the product. The validation studies must address the ability of the procedure to inactivate or remove any potential contaminants such as pathogens that could be transmitted from the donor to the recipient target species and infectious agents such as those that cause ubiquitous infections in the donor animals and cannot be readily eliminated from these donor animals.

For purified immunosera, the globulins containing the immune substances may be obtained from the crude immunoserum by enzyme treatment and fractional precipitation or by other suitable chemical or physical methods

**Antimicrobial preservatives**. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during use of a product. Antimicrobial preservatives are not included in freeze-dried products but, if justified, taking into account the maximum recommended period of use after reconstitution, they may be included in the diluent for multidose freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not normally acceptable, but may be acceptable, for example where the same product is filled in single-dose and multidose containers and is for use in non-food producing species. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after broaching of the container.

During development studies the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in chapter *5.1.3*; for a multidose preparation, additional samples are taken, to monitor the effect of the antimicrobial preservative over the proposed in-use shelf-life. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to antisera for veterinary use: bacteria, no increase at 24 h and 7 days, 3 $\log_{10}$ reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

Addition of antibiotics as antimicrobial preservative is not acceptable.

Unless otherwise prescribed in the monograph, the final bulk is distributed aseptically into sterile, tamper-evident containers which are then closed so as to exclude contamination.

The preparation may be freeze-dried.

*In-process tests*. Suitable tests are carried out in-process, such as on samples from collections before pooling to form a bulk.

*BATCH TESTS*

The tests that are necessary to demonstrate the suitability of a batch of a product will vary and are influenced by a number of factors, including the detailed method of production. The tests to be conducted by the manufacturer on a particular product are agreed with the competent authority. If a product is treated by a validated procedure for inactivation of extraneous agents, the test for extraneous agents can be omitted on that product with the agreement of the competent authority. If a product is treated by a validated procedure for inactivation of mycoplasmas, the test for mycoplasmas can be omitted on that product with the agreement of the competent authority.

Only a batch that complies with each of the relevant requirements given below under Identification, Tests and Potency and/or in the relevant specific monograph may be released for use. With the agreement of the competent authority, certain tests may be omitted where in-process tests give an equal or better guarantee that the batch would comply or where alternative tests validated with respect to the Pharmacopoeia method have been carried out.

Certain tests, e.g. for antimicrobial preservatives, for foreign proteins and for albumin, may be carried out by the manufacturer on the final bulk rather than on the batch, batches or sub-batches of finished product prepared from it.

In some circumstances, e.g. when collections are made into plasmapheresis bags and each one is, essentially, a batch, pools of samples may be tested, with the agreement of the competent authority.

It is recognised that, in accordance with General Notices (section 1.1. General statements), for an established antiserum the routine application of the safety test will be waived by the competent authority in the interests of animal welfare when a sufficient number of consecutive batches have been produced and found to comply with this test, thus demonstrating consistency of the manufacturing process. Significant changes to the manufacturing process may require resumption of routine testing to re-establish consistency. The number of consecutive batches to be tested depends on a number of factors such as the type of antiserum, the frequency of production of batches, and experience with the immunoserum during developmental safety testing and during application of the batch safety test. Without prejudice to the decision of the competent authority in the light of information available for a given antiserum, testing of 10 consecutive batches is likely to be sufficient for the majority of products. For products with an inherent safety risk, it may be necessary to continue to conduct the safety test on each batch.

*Animal tests.* In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in the light of this. For example, if it is indicated that an animal is considered to show positive, infected etc. when typical clinical signs occur then as soon as sufficient indication of a positive result is obtained the animal in question shall be either euthanised or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering.

**pH** (*2.2.3*). The pH of crude and purified immunosera is shown to be within the limits set for the products.

**Formaldehyde**. If formaldehyde is used for production of immunoserum, a test for free formaldehyde is carried out as prescribed under Tests.

**Other inactivating agents**. When other inactivation methods are used, appropriate tests are carried out to demonstrate that the inactivating agent has been removed or reduced to an acceptable residual level.

**Batch potency test**. If a specific monograph exists for the product, the test described under Potency is not necessarily carried out for routine testing of batches of antiserum. The type of batch potency test to be carried out will depend on the claims being made for the product. Wherever possible, *in vitro* tests must be used. The type of test required may include measurement of antibodies against specific infectious organisms, determination of the type of antibody (e.g. neutralising or opsonising). All tests must be validated. The criteria for acceptance must be set with reference to a batch that has been shown to comply with the requirements specified under Potency if a specific monograph exists for the product, and which has been shown to have satisfactory efficacy, in accordance with the claims being made for the product.

**Total immunoglobulins**. A test for the quantities of total immunoglobulins and/or total gammaglobulins and/or specific immunoglobulin classes is carried out. The results obtained must be within the limits set for the product and agreed with the competent authority. The batch contains not more than the level shown to be safe in the safety studies and, unless the batch potency test specifically covers all appropriate immunoglobulins, the level in the batch is not less than that in the batch or batches shown to be effective in the efficacy studies.

**Total protein**. For products where claims are being made which relate to the protein content, as well as demonstrating that the batch contains not more than the stated upper limit, the batch shall be shown to contain not less than that in the batch or batches shown to be effective in the efficacy studies.

**Extraneous agents**. In addition to the test described under Tests, specific tests may be required depending on the nature of the preparation, its risk of contamination and the use of the product. In particular, specific tests for important potential pathogens may be required when the donor and recipient species are the same and when these agents would not be detected reliably by the general screening test described under Tests.

**Water**. Where applicable, the freeze-drying process is checked by a determination of water and shown to be within the limits set for the product.

## IDENTIFICATION

The identity of the product is established by immunological tests and, where necessary, by determination of biological activity. The potency test may also serve for identification.

## TESTS

*The following requirements refer to liquid immunosera and reconstituted freeze-dried immunosera.*

**Foreign proteins**. When examined by precipitation tests with specific antisera against plasma proteins of a suitable range of species, only protein from the declared animal species is shown to be present.

**Albumin**. Purified immunosera comply with a test for albumin. Unless otherwise prescribed in the monograph, when examined electrophoretically, purified immunosera show not more than a trace of albumin, and the content of albumin is in any case not greater than 30 g/L of the reconstituted preparation, where applicable.

**Total protein**. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2 mL of this solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, discard the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying by 6.25. The results obtained are not greater than the upper limit stated on the label.

**Antimicrobial preservative**. Determine the amount of antimicrobial preservative by a suitable physicochemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Formaldehyde** (*2.4.18*). Where formaldehyde has been used in the preparation, the concentration of free formaldehyde is not greater than 0.5 g/L, unless a higher amount has been shown to be safe.

**Sterility** (*2.6.1*). Immunosera for veterinary use comply with the test for sterility. When the volume of liquid in a container is greater than 100 mL, the method of membrane filtration is used wherever possible. If this method is used, incubate the media for not less than 14 days. Where the method of membrane filtration cannot be employed, the method of direct inoculation may be used. Where the volume of liquid in each container is at least 20 mL, the minimum volume to be used for each culture medium is 10 per cent of the contents of the

container or 5 mL, whichever is the least. The appropriate number of items to be tested (*2.6.1*) is 1 per cent of the batch with a minimum of 4 and a maximum of 10.

**Mycoplasmas** (*2.6.7*). Immunosera for veterinary use comply with the test for mycoplasmas.

**Safety**. A test is conducted in one of the species for which the product is recommended. Unless an overdose is specifically contraindicated on the label, twice the maximum recommended dose for the species used is administered by a recommended route. If there is a warning against administration of an overdose, a single dose is administered. For products to be used in mammals, use 2 animals of the minimum age for which the product is recommended. For avian products, use not fewer than 10 birds of the minimum age recommended. The birds are observed for 21 days. The other species are observed for 14 days. No abnormal local or systemic reaction occurs.

**Extraneous agents**. A test for extraneous agents is conducted by inoculation of cell cultures sensitive to pathogens of the species of the donor animal and into cells sensitive to pathogens of each of the recipient target species stated on the label (*2.6.25*). Observe the cells for 14 days. During this time, carry out at least one passage. The cells are checked daily for cytopathic effect and are checked at the end of 14 days for the presence of a haemadsorbing agent. The batch complies with the test if there is no evidence of the presence of an extraneous agent.

For immunosera of avian origin, if a test in cell culture is insufficient to detect potential extraneous agents, a test is conducted by inoculation of embryonated eggs from flocks free from specified pathogens (*5.2.2*) or by some other suitable method (polymerase chain reaction (PCR) for example).

POTENCY

Carry out a suitable test for potency.

Where a specific monograph exists, carry out the biological assay prescribed in the monograph and express the result in International Units per millilitre when such exist.

STORAGE

Protected from light, at a temperature of 5 ± 3 °C. Liquid immunosera must not be allowed to freeze.

LABELLING

The label states:

- that the preparation is for veterinary use;
- whether or not the preparation is purified;
- the minimum number of International Units per millilitre, where such exist;
- the volume of the preparation in the container;
- the indications for the product;
- the instructions for use including the interval between any repeat administrations and the maximum number of administrations that is recommended;
- the recipient target species for the immunoserum;
- the dose recommended for different species;
- the route(s) of administration;
- the name of the species of the donor animal;
- the maximum quantity of total protein;
- the name and amount of any antimicrobial preservative or any other excipient;
- any contra-indications to the use of the product including any required warning on the dangers of administration of an overdose;
- for freeze-dried immunosera:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the period within which the immunoserum is to be used after reconstitution.

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**corrected 10.0**

# LIVE BIOTHERAPEUTIC PRODUCTS FOR HUMAN USE

## Producta biotherapeutica viva ad usum humanum

DEFINITION

Live biotherapeutic products (LBP) are medicinal products containing live micro-organisms (bacteria or yeasts) for human use.

LBP are administered orally or vaginally and are available in different pharmaceutical forms.

LBP may contain one or multiple microbial strains from the same or different species of micro-organisms. The quantity of living bacteria or yeasts is determined by counting viable and culturable micro-organisms of the intended strain(s).

Faecal microbiota transplantation (FMT) and products intended as gene therapy agents are excluded from this monograph.

PRODUCTION

*GENERAL PROVISIONS*

The production method for a given product must have been shown to yield consistently an LBP comparable with the batch(es) of proven clinical efficacy and safety in man. Suitable in-process controls ensure that the production process is under control and consistently produces LBP of defined quality.

LBP are produced using a seed-lot system. The methods of preparation are designed to maintain the viability of the micro-organisms. Stabilisers and other excipients of controlled microbiological quality may be added.

Where LBP are produced using substances of human or animal origin, the requirements of general chapter *5.1.7. Viral safety* apply. The production process is optimised to consistently minimise or remove adventitious agents and toxic or other impurities.

In the production of a final lot of LBP, the number of subcultures of a bacterium or yeast, from the master seed lot, shall not exceed that used for production of the LBP shown to be satisfactory in clinical trials with respect to quality, safety and efficacy.

Consistency of production is important to ensure the quality of LBP. Limits for process parameters and for tests carried out during production and on the final lot may be in the form of maximum values, minimum values, or tolerances around a given value. Limits are based on the results found for batches tested clinically and those used to demonstrate consistency of production. These limits may subsequently be refined on a statistical basis in the light of production data.

*CHARACTERISATION OF THE MICRO-ORGANISM(S)*

The characterisation of the micro-organism(s) is carried out at the strain level. The strain used for master seed lots is identified by historical records that include information on its origin, subsequent manipulation and the tests used to characterise the strain. Characterisation includes determination of the phenotype and genotype of the strain, using methods such as macroscopic and microscopic methods, biochemical tests, molecular genetic tests, sequencing or mass spectrometry. The strain must be phenotypically and genotypically stable. Antimicrobial resistance is determined and any resistance transferable from the micro-organism

to the relevant microbiota is investigated and excluded. The presence of virulence factors must be investigated and evaluated with respect to safety.

For products to be administered orally, survivability of the micro-organism(s) in the human gut is demonstrated by *in vitro* gastric acid and bile resistance testing.

*SEED-LOT SYSTEM*

A seed-lot system is used. Suitable measures are taken to ensure that adventitious agents and toxic or other impurities are not present in master or working seed-lots. When a master seed-lot is replaced, the new master seed-lot must be fully characterised and the critical steps in the production process revalidated to the extent required to demonstrate that no adverse change has occurred in the quality, safety and efficacy of the product.

*CULTURE MEDIA*

Culture media are sterile and, as far as possible, free from ingredients known to cause toxic, allergic or other undesirable reactions in humans; if inclusion of such ingredients is necessary, it is demonstrated that the residual amount in the final lot does not adversely affect product safety.

*GROWTH AND HARVESTING*

Each strain is cultured and the culture is examined for purity by suitable tests. These tests may include inoculation into suitable media, examination of colony morphology and microscopic examination. The culture is harvested under defined conditions and may be stored under appropriate conditions.

*INTERMEDIATE PRODUCTS*

When intermediate products are stored, a storage period justified by stability data is established for each intermediate.

**Test for microbial contamination**. Each intermediate is shown to comply with defined in-process limits using a suitable method.

*FINAL BULK*

The final bulk may contain a single strain or a mixture of 2 or more strains. Stabilisers and other excipients may be added depending on the dosage form.

*FINAL LOT*

When the final lot is an aqueous suspension, it is distributed into sterile, tamper-evident containers (*3.2*).

The final lot complies with the requirements under Identification, Tests and Assay. Certain tests prescribed for the final lot may be carried out on the final bulk, provided it has been demonstrated that subsequent manufacturing operations do not affect compliance.

IDENTIFICATION

Each micro-organism is identified by a suitable method.

TESTS

**Appearance**. It complies with the established specification.

**Microbial contamination**. Microbiological examination of LBP is performed according to the methods given in general chapters *2.6.36* and *2.6.38*.

When an acceptance criterion for microbial contamination is prescribed it is interpreted as follows:

– $10^1$ CFU: maximum acceptable count = 20;

– $10^2$ CFU: maximum acceptable count = 200;

– $10^3$ CFU: maximum acceptable count = 2000.

Acceptance criteria based upon the aerobic microbial contamination count (AMCC) and the combined yeasts/moulds contamination count (YMCC) are given in Table 3053.-1.

Table 3053.-1. – *Acceptance criteria for microbiological quality of LBP*

| Route of administration | AMCC* (CFU/g or CFU/mL) | YMCC** (CFU/g or CFU/mL) | Specified micro-organisms*** |
|---|---|---|---|
| Non-aqueous preparations for oral use | $10^3$ | $10^2$ | Absence of *Escherichia coli***** (1 g or 1 mL) |
| Aqueous preparations for oral use | $10^2$ | $10^1$ | Absence of *Escherichia coli***** (1 g or 1 mL) |
| Vaginal use | $10^2$ | $10^1$ | Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)<br>Absence of *Staphylococcus aureus* (1 g or 1 mL)<br>Absence of *Candida albicans* (1 g or 1 mL) |

* If it is not possible to determine the AMCC due to the absence of a suitable method for the LBP, then test for the absence of the following specified micro-organisms using the methods in general chapter *2.6.38*: bile-tolerant Gram-negative bacteria(1) (1 g or 1 mL), *P. aeruginosa* (1 g or 1 mL), *S. aureus* (1 g or 1 mL), *Salmonella* spp. (10 g or 10 mL). Tests for other pathogens may be carried out based on a risk assessment (e.g. specific environmental contaminants).

(1) If the LBP consists of *E. coli*, test for the absence of Enterobacteriaceae and other Gram-negative bacteria using suitable culture media instead of testing for the absence of bile-tolerant Gram-negative bacteria.

** If it is not possible to determine the YMCC due to the absence of a suitable method for the LBP, then test for the absence of *C. albicans* and test for pathogenic moulds based on a risk assessment.

*** If the LBP consists of *Bacillus* spp. spores, test for the absence of *Bacillus cereus* in addition to the tests described in the table.

**** If the LBP consists of *E. coli*, the test for *E. coli* should be replaced by another test for the absence of faecal contaminants by testing for *Enterococcus faecalis* and *Enterococcus faecium* as indicators. A search for other pathogens may be carried out based on a risk assessment (e.g. pathogenic *E. coli*).

In addition to the micro-organisms listed in Table 3053.-1, tests for other pathogens may be carried out based on a risk assessment (e.g. specific environmental contaminants, anaerobic contaminants (e.g. Clostridia)).

The significance of other micro-organisms recovered is evaluated in terms of:

– intended recipient: risk may differ for neonates, infants, the debilitated;

– use of immunosuppressive agents, corticosteroids;

– presence of disease, wounds, organ damage.

**pH** (*2.2.3*). It complies with the limit approved for the particular product.

**Water** (*2.5.12*)**, Loss on drying** (*2.2.32*) **or Water activity** (*2.9.39*). It complies with the limit approved for the particular product.

ASSAY

**Number of live micro-organisms**. The number of micro-organisms, expressed in CFU, is determined by a suitable microbial enumeration test, for example by the pour-plate method or surface-spread method.

The potency of each strain is expressed in CFU/g, CFU/mL, or CFU/unit; it is not less than the stated value or it is within the stated range.

The potency may be expressed in number of viable cells/mL based on viable-cell assay, in agreement with the competent authority.

STORAGE

Liquid LBP are not to be frozen.

LABELLING

The label states:

- the name of the strain(s);
- the route of administration;
- the storage conditions;
- the expiry date;
- the potency of each strain expressed in CFU/mL, CFU/g, CFU/unit or viable cells/mL;
- the name of any stabilisers and other excipients;
- where applicable, for freeze-dried preparations to be reconstituted before use:
  - the name, composition and volume of the reconstituting liquid to be added;
  - the storage conditions and the period of time within which the LBP is to be used after reconstitution.

**01/2012:2031**



# MONOCLONAL ANTIBODIES FOR HUMAN USE

## Anticorpora monoclonalia ad usum humanum

DEFINITION

Monoclonal antibodies for human use are preparations of an immunoglobulin or a fragment of an immunoglobulin, for example, F(ab′)2, with defined specificity, produced by a single clone of cells. They may be conjugated to other substances, including for radiolabelling.

They can be obtained from immortalised B lymphocytes that are cloned and expanded as continuous cell lines or from rDNA-engineered cell lines.

Examined under suitable conditions of visibility, they are practically free from particles.

Currently available rDNA-engineered antibodies include the following antibodies.

*Chimeric monoclonal antibodies*: the variable heavy- and light-chain domains of a human antibody are replaced by those of a non-human species that possess the desired antigen specificity.

*Humanised monoclonal antibodies*: the 3 short hypervariable sequences (the complementarity-determining regions) of non-human variable domains for each chain are engineered into the variable domain framework of a human antibody; other sequence changes may be made to improve antigen binding.

*Recombinant human monoclonal antibodies*: the variable heavy- and light-chain domains of a human antibody are combined with the constant region of a human antibody.

Monoclonal antibodies obtained from cell lines modified by recombinant DNA technology also comply with the requirements of the monograph *Products of recombinant DNA technology (0784)*.

This monograph applies to monoclonal antibodies, including conjugates, for therapeutic and prophylactic use and for use as *in vivo* diagnostics. It does not apply to monoclonal antibodies used as reagents in the manufacture of medicinal products. Nor does it apply to monoclonal antibodies produced in ascites, for which requirements are decided by the competent authority.

PRODUCTION

*GENERAL PROVISIONS*

Production is based on a seed-lot system using a master cell bank and, if applicable, a working cell bank derived from the cloned cells. The production method is validated during development studies in order to prevent transmission of infectious agents by the final product. All biological materials and cells used in the production are characterised and are in compliance with chapter *5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products*. Where monoclonal antibodies for human use are manufactured using materials of human or animal origin, the requirements of chapter *5.1.7. Viral safety* also apply. Where an immunogen is used, it is characterised and the method of immunisation is documented.

**Process validation**. During development studies, the production method is validated for the following aspects:

- consistency of the production process including cell-culture/fermentation, purification and, where applicable, fragmentation method;
- removal or inactivation of infectious agents;
- adequate removal of product- and process-related impurities (for example, host-cell protein and DNA, protein A, antibiotics, cell-culture components);
- specificity and biological activity of the monoclonal antibody;
- absence of non-endotoxin pyrogens, where applicable;
- reusability of purification components (for example, column material), limits or acceptance criteria being set as a function of the validation;
- methods used for conjugation, where applicable.

**Product characterisation**. The product is characterised to obtain adequate information including: structural integrity, isotype, amino-acid sequence, secondary structure, carbohydrate moiety, disulfide bridges, conformation, specificity, affinity, biological activity and heterogeneity (characterisation of isoforms).

A battery of suitable analytical techniques is used including chemical, physical, immunochemical and biological tests (for example, peptide mapping, *N*- and *C*-terminal amino-acid sequencing, mass spectrometry, chromatographic, electrophoretic and spectroscopic techniques). Additional tests are performed to obtain information on cross-reactivity with human tissues.

For those products that are modified by fragmentation or conjugation, the influence of the methods used on the antibody is characterised.

**Process intermediates**. Where process intermediates are stored, an expiry date or a storage period justified by stability data is established for each.

**Biological assay**. The biological assay is chosen in terms of its correlation with the intended mode of action of the monoclonal antibody.

**Reference preparation**. A batch shown to be stable and shown to be suitable in clinical trials, or a batch representative thereof, is used as a reference preparation for the identification, tests and assay. The reference preparation is appropriately characterised as defined under Product characterisation, except that it is not necessary to examine cross-reactivity for each batch of reference preparation.

**Definition of a batch**. Definition of a batch is required throughout the process.

*SOURCE CELLS*

Source cells include fusion partners, lymphocytes, myeloma cells, feeder cells and host cells for the expression of the recombinant monoclonal antibody.

The origin and characteristics of the parental cell are documented, including information on the health of the donors, and on the fusion partner used (for example, myeloma cell line, human lymphoblastoid B-cell line).

Wherever possible, source cells undergo suitable screening for extraneous agents and endogenous agents. The choice of viruses for the tests is dependent on the species and tissue of origin.

*CELL LINE PRODUCING THE MONOCLONAL ANTIBODY*

The suitability of the cell line producing the monoclonal antibody is demonstrated by:

- documentation on the history of the cell line including description of the cell fusion, immortalisation or transfection and cloning procedure;
- characterisation of the cell line (for example, phenotype, isoenzyme analysis, immunochemical markers and cytogenetic markers);
- characterisation of relevant features of the antibody;
- consistency of critical quality attributes for the antibody up to or beyond the population doubling level or generation number used for routine production;
- for recombinant DNA products, consistency of the coding sequence of the expression construct in cells cultivated to the limit of *in vitro* cell age for production use or beyond, by either nucleic acid testing or product analysis.

*CELL BANKS*

The master cell bank is a homogeneous suspension of the cell line producing the monoclonal antibody, distributed in equal volumes in a single operation into individual containers for storage.

A working cell bank is a homogeneous suspension of the cell material derived from the master cell bank at a finite passage level, distributed in equal volumes in a single operation into individual containers for storage.

Post-production cells are cells cultured up to or beyond the population doubling level or generation number used for routine production.

The following tests are performed on the master cell bank: viability, identity, absence of bacterial, fungal and mycoplasmal contamination, characterisation of the monoclonal antibody produced. Adventitious viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination is tested using a suitable range of *in vitro* tests.

The following tests are performed on the working cell bank: viability, identity, absence of bacterial, fungal and mycoplasmal contamination. Adventitious viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. For the first working cell bank, these tests are performed on post-production cells, generated from that working cell bank; for working cell banks subsequent to the first working cell bank, a single *in vitro* and *in vivo* test can be done either directly on the working cell bank or on post-production cells.

For the master cell bank and working cell bank, tests for specific viruses are carried out when potentially contaminated biological material has been used during preparation of the cell banks, taking into account the species of origin of this material. This may not be necessary when this material is inactivated using validated procedures.

The following tests are performed on the post-production cells: absence of bacterial, fungal and mycoplasmal contamination. Adventitious viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination is tested using a suitable range of *in vitro* tests.

*CULTURE AND HARVEST*

**Production at finite passage level (single harvest)**. Cells are cultivated up to a defined maximum number of passages or population doublings, or up to a fixed harvest time (in accordance with the stability of the cell line). Product is harvested in a single operation.

**Continuous-culture production (multiple harvest)**. Cells are continuously cultivated for a defined period (in accordance with the stability of the system and production consistency). Monitoring is necessary throughout the life of the culture; the required frequency and type of monitoring will depend on the nature of the production system.

Each harvest is tested for antibody content, bioburden, endotoxin and mycoplasmas. General or specific tests for adventitious viruses are carried out at a suitable stage depending on the nature of the manufacturing process and the materials used. For processes using production at finite passage level (single harvest), at least 3 harvests are tested for adventitious viruses using a suitable range of *in vitro* methods.

The acceptance criteria for harvests for further processing are clearly defined and linked to the schedule of monitoring applied. If any adventitious viruses are detected, the process is carefully investigated to determine the cause of the contamination and the harvest is not further processed. Harvests in which an endogenous virus has been detected are not used for purification unless an appropriate action plan has been defined to prevent transmission of infectious agents.

*PURIFICATION*

Harvests or intermediate pools may be pooled before further processing. The purification process includes steps that remove and/or inactivate non-enveloped and enveloped viruses. A validated purification process, for which removal and/or inactivation of infectious agents and removal of product- and process-related impurities has been demonstrated, is used. Defined steps of the process lead to a purified monoclonal antibody (active substance) of consistent quality and biological activity.

*ACTIVE SUBSTANCE*

The test programme for the active substance depends on the validation of the process, on demonstration of consistency and on the expected level of product- and process-related impurities. The active substance is tested for appearance, identity, bioburden and bacterial endotoxins, product-related substances, product- and process-related impurities including tests for host-cell-derived proteins and host-cell- and vector-derived DNA, as well as structural integrity, protein content and biological activity by suitable analytical methods, comparing with the reference preparation where necessary. When the active substance is a conjugated or transformed antibody, appropriate tests must be performed before and after the antibody conjugation/modification.

If storage of intermediates is intended, adequate stability of these preparations and its impact on quality or shelf-life of the finished product are evaluated.

*FINAL BULK*

One or more batches of active substance may be combined to produce the final bulk. Suitable stabilisers and other excipients may be added during preparation of the final bulk.

The final bulk must be stored under validated conditions with respect to bioburden and stability.

*FINAL LOT*

The final bulk is sterile-filtered and distributed under aseptic conditions into sterile containers, which may subsequently be freeze-dried.

As part of the in-process control each container (vial, syringe or ampoule) is inspected after filling to eliminate containers that contain visible particles. During development of the product it must be demonstrated that either the process will

not generate visible proteinaceous particles in the final lot or such particles are reduced to a low level as justified and authorised.

CHARACTERS

Liquid preparations are clear or slightly opalescent, colourless or slightly coloured liquids. Freeze-dried products are white or slightly coloured powders or solid friable masses. After reconstitution they show the same characteristics as liquid preparations.

IDENTIFICATION

The identity is established by suitable validated methods comparing the product with the reference preparation, where appropriate. The assay also contributes to identification.

TESTS

**Appearance**. Liquid or reconstituted freeze-dried preparations comply with the limits approved for the particular product with regard to degree of opalescence (*2.2.1*) and degree of coloration (*2.2.2*). They are without visible particles, unless otherwise justified and authorised.

**Solubility**. Freeze-dried preparations dissolve completely in the prescribed volume of reconstituting liquid, within a defined time, as approved for the particular product.

**pH** (*2.2.3*). It complies with the limits approved for the particular product.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg, unless otherwise justified and authorised.

**Extractable volume** (*2.9.17*). It complies with the test for extractable volume.

**Total protein** (*2.5.33*). It complies with the limits approved for the particular product.

**Molecular-size distribution**. Molecular-size distribution is determined by a suitable method, for example size-exclusion chromatography (*2.2.30*). It complies with the limits approved for the particular product.

**Molecular identity and structural integrity**. Depending on the nature of the monoclonal antibody, its microheterogeneity and isoforms, a number of different tests can be used to demonstrate molecular identity and structural integrity. These tests may include peptide mapping, isoelectric focusing, ion-exchange chromatography, hydrophobic interaction chromatography, oligosaccharide mapping, monosaccharide content and mass spectrometry.

**Purity**. Tests for process- and product-related impurities are carried out by suitable validated methods. Provided that tests for process-related impurities have been carried out on the active substance or on the final bulk with satisfactory results, they may be omitted on the final lot.

**Stabiliser**. Where applicable, it complies with the limits approved for the particular product.

**Water** (*2.5.12*). Freeze-dried products comply with the limits approved for the particular product.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). It complies with the limits approved for the particular product.

**Tests applied to modified antibodies**. Suitable tests are carried out depending on the type of modification.

ASSAY

Carry out a suitable biological assay compared to the reference preparation. Design of the assay and calculation of the results are made according to the usual principles (for example, *5.3*).

STORAGE

As stated on the label.

*Expiry date*. The expiry date is calculated from the date of sterile filtration, the date of filling (for liquid preparations) or the date of freeze-drying (where applicable).

LABELLING

The label states:

- the number of units per millilitre, where applicable;
- the quantity of protein per container;
- the quantity of monoclonal antibody in the container;
- for liquid preparations, the volume of the preparation in the container;
- for freeze-dried preparations:
  - the name and the volume of the reconstitution liquid to be added;
  - the period of time within which the monoclonal antibody is to be used after reconstitution;
- the dilution to be made before use of the product, where applicable.

**04/2019:2619**

# PHARMACEUTICAL PREPARATIONS

## Pharmaceutica

INTRODUCTION

This monograph is intended to be a reference source of standards in the European Pharmacopoeia on active substances, excipients and dosage forms, which are to be applied in the manufacture/preparation of pharmaceuticals, but not a guide on how to manufacture as there is specific guidance available covering methods of manufacture and associated controls.

It does not cover investigational medicinal products, but competent authorities may refer to pharmacopoeial standards when authorising clinical trials using investigational medicinal products.

DEFINITION

Pharmaceutical preparations are medicinal products generally consisting of active substances that may be combined with excipients, formulated into a dosage form suitable for the intended use, where necessary after reconstitution, presented in a suitable and appropriately labelled container.

Pharmaceutical preparations may be licensed by the competent authority, or unlicensed and made to the specific needs of patients according to legislation. There are 2 categories of unlicensed pharmaceutical preparations:

- extemporaneous preparations, i.e. pharmaceutical preparations individually prepared for a specific patient or patient group, supplied after preparation;
- stock preparations, i.e. pharmaceutical preparations prepared in advance and stored until a request for a supply is received.

In addition to this monograph, pharmaceutical preparations also comply with the General Notices and with the relevant general chapters of the Pharmacopoeia. General chapters are normally given for information and become mandatory when referred to in a general or specific monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

Where relevant, pharmaceutical preparations also comply with the dosage form monographs (e.g. *Capsules (0016)*, *Tablets (0478)*) and general monographs relating to pharmaceutical preparations (e.g. *Allergen products (1063)*, *Herbal teas (1435)*, *Homoeopathic preparations (1038)*,

*Homoeopathic pillules, coated (2786)*, *Homoeopathic pillules, impregnated (2079)*, *Immunosera for human use, animal (0084)*, *Immunosera for veterinary use (0030)*, *Live biotherapeutic products for human use (3053)*, *Monoclonal antibodies for human use (2031)*, *Radiopharmaceutical preparations (0125)*, *Vaccines for human use (0153)*, *Vaccines for veterinary use (0062)*).

## ETHICAL CONSIDERATIONS AND GUIDANCE IN THE PREPARATION OF UNLICENSED PHARMACEUTICAL PREPARATIONS

The underlying principle of legislation for pharmaceutical preparations is that, subject to specific exemptions, no pharmaceutical preparation may be placed on the market without an appropriate marketing authorisation. The exemptions from the formal licensing requirement allow the supply of unlicensed products to meet the special needs of individual patients. However, when deciding to use an unlicensed preparation all health professionals involved (e.g. the prescribing practitioners and/or the preparing pharmacists) have, within their area of responsibilities, a duty of care to the patient receiving the pharmaceutical preparation.

In considering the preparation of an unlicensed pharmaceutical preparation, a suitable level of risk assessment is undertaken.

The risk assessment identifies:

- the criticality of different parameters (e.g. quality of active substances, excipients and containers; design of the preparation process; extent and significance of testing; stability of the preparation) to the quality of the preparation; and
- the risk that the preparation may present to a particular patient group.

Based on the risk assessment, the person responsible for the preparation must ensure, with a suitable level of assurance, that the pharmaceutical preparation is, throughout its shelf-life, of an appropriate quality and suitable and fit for its purpose. For stock preparations, storage conditions and shelf-life have to be justified on the basis of, for example, analytical data or professional judgement, which may be based on literature references.

## PRODUCTION

Manufacture/preparation must take place within the framework of a suitable quality system and be compliant with the standards relevant to the type of product being made. Licensed products must comply with the requirements of their licence. For unlicensed products a risk assessment as outlined in the section 'Ethical considerations and guidance in the preparation of unlicensed pharmaceutical preparations' is of special importance, as these products are not previously assessed by the competent authority.

Where pharmaceutical preparations are manufactured/prepared using materials of human or animal origin, the general requirements of general chapters *5.1.7. Viral safety* and *5.2.6. Evaluation of safety of veterinary vaccines and immunosera* and of the general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)* apply, where appropriate.

**Formulation**. During pharmaceutical development or prior to manufacture/preparation, suitable ingredients, processes, tests and specifications are identified and justified in order to ensure the suitability of the product for the intended purpose. This includes consideration of the properties required in order to identify whether specific ingredient properties or process steps are critical to the required quality of the pharmaceutical preparation.

**Active substances and excipients**. Active substances and excipients used in the formulation of pharmaceutical preparations comply with the requirements of the relevant general monographs, e.g. *Substances for pharmaceutical use (2034)*, *Essential oils (2098)*, *Herbal drug extracts (0765)*, *Herbal drugs (1433)*, *Herbal drug preparations (1434)*, *Herbal drugs for homoeopathic preparations (2045)*, *Mother tinctures for homoeopathic preparations (2029)*, *Methods of preparation of homoeopathic stocks and potentisation (2371)*, *Products of fermentation (1468)*, *Products of recombinant DNA technology (0784)*, *Vegetable fatty oils (1579)*.

In addition, where specific monographs exist, the quality of the active substances and excipients used complies with the corresponding monographs.

Where no specific monographs exist, the required quality must be defined, taking into account the intended use and the involved risk.

When physicochemical characteristics of active substances and functionality-related characteristics (FRCs) of excipients (e.g. particle-size distribution, viscosity, polymorphism) are critical in relation to their role in the manufacturing process and quality attributes of the pharmaceutical preparation, they must be identified and controlled.

Detailed information on FRCs is given in general chapter *5.15. Functionality-related characteristics of excipients*.

**Microbiological quality**. The formulation of the pharmaceutical preparation and its container must ensure that the microbiological quality is suitable for the intended use.

During development, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives, or by the selection of an appropriate container, provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during the storage and use of the preparation. A suitable test method together with criteria for evaluating the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

If preparations do not have adequate antimicrobial efficacy and do not contain antimicrobial preservatives they are supplied in single-dose containers, or in multidose containers that prevent microbial contamination of the contents after opening.

In the manufacture/preparation of non-sterile pharmaceutical preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapters *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* and *5.1.8. Microbiological quality of herbal medicinal products for oral use and extracts used in their preparation*.

Sterile preparations are manufactured/prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

**Containers**. A suitable container is selected. Consideration is given to the intended use of the preparation, the properties of the container, the required shelf-life, and product/container incompatibilities. Where applicable, containers for pharmaceutical preparations comply with the requirements for containers (*3.2* and subsections) and materials used for the manufacture of containers (*3.1* and subsections).

**Stability**. Stability requirements of pharmaceutical preparations are dependent on their intended use and on the desired storage time.

Where applicable, the probability and criticality of possible degradation products of the active substance(s) and/or reaction products of the active substance(s) with an excipient and/or the immediate container must be assessed. Depending on the result of this assessment, limits of degradation and/or reaction products are set and monitored in the pharmaceutical preparation. Licensed products require a stability exercise.

Methods used for the purpose of stability testing for all relevant characteristics of the preparation are validated as stability indicating, i.e. the methods allow the quantification of the relevant degradation products and physical characteristic changes.

TESTS

Relevant tests to apply in order to ensure the appropriate quality of a particular dosage form are described in the specific dosage form monographs.

Where it is not practical, for unlicensed pharmaceutical preparations, to carry out the tests (e.g. batch size, time restraints), other suitable methods are implemented to ensure that the appropriate quality is achieved in accordance with the risk assessment carried out and any local guidance or legal requirements.

Stock preparations are normally tested to a greater extent than extemporaneous preparations.

The following tests are applicable to many preparations and are therefore listed here.

**Appearance**. The appearance (e.g. size, shape and colour) of the pharmaceutical preparation is controlled.

**Identity and purity tests**. Where applicable, the following tests are carried out on the pharmaceutical preparation:

- identification of the active substance(s);
- identification of specific excipient(s), such as preservatives;
- purity tests (e.g. investigation of degradation products, residual solvents (*2.4.24*) or other related impurities, sterility (*2.6.1*));
- safety tests (e.g. safety tests for biological products).

**Elemental impurities**. General chapter *5.20*. *Elemental impurities* applies to pharmaceutical preparations except products for veterinary use, unlicensed preparations and other products that are excluded from the scope of this chapter.

For pharmaceutical preparations outside the scope of general chapter *5.20*, manufacturers of these products remain responsible for controlling the levels of elemental impurities using the principles of risk management.

If appropriate, testing is performed using suitable analytical procedures according to general chapter *2.4.20*. *Determination of elemental impurities*.

**Uniformity** (*2.9.40* or *2.9.5*/*2.9.6*). Pharmaceutical preparations presented in single-dose units comply with the test(s) as prescribed in the relevant specific dosage form monograph. If justified and authorised, general chapter *2.9.40* can be applicable only at the time of release.

Special uniformity requirements apply in the following cases:

- for herbal drugs and herbal drug preparations, compliance with general chapter *2.9.40* is not required;
- for homoeopathic preparations, the provisions of general chapters *2.9.6* and *2.9.40* are normally not appropriate, however in certain circumstances compliance with these chapters may be required by the competent authority;
- for single- and multivitamin and trace-element preparations, compliance with general chapters *2.9.6* and *2.9.40* (*content uniformity only*) is not required;
- in justified and authorised circumstances, for other preparations, compliance with general chapters *2.9.6* and *2.9.40* may not be required by the competent authority.

**Reference standards**. Reference standards may be needed at various stages for quality control of pharmaceutical preparations. They are established and monitored taking due account of general chapter *5.12. Reference standards*.

ASSAY

Unless otherwise justified and authorised, contents of active substances and specific excipients such as preservatives are determined in pharmaceutical preparations. Limits must be defined and justified.

Suitable and validated methods are used. If assay methods prescribed in the respective active substance monographs are used, it must be demonstrated that they are not affected by the presence of the excipients and/or by the formulation.

**Reference standards**. See Tests.

LABELLING AND STORAGE

The relevant labelling requirements given in the general dosage form monographs apply. In addition, relevant European Union or other applicable regulations apply.

GLOSSARY

**Formulation**: the designing of an appropriate formula (including materials, processes, etc.) that will ensure that the patient receives the suitable pharmaceutical preparation in an appropriate form that has the required quality and that will be stable and effective for the required length of time.

**Licensed pharmaceutical preparation**: a medicinal product that has been granted a marketing authorisation by a competent authority. Synonym: authorised pharmaceutical preparation.

**Manufacture**: all operations of purchase of materials and products, Production, Quality Control, release, storage, distribution of medicinal products and the related controls.

**Preparation (of an unlicensed pharmaceutical preparation)**: the 'manufacture' of unlicensed pharmaceutical preparations by or at the request of pharmacies or other healthcare establishments (the term 'preparation' is used instead of 'manufacture' in order clearly to distinguish it from the industrial manufacture of licensed pharmaceutical preparations).

**Reconstitution**: manipulation to enable the use or application of a medicinal product with a marketing authorisation in accordance with the instructions given in the summary of product characteristics or the patient information leaflet.

**Risk assessment**: the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

**Unlicensed pharmaceutical preparation**: a medicinal product that is exempt from the need of having a marketing authorisation issued by a competent authority but is made for specific patients' needs according to legislation.

**04/2018:1468**

# PRODUCTS OF FERMENTATION

## Producta ab fermentatione

*This monograph applies to indirect gene products obtained by fermentation. It is not applicable to*:

- *monographs in the Pharmacopoeia concerning vaccines for human or veterinary use*;
- *products derived from continuous cell lines of human or animal origin*;
- *direct gene products that result from the transcription and translation from nucleic acid to protein, whether or not subject to post-translational modification*;
- *products obtained by semi-synthesis from a product of fermentation and those obtained by biocatalytic transformation*;

– *whole broth concentrates or raw fermentation products.*

*This monograph provides general requirements for the development and manufacture of products of fermentation. These requirements are not necessarily comprehensive in a given case and requirements complementary or additional to those prescribed in this monograph may be imposed in an individual monograph or by the competent authority.*

## DEFINITION

For the purposes of this monograph, products of fermentation are active or inactive pharmaceutical substances produced by controlled fermentation as indirect gene products. They are primary or secondary metabolites of micro-organisms such as bacteria, yeasts, fungi and micro-algae, whether or not modified by traditional procedures or recombinant DNA (rDNA) technology. Such metabolites include vitamins, amino acids, antibiotics, alkaloids and polysaccharides.

They may be obtained by batch or continuous fermentation processes followed by procedures such as extraction, concentration, purification and isolation.

## PRODUCTION

Production is based on a process that has been validated and shown to be suitable. The extent of validation depends on the critical nature of the respective process step.

## CHARACTERISATION OF THE PRODUCER MICRO-ORGANISM

The history of the micro-organism used for production is documented. The micro-organism is adequately characterised. This may include determination of the phenotype of the micro-organism, macroscopic and microscopic methods and biochemical tests and, if appropriate, determination of the genotype of the micro-organism and molecular genetic tests.

## PROCESSES USING A SEED-LOT SYSTEM

The *master cell bank* is a homogeneous suspension or lyophilisate of the original cells distributed into individual containers for storage. The viability and productivity of the cells under the selected storage conditions and their suitability for initiating a satisfactory production process after storage must be demonstrated.

Propagation of the master cell bank may take place through a seed-lot system that uses a working cell bank.

The *working cell bank* is a homogeneous suspension or lyophilisate of the cell material derived from the master cell bank, distributed in equal volumes into individual containers for storage (for example, in liquid nitrogen).

Production may take place by batch or continuous culture and may be terminated under defined conditions.

All containers in a cell bank are stored under identical conditions. Once removed from storage, the individual ampoules, vials or culture straws are not returned to the cell bank.

## PROCESSES USING STAGED GROWTH IN CULTURES

The contents of a container of the working cell bank are used, if necessary after resuspension, to prepare an inoculum in a suitable medium. After a suitable period of growth, the cultures are used to initiate the fermentation process, if necessary following preculture in a prefermentor. The conditions to be used at each stage of the process are defined and must be met with each production run.

## CHANGE CONTROL

If the production process is altered in a way that causes a significant change in the impurity profile of the product, the critical steps associated with this change in impurity profile are revalidated.

If a significant change has taken place in the micro-organism used for production that causes a significant change in the impurity profile of the product, the critical steps of the production process associated with this change, particularly the procedure for purification and isolation, are revalidated.

Revalidation includes demonstration that new impurities present in the product as a result of the change are adequately controlled by the test procedures. If necessary, additional or alternative tests must be introduced with appropriate limits. If the change in the process or in the micro-organism results in an increase in the level of an impurity already present, the acceptability of such an increase is addressed.

When a master cell bank is replaced, the critical steps of the production process must be revalidated to the extent necessary to demonstrate that no adverse change has occurred in the quality and safety of the product. Particular attention must be given to possible changes in the impurity profile of the product if a modified or new micro-organism is introduced into the process.

## RAW MATERIALS

The raw materials employed in the fermentation and/or down-stream processing are of suitable quality for the intended purpose. They are tested to ensure that they comply with written specifications. Special attention must be paid to the levels of free histidine in fish peptones as the presence of free histidine may lead to histamine formation in certain conditions.

Levels of bioburden in media or in the inlet air for aeration are reduced to an adequately low level to ensure that if microbial contamination occurs, it does not adversely affect the quality, purity and safety of the product. Addition of components such as nutrients, precursors, and substrates during fermentation takes place aseptically.

## IN-PROCESS CONTROLS

In-process controls are in place to ensure the consistency of the conditions during fermentation and down-stream processing and of the quality of the isolated product. Particular attention must be paid to ensure that any microbial contamination that adversely affects the quality, purity and safety of the product is detected by the controls applied.

Production conditions may be monitored, as appropriate, by suitable procedures for example to control and check:

– temperature,
– pH,
– rate of aeration,
– rate of agitation,
– pressure,

and to monitor the concentration of the required product.

## DOWN-STREAM PROCESSING

At the end of fermentation, the producer micro-organism is inactivated or removed. Further processing is designed to reduce residues originating from the culture medium to an acceptable level and to ensure that the desired product is recovered with consistent quality.

Various purification processes may be used, for example, charcoal treatment, ultrafiltration and solvent extraction. It must be demonstrated that the process or processes chosen reduce to a minimum or remove:

– residues from the producer micro-organism, culture media, substrates and precursors,
– unwanted transformation products of substrates and precursors.

If necessary, suitable tests are performed either as in-process controls or on the isolated product of fermentation.

IDENTIFICATION, TESTS AND ASSAY

The requirements with which the product must comply throughout its period of validity, as well as specific test methods, are stated in the individual monographs.

**01/2008:1483**

# PRODUCTS WITH RISK OF TRANSMITTING AGENTS OF ANIMAL SPONGIFORM ENCEPHALOPATHIES

## Producta cum possibili transmissione vectorium enkephalopathiarum spongiformium animalium

DEFINITION

Products with risk of transmitting agents of animal spongiform encephalopathies are those derived from tissues or secretions of animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge. This monograph applies to all substances or preparations obtained from such animals and to all substances or preparations where products obtained from such animals are included as active substances or excipients or have been used during production, for example as raw or source materials, starting materials or reagents.

PRODUCTION

Production complies with chapter *5.2.8. Minimising the risk of transmitting animal spongiform encephalophathy agents via human and veterinary medicinal products*.

**07/2016:0125**

# RADIOPHARMACEUTICAL PREPARATIONS

## Radiopharmaceutica

DEFINITIONS

Radiopharmaceutical preparations or radiopharmaceuticals are medicinal products which, when ready for use, contain 1 or more radionuclides (radioactive isotopes) included for a medicinal purpose.

For the purpose of this general monograph, radiopharmaceutical preparations also cover:

- radionuclide generators: any system incorporating a fixed parent radionuclide from that is produced a daughter radionuclide that is to be obtained by elution or by any other method and used in a radiopharmaceutical preparation;
- kits for radiopharmaceutical preparation: any preparation to be reconstituted or combined with radionuclides in the final radiopharmaceutical preparation, usually prior to its administration;
- radionuclide precursors: any radionuclide produced for radiolabelling of another substance prior to administration.

Radionuclide precursors may be supplied as solutions for radiolabelling.

A nuclide is a species of atom characterised by the number of protons and neutrons in its nucleus (and hence by its atomic number Z and mass number A) and also by its nuclear energy state. Isotopes of an element are nuclides with the same atomic number but different mass numbers. Nuclides containing an unstable arrangement of protons and neutrons will transform spontaneously to either a stable or another unstable combination of protons and neutrons with a constant statistical probability. Such nuclides are said to be radioactive and are called radionuclides. The initial unstable nuclide is referred to as the parent radionuclide and the resulting nuclide as the daughter nuclide.

Decay or transformation of radionuclides may involve the emission of charged particles, electron capture (EC) or isomeric transition (IT). The charged particles emitted from nuclei may be alpha particles (nuclei of $^4He$) or beta particles (negatively charged, generally called electrons, or positively charged, generally called positrons). Alpha decay usually concerns heavy nuclei ($Z > 82$). Radionuclides with a deficit of protons usually decay by emitting electrons. Radionuclides with a deficit of neutrons usually decay by electron capture or by emitting positrons. In the latter case, radionuclides are called positron emitters. Positrons are annihilated after interaction with electrons in the surrounding matter. The annihilation results in the emission of 2 gamma photons, each with energy of 0.511 MeV, generally emitted at 180° to each other (annihilation radiation). All decay modes may be accompanied by an emission of gamma rays. The emission of gamma rays may be partly or completely replaced by the ejection of electrons, known as internal conversion electrons. This phenomenon, like the process of electron capture, causes a secondary emission of X-rays (due to a reorganisation of the electrons in the atom). This secondary emission may itself be partly replaced by the ejection of electrons, known as Auger electrons.

**Radioactivity**: generally the term 'radioactivity' is used both to describe the phenomenon of radioactive decay and to express the physical quantity of this phenomenon.

The radioactivity of a preparation is the number of nuclear disintegrations or transformations per unit time.

In the International System (SI), radioactivity is expressed in becquerel (Bq), which is 1 nuclear transformation per second. Absolute radioactivity measurements require a specialised laboratory but identification of radioactivity and quantitative measurement of radioactivity can be carried out relatively by comparing the measured samples with standardised preparations provided by laboratories recognised by the competent authority or by using a calibrated instrument.

**Radioactive decay**: any radionuclide decays at an exponential rate with its characteristic decay constant.

The curve of exponential decay (decay curve) is described by the following expression:

$$A_t = A_0 e^{-\lambda t}$$

$A_t$ = the radioactivity at time $t$;

$A_0$ = the radioactivity at time $t = 0$;

$\lambda$ = the decay constant, characteristic of each radionuclide;

$e$ = the base of natural logarithms.

The half-life ($T_{1/2}$) is the time in which a given radioactivity (amount) of a radionuclide decays to half its initial value.

It is related to the decay constant ($\lambda$) by the following equation:

$$T_{1/2} = \frac{ln\,2}{\lambda}$$

The equation of exponential decay can thus be expressed also in the following way, useful for the fast estimation of the radioactivity left after elapsing time $t$:

$$A_t = A_0 \left(\frac{1}{2}\right)^{\frac{t}{T_{1/2}}}$$

The penetrating power of each radiation varies considerably according to its nature and its energy. Alpha particles are completely absorbed in a thickness of a few micrometres to some tens of micrometres of matter. Beta particles are completely absorbed in a thickness of several millimetres to several centimetres of matter. Gamma rays are not completely absorbed but only attenuated and a tenfold reduction may require, for example, several centimetres of lead. The denser the absorbent, the shorter the range of alpha and beta particles and the greater the attenuation of gamma rays.

Each radionuclide is characterised by an invariable half-life, expressed in units of time and by the nature and energy of its radiation or radiations. The energy is expressed in electronvolts (eV), kilo-electronvolts (keV) or mega-electronvolts (MeV).

**Radionuclidic purity**: the ratio, expressed as a percentage, of the radioactivity of the radionuclide concerned to the total radioactivity of the radiopharmaceutical preparation. The relevant potential radionuclidic impurities are listed with their limits in the individual monographs.

**Radiochemical purity**: the ratio, expressed as a percentage, of the radioactivity of the radionuclide concerned which is present in the radiopharmaceutical preparation in the stated chemical form, to the total radioactivity of that radionuclide present in the radiopharmaceutical preparation. The relevant potential radiochemical impurities are listed with their limits in the individual monographs.

**Chemical purity**: in monographs on radiopharmaceutical preparations, chemical purity is controlled by specifying limits for chemical impurities.

**Isotopic carrier**: a stable isotope of the element concerned either present in or added to the radioactive preparation in the same chemical form as that in which the radionuclide is present.

**Carrier-free preparation**: a preparation free from stable isotopes of the same element as the radionuclide concerned present in the preparation in the stated chemical form or at the position of the radionuclide in the molecule concerned.

**No-carrier-added preparation**: a preparation to which no stable isotopes of the same element as the radionuclide concerned are intentionally added in the stated chemical form or at the position of the radionuclide in the molecule concerned.

**Specific radioactivity**: the radioactivity of a radionuclide per unit mass of the element or of the chemical form concerned, e.g. becquerel per gram or becquerel per mole.

**Radioactivity concentration**: the radioactivity of a radionuclide per unit volume or unit mass of the preparation. For radiopharmaceutical solutions, it is expressed as radioactivity per unit volume of the preparation.

**Total radioactivity**: the radioactivity of the radionuclide, expressed per unit (vial, capsule, ampoule, generator, etc).

**Period of validity**: the time during which specifications described in the monograph must be fulfilled.

## PRODUCTION

A radiopharmaceutical preparation contains its radionuclide:

- as an element in atomic or molecular form, e.g. $^{133}Xe$, $[^{15}O]O_2$;
- as an ion, e.g. $[^{131}I]$iodide, $[^{99m}Tc]$pertechnetate;
- included in, adsorbed on or attached to molecules by chelation, e.g. $[^{111}In]$indium oxine, or by covalent bonding, e.g. 2-$[^{18}F]$fluoro-2-deoxy-D-glucose.

Radionuclides can be produced in the following ways:

- in reactions of neutrons (target irradiation in nuclear reactors);
- in reactions of charged particles (target irradiation using accelerators, in particular cyclotrons);
- by its separation from radionuclide generators.

The probability of nuclear reaction occurrence depends on the nature and energy of the incident particles (protons, neutrons, deuterons etc.) and on the nature of the nucleus that is irradiated by them. The rate of production (yield) of a given radionuclide resulting from the irradiation depends in addition on the isotopic composition of the target material and its chemical purity, and in the case of neutrons on their flux, and in the case of charged particles on beam current.

In addition to the desired nuclear reaction, simultaneous transformations usually occur. Probability of their occurrence is given by the same factors as mentioned in the previous paragraph. Such simultaneous transformations may give rise to radionuclidic impurities.

The nuclear reaction (transformation) can be written in the form: target nucleus (incident particle, emitted particle) produced nucleus.

Examples: $^{58}Fe(n,\gamma)^{59}Fe$

$^{18}O(p,n)^{18}F$

*NEUTRON IRRADIATION*

Irradiation of stable radionuclides in nuclear reactors usually results in proton-deficient nuclei, i.e. electron emitters that are formed in (n,γ) reactions (so-called radiative capture). The product is isotopic with the target nucleus and it may thus contain a considerable amount of carrier.

A number of nuclides with high atomic number are fissionable by neutrons. Nuclear fission, denoted as (n, f) reaction, results in a large number of radionuclides of various masses and half-lives. The most frequently used fission is that of $^{235}U$. Iodine-131, molybdenum-99 and xenon-133 can be produced by irradiation of $^{235}U$ in nuclear reactors and by their separation from more than 200 radionuclides formed in that process.

*CHARGED PARTICLE IRRADIATION*

Irradiation of stable radionuclides with charged particles usually results in neutron-deficient nuclei that decay either by electron capture or by positron emission. They are formed in particular in (p, xn) reactions (where x is the number of emitted neutrons). The product is not isotopic with the target nucleus and its specific radioactivity might be close to that of a carrier-free preparation.

*RADIONUCLIDE GENERATORS*

Radionuclide generator systems use a parent radionuclide which decays to a daughter radionuclide with a shorter half-life.

By separating the daughter radionuclide from the parent radionuclide by a chemical or physical process, it is possible to use the daughter radionuclide at a considerable distance from the production site of the generator despite its short half-life.

*TARGET MATERIALS*

The isotopic composition and purity of the target material together with other factors such as the nature and energy of incident particles will determine the relative percentages of the principal radionuclide and radionuclidic impurities produced by irradiation. The use of isotopically enriched target material in which the abundance of the required target nuclide has been artificially increased, can improve the production yield and the purity of the desired radionuclide.

The chemical form, the purity and the physical state of the target material and the chemical additives, as well as the irradiation conditions and the direct physical and chemical environment, determine the chemical state and chemical purity of the radionuclides that are produced. In the production of radionuclides, and particularly of radionuclides with a short half-life, it may not be possible to determine any of these quality criteria before further processing and manufacture of radiopharmaceutical preparations. Therefore

the quality of each batch of target material is assessed before its use in routine radionuclide production and manufacture of radiopharmaceutical preparations.

The target material is contained in a holder in gaseous, liquid or solid state, in order to be irradiated by a beam of particles. For neutron irradiation, the target material is commonly contained in quartz ampoules or high-purity aluminium or titanium containers. It is necessary to ascertain that no interaction can occur between the container and its contents under the irradiation conditions.

For charged particle irradiation, the holder for target material is constructed of an appropriate metal, possibly with inlet and outlet ports, a surrounding cooling system and usually a thin metal foil target window.

To evaluate all effects on the efficiency of the production of the radionuclide in terms of quality and quantity, the production procedure must clearly describe and take into consideration: the target material, the construction of the holder for target material, method of irradiation and separation of the desired radionuclide.

CHARACTERS

The general chapter *5.7 Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia* summarises the most commonly accepted physical characteristics of radionuclides used in preparations that are the subject of monographs in the European Pharmacopoeia. In addition, the Table states the physical characteristics of the main potential radionuclidic impurities of the radionuclides mentioned in the monographs.

The term 'transition probability' means the probability of the transformation of a nucleus in a given energy state, via the transition concerned. Instead of 'probability' the term 'abundance' is also used.

The term 'emission probability' means the probability that an atom of a radionuclide gives rise to the emission of the particles or radiation concerned.

Irrespective of which meaning is intended, probability is usually stated as a percentage.

IDENTIFICATION

A radionuclide is generally identified by its half-life or by the nature and energy of its radiation or radiations or by both, as prescribed in the monograph.

**Approximate half-life**: the half-life as determined over a relatively short time period to allow release for use of radiopharmaceutical preparations.

The calculated approximate half-life is within the range of the values stated in the individual monograph.

**Determination of the nature and energy of the radiation**. The nature and energy of the radiation emitted are determined using spectrometry. The nature and energy of the radiation of positron emitters is usually not determined; their identification is performed by determination of their half-life and gamma-ray spectrum.

TESTS

*It is sometimes difficult to carry out some of the following tests before releasing the batch for use when the half-life of the radionuclide in the preparation is short. The individual monograph indicates the tests that need not be completed before release for use. These tests then constitute a control of the quality of production.*

**Non-radioactive substances and related substances**. This section prescribes the determination of non-radioactive substances and related substances that can be present.

**Residual solvents**. Residual solvents are limited according to general chapter *5.4. Residual solvents*, using the methods given in general chapter *2.4.24. Identification and control of residual solvents* or another suitable method.

*RADIONUCLIDIC PURITY*

Radionuclidic impurities may arise during the production and decay of a radionuclide. Potential radionuclidic impurities may be mentioned in the monographs and their characteristics are described in general chapter *5.7. Table of physical characteristics of radionuclides mentioned in the European Pharmacopoeia*.

In most cases, to establish the radionuclidic purity of a radiopharmaceutical preparation, the identity of every radionuclide present and its radioactivity must be known. Generally, the most useful method for examination of the radionuclidic purity of gamma- and X-ray emitting radionuclides is gamma-ray spectrometry. The use of sodium iodide detectors may cause a problem: the peaks due to gamma-ray emitting impurities may be concealed in the spectrum of the principal radionuclide or left unresolved from peaks of other radionuclidic impurities in the preparation. Alpha- and beta-particle emitting impurities that do not emit gamma- or X-rays cannot be detected in this way. For alpha- and beta-emitters other methods must be employed.

The individual monographs prescribe the radionuclidic purity required and may set limits for specific radionuclidic impurities (for example, molybdenum-99 in technetium-$99^{m}$). While these requirements are necessary, they are not in themselves sufficient to ensure that the radionuclidic purity of a preparation is sufficient for its clinical use. The manufacturer must examine the product in detail and especially must examine preparations of radionuclides with a short half-life for impurities with a long half-life after a suitable period of decay. In this way, information on the suitability of the manufacturing processes and the adequacy of the testing procedures is obtained. In cases where 2 or more positron-emitting radionuclides need to be identified and/or differentiated, for example the presence of $^{18}$F-impurities in $^{13}$N-preparations, half-life determinations need to be carried out in addition to gamma-ray spectrometry.

Due to differences in the half-lives of the different radionuclides present in a radiopharmaceutical preparation, the radionuclidic purity changes with time.

*RADIOCHEMICAL PURITY*

Radiochemical impurities may originate from:

- radionuclide production;
- subsequent chemical procedures;
- incomplete preparative separation;
- chemical changes during storage.

The determination of radiochemical purity requires separation of the different chemical substances containing the radionuclide and determination of the percentage of radioactivity of the radionuclide concerned associated with the stated chemical form. The radiochemical purity section of an individual monograph may include limits for specified radiochemical impurities, including isomers.

In principle, any method of analytical separation may be used in the determination of radiochemical purity. For example, the monographs for radiopharmaceutical preparations may include paper chromatography (*2.2.26*), thin-layer chromatography (*2.2.27*), electrophoresis (*2.2.31*), size-exclusion chromatography (*2.2.30*), gas chromatography (*2.2.28*) and liquid chromatography (*2.2.29*). The technical description of these analytical methods is set out in the monographs. Moreover, certain precautions special to radiopharmaceuticals must also be considered, such as radiation protection, measurement geometry, detector linearity, use of carriers, dilution of the preparation.

**Specific radioactivity**

Specific radioactivity is usually calculated taking into account the radioactivity concentration and the concentration of the chemical substance being studied, after verification that the radioactivity is attributable only to the radionuclide

(radionuclidic purity) and the chemical species (radiochemical purity) concerned.

Specific radioactivity changes with time. The statement of the specific radioactivity therefore includes reference to a date and, if necessary, time.

**Physiological distribution**

Tests involving animals should be avoided wherever possible. Where the tests for identity and for radiochemical purity are not adequate to completely define and control the radiochemical species in a radiopharmaceutical preparation, a physiological distribution test may be required. The distribution pattern of radioactivity observed in specified organs, tissues or other body compartments of an appropriate animal species can be a reliable indication of the suitability for the intended purpose.

Alternatively, a physiological distribution test can serve to establish the biological equivalence of the preparation under test with similar preparations known to be clinically effective.

The individual monograph prescribes the details concerning the conduct of the test and the physiological distribution requirements that must be met.

In general, the test is performed as follows.

Each of 3 animals is injected intravenously with the preparation. In some cases, dilution immediately before injection may be necessary.

Immediately after injection each animal is placed in a separate cage for collection of excreta and prevention of contamination of the body surface of the animal. At the specified time after injection, the animals are euthanised by an appropriate method and dissected. Selected organs and tissues are assayed for their radioactivity. The physiological distribution is then calculated and expressed in terms of the percentage of the administered radioactivity that is found in each of the selected organs or tissues, taking into account corrections for radioactive decay. For some radiopharmaceutical preparations it is necessary to determine the ratio of the radioactivity in weighed samples of selected tissues (radioactivity/mass).

A preparation meets the requirements of the test if the distribution of radioactivity in at least 2 of the 3 animals complies with all the specified criteria.

Disregard the results from any animal showing evidence of extravasation of the injection (observed at the time of injection or revealed by subsequent assay of tissue radioactivity). In that case the test may be repeated.

**Sterility**

Radiopharmaceutical preparations for parenteral administration comply with the test for sterility. They must be prepared using precautions designed to exclude microbial contamination and to ensure sterility. The test for sterility is carried out as described in the general method (*2.6.1*). Special difficulties arise with radiopharmaceutical preparations because of the short half-life of some radionuclides, the small size of batches and the radiation hazards. In the case that the monograph states that the preparation can be released for use before completion of the test for sterility, the sterility test must be started as soon as practically possible in relation to the radiation. If not started immediately, samples are stored under conditions that are shown to be appropriate in order to prevent false negative results. Parametric release (*5.1.1*) of the product manufactured by a fully validated process is the method of choice in such cases. When aseptic manufacturing is used, the test for sterility has to be performed as a control of the quality of production.

When the size of a batch of the radiopharmaceutical preparation is limited to 1 or a few samples, sampling the batch for sterility testing according to the recommendations of the general method (*2.6.1*) may not be applicable.

When the half-life of the radionuclide is less than 5 min, the administration of the radiopharmaceutical preparation to the patient is generally on-line with a validated production system. For safety reasons (high level of radioactivity) it is not possible to use the quantity of radiopharmaceutical preparations as required in the test for sterility (*2.6.1*). The method of membrane filtration is preferred to limit irradiation of personnel.

Notwithstanding the requirements concerning the use of antimicrobial preservatives in the monograph *Parenteral preparations (0520)*, their addition to radiopharmaceutical preparations in multidose containers is not obligatory, unless prescribed in the monograph.

**Bacterial endotoxins - pyrogens**

Radiopharmaceuticals for parenteral administration comply with the test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*).

Eluates of radionuclide generators, solutions for radiolabelling and kits for radiopharmaceutical preparations also comply with the test for bacterial endotoxins if they are intended for the preparation of radiopharmaceuticals for parenteral administration without further purification.

Radionuclide precursors comply with the test for bacterial endotoxins if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

The test for bacterial endotoxins is carried out as described in the general method (*2.6.14*), taking the necessary precautions to limit irradiation of the personnel carrying out the test. The limit for bacterial endotoxins is indicated in the individual monograph or calculated according to general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*.

When the nature of the radiopharmaceutical preparation results in interference in the test for bacterial endotoxins by inhibition or activation and it is not possible to eliminate the interfering factor(s), the test for pyrogens (*2.6.8*) may be specifically prescribed.

STORAGE

Store preparations containing radioactive substances in an airtight container that is sufficiently shielded to protect personnel from irradiation by primary or secondary emissions and that complies with national and international regulations concerning the storage of radioactive substances. During storage, containers may darken due to irradiation. Such darkening does not necessarily involve deterioration of the preparations.

LABELLING

The labelling of radiopharmaceutical preparations complies with the relevant national and European legislation.

For preparations prepared at the site of use, the labelling can be modified.

The radioactivity of a preparation is stated at a given date. If the half-life is less than 70 days the time is also indicated, with reference to a time zone. The radioactivity at other times may be calculated from the decay equation or from tables.

In addition to the above, the label on the container, the package, a leaflet accompanying the package or a certificate of analysis accompanying the radiopharmaceutical preparation states:

- the route of administration;
- if applicable, the maximum recommended dose in millilitres;
- the name and concentration of any added antimicrobial preservative;
- where applicable, any special storage conditions.

DETECTION AND MEASUREMENT OF RADIOACTIVITY

Detection and measurement of radioactivity are carried out according to general chapter *2.2.66. Detection and measurement of radioactivity*.

04/2019:0784

# RECOMBINANT DNA TECHNOLOGY, PRODUCTS OF

## Producta ab arte ADN recombinandorum

*This monograph provides general requirements for the manufacture and control of finished products derived from recombinant DNA (rDNA) technology, and includes requirements for the active substance in these products. Active substances produced by rDNA technology are expected to meet the requirements given in the corresponding section of the present monograph. These requirements are not necessarily comprehensive in a given case and complementary or additional requirements may be prescribed in an individual monograph or imposed by the competent authority.*

*The monograph is also applicable to vaccine antigens produced by rDNA technology, with more detailed requirements provided in the general monograph Vaccines for human use (0153) and in individual vaccine monographs.*

*Certain aspects of the monograph may apply to products produced in transgenic animals and plants.*

*The monograph is not applicable to recombinant organisms that are intended to be used directly in man and animals, for example as live recombinant vectors or vaccines.*



DEFINITION

Products of rDNA technology are produced by genetic modification in which DNA coding for the required product is introduced, usually by means of a plasmid or a viral vector, into suitable micro-organisms such as bacteria and yeast, or a suitable cell line of mammalian (including human), insect or plant origin. The DNA introduced can then be expressed as a protein.

The desired product is then recovered by extraction and purification. The cell or micro-organism used to harbour the vector is referred to as the host cell, and the stable association of the two used in the manufacturing process is referred to as the host-vector system.

Products of rDNA technology can also undergo intentional modifications such as pegylation or conjugation.

PRODUCTION

*GENERAL PROVISIONS*

Production of rDNA products is based on a validated seed-lot system using a host-vector combination that has been shown suitable.

The seed-lot system typically uses a master cell bank and a working cell bank. The host cell, the vector, the host-vector system, the master cell bank and the working cell bank, as well as their establishment, maintenance and cultivation are to be described in detail.

Where products of rDNA technology are manufactured using materials of human or animal origin, the requirements of general chapter *5.1.7. Viral safety* apply.

The production process is validated for the following aspects:

- process consistency, including cell-culture/fermentation, purification and any subsequent intentional modification of the active substance where applicable;
- removal or inactivation of extraneous agents;
- removal of product- and process-related impurities (e.g. unwanted variants, host-cell proteins and DNA, antibiotics, cell-culture components);
- removal of pyrogenic substances where applicable.

A batch shown to be stable and representative of batches tested clinically is used as a reference preparation for identification, tests and assay. The reference preparation is appropriately characterised.

*EXPRESSION VECTOR AND HOST CELL*

The starting materials to be used for the production of the rDNA product, including the expression vector, the host-cell and the pool of transformed or transfected cells from which the initial clone will be derived, are documented.

Information on the source and history of the host cell and of the expression vector is recorded. The gene coding for the protein of interest as well as other functional sequences of the vector should be characterised (e.g. nucleotide sequence of the gene of interest and its flanking regions, restriction map).

The suitability of the host-vector system, particularly with regard to microbiological purity, is demonstrated by:

- phenotypic and genotypic characterisation of the host cell;
- characterisation of the expression vector within the host cell (e.g. integration, copy number, nucleotide sequence);
- documentation on the raw materials, the transformation/transfection of the host cell by the expression vector and the strategy for deriving the clone used to establish the cell bank system.

*CELL BANKS*

The master cell bank (MCB) is a homogeneous suspension of the original cells already transformed or transfected by the expression vector containing the desired gene, distributed as equal volumes in a single operation into individual containers for storage (e.g. in liquid nitrogen). In some cases, it may be necessary to establish separate master cell banks for the expression vector and the host cells.

The working cell bank (WCB) is a homogeneous suspension of cells derived from the MCB(s) at a finite passage level, distributed as equal volumes in a single operation into individual containers for storage (e.g. in liquid nitrogen).

For each type of cell bank, all containers are treated identically during storage and once removed from storage, are not returned to the cell stock.

The characterisation and testing of eukaryotic and prokaryotic cell banks are critical components of the control of rDNA-derived biological products. Cell banks are tested to confirm identity, purity and suitability of the cell substrate for the intended manufacturing use. The strategy for testing the cell banks may vary according to the nature and biological properties of the cells (e.g. growth properties) and their cultivation history (e.g. use of animal-derived raw material). Molecular methods are used to analyse the expression vector for copy number, insertions or deletions, and the number of integration sites. The nucleotide sequences of the coding region, flanking regions and promoters are shown to be identical to those determined for the expression vector. The coding region corresponds to that expected for the protein sequence. If any differences in nucleic acid sequences are identified, these must be clearly defined and the expression vector shown to be stable and capable of expressing the expected product consistently.

Cell banks must be characterised and tested at different stages including the MCB, the WCB and cells at or beyond the maximum population doubling level used for production.

For prokaryotic and yeast cell banks, characterisation and testing includes molecular identity of the gene being expressed, identity and purity of the cells including strain identification (e.g. by biochemical, genetic, or proteomic methods), phenotypic and genotypic strain characterisation, viability, presence of the plasmid (e.g. sequence, copy number, restriction map, percentage of cells retaining the plasmid), microbiological purity and, if appropriate, a test for bacteriophage. Additional tests may be relevant and shall be addressed on a case-by-case basis.

For animal cell banks, characterisation and testing includes morphology, identity, viability, genetic stability of the cells (e.g. copy number, integrity of the expression cassette) and testing for extraneous agents. Animal cell substrates have the capacity to propagate extraneous agents such as mycoplasma and viruses. In addition, animal cells may contain endogenous agents such as endogenous retroviruses. Consequently, a testing strategy with regard to extraneous agents should be developed based on a risk assessment taking into account the nature and the history of the cell line.

*CULTURE MEDIA AND OTHER RAW MATERIALS*

The quality of media and other raw materials used in the production of rDNA proteins is controlled, with consideration given to the impact of the raw material on the quality, safety and efficacy of the final medicinal product. In particular, the origin of the materials must be known and their traceability documented.

*CULTURE AND HARVEST*

The cell bank (e.g. 1 or more vials(s) of the WCB) is used to start the culture process. In-process controls (e.g. for population doubling levels, cell concentration, volumes, pH, cultivation times, temperature, microbial tests) are established to ensure suitable performance and consistency with regard to the culture process.

Criteria for harvesting and terminating the production must be defined by the manufacturer, and take into account the limit for *in vitro* cell age (e.g. limited number of passages or population doublings) for which the cell substrate has been demonstrated as stable and capable of producing the desired product/intermediate. Each harvest is tested to ensure absence of contamination.

When prokaryotic or other microbial cells are used to manufacture the product, absence of contamination of the harvest is demonstrated primarily by establishing microbial purity. When animal cells are used, the absence of extraneous agents is verified using appropriate *in vitro* cell culture methods and/or molecular methods.

*PURIFICATION*

The extraction and purification procedure must be shown to yield the desired intermediates at a suitable purity. In this respect, an analysis of the step(s) taken to control the product-related impurities, and to remove or inactivate process-related impurities or contaminants is conducted. In-process controls (e.g. for yield, volume, pH, processing time, temperature, elution profile and fraction selection, microbial tests) are established to ensure suitable performance and consistency with regard to the purification process.

Holding times and storage conditions of intermediates are to be defined where appropriate.

*CHARACTERISATION*

During development studies, the active substance is characterised extensively in order to determine its structure, physico-chemical properties, biological activity, immunochemical properties and purity.

Characterisation is necessary to identify the quality attributes that may be important for the safety and efficacy of the product. It provides the basis for release specifications, stability evaluation and for any testing that may have to be performed in support of process changes.

**Structure**. The characterisation should determine, as far as possible, the primary and higher-order structure of the protein, any post-translational modifications such as glycosylation, and any other intentional modification.

The amino-acid sequence is deduced from the DNA sequence of the expression vector and confirmed by analysis of the produced protein. The amino-acid sequence and the disulfide bridges of the substance are determined using a combination of techniques such as peptide mapping (*2.2.55*) and mass spectrometry (*2.2.43*).

The analysis of pegylated proteins should include, but not be limited to, the location of modification and the degree of site occupancy.

Glycosylation is characterised, and covers overall monosaccharide composition (neutral sugars, amino sugars and sialic acids), glycosylation site(s) and type (e.g. N- or O-linked), degree of site occupancy, and oligosaccharide structures of glycan chains (extensions, branching and linkage) using, for example, the principles and techniques described in general chapter *2.2.59. Glycan analysis of glycoproteins*.

Due to their possible immunogenicity, particular attention is paid to glycan structures that are not known to be present in natural human proteins.

The higher-order structure of the substance is examined using physico-chemical methods such as circular dichroism spectroscopy, Fourier transform infrared spectroscopy, fluorescence, differential scanning calorimetry, proton nuclear magnetic resonance spectrometry and/or hydrogen-deuterium exchange mass spectrometry.

Biological assays based on functional activity may also serve as additional confirmation of the higher-order structure.

**Biological activity**. The biological activity (i.e. the specific ability of a product to achieve a defined biological effect) is assessed by biological, biochemical (including immunochemical assays) or physico-chemical assays, as appropriate.

The mechanism of action is investigated and preferably reflected in the potency assay.

**Immunochemical properties**. Where relevant for the mechanism of action, e.g. in the case of antibodies, immunochemical properties (such as Fc-effector functions) are characterised extensively.

**Product-related substances and impurities**. Products of rDNA technology commonly display several sources of heterogeneity (e.g. N- or C-terminal processing, N-terminal pyroglutamation, deamidation, oxidation, isomerisation, fragmentation, disulfide bond mismatch, N-linked and O-linked glycosylation, glycation, aggregation), which leads to a complex product profile consisting of several molecular entities or variants. When the activity, safety and efficacy of these variants are comparable to those of the desired product, the variants are regarded as product-related substances. Other variants are considered as product-related impurities.

The methods used to assess product-related substances and impurities should be capable of detecting structural variants with different physico-chemical properties, e.g. charge, size and hydrophobicity. Typically, a combination of orthogonal methods is applied, e.g. chromatographic, electrophoretic and spectroscopic techniques.

Characterisation of charged variants, such as differentially sialylated or deamidated variants, is performed using appropriate methods (e.g. capillary electrophoresis, isoelectric focusing, ion-exchange chromatography), which may be coupled to other techniques such as mass spectrometry.

High-molecular-mass forms such as dimers and higher oligomers can be resolved and quantified by size-based separation procedures (e.g. size-exclusion chromatography, field flow fractionation, analytical ultracentrifugation) coupled with suitable detection methods (e.g. ultraviolet, fluorescence, light scattering).

**Process-related impurities and contaminants**.

Process-related impurities derived from the upstream process may include host-cell proteins, host-cell DNA, or other media components (e.g. inducers, antibiotics, serum). They are to be evaluated qualitatively and quantitatively.

Host-cell proteins are investigated using a sensitive assay capable of detecting a wide range of protein impurities, and taking into account the recommendations given in general chapter *2.6.34. Host-cell protein assays*.

Residual host-cell DNA is determined using a suitably sensitive assay.

Process-related impurities derived from the downstream process may include enzymes, processing reagents (e.g. guanidine, dyes, oxidising and reducing agents), salts (e.g. heavy metals, non-metallic ions), solvents, carriers, ligands (e.g. protein A) and other leachables.

Contaminants include all adventitiously introduced materials not intended to be part of the manufacturing process (e.g. micro-organisms, microbial proteases, bacterial endotoxins).

Process-related impurities and contaminants are controlled using appropriate strategies based on the principles of risk management.

**Content**. Content is determined by use of an appropriate physico-chemical or immunochemical assay. The total protein content (expressed in mass units) can be determined as described in general chapter *2.5.33. Total protein*. The protein content is determined using several methods, including absolute method(s).

*CONTROL STRATEGY*

Based on the understanding of the product and process, a planned set of controls that ensures process performance and product quality (i.e. a control strategy) is established. This control strategy includes the control of process parameters, in-process controls, control of raw materials, intermediates, active substance and finished product, as well as the methods used and the frequency of control.

The control strategy should ensure that the quality attributes relevant to the safety and efficacy of the product are within the appropriate range, limit or distribution to ensure the desired product quality.

The selection of tests and the stages at which they are performed are based on knowledge of the manufacturing process and extensive characterisation of the active substance and finished product. Some characterisation tests may be selected to become part of the specifications. The specifications for the active substance and finished product are only 1 part of the overall control strategy.

*ACTIVE SUBSTANCE*

The active substance is tested for appearance, identity, microbial quality and bacterial endotoxins, product-related substances, product- and process-related impurities, structural integrity, protein content and biological activity, with comparison to suitable reference standards where appropriate. When the active substance is a conjugated or chemically modified protein, e.g. a pegylated protein, appropriate tests must be performed on both the modified and unmodified protein. Tests for product-related variants (e.g. proportion of modified and unmodified protein) and process-related impurities derived from the modification procedure (e.g. by-products of the modification reaction, reagents) are carried out and acceptance criteria should be established.

*FINISHED PRODUCT*

One or more batches of active substance may be combined to obtain the finished product. Suitable stabilisers and excipients may be added.

The finished product complies with the relevant dosage form monographs, with the relevant individual monograph as appropriate, and with the following considerations.

IDENTIFICATION

The identity test(s) must be specific and must be based on unique aspects of the product's molecular structure or other specific properties such as the size of the molecule, its primary sequence, its isoelectric profile, its chromatographic properties and its functional conformation, with comparison of the product to a suitable reference standard where appropriate. Methods used in the determination of potency or purity may also contribute to the identification.

TESTS

Methods capable of determining process-related impurities and product-related substances and impurities (e.g. resulting from product truncation, fragmentation, aggregation, oxidation, deamidation) are applied and acceptance criteria established, as appropriate. Where impurities are controlled at a suitable earlier stage, the tests may be omitted at the finished product stage.

**Tests applied to modified proteins**. Suitable tests are carried out depending on the type of modification and the product complies with the approved limits.

ASSAY

**Content**. It complies with the limits approved for the particular product. It is usually based on protein content and expressed in mass units. The procedures described in general chapter *2.5.33. Total protein* may be applied. When appropriate, other methods using a suitable reference standard such as liquid chromatography (*2.2.29*) may also be used. For modified proteins, the content refers to the protein part of the molecule.

**Potency**. A potency assay is established using a suitable reference standard and carried out against this reference standard. General chapter *5.3. Statistical analysis of results of biological assays and tests* may be used to design the assay and calculate the results.

**01/2018:2034**

# SUBSTANCES FOR PHARMACEUTICAL USE

## Corpora ad usum pharmaceuticum

DEFINITION

Substances for pharmaceutical use are any organic or inorganic substances that are used as active substances or excipients for the production of medicinal products for human or veterinary use. They may be obtained from natural sources or produced by extraction from raw materials, fermentation or synthesis.

This general monograph does not apply to herbal drugs, herbal drugs for homoeopathic preparations, herbal drug preparations, herbal drug extracts, or mother tinctures for homoeopathic preparations, which are the subject of separate general monographs (*Herbal drugs (1433)*, *Herbal drugs for homoeopathic preparations (2045)*, *Herbal drug preparations (1434)*, *Herbal drug extracts (0765)*, *Mother tinctures for homoeopathic preparations (2029)*). It does not apply to raw materials for homoeopathic preparations, except where there is an individual monograph for the substance in the non-homoeopathic part of the Pharmacopoeia.

This monograph does not apply to chemical precursors for radiopharmaceutical preparations which are the subject of a separate monograph (*Chemical precursors for radiopharmaceutical preparations (2902)*).

Where a substance for pharmaceutical use not described in an individual monograph of the Pharmacopoeia is used in a medicinal product prepared for the special needs of individual patients, the need for compliance with the present general monograph is decided in the light of a risk assessment that takes account of the available quality of the substance and its intended use.

Where medicinal products are manufactured using substances for pharmaceutical use of human or animal origin, the requirements of chapter *5.1.7. Viral safety* apply.

Substances for pharmaceutical use may be used as such or as starting materials for subsequent formulation to prepare medicinal products. Depending on the formulation, certain substances may be used either as active substances or as excipients. Solid substances may be compacted, coated, granulated, powdered to a certain fineness, or processed in other ways. A monograph is applicable to a substance processed with an excipient only where such processing is mentioned in the definition section of the monograph.

*Substance for pharmaceutical use of special grade*. Unless otherwise indicated or restricted in the individual monographs, a substance for pharmaceutical use is intended for human and veterinary use, and is of appropriate quality for the manufacture of all dosage forms in which it can be used.

*Polymorphism*. Individual monographs do not usually specify crystalline or amorphous forms, unless bioavailability is affected. All forms of a substance for pharmaceutical use comply with the requirements of the monograph, unless otherwise indicated.

## PRODUCTION

Substances for pharmaceutical use are manufactured by procedures that are designed to ensure a consistent quality and comply with the requirements of the individual monograph or approved specification.

The manufacture of active substances must take place under conditions of good manufacturing practice.

The provisions of general chapter *5.10* apply to the control of impurities in substances for pharmaceutical use.

Whether or not it is specifically stated in the individual monograph that the substance for pharmaceutical use:

- is a recombinant protein or another substance obtained as a direct gene product based on genetic modification, where applicable, the substance also complies with the requirements of the general monograph *Products of recombinant DNA technology (0784)*;
- is obtained from animals susceptible to transmissible spongiform encephalopathies other than by experimental challenge, where applicable, the substance also complies with the requirements of the general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*;
- is a substance derived from a fermentation process, whether or not the micro-organisms involved are modified by traditional procedures or recombinant DNA (rDNA) technology, where applicable, the substance also complies with the requirements of the general monograph *Products of fermentation (1468)*.

If solvents are used during production, they are of suitable quality. In addition, their toxicity and their residual level are taken into consideration (*5.4*). If water is used during production, it is of suitable quality.

The identity of elemental impurities derived from intentionally added catalysts and reagents is known, and strategies for controlling them should be established using the principles of risk management.

If substances are produced or processed to yield a certain form or grade, that specific form or grade of the substance complies with the requirements of the monograph. Certain functionality-related tests may be described to control properties that may influence the suitability of the substance and subsequently the properties of dosage forms prepared from it.

*Powdered substances* may be processed to obtain a certain degree of fineness (*2.9.35*).

*Compacted substances* are processed to increase the particle size or to obtain particles of a specific form and/or to obtain a substance with a higher bulk density.

*Coated active substances* consist of particles of the active substance coated with one or more suitable excipients.

*Granulated active substances* are particles of a specified size and/or form produced from the active substance by granulation directly or with one or more suitable excipients.

If substances are processed with excipients, these excipients comply with the requirements of the relevant monograph or, where no such monograph exists, the approved specification.

Where active substances have been processed with excipients to produce, for example, coated or granulated substances, the processing is carried out under conditions of good manufacturing practice and the processed substances are regarded as intermediates in the manufacture of a medicinal product.

## CHARACTERS

The statements under the heading Characters (e.g. statements about the solubility or a decomposition point) are not to be interpreted in a strict sense and are not requirements. They are given for information.

Where a substance may show polymorphism, this may be stated under Characters in order to draw this to the attention of the user who may have to take this characteristic into consideration during formulation of a preparation.

## IDENTIFICATION

Where under Identification an individual monograph contains subdivisions entitled 'First identification' and 'Second identification', the test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be used in pharmacies provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the Tests section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

## TESTS

**Polymorphism** (*5.9*). If the nature of a crystalline or amorphous form imposes restrictions on its use in preparations, the nature of the specific crystalline or amorphous form is identified, its morphology is adequately controlled and its identity is stated on the label.

**Related substances**. Unless otherwise prescribed or justified and authorised, organic impurities in active substances are to be reported, identified wherever possible, and qualified as indicated in Table 2034.-1 or in Table 2034.-2 for peptides obtained by chemical synthesis.

Table 2034.-1. – *Reporting, identification and qualification of organic impurities in active substances*

| Use | Maximum daily dose | Reporting threshold | Identification threshold | Qualification threshold |
|---|---|---|---|---|
| Human use or human and veterinary use | ≤ 2 g/day | > 0.05 per cent | > 0.10 per cent or a daily intake of > 1.0 mg (whichever is the lower) | > 0.15 per cent or a daily intake of > 1.0 mg (whichever is the lower) |
| Human use or human and veterinary use | > 2 g/day | > 0.03 per cent | > 0.05 per cent | > 0.05 per cent |
| Veterinary use only | Not applicable | > 0.10 per cent | > 0.20 per cent | > 0.50 per cent |

Table 2034.-2. – *Reporting, identification and qualification of organic impurities in peptides obtained by chemical synthesis*

| Reporting threshold | Identification threshold | Qualification threshold |
|---|---|---|
| > 0.1 per cent | > 0.5 per cent | > 1.0 per cent |

Specific thresholds may be applied for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects.

For DNA reactive impurities, the requirements of ICH Guideline M7 *Assessment and Control of DNA Reactive (Mutagenic) Impurities in Pharmaceuticals to Limit Potential Carcinogenic Risk* must be complied with for active substances to be used in medicinal products for human use, in cases defined in the scope of the guideline.

If the individual monograph does not provide suitable control for a new impurity, a suitable test for control must be developed and included in the specification for the substance.

The requirements above do not apply to biological and biotechnological products, oligonucleotides, products of fermentation and semi-synthetic products derived therefrom, to crude products of animal or plant origin or herbal products.

**Elemental impurities**. Permitted daily exposures for elemental impurities (e.g. as included in the ICH Q3D guideline, the principles of which are reproduced in general chapter *5.20. Elemental impurities*) apply to the medicinal product. Individual monographs on substances for pharmaceutical use therefore do not contain specifications for elemental impurities unless otherwise prescribed.

**Residual solvents** are limited according to the principles defined in chapter *5.4*, using general method *2.4.24* or another suitable method. Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for calculation of the assay content of the substance, the specific optical rotation and the specific absorbance.

**Microbiological quality**. Individual monographs give acceptance criteria for microbiological quality wherever such control is necessary. Table 5.1.4.-2. – *Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use* in chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use* gives recommendations on microbiological quality that are of general relevance for substances subject to microbial contamination. Depending on the nature of the substance and its intended use, different acceptance criteria may be justified.

**Sterility** (*2.6.1*). If intended for use in the manufacture of sterile dosage forms without a further appropriate sterilisation procedure, or if offered as sterile grade, the substance for pharmaceutical use complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The substance for pharmaceutical use complies with the test for bacterial endotoxins if it is labelled as a bacterial endotoxin-free grade or if it is intended for use in the manufacture of parenteral preparations or preparations for irrigation without a further appropriate procedure for the removal of bacterial endotoxins. The limit, when not indicated in the individual monograph, is determined in accordance with the recommendations of general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*.

**Pyrogens** (*2.6.8*). If the test for pyrogens is justified rather than the test for bacterial endotoxins and if a pyrogen-free grade is offered, the substance for pharmaceutical use complies with the test for pyrogens. The limit and test method are stated in the individual monograph or approved by the competent authority. Based on appropriate test validation for bacterial endotoxins and pyrogens, the test for bacterial endotoxins may replace the test for pyrogens.

**Additional properties**. Control of additional properties (e.g. physical characteristics, functionality-related characteristics) may be necessary for individual manufacturing processes or formulations. Grades (such as sterile, endotoxin-free, pyrogen-free) may be produced with a view to manufacture of preparations for parenteral administration or other dosage forms and appropriate requirements may be specified in an individual monograph.

ASSAY

Unless justified and authorised, contents of substances for pharmaceutical use are determined. Suitable methods are used.

LABELLING

In general, labelling is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling therefore are not comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term 'label' is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package or a certificate of analysis accompanying the article, as decided by the competent authority.

Where appropriate, the label states that the substance is:

- intended for a specific use;
- of a distinct crystalline form;
- of a specific degree of fineness;
- compacted;
- coated;
- granulated;
- sterile;
- free from bacterial endotoxins;
- free from pyrogens;
- containing gliding agents.

Where applicable, the label states:

- the degree of hydration;
- the name and concentration of any excipient.

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**corrected 10.0**

# VACCINES FOR HUMAN USE

## Vaccina ad usum humanum

DEFINITION

Vaccines for human use are preparations containing antigens capable of inducing a specific and active immunity in man against an infecting agent or the toxin or antigen elaborated by it. Immune responses include the induction of the innate and the adaptive (cellular, humoral) parts of the immune system. Vaccines for human use shall have been shown to have acceptable immunogenic activity and safety in man with the intended vaccination schedule.

Vaccines for human use may contain: whole micro-organisms (bacteria, viruses or parasites), inactivated by chemical or physical means that maintain adequate immunogenic properties; whole live micro-organisms that are naturally avirulent or that have been treated to attenuate their virulence whilst retaining adequate immunogenic properties; antigens extracted from the micro-organisms or secreted by the micro-organisms or produced by genetic engineering or chemical synthesis. The antigens may be used in their native state or may be detoxified or otherwise modified by chemical or physical means and may be aggregated, polymerised or conjugated to a carrier to increase their immunogenicity. Vaccines may contain an adjuvant. Where the antigen is adsorbed on a mineral adjuvant, the vaccine is referred to as 'adsorbed'.

Terminology used in monographs on vaccines for human use is defined in general chapter *5.2.1*.

*Bacterial vaccines containing whole cells* are suspensions of various degrees of opacity in colourless or almost colourless liquids, or may be freeze-dried. They may be adsorbed. The concentration of living or inactivated bacteria is expressed in terms of International Units of opacity or, where appropriate, is determined by direct cell count or, for live bacteria, by viable count.

*Bacterial vaccines containing bacterial components* are suspensions or freeze-dried products. They may be adsorbed. The antigen content is determined by a suitable validated assay.

*Bacterial toxoids* are prepared from toxins by diminishing their toxicity to an acceptable level or by completely eliminating it by physical or chemical procedures whilst retaining adequate immunogenic properties. The toxins are obtained from selected strains of micro-organisms. The method of production is such that the toxoid does not revert to toxin. The toxoids are purified. Purification is performed before and/or after detoxification. Toxoid vaccines may be adsorbed.

*Viral vaccines* are prepared from viruses grown in animals, in fertilised eggs, in suitable cell cultures or in suitable tissues, or by culture of genetically engineered cells. They are liquids that vary in opacity according to the type of preparation or may be freeze-dried. They may be adsorbed. Liquid preparations and freeze-dried preparations after reconstitution may be coloured if a pH indicator such as phenol red has been used in the culture medium.

*Synthetic antigen vaccines* are generally clear or colourless liquids. The concentration of the components is usually expressed in terms of specific antigen content.

*Combined vaccines* are multicomponent preparations formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or against different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use. Where there is no monograph to cover a particular combination, the vaccine complies with the monograph for each individual component, with any necessary modifications approved by the competent authority.

*Adsorbed vaccines* are suspensions and may form a sediment at the bottom of the container.

PRODUCTION

**General provisions**. The production method for a given product must have been shown to yield consistently batches comparable with the batch of proven clinical efficacy, immunogenicity and safety in man. Product specifications including in-process testing should be set. Specific requirements for production including in-process testing are included in individual monographs. Where justified and authorised, certain tests may be omitted where it can be demonstrated, for example by validation studies, that the production process consistently ensures compliance with the test.

Unless otherwise justified and authorised, vaccines are produced using a seed-lot system. The methods of preparation are designed to maintain adequate immunogenic properties, to render the preparation harmless and to prevent contamination with extraneous agents.

Where vaccines for human use are manufactured using materials of human or animal origin, the general requirements of general chapter *5.1.7. Viral safety* apply in conjunction with the more specific requirements relating to viral safety in this monograph, in general chapters *5.2.2. Chicken flocks free from specified pathogens for the production and quality control of vaccines*, *5.2.3. Cell substrates for the production of vaccines for human use* and *2.6.16. Tests for extraneous agents in viral vaccines for human use*, and in individual monographs.

Unless otherwise justified and authorised, in the production of a final lot of vaccine, the number of passages of a virus, or the number of subcultures of a bacterium, from the master seed lot shall not exceed that used for production of the vaccine shown to be satisfactory in clinical trials with respect to safety and efficacy or immunogenicity.

Vaccines are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man. Suitable additives, including stabilisers and adjuvants may be incorporated. Penicillin and streptomycin are neither used at any stage of production nor added to the final product; however, master seed lots prepared with media containing penicillin or streptomycin may, where justified and authorised, be used for production.

Consistency of production is an important feature of vaccine production. Monographs on vaccines for human use give limits for various tests carried out during production and on the final lot. These limits may be in the form of maximum values, minimum values, or minimum and maximum tolerances around a given value. While compliance with these limits is required, it is not necessarily sufficient to ensure consistency of production for a given vaccine. For relevant tests, the manufacturer must therefore define for each product a suitable action or release limit or limits to be applied in view of the results found for batches tested clinically and those used to demonstrate consistency of production. These limits may subsequently be refined on a statistical basis in light of production data.

**Substrates for propagation**. Substrates for propagation comply with the relevant requirements of the Pharmacopoeia (*5.2.2*, *5.2.3*) or in the absence of such requirements with those of the competent authority. Processing of cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions shall be shown to be free from extraneous agents.

**Seed lots/cell banks**. The master seed lot or cell bank is identified by historical records that include information on its origin and subsequent manipulation. Suitable measures are taken to ensure that no extraneous agent or undesirable substance is present in a master or working seed lot or a cell bank.

**Culture media**. Culture media are as far as possible free from ingredients known to cause toxic, allergic or other undesirable reactions in man; if inclusion of such ingredients is necessary, it shall be demonstrated that the amount present in the final lot is reduced to such a level as to render the product safe. Approved animal (but not human) serum may be used in the growth medium for cell cultures but the medium used for maintaining cell growth during virus multiplication shall not contain serum, unless otherwise stated. Cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration, although it is preferable to have a medium free from antibiotics during production.

**Propagation and harvest**. The seed cultures are propagated and harvested under defined conditions. The purity of the harvest is verified by suitable tests as defined in the monograph.

**Control cells**. For vaccines produced in cell cultures, control cells are maintained and tested as prescribed. In order to provide a valid control, these cells must be maintained in conditions that are essentially equivalent to those used for the production cell cultures, including use of the same batches of media and media changes.

**Control eggs**. For live vaccines produced in eggs, control eggs are incubated and tested as prescribed in the monograph.

**Purification**. Where applicable, validated purification procedures may be applied.

**Inactivation**. Inactivated vaccines are produced using a validated inactivation process whose effectiveness and consistency have been demonstrated. Where it is recognised that extraneous agents may be present in a harvest, for example in vaccines produced in eggs from healthy, non-SPF flocks, the inactivation process is also validated with respect to a panel of model extraneous agents representative of the potential extraneous agents. A test for effectiveness of the inactivation process is carried out as soon as possible after the inactivation process.

**Carrier proteins**. Bacterial polysaccharide antigens may be conjugated with carrier proteins to improve their immunogenicity to enable the induction of a protective response in infants. Carrier proteins comply with the relevant requirements of general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*.

**Test for sterility of intermediates prior to final bulk**. Individual monographs on vaccines for human use may prescribe a test for sterility for intermediates.

In agreement with the competent authority, replacement of the sterility test by a bioburden test with a low bioburden limit based on batch data and process validation may be acceptable for intermediates preceding the final bulk, provided that a sterilising filtration is performed later in the production process.

It is a prerequisite that the intermediate is filtered through a bacteria-retentive filter prior to storage, that authorised pre-filtration bioburden limits have been established for this filtration, and that adequate measures are in place to avoid contamination and growth of micro-organisms during storage of the intermediate.

**Final bulk**. The final bulk is prepared by aseptically blending the ingredients of the vaccine. For non-liquid vaccines for administration by a non-parenteral route, the final bulk is prepared by blending the ingredients of the vaccine under suitable conditions.

*Adjuvants*. One or more adjuvants may be included in the formulation of a vaccine to potentiate and/or modulate the immune response to the antigen(s). Adjuvants may be included in the formulation of the final vaccine or presented separately. Suitable characterisation and quality control of the adjuvant(s), alone and in combination with the antigen(s), is essential for consistent production. Quality specifications are established for each adjuvant, alone and in combination with the antigen(s).

*Adsorbents as adjuvants*. Vaccines may be adsorbed on aluminium hydroxide, aluminium phosphate, calcium phosphate or other suitable adsorbents. The adsorbents are prepared in special conditions that confer the appropriate physical form and adsorptive properties.

Where an adsorbent is used as an adjuvant and is generated *in situ* during production of the vaccine, quality specifications are established for each of the ingredients and for the generated adsorbent in the vaccine. Quality specifications are intended to control, in particular:

- qualitative and quantitative chemical composition;
- physical form and associated adsorptive properties, where relevant, and particularly where the adjuvant will be present as an adsorbent;
- interaction between adjuvant and antigen;
- purity, including bacterial endotoxin content and microbiological quality;
- any other parameters identified as being critical for functionality.

The stability of each adjuvant, alone and in combination with the antigen(s), particularly for critical parameters, is established during development studies.

*Antimicrobial preservatives*. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during the use of a vaccine. Antimicrobial preservatives are not included in freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not normally acceptable. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after broaching of the container. If an antimicrobial preservative is used, it shall be shown that it does not impair the safety or efficacy of the vaccine. Addition of antibiotics as antimicrobial preservatives is not normally acceptable.

During development studies, the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in general chapter *5.1.3*. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to vaccines for human use: bacteria, no increase at 24 h and 7 days, 3 $\log_{10}$ reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

**Stability of intermediates**. During production of vaccines, intermediates are obtained at various stages and are stored, sometimes for long periods. Such intermediates include:

- seed lots and cell banks;
- live or inactivated harvests;
- purified harvests that may consist of toxins or toxoids, polysaccharides, bacterial or viral suspensions;
- purified antigens;
- adsorbed antigens;

- conjugated polysaccharides;
- final bulk vaccine;
- vaccine in the final closed container stored at a temperature lower than that used for final-product stability studies and intended for release without re-assay.

Except where they are used within a short period of time, stability studies are carried out on the intermediates in the intended storage conditions to establish the expected extent of degradation. For final bulk vaccine, stability studies may be carried out on representative samples in conditions equivalent to those intended to be used for storage. For each intermediate (except for seed lots and cell banks), a period of validity applicable for the intended storage conditions is established, where appropriate in light of stability studies.

**Final lot**. The final lot is prepared by aseptically distributing the final bulk into sterile, tamper-evident containers, which, after freeze-drying where applicable, are closed so as to exclude contamination. For non-liquid vaccines for administration by a non-parenteral route, the final lot is prepared by distributing the final bulk under suitable conditions into sterile, tamper-evident containers. Where justified and authorised, certain tests prescribed for the final lot may be carried out on the final bulk, if it has been demonstrated that subsequent manufacturing operations do not affect compliance.

**Appearance**. Unless otherwise justified and authorised, each container (vial, syringe or ampoule) in each final lot is inspected visually or mechanically for acceptable appearance.

*Degree of adsorption*. For an adsorbed vaccine, unless otherwise justified and authorised, a release specification for the degree of adsorption is established in light of results found for batches used in clinical trials. From the stability data generated for the vaccine it must be shown that at the end of the period of validity the degree of adsorption is not less than for batches used in clinical trials.

*Thermal stability*. When the thermal stability test is prescribed in a monograph for a live attenuated vaccine, the test is carried out on the final lot to monitor the lot-to-lot consistency in heat-sensitivity of viral/bacterial particles in the product. Suitable conditions are indicated in the individual monograph. The test may be omitted as a routine test for a given product once the consistency of the production process has been demonstrated, in agreement with the competent authority, using relevant parameters, such as consistency in yield, ratio of infectious viruses (viable bacteria) before and after freeze-drying, potency at release and real-time stability under the prescribed conditions as well as thermal stability. Where there is a significant change in the manufacturing procedure of the antigen(s) or formulation, the need for re-introduction of the test is considered.

*Stability*. During development studies, maintenance of potency of the final lot throughout the period of validity shall be demonstrated; the loss of potency in the recommended storage conditions is assessed. Excessive loss even within the limits of acceptable potency may indicate that the vaccine is unacceptable.

*Expiry date*. Unless otherwise stated, the expiry date is calculated from the beginning of the assay or from the beginning of the first assay for a combined vaccine. For vaccines stored at a temperature lower than that used for stability studies and intended for release without re-assay, the expiry date is calculated from the date of removal from cold storage. If, for a given vaccine, an assay is not carried out, the expiry date for the final lot is calculated from the date of an approved stability-indicating test or, failing this, from the date of freeze-drying or the date of filling into the final containers. For a combined vaccine where components are presented in separate containers, the expiry date is that of the component which expires first.

The expiry date applies to vaccines stored in the prescribed conditions.

**Animal tests**. In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in light of this. For example, if it is indicated that an animal is considered to be positive, infected, etc. when typical clinical signs or death occur, then as soon as sufficient indication of a positive result is obtained the animal in question shall be either euthanised or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering. Guidance on how to substitute *in vivo* methods by *in vitro* methods, in cases where a direct head-to-head comparison is not possible, can be found in general chapter *5.2.14*.

## TESTS

Vaccines comply with the tests prescribed in individual monographs including, where applicable, the following:

**pH** (*2.2.3*). Liquid vaccines, after reconstitution where applicable, comply with the limits for pH approved for the particular preparation.

**Adjuvant**. If the vaccine contains an adjuvant, the amount is determined and shown to be within acceptable limits with respect to the expected amount (see also the tests for aluminium and calcium below).

**Aluminium** (*2.5.13*): maximum 1.25 mg of aluminium (Al) per single human dose where an aluminium adsorbent has been used in the vaccine, unless otherwise stated.

**Calcium** (*2.5.14*): maximum 1.3 mg of calcium (Ca) per single human dose where a calcium adsorbent has been used in the vaccine, unless otherwise stated.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L of free formaldehyde in the final product where formaldehyde has been used in the preparation of the vaccine, unless otherwise stated.

**Phenol** (*2.5.15*): maximum 2.5 g/L in the final product where phenol has been used in the preparation of the vaccine, unless otherwise stated.

**Water** (*2.5.12*): maximum 3.0 per cent *m/m* for freeze-dried vaccines, unless otherwise stated.

**Extractable volume** (*2.9.17*). Unless otherwise justified and authorised, it complies with the requirement for extractable volume.

**Bacterial endotoxins**. Unless otherwise justified and authorised, a test for bacterial endotoxins is carried out on the final product. Where no limit is specified in the individual monograph, the content of bacterial endotoxins determined by a suitable method (*2.6.14*) is less than the limit approved for the particular product.

## STORAGE

Store protected from light. Unless otherwise stated, the storage temperature is 5 ± 3 °C; liquid adsorbed vaccines must not be allowed to freeze.

## LABELLING

The label states:

- the name of the preparation;
- a reference identifying the final lot;
- the recommended human dose and route of administration;
- the storage conditions;
- the expiry date;
- the name and amount of any antimicrobial preservative;

- the name of any antibiotic, adjuvant, flavour or stabiliser present in the vaccine;
- where applicable, that the vaccine is adsorbed;
- the name of any constituent that may cause adverse reactions and any contra-indications to the use of the vaccine;
- for freeze-dried vaccines:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the vaccine is to be used after reconstitution.

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**corrected 10.0**

# VACCINES FOR VETERINARY USE

## Vaccina ad usum veterinarium

*In the case of combined vaccines, for each component that is the subject of a monograph in the Pharmacopoeia, the provisions of that monograph apply to that component, modified where necessary as indicated (see general chapters 5.2.6. Evaluation of safety of veterinary vaccines and immunosera and 5.2.7. Evaluation of efficacy of veterinary vaccines and immunosera).*

*If an immunological product for veterinary use is intended for minor use, certain tests may be excluded, subject to approval by the competent authority*[(1)].

1. DEFINITION

Vaccines for veterinary use are preparations containing antigenic substances and are administered for the purpose of inducing a specific and active immunity against disease provoked by bacteria, toxins, viruses, fungi or parasites. The vaccines, live or inactivated, confer active immunity that may be transferred passively via maternal antibodies against the immunogens they contain and sometimes also against antigenically related organisms. Vaccines may contain living or inactivated micro-organisms (bacteria, viruses or fungi), parasites, or antigenic fractions or substances produced by these organisms (e.g. toxins), rendered harmless whilst retaining all or part of their antigenic properties; vaccines may also contain combinations of these constituents. The antigens may be produced by recombinant DNA technology. Suitable adjuvants may be included to enhance the immunising properties of the vaccines.

Terminology used in monographs on vaccines for veterinary use is defined in general chapter *5.2.1*.

1-1. *BACTERIAL VACCINES AND BACTERIAL TOXOIDS*

Bacterial vaccines and bacterial toxoids are prepared from cultures grown on suitable solid or liquid media, or by other suitable means; the requirements of this section do not apply to bacterial vaccines prepared in cell cultures or in live animals. The strain of bacterium used may have been modified by genetic engineering. The identity, antigenic potency and purity of each bacterial culture used is carefully controlled.

Bacterial vaccines contain inactivated or live bacteria or their antigenic components; they are liquid preparations of various degrees of opacity or they may be freeze-dried.

Bacterial toxoids are prepared from toxins by diminishing their toxicity to a very low level or by completely eliminating it by physical or chemical means whilst retaining adequate immunising potency. The toxins are obtained from selected strains of specified micro-organisms grown in suitable media or are obtained by other suitable means, for example, chemical synthesis.

The toxoids may be:

- liquid;
- precipitated with alum or another suitable agent;
- purified and/or adsorbed on aluminium phosphate, aluminium hydroxide, calcium phosphate or another adsorbent prescribed in the monograph.

Bacterial toxoids are clear or slightly opalescent liquids. Adsorbed toxoids are suspensions or emulsions. Certain toxoids may be freeze-dried.

Unless otherwise indicated, statements and requirements given below for bacterial vaccines apply equally to bacterial vaccines, bacterial toxoids and products containing a combination of bacterial cells and toxoid.

1-2. *VIRAL VACCINES*

Viral vaccines are prepared by growth in suitable cell cultures (*5.2.4*), in tissues, in micro-organisms, in fertilised eggs or, where no other possibility is available, in live animals, or by other suitable means. The strain of virus used may have been modified by genetic engineering. They are liquid or freeze-dried preparations of one or more viruses or viral subunits or peptides.

Live viral vaccines are prepared from viruses of attenuated virulence or of natural low virulence for the target species.

Inactivated viral vaccines are treated by a validated procedure for inactivation of the virus and may be purified and concentrated.

1-3. *VECTOR VACCINES*

Vector vaccines are liquid or freeze-dried preparations of one or more types of live micro-organisms (bacteria, viruses or fungi) that are non-pathogenic or have low pathogenicity for the target species and in which have been inserted one or more genes encoding antigens that stimulate an immune response protective against other micro-organisms.

2. PRODUCTION

**General provisions**. Production is designed to provide a finished product that complies with the approved requirements. Compliance with these requirements is demonstrated by safety and efficacy studies carried out on batches during development and by the control strategy. The tests to be applied are outlined below and in individual monographs. In accordance with the General Notices, performance of all the tests in a monograph is not necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. Therefore, routinely used *in vivo* tests can ultimately be replaced in accordance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, if the product profile is well defined by a set of parameters, including antigen content and antigen quality, established to verify that the manufacturing process consistently produces final batches equivalent to a final batch that fulfils the criteria of the European Pharmacopoeia.

2-1. *STARTING MATERIAL*

Monographs prescribe a set of measures that, taken together, give an acceptable degree of assurance that the final product does not contain infectious extraneous agents. These measures include:

1. production within a seed-lot system and a cell-seed system, where applicable;
2. extensive testing of seed lots and cell seed for extraneous agents;

(1) NOTE: Guideline on data requirements for immunological veterinary medicinal products intended for minor use or minor species/limited markets (EMA/CVMP/IWP/123243/2006, including any subsequent revision of this document).

3. requirements for SPF flocks used for providing substrates for vaccine production;
4. testing of substances of animal origin, which must, wherever possible, undergo an inactivation procedure.

Substances of animal origin used in the production of vaccines for veterinary use comply with the requirements of general chapter *5.2.5*. Other substances used in the preparation of vaccines for veterinary use comply with requirements of the Pharmacopoeia (where a relevant monograph exists) and are prepared in a manner that avoids contamination of the vaccine.

2-1-1. **Substrates for production**. Cell cultures used in the production of vaccines for veterinary use comply with the requirements of general chapter *5.2.4*.

Where a monograph refers to chicken flocks free from specified pathogens (SPF), these flocks comply with the requirements prescribed in general chapter *5.2.2*.

For production of inactivated vaccines, where vaccine organisms are grown in embryonated hens' eggs, such eggs are derived either from SPF flocks (*5.2.2*) or from healthy non-SPF flocks (*5.2.13*). It may be necessary to demonstrate that the inactivation process is effective against specified potential contaminants. For the production of a master seed lot and for all passages of a micro-organism up to and including the working seed lot, eggs from SPF flocks (*5.2.2*) are used.

Where it is unavoidable to use animals or animal tissues in the production of veterinary vaccines, such animals shall be free from specified pathogens, as appropriate to the source species and the target animal for the vaccine.

2-1-2. **Media used for seed culture preparation and for production**. Media used for seed culture preparation and for production are prepared following a standard formulation. The media composition is described in the manufacturing process. The qualitative and quantitative composition of all media used must be recorded. Where media or ingredients are claimed as proprietary, this is indicated and an appropriate description recorded. Ingredients that are derived from animals are specified as to the source species and country of origin, and must comply with the criteria described in general chapter *5.2.5*. Preparation processes for media used, including sterilisation procedures, are documented.

The addition of antibiotics during the manufacturing process is normally restricted to cell culture fluids and other media, egg inocula and material harvested from tissues and embryonated eggs.

2-1-3. **Seed lots**

2-1-3-1. *Bacterial seed lots*

2-1-3-1-1. General requirements. The genus and species (and varieties where appropriate) of the bacteria used in the vaccine are stated. Bacteria used in manufacture are handled in a seed-lot system wherever possible. Each master seed lot is tested as described below. A record of the origin, date of isolation, passage history (including purification and characterisation procedures) and storage conditions is maintained for each master seed lot. Each master seed lot is assigned a specific code for identification purposes.

2-1-3-1-2. Propagation. The minimum and maximum number of subcultures of each master seed lot prior to the production stage are specified. The methods used for the preparation of seed cultures, preparation of suspensions for seeding, techniques for inoculation of seeds, titre and concentration of inocula and the media used, are documented. It shall be demonstrated that the characteristics of the seed material (for example, dissociation or antigenicity) are not changed by these subcultures. The conditions under which each seed lot is stored are documented.

2-1-3-1-3. Identity and purity. Each master seed lot is shown to contain only the species and strain of bacterium stated. A brief description of the method of identifying each strain by biochemical, serological and morphological characteristics and distinguishing it as far as possible from related strains is recorded, as is also the method of determining the purity of the strain. If the master seed lot is shown to contain living organisms of any kind other than the species and strain stated, then it is unsuitable for vaccine production.

2-1-3-2. *Virus seed lots*

2-1-3-2-1. General requirements. Viruses used in manufacture are handled in a seed-lot system. Each master seed lot is tested as described below. A record of the origin, date of isolation, passage history (including purification and characterisation procedures) and storage conditions is maintained for each seed lot. Each master seed lot is assigned a specific code for identification purposes. Production of vaccine is not normally undertaken using virus more than 5 passages from the master seed lot. In the tests on the master seed lot described below, the organisms used are not normally more than 5 passages from the master seed lot at the start of the tests, unless otherwise indicated.

Where the master seed lot is contained within a permanently infected master cell seed, the following tests are carried out on an appropriate volume of virus from disrupted master cell seed. Where relevant tests have been carried out on disrupted cells to validate the suitability of the master cell seed, these tests need not be repeated.

2-1-3-2-2. Propagation. The master seed lot and all subsequent passages are propagated on cells, on embryonated eggs or in animals that have been shown to be suitable for vaccine production (see above), and, where applicable, using substances of animal origin that meet the requirements prescribed in general chapter *5.2.5*.

2-1-3-2-3. Identification. A suitable method to identify the vaccine strain and to distinguish it as far as possible from related strains must be used.

2-1-3-2-4. Bacteria and fungi. The master seed lot complies with the test for sterility (*2.6.1*).

2-1-3-2-5. Mycoplasmas (*2.6.7*). The master seed lot complies with the test for mycoplasmas (culture method and indicator cell culture method).

2-1-3-2-6. Absence of extraneous viruses. Monographs may contain requirements for freedom from extraneous agents, otherwise the requirements stated below apply.

Preparations of monoclonal or polyclonal antibodies containing high levels of neutralising antibody to the virus of the seed lot are made on a batch basis, using antigen that is not derived from any passage level of the virus isolate giving rise to the master seed virus. Each batch of serum is maintained at 56 °C for 30 min to inactivate complement. Each batch is shown to be free of antibodies to potential contaminants of the seed virus and is shown to be free of any non-specific inhibiting effects on the ability of viruses to infect and propagate within cells (or eggs, where applicable). If such a serum cannot be obtained, other methods are used to remove or neutralise the seed virus specifically.

If the seed lot virus would interfere with the conduct and sensitivity of a test for extraneous viruses, a sample of the master seed lot is treated with a minimum amount of the monoclonal or polyclonal antibody so that the vaccine virus is neutralised as far as possible or removed. The final virus-serum mixture shall, if possible, contain at least the virus content of 10 doses of vaccine per 0.1 mL for avian vaccines and per millilitre for other vaccines. For avian vaccines, the testing to be carried out on seed lots is given in general chapter *2.6.24*. For mammalian vaccines, the seed lot or the mixture of seed lot and antiserum is tested for freedom from extraneous agents as follows.

The mixture is inoculated onto cultures of at least 70 $cm^2$ of the required cell types. The cultures may be inoculated at any suitable stage of growth up to 70 per cent confluency. At least 1 monolayer of each type must be retained as a control. The cultures must be monitored daily for a week. At the end of

this period the cultures are freeze thawed 3 times, centrifuged to remove cell debris and re-inoculated onto the same cell type as above. This is repeated twice. The final passage must produce sufficient cells in appropriate vessels to carry out the tests below.

Cytopathic and haemadsorbing agents are tested for using the methods described in the relevant sections on testing cell cultures (*5.2.4*) and techniques such as immunofluorescence are used for detection of specific contaminants for the tests in cell cultures. The master seed lot is inoculated onto:

- primary cells of the species of origin of the virus;
- cells sensitive to viruses pathogenic for the species for which the vaccine is intended;
- cells sensitive to pestiviruses.

If the master seed lot is shown to contain living organisms of any kind, other than the virus of the species and strain stated, or foreign viral antigens, then it is unsuitable for vaccine production.

2-2. *CHOICE OF VACCINE COMPOSITION AND CHOICE OF VACCINE STRAIN*

For the choice of vaccine composition and choice of vaccine strain, important aspects to be evaluated include safety, efficacy and stability.

2-2-1. **Development studies on safety and efficacy**. General requirements for evaluation of safety and efficacy are given in general chapters *5.2.6* and *5.2.7*. These requirements may be made more explicit or supplemented by the requirements of individual monographs.

2-2-1-1. *Potency and immunogenicity*. The tests given under the headings Potency and Immunogenicity in monographs serve 2 purposes:

- the Potency section establishes, by a well-controlled test in experimental conditions, the minimum acceptable vaccinating capacity for all vaccines within the scope of the definition, which must be guaranteed throughout the period of validity;
- well-controlled experimental studies are normally a part of the overall demonstration of efficacy of a vaccine (see general chapter *5.2.7*); the test referred to in the Immunogenicity section (to which the Potency section usually cross-refers) is suitable as a part of this testing.

2-2-1-2. *Information for performing the safety and efficacy studies.*

During development of a vaccine, safety and immunogenicity are demonstrated for each route and for each method of administration to be recommended. The following is a non-exhaustive list of such routes of administration:

- intramuscular;
- subcutaneous;
- intravenous;
- ocular;
- oral;
- nasal;
- foot-stab;
- wing web;
- intradermal;
- intraperitoneal;
- *in ovo*.

The following is a non-exhaustive list of such methods of administration:

- injection;
- drinking water;
- spray;
- eye-drop;
- scarification;
- implantation;
- immersion.

Monographs may indicate that a given test is to be carried out for each category of animal of the target species for which the product is recommended or is to be recommended. The following is a non-exhaustive list of categories that are to be taken into account.

- *Mammals*:
  - pregnant animals/non-pregnant animals;
  - animals raised primarily for breeding/animals raised primarily for food production;
  - animals of the minimum age or size recommended for vaccination.
- *Avian species*:
  - birds raised primarily for egg production/birds raised primarily for production of meat;
  - birds before point of lay/birds after onset of lay.
- *Fish*:
  - broodstock fish/fish raised primarily for food production.

2-2-2. **Antimicrobial preservatives**. Antimicrobial preservatives are used to prevent spoilage or adverse effects caused by microbial contamination occurring during use of a vaccine which is expected to be no longer than 10 h after first broaching. Antimicrobial preservatives are not included in freeze-dried products but, if justified, taking into account the maximum recommended period of use after reconstitution, they may be included in the diluent for multi-dose freeze-dried products. For single-dose liquid preparations, inclusion of antimicrobial preservatives is not acceptable unless justified and authorised, but may be acceptable, for example where the same vaccine is filled in single-dose and multidose containers and is used in non-food-producing species. For multidose liquid preparations, the need for effective antimicrobial preservation is evaluated taking into account likely contamination during use and the maximum recommended period of use after broaching of the container.

During development studies the effectiveness of the antimicrobial preservative throughout the period of validity shall be demonstrated to the satisfaction of the competent authority.

The efficacy of the antimicrobial preservative is evaluated as described in general chapter *5.1.3* and in addition samples are tested at suitable intervals over the proposed in-use shelf-life. If neither the A criteria nor the B criteria can be met, then in justified cases the following criteria are applied to vaccines for veterinary use: bacteria, no increase from 24 h to 7 days, 3 $\log_{10}$ reduction at 14 days, no increase at 28 days; fungi, no increase at 14 days and 28 days.

Addition of antibiotics as antimicrobial preservative is generally not acceptable.

2-2-3. **Stability**. Evidence of stability is obtained to justify the proposed period of validity. This evidence takes the form of the results of virus titrations, bacterial counts or potency tests carried out at regular intervals until 3 months beyond the end of the shelf life on not fewer than 3 representative consecutive batches of vaccine kept under recommended storage conditions together with results from studies of moisture content (for freeze-dried products), physical tests on the adjuvant, chemical tests on substances such as the adjuvant constituents and preservatives, and pH, as appropriate.

Where applicable, studies on the stability of the reconstituted vaccine are carried out, using the product reconstituted in accordance with the proposed recommendations.

The variations in the results obtained during the stability study are taken into account when defining appropriate formulation and release specifications to ensure the conformity of the product for the claimed shelf-life.

2-2-4. **Formulation**. The minimum antigen content, virus titre or bacterial count acceptable from the point of view of efficacy (i.e. gives satisfactory results in the potency test and other efficacy studies) is established during development studies. The antigen formulation, where applicable the adjuvant formulation, and the release specifications are set based on this minimum value and based on the results of the stability studies.

A maximum antigen content, virus titre or bacterial count, acceptable from the point of view of safety, is established during development studies.

For live vaccines, this is also used as the maximum acceptable titre for each batch of vaccine at release.

2-3. *PREPARATION OF THE VACCINE*

The methods of preparation, which vary according to the type of vaccine, are such as to maintain the integrity and immunogenicity of the antigen, to ensure freedom from contamination with extraneous agents and to ensure production of vaccine batches of consistent quality.
For each individual product, relevant in-process and finished product controls are established to verify the production process and the batch-to-batch quality of the product. The results are within the approved limits defined for the particular product.

2-3-1. **Propagation and harvest of bacterial and viral antigens**. Each strain of a multivalent vaccine is cultivated and harvested separately.

The working seed materials are propagated in suitable media/substrates for production. The conditions of these propagation steps are described and monitored by recording appropriate parameters, e.g. temperature, pH, duration, turbidity and oxygen saturation. The results are within the approved limits defined for the particular product.

During production, where possible, growth rate is monitored by suitable methods and the values are recorded and within the approved limits defined for the particular product. The antigen may then be inactivated and/or purified and/or concentrated.

2-3-2. **Inactivation**. Inactivated vaccines are subjected to a validated inactivation procedure. The testing of the inactivation kinetics described below is carried out once for a given production process. The rest of this section applies to each production run. When conducting tests for inactivation, it is essential to take account of the possibility that under the conditions of manufacture, organisms may be physically protected from inactivant.

2-3-2-1. *Inactivation kinetics*. The inactivating agent and the inactivation procedure shall be shown, under conditions of manufacture, to inactivate the vaccine micro-organism. Adequate data on inactivation kinetics shall be obtained. Normally, the time required for inactivation shall be not more than 67 per cent of the duration of the inactivation process. The maximum titre of the vaccine micro-organism capable of being inactivated by the selected method is established based on the inactivation kinetics data.

2-3-2-2. *Residues of inactivating agents*. Appropriate tests are carried out to demonstrate that the inactivating agent has been removed or reduced to an acceptable residual level.

If an aziridine compound is used as the inactivating agent, this may be accomplished by neutralising it with thiosulfate and demonstrating residual thiosulfate in the inactivated harvest at the completion of the inactivation procedure.

If formaldehyde is used as the inactivating agent, then a test for free formaldehyde is carried out as prescribed under 3. Batch tests.

2-3-2-3. *Residual live virus/bacteria and/or detoxification testing*. A test for complete inactivation and/or detoxification is performed immediately after the inactivation and/or detoxification procedure and, if applicable, the neutralisation or removal of the inactivating or the test for detoxifying agent. Validation of the test for residual live virus/bacteria or the test for detoxification shall focus on the level of detection of the live virus/bacteria or toxin.

2-3-2-3-1. Bacterial vaccines. The test selected shall be appropriate to the vaccine bacteria being used and shall consist of at least 2 passages in production medium or, if solid medium has been used for production, in a suitable liquid medium or in the medium prescribed in the monograph. The product complies with the test if no evidence of any live micro-organism is observed.

2-3-2-3-2. Bacterial toxoids. The test selected shall be appropriate to the toxin or toxins present and shall be the most sensitive available.

2-3-2-3-3. Viral vaccines. The test selected shall be appropriate to the vaccine virus being used and must consist of at least 2 passages in cells, embryonated eggs or, where no other suitably sensitive method is available, in animals. The quantity of cell samples, eggs or animals shall be sufficient to ensure appropriate sensitivity of the test. For tests in cell cultures, not less than 150 $cm^2$ of cell culture monolayer is inoculated with 1.0 mL of inactivated harvest. The product complies with the test if no evidence of the presence of any live virus or other micro-organism is observed.

2-3-3. **Final bulk and final batch**. The final bulk vaccine is prepared by combining one or more batches of antigen, that comply with all the relevant requirements, with any auxiliary substances, such as adjuvants, stabilisers, antimicrobial preservatives and diluents.

The vaccine is blended according to a defined formulation.

Unless otherwise prescribed in the individual monograph or otherwise justified and authorised, the final bulk vaccine is distributed aseptically with or without freeze-drying, into sterile, tamper-evident containers, which are then closed so as to prevent contamination. This constitutes the final batch.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Antigen content**. The formulation of the vaccine is based, whenever possible, on the antigen content determined on the harvest before or after inactivation and/or downstream processing, if applicable.

2-4-2. **Batch potency test**. For most vaccines, the tests cited under Potency or Immunogenicity are not suitable for the routine testing of batches.

If the test described under Potency is not used for routine testing, a batch potency test is established during development. The aim of the batch potency test is to ensure that each batch of vaccine would, if tested, comply with the test described under Potency and Immunogenicity. The acceptance criteria for the batch potency test are therefore established by correlation with the test described under Potency. Where a batch potency test is described in a monograph, this is given as an example of a test that is considered suitable, after establishment of correlation with the potency test; other test models can also be used.

For live vaccines, virus titre or bacterial count is generally appropriate as a batch potency test.

For inactivated vaccines, development of *in vitro* methods is recommended, provided that:

- key in-process parameters are defined and monitored;
- in-process control tests (including antigen quantification after inactivation and/or concentration, if applicable) and target formulation of the final product are performed.

*Antigen content*. The quantity of appropriate antigen per dose, determined by a suitable method, is not significantly lower than that of a batch of vaccine that has given satisfactory results in the test described under Potency.

*Adjuvant*. If the test for antigen content is performed and if the vaccine is adjuvanted, the identity of the adjuvant is verified by suitable chemical methods and the adjuvant is tested as described in section 3. Batch tests. The quality and

quantity of the adjuvant is not significantly different from that of a batch of vaccine that has given satisfactory results in the test described under Potency.

2-5. *IN-PROCESS STABILITY*

During production of vaccines, intermediate products are obtained at various stages and may be stored. The intended conditions and duration of storage are defined in light of the stability data.

3. BATCH TESTS

*The individual monographs also indicate tests to be carried out on each particular vaccine.*

Certain tests may be carried out on the final bulk vaccine rather than on the batch or batches prepared from it; such tests include those for antimicrobial preservatives, free formaldehyde and the potency determination for inactivated vaccines.

Under particular circumstances (i.e. significant changes to the manufacturing process, as well as reports of unexpected adverse reactions observed in the field or reports that the final batches do not comply with the former data provided during licensing), other tests, including tests on animals, may be needed on an *ad hoc* basis; they are carried out in agreement with or at the request of the competent authority. For safety testing, one or more of the tests described in general chapter *5.2.6* may be carried out.

Only a batch that complies with each of the requirements given below, completed or amended by the requirements given in the relevant individual monograph, may be released for use.

*Animal tests.* In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The criteria for judging tests in monographs must be applied in light of this. For example, if it is indicated that an animal is considered to be positive, infected etc. when typical clinical signs occur then as soon as it is clear that result will not be affected the animal in question shall be either euthanised or given suitable treatment to prevent unnecessary suffering. In accordance with the General Notices, alternative test methods may be used to demonstrate compliance with the monograph and the use of such tests is particularly encouraged when this leads to replacement or reduction of animal use or reduction of suffering. Guidance on how to substitute *in vivo* methods by *in vitro* methods, in cases where a direct head-to-head comparison is not possible, can be found in general chapter *5.2.14*.

Taking into account the quality systems in place and advances in scientific knowledge and understanding of the products, manufacturing processes and their controls, the choice of tests to be performed may be reconsidered when assessing compliance with Pharmacopoeia monographs, in accordance with the General Notices. On a case-by-case basis, with the agreement of the competent authority, the choice and necessity of certain final product tests may be reconsidered, where in-process tests give at least an equal guarantee that the batch would comply if tested, or where alternative tests validated with respect to the Pharmacopoeia method have been carried out.

All hen eggs, chickens and chicken cell cultures for use in quality control tests shall be derived from an SPF flock (*5.2.2*).

3-1. **Identification**. The antigen is identified by suitable methods such as nucleic acid amplification techniques (*2.6.21*). For inactivated vaccines, the identification test may be combined with the batch potency test.

3-2. **Physical tests**. A vaccine with an oily adjuvant is tested for viscosity by a suitable method and shown to be within the limits set for the product. The stability of the emulsion shall be demonstrated.

3-3. **Chemical tests**. Tests for the concentrations of appropriate substances such as aluminium and preservatives are carried out to show that these are within the limits set for the product.

3-4. **pH**. The pH of liquid products and diluents is measured if possible and shown to be within the limits set for the product.

3-5. **Water**. Where applicable, the freeze-drying process is checked by a determination of water and shown to be within the limits set for the product.

3-6. **Formaldehyde** (*2.4.18*; use Method B if sodium metabisulfite has been used to neutralise excess formaldehyde). Where formaldehyde has been used in the preparation, the concentration of free formaldehyde is not greater than 0.5 g/L, unless a higher amount has been shown to be safe.

3-7. **Phenol** (*2.5.15*). When the vaccine contains phenol, the concentration is not greater than 5 g/L.

3-8. **Bacteria and fungi**. Vaccines comply with the test for sterility (*2.6.1*). Where the volume of liquid in a container is greater than 100 mL, the method of membrane filtration is used wherever possible. Where the method of membrane filtration cannot be used, the method of direct inoculation may be used. Where the volume of liquid in each container is at least 20 mL, the minimum volume to be used for each culture medium is 10 per cent of the contents or 5 mL, whichever is less. The appropriate number of items to be tested (*2.6.1*) is 1 per cent of the batch with a minimum of 4 and a maximum of 10.

For live bacterial and for live fungal vaccines, the absence of micro-organisms other than the vaccine strain is demonstrated by suitable methods such as microscopic examination and inoculation of suitable media.

For frozen or freeze-dried avian live viral vaccines produced in embryonated eggs, for non-parenteral use only, the requirement for sterility is usually replaced by requirements for absence of pathogenic micro-organisms and for a maximum of 1 non-pathogenic micro-organism per dose.

3-9. **Extraneous agents**. Avian live viral vaccines comply with the tests for extraneous agents in batches of finished product (*2.6.25*).

Mammalian live viral vaccines are tested for extraneous viruses using an appropriate method.

In case of doubt, the tests intended for the seed lot of a live vaccine may also be applied to the final product. If an extraneous agent is found in such a test, the vaccine does not comply with the monograph.

3-10. **Residual live virus/bacteria and/or detoxification testing**. For inactivated vaccines and bacterial toxoids, the tests described in section 2-3-2-3 are performed. Where auxiliary substances would interfere with a test for inactivation and/or detoxification, such a test is carried out during preparation of the final bulk, after the different batches of antigen have been combined, but before addition of the auxiliary substances; the test for inactivation or detoxification may then be omitted on the final bulk and the final batch.

The test for residual live virus/bacteria may be omitted for batch release provided that:

1. a titration is performed on each harvest prior to inactivation and the titre is not greater than the maximum titre established based on studies of the inactivation kinetics;
2. suitable test sensitivity for residual live virus/bacteria has been demonstrated;
3. the test for residual live virus/bacteria is performed with satisfactory results on each harvest.

Where there is a risk of reversion to toxicity, the test for detoxification performed at the latest stage of the production process at which the sensitivity of the test is not compromised

(e.g. after the different batches of antigen have been combined but before the addition of auxiliary substances) is important to demonstrate a lack of reversion to toxicity.

3-11. **Mycoplasmas** (*2.6.7*). Live viral vaccines comply with the test for mycoplasmas (culture method).

3-12. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (see section 2-2-1-1) when administered by a recommended route and method.

4. STORAGE

Store protected from light at a temperature of 5 ± 3 °C, unless otherwise indicated. Liquid preparations are not to be allowed to freeze, unless otherwise indicated.

*Expiry date*. Unless otherwise stated, the expiry date is calculated from the beginning of the virus titration or bacterial count (for live vaccines) or the beginning of the potency test (for other vaccines). For combined vaccines, the expiry date is that of the component which expires first. For vaccines stored by the manufacturer at a temperature lower than that stated on the label, the stability for the entire storage period is demonstrated by an appropriate study. The expiry date is then calculated from the date that the vaccine is stored in the conditions stated on the label.

The expiry date applies to vaccines stored in the prescribed conditions.

5. LABELLING

The label states:

- that the preparation is for veterinary use;
- the volume of the preparation and the number of doses in the container;
- the route of administration;
- the type or types of bacteria (and where applicable the antigenic components) or viruses used and for live vaccines the minimum and the maximum number of live bacteria or the minimum and the maximum virus titre;
- where applicable, for inactivated vaccines, the minimum potency in International Units;
- where applicable, the name of any antimicrobial preservative or other substance added to the vaccine;
- the name of any substance that may cause an adverse reaction;
- for freeze-dried vaccines:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the period within which the vaccine is to be used after reconstitution;
- for vaccines with an oily adjuvant, that if the vaccine is accidentally injected into man, urgent medical attention is necessary;
- the animal species for which the vaccine is intended;
- the indications for the vaccine;
- the instructions for use;
- any contra-indications to the use of the product including any required warning on the dangers of administration of an overdose;
- the doses recommended for different species.

**01/2008:1579**
**corrected 6.4**

# VEGETABLE FATTY OILS

## Olea herbaria

DEFINITION

Vegetable fatty oils are mainly solid or liquid triglycerides of fatty acids. They may contain small amounts of other lipids such as waxes, free fatty acids, partial glycerides or unsaponifiable matters. Vegetable fatty oils are obtained from the seeds, the fruit or the pit/stone/kernel of various plants by expression and/or solvent extraction, then possibly refined and hydrogenated. A suitable antioxidant may be added if necessary.

*Virgin oil*: an oil obtained from raw materials of special quality by mechanical procedures (e.g. by cold expression or centrifugation).

*Refined oil*: an oil obtained by expression and/or solvent extraction, and subsequently either alkali refining (followed by bleaching and any deodorisation) or physical refining.

*Hydrogenated oil*: an oil obtained by expression and/or solvent extraction, and subsequently either alkali refining or physical refining, then possible bleaching, followed by drying, hydrogenation and subsequent bleaching and deodorisation.

Only alkali-refined oils are used in the manufacture of parenteral preparations.

PRODUCTION

Measures are taken to ensure that the oil complies with the limit for benzo[a]pyrene decided by the competent authority. A limit of 2.0 ppb is set in Commission Regulation (EC) No. 208/2005.

*OBTENTION OF A CRUDE OIL*

Where the plant has a high oil content, the oil is generally obtained by expression under heating followed by an extraction; where the plant has a low oil content, the oil is generally obtained by direct extraction.

**Mechanical procedures**

A. Expression

*High-pressure screw-pressing*. It consists of some or all of the following steps: cleaning, drying, dehulling or decorticating, grinding, cooking and flaking.

During *cleaning* the foreign matter is eliminated. *Drying* may be necessary if the seed moisture content is higher than desirable for downstream processing. *Decorticating* is useful to obtain a high-protein meal by reduction of fibre and to reduce impurities in the oil. *Cooking* serves various purposes: completion of the breakdown of oil cells, lowering of the viscosity of the oil, coagulation of the protein in the meal, adjustment of the moisture level, sterilisation of the seed, detoxifying undesirable seed constituents (gossypol for cottonseed) and fixing certain phosphatides in the cake thus lowering subsequent refining losses. The efficacy of the expression process is such that only 3 per cent to 6 per cent of the oil is left in the cake.

*Wet screw-pressing*. The bunches are loaded into cages (for palm fruit) and moved into a horizontal steriliser with application of live steam and heating. The purposes of this steriliser are inactivation of enzymes, loosening of the fruit on the bunch, coagulation of proteins, etc. After heating in a digester, the pulp is fed to a screw-press. The oil is centrifugally clarified and vacuum-dried.

*Pre-pressing followed by solvent extraction*. The same sequence of steps is performed as above. The main function of pre-pressing is to obtain a cake of excellent

permeability for the following solvent extraction stage. The extraction is performed either in a percolation-type or in an immersion-type apparatus. The efficacy of the solvent extraction process is such that residual oil levels in meal are generally below 1 per cent.

B. Centrifugation

Centrifugation separates the oily phase from the aqueous phase, which contains water-soluble components and residual solid particles. This operation can be carried out using:

- self-cleaning bowl or disc centrifuges;
- super-decanters, which are horizontal turbines equipped with a cylindrical bowl that tapers slightly at one end and which contains a continuously turning screw that scrapes the sides of the bowl; the screw and the bowl rotate at different speeds; the solid particles are discarded from the tapered end of the bowl and the oil flows out from the other end.

**Solvent extraction**. Prior to extraction, the following steps are carried out: the seeds are tempered for about a week at a temperature below 24 °C in order to loosen the hull from the seed and allow the seed moisture to attain equilibrium, then the seeds are cleaned, ground, dehulled and flaked. The most widely used solvent is a mixture of mainly *n*-hexane and methylpentanes (bp: 65-70 °C) commonly referred to as 'hexane'. Due to the major fire and explosive risks of this mixture, liquified gases and supercritical gases may also be used.

*REFINING*

The objective of refining is to remove impurities and contaminants of the oil with the least possible damage to the triglycerides and with minimal loss of oil. The contents of the following substances are reduced:

- free fatty acids, which may cause deterioration of the oil by oxidation, a smoked taste when heated and a sharp flavour (by alkali refining);
- water, which favours the enzymatic hydrolysis reactions (by alkali refining, drying);
- partial glycerides, which may cause foaming and a bitter taste (by neutralisation, washing);
- phosphatides and phosphorous compounds, which have emulsifying properties and may cause deposits, a darkening of the oil when heated, a cloudy appearance and bad organoleptic stability (by alkali refining);
- colouring matters such as chlorophyll (by alkali refining) and carotenoids (by bleaching);
- glycolipids, which may form colloidal solutions with water;
- free hydrocarbons, paraffin, waxes and resinous materials;
- metals (Fe, Cu, Pb, Sn, Pt, Pd, etc.), which are strong oxidation catalysts;
- pigments such as gossypol (in cottonseed oil) or mycotoxins such as aflatoxin (mainly in arachis seeds);
- pesticides;
- oxidation products (aldehydes, peroxides);
- proteins having possible allergic reactions;
- unsaponifiable matters (sterols, tocopherols and other vitamins);
- polycyclic aromatic hydrocarbons.

**Alkali refining**. It involves the following steps: degumming if necessary, neutralisation using alkali, washing and drying.

*Degumming.* During this step of the refining, i.e. treatment with water and/or phosphoric acid and/or sodium chloride, the phosphatides, phosphorous compounds and metals are eliminated. The use of this step depends on the nature of the oil.

*Neutralisation with alkali.* This step reduces the free-fatty-acid content below 0.1 per cent; the fatty acids are converted into oil-insoluble soaps, also called 'soapstocks'. Other substances may be removed by adsorption on these soaps: mucilaginous substances, phosphatides, oxidation products, colouring matters, etc. All substances that become insoluble in the oil on hydration are removed. Neutralisation with alkali has the disadvantage of saponifying a portion of neutral oil if the neutralisation is not well conducted.

*Washing.* This operation consists in removing the excess of soaps and alkali as well as the remaining traces of metals, phosphatides and other impurities, using hot water.

*Drying.* The remaining water is eliminated under vacuum before any further steps, such as bleaching.

**Physical refining**. It involves a steam treatment of the oil under high vacuum at a temperature greater than 235 °C. This technique can only be applied to oils naturally low in phosphatides and metals (palm and coconut) or from which phosphatides and metals have been removed by an acid treatment using concentrated phosphoric acid followed by an adsorptive treatment with activated bleaching earth (for sunflower, rapeseed, soya-bean). Moreover, it cannot be used for heat-sensitive oils (cottonseed oil), which darken.

**Bleaching**. The common method of bleaching is by adsorption treatment of the oil, which is generally heated at 90 °C for 30 min under vacuum, with bleaching earth (natural or activated) or carbon (activated or not); synthetic silica adsorbents may also be added. Substances that have not been totally removed during refining are eliminated, for example carotenoids and chlorophyll.

**Deodorisation**. Deodorisation eliminates odours, volatile substances and any residual extraction solvents; it involves injecting dry vapour into the oil, which is kept under vacuum at a high temperature. Different temperatures are used according to the oil: 200-235 °C for 1.5-3 h or greater than 240 °C for 30 min.

One of the main side reactions is thermic decolourisation due to the destruction of carotenoids when the temperature is greater than 150 °C. This technique provokes a loss of substances that may be distilled (free fatty acids, sterols, tocopherols, part of the refined oil), and may cause *cis-trans* isomerisation of the unsaturated fatty-acid double bonds.

*WINTERISATION*

Elimination of solids and waxes by filtration at low temperature (also called dewaxing). These solids and waxes could affect the appearance of the oil and cause deposits.

*HYDROGENATION*

The hydrogenation of the dried and/or bleached oil is performed using a catalyst (e.g. Ni, Pt, Pd), at a temperature of about 100-200 °C under hydrogen pressure. The catalyst is then removed by filtration at 90 °C. The hydrogen must be pure: free of poisons for the catalyst, water-free, and low in carbon dioxide, methane and nitrogen contents. Small amounts of polymers may be obtained. *Trans*-fatty acids are formed during partial hydrogenation.

*CHROMATOGRAPHIC PURIFICATION*

In high-purity applications, mainly for parenteral uses, the oil may be further purified by passing the oil through a column containing an activated earth. A solvent may sometimes be used to improve the efficiency. High-polarity molecules, such as oxidised materials, acids, alcohols, partial glycerides and free sterols, are preferentially removed.

When the oil is used in the manufacture of parenteral preparations, the limits set in the monograph for the acid value, the peroxide value and the water content may be different.

LABELLING

The label states:

- where applicable, that the oil was obtained by expression or extraction;
- where applicable, that the oil is suitable for use in the manufacture of parenteral preparations.

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**07/2017:1502**

# GLOSSARY

## Glossa

*This glossary provides definitions and/or explanations of terms that may be found in, or used in association with, the general monographs on dosage forms and the corresponding chapters on Pharmaceutical technical procedures (2.9), but that are not defined within them. Where relevant, reference is made to other equivalent terms that may be found in other publications or contexts.*

*This glossary is published for information.*

**Active substance**

Equivalent terms: active ingredient, drug substance, medicinal substance, active pharmaceutical ingredient.

**Basis**

Basis is an alternative term for 'vehicle' in a semi-solid or moulded solid preparation. The basis may be single-phase or multiphase.

**Conventional-release dosage form**

A conventional-release dosage form is a preparation showing a release of the active substance(s) that is not deliberately modified by a special formulation design and/or manufacturing method. In the case of a solid dosage form, the dissolution profile of the active substance depends essentially on its intrinsic properties. Equivalent term: immediate-release dosage form.

**Delayed-release dosage form**

A delayed-release dosage form is a modified-release dosage form, usually administered orally, where the onset of release of the active substance(s) is adjusted to take place after a specific time or at a specific location in the gastrointestinal tract. Delayed release is achieved by a special formulation design and/or manufacturing method. Delayed-release dosage forms include gastro-resistant preparations as defined in the general monographs on solid oral dosage forms.

**Dispersion**

From a Ph. Eur. perspective, a dispersion is a system consisting of 2 or more phases.

– *Colloidal dispersion*

  A colloidal dispersion is a system in which particles of colloidal size (a nominal diameter between approximately 1 nm and 1 µm) of any nature (solid, liquid or gas, including liposomes and nanosystems) are dispersed in a continuous phase of a different composition and/or state. The use of 'nominal diameter' is intended to convey that not all particles are spherical. Dispersions of particles below 1 µm are considered less likely to undergo sedimentation because of the effect of Brownian motion.

– *Suspension*

  A suspension is a dispersed system comprising solid particles of a nominal diameter greater than approximately 1 µm, dispersed in a liquid or semi-solid continuous phase. If the solid particles are of a nominal diameter lower than approximately 1 µm, the system is a colloidal suspension.

– *Emulsion*

  An emulsion is a dispersed system consisting of a mixture of at least 2 liquids that are not miscible with each other. One of the liquids is dispersed in the other as droplets of a diameter greater than approximately 1 µm. If the droplets are of a nominal diameter lower than approximately 1 µm, the system is a colloidal emulsion.

**Large-volume parenteral**

A large-volume parenteral is an infusion or injection supplied in a container with a nominal content of more than 100 mL.

**Modified-release dosage form**

A modified-release dosage form is a preparation where the onset, rate and/or place of release of the active substance(s) is different from that of a conventional-release dosage form administered by the same route. This deliberate modification is achieved by a special formulation design and/or manufacturing method. Modified-release dosage forms include prolonged-release, delayed-release and pulsatile-release dosage forms.

**Prolonged-release dosage form**

A prolonged-release dosage form is a modified-release dosage form showing a slower release of the active substance(s) than that of a conventional-release dosage form administered by the same route. Prolonged release is achieved by a special formulation design and/or manufacturing method. Equivalent term: extended-release dosage form.

**Pulsatile-release dosage form**

A pulsatile-release dosage form is a modified-release dosage form showing a sequential, intermittent release of the active substance(s). Pulsatile release is achieved by a special formulation design and/or manufacturing method.

**Small-volume parenteral**

A small-volume parenteral is an infusion or injection supplied in a container with a nominal content of 100 mL or less.

**Solution**

A solution is a homogeneous mixture forming a single phase containing one or more dissolved substances, i.e. substances in a molecular or ionic state dispersed in a solvent or in miscible solvents. The dissolved substances can be solids, liquids or gases.

**Spheroids**

Spheroids are considered to be spherical or approximately spherical granules with a usually increased mechanical resistance compared to conventional *Granules (0499)*. They possess a smooth, uniform surface, with a typical size range of 200 µm to 2.8 mm. Spheroids may be prepared by any suitable method.

**Standard Term**

Standard Terms are approved terms for describing certain aspects of a medicinal product, namely the dosage form and route or method of administration, and certain important packaging items such as the container, closure and administration device. They are established by the European Pharmacopoeia Commission, which started working on the lists of Standard Terms in response to a request made by the EU Commission for use in the marketing authorisation application (MAA), summary of product characteristics (SmPC), labelling, and electronic communications. Standard Terms have the double purpose of bringing information to the patient/user/prescriber and distinguishing medicinal products having the same trade name, and are published in a specific Standard Terms database.

**Vehicle**

A vehicle is the carrier, composed of one or more excipients, in which the active substance(s) are contained, usually when referring to a liquid or semi-solid preparation.

04/2018:0016

# CAPSULES

## Capsulae

*The requirements of this monograph do not necessarily apply to preparations that are presented as capsules intended for use other than by oral administration. Requirements for such preparations may be found, where appropriate, in other general monographs, for example Rectal preparations (1145) and Vaginal preparations (1164).*

DEFINITION

Capsules are solid preparations with hard or soft shells of various shapes and capacities, containing a single dose of active substance(s). They are usually intended for oral administration.

The capsule shells are made of gelatin or other substances, the consistency of which may be adjusted by the addition of substances such as glycerol or sorbitol. Excipients such as surface-active agents, opaque fillers, antimicrobial preservatives, sweeteners, colouring matter authorised by the competent authority and flavouring substances may be added. The capsules may bear surface markings.

The contents of capsules may be solid, semi-solid or liquid. They consist of one or more active substances with or without excipients such as solvents, diluents, lubricants and disintegrating agents. The contents do not cause deterioration of the shell. The shell, however, is attacked by the digestive fluids and the contents are released.

Where applicable, containers for capsules comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of capsules may be distinguished:

- hard capsules;
- soft capsules;
- gastro-resistant capsules;
- modified-release capsules;
- cachets.

PRODUCTION

In the manufacture, packaging, storage and distribution of capsules, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Capsules comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, capsules with a content of active substance less than 2 mg or less than 2 per cent of the fill mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those ingredients that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Capsules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out, for example one of the tests described in general chapter *2.9.3. Dissolution test for solid dosage forms*.

Where a dissolution test is prescribed, a disintegration test may not be required.

STORAGE

Store at a temperature not exceeding 30 °C.

LABELLING

The label states the name of any added preservative.

## Hard capsules

DEFINITION

Hard capsules are solid single-dose preparations. They consist of a hard shell, the capacity of which can be varied, containing a solid, semi-solid or liquid preparation. The shell is made of gelatin or other substances, and consists of 2 prefabricated cylindrical sections that are open at one end and rounded and closed at the other. The contents are filled into one of the sections and the capsule is closed by slipping the other section over it.

PRODUCTION

The active substance(s), usually in solid form (powder or granules), are filled into one of the sections that is then closed by slipping the other section over it. The security of the closure may be strengthened by suitable means.

TESTS

**Disintegration** (*2.9.1*). Hard capsules comply with the test. Use *water R* as the liquid medium. When justified and authorised, *0.1 M hydrochloric acid* or *artificial gastric juice R* may be used as the liquid medium. If the capsules float on the surface of the water, a disc may be added. Operate the apparatus for 30 min, unless otherwise justified and authorised.

## Soft capsules

DEFINITION

Soft capsules are solid single-dose preparations. They consist of a soft shell, the capacity and shape of which can be varied, containing a semi-solid or liquid preparation. The shell is made of gelatin or other substances and may have one or more solid active substances incorporated into it. The shell is usually thicker than that of a hard capsule and consists of one part, as soft capsules are generally formed, filled and sealed in a single operation.

PRODUCTION

Soft capsules are usually formed, filled and sealed in one operation, but for extemporaneous use the shell may be prefabricated. The shell material may contain an active substance.

Liquids may be enclosed directly; solids are usually dissolved or dispersed in a suitable vehicle to give a solution or dispersion of a paste-like consistency.

There may be partial migration of the constituents from the capsule contents into the shell and vice versa because of the nature of the materials and the surfaces in contact.

TESTS

**Disintegration** (*2.9.1*). Soft capsules comply with the test. Use *water R* as the liquid medium. When justified and authorised, *0.1 M hydrochloric acid* or *artificial gastric juice R* may be used

as the liquid medium. Add a disc to each tube. Liquid active substances dispensed in soft capsules may attack the disc; in such circumstances and where authorised, the disc may be omitted. Operate the apparatus for 30 min, unless otherwise justified and authorised. If the capsules fail to comply because of adherence to the discs, the results are invalid. Repeat the test on a further 6 capsules omitting the discs.

### Modified-release capsules

DEFINITION

Modified-release capsules are hard or soft capsules in which the contents or the shell or both contain special excipients or are prepared by a special process designed to modify the rate, the place or the time at which the active substance(s) are released.

Modified-release capsules include prolonged-release capsules, delayed-release capsules and pulsatile-release capsules.

### Gastro-resistant capsules

DEFINITION

Gastro-resistant capsules are delayed-release capsules that are intended to resist the gastric fluid and to release their active substance or substances in the intestinal fluid. Usually they are prepared by filling capsule shells with granules or with particles covered with a gastro-resistant coating. In other cases, the shell of the capsule is covered with a gastro-resistant coating or the shell itself has gastro-resistant properties.

TESTS

**Disintegration** (*2.9.1*). Capsules with a gastro-resistant shell comply with the test with the following modifications. Use *0.1 M hydrochloric acid* as the liquid medium and operate the apparatus for 2 h, or other such time as may be authorised, without the discs. Examine the state of the capsules. The time of resistance to the acid medium varies according to the formulation of the capsules to be examined. It is typically 2 h to 3 h but even with authorised deviations it must not be less than 1 h. No capsule shows signs of disintegration or rupture permitting the escape of the contents. Replace the acid by *phosphate buffer solution pH 6.8 R*. When justified and authorised, a buffer solution of pH 6.8 with added pancreas powder (for example, 0.35 g of *pancreas powder R* per 100 mL of buffer solution) may be used. Add a disc to each tube. Operate the apparatus for 60 min. If the capsules fail to comply because of adherence to the discs, the results are invalid. Repeat the test on a further 6 capsules omitting the discs.

### Cachets

DEFINITION

Cachets are solid preparations consisting of a hard shell containing a single dose of one or more active substances. The cachet shell is made of unleavened bread usually from rice flour and consists of 2 prefabricated flat cylindrical sections. Before administration, the cachets are immersed in water for a few seconds, placed on the tongue and swallowed with a draught of water.

**01/2018:1239**

## CHEWING GUMS, MEDICATED

### Masticabilia gummis medicata

DEFINITION

Medicated chewing gums are solid, single-dose preparations with a base consisting mainly of gum that are intended to be chewed but not swallowed.

They contain one or more active substances that are released by chewing. After dissolution or dispersion of the active substances in saliva, chewing gums are intended to be used for:

- local treatment of mouth diseases;
- systemic delivery after absorption through the buccal mucosa or from the gastrointestinal tract.

PRODUCTION

Medicated chewing gums are made with a tasteless masticatory gum base that consists of natural or synthetic elastomers. They may contain other excipients such as fillers, softeners, sweetening agents, flavouring substances, stabilisers and plasticisers and authorised colouring matter.

Medicated chewing gums are manufactured by compression or by softening or melting the gum bases and adding successively the other substances. In the latter case, chewing gums are then further processed to obtain the desired gum presentation. The medicated chewing gums may be coated, for example, if necessary to protect from humidity and light.

In the manufacture, packaging, storage and distribution of medicated chewing gums, suitable measures must be taken to ensure their microbial quality; recommendations related to this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Medicated chewing gums comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, medicated chewing gums with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those active substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Uncoated medicated chewing gums and, unless otherwise justified and authorised, coated medicated chewing gums comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example the test described in general chapter *2.9.25. Dissolution test for medicated chewing gums*.

STORAGE

Where appropriate, store uncoated medicated chewing gums protected from humidity and light.

04/2011:0652
corrected 10.0

# EAR PREPARATIONS

## Auricularia

DEFINITION

Ear preparations are liquid, semi-solid or solid preparations intended for instillation, for spraying, for insufflation, for application to the auditory meatus or as an ear wash.

Ear preparations usually contain 1 or more active substances in a suitable vehicle. They may contain excipients, for example, to adjust tonicity or viscosity, to adjust or stabilise the pH, to increase the solubility of the active substances, to stabilise the preparation or to provide adequate antimicrobial properties. The excipients do not adversely affect the intended medicinal action of the preparation or, at the concentrations used, cause toxicity or undue local irritation.

Preparations for application to the injured ear, particularly where the eardrum is perforated, or prior to surgery are sterile and, unless otherwise justified and authorised, free from antimicrobial preservatives and supplied in single-dose containers.

Ear preparations are supplied in multidose or single-dose containers, provided, if necessary, with a suitable administration device, which may be designed to avoid the introduction of contaminants.

Unless otherwise justified and authorised, aqueous ear preparations supplied in multidose containers contain a suitable antimicrobial preservative at a suitable concentration, except where the preparation itself has adequate antimicrobial properties.

Where applicable, containers for ear preparations comply with the requirements of chapters *3.1. Materials used for the manufacture of containers* (and subsections) and *3.2. Containers* (and subsections).

Several categories of ear preparations may be distinguished:

- ear drops and sprays;
- semi-solid ear preparations;
- ear powders;
- ear washes;
- ear tampons.

PRODUCTION

During development of an ear preparation whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in chapter *5.1.3. Efficacy of antimicrobial preservation*.

During development of ear washes, it must be demonstrated that the nominal content can be withdrawn from the container of preparations presented in single-dose containers.

In the manufacture, packaging, storage and distribution of ear preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Sterile ear preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in chapter *5.1.1. Methods of preparation of sterile products*.

In the manufacture of ear preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Uniformity of dosage units**. Single-dose ear preparations comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose ear preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those ingredients that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose ear preparations comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Sterility** (*2.6.1*). Where the label indicates that the ear preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name of any added antimicrobial preservative;
- where applicable, that the preparation is sterile;
- for multidose containers, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

## Ear drops and ear sprays

DEFINITION

Ear drops and ear sprays are solutions, emulsions or suspensions of one or more active substances in liquids suitable for application to the auditory meatus without exerting harmful pressure on the eardrum (for example, water, glycols or fatty oils). They may also be placed in the auditory meatus by means of a tampon impregnated with the liquid.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment, which is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Ear drops are usually supplied in multidose containers of glass or suitable plastic material that are fitted with an integral dropper or with a screw cap of suitable materials incorporating a dropper and rubber or plastic teat. Alternatively, such a cap assembly is supplied separately. Ear sprays are usually supplied in multidose containers fitted with an appropriate applicator. When ear sprays are supplied in pressurised containers, these comply with the requirements of the monograph *Pressurised pharmaceutical preparations (0523)*.

## Semi-solid ear preparations

DEFINITION

Semi-solid ear preparations are intended for application to the external auditory meatus, if necessary by means of a tampon impregnated with the preparation.

Semi-solid ear preparations comply with the requirements of the monograph *Semi-solid preparations for cutaneous application (0132)*.

They are supplied in containers fitted with a suitable applicator.

## Ear powders

DEFINITION

Ear powders are intended for application or insufflation into the external auditory meatus. They comply with the requirements of the monograph *Powders for cutaneous application (1166)*.

They are supplied in containers fitted with a suitable device for application or insufflation.

## Ear washes

DEFINITION

Ear washes are preparations intended to cleanse the external auditory meatus. They are usually aqueous solutions with a pH within physiological limits.

Ear washes intended for application to injured parts or prior to a surgical operation are sterile.

## Ear tampons

DEFINITION

Ear tampons are intended to be inserted into the external auditory meatus. They comply with the requirements of the monograph *Medicated tampons (1155)*.

**01/2008:1163**
**corrected 10.0**

# EYE PREPARATIONS

## Ophthalmica

DEFINITION

Eye preparations are sterile liquid, semi-solid or solid preparations intended for administration upon the eyeball and/or to the conjunctiva, or for insertion in the conjunctival sac.

Where applicable, containers for eye preparations comply with the requirements of materials used for the manufacture of containers (*3.1* and subsections) and containers (*3.2* and subsections).

Several categories of eye preparations may be distinguished:

- eye drops;
- eye lotions;
- powders for eye drops and powders for eye lotions;
- semi-solid eye preparations;
- ophthalmic inserts.

PRODUCTION

During the development of an eye preparation whose formulation contains an antimicrobial preservative, the necessity for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in chapter *5.1.3. Efficacy of antimicrobial preservation*.

Eye preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in chapter *5.1.1. Methods of preparation of sterile products*.

In the manufacture of eye preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

During development, it must be demonstrated that the nominal contents can be withdrawn from the container of liquid and semi-solid eye preparations supplied in single-dose containers.

TESTS

**Sterility** (*2.6.1*). Eye preparations comply with the test. Applicators supplied separately also comply with the test. Remove the applicator with aseptic precautions from its package and transfer it to a tube of culture medium so that it is completely immersed. Incubate and interpret the results as described in the test.

STORAGE

Unless otherwise justified and authorised, store in a sterile, tamper-evident container.

LABELLING

The label states the name of any added antimicrobial preservative.

## Eye drops

DEFINITION

Eye drops are sterile aqueous or oily solutions, emulsions or suspensions of one or more active substances intended for instillation into the eye.

Eye drops may contain excipients, for example, to adjust the tonicity or the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance, or to stabilise the preparation. These substances do not adversely affect the intended medicinal action or, at the concentrations used, cause undue local irritation.

Aqueous preparations supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen must be compatible with the other ingredients of the preparation and must remain effective throughout the period of time during which eye drops are in use.

If eye drops do not contain antimicrobial preservatives they are supplied in single-dose containers or in multidose containers preventing microbial contamination of the contents after opening.

Eye drops intended for use in surgical procedures do not contain antimicrobial preservatives.

Eye drops that are solutions, examined under suitable conditions of visibility, are practically clear and practically free from particles.

Eye drops that are suspensions may show a sediment that is readily redispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Multidose preparations are supplied in containers that allow successive drops of the preparation to be administered. The containers contain at most 10 mL of the preparation, unless otherwise justified and authorised.

TESTS

**Particle size**. Unless otherwise justified and authorised, eye drops in the form of a suspension comply with the following test: introduce a suitable quantity of the suspension into a counting cell or with a micropipette onto a slide, as appropriate, and scan under a microscope an area corresponding to 10 µg of the solid phase. For practical reasons, it is recommended that the whole sample is first scanned at low magnification (e.g. × 50) and particles greater than 25 µm are identified. These larger particles can then

be measured at a larger magnification (e.g. × 200 to × 500). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

LABELLING

The label states, for multidose containers, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

## Eye lotions

DEFINITION

Eye lotions are sterile aqueous solutions intended for use in rinsing or bathing the eye or for impregnating eye dressings.

Eye lotions may contain excipients, for example to adjust the tonicity or the viscosity of the preparation or to adjust or stabilise the pH. These substances do not adversely affect the intended action or, at the concentrations used, cause undue local irritation.

Eye lotions supplied in multidose containers contain a suitable antimicrobial preservative in appropriate concentration except when the preparation itself has adequate antimicrobial properties. The antimicrobial preservative chosen is compatible with the other ingredients of the preparation and remains effective throughout the period of time during which the eye lotions are in use.

If eye lotions do not contain antimicrobial preservatives, they are supplied in single-dose containers. Eye lotions intended for use in surgical procedures or in first-aid treatment do not contain an antimicrobial preservative and are supplied in single-dose containers.

Eye lotions, examined under suitable conditions of visibility, are practically clear and practically free from particles.

The containers for multidose preparations do not contain more than 200 mL of eye lotion, unless otherwise justified and authorised.

LABELLING

The label states:

- where applicable, that the contents are to be used on one occasion only;
- for multidose containers, the period after opening the container after which the contents must not be used; this period does not exceed 4 weeks, unless otherwise justified and authorised.

## Powders for eye drops and powders for eye lotions

DEFINITION

Powders for the preparation of eye drops and eye lotions are supplied in a dry, sterile form to be dissolved or suspended in an appropriate liquid vehicle at the time of administration. They may contain excipients to facilitate dissolution or dispersion, to prevent caking, to adjust the tonicity, to adjust or stabilise the pH or to stabilise the preparation.

After dissolution or suspension in the prescribed liquid, they comply with the requirements for eye drops or eye lotions, as appropriate.

TESTS

**Uniformity of dosage units** (*2.9.40*). Single-dose powders for eye drops and eye lotions comply with the test or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose powders for eye drops and eye lotions with a content of active substance less then 2 mg or less than 2 per cent of the total mass comply with test B. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above condition.

**Uniformity of mass** (*2.9.5*). Single-dose powders for eye drops and eye lotions comply with the test. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

## Semi-solid eye preparations

DEFINITION

Semi-solid eye preparations are sterile ointments, creams or gels intended for application to the conjunctiva or to the eyelids. They contain one or more active substances dissolved or dispersed in a suitable basis. They have a homogeneous appearance.

Semi-solid eye preparations comply with the requirements of the monograph *Semi-solid preparations for cutaneous application (0132)*. The basis is non-irritant to the conjunctiva.

Semi-solid eye preparations are packed in small, sterilised collapsible tubes fitted or provided with a sterilised cannula. The containers contain at most 10 g of the preparation, unless otherwise justified and authorised. The tubes must be well-closed to prevent microbial contamination. Semi-solid eye preparations may also be packed in suitably designed single-dose containers. The containers, or the nozzles of tubes, are of such a shape as to facilitate administration without contamination.

TESTS

**Particle size**. Semi-solid eye preparations containing dispersed solid particles comply with the following test: spread gently a quantity of the preparation corresponding to at least 10 µg of solid active substance as a thin layer. Scan under a microscope the whole area of the sample. For practical reasons, it is recommended that the whole sample is first scanned at a small magnification (e.g. × 50) and particles greater than 25 µm are identified. These larger particles can then be measured at a larger magnification (e.g. × 200 to × 500). For each 10 µg of solid active substance, not more than 20 particles have a maximum dimension greater than 25 µm, and not more than 2 of these particles have a maximum dimension greater than 50 µm. None of the particles has a maximum dimension greater than 90 µm.

LABELLING

The label states, for multidose containers, the period after opening the container after which the contents must not be used. This period does not exceed 4 weeks, unless otherwise justified and authorised.

## Ophthalmic inserts

DEFINITION

Ophthalmic inserts are sterile, solid or semi-solid preparations of suitable size and shape, designed to be inserted in the conjunctival sac, to produce an ocular effect. They generally consist of a reservoir of active substance embedded in a matrix or bounded by a rate-controlling membrane. The active substance, which is more or less soluble in lacrymal liquid, is released over a determined period of time.

Ophthalmic inserts are individually distributed into sterile containers.

PRODUCTION

In the manufacture of ophthalmic inserts, measures are taken to ensure a suitable dissolution behaviour.

TESTS

**Uniformity of dosage units** (*2.9.40*). Ophthalmic inserts comply with the test or, where justified and authorised, with the test for uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Ophthalmic inserts comply, where applicable, with test A.

LABELLING

The label states:

- where applicable, the total quantity of active substance per insert;
- where applicable, the dose released per unit time.

**01/2008:1105**

# FOAMS, MEDICATED

## Musci medicati

*Additional requirements for medicated foams may be found, where appropriate, in other general monographs, for example on Rectal preparations (1145), Vaginal preparations (1164) and Liquid preparations for cutaneous application (0927).*

DEFINITION

Medicated foams are preparations consisting of large volumes of gas dispersed in a liquid generally containing one or more active substances, a surfactant ensuring their formation and various other excipients. Medicated foams are usually intended for application to the skin or mucous membranes.

Medicated foams are usually formed at the time of administration from a liquid preparation in a pressurised container. The container is equipped with a device consisting of a valve and a push button suitable for the delivery of the foam.

Medicated foams intended for use on severely injured skin and on large open wounds are sterile.

Medicated foams supplied in pressurised containers comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

PRODUCTION

Sterile medicated foams are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

TESTS

**Relative foam density**. Maintain the container at about 25 °C for at least 24 h. Taking care not to warm the container, fit a rigid tube 70 mm to 100 mm long and about 1 mm in internal diameter onto the push button. Shake the container to homogenise the liquid phase of the contents and dispense 5 mL to 10 mL of foam to waste. Tare a flat-bottomed dish with a volume of about 60 mL and about 35 mm high. Place the end of the rigid tube attached to the push button in the corner of the dish, press the push button and fill the dish uniformly, using a circular motion. After the foam has completely expanded, level off by removing the excess foam with a slide. Weigh. Determine the mass of the same volume of *water R* by filling the same dish with *water R*.

The relative foam density is equivalent to the ratio:

$$\frac{m}{e}$$

$m$ = mass of the test sample of foam, in grams;

$e$ = mass of same volume of *water R*, in grams.

Carry out three measurements. None of the individual values deviate by more than 20 per cent from the mean value.

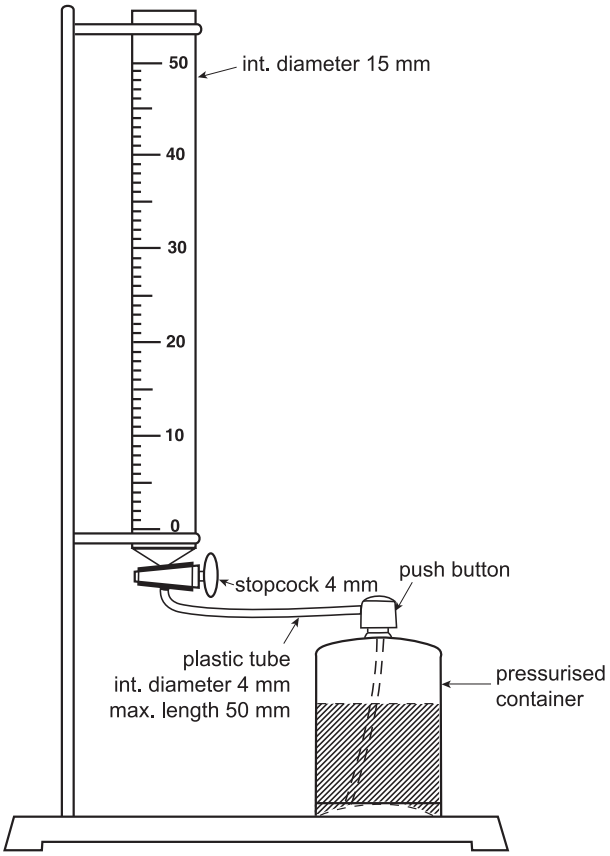


Figure 1105.-1. – *Apparatus for the determination of the duration of expansion*

**Duration of expansion**. The apparatus (Figure 1105.-1) consists of a 50 mL burette, 15 mm in internal diameter, with 0.1 mL graduations and fitted with a 4 mm single bore stopcock. The graduation corresponding to 30 mL is at least 210 mm from the axis of the stopcock. The lower part of the burette is connected by means of a plastic tube not longer than 50 mm and 4 mm in internal diameter to the foam-generating container equipped with a push button fitted to this connection. Maintain the container at about 25 °C for at least 24 h. Shake the container, taking care not to warm it, to homogenise the liquid phase of the contents and dispense 5 mL to 10 mL of the foam to waste. Connect the push button to the outlet of the burette. Press the button and introduce about 30 mL of foam in a single delivery. Close the stopcock and at the same time start the chronometer and read the volume of foam in the burette. Every 10 s read the growing volume until the maximum volume is reached.

Carry out three measurements. None of the times needed to obtain the maximum volume is more than 5 min.

**Sterility** (*2.6.1*). When the label indicates that the preparation is sterile, it complies with the test for sterility.

LABELLING

The label states, where applicable, that the preparation is sterile.

01/2019:0499

# GRANULES

## Granulata

*Requirements for granules to be used for the preparation of oral solutions or suspensions are given in the monograph Liquid preparations for oral use (0672). Where justified and authorised, the requirements of this monograph do not apply to granules for veterinary use.*

DEFINITION

Granules are preparations consisting of solid, dry aggregates of powder particles sufficiently robust to withstand handling. They are intended for oral administration. Some are swallowed as such, some are chewed and some are dissolved or dispersed in water or another suitable liquid before being administered.

For reasons of patient safety and to ensure the correct administration of the medicinal product, this term may also be used where very small tablets (rather than granules) are presented in a sachet, and where the entire contents of the sachet are intended for oral administration as a single dose.

Granules contain one or more active substances with or without excipients and, if necessary, colouring matter authorised by the competent authority and flavouring substances.

Granules are presented as single-dose or multidose preparations. Each dose of a multidose preparation is administered by means of a device suitable for measuring the quantity prescribed. For single-dose granules, each dose is enclosed in an individual container, for example a sachet or a vial.

Where applicable, containers for granules comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of granules may be distinguished:

- effervescent granules;
- coated granules;
- gastro-resistant granules;
- modified-release granules.

PRODUCTION

In the manufacture, packaging, storage and distribution of granules, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Single-dose granules comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose granules with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose granules except for coated granules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Uniformity of mass of delivered doses from multidose containers** (*2.9.27*). Granules supplied in multidose containers comply with the test.

STORAGE

If the preparation contains volatile ingredients or the contents have to be protected, store in an airtight container.

## Effervescent granules

DEFINITION

Effervescent granules are uncoated granules generally containing acid substances and carbonates or hydrogen carbonates which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

TESTS

**Disintegration**. Place 1 dose of the effervescent granules in a beaker containing 200 mL of *water R* at 15-25 °C; numerous bubbles of gas are evolved. When the evolution of gas around the individual grains ceases, the granules have disintegrated, being either dissolved or dispersed in the water. Repeat the operation on 5 further doses. The preparation complies with the test if each of the 6 doses used disintegrates within 5 min.

STORAGE

In an airtight container.

## Coated granules

DEFINITION

Coated granules are usually multidose preparations and consist of granules coated with one or more layers of mixtures of various excipients.

PRODUCTION

The substances used as coatings are usually applied as a solution or suspension in conditions in which evaporation of the vehicle occurs.

TESTS

**Dissolution**. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in general chapter *2.9.3. Dissolution test for solid dosage forms*.

## Modified-release granules

DEFINITION

Modified-release granules are coated or uncoated granules that contain special excipients or that are prepared by special procedures, or both, designed to modify the rate, the place or the time at which the active substance or substances are released.

Modified-release granules include prolonged-release granules and delayed-release granules.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

**Dissolution**. Carry out a suitable test to demonstrate the appropriate release of the active substance(s), for example the test described in general chapter *2.9.3. Dissolution test for solid dosage forms*.

## Gastro-resistant granules

DEFINITION

Gastro-resistant granules are delayed-release granules that are intended to resist the gastric fluid and to release the active substance(s) in the intestinal fluid. These properties are achieved by covering the granules with a gastro-resistant material or by other suitable means.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

TESTS

**Dissolution**. Carry out a suitable test to demonstrate the appropriate release of the active substance(s), for example the test described in general chapter *2.9.3. Dissolution test for solid dosage forms*.

**01/2008:0945**
**corrected 10.0**

# INTRAMAMMARY PREPARATIONS FOR VETERINARY USE

## Praeparationes intramammariae ad usum veterinarium

DEFINITION

Intramammary preparations for veterinary use are sterile preparations intended for introduction into the mammary gland via the teat canal. There are two main categories: those intended for administration to lactating animals, and those intended for administration to animals at the end of lactation or to non-lactating animals for the treatment or prevention of infection.

Intramammary preparations for veterinary use are solutions, emulsions or suspensions or semi-solid preparations containing one or more active substances in a suitable vehicle. They may contain excipients such as stabilising, emulsifying, suspending and thickening agents. Suspensions may show a sediment which is readily dispersed on shaking. Emulsions may show evidence of phase separation but are readily redispersed on shaking.

Unless otherwise justified and authorised, intramammary preparations for veterinary use are supplied in containers for use on one occasion only for introduction in a single teat canal of an animal.

If supplied in multidose containers, aqueous preparations contain a suitable antimicrobial preservative at a suitable concentration, except where the preparation itself has adequate antimicrobial properties. Precautions for administration and for storage between administrations must be taken.

Where applicable, containers for intramammary preparations for veterinary use comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

PRODUCTION

During the development of a intramammary preparation for veterinary use, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (*5.1.3*).

Intramammary preparations for veterinary use are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

In the manufacture of intramammary preparations for veterinary use containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Deliverable mass or volume**. Squeeze out as much as possible of the contents of ten containers according to the instructions on the label. The mean mass or volume does not differ by more than 10 per cent from the nominal mass or volume.

**Sterility** (*2.6.1*). Intramammary preparations for veterinary use comply with the test for sterility; use the technique of membrane filtration or, in justified cases, direct inoculation of the culture media. Squeeze out the contents of ten containers and mix thoroughly. For each medium, use 0.5 g to 1 g (or 0.5 mL to 1 mL as appropriate) taken from the mixed sample.

STORAGE

Store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name of the active substance(s) and the mass or number of International Units of the active substance(s) that may be delivered from the container using normal technique;
- whether the preparation is intended for use in a lactating animal or a non-lactating animal;
- in the case of multidose containers, the name of any added antimicrobial preservative.

**01/2018:1228**

# INTRARUMINAL DELIVERY SYSTEMS

## Praeparationes intraruminales

*The requirements of this monograph do not apply to preparations (sometimes known as boluses) such as large conventional tablets, capsules or moulded dosage forms that give immediate or prolonged release of the active substance(s). Such preparations comply with the relevant parts of the monographs Capsules (0016) or Tablets (0478).*

DEFINITION

Intraruminal delivery systems are solid preparations each containing one or more active substances. They are intended for oral administration to ruminant animals, and may be administered by means of a suitable device. They are designed to be retained in the rumen to deliver the active substance(s) in a continuous or pulsatile manner. The period of release of the active substance(s) may vary from days to weeks according to the nature of the formulation and/or the delivery system.

Some intraruminal delivery systems are intended to float on the surface of the ruminal fluid while others are intended to remain on the floor of the rumen or reticulum. Each delivery system has a density appropriate for its intended purpose.

PRODUCTION

For continuous release, the intraruminal delivery system is designed to release the active substance(s) at a defined rate over a defined period of time. This may be achieved by erosion, corrosion, diffusion, osmotic pressure or any other suitable chemical, physical or physico-chemical means.

For pulsatile release, the intraruminal delivery system is designed to release a specific quantity of active substance(s) at one or several defined intermediate times. This may be achieved by corrosion by ruminal fluids of the metallic elements of the intraruminal delivery system, leading to sequential release of the constituent units, which are usually in the form of tablets.

In the manufacture of intraruminal delivery systems, measures are taken to ensure an appropriate release of the active substance(s).

In the manufacture, packaging, storage and distribution of intraruminal delivery systems, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Constituent dosage units of intraruminal delivery systems comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise justified and authorised, constituent dosage units of intraruminal delivery systems in which the active substances are present at levels less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Unless otherwise justified and authorised, the constituent dosage units of intraruminal delivery systems comply with the test for uniformity of mass. If the test for uniformity of content is prescribed for all active substances, the test for uniformity of mass is not required.

LABELLING

The label states:

- for continuous-release delivery systems, the dose released per unit time;
- for pulsatile-release delivery systems, the dose released at specified times.

**01/2008:1806**
**corrected 6.3**

# INTRAUTERINE PREPARATIONS FOR VETERINARY USE

## Praeparationes intra-uterinae ad usum veterinarium

DEFINITION

Intrauterine preparations for veterinary use are liquid, semi-solid or solid preparations intended for the direct administration to the uterus (cervix, cavity or fundus), usually in order to obtain a local effect. They contain 1 or more active substances in a suitable basis.

Where appropriate, containers for intrauterine preparations for veterinary use comply with the requirements for *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of intrauterine preparations for veterinary use may be distinguished:

- intrauterine tablets;
- intrauterine capsules;
- intrauterine solutions, emulsions and suspensions, concentrates for intrauterine solutions;
- tablets for intrauterine solutions and suspensions;
- semi-solid intrauterine preparations;
- intrauterine foams;
- intrauterine sticks.

PRODUCTION

During the development of an intrauterine preparation for veterinary use, the effectiveness of any added antimicrobial preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided under *Efficacy of antimicrobial preservation* (*5.1.3*).

In the manufacture, packaging, storage and distribution of intrauterine preparations for veterinary use, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*, see Table 5.1.4.-1. – *Cutaneous use*.

Sterile intrauterine preparations for veterinary use are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

During development, it must be demonstrated that the nominal content can be withdrawn from the container of liquid and semi-solid intrauterine preparations for veterinary use presented in single-dose containers.

TESTS

**Uniformity of dosage units**. Single-dose intrauterine preparations for veterinary use comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, solid single-dose preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (intrauterine tablets) or test B (intrauterine capsules) for uniformity of content of single-dose preparations. If the preparation has more than 1 active substance, the requirement applies only to those substances which correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Solid single-dose intrauterine preparations for veterinary use comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed or justified and authorised for all the active substances, the test for uniformity of mass is not required.

**Dissolution**. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s) from solid single-dose intrauterine preparations for veterinary use, for example one of the tests described in *Dissolution test for solid dosage forms* (*2.9.3*).

When a dissolution test is prescribed, a disintegration test may not be required.

**Sterility** (*2.6.1*). Sterile intrauterine preparations for veterinary use comply with the test for sterility. Applicators supplied with the preparation also comply with the test for

sterility. Remove the applicator with aseptic precautions from its package and transfer it to a tube of culture medium so that it is completely immersed. Incubate and interpret the results as described in the test for sterility.

LABELLING

The label states:

- the name of any added antimicrobial preservative;
- where applicable, that the preparation is sterile.

## Intrauterine tablets

DEFINITION

Intrauterine tablets are solid preparations each containing a single dose of 1 or more active substances. They generally conform to the definition given in the monograph on *Tablets (0478)*.

A suitable applicator may be used for application into the uterus.

TESTS

**Disintegration**. Unless intended for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (*2.9.2*). Examine the state of the tablets after 30 min, unless otherwise justified and authorised.

## Intrauterine capsules

DEFINITION

Intrauterine capsules are solid, single-dose preparations. They are generally similar to soft capsules, differing only in their shape and size. Intrauterine capsules have various shapes, usually ovoid. They are smooth and have a uniform external appearance.

A suitable applicator may be used for application into the uterus.

TESTS

**Disintegration**. Unless intended for prolonged local action, they comply with the test for disintegration of suppositories and pessaries (*2.9.2*). Examine the state of the capsules after 30 min, unless otherwise justified and authorised.

## Intrauterine solutions, suspensions and emulsions Concentrates for intrauterine solutions

DEFINITION

Intrauterine solutions, suspensions and emulsions are liquid preparations. Concentrates for intrauterine solutions are intended for administration after dilution.

They may contain excipients, for example to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance(s) or to stabilise the preparation. The excipients do not adversely affect the intended medical action, or, at the concentrations used, cause undue local irritation.

Intrauterine emulsions may show evidence of phase separation, but are readily redispersed on shaking. Intrauterine suspensions may show a sediment that is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable a homogeneous preparation to be delivered.

They may be supplied in single-dose containers. The container is adapted to deliver the preparation to the uterus or it may be accompanied by a suitable applicator.

PRODUCTION

In the manufacture of intrauterine suspensions, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

## Tablets for intrauterine solutions and suspensions

DEFINITION

Tablets intended for the preparation of intrauterine solutions and suspensions are single-dose preparations which are dissolved or dispersed in water at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent caking.

Tablets for intrauterine solutions or suspensions conform with the definition given in the monograph on *Tablets (0478)*.

After dissolution or dispersion, they comply with the requirements for intrauterine solutions or intrauterine suspensions, as appropriate.

TESTS

**Disintegration** (*2.9.1*). Tablets for intrauterine solutions or suspensions disintegrate within 3 min using *water R* at 15-25 °C.

LABELLING

The label states:

- the method of preparation of the intrauterine solution or suspension;
- the conditions and duration of storage of the solution or suspension after reconstitution.

## Semi-solid intrauterine preparations

DEFINITION

Semi-solid preparations for intrauterine use are ointments, creams or gels.

Semi-solid preparations for intrauterine use comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application (0132)*.

They are often supplied in single-dose containers. The container is adapted to deliver the preparation to the uterus or it may be accompanied by a suitable applicator.

## Intrauterine foams

DEFINITION

Intrauterine foams comply with the requirements of the monograph on *Medicated foams (1105)*.

They are supplied in multidose containers. The container is adapted to deliver the preparation to the uterus or it may be accompanied by a suitable applicator.

## Intrauterine sticks

DEFINITION

Intrauterine sticks comply with the requirements of the monograph on *Sticks (1154)*. They often produce a foam when coming into contact with physiological fluids.

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**corrected 10.0**

# LIQUID PREPARATIONS FOR CUTANEOUS APPLICATION

## Praeparationes liquidae ad usum dermicum

*Where justified and authorised, the requirements of this monograph do not apply to preparations intended for systemic and veterinary use.*

DEFINITION

Liquid preparations for cutaneous application are preparations of a variety of viscosities intended for local or transdermal delivery of active ingredients. They are solutions, emulsions or suspensions that may contain 1 or more active substances in a suitable vehicle. They may contain suitable antimicrobial preservatives, antioxidants and other excipients such as stabilisers, emulsifiers and thickeners.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment that is readily dispersed on shaking to give a suspension that is sufficiently stable to enable a homogeneous preparation to be delivered.

Where applicable, containers for liquid preparations for cutaneous application comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

When liquid preparations for cutaneous application are dispensed in pressurised containers, the containers comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

Preparations specifically intended for use on severely injured skin are sterile.

Several categories of liquid preparations for cutaneous application may be distinguished, for example:

– shampoos;
– cutaneous foams.

PRODUCTION

During development of a liquid preparation for cutaneous application whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (*5.1.3*).

During development, it must be demonstrated that the nominal content can be withdrawn from the container of liquid preparations for cutaneous application presented in single-dose containers.

In the manufacture, packaging, storage and distribution of liquid preparations for cutaneous application, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use.*

Sterile liquid preparations for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

In the manufacture of liquid preparations for cutaneous application containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Sterility** (*2.6.1*). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

– the name of any added antimicrobial preservative;
– where applicable, that the preparation is sterile.

## Shampoos

DEFINITION

Shampoos are liquid or, occasionally, semi-solid preparations intended for application to the scalp and subsequent washing away with water. Upon rubbing with water they usually form a foam.

They are emulsions, suspensions or solutions. Shampoos normally contain surface active agents.

## Cutaneous foams

DEFINITION

Cutaneous foams comply with the requirements of the monograph on *Medicated foams (1105)*.

**01/2018:0672**

# LIQUID PREPARATIONS FOR ORAL USE

## Praeparationes liquidae peroraliae

*Where justified and authorised, the requirements of this monograph do not apply to liquid preparations for oral use intended for veterinary use.*

DEFINITION

Liquid preparations for oral use are usually solutions, emulsions or suspensions containing one or more active substances in a suitable vehicle; they may, however, consist of liquid active substances used as such (oral liquids).

Some preparations for oral use are prepared by dilution of concentrated liquid preparations, or from powders or granules for the preparation of oral solutions or suspensions, for oral drops or for syrups, using a suitable vehicle.

The vehicle for any preparation for oral use is chosen having regard to the nature of the active substance(s) and to provide organoleptic characteristics appropriate to the intended use of the preparation.

Liquid preparations for oral use may contain suitable antimicrobial preservatives, antioxidants and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilising, stabilising, flavouring and sweetening agents and colouring matter, authorised by the competent authority.

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment, which is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Where applicable, containers for liquid preparations for oral use comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of preparations may be distinguished;

- oral solutions, emulsions and suspensions;
- powders and granules for oral solutions and suspensions;
- oral drops;
- powders for oral drops;
- syrups;
- powders and granules for syrups.

PRODUCTION

During development of a preparation for oral use whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

During development, it must be demonstrated that the nominal content can be withdrawn from the container, for liquid preparations for oral use presented in single-dose containers.

In the manufacturing, packaging, storage and distribution of liquid preparations for oral use, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

In the manufacture of liquid preparations for oral use containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Uniformity of dosage units**. Solutions, suspensions and emulsions in single-dose containers comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose preparations that are suspensions or emulsions comply with the following test. After shaking, empty each container as completely as possible and carry out the test on the individual contents. They comply with test B for uniformity of content of single-dose preparations.

**Uniformity of mass**. Single-dose preparations that are solutions comply with the following test: weigh individually the contents of 20 containers, emptied as completely as possible, and determine the average mass. Not more than 2 of the individual masses deviate by more than 10 per cent from the average mass and none deviate by more than 20 per cent.

**Dose and uniformity of dose of oral drops**. Into a suitable graduated cylinder, introduce by means of the dropping device the number of drops usually prescribed for one dose, or introduce by means of the measuring device the usually prescribed quantity. The dropping speed does not exceed 2 drops per second. Weigh the liquid, repeat the addition, weigh again and carry on repeating the addition and weighing until a total of 10 masses are obtained. No single mass deviates by more than 10 per cent from the average mass.

If the labelling defines a mass, the total of 10 masses does not differ by more than 15 per cent from the nominal mass of 10 doses defined on the labelling. If the labelling defines a volume, measure the total volume of 10 doses. The volume does not differ by more than 15 per cent from the nominal volume of 10 doses defined on the labelling.

**Uniformity of mass of delivered doses from multidose containers** (*2.9.27*). Liquid preparations for oral use supplied in multidose containers comply with the test. Oral drops are not subject to the provisions of this test.

LABELLING

The label states the name of any added preservative.

## Oral solutions, emulsions and suspensions

DEFINITION

Oral solutions, emulsions and suspensions are supplied in single-dose or multidose containers. Each dose from a multidose container is administered by means of a device suitable for measuring the prescribed volume. The device is usually a spoon or a cup for volumes of 5 mL or multiples thereof or an oral syringe for other volumes.

## Powders and granules for oral solutions and suspensions

DEFINITION

Powders and granules for the preparation of oral solutions or suspensions are intended to be reconstituted with the prescribed liquid to produce a liquid preparation for oral use. They may contain excipients, in particular to facilitate dispersion or dissolution and to prevent caking.

After dissolution or suspension, they comply with the requirements for oral solutions or oral suspensions, as appropriate.

TESTS

**Uniformity of dosage units**. Single-dose powders and single-dose granules comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose powders and single-dose granules with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose powders and single-dose granules comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

LABELLING

The label states:

- the method of preparation of the solution or suspension;
- the conditions and the duration of storage after reconstitution.

## Oral drops

DEFINITION

Oral drops are solutions, emulsions or suspensions that are administered in small volumes such as drops by means of a suitable device.

LABELLING

The label states the number of drops per millilitre of preparation or per gram of preparation if the dose is measured in drops.

## Powders for oral drops

DEFINITION

Powders for the preparation of oral drops are intended to be reconstituted with the prescribed liquid to produce a liquid preparation for oral use. They may contain excipients to facilitate dissolution or suspension in the prescribed liquid or to prevent caking.

After dissolution or suspension, they comply with the requirements for oral drops.

TESTS

**Uniformity of dosage units**. Single-dose powders for oral drops comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose powders for oral drops with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose powders for oral drops comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

## Syrups

DEFINITION

Syrups are aqueous preparations characterised by a sweet taste and a viscous consistency. They may contain sucrose at a concentration of at least 45 per cent *m/m*. The sweet taste can also be obtained by using other polyols or sweetening agents. Syrups usually contain aromatic or other flavouring agents. Each dose from a multidose container is administered by means of a device suitable for measuring the prescribed volume. The device is usually a spoon or a cup for volumes of 5 mL or multiples thereof.

LABELLING

The label states the name and concentration of the polyol or sweetening agent.

## Powders and granules for syrups

DEFINITION

Powders and granules for syrups are intended to be reconstituted with the prescribed liquid to produce a liquid preparation for oral use. They may contain excipients to facilitate dissolution.

After dissolution, they comply with the requirements for syrups.

TESTS

**Uniformity of dosage units**. Single-dose powders and granules for syrups comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose powders and granules for syrups with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose powders and granules for syrups comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

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**corrected 10.0**

# NASAL PREPARATIONS

## Nasalia

DEFINITION

Nasal preparations are liquid, semi-solid or solid preparations intended for administration to the nasal cavities to obtain a systemic or local effect. They contain one or more active substances. Nasal preparations are as far as possible non-irritating and do not adversely affect the functions of the nasal mucosa and its cilia. Aqueous nasal preparations are usually isotonic and may contain excipients, for example, to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance, or to stabilise the preparation.

Nasal preparations are supplied in multidose or single-dose containers, provided, if necessary, with a suitable administration device, which may be designed to avoid the introduction of contaminants.

Unless otherwise justified and authorised, aqueous nasal preparations supplied in multidose containers contain a suitable antimicrobial preservative in an appropriate concentration, except where the preparation itself has adequate antimicrobial properties.

Where applicable, the containers comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of nasal preparations may be distinguished:

- nasal drops and liquid nasal sprays;
- nasal powders;
- semi-solid nasal preparations;
- nasal washes;
- nasal sticks.

PRODUCTION

During the development of a nasal preparation whose formulation contains an antimicrobial preservative, the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in the text on *Efficacy of antimicrobial preservation* (*5.1.3*).

In the manufacture, packaging, storage and distribution of nasal preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Sterile nasal preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

In the manufacture of nasal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Sterility** (*2.6.1*). Where the label states that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name of any added antimicrobial preservative;
- where applicable, that the preparation is sterile.

## Nasal drops and liquid nasal sprays

DEFINITION

Nasal drops and liquid nasal sprays are solutions, emulsions or suspensions intended for instillation or spraying into the nasal cavities.

Emulsions may show evidence of phase separation but are easily redispersed on shaking. Suspensions may show a sediment, which is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Nasal drops are usually supplied in multidose containers provided with a suitable applicator.

Liquid nasal sprays are supplied in containers with atomising devices or in pressurised containers fitted with a suitable adapter and with or without a metering dose valve, which comply with the requirements of the monograph on *Pressurised pharmaceutical preparations (0523)*.

The size of droplets of the spray is such as to localise their deposition in the nasal cavity.

TESTS

Unless otherwise prescribed or justified and authorised, nasal drops supplied in single-dose containers and single doses of metered-dose nasal sprays, both intended for systemic action, comply with the following tests.

*NASAL DROPS IN SINGLE-DOSE CONTAINERS*

**Uniformity of dosage units**. Nasal drops in single-dose containers comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of mass or uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of mass**. *Nasal drops that are solutions comply with the following test.* Weigh individually the contents of 10 containers emptied as completely as possible, and determine the average mass. Not more than 2 of the individual masses deviate by more than 10 per cent from the average mass, and none deviate by more than 20 per cent.

**Uniformity of content** (*2.9.6*). *Nasal drops that are suspensions or emulsions comply with the following test.* Empty each container as completely as possible and carry out the test on the individual contents. They comply with test B for uniformity of content.

*METERED-DOSE NASAL SPRAYS*

**Uniformity of dosage units**. Metered-dose nasal sprays comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of mass or the test for uniformity of delivered dose shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

*In the case of metered-dose nasal sprays that are solutions, proceed as follows.* Discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers. Determine the mass variation (*2.9.40*).

*In the case of metered-dose nasal sprays that are suspensions or emulsions, proceed as follows.* Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device. Shake the container for 5 s and discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 s, fire 1 dose of the metered-dose nasal spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers. Determine the content uniformity (*2.9.40*).

**Uniformity of mass**. *Metered-dose nasal sprays that are solutions comply with the following test.* Discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers.

The preparation complies with the test if not more than 2 of the individual values deviate by more than 25 per cent from the average value and none deviate by more than 35 per cent.

**Uniformity of delivered dose**. *Metered-dose nasal sprays that are suspensions or emulsions comply with the following test.* Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device. Shake the container for 5 s and discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 s, fire 1 dose of the metered-dose nasal spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers.

Unless otherwise justified and authorised, the preparation complies with the test if not more than 1 of the individual contents is outside the limits of 75 per cent to 125 per cent and none are outside the limits of 65 per cent to 135 per cent of the average content.

If 2 or at most 3 individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 20 more containers. The preparation complies with the test if not more than 3 individual contents of the 30 individual contents are outside the limits of 75 per cent to 125 per cent and none are outside the limits of 65 per cent to 135 per cent of the average content.

## Nasal powders

DEFINITION

Nasal powders are powders intended for insufflation into the nasal cavity by means of a suitable device.

They comply with the requirements of the monograph on *Powders for cutaneous application (1166)*.

The size of the particles is such as to localise their deposition in the nasal cavity and is verified by adequate methods of particle-size determination.

## Semi-solid nasal preparations

DEFINITION

Semi-solid nasal preparations comply with the requirements of the monograph on *Semi-solid preparations for cutaneous application (0132)*.

The containers are adapted to deliver the product to the site of application.

## Nasal washes

DEFINITION

Nasal washes are generally aqueous isotonic solutions intended to cleanse the nasal cavities.

Nasal washes intended for application to injured parts or prior to a surgical operation are sterile.

PRODUCTION

During development, it must be demonstrated that the nominal content can be withdrawn from the container, for nasal washes presented in single-dose containers.

## Nasal sticks

DEFINITION

Nasal sticks comply with the monograph on *Sticks (1154)*.



**01/2018:1807**

# OROMUCOSAL PREPARATIONS

## Praeparationes buccales

*This monograph does not apply to dental preparations or to preparations such as chewable tablets (0478), medicated chewing gums (1239), oral lyophilisates and other solid or semi-solid preparations that are not specifically listed in this monograph. Where justified and authorised, this monograph does not apply to preparations for veterinary use.*

DEFINITION

Oromucosal preparations are solid, semi-solid or liquid preparations, containing one or more active substances intended for administration to the oral cavity and/or the throat to obtain a local or systemic effect. Preparations intended for a local effect may be designed for application to a specific site within the oral cavity such as the gums (gingival preparations) or the throat (oropharyngeal preparations). Preparations intended for a systemic effect are designed to be absorbed primarily at one or more sites on the oral mucosa (e.g. sublingual preparations). Mucoadhesive preparations are intended to be retained in the oral cavity by adhesion to the mucosal epithelium and may modify systemic drug absorption at the site of application. For many oromucosal preparations, it is likely that some proportion of the active substance(s) will be swallowed and may be absorbed via the gastrointestinal tract.

Oromucosal preparations may contain suitable antimicrobial preservatives and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilising, stabilising, flavouring and sweetening agents. Solid preparations may in addition contain glidants, lubricants and excipients capable of modifying the release of the active substance(s).
Where applicable, containers for oromucosal preparations comply with the requirements for *Materials used for the manufacture of containers* (*3.1 and subsections*) and *Containers* (*3.2 and subsections*).

Several categories of preparations for oromucosal use may be distinguished:

- gargles;
- mouthwashes;
- gingival solutions;
- oromucosal solutions and oromucosal suspensions;
- semi-solid oromucosal preparations (including for example gingival gel, gingival paste, oromucosal gel, oromucosal paste);
- oromucosal drops, oromucosal sprays and sublingual sprays (including oropharyngeal sprays);
- lozenges and pastilles;
- compressed lozenges;
- sublingual tablets and buccal tablets;
- oromucosal capsules;
- mucoadhesive preparations;
- orodispersible films.

PRODUCTION

During the development of an oromucosal preparation containing an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with the criteria for judging the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

In the manufacture, packaging, storage and distribution of oromucosal preparations, suitable measures are taken to ensure their microbiological quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

In the manufacture of semi-solid and liquid oromucosal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Uniformity of dosage units**. Single-dose oromucosal preparations comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose oromucosal preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (compressed and moulded dosage forms) or test B (capsules) for the uniformity of content of single-dose preparations. If the preparation contains more than one active substance, this requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Solid single-dose oromucosal preparations comply with the test for uniformity of mass. If the test for the uniformity of content is prescribed, or justified and authorised, for all active substances, the test for uniformity of mass is not required.

LABELLING

The label states the name of any added preservative.

## Gargles

DEFINITION

Gargles are aqueous solutions intended for gargling to obtain a local effect. They are not to be swallowed. They are supplied as ready-to-use solutions or concentrated solutions to be diluted. They may also be prepared from powders or tablets to be dissolved in water before use.

Gargles may contain excipients to adjust the pH which, as far as possible, is neutral.

## Mouthwashes

DEFINITION

Mouthwashes are aqueous solutions intended for use in contact with the mucous membrane of the oral cavity. They are not to be swallowed. They are supplied as ready-to-use solutions or concentrated solutions to be diluted. They may also be prepared from powders or tablets to be dissolved in water before use.

Mouthwashes may contain excipients to adjust the pH which, as far as possible, is neutral.

## Gingival solutions

DEFINITION

Gingival solutions are liquid, usually multidose preparations intended for administration to the gingivae by means of a suitable applicator.

## Oromucosal solutions and oromucosal suspensions

DEFINITION

Oromucosal solutions and oromucosal suspensions are liquid preparations intended for administration to the oral cavity by means of a suitable applicator.

Oromucosal suspensions may show a sediment which is readily dispersible on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

## Semi-solid oromucosal preparations

DEFINITION

Semi-solid oromucosal preparations are hydrophilic gels, creams, ointments or pastes intended for administration to the oral cavity or to a specific part of the oral cavity such as the gingivae (gingival gel, gingival cream, gingival ointment, gingival paste). They may be provided as single-dose preparations.

Semi-solid oromucosal preparations comply with the requirements of the monograph *Semi-solid preparations for cutaneous use (0132)*.

## Oromucosal drops, oromucosal sprays and sublingual sprays

DEFINITION

Oromucosal drops, oromucosal sprays and sublingual sprays are solutions, emulsions or suspensions intended for local or systemic effect. They are applied by instillation or spraying into the oral cavity or onto a specific part of the oral cavity such as spraying under the tongue (sublingual spray) or into the throat (oropharyngeal spray).

Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be delivered.

Liquid oromucosal sprays are supplied in containers with atomising devices or in pressurised containers having a suitable adaptor, with or without a metering dose valve, which comply with the requirements of the monograph *Pressurised pharmaceutical preparations (0523)*.

The size of the droplets of the spray is such as to localise their deposition in the oral cavity or the throat as intended.

TESTS

Unless otherwise prescribed or justified and authorised, oromucosal drops supplied in single-dose containers, single doses of metered-dose oromucosal sprays and sublingual sprays, all intended for systemic action, comply with the following tests.

*OROMUCOSAL DROPS IN SINGLE-DOSE CONTAINERS*

**Uniformity of dosage units**. Oromucosal drops in single-dose containers comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of mass or uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of mass**. *Oromucosal drops that are solutions comply with the following test.* Weigh individually the contents of 10 containers emptied as completely as possible, and determine the average mass. Maximum 2 of the individual masses deviate by more than 10 per cent from the average mass and none deviates by more than 20 per cent.

**Uniformity of content** (*2.9.6*). *Oromucosal drops that are suspensions or emulsions comply with the following test.* Empty each container as completely as possible and carry out the test on the individual contents. They comply with test B of uniformity of content.

*METERED-DOSE OROMUCOSAL SPRAYS AND SUBLINGUAL SPRAYS*

**Uniformity of dosage units**. Metered-dose oromucosal sprays and sublingual sprays comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of mass or the test for uniformity of delivered dose shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

*In the case of metered-dose oromucosal sprays and sublingual sprays that are solutions, proceed as follows.* Discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers. Determine the mass variation (*2.9.40*).

**Uniformity of mass**. *Metered-dose oromucosal sprays and sublingual sprays that are solutions comply with the following test.* Discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers.

The preparation complies with the test if maximum 2 of the individual values deviate by more than 25 per cent from the average value and none deviates by more than 35 per cent.

**Uniformity of delivered dose**. *Metered-dose oromucosal sprays and sublingual sprays that are suspensions or emulsions comply with the following test*. Use an apparatus capable of quantitatively retaining the dose leaving the actuator of the atomising device.

Shake the container for 5 s and discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 s, fire 1 dose of the metered-dose spray into the collecting vessel by actuating the atomising device. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers.

Unless otherwise justified and authorised, the preparation complies with the test if maximum 1 of the individual contents is outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

If 2 or maximum 3 individual contents are outside the limits of 75 per cent to 125 per cent but within the limits of 65 per cent to 135 per cent, repeat the test for 20 more containers. The preparation complies with the test if maximum 3 individual contents of the 30 individual contents are outside the limits of 75 per cent to 125 per cent and none is outside the limits of 65 per cent to 135 per cent of the average content.

## Lozenges and pastilles

DEFINITION

Lozenges and pastilles are solid, single-dose preparations intended to be sucked to obtain, usually, a local effect in the oral cavity and the throat. They contain one or more active substances, usually in a flavoured and sweetened base, and are intended to dissolve or disintegrate slowly in the mouth when sucked.

Lozenges are hard preparations prepared by moulding. Pastilles are soft, flexible preparations prepared by moulding of mixtures containing natural or synthetic polymers or gums and sweeteners.

## Compressed lozenges

DEFINITION

Compressed lozenges are solid, single-dose preparations intended to be sucked to obtain a local or systemic effect. They are prepared by compression and are often rhomboid in shape.

Compressed lozenges conform with the general definition of tablets.

PRODUCTION

In the manufacture of compressed lozenges, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the *Friability of uncoated tablets* (*2.9.7*) and the *Resistance to crushing of tablets* (*2.9.8*).

TESTS

**Dissolution**. Unless otherwise justified and authorised, for compressed lozenges intended for a systemic effect, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in general chapter *2.9.3. Dissolution of solid dosage forms*.

## Sublingual tablets and buccal tablets

DEFINITION

Sublingual tablets and buccal tablets are solid, single-dose preparations to be applied under the tongue or to the buccal cavity, respectively, to obtain a systemic effect. They are prepared by compression of mixtures of powders or granulations into tablets with a shape suited for the intended use.

Sublingual tablets and buccal tablets conform to the general definition of tablets.

PRODUCTION

In the manufacture of sublingual tablets and buccal tablets, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. This may be demonstrated by examining the *Friability of uncoated tablets* (*2.9.7*) and the *Resistance to crushing of tablets* (*2.9.8*).

TESTS

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in general chapter *2.9.3. Dissolution of solid dosage forms*.

## Oromucosal capsules

DEFINITION

Oromucosal capsules are soft capsules to be chewed or sucked.

## Mucoadhesive preparations

DEFINITION

Mucoadhesive preparations contain one or more active substances intended for systemic absorption through the buccal mucosa over a prolonged period of time. They may be supplied as mucoadhesive buccal tablets, as buccal films or as other mucoadhesive solid or semi-solid preparations. They usually contain hydrophilic polymers, which on wetting with the saliva produce a hydrogel that adheres to the buccal mucosa; in addition, buccal films may dissolve.

Mucoadhesive buccal tablets are prepared by compression and may be single- or multilayer tablets.

Buccal films are single- or multilayer sheets of suitable materials.

PRODUCTION

In the manufacture of mucoadhesive buccal tablets and of buccal films, measures are taken to ensure that they possess suitable mechanical strength to resist handling without crumbling or breaking. For mucoadhesive buccal tablets this may be demonstrated by examining the *Friability of uncoated tablets* (*2.9.7*) and the *Resistance to crushing of tablets* (*2.9.8*).

TESTS

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in general chapter *2.9.3. Dissolution of solid dosage forms*.

## Orodispersible films

DEFINITION

Orodispersible films are single- or multilayer sheets of suitable materials, to be placed in the mouth where they disperse rapidly.

PRODUCTION

In the manufacture of orodispersible films, measures are taken to ensure that they possess suitable mechanical strength to resist handling without being damaged.

TESTS

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out to demonstrate the appropriate release of the active substance(s), for example one of the tests described in general chapter *2.9.3. Dissolution of solid dosage forms*.

**04/2015:0520**
**corrected 10.0**

# PARENTERAL PREPARATIONS

## Parenteralia

*The requirements of this monograph do not necessarily apply to products derived from human blood, to immunological preparations, or radiopharmaceutical preparations. Special requirements may apply to preparations for veterinary use depending on the species of animal for which the preparation is intended.*

DEFINITION

Parenteral preparations are sterile preparations intended for administration by injection, infusion or implantation into the human or animal body.

Parenteral preparations may require the use of excipients, for example to make the preparation isotonic with respect to blood, to adjust the pH, to increase solubility, to prevent deterioration of the active substances or to provide adequate antimicrobial properties, but not to adversely affect the intended medicinal action of the preparation or, at the concentrations used, to cause toxicity or undue local irritation.

Containers for parenteral preparations are made as far as possible from materials that are sufficiently transparent to permit the visual inspection of the contents, except for implants and in other justified and authorised cases.

Where applicable, the containers for parenteral preparations comply with the requirements for *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Parenteral preparations intended for chronic use or total parenteral nutrition should have appropriate limits for specific components or elements, taking long-term toxicity into account.

Parenteral preparations are supplied in glass containers (*3.2.1*) or in other containers such as plastic containers (*3.2.2*, *3.2.2.1* and *3.2.9*) and prefilled syringes. The tightness of the container is ensured by suitable means. Closures ensure a good seal, prevent the access of micro-organisms and other contaminants and usually permit the withdrawal of a part or the whole of the contents without removal of the closure. The plastic materials or elastomers (*3.2.9*) used to manufacture the closures are sufficiently firm and elastic to allow the passage of a needle with the least possible shedding of particles. Closures for multidose containers are sufficiently elastic to ensure that the puncture is resealed when the needle is withdrawn.

Several categories of parenteral preparations may be distinguished:

- injections;
- infusions;
- concentrates for injections or infusions;
- powders for injections or infusions;
- gels for injections;
- implants.

PRODUCTION

During the development of a parenteral preparation, the formulation for which contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided under *Efficacy of antimicrobial preservation* (*5.1.3*).

Parenteral preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms. Recommendations on this aspect are provided in the text *Methods of preparation of sterile products* (*5.1.1*).

Water used in the manufacture of parenteral preparations complies with the requirements of water for injections in bulk stated in the monograph *Water for injections (0169)*.

TESTS

**Particulate contamination: sub-visible particles** (*2.9.19*). For preparations for human use, solutions for infusion or solutions for injection comply with the test.

In the case of preparations for subcutaneous or intramuscular injection, higher limits may be appropriate. Radiopharmaceutical preparations are exempt from these requirements. Preparations for which the label states that the product is to be used with a final filter are exempt from these requirements, providing it has been demonstrated that the filter delivers a solution that complies with the test.

For preparations for veterinary use, when supplied in containers with a nominal content of more than 100 mL and when the content is equivalent to a dose of more than 1.4 mL per kilogram of body mass, solutions for infusion or solutions for injection comply with the test for particulate contamination: sub-visible particles.

**Sterility** (*2.6.1*). Parenteral preparations comply with the test for sterility.

STORAGE

In a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name and concentration of any added antimicrobial preservative;
- where applicable, that the solution is to be used in conjunction with a final filter;
- where applicable, that the preparation is free from bacterial endotoxins or that it is apyrogenic.

## Injections

DEFINITION

Injections are sterile solutions, emulsions or suspensions. They are prepared by dissolving, emulsifying or suspending the active substance(s) and any added excipients in water, in a suitable non-aqueous liquid, that may be non-sterile where justified, or in a mixture of these vehicles.

Solutions for injection, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for injection do not show any evidence of phase separation. Suspensions for injection may show a sediment which is readily dispersed on shaking to give a suspension which remains sufficiently stable to enable the correct dose to be withdrawn.

*Multidose preparations.* Multidose aqueous injections contain a suitable antimicrobial preservative at an appropriate concentration except when the preparation itself has adequate antimicrobial properties. When a preparation for parenteral administration is presented in a multidose container, the precautions to be taken for its administration and more particularly for its storage between successive withdrawals are given.

*Antimicrobial preservatives.* Aqueous preparations which are prepared using aseptic precautions and which cannot be terminally sterilised may contain a suitable antimicrobial preservative in an appropriate concentration.

No antimicrobial preservative is added when:

- the volume to be injected in a single dose exceeds 15 mL, unless otherwise justified;
- the preparation is intended for administration by routes where, for medical reasons, an antimicrobial preservative is not acceptable, such as intracisternally, epidurally, intrathecally or by any route giving access to the cerebrospinal fluid, or intra- or retro-ocularly.

Such preparations are presented in single-dose containers.

PRODUCTION

In the manufacture of injections containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

*Single-dose preparations.* The volume of the injection in a single-dose container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique (*2.9.17*).

TESTS

**Uniformity of dosage units**. Single-dose suspensions for injection comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose suspensions for injection with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Bacterial endotoxins - pyrogens**. A test for bacterial endotoxins (*2.6.14*) is carried out or, where justified and authorised, the test for pyrogens (*2.6.8*). Recommendations on the limits for bacterial endotoxins are given in general chapter *5.1.10*.

*Preparations for human use.* The preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*).

*Preparations for veterinary use.* When the volume to be injected in a single dose is 15 mL or more and is equivalent to a dose of 0.2 mL or more per kilogram of body mass, the preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*).

*Any preparation.* Where the label states that the preparation is free from bacterial endotoxins or apyrogenic, respectively, the preparation complies with a test for bacterial endotoxins (*2.6.14*) or with the test for pyrogens (*2.6.8*), respectively.

## Infusions

DEFINITION

Infusions are sterile, aqueous solutions or emulsions with water as the continuous phase. They are usually made isotonic with respect to blood. They are principally intended for administration in large volume. Infusions do not contain any added antimicrobial preservative.

Solutions for infusion, examined under suitable conditions of visibility, are clear and practically free from particles.

Emulsions for infusion do not show any evidence of phase separation.

PRODUCTION

In the manufacture of infusions containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

The volume of the infusion in the container is sufficient to permit the withdrawal and administration of the nominal dose using a normal technique (*2.9.17*).

TESTS

**Bacterial endotoxins - pyrogens**. They comply with a test for bacterial endotoxins (*2.6.14*) or, where justified and authorised, with the test for pyrogens (*2.6.8*). For the latter test inject 10 mL per kilogram of body mass into each rabbit, unless otherwise justified and authorised.

## Concentrates for injections or infusions

DEFINITION

Concentrates for injections or infusions are sterile solutions intended for injection or infusion after dilution. They are diluted to a prescribed volume with a prescribed liquid before administration. After dilution, they comply with the requirements for injections or for infusions.

TESTS

**Bacterial endotoxins - pyrogens**. They comply with the requirements prescribed for injections or for infusions, after dilution to a suitable volume.

## Powders for injections or infusions

DEFINITION

Powders for injections or infusions are solid, sterile substances distributed in their final containers and which, when shaken with the prescribed volume of a prescribed sterile liquid rapidly form either clear and practically particle-free solutions or uniform suspensions. After dissolution or suspension, they comply with the requirements for injections or for infusions.

Freeze-dried products for parenteral administration are considered as powders for injections or infusions.

PRODUCTION

The uniformity of content and uniformity of mass of freeze-dried products for parenteral administration are ensured by the in-process control of the amount of the solution prior to freeze-drying.

TESTS

**Uniformity of dosage units**. Powders for injections or infusions comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, powders for injections or infusions with a content of active substance less than 2 mg or less than 2 per cent of the total mass, or with a unit mass equal to or less than 40 mg comply with test A for uniformity of content of single-dose preparations. If the preparation contains more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Powders for injections or infusions comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Bacterial endotoxins - pyrogens**. They comply with the requirements prescribed for injections or for infusions, after dissolution or suspension in a suitable volume of liquid.

LABELLING

The label states the instructions for the preparation of injections and infusions.

## Gels for injections

DEFINITION

Gels for injections are sterile gels with a viscosity suitable to guarantee a modified release of the active substance(s) at the site of injection.

## Implants

DEFINITION

Implants are sterile, solid preparations of a size and shape suitable for parenteral implantation and release of the active substance(s) over an extended period of time. Each dose is provided in a sterile container.

TESTS

A suitable test is carried out to demonstrate the appropriate release of the active substance(s).

**01/2008:1011**

# PATCHES, TRANSDERMAL

## Emplastra transcutanea

DEFINITION

Transdermal patches are flexible pharmaceutical preparations of varying sizes, containing one or more active substances. They are intended to be applied to the unbroken skin in order to deliver the active substance(s) to the systemic circulation after passing through the skin barrier.

Transdermal patches normally consist of an outer covering which supports a preparation which contains the active substance(s). The transdermal patches are covered on the site of the release surface of the preparation by a protective liner, which is removed before applying the patch to the skin.

The outer covering is a backing sheet impermeable to the active substance(s) and normally impermeable to water, designed to support and protect the preparation. The outer covering may have the same dimensions as the preparation or it may be larger. In the latter case the overlapping border of the outer covering is covered by pressure-sensitive adhesive substances which assure the adhesion of the patch to the skin.

The preparation contains the active substance(s) together with excipients such as stabilisers, solubilisers or substances intended to modify the release rate or to enhance transdermal absorption. It may be a single layer or multi-layer solid or semi-solid matrix, and in this case it is the composition and structure of the matrix which determines the diffusion pattern of the active substance(s) to the skin. The matrix may contain pressure-sensitive adhesives which assure the adhesion of the preparation to the skin. The preparation may exist as a semi-solid reservoir one side of which is a membrane which may control the release and the diffusion of the active substance(s) from the preparation. The pressure-sensitive adhesive substances may, in this case, be applied to some or all parts of the membrane, or only around the border of the membrane of the outer covering.

When applied to the dried, clean and unbroken skin, the transdermal patch adheres firmly to the skin by gentle pressure of the hand or the fingers and can be peeled off without causing appreciable injury to the skin or detachment of the preparation from the outer covering. The patch must not be irritant or sensitising to the skin, even after repeated applications.

The protective liner generally consists of a sheet of plastic or metal material. When removed, the protective liner does not detach the preparation (matrix or reservoir) or the adhesive from the patch.

Transdermal patches are normally individually enclosed in sealed sachets.

PRODUCTION

In the manufacture, packaging, storage and distribution of transdermal patches suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in the text on *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Transdermal patches comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, transdermal patches comply with test C for uniformity of content of single-dose preparations.

**Dissolution**. A suitable test may be required to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for transdermal patches* (*2.9.4*). The disc assembly method, the cell method or the rotating cylinder method may be used, as suitable, according to the composition, dimensions and shape of the patch.

A membrane may be used. It can be of various materials, such as inert porous cellulose or silicones, and must not affect the release kinetics of the active substance(s) from the patch. Furthermore, it must be free of substances that may interfere with its performance (for example grease). The membrane may be suitably treated before the tests, for example, by maintaining it in the medium to be used in the test for 24 h. Apply the membrane above the releasing surface of the patch, avoiding the formation of air bubbles.

The test conditions and the requirements are to be authorised by the competent authority.

STORAGE

Store at room temperature, unless otherwise indicated.

LABELLING

The label states, where applicable, the total quantity of active substance(s) per patch, the dose released per unit time and the area of the releasing surface.

**01/2017:1166**

# POWDERS FOR CUTANEOUS APPLICATION

## Pulveres ad usum dermicum

*Where justified and authorised, the requirements of this monograph do not apply to powders for cutaneous application intended for veterinary use.*

DEFINITION

Powders for cutaneous application are preparations consisting of solid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients and, if necessary, colouring matter authorised by the competent authority.

Powders for cutaneous application are presented as single-dose powders or multidose powders. They are free from grittiness. Powders specifically intended for use on large open wounds or on severely injured skin are sterile.

Multidose powders for cutaneous application may be dispensed in sifter-top containers, containers equipped with a mechanical spraying device or in pressurised containers.

Powders dispensed in pressurised containers comply with the requirements of *Pressurised pharmaceutical preparations (0523)*.

Where applicable, containers for powders comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

PRODUCTION

In the manufacture of powders for cutaneous application, measures are taken to ensure a suitable particle size with regard to the intended use.

In the manufacture, packaging, storage and distribution of powders for cutaneous application, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Sterile powders for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

TESTS

**Fineness**. If prescribed, the fineness of a powder is determined by the sieve test (*2.9.35*) or another appropriate method.

**Uniformity of dosage units**. Single-dose powders for cutaneous application comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose powders for cutaneous application with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose powders for cutaneous application comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Sterility** (*2.6.1*). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

LABELLING

The label states, where applicable, that the preparation is sterile.

**01/2020:1165**

# POWDERS, ORAL

## Pulveres perorales

*Requirements for powders to be used for the preparation of oral solutions or suspensions are given in the monograph Liquid preparations for oral use (0672). Where justified and authorised, the requirements of this monograph do not apply to oral powders intended for veterinary use.*

DEFINITION

Oral powders are preparations consisting of solid, loose, dry particles of varying degrees of fineness. They contain one or more active substances, with or without excipients and, if necessary, colouring matter authorised by the competent authority and flavouring substances. They are generally administered in or with water or another suitable liquid. They may also be swallowed directly. They are presented as single-dose or multidose preparations.

Where applicable, containers for oral powders comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Multidose oral powders require the provision of a measuring device capable of delivering the quantity prescribed. Each dose of a single-dose powder is enclosed in an individual container, for example a sachet or a vial.

PRODUCTION

In the manufacture of oral powders, measures are taken to ensure a suitable particle size with regard to the intended use.

In the manufacture, packaging, storage and distribution of oral powders, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Uniformity of dosage units**. Single-dose oral powders comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose oral powders with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B for uniformity of content of single-dose preparations. If the preparation has more than one active substance, the requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose oral powders comply with the test for uniformity of mass of single-dose preparations. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Uniformity of mass of delivered doses from multidose containers** (*2.9.27*). Oral powders supplied in multidose containers comply with the test.

STORAGE

If the preparation contains volatile ingredients, or the contents have to be protected, store in an airtight container.

## Effervescent powders

DEFINITION

Effervescent powders are presented as single-dose or multidose preparations and generally contain acid substances and carbonates or hydrogen carbonates that react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

TESTS

Unless otherwise justified and authorised, the preparation complies with the following test.

**Disintegration**. Place 1 dose of the effervescent powder in a beaker containing 200 mL of *water R* at 15-25 °C; numerous bubbles of gas are evolved. When the evolution of gas around the particles ceases, the powder has disintegrated, being either dissolved or dispersed in the water. Repeat the operation on 5 further doses. The preparation complies with the test if each of the 6 doses used disintegrates within 5 min.

STORAGE

In an airtight container.

**01/2014:1037**

# PREMIXES FOR MEDICATED FEEDING STUFFS FOR VETERINARY USE

## Praeadmixta ad alimenta medicata ad usum veterinarium

DEFINITION

Mixtures of one or more active substances, usually in a suitable basis or vehicle, that are prepared to facilitate feeding the active substances to animals. They are used exclusively in the preparation of medicated feeding stuffs.

Premixes occur in granulated, powdered, semi-solid or liquid form. Used as powders or granules, they are free-flowing and homogeneous; any aggregates break apart during normal handling. Used in liquid form, they are homogeneous suspensions or solutions that may be obtained from thixotropic gels or structured liquids. The particle size and other properties are such as to ensure uniform distribution of the active substance(s) in the final feed. Unless otherwise justified and authorised, the instructions for use state that the concentration of a premix in granulated or powdered form is at least 0.5 per cent in the medicated feeding stuff.

PRODUCTION

In the manufacture, packaging, storage and distribution of premixes for medicated feeding stuffs for veterinary use, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

**Active substance**. Unless already otherwise justified and authorised for existing premixes, an active substance intended for incorporation into a medicated premix:

- complies with the requirements of the relevant monograph of the European Pharmacopoeia;
- in the case of a fermentation product that is not the subject of a monograph of the European Pharmacopoeia, complies with the monograph *Products of fermentation (1468)*, notably with the section Down-stream processing.

TESTS

**Loss on drying** (*2.2.32*): unless otherwise justified and authorised, for premixes occurring in granulated or powdered form, maximum 15.0 per cent, determined on 3.000 g by drying in an oven at 105 °C for 2 h.

LABELLING

The label states the instructions for the preparation of the medicated feeding stuffs from the premix and the basic feed, including information on whether or not the premix can be granulated with the feed and the critical parameters (e.g. maximum temperature) that may be applied during the process.

**04/2018:0671**

# PREPARATIONS FOR INHALATION

## Inhalanda

DEFINITION

Preparations for inhalation are liquid, semi-solid or solid preparations intended for administration as vapours or aerosols to the lung in order to obtain a local or systemic effect. They contain one or more active substances that may be dissolved or dispersed in a suitable vehicle.

Preparations for inhalation may, depending on the type of preparation, contain propellants, cosolvents, diluents, antimicrobial preservatives, carriers, solubilising and stabilising agents, etc. These excipients do not adversely affect the functions of the mucosa of the respiratory tract or its cilia.

Suspensions and emulsions are readily dispersible on shaking and they remain sufficiently stable to enable the correct dose to be delivered.

Preparations for inhalation are supplied in multidose or single-dose containers. When supplied in pressurised containers, they comply with the requirements of the monograph *Pressurised pharmaceutical preparations (0523)*.

Preparations intended to be administered as aerosols (dispersions of solid or liquid particles in a gas) are administered by one of the following devices:

- a nebuliser;
- an inhaler (pressurised metered-dose inhaler, non-pressurised metered-dose inhaler or powder inhaler).

Several categories of preparations for inhalation may be distinguished:

- preparations to be converted into vapour;
- liquid preparations for nebulisation;
- pressurised metered-dose preparations for inhalation;
- non-pressurised metered-dose preparations for inhalation;
- inhalation powders.

PRODUCTION

During the development of a preparation for inhalation that contains an antimicrobial preservative, the effectiveness of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with the criteria for judging the preservative properties of the formulation are described in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

In the manufacture, packaging, storage and distribution of preparations for inhalation, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Uniformity of delivered dose of a multidose inhaler must be ensured within a device (intra-inhaler) and between devices (inter-inhaler). For intra-inhaler testing, the uniformity of delivered dose tests are described in the Tests sections of the various preparation categories in this monograph. For inter-inhaler testing, an example of a suitable procedure is to take 10 inhalers and collect a single dose from each inhaler, collecting the dose at the beginning (from 3 inhalers), middle (from 4 inhalers) and end (from 3 inhalers) of the number of doses stated on the label. Other inter-inhaler testing procedures are possible, where justified.

LABELLING

For preparations administered by an inhaler, the label states:

- the delivered dose or, if justified and authorised (e.g. where the dose has been established as a metered dose or as a pre-metered dose), the metered dose or the pre-metered dose;
- where applicable, the number of deliveries from the inhaler to provide the minimum recommended dose;
- the number of deliveries per inhaler.

The label states, where applicable, the name of any added antimicrobial preservative.

## Preparations to be converted into vapour

DEFINITION

Preparations intended to be converted into vapour are liquids, solutions, suspensions, emulsions, or semi-solid or solid preparations. They are usually added to hot water and the vapour generated is inhaled.

## Liquid preparations for nebulisation

DEFINITION

Liquid preparations for nebulisation are solutions, suspensions or emulsions intended to be converted into aerosols by nebulisers.

Liquid preparations for nebulisation in concentrated form are diluted to the prescribed volume with the prescribed liquid before use. Liquid preparations for nebulisation may also be prepared from powders by reconstitution in the prescribed liquid.

The pH of liquid preparations for nebulisation is not lower than 3 and not higher than 10.

Liquid preparations for nebulisation supplied in multidose containers may contain a suitable antimicrobial preservative at a suitable concentration except where the preparation itself has adequate antimicrobial properties.

Liquid preparations for nebulisation supplied in multidose containers that do not contain an antimicrobial preservative, and where the preparation itself does not have adequate antimicrobial properties, are sterile and are supplied in containers preventing microbial contamination of the contents during storage and use.

Liquid preparations for nebulisation supplied in single-dose containers are sterile and preservative-free, unless otherwise justified and authorised.

Nebulisers are devices that convert liquids into aerosols by high-pressure gases, ultrasonic vibration, extrusion through a mesh or other methods. They allow the dose to be inhaled at an appropriate active-substance delivery rate over an extended period of time involving consecutive inhalations and with a particle size that allows deposition of the preparation in the lungs.

Nebulisers may be breath-triggered or use other means to synchronise or modify the nebuliser operation with the patient's breathing.

PRODUCTION

The active substance delivery rate, the total active substance delivered and the particle-/droplet-size distribution are determined using the methods described in general chapter *2.9.44. Preparations for nebulisation: characterisation*. Where justified and authorised, different apparatus and procedures may be used.

TESTS

*Prepare the liquid preparation for nebulisation as directed in the instructions to the patient.*

**Uniformity of dosage units**. Solutions, suspensions and emulsions in single-dose containers comply with the test for uniformity of dosage units (*2.9.40*) or, where justified and authorised, with the test for uniformity of content or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose preparations that are emulsions or suspensions comply with the following test: after shaking, empty each container as completely as possible and carry out the test on the individual contents; they comply with test B for uniformity of content of single-dose preparations.

**Uniformity of mass** (*2.9.5*). Single-dose preparations that are solutions comply with the following test: weigh individually the contents of 20 containers, emptied as completely as possible, and determine the average mass; not more than 2 of the individual masses deviate by more than 10 per cent from the average mass and none deviate by more than 20 per cent.

**Aerodynamic assessment of nebulised aerosols**. For liquid preparations for nebulisation that are suspensions, determine fine-particle mass using an apparatus and procedure described in general chapter *2.9.44. Preparations for nebulisation: characterisation*. Where justified and authorised, a different apparatus and procedure may be used.

## Pressurised metered-dose preparations for inhalation

DEFINITION

Pressurised metered-dose preparations for inhalation are solutions, suspensions or emulsions supplied in containers equipped with a metering valve and which are held under pressure with (a) suitable propellant(s), which can act also as a solvent.

The delivered dose is the dose delivered from the inhaler. For some preparations the dose has been established as a metered dose. The metered dose is determined by adding the amount deposited on the inhaler to the delivered dose. It may also be determined directly.

PRODUCTION

The size of aerosol particles to be inhaled is controlled so that a consistent portion is deposited in the lungs. The fine-particle characteristics of pressurised metered-dose preparations for inhalation are determined using the method described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles*.

TESTS

*For breath-triggered pressurised metered-dose inhalers, the test conditions described below may need to be modified to ensure that actuation occurs for the inhaler under test.*

*Prepare and use the inhaler as directed in the instructions to the patient.*

**Uniformity of delivered dose**. Pressurised metered-dose inhalers usually operate in a valve-down position. For inhalers that operate in a valve-up position, an equivalent test is applied using methods that ensure the complete collection of the delivered dose.

The dose collection apparatus must be capable of quantitatively capturing the delivered dose.

The following apparatus (Figure 0671.-1) and procedure may be used.

The apparatus consists of a filter-support base with an open-mesh filter-support, such as a stainless steel screen, a collection tube that is clamped or screwed to the filter-support base, and a mouthpiece adapter to ensure an airtight seal between the collection tube and the mouthpiece. Use a mouthpiece adapter that ensures that the front face of the inhaler mouthpiece is flush with the front face or the 2.5 mm indented shoulder of the sample collection tube, as appropriate. The vacuum connector is connected to a system comprising a vacuum source and a flow regulator. The source is adjusted to draw air through the complete assembly, including the filter and the inhaler to be tested, at 28.3 L/min (± 5 per cent). Air should be drawn continuously through the apparatus to avoid loss of the active substance into the atmosphere. The filter-support base is designed to accommodate 25 mm diameter filter disks. The filter disk and other materials used in the construction of the apparatus must be compatible with the active substance and solvents that are used to extract the active substance from the filter. One end of the collection tube is designed to hold the filter disk tightly against the filter-support base. When assembled, the joints between the components of the apparatus are airtight so that when a vacuum is applied to the base of the filter, all of the air drawn through the collection tube passes through the inhaler.

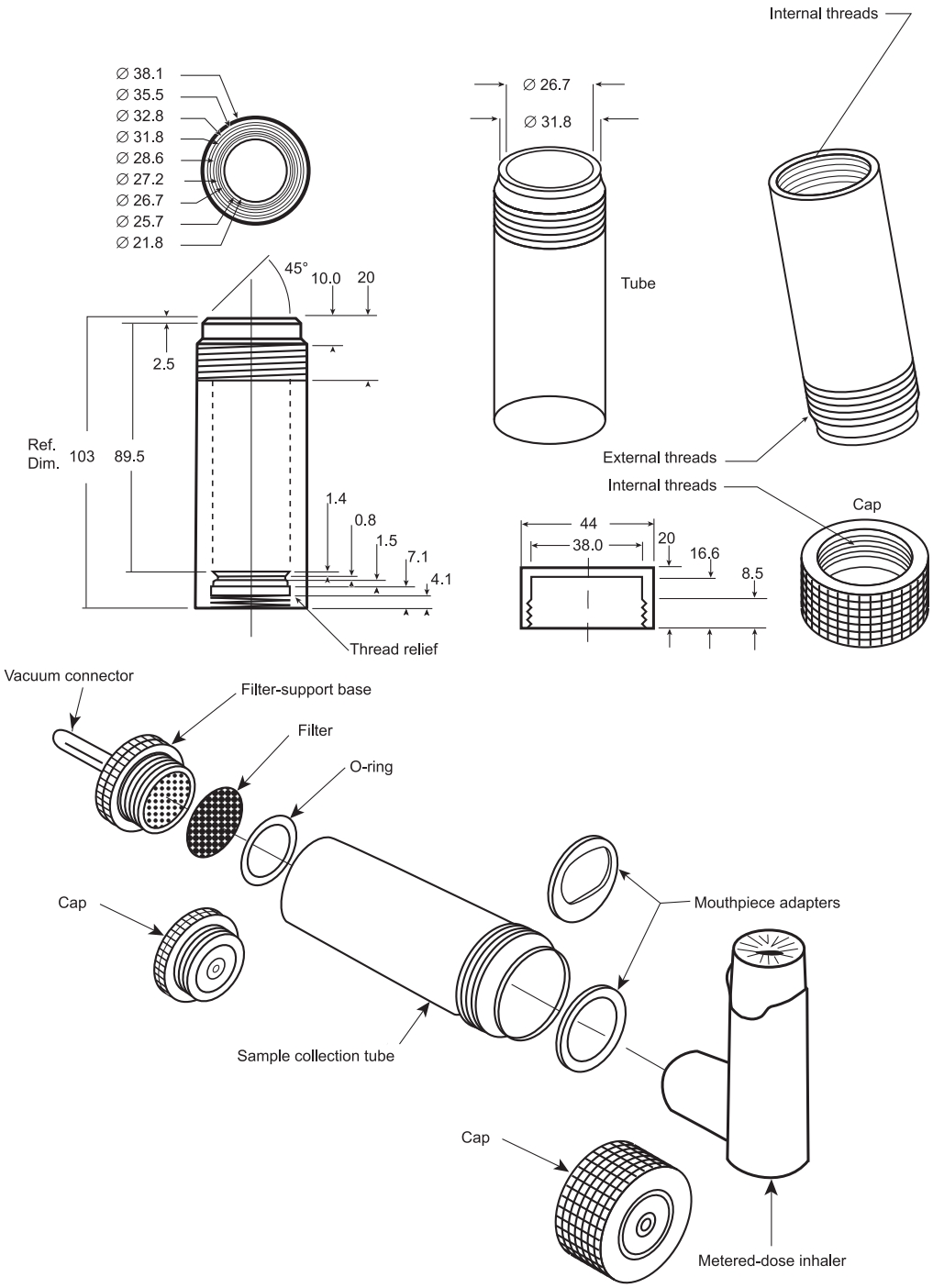


Figure 0671.-1. – *Dose collection apparatus for pressurised metered-dose inhalers*
*Dimensions in millimetres*

Unless otherwise prescribed in the instructions to the patient, shake the inhaler for 5 s and discharge 1 delivery to waste. Discharge the inverted inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Repeat the procedure until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 2 doses.

Discharge the inhaler to waste, waiting not less than 5 s between actuations, until ($n$/2) + 1 deliveries remain, where $n$ is the number of deliveries stated on the label. Collect 4 doses using the procedure described above.

Discharge the inhaler to waste, waiting not less than 5 s between actuations, until 3 doses remain. Collect these 3 doses using the procedure described above.

For preparations containing more than 1 active substance, carry out the test for uniformity of delivered dose for each active substance.

Unless otherwise justified and authorised, the preparation complies with the test if 9 out of 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent. If 2 or 3 values lie outside the limits of 75 per cent to 125 per cent, repeat the test for 2 more inhalers. Not more than 3 of the 30 values lie outside the limits of 75 per cent to 125 per cent and no value lies outside the limits of 65 per cent to 135 per cent. Unless otherwise authorised, the mean value must be between 85 per cent and 115 per cent of the label claim for delivered dose.

**Fine particle dose**. Using an apparatus and procedure described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles* (apparatus C, D or E), calculate the fine particle dose.

**Number of deliveries per inhaler**. Take 1 inhaler and discharge the contents to waste, actuating the valve at intervals of not less than 5 s. The total number of deliveries so discharged from the inhaler is not less than the number stated on the label (this test may be combined with the test for uniformity of delivered dose).

**Leak rate**. Take a suitable number of containers, for example 1 container, remove any labels and record the date and time to the nearest half hour. Weigh the container to the nearest milligram and record the mass ($M_1$) in milligrams. Allow the container to stand in an upright position at a temperature of 25.0 ± 2.0 °C for not less than 3 days, and again weigh the container, recording the mass ($M_2$) in milligrams, and recording the date and time to the nearest half hour. Determine the time ($T$), in hours, during which the container was under test.

Calculate the total loss of mass, in milligrams, over the entire shelf life ($D$), in months, of the container, using the following expression:

$$\frac{730 \times D}{T} \times (M_1 - M_2)$$

Unless otherwise justified, the preparation complies if the total loss of mass over the entire shelf life is not more than 10 per cent ($m/m$) of the nominal fill mass of the container.

## Non-pressurised metered-dose preparations for inhalation

DEFINITION

Non-pressurised metered-dose preparations for inhalation are solutions, suspensions or emulsions for use with inhalers that convert liquids into aerosols using single or multiple liquid jets, ultrasonic vibration or other methods. The volume of liquid to be converted into an aerosol is pre-metered or metered by the inhaler so that the dose delivered from the inhaler can be inhaled with 1 or more inspirations.

Non-pressurised metered-dose preparations for inhalation supplied in multidose containers may contain a suitable antimicrobial preservative at a suitable concentration except where the preparation itself has adequate antimicrobial properties.

Non-pressurised metered-dose preparations for inhalation supplied in multidose containers that do not contain an antimicrobial preservative, and where the preparation itself does not have adequate antimicrobial properties, are sterile and are supplied in containers preventing microbial contamination of the contents during storage and use.

Non-pressurised metered-dose preparations for inhalation supplied in single-dose containers are sterile and preservative-free, unless otherwise justified and authorised.

PRODUCTION

The size of aerosol particles to be inhaled is controlled so that a consistent portion is deposited in the lung. The fine-particle characteristics of non-pressurised metered-dose preparations for inhalation are determined using the method described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles*. Alternatively, laser diffraction analysis may be used, when properly validated against method *2.9.18* (apparatus C, D or E).

TESTS

*For breath-triggered non-pressurised metered-dose inhalers, the test conditions described below may need to be modified to ensure that actuation occurs for the inhaler under test.*

*Prepare and use the inhaler as directed in the instructions to the patient.*

**Uniformity of delivered dose**. The dose collection apparatus must be capable of quantitatively capturing the delivered dose. The apparatus described in the test for uniformity of delivered dose for pressurised metered-dose preparations may be used.

Discharge the inhaler into the apparatus. Repeat the procedure until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 2 doses.

Discharge the inhaler to waste until ($n$/2) + 1 deliveries remain, where $n$ is the number of deliveries stated on the label. Collect 4 doses using the procedure described above.

Discharge the inhaler to waste until 3 doses remain. Collect these 3 doses using the procedure described above.

For preparations containing more than 1 active substance, carry out the test for uniformity of delivered dose for each active substance.

Unless otherwise justified and authorised, the preparation complies with the test if 9 out of 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent. If 2 or 3 values lie outside the limits of 75 per cent to 125 per cent, repeat the test for 2 more inhalers. Not more than 3 of the 30 values lie outside the limits of 75 per cent to 125 per cent and no value lies outside the limits of 65 per cent to 135 per cent. Unless otherwise authorised, the mean value must be between 85 per cent and 115 per cent of the label claim for delivered dose.

Where justified and authorised, another apparatus and procedure may be used.

**Fine particle dose**. Using an apparatus and procedure described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles* (apparatus C, D or E), calculate the fine particle dose. Use the same procedure as for pressurised inhalers with appropriate adaptation of the methodology to non-pressurised inhalers. Depending on the characteristics of the non-pressurised metered-dose preparations for inhalation, relative humidity and/or temperature may need to be controlled during the test.

**Number of deliveries per inhaler**. Take 1 inhaler and discharge the contents to waste. The total number of deliveries so discharged from the inhaler is not less than the number stated on the label (this test may be combined with the test for uniformity of delivered dose).

## Inhalation powders

DEFINITION

Inhalation powders are supplied in single-dose or multidose containers. Pre-metered inhalers are loaded with powders pre-dispensed in capsules or other suitable dosage forms. Device-metered inhalers may use a powder reservoir, where the dose is created by a metering mechanism within the inhaler.

To facilitate the use of inhalation powders, active substances may be combined with suitable excipients, e.g. a carrier substance.

The delivered dose is the dose delivered from the inhaler. For some preparations, the labelled dose has been established as a metered dose or as a pre-metered dose. The metered dose is determined by adding the amount deposited on the inhaler to the delivered dose. It may also be determined directly.

PRODUCTION

The size of aerosol particles to be inhaled is controlled so that a consistent portion is deposited in the lung. The fine-particle characteristics of powders for inhalation are determined using the method described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles*.

TESTS

*Prepare and use the inhaler as directed in the instructions to the patient.*

**Uniformity of delivered dose**. The dose collection apparatus must be capable of quantitatively capturing the delivered dose. A dose collection apparatus similar to that described for the evaluation of pressurised metered-dose inhalers may be used provided that the dimensions of the tube and the filter can accommodate the measured flow rate. A suitable tube is defined in Table 0671.-1. Connect the tube to a flow system according to the scheme specified in Figure 0671.-2 and Table 0671.-1.

Unless otherwise stated, determine the test flow rate and duration using the dose collection tube, the associated flow system, a suitable differential pressure meter and a suitable volumetric flowmeter, calibrated for the flow leaving the meter, according to the following procedure.

Table 0671.-1. – *Specifications of the apparatus used for powder inhalers described in Figure 0671.-2*

| Code | Item | Description |
|---|---|---|
| A | Sample collection tube | Capable of quantitatively capturing the delivered dose, e.g. dose collection tube similar to that described in Figure 0671.-1 with dimensions of 34.85 mm ID × 12 cm length (e.g. product number XX40 047 00, Millipore Corporation, Bedford, MA 01732, USA with modified exit tube, ID ≥ 8 mm, fitted with Gelman product number 61631), or equivalent. |
| B | Filter | 47 mm filter, e.g. A/E glass fibre filter (Gelman Sciences, Ann Arbor, MI 48106, USA), or equivalent. |
| C | Connector | ID ≥ 8 mm, e.g., short metal coupling, with low-diameter branch to P3. |
| D | Vacuum tubing | A length of suitable tubing having an ID ≥ 8 mm and an internal volume of 25 ± 5 mL. |
| E | 2-way solenoid valve | A 2-way, 2-port solenoid valve having a minimum airflow resistance orifice with ID ≥ 8 mm and an opening time ≤ 100 ms (e.g. type 256-A08, Bürkert GmbH, 74653 Ingelfingen, Deutschland), or equivalent. |
| F | Vacuum pump | Pump must be capable of drawing the required flow rate through the assembled apparatus with the powder inhaler in the mouthpiece adapter (e.g. product type 1023, 1423 or 2565, Gast Manufacturing Inc., Benton Harbor, MI 49022, USA), or equivalent. Connect the pump to the 2-way solenoid valve using short and/or wide (≥ 10 mm ID) vacuum tubing and connectors to minimise pump capacity requirements. |
| G | Timer | Timer capable of driving the 2-way solenoid valve for the required time period (e.g. type G814, RS Components International, Corby, NN17 9RS, UK), or equivalent. |
| P1 | Pressure tap | 2.2 mm ID, 3.1 mm OD, flush with internal surface of the sample collection tube, centred and burr-free, 59 mm from its inlet. The pressure tap P1 must never be open to the atmosphere. Differential pressure to atmosphere is measured at P1. |
| P2<br>P3 | Pressure measurements | Absolute pressures. |
| H | Flow control valve | Adjustable regulating valve with maximum $C_V \geq 1$ (e.g. type 8FV12LNSS, Parker Hannifin plc., Barnstaple, EX31 1NP, UK), or equivalent. |

Prepare the inhaler for use and connect it to the inlet of the apparatus using a mouthpiece adapter to ensure an airtight seal. Use a mouthpiece adapter that ensures that the front face of the inhaler mouthpiece is flush with the front face of the sample collection tube. Connect one port of a differential pressure meter to the pressure reading point P1 in

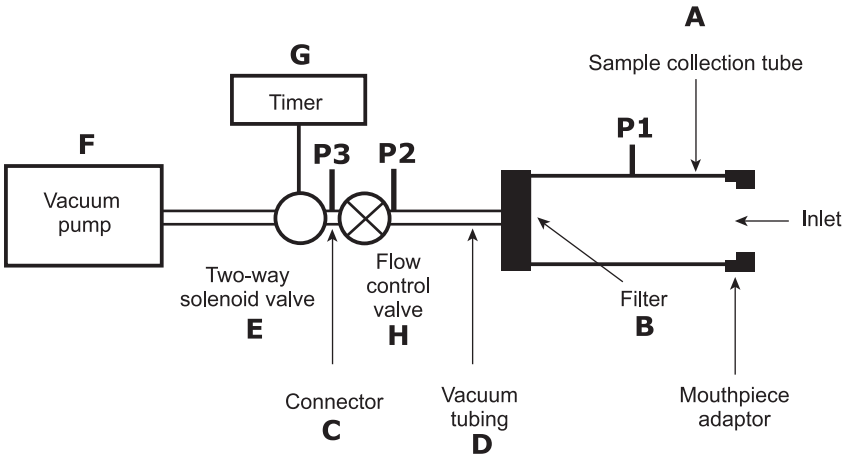


Figure 0671.-2. – *Apparatus suitable for measuring the uniformity of delivered dose for powder inhalers*

Figure 0671.-2, and let the other be open to the atmosphere. Switch on the pump, open the 2-way solenoid valve and adjust the flow control valve until the pressure drop across the inhaler is 4.0 kPa (40.8 cm $H_2O$) as indicated by the differential pressure meter. Remove the inhaler from the mouthpiece adapter and, without touching the flow control valve, connect a flowmeter to the inlet of the sampling apparatus. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter ($Q_{out}$) using the ideal gas law. For a meter calibrated for the entering volumetric flow ($Q_{in}$), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

$P_0$ = atmospheric pressure;

$\Delta P$ = pressure drop over the meter.

If the flow rate is above 100 L/min adjust the flow control valve to obtain a flow rate of 100 L/min (± 5 per cent). Note the volumetric airflow rate exiting the meter and define this as the test flow rate, $Q_{out}$, in litres per minute. Define the test flow duration, $T$, in seconds so that a volume of 4 L of air is drawn from the mouthpiece of the inhaler at the test flow rate, $Q_{out}$.

Ensure that critical flow occurs in the flow control valve by the following procedure: with the inhaler in place and the test flow rate $Q_{out}$, measure the absolute pressure on both sides of the control valve (pressure reading points P2 and P3 in Figure 0671.-2); a ratio P3/P2 of less than or equal to 0.5 indicates critical flow; switch to a more powerful pump and re-measure the test flow rate if critical flow is not indicated.

*Pre-metered inhalers.* Connect the inhaler to the apparatus using an adapter that ensures a good seal. Draw air through the inhaler using the predetermined conditions. Repeat the procedure until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 9 doses.

*Device-metered inhalers.* Connect the inhaler to the apparatus using an adapter that ensures a good seal. Draw air through the inhaler under the predetermined conditions. Repeat the procedure until the number of deliveries that constitute the minimum recommended dose have been sampled. Quantitatively collect the contents of the apparatus and determine the amount of active substance.

Repeat the procedure for a further 2 doses.

Discharge the inhaler to waste until ($n$/2) + 1 deliveries remain, where $n$ is the number of deliveries stated on the label. If necessary, store the inhaler to discharge electrostatic charges. Collect 4 doses using the procedure described above.

Discharge the inhaler to waste until 3 doses remain. If necessary, store the inhaler to discharge electrostatic charges. Collect 3 doses using the procedure described above.

For preparations containing more than 1 active substance, carry out the test for uniformity of delivered dose for each active substance.

*Results.* The preparation complies with the test if 9 out of 10 results lie between 75 per cent and 125 per cent of the mean value and all lie between 65 per cent and 135 per cent. If 2 or 3 values lie outside the limits of 75 per cent to 125 per cent, repeat the test for 2 more inhalers. Not more than 3 of the 30 values lie outside the limits of 75 per cent to 125 per cent and no value lies outside the limits of 65 per cent to 135 per cent.

In justified and authorised cases, these ranges may be extended but no value should be greater than 150 per cent or less than 50 per cent of the mean value. Unless otherwise authorised, the mean value must be between 85 per cent and 115 per cent of the label claim for delivered dose.

**Fine particle dose.** Using an apparatus and procedure described in general chapter *2.9.18. Preparations for inhalation: aerodynamic assessment of fine particles* (apparatus C, D or E), calculate the fine particle dose.

**Number of deliveries per inhaler for multidose inhalers.** Discharge doses from the inhaler until empty, at the predetermined flow rate. Record the deliveries discharged. The total number of deliveries so discharged from the inhaler is not less than the number stated on the label (this test may be combined with the test for uniformity of delivered dose).

**01/2008:1116**

# PREPARATIONS FOR IRRIGATION

## Praeparationes ad irrigationem

DEFINITION

Preparations for irrigation are sterile, aqueous, large-volume preparations intended to be used for irrigation of body cavities, wounds and surfaces, for example during surgical procedures.

Preparations for irrigation are either solutions prepared by dissolving one or more active substances, electrolytes or osmotically active substances in water complying with the requirements for *Water for injections (0169)* or they consist of such water alone. In the latter case, the preparation may be labelled as 'water for irrigation'. Irrigation solutions are usually adjusted to make the preparation isotonic with respect to blood.

Examined in suitable conditions of visibility, preparations for irrigation are clear and practically free from particles.

Preparations for irrigation are supplied in single-dose containers. The containers and closures comply with the requirements for containers for preparations for parenteral administration (*3.2.1* and *3.2.2*), but the administration port of the container is incompatible with intravenous administration equipment and does not allow the preparation for irrigation to be administered with such equipment.

PRODUCTION

Preparations for irrigation are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

During development, it must be demonstrated that the nominal content can be withdrawn from the container.

TESTS

**Sterility** (*2.6.1*). Preparations for irrigation comply with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mL.

**Pyrogens** (*2.6.8*). Preparations for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the preparation, unless otherwise justified and authorised.

LABELLING

The label states:

- that the preparation is not to be used for injection;
- that the preparation is to be used on one occasion only and that any unused portion of the preparation is to be discarded.

**01/2019:0523**

# PRESSURISED PHARMACEUTICAL PREPARATIONS

## Praeparationes pharmaceuticae in vasis cum pressu

*Additional requirements for preparations presented in pressurised containers may be found, where appropriate, in other general monographs, for example Preparations for inhalation (0671), Liquid preparations for cutaneous application (0927), Powders for cutaneous application (1166), Nasal preparations (0676) and Ear preparations (0652).*

DEFINITION

Pressurised pharmaceutical preparations are usually multidose preparations presented in special containers under pressure of a gas and often fitted with a metering valve, and contain one or more active substances. The preparations are released from the container, upon actuation of an appropriate valve, in the form of an aerosol (dispersion of solid or liquid particles in a gas, the size of the particles being adapted to the intended use) or of a liquid or semisolid jet such as a foam. The pressure for the release is generated by suitable propellants.

The preparations are intended for local application to the skin or to mucous membranes of various body orifices, or for inhalation. Pressurised pharmaceutical preparations are provided with a delivery device appropriate for the intended application. Pressurised pharmaceutical preparations intended to be used on severely injured skin or large open wounds are sterile and comply with the test for sterility. Suitable excipients may also be used, for example solvents, solubilisers, emulsifying agents, suspending agents and lubricants for the valve to prevent clogging.

Pressurised pharmaceutical preparations may have the following components.

*Propellants.* The propellants are either gases liquefied under pressure or compressed gases or low-boiling liquids. Liquefied gases are, for example, fluorinated hydrocarbons and low-molecular-mass hydrocarbons (such as propane and butane). Compressed gases are, for example, carbon dioxide, nitrogen and nitrous oxide.

Mixtures of these propellants may be used to obtain optimal solution properties and desirable pressure, delivery and spray characteristics.

*Containers.* The containers are tight and resistant to the internal pressure and may be made of metal, glass, plastic or combinations of these materials. They are compatible with their contents. Glass containers are protected with a plastic coating.

*Spraying device.* The valve keeps the container tightly closed when not in use and regulates the delivery of the contents during use. The spray characteristics are influenced by the type of spraying device, in particular by the dimensions, number and location of orifices. Some valves provide a continuous release, others (metering valves) deliver a defined quantity of product upon each valve actuation.

The various valve materials in contact with the contents are compatible with them.

PRODUCTION

Special requirements may be necessary regarding the selection of propellants, the particle size and the dose delivered by the metering valve, depending on the intended application. Sterile pressurised preparations are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

TESTS

**Sterility** (*2.6.1*). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

LABELLING

The label states:
- where applicable, that the preparation is sterile;
- the method of use;
- any precautions to be taken;
- for a container with a metering valve, the quantity of active substance delivered per actuation.

**01/2008:1145**
**corrected 10.0**

# RECTAL PREPARATIONS

## Rectalia

DEFINITION

Rectal preparations are intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

Where applicable, containers for rectal preparations comply with the requirements for materials used for the manufacture of containers (*3.1* and subsections) and containers (*3.2* and subsections).

Several categories of rectal preparations may be distinguished:
- suppositories;
- rectal capsules;
- rectal solutions, emulsions and suspensions;
- powders and tablets for rectal solutions and suspensions;
- semi-solid rectal preparations;
- rectal foams;
- rectal tampons.

PRODUCTION

During the development of a rectal preparation whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in chapter *5.1.3. Efficacy of antimicrobial preservation*.

During development, it must be demonstrated that the nominal contents can be withdrawn from the container of liquid and semi-solid rectal preparations presented in single-dose containers.

In the manufacture, packaging, storage and distribution of rectal preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

In the manufacture of semi-solid and liquid rectal preparations containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Uniformity of dosage units** (*2.9.40*). Liquid and semi-solid single-dose rectal preparations comply with the test. Solid single-dose rectal preparations comply with the test or, where

justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, solid single-dose rectal preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (tablets) or test B (suppositories, rectal capsules). If the preparation contains more than one active substance, this requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Solid single-dose rectal preparations comply with the test. If the test for uniformity of content is prescribed for all active substances, the test for uniformity of mass is not required.

**Dissolution**. A suitable test may be required to demonstrate the appropriate release of the active substance(s) from solid single-dose rectal preparations, for example *2.9.42. Dissolution test for lipophilic solid dosage forms*.

Where a dissolution test is prescribed, a disintegration test may not be required.

LABELLING

The label states the name of any added antimicrobial preservative.

## Suppositories

DEFINITION

Suppositories are solid, single-dose preparations. The shape, volume and consistency of suppositories are suitable for rectal administration.

They contain 1 or more active substances dispersed or dissolved in a suitable basis that may be soluble or dispersible in water or may melt at body temperature. Excipients such as diluents, adsorbents, surface-active agents, lubricants, antimicrobial preservatives and colouring matter, authorised by the competent authority, may be added if necessary.

PRODUCTION

Suppositories are prepared by compression or moulding. If necessary, the active substance(s) are previously ground and sieved through a suitable sieve. When prepared by moulding, the medicated mass, sufficiently liquefied by heating, is poured into suitable moulds. The suppository solidifies on cooling. Various excipients are available for this process, such as hard fat, macrogols, cocoa butter, and various gelatinous mixtures consisting of, for example, gelatin, water and glycerol. The determination of the softening time of lipophilic suppositories (*2.9.22*) is carried out.

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from suppositories intended for modified release or for prolonged local action.

In the manufacture of suppositories containing dispersed active substances, measures are taken to ensure a suitable and controlled particle size.

TESTS

**Disintegration** (*2.9.2*). Unless intended for modified release or for prolonged local action, they comply with the test. For suppositories with a fatty base, examine after 30 min, and for suppositories with a water-soluble base, examine after 60 min, unless otherwise justified and authorised.

## Rectal capsules

DEFINITION

Rectal capsules (shell suppositories) are solid, single-dose preparations generally similar to soft capsules as defined in the monograph *Capsules (0016)* except that they may have lubricating coatings. They are of elongated shape, are smooth and have a uniform external appearance.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from rectal capsules intended for modified release or for prolonged local action.

TESTS

**Disintegration** (*2.9.2*). Unless intended for modified release or for prolonged local action, they comply with the test. Examine the state of the capsules after 30 min, unless otherwise justified and authorised.

## Rectal solutions, emulsions and suspensions

DEFINITION

Rectal solutions, emulsions and suspensions are liquid preparations intended for rectal use in order to obtain a systemic or local effect, or they may be intended for diagnostic purposes.

Rectal solutions, emulsions and suspensions are supplied in single-dose containers and contain 1 or more active substances dissolved or dispersed in water, glycerol or macrogols or other suitable solvents. Emulsions may show evidence of phase separation but are readily redispersed on shaking. Suspensions may show a sediment that is readily dispersible on shaking to give a suspension that remains sufficiently stable to enable the correct dose to be delivered.

Rectal solutions, emulsions and suspensions may contain excipients, for example to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance(s) or to stabilise the preparation. These substances do not adversely affect the intended medical action or, at the concentrations used, cause undue local irritation.

Rectal solutions, emulsions and suspensions are supplied in containers containing a volume in the range of 2.5 mL to 2000 mL. The container is adapted to deliver the preparation to the rectum or is accompanied by a suitable applicator.

## Powders and tablets for rectal solutions and suspensions

DEFINITION

Powders and tablets intended for the preparation of rectal solutions or suspensions are single-dose preparations that are dissolved or dispersed in water or other suitable solvents at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent aggregation of the particles.

After dissolution or suspension, they comply with the requirements for rectal solutions or rectal suspensions, as appropriate.

TESTS

**Disintegration** (*2.9.1*). Tablets for rectal solutions or suspensions disintegrate within 3 min, using *water R* at 15-25 °C as the liquid medium.

LABELLING

The label states:

– the method of preparation of the rectal solution or suspension;

– the conditions and duration of storage of the solution or suspension after constitution.

## Semi-solid rectal preparations

DEFINITION

Semi-solid rectal preparations are ointments, creams or gels.

They are often supplied as single-dose preparations in containers provided with a suitable applicator.

Semi-solid rectal preparations comply with the requirements of the monograph *Semi-solid preparations for cutaneous application (0132)*.

## Rectal foams

DEFINITION

Rectal foams comply with the requirements of the monograph *Medicated foams (1105)*.

## Rectal tampons

DEFINITION

Rectal tampons are solid, single-dose preparations intended to be inserted into the lower part of the rectum for a limited time.

They comply with the requirements of the monograph *Medicated tampons (1155)*.

**04/2010:0132**
**corrected 10.0**

# SEMI-SOLID PREPARATIONS FOR CUTANEOUS APPLICATION

## Praeparationes molles ad usum dermicum

*The requirements of this monograph apply to all semi-solid preparations for cutaneous application. Where appropriate, additional requirements specific to semi-solid preparations intended to be applied to particular surfaces or mucous membranes may be found in other general monographs, for example Ear preparations (0652), Nasal preparations (0676), Rectal preparations (1145), Eye preparations (1163) and Vaginal preparations (1164).*

DEFINITION

Semi-solid preparations for cutaneous application are intended for local or transdermal delivery of active substances, or for their emollient or protective action. They are of homogeneous appearance.

Semi-solid preparations for cutaneous application consist of a simple or compound basis in which, usually, 1 or more active substances are dissolved or dispersed. According to its composition, the basis may influence the activity of the preparation.

The basis may consist of natural or synthetic substances and may be single phase or multiphase. According to the nature of the basis, the preparation may have hydrophilic or hydrophobic properties; it may contain suitable excipients such as antimicrobial preservatives, antioxidants, stabilisers, emulsifiers, thickeners and penetration enhancers.

Semi-solid preparations for cutaneous application intended for use on severely injured skin are sterile.

Where applicable, containers for semi-solid preparations for cutaneous application comply with the requirements of *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

Several categories of semi-solid preparations for cutaneous application may be distinguished:

– ointments;
– creams;
– gels;
– pastes;
– poultices;
– medicated plasters;
– cutaneous patches.

According to their structure, ointments, creams and gels generally show viscoelastic behaviour and are non-Newtonian in character, e.g. plastic, pseudoplastic or thixotropic type flow at high shear rates. Pastes frequently exhibit dilatancy.

PRODUCTION

During development of semi-solid preparations for cutaneous application whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in *Efficacy of antimicrobial preservation* (*5.1.3*). In the manufacture, packaging, storage and distribution of semi-solid preparations for cutaneous application, suitable measures are taken to ensure their microbiological quality; recommendations on this are provided in *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*. Sterile semi-solid preparations for cutaneous application are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this are provided in *Methods of preparation of sterile products* (*5.1.1*).

During development, it must be demonstrated that the nominal content can be withdrawn from the container of semi-solid preparations for cutaneous application presented in single-dose containers.

In the manufacture of semi-solid preparations for cutaneous application, suitable measures are taken to ensure that the defined rheological properties are fulfilled. Where appropriate, the following non-mandatory tests may be carried out: measurement of consistency by penetrometry (*2.9.9*), viscosity (apparent viscosity) (*2.2.10*) and a suitable test to demonstrate the appropriate release of the active substance(s).

In the manufacture of semi-solid preparations for cutaneous application containing 1 or more active substances that are not dissolved in the basis (e.g. emulsions or suspensions), measures are taken to ensure appropriate homogeneity of the preparation to be delivered.

In the manufacture of semi-solid preparations for cutaneous application containing dispersed particles, measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

TESTS

**Uniformity of dosage units**. Semi-solid preparations that are supplied either in single-dose containers that represent 1 dose of medicinal product or in metered-dose containers, and that are intended for transdermal delivery of the active substance(s) in view of a systemic effect, comply with the test for uniformity of dosage units (*2.9.40*). Semi-solid preparations in which the active substance(s) are dissolved comply with the test for mass variation; semi-solid preparations in which the active substance(s) are suspended comply with the test for content uniformity. Follow the procedure described for liquid dosage forms. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

For semi-solid preparations presented in metered-dose containers and in which the active substance(s) are dissolved,

proceed as follows. Discharge once to waste. Wait for a minimum of 5 s, shake for 5 s if necessary, and discharge again to waste. Repeat this procedure for a further 3 actuations. Weigh the container, discharge once to waste and weigh the container again. Calculate the difference between the 2 masses. Repeat the procedure for a further 9 containers. Determine the mass variation (*2.9.40*).

For semi-solid preparations supplied in metered-dose containers and in which the active substance(s) are suspended, proceed as follows. Use an apparatus capable of quantitatively retaining the dose leaving the metered-dose container. Shake 1 container for 5 s and discharge once to waste. Wait for a minimum of 5 s, shake for 5 s and discharge again to waste. Repeat this procedure for a further 3 actuations. After 2 s, fire 1 dose from the metered-dose container into the collecting vessel. Collect the contents of the collecting vessel by successive rinses. Determine the content of active substance in the combined rinses. Repeat the procedure for a further 9 containers. Determine the content uniformity (*2.9.40*).

**Sterility** (*2.6.1*). Where the label indicates that the preparation is sterile, it complies with the test for sterility.

STORAGE

If the preparation contains water or other volatile ingredients, store in an airtight container. If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the name of any excipient;
- where applicable, that the preparation is sterile.

## Ointments

DEFINITION

An ointment consists of a single-phase basis in which solids or liquids may be dispersed.

*Hydrophobic ointments*

Hydrophobic ointments can absorb only small amounts of water. Typical bases used for their formulation are hard, liquid and light liquid paraffins, vegetable oils, animal fats, synthetic glycerides, waxes and liquid polyalkylsiloxanes.

*Water-emulsifying ointments*

Water-emulsifying ointments can absorb larger amounts of water and thereby produce water-in-oil or oil-in-water emulsions after homogenisation, depending on the nature of the emulsifiers: water-in-oil emulsifying agents such as wool alcohols, sorbitan esters, monoglycerides and fatty alcohols, or oil-in-water emulsifying agents such as sulfated fatty alcohols, polysorbates, macrogol cetostearyl ether or esters of fatty acids with macrogols may be used for this purpose. Their bases are those of the hydrophobic ointments.

*Hydrophilic ointments*

Hydrophilic ointments are preparations having bases that are miscible with water. The bases usually consist of mixtures of liquid and solid macrogols (polyethylene glycols). They may contain appropriate amounts of water.

## Creams

DEFINITION

Creams are multiphase preparations consisting of a lipophilic phase and an aqueous phase.

*Lipophilic creams*

Lipophilic creams have as the continuous phase the lipophilic phase. They usually contain water-in-oil emulsifying agents such as wool alcohols, sorbitan esters and monoglycerides.

*Hydrophilic creams*

Hydrophilic creams have as the continuous phase the aqueous phase. They contain oil-in-water emulsifying agents such as sodium or trolamine soaps, sulfated fatty alcohols, polysorbates and polyoxyl fatty acid and fatty alcohol esters combined, if necessary, with water-in-oil emulsifying agents.

## Gels

DEFINITION

Gels consist of liquids gelled by means of suitable gelling agents.

*Lipophilic gels*

Lipophilic gels (oleogels) are preparations whose bases usually consist of liquid paraffin with polyethylene or fatty oils gelled with colloidal silica or aluminium or zinc soaps.

*Hydrophilic gels*

Hydrophilic gels (hydrogels) are preparations whose bases usually consist of water, glycerol or propylene glycol gelled with suitable gelling agents such as poloxamers, starch, cellulose derivatives, carbomers and magnesium-aluminium silicates.

## Pastes

DEFINITION

Pastes are semi-solid preparations for cutaneous application containing large proportions of solids finely dispersed in the basis.

## Poultices

DEFINITION

Poultices consist of a hydrophilic heat-retentive basis in which solid or liquid active substances are dispersed. They are usually spread thickly on a suitable dressing and heated before application to the skin.

## Medicated plasters

DEFINITION

Medicated plasters are flexible preparations containing 1 or more active substances. They are intended to be applied to the skin. They are designed to maintain the active substance(s) in close contact with the skin such that these may be absorbed slowly, or act as protective or keratolytic agents.

Medicated plasters consist of an adhesive basis, which may be coloured, containing 1 or more active substances, spread as a uniform layer on an appropriate support made of natural or synthetic material. They are not irritant or sensitising to the skin. The adhesive layer is covered by a suitable protective liner, which is removed before applying the plaster to the skin. When removed, the protective liner does not detach the preparation from the outer, supporting layer.

Medicated plasters are presented in a range of sizes directly adapted to their intended use or as larger sheets to be cut before use. Medicated plasters adhere firmly to the skin when gentle pressure is applied and can be peeled off without causing appreciable injury to the skin or detachment of the preparation from the outer, supporting layer.

TESTS

**Dissolution**. A suitable test may be required to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for transdermal patches* (*2.9.4*).

## Cutaneous patches

DEFINITION

Cutaneous patches are flexible preparations containing 1 or more active substances. They are intended to be applied to the skin. They are designed to maintain the active substance(s) in close contact with the skin such that these may act locally.

Cutaneous patches consist of an adhesive basis, which may be coloured, containing 1 or more active substances, spread as a uniform layer on an appropriate support made of natural or synthetic material. The adhesive basis is not irritant or sensitising to the skin. The adhesive layer is covered by a suitable protective liner, which is removed before applying the patch to the skin. When removed, the protective liner does not detach the preparation from the outer, supporting layer.

Cutaneous patches are presented in a range of sizes adapted to their intended use. They adhere firmly to the skin when gentle pressure is applied and can be peeled off without causing appreciable injury to the skin or detachment of the preparation from the outer, supporting layer.

TESTS

**Dissolution**. A suitable test may be required to demonstrate the appropriate release of the active substance(s), for example one of the tests described in *Dissolution test for transdermal patches* (*2.9.4*).

**01/2019:1154**

# STICKS

## Styli

*Additional requirements for sticks may be found, where appropriate, in other general monographs, for example Nasal preparations (0676).*

DEFINITION

Sticks are solid preparations intended for local application. They may be single-dose or multidose preparations. They are rod-shaped or conical preparations consisting of one or more active substances, either alone or dissolved or dispersed in a suitable basis, and are usually intended to dissolve or melt at body temperature. They may be inserted into a body cavity or wound, or be applied cutaneously.

Urethral sticks and sticks for insertion into wounds are sterile.

PRODUCTION

In the manufacture, packaging, storage and distribution of sticks, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

Urethral sticks and other sterile sticks are prepared using materials and methods designed to ensure sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in general chapter *5.1.1. Methods of preparation of sterile products*.

TESTS

**Uniformity of dosage units** (*2.9.40*). Single-dose sticks comply with the test or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, single-dose sticks with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test B. If the preparation contains more than one active substance, this requirement applies only to those substances that correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Single-dose sticks comply with the test. If the test for uniformity of content is prescribed for all active substances, the test for uniformity of mass is not required.

**Sterility** (*2.6.1*). Urethral sticks and sticks for insertion into wounds comply with the test for sterility.

LABELLING

The label states, for urethral sticks and sticks to be inserted into wounds, that they are sterile.

**01/2018:0478**

# TABLETS

## Compressi

*The requirements of this monograph do not necessarily apply to preparations that are presented as tablets intended for use other than by oral administration. Requirements for such preparations may be found, where appropriate, in other general monographs; for example Rectal preparations (1145), Vaginal preparations (1164) and Oromucosal preparations (1807). This monograph does not apply to lozenges, oral pastes and oral gums. Where justified and authorised, the requirements of this monograph do not apply to tablets for veterinary use. Tablets for use in the mouth comply with the requirements of the monograph Oromucosal preparations (1807).*

DEFINITION

Tablets are solid preparations each containing a single dose of one or more active substances. They are obtained by compressing uniform volumes of particles or by another suitable manufacturing technique, such as extrusion, moulding or freeze-drying (lyophilisation). Tablets are intended for oral administration. Some are swallowed whole, some after being chewed, some are dissolved or dispersed in water before being administered and some are retained in the mouth where the active substance is liberated.

The particles consist of one or more active substances with or without excipients such as diluents, binders, disintegrating agents, glidants, lubricants, substances capable of modifying the behaviour of the preparation in the digestive tract, colouring matter authorised by the competent authority and flavouring substances.

Tablets are usually straight, solid cylinders, the end surfaces of which are flat or convex and the edges of which may be bevelled. They may have break-marks and may bear a symbol or other markings. Tablets may be coated.

Where applicable, containers for tablets comply with the requirements for materials used for the manufacture of containers (*3.1* and subsections) and containers (*3.2* and subsections).

Several categories of tablets for oral use may be distinguished:

- uncoated tablets;
- coated tablets;
- gastro-resistant tablets;
- modified-release tablets;
- effervescent tablets;
- soluble tablets;
- dispersible tablets;

- orodispersible tablets;
- chewable tablets;
- oral lyophilisates.

PRODUCTION

Tablets are usually prepared by compressing uniform volumes of particles or particle aggregates produced by granulation methods. In the manufacture of tablets, measures are taken to ensure that they possess a suitable mechanical strength to avoid crumbling or breaking on handling or subsequent processing. This may be demonstrated using the tests described in general chapters *2.9.7. Friability of uncoated tablets* and *2.9.8. Resistance to crushing of tablets*.

In the manufacture, packaging, storage and distribution of tablets, suitable measures are taken to ensure their microbiological quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

*Subdivision of tablets*. Tablets may bear a break-mark or break-marks for the purpose of being subdivided into parts, either to ease the intake of the medicinal product or to deliver fractional doses. In cases where fractions of tablets are necessary to deliver the intended dose stated on the label, the efficacy of the breakmark is assessed during product development or for validation purposes by determining the uniformity of mass of the sub-divided parts using the following test.

Take 30 tablets at random, break them by hand and, from all the parts obtained from 1 tablet, take 1 part for the test and reject the other part(s). Weigh each of the 30 parts individually and calculate the average mass. The tablets comply with the test if not more than 1 individual mass is outside the limits of 85 per cent to 115 per cent of the average mass. The tablets fail to comply with the test if more than 1 individual mass is outside these limits, or if 1 individual mass is outside the limits of 75 per cent to 125 per cent of the average mass.

TESTS

**Uniformity of dosage units** (*2.9.40*). Tablets comply with the test or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, tablets with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A. If the preparation has more than 1 active substance, the requirement applies only to those substances that correspond to the above conditions.

Unless otherwise justified and authorised, coated tablets other than film-coated tablets comply with test A irrespective of their content of active substance(s).

**Uniformity of mass** (*2.9.5*). Uncoated tablets and, unless otherwise justified and authorised, film-coated tablets comply with the test. If the test for uniformity of content is prescribed or justified and authorised for all the active substances, the test for uniformity of mass is not required.

**Dissolution**. Unless otherwise justified and authorised, a suitable test is carried out, for example one of the tests described in general chapter *2.9.3. Dissolution test for solid dosage forms*.

Where a dissolution test is prescribed, a disintegration test may not be required.

## Uncoated tablets

DEFINITION

Uncoated tablets include single-layer tablets resulting from a single compression of particles and multi-layer tablets consisting of concentric or parallel layers obtained by successive compression of particles of different composition. The excipients used are not specifically intended to modify the release of the active substance in the digestive fluids.

Uncoated tablets conform to the general definition of tablets. A broken section, when examined under a lens, shows either a relatively uniform texture (single-layer tablets) or a stratified texture (multi-layer tablets) but no signs of coating.

TESTS

**Disintegration** (*2.9.1*). Uncoated tablets comply with the test using *water R* as the liquid medium. Add a disc to each tube. Operate the apparatus for 15 min, unless otherwise justified and authorised, and examine the state of the tablets. If the tablets fail to comply because of adherence to the discs, the results are invalid. Repeat the test on a further 6 tablets, omitting the discs.

## Coated tablets

DEFINITION

Coated tablets are tablets covered with one or more layers of mixtures of various substances such as natural or synthetic resins, gums, gelatin, inactive and insoluble fillers, sugars, plasticisers, polyols, waxes, colouring matter authorised by the competent authority and sometimes flavouring substances and active substances. The substances used as coatings are usually applied as a solution or suspension in conditions in which evaporation of the vehicle occurs. When the coating is a very thin polymeric coating, the tablets are known as film-coated tablets.

Coated tablets have a smooth surface, which is often coloured and may be polished; a broken section, when examined under a lens, shows a core surrounded by one or more continuous layers with a different texture.

PRODUCTION

Where justified, uniformity of mass or uniformity of content of coated tablets other than film-coated tablets may be ensured by control of the cores.

TESTS

**Disintegration** (*2.9.1*). Coated tablets other than film-coated tablets comply with the test using *water R* as the liquid medium. Add a disc to each tube. Operate the apparatus for 60 min, unless otherwise justified and authorised, and examine the state of the tablets. If any of the tablets has not disintegrated, repeat the test on a further 6 tablets, replacing *water R* with *0.1 M hydrochloric acid*. If 1 or 2 tablets fail to disintegrate, repeat the test on 12 additional tablets.

The requirements of the test are met if not fewer than 16 of the 18 tablets tested have disintegrated.

Film-coated tablets comply with the disintegration test prescribed above except that the apparatus is operated for 30 min, unless otherwise justified and authorised.

If coated tablets or film-coated tablets fail to comply because of adherence to the discs, the results are invalid. Repeat the test on a further 6 tablets, omitting the discs.

## Gastro-resistant tablets

DEFINITION

Gastro-resistant tablets are delayed-release tablets that are intended to resist the gastric fluid and to release their active substance(s) in the intestinal fluid. Usually they are prepared

by covering tablets with a gastro-resistant coating or from granules or particles already covered with a gastro-resistant coating.

Tablets covered with a gastro-resistant coating conform to the definition of coated tablets.

TESTS

**Disintegration** (*2.9.1*). Tablets covered with a gastro-resistant coating comply with the test with the following modifications. Use *0.1 M hydrochloric acid* as the liquid medium. Operate the apparatus for 2 h, or another such time as may be justified and authorised, without the discs, and examine the state of the tablets. The time of resistance to the acid medium varies according to the formulation of the tablets to be examined. It is typically 2 h to 3 h but even with authorised deviations is not less than 1 h. No tablet shows signs of either disintegration (apart from fragments of coating) or cracks that would allow the escape of the contents. Replace the acid by *phosphate buffer solution pH 6.8 R* and add a disc to each tube. Operate the apparatus for 60 min and examine the state of the tablets. If the tablets fail to comply because of adherence to the discs, the results are invalid. Repeat the test on a further 6 tablets, omitting the discs.

## Modified-release tablets

DEFINITION

Modified-release tablets are coated or uncoated tablets that contain special excipients or are prepared by special procedures, or both, designed to modify the rate, the place or the time at which the active substance(s) are released.

Modified-release tablets include prolonged-release tablets, delayed-release tablets and pulsatile-release tablets.

## Effervescent tablets

DEFINITION

Effervescent tablets are uncoated tablets generally containing acid substances and carbonates or hydrogen carbonates, which react rapidly in the presence of water to release carbon dioxide. They are intended to be dissolved or dispersed in water before administration.

TESTS

**Disintegration**. Place 1 tablet in a beaker containing 200 mL of *water R* at 15-25 °C; numerous bubbles of gas are evolved. When the evolution of gas around the tablet or its fragments ceases the tablet has disintegrated, being either dissolved or dispersed in the water so that no agglomerates of particles remain. Repeat the operation on 5 other tablets. The tablets comply with the test if each of the 6 tablets used disintegrates in the manner prescribed within 5 min, unless otherwise justified and authorised.

## Soluble tablets

DEFINITION

Soluble tablets are uncoated or film-coated tablets. They are intended to be dissolved in water before administration. The solution produced may be slightly opalescent due to the added excipients used in the manufacture of the tablets.

TESTS

**Disintegration** (*2.9.1*). Soluble tablets disintegrate within 3 min, using *water R* at 15-25 °C as the liquid medium.

## Dispersible tablets

DEFINITION

Dispersible tablets are uncoated or film-coated tablets intended to be dispersed in water before administration, giving a homogeneous dispersion.

TESTS

**Disintegration** (*2.9.1*). Dispersible tablets disintegrate within 3 min, using *water R* at 15-25 °C as the liquid medium.

**Fineness of dispersion**. Place 2 tablets in 100 mL of *water R* and stir until completely dispersed. A smooth dispersion is produced, which passes through a sieve screen with a nominal mesh aperture of 710 µm.

## Orodispersible tablets

DEFINITION

Orodispersible tablets are uncoated tablets intended to be placed in the mouth where they disperse rapidly before being swallowed.

TESTS

**Disintegration** (*2.9.1*). Orodispersible tablets disintegrate within 3 min, using *water R* as the liquid medium.

## Chewable tablets

DEFINITION

Chewable tablets are intended to be chewed before being swallowed.

PRODUCTION

Chewable tablets are prepared to ensure that they are easily crushed by chewing.

## Oral lyophilisates

DEFINITION

Oral lyophilisates are solid single-dose preparations made by freeze-drying of a liquid or semi-solid preparation. These fast-releasing preparations are intended to be placed in the mouth where their contents are released in saliva and swallowed or, alternatively, are intended to be dissolved or dispersed in water before oral administration.

PRODUCTION

Oral lyophilisates are obtained by freeze-drying (lyophilisation), involving division into single doses, freezing, sublimation and drying of usually aqueous, liquid or semi-solid preparations.

TESTS

**Disintegration**. Place 1 oral lyophilisate in a beaker containing 200 mL of *water R* at 15-25 °C. It disintegrates within 3 min. Repeat the test on 5 other oral lyophilisates. They comply with the test if all 6 have disintegrated.

**Water** (*2.5.12*). Oral lyophilisates comply with the test; the limits are approved by the competent authority.

01/2017:1155

# TAMPONS, MEDICATED

## Tamponae medicatae

*Additional requirements for medicated tampons may be found, where appropriate, in other general monographs, for example Rectal preparations (1145), Vaginal preparations (1164) and Ear preparations (0652).*

DEFINITION

Medicated tampons are solid, single-dose preparations intended to be inserted into the body cavities for a limited period of time, usually to obtain a local effect. They consist of a suitable material such as cellulose, collagen or silicone impregnated with one or more active substances.

PRODUCTION

In the manufacture, packaging, storage and distribution of medicated tampons, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use.*

LABELLING

The label states the quantity of active substance(s) per tampon.

01/2008:1164
corrected 10.0

# VAGINAL PREPARATIONS

## Vaginalia

DEFINITION

Vaginal preparations are liquid, semi-solid or solid preparations intended for administration to the vagina usually in order to obtain a local effect. They contain 1 or more active substances in a suitable basis.

Where appropriate, containers for vaginal preparations comply with the requirements for materials used for the manufacture of containers (*3.1* and subsections) and containers (*3.2* and subsections).

Several categories of vaginal preparations may be distinguished:

- pessaries;
- vaginal tablets;
- vaginal capsules;
- vaginal solutions, emulsions and suspensions;
- tablets for vaginal solutions and suspensions;
- semi-solid vaginal preparations;
- vaginal foams;
- medicated vaginal tampons.

PRODUCTION

During development, it must be demonstrated that the nominal contents can be withdrawn from the container of liquid and semi-solid vaginal preparations presented in single-dose containers.

In the manufacturing, packaging, storage and distribution of vaginal preparations, suitable measures are taken to ensure their microbial quality; recommendations on this aspect are provided in chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use.*

TESTS

**Uniformity of dosage units** (*2.9.40*). Liquid and semi-solid single-dose vaginal preparations comply with the test. Solid single-dose vaginal preparations comply with the test or, where justified and authorised, with the tests for uniformity of content and/or uniformity of mass shown below. Herbal drugs and herbal drug preparations present in the dosage form are not subject to the provisions of this paragraph.

**Uniformity of content** (*2.9.6*). Unless otherwise prescribed or justified and authorised, solid single-dose vaginal preparations with a content of active substance less than 2 mg or less than 2 per cent of the total mass comply with test A (vaginal tablets) or test B (pessaries, vaginal capsules). If the preparation has more than one active substance, the requirement applies only to those substances which correspond to the above conditions.

**Uniformity of mass** (*2.9.5*). Solid single-dose vaginal preparations comply with the test. If the test for uniformity of content is prescribed for all the active substances, the test for uniformity of mass is not required.

**Dissolution**. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s) from solid single-dose vaginal preparations, for example one of the tests described in chapter *2.9.3. Dissolution test for solid dosage forms* or in *2.9.42. Dissolution test for lipophilic solid dosage forms*.

When a dissolution test is prescribed, a disintegration test may not be required.

## Pessaries

DEFINITION

Pessaries are solid, single-dose preparations. They have various shapes, usually ovoid, with a volume and consistency suitable for insertion into the vagina. They contain 1 or more active substances dispersed or dissolved in a suitable basis that may be soluble or dispersible in water or may melt at body temperature. Excipients such as diluents, adsorbents, surface-active agents, lubricants, antimicrobial preservatives and colouring matter authorised by the competent authority may be added, if necessary.

PRODUCTION

Pessaries are usually prepared by moulding. Where appropriate in the manufacture of pessaries, measures are taken to ensure a suitable and controlled particle size of the active substance(s). If necessary, the active substance(s) are previously ground and sieved through a suitable sieve.

When prepared by moulding, the medicated mass, sufficiently liquefied by heating, is poured into suitable moulds. The pessary solidifies on cooling. Various excipients are available for this process, such as hard fat, macrogols, cocoa butter, and various gelatinous mixtures consisting, for example, of gelatin, water and glycerol.

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from pessaries intended for prolonged local action.

TESTS

**Disintegration** (*2.9.2*). Unless intended for prolonged local action, they comply with the test. Examine the state of the pessaries after 60 min, unless otherwise justified and authorised.

## Vaginal tablets

DEFINITION

Vaginal tablets are solid, single-dose preparations. They generally conform to the definitions of uncoated or film-coated tablets given in the monograph *Tablets (0478)*.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from vaginal tablets intended for prolonged local action.

TESTS

**Disintegration** (*2.9.2*). Unless intended for prolonged local action, they comply with the test (special method for vaginal tablets). Examine the state of the tablets after 30 min, unless otherwise justified and authorised.

## Vaginal capsules

DEFINITION

Vaginal capsules (shell pessaries) are solid, single-dose preparations. They are generally similar to soft capsules as defined in the monograph *Capsules (0016)*, differing only in their shape and size. Vaginal capsules have various shapes, usually ovoid. They are smooth and have a uniform external appearance.

PRODUCTION

A suitable test is carried out to demonstrate the appropriate release of the active substance(s) from vaginal capsules intended for prolonged local action.

TESTS

**Disintegration** (*2.9.2*). Unless intended for prolonged local action, they comply with the test. Examine the state of the capsules after 30 min, unless otherwise justified and authorised.

## Vaginal solutions, emulsions and suspensions

DEFINITION

Vaginal solutions, emulsions and suspensions are liquid preparations intended for a local effect, for irrigation or for diagnostic purposes. They may contain excipients, for example to adjust the viscosity of the preparation, to adjust or stabilise the pH, to increase the solubility of the active substance(s) or to stabilise the preparation. The excipients do not adversely affect the intended medical action or, at the concentrations used, cause undue local irritation.

Vaginal emulsions may show evidence of phase separation but are readily redispersed on shaking. Vaginal suspensions may show a sediment that is readily dispersed on shaking to give a suspension that remains sufficiently stable to enable a homogeneous preparation to be delivered.

They are supplied in single-dose containers. The container is adapted to deliver the preparation to the vagina or it is accompanied by a suitable applicator.

PRODUCTION

In the manufacture of vaginal suspensions measures are taken to ensure a suitable and controlled particle size with regard to the intended use.

## Tablets for vaginal solutions and suspensions

DEFINITION

Tablets intended for the preparation of vaginal solutions and suspensions are single-dose preparations that are dissolved or dispersed in water at the time of administration. They may contain excipients to facilitate dissolution or dispersion or to prevent caking.

Apart from the test for disintegration, tablets for vaginal solutions or suspensions conform with the definition for *Tablets (0478)*.

After dissolution or dispersion, they comply with the requirements for vaginal solutions or vaginal suspensions, as appropriate.

TESTS

**Disintegration** (*2.9.1*). Tablets for vaginal solutions or suspensions disintegrate within 3 min, using *water R* at 15-25 °C as the liquid medium.

LABELLING

The label states:

- the method of preparation of the vaginal solution or suspension;
- the conditions and duration of storage of the solution or suspension after constitution.

## Semi-solid vaginal preparations

DEFINITION

Semi-solid vaginal preparations are ointments, creams or gels.

They are often supplied in single-dose containers. The container is provided with a suitable applicator.

Semi-solid vaginal preparations comply with the requirements of the monograph *Semi-solid preparations for cutaneous application (0132)*.

## Vaginal foams

DEFINITION

Vaginal foams comply with the requirements of the monograph *Medicated foams (1105)*.

## Medicated vaginal tampons

DEFINITION

Medicated vaginal tampons are solid, single-dose preparations intended to be inserted in the vagina for a limited time.

They comply with the requirements of the monograph *Medicated tampons (1155)*.

**01/2018:1808**

# VETERINARY LIQUID PREPARATIONS FOR CUTANEOUS APPLICATION

## Praeparationes liquidae veterinariae ad usum dermicum

*Unless otherwise justified and authorised, veterinary liquid preparations for cutaneous application comply with the requirements of the monograph Liquid preparations for cutaneous application (0927). In addition to these requirements, the following statements apply to veterinary liquid preparations for cutaneous application.*

DEFINITION

Veterinary liquid preparations for cutaneous application are liquid preparations intended to be applied to the skin to obtain a local and/or systemic effect. They are solutions, suspensions or emulsions that may contain one or more active substances in a suitable vehicle. They may be presented as concentrates in the form of wettable powders, pastes, solutions or suspensions, which are used to prepare diluted solutions, suspensions or emulsions of active substances. They may contain suitable antimicrobial preservatives, antioxidants and other excipients such as stabilisers, emulsifiers and thickeners.

Several categories of veterinary liquid preparations for cutaneous application may be distinguished:

- cutaneous foams (see *Liquid preparations for cutaneous application (0927)*);
- dip concentrates;
- pour-on preparations;
- shampoos (see *Liquid preparations for cutaneous application (0927)*);
- spot-on preparations;
- sprays;
- teat dips;
- teat sprays;
- udder-washes.

## Dip concentrates

DEFINITION

Dip concentrates are preparations containing one or more active substances, usually in the form of wettable powders, pastes, solutions or suspensions, which are used to prepare diluted solutions, suspensions or emulsions of active substances. The diluted preparations are applied by complete immersion of the animal.

## Pour-on preparations

DEFINITION

Pour-on preparations contain one or more active substances for the prevention and treatment of ectoparasitic and/or endoparasitic infestations of animals. They are applied in volumes that are usually greater than 5 mL by pouring along the animal's dorsal midline.

## Spot-on preparations

DEFINITION

Spot-on preparations contain one or more active substances for the prevention and treatment of ectoparasitic and/or endoparasitic infestations of animals. They are applied in volumes that are usually less than 10 mL, to a small area on the head or back, as appropriate, of the animal.

## Sprays

DEFINITION

Sprays contain one or more active substances that are intended to be applied externally for therapeutic or prophylactic purposes. They are delivered in the form of an aerosol by the actuation of an appropriate valve or by means of a suitable atomising device that is either an integral part of the container or is supplied separately.

Sprays may be presented in pressurised containers (see *Pressurised pharmaceutical preparations (0523)*). When so presented, sprays usually consist of one or more active substances in a suitable vehicle held under pressure with suitable propellants or suitable mixtures of propellants. When otherwise presented, sprays are supplied in well-closed containers.

PRODUCTION

During the development and manufacture of a spray, measures are taken to ensure that the assembled product conforms to a defined spray rate and spray pattern.

## Teat dips

DEFINITION

Teat dips contain one or more active substances, usually in the form of solutions into which the teats of an animal are dipped pre- and post-milking, as appropriate, to reduce the population of pathogenic micro-organisms on the surfaces. Teat dips may be supplied/presented as ready-to-use preparations or they may be prepared by dilution of teat dip concentrates. Pre- and post-milking teat dips often differ in formulation. Teat dips usually contain emollients to promote skin hydration, to soften the skin and allow healing of lesions that would otherwise harbour bacteria.

## Teat sprays

DEFINITION

Teat sprays contain one or more active substances, usually in the form of solutions that are sprayed onto the teats of an animal pre- and post-milking, as appropriate, to reduce the population of pathogenic micro-organisms on the surfaces. Teat sprays may be supplied/presented as ready-to-use preparations or they may be prepared by dilution of teat spray concentrates. Pre- and post-milking sprays often differ in formulation. Teat sprays usually contain emollients to promote skin hydration, to soften the skin and allow healing of lesions that would otherwise harbour bacteria.

## Udder-washes

DEFINITION

Udder-washes contain one or more active substances, usually in the form of solutions that are sprayed onto the udder and teats of an animal to remove mud and faecal contamination before the application of teat dips or sprays. Udder-washes are usually prepared by the dilution either of concentrated preparations or of ready-to-use teat dips or teat sprays.

**04/2014:2638**

# VETERINARY SEMI-SOLID PREPARATIONS FOR ORAL USE

## Praeparationes molles veterinariae peroraliae

DEFINITION

Veterinary semi-solid preparations for oral use (usually pastes or gels) contain one or more active substances dissolved or dispersed in a suitable basis. They are administered to the oral cavity and are intended to be swallowed for delivery of active substances to the gastrointestinal tract.

Veterinary semi-solid preparations for oral use may contain suitable antimicrobial preservatives and other excipients such as dispersing, suspending, thickening, emulsifying, buffering, wetting, solubilising, stabilising, flavouring and sweetening agents.

Veterinary semi-solid preparations for oral use are usually supplied in multidose containers such as oral syringes, which are designed to allow the accurate dosing of animals according to their bodyweight.

Where applicable, containers for veterinary semi-solid preparations for oral use comply with the requirements described in *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections).

PRODUCTION

During the development of veterinary semi-solid preparations for oral use whose formulation contains an antimicrobial preservative, the need for and the efficacy of the chosen preservative shall be demonstrated to the satisfaction of the competent authority. A suitable test method together with criteria for judging the preservative properties of the formulation are provided in general chapter *5.1.3. Efficacy of antimicrobial preservation*.

In the manufacture, packaging, storage and distribution of veterinary semi-solid preparations for oral use, suitable measures are taken to ensure their microbiological quality; recommendations on this are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

TESTS

**Dissolution**. A suitable test may be carried out to demonstrate the appropriate release of the active substance(s).

**Uniformity of mass of delivered doses from multidose containers** (*2.9.27*). *Unless otherwise justified and authorised, the minimum and the maximum labelled doses are tested, where possible using the same container and dosing device.*

Discharge once to waste in order to prime the system, if so stated on the label and depending on the type and shape of the container.

Unless otherwise justified and authorised, the preparation complies with the test.

STORAGE

If the preparation contains water or other volatile ingredients, store in an airtight container.

LABELLING

The label states:

- the name of any added antimicrobial preservative;
- if it is necessary to prime the dosing device before administration.

# Vaccines for human use

Anthrax vaccine for human use (adsorbed, prepared from culture filtrates)..... 947
BCG for immunotherapy..... 948
BCG vaccine, freeze-dried..... 949
Cholera vaccine (inactivated, oral)..... 951
Diphtheria and tetanus vaccine (adsorbed)..... 952
Diphtheria and tetanus vaccine (adsorbed, reduced antigen(s) content)..... 953
Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed)..... 954
Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed)..... 955
Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed, reduced antigen(s) content)..... 956
Diphtheria, tetanus and pertussis (whole cell) vaccine (adsorbed)..... 958
Diphtheria, tetanus and poliomyelitis (inactivated) vaccine (adsorbed, reduced antigen(s) content)..... 959
Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed)..... 961
Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed)..... 963
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed)..... 964
Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed, reduced antigen(s) content)..... 966
Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)..... 968
Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)..... 970
Diphtheria, tetanus, pertussis (whole cell) and poliomyelitis (inactivated) vaccine (adsorbed)..... 973
Diphtheria, tetanus, pertussis (whole cell), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed)..... 974
Diphtheria vaccine (adsorbed)..... 977
Diphtheria vaccine (adsorbed, reduced antigen content)..... 978
Haemophilus type b and meningococcal group C conjugate vaccine..... 979
Haemophilus type b conjugate vaccine..... 980
Hepatitis A (inactivated, adsorbed) and typhoid polysaccharide vaccine..... 982
Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed)..... 983
Hepatitis A vaccine (inactivated, adsorbed)..... 984
Hepatitis A vaccine (inactivated, virosome)..... 985
Hepatitis B vaccine (rDNA)..... 988
Human papillomavirus vaccine (rDNA)..... 989
Influenza vaccine (live, nasal)..... 992
Influenza vaccine (split virion, inactivated)..... 995
Influenza vaccine (surface antigen, inactivated)..... 996
Influenza vaccine (surface antigen, inactivated, prepared in cell cultures)..... 998
Influenza vaccine (surface antigen, inactivated, virosome)..... 1000
Influenza vaccine (whole virion, inactivated)..... 1002
Influenza vaccine (whole virion, inactivated, prepared in cell cultures)..... 1003
Measles, mumps and rubella vaccine (live)..... 1005
Measles, mumps, rubella and varicella vaccine (live)..... 1006
Measles vaccine (live)..... 1007
Meningococcal group A, C, W135 and Y conjugate vaccine..... 1009
Meningococcal group C conjugate vaccine..... 1010
Meningococcal polysaccharide vaccine..... 1012
Mumps vaccine (live)..... 1014
Pertussis vaccine (acellular, component, adsorbed)..... 1015
Pertussis vaccine (acellular, co-purified, adsorbed)..... 1016
Pertussis vaccine (whole cell, adsorbed)..... 1018
Pneumococcal polysaccharide conjugate vaccine (adsorbed)..... 1019
Pneumococcal polysaccharide vaccine..... 1021
Poliomyelitis vaccine (inactivated)..... 1023
Poliomyelitis vaccine (oral)..... 1026
Rabies vaccine for human use prepared in cell cultures..... 1030
Rotavirus vaccine (live, oral)..... 1033
Rubella vaccine (live)..... 1035
Shingles (herpes zoster) vaccine (live)..... 1036
Smallpox vaccine (live)..... 1037
Tetanus vaccine (adsorbed)..... 1042
Tick-borne encephalitis vaccine (inactivated)..... 1043
Typhoid polysaccharide vaccine..... 1044
Typhoid vaccine..... 1046
Typhoid vaccine (live, oral, strain Ty 21a)..... 1046
Varicella vaccine (live)..... 1048
Yellow fever vaccine (live)..... 1049

**01/2019:2188**
**corrected 10.0**

# ANTHRAX VACCINE FOR HUMAN USE (ADSORBED, PREPARED FROM CULTURE FILTRATES)

## Vaccinum anthracis adsorbatum ab colato culturarum ad usum humanum

DEFINITION

Anthrax vaccine for human use (adsorbed, prepared from culture filtrates) is a preparation of *Bacillus anthracis* antigens precipitated by aluminium potassium sulfate. The antigens are prepared from a sterile culture filtrate produced by a non-encapsulated strain, either avirulent or attenuated, of *B. anthracis*.

The main virulence components of *B. anthracis* are the polyglutamic aicd capsule and 2 binary anthrax toxins, namely lethal toxin and oedema toxin, formed from the respective combination of protective antigen (PA) with either lethal factor (LF) or oedema factor (EF).

LF is a zinc-dependent endopeptidase and EF is a potent calmodulin and calcium-dependent adenylate cyclase. Cell-free cultures of *B. anthracis* contain PA and because expression of the 3 toxin-component genes is co-ordinately regulated, LF and EF are also present. In addition, the vaccine is likely to contain many other *B. anthracis* antigens, including membrane proteins, secreted proteins, cytoplasmic proteins, peptidoglycans, nucleic acids and carbohydrates.

PRODUCTION

*GENERAL PROVISIONS*

Cultures are managed in a seed-lot system. The vaccine strain is toxigenic but lacks the plasmid with the necessary genes for synthesis of the capsule, an important virulence factor.

The production method must be shown to yield a consistent and active product with a safety and efficacy profile that is adequate or equivalent to previous lots. The vaccine must show a level of protection against a virulent strain of *B. anthracis*, in a suitable animal infection model, that is equal to or greater than that of a reference vaccine. The vaccine must not show a level of toxicity that exceeds that of a reference vaccine.

The production method and stability of the final lot and relevant intermediates are evaluated using one or more indicator tests. Such tests include potency and specific toxicity, and may be supported by tests confirming the presence of relevant antigens and associated proteins. Release and shelf-life specifications are established based upon the results of stability testing so as to ensure satisfactory product performance during the approved period of validity.

*SEED LOTS*

The attenuated non-encapsulated strain of *B. anthracis* used is identified by historical records that include information on its origin and subsequent manipulation and the tests used to characterise the strain. These include morphological, cultural, biochemical and genetic properties of the strain. Only a master seed lot or, where applicable, working seed lots, that comply with the following requirements may be used.

**Identification**. Each seed lot is identified as containing *B. anthracis*.

**Phenotypic parameters**. Each seed lot must have a known biochemical and enzymatic profile and have a known history of absence of antibiotic resistance.

**Microbial purity**. Each seed lot complies with the requirements for absence of contaminating organisms. Purity of bacterial cultures is verified by methods of suitable sensitivity.

**Virulence test**. The absence of bacterial capsule is demonstrated for each seed lot by McFadyean stain and the specific toxicity (oedema) test.

*REFERENCE PREPARATION*

The potency and toxicity of the vaccine bulk are verified using reference standards derived from representative vaccine batches. These batches are extensively characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their stability.

*PROPAGATION AND HARVEST*

The attenuated strain is grown using suitable liquid media. At the end of cultivation, the purity of the culture is tested. The culture medium is separated from the bacterial mass by filtration. The pH of the filtrate is determined after dilution with a 0.9 g/L solution of *sodium chloride R* and is shown to be within limits suitable for stability. A suitable test for absence of live *B. anthracis*, including spores, is carried out. Aluminium potassium sulfate or an alternative adjuvant may be added at this stage. An antimicrobial preservative may be added to the suspension to form the purified harvest.

Only a purified harvest that complies with the following requirements may be used in the preparation of the final lot.

**Immunological identity**. Confirm the presence of *B. anthracis* protective antigen by a suitable immunochemical method (*2.7.1*).

**Antimicrobial preservative**. Determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

*FINAL BULK VACCINE*

The purified harvest is diluted aseptically with sterile saline solution to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident glass ampoules and heat-sealed to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the potency assay, the specific toxicity (oedema) test and the test for antimicrobial preservative have been carried out with satisfactory results on the purified harvest, they may be omitted on the final lot.

IDENTIFICATION

The presence of *B. anthracis* protective antigen is confirmed by a suitable immunochemical method (*2.7.1*).

TESTS

**Specific toxicity (oedema) test**. Use not fewer than 2 rabbits per test. Prepare serial two-fold dilutions of vaccine with normal saline, corresponding to 4, 2, 1, 0.5 and 0.25 human doses. Inject intradermally 0.1 mL of each dilution of the test and of the reference vaccine into the shaved flanks of 2 rabbits. Each rabbit receives the 10 previously prepared injections (5 dilutions of the test vaccine and 5 dilutions of the reference vaccine). In one of the rabbits, the lower concentrations are injected at the anterior end and the higher concentrations at the posterior end. The reverse is used for the 2nd rabbit. The rabbits are monitored for 24 h for signs of oedema at

the injection site. The vaccine complies with the test if the oedematous reaction is not greater than that observed with the reference vaccine.

Alternatively, specific *in vitro* assays for lethal factor and adenylate cyclase activity may be used, subject to validation.

**Antimicrobial preservative**. Determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the intended content.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

The potency of the anthrax vaccine is determined by comparing the dose required to protect guinea-pigs against intradermal challenge by a virulent strain of *B. anthracis* with the dose of a suitable reference preparation that gives the same protection. Use 9 groups of not fewer than 16 female guinea-pigs, each weighing 250-350 g. Prepare 4 dilutions of the vaccine and of the reference preparation containing 1.5, 0.5, 0.17 and 0.05 human doses in 0.5 mL. Allocate each dilution to a separate group. The remaining group receives 0.5 mL of saline and is used to verify the challenge dose. Inject subcutaneously into each guinea-pig 0.5 mL of the dilution allocated to its group on each of 2 occasions, 1 week apart. 7 days after the 2nd injection, inject intradermally into each guinea-pig 2000 spores of a virulent strain of *B. anthracis* (Vollum) in 0.1 mL. Observe the animals for 10 days and record the number of deaths per group. The test is not valid unless all the control animals die within 5 days of challenge. Using the proportions of animals that survive in each of the vaccinated groups, calculate the potency of the vaccine relative to the reference preparation using the usual statistical methods (*5.3*). The vaccine complies with the test if:

- the relative potency estimate exceeds 1.0, or;
- the 95 per cent confidence interval for the relative potency includes 1.0, and the lower 95 per cent confidence limit is not less than 50 per cent of the relative potency estimate.

LABELLING

The label states that the vaccine is not to be frozen.

**01/2009:1929**

# BCG FOR IMMUNOTHERAPY

## BCG ad immunocurationem

DEFINITION

BCG for immunotherapy is a freeze-dried preparation of live bacteria derived from a culture of the bacillus of Calmette and Guérin (*Mycobacterium bovis* BCG) whose capacity for treatment has been established.

It complies with the monograph *Vaccines for human use (0153)*.

PRODUCTION

*GENERAL PROVISIONS*

BCG for immunotherapy shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, nor shall they be exposed to a known risk of tuberculosis infection. Staff are examined periodically for tuberculosis. BCG for immunotherapy is susceptible to sunlight: the procedures for production shall be so designed that all products are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production is based on a seed-lot system. The production method shall have been shown to yield consistently BCG products that can be used for treatment of superficial bladder cancer and are safe. The product is prepared from cultures which are separated from the master seed lot by as few subcultures as possible and in any case not more than 8 subcultures. During the course of these subcultures the preparation is not freeze-dried more than once.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

*SEED LOTS*

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics, its capacity to treat and prevent superficial bladder cancer, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation. Before establishment of a working seed lot a batch is prepared and reserved for use as the comparison product. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of product prepared from the new working seed lot; the product is shown to be not significantly different in activity from the comparison product. Antimicrobial agent sensitivity testing is also carried out.

Only a working seed lot that complies with the following requirements may be used for propagation.

**Identification**. The bacteria in the working seed lot are identified as *Mycobacterium bovis* BCG using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium. The working seed lot complies with the test for sterility, except for the presence of mycobacteria.

**Virulent mycobacteria**. Examine the working seed lot as prescribed under Tests, using 10 guinea-pigs.

*PROPAGATION AND HARVEST*

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium does not contain substances known to cause toxic or allergic reactions in human beings or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the culture as determined by a suitable method of viable count.

*FINAL BULK*

The final bulk is prepared from a single harvest or by pooling a number of single harvests. A stabiliser may be added; if the stabiliser interferes with the determination of bacterial concentration on the final bulk, the determination is carried out before addition of the stabiliser.

Only final bulk that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL of final bulk for each medium. The final bulk complies with the test for sterility, except for the presence of mycobacteria.

**Count of viable units**. Determine the number of viable units per millilitre by viable count on solid medium using a method suitable for the product to be examined or by a suitable biochemical method. Carry out the test in parallel on a reference preparation of the same strain.

**Bacterial concentration**. Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the micro-organisms; if the bacterial concentration is determined before addition of a stabiliser, the concentration in the final bulk is established by calculation. The total bacterial concentration is within the limits approved for the particular product.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product.

*FINAL LOT*

The final bulk is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the product; the containers are closed either under vacuum or under an inert gas.

Except where the filled and closed containers are stored at a temperature of – 20 °C or lower, the expiry date is not later than 4 years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

**Count of viable units**. Determine the number of viable units per millilitre of the reconstituted product by viable count on solid medium using a method suitable for the product to be examined, or by a suitable biochemical method. The ratio of the count of viable units after freeze-drying to that before is not less than that approved for the particular product.

IDENTIFICATION

BCG for immunotherapy is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance of colonies grown on solid medium. Alternatively, molecular biology techniques (for example, nucleic acid amplification) may be used.

TESTS

**Virulent mycobacteria**. Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing 250-400 g and having received no treatment likely to interfere with the test, a quantity of the product to be examined equivalent to at least 1/25 of 1 human dose. Observe the animals for at least 42 days. At the end of this period, euthanise the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The product complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than 1 animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis, repeat the test on 6 other guinea-pigs. The product complies with the test if not more than 1 animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

**Bacterial and fungal contamination**. The reconstituted product complies with the test for sterility (*2.6.1*) except for the presence of mycobacteria.

**Water**. Not more than the limit approved for the particular product, determined by a suitable method.

ASSAY

Determine the number of viable units in the reconstituted product by viable count on solid medium using a method suitable for the product to be examined or by a suitable validated biochemical method. The number is within the range stated on the label. Determine the number of viable units in the comparison control in parallel.

LABELLING

The label states:

- the minimum and the maximum number of viable units per dose in the reconstituted product;
- that the product must be protected from direct sunlight.

**01/2012:0163**

# BCG VACCINE, FREEZE-DRIED

## Vaccinum tuberculosis (BCG) cryodesiccatum

DEFINITION

Freeze-dried BCG vaccine is a preparation of live bacteria derived from a culture of the bacillus of Calmette and Guérin (*Mycobacterium bovis* BCG) whose capacity to protect against tuberculosis has been established.

PRODUCTION

*GENERAL PROVISIONS*

BCG vaccine shall be produced by a staff consisting of healthy persons who do not work with other infectious agents; in particular they shall not work with virulent strains of *Mycobacterium tuberculosis*, nor shall they be exposed to a known risk of tuberculosis infection. Staff are examined periodically for tuberculosis. BCG vaccine is susceptible to sunlight: the procedures for the preparation of the vaccine shall be designed so that all cultures and vaccines are protected from direct sunlight and from ultraviolet light at all stages of manufacture, testing and storage.

Production of the vaccine is based on a seed-lot system. The production method shall have been shown to yield consistently BCG vaccines that induce adequate sensitivity to tuberculin in man, that have acceptable protective potency in animals and are safe. The vaccine is prepared from cultures which are derived from the master seed lot by as few subcultures as possible and in any case not more than 8 subcultures. During the course of these subcultures the preparation is not freeze-dried more than once.

If a bioluminescence test or other biochemical method is used instead of viable count, the method is validated against the viable count for each stage of the process at which it is used.

*BACTERIAL SEED LOTS*

The strain used to establish the master seed lot is chosen for and maintained to preserve its characteristics, its capacity to sensitise man to tuberculin and to protect animals against tuberculosis, and its relative absence of pathogenicity for man and laboratory animals. The strain used shall be identified by historical records that include information on its origin and subsequent manipulation.

A suitable batch of vaccine is prepared from the first working seed lot and is reserved for use as the comparison vaccine. When a new working seed lot is established, a suitable test for delayed hypersensitivity in guinea-pigs is carried out on a batch of vaccine prepared from the new working seed lot; the vaccine is shown to be not significantly different in activity from the comparison vaccine. Antimicrobial agent sensitivity testing is also carried out.

Only a working seed lot that complies with the following requirements may be used for propagation.

**Identification**. The bacteria in the working seed lot are identified as *Mycobacterium bovis* BCG using microbiological techniques, which may be supplemented by molecular biology techniques (for example, nucleic acid amplification and restriction-fragment-length polymorphism).

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

**Virulent mycobacteria**. Examine the working seed lot as prescribed under Tests, using 10 guinea-pigs.

*PROPAGATION AND HARVEST*

The bacteria are grown in a suitable medium for not more than 21 days by surface or submerged culture. The culture medium does not contain substances known to cause toxic or allergic reactions in humans or to cause the bacteria to become virulent for guinea-pigs. The culture is harvested and suspended in a sterile liquid medium that protects the viability of the vaccine as determined by a suitable method of viable count.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from a single harvest or by pooling a number of single harvests. A stabiliser may be added; if the stabiliser interferes with the determination of bacterial concentration in the final bulk vaccine, the determination is carried out before addition of the stabiliser.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium. The final bulk vaccine complies with the test for sterility except for the presence of mycobacteria.

**Count of viable units**. Determine the number of viable units per millilitre by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable biochemical method. Carry out the test in parallel on a reference preparation of the same strain.

**Bacterial concentration**. Determine the total bacterial concentration by a suitable method, either directly by determining the mass of the micro-organisms, or indirectly by an opacity method that has been calibrated in relation to the mass of the organisms; if the bacterial concentration is determined before addition of a stabiliser, the concentration in the final bulk vaccine is established by calculation. The total bacterial concentration is within the limits approved for the particular product.

The ratio of the count of viable units to the total bacterial concentration is not less than that approved for the particular product.

*FINAL LOT*

The final bulk vaccine is distributed into sterile containers and freeze-dried to a moisture content favourable to the stability of the vaccine; the containers are closed either under vacuum or under an inert gas.

Except where the filled and closed containers are stored at a temperature of – 20 °C or lower, the expiry date is not later than 4 years from the date of harvest.

Only a final lot that complies with the following requirement for count of viable units and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for virulent mycobacteria has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. Provided the test for excessive dermal reactivity has been carried out with satisfactory results on the working seed lot and on 5 consecutive final lots produced from it, the test may be omitted on the final lot.

**Count of viable units**. Determine the number of viable units per millilitre of the reconstituted vaccine by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable biochemical method. The ratio of the count of viable units after freeze-drying to that before is not less than that approved for the particular product.

**Thermal stability**. Maintain containers of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 4 weeks. Determine the number of viable units as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The number of viable units in the heated vaccine is not less than 20 per cent of that in the unheated vaccine.

## IDENTIFICATION

BCG vaccine is identified by microscopic examination of the bacilli in stained smears demonstrating their acid-fast property and by the characteristic appearance of colonies grown on solid medium. Alternatively, molecular biology techniques (for example nucleic acid amplification) may be used.

## TESTS

**Virulent mycobacteria**. Inject subcutaneously or intramuscularly into each of 6 guinea-pigs, each weighing 250-400 g and having received no treatment likely to interfere with the test, a quantity of vaccine equivalent to at least 50 human doses. Observe the animals for at least 42 days. At the end of this period, euthanise the guinea-pigs and examine by autopsy for signs of infection with tuberculosis, ignoring any minor reactions at the site of injection. Animals that die during the observation period are also examined for signs of tuberculosis. The vaccine complies with the test if none of the guinea-pigs shows signs of tuberculosis and if not more than 1 animal dies during the observation period. If 2 animals die during this period and autopsy does not reveal signs of tuberculosis repeat the test on 6 other guinea-pigs. The vaccine complies with the test if not more than 1 animal dies during the 42 days following the injection and autopsy does not reveal any sign of tuberculosis.

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*) except for the presence of mycobacteria.

**Excessive dermal reactivity**. Use 6 healthy, white or pale-coloured guinea-pigs, each weighing not less than 250 g and having received no treatment likely to interfere with the test. Inject intradermally into each guinea-pig, according to a randomised plan, 0.1 mL of the reconstituted vaccine and of 2 tenfold serial dilutions of the vaccine and identical doses of the comparison vaccine. Observe the lesions formed at the site of the injection for 4 weeks. The vaccine complies with the test if the reaction it produces is not markedly different from that produced by the comparison vaccine.

**Water**. Not more than the limit approved for the particular product, determined by a suitable method.

## ASSAY

Determine the number of viable units in the reconstituted vaccine by viable count on solid medium using a method suitable for the vaccine to be examined or by a suitable validated biochemical method. The number is within the range stated on the label. Determine the number of viable units in the comparison vaccine in parallel.

## LABELLING

The label states:

- the minimum and maximum number of viable units per millilitre in the reconstituted vaccine,
- that the vaccine must be protected from direct sunlight.

**01/2008:2327**

# CHOLERA VACCINE (INACTIVATED, ORAL)

## Vaccinum cholerae perorale inactivatum

DEFINITION

Cholera vaccine (inactivated, oral) is a homogeneous suspension of inactivated suitable strains of *Vibrio cholerae* serogroup O1, representing serotypes and biotypes of epidemic strains. The vaccine may contain the B subunit of cholera toxin (CTB). Just prior to ingestion, one dose of vaccine suspension is mixed with a suitable buffer as stated on the label.

PRODUCTION

*GENERAL PROVISIONS*

The production method must be validated to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The production process must be validated to show that no clinically significant quantities of active toxin are present in the product.

*CHOICE OF VACCINE STRAIN*

The vaccine consists of a mixture of epidemic *V. cholerae* strains inactivated by a suitable method such as heat or formalin inactivation. All strains express smooth lipopolysaccharide (LPS). The CTB is produced by recombinant DNA technology in a strain that lacks the gene for cholera toxin subunit A (*ctxA*$^-$). Selected *V. cholerae* strains are low cholera-toxin producers.

The World Health Organization (WHO) can recommend new vaccine strains or antigens that may be used if necessary, in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia.

*SEED LOTS*

The strains of *V. cholerae* used shall be identified by historical records that include information on the origin of the strains and their subsequent manipulation. Characterisation and maintenance of the recombinant strains and plasmids used for production of the recombinant B subunit of cholera toxin (rCTB) and the origin of the gene for cholera toxin subunit B (*ctxB*) are documented. The stability of the rCTB plasmid in the recombinant strain during storage and beyond the passage level used in production is confirmed.

Characterisation of the rCTB is undertaken using a variety of analytical techniques including determination of molecular size, charge and amino acid composition. Techniques suitable for such purposes include sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and different liquid chromatographies. The identity of the product is confirmed by at least partial *N*-terminal and *C*-terminal amino acid sequencing.

Master seed lots are grown on agar plates, which may contain appropriate antibiotics. Colonies are used to produce working seed lots in liquid media that are free from antibiotics. Cultures derived from the working seed lot must have the same characteristics as the cultures of the strain from which the master seed lot was derived.

Only a seed lot that complies with the following requirements may be used in the preparation of the monovalent cell harvest.

**Identification**. Master seed lots are identified by colony morphology, and by biochemical characterisation, using suitable molecular assays or immunoassays. Working seed lots are identified by colony morphology and by molecular assays or immunoassays.

**Purity**. Purity of master seed lots and working seed lots is verified by methods of suitable sensitivity.

*PROPAGATION AND HARVEST*

Each strain is grown separately from the working seed lot.

Cultures are checked at different stages of fermentation (subcultures and main culture) for purity, identity, cell opacity, pH and biochemical characteristics. Unsatisfactory cultures must be discarded.

Production cultures are shown to be consistent in respect of growth rate, pH and yield of cells or cell products.

*MONOVALENT CELL HARVEST*

Only a monovalent harvest that complies with established specifications for the following tests may be used.

**pH** (*2.2.3*): within the range approved for the particular product.

**Identification**. Relevant antigenic characteristics are verified by suitable immunological or biochemical assays.

**Purity**. Samples of culture are examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

**Opacity**. The absorbance at 600 nm (*2.2.25*) is within the range approved for the particular product.

*INACTIVATED MONOVALENT CELL BULK*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. Bacteria are inactivated after washing, either by treatment with formaldehyde or by heating under conditions that ensure inactivation.

Only an inactivated monovalent cell bulk that complies with established specifications for the following tests may be used in the preparation of the final bulk.

**pH** (*2.2.3*): within the range approved for the particular product.

**Identification**: verified by slide agglutination.

**Inactivation**. Complete inactivation is verified by a suitable culture method.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**Opacity**. The inactivation process may affect the accuracy of opacity measurements.

**Purity**. Samples of culture are examined by microscopy of Gram-stained smears, by inoculation of appropriate culture media or by another suitable procedure.

**Smooth LPS content**: verified by a suitable immunoassay (*2.7.1*).

**Residual cholera toxin**. The absence of residual cholera toxin is verified by a suitable immunoassay (*2.7.1*) or biochemical assay.

**Free formaldehyde** (*2.4.18*): content to be determined where formaldehyde is used for inactivation.

*PURIFIED rCTB*

Production of the rCTB follows the guidelines for assuring the quality of pharmaceutical and biological products prepared by recombinant technology and is covered by the monograph *Products of recombinant DNA technology (0784)*. Prior to harvest, the cell culture is checked for purity and opacity. rCTB is harvested by suitable filtration, concentrated by diafiltration, purified by chromatography, filter-sterilised and stored under suitable conditions. The pH of the pooled eluate is adjusted prior to buffer exchange.

Only purified rCTB that complies with established specifications for the following tests may be used in the preparation of the final bulk.

**pH** (*2.2.3*): within the range approved for the particular product.

**Purity**: verified by SDS-PAGE (*2.2.31*) and an appropriate liquid chromatography method (*2.2.29*).

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**rCTB**. The amount of rCTB is determined by a suitable immunoassay (*2.7.1*).

*FINAL BULK*

The final bulk vaccine is prepared by aseptically mixing a suitable buffer with monovalent cell bulks. Where used, the rCTB bulk is added in appropriate amounts. Preservatives, if used, may be added at this stage.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

*FINAL LOT*

The final bulk is mixed to homogeneity and filled aseptically into suitable containers.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

Serotypes are detected by a suitable immunoassay (*2.7.1*) or molecular assay. rCTB is detected by a suitable immunoassay (*2.7.1*). The antigen-content assays may also serve as an identity test.

TESTS

**pH** (*2.2.3*): within the range approved for the particular product.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

ASSAY

**Antigen content**. The amount of smooth LPS, and where applicable, the amount of rCTB, are within the limits approved for the particular product, determined by a suitable immunoassay (*2.7.1*).

LABELLING

The label states:

- the method of inactivation;
- the serogroup, serotypes and biotypes of vaccine strains;
- the number of bacteria per human dose;
- the amount of rCTB.

**01/2008:0444**
**corrected 10.0**

# DIPHTHERIA AND TETANUS VACCINE (ADSORBED)

## Vaccinum diphtheriae et tetani adsorbatum

DEFINITION

Diphtheria and tetanus vaccine (adsorbed) is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED DIPHTHERIA AND TETANUS TOXOIDS*

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs on *Diphtheria vaccine (adsorbed) (0443)* and *Tetanus vaccine (adsorbed) (0452)* and comply with the requirements prescribed therein.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.
Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.
Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

**01/2015:0647**
**corrected 10.0**

# DIPHTHERIA AND TETANUS VACCINE (ADSORBED, REDUCED ANTIGEN(S) CONTENT)

## Vaccinum diphtheriae et tetani, antigeni-o(-is) minutum, adsorbatum

DEFINITION

Diphtheria and tetanus vaccine (adsorbed, reduced antigen(s) content) is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively. The amount of diphtheria toxoid per single human dose is reduced compared to vaccines generally used for primary vaccination; the amount of tetanus toxoid may also be reduced.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than one animal dies from non-specific causes, repeat the test once; if more than one animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED DIPHTHERIA TOXOID AND TETANUS TOXOIDS*

The bulk purified diphtheria and tetanus toxoids are prepared as described in the monographs *Diphtheria vaccine (adsorbed) (0443)* and *Tetanus vaccine (adsorbed) (0452)* and comply with the requirements prescribed therein.

*FINAL BULK VACCINE*

The vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoids or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 mL of the vaccine to be examined and suspend the residue in 5 mL of a freshly prepared mixture of 1 volume of a 56 g/L solution of *sodium edetate R* and

49 volumes of *disodium hydrogen phosphate solution R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 2 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 20 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.



**01/2008:2062**

# DIPHTHERIA, TETANUS AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani et hepatitidis B (ADNr) adsorbatum

DEFINITION

Diphtheria, tetanus and hepatitis B (rDNA) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; hepatitis B surface antigen (HBsAg); a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

HBsAg is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

The content of bacterial endotoxins (*2.6.14*) in the bulk purified diphtheria toxoid and tetanus toxoid is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and in any case the contents are such that the final vaccine contains less than 100 IU per single human dose.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)* and *Hepatitis B vaccine (rDNA) (1056)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and HBsAg onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria and tetanus components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Pyrogens** (*2.6.8*). It complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Hepatitis B component**. It complies with the assay of hepatitis B vaccine (*2.7.15*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose,
- the amount of HBsAg per single human dose,
- the type of cells used for production of the HBsAg component,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.

01/2020:1931

# DIPHTHERIA, TETANUS AND PERTUSSIS (ACELLULAR, COMPONENT) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani et pertussis sine cellulis ex elementis praeparatum adsorbatum

DEFINITION

Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in the bulk purified diphtheria toxoid, tetanus toxoid and pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict

adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)* and *Pertussis vaccine (acellular, component, adsorbed) (1356)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and pertussis components separately or together onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for free formaldehyde and antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2020:2764**

# DIPHTHERIA, TETANUS AND PERTUSSIS (ACELLULAR, COMPONENT) VACCINE (ADSORBED, REDUCED ANTIGEN(S) CONTENT)

## Vaccinum diphtheriae, tetani et pertussis sine cellulis ex elementis praeparatum, antigeni-o(-is) minutum, adsorbatum

DEFINITION

Diphtheria, tetanus and pertussis (acellular, component) vaccine (adsorbed, reduced antigen(s) content) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The amount of diphtheria toxoid per single human dose is reduced compared to vaccines generally used for primary vaccination; the amounts of tetanus toxoid and pertussis components may also be reduced.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

## PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

*Reference vaccine(s)*. Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)* and *Pertussis vaccine (acellular, component, adsorbed) (1356)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and acellular pertussis components. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

## IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 mL of the vaccine to be examined and suspend the residue in 5 mL of a freshly prepared mixture of 1 volume of a 56 g/L solution of *sodium edetate R* and 49 volumes of *disodium hydrogen phosphate solution R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

## TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 2 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 20 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**07/2012:0445**
**corrected 10.0**

# DIPHTHERIA, TETANUS AND PERTUSSIS (WHOLE CELL) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani et pertussis ex cellulis integris adsorbatum

DEFINITION

Diphtheria, tetanus and pertussis (whole cell) vaccine (adsorbed) is a preparation of diphtheria formol toxoid and tetanus formol toxoid with a mineral adsorbent to which a suspension of inactivated *Bordetella pertussis* has been added. The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED DIPHTHERIA AND TETANUS TOXOIDS, BULK INACTIVATED B. PERTUSSIS SUSPENSION*

The bulk purified diphtheria and tetanus toxoids and the inactivated *B. pertussis* suspension are prepared as described in the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)* and *Pertussis vaccine (whole cell, adsorbed) (0161)*, respectively, and comply with the requirements prescribed therein.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide and admixture of an appropriate quantity of a suspension of inactivated *B. pertussis*; the resulting mixture is approximately isotonic with blood. The *B. pertussis* concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. Suitable antimicrobial preservatives may be added to the bulk vaccine. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity of the pertussis component, antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained; reserve the precipitate for identification test C. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge to obtain a bacterial precipitate. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay.

TESTS

**Specific toxicity of the pertussis component**. Use not fewer than 5 mice each weighing 14 - 16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 mL, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 mL of a 9 g/L sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

If the test is carried out in guinea-pigs, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 60 IU per single human dose.

**Pertussis component**. Carry out the assay of pertussis vaccine (whole cell) (*2.7.7*).

The estimated potency is not less than 4.0 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2.0 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units of each component per single human dose;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2008:2328**
**corrected 10.0**

# DIPHTHERIA, TETANUS AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED, REDUCED ANTIGEN(S) CONTENT)

## Vaccinum diphtheriae, tetani et poliomyelitidis inactivatum, antigeni-o(-is) minutum, adsorbatum

DEFINITION

Diphtheria, tetanus and poliomyelitis (inactivated) vaccine (adsorbed, reduced antigen(s) content) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The amount of diphtheria toxoid per single human dose is reduced compared to vaccines generally used for primary vaccination; the amount of tetanus toxoid may also be reduced.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than one animal dies from non-specific causes, repeat the test once; if more than one animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid and inactivated monovalent poliovirus harvests is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs on *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)* and *Poliomyelitis vaccine (inactivated) (0214)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid and tetanus toxoid, and an admixture of suitable quantities of purified monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) after virus harvest and before addition of the adsorbent in the preparation of the final bulk vaccine, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria and tetanus components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided the determination of D-antigen content cannot be carried out on the final lot, it is carried out during preparation of the final bulk before addition of the adsorbent.

Provided the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given vaccine and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 mL of the vaccine to be examined and suspend the residue in 5 mL of a freshly prepared mixture of 1 volume of a 56 g/L solution of *sodium edetate R* and 49 volumes of *disodium hydrogen phosphate solution R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 20 IU per single human dose.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

– the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;

- the types of poliovirus contained in the vaccine;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2020:1932**

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et haemophili stirpis b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral absorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented either as a tetravalent liquid formulation in the same container, or as a trivalent liquid formulation with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{11}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Where the haemophilus component is presented in a separate container, as part of consistency studies the assays of the diphtheria, tetanus and pertussis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine; where the haemophilus component is presented in a separate container, the contents of the diphtheria, tetanus and pertussis antigens are in any case such that the final vial for these components contains less than 100 IU per single human dose.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the conjugate are not affected.

Where the haemophilus component is presented in a separate container, the production method is validated to demonstrate that the haemophilus component, if tested, would comply with the test for pyrogens (*2.6.8*), carried out as follows: inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP (meningococcal group B outer membrane protein complex) as carrier.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)* and *Haemophilus type b conjugate vaccine (1219)*.

*FINAL BULK VACCINE*

Different methods of preparation may be used: a final bulk vaccine may be prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and PRP conjugate onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate; or 2 final bulks may be prepared and filled separately, one containing the diphtheria, tetanus and pertussis components, the other the haemophilus component, which may be freeze-dried. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

**Osmolality** (*2.2.35*). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

**pH** (*2.2.3*). The pH of the vaccine, reconstituted if necessary, is within the range approved for the particular product.

**Free PRP**. Unbound PRP is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

*Where the haemophilus component is presented in a separate container: identification tests A, B and C are carried out using the container containing the diphtheria, tetanus and pertussis components; identification test D is carried out on the container containing the haemophilus component.*

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

D. The haemophilus component is identified by a suitable immunochemical method (*2.7.1*) for PRP.

TESTS

*Where the product is presented with the haemophilus component in a separate container: the tests for aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus and pertussis components; the tests for PRP content, water (where applicable), sterility and bacterial endotoxins are carried out on the container with the haemophilus component.*

*If the haemophilus component is freeze-dried, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.*

**PRP**: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Water** (*2.5.12*): maximum 3.0 per cent for the freeze-dried haemophilus component.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the haemophilus component of the particular product. If any components of the vaccine prevent the determination of endotoxin, a test for pyrogens is carried out as described under General provisions.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the number of micrograms of PRP per single human dose;
- the type and nominal amount of carrier protein per single human dose;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

01/2020:1933

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et hepatitidis B (ADNr) adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and hepatitis B (rDNA) vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani*, respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in the bulk purified diphtheria toxoid, tetanus toxoid and pertussis components is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine.

*Reference vaccine(s)*. Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)* and *Hepatitis B vaccine (rDNA) (1056)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the content of free formaldehyde has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

D. The assay or, where applicable, the electrophoretic profile, serves also to identify the hepatitis B component of the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Pyrogens** (*2.6.8*). The vaccine complies with the test for pyrogens. Inject the equivalent of 1 human dose into each rabbit.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

**Hepatitis B component**. The vaccine complies with the assay of hepatitis B vaccine (*2.7.15*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the amount of HBsAg per single human dose;
- the type of cells used for production of the hepatitis B component;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.



01/2020:1934

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et poliomyelitidis inactivatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and purified, inactivated monovalent poliovirus harvests is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between

the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)* and *Poliomyelitis vaccine (inactivated) (0214)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and admixture of suitable quantities of purified monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) after virus harvest and before addition of the adsorbent in the preparation of the final bulk vaccine, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided that the determination of D-antigen content has been carried out with satisfactory results during preparation of the final bulk before addition of the adsorbent, it may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with specific antisera to the pertussis components of the vaccine.

D. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia

Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the types of poliovirus contained in the vaccine;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2020:2329**

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT) AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED, REDUCED ANTIGEN(S) CONTENT)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum et poliomyelitidis inactivatum, antigeni-o(-is) minutum, adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component) and poliomyelitis (inactivated) vaccine (adsorbed, reduced antigen(s) content) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The amount of diphtheria toxoid per single human dose is reduced compared to vaccines generally used for primary vaccination; the amounts of tetanus toxoid and pertussis components may also be reduced.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components and inactivated monovalent poliovirus harvests is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved for the particular vaccine and, in any case, the contents are such that the final vaccine contains less than 100 IU per single human dose.

*Reference vaccine(s)*. Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)* and *Poliomyelitis vaccine (inactivated) (0214)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid and acellular pertussis components, and an admixture of suitable quantities of purified monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) after virus harvest and before addition of the adsorbent in the preparation of the final bulk vaccine, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided the determination of D-antigen content cannot be carried out on the final lot, it is carried out during preparation of the final bulk before addition of the adsorbent.

Provided the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given vaccine and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 mL of the vaccine to be examined and suspend the residue in 5 mL of a freshly prepared mixture of 1 volume of a 56 g/L solution of *sodium edetate R* and 49 volumes of a *disodium hydrogen phosphate solution R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained as described in identification test A reacts with a specific antisera to the pertussis components of the vaccine.

D. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 2 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 20 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the types of poliovirus contained in the vaccine;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

01/2020:2067

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT), HEPATITIS B (rDNA), POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum, hepatitidis B (ADNr), poliomyelitidis inactivatum et haemophili stirpis b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component), hepatitis B (rDNA), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; hepatitis B surface antigen (HBsAg); human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein. The antigens in the vaccine may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented either as a hexavalent liquid formulation in the same container, or as a pentavalent liquid formulation with the haemophilus component in a separate container, the contents of which are mixed with the other components immediately before or during use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

Hepatitis B surface antigen is a component protein of hepatitis B virus; the antigen is obtained by recombinant DNA technology.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{11}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

If the vaccine is presented with the haemophilus component in a separate container, as part of consistency studies the assays of the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid and pertussis components, hepatitis B surface antigen, purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is not greater than the limit approved.

During development studies and wherever revalidation is necessary, a test for pyrogens in rabbits (*2.6.8*) is carried out by injection of a suitable dose of the final lot. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the conjugate are not affected.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. For the haemophilus component, such tests may include determination of molecular size, determination of free PRP in the conjugate and kinetics of depolymerisation. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)*, *Hepatitis B vaccine (rDNA) (1056)*, *Poliomyelitis vaccine (inactivated) (0214)* and *Haemophilus type b conjugate vaccine (1219)*.

*FINAL BULKS*

*Vaccine with all components in the same container.* The final bulk is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface

antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of a suitable quantity of PRP conjugate and suitable quantities of purified and inactivated, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

*Vaccine with the haemophilus component in a separate container*. The final bulk of diphtheria, tetanus, pertussis, hepatitis B and poliovirus component is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, tetanus toxoid, acellular pertussis components and hepatitis B surface antigen onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of purified and inactivated, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable pool of such monovalent harvests. This final bulk is filled separately. Suitable antimicrobial preservatives may be added. The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) after purification of the harvests and before preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Where the haemophilus component is in a separate container, the final bulk of the haemophilus component is freeze-dried. Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for osmolality, the test for antimicrobial preservative and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided that the test for bovine serum albumin has been carried out with satisfactory results on the trivalent pool of inactivated monovalent harvests of polioviruses or on the final bulk vaccine, it may be omitted on the final lot.

If an *in vivo* assay is used for the hepatitis B component, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

Provided the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Free PRP**. For vaccines with all components in the same container, the free PRP content is determined on the non-absorbed fraction. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

**Bacterial endotoxins** (*2.6.14*): less than the limit approved for the product concerned.

**Osmolality** (*2.2.35*). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

IDENTIFICATION

*If the vaccine is presented with the haemophilus component in a separate container: identification tests A, B, C, D and E are carried out using the container with the diphtheria, tetanus, pertussis, hepatitis B and poliomyelitis components; identification test F is carried out on the container with the haemophilus components.*

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The clear supernatant obtained during identification test A reacts with specific antisera to the pertussis components of the vaccine when examined by suitable immunochemical methods (*2.7.1*).

D. The hepatitis B component is identified by a suitable immunochemical method (*2.7.1*), for example the *in vitro* assay, or by a suitable electrophoretic method (*2.2.31*).

E. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

F. The PRP and its carrier protein are identified by a suitable immunochemical method (*2.7.1*).

TESTS

*If the product is presented with the haemophilus component in a separate container, the tests for free formaldehyde, aluminium, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis, poliomyelitis and hepatitis B components; the tests for PRP, water, antimicrobial preservative (where applicable), aluminium (where applicable) and sterility are carried out on the container with the haemophilus component.*

*Some tests for the haemophilus component are carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.*

**PRP**: minimum 80 per cent of the amount of PRP stated on the label, for a vaccine with the haemophilus component in a separate container.

For a vaccine with all components in the same container: the PRP content determined on the non-absorbed fraction is not less than that approved for the product.

PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L of free formaldehyde per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Water** (*2.5.12*): maximum 3.0 per cent for the freeze-dried haemophilus component.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than the minimum potency stated on the label.

Unless otherwise justified and authorised, the minimum potency stated on the label is 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

**Hepatitis B component**. The vaccine complies with the assay of hepatitis B vaccine (*2.7.15*).

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the amount of HBsAg per single human dose;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the types of cells used for production of the poliomyelitis and the hepatitis B components;
- the number of micrograms of PRP per single human dose;
- the type and nominal amount of carrier protein per single human dose;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2020:2065**

# DIPHTHERIA, TETANUS, PERTUSSIS (ACELLULAR, COMPONENT), POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis sine cellulis ex elementis praeparatum, poliomyelitidis inactivatum et haemophili stirpis b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (acellular, component), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; individually purified antigenic components of *Bordetella pertussis*; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented either as a pentavalent liquid formulation in the same container, or as a tetravalent liquid formulation with the freeze-dried haemophilus component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties produced by expression of a genetically modified form of the corresponding gene. The acellular pertussis component may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{11}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b.

The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

The content of bacterial endotoxins (*2.6.14*) in bulk purified diphtheria toxoid, tetanus toxoid, pertussis components, purified, inactivated monovalent poliovirus harvests and bulk PRP conjugate is determined to monitor the purification procedure and to limit the amount in the final vaccine. For each component, the content of bacterial endotoxins is less than the limit approved by the competent authority for the particular vaccine.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the conjugate are not affected.

Where the haemophilus component is presented in a separate container, and as part of consistency studies, the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

Where the haemophilus component is presented in a separate container, the production method is validated to demonstrate that the haemophilus component, if tested, would comply with the test for pyrogens (*2.6.8*), carried out as follows: inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP (meningococcal group B outer membrane protein complex) as carrier.

*Reference vaccine(s)*. Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of differences in composition between the monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (acellular, component, adsorbed) (1356)*, *Poliomyelitis vaccine (inactivated) (0214)* and *Haemophilus type b conjugate vaccine (1219)*.

*FINAL BULKS*

The final tetravalent bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, bulk purified tetanus toxoid and bulk purified acellular pertussis components onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, and admixture of suitable quantities of purified, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

Where the vaccine is presented with all 5 components in the same container, the final bulk is prepared by addition of a suitable quantity of the haemophilus bulk conjugate to the tetravalent bulk. Where the haemophilus component is presented in a separate container, the final bulk is prepared by dilution of the bulk conjugate with suitable diluents for freeze-drying. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Where the haemophilus component is presented in a separate container, the final bulk of the haemophilus component is freeze-dried.

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens and the purified monovalent harvests or the trivalent pool of polioviruses or the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

If the *in vivo* assay for the poliomyelitis component is used, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

**Free PRP**. Where the haemophilus component is presented in liquid formulation, the presence of other components may interfere in the assay and it may not be possible to separate the PRP from the adjuvant. The presence of free PRP may be determined on the bulk conjugate prior to the addition of other components or on the non-adsorbed fraction in the final combination.

Where the haemophilus component is presented in a separate container, a number of methods have been used to separate free PRP from the conjugate, including precipitation, gel filtration, size-exclusion, anion-exchange and hydrophobic chromatography, ultrafiltration and ultracentrifugation. The free PRP can then be quantified by a range of techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and immunoassays with anti-PRP antibodies. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

*Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis components; identification test E is carried out either on the vial containing all 5 components, or on the vial containing the haemophilus component alone.*

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The pertussis components are identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with specific antisera to the pertussis components of the vaccine.

D. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

E. The haemophilus component is identified by a suitable immunochemical method (*2.7.1*) for PRP.

TESTS

*Where the haemophilus component is presented in a separate container, the tests for aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with the diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP, water, sterility and bacterial entodoxins are carried out on the container with the haemophilus component alone.*

*Where the haemophilus component is presented in a separate container, some tests may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.*

**PRP**: not less than 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Water** (*2.5.12*): maximum 3.0 per cent for the freeze-dried haemophilus component.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the haemophilus component of the particular product. If any components of the vaccine prevent the determination of endotoxin, a test for pyrogens is carried out as described under General provisions.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

Unless otherwise justified and authorised, the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose.

**Pertussis component**. Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the names and amounts of the pertussis components per single human dose;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- the number of micrograms of PRP per single human dose;

- the type and nominal amount of carrier protein per single human dose;
- the name and the amount of the adsorbent;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**07/2012:2061**

# DIPHTHERIA, TETANUS, PERTUSSIS (WHOLE CELL) AND POLIOMYELITIS (INACTIVATED) VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis ex cellulis integris et poliomyelitidis inactivatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (whole cell) and poliomyelitis (inactivated) vaccine (adsorbed) is a combined vaccine containing: diphtheria formol toxoid; tetanus formol toxoid; an inactivated suspension of *Bordetella pertussis*; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

**Specific toxicity of the diphtheria and tetanus components.** The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (whole cell, adsorbed) (0161)* and *Poliomyelitis vaccine (inactivated) (0214)*.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate, separately or together, of suitable quantities of bulk purified diphtheria toxoid and bulk purified tetanus toxoid and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and purified monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such purified monovalent harvests. Suitable antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to the test for osmolality and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for specific toxicity of the pertussis component and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided that the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided that the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine is within the limits approved for the particular preparation.

IDENTIFICATION

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an

example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.

D. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

TESTS

**Specific toxicity of the pertussis component**. Use not fewer than 5 healthy mice each weighing 14-16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 mL, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 mL of a 9 g/L sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

If the test is carried out in guinea pigs, the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 60 IU per single human dose.

**Pertussis component**. Carry out the assay of pertussis vaccine (whole cell) (*2.7.7*).

The estimated potency is not less than 4.0 IU per single human dose and the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 2.0 IU per single human dose.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption, using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the minimum number of International Units of pertussis vaccine per single human dose;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**07/2018:2066**

# DIPHTHERIA, TETANUS, PERTUSSIS (WHOLE CELL), POLIOMYELITIS (INACTIVATED) AND HAEMOPHILUS TYPE b CONJUGATE VACCINE (ADSORBED)

## Vaccinum diphtheriae, tetani, pertussis ex cellulis integris, poliomyelitidis inactivatum et haemophili stirpis b coniugatum adsorbatum

DEFINITION

Diphtheria, tetanus, pertussis (whole cell), poliomyelitis (inactivated) and haemophilus type b conjugate vaccine (adsorbed) is a combined vaccine composed of: diphtheria formol toxoid; tetanus formol toxoid; an inactivated suspension of *Bordetella pertussis*; suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a suitable method; polyribosylribitol phosphate (PRP) covalently bound to a carrier protein; a mineral adsorbent such as aluminium hydroxide or hydrated aluminium phosphate. The product is presented with the

haemophilus component in a separate container, the contents of which are mixed with the other components immediately before use.

The formol toxoids are prepared from the toxins produced by the growth of *Corynebacterium diphtheriae* and *Clostridium tetani* respectively.

PRP is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size and derived from a suitable strain of *Haemophilus influenzae* type b. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the conjugate are not affected.

As part of consistency studies the assays of the diphtheria, tetanus, pertussis and poliomyelitis components are carried out on a suitable number of batches of vaccine reconstituted as for use. For subsequent routine control, the assays of these components may be carried out without mixing with the haemophilus component.

For the haemophilus component, the production method is validated to demonstrate that the haemophilus component, if tested, would comply with the test for pyrogens (*2.6.8*), carried out as follows: inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP (meningococcal group B outer membrane protein complex) as carrier.

*Reference vaccine(s).* Provided valid assays can be performed, monocomponent reference vaccines may be used for the assays on the combined vaccine. If this is not possible because of interaction between the components of the combined vaccine or because of the difference in composition between monocomponent reference vaccine and the test vaccine, a batch of combined vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine may be stabilised by a method that has been shown to have no effect on the assay procedure.

**Specific toxicity of the diphtheria and tetanus components**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia or tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*PRODUCTION OF THE COMPONENTS*

The production of the components complies with the requirements of the monographs *Diphtheria vaccine (adsorbed) (0443)*, *Tetanus vaccine (adsorbed) (0452)*, *Pertussis vaccine (whole cell, adsorbed) (0161)*, *Poliomyelitis vaccine (inactivated) (0214)* and *Haemophilus type b conjugate vaccine (1219)*.

*FINAL BULKS*

The final bulk of the diphtheria, tetanus, pertussis and poliomyelitis components is prepared by adsorption, separately or together, of suitable quantities of bulk purified diphtheria toxoid, and bulk purified tetanus toxoid onto a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate and admixture of suitable quantities of an inactivated suspension of *B. pertussis* and of purified, monovalent harvests of human poliovirus types 1, 2 and 3 or a suitable quantity of a trivalent pool of such monovalent harvests. Suitable antimicrobial preservatives may be added.

The final bulk of the haemophilus component is prepared by dilution of the bulk conjugate to the final concentration with a suitable diluent. A stabiliser may be added.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

**Bovine serum albumin**. Determined on the poliomyelitis components by a suitable immunochemical method (*2.7.1*) during preparation of the final bulk vaccine, before addition of the adsorbent, the amount of bovine serum albumin is such that the content in the final vaccine will be not more than 50 ng per single human dose.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk of the haemophilus component is freeze-dried.

Only a final lot that is satisfactory with respect to the test for osmolality shown below and with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for specific toxicity of the pertussis component and antimicrobial preservative, and the assays for the diphtheria, tetanus and pertussis components have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens, the inactivated *B. pertussis* suspension and the purified monovalent harvests or the trivalent pool of polioviruses or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

Provided the *in vivo* assay for the poliomyelitis component has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

The *in vivo* assay for the poliomyelitis component may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

**Osmolality** (*2.2.35*). The osmolality of the vaccine, reconstituted where applicable, is within the limits approved for the particular preparation.

**Free PRP**. Unbound PRP is determined on the haemophilus component after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

*Identification tests A, B, C and D are carried out using the vial containing the diphtheria, tetanus, pertussis and poliomyelitis components; identification test E is carried out on the vial containing the haemophilus component.*

A. Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

B. Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. The clear supernatant obtained during identification test A reacts with a suitable tetanus antitoxin, giving a precipitate.

C. The centrifugation residue obtained in identification A may be used. Other suitable methods for separating the bacteria from the adsorbent may also be used. Identify pertussis vaccine by agglutination of the bacteria from the resuspended precipitate by antisera specific to *B. pertussis* or by the assay of the pertussis component prescribed under Assay.

D. The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*), such as determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

E. The haemophilus component is identified by a suitable immunochemical method (*2.7.1*) for PRP.

TESTS

*The tests for specific toxicity of the pertussis component, aluminium, free formaldehyde, antimicrobial preservative and sterility are carried out on the container with diphtheria, tetanus, pertussis and poliomyelitis components; the tests for PRP, water, sterility and bacterial endotoxins are carried out on the container with the haemophilus component.*

*Some tests for the haemophilus component may be carried out on the freeze-dried product rather than on the bulk conjugate where the freeze-drying process may affect the component to be tested.*

**Specific toxicity of the pertussis component**. Use not fewer than 5 healthy mice each weighing 14-16 g, for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Allow the animals access to food and water for at least 2 h before injection and during the test. Inject each mouse of the vaccine group intraperitoneally with 0.5 mL, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 mL of a 9 g/L sterile solution of *sodium chloride R*, preferably containing the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the total mass of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. The test may be repeated and the results of the tests combined.

**PRP**: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Water** (*2.5.12*): maximum 3.0 per cent for the haemophilus component.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the haemophilus component of the particular product. If any components of the vaccine prevent the determination of endotoxin, a test for pyrogens is carried out as described under General provisions.

ASSAY

**Diphtheria component**. Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 30 IU per single human dose.

**Tetanus component**. Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

If the test is carried out in guinea-pigs, the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 40 IU per single human dose; if the test is carried out in mice, the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 60 IU per single human dose.

**Pertussis component**. Carry out the assay of pertussis vaccine (whole cell) (*2.7.7*).

The estimated potency is not less than 4.0 IU per single human dose and the lower confidence limit ($P$ = 0.95) of the estimated potency is not less than 2.0 IU per single human dose.

**Poliomyelitis component**

*D-antigen content*. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) following desorption using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

*In vivo test*. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

LABELLING

The label states:

- the minimum number of International Units of diphtheria and tetanus toxoid per single human dose;
- the minimum number of International Units of pertussis vaccine per single human dose;
- the nominal amount of poliovirus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the type of cells used for production of the poliomyelitis component;
- the number of micrograms of PRP per single human dose;

- the type and nominal amount of carrier protein per single human dose;
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2008:0443**
**corrected 10.0**

# DIPHTHERIA VACCINE (ADSORBED)

## Vaccinum diphtheriae adsorbatum

DEFINITION

Diphtheria vaccine (adsorbed) is a preparation of diphtheria formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED TOXOID*

For the production of diphtheria toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *Corynebacterium diphtheriae* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is separated aseptically from the bacterial mass as soon as possible. The toxin content (Lf per millilitre) is checked (*2.7.27*) to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenic potency of the toxoid and reversion of the toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

**Absence of toxin and irreversibility of toxoid**. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at 100 Lf/mL. Divide the solution into 2 equal parts. Maintain 1 part at 5 ± 3 °C and the other at 37 °C for 6 weeks. Carry out a test in Vero cells for active diphtheria toxin using 50 µL/well of both samples. The sample should not contain antimicrobial preservatives and detoxifying agents should be determined to be below the concentration toxic to Vero cells. Non-specific toxicity may be eliminated by dialysis.

Use freshly trypsinised Vero cells at a suitable concentration, for example $2.5 \times 10^5$ $mL^{-1}$ and a reference diphtheria toxin diluted in 100 Lf/mL diphtheria toxoid. A suitable reference diphtheria toxin will contain either not less than 100 $LD_{50}$/mL or 67 to 133 lr/100 in 1 Lf and 25 000 to 50 000 minimal reacting doses for guinea-pig skin in 1 Lf (*diphtheria toxin BRP* is suitable for use as the reference toxin). Dilute the toxin in 100 Lf/mL diphtheria toxoid to a suitable concentration, for example $2 \times 10^{-4}$ Lf/mL. Prepare serial twofold dilutions of the diluted diphtheria toxin and use undiluted test samples (50 µL/well). Distribute them in the wells of a sterile tissue culture plate containing a medium suitable for Vero cells. To ascertain that any cytotoxic effect noted is specific to diphtheria toxin, prepare in parallel dilutions where the toxin is neutralised by a suitable concentration of diphtheria antitoxin, for example 100 IU/mL. Include control wells without toxoid or toxin and with non-toxic toxoid at 100 Lf/mL on each plate to verify normal cell growth. Add cell suspension to each well, seal the plates and incubate at 37 °C for 5-6 days. Cytotoxic effect is judged to be present where there is complete metabolic inhibition of the Vero cells, indicated by the pH indicator of the medium. Confirm cytopathic effect by microscopic examination or suitable staining such as MTT dye. The test is invalid if $5 \times 10^{-5}$ Lf/mL of reference diphtheria toxin in 100 Lf/mL toxoid has no cytotoxic effect on Vero cells or if the cytotoxic effect of this amount of toxin is not neutralised in the wells containing diphtheria antitoxin. The bulk purified toxoid complies with the test if no toxicity neutralisable by antitoxin is found in either sample.

**Antigenic purity** (*2.7.27*). Not less than 1500 Lf per milligram of protein nitrogen.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified antigens or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the absorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 30 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- where applicable, that the vaccine is intended for primary vaccination of children and is not necessarily suitable for reinforcing doses or for administration to adults,
- the name and the amount of the adsorbent,
- that the vaccine must be shaken before use,
- that the vaccine is not to be frozen.



**01/2015:0646**
**corrected 10.0**

# DIPHTHERIA VACCINE (ADSORBED, REDUCED ANTIGEN CONTENT)

## Vaccinum diphtheriae, antigeniis minutum, adsorbatum

DEFINITION

Diphtheria vaccine (adsorbed, reduced antigen content) is a preparation of diphtheria formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Corynebacterium diphtheriae*. The amount of diphtheria toxoid per single human dose is reduced compared to vaccines generally used for primary vaccination.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 42 days of the injection any of the animals shows signs of or dies from diphtheria toxaemia, the vaccine does not comply with the test. If more than one animal dies from non-specific causes, repeat the test once; if more than one animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED TOXOID*

The bulk purified toxoid is prepared as described in the monograph *Diphtheria vaccine (adsorbed) (0443)* and complies with the requirements prescribed therein.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Diphtheria toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate. If a satisfactory result is not obtained with a vaccine adsorbed on aluminium hydroxide, carry out the test as follows. Centrifuge 15 mL of the vaccine to be examined and suspend the residue in 5 mL of a freshly prepared mixture of 1 volume of a 56 g/L solution of *sodium edetate R* and 49 volumes of *disodium hydrogen phosphate solution R*. Maintain at 37 °C for not less than 6 h and centrifuge. The clear supernatant reacts with a suitable diphtheria antitoxin, giving a precipitate.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of diphtheria vaccine (adsorbed) (*2.7.6*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**04/2016:2622**

# HAEMOPHILUS TYPE b AND MENINGOCOCCAL GROUP C CONJUGATE VACCINE

## Vaccinum haemophili stirpi b et meningococcale classis C coniugatum

DEFINITION

Haemophilus type b and meningococcal group C conjugate vaccine is a combined vaccine composed of a preparation of a polysaccharide derived from a suitable strain of *Haemophilus influenzae* type b covalently bound to a carrier protein and a preparation of purified capsular polysaccharide derived from a suitable strain of *Neisseria meningitidis* group C covalently bound to a carrier protein. The haemophilus type b polysaccharide, polyribosylribitol phosphate (referred to as PRP), is a linear copolymer composed of repeating units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size. Meningococcal group C polysaccharide consists of partly *O*-acetylated or *O*-deacetylated repeating units of sialic acids, linked with 2α→9 glycosidic bonds. The carrier proteins, when conjugated to PRP and group C polysaccharide, are capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to consistently yield combined haemophilus type b and meningococcal group C conjugate vaccines of adequate safety and immunogenicity in man. The production of PRP, group C polysaccharide and the carrier protein(s) are based on seed-lot systems.

During development studies and wherever revalidation is necessary, a test for pyrogens (*2.6.8*) is carried out by injection of a suitable dose of the final lot as follows: inject, per kilogram of the rabbit's mass, a quantity of the vaccine equivalent to 0.1 µg of PRP. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

During development studies and wherever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the final lot and relevant intermediates is evaluated using 1 or more indicator tests. Such tests may include determination of molecular size, determination of free polysaccharide in the conjugate and the immunogenicity test in mice.

*BACTERIAL SEED LOTS*

The bacterial strains used for master seed lots shall be identified by historical records that include information on their origin and the tests used to characterise the strain. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

The seed lots are shown to be free from contamination by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

No complex products of animal origin are included in the medium used for preservation of strain viability, either for freeze-drying or for frozen storage.

It is recommended that PRP produced by the seed lot be characterised using nuclear magnetic resonance spectrometry (*2.2.33*).

*PRODUCTION OF THE COMPONENTS*

The production of the bulks and bulk conjugate components complies with the requirements of the monographs *Haemophilus type b conjugate vaccine (1219)*, *Meningococcal group C conjugate vaccine (2112)* and the general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use.*

*FINAL BULK VACCINE*

Only a final bulk vaccine that complies with the following requirement and is within the limits approved for the particular product may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

Both antigens are identified by a suitable immunochemical method (*2.7.1*) for PRP and group C polysaccharide or other suitable method.

TESTS

**pH** (*2.2.3*). The pH of the vaccine, reconstituted if necessary, is within the limits approved for the particular product.

**Water** (*2.5.12*): maximum 3.0 per cent for freeze-dried vaccines.

**Free PRP**. A number of methods are used to separate free PRP from the conjugate, including precipitation, gel filtration, size-exclusion, anion-exchange and hydrophobic chromatography, ultrafiltration and ultracentrifugation. The free PRP can then be quantified by a range of techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and immunoassays (*2.7.1*) with anti-PRP antibodies. The amount of free PRP is not greater than that approved for the particular product.

**Free saccharide**. Unbound group C polysaccharide is determined after removal of the conjugate, for example by anion-exchange liquid chromatography, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. The amount of free group C polysaccharide is not greater than that approved for the particular product.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the particular product.

ASSAY

**PRP**: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Saccharide**: minimum 80 per cent of the amount of group C polysaccharide stated on the label. The group C polysaccharide content is determined by a suitable validated assay, for example sialic acid assay (*2.5.23*) or anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

LABELLING

The label states:

- the number of micrograms of PRP and group C polysaccharide per single human dose;
- the type and nominal amount of carrier protein per single human dose.

**01/2019:1219**

# HAEMOPHILUS TYPE b CONJUGATE VACCINE

## Vaccinum haemophili stirpis b coniugatum

DEFINITION

Haemophilus type b conjugate vaccine is a liquid or freeze-dried preparation of a polysaccharide, derived from a suitable strain of *Haemophilus influenzae* type b, covalently bound to a carrier protein. The polysaccharide, polyribosylribitol phosphate, referred to as PRP, is a linear copolymer composed of repeated units of 3-β-D-ribofuranosyl-(1→1)-ribitol-5-phosphate $[(C_{10}H_{19}O_{12}P)_n]$, with a defined molecular size. The carrier protein, when conjugated to PRP, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently haemophilus type b conjugate vaccines of adequate safety and immunogenicity in man. The production of PRP and of the carrier protein are based on seed-lot systems.

The production method is validated to demonstrate that the product, if tested, would comply with the test for pyrogens (*2.6.8*), carried out as follows: inject per kilogram of the rabbit's mass a quantity of the vaccine equivalent to: 1 µg of PRP for a vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier; 0.1 µg of PRP for a vaccine with tetanus toxoid as carrier; 0.025 µg of PRP for a vaccine with OMP (meningococcal group B outer membrane protein complex) as carrier.

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to PRP. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the vaccine are not affected.

The stability of the final lot and relevant intermediates is evaluated using one or more indicator tests. Such tests may include determination of molecular size, determination of free PRP in the conjugate and the immunogenicity test in mice. Taking account of the results of the stability testing, release requirements are set for these indicator tests to ensure that the vaccine will be satisfactory at the end of the period of validity.

*BACTERIAL SEED LOTS*

The seed lots of *H. influenzae* type b are shown to be free from contamination by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

No complex products of animal origin are included in the medium used for preservation of strain viability, either for freeze-drying or for frozen storage.

It is recommended that PRP produced by the seed lot be characterised using nuclear magnetic resonance spectrometry (*2.2.33*).

*H. INFLUENZAE TYPE b POLYSACCHARIDE (PRP)*

*H. influenzae* type b is grown in a liquid medium that does not contain high-molecular-mass polysaccharides; if any ingredient of the medium contains blood-group substances, the process shall be validated to demonstrate that after the purification step they are no longer detectable. The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera. The culture may be inactivated. PRP is separated from the culture medium and purified by a suitable method. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method; the result is used to calculate the results of certain tests with reference to the dried substance, as prescribed below.

Only PRP that complies with the following requirements may be used in the preparation of the conjugate.

**Identification**. PRP is identified by an immunochemical method (*2.7.1*) or other suitable method, for example $^1$H nuclear magnetic resonance spectrometry (*2.2.33*).

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. Where applicable, the molecular-size distribution is also determined after chemical modification of the polysaccharide. An acceptable value is established for the PRP polysaccharide. Each batch must be shown to comply with this limit.

**Ribose** (*2.5.31*): within the limits approved by the competent authority for the particular product, calculated with reference to the dried substance.

**Phosphorus** (*2.5.18*): within the limits approved by the competent authority for the particular product, calculated with reference to the dried substance.

**Protein** (*2.5.16*): maximum 1.0 per cent, calculated with reference to the dried substance. Use sufficient PRP to allow detection of proteins at concentrations of 1 per cent or greater.

**Nucleic acid** (*2.5.17*): maximum 1.0 per cent, calculated with reference to the dried substance.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU per microgram of PRP.

**Residual reagents**. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of PRP must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on PRP may be omitted.

*CARRIER PROTEIN*

The production and characteristics of the carrier proteins are described in general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for*

*human use*. Only a carrier protein that complies with the requirements of this chapter may be used in the preparation of the conjugate.

*BULK CONJUGATE*

PRP is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. Reactive functional groups or spacers may be introduced into the appropriate carrier protein or PRP prior to conjugation. As a measure of consistency, the extent of derivatisation is monitored. The conjugate is obtained by the covalent binding of PRP and the appropriate carrier protein. Where applicable, unreacted but potentially reactogenic functional groups are made unreactive by means of capping agents; the conjugate is purified to remove reagents.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits. For a freeze-dried vaccine, some of the tests may be carried out on the final lot rather than on the bulk conjugate where the freeze-drying process may affect the component being tested.

**PRP**. The PRP content is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Protein**. The protein content is determined by a suitable chemical method (for example, *2.5.16*).

**PRP-to-protein ratio**. Determine the ratio by calculation.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for the bulk conjugate. Each batch must be shown to comply with this limit.

**Free PRP**. A number of methods are used to separate free PRP from the conjugate, including precipitation, gel filtration, size-exclusion, anion-exchange and hydrophobic chromatography, ultrafiltration and ultracentrifugation. The free PRP can then be quantified by a range of techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and immunoassays with anti-PRP antibodies.

**Free carrier protein**. Determine the content by a suitable method, either directly or by deriving the content by calculation from the results of other tests. The amount is within the limits approved for the particular product.

**Unreacted functional groups**. No unreacted functional groups are detectable in the bulk conjugate unless process validation has shown that unreacted functional groups detectable at this stage are removed during the subsequent manufacturing process (for example, owing to short half-life).

**Residual reagents**. Removal of residual reagents such as cyanide, EDAC (ethyldimethylaminopropylcarbodiimide) and phenol is confirmed by suitable tests or by validation of the process.

**Sterility** (*2.6.1*). Carry out the test using for each medium 10 mL or the equivalent of 100 doses, whichever is less.

*FINAL BULK VACCINE*

An adjuvant, an antimicrobial preservative and a stabiliser may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to each of the following requirements and the requirements given below under Identification and Tests may be released for use. Provided the test for antimicrobial preservative has been carried out on the final bulk vaccine, it may be omitted on the final lot.

**pH** (*2.2.3*). The pH of the vaccine, reconstituted if necessary, is within the limits approved for the particular product.

**Free PRP**. A number of methods are used to separate free PRP from the conjugate, including precipitation, gel filtration, size-exclusion, anion-exchange and hydrophobic chromatography, ultrafiltration and ultracentrifugation. The free PRP can then be quantified by a range of techniques, including HPAEC-PAD and immunoassays with anti-PRP antibodies. The amount of free PRP is not greater than that approved for the particular product.

IDENTIFICATION

The vaccine is identified by a suitable immunochemical method (*2.7.1*) for PRP.

TESTS

**PRP**: minimum 80 per cent of the amount of PRP stated on the label. PRP is determined either by assay of ribose (*2.5.31*) or phosphorus (*2.5.18*), by an immunochemical method (*2.7.1*) or by anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not greater than 115 per cent of the quantity stated on the label.

**Water** (*2.5.12*): maximum 3.0 per cent for freeze-dried vaccines.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the particular product. If any components of the vaccine prevent the determination of endotoxin, a test for pyrogens is carried out as described under General provisions.

LABELLING

The label states:

- the number of micrograms of PRP per single human dose;
- the type and nominal amount of carrier protein per single human dose.

01/2019:2597

# HEPATITIS A (INACTIVATED, ADSORBED) AND TYPHOID POLYSACCHARIDE VACCINE

## Vaccinum hepatitidis A inactivatum adsorbatum et febris typhoidis polysaccharidicum

DEFINITION

Hepatitis A (inactivated, adsorbed) and typhoid polysaccharide vaccine is a suspension consisting of a suitable strain of hepatitis A virus, grown in cell cultures and inactivated by a validated method, and of purified Vi capsular polysaccharide obtained from *Salmonella typhi* Ty 2 strain or some other suitable strain that has the capacity to produce Vi polysaccharide.

The hepatitis A antigen is adsorbed on a mineral carrier, such as aluminium hydroxide, and the Vi capsular polysaccharide consists of partly 3-*O*-acetylated repeated units of 2-acetylamino-2-deoxy-D-galactopyranuronic acid with α-(1→4) linkages.

The product is presented either as a liquid mixture containing the hepatitis A component and the typhoid Vi polysaccharide component or as 2 separate liquids, one containing the hepatitis A component and the other the typhoid Vi polysaccharide component, which are mixed together immediately before use.

PRODUCTION

*GENERAL PROVISIONS*

The 2 components are prepared as described in the monographs *Hepatitis A vaccine (inactivated, adsorbed) (1107)* and *Typhoid polysaccharide vaccine (1160)* and comply with the requirements prescribed therein.

*Reference preparation.* The hepatitis A reference preparation is part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course primary immunisation. An antibody level not less than 20 mIU/mL determined by enzyme-linked immunosorbent assay is recognised as being protective.

*FINAL BULKS*

The hepatitis A final bulk is prepared from 1 or more inactivated harvests of hepatitis A virus. Approved adjuvants, stabilisers and antimicrobial preservatives may be added.

The Vi polysaccharide final bulk is prepared from 1 or more batches of purified Vi polysaccharide which are dissolved in a suitable solvent, which may contain an antimicrobial preservative, so that the volume corresponding to 1 dose contains 25 μg of polysaccharide and the solution is isotonic with blood (250-350 mosmol/kg).

Where the vaccine is presented as a liquid mixture of both components, the final bulk is prepared by addition of a suitable quantity of the Vi capsular polysaccharide bulk to the hepatitis A bulk.

Only final bulks that comply with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulks are distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable), antimicrobial preservative (where applicable) and bacterial endotoxins have been carried out on the final bulks with satisfactory results, they may be omitted on the final lot. If the assay of the hepatitis A component is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk containing the hepatitis A component, it may be omitted on the final lot.

CHARACTERS

*If the vaccine is presented as 2 separate liquids, test A is carried out using the hepatitis A component and test B is carried out using the typhoid Vi polysaccharide component. Test C is carried out if the vaccine is presented as a liquid mixture of both components or immediately after mixing both components if the vaccine is presented as 2 separate liquids.*

A. Whitish, cloudy suspension.

B. Clear, colourless liquid, free from visible particles.

C. Turbid liquid with a slow settling white deposit.

IDENTIFICATION

*If the vaccine is presented as 2 separate liquids, identification test A is carried out using the hepatitis A component and identification test B is carried out using the typhoid Vi polysaccharide component. If the vaccine is presented as a liquid mixture, tests A and B are carried out.*

A. The assay (*2.7.14*) serves also to identify the vaccine.

B. Typhoid Vi polysaccharide is identified by a suitable immunochemical method (*2.7.1*) using specific antibodies.

TESTS

*If the vaccine is presented as 2 separate liquids, the tests for pH, antimicrobial preservative and bacterial endotoxins are carried out on both components; the test for aluminium is carried out using the hepatitis A component and the test for O-acetyl groups is carried out using the typhoid Vi polysaccharide component; the tests for pH, free formaldehyde, osmolality and sterility are carried out immediately after mixing both components. If the vaccine is presented as a liquid mixture, the test for O-acetyl groups is carried out before the 2 components are mixed.*

**pH** (*2.2.3*): 6.8 to 7.8 for the hepatitis A component and 6.5 to 7.5 for the typhoid Vi polysaccharide component; 6.6 to 7.6 for the vaccine presented as a liquid mixture or immediately after mixing both components if the vaccine is presented as 2 separate liquids.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the amount stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Osmolality** (*2.2.35*). Where applicable, the osmolality of the vaccine is within the limits approved for the particular product.

**Bacterial endotoxins** (*2.6.14*). The bacterial endotoxins content is less than 2 IU per human dose for the hepatitis A component and within the limit approved for the typhoid Vi polysaccharide component. If the vaccine is presented as

a liquid mixture of hepatitis A component and typhoid Vi polysaccharide component the bacterial endotoxins content is within the limit approved for the specific product.

***O*-Acetyl groups** (*2.5.19*): 0.085 µmol (± 25 per cent) per dose (25 µg of polysaccharide).

ASSAY

**Hepatitis A component**. The vaccine complies with the assay of hepatitis A vaccine (*2.7.14*).

**Typhoid Vi polysaccharide component**. Determine Vi polysaccharide by a suitable immunochemical method (*2.7.1*), using a reference purified polysaccharide. The estimated amount of polysaccharide per dose is 80 per cent to 120 per cent of the content stated on the label. The confidence limits ($P$ = 0.95) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

LABELLING

The label states:

- the amount of hepatitis A virus antigen per human dose;
- the number of micrograms of polysaccharide per human dose (25 µg);
- the total quantity of polysaccharide in the container;
- the type of cells used for production of the vaccine;
- the name and amount of the adsorbent used;
- that the vaccine must be shaken before use;
- that the vaccine must not be frozen.

**01/2019:1526**

# HEPATITIS A (INACTIVATED) AND HEPATITIS B (rDNA) VACCINE (ADSORBED)

## Vaccinum hepatitidis A inactivatum et hepatitidis B (ADNr) adsorbatum

DEFINITION

Hepatitis A (inactivated) and hepatitis B (rDNA) vaccine (adsorbed) is a suspension consisting of a suitable strain of hepatitis A virus, grown in cell cultures and inactivated by a validated method, and of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus obtained by recombinant DNA technology; the antigens are adsorbed on a mineral carrier, such as aluminium hydroxide or hydrated aluminium phosphate.

PRODUCTION

*GENERAL PROVISIONS*

The two components are prepared as described in the monographs on *Hepatitis A vaccine (inactivated, adsorbed) (1107)* and *Hepatitis B vaccine (rDNA) (1056)* and comply with the requirements prescribed therein.

*Reference preparation*. The reference preparation is part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody recognised to be protective, after a full-course primary immunisation. For hepatitis A, an antibody level not less than 20 mIU/mL determined by enzyme-linked immunosorbent assay is recognised as being protective. For hepatitis B, an antibody level not less than 10 mIU/mL against HBsAg is recognised as being protective.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more inactivated harvests of hepatitis A virus and one or more batches of purified antigen.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay of the hepatitis A and/or the hepatitis B component is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

**Hepatitis A component**. The assay (*2.7.14*) serves also to identify the vaccine.

**Hepatitis B component**. The assay (*2.7.15*) or, where applicable, the electrophoretic profile, serves also to identify the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 2 IU per human dose.

ASSAY

**Hepatitis A component**. The vaccine complies with the assay of hepatitis A vaccine (*2.7.14*).

**Hepatitis B component**. The vaccine complies with the assay of hepatitis B vaccine (rDNA) (*2.7.15*).

LABELLING

The label states:

- the amount of hepatitis A virus antigen and hepatitis B surface antigen per container;
- the type of cells used for production of the vaccine;
- the name and amount of the adsorbent used;
- that the vaccine must be shaken before use;
- that the vaccine must not be frozen.

01/2019:1107

# HEPATITIS A VACCINE (INACTIVATED, ADSORBED)

## Vaccinum hepatitidis A inactivatum adsorbatum

DEFINITION

Hepatitis A vaccine (inactivated, adsorbed) is a suspension consisting of a suitable strain of hepatitis A virus grown in cell cultures, inactivated by a validated method and adsorbed on a mineral carrier.

PRODUCTION

*GENERAL PROVISIONS*

Production of the vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to consistently yield vaccines that comply with the requirements for immunogenicity, safety and stability.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

*Reference preparation*. A part of a batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course primary immunisation is used as a reference preparation. An antibody level of 20 mIU/mL determined by enzyme-linked immunosorbent assay is recognised as being protective.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in a human diploid cell line (*5.2.3*) or in a continuous cell line approved by the competent authority.

*SEED LOTS*

The strain of hepatitis A virus used to prepare the master seed lot shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. Each master and working seed lot is identified as hepatitis A virus using specific antibodies.

**Virus concentration**. The virus concentration of each master and working seed lot is determined to monitor consistency of production.

**Extraneous agents**. The working seed lot complies with the requirements for seed lots for virus vaccines (*2.6.16*). In addition, if primary monkey cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus and filoviruses.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator, such as phenol red, and antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. When the determination of the ratio of virus concentration to antigen content has been carried out on a suitable number of single harvests to demonstrate production consistency, it may subsequently be omitted as a routine test.

**Identification**. The test for antigen content also serves to identify the single harvest.

**Bacterial and fungal contamination**. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). The single harvest complies with the test for mycoplasmas, carried out using 1 mL for each medium.

**Control cells**. The control cells of the production cell culture comply with a test for identification and the requirements for extraneous agents (*2.6.16*).

**Antigen content**. Determine the hepatitis A antigen content by a suitable immunochemical method (*2.7.1*) to monitor production consistency; the content is within the limits approved for the particular product.

**Ratio of virus concentration to antigen content**. The consistency of the ratio of the concentration of infectious virus, determined by a suitable cell culture method, to antigen content is established by validation on a suitable number of single harvests.

*PURIFICATION AND PURIFIED HARVEST*

The harvest, which may be a pool of several single harvests, is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the level of host-cell DNA.

Only a purified harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

**Virus concentration**. The concentration of infectious virus in the purified harvest is determined by a suitable cell culture method to monitor production consistency and as a starting point for monitoring the inactivation curve.

**Antigen:total protein ratio**. Determine the hepatitis A virus antigen content by a suitable immunochemical method (*2.7.1*). Determine the total protein by a validated method. The ratio of hepatitis A virus antigen content to total protein content is within the limits approved for the particular product.

**Bovine serum albumin**. Not more than 50 ng in the equivalent of a single human dose, determined by a suitable immunochemical method (*2.7.1*). Where appropriate in view of the manufacturing process, other suitable protein markers may be used to demonstrate effective purification.

**Residual host-cell DNA**. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, is not greater than 100 pg in the equivalent of a single human dose.

**Residual chemicals**. If chemical substances are used during the purification process, tests for these substances are carried out on the purified harvest (or on the inactivated harvest), unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved for the particular product.

*INACTIVATION AND INACTIVATED HARVEST*

Several purified harvests may be pooled before inactivation. In order to avoid interference with the inactivation process, virus aggregation must be prevented or aggregates must be removed immediately before and/or during the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating hepatitis A virus without destroying the antigenic

and immunogenic activity; for each inactivation procedure, an inactivation curve is plotted representing residual live virus concentration measured at not fewer than 3 points in time (for example, on days 0, 1 and 2 of the inactivation process). If formaldehyde is used for inactivation, the presence of excess free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Inactivation**. Carry out an amplification test for residual infectious hepatitis A virus by inoculating a quantity of the inactivated harvest equivalent to 5 per cent of the batch or, if the harvest contains the equivalent of 30 000 doses or more, not less than 1500 doses of vaccine into cell cultures of the same type as those used for production of the vaccine; incubate for a total of not less than 70 days making not fewer than one passage of cells within that period. At the end of the incubation period, carry out a test of suitable sensitivity for residual infectious virus. No evidence of hepatitis A virus multiplication is found in the samples taken at the end of the inactivation process. Use infectious virus inocula concurrently as positive controls to demonstrate cellular susceptibility and absence of interference.

**Sterility** (*2.6.1*). The inactivated viral harvest complies with the test for sterility, carried out using 10 mL for each medium.

**Bacterial endotoxins** (*2.6.14*): less than 2 IU in the equivalent of a single human dose.

**Antigen content**. Determine the hepatitis A virus antigen content by a suitable immunochemical method (*2.7.1*).

**Residual chemicals**. See under Purification and purified harvest.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more inactivated harvests. Approved adjuvants, stabilisers and antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, these tests may be omitted on the final lot. If the assay is carried out using mice or other animals, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The assay (*2.7.14*) serves also to identify the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

The vaccine complies with the assay of hepatitis A vaccine (*2.7.14*).

LABELLING

The label states the biological origin of the cells used for the preparation of the vaccine.

**01/2019:1935**

# HEPATITIS A VACCINE (INACTIVATED, VIROSOME)

## Vaccinum hepatitidis A inactivatum virosomale

DEFINITION

Hepatitis A vaccine (inactivated, virosome) is a suspension of a suitable strain of hepatitis A virus grown in cell cultures and inactivated by a validated method. Virosomes composed of influenza proteins of a strain approved for the particular product and phospholipids are used as adjuvants.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

*Reference preparation.* A reference preparation of inactivated hepatitis A antigen is calibrated against a batch of hepatitis A vaccine (inactivated, virosome) that, in clinical studies in young healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of neutralising antibody accepted to be protective, after a full-course primary immunisation. An antibody level not less than 20 mIU/mL determined by enzyme-linked immunosorbent assay is recognised as being protective.

*PREPARATION OF HEPATITIS A ANTIGEN*

Production of the hepatitis A antigen is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to consistently yield vaccines that comply with the requirements for immunogenicity, safety and stability.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

*SUBSTRATE FOR PROPAGATION OF HEPATITIS A VIRUS*

The virus is propagated in a human diploid cell line (*5.2.3*).

*SEED LOTS OF HEPATITIS A VIRUS*

The strain of hepatitis A virus used to prepare the master seed lot shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. Each master and working seed lot is identified as hepatitis A virus using specific antibodies.

**Virus concentration**. The virus concentration of each master and working seed lot is determined to monitor consistency of production.

**Extraneous agents**. The working seed lot complies with the requirements for seed lots for virus vaccines (*2.6.16*).

*PROPAGATION AND HARVEST OF HEPATITIS A VIRUS*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. When the determination of the ratio of virus concentration to antigen content has been carried out on a suitable number of single harvests to demonstrate consistency, it may subsequently be omitted as a routine test.

**Identification**. The test for antigen content also serves to identify the single harvest.

**Bacterial and fungal contamination**. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). The single harvest complies with the test for mycoplasmas.

**Control cells**. The control cells of the production cell culture comply with a test for identity and the requirements for extraneous agents (*2.6.16*).

**Antigen content**. Determine the hepatitis A antigen content by a suitable immunochemical method (*2.7.1*) to monitor production consistency; the content is within the limits approved for the particular product.

**Ratio of virus concentration to antigen content**. The consistency of the ratio of the concentration of infectious virus, as determined by a suitable cell culture method, to antigen content is established by validation on a suitable number of single harvests.

*PURIFICATION AND PURIFIED HARVEST OF HEPATITIS A VIRUS*

The harvest, which may be a pool of several single harvests, is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the level of host-cell DNA.

Only a purified harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

**Virus concentration**. The concentration of infective virus in the purified harvest is determined by a suitable cell culture method to monitor production consistency and as a starting point for monitoring the inactivation curve.

**Ratio of antigen to total protein**. Determine the hepatitis A virus antigen content by a suitable immunochemical method (*2.7.1*). Determine the total protein by a validated method. The ratio of hepatitis A virus antigen content to total protein content is within the limits approved for the particular product.

**Bovine serum albumin**: maximum 50 ng per single human dose if foetal bovine serum is used, determined by a suitable immunochemical method (*2.7.1*). Where appropriate in view of the manufacturing process, other suitable protein markers may be used to demonstrate effective purification.

**Residual chemicals**. If chemical substances are used during the purification process, tests for these substances are carried out on the purified harvest (or on the inactivated harvest), unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved for the particular product.

*INACTIVATION AND INACTIVATED HARVEST OF HEPATITIS A VIRUS*

Several purified harvests may be pooled before inactivation. In order to avoid interference with the inactivation process, virus aggregation must be prevented or aggregates must be removed immediately before and/or during the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating hepatitis A virus without destroying the antigenic and immunogenic activity; for each inactivation procedure, an inactivation curve is plotted representing residual live virus concentration measured on at least 3 occasions (for example, on days 0, 1 and 2 of the inactivation process). If formaldehyde is used for inactivation, the presence of excess free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Inactivation**. Carry out an amplification test for residual infectious hepatitis A virus by inoculating a quantity of the inactivated harvest equivalent to 5 per cent of the batch or, if the harvest contains the equivalent of 30 000 doses or more, not less than 1500 doses of vaccine into cell cultures of the same type as those used for production of the vaccine; incubate for a total of not less than 70 days making not fewer than 1 passage of cells within that period. At the end of the incubation period, carry out a test of suitable sensitivity for residual infectious virus. No evidence of hepatitis A virus multiplication is found in the samples taken at the end of the inactivation process. Use infective virus inocula concurrently as positive controls to demonstrate cellular susceptibility and absence of interference.

**Sterility** (*2.6.1*). The inactivated viral harvest complies with the test for sterility, carried out using 10 mL for each medium.

**Bacterial endotoxins** (*2.6.14*): less than 2 IU of endotoxin in the equivalent of a single human dose.

**Antigen content**. Determine the hepatitis A virus antigen content by a suitable immunochemical method (*2.7.1*).

**Residual chemicals**. See under Purification and purified harvest.

*PREPARATION OF INACTIVATED INFLUENZA VIRUS*

The production of influenza viruses is based on a seed-lot system. Working seed lots represent not more than 15 passages from the approved reassorted virus or the approved virus isolate. The final production represents 1 passage from the working seed lot. The strain of influenza virus to be used is approved by the competent authority.

*SUBSTRATE FOR PROPAGATION OF INFLUENZA VIRUS*

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (*5.2.2*) or in suitable cell cultures (*5.2.4*), such as chick-embryo fibroblasts or chick kidney cells obtained from chicken flocks free from specified pathogens (*5.2.2*). For production, the virus is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

*SEED LOTS OF INFLUENZA VIRUS*

The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL.

*PROPAGATION AND HARVEST OF INFLUENZA VIRUS*

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form the monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

*POOLED HARVEST OF INFLUENZA VIRUS*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying antigenicity of haemagglutinin. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/L of $CH_2O$ at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent *V/V* at any time during inactivation.

Only a pooled harvest that complies with the following requirements may be used in the preparation of the virosomes.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Viral inactivation**. Inoculate 0.2 mL of the harvest into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

**Ovalbumin**: maximum 1 µg of ovalbumin in the equivalent of 1 human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Residual chemicals**. Tests are carried out on the monovalent pooled harvest for the chemicals used for inactivation, the limits being approved by the competent authority.

*PREPARATION OF VIROSOMES*

Inactivated influenza virions are solubilised using a suitable detergent and are purified by high-speed centrifugation in order to obtain supernatants containing mainly influenza antigens. After the addition of suitable phospholipids, virosomes are formed by removal of the detergent either by adsorption chromatography or another suitable technique

Only virosomes that comply with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin content**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it.

**Phospholipids**. The content and identity of the phospholipids are determined by suitable immunochemical or physico-chemical methods.

**Ratio of phospholipid to haemagglutinin**. The ratio of phospholipid content to haemagglutinin content is within the limits approved for the particular product.

**Residual chemicals**. Tests are carried out for the chemicals used during the process. The concentration of each residual chemical is within the limits approved for the particular product.

*FINAL BULK VACCINE*

The bulk vaccine is prepared by adding virosomes to inactivated hepatitis A viruses to yield an approved hepatitis A antigen:haemagglutinin ratio. Several bulks may be pooled, and approved stabilisers and antimicrobial preservatives may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Protein content**. The amount of protein is determined using a suitable technique, the limits being approved by the competent authorithy.

**Phospholipids**. The content and identity of the phospholipids are determined by suitable immunochemical or physico-chemical methods. The amount of phospholipids complies with the limits approved for the particular product.

**Haemagglutinin content**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*). The amount of haemagglutinin must not exceed the limits approved for the particular product.

**Hepatitis A antigen content**. Determine the hepatitis A antigen content by a suitable immunochemical method. The amount of antigen must not exceed the limits approved for the particular product.

**Ratio of hepatitis A antigen to haemagglutinin**. The ratio of hepatitis A antigen content to haemagglutinin content is within the limits approved for the particular product.

**Ovalbumin**: maximum 1 µg of ovalbumin per human dose, determined by a suitable technique using a suitable reference preparation of ovalbumin.

**Virosome size**. The size distribution of the virosome-hepatitis A virus mixture is within the limits approved for the particular product.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Residual chemicals**. If chemical substances are used during the formulation process, tests for these substances are carried out, the limits being approved by the competent authorithy.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, these tests may be omitted

on the final lot. If the assay is carried out *in vivo*, provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The assay (*2.7.14*) serves also to identify the vaccine.

TESTS

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 2 IU of endotoxin per human dose.

ASSAY

The vaccine complies with the assay of hepatitis A vaccine (*2.7.14, Method A*).

LABELLING

The label states:

- the biological origin of the cells used for the preparation of the vaccine;
- that the carrier contains influenza proteins prepared in eggs;
- that the vaccine is to be shaken before use;
- that the vaccine is not to be frozen.

**01/2019:1056**

# HEPATITIS B VACCINE (rDNA)

## Vaccinum hepatitidis B (ADNr)

DEFINITION

Hepatitis B vaccine (rDNA) is a preparation of hepatitis B surface antigen (HBsAg), a component protein of hepatitis B virus; the antigen may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The vaccine may also contain the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A. The antigen is obtained by recombinant DNA technology.

PRODUCTION

*GENERAL PROVISIONS*

The vaccine shall have been shown to induce specific, protective antibodies in man. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity and safety.

Hepatitis B vaccine (rDNA) is produced by the expression of the viral gene coding for HBsAg in yeast (*Saccharomyces cerevisiae*) or mammalian cells (Chinese hamster ovary (CHO) cells or other suitable cell lines), purification of the resulting HBsAg and the rendering of this antigen into an immunogenic preparation. The suitability and safety of the cells are approved by the competent authority.

The vaccine may contain the product of the S gene (major protein), a combination of the S gene and pre-S2 gene products (middle protein) or a combination of the S gene, the pre-S2 gene and pre-S1 gene products (large protein).

*Reference preparation*: part of a representative batch shown to be at least as immunogenic in animals as a batch that, in clinical studies in young, healthy adults, produced not less than 95 per cent seroconversion, corresponding to a level of HBsAg neutralising antibody recognised to be protective, after a full-course primary immunisation. An antibody level not less than 10 mIU/mL is recognised as being protective.

*CHARACTERISATION OF THE SUBSTANCE*

Development studies are carried out to characterise the antigen. The complete protein, lipid and carbohydrate structure of the antigen is established. The morphological characteristics of the antigen particles are established by electron microscopy. The mean buoyant density of the antigen particles is determined by a physico-chemical method, such as gradient centrifugation. The antigenic epitopes are characterised. The protein fraction of the antigen is characterised in terms of the primary structure (for example, by determination of the amino-acid composition, by partial amino-acid sequence analysis and by peptide mapping).

*CULTURE AND HARVEST*

Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages. If mammalian cells are used, tests for extraneous agents and mycoplasmas are performed in accordance with general chapter *2.6.16. Tests for extraneous agents in viral vaccines for human use*, but using 200 mL of harvest in the test in cell culture for other extraneous agents.

*PURIFIED ANTIGEN*

Only a purified antigen that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Total protein**. The total protein is determined by a validated method. The content is within the limits approved for the specific product.

**Antigen content and identification**. The quantity and specificity of HBsAg is determined in comparison with the International Standard for HBsAg subtype *ad* or an in-house reference, by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is within the limits approved for the specific product.

The molecular weight of the major band revealed following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing conditions corresponds to the value expected from the known nucleic acid and polypeptide sequences and possible glycosylation.

**Antigenic purity**. The purity of the antigen is determined by comparison with a reference preparation using liquid chromatography or other suitable methods such as SDS-PAGE with staining by acid blue 92 and silver. A suitable method is sensitive enough to detect a potential contaminant at a concentration of 1 per cent of total protein. Not less than 95 per cent of the total protein consists of hepatitis B surface antigen.

**Composition**. The content of proteins, lipids, nucleic acids and carbohydrates is determined.

**Host-cell- and vector-derived DNA**. If mammalian cells are used for production, not more than 10 pg of DNA in the quantity of purified antigen equivalent to a single human dose of vaccine.

**Caesium**. If a caesium salt is used during production, a test for residual caesium is carried out on the purified antigen. The content is within the limits approved for the specific product.

**Sterility** (*2.6.1*). The purified antigen complies with the test, carried out using 10 mL for each medium.

Additional tests on the purified antigen may be required depending on the production method used: for example, a test for residual animal serum where mammalian cells are used for production or tests for residual chemicals used during extraction and purification.

*ADSORBED 3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A BULK*

If 3-*O*-desacyl-4′-monophosphoryl lipid A is included in the vaccine it complies with the monograph *3-O-desacyl-4′-monophosphoryl lipid A (2537)*. Where 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk is adsorbed prior to inclusion in the vaccine, the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk complies with the following requirements.

**Degree of adsorption of 3-*O*-desacyl-4′-monophosphoryl lipid A**. The content of non-adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A in the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk is determined by a suitable method, for example gas chromatographic quantification of the *3-O-desacyl-4′-monophosphoryl lipid A (2537)* fatty acids in the supernatant, evaporated to dryness, after centrifugation.

**pH** (*2.2.3*). The pH is within the limits approved for the particular preparation.

**Sterility** (*2.6.1*). It complies with the test, carried out using 10 mL for each medium.

*FINAL BULK VACCINE*

An antimicrobial preservative, a mineral carrier, such as aluminium hydroxide or hydrated aluminium phosphate, and the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A may be included in the formulation of the final bulk.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde (where applicable) and antimicrobial preservative content (where applicable) have been carried out on the final bulk vaccine with satisfactory results, they may be omitted on the final lot. If the assay is carried out *in vivo*, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Degree of adsorption**. The degree of adsorption of the antigen and, where applicable, 3-*O*-desacyl-4′-monophosphoryl lipid A is assessed.

IDENTIFICATION

The assay or, where applicable, the electrophoretic profile, serves also to identify the vaccine. In addition, where applicable, the test for 3-*O*-desacyl-4′-monophosphoryl lipid A content also serves to identify the 3-*O*-desacyl-4′-monophosphoryl lipid A-containing vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**3-*O*-Desacyl-4′-monophosphoryl lipid A**: minimum 80 per cent and maximum 120 per cent of the intended amount.

Where applicable, determine the content of 3-*O*-desacyl-4′-monophosphoryl lipid A by a suitable method, for example gas chromatography (*2.2.28*).

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the content of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test.

**Pyrogens** (*2.6.8*). The vaccine complies with the test for pyrogens. Inject the equivalent of one human dose into each rabbit or, if the vaccine contains 3-*O*-desacyl-4′-monophosphoryl lipid A, inject per kilogram of the rabbit's mass an amount of the vaccine containing 2.5 µg of 3-*O*-desacyl-4′-monophosphoryl lipid A.

ASSAY

The vaccine complies with the assay of hepatitis B vaccine (rDNA) (*2.7.15*).

LABELLING

The label states:

- the amount of HBsAg per container;
- the type of cells used for production of the vaccine;
- the name and amount of the adjuvant and/or adsorbent used;
- that the vaccine must be shaken before use;
- that the vaccine must not be frozen.

**01/2019:2441**

# HUMAN PAPILLOMAVIRUS VACCINE (rDNA)

## Vaccinum papillomaviri humani (ADNr)

DEFINITION

Human papillomavirus vaccine (rDNA) is a preparation of purified virus-like particles (VLPs) composed of the major capsid protein (L1) of one or more human papillomavirus (HPV) genotypes; the antigens may be adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate. The vaccine may also contain the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A. The antigens are obtained by recombinant DNA technology.

PRODUCTION

*GENERAL PROVISIONS*

The vaccine shall have been shown to induce specific neutralising antibodies in man. The production method shall have been shown to yield consistently vaccines comparable in quality with the vaccine of proven clinical efficacy and safety in man.

The vaccine is produced by the expression of the viral genes coding for the capsid proteins in yeast or in an insect cell/baculovirus expression vector system, purification of the resulting VLPs and the rendering of these particles into an immunogenic preparation. The suitability and safety of the expression systems are approved by the competent authority. Production of the vaccine is based on a seed lot/cell bank system. Unless otherwise justified and authorised, the virus and cells used for vaccine production shall not have undergone more passages from the master seed lot/cell bank than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

*Reference preparation*. A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. The reference vaccine is preferably stabilised and the stabilisation method shall have been shown to have no significant effect on the assay validity.

*CHARACTERISATION*

Characterisation of the VLPs is performed on lots produced during vaccine development, including the process validation batches. Characterisation includes protein composition, for example using techniques such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting or mass spectrometry, peptide mapping and/or terminal amino acid sequence analysis. Morphological characteristics of the VLPs and degree of aggregation are determined to confirm the presence of the conformational epitopes that are essential for efficacy. VLP characterisation may be done by atomic force microscopy and transmission electron microscopy, dynamic light scattering, epitope mapping and reactivity with neutralising monoclonal antibodies. In addition, the protein, lipid, nucleic acid and carbohydrate content are measured where applicable. The level of residual host-cell protein derived from insect cells meets acceptable safety criteria as set by the competent authority.

*CELL BANKS AND SEED LOTS*

*Production in recombinant yeast cells*. Only cell banks that have been satisfactorily characterised for identity, microbial purity, growth characteristics and stability shall be used for production. Gene homogeneity is studied for the master and working cell banks. A full description of the biological characteristics of the host cell and expression vectors is given. The physiological measures used to promote and control the expression of the cloned gene in the host cell are described in detail. This includes genetic markers of the host cell, the construction, genetics and structure of the expression vector, and the origin and identification of the gene that is being cloned. The nucleotide sequence of the gene insert and of adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert are provided. Data that demonstrates the stability of the expression system during storage of the recombinant working cell bank up to or beyond the passage level used for production is provided.

*Production in an insect cell/baculovirus expression vector system*

- *Insect cell substrate*. Only cell banks that have been satisfactorily characterised for identity, purity, growth characteristics, stability, extraneous agents and tumorigenicity shall be used for production. Such characterisation is performed at suitable stages of production in accordance with general chapters *5.2.3. Cell substrates for the production of vaccines for human use* and *2.6.16. Tests for extraneous agents in viral vaccines for human use*. Special attention is given to insect-borne viruses, in particular insect-borne potential human pathogens (e.g. arboviruses). Adventitious infectious agents of insect cells may be without cytopathic effect. Tests therefore include nucleic acid amplification techniques, and other tests such as electron microscopy and co-cultivation.
- *Recombinant baculovirus*. The use of the recombinant baculovirus vector is based on a seed-lot system with a defined number of passages between the original virus and the master and the working seed-lots, as approved by the competent authorities. The recombinant baculovirus expression vector contains the coding sequence of the HPV L1 antigen. Segments of the expression construct are analysed using nucleic acid amplification techniques in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the expressed HPV L1 antigens. The recombinant baculovirus used in the production of HPV vaccines is identified by historical records, which include information on the origin and identity of the gene being cloned as well as the construction, genetics and structure of the baculovirus expression vector(s). The genetic stability of the expression construct is demonstrated from the baculovirus master seed up to at least the highest level used in production and preferably beyond this level.

Recombinant baculovirus seed lots are prepared in large quantities and stored at temperatures favourable for stability.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified by the HPV type of the inserted gene of origin, by an appropriate method such as nucleic acid amplification techniques (NAT) (*2.6.21*).

**Virus concentration**. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots and control cells. Special attention is given to *Spiroplasma* spp. and insect-borne viruses, in particular insect-borne potential human pathogens (e.g. arboviruses).

*PROPAGATION AND HARVEST*

All processing of the cell banks and baculovirus seed lots and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled.

Where justified and authorised for production in an insect cell/baculovirus expression vector system, a stored virus intermediate culture that complies with the 5 following tests may be used for virus propagation.

**Identification**. Each stored virus intermediate culture is identified by HPV type, by an immunological assay using specific antibodies or by a molecular identity test such as NAT (*2.6.21*).

**Bacterial and fungal contamination**. Each stored virus intermediate culture complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Virus concentration**. The virus concentration of each stored baculovirus intermediate culture is determined by a suitable method such as plaque assay or NAT (*2.6.21*) in order to monitor consistency of production.

**Extraneous agents** (*2.6.16*). Each stored virus intermediate culture complies with the tests for extraneous agents.

**Control cells**. The control cells of the production cell culture from which each stored virus intermediate is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

*Production in recombinant yeast cells*. Identity, microbial purity, plasmid retention and consistency of yield are determined at suitable production stages.

*Production in an insect cell/baculovirus expression vector system*. Insect cell cultures are inoculated with recombinant baculovirus at a defined multiplicity of infection as approved by the competent authority. Several single harvests may be pooled before testing. No antibiotics are added at the time of harvesting or at any later stage of manufacturing.

*SINGLE HARVESTS*

Only a single harvest or a pool of single harvests that complies with the following requirements may be used in the preparation of the purified monovalent antigen.

**Identification**. Each single harvest is identified as the appropriate HPV type by immunological assay or by a molecular biology-based assay, for example hybridisation or polymerase chain reaction (PCR).

**Bacterial and fungal contamination**. In case of production in an insect cell/baculovirus expression vector system the single harvest complies with the test for sterility (*2.6.1*). In case of production in yeast cells the single harvest is tested for culture purity by inoculation of suitable medium to ensure no growth other than the host organism.

**Extraneous agents** (*2.6.16*). In case of production in an insect cell/baculovirus expression vector system the single harvest complies with the tests for extraneous agents. Special attention is given to insect-borne viruses as mentioned under Cell banks and seed lots.

**Control cells**. In case of production in an insect cell/baculovirus expression vector system the control cells comply with a test for identification and with the requirements for extraneous agents (*2.6.16*). Special attention is given to insect-borne viruses as mentioned under Cell banks and seed lots.

*PURIFIED MONOVALENT ANTIGEN*

Harvests are purified using validated methods. When an insect cell/baculovirus expression vector system substrate is used, the production process is validated for its capacity to eliminate (by removal and/or inactivation) adventitious viruses and recombinant baculoviruses.

Only a purified monovalent antigen that complies with the following requirements may be used in the preparation of the final bulk. In agreement with the competent authority one or more of the tests mentioned below may be omitted if performed on the adsorbed monovalent antigen.

**Total protein**. The total protein is determined by a validated method. The content is within the limits approved for the particular product.

**Antigen content and identification**. The quantity and specificity of each antigen type is determined by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio may be determined and is within the limits approved for the particular product.

**Antigenic purity**. The purity of each purified monovalent antigen is determined by a suitable method, such as SDS-PAGE with quantification by densitometric analysis, the limit of detection being 1 per cent of impurities or better with respect to total protein. A reference preparation is used to validate each test. The protein purity is calculated as the ratio of the L1 protein-related bands relative to the total protein bands, expressed as a percentage. For the genotypes included in the vaccine, the value calculated for purity is within the limits approved for the particular product.

**Percent intact L1 monomer**. The antigenic purity assay serves also to assess the integrity of the L1 monomer. The percent intact L1 monomer is the ratio of the intact L1 monomer to the total protein, expressed as a percentage.

**VLP size and structure**. The size and structure of the VLPs is established and monitored by a suitable method such as dynamic light scattering. The size is within the limits approved for the particular product.

**Composition**. The protein, lipid, nucleic acid and carbohydrate contents are determined, where applicable.

**Host-cell and vector-derived DNA**: maximum 10 ng of DNA in a quantity of purified antigen equivalent to a single human dose of vaccine, determined in each monovalent purified antigen by sensitive methods.

**Residual host-cell proteins**. Tests for residual host-cell proteins are carried out. The content is within the limits approved for the particular product.

**Chemicals used for disruption and purification**. Tests for the chemicals used for purification or other stages of production are carried out. The content is within the limits approved for the particular products.

**Sterility** (*2.6.1*). Each purified monovalent antigen complies with the test, carried out using 10 mL for each medium.

*ADSORBED MONOVALENT ANTIGEN*

The purified monovalent antigens may be adsorbed onto a mineral vehicle such as an aluminium salt.

Only an adsorbed monovalent antigen that complies with the following requirements may be used in the preparation of the final bulk.

**Sterility** (*2.6.1*). Each adsorbed monovalent antigen complies with the test, carried out using 10 mL for each medium.

**Bacterial endotoxins** (*2.6.14*). Each adsorbed monovalent antigen is tested for bacterial endotoxins. The content is within the limits approved for the particular product.

**Antigen content and identification**. Each antigen type is identified by a suitable immunochemical method (*2.7.1*) such as radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), immunoblot (preferably using a monoclonal antibody directed against a protective epitope) or single radial diffusion. The antigen/protein ratio is determined.

**Mineral vehicle concentration.** Where applicable, each adsorbed monovalent antigen is tested for the content of mineral vehicle. The content is within the limits approved for the particular product.

*ADSORBED 3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A BULK*

If 3-*O*-desacyl-4′-monophosphoryl lipid A is included in the vaccine it complies with the monograph *3-O-desacyl-4′-monophosphoryl lipid A (2537)*. Where 3-*O*-desacyl-4′-monophosphoryl lipid A is adsorbed prior to inclusion in the vaccine, the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk complies with the following requirements.

**Degree of adsorption of 3-*O*-desacyl-4′-monophosphoryl lipid A**. The content of non-adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A in the adsorbed 3-*O*-desacyl-4′-monophosphoryl lipid A bulk is determined by a suitable method, for example gas chromatographic quantification of the *3-O-desacyl-4′-monophosphoryl lipid A (2537)* fatty acids in the supernatant, evaporated to dryness, after centrifugation.

**pH** (*2.2.3*). The pH is within the limits approved for the particular preparation.

**Sterility** (*2.6.1*). It complies with the test, carried out using 10 mL for each medium.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared directly from each purified monovalent antigen HPV type or adsorbed purified monovalent antigen HPV type. An antimicrobial preservative, a mineral vehicle such as an aluminium salt and the adjuvant 3-*O*-desacyl-4′-monophosphoryl lipid A may be included in the formulation of the final bulk.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for antimicrobial preservative content (where applicable) has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot. If an *in vivo* assay is carried out, then provided it has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Adjuvants**. If the vaccine contains an adjuvant, the amount is determined and shown to be within acceptable limits with respect to the expected amount. A suitable method for 3-*O*-desacyl-4′-monophosphoryl lipid A is, for example, gas chromatography.

**Degree of adsorption**. The degree of adsorption of each antigen and, where applicable, 3-*O*-desacyl-4′-monophosphoryl lipid A is assessed.

IDENTIFICATION

The vaccine is shown to contain the different types of HPV antigen by a suitable immunochemical method (*2.7.1*). The *in vitro* assay may serve to identify the vaccine. In addition, where applicable, the test for 3-*O*-desacyl-4′-monophosphoryl lipid A content also serves to identify the 3-*O*-desacyl-4′-monophosphoryl lipid A-containing vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**3-*O*-Desacyl-4′-monophosphoryl lipid A**: minimum 80 per cent and maximum 120 per cent of the intended amount.

Where applicable, determine the content of 3-*O*-desacyl-4′-monophosphoryl lipid A by a suitable method, for example gas chromatography (*2.2.28*).

**Antimicrobial preservative**. Where applicable, determine the content of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test.

**Bacterial endotoxins** (*2.6.14*): maximum 5 IU per single human dose. If the adjuvant prevents the determination of endotoxin, a suitable in-process test is carried out.

ASSAY

The assay is performed by an *in vivo* test or an *in vitro* test having acceptance criteria established by correlation studies against an *in vivo* test.

***In vivo* test**. A suitable *in vivo* assay method consists of the injection of not fewer than 3 dilutions of the vaccine to be examined and of a reference vaccine preparation, using for each dilution a group of a suitable number of female mice of a suitable strain. The vaccine is diluted in a solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine production. The mice are 6-8 weeks old at the time of injection, and each mouse is given a 0.5 mL injection. A preimmunisation serum sample is taken prior to inoculation, and a final serum sample is taken at a defined time between days 21 and 28. Assay the individual sera for specific neutralising antibodies against each HPV type by a suitable immunochemical method (*2.7.1*).

The test is not valid unless:

- for both the vaccine to be examined and the reference vaccine, the $ED_{50}$ lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are within the limits approved for the particular product.

***In vitro* test**. Carry out an immunochemical determination (*2.7.1*) of each antigen genotype content. Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of the L1 protein have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and a manufacturer's reference preparation are used and a suitable model is used to analyse the data. For each type, the antigen content is within the limits approved for the particular product.

LABELLING

The label states:

- the amount of L1 proteins and the genotype of HPV contained in the vaccine;
- the cell substrate used for production of the vaccine;
- the name and amount of the adjuvant and/or adsorbent used;
- that the vaccine must not be frozen.

**07/2015:2772**
**corrected 10.0**

# INFLUENZA VACCINE (LIVE, NASAL)

## Vaccinum influenzae vivum pernasale

DEFINITION

Influenza vaccine (live, nasal) is an aqueous suspension of a live attenuated strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs. The vaccine is presented in a form suitable for nasal administration. The vaccine is a colourless slightly opalescent liquid and may contain white particles.

PRODUCTION

*GENERAL PROVISIONS*

Production of the vaccine is based on a virus seed-lot system. The production method shall have been shown to consistently yield influenza vaccine (live) that complies with the requirements for immunogenicity, safety and stability.

*CHOICE OF VACCINE STRAIN*

The World Health Organization reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia.

The attenuated donor virus strain and the attenuated vaccine virus strain may be generated by the manufacturer itself by classical reassortant methods or reverse genetics (e.g. plasmid rescue). The wild type virus strains used for the production of the attenuated vaccine virus seed lots must have been approved by the competent authority.

The complete history of production of the attenuated vaccine virus strain including description of the derivation of the seeds from the attenuated donor virus strain(s) and the WHO recommended wild virus strain(s) shall be approved by the competent authority.

During development studies and whenever a new HA subtype of influenza A virus (i.e. non-H1, non-H3 subtype) or a new influenza B virus type differing from the currently circulating genetic lineages is included in the vaccine, the neurovirulence of the master virus seed lots is assessed using suitable animal models (e.g. in mice) with the attenuated donor virus strain as a comparator. The new strain shall not be more neurovirulent than the comparator.

Genotypic and phenotypic characterisations of attenuated donor virus strain(s) are undertaken using techniques for identification of attenuation markers and nucleotide sequences.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus seed and all vaccine batches are propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*).

*VIRUS SEED LOTS*

The production of vaccine is based on a seed-lot system. The attenuated donor virus strains and the wild type virus strains used for the production of the attenuated master seed lots are identified by historical records that include information on their origins and the tests used in their characterisations. Only an attenuated master donor virus strain that has been demonstrated by a suitable method (e.g. multiplex PCR assay) to be free from human respiratory pathogens which are able to replicate in eggs could be used for the production of attenuated master virus seed lots. This assay is omitted if reverse genetics method (e.g. plasmid rescue) is used.

The production of the attenuated master virus seed lot has to be approved by the competent authority. The attenuated master virus seed lot must have the same characteristics as the attenuated donor virus strain. The number of passages required to produce the attenuated master virus seed lot from the attenuated donor virus strain is limited and approved by the competent authority. Unless otherwise justified and authorised, the inoculum for infecting the eggs used in the production of a vaccine lot shall be a virus harvest without intermediate passage, so that no vaccine virus is more than 1 passage from an attenuated master virus seed lot that has passed all safety tests.

Each virus seed lot used for propagation must have been filtrated through a bacteria retentive filter.

The attenuated master virus seed lot has to express the haemagglutinin and the neuraminidase from the wild type virus strain and other proteins from the attenuated donor virus strain.

The attenuated master virus seed lot characterisation shall include the following tests:

- genotype analyses using validated nucleic acid amplification techniques (*2.6.21*);
- virus sequencing of the seed lot and comparison of the coding sequences as follows; the sequences of the haemagglutinin and neuraminidase genes with those of the recommended strains and the sequences of the 6 remaining genes with those of the attenuated donor strain.
- genetic stability by sequencing, cold adapted and temperature sensitive phenotypes determination and attenuation test upon several passages in the substrate.

Only an attenuated master virus seed lot that complies with the following requirements may be used in the preparation of the harvest.

**Identification**. For each attenuated master virus seed lot the haemagglutinin and the neuraminidase antigens are identified using suitable methods.

**Cold adapted and temperature sensitive phenotype**. For each attenuated master virus seed lot a test is carried out in cell cultures to demonstrate the cold adapted and temperature sensitive phenotypes of the seed lot. The attenuated master virus seed lot complies with the test:

- For the cold adaptation if the loss of virus titre between the incubation at + 25 °C and + 33 °C is not more than 2.0 $\log_{10}$ of infectious units as expressed in Fluorescent Focus Unit (FFU).
- For the temperature sensitivity if the loss of virus titre between the incubation at + 33 °C and +37 °C (for strains B) or 39 °C (for strains A) is not less than 2.0 $\log_{10}$ of infectious units as expressed in Fluorescent Focus Unit (FFU).

**Attenuation**. For each attenuated master virus seed lot, an *in vivo* attenuation test is carried out on ferrets. The conditions of the test such as inoculation dose and observation period are established in validation studies. The attenuation test is performed by intranasal inoculation of ferrets, free from antibodies against influenza virus, with the attenuated master virus seed lot. The animals are monitored for a defined number of days post-inoculation for signs of influenza-like illness, including nasal discharge, frequent sneezing, severe lethargy, or fever.

At the conclusion of the monitoring period, animals are euthanized. Nasal turbinate and lung tissues are collected and analysed for the presence of infectious virus using a suitable infectivity assay.

For a master virus seed lot to be identified as attenuated, the virus must be detected in samples of nasal turbinate tissues and samples from lung tissues from individual animals, and must demonstrate that the virus growth is restricted or shows no virus replication. In addition, there are no signs of influenza-like illness in the inoculated animals.

**Virus concentration**. The virus concentration of each attenuated master virus seed lot is determined by titration in cell cultures using a suitable validated *in vitro* cell based assay (e.g. fluorescent focus assay) to monitor the consistency of production.

**Extraneous agents** (*2.6.16*). Each attenuated master virus seed lot complies with the requirements for virus seed lots.

**Avian leucosis viruses** (*2.6.24*). Each attenuated master virus seed lot complies with the test for avian leucosis viruses.

*PROPAGATION AND HARVEST*

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. After inoculation and incubation at a controlled temperature, only eggs containing living and typical chick embryos are harvested. The percentage of rejected eggs is recorded. After homogenisation and clarification by centrifugation, the clarified allantoic fluid is tested as described below and kept at − 70 °C or colder until further processing. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest or a monovalent pooled harvest that comply with the following requirements may be used in the preparation of the monovalent bulk.

**Extraneous agents** (*2.6.16*). Each single harvest or monovalent pooled harvests comply with the tests for extraneous agents with the exception of the tests for mycobacteria and sterility which are not required at this stage of production.

**Avian leucosis viruses** (*2.6.24*). Each single harvest or a monovalent pooled harvest comply with the test for avian leucosis viruses.

**Microbiological contamination**. The bioburden test using a membrane filtration is carried out on each single harvest or on each monovalent pooled harvest to determine the total viable aerobic count and to verify the absence of yeast and mould using selective media. The total viable aerobic count is within the limit approved by the competent authority. Verification of absence of *Vibrio*, *Shigella* and *Salmonella* is carried out using supplementary specific validated techniques approved by the competent authority.

*MONOVALENT BULK*

Monovalent bulks are prepared by pooling a number of satisfactory single harvests or monovalent pooled harvests of the same virus type. The monovalent bulk is concentrated and purified by high-speed centrifugation or other suitable method then filtered through a bacteria retentive filter.

Only a monovalent bulk that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. Each monovalent bulk is identified as influenza virus of the given type using suitable haemagglutinin type specific assay.

**Virus concentration**. The virus concentration of each monovalent bulk is determined by titration using a suitable validated *in vitro* assay (e.g. fluorescent focus assay).

**Cold adapted and temperature sensitive phenotype**. Each monovalent bulk complies with the test as described under Virus seed lots.

**Attenuation test**. The attenuation test is performed by intranasal inoculation of ferrets, free from antibodies against influenza virus, with each monovalent bulk test sample as described under Virus seed lots.

If sufficient consistency data are available, and approved by the competent authority, only the first 3 monovalent bulks following the introduction of a new attenuated master virus seed lot are tested on ferrets.

Wherever possible in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, manufacturers are encouraged to develop validated *in vitro* alternative methods to the animal test for monovalent bulks using appropriate tools such as molecular methods or other suitable methods for determination of viral attenuation markers.

**Genotyping**. The genotype of each monovalent bulk is verified using suitable validated nucleic acid amplification techniques (*2.6.21*).

**Bacterial and fungal contamination**. Each monovalent bulk complies with the test for sterility (*2.6.1*), carried out using 10 mL of each medium.

**Total protein content**: maximum 0.25 mg per human dose before the addition of any stabiliser.

*FINAL BULK VACCINE*

A final bulk vaccine is formulated aseptically from appropriate quantities of the monovalent bulks of each virus strain. The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirements and is within the limits approved for the particular product. A suitable stabiliser may be added. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

*FINAL LOT*

An approved minimum virus concentration for release of the product is established for each virus strain to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity. Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

**Thermal stability**. Maintain not fewer than 3 containers of the final lot at an elevated temperature for a defined period of time, using conditions found suitable for the particular product as approved by the competent authority. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. For each virus strain, the virus concentration of the containers that have been heated does not decrease by more than an approved amount during the period of exposure.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Ovalbumin**. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

**Total protein**. Not more than the quantity stated on the label and in any case not more than 2.2 mg per human dose.

**Bacterial and fungal contamination**. It complies with the test for sterility (*2.6.1*).

**Bacterial endotoxins** (*2.6.14*): less than 6 IU per single human dose.

ASSAY

Titrate the vaccine for infective virus in cell cultures using at least 3 separate containers of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established for each virus strain on a historical basis by each laboratory. If the vaccine contains more than one influenza virus strain, titrate each virus strain separately, using an appropriate type-specific antiserum.

Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). For each virus strain, the combined virus concentration for the 3 containers of vaccine is within the range stated on the label.

The assay is not valid if:

- for each virus strain, the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than $\pm 0.3$ $\log_{10}$ infectious units as expressed in FFU;
- for each virus strain, the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ infectious units as expressed in FFU from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than $\pm 0.3$ $\log_{10}$ infectious units as expressed in FFU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than $\pm 0.3$ $\log_{10}$ infectious units as expressed in FFU.

LABELLING

The label states:

- that the vaccine has been prepared on eggs,
- the strain or strains of influenza virus used in preparation of the vaccine,
- the minimum and maximum virus strain concentration per human dose,
- the maximum amount of ovalbumin,
- the season during which the vaccine is intended to protect.

**07/2019:0158**
**corrected 10.0**

# INFLUENZA VACCINE (SPLIT VIRION, INACTIVATED)

## Vaccinum influenzae inactivatum ex virorum fragmentis praeparatum

DEFINITION

Influenza vaccine (split virion, inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs, inactivated and treated so that the integrity of the virus particles has been disrupted without diminishing the antigenic properties of the haemagglutinin and neuraminidase antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

The vaccine is a slightly opalescent liquid.

PRODUCTION

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends the strains that correspond to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.4*), such as chick-embryo fibroblasts or chick kidney cells obtained from SPF chicken flocks (*5.2.2*). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

*VIRUS SEED LOT*

The production of vaccine is based on a seed-lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL.

*VIRUS PROPAGATION AND HARVEST*

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

*MONOVALENT POOLED HARVEST*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/L of $CH_2O$ at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent *V/V* at any time during inactivation.

Before or after the inactivation procedure, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method and the virus particles are disrupted into component subunits by the use of approved procedures. For each new strain, a validation test is carried out to show that the monovalent bulk consists predominantly of disrupted virus particles.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[(1)]. Carry out the test at 20-25 °C.

For some vaccines, the physical form of the haemagglutinin particles prevents quantitative determination by immunodiffusion after inactivation of the virus. For these vaccines, a determination of haemagglutinin antigen is made on the monovalent pooled harvest before inactivation. The production process is validated to demonstrate suitable conservation of haemagglutinin antigen and a suitable tracer is used for formulation, for example, protein content.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual infectious virus**. Carry out the test described below under Tests.

**Chemicals used for disruption**. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption, the limits being approved by the competent authority.

*FINAL BULK VACCINE*

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

(1) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Residual infectious virus**. Inoculate 0.2 mL of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Ovalbumin**. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

**Total protein**. Not more than 6 times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[(1)]. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated haemagglutinin antigen content. The lower confidence limit ($P$ = 0.95) is not less than 80 per cent of the amount stated on the label for each strain.

For some vaccines, quantitative determination of haemagglutinin antigen with respect to available reference preparations is not possible. An immunological identification of the haemagglutinin antigen and a semi-quantitative determination are carried out instead by suitable methods.

LABELLING

The label states:

- that the vaccine has been prepared on eggs;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content in micrograms per virus strain per dose;
- the maximum amount of ovalbumin;
- the season during which the vaccine is intended to protect.

**07/2019:0869**
**corrected 10.0**

# INFLUENZA VACCINE (SURFACE ANTIGEN, INACTIVATED)

## Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum

DEFINITION

Influenza vaccine (surface antigen, inactivated) is a sterile suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs, inactivated and treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens, without diminishing the antigenic properties of these antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount. The vaccine may contain an adjuvant.

PRODUCTION

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends the strains that correspond to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.4*), such as chick-embryo fibroblasts or chick kidney cells obtained from SPF chicken flocks (*5.2.2*). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

*VIRUS SEED LOT*

The production of vaccine is based on a seed-lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot. The

haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL.

*VIRUS PROPAGATION AND HARVEST*

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

*MONOVALENT POOLED HARVEST*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/L of $CH_2O$ at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent *V/V* at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method. Virus particles are disrupted into component subunits by approved procedures and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[(2)]. Carry out the test at 20-25 °C.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual infectious virus**. Carry out the test described below under Tests.

**Purity**. The purity of the monovalent pooled harvest is examined by polyacrylamide gel electrophoresis or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens shall be present.

**Chemicals used for disruption and purification**. Tests are carried out on the monovalent pooled harvest for the chemicals used for disruption and purification, the limits being approved by the competent authority.

*FINAL BULK VACCINE*

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine. An adjuvant may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

If the ovalbumin and formaldehyde content cannot be determined on the final lot, owing to interference from the adjuvant, they are determined on the monovalent pooled harvest, the acceptance limits being set to ensure that the limits for the final product will not be exceeded.

If the vaccine contains an adjuvant, suitable tests for identity and other relevant quality criteria are carried out on the final lot. These tests may include chemical and physical analysis, determination of particle size and determination of the number of particles per unit volume.

## IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

## TESTS

**Residual infectious virus**. Inoculate 0.2 mL of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Ovalbumin**. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

(2) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

**Total protein**. Not more than 40 µg of protein other than haemagglutinin per virus strain per human dose and not more than a total of 120 µg of protein other than haemagglutinin per human dose.

**Sterility**. It complies with the test for sterility (*2.6.1*).

**Bacterial endotoxins** (*2.6.14*): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[(2)]. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated content. The lower confidence limit ($P$ = 0.95) haemagglutinin antigen is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content, in micrograms per virus strain per dose;
- the season during which the vaccine is intended to protect;
- the maximum amount of ovalbumin;
- where applicable, the name and the quantity of adjuvant used.

**01/2020:2149**

# INFLUENZA VACCINE (SURFACE ANTIGEN, INACTIVATED, PREPARED IN CELL CULTURES)

## Vaccinum influenzae inactivatum ex cellulis corticisque antigeniis praeparatum

DEFINITION

Influenza vaccine (surface antigen, inactivated, prepared in cell cultures) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in cell cultures, inactivated and treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens, preserving adequate antigenic properties of these antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount. The vaccine is a clear or slightly opalescent liquid. The vaccine may contain an adjuvant. This monograph applies to vaccines produced in diploid or continuous cell lines of mammalian origin.

PRODUCTION

*GENERAL PROVISIONS*

Production of the vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability.

The production method is validated to demonstrate suitable reduction of residual host-cell protein. With the agreement of the competent authority and for each specific product, routine testing for residual host-cell proteins may be omitted based on the results of validation studies for the product. Guidance on the principles of such validation studies is given, for example, in the monograph *Products of recombinant DNA technology (0784)*, in particular in the sections 'Validation of the production process - Extraction and purification' and 'Production consistency - Host-cell-derived proteins'.

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus used in the preparation of seed lots is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.3*), such as chick-embryo fibroblasts, chick kidney cells obtained from SPF chicken flocks (*5.2.2*), or a diploid or continuous cell line. The final passage for establishment of the working seed lot is prepared in the cell line used for routine production. For this production, the virus of each strain is propagated in a diploid or continuous cell line (*5.2.3*).

*VIRUS SEED LOT*

The production of vaccine is based on a seed-lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Each of the strains of influenza virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The haemagglutinin and neuraminidase antigens of each master and working seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

**Virus concentration**. The virus concentration of each working seed lot is determined. Where applicable, the virus concentration of each master seed lot is determined.

**Extraneous agents** (*2.6.16*). The working seed lots comply with the requirements for seed lots. It is recognised that due to a seasonal change in one or more of the influenza vaccine strains, timely testing of a virus seed for extraneous agents using *in vitro* tests in cell culture and *in vivo* tests may be problematic (e.g. duration of *in vivo* tests, timely availability of specific neutralising antisera). In agreement with the competent authority, and in light of a risk assessment, rapid assays (e.g. multiplex PCR; high-throughput sequencing methods) may be applied following validation.

Such risk assessment and validation includes more general considerations on potential contaminants of the virus isolates, the susceptibility of the cell substrate to such viruses and the capacity of the production process for viral removal or inactivation; validation also includes comparative data on testing of seeds using *in vitro* tests in cell culture and/or *in vivo* tests according to general chapter *2.6.16* and the proposed rapid assays. Each rapid assay must be shown to be suitable for its intended use by appropriate analytical validation. The risk assessment is reviewed when new information becomes available on potential viral contaminants. Where PCR is used, the justification of the chosen panel of extraneous agents tested for is provided to the competent authority within the annual

update. This update also includes vaccine strain-specific aspects such as specific PCR inhibitory effects.

If an agent is detected in a virus seed and the mammalian cells used for production are shown to be susceptible to this agent, the virus seed is not used for vaccine production.

If an agent is detected in a virus seed and the mammalian cells are not susceptible to the agent, validation of the production process to demonstrate removal or inactivation of the agent is carried out. If removal or inactivation cannot be demonstrated, the inactivated monovalent harvest is tested to demonstrate absence of any contaminant identified in the virus seed.

*PROPAGATION AND SINGLE HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled at the same time. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions or media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator, such as phenol red, and antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells).

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine.

**Identification**. The test for antigen content also serves to identify the single harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL for each medium.

**Control cells**. The control cells of the production cell culture comply with a test for identification and the requirements for extraneous agents (*2.6.16*).

**Haemagglutinin antigen**. Determine the haemagglutinin antigen content by a suitable immunochemical method (*2.7.1*).

*INACTIVATED AND PURIFIED MONOVALENT HARVEST*

The harvest, which may be a pool of several single harvests of the same strain, is inactivated and purified by validated methods. Before or after the inactivation process, the monovalent harvest is concentrated and purified by high-speed centrifugation or another suitable method. The influenza virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process is designed so as to cause minimum alteration of the haemagglutinin and neuraminidase antigens.

Virus particles are disrupted into component subunits by approved procedures and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens.

If continuous cell lines are used for production, the purification process shall have been validated to reduce consistently host-cell DNA to a suitable level.

Only an inactivated, purified monovalent harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the haemagglutinin antigen content by a suitable immunochemical method (*2.7.1*).

**Antigen/total protein ratio**. Determine the haemagglutinin antigen content by a suitable immunodiffusion test. Determine the total protein by a validated method. The ratio of haemagglutinin antigen content to total protein content is within the limits approved for the particular product.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent harvests from each working seed lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual infectious virus**. Carry out the test described below under Tests.

**Purity**. The purity of the monovalent harvest is examined by polyacrylamide gel electrophoresis or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens are present.

**Chemicals used for disruption and purification**. Tests are carried out on the monovalent harvest for the chemicals used for disruption and purification, unless validation of the process has demonstrated total clearance. The concentration must not exceed the limits approved by the competent authority for the particular product.

*FINAL BULK VACCINE*

Appropriate quantities of the inactivated, purified monovalent pooled harvests are blended to make the final bulk vaccine. An adjuvant may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual host-cell DNA**. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, is not greater than 10 ng in the equivalent of a single human dose.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each inactivated and purified monovalent harvest and that the tests for free formaldehyde, bovine serum albumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

If the vaccine contains an adjuvant, suitable tests for identity and other relevant quality criteria are carried out on the final lot. These tests may include chemical and physical analysis, determination of particle size and determination of the number of particles per unit volume.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Residual infectious virus**. Carry out an amplification test for residual infectious influenza virus by inoculating not less than 0.2 mL of the vaccine into cell cultures of the same type as used for production of the vaccine; incubate for not less than 4 days at 37 °C. Inoculate not less than 0.2 mL of the cell culture harvested medium into a new semiconfluent cell culture and incubate as before. At the end of the incubation period, examine for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage on cell cultures and test for haemagglutination; no haemagglutination occurs.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Bovine serum albumin**: maximum 50 ng per human dose, determined by a suitable immunochemical method (*2.7.1*).

**Total protein**: maximum 40 µg of protein other than haemagglutinin per virus strain per human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation[3] or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated content. The lower confidence limit ($P$ = 0.95) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- the biological origin of the cells used for the preparation of the vaccine;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content, in micrograms per virus strain per dose;
- the season during which the vaccine is intended to protect;
- where applicable, the name and the quantity of adjuvant used.



**07/2019:2053 corrected 10.0**

# INFLUENZA VACCINE (SURFACE ANTIGEN, INACTIVATED, VIROSOME)

## Vaccinum influenzae inactivatum ex corticis antigeniis praeparatum virosomale

DEFINITION

Influenza vaccine (surface antigen, inactivated, virosome) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs, inactivated and treated so that the preparation consists predominantly of haemagglutinin and neuraminidase antigens reconstituted to virosomes and without diminishing the antigenic properties of the antigens. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

The vaccine is a slightly opalescent liquid.

PRODUCTION

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends the strains that correspond to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.4*), such as chick-embryo fibroblasts or chick kidney cells obtained from SPF chicken flocks (*5.2.2*). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

*VIRUS SEED LOT*

The production of vaccine is based on a seed lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL.

*VIRUS PROPAGATION AND HARVEST*

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest.

*MONOVALENT POOLED HARVEST*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process is designed so as to cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at a temperature of 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/L of $CH_2O$ at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent *V/V* at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or another suitable method.

Only a monovalent pooled harvest that complies with the following requirements may be used for the preparation of virosomes.

(3) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

Provided the tests for haemagglutinin antigen, neuraminidase antigen and residual infectious virus have been carried out with satisfactory results on the monovalent virosomal preparation, they may be omitted on the monovalent pooled harvest when the manufacturing process is continuous between the monovalent pooled harvest and the monovalent virosomal preparation.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation[(4)] or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

**Residual infectious virus**. Carry out the test described under Tests.

*PREPARATION OF MONOVALENT VIROSOMES*

Virus particles are disrupted into component subunits by approved procedures and further purified so that the monovalent bulk consists mainly of haemagglutinin and neuraminidase antigens. Additional phospholipids may be added and virosomes may be formed by removal of the detergent either by adsorption chromatography or another suitable technique. Several monovalent virosomal preparations may be pooled.

Only a monovalent virosomal preparation that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation[(4)] or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 virosomal preparations from each working seed lot.

**Residual infectious virus**. Carry out the test described under Tests. Provided this test has been carried out with satisfactory results on the monovalent pooled harvest, it may be omitted on the preparation of monovalent virosomes.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Purity**. The purity of the monovalent virosomal preparation is examined by polyacrylamide gel electrophoresis (*2.2.31*) or by other approved techniques. Mainly haemagglutinin and neuraminidase antigens are present.

**Chemicals used for disruption and purification**. Tests for the chemicals used for disruption and purification are carried out on the monovalent virosomal preparation, the limits being approved by the competent authority.

**Phospholipids**. The content and identity of the phospholipids are determined by suitable immunochemical or physico-chemical methods.

**Ratio of haemagglutinin to phospholipid**. The ratio of haemagglutinin content to phospholipid content is within the limits approved for the particular product.

**Virosome size**. The average virosome diameter, determined by a suitable method such as photon-correlation spectroscopy, is not less than 100 nm and not greater than 300 nm. The polydispersity index is not greater than 0.4.

*FINAL BULK VACCINE*

Appropriate quantities of the monovalent virosomal preparations are blended to make the final bulk vaccine. Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each monovalent pooled harvest or, where appropriate, on the monovalent virosomal preparations, and that the tests for phospholipids, ratio of haemagglutinin to phospholipid, free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Residual infectious virus**. Inoculate 0.2 mL of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

**pH** (*2.2.3*): 6.5 to 7.8

**Phospholipids**. The content and identity of the phospholipids is determined by a suitable immunochemical or physico-chemical method.

**Ratio of haemagglutinin to phospholipid**. The ratio of haemagglutinin content to phospholipid content is within the limits approved for the particular product.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Ovalbumin**. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

**Total protein**. Not more than 40 µg of protein other than haemagglutinin per virus strain per human dose, and not more than a total of 120 µg of protein other than hemagglutinin per human dose.

(4) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Virosome size**. The average virosome diameter, determined by a suitable method such as photon-correlation spectroscopy, is not less than 100 nm and not greater than 300 nm. The polydispersity index is not greater than 0.4.

**Bacterial endotoxins** (*2.6.14*): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation[4] or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated haemagglutinin antigen content. The lower confidence limit ($P$ = 0.95) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content, in micrograms per virus strain per dose;
- the maximum amount of ovalbumin;
- the season during which the vaccine is intended to protect.

**07/2019:0159**
**corrected 10.0**

Vaccines

# INFLUENZA VACCINE (WHOLE VIRION, INACTIVATED)

## Vaccinum influenzae inactivatum ex viris integris praeparatum

DEFINITION

Influenza vaccine (whole virion, inactivated) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in fertilised hens' eggs and inactivated in such a manner that their antigenic properties are retained. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount.

The vaccine is a slightly opalescent liquid.

PRODUCTION

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends the strains that correspond to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory States of the Convention on the Elaboration of a European Pharmacopoeia. It is now common practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus seed to be used in the production of vaccine is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.4*), such as chick-embryo fibroblasts or chick kidney cells obtained from SPF chicken flocks (*5.2.2*). For production, the virus of each strain is grown in the allantoic cavity of fertilised hens' eggs from healthy flocks.

*VIRUS SEED LOT*

The production of vaccine is based on a seed-lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot. The haemagglutinin and neuraminidase antigens of each seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

Only a working virus seed lot that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL.

*VIRUS PROPAGATION AND HARVEST*

An antimicrobial agent may be added to the inoculum. After incubation at a controlled temperature, the allantoic fluids are harvested and combined to form a monovalent pooled harvest. An antimicrobial agent may be added at the time of harvest. At no stage in the production is penicillin or streptomycin used.

*MONOVALENT POOLED HARVEST*

To limit the possibility of contamination, inactivation is initiated as soon as possible after preparation. The virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process should cause minimum alteration of the haemagglutinin and neuraminidase antigens. The inactivation process shall also have been shown to be capable of inactivating avian leucosis viruses and mycoplasmas. If the monovalent pooled harvest is stored after inactivation, it is held at 5 ± 3 °C. If formaldehyde solution is used, the concentration does not exceed 0.2 g/L of $CH_2O$ at any time during inactivation; if betapropiolactone is used, the concentration does not exceed 0.1 per cent *V/V* at any time during inactivation.

Before or after the inactivation process, the monovalent pooled harvest is concentrated and purified by high-speed centrifugation or other suitable method.

Only a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[5]. Carry out the test at 20-25 °C.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent pooled harvests from each working seed lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

(5) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

**Residual infectious virus**. Carry out the test described below under Tests.

*FINAL BULK VACCINE*

Appropriate quantities of the monovalent pooled harvests are blended to make the final bulk vaccine.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each monovalent pooled harvest and that the tests for free formaldehyde, ovalbumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Residual infectious virus**. Inoculate 0.2 mL of the vaccine into the allantoic cavity of each of 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 fertilised eggs and incubate at 33-37 °C for 3 days. The test is not valid unless at least 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; no haemagglutination occurs.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Ovalbumin**. Not more than the quantity stated on the label and in any case not more than 1 µg per human dose, determined by a suitable immunochemical method (*2.7.1*) using a suitable reference preparation of ovalbumin.

**Total protein**. Not more than 6 times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose and not more than a total of 300 µg of protein per human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 100 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation or with an antigen preparation calibrated against it[5]. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated haemagglutinin antigen content. The lower confidence limit ($P$ = 0.95) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- that the vaccine has been prepared on eggs;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content, in micrograms per virus strain per dose;
- the maximum amount of ovalbumin;
- the season during which the vaccine is intended to protect.

**01/2020:2308**

# INFLUENZA VACCINE (WHOLE VIRION, INACTIVATED, PREPARED IN CELL CULTURES)

## Vaccinum influenzae inactivatum ex cellulis virisque integris praeparatum

DEFINITION

Influenza vaccine (whole virion, inactivated, prepared in cell cultures) is a sterile, aqueous suspension of a strain or strains of influenza virus, type A or B, or a mixture of strains of the 2 types grown individually in cell cultures and inactivated in such a manner that their antigenic properties are retained. The stated amount of haemagglutinin antigen for each strain present in the vaccine is 15 µg per dose, unless clinical evidence supports the use of a different amount. The vaccine is a slightly opalescent or opalescent liquid. The vaccine may contain an adjuvant. This monograph applies to vaccines produced in diploid or continuous cell lines of mammalian origin.

PRODUCTION

*GENERAL PROVISIONS*

Production of the vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability.

The production method is validated to demonstrate suitable reduction of residual host-cell protein. With the agreement of the competent authority and for each specific product, routine testing for residual host-cell proteins may be omitted based on the results of validation studies for the product. Guidance on the principles of such validation studies is given, for example, in the monograph *Products of recombinant DNA technology (0784)*, in particular in the sections 'Validation of the production process - Extraction and purification' and 'Production consistency - Host-cell-derived proteins'.

*CHOICE OF VACCINE STRAIN*

The World Health Organization (WHO) reviews the world epidemiological situation annually and if necessary recommends new strains corresponding to this epidemiological evidence.

Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. It is now common

practice to use reassorted strains giving high yields of the appropriate surface antigens. The origin and passage history of virus strains shall be approved by the competent authority.

*SUBSTRATE FOR VIRUS PROPAGATION*

Influenza virus used in the preparation of seed lots is propagated in fertilised eggs from chicken flocks free from specified pathogens (SPF) (*5.2.2*) or in suitable cell cultures (*5.2.3*), such as chick-embryo fibroblasts, chick kidney cells obtained from SPF chicken flocks (*5.2.2*), or a diploid or continuous cell line. The final passage for establishment of the working seed lot is prepared in the cell line used for routine production. For this production, the virus of each strain is propagated in a diploid or continuous cell line (*5.2.3*).

*VIRUS SEED LOT*

The production of vaccine is based on a seed-lot system established by subculture of the candidate vaccine virus (CVV). This CVV is the approved virus isolate or reassorted virus supplied by WHO designated laboratories, or established by vaccine manufacturers. Each of the strains of influenza virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Working seed lots represent not more than 15 passages from the CVV. The final vaccine represents 1 passage from the working seed lot.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The haemagglutinin and neuraminidase antigens of each master and working seed lot are identified as originating from the correct strain of influenza virus by suitable methods.

**Virus concentration**. The virus concentration of each working seed lot is determined. Where applicable, the virus concentration of each master seed lot is determined.

**Extraneous agents** (*2.6.16*). The working seed lots comply with the requirements for seed lots. It is recognised that due to a seasonal change in one or more of the influenza vaccine strains, timely testing of a virus seed for extraneous agents using *in vitro* tests in cell culture and *in vivo* tests may be problematic (e.g. duration of *in vivo* tests, timely availability of specific neutralising antisera). In agreement with the competent authority, and in light of a risk assessment, rapid assays (e.g. multiplex PCR; high-throughput sequencing methods) may be applied following validation.

Such risk assessment and validation includes more general considerations on potential contaminants of the virus isolates, the susceptibility of the cell substrate to such viruses and the capacity of the production process for viral removal or inactivation; validation also includes comparative data on testing of seeds using *in vitro* tests in cell culture and/or *in vivo* tests according to general chapter *2.6.16* and the proposed rapid assays. Each rapid assay must be shown to be suitable for its intended use by appropriate analytical validation. The risk assessment is reviewed when new information becomes available on potential viral contaminants. Where PCR is used, the justification of the chosen panel of extraneous agents tested for is provided to the competent authority within the annual update. This update also includes vaccine strain-specific aspects such as specific PCR inhibitory effects.

If an agent is detected in a virus seed and the mammalian cells used for production are shown to be susceptible to this agent, the virus seed is not used for vaccine production.

If an agent is detected in a virus seed and the mammalian cells are not susceptible to the agent, validation of the production process to demonstrate removal or inactivation of the agent is carried out. If removal or inactivation cannot be demonstrated, the inactivated monovalent harvest is tested to demonstrate absence of any contaminant identified in the virus seed.

*PROPAGATION AND SINGLE HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled at the same time. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions or media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator, such as phenol red, and antibiotics at the lowest effective concentration. A sufficient quantity of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells).

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine.

**Identification**. The test for antigen content also serves to identify the single harvest.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). Carry out the test for mycoplasmas, using 10 mL for each medium.

**Control cells**. The control cells of the production cell culture comply with a test for identification and the requirements for extraneous agents (*2.6.16*).

**Haemagglutinin antigen**. Determine the haemagglutinin antigen content by a suitable immunochemical method (*2.7.1*).

*INACTIVATED AND PURIFIED MONOVALENT HARVEST*

The harvest, which may be a pool of several single harvests of the same strain, is inactivated and purified by validated methods. Before or after the inactivation process, the monovalent harvest is concentrated and purified by high-speed centrifugation or another suitable method. The influenza virus is inactivated by a method that has been demonstrated on 3 consecutive batches to be consistently effective for the manufacturer. The inactivation process shall have been shown to be capable of inactivating the influenza virus without destroying its antigenicity; the process is designed so as to cause minimum alteration of the haemagglutinin and neuraminidase antigens.

If continuous cell lines are used for production, the purification process shall have been validated to reduce consistently host-cell DNA to a suitable level.

Only an inactivated, purified monovalent harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Haemagglutinin antigen**. Determine the haemagglutinin antigen content by a suitable immunochemical method (*2.7.1*).

**Antigen/total protein ratio**. Determine the haemagglutinin antigen content by a suitable immunodiffusion test. Determine the total protein by a validated method. The ratio of haemagglutinin antigen content to total protein content is within the limits approved for the particular product.

**Neuraminidase antigen**. The presence and type of neuraminidase antigen are confirmed by suitable enzymatic or immunological methods on the first 3 monovalent harvests from each working seed lot.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual infectious virus**. Carry out the test described below under Tests.

*FINAL BULK VACCINE*

Appropriate quantities of the inactivated, purified monovalent pooled harvests are blended to make the final bulk vaccine. An adjuvant may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility, using 10 mL for each medium.

**Residual host-cell DNA**. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, is not greater than 10 ng in the equivalent of a single human dose.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Tests and Assay may be released for use. Provided that the test for residual infectious virus has been performed with satisfactory results on each inactivated and purified monovalent harvest and that the tests for free formaldehyde, bovine serum albumin and total protein have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

If the vaccine contains an adjuvant, suitable tests for identity and other relevant quality criteria are carried out on the final lot. These tests may include chemical and physical analysis, determination of particle size and determination of the number of particles per unit volume.

IDENTIFICATION

The assay serves to confirm the antigenic specificity of the vaccine.

TESTS

**Residual infectious virus**. Carry out an amplification test for residual infectious influenza virus by inoculating not less than 4 mL of the vaccine into cell cultures of the same type as used for production of the vaccine; incubate for not less than 7 days at 32 ± 2 °C. Inoculate not less than 10 mL of the cell culture harvested medium into a new semi-confluent cell culture and incubate as before. At the end of the incubation period, examine for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage on cell cultures and test for haemagglutination; no haemagglutination occurs.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Bovine serum albumin**: maximum 50 ng per human dose, determined by a suitable immunochemical method (*2.7.1*).

**Total protein**. Not more than 6 times the total haemagglutinin content of the vaccine as determined in the assay, but in any case, not more than 100 µg of protein per virus strain per human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU per human dose.

ASSAY

Determine the content of haemagglutinin antigen by an immunodiffusion test (*2.7.1*), by comparison with a haemagglutinin antigen reference preparation[6] or with an antigen preparation calibrated against it. Carry out the test at 20-25 °C. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated content. The lower confidence limit ($P$ = 0.95) is not less than 80 per cent of the amount stated on the label for each strain.

LABELLING

The label states:

- the biological origin of the cells used for the preparation of the vaccine;
- the strain or strains of influenza virus used to prepare the vaccine;
- the method of inactivation;
- the haemagglutinin antigen content, in micrograms per virus strain per dose;
- the season during which the vaccine is intended to protect;
- where applicable, the name and the quantity of adjuvant used.

**01/2019:1057**

# MEASLES, MUMPS AND RUBELLA VACCINE (LIVE)

## Vaccinum morbillorum, parotitidis et rubellae vivum

DEFINITION

Measles, mumps and rubella vaccine (live) is a freeze-dried preparation of suitable attenuated strains of measles virus, mumps virus and rubella virus.

The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The 3 components are prepared as described in the monographs *Measles vaccine (live) (0213)*, *Mumps vaccine (live) (0538)* and *Rubella vaccine (live) (0162)* and comply with the requirements prescribed therein.

*FINAL BULK VACCINE*

Virus harvests for each component are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate. Suitable quantities of the pooled harvest for each component are mixed.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

*FINAL LOT*

For each component, a minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration of each component for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the tests for bovine serum albumin and, where applicable, for ovalbumin have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

**Thermal stability**. Maintain at least 3 vials of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay

(6) Reference haemagglutinin antigens are available from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, Great Britain.

in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. For each component, the virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with antibodies specific for measles virus, mumps virus and rubella virus, it is no longer able to infect cell cultures susceptible to these viruses. When the vaccine reconstituted as stated on the label is mixed with quantities of specific antibodies sufficient to neutralise any 2 viral components, the 3rd viral component infects susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Ovalbumin**. If the mumps component is produced in chick embryos, the vaccine contains not more than 1 µg of ovalbumin per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

The cell lines and/or neutralising antisera are chosen to ensure that each component is assayed without interference from the other 2 components.

Titrate the vaccine for infective measles, mumps and rubella virus, using at least 3 separate vials of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 vial of the appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*).

The combined estimates of the measles, mumps and rubella virus concentrations for the 3 vials of vaccine are not less than that stated on the label; the minimum measles virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose; the minimum mumps virus concentration stated on the label is not less than 3.7 $\log_{10}$ $CCID_{50}$ per single human dose; the minimum rubella virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

*Measles vaccine (live) BRP* is suitable for use as a reference preparation.

*Mumps vaccine (live) BRP* is suitable for use as a reference preparation.

*Rubella vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strains of virus used in the preparation of the vaccine;
- where applicable, that chick embryos have been used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration for each component of the vaccine;
- that contact between the vaccine and disinfectants is to be avoided.

**01/2019:2442**
**corrected 10.0**

# MEASLES, MUMPS, RUBELLA AND VARICELLA VACCINE (LIVE)

## Vaccinum morbillorum, parotitidis, rubellae et varicellae vivum

DEFINITION

Measles, mumps, rubella and varicella vaccine (live) is a freeze-dried preparation of suitable attenuated strains of measles virus, mumps virus, rubella virus and human herpesvirus 3. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The 4 components are prepared as described in the monographs *Measles vaccine (live) (0213)*, *Mumps vaccine (live) (0538)*, *Rubella vaccine (live) (0162)* and *Varicella vaccine (live) (0648)* and comply with the requirements prescribed therein.

*FINAL BULK VACCINE*

Virus harvests for each component are pooled and clarified to remove cells. A suitable stabiliser may be added and for each component the pooled harvests diluted as appropriate. Suitable quantities of the pooled harvest for each component are mixed.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

*FINAL LOT*

For each component, a minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity. The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that complies with the requirements for minimum virus concentration of each component for release, with the following requirements for thermal stability, bovine serum albumin and water, and with each of the requirements given under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Thermal stability**. For the measles, mumps and rubella components maintain at least 3 containers of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. For each component, the virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

**Bovine serum albumin**. Not more than the amount approved by the competent authority, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*). Not more than the amount shown to ensure stability of the vaccines as approved by the competent authority, determined by the semi-micro determination of water.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with antibodies specific for measles virus, mumps virus, rubella virus and human herpesvirus 3, it is no longer able to infect cell cultures susceptible to these viruses. When the vaccine reconstituted as stated on the label is mixed with quantities of specific antibodies sufficient to neutralise any 3 viral components, the 4$^{th}$ viral component infects susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

ASSAY

The cell lines and/or neutralising antisera are chosen to ensure that each component is assayed without interference from the other 3 components.

Titrate the vaccine for infective measles virus, mumps virus, rubella virus and human herpesvirus 3 using at least 3 separate containers of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 container of the appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Unless otherwise justified and authorised, for the measles, mumps, rubella and human herpesvirus 3 viruses the relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*).

The combined estimates of the measles virus, mumps virus, rubella virus and human herpesvirus 3 concentrations for the 3 containers of vaccine are not less than that stated on the label; the minimum measles virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose; the minimum mumps virus concentration stated on the label is not less than 3.7 $\log_{10}$ $CCID_{50}$ per single human dose; the minimum rubella virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (measles virus, mumps virus and rubella virus) or ± 0.3 $\log_{10}$ PFU (human herpesvirus 3);
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ (measles virus, mumps virus and rubella virus) or 0.5 $\log_{10}$ PFU (human herpesvirus 3) from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (measles virus, mumps virus and rubella virus) or ± 0.3 $\log_{10}$ PFU (human herpesvirus 3); data obtained from valid assays only are combined by using the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (measles virus, mumps virus and rubella virus) or ± 0.3 $\log_{10}$ PFU (human herpesvirus 3).

*Measles vaccine (live) BRP* is suitable for use as a reference preparation.

*Mumps vaccine (live) BRP* is suitable for use as a reference preparation.

*Rubella vaccine (live) BRP* is suitable for use as a reference preparation.

*Varicella vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strains of virus used in the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration for each component of the vaccine;
- that contact between the vaccine and disinfectants is to be avoided.



**01/2019:0213**

# MEASLES VACCINE (LIVE)

## Vaccinum morbillorum vivum

DEFINITION

Measles vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of measles virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live measles vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to

be satisfactory with respect to safety and efficacy; even with authorised exceptions, the number of passages beyond the level used for clinical studies shall not exceed 5.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*).

*SEED LOT*

The strain of measles virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below – 20 °C if freeze-dried, or below – 60 °C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration of the master and working seed lots is determined to monitor consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots.

*PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled during production. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cells during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 mL of the production cell cultures is set aside as uninfected cell cultures (control cells). The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The single harvest contains virus that is identified as measles virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents.

**Control cells**. If human diploid cells are used for production, the control cells comply with a test for identification. They comply with the tests for extraneous agents (*2.6.16*).

*FINAL BULK VACCINE*

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

*FINAL LOT*

A minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Thermal stability**. Maintain at least 3 vials of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific measles antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

*Measles vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

**07/2019:3066**

# MENINGOCOCCAL GROUP A, C, W135 AND Y CONJUGATE VACCINE

## Vaccinum meningococcale classium A, C, W135 et Y coniugatum

DEFINITION

Meningococcal group A, C, W135 and Y conjugate vaccine is a freeze-dried preparation, or a preparation consisting of freeze-dried and liquid components, of purified capsular polysaccharides covalently linked to a carrier protein. The polysaccharides are obtained from suitable strains of *Neisseria meningitidis* group A, group C, group W135 and group Y that are capable of consistently producing polysaccharides.

*N. meningitidis* group A polysaccharide consists of partly *O*-acetylated repeating units of *N*-acetylmannosamine, linked with α-(1→6) phosphodiester bonds.

*N. meningitidis* group C polysaccharide consists of partly *O*-acetylated repeating units of sialic acid, linked with α-(2→9) glycosidic bonds.

*N. meningitidis* group W135 polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-galactose, linked with α-(2→6) and α-(1→4) glycosidic bonds.

*N. meningitidis* group Y polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-glucose, linked with α-(2→6) and α-(1→4) glycosidic bonds.

The carrier protein used may vary for the various polysaccharide conjugates contained in a multivalent vaccine.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently meningococcal group A, C, W135 and Y conjugate vaccines of adequate safety and immunogenicity in man. The production of the meningococcal polysaccharides and of the carrier protein(s) is based on a seed-lot system.

The production method is validated to demonstrate that the product, if tested, would comply with the test for pyrogens (*2.6.8*).

During development studies, it shall be demonstrated that the vaccine consistently induces a T-cell-dependent B-cell immune response to each polysaccharide. If the manufacturing process is modified, it shall be demonstrated by appropriate *in vitro* methods that the characteristic properties of the vaccine are not affected.

The stability of the conjugated bulks and/or final lot and meningococcal polysaccharides is evaluated using suitable indicator tests. Such tests may include determination of molecular size, quantification of polysaccharide content and free polysaccharide content in the conjugate.

*PRODUCTION OF THE COMPONENTS*

The production of the seed lots and bulk polysaccharides complies with the requirements of the monograph *Meningococcal polysaccharide vaccine (0250)*.

The production of the carrier protein(s) complies with the requirements of the general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*.

*PURIFIED POLYSACCHARIDES*

The polysaccharides are purified, after dissociation of the complex of polysaccharide and cetrimonium bromide, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides.

The final purification step consists of ethanol precipitation of the polysaccharides, which are then dried *in vacuo* and stored at − 20 °C. The loss on drying is determined by thermogravimetry (*2.2.34*) or another suitable method and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

Only purified polysaccharides that comply with the following requirements may be used in the preparation of the conjugate.

**Protein** (*2.5.16*): maximum 10 mg per gram of purified polysaccharide, calculated with reference to the dried substance.

**Nucleic acids** (*2.5.17*): maximum 10 mg per gram of purified polysaccharide, calculated with reference to the dried substance.

***O*-Acetyl groups** (*2.5.19*): minimum 2 mmol per gram of purified polysaccharide for group A, minimum 1.5 mmol per gram of purified polysaccharide for group C, minimum 0.3 mmol per gram of purified polysaccharide for groups W135 and Y, all calculated with reference to the dried substance.

**Phosphorus** (*2.5.18*): minimum 80 mg per gram of purified polysaccharide for group A, calculated with reference to the dried substance.

**Sialic acid** (*2.5.23*): minimum 800 mg per gram of purified polysaccharide for group C and minimum 560 mg per gram of purified polysaccharide for groups W135 and Y, all calculated with reference to the dried substance, using *N-acetylneuraminic acid R* to prepare the reference solution.

**Calcium**. If a calcium salt is used during purification, a determination of calcium is carried out on the purified polysaccharide; the content is within the limits approved for the particular product.

**Residual reagents**. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on purified polysaccharides may be omitted.

**Molecular-size or molecular-mass distribution**. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each purified polysaccharide. Each batch must be shown to comply with this limit.

**Identification and serological specificity**. The identity and serological specificity are determined by a suitable immunochemical method (*2.7.1*) or another suitable method, for example $^{1}$H nuclear magnetic resonance spectrometry (*2.2.33*). Identity and purity of each polysaccharide shall

be confirmed; it shall be shown that there is not more than 1 per cent *m/m* of group-heterologous *N. meningitidis* polysaccharide.

**Bacterial endotoxins** (*2.6.14*). The content is within the limits approved by the competent authority for the particular product.

**Water** (*2.5.12*). Where applicable, the values are within the limits approved for each group, determined by a suitable method.

*MODIFIED MENINGOCCOCAL POLYSACCHARIDES*

Before conjugation, the polysaccharides can be depolymerised by chemical or mechanical means followed by a concentration step to obtain polysaccharides of a desired molecular size range. Polysaccharides or depolymerised polysaccharides are modified by an activation process.

*MONOVALENT BULK CONJUGATE*

The conjugate is obtained by the covalent binding of activated polysaccharides to the appropriate carrier protein.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits.

**Saccharide**. The polysaccharide content is determined by a suitable physical or chemical method or by an immunochemical method (*2.7.1*). The value complies with the requirement approved for each conjugate.

**Protein**. The protein content is determined by a suitable physical or chemical method (for example, *2.5.16*). The value complies with the requirement approved for each conjugate.

**Saccharide-to-protein ratio**. Determine the ratio by calculation. The value complies with the requirement approved for each conjugate.

**Free saccharide**. The free polysaccharide content is determined after separation from the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration, or other validated methods. The value complies with the requirement approved for each conjugate.

**Free carrier protein**. The free carrier protein content is determined by a suitable method, either directly or by calculation from the results of other tests. The value complies with the requirement approved for each conjugate.

**Residual reagents**. Where applicable, suitable tests are carried out to determine residues of reagents used during conjugation. An acceptable value for each reagent is established for the particular product and each batch of monovalent bulk conjugate must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on monovalent bulk conjugate may be omitted.

**Molecular size or molecular-mass distribution**. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each conjugate. Each batch must be shown to comply with this limit.

**Identification**. Each polysaccharide conjugate is identified by an immunochemical method (*2.7.1*) or another suitable method.

*FINAL BULK VACCINE*

Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirement and is within the limits approved for the particular product.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL or the equivalent of 100 doses for each medium, whichever is less.

*FINAL LOT*

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

Conjugate from each group present in the vaccine is identified by a suitable immunochemical method (*2.7.1*).

TESTS

**Free saccharide**. The free polysaccharide content for each group is determined by a suitable physico-chemical (for example liquid chromatography (*2.2.29*)) or immunochemical (for example enzyme-linked immunosorbent assay (ELISA) (*2.7.1*)) method. The free polysaccharide content is within the limits approved by the competent authority for the particular product. Where justified and authorised, the test may be carried out on the monovalent bulk conjugate only.

**Molecular-size or molecular-mass distribution**. The molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each conjugate. Where justified and authorised, the test may be carried out on the monovalent bulk conjugate only.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

**Saccharide**. The total polysaccharide content for each group is determined by a suitable physico-chemical (for example liquid chromatography (*2.2.29*)) or immunochemical (for example enzyme-linked immunosorbent assay (ELISA) (*2.7.1*)) method.

The content of each group is within the limits approved by the competent authority for the particular product.

LABELLING

The label states:

- the nominal amount of polysaccharide for each group (A, C, W135 and Y) per single human dose;
- the type and amount of carrier protein per single human dose.

**01/2019:2112**

# MENINGOCOCCAL GROUP C CONJUGATE VACCINE

## Vaccinum meningococcale classis C coniugatum

DEFINITION

Meningococcal group C conjugate vaccine is a liquid or freeze-dried preparation of purified capsular polysaccharide derived from a suitable strain of *Neisseria meningitidis* group C covalently linked to a carrier protein. Meningococcal group C polysaccharide consists of partly *O*-acetylated or *O*-deacetylated repeating units of sialic acids, linked with 2α→9 glycosidic bonds. The carrier protein, when conjugated to group C polysaccharide, is capable of inducing a T-cell-dependent B-cell immune response to the polysaccharide. The vaccine may contain an adjuvant.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall consistently have been shown to yield meningococcal group C conjugate vaccines of satisfactory immunogenicity and safety in man. The production of meningococcal group C polysaccharide and of the carrier protein are based on seed-lot systems.

During development studies and wherever revalidation is necessary, a test for pyrogens in rabbits (*2.6.8*) is carried out by injection of a suitable dose of the final lot. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

During development studies and wherever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the final lot and relevant intermediates is evaluated using 1 or more indicator tests. Such tests may include determination of molecular size, determination of free saccharide in the conjugate or an immunogenicity test in animals.

*BACTERIAL SEED LOTS*

The bacterial strains used for master seed lots shall be identified by historical records that include information on their origin and the tests used to characterise the strain. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

*MENINGOCOCCAL GROUP C POLYSACCHARIDE*

*N. meningitidis* is grown in a liquid medium that does not contain high-molecular-mass polysaccharides and is free from ingredients that will form a precipitate upon addition of cetyltrimethylammonium bromide (CTAB). The culture may be inactivated by heat and filtered before the polysaccharide is precipitated by addition of CTAB. The precipitate is further purified using suitable methods to remove nucleic acids, proteins and lipopolysaccharides and the final purification step consists of ethanol precipitation. An *O*-deacetylation step may also be included. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method such as thermogravimetry (*2.2.34*). The value is used to calculate the results of other tests with reference to the dried substance, as prescribed below.

Only meningococcal group C polysaccharide that complies with the following requirements may be used in the preparation of the conjugate.

**Protein** (*2.5.16*): maximum 1.0 per cent, calculated with reference to the dried substance.

**Nucleic acid** (*2.5.17*): maximum 1.0 per cent, calculated with reference to the dried substance.

***O*-acetyl groups**. Examine by a suitable method (for example *2.5.19*). An acceptable value is established for the particular product and each batch of meningococcal group C polysaccharide must be shown to comply with this limit.

**Sialic acid** (*2.5.23*): minimum 0.800 g of sialic acid per gram of meningococcal group C polysaccharide using *N-acetylneuraminic acid R* to prepare the reference solution.

**Residual reagents**. Where applicable, tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of meningococcal group C polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of a residual reagent, the test on purified meningococcal group C polysaccharide may be omitted.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. Where applicable, the molecular-size distribution is also determined after chemical modification of the polysaccharide. An acceptable value is established for the meningococcal group C polysaccharide. Each batch must be shown to comply with this limit.

**Identification and serological specificity**. The identity and serological specificity are determined by a suitable immunochemical method (*2.7.1*) or other suitable method, for example $^{1}$H nuclear magnetic resonance spectrometry (*2.2.33*).

**Bacterial endotoxins** (*2.6.14*): less than 100 IU per microgram of meningococcal group C polysaccharide.

*CARRIER PROTEIN*

The production and characteristics of the carrier proteins are described in general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*. Only a carrier protein that complies with the requirements of this chapter may be used in the preparation of the conjugate.

*BULK CONJUGATE*

Meningococcal group C polysaccharide is chemically modified to enable conjugation; it is usually partly depolymerised either before or during this procedure. The conjugate is obtained by the covalent binding of activated meningococcal group C oligosaccharide and the appropriate carrier protein. The conjugate purification procedures are designed to remove residual reagents used for conjugation. The removal of residual reagents and reaction by-products is confirmed by suitable tests or by validation of the purification process.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test and for each particular product, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for the bulk conjugate. Each batch must be shown to comply with this limit.

**Saccharide**. The saccharide content is determined by a suitable validated assay (for example *2.5.23*). Anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection may also be used for determination of saccharide content. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

**Protein**. The protein content is determined by a suitable chemical method (for example *2.5.16*). An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

**Saccharide-to-protein ratio**. Determine the ratio by calculation.

**Free saccharide**. Unbound saccharide is determined after removal of the conjugate, for example by anion-exchange liquid chromatography, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

**Free carrier protein**. Determine the content, either directly by a suitable method or by deriving the content by calculation from the results of other tests. An acceptable value is established for the particular product and each batch of bulk conjugate must be shown to comply with this limit.

**Residual reagents**. Removal of residual reagents such as cyanide is confirmed by suitable tests or by validation of the process.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium or the equivalent of 100 doses, whichever is less.

*FINAL BULK VACCINE*

An adjuvant and a stabiliser may be added to the bulk conjugate before dilution to the final concentration with a suitable diluent.

Only a final bulk vaccine that complies with the following requirement and is within the limits approved for the particular product may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

The vaccine is identified by a suitable immunochemical method (*2.7.1*).

TESTS

**pH** (*2.2.3*). The pH of the vaccine, reconstituted if necessary, is within the limits approved for the particular product.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Water** (*2.5.12*): maximum 3.0 per cent for freeze-dried vaccines.

**Free saccharide**. Unbound saccharide is determined after removal of the conjugate, for example by anion-exchange liquid chromatography, size-exclusion or hydrophobic chromatography, ultrafiltration or other validated methods. An acceptable value consistent with adequate immunogenicity, as shown in clinical trials, is established for the particular product and each final lot must be shown to comply with this limit.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU per single human dose.

ASSAY

**Saccharide**: minimum 80 per cent of the amount of meningococcal group C polysaccharide stated on the label. The saccharide content is determined by a suitable validated assay, for example sialic acid assay (*2.5.23*) or anion-exchange liquid chromatography (*2.2.29*) with pulsed amperometric detection.

LABELLING

The label states:

- the number of micrograms of meningococcal group C polysaccharide per single human dose;
- the type and nominal amount of carrier protein per single human dose.



**01/2019:0250**

# MENINGOCOCCAL POLYSACCHARIDE VACCINE

## Vaccinum meningococcale polysaccharidicum

DEFINITION

Meningococcal polysaccharide vaccine is a freeze-dried preparation of one or more purified capsular polysaccharides obtained from one or more suitable strains of *Neisseria meningitidis* group A, group C, group Y and group W135 that are capable of consistently producing polysaccharides.

*N. meningitidis* group A polysaccharide consists of partly *O*-acetylated repeating units of *N*-acetylmannosamine, linked with 1α→6 phosphodiester bonds.

*N. meningitidis* group C polysaccharide consists of partly *O*-acetylated repeating units of sialic acid, linked with 2α→9 glycosidic bonds.

*N. meningitidis* group Y polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-glucose, linked with 2α→6 and 1α→4 glycosidic bonds.

*N. meningitidis* group W135 polysaccharide consists of partly *O*-acetylated alternating units of sialic acid and D-galactose, linked with 2α→6 and 1α→4 glycosidic bonds.

The polysaccharide component or components stated on the label together with calcium ions and residual moisture account for over 90 per cent of the mass of the preparation.

PRODUCTION

Production of the meningococcal polysaccharides is based on a seed-lot system. The production method shall have been shown to yield consistently meningococcal polysaccharide vaccines of satisfactory immunogenicity and safety in man.

*SEED LOTS*

The strains of *N. meningitidis* used for the master seed lots shall be identified by historical records that include information on their origin and by their biochemical and serological characteristics.

Cultures from each working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot. The strains have the following characteristics:

- colonies obtained from a culture are rounded, uniform in shape and smooth with a mucous, opalescent, greyish appearance;
- Gram staining reveals characteristic Gram-negative diplococci in 'coffee-bean' arrangement;
- the oxidase test is positive;
- the culture utilises glucose and maltose;
- suspensions of the culture agglutinate with suitable specific antisera.

Purity of bacterial strains used for the seed lots is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

*PROPAGATION AND HARVEST*

The working seed lots are cultured on solid media that do not contain blood-group substances or ingredients of mammalian origin. The inoculum may undergo 1 or more subcultures in liquid medium before being used for inoculating the final medium. The liquid media used and the final medium are semi-synthetic and free from substances precipitated

by cetrimonium bromide (hexadecyltrimethylammonium bromide) and do not contain blood-group substances or high-molecular-mass polysaccharides.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The cultures are centrifuged and the polysaccharides precipitated from the supernatant by addition of cetrimonium bromide. The precipitate obtained is harvested and may be stored at − 20 °C awaiting further purification.

*PURIFIED POLYSACCHARIDES*

The polysaccharides are purified, after dissociation of the complex of polysaccharide and cetrimonium bromide, using suitable procedures to remove successively nucleic acids, proteins and lipopolysaccharides.

The final purification step consists of ethanol precipitation of the polysaccharides which are then dried and stored at − 20 °C. The loss on drying is determined by thermogravimetry (*2.2.34*) and the value is used to calculate the results of the other chemical tests with reference to the dried substance.

Only purified polysaccharides that comply with the following requirements may be used in the preparation of the final bulk vaccine.

**Protein** (*2.5.16*). Not more than 10 mg of protein per gram of purified polysaccharide, calculated with reference to the dried substance.

**Nucleic acids** (*2.5.17*). Not more than 10 mg of nucleic acids per gram of purified polysaccharide, calculated with reference to the dried substance.

***O*-Acetyl groups** (*2.5.19*). Not less than 2 mmol of *O*-acetyl groups per gram of purified polysaccharide for group A, not less than 1.5 mmol per gram of polysaccharide for group C, not less than 0.3 mmol per gram of polysaccharide for groups Y and W135, all calculated with reference to the dried substance.

**Phosphorus** (*2.5.18*). Not less than 80 mg of phosphorus per gram of group A purified polysaccharide, calculated with reference to the dried substance.

**Sialic acid** (*2.5.23*). Not less than 800 mg of sialic acid per gram of group C polysaccharide and not less than 560 mg of sialic acid per gram of purified polysaccharide for groups Y and W135, all calculated with reference to the dried substance. Use the following reference solutions.

Group C polysaccharide: a 150 mg/L solution of *N-acetylneuraminic acid R*.

Group Y polysaccharide: a solution containing 95 mg/L of *N-acetylneuraminic acid R* and 55 mg/L of *glucose R*.

Group W135 polysaccharide: a solution containing 95 mg/L of *N-acetylneuraminic acid R* and 55 mg/L of *galactose R*.

**Calcium**. If a calcium salt is used during purification, a determination of calcium is carried out on the purified polysaccharide; the content is within the limits approved for the particular product.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each purified polysaccharide. Each batch must be shown to comply with this limit.

**Identification and serological specificity**. The identity and serological specificity are determined by a suitable immunochemical method (*2.7.1*). Identity and purity of each polysaccharide shall be confirmed; it shall be shown that there is not more than 1 per cent *m/m* of group-heterologous *N. meningitidis* polysaccharide.

**Pyrogens** (*2.6.8*). The polysaccharide complies with the test for pyrogens. Inject into each rabbit per kilogram of body mass 1 mL of a solution containing 0.025 μg of purified polysaccharide per millilitre.

*FINAL BULK VACCINE*

One or more purified polysaccharides of 1 or more *N. meningitidis* groups are dissolved in a suitable solvent that may contain a stabiliser. When dissolution is complete, the solution is filtered through a bacteria-retentive filter.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile containers. The containers are then closed so as to avoid contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay may be released for use.

CHARACTERS

A white or cream-coloured powder or pellet, freely soluble in water.

IDENTIFICATION

Carry out an identification test for each polysaccharide present in the vaccine by a suitable immunochemical method (*2.7.1*).

TESTS

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each purified polysaccharide. Each batch must be shown to comply with this limit.

**Water** (*2.5.12*). Not more than 3.0 per cent.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Pyrogens** (*2.6.8*). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 mL of a solution containing:

- 0.025 μg of polysaccharide for a monovalent vaccine;
- 0.050 μg of polysaccharide for a divalent vaccine;
- 0.10 μg of polysaccharide for a tetravalent vaccine.

ASSAY

Carry out an assay of each polysaccharide present in the vaccine.

For a divalent vaccine (group A + group C), use measurement of phosphorus (*2.5.18*) to determine the content of polysaccharide A and measurement of sialic acid (*2.5.23*) to determine the content of polysaccharide C. To determine sialic acid, use as reference solution a 150 mg/L solution of *N-acetylneuraminic acid R*.

For a tetravalent vaccine (group A + group C + group Y + group W135) a suitable immunochemical method (*2.7.1*) is used with a reference preparation of purified polysaccharide for each group.

The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity of each polysaccharide stated on the label.

LABELLING

The label states:

- the group or groups of polysaccharides (A, C, Y or W135) present in the vaccine;
- the number of micrograms of polysaccharide per single human dose.

01/2019:0538

# MUMPS VACCINE (LIVE)

## Vaccinum parotitidis vivum

DEFINITION

Mumps vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of mumps virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and, if the virus is propagated in human diploid cells, a cell-bank system. The production method shall have been shown to yield consistently live mumps vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*) or in chick-embryo cells or in the amniotic cavity of chick embryos derived from a chicken flock free from specified pathogens (*5.2.2*).

*SEED LOT*

The strain of mumps virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below − 20 °C if freeze-dried, or below − 60 °C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as mumps virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots.

*PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled during the production. Suitable animal (but not human) serum may be used in the culture media. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 mL of the production cell cultures is set aside as uninfected cell cultures (control cells). If the virus is propagated in chick embryos, 2 per cent but not less than 20 eggs are set aside as uninfected control eggs. The viral suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The single harvest contains virus that is identified as mumps virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents.

**Control cells or eggs**. If human diploid cells are used for production, the control cells comply with a test for identification; the control cells and the control eggs comply with the tests for extraneous agents (*2.6.16*).

*FINAL BULK VACCINE*

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

*FINAL LOT*

A minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the tests for bovine serum albumin and, where applicable, for ovalbumin have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

**Thermal stability**. Maintain at least 3 vials of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific mumps antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Ovalbumin**. If the vaccine is produced in chick embryos, it contains not more than 1 µg of ovalbumin per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 3.7 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

*Mumps vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- that the vaccine has been prepared in chick embryos or the type and origin of cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

**01/2020:1356**

# PERTUSSIS VACCINE (ACELLULAR, COMPONENT, ADSORBED)

## Vaccinum pertussis sine cellulis ex elementis praeparatum adsorbatum

DEFINITION

Pertussis vaccine (acellular, component, adsorbed) is a preparation of individually prepared and purified antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains either pertussis toxoid (chemically detoxified pertussis toxin) or a pertussis toxin-like protein free from toxic properties, produced by expression of a genetically modified form of the corresponding gene. The vaccine may also contain filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The latter 2 antigens may be co-purified. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Where a genetically modified form of *B. pertussis* is used, production consistency and genetic stability shall be established in conformity with the requirements of the monograph *Products of recombinant DNA technology (0784)*.

*Reference vaccine.* A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference vaccine is preferably stabilised by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

*CHARACTERISATION OF COMPONENTS*

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently individual components that comply with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

**Adenylate cyclase**. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

**Tracheal cytotoxin**. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (*2.2.29*).

**Absence of residual dermonecrotic toxin**. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 mL, the amount of component or antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 h. No dermonecrotic reaction is demonstrable.

**Specific properties**. The components of the vaccine are analysed by one or more of the methods shown below in order to determine their identity and specific properties (activity per unit amount of protein) in comparison with reference preparations.

*Pertussis toxin.* Chinese hamster ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP-ribosyl transferase activity using transducin as the acceptor.

*Filamentous haemagglutinin.* Haemagglutination and inhibition by a specific antibody.

*Pertactin, fimbrial-2 and fimbrial-3 antigens.* Reactivity with specific antibodies.

*Pertussis toxoid.* The toxoid induces in animals the production of antibodies capable of inhibiting all the properties of pertussis toxin.

*PURIFIED COMPONENTS*

Production of each component is based on a seed-lot system. The seed cultures from which toxin is prepared are managed to conserve or, where necessary, restore toxinogenicity by deliberate selection.

None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed lots and inocula may contain blood or blood products of animal origin.

Pertussis toxin and, where applicable, filamentous haemagglutinin and pertactin are purified and, after appropriate characterisation, detoxified using suitable chemical reagents. The detoxification process for the pertussis toxin is validated to demonstrate its ability to consistently produce antigens that are immunogenic and stably detoxified. Other components such as fimbrial-2 and fimbrial-3 antigens are purified either separately or together, characterised and shown to be free from toxic substances. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins (*2.6.14*) is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied for the individual components are such that the final vaccine contains less than 100 IU per single human dose.

Before detoxification, the purity of the components is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) or liquid chromatography. SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only purified components that comply with the following requirements may be used in the preparation of the final bulk vaccine.

**Sterility** (*2.6.1*). Carry out the test for sterility using for each medium a quantity of purified component equivalent to not less than 100 doses.

**Residual pertussis toxin** (*2.6.33*). The limit is approved by the competent authority.

**Residual detoxifying agents and other reagents**. The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

**Antigen content**. Determine the antigen content by a suitable immunochemical method (*2.7.1*) and protein nitrogen by sulfuric acid digestion (*2.5.9*) or another suitable method. The ratio of antigen content to protein nitrogen is within the limits established for the product.

*FINAL BULK VACCINE*

The vaccine is prepared by adsorption of suitable quantities of purified components, separately or together, onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for antimicrobial preservative and free formaldehyde, and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 10 g/L solution; maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. Examined by a suitable immunochemical method (*2.7.1*), the clear supernatant reacts with specific antisera to the components stated on the label.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

LABELLING

The label states:

- the names and amounts of the antigenic components present in the vaccine;
- where applicable, that the vaccine contains a pertussis toxin-like protein produced by genetic modification;
- the name and amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2020:1595**

# PERTUSSIS VACCINE (ACELLULAR, CO-PURIFIED, ADSORBED)

## Vaccinum pertussis sine cellulis copurificatum adsorbatum

DEFINITION

Pertussis vaccine (acellular, co-purified, adsorbed) is a preparation of antigenic components of *Bordetella pertussis* adsorbed on a mineral carrier such as aluminium hydroxide or hydrated aluminium phosphate.

The vaccine contains an antigenic fraction purified without separation of the individual components. The antigenic fraction is composed of pertussis toxoid (chemically detoxified pertussis toxin), filamentous haemagglutinin, pertactin (a 69 kDa outer-membrane protein) and other defined components of *B. pertussis* such as fimbrial-2 and fimbrial-3 antigens. The antigenic composition and characteristics are based on evidence of protection and freedom from unexpected reactions in the target group for which the vaccine is intended.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

*Reference vaccine.* A batch of vaccine shown to be effective in clinical trials or a batch representative thereof is used as a reference vaccine. For the preparation of a representative batch, strict adherence to the production process used for the batch tested in clinical trials is necessary. The reference

vaccine is preferably stabilised, by a method that has been shown to have no significant effect on the assay procedure when the stabilised and non-stabilised batches are compared.

*CHARACTERISATION OF COMPONENTS*

During development of the vaccine, the production process shall be validated to demonstrate that it yields consistently an antigenic fraction that complies with the following requirements; after demonstration of consistency, the tests need not be applied routinely to each batch.

**Adenylate cyclase**. Not more than 500 ng in the equivalent of 1 dose of the final vaccine, determined by immunoblot analysis or another suitable method.

**Tracheal cytotoxin**. Not more than 2 pmol in the equivalent of 1 dose of the final vaccine, determined by a suitable method such as a biological assay or liquid chromatography (*2.2.29*).

**Absence of residual dermonecrotic toxin**. Inject intradermally into each of 3 unweaned mice, in a volume of 0.1 mL, the amount of antigenic fraction equivalent to 1 dose of the final vaccine. Observe for 48 h. No dermonecrotic reaction is demonstrable.

**Specific properties**. The antigenic fraction is analysed by one or more of the methods shown below in order to determine the identity and specific properties (activity per unit amount of protein) of its components in comparison with reference preparations.

*Pertussis toxin*. Chinese hamster ovary (CHO) cell-clustering effect and haemagglutination as *in vitro* methods; lymphocytosis-promoting activity and insulin secretory activity as *in vivo* methods. The toxin shows ADP-ribosyl transferase activity using transducin as the acceptor.

*Filamentous haemagglutinin*. Haemagglutination and inhibition by a specific antibody.

*Pertactin, fimbrial-2 and fimbrial-3 antigens*. Reactivity with specific antibodies.

*Pertussis toxoid*. The toxoid induces in animals the production of antibodies capable of inhibiting all the properties of pertussis toxin.

*PURIFIED ANTIGENIC FRACTION*

Production of the antigenic fraction is based on a seed-lot system. The seed cultures are managed to conserve or, where necessary, restore toxinogenicity by deliberate selection.

None of the media used at any stage contains blood or blood products of human origin. Media used for the preparation of seed batches and inocula may contain blood or blood products of animal origin.

The antigenic fraction is purified and, after appropriate characterisation, detoxified using suitable reagents. The detoxification process for the pertussis toxin is validated to demonstrate its ability to consistently produce antigens that are immunogenic and stably detoxified. The purification procedure is validated to demonstrate appropriate clearance of substances used during culture or purification.

The content of bacterial endotoxins (*2.6.14*) is determined to monitor the purification procedure and to limit the amount in the final vaccine. The limits applied are such that the final vaccine contains not more than 100 IU per single human dose.

Before detoxification, the purity of the antigenic fraction is determined by a suitable method such as polyacrylamide gel electrophoresis (PAGE) or liquid chromatography. SDS-PAGE or immunoblot analysis with specific monoclonal or polyclonal antibodies may be used to characterise subunits. Requirements are established for each individual product.

Only a purified antigenic fraction that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Sterility** (*2.6.1*). Carry out the test for sterility using for each medium a quantity of purified antigenic fraction equivalent to not less than 100 doses of the final vaccine.

**Residual pertussis toxin** (*2.6.33*). The limit is approved by the competent authority.

**Residual detoxifying agents and other reagents**. The content of residual detoxifying agents and other reagents is determined and shown to be below approved limits unless validation of the process has demonstrated acceptable clearance.

**Antigen content**. Determine the complete quantitative antigen composition of the antigenic fraction by suitable immunochemical methods (*2.7.1*) and protein nitrogen by sulfuric acid digestion (*2.5.9*) or another suitable method. The ratio of total antigen content to protein nitrogen is within the limits established for the product.

*FINAL BULK VACCINE*

The vaccine is prepared by adsorption of a suitable quantity of the antigenic fraction onto aluminium hydroxide or hydrated aluminium phosphate. A suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended content.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided the tests for antimicrobial preservative and free formaldehyde, and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

Subject the vaccine to a suitable desorption procedure such as the following: dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 10 g/L solution; maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. Examined by a suitable immunochemical method (*2.7.1*), the clear supernatant reacts with specific antisera to the components in the vaccine.

TESTS

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of pertussis vaccine (acellular) (*2.7.16*). The vaccine complies with the limit approved for the particular product.

LABELLING

The label states:

- the names and amounts of the antigenic components present in the vaccine;
- the name and amount of the adsorbent;
- that the vaccine must be shaken before use;

– that the vaccine is not to be frozen.

**01/2010:0161**
**corrected 7.2**

# PERTUSSIS VACCINE (WHOLE CELL, ADSORBED)

## Vaccinum pertussis ex cellulis integris adsorbatum

DEFINITION

Pertussis vaccine (whole cell, adsorbed) is a sterile suspension of inactivated whole cells of one or more strains of *Bordetella pertussis*, treated to minimise toxicity and retain potency. The vaccine contains a mineral adsorbent such as hydrated aluminium phosphate or aluminium hydroxide.

PRODUCTION

*GENERAL PROVISIONS*

The production process shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man.

Levels of pertussis toxin, active heat-labile toxin (dermonecrotic toxin) or tracheal cytotoxin must be comparable to the levels present in the vaccine of proven clinical efficacy and safety in man and be approved by the competent authority.

*CHOICE OF VACCINE STRAIN*

The vaccine consists of a mixture of one or more strains of *B. pertussis*. Strains of *B. pertussis* used in preparing vaccines are well characterised and chosen in such a way that the final vaccine contains predominantly phase I cells that display fimbriae 2 and 3, as determined by an agglutination test or other suitable immunochemical method (*2.7.1*).

*SEED LOTS*

The production of pertussis vaccine is based on a seed-lot system.

The strains of *B. pertussis* used are identified by a full historical record, including information on the origin of the strain and its subsequent manipulation, characteristics on isolation, and particularly on all tests carried out periodically to verify the strain's characters.

The media chosen for growing *B. pertussis* are carefully selected and enable the micro-organism to retain phase I characteristics.

When animal blood or animal blood products are used, they are removed by washing the harvested bacteria.

Human blood or human blood products are not used in any culture media for propagating bacteria, either for seed or for vaccine.

*PROPAGATION AND HARVEST*

Each strain is grown separately from the working seed lot.

Cultures are checked at different stages of fermentation (subcultures and main culture) for purity, identity, cell opacity and pH. Unsatisfactory cultures must be discarded.

Production cultures are shown to be consistent in respect of growth rate, pH and yield of cells or cell products.

The bacteria are harvested and may be washed to remove substances derived from the medium and suspended in a 9 g/L solution of sodium chloride or other suitable isotonic solution.

*MONOVALENT CELL HARVEST*

Consistency of production is monitored in respect of growth rate, pH, yield and demonstration of characteristics of phase I organisms in the culture, such as presence of fimbriae 2 and 3 and haemolytic activity. Single harvests are not used for the final bulk vaccine unless they have been shown to contain *B. pertussis* cells with the same characteristics with regard to growth and agglutinogens as the parent strain, and to be free from contaminating bacteria and fungi.

Only a monovalent harvest that complies with the following requirements may be used in further production.

**Purity**. Samples of single harvests taken before inactivation are examined by microscopy of Gram-stained smears or by inoculation into appropriate culture media or by another suitable procedure.

**Opacity**. The opacity of each single harvest is measured not later than 2 weeks after harvest and before the bacterial suspension has been subjected to any process capable of altering its opacity, by comparison with the International Reference Preparation of Opacity, and used as the basis of calculation for subsequent stages in vaccine preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

A spectrophotometric method validated against the opacity reference preparation may be used and absorbance may, for example, be measured at 600 nm (*2.2.25*).

*INACTIVATION AND DETOXIFICATION OF B. PERTUSSIS SUSPENSION*

Inactivation is initiated as soon as possible after taking samples of single harvests for purity control and opacity measurement. The bacteria are killed and detoxified in controlled conditions by means of a suitable chemical agent or by heating or by a combination of these methods. The suspension is maintained at 5 ± 3 °C for a suitable period to diminish its toxicity.

Only an inactivated monovalent cell bulk that complies with established specifications for the following tests may be used in the preparation of the final bulk.

**Residual live *B. pertussis***. Inactivation of the whole cells of *B. pertussis* is verified by a suitable culture medium.

**Pertussis toxin**. Presence of pertussis toxin is measured by a CHO cell culture assay using a semi-quantitative technique and range determined for the particular product.

**pH** (*2.2.3*): within the range approved for the particular product.

**Identification**: verified by agglutination assay or suitable immunodiffusion assay.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**Opacity**. The opacity of each single harvest is measured in the final phase, at the end of the main fermentation process, by comparison with the International Reference Preparation of Opacity. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization. The absorbance, for example measured at 600 nm (*2.2.25*), is within the range approved for the particular product.

*FINAL BULK*

The final bulk vaccine is prepared by aseptically mixing suitable quantities of the inactivated single harvests.

If 2 or more strains of *B. pertussis* are used, the composition of consecutive lots of the final bulk vaccine shall be consistent with respect to the proportion of each strain as measured in opacity units. The bacterial concentration of the final bulk vaccine does not exceed that corresponding to an opacity of 20 IU per single human dose. The opacity measured on the single harvests is used to calculate the bacterial concentration in the final bulk. A mineral adsorbent such as hydrated aluminium phosphate or aluminium hydroxide is added to the cell suspension. Suitable antimicrobial preservatives may be added. Phenol is not used as a preservative.

Only a final bulk that complies with the following requirements may be used in the preparation of the final lot.

**Fimbriae**. Each bulk is examined, before adsorbent is added, for the presence of fimbriae 2 and 3 to ensure that appropriate expression has occurred during bacterial growth.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

*FINAL LOT*

The final bulk is mixed to homogeneity and filled aseptically into suitable containers.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the tests for specific toxicity, free formaldehyde and antimicrobial preservative and the determination of potency have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge to obtain a bacterial precipitate. Identity of pertussis vaccine is based on an immunological reaction, for example agglutination of the resuspended bacteria with a specific anti-pertussis serum or another suitable immunochemical method (*2.7.1*).

TESTS

**Specific toxicity**. Use not fewer than 5 healthy mice each weighing 14-16 g for the vaccine group and for the saline control. Use mice of the same sex or distribute males and females equally between the groups. Inject each mouse of the vaccine group intraperitoneally with 0.5 mL, containing a quantity of the vaccine equivalent to not less than half the single human dose. Inject each mouse of the control group with 0.5 mL of a 9 g/L sterile solution of *sodium chloride R*, preferably containing, where applicable, the same amount of antimicrobial preservative as that injected with the vaccine. Weigh the groups of mice immediately before the injection and 72 h and 7 days after the injection. The vaccine complies with the test if: (a) at the end of 72 h the average weight of the group of vaccinated mice is not less than that preceding the injection; (b) at the end of 7 days the average increase in mass per vaccinated mouse is not less than 60 per cent of that per control mouse; and (c) not more than 5 per cent of the vaccinated mice die during the test. If the test is carried out using 5 mice and 1 vaccinated mouse dies, the test may be repeated using 15 mice and the results of both tests combined.

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L, where applicable.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

Carry out the assay of pertussis vaccine (whole cell) (*2.7.7*).

The estimated potency is not less than 4.0 IU per single human dose and the lower confidence limit ($P = 0.95$) of the estimated potency is not less than 2.0 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose;
- the method used for inactivation;
- the name and the amount of the adsorbent;
- that the vaccine must be shaken before use;
- that the vaccine is not to be frozen.

**01/2019:2150**

# PNEUMOCOCCAL POLYSACCHARIDE CONJUGATE VACCINE (ADSORBED)

## Vaccinum pneumococcale polysaccharidicum coniugatum adsorbatum

DEFINITION

Pneumococcal polysaccharide conjugate vaccine (adsorbed) is a sterile suspension of purified capsular polysaccharides obtained from *Streptococcus pneumoniae* serotypes individually conjugated to a carrier protein. The carrier protein used may vary for the various polysaccharide conjugates contained in a multivalent vaccine. The vaccine may be adsorbed on a suitable adjuvant or adsorbant.

Each serotype, produced from suitable pathogenic strains of *S. pneumoniae*, is grown in an appropriate medium.

The individual polysaccharides are purified through suitable purification methods (for example centrifugation, precipitation, ultrafiltration and column chromatography).

Each polysaccharide has a defined composition and a defined molecular size range.

The choice of polysaccharide depends on the frequency of the serotypes responsible for acute pathologies and their geographical distribution. The vaccine contains immunochemically different capsular polysaccharides.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently *S. pneumoniae* conjugate vaccines of adequate safety and immunogenicity in man. The production of polysaccharides and of the carrier(s) is based on a seed-lot system.

During development studies and wherever revalidation is necessary, a test for pyrogens in rabbits (*2.6.8*) is carried out. The vaccine is shown to be acceptable with respect to absence of pyrogenic activity.

During development studies and whenever revalidation of the manufacturing process is necessary, it shall be demonstrated by tests in animals that the vaccine consistently induces a T-cell-dependent B-cell immune response.

The stability of the conjugated bulk and/or final lot and pneumococcal saccharide is evaluated using suitable indicator tests. Such tests may include determination of molecular size, quantification of saccharide content and free polysaccharide content in the conjugate.

*BACTERIAL SEED LOTS*

The bacterial strains used for master seed lots shall be identified by historical records that include information on their origin and the tests used to characterise the strain.

Cultures obtained from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

*PNEUMOCOCCAL POLYSACCHARIDES*

Each strain of *S. pneumoniae* serotypes is individually grown in a liquid medium that does not contain high-molecular-mass polysaccharides; if any ingredient of the medium contains blood-group substances, the process is validated to demonstrate that after the purification step they are no longer detectable. The bacterial purity of the culture is verified by suitable methods. The culture is then inactivated. Each polysaccharide is separated from the liquid culture and purified by suitable methods. Volatile matter, including water, in the purified polysaccharide is determined by a suitable method such as thermogravimetry (*2.2.34*), semi-micro determination of water (*2.5.12*) or, where applicable, determination of solvent and/or alcohol content by spectrometry. The values are used to calculate the results of other chemical tests with reference to the dried substance, as prescribed below.

Only polysaccharides that comply with the following requirements may be used in the preparation of the conjugate.

**Identification**. Each polysaccharide is identified by an immunochemical method (*2.7.1*) or other suitable methods, for example $^{1}$H nuclear magnetic resonance spectrometry (*2.2.33*).

**Protein** (*2.5.16*): depending on the serotype used, not more than the limit approved for the product, calculated with reference to the dried substance.

**Nucleic acid** (*2.5.17*): depending on the serotype used, not more than the limit approved for the product, calculated with reference to the dried substance.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each pneumococcal polysaccharide. Each batch must be shown to comply with this limit.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU per microgram of polysaccharide.

**Residual reagents**. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of polysaccharide must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on polysaccharides may be omitted.

**Water**. Where applicable, the values are within the limits approved for each serotype, determined by a suitable method.

Depending on the chemical composition of a pneumococcal polysaccharide serotype, not all of the following tests may be applicable. The values are within the limits approved. Suitable limits for some pneumococcal polysaccharide serotypes are given in the monograph *Pneumococcal polysaccharide vaccine (0966)*.

**Total nitrogen** (*2.5.9*).

**Phosphorus** (*2.5.18*).

**Uronic acids** (*2.5.22*).

**Hexosamines** (*2.5.20*).

**Methylpentoses** (*2.5.21*).

***O*-Acetyl groups** (*2.5.19*).

*MODIFIED PNEUMOCOCCAL POLYSACCHARIDES*

Before conjugation, the polysaccharide can be depolymerised by chemical or mechanical means followed by a concentration step to obtain polysaccharides of a desired molecular size range. Polysaccharides or depolymerised polysaccharides are modified by an activation process.

Only modified polysaccharides that comply with the following requirements may be used in the preparation of the conjugate.

**Molecular-size or molecular-mass distribution**. In the case of a size-reduced modified pneumococcal polysaccharide, the molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each modified pneumococcal polysaccharide. Each batch must be shown to comply with this limit.

**Degree of oxidation**. Where applicable, the degree of oxidation is represented by the ratio of moles of saccharide repeat unit per mole of aldehyde and determined by a suitable method. The values are within the limits approved for each serotype.

*CARRIER PROTEIN*

The production and characteristics of the carrier proteins are described in general chapter *5.2.11. Carrier proteins for the production of conjugated polysaccharide vaccines for human use*. Only a carrier protein that complies with the requirements of this chapter may be used in the preparation of the conjugate.

*MONOVALENT BULK CONJUGATE*

The conjugate is obtained by the covalent binding of activated polysaccharides to the appropriate carrier protein.

The conjugate purification procedures are designed to remove residual reagents used for conjugation. The removal of residual reagents is confirmed by suitable tests or by validation of the purification process.

Only a bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine. For each test, limits of acceptance are established and each batch of conjugate must be shown to comply with these limits.

**Saccharide**. The polysaccharide content is determined by a suitable physical or chemical method or by an immunochemical method (*2.7.1*). The value complies with the requirement approved for each serotype.

**Protein**. The protein content is determined by a suitable physical or chemical method (for example, *2.5.16*). The value complies with the requirement approved for each serotype.

**Saccharide-to-protein ratio**. Determine the saccharide-to-protein ratio by calculation. The value complies with the requirement approved for each serotype.

**Free saccharide**. Unbound polysaccharide is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic chromatography, ultrafiltration, or other validated methods. A value consistent with adequate immunogenicity as shown in clinical trials is established for each serotype and each lot must be shown to comply with this limit.

**Free carrier protein**. Determine the content by a suitable method, either directly or by deriving the content by calculation from the results of other tests. The value complies with the requirement approved for each serotype.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for the bulk conjugate of each polysaccharide. Each batch must be shown to comply with this limit.

**Residual reagents**. Where applicable, suitable tests are carried out to determine residues of reagents used during inactivation and purification. An acceptable value for each reagent is established for the particular product and each batch of conjugate must be shown to comply with this limit. Where validation studies have demonstrated removal of residual reagents, the test on conjugate polysaccharides may be omitted.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium or the equivalent of 100 doses for each medium, whichever is less.

**Bacterial endotoxins** (*2.6.14*): less than 0.75 IU per microgram of polysaccharide.

*ADSORBED MONOVALENT BULK CONJUGATE*

An aluminium-containing adjuvant may be added to each of the monovalent bulk conjugates prior to formulation of the final bulk. Once the conjugates are adsorbed on a sterile adjuvant, sterility is assured by aseptic processing.

Only an adsorbed monovalent bulk conjugate that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. Each adsorbed polysaccharide conjugate is identified by an immunochemical method (*2.7.1*) or other suitable methods.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL or the equivalent of 100 doses for each medium, whichever is less.

**Saccharide**. The polysaccharide content is determined by a suitable physical or chemical method or by an immunochemical method (*2.7.1*). The value complies with the requirement approved for each serotype.

**Free saccharide**. Centrifuge the adsorbed monovalent bulk conjugate. In the supernatant the unbound polysaccharide is determined after removal of the conjugate, for example by anion-exchange, size-exclusion or hydrophobic liquid chromatography, ultrafiltration, or other validated methods. An acceptable value consistent with adequate immunogenicity as shown in clinical trials is established for each serotype and each lot must be shown to comply with this limit.

**Degree of adsorption**. The degree of adsorption of each polysaccharide conjugate is assessed.

*FINAL BULK VACCINE*

A final bulk vaccine may be formulated from the individually adsorbed monovalent bulk conjugates, or from the mixture of the monovalent bulk conjugates that is adsorbed on an aluminium-containing adjuvant.

Where a final bulk vaccine is formulated as a release intermediate, it complies with the following requirements and is within the limits approved for the particular product.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL or the equivalent of 100 doses for each medium, whichever is less.

*FINAL LOT*

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION

Each polysaccharide present in the vaccine is identified by a suitable immunochemical method (*2.7.1*).

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 12.5 IU per single human dose, unless otherwise justified and authorised.

ASSAY

**Saccharide content**. The polysaccharide content for each serotype is determined by a suitable immunochemical method (for example, nephelometry assay or enzyme-linked immunosorbent assay (ELISA)). The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity stated on the label for each polysaccharide. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated content.

LABELLING

The label states:

- the pneumococcal serotype and carrier protein present in each single human dose;
- the number of micrograms of each polysaccharide per single human dose;
- the type and nominal amount of carrier protein per single human dose;
- if applicable, the name and amount of adsorbent;
- if applicable, that the vaccine must be shaken before use;
- if applicable, that the vaccine must not be frozen.

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# PNEUMOCOCCAL POLYSACCHARIDE VACCINE

## Vaccinum pneumococcale polysaccharidicum

DEFINITION

Pneumococcal polysaccharide vaccine consists of a mixture of equal parts of purified capsular polysaccharide antigens prepared from suitable pathogenic strains of *Streptococcus pneumoniae* whose capsules have been shown to be made up of polysaccharides that are capable of inducing satisfactory levels of specific antibodies in man. It contains the 23 immunochemically different capsular polysaccharides listed in Table 0966.-1.

The vaccine is a clear, colourless liquid.

PRODUCTION

Production of the vaccine is based on a seed-lot system for each type. The production method shall have been shown to yield consistently pneumococcal polysaccharide vaccines of adequate safety and immunogenicity in man.

*MONOVALENT BULK POLYSACCHARIDES*

The bacteria are grown in a suitable liquid medium that does not contain blood-group substances or high-molecular-mass polysaccharides. The bacterial purity of the culture is verified and the culture is inactivated with phenol. Impurities are removed by such techniques as fractional precipitation, enzymatic digestion and ultrafiltration. The polysaccharide is obtained by fractional precipitation, washed, and dried in a vacuum to a residual moisture content shown to be favourable to the stability of the polysaccharide. The residual moisture content is determined by drying under reduced pressure over diphosphorus pentoxide or by thermogravimetric analysis and the value obtained is used to calculate the results of the tests shown below with reference to the dried substance. The monovalent bulk polysaccharide is stored at a suitable temperature in conditions that avoid the uptake of moisture.

Only a monovalent bulk polysaccharide that complies with the following requirements may be used in the preparation of the final bulk vaccine. Percentage contents of components, determined by the methods prescribed below, are shown in Table 0966.-1.

**Protein** (*2.5.16*).

**Nucleic acids** (*2.5.17*).

**Total nitrogen** (*2.5.9*).

**Phosphorus** (*2.5.18*).

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for each purified polysaccharide. Each batch must be shown to comply with this limit.

**Uronic acids** (*2.5.22*).

**Hexosamines** (*2.5.20*).

**Methylpentoses** (*2.5.21*).

***O*-Acetyl groups** (*2.5.19*).

**Identification** (*2.7.1*). Confirm the identity of the monovalent bulk polysaccharide by double immunodiffusion or electroimmunodiffusion (except for polysaccharides 7F, 14 and 33F), using specific antisera.

**Specificity**. No reaction occurs when the antigens are tested against all the antisera specific for the other polysaccharides of the vaccine, including factor sera for distinguishing types within groups. The polysaccharides are tested at a concentration of 50 µg/mL using a method capable of detecting 0.5 µg/mL.

*FINAL BULK VACCINE*

The final bulk vaccine is obtained by aseptically mixing the different polysaccharide powders. The uniform mixture is aseptically dissolved in a suitable isotonic solution so that one human dose of 0.50 mL contains 25 µg of each polysaccharide. An antimicrobial preservative may be added. The solution is sterilised by filtration through a bacteria-retentive filter.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for phenol and for antimicrobial preservative have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot. When consistency of production has been established on a suitable number of consecutive batches, the assay may be replaced by a qualitative test that identifies each polysaccharide, provided that an assay has been performed on each monovalent bulk polysaccharide used in the preparation of the final lot.

Table 0966.-1 – *Percentage contents of components of monovalent bulk polysaccharides*

| Molecular type* | Protein | Nucleic acids | Total nitrogen | Phosphorus | Uronic acids | Hexosamines | Methyl-pentoses | *O*-acetyl Groups |
|---|---|---|---|---|---|---|---|---|
| 1 | ≤ 2 | ≤ 2 | 3.5-6 | 0-1.5 | ≥ 45 | | | ≥ 1.8 |
| 2 | ≤ 2 | ≤ 2 | 0-1 | 0-1.0 | ≥ 15 | | ≥ 38 | |
| 3 | ≤ 5 | ≤ 2 | 0-1 | 0-1.0 | ≥ 40 | | | |
| 4 | ≤ 3 | ≤ 2 | 4-6 | 0-1.5 | | ≥ 40 | | |
| 5 | ≤ 7.5 | ≤ 2 | 2.5-6.0 | ≤ 2 | ≥ 12 | ≥ 20 | | |
| 6B | ≤ 2 | ≤ 2 | 0-2 | 2.5-5.0 | | | ≥ 15 | |
| 7F | ≤ 5 | ≤ 2 | 1.5-4.0 | 0-1.0 | | | ≥13 | |
| 8 | ≤ 2 | ≤ 2 | 0-1 | 0-1.0 | ≥ 25 | | | |
| 9N | ≤ 2 | ≤ 1 | 2.2-4 | 0-1.0 | ≥ 20 | ≥ 28 | | |
| 9V | ≤ 2 | ≤ 2 | 0.5-3 | 0-1.0 | ≥ 15 | ≥ 13 | | |
| 10A | ≤ 7 | ≤ 2 | 0.5-3.5 | 1.5-3.5 | | ≥ 12 | | |
| 11A | ≤ 3 | ≤ 2 | 0-2.5 | 2.0-5.0 | | | | ≥ 9 |
| 12F | ≤ 3 | ≤ 2 | 3-5 | 0-1.0 | | ≥ 25 | | |
| 14 | ≤ 5 | ≤ 2 | 1.5-4 | 0-1.0 | | ≥ 20 | | |
| 15B | ≤ 3 | ≤ 2 | 1-3 | 2.0-4.5 | | ≥ 15 | | |
| 17A or17F | ≤ 2 | ≤ 2 | 0-1.5 | 0-3.5 | | | ≥ 20 | |
| 18C | ≤ 3 | ≤ 2 | 0-1 | 2.4-4.9 | | | ≥ 14 | |
| 19A | ≤ 2 | ≤ 2 | 0.6-3.5 | 3.0-7.0 | | ≥ 12 | ≥ 20 | |
| 19F | ≤ 3 | ≤ 2 | 1.4-3.5 | 3.0-5.5 | | ≥ 12.5 | ≥ 20 | |
| 20 | ≤ 2 | ≤ 2 | 0.5-2.5 | 1.5-4.0 | | ≥ 12 | | |
| 22F | ≤ 2 | ≤ 2 | 0-2 | 0-1.0 | ≥ 15 | | ≥ 25 | |
| 23F | ≤ 2 | ≤ 2 | 0-1 | 3.0-4.5 | | | ≥ 37 | |
| 33F | ≤ 2.5 | ≤ 2 | 0-2 | 0-1.0 | | | | |

* The different types are indicated using the Danish nomenclature.

IDENTIFICATION

The assay serves also to identify the vaccine.

TESTS

**pH** (*2.2.3*). The pH of the vaccine is 4.5 to 7.4.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Phenol** (*2.5.15*). Not more than 2.5 g/L.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Pyrogens** (*2.6.8*). It complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 mL of a dilution of the vaccine containing 2.5 µg/mL of each polysaccharide.

ASSAY

Determine the content of each polysaccharide by a suitable immunochemical method (*2.7.1*), using antisera specific for each polysaccharide contained in the vaccine, including factor sera for types within groups, and purified polysaccharides of each type as standards.

The vaccine contains not less than 70 per cent and not more than 130 per cent of the quantity stated on the label for each polysaccharide. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated content.

LABELLING

The label states:

- the number of micrograms of each polysaccharide per single human dose;
- the total amount of polysaccharide in the container.

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# POLIOMYELITIS VACCINE (INACTIVATED)

## Vaccinum poliomyelitidis inactivatum

DEFINITION

Poliomyelitis vaccine (inactivated) is a liquid preparation of suitable strains of human poliovirus types 1, 2 and 3 grown in suitable cell cultures and inactivated by a validated method. It is a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production method shall have been shown to yield consistently vaccines of acceptable safety and immunogenicity in man.

Production of the vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary, secondary or tertiary monkey kidney cells are used, production complies with the requirements indicated below.

Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than was used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in a human diploid cell line (*5.2.3*), in a continuous cell line (*5.2.3*) or in primary, secondary or tertiary monkey kidney cells.

**Primary, secondary or tertiary monkey kidney cells**. The following special requirements for the substrate for virus propagation apply to primary, secondary or tertiary monkey kidney cells.

*Monkeys used in the preparation of kidney cell cultures for production and control of the vaccine.* The animals used are of a species approved by the competent authority, in good health and, unless otherwise justified and authorised, have not been previously employed for experimental purposes. Kidney cells used for vaccine production and control are derived from monitored, closed colonies of monkeys bred in captivity, not from animals caught in the wild; a previously approved seed lot prepared using virus passaged in cells from wild monkeys may, subject to approval by the competent authority, be used for vaccine production if historical data on safety justify this.

*Monitored, closed colonies of monkeys.* The monkeys are kept in groups in cages. Freedom from extraneous agents is achieved by the use of animals maintained in closed colonies that are subject to continuous and systematic veterinary and laboratory monitoring for the presence of infectious agents. The supplier of animals is certified by the competent authority. Each monkey is tested serologically at regular intervals during a quarantine period of not less than 6 weeks imposed before entering the colony, and then during its stay in the colony.

The monkeys used are shown to be tuberculin-negative and free from antibodies to simian virus 40 (SV40) and simian immunodeficiency virus. The blood sample used in testing for SV40 antibodies must be taken as close as possible to the time of removal of the kidneys. If *Macaca* sp. monkeys are used for production, the monkeys are also shown to be free from antibodies to herpesvirus B (cercopithecine herpesvirus 1) infection. Human herpesvirus 1 has been used as an indicator for freedom from herpesvirus B antibodies on account of the danger of handling herpesvirus B (cercopithecine herpesvirus 1).

Monkeys from which kidneys are to be removed are thoroughly examined, particularly for evidence of tuberculosis and herpesvirus B (cercopithecine herpesvirus 1) infection. If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it is not to be used nor are any of the remaining monkeys of the group concerned unless it is evident that their use will not impair the safety of the product.

All the operations described in this section are conducted outside the area where the vaccine is produced.

*Monkey cell cultures for vaccine production.* Kidneys that show no pathological signs are used for preparing cell cultures. Each group of cell cultures derived from a single monkey forms a separate production cell culture giving rise to a separate single harvest.

The primary monkey kidney cell suspension complies with the test for mycobacteria (*2.6.2*); disrupt the cells before carrying out the test.

If secondary or tertiary cells are used, it shall be demonstrated by suitable validation tests that cell cultures beyond the passage level used for production are free from tumorigenicity.

*SEED LOTS*

Each of the 3 strains of poliovirus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Only a working seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. Each working seed lot is identified as human poliovirus types 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

**Virus concentration**. The virus concentration of each working seed lot is determined to define the quantity of virus to be used for inoculation of production cell cultures.

**Extraneous agents**. The working seed lot complies with the requirements for seed lots for virus vaccines (*2.6.16*). In addition, if primary, secondary or tertiary monkey kidney cells have been used for isolation of the strain, measures are taken to ensure that the strain is not contaminated with simian viruses such as simian immunodeficiency virus, simian virus 40, filoviruses and herpesvirus B (cercopithecine herpesvirus 1). A working seed lot produced in primary, secondary or tertiary monkey kidney cells complies with the requirements given below under Virus propagation and harvest for single harvests produced in such cells.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell bank and cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Approved animal serum (but not human serum) may be used in the cell culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells); where continuous cell lines in a fermenter are used for production, $200 \times 10^6$ cells are set aside to prepare control cells; where primary, secondary or tertiary monkey kidney cells are used for production, a cell sample equivalent to at least 500 mL of the cell suspension, at the concentration employed for vaccine production, is taken to prepare control cells.

Only a single harvest that complies with the following requirements may be used in the preparation of the vaccine. The tests for identification and bacterial and fungal contamination may be carried out instead on the purified, pooled monovalent harvest. After demonstration of consistency of production at the stage of the single harvest, the test for virus concentration may be carried out instead on the purified, pooled monovalent harvest.

**Control cells**. The control cells of the production cell culture comply with a test for identification (if a cell-bank system is used for production) and with the requirements for extraneous agents (*2.6.16*; where primary, secondary or tertiary monkey kidney cells are used, the tests in cell cultures are carried out as shown below under Test in rabbit kidney cell cultures and Test in cercopithecus kidney cell cultures).

*Test in rabbit kidney cell cultures*. Test a sample of at least 10 mL of the pooled supernatant fluid from the control cultures for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures. The dilution of supernatant in the nutrient medium is not greater than 1/4 and the area of the cell layer is at least 3 $cm^2$ per millilitre of inoculum. Set aside one or more containers of each batch of cells with the same medium as non-inoculated control cells. Incubate the cultures at 37 °C and observe for at least 2 weeks. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

*Test in cercopithecus kidney cell cultures*. Test a sample of at least 10 mL of the pooled supernatant fluid from the control cultures for the absence of SV40 virus and other extraneous agents by inoculation onto cell cultures prepared from the kidneys of cercopithecus monkeys, or other cells shown to be at least as sensitive for SV40, by the method described under Test in rabbit kidney cell cultures. The test is not valid if more than 20 per cent of the control cell cultures are discarded for non-specific, accidental reasons.

**Identification**. The single harvest is identified as containing human poliovirus types 1, 2 or 3 by virus neutralisation in cell cultures using specific antibodies.

**Virus concentration**. The virus concentration of each single harvest is determined by titration of infectious virus in cell cultures.

**Bacterial and fungal contamination**. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). The single harvest complies with the test for mycoplasmas, carried out using 10 mL.

**Test in rabbit kidney cell cultures**. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 mL of the single harvest for the absence of herpesvirus B (cercopithecine herpesvirus 1) and other viruses by inoculation onto rabbit kidney cell cultures as described above for the control cells.

**Test in cercopithecus kidney cell cultures**. Where primary, secondary or tertiary monkey kidney cells are used for production, test a sample of at least 10 mL of the single harvest for the absence of SV40 virus and other extraneous agents. Neutralise the sample by a high-titre antiserum against the specific type of poliovirus. Test the sample in primary cercopithecus kidney cell cultures or cells that have been demonstrated to be at least as susceptible for SV40. Incubate the cultures at 37 °C and observe for 14 days. At the end of this period, make at least one subculture of fluid in the same cell culture system and observe both primary cultures and subcultures for an additional 14 days.

*PURIFICATION AND PURIFIED MONOVALENT HARVEST*

Several single harvests of the same type may be pooled and may be concentrated. The monovalent harvest or pooled monovalent harvest is purified by validated methods. If continuous cell lines are used for production, the purification process shall have been shown to reduce consistently the content of substrate-cell DNA to not more than 100 pg per single human dose.

Only a purified monovalent harvest that complies with the following requirements may be used for the preparation of the inactivated monovalent harvest.

**Identification**. The virus is identified by virus neutralisation in cell cultures using specific antibodies or by determination of D-antigen.

**Virus concentration**. The virus concentration is determined by titration of infectious virus.

**Specific activity**. The ratio of the virus concentration or the D-antigen content, determined by a suitable immunochemical method (*2.7.1*), to the total protein content (specific activity) of the purified monovalent harvest is within the limits approved for the particular product.

*INACTIVATION AND INACTIVATED MONOVALENT HARVEST*

Several purified monovalent harvests of the same type may be mixed before inactivation. To avoid failures in inactivation caused by the presence of virus aggregates, filtration is carried out before and during inactivation; inactivation is started within a suitable period, preferably not more than 24 h and in any case not more than 72 h, of the prior filtration. The virus suspension is inactivated by a validated method that has been shown to inactivate poliovirus without destruction of immunogenicity; during validation studies, an inactivation curve with at least 4 points (for example, time 0 h, 24 h, 48 h and 96 h) is established showing the decrease in concentration of live virus with time. If formaldehyde is used for inactivation, the presence of an excess of formaldehyde at the end of the inactivation period is verified. The inactivation kinetics tests mentioned below are carried out on each batch to ensure consistency of the inactivation process.

Only an inactivated monovalent harvest that complies with the following requirements may be used in the preparation of a trivalent pool of inactivated monovalent harvests or a final bulk vaccine.

**Test for effective inactivation**. After neutralisation of the formaldehyde with sodium bisulfite (where applicable), verify the absence of residual live poliovirus by inoculation

on suitable cell cultures of 2 samples of each inactivated monovalent harvest, corresponding to at least 1500 human doses. Cells used for the test must be of optimal sensitivity regarding residual infectious poliovirus, for example kidney cells from certain monkey species (*Macaca*, *Cercopithecus* or *Papio*), or Hep-2 cells. If other cells are used, they must have been shown to possess at least the same sensitivity as those specified above. Take one sample not later than 3/4 of the way through the inactivation period and the other at the end. Inoculate the samples in cell cultures such that the dilution of vaccine in the nutrient medium is not greater than 1/4 and the area of the cell layer is at least 3 $cm^2$ per millilitre of inoculum. Set aside one or more containers with the same medium as non-inoculated control cells. Observe the cell cultures for at least 3 weeks. Make not fewer than 2 passages from each container, one at the end of the observation period and the other 1 week before; for the passages, use cell culture supernatant and inoculate as for the initial sample. Observe the subcultures for at least 2 weeks. No sign of poliovirus multiplication is present in the cell cultures. At the end of the observation period, test the susceptibility of the cell culture used by inoculation of live poliovirus of the same type as that present in the inactivated monovalent harvest.

**Inactivation kinetics**. Kinetics of inactivation are established and approved by the competent authority. Adequate data on inactivation kinetics are obtained and consistency of the inactivation process is monitored.

**Sterility** (*2.6.1*). The inactivated monovalent harvest complies with the test for sterility, carried out using 10 mL for each medium.

**D-antigen content**. The content of D-antigen determined by a suitable immunochemical method (*2.7.1*) is within the limits approved for the particular preparation.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared directly from the inactivated monovalent harvests of human poliovirus types 1, 2 and 3 or from a trivalent pool of inactivated monovalent harvests. A suitable stabiliser and a suitable antimicrobial preservative may be added.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

*FINAL LOT*

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde and antimicrobial preservative and the *in vivo* assay have been performed with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

The *in vivo* assay may be omitted once it has been demonstrated for a given product and for each poliovirus type that the acceptance criteria for the D-antigen determination are such that it yields the same result as the *in vivo* assay in terms of acceptance or rejection of a batch. This demonstration must include testing of subpotent batches, produced experimentally if necessary, for example by heat treatment or other means of diminishing the immunogenic activity. Where there is a significant change in the manufacturing process of the antigens or their formulation, any impact on the *in vivo* and *in vitro* assays must be evaluated, and the need for revalidation considered.

Provided that the protein content has been determined on the purified monovalent harvests or on the inactivated monovalent harvests and that it has been shown that the content in the final lot will not exceed 10 µg per single human dose, the test for protein content may be omitted on the final lot.

Provided that the test for bovine serum albumin has been performed with satisfactory results on the trivalent pool of inactivated monovalent harvests or on the final bulk vaccine, it may be omitted on the final lot.

## IDENTIFICATION

The vaccine is shown to contain human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) such as the determination of D-antigen by enzyme-linked immunosorbent assay (ELISA).

## TESTS

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The amount is not less than the minimum amount shown to be effective and is not greater than 115 per cent of that stated on the label.

**Protein content** (*2.5.33, Method 2*): maximum 10 µg per single human dose.

**Bovine serum albumin**: maximum 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Sterility** (*2.6.1*). It complies with the test.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU per single human dose.

## ASSAY

**D-antigen content**. As a measure of consistency of production, determine the D-antigen content for human poliovirus types 1, 2 and 3 by a suitable immunochemical method (*2.7.1*) using a reference preparation calibrated in European Pharmacopoeia Units of D-antigen. For each type, the content, expressed with reference to the amount of D-antigen stated on the label, is within the limits approved for the particular product. *Poliomyelitis vaccine (inactivated) BRP* is calibrated in European Pharmacopoeia Units and intended for use in the assay of D-antigen. The European Pharmacopoeia Unit and the International Unit are equivalent.

***In vivo* test**. The vaccine complies with the *in vivo* assay of poliomyelitis vaccine (inactivated) (*2.7.20*).

## LABELLING

The label states:

- the types of poliovirus contained in the vaccine;
- the nominal amount of virus of each type (1, 2 and 3), expressed in European Pharmacopoeia Units of D-antigen, per single human dose;
- the cell substrate used to prepare the vaccine.

01/2020:0215

# POLIOMYELITIS VACCINE (ORAL)

## Vaccinum poliomyelitidis perorale

DEFINITION

Oral poliomyelitis vaccine is a preparation of approved strains of live attenuated poliovirus type 1, 2 or 3 grown in *in vitro* cultures of approved cells, containing either a single type, a combination of type 1 and type 3 or a combination of the 3 types of Sabin strains, presented in a form suitable for oral administration.

The vaccine is a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The vaccine strains and the production method shall have been shown to consistently yield vaccines that are both immunogenic and safe in man.

The production of vaccine is based on a virus seed-lot system. Cell lines are used according to a cell-bank system. If primary monkey kidney cell cultures are used, production complies with the requirements indicated below. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more than 2 passages from the master seed lot.

REFERENCE STANDARDS

The International Standards for poliovirus type 1 (Sabin), poliovirus type 2 (Sabin) for MAPREC (Mutant Analysis by PCR and Restriction Enzyme Cleavage) assays and poliovirus type 3 (Sabin) synthetic DNA for MAPREC assays are suitable for use in the tests for genetic markers and the molecular tests for consistency of production.

Reference preparations of each poliovirus type at the Sabin Original + 2 passage level, namely WHO (SO + 2)/I for type 1 virus, WHO (SO + 2)/II for type 2 virus and WHO (SO + 2)/III for type 3 virus are available for comparison of the *in vivo* neurovirulence with that of homotypic vaccines. Requests for the WHO reference preparations for *in vivo* neurovirulence tests are to be directed to the World Health Organization (WHO), Biologicals, Geneva, Switzerland.

A suitable reference preparation is to be included in each test.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*), in continuous cell lines (*5.2.3*) or in primary monkey kidney cell cultures (including serially passaged cells from primary monkey kidney cells).

**Primary monkey kidney cell cultures**. *The following special requirements for the substrate for virus propagation apply to primary monkey kidney cell cultures.*

*Monkeys used for preparation of primary monkey kidney cell cultures and for testing of virus.* If the vaccine is prepared in primary monkey kidney cell cultures, animals of a species approved by the competent authority, in good health, kept in closed or intensively monitored colonies and not previously employed for experimental purposes shall be used.

The monkeys shall be kept in well-constructed and adequately ventilated animal rooms in cages spaced as far apart as possible. Adequate precautions shall be taken to prevent cross-infection between cages. Not more than 2 monkeys shall be housed per cage and cage-mates shall not be interchanged. The monkeys shall be kept in the country of manufacture of the vaccine in quarantine groups for a period of not less than 6 weeks before use. A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities, and having no contact with other monkeys during the quarantine period. If at any time during the quarantine period the overall death rate of a shipment consisting of one or more groups reaches 5 per cent (excluding deaths from accidents or where the cause was specifically determined not to be an infectious disease), monkeys from that entire shipment shall continue in quarantine from that time for a minimum of 6 weeks. The groups shall be kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the monkeys are used. After the last monkey of a group has been taken, the room that housed the group shall be thoroughly cleaned and decontaminated before being used for a fresh group. If kidneys from near-term monkeys are used, the mother is quarantined for the term of pregnancy.

Monkeys from which kidneys are to be removed shall be anaesthetised and thoroughly examined, particularly for evidence of tuberculosis and cercopithecid herpesvirus 1 (B virus) infection.

If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of a seed lot or vaccine, it shall not be used, nor shall any of the remaining monkeys of the quarantine group concerned be used unless it is evident that their use will not impair the safety of the product.

All the operations described in this section shall be conducted outside the areas where the vaccine is produced.

The monkeys used shall be shown to be free from antibodies to simian virus 40 (SV40), simian immunodeficiency virus and spumaviruses. The blood sample used in testing for SV40 antibodies must be taken as close as possible to the time of removal of the kidneys. If *Macaca* spp. are used for production, the monkeys shall also be shown to be free from antibodies to cercopithecid herpesvirus 1 (B virus). Human herpesvirus has been used as an indicator for freedom from B virus antibodies on account of the danger of handling cercopithecid herpesvirus 1 (B virus). Monkeys used for the production of new seed lots are shown to be free from antibodies to simian cytomegalovirus (sCMV).

*Primary monkey kidney cell cultures for vaccine production.* Kidneys that show no pathological signs are used for preparing cell cultures. If the monkeys are from a colony maintained for vaccine production, serially passaged monkey kidney cell cultures from primary monkey kidney cells may be used for virus propagation, otherwise the monkey kidney cells are not propagated in series. Virus for the preparation of vaccine is grown by aseptic methods in such cultures. If animal serum is used in the propagation of the cells, the maintenance medium after virus inoculation shall contain no added serum.

Each group of cell cultures derived from a single monkey or from foetuses from no more than 10 near-term monkeys is prepared and tested as an individual group.

*VIRUS SEED LOTS*

The strains of poliovirus used shall be identified by historical records that include information on the origin and subsequent manipulation of the strains.

Working seed lots are prepared by a single passage from a master seed lot and at an approved passage level from the original Sabin virus. Virus seed lots are prepared in large quantities and stored at a temperature below – 60 °C.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. Each working seed lot is identified as poliovirus of the given type, using specific antibodies.

**Virus concentration**. Determined by the method described below, the virus concentration is the basis for the quantity of virus used in the neurovirulence test.

**Extraneous agents** (*2.6.16*). If the working seed lot is produced in human diploid cells or in a continuous cell line, it complies with the requirements for seed lots for virus vaccines. If the working seed lot is produced in primary monkey kidney

cell cultures, it complies with the requirements given below under Virus propagation and harvest, and under Monovalent bulk.

In addition to the requirements in chapter *2.6.16*, for vaccines produced in cell lines and when the seed lot was produced in primary monkey kidney cell cultures, a validated test for sCMV is performed.

Working seed lots shall be free from detectable DNA sequences from SV40.

**Neurovirulence**. Each master and working seed lot complies with the test for neurovirulence of poliomyelitis vaccine (oral) in transgenic mice or monkeys. Suitable procedures for the tests in mice (*Neurovirulence test of types 1, 2 or 3 live poliomyelitis vaccine (oral) in transgenic mice susceptible to poliovirus*) and in monkeys (*Neurovirulence test of types 1, 2 or 3 live poliomyelitis vaccine (oral) in monkeys*) are available from the WHO.

In addition, at least the first 3 consecutive batches of monovalent bulk prepared from a new seed lot shall be shown to comply with the test for neurovirulence of poliomyelitis vaccine (oral) before the seed lot is deemed suitable for use. Furthermore, the seed lots shall cease to be used in vaccine production if the frequency of failure of the monovalent bulks produced from it is greater than predicted statistically. This statistical prediction is calculated after each test on the basis of all the monovalent bulks tested; it is equal to the probability of false rejection on the occasion of a first test (i.e. 1 per cent), the probability of false rejection on retest being negligible.

**Phenotypic or genotypic markers**. Each virus seed lot complies with the requirements of the MAPREC assay. A validated MAPREC assay is carried out for each master and working seed lot to establish a profile (i.e. percentage of mutant content). A suitable procedure (*Mutant analysis by PCR and restriction enzyme cleavage (MAPREC) for oral poliovirus (Sabin) vaccine types 1, 2 or 3*) is available from the WHO. Pending validation of MAPREC assays, each master and working seed lot is tested for its replicating properties at temperatures ranging from 36 °C to 40 °C as described under Monovalent bulk.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell banks and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled during the production. Suitable animal (but not human) serum may be used in the culture media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from live extraneous agents. The cell-culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. On the day of inoculation with the virus working seed lot, not less than 5 per cent or 1000 mL (whichever is lower) of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells). Special requirements, given below, apply to control cells when the vaccine is produced in primary monkey kidney cell cultures. The virus suspension is harvested no later than 4 days after virus inoculation. After inoculation of the production cell culture with the virus working seed lot, inoculated cells are maintained at a fixed temperature, shown to be suitable, within the range 33-35 °C; the temperature is maintained constant to ± 0.5 °C; control cell cultures are maintained at 33-35 °C for the relevant incubation periods.

Only a single virus harvest that complies with the following requirements may be used in the preparation of the monovalent bulk.

**Virus concentration**. The virus concentration of virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Molecular tests for consistency of production**. A validated MAPREC assay is performed on each virus harvest unless otherwise authorised and justified. The acceptance/rejection criteria for consistency of production are determined for each manufacturer and for each working seed. These criteria are periodically reviewed and updated to the satisfaction of the competent authority. An investigation of consistency occurs if a virus harvest gives results that are inconsistent with previous production history.

**Control cells**. The control cells of the production cell culture from which the virus harvest is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*) or, where primary monkey kidney cell cultures are used, as shown below.

**Primary monkey kidney cell cultures**. *The following special requirements apply to virus propagation and harvest in primary monkey kidney cell cultures.*

*Cell cultures.* On the day of inoculation with the virus working seed lot, each cell culture is examined for degeneration caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any extraneous agent, the entire group of cultures concerned shall be rejected.

On the day of inoculation with the virus working seed lot, a sample of at least 30 mL of the pooled fluid removed from the cell cultures of the kidneys of each single monkey or from foetuses from not more than 10 near-term monkeys is divided into 2 equal portions. 1 portion of the pooled fluid is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other portion of the pooled fluid is, where necessary, tested in monkey kidney cell cultures from another species so that tests on the pooled fluids are done in cell cultures from at least 1 species known to be sensitive to SV40. The pooled fluid is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 $cm^2$/mL of pooled fluid. At least 1 bottle of each type of cell culture remains uninoculated to serve as a control. If the monkey species used for vaccine production is known to be sensitive to SV40, a test in a $2^{nd}$ species is not required. Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium after inoculation of test material contains no added serum except as described below.

The cultures are incubated at a temperature of 35-37 °C and are observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks' incubation, at least 1 subculture of fluid is made from each of these cultures in the same cell culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain SV40 antibody.

Fluorescent-antibody techniques may be useful for detecting SV40 and other viruses in the cells.

A further sample of at least 10 mL of the pooled fluid is tested for cercopithecid herpesvirus 1 (B virus) and other viruses in rabbit kidney cell cultures. Serum used in the nutrient medium of these cultures shall have been shown to be free from inhibitors of B virus. Human herpesvirus has been used as an indicator for freedom from B virus inhibitors on account of the danger of handling cercopithecid herpesvirus 1 (B virus). The sample is inoculated into bottles of these cell cultures in such a way that the dilution of the pooled fluid in

the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 $cm^2$/mL of pooled fluid. At least 1 bottle of the cell cultures remains uninoculated to serve as a control.

The cultures are incubated at a temperature of 35-37 °C and observed for at least 2 weeks.

A further sample of 10 mL of the pooled fluid removed from the cell cultures on the day of inoculation with the seed lot virus is tested for the presence of extraneous agents by inoculation into human cell cultures sensitive to measles virus.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an extraneous agent, the single harvest from the whole group of cell cultures concerned is rejected.

If the presence of cercopithecid herpesvirus 1 (B virus) is demonstrated, the manufacture of oral poliomyelitis vaccine shall be discontinued and the competent authority shall be informed. Manufacturing shall not be resumed until a thorough investigation has been completed and precautions have been taken against any reappearance of the infection, and then only with the approval of the competent authority.

If these tests are not done immediately, the samples of pooled cell-culture fluid shall be kept at a temperature of − 60 °C or below, with the exception of the sample for the test for B virus, which may be held at 4 °C, provided that the test is done not more than 7 days after it has been taken.

*Control cell cultures*. On the day of inoculation with the virus working seed lot, 25 per cent (but not more than 2.5 L) of the cell suspension obtained from the kidneys of each single monkey or from not more than 10 near-term monkeys is taken to prepare uninoculated control cell cultures. These control cell cultures are incubated in the same conditions as the inoculated cultures for at least 2 weeks and are examined during this period for evidence of cytopathic changes. The tests are not valid if more than 20 per cent of the control cell cultures have been discarded for non-specific, accidental reasons. At the end of the observation period, the control cell cultures are examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any extraneous agent, the poliovirus grown in the corresponding inoculated cultures from the same group shall be rejected.

*Tests for haemadsorbing viruses*. At the time of harvest or within 4 days of inoculation of the production cultures with the virus working seed lot, a sample of 4 per cent of the control cell cultures is taken and tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures are similarly tested. The tests are carried out as described in chapter *2.6.16*.

*Tests for other extraneous agents*. At the time of harvest, or within 7 days of the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 mL of the pooled fluid from each group of control cultures is taken and tested in 2 kinds of monkey kidney cell culture, as described above.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid are taken and the tests referred to in this section in the 2 kinds of monkey kidney cell culture and in the rabbit cell cultures are repeated, as described above under Cell cultures.

If the presence of cercopithecid herpesvirus 1 (B virus) is demonstrated, the production cell cultures shall not be used and the measures concerning vaccine production described above must be undertaken.

The fluids collected from the control cell cultures at the time of virus harvest and at the end of the observation period may be pooled before testing for extraneous agents. A sample of 2 per cent of the pooled fluid is tested in each of the cell culture systems specified.

*Single harvests*

*Tests for neutralised single harvests in primary monkey kidney cell cultures*. A sample of at least 10 mL of each single harvest is neutralised by a type-specific poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this purpose, the immunising antigens used shall be prepared in non-simian cells.

Half of the neutralised suspension (corresponding to at least 5 mL of single harvest) is tested in monkey kidney cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralised suspension is tested, if necessary, in monkey kidney cell cultures from another species so that the tests on the neutralised suspension are done in cell cultures from at least 1 species known to be sensitive to SV40.

The neutralised suspensions are inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell sheet is at least 3 $cm^2$/mL of neutralised suspension. At least 1 bottle of each type of cell culture remains uninoculated to serve as a control and is maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralisation.

Animal serum may be used in the propagation of the cells, provided that it does not contain SV40 antibody, but the maintenance medium, after the inoculation of the test material, contains no added serum other than the poliovirus neutralising antiserum, except as described below.

The cultures are incubated at a temperature of 35-37 °C and observed for a total period of at least 4 weeks. During this observation period and after not less than 2 weeks' incubation, at least 1 subculture of fluid is made from each of these cultures in the same cell-culture system. The subcultures are also observed for at least 2 weeks.

Serum may be added to the original cultures at the time of subculturing, provided that the serum does not contain SV40 antibody.

Additional tests are made for extraneous agents on a further sample of the neutralised single harvests by inoculation of 10 mL into human cell cultures sensitive to measles virus. This test is also validated for the detection of sCMV.

Fluorescent-antibody techniques may be useful for detecting SV40 and other viruses in the cells.

The tests are not valid if more than 20 per cent of the culture vessels have been discarded for non-specific accidental reasons by the end of the respective test periods.

If any cytopathic changes occur in any of the cultures, the causes of these changes are investigated. If the cytopathic changes are shown to be due to unneutralised poliovirus, the test is repeated. If there is evidence of the presence of SV40 or other extraneous agents attributable to the single harvest, that single harvest is rejected.

*MONOVALENT BULK*

Monovalent bulks may be prepared by pooling a number of satisfactory single harvests of the same virus type. Monovalent bulks from continuous cell lines may be purified. Each monovalent bulk is filtered through a bacteria-retentive filter.

Only a monovalent bulk that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. Each monovalent bulk is identified as poliovirus of the given type, using specific antibodies.

**Virus concentration**. The virus concentration is determined by the method described below and serves as the basis for calculating the dilutions for preparation of the final bulk, for the quantity of virus used in the neurovirulence test and to establish and monitor production consistency.

**Phenotypic or genotypic markers**. A validated MAPREC assay is carried out for Sabin poliovirus types 1, 2 or 3 using the procedure as described under Virus seed lots. In this analysis, the amount of mutation at positions 480 and 525 of the genome (480-A; 525-C) for type 1, position 481 of the genome (481-G) for type 2, and position 472 of the genome (472-C) for type 3 is estimated and expressed as a ratio relative to the International Standard for MAPREC analysis of each related poliovirus type (Sabin). As the MAPREC assay for type 3 poliovirus (Sabin) is highly predictive of *in vivo* neurovirulence, a poliovirus type 3 monovalent bulk found to have a level of mutation greater than 1.0 per cent fails the assay.

For the monovalent bulk of poliovirus types 1 or 2, the limits for the level of mutation are approved by the competent authority.

Acceptance/rejection criteria for assessment of consistency of production are determined for each manufacturer and for each working seed lot by agreement with the competent authority. These criteria are updated as each new bulk is prepared and analysed. An investigation of consistency occurs if a monovalent bulk gives results that are inconsistent with previous production history.

If a filtered monovalent bulk of poliovirus (Sabin) fails the MAPREC assay then this triggers an investigation of the consistency of the manufacturing process. In the case of a new working seed lot, this investigation also includes a consideration of the suitability of this seed lot.

Monovalent bulks passing the MAPREC assay are subsequently tested for *in vivo* neurovirulence.

Pending validation of MAPREC assays, monovalent bulks are tested for the virus property of reproducing at temperatures of 36 °C and 40 °C. A ratio of the replication capacities of the virus in the monovalent bulk is obtained over a temperature range between 36 °C and 40 °C in comparison with the seed lot or a reference preparation for the marker tests and with appropriate rct/40− and rct/40+ strains of poliovirus of the same type. The incubation temperatures used in this test are controlled to within ± 0.1 °C. The monovalent bulk passes the test if, for both the virus in the harvest and the appropriate reference material, the titre determined at 36 °C is at least 5.0 $\log_{10}$ greater than that determined at 40 °C. If growth at 40 °C is so low that a valid comparison cannot be established, a temperature in the region of 39.0-39.5 °C is used, at which temperature the reduction in titre of the reference material must be in the range 3.0-5.0 $\log_{10}$ of its value at 36 °C; the acceptable minimum reduction is determined for each virus strain at a given temperature. If the titres obtained for 1 or more of the reference viruses are not concordant with the expected values, the test must be repeated.

**Neurovirulence**. Each monovalent bulk complies with the test as described under Virus seed lots.

**Primary monkey kidney cell cultures**. *The following special requirements apply to monovalent pooled harvests derived from primary monkey kidney cell cultures.*

*Retroviruses*. The monovalent pooled harvest is examined using a reverse transcriptase assay. No indication of the presence of retroviruses is found.

*Test in rabbits*. A sample of the monovalent pooled harvest is tested for cercopithecid herpesvirus 1 (B virus) and other viruses by injection of not less than 100 mL into not fewer than 10 healthy rabbits each weighing 1.5-2.5 kg. Each rabbit receives not less than 10 mL and not more than 20 mL, of which 1 mL is given intradermally at multiple sites since the maximum volume to be given intradermally at each site is 0.1 mL, and the remainder subcutaneously. The rabbits are observed for at least 3 weeks for death or signs of illness.

All rabbits that die after the first 24 h of the test and those showing signs of illness are examined by autopsy, and the brain and organs removed for detailed examination to establish the cause of death.

The test is not valid if more than 20 per cent of the inoculated rabbits show signs of intercurrent infection during the observation period. The monovalent pooled harvest passes the test if none of the rabbits shows evidence of infection with B virus or with other extraneous agents or lesions of any kind attributable to the bulk suspension.

If the presence of B virus is demonstrated, the measures concerning vaccine production described above under Cell cultures are taken.

*Test in guinea-pigs. If the primary monkey kidney cell cultures are not derived from monkeys kept in a closed colony, the monovalent pooled harvest shall be shown to comply with the following test*. Administer to each of not fewer than 5 guinea-pigs, each weighing 350-450 g, 0.1 mL of the monovalent pooled harvest by intracerebral injection (0.05 mL in each cerebral hemisphere) and 0.5 mL by intraperitoneal injection. Measure the rectal temperature of each animal on each working day for 6 weeks. At the end of the observation period carry out autopsy on each animal.

In addition, administer to not fewer than 5 guinea-pigs 0.5 mL by intraperitoneal injection and observe as described above for 2-3 weeks. At the end of the observation period, carry out a passage from these animals to not fewer than 5 guinea-pigs using blood and a suspension of liver or spleen tissue. Measure the rectal temperature of the latter guinea-pigs for 2-3 weeks. Examine by autopsy all animals that, after the first day of the test, die or are euthanised because they show disease, or show on 3 consecutive days a body temperature higher than 40.1 °C; carry out histological examination to detect infection with filoviruses; in addition, inject a suspension of liver or spleen tissue or of blood intraperitoneally into not fewer than 3 guinea-pigs. If any signs of infection with filoviruses are noted, confirmatory serological tests are carried out on the blood of the affected animals. The monovalent pooled harvest complies with the test if not fewer than 80 per cent of the guinea-pigs survive to the end of the observation period and remain in good health, and no animal shows signs of infection with filoviruses.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more satisfactory monovalent bulks and may contain more than one virus type. Suitable flavouring substances and stabilisers may be added.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium.

*FINAL LOT*

Only a final lot that complies with the following requirement for thermal stability and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

**Thermal stability**. Maintain not fewer than 3 containers of the final lot at 37 ± 1 °C for 48 h. Determine the total virus concentration as described under Assay in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. The total virus concentration of the heated vaccine is not more than 0.5 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

The vaccine is shown to contain poliovirus of each type stated on the label, using specific antibodies.

TESTS

**Bacterial and fungal contamination**. The vaccine complies with the test for sterility (*2.6.1*).

ASSAY

Titrate the vaccine for infectious virus, using not fewer than 3 separate containers of vaccine, following the method described below. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. If the vaccine contains more than one poliovirus type, titrate each type separately, using an appropriate type-specific antiserum (or preferably a monoclonal antibody) to neutralise each of the other types present.

Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*).

For a trivalent vaccine, the combined estimated virus titres per single human dose must be:

- not less than 6.0 $\log_{10}$ infectious virus units ($CCID_{50}$) for type 1;
- not less than 5.0 $\log_{10}$ infectious virus units ($CCID_{50}$) for type 2; and
- not less than 5.5 $\log_{10}$ infectious virus units ($CCID_{50}$) for type 3.

For a monovalent or divalent vaccine, the minimum virus titres are decided by the competent authority.

*Method*. Inoculate a suitable number of wells in a microtitre plate with a suitable volume of each of the selected dilutions of virus followed by a suitable volume of a cell suspension of the Hep-2 (Cincinnati) line. Examine the cultures between days 7 and 9.

The assay is not valid if:

- the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the types of poliovirus contained in the vaccine;
- the minimum amount of virus of each type contained in a single human dose;
- the cell substrate used for the preparation of the vaccine.

**01/2019:0216**

# RABIES VACCINE FOR HUMAN USE PREPARED IN CELL CULTURES

## Vaccinum rabiei ex cellulis ad usum humanum

DEFINITION

Rabies vaccine for human use prepared in cell cultures is a freeze-dried preparation of a suitable strain of fixed rabies virus grown in cell cultures and inactivated by a validated method.

The vaccine is reconstituted immediately before use as stated on the label to give a clear or opalescent liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

*GENERAL PROVISIONS*

The production of the vaccine is based on a virus seed-lot system and, if a cell line is used for virus propagation, a cell-bank system. The production method shall have been shown to yield consistently vaccines that comply with the requirements for immunogenicity, safety and stability. Unless otherwise justified and authorised, the virus in the final vaccine must not have undergone more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy; even with authorised exceptions, the number of passages beyond the level used for clinical studies must not exceed 5.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in a human diploid cell line, or in a continuous cell line (*5.2.3*) approved by the competent authority, or in cultures of chick-embryo cells derived from a flock free from specified pathogens (*5.2.2*).

*SEED LOTS*

The strain of rabies virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation.

Working seed lots are prepared by not more than 5 passages from the master seed lot.

Only a working seed lot that complies with the following tests may be used for virus propagation.

**Identification**. Each working seed lot is identified as rabies virus using specific antibodies.

**Virus concentration**. The virus concentration of each working seed lot is determined by a cell-culture method using immunofluorescence, to ensure consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for virus seed lots. If the virus has been passaged in mouse brain, specific tests for murine viruses are carried out.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled. Approved animal (but not human) serum may be used in the media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum; the media may contain human albumin. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. Not less than 500 mL of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control

cells). The virus suspension is harvested on one or more occasions during incubation. Successive harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the inactivated viral harvest.

**Identification**. The single harvest contains virus that is identified as rabies virus using specific antibodies.

**Virus concentration**. Titrate for infective virus in cell cultures; the titre is used to monitor consistency of production.

**Control cells**. The control cells of the production cell culture from which the single harvest is derived comply with a test for identification and with the requirements for extraneous agents (*2.6.16*).

*PURIFICATION AND INACTIVATION*

The virus harvest may be concentrated and/or purified by suitable methods; the virus harvest is inactivated by a validated method at a fixed, well-defined stage of the process, which may be before, during or after any concentration or purification. In order to ensure that the virus inactivation process is effective, conditions that could lead to virus aggregation should be avoided at process steps preceding virus inactivation. Any aggregates present in the preparation to be inactivated must be removed immediately before the inactivation process, for example by a suitable filtration method. It shall have been demonstrated in process validation studies that the inactivation process is consistently effective in inactivating rabies virus in such a way that it assures consistent protective immunogenic activity.

The demonstration of consistency must be based on the following:

- the inactivation kinetics are shown to be consistent using at least 5 consecutive batches. Samples of virus, collected at appropriate times, are inoculated into a sensitive substrate to establish the inactivation curve. If necessary, the agent used for inactivation is neutralised prior to inoculation;
- the time needed to achieve complete inactivation is determined in order to define the required inactivation time. The test for residual infectious virus is used for this purpose. The total inactivation time used in routine production must be at least twice the time needed for complete virus inactivation.

If betapropiolactone is used, the concentration shall at no time exceed 1:3500 *V*/*V*.

Only an inactivated viral suspension that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Residual infectious virus**. Carry out an amplification test for residual infectious rabies virus immediately after inactivation or using a sample frozen immediately after inactivation and stored at − 70 °C. Inoculate a quantity of inactivated viral suspension equivalent to not less than 25 mL of bulk vaccine corresponding to at least 25 human doses of vaccine into cell cultures of the same type as those used for production of the vaccine or of another approved cell type. Cells used for the test must be of optimal sensitivity regarding residual infectious rabies virus, for example, Vero, BHK-21 or neuroblastoma cells that are known to be highly sensitive to rabies virus may be used. If other cells are used, they must have been shown to possess at least the same sensitivity as those specified. A passage may be made after 7 days. Maintain the cultures for a total of 21 days and then examine the cell cultures for rabies virus using an immunofluorescence test or another suitable method of comparable sensitivity. The inactivated virus harvest complies with the test if no replicating infectious rabies virus is detected.

**Residual host-cell DNA**. If a continuous cell line is used for virus propagation, the content of residual host-cell DNA, determined using a suitable method, is not greater than 10 ng per single human dose.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more inactivated viral suspensions. An approved stabiliser may be added to maintain the activity of the product during and after freeze-drying.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Glycoprotein content**. Determine the glycoprotein content by a suitable immunochemical method (*2.7.1*), for example, single-radial immunodiffusion, enzyme-linked immunosorbent assay or an antibody-binding test. The content is within the limits approved for the particular product.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to avoid contamination and the introduction of moisture.

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain rabies virus antigen by a suitable immunochemical method (*2.7.1*) using specific antibodies, preferably monoclonal; alternatively, the assay serves also to identify the vaccine.

TESTS

**Glycoprotein content**. Determine the glycoprotein content by a suitable immunochemical method (*2.7.1*), for example, single-radial immunodiffusion, enzyme-linked immunosorbent assay or an antibody-binding test. The content is within the limits approved for the particular product.

**Bovine serum albumin**: maximum 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Sterility** (*2.6.1*). It complies with the test.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU per single human dose.

**Pyrogens** (*2.6.8*). The test for pyrogens is performed only in cases of evidence of the presence of non-endotoxin pyrogenic substances. It complies with the test. Unless otherwise justified and authorised, inject into each rabbit 1 mL of a single human dose of the vaccine diluted to 10-100 times its volume.

**Water** (*2.5.12*): maximum 3.0 per cent.

ASSAY

The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of rabies virus, administered intracerebrally, with the quantity of a reference preparation of rabies vaccine necessary to provide the same protection. For this comparison a reference preparation of rabies vaccine, calibrated in International Units, and a suitable preparation of rabies virus for use as the challenge preparation are necessary. Alternatively, in the interest of animal welfare, a validated serology potency assay or an immunochemical assay (*2.7.1*)

for a native glycoprotein content is recommended. The alternative method is validated against a challenge assay and approved for a given product by the competent authority.

The International Unit is the activity contained in a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The challenge test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation. Once the analyst has experience with the method for a given vaccine, it is possible to carry out a simplified test using a single dilution of the vaccine to be examined and of the reference preparation. Such a test enables the analyst to determine that the vaccine has a potency significantly higher than the required minimum, but does not give full information on the validity of each individual potency determination. The use of a single dilution allows a considerable reduction in the number of animals required for the test and must be considered by each laboratory in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

*Selection and distribution of the test animals.* Use healthy female mice, about 4 weeks old, each weighing 11-15 g, and from the same stock. Distribute the mice into 6 groups of a size suitable to meet the requirements for validity of the test and, for titration of the challenge suspension, 4 groups of 5.

*Preparation of the challenge suspension.* Inoculate mice intracerebrally with the Challenge Virus Standard (CVS) strain of rabies virus and when the mice show signs of rabies, but before they die, euthanise them, then remove the brains and prepare a homogenate of the brain tissue in a suitable diluent. Separate gross particulate matter by centrifugation and use the supernatant as the challenge suspension. Distribute the suspension in small volumes in ampoules, seal and store at a temperature below – 60 °C. Thaw 1 ampoule of the suspension and make serial dilutions in a suitable diluent. Allocate each dilution to a group of 5 mice and inject intracerebrally into each mouse 0.03 mL of the dilution allocated to its group. Observe the mice for 14 days. Calculate the $LD_{50}$ of the undiluted suspension using the number in each group that, between the 5$^{th}$ and 14$^{th}$ days, die or develop signs of rabies.

*Determination of potency of the vaccine.* Prepare 3 fivefold serial dilutions of the vaccine to be examined and 3 fivefold serial dilutions of the reference preparation. Prepare the dilutions such that the most concentrated suspensions may be expected to protect more than 50 per cent of the animals to which they are administered and the least concentrated suspensions may be expected to protect less than 50 per cent of the animals to which they are administered. Allocate the 6 dilutions, 1 to each of the 6 groups of mice, and inject by the intraperitoneal route into each mouse 0.5 mL of the dilution allocated to its group. After 7 days, prepare 3 identical dilutions of the vaccine to be examined and of the reference preparation and repeat the injections. Seven days after the second injection, prepare a suspension of the challenge virus such that, on the basis of the preliminary titration, 0.03 mL contains about 50 $LD_{50}$. Inject intracerebrally into each vaccinated mouse 0.03 mL of this suspension. Prepare 3 suitable serial dilutions of the challenge suspension. Allocate the challenge suspension and the 3 dilutions, 1 to each of the 4 groups of 5 control mice, and inject intracerebrally into each mouse 0.03 mL of the suspension or dilution allocated to its group. Observe the animals in each group for 14 days and record the number in each group that die or show signs of rabies in the period 5-14 days after challenge.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose lies between the largest and smallest doses given to the mice;
- the titration of the challenge suspension shows that 0.03 mL of the suspension contained not less than 10 $LD_{50}$;
- the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response curves;
- the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

The vaccine complies with the test if the estimated potency is not less than 2.5 IU per human dose.

*Application of alternative end-points.* Once a laboratory has established the above assay for routine use, the lethal end-point is replaced by an observation of clinical signs and application of an end-point earlier than death to reduce animal suffering. The following is given as an example.

The progress of rabies infection in mice following intracerebral injection can be represented by 5 stages defined by typical clinical signs:

Stage 1: ruffled fur, hunched back;

Stage 2: slow movements, loss of alertness (circular movements may also occur);

Stage 3: shaky movements, trembling, convulsions;

Stage 4: signs of paresis or paralysis;

Stage 5: moribund state.

Mice are observed at least twice daily from day 4 after challenge. Clinical signs are recorded using a chart such as that shown in Table 0216.-1. Experience has shown that using stage 3 as an end-point yields assay results equivalent to those found when a lethal end-point is used. This must be verified by each laboratory by scoring a suitable number of assays using both the clinical signs and the lethal end-point.

Table 0216.-1. – *Example of a chart used to record clinical signs in the rabies vaccine potency test*

| | **Days after challenge** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Clinical signs** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| Ruffled fur<br>Hunched back | | | | | | | | |
| Slow movements<br>Loss of alertness<br>Circular movements | | | | | | | | |
| Shaky movements<br>Trembling<br>Convulsions | | | | | | | | |
| Paresis<br>Paralysis | | | | | | | | |
| Moribund state | | | | | | | | |

LABELLING

The label states the biological origin of the cells used for the preparation of the vaccine.

**01/2012:2417**

# ROTAVIRUS VACCINE (LIVE, ORAL)

## Vaccinum rotaviri vivum perorale

DEFINITION

Rotavirus vaccine (live, oral) is a preparation of one or more suitable virus serotypes, grown in an approved cell substrate and presented in a form suitable for oral administration.

The vaccine is a clear liquid or it may be a freeze-dried preparation to be reconstituted immediately before use, as stated on the label, to give a slightly turbid liquid. The vaccine ready for administration may be coloured owing to the presence of a pH indicator.

PRODUCTION

*GENERAL PROVISIONS*

The vaccine strains and the production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man. The vaccine is formulated so as to avoid inactivation by gastric fluids. Where the vaccine is freeze-dried, the antacid capacity of the solvent and its stability are established.

The production of vaccine is based on a virus seed-lot system and a cell-bank system. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

If purification steps are present, the reduction of selected process-related impurities and residuals such as residual host-cell proteins, residual cellular DNA, endotoxins, bovine serum, trypsin, and antibiotics is monitored to establish consistency of the purification process.

*REFERENCE PREPARATION*

A suitable reference preparation that is representative of batches of vaccine shown to be effective in clinical trials is established for use in tests to determine virus concentration. The differences in the composition and characteristics of rotavirus vaccines mean that there will be a specific reference preparation for each one.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in a suitable cell line (*5.2.3*).

*VIRUS SEED LOTS*

The strain(s) of rotavirus used shall be identified by historical records that include information on the origin of each strain and its subsequent manipulation including the method of attenuation, whether the strains have been biologically cloned prior to generation of the master seed lot, genetic sequence information, the phenotypic and genotypic stability of the master and working seed lots when passaged up to the single harvest level, and the passage level at which attenuation for humans was demonstrated by clinical trials. Virus seed lots are stored at temperatures below – 20 °C if freeze-dried, or below – 60 °C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are shown to be of the required rotavirus type by an immunological assay using specific antibodies or by a molecular identity test such as polyacrylamide gel electrophoresis of RNA, RNA/RNA hybridisation, or restriction-enzyme mapping of genetic sequences of polymerase chain reaction (PCR)-amplified VP7 gene segments.

**Virus concentration**. The virus concentration of the master and working seed lots is determined to monitor consistency of production. Direct cell-culture based methods and nucleic acid amplification techniques (NAT) (*2.6.21*) such as PCR quantification of virus replication in cell culture may be used.

**Extraneous agents** (*2.6.16*). Each working seed lot complies with the requirements for virus seed lots.

*VIRUS PROPAGATION, SINGLE HARVEST, MONOVALENT POOLED HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are being handled. Suitable animal (but not human) serum may be used in the culture media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production.

*STORED VIRUS INTERMEDIATE CULTURE*

Where a stored virus intermediate culture, prepared from the working seed lot, is used for inoculation, on the day of inoculation not less than 5 per cent or 500 mL of the cell cultures employed, whichever is greater, are set aside as uninfected cell cultures (control cells). Stored virus intermediate cultures are harvested at a time appropriate to the strain of virus and stored at temperatures below – 60°C.

Only a stored virus intermediate culture that complies with the following requirements may be used for virus propagation.

**Identification**. Each stored virus intermediate culture is identified by rotavirus type by an immunological assay using specific antibodies or by a molecular identity test such as NAT (*2.6.21*).

**Bacterial and fungal contamination**. Each stored virus intermediate culture complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Virus concentration**. The virus concentration of each stored virus intermediate culture is determined as prescribed under Assay to monitor consistency of production. Both direct cell-culture based methods and NAT (*2.6.21*) such as PCR quantification of virus replication in cell culture may be used.

**Extraneous agents** (*2.6.16*). Each stored virus intermediate culture complies with the tests for extraneous agents.

**Control cells**. The control cells of the production cell culture from which each stored virus intermediate culture is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

*VIRUS PROPAGATION AND SINGLE HARVEST*

On the day of inoculation with the virus working seed lot or stored virus intermediate culture, cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells). If bioreactor technology is used, the size and handling of the cell sample to be examined is approved by the competent authority. The virus suspensions are harvested at a time appropriate to the strain of virus being used.

Only a single virus harvest that complies with the following requirements may be used for further processing.

**Bacterial and fungal contamination**. Each single virus harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Control cells**. The control cells of the production cell culture from which each single harvest is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

*MONOVALENT POOLED HARVEST*

Monovalent pooled harvests are prepared by pooling a number of single harvests of the same virus type. If no monovalent pooled harvest is prepared, the tests below are carried out on each single harvest.

Only a single harvest or a monovalent pooled harvest that complies with the following requirements may be used in the preparation of the purified monovalent harvest.

**Identification**. Each single harvest or monovalent pooled harvest is identified by rotavirus type by an immunological assay using specific antibodies or by a molecular identity test such as NAT (*2.6.21*).

**Bacterial and fungal contamination**. Each single harvest or monovalent pooled harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Virus concentration**. The virus concentration of each single harvest or monovalent pooled harvest is determined as prescribed under Assay to monitor consistency of production. Both direct cell-culture based methods and NAT (*2.6.21*) such as PCR quantification of virus replication in cell culture may be used.

**Extraneous agents** (*2.6.16*). Each single harvest or monovalent pooled harvest complies with the tests for extraneous agents.

*PURIFIED MONOVALENT HARVEST*

The purified monovalent harvest is prepared from a single harvest or a pooled monovalent harvest. The single harvest or pooled monovalent harvest is clarified to remove cell debris and may be further purified.

Only a purified monovalent harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Bacterial and fungal contamination**. The purified monovalent harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Virus concentration**. The virus concentration of the purified monovalent harvest is determined as prescribed under Assay to monitor consistency of production. Both direct cell-culture based methods and NAT (*2.6.21*) such as PCR quantification of virus replication in cell culture may be used.

**Residual cellular DNA**: maximum 100 µg of cellular DNA per human dose for viruses grown in continuous cells lines.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more satisfactory purified monovalent harvests and may contain more than one virus type. Suitable stabilisers may be added.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile containers and may be freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to avoid contamination and the introduction of moisture.

An approved minimum virus concentration for release of the product is established for each virus type to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

For freeze-dried vaccines, tests for identity, pH, volume, sterility and content of key components are carried out on the solvent.

Only a final lot that complies with the following requirement for thermal stability and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

**Thermal stability**. Maintain not fewer than 3 containers of the final lot at an elevated temperature for a defined time period, using conditions found suitable for the particular product as approved by the competent authority. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine maintained at the temperature recommended for storage. The virus concentration of the containers that have been heated does not decrease by more than an approved amount during the period of exposure. For a multivalent vaccine, if there is no significant difference in the virus loss between serotypes, the loss may be determined from total virus concentration.

IDENTIFICATION

The vaccine is shown to contain rotavirus of each type stated on the label by an immunological assay using specific antibodies or by a molecular identity test. If PCR is used for the assay, this may serve as the identity test.

TESTS

**Bacterial and fungal contamination**. The vaccine complies with the test for sterility (*2.6.1*).

**Water** (*2.5.12*): maximum 3.0 per cent for each final lot of freeze-dried vaccine.

ASSAY

The assay of rotavirus vaccine is carried out by inoculation of suitable cell cultures with dilutions of the vaccine and evaluation of the rotavirus concentration, either by visualisation of infected areas of a cell monolayer or by comparison of the capacity of the vaccine to produce viral RNA following infection of cells with the corresponding capacity of an approved reference preparation.

*For the assay based on visualisation of infected areas of a cell monolayer*, titrate the vaccine for infective virus using at least 3 separate containers. Titrate the contents of 1 container of an appropriate virus reference preparation in triplicate to validate each assay. If the vaccine contains more than 1 rotavirus type, titrate each type separately using a method of suitable specificity. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory.

Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*).

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay);
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay) from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay); data generated from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$ (or an equivalent value expressed with a unit suitable for the method used for the assay).

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

*For the assay based on comparison of the capacity of the vaccine to produce viral RNA* following infection of cells with the corresponding capacity of an approved reference preparation, a suitable number of cell cultures in a microtitre plate are infected in parallel with serial dilutions of the vaccine and the reference preparation. After incubation to allow virus replication, viral RNA in the individual wells is released from the cells and quantified by NAT (*2.6.21*), such as real-time quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) technology.

Not fewer than 3 separate containers of the vaccine are assayed against a container of the reference preparation titrated in triplicate.

Calculate the individual virus concentration for each container of vaccine against the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*).

The combined estimate of the virus concentration for the 3 containers of vaccine is not less than that stated on the label.

The assay is not valid unless:

- the negative external NAT control is unambiguously negative;
- the positive external NAT control is unambiguously positive;
- the negative matrix control (uninfected cells) is unambiguously negative;
- the positive matrix control (cells spiked with viral RNA) is unambiguously positive;
- the statistical analysis shows a significant slope and no significant deviations from linearity or parallelism of the dose-response curves.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than $\pm 0.3 \log_{10}$ infectious units; data generated from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than $\pm 0.3 \log_{10}$ infectious units.

LABELLING

The label states:

- the type or types of rotavirus contained in the vaccine;
- the minimum amount of each type of virus contained in 1 single human dose;
- the cell substrate used for the preparation of the vaccine.

**01/2019:0162**

# RUBELLA VACCINE (LIVE)

## Vaccinum rubellae vivum

DEFINITION

Rubella vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of rubella virus. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live rubella vaccines of adequate immunogenicity and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall have undergone no more passages from the master seed lot than were used to prepare the vaccine shown in clinical studies to be satisfactory with respect to safety and efficacy.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*).

*SEED LOT*

The strain of rubella virus used shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at temperatures below − 20 °C if freeze-dried, or below − 60 °C if not freeze-dried.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as rubella virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration of the master and working seed lots is determined to ensure consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots.

*PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled during the production. Suitable animal (but not human) serum may be used in the growth medium, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and culture media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. Not less than 500 mL of the production cell cultures is set aside as uninfected cell cultures (control cells). The temperature of incubation is controlled during the growth of the virus. The virus suspension is harvested, on one or more occasions, within 28 days of inoculation. Multiple harvests from the same production cell culture may be pooled and considered as a single harvest.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The single harvest contains virus that is identified as rubella virus by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration in the single harvest is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents.

**Control cells**. The control cells comply with a test for identification and with the tests for extraneous agents (*2.6.16*).

*FINAL BULK VACCINE*

Single harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

*FINAL LOT*

A minimum virus concentration for release of the product is established such as to ensure, in light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification and Tests may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Thermal stability**. Maintain at least 3 vials of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 7 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific rubella antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**. Not more than 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*). Not more than 3.0 per cent, determined by the semi-micro determination of water.

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine and inoculating a suitable number of wells for each dilution step. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label; the minimum virus concentration stated on the label is not less than 3.0 $\log_{10}$ $CCID_{50}$ per single human dose.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ $CCID_{50}$;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ $CCID_{50}$ from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ $CCID_{50}$; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ $CCID_{50}$.

*Rubella vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

**01/2019:2418**
**corrected 10.0**

# SHINGLES (HERPES ZOSTER) VACCINE (LIVE)

## Vaccinum zonae vivum

DEFINITION

Shingles (herpes zoster) vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of human herpesvirus 3. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear or slightly opalescent liquid, almost white suspension or pale yellow liquid that may be coloured owing to the presence of a pH indicator. It is intended for administration to adults.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live shingles vaccines of adequate immunogenicity and safety in man. The virus in the final vaccine shall not have been passaged in cell cultures beyond a defined number of passages approved by the competent authority from the original isolated virus.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*).

*VIRUS SEED LOT*

The strain of human herpesvirus 3 shall be identified as being suitable by historical records that include information on the origin of the strain and its subsequent manipulation. The virus shall at no time have been passaged in continuous cell lines. Seed lots are prepared in the same kind of cells as those used for the production of the final vaccine. Virus seed lots are prepared in large quantities and stored at temperatures below − 20 °C if freeze-dried, or below − 60 °C if not freeze-dried.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as human herpesvirus 3 by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration of the master and working seed lots is determined as prescribed under Assay to monitor consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots for live virus vaccines; a sample of 50 mL is taken for the test in cell cultures.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells or virus are being handled. Approved animal (but not human) serum may be used in the culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. 5 per cent, but not less than 50 mL, of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). The infected cells constituting a single harvest are washed, released from the support surface and pooled. The cell suspension is disrupted by sonication.

Only a virus harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The virus harvest contains virus that is identified as human herpesvirus 3 by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The concentration of infective virus in virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Extraneous agents** (*2.6.16*). Use 50 mL for the test in cell cultures.

**Control cells**. The control cells of the production cell culture from which the single harvest is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

*FINAL BULK VACCINE*

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*) using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to the test for water and each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Water** (*2.5.12*). Not more than the amount shown to ensure stability of the vaccine as approved by the competent authority, determined by the semi-micro determination of water.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific human herpesvirus 3 antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**: maximum 0.65 µg per human dose, determined by a suitable immunochemical method (*2.7.1*).

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label.

The assay is not valid if:

- the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than $\pm 0.3$ $\log_{10}$ PFU;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ PFU from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than $\pm 0.3$ $\log_{10}$ PFU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than $\pm 0.3$ $\log_{10}$ PFU.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided;
- that the vaccine is not to be administered to pregnant women.

**01/2019:0164**

# SMALLPOX VACCINE (LIVE)

## Vaccinum variolae vivum

DEFINITION

Smallpox vaccine (live) is a liquid or freeze-dried preparation of live vaccinia virus grown *in ovo* in the membranes of the chick embryo, in cell cultures or in the skin of living animals.

This monograph applies to vaccines produced using strains of confirmed efficacy in man, in particular those used during eradication of smallpox, for example the Lister strain (sometimes referred to as the Lister/Elstree strain) and the New York City Board of Health (NYCBOH) strain. It does not apply to non-replicative strains such as Modified Virus Ankara (MVA).

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently smallpox vaccines of adequate safety and immunogenicity in man. The strain used shall have been shown to produce typical vaccinia skin lesions in man. Production is based on a seed-lot system.

The International Reference Preparation for smallpox vaccine is suitable for use as a reference preparation in virus titration.

*SUBSTRATE FOR VIRUS PROPAGATION*

**Animals used for production of skin-derived vaccines**. If the vaccine is prepared in animals skins, the animals used are of a species approved by the competent authority, are in good health, are kept in closed or intensively monitored colonies, and have not previously been employed for experimental purposes. Only animals susceptible to infection by dermal inoculations with vaccinia virus may be used for vaccine production.

The animals are kept in well-constructed and adequately ventilated animals rooms with cages spaced as far apart as possible. Adequate precautions are taken to prevent cross-infection between cages. Not more than 1 large animal is housed per stall. Not more than 2 small animals are housed per cage and cage-mates must not be interchanged. The animals must be kept in the country of production of the vaccine in quarantine groups for a period of not less than 6 weeks before use.

If at any time during the quarantine period the overall death rate of the group reaches 5 per cent, no animals from that entire group may be used for vaccine production.

The groups are kept continuously in isolation, as in quarantine, even after completion of the quarantine period, until the animals are used. After the last animal of a group has been taken, the room that housed the group is thoroughly cleaned and decontaminated before receiving a new group.

Animals that are to be inoculated are anaesthetised and thoroughly examined. If an animal shows any pathological lesion, it is not used in the preparation of a seed lot or a vaccine, nor are any of the remaining animals of the quarantine group concerned unless it is evident that their use will not impair the safety of the product.

The prophylactic and diagnostic measures adopted to exclude the presence of infectious disease are approved by the competent authority. According to the species of animals used and the diseases to which that animal is liable in the country where the vaccine is being produced, these measures may vary. Consideration must also be given to the danger of spreading diseases to other countries to which the vaccine may be shipped. Special attention must always be given to foot-and-mouth disease, brucellosis, Q fever, tuberculosis and dermatomycosis, and it may also be necessary to consider diseases such as contagious pustular dermatitis (orf), anthrax, rinderpest, haemorrhagic septicaemia, Rift valley fever and others.

**Embryonated eggs**. Embryonated eggs used for production are obtained from a flock free from specified pathogens (SPF) (*5.2.2*).

**Human diploid cells, continuous cell lines**. Human diploid cells and continuous cell lines comply with the requirements for cell substrates (*5.2.3*).

**Primary chick embryo cells**. Primary chick embryo cells are derived from an SPF flock (*5.2.2*).

**Primary rabbit kidney cells**. Only healthy rabbits derived from a closed colony approved by the competent authority are used as a source. The animals, preferably 2-4 weeks old, are tested to ensure freedom from specified pathogens or their antibodies.

Where new animals are introduced into the colony, they are maintained in quarantine for a minimum of 2 months and shown to be free from specified pathogens. Animals to be used to provide kidneys shall not have been previously employed for experimental purposes, especially those involving infectious agents. The colony is monitored for zoonotic viruses and markers of contamination at regular intervals.

At the time the colony is established, all animals are tested to determine freedom from antibodies to possible viral contaminants for which there is evidence of capacity for infecting humans or evidence of capacity to replicate *in vitro* in cells of human origin. A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase assay is also included. Nucleic acid amplification tests (*2.6.21*) for retroviruses may also be used.

After the colony is established, it is monitored by testing a representative group of at least 5 per cent of the animals, which are then bled at suitable (for example monthly) intervals. In addition, the colony is screened for pathogenic micro-organisms, including mycobacteria, fungi and mycoplasmas. The screening programme is designed to ensure that all animals are tested within a given period of time.

Any animal that dies is examined to determine the cause of death. If the presence of a causative infectious agent is demonstrated in the colony, the production of smallpox vaccine is discontinued.

At the time of kidney harvest, the animals are examined for the presence of abnormalities and, if any are noted, the animals are not used for vaccine production.

Each set of control cultures derived from a single group of animals used to produce a single virus harvest must remain identifiable as such until all testing, especially for extraneous agents, is completed.

*VIRUS SEED LOT*

The vaccinia virus isolate used for the master seed lot is identified by historical records that include information on its origin and the tests used in its characterisation.

Virus from the working seed lot must have the same characteristics as the strain that was used to prepare the master seed lot. The number of passages required to produce single harvests from the original isolate is limited and approved by the competent authority. Vaccine is produced from the working seed with a minimum number of intervening passages.

Since cell culture production and clonal selection (for example, plaque purification) may lead to altered characteristics of the virus, the master seed virus must be characterised as fully as possible, for example by comparing the safety profile and biological characteristics of the strain with that of the parental isolate. The characterisation shall include the following:

- antigenic analyses using specific antisera and/or monoclonal antibodies;
- biological studies such as infectivity titre, chorioallantoic membrane (CAM) assay, *in vitro* yield and *in vivo* growth characteristics in a suitable animal model;
- genetic analyses such as restriction mapping/southern blotting, PCR analyses and limited sequencing studies;
- phenotypic and genetic stability upon passage in the substrate;
- neurovirulence testing and immunogenicity studies.

The characterisation tests are also carried out on each working seed lot and on 3 batches of vaccine from the first working seed lot to verify genetic stability of the vaccine strain.

Only a virus seed that complies with the following requirements may be used for virus propagation.

**Identification**. Each working seed lot is identified as vaccinia virus using specific antibodies and molecular tests. Suitable tests are conducted to exclude the presence of variola virus and other orthopoxviruses.

**Virus concentration**. Determine by the CAM assay or by a suitable validated *in vitro* assay (plaque assay or $CCID_{50}$ assay). The virus concentration is the basis for the quantity of virus used in the neurovirulence test.

**Extraneous agents** (*2.6.16*). If the working seed lot is produced in embryonated eggs, human diploid cells, or in a continuous cell line, it complies with the requirements for seed lots for virus vaccines. Seed lots produced in embryonated eggs and seed lots produced in primary cell cultures comply with the additional requirements described below.

Where the tests prescribed cannot be carried out because complete neutralisation of the seed virus is not possible, the seed lot may be diluted to a concentration equivalent to that of the dilution used as inoculum for production of vaccine prior to testing for extraneous viruses. Supplementary specific testing for extraneous viruses using validated nucleic acid amplification techniques (*2.6.21*) or immunochemical methods (*2.7.1*) may be envisaged. Where the indicator cell culture method for mycoplasma detection (*2.6.7*) cannot be carried out, nucleic acid amplification testing is performed instead.

Seed lots to be used for embryonated egg or cell culture production are in addition to be tested for carry-over of potential extraneous agents from the original seed. Given that the complete passage history of the original seed is unlikely to be known and that more than one species may have been used, this additional testing must at least cover important extraneous agents of concern.

The bioburden of master and working seed lots prepared in animal skins is limited by meticulous controls of facilities, personnel, and animals used for production, and by specific tests on the seeds. However, it may be difficult to ensure that seed lots produced in animal skins are totally free from extraneous agents, and consideration must be given to production procedures which remove or reduce them. Such lots must comply with the requirements indicated below. The absence of specific human pathogens is confirmed by additional testing procedures, for example, bacterial and fungal cultures, virus culture, nucleic acid amplification testings (*2.6.21*) for viral agents.

**Neurovirulence**. The neurovirulence of master and working seed lots is assessed using a suitable animal model, for example in monkeys or mice. The parental isolate is used as comparator. Where the original isolate is not available for this purpose, equivalent materials may be used.

*VIRUS PROPAGATION AND HARVEST*

**VACCINE PRODUCED IN LIVING ANIMALS**

Before inoculation the animals are cleaned and thereafter kept in scrupulously clean stalls until the vaccinia material is harvested. For 5 days before inoculation and during incubation the animals remain under veterinary supervision and must remain free from any sign of disease; daily rectal temperatures are recorded. If any abnormal rise in temperature occurs or any clinical sign of disease is observed, the production of vaccine from the group of animals concerned must be suspended until the cause has been resolved.

The inoculation of seed virus is carried out on such parts of the animal that are not liable to be soiled by urine and faeces. The surface used for inoculation is shaved and cleaned so as to achieve conditions that are as close as possible to surgical asepsis. If any antiseptic substance deleterious to the virus is used in the cleaning process it is removed by thorough rinsing with sterile water prior to inoculation. During inoculation the exposed surface of the animal not used for inoculation is covered with a sterile covering. By historical experience the ventral surface of female animals is appropriate for inoculation and inoculation of male animals is more appropriate on the flank.

Before the collection of the vaccinia material, any antibiotic is removed and the inoculated area is cleaned. The uninoculated surfaces are covered with a sterile covering. Before harvesting the animals are euthanised and exsanguinated to avoid heavy mixtures of the vaccinia material with blood. The vaccinia material from each animal is collected separately with aseptic precautions. All animals used in the production of vaccine are examined by autopsy. If evidence of any generalised or systemic disease other than vaccinia is found, the vaccinia material from that animal is discarded. If the disease is considered to be a communicable one, the harvest from the entire group of animals exposed must be discarded unless otherwise justified and authorised.

**VACCINE PRODUCED IN EGGS**

All processing of embryonated eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. After inoculation and incubation at a controlled temperature only living and suitable chick embryos are harvested. The age of the embryos at the time of virus harvest is reckoned from the initial introduction of the egg into the incubator and shall be not more than 12 days. After homogenisation and clarification by centrifugation, the extract of embryonic pulp is tested as described below and kept at − 70 °C or below until further processing. Virus harvests that comply with the prescribed tests may be pooled. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Control eggs**. Control eggs comply with the tests for extraneous agents (*2.6.16*). A sample of 2 per cent of uninoculated embryonated eggs (not less than 20 and not more than 50) from the batch used for vaccine production shall be incubated under the same conditions as the inoculated eggs. At the time of virus harvest the uninoculated eggs are processed in the same manner as the inoculated eggs.

**Sterility** (*2.6.1*). It complies with the test for sterility, carried out using 10 mL for each medium.

**VACCINE PRODUCED IN CELL CULTURES (PRIMARY CHICK EMBRYO CELLS, PRIMARY RABBIT KIDNEY CELLS, HUMAN DIPLOID CELLS OR CONTINUOUS CELL LINES)**

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells are handled at the same time during production. Suitable animal (but not human) serum may be used in the culture media, but the final medium for maintaining cell growth during virus multiplication does not contain animal serum. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. On the day of inoculation with the virus working seed lot, not less than 5 per cent or 1000 mL, whichever is the least, of the cell cultures employed for vaccine production are set aside as uninfected cell cultures (control cells); special requirements, given below, apply to control cells when the vaccine is produced in primary rabbit kidney cell cultures.

After inoculation of the production cell culture with the working seed lot, inoculated cells are maintained at a suitable fixed temperature, and the virus suspension is harvested after a suitable incubation period.

Only a single harvest that complies with the following requirements may be used in the preparation of the monovalent pooled harvest.

**Control cells**. The control cells of the production cell culture from which the virus harvest is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*) or, where primary rabbit kidney cells cultures are

used, with specific tests as mentioned hereafter. The test is invalid if more than 20 per cent of the control cell cultures have been discarded at the end of the observation period.

**Extraneous agents** (*2.6.16*). The single harvest complies with the tests for extraneous agents. Complete neutralisation of vaccinia virus may be difficult to achieve at high virus concentration. In this case specific tests such as nucleic acid amplification (*2.6.21*) and immunochemical tests (*2.7.1*) can replace non-specific testing in cell culture or eggs. To save biological reagents such as vaccinia neutralising antisera, testing for extraneous agents may be performed on the final bulk instead of on the single harvests.

*Vaccine prepared in primary chick embryo cells.* A sample of fluids pooled from the control cultures is tested for adenoviruses and for avian retroviruses such as avian leukosis virus. In addition, a volume of each neutralised virus pool equivalent to 100 human doses of vaccine or 10 mL, whichever is the greater, is tested in a group of fertilised eggs by the allantoic route of inoculation, and a similar sample is tested in a separate group of eggs by the yolk-sac route of inoculation. In both cases 0.5 mL of inoculum is used per egg. The virus pool passes the test if, after 3-7 days, there is no evidence of the presence of any extraneous agent.

*Vaccine prepared in primary rabbit kidney cell cultures.* The following special requirements apply to virus propagation, harvest and testing. On the day of inoculation with virus working seed, a sample of at least 30 mL of the pooled fluid is removed from the cell cultures of the kidneys of each group of animals used to prepare the primary cell suspension. The pooled fluid is inoculated in primary kidney cell cultures in such a way that the dilution of the pooled fluid does not exceed 1 in 4. The cultures are incubated at a temperature of 34-36 °C and observed for a period of at least 4 weeks. During this observation period and after not less than 2 weeks of incubation, at least 1 subculture of fluid is made from each of these cultures and observed also for a period of 2 weeks. The test is invalid if more than 20 per cent of the cultures are discarded. If evidence is found of the presence of an extraneous agent, no cell cultures from the entire group may be used for vaccine production.

- *Control cell cultures.* Cultures prepared on the day of inoculation with the working virus seed lot from 25 per cent of the cell suspensions obtained from the kidneys of each group of animals are maintained as controls. These control cell cultures are incubated under the same conditions as the inoculated cultures for at least 2 weeks. The test is invalid if more than 20 per cent of the control cell cultures are discarded for non-specific reasons.
- *Test for haemadsorbing viruses.* At the time of harvest or not more than 4 days after the day of inoculation of the production cultures with the virus working seed, a sample of 4 per cent of the control cell cultures is tested for haemadsorbing viruses by addition of guinea-pig red blood cells.
- *Test for other extraneous agents.* At the time of harvest or not more than 7 days after the day of inoculation of the production cultures with the virus working seed, a sample of at least 20 mL of the pooled fluid from each group of control cultures is tested for other extraneous agents.
- *Tests of neutralised single harvest in primary rabbit kidney cell cultures.* Each neutralised single harvest is additionally tested in primary kidney cell cultures prepared from a different group of animals to that used for production.

*POOLED HARVEST*

Only a pooled harvest that complies with the following requirements and is within the limits approved for the product may be used in the preparation of the final lot.

**Identity**. The vaccinia virus in the pooled harvest is identified by serological methods, which may be supplemented by molecular methods. Molecular tests such as restriction fragment length polymorphism or partial sequencing, especially of terminal DNA sequences which show the greatest variation between vaccinia strains, may be useful.

**Virus concentration**. The vaccinia virus concentration of the pooled harvest is determined by chick egg CAM assay or in cell cultures. A reference preparation is assayed in the same system in parallel for validation of the pooled harvest titration. The virus concentration serves as the basis for the quantity of virus used in the neurovirulence test in mice.

**Consistency of virus characteristics**. Vaccinia virus in the pooled harvest or the final bulk is examined by tests that are able to determine that the phenotypic and genetic characteristics of the vaccinia virus have not undergone changes during the multiplication in the production system. The master seed or an equivalent preparation is used as a comparator in these tests and the comparator and the tests to be used are approved by the competent authority.

**Neurovirulence**. The neurovirulence of the pooled harvest is assessed versus a comparator original seed (or equivalent) by intracerebral inoculation into suckling mice. Other tests may be useful to discriminate between acceptable and unacceptable batches.

**Residual DNA**. For viruses grown in continuous cells the pooled harvest is tested for residual DNA. The production process demonstrates a level of cellular DNA of less than 10 ng per human dose.

**Bacterial and fungal contamination**. For vaccines other than those prepared on animal skins, the final bulk complies with the test for sterility (*2.6.1*) using 10 mL for each medium.

**Mycoplasma** (*2.6.7*). For vaccines other than those prepared on animal skins, the final bulk complies with the test for mycoplasma, carried out using 10 mL.

*FINAL BULK VACCINE*

A minimum virus concentration for release of the product is established such as to ensure, in the light of stability data, that the minimum concentration stated on the label will be present at the end of the period of validity.

#### VACCINE PRODUCED IN LIVING ANIMALS

The pooled harvest is centrifuged. If the vaccine is intended for issue in the liquid form, treatment to reduce the presence of extraneous agents may consist of the addition of glycerol or another suitable diluent, with or without an antimicrobial substance, and temporary storage at a suitable temperature. If the vaccine is intended for issue in the dried form, the treatment may consist of the addition of a suitable antimicrobial substance. The following special requirements apply to the bulk vaccine for vaccines produced in living animals.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Total bacterial count**: for vaccines produced on animal skins only, maximum 50 per millilitre, determined by plate count using a suitable volume of the final bulk vaccine.

***Escherichia coli***. At least 1 mL samples of a 1:100 dilution of the final bulk vaccine is cultured on plates of a medium suitable for differentiating *E. coli* from other bacteria. The plates are incubated at 35-37 °C for 48 h. If *E. coli* is detected the final bulk is discarded or, subject to approval by the competent authority, processed further.

**Haemolytic streptococci, coagulase-positive staphylococci or any other pathogenic micro-organisms which are known to be harmful to man by vaccination**. At least 1 mL samples of a 1:100 dilution of the final bulk vaccine are cultured on blood agar. The plates are incubated at 35-37 °C for 48 h. If micro-organisms are detected, the final bulk vaccine is discarded.

***Bacillus anthracis***. Any colony seen on any of the plates that morphologically resembles *B. anthracis* is examined. If the organisms contained in the colony are non-motile, further tests for the cultural character of *B. anthracis* are carried out, including pathogenicity tests in suitable animals. If *B. anthracis* is found to be present, the final bulk vaccine and any other associated bulks are discarded. Additional validated molecular testing may be performed.

***Clostridium tetani* and other pathogenic spore-forming anaerobes**. A total volume of not less than 10 mL of the final bulk vaccine is distributed in equal amounts into 10 tubes, each containing not less than 10 mL of suitable medium for the growth of anaerobic micro-organisms. The tubes are kept at 65 °C for 1 h in order to reduce the content of non-spore-forming organisms, after which they are anaerobically incubated at 35-37 °C for at least 1 week. From every tube or plate showing growth, subcultures are made on plates of a suitable medium. Tubes and plates are incubated anaerobically at the same temperature. All anaerobic colonies are examined and identified and if *C. tetani* or other pathogenic spore-forming anaerobes are present, the final bulk is discarded.

#### VACCINE PRODUCED IN EGGS

The pooled harvest is clarified and may be further purified.

#### VACCINE PRODUCED IN CELL CULTURES (PRIMARY CHICK EMBRYOS FIBROBLASTS, HUMAN DIPLOID CELLS OR CONTINUOUS CELL LINES)

The pooled harvest is clarified to remove cells and may be further purified.

*FINAL LOT*

Only a final lot that complies with the requirements for minimum virus concentration for release, with the following requirement for thermal stability and with each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for antimicrobial preservative, protein content, bovine serum albumin and ovalbumin have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

**Thermal stability**

For liquid products, maintain not fewer than 3 containers of the final lot at an elevated temperature for a defined time period, using conditions found suitable for the particular product as approved by the competent authority. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the containers that have been heated does not decrease by more than an approved amount during the period of exposure. The conditions of the test and the requirements are approved by the competent authority.

For freeze-dried products, maintain at least 3 containers of the final lot in the dry state at 37 ± 1 °C for 28 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

## IDENTIFICATION

The vaccinia virus is identified by an appropriate method.

## TESTS

**Antimicrobial preservative**. Where applicable determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Phenol** (*2.5.15*): maximum 0.5 per cent, if phenol is used.

**Protein content**. The protein content of each filling lot, if not done on the final bulk, is determined and is within the limits approved by the competent authority.

**Bovine serum albumin**: maximum 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*), where bovine serum albumin is used during cell culture.

**Ovalbumin**. For vaccines produced in embryonated eggs, the ovalbumin content is within the limits approved by the competent authority.

**Residual moisture**. The residual moisture content of each final lot of freeze-dried vaccines is within the limits approved by the competent authority.

**Bacterial count**. For skin-derived vaccines, examine the vaccine by suitable microscopic and culture methods for micro-organisms pathogenic for man and, in particular, haemolytic streptococci, staphylococci, pathogenic spore-bearing organisms, especially *B. anthracis*, and *E. coli*. The vaccine is free from such contaminants. The total number of non-pathogenic bacteria does not exceed 50 per millilitre.

**Sterility** (*2.6.1*). Except for skin-derived vaccines, the vaccine complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*). The vaccine complies with the specification approved by the competent authority.

## ASSAY

Reconstitue the vaccine if necessary and titrate for infectious virus using at least 3 separate containers of vaccine. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined virus concentration for the 3 containers of vaccine is not less than 8.0 $\log_{10}$ pock-forming units per millilitre or the validated equivalent in plaque-forming units or 50 per cent cell culture infective doses, unless a lower titre is justified by clinical studies.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.5 $\log_{10}$ infectious units;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ infectious units from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.5 $\log_{10}$ infectious units; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.5 $\log_{10}$ infectious units.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

## LABELLING

The label states:

- the designation of the vaccinia virus strain;
- the minimum amount of virus per millilitre;
- the substrate used for the preparation of the vaccine;
- the nature and amount of stabiliser, preservative or additive present in the vaccine and/or in the diluent.

01/2008:0452
corrected 10.0

# TETANUS VACCINE (ADSORBED)

## Vaccinum tetani adsorbatum

DEFINITION

Tetanus vaccine (adsorbed) is a preparation of tetanus formol toxoid with a mineral adsorbent. The formol toxoid is prepared from the toxin produced by the growth of *Clostridium tetani*.

PRODUCTION

*GENERAL PROVISIONS*

**Specific toxicity**. The production method is validated to demonstrate that the product, if tested, would comply with the following test: inject subcutaneously 5 times the single human dose stated on the label into each of 5 healthy guinea-pigs, each weighing 250-350 g, that have not previously been treated with any material that will interfere with the test. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test once; if more than 1 animal dies in the second test, the vaccine does not comply with the test.

*BULK PURIFIED TOXOID*

For the production of tetanus toxin, from which toxoid is prepared, seed cultures are managed in a defined seed-lot system in which toxinogenicity is conserved and, where necessary, restored by deliberate reselection. A highly toxinogenic strain of *Clostridium tetani* with known origin and history is grown in a suitable liquid medium. At the end of cultivation, the purity of each culture is tested and contaminated cultures are discarded. Toxin-containing culture medium is collected aseptically. The toxin content (Lf per millilitre) is checked (*2.7.27*) to monitor consistency of production. Single harvests may be pooled to prepare the bulk purified toxoid. The toxin is purified to remove components likely to cause adverse reactions in humans. The purified toxin is detoxified with formaldehyde by a method that avoids destruction of the immunogenic potency of the toxoid and reversion of toxoid to toxin, particularly on exposure to heat. Alternatively, purification may be carried out after detoxification.

Only bulk purified toxoid that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

**Absence of toxin and irreversibility of toxoid**. Using the same buffer solution as for the final vaccine, without adsorbent, prepare a solution of bulk purified toxoid at the same concentration as in the final vaccine. Divide the dilution into 2 equal parts. Keep one of them at 5 ± 3 °C and the other at 37 °C for 6 weeks. Test both dilutions as described below. Use 15 guinea-pigs, each weighing 250-350 g and that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each of 5 guinea-pigs 5 mL of the dilution incubated at 5 ± 3 °C. Inject subcutaneously into each of 5 other guinea-pigs 5 mL of the dilution incubated at 37 °C. Inject subcutaneously into each of 5 guinea-pigs at least 500 Lf of the non-incubated bulk purified toxoid in a volume of 1 mL. The bulk purified toxoid complies with the test if during the 21 days following the injection no animal shows signs of or dies from tetanus. If more than 1 animal dies from non-specific causes, repeat the test; if more than 1 animal dies in the second test, the toxoid does not comply with the test.

**Antigenic purity** (*2.7.27*). Not less than 1000 Lf per milligram of protein nitrogen.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by adsorption of a suitable quantity of bulk purified toxoid onto a mineral carrier such as hydrated aluminium phosphate or aluminium hydroxide; the resulting mixture is approximately isotonic with blood. Suitable antimicrobial preservatives may be added. Certain antimicrobial preservatives, particularly those of the phenolic type, adversely affect the antigenic activity and must not be used.

Only final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

**Sterility** (*2.6.1*). Carry out the test for sterility using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers. The containers are closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided the test for antimicrobial preservative and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

Provided the free formaldehyde content has been determined on the bulk purified toxoid or on the final bulk and it has been shown that the content in the final lot will not exceed 0.2 g/L, the test for free formaldehyde may be omitted on the final lot.

IDENTIFICATION

Tetanus toxoid is identified by a suitable immunochemical method (*2.7.1*). The following method, applicable to certain vaccines, is given as an example. Dissolve in the vaccine to be examined sufficient *sodium citrate R* to give a 100 g/L solution. Maintain at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The clear supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical method. The content is not less than the minimum amount shown to be effective and is not greater than 115 per cent of the quantity stated on the label.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Carry out one of the prescribed methods for the assay of tetanus vaccine (adsorbed) (*2.7.8*).

The lower confidence limit ($P = 0.95$) of the estimated potency is not less than 40 IU per single human dose.

LABELLING

The label states:

- the minimum number of International Units per single human dose,
- the name and the amount of the adsorbent,

– that the vaccine must be shaken before use,
– that the vaccine is not to be frozen.

**01/2019:1375**

# TICK-BORNE ENCEPHALITIS VACCINE (INACTIVATED)

## Vaccinum encephalitidis ixodibus advectae inactivatum

DEFINITION

Tick-borne encephalitis vaccine (inactivated) is a liquid preparation of a suitable strain of tick-borne encephalitis virus grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by a suitable, validated method.

PRODUCTION

*GENERAL PROVISIONS*

Production of the vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently vaccines comparable with the vaccine of proven clinical efficacy and safety in man. Unless otherwise justified and authorised, the virus in the final vaccine shall not have undergone more passages from the master seed lot than the virus in the vaccine used in clinical trials.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in chick embryo cells prepared from eggs derived from a chicken flock free from specified pathogens (*5.2.2*) or in other suitable cell cultures (*5.2.3*).

*SEED LOTS*

The strain of virus used is identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are stored at or below – 60 °C.

Only a seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. Each seed lot is identified as containing the vaccine strain of tick-borne encephalitis virus by a suitable immunochemical method (*2.7.1*), preferably using monoclonal antibodies.

**Virus concentration**. The virus concentration of each seed lot is determined by titration in suitable cell cultures to monitor consistency of production.

**Extraneous agents** (*2.6.16*). Each seed lot complies with the requirements for extraneous agents in viral vaccines for human use. For neutralisation of the vaccine virus, the use of monoclonal antibodies is preferable.

*VIRUS PROPAGATION AND HARVEST*

If the virus has been passaged in mouse brain during preparation of the master seed lot, not fewer than 2 passages of the master seed virus in cell culture are made before inoculation of the production cell culture.

All processing of the cell cultures is performed under aseptic conditions in an area where no other cells are being handled. Serum and trypsin used in the preparation of cell suspensions and media used must be shown to be free from extraneous agents. The cell culture media may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. At least 500 mL of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells).

Only a single harvest that complies with the following requirements may be used in the preparation of the inactivated harvest.

**Identification**. The single harvest is shown to contain tick-borne encephalitis virus by a suitable immunochemical method (*2.7.1*), preferably using monoclonal antibodies, or by virus neutralisation in cell cultures.

**Bacterial and fungal contamination**. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). The single harvest complies with the test for mycoplasmas carried out using 1 mL for each medium.

**Control cells**. The control cells comply with the tests for extraneous agents (*2.6.16*). If the vaccine is produced using a cell-bank system, the control cells comply with a test for identification.

**Virus concentration**. Determine the virus concentration by titration in suitable cell cultures to monitor consistency of production.

*INACTIVATION*

To avoid interference, viral aggregates are removed, where necessary, by filtration immediately before the inactivation process. The virus suspension is inactivated by a validated method; the method shall have been shown to be consistently capable of inactivating tick-borne encephalitis virus without destroying the antigenic and immunogenic activity; as part of the validation studies, an inactivation curve is plotted representing residual live virus concentration measured on not fewer than 3 occasions. If formaldehyde is used for inactivation, the presence of an excess of free formaldehyde is verified at the end of the inactivation process.

Only an inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Residual infective virus**. Inoculate a quantity of the inactivated harvest equivalent to not less than 10 human doses of vaccine in the final lot into primary chicken fibroblast cell cultures, or other cells shown to be at least as sensitive to tick-borne encephalitis virus, with not less than 3 $cm^2$ of cell sheet per millilitre of inoculum. Incubate at 37 ± 1 °C for 14 days. No cytopathic effect is detected at the end of the incubation period. Collect the culture fluid and examine for the presence of infective tick-borne encephalitis virus by the following test in mice or by a validated *in vitro* method: inoculate 0.03 mL intracerebrally into each of not fewer than 10 mice about 4 weeks old. Observe the mice for 14 days. They show no evidence of tick-borne encephalitis virus infection.

*PURIFICATION*

Several inactivated single harvests may be pooled before concentration and purification by suitable methods, preferably by continuous-flow, sucrose density-gradient centrifugation.

Several purified inactivated harvests may be pooled.

Only a purified, inactivated harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Sterility** (*2.6.1*). The purified, inactivated harvest complies with the test for sterility carried out using 10 mL for each medium.

**Specific activity**. Determine the antigen content of the purified, inactivated harvest by a suitable immunochemical method (*2.7.1*). Determine the total protein content by a suitable method. The specific activity, calculated as the antigen content per unit mass of protein, is within the limits approved for the specific product.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared from one or more purified, inactivated harvests.

Only a final bulk vaccine that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the tests for free formaldehyde, bovine serum albumin (where applicable) and pyrogens and the assay have been carried out with satisfactory results on the final bulk vaccine, they may be omitted on the final lot.

IDENTIFICATION

The vaccine is shown to contain tick-borne encephalitis virus antigen by a suitable immunochemical method (*2.7.1*) using specific antibodies. The assay also serves to identify the vaccine.

TESTS

**Aluminium** (*2.5.13*): maximum 1.25 mg per single human dose, if aluminium hydroxide or hydrated aluminium phosphate is used as the adsorbent.

**Free formaldehyde** (*2.4.18*): maximum 0.1 g/L.

**Bovine serum albumin**: maximum 50 ng per single human dose, determined by a suitable immunochemical method (*2.7.1*), if bovine serum albumin has been used during production.

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

**Pyrogens** (*2.6.8*). The vaccine complies with the test for pyrogens. Inject into each rabbit, per kilogram of body mass, 1 dose of vaccine.

ASSAY

The potency is determined by comparing the dose necessary to protect a given proportion of mice against the effects of a lethal dose of tick-borne encephalitis virus, administered intraperitoneally, with the quantity of a reference preparation of tick-borne encephalitis vaccine necessary to provide the same protection. For this comparison an approved reference preparation and a suitable preparation of tick-borne encephalitis virus from an approved strain for use as the challenge preparation are necessary.

*The following is cited as an example of a method that has been found suitable for a given vaccine.*

*Selection and distribution of test animals.* Use healthy mice weighing 11-17 g and derived from the same stock. Distribute the mice into not fewer than 6 groups of a suitable size to meet the requirements for validity of the test; for titration of the challenge suspension, use not fewer than 4 groups of 10 mice. Use mice of the same sex or distribute males and females equally between groups.

*Determination of potency of the vaccine.* Prepare not fewer than 3 suitable dilutions of the vaccine to be examined and of the reference preparation; in order to comply with validity criteria 4 or 5 dilutions will usually be necessary. Prepare dilutions such that the most concentrated suspension is expected to protect more than 50 per cent of the animals and the least concentrated suspension less than 50 per cent. Allocate each dilution to a different group of mice and inject subcutaneously into each mouse 0.2 mL of the dilution allocated to its group. 7 days later make a second injection using the same dilution scale. 14 days after the second injection prepare a suspension of the challenge virus containing not less than 100 $LD_{50}$ in 0.2 mL. Inject 0.2 mL of this virus suspension intraperitoneally into each vaccinated mouse. To verify the challenge dose, prepare a series of not fewer than 3 dilutions of the challenge virus suspension at not greater than one-hundredfold intervals. Allocate the challenge suspension and all of the dilutions, one to each of the groups of 10 mice, and inject intraperitoneally into each mouse 0.2 mL of the challenge suspension or the dilution allocated to its group. Observe the animals for 21 days after the challenge and record the number of mice that die in the period between 7 and 21 days after the challenge. Humane endpoints may be used to avoid unnecessary suffering of animals after the virulent challenge.

*Calculations.* Calculate the results for an assay with quantal responses by the usual statistical methods (for example, *5.3*).

*Validity criteria.* The test is not valid unless:

- the concentration of the challenge virus is not less than 100 $LD_{50}$,
- for both the vaccine to be examined and the reference preparation the 50 per cent protective dose ($PD_{50}$) lies between the largest and smallest doses given to the mice,
- the statistical analysis shows a significant slope and no significant deviation from linearity and parallelism of the dose-response lines,
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

*Potency requirement.* Include all valid tests to estimate the mean potency and the confidence limits ($P = 0.95$) for the mean potency; compute weighted means with the inverse of the squared standard error as weights. The vaccine complies with the test if the estimated potency is not less than that approved by the competent authority, based on data from clinical efficacy trials.

LABELLING

The label states:

- the strain of virus used in preparation,
- the type of cells used for production of the vaccine.

**01/2019:1160**
**corrected 10.0**

# TYPHOID POLYSACCHARIDE VACCINE

## Vaccinum febris typhoidis polysaccharidicum

DEFINITION

Typhoid polysaccharide vaccine is a preparation of purified Vi capsular polysaccharide obtained from *Salmonella typhi* Ty 2 strain or some other suitable strain that has the capacity to produce Vi polysaccharide.

Capsular Vi polysaccharide consists of partly 3-*O*-acetylated repeated units of 2-acetylamino-2-deoxy-D-galactopyranuronic acid with α-(1→4) linkages.

PRODUCTION

The production of Vi polysaccharide is based on a seed-lot system. The method of production shall have been shown to yield consistently typhoid polysaccharide vaccines of adequate immunogenicity and safety in man.

*BACTERIAL SEED LOTS*

The strain of *S. typhi* used for the master seed lot shall be identified by historical records that include information on its origin and by its biochemical and serological characteristics. Cultures from the working seed lot shall have the same characteristics as the strain that was used to prepare the master seed lot.

Only a strain that has the following characteristics may be used in the preparation of the vaccine: (a) stained smears from a culture are typical of enterobacteria; (b) the culture utilises glucose without production of gas; (c) colonies on agar are oxidase-negative; (d) a suspension of the culture agglutinates specifically with a suitable Vi antiserum or colonies form haloes on an agar plate containing a suitable Vi antiserum.

The purity of the bacterial strain used for the seed lot is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

*CULTURE AND HARVEST*

The working seed lot is cultured on a solid medium, which may contain blood-group substances, or a liquid medium; the inoculum obtained is transferred to a liquid medium which is used to inoculate the final medium. The liquid medium used and the final medium are semi-synthetic, free from substances that are precipitated by cetrimonium bromide and do not contain blood-group substances or high-molecular-mass polysaccharides, unless it has been demonstrated that they are removed by the purification process.

The bacterial purity of the culture is verified by methods of suitable sensitivity. These may include inoculation into suitable media, examination of colony morphology, microscopic examination of Gram-stained smears and culture agglutination with suitable specific antisera.

The culture is then inactivated at the beginning of the stationary phase by the addition of formaldehyde. Bacterial cells are eliminated by centrifugation; the polysaccharide is precipitated from the culture medium by addition of hexadecyltrimethylammonium bromide (cetrimonium bromide). The precipitate is harvested and may be stored at − 20 °C before purification.

*PURIFIED Vi POLYSACCHARIDE*

The polysaccharide is purified, after dissociation of the polysaccharide/cetrimonium bromide complex, using suitable procedures to eliminate successively nucleic acids, proteins and lipopolysaccharides. The polysaccharide is precipitated as the calcium salt in the presence of ethanol and dried at 2-8 °C; the powder obtained constitutes the purified Vi polysaccharide. The loss on drying is determined by thermogravimetry (*2.2.34*) and is used to calculate the results of the chemical tests shown below with reference to the dried substance.

Only a purified Vi polysaccharide that complies with the following requirements may be used in the preparation of the final bulk.

**Protein** (*2.5.16*): maximum 10 mg per gram of polysaccharide, calculated with reference to the dried substance.

**Nucleic acids** (*2.5.17*): maximum 20 mg per gram of polysaccharide, calculated with reference to the dried substance.

***O*-Acetyl groups** (*2.5.19*): minimum 2 mmol per gram of polysaccharide, calculated with reference to the dried substance.

**Molecular-size or molecular-mass distribution**. Molecular-size or molecular-mass distribution is determined by size-exclusion chromatography (*2.2.30*) combined with an appropriate detection system. An acceptable value is established for the purified polysaccharide. Each batch must be shown to comply with this limit.

**Identification**. Carry out an identification test using a suitable immunochemical method (*2.7.1*).

**Bacterial endotoxins**. The content of bacterial endotoxins determined by a suitable method (*2.6.14*) is within the limits approved for the specific product.

*FINAL BULK VACCINE*

One or more batches of purified Vi polysaccharide are dissolved in a suitable solvent, which may contain an antimicrobial preservative, so that the volume corresponding to 1 dose contains 25 µg of polysaccharide and the solution is isotonic with blood (250 mosmol/kg to 350 mosmol/kg).

Only a final bulk vaccine that complies with the following tests may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk vaccine complies with the test for sterility, carried out using 10 mL for each medium.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable physico-chemical method. The amount is not less than 85 per cent and not greater than 115 per cent of the intended amount.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile tamper-evident containers that are then closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements prescribed below under Identification, Tests and Assay and with the requirement for bacterial endotoxins may be released for use. Provided the tests for free formaldehyde and antimicrobial preservative have been carried out on the final bulk vaccine, they may be omitted on the final lot.

**Bacterial endotoxins**. The content of bacterial endotoxins determined by a suitable method (*2.6.14*) is within the limit approved for the specific product.

CHARACTERS

Clear colourless liquid, free from visible particles.

IDENTIFICATION

Carry out an identification test using a suitable immunochemical method (*2.7.1*).

TESTS

**pH** (*2.2.3*): 6.5 to 7.5.

***O*-Acetyl groups**: 0.085 (± 25 per cent) µmol per dose (25 µg of polysaccharide).

*Test solution.* Place 3 mL of the vaccine in each of 3 tubes (2 reaction solutions and 1 correction solution).

*Reference solutions.* Dissolve 0.150 g of *acetylcholine chloride R* in 10 mL of *water R* (stock solution containing 15 g/L of acetylcholine chloride). Immediately before use, dilute 0.5 mL of the stock solution to 50 mL with *water R* (working dilution containing 150 µg/mL of acetylcholine chloride). In 10 tubes, place in duplicate (reaction and correction solutions) 0.1 mL, 0.2 mL, 0.5 mL, 1.0 mL and 1.5 mL of the working dilution.

Prepare a blank using 3 mL of *water R*.

Make up the volume in each tube to 3 mL with *water R*. Add 0.5 mL of a mixture of 1 volume of *water R* and 2 volumes of *dilute hydrochloric acid R* to each of the correction tubes and to the blank. Add 1.0 mL of *alkaline hydroxylamine solution R* to each tube. Allow the reaction to proceed for exactly 2 min and add 0.5 mL of a mixture of 1 volume of *water R* and 2 volumes of *dilute hydrochloric acid R* to each of the reaction tubes. Add 0.5 mL of a 200 g/L solution of *ferric chloride R* in *0.2 M hydrochloric acid* to each tube, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (*2.2.25*) of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the 5 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 2 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

**Free formaldehyde** (*2.4.18*): maximum 0.2 g/L.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than

115 per cent of the content stated on the label. If phenol has been used in the preparation, the content is not more than 2.5 g/L (*2.5.15*).

**Sterility** (*2.6.1*). The vaccine complies with the test for sterility.

ASSAY

Determine Vi polysaccharide by a suitable immunochemical method (*2.7.1*), using a reference purified polysaccharide. The estimated amount of polysaccharide per dose is 80 per cent to 120 per cent of the content stated on the label. The confidence limits ($P$ = 0.95) of the estimated amount of polysaccharide are not less than 80 per cent and not more than 120 per cent.

LABELLING

The label states:

- the number of micrograms of polysaccharide per single human dose (25 µg);
- the total quantity of polysaccharide in the container.

**01/2019:0156**

# TYPHOID VACCINE

## Vaccinum febris typhoidis

DEFINITION

Typhoid vaccine is a sterile suspension of inactivated *Salmonella typhi* containing not less than $5 \times 10^8$ and not more than $1 \times 10^9$ bacteria (*S. typhi*) per human dose. The human dose does not exceed 1.0 mL.

PRODUCTION

The vaccine is prepared using a seed-lot system from a suitable strain, such as Ty 2[(7)], of *S. typhi*. The final vaccine represents not more than 3 subcultures from the strain on which were made the laboratory and clinical tests that showed it to be suitable. The bacteria are inactivated by acetone, by formaldehyde, by phenol or by heating or by a combination of the last 2 methods.

IDENTIFICATION

It is identified by specific agglutination.

TESTS

**Phenol** (*2.5.15*). If phenol has been used in the preparation, the concentration is not more than 5 g/L.

**Antigenic power**. When injected into susceptible laboratory animals, it elicits anti-O, anti-H and, to a lesser extent, anti-Vi agglutinins.

**Sterility** (*2.6.1*). It complies with the test for sterility.

LABELLING

The label states:

- the method used to inactivate the bacteria;
- the number of bacteria per human dose.

**07/2015:1055**

# TYPHOID VACCINE (LIVE, ORAL, STRAIN Ty 21a)

## Vaccinum febris typhoidis vivum perorale (stirpis Ty 21a)

DEFINITION

Typhoid vaccine (live, oral, strain Ty 21a) is a freeze-dried preparation of live *Salmonella typhi* strain Ty 21a grown in a suitable medium. When presented in capsules, the vaccine complies with the monograph *Capsules (0016)*.

PRODUCTION

*CHOICE OF VACCINE STRAIN*

The main characteristic of the strain is the defect of the enzyme uridine diphosphate-galactose-4-epimerase. The activities of galactopermease, galactokinase and galactose-1-phosphate uridyl-transferase are reduced by 50 per cent to 90 per cent. Whatever the growth conditions, the strain does not contain Vi antigen. The strain agglutinates to anti-O:9 antiserum only if grown in medium containing galactose. It contains the flagellar H:d antigen and does not produce hydrogen sulfide on Kligler iron agar. The strain is nonvirulent for mice. Cells of strain Ty 21a lyse if grown in the presence of 1 per cent of galactose.

*BACTERIAL SEED LOTS*

The vaccine is prepared using a seed-lot system. The working seed lots represent not more than one subculture from the master seed lot. The final vaccine represents not more than four subcultures from the original vaccine on which were made the laboratory and clinical tests showing the strain to be suitable.

Only a master seed lot that complies with the following requirements may be used in the preparation of working seed lots.

**Galactose metabolism**. In a spectrophotometric assay, no activity of the enzyme uridine diphosphate-galactose-4-epimerase is found in the cytoplasm of strain Ty 21a compared to strain Ty 2.

**Biosynthesis of lipopolysaccharide**. Lipopolysaccharides are extracted by the hot-phenol method and examined by size-exclusion chromatography. Strain Ty 21a grown in medium free of galactose shows only the rough (R) type of lipopolysaccharide.

**Serological characteristics**. Strain Ty 21a grown in a synthetic medium without galactose does not agglutinate to specific anti-O:9 antiserum. Whatever the growth conditions, strain Ty 21a does not agglutinate to Vi antiserum. Strain Ty 21a agglutinates to H:d flagellar antiserum.

**Biochemical markers**. Strain Ty 21a does not produce hydrogen sulfide on Kligler iron agar. This property serves to distinguish Ty 21a from other galactose-epimerase-negative *S. typhi* strains.

**Cell growth**. Strain Ty 21a cells lyse when grown in the presence of 1 per cent of galactose.

*BACTERIAL PROPAGATION AND HARVEST*

The bacteria from the working seed lot are multiplied in a preculture, subcultured once and are then grown in a suitable medium containing 0.001 per cent of galactose at 30 °C for 13 h to 15 h. The bacteria are harvested. The harvest must be free from contaminating micro-organisms.

(7) This strain is issued by the World Health Organization Collaborating Centre for Reference and Research on Bacterial Vaccines, Human Serum and Vaccine Institute, Szallas Utea 5, H-1107, Budapest, Hungary.

Only a single harvest that complies with the following requirements may be used for the preparation of the freeze-dried harvest.

**pH**. The pH of the culture is 6.8 to 7.5.

**Optical density**. The optical density of the culture, measured at 546 nm, is 6.5 to 11.0. Before carrying out the measurement, dilute the culture so that a reading in the range 0.1 to 0.5 is obtained and correct the reading to take account of the dilution.

**Identification**. Culture bacteria on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

*FREEZE-DRIED HARVEST*

The harvest is mixed with a suitable stabiliser and freeze-dried by a process that ensures the survival of at least 10 per cent of the bacteria and to a water content shown to be favourable to the stability of the vaccine. No antimicrobial preservative is added to the vaccine.

Only a freeze-dried harvest that complies with the following tests may be used for the preparation of the final bulk.

**Identification**. Culture bacteria are examined on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

**Number of live bacteria**. Not fewer than $1 \times 10^{11}$ live *S. typhi* strain Ty 21a per gram.

**Water** (*2.5.12*): 1.5 per cent to 4.0 per cent, determined by the semi-micro determination of water.

*FINAL BULK VACCINE*

The final bulk vaccine is prepared by mixing under suitable conditions one or more freeze-dried harvests with suitable excipients.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

**Number of live bacteria**. Not fewer than $40 \times 10^9$ live *S. typhi* strain Ty 21a per gram.

*FINAL LOT*

The final bulk vaccine is distributed under suitable conditions into capsules with a gastro-resistant shell or into suitable containers.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Number of live bacteria may be released for use, except that in the determination of the number of live bacteria each dosage unit must contain not fewer than $4 \times 10^9$ live bacteria.

## IDENTIFICATION

Culture bacteria from the vaccine to be examined on an agar medium containing 1 per cent of galactose and bromothymol blue. Light blue, concave colonies, transparent due to lysis of cells, are formed. No yellow colonies (galactose-fermenting) are found.

## TESTS

**Microbial contamination**. Inoculate at least a total of $2 \times 10^9$ live *S. typhi* Ty 21a on at least 10 plates of casein soya bean digest agar and on at least 10 plates of Sabouraud-dextrose agar. Incubate in aerobic conditions the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Observe the plates for conspicuous colonies of micro-organisms in a lawn of weakly growing *S. typhi* Ty 21a. The number of contaminating micro-organisms per dosage unit is not greater than $10^2$ bacteria and 20 fungi.

Carry out the test for specified micro-organisms on selective media and under cultivation conditions as indicated in Table 1055.-1. Test a dose equivalent to at least $2 \times 10^9$ live *S. typhi* Ty 21a.

Growth-promoting and inhibitory properties of the media used and suitability of the testing conditions are demonstrated.

Composition of culture media listed in Table 1055.-1 that are not described in general chapter *2.6.13. Microbiological examination of non-sterile products: test for specified micro-organisms.*

*Mannitol salt broth*

| | |
|---|---|
| Proteose peptone | 10.0 g |
| Beef extract | 1.5 g |
| Sodium chloride | 75.0 g |
| D-Mannitol | 10.0 g |
| Purified water | 1000 mL |

*Acetamide broth*

| | |
|---|---|
| Acetamide | 2.0 g |
| Magnesium sulfate | 0.2 g |
| Sodium chloride | 0.2 g |
| Ferric sulfate | 0.0005 g |
| Potassium dihydrogen phosphate | 1.0 g |
| Sodium molybdate | 0.005 g |
| Purified water | 1000 mL |

*Bromothymol blue galactose broth*

| | |
|---|---|
| Heart infusion broth | 25.0 g |
| Sodium chloride | 5.0 g |
| Purified water | 930 mL |

Sterilise the medium and cool to 45 °C, then add 1.0 g of sodium thiosulfate, 40 mL of bromothymol blue solution and 30 mL of galactose solution.

*Bromothymol blue galactose agar*

| | |
|---|---|
| Heart infusion agar | 40.0 g |
| Sodium chloride | 5.0 g |
| Purified water | 930 mL |

Sterilise the medium and cool to 45 °C, then add 1.0 g of sodium thiosulfate, 40 mL of bromothymol blue solution and 30 mL of galactose solution.

*Bromothymol blue solution*

| | |
|---|---|
| Bromothymol blue | 2.0 g |
| 0.1 M sodium hydroxide | 50 mL |
| Purified water | 950 mL |

*Galactose solution*

| | |
|---|---|
| D-galactose | 33.0 g |
| Purified water | 100 mL |

The product complies with the test if no colonies of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* or clostridia, and no salmonella other than strain Ty 21a, are found.

**Water** (*2.5.12*): 1.5 per cent to 4.0 per cent, determined on the contents of the capsule or of the container by the semi-micro determination of water.

NUMBER OF LIVE BACTERIA

Carry out the test using not fewer than 5 dosage units. Homogenise the contents of the dosage units in a 9 g/L solution of *sodium chloride R* at 4 °C using a mixer in a cold room with sufficient glass beads to emerge from the liquid. Immediately after homogenisation prepare a suitable dilution of the suspension using cooled diluent and inoculate brain heart infusion agar; incubate at 36 ± 1 °C for 20 h to 36 h. The vaccine contains not fewer than $2 \times 10^9$ live *S. typhi* Ty 21a bacteria per dosage unit.

LABELLING

The label states:

– the minimum number of live bacteria per dosage unit;

– that the vaccine is for oral use only.

Table 1055.-1. – *Test for specified micro-organisms: pre-incubation and subculture conditions*

| Micro-organism | Pre-incubation 1 | Pre-incubation 2 | Subculture |
|---|---|---|---|
| *Staphylococcus aureus* | Mannitol salt broth<br>30-35 °C<br>40-48 h | | Mannitol salt agar*<br>30-35 °C<br>18-72 h |
| *Pseudomonas aeruginosa* | Acetamide broth<br>30-35 °C<br>40-48 h | | Cetrimide agar*<br>30-35 °C<br>18-72 h |
| *Escherichia coli* | Casein soya bean digest broth*<br>30-35 °C<br>18-24 h | MacConkey broth*<br>41-45 °C<br>18-24 h | MacConkey agar*<br>30-35 °C<br>18-72 h |
| *Salmonella* spp. | Bromothymol blue galactose broth<br>30-35 °C<br>18-24 h | | Bromothymol blue galactose agar<br>30-35 °C<br>18-72 h |
| Clostridia | Reinforced medium for clostridia*<br>30-35 °C<br>40-48 h<br>Unheated and heated at 80 °C for 10 min in buffered sodium chloride-peptone solution pH 7.0*<br>Anaerobic conditions | | Columbia agar*<br>30-35 °C<br>18-72 h<br>Anaerobic conditions |

*Culture media recommended for identification of specified micro-organisms (see general chapter *2.6.13* for composition)

**01/2019:0648**
**corrected 10.0**

# VARICELLA VACCINE (LIVE)

## Vaccinum varicellae vivum

DEFINITION

Varicella vaccine (live) is a freeze-dried preparation of a suitable attenuated strain of human herpesvirus 3. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid that may be coloured owing to the presence of a pH indicator.

PRODUCTION

The production of vaccine is based on a virus seed-lot system and a cell-bank system. The production method shall have been shown to yield consistently live varicella vaccines of adequate immunogenicity and safety in man. The virus in the final vaccine shall not have been passaged in cell cultures beyond a defined number of passages approved by the competent authority from the original isolated virus.

The potential neurovirulence of the vaccine strain is considered during preclinical development, based on available epidemiological data on neurovirulence and neurotropism, primarily for the wild-type virus. In light of this, a risk analysis is carried out. Where necessary and if available, a test is carried out on the vaccine strain using an animal model that differentiates wild-type and attenuated virus; tests on strains of intermediate attenuation may also be needed.

*SUBSTRATE FOR VIRUS PROPAGATION*

The virus is propagated in human diploid cells (*5.2.3*).

*VIRUS SEED LOT*

The strain of human herpesvirus 3 used shall be identified as being suitable by historical records that include information on the origin of the strain and its subsequent manipulation. The virus shall at no time have been passaged in continuous cell lines. Seed lots are prepared in the same kind of cells as those used for the production of the final vaccine. Virus seed lots are prepared in large quantities and stored at temperatures below – 20 °C if freeze-dried, or below – 60 °C if not freeze-dried.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as human herpesvirus 3 by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The virus concentration of the master and working seed lots is determined as prescribed under Assay to monitor consistency of production.

**Extraneous agents** (*2.6.16*). The working seed lot complies with the requirements for seed lots for live virus vaccines; a sample of 50 mL is taken for the test in cell cultures.

*VIRUS PROPAGATION AND HARVEST*

All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area where no other cells or viruses are being handled. Approved animal (but not human) serum may be used in the culture media. Serum and trypsin used in the preparation of cell suspensions and media are shown to be free from extraneous agents. The cell culture medium may contain a pH indicator such as phenol red and approved antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production. 5 per cent, but not less than 50 mL, of the cell cultures employed for vaccine production is set aside as uninfected cell cultures (control cells). The infected cells

constituting a single harvest are washed, released from the support surface and pooled. The cell suspension is disrupted by sonication.

Only a virus harvest that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The virus harvest contains virus that is identified as human herpesvirus 3 by serum neutralisation in cell culture, using specific antibodies.

**Virus concentration**. The concentration of infective virus in virus harvests is determined as prescribed under Assay to monitor consistency of production and to determine the dilution to be used for the final bulk vaccine.

**Extraneous agents** (*2.6.16*). Use 50 mL for the test in cell cultures.

**Control cells**. The control cells of the production cell culture from which the single harvest is derived comply with a test for identity and with the requirements for extraneous agents (*2.6.16*).

*FINAL BULK VACCINE*

Virus harvests that comply with the above tests are pooled and clarified to remove cells. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*) using 10 mL for each medium.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to the test for water and each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for bovine serum albumin has been carried out with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Water** (*2.5.12*). Not more than the amount shown to ensure stability of the vaccines as approved by the competent authority, determined by the semi-micro determination of water.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific human herpesvirus 3 antibodies, it is no longer able to infect susceptible cell cultures.

TESTS

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bovine serum albumin**: maximum 0.5 µg per human dose, determined by a suitable immunochemical method (*2.7.1*).

ASSAY

Titrate the vaccine for infective virus, using at least 3 separate vials of vaccine. Titrate 1 vial of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. The relation with the appropriate European Pharmacopoeia Biological Reference Preparation is established and monitored at regular intervals if a manufacturer's reference preparation is used. Calculate the individual virus concentration for each vial of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations, using the usual statistical methods (for example, *5.3*). The combined estimate of the virus concentration for the 3 vials of vaccine is not less than that stated on the label.

The assay is not valid if:

- the confidence interval ($P = 0.95$) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ PFU;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ PFU from the established value.

The assay is repeated if the confidence interval ($P = 0.95$) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ PFU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P = 0.95$) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ PFU.

*Varicella vaccine (live) BRP* is suitable for use as a reference preparation.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used for the preparation of the vaccine;
- the type and origin of the cells used for the preparation of the vaccine;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

**01/2020:0537**

# YELLOW FEVER VACCINE (LIVE)

## Vaccinum febris flavae vivum

DEFINITION

Yellow fever vaccine (live) is a freeze-dried preparation of yellow fever virus derived from the 17D strain and grown in fertilised hen eggs. The vaccine is reconstituted immediately before use, as stated on the label, to give a clear liquid.

PRODUCTION

The production of vaccine is based on a virus seed-lot system. The production method shall have been shown to yield consistently yellow fever vaccine (live) of acceptable immunogenicity and safety for man.

*Reference preparation*. In the test for neurotropism, a suitable batch of vaccine known to have satisfactory properties in man is used as the reference preparation.

A reference preparation calibrated in International Units per ampoule is used to verify the titre of the virus inoculum in the tests for viraemia (viscerotropism) and immunogenicity, and to titrate the vaccine batch in the potency assay.

The International Unit is the activity contained in a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*SUBSTRATE FOR VIRUS PROPAGATION*

Virus for the preparation of master and working seed lots and of all vaccine batches is grown in the tissues of chick embryos from a flock free from specified pathogens (SPF) (*5.2.2*).

*SEED LOTS*

The 17D strain shall be identified by historical records that include information on the origin of the strain and its subsequent manipulation. Virus seed lots are prepared in large quantities and stored at a temperature below – 60 °C. Master and working seed lots shall not contain any human protein, added serum or antibiotics.

Unless otherwise justified and authorised, the virus in the final vaccine shall be between passage levels 204 and 239 from the original isolate of strain 17D. A working seed lot shall be only 1 passage from a master seed lot. A working seed lot shall be used without intervening passage as the inoculum for infecting the tissues used in the production of a vaccine lot, so that no vaccine virus is more than 1 passage from a seed lot that has passed all the safety tests.

Only a virus seed lot that complies with the following requirements may be used for virus propagation.

**Identification**. The master and working seed lots are identified as containing yellow fever virus by serum neutralisation in cell culture using specific antibodies, or by molecular methods (e.g. nucleic acid amplification techniques (NAT), sequencing).

**Extraneous agents** (*2.6.16*). Each master seed lot complies with the following tests:

- bacterial and fungal sterility (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycoplasmas (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycobacteria (as described in chapter *2.6.16* under Virus seed lot and virus harvests).

**Avian leucosis viruses** (*2.6.24*). Each master seed lot complies with the test for avian leucosis viruses.

**Extraneous agents** (*2.6.16*). Each working seed lot complies with the following tests:

- bacterial and fungal sterility (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycoplasmas (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- mycobacteria (as described in chapter *2.6.16* under Virus seed lot and virus harvests);
- test in cell culture for other extraneous agents: a neutralised sample of 5 mL of working seed lot, representing at least 500 000 (5.7 $\log_{10}$) IU, is tested for the presence of extraneous agents by inoculation into continuous simian kidney and human cell cultures as well as into primary chick-embryo-fibroblast cells; the cells are incubated at 36 ± 1 °C and observed for a period of 14 days; the working seed lot passes the test if there is no evidence of the presence of any extraneous agents; the test is not valid unless at least 80 per cent of the cell cultures remain viable;
- avian viruses: a neutralised sample of 1 mL of working seed lot, representing at least 100 000 (5.0 $\log_{10}$) IU, is tested for the presence of avian viruses by inoculation by the allantoic route into a group of at least 20 fertilised, 9- to 11-day-old, SPF eggs (*5.2.2*), and by inoculation into the yolk sac of a group of at least 20 fertilised, 5- to 7-day-old, SPF eggs (*5.2.2*); incubate for 7 days; the working seed lot complies if the allantoic and yolk sac fluids show no signs of haemagglutinating agents and if the embryos and chorio-allantoic membranes examined to detect any macroscopic pathology are typical; the test is not valid unless at least 80 per cent of the inoculated eggs survive during the 7-day observation period.

**Avian leucosis viruses** (*2.6.24*). Each working seed lot complies with the test for avian leucosis viruses.

**Tests in monkeys**. Each master and working seed lot complies with the following tests in monkeys for viraemia (viscerotropism), immunogenicity and neurotropism.

The monkeys shall be *Macaca* sp. susceptible to yellow fever virus and shall have been shown to be non-immune to yellow fever at the time of injecting the seed virus. They shall be healthy and shall not have received previously intracerebral or intraspinal inoculation. Furthermore, they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever virus. Not fewer than 10 monkeys are used for each test.

Use a test dose of 0.25 mL containing the equivalent of not less than 5000 (3.7 $\log_{10}$) IU and not more than 50 000 (4.7 $\log_{10}$) IU, determined by an *in vitro* titration for infectious virus in cell culture. Inject the test dose into 1 frontal lobe of each monkey under anaesthesia and observe the monkeys for not less than 30 days.

*Viraemia (Viscerotropism)*. Viscerotropism is indicated by the amount of virus present in serum. Take blood from each of the test monkeys on the 2nd, 4th and 6th days after inoculation and prepare serum from each sample. Prepare 1:10, 1:100 and 1:1000 dilutions from each serum and inoculate each dilution into a group of at least 4 cell culture vessels used for the determination of the virus concentration. The seed lot complies with the test if none of the sera contains more than the equivalent of 500 (2.7 $\log_{10}$) IU in 0.03 mL and at most 1 serum contains more than the equivalent of 100 (2.0 $\log_{10}$) IU in 0.03 mL.

*Immunogenicity*. Take blood from each monkey 30 days after the injection of the test dose and prepare serum from each sample. The seed lot complies with the test if at least 90 per cent of the test monkeys are shown to be immune, as determined by examining their sera in the test for neutralisation of yellow fever virus described below.

It has been shown that a low dilution of serum (for example, 1:10) may contain non-specific inhibitors that influence this test; such serum shall be treated to remove inhibitors. Mix dilutions of at least 1:10, 1:40 and 1:160 of serum from each monkey with an equal volume of 17D vaccine virus at a dilution that will yield an optimum number of plaques with the titration method used. Incubate the serum-virus mixtures in a water-bath at 37 °C for 1 h and then cool in iced water; add 0.2 mL of each serum-virus mixture to each of 4 cell culture plates and proceed as for the determination of virus concentration. Inoculate similarly 10 plates with the same amount of virus, plus an equal volume of a 1:10 dilution of monkey serum known to contain no neutralising antibodies to yellow fever virus. At the end of the observation period, compare the mean number of plaques in the plates receiving virus plus non-immune serum with the mean number of plaques in the plates receiving virus plus dilutions of each monkey serum. Not more than 10 per cent of the test monkeys have serum that fails to reduce the number of plaques by 50 per cent at the 1:10 dilution.

*Neurotropism*. Neurotropism is assessed from clinical evidence of encephalitis, from incidence of clinical manifestations and by evaluation of histological lesions, in comparison with 10 monkeys injected with the reference preparation. The seed lot is not acceptable if either the onset and duration of the febrile reaction or the clinical signs of encephalitis and pathological findings are such as to indicate a change in the properties of the virus.

Clinical evaluation

The monkeys are examined daily for 30 days by personnel familiar with clinical signs of encephalitis in primates (if necessary, the monkeys are removed from their cage and examined for signs of motor weakness or spasticity). The seed lot is not acceptable if in the monkeys injected with it the incidence of severe signs of encephalitis, such as paralysis or inability to stand when stimulated, or mortality is greater than for the reference vaccine. These and other signs of

encephalitis, such as paresis, incoordination, lethargy, tremors or spasticity are assigned numerical values for the severity of symptoms by a grading method. Each day each monkey in the test is given a score based on the following scale:

- grade 1: rough coat, not eating;
- grade 2: high-pitched voice, inactive, slow moving;
- grade 3: shaky movements, tremors, incoordination, limb weakness;
- grade 4: inability to stand, limb paralysis or death (a dead monkey receives a daily score of 4 from the day of death until day 30).

A clinical score for a particular monkey is the average of its daily scores; the clinical score for the group is the arithmetic mean of the individual monkey scores. The seed lot is not acceptable if the mean of the clinical severity scores for the group of monkeys inoculated with it is significantly greater ($P = 0.95$) than the mean for the group of monkeys injected with the reference preparation. In addition, special consideration is given to any animal showing unusually severe signs when deciding on the acceptability of the seed lot.

Histological evaluation

5 levels of the brain are examined including:

- block I: the corpus striatum at the level of the optic chiasma;
- block II: the thalamus at the level of the mamillary bodies;
- block III: the mesencephalon at the level of the superior colliculi;
- block IV: the pons and cerebellum at the level of the superior olives;
- block V: the medulla oblongata and cerebellum at the level of the mid-inferior olivary nuclei.

Cervical and lumbar enlargements of the spinal cord are each divided equally into 6 blocks; 15 µm sections are cut from the tissue blocks embedded in paraffin wax and stained with gallocyanin. Numerical scores are given to each hemisection of the cord and to structures in each hemisection of the brain as listed below. Lesions are scored as follows:

- grade 1 - minimal: 1 to 3 small focal inflammatory infiltrates; degeneration or loss of a few neurons;
- grade 2 - moderate: 4 or more focal inflammatory infiltrates; degeneration or loss of neurons affecting not more than one third of cells;
- grade 3 - severe: moderate focal or diffuse inflammatory infiltration; degeneration or loss of 33-90 per cent of the neurons;
- grade 4 - overwhelming: variable but often severe inflammatory reaction; degeneration or loss of more than 90 per cent of neurons.

It has been found that inoculation of yellow fever vaccine into the monkey brain causes histological lesions in different anatomical formations of the central nervous system with varying frequency and severity (I. S. Levenbook *et al.*, *Journal of Biological Standardization*, 1987, 15, 305-313). Based on these 2 indicators, the anatomical structures can be divided into target, spared and discriminator areas. Target areas are those that show more severe specific lesions in a majority of monkeys irrespective of the degree of neurovirulence of the seed lot. Spared areas are those that show only minimal specific lesions and in a minority of monkeys. Discriminator areas are those where there is a significant increase in the frequency of more severe specific lesions with seed lots having a higher degree of neurovirulence. Discriminator and target areas for *Macaca cynomolgus* and *Macaca rhesus* monkeys are shown in the table below.

| Type of monkey | Discriminator areas | Target areas |
|---|---|---|
| *Macaca cynomolgus* | Globus pallidus | Substantia nigra |
| | Putamen | |
| | Anterior/median thalamic nucleus | |
| | Lateral thalamic nucleus | |
| *Macaca rhesus* | Caudate nucleus | Substantia nigra |
| | Globus pallidus | Cervical enlargement |
| | Putamen | Lumbar enlargement |
| | Anterior/median thalamic nucleus | |
| | Lateral thalamic nucleus | |
| | Cervical enlargement | |
| | Lumbar enlargement | |

Scores for discriminator and target areas are used for the final evaluation of the seed lot. The individual monkey score is calculated from the sum of individual target area scores in each hemisection divided by the number of areas examined. A separate score is calculated similarly for the discriminator areas.

Mean scores for the test group are calculated in 2 ways: (1) by dividing the sum of the individual monkey discriminator scores by the number of monkeys; and (2) by dividing the sum of the individual monkey target and discriminator scores by the number of monkeys. These 2 mean scores are taken into account when deciding on the acceptability of the seed lot. The seed lot is not acceptable if either of the mean lesion scores is significantly greater ($P = 0.95$) than for the reference preparation.

*PROPAGATION AND HARVEST*

All processing of the fertilised eggs is done under aseptic conditions in an area where no other infectious agents or cells are handled at the same time. At least 2 per cent but not fewer than 20 and not more than 80 eggs are maintained as uninfected control eggs. After inoculation and incubation at a controlled temperature, only living and typical chick embryos are harvested. At the time of harvest, the control eggs are treated in the same way as the inoculated eggs to obtain a control embryonic pulp. The age of the embryo at the time of virus harvest is reckoned from the initial introduction of the egg into the incubator and shall be not more than 12 days. After homogenisation and clarification by centrifugation, the extract of embryonic pulp is tested as described below and kept at − 70 °C or colder until further processing. Virus harvests may be pooled. No human protein is added to the virus suspension at any stage during production. If stabilisers are added, they shall have been shown to have no antigenic or sensitising properties for man.

Only a single harvest or, where applicable, a pool of single harvests that complies with the following requirements may be used in the preparation of the final bulk vaccine.

**Identification**. The single harvest or pool of single harvests contains virus that is identified as yellow fever virus by serum neutralisation in cell culture using specific antibodies, or by molecular methods (e.g. NAT, sequencing).

**Bacterial and fungal contamination**. The single harvest complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). The single harvest or pool of single harvests complies with the test for mycoplasmas, carried out using 10 mL.

**Mycobacteria** (*2.6.2*). A 5 mL sample of the single harvest or pool of single harvests is tested for the presence of *Mycobacterium* spp. by culture methods known to be sensitive for the detection of these organisms.

**Embryonic pulp of control eggs**. The extract of the control eggs shows no evidence of the presence of any extraneous agents in the tests described below.

*Test in cell culture for other extraneous agents.* Inoculate a 5 mL sample of embryonic pulp of the control eggs into continuous simian kidney and human cell cultures as well as into primary chick-embryo-fibroblast cells. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. The embryonic pulp of the control eggs passes the test if there is no evidence of the presence of any extraneous agents. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

*Avian viruses.* Using 0.1 mL per egg, inoculate the embryonic pulp of control eggs: by the allantoic route into a group of 10 fertilised, 9- to 11-day-old, SPF eggs (*5.2.2*); and into the yolk sac of a group of 10 fertilised, 5- to 7-day-old, SPF eggs (*5.2.2*). Incubate for 7 days. The embryonic pulp lot of the control eggs complies if the allantoic and yolk sac fluids show no signs of haemagglutinating agents and if the embryos and chorio-allantoic membranes examined to detect any macroscopic pathology are typical. The test is not valid unless at least 80 per cent of the inoculated eggs survive during the 7 day observation period.

**Virus concentration**. In order to calculate the dilution for formulation of the final bulk, each single harvest is titrated as described under Assay.

*FINAL BULK VACCINE*

Single harvests or pools of single harvests that comply with the tests prescribed above are pooled and clarified again. A test for protein nitrogen content is carried out. A suitable stabiliser may be added and the pooled harvests diluted as appropriate.

Only a final bulk vaccine that complies with the following requirements may be used in the preparation of the final lot.

**Bacterial and fungal contamination**. The final bulk vaccine complies with the test for sterility (*2.6.1*), carried out using 10 mL for each medium.

**Protein nitrogen content**: maximum 0.25 mg per human dose before the addition of any stabiliser.

*FINAL LOT*

The final bulk vaccine is distributed aseptically into sterile, tamper-evident containers and freeze-dried to a moisture content shown to be favourable to the stability of the vaccine. The containers are then closed so as to prevent contamination and the introduction of moisture.

Only a final lot that is satisfactory with respect to thermal stability and each of the requirements given below under Identification, Tests and Assay may be released for use. Provided that the test for ovalbumin has been performed with satisfactory results on the final bulk vaccine, it may be omitted on the final lot.

**Thermal stability**. Maintain at least 3 containers of the final lot of freeze-dried vaccine in the dry state at 37 ± 1 °C for 14 days. Determine the virus concentration as described under Assay in parallel for the heated vaccine and for vaccine stored at the temperature recommended for storage. The virus concentration of the heated vaccine is not more than 1.0 $\log_{10}$ lower than that of the unheated vaccine.

IDENTIFICATION

When the vaccine reconstituted as stated on the label is mixed with specific yellow fever virus antibodies, there is a significant reduction in its ability to infect susceptible cell cultures. Alternatively, the vaccine reconstituted as stated on the label contains virus that is identified as yellow fever virus by molecular methods (e.g. NAT, sequencing).

TESTS

**Ovalbumin**: maximum 5 µg of ovalbumin per human dose, determined by a suitable immunochemical method (*2.7.1*).

**Water** (*2.5.12*): maximum 3.0 per cent.

**Bacterial and fungal contamination**. The reconstituted vaccine complies with the test for sterility (*2.6.1*).

**Bacterial endotoxins** (*2.6.14*): less than 5 IU per single human dose.

ASSAY

Titrate for infective virus in cell cultures using at least 3 separate containers of vaccine. Titrate 1 container of an appropriate virus reference preparation in triplicate to validate each assay. The virus concentration of the reference preparation is monitored using a control chart and a titre is established on a historical basis by each laboratory. Calculate the individual virus concentration for each container of vaccine and for each replicate of the reference preparation as well as the corresponding combined virus concentrations using the usual statistical methods (for example, *5.3*). The combined virus concentration for the 3 containers of vaccine is compared to the results of the reference preparation titrated in parallel, to obtain results in International Units. The combined virus concentration of the vaccine is not less than 3.0 $\log_{10}$ IU per human dose and not more than the upper limit approved for the particular product by the competent authority.

The assay is not valid if:

- the confidence interval ($P$ = 0.95) of the estimated virus concentration of the reference preparation for the 3 replicates combined is greater than ± 0.3 $\log_{10}$ IU;
- the virus concentration of the reference preparation differs by more than 0.5 $\log_{10}$ IU from the established value.

The assay is repeated if the confidence interval ($P$ = 0.95) of the combined virus concentration of the vaccine is greater than ± 0.3 $\log_{10}$ IU; data obtained from valid assays only are combined by the usual statistical methods (for example, *5.3*) to calculate the virus concentration of the sample. The confidence interval ($P$ = 0.95) of the combined virus concentration is not greater than ± 0.3 $\log_{10}$ IU.

Where justified and authorised, different assay designs may be used; this may imply the application of different validity and acceptance criteria. However, the vaccine must comply if tested as described above.

LABELLING

The label states:

- the strain of virus used in preparation of the vaccine;
- that the vaccine has been prepared in chick embryos;
- the minimum virus concentration;
- that contact between the vaccine and disinfectants is to be avoided.

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**04/2013:0441**

# ANTHRAX SPORE VACCINE (LIVE) FOR VETERINARY USE

## Vaccinum anthracis vivum ad usum veterinarium

1. DEFINITION

Anthrax spore vaccine (live) for veterinary use is a preparation of live spores of a suitable attenuated, non-capsulated strain of *Bacillus anthracis*. This monograph applies to vaccines intended for the active immunisation of animals against disease caused by *B. anthracis*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*B. anthracis* is grown in an appropriate medium. At the end of growth the spores are suspended in a stabilising solution and counted. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE STRAIN*

The strain used is:

– not lethal to the guinea-pig or the mouse,
– or lethal to the guinea-pig but not to the rabbit,
– or lethal to some rabbits.

The vaccine strain is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended.

The following test for immunogenicity (2-2-1) may be used during the demonstration of efficacy.

2-2-1. **Immunogenicity**. For a strain of *B. anthracis* which is not lethal to the guinea-pig or the mouse, the test may be carried out in guinea-pigs. For a strain which is lethal to the guinea-pig but not to the rabbit, the test may be carried out in rabbits. For a strain which is lethal to some rabbits, carry out the test in sheep.

If the test is carried out in guinea-pigs or in rabbits, use not fewer than 13 healthy animals (group a). Inject by the subcutaneous or the intradermal route into each of not fewer than 10 animals $1/10^{th}$ of the smallest dose to be recommended for sheep. Maintain not fewer than 3 animals of the same species and the same origin as controls. Observe the animals at least daily for 21 days. If more than 2 animals die from non-specific causes, repeat the test.

If the test is carried out in sheep, use not fewer than 8 healthy sheep (group b). Vaccinate by the subcutaneous or the intradermal route each of not fewer than 5 sheep 1/10 of the smallest dose of the vaccine stated on the label for sheep. Maintain not fewer than 3 sheep of the same origin as controls. Observe the sheep at least daily for 21 days.

Challenge each vaccinated animal of group (a) or group (b) by a subcutaneous route with at least 100 MLD, and challenge each control animal by a subcutaneous route with at least 10 MLD of a strain of *B. anthracis* pathogenic for the species of animal used in the test. Observe all the animals at least daily for 10 days after challenge.

The vaccine complies with the test if during the observation period after challenge, all the vaccinated animals survive and all the controls die from anthrax. If a vaccinated animal dies after the challenge, repeat the test. If in the second test a vaccinated animal dies, the vaccine fails the test.

3. BATCH TESTS

3-1. **Identification**. *B. anthracis* present in the vaccine is identified by means of morphological and serological tests, culture and biochemical tests.

3-2. **Bacteria and fungi**. Carry out the test by microscopic examination and by inoculation of suitable media. The vaccine, including where applicable, the diluent supplied for reconstitution, does not contain contaminating bacteria and fungi.

3-3. **Live spores**. Make a count of live spores by plate count. The vaccine complies with the test if the number of live spores is not less than 80 per cent of that stated on the label.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-2-1). It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum number of live spores stated on the label.

**01/2017:0744**

# AUJESZKY'S DISEASE VACCINE (INACTIVATED) FOR PIGS

## Vaccinum morbi Aujeszkyi ad suem inactivatum

1. DEFINITION

Aujeszky's disease vaccine (inactivated) for pigs is a preparation of a suitable strain of Aujeszky's disease virus, inactivated while maintaining adequate immunogenic properties, or a preparation of an inactivated fraction of the virus having adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of pigs and for passive protection of their progeny against Aujeszky's disease.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The viral suspension is harvested and inactivated; it may be treated to fragment the virus and the viral fragments may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

2-3-1-1. *Laboratory tests.* Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended (sows, fattening pigs), using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

2-3-1-1-1. General safety. For each test, use not fewer than 8 pigs that do not have antibodies against Aujeszky's disease virus. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a $2^{nd}$ dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until 14 days after the last administration. If the test is carried out in pregnant sows, observe the sows until 1 day after farrowing.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine during the test. If the test is carried out in pregnant sows, no adverse effects on gestation or the offspring are noted.

2-3-1-1-2. Safety in the pigs used in tests 2-3-2 for immunogenicity. The pigs used in the tests for immunogenicity are also used to evaluate safety. Measure the body temperature of each vaccinated pig at the time of vaccination and 6 h, 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

- a temperature rise greater than 1.5 °C and the number of pigs showing a temperature greater than 41 °C does not exceed 10 per cent of the group;
- other systemic reactions (for example, anorexia);
- abnormal local reactions attributable to the vaccine.

2-3-1-2. *Field studies.* The pigs used for field trials are also used to evaluate safety. Carry out a test in each category of pigs for which the vaccine is intended (sows, fattening pigs). Use not fewer than 3 groups each of not fewer than 20 pigs with corresponding groups of not fewer than 10 controls. Measure the body temperature of each vaccinated pig at the time of vaccination and 6 h, 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

- a temperature rise greater than 1.5 °C and the number of pigs showing a temperature greater than 41 °C does not exceed 25 per cent of the group;
- abnormal local reactions attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case pigs of the age to be recommended for vaccination. The vaccine administered to each pig is of minimum potency.

2-3-2-1. *Vaccines intended for active immunisation.* Use for the test not fewer than 15 fattening pigs that do not have antibodies against Aujeszky's disease virus. The body mass of none of the pigs differs from the average body mass of the group by more than 20 per cent. Vaccinate not fewer than 10 pigs, according to the schedule to be recommended. Maintain not fewer than 5 pigs as controls. At the end of the fattening period (80-90 kg), weigh and challenge each pig by the intranasal route with a sufficient quantity of virulent Aujeszky's disease virus (challenge with at least $10^6$ $CCID_{50}$ of a virulent strain having undergone not more than 3 passages and administered in not less than 4 mL of diluent has been found to be satisfactory). The titre of excreted virus is determined in swabs taken from the nasal cavity of each pig daily from the day before challenge until virus is no longer detected. Each pig is weighed 7 days after challenge or at the time of death if this occurs earlier and the average daily gain is calculated as a percentage. For each group (vaccinated and controls), the average of the average daily gains is calculated.

The test is invalid unless all the control pigs display signs of Aujeszky's disease and the average of their daily gains is less than – 0.5 kg. The vaccine complies with the test if:

- all the vaccinated pigs survive and the difference between the averages of the daily gains for the 2 groups is not less than 1.5 kg;
- the geometrical mean titres and the duration of excretion of the challenge virus are significantly lower in vaccinates than in controls.

2-3-2-2. *Vaccines intended for passive immunisation.* If the vaccine is intended for use in sows for the passive protection of piglets, the suitability of the strain for this purpose may be demonstrated by the following method.

Use for the test not fewer than 12 sows that do not have antibodies against Aujeszky's disease virus. Vaccinate not fewer than 8 sows, according to the schedule to be recommended. Maintain not fewer than 4 sows as controls. At 6-10 days of age, challenge the piglets from the sows with a sufficient quantity of virulent Aujeszky's disease virus. Observe the piglets at least daily for 21 days.

The test is not valid if the average number of piglets per litter for each group is less than 6. The vaccine complies with the test if not less than 80 per cent protection against mortality is found in the piglets from the vaccinated sows compared to those from the control sows.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in the same type of cell culture as that used in the production of the vaccine or cells shown to be at least as sensitive. The quantity of inactivated virus harvest used in the test is equivalent to not less than 25 doses of the vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-5) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. The test described under Potency is carried out for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where this test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release as stated in the monograph Vaccines for veterinary use (0062).*

Wherever possible, carry out a suitable test for residual live Aujeszky's disease virus using 2 passages in the same type of cell culture as used in the production of the vaccine or cells shown to be at least as sensitive. Otherwise, inject 1 dose of the vaccine subcutaneously into each of 5 healthy non-immunised rabbits. Observe the rabbits for 14 days after the injection. The vaccine complies with the test if no abnormal reaction (in particular a local rash) occurs. If the vaccine strain is not pathogenic for the rabbit, carry out the test in 2 sheep.

3-4. **Specified extraneous agents**. Use not fewer than 2 pigs that do not have antibodies against Aujeszky's disease virus and against pestiviruses. Administer to each pig by a recommended route a double dose of the vaccine, then another dose after 14 days. 14 days after the last administration, carry out tests for antibodies. The vaccine complies with the test if it does not stimulate the formation of antibodies against pestiviruses.

3-5. **Potency**. The vaccine complies with the requirements of the test described below when administered by a recommended route and method.

Use for the test not fewer than 10 pigs weighing 15-35 kg and that do not have antibodies against Aujeszky's disease virus or against a fraction of the virus. The body mass of none of the pigs differs from the average body mass of the group by more than 25 per cent. Vaccinate not fewer than 5 pigs with 1 dose of the vaccine. Maintain not fewer than 5 pigs as controls. After 3 weeks, weigh each pig, then challenge them by the intranasal route with a sufficient quantity of virulent Aujeszky's disease virus. Weigh each animal 7 days after challenge or at the time of death if this occurs earlier and calculate the average daily gain as a percentage. For each group (vaccinated and controls), calculate the average of the average daily gains.

The test is invalid unless all the control pigs display signs of Aujeszky's disease and the average of their daily gains is less than – 0.5 kg. The vaccine complies with the test if the vaccinated pigs survive and the difference between the averages of the daily gains for the 2 groups is not less than 1.1 kg.

4. LABELLING

The label states whether the vaccine strain is pathogenic for the rabbit.

**01/2014:0745**

# AUJESZKY'S DISEASE VACCINE (LIVE) FOR PIGS FOR PARENTERAL ADMINISTRATION

## Vaccinum morbi Aujeszkyi vivum ad suem ad usum parenteralem

1. DEFINITION

Aujeszky's disease vaccine (live) for pigs for parenteral administration is a preparation of a suitable strain of Aujeszky's disease virus. This monograph applies to vaccines intended for the active immunisation of pigs and for passive protection of their progeny against Aujeszky's disease. The vaccine may be administered after mixing with an adjuvant.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for the production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended. The virus may have a genetic marker.

The following tests for safety (section 2-3-1), virus excretion (section 2-3-2), non-transmissibility, including transmission across the placenta and by semen (section 2-3-3), increase in virulence (section 2-3-4) and immunogenicity (section 2-3-5), may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

2-3-1-1. *Safety test in piglets*. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case piglets 3-4 weeks old. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine.

For each test, use not fewer than 20 piglets that do not have antibodies against Aujeszky's disease virus. Administer to not fewer than 10 piglets a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Maintain not fewer than 10 piglets as controls. Observe the piglets at least daily for at least 21 days.

The vaccine virus complies with the test if the weight curve of the vaccinated piglets does not differ significantly from that of the controls and if no piglet shows signs of disease or dies from causes attributable to the vaccine virus.

2-3-1-2. *Safety of the pigs used in tests 2-3-5 for immunogenicity*. The pigs used in the tests for immunogenicity are also used to evaluate safety. Measure the body temperature of each vaccinated pig at the time of vaccination and 6 h, 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine virus complies with the test if no pig shows:

- a temperature rise greater than 1.5 °C and the number of pigs showing a temperature greater than 41 °C does not exceed 10 per cent of the group;
- other systemic reactions (for example, anorexia);
- abnormal local reactions attributable to the vaccine virus.

2-3-1-3. *Safety in field studies*. The pigs used for field trials are also used to evaluate safety. Carry out a test in each category of pigs for which the vaccine is intended (sows, fattening pigs). Use not fewer than 3 groups, each of not fewer than 20 pigs, with corresponding groups of not fewer than 10 controls. Measure the body temperature of each pig at the time of vaccination and 6 h, 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine virus complies with the test if no pig shows:

- a temperature rise greater than 1.5 °C and the number of pigs showing a temperature greater than 41 °C does not exceed 25 per cent of the group;
- abnormal local reactions attributable to the vaccine virus.

2-3-1-4. *Neurological safety*. Use for the test not fewer than 10 piglets, 3-5 days old and that do not have antibodies against Aujeszky's disease virus. Administer to each piglet by the intranasal route a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets at least daily for at least 21 days.

The vaccine virus complies with the test if none of the piglets dies or shows signs of neurological disorder attributable to the vaccine virus.

2-3-1-5. *Neurological safety for strains other than gE-negative*. This test is not necessary for gE-negative strains. Administer to not fewer than 5 piglets, 3-5 days old, by the intracerebral route, $10^{4.5}$ $CCID_{50}$ of vaccine virus.

The vaccine virus complies with the test if none of the piglets dies or shows signs of neurological disorder.

2-3-1-6. *Absence of latent infections*. Use for the test not fewer than 10 piglets, 3-4 weeks old and that do not have antibodies against Aujeszky's disease virus. Administer to each piglet a daily injection of 2 mg of prednisolone per kilogram of body mass for 5 consecutive days. On the 3rd day administer to each piglet a quantity of vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine by a route to be recommended. Antimicrobial agents may be administered to prevent aspecific signs. Observe the piglets at least daily for at least 21 days.

The vaccine virus complies with the test if no piglet shows signs of disease or dies from causes attributable to the vaccine virus.

2-3-1-7. *Safety in pregnant sows and absence of transmission across the placenta*. Use for the test not fewer than 15 pregnant sows that do not have antibodies against Aujeszky's disease virus. Administer to not fewer than 5 sows, by a route to be recommended, a quantity of vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine during the 4th or 5th week of gestation. Administer to not fewer than 5 other sows the same dose of the virus by the same route during the 10th or 11th weeks of gestation. Maintain not fewer than 5 other pregnant sows as controls. For the piglets from vaccinated sows: carry out tests for serum antibodies against Aujeszky's disease virus; carry out tests for Aujeszky's disease virus antigen in the liver and lungs of those piglets showing abnormalities and in a quarter of the remaining healthy piglets.

The vaccine virus complies with the test if:

- the number of piglets born to the vaccinated sows, any abnormalities in the piglets and the duration of gestation do not differ significantly from those of the controls;
- no Aujeszky's disease virus antigen is found in piglets born to the vaccinated sows;

– no antibodies against Aujeszky's disease virus are found in the serum taken before ingestion of the colostrum.

2-3-2. **Virus excretion**. Use for the test not fewer than 18 pigs, 3-4 weeks old and that do not have antibodies against Aujeszky's disease virus. Administer to not fewer than 14 pigs a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine by a route and a site to be recommended. Maintain not fewer than 4 pigs as contact controls. Carry out suitably sensitive tests for the virus individually on the nasal and oral secretions as follows: collect nasal and oral swabs daily from the day before vaccination until 10 days after vaccination.

The vaccine complies with the test if the virus is not isolated from the secretions collected.

2-3-3. **Non-transmissibility**. Carry out the test on 4 separate occasions. Each time, administer to not fewer than 4 piglets, 3-4 weeks old and that do not have antibodies against Aujeszky's disease virus, by a route to be recommended, a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. After 1 day, keep not fewer than 2 other piglets of the same age and that do not have antibodies against Aujeszky's disease virus close together with them. After 5 weeks, test all the piglets for the presence of antibodies against Aujeszky's disease virus.

The test is not valid if any vaccinated piglet does not show an antibody response. The vaccine virus complies with the test if no antibodies against Aujeszky's disease virus are detected in any group of contact controls and if all the vaccinated piglets show an antibody response.

2-3-4. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using piglets 3-5 days old and that do not have antibodies against Aujeszky's disease virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each piglet of the 1st group by the intranasal route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. After 3-5 days, prepare a suspension from the brain, lung, tonsils and local lymph glands of each piglet and pool the samples. Administer 1 mL of the suspension of pooled samples by the intranasal route to each piglet of the next group. Carry out this passage operation not fewer than 4 times, verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-5. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination. The quantity of vaccine virus to be administered to each pig is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

2-3-5-1. *Vaccines intended for active immunisation.* Use for the test not fewer than 15 fattening pigs of the age to be recommended and that do not have antibodies against Aujeszky's disease virus. The body mass of none of the pigs differs from the average body mass of the group by more than 20 per cent. Vaccinate not fewer than 10 pigs, according to the schedule to be recommended. Maintain not fewer than 5 pigs as controls. At the end of the fattening period (80-90 kg), weigh and challenge each pig by the intranasal route with a sufficient quantity of virulent Aujeszky's disease virus (challenge with at least $10^6$ $CCID_{50}$ of a virulent strain having undergone not more than 3 passages and administered in not less than 4 mL of diluent has been found to be satisfactory). Determine the titre of virus in swabs taken from the nasal cavity of each pig daily from the day before challenge until virus is no longer detected. Weigh each pig 7 days after challenge or at the time of death if this occurs earlier and calculate the average daily gain as a percentage. For each group (vaccinated and controls), calculate the average of the average daily gains.

The test is invalid unless all the control pigs display signs of Aujeszky's disease and the average of their average daily gains is less than – 0.5 kg. The vaccine complies with the test if:

– all the vaccinated pigs survive and the difference between the averages of the average daily gains for the 2 groups is not less than 1.5 kg;
– the geometrical mean titres and the duration of excretion of the challenge virus are significantly lower in vaccinates than in controls.

2-3-5-2. *Vaccines intended for passive protection.* If the vaccine is intended for use in sows for the passive protection of piglets, the suitability of the vaccine virus for this purpose may be demonstrated by the following method.

Use for the test not fewer than 12 sows that do not have antibodies against Aujeszky's disease virus. Vaccinate not fewer than 8 sows, according to the schedule to be recommended. Maintain not fewer than 4 sows as controls. At 6-10 days of age, challenge the piglets from the sows with a sufficient quantity of virulent Aujeszky's disease virus. Observe the piglets at least daily for 21 days.

The test is not valid if the average number of piglets per litter for each group is less than 6.

The vaccine complies with the test if not less than 80 per cent protection against mortality is found in the piglets from the vaccinated sows compared to those from the control sows.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Batch potency test**. It is not necessary to carry out the Potency test (section 3-6) for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label. The test described under Potency is carried out for a given vaccine, on one or more occasions, as decided by or with the agreement of the competent authority. Where this test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine virus is identified by a suitable method, e.g. when mixed with a monospecific antiserum, the vaccine virus is no longer able to infect susceptible cell cultures into which it is inoculated.

3-2. **Bacteria and fungi**. The vaccine, including where applicable, the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum or monoclonal antibodies against Aujeszky's disease virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for pigs and to pestiviruses. Carry out at least 1 passage and maintain the cultures for 14 days. The vaccine complies with the test

if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test described below when administered by a recommended route and method.

Use not fewer than 10 pigs weighing 15-35 kg and that do not have antibodies against Aujeszky's disease virus. The body mass of none of the pigs differs from the average body mass of the group by more than 25 per cent. Vaccinate not fewer than 5 pigs with 1 dose of the vaccine. Maintain not fewer than 5 pigs as controls. After 3 weeks, weigh each pig, then challenge them by the intranasal route with a sufficient quantity of virulent Aujeszky's disease virus. Weigh each pig 7 days after challenge or at the time of death if this occurs earlier and calculate the average daily gain as a percentage. For each group (vaccinated and controls), calculate the average of the average daily gains.

The test is invalid unless all the control pigs display signs of Aujeszky's disease and the average of their average daily gains is less than – 0.5 kg. The vaccine complies with the test if all the vaccinated pigs survive and the difference between the averages of the average daily gains for the 2 groups is not less than 1.6 kg.

**01/2017:0959**

# AVIAN INFECTIOUS BRONCHITIS VACCINE (INACTIVATED)

## Vaccinum bronchitidis infectivae aviariae inactivatum

1. DEFINITION

Avian infectious bronchitis vaccine (inactivated) is a preparation of one or more suitable strains of one or more serotypes of avian infectious bronchitis virus, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended to protect birds against a drop in egg production or quality; for vaccines also intended for protection against respiratory signs, a demonstration of efficacy additional to that described under Potency is required.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is propagated in fertilised hens' eggs or in cell cultures. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from healthy flocks (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the test for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended.

The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination and for each avian species for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 birds not older than the minimum age to be recommended for vaccination. In the case of chickens, use chickens from a flock free from specified pathogens (SPF) (*5.2.2*) and if the vaccine is used for species other than chickens, they have not been vaccinated and do not have antibodies against avian infectious bronchitis virus. Administer by a route to be recommended and method to each bird 1 dose of the vaccine. Observe the birds for at least daily for at least 14 days after the administration of the vaccine.

The test is not valid if non-specific mortality occurs. The vaccine complies with the test if no bird shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case chickens from an SPF flock (*5.2.2*) and for each serotype in the vaccine. The vaccine administered to each chicken is of minimum potency.

Use for the test 4 groups of not fewer than 30 chickens treated as follows:

- group A: unvaccinated controls;
- group B: vaccinated with inactivated avian infectious bronchitis vaccine;
- group C: vaccinated with live avian infectious bronchitis vaccine and inactivated avian infectious bronchitis vaccine according to the schedule to be recommended;
- group D: vaccinated with live avian infectious bronchitis vaccine.

Monitor egg production and quality in all chickens from point of lay until at least 4 weeks after challenge. At the peak of lay, challenge all groups with a quantity of virulent avian infectious bronchitis virus sufficient to cause a drop in egg production or quality over 3 consecutive weeks during the 4 weeks following challenge. The test is invalid unless there is a drop in egg production in group A compared to the normal level noted before challenge of at least 35 per cent where challenge has been made with a Massachusetts-type strain; where it is necessary to carry out a challenge with a strain of another serotype for which there is documented evidence that the strain will not cause a 35 per cent drop in egg production, the challenge must produce a drop in egg production commensurate with the documented evidence and in any case not less than 15 per cent. The vaccine complies with the test if egg production or quality is significantly better in group C than in group D and significantly better in group B than in group A.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Residual live virus**. An amplification test for residual live avian infectious bronchitis virus is carried out on each batch of antigen immediately after inactivation and on the final bulk vaccine or, if the vaccine contains an adjuvant, on the bulk antigen or mixture of bulk antigens immediately before the addition of adjuvant; the test is carried out in embryonated hen eggs from SPF flocks (*5.2.2*) or in suitable cell cultures (*5.2.4*), whichever is the most sensitive for the vaccine strain. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-5-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Administer 1 dose of vaccine by the intramuscular route to each of not fewer than 10 chickens, between 2 weeks of age and the minimum age stated for vaccination and from an SPF flock (*5.2.2*), and maintain 5 hatch mates as unvaccinated controls. Collect serum samples from each chicken just before administration of the vaccine and after the period defined when testing the reference vaccine; determine the antibody titre of each serum, for each serotype in the vaccine, by a suitable serological method, for example, serum neutralisation. The test is invalid unless the sera collected from the unvaccinated controls and from the chickens just before the administration of the vaccine are free from detectable specific antibody. The vaccine complies with the test if the antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

A test for residual live virus is carried out to confirm inactivation of avian infectious bronchitis virus.

A. For vaccine prepared with embryo-adapted strains of virus, inject 2/5 of a dose into the allantoic cavity of ten 9- to 11-day-old embryonated hens' eggs from an SPF flock (*5.2.2*) and incubate. Observe for 5-6 days and pool separately the allantoic liquid from eggs containing live embryos and that from eggs containing dead embryos, excluding those that die within the first 24 h after injection. Examine for abnormalities all embryos which die after 24 h of injection or which survive 5-6 days. No death or abnormality attributable to the vaccine virus occurs. Inject into the allantoic cavity of each of ten 9- to 11-day-old embryonated hens' eggs from an SPF flock (*5.2.2*) 0.2 mL of the pooled allantoic liquid from the live embryos and into each of 10 similar eggs 0.2 mL of the pooled liquid from the dead embryos and incubate for 5-6 days. Examine for abnormalities all embryos which die after 24 h of injection or which survive 5-6 days. If more than 20 per cent of the embryos die at either stage repeat the test from that stage. The vaccine complies with the test if there is no death or abnormality attributable to the vaccine virus.

B. For vaccine prepared with cell-culture-adapted strains of virus, inoculate 10 doses of the vaccine into suitable cell cultures. If the vaccine contains an oil adjuvant, eliminate it by suitable means. Incubate at 38 ± 1 °C for 7 days. Make a passage on another set of cell cultures and incubate at 38 ± 1 °C for 7 days. The vaccine complies with the test if none of the cultures show signs of infection.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

4. LABELLING

The label states whether the strain in the vaccine is embryo-adapted or cell-culture-adapted.

**04/2013:0442**

# AVIAN INFECTIOUS BRONCHITIS VACCINE (LIVE)

## Vaccinum bronchitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious bronchitis vaccine (live) is a preparation of one or more suitable strains of different types of avian infectious bronchitis virus. This monograph applies to vaccines intended for administration to chickens for active immunisation against respiratory disease caused by avian infectious bronchitis virus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more that 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**

2-4-1-1. *Safety for the respiratory tract and kidneys.* Carry out the test in chickens not older than the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine.

Use not fewer than 15 chickens of the same origin and from an SPF flock (*5.2.2*). Administer to each chicken by the oculonasal route a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. On each of days 5, 7 and 10 after administration of the virus, euthanise not fewer than 5 of the chickens and take samples of trachea and kidney. Fix kidney samples for histological examination. Remove the tracheas and prepare 3 transverse sections from the upper part, 4 from the middle part and 3 from the lower part of the trachea of each chicken; examine all tracheal explants as soon as possible and at the latest 2 h after sampling by low-magnification microscopy for ciliary activity. Score for ciliostasis on a scale from 0 (100 per cent ciliary activity) to 4 (no activity, complete ciliostasis); calculate the mean ciliostasis score (the maximum for each trachea being 40) for the 5 chickens euthanised on each of days 5, 7 and 10.

The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccine virus.

The vaccine virus complies with the test if:

– no chicken shows notable clinical signs of avian infectious bronchitis or dies from causes attributable to the vaccine virus;

– any inflammatory lesions seen during the kidney histological examination are, at most, moderate.

A risk/benefit analysis is carried out, taking into account the average ciliostasis scores obtained and the benefits expected from the use of the vaccine.

2-4-1-2. *Safety for the reproductive tract.* If the recommendations for use state or imply that the vaccine may be used in females less than 3 weeks old that are subsequently kept to sexual maturity, it shall be demonstrated that there is no damage to the development of the reproductive tract when the vaccine is given to chickens of the minimum age to be recommended for vaccination.

The following test may be carried out: use not fewer than 40 female chickens from an SPF flock (*5.2.2*) that are not older than the minimum age to be recommended for vaccination; use the vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine; administer to each chicken by a route to be recommended a quantity of virus equivalent to not less than the maximum titre likely to be present in 1 dose of vaccine; at least 10 weeks after administration of the vaccine virus, euthanise the chickens and carry out a macroscopic examination of the oviducts. The vaccine virus complies with the test if abnormalities are present in not more than 5 per cent of the oviducts.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using 2-week-old SPF chickens (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise, passage as described below is carried out.

Administer to each chicken of the 1st group by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. 2-4 days after administration of the vaccine virus, prepare a suspension from the mucosa of the trachea of each chicken and pool these samples. Administer 0.05 mL of the pooled samples by eye-drop to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens. Carry out the test for safety for the respiratory tract and kidneys (section 2-4-1-1) and, where applicable, the test for safety for the reproductive tract (section 2-4-1-2) using the material used for the 1st passage and the virus at the final passage level. Administer the virus by the route to be recommended for vaccination that is likely to be the least safe.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. Immunogenicity is demonstrated for each strain of virus to be included in the vaccine. A test is carried out for each route and method of administration to be recommended using in each case chickens from an SPF flock (*5.2.2*) that are not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

Either or both of the tests below may be used during the demonstration of immunogenicity.

2-4-3-1. *Ciliary activity of tracheal explants.* Use not fewer than 25 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to berecommended not fewer than 20 chickens. Maintain not fewer than 5 chickens as controls. Challenge each chicken after 21 days by eye-drop with a sufficient quantity of virulent avian infectious bronchitis virus of the same type as the vaccine virus to be tested. Euthanise the chickens 4-7 days after challenge and prepare 3 transverse sections from the upper part, 4 from the middle part, and 3 from the lower part of the trachea of each chicken. Examine all tracheal explants as soon as possible and at the latest 2 h after sampling by low-magnification microscopy for ciliary activity. For a given tracheal section, ciliary activity is considered as normal when at least 50 per cent of the internal ring shows vigorous ciliary movement. A chicken is considered not affected if not fewer than 9 out of 10 rings show normal ciliary activity.

The test is not valid if:

– fewer than 80 per cent of the control chickens show cessation or extreme loss of vigour of ciliary activity;

– and/or during the period between the vaccination and challenge, more than 10 per cent of vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if not fewer than 80 per cent of the vaccinated chickens show normal ciliary activity.

2-4-3-2. *Virus recovery from tracheal swabs.* Use not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by eye-drop with a sufficient quantity of virulent avian infectious bronchitis virus of the same type as the vaccine virus to be tested. Euthanise the chickens 4-7 days after challenge and prepare a suspension from swabs of the tracheal mucosa of each chicken. Inoculate 0.2 mL of the suspension into the allantoic cavity of each of 5 embryonated hens' eggs, 9-11 days old, from an SPF flock (*5.2.2*). Incubate the eggs for 6-8 days after inoculation. Eggs that after 1 day of incubation do not contain a live embryo are eliminated and considered as non-specific deaths. Record the other eggs containing a dead embryo and after 6-8 days' incubation examine each egg containing a live embryo for lesions characteristic of avian infectious bronchitis. Make successively 3 such passages. If 1 embryo of a series of eggs dies or shows characteristic lesions, the inoculum is considered to be a carrier of avian infectious bronchitis virus. The examination of a series of eggs is considered to be definitely negative if no inoculum concerned is a carrier.

The test is not valid if:

– the challenge virus is re-isolated from fewer than 80 per cent of the control chickens;

– and/or during the period between vaccination and challenge, more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine;

– and/or more than 1 egg in any group is eliminated because of non-specific embryo death.

The vaccine virus complies with the test if the challenge virus is re-isolated from not more than 20 per cent of the vaccinated chickens.

3. BATCH TESTS

3-1. **Identification**

3-1-1. *Vaccines containing one type of virus.* The vaccine, diluted if necessary and mixed with avian infectious bronchitis virus antiserum specific for the virus type, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-1-2. *Vaccines containing more than one type of virus*. The vaccine, diluted if necessary and mixed with type-specific antisera against each strain present in the vaccine except that to be identified, infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated, whereas after further admixture with type-specific antiserum against the strain to be identified it no longer produces such infection.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection comply either with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). If the vaccine contains more than 1 strain of virus, titrate each strain after having neutralised the others with type-specific avian infectious bronchitis antisera. The vaccine complies with the test if 1 dose contains for each vaccine virus not less than the minimum titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of 1 of the tests prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.



**01/2017:0960**

# AVIAN INFECTIOUS BURSAL DISEASE VACCINE (INACTIVATED)

## Vaccinum bursitidis infectivae aviariae inactivatum

1. DEFINITION

Avian infectious bursal disease vaccine (inactivated) is a preparation of a suitable strain of avian infectious bursal disease virus type 1, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for use in breeding chickens to protect their progeny from avian infectious bursal disease.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from healthy flocks (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended.

The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 chickens not older than the minimum age to be recommended for vaccination and from a flock free from specified pathogens (SPF) (*5.2.2*). Administer by a route and method to be recommended to each chicken 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days after the administration of the vaccine.

The test is not valid if non-specific mortality occurs. The vaccine complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using in each case chickens from an SPF flock (*5.2.2*) and not older than the minimum age to be recommended for vaccination (close to the point of lay). The dose of vaccine administered to each chicken contains not more than the minimum potency to be stated on the label.

Where a challenge test is to be carried out, the following test may be used. Use 2 groups of not less than 20 hens treated as follows:

- group A: unvaccinated controls;
- group B: vaccinated with inactivated avian infectious bursal disease vaccine.

Serum samples are collected from each unvaccinated control (group A) hen just before administration of the vaccine, 4-6 weeks later, and at the time of egg collection for hatching. If a serological test is to be carried out for demonstration of immunogenicity by other routes, serum samples are also collected from each vaccinated (group B) hen at the time of egg collection for hatching. The antibody response is measured in a serum-neutralisation test.

Eggs are collected for hatching not less than 5 weeks after vaccination and the test described below is carried out with chickens at least 3 weeks old from that egg collection.

25 chickens from vaccinated (group B) hens and 10 control chickens of the same breed and age from unvaccinated (group A) hens are challenged with an eye-drop application of a quantity of a virulent strain of avian infectious bursal disease virus sufficient to produce severe signs of disease, including lesions of the bursa of Fabricius, in all unvaccinated chickens. 3-4 days after challenge, the bursa of Fabricius is removed from each chicken. The bursae are examined for evidence of infection by histological examination and by testing for the presence of avian infectious bursal disease antigen by a suitable method. The vaccine complies with the test if 3 or fewer of the

chickens from group B hens show evidence of avian infectious bursal disease. The test is invalid unless all the chickens from group A hens show evidence of avian infectious bursal disease.

Where there is more than one recommended route of administration, the test described under Potency is carried out in parallel with the above immunogenicity test, using different groups of birds for each recommended route. The serological response of the birds inoculated by routes other than that used in the immunogenicity test is not significantly less than that of the group vaccinated by that route.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Residual live virus**. An amplification test for residual live avian infectious bursal disease virus is carried out on each batch of antigen immediately after inactivation to confirm inactivation; the test is carried out in embryonated hens' eggs or in suitable cell cultures (*5.2.4*), whichever is the most sensitive for the vaccine strain; the quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of the vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-5-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Vaccinate each of not fewer than 10 chickens, 14-28 days old and from an SPF flock (*5.2.2*), with 1 dose of vaccine by a recommended route. 4-6 weeks later, collect serum samples from each bird and 10 unvaccinated control birds of the same age and from the same source. Measure the antibody response in a serum-neutralisation test.

The test is not valid if there are specific antibodies in the sera of the unvaccinated birds. The vaccine complies with the test if the mean antibody titre in the sera from the vaccinated birds is equal to or greater than the titres obtained with a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

A test for residual live virus is carried out to confirm inactivation of avian infectious bursal disease type 1.

A. For vaccine prepared with embryo-adapted strains of virus, inject 2/5 of a dose into the allantoic cavity or onto the chorio-allantoic membrane of ten 9- to 11-day-old embryonated hen eggs from an SPF flock (*5.2.2*). Incubate the eggs and observe at least daily for 6 days. Pool separately the allantoic liquid or membranes from eggs containing live embryos, and that from eggs containing dead embryos, excluding those that die from non-specific causes within 24 h of the injection.

   Inject into the allantoic cavity or onto the chorio-allantoic membrane of each of ten 9- to 11-day-old SPF eggs 0.2 mL of the pooled allantoic liquid or crushed chorio-allantoic membranes from the live embryos and, into each of 10 similar eggs, 0.2 mL of the pooled liquid or membranes from the dead embryos and incubate for 6 days. Examine each embryo for lesions of avian infectious bursal disease. If more than 20 per cent of the embryos die at either stage repeat that stage.

   The vaccine complies with the test if there is no evidence of lesions of avian infectious bursal disease and if, in any repeat test, not more than 20 per cent of the embryos die from non-specific causes.

   Antibiotics may be used in the test to control extraneous bacterial infection.

B. For vaccine prepared with cell-culture-adapted strains of virus, inoculate 10 doses of the vaccine into suitable cell cultures. If the vaccine contains an oil adjuvant, eliminate it by suitable means. Incubate at 38 ± 1 °C for 7 days. Make a passage on another set of cell cultures and incubate at 38 ± 1 °C for 7 days.

   The vaccine complies with the test if the cultures show no signs of infection.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

4. LABELLING

The label states whether the strain in the vaccine is embryo-adapted or cell-culture-adapted.

**07/2019:0587**

# AVIAN INFECTIOUS BURSAL DISEASE VACCINE (LIVE)

## Vaccinum bursitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious bursal disease vaccine (live) [Gumboro disease vaccine (live)] is a preparation of a suitable strain of infectious bursal disease virus type 1. This monograph applies to vaccines intended for administration to chickens for active immunisation; it applies to vaccines containing strains of low virulence but not to those containing strains of higher virulence that may be needed for disease control in certain epidemiological situations.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), damage to the bursa of Fabricius (section 2-4-2), immunosuppression (section 2-4-3), increase in virulence (section 2-4-4) and immunogenicity (section 2-4-5) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For chickens older than 3 weeks of age, the test in not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-2. **Damage to the bursa of Fabricius**. Carry out the test for the route to be recommended for vaccination likely to be the least safe using chickens not older than the minimum age to be recommended for vaccination. Use virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Use not fewer than 20 chickens from an SPF flock (*5.2.2*). Administer to each chicken a quantity of the vaccine virus equivalent to 10 times the maximum titre likely to be contained in a dose of the vaccine. On each of days 7, 14, 21 and 28 after administration of the vaccine virus, euthanise not fewer than 5 chickens and prepare a section from the site with the greatest diameters of the bursa of Fabricius of each chicken. Carry out histological examination of the section and score the degree of bursal damage using the following scale.

0 No lesion, normal bursa.

1 1 per cent to 25 per cent of the follicles show lymphoid depletion (i.e., less than 50 per cent depletion in 1 affected follicle); influx of heterophils in lesions.

2 26 per cent to 50 per cent of the follicles show nearly complete lymphoid depletion (i.e., more than 75 per cent depletion in 1 affected follicle); affected follicles show necrosis and severe influx of heterophils may be detected.

3 51 per cent to 75 per cent of the follicles show lymphoid depletion; affected follicles show necrosis and severe influx of heterophils is detected.

4 76 per cent to 100 per cent of the follicles show nearly complete lymphoid depletion, hyperplasia and cyst structures are detected; affected follicles show necrosis and severe influx of heterophils is detected.

5 100 per cent of the follicles show nearly complete lymphoid depletion; complete loss of follicular structure, thickened and folded epithelium, fibrosis of bursal tissue.

Calculate the average score for each group of chickens. The vaccine virus complies with the test if:

- no chicken shows notable clinical signs of disease or dies from causes attributable to the vaccine virus,
- the average score for bursal damage 21 days after administration of the vaccine virus is less than or equal to 2.0 and 28 days after administration is less than or equal to 0.6,
- during the 21 days after administration a notable repopulation of the bursae by lymphocytes has taken place.

2-4-3. **Immunosuppression**. Carry out the tests for the route to be recommended for vaccination likely to be the least safe using chickens not older than the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Use not fewer than 30 chickens from an SPF flock (*5.2.2*). Divide them randomly into 3 groups each of not fewer than 10 and maintain the groups separately. Administer to each chicken of 1 group a quantity of the vaccine virus equivalent to not less than the maximum titre likely to be contained in 1 dose of the vaccine. At the time after administration when maximal bursal damage is likely to be present, as judged from the results obtained in the test for damage to the bursa of Fabricius (section 2-4-2), administer by eye-drop to each vaccinated chicken and to each chicken of another group 1 dose of Hitchner B1 strain Newcastle disease vaccine (live). Determine the seroresponse of each chicken of the 2 groups to the Newcastle disease virus 14 days after administration. Challenge each chicken of the 3 groups by the intramuscular route with not less than $10^5$ $EID_{50}$ of virulent Newcastle disease virus and note the degree of protection in the 2 groups vaccinated with Hitchner B1 strain Newcastle vaccine compared with the non-vaccinated group.

The test is not valid if 1 or more of the non-vaccinated chickens does not die within 7 days of challenge. The degree of immunosuppression is estimated from the comparative seroresponses and protection rates of the 2 Hitchner B1 vaccinated groups.

The vaccine complies with the test if there is no significant difference between the 2 groups.

2-4-4. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using chickens from an SPF flock (*5.2.2*) and not older than the minimum age to be recommended for vaccination. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each chicken of the 1st group by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Prepare 3 to 4 days after administration a suspension from the bursa of Fabricius of each chicken and pool these samples. Administer 0.05 mL of the pooled samples by eye-drop to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens.

Carry out the test for damage to the bursa of Fabricius (section 2-4-2) using the material used for the 1st passage and the virus at the final passage. Administer the virus by the route to be recommended for vaccination likely to be the least safe.

The vaccine virus complies with the test if no indication of increasing virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-5. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the minimum age to be recommended for vaccination. The quantity of vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most

attenuated passage level that will be present in a batch of the vaccine. Use not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 14 days by eye-drop with a sufficient quantity of virulent avian infectious bursal disease virus. Observe the chickens at least daily for 10 days after challenge. Record the deaths due to infectious bursal disease and the surviving chickens that show clinical signs of disease. At the end of the observation period, euthanise all the surviving chickens and carry out histological examination for lesions of the bursa of Fabricius.

The test is not valid if one or more of the following applies:

- during the observation period following challenge, fewer than 50 per cent of the control chickens show characteristic signs of avian infectious bursal disease;
- 1 or more of the surviving control chickens does not show degree 3 lesions of the bursa of Fabricius;
- during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease nor degree 3 lesions of the bursa of Fabricius.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific infectious bursal disease virus type 1 antiserum, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-5) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:0588**

# AVIAN INFECTIOUS ENCEPHALOMYELITIS VACCINE (LIVE)

## Vaccinum encephalomyelitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious encephalomyelitis vaccine (live) is a preparation of a suitable strain of avian encephalomyelitis virus. This monograph applies to vaccines intended for administration to non-laying breeder chickens to protect passively their future progeny and/or to prevent vertical transmission of virus via the egg.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case non-laying breeder chickens not older than the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 chickens from an SPF flock (*5.2.2*). Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 21 days.

The test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-2. **Increase in virulence** Carry out the test according to general chapter *5.2.6* using 1-day-old chickens from an SPF flock (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each chicken of the 1st group by a route and method to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described

below. 5-7 days later, prepare a suspension from the brain of each chicken and pool these samples. Administer a suitable volume of the pooled samples by the oral route to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens.

If the 5th group of chickens shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 chickens receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. If the vaccine is recommended for passive protection of future progeny carry out test 2-4-3-1. If the vaccine is recommended for prevention of vertical transmission of virus via the egg, carry out test 2-4-3-2. A test is carried out for each route and method of administration to be recommended, using in each case chickens from an SPF flock (*5.2.2*) not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-3-1. *Passive immunity in chickens.* Vaccinate not fewer than 20 breeder chickens from an SPF flock (*5.2.2*). Maintain separately not fewer than 10 breeder chickens of the same age and origin as controls. At the peak of lay, hatch not fewer than 25 chickens from eggs from vaccinated breeder chickens and 10 chickens from non-vaccinated breeder chickens. At 2 weeks of age, challenge each chicken by the intracerebral route with a sufficient quantity of virulent avian encephalomyelitis virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease.

The test is not valid if:

- during the observation period after challenge fewer than 80 per cent of the control chickens die or show severe clinical signs of avian infectious encephalomyelitis,
- and/or during the period between the vaccination and challenge more than 15 per cent of control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 80 per cent of the progeny of vaccinated chickens survive and show no notable clinical signs of disease.

2-4-3-2. *Passive immunity in embryos.* Vaccinate not fewer than 20 breeder chickens from an SPF flock (*5.2.2*). Maintain separately not fewer than 10 breeder chickens of the same age and origin as controls. At the peak of lay, incubate not fewer than 36 eggs from the 2 groups, vaccinated and controls, and carry out an embryo sensitivity test. On the sixth day of incubation inoculate 100 $EID_{50}$ of the Van Roekel strain of avian encephalomyelitis virus into the yolk sacs of the eggs. 12 days after inoculation examine the embryos for specific lesions of avian encephalomyelitis (muscular atrophy). Deaths during the first 24 h are considered to be non-specific.

The test is not valid if fewer than 80 per cent of the control embryos show lesions of avian encephalomyelitis. The test is not valid if fewer than 80 per cent of the embryos can be given an assessment.

The vaccine virus complies with the test if not fewer than 80 per cent of the embryos in the vaccinated group show no lesions of avian encephalomyelitis.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific avian encephalomyelitis virus antiserum, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. Depending on the indications, the vaccine complies with the requirements of 1 or both of the tests prescribed under Immunogenicity (section 2-4-3-1, 2-4-3-2), when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:1068**

# AVIAN INFECTIOUS LARYNGOTRACHEITIS VACCINE (LIVE)

## Vaccinum laryngotracheitidis infectivae aviariae vivum

1. DEFINITION

Avian infectious laryngotracheitis vaccine (live) is a preparation of a suitable strain of avian infectious laryngotracheitis virus (gallid herpesvirus 1). This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for index of respiratory virulence (section 2-4-1), safety (section 2-4-2), increase in virulence (section 2-4-3) and immunogenicity (section 2-4-4) may be used during the demonstration of safety and efficacy.

2-4-1. **Index of respiratory virulence**. Use for the test not fewer than sixty 10-day-old chickens from an SPF flock (*5.2.2*). Divide them randomly into 3 groups, maintained separately. Prepare 2 tenfold serial dilutions starting from a suspension of the vaccine virus having a titre of $10^5$ $EID_{50}$ or $10^5$ $CCID_{50}$ per 0.2 mL or, if not possible, having the maximum attainable titre. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Allocate the undiluted virus suspension and the 2 virus dilutions each to a different group of chickens. Administer by the intratracheal route to each chicken 0.2 mL of the virus suspension attributed to its group. Observe the chickens for 10 days after administration and record the number of deaths. The index of respiratory virulence is the total number of deaths in the 3 groups divided by the total number of chickens. The vaccine virus complies with the test if its index of respiratory virulence is not greater than 0.33.

2-4-2. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine. For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For chickens older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-3. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using chickens not more than 2 weeks old, from an SPF flock (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each chicken of the 1st group by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. After the period shown to correspond to maximum replication of the virus, prepare a suspension from the mucosae of suitable parts of the respiratory tract of each chicken and pool these samples. Administer 0.05 mL of the pooled samples by eye-drop to each 2 week-old SPF chicken (*5.2.2*) of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens. Determine the index of respiratory virulence (section 2-4-1) using the material used for the 1st passage and the virus at the final passage level; if the titre of the final passaged virus is less than $10^5$ $EID_{50}$ or $10^5$ $CCID_{50}$, prepare the tenfold, serial dilutions using the highest titre available.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-4. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case chickens not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the intratracheal route with a sufficient quantity of virulent infectious laryngotracheitis virus. Observe the chickens at least daily for 7 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. At the end of the observation period euthanise all the surviving chickens and carry out examination for macroscopic lesions: mucoid, haemorrhagic and pseudomembraneous inflammation of the trachea and orbital sinuses.

The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the control chickens die or show severe clinical signs of avian infectious laryngotracheitis or notable macroscopic lesions of the trachea and orbital sinuses;
- or if during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control chickens show notable clinical signs of disease or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease and/or macroscopical lesions of the trachea and orbital sinuses.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific infectious laryngotracheitis virus antiserum, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-4) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:1392**

# AVIAN PARAMYXOVIRUS 3 VACCINE (INACTIVATED) FOR TURKEYS

## Vaccinum paramyxoviris 3 aviarii inactivatum ad meleagrem

1. DEFINITION

Avian paramyxovirus 3 vaccine (inactivated) for turkeys is a preparation of a suitable strain of avian paramyxovirus 3, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for protection of turkeys against a drop in egg production and loss of egg quality.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is propagated in embryonated eggs or in cell cultures. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated eggs**. If the vaccine virus is grown in embryonated eggs, they are obtained from healthy flocks (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for each category of turkeys for which it is intended.

The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 turkeys not older than the minimum age to be recommended for vaccination, that have not been vaccinated and that do not have antibodies against avian paramyxovirus 3. Administer by a recommended route and method to each turkey 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose to each turkey after an interval of at least 14 days. Observe the turkeys at least daily for at least 14 days after the last administration of the vaccine.

The test is not valid if non-specific mortality occurs. The vaccine complies with the test if no turkey shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case turkeys of the minimum age to be recommended for vaccination. The vaccine administered to each turkey is of minimum potency.

Use for the test 2 groups each of not fewer than 20 turkeys of the same origin and of the same age, that do not have antibodies against avian paramyxovirus 3. Vaccinate one group in accordance with the recommendations stated on the label. Maintain the other group as controls.

The test is not valid if serological tests carried out on serum samples obtained at the time of first vaccination show the presence of antibodies against avian paramyxovirus 3 in either vaccinates or controls or if tests carried out at the time of challenge show such antibodies in controls.

At the egg-production peak, challenge the 2 groups by the oculonasal route with a sufficient quantity of a virulent strain of avian paramyxovirus 3. For not less than 6 weeks after challenge, record the number of eggs laid weekly for each group, distinguishing between normal and abnormal eggs. The vaccine complies with the test if egg production and quality are significantly better in the vaccinated group than in the control group.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Residual live virus**. The test for residual live virus is carried out in embryonated eggs or suitable cell cultures (*5.2.4*), whichever is the most sensitive for the vaccine strain. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-5-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

A test for residual live virus is carried out to confirm inactivation of avian paramyxovirus 3.

Inject 2/5 of a dose into the allantoic cavity of each of not fewer than 10 embryonated hen eggs 9-11 days old, from flocks free from specified pathogens (SPF) (*5.2.2*) and incubate. Observe

for 6 days and pool separately the allantoic fluid from eggs containing live embryos, and that from eggs containing dead embryos, excluding those dying within 24 h of the injection. Examine embryos that die within 24 h of injection for the presence of avian paramyxovirus 3.

The vaccine does not comply with the test if avian paramyxovirus 3 is found.

Inject into the allantoic cavity of each of not fewer than ten 9- to 11-day-old fertilised hen eggs from an SPF flock (*5.2.2*), 0.2 mL of the pooled allantoic fluid from the live embryos and, into each of 10 similar eggs, 0.2 mL of the pooled fluid from the dead embryos, and incubate for 5-6 days. Test the allantoic fluid from each egg for the presence of haemagglutinins using chicken erythrocytes.

The vaccine complies with the test if there is no evidence of haemagglutinating activity and if not more than 20 per cent of the embryos die at either stage. If more than 20 per cent of the embryos die at one of the stages, repeat that stage; the vaccine complies with the test if there is no evidence of haemagglutinating activity and not more than 20 per cent of the embryos die at that stage.

Antibiotics may be used in the test to control extraneous bacterial infection.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

**04/2013:1956**

# AVIAN VIRAL TENOSYNOVITIS VACCINE (LIVE)

## Vaccinum tenosynovitidis viralis aviariae vivum

1. DEFINITION

Avian viral tenosynovitis vaccine (live) is a preparation of a suitable strain of avian tenosynovitis virus (avian orthoreovirus). This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. Cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the minimum age to be recommended for vaccination and from a flock free from specified pathogens (SPF) (*5.2.2*). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 21 days. Carry out histological examination of the joints and tendon sheaths of the legs and feet at the end of the observation period (as a basis for comparison in the test for increase in virulence).

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine virus. For chickens older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using 1-day-old chickens from an SPF flock (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each chicken of the 1st group by a suitable route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Euthanise the chickens at the moment when the virus concentration in the most suitable material (for example, tendons, tendon sheaths and liquid exudates from the hock joints, spleen) is sufficient. Prepare a suspension from this material from each chicken and pool these samples. Administer 0.1 mL of the pooled samples by the route of administration most likely to lead to increase in virulence to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens.

If the 5th group of chickens shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 chickens receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus at the final passage level compared with the material used for the 1st passage is observed. If the virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case chickens not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Administer the vaccine by a route to be recommended to not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by a suitable route with a sufficient quantity of virulent avian tenosynovitis virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the surviving chickens that show clinical signs of disease. If the challenge is administered by the foot pad, any transient swelling of the foot

pad during the first 5 days after challenge may be considered non-specific. At the end of the observation period, euthanise all the surviving chickens and carry out macroscopic and/or microscopic examination for lesions of the joints and tendon sheaths of the legs and feet, e.g. exudate and swelling.

The test is not valid if:

- during the observation period after challenge fewer than 80 per cent of the control chickens die or show severe clinical signs of avian viral tenosynovitis or show macroscopical and/or microscopical lesions in the joints and tendon sheaths of the legs and feet,
- or if during the period between vaccination and challenge more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease or show macroscopical and/or microscopical lesions in the joints and tendon sheaths of the legs and feet.

3. BATCH TESTS

3-1. **Identification**. Carry out an immunostaining test in cell cultures to identify the vaccine virus.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:2525**

# BORDETELLA BRONCHISEPTICA VACCINE (LIVE) FOR DOGS

## Vaccinum Bordetellae bronchisepticae vivum ad canem

1. DEFINITION

*Bordetella bronchiseptica* vaccine (live) for dogs is a preparation of a suitable strain of *Bordetella bronchiseptica*. This monograph applies to vaccines intended for the active immunisation of dogs against respiratory disease caused by *B. bronchiseptica*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine strain is grown in a suitable medium.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine strain is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-2-1), excretion and transmission of the vaccine strain (section 2-2-2), increase in virulence (section 2-2-3) and immunogenicity (section 2-2-4) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case dogs not older than the minimum age to be recommended for vaccination. The vaccine strain to be administered is at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 dogs, shown to be free from *B. bronchiseptica* and that do not have antibodies against *B. bronchiseptica*. Administer to each dog a quantity of the vaccine strain equivalent to not less than 10 times the maximum number of live bacteria likely to be contained in 1 dose of the vaccine. Observe the dogs at least daily for at least 14 days.

The vaccine strain complies with the test if no dog shows abnormal local or systemic reactions or dies from causes attributable to the vaccine strain.

2-2-2. **Excretion and transmission of the vaccine strain**. Use dogs not older than 10 weeks of age. Administer the strain by the route to be recommended for vaccination most likely to lead to excretion. The vaccine strain to be administered is at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 dogs, shown to be free from *B. bronchiseptica* and that do not have antibodies against *B. bronchiseptica*. Administer to not fewer than 4 dogs a quantity of the vaccine strain equivalent to not less than the maximum number of live bacteria likely to be contained in 1 dose of the vaccine. 2 days after vaccination add 4 dogs to the group of vaccinated dogs. Observe the animals for 70 days. Collect nasal swabs or washings from each dog at weekly intervals. Verify the presence of the excreted vaccine strain with a suitable method.

The vaccine strain complies with the test if no dog shows abnormal local or systemic reactions or dies from causes attributable to the vaccine strain.

The results are noted and used to formulate the label statement (whether the vaccinated strain is excreted, the period over which there is excretion and whether or not the vaccine strain spreads to in-contact dogs).

2-2-3. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using dogs not older than 10 weeks of age, which are free from *B. bronchiseptica* and that do not have antibodies against *B. bronchiseptica*. If the properties of the vaccine strain allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each dog by a route to be recommended a quantity of the vaccine strain that will allow recovery of bacteria for the passages described below. Administer the strain by the route to be recommended for vaccination most likely to lead to reversion to virulence. On one occasion between 4 and 6 days after administration, collect nasal swabs or washings from each dog, verify the presence of bacteria and pool positive samples. Administer 1 mL of the pooled samples by a suitable route (for example, the intranasal route) to each dog of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the bacteria at each passage. If the bacteria are not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5$^{th}$ group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1$^{st}$ passage and another similar group receiving the bacteria at the final passage level.

The vaccine strain complies with the test if no indication of increased virulence of the bacteria recovered for the final passage compared with the material used for the 1$^{st}$ passage is observed. If bacteria are not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine strain also complies with the test.

2-2-4. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case dogs of the minimum age to be recommended. The quantity of vaccine strain to be administered to each dog is not greater than the minimum number of live bacteria to be stated on the label and the strain is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 15 dogs which are free from *B. bronchiseptica* and that do not have antibodies against *B. bronchiseptica*. Vaccinate not fewer than 10 dogs, according to the schedule to be recommended. Maintain not fewer than 5 dogs as controls. Challenge each dog after 20-22 days by the intranasal route with a quantity of a suspension of virulent *B. bronchiseptica* sufficient to cause typical signs of respiratory disease in a dog that does not have antibodies against *B. bronchiseptica*. Observe the dogs at least daily for 14 days after challenge. Collect nasal swabs or washings from each dog daily from day 2 to 14 after challenge and determine the number of excreted *B. bronchiseptica* in each sample. Use a scoring system to record the signs of respiratory disease in each dog.

The test is invalid if more than 20 per cent of the controls show no typical signs of the disease.

The vaccine complies with the test if there is a significant decrease in the score for respiratory signs and in the number of *B. bronchiseptica* excreted in vaccinates compared to controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine strain is identified by suitable methods.

3-2. **Bacteria and fungi**. Carry out the test by inoculation of suitable media. The vaccine complies with the test if it does not contain extraneous micro-organisms. Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Live bacteria**. Make a count of live bacteria on a solid medium suitable for the culture of *B. bronchiseptica*. The vaccine complies with the test if 1 dose contains not less than the minimum number of live *B. bronchiseptica* stated on the label.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-2-4) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum number of live *B. bronchiseptica* stated on the label.

4. LABELLING

The label states:
- where applicable, the period after vaccination during which the vaccine is excreted;
- where applicable, that the vaccine strain may be transmitted to other dogs.

**01/2017:1939**

# BOVINE LEPTOSPIROSIS VACCINE (INACTIVATED)

## Vaccinum leptospirosis bovinae inactivatum

1. DEFINITION

Bovine leptospirosis vaccine (inactivated) is a preparation of inactivated whole organisms and/or antigenic extract(s) of one or more suitable strains of one or more of *Leptospira borgpetersenii* serovar hardjo, *Leptospira interrogans* serovar hardjo or other *L. interrogans* serovars, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of cattle against leptospirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvest is inactivated by a suitable method. The antigen may be concentrated. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests.* Carry out the test for each route and method of administration to be recommended for vaccination and in cattle of each category for which the vaccine is intended (for example, young calves, pregnant cattle). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cattle that do not have antibodies against *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Administer to each animal 1 dose of the vaccine. If the schedule to be recommended

requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the cattle at least daily for at least 14 days after the last administration. Record body temperatures the day before each vaccination, at vaccination, 4 h later and daily for 4 days.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions, signs of disease, or dies from causes attributable to the vaccine.

2-2-1-2. *Field studies*. The cattle used for the field trials are also used to evaluate safety. Use not fewer than 3 groups of 20 cattle with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine. In addition, if the vaccine is for use in pregnant cattle, no adverse effects on the pregnancy and offspring are noted.

2-2-2. **Immunogenicity**. Carry out a separate test for each of the serovars for which a claim is made for a beneficial effect on the rates of infection and urinary excretion. If claims are to be made for protection against reproductive or production losses, further specific studies will be required.

Each test is carried out for each route and method of administration to be recommended, using in each case cattle of the minimum age to be recommended for vaccination. The vaccine administered to each animal is of minimum potency.

2-2-2-1. *Immunogenicity against L. borgpetersenii serovar hardjo*. Use not fewer than 15 cattle that do not have antibodies against *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Vaccinate not fewer than 10 cattle according to the schedule to be recommended. Maintain not fewer than 5 cattle as controls. 20-22 days after the last vaccination, challenge all the cattle by a suitable mucosal route with a sufficient quantity of a virulent strain of the relevant serovar. Observe the cattle at least daily for a further 35 days. Collect urine samples from each animal on days 0, 14, 21, 28 and 35 post-challenge. Euthanise surviving cattle at the end of the observation period. Carry out post-mortem examination on any animal that dies and on those euthanised at the end of the observation period. In particular, examine the kidneys for macroscopic and microscopic signs of leptospira infection. A sample of each kidney is collected and each urine and kidney sample is tested for the presence of the challenge organisms by re-isolation or by another suitable method.

For the test conducted with *L. borgpetersenii* serovar hardjo, control cattle are regarded as infected if the challenge organisms are re-isolated from at least 2 samples. The test is not valid if infection has been established in fewer than 80 per cent of the control cattle.

The vaccine complies with the test if the challenge organisms are re-isolated from any urine or kidney sample from not more than 20 per cent of the vaccinated cattle.

2-2-2-2. *Immunogenicity against other leptospira species*. For leptospiral species other than *L. borgpetersenii* serovar hardjo, the test is conducted as described in section 2-2-2-1 but urine samples are collected on appropriate days, determined by the characteristics of the challenge model. In the case of serovars for which there is published evidence that the serovar has a lower tropism for the urinary tract, a lower rate of infection may be justified. Depending on their tissue tropism, for some leptospira serovars, samples from other tissues/body fluids can be used to establish whether the cattle are infected or not by the challenge organism.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual live bacteria**. Carry out a test for residual live leptospirae by inoculation of a specific medium. Inoculate 1 mL of the bulk antigen into 100 mL of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: the bulk antigen complies with the test if no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the bulk antigen together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: the test is not valid if growth of leptospirae does not occur within 14 days.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following tests may be used. However, in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, an alternative validated method, preferably the 1st option proposed below, should be used for routine testing.

2-3-2-1. For each of the serovars present in the vaccine, a suitable validated *in vitro* test may be carried out to determine the content of one or more antigenic components which are indicators of protection and which are specific for that serovar. The criteria for acceptance are set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. Methods using lipopolysaccharide (LPS)-based antigen quantification have been shown to be suitable.

2-3-2-2. For each of the serovars for which protection is claimed, the antibody response from vaccinated animals is measured. Use not fewer than 12 guinea-pigs weighing 250-350 g that do not have antibodies against *L. borgpetersenii* serovar hardjo and the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis) and that have been obtained from a regularly tested and certified leptospira-free source. The dose to be administered to the guinea-pigs is that fraction of a cattle dose which has been shown in the validation studies to provide a suitably sensitive test. Vaccinate each of 10 guinea-pigs with the suitable dose. Maintain not fewer than 2 guinea-pigs as controls. At a given interval within the range of 19-23 days after the injection, collect blood from each guinea-pig and prepare serum samples. Use a suitable validated method such as a micro-agglutination test to measure the antibodies in each sample.

The vaccine complies with the test if antibody levels are equal to or greater than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition. If test 2-3-2-1 is used for the batch potency test, it also serves to identify the vaccine.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live bacteria**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live leptospirae by inoculation of a specific medium. Inoculate 1 mL of the vaccine into 100 mL

of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: the vaccine complies with the test if no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the vaccine together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: the test is not valid if growth of leptospirae does not occur within 14 days.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:1176**

# BOVINE PARAINFLUENZA VIRUS VACCINE (LIVE)

## Vaccinum viri parainfluenzae bovini vivum

1. DEFINITION

Bovine parainfluenza virus vaccine (live) is a preparation of a suitable strain of bovine parainfluenza 3 virus. This monograph applies to vaccines intended for the active immunisation of cattle against infection with bovine parainfluenza virus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 calves of the minimum age to be recommended for vaccination and preferably that do not have antibodies against bovine parainfluenza 3 virus or, where justified, use calves with a very low level of such antibodies as long as they have not been vaccinated against bovine parainfluenza virus and administration of the vaccine does not cause an anamnestic response. Administer to each calf a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the calves at least daily for at least 14 days. Measure the body temperature of each calf on the day before vaccination, at the time of vaccination and for the 4 subsequent days.

The vaccine virus complies with the test if no abnormal effect on body temperature occurs and if no calf shows abnormal, local or systemic reactions or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to chapter *5.2.6. Evaluation of safety of veterinary vaccines and immunosera*, using calves that do not have antibodies against bovine parainfluenza 3 virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each calf of the 1st group by the intranasal route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. On each of days 3 to 7 after administration of the virus, take nasal swabs from each calf and collect in not more than 5 mL of a suitable medium, which is then used to inoculate cell cultures to verify the presence of virus. Administer about 1 mL of the suspension from the swabs that contain the maximum amount of virus, as indicated by the titration of cell cultures, to each calf of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed; account is taken of the titre of excreted virus in the nasal swabs. If virus is not recovered after an initial passage in 2 animals and a subsequent repeated passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case calves of the minimum age to be recommended. The quantity of vaccine to be administered to each calf is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 10 calves that do not have antibodies against bovine parainfluenza 3 virus; calves having low levels of such antibodies may be used if it has been demonstrated that valid results are obtained in these conditions. Collect sera from the calves before vaccination, 7 days and 14 days after the time of vaccination and just before challenge. Vaccinate not fewer than 5 calves, according to the schedule to be recommended. Maintain not fewer than 5 calves as controls. Challenge each calf after 20-22 days by a respiratory tract route with a sufficient quantity of a suspension of a low-passage virulent bovine parainfluenza 3 virus. Observe the calves at least daily for 14 days after challenge and monitor each of them for signs, in particular respiratory signs and virus shedding (by nasal swabs or tracheobronchial washing).

The test is not valid if tests for antibodies against bovine parainfluenza 3 virus on the sera indicate that there was intercurrent infection with the virus during the test or if, during the observation period after challenge, more than 2 of the 5 control calves show no excretion of the challenge virus, as shown by nasal swabs or samples harvested by tracheobronchial washing.

The vaccine virus complies with the test if, during the observation period after challenge, in vaccinated calves compared to controls there is a significant reduction in mean titre and in mean duration of virus excretion, and a notable reduction in general and local signs (if the challenge virus used produces such signs).

3. BATCH TESTS

3-1. **Identification**. Carry out an immunostaining test in suitable cell cultures, using a monospecific antiserum.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against bovine parainfluenza 3 virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for cattle. Carry out at least 1 passage and maintain the cultures for 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:1177**

# BOVINE RESPIRATORY SYNCYTIAL VIRUS VACCINE (LIVE)

## Vaccinum viri syncytialis meatus spiritus bovini vivum



1. DEFINITION

Bovine respiratory syncytial virus vaccine (live) is a preparation of a suitable strain of bovine respiratory syncytial virus. This monograph applies to vaccines intended for the active immunisation of cattle against infection with bovine respiratory syncytial virus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case calves of the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

2-3-1-1. *Laboratory test*. For each test, use not fewer than 5 calves that do not have antibodies against bovine respiratory syncytial virus. Administer to each calf a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the calves at least daily for at least 14 days. Measure the body temperature of each calf on the day before vaccination, at the time of vaccination and daily for the following 7 days.

The vaccine virus complies with the test if no abnormal effect on body temperature occurs and if no calf shows abnormal local or systemic reactions or dies from causes attributable to the vaccine virus.

2-3-1-2. *Field studies*. The calves used for the field trials are also used to evaluate the incidence of hypersensitivity reactions in vaccinated calves following subsequent exposure to the vaccine or to wild virus. The vaccine complies with the test if it is not associated with an abnormal incidence of immediate hypersensitivity reactions.

2-3-2. **Increase in virulence**. Carry out the test according to chapter *5.2.6. Evaluation of safety of veterinary vaccines and immunosera*, using calves that do not have antibodies against bovine respiratory syncytial virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each calf of the 1st group by the intranasal route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. On each of days 3 to 7 after administration of the virus, take nasal swabs from each calf and collect in not more than 5 mL of a suitable medium, which is then used to inoculate cell cultures to verify the presence of virus. Administer about 1 mL of the suspension from the swabs that contain the maximum amount of virus, as indicated by the titration of cell cultures, to each calf of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of calves shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no calf shows signs attributable to the vaccine virus and no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed; account is taken of the titre of excreted virus in the nasal swabs. If virus is not recovered after an initial passage in 2 animals and a subsequent repeated passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case calves of the minimum age to be recommended. The quantity of vaccine to be administered to each calf is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 10 calves that do not have antibodies against bovine respiratory syncytial virus. Collect sera from the calves before the time of vaccination, 7 and 14 days after the time of vaccination and just before challenge. Vaccinate not fewer than 5 calves, according to the schedule to be recommended. Maintain not fewer than 5 calves as controls. Challenge each calf after 20-22 days by a respiratory tract route with a sufficient quantity of a suspension of a low-passage virulent bovine respiratory syncytial virus. Observe the calves at least daily for 14 days after challenge and monitor each of them for signs, in particular respiratory signs and virus shedding (by nasal swabs or tracheobronchial washing).

The test is not valid if antibodies against bovine respiratory syncytial virus are detected in any sample from control calves before challenge or if more than 2 of the 5 control calves show no excretion of the challenge virus, as shown by nasal swabs or samples harvested by tracheobronchial washing.

The vaccine virus complies with the test if, during the observation period after challenge, there is a significant reduction in mean titre and in mean duration of virus excretion in vaccinates compared to controls, and a notable reduction in general and local signs in vaccinated calves (if the challenge virus used produces such signs).

3. BATCH TESTS

3-1. **Identification**. Identify the vaccine by an immunostaining test in suitable cell cultures using a monospecific antiserum.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against bovine respiratory syncytial virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for cattle. Carry out at least one passage and maintain the cultures for 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents. Carry out a specific test for pestiviruses.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:1952**

# BOVINE VIRAL DIARRHOEA VACCINE (INACTIVATED)

## Vaccinum diarrhoeae viralis bovinae inactivatum

1. DEFINITION

Bovine viral diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine diarrhoea virus inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of heifers and cows for protection of their progeny against transplacental infection.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The viral suspensions of each vaccine virus are harvested separately and inactivated by a method that maintains immunogenicity. The viral suspensions may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and in each category of cattle for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

2-3-1-1. *General safety*. For each test, use not fewer than 8 cattle of the minimum age to be recommended for vaccination and that do not have bovine diarrhoea virus or antibodies against the virus. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the cattle at least daily for at least 14 days.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-1-2. *Safety in pregnant cattle*. If the vaccine is intended for use in pregnant cattle, use not fewer than 8 cattle at the beginning of each semester for which use is not contraindicated. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the cattle at least daily until calving.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no adverse effects on gestation or the offspring are noted.

2-3-1-3. *Examination of reproductive performance*. If the vaccine is intended for administration shortly before or at insemination, absence of undesirable effects on conception rate must be demonstrated.

2-3-2. **Immunogenicity**. The following test is suitable to demonstrate the immunogenicity of the vaccine with respect to bovine diarrhoea virus of genotype 1; if protection against bovine diarrhoea virus of genotype 2 is claimed, an additional test, similar to that described below, but using bovine diarrhoea virus of genotype 2 for challenge, is carried out.

A test is carried out for each route and method of administration to be recommended. The vaccine administered to each heifer is of minimum potency.

Use for the test not fewer than 20 heifers free from bovine diarrhoea virus and that do not have antibodies against bovine diarrhoea virus. Vaccinate not fewer than 13 heifers according to the schedule to be recommended. Maintain not fewer than 7 heifers as controls. Keep all the animals as one group. Inseminate the heifers. Take a blood sample from non-vaccinated heifers shortly before challenge. The test is discontinued if fewer than 10 vaccinated heifers or 5 non-vaccinated heifers are pregnant at the time of challenge. Challenge each heifer between the 60th and 90th days of gestation. For both test models described (observation until calving and harvest of foetuses at 28 days), challenge may be made by the intranasal route with a sufficient quantity of a non-cytopathic strain of bovine diarrhoea virus or alternatively, where the heifers are observed until calving, challenge may be made by contact with a persistently viraemic animal. Observe the heifers clinically at least daily from challenge either until the end of gestation or until harvest of foetuses after 28 days. If abortion occurs, examine the aborted foetus for bovine diarrhoea virus by suitable methods. If cattle

are observed until calving, immediately after birth and prior to ingestion of colostrum, examine all calves for viraemia and antibodies against bovine diarrhoea virus. If foetuses are harvested 28 days after challenge, examine the foetuses for bovine diarrhoea virus by suitable methods. Transplacental infection is considered to have occurred if virus is detected in foetal organs or in the blood of newborn calves or if antibodies are detected in precolostral sera of calves.

The test is not valid if any of the control heifers have neutralising antibody before challenge or if transplacental infection fails to occur in more than 10 per cent of the calves from the control heifers. The vaccine complies with the test if at least 90 per cent of the calves from the vaccinated heifers are protected from transplacental infection.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using a quantity of inactivated virus harvest equivalent to not less than 25 doses of vaccine in cells of the same type as those used for production of the vaccine or cells shown to be at least as sensitive; the cells are passaged after 7 days and observed for a total of not less than 14 days. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use for the test 7 suitable laboratory animals or calves that do not have antibodies against bovine diarrhoea virus. Administer by the subcutaneous route to 5 animals a suitable dose of the vaccine. Maintain 2 animals as controls. A 2nd dose of vaccine may be administered after a suitable interval if this has been shown to provide a suitably discriminating test system. Collect blood samples before the 1st vaccination and at a given interval between 14 and 21 days after the last vaccination. Determine the antibody titres against bovine diarrhoea virus by seroneutralisation on suitable cell cultures.

The test is not valid if the control animals show antibodies against bovine diarrhoea virus. The vaccine complies with the test if the level of antibodies in the vaccinates is not lower than that found for a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live bovine diarrhoea virus by inoculating not less than 10 doses onto cells known to be sensitive to bovine diarrhoea virus; passage the cells after 7 days and observe the 2nd culture for not less than 7 days. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant if possible from the liquid phase by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**01/2016:0793**

# BRUCELLOSIS VACCINE (LIVE) (BRUCELLA MELITENSIS REV. 1 STRAIN) FOR VETERINARY USE

## Vaccinum brucellosis (Brucella melitensis stirps Rev. 1) vivum ad usum veterinarium

1. DEFINITION

Brucellosis vaccine (live) (Brucella melitensis Rev. 1 strain) for veterinary use is a suspension of live *Brucella melitensis* Rev. 1 strain. The vaccine contains not fewer than $0.5 \times 10^9$ and not more than $4 \times 10^9$ live bacteria per dose. This monograph applies to vaccines intended for the active immunisation of sheep and goats against disease caused by *B. melitensis*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*B. melitensis* Rev. 1 strain is cultured in a suitable medium. The method of culture is such as to avoid bacterial dissociation and thus maintain the smooth characteristic of the culture. The bacteria are suspended in a buffer solution that may contain a suitable stabiliser. The suspension is distributed into containers.

2-2. *CHOICE OF VACCINE STRAIN*

The vaccine strain is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the sheep and goats for which it is intended.

The following tests for safety (section 2-2-1), residual virulence (section 2-2-2), determination of dissociation phase of master seed lot (section 2-2-3) and immunogenicity in mice (section 2-2-4) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. Use 8 sheep, 4-6 months old, that do not have antibodies against *B. melitensis*. Administer to each sheep by a route to be recommended 3 doses of the vaccine. Observe the sheep at least daily for at least 14 days.

The vaccine complies with the test if no sheep shows notable signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Residual virulence**. The test is carried out on the master seed lot and on a representative batch of vaccine. If the quantity of the master seed sufficient for performing the test is not available, the lowest passage seed used for the production that is available in sufficient quantity may be used.

Use 32 female CD1 mice, 5-6 weeks old. Vaccinate each mouse by the subcutaneous route with a suspension (0.1 mL) containing $10^8$ live bacteria. Euthanise the mice in groups of 8, selected at random, 3, 6, 9 and 12 weeks later. Remove the spleens and homogenise individually and aseptically in 1 mL of *phosphate buffered saline pH 6.8 R*. Spread the entire suspension on plates containing a suitable culture medium (lower limit of detection: 1 bacterium per spleen). Carry out in parallel a similar test using a suitable reference strain of *Brucella melitensis* Rev. 1. Calculate the 50 per cent persistence time by the usual statistical methods (*5.3*) for probit analysis.

The product complies with the test if the 50 per cent persistence time for the vaccine strain does not differ significantly from that of the reference strain.

2-2-3. **Determination of dissociation phase of the master seed lot**. Examine not fewer than 200 colonies by a suitable technique. The culture of the vaccine strain is seen to be in the smooth (S) phase.

The seed lot complies with the test if not fewer than 99 per cent of the colonies are of the smooth type.

2-2-4. **Immunogenicity in mice**. The test is carried out on the master seed lot and on a representative batch of vaccine. If the quantity of the master seed sufficient for performing the test is not available, the lowest passage seed used for the production that is available in sufficient quantity may be used.

Use for the test healthy CD1 female mice, 5-7 weeks old and from the same stock. Distribute the mice into 3 groups of 6 mice. Dilute the vaccine strain and a suitable reference strain of *Brucella melitensis* Rev. 1 to a concentration of $10^6$ CFU/mL.

Vaccinate by the subcutaneous route the mice of the 1st group with 0.1 mL of the diluted vaccine strain and the mice of the 2nd group with 0.1 mL of the diluted reference strain; keep the 3rd group as the unvaccinated control. After 30 days, challenge all the mice with $2 \times 10^5$ bacteria of *B. abortus* strain 544 ($CO_2$-dependent). Euthanise the mice 15 days later and remove the spleen for *B. abortus* isolation. Record the number of *B. abortus* per spleen (X) and transform this value to obtain $Y = \log_{10} (X/\log_{10} X)$. Then calculate the mean and standard deviation of each group.

The test is valid if:

- the mean of the unvaccinated control group is at least 4.5 (mean of Y);
- the mean of the group receiving the reference strain is lower than 2.5 (mean of Y); and
- the standard deviation of each group is lower than 0.8.

Carry out a statistical comparison of the immunogenicity values of the 3 groups using the least significant differences test. The vaccine strain complies with the test if:

- the immunogenicity value of the group receiving the vaccine strain is significantly lower than the immunogenicity value of the control group; and
- the immunogenicity value of the group receiving the vaccine strain is not significantly different from the immunogenicity value of the group receiving the reference strain.

3. BATCH TESTS

3-1. **Identification**. *B. melitensis* present in the vaccine is identified by suitable morphological, serological and biochemical tests and by culture: Rev. 1 strain is inhibited by addition to the suitable culture medium of either benzylpenicillin sodium (3 µg/mL), thionin (20 µg/mL) or basic fuchsin (20 µg/mL); the strain grows on agar containing 2.5 µg of streptomycin per millilitre.

3-2. **Determination of dissociation phase**. Examine not fewer than 200 colonies by a suitable technique. The culture of the vaccine strain is seen to be in the smooth (S) phase.

The vaccine complies with the test if not fewer than 95 per cent of the colonies are of the smooth type.

3-3. **Bacteria and fungi**. The vaccine complies with the test if it does not contain extraneous micro-organisms. Verify the absence of micro-organisms other than *B. melitensis* Rev. 1 strain as described in the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-4. **Live bacteria**. Make a count of live bacteria on a solid medium suitable for the culture of *B. melitensis* Rev. 1 strain.

The vaccine complies with the test if it contains not fewer than $0.5 \times 10^9$ and not more than $4 \times 10^9$ live bacteria per dose.

4. LABELLING

The label states:

- that the vaccine may be dangerous for man;
- that the vaccine is not to be used in pregnant animals;
- that the vaccine may be dangerous for cattle and that they are not to be kept in contact with sheep or goats vaccinated less than 24 h previously.

**01/2017:1953**

# CALF CORONAVIRUS DIARRHOEA VACCINE (INACTIVATED)

## Vaccinum inactivatum diarrhoeae vituli coronaviro illatae

1. DEFINITION

Calf coronavirus diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine coronavirus, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of dams for passive protection of their progeny against coronavirus diarrhoea during the first few weeks of life.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Each vaccine virus is grown separately in cell cultures. The viral suspensions of each vaccine virus are harvested separately and inactivated by a method that maintains immunogenicity. The viral suspensions may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pregnant cows for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety in pregnant cows**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case pregnant cows that have not been vaccinated against bovine coronavirus. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cows per group at the stage or at different stages of pregnancy according to the schedule to be recommended. Administer to each pregnant animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. After each injection, measure the body temperature on the day of the injection and on the 4 following days. Observe the pregnant cows at least daily until calving.

The vaccine complies with the test if no pregnant cow shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no adverse effects on gestation or the offspring are noted.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended. The vaccine administered to each cow is of minimum potency.

Use for the test not fewer than 15 pregnant cows, preferably that do not have antibodies against bovine coronavirus. Where such cows are not available, use cows that: have not been vaccinated against bovine coronavirus; come from a farm where there is no recent history of infection with bovine coronavirus; and have a low level of antibodies against bovine coronavirus, the levels being comparable in all cows. Vaccinate not fewer than 10 pregnant cows according to the schedule to be recommended. Maintain not fewer than 5 pregnant cows as controls. Starting at calving, take the colostrum and then milk

from each cow and keep it in suitable conditions. Determine individually the protective activity of the colostrum and milk from each cow using calves born from healthy cows, and which may be born by Caesarean section, and maintained in an environment where they are not exposed to infection by bovine coronavirus. Feed colostrum and then milk to each calf every 6 h or according to the schedule to be recommended. At 5-7 days after birth, challenge each calf by the oral route with a sufficient quantity of a virulent strain of bovine coronavirus. Observe the calves at least daily for 7 days. Note the incidence, severity and duration of diarrhoea and the duration and quantity of virus excretion.

The vaccine complies with the test if there is a significant reduction in diarrhoea and virus excretion in calves given colostrum and milk from vaccinated cows compared to those given colostrum and milk from controls.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in cell cultures of the same type as those used for production or in cells shown to be at least as sensitive. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-5) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose required, carry out the test as follows. Use for the test not fewer than 7 animals of a suitable species and that do not have specific antibodies against bovine coronavirus. Vaccinate not fewer than 5 animals using 1 injection of a suitable dose. Maintain not fewer than 2 animals as controls. Where the recommended schedule requires a booster injection, this may be administered provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval not less than 14 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test to measure the antibody response. The vaccine complies with the test if the antibody level in the vaccinates is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for production of the vaccine or other cell cultures of suitable sensitivity. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Specified extraneous agents**. Use 2 cattle not less than 6 months old and that do not have antibodies against bovine herpesvirus 1 (BHV1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV). Administer to each animal by a recommended route a double dose of the vaccine, then another dose after 14 days. Observe the cattle at least daily until 14 days after the last administration. Take a blood sample at the end of the observation period. The vaccine complies with the test if it does not stimulate the formation of antibodies against bovine herpesvirus 1 (BHV1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV).

3-5. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

4. LABELLING

The label states the recommended schedule for administering colostrum and milk, post-partum.

**01/2017:1954**

# CALF ROTAVIRUS DIARRHOEA VACCINE (INACTIVATED)

## Vaccinum inactivatum diarrhoeae vituli rotaviro illatae

1. DEFINITION

Calf rotavirus diarrhoea vaccine (inactivated) is a preparation of one or more suitable strains of bovine rotavirus, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of dams for passive protection of their progeny against rotavirus diarrhoea during the first few weeks of life.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Each vaccine virus is grown separately in cell cultures. The viral suspensions of each vaccine virus are harvested separately and inactivated by a method that maintains immunogenicity. The viral suspensions may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pregnant cows for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety in pregnant cows**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case pregnant cows that have not been vaccinated against bovine rotavirus. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cows per group at the stage or at different stages of pregnancy according to the schedule to be recommended. Administer to each pregnant animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. After each injection, measure the body temperature

on the day of the injection and on the 4 following days. Observe the pregnant cows at least daily until calving.

The vaccine complies with the test if no pregnant cow shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no adverse effects on gestation or the offspring are noted.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended. The vaccine administered to each cow is of minimum potency.

Use for the test not fewer than 15 pregnant cows, preferably that do not have antibodies against bovine rotavirus. Where such cows are not available, use cows that: have not been vaccinated against bovine rotavirus; come from a farm where there is no recent history of infection with bovine rotavirus; and have a low level of antibodies against bovine rotavirus, the levels being comparable in all cows. Vaccinate not fewer than 10 pregnant cows according to the schedule to be recommended. Maintain not fewer than 5 pregnant cows as controls. Starting at calving, take the colostrum and then milk from each cow and keep it in suitable conditions. Determine individually the protective activity of the colostrum and milk from each cow using calves born from healthy cows, and which may be born by Caesarean section, and maintained in an environment where they are not exposed to infection by bovine rotavirus. Feed colostrum and then milk to each calf every 6 h or according to the schedule to be recommended. At 5-7 days after birth, challenge each calf by the oral route with a sufficient quantity of a virulent strain of bovine rotavirus. Observe the calves at least daily for 7 days. Note the incidence, severity and duration of diarrhoea and the duration and quantity of virus excretion.

The vaccine complies with the test if there is a significant reduction in diarrhoea and virus excretion in calves given colostrum and milk from vaccinated cows compared to those given colostrum and milk from controls.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in cell cultures of the same type as those used for production or in cells shown to be at least as sensitive. The quantity of inactivated virus harvest used in the test is equivalent to not less than 100 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-5) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose required, carry out the test as follows. Use for the test not fewer than 7 animals of a suitable species and that do not have antibodies against bovine rotavirus. Vaccinate not fewer than 5 animals using 1 injection of a suitable dose. Maintain not fewer than 2 animals as controls. Where the recommended schedule requires a booster injection, this may be given provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval not less than 14 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test to measure the antibody response. The vaccine complies with the test if the antibody level in the vaccinates is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for production of the vaccine or other cell cultures of suitable sensitivity. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Specified extraneous agents**. Use 2 cattle not less than 6 months old and that do not have antibodies against bovine herpesvirus 1 (BHV1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV). Administer to each animal by a recommended route a double dose of the vaccine, then another dose after 14 days. Observe the cattle at least daily until 14 days after the last administration. Take a blood sample at the end of the observation period. The vaccine complies with the test if it does not stimulate the formation of antibodies against bovine herpesvirus 1 (BHV1), bovine leukaemia virus (BLV) and bovine viral diarrhoea virus (BVDV).

3-5. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

4. LABELLING

The label states the recommended schedule for administering colostrum and milk, post-partum.

**01/2017:1298**

# CANINE ADENOVIRUS VACCINE (INACTIVATED)

## Vaccinum adenovirosis caninae inactivatum

1. DEFINITION

Canine adenovirus vaccine (inactivated) is a preparation of one or more suitable strains of canine adenovirus 1 (canine contagious hepatitis virus) and/or canine adenovirus 2, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of dogs against canine contagious hepatitis and/or respiratory disease caused by canine adenovirus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 dogs of the minimum age to be recommended and that do not have antibodies against canine adenovirus 1 or 2. Administer to each dog 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the dogs at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no dog shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. For vaccines intended to protect against hepatitis, the test described below is suitable for demonstration of immunogenicity. If the vaccine is indicated for protection against respiratory signs, a further test to demonstrate immunogenicity for this indication is also necessary.

A test is carried out for each route and method of administration to be recommended for vaccination, using in each case dogs of the minimum age to be recommended. The vaccine administered to each dog is of minimum potency.

Use for the test not fewer than 7 dogs that do not have antibodies against canine adenovirus. Vaccinate not fewer than 5 dogs, according to the schedule to be recommended. Maintain not fewer than 2 dogs as controls. Challenge each dog after 20-22 days by the intravenous route with a sufficient quantity of a suspension of pathogenic canine adenovirus. Observe the dogs at least daily for 21 days after challenge. Dogs displaying typical signs of serious infection with canine adenovirus are euthanised to avoid unnecessary suffering.

The test is not valid if, during the observation period after challenge, fewer than 100 per cent of the control dogs die from or show typical signs of serious infection with canine adenovirus. The vaccine complies with the test if, during the observation period, all the vaccinated dogs survive and show no signs of disease.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using a quantity of inactivated virus harvest equivalent to at least 10 doses of vaccine with 2 passages in cell cultures of the same type as those used for production or in cell cultures shown to be at least as sensitive. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency**. It is not necessary to carry out the Potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live canine adenovirus using 10 doses of vaccine by inoculation into sensitive cell cultures; make a passage after 6-8 days and maintain the cultures for 14 days. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant from the liquid phase by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**04/2013:1951**

# CANINE ADENOVIRUS VACCINE (LIVE)

## Vaccinum adenovirosidis caninae vivum

1. DEFINITION

Canine adenovirus vaccine (live) is a preparation of a suitable strain of canine adenovirus 2. This monograph applies to vaccines intended for the active immunisation of dogs against canine contagious hepatitis and/or respiratory disease caused by canine adenovirus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 dogs of the minimum age to be recommended for vaccination and that do not have antibodies against canine adenoviruses. Administer to each dog a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the dogs at least daily for at least 14 days.

The vaccine virus complies with the test if no dog shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using dogs 5-7 weeks old, that do not have antibodies against canine adenoviruses. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each dog of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion of virulence. After 4-6 days, prepare a suspension from the nasal and pharyngeal mucosa, tonsils, lung, spleen and if they are likely to contain virus, liver and kidney of each dog and pool the samples. Administer

1 mL of the pooled samples by a suitable route – for example, the intranasal route – to each dog of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case dogs of the minimum age to be recommended. The quantity of vaccine virus to be administered to each dog is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

2-3-3-1. *Vaccines intended to protect against hepatitis.* Use for the test not fewer than 7 dogs that do not have antibodies against canine adenoviruses. Vaccinate not fewer than 5 dogs, according to the schedule to be recommended. Maintain not fewer than 2 dogs as controls. Challenge each dog after 20-22 days by the intravenous route with a sufficient quantity of a suspension of virulent canine adenovirus 1 (canine contagious hepatitis virus). Observe the dogs at least daily for 21 days after challenge. Dogs displaying typical signs of serious infection with canine adenovirus are euthanised to avoid unnecessary suffering.

The test is not valid if during the observation period after challenge, fewer than 100 per cent of the control dogs die or show notable signs of canine adenovirosis.

The vaccine virus complies with the test if during the observation period after challenge, all the vaccinated dogs survive and show no signs of disease except for a possible transient elevated rectal temperature.

2-3-3-2. *Vaccine intended to protect against respiratory signs.* Use for the test not fewer than 20 dogs that do not have antibodies against canine adenoviruses. Vaccinate not fewer than 10 dogs, according to the schedule to be recommended. Maintain not fewer than 10 dogs as controls. Challenge each dog after 20-22 days by the intranasal route with a quantity of a suspension of virulent canine adenovirus 2 sufficient to cause typical signs of respiratory disease in a dog that does not have antibodies against canine adenoviruses. Observe the dogs at least daily for 10 days after challenge. Record the incidence of signs of respiratory and general disease in each dog (for example, sneezing, coughing, nasal and lachrymal discharge, loss of appetite). Collect nasal swabs or washings from each dog daily from days 2 to 10 after challenge and test these samples to determine the presence and titre of excreted virus.

The vaccine complies with the test if there is a notable decrease in the incidence and severity of signs and in virus excretion in vaccinates compared to controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine mixed with monospecific antiserum against canine adenovirus 2 no longer infects susceptible cell cultures.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against canine adenovirus 2 and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6-8 days and maintain the cultures for a total of 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of one or both of the tests prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:0448**

# CANINE DISTEMPER VACCINE (LIVE)

## Vaccinum morbi Carrei vivum ad canem

1. DEFINITION

Canine distemper vaccine (live) is a preparation of a suitable strain of distemper virus. This monograph applies to vaccines intended for the active immunisation of dogs against canine distemper.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 dogs of the minimum age to be recommended for vaccination and that do not have antibodies against canine distemper virus. Administer to each dog a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained

in 1 dose of the vaccine. Observe the dogs at least daily for 42 days.

The vaccine virus complies with the test if no dog shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using dogs 5-7 weeks old, that do not have antibodies against canine distemper virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each dog of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion to virulence. After 5-10 days, prepare a suspension from the nasal mucosa, tonsils, thymus, spleen and the lungs and their local lymph nodes of each dog and pool the samples. Administer 1 mL of the pooled samples by the intranasal route to each dog of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case dogs 8-16 weeks old. The quantity of vaccine virus to be administered to each dog is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 7 dogs that do not have antibodies against canine distemper virus. Vaccinate not fewer than 5 dogs according to the schedule to be recommended. Maintain not fewer than 2 dogs as controls. Challenge each dog after 20-22 days by the intravenous route with a sufficient quantity of a suspension of virulent canine distemper virus. Observe the dogs at least daily for 21 days after challenge. Dogs displaying typical signs of serious infection with canine distemper virus are euthanised to avoid unnecessary suffering.

The test is not valid if during the observation period after challenge, fewer than 100 per cent of the control dogs die or show notable signs of canine distemper.

The vaccine virus complies with the test if during the observation period after challenge, all the vaccinated dogs survive and show no signs of disease.

3. BATCH TESTS

3-1. **Identification**. The vaccine mixed with a monospecific distemper antiserum against canine distemper virus no longer provokes cytopathic effects in susceptible cell cultures.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against canine distemper virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6-8 days and maintain the cultures for 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:0447**

# CANINE LEPTOSPIROSIS VACCINE (INACTIVATED)

## Vaccinum leptospirosis caninae inactivatum

1. DEFINITION

Canine leptospirosis vaccine (inactivated) is a preparation of inactivated whole organisms and/or antigenic extract(s) of one or more suitable strains of one or more of *Leptospira interrogans* serovar canicola, serovar icterohaemorrhagiae or any other epidemiologically appropriate serovar, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of dogs against leptospirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvests are collected separately and inactivated by a suitable method. The antigen may be concentrated. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and in dogs of each category for which the vaccine is to be intended, using in each case dogs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 dogs that do not have antibodies against the principal *L. interrogans* serovars (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Administer to each dog 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after

the recommended interval. Observe the dogs at least daily for at least 14 days after the last administration. Record body temperatures the day before each vaccination, at vaccination, 4 h later and daily for 4 days.

The vaccine complies with the test if no dog shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. For each type of the serovars against which protective immunity is claimed on the label, carry out a separate test with a challenge strain representative of that serovar.

Each test is carried out for each route and method of administration to be recommended for vaccination, using in each case dogs of the minimum age to be recommended. The vaccine administered to each dog is of minimum potency.

Use for the test not fewer than 12 dogs that do not have antibodies against the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis). Vaccinate not fewer than 6 dogs, according to the schedule to be recommended. Maintain not fewer than 6 dogs as controls. Challenge each dog after 25-28 days by the conjunctival and/or intraperitoneal route with a sufficient quantity of a suspension of the relevant pathogenic *L. interrogans* serovar. Observe the dogs at least daily for 28 days after challenge.

Examine the dogs daily and record and score clinical signs observed post-challenge and any deaths that occur. If a dog shows marked signs of disease, it is euthanised. Monitor body temperatures each day for the first week after challenge. Collect blood samples from each dog on days 0, 2, 3, 4, 5, 8 and 11 post challenge. Collect urine samples from each dog on days 0, 3, 5, 8, 11, 14, 21 and 28 post challenge. Euthanise surviving dogs at the end of the observation period. Carry out post-mortem examination on any dog that dies during the observation period and on the remainder when euthanised at the end of the observation period. In particular, examine the liver and kidneys for macroscopic and microscopic signs of leptospira infection. Take a sample of each kidney and test each blood, urine and kidney sample for the presence of challenge organisms by re-isolation or by another suitable method. Analyse blood samples to detect biochemical and haematological changes indicative of infection and score these.

The test is not valid if: samples give positive results on day 0; *L. interrogans* serovar challenge strain is re-isolated from or demonstrated by another suitable method to be present in fewer than 2 samples on fewer than 2 different days, to show infection has been established in fewer than 80 per cent of the control dogs.

The vaccine complies with the test if: at least 80 per cent of the vaccinates show no more than mild signs of disease (for example, transient hyperthermia) and, depending on the *L. interrogans* serovar used for the challenge, one or more of the following is also shown:

- where the vaccine is intended to have a beneficial effect against signs of disease, the clinical scores and haematological and biochemical scores are statistically lower for the vaccinates than for the controls,
- where the vaccine is intended to have a beneficial effect against infection, the number of days that the organisms are detected in the blood is statistically lower for the vaccinates than for the controls,
- where the vaccine is intended to have a beneficial effect against urinary tract infection and excretion, the number of days that the organisms are detected in the urine and the number of kidney samples in which the organisms are detected is statistically lower for the vaccinates than for the controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual live bacteria**. Carry out a test for residual live leptospirae by inoculation of a specific medium. Inoculate 1 mL of the bulk antigen into 100 mL of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: the bulk antigen complies with the test if no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the bulk antigen together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: the test is not valid if growth of leptospirae does not occur within 14 days.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following tests may be used. However, in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, an alternative validated method, preferably the 1st option proposed below, should be used for routine testing.

2-3-2-1. For each of the serovars present in the vaccine, a suitable validated *in vitro* test may be carried out to determine the content of one or more antigenic components which are indicators of protection and which are specific for that serovar. The criteria for acceptance are set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. Methods using lipopolysaccharide (LPS)-based antigen quantification have been shown to be suitable.

2-3-2-2. A suitable validated sero-response test may be carried out. Vaccinate each animal in a group of experimental animals with a suitable dose. Collect blood samples after a suitable, fixed time after vaccination. For each of the serovars present in the vaccine, an *in vitro* test is carried out on individual blood samples to determine the antibody response to one or more antigenic components which are indicators of protection and which are specific for that serovar. The criteria for acceptance are set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

2-3-2-3. If leptospira from more than one serovar (for example *L. interrogans* serovar canicola and serovar icterohaemorrhagiae) has been used to prepare the vaccine, carry out a batch potency test for each serovar against which protective immunity is claimed on the label. Use for the test 10 healthy hamsters not more than 3 months old, that do not have antibodies against the principal serovars of *L. interrogans* (icterohaemorrhagiae, canicola, grippotyphosa, sejroe, hardjo, hebdomonadis, pomona, australis and autumnalis) and which have been obtained from a regularly tested and certified leptospira-free source. Administer 1/40 of the dose for dogs by the subcutaneous route to 5 hamsters. Maintain 5 hamsters as controls. Challenge each hamster after 15-20 days by the intraperitoneal route with a sufficient quantity of a virulent culture of leptospirae of the serovar against which protective immunity is claimed on the label. The vaccine complies with the test if not fewer than 4 of the 5 control hamsters die showing typical signs of leptospira infection within 14 days of receiving the challenge suspension and if not fewer than 4 of the 5 vaccinated hamsters remain in good health for 14 days after the death of 4 control hamsters.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition. If test 2-3-2-1 is used for the batch potency test, it also serves to identify the vaccine.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live bacteria**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live leptospirae by inoculation of a specific medium. Inoculate 1 mL of the vaccine into 100 mL of the medium. Incubate at 30 °C for 14 days, subculture into a further quantity of the medium and incubate both media at 30 °C for 14 days: the vaccine complies with the test if no growth occurs in either medium. At the same time, carry out a control test by inoculating a further quantity of the medium with the vaccine, together with a quantity of a culture containing approximately 100 leptospirae and incubating at 30 °C: the test is not valid if growth of leptospirae does not occur within 14 days.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:1955**

# CANINE PARAINFLUENZA VIRUS VACCINE (LIVE)

## Vaccinum parainfluenzae viri canini vivum

1. DEFINITION

Canine parainfluenza virus vaccine (live) is a preparation of a suitable strain of parainfluenza virus of canine origin. This monograph applies to vaccines intended for the active immunisation of dogs against respiratory signs of infection with parainfluenza virus of canine origin.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 dogs of the minimum age to be recommended for vaccination and that do not have antibodies against parainfluenza virus of canine origin. Administer to each dog a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the dogs at least daily for at least 14 days.

The vaccine virus complies with the test if no dog shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using dogs 5-7 weeks old, that do not have antibodies against parainfluenza virus of canine origin. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each dog of the 1st group by the intranasal route and by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion to virulence. After 3-10 days, prepare a suspension from nasal swabs of each dog. Administer 1 mL of the suspension from the swabs that contain the maximum amount of virus by the intranasal route to each dog of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case dogs of the minimum age to be recommended. The quantity of vaccine virus to be administered to each dog is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 15 dogs that do not have antibodies against parainfluenza virus of canine origin. Vaccinate not fewer than 10 dogs according to the schedule to be recommended. Maintain not fewer than 5 dogs as controls. Challenge each dog after not less than 20-22 days by the intratracheal or intranasal route with a sufficient quantity of a suspension of virulent parainfluenza virus of canine origin. Observe the dogs at least daily for 14 days after challenge. Collect nasal swabs or washings from each dog daily from day 2 to 10 after challenge and test these samples for the presence of excreted virus. Use a scoring system to record the incidence of coughing in each dog.

The test is not valid if more than 1 of the control dogs shows neither coughing nor the excretion of the challenge virus.

The vaccine complies with the test if the scores for coughing or virus excretion for the vaccinated dogs are significantly lower than in the controls.

3. BATCH TESTS

3-1. **Identification**. Carry out an immunofluorescence test in suitable cell cultures, using a monospecific antiserum.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against parainfluenza virus of canine origin and inoculate into cell cultures known for

their susceptibility to viruses pathogenic for the dog. Carry out a passage after 6-8 days and maintain the cultures for a total of 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:0795**

# CANINE PARVOVIROSIS VACCINE (INACTIVATED)

## Vaccinum parvovirosis caninae inactivatum

1. DEFINITION

Canine parvovirosis vaccine (inactivated) is a preparation of a suitable strain of canine parvovirus inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of dogs against canine parvovirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 dogs not older than the minimum age to be recommended for vaccination and that do not have antibodies against canine parvovirus. Administer to each dog 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the dogs at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no dog shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case dogs of the minimum age to be recommended. The vaccine administered to each dog is of minimum potency.

Use for the test not fewer than 7 dogs that do not have antibodies against canine parvovirus. Vaccinate not fewer than 5 dogs according to the schedule to be recommended. Maintain not fewer than 2 dogs as controls. Challenge each dog after 20-22 days by the oronasal route with a sufficient quantity of a suspension of pathogenic canine parvovirus. Observe the dogs at least daily for 14 days after challenge. At the end of the observation period, carry out haemagglutination tests for and titration of the virus in the faeces.

The test is not valid if fewer than 100 per cent of the control dogs show notable signs of the disease or leucopenia and excretion of the virus. The vaccine complies with the test if all the vaccinated dogs survive and show no signs of disease nor leucopenia and if the maximum titre of virus excreted in the faeces is less than 1/100 of the geometric mean of the maximum titres found in the controls.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. A test for residual live virus is carried out on the bulk harvest of each batch. The quantity of inactivated virus harvest used in the test is equivalent to not less than 100 doses of the vaccine. The inactivated virus harvest is inoculated into suitable non-confluent cells; after incubation for 8 days, a subculture is made using trypsinised cells. After incubation for a further 8 days, the cultures are examined for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. The inactivated virus harvest complies with the test if no live virus is detected.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus by inoculating not less than 10 doses of vaccine into suitable non-confluent cells; after incubation for 8 days, a subculture is made using trypsinised cells. After incubation for a further 8 days, the cultures are examined for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. Carry out test 3-4-1 or test 3-4-2.

3-4-1. *Test in guinea-pigs for haemagglutination-inhibiting antibodies*. Use for the test not fewer than 5 guinea-pigs that do not have specific antibodies. Administer to each guinea-pig by the subcutaneous route half of the dose to be recommended. After 14 days, inject again half of the dose to be recommended. 14 days later, collect blood samples and separate the serum. Inactivate each serum by heating at 56 °C for 30 min. To 1 volume of each serum add 9 volumes of a 200 g/L suspension of *light kaolin R* in *phosphate buffered saline pH 7.4 R*. Shake each mixture for 20 min. Centrifuge, collect the supernatant and mix with 1 volume

of a concentrated suspension of pig erythrocytes. Allow to stand at 4 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:10. Using each serum, prepare a series of twofold dilutions. To 0.025 mL of each of the latter dilutions add 0.025 mL of a suspension of canine parvovirus antigen containing 4 haemagglutinating units. Allow to stand at 37 °C for 30 min and add 0.05 mL of a suspension of pig erythrocytes containing $30 \times 10^6$ cells per millilitre. Allow to stand at 4 °C for 90 min and note the last dilution of serum that still completely inhibits haemagglutination. The vaccine complies with the test if the median antibody titre of the sera collected after the second vaccination is not less than 1/80.

3-4-2. *Test in dogs for virus-neutralising antibodies*. Use for the test not fewer than 2 healthy dogs, 8-12 weeks old, that have antibody titres less than 4 $ND_{50}$ per 0.1 mL of serum, measured by the method described below. Vaccinate each dog according to the recommended schedule. 14 days after vaccination, examine the serum of each dog as follows. Heat the serum at 56 °C for 30 min and prepare serial dilutions using a medium suitable for canine cells. Add to each dilution an equal volume of a virus suspension containing an amount of virus such that when the volume of serum-virus mixture appropriate for the assay system is inoculated into cell cultures, each culture receives approximately $10^4$ $CCID_{50}$. Incubate the mixtures at 37 °C for 1 h and inoculate 4 canine cell cultures with a suitable volume of each mixture. Incubate the cell cultures at 37 °C for 7 days, passage and incubate for a further 7 days. Examine the cultures for evidence of specific cytopathic effects and calculate the antibody titre. The vaccine complies with the test if the mean titre is not less than 32 $ND_{50}$ per 0.1 mL of serum. If one dog fails to respond, repeat the test using 2 more dogs and calculate the result as the mean of the titres obtained from all of the 3 dogs that have responded.

**04/2013:0964**

# CANINE PARVOVIROSIS VACCINE (LIVE)

## Vaccinum parvovirosis caninae vivum

1. DEFINITION

Canine parvovirosis vaccine (live) is a preparation of a suitable strain of canine parvovirus. This monograph applies to vaccines intended for the active immunisation of dogs against canine parvovirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the dogs for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case dogs of the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

2-3-1-1. *General safety*. For each test, use not fewer than 5 dogs that do not have haemagglutination-inhibiting antibodies against canine parvovirus. A count of white blood cells in circulating blood is made on days 4, 2 and 0 before injection of the vaccine strain. Administer to each dog a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the dogs at least daily for at least 14 days. A count of white blood cells in circulating blood is made on days 3, 5, 7 and 10 after the injection.

The test is not valid if a diminution in the number of circulating white blood cells greater than 50 per cent of the initial number - determined as the average of the 3 values found before injection of the vaccine strain – is noted. The vaccine virus complies with the test if no dog shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus and if, for each dog and each blood count, after vaccination, the number of leucocytes is not less than 50 per cent of the initial value.

2-3-1-2. *Effects on the thymus*. For each test, use not fewer than 8 dogs that do not have haemagglutination-inhibiting antibodies against canine parvovirus. Administer to each of not fewer than 4 dogs a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Maintain not fewer than 4 dogs as controls. Observe the dogs at least daily. After 14 days, euthanise 2 dogs from each group and after 21 days, the remaining dogs from each group. Carry out histological examination of the thymus of each dog.

The vaccine virus complies with the test if there is no more than slight hypoplasia of the thymus after 14 days and no damage is evident after 21 days.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using dogs of the minimum age to be recommended for vaccination, that do not have haemagglutination-inhibiting antibodies against canine parvovirus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each dog of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Collect the faeces from each dog from the 2nd to the 10th day after administration of the virus, check them for the presence of the virus and pool the faeces containing virus. Administer 1 mL of the suspension of pooled faeces by the oronasal route to each dog of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed; account is taken, notably, of the count of white blood cells, of results of histological examination of the thymus and of the titre of excreted virus. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using in each case dogs of the minimum age to be recommended for vaccination. The quantity of vaccine virus to be administered to each dog is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 7 dogs that do not have haemagglutination-inhibiting antibodies against canine parvovirus. Vaccinate not fewer than 5 dogs. Maintain not fewer than 2 dogs as controls. Challenge each dog after 20-22 days by the oronasal route with a sufficient quantity of a suspension of virulent canine parvovirus. Observe the dogs at least daily for 14 days after challenge. At the end of the observation period, carry out a haemagglutination test for and titration of the virus in the faeces.

The test is not valid if fewer than 100 per cent of the control dogs show typical signs of the disease and/or leucopenia and excretion of the virus.

The vaccine virus complies with the test if all the vaccinated dogs survive and show no sign of disease nor leucopenia and if the maximum titre of virus excreted in the faeces is less than 1/100 of the geometric mean of the maximum titres found in the controls.

3. BATCH TESTS

3-1. **Identification**. The vaccine is grown in a susceptible cell line in a substrate suitable for presenting for fluorescent antibody or immunoperoxidase tests. Suitable controls are included. A proportion of the cells is tested with a monoclonal antibody specific for canine parvovirus and a proportion of the cells tested with a monoclonal antibody specific for feline parvovirus. Canine parvovirus antigen is detected but no feline parvovirus is detected in the cells inoculated with the vaccine.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against canine parvovirus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the dog.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of haemagglutinating or haemadsorbing agents and no other sign of the presence of extraneous viruses.

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into suitable cell cultures. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:0360**

# CLOSTRIDIUM BOTULINUM VACCINE FOR VETERINARY USE

## Vaccinum Clostridii botulini ad usum veterinarium

1. DEFINITION

Clostridium botulinum vaccine for veterinary use is prepared from liquid cultures of suitable strains of *Clostridium botulinum* type C or type D or a mixture of these types. The whole culture or its filtrate or a mixture of the two is inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of animals against botulism.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. botulinum* used for production is grown in an appropriate liquid medium.
The preparation may be adsorbed, precipitated or concentrated. It may be treated with a suitable adjuvant and may be freeze-dried.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended.
The following test for safety (section 2-2-1) may be used during the demonstration of safety.

2-2-1. **Safety**. Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against *C. botulinum*. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

*The identification, the tests and the determination of potency apply to the liquid preparation and to the freeze-dried preparation reconstituted as stated on the label.*

3-1. **Identification**. When injected into a healthy animal free from antibodies against the type or types of *C. botulinum* from which the vaccine was prepared, the vaccine stimulates the production of such antibodies.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Inject 0.5 mL of the vaccine by the subcutaneous route into each of 5 mice, each weighing 17-22 g. Observe the animals at least daily for 7 days.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine.

3-4. **Potency**. Use for the test healthy white mice from a uniform stock, each weighing 18-20 g. Use as challenge dose a quantity of a toxin of *C. botulinum* of the same type as that used in the preparation of the vaccine corresponding to 25 times the paralytic dose 50 per cent, a paralytic dose 50 per cent being the quantity of toxin that, when injected by the intraperitoneal route into mice, causes paralysis in 50 per cent of the animals within an observation period of 7 days. If 2 types of *C. botulinum* have been used in the preparation of the vaccine, carry out the potency determination for each. Dilute the vaccine to be examined 8-fold using a 9 g/L solution of *sodium chloride R*. Inject 0.2 mL of the dilution subcutaneously into each of 20 mice. After 21 days, inject the challenge dose by the intraperitoneal route into each of the vaccinated mice and into each of 10 control mice. Observe the mice for 7 days and record the number of animals that show signs of botulism.

The test is not valid unless all the control mice show signs of botulism during the observation period. The vaccine complies with the test if not fewer than 80 per cent of the vaccinated mice are protected.

4. LABELLING

The label states:

- the type or types of *C. botulinum* from which the vaccine has been prepared;
- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture or a mixture of the two.

**04/2013:0361**

# CLOSTRIDIUM CHAUVOEI VACCINE FOR VETERINARY USE

## Vaccinum Clostridii chauvoei ad usum veterinarium

1. DEFINITION

Clostridium chauvoei vaccine for veterinary use is prepared from liquid cultures of one or more suitable strains of *Clostridium chauvoei*. The whole culture is inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of animals against disease caused by *C. chauvoei*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. chauvoei* used for production is grown in an appropriate liquid medium. Inactivated cultures may be treated with a suitable adjuvant.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended.

The following test for safety (section 2-2-1) may be used during the demonstration of safety.

2-2-1. **Safety**. Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against *C. chauvoei*. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

3. BATCH TESTS

3-1. **Identification**. The vaccine protects susceptible animals against infection with *C. chauvoei*. The potency test may also serve for identification.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. Use for the test not fewer than 10 healthy guinea-pigs, each weighing 350-450 g. Administer to each guinea-pig by the subcutaneous route a quantity of the vaccine not greater than the minimum dose stated on the label as the 1st dose. After 28 days, administer to the same animals a quantity of the vaccine not greater than the minimum dose stated on the label as the 2nd dose. 14 days after the 2nd vaccination, inoculate by the intramuscular route each of the vaccinated guinea-pigs and each of 5 control animals with a suitable quantity of a virulent culture, or of a spore suspension, of *C. chauvoei*, activated if necessary with an activating agent such as calcium chloride.

The vaccine complies with the test if not more than 10 per cent of the vaccinated guinea-pigs die from *C. chauvoei* infection within 5 days and all the control animals die from *C. chauvoei* infection within 48 h of challenge or within 72 h if a spore suspension was used for the challenge. If more than 10 per cent but not more than 20 per cent of the vaccinated animals die, repeat the test. The vaccine complies with the test if not more than 10 per cent of the 2nd group of vaccinated animals die within 5 days and all of the 2nd group of control animals die within 48 h of challenge or within 72 h if a spore suspension was used for the challenge. To avoid unnecessary suffering following virulent challenge, moribund animals are euthanised and are then considered to have died from *C. chauvoei* infection.

**04/2013:0362**

# CLOSTRIDIUM NOVYI (TYPE B) VACCINE FOR VETERINARY USE

## Vaccinum Clostridii novyi B ad usum veterinarium

1. DEFINITION

Clostridium novyi (type B) vaccine for veterinary use is prepared from a liquid culture of a suitable strain of *Clostridium novyi* (type B).

The whole culture or its filtrate or a mixture of the two is inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. This monograph applies to vaccines

intended for active immunisation of animals and/or to protect passively their progeny against disease caused by *C. novyi* (type B).

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. novyi* (type B) used for production is grown in an appropriate liquid medium. Toxoids and/or inactivated cultures may be treated with a suitable adjuvant, after concentration if necessary.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended. For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the schedule to be recommended, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

The following test for safety (section 2-2-1) may be used during the demonstration of safety.

2-2-1. **Safety**. Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against *C. novyi* (type B). Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine. If the test is carried out in pregnant animals, no adverse effects on gestation or the offspring are noted.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual toxicity**. A test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a 2nd test is carried out at as late a stage as possible during the production process. The test for residual toxicity (section 3-3) may be omitted by the manufacturer.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency.

Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test under Potency (section 3-4) has been established.

Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the alpha toxin of *C. novyi* in the individual sera by a suitable method such as an immunochemical method (*2.7.1*) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. novyi* alpha antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum.

The vaccine complies with the test if the level of antibodies is not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

3. BATCH TESTS

3-1. **Identification**. When injected into animals that do not have novyi alpha antitoxin, the vaccine stimulates the formation of such antitoxins.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Administer 0.5 mL of the vaccine by the subcutaneous route into each of 5 mice, each weighing 17-22 g. Observe the animals at least daily for 7 days.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine.

3-4. **Potency**. Use for the test not fewer than 10 healthy rabbits, 3-6 months old. Administer to each rabbit by the subcutaneous route a quantity of vaccine not greater than the minimum dose stated on the label as the 1st dose. After 21-28 days, administer to the same animals a quantity of the vaccine not greater than the minimum dose stated on the label as the 2nd dose. 10-14 days after the 2nd injection, bleed the rabbits and pool the sera.

The vaccine complies with the test if the potency of the pooled sera is not less than 3.5 IU/mL.

The International Unit is the specific neutralising activity for *C. novyi* alpha toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *C. novyi* alpha toxin with the quantity of a reference preparation of Clostridium novyi alpha antitoxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. novyi* alpha toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the serum to be examined is determined in relation to the reference preparation using the test toxin.

*Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum.

3-4-1. *Preparation of test toxin*. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *C. novyi* type B and dry by a suitable method. Select the test toxin by determining for mice the L+/10 dose and the $LD_{50}$, the observation period being 72 h.

A suitable alpha toxin contains not less than 1 L+/10 dose in 0.05 mg and not less than 10 $LD_{50}$ in each L+/10 dose.

3-4-2. *Determination of test dose of toxin*. Prepare a solution of the reference preparation in a suitable liquid so that it contains 1 IU/mL. Prepare a solution of the test toxin in a suitable liquid so that 1 mL contains a precisely known amount such as 1 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 1.0 mL of the solution of the reference preparation (1 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice, each weighing 17-22 g, for each mixture, inject a dose of 0.2 mL by the intramuscular or the subcutaneous route into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.2 mL of the mixture is in excess of the test dose. If none of the mice die, the amount of toxin present in 0.2 mL of the mixture is less than the test dose. Prepare fresh mixtures such that 2.0 mL of each mixture contains 1.0 mL of the solution of the reference preparation (1 IU) and one of a series of graded volumes of the solution of

the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.2 mL by the intramuscular or the subcutaneous route into each mouse. Observe the mice for 72 h. Repeat the determination at least once and combine the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition.

The test dose of toxin is the amount present in 0.2 mL of that mixture which causes the death of one half of the total number of mice injected with it.

3-4-3. *Determination of the potency of the serum obtained from rabbits*

*Preliminary test.* Dissolve a quantity of the test toxin in a suitable liquid so that 1.0 mL contains 10 times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that each mixture contains 1.0 mL of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 2.0 mL. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.2 mL by the intramuscular or the subcutaneous route into each mouse. Observe the mice for 72 h. If none of the mice die, 0.2 mL of the mixture contains more than 0.1 IU. If all the mice die, 0.2 mL of the mixture contains less than 0.1 IU.

*Final test.* Prepare a series of mixtures of the solution of the test toxin and the serum to be examined such that 2.0 mL of each mixture contains 1.0 mL of the solution of the test toxin and one of a series of graded volumes of the serum to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 2.0 mL of each mixture contains 1.0 mL of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation, in order to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, proceed as described in the preliminary test.

The test mixture that contains 0.1 IU in 0.2 mL is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.1 IU in 0.2 mL. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used;
- 91.5 per cent and 109 per cent when 4 animals per dose are used;
- 93 per cent and 108 per cent when 6 animals per dose are used.

4. LABELLING

The label states:

- whether the product is a toxoid, a vaccine prepared from a whole inactivated culture or a mixture of the two;
- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

**04/2013:0363**

# CLOSTRIDIUM PERFRINGENS VACCINE FOR VETERINARY USE

## Vaccinum Clostridii perfringentis ad usum veterinarium

1. DEFINITION

Clostridium perfringens vaccine for veterinary use is prepared from liquid cultures of suitable strains of *Clostridium perfringens* type B, *C. perfringens* type C or *C. perfringens* type D or a mixture of these types.

The whole cultures or their filtrates or a mixture of the two are inactivated to eliminate their toxicity while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of animals and/or to protect passively their progeny against disease caused by *C. perfringens*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. perfringens* used for production is grown in an appropriate liquid medium. Toxoids and/or inactivated cultures may be treated with a suitable adjuvant.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended. For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the schedule to be recommended, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

The following test for safety (section 2-2-1) may be used during the demonstration of safety.

2-2-1. **Safety**. Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against *C. perfringens*. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine. If the test is carried out in pregnant animals, no adverse effects on gestation or the offspring are noted.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual toxicity**. A test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a 2nd test is carried out at as late a stage as possible during the production process. The test for residual toxicity (section 3-3) may be omitted by the manufacturer.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory

results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test under Potency (section 3-4) has been established.

Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the beta and/or epsilon toxins of *C. perfringens* in the individual sera by a suitable method such as an immunochemical method (*2.7.1*) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. perfringens* beta and/or epsilon antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum.

The vaccine complies with the test if the level or levels of antibodies are not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

3. BATCH TESTS

3-1. **Identification**

*Type B.* When injected into animals that do not have beta and epsilon antitoxins, the vaccine stimulates the formation of such antitoxins.

*Type C.* When injected into animals that do not have beta antitoxin, the vaccine stimulates the formation of such antitoxin.

*Type D.* When injected into animals that do not have epsilon antitoxin, the vaccine stimulates the formation of such antitoxin.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Administer 0.5 mL of the vaccine by the subcutaneous route to each of 5 mice, each weighing 17-22 g. Observe the mice at least daily for 7 days.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine.

3-4. **Potency**. Use for the test not fewer than 10 healthy rabbits, 3-6 months old. Administer to each rabbit by the subcutaneous route a quantity of vaccine not greater than the minimum dose stated on the label as the 1st dose. After 21-28 days, administer to the same animals a quantity of the vaccine not greater than the minimum dose stated on the label as the 2nd dose. 10-14 days after the 2nd injection, bleed the rabbits and pool the sera.

*Type B.* The vaccine complies with the test if the potency of the pooled sera is not less than 10 IU of beta antitoxin and not less than 5 IU of epsilon antitoxin per millilitre.

*Type C.* The vaccine complies with the test if the potency of the pooled sera is not less than 10 IU of beta antitoxin per millilitre.

*Type D.* The vaccine complies with the test if the potency of the pooled sera is not less than 5 IU of epsilon antitoxin per millilitre.

3-4-1. *International standard for Clostridium perfringens beta antitoxin*

The International Unit is the specific neutralising activity for *C. perfringens* beta toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

3-4-2. *International standard for Clostridium perfringens epsilon antitoxin*

The International Unit is the specific neutralising activity for *C. perfringens* epsilon toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard, is stated by the World Health Organization.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *C. perfringens* beta toxin or *C. perfringens* epsilon toxin with the quantity of a reference preparation of Clostridium perfringens beta antitoxin or Clostridium perfringens epsilon antitoxin, as appropriate, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. perfringens* beta or epsilon toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the appropriate reference preparation; the potency of the serum to be examined is determined in relation to the appropriate reference preparation using the appropriate test toxin.

*Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum.

3-4-3. *Preparation of test toxin*. Prepare the test toxin from a sterile filtrate of an early culture in liquid medium of *C. perfringens* type B, type C or type D as appropriate and dry by a suitable method. Use a beta or epsilon toxin as appropriate. Select the test toxin by determining for mice the L+ and the $LD_{50}$ for the beta toxin and the L+/10 dose and the $LD_{50}$ for the epsilon toxin, the observation period being 72 h.

A suitable beta toxin contains not less than 1 L+ in 0.2 mg and not less than 25 $LD_{50}$ in 1 L+ dose. A suitable epsilon toxin contains not less than 1 L+/10 dose in 0.005 mg and not less than 20 $LD_{50}$ in 1 L+/10 dose.

3-4-4. *Determination of test dose of toxin*. Prepare a solution of the reference preparation in a suitable liquid so that it contains 5 IU/mL for Clostridium perfringens beta antitoxin and 0.5 IU/mL for Clostridium perfringens epsilon antitoxin. Prepare a solution of the test toxin in a suitable liquid so that 1 mL contains a precisely known amount such as 10 mg for beta toxin and 1 mg for epsilon toxin. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice, each weighing 17-22 g, for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 mL of the mixture is in excess of the test dose. If none of the mice die the amount of toxin present in 0.5 mL of the mixture is less than the test dose. Prepare fresh mixtures such that 5.0 mL of each mixture contains 2.0 mL of the solution of the reference preparation and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. Repeat the determination at least once and add together the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of given composition.

The test dose of toxin is the amount present in 0.5 mL of that mixture which causes the death of one half of the total number of mice injected with it.

3-4-5. *Determination of the potency of the serum obtained from rabbits*

*Preliminary test.* Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 mL contains 10 times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined

such that each contains 2.0 mL of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 mL. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. If none of the mice die, 0.5 mL of the mixture contains more than 1 IU of beta antitoxin or 0.1 IU of epsilon antitoxin. If all the mice die, 0.5 mL of the mixture contains less than 1 IU of beta antitoxin or 0.1 IU of epsilon antitoxin.

*Final test.* Prepare a series of mixtures of the solution of the test toxin and the serum to be examined such that 5.0 mL of each mixture contains 2.0 mL of the solution of the test toxin and one of a series of graded volumes of the serum to be examined separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 5.0 mL of each mixture contains 2.0 mL of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation, in order to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 30 min. Using not fewer than 2 mice for each mixture proceed as described in the preliminary test.

*Beta antitoxin.* The test mixture that contains 1 IU in 0.5 mL is that mixture which kills the same or almost the same number of mice as the reference mixture containing 1 IU in 0.5 mL.

*Epsilon antitoxin.* The test mixture that contains 0.1 IU in 0.5 mL is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.1 IU in 0.5 mL. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P$ = 0.95) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used;
- 91.5 per cent and 109 per cent when 4 animals per dose are used;
- 93 per cent and 108 per cent when 6 animals per dose are used.

4. LABELLING

The label states:

- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture or a mixture of the two;
- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

**04/2013:0364**

# CLOSTRIDIUM SEPTICUM VACCINE FOR VETERINARY USE

## Vaccinum Clostridii septici ad usum veterinarium

1. DEFINITION

Clostridium septicum vaccine for veterinary use is prepared from a liquid culture of a suitable strain of *Clostridium septicum*.

The whole culture or its filtrate or a mixture of the two is inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of animals and/or to protect passively their progeny against disease caused by *C. septicum*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. septicum* used for production is grown in an appropriate liquid medium. Toxoid and/or inactivated cultures may be treated with a suitable adjuvant.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended. For the latter, it shall be demonstrated that for each target species the vaccine, when administered according to the schedule to be recommended, stimulates an immune response (for example, induction of antibodies) consistent with the claims made for the product.

The following test for safety (section 2-2-1) may be used during the demonstration of safety.

2-2-1. **Safety**. Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against *C. septicum*. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine. If the test is carried out in pregnant animals, no adverse effets on gestation or the offspring are noted.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual toxicity**. A test for detoxification is carried out immediately after the detoxification process and, when there is risk of reversion, a 2nd test is carried out at as late a stage as possible during the production process. The test for residual toxicity (section 3-3) may be omitted by the manufacturer.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species. The following test may be used after a satisfactory correlation with the test under Potency (section 3-4) has been established.

Vaccinate rabbits as described under Potency and prepare sera. Determine the level of antibodies against the toxin of *C. septicum* in the individual sera by a suitable method such as an immunochemical method (*2.7.1*) or neutralisation in cell cultures. Use a homologous reference serum calibrated in International Units of *C. septicum* antitoxin. *Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum. The vaccine complies with the test if the level of antibodies is not less than that found for a batch of vaccine that has given satisfactory results in the test described under Potency and that has been shown to be satisfactory with respect to immunogenicity in the target species.

3. BATCH TESTS

3-1. **Identification**. When injected into animals that do not have *C. septicum* antitoxin, the vaccine stimulates the formation of such antitoxin.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Inject 0.5 mL of the vaccine by the subcutaneous route into each of 5 mice, each weighing 17-22 g. Observe the mice at least daily for 7 days.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine.

3-4. **Potency**. Use for the test not fewer than 10 healthy rabbits, 3-6 months old. Administer to each rabbit by the subcutaneous route a quantity of vaccine not greater than the minimum dose stated on the label as the 1st dose. After 21-28 days, administer to the same animals a quantity of the vaccine not greater than the minimum dose stated on the label as the 2nd dose. 10-14 days after the 2nd injection, bleed the rabbits and pool the sera.

The vaccine complies with the test if the potency of the pooled sera is not less than 2.5 IU/mL.

The International Unit is the specific neutralising activity for *C. septicum* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The potency of the pooled sera obtained from the rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a dose of *C. septicum* toxin with the quantity of a reference preparation of Clostridium septicum antitoxin, calibrated in International Units, necessary to give the same protection. For this comparison, a suitable preparation of *C. septicum* toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the serum to be examined is determined in relation to the reference preparation using the test toxin.

*Clostridia (multicomponent) rabbit antiserum BRP* is suitable for use as a reference serum.

3-4-1. *Preparation of test toxin*. Prepare the test toxin from a sterile filtrate of a 1- to 3-day culture of *C. septicum* in liquid medium and dry by a suitable method. Select the test toxin by determining for mice the L+/5 dose and the $LD_{50}$, the observation period being 72 h.

A suitable toxin contains not less than 1 L+/5 dose in 1.0 mg and not less than 10 $LD_{50}$ in each L+/5 dose.

3-4-2. *Determination of test dose of toxin*. Prepare a solution of the reference preparation in a suitable liquid so that it contains 1.0 IU/mL. Prepare a solution of the test toxin in a suitable liquid so that 1 mL contains a precisely known amount, such as 4 mg. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each mixture contains 2.0 mL of the solution of the reference preparation (2 IU), one of a series of graded volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice, each weighing 17-22 g, for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. If all the mice die, the amount of toxin present in 0.5 mL of the mixture is in excess of the test dose. If none of the mice die, the amount of toxin present in 0.5 mL of the mixture is less than the test dose. Prepare fresh mixtures such that 5.0 mL of each mixture contains 2.0 mL of the reference preparation (2 IU) and one of a series of graded volumes of the solution of the test toxin separated from each other by steps of not more than 20 per cent and covering the expected end-point. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. Repeat the determination at least once and add together the results of the separate tests that have been made with mixtures of the same composition so that a series of totals is obtained, each total representing the mortality due to a mixture of a given composition.

The test dose of toxin is the amount present in 0.5 mL of that mixture which causes the death of one half of the total number of mice injected with it.

3-4-3. *Determination of the potency of the serum obtained from rabbits*

*Preliminary test.* Dissolve a quantity of the test toxin in a suitable liquid so that 2.0 mL contains 10 times the test dose (solution of the test toxin). Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a series of graded volumes of the serum to be examined and sufficient of a suitable liquid to bring the final volume to 5.0 mL. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture, inject a dose of 0.5 mL by the intravenous or the intraperitoneal route into each mouse. Observe the mice for 72 h. If none of the mice die, 0.5 mL of the mixture contains more than 0.2 IU. If all the mice die, 0.5 mL of the mixture contains less than 0.2 IU.

*Final test.* Prepare a series of mixtures of the solution of the test toxin and of the serum to be examined such that 5.0 mL of each mixture contains 2.0 mL of the solution of the test toxin and one of a series of graded volumes of the serum to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined by the preliminary test. Prepare further mixtures of the solution of the test toxin and of the solution of the reference preparation such that 5.0 mL of each mixture contains 2.0 mL of the solution of the test toxin and one of a series of graded volumes of the solution of the reference preparation to confirm the test dose of the toxin. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 mice for each mixture proceed as described in the preliminary test. The test mixture which contains 0.2 IU in 0.5 mL is that mixture which kills the same or almost the same number of mice as the reference mixture containing 0.2 IU in 0.5 mL. Repeat the determination at least once and calculate the average of all valid estimates. The test is valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P = 0.95$) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used;
- 91.5 per cent and 109 per cent when 4 animals per dose are used;
- 93 per cent and 108 per cent when 6 animals per dose are used.

4. LABELLING

The label states:

- whether the preparation is a toxoid or a vaccine prepared from a whole inactivated culture, or a mixture of the two;
- for each target species, the immunising effect produced (for example, antibody production, protection against signs of disease or infection).

04/2013:2326
corrected 8.1

# COCCIDIOSIS VACCINE (LIVE) FOR CHICKENS

## Vaccinum coccidiosidis vivum ad pullum

### 1. DEFINITION

Coccidiosis vaccine (live) for chickens is a preparation of sporulated oocysts of a suitable line or lines of species of coccidial parasites (*Eimeria* spp.). This monograph applies to vaccines intended for administration to chickens for active immunisation.

### 2. PRODUCTION

#### 2-1. *PREPARATION OF THE VACCINE*

Oocysts are produced in chickens from a flock free from specified pathogens (SPF) (*5.2.2*) or in embryonated hens' eggs from an SPF flock (*5.2.2*). The eggs must be subject to disinfection and/or incubation conditions validated to ensure the inactivation of any *Eimeria* that may be on the shells. The hatched chickens must then be reared in disinfected premises, in isolation conditions that ensure no infection with *Eimeria*. The chickens must not have been treated with coccidiostats. Oocysts are collected from faeces or contents of the intestinal tract of infected chickens during the patent period. Oocysts of different *Eimeria* lines are produced separately. Oocysts are isolated, purified, disinfected, sporulated and counted. The vaccine is produced by blending defined numbers of sporulated oocysts of each line in a suitable medium.

#### 2-2. *SEED LOTS*

2-2-1. **Identification**. The identity of each *Eimeria* master seed is established from the characteristics of the coccidia produced from it, based on an appropriate selection of the following characteristics: size and shape of the oocyst; localisation of the developmental stages in the chicken intestine; pathognomonic lesions (*E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima* and *E. brunetti*) and lack of macroscopic lesions (*E. praecox* and *E. mitis*); size of schizonts in the intestinal mucosa; size of gametocytes in the mucosa; differences in the electrophoretic mobilities of certain isoenzymes, e.g. lactate dehydrogenase and glucose phosphate isomerase; and by the use of molecular biology techniques. Artificially attenuated lines may be distinguished from the parent strains by studying parameters appropriate to the method of attenuation.

2-2-2. **Extraneous agents**. Carry out tests 1-6 of chapter *2.6.24. Avian viral vaccines: tests for extraneous agents in seed lots*. General provisions *a-d*, *f* and *h* and section 7 of chapter *2.6.24* are also applicable. In these tests on the master seed lot, use organisms that are not more than 5 passages from the master seed lot at the start of the tests. Each master seed lot complies with the requirements of each test.

#### 2-3. *CHOICE OF VACCINE COMPOSITION*

Only coccidial lines shown to be satisfactory with respect to residual pathogenicity and increase in virulence may be used in the preparation of the vaccine, and the tests described below (sections 2-3-2 and 2-3-3) may be used to demonstrate this. The vaccine shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended. The following tests under Specific test for the safety of the vaccine composition (section 2-3-1) and Immunogenicity (section 2-3-4) may be used during the demonstration of safety and efficacy.

2-3-1. **Specific test for the safety of the vaccine composition**. Carry out the test with a preparation containing oocysts of each species at the least attenuated passage level that will be present in a batch of vaccine. Use not fewer than 10 chickens from an SPF flock (*5.2.2*). The chickens must be hatched and reared as described in section 2-1 and must not have been treated with coccidiostats. Use chickens of the category that is expected to be the most sensitive, i.e. 14-day-old chickens. During the test, chickens are housed in suitable conditions with the use of floor pens or cages with solid floors to favour reinfection with oocysts. Administer by gavage or another suitable route to each chicken a quantity of vaccinal oocysts consisting of the equivalent of not less than 10 times the maximum quantity of oocysts of each coccidial species likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the vaccinated chickens die from causes not attributable to the vaccinal oocysts.

The vaccine complies with the test if no vaccinated chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-3-2. **Test for residual pathogenicity**. Carry out a separate test with each coccidial species and line to be included in the vaccine. Use in each case a preparation containing oocysts at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. For each test use not fewer than 20 chickens from an SPF flock (*5.2.2*). The chickens must be hatched and reared as described in section 2-1 and must not have been treated with coccidiostats. Use chickens of the category that is expected to be the most sensitive, i.e. 14-day-old chickens. During the test, the chickens are placed in cages (or any other suitable accomodation that prevents reinfection and allows collection of faeces). Administer by gavage or another suitable route to each chicken the equivalent of not less than 10 times the maximum quantity of the vaccinal oocysts likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for 14 days. The test is not valid if more than 10 per cent of the chickens die from causes not attributable to the vaccinal oocysts. Collect faeces and determine oocyst production daily from day 3 until day 14. On one day between days 4 and 8, depending on the length of the pre-patent period, when lesions are expected to be maximal, and on day 14, euthanise not fewer than 9 chickens and examine the intestinal tract for specific lesions indicative of infection with the coccidial species or, for species known not to induce macroscopic lesions (*E. mitis* and *E. praecox*), microscopic evidence of infection such as demonstration of oocysts or developing oocysts in the intestinal contents or scrapings of the intenstinal wall. For species that have the potential to produce relevant macroscopic pathological changes if not attenuated, a scoring system with a scale from 0 to 4 is used to record the species-specific lesions visible in the intestine as follows.

*Eimeria acervulina*

0 No gross lesions.

1 Scattered, white, plaque-like lesions containing developing oocysts are confined to the duodenum. These lesions are elongated with the longer axis transversely oriented on the intestinal walls like the rungs of a ladder. They may be seen on either the serosal or mucosal intestinal surfaces. There may be up to a maximum of 5 lesions per square centimetre.

2 Lesions are much closer together, but not coalescent; lesions may extend as far posterior as 20 cm below the duodenum in 3-week-old birds. The intestinal walls show no thickening. Digestive tract contents are normal.

3 Lesions are numerous enough to cause coalescence with reduction in lesion size and give the intestine a coated appearance. The intestinal wall is thickened and the contents are watery. Lesions may extend as far posterior as the yolk sac diverticulum.

4 The mucosal wall is greyish with completely coalescent colonies. Congestion may be confined to small petechiae or, in extremely heavy infections, the entire mucosa may be bright red in colour. Individual lesions may be indistinguishable in the upper intestine. Typical ladder-like lesions appear in the middle part of the intestine. The intestinal wall is very much thickened and the intestine is filled with a creamy exudate, which may bear large numbers of oocysts. Birds dying of coccidiosis are scored as 4.

*Eimeria brunetti*

0 No gross lesions.

1 No gross lesions. In the absence of distinct lesions, the presence of parasites may go undetected unless scrapings from suspicious areas are examined microscopically.

2 The intestinal wall may appear grey in colour. The lower portion may be thickened and flecks of pink material sloughed from the intestine are present.

3 The intestinal wall is thickened and a blood-tinged catarrhal exudate is present. Transverse red streaks may be present in the lower rectum and lesions occur in the caecal tonsils. Soft mucous plugs may be present in this latter area.

4 Extensive coagulation necrosis of the mucosal surface of the lower intestine may be present. In some birds a dry necrotic membrane may line the intestine and caseous cores may plug the entrance to the caeca. Lesions may extend into the middle or upper intestine. Birds dying of coccidiosis are scored as 4.

*Eimeria maxima*

0 No gross lesions.

1 Small red petechiae may appear on the serosal side of the mid-intestine. There is no ballooning or thickening of the intestine, though small amounts of orange mucous may be present.

2 The serosal surface may be speckled with numerous red petechiae. The intestine may be filled with orange mucous, but there is little or no ballooning of the intestine. There is thickening of the wall.

3 The intestinal wall is ballooned and thickened. The mucosal surface is roughened. Intestinal contents are composed of pinpoint blood clots and mucous.

4 The intestinal wall may be ballooned for most of its length. It contains numerous blood clots and digested red blood cells giving a characteristic colour and putrid odour. The wall is greatly thickened. Birds dying of coccidiosis are scored as 4.

*Eimeria necatrix*

0 No gross lesions.

1 Small scattered petechiae and white spots are easily seen on the serosal surface. There is little, if any, damage apparent on the mucosal surface.

2 Numerous petechiae are seen on the serosal surface. Slight ballooning confined to the midgut area may be present.

3 There is extensive haemorrhage into the lumen of the intestine. The serosal surface is covered with red petechiae and/or white plaques, and is rough and thickened with many pinpoint haemorrhages. Normal intestinal contents are lacking. Ballooning extends over the lower half of the small intestine.

4 Extensive haemorrhage gives the intestine a dark colour, and the intestinal contents consist of red or brown mucous. Ballooning may extend throughout much of the length of the intestine. Birds dying of coccidiosis are scored as 4.

*Eimeria tenella*

0 No gross lesions.

1 Very few scattered petechiae are seen on the caecal wall, and there is no thickening of the caecal walls. Normal caecal contents are present.

2 Lesions are more numerous with noticeable blood in the caecal contents, and the caecal wall is somewhat thickened. Normal caecal contents are present.

3 Large amounts of blood or caecal cores are present, and the caecal walls are greatly thickened. There is little if any normal faecal content in the caeca.

4 The caecal wall is greatly distended with blood or large caseous cores. Faecal debris is lacking or included in the cores. Birds dying of coccidiosis are scored as 4.

The species and line comply with the test for attenuation if no more than mild coccidial lesions or limited signs of infection are observed; where the scoring system described above is appropriate, the average lesion score on the day of sampling between days 4 and 8 and on day 14 is not greater than 1.5 points and no individual score is greater than 3 points. The quantity and time of oocyst production is determined.

2-3-3. **Increase in virulence**. Carry out a separate test according to general chapter *5.2.6* with each coccidial species and line to be included in the vaccine. Use a preparation containing oocysts at the master seed lot level. If the quantity of the master seed sufficient for performing the test is not available, the lowest passage seed used for the production that is available in sufficient quantity may be used. For each test use 14-day-old chickens from an SPF flock (*5.2.2*). If the properties of the vaccinal oocysts allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise, passage as described below is carried out. The chickens must be hatched and reared as described in section 2-1 and must not have been treated with coccidiostats. During the test, the chickens are placed in cages (or any other suitable accomodation that prevents reinfection and allows collection of faeces). Administer by gavage or another suitable route to each chicken of the 1st group a quantity of oocysts that will allow recovery of the oocysts for the passages described below. Collect faeces daily from day 2 to day 14 after infection and prepare a pooled suspension of sporulated oocysts from the 5 chickens. Administer a suitable quantity by gavage or another suitable route to each chicken of the next group. Carry out this passage operation not fewer than 4 times, verifying the presence of oocysts at each passage. If the vaccinal oocysts are not found at a passage level, repeat the passage by administration to a group of 10 chickens. Carry out the test for residual pathogenicity (section 2-3-2), using the material used for the 1st passage and the oocysts that have been recovered in the final passage. Compare the results obtained for signs of lesions or infection in the intestinal tract and oocyst output from administration of passaged and unpassaged oocysts.

The line complies with the test if no indication of an increase in virulence of the maximally passaged oocysts compared with the unpassaged oocysts is observed.

The test is not valid if oocysts are not recovered at any passage level.

2-3-4. **Immunogenicity**. The efficacy of each coccidial species and line included in the vaccine is determined in a separate study with an appropriate challenge strain. For each component, a test is carried out with vaccine administered by each route and method of administration to be recommended, using in each case chickens not older than the minimum age to be recommended for vaccination. The quantity of each of the components in the batch of vaccine administered to each chicken is not greater than the minimum number of oocysts to be stated on the label and the oocysts are at the most attenuated passage level that will be present in a batch of vaccine. Use for the test not fewer than 40 chickens from an SPF flock (*5.2.2*). The chickens must be hatched and reared as described in section 2-1 and must not have been treated with coccidiostats. Vaccinate not fewer than 20 chickens and maintain not fewer than 20 chickens as controls. For the evaluation of weight gain with *Eimeria* strains showing a low pathogenicity, the number of chickens used may be higher. The test may require different challenge doses for different test parameters and so may be assessed as separate challenge groups. For example, a lower challenge dose may be needed to determine the effect on oocyst output than the dose needed to determine the effect on weight gain and lesion scoring. After vaccination, the chickens are housed in suitable conditions with the use of floor pens or cages with solid floors to favour reinfection with oocysts. On a suitable day between days 14 and 21 after vaccination, weigh each chicken, move them to cages (or any other suitable accomodation that prevents reinfection and allows collection of faeces) and challenge each chicken by gavage or another suitable route with a sufficient quantity of virulent coccidia to induce in the unvaccinated controls signs of disease characteristic of the *Eimeria* challenge species. Observe the chickens at least daily until the end of the test. Record deaths and the number of surviving chickens that show clinical signs of disease. Collect faeces and determine oocyst production from day 3 after challenge until the end of the test. On an appropriate day between days 4 and 8 after challenge, depending on the length of the pre-patent period of the challenge species, weigh each chicken. Euthanise 10 chickens from each group and examine them for lesions in the intestinal tract. Where appropriate, record the specific lesions indicative of the coccidial challenge species (using the scoring system described in section 2-3-2). For species known not to induce macroscopic lesions (*E. mitis* and *E. praecox*), examine the chickens for microscopic evidence of infection such as demonstration of oocysts or developing oocysts in the intestinal contents or scrapings of the intestinal wall. On day 14 after challenge, weigh each of the remaining chickens.

The test is not valid if:

- during the period between vaccination and challenge, more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine;
- for challenges with *E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima* or *E. brunetti*, fewer than 80 per cent of the control chickens euthanised between days 4 and 8 have marked characteristic lesions of the challenge infection in the intestine at post-mortem examination (e.g. lesion scores not less than 2);
- for challenges with *E. mitis* or *E. praecox*, fewer than 80 per cent of the control chickens euthanised between days 4 and 8 are infected.

The vaccine complies with the test if:

- for all the *Eimeria* challenge species, the production of the oocysts is significantly decreased in vaccinates compared with controls;
- for all the *Eimeria* challenge species, no vaccinated chicken dies due to the challenge infection;
- for challenge with *E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima* or *E. brunetti*, at least 80 per cent of the vaccinates show no more than mild signs of disease and these are less marked than those in the controls;
- for challenges with *E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima* or *E. brunetti*, at least 80 per cent of the vaccinates have no or minimal lesions in the intestine (e.g. mean lesion scores not greater than 1) and no bird has a lesion score of 4;
- for challenges with *E. tenella*, *E. acervulina*, *E. necatrix*, *E. maxima*, *E. brunetti*, *E. mitis*, or *E. praecox*, the growth rate in the vaccinates is significantly greater than in the controls.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **In-process test for sporulation rate and oocyst count**. A sample of each oocyst bulk is examined microscopically after the sporulation step and before blending to determine the percentage of sporulated oocysts and the oocyst count. The values obtained are within the limits shown to allow preparation of a satisfactory vaccine.

2-4-2. **Batch potency test for each *Eimeria* species in the vaccine**. It is not necessary to carry out the potency test (section 3-6) for each batch of the vaccine if it has been carried out using a batch or batches of vaccine with minimum potency and sporulated oocyst content. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance for each component being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

2-4-3. **Freedom from extraneous agents**. The disinfection method applied during the preparation of the final product from the harvested oocysts may be validated to show effective inactivation of certain potential extraneous agents. Where relevant validation data is available and where justified and authorised, some or all of the tests indicated under Extraneous agents (section 3-4) may be omitted as routine tests on each batch.

3. BATCH TESTS

3-1. **Identification**

3-1-1. Microscopical examination is used to confirm the presence of coccidial oocysts in the batch of vaccine.

3-1-2. The potency test (or batch potency test) is used to confirm the presence of oocysts of each of the *Eimeria* species stated on the label.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* and comply with the test with a medium selective for *Campylobacter* spp.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. Carry out tests 1-6 of chapter *2.6.25. Avian live virus vaccines: tests for extraneous agents in batches of finished product*. General provisions *a-d*, *g* and *h* are also applicable. The vaccine complies with the requirements of each test.

3-5. **Sporulated oocyst count**. The sporulated oocysts content per dose is determined by counting the sporulated oocysts in a suitable counting chamber, under the microscope. The contents are not less than the minimum and not more than the maximum content of sporulated oocysts stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-4) using 1 dose of the vaccine administered by a recommended route.

4. LABELLING

The label states the minimum and maximum number of sporulated oocysts per dose.

**04/2013:0449**

# DISTEMPER VACCINE (LIVE) FOR MUSTELIDS

## Vaccinum morbi Carrei vivum ad mustelidas

1. DEFINITION

Distemper vaccine (live) for mustelids is a preparation of a suitable strain of distemper virus that is attenuated for ferrets, or for ferrets and minks. This monograph applies to vaccines intended for the active immunisation of ferrets, or ferrets and minks, against disease caused by distemper virus.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the ferrets, or for the ferrets and minks for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy. The tests are performed in each species for which the vaccine is intended.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 ferrets and/or minks of the minimum age to be recommended for vaccination and that do not have antibodies against distemper virus. Administer to each ferret and/or mink a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the animals at least daily for 42 days.

The vaccine complies with the test if no animal shows abnormal local or systematic reactions, signs of disease or dies from causes attributable to the vaccine.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using animals of the most susceptible target species. Use animals that do not have antibodies against distemper virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each animal of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion to virulence. After 5-10 days, prepare a suspension from, for example, the nasal mucosa, tonsils, thymus, spleen and the lungs and their local lymph nodes of each animal and pool the samples. Administer 1 mL of the pooled samples by the intranasal route to each animal of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using animals of the target species (ferrets and/or minks) for which the vaccine is intended. Use animals not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each animal is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 7 ferrets and/or minks that do not have antibodies against distemper virus. Vaccinate not fewer than 5 animals, according to the schedule to be recommended. Maintain not fewer than 2 animals as controls. Challenge each animal after 20-22 days by the intramuscular route with a quantity of a suspension of virulent distemper virus sufficient to cause the death of a ferret and/or a mink. Observe the animals at least daily for 21 days after challenge. Animals displaying typical signs of serious infection with distemper virus are euthanised to avoid unecessary suffering.

The test is not valid if 1 or both of the control animals do not die of distemper. The vaccine virus complies with the test if the vaccinated animals remain in normal health.

3. BATCH TESTS

3-1. **Identification**. The vaccine mixed with a specific distemper antiserum no longer provokes cytopathic effects in susceptible cell cultures or lesions on the chorio-allantoic membranes of fertilised hen eggs 9-11 days old.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against distemper virus and inoculate into susceptible cell cultures. The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemagglutinating or haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures or fertilised hens' eggs 9-11 days old. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

04/2013:1938

# DUCK PLAGUE VACCINE (LIVE)

## Vaccinum pestis anatis vivum

1. DEFINITION

Duck plague vaccine (live) is a preparation of a suitable strain of duck plague virus (anatid herpesvirus 1). This monograph applies to vaccines intended for the active immunisation of ducks.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures. The vaccine may be freeze-dried.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the test for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the ducks for which the vaccine is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case ducks from a species considered to be the most susceptible among the species to be recommended for vaccination, not older than the minimum age to be recommended for vaccination and that do not have antibodies against duck plague virus. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in ducks younger than 3 weeks of age, use not fewer than 10 susceptible ducks. For each test performed in ducks older than 3 weeks of age, use not fewer than 8 susceptible ducks. Administer to each duck a quantity of vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the ducks at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the ducks younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For ducks older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no duck shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using domestic ducks that do not have antibodies against duck plague virus and of an age suitable for the multiplication of the virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out. Administer to each duck of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. 2 to 4 days later, take samples of liver and spleen from each duck and pool all samples. Administer 0.1 mL of the pooled suspension by the oro-nasal or a parenteral route to each duck of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 ducks.

If the 5th group of ducks shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 ducks receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus at the final passage level compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 ducks and a subsequent repeat passage in 10 ducks, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case domestic ducks not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each duck is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. For each test, use not fewer than 30 ducks of the same origin and that do not have antibodies against duck plague virus. Vaccinate by a route to be recommended not fewer than 20 ducks. Maintain not fewer than 10 ducks as controls. After 5 days, challenge each duck by a suitable route with a sufficient quantity of virulent duck plague virus. Observe the ducks at least daily for 14 days after challenge. Record the deaths and the number of surviving ducks that show clinical signs of disease.

The test is not valid if during the observation period after challenge fewer than 80 per cent of the control ducks die or show typical signs of duck plague and/or if during the period between the vaccination and challenge more than 10 per cent of control or vaccinated ducks show abnormal clinical signs of disease or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 80 per cent of the vaccinated ducks survive and show no notable clinical signs of duck plague.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific duck plague virus antiserum, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the test prescribed under immunogenicity (section 2-4-3), when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:1315**

# DUCK VIRAL HEPATITIS TYPE I VACCINE (LIVE)

## Vaccinum hepatitidis viralis anatis stirpis I vivum

1. DEFINITION

Duck viral hepatitis type I vaccine (live) is a preparation of a suitable strain of duck hepatitis virus type I. This monograph applies to vaccines intended for the active immunisation of breeder ducks in order to protect passively their progeny and/or for the active immunisation of ducklings.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the ducks for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case susceptible domestic ducks (*Anas platyrhynchos*) not older than the minimum age to be recommended for vaccination and that do not have antibodies against duck hepatitis virus type I. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in ducklings younger than 3 weeks of age, use not fewer than 10 ducklings. For each test performed in ducklings older than 3 weeks of age, use not fewer than 8 ducklings. Administer to each duckling a quantity of vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of vaccine. Observe the ducklings at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the ducklings younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For ducklings older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no duckling shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using 1-day-old domestic ducklings that do not have antibodies against duck hepatitis virus type I. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each duckling of the 1st group by the oro-nasal route a quantity of vaccine virus that will allow recovery of virus for the passages described below. 2 to 4 days later, take samples of liver from each duckling and pool the samples. Administer 1 mL of the pooled liver suspension by the oro-nasal route to each duckling of the next group. Carry out this operation 4 times. Verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 ducklings. Observe the ducklings given the last passage at least daily for 21 days.

If the 5th group of ducklings shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 ducklings receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increase in virulence of the virus at the final passage level compared with the material used for the 1st passage is observed. If the virus is not recovered after an initial passage in 5 ducklings and a subsequent repeat passage in 10 ducklings, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case domestic ducks not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each bird is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-3-1. *Vaccines for passive immunisation of ducklings*. Use for the test not fewer than 15 laying ducks or ducks intended for laying, as appropriate, of the same origin and that do not have antibodies against duck hepatitis virus type I. Vaccinate by a route to be recommended not fewer than 10 ducks using the schedule to be recommended. Maintain not fewer than 5 ducks as controls. Starting from 4 weeks after onset of lay, collect embryonated eggs from vaccinated and control ducks and incubate them. Challenge not fewer than twenty 1-week-old ducklings representative of the vaccinated group and not fewer than 10 from the control group by the oro-nasal route with a sufficient quantity of virulent duck hepatitis virus type I. Observe the ducklings at least daily for 14 days after challenge. Record the deaths and the number of surviving ducklings that show clinical signs of disease.

The test is not valid if:

- during the observation period after challenge fewer than 70 per cent of the challenged ducklings from the control ducks die or show typical signs of the disease,
- and/or during the period between vaccination and collection of the eggs more than 10 per cent of the control or vaccinated ducks show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge the percentage relative protection calculated using the following expression is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

$V$ = percentage of challenged ducklings from vaccinated ducks that survive to the end of the observation period without clinical signs of the disease;

$C$ = percentage of challenged ducklings from unvaccinated control ducks that survive to the end of the observation period without clinical signs of the disease.

2-4-3-2. *Vaccines for active immunisation of ducklings*. Use for the test not fewer than 30 ducklings of the same origin and that do not have antibodies against duck hepatitis virus type I. Vaccinate by a route to be recommended not fewer than 20 ducklings. Maintain not fewer than 10 ducklings as controls. Challenge each duckling after at least 5 days by the oro-nasal route with a sufficient quantity of virulent duck hepatitis virus type I. Observe the ducklings at least daily for 14 days after challenge. Record the deaths and the number of surviving ducklings that show clinical signs of disease.

The test is not valid if:

– during the observation period after challenge fewer than 70 per cent of the control ducklings die or show typical signs of the disease;

– and/or during the period between vaccination and challenge more than 10 per cent of the control or vaccinated ducklings show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge the percentage relative protection calculated using the following expression is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

$V$ = percentage of challenged vaccinated ducklings that survive to the end of the observation period without clinical signs of the disease;

$C$ = percentage of challenged unvaccinated control ducklings that survive to the end of the observation period without clinical signs of the disease.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific duck hepatitis virus type I antiserum, no longer infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. Depending on the indications, the vaccine complies with 1 or both of the tests prescribed under Immunogenicity (section 2-4-3), when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

4. LABELLING

If it has been found that the vaccine may show reversion to virulence, the label indicates the precautions necessary to avoid transmission of virulent virus to unvaccinated ducklings.

**01/2017:1202**

# EGG DROP SYNDROME ′76 VACCINE (INACTIVATED)

## Vaccinum morbi partus diminutionis MCMLXXVI inactivatum ad pullum

1. DEFINITION

Egg drop syndrome ′76 vaccine (inactivated) is a preparation of a suitable strain of egg drop syndrome ′76 virus (haemagglutinating avian adenovirus), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for protection of laying birds against a drop in egg production and/or for prevention of loss of egg quality.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine strain is propagated in embryonated hens' or ducks' eggs or in cell cultures. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' or ducks' eggs**. If the vaccine virus is grown in embryonated hens' or ducks' eggs, they are obtained from healthy flocks (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the test for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended.

The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 hens not older than the minimum age to be recommended for vaccination and from a flock free from specified pathogens (SPF) (*5.2.2*). Administer by a route and method to be recommended to each hen 1 dose of the vaccine. Observe the hens at least daily for at least 14 days after the administration of the vaccine.

The test is not valid if non-specific mortality occurs. The vaccine complies with the test if no hen shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case hens from an SPF flock (*5.2.2*) and of the age at which vaccination is recommended. The vaccine administered to each hen is of minimum potency.

Vaccinate each of 2 groups of 30 hens. Maintain 2 control groups one of 10 hens and the other of 30 hens, of the same age and from the same source as the vaccinates. Maintain egg production records from point of lay until 4 weeks after challenge. At 30 weeks of age, challenge each hen from 1 group of 30 vaccinates and the group of 10 control hens with a quantity of egg drop syndrome '76 virus sufficient to cause a well marked drop in egg production and/or quality. The test is invalid unless there is a well marked drop in egg production and/or quality in the control hens. The vaccine complies with the test if the vaccinated hens show no marked drop in egg production and/or quality.

When the second group of vaccinated hens and the group of 30 control hens are nearing the end of lay, challenge these hens, as before. The test is invalid unless there is a well marked drop in egg production and/or quality in the control hens. The vaccine complies with the test if the vaccinated hens show no marked drop in egg production and/or quality.

Carry out serological tests on serum samples obtained at the time of vaccination, 4 weeks later and just prior to challenge. The test is not valid if antibodies against egg drop syndrome '76 virus are detected in any sample from control hens.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Residual live virus**. The test for residual live virus is carried out in embryonated ducks' eggs from a flock free from egg drop syndrome '76 virus infection, or in embryonated hens' eggs from an SPF flock (*5.2.2*), or in suitable cell cultures, whichever is the most sensitive for the vaccine strain. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of the vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-5-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Vaccinate not fewer than ten 14- to 28-day-old chickens from an SPF flock (*5.2.2*) with 1 dose of vaccine by one of the recommended routes. 4 weeks later, collect serum samples from each bird and from 5 unvaccinated control birds of the same age and from the same source. Measure the antibody response in a haemagglutination (HA) inhibition test on each serum using 4 HA units of antigen and chicken erythrocytes. The test is not valid if there are specific antibodies in the sera of the unvaccinated birds. The vaccine complies with the test if the mean titre of the vaccinated group is not less than that found previously for a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

A test for residual live virus is carried out to confirm inactivation of egg drop syndrome '76 virus.

A. For a vaccine prepared in eggs, carry out the test in embryonated ducks' eggs from a flock free from egg drop syndrome '76 virus infection or, if it is known to provide a more sensitive test system, in hens' eggs from an SPF flock (*5.2.2*). Inject 2/5 of a dose into the allantoic cavity of each of ten 10- to 14-day-old embryonated eggs that are free from parental antibodies to egg drop syndrome '76 virus. Incubate the eggs and observe for 8 days. Pool separately the allantoic fluid from eggs containing live embryos, and that from eggs containing dead embryos, excluding those that die from non-specific causes within 24 h of the injection. Inject into the allantoic cavity of each of ten 10- to 14-day-old embryonated eggs that do not have parental antibodies to egg drop syndrome '76 virus, 0.2 mL of the pooled allantoic fluid from the live embryos and into each of 10 similar eggs, 0.2 mL of the pooled allantoic fluid from the dead embryos and incubate for 8 days. Examine the allantoic fluid from each egg for the presence of haemagglutinating activity using chicken erythrocytes. If more than 20 per cent of the embryos die at either stage, repeat that stage. The vaccine complies with the test if there is no evidence of haemagglutinating activity and if, in any repeat test, not more than 20 per cent of the embryos die from non-specific causes.

   Antibiotics may be used in the test to control extraneous bacterial infection.

B. For a vaccine adapted to growth in cell cultures, inoculate 10 doses into suitable cell cultures. If the vaccine contains an oily adjuvant, eliminate it by suitable means. Incubate the cultures at 38 ± 1 °C for 7 days. Make a passage on another set of cell cultures and incubate at 38 ± 1 °C for 7 days. Examine the cultures regularly and at the end of the incubation period examine the supernatant for the presence of haemagglutinating activity. The vaccine complies with the test if the cell cultures show no sign of infection and if there is no haemagglutinating activity in the supernatant.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

4. LABELLING

The label states whether the strain in the vaccine is duck- or hen-embryo-adapted or cell-culture-adapted.

01/2020:1950

# ENTERIC REDMOUTH DISEASE VACCINE (INACTIVATED) FOR RAINBOW TROUT

## Vaccinum morbi oris rubri inactivatum ad Oncorhynchum mykissem

1. DEFINITION

Enteric redmouth (ERM) disease vaccine (inactivated) for rainbow trout is prepared from cultures of serovars 1 or 2 of *Yersinia ruckeri*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for administration by injection or immersion for the active immunisation of rainbow trout against enteric redmouth disease.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The strains of *Y. ruckeri* are harvested and inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium.

2-2. *CHOICE OF VACCINE COMPOSITION*

The strains of *Y. ruckeri* used are shown to be suitable with respect to the production of antigens of assumed protective importance. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*).

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Safety is tested using test 2-2-1-1-1, test 2-2-1-1-2, or both, depending on the recommendations for use.

Carry out the test using rainbow trout with an average body mass corresponding to the minimum body mass to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. The test is carried out under the conditions to be recommended for use of the vaccine with a water temperature not less than 10 °C.

2-2-1-1-1. *Vaccines intended for administration by injection*. Use not fewer than 50 fish from a population that does not have specific antibodies against the relevant serovars of *Y. ruckeri* and has not been vaccinated against or exposed to ERM disease. Where the size of the fish for the test is such that a blood sample cannot be removed for antibody testing, a number of larger fish may be kept with the group for this purpose. Administer to each fish by the intraperitoneal route 1 dose of the vaccine. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-1-2. *Vaccines intended for administration by immersion*. Use not fewer than 50 fish from a population that does not have specific antibodies against the relevant serovars of *Y. ruckeri* and has not been vaccinated against or exposed to ERM disease. Where the size of the fish for the test is such that a blood sample cannot be removed for antibody testing, a number of larger fish may be kept with the group for this purpose. Prepare an immersion bath at twice the concentration to be recommended. Bathe the fish for twice the time to be recommended. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. Carry out a test for each serovar included in the vaccine, according to a protocol defining water source, water flow and temperature limits, and preparation of a standardised challenge. Each test is carried out for each route and method of administration to be recommended. Where the size of the fish for the test is such that a blood sample cannot be removed for antibody testing, a number of larger fish of the same origin may be selected for this purpose. The vaccine administered to each fish is of minimum potency.

Use for the test not fewer than 60 fish from a population that does not have specific antibodies against the relevant serovars of *Y. ruckeri* and has not been vaccinated against or exposed to ERM disease. The average body mass of the fish corresponds to the minimum body mass to be recommended for vaccination. Vaccinate not fewer than 30 fish according to the instructions for use. Perform mock vaccination on a control group of not fewer than 30 fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than 1 tank is used. Where justified and when fish cannot be marked, non-marked fish may be used. Vaccinates and controls may then be kept in the same tank but physically separated (for example by fishing nets). Challenge each fish at a fixed interval after vaccination, corresponding to the onset of immunity claimed, by injection or immersion, with a sufficient quantity of cultures of *Y. ruckeri* of which the virulence has been verified or, where all fish are kept in the same tank, with a sufficient challenge by cohabitation. Observe the fish at least daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time and determine by interpolation the time corresponding to 60 per cent specific mortality in controls.

The test is not valid if the specific mortality is less than 60 per cent in the control group 21 days after the 1st death in the fish. Read from the curve for vaccinates the mortality ($M$) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) using the following expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

For serovar 1 vaccines, the vaccine complies with the test if the RPS is not less than 75 per cent for vaccines administered by immersion and 90 per cent for vaccines administered by injection.

For serovar 2 vaccines, the vaccine complies with the test if the RPS is not less than 60 per cent for vaccines administered by immersion and 85 per cent for vaccines administered by injection.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. The potency test (section 3-3) may be carried out for each batch of the vaccine. Where the test is not carried out, an alternative validated method in fish or other vertebrate animals may be used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 25 fish from a population that does not have specific antibodies against the relevant serovars of *Y. ruckeri* and that are within specified limits for body mass. Carry out the test at a defined temperature. Inject into each of not fewer than 20 fish 1 dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not

fewer than 5 fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against the relevant serovars of *Y. ruckeri* included in the vaccine by a suitable immunochemical method (*2.7.1*).

The test is not valid if the control group shows antibodies against the relevant serovars of *Y. ruckeri*. The vaccine complies with the test if the mean level of antibodies in the vaccinates is not significantly lower than that found for a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

4. LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

**01/2017:1613**

# EQUINE HERPESVIRUS VACCINE (INACTIVATED)

## Vaccinum herpesviris equini inactivatum

1. DEFINITION

Equine herpesvirus vaccine (inactivated) is a preparation of one or more suitable strains of equid herpesvirus 1 and/or equid herpesvirus 4, inactivated while maintaining adequate immunogenic properties or a suspension of an inactivated fraction of the virus. This monograph applies to vaccines intended for the active immunisation of horses against disease caused by equid herpesvirus 1 and/or equid herpesvirus 4.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Each strain of vaccine virus is grown separately in cell cultures. The viral suspensions may be purified and concentrated and are inactivated; they may be treated to fragment the virus and the viral fragments may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the horses for which it is intended. Where a particular breed of horse is known to be especially sensitive to the vaccine, horses from that breed are included in the test for safety.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and in horses of each category for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

Use for the test not fewer than 8 horses that have not been previously vaccinated with an equine herpesvirus vaccine, that have at most a low antibody titre not indicative of recent infection and that do not excrete equid herpesvirus. Administer to each horse 1 dose of the vaccine, then another dose after 14 days. Observe the horses at least daily until at least 14 days after the last administration.

The vaccine complies with the test if no horse shows abnormal local or systemic reactions or dies from causes attributable to the vaccine during the 28 days of the test.

2-3-2. **Immunogenicity**. The type of immunogenicity test depends on the claims for the product. For vaccines intended to protect against the disease of the respiratory tract, carry out test 2-3-2-1, using equid herpesvirus 1 and/or equid herpesvirus 4 depending on the claims for protection. For vaccines intended to protect against abortion carry out test 2-3-2-2.

A test is carried out for each route and method of administration to be recommended, using in each case horses that have not been vaccinated with an equine herpesvirus vaccine, that have at most a low antibody titre not indicative of recent infection, and that do not excrete equid herpesvirus. To demonstrate that no recent infection occurs, immediately before vaccination: draw a blood sample from each horse and test individually for antibodies against equid herpesviruses 1 and 4; collect 10 mL of heparinised blood and test the washed leucocytes for equid herpesviruses 1 and 4; collect a nasopharyngeal swab and test for equid herpesviruses 1 and 4. There is no indication of an active infection. Immediately before challenge collect a nasopharyngeal swab and test for equid herpesviruses 1 and 4. If there is an indication of virus excretion remove the horse from the test. Keep the horses in strict isolation. The vaccine administered to each horse is of minimum potency.

2-3-2-1. *Vaccines intended for protection against disease of the respiratory tract*. Use for the test not fewer than 10 horses, not less than 6 months old. Vaccinate not fewer than 6 horses according to the schedule to be recommended. Maintain not fewer than 4 horses as controls. At least 2 weeks after the last vaccination, challenge each horse by nasal instillation with a quantity of equid herpesvirus 1 or 4, sufficient to produce in a susceptible horse characteristic signs of the disease such as pyrexia and virus excretion (and possibly nasal discharge and coughing). Observe the horses at least daily for 14 days. Collect nasopharyngeal swabs daily from each individual horse to isolate the virus.

The vaccine complies with the test if the vaccinated horses show no more than slight signs; the signs in vaccinates are less severe than in controls. The average number of days on which virus is excreted and the respective virus titres are significantly lower in vaccinated horses than in controls.

2-3-2-2. *Vaccines intended for protection against abortion*. Use not fewer than 10 pregnant horses. In addition to the testing described above, 6, 4, 3, 2 and 1 month before the first vaccination draw a blood sample from each horse and test individually for antibodies against equid herpesvirues 1 and 4. There is no evidence of recent infection or virus excretion. Vaccinate not fewer than 6 horses according to the schedule to be recommended. Maintain not fewer than 4 horses as controls. Between day 260 and day 290 of pregnancy but not earlier than 3 weeks after the last vaccination, challenge each horse, by nasal instillation, with a quantity of equid herpesvirus 1 sufficient to produce abortion in susceptible horses. Observe the horses at least daily up to foaling or abortion. Collect samples of fetal lung and liver tissues from aborted fetuses and carry out tests for virus in cell cultures.

The test is not valid if more than one control horse gives birth to a healthy foal and if the challenge virus is not isolated from the aborted fetuses. The vaccine complies with the test if not more than one vaccinated horse aborts.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in the same type of cell culture as that used in the production or in cell cultures shown to be at least as sensitive. The quantity of inactivated virus harvest used in the test is equivalent to not less than 25 doses of the vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Vaccinate not fewer than 5 rabbits, guinea-pigs or mice with a single injection of a suitable dose. Where the recommended schedule requires a 2nd injection, this may be given provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay to measure the response to each of the antigens stated on the label. The vaccine complies with the test if the antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition. The method used must distinguish between antibodies against equid herpesviruses 1 and 4.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus using not less than 25 doses of vaccine by inoculating cell cultures sensitive to equid herpesviruses 1 and 4; make a passage after 5-7 days and maintain the cultures for 14 days. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant, separate the adjuvant from the liquid phase, by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses, or carry out a test for inactivation on the mixture of bulk antigens before addition of the adjuvant.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**07/2019:0249**

# EQUINE INFLUENZA VACCINE (INACTIVATED)

## Vaccinum influenzae equinae inactivatum

1. DEFINITION

Equine influenza vaccine (inactivated) is a preparation of one or more suitable strains of equine influenza virus, inactivated while maintaining adequate immunogenic properties. Suitable strains contain both haemagglutinin and neuraminidase. This monograph applies to vaccines intended for the active immunisation of horses against equine influenza.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Each strain of virus is grown separately in embryonated hens' eggs or in cell cultures. The viral suspensions may be purified and concentrated. The antigen content of the vaccine is based on the haemagglutinin content of the viral suspensions determined as described under Manufacturer's tests; the amount of haemagglutinin for each strain is not less than that in the vaccine shown to be satisfactory in the test for potency. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from a healthy flock (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The choice of strains used in the vaccine is based on epidemiological data. The World Organisation for Animal Health (OIE, formerly the *Office international des épizooties*) reviews the epidemiological data periodically and if necessary recommends new strains corresponding to prevailing epidemiological evidence. Such strains are used in accordance with the regulations in force in the signatory states of the Convention on the Elaboration of a European Pharmacopoeia. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the horses for which it is intended. Where a particular breed of horse is known to be especially sensitive to the vaccine, horses from that breed are included in the tests for safety.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and in horses of each category for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

Use for the test not fewer than 8 horses that preferably do not have antibodies against equine influenza virus or, where justified, use horses with a low level of such antibodies as long as they have not been vaccinated against equine influenza and administration of the vaccine does not cause an anamnestic response. Administer to each horse 1 dose of the vaccine, then another dose after at least 14 days. Observe the horses at least daily until at least 14 days after the last administration.

The vaccine complies with the test if no horse shows abnormal local or systemic reactions or dies from causes attributable to the vaccine during the 28 days of the test.

2-3-2. **Immunogenicity**. The test described under 2-3-2-1 is suitable to demonstrate the immunogenicity of the strains present in the vaccine.

A test with virulent challenge is carried out for at least one vaccine strain (see test under 2-3-2-1). For other strains in the vaccine, demonstration of immunogenicity may, where justified, be based on the serological response induced in horses by the vaccine (see tests under 2-3-2-2); justification for protection against these strains may be based on published data on the correlation of the antibody titre with protection against antigenically related strains.

Where serology is used, the test is carried out as described under 2-3-2-1 but instead of virulent challenge, a blood sample is drawn 2 weeks after the last vaccination and the antibody titre of each serum is determined by a suitable immunochemical method (*2.7.1*), such as the single radial haemolysis test or the haemagglutination-inhibition test shown below; a reference serum is used to validate the test. The acceptance criteria depend on the strain and are based on available data; for A/equine-2 virus, vaccines have usually been found satisfactory if the antibody titre of each serum is not less than 85 $mm^2$ where the single radial haemolysis test is used, or not less than 1:64 (before mixture with the suspension of antigen and erythrocytes) where the haemagglutination-inhibition test is used.

*Equine influenza subtype 1 strain A/eq/Newmarket/77 horse antiserum BRP*, *equine influenza subtype 2 European-like strain A/eq/Newmarket/2/93 horse antiserum BRP*, *equine influenza subtype 2 American-like strain A/eq/Newmarket/1/93 horse antiserum BRP*, *equine influenza subtype 2 American-like strain A/eq/South Africa/4/03 horse antiserum BRP* and *equine influenza subtype 2 American-like strain A/eq/Richmond/1/2007 horse antiserum BRP* are suitable for use as reference sera for the single radial haemolysis test.

The claims for the product reflect the type of immunogenicity demonstrated (protection against challenge or antibody production).

2-3-2-1. *Protection from signs of disease and reduction of virus excretion*. Carry out the immunogenicity test using a challenge strain against which the vaccine is stated to provide protection. Use where possible a recent isolate.

A test is carried out for each route and method of administration to be recommended, using in each case horses not less than 6 months old. The vaccine administered to each horse is of minimum potency.

Use for the test not fewer than 10 horses that do not have antibodies against equine influenza virus. Draw a blood sample from each horse and test individually for antibodies against equine influenza virus to determine seronegativity. Vaccinate not fewer than 6 horses according to the schedule to be recommended. Maintain not fewer than 4 horses as controls. Draw a second blood sample from each vaccinated horse 7 days after the first vaccination and test individually for antibodies against equine influenza virus, to detect an anamnestic sero-response. Horses showing sero-conversion at this stage are excluded from the test. At least 2 weeks after the last vaccination, challenge each horse by aerosol with a quantity of equine influenza virus sufficient to produce characteristic signs of disease such as fever, nasal discharge and coughing in a susceptible horse. Observe the horses at least daily for 14 days. Collect nasal swabs daily from each individual horse to isolate the virus.

The vaccine complies with the test if the vaccinated horses show no more than slight signs; the controls show characteristic signs. The average number of days on which virus is excreted and the respective virus titres are significantly lower in vaccinated horses than in control horses.

2-3-2-2. *Presence of antibodies after vaccination*

2-3-2-2-1. Single radial haemolysis. Heat each serum at 56 °C for 30 min. Perform tests on each serum using respectively the antigen or antigens prepared from the strain(s) used in the production of the vaccine. Mix 1 mL of sheep erythrocyte suspension in barbital buffer solution (1 volume of erythrocytes in 10 volumes of final suspension) with 1 mL of a suitable dilution of the influenza virus strain in barbital buffer solution and incubate the mixture at 4 °C for 30 min. To 2 mL of the virus/erythrocyte mixture, add 1 mL of a 3 g/L solution of *chromium(III) trichloride hexahydrate R*, mix and allow to stand for 10 min. Heat the sensitised erythrocytes to 47 °C in a water-bath. Mix 15 mL of a 10 g/L solution of *agarose for electrophoresis R* in barbital buffer solution, 0.7 mL of sensitised erythrocyte suspension and the appropriate amount of diluted guinea-pig complement in barbital buffer solution at 47 °C. Pour the mixture into Petri dishes and allow the agar to set. Punch holes in the agar layer and place in each hole 5 µL of the undiluted serum to be tested or the control serum. Incubate the Petri dishes at 37 °C for 18 h. Measure the diameter of the haemolysis zone and calculate its area, which expresses the antibody titre, in square millimetres.

*Equine influenza subtype 1 strain A/eq/Newmarket/77 horse antiserum BRP*, *equine influenza subtype 2 European-like strain A/eq/Newmarket/2/93 horse antiserum BRP*, *equine influenza subtype 2 American-like strain A/eq/Newmarket/1/93 horse antiserum BRP*, *equine influenza subtype 2 American-like strain A/eq/South Africa/4/03 horse antiserum BRP* and *equine influenza subtype 2 American-like strain A/eq/Richmond/1/2007 horse antiserum BRP* are suitable for use as reference sera for the single radial haemolysis test.

2-3-2-2-2. Haemagglutination-inhibition test. Inactivate each serum by heating at 56 °C for 30 min. To 1 volume of each serum add 3 volumes of *phosphate buffered saline pH 7.4 R* and 4 volumes of a 250 g/L suspension of *light kaolin R* in the same buffer solution. Shake each mixture for 10 min. Centrifuge, collect the supernatant and mix with a concentrated suspension of chicken erythrocytes. Allow to stand at 37 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:8. Perform tests on each serum using each antigen prepared from the strains used in the production of the vaccine. Using each diluted serum, prepare a series of 2-fold dilutions. To 0.025 mL of each of the latter dilutions add 0.025 mL of a suspension of antigen treated with *ether R* and containing 4 haemagglutinating units. Allow the mixture to stand for 30 min and add 0.05 mL of a suspension of chicken erythrocytes containing $2 \times 10^7$ erythrocytes/mL. Allow to stand for 1 h and note the last dilution of serum that still completely inhibits haemagglutination.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using method 2-4-1-1 or method 2-4-1-2, whichever is more sensitive. The quantity of inactivated virus harvest used is equivalent to not less than 10 doses of vaccine.

2-4-1-1. *Test in cell cultures*. Inoculate the vaccine into suitable cells; after incubation for 8 days, make a subculture. Incubate for a further 6-8 days. Harvest about 0.1 mL of the supernatant and examine for live virus by a haemagglutination test. If haemagglutination is found, carry out a further passage in cell culture and test for haemagglutination; the inactivated virus harvest complies with the test if no haemagglutination occurs.

2-4-1-2. *Test in embryonated eggs*. Inoculate 0.2 mL into the allantoic cavity of each of 10 embryonated eggs and incubate at 33-37 °C for 3-4 days. The test is invalid unless not fewer than 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 embryonated eggs and incubate at 33-37 °C for 3-4 days. The test is invalid unless at least 8 of the 10 embryonated embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids,

carry out a further passage of that fluid in eggs and test for haemagglutination; the inactivated virus harvest complies with the test if no haemagglutination occurs.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test(s) described under Potency. The following test may be used.

Use 5 guinea-pigs that do not have specific antibodies. Vaccinate each guinea-pig by the subcutaneous route with one dose of vaccine. 21 days later, collect blood samples and separate the serum. Carry out tests on the serum for specific antibodies by a suitable immunochemical method (*2.7.1*) such as single radial haemolysis or haemagglutination-inhibition, using reference sera to validate the test. The vaccine complies with the test if the antibody titres are not significantly lower than those obtained in guinea-pigs with a reference batch of vaccine shown to have satisfactory potency in horses.

2-4-3. **Bacterial endotoxins**. For vaccines produced in eggs, the content of bacterial endotoxins is determined on the virus harvest to monitor production.

2-4-4. **Haemagglutinin content**. The content of haemagglutinin in the inactivated virus suspension, after purification and concentration where applicable, is determined by a suitable immunochemical method (*2.7.1*), such as single radial immunodiffusion, using a suitable haemagglutinin reference preparation; the inactivated virus suspension complies with the test if the content is shown to be within the limits shown to allow preparation of a satisfactory vaccine.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Inoculate 0.2 mL of the vaccine into the allantoic cavity of each of 10 embryonated eggs and incubate at 33-37 °C for 3-4 days. The test is invalid unless at least 8 of the 10 embryos survive. Harvest 0.5 mL of the allantoic fluid from each surviving embryo and pool the fluids. Inoculate 0.2 mL of the pooled fluid into a further 10 embryonated eggs and incubate at 33-37 °C for 3-4 days. The test is invalid unless not fewer than 8 of the 10 embryos survive. Harvest about 0.1 mL of the allantoic fluid from each surviving embryo and examine each individual harvest for live virus by a haemagglutination test. If haemagglutination is found for any of the fluids, carry out for that fluid a further passage in eggs and test for haemagglutination; the vaccine complies with the test if no haemagglutination occurs.

3-4. **Potency**. The vaccine complies with the requirements of the test(s) mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

01/2017:1101

# FELINE CALICIVIROSIS VACCINE (INACTIVATED)

## Vaccinum calicivirosis felinae inactivatum

1. DEFINITION

Feline calicivirosis vaccine (inactivated) is a preparation of one or more suitable strains of feline calicivirus inactivated while maintaining adequate immunogenic properties or of fractions of one or more strains of feline calicivirus with adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of cats against feline calicivirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated; the virus may be disrupted and the fractions purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline calicivirus. Administer to each cat 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days after the last administration. Observe the cats at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no cat shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each strain of feline calicivirus in the vaccine and for each route and method of administration to be recommended for vaccination, using in each case cats 8-12 weeks old. The vaccine administered to each cat is of minimum potency.

Use for the test not fewer than 20 cats that do not have antibodies against feline calicivirus. Vaccinate not fewer than 10 cats, according to the schedule to be recommended. Maintain not fewer than 10 cats as controls. Challenge each cat after 4 weeks by the intranasal route with a sufficient quantity of a suspension of virulent feline calicivirus. Observe the cats at least daily for 14 days after challenge. Collect nasal washings daily on days 2 to 14 to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below.

The test is not valid if during the observation period after challenge, fewer than 80 per cent of the control cats show notable signs of feline calicivirosis (hyperthermia, buccal ulcers, respiratory signs). The vaccine complies with the test if during the observation period after challenge, the score for the vaccinated cats is significantly lower than that for the controls.

| **Observed signs** | **Score** |
|---|---|
| Death | 10 |
| Depressed state | 2 |
| Temperature ≥ 39.5 °C | 1 |
| Temperature ≤ 37 °C | 2 |
| Ulcer (nasal or oral) | |
| – small and few in number | 1 |
| – large and numerous | 3 |
| Nasal discharge | |
| – slight | 1 |
| – copious | 2 |
| Ocular discharge | 1 |
| Weight loss | 2 |
| Virus excretion (total number of days): | |
| ≤ 4 days | 1 |
| 5-7 days | 2 |
| > 7 days | 3 |

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live calicivirus is carried out using 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive; the quantity of inactivated virus harvest used in the test is equivalent to not less than 25 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the Potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use for the test groups of 15 seronegative mice. Administer to each mouse half a dose of the vaccine and 7 days later, repeat the administration. After 21 days following the first injection, take blood samples and determine the level of antibodies against feline calicivirus by an immunofluorescence technique using pools of serum from groups of 3 mice. The vaccine complies with the test if the antibody levels are not significantly lower than those obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live calicivirus using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that would interfere with the test, where possible separate the adjuvant from the liquid phase by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**04/2013:1102**

# FELINE CALICIVIROSIS VACCINE (LIVE)

## Vaccinum calicivirosis felinae vivum

1. DEFINITION

Feline calicivirosis vaccine (live) is a preparation of one or more suitable strains of feline calicivirus. This monograph applies to vaccines intended for the active immunisation of cats against feline calicivirosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline calicivirus. Administer to each cat a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the cats at least daily for at least 14 days.

The vaccine virus complies with the test if no cat shows abnormal local or systemic reactions, or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using cats that do not have antibodies against feline calicivirus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each cat of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion of virulence. After 5 days, remove the nasal mucus, tonsils and trachea of each cat. Mix, homogenise in 10 mL of buffered saline and allow the solids to settle. Administer the supernatant by the intranasal route to each cat of the next group. Carry out this passage operation 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the first passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the first passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each strain of feline calicivirus in the vaccine, for each route and method of administration to be recommended for vaccination. The quantity of vaccine virus to be administered to each cat is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 20 cats, 8-12 weeks old, that do not have antibodies against feline calicivirus. Vaccinate not fewer than 10 cats, according to the schedule to be recommended. Maintain not fewer than 10 cats as controls. Challenge each cat after 4 weeks by the intranasal route with a sufficient quantity of a suspension of virulent feline calicivirus virus. Observe the cats at least daily for 14 days after challenge. Collect nasal washings daily on days 2 to 14 to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below.

The test is not valid if during the observation period after challenge, fewer than 80 per cent of the control cats show notable signs of feline calicivirosis (hyperthermia, buccal ulcers, respiratory signs).

The vaccine virus complies with the test if during the observation period after challenge, the score for the vaccinated cats is significantly lower than that for the controls.

| **Observed signs** | **Score** |
|---|---|
| Death | 10 |
| Depressed state | 2 |
| Temperature ≥ 39.5 °C | 1 |
| Temperature ≤ 37 °C | 2 |
| Ulcer (nasal or oral) | |
| – small and few in number | 1 |
| – large and numerous | 3 |
| Nasal discharge | |
| – slight | 1 |
| – copious | 2 |
| Ocular discharge | 1 |
| Weight loss | 2 |
| Virus excretion (total number of days): | |
| ≤ 4 days | 1 |
| 5-7 days | 2 |
| > 7 days | 3 |

3. BATCH TESTS

3-1. **Identification**. When neutralised by one or more monospecific antisera, the vaccine no longer infects susceptible cell cultures into which it is inoculated.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with one or more suitable monospecific antisera against feline calicivirus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the cat. Carry out at least 1 passage and maintain the cultures for 14 days. The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures at a temperature favourable to replication of the virus. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:2324**

# FELINE CHLAMYDIOSIS VACCINE (INACTIVATED)

## Vaccinum chlamydiosidis felinae inactivatum

1. DEFINITION

Feline chlamydiosis vaccine (inactivated) is a preparation of one or more suitable strains of *Chlamydophila felis*, which have been inactivated by a suitable method. This monograph applies to vaccines intended for administration to cats for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in embryonated hens' eggs from a healthy flock (*5.2.13*) or in suitable cell cultures (*5.2.4*). If the vaccine contains more than one strain of bacterium, the different strains are grown and harvested separately. The bacterial harvests are inactivated using suitable and validated methods. The suspensions may be treated to fragment the micro-organisms and the fragments may be purified and concentrated. The vaccine may contain adjuvants.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in cats for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against *C. felis*. Administer to each cat 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days.

Observe the cats at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no cat shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case cats not older than the minimum age to be recommended for vaccination. The vaccine to be administered to each cat is of minimum potency.

Vaccinate 10 cats that are free from antibodies against *C. felis* and keep 10 cats as controls. Not later than 4 weeks after the last administration of vaccine, administer by a suitable route to each cat a quantity of a virulent strain of *C. felis* sufficient to produce in susceptible cats typical signs of disease such as conjunctivitis and nasal discharge. Observe the cats for 28 days. Where reduction of chlamydophila excretion is to be claimed, collect nasal washings and/or conjunctival swabs on days 7, 14, 17, 21, 24 and 28 after challenge to test for chlamydophila excretion. The duration of excretion for the vaccinated animals is significantly lower than for the controls. Note daily the body temperature and signs of disease using a suitable scoring system. The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual live chlamydophila**. A validated test for residual live chlamydophila is carried out. The vaccine complies with the test if no live chlamydophila is detected.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out on a batch, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the potency test (section 3-4). The following test may be used.

Inject a suitable dose by a suitable route into each of 5 seronegative cats or another suitable species. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. Before the vaccination and at a given interval usually within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Determine individually for each serum the titre of antibodies against each strain stated on the label, using a suitable test such as enzyme-linked immunosorbent assay (*2.7.1*). The vaccine complies with the test if the antibody levels are not significantly lower than those obtained for a batch that has given satisfactory results in the potency test (section 3-4).

2-3-3. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown to be satisfactory in the safety test (section 2-2-1). The method chosen for determining the maximum acceptable amount of bacterial endotoxins is subsequently used for the testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Residual live chlamydophila**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a validated test for residual live chlamydophila to confirm inactivation of *C. felis*. The vaccine complies with the test if no live chlamydophila is detected.

3-3. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-4. **Potency**. The vaccine complies with the test for immunogenicity (section 2-2-2).

**01/2017:0794**

# FELINE INFECTIOUS ENTERITIS (FELINE PANLEUCOPENIA) VACCINE (INACTIVATED)

## Vaccinum panleucopeniae felinae infectivae inactivatum

1. DEFINITION

Feline infectious enteritis (feline panleucopenia) vaccine (inactivated) is a preparation of a suitable strain of feline panleucopenia virus or canine parvovirus inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of cats against feline infectious enteritis (feline panleucopenia).

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline panleucopenia virus. Administer to each cat 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the cats at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no cat shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case cats 8-12 weeks old. The vaccine administered to each cat is of minimum potency.

Use for the test not fewer than 10 cats that do not have antibodies against feline panleucopenia virus and canine parvovirus. Vaccinate not fewer than 5 cats, according to the schedule to be recommended. Maintain not fewer than 5 cats as controls. Carry out leucocyte counts 8 days and 4 days before challenge and calculate the mean of the 2 counts to serve as the initial value. Challenge each cat after 20-22 days by the intraperitoneal route with a sufficient quantity of a suspension of virulent feline panleucopenia virus. Observe the cats at least daily for 14 days after challenge. Carry out leucocyte counts on the $4^{th}$, $6^{th}$, $8^{th}$ and $10^{th}$ days after challenge.

The test is not valid if during the observation period after challenge, fewer than 100 per cent of the control cats show on not fewer than one occasion a diminution in the number of leucocytes of at least 75 per cent of the initial value or die from panleucopenia. The vaccine complies with the test if during the observation period after challenge, all the vaccinated cats survive and show no signs of disease nor leucopenia; that is to say, the diminution in the number of leucocytes does not exceed, in any of the 4 counts, 50 per cent of the initial value.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using a quantity of inactivated virus harvest equivalent to not less than 100 doses of the vaccine by a validated method such as the following: inoculate into suitable non-confluent cells and after incubation for 8 days, make a subculture using trypsinised cells. After incubation for a further 8 days, examine the cultures for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. For routine testing of batches of vaccine, a test based on production of haemagglutination-inhibiting antibodies in guinea-pigs may be used instead of test 3-4-1 or 3-4-2 described under Potency if a satisfactory correlation with the test for immunogenicity has been established.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus by inoculating not less than 10 doses of vaccine into suitable non-confluent cells; after incubation for 8 days, a subculture is made using trypsinised cells. After incubation for a further 8 days, the cultures are examined for residual live parvovirus by an immunofluorescence test. The immunofluorescence test may be supplemented by a haemagglutination test or other suitable tests on the supernatant of the cell cultures. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that interferes with the test, separate it if possible from the liquid phase of the vaccine by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. Carry out test 3-4-1 or test 3-4-2.

3-4-1. *Test in cats for haemagglutination-inhibiting antibodies.* Use for the test not fewer than 4 cats, 8-12 weeks old, that do not have antibodies against feline panleucopenia virus and canine parvovirus. Vaccinate not fewer than 2 cats with 1 dose of the vaccine. Maintain not fewer than 2 cats as controls. After 21 days, draw a blood sample from each cat and separate the serum from each sample. Inactivate each serum by heating at 56 °C for 30 min. To 1 volume of each serum add 9 volumes of a 200 g/L suspension of *light kaolin R* in *phosphate buffered saline pH 7.4 R*. Shake each mixture for 20 min. Centrifuge, collect the supernatant and mix with 1 volume of a concentrated suspension of pig erythrocytes. Allow to stand at 4 °C for 60 min and centrifuge. The dilution of the serum obtained is 1:10. Using each serum, prepare a series of twofold dilutions. To 0.025 mL of each of the latter dilutions add 0.025 mL of a suspension of canine parvovirus or feline panleucopenia virus antigen containing 4 haemagglutinating units. Allow to stand at 37 °C for 30 min and add 0.05 mL of a suspension of pig erythrocytes containing $30 \times 10^6$ cells per millilitre. Allow to stand at 4 °C for 90 min and note the last dilution of serum that still completely inhibits haemagglutination.

The test is not valid if either control cat develops antibodies against canine parvovirus or feline panleucopenia virus. The vaccine complies with the test if both vaccinated cats have developed titres of at least 1:20.

3-4-2. *Test in cats for virus-neutralising antibodies.* Use for the test not fewer than 2 cats, 8-12 weeks old, that have antibody titres less than $4ND_{50}$ per 0.1 mL of serum measured by the method described below. Vaccinate each cat according to the recommended schedule. 14 days after vaccination, examine the serum of each cat as follows. Heat the serum at 56 °C for 30 min and prepare serial dilutions using a medium suitable for feline cells. Add to each dilution an equal volume of a virus suspension containing an amount of virus such that when the volume of serum-virus mixture appropriate for the assay system is inoculated into cell cultures, each culture receives approximately $10^4$ $CCID_{50}$. Incubate the mixtures at 37 °C for 1 h and inoculate 4 feline cell cultures with a suitable volume of each mixture. Incubate the cell cultures at 37 °C for 7 days, passage and incubate for a further 7 days. Examine the cultures for evidence of specific cytopathic effects and calculate the antibody titre.

The vaccine complies with the test if the mean titre is not less than 32 $ND_{50}$ per 0.1 mL of serum. If one cat fails to respond, repeat the test using 2 more cats and calculate the result as the mean of the titres obtained from all of the 3 cats that have responded.

**04/2013:0251**

# FELINE INFECTIOUS ENTERITIS (FELINE PANLEUCOPENIA) VACCINE (LIVE)

## Vaccinum panleucopeniae felinae infectivae vivum

1. DEFINITION

Feline infectious enteritis (feline panleucopenia) vaccine (live) is a preparation of a suitable strain of feline panleucopenia virus. This monograph applies to vaccines intended for the active immunisation of cats against feline infectious enteritis (feline panleucopenia).

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended, including safety for pregnant queens if the vaccine may be used in such queens. If the virus is excreted in the faeces, the effect in pregnant queens must be documented.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

2-3-1-1. *General safety*. For each test, use not fewer than 5 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline panleucopenia virus and canine parvovirus. Make counts of leucocytes in circulating blood on days 8 and 4 before injection of the vaccine virus and calculate the mean of the 2 counts to serve as the initial value. Administer to each cat a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the cats at least daily for at least 14 days. Make leucocyte counts on the 4$^{th}$, 6$^{th}$, 8$^{th}$ and 10$^{th}$ days after inoculation.

The vaccine virus complies with the test if no cat shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus and if, for each cat and each blood count, the number of leucocytes is not less than 50 per cent of the initial value.

2-3-1-2. *Safety in pregnant queens*. If the vaccine is intended for use or may be used in pregnant queens, use not fewer than 5 queens per group, at the stage of pregnancy to be recommended or at a range of stages of pregnancy according to the schedule to be recommended. Administer to each queen a quantity of vaccine virus at least equivalent to the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the queens at least daily until 1 day after parturition and observe their kittens until at least the age of 3 weeks.

The vaccine virus complies with the test if no queen shows abnormal local or systemic reactions, signs of disease or dies from causes attributable to the vaccine virus and if no adverse effects on the pregnancy or the offspring, such as foetal resorption or ataxia in the kittens, are noted.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline panleucopenia virus and canine parvovirus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each cat of the 1$^{st}$ group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Collect the faeces from each cat from the 2$^{nd}$ to the 10$^{th}$ day after administration of the virus, check them for the presence of the virus and pool the faeces containing virus. Administer 1 mL of the suspension of pooled faeces by either the oral or the intranasal route to each cat of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5$^{th}$ group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters (count of white blood cells, results of histological examination of the thymus and titre of excreted virus) in a group of at least 8 animals receiving the material used for the 1$^{st}$ passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no cat dies or shows signs attributable to the vaccine virus and no indication of increasing virulence of the virus recovered for the final passage compared with the material used for the 1$^{st}$ passage is observed. Account is taken, notably, of the count of white blood cells, of results of histological examination of the thymus and of the titre of excreted virus. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination. The quantity of vaccine virus to be administered to each cat is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 10 cats, 8-12 weeks old, that do not have antibodies against feline panleucopenia virus and canine parvovirus. Vaccinate not fewer than 5 cats, according to the schedule to be recommended. Maintain not fewer than 5 cats as controls. Carry out leucocyte counts 8 days and 4 days before challenge and calculate the mean of the 2 counts to serve as the initial value. Challenge each cat after 20-22 days by the intraperitoneal route with a sufficient quantity of a suspension of virulent feline panleucopenia virus. Observe the cats at least daily for 14 days after challenge. Carry out leucocyte counts on the 4$^{th}$, 6$^{th}$, 8$^{th}$ and 10$^{th}$ days after challenge.

The test is not valid if during the observation period after challenge, fewer than 100 per cent of the control cats show, on fewer than one occasion, a diminution in the number of leucocytes of at least 75 per cent of the initial value or die from feline panleucopenia. The vaccine virus complies with the test if during the observation period after challenge, all the vaccinated cats survive and show no signs of disease nor leucopenia; that is to say, the diminution in the number of leucocytes does not exceed, in any of the 4 counts, 50 per cent of the initial value.

3. BATCH TESTS

3-1. **Identification**. Carry out replication of the vaccine virus in a susceptible cell line in a substrate suitable for a fluorescent antibody test or peroxidase test. Prepare suitable controls. Test a proportion of the cells with monoclonal antibodies specific for feline panleucopenia virus and a proportion with monoclonal antibodies specific for canine parvovirus. Feline panleucopenia virus is detected but no canine parvovirus is detected in the cells inoculated with the vaccine.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against feline panleucopenia virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the cat. Carry out at least 1 passage and maintain the cultures for 14 days. The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:1321**

# FELINE LEUKAEMIA VACCINE (INACTIVATED)

## Vaccinum leucosis felinae inactivatum

1. DEFINITION

Feline leukaemia vaccine (inactivated) is a preparation of immunogens from a suitable strain of feline leukaemia virus. This monograph applies to vaccines intended for the active immunisation of cats against feline leukaemia.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The immunogens consist either of a suitable strain of feline leukaemia virus inactivated while maintaining adequate immunogenic properties or of a fraction of the virus with adequate immunogenic properties; the immunogenic fraction may be produced by recombinant DNA technology. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

2-2-1-1. *General safety and immunosuppression.* Use for the test not fewer than 15 cats of the minimum age to be recommended and that do not have antibodies against gp 70 antigen of feline leukaemia virus nor display viraemia or antigenaemia at the time of the test; absence of antibodies and antigen is demonstrated by enzyme-linked immunosorbent assay (*2.7.1*). Administer to each of not fewer than 10 cats 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Maintain not fewer than 5 cats as controls. Record the body temperature of each cat on the day before each vaccination, at the time of vaccination, 4 h and 8 h later, and once per day during the 4 following days. Observe the cats at least daily for not less than 4 weeks after the last vaccination. 1, 2 and 4 weeks after the last vaccination, submit the cats to suitable tests for evidence of an immunosuppressive effect.

The vaccine complies with the test if no cat shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no significant difference is observed in vaccinated cats compared with controls regarding immunosuppressive effect.

2-2-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case cats of the minimum age to be recommended for vaccination. The vaccine administered to each cat is of minimum potency.

Use for the test not fewer than 25 cats that do not have antibodies against the antigens of feline leukaemia virus and against the feline oncogene membrane antigen (anti-FOCMA antibodies), and showing no viraemia or antigenaemia at the time of the test. Vaccinate not fewer than 15 cats according to the schedule to be recommended. Maintain not fewer than 10 cats as controls. Challenge each cat after 14 days by the peritoneal or oronasal route, on one or several occasions, with a sufficient quantity of suspension of an epidemiologically relevant virulent strain of feline leukaemia virus, consisting predominantly of type A virus. Observe the cats at least daily for 15 weeks and, from the 3rd week onwards, test each week for viraemia or antigenaemia (p27 protein) by suitable methods such as immunofluorescence on circulating leucocytes or enzyme-linked immunosorbent assay. A cat is considered persistently infected if it shows positive viraemia or antigenaemia for 3 consecutive weeks or on 5 occasions, consecutively or not, between the 3rd and the 15th week.

The test is not valid if during the observation period after challenge, fewer than 80 per cent of the control cats show persistant viraemia or antigenaemia. The vaccine complies with the test if during the observation period after challenge, not fewer than 80 per cent of the vaccinated cats show no persistent infection.

2-3. *IN-PROCESS CONTROL TESTS*

During production, suitable immunochemical tests are carried out for the evaluation of the quality and purity of the viral antigens included in the vaccine composition. The values found are within the limits approved for the particular vaccine.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. Where applicable, the test for residual live virus is carried out using a quantity of inactivated virus harvest equivalent to not less than 25 doses of vaccine and 2 passages in the same type of cell cultures as used for the production of the vaccine or in cell cultures shown to be at least as sensitive. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the Potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

2-4-3. **Bacterial endotoxins**. For vaccines produced by recombinant DNA technology with a bacterial host cell such as *Escherichia coli*, a test for bacterial endotoxins (*2.6.14*) is carried out on each final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the antigen immediately before addition of the adjuvant. The value found is within the limit approved for the particular vaccine and which has been shown to be safe for cats.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

If the vaccine contains inactivated virus, carry out a test for residual live feline leukaemia virus by making 2 passages on susceptible cell cultures. The vaccine complies with the test if no virus is detected. If the vaccine contains an adjuvant, if possible separate the adjuvant from the liquid phase by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**01/2017:1207**

# FELINE VIRAL RHINOTRACHEITIS VACCINE (INACTIVATED)

## Vaccinum rhinotracheitidis viralis felinae inactivatum

1. DEFINITION

Feline viral rhinotracheitis vaccine (inactivated) is a preparation of a suitable strain of feline rhinotracheitis virus (feline herpesvirus 1), inactivated while maintaining adequate immunogenic properties, or of an inactivated fraction of the virus having adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of cats against feline viral rhinotracheitis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated; the virus may be disrupted and the fractions purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline herpesvirus 1 or against a fraction of the virus. Administer to each cat 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days after the last administration. Observe the cats at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no cat shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case cats 8-12 weeks old. The vaccine administered to each cat is of minimum potency.

Use for the test not fewer than 20 cats that do not have antibodies against feline herpesvirus 1 or against a fraction of the virus. Vaccinate not fewer than 10 cats, according to the schedule to be recommended. Maintain not fewer than 10 cats as controls. Challenge each cat after 4 weeks by the intranasal route with a quantity of a suspension of virulent feline herpesvirus 1 sufficient to produce typical signs of the disease such as fever, nasal discharge and cough in a cat that does not have antibodies against feline herpesvirus 1 or a fraction of the virus. Observe the cats at least daily for 14 days after challenge. Collect nasal washings daily on days 2 to 14 after challenge to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below.

The vaccine complies with the test if the score for the vaccinated cats is significantly lower than that for the controls.

| Sign | Score |
|---|---|
| Death | 10 |
| Depressed state | 2 |
| Temperature: | |
| 39.5 °C - 40.0 °C | 1 |
| ≥ 40.0 °C | 2 |
| ≤ 37.0 °C | 3 |
| Glossitis | 3 |
| Nasal discharge, slight | 1 |
| Nasal discharge, copious | 2 |
| Cough | 2 |
| Sneezing | 1 |
| Sneezing, paroxysmal | 2 |
| Ocular discharge, slight | 1 |
| Ocular discharge, serious | 2 |
| Conjunctivitis | 2 |
| Weight loss ≥ 5.0 per cent | 5 |
| Virus excretion (total number of days): | |
| ≤ 4 days | 1 |
| 5-7 days | 2 |
| > 7 days | 3 |

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in cell cultures of the same type as those used for preparation of the vaccine or in cell cultures shown to be at least as sensitive; the quantity of inactivated virus harvest used in the test is equivalent to not less than 25 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use for the test a group of 15 seronegative mice. Administer to each mouse half a dose of the vaccine and, 7 days later, repeat the administration. 21 days after the first injection, take blood samples and determine the level of antibodies against feline herpesvirus 1 by a suitable immunochemical method (*2.7.1*), such as an immunofluorescence technique using pools of serum from groups of 3 mice. The vaccine complies with the test if the antibody levels are not significantly lower than those obtained with a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live feline herpesvirus 1 using 10 doses of vaccine and 2 passages in cell cultures of the same type as those used for preparation of the vaccine, or in other suitably sensitive cell cultures. The vaccine complies with the test if no live virus is detected. If the vaccine contains an adjuvant that interferes with the test, where possible separate the adjuvant from the liquid phase by a method that does not inactivate the virus or otherwise interfere with the detection of live viruses.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**04/2013:1206**

# FELINE VIRAL RHINOTRACHEITIS VACCINE (LIVE)

## Vaccinum rhinotracheitidis viralis felinae vivum

1. DEFINITION

Feline viral rhinotracheitis vaccine (live) is a preparation of a suitable strain of feline rhinotracheitis virus (feline herpesvirus 1). This monograph applies to vaccines intended for the active immunisation of cats against feline viral rhinotracheitis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cats for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (section 2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 cats of the minimum age to be recommended for vaccination and that do not have antibodies against feline herpesvirus 1. Administer to each cat a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the cats at least daily for at least 14 days.

The vaccine virus complies with the test if no cat shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine virus.

2-3-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using cats that do not have antibodies against feline herpesvirus 1. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each cat of the 1st group by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion of virulence. After 2-4 days, remove the nasal mucus, tonsils and local lymphatic ganglia and the trachea of each cat. Mix, homogenise in 10 mL of buffered saline and allow the solids to settle. Administer 1 mL of the supernatant by the intranasal route to each cat of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the first passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the first passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination. The quantity of vaccine virus to be administered to each cat is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 20 cats, 8-12 weeks old, that do not have antibodies against feline herpesvirus 1. Vaccinate not fewer than 10 cats, according to the schedule to be recommended. Maintain not fewer than 10 cats as controls. Challenge each cat after 4 weeks by the intranasal route with a quantity of a suspension of virulent feline herpesvirus 1 sufficient to cause typical signs of disease such as fever, nasal discharge and cough. Observe the cats at least daily for 14 days after challenge. Collect nasal washings daily on days 2 to 14 after challenge to test for virus excretion. Note daily the body temperature and signs of disease using the scoring system shown below.

The vaccine virus complies with the test if, during the observation period after challenge, the score for the vaccinated cats is significantly lower than that for the controls.

| Sign | Score |
|---|---|
| Death | 10 |
| Depressed state | 2 |
| Temperature: | |
| 39.5 °C - 40.0 °C | 1 |
| ≥ 40.0 °C | 2 |
| ≤ 37.0 °C | 3 |
| Glossitis | 3 |
| Nasal discharge, slight | 1 |
| Nasal discharge, copious | 2 |
| Cough | 2 |
| Sneezing | 1 |
| Sneezing, paroxysmal | 2 |

| Sign | Score |
|---|---|
| Ocular discharge, slight | 1 |
| Ocular discharge, serious | 2 |
| Conjunctivitis | 2 |
| Weight loss ≥ 5.0 per cent | 5 |
| Virus excretion (total number of days): | |
| ≤ 4 days | 1 |
| 5-7 days | 2 |
| > 7 days | 3 |

3. BATCH TESTS

3-1. **Identification**. When mixed with a monospecific antiserum, the vaccine no longer infects susceptible cell cultures into which it is inoculated.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against feline herpesvirus 1 and inoculate into cell cultures known for their susceptibility to viruses pathogenic for the cat. Carry out at least 1 passage and maintain the cultures for 14 days. The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures and at a temperature favourable to replication of the virus. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:0063**
**corrected 9.5**

# FOOT-AND-MOUTH DISEASE (RUMINANTS) VACCINE (INACTIVATED)

## Vaccinum aphtharum epizooticarum inactivatum ad ruminantes

1. DEFINITION

Foot-and-mouth disease (ruminants) vaccine (inactivated) is a preparation containing one or more suitable strains of foot-and-mouth disease virus inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of ruminants against foot-and-mouth disease.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures and then separated from cellular material by filtration or other suitable procedures. The harvested virus is inactivated in suitable conditions and may be concentrated and purified. It is used for the preparation of vaccine immediately or after storage at a temperature shown to be consistent with antigen stability. The vaccine is prepared from inactivated virus by blending with one or more adjuvants. For a given strain, the quantity of 146S antigen blended in each batch of vaccine is not lower than that of a batch of vaccine that has been found satisfactory with respect to Immunogenicity.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *VALIDATION OF THE INACTIVATION PROCEDURE*

During inactivation, the virus titre is monitored by a sensitive and reproducible technique. The inactivation procedure is not satisfactory unless the decrease in virus titre, plotted logarithmically, is linear and extrapolation indicates that there is less than 1 infectious virus unit per $10^4$ L of liquid preparation at the end of inactivation.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for each species for which it is intended.

The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**

Carry out the test for each route and method of administration to be recommended for vaccination and in each category of each species for which the vaccine is intended, using in each case animals of the minimum age to be recommended. Use a representative batch of vaccine containing not less than the maximum antigen content that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals that do not have antibodies against foot-and-mouth disease virus. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the animals at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions, or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. Carry out an immunogenicity test for each strain of foot-and-mouth disease virus that may be included in the vaccine.

Each test is carried out for each route and method of administration to be recommended for vaccination, using in each case cattle not less than 6 months old. The vaccine administered to each cattle is of minimum antigen content.

Either of the following 2 tests is suitable to demonstrate immunogenicity of the vaccine for cattle.

2-4-2-1. *$PD_{50}$ challenge test*. The potency of the vaccine is expressed as the number of 50 per cent cattle protective doses ($PD_{50}$) contained in the dose stated on the label. The $PD_{50}$ is determined in cattle given primary vaccination and challenged by the inoculation of 10 000 $ID_{50}$ of virulent bovine virus of the same strain as that used in the preparation of the vaccine in the conditions described below. The vaccine virus may be used for challenge.

Use for the test not fewer than 17 cattle obtained from areas free from foot-and-mouth disease, that have never been vaccinated against foot-and-mouth disease and do not have antibodies neutralising the different strains of foot-and-mouth disease virus. Vaccinate not fewer than 3 groups of not fewer than 5 cattle per group, using a different dose of the vaccine for each group. Administer the different doses by injecting different volumes of the vaccine and not by dilution of the vaccine. Maintain 2 cattle as controls. For example, if the

label states that the injection of 2 mL corresponds to the administration of 1 dose of vaccine, a 1/4 dose of vaccine would be obtained by injecting 0.5 mL, and a 1/10 dose would be obtained by injecting 0.2 mL. Challenge all the cattle after 20-22 days by the intradermal route, into at least 2 sites on the upper surface of the tongue (0.1 mL per site), with a dose equivalent to approximately 10 000 $ID_{50}$ of a suspension of a fully virulent virus, obtained from cattle and of the same strain as that used in the preparation of the vaccine. Observe the cattle at least daily for 8 days. In the interest of animal welfare, individual animals may be euthanised before the end of the observation period and considered as unprotected if a vaccinated animal shows lesions of foot-and-mouth disease on at least 1 foot or if a control animal shows lesions of foot-and-mouth disease on at least 3 feet. Unprotected cattle show lesions at sites other than the tongue. Protected cattle may display lingual lesions.

The test is not valid if both control cattle do not show lesions on at least 3 feet. From the number of protected cattle in each group, calculate the $PD_{50}$ content of the vaccine.

The vaccine complies with the test if the potency is not less than that to be stated on the label; the minimum potency to be stated on the label is not less than 3 $PD_{50}$ per dose for cattle.

2-4-2-2. *PPG test*. The following test could also be used to demonstrate immunogenicity of the vaccine for cattle (referred to as the 'Percentage of protection against generalised foot infection' (PPG test)).

The potency of the vaccine is expressed as the percentage of cattle that do not show lesions on any feet. The PPG is determined in cattle given primary vaccination and challenged by the inoculation of 10 000 $ID_{50}$ of virulent virus of the same strain as that used in the preparation of the vaccine under the conditions described below. The vaccine virus may be used for challenge. Use for the test not fewer than 18 cattle obtained from areas free from foot-and-mouth disease, that have never been vaccinated against foot-and-mouth disease and do not have antibodies neutralising the different strains of foot-and-mouth disease virus. Vaccinate not fewer than 16 cattle with 1 full dose. Maintain 2 cattle as controls. Challenge all the cattle after 20-22 days by the intradermal route, into at least 2 sites on the upper surface of the tongue (0.1 mL per site), with a dose equivalent to approximately 10 000 $ID_{50}$ of a suspension of a fully virulent virus of the same strain as that used in the preparation of the vaccine. Observe the cattle at least daily for 8 days. In the interest of animal welfare, individual animals may be euthanised before the end of the observation period and considered as unprotected if a vaccinated animal shows lesions of foot-and-mouth disease on at least 1 foot or if a control animal shows lesions of foot-and-mouth disease on at least 3 feet. Unprotected cattle show lesions at sites other than the tongue. Protected cattle may display lingual lesions.

The test is not valid if both control cattle do not show lesions on at least 3 feet. From the number of protected cattle in the vaccinated group, calculate the percentage of protected cattle.

The vaccine complies with the test if the potency is not less than that to be stated on the label; the minimum potency to be stated on the label is not less than 75 per cent.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Identification**. The bulk inactivated antigen is identified by a suitable immunochemical method (*2.7.1*).

2-5-2. **Residual live virus**. The limit of detection of the cell cultures to be used with respect to the virus to be tested is established by determining the number of $CCID_{50}$ and the 146S antigen content of a sample of live virus. The cells are not suitable if an amount of virus corresponding to 1 µg of 146S antigen has less than $10^6$ $CCID_{50}$. A proportion of each batch of bulk inactivated antigen representing at least 200 doses is tested for freedom from live virus by inoculation into suitable cell cultures. A passage is made during culture of the cells. For this purpose, the sample of the inactivated antigen may be concentrated to allow testing of such large samples in cell cultures. It must be shown that the selected concentration and assay systems are not detrimental to detection of infectious virus within the test sample and that the concentrated inactivated antigen does not interfere with virus replication or cause toxic changes. A positive control is included in each test.

2-5-3. **Antigen content**. The 146S antigen content of each batch of bulk inactivated antigen is determined by an *in vitro* method (for example, by sucrose density gradient centrifugation and ultraviolet spectrophotometry at 259 nm).

2-5-4. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum antigen content. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency and has been shown to be satisfactory with respect to immunogenicity in the target species.

The following test may be used after a satisfactory pass level for a given strain has been established. Once a pass level has been established for a given strain, the same level of antigen may be used when this strain is formulated in combination with any other antigen provided that the formulation of the vaccine differs only in the strains included.

2-5-4-1. *Vaccines for use in cattle*. Use cattle of the minimum age recommended for vaccination obtained from areas free from foot-and-mouth disease, that have never been vaccinated against foot-and-mouth disease and do not have antibodies neutralising the different strains of foot-and-mouth disease virus. Vaccinate not fewer than 5 cattle by a recommended route. Use a suitable dose of the vaccine for each animal. After a defined period, not greater than 28 days following vaccination, draw a blood sample and determine individually in each serum the level of antibodies against each strain used in the preparation of the vaccine by a validated technique (e.g. sero-neutralisation test, ELISA). The vaccine complies with the test if the geometric mean of the antibody titre in cattle is not significantly lower than the pass level.

2-5-4-2. *Vaccines for use in other ruminants*. The potency of each batch shall be demonstrated in a suitable, validated test.

EMERGENCY USE: in situations of extreme urgency and subject to agreement by the competent authority, a batch of vaccine may be released before completion of the tests and the determination of potency if a test for sterility has been carried out on the bulk inactivated antigen and all other components of the vaccine and if the determination of potency has been carried out on a representative batch of vaccine prepared from the same bulk inactivated antigen. In this context, a batch is not considered to be representative unless it has been prepared with not more than the amount of antigen or antigens and with the same formulation as the batch to be released.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine and, where applicable, the liquid supplied with it, comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

**01/2017:1945**

# FOWL CHOLERA VACCINE (INACTIVATED)

## Vaccinum cholerae aviariae inactivatum

### 1. DEFINITION

Fowl cholera vaccine (inactivated) is a preparation of one or more suitable strains of one or more serovars of *Pasteurella multocida*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of chickens, turkeys, ducks and geese against acute fowl cholera.

### 2. PRODUCTION

#### 2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium. If the vaccine contains more than one strain of bacterium, the different strains are grown and harvested separately. The bacterial harvests are inactivated. The vaccine may be adjuvanted.

#### 2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the species for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination and for each avian species for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test performed in birds younger than 3 weeks of age, use not fewer than 10 birds not older than the minimum age to be recommended for vaccination. For each test performed in birds older than 3 weeks of age, use not fewer than 8 birds not older than the minimum age to be recommended for vaccination. In the case of chickens, use chickens from a flock free from specified pathogens (SPF) (*5.2.2*) and in the case of turkeys, ducks or geese, use birds that have not been vaccinated and that do not have antibodies against *P. multocida*. Administer by a route and method to be recommended to each bird 1 dose of vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose to each bird after an interval of at least 14 days. Observe the birds at least daily for at least 14 days for the last administration of the vaccine.

The test is not valid if more than 10 per cent of the birds younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For birds older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine complies with the test if no bird shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. The test is carried out for each route and method of administration to be recommended for vaccination, for each avian species for which the vaccine is intended and for each serovar of *P. multocida* against which protection is claimed. Use for each test not fewer than 30 birds not older than the minimum age to be recommended for vaccination. Use birds that have not been vaccinated and that are free from antibodies against *P. multocida*. For each test, administer to each of not fewer than 20 birds a quantity of the vaccine not greater than 1 dose. If re-vaccination is recommended, repeat this operation after the recommended interval. Maintain not fewer than 10 birds as controls. Challenge each of the birds of both groups 21 days after the last administration by the intramuscular route with a sufficient quantity of virulent *P. multocida*. Observe the birds for 14 days after challenge.

The test is not valid if during the observation period after challenge, fewer than 70 per cent of the control birds die or show signs of infection (such as either clinical signs or bacterial re-isolation in organs) or if during the period before challenge, more than 10 per cent of the birds from the control group or from the vaccinated group show abnormal signs of disease or die from causes not attributable to the vaccine.

The vaccine complies with the test if, at the end of the observation period after challenge, not fewer than 70 per cent of the birds from the vaccinated group survive and show no signs of disease. Mild signs that do not persist beyond the observation period may be tolerated.

#### 2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 15 SPF chickens (*5.2.2*), 3-4 weeks old. Collect serum samples from each vaccinated and control chicken just before vaccination and check for the absence of antibodies against each serovar of *P. multocida* in the vaccine. Administer to each of 10 chickens 1 dose of the vaccine by the subcutaneous route. Maintain 5 chickens as controls. Collect serum samples 5 weeks after vaccination from each vaccinated and control chicken. Measure the titres of serum antibodies against each serovar of *P. multocida* stated on the label using a suitable validated serological method. Calculate the mean titres for the group of vaccinates.

The test is not valid if specific *P. multocida* antibodies are found: before vaccination in 1 or more sera from chickens to be vaccinated or from controls; in 1 or more sera from control chickens 5 weeks after the time of administration of the vaccine.

The vaccine complies with the test if the mean antibody titres of the group of vaccinates are equal to or greater than the titres obtained with a batch that has given satisfactory results in the test described under Potency.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test (section 2-2-1). The method chosen for determining the maximum acceptable amount of bacterial endotoxins is used subsequently for testing each batch.

### 3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

LABELLING

The label states:

- the serovar(s) used to prepare the vaccine;
- the serovar(s) against which protection is claimed.

04/2013:0649

# FOWL-POX VACCINE (LIVE)

## Vaccinum variolae gallinaceae vivum

1. DEFINITION

Fowl-pox vaccine (live) is a preparation of a suitable strain of avian pox virus. This monograph applies to vaccines intended for administration to chickens for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination using in each case chickens not older than the minimum age to be recommended for vaccination from an SPF flock (*5.2.2*). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For chickens older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise, passage as described below is carried out.

Administer to each chicken of the 1st group by a suitable route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Prepare 4-7 days after administration a suspension from the induced skin lesions of each chicken and pool these samples. Administer 0.2 mL of the pooled samples by cutaneous scarification of the comb or other unfeathered part of the body, or by another suitable method to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens.

If the 5th group of chickens shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 chickens receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increase in virulence of the virus at the final passage level compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using in each case chickens not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine. Use for the test not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the feather-follicle route with a sufficient quantity of virulent fowl-pox virus. Observe the chickens at least daily for 21 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. Examine each surviving chicken for macroscopic lesions: cutaneous lesions of the comb, wattle and other unfeathered areas of the skin and diphtherical lesions of the mucous membranes of the oro-pharyngeal area.

The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the control chickens die or show severe clinical signs of fowl pox, including notable macroscopical lesions of the skin or mucous membranes of the oro-pharyngeal area,
- and/or during the period between vaccination and challenge, more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not less than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of disease, including macroscopical lesions of the skin and mucous membranes of the oro-pharyngeal area.

3. BATCH TESTS

3-1. **Identification**. Carry out an immunostaining test in cell cultures to demonstrate the presence of the vaccine virus. For egg adapted strains, inoculate the vaccine into eggs and notice the characteristic lesions.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection, scarification or wing web piercing comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection, scarification or wing web piercing either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**07/2017:1521**

# FURUNCULOSIS VACCINE (INACTIVATED, OIL-ADJUVANTED, INJECTABLE) FOR SALMONIDS

## Vaccinum furunculosidis inactivatum ad salmonidas cum adiuvatione oleosa ad iniectionem

### 1. DEFINITION

Furunculosis vaccine (inactivated, oil-adjuvanted, injectable) for salmonids is prepared from cultures of one or more suitable strains of *Aeromonas salmonicida* subsp. *salmonicida*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of salmonids against furunculosis.

### 2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The strains of *A. salmonicida* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium. The vaccine contains an oily adjuvant.

2-2. *CHOICE OF VACCINE STRAIN*

The strains included in the vaccine are shown to be suitable with respect to the production of antigens of assumed protective importance. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in the species of fish for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory test*. Carry out the test in each species of fish for which the vaccine is intended, using fish of the minimum body mass to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

Use not fewer than 50 fish from a population that does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* and has not been vaccinated against or exposed to furunculosis. The test is carried out in the conditions to be recommended for the use of the vaccine with a water temperature not less than 10 °C. Administer to each fish by the intraperitoneal route 1 dose of the vaccine. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-2. *Field studies*. Safety is also demonstrated in field trials by administering the intended dose to a sufficient number of fish in not fewer than 2 sets of premises. Samples of 30 fish are taken on 3 occasions (after vaccination, at the middle of the rearing period and at slaughter) and examined for local reactions in the body cavity. Moderate lesions involving localised adhesions between viscera or between viscera and the abdominal wall and slight opaqueness and/or sparse pigmentation of the peritoneum are acceptable. Extensive lesions including adhesions between greater parts of the abdominal organs, massive pigmentation and/or obvious thickening and opaqueness of greater areas of the peritoneum are unacceptable if they occur in more than 10 per cent of the fish in any sample. Such lesions include adhesions that give the viscera a 'one-unit' appearance and/or lead to manifest laceration of the peritoneum following evisceration.

2-2-2. **Immunogenicity**. Carry out the test according to a protocol defining limits of body mass for the fish, water source, water flow and temperature limits, and preparation of a standardised challenge. A test is carried out for the route and method of administration to be recommended. The vaccine administered to each fish is of minimum potency.

Use for the test not fewer than 60 fish from a population that does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* and has not been vaccinated against or exposed to furunculosis. Vaccinate not fewer than 30 fish according to the instructions for use. Perform mock vaccination on a control group of not fewer than 30 fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than one tank is used. Where justified and when fish cannot be marked, non-marked fish may be used. Vaccinates and controls may then be kept in the same tank but physically separated (for example by fishing nets). Challenge each fish, by injection, at a fixed interval after vaccination corresponding to the onset of immunity claimed, with a sufficient quantity of a culture of *A. salmonicida* subsp. *salmonicida* whose virulence has been verified. Observe the fish at least daily until the end of mortality is reached in the control group (no fish have died over a period of 2 days).

The test is not valid if the specific mortality is less than 60 per cent in the control group 21 days after the 1st death in the fish. Calculate the relative percentage survival (RPS) using the following expression:

$$\left(1 - \frac{V}{C}\right) \times 100$$

$V$ = percentage of mortality in vaccinates;

$C$ = percentage of mortality in controls.

The vaccine complies with the test if the RPS is not less than 70 per cent.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. The potency test (section 3-3) may be carried out for each batch of vaccine, using fish of one of the species for which the vaccine is intended. Where the test is not carried out, an alternative validated method may be used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 35 fish from a population that does not have specific antibodies against *A. salmonicida* subsp. *salmonicida* and that are within specified limits for body mass. Carry out the test at a defined temperature not less than 12 °C. Inject intraperitoneally into each of not fewer than 25 fish 1 dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than 10 fish. Collect blood samples from vaccinates and controls at a defined time not less than 500 degree days after vaccination. Determine for each sample the level of specific antibodies against *A. salmonicida* subsp. *salmonicida* by a suitable immunochemical method (*2.7.1*).

The test is not valid if the control group shows antibodies against *A. salmonicida* subsp. *salmonicida*.

The vaccine complies with the test if the mean level of antibodies in the vaccinates is not significantly lower than that found for a batch that gave satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by the recommended route and method.

4. LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

**01/2017:2674**

# INFECTIOUS BOVINE RHINOTRACHEITIS VACCINE (INACTIVATED)

## Vaccinum rhinotracheitidis infectivae bovinae inactivatum

1. DEFINITION

Infectious bovine rhinotracheitis vaccine (inactivated) is a preparation of a suitable strain of infectious bovine rhinotracheitis virus, i.e. bovine herpesvirus 1 (BHV1), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of cattle against bovine rhinotracheitis caused by bovine herpesvirus 1 (BHV1).

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus harvest is inactivated by a suitable method. The viral suspensions may be purified and concentrated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, and in each category of cattle for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency or maximum antigen content that may be expected in a batch of vaccine.

For each test, use not fewer than 8 calves of the minimum age to be recommended for vaccination and that do not have antibodies against infectious bovine rhinotracheitis virus. Administer to each calf 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the calves at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no calf shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination, using in each case calves that do not have antibodies against bovine rhinotracheitis virus. The vaccine administered to each calf is of minimum potency or minimum antigen content.

Use for the test not fewer than 10 calves of the minimum age to be recommended for vaccination. Vaccinate not fewer than 5 calves according to the schedule to be recommended. Maintain not fewer than 5 calves as controls. Challenge each animal at least 2 weeks after the last vaccination by a respiratory tract route with a sufficient quantity of virulent infectious bovine rhinotracheitis virus. Observe the calves at least daily for 14 days, in particular for respiratory signs and virus shedding (by nasal swabs or tracheobronchial washing).

The test is not valid if more than 1 of the 5 controls does not show virus excretion and typical signs of disease (e.g. fever, ocular and nasal discharge, coughing).

The vaccine complies with the test if, during the observation period after challenge:

– the vaccinated calves show no more than mild signs of disease;
– over the whole period of virus excretion by the controls, the average virus titre found in the nasal mucus of vaccinated calves is at least 100 times lower than the average virus titre found in the control calves.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. The test for residual live virus is carried out using 2 passages in the same type of cell culture as that used for production, or in cell cultures shown to be at least as sensitive. The quantity of inactivated harvest used is sufficient to ensure a test of suitable sensitivity. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Antigen content**. The content of an appropriate bovine herpesvirus glycoprotein is determined by a suitable immunochemical method (*2.7.1*).

2-4-3. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following assay may be used.

*Serological assay*. Vaccinate not fewer than 5 animals that do not have antibodies against bovine herpesvirus 1 (BHV1) with a single injection of a suitable dose. Where the recommended schedule requires a 2nd injection, this may be given provided it has been demonstrated that this will still provide a suitably sensitive system. At a given interval within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated method such as seroneutralisation to measure the response to the antigen.

The vaccine complies with the test if the antibody levels are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency.

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, manufacturers are encouraged to develop alternative *in vitro* methods to the animal test for batch release using appropriate tools such as the monitoring of production consistency (as stated in the General Notices, section 1.1) and the quantification of herpesvirus glycoprotein in the final product.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a test for residual live virus using 2 passages in the same type of cell culture as that used in the production, or in cell cultures shown to be at least as sensitive. The quantity of vaccine used is sufficient to ensure a test of suitable sensitivity. The vaccine complies with the test if no live virus is detected.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**04/2013:0696**

# INFECTIOUS BOVINE RHINOTRACHEITIS VACCINE (LIVE)

## Vaccinum rhinotracheitidis infectivae bovinae vivum

1. DEFINITION

Infectious bovine rhinotracheitis vaccine (live) is a preparation of one or more suitable strains of infectious bovine rhinotracheitis virus (bovine herpesvirus 1). This monograph applies to vaccines intended for the active immunisation of cattle against bovine rhinotracheitis caused by bovine herpesvirus 1.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-3-1), abortigenicity and passage through the placenta (section 2-3-2), increase in virulence (section 2-3-3) and immunogenicity (section 2-3-4) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 5 calves, 3 months old or of the minimum age to be recommended for vaccination if this is less than 3 months, and that do not have antibodies against infectious bovine rhinotracheitis virus. Administer to each calf a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the calves at least daily for at least 14 days.

The vaccine virus complies with the test if no calf shows abnormal local or systemic reactions or dies from causes attributable to the vaccine virus.

2-3-2. **Abortigenicity and passage through the placenta**. Use not fewer than 24 pregnant cows that do not have antibodies against infectious bovine rhinotracheitis virus: 8 of the cows are in the 4th month of pregnancy, 8 in the 5th and 8 in the 6th or 7th month. Administer to each cow by a route to be recommended a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the cows at least daily until the end of pregnancy.

The vaccine virus complies with the test if:

– where abortion occurs, tests show that neither virus nor viral antigens are present in the foetus or placenta;
– on calves born at term before ingestion of colostrum, a test for antibodies against infectious bovine rhinotracheitis virus indicates no such antibodies are found.

2-3-3. **Increase in virulence**. Carry out the test according to chapter *5.2.6. Evaluation of safety of veterinary vaccines and immunosera* using calves 3 months old or of the minimum

age to be recommended for vaccination if this is less than 3 months, and that do not have antibodies against infectious bovine rhinotracheitis virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Take suitable samples from the calves used for the test for safety at a time when the vaccinal virus can be easily detected, verify the presence and titre of the virus in the samples and mix them. Administer to each calf of the 1st group by the intranasal route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the intranasal route to each calf of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5th group of calves shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeated passage in 10 animals, the vaccine virus also complies with the test.

2-3-4. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case calves 2-3 months old. The quantity of vaccine to be administered to each calf is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine. Use for the test not fewer than 7 calves that do not have antibodies against infectious bovine rhinotracheitis virus. Vaccinate not fewer than 5 calves, according to the schedule to be recommended. Maintain not fewer than 2 calves as controls. Challenge each calf after 20-22 days by the intranasal route with a sufficient quantity of a virulent infectious bovine rhinotracheitis virus. Observe the calves at least daily for 21 days after challenge, in particular for respiratory signs and virus shedding (by nasal swabs or tracheobronchial washing).

The test is not valid if the controls do not show typical signs of disease such as fever, ocular and nasal discharge and ulceration of the nasal mucosa.

The vaccine virus complies with the test if, during the observation period after challenge:

- the vaccinated calves show no more than mild signs;
- in not fewer than 4 of the 5 vaccinated calves, the maximum virus titre found in the nasal mucus is at least 100 times lower than the average of the maximum titres found in the control calves; and
- the average number of days on which virus is excreted is at least 3 days less in vaccinated calves than in the control calves.

3. BATCH TESTS

3-1. **Identification**

3-1-1. When mixed with a suitable quantity of a monospecific antiserum, the vaccine is no longer able to infect susceptible cell cultures into which it is inoculated.

3-1-2. Any markers of the strain are verified.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus with a suitable monospecific antiserum against bovine rhinotracheitis virus and inoculate into cell cultures known for their susceptibility to viruses pathogenic for cattle. Carry out 1 passage at 7 days and maintain the cultures for 14 days.

The vaccine complies with the test if no cytopathic effect develops and there is no sign of the presence of haemadsorbing agents.

3-5. **Virus titre**. Titrate the vaccine virus in susceptible cell cultures at a temperature favourable to replication of the virus. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-4) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:2038**
**corrected 9.5**

# INFECTIOUS CHICKEN ANAEMIA VACCINE (LIVE)

## Vaccinum anaemiae infectivae pulli vivum

1. DEFINITION

Infectious chicken anaemia vaccine (live) is a preparation of a suitable strain of chicken anaemia virus. This monograph applies to vaccines intended for administration to breeder chickens for active immunisation, to prevent excretion of the virus, to prevent or reduce egg transmission and to protect passively their future progeny.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the test for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination in chickens not older than the minimum age to be recommended

for vaccination and from an SPF flock (*5.2.2*). Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

2-4-1-1. *General safety*. For each test, use not fewer than 8 chickens. Administer to each chicken a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. 14 days after vaccination, collect blood samples from half of the chickens and determine the haematocrit value. Euthanise these chickens and carry out post-mortem examination. Note any pathological changes attributable to chicken anaemia virus, such as thymic atrophy and specific bone-marrow lesions. Observe the remaining chickens at least daily, for at least 21 days after vaccination.

The test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if during the observation period no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine virus.

2-4-1-2. *Safety for young chickens*. Use not fewer than twenty 1-day-old chickens from an SPF flock (*5.2.2*). Administer to each chicken by the oculonasal route a quantity of the vaccine virus equivalent to not less than the maximum titre likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily. Record the incidence of any signs attributable to the vaccine virus, such as depression, and any deaths. 14 days after vaccination, collect blood samples from half of the chickens and determine the haematocrit value. Euthanise these chickens and carry out post-mortem examination. Note any pathological changes attributable to chicken anaemia virus, such as thymic atrophy and specific bone marrow lesions. Observe the remaining chickens at least daily, for at least 21 days after vaccination. Assess the extent to which the vaccine strain is pathogenic for 1-day-old susceptible chickens from the results of the clinical observations and mortality rates and the proportion of chickens examined at 14 days that show anaemia (haematocrit value less than 27 per cent) and signs of infectious chicken anaemia on post-mortem examination. The results are used to formulate the label statement on safety for young chickens.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using 1-day-old chickens from an SPF flock (*5.2.2*). If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each chicken of the 1st group by the intramuscular route a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Prepare 7-9 days after administration a suspension from the liver of each chicken and pool these samples. Depending on the tropism of the virus, other tissues such as spleen or bone marrow may be used. Administer 0.1 mL of the pooled samples by the intramuscular route to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 chickens.

If the 5th group of chickens shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 10 chickens receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus at the final passage level compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens and a subsequent repeat passage in 10 chickens, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*). The test for prevention of virus excretion is intended to demonstrate reduction of egg transmission through viraemia and virus excretion in the faeces. The quantity of the vaccine virus to be administered to each chicken is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

2-4-3-1. *Passive immunisation of chickens*. Vaccinate according to the schedule to be recommended not fewer than 10 breeder chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*); keep not fewer than 10 unvaccinated breeder chickens of the same origin and from an SPF flock (*5.2.2*) as controls that have no contact with the vaccinated chickens. At a suitable time, after excretion of vaccine virus has ceased, collect fertilised eggs from the vaccinated and control breeder chickens and incubate them. Challenge at least 30 1-day-old chickens from each of the vaccinated and control groups by intramuscular administration of a sufficient quantity of virulent chicken anaemia virus. Observe the chickens at least daily for 14 days after challenge. Record the deaths and the surviving chickens that show signs of disease. At the end of the observation period determine the haematocrit value of each surviving chicken. Euthanise these chickens and carry out post-mortem examination. Note any pathological signs attributable to chicken anaemia virus, such as thymic atrophy and specific bone-marrow lesions.

The test is not valid if:

- the laying rate in the vaccinated and control breeder chickens is significantly different;
- during the observation period after challenge fewer than 90 per cent of the chickens of the control breeder chickens die or show severe signs of infectious chicken anaemia, including haematocrit value under 27 per cent, and/or notable macroscopic lesions of the bone marrow and thymus;
- and/or during the period between vaccination and egg collection more than 10 per cent of vaccinated or control breeder chickens show notable signs of disease or die from causes not attributable to the vaccine.

The vaccine complies with the test if during the observation period after challenge not fewer than 90 per cent of the chickens of the vaccinated breeder chickens survive and show no notable signs of disease and/or macroscopic lesions of the bone marrow and thymus.

2-4-3-2. *Prevention of virus excretion*. Vaccinate according to the schedule to be recommended not fewer than 10 chickens not older than the minimum age to be recommended for vaccination and from an SPF flock (*5.2.2*). Maintain not fewer than 10 chickens of the same age and origin as controls that have no contact with the vaccinated chickens. At a suitable time after excretion of vaccine virus has ceased, challenge all the chickens by intramuscular administration of a sufficient quantity of virulent chicken anaemia virus. Collect blood samples from the chickens on days 3, 5 and 7 after challenge and faecal samples from the chickens on days 7, 14 and 21 after challenge and carry out a test for presence of virus to determine whether or not the chickens are viraemic and are excreting the virus.

The test is not valid if:

- fewer than 70 per cent of the control chickens are viraemic and excrete the virus at one or more times of sampling;
- and/or during the period between vaccination and challenge more than 10 per cent of control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine complies with the test if not fewer than 90 per cent of the vaccinated chickens do not develop viraemia or excrete the virus.

3. BATCH TESTING

3-1. **Identification**. The vaccine, diluted if necessary and mixed with a monospecific chicken anaemia virus antiserum, no longer infects susceptible cell cultures or eggs from an SPF flock (*5.2.2*) into which it is inoculated.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into suitable cell cultures (*5.2.4*) or eggs from an SPF flock (*5.2.2*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the tests prescribed under Immunogenicity (sections 2-4-3-1 and 2-4-3-2) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

4. LABELLING

The label states to which extent the vaccine virus causes disease if it spreads to susceptible young chickens.



**01/2020:3063**

# INFECTIOUS PANCREATIC NECROSIS VACCINE (INACTIVATED, OIL-ADJUVANTED, INJECTABLE) FOR SALMONIDS

## Vaccinum necrosis pancreaticae infectivae inactivatum ad salmonidas cum adiuvatione oleosa ad iniectionem

1. DEFINITION

Infectious pancreatic necrosis (IPN) vaccine (inactivated, oil-adjuvanted, injectable) for salmonids is prepared from cultures of a suitable strain of infectious pancreatic necrosis virus, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for administration by injection for the active immunisation of salmonids against IPN.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The harvest is inactivated by a suitable method. The virus suspension may be purified and/or concentrated. The vaccine contains an oily adjuvant.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The strain included in the vaccine is shown to be suitable with respect to the production of antigens of protective importance. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in the species of fish for which it is intended. The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

2-3-1-1. *Laboratory test*. Carry out the test in each species of fish for which the vaccine is intended. The average body mass of the fish corresponds to the minimum body mass to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency or maximum antigen content that may be expected in a batch of vaccine.

Use not fewer than 50 fish from a population that does not have specific antibodies against IPN virus and has not been vaccinated against IPN or exposed to IPN virus. The test is carried out under the conditions to be recommended for use of the vaccine with a water temperature not less than 10 °C. Administer to each fish by the intraperitoneal route 1 dose of the vaccine. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-1-2. *Field studies*. Safety is also demonstrated in field trials by administering the intended dose to a sufficient number of fish in not fewer than 2 sets of premises. Samples of 30 fish are taken on 3 occasions (after vaccination, at the middle of the rearing period and at slaughter) and examined for local reactions in the body cavity. Moderate lesions involving localised adhesions between viscera or between viscera and the abdominal wall and slight opaqueness and/or sparse pigmentation of the peritoneum are acceptable. Extensive lesions including adhesions between greater parts of the abdominal organs, massive pigmentation and/or obvious thickening and opaqueness of greater areas of the peritoneum are unacceptable if they occur in more than 10 per cent of the fish in any sample. Such lesions include adhesions that give the viscera a 'one-unit' appearance and/or lead to manifest laceration of the peritoneum following evisceration.

2-3-2. **Immunogenicity**. Carry out the test according to a protocol defining limits of body mass for the fish, water source, water flow and temperature limits, and preparation of a standardised challenge. A test is carried out after intraperitoneal administration of the vaccine. The vaccine administered to each fish is of minimum potency or minimum antigen content.

Use for the test not fewer than 60 fish from a population that does not have specific antibodies against IPN virus and has not been vaccinated against IPN or exposed to IPN virus. The average body mass of the fish corresponds to the minimum body mass to be recommended for vaccination. Vaccinate not fewer than 30 fish according to the instructions for use. Perform mock vaccination on a control group of not fewer than 30 fish (negative controls); mark vaccinated and negative-control fish for identification. Keep all the fish in the same tank or mix equal numbers of negative controls and

vaccinates in each tank if more than 1 tank is used. Challenge each fish at a fixed interval after vaccination, corresponding to the onset of immunity claimed, by a suitable route, with a sufficient quantity of IPN virus of which the virulence has been verified. Cohabitation challenge may be used, whereby shedder fish are injected with a virulent IPN virus strain in order to obtain viral shedding fish that will induce mortality in susceptible cohabiting fish. Add the shedder fish to the tank(s) and observe the fish at least daily until the end of mortality is reached in the control group (determined as the point in time when no negative-control fish have died over a period of 2 consecutive days). If co-habitation challenge is used, the shedder fish are added to the tank(s) at an appropriate ratio to the susceptible cohabiting fish to permit a valid challenge. Shedder and cohabiting fish are marked for individual or group identification.

The test is not valid if the specific mortality is less than 50 per cent in the control group. Calculate the relative percentage survival (RPS) using the following expression:

$$\left(1 - \frac{V}{C}\right) \times 100$$

$V$ = percentage of mortality in vaccinated fish;

$C$ = percentage of mortality in negative-control fish.

The vaccine complies with the test if the RPS is not less than 50 per cent at 50 per cent control mortality.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method may be used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The test for antigen content (section 2-4-1-1) together with the test for adjuvant (section 2-4-1-2) may be carried out.

2-4-1-1. *Antigen content*. The quantity of virus antigen per dose is determined by a suitable immunochemical method (*2.7.1*), which can include but is not limited to an enzyme-linked immunosorbent assay (ELISA) test for aqueous antigen content of a split emulsion. The antigen content is not significantly lower than that found for a batch that has given satisfactory results in the test described under Immunogenicity (section 2-3-2).

2-4-1-2. *Adjuvant*. If the immunochemical assay (section 2-4-1-1) is performed, the adjuvant is tested, in accordance with the monograph *Vaccines for veterinary use (0062)*, by suitable physical and chemical methods. If the adjuvant cannot be adequately characterised, the antigen content determination cannot be used as the batch potency test.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by the recommended route and method.

4. LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

**01/2017:1944**

# MANNHEIMIA VACCINE (INACTIVATED) FOR CATTLE

## Vaccinum mannheimiae bovinae inactivatum

1. DEFINITION

Mannheimia vaccine (inactivated) for cattle is a preparation from cultures of one or more suitable strains of *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of cattle of different ages against respiratory diseases caused by *M. haemolytica*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The choice of composition and the strains to be included in the vaccine is based on epidemiological data on the prevalence of the different serovars of *M. haemolytica* and on the claims being made.

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the cattle for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Carry out the test for each route and method of administration to be recommended for vaccination and in cattle of each category for which the vaccine is intended (for example, young calves, pregnant cattle). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 cattle that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified, cattle with a known history of no previous mannheimia vaccination and with low antibody titres (measured in a sensitive test system such as ELISA) may be used. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the cattle at least daily for at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine, if the average body temperature increase for all cattle does not exceed 1.5 °C, and if no animal shows a rise greater than 2.0 °C.

2-2-1-2. *Field studies*. The cattle used for the field trials are also used to evaluate safety. Carry out a test in each category of cattle for which the vaccine is intended. Use not fewer than 3 groups of 20 cattle with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine. The average body temperature increase for all cattle does not exceed 1.5 °C and no animal shows a rise greater than 2.0 °C. In addition, if the vaccine is intended for use in pregnant cows, no significant effects on gestation or the offspring are demonstrated.

2-2-2. **Immunogenicity**. Carry out a test for each serovar for which protection is claimed on the label.

Each test is carried out for each route and method of administration to be recommended, using in each case cattle of the minimum age to be recommended for vaccination. The vaccine administered to each animal is of minimum potency.

Use not fewer than 16 cattle that do not have antibodies against *M. haemolytica* and against the leucotoxin of *M. haemolytica*. Vaccinate not fewer than 8 of the cattle according to the schedule to be recommended. Maintain not fewer than 8 cattle as controls. Challenge each animal 20-22 days after the last vaccination by the intratracheal route or by another appropriate route, with a sufficient quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Observe the cattle at least daily for a further 7 days; to avoid unnecessary suffering, severely ill cattle are euthanised and are then considered to have died from the disease. During the observation period, examine the cattle for signs of disease (for example, increased body temperature, dullness, abnormal breathing) and record the mortality. Euthanise surviving cattle at the end of the observation period. Carry out post-mortem examination on any animal that dies and those euthanised at the end of the observation period. Examine the lungs and evaluate the extent of lung lesions due to mannheimiosis. Collect samples of lung tissue for re-isolation of the challenge organisms. Score the clinical observations and lung lesions and compare the results obtained for these parameters and the bacterial re-isolation results for the 2 groups.

The test is not valid if signs of *M. haemolytica* infection occur in less than 70 per cent of the control cattle. The vaccine complies with the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test 2-2-1-1 given under Choice of vaccine composition. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**01/2017:1946**

# MANNHEIMIA VACCINE (INACTIVATED) FOR SHEEP

## Vaccinum mannheimiae inactivatum ad ovem

1. DEFINITION

Mannheimia vaccine (inactivated) for sheep is a preparation of one or more suitable strains of *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of sheep and/or for the passive protection of their progeny against disease caused by *M. haemolytica*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The choice of composition and the strains to be included in the vaccine are based on epidemiological data on the prevalence of the different serovars of *M. haemolytica* and on the claims being made for the product, for example active and/or passive protection.

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the sheep for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Carry out the tests for each route and method of administration to be recommended for vaccination and in sheep of each category for which the vaccine is intended (for example, young sheep, pregnant ewes). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 sheep that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified,

sheep with a known history of no previous mannheimia vaccination and with low antibody titres (measured in a sensitive test system such as ELISA) may be used. Administer to each sheep 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the sheep at least daily for at least 14 days after the last administration. If the test is carried out in pregnant ewes, observe the ewes until 1 day after lambing. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each sheep.

The vaccine complies with the test if:

- no sheep shows abnormal local reactions or notable signs of disease, or dies from causes attributable to the vaccine,
- the average body temperature increase for all sheep does not exceed 1.5 °C and no sheep shows a rise greater than 2.0 °C, and if
- no adverse effects on gestation or the offspring are noted if the test is carried out in pregnant ewes.

2-2-1-2. *Field studies.* The sheep used for the field trials are also used to evaluate safety. Carry out a test in each category of sheep for which the vaccine is intended. Use not fewer than 3 groups of 20 sheep with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination.

The vaccine complies with the test if no sheep shows abnormal local or systemic reactions or notable signs of disease, or dies from causes attributable to the vaccine. The average body temperature increase for all sheep does not exceed 1.5 °C and no sheep shows a rise greater than 2.0 °C. In addition, if the vaccine is intended for use in pregnant ewes, no adverse effects on the gestation or offspring are demonstrated.

2-2-2. **Immunogenicity**

2-2-2-1. *Active immunisation.* For vaccines with claims for active immunisation against mannheimiosis, carry out a test for each serovar of *M. haemolytica* for which protection is to be claimed on the label.

A test is carried out for each route and method of administration to be recommended, using in each case lambs of the minimum age to be recommended for vaccination. The vaccine administered to each lamb is of minimum potency.

Use not fewer than 20 lambs that do not have antibodies against *M. haemolytica* and against the leucotoxin of *M. haemolytica*. Vaccinate not fewer than 10 lambs according to the schedule to be recommended. Maintain not fewer than 10 lambs as controls. 20-22 days after the last vaccination, challenge each lamb by the intratracheal route or by another appropriate route, with a sufficient quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Where necessary for a given serovar, prechallenge with parainfluenza type 3 (PI3) virus or another appropriate respiratory pathogen may be used. Observe the lambs for a further 7 days; to avoid unnecessary suffering, severely ill lambs are euthanised and are then considered to have died from the disease. During the observation period, examine the lambs for signs of disease (for example, increased body temperature, dullness, abnormal respiration) and record the mortality. Euthanise surviving lambs at the end of the observation period. Carry out post-mortem examination on any lamb that dies and those euthanised at the end of the observation period. Examine the lungs and evaluate the extent of lung lesions due to mannheimiosis. Collect samples of lung tissue for re-isolation of the challenge organisms. Score the clinical observations and lung lesions and compare the results obtained for these parameters and the bacterial re-isolation results for the 2 groups.

The test is not valid if signs of *M. haemolytica* infection occur in less than 70 per cent of the control lambs. The vaccine complies with the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

2-2-2-2. *Passive protection.* For vaccines with claims for passive protection against mannheimiosis carry out a test for each serovar of *M. haemolytica* for which protection is to be claimed on the label.

A test is carried out for each route and method of administration to be recommended for vaccination. The vaccine administered to each ewe is of minimum potency.

Use not fewer than 6 ewes that preferably do not have antibodies against the serovars of *M. haemolytica* or against the leucotoxin present in the vaccine. Where justified, ewes with a known history of no previous mannheimia vaccination, from a source with a low incidence of respiratory disease and with low antibody titres (measured in a sensitive test system such as ELISA) may be used. Vaccinate the ewes at the stages of pregnancy and according to the schedule to be recommended. A challenge study is conducted with 20 newborn, colostrum-deprived lambs. 10 of these lambs are given colostrum from the vaccinated ewes and 10 control lambs are given colostrum or colostrum substitute without detectable antibodies to *M. haemolytica*. When the lambs are at the age to be claimed for the duration of the passive protection, challenge each by the intratracheal route with a sufficient quantity of a low-passage, virulent strain of a serovar of *M. haemolytica*. Observe the lambs for a further 7 days; to avoid unnecessary suffering, severely ill lambs are euthanised and are then considered to have died from the disease. Observe the lambs and assess the effect of the challenge on the offspring of the vaccinates and the controls as described in the test for active immunisation.

The test is not valid if signs or lesions of *M. haemolytica* infection occur in less than 70 per cent of the control lambs. The vaccine complies with the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the lambs from the vaccinates compared to those from the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the lambs from the vaccinates compared to those from the controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the relevant potency test or tests (section 3-3) for each batch of vaccine if they have been carried out using a batch of vaccine with a minimum potency. Where the relevant test or tests are not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test(s) described under Potency.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety tests 2-2-1-1 given under Choice of vaccine composition. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test or test(s) mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:0589**

# MAREK'S DISEASE VACCINE (LIVE)

## Vaccinum morbi Marek vivum

1. DEFINITION

Marek's disease vaccine (live) is a preparation of a suitable strain or strains of Marek's disease virus (gallid herpesvirus 2 or 3) and/or turkey herpesvirus (meleagrid herpesvirus 1). This monograph applies to vaccines intended for administration to chickens and/or chicken embryos for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. If the vaccine contains more than one type of virus, the different types are grown separately. The vaccine may be freeze-dried or stored in liquid nitrogen.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens and/or chicken embryos for which it is intended.
The tests shown below for residual pathogenicity of the strain (section 2-4-1-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy. Additional testing may be needed to demonstrate safety in breeds of chickens known to be particularly susceptible to Marek's disease virus, unless the vaccine is to be contra-indicated.

2-4-1. **Safety**

2-4-1-1. *Residual pathogenicity of the strain*. Carry out the test for the route to be recommended for vaccination that is likely to be the least safe and in the category of chickens for which the vaccine is intended that is likely to be the most susceptible for Marek's disease.

Carry out the test in chickens if the vaccine is intended for chickens; carry out the test in chicken embryos if the vaccine is intended for chicken embryos; carry out the test in chickens and in chicken embryos if the vaccine is intended for both.

Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine.

*Vaccines intended for use in chickens*. Use not fewer than 80 1-day-old chickens from a flock free from specified pathogens (SPF) (*5.2.2*). Divide them randomly into 2 groups of not fewer than 40 chickens and maintain the groups separately. Administer by a suitable route to each chicken of one group (I) a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Administer by a suitable route to each chicken of the other group (II) a quantity of virulent Marek's disease virus that will cause mortality and/or severe macroscopic lesions of Marek's disease in not fewer than 70 per cent of the effective number of chickens within 70 days (initial number reduced by the number that die within the first 7 days of the test).

*Vaccines intended for use in chicken embryos*. Use not fewer than 150 embryonated eggs from an SPF flock (*5.2.2*). Divide them randomly into 3 groups of not fewer than 50 embryonated eggs and maintain the groups separately but under identical incubation conditions. Not later than the recommended day of vaccination, administer by the method to be recommended to each embryonated egg of one group (I) a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Administer by a suitable route to each embryonated egg of another group (II) a quantity of virulent Marek's disease virus that will cause mortality and/or severe macroscopic lesions of Marek's disease in not fewer than 70 per cent of the effective number of hatched chickens within 70 days (initial number reduced by the number that die within the first 7 days after hatching). Keep the last group (III) non-inoculated. The test is not valid if there is a significant difference in hatchability between groups I and III and the hatchability in any of the 3 groups is less than 80 per cent.

Provided that the chickens and chicken embryos are derived from the same flock, a common control group for *in ovo* and parenteral administration can be used.

Irrespective of whether the vaccine was administered to chickens or chicken embryos, observe the chickens of group II at least daily for 70 days and those of group I at least daily for 120 days.

The test is not valid if one or more of the following apply:

- more than 10 per cent of the chickens in any of the 3 groups die within the first 7 days;
- fewer than 70 per cent of the effective number of chickens in group II show macroscopic lesions of Marek's disease;

The vaccine virus complies with the test if:

- no chicken of group I shows notable clinical signs or macroscopic lesions of Marek's disease or dies from causes attributable to the vaccine virus;
- at 120 days the number of surviving chickens of group I is not fewer than 80 per cent of the effective number.

2-4-2. **Increase in virulence**. The test for increase in virulence is required for Marek's disease virus vaccine strains but not for turkey herpesvirus vaccine strains, which are naturally apathogenic.

Carry out the test according to general chapter *5.2.6*.

*Vaccines intended for use in chickens*. Administer to each 1-day-old SPF chicken (*5.2.2*) by the intramuscular route a quantity of the vaccine virus that will allow recovery of virus for the passages described below.

*Vaccines intended for use only in chicken embryos or intended for use in chickens and in chicken embryos*. Administer to each embryonated egg not later than the recommended day for vaccination by the *in ovo* route, using the recommended method, a quantity of the vaccine virus that will allow recovery of virus for the passages described below.

If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

5-7 days after administering the vaccine to chickens or 5-7 days after hatching when the vaccine has been administered *in ovo*, prepare a suspension of white blood cells from each chicken and pool these samples. Administer a suitable volume of the pooled samples by the intraperitoneal route to each 1-day-old SPF chicken (*5.2.2*) of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals. Carry out the test for residual pathogenicity (section 2-4-1-1) using the material used for the 1st passage and the virus at the final passage level. Administer the virus by the route to be recommended for vaccination that is likely to be the least safe for use in these chickens or chicken embryos.

The vaccine virus complies with the test if no indication of increase in virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 chickens or chicken embryos and a subsequent repeat passage in 10 chickens or chicken embryos, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case chickens of the minimum age to be recommended for vaccination or chicken embryos. The quantity of the vaccine virus administered to each chicken or chicken embryo is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

*Vaccines intended for use in chickens.* Use not fewer than 60 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 30 chickens. Maintain not fewer than 30 chickens as controls.

*Vaccines intended for use in chicken embryos.* Use embryonated chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by the *in ovo* route using the method to be recommended, 50 per cent of the embryonated eggs. Maintain 50 per cent of the embryonated eggs as controls. The test is not valid if any group consists of fewer than 30 hatched chicks.

Irrespective of whether the vaccine was administered to chickens or chicken embryos, challenge each chicken not later than 9 days after vaccination by a suitable route with a sufficient quantity of virulent Marek's disease virus. Observe the chickens at least daily for 70 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease. At the end of the observation period, euthanise all the surviving chickens and carry out an examination for macroscopic lesions of Marek's disease.

The test is not valid if:

- during the observation period after challenge, fewer than 70 per cent of the control chickens die or show severe clinical signs or macroscopic lesions of Marek's disease;
- and/or, during the period between the vaccination and challenge, more than 10 per cent of the control or vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if the relative protection percentage, calculated using the following expression, is not less than 80 per cent:

$$\frac{V - C}{100 - C} \times 100$$

$V$ = percentage of challenged vaccinated chickens that survive to the end of the observation period without notable clinical signs or macroscopic lesions of Marek's disease;

$C$ = percentage of challenged control chickens that survive to the end of the observation period without notable clinical signs or macroscopic lesions of Marek's disease.

3. BATCH TESTS

3-1. **Identification**. Carry out an immunostaining test in cell cultures using monoclonal antibodies to demonstrate the presence of each type of virus stated on the label.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**

3-5-1. *Vaccines containing one type of virus.* Titrate the vaccine virus by inoculation into suitable cell cultures (*5.2.4*). If the virus titre is determined in plaque-forming units (PFU), only primary plaques are taken into consideration. The vaccine complies with the test if one dose contains not less than the minimum virus titre stated on the label.

3-5-2. *Vaccines containing more than one type of virus.* For vaccines containing more than one type of virus, titrate each virus by inoculation into suitable cell cultures (*5.2.4*), reading the results by immunostaining using antibodies. The vaccine complies with the test if one dose contains for each vaccine virus not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the test for immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:1942**

# MYCOPLASMA GALLISEPTICUM VACCINE (INACTIVATED)

## Vaccinum Mycoplasmatis galliseptici inactivatum

1. DEFINITION

Mycoplasma gallisepticum vaccine (inactivated) is a preparation of one or more suitable strains of *Mycoplasma gallisepticum* that have been inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of chickens and/or turkeys.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable solid and/or liquid medium to ensure optimal growth under the chosen incubation conditions. Each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity and identity of the harvest are verified using suitable methods. After cultivation, the mycoplasma suspensions are collected separately and inactivated by a suitable method. The mycoplasma suspensions may be treated to fragment the mycoplasmas and the fragments may be purified and concentrated. The vaccine may contain an adjuvant.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in the target animals. The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy. If the indications for the vaccine include protection against a drop in laying performance or protection against infectious sinusitis in turkeys, further suitable immunogenicity testing is necessary.

2-2-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination and for each avian species for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test performed in birds younger than 3 weeks of age, use not fewer than 10 birds not older than the minimum age to be recommended for vaccination. For each test performed in birds older than 3 weeks of age, use not fewer than 8 birds not older than the minimum age to be recommended for vaccination. In the case of chickens, use chickens from a flock free from specified pathogens (SPF) (*5.2.2*) and in the case of turkeys, use birds that have not been vaccinated and that do not have antibodies against *M. gallisepticum*. Administer by a route and method to be recommended to each bird 1 dose of vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose to each bird after an interval of at least 14 days. Observe the birds at least daily for at least 14 days after the last administration of the vaccine.

The test is not valid if more than 10 per cent of the birds younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For birds older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine complies with the test if no bird shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. The test is carried out for each recommended route of administration and for each avian species for which the vaccine is intended. Use for each test not fewer than 40 birds not older than the minimum age to be recommended for vaccination. Use chickens from an SPF flock (*5.2.2*) or turkeys that have not been vaccinated and are free from antibodies against *M. gallisepticum*. For each test, administer to each of not fewer than 20 birds a quantity of the vaccine not greater than a single dose. If re-vaccination is recommended, repeat this operation after the recommended interval. Maintain not fewer than 20 birds as controls. Challenge each bird from both groups not more than 28 days after the last administration by a suitable route with a sufficient quantity of virulent *M. gallisepticum* (R-strain). Observe the birds at least daily for 14 days after challenge. Evaluation is carried out 14 days after challenge, at which point the birds are euthanised. Record the deaths and the number of surviving birds that show clinical signs of disease (e.g. respiratory distress, nasal discharge), and record air sac lesions.

The test is not valid if:

- during the observation period after challenge, fewer than 70 per cent of the controls die or show lesions or clinical signs of disease; and/or
- during the period between vaccination and challenge, more than 10 per cent of the birds from the control group or from the vaccinated group show abnormal clinical signs of disease or die from causes not attributable to the vaccine.

Thoracic and abdominal air sacs are evaluated individually on each side of the animal. The scoring system presented below may be used. The vaccine complies with the test if the score for the vaccinated birds is significantly lower than that for the controls and if the reduction is not less than 30 per cent.

0 no air sac lesions

1 in a limited area of 1 or 2 air sacs: cloudiness with slight thickening of the air sac membrane or flecks of yellowish exudate

2 in 1 air sac or portions of 2 air sacs: greyish or yellow, sometimes foamy exudate, with thickening of the air sac membrane

3 in 3 air sacs: extensive exudate, with clear thickening of most air sacs

4 severe air-sacculitis with considerable exudate and thickening of most air sacs.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual live mycoplasmas**. A validated test for residual live mycoplasmas is carried out using a culture method (for example *2.6.7*, using media shown to be suitable for *M. gallisepticum*). The inactivated mycoplasma harvest complies with the test if no live mycoplasmas are detected.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out on a batch, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the potency test (section 3-4). The following test may be used.

Use not fewer than 15 chickens, 3-4 weeks old, from an SPF flock (*5.2.2*) or not fewer than 15 turkeys, 3-4 weeks old, that have not been vaccinated against *M. gallisepticum*, do not have antibodies against *M. gallisepticum*, and are obtained from a healthy flock. Collect serum samples from each vaccinate and control bird just before vaccination and check for the absence of antibodies against *M. gallisepticum*. Administer to each of not fewer than 10 birds 1 dose of the vaccine by a recommended route. Maintain not fewer than 5 birds as controls. Collect serum samples 5 weeks after vaccination from each vaccinated and control bird. Measure the titres of serum antibodies against *M. gallisepticum* using a suitable method. Calculate the mean titres for the group of vaccinates. The test is not valid if specific *M. gallisepticum* antibodies are found in any serum samples from the control birds 5 weeks after the time of administration of the vaccine. The vaccine complies with the test if the mean antibody titres of the group of vaccinates are equal to or greater than the titres obtained with a batch that has given satisfactory results in the potency test (section 3-4).

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live mycoplasmas**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a validated test for residual live mycoplasmas to confirm inactivation of *M. gallisepticum* using a culture method (for example *2.6.7*, using media shown to be suitable for *M. gallisepticum*). The vaccine complies with the test if no live mycoplasmas are detected.

3-4. **Potency**. The vaccine complies with the test for immunogenicity (section 2-2-2).

**04/2013:1943**

# MYXOMATOSIS VACCINE (LIVE) FOR RABBITS

## Vaccinum myxomatosidis vivum ad cuniculum

1. DEFINITION

Myxomatosis vaccine (live) for rabbits is a preparation of a suitable strain of either myxoma virus that is attenuated for rabbits or Shope fibroma virus. This monograph applies to vaccines intended for the active immunisation of rabbits against myxomatosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The viral suspension is harvested and titrated and may be mixed with a suitable stabilising solution.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the rabbits for which it is intended.

The following tests for safety (section 2-3-1), increase in virulence (section 2-3-2) and immunogenicity (2-3-3) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination, using in each case rabbits of the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test, use not fewer than 8 rabbits that do not have antibodies against myxoma virus. Administer to each rabbit a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the rabbits at least daily for 28 days. Record the body temperature the day before vaccination, at vaccination, 4 h after vaccination and then daily for 4 days; note the maximum temperature increase for each rabbit.

The vaccine virus complies with the test if no rabbit shows notable signs of disease or dies from causes attributable to the vaccine virus; the average temperature increase does not exceed 1.0 °C and no rabbit shows a rise greater than 2.0 °C. A local reaction lasting less than 28 days may occur.

2-3-2. **Increase in virulence**. (*This test is performed only for vaccines based on attenuated strains of myxoma virus*). Carry out the test according to general chapter *5.2.6. Evaluation of safety of veterinary vaccines and immunosera*, using rabbits 5-7 weeks old that do not have antibodies against myxoma virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each rabbit by a route to be recommended a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Administer the virus by the route to be recommended for vaccination most likely to lead to reversion of virulence. Euthanise the rabbits 5-10 days after inoculation and remove from each rabbit organs or tissues with sufficient virus to allow passage; homogenise the organs and tissues in a suitable buffer solution, centrifuge the suspension and use the supernatant for further passages. Inoculate the supernatant into suitable cell culture to verify the presence of virus. Administer by an appropriate route, at a suitable rate, a suitable volume of the supernatant to each rabbit of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

If the 5$^{th}$ group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1$^{st}$ passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increased virulence of the virus recovered for the final passage compared with the material used for the 1$^{st}$ passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals , the vaccine virus also complies with the test.

2-3-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination using in each case rabbits of the minimum age to be recommended. The quantity of vaccine virus to be administered to each rabbit is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 15 rabbits that do not have antibodies against myxoma virus and are reared in suitable isolation conditions to ensure absence of contact with myxoma virus. Administer 1 dose of vaccine to each of not fewer than 10 of the rabbits according to the schedule to be recommended. Maintain not fewer than 5 rabbits as controls. Challenge each rabbit not less than 21 days after the last vaccination by a suitable route with a quantity of a virulent strain of myxoma virus sufficient to cause typical signs of myxomatosis in a rabbit. Observe the rabbits at least daily for a further 21 days after challenge and monitor each of them.

The test is not valid if fewer than 90 per cent of the control rabbits display typical signs of myxomatosis. A vaccine containing myxoma virus complies with the test if, during the observation period after challenge, not fewer than 90 per cent of vaccinated rabbits show no signs of myxomatosis. A vaccine containing Shope fibroma virus complies with the test if, during the observation period after challenge, not fewer than 75 per cent of vaccinated rabbits show no signs of myxomatosis.

3. BATCH TESTS

3-1. **Identification**. Carry out an immunofluorescence test in suitable cell cultures, using a monospecific antiserum.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Specified extraneous agents**. Use not fewer than 2 rabbits that are not older than the minimum age recommended for vaccination, that do not have antibodies against myxoma virus and rabbit haemorrhagic disease virus and that have been reared in suitable isolation conditions to avoid contact with myxoma virus. Administer to each rabbit by a recommended route 10 doses of the vaccine. Observe the rabbits at least daily for 14 days. At the end of the observation period administer by a recommended route to each rabbit a further 10 doses of vaccine. After 14 days take a blood sample from

each rabbit and carry out a test for antibodies against rabbit haemorrhagic disease virus. The vaccine complies with the test if no antibodies are found.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3.) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**01/2017:0962**

# NEONATAL PIGLET COLIBACILLOSIS VACCINE (INACTIVATED)

## Vaccinum colibacillosis fetus a partu recentis inactivatum ad suem

1. DEFINITION

Neonatal piglet colibacillosis vaccine (inactivated) is a preparation from cultures of one or more suitable strains of *Escherichia coli*, carrying one or more adhesins or enterotoxins. This monograph applies to vaccines intended for the active immunisation of sows and gilts for passive protection of their newborn progeny against enteric forms of colibacillosis, administered by injection.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The *E. coli* strains used for production are cultured separately in a suitable medium. The cells or toxins are processed to render them safe while maintaining adequate immunogenic properties and are blended. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The *E. coli* strains used in the production of the vaccine are shown to be satisfactory with respect to expression of antigens and the vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the sows and gilts for which it is intended.

The following tests for expression of antigens (section 2-2-1), safety (section 2-2-2) and immunogenicity (section 2-2-3) may be used during the demonstration of safety and efficacy.

2-2-1. **Expression of antigens**. The expression of antigens that stimulate a protective immune response is verified by a suitable immunochemical method (*2.7.1*) carried out on the antigen obtained from each of the vaccine strains under the conditions used for the production of the vaccine.

2-2-2. **Safety**

2-2-2-1. *Safety in pregnant sows*. Carry out the test for each route and method of administration to be recommended for vaccination and in pregnant sows. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 pregnant sows per group that have not been vaccinated against colibacillosis, at the relevant stages of pregnancy in accordance with the schedule to be recommended or at different stages of pregnancy. Administer to each sow 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the sows at least daily until farrowing. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each sow.

The vaccine complies with the test if:

- no sow shows abnormal local or systemic reactions or dies from causes attributable to the vaccine;
- the average temperature increase for all sows does not exceed 1.5 °C and no sow shows a rise greater than 2.0 °C; and
- no adverse effects on gestation or the offspring are noted.

2-2-2-2. *Field studies*. The pigs used for field trials are also used to evaluate safety. Use not fewer than 3 groups each of not fewer than 20 pigs with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test 2-2-2-1, and daily during the 2 days following vaccination; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine, the average temperature increase for all pigs does not exceed 1.5 °C, and no pig shows a rise greater than 2.0 °C.

2-2-3. **Immunogenicity**. Carry out the test with a challenge strain representing each type of antigen against which the vaccine is intended to protect: if a single strain with all the necessary antigens is not available, repeat the test using different challenge strains.

Each test is carried out for each route and method of administration to be recommended for vaccination. The vaccine administered to each gilt is of minimum potency.

Use not fewer than 8 gilts susceptible to *E. coli* infections and that do not have antibodies against the antigens to be stated on the label. Take not fewer than 4 at random and vaccinate these at the stage of pregnancy and according to the schedule to be recommended. Maintain not fewer than 4 gilts as controls. Within 12 h of their giving birth, take not fewer than 15 healthy piglets from the vaccinated gilts and 15 healthy piglets from the controls, taking at least 3 from each litter. Challenge each piglet by the oral route with a sufficient quantity of a virulent strain of *E. coli* before or after colostrum feeding and using the same conditions for vaccinated piglets and controls. The strain used must not be one used in the manufacture of the vaccine. Return the piglets to their dam and observe at least daily for 8 days.

On each day, note signs in each piglet and score using the following scale.

0 no signs

1 slight diarrhoea

2 marked diarrhoea (watery faeces)

3 dead

Calculate total scores for each piglet over 8 days.

The test is not valid if fewer than 40 per cent of the piglets from the control gilts die and more than 15 per cent of the piglets from the control gilts show no signs of illness. The vaccine complies with the test if there is a significant reduction in score in the group of piglets from the vaccinated gilts compared with the group from the unvaccinated controls.

For some adhesins (for example, F5 and F41), there is published evidence that high mortality cannot be achieved under experimental conditions. If challenge has to be carried out with a strain having such adhesins: the test is not valid if fewer than 70 per cent of the control piglets show signs expected with the challenge strain; the vaccine complies with the test if there is a significant reduction in score in the group of piglets from the vaccinated gilts compared with the group from the unvaccinated controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use 7 pigs not less than 3 weeks old and that do not have antibodies against the antigens stated on the label. Vaccinate each of 5 pigs by the recommended route and according to the recommended schedule. Maintain 2 pigs as controls. Alternatively, if the nature of the antigens allows reproducible results to be obtained, a test in laboratory animals (for example, guinea-pigs, mice, rabbits or rats) may be carried out. To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose, carry out the test as follows. Vaccinate not fewer than 5 animals with a single injection of a suitable dose. Maintain not fewer than 2 animals as controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay (*2.7.1*) to measure the antibody response to each of the antigens stated on the label. The vaccine complies with the test if the antibody levels in the vaccinates are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

Where animals that do not have antibodies against the antigens stated on the label are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of possible prevaccination titres and establish the acceptable minimum titre rise after vaccination in relation to these.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test 2-2-2-1 given under Choice of vaccine composition. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-3) when administered by a recommended route and method.

**01/2017:0961**

# NEONATAL RUMINANT COLIBACILLOSIS VACCINE (INACTIVATED)

## Vaccinum colibacillosis fetus a partu recentis inactivatum ad ruminantes

1. DEFINITION

Neonatal ruminant colibacillosis vaccine (inactivated) is a preparation from cultures of one or more suitable strains of *Escherichia coli*, carrying one or more adhesin factors or enterotoxins. This monograph applies to vaccines intended for the active immunisation of dams for passive protection of their newborn progeny against enteric forms of colibacillosis, administered by injection.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The *E. coli* strains used for production are cultured separately in a suitable medium. The cells or toxins are processed to render them safe while maintaining adequate immunogenic properties and are blended. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The *E. coli* strains used in the production of the vaccine are shown to be satisfactory with respect to expression of antigens and the vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the ruminants for which it is intended.

The following tests for expression of antigens (section 2-2-1), safety (section 2-2-2) and immunogenicity (section 2-2-3) may be used during the demonstration of safety and efficacy.

2-2-1. **Expression of antigens**. The expression of antigens that stimulate a protective immune response is verified by a suitable immunochemical method (*2.7.1*) carried out on the antigen obtained from each of the vaccine strains under the conditions used for the production of the vaccine.

2-2-2. **Safety**

2-2-2-1. *Safety in pregnant animals*. Carry out the test for each route and method of administration to be recommended for vaccination and in pregnant animals of each species for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 pregnant animals per group that have not been vaccinated against colibacillosis. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until parturition. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each animal.

The vaccine complies with the test if:

- no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine;
- the average temperature increase for all animals does not exceed 1.5 °C and no animal shows a rise greater than 2.0 °C; and
- no adverse effects on gestation or the offspring are noted.

2-2-2-2. *Field studies*. Safety is demonstrated in field trials for each species for which the vaccine is intended. Administer the dose to be recommended to not fewer than 60 animals from 3 different stocks by the route and according to the schedule to

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be recommended. Assign not fewer than 30 animals from the same stocks to control groups. Observe the animals at least daily for 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no rise in temperature of more than 1.5 °C occurs within 2 days of administration of each dose of the vaccine.

2-2-3. **Immunogenicity**. Carry out the test with a challenge strain representing each type of antigen against which the vaccine is intended to protect: if a single strain with all the necessary antigens is not available, repeat the test using different challenge strains. Each test is carried out for each route and method of administration to be recommended for vaccination, using in each case animals of each species for which the vaccine is intended. The vaccine administered to each animal is of minimum potency.

For each test, use not fewer than 15 animals that do not have antibodies against the antigens to be stated on the label. Take not fewer than 10 at random and vaccinate these at the stage of pregnancy and according to the schedule to be recommended. Maintain not fewer than 5 animals as controls. Collect colostrum from all animals after parturition and store the samples individually in conditions that maintain antibody levels. Take not fewer than 15 newborn unsuckled animals and house them in an environment ensuring absence of enteric pathogens. Allocate a colostrum sample from not fewer than 10 vaccinated dams and not fewer than 5 controls to the offspring. After birth, feed the animals with the colostrum sample allocated to it. After feeding the colostrum and within 12 h of birth, challenge all the animals by the oral route with a sufficient quantity of a virulent strain of *E. coli* and observe at least daily for 10 days. The strain must not be one used in the manufacture of the vaccine.

On each day, note daily signs in each animal and score using the following scale.

0 no signs

1 slight diarrhoea

2 marked diarrhoea (watery faeces)

3 dead

Calculate total scores for each animal over 10 days.

The test is not valid if fewer than 80 per cent of the animals given colostrum from the controls die or show severe signs of disease. The vaccine complies with the test if there is a significant reduction in score in the group of animals given colostrum from vaccinated dams compared with the group given colostrum from the unvaccinated controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different dose. For each dose required, carry out the test as follows. Use not fewer than 7 animals (for example rabbits, guinea-pigs, rats or mice) that do not have antibodies against the antigens stated on the label. Vaccinate not fewer than 5 animals, using 1 injection of a suitable dose. Maintain 2 animals as controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14-21 days after the last injection, collect blood from each animal and prepare serum samples. Use a suitable validated test such as an enzyme-linked immunosorbent assay (*2.7.1*) to measure the antibody response to each of the protective antigens stated on the label. The vaccine complies with the test if the antibody levels in the vaccinates are not significantly less than those obtained with a batch that has given satisfactory results in the test described under Potency and there is no significant increase in antibody titre in the controls.

Where animals that do not have antibodies against the antigens stated on the label are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of possible prevaccination titres and establish the acceptable minimum titre rise after vaccination in relation to these.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test 2-2-2-1 given under Choice of vaccine composition. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxins is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-3) when administered by a recommended route and method.

**01/2017:0870**

# NEWCASTLE DISEASE VACCINE (INACTIVATED)

## Vaccinum pseudopestis aviariae inactivatum

1. DEFINITION

Newcastle disease vaccine (inactivated) (also known as avian paramyxovirus 1 vaccine (inactivated) for vaccines intended for some species) is a preparation of a suitable strain of Newcastle disease virus (avian paramyxovirus 1), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of birds against Newcastle disease.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures. The virus harvest is inactivated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from healthy flocks (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the test for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for each species and category of birds for which it is intended.
The following tests for safety (section 2-4-1) and immunogenicity (section 2-4-2) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination and for each avian species for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test performed in birds younger than 3 weeks of age, use not fewer than 10 birds not older than the minimum age to be recommended for vaccination. For each test performed in birds older than 3 weeks of age, use not fewer than 8 birds not older than the minimum age to be recommended for vaccination. In the case of chickens, use chickens from a flock free from specified pathogens (SPF) (*5.2.2*) and if the vaccine is used for species other than chickens, they have not been vaccinated and do not have antibodies against Newcastle disease virus. Administer by a route and method to be recommended to each bird 1 dose of vaccine. Observe the birds at least daily for at least 14 days after the last administration of the vaccine.

The test is not valid if more than 10 per cent of the birds younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For birds older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine complies with the test if no bird shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended; the vaccine administered to each bird is of minimum potency.

For chickens, the test for vaccines for use in chickens (section 2-4-2-1) is suitable for demonstrating immunogenicity. For other species of birds (for example, pigeons or turkeys), the test for vaccines for use in species other than the chicken (section 2-4-2-2) is suitable for demonstrating immunogenicity.

2-4-2-1. *Vaccines for use in chickens.* Use not fewer than 70 chickens, 21-28 days old, of the same origin and from an SPF flock (*5.2.2*). For vaccination, use not fewer than 3 groups, each of not fewer than 20 chickens. Choose a number of different volumes of the vaccine corresponding to the number of groups: for example, volumes equivalent to 1/25, 1/50 and 1/100 of a dose. Allocate a different volume to each vaccination group. Vaccinate each chicken by the intramuscular route with the volume of vaccine allocated to its group. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 17-21 days by the intramuscular route with 6 $\log_{10}$ embryo $LD_{50}$ of the Herts (Weybridge 33/56) strain of avian paramyxovirus 1. Observe the chickens at least daily for 21 days after challenge. At the end of the observation period, calculate the $PD_{50}$ by standard statistical methods from the number of chickens that survive in each vaccinated group without showing any signs of Newcastle disease during the 21 days.

The test is invalid unless all the control birds die within 6 days of challenge.

The vaccine complies with the test if the smallest dose stated on the label corresponds to not less than 50 $PD_{50}$ and the lower confidence limit is not less than 35 $PD_{50}$ per dose. If the lower confidence limit is less than 35 $PD_{50}$ per dose, repeat the test; the vaccine must be shown to contain not less than 50 $PD_{50}$ in the repeat test.

2-4-2-2. *Vaccines for use in species other than the chicken.* Use not fewer than 30 birds of the target species, of the same origin and of the same age, that do not have antibodies against avian paramyxovirus 1. Vaccinate in accordance with the recommendations for use not fewer than 20 birds. Maintain not fewer than 10 birds as controls. Challenge each bird after 4 weeks by the intramuscular route with a sufficient quantity of virulent avian paramyxovirus 1.

The test is not valid if serum samples obtained at the time of the first vaccination show the presence of antibodies against avian paramyxovirus 1 in either vaccinates or controls, or if tests carried out at the time of challenge show such antibodies in controls.

The test is not valid if fewer than 70 per cent of the control birds die or show serious signs of Newcastle disease.

The vaccine complies with the test if not fewer than 90 per cent of the vaccinated birds survive and show no serious signs of avian paramyxovirus 1 infection.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Residual live virus**. The test is carried out in embryonated eggs or suitable cell cultures (*5.2.4*), whichever is the most sensitive for the vaccine strain. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-5-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following tests may be used. Wherever possible, carry out the test for antigen content (section 2-5-2-1) together with the test for adjuvant (section 2-5-2-2).

*Vaccines for use in chickens.* The test for antigen content (section 2-5-2-1) together with the test for adjuvant (section 2-5-2-2) may be carried out; if the nature of the product does not allow valid results to be obtained with these tests, or if the vaccine does not comply, the test for serological assay (section 2-5-2-3) may be carried out. If the vaccine does not comply with the latter test, the test for vaccines for use in chickens (section 2-4-2-1) may be carried out. A test using fewer than 20 birds per group and a shorter observation period after challenge may be used if this has been shown to give a valid potency test.

*Vaccines for use in species other than the chicken.* Carry out a suitable test for which a satisfactory correlation has been established with the test for vaccines for use in species other than the chicken (section 2-4-2-2), the criteria for acceptance being set with reference to a batch that has given satisfactory results in the latter test. A test in chickens from an SPF flock (*5.2.2*) consisting of a measure of the serological response to graded amounts of vaccine (for example, 1/25, 1/50 and 1/100 of a dose with serum sampling 17-21 days later) may be used. Alternatively, the test for antigen content (section 2-5-2-1) together with the test for adjuvant (section 2-5-2-2) may be conducted if shown to provide a valid potency test.

2-5-2-1. *Antigen content*. The relative antigen content is determined by comparing the content of haemagglutinin-neuraminidase antigen per dose of vaccine with a haemagglutinin-neuraminidase antigen reference preparation, by enzyme-linked immunosorbent assay (*2.7.1*). For this comparison, *Newcastle disease virus reference antigen BRP*, *Newcastle disease virus control antigen BRP*, *Newcastle disease virus coating antibody BRP* and *Newcastle disease virus conjugated detection antibody BRP* are suitable. Before estimation, the antigen may be extracted from the emulsion using *isopropyl myristate R* or another suitable method. The vaccine complies with the test if the estimated antigen content is not significantly lower than that of a batch that has been found to be satisfactory with respect to immunogenicity (section 2-4-2).

2-5-2-2. *Adjuvant*. If the immunochemical assay (section 2-5-2-1) is performed and if the vaccine is adjuvanted, the adjuvant is tested by suitable physical and chemical methods. For oil-adjuvanted vaccines, the adjuvant is tested in accordance with the monograph *Vaccines for veterinary use (0062)*. If the adjuvant cannot be adequately characterised, the antigen content determination cannot be used as the batch potency test.

2-5-2-3. *Serological assay*. Use not fewer than 15 chickens, 21-28 days old, of the same origin and from an SPF flock (*5.2.2*). Vaccinate by the intramuscular route not fewer than 10 chickens with a volume of the vaccine equivalent to 1/50 of a dose. Maintain not fewer than 5 chickens as controls. Collect serum samples from each chicken after 17-21 days. Measure the antibody levels in the sera by the haemagglutination-inhibition (HI) test using the technique described below or an equivalent technique with the same numbers of haemagglutinating units and red blood cells. The test system used must include negative and positive control sera, the latter having an HI titre of 5.0 $\log_2$ to 6.0 $\log_2$. The vaccine complies with the test if the mean HI titre of the vaccinated group is equal to or greater than 4.0 $\log_2$ and that of the unvaccinated group is 2.0 $\log_2$ or less. If the HI titres are not satisfactory, carry out the test for vaccines for use in chickens (section 2-4-2-1).

**Haemagglutination inhibition**. Inactivate the test sera by heating at 56 °C for 30 min. Add 25 µL of inactivated serum to the first row of wells in a microtitre plate. Add 25 µL of a buffered 9 g/L solution of *sodium chloride R* at pH 7.2-7.4 to the rest of the wells. Prepare twofold dilutions of the sera across the plate. To each well add 25 µL of a suspension containing 4 haemagglutinating units of inactivated Newcastle disease virus. Incubate the plate at 4 °C for 1 h. Add 25 µL of a 1 per cent *V/V* suspension of red blood cells collected from chickens that are 3-4 weeks old and free from antibodies against Newcastle disease virus. Incubate the plate at 4 °C for 1 h. The HI titre is equal to the highest dilution that produces complete inhibition.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

A test for residual live virus is carried out to confirm inactivation of Newcastle disease virus.

Inject 2/5 of a dose into the allantoic cavity of each of 10 embryonated hen eggs that are 9-11 days old and from SPF flocks (*5.2.2*) (SPF eggs), and incubate. Observe for 6 days and pool separately the allantoic fluid from eggs containing live embryos and that from eggs containing dead embryos, excluding those dying within 24 h of the injection. Examine embryos that die within 24 h of injection for the presence of Newcastle disease virus: the vaccine does not comply with the test if Newcastle disease virus is found.

Inject into the allantoic cavity of each of 10 SPF eggs, 9-11 days old, 0.2 mL of the pooled allantoic fluid from the live embryos and, into each of 10 similar eggs, 0.2 mL of the pooled fluid from the dead embryos and incubate for 5-6 days. Test the allantoic fluid from each egg for the presence of haemagglutinins using chicken erythrocytes.

The vaccine complies with the test if there is no evidence of haemagglutinating activity and if not more than 20 per cent of the embryos die at either stage. If more than 20 per cent of the embryos die at one of the stages, repeat that stage; the vaccine complies with the test if there is no evidence of haemagglutinating activity and not more than 20 per cent of the embryos die at that stage.

Antibiotics may be used in the test to control extraneous bacterial infection.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-4-2) when administered by a recommended route and method.

**04/2013:0450**

# NEWCASTLE DISEASE VACCINE (LIVE)

## Vaccinum pseudopestis aviariae vivum

1. DEFINITION

Newcastle disease vaccine (live) is a preparation of a suitable strain of Newcastle disease virus (avian paramyxovirus 1). This monograph applies to vaccines intended for administration to chickens and/or other avian species for active immunisation.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from flocks free from specified pathogens (SPF) (*5.2.2*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the tests.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended.

The following tests for intracerebral pathogenicity index (section 2-4-1), amino-acid sequence (section 2-4-2), safety (section 2-4-3), increase in virulence (section 2-4-4) and immunogenicity (section 2-4-5) may be used during the demonstration of safety and efficacy.

2-4-1. **Intracerebral pathogenicity index**. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Inoculate the vaccine virus into the allantoic cavity of embryonated hens'

eggs, 9- to 11- days-old, from an SPF flock (*5.2.2*). Incubate the inoculated eggs for a suitable period and harvest and pool the allantoic fluids. Use not fewer than ten 1-day-old chickens (i.e. more than 24 h but less than 40 h after hatching), from an SPF flock (*5.2.2*). Administer by the intracerebral route to each chick 0.05 mL of the pooled allantoic fluids containing not less than $10^{8.0}$ $EID_{50}$ or, if this virus quantity cannot be achieved, not less than $10^{7.0}$ $EID_{50}$. Observe the chickens at least daily for 8 days after administration and score them once every 24 h. A score of 0 is attributed to a chicken if it is clinically normal, 1 if it shows clinical signs of disease and 2 if it is dead. The intracerebral pathogenicity index is the mean of the scores per chicken per observation over the 8-day period.

If an inoculum of not less than $10^{8.0}$ $EID_{50}$ is used, the vaccine virus complies with the test if its intracerebral pathogenicity index is not greater than 0.5; if an inoculum of not less than $10^{7.0}$ $EID_{50}$ but less than $10^{8.0}$ $EID_{50}$ is used, the vaccine virus complies with the test if its intracerebral pathogenicity index is not greater than 0.4.

2-4-2. **Amino-acid sequence**. Determine the sequence of a fragment of RNA from the vaccine virus containing the region encoding for the F0 cleavage site by a suitable method. The encoded amino-acid sequence is shown to be one of the following:

| | | F2 | | | | | | Cleavage site | F1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Site | | 111 | 112 | 113 | 114 | 115 | 116 | ∨ | 117 | 118 | 119 |
| | | Gly | Gly | Lys | Gln | Gly | Arg | | Leu | Ile | Gly |
| | or | Gly | Gly | Arg | Gln | Gly | Arg | | Leu | Ile | Gly |
| | or | Gly | Glu | Arg | Gln | Glu | Arg | | Leu | Val | Gly |

or equivalent with leucine at 117 and no basic amino acids at sites 111, 112, 114 and 115.

2-4-3. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and in each avian species for which the vaccine is intended, using in each case birds not older than the minimum age to be recommended for vaccination. If the test is performed in chickens, use chickens from an SPF flock (*5.2.2*). If the test is performed in birds other than chickens, use birds that do not have antibodies against Newcastle disease virus. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in birds younger than 3 weeks of age, use not fewer than 10 birds. For each test performed in birds older than 3 weeks of age, use not fewer than 8 birds. Administer to each bird a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the birds at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the birds younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine virus. For birds older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no bird shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-4-4. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using birds not more than 2 weeks old. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out. Carry out the test in a target species, using the chicken if it is one of the target species. For the test in chickens, use chickens from an SPF flock (*5.2.2*). For other species, carry out the test in birds that do not have antibodies against Newcastle disease virus. Administer to each bird of the 1st group by eye-drop a quantity of the vaccine virus that will allow recovery of virus for the passages described below. Observe the birds for the period shown to correspond to maximum replication of the vaccine virus, euthanise them and prepare a suspension from the brain of each bird and from a suitable organ depending on the tropism of the strain (for example, mucosa of the entire trachea, intestine, pancreas); pool the samples. Administer 0.05 mL of the pooled samples by eye-drop to each bird of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 birds.

A. Carry out the test for intracerebral pathogenicity index (section 2-4-1) using the material used for the 1st passage and the virus at the final passage level.

B. Carry out the test for amino-acid sequence (section 2-4-2) using unpassaged vaccine virus the material used for the 1st passage and the virus at the final passage level.

C. Carry out the test for safety (section 2-4-3) using the material used for the 1st passage and the virus at the final passage level.

   Administer the virus by the route to be recommended for vaccination likely to be the least safe and to the avian species for which the vaccine is intended that is likely to be the most susceptible to Newcastle disease.

The vaccine virus complies with the test if, in the tests 2-4-4A, 2-4-4B and 2-4-4C, no indication of increase in virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 5 birds and a subsequent repeat passage in 10 birds, the vaccine virus also complies with the test.

2-4-5. **Immunogenicity**. For each avian species for which the vaccine is intended, a test is carried out for each route and method of administration to be recommended using in each case birds not older than the minimum age to be recommended for vaccination. The quantity of the vaccine virus administered to each bird is not greater than the minimum titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

2-4-5-1. *Vaccines for use in chickens*. Use not fewer than 30 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate by a route to be recommended not fewer than 20 chickens. Maintain not fewer than 10 chickens as controls. Challenge each chicken after 21 days by the intramuscular route with not less than $10^{5.0}$ embryo $LD_{50}$ of the Herts (Weybridge 33/56) strain of Newcastle disease virus. Observe the chickens at least daily for 14 days after challenge. Record the deaths and the number of surviving chickens that show clinical signs of disease.

The test is not valid if 6 days after challenge fewer than 100 per cent of the control chickens have died or if during the period between vaccination and challenge more than 10 per cent of the vaccinated or control chickens show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated chickens survive and show no notable clinical signs of Newcastle disease.

2-4-5-2. *Vaccines for use in avian species other than the chicken*. Use not fewer than 30 birds of the species for which the vaccine is intended for Newcastle disease, of the same origin and that do not have antibodies against avian paramyxovirus 1. Vaccinate by a route to be recommended not fewer than 20 birds. Maintain not fewer than 10 birds as controls. Challenge each bird after 21 days by the intramuscular route with a sufficient quantity of virulent avian paramyxovirus 1. Observe the birds at least daily for 21 days after challenge. Record the deaths and the surviving birds that show clinical signs of disease.

The test is not valid if:

- during the observation period after challenge fewer than 90 per cent of the control birds die or show severe clinical signs of Newcastle disease;
- or if during the period between the vaccination and challenge more than 10 per cent of the vaccinated or control birds show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated birds survive and show no notable clinical signs of Newcastle disease. For species where there is published evidence that it is not possible to achieve this level of protection, the vaccine complies with the test if there is a significant reduction in morbidity and mortality of the vaccinated birds compared with the control birds.

3. BATCH TESTS

3-1. **Identification**

3-1-1. *Identification of the vaccine virus.* The vaccine, diluted if necessary and mixed with a monospecific Newcastle disease virus antiserum, no longer provokes haemagglutination of chicken red blood cells or infects embryonated hens' eggs from an SPF flock (*5.2.2*) or susceptible cell cultures (*5.2.4*) into which it is inoculated.

3-1-2. *Identification of the virus strain.* The strain of vaccine virus is identified by a suitable method, for example using monoclonal antibodies.

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Frozen or freeze-dried vaccines produced in embryonated eggs and not intended for administration by injection either comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)* or with the following test: carry out a quantitative test for bacterial and fungal contamination; carry out identification tests for micro-organisms detected in the vaccine; the vaccine does not contain pathogenic micro-organisms and contains not more than 1 non-pathogenic micro-organism per dose.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into embryonated hens' eggs from an SPF flock (*5.2.2*) or into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. Depending on the indications, the vaccine complies with 1 or both of the tests prescribed under Immunogenicity (section 2-4-5) when administered according to the recommended schedule by a recommended route and method. If the test in section 2-4-5-2. *Vaccine for use in avian species other than the chicken* is conducted and the vaccine is recommended for use in more than 1 avian species, the test is carried out with birds of that species for which the vaccine is recommended which is likely to be the most susceptible to avian paramyxovirus 1. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.



# PASTEURELLA VACCINE (INACTIVATED) FOR SHEEP

## Vaccinum pasteurellae inactivatum ad ovem

1. DEFINITION

Pasteurella vaccine (inactivated) for sheep is a preparation of one or more suitable strains of *Pasteurella trehalosi*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of sheep against disease caused by *P. trehalosi*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable medium; each strain is cultivated separately and identity is verified using a suitable method. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity of the harvest are verified using suitable methods. After cultivation, the bacterial suspensions are collected separately and inactivated by a suitable method. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The choice of composition and the strains to be included in the vaccine are based on epidemiological data on the prevalence of the different serovars of *P. trehalosi*.

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the sheep for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests.* Carry out the tests for each route and method of administration to be recommended for vaccination and in sheep of each category for which the vaccine is intended (for example, young sheep, pregnant ewes). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 sheep that preferably do not have antibodies against the serovars of *P. trehalosi* or against the leucotoxin present in the vaccine. Where justified, sheep with a known history of no previous pasteurella vaccination and with low antibody titres (measured in a sensitive test system such as ELISA) may be used. Administer to each sheep 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the sheep at least daily for at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each sheep.

The vaccine complies with the test if no sheep shows abnormal local reactions, notable signs of disease or dies from causes attributable to the vaccine, if the average body temperature increase for all sheep does not exceed 1.5 °C and no sheep shows a rise greater than 2.0 °C.

2-2-1-2. *Field studies.* The sheep used for the field trials are also used to evaluate safety. Carry out a test in each category of sheep for which the vaccine is intended. Use not fewer than 3 groups of 20 sheep with corresponding groups of not fewer than 10 controls in 3 different locations. Examine the

injection sites for local reactions after vaccination. Record body temperatures the day before vaccination, at vaccination and on the 2 days following vaccination.

The vaccine complies with the test if no sheep shows abnormal local or systemic reactions, notable signs of disease or dies from causes attributable to the vaccine. The average body temperature increase for all sheep does not exceed 1.5 °C and no sheep shows a rise greater than 2.0 °C. In addition, if the vaccine is intended for use in pregnant ewes, no adverse effects on the pregnancy and offspring are demonstrated.

2-2-2. **Immunogenicity**. Carry out a test for each serovar of *P. trehalosi* for which protection is to be claimed on the label.

A test is carried out for each route and method of administration to be recommended, using in each case lambs of the minimum age to be recommended for vaccination. The vaccine administered to each lamb is of minimum potency.

Use not fewer than 20 lambs that do not have antibodies against *P. trehalosi* and against the leucotoxin of *P. trehalosi*. Vaccinate not fewer than 10 lambs according to the schedule to be recommended. Maintain not fewer than 10 lambs as controls. 20-22 days after the last vaccination, challenge each lamb by the subcutaneous or another suitable route, with a sufficient quantity of a low-passage, virulent strain of a serovar of *P. trehalosi*. Observe the lambs for a further 7 days; to avoid unnecessary suffering, severely ill lambs are euthanised and are then considered to have died from the disease. During the observation period, examine the lambs for any signs of disease (for example, severe dullness, excess salivation) and record the mortality. Euthanise surviving lambs at the end of the observation period. Carry out post-mortem examination on any lamb that dies and those euthanised at the end of the observation period. Examine the lungs, pleura, liver and spleen for haemorrhages and evaluate the extent of lung consolidation due to pasteurellosis. Collect samples of lung, liver and spleen tissue for re-isolation of the challenge organisms. Score the mortality, clinical observations and the post-mortem lesions and compare the results obtained for these parameters and the bacterial re-isolation results for the 2 groups.

The test is not valid if signs or lesions of *P. trehalosi* infection occur in less than 70 per cent of the control lambs. The vaccine complies with the test if there is a significant difference between the scores obtained for the clinical and post-mortem observations in the vaccinates compared to the controls. For vaccines with a claim for a beneficial effect on the extent of infection against the serovar, the results for the infection rates are also significantly better for the vaccinates compared to the controls.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final lot or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety tests 2-2-1-1 given under Choice of vaccine composition. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:1360**

# PORCINE ACTINOBACILLOSIS VACCINE (INACTIVATED)

## Vaccinum actinobacillosidis inactivatum ad suem

1. DEFINITION

Porcine actinobacillosis vaccine (inactivated) is a preparation which has one or more of the following components: inactivated *Actinobacillus pleuropneumoniae* of a suitable strain or strains; toxins, proteins or polysaccharides derived from suitable strains of *A. pleuropneumoniae*, and treated to render them harmless while maintaining adequate immunogenic properties; fractions of toxins derived from suitable strains of *A. pleuropneumoniae* and treated if necessary to render them harmless while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of pigs against actinobacillosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate, protein content and quantity of relevant antigens are monitored by suitable methods; the values are within the limits approved for the particular product. Purity and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvests are collected separately and inactivated by a suitable method. They may be detoxified, purified and concentrated. The vaccine may be adjuvanted.

2-2. *CHOICE OF VACCINE COMPOSITION*

The choice of strains is based on epidemiological data. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Carry out the test for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended, using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 pigs that do not have antibodies against the serotypes of *A. pleuropneumoniae* or its toxins present in the vaccine. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until 14 days after the

last administration. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine, and if the average temperature increase for all pigs does not exceed 1.5 °C and no pig shows a rise greater than 2.0 °C.

2-2-1-2. *Field studies*. The pigs used for field trials are also used to evaluate safety. Carry out a test in each category of pigs for which the vaccine is intended. Use not fewer than 3 groups each of not fewer than 20 pigs with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test 2-2-1-1, and daily during the 2 days following vaccination; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine, and if the average temperature increase for all pigs does not exceed 1.5 °C and no pig shows a rise greater than 2.0 °C.

2-2-2. **Immunogenicity**

The challenge strain for the following test is chosen to ensure challenge with each Ap toxin[(1)] produced by the serotypes to be stated on the label; it may be necessary to carry out more than one test using a different challenge strain for each test.

Each test is carried out for each route and method of administration to be recommended. The vaccine administered to each pig is of minimum potency.

For each test, use not fewer than 14 pigs that do not have antibodies against *A. pleuropneumoniae* and Ap toxins. Vaccinate not fewer than 7 pigs according to the schedule to be recommended. Maintain not fewer than 7 pigs as controls. 3 weeks after the last vaccination, challenge all the pigs by the intranasal or intratracheal route or by aerosol with a sufficient quantity of a virulent serotype of *A. pleuropneumoniae*. Observe the pigs at least daily for 7 days; to avoid unnecessary suffering, severely ill control pigs are euthanised and are then considered to have died from the disease. Euthanise all surviving pigs at the end of the observation period. Carry out a post-mortem examination on all pigs. Examine the lungs, the tracheobronchial lymph nodes and the tonsils for the presence of *A. pleuropneumoniae*. Evaluate the extent of lung lesions at post-mortem examination. Each of the 7 lobes of the lungs is allotted a maximum possible lesion score[(2)] of 5. The area showing pneumonia and/or pleuritis of each lobe is assessed and expressed on a scale of 0 to 5 to give the pneumonic score per lobe (the maximum total score possible for each complete lung is 35). Calculate separately for the vaccinated and the control pigs the total score (the maximum score per group is 245, if 7 pigs are used per group).

The vaccine complies with the test if the vaccinated pigs, when compared with controls, show lower incidence of: mortality; typical signs (dyspnoea, coughing and vomiting); typical lung lesions; re-isolation of *A. pleuropneumoniae* from the lungs, the tracheobronchial lymph nodes and the tonsils. Where possible, the incidence is analysed statistically and shown to be significantly lower for vaccinates.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use 5 mice weighing 18-20 g and that do not have antibodies against the serotypes of *A. pleuropneumoniae* or its toxins present in the vaccine. Vaccinate each mouse by the subcutaneous route with a suitable dose. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. Before the vaccination and at a given interval within the range of 14-21 days after the last injection, collect blood from each mouse and prepare serum samples. Determine individually for each serum the titre of specific antibodies against each antigenic component stated on the label, using a suitable validated test such as enzyme-linked immunosorbent assay (*2.7.1*). The vaccine complies with the test if the antibody levels are not significantly lower than those obtained for a batch that has given satisfactory results in the test described under Potency.

2-3-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the final bulk or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine that has been shown satisfactory in safety test 2-2-1-1 described under Choice of vaccine composition or in the residual toxicity test described under Batch tests, carried out using 10 pigs. Where the latter test is used, note the maximum temperature increase for each animal; the vaccine complies with the test if the average temperature increase for all animals does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for batch testing.

3. BATCH TESTS

3-1. **Identification**. When injected into healthy animals that do not have specific antibodies against the antigenic components of *A. pleuropneumoniae* stated on the label, the vaccine stimulates the production of such antibodies.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Use 2 pigs of the minimum age recommended for vaccination and that do not have antibodies against the serotypes of *A. pleuropneumoniae* or its toxins present in the vaccine. Administer to each pig by a recommended route a double dose of the vaccine. Observe the pigs at least daily for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days.

It is recommended to use the mean temperature of the days before administration of the vaccine (e.g. day – 3 to day 0) as the baseline temperature to have clear guidance for evaluation of the test.

The vaccine complies with the test if no pig shows notable signs of disease or dies from causes attributable to the vaccine; a transient temperature increase not exceeding 2.0 °C may occur.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

(1) The nomenclature of the toxins of *A. pleuropneumoniae* is described by J. Frey *et al.*, *Journal of General Microbiology*, 1993, 139, 1723-1728.
(2) The system of lung scores is described in detail by P.C.T. Hannan, B.S. Bhogal, J.P. Fish, *Research in Veterinary Science*, 1982, 33, 76-88.

**01/2017:2448**

# PORCINE ENZOOTIC PNEUMONIA VACCINE (INACTIVATED)

## Vaccinum pneumoniae enzooticae suillae inactivatum

1. DEFINITION

Porcine enzootic pneumonia vaccine (inactivated) is a preparation of a suitable strain of *Mycoplasma hyopneumoniae* that has been inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of pigs against enzootic pneumonia caused by *M. hyopneumoniae*.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

Production of the vaccine is based on a seed-lot system. The seed material is cultured in a suitable solid and/or liquid medium to ensure optimal growth under the chosen incubation conditions. The identity of the strain is verified using a suitable method.

During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity of the harvest is verified using a suitable method.

After cultivation, the mycoplasma suspension is collected and inactivated by a suitable method. The vaccine may contain an adjuvant.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Carry out the test for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended, using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 pigs that do not have antibodies against *M. hyopneumoniae*. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until at least 14 days after the last administration. Record body temperature the day before vaccination, at vaccination, 4 h later and then daily for 4 days; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows notable signs of disease or dies from causes attributable to the vaccine, and, in particular, if the average body temperature increase for all pigs does not exceed 1.5 °C and no pig shows a rise greater than 2.0 °C.

2-2-1-2. *Field studies*. The animals used for field trials are also used to evaluate safety. Carry out a test in each category of animals for which the vaccine is intended. Use not fewer than 3 groups each of not fewer than 20 animals with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test 2-2-1-1, and daily during the 2 days following vaccination; note the maximum temperature increase for each animal.

The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine, the average body temperature increase for all animals does not exceed 1.5 °C, and no animal shows a rise in body temperature greater than 2.0 °C.

2-2-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using in each case pigs not older than the minimum age to be recommended for vaccination. The vaccine to be administered to each pig is of minimum potency.

Use not fewer than 20 pigs that do not have antibodies against *M. hyopneumoniae* and that are from a herd or herds where there are no signs of enzootic pneumonia and that have not been vaccinated against *M. hyopneumoniae*. Vaccinate not fewer than 12 pigs according to the schedule to be recommended. Maintain not fewer than 8 non-vaccinated pigs as controls. Challenge each pig at least 14 days after the last vaccination by the intranasal or intratracheal route or by aerosol with a sufficient quantity of a virulent strain of *M. hyopneumoniae*. The challenge strain used is different from the vaccine strain. 21-30 days after challenge, euthanise the pigs. Conduct a post-mortem examination on each pig in order to evaluate the extent of lung lesions using a validated lung lesion scoring system that is adapted to the age of the animals. The following scoring system may be used.

A weighted score is allocated to each of the 7 lobes of the lungs according to the relative weight of the lung lobes.

| Lobes | Left | Right |
|---|---|---|
| Apical | 5 | 11 |
| Cardiac | 6 | 10 |
| Diaphragmatic | 29 | 34 |
| Intermediate | 5 | |

The vaccine complies with the test if the vaccinated pigs, when compared with controls, show a significant reduction in the lung lesion score.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Residual live mycoplasmas**. A validated test for residual live mycoplasmas is carried out using a culture method (for example *2.6.7*, using media shown to be suitable for *M. hyopneumoniae*). The inactivated mycoplasma harvest complies with the test if no live mycoplasmas are detected.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. A quantification of the antigen (i.e. an *in vitro* test using a reference vaccine that has given satisfactory results in the test described under Potency) together with a test for adjuvant quantification may be used as an alternative method provided the antigen that is measured has been proven to be protective and/or immunorelevant.

Alternatively, a test measuring induction of antibody response in laboratory animals may be used. The following method is given as an example.

Use at least 5 mice weighing 18-20 g and that do not have antibodies against *M. hyopneumoniae*. Vaccinate each mouse by the subcutaneous route with a suitable dose. Maintain not fewer than 5 mice as controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test

system. Before the vaccination and at a given interval within the range of 14-21 days after the last injection, collect blood from each mouse and prepare serum samples. Determine individually for each serum the titre of specific antibodies against each antigenic component stated on the label, using a suitable validated test such as enzyme-linked immunosorbent assay (*2.7.1*).

The vaccine complies with the test if the mean antibody levels are not significantly lower than those obtained for a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live mycoplasmas**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out a validated test for residual live mycoplasmas to confirm inactivation of *M. hyopneumoniae* using a culture method (for example *2.6.7*, using media shown to be suitable for *M. hyopneumoniae*). The vaccine complies with the test if no live mycoplasmas are detected.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**01/2017:0963**

# PORCINE INFLUENZA VACCINE (INACTIVATED)

## Vaccinum influenzae inactivatum ad suem

1. DEFINITION

Porcine influenza vaccine (inactivated) is a preparation of one or more suitable strains of swine or human influenza virus inactivated while maintaining adequate immunogenic properties. Suitable strains contain both haemagglutinin and neuraminidase. This monograph applies to vaccines intended for the active immunisation of pigs against porcine influenza.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in embryonated hens' eggs or in cell cultures. Each virus strain is cultivated separately. After cultivation, the viral suspensions are collected separately and inactivated by a suitable method. If necessary, they may be purified. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Embryonated hens' eggs**. If the vaccine virus is grown in embryonated hens' eggs, they are obtained from a healthy flock (*5.2.13*).

2-2-2. **Cell cultures**. If the vaccine virus is grown in cell cultures, they comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The choice of strains is based on the antigenic types and sub-types observed in Europe. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

2-3-1-1. *Laboratory tests.* Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended (sows, fattening pigs), using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

2-3-1-1-1. General safety. For each test, use not fewer than 8 pigs that do not have antibodies against swine influenza virus. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until 14 days after the last administration.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine during the test.

2-3-1-1-2. Safety in the pigs used in test 2-3-2 for immunogenicity. The pigs used in the test for immunogenicity are also used to evaluate safety. Measure the body temperature of each vaccinated pig at the time of vaccination and 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

– abnormal body temperature;
– other systemic reactions (for example, anorexia);
– abnormal local reactions attributable to the vaccine.

2-3-1-2. *Field studies.* The pigs used for field trials are also used to evaluate safety. Carry out a test in each category of pigs for which the vaccine is intended (sows, fattening pigs). Use not fewer than 3 groups each of not fewer than 20 pigs in at least 2 locations with corresponding groups of not fewer than 10 controls. Measure the body temperature of each vaccinated pig at the time of vaccination and 24 h and 48 h later. Examine the injection site at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

– abnormal body temperature;
– abnormal local reactions attributable to the vaccine.

2-3-2. **Immunogenicity**. The following test carried out using an epidemiologically relevant challenge strain or strains is suitable to demonstrate the immunogenicity of the vaccine. It is carried out for each subtype used in the preparation of the vaccine.

A test is carried out for each route and method of administration to be recommended, using in each case pigs of the minimum age to be recommended for vaccination. The vaccine administered to each pig is of minimum potency.

Use for the test not fewer than 20 pigs that do not have antibodies against swine influenza virus. Vaccinate not fewer than 10 pigs according to the schedule to be recommended. Maintain not fewer than 10 pigs as controls. Take a blood sample from all control pigs immediately before challenge. 3 weeks after the last administration of vaccine, challenge all the pigs by the intratracheal route with a sufficient quantity of a virulent influenza field virus. Euthanise half of the vaccinated and control pigs 24 h after challenge and the other half 72 h after challenge. For each pig, measure the quantity of influenza virus in 2 lung tissue homogenates, one from the left apical, cardiac and diaphragmatic lobes, and the other from the corresponding right lung lobes. Take equivalent samples from each pig.

The test is not valid if antibodies against influenza virus are found in any control pig immediately before challenge. The vaccine complies with the test if, at both times of measurement, the mean virus titre in the pooled lung tissue

samples of vaccinated pigs is significantly lower than that for control pigs, when analysed by a suitable statistical method such as the Wilcoxon Mann-Whitney test.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. An amplification test for residual live virus is carried out on each batch of antigen immediately after inactivation by passage in the same type of substrate as that used for production (eggs or cell cultures) or a substrate shown to be at least as sensitive. The quantity of inactivated virus harvest used in the test is equivalent to not less than 10 doses of the vaccine. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use 5 guinea-pigs, 5-7 weeks old and that do not have antibodies against swine influenza virus. Vaccinate each guinea-pig by the subcutaneous route with a quarter of the recommended dose. Collect blood samples before the vaccination and 21 days after vaccination. Determine for each sample the level of specific antibodies against each virus subtype in the vaccine by haemagglutination-inhibition or another suitable test. The vaccine complies with the test if the level of antibodies is not lower than that found for a batch of vaccine that gave satisfactory results in the potency test in pigs (see Potency).

2-4-3. **Bacterial endotoxins**. For vaccines produced in eggs, the content of bacterial endotoxins is determined on the virus harvest to monitor production.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

3-3-1. *Vaccines prepared in eggs.* If the vaccine has been prepared in eggs, inoculate 0.2 mL into the allantoic cavity of each of 10 fertilised hen eggs, 9-11 days old. Incubate at a suitable temperature for 3 days. The death of any embryo within 24 h of inoculation is considered as non-specific mortality and the egg is discarded. The test is not valid if fewer than 80 per cent of the eggs survive. Collect the allantoic fluid of each egg, pool equal quantities and carry out a 2nd passage on fertilised eggs in the same manner. Incubate for 4 days; the vaccine complies with the test if the allantoic fluid of these eggs shows no haemagglutinating activity.

3-3-2. *Vaccines prepared in cell cultures.* If the vaccine has been prepared in cell cultures, carry out a suitable test for residual live virus using 2 passages in the same type of cell culture as used in the production of vaccine. The vaccine complies with the test if no live virus is detected. If the vaccine contains an oily adjuvant that interferes with this test, where possible separate the aqueous phase from the vaccine by means that do not diminish the capacity to detect residual live virus.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**01/2017:0965**

# PORCINE PARVOVIROSIS VACCINE (INACTIVATED)

## Vaccinum parvovirosis inactivatum ad suem

1. DEFINITION

Porcine parvovirosis vaccine (inactivated) is a preparation of a suitable strain of porcine parvovirus, inactivated while maintaining adequate immunogenic properties, or of a non infectious fraction of the virus. This monograph applies to vaccines intended for the active immunisation of sows and gilts for protection of their progeny against transplacental infection.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The viral suspension is harvested; the virus is inactivated by a suitable method and may be fragmented (inactivation may be by fragmentation); the virus or viral fragments may be purified and concentrated at a suitable stage of the process. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) (including absence of adverse effects on fertility, gestation, farrowing or offspring) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

2-3-1-1. *Laboratory tests.* Carry out the tests for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended, using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

2-3-1-1-1. General safety. For each test, use not fewer than 8 pigs that do not have antibodies against porcine parvovirus or against a fraction of the virus. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until 14 days after the last administration.

The vaccine complies with the test if no pig shows notable signs of disease or dies from causes attributable to the vaccine during the test.

2-3-1-1-2. Safety in pregnant sows. If the vaccine is intended for use in pregnant sows, use for the test not fewer than 8 pregnant sows at the stage or at different stages of pregnancy according to the recommended schedule. Administer to each sow 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the sows at least daily until farrowing.

The vaccine complies with the test if no sow shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if no adverse effects on gestation or the offspring are noted.

2-3-1-1-3. Safety in the pigs used in test 2-3-2 for immunogenicity. The pigs used in the test for immunogenicity are also used to evaluate safety. Measure the body temperature of each vaccinated pig at the time of vaccination 24 h and 48 h later. Examine the injection site after vaccination and at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

– abnormal body temperature;

– other systemic reactions (for example, anorexia);

– abnormal local reactions attributable to the vaccine.

2-3-1-2. *Field studies*. The pigs used for field trials are also used to evaluate safety. Carry out a test in each category of pigs for which the vaccine is intended (sows, gilts). Use not fewer than 3 groups each of not fewer than 20 pigs with corresponding groups of not fewer than 10 controls. Measure the body temperature of each vaccinated pig at the time of vaccination, 24 h and 48 h later. Examine the injection site after vaccination and at slaughter for local reactions.

The vaccine complies with the test if no pig shows:

– abnormal body temperature;

– abnormal local reactions attributable to the vaccine.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended, using in each case gilts of 5-6 months old. The vaccine administered to each gilt is of minimum potency.

Use for the test not fewer than 12 gilts that do not have antibodies against porcine parvovirus or against a fraction of the virus. Vaccinate not fewer than 7 gilts according to the schedule to be recommended. Maintain not less than 5 unvaccinated gilts of the same age as controls. The interval between vaccination and service is that to be recommended. Mate all the gilts on 2 consecutive days immediately following signs of oestrus. At about the 40$^{th}$ day of gestation, challenge each gilt with a suitable quantity of a virulent strain of porcine parvovirus. Euthanise the gilts at about the 90$^{th}$ day of gestation and examine their foetuses for infection with porcine parvovirus as demonstrated by the presence of either virus or antibodies.

The test is not valid if:

– fewer than 7 vaccinated gilts and 5 control gilts are challenged;

– fewer than 90 per cent of piglets from the control gilts are infected;

– and the average number of piglets per litter for the vaccinated gilts is fewer than 6.

The vaccine complies with the test if not fewer than 80 per cent of the total number of piglets from vaccinated gilts are protected from infection.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. A test for residual live virus is carried out on each batch of antigen immediately after inactivation. The quantity of inactivated virus harvest used in the test is equivalent to not less than 100 doses of the vaccine. The bulk harvest is inoculated into suitable non-confluent cells; after incubation for 7 days, a subculture is made using trypsinised cells. After incubation for a further 7 days, the cultures are examined for residual live parvovirus by an immunofluorescence test. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-5) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 5 guinea-pigs, 5-7 weeks old and that do not have antibodies against porcine parvovirus or against a fraction of the virus. Vaccinate each guinea-pig by the subcutaneous route with a quarter of the prescribed dose volume. Take blood samples after the period corresponding to maximum antibody production and carry out tests on the serum for specific antibodies by a haemagglutination-inhibition test or other suitable test. The vaccine complies with the test if the level of antibodies is not lower than that found for a batch of vaccine that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Use a quantity of vaccine equivalent to 10 doses. If the vaccine contains an oily adjuvant, break the emulsion and separate the phases. If the vaccine contains a mineral adjuvant, carry out an elution to liberate the virus. Concentrate the viral suspension 100 times by ultrafiltration or ultracentrifugation. None of the above procedures must be such as to inactivate the virus or otherwise interfere with the detection of live viruses. Carry out a test for residual live virus in suitable non-confluent cells; after incubation for 7 days, make a subculture using trypsinised cells. After incubation for a further 7 days, examine the cultures for residual live parvovirus by an immunofluorescence test. The vaccine complies with the test if no live virus is detected.

3-4. **Specified extraneous agents**. Use 2 pigs that do not have antibodies against porcine parvovirus or against a fraction of the virus, against Aujeszky's disease virus or against pestiviruses. Administer to each pig by a recommended route a double dose of the vaccine, then another dose after 14 days. 14 days after the last administration, carry out tests for antibodies. The vaccine complies with the test if it does not stimulate the formation of antibodies against pestiviruses and against Aujeszky's disease virus.

3-5. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2) when administered by a recommended route and method.

**04/2013:1361**

# PORCINE PROGRESSIVE ATROPHIC RHINITIS VACCINE (INACTIVATED)

## Vaccinum rhinitidis atrophicantis ingravescentis suillae inactivatum

1. DEFINITION

Porcine progressive atrophic rhinitis vaccine (inactivated) is a preparation containing either the dermonecrotic exotoxin of *Pasteurella multocida*, treated to render it harmless while maintaining adequate immunogenic properties, or a genetically modified form of the exotoxin that has adequate immunogenic properties and that is free from toxic properties; the vaccine may also contain cells and/or antigenic

components of one or more suitable strains of *P. multocida* and/or *Bordetella bronchiseptica*. This monograph applies to vaccines intended for the active immunisation of sows and gilts for passive protection of their progeny against porcine progressive atrophic rhinitis.

## 2. PRODUCTION

### 2-1. *PREPARATION OF THE VACCINE*

The bacterial strains used for production are cultured separately in suitable media. The toxins and/or cells are treated to render them safe. The vaccine may be adjuvanted.

### 2-2. *DETOXIFICATION*

A test for detoxification of the dermonecrotic exotoxin of *P. multocida* is carried out immediately after detoxification. The concentration of detoxified exotoxin used in the test is not less than that in the vaccine. The suspension complies with the test if no toxic dermonecrotic exotoxin is detected. The test for detoxification is not required where the vaccine is prepared using a toxin-like protein free from toxic properties, produced by expression of a modified form of the corresponding gene.

### 2-3. *ANTIGEN CONTENT*

The content of the dermonecrotic exotoxin of *P. multocida* in the detoxified suspension or the toxin-like protein in the harvest is determined by a suitable immunochemical method (*2.7.1*), such as an enzyme-linked immunosorbent assay, and the value found is used in the formulation of the vaccine. The content of other antigens stated on the label is also determined (*2.7.1*).

### 2-4. *CHOICE OF VACCINE COMPOSITION*

The strains used for the preparation of the vaccine are shown to be satisfactory with respect to the production of the dermonecrotic exotoxin and the other antigens claimed to be protective. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the sows and gilts for which it is intended.

The following tests for production of antigens (section 2-4-1), safety (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Production of antigens**. The production of antigens claimed to be protective is verified by a suitable bioassay or immunochemical method (*2.7.1*), carried out on the antigens obtained from each of the vaccine strains under the conditions to be used for the production of the vaccine.

2-4-2. **Safety**

2-4-2-1. *Safety in pregnant sows*. Carry out the test for each route and method of administration to be recommended for vaccination using in each case pregnant sows or gilts that do not have antibodies against the components of the vaccine, from a herd or herds where there are no signs of atrophic rhinitis and that have not been vaccinated against atrophic rhinitis. Use a batch containing not less than the maximum potency that may be expected in a batch of vaccine.

Use not fewer than 8 pregnant sows or gilts per group, at the stage or at different stages of pregnancy according to the schedule to be recommended. Administer to each pregnant sow or gilt 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pregnant sows or gilts at least daily until farrowing. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 4 days; note the maximum temperature increase for each pregnant sow or gilt.

The vaccine complies with the test if no pregnant sow or gilt shows abnormal local or systemic reactions or dies from causes attributable to the vaccine, if the average temperature increase for all pregnant sows or gilts does not exceed 1.5 °C and no pregnant sow or gilt shows a rise greater than 2.0 °C, and if no adverse effects on gestation and offspring are noted.

2-4-2-2. *Field studies*. The pigs used for field trials are also used to evaluate safety. Use not fewer than 3 groups each of not fewer than 20 pigs with corresponding groups of not fewer than 10 controls. Examine the injection site for local reactions after vaccination. Record body temperature the day before vaccination, at vaccination, at the time interval after which a rise in temperature, if any, was seen in test 2-4-2-1, and daily during the 2 days following vaccination; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or dies from causes attributable to the vaccine and if the average temperature increase for all pigs does not exceed 1.5 °C and no pig shows a rise greater than 2.0 °C.

2-4-3. **Immunogenicity**. Each test is carried out for each route and method of administration to be recommended, using in each case pigs that do not have antibodies against the components of the vaccine, that are from a herd or herds where there are no signs of atrophic rhinitis and that have not been vaccinated against atrophic rhinitis. The vaccine administered to each pig is of minimum potency.

2-4-3-1. *Vaccines containing dermonecrotic exotoxin of* P. multocida *(with or without cells of* P. multocida*)*. Use not fewer than 12 breeder pigs. Vaccinate not fewer than 6 randomly chosen pigs at the stage of pregnancy or non-pregnancy and according to the schedule to be recommended. Maintain not fewer than 6 pigs as controls. From birth allow all the piglets from the vaccinated and unvaccinated breeder pigs to feed from their own dam. Constitute from the progeny 2 challenge groups each of not fewer than 30 piglets chosen randomly, taking not fewer than 3 piglets from each litter. On the 2 consecutive days preceding challenge, the mucosa of the nasal cavity of the piglets may be treated by instillation of 0.5 mL of a solution of acetic acid (10 g/L $C_2H_4O_2$) in isotonic buffered saline pH 7.2.

Challenge each piglet at 10 days of age by the intranasal route with a sufficient quantity of a toxigenic strain of *P. multocida*. At the age of 42 days, euthanise the piglets of the 2 groups and dissect the nose of each of them transversally at premolar-1. Examine the ventral and dorsal turbinates and the nasal septum for evidence of atrophy or distortion and grade the observations on the following scales.

*Turbinates*

0 no atrophy

1 slight atrophy

2 moderate atrophy

3 severe atrophy

4 very severe atrophy with almost complete disappearance of the turbinate

The maximum score is 4 for each turbinate and 16 for the sum of the 2 dorsal and 2 ventral turbinates.

*Nasal septum*

0 no deviation

1 very slight deviation

2 deviation of the septum

The maximum total score for the turbinates and the nasal septum is 18.

The test is not valid if fewer than 80 per cent of the progeny of each litter of the unvaccinated breeder pigs have a total score of at least 10. The vaccine complies with the test if a significant reduction in the total score has been demonstrated in the group from the vaccinated breeder pigs compared to that from the unvaccinated breeder pigs.

2-4-3-2. *Vaccines containing* P. multocida *dermonecrotic exotoxin (with or without cells of* P. multocida*) and cells and/or antigenic components of* B. bronchiseptica. Use not fewer than 24 breeder pigs. Vaccinate not fewer than 12 randomly chosen pigs at the stage of pregnancy or non-pregnancy and according to the schedule to be recommended. Maintain not fewer than 12 pigs as controls. From birth allow all the piglets from the vaccinated and unvaccinated breeder pigs to feed from their own dam. Using groups of not fewer than 6 pigs, constitute from their progeny 2 challenge groups from vaccinated pigs and 2 groups from control pigs each group consisting of not fewer than 30 piglets chosen randomly, taking not fewer than 3 piglets from each litter. On the 2 consecutive days preceding challenge, the mucosa of the nasal cavity of the piglets may be treated by instillation of 0.5 mL of a solution of acetic acid (10 g/L $C_2H_4O_2$) in isotonic buffered saline pH 7.2. For a group of piglets from not fewer than 6 vaccinated pigs and a group from not fewer than 6 controls, challenge each piglet by the intranasal route at 10 days of age with a sufficient quantity of a toxigenic strain of *P. multocida*. For the other group of piglets from not fewer than 6 vaccinated pigs and the other group from not fewer than 6 controls, challenge each piglet at 7 days of age by the intranasal route with a sufficient quantity of *B. bronchiseptica*. In addition, challenge each piglet at 10 days of age by the intranasal route with a sufficient quantity of a toxigenic strain of *P. multocida*. At the age of 42 days, euthanise the piglets of the 4 groups and dissect the nose of each of them transversally at premolar-1. Examine the ventral and dorsal turbinates and the nasal septum for evidence of atrophy or distortion and grade the observations on the scale described above.

The test is not valid if fewer than 80 per cent of the progeny of each litter of the unvaccinated breeder pigs have a total score of at least 10. The vaccine complies with the test if a significant reduction in the total score has been demonstrated in the groups from the vaccinated breeder pigs compared to the corresponding group from the unvaccinated breeder pigs.

2-5. *MANUFACTURER'S TESTS*

2-5-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 7 pigs not less than 3 weeks old and that do not have antibodies against the components of the vaccine. Vaccinate not fewer than 5 pigs by a recommended route and according to the recommended schedule. Maintain not fewer than 2 pigs of the same origin as controls under the same conditions. Alternatively, if the nature of the antigens allows reproducible results to be obtained, a test in laboratory animals that do not have antibodies against the components of the vaccine may be carried out. To obtain a valid assay, it may be necessary to carry out a test using several groups of animals, each receiving a different quantity of vaccine. For each quantity of vaccine, carry out the test as follows: vaccinate not fewer than 5 animals with a suitable quantity of vaccine. Maintain not fewer than 2 animals of the same species and origin as controls. Where the recommended schedule requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval within the range of 14-21 days after the last administration, collect blood from each animal and prepare serum samples. Use a validated test such as an enzyme-linked immunosorbent assay to measure the antibody response to each of the antigens stated on the label.

The test is not valid if there is a significant antibody titre in the controls. The vaccine complies with the test if the antibody responses of the vaccinated animals are not significantly less than those obtained with a batch of vaccine that has given satisfactory results in the test or tests (as applicable) described under Potency.

Where animals that do not have antibodies against the antigens stated on the label are not available, seropositive animals may be used in the above test. During the development of a test with seropositive animals, particular care will be required during the validation of the test system to establish that the test is suitably sensitive and to specify acceptable pass, fail and retest criteria. It will be necessary to take into account the range of prevaccination antibody titres and to establish the acceptable minimum antibody titre rise after vaccination in relation to these.

2-5-2. **Bacterial endotoxins**. A test for bacterial endotoxins (*2.6.14*) is carried out on the batch or, where the nature of the adjuvant prevents performance of a satisfactory test, on the bulk antigen or the mixture of bulk antigens immediately before addition of the adjuvant. The maximum acceptable amount of bacterial endotoxins is that found for a batch of vaccine shown satisfactory in safety test 2-4-2-1 given under Choice of vaccine composition or in the residual toxicity test described under Batch tests, carried out using 10 pigs. Where the latter test is used, note the maximum temperature increase for each pig; the vaccine complies with the test if the average temperature increase for all pigs does not exceed 1.5 °C. The method chosen for determining the amount of bacterial endotoxin present in the vaccine batch used in the safety test for determining the maximum acceptable level of endotoxin is used subsequently for testing of each batch.

3. BATCH TESTS

3-1. **Identification**. In animals that do not have specific antibodies against the antigens stated on the label, the vaccine stimulates the production of such antibodies.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Use not fewer than 2 pigs that do not have antibodies against *P. multocida* and that preferably do not have antibodies against *B. bronchiseptica*. Administer to each pig by a recommended route a double dose of the vaccine. Observe the pigs at least daily for 14 days. Record body temperature the day before vaccination, at vaccination, 2 h, 4 h and 6 h later and then daily for 2 days.

It is recommended to use the mean temperature of the days before administration of the vaccine (e.g. day − 3 to day 0) as the baseline temperature to have clear guidance for evaluation of the test.

The vaccine complies with the test if no pig shows notable signs of disease or dies from causes attributable to the vaccine; a transient temperature increase not exceeding 2.0 °C may occur.

3-4. **Potency**. The vaccine complies with the requirements of the tests mentioned under Immunogenicity (section 2-4-3) when administered by a recommended route and method.

**01/2017:2325**

# RABBIT HAEMORRHAGIC DISEASE VACCINE (INACTIVATED)

## Vaccinum morbi haemorrhagici cuniculi inactivatum

1. DEFINITION

Rabbit haemorrhagic disease vaccine (inactivated) is a preparation of a suitable strain of rabbit haemorrhagic disease virus (RHDV), inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for active immunisation of rabbits.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in rabbits. The rabbits must be healthy, not vaccinated against RHDV, free from antibodies against RHDV, not treated with antibiotics within at least 15 days of their use and from a healthy and monitored breeding unit. A suspension is prepared from a homogenate of suitable internal organs of those rabbits that are euthanised or that succumb to the infection within 120 h of inoculation. The virus in the suspension may be purified and concentrated, and is inactivated by a suitable method.

2-2. *SEED LOTS*

2-2-1. **Extraneous agents**. Each master seed lot complies with the tests for extraneous agents in seed lots prescribed in the monograph *Vaccines for veterinary use (0062)*.

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the rabbits for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy.

2-3-1. **Safety**

Carry out the test for each route and method of administration to be recommended for vaccination and in rabbits of each category for which the vaccine is intended. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 healthy rabbits from the same stock, not older than the minimum age to be recommended for vaccination and free from antibodies against RHDV. Administer to each rabbit 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the animals for at least 14 days after the last administration. Record the body temperature the day before vaccination, at vaccination, 4 h after vaccination and then daily for 4 days; note the maximum temperature increase for each animal.

The vaccine complies with the test if no rabbit shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine, the average body temperature increase for all animals does not exceed 1.5 °C, and no animal shows a temperature rise greater than 2.0 °C.

2-3-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination.

The test is carried out using in each case rabbits not less than 10 weeks old. The vaccine administered to each rabbit is of minimum potency.

Use not fewer than 15 healthy, susceptible rabbits, free from antibodies against RHDV, from the same healthy stock, and reared in suitable isolation conditions to ensure absence of contact with RHDV. Administer 1 dose of vaccine to each of not fewer than 10 of the rabbits according to the instructions for use to be stated on the label. Maintain not fewer than 5 other rabbits as controls. Not less than 7 days after vaccination, challenge each rabbit by a suitable route with a quantity of a virulent strain of RHDV sufficient to cause signs of rabbit haemorrhagic disease (RHD) in a susceptible rabbit. Observe the rabbits for a further 14 days.

The test is not valid if fewer than 80 per cent of control rabbits die with typical signs of RHD within 120 h of challenge.

The vaccine complies with the test if not fewer than 90 per cent of vaccinated rabbits show no signs of RHD.

2-4. *MANUFACTURER'S TESTS*

2-4-1. **Residual live virus**. A test for residual live virus is carried out on the bulk harvest of each batch to confirm inactivation of the RHDV. The test for inactivation is carried out in healthy, susceptible rabbits, not less than 10 weeks old, free from antibodies against RHDV and from the same healthy stock. 5 rabbits are inoculated by a suitable parenteral route (subcutaneous or intramuscular) with at least a 5 mL dose of the suspension. The rabbits are observed for not less than 7 days. At the end of the observation period, the animals are euthanised and liver extracts are tested by a suitable method for freedom from RHDV.

The inactivated virus harvest complies with the test if no rabbit dies and no RHDV antigen is detected in the livers.

2-4-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

The following method is given as an example. Administer 1 dose of vaccine intramuscularly to each of 5 healthy rabbits, 10 weeks old, free from antibodies against RHDV and from the same healthy stock. Maintain 2 rabbits as unvaccinated controls. Collect serum samples from each rabbit just before administration of the vaccine and after the period defined when testing the reference vaccine; determine the antibody titre of each serum by a suitable immunological method, for example, ELISA. The antibody levels are not significantly lower than those obtained with a batch that has given satisfactory results in the test described under Potency.

The test is not valid if the sera collected from the unvaccinated controls and from the rabbits just before the administration of the vaccine show detectable specific antibodies.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Use not fewer than 2 healthy rabbits, not less than 10 weeks old, free from antibodies against RHDV and from the same healthy stock. Administer by a recommended route to each rabbit 2 doses of vaccine. Observe the rabbits for 14 days.

The vaccine complies with the test if no rabbit shows notable signs of disease or dies from causes attributable to the vaccine.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-2), when administered by a recommended route and method.

01/2017:0451

# RABIES VACCINE (INACTIVATED) FOR VETERINARY USE

## Vaccinum rabiei inactivatum ad usum veterinarium

1. DEFINITION

Rabies vaccine (inactivated) for veterinary use is a preparation of a suitable strain of fixed rabies virus, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of animals against rabies.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine is prepared from virus grown either in suitable cell lines or in primary cell cultures from healthy animals (*5.2.4*). The virus suspension is harvested on one or more occasions within 28 days of inoculation. Multiple harvests from a single production cell culture may be pooled and considered as a single harvest.

The virus harvest is inactivated. The vaccine may be adjuvanted.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE COMPOSITION*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the species for which it is intended.

The following tests for safety (section 2-3-1) and immunogenicity (section 2-3-2) may be used during the demonstration of safety and efficacy in cats and dogs.

The suitability of the vaccine with respect to immunogenicity (section 2-3-2) for carnivores (cats and dogs) is demonstrated by direct challenge. For other species, if a challenge test has been carried out for the vaccine in cats or dogs, an indirect test is carried out by determining the antibody level following vaccination of not fewer than 20 animals according to the schedule to be recommended; the vaccine is satisfactory if, after the period to be claimed for protection, the mean rabies virus antibody level in the serum of the animals is not less than 0.5 IU/mL and if not more than 10 per cent of the animals have an antibody level less than 0.1 IU/mL.

2-3-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 animals of the minimum age to be recommended and that do not have antibodies against rabies virus. Administer to each animal 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose after an interval of at least 14 days. Observe the animals at least daily for at least 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-3-2. **Immunogenicity**. Each test is carried out for each route and method of administration to be recommended, using in each case animals of the minimum age to be recommended for vaccination. The vaccine administered to each animal is of minimum potency.

Use for the test not fewer than 35 animals. Take a blood sample from each animal and test individually for antibodies against rabies virus to determine susceptibility. Vaccinate not fewer than 25 animals, according to the schedule to be recommended. Maintain not fewer than 10 animals as controls. Observe all the animals for a period equal to the claimed duration of immunity. No animal shows signs of rabies. On the last day of the claimed period for duration of immunity or later, challenge each animal by intramuscular injection with a sufficient quantity of virulent rabies virus of a strain approved by the competent authority. Observe the animals at least daily for 90 days after challenge. Animals that die from causes not attributable to rabies are eliminated. The test is not valid if the number of such deaths reduces the number of vaccinated animals in the test to fewer than 25 and the test is invalid unless at least 8 control animals (or a statistically equivalent number if more than 10 control animals are challenged) show signs of rabies and the presence of rabies virus in their brain is demonstrated by the fluorescent-antibody test or some other suitable method. The vaccine complies with the test if not more than 2 of the 25 vaccinated animals (or a statistically equivalent number if more than 25 vaccinated animals are challenged) show signs of rabies.

2-4. MANUFACTURER'S TESTS

2-4-1. **Residual live virus**. The test for residual live virus is carried out by inoculation of the inactivated virus into the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The quantity of inactivated virus harvest used is equivalent to not less than 25 doses of the vaccine. After incubation for 4 days, a subculture is made using trypsinised cells; after incubation for a further 4 days, the cultures are examined for residual live rabies virus by an immunofluorescence test. The inactivated virus harvest complies with the test if no live virus is detected.

2-4-2. **Antigen content of the harvest**. The content of rabies virus glycoprotein is determined by a suitable immunochemical method (*2.7.1*). The content is within the limits approved for the particular preparation.

2-4-3. **Antigen content of the pooled harvest**. The quantity of rabies virus glycoprotein per dose, determined by a suitable immunochemical method (*2.7.1*) on the pooled harvest immediately before blending, is not significantly lower than that of a batch of vaccine that gave satisfactory results in the test described under Potency.

2-4-4. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, such an alternative validated method should preferably be used for routine testing. The following serological assay has been shown to be suitable[(3)] and may

(3) B. Krämer *et al.* Collaborative Study for Validation of a Serological Potency Assay for Rabies Vaccines (inactivated) for Veterinary Use, *Pharmeur Bio Sci Notes* 2010(2):37-55.

be used provided the test for antigen content of the pooled harvest (section 2-4-3) has been carried out with satisfactory results.

Use groups of not fewer than 8 female mice (strain NMRI), each weighing 18-20 g. Prepare a 1 IU/mL suspension of *rabies vaccine (inactivated) for veterinary use BRP* using phosphate-buffered saline (PBS) for dilution. Vaccines with a minimum potency requirement of 1 IU/mL are used without further dilution. Vaccines with a minimum potency requirement of more than 1 IU/mL are diluted with PBS to contain approximately, but not less than, 1 IU/mL. Administer by the intraperitoneal route to each mouse of one group 0.2 mL of the vaccine, diluted where necessary, and to each mouse of another group 0.2 mL of the suspension of *rabies vaccine (inactivated) for veterinary use BRP*. Take blood samples 14 days after the injection and test the sera individually for rabies antibody using a suitable virus neutralisation test, for example the rapid fluorescent focus inhibition test (RFFIT) described for *Human rabies immunoglobulin (0723)* or a suitable validated modification of the RFFIT[(4)].

The test is not valid if more than 2 mice injected with the suspension of *rabies vaccine (inactivated) for veterinary use BRP* show no antibodies in their serum.

Individual serum titres are determined with an appropriate anti-rabies immunoglobulin reference.

The antibody titre of mice receiving the suspension of *rabies vaccine (inactivated) for veterinary use BRP* is compared to the antibody titre of mice receiving the vaccine using a suitable statistical approach (*5.3*).

The vaccine complies with the test if the antibody titre of mice injected with the vaccine is significantly higher than that of mice injected with the suspension of *rabies vaccine (inactivated) for veterinary use BRP*.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual live virus**. *This test may be omitted for batch release, as stated in the monograph Vaccines for veterinary use (0062).*

Carry out the test using a pool of the contents of 5 containers.

For vaccines which do not contain an adjuvant, carry out a suitable amplification test for residual live virus using the same type of cell culture as that used in the production of the vaccine or a cell culture shown to be at least as sensitive. The vaccine complies with the test if no live virus is detected.

For vaccines that contain an adjuvant, inject intracerebrally into each of not fewer than 10 mice, each weighing 11-15 g, 0.03 mL of a pool of at least 5 times the smallest stated dose. To avoid interference from any antimicrobial preservative or the adjuvant, the vaccine may be diluted not more than 10 times before injection. In this case or if the vaccine strain is pathogenic only for unweaned mice, carry out the test on mice 1-4 days old. Observe the animals for 21 days. If more than 2 animals die during the first 48 h, repeat the test. The vaccine complies with the test if, from the 3rd to the 21st days following the injection, the animals show no signs of rabies and immunofluorescence tests carried out on the brains of the animals show no indication of the presence of rabies virus.

3-4. **Potency**. The potency of rabies vaccine is determined by comparing the dose necessary to protect mice against the clinical effects of the dose of rabies virus defined below, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, necessary to provide the same protection.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Rabies vaccine (inactivated) for veterinary use BRP* is calibrated in International Units against the International Standard.

The test described below uses a parallel-line model with at least 3 points for the vaccine to be examined and the reference preparation. Once the analyst has experience with the method for a given vaccine, it is possible to carry out a simplified test using 1 dilution of the vaccine to be examined. Such a test enables the analyst to determine that the vaccine has a potency significantly higher than the required minimum but will not give full information on the validity of each individual potency determination. It allows a considerable reduction in the number of animals required for the test and should be considered by each laboratory in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

*Selection and distribution of the test animals.* Use in the test healthy female mice about 4 weeks old and from the same stock. Distribute the mice into at least 10 groups of not fewer than 10 mice.

*Preparation of the challenge suspension.* Inoculate a group of mice intracerebrally with the CVS strain of rabies virus and when the mice show signs of rabies, but before they die, euthanise the mice and remove the brains and prepare a homogenate of the brain tissue in a suitable diluent. Separate gross particulate matter by centrifugation and use the supernatant as challenge suspension. Distribute the suspension in small volumes in ampoules, seal and store at a temperature below – 60 °C. Thaw 1 ampoule of the suspension and make serial dilutions in a suitable diluent. Allocate each dilution to a group of mice and inject intracerebrally into each mouse 0.03 mL of the dilution allocated to its group. Observe the animals at least daily for 14 days and record the number in each group that, between the 5th and the 14th days, develop signs of rabies. Calculate the $ID_{50}$ of the undiluted suspension.

*Determination of potency of the vaccine to be examined.* Prepare at least 3 serial dilutions of the vaccine to be examined and 3 similar dilutions of the reference preparation. Prepare the dilutions such that those containing the largest quantity of vaccine may be expected to protect more than 50 per cent of the animals into which they are injected and those containing the smallest quantities of vaccine may be expected to protect less than 50 per cent of the animals into which they are injected. Allocate each dilution to a different group of mice and inject by the intraperitoneal route into each mouse 0.5 mL of the dilution allocated to its group. 14 days after the injection prepare a suspension of the challenge virus such that, on the basis of the preliminary titration, it contains about 50 $ID_{50}$ in each 0.03 mL. Inject intracerebrally into each vaccinated mouse 0.03 mL of this suspension. Prepare 3 suitable serial dilutions of the challenge suspension. Allocate the challenge suspension and the 3 dilutions one to each of 4 groups of 10 unvaccinated mice and inject intracerebrally into each mouse 0.03 mL of the suspension or one of the dilutions allocated to its group. Observe the animals in each group at least daily for 14 days. The test is not valid if more than 2 mice of any group die within the first 4 days after challenge. Record the numbers in each group that show signs of rabies in the period 5 days to 14 days after challenge.

(4) B. Krämer *et al.* The rapid fluorescent focus inhibition test is a suitable method for batch potency testing of inactivated rabies vaccine. *Biologicals* 2009;**37**:119-126.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the smallest and the largest dose given to the mice;
- the titration of the challenge suspension shows that 0.03 mL of the suspension contained at least 10 $ID_{50}$;
- the confidence limits ($P$ = 0.95) are not less than 25 per cent and not more than 400 per cent of the estimated potency; when this validity criteria is not met, the lower limit of the estimated potency must be at least 1 IU in the smallest prescribed dose;
- the statistical analysis shows a significant slope ($P$ = 0.95) and no significant deviations from linearity or parallelism of the dose-response lines ($P$ = 0.99).

The vaccine complies with the test if the estimated potency is not less than 1 IU in the smallest prescribed dose.

*Application of alternative end-points.* Once a laboratory has established the above assay for routine use, the lethal end-point is replaced by an observation of clinical signs and application of an end-point earlier than death to reduce animal suffering. The following is given as an example.

The progress of rabies infection in mice following intracerebral injection can be represented by 5 stages defined by typical clinical signs:

Stage 1: ruffled fur, hunched back;

Stage 2: slow movements, loss of alertness (circular movements may also occur);

Stage 3: shaky movements, trembling, convulsions;

Stage 4: signs of paresis or paralysis;

Stage 5: moribund state.

Mice are observed at least twice daily from day 4 after challenge. Clinical signs are recorded using a chart such as that shown in Table 0451.-1. Experience has shown that using stage 3 as an end-point yields assay results equivalent to those found when a lethal end-point is used. This must be verified by each laboratory by scoring a suitable number of assays using both clinical signs and the lethal end-point.

Table 0451.-1. – *Example of a chart used to record clinical signs in the rabies vaccine potency test*

| | Days after challenge | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Clinical signs** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| Ruffled fur<br>Hunched back | | | | | | | | |
| Slow movements<br>Loss of alertness<br>Circular movements | | | | | | | | |
| Shaky movements<br>Trembling<br>Convulsions | | | | | | | | |
| Paresis<br>Paralysis | | | | | | | | |
| Moribund state | | | | | | | | |

4. LABELLING

The label states:

- the type of cell culture used to prepare the vaccine and the species of origin;
- the minimum number of International Units per dose;
- the minimum period for which the vaccine provides protection.

**01/2014:0746**

# RABIES VACCINE (LIVE, ORAL) FOR FOXES AND RACCOON DOGS

## Vaccinum rabiei perorale vivum ad vulpem et nyctereutem

1. DEFINITION

Rabies vaccine (live, oral) for foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*) is a preparation of a suitable immunogenic strain of an attenuated rabies virus. The virus strain has one or more stable genetic markers that discriminates the vaccine strain from other rabies virus strains. The vaccine is incorporated in bait in such a manner as to enable the tests prescribed below to be performed aseptically. The bait casing, attractive to the target species, may contain a biomarker (e.g. tetracycline). This monograph applies to vaccines intended for the active immunisation of foxes, or foxes and raccoon dogs against rabies.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures. The virus suspension is harvested on one or more occasions within 14 days of inoculation. Multiple harvests from a single cell lot may be pooled and considered as a single harvest. It may be mixed with a suitable stabiliser.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*); if the cell cultures are of mammalian origin, they are shown to be free from rabies virus.

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) for the target species and the non-target species, and efficacy (*5.2.7*) for the species for which it is intended. The vaccine strain is genetically characterised by gene sequencing. The following tests for safety of the virus strain (section 2-3-1), stability of the genetic marker (section 2-3-2) and immunogenicity (2-3-3) may be used during the demonstration of safety and efficacy.

In natural and experimental conditions, the virus strain does not spread from one animal to another in wild rodents.

2-3-1. **Safety of the virus strain**. Administer the virus strain by the oral route. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

For each test performed in the target species (foxes, or foxes and raccoon dogs), use not fewer than 20 animals that do not have antibodies against rabies virus. Administer orally to each animal a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 vaccine bait. Observe the animals at least daily for 180 days.

For each test performed in the non-target species (dogs, cats, and if appropriate, raccoon dogs), use not fewer than 10 animals that do not have antibodies against rabies virus. Administer orally to each animal a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 vaccine bait. Observe the animals at least daily for 180 days.

The vaccine virus complies with the test if no animal shows signs of disease and if the presence of the vaccine virus is not demonstrated in the brain of any animal. The presence of rabies virus in the brain is tested using reference diagnostic tests (immunofluorescence test and cell-culture test).

2-3-2. **Stability of the genetic marker**. Carry out the test using suckling mice that have not been vaccinated against rabies. Passage the vaccine virus sequentially through 5 groups via the intracerebral route.

Inoculate each of the 5 mice of the 1st group with a quantity of the vaccine virus that will allow recovery of virus for the passages described below (e.g. not more than 0.02 mL). When the mice show signs of rabies, but not later than 14 days after inoculation, euthanise the mice and remove the brain of each mouse. Prepare a suspension from the brain of each mouse and pool the samples. Administer not more than 0.02 mL of the pooled samples to each mouse of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 animals.

Verify the genetic marker in the vaccine virus recovered from the last passage.

The vaccine virus complies with the test if the genetic marker remains stable.

2-3-3. **Immunogenicity**. A test is carried out for the oral route of administration and with the bait to be stated on the label using animals of the target species (foxes, or foxes and raccoon dogs) at least 3 months old. The quantity of vaccine virus to be administered to each fox, or fox and raccoon dog, is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of vaccine.

Use for the test not fewer than 35 animals of each target species, that do not have antibodies against rabies virus. In each target species, apply the following protocol, validity criteria and acceptance limits.

Vaccinate not fewer than 25 animals, according to the schedule to be recommended. Maintain not fewer than 10 animals as controls. Observe the animals for 180 days after vaccination. The test is not valid if fewer than 25 vaccinated animals survive after this observation period. Challenge all the animals at least 180 days after vaccination by intramuscular injection of a sufficient quantity of a virulent rabies virus strain approved by the competent authority. Observe the animals at least daily for 90 days after challenge. Animals that die from causes not attributable to rabies are eliminated.

The test is not valid if the number of such deaths reduces the number of vaccinated animals in the test to fewer than 25 and the test is invalid unless at least 9 control animals (or a statistically equivalent number if more than 10 control animals are challenged) show signs of rabies and the presence of rabies virus in their brain is demonstrated by the immunofluorescence test or some other reliable method.

The vaccine virus complies with the test if not more than 2 of 25 vaccinated animals (or a statistically equivalent number if more than 25 vaccinated animals are challenged) show signs of rabies.

2-4. *BAIT STABILITY*

Incubate the bait at 25 °C for 5 days. Titrate the vaccine. The virus titre must be at least the minimum virus titre stated on the label. Heat the bait at 40 °C for 1 h. The bait casing complies with the test if it remains in its original shape and adheres to the vaccine container.

3. BATCH TESTS

3-1. **Identification**

3-1-1. The vaccine virus is identified by a suitable method, e.g. when mixed with a monospecific rabies antiserum, the vaccine is no longer able to infect susceptible cell cultures into which it is inoculated.

3-1-2. A test is carried out to demonstrate the presence of the genetic marker.

3-2. **Bacteria and fungi**. The vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**

3-4-1. Neutralise the vaccine virus with a suitable monospecific neutralising rabies virus antiserum and inoculate into susceptible cell cultures. The vaccine complies with the test if it no longer provokes cytopathic effects in susceptible cell cultures, and it shows no evidence of haemagglutinating or haemadsorbing agents.

3-4-2. Inoculate 1 in 10 and 1 in 1000 dilutions of the vaccine into susceptible cell cultures. Incubate at 37 °C. After 2, 4 and 6 days, stain the cells with a panel of monoclonal antibodies that do not react with the vaccine strain but that react with other strains of rabies vaccine (for example, street virus, Pasteur strain). The vaccine complies with the test if it shows no evidence of contaminating rabies virus.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures. The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-3) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

3-7. **Biomarker**. If the bait contains a biomarker, the stability of the biomarker is verified by a suitable method. When tetracycline is used, the vaccine complies with the test if chemical analysis of the bait casing shows less than 30 per cent conversion of the total amount of tetracycline into the epitetracycline isomer.

4. LABELLING

The label states:

- the nature of the genetic marker of the virus strain;
- where applicable, the nature of the biomarker of the bait.

**01/2017:1947**

# SALMONELLA ENTERITIDIS VACCINE (INACTIVATED) FOR CHICKENS

## Vaccinum Salmonellae Enteritidis inactivatum ad pullum

1. DEFINITION

Salmonella Enteritidis vaccine (inactivated) for chickens is a preparation of a suitable strain or strains of *Salmonella enterica* Enteritidis, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for administration to chickens for reducing *S. enterica* Enteritidis colonisation and faecal excretion of *S. enterica* Enteritidis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity of the cultures and identity are verified on the harvest using suitable methods. After

cultivation, the bacterial harvests are collected separately, inactivated by a suitable method, and blended. The vaccine may contain adjuvants.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended. The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination, using in each case chickens not older than the minimum age to be recommended for vaccination and from a flock free from specified pathogens (SPF) (*5.2.2*). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer by a route and method to be recommended to each chicken 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose to each chicken after an interval of at least 14 days. Observe the chickens at least daily for at least 14 days after the last administration of the vaccine.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For chickens older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination. The vaccine administered to each animal is of minimum potency.

Use for the test not fewer than 60 SPF chickens (*5.2.2*) not older than the minimum age to be recommended for vaccination. Vaccinate not fewer than 30 chickens with no more than the minimum recommended number of doses of vaccine. Maintain not fewer than 30 chickens as controls for each group of vaccinates. Challenge both groups, 4 weeks after the last administration of vaccine, by oral administration to each chicken of a sufficient quantity of a strain of *S. enterica* Enteritidis that is able to colonise chickens. Take blood samples from control chickens on the day before challenge. Observe the chickens at least daily for 4 weeks. Take individual fresh faeces samples on day 1 after challenge and at least twice weekly (including day 7) until 14 days after challenge. Test the fresh faeces samples for the presence of *S. enterica* Enteritidis by direct plating. Euthanise all surviving chickens at the end of the observation period, take samples of liver and spleen and test for the presence of *S. enterica* Enteritidis by an appropriate method.

The test is not valid if antibodies against *S. enterica* Enteritidis are found in any control chicken before challenge.

The vaccine complies with the test if:

- the number of *S. enterica* Enteritidis in fresh faeces samples from vaccinated chickens after challenge at the different days of sampling is significantly lower in vaccinates than in controls and remains lower until the end of the test;
- the number of positive samples of liver and spleen is significantly lower in vaccinates than in controls.

2-3. *MANUFACTURER'S TEST*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 15 SPF chickens (*5.2.2*). Maintain not fewer than 5 SPF chickens as controls. Administer to each of 10 chickens 1 dose of vaccine by a recommended route. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval after the last injection, collect blood from each vaccinated and control chicken and prepare serum samples. Measure the titre of antibodies against *S. enterica* Enteritidis in each serum sample using a suitable validated serological method. Calculate the titre for the group of vaccinates.

The test is not valid if specific *S. enterica* Enteritidis antibodies are found in 1 or more sera from control chickens at a given interval after the time of administration of the vaccine in the vaccinated group.

The vaccine complies with the test if the antibody titres of the group of vaccinates at a given interval after each vaccination, where applicable, are not significantly lower than the value obtained with a batch that has given satisfactory results in the test described under Potency (section 3-3).

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:2520**

# SALMONELLA ENTERITIDIS VACCINE (LIVE, ORAL) FOR CHICKENS

## Vaccinum Salmonellae Enteritidis vivum perorale ad pullum

1. DEFINITION

Salmonella Enteritidis vaccine (live, oral) for chickens is a preparation of a suitable strain of live *Salmonella enterica* Enteritidis. This monograph applies to vaccines intended for the active immunisation of chickens against colonisation by and faecal excretion of *S. enterica* Enteritidis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine strain is cultured in a suitable medium. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity and identity of the cultures are verified on the harvest using a combination of methods such as morphological, serological and biochemical methods and culture on appropriate selective media. Suitable test(s) are conducted to confirm the presence of relevant marker(s). The harvests are shown to be pure and the results obtained from the tests for identity are in accordance with the documented characteristics of the strain.

2-2. *CHOICE OF VACCINE STRAIN*

The vaccine strain is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended. During development, the safety of the vaccine for the persons handling the vaccine or vaccinated chickens has to be addressed as well as, in accordance with the requirements of general chapter *5.2.6*, the safety of the spread of the vaccine to other susceptible species. The strain has a stable marker or markers to distinguish it from wild-type strains.

The following tests described under General safety (section 2-2-1-1), Excretion, duration of excretion and survival in the environment (section 2-2-1-2), Spread of the vaccine strain (section 2-2-1-3), Dissemination and survival of the vaccine strain in vaccinated chickens after each vaccination (section 2-2-1-4), Increase in virulence (section 2-2-1-5), Field trials (section 2-2-1-6), and Immunogenicity (sections 2-2-2-1 and 2-2-2-2) may be used during the demonstration of safety and efficacy.

For vaccines intended to prevent and/or reduce colonisation by and faecal excretion of *S. enterica* Enteritidis, the test for immunogenicity (section 2-2-2-1) is suitable to demonstrate that the vaccine is suitably immunogenic.

When the vaccine is recommended for use in laying chickens, the continuing immunogenicity of the vaccine until the end of the laying period has to be demonstrated and the test for immunogenicity at the end of the laying period (section 2-2-2-2) is suitable.

2-2-1. **Safety**. Unless otherwise indicated below, carry out each test by the oral route of administration, using chickens from a flock free from specified pathogens (SPF) (*5.2.2*) not older than the minimum age to be recommended for vaccination and that are free from antibodies against *Salmonella* spp. Where the vaccine is recommended for administration to 1-day-old chickens, the vaccine is administered before food is provided. Use vaccine bacteria at the least attenuated passage level that will be present in a batch of vaccine.

Measures taken to ensure absence of contamination by *Salmonella* spp. from the environment before the start of the test and on a regular ongoing basis are described and justified.

Whenever possible, items taken into the facilities are sterilised.

For re-isolation of the vaccine strain, suitably sensitive validated methods that are optimal for the vaccine strain concerned are used. The presence of relevant markers is confirmed to demonstrate that the organisms isolated are vaccine-derived and not wild-type contaminants.

2-2-1-1. *General safety*. For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens that are free from antibodies against *Salmonella* spp. Administer orally to each chicken a quantity of the vaccine strain equivalent to not less than 10 times the maximum titre(s) likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs or die from causes not attributable to the vaccine.

The vaccine complies with the test if no chicken shows notable signs of disease or dies from causes attributable to the vaccine.

2-2-1-2. *Excretion, duration of excretion and survival in the environment*. The same animals can be used for the test for spread of the vaccine strain (section 2-2-1-3) provided they are of the minimum age to be recommended for vaccination. Use for the test not fewer than 10 chickens. Administer orally to each chicken a quantity of the vaccine equivalent to not less than the maximum titre of the strain under study likely to be contained in 1 dose of the vaccine. Samples are collected for re-isolation of vaccine from cloacal swabs from each chicken and floor faeces on days 3, 7, 10 and 14 after vaccination and then weekly until 3 consecutive negative weekly samples are obtained from all vaccinated chickens. Samples are collected for re-isolation of the vaccine strain from the caeca of vaccinates at the end of the test.

The test is not valid if more than 10 per cent of vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The results are noted and used to formulate the label statement on the length of time of excretion of the vaccine strain.

2-2-1-3. *Spread of the vaccine strain*. The same animals can be used for the test for excretion, duration of excretion and survival in the environment (section 2-2-1-2). Use for the test not fewer than 10 chickens of the minimum age to be recommended for vaccination. Use 10 chickens as controls.

Administer orally to each chicken a quantity of the vaccine strain equivalent to not less than the maximum titre of the strain likely to be contained in 1 dose of the vaccine. 1 day after vaccination, mix the 10 vaccinates with at least 10 non-vaccinated chickens of the same age and source. Samples are collected for the re-isolation of the vaccine strain from cloacal swabs from each chicken and floor faeces on days 3, 7, 10 and 14 after vaccination and then weekly until 3 consecutive negative weekly samples are obtained from all chickens. Collect samples of the caeca and spleens for re-isolation of the vaccine strain from 10 in-contact control chickens at the end of the test. The results are noted and used to formulate the label statement on the extent to which the vaccine spreads to in-contact non-vaccinated chickens.

2-2-1-4. *Dissemination and survival of the vaccine strain in vaccinated chickens after each vaccination*. Conduct the test after each vaccination as prescribed by the vaccination schedule to be recommended in chickens of each category for which the vaccine is intended with oral administration of the test preparation. Use a sufficient number of chickens to conduct the sampling described below, the number of chickens required being dependent on the number of vaccinations to be recommended, the interval between vaccinations and the length of time chickens are maintained after the last vaccination. Administer to each chicken a quantity of the vaccine strain equivalent to not less than the maximum titre of the strain under study likely to be contained in 1 dose of the vaccine. Collect cloacal swabs from each chicken for re-isolation of the vaccine strain on days 7 and 14 after each vaccination and at later appropriate stages and with sufficient frequency to determine the duration of dissemination.

For example, for broilers, samples are collected from 5 chickens for re-isolation of the vaccine strain on days 7 and 14 after each vaccination and weekly until 8 weeks of age. At the 7- and 14-day sampling points, samples are taken from the liver, caecum and spleen of 5 chickens.

In the case of chickens intended for laying, samples are collected from 5 chickens on days 7 and 14 after each vaccination and weekly until 3 consecutive negative weekly samples are obtained or the time of the next vaccination is reached, whichever is the sooner. At the 7- and 14-day sampling points, samples are taken from the liver, caecum and spleen of 5 chickens. In addition, samples are collected from ovaries and oviducts where dissemination in vaccinated future layers is being investigated.

The test is not valid if more than 10 per cent of vaccinated chickens in any group show abnormal clinical signs or die from causes not attributable to the vaccine. The results are noted and used to formulate the label statement on the length of time the vaccine strain survives in the body and to define a suitable withdrawal period.

2-2-1-5. *Increase in virulence*. Carry out the test according to general chapter *5.2.6* using SPF chickens (*5.2.2*) not older than the minimum age to be recommended for vaccination.

Administer orally to each chicken of the 1st group a quantity of the vaccine strain of the strain under study that will allow recovery of bacteria for the passages described below. 4 to 7 days after administration of the vaccine strain, prepare a

suspension from the liver, spleen and caecum of chickens and pool these samples. Administer pooled samples orally to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the bacteria at each passage. If the bacteria are not found at a passage level, repeat the passage by administration to a group of 10 chickens. Any mortalities are investigated for the presence of the vaccine strain and the properties of any re-isolated vaccine strain determined.

Carry out the test for excretion, duration of excretion and survival in the environment (section 2-2-1-2) and, if the last group of birds from which the bacteria was recovered shows evidence of an increase in virulence indicative of reversion during the observation period, carry out the test for general safety (section 2-2-1-1), using the material used for the 1st passage and the bacteria at the last passage level where it was recovered. Test the bacteria recovered for the final passage for the presence and stability of the marker(s).

The vaccine strain complies with the test if no indication of increased virulence of the bacteria recovered for the final passage compared with the material used for the 1st passage is observed and the presence of the marker(s) is confirmed in the bacteria recovered for the final passage and remains identical to the material used for the 1st passage. If the bacteria are not recovered after an initial passage in 5 animals and a subsequent repeat passage in 10 animals, the vaccine also complies with the test.

2-2-1-6. *Field trials*. The chickens used for field trials are also used to evaluate safety. A trial is carried out in each category of chickens for which the vaccine strain is intended, in not fewer than 2 sets of premises. Samples are taken from a significant number of chickens for re-isolation of bacteria to provide information on the persistence, dissemination and spread of the bacteria, which can be used, with the data from the laboratory studies, to formulate the statements on the label. The samples include cloacal swabs, floor faeces, spleen and liver and, in laying chickens, samples of ovaries and oviducts. Environmental samples are also tested at regular intervals.

2-2-2. **Immunogenicity**. The tests described in section 2-2-2-1 and, if appropriate, in section 2-2-2-2 are carried out using chickens not older than the minimum age to be recommended for vaccination and that are free from antibodies against *Salmonella* spp. The quantity of the vaccine strain to be administered orally to each chicken is not greater than the minimum titre to be stated on the label.

Measures taken to ensure absence of contamination by *Salmonella* spp. from the environment before the start of the test and on a regular ongoing basis are described and justified.

Whenever possible, items taken into the facilities are sterilised.

Suitably sensitive validated methods are used for re-isolation of bacteria derived from the challenge and for distinguishing these from the vaccine strain.

2-2-2-1. *Immunogenicity*. Use for the test not fewer than 40 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate according to the schedule to be recommended not fewer than 20 chickens with a single dose of vaccine. Maintain not fewer than 20 chickens as controls. Challenge each chicken after 14 days by a suitable route with a sufficient quantity of a virulent strain of *S. enterica* Enteritidis to give a valid test. Collect cloacal swabs from vaccinates and controls on days 3, 5, 7, 10 and 14 post-challenge. Samples of caecum, liver and spleen are collected from 10 chickens of each group on days 7 and 14 post-challenge for re-isolation of challenge bacteria. Collect the same samples of internal organs from any chicken that dies. Examine the samples for the presence of the challenge organisms using a suitable sensitive culture medium and compare results for vaccinates and controls.

The test is not valid if, during the observation period, fewer than 80 per cent of the control chickens die or challenge organisms are re-isolated from fewer than 80 per cent of the control chickens.

The vaccine complies with the test if there is a significant reduction in the total number of cloacal swabs from vaccinates containing challenge organisms compared with the number from the controls and there is a significant reduction in the number of samples from internal organs from vaccinates containing challenge bacteria compared with the number from the controls.

2-2-2-2. *Immunogenicity at the end of the laying period*. Use for the test not fewer than 40 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate not fewer than 20 chickens according to the schedule to be recommended. Maintain not fewer than 20 chickens as controls. At the end of the laying period, take serum samples and cloacal swabs from the chickens and environmental samples from the housing area. Test each serum sample individually for the presence of antibodies to *S. enterica* Enteritidis and each cloacal swab and environmental sample for the presence of *Salmonella* spp. Challenge each chicken by a suitable route with a sufficient quantity of a virulent strain of *S. enterica* Enteritidis to give a valid test. Collect cloacal swabs from vaccinates and controls on days 3, 5, 7, 10 and 14 post-challenge. Samples of caecum, liver, spleen, ovaries and oviducts are collected from 10 chickens of each group for re-isolation of challenge bacteria on days 7 and 14 post-challenge. Collect the same samples of internal organs from any chicken that dies during the observation period. Examine the samples for the presence of the challenge organisms with growth in a suitable medium and compare results for vaccinates and controls.

The test is not valid if, before the challenge, antibodies to *Salmonella* spp. are found in the serum of the controls or *Salmonella* spp. bacteria are isolated from any of the chickens. The test is also not valid if the challenge organisms are re-isolated from fewer than 80 per cent of the control chickens.

The vaccine complies with the test if there is a significant reduction in the number of cloacal swabs from vaccinates containing challenge organisms compared with the number from the controls and there is a significant decrease in the number of samples of internal organs from vaccinates containing challenge bacteria compared with the number from the controls.

## 3. BATCH TESTS

3-1. **Identification**. The strain present in the vaccine is identified by a combination of methods such as morphological, serological and biochemical methods and culture on appropriate selective media. Suitable test(s) are conducted to confirm the presence of the relevant marker(s).

3-2. **Bacteria and fungi**. Carry out the test by microscopic examination and by inoculation of suitable media, or verify the absence of micro-organisms other than the vaccine strain present in the vaccine as described in the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*. The vaccine complies with the test if it does not contain extraneous micro-organisms.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Live bacteria**. Titrate the vaccine strain using a suitable medium for the culture of the strain. The vaccine complies with the test if it contains not less than the titre stated on the label.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-2-2-1) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum titre stated on the label.

4. LABELLING

The label states:

- the nature of the markers allowing the vaccine to be distinguished from wild-type strains;
- the extent to which the vaccine spreads and is transmitted to non-vaccinated chickens and the time over which this could occur;
- the time that the vaccine survives in the body;
- the length of time of excretion and the time that the vaccine survives in the environment;
- the potential for spread to other susceptible species including humans;
- the withdrawal period.

**01/2017:2361**

# SALMONELLA TYPHIMURIUM VACCINE (INACTIVATED) FOR CHICKENS

## Vaccinum Salmonellae Typhimurium inactivatum ad pullum

1. DEFINITION

Salmonella Typhimurium vaccine (inactivated) for chickens is a preparation of a suitable strain or strains of *Salmonella enterica* Typhimurium, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for administration to chickens for reducing *S. enterica* Typhimurium colonisation and faecal excretion of *S. enterica* Typhimurium.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The seed material is cultured in a suitable medium; each strain is cultivated separately. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity of the cultures and identity are verified on the harvest using suitable methods. After cultivation, the bacterial harvests are collected separately, inactivated by a suitable method, and blended. The vaccine may contain adjuvants.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the birds for which it is intended. The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**. The test is carried out for each route of administration to be recommended for vaccination, using in each case chickens not older than the minimum age to be recommended for vaccination and from a flock free from specified pathogens (SPF) (*5.2.2*). Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens. For each test performed in chickens older than 3 weeks of age, use not fewer than 8 chickens. Administer by a route and method to be recommended to each chicken 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer 1 dose to each chicken after an interval of at least 14 days.

Observe the chickens at least daily for at least 14 days after the last administration of the vaccine.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine. For chickens older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended for vaccination. The vaccine administered to each animal is of minimum potency.

Use for the test not fewer than 60 SPF chickens (*5.2.2*) not older than the minimum age to be recommended for vaccination. Vaccinate not fewer than 30 chickens with no more than the minimum number of doses of vaccine to be recommended. Maintain not fewer than 30 chickens as controls for each group of vaccinates. Challenge both groups, 4 weeks after the last administration of vaccine, by oral administration to each chicken of a sufficient quantity of a strain of *S. enterica* Typhimurium that is able to colonise chickens. Take blood samples from control chickens on the day before challenge. Observe the chickens at least daily for 4 weeks. Take individual fresh faeces samples on day 1 after challenge and at least twice weekly (including day 7) until 14 days after challenge. Test the fresh faeces samples for the presence of *S. enterica* Typhimurium by direct plating. Euthanise all surviving chickens at the end of the observation period, take samples of liver and spleen and test for the presence of *S. enterica* Typhimurium by an appropriate method.

The test is not valid if antibodies against *S. enterica* Typhimurium are found in any control chicken before challenge.

The vaccine complies with the test if:

- the number of *S. enterica* Typhimurium in fresh faeces samples from vaccinated chickens after challenge at the different days of sampling is significantly lower in vaccinates than in controls and remains lower until the end of the test;
- the number of positive samples of liver and spleen is significantly lower in vaccinates than in controls.

2-3. *MANUFACTURER'S TEST*

2-3-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of the vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 15 SPF chickens (*5.2.2*). Maintain not fewer than 5 SPF chickens as controls. Administer to each of 10 chickens 1 dose of vaccine by a recommended route. Where the schedule stated on the label requires a booster injection to be given, a booster vaccination may also be given in this test provided it has been demonstrated that this will still provide a suitably sensitive test system. At a given interval after the last injection, collect blood from each vaccinated and control chicken and prepare serum samples. Measure the titre of antibodies against *S. enterica* Typhimurium in each serum sample using a suitable validated serological method. Calculate the titre for the group of vaccinates.

The test is not valid if specific *S. enterica* Typhimurium antibodies are found in 1 or more sera from control chickens at a given interval after the time of administration of the vaccine in the vaccinated group.

The vaccine complies with the test if the antibody titres of the group of vaccinates at a given interval after each vaccination, where applicable, are not significantly lower than the value obtained with a batch that has given satisfactory results in the test described under Potency (section 3-3).

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

**04/2013:2521**

# SALMONELLA TYPHIMURIUM VACCINE (LIVE, ORAL) FOR CHICKENS

## Vaccinum Salmonellae Typhimurium vivum perorale ad pullum

1. DEFINITION

Salmonella Typhimurium vaccine (live, oral) for chickens is a preparation of a suitable strain of live *Salmonella enterica* Typhimurium. This monograph applies to vaccines intended for the active immunisation of chickens against colonisation by and faecal excretion of *S. enterica* Typhimurium.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine strain is cultured in a suitable medium. During production, various parameters such as growth rate are monitored by suitable methods; the values are within the limits approved for the particular vaccine. Purity and identity of the cultures are verified on the harvest using a combination of methods such as morphological, serological and biochemical methods and culture on appropriate selective media. Suitable test(s) are conducted to confirm the presence of relevant marker(s). The harvests are shown to be pure and the results obtained from the tests for identity are in accordance with the documented characteristics of the strain.

2-2. *CHOICE OF VACCINE STRAIN*

The vaccine strain is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the chickens for which it is intended. During development, the safety of the vaccine for the persons handling the vaccine or vaccinated chickens has to be addressed as well as, in accordance with the requirements of general chapter *5.2.6*, the safety of the spread of the vaccine to other susceptible species. The strain has a stable marker or markers to distinguish it from wild-type strains.

The following tests described under General safety (section 2-2-1-1), Excretion, duration of excretion and survival in the environment (section 2-2-1-2), Spread of the vaccine strain (section 2-2-1-3), Dissemination and survival of the vaccine strain in vaccinated chickens after each vaccination (section 2-2-1-4), Increase in virulence (section 2-2-1-5), Field trials (section 2-2-1-6), Immunogenicity (sections 2-2-2-1 and 2-2-2-2) may be used during the demonstration of safety and efficacy.

For vaccines intended to prevent and/or reduce colonisation by and faecal excretion of *S. enterica* Typhimurium, the test for immunogenicity (section 2-2-2-1) is suitable to demonstrate that the vaccine is suitably immunogenic.

When the vaccine is recommended for use in laying chickens, the continuing immunogenicity of the vaccine until the end of the laying period has to be demonstrated and the test for immunogenicity at the end of the laying period (section 2-2-2-2) is suitable.

2-2-1. **Safety**. Unless otherwise indicated below, carry out each test by the oral route of administration, using chickens from a flock free from specified pathogens (SPF) (*5.2.2*) not older than the minimum age to be recommended for vaccination and that are free from antibodies against *Salmonella* spp. Where the vaccine is recommended for administration to 1-day-old chickens, the vaccine is administered before food is provided. Use vaccine bacteria at the least attenuated passage level that will be present in a batch of vaccine.

Measures taken to ensure absence of contamination by *Salmonella* spp. from the environment before the start of the test and on a regular ongoing basis are described and justified.

Whenever possible, items taken into the facilities are sterilised.

For re-isolation of the vaccine strain, suitably sensitive validated methods that are optimal for the vaccine strain concerned are used. The presence of relevant markers is confirmed to demonstrate that the organisms isolated are vaccine-derived and not wild-type contaminants.

2-2-1-1. *General safety*. For each test performed in chickens younger than 3 weeks of age, use not fewer than 10 chickens that are free from antibodies against *Salmonella* spp. Administer orally to each chicken a quantity of the vaccine strain equivalent to not less than 10 times the maximum titre(s) likely to be contained in 1 dose of the vaccine. Observe the chickens at least daily for at least 14 days.

The test is not valid if more than 10 per cent of the chickens younger than 3 weeks of age show abnormal signs or die from causes not attributable to the vaccine.

The vaccine complies with the test if no chicken shows abnormal signs of disease or dies from causes attributable to the vaccine.

2-2-1-2. *Excretion, duration of excretion and survival in the environment*. The same animals can be used for the test for spread of the vaccine strain (section 2-2-1-3) provided they are of the minimum age to be recommended for vaccination. Use for the test not fewer than 10 chickens. Administer orally to each chicken a quantity of the vaccine equivalent to not less than the maximum titre of the strain under study likely to be contained in 1 dose of the vaccine. Samples are collected for reisolation of vaccine from cloacal swabs from each chicken and floor faeces on days 3, 7, 10 and 14 after vaccination and then weekly until 3 consecutive negative weekly samples are obtained from all vaccinated chickens. Samples are collected for re-isolation of the vaccine strain from the caeca of vaccinates at the end of the test.

The test is not valid if more than 10 per cent of vaccinated chickens show abnormal clinical signs or die from causes not attributable to the vaccine. The results are noted and used to formulate the label statement on the length of time of excretion of the vaccine strain.

2-2-1-3. *Spread of the vaccine strain*. The same animals can be used for the test for excretion, duration of excretion and survival in the environment (section 2-2-1-2). Use for the test not fewer than 10 chickens of the minimum age to be recommended for vaccination. Use 10 chickens as controls. Administer orally to each chicken a quantity of the vaccine strain equivalent to not less than the maximum titre of the strain likely to be contained in 1 dose of the vaccine. 1 day after vaccination, mix the 10 vaccinates with at least 10 non-vaccinated chickens of the same age and source. Samples are collected for the re-isolation of the vaccine

strain from cloacal swabs from each chicken and floor faeces on days 3, 7, 10 and 14 after vaccination and then weekly until 3 consecutive negative weekly samples are obtained from all chickens. Collect samples of the caeca and spleens for re-isolation of the vaccine strain from 10 in-contact control chickens at the end of the test. The results are noted and used to formulate the label statement on the extent to which the vaccine spreads to in-contact non-vaccinated chickens.

2-2-1-4. *Dissemination and survival of the vaccine strain in vaccinated chickens after each vaccination.* Conduct the test after each vaccination as prescribed by the vaccination schedule to be recommended in chickens of each category for which the vaccine is intended with oral administration of the test preparation. Use a sufficient number of chickens to conduct the sampling described below, the number of chickens required being dependent on the number of vaccinations recommended, the interval between vaccinations and the length of time chickens are maintained after the last vaccination. Administer to each chicken a quantity of the vaccine strain equivalent to not less than the maximum titre of the strain under study likely to be contained in 1 dose of the vaccine. Collect cloacal swabs from each chicken for re-isolation of vaccinal bacteria on days 7 and 14 after each vaccination and at later appropriate stages and with sufficient frequency to determine the duration of dissemination.

For example, for broilers, samples are collected from 5 chickens for re-isolation of the vaccine strain on days 7 and 14 after each vaccination and weekly until 8 weeks of age. At the 7- and 14-day sampling points, samples are taken from the liver, caecum and spleen of 5 chickens.

In the case of chickens intended for laying, samples are collected from 5 chickens on days 7 and 14 after each vaccination and weekly until 3 consecutive negative weekly samples are obtained or the time of the next vaccination is reached, whichever is the sooner. At the 7- and 14- day sampling points, samples are taken from the liver, caecum and spleen of 5 chickens. In addition, samples are collected from ovaries and oviducts where dissemination in vaccinated future layers is being investigated.

The test is not valid if more than 10 per cent of vaccinated chickens in any group show abnormal clinical signs or die from causes not attributable to the vaccine. The results are noted and used to formulate the label statement on the length of time the vaccine strain survives in the body and to define a suitable withdrawal period.

2-2-1-5. *Increase in virulence.* Carry out the test according to general chapter *5.2.6* using SPF chickens (*5.2.2*) not older than the minimum age to be recommended for vaccination.

Administer orally to each chicken of the 1st group a quantity of the vaccine strain under study that will allow recovery of bacteria for the passages described below. 4 to 7 days after administration of the vaccine strain, prepare a suspension from the liver, spleen and caecum of chickens and pool these samples. Administer pooled samples orally to each chicken of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the bacteria at each passage. If the bacteria are not found at a passage level, repeat the passage by administration to a group of 10 chickens. Any mortalities are investigated for the presence of the vaccine strain and the properties of any re-isolated vaccine strain determined.

Carry out the test for excretion, duration of excretion and survival in the environment (section 2-2-1-2) and, if the last group of birds from which the bacteria was recovered shows evidence of an increase in virulence indicative of reversion during the observation period, carry out the test for general safety (section 2-2-1-1), using the material used for the 1st passage and the bacteria at the last passage level where it was recovered. Test the bacteria recovered for the final passage for the presence and stability of the marker(s).

The vaccine strain complies with the test if no indication of increased virulence of the bacteria recovered for the final passage compared with the material used for the 1st passage is observed and the presence of the marker(s) is confirmed in the bacteria recovered for the final passage and remains identical to the material used for the 1st passage. If the bacteria are not recovered after an initial passage in 5 animals and a subsequent repeat passage in 10 animals, the vaccine also complies with the test.

2-2-1-6. *Field trials.* The chickens used for field trials are also used to evaluate safety. A trial is carried out in each category of chickens for which the vaccine strain is intended, in not fewer than 2 sets of premises. Samples are taken from a significant number of chickens for re-isolation of bacteria to provide information on the persistence, dissemination and spread of the bacteria, which can be used, with the data from the laboratory studies, to formulate the statements on the label. The samples include cloacal swabs, floor faeces, spleen and liver and, in laying chickens, samples of ovaries and oviducts. Environmental samples are also tested at regular intervals.

2-2-2. **Immunogenicity**. The tests described in section 2-2-2-1 and, if appropriate, in section 2-2-2-2, are carried out using chickens not older than the minimum age to be recommended for vaccination and that are free from antibodies against *Salmonella* spp. The quantity of the vaccine strain to be administered orally to each chicken is not greater than the minimum titre to be stated on the label.

Measures taken to ensure absence of contamination by *Salmonella* spp. from the environment before the start of the test and on a regular ongoing basis are described and justified.

Suitably sensitive validated methods are used for re-isolation of bacteria derived from the challenge and for distinguishing these from the vaccine strain.

2-2-2-1. *Immunogenicity.* Use for the test not fewer than 40 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate according to the schedule to be recommended not fewer than 20 chickens with a single dose of vaccine. Maintain not fewer than 20 chickens as controls. Challenge each chicken after 14 days by a suitable route with a sufficient quantity of a virulent strain of *S. enterica* Typhimurium to give a valid test. Collect cloacal swabs from vaccinates and controls on days 3, 5, 7, 10 and 14 post-challenge. Samples of caecum, liver and spleen are collected from 10 chickens of each group on days 7 and 14 post-challenge for re-isolation of challenge bacteria. Collect the same samples of internal organs from any chicken that dies. Examine the samples for the presence of the challenge organisms using a suitable sensitive culture medium and compare results for vaccinates and controls.

The test is not valid if, during the observation period, fewer than 80 per cent of the control chickens die or challenge organisms are re-isolated from fewer than 80 per cent of the control chickens.

The vaccine complies with the test if there is a significant reduction in the number of cloacal swabs from vaccinates containing challenge organisms compared with the number from the controls and there is a significant reduction in the number of samples from internal organs from vaccinates containing challenge bacteria compared with the number from the controls.

2-2-2-2. *Immunogenicity at the end of the laying period.* Use for the test not fewer than 40 chickens of the same origin and from an SPF flock (*5.2.2*). Vaccinate not fewer than 20 chickens according to the schedule to be recommended. Maintain not fewer than 20 chickens as controls. At the end of the laying period, take serum samples and cloacal swabs from the chickens and environmental samples from the housing area. Test each serum sample individually for the presence of antibodies to *S. enterica* Typhimurium and each cloacal swab and fresh environmental sample for the presence of *Salmonella* spp. Challenge each chicken by a suitable route with a sufficient quantity of a virulent strain

of *S. enterica* Typhimurium to give a valid test. Collect cloacal swabs from vaccinates and controls on days 3, 5, 7, 10 and 14 post-challenge. Samples of caecum, liver, spleen, ovaries and oviducts are collected from 10 chickens of each group for re-isolation of challenge bacteria on days 7 and 14 post-challenge. Collect the same samples of internal organs from any chicken that dies during the observation period. Examine the samples for the presence of the challenge organisms with growth in a suitable medium and compare results for vaccinates and controls.

The test is not valid if, before the challenge, antibodies to *Salmonella* spp. are found in the serum of the controls or *Salmonella* spp. bacteria are isolated from any of the chickens. The test is also not valid if the challenge organisms are re-isolated from fewer than 80 per cent of the control chickens.

The vaccine complies with the test if there is a significant reduction in the number of cloacal swabs from vaccinates containing challenge organisms compared with the number from the controls and there is a significant decrease in the number of samples of internal organs from vaccinates containing challenge bacteria compared with the number from the controls.

3. BATCH TESTS

3-1. **Identification**. The strain present in the vaccine is identified by a combination of methods such as morphological, serological and biochemical methods and culture on appropriate selective media. Suitable test(s) are conducted to confirm the presence of the relevant marker(s).

3-2. **Bacteria and fungi**. Carry out the test by microscopic examination and by inoculation of suitable media, or verify the absence of micro-organisms other than the vaccine strain present in the vaccine as described in the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*. The vaccine complies with the test if it does not contain extraneous micro-organisms.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Live bacteria**. Titrate the vaccine strain using a suitable medium for the culture of the strain. The vaccine complies with the test if it contains not less than the titre stated on the label.

3-4. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-2-2-1) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum titre stated on the label.

4. LABELLING

The label states:

- the nature of the markers allowing the vaccine to be distinguished from wild-type strains;
- the extent to which the vaccine spreads and is transmitted to non-vaccinated chickens and the time over which this could occur;
- the time that the vaccine survives in the body;
- the length of time of excretion and the time that the vaccine survives in the environment;
- the potential for spread to other susceptible species including humans;
- the withdrawal period.

**01/2017:0064**

# SWINE ERYSIPELAS VACCINE (INACTIVATED)

## Vaccinum erysipelatis suillae inactivatum

1. DEFINITION

Swine erysipelas vaccine (inactivated) is a preparation of one or more suitable strains of *Erysipelothrix rhusiopathiae*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for the active immunisation of pigs against swine erysipelas.

2. PRODUCTION

The vaccine may be adjuvanted.

2-1. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the pigs for which it is intended.

The following tests for safety (section 2-1-1) and immunogenicity (section 2-1-2) may be used during the demonstration of safety and efficacy.

2-1-1. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and where applicable, in pigs of each category for which the vaccine is intended, using in each case pigs not older than the minimum age to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test, use not fewer than 8 pigs that do not have antibodies against swine erysipelas. Administer to each pig 1 dose of the vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the pigs at least daily until at least 14 days after the last administration. Record body temperatures the day before each vaccination, at vaccination, 2 h, 4 h and 6 h later and daily for 4 days; note the maximum temperature increase for each pig.

The vaccine complies with the test if no pig shows abnormal local or systemic reactions or signs of disease, or dies from causes attributable to the vaccine, if the average body temperature increase for all pigs does not exceed 1.5 °C, and if no pig shows a rise greater than 2.0 °C.

2-1-2. **Immunogenicity**. The test described below is suitable to demonstrate immunogenicity of the vaccine with respect to *E. rhusiopathiae* serotypes 1 and 2. If claims are made concerning another serotype, then a further test to demonstrate immunogenicity against this serotype is necessary.

If the vaccine contains more than 1 serotype, a test for 2 serotypes may be carried out on a single group by injecting each challenge serotype on different flanks of the pigs. Validation and acceptance criteria are applied separately to the respective injection sites. If the vaccine contains more than 1 serotype, the immunogenicity test may also be carried out using a separate group for each serotype.

A test is carried out for each route and method of administration to be recommended, using in each case pigs not less than 12 weeks old and weighing not less than 20 kg. The vaccine administered to each pig is of minimum potency.

For each test, use not fewer than 15 pigs that do not have antibodies against swine erysipelas. Divide the pigs into 2 groups. Vaccinate a group of not fewer than 10 pigs according to the schedule to be recommended. Maintain a group of not fewer than 5 pigs as controls. Challenge each pig 3 weeks after vaccination by the intradermal route by separate

injections of 0.1 mL of a virulent strain of each of serotype 1 and serotype 2 of *E. rhusiopathiae*. Observe the pigs at least daily for 7 days.

The test is not valid if fewer than 80 per cent of control pigs show typical signs of disease, i.e. diamond skin lesions at the injection sites. The vaccine complies with the test if not fewer than 90 per cent of the vaccinated pigs remain free from diamond skin lesions at the injection site.

*Swine erysipelas bacteria serotype 1 BRP* and *swine erysipelas bacteria serotype 2 BRP* are suitable for use as challenge strains.

2-2. *MANUFACTURER'S TESTS*

2-2-1. **Batch potency test**. It is not necessary to carry out the potency test (section 3-3) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use 10 mice of a suitable strain (for example, NMRI) weighing 17-20 g, from a uniform stock and that do not have antibodies against swine erysipelas. Vaccinate each mouse by the subcutaneous route with a suitable dose (usually 1/10 of the pig dose). At a given interval (for example, 21-28 days), depending on the vaccine to be examined, bleed the mice under anaesthesia. Pool the sera, using an equal volume from each mouse. Determine the level of antibodies by a suitable immunochemical method (*2.7.1*), for example, enzyme-linked immunosorbent assay with *erysipelas ELISA coating antigen BRP*. The vaccine complies with the test if the antibody level is not significantly less than that obtained with a batch that has given satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the tests mentioned under Immunogenicity (section 2-1-2) when administered by a recommended route and method.

**04/2013:0065**

# SWINE-FEVER VACCINE (LIVE, PREPARED IN CELL CULTURES), CLASSICAL

## Vaccinum pestis classicae suillae vivum ex cellulis

1. DEFINITION

Classical swine-fever vaccine (live, prepared in cell cultures) is a preparation obtained from a strain of classical swine-fever virus that has lost its pathogenicity for the pig by *in vivo* and/or *in vitro* passage and has been adapted to growth in cell cultures.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

**Cell cultures**. Cell cultures comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *CHOICE OF VACCINE VIRUS*

The vaccine virus is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the swine for which it is intended.

The following tests described under Safety test in piglets (section 2-3-1), Safety test in pregnant sows and test for transplacental transmission (section 2-3-2), Non-transmissibility (section 2-3-3), Increase in virulence (section 2-3-4) and Immunogenicity (section 2-3-5) may be used during the demonstration of safety and immunogenicity.

2-3-1. **Safety test in piglets**. Carry out the test for each route to be recommended using in each case piglets not older than the minimum age to be recommended for vaccination. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Use not fewer than 8 healthy piglets that do not have antibodies against pestiviruses. Administer to not fewer than 8 piglets a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the piglets at least daily for at least 14 days. The body temperature of each vaccinated piglet is measured on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 h after and then daily for at least 14 days. The vaccine complies with the test if the average body temperature increase for all piglets does not exceed 1.5 °C, no piglet shows a temperature rise greater than 1.5 °C for a period exceeding 3 days, and no piglet shows notable signs of disease or dies from causes attributable to the vaccine.

2-3-2. **Safety test in pregnant sows and test for transplacental transmission**. Carry out the test by a route to be recommended using not fewer than 8 healthy sows or gilts of the same age and origin, between the 55$^{th}$ and 80$^{th}$ days of gestation, and that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present in a batch of the vaccine.

Administer to not fewer than 8 sows or gilts a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Record the body temperature on at least the 3 days preceding administration of the vaccine, at the time of administration, 4 h after and then daily for at least 15 days. Observe until farrowing.

Carry out tests for serum antibodies against classical swine-fever virus. No antibodies against classical swine-fever virus are found in sera taken from the newborn piglets before ingestion of colostrum. The test is not valid if the vaccinated sows do not seroconvert. The vaccine virus complies with the test if no abnormalities in the gestation or in the piglets are noted, no sow or gilt shows a temperature rise greater than 1.5 °C for a period exceeding 5 days, and no sow or gilt shows notable signs of disease or dies from causes attributable to the vaccine.

2-3-3. **Non-transmissibility**. Keep together for the test not fewer than 12 healthy piglets, 6-10 weeks old and of the same origin, and that do not have antibodies against pestiviruses. Use vaccine virus at the least attenuated passage level that will be present between the master seed lot and a batch of the vaccine. Administer by a route to be recommended to not fewer than 6 piglets a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Maintain not fewer than 6 piglets as contact controls. The mixing of vaccinated piglets and contact piglets is done 24 h after vaccination.

After 45 days, euthanise all piglets. Carry out appropriate tests on the piglets to detect antibodies to classical swine-fever. Carry out appropriate tests on the control piglets to detect

classical swine-fever virus in the tonsils. The vaccine complies with the test if antibodies are found in all vaccinated piglets and if no antibodies and no virus are found in the control piglets.

2-3-4. **Increase in virulence**. Carry out the test according to chapter *5.2.6. Evaluation of safety of veterinary vaccines and immunosera*, using piglets 6-10 weeks old that do not have antibodies against pestiviruses. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise passage as described below is carried out.

Administer to each piglet of the 1st group by a route to be recommended a quantity of the vaccine virus equivalent to not less than the maximum virus titre likely to be contained in 1 dose of the vaccine. Collect an appropriate quantity of blood from each piglet daily between day 2 and day 7 after administration of the vaccine virus, and pool the samples taken on the same day. Administer 2 mL of the pooled blood with the highest virus titre by a route to be recommended to each piglet of the next group. Carry out this passage operation not fewer than 4 times, verifying the presence of the virus at each passage. If no virus is found, repeat the test once. If virus is found, carry out a 2nd series of passages by administering 2 mL of positive blood by a route to be recommended to each piglet of a group of 10 animals.

If the 5th group of animals shows no evidence of an increase in virulence indicative of reversion during the observation period, further testing is not required. Otherwise, carry out an additional safety test and compare the clinical signs and any relevant parameters in a group of at least 8 animals receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of increasing virulence of the virus recovered for the final passage compared with the material used for the 1st passage is observed. If virus is not recovered after an initial passage in 2 animals and a subsequent repeat passage in 10 animals, the vaccine virus also complies with the test.

2-3-5. **Immunogenicity**

2-3-5-1. *Protective dose*. The efficacy of the vaccine is expressed by the number of 50 per cent protective doses ($PD_{50}$) for pigs contained in the vaccinal dose as indicated on the label. The vaccine contains at least 100 $PD_{50}$ per dose.

Use 1 or more groups of piglets aged 6-10 weeks and that do not have antibodies against pestiviruses, and use an additional group of piglets of the same age and origin as controls. Each group of piglets is vaccinated with 1 dilution of the vaccine dose. 14 days after the single injection of vaccine, challenge the piglets by a suitable route with a suitable strain of virulent virus and a dose that kills not fewer than 50 per cent of the non-vaccinated piglets in less than 21 days. Observe the piglets for 21 days and record the body temperature 3 days before challenge and daily after challenge for 21 days. The $PD_{50}$ is calculated by the usual statistical methods (for example, *5.3*), taking into account the surviving piglets that have no clinical signs of swine fever, including cutaneous lesions or an increase in body temperature.

The test is not valid if fewer than 50 per cent of the control piglets display typical signs of serious infection with swine-fever virus, including cutaneous lesions, or die, and if fewer than 100 per cent of the control piglets show clinical signs of disease within the 21 days following challenge. The vaccine complies with the test if the minimum dose stated on the label corresponds to not less than 100 $PD_{50}$.

2-3-5-2. *Protection against transplacental infection*. Use at least 8 sows that do not have antibodies against pestiviruses, randomly allocated to either the vaccine group ($n$ = 6) or the control group ($n$ = 2).

Between the 40th and 50th day of gestation, all sows allocated to the vaccine group are vaccinated once with 1 dose of vaccine containing not more than the minimum titre stated on the label. On day 60 of gestation, all sows are challenged by a route to be recommended with a suitable strain of virulent virus. Just before farrowing and about 5-6 weeks after challenge, the sows are euthanised and their foetuses are examined for classical swine-fever virus. Serum samples from sows and foetuses are tested for the presence of antibodies against classical swine-fever virus. Isolation of classical swine-fever virus is carried out from blood of the sows (collected 7 and 9 days after challenge and at euthanasia), and from homogenised organ material (spleen, kidneys, lymph nodes) of the foetuses.

The test is not valid if one or more of the vaccinated sows do not seroconvert after the vaccination and the control sows do not seroconvert after the challenge, or if no virus is found in more than 50 per cent of the foetuses from the control sows (excluding mummified foetuses).

The vaccine complies with the test if no virus is found in the blood of vaccinated sows and in foetuses from the vaccinated sows, and no antibodies against classical swine-fever virus are found in the serum of the foetuses from the vaccinated sows.

3. BATCH TESTS

3-1. **Identification**

Specific classical swine-fever monoclonal antibodies are used to identify the vaccinal strain.

3-2. **Bacteria and fungi**. The vaccine, including where applicable, the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas** (*2.6.7*). The vaccine complies with the test for mycoplasmas.

3-4. **Extraneous agents**. Neutralise the vaccine virus using monoclonal antibodies to the vaccine virus. Inoculate into cell cultures known to be sensitive to viruses pathogenic for pigs and to pestiviruses. Maintain these cultures for not less than 14 days and carry out at least 3 passages during this period.

The vaccine complies with the test if no cytopathic effect is produced and if the cells show no evidence of the presence of haemadsorbing agents.

Use monoclonal antibodies that can identify possible contamination with pestiviruses. No virus is detected by an appropriate method.

3-5. **Virus titre**. Titrate the vaccine virus in suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum virus titre stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-3-5) when administered by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**04/2013:0697**
**corrected 8.3**

# TETANUS VACCINE FOR VETERINARY USE

## Vaccinum tetani ad usum veterinarium

1. DEFINITION

Tetanus vaccine for veterinary use is a preparation of the neurotoxin of *Clostridium tetani* inactivated to eliminate its toxicity while maintaining adequate immunogenic properties. The vaccine may be used to induce active and/or passive immunity.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

*C. tetani* used for production is grown in an appropriate liquid medium. The toxin is purified and then detoxified or it may be detoxified before purification. The antigenic purity is determined in Lf units of tetanus toxoid per milligram of protein and shown to be not less than the value approved for the particular product.

2-2. *CHOICE OF VACCINE COMPOSITION*

The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the animals for which it is intended. The following tests for production of antigens (section 2-2-1), safety (section 2-2-2) and immunogenicity (section 2-2-3) may be used during demonstration of safety and efficacy.

The *C. tetani* strain used in the preparation of the vaccine is shown to be satisfactory with respect to the production of the neurotoxin.

2-2-1. **Production of antigens**. The production of the neurotoxin of *C. tetani* is verified by a suitable immunochemical method (*2.7.1*) carried out on the neurotoxin obtained from the vaccine strain under the conditions used for the production of the vaccine.

2-2-2. **Safety**. Carry out the test for each route and method of administration to be recommended for vaccination and where applicable, in animals of each category for which the vaccine is intended, using in each case animals not older than the minimum age to be recommended for vaccination and of the most sensitive category for the species. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine.

For each test use not fewer than 8 animals, free from antitoxic antibodies. Administer to each animal 1 dose of vaccine. If the schedule to be recommended requires a 2nd dose, administer another dose after an interval of at least 14 days. Observe the animals at least daily until at least 14 days after the last administration.

The vaccine complies with the test if no animal shows abnormal local or systemic reactions or dies from causes attributable to the vaccine. If the test is carried out in pregnant animals, no adverse effects on gestation or the offspring are noted.

2-2-3. **Immunogenicity**

2-2-3-1. *Immunogenicity test in the target species*

It shall be demonstrated for each target species that the vaccine, when administered according to the schedule to be recommended and by the route to be recommended, stimulates an immune response (for example, induction of antitoxic antibodies or induction of protective levels of antitoxic antibodies) consistent with the claims made for the product.

2-2-3-2. *Immunogenicity test in guinea-pigs*

Administer 1 dose of vaccine by the subcutaneous route to each of at least 5 guinea-pigs that do not have antibodies against the neurotoxin of *C. tetani*. After 28 days, administer again to each guinea-pig 1 dose by the subcutaneous route. 14 days after the 2nd dose, collect blood from each guinea-pig and prepare serum samples. Determine for each serum the titre of antibodies against the neurotoxin of *C. tetani* using a suitable immunochemical method (*2.7.1*) such as a toxin-binding-inhibition test (ToBI test) and a homologous reference serum. Determine the average antibody titre of the serum samples.

*Clostridium tetani guinea-pig antiserum for vaccines for veterinary use BRP* is suitable for use as a reference serum.

Tetanus vaccine intended for use in animals other than horses complies with the test if the average antibody titre is not less than 7.5 IU/mL.

Tetanus vaccine intended for use in horses complies with the test if the average antibody titre is not less than 30 IU/mL.

For tetanus vaccine presented as a combined vaccine for use in animals other than horses, the above test may be carried out in susceptible rabbits instead of guinea-pigs. The vaccine complies with the test if the average antibody titre of the sera of the vaccinated rabbits is not less than 2.5 IU/mL.

*Clostridia (multicomponent) rabbit antiserum BRP* and *Clostridium tetani rabbit antiserum BRP* are suitable for use as reference sera.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Absence of toxin and irreversibility of toxoid**. Carry out a test for reversion to toxicity on the detoxified harvest using 2 groups of 5 guinea-pigs, each weighing 350-450 g; if the vaccine is adsorbed, carry out the test with the shortest practical time interval before adsorption. Prepare a dilution of the detoxified harvest so that the guinea-pigs each receive 10 times the amount of toxoid (measured in Lf units) that will be present in a dose of vaccine. Divide the dilution into 2 equal parts. Keep 1 part at 5 ± 3 °C and the other at 37 °C for 6 weeks. Attribute each dilution to a separate group of guinea-pigs and inject into each guinea-pig the dilution attributed to its group. Observe the animals at least daily for 21 days. The toxoid complies with the test if no guinea-pig shows signs of disease or dies from causes attributable to the neurotoxin of *C. tetani*.

2-3-2. **Batch potency test**. It is not necessary to carry out the potency test (section 3-4) for each batch of vaccine if it has been carried out using a batch of vaccine with a minimum potency. Where the test is not carried out, an alternative validated method is used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency.

Where the test described under Potency is used as the batch potency test, the vaccine complies with the test if the antibody titre in International Units is not less than that found for a batch of vaccine shown to be satisfactory with respect to immunogenicity in the target species.

3. BATCH TESTS

3-1. **Identification**

*If the nature of the adjuvant allows it, carry out test A. Otherwise carry out test B.*

A. Dissolve in the vaccine sufficient *sodium citrate R* to give a 100 g/L solution. Maintain the solution at 37 °C for about 16 h and centrifuge until a clear supernatant is obtained. The supernatant reacts with a suitable tetanus antitoxin, giving a precipitate.

B. When injected into animals that do not have antibodies against the neurotoxin of *C. tetani*, the vaccine stimulates the production of such antibodies.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Residual toxicity**. Administer 5 mL of the vaccine by the subcutaneous route, as 2 equal divided doses at separate sites, into each of 5 healthy guinea-pigs, each weighing 350-450 g, that have not previously been treated with any material that will interfere with the test. The vaccine complies with the test if no animal shows notable signs of disease or dies from causes attributable to the vaccine. If within 21 days of the injection any of the animals shows signs of or dies from tetanus, the vaccine does not comply with the test. If more than 1 animal dies from non-specific causes, repeat the test. If any animal dies in the 2nd test, the vaccine does not comply with the test.

3-4. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-3-2).

**04/2013:2461**

# TURKEY INFECTIOUS RHINOTRACHEITIS VACCINE (LIVE)

## Vaccinum rhinotracheitidis infectivae vivum ad meleagrem

1. DEFINITION

Turkey infectious rhinotracheitis vaccine (live) is a preparation of a suitable strain of turkey rhinotracheitis virus. This monograph applies to vaccines intended for administration to turkeys for active immunisation against turkey infectious rhinotracheitis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The vaccine virus is grown in cell cultures.

2-2. *SUBSTRATE FOR VIRUS PROPAGATION*

2-2-1. **Cell cultures**. The vaccine virus is grown in cell cultures that comply with the requirements for cell cultures for production of veterinary vaccines (*5.2.4*).

2-3. *SEED LOTS*

2-3-1. **Extraneous agents**. The master seed lot complies with the tests for extraneous agents in seed lots (*2.6.24*). In these tests on the master seed lot, the organisms used are not more than 5 passages from the master seed lot at the start of the test.

2-4. *CHOICE OF VACCINE VIRUS*

The vaccine virus shall be shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) for the turkeys for which it is intended.

The following tests for safety (section 2-4-1), increase in virulence (section 2-4-2) and immunogenicity (section 2-4-3) may be used during the demonstration of safety and efficacy.

2-4-1. **Safety**

*Safety for the respiratory tract*. Carry out the test using turkeys not older than the minimum age to be recommended for vaccination and free from antibodies against turkey rhinotracheitis virus. Use vaccine virus at the least attenuated passage level that will be present in a batch of vaccine.

For each test performed in turkeys younger than 3 weeks of age, use not fewer than 10 turkeys. For each test performed in turkeys older than 3 weeks of age, use not fewer than 8 turkeys.

Administer to each turkey, by the oculonasal route, a quantity of the vaccine virus equivalent to not less than 10 times the maximum virus titre likely to be contained in 1 dose of the vaccine. Observe the turkeys at least daily for at least 14 days and monitor clinical signs individually by a suitable scoring system. Mortality should be taken into account when calculating clinical scores. Record the death of any turkey and check for lesions of the respiratory tract.

The test is not valid if more than 10 per cent of the turkeys younger than 3 weeks of age show abnormal signs of disease or die from causes not attributable to the vaccine virus. For turkeys older than 3 weeks of age, the test is not valid if non-specific mortality occurs.

The vaccine virus complies with the test if no vaccinated turkey shows notable signs of disease or dies from causes attributable to the vaccine virus.

The clinical scores are used in the test described under 2-4-2.

2-4-2. **Increase in virulence**. Carry out the test according to general chapter *5.2.6* using turkeys younger than 3 weeks of age and free from antibodies against turkey rhinotracheitis virus. If the properties of the vaccine virus allow sequential passage through 5 groups via natural spreading, this method may be used, otherwise, passage as described below is carried out.

Administer to each turkey of the 1st group, by the oculonasal route, a quantity of the vaccine virus that will allow recovery of virus for the passages described below. 2-6 days after administration of the vaccine virus, prepare a suspension from the mucosa of the turbinates or the upper trachea, or from an oro-pharyngal or tracheal swab from not less than 5 inoculated turkeys and pool these samples. Administer 0.1 mL of the pooled samples by the oculonasal route to each turkey of the next group. Carry out this passage operation not fewer than 4 times; verify the presence of the virus at each passage. If the virus is not found at a passage level, repeat the passage by administration to a group of 10 turkeys.

If the 5th group of turkeys shows no evidence of an increase in virulence during the observation period, further testing is not required. Otherwise, carry out an additional safety test for the respiratory tract and compare the clinical signs and any relevant parameters in a group of at least 10 turkeys receiving the material used for the 1st passage and another similar group receiving the virus at the final passage level.

The vaccine virus complies with the test if no indication of an increased virulence of the virus at the final passage level compared with the material used for the 1st passage is observed or a slight increase in virulence of the virus at the final passage level may be observed for a vaccine which complies with the safety test (section 2-4-1). If virus is not recovered after an initial passage in 5 turkeys and a subsequent repeat passage in 10 turkeys, the vaccine virus also complies with the test.

2-4-3. **Immunogenicity**. A test is carried out for each route and method of administration to be recommended using turkeys not older than the minimum age to be recommended for vaccination and that are free from antibodies against turkey rhinotracheitis virus. The quantity of vaccine virus to be administered to each turkey is not greater than the minimum virus titre to be stated on the label and the virus is at the most attenuated passage level that will be present in a batch of the vaccine.

*Clinical protection against virulent challenge*. Use not fewer than 30 turkeys of the same origin and free from antibodies against turkey rhinotracheitis virus. Vaccinate by a route to be recommended not fewer than 20 turkeys according to the schedule to be recommended. Maintain not fewer than 10 turkeys as controls. Challenge each turkey after 21 days by the oculonasal route with a sufficient quantity of a suitable strain of virulent turkey rhinotracheitis virus. Observe the turkeys at least daily for 10 days and monitor clinical signs individually. Record the death of any turkey and check for lesions of the respiratory tract.

The test is not valid if one or more of the following applies:

– fewer than 80 per cent of the unvaccinated turkeys show typical signs of respiratory disease following challenge with the virulent turkey rhinotracheitis virus;

– during the period between vaccination and challenge, more than 10 per cent of vaccinated or control turkeys show abnormal clinical signs or die from causes not attributable to the vaccine.

The vaccine virus complies with the test if during the observation period after challenge not fewer than 90 per cent of the vaccinated turkeys survive and show no typical clinical signs or lesions of an infection with turkey rhinotracheitis virus.

3. BATCH TESTS

3-1. **Identification**. The vaccine, diluted if necessary and mixed with turkey rhinotracheitis virus antiserum specific for the virus subgroup, no longer infects susceptible cell cultures (*5.2.4*) into which it is inoculated. The vaccine may also be identified using appropriate molecular biology techniques (for example RT-PCR).

3-2. **Bacteria and fungi**. Vaccines intended for administration by injection comply with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

Any diluent supplied for reconstitution of the vaccine complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Mycoplasmas**. The vaccine complies with the test for mycoplasmas (*2.6.7*).

3-4. **Extraneous agents**. The vaccine complies with the tests for extraneous agents in batches of finished product (*2.6.25*).

3-5. **Virus titre**. Titrate the vaccine virus by inoculation into suitable cell cultures (*5.2.4*). The vaccine complies with the test if 1 dose contains not less than the minimum titre of vaccine virus stated on the label.

3-6. **Potency**. The vaccine complies with the requirements of the test prescribed under Immunogenicity (section 2-4-3) when administered according to the recommended schedule by a recommended route and method. It is not necessary to carry out the potency test for each batch of the vaccine if it has been carried out on a representative batch using a vaccinating dose containing not more than the minimum virus titre stated on the label.

**07/2017:1580**

# VIBRIOSIS (COLD-WATER) VACCINE (INACTIVATED) FOR SALMONIDS

## Vaccinum vibriosidis aquae frigidae inactivatum ad salmonidas

1. DEFINITION

Cold-water vibriosis vaccine (inactivated) for salmonids is prepared from cultures of one or more suitable strains of *Vibrio salmonicida*, inactivated while maintaining adequate immunogenic properties. This monograph applies to vaccines intended for administration by injection or immersion for the active immunisation of salmonids against cold-water vibriosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The strains of *V. salmonicida* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium.

2-2. *CHOICE OF VACCINE COMPOSITION*

The strain or strains of *V. salmonicida* used are shown to be suitable with respect to production of antigens of assumed protective importance. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in the species of fish for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Safety is tested using test 2-2-1-1-1, test 2-2-1-1-2, or both, depending on the recommendations for use.

Carry out the test in each species of fish for which the vaccine is intended, using fish of the minimum body mass to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. The test is carried out in the conditions to be recommended for use of the vaccine with a water temperature not less than 10 °C.

2-2-1-1-1. *Vaccines intended for administration by injection*. Use not fewer than 50 fish from a population that does not have specific antibodies against *V. salmonicida* and has not been vaccinated against or exposed to cold-water vibriosis. Administer to each fish by the intraperitoneal route 1 dose of the vaccine. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-1-2. *Vaccines intended for administration by immersion*. Use not fewer than 50 fish from a population that does not have specific antibodies against *V. salmonicida* and has not been vaccinated against or exposed to cold-water vibriosis. Prepare an immersion bath at twice the concentration to be recommended. Bathe the fish for twice the time to be recommended. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-2. *Field studies*. Safety is demonstrated in addition in field trials by administering the dose to be recommended to a sufficient number of fish distributed in not fewer than 2 sets of premises.

The vaccine complies with the test if no fish shows abnormal reactions or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. Carry out a separate test for each fish species and each strain included in the vaccine, according to a protocol defining water source, water flow, temperature limits, and preparation of a standardised challenge. Each test is carried out for each route and method of administration to be recommended. The vaccine administered to each fish is of minimum potency.

Use for the test not fewer than 60 fish of the minimum body mass to be recommended for vaccination, from a population that does not have specific antibodies against *V. salmonicida* and has not been vaccinated against or exposed to cold-water vibriosis. Vaccinate not fewer than 30 fish according to the instructions for use. Perform mock vaccination on a control group of not fewer than 30 fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or

mix equal numbers of controls and vaccinates in each tank if more than 1 tank is used. Where justified and when fish cannot be marked, non-marked fish may be used. Vaccinates and controls may then be kept in the same tank but physically separated (for example by fishing nets). Challenge each fish at a fixed interval after vaccination, corresponding to the onset of immunity claimed, by a suitable route, with a sufficient quantity of a culture of *V. salmonicida* whose virulence has been verified. Observe the fish at least daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time from challenge and determine by interpolation the time corresponding to 60 per cent specific mortality in controls.

The test is not valid if the specific mortality is less than 60 per cent in the control group 21 days after the 1st death in the fish. Read from the curve for vaccinates the mortality ($M$) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) using the following expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

The vaccine complies with the test if the RPS is not less than 60 per cent for vaccines administered by immersion and 90 per cent for vaccines administered by injection.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. The potency test (section 3-3) may be carried out for each batch of vaccine using fish of one of the species for which the vaccine is intended. Where the test is not carried out, an alternative validated method may be used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 35 fish from a population that does not have specific antibodies against *V. salmonicida* and that are within specified limits for body mass. Carry out the test at a defined temperature. Inject into each of not fewer than 25 fish 1 dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than 10 fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against *V. salmonicida* by a suitable immunochemical method (*2.7.1*). The test is not valid if the control group shows antibodies against *V. salmonicida*. The vaccine complies with the test if the mean level of antibodies in the vaccinates is not significantly lower than that found for a batch that gave satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

4. LABELLING

The label states information on the time needed for development of immunity after vaccination under the range of conditions corresponding to the recommended use.

07/2017:1581

# VIBRIOSIS VACCINE (INACTIVATED) FOR SALMONIDS

## Vaccinum vibriosidis inactivatum ad salmonidas

1. DEFINITION

Vibriosis vaccine (inactivated) for salmonids is prepared from cultures of one or more suitable strains or serovars of *Listonella anguillarum (Vibrio anguillarum)*, inactivated while maintaining adequate immunogenic properties; the vaccine may also include *Vibrio ordalii*. This monograph applies to vaccines intended for administration by injection or immersion for the active immunisation of salmonids against vibriosis.

2. PRODUCTION

2-1. *PREPARATION OF THE VACCINE*

The strains of *L. anguillarum* and *V. ordalii* are cultured and harvested separately. The harvests are inactivated by a suitable method. They may be purified and concentrated. Whole or disrupted cells may be used and the vaccine may contain extracellular products of the bacterium released into the growth medium.

2-2. *CHOICE OF VACCINE COMPOSITION*

The strains of *L. anguillarum* and *V. ordalii* used are shown to be suitable with respect to production of antigens of assumed protective importance. The vaccine is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*) in the species of fish for which it is intended.

The following tests for safety (section 2-2-1) and immunogenicity (section 2-2-2) may be used during the demonstration of safety and efficacy.

2-2-1. **Safety**

2-2-1-1. *Laboratory tests*. Safety is tested using test 2-2-1-1-1, test 2-2-1-1-2, or both, depending on the recommendations for use.

Carry out the test in each species of fish for which the vaccine is intended, using fish of the minimum body mass to be recommended for vaccination. Use a batch of vaccine containing not less than the maximum potency that may be expected in a batch of vaccine. The test is carried out in the conditions to be recommended for use of the vaccine with a water temperature not less than 10 °C.

2-2-1-1-1. *Vaccines intended for administration by injection*. Use not fewer than 50 fish from a population that does not have specific antibodies against *L. anguillarum* or where applicable *V. ordalii* and has not been vaccinated against or exposed to vibriosis. Administer to each fish by the intraperitoneal route 1 dose of the vaccine. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-1-2. *Vaccines intended for administration by immersion*. Use not fewer than 50 fish from a population that does not have specific antibodies against *L. anguillarum* or where applicable *V. ordalii* and has not been vaccinated against or exposed to vibriosis. Prepare an immersion bath at twice the concentration to be recommended. Bathe the fish for twice the time to be recommended. Observe the fish at least daily for 21 days.

The test is not valid if more than 6 per cent of the fish die from causes not attributable to the vaccine. The vaccine complies with the test if no fish shows abnormal local or systemic reactions or dies from causes attributable to the vaccine.

2-2-1-2. *Field studies*. Safety is demonstrated in addition in field trials by administering the dose to be recommended to a sufficient number of fish distributed in not fewer than 2 sets of premises.

The vaccine complies with the test if no fish shows abnormal reactions or dies from causes attributable to the vaccine.

2-2-2. **Immunogenicity**. Carry out a separate test for each fish species and each serovar included in the vaccine, according to a protocol defining water source, water flow and temperature limits, and preparation of a standardised challenge. Each test is carried out for each route and method of administration to be recommended. The vaccine administered to each fish is of minimum potency.

Use for the test not fewer than 60 fish of the minimum body mass to be recommended for vaccination, from a population that does not have specific antibodies against *L. anguillarum* or where applicable *V. ordalii* and has not been vaccinated against or exposed to vibriosis. Vaccinate not fewer than 30 fish according to the instructions for use. Perform mock vaccination on a control group of not fewer than 30 fish; mark vaccinated and control fish for identification. Keep all the fish in the same tank or mix equal numbers of controls and vaccinates in each tank if more than 1 tank is used. Where justified and when fish cannot be marked, non-marked fish may be used. Vaccinates and controls may then be kept in the same tank but physically separated (for example by fishing nets). Challenge each fish at a fixed interval after vaccination, corresponding to the onset of immunity claimed, by a suitable route with a sufficient quantity of cultures of *L. anguillarum* or *V. ordalii* whose virulence has been verified. Observe the fish at least daily until at least 60 per cent specific mortality is reached in the control group. Plot for both vaccinates and controls a curve of specific mortality against time from challenge and determine by interpolation the time corresponding to 60 per cent specific mortality in controls.

The test is not valid if the specific mortality is less than 60 per cent in the control group 21 days after the 1st death in the fish. Read from the curve for vaccinates the mortality ($M$) at the time corresponding to 60 per cent mortality in controls. Calculate the relative percentage survival (RPS) using the following expression:

$$\left(1 - \frac{M}{60}\right) \times 100$$

The vaccine complies with the test if the RPS is not less than 60 per cent for vaccines administered by immersion and 75 per cent for vaccines administered by injection.

2-3. *MANUFACTURER'S TESTS*

2-3-1. **Batch potency test**. The potency test (section 3-3) may be carried out for each batch of vaccine, using fish of one of the species for which the vaccine is intended. Where the test is not carried out, an alternative validated method may be used, the criteria for acceptance being set with reference to a batch of vaccine that has given satisfactory results in the test described under Potency. The following test may be used.

Use not fewer than 35 fish from a population that does not have specific antibodies against *L. anguillarum* included in the vaccine and where applicable against *V. ordalii*, and that are within specified limits for body mass. Carry out the test at a defined temperature. Inject into each of not fewer than 25 fish 1 dose of vaccine, according to the instructions for use. Perform mock vaccination on a control group of not fewer than 10 fish. Collect blood samples at a defined time after vaccination. Determine for each sample the level of specific antibodies against *L. anguillarum* included in the vaccine and where applicable against *V. ordalii*, by a suitable immunochemical method (*2.7.1*). The test is not valid if the control group shows antibodies against *L. anguillarum* or, where applicable, against *V. ordalii*. The vaccine complies with the test if the mean level of antibodies in the vaccinates is not significantly lower than that found for a batch that gave satisfactory results in the test described under Potency.

3. BATCH TESTS

3-1. **Identification**. The vaccine contains the antigen or antigens stated under Definition.

3-2. **Bacteria and fungi**. The vaccine, including where applicable the diluent supplied for reconstitution, complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

3-3. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-2-2) when administered by a recommended route and method.

4. LABELLING

The label states information on the time needed for the development of immunity after vaccination under the range of conditions corresponding to the recommended use.

# Immunosera for human use

Botulinum antitoxin.. ..... 1169
Diphtheria antitoxin.. ..... 1170
Gas-gangrene antitoxin, mixed.. ..... 1170
Gas-gangrene antitoxin (novyi).. ..... 1171
Gas-gangrene antitoxin (perfringens).. ..... 1171
Gas-gangrene antitoxin (septicum).. ..... 1172
Tetanus antitoxin for human use.. ..... 1173
Viper venom antiserum, European.. ..... 1174

**01/2020:0085**

# BOTULINUM ANTITOXIN

## Immunoserum botulinicum

DEFINITION

Liquid or freeze-dried preparation containing purified immunoglobulin fragments obtained from serum or plasma of immunised animals that have the power of specifically neutralising the toxins formed by *Clostridium botulinum* type A, type B or type E, or any mixture of these types.

PRODUCTION

Botulinum antitoxin is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *C. botulinum* type A, type B and type E toxins.

IDENTIFICATION

Botulinum antitoxin specifically neutralises the types of *C. botulinum* toxins stated on the label, rendering them harmless to susceptible animals. Alternatively, botulinum antitoxin can be identified by a suitable immunochemical method (*2.7.1*).

POTENCY

Not less than 500 IU of antitoxin per millilitre for each of types A and B and not less than 50 IU of antitoxin per millilitre for type E.

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The assay described below is associated with severe suffering of animals and manufacturers are strongly encouraged to develop and validate assays that will reduce the number of animals used, or refine or replace the test procedure with the goal of promoting animal welfare.

The potency of botulinum antitoxin is determined by comparing the dose necessary to protect mice against the lethal effects of a fixed dose of botulinum toxin with the quantity of the standard preparation of botulinum antitoxin necessary to give the same protection. For this comparison a reference preparation of each type of botulinum antitoxin, calibrated in International Units, and suitable preparations of botulinum toxins, for use as test toxins, are required. The potency of each test toxin is determined in relation to the specific reference preparation; the potency of the botulinum antitoxin to be examined is determined in relation to the potency of the test toxins by the same method.

International Units of the antitoxin are the specific neutralising activity for botulinum toxin type A, type B and type E contained in stated amounts of the International Standards that consist of dried immune horse sera of types A, B and E. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Selection of animals.* Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

*Preparation of test toxins. CAUTION: botulinum toxin is extremely toxic; exceptional care must be taken in any procedure in which it is employed.* Prepare types A, B and E toxins from sterile filtrates of approximately 7-day cultures in liquid medium of *C. botulinum* types A, B and E. To the filtrates add 2 volumes of glycerol, concentrate if necessary by dialysis against glycerol, and store at or slightly below 0 °C.

*Selection of test toxins.* Select toxins of each type for use as test toxins by determining for mice the L+/10 dose and the $LD_{50}$, the observation period being 96 h. The test toxins contain at least 1000 $LD_{50}$ in an L+/10 dose.

*Determination of test doses of the toxins* (L+/10 dose). Prepare solutions of the reference preparations in a suitable liquid such that each contains 0.25 IU of antitoxin per millilitre. Using each solution in turn, determine the test dose of the corresponding test toxin.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of a graded series of volumes of the test toxin and a sufficient volume of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using 4 mice for each mixture, inject a dose of 1.0 mL intraperitoneally into each mouse. Observe the mice for 96 h.

The test dose of toxin is the quantity in 1.0 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all 4 mice injected with the mixture within the observation period.

*Determination of potency of the antitoxin.* Prepare solutions of each reference preparation in a suitable liquid such that each contains 0.25 IU of antitoxin per millilitre.

Prepare solutions of each test toxin in a suitable liquid such that each contains 2.5 test doses per millilitre.

Using each toxin solution and the corresponding reference preparation in turn, determine the potency of the antitoxin. Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined, and a sufficient volume of a suitable liquid to bring the total volume to 5.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 mL) that contains 0.5 IU, and a sufficient volume of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using 4 mice for each mixture, inject a dose of 1.0 mL intraperitoneally into each mouse. Observe the mice for 96 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 0.5 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 mL or less of the solution of the reference preparation die and all those injected with mixtures containing more survive.

After validation with respect to the $LD_{50}$ method, the product potency may be assessed by other methods, for example mouse bioassays using paralysis or digit abduction as an end point, *ex vivo* assays using mouse phrenic nerve diaphragm, or cell-based or other suitable *in vitro* methods. Potency is calculated with respect to a suitable reference preparation calibrated in International Units.

LABELLING

The label states the types of *C. botulinum* toxin neutralised by the preparation.

01/2008:0086

# DIPHTHERIA ANTITOXIN

## Immunoserum diphthericum

DEFINITION

Diphtheria antitoxin is a preparation containing antitoxic globulins that have the power of specifically neutralising the toxin formed by *Corynebacterium diphtheriae*.

PRODUCTION

It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against diphtheria toxin.

IDENTIFICATION

It specifically neutralises the toxin formed by *C. diphtheriae*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1000 IU of antitoxin per millilitre for antitoxin obtained from horse serum. Not less than 500 IU of antitoxin per millilitre for antitoxin obtained from the serum of other mammals.

The potency of diphtheria antitoxin is determined by comparing the dose necessary to protect guinea-pigs or rabbits against the erythrogenic effects of a fixed dose of diphtheria toxin with the quantity of the standard preparation of diphtheria antitoxin necessary to give the same protection. For this comparison a reference preparation of diphtheria antitoxin, calibrated in International Units, and a suitable preparation of diphtheria toxin, for use as a test toxin, are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the diphtheria antitoxin to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for diphtheria toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Preparation of test toxin.* Prepare diphtheria toxin from cultures of *C. diphtheriae* in a liquid medium. Filter the culture to obtain a sterile toxic filtrate and store at 4 °C.

*Selection of test toxin.* Select a toxin for use as a test toxin by determining for guinea-pigs or rabbits the lr/100 dose and the minimal reacting dose, the observation period being 48 h. The test toxin has at least 200 minimal reacting doses in the lr/100 dose.

*Minimal reacting dose.* This is the smallest quantity of toxin which, when injected intracutaneously into guinea-pigs or rabbits, causes a small, characteristic reaction at the site of injection within 48 h.

The test toxin is allowed to stand for some months before being used for the assay of antitoxin. During this time its toxicity declines and the lr/100 dose may be increased. Determine the minimal reacting dose and the lr/100 dose at frequent intervals. When experiment shows that the lr/100 dose is constant, the test toxin is ready for use and may be used for a long period. Store the test toxin in the dark at 0 °C to 5 °C. Maintain its sterility by the addition of toluene or other antimicrobial preservative that does not cause a rapid decline in specific toxicity.

*Determination of test dose of toxin* (lr/100 dose). Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.1 IU of antitoxin per millilitre.

Prepare mixtures of the solution of the reference preparation and of the test toxin such that each contains 1.0 mL of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 15 min to 60 min. Using two animals for each mixture, inject a dose of 0.2 mL intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 h.

The test dose of toxin is the quantity in 0.2 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, a small but characteristic erythematous lesion at the site of injection.

*Determination of potency of the antitoxin.* Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.125 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains 12.5 test doses per millilitre.

Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined such that each contains 0.8 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 0.8 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (0.8 mL) that contains 0.1 IU and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 15 min to 60 min. Using two animals for each mixture, inject a dose of 0.2 mL intracutaneously into the shaven or depilated flanks of each animal. Observe the animals for 48 h.

The mixture that contains the largest volume of antitoxin that fails to protect the guinea-pigs from the erythematous effects of the toxin contains 0.1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the sites injected with mixtures containing 0.8 mL or less of the solution of the reference preparation show erythematous lesions and at all those injected with mixtures containing more there are no lesions.

01/2008:0090

# GAS-GANGRENE ANTITOXIN, MIXED

## Immunoserum gangraenicum mixtum

DEFINITION

Mixed gas-gangrene antitoxin is prepared by mixing gas-gangrene antitoxin (novyi), gas-gangrene antitoxin (perfringens) and gas-gangrene antitoxin (septicum) in appropriate quantities.

IDENTIFICATION

It specifically neutralises the alpha toxins formed by *Clostridium novyi* (former nomenclature: *Clostridium oedematiens*), *Clostridium perfringens* and *Clostridium septicum*, rendering them harmless to susceptible animals.

ASSAY

Gas-gangrene antitoxin (novyi), not less than 1000 IU of antitoxin per millilitre; gas-gangrene antitoxin (perfringens), not less than 1000 IU of antitoxin per millilitre; gas-gangrene antitoxin (septicum) not less than 500 IU of antitoxin per millilitre.

Carry out the assay for each component, as prescribed in the monographs on *Gas-gangrene antitoxin (novyi) (0087)*, *Gas-gangrene antitoxin (perfringens) (0088)* and *Gas-gangrene antitoxin (septicum) (0089)*.

**01/2008:0087**

# GAS-GANGRENE ANTITOXIN (NOVYI)

## Immunoserum gangraenicum (Clostridium novyi)

DEFINITION

Gas-gangrene antitoxin (novyi) is a preparation containing antitoxic globulins that have the power of neutralising the alpha toxin formed by *Clostridium novyi* (Former nomenclature: *Clostridium oedematiens*). It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. novyi* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. novyi*, rendering it harmless to susceptible animals.

ASSAY

Not less than 3750 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (novyi) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. novyi* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (novyi) necessary to give the same protection. For this comparison a reference preparation of gas-gangrene antitoxin (novyi), calibrated in International Units, and a suitable preparation of *Cl. novyi* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (novyi) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. novyi* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Selection of animals*. Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

*Preparation of test toxin*. Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. novyi*. Treat the filtrate with *ammonium sulfate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

*Selection of test toxin*. Select a toxin for use as a test toxin by determining for mice the L+ dose and the $LD_{50}$, the observation period being 72 h. The test toxin has an L+ dose of 0.5 mg or less and contains not less than 25 $LD_{50}$ in each L+ dose.

*Determination of test dose of toxin (L+ dose)*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 12.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 mL contains a precisely known amount such as 10 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 0.8 mL of the solution of the reference preparation, one of a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.2 mL intramuscularly into each mouse. Observe the mice for 72 h.

The test dose of toxin is the quantity in 0.2 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

*Determination of potency of the antitoxin*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 12.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains 12.5 test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 0.8 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 0.8 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (0.8 mL) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 2.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.2 mL intramuscularly into each mouse. Observe the mice for 72 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 0.8 mL or less of the solution of the reference preparation die and all those injected with mixtures containing a larger volume survive.

**01/2008:0088**

# GAS-GANGRENE ANTITOXIN (PERFRINGENS)

## Immunoserum gangraenicum (Clostridium perfringens)

DEFINITION

Gas-gangrene antitoxin (perfringens) is a preparation containing antitoxic globulins that have the power of specifically neutralising the alpha toxin formed by *Clostridium perfringens*. It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. perfringens* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. perfringens*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1500 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (perfringens) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. perfringens* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (perfringens) necessary to give the same protection. For this comparison a

reference preparation of gas-gangrene antitoxin (perfringens), calibrated in International Units, and a suitable preparation of *Cl. perfringens* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (perfringens) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. perfringens* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Selection of animals.* Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

*Preparation of test toxin.* Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. perfringens*. Treat the filtrate with *ammonium sulfate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

*Selection of test toxin.* Select a toxin for use as a test toxin by determining for mice the L+ dose and the $LD_{50}$, the observation period being 48 h. The test toxin has an L+ dose of 4 mg or less and contains not less than 20 $LD_{50}$ in each L+ dose.

*Determination of test dose of toxin (L+ dose).* Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 mL contains a precisely known amount such as 10 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 mL intravenously into each mouse. Observe the mice for 48 h.

The test dose of toxin is the quantity in 0.5 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

*Determination of potency of the antitoxin.* Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 mL) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 mL intravenously into each mouse. Observe the mice for 48 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 mL or less of the solution of the reference preparation die and all those injected with mixtures containing a larger volume survive.

**01/2008:0089**

# GAS-GANGRENE ANTITOXIN (SEPTICUM)

## Immunoserum gangraenicum (Clostridium septicum)

DEFINITION

Gas-gangrene antitoxin (septicum) is a preparation containing antitoxic globulins that have the power of specifically neutralising the alpha toxin formed by *Clostridium septicum*. It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against *Cl. septicum* alpha toxin.

IDENTIFICATION

It specifically neutralises the alpha toxin formed by *Cl. septicum*, rendering it harmless to susceptible animals.

ASSAY

Not less than 1500 IU of antitoxin per millilitre.

The potency of gas-gangrene antitoxin (septicum) is determined by comparing the dose necessary to protect mice or other suitable animals against the lethal effects of a fixed dose of *Cl. septicum* toxin with the quantity of the standard preparation of gas-gangrene antitoxin (septicum) necessary to give the same protection. For this comparison a reference preparation of gas-gangrene antitoxin (septicum), calibrated in International Units, and a suitable preparation of *Cl. septicum* toxin for use as a test toxin are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the gas-gangrene antitoxin (septicum) to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for *Cl. septicum* toxin contained in a stated amount of the International Standard, which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Selection of animals.* Use mice having body masses such that the difference between the lightest and the heaviest does not exceed 5 g.

*Preparation of test toxin.* Prepare the test toxin from a sterile filtrate of an approximately 5-day culture in liquid medium of *Cl. septicum*. Treat the filtrate with *ammonium sulfate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry.

*Selection of test toxin.* Select a toxin for use as a test toxin by determining for mice the L+ dose and the $LD_{50}$, the observation period being 72 h. The test toxin has an L+ dose of 0.5 mg or less and contains not less than 25 $LD_{50}$ in each L+ dose.

*Determination of test dose of toxin (L+ dose).* Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that 1 mL contains a precisely known amount such as 20 mg.

Prepare mixtures of the solution of the reference preparation and the solution of the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of

a graded series of volumes of the solution of the test toxin and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 mL intravenously into each mouse. Observe the mice for 72 h.

The test dose of toxin is the quantity in 0.5 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, the death of all six mice injected with the mixture within the observation period.

*Determination of potency of the antitoxin*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 mL) that contains 10 IU and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 mL intravenously into each mouse. Observe the mice for 72 h.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from death contains 10 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 mL or less of the solution of the reference preparation die and all those injected with mixtures containing more survive.

**01/2008:0091**

# TETANUS ANTITOXIN FOR HUMAN USE

## Immunoserum tetanicum ad usum humanum

DEFINITION

Tetanus antitoxin for human use is a preparation containing antitoxic globulins that have the power of specifically neutralising the toxin formed by *Clostridium tetani*.

PRODUCTION

It is obtained by fractionation from the serum of horses, or other mammals, that have been immunised against tetanus toxin.

IDENTIFICATION

It specifically neutralises the toxin formed by *Cl. tetani*, rendering it harmless to susceptible animals.

POTENCY

Not less than 1000 IU of antitoxin per millilitre when intended for prophylactic use. Not less than 3000 IU of antitoxin per millilitre when intended for therapeutic use.

The potency of tetanus antitoxin is determined by comparing the dose necessary to protect guinea-pigs or mice against the paralytic effects of a fixed dose of tetanus toxin with the quantity of the standard preparation of tetanus antitoxin necessary to give the same protection. In countries where the paralysis method is not obligatory the lethal method may be used. For this method the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than the onset of paralysis and the L+/10 dose is used instead of the Lp/10 dose. For this comparison a reference preparation of tetanus antitoxin, calibrated in International Units, and a suitable preparation of tetanus toxin, for use as a test toxin, are required. The potency of the test toxin is determined in relation to the reference preparation; the potency of the tetanus antitoxin to be examined is determined in relation to the potency of the test toxin by the same method.

The International Unit of antitoxin is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Selection of animals*. If mice are used, the body masses should be such that the difference between the lightest and the heaviest does not exceed 5 g.

*Preparation of test toxin*. Prepare the test toxin from a sterile filtrate of an approximately 9-day culture in liquid medium of *Cl. tetani*. To the filtrate add 1 to 2 volumes of glycerol and store slightly below 0 °C. Alternatively, treat the filtrate with *ammonium sulfate R*, collect the precipitate, which contains the toxin, dry *in vacuo* over *diphosphorus pentoxide R*, powder and store dry, either in sealed ampoules or *in vacuo* over *diphosphorus pentoxide R*.

*Determination of test dose of toxin (Lp/10 dose)*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre.

If the test toxin is stored dry, reconstitute it using a suitable liquid.

Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject a dose of 0.5 mL subcutaneously into each mouse. Observe the mice for 96 h. Mice that become paralysed may be euthanised.

The test dose of toxin is the quantity in 0.5 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis in all six mice injected with the mixture within the observation period.

*Determination of potency of the antitoxin*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre.

Prepare a solution of the test toxin in a suitable liquid such that it contains five test doses per millilitre.

Prepare mixtures of the solution of the test toxin and the antitoxin to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the antitoxin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 mL) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand at room temperature, protected from light, for 60 min. Using six mice for each mixture, inject into each mouse subcutaneously a dose of 0.5 mL. Observe the mice for 96 h. Mice that become paralysed may be euthanised.

The mixture that contains the largest volume of antitoxin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the antitoxin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 mL or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

**01/2008:0145**
**corrected 10.0**

# VIPER VENOM ANTISERUM, EUROPEAN

## Immunoserum contra venena viperarum europaearum

DEFINITION

European viper venom antiserum is a preparation containing antitoxic globulins that have the power of neutralising the venom of one or more species of viper. The globulins are obtained by fractionation of the serum of animals that have been immunised against the venom or venoms.

IDENTIFICATION

It neutralises the venom of *Vipera ammodytes*, or *Vipera aspis*, or *Vipera berus*, or *Vipera ursinii* or the mixture of these venoms stated on the label, rendering them harmless to susceptible animals.

ASSAY

Each millilitre of the preparation to be examined contains sufficient antitoxic globulins to neutralise not less than 100 mouse $LD_{50}$ of *Vipera ammodytes* venom or *Vipera aspis* venom and not less than 50 mouse $LD_{50}$ of the venoms of other species of viper.

The potency of European viper venom antiserum is determined by estimating the dose necessary to protect mice against the lethal effects of a fixed dose of venom of the relevant species of viper.

*Selection of test venoms.* Use venoms which have the normal physico-chemical, toxicological and immunological characteristics of venoms from the particular species of vipers. They are preferably freeze-dried and stored in the dark at 5 ± 3 °C.

Select a venom for use as a test venom by determining the $LD_{50}$ for mice, the observation period being 48 h.

*Determination of the test dose of venom.* Prepare graded dilutions of the reconstituted venom in a 9 g/L solution of *sodium chloride R* or other isotonic diluent in such a manner that the middle dilution contains in 0.25 mL the dose expected to be the $LD_{50}$. Dilute with an equal volume of the same diluent. Using at least four mice, each weighing 18 g to 20 g, for each dilution, inject 0.5 mL intravenously into each mouse. Observe the mice for 48 h and record the number of deaths. Calculate the $LD_{50}$ using the usual statistical methods.

*Determination of the potency of the antiserum to be examined.* Dilute the reconstituted test venom so that 0.25 mL contains the test dose of 5 $LD_{50}$ (test venom solution).

Prepare serial dilutions of the antiserum to be examined in a 9 g/L solution of *sodium chloride R* or other isotonic diluent, the dilution factor being 1.5 to 2.5. Use a sufficient number and range of dilutions to enable a mortality curve between 20 per cent and 80 per cent mortality to be established and to permit an estimation of the statistical variation.

Prepare mixtures such that 5 mL of each mixture contains 2.5 mL of one of the dilutions of the antiserum to be examined and 2.5 mL of the test venom solution. Allow the mixtures to stand in a water-bath at 37 °C for 30 min. Using not fewer than six mice, each weighing 18 g to 20 g, for each mixture, inject 0.5 mL intravenously into each mouse. Observe the mice for 48 h and record the number of deaths. Calculate the $PD_{50}$, using the usual statistical methods. At the same time verify the number of $LD_{50}$ in the test dose of venom, using the method described above. Calculate the potency of the antiserum using the following expression:

$$\frac{(T_v - 1)}{PD_{50}}$$

$T_v$ = number of $LD_{50}$ in the test dose of venom.

In each mouse dose of the venom-antiserum mixture at the end point there is one $LD_{50}$ of venom remaining unneutralised by the antiserum and it is this unneutralised venom that is responsible for the deaths of 50 per cent of the mice inoculated with the mixture. The amount of venom neutralised by the antiserum is thus one $LD_{50}$ less than the total amount contained in each mouse dose. Therefore, as the potency of the antiserum is defined in terms of the number of $LD_{50}$ of venom that are neutralised. rather than the number of $LD_{50}$ in each mouse dose, the expression required in the calculation of potency is $T_v - 1$ rather than $T_v$.

Alternatively, the quantity of test venom in milligrams that is neutralised by 1 mL or some other defined volume of the antiserum to be examined may be calculated.

LABELLING

The label states the venom or venoms against which the antiserum is effective.

*CAUTION: because of the allergenic properties of viper venoms, inhalation of venom dust should be avoided by suitable precautions.*

# Immunosera for veterinary use

Tetanus antitoxin for veterinary use..... 1177

**01/2008:0343**

# TETANUS ANTITOXIN FOR VETERINARY USE

## Immunoserum tetanicum ad usum veterinarium

DEFINITION

Tetanus antitoxin for veterinary use is a preparation containing principally the globulins that have the power of specifically neutralising the neurotoxin formed by *Clostridium tetani*. It consists of the serum or a preparation obtained from the serum of animals immunised against tetanus toxin.

PRODUCTION

*CHOICE OF COMPOSITION*

The antitoxin is shown to be satisfactory with respect to safety (*5.2.6*) and efficacy (*5.2.7*). For the latter, it shall be demonstrated, for each target species, that the product, when administered at the minimum recommended dose and according to the recommended schedule(s), provides a response or responses consistent with the claims made for the product. The ability of the product to neutralise the neurotoxin formed by *C. tetani* must also be demonstrated, e.g. by conducting the test in mice as described below.

**Demonstration of neurotoxin neutralisation**. The ability of tetanus antitoxin to neutralise the neurotoxin of *C. tetani* is determined by establishing the dose necessary to protect mice (or guinea-pigs) against the toxic effects of a fixed dose of tetanus toxin. The test must be conducted in parallel with a test of a reference preparation of tetanus antitoxin, calibrated in International Units, using a quantity expected to give the same protection. The ability of the test antitoxin to neutralise the neurotoxin (potency) can then be expressed in International Units. For this study, a suitable preparation of tetanus toxin for use as a test toxin is required. The dose of the test toxin is determined in relation to the reference preparation; the potency of the antitoxin to be examined is determined in relation to the reference preparation using the test toxin.

**Preparation of test toxin**. Prepare the test toxin from a sterile filtrate of an 8-10 day culture in liquid medium of *C. tetani*. A test toxin may be prepared by adding this filtrate to *glycerol R* in the proportion of 1 volume of filtrate to 1 to 2 volumes of *glycerol R*. The solution of test toxin may be stored at or slightly below 0 °C. The toxin may also be dried by a suitable method. Select the test toxin by determining for mice the Lp/10 dose and the paralytic dose 50 per cent. A suitable toxin contains not less than 1000 times the paralytic dose 50 per cent in 1 Lp/10 dose.

**Lp/10 dose (Limes paralyticum)**. This is the smallest quantity of toxin which when mixed with 0.1 IU of antitoxin and injected subcutaneously into mice (or guinea-pigs) causes tetanic paralysis in the animals on or before the 4$^{th}$ day after injection.

**Paralytic dose 50 per cent**. This is the quantity of toxin which when injected subcutaneously into mice (or guinea-pigs) causes tetanic paralysis in one half of the animals on or before the 4$^{th}$ day after injection.

**Determination of test dose of toxin**. Reconstitute or dilute the reference preparation with a suitable liquid so that it contains 0.5 IU/mL. Measure or weigh a quantity of the test toxin and dilute with or dissolve in a suitable liquid. Prepare mixtures of the solution of the reference preparation and the solution of the test toxin so that each mixture will contain 0.1 IU of antitoxin in the volume chosen for injection and one of a series of graded volumes of the solution of the test toxin, separated from each other by steps of not more than 20 per cent and covering the expected end-point. Adjust each mixture with a suitable liquid to the same final volume (0.4 mL to 0.6 mL if mice are used for the test or 4.0 mL if guinea-pigs are used). Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. Repeat the test at least once and calculate the test dose as the mean of the different tests. The test dose of the toxin is the amount present in that mixture which causes tetanic paralysis in one half of the total number of animals injected with it.

**Determination of the neutralising ability of the antitoxin to be examined**

*Preliminary test*. Measure or weigh a quantity of the test toxin and dilute with or dissolve in a suitable liquid so that the solution contains 5 test doses per millilitre (solution of the test toxin). Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined so that for each mixture the volume chosen for injection contains the test dose of toxin and one of a series of graded volumes of the antitoxin to be examined. Adjust each mixture to the same final volume with a suitable liquid. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. Using the results, select suitable mixtures for the final test.

*Final test*. Prepare mixtures of the solution of the test toxin and of the antitoxin to be examined so that for each mixture the volume chosen for the injection contains the test dose of toxin and one of a series of graded volumes of the antitoxin to be examined, separated from each other by steps of not more than 20 per cent and covering the expected end-point as determined in the preliminary test. Prepare further mixtures with the same amount of test toxin and graded volumes of the reference preparation, centred on 0.1 IU in the volume chosen for injection, to confirm the test dose of the toxin. Adjust each mixture to the same final volume with a suitable liquid. Allow the mixtures to stand at room temperature for 60 min. Using not fewer than 2 animals for each mixture, inject the chosen volume subcutaneously into each animal. Observe the animals for 96 h and make daily records of the degree of tetanus developing in each group of animals. The test mixture which contains 0.1 IU in the volume injected is that mixture which causes tetanic paralysis in the same, or almost the same, number of animals as the reference mixture containing 0.1 IU in the volume injected. Repeat the determination at least once and calculate the mean of all valid estimates. Estimates are valid only if the reference preparation gives a result within 20 per cent of the expected value.

The confidence limits ($P$ = 0.95) have been estimated to be:

- 85 per cent and 114 per cent when 2 animals per dose are used;
- 91.5 per cent and 109 per cent when 3 animals per dose are used;
- 93 per cent and 108 per cent when 6 animals per dose are used.

IDENTIFICATION

The antitoxin is shown, by a suitable immunochemical method (*2.7.1*), to react specifically with the neurotoxin formed by *C. tetani*. The potency test may also serve for identification.

POTENCY

Determine the titre of antibodies against the neurotoxin formed by *C. tetani* using a suitable immunochemical method (*2.7.1*) such as a toxin-binding-inhibition test (ToBI test) and a homologous reference serum, calibrated in International Units per millilitre.

The International Unit is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard which consists of a quantity of dried immune horse serum. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The potency of the finished product is expressed in International Units per millilitre and is shown to be not less than the minimum number stated on the label.

# Radiopharmaceutical preparations and starting materials for radiopharmaceutical preparations


Alovudine ($^{18}$F) injection.. 1181
Ammonia ($^{13}$N) injection.. 1183
Carbon monoxide ($^{15}$O).. 1184
Choline ([$^{11}$C]methyl) injection.. 1185
Chromium ($^{51}$Cr) edetate injection.. 1186
Copper tetramibi tetrafluoroborate for radiopharmaceutical preparations.. 1187
Cyanocobalamin ($^{57}$Co) capsules.. 1188
Cyanocobalamin ($^{57}$Co) solution.. 1188
Cyanocobalamin ($^{58}$Co) capsules.. 1189
Cyanocobalamin ($^{58}$Co) solution.. 1189
Fludeoxyglucose ($^{18}$F) injection.. 1190
Flumazenil (*N*-[$^{11}$C]methyl) injection.. 1192
Fluoride ($^{18}$F) solution for radiolabelling.. 1194
Fluorocholine ($^{18}$F) injection.. 1195
Fluorodopa ($^{18}$F) (prepared by electrophilic substitution) injection.. 1197
Fluorodopa ($^{18}$F) (prepared by nucleophilic substitution) injection.. 1198
Fluoroethyl-L-tyrosine ($^{18}$F) injection.. 1201
Fluoromisonidazole ($^{18}$F) injection.. 1204
Gallium ($^{67}$Ga) citrate injection.. 1206
Gallium ($^{68}$Ga) chloride solution for radiolabelling.. 1206
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Raclopride ([$^{11}$C]methoxy) injection.. 1224
Sodium acetate ([1-$^{11}$C]) injection.. 1225
Sodium chromate ($^{51}$Cr) sterile solution.. 1226
Sodium fluoride ($^{18}$F) injection.. 1227
Sodium iodide ($^{123}$I) injection.. 1228
Sodium iodide ($^{123}$I) solution for radiolabelling.. 1228
Sodium iodide ($^{131}$I) capsules for diagnostic use.. 1229
Sodium iodide ($^{131}$I) capsules for therapeutic use.. 1230
Sodium iodide ($^{131}$I) solution.. 1231
Sodium iodide ($^{131}$I) solution for radiolabelling.. 1232
Sodium iodohippurate ($^{123}$I) injection.. 1233
Sodium iodohippurate ($^{131}$I) injection.. 1234
Sodium iodohippurate dihydrate for radiopharmaceutical preparations.. 1234
Sodium molybdate ($^{99}$Mo) solution (fission).. 1235
Sodium pertechnetate ($^{99m}$Tc) injection (accelerator-produced).. 1237
Sodium pertechnetate ($^{99m}$Tc) injection (fission).. 1238
Sodium pertechnetate ($^{99m}$Tc) injection (non-fission).. 1240
Sodium phosphate ($^{32}$P) injection.. 1240
Sodium pyrophosphate decahydrate for radiopharmaceutical preparations.. 1241
Strontium ($^{89}$Sr) chloride injection.. 1242
Technetium ($^{99m}$Tc) bicisate injection.. 1243
Technetium ($^{99m}$Tc) colloidal rhenium sulfide injection ... 1244
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Technetium ($^{99m}$Tc) etifenin injection.. 1246
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Technetium ($^{99m}$Tc) gluconate injection.. 1248
Technetium ($^{99m}$Tc) human albumin injection.. 1249
Technetium ($^{99m}$Tc) macrosalb injection.. 1251
Technetium ($^{99m}$Tc) mebrofenin injection.. 1252
Technetium ($^{99m}$Tc) medronate injection.. 1253
Technetium ($^{99m}$Tc) mertiatide injection.. 1254
Technetium ($^{99m}$Tc) microspheres injection.. 1255
Technetium ($^{99m}$Tc) oxidronate injection.. 1256
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Technetium ($^{99m}$Tc) tin pyrophosphate injection.. 1260
Tetra-*O*-acetyl-mannose triflate for radiopharmaceutical preparations.. 1261
Thallous ($^{201}$Tl) chloride injection.. 1262
Tritiated ($^{3}$H) water injection.. 1263
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**01/2014:2460**

# ALOVUDINE ($^{18}$F) INJECTION

## Alovudini ($^{18}$F) solutio iniectabilis

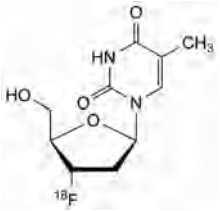

$C_{10}H_{13}{}^{18}FN_2O_4$ $M_r$ 243.2

DEFINITION

Sterile solution containing 1-[(2*R*,4*S*,5*R*)-4-[$^{18}$F]fluoro-5-(hydroxymethyl)tetrahydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (3′-deoxy-3′-[$^{18}$F]fluorothymidine, [$^{18}$F]fluorodeoxythymidine, [$^{18}$F]FLT). It may contain a suitable buffer.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label;
– *alovudine*: maximum 0.1 mg per maximum recommended dose in millilitres.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for [$^{18}$F]alovudine under radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH**: 4.5 to 8.5, using a *pH indicator strip R*.

**Impurity A**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity A) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; as an additional spot, apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

– the spot due to the application of both the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity A) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;
– the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;
– the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

– the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.170 g of *tetrabutylammonium hydroxide R* in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80.0 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 0.95 g/L solution of *toluenesulfonic acid R*, *acetonitrile R* (25:75 *V*/*V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity B.

*Retention time*: impurity B = about 3.3 min.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak;
– *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**Alovudine and related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 5.0 mg of *alovudine R* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 5.0 mg of *stavudine R* (impurity C) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

*Blank solution.* Prepare a solution containing each excipient at the concentration used in the preparation.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *carbon dioxide-free water R*, protected from the atmosphere during chromatography;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 90 | 10 |
| 10 - 20 | 90 → 5 | 10 → 95 |
| 20 - 30 | 5 | 95 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 270 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Relative retention* with reference to alovudine (retention time = about 8 min): impurity C = about 0.6.

*System suitability*: reference solution (c) using the spectrophotometer:

– *resolution*: minimum 5.0 between the peaks due to impurity C and alovudine.

*Limits*: in the chromatogram obtained with the spectrophotometer:

– *alovudine*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);

– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 mg/*V*);

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 mg/*V*);

– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 mg/*V*).

**Ethanol** (*2.4.24* or another suitable, validated method): maximum 10 per cent *V/V* and maximum 2.5 g per administration, taking the density (*2.2.5*) to be 0.790 g/mL.

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]Alovudine**. Liquid chromatography (*2.2.29*) as described in the test for alovudine and related substances. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to [$^{18}$F]alovudine by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer.

*Limit*:

– *[$^{18}$F]alovudine*: minimum 95 per cent of the total radioactivity due to fluorine-18.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (5:95 *V/V*).

*Application*: about 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity D = about 0; [$^{18}$F]alovudine = about 0.7.

*Limit*:

– *impurity D*: maximum 5 per cent of the total radioactivity due to fluorine-18.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the percentage content of ethanol in the preparation.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance)*: E, F.*

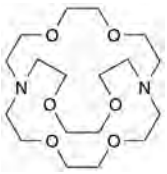

A. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (aminopolyether),

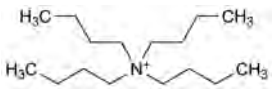

B. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

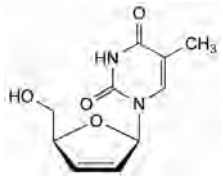

C. 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (stavudine),

D. [$^{18}$F]fluoride,

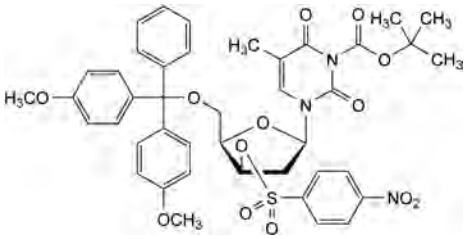

E. *tert*-butyl 3-[(2*R*,4*R*,5*R*)-5-[[bis(4-methoxyphenyl)phenylmethoxy]methyl]-4-[[(4-nitrophenyl)sulfonyl]oxy]tetrahydrofuran-2-yl]-5-methyl-2,6-dioxo-3,6-dihydropyrimidine-1(2*H*)-carboxylate,

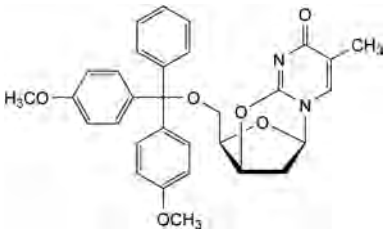

F. (2*R*,3*R*,5*R*)-3-[[bis(4-methoxyphenyl)phenylmethoxy]-methyl]-8-methyl-2,3-dihydro-9*H*-2,5-methanopyrimido-[2,1-*b*][1,5,3]dioxazepin-9-one.

**01/2008:1492**
**corrected 7.0**

# AMMONIA ($^{13}$N) INJECTION

## Ammoniae ($^{13}$N) solutio iniectabilis

DEFINITION

Sterile solution of [$^{13}$N]ammonia for diagnostic use.

*Nitrogen-13*: 90 per cent to 110 per cent of the declared nitrogen-13 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of nitrogen-13*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Test A for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution has approximately the same retention time as the principal peak in the radiochromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 5.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

**Aluminium**: maximum 2 ppm. The preparation may be released for use before completion of the test.

*Test solution.* In a test-tube about 12 mm in internal diameter, mix 1 mL of *acetate buffer solution pH 4.6 R* and 2 mL of a 1 in 20 dilution of the preparation to be examined in *water R*. Add 0.05 mL of a 10 g/L solution of *chromazurol S R*.

*Reference solution*. Prepare at the same time and in the same manner as the test solution using 2 mL of a 1 in 20 dilution of *aluminium standard solution (2 ppm Al) R*.

After 3 min, the colour of the test solution is not more intense than that of the reference solution.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of tests A and B.

A. *Half-life*. The half-life is between 9 min and 11 min.

B. *Gamma emitting impurities*: maximum 1.0 per cent of the total radioactivity.

*Gamma-ray spectrometry*. Retain a sample of the preparation to be examined for 2 h. Examine the gamma-ray spectrum of the decayed material for the presence of radionuclidic impurities, which should, where possible, be identified and quantified.

*RADIOCHEMICAL PURITY*

**[$^{13}$N]Ammonia**. Liquid chromatography (*2.2.29*).

The preparation may be released for use before completion of the test.

*Test solution.* The preparation to be examined.

*Reference solution.* Dilute 1.0 mL of *dilute ammonia R2* to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.04 m, Ø = 4.0 mm;
– *stationary phase*: *cation-exchange resin R* (10 µm);
– *temperature*: constant at 20-30 °C.

*Mobile phase*: *0.002 M nitric acid*.

*Flow rate*: 2 mL/min.

*Detection*: suitable radioactivity detector and conductivity detector.

*System suitability*: the chromatogram obtained with the test solution and the radioactivity detector shows a principal peak with approximately the same retention time as the peak in the chromatogram obtained with the reference solution and the conductivity detector.

*Limit*:

– *[$^{13}$N]ammonia*: minimum 99 per cent of the total radioactivity due to nitrogen-13.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{13}$N]$O_2^-$,

B. [$^{13}$N]$O_3^-$,

C. [$^{18}$F$^-$],

D. $H_2$[$^{15}$O].

**01/2008:1607**

# CARBON MONOXIDE ($^{15}$O)

## Carbonei monoxidum ($^{15}$O)

DEFINITION

Mixture of carbon [$^{15}$O]monoxide in the gaseous phase and a suitable vehicle such as *Medicinal air (1238)*, for diagnostic use.

*Purity*:

- minimum 99 per cent of the total radioactivity corresponds to oxygen-15,
- minimum 97 per cent of the total radioactivity corresponds to oxygen-15 in the form of carbon monoxide (CO).

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Oxygen-15 is a radioactive isotope of oxygen which may be produced by various nuclear reactions such as proton irradiation of nitrogen-15 or deuteron irradiation of nitrogen-14.

*RADIOCHEMICAL SYNTHESIS*

In order to recover oxygen-15 as molecular oxygen from the nitrogen target gas, carrier oxygen is added at concentrations generally ranging from 0.2 per cent *V/V* to 1.0 per cent *V/V*. After irradiation, the target gas is usually reacted with activated charcoal at a temperature of about 950 °C. The activated charcoal is preconditioned before use by flushing an inert gas at the production flow rate at a temperature of about 950 °C for not less than 1 h. The carbon [$^{15}$O]monoxide obtained is purified by passage through a carbon dioxide scavenger, such as soda lime, before mixing with the vehicle.

CHARACTERS

*Appearance*: colourless gas.

*Half-life and nature of radiation of oxygen-15*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peaks in the chromatogram obtained with the test gas using the radioactivity detector are similar in retention times to the principal peaks corresponding to carbon monoxide in the chromatogram obtained with reference gas (a) using the thermal conductivity detector.

TESTS

*The following tests are performed on carbon [$^{15}$O]monoxide as described under radiochemical synthesis before mixing with the vehicle.*

**Carbon monoxide**. Gas chromatography (*2.2.28*) as described in the test for radiochemical purity.

The concentration of carbon monoxide in the test sample is determined before administration and is used to calculate the amount of carbon monoxide to be administered to the patient.

*Injection*: test sample, reference gas (b).

Examine the chromatogram obtained with the thermal conductivity detector and calculate the content of carbon monoxide.

*RADIONUCLIDIC PURITY*

**Oxygen-15**: minimum 99 per cent of the total radioactivity.

A. Gamma spectrometry.

*Comparison*: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 1.9 min to 2.2 min.

The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Carbon [$^{15}$O]monoxide**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test sample.* Carbon [$^{15}$O]monoxide as described under radiochemical synthesis.

*Reference gas (a). Nitrogen gas mixture R.*

*Reference gas (b). Nitrogen R*, containing 2.0 per cent *V/V* of *carbon monoxide R1*.

*Column*:

- *size*: *l* = 1.8 m, Ø1 = 6.3 mm and Ø2 = 3.2 mm,
- *stationary phase*: *GC concentrical column R*,

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 65 mL/min.

*Temperature*:

- *column*: 40 °C,
- *injection port*: 40 °C,
- *thermal conductivity detector*: 70 °C.

*Detection*: thermal conductivity detector and radioactivity detector connected in series.

*Injection*: loop injector.

*Run time*: 10 min.

*Retention times*: oxygen, nitrogen and carbon monoxide eluting from the inner column = about 0.4 min; carbon dioxide eluting from the inner column = about 0.8 min; oxygen eluting from the outer column = about 2.1 min; nitrogen eluting from the outer column = about 3.1 min; carbon monoxide eluting from the outer column = about 6.2 min.

*System suitability*: reference gas (a):

- 5 clearly separated principal peaks are observed in the chromatogram obtained using the thermal conductivity detector,
- *resolution*: minimum of 1.5 between the peaks due to carbon dioxide eluting from the inner column and oxygen eluting from the outer column, in the chromatogram obtained using the thermal conductivity detector.

*Limits*: examine the chromatogram obtained with the radioactivity detector and calculate the percentage content of oxygen-15 substances from the peak areas.

- *carbon [$^{15}$O]monoxide*: minimum 97 per cent of the total radioactivity.
- *disregard* the first peak corresponding to components co-eluting from the inner column.

RADIOACTIVITY

The radioactive concentration is determined before administration.

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

**04/2018:2462**
**corrected 10.0**

# CHOLINE ([$^{11}$C]METHYL) INJECTION

## Cholini ([$^{11}$C]methyl) solutio iniectabilis

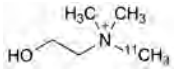

$^{11}C_1C_4H_{14}NO^+$ $M_r$ 103.2
[94793-58-5]

DEFINITION

Sterile solution containing 2-hydroxy-*N*-[$^{11}$C]methyl-*N*,*N*-dimethylethan-1-aminium ([methyl-$^{11}$C]choline) ions. It may contain a suitable buffer.

*Content*:

– *carbon-11*: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of carbon-11*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Approximate half-life: 19.0 min to 21.8 min.

C. Examine the chromatograms obtained with reference solution (d) in the test for [methyl-$^{11}$C]choline under Radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram is similar in retention time to the principal positive peak in the chromatogram obtained with the spectrophotometer.

TESTS

**pH** (*2.2.4*): 4.5 to 8.5.

**Impurity A**. Gas chromatography (*2.2.28*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 10.0 mg of *dimethylaminoethanol R* (impurity A) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b)*. To 0.1 mL of *ethanol R* add 0.1 mL of *acetonitrile R* and dilute to 10 mL with *water R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 60 |
| | 1 - 5 | 60 → 160 |
| | 5 - 13 | 160 |
| Injection port | | 250 |
| Detector | | 275 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to ethanol and acetonitrile.

*Calculation of content*:

– for impurity A, use the concentration of impurity A in reference solution (a).

*Limit*:

– *impurity A*: maximum 1.0 mg/*V*.

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/ mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Carbon-11**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limits*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of carbon-11 and radionuclidic impurities with a half-life longer than 20 min. For the detection and quantification of impurities, retain the preparation to be examined for at least 3 h to allow the carbon-11 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[Methyl-$^{11}$C]choline**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 5 mg of *choline chloride R* in *water R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *fluorocholine chloride R* in *water R* and dilute to 20 mL with the same solvent.

*Reference solution (c)*. Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

*Reference solution (d)*. Mix 1 mL of the test solution and 1 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 20.7 g of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R* and add 80 µL of *anhydrous pyridine R*. Adjust to pH 2.5 with *phosphoric acid R* and dilute to 1 L with *water for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 270 nm and radioactivity detector connected in series.

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*Run time*: 14 min.

*Relative retention* with reference to choline: fluorocholine = about 0.9.

*System suitability*: reference solution (c) using the spectrophotometer:

– *resolution*: baseline separation between the peaks due to fluorocholine and choline.

Examine the radiochromatogram obtained with the test solution.

*Limit*:

– *[methyl-$^{11}$C]choline*: minimum 95 per cent of the total radioactivity due to carbon-11.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

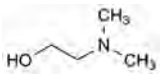

A. 2-(dimethylamino)ethan-1-ol,

B. iodo[$^{11}$C]methane.

**07/2008:0266**
**corrected 7.0**

# CHROMIUM ($^{51}$Cr) EDETATE INJECTION

## Chromii ($^{51}$Cr) edetatis solutio iniectabilis

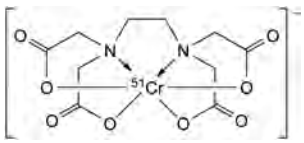

DEFINITION

Sterile solution containing chromium-51 in the form of a complex of chromium(III) with (ethylenedinitrilo)tetraacetic acid, the latter being present in excess. It may be made isotonic by the addition of sodium chloride and may contain a suitable antimicrobial preservative such as benzyl alcohol.

*Chromium-51*: 90 per cent to 110 per cent of the declared chromium-51 radioactivity at the date and time stated on the label.

*Chromium*: maximum 1 mg/mL.

CHARACTERS

*Appearance*: clear, violet solution.

*Half-life and nature of radiation of chromium-51*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Radionuclidic purity (see Tests).

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retardation factor to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 3.5 to 6.5.

**Chromium**: maximum 1 mg/mL.

Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 0.96 g of *chromic potassium sulfate R* and 2.87 g of *sodium edetate R* in 50 mL of *water R*, boil for 10 min, cool, adjust to pH 3.5-6.5 with *dilute sodium hydroxide solution R* and dilute to 100.0 mL with *water R*.

Measure the absorbance of the test solution and the reference solution at the absorption maximum at 560 nm.

*Result*: the absorbance of the test solution is not greater than that of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Chromium-51**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.320 MeV.

B. Gamma-ray spectrometry.

Determine the relative amount of radionuclidic impurities.

*Results*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{51}$Cr]Chromium edetate**. Descending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Reference solution.* Use the reference solution from the test for chromium.

*Chromate carrier solution.* Dissolve 0.1 g of *potassium chromate R* in 1 mL of *concentrated ammonia R1* and dilute to 100 mL with *water R*.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *concentrated ammonia R1*, *ethanol (96 per cent) R*, *water R* (1:2:5 *V/V/V*).

*Application.* Apply a band of a 50 g/L solution of *lead acetate R* to the paper at about 4 cm from the origin and dry in hot air. Apply 10 µL of the chromate carrier solution at the origin, followed by 10 µL of the test solution on the same spot. On a separate sheet, repeat the above procedure, applying 10 µL of the reference solution instead of the test solution.

*Development*: immediately, over a path of 14 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0; impurity B = 0.2 to 0.4; [$^{51}$Cr]chromium edetate = 0.8 to 0.9.

*System suitability.* The band of lead acetate turns yellow due to reaction with the chromate carrier solution. The retardation factor of the radioactive spot due to [$^{51}$Cr]chromium edetate in the radiochromatogram obtained with the test solution is similar to that of the violet spot in the chromatogram obtained with the reference solution.

*Limit*:

– *[$^{51}$Cr]chromium edetate*: minimum 97.0 per cent of the total radioactivity due to chromium-51.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{51}$Cr]chromium(III) ion,

B. [$^{51}$Cr]chromate ion.

**07/2017:2547**
**corrected 10.0**

# COPPER TETRAMIBI TETRAFLUOROBORATE FOR RADIOPHARMACEUTICAL PREPARATIONS

## Cupri tetramibi tetrafluoroboras ad radiopharmaceutica

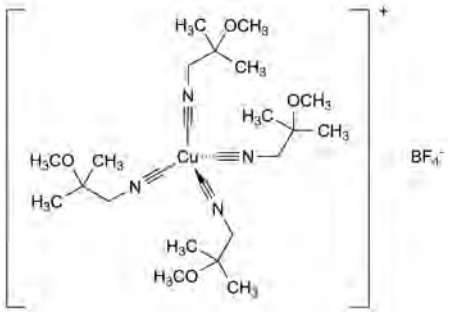

$C_{24}H_{44}BCuF_4N_4O_4$ $M_r$ 603
[103694-84-4]

DEFINITION

(*T*-4)-Tetrakis[1-(isocyano-κ*C*)-2-methoxy-2-methylpropane]copper(I) tetrafluoroborate.

*Content*: 10.04 per cent to 11.04 per cent of Cu ($A_r$ 63.5) (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *copper tetramibi tetrafluoroborate CRS*.

TESTS

**Solution S**. Dissolve 25 mg in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**pH** (*2.2.3*): 4.5 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (5 µm);

– *temperature*: 20 °C.

*Mobile phase*: mix 40 volumes of *acetonitrile R1* and 60 volumes of a 20.4 g/L solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 6.0 with a 56.1 g/L solution of *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of copper tetramibi.

*Retention time*: copper tetramibi = about 5 min.

*System suitability*: reference solution:

– *symmetry factor*: maximum 1.35 for the principal peak.

*Calculation of percentage contents*:

– for each impurity, use the concentration of copper tetramibi tetrafluoroborate in the reference solution.

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g. Use direct sample introduction.

ASSAY

Inductively coupled plasma-mass spectrometry (*2.2.58*).

*Test solution*. Place 20.0 mg of the substance to be examined in a polytetrafluoroethylene tube for microwave digestion and add 4.5 mL of *heavy metal-free nitric acid R* and 1.5 mL of *strong hydrogen peroxide solution R*. Treat by digestion in a microwave oven and dilute to 25.0 mL with *water R*.

*Copper stock solution (1 mg/L)*. To 50 mL of *water R* in a 100 mL plastic volumetric flask add 1.0 mL of *heavy metal-free nitric acid R* and 100 µL of *copper standard solution (0.1 per cent Cu) for ICP R* and dilute to 100.0 mL with *water R*.

*Scandium internal standard (10 mg/L)*. To 50 mL of *water R* in a 100 mL plastic volumetric flask add 1.0 mL of *heavy metal-free nitric acid R* and 1000 µL of *scandium standard solution (0.1 per cent Sc) for ICP R* and dilute to 100.0 mL with *water R*.

*Reference solutions*. Dilute the copper stock solution (1 mg/L) with *water R* to prepare a range of reference solutions spanning 80-120 per cent of the expected concentration of copper in the substance to be examined.

*Procedure*. To 50 µL of the digested sample solution and each of the reference solutions, add 1.0 mL of the scandium internal standard (10 mg/L) and 1.0 mL of *heavy metal-free nitric acid R* and dilute to 100.0 mL with *water R*.

*Detection*:

– copper *m/z* = 63;

– scandium *m/z* = 45.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2008:0710**
**corrected 7.0**

# CYANOCOBALAMIN ($^{57}$Co) CAPSULES

## Cyanocobalamini ($^{57}$Co) capsulae

DEFINITION

Capsules containing [$^{57}$Co]-α-(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide; they may contain suitable excipients.

The capsules comply with the requirements for hard capsules prescribed in the monograph *Capsules (0016)*, unless otherwise justified and authorised.

*Cobalt-57*: 90 per cent to 110 per cent of the declared cobalt-57 radioactivity at the date stated on the label.

CHARACTERS

*Appearance*: hard, gelatin capsules.

*Half-life and nature of radiation of cobalt-57*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of cobalt-57 has an energy of 0.122 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Disintegration**. The capsules comply with the test for disintegration of tablets and capsules (*2.9.1*), except that 1 capsule is used in the test instead of 6.

**Uniformity of content**. Determine, by measurement in a suitable counting assembly and under identical geometrical conditions, the radioactivity of each of not fewer than 10 capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average. The relative standard deviation is less than 3.5 per cent.

*RADIONUCLIDIC PURITY*

**Cobalt-57**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of cobalt-57, cobalt-56 and cobalt-58 present.

*RADIOCHEMICAL PURITY*

**[$^{57}$Co]Cyanocobalamin**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve the contents of a capsule in 1.0 mL of *water R* and allow to stand for 10 min. Centrifuge at 2000 r/min for 10 min. Use the supernatant.

*Reference solution.* Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 2 mL of this solution to 100 mL with the mobile phase. *Use within 1 h of preparation.*

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/L solution of *disodium hydrogen phosphate dodecahydrate R* adjusted to pH 3.5 using *phosphoric acid R*. *Use within 2 days of preparation.*

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector adjusted for cobalt-57 and spectrophotometer at 361 nm.

*Injection*: 100 µL.

*Run time*: 3 times the retention time of cyanocobalamin for the test solution; 30 min for the reference solution.

*Limit*:

– *[$^{57}$Co]cyanocobalamin*: minimum 90 per cent of the total radioactivity due to cobalt-57.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

A. cobalt-56,

B. cobalt-58.

**01/2008:0269**
**corrected 7.0**

# CYANOCOBALAMIN ($^{57}$Co) SOLUTION

## Cyanocobalamini ($^{57}$Co) solutio

DEFINITION

Solution of [$^{57}$Co]-α-(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain a stabiliser and an antimicrobial preservative.

*Cobalt-57*: 90 per cent to 110 per cent of the declared cobalt-57 radioactivity at the date stated on the label.

CHARACTERS

*Appearance*: clear, colourless or slightly pink solution.

*Half-life and nature of radiation of cobalt-57*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of cobalt-57 has an energy of 0.122 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

*RADIONUCLIDIC PURITY*

**Cobalt-57**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of cobalt-57, cobalt-56 and cobalt-58 present.

*RADIOCHEMICAL PURITY*

**[$^{57}$Co]Cyanocobalamin**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 2 mL of this solution to 100 mL with the mobile phase. *Use within 1 h after preparation.*

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/L solution of *disodium hydrogen phosphate dodecahydrate R* adjusted to pH 3.5 using *phosphoric acid R* (use within 2 days after preparation).

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector adjusted for cobalt-57 and spectrophotometer at 361 nm.

*Injection*: 100 µL.

*Run time*: 3 times the retention time of cyanocobalamin for the test solution; 30 min for the reference solution.

*Limit*:
- *[$^{57}$Co]cyanocobalamin*: minimum 90 per cent of the radioactivity due to cobalt-57.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

A. cobalt-56,

B. cobalt-58.

**01/2008:1505**
**corrected 7.0**

# CYANOCOBALAMIN ($^{58}$Co) CAPSULES

## Cyanocobalamini ($^{58}$Co) capsulae

DEFINITION

Capsules containing [$^{58}$Co]-α-(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide; they may contain suitable excipients.

The capsules comply with the requirements for hard capsules in the monograph *Capsules (0016)*, unless otherwise justified and authorised.

*Cobalt-58*: average between 90 per cent and 110 per cent of the declared cobalt-58 radioactivity at the date stated on the label.

CHARACTERS

*Appearance*: hard gelatin capsules.

*Half-life and nature of radiation of cobalt-58*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photons of cobalt-58 have energies of 0.511 MeV (annihilation radiation) and 0.811 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Disintegration**. The capsules comply with the test for disintegration of tablets and capsules (*2.9.1*) except that 1 capsule is used in the test instead of 6.

**Uniformity of content**. Determine by measurement in a suitable counting assembly and under identical geometrical conditions the radioactivity of each of not less than 10 capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average. The relative standard deviation is less than 3.5 per cent.

*RADIONUCLIDIC PURITY*

**Cobalt-58**: minimum 98 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of cobalt-58, cobalt-57 and cobalt-60 present.

*Result*:
- *cobalt-60*: maximum 1 per cent of the total radioactivity.

*RADIOCHEMICAL PURITY*

**[$^{58}$Co]Cyanocobalamin**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve the contents of a capsule in 1.0 mL of *water R* and allow to stand for 10 min. Centrifuge at 2000 r/min for 10 min. Use the supernatant.

*Reference solution.* Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 2 mL of this solution to 100 mL with the mobile phase. *Use within 1 h after preparation.*

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, adjusted to pH 3.5 with *phosphoric acid R* (use within 2 days).

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector adjusted for cobalt-58 and spectrophotometer at 361 nm.

*Injection*: 100 µL.

*Run time*: 3 times the retention time of cyanocobalamin for the test solution; 30 min for the reference solution.

*Limit*:
- *[$^{58}$Co]cyanocobalamin*: minimum 84 per cent of the total radioactivity due to cobalt-58.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

A. cobalt-57,

B. cobalt-60.

**01/2008:0270**
**corrected 7.0**

# CYANOCOBALAMIN ($^{58}$Co) SOLUTION

## Cyanocobalamini ($^{58}$Co) solutio

DEFINITION

Solution of [$^{58}$Co]-α-(5,6-dimethylbenzimidazol-1-yl)cobamide cyanide and may contain a stabiliser and an antimicrobial preservative.

*Cobalt-58*: 90 per cent to 110 per cent of the declared cobalt-58 radioactivity at the date stated on the label.

CHARACTERS

*Appearance*: clear, colourless or slightly pink solution.

*Half-life and nature of radiation of cobalt-58*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photons of cobalt-58 have energies of 0.511 MeV (annihilation radiation) and 0.811 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

*RADIONUCLIDIC PURITY*

**Cobalt-58**: minimum 98 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of cobalt-58, cobalt-57 and cobalt-60 present.

*Result*:

– *cobalt-60*: maximum 1 per cent of the total radioactivity.

*RADIOCHEMICAL PURITY*

**[$^{58}$Co]Cyanocobalamin**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 10 mg of *cyanocobalamin CRS* in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 2 mL of this solution to 100 mL with the mobile phase. *Use within 1 h after preparation.*

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/L solution of *disodium hydrogen phosphate dodecahydrate R* adjusted to pH 3.5 using *phosphoric acid R* (use within 2 days).

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector adjusted for cobalt-58 and spectrophotometer at 361 nm.

*Injection*: 100 µL.

*Run time*: 3 times the retention time of cyanocobalamin for the test solution; 30 min for the reference solution.

*Limit*:

– *[$^{58}$Co]cyanocobalamin*: minimum 90 per cent of the radioactivity due to cobalt-58.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

A. cobalt-57,

B. cobalt-60.

**01/2014:1325**
**corrected 8.2**

# FLUDEOXYGLUCOSE ($^{18}$F) INJECTION

## Fludeoxyglucosi ($^{18}$F) solutio iniectabilis

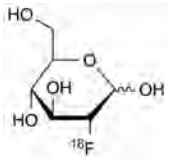


$C_6H_{11}{}^{18}FO_5$ $M_r$ 181.1

DEFINITION

Sterile solution containing 2-[$^{18}$F]fluoro-2-deoxy-D-glucopyranose (2-[$^{18}$F]fluoro-2-deoxy-D-glucose) prepared by nucleophilic substitution. It may also contain 2-[$^{18}$F]fluoro-2-deoxy-D-mannose.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label.

– *2-fluoro-2-deoxy-D-glucose*: maximum 0.5 mg per maximum recommended dose in millilitres.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained in test A for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

*Particular tests for chemical impurities may be omitted if the substances mentioned are not used or cannot be formed in the production process.*

**pH** (*2.2.3*): 4.5 to 8.5.

**2-Fluoro-2-deoxy-D-glucose and impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 1.0 mg of *2-fluoro-2-deoxy-D-glucose R* in *water R* and dilute to 2.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 1.0 mg of *2-chloro-2-deoxy-D-glucose R* (impurity A) in *water R* and dilute to 2.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Dissolve 1.0 mg of *2-fluoro-2-deoxy-D-mannose R* in *water R* and dilute to 20.0 mL with the same solvent. Mix 0.5 mL of this solution with 0.5 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm);

– *temperature*: 25 °C.

*Mobile phase*: 4 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*, protected from the atmosphere during chromatography.

*Flow rate*: 1 mL/min.

*Detection*: detector suitable for carbohydrates in the required concentration range, such as a pulsed amperometric detector and radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: twice the retention time of 2-fluoro-2-deoxy-D-glucose.

*Relative retention* with reference to 2-fluoro-2-deoxy-D-glucose (retention time = about 12 min): 2-fluoro-2-deoxy-D-mannose = about 0.9; impurity A = about 1.1.

*System suitability*: reference solution (c) using the carbohydrate detector:

– *resolution*: minimum 1.5 between the peaks due to 2-fluoro-2-deoxy-D-mannose and 2-fluoro-2-deoxy-D-glucose;

– *signal-to-noise ratio*: minimum 10 for the peak due to 2-fluoro-2-deoxy-D-glucose.

*Limits*: in the chromatogram obtained with the carbohydrate detector:

– *2-fluoro-2-deoxy-D-glucose*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 mg/*V*);

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 mg/*V*).

**Impurity B**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity B) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; in addition, apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

– the spot due to the successive application of the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity B) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;

– the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;

– the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

– the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity C**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.170 g of *tetrabutylammonium hydroxide R* in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80.0 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 25 volumes of a 0.95 g/L solution of *toluenesulfonic acid R* and 75 volumes of *acetonitrile R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity C.

*Retention time*: impurity C = about 3.3 min.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak;

– *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**Impurity D**: maximum 0.02 mg/*V*.

Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 20.0 mg of *4-(4-methylpiperidin-1-yl)pyridine R* (impurity D) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 0.1 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

Measure the absorbance of the test solution and the reference solution at the absorption maximum of 263 nm.

*Result*: the absorbance of the test solution is not greater than that of the reference solution.

**Residual solvents**: limited according to the principles defined in general chapter *5.4.* The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125).* The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Results*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

A. Liquid chromatography (*2.2.29*) as described in the test for 2-fluoro-2-deoxy-D-glucose and impurity A. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

*Injection*: test solution and reference solutions (a) and (c).

*Relative retention* with reference to 2-[$^{18}$F]fluoro-2-deoxy-D-glucose (retention time = about 12 min): 2-[$^{18}$F]fluoro-2-deoxy-D-mannose = about 0.9. Partially or fully acetylated derivatives of both compounds hydrolyse under the chromatographic conditions and therefore elute as 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose.

Locate the peaks due to 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose using the chromatograms obtained with the carbohydrate detector and reference solutions (a) and (c).

*Limits*:

– *[$^{18}$F]fluorine in the form of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose*: minimum 95 per cent of the total radioactivity due to fluorine-18;

– *2-[$^{18}$F]fluoro-2-deoxy-D-mannose*: maximum 10 per cent of the total radioactivity due to 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve, with gentle heating, 30 mg of *1,2,3,4-tetra-O-acetyl-β-D-glucopyranose R* and 20 mg of *glucose R* in 1 mL of *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (5:95 *V/V*).

*Application*: about 5 µL.

*Development*: over a path of 8 cm.

*Drying*: in air for 15 min.

*Detection*: suitable detector to determine the distribution of radioactivity; immerse the plate in a 75 g/L solution of *sulfuric acid R* in *methanol R* and dry with a heat gun or at 150 °C until the appearance of dark spots in the chromatogram obtained with the reference solution.

*Retardation factors*: [$^{18}$F]fluoride = about 0; 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose = about 0.45; partially or fully acetylated derivatives of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose = about 0.8 to 0.95.

*System suitability*: reference solution:

– the chromatogram shows 2 clearly separated spots.

*Limits*:

– *[$^{18}$F]fluorine in the form of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose*: minimum 95 per cent of the total radioactivity due to fluorine-18;

– *[$^{18}$F]fluorine in the form of fluoride and partially or fully acetylated derivatives of 2-[$^{18}$F]fluoro-2-deoxy-D-glucose and 2-[$^{18}$F]fluoro-2-deoxy-D-mannose*: maximum 5 per cent of the total radioactivity due to fluorine-18.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

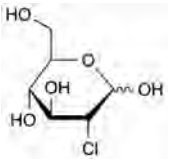

A. 2-chloro-2-deoxy-D-glucopyranose (2-chloro-2-deoxy-D-glucose),

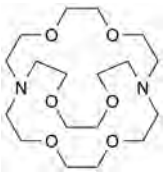

B. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (aminopolyether),

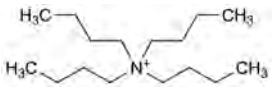

C. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

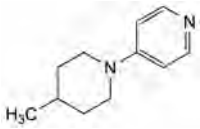

D. 4-(4-methylpiperidin-1-yl)pyridine,

E. [$^{18}$F]fluoride.

**01/2008:1917**

# FLUMAZENIL (*N*-[$^{11}$C]METHYL) INJECTION

## Flumazenili (*N*-[$^{11}$C]methyl) solutio iniectabilis

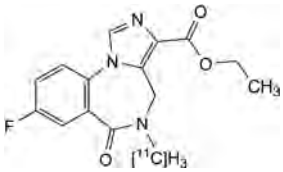

DEFINITION

Sterile solution of ethyl 8-fluoro-5-[$^{11}$C]methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate which may contain a stabiliser such as ascorbic acid.

*Content*: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

*Content of flumazenil*: maximum 50 µg in the maximum recommended dose in millilitres.

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [$^{11}$C]carbon dioxide or [$^{11}$C]methane, respectively.

*RADIOCHEMICAL SYNTHESIS*

[5-Methyl-$^{11}$C]flumazenil may be prepared by *N*-alkylation of ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (demethylflumazenil) with iodo[$^{11}$C]methane or [$^{11}$C]methyl trifluoromethanesulfonate.

**Synthesis of iodo[$^{11}$C]methane**

Iodo[$^{11}$C]methane may be produced from [$^{11}$C]carbon dioxide or from [$^{11}$C]methane. The most frequently used method is reduction of [$^{11}$C]carbon dioxide with lithium aluminium hydride. The [$^{11}$C]methanolate formed is reacted with hydriodic acid. Alternatively [$^{11}$C]methane, either obtained directly in the target or by on-line processes from [$^{11}$C]carbon dioxide, is reacted with iodine.

**Synthesis of [$^{11}$C]methyl trifluoromethanesulfonate**

[$^{11}$C]methyl trifluoromethanesulfonate may be prepared from iodo[$^{11}$C]methane using a solid support such as graphitised carbon, impregnated with silver trifluoromethanesulfonate.

**Synthesis of [5-methyl-$^{11}$C]flumazenil**

The most widely used method to obtain [5-methyl-$^{11}$C]-flumazenil is the *N*-alkylation of demethylflumazenil with iodo[$^{11}$C]methane in alkaline conditions in a solvent such as dimethylformamide or acetone. The resulting [5-methyl-$^{11}$C]flumazenil can be purified by semi-preparative liquid chromatography. For example, a column packed with octadecylsilyl silica gel for chromatography eluted with a mixture of ethanol and water is suitable.

*PRECURSOR FOR SYNTHESIS*

**Demethylflumazenil**

Melting point (*2.2.14*): 286 °C to 289 °C.

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of demethylflumazenil.*

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of carbon-11*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 6.0 to 8.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

**Flumazenil and impurity A**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 2.5 mg of *flumazenil R* in 5 mL of *methanol R*.

*Reference solution (b)*. Dissolve 2.5 mg of *demethylflumazenil R* in 50 mL of *methanol R*.

*Reference solution (c)*. To 0.1 mL of reference solution (a) add 0.1 mL of reference solution (b) and dilute to *V* with a 0.9 g/L solution of *sodium chloride R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (d)*. Dilute 0.1 mL of reference solution (a) to 50 mL with *methanol R*. Dilute 1.0 mL of this solution to *V* with a 0.9 g/L solution of *sodium chloride R*, *V* being the maximum recommended dose in millilitres.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm,

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 440 m$^2$/g, a pore size of 100 nm and a carbon loading of 19 per cent,

– *temperature*: maintain at a constant temperature between 20-30 °C.

*Mobile phase*: *methanol R*, *water R* (45:55 *V*/*V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm and radioactivity detector connected in series.

*Injection*: 100 µL.

*Run time*: 10 min.

*Relative retention* with reference to flumazenil: impurity A = about 0.74.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to flumazenil and impurity A.

*Limits*: examine the chromatogram obtained with the spectrophotometer:

– *flumazenil*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (50 µg/*V*),

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (5 µg/*V*),

– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1 µg/*V*).

**Residual solvents** are limited according to the principles defined in the general chapter (*5.4*), using the general method (*2.4.24*). The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Carbon-11**: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

*RADIOCHEMICAL PURITY*

Liquid chromatography (*2.2.29*) as described in the test for flumazenil and impurity A, with the following modifications.

*Injection*: test solution and reference solution (a); if necessary, dilute the test solution to a radioactivity concentration suitable for the detector.

*Limit*: examine the chromatogram obtained with the radioactivity detector:

– *[5-methyl-$^{11}$C]flumazenil*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the maximum recommended dose in millilitres.

IMPURITIES

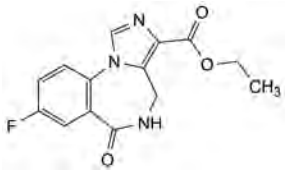

A. ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*]-[1,4]benzodiazepine-3-carboxylate (demethylflumazenil),

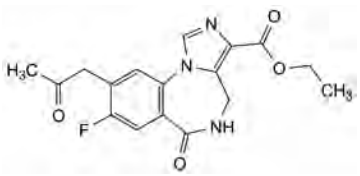

B. ethyl 8-fluoro-6-oxo-9-(2-oxopropyl)-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (acetone addition compound of demethylflumazenil).

**01/2011:2390**

# FLUORIDE ($^{18}$F) SOLUTION FOR RADIOLABELLING

## Fluoridi ($^{18}$F) solutio ad radio-signandum

DEFINITION

Alkaline solution containing fluorine-18 in the form of [$^{18}$F]fluoride.

*Content*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution; in the chromatogram obtained with the reference solution, the signal due to fluoride is negative.

TESTS

**pH**: 8.0 to 14.0, using a *pH indicator strip R*.

**Bacterial endotoxins** (*2.6.14*): less than 20 IU/mL, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry. Preliminary test.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]fluoride**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

*Reference solution*. Dissolve 10 mg of *potassium fluoride R* in *water R* and dilute to 10 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm).

*Mobile phase*: 4 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*, protected from atmospheric carbon dioxide.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm and a radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 12 min.

*System suitability*: reference solution:

– *signal-to-noise ratio*: minimum 10 for the principal peak;
– *retention time of fluoride*: minimum 3 times the hold-up time.

Examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to fluoride by comparison with the chromatogram obtained with the reference solution using the spectrophotometer.

*Limit*:

– *[$^{18}$F]fluoride*: minimum 98.5 per cent of the total radioactivity due to fluorine-18.

*RADIOACTIVITY*

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

– that the solution is not for direct administration to humans;
– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**07/2016:2793 corrected 10.0**

# FLUOROCHOLINE ($^{18}$F) INJECTION

## Fluorocholini ($^{18}$F) solutio iniectabilis

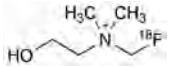

$C_5H_{13}{}^{18}FNO^+$ $M_r$ 121.2
[475572-73-7]

DEFINITION

Sterile solution containing *N*-([$^{18}$F]fluoromethyl)-2-hydroxy-*N*,*N*-dimethylethan-1-aminium ([$^{18}$F]fluorocholine) ions. It may contain a suitable buffer.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained with reference solution (d) in the test for [$^{18}$F]fluorocholine under Radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram is similar in retention time to the principal positive peak in the chromatogram obtained with the spectrophotometer.

TESTS

**pH**: 4.5 to 8.5, using a *pH indicator strip R*.

**Impurity A**. Gas chromatography (*2.2.28*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 10.0 mg of *dimethylaminoethanol R* (impurity A) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* To 0.1 mL of *anhydrous ethanol R*, add 0.1 mL of *acetonitrile R* and dilute to 10 mL with *water R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 60 |
| | 1 - 5 | 60 → 160 |
| | 5 - 13 | 160 |
| Injection port | | 250 |
| Detector | | 275 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to ethanol and acetonitrile.

*Calculation of content*:

– for impurity A, use the concentration of impurity A in reference solution (a).

*Limit*:

– *impurity A*: maximum 1.0 mg/*V*.

**Impurity B** (*2.4.24* or another suitable, validated method).

The following solutions or dilutions therefrom may be used:

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 10.0 mg of *dibromomethane R* (impurity B) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Limit*:

– *impurity B*: maximum 0.1 mg/*V*.

**Impurity C**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity C) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; also apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

– the spot due to the successive application of the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity C) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;
– the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;
– the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

– the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.170 g of *tetrabutylammonium hydroxide R* (impurity D) in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to 25 mL with *water R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 0.95 g/L solution of *toluenesulfonic acid R*, *acetonitrile R* (25:75 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity D.

*Retention time*: impurity D = about 3.3 min.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak;
– *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limits*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]Fluorocholine**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 5 mg of *fluorocholine chloride R* in *water R* and dilute to 20 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *fluoroethyl(2-hydroxyethyl)dimethylammonium chloride R* in *water R* and dilute to 20 mL with the same solvent.

*Reference solution (c).* Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

*Reference solution (d).* Mix 1 mL of the test solution and 1 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 20.7 g of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R*, add 80 µL of *anhydrous pyridine R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm and radioactivity detector connected in series.

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*Run time*: 20 min.

*Relative retention* with reference to fluorocholine (retention time = about 8 min): fluoroethyl(2-hydroxyethyl)dimethylammonium = about 1.1.

*System suitability*: reference solution (c) using the spectrophotometer:

– *resolution*: baseline separation between the peaks due to fluorocholine and fluoroethyl(2-hydroxyethyl)dimethylammonium.

Examine the radiochromatogram obtained with the test solution.

*Limit*:

– *[$^{18}$F]fluorocholine*: minimum 95 per cent of the total radioactivity due to fluorine-18.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetonitrile R*, 9 g/L solution of *sodium chloride R* (50:50 *V/V*).

*Application*: about 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity E = about 0.0; [$^{18}$F]fluorocholine = about 0.5.

*Limit*:

– *impurity E*: maximum 5 per cent of the total radioactivity due to fluorine-18.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

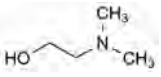


A. 2-(dimethylamino)ethan-1-ol,

B. dibromomethane,

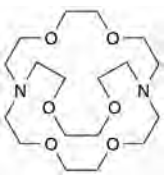


C. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (aminopolyether),

Radiopharmaceutical preparations

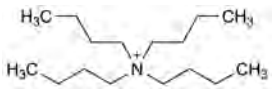

D. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

E. [$^{18}$F]fluoride.

**01/2008:1918**

# FLUORODOPA ($^{18}$F) (PREPARED BY ELECTROPHILIC SUBSTITUTION) INJECTION

## Fluorodopae ($^{18}$F) ab electrophila substitutione solutio iniectabilis

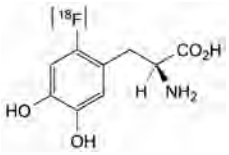

DEFINITION

Sterile solution of (2*S*)-2-amino-3-(2-([$^{18}$F]fluoro)-4,5-dihydroxyphenyl)propanoic acid (6-[$^{18}$F]fluorolevodopa). It may contain stabilisers such as ascorbic acid and edetic acid.

This monograph applies to an injection containing 6-[$^{18}$F]fluorolevodopa produced by electrophilic substitution.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label;

– *dopa*: maximum 1 mg per maximum recommended dose in millilitres;

– *6-fluorolevodopa*: maximum 15 mg per maximum recommended dose in millilitres.

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Fluorine-18 is a radioactive isotope of fluorine that may be produced by various nuclear reactions induced by proton irradiation of oxygen-18, deuteron irradiation of neon-20, or helium-3 or helium-4 irradiation of oxygen-16.

In order to obtain fluorine-18 in a chemical form suitable for electrophilic substitution reactions, such as fluorine gas or gaseous acetylhypofluorite, a small amount of non-radioactive fluorine gas (0.3-0.8 per cent of the target gas volume) must be added as a carrier at some step in the production process.

*RADIOCHEMICAL SYNTHESIS*

6-[$^{18}$F]Fluorolevodopa may be prepared by various radiochemical synthetic pathways, which lead to different products in terms of yield, specific radioactivity, by-products and possible impurities. Electrophilic pathways for production of 6-[$^{18}$F]fluorolevodopa may proceed by fluorodemetallation of a stannylated derivative of levodopa, with molecular [$^{18}$F]fluorine or [$^{18}$F]acetylhypofluorite, followed by hydrolysis of protecting groups and final purification by semipreparative liquid chromatography. Pathways using demercuration or dethallation must not be used.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Test A for radionuclidic purity (see Tests).

B. Determine the approximate half-life by at least 3 measurements of the activity of a sample in the same geometrical conditions over a suitable period of time, for example 30 min.

*Results*: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the peak due to 6-fluorolevodopa in the chromatogram obtained with reference solution (a).

D. Examine the chromatograms obtained in the test for impurities C and D (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retardation factor to the peak due to 6-fluorolevodopa in the chromatogram obtained with reference solution (b).

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

**6-Fluorolevodopa, dopa, impurity A and impurity B**. Liquid chromatography (*2.2.29*). *Prepare the reference solutions immediately before use.*

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 18.0 mg of *6-fluorolevodopa hydrochloride R* in 5.0 mL of the mobile phase and dilute to *V* with the mobile phase, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 1.0 mg of *levodopa R* in 5 mL of the mobile phase and dilute to *V* with the mobile phase, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Dissolve 1.0 mg of *trimethyltin chloride R* (impurity A) in 2.0 mL of the mobile phase. Dilute 1.0 mL of this solution to *V* with the mobile phase, *V* being the maximum recommended dose in millilitres.

*Reference solution (d).* Mix equal volumes of reference solutions (b) and (c).

*Reference solution (e).* Dissolve 2.0 mg of *6-hydroxydopa R* (impurity B) in 20.0 mL of the mobile phase. Dilute 0.25 mL of this solution to *V* with the mobile phase, *V* being the maximum recommended dose in millilitres.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R*;

– *temperature*: maintain at a constant temperature between 20 °C and 30 °C.

*Mobile phase*: 6.9 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 2.4 with a 4.8 g/L solution of *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 200 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 15 min.

*Relative retention* with reference to 6-fluorolevodopa (retention time = about 6 min): impurity A and impurity B = about 0.7; dopa = about 0.8.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to dopa and impurity A.

*Limits*: examine the chromatograms obtained with the spectrophotometer:

– *6-fluorolevodopa*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (15 mg/*V*);
– *dopa*: not more than the area of the peak due to levodopa in the chromatogram obtained with reference solution (b) (1.0 mg/*V*);
– *sum of impurities A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (corresponding to a limit of 0.5 mg/*V* of impurity A or a limit of 0.025 mg/*V* of impurity B, or to lower limits of each if both impurities are present).

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity. The preparation may be released for use before completion of test B.

A. Gamma-ray spectrometry

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Gamma-ray spectrometry

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for a sufficient time to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from a background spectrum.

*RADIOCHEMICAL PURITY*

Liquid chromatography (*2.2.29*) as described in the test for 6-fluorolevodopa, dopa, impurity A and impurity B. Examine the chromatogram recorded using the radioactivity detector and locate the peak due to 6-[$^{18}$F]fluorolevodopa by comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

*Limit*:

– *6-[$^{18}$F]fluorolevodopa*: minimum 95 per cent of the total radioactivity due to fluorine-18.

**Impurities C and D**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 2 mg of *DL-6-fluorodopa hydrochloride R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 2 mg of *6-fluorolevodopa hydrochloride R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate for chiral separations R*.

*Mobile phase*: *methanol R, water R* (50:50 *V/V*).

*Application*: 2 µL.

*Development*: over a path of 10 cm.

*Drying*: in air for 5 min.

*Detection*: spray with a 2 g/L solution of *ninhydrin R* in *anhydrous ethanol R* and heat at 60 °C for 10 min; determine the distribution of radioactivity using a suitable detector.

*Retardation factors*: impurity D = about 0; 6-[$^{18}$F]fluorolevodopa = about 0.3; impurity C = about 0.5.

*System suitability*: reference solution (a):

– the chromatogram shows 2 clearly separated spots.

*Limits*:

– *impurity C*: maximum 2 per cent of the total radioactivity due to fluorine-18;
– *impurity D*: maximum 4 per cent of the total radioactivity due to fluorine-18.

RADIOACTIVITY

Measure the radioactivity using a calibrated instrument.

LABELLING

The label states the maximum recommended dose in millilitres.

IMPURITIES

A. $Cl\text{-}Sn(CH_3)_3$: chlorotrimethylstannane (trimethyltin chloride),

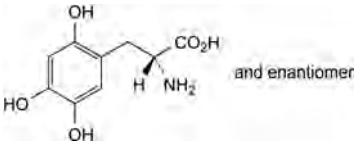


B. (2*RS*)-2-amino-3-(2,4,5-trihydroxyphenyl)propanoic acid (6-hydroxydopa),

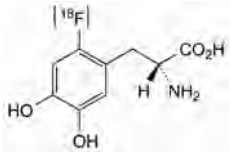


C. (2*R*)-2-amino-3-(2-[$^{18}$F]fluoro-4,5-dihydroxyphenyl)-propanoic acid (6-[$^{18}$F]fluorodextrodopa),

D. [$^{18}$F]fluoride.

**04/2019:2481**

# FLUORODOPA ($^{18}$F) (PREPARED BY NUCLEOPHILIC SUBSTITUTION) INJECTION

## Fluorodopae ($^{18}$F) ab nucleophila substitutione solutio iniectabilis

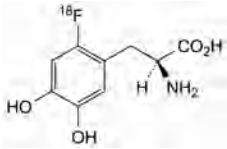


$C_9H_{10}{}^{18}FNO_4$ $M_r$ 214.2
[92812-82-3]

DEFINITION

Sterile solution of (2*S*)-2-amino-3-(2-[$^{18}$F]fluoro-4,5-dihydroxyphenyl)propanoic acid (6-[$^{18}$F]fluorolevodopa). It may contain stabilisers such as ascorbic acid or edetic acid.

This monograph applies to an injection containing 6-[$^{18}$F]fluorolevodopa produced by nucleophilic substitution.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label;
– *6-fluorolevodopa*: maximum 0.1 mg per maximum recommended dose in millilitres.

Radiopharmaceutical preparations

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Approximate half-life: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for 6-[$^{18}$F]fluorolevodopa under Radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

D. Examine the chromatograms obtained in the test for enantiomeric purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.4*): 4.0 to 5.5.

**Impurity A**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity A) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; also apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

- the spot due to the application of both the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity A) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;
- the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;
- the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

- the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 170.0 mg of *tetrabutylammonium hydroxide R* (impurity B) in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80.0 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 0.95 g/L solution of *toluenesulfonic acid R*, *acetonitrile R* (25:75 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity B.

*Retention time*: impurity B = about 3.3 min.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 10 for the principal peak;
- *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**6-Fluorolevodopa and related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 2.3 mg of *6-fluorolevodopa hydrochloride R* in mobile phase A and dilute to 20.0 mL with mobile phase A. Dilute 1.0 mL of the solution to *V* with mobile phase A, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 2 mg of *levodopa R* (L-form of impurity D) in mobile phase A and dilute to 20 mL with mobile phase A. Dilute 1 mL of the solution to *V* with mobile phase A, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Dissolve 2 mg of *6-hydroxydopa R* (impurity E) in mobile phase A and dilute to 20 mL with mobile phase A. Dilute 1 mL of the solution to *V* with mobile phase A, *V* being the maximum recommended dose in millilitres.

*Reference solution (d).* Mix equal volumes of reference solutions (a), (b) and (c).

*Blank solution.* Prepare a solution in mobile phase A containing each excipient at the concentration used in the preparation.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 1.22 g/L solution of *trifluoroacetic acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 98 | 2 |
| 10 - 13 | 98 → 95 | 2 → 5 |
| 13 - 20 | 95 | 5 |
| 20 - 35 | 95 → 5 | 5 → 95 |
| 35 - 37 | 5 | 95 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 283 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Relative retention* with reference to 6-fluorolevodopa (retention time = about 10 min): impurity E = about 0.7; impurity D = about 0.8.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.5 between the peaks due to impurities E and D; minimum 5 between the peaks due to impurity D and 6-fluorolevodopa.

*Limits*:

– *6-fluorolevodopa*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);
– *impurity E*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 mg/*V*);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 mg/*V*);
– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 mg/*V*); disregard any peak due to the blank.

**Ethanol** (*2.4.24* or another suitable, validated method): maximum 10 per cent *V/V* and maximum 2.5 g per administration, taking the density (*2.2.5*) to be 0.790 g/mL.

**Residual solvents**: limited according to the principles defined in the general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**6-[$^{18}$F]Fluorolevodopa**: minimum 95 per cent of the total radioactivity due to fluorine-18.

Liquid chromatography (*2.2.29*) as described in the test for 6-fluorolevodopa and related substances. If necessary, dilute the test solution with mobile phase A to obtain a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to 6-[$^{18}$F]fluorolevodopa by comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

**Impurity I**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetic acid R*, *methanol R* (10:90 *V/V*).

*Application*: about 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity I = about 0; 6-[$^{18}$F]fluorolevodopa = about 0.4.

*Limit*:

– *impurity I*: maximum 5 per cent of the total radioactivity due to fluorine-18.

*ENANTIOMERIC PURITY*

Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 2 mg of *6-fluorolevodopa hydrochloride R* in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *DL-6-fluorodopa hydrochloride R* in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.0 mm;
– *stationary phase*: *crown-ether silica gel for chiral separation R* (5 µm).

*Mobile phase*: 2.9 g/L solution of *perchloric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 283 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: twice the retention time of 6-[$^{18}$F]fluorolevodopa.

*Relative retention* with reference to 6-[$^{18}$F]fluorolevodopa (retention time = about 7 min): impurity F = about 0.7.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to 6-[$^{18}$F]fluorolevodopa by comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to 6-fluorodextrodopa (non-radioactive analogue of impurity F) and 6-fluorolevodopa.

*Limits*:

– *6-[$^{18}$F]fluorolevodopa*: minimum 96 per cent of the total radioactivity;
– *impurity F*: maximum 4 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

*Specified impurities: A, B, E, F, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance)*: D, G, H, J, K, L, M, N, O, P, Q.*

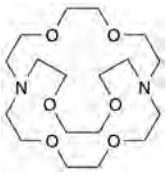


A. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (aminopolyether),

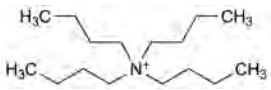

B. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

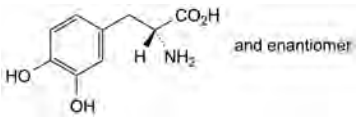


D. (2*RS*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid (DL-dopa),

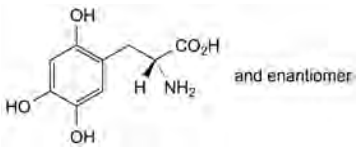


E. (2*RS*)-2-amino-3-(2,4,5-trihydroxyphenyl)propanoic acid (6-hydroxy-DL-dopa),

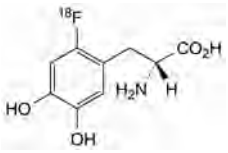

F. (2*R*)-2-amino-3-(2-[$^{18}$F]fluoro-4,5-dihydroxyphenyl)-propanoic acid (6-[$^{18}$F]fluorodextrodopa),

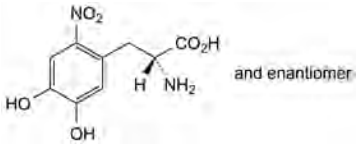


G. (2*RS*)-2-amino-3-(4,5-dihydroxy-2-nitrophenyl)propanoic acid (6-nitro-DL-dopa),

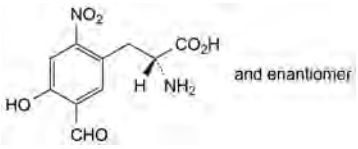


H. (2*RS*)-2-amino-3-(5-formyl-4-hydroxy-2-nitrophenyl)-propanoic acid (3-formyl-6-nitro-DL-dopa),

I. [$^{18}$F]fluoride,

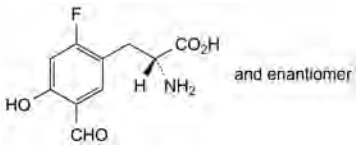


J. (2*RS*)-2-amino-3-(2-fluoro-5-formyl-4-hydroxyphenyl)-propanoic acid (6-fluoro-3-formyl-DL-dopa),

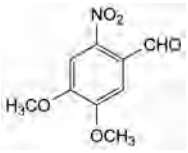

K. 4,5-dimethoxy-2-nitrobenzaldehyde,

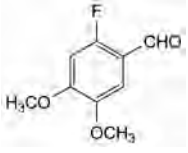

L. 2-fluoro-4,5-dimethoxybenzaldehyde,

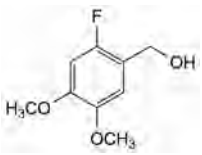

M. (2-fluoro-4,5-dimethoxyphenyl)methanol,

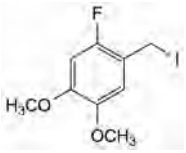

N. 1-fluoro-2-(iodomethyl)-4,5-dimethoxybenzene,

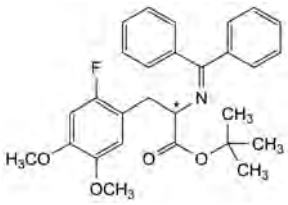

O. *tert*-butyl (2Ξ)-2-[(diphenylmethylidene)amino]-3-(2-fluoro-4,5-dimethoxyphenyl)propanoate,

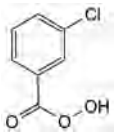

P. 3-chlorobenzene-1-carboperoxoic acid,

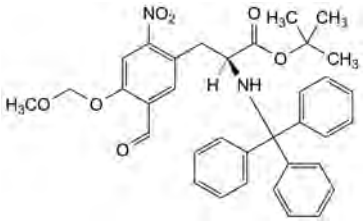

Q. *tert*-butyl (2*S*)-3-[5-formyl-4-(methoxymethoxy)-2-nitrophenyl]-2-[(triphenylmethyl)amino]propanoate.

**07/2015:2466**
**corrected 9.3**

# FLUOROETHYL-L-TYROSINE ($^{18}$F) INJECTION

## Fluoroethyl-L-tyrosini ($^{18}$F) solutio iniectabilis

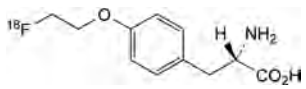

$C_{11}H_{14}{}^{18}FNO_3$ $M_r$ 226.2

DEFINITION

Sterile solution containing (2*S*)-2-amino-3-[4-(2-[$^{18}$F]fluoroethoxy)phenyl]propanoic acid (*O*-(2-[$^{18}$F]fluoroethyl)-L-tyrosine, [$^{18}$F]FET). It may contain a suitable buffer.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label;

Radiopharmaceutical preparations

– *fluoroethyl-L-tyrosine*: maximum 0.1 mg per maximum recommended dose in millilitres.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for [$^{18}$F]fluoroethyl-L-tyrosine under radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH**: 4.5 to 8.5, using a *pH indicator strip R*.

**Impurity A**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity A) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; as an additional spot, apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

– the spot due to the application of both the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity A) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;
– the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;
– the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

– the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.170 g of *tetrabutylammonium hydroxide R* in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80.0 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 0.95 g/L solution of *toluenesulfonic acid R*, *acetonitrile R* (25:75 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity B.

*Retention time*: impurity B = about 3.3 min.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak;
– *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**Fluoroethyl-L-tyrosine and related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 11.6 mg of *fluoroethyl-L-tyrosine hydrochloride R* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Dissolve 10 mg of *3,4-dimethoxy-L-phenylalanine R* in *water R* and dilute to 20 mL with the same solvent. To 1 mL of the solution add 1 mL of reference solution (a).

*Blank solution.* Prepare a solution containing 2 mg/mL of each excipient stated on the label.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *carbon dioxide-free water R*, protected from the atmosphere during chromatography;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 90 | 10 |
| 10 - 20 | 90 → 5 | 10 → 95 |
| 20 - 30 | 5 | 95 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Relative retention* with reference to fluoroethyl-L-tyrosine (retention time = about 6 min): 3,4-dimethoxy-L-phenylalanine = about 0.8.

*System suitability*: reference solution (c) using the spectrophotometer:

– *resolution*: minimum 2.0 between the peaks due to fluoroethyl-L-tyrosine and 3,4-dimethoxy-L-phenylalanine.

*Limits*: in the chromatogram obtained with the spectrophotometer:

- *fluoroethyl-L-tyrosine*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 mg/*V*);
- *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 mg/*V*);
- *total of fluoroethyl-L-tyrosine and impurities*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 mg/*V*);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 mg/*V*).

**Ethanol** (*2.4.24* or another suitable, validated method): maximum 10 per cent *V/V* and maximum 2.5 g per administration, taking the density (*2.2.5*) to be 0.790 g/mL.

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 hours to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]Fluoroethyl-L-tyrosine**. Liquid chromatography (*2.2.29*) as described in the test for fluoroethyl-L-tyrosine and related substances. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to [$^{18}$F]fluoroethyl-L-tyrosine by comparison with the chromatogram obtained with reference solution (b) and the spectrophotometer.

*Limit*:

- *[$^{18}$F]fluoroethyl-L-tyrosine*: minimum 95 per cent of the total radioactivity due to fluorine-18.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetic acid R*, *methanol R* (10:90 *V/V*).

*Application*: about 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity D = about 0; [$^{18}$F]fluoroethyl-L-tyrosine = about 0.7.

*Limit*:

- *impurity D*: maximum 5 per cent of the total radioactivity due to fluorine-18.

*ENANTIOMERIC PURITY*

Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Reference solution (b) of the test for fluoroethyl-L-tyrosine and related substances. Add 0.01 mL of a 2.9 g/L solution of *perchloric acid R*.

*Reference solution (b).* Dissolve 1 mg of *fluoroethyl-D-tyrosine hydrochloride R* (non-radioactive analog of impurity C) in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres. Add 0.01 mL of a 2.9 g/L solution of *perchloric acid R*.

*Reference solution (c).* Mix 1.5 mL of reference solution (a) and 1.5 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *crown-ether silica gel for chiral separation R* (5 µm).

*Mobile phase*: *methanol R*, 2.9 g/L solution of *perchloric acid R* (10:90 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm and radioactivity detector connected in series.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: 2.5 times the retention time of [$^{18}$F]fluoroethyl-L-tyrosine.

*Relative retention* with reference to [$^{18}$F]fluoroethyl-L-tyrosine (retention time = about 14 min): impurity D = about 0.1 [unretained]; impurity C = about 0.7.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to [$^{18}$F]fluoroethyl-L-tyrosine by comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity C and fluoroethyl-L-tyrosine.

*Limits*:

- *[$^{18}$F]fluoroethyl-L-tyrosine*: minimum 95 per cent of the total radioactivity;
- *sum of impurities C and D*: maximum 5 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the name of each excipient and the percentage content of ethanol in the preparation.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance): *E.*

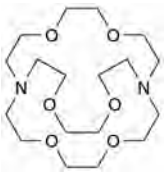

A. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane (aminopolyether),

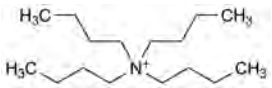

B. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

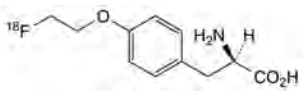

C. (2*R*)-2-amino-3-[4-(2-[$^{18}$F]fluoroethoxy)phenyl]propanoic acid ([$^{18}$F]fluoroethyl-D-tyrosine),

D. [$^{18}$F]fluoride,

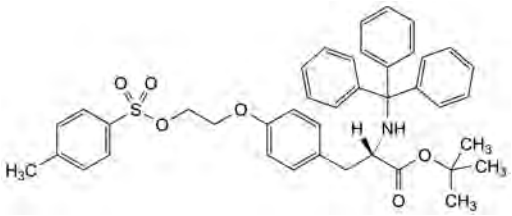

E. 1,1-dimethylethyl (2*S*)-3-[4-[2-[[(4-methylphenyl)-sulfonyl]oxy]ethoxy]phenyl]-2-[(triphenylmethyl)-amino]propanoate.

**01/2014:2459**

# FLUOROMISONIDAZOLE ($^{18}$F) INJECTION

## Fluoromisonidazoli ($^{18}$F) solutio iniectabilis

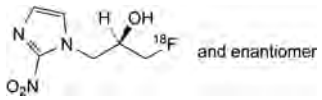


$C_6H_8{}^{18}FN_3O_3$ $M_r$ 188.1

DEFINITION

Sterile solution containing (2*RS*)-1-[$^{18}$F]fluoro-3-(2-nitro-1*H*-imidazol-1-yl)propan-2-ol ([$^{18}$F]FMISO). It may contain a suitable buffer.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and time stated on the label;

– *fluoromisonidazole*: maximum 0.1 mg per maximum recommended dose in millilitres.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 30 min).

*Result*: 105 min to 115 min.

C. Examine the chromatograms obtained in the test for [$^{18}$F]fluoromisonidazole under radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH**: 4.5 to 8.5, using a *pH indicator strip R*.

**Impurity A**. Spot test.

*Test solution.* To 100 µL of the preparation to be examined add 400 µL of *water R* and mix.

*Reference solution (a)*: *water R*.

*Reference solution (b).* Dissolve 11.0 mg of *aminopolyether R* (impurity A) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Plate*: *TLC silica gel plate for aminopolyether test R*.

*Application*: 2.5 µL; as an additional spot, apply 2.5 µL of the test solution and then 2.5 µL of reference solution (b) at the same place.

*Detection*: visually compare the spots 1 min after application.

*System suitability*:

– the spot due to the application of both the test solution and reference solution (b) is similar in appearance to the spot due to reference solution (b), which is characterised by a number of concentric circles; the darker innermost circle (of intensity proportional to the concentration of impurity A) may be surrounded by a bluish-black ring, outside of which is a lighter circle surrounded by a peripheral dark edge;

– the spot due to reference solution (a) has a more diffuse inner circle, which is brownish-pink and without a distinct margin between it and the surrounding lighter zone;

– the spot due to reference solution (b) is clearly different from the spot due to reference solution (a).

*Limit*:

– the central portion of the spot due to the test solution is not more intense than that of the spot due to reference solution (b) (2.2 mg/*V*).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.170 g of *tetrabutylammonium hydroxide R* in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dissolve 80.0 mg of *tetrabutylammonium hydroxide R* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: 0.95 g/L solution of *toluenesulfonic acid R*, *acetonitrile R* (25:75 *V*/*V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of impurity B.

*Retention time*: impurity B = about 3.3 min.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 10 for the principal peak;
- *symmetry factor*: maximum 1.8 for the principal peak.

*Limit*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2.6 mg/*V*).

**Fluoromisonidazole and related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 5.0 mg of *fluoromisonidazole R* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b)*. Dissolve 5.0 mg of *desmethylmisonidazole R* (impurity C) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (c)*. Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

*Blank solution*. Prepare a solution containing each excipient at the concentration used in the preparation.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *carbon dioxide-free water R*, protected from the atmosphere during chromatography;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 90 | 10 |
| 10 - 20 | 90 → 5 | 10 → 95 |
| 20 - 30 | 5 | 95 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 320 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Relative retention* with reference to fluoromisonidazole (retention time = about 6 min): impurity C = about 0.6.

*System suitability*: reference solution (c) using the spectrophotometer:

- *resolution*: minimum 5.0 between the peaks due to impurity C and fluoromisonidazole.

*Limits*: in the chromatogram obtained with the spectrophotometer:

- *fluoromisonidazole*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 mg/*V*);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 mg/*V*);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 mg/*V*);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 mg/*V*).

**Ethanol** (*2.4.24* or another suitable, validated method): maximum 10 per cent *V/V* and maximum 2.5 g per administration, taking the density (*2.2.5*) to be 0.790 g/mL.

**Residual solvents**: limited according to the principles defined in general chapter *5.4*. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma spectrum corresponding to photons with an energy different from 0.511 MeV or 1.022 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for at least 24 h to allow the fluorine-18 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to radionuclidic impurities is not more than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]Fluoromisonidazole**. Liquid chromatography (*2.2.29*) as described in the test for fluoromisonidazole and related substances. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to [$^{18}$F]fluoromisonidazole by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer.

*Limit*:

- *[$^{18}$F]fluoromisonidazole*: minimum 95 per cent of the total radioactivity due to fluorine-18.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (5:95 *V/V*).

*Application*: about 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity D = about 0; [$^{18}$F]fluoromisonidazole = about 0.8.

*Limit*:

- *impurity D*: maximum 5 per cent of the total radioactivity due to fluorine-18.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the percentage content of ethanol in the preparation.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance)*: E.*

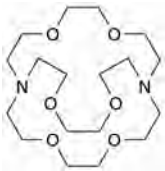

A. 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (aminopolyether),

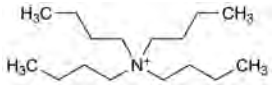

B. *N*,*N*,*N*-tributylbutan-1-aminium (tetrabutylammonium),

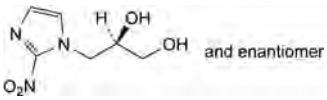


C. (2*RS*)-3-(2-nitro-1*H*-imidazol-1-yl)propane-1,2-diol (desmethylmisonidazole),

D. [$^{18}$F]fluoride,

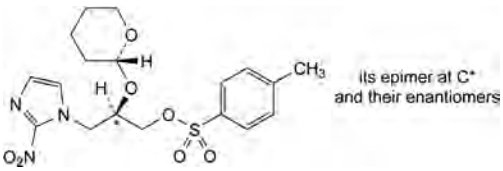


E. mixture of the 4 stereoisomers of 3-(2-nitro-1*H*-imidazol-1-yl)-2-(tetrahydro-2*H*-pyran-2-yloxy)propyl 4-methylbenzenesulfonate.

**01/2008:0555**
**corrected 7.0**

# GALLIUM ($^{67}$Ga) CITRATE INJECTION

## Gallii ($^{67}$Ga) citratis solutio iniectabilis

DEFINITION

Sterile solution of gallium-67 in the form of gallium citrate. It may be made isotonic by the addition of sodium chloride and sodium citrate and may contain a suitable antimicrobial preservative such as benzyl alcohol.

*Gallium-67*: 90 per cent to 110 per cent of the declared gallium-67 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of gallium-67*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photons have energies of 0.093 MeV, 0.185 MeV and 0.300 MeV.

B. To 0.2 mL of the preparation to be examined add 0.2 mL of a solution containing 1 g/L of *ferric chloride R* and 0.1 per cent *V/V* of *hydrochloric acid R* and mix.

Compare the colour with that of a solution containing 7 g/L of *sodium chloride R* and 9 g/L of *benzyl alcohol R* treated in the same manner. A yellow colour develops in the test solution only.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

**Zinc**: maximum 5 ppm.

*Test solution*. To 0.1 mL of the preparation to be examined add 0.9 mL of *water R*, 1 mL of a 250 g/L solution of *sodium thiosulfate R*, 5 mL of *acetate buffer solution pH 4.7 R* and 5.0 mL of a dithizone solution prepared as follows: dissolve 10 mg of *dithizone R* in 100 mL of *methyl ethyl ketone R* allow to stand for 5 min, filter and immediately before use dilute the solution to 10 times its volume with *methyl ethyl ketone R*. Shake vigorously for 2 min and allow the organic layer to separate.

*Reference solution*. 0.1 mL of *zinc standard solution (5 ppm Zn) R* treated in the same manner as the test solution.

Measure the absorbance (*2.2.25*) of the organic layers at 530 nm, using the organic layer of a blank solution as the compensation liquid.

*Results*: the absorbance of the organic layer obtained with the test solution is not greater than that of the organic layer obtained with the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Gallium-67**: minimum 99.8 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of gallium-66 and other radionuclidic impurities present.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. gallium-66.

**07/2013:2464**

# GALLIUM ($^{68}$Ga) CHLORIDE SOLUTION FOR RADIOLABELLING

## Gallii ($^{68}$Ga) chloridi solutio ad radio-signandum

$^{68}GaCl_3$ $M_r$ 174.3

DEFINITION

Solution containing gallium-68 in the form of gallium chloride in dilute hydrochloric acid. The preparation may contain acetone.

*Content*:

– *gallium-68*: 90 per cent to 110 per cent of the declared gallium-68 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of gallium-68*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have energies of 0.511 MeV and 1.077 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 15 min).

*Result*: 62 min to 74 min.

C. pH (see Tests).

D. To a volume of 20-100 µL of the solution to be examined add 1 mL of a 1.03 g/L solution of *hydrochloric acid R*. Apply this solution to the top of a column containing *strong cation-exchange resin R*, push 5 mL of air through the column and collect the eluate. Determine the radioactivity of the eluate (A1). Elute the column with 1 mL of a 1.03 g/L solution of *hydrochloric acid R*. Determine the radioactivity of the eluate (A2). Elute the column with 1 mL of a mixture of 2 volumes of *hydrochloric acid R* and 98 volumes of *acetone R* and push 5 mL of air through the column. Determine the radioactivity of the eluate (A3) and the residual activity on the column (A4).

Calculate the percentage of radioactivity in the A3 eluate using the following expression:

$$A3 \times 100 / (A1 + A2 + A3 + A4)$$

*Result*: the percentage of radioactivity in the A3 eluate is not less than 90 per cent.

E. To 100 µL of *silver nitrate solution R2* add 50 µL of the solution to be examined. A white precipitate is formed.

TESTS

**pH**: maximum 2, using a *pH indicator strip R*.

**Iron**: maximum 10 µg/GBq.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Modifier solution*: 14 g/L solution of *magnesium nitrate R*.

*Test solution*. Dilute the solution to be examined with a 1 per cent *V/V* solution of *nitric acid R* to obtain a radioactivity concentration of 2.5 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluting with a 1 per cent *V/V* solution of *nitric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: graphite furnace.

An example of the injection and instrument parameters for the graphic furnace atomic absorption analysis is shown below.

*Internal and external protective gas*: *argon R*.

*Injection*: 20 µL of the test solution and the reference solutions, and 1 µL of the modifier solution.

*Injection temperature*: 20 °C.

*Furnace programme*:

| Step | Final temperature (°C) | Ramp time (s) | Hold time (s) | Internal protective gas flow rate (mL/min) |
|---|---|---|---|---|
| Drying | 110 | 1 | 30 | 250 |
| Drying | 130 | 15 | 30 | 250 |
| Pyrolysis | 1400 | 10 | 20 | 250 |
| Atomisation | 2100 | 0 | 5 | 0 |
| Cleaning | 2450 | 1 | 3 | 250 |

The solution may be released for use before completion of the test.

**Zinc**: maximum 10 µg/GBq.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dilute the solution to be examined with a 1 per cent *V/V* solution of *nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with a 1 per cent *V/V* solution of *nitric acid R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

The solution may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175 IU/*V*, *V* being the maximum volume to be used for the preparation of a single patient dose, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. The solution may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The solution may be released for use before completion of test B.

**Gallium-68**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma-ray spectrum corresponding to photons with an energy different from 0.511 MeV, 1.077 MeV, 1.022 MeV and 1.883 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Germanium-68 and gamma-ray-emitting impurities. Gamma-ray spectrometry.

Determine the amount of gallium-68, germanium-68 and radionuclidic impurities with a half-life longer than 5 h. For the detection and quantification of germanium-68 and gamma-ray-emitting impurities, retain the solution to be examined for at least 48 h to allow the gallium-68 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to germanium-68 and gamma-ray-emitting impurities is not more than 0.001 per cent.

*RADIOCHEMICAL PURITY*

**[$^{68}$Ga]Gallium(III) ion**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Adjust the solution to be examined to obtain a concentration of *hydrochloric acid R* of 10.3 g/L.

*Reference solution (a)*. To 0.2 mL of the test solution add 0.3 mL of a 4 g/L solution of *sodium hydroxide R*. Use within 30 min of preparation.

*Reference solution (b)*. To 1 mL of the test solution add 1 mL of a 10 g/L solution of *pentetic acid R* in a 4 g/L solution of *sodium hydroxide R*. Use within 30 min of preparation.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 77 g/L solution of *ammonium acetate R*, *methanol R* (50:50 *V/V*).

*Application*: about 5 µL.

*Development*: immediately, over a path of at least 10 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factor*: [$^{68}$Ga]Gallium(III) ion = 0-0.2.

*System suitability*: the retardation factor of the principal peak in the chromatogram obtained with reference solution (a) is not more than 0.1; the retardation factor of the principal peak in the chromatogram obtained with reference solution (b) is not less than 0.7.

*Limit*:

– *[$^{68}$Ga]gallium(III) ion*: minimum 95 per cent of the total radioactivity due to gallium-68.

*RADIOACTIVITY*

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

– that the solution is not intended for direct administration to humans;
– the maximum volume that can be used for the preparation of a single patient dose;
– the concentration of hydrochloric acid;
– the concentration of acetone, if present;
– that the solution is intended for use in the preparation of gallium-68-labelled radiopharmaceuticals;
– a procedure to reduce the level of germanium-68 below 0.001 per cent of the total radioactivity.

IMPURITIES

A. germanium-68.

**01/2013:2482**
**corrected 9.6**

# GALLIUM ($^{68}$Ga) EDOTREOTIDE INJECTION

## Gallii ($^{68}$Ga) edotreotidi solutio iniectabilis

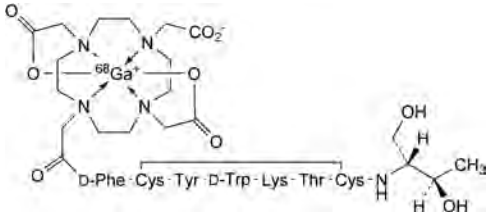


$C_{65}H_{89}{}^{68}GaN_{14}O_{18}S_2$ $M_r$ 1487

DEFINITION

Sterile solution of a complex of gallium-68 with edotreotide (*N*-[[4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl]-D-phenylalanyl-L-cysteinyl-L-tyrosyl-D-tryptophyl-L-lysyl-L-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide) (gallium-68 DOTATOC).

*Content*:

– *gallium-68*: 90 per cent to 110 per cent of the declared gallium-68 radioactivity at the date and time stated on the label;
– *edotreotide*: maximum 50 µg per maximum recommended dose in millilitres.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of gallium-68*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the principal gamma photons have energies of 0.511 MeV and 1.077 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Determine the approximate half-life by no fewer than 3 measurements of the activity of a sample in the same geometrical conditions within a suitable period of time (for example, 15 min).

*Result*: 62 min to 74 min.

C. Examine the chromatograms obtained in the test for other radiochemical impurities (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution has a relative retention of 1.3 with reference to the principal peak in the chromatogram obtained with reference solution (a) using the spectrophotometer.

TESTS

**pH**: 4.0 to 8.0, using a *pH indicator strip R*.

**Edotreotide, gallium edotreotide and other related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Prepare a 50 µg/*V* solution of *edotreotide R* in a 10.3 g/L solution of *hydrochloric acid R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Prepare a 50 µg/*V* solution of *octreotide acetate R* in a 10.3 g/L solution of *hydrochloric acid R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (c).* Mix 0.1 mL of reference solution (a) and 0.1 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile for chromatography R* (0.1:99.9 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 76 | 24 |
| 8 - 9 | 76 → 40 | 24 → 60 |
| 9 - 14 | 40 | 60 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: 20 µL.

*Relative retention* with reference to edotreotide (retention time = about 3.3 min): gallium edotreotide = about 1.3; octreotide = about 2.6.

*System suitability*: reference solution (c) using the spectrophotometer:

– *resolution*: minimum 5.0 between the peaks due to edotreotide and octreotide.

*Limits:* in the chromatogram obtained with the spectrophotometer:

– *edotreotide and metal complexes of edotreotide* (sum of the areas of the peaks with a relative retention with reference to edotreotide between 0.8 and 1.4): not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (50 µg/*V*);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (50 µg/*V*); disregard any peak with a relative retention with reference to edotreotide of 0.5 or less.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve 10 mg of *HEPES R* (impurity D) in *water R* and dilute to *V* with the same solvent, *V* being the maximum recommended dose in millilitres. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*; use an aluminium plate.

*Mobile phase*: *water R*, *acetonitrile R* (25:75 *V/V*).

*Application*: (*V*/1000) mL, *V* being the maximum recommended dose in millilitres; apply portions of 1 µL and dry with a current of warm air after each application.

*Development*: over 2/3 of the plate.

*Detection*: expose to iodine vapour for 4 min.

*Retardation factor*: impurity D = about 0.3.

*System suitability*: reference solution:

– the chromatogram shows a clearly visible spot.

*Limit*:

– *impurity D*: any spot due to impurity D is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (200 µg/*V*).

**Ethanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1 mL of *propanol R* to 1000 mL with *water R*.

*Test solution.* Dilute 0.10 mL of the preparation to be examined to 10.0 mL with the internal standard solution.

*Reference solution.* Dilute 1.0 mL of *anhydrous ethanol R* to 100.0 mL with the internal standard solution. Dilute 1.0 mL of this solution to 10.0 mL with the internal standard solution.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 10 mL/min.

*Split ratio*: 1:10.

*Temperature*:

– *column*: 35 °C;
– *injection port*: 140 °C;
– *detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*System suitability*: reference solution:

– *retention time*: ethanol = 2 min to 4 min;
– *resolution*: minimum 5.0 between the peaks due to ethanol and propanol.

*Limit*:

– *ethanol*: maximum 10 per cent *V/V* and maximum 2.5 g per administration, taking the density (*2.2.5*) to be 0.790 g/mL.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of test B.

**Gallium-68**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray spectrometry.

*Limit*: peaks in the gamma-ray spectrum corresponding to photons with an energy different from 0.511 MeV, 1.077 MeV, 1.022 MeV and 1.883 MeV represent not more than 0.1 per cent of the total radioactivity.

B. Germanium-68 and gamma-ray-emitting impurities. Gamma-ray spectrometry.

Determine the amount of gallium-68, germanium-68 and radionuclidic impurities with a half-life longer than 5 h. For the detection and quantification of germanium-68 and gamma-ray-emitting impurities, retain the preparation to be examined for at least 48 h to allow the gallium-68 to decay to a level that permits the detection of impurities.

*Result*: the total radioactivity due to germanium-68 and gamma-ray-emitting impurities is not more than 0.001 per cent.

*RADIOCHEMICAL PURITY*

– *[$^{68}$Ga]gallium edotreotide*: minimum 91 per cent of the total radioactivity due to gallium-68.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dilute *gallium ($^{68}$Ga) chloride solution R* with *water R* to obtain a final concentration of 10 g/L of *hydrochloric acid R*. To 1 mL of this solution add 1.5 mL of a 4 g/L solution of *sodium hydroxide R*. Use within 30 min of preparation.

*Reference solution (b).* Dilute *gallium ($^{68}$Ga) chloride solution R* with *water R* to obtain a final concentration of 10 g/L of *hydrochloric acid R*. To 1 mL of this solution add 1 mL of a solution containing 10 g/L of *pentetic acid R* and 4 g/L of *sodium hydroxide R*. Use within 30 min after preparation.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 77 g/L solution of *ammonium acetate R* in *water R*, *methanol R* (50:50 *V/V*).

*Application*: 5 µL.

*Development*: immediately, over 2/3 of the plate.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0-0.1; [$^{68}$Ga]gallium edotreotide = 0.8-1.0.

*System suitability*: the retardation factor of the principal signal in the chromatogram obtained with reference solution (a) is not more than 0.1; the retardation factor of the principal signal in the chromatogram obtained with reference solution (b) is more than 0.7.

*Limit*:

– *impurity A*: not more than 3 per cent of the total radioactivity due to gallium-68.

**Other radiochemical impurities**. Liquid chromatography (*2.2.29*) as described in the test for edotreotide, gallium edotreotide and other related substances. If necessary, dilute the test solution with *water R* to a radioactivity concentration suitable for the radioactivity detector.

Examine the chromatogram recorded using the radioactivity detector and locate the peak due to [$^{68}$Ga]gallium edotreotide by comparison with the chromatogram obtained with reference solution (a) and the spectrophotometer.

*Relative retention* with reference to [$^{68}$Ga]gallium edotreotide (retention time = about 4.2 min): impurity B = about 0.3.

*Limit*:

– *impurity B*: not more than 2 per cent of the total radioactivity due to gallium-68.

Calculate the percentage of radioactivity due to [$^{68}$Ga]gallium edotreotide using the following expression:

$$(100 - A) \times T$$

$A$ = percentage of radioactivity due to impurity A determined in the test for impurity A under radiochemical purity;

$T$ = proportion of the area of the peak due to [$^{68}$Ga]gallium edotreotide relative to the total areas of the peaks in the chromatogram obtained with the test solution.

*RADIOACTIVITY*

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the percentage content of ethanol in the preparation.

IMPURITIES

*Specified impurities: A, B, C, D.*

A. [$^{68}$Ga]gallium in colloidal form,

B. [$^{68}$Ga]gallium(III) ion,

C. germanium-68,

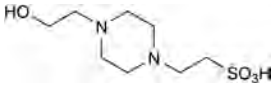


D. 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES).

**01/2008:1922**

# HUMAN ALBUMIN INJECTION, IODINATED ($^{125}$I)

## Iodinati ($^{125}$I) humani albumini solutio iniectabilis

DEFINITION

Sterile, endotoxin-free solution of human albumin labelled with iodine-125. It may contain a suitable buffer and an antimicrobial preservative. The human albumin used complies with the requirements of the monograph on *Human albumin solution (0255)*.

*Content*: 90 per cent to 110 per cent of the declared iodine-125 radioactivity at the date stated on the label.

*Purity*:

– minimum of 99.0 per cent of the total radioactivity corresponds to iodine-125,

– minimum of 80 per cent of the total radioactivity is associated with the albumin fractions II to V,

– maximum of 5 per cent of the total radioactivity corresponds to unbound iodide.

*Content of albumin*: 95 per cent to 105 per cent of the declared albumin content stated on the label.

CHARACTERS

*Appearance*: clear, colourless to yellowish solution.

*Half-life and nature of radiation of iodine-125*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Comparison*: standardised iodine-125 solution, or by using a calibrated instrument. Standardised iodine-125 solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised iodine-125 solution, apart from any differences attributable to the presence of iodine-126. The most prominent photon has an energy of 0.027 MeV, corresponding to the characteristic X-ray of tellurium, gamma photons of an energy of 0.035 MeV are also present. Iodine-126 has a half-life of 13.11 days and its most prominent gamma photons have energies of 0.388 MeV and 0.666 MeV.

B. Examine by a suitable immunoelectrophoresis technique (*2.7.1*). Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted if necessary. The main component of the preparation to be examined corresponds to the main component of the normal human serum. The diluted solution may show the presence of small quantities of other plasma proteins.

TESTS

**pH** (*2.2.3*): 5.0 to 9.0.

**Albumin**

*Reference solution.* Dilute *human albumin solution R* with a 9 g/L solution of *sodium chloride R* to a concentration of 5 mg of albumin per millilitre.

To 1.0 mL of the preparation to be examined and to 1.0 mL of the reference solution add 4.0 mL of *biuret reagent R* and mix. After exactly 30 min, measure the absorbance (*2.2.25*) of each solution at 540 nm, using as the compensation liquid a 9 g/L solution of *sodium chloride R* treated in the same manner. From the absorbances measured, calculate the content of albumin in the injection to be examined in milligrams per millilitre.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*.

**Bacterial endotoxins** (*2.6.14*): less than 175/$V$ IU/mL, $V$ being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

**Iodine-125**: minimum 99.0 per cent of the total radioactivity.

Gamma-ray and X-ray spectroscopy.

*Comparison*: standardised solution of iodine-125.

Determine the relative amounts of iodine-125 and iodine-126 present.

*RADIOCHEMICAL PURITY*

**Iodine-125 in albumin fractions II to V, iodine-125 corresponding to unbound iodide**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Mix 0.25 mL of the preparation to be examined with 0.25 mL of the mobile phase. Use immediately after mixing.

*Reference solution. Human albumin solution R* or another appropriate human albumin standard diluted with the mobile phase to a suitable albumin concentration.

*Column*:

– *size*: $l$ = 0.6 m, Ø = 7.5 mm,

– *stationary phase*: *silica gel for size-exclusion chromatography R*,

– *temperature*: 25 °C.

*Mobile phase*: dissolve 11.24 g of *potassium dihydrogen phosphate R*, 42.0 g of *disodium hydrogen phosphate dodecahydrate R*, 11.70 g of *sodium chloride R* in 2000 mL of *water R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 280 nm and radioactivity detector set for iodine-125 connected in series.

*Injection*: loop injector.

*Run time*: 85 min.

*Retention times*:

| Peak No. | Fraction | Description of the compound | Retention time (min) |
|---|---|---|---|
| 1 | I | High molecular mass compound | 18 - 20 |
| 2 | II | Poly III albumin | 23 - 24 |
| 3 | III | Poly II albumin | 25 - 26 |
| 4 | IV | Poly I albumin | 28 |
| 5 | V | Human serum albumin | 29 - 31 |
| 6 | VI | Iodide | 43 - 45 |

The main peak in the chromatogram obtained with the reference solution corresponds to fraction V.

*Limits*:

- *radioactivity in fractions II to V*: minimum 80 per cent of the total radioactivity applied to the column,
- *iodine-125 in fraction VI*: maximum 5 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised iodine-125 solution or by measurement with a calibrated instrument.

LABELLING

The label states:

- the amount of albumin,
- the maximum volume to be injected.

**01/2008:1227**
**corrected 7.0**

# INDIUM ($^{111}$In) CHLORIDE SOLUTION

## Indii ($^{111}$In) chloridi solutio

DEFINITION

Sterile solution of indium-111 as the chloride in aqueous hydrochloric acid containing no additives.

*Indium-111*: 90 per cent to 110 per cent of the declared indium-111 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 1.85 GBq of indium-111 per microgram of indium.

PRODUCTION

No carrier indium is added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of indium-111*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry. *Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay.*

   *Results*: the most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.

B. To 100 µL of *silver nitrate solution R2* add 50 µL of the preparation to be examined. A white precipitate is formed.

C. pH (see Tests).

D. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

   *Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is 0.5 to 0.8.

TESTS

**pH** (*2.2.3*): 1.0 to 2.0.

**Cadmium**: maximum 0.40 µg/mL.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 0.05 mL of the preparation to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with the same concentration of *hydrochloric acid R* as in the test solution.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: electrothermal.

**Copper**: maximum 0.15 µg/mL.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 0.1 mL of the preparation to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R* diluting with the same concentration of *hydrochloric acid R* as in the test solution.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Atomisation device*: electrothermal.

**Iron**: maximum 0.60 µg/mL.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 0.1 mL of the preparation to be examined to a suitable volume with a suitable concentration of *hydrochloric acid R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (0.1 per cent Fe) R* diluting with the same concentration of *hydrochloric acid R* as in the test solution.

*Source*: iron hollow-cathode lamp

*Wavelength*: 248.3 nm.

*Atomisation device*: electrothermal.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Indium-111**. Gamma-ray and X-ray spectrometry.

*Comparison*: standardised indium-111 solution.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised indium-111 solution apart from any differences due to the presence of indium-114m.

**Impurity A**: maximum 0.25 per cent of the total radioactivity.

Gamma-ray spectrometry. *Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay.*

Take a volume equivalent to 30 MBq and record the gamma-ray spectrum using a suitable detector with a shield of lead, 6 mm thick, placed between the sample and the detector.

*Results*: the response in the region corresponding to the 0.558 MeV photon and the 0.725 MeV photon of indium-114m does not exceed that obtained using 75 kBq of a standardised indium-114m solution measured under the same conditions, when all measurements are calculated with reference to the date and time of administration.

*RADIOCHEMICAL PURITY*

**[$^{111}$In]Indium(III) ion**. Thin-layer chromatography (*2.2.27*)

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*. Use silica gel as the coating substance on a glass-fibre sheet.

*Mobile phase*: 9.0 g/L solution of *sodium chloride R* adjusted to pH 2.3 ± 0.05 with *dilute hydrochloric acid R*.

*Application*: 5 µL.

*Development*: immediately over a path of 15 cm.

*Drying*: in a current of cold air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factor*: [$^{111}$In]indium(III) ion = 0.5 to 0.8.

*Limit*:

– *[$^{111}$In]indium(III) ion* : minimum 95 per cent of the radioactivity due to indium-111.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. indium-114m.

**01/2008:1109**
**corrected 7.0**

# INDIUM ($^{111}$In) OXINE SOLUTION

## Indii ($^{111}$In) oxini solutio

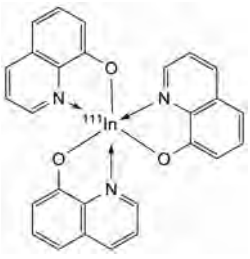

$C_{27}H_{18}[^{111}In]N_3O_3$ $M_r$ 543.5

DEFINITION

Sterile solution of indium-111 in the form of a complex with 8-hydroxyquinoline. It may contain suitable surface active agents and may be made isotonic by the addition of sodium chloride and a suitable buffer.

*Indium-111*: 90 per cent to 110 per cent of the declared indium-111 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 1.85 GBq of indium-111 per microgram of indium.

PRODUCTION

No carrier indium is added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of indium-111*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry. *Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay.*

   *Results*: the most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.

B. Place 5-10 mg of *magnesium oxide R* in a glass container about 20 mm in internal diameter. Add 20 µL of the preparation to be examined. Examine in ultraviolet light at 365 nm. Bright yellow fluorescence is produced.

C. The distribution of radioactivity between the organic and aqueous phases in the test for radiochemical purity (see Tests) contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 6.0 to 7.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Indium-111**. Gamma-ray and X-ray spectrometry.

*Comparison*: standardised indium-111 solution.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised indium-111 solution, apart from any differences due to the presence of indium-114m.

**Impurity A**: maximum 0.25 per cent of the total radioactivity.

Gamma-ray spectrometry. *Carry out the test after allowing sufficient time for short-lived impurities such as indium-110m to decay.*

Take a volume equivalent to 30 MBq and record the gamma-ray spectrum using a suitable detector with a shield of lead, 6 mm thick, placed between the sample and the detector.

*Results*: the response in the region corresponding to the 0.558 MeV photon and the 0.725 MeV photon of indium-114m does not exceed that obtained using 75 kBq of a standardised indium-114m solution measured under the same conditions, when all measurements are calculated with reference to the date and time of administration.

*RADIOCHEMICAL PURITY*

**[$^{111}$In]Indium oxine**. To a silanised separating funnel containing 3 mL of a 9 g/L solution of *sodium chloride R* add 100 µL of the preparation to be examined and mix. Add 6 mL of *octanol R* and shake vigorously. Allow the phases to separate and then run the lower layer into a suitable vial for counting. Allow the upper layer to drain completely into a similar vial. Add 1 mL of *octanol R* to the separating funnel, shake vigorously and drain into the vial containing the organic fraction. Add 5 mL of *dilute hydrochloric acid R* to the separating funnel, shake vigorously and drain these rinsings into a 3rd vial. Seal each vial and, using a suitable instrument, measure the radioactivity in each. Calculate the radiochemical purity by expressing the radioactivity of the [$^{111}$In]indium oxine, found in the organic phase, as a percentage of the total radioactivity due to indium-111 measured in the 3 solutions.

*Result*: minimum 90 per cent of the radioactivity due to indium-111.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states that the solution is not for direct administration to humans.

IMPURITIES

A. indium-114m.

**01/2008:0670**
**corrected 7.0**

# INDIUM ($^{111}$In) PENTETATE INJECTION

## Indii ($^{111}$In) pentetatis solutio iniectabilis

DEFINITION

Sterile solution containing indium-111 in the form of indium diethylenetriaminepenta-acetate. It may contain calcium and may be made isotonic by the addition of sodium chloride and a suitable buffer.

*Indium-111*: 90 per cent to 110 per cent of the declared indium-111 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of Indium-111*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.
*Results*: the most prominent gamma photons of indium-111 have energies of 0.171 MeV and 0.245 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests). The distribution of radioactivity contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 7.0 to 8.0.

**Cadmium**: maximum 5 µg/mL.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Mix 0.1 mL of the preparation to be examined with 0.9 mL of a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *water R*.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *water R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

**Uncomplexed diethylenetriaminepenta-acetic acid**: maximum 0.4 mg/mL.

In a micro test-tube, mix 100 µL of the preparation to be examined with 100 µL of a freshly prepared 1 g/L solution of *hydroxynaphthol blue, sodium salt R* in a 42 g/L solution of *sodium hydroxide R*. Add 50 µL of a 0.15 g/L solution of *calcium chloride R*. The solution remains pinkish-violet or changes from blue to pinkish-violet.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 14/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

**Indium-111**. Gamma-ray and X-ray spectrometry.

*Comparison*: standardised indium-111 solution.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised indium-111 solution apart from any differences due to the presence of indium-114m.

**Impurity A**: maximum 0.2 per cent of the total radioactivity at the date and time of administration.

Gamma-ray spectrometry.

Retain a sample of the preparation to be examined for a sufficient time to allow the indium-111 radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. Record the gamma-ray spectrum of the decayed material in a suitable instrument calibrated with the aid of a standardised indium-114m solution.

*Result*: indium-114m has a half-life of 49.5 days and its most prominent gamma photon has an energy of 0.190 MeV.

*RADIOCHEMICAL PURITY*

**[$^{111}$In]Indium pentetate**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: 5-10 µL.

*Development*: over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Identification of spots*: [$^{111}$In]indium pentetate migrates near to the solvent front.

*Limit*:

– *[$^{111}$In]indium pentetate* : minimum 95 per cent of the radioactivity due to indium-111.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. indium-114m.

**01/2008:1113**
**corrected 7.0**

# IOBENGUANE ($^{123}$I) INJECTION

## Iobenguani ($^{123}$I) solutio iniectabilis

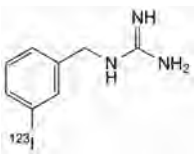


$C_8H_{10}[^{123}I]N_3$

DEFINITION

Sterile, bacterial endotoxin-free solution of 1-(3-[$^{123}$I]iodobenzyl)guanidine or its salts. It may contain a suitable buffer, a suitable labelling catalyst such as ionic copper, a suitable labelling stabiliser such as ascorbic acid and antimicrobial preservatives.

*Iodine-123*: 90 per cent to 110 per cent of the declared iodine-123 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 10 GBq of iodine-123 per gram of iobenguane base.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of iodine-123*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Result*: the energy of the most prominent gamma photon of iodine-123 is 0.159 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests). The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 3.5 to 8.0.

**Specific radioactivity**. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulfate from the areas of the peaks due to iobenguane in the chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the test by 0.85.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of the test.

**Radionuclides other than iodine-123**: maximum 0.35 per cent of the total radioactivity.

Gamma-ray and X-ray spectrometry.

Record the gamma-ray spectrum and the X-ray spectrum using a suitable instrument.

Determine the relative amounts of iodine-125, tellurium-121 and other radionuclidic impurities present. For their determination, retain the preparation to be examined for a sufficient time to allow iodine-123 to decay to a level which permits the detection of radionuclidic impurities. No radionuclides with a half-life longer than that of iodine-125 are detected.

*RADIOCHEMICAL PURITY*

**[$^{123}$I]Iobenguane**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.100 g of *sodium iodide R* in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *iobenguane sulfate CRS* in 25 mL of the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: 80 g/L solution of *ammonium nitrate R*, *dilute ammonia R2*, *methanol R* (1:2:27 *V*/*V*/*V*).

*Flow rate*: 1.0 mL/min.

*Detection*: suitable detector to determine the distribution of radioactivity and spectrophotometer at 254 nm, provided with a flow-cell.

*Injection*: 10 µL.

*Limits*:

– *[$^{123}$I]iobenguane*: minimum 95 per cent of the radioactivity due to iodine-123;

– *impurity A*: maximum 4 per cent of the radioactivity due to iodine-123;

– *other impurities*: maximum 1 per cent of the radioactivity due to iodine-123.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

LABELLING

The label states the specific radioactivity expressed in GBq of iodine-123 per gram of iobenguane base.

IMPURITIES

A. [$^{123}$I]iodide,

B. iodine-125,

C. tellurium-121.

**01/2008:1111**
**corrected 7.0**

# IOBENGUANE ($^{131}$I) INJECTION FOR DIAGNOSTIC USE

## Iobenguani ($^{131}$I) solutio iniectabilis ad usum diagnosticum

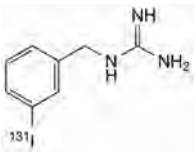


$C_8H_{10}[^{131}I]N_3$

DEFINITION

Sterile, bacterial endotoxin-free solution of 1-(3-[$^{131}$I]iodobenzyl)guanidine or its salts. It may contain a suitable buffer, a suitable labelling catalyst such as ionic copper, a suitable labelling stabiliser such as ascorbic acid, and antimicrobial preservatives.

*Iodine-131*: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 20 GBq of iodine-131 per gram of iobenguane base.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests). The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 3.5 to 8.0.

**Specific radioactivity**. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulfate from the areas of the peaks due to iobenguane in the chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the test by 0.85.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iobenguane**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.100 g of *sodium iodide R* in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *iobenguane sulfate CRS* in 25 mL of the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: 80 g/L solution of *ammonium nitrate R*, *dilute ammonia R2*, *methanol R* (1:2:27 *V*/*V*/*V*).

*Flow rate*: 1.0 mL/min.

*Detection*: suitable detector to determine the distribution of radioactivity and spectrophotometer at 254 nm, provided with a flow-cell.

*Injection*: 10 µL.

*Limits*:

– *[$^{131}$I]iobenguane*: minimum 94 per cent of the radioactivity due to iodine-131;

– *impurity A*: maximum 5 per cent of the radioactivity due to iodine-131;

– *other impurities*: maximum 1 per cent of the radioactivity due to iodine-131.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

LABELLING

The label states the specific radioactivity expressed in GBq of iodine-131 per gram of iobenguane base.

IMPURITIES

A. [$^{131}$I]iodide,

B. iodine-133,

C. iodine-135.

**01/2008:1112**
**corrected 7.0**

# IOBENGUANE ($^{131}$I) INJECTION FOR THERAPEUTIC USE

## Iobenguani ($^{131}$I) solutio iniectabilis ad usum therapeuticum

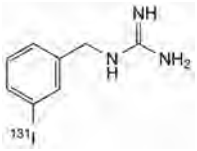


$C_8H_{10}$ [$^{131}$I] $N_3$

DEFINITION

Sterile, bacterial endotoxin-free solution of 1-(3-[$^{131}$I]iodobenzyl)guanidine or its salts. It may contain a suitable buffer, a suitable labelling catalyst such as ionic copper, a suitable labelling stabiliser such as ascorbic acid, and antimicrobial preservatives.

*Iodine-131*: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 400 GBq of iodine-131 per gram of iobenguane base.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatogram obtained in the test for radio-chemical purity (see Tests). The distribution of the radioactivity contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 3.5 to 8.0.

**Specific radioactivity**. The specific radioactivity is calculated from the results obtained in the test for radiochemical purity. Determine the content of iobenguane sulfate from the areas of the peaks due to iobenguane in the chromatograms obtained with the test solution and reference solution (b). Calculate the concentration as iobenguane base by multiplying the result obtained in the test by 0.85.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iobenguane**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* Dissolve 0.100 g of *sodium iodide R* in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *iobenguane sulfate CRS* in 25 mL of the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: 80 g/L solution of *ammonium nitrate R*, *dilute ammonia R2*, *methanol R* (1:2:27 *V*/*V*/*V*).

*Flow rate*: 1.0 mL/min.

*Detection*: suitable detector to determine the distribution of radioactivity and spectrophotometer at 254 nm, provided with a flow-cell.

*Injection*: 10 µL.

*Limits*:

– *[$^{131}$I]iobenguane*: minimum 92 per cent of the radioactivity due to iodine-131;

– *impurity A*: maximum 7 per cent of the radioactivity due to iodine-131;

– *other impurities*: maximum 1 per cent of the radioactivity due to iodine-131.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

LABELLING

The label states the specific radioactivity expressed in GBq of iodine-131 per gram of iobenguane base.

IMPURITIES

A. [$^{131}$I]iodide,

B. iodine-133,

C. iodine-135.

**07/2017:2351**

# IOBENGUANE SULFATE FOR RADIOPHARMACEUTICAL PREPARATIONS

## Iobenguani sulfas ad radiopharmaceutica

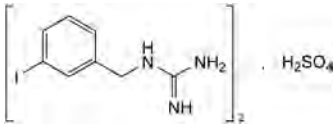

$C_{16}H_{22}I_2N_6O_4S$ $M_r$ 648

[87862-25-7]

DEFINITION

Bis[[(3-iodophenyl)methyl]guanidine] sulfate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystals.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *iobenguane sulfate CRS*.

B. Dissolve about 10 mg in 1 mL of *water R* with gentle heating. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Impurity A**. Liquid chromatography (*2.2.29*). *Prepare the solutions and the mobile phase immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 1 mL of the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *iobenguane sulfate CRS* in 1 mL of the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 23.1 mg of *3-iodobenzylammonium chloride R* (salt of impurity A) in 1 mL of the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c).* Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

*Reference solution (d).* Dilute 0.1 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm);

– *temperature*: maintain at a constant temperature between 20 °C and 30 °C.

*Mobile phase*: mix 40 mL of an 80 g/L solution of *ammonium nitrate R*, 80 mL of *dilute ammonia R2* and 1080 mL of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*Run time*: 15 min.

*Relative retention* with reference to iobenguane (retention time = about 7 min): impurity A = about 0.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 4.0 between the peaks due to impurity A and iobenguane.

*Limit*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (1.0 per cent).

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 0.100 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{16}H_{22}I_2N_6O_4S$ from the declared content of *iobenguane sulfate CRS*.

STORAGE

Protected from light, at a temperature below 25 °C.

IMPURITIES

*Specified impurities: A.*

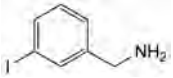

A. 1-(3-iodophenyl)methanamine.

**01/2008:0939**
**corrected 7.0**

# IODOMETHYLNORCHOLESTEROL ($^{131}$I) INJECTION

## Iodomethylnorcholesteroli ($^{131}$I) solutio iniectabilis

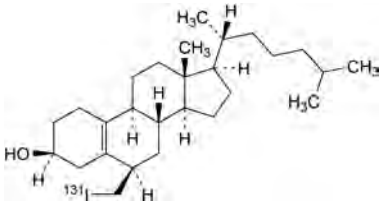

DEFINITION

Sterile solution of 6β-[$^{131}$I]iodomethyl-19-norcholest-5(10)-en-3β-ol. It may contain a suitable emulsifier such as polysorbate 80 and a suitable antimicrobial preservative such as benzyl alcohol.

*Iodine-131*: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and time stated on the label.

*Specific radioactivity*: 3.7 GBq to 37 GBq per gram of 6β-iodomethylnorcholesterol.

CHARACTERS

*Appearance*: clear or slightly turbid, colourless or pale yellow solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity 6β-[$^{131}$I]iodomethyl-19-norcholest-5(10)-en-3β-ol (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is about 0.5.

TESTS

**pH** (*2.2.3*): 3.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**6β-[$^{131}$I]Iodomethyl-19-norcholest-5(10)-en-3β-ol**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Carrier solution.* Dissolve 10 mg of *potassium iodide R*, 20 mg of *potassium iodate R* and 0.1 g of *sodium hydrogen carbonate R* in *distilled water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel GF$_{254}$ plate R.*

*Mobile phase*: *chloroform R.*

*Application*: up to 5 µL of the test solution and 10 µL of the carrier solution on the same spot.

*Development*: over a path of 15 cm in about 60 min.

*Drying*: in air.

*Detection*: ultraviolet light at 254 nm and suitable detector to determine the distribution of radioactivity.

*Retardation factor*: 6β-[$^{131}$I]iodomethyl-19-norcholest-5(10)-en-3β-ol = about 0.5.

*Identification of spots*: impurity C remains near the point of application.

*Limit*:

– *6β-[$^{131}$I]iodomethyl-19-norcholest-5(10)-en-3β-ol*: minimum 85 per cent of the total radioactivity due to iodine-131.

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Carrier solution.* Dissolve 10 mg of *potassium iodide R*, 20 mg of *potassium iodate R* and 0.1 g of *sodium hydrogen carbonate R* in *distilled water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel GF$_{254}$ plate R.*

*Mobile phase*: *chloroform R*, *anhydrous ethanol R* (50:50 *V*/*V*).

*Application*: 10 µL of the carrier solution and then up to 5 µL of the test solution on the same spot.

*Development*: over a path of 15 cm in about 90 min.

*Drying*: in air.

*Detection*: ultraviolet light at 254 nm for 5 min and suitable detector to determine the distribution of radioactivity.

*Retardation factor*: impurity C (yellow spot) = about 0.5.

*Identifications of spots*: the principal peak of radioactivity is near to the solvent front; other iodocholesterols migrate near the solvent front.

*Limit*:

– *impurity C*: maximum 5 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light, at − 18 °C or below.

IMPURITIES

A. iodine-133,

B. iodine-135,

C. [$^{131}$I]iodide.

**01/2008:1533**

# KRYPTON ($^{81m}$Kr) INHALATION GAS

## Kryptonum ($^{81m}$Kr) ad inhalationem

DEFINITION

Gaseous mixture of krypton-81m and a suitable vehicle such as air.

PRODUCTION

Krypton-81m is formed by decay of its parent radionuclide rubidium-81. Rubidium-81 has a half-life of 4.58 h.

The krypton-81m formed is separated from the rubidium-81 with a flow of a suitable gas in a rubidium/krypton generator. Rubidium-81 is produced by proton irradiation of krypton isotopes or by helium-3 or helium-4 irradiation of bromine. After separation of rubidium-81 from the target, it is retained by a suitable support.

Krypton-81m is eluted at a suitable flow rate with a vehicle such as air. The level of moisture required in the eluent depends on the type of generator used. The transport tube for administration has a defined length and inner diameter. The radioactivity concentration is determined before administration.

CHARACTERS

*Appearance*: clear, colourless gas.

*Half-life and nature of radiation of krypton-81m*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Result*: the gamma photon of krypton-81m has an energy of 0.190 MeV.

B. The half-life of krypton-81m is 11.8 s to 14.4 s.

TESTS

*RADIONUCLIDIC PURITY*

**Radionuclides other than krypton-81m**: maximum 0.1 per cent of the radioactivity passed through the absorber, calculated with reference to the date and time of administration.

*Gamma-ray and X-ray spectrometry*.

Elute the generator as prescribed. Pass a sufficient amount (2 L to 10 L) of eluate at a suitable flow rate through a suitable absorber such as water. Determine the amount of radioactivity eluted. Allow the krypton-81m to decay for 5 min and record the gamma and X-ray spectrum of the residual radioactivity on the absorber using a suitable instrument. Examine the gamma-ray and X-ray spectrum of the absorber for the presence of radioactive impurities, which must be identified and quantified.

RADIOACTIVITY

Determine the radioactive concentration of the preparation using suitable equipment such as an ionisation chamber or a gamma ray spectrometer. The radioactivity is measured under defined operating conditions, such as gas flow rate and measurement geometry, that are identical to those used for the calibration of the instrument.

STORAGE

The storage conditions apply to the generator.

LABELLING

The labelling conditions apply to the generator.

**07/2017:2798**
**corrected 9.3**

# LUTETIUM ($^{177}$Lu) SOLUTION FOR RADIOLABELLING

## Lutetii ($^{177}$Lu) solutio ad radio-signandum

$^{177}Lu^{3+}$ $M_r$ 176.9

DEFINITION

Solution containing lutetium-177 in the form of lutetium(III) ion in dilute hydrochloric acid.

*Content*:

– *lutetium-177*: 90 per cent to 110 per cent of the declared lutetium-177 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of lutetium-177*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of lutetium-177 has an energy of 0.208 MeV. Gamma photons with an energy of 0.113 MeV are also present.

B. pH (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is 0.4 to 0.7.

TESTS

**pH**: 1.0 to 2.0, using a *pH indicator strip R*.

**Lutetium**. Inductively coupled plasma-atomic emission spectrometry (*2.2.57*), for determination of specific radioactivity.

*Test solution*. Dilute the preparation to be examined with a 48.3 g/L solution of *heavy metal-free nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *lutetium standard solution (20 ppm Lu) R*, diluting with a 48.3 g/L solution of *heavy metal-free nitric acid R*.

*Wavelength*: 261.542 nm.

**Copper**: maximum 1.0 µg/GBq.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Dilute the preparation to be examined with a 48.3 g/L solution of *heavy metal-free nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *copper standard solution (10 ppm Cu) R*, diluting with a 48.3 g/L solution of *heavy metal-free nitric acid R*.

*Wavelength*: 327.393 nm.

**Iron**: maximum 0.5 µg/GBq.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Dilute the preparation to be examined with a 48.3 g/L solution of *heavy metal-free nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluting with a 48.3 g/L solution of *heavy metal-free nitric acid R*.

*Wavelength*: 238.204 nm.

**Lead**: maximum 0.5 µg/GBq.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution*. Dilute the preparation to be examined with a 48.3 g/L solution of *heavy metal-free nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions*. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluting with a 48.3 g/L solution of *heavy metal-free nitric acid R*.

*Wavelength*: 220.353 nm.

**Zinc**: maximum 1.0 µg/GBq.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Dilute the preparation to be examined with a 48.3 g/L solution of *heavy metal-free nitric acid R* to obtain a radioactivity concentration of 50 MBq/mL.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with a 48.3 g/L solution of *heavy metal-free nitric acid R*.

*Wavelength*: 213.855 nm.

**Sterility**. If intended for use in the manufacture of parenteral preparations without a further appropriate sterilisation procedure, it complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175 IU/*V*, *V* being the maximum volume to be used for the preparation of a single patient dose, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

*RADIONUCLIDIC PURITY*

**Lutetium-177**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of lutetium-177m, ytterbium-175 and other radionuclidic impurities present.

*Results*:

- the total radioactivity due to ytterbium-175 (impurity B) is not more than 0.1 per cent;
- the total radioactivity due to lutetium-177m (impurity A) is not more than 0.07 per cent;
- the total radioactivity due to radionuclidic impurities other than A and B is not more than 0.01 per cent.

*RADIOCHEMICAL PURITY*

**[$^{177}$Lu]lutetium(III) ion**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution.* To 1 mL of the test solution add 0.4 mL of a 10 g/L solution of *pentetic acid R* in a 4 g/L solution of *sodium hydroxide R*.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 9.0 g/L solution of *sodium chloride R* adjusted to pH 2.30 ± 0.05 with *dilute hydrochloric acid R*.

*Application*: 5 µL.

*Development*: immediately, over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{177}$Lu]lutetium(III) ion = about 0.4-0.7; [$^{177}$Lu]lutetium pentetate = minimum 0.9.

*System suitability*: reference solution:

- the chromatogram shows 2 clearly separated spots.

*Limit*:

- *[$^{177}$Lu]lutetium(III) ion*: minimum 99 per cent of the total radioactivity due to lutetium-177.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- that the solution is not for direct administration to humans;
- the maximum volume that can be used for the preparation of a single patient dose;
- the concentration of hydrochloric acid;
- that the solution is intended for use in the preparation of lutetium-177-labelled radiopharmaceuticals;
- the specific radioactivity.

IMPURITIES

A. lutetium-177m,

B. ytterbium-175.

**07/2017:2350**

# MEDRONIC ACID FOR RADIOPHARMACEUTICAL PREPARATIONS

## Acidum medronicum ad radiopharmaceutica

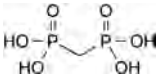

$CH_6O_6P_2$ $M_r$ 176.0

[1984-15-2]

DEFINITION

Methylenediphosphonic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline, hygroscopic powder.

*Solubility*: very soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A.*

*Second identification: B.*

A. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Preparation*: 100 g/L solution in *deuterium oxide R*.

*Comparison*: 100 g/L solution of *medronic acid CRS* in *deuterium oxide R*.

*Results*: the $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *medronic acid CRS*.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *medronic acid CRS*.

TESTS

**Impurities A and B**. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Test solution.* To 1.0 g of the substance to be examined add 10 mL of *deuterated chloroform R*. Stir for 1 hour. Pass the resulting solution through a sintered-glass filter to remove the precipitate containing medronic acid. Evaporate the filtrate to about 0.5 mL.

*Reference solution (a).* Mix 10 µL of *medronic acid impurity A CRS* with 1.0 mL of *deuterated chloroform R*.

*Reference solution (b).* Mix 10 µL of *medronic acid impurity B CRS* with 1.0 mL of *deuterated chloroform R*.

*Reference solution (c).* After recording the NMR spectrum of the test solution, add 10 µL of *medronic acid impurity A CRS* and 10 µL of *medronic acid impurity B CRS* to the test solution.

*Apparatus*: NMR spectrometer operating at minimum 250 MHz.

Record the $^1$H NMR spectra of the test solution and the reference solutions, if necessary using *tetramethylsilane R* as a chemical shift internal reference compound.

*Position of the signals*: deuterated chloroform = about 7.3 ppm; impurity A = about 4.4 ppm and 1.3 ppm; impurity B = about 4.7 ppm, 2.4 ppm and 1.3 ppm.

*System suitability*:

– the positions of the signals due to impurities A and B in the spectrum obtained with reference solution (c) do not differ significantly from those in the spectra obtained with reference solutions (a) and (b).

*Limits*:

– *integration*: integrate the multiplet at 4.4 ppm due to impurity A and the multiplet at 2.4 ppm due to impurity B in the spectra obtained with the test solution and reference solution (c) to obtain the areas of the peaks used in the comparison of impurity contents;
– *impurities A, B*: for each impurity, not more than 0.5 times the area of the corresponding peak in the spectrum obtained with reference solution (c) (1 per cent).

**Phosphates** (*2.4.11*): maximum 1.0 per cent.

Dissolve 0.100 g in 10 mL of *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 75 mg in *water R* and dilute to 50 mL with the same solvent. Titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *bromocresol green solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 8.80 mg of $CH_6O_6P_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

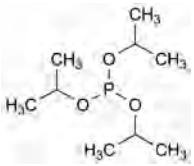

A. tris[(propan-2-yl)oxy]phosphane,

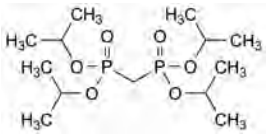

B. tetrakis(propan-2-yl) methylenediphosphonate.

**01/2008:1617**

# L-METHIONINE ([11C]METHYL) INJECTION

## L-Methionini ([11C]methyl) solutio iniectabilis

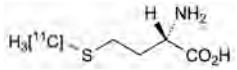

DEFINITION

Sterile solution of (2*S*)-2-amino-4-([11C]methylsulfanyl)-butanoic acid for diagnostic use.

*Content*: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

*Purity*:

– minimum of 99 per cent of the total radioactivity corresponds to carbon-11,
– minimum of 95 per cent of the total radioactivity corresponds to carbon-11 in the form of L-[*methyl*-11C]methionine and D-[*methyl*-11C]methionine,
– maximum of 10 per cent of the total radioactivity corresponds to carbon-11 in the form of D-[*methyl*-11C]methionine.

*Content of methionine*: maximum of 2 mg per maximum recommended dose in millilitres.

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [11C]carbon dioxide or [11C]methane.

*RADIOCHEMICAL SYNTHESIS*

L-[*Methyl*-11C]methionine can be prepared by various chemical synthetic pathways. All methods rely on the alkylation of the sulfide anion of L-homocysteine with [11C]methyl iodide or [11C]methyl triflate. Variations in the procedures used to generate the sulfide anion of L-homocysteine and methods to obtain [11C]methyl iodide lead to negligible differences with respect to quality in terms of specific radioactivity, enantiomeric purity and possible chemical and radiochemical impurities.

**Synthesis of [11C]methyl iodide**

[11C]Methyl iodide can be obtained either starting from [11C]carbon dioxide or from [11C]methane. The most frequently used method is the reduction of [11C]carbon dioxide with lithium aluminium hydride. The formed [11C]methanol is reacted with hydroiodic acid. Alternatively [11C]methane, either obtained directly in the target or by on-line processes from [11C]carbon dioxide, is reacted with iodine.

**Synthesis of [11C]methyl triflate**

[11C]methyl triflate can be prepared from [11C]methyl iodide using a silver triflate-impregnated solid support such as graphitised carbon.

**Synthesis of L-[*methyl*-11C]methionine**

The most widely used method to obtain L-[*methyl*-11C]-methionine is the alkylation of the sulfide anion, generated from L-homocysteine thiolactone, with [11C]methyl iodide or [11C]methyl triflate in alkaline conditions in a solvent such as acetone. The L-[*methyl*-11C]methionine obtained can be purified by semi-preparative liquid chromatography. For example, a column packed with octadecylsilyl silica gel for chromatography eluted with a 9 g/L solution of sodium chloride is suitable.

**L-Homocysteine thiolactone hydrochloride**

Specific optical rotation (*2.2.7*): + 20.5 to + 21.5, determined on a 10 g/L solution at 25 °C.

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of L-homocysteine thiolactone hydrochloride.*

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of carbon-11*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**pH** (*2.2.3*): 4.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

*CHEMICAL PURITY*

**Impurity A, impurity B and methionine**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 0.6 mg of *L-homocysteine thiolactone hydrochloride R*, 2 mg of *DL-homocysteine R* and 2 mg of *DL-methionine R* in *water R* and dilute to *V*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b)*. Dissolve 2 mg of *L-methionine R* in the same solvent as used in the test solution and dilute to 10 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface of 220 $m^2$/g, a pore size of 8 nm and a carbon loading of 6.2 per cent,

– *temperature*: 25 °C.

*Mobile phase*: 1.4 g/L solution of *potassium dihydrogen phosphate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm and radioactivity detector connected in series.

*Injection*: loop injector.

*Run time*: 10 min.

*Relative retention* with reference to methionine (retention time = about 2.6 min): impurity B = about 0.8, impurity A = about 2.7.

*System suitability*: reference solution (a):

– *resolution*: minimum of 2.5 between the peaks due to methionine and impurity B.

*Limits*: examine the chromatogram obtained with the spectrophotometer:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.6 mg/*V*),

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2 mg/*V*),

– *methionine*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2 mg/*V*).

**Residual solvents** (*2.4.24*): maximum 50 mg/*V* for the concentration of acetone, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Carbon-11**: minimum 99 per cent of the total radioactivity.

A. Gamma-ray spectroscopy.

*Comparison*: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**L-[*Methyl*-$^{11}$C]methionine and impurity E**. Liquid chromatography (*2.2.29*) as described in the test for impurity A, impurity B and methionine.

*Injection*: test solution and reference solution (b).

*Limits*: examine the chromatogram obtained with the radioactivity detector:

– *total of L-[methyl-$^{11}$C]methionine and impurity E*: minimum of 95 per cent of the total radioactivity,

– other peaks in the chromatogram may be due to impurity C, impurity D and impurity F.

*ENANTIOMERIC PURITY*

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 2 mg of *L-methionine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 4 mg of *DL-methionine R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate for chiral separations R*.

*Mobile phase*: *methanol R*, *water R* (50:50 *V*/*V*).

*Application*: 2-10 µL.

*Development*: over a path of 8 cm.

*Drying*: in air for 5 min.

*Detection*: spray with a 2 g/L solution of *ninhydrin R* in *ethanol R* and heat at 60 °C for 10 min. Determine the distribution of radioactivity using a suitable detector.

*Retardation factors*: L-[*methyl*-$^{11}$C]methionine = about 0.58; impurity E = about 0.51.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Limits*:

– *total of L-[methyl-$^{11}$C]methionine and impurity E*: minimum 95 per cent of the total radioactivity,

– *impurity E*: maximum 10 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

IMPURITIES

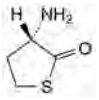

A. (3*S*)-3-aminodihydrothiophen-2(3*H*)-one (L-homocysteine thiolactone),

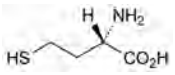

B. (2*S*)-2-amino-4-sulfanylbutanoic acid (L-homocysteine),

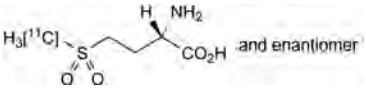


C. (2*RS*)-2-amino-4-([$^{11}$C]methylsulfonyl)butanoic acid (DL-[*methyl*-$^{11}$C]methiononine *S*,*S*-dioxide),

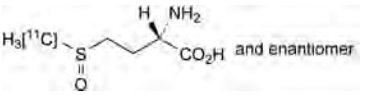


D. (2*RS*)-2-amino-4-([$^{11}$C]methylsulfinyl)butanoic acid (DL-[*methyl*-$^{11}$C]methiononine *S*-oxide),

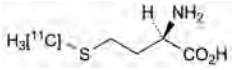

E. (2*R*)-2-amino-4-([$^{11}$C]methylsulfanyl)butanoic acid (D-[*methyl*-$^{11}$C]methionine),

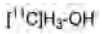

F. [$^{11}$C]methanol

**01/2008:1620**

# OXYGEN ($^{15}$O)

## Oxygenium ($^{15}$O)

DEFINITION

Mixture of [$^{15}$O]oxygen in the gaseous phase and a suitable vehicle such as *Medicinal air (1238)*, for diagnostic use.

*Purity*:

- minimum 99 per cent of the total radioactivity corresponds to oxygen-15,
- minimum 97 per cent of the total radioactivity corresponds to oxygen-15 in the form of oxygen ($O_2$).

PRODUCTION

*RADIONUCLIDIC PRODUCTION*

Oxygen-15 is a radioactive isotope of oxygen which may be produced by various nuclear reactions such as proton irradiation of nitrogen-15 or deuteron irradiation of nitrogen-14.

*RADIOCHEMICAL SYNTHESIS*

In order to recover oxygen-15 as molecular oxygen from the nitrogen target gas, carrier oxygen is added at concentrations generally ranging from 0.2 per cent *V/V* to 1.0 per cent *V/V*. After irradiation, the target gas is usually passed through activated charcoal and a carbon dioxide scavenger, such as soda lime, before mixing with the vehicle.

CHARACTERS

*Appearance*: colourless gas.

*Half-life and nature of radiation of oxygen-15*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the retention times of the principal peaks in the chromatogram obtained with the test gas using the radioactivity detector are similar to those of the principal peaks corresponding to oxygen in the chromatogram obtained with the reference gas using the thermal conductivity detector.

TESTS

*The following tests are performed on [$^{15}$O]oxygen as described under radiochemical synthesis before mixing with the vehicle.*

*RADIONUCLIDIC PURITY*

**Oxygen-15**: minimum 99 per cent of the total radioactivity.

A. Gamma spectrometry.

*Comparison*: standardised fluorine-18 solution, or by using an instrument calibrated with the aid of such a solution. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 1.9 min to 2.2 min.

The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Oxygen-15 in the form of $O_2$.** Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test sample.* [$^{15}$O]oxygen as described under radiochemical synthesis.

*Reference gas. Nitrogen gas mixture R.*

*Column*:

- *size*: l = 1.8 m, Ø1 = 6.3 mm and Ø2 = 3.2 mm,
- *stationary phase*: *GC concentrical column R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 65 mL/min.

*Temperature*:

- *column*: 40 °C,
- *injection port*: 40 °C,
- *thermal conductivity detector*: 70 °C.

*Detection*: thermal conductivity detector and radioactivity detector connected in series.

*Injection*: loop injector.

*Run time*: 10 min.

*Retention times*: oxygen, nitrogen and carbon monoxide eluting from the inner column = about 0.4 min; carbon dioxide eluting from the inner column = about 0.8 min; oxygen eluting from the outer column = about 2.1 min; nitrogen eluting from the outer column = about 3.1 min; carbon monoxide eluting from the outer column = about 6.2 min.

*System suitability*: reference gas:

- 5 clearly separated principal peaks are observed in the chromatogram obtained using the thermal conductivity detector,
- *resolution*: minimum of 1.5 between the peaks due to carbon dioxide eluting from the inner column and oxygen eluting from the outer column, in the chromatogram obtained using the thermal conductivity detector.

*Limits*: examine the chromatogram obtained with the radioactivity detector and calculate the percentage content of oxygen-15 substances from the peak areas.

- *oxygen-15 gas in the form of $O_2$*: minimum 97 per cent of the total radioactivity,
- disregard the first peak corresponding to components co-eluting from the inner column.

RADIOACTIVITY

The radioactive concentration is determined before administration.

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement in an instrument calibrated with the aid of such a solution.

**01/2018:2353**

# PENTETATE SODIUM CALCIUM FOR RADIOPHARMACEUTICAL PREPARATIONS

## Natrii calcii pentetas ad radiopharmaceutica

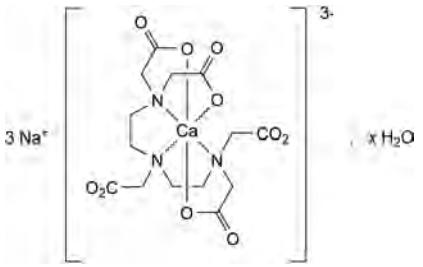


$C_{14}H_{18}CaN_3Na_3O_{10},xH_2O$ $M_r$ 497.4 (anhydrous substance)

DEFINITION

Trisodium [1,1′,1″,1‴-[[(carboxylatomethyl)imino]-bis(ethylenenitrilo)]tetraacetato]calciate(3-).

It is a starting material for the preparation of technetium ($^{99m}$Tc) pentetate injection.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder or crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pentetate sodium calcium CRS*.

B. Ignite. The residue gives reaction (b) of calcium (*2.3.1*).

C. The substance to be examined gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 8.0 to 9.5 for solution S.

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*. Dissolve 10 g of *ferric sulfate pentahydrate R* in 20 mL of *0.5 M sulfuric acid* and add 780 mL of *water R*. Adjust to pH 2.0 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 0.100 g of *sodium calcium edetate R* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 40.0 mg of *nitrilotriacetic acid R* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. To 10.0 mL of the solution add 1 mL of reference solution (a) and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: spherical *graphitised carbon for chromatography R1* (5 µm) with a specific surface area of 120 $m^2$/g and a pore size of 25 nm.

*Mobile phase*: dissolve 50 mg of *ferric sulfate pentahydrate R* in 50 mL of *0.5 M sulfuric acid* and add 750 mL of *water R*; adjust to pH 1.5 with *0.5 M sulfuric acid* or *1 M sodium hydroxide*, add 20 mL of *ethylene glycol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL of the test solution and reference solution (b); filter the solutions and inject immediately.

*Run time*: 4 times the retention time of the iron complex of impurity A.

*Retention time*: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min; the iron complex of pentetic acid elutes with the void volume.

*System suitability*: reference solution (b):

- *resolution*: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid;
- *signal-to-noise ratio*: minimum 50 for the peak due to the iron complex of impurity A.

*Limit*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Impurity B**: maximum 1.0 per cent.

Dissolve 5.0 g of the substance to be examined in 250 mL of *water R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and 50 mg of *mordant black 11 triturate R*. Not more than 1.3 mL of *0.1 M magnesium chloride* is required to change the colour of the indicator to violet.

**Chlorides**: maximum 0.1 per cent.

Dissolve 0.7 g in *water R* and dilute to 20 mL with the same solvent. Add 30 mL of *dilute nitric acid R*, allow to stand for 30 min and filter. Dilute 10 mL of the filtrate to 50 mL with *water R*. Use this solution as the test solution. Prepare the reference solution using 0.40 mL of *0.01 M hydrochloric acid*, add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Filter both solutions if necessary. Add 1 mL of *silver nitrate solution R2* to the test solution and the reference solution. Mix and allow to stand for 5 min protected from light. Any opalescence in the test solution is not more intense than that in the reference solution.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 2.5 mL of solution S to 10 mL with *water R*. Add 0.25 g of *calcium chloride R* to the test solution and the standard before the addition of the *thioglycollic acid R*.

**Water** (*2.5.12*): maximum 15.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 50.0 mL with the same solvent. To 25.0 mL of this solution add 80 mL of *water R* and adjust to pH 2.3 with *dilute nitric acid R*. Titrate with *0.01 M bismuth nitrate* using 0.1 mL of a 1 g/L solution of *xylenol orange R* as indicator. The colour of the solution changes from yellow to red.

1 mL of *0.01 M bismuth nitrate* is equivalent to 4.974 mg of $C_{14}H_{18}CaN_3Na_3O_{10}$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

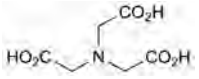

A. nitrilotriacetic acid,

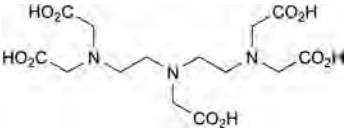

B. [[(carboxymethyl)imino]bis(ethylenenitrilo)]tetraacetic acid (pentetic acid).

**01/2008:1924**

# RACLOPRIDE ([$^{11}$C]METHOXY) INJECTION

## Raclopridi ([$^{11}$C]methoxy) solutio iniectabilis

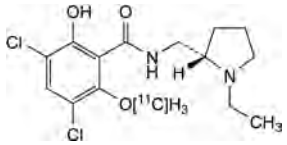

DEFINITION

Sterile solution of 3,5-dichloro-*N*-[[(2*S*)-1-ethylpyrrolidin-2-yl]methyl]-2-hydroxy-6-([$^{11}$C]methoxy)benzamide.

*Content*: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

*Purity*:

- minimum of 99 per cent of the total radioactivity corresponds to carbon-11,
- minimum of 95 per cent of the total radioactivity corresponds to carbon-11 in the form of [*methoxy*-$^{11}$C]raclopride.

*Content of raclopride*: maximum of 10 µg per maximum recommended dose in millilitres.

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Carbon-11 is a radioactive isotope of carbon most commonly produced by proton irradiation of nitrogen. Depending on the addition of either trace amounts of oxygen or small amounts of hydrogen, the radioactivity is obtained as [$^{11}$C]carbon dioxide or [$^{11}$C]methane, respectively.

*RADIOCHEMICAL SYNTHESIS*

[*Methoxy*-$^{11}$C]raclopride may be prepared by *O*-alkylation of the corresponding phenolate anion (*S*)-3,5-dichloro-2,6-dihydroxy-*N*-[(1-ethylpyrrolidin-2-yl)methyl]benzamide with iodo[$^{11}$C]methane or [$^{11}$C]methyl trifluoromethanesulfonate.

**Synthesis of iodo[$^{11}$C]methane**

Iodo[$^{11}$C]methane may be produced from [$^{11}$C]carbon dioxide or from [$^{11}$C]methane. The most frequently used method is reduction of [$^{11}$C]carbon dioxide with lithium aluminium hydride. The lithium aluminium [$^{11}$C]methanolate formed is reacted with hydroiodic acid to iodo[$^{11}$C]methane via [$^{11}$C]methanol. Alternatively [$^{11}$C]methane, either obtained directly in the target or by on-line processes from [$^{11}$C]carbon dioxide, is reacted with iodine.

**Synthesis of [$^{11}$C]methyl trifluoromethanesulfonate**

[$^{11}$C]Methyl trifluoromethanesulfonate may be prepared from iodo[$^{11}$C]methane using a solid support such as graphitised carbon impregnated with silver trifluoromethanesulfonate.

**Synthesis of [*methoxy*-$^{11}$C]raclopride**

Methylation with iodo[$^{11}$C]methane is performed under alkaline conditions in a solvent such as dimethyl sulfoxide. The methylation with [$^{11}$C]methyl trifluoromethanesulfonate is performed in a solvent such as dimethylformamide or acetone. The resulting [*methoxy*-$^{11}$C]raclopride may be purified by semi-preparative liquid chromatography using, for example, a column packed with octadecylsilyl silica gel for chromatography eluted with a mixture of 25 volumes of acetonitrile and 75 volumes of 0.01 M phosphoric acid.

*PRECURSOR FOR SYNTHESIS*

**(*S*)-3,5-Dichloro-2,6-dihydroxy-*N*-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide**

Melting point (*2.2.14*): 211 °C to 213 °C.

Specific optical rotation (*2.2.7*): + 11.3 to + 11.5, determined on a 15.0 g/L solution in *ethanol R* at 22 °C.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of carbon-11*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**pH** (*2.2.3*): 4.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

*CHEMICAL PURITY*

**Raclopride and impurity A**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. Dissolve 7.2 mg of *raclopride tartrate R* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dissolve 1.2 mg of *(S)-3,5-dichloro-2,6-dihydroxy-N-[(1-ethylpyrrolidin-2-yl)methyl]benzamide hydrobromide R* in *methanol R* and dilute to 100 mL with the same solvent.

*Reference solution (c).* To 0.1 mL of reference solution (a) add 0.1 mL of reference solution (b) and dilute to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.05 m, Ø = 4.6 mm,

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm) with a specific surface area of 175 $m^2$/g, a pore size of 12.5 nm, a pore volume of 0.7 $cm^3$/g and a carbon loading of 15 per cent,

– *temperature*: 30 °C.

*Mobile phase*: dissolve 2 g of *sodium heptanesulfonate R* in 700 mL of *water R*, adjust to pH 3.9 with *phosphoric acid R* and dilute to 1000 mL with *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: loop injector; inject the test solution and reference solutions (b) and (c).

*Run time*: 10 min.

*Relative retention* with reference to raclopride: impurity A = about 0.46.

*System suitability*: reference solution (c):

– *resolution*: minimum of 5 between the peaks due to raclopride and to impurity A.

*Limits*: examine the chromatogram obtained with the spectrophotometer:

– *raclopride*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (10 µg/*V*),

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1 µg/*V*).

**Residual solvents** are limited according to the principles defined in the general chapter (*5.4*), using the general method (*2.4.24*). The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Carbon-11**: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the test.

A. Gamma-ray spectrometry.

*Comparison*: standardised fluorine-18 solution, or by using a calibrated instrument. Standardised fluorine-18 solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life. 19.9 min to 20.9 min.

*RADIOCHEMICAL PURITY*

Liquid chromatography (*2.2.29*) as described in the test for raclopride and impurity A with the following modifications.

*Injection*: test solution and reference solution (d).

*Limits*: examine the chromatogram obtained with the radioactivity detector:

– [*Methoxy-$^{11}$C] raclopride*: minimum of 95 per cent of the total radioactivity.

RADIOACTIVITY

Mesure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by using a calibrated instrument.

LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

IMPURITIES

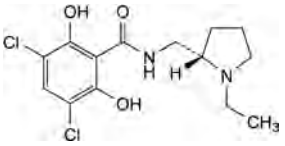


A. 3,5-dichloro-*N*-[[(2*S*)-1-ethylpyrrolidin-2-yl]methyl]-2,6-dihydroxybenzamide.

**01/2008:1920**

# SODIUM ACETATE ([1-$^{11}$C]) INJECTION

## Natrii acetatis ([1-$^{11}$C]) solutio iniectabilis

$CH_3{}^{11}COONa$

DEFINITION

Sterile solution of sodium [1-$^{11}$C]acetate, in equilibrium with [1-$^{11}$C]acetic acid.

*Content*: 90 per cent to 110 per cent of the declared carbon-11 radioactivity at the date and time stated on the label.

PRODUCTION

*RADIONUCLIDE PRODUCTION*

Carbon-11 is a radioactive isotope of carbon which is most commonly produced by proton irradiation of nitrogen. By the addition of trace amounts of oxygen, the radioactivity is obtained as [$^{11}$C]carbon dioxide.

*RADIOCHEMICAL SYNTHESIS*

[$^{11}$C]Carbon dioxide may be separated from the target gas mixture by cryogenic trapping or by trapping on a molecular sieve at room temperature. [$^{11}$C]Carbon dioxide is then released from the trap using an inert gas such as nitrogen at a temperature higher than the trapping temperature. [1-$^{11}$C]Acetate is usually prepared by reaction of [$^{11}$C]carbon dioxide with methylmagnesium bromide in organic solvents such as ether or tetrahydrofuran.

Hydrolysis of the product yields [1-$^{11}$C]acetic acid. It is purified by chromatographic procedures. The eluate is diluted with sodium chloride solution.

*PRECURSOR FOR SYNTHESIS*

**Methylmagnesium bromide**. The reactivity of methylmagnesium bromide is tested by decomposition of a defined amount with water. The amount of methane released during this reaction is not less than 90 per cent of the theoretical value.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of carbon-11*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

*CHEMICAL PURITY*

**Acetate**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution*. Dissolve 28 mg of *sodium acetate R* in *water R* and dilute to *V*, *V* being the maximum recommended dose in millilitres.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm);

– *temperature*: 25 °C.

*Mobile phase*: *0.1 M sodium hydroxide* protected from atmospheric carbon dioxide.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: loop injector.

*Run time*: 10 min.

*System suitability*: reference solution:

– *resolution*: minimum 4.0 between the peaks due to hold-up volume and acetate.

*Limit*: examine the chromatograms obtained with the spectrophotometer:

– *acetate*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (20 mg per *V*).

**Residual solvents** are limited according to the principles defined in the general chapter (*5.4*), using the general method (*2.4.24*). The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Carbon-11**: minimum 99 per cent of the total radioactivity.

The preparation may be released for use before completion of the tests.

A. Gamma-ray spectrometry.

*Comparison*: standardised fluorine-18 solution, or by using a calibrated instrument. Standardised fluorine-18 solutions and/or standardisation services are available from laboratories recognised by the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution.

B. Half-life: 19.9 min to 20.9 min.

*RADIOCHEMICAL PURITY*

**[1-$^{11}$C]Acetate**. Liquid chromatography (*2.2.29*) as described in the test for acetate.

*Limit*: examine the chromatograms obtained with the spectrophotometer and the radioactivity detector:

– *total of [1-$^{11}$C]acetate*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised fluorine-18 solution or by measurement with a calibrated instrument.

LABELLING

The accompanying information specifies the maximum recommended dose in millilitres.

**01/2008:0279**
**corrected 7.0**

# SODIUM CHROMATE ($^{51}$Cr) STERILE SOLUTION

## Natrii chromatis ($^{51}$Cr) solutio sterilis

DEFINITION

Sterile solution of sodium [$^{51}$Cr]chromate made isotonic by the addition of sodium chloride.

*Chromium-51*: 90 per cent to 110 per cent of the declared chromium-51 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 370 MBq of chromium-51 per milligram of chromate ion.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of chromium-51*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the only gamma photon of chromium-51 has an energy of 0.320 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is about 0.9.

TESTS

**pH** (*2.2.3*): 6.0 to 8.5.

**Total chromate**: maximum 2.7 µg of chromate ion ($CrO_4^{2-}$) per MBq.

*Test solution*. The preparation to be examined.

*Reference solution*. 1.7 mg/L solution of *potassium chromate R*.

Measure the absorbance of the solutions (*2.2.25*) at the absorption maximum at 370 nm. If necessary, adjust the test solution and the reference solution to pH 8.0 by adding *sodium hydrogen carbonate solution R*. Calculate the content of chromate in the preparation to be examined using the measured absorbances.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Chromium-51**. Gamma-ray spectrometry.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised chromium-51 solution.

*RADIOCHEMICAL PURITY*

**[$^{51}$Cr]Chromate ion**. Ascending paper chromatography (*2.2.26*).

*Test solution*. The preparation to be examined.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *ammonia R*, *ethanol (96 per cent) R*, *water R* (25:50:125 *V*/*V*/*V*).

*Application*: a volume of the solution sufficient for the detection method.

*Development*: immediately, for 2.5 h.

*Detection*: suitable detector to determine the distribution of the radioactivity.

*Retardation factor*: impurity A = 0.0 to 0.1; chromate ion = about 0.9.

*Limit*:

– *[$^{51}$Cr]chromate ion*: minimum 90 per cent of the total radioactivity due to chromium-51.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{51}$Cr]chromium(III) ion.

**01/2008:2100**

# SODIUM FLUORIDE ($^{18}$F) INJECTION

## Natrii fluoridi ($^{18}$F) solutio iniectabilis

DEFINITION

Sterile solution containing fluorine-18 in the form of sodium fluoride. It may contain carrier fluoride and a suitable buffer.

*Content*:

– *fluorine-18*: 90 per cent to 110 per cent of the declared fluorine-18 radioactivity at the date and hour stated on the label,

– *fluoride*: maximum 4.52 mg per maximum recommended dose in millilitres.

PRODUCTION

The radionuclide fluorine-18 is most commonly produced by proton irradiation of water enriched in oxygen-18. Fluorine-18 in the form of fluoride is recovered from the target water, generally by adsorption and desorption from anion-exchange resins or electrochemical deposition and redissolution.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of fluorine-18*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the only gamma photons have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. It complies with test B for radionuclidic purity (see Tests).

C. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution. In the chromatogram obtained with the reference solution, the peak due to fluoride is negative.

TESTS

**pH** (*2.2.3*): 5.0 to 8.5.

**Fluoride**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution*. Dissolve 10 mg of *sodium fluoride R* in *water R* and dilute to *V* with the same solvent, *V* being the maximum recommended dose in millilitres.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm,

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm),

– *temperature*: constant, between 20 °C and 30 °C.

*Mobile phase*: 4 g/L solution of *sodium hydroxide R*, protected from atmospheric carbon dioxide.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm and a radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 15 min.

*System suitability*: examine the chromatogram obtained with the reference solution using the spectrophotometer:

– *signal-to-noise ratio*: minimum 10 for the principal peak,

– *retention time of fluoride*: minimum 3 times the hold-up time.

*Limit*: examine the chromatogram obtained with the spectrophotometer:

– *fluoride*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (4.52 mg/*V*).

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The injection may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Fluorine-18**: minimum 99.9 per cent of the total radioactivity.

The preparation may be released for use before completion of the tests.

A. Gamma-ray spectrometry.

Determine the amount of fluorine-18 and radionuclidic impurities with a half-life longer than 2 h. For the detection and quantification of impurities, retain the preparation to be examined for a sufficient time to allow the fluorine-18 to decay to a level which permits the detection of impurities.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a background spectrum.

B. Half-life: 105 min to 115 min.

*RADIOCHEMICAL PURITY*

**[$^{18}$F]fluoride**. Liquid chromatography (*2.2.29*) as described in the test for fluoride. If necessary, dilute the test solution with *water R* to obtain a radioactivity concentration suitable for the radioactivity detector.

*Limit*: examine the chromatogram obtained with the radioactivity detector:

– *[$^{18}$F]fluoride*: minimum 98.5 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the maximum recommended dose in millilitres.

**01/2008:0563**

# SODIUM IODIDE ($^{123}$I) INJECTION

## Natrii iodidi ($^{123}$I) solutio iniectabilis

DEFINITION

Sterile solution containing iodine-123 in the form of sodium iodide; it may contain sodium thiosulfate or some other suitable reducing agent and a suitable buffer.

*Content*: 90 per cent to 110 per cent of the declared iodine-123 radioactivity at the date and hour stated on the label.

PRODUCTION

Iodine-123 is obtained by proton irradiation of xenon enriched in xenon-124 (minimum 98 per cent) followed by the decay of xenon-123 which is formed directly and by the decay of caesium-123. No carrier iodide is added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-123*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-123 solution. The most prominent gamma photon has an energy of 0.159 MeV and is accompanied by the principal X-ray of 0.027 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 7.0 to 10.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Iodine-123**: minimum 99.65 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-123, iodine-125, tellurium-121 and other radionuclidic impurities present. For the detection of tellurium-121 and iodine-125, retain the preparation to be examined for a sufficient time to allow iodine-123 to decay to a level which permits the detection of radionuclidic impurities. No radionuclides with a half-life longer than that of iodine-125 are detected.

The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**[$^{123}$I]Iodide**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the preparation to be examined with a 2 g/L solution of *sodium hydroxide R* to a radioactive concentration suitable for the detector. Add an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R* and mix.

*Reference solution (a)*. Dilute 1 mL of a 26.2 mg/L solution of *potassium iodide R* to 10 mL with *water R*.

*Reference solution (b)*. Dilute 1 mL of a 24.5 mg/L solution of *potassium iodate R* to 10 mL with *water R*. Mix equal volumes of this solution and reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: constant between 20 °C and 30 °C.

*Mobile phase*: dissolve 5.85 g of *sodium chloride R* in 1000 mL of *water R*, add 0.65 mL of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and a radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram recorded with the spectrophotometer.

*Limit*: examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer:

- *[$^{123}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the name of any excipient.

IMPURITIES

A. [$^{123}$I]iodate ion.

**01/2008:2314**

# SODIUM IODIDE ($^{123}$I) SOLUTION FOR RADIOLABELLING

## Natrii iodidi ($^{123}$I) solutio ad radio-signandum

DEFINITION

Strongly alkaline solution containing iodine-123 in the form of sodium iodide.

*Content*: 90 per cent to 110 per cent of the declared iodine-123 radioactivity at the date and hour stated on the label.

PRODUCTION

Iodine-123 is obtained by proton irradiation of xenon highly enriched in xenon-124 followed by the decay of directly formed xenon-123 and by the decay of caesium-123. No carrier iodide or reducing agents are added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-123*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photon of iodine-123 has an energy of 0.159 MeV and is accompanied by the principal X-ray of 0.027 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Alkalinity** (*2.2.4*). The solution is strongly alkaline.

*RADIONUCLIDIC PURITY*

**Iodine-123**: minimum 99.7 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-123, iodine-125, tellurium-121 and other radionuclidic impurities present. For the detection of tellurium-121 and iodine-125, retain the solution to be examined for a sufficient time to allow iodine-123 to decay to a level which permits the detection of radionuclidic impurities. No radionuclides with a half-life longer than that of iodine-125 are detected.

The solution may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**[$^{123}$I]Iodide**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the solution to be examined with an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R* and mix. If necessary, first dilute the solution to be examined with a 2 g/L solution of *sodium hydroxide R* to ensure that the final mixture has a radioactivity concentration suitable for the radioactivity detector.

*Reference solution (a)*. Dissolve 10 mg of *potassium iodide R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *potassium iodate R* in *water R* and dilute to 10 mL with the same solvent. Mix equal volumes of this solution and reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: constant, between 20 °C and 30 °C.

Use stainless steel tubing.

*Mobile phase*: dissolve 5.85 g of *sodium chloride R* in 1000 mL of *water R*, add 0.65 mL of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and a radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram recorded with the spectrophotometer.

Examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer.

*Limit*:

- *[$^{123}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- the name of any excipient;
- that the solution is not for direct administration to humans.

IMPURITIES

A. iodine-125,

B. tellurium-121,

C. [$^{123}$I]iodate ion.

**01/2008:0938**

# SODIUM IODIDE ($^{131}$I) CAPSULES FOR DIAGNOSTIC USE

## Natrii iodidi ($^{131}$I) capsulae ad usum diagnosticum

DEFINITION

Capsules for diagnostic use containing iodine-131 in the form of sodium iodide on a solid support; they may contain sodium thiosulfate or some other suitable reducing agents and a suitable buffering substance. A package contains 1 or more capsules.

*Content*:

- iodine-131: maximum 37 MBq per capsule; the average radioactivity determined in the test for uniformity of content is 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label;
- iodide: maximum 20 µg per capsule.

PRODUCTION

Iodine-131 is obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with test solution (b) is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Disintegration**: the contents of the capsule dissolve completely within 15 min.

In a water-bath at 37 °C, warm in a small beaker about 20 mL of a 2.0 g/L solution of *potassium iodide R*. Add a capsule to be examined. Stir magnetically at 20 r/min.

**Uniformity of content**. Determine the radioactivity of each of not fewer than 10 capsules. Calculate the average radioactivity per capsule. The radioactivity of no capsule differs by more than 10 per cent from the average, the relative standard deviation is not greater than 3.5 per cent.

**Iodide**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve a capsule to be examined in 10 mL of *water R*. Filter through a 0.2 µm filter.

*Test solution (b).* Dissolve a capsule to be examined in *water R*. Filter through a 0.2 µm filter and dilute the filtrate with a 2 g/L solution of *sodium hydroxide R* to a radioactive concentration suitable for the detector. Add an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R* and mix.

*Reference solution (a).* Dilute 1 mL of a 26.2 mg/L solution of *potassium iodide R* to 10 mL with *water R*.

*Reference solution (b).* Dilute 1 mL of a 24.5 mg/L solution of *potassium iodate R* to 10 mL with *water R*. Mix equal volumes of this solution and reference solution (a).

*Blank solution.* Prepare a solution containing 2 mg/mL of each constituent stated on the label, apart from iodide.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: constant between 20 °C and 30 °C.

*Mobile phase*: dissolve 5.85 g of *sodium chloride R* in 1000 mL of *water R*, add 0.65 mL of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: 20 µL of test solution (a), reference solutions (a) and (b) and the blank solution.

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*:

- in the chromatogram obtained with the blank solution, none of the peaks has a retention time similar to that of the peak due to iodide,
- *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram obtained with reference solution (b) recorded with the spectrophotometer.

*Limit*: examine the chromatograms obtained with the spectrophotometer:

- *iodide*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (20 µg/capsule).

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iodide**. Liquid chromatography (*2.2.29*) as described in the test for iodide with the following modifications.

*Injection*: 20 µL of test solution (b) and reference solution (a).

*Limit*: examine the chromatogram obtained with the test solution using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer:

- *[$^{131}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity of the package using a calibrated instrument.

LABELLING

The label states the name of any excipient and the number of capsules in the package.

IMPURITIES

A. [$^{131}$I]iodate ion.

**01/2008:2116**

# SODIUM IODIDE ($^{131}$I) CAPSULES FOR THERAPEUTIC USE

## Natrii iodidi ($^{131}$I) capsulae ad usum therapeuticum

DEFINITION

Capsules for therapeutic use containing iodine-131 in the form of sodium iodide on a solid support; they contain sodium thiosulfate or some other suitable reducing agents and a suitable buffering substance.

*Content*:

- iodine-131: 90 per cent to 110 per cent of the declared radioactivity at the date and hour stated on the label,
- iodide: maximum 20 µg per capsule.

PRODUCTION

Iodine-131 is obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with test solution (b) is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Disintegration**: the contents of the capsule dissolve completely within 15 min.

In a water-bath at 37 °C, warm in a small beaker about 20 mL of a 2.0 g/L solution of *potassium iodide R*. Add a capsule to be examined. Stir magnetically at a rotation rate of 20 r/min.

**Iodide**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve a capsule to be examined in 10 mL of *water R*. Filter through a 0.2 µm filter.

*Test solution (b).* Dissolve a capsule to be examined in *water R*. Filter through a 0.2 µm filter and dilute the filtrate with an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R*. If necessary, first dilute the filtrate with a 2 g/L solution of *sodium hydroxide R* to ensure that the final mixture has a radioactivity concentration suitable for the radioactivity detector.

*Reference solution (a).* Dilute 1.0 mL of a 26.2 mg/L solution of *potassium iodide R* to 10.0 mL with *water R*.

*Reference solution (b).* Dilute 1 mL of a 24.5 mg/L solution of *potassium iodate R* to 10 mL with *water R*. Mix equal volumes of this solution with reference solution (a).

*Blank solution.* Prepare a solution containing 2 mg/mL of each excipient stated on the label, apart from iodide.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
– *temperature*: constant, between 20 °C and 30 °C.

Use stainless steel tubing.

*Mobile phase*: dissolve 5.85 g of *sodium chloride R* in 1000 mL of *water R*, add 0.65 mL of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: 20 µL of test solution (a), reference solutions (a) and (b) and the blank solution.

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*:

– in the chromatogram obtained with the blank solution, none of the peaks has a retention time similar to that of the peak due to iodide;
– *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram obtained with reference solution (b) using the spectrophotometer.

*Limits*: examine the chromatograms obtained with the spectrophotometer; locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a):

– *iodide*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (20 µg/capsule).

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-130, iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iodide**. Liquid chromatography (*2.2.29*) as described in the test for iodide with the following modifications.

*Injection*: 20 µL of test solution (b) and reference solution (a).

*Limits*: examine the chromatogram obtained with test solution (b) using the radioactivity detector and locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a) using the spectrophotometer:

– *[$^{131}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity of each capsule using a calibrated instrument.

LABELLING

The label states the name of any excipient.

IMPURITIES

A. [$^{131}$I]iodate ion,

B. iodine-130,

C. iodine-133,

D. iodine-135.

**01/2008:0281**

# SODIUM IODIDE ($^{131}$I) SOLUTION

## Natrii iodidi ($^{131}$I) solutio

DEFINITION

Solution containing iodine-131 in the form of sodium iodide and also sodium thiosulfate or some other suitable reducing agent. It may contain a suitable buffer.

*Content*:

– *iodine-131*: 90 per cent to 110 per cent of the declared radioactivity at the date and hour stated on the label,
– *iodide*: maximum 20 µg in the maximum recommended dose in millilitres.

PRODUCTION

Iodine-131 is a radioactive isotope of iodine and may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for iodide.

*Results*: the principal peak in the radiochromatogram obtained with test solution (a) is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 7.0 to 10.0.

**Sterility**. If intended for parenteral administration, it complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The solution may be released for use before completion of the test.

**Iodide**. Liquid chromatography (*2.2.29*).

*Test solution (a).* The preparation to be examined.

*Test solution (b).* Dilute the preparation to be examined with *0.05 M sodium hydroxide* until the radioactivity is equivalent to about 74 MBq/mL. Add an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R* and mix.

*Reference solution (a).* Dilute 1 mL of a 26.2 mg/L solution of *potassium iodide R* to *V* with *water R*, *V* being the maximum recommended dose in millilitres.

*Reference solution (b).* Dilute 1 mL of a 24.5 mg/L solution of *potassium iodate R* to *V* with *water R*, *V* being the maximum recommended dose in millilitres. Mix equal volumes of this solution and of reference solution (a).

*Blank solution.* Prepare a solution containing 2 mg/mL of each of the components stated on the label, apart from iodide.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: maintain at a constant temperature between 20 °C and 30 °C.

Use stainless steel tubing.

*Mobile phase*: dissolve 5.844 g of *sodium chloride R* in 1000 mL of *water R*, add 650 µL of *octylamine R* and adjust to pH 7.0 with *phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and radioactivity detector connected in series.

*Injection*: 25 µL; inject test solution (a), the blank solution and reference solutions (a) and (b).

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*:
- in the chromatogram obtained with the blank solution, none of the peaks shows a retention time similar to that of the peak due to iodide,
- *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram obtained with reference solution (b) recorded with the spectrophotometer.

*Limit*: examine the chromatogram obtained with the spectrophotometer; locate the peak due to iodide by comparison with the chromatogram obtained with reference solution (a):
- *iodide*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a).

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iodide**. Liquid chromatography (*2.2.29*) as described in the test for iodide with the following modification.

*Injection*: test solution (b).

*Limit*: examine the chromatogram obtained with the radioactivity detector:
- *[$^{131}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised iodine-131 solution or by using a calibrated instrument.

LABELLING

The label states:
- the name of any excipient,
- the maximum recommended dose, in millilitres,
- where applicable, that the preparation is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

A. [$^{131}$I]iodate ion.

**01/2008:2121**

# SODIUM IODIDE ($^{131}$I) SOLUTION FOR RADIOLABELLING

## Natrii iodidi ($^{131}$I) solutio ad radio-signandum

DEFINITION

Strongly alkaline solution containing iodine-131 in the form of sodium iodide. It does not contain a reducing agent.

*Content*: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and hour stated on the label.

PRODUCTION

Iodine-131 may be obtained by neutron irradiation of tellurium or by extraction from uranium fission products. No carrier iodide is added.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the preparation to be examined does not differ significantly from that of a standardised iodine-131 solution. The most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Alkalinity** (*2.2.4*). The preparation is strongly alkaline.

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-130, iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**[$^{131}$I]Iodide**. Liquid chromatography (*2.2.29*).

*Test solution.* Dilute the preparation to be examined with an equal volume of a solution containing 1 g/L of *potassium iodide R*, 2 g/L of *potassium iodate R* and 10 g/L of *sodium hydrogen carbonate R* and mix. If necessary, first dilute the preparation to be examined with a 2 g/L solution of *sodium hydroxide R* to ensure that the final mixture has a radioactivity concentration suitable for the radioactivity detector.

*Reference solution (a).* Dissolve 10 mg of *potassium iodide R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *potassium iodate R* in *water R* and dilute to 10 mL with the same solvent. Mix equal volumes of this solution and reference solution (a).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: constant, between 20 °C and 30 °C.

Use stainless steel tubing.

*Mobile phase*: dissolve 5.85 g of *sodium chloride R* in 1000 mL of *water R*, add 0.65 mL of *octylamine R* and adjust to pH 7.0 with *dilute phosphoric acid R*; add 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm and a radioactivity detector connected in series.

*Injection*: 20 µL.

*Run time*: 12 min.

*Relative retention* with reference to iodide (retention time = about 5 min): iodate = 0.2 to 0.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 2 between the peaks due to iodide and iodate in the chromatogram recorded with the spectrophotometer.

*Limit*: examine the chromatogram obtained with the radioactivity detector:

– *[$^{131}$I]iodide*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

– the method of production of iodine-131,
– the name of any excipient,
– that the preparation is not for direct human use.

IMPURITIES

A. [$^{131}$I]iodate ion.

**01/2008:0564**
**corrected 7.0**

# SODIUM IODOHIPPURATE ($^{123}$I) INJECTION

## Natrii iodohippurati ($^{123}$I) solutio iniectabilis

DEFINITION

Sterile solution of sodium (2-[$^{123}$I]iodobenzamido)acetate. It may contain a suitable buffer and a suitable antimicrobial preservative such as benzyl alcohol.

*Iodine-123*: 90 per cent to 110 per cent of the declared iodine-123 radioactivity at the date and time stated on the label.

*Specific radioactivity*: 0.74 GBq to 10.0 GBq of iodine-123 per gram of sodium 2-iodohippurate.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-123*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Results*: the most prominent gamma photon has an energy of 0.159 MeV and is accompanied by an X-ray of 0.027 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal spot in the radiochromatogram obtained with the test solution is similar in retardation factor to the spot corresponding to 2-iodohippuric acid in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 3.5 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of the test.

**Radionuclides other than iodine-123**: maximum 0.35 per cent of the total radioactivity.

Gamma-ray and X-ray spectrometry.

Determine the relative amounts of iodine-125, tellurium-121 and other radionuclidic impurities present. For their detection, retain the preparation to be examined for a sufficient time to allow iodine-123 to decay to a level which permits the detection of radionuclidic impurities. Record the gamma-ray spectrum and X-ray spectrum of the decayed material. No radionuclides with a half-life longer than that of iodine-125 are detected.

*RADIOCHEMICAL PURITY*

**2-[$^{123}$I]Iodohippuric acid**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1 g of *potassium iodide R* in 10 mL of *water R*, add 1 volume of this solution to 10 volumes of the preparation to be examined and use within 10 min of mixing. If necessary, dilute with the reference solution (carrier) to give a radioactive concentration sufficient for the detection method, for example 3.7 MBq per millilitre.

*Reference solution* (*carrier*). Dissolve 40 mg of *2-iodobenzoic acid R* and 40 mg of *2-iodohippuric acid R* in 4 mL of a 4 g/L solution of *sodium hydroxide R*, add 10 mg of *potassium iodide R* and dilute to 10 mL with *water R*.

*Plate*: *TLC silica gel GF$_{254}$ plate R*.

*Mobile phase*: *water R*, *glacial acetic acid R*, *butanol R*, *toluene R* (1:4:20:80 *V*/*V*/*V*/*V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm in about 75 min.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm and determine the distribution of radioactivity using a suitable detector.

*Identification of spots*: the chromatogram obtained with the reference solution shows a spot corresponding to 2-iodohippuric acid and nearer to the solvent front a spot corresponding to impurity D; impurity C remains near the point of application.

*Limits*:

– *2-[$^{123}$I]iodohippuric acid*: minimum 96 per cent of the total radioactivity due to iodine-123;
– *impurity C*: maximum 2 per cent of the total radioactivity due to iodine-123;
– *impurity D*: maximum 2 per cent of the total radioactivity due to iodine-123.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

LABELLING

The label states whether or not the preparation is suitable for renal plasma-flow studies.

IMPURITIES

A. iodine-125,

B. tellurium-121,

C. [$^{123}$I]iodide,

D. 2-[$^{123}$I]iodobenzoic acid.

**01/2008:0282**
**corrected 7.0**

# SODIUM IODOHIPPURATE ($^{131}$I) INJECTION

## Natrii iodohippurati ($^{131}$I) solutio iniectabilis

DEFINITION

Sterile solution of sodium 2-(2-[$^{131}$I]iodobenzamido)acetate. It may contain a suitable buffer and a suitable antimicrobial preservative such as benzyl alcohol.

*Iodine-131*: 90 per cent to 110 per cent of the declared iodine-131 radioactivity at the date and time stated on the label.

*Specific radioactivity*: 0.74 GBq to 7.4 GBq of iodine-131 per gram of sodium 2-iodohippurate.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of iodine-131*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of iodine-131 has an energy of 0.365 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution has a similar retardation factor as the spot corresponding to 2-iodohippuric acid in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 6.0 to 8.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Iodine-131**: minimum 99.9 per cent of the total radioactivity.

Gamma-ray spectrometry.

Determine the relative amounts of iodine-131, iodine-133, iodine-135 and other radionuclidic impurities present.

*RADIOCHEMICAL PURITY*

**2-[$^{131}$I]Iodohippuric acid**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1 g of *potassium iodide R* in 10 mL of *water R*, add 1 volume of this solution to 10 volumes of the preparation to be examined and use within 10 min of mixing. If necessary dilute with the reference solution (carrier) to give a radioactive concentration sufficient for the detection method, for example 3.7 MBq/mL.

*Reference solution* (carrier). Dissolve 40 mg of *2-iodobenzoic acid R* and 40 mg of *2-iodohippuric acid R* in 4 mL of a 4 g/L solution of *sodium hydroxide R*, add 10 mg of *potassium iodide R* and dilute to 10 mL with *water R*.

*Plate*: *TLC silica gel GF$_{254}$ plate R*.

*Mobile phase*: *water R*, *glacial acetic acid R*, *butanol R*, *toluene R* (1:4:20:80 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm in about 75 min.

*Drying*: in air.

*Detection*: in ultraviolet light at 254 nm and with a suitable detector to determine the distribution of radioactivity.

*Identification of spots*: the chromatogram obtained with the reference solution shows a spot corresponding to 2-iodohippuric acid and nearer to the solvent front a spot corresponding to impurity C; impurity D remains near the point of application.

*Limits*:

- *2-[$^{131}$I]iodohippuric acid*: minimum 96 per cent of the total radioactivity due to iodine-131;
- *impurity C*: maximum 2 per cent of the total radioactivity due to iodine-131;
- *impurity D*: maximum 2 per cent of the total radioactivity due to iodine-131.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

LABELLING

The label states that the preparation is not necessarily suitable for renal plasma-flow studies.

IMPURITIES

A. iodine-133,

B. iodine-135,

C. 2-[$^{131}$I]iodobenzoic acid,

D. [$^{131}$I]iodide.

**07/2017:2352**

# SODIUM IODOHIPPURATE DIHYDRATE FOR RADIOPHARMACEUTICAL PREPARATIONS

## Natrii iodohippuras dihydricus ad radiopharmaceutica

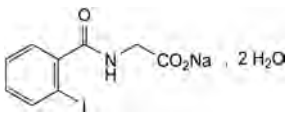


$C_9H_7INNaO_3,2H_2O$ $M_r$ 363.1

[5990-94-3]

DEFINITION

Sodium (2-iodobenzamido)acetate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium iodohippurate CRS*.

B. It gives reaction (b) of sodium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *2-iodobenzoic acid R* (impurity A) in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 10 mg of *benzoic acid R* in the mobile phase, add 1 mL of the test solution and dilute to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*: *acetic acid R*, *methanol R*, *water R* (1:50:50 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of 2-iodohippuric acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to 2-iodohippuric acid (retention time = about 4.5 min): benzoic acid = about 1.6; impurity A = about 2.1.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to 2-iodohippuric acid and benzoic acid.

*Limits*:

- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 8.0 per cent to 12.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.250 g in 20 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.71 mg of $C_9H_7INNaO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A*.

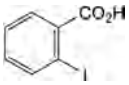


A. 2-iodobenzoic acid.

**01/2008:1923**

# SODIUM MOLYBDATE ($^{99}$Mo) SOLUTION (FISSION)

## Natrii molybdatis ($^{99}$Mo) fissione formati solutio

DEFINITION

Alkaline solution of sodium [$^{99}$Mo]molybdate obtained by extraction of fission products of uranium-235. It may contain stabilisers.

*Content*: 90 per cent to 110 per cent of the declared molybdenum-99 radioactivity at the date and time stated on the label.

PRODUCTION

Molybdenum-99 is usually produced by fission of uranium enriched in uranium-235, which is caused by the absorption of a thermal neutron, resulting in high-specific-activity molybdenum-99. By the fission of uranium after neutron capture, more than 200 different radionuclides are produced. In approximately 6 per cent of the fissions, molybdenum-99 is formed after decay of a number of short-lived parent radionuclides. After dissolution of the target, the molybdenum-99 is separated from the mixture of nuclides and purified by using chromatographic processes in order to obtain molybdenum-99 with a high level of radionuclidic purity.

CHARACTERS

*Appearance*: clear, colourless or almost colourless solution.

*Half-life and nature of radiation of molybdenum-99*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.
*Results*: the most prominent gamma photon of molybdenum-99 has an energy of 0.740 MeV; a peak with an energy of 0.141 MeV, due to technetium-99-m is also visible.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).
*Results*: the principal peak in the radiochromatogram obtained with the test solution has a similar retardation factor to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dilute the preparation to be examined to a radioactivity concentration of approximately 370 MBq/mL with a 2.42 g/L solution of *sodium molybdate R*.

**Alkalinity**. The preparation is alkaline (*2.2.4*).

*RADIONUCLIDIC PURITY*

**Iodine-131, ruthenium-103 and tellurium-132**:

- *iodine-131*: maximum $5 \times 10^{-3}$ per cent of the total radioactivity;
- *ruthenium-103*: maximum $5 \times 10^{-3}$ per cent of the total radioactivity;

Radiopharmaceutical preparations

– *tellurium-132*: maximum $5 \times 10^{-3}$ per cent of the total radioactivity.

The following method has been found to be suitable; other validated methods, approved by the competent authority, may be used.

Gamma-ray spectrometry.

Condition a column with an internal volume of approximately 1.5 mL of *strongly basic anion-exchange resin R* with a mixture of equal volumes of *glacial acetic acid R* and *water R*. All elutions of the column are made at a flow rate not exceeding 1 mL/min.

*Test solution.* In a test-tube, successively add, with mixing, 1 mL of a 24.2 g/L solution of *sodium molybdate R*, 0.5 mL of *strong hydrogen peroxide solution R*, 2.5 mL of *glacial acetic acid R*, 1.0 mL of *iodine-123 and ruthenium-106 spiking solution R* and 1.0 mL of solution S. Allow to stand for 30 min at room temperature.

*Reference solution.* Mix 1.0 mL of *iodine-123 and ruthenium-106 spiking solution R* and 4.0 mL of *water R*.

Apply the test solution to the column and elute. Just before the disappearance of the liquid from the top of the column, add 6 mL of a mixture of equal volumes of *glacial acetic acid R* and *water R* and elute. Transfer 5.0 mL of the combined eluates to a counting tube. Determine the radioactivity of iodine-123, iodine-131, ruthenium-103, ruthenium-106 and iodine-132 at the gamma-ray energies of 0.159 MeV for iodine-123, 0.365 MeV for iodine-131, 0.497 MeV for ruthenium-103, 0.512 MeV for ruthenium-106 and 0.668 MeV for iodine-132. Determine in the same way the radioactivity of iodine-123 and ruthenium-106 in the reference solution and calculate the recovery of iodine-123 and ruthenium-106 in the combined eluates.

Calculate the radioactivity of iodine-131, iodine-132 and ruthenium-103 in the combined eluates, taking into account the recovery, the fraction of eluate used, the counting efficiency and the radioactive decay. From the radioactivity of iodine-132 (daughter radionuclide of tellurium-132), calculate the radioactivity of tellurium-132, taking into account the time of the test and the time of separation of molybdenum-99.

**Total radioactivity due to strontium-89 and strontium-90**: maximum $6 \times 10^{-5}$ per cent of the total radioactivity.

The following method has been found to be suitable; other validated methods, approved by the competent authority, may be used.

Liquid scintillation spectrometry.

Connect 2 columns, each with an internal volume of approximately 1.5 mL of *strongly basic anion-exchange resin R*, in series and condition the columns with 10 mL of a 4 g/L solution of *sodium hydroxide R*. All elutions of the columns are made at a flow rate not exceeding 1 mL/min.

*Test solution.* In a test-tube, successively add, with mixing, 1.0 mL of solution S, 50 µL of *strontium-85 spiking solution R* and 0.05 mL of *strong sodium hypochlorite solution R*. Allow to stand for 10 min at room temperature.

*Reference solution.* Mix 50 µL of *strontium-85 spiking solution R* with 5.0 mL of a 9.5 g/L solution of *nitric acid R* in a vial for liquid scintillation counting and add 10 mL of *liquid scintillation cocktail R*.

Apply the test solution to the upper of the 2 columns and elute. Just before the disappearance of the liquid from the top of the upper column, add 3 mL of a 4 g/L solution of *sodium hydroxide R* and elute until the columns are dry. Combine the eluates and add 4 mL of a 947 g/L solution of *nitric acid R* (molybdenum-poor eluate). Determine the radioactivity due to molybdenum-99 using gamma-ray spectrometry. If the radioactivity due to molybdenum-99 is higher than $6 \times 10^{-7}$ per cent of the radioactivity due to molybdenum-99 in 1 mL of solution S, repeat the above procedure using 2 new columns.

Condition a column with an internal volume of approximately 2 mL of *strontium selective extraction resin R* with 5 mL of a 473 g/L solution of *nitric acid R* and dry the column. All elutions of the column are made at a flow rate not exceeding 1 mL/min. Apply to the column the molybdenum-poor eluate and elute. Just before the disappearance of the liquid from the top of the column, add 20 mL of a 473 g/L solution of *nitric acid R* and elute until the column is dry. Rinse the column with 2 mL of a 9.5 g/L solution of *nitric acid R*, dry the column and discard the eluate. Elute the column with 8.0 mL of a 9.5 g/L solution of *nitric acid R* until the column is dry. Transfer 5.0 mL of the eluate into a vial for liquid scintillation counting and add 10 mL of *liquid scintillation cocktail R*.

Determine the total radioactivity due to strontium-89 and strontium-90 in this solution by liquid scintillation spectrometry, and the radioactivity due to strontium-85 by gamma-ray spectrometry. Determine the radioactivity due to strontium-85 in the reference solution by gamma-ray spectrometry. Calculate the recovery of strontium-85 in the eluate. Calculate the measured total radioactivity of strontium-89 and strontium-90 in the eluate, taking into account the recovery of strontium and the fraction of eluate used.

**Total radioactivity due to alpha-particle-emitting impurities**: maximum $1 \times 10^{-7}$ per cent of the total radioactivity.

The following method has been found to be suitable; other validated methods, approved by the competent authority, may be used.

Alpha-ray spectrometry.

*Test solution.* To 0.2 mL of the preparation to be examined add 1.0 mL of *plutonium-242 spiking solution R*, 1.0 mL of *americium-243 spiking solution R* and 9.0 mL of a 927 g/L solution of *hydrochloric acid R*. Evaporate the sample to dryness. Dissolve the residue in 2 mL of a 927 g/L solution of *hydrochloric acid R*. Evaporate again to dryness. Dissolve the residue in 2 mL of a 10.3 g/L solution of *hydrochloric acid R*.

Apply the test solution to a column containing 0.7 g of *anion-exchange resin R1*. Collect the eluate and wash the column with 1 mL of a 10.3 g/L solution of *hydrochloric acid R*. Evaporate the combined eluates to dryness and dissolve the residue in 2 mL of a 10.3 g/L solution of *hydrochloric acid R*. Apply this solution to a 2nd column containing 0.7 g of *anion-exchange resin R1*. Collect the eluate and wash the column with 1 mL of a 10.3 g/L solution of *hydrochloric acid R*. Evaporate the combined eluates to dryness and dissolve the residue in 1 mL of *nitric acid R*. Evaporate to dryness. Dissolve the residue again in 1 mL of *nitric acid R*.

Add 1 mL of a 42.6 g/L solution of *anhydrous sodium sulfate R* and evaporate to dryness. Add 0.3 mL of *sulfuric acid R*. Warm until the residue is dissolved. Add 4 mL of *distilled water R* and 0.01 mL of *thymol blue solution R*. Add *concentrated ammonia R* dropwise until the colour changes from red to yellow.

Prepare an electrodeposition cell as follows. An electropolished stainless steel planchet is fitted in the cap of a 20 mL polyethylene scintillation vial. The bottom of the vial has been cut off and a hole has been drilled through the centre of the cap for electrical connection to the planchet cathode. The planchet, 20 mm in diameter and 0.5 mm thick, is rinsed with *acetone R* and *water R* prior to use. The anode, a platinum spiral, is introduced through the bottom of the vial and fitted 5 mm from the cathode.

Pour the solution prepared as described above into the electrodeposition cell and rinse the container with a total of 5 mL of a 10 g/L solution of *sulfuric acid R* (the solution becomes slightly pink). Adjust to pH 2.1-2.4. with *concentrated ammonia R* or with a 200 g/L solution of *sulfuric acid R*. Electrolyse at 1.2 A for 75 min without stirring.

Add 1 mL of *concentrated ammonia R* about 1 min prior to switching off the current. Rinse the planchet with a 57 g/L solution of *ammonia R*. Rinse the planchet with *acetone R* and remove any residual solvent by patting the planchet with absorbent paper. Heat the planchet on a hot plate at 180 °C for 10 min.

Determine the radioactivity of alpha emitters by alpha-ray spectrometry, taking into account the recovery of the alpha-particle-emitting radionuclides (measured using the plutonium-242 and americium-243 spiking solutions).

**Total of gamma-ray-emitting radionuclides other than molybdenum-99, technetium-99m, iodine-131, ruthenium-103 and tellurium-132**: maximum $1 \times 10^{-2}$ per cent of the total radioactivity.

The following method has been found to be suitable; other validated methods, approved by the competent authority, may be used.

Gamma-ray spectrometry.

Allow the preparation to decay for 4-6 weeks. Examine the gamma-ray spectrum for the presence of other gamma-ray-emitting impurities. Identify and quantify other gamma-ray-emitting impurities. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**[$^{99}$Mo]Molybdate**

The following method has been found to be suitable; other validated methods, approved by the competent authority, may be used.

Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute the preparation to be examined with a 4.0 g/L solution of *sodium hydroxide R* to a radioactivity concentration suitable for the detector.

*Reference solution*: 50 g/L solution of *sodium molybdate R* in a 4.0 g/L solution of *sodium hydroxide R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: 10.6 g/L solution of *anhydrous sodium carbonate R*.

*Application*: 5 µL of the test solution and 2 µL of the reference solution.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: determine the distribution of radioactivity using a suitable detector and spray with a 2 g/L solution of *phenylhydrazine R* in *glacial acetic acid R*; heat at 100-105 °C for 5 min.

*Retardation factor*: molybdate and pertechnetate = about 0.9.

*Limit*:

– *sum of [$^{99}$Mo]molybdate and [$^{99m}$Tc]pertechnetate*: minimum 95 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states that the preparation is only suitable for the preparation of technetium-99m generators.

IMPURITIES

A. iodine-131,

B. ruthenium-103,

C. tellurium-132,

D. strontium-89,

E. strontium-90.

**01/2018:2891**

# SODIUM PERTECHNETATE ($^{99m}$Tc) INJECTION (ACCELERATOR-PRODUCED)

## Natrii pertechnetatis ($^{99m}$Tc) acceleratore formati solutio iniectabilis

DEFINITION

Sterile solution containing technetium-99m in the form of pertechnetate ion prepared by proton irradiation of highly enriched molybdenum-100, made isotonic by the addition of sodium chloride.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photons of technetium-99m have an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is about 0.7.

TESTS

**pH** (*2.2.4*): 4.0 to 8.0.

**Aluminium**: maximum 5 ppm.

Determine the content of aluminium using an *aluminium test strip R*, *aluminium standard solution (5 ppm Al) R* and following the instructions given by the test strip manufacturer.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**RADIONUCLIDIC PURITY**

The preparation may be released for use before completion of the test for long-lived radionuclidic impurities.

**Short-lived impurities**. Gamma-ray spectrometry.

Take a volume of the preparation to be examined equivalent to 40–100 MBq and record the gamma and X-ray spectrum using a high purity germanium detector with a 6 mm thick lead shield interposed between the sample and the detector.

*Limits*:

– *Impurity A*: maximum 0.005 per cent of the total radioactivity.

*Identification of impurity A*: the most prominent gamma photons have energies of 0.181 MeV and 0.740 MeV; use the most prominent gamma line at 0.740 MeV for the radioactivity calculation; molybdenum-99 has a half-life of 65.94 h.

– *Impurity B*: maximum 0.005 per cent of the total radioactivity.

*Identification of impurity B*: the most prominent gamma photons have energies of 0.460 MeV, 0.569 MeV, 0.778 MeV and 1.091 MeV; use the gamma line at 0.569 MeV for the radioactivity calculation; niobium-96 has a half-life of 23.35 h.

– *Impurity C*: maximum 0.005 per cent of the total radioactivity.

*Identification of impurity C*: the most prominent gamma photons have an energy of 0.658 MeV; use this gamma line for the radioactivity calculation; niobium-97 has a half-life of 72.1 min.

– *Impurity D*: maximum 0.01 per cent of the total radioactivity.

*Identification of impurity D*: the most prominent gamma photons have an energy of 0.392 MeV; use this gamma line for the radioactivity calculation; technetium-93m has a half-life of 43.5 min.

– *Impurity E*: maximum 0.04 per cent of the total radioactivity.

*Identification of impurity E*: the most prominent gamma photons have energies of 1.363 MeV and 1.520 MeV; use the gamma line at 1.363 MeV for the radioactivity calculation; technetium-93 has a half-life of 2.75 h.

– *Impurity F*: maximum 0.02 per cent of the total radioactivity.

*Identification of impurity F*: the most prominent gamma photons have energies of 0.871 MeV and 1.869 MeV; use the gamma line at 1.869 MeV for the radioactivity calculation; technetium-94m has a half-life of 52 min.

– *Impurity G*: maximum 0.04 per cent of the total radioactivity.

*Identification of impurity G*: the most prominent gamma photons have energies of 0.703 MeV, 0.850 MeV and 0.871 MeV; use the gamma line at 0.703 MeV for the radioactivity calculation; technetium-94 has a half-life of 4.883 h.

– *Impurity H*: maximum 0.07 per cent of the total radioactivity.

*Identification of impurity H*: the most prominent gamma photons have an energy of 0.766 MeV; use this gamma line for the radioactivity calculation; technetium-95 has a half-life of 20.0 h.

– *Impurity I*: maximum 0.07 per cent of the total radioactivity.

*Identification of impurity I*: the most prominent gamma photons have energies of 0.778 MeV, 0.813 MeV and 0.850 MeV; use the gamma line at 0.813 MeV for the radioactivity calculation; technetium-96 has a half-life of 4.28 days.

**Long-lived impurities**. Gamma-ray spectrometry.

Retain the sample used for determining short-lived impurities for a sufficient time (> 132 h) allowing for detection of radionuclidic impurities without lead shielding. Record its gamma and X-ray spectrum using a high purity germanium detector.

*Limits*:

– *Impurity J*: maximum 0.005 per cent of the total radioactivity.

*Identification of impurity J*: the most prominent gamma photons have energies of 0.204 MeV, 0.582 MeV and 0.835 MeV; use the gamma line at 0.204 MeV for the radioactivity calculation; technetium-95m has a half-life of 61 days.

– *Impurity K*: maximum 0.01 per cent of the total radioactivity.

*Identification of impurity K*: the most prominent gamma photons have an energy of 0.097 MeV; use this gamma line for the radioactivity calculation; technetium-97m has a half-life of 91.0 days.

– *Other gamma-ray emitting impurities*: maximum 0.02 per cent of the total radioactivity.

Examine the gamma-ray spectrum of the shielded and that of the decayed sample for the presence of other radionuclidic impurities. Identify and quantify these impurities.

*NOTE: impurities A and I may also be identified and quantified in the decayed sample.*

**RADIOCHEMICAL PURITY**

**[$^{99m}$Tc]Pertechnetate ion**. Ascending paper chromatography (*2.2.26*).

*Test solution*. Dilute the preparation to be examined with *water R* to a suitable radioactivity concentration.

*Paper*: *paper for chromatography R*.

*Mobile phase*: a 9 g/L solution of *sodium chloride R* in *water R*.

*Application*: 1-2 µL.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]pertechnetate ion = about 0.7; impurity L = about 0.

*Limit*:

– *[$^{99m}$Tc]pertechnetate ion*: minimum 95 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. molybdenum-99,
B. niobium-96,
C. niobium-97,
D. technetium-93m,
E. technetium-93,
F. technetium-94m,
G. technetium-94,
H. technetium-95,
I. technetium-96,
J. technetium-95m,
K. technetium-97m,
L. technetium-99m in colloidal form.

**01/2008:0124**
**corrected 7.0**

# SODIUM PERTECHNETATE ($^{99m}$Tc) INJECTION (FISSION)

## Natrii pertechnetatis ($^{99m}$Tc) fissione formati solutio iniectabilis

*This monograph applies to sodium pertechnetate ($^{99m}$Tc) injection obtained from molybdenum-99 extracted from fission products of uranium. Sodium pertechnetate ($^{99m}$Tc) injection obtained from molybdenum-99 produced by neutron irradiation of molybdenum is described in the monograph Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283).*

DEFINITION

Sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride. The injection may be prepared from a sterile preparation of molybdenum-99 under aseptic conditions.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

TESTS

**pH** (*2.2.3*): 4.0 to 8.0.

**Aluminium**: maximum 5 ppm.

*Test solution*. In a test tube about 12 mm in internal diameter, mix 1 mL of *acetate buffer solution pH 4.6 R* and 2 mL of a 1 in 2.5 dilution of the preparation to be examined in *water R*. Add 0.05 mL of a 10 g/L solution of *chromazurol S R*.

*Reference solution*. Prepare at the same time and in the same manner as the test solution and using 2 mL of *aluminium standard solution (2 ppm Al) R*.

After 3 min, the colour of the test solution is not more intense than that of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

*Preliminary test*. To obtain an approximate estimate before use of the preparation, take a volume equivalent to 37 MBq and determine the gamma-ray spectrum using a sodium iodide detector with a shield of lead, of thickness 6 mm, interposed between the sample and the detector. The response in the region corresponding to the 0.740 MeV photon of molybdenum-99 does not exceed that obtained using 37 kBq of a standardised molybdenum-99 solution measured under the same conditions, when all measurements are expressed with reference to the date and time of administration.

*Definitive test*. Retain a sample of the preparation to be examined for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and time of administration.

– *Impurity A*: maximum $5 \times 10^{-3}$ per cent of the total radioactivity.

  Gamma-ray spectrometry. *Record the spectrum of the decayed material.*

  *Comparison*: suitable instrument calibrated with the aid of a standardised iodine-131 solution.

  *Results*: the most prominent photon has an energy of 0.365 MeV; iodine-131 has a half-life of 8.04 days.

– *Impurity B*: maximum 0.1 per cent of the total radioactivity.

  Gamma-ray spectrometry. *Record the spectrum of the decayed material.*

  *Comparison*: suitable instrument calibrated with the aid of a standardised molybdenum-99 solution.

  *Results*: the most prominent photons have energies of 0.181 MeV, 0.740 MeV and 0.778 MeV; molybdenum-99 has a half-life of 66.0 h.

– *Impurity C*: maximum $5 \times 10^{-3}$ per cent of the total radioactivity.

  Gamma-ray spectrometry. *Record the spectrum of the decayed material.*

  *Comparison*: suitable instrument calibrated using a standardised ruthenium-103 solution.

  *Results*: the most prominent photon has an energy of 0.497 MeV; ruthenium-103 has a half-life of 39.3 days.

– *Impurity D*: maximum $6 \times 10^{-5}$ per cent of the total radioactivity.

  Determine the presence of strontium-89 in the decayed material with an instrument suitable for the detection of beta rays. It is usually necessary first to carry out chemical separation of the strontium so that the standard and the sample may be compared in the same physical and chemical form.

  *Comparison*: standardised strontium-89 solution.

  *Results*: strontium-89 decays with a beta emission of 1.492 MeV maximum energy and has a half-life of 50.5 days.

– *Impurity E*: maximum $6 \times 10^{-6}$ per cent of the total radioactivity.

  Determine the presence of strontium-90 in the decayed material with an instrument suitable for the detection of beta rays. To distinguish strontium-90 from strontium-89, compare the radioactivity of yttrium-90, the daughter nuclide of strontium-90, with an yttrium-90 standard after the chemical separation of the yttrium. If prior chemical separation of the strontium is necessary, the conditions of radioactive equilibrium must be ensured. The yttrium-90 standard and the sample must be compared in the same physical and chemical form.

  *Results*: strontium-90 and yttrium-90 decay with respective beta emissions of 0.546 MeV and 2.284 MeV maximum energy and half-lives of 29.1 years and 64.0 h.

– *Other gamma-emitting impurities*: maximum 0.01 per cent of the total radioactivity.

  Gamma-ray spectrometry.

  Examine the spectrum of the decayed material for the presence of other radionuclidic impurities, which should, where possible, be identified and quantified.

– *Alpha-emitting impurities*: maximum $1 \times 10^{-7}$ per cent of the total radioactivity.

  Measure the alpha radioactivity of the decayed material to detect any alpha-emitting radionuclidic impurities, which should, where possible, be identified and quantified.

*RADIOCHEMICAL PURITY*

**[$^{99m}$Tc]Pertechnetate ion**. Descending paper chromatography (*2.2.26*).

*Test solution*. Dilute the preparation to be examined with *water R* to a suitable radioactive concentration.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *water R*, *methanol R* (20:80 *V/V*).

*Application*: 5 µL.

*Development*: for 2 h.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factor*: [$^{99m}$Tc]pertechnetate ion = about 0.6.

*Limit*:

– *[$^{99m}$Tc]pertechnetate ion*: minimum 95 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. iodine-131,

B. molybdenum-99,

C. ruthenium-103,

D. strontium-89,

E. strontium-90.

**01/2008:0283**
**corrected 7.0**

# SODIUM PERTECHNETATE ($^{99m}$Tc) INJECTION (NON-FISSION)

## Natrii pertechnetatis ($^{99m}$Tc) sine fissione formati solutio iniectabilis

*This monograph applies to sodium pertechnetate ($^{99m}$Tc) injection obtained from molybdenum-99 produced by neutron irradiation of molybdenum. Sodium pertechnetate ($^{99m}$Tc) injection obtained from molybdenum-99 extracted from fission products of uranium is described in the monograph Sodium pertechnetate($^{99m}$Tc) injection (fission) (0124).*

DEFINITION

Sterile solution containing technetium-99m in the form of pertechnetate ion and made isotonic by the addition of sodium chloride.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is about 0.6.

TESTS

**pH** (*2.2.3*): 4.0 to 8.0.

**Aluminium**: maximum 5 ppm.

*Test solution*. In a test tube about 12 mm in internal diameter, mix 1 mL of *acetate buffer solution pH 4.6 R* and 2 mL of a 1 in 2.5 dilution of the preparation to be examined in *water R*. Add 0.05 mL of a 10 g/L solution of *chromazurol S R*.

*Reference solution*. Prepare at the same time and in the same manner as the test solution and using 2 mL of *aluminium standard solution (2 ppm Al) R*.

After 3 min, the colour of the test solution is not more intense than that of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

*Preliminary test*. To obtain an approximate estimate before use of the preparation, take a volume equivalent to 37 MBq and record the gamma-ray spectrum using a sodium iodide detector with a shield of lead, 6 mm thick, interposed between the sample and the detector. The response in the region corresponding to the 0.740 MeV photon of molybdenum-99 does not exceed that obtained using 37 kBq of a standardised molybdenum-99 solution measured under the same conditions, when all measurements are expressed with reference to the date and time of administration.

*Definitive test*. Retain a sample of the preparation to be examined for a sufficient time to allow the technetium-99m radioactivity to decay to a sufficiently low level to permit the detection of radionuclidic impurities. All measurements of radioactivity are expressed with reference to the date and time of administration.

– *Impurity A*: maximum 0.1 per cent of the total radioactivity.

Gamma-ray spectrometry. Record the gamma-ray spectrum of the decayed material.

*Comparison*: standardised molybdenum-99 solution.

*Results*: the most prominent gamma photons have energies of 0.181 MeV, 0.740 MeV and 0.778 MeV; molybdenum-99 has a half-life of 66.0 h.

– *Other gamma-emitting impurities*: maximum 0.01 per cent of the total radioactivity.

Gamma-ray spectrometry. Examine the gamma-ray spectrum of the decayed material for the presence of other radionuclidic impurities, which should, where possible, be identified and quantified.

*RADIOCHEMICAL PURITY*

**[$^{99m}$Tc]Pertechnetate ion**. Descending paper chromatography (*2.2.26*).

*Test solution*. Dilute the preparation to be examined with *water R* to a suitable radioactive concentration.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *water R*, *methanol R* (20:80 *V/V*).

*Application*: 5 µL.

*Development*: for 2 h.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factor*: [$^{99m}$Tc]pertechnetate ion = about 0.6.

*Limit*:

– *[$^{99m}$Tc]pertechnetate ion*: minimum 95 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. molybdenum-99.

**01/2008:0284**
**corrected 7.0**

# SODIUM PHOSPHATE ($^{32}$P) INJECTION

## Natrii phosphatis ($^{32}$P) solutio iniectabilis

DEFINITION

Sterile solution of disodium and monosodium ($^{32}$P) orthophosphates made isotonic by the addition of sodium chloride.

*Phosphorus-32*: 90 per cent to 110 per cent of the declared phosphorus-32 radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 11.1 MBq of phosphorus-32 per milligram of orthophosphate ion.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of phosphorus-32*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Beta-ray spectrometry.

*Result*: the maximum energy of the beta radiation is 1.71 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retardation factor to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 6.0 to 8.0.

**Phosphates**: maximum 89 µg/MBq.

*Test solution.* Dilute the preparation to be examined with *water R* to give a radioactive concentration of 370 kBq of phosphorus-32 per millilitre. Mix in a volumetric flask, with shaking, 1.0 mL of this solution with a mixture of 0.5 mL of *ammonium molybdate solution R*, 0.5 mL of a 2.5 g/L solution of *ammonium vanadate R* and 1 mL of *perchloric acid R*, and dilute to 5.0 mL with *water R*.

*Reference solution.* Prepare at the same time and in the same manner as the test solution, using 1.0 mL of a solution containing 33 mg of orthophosphate ion per litre.

After 30 min, the test solution is not more intensely coloured than the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

Beta-ray spectrometry.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained under the same conditions with a standardised phosphorus-32 solution.

*RADIOCHEMICAL PURITY*

**[$^{32}$P]Phosphate**. Ascending paper chromatography (*2.2.26*).

*Test solution.* Dilute the preparation to be examined with *water R* until the radioactivity is equivalent to 10 000-20 000 counts per minute per 10 µL.

*Reference solution.* A solution of *phosphoric acid R* containing 2 mg of phosphorus per millilitre.

*Paper*: *paper for chromatography R*; use a strip of paper 25 mm wide and about 300 mm long.

*Mobile phase*: mixture of 0.3 mL of *ammonia R*, 5 g of *trichloroacetic acid R*, 25 mL of *water R* and 75 mL of *2-propanol R*.

*Application*: 10 µL of the reference solution, then apply to the same point of application 10 µL of the test solution.

*Development*: for 16 h.

*Drying*: in air.

*Detection*: determine the position of the non-radioactive phosphoric acid by spraying with a 50 g/L solution of *perchloric acid R* and then with a 10 g/L solution of *ammonium molybdate R*. Expose the paper to *hydrogen sulfide R*. A blue colour develops. Determine the distribution of radioactivity using a suitable detector.

*Limit*:

– *[$^{32}$P]phosphate*: minimum 95 per cent of the total radioactivity due to phosphorus-32.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

**07/2017:2552**

# SODIUM PYROPHOSPHATE DECAHYDRATE FOR RADIOPHARMACEUTICAL PREPARATIONS

## Natrii pyrophosphas decahydricus ad radiopharmaceutica

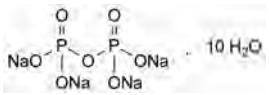


$Na_4O_7P_2,10H_2O$ $M_r$ 446.1

[13472-36-1]

DEFINITION

Tetrasodium diphosphate decahydrate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: colourless or white or almost white, slightly efflorescent crystals.

*Solubility*: soluble in water, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. To 5 mL of solution S (see Tests), neutralised if necessary, add 5 mL of *silver nitrate solution R1*. A white precipitate is formed.

B. Solution S gives reaction (a) of sodium (*2.3.1*).

C. To 1 mL of a 10 g/L solution of the substance to be examined add 1 mL of a 72 g/L solution of *zinc sulfate R*; a white precipitate is formed. To 1 mL of the suspension add 1 mL of *dilute acetic acid R*; the precipitate remains. To the rest of the suspension add *dilute ammonia R1* until alkaline; the precipitate dissolves. Heat the resulting solution to boiling; a white precipitate is formed.

D. pH (see Tests).

E. Assay (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.5 to 11.0 for solution S.

**Reducing substances**. To 5 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of a 3.2 g/L solution of *potassium permanganate R*. Heat on a water bath for 5 min. A slight red colour persists.

**Phosphates**: maximum 0.1 per cent.

*Ammonium molybdate solution.* Dissolve 2 g of *ammonium molybdate R* in 100 mL of *water R* and adjust to pH 1.7-1.9 with *hydrochloric acid R*.

Dissolve 1.0 g in *water R* and dilute to 300 mL with the same solvent. Adjust 30 mL of the solution to pH 1.7-1.9 with *dilute hydrochloric acid R* and add 12.5 mL of *2-butanol R1*, 12.5 mL of *ether R* and 25 mL of the ammonium molybdate solution. Shake for 20 s and discard the lower layer. Add 10 mL of a 1 g/L solution of *hydrochloric acid R*, shake for 10 s and discard the lower layer. To the upper layer add 0.2 mL of *stannous chloride solution R1*.

Prepare a reference solution in the same manner using a mixture of 20 mL of *phosphate standard solution (5 ppm $PO_4$) R* and 10 mL of *water R*. Compare the colours after 10 min.

Any colour in the test solution is not more intense than that in the reference solution.

**Water** (*2.5.32*): 38.0 per cent to 42.0 per cent, determined on 30.0 mg using the evaporation technique at 100-110 °C.

ASSAY

Dissolve 2.0 g in *water R* and dilute to 50 mL with the same solvent. Carry out a potentiometric titration (*2.2.20*), using *1 M hydrochloric acid*. Read the volume added at the 1st point of inflexion.

1 mL of *1 M hydrochloric acid* is equivalent to 223.0 mg of $Na_4O_7P_2,10H_2O$.

STORAGE

In an airtight container.

**01/2008:1475**
**corrected 7.0**

# STRONTIUM ($^{89}$Sr) CHLORIDE INJECTION

## Strontii ($^{89}$Sr) chloridi solutio iniectabilis

DEFINITION

Sterile solution of [$^{89}$Sr]strontium chloride.

*Strontium-89*: 90 per cent to 110 per cent of the declared strontium-89 radioactivity at the date stated on the label.

*Specific radioactivity*: minimum 1.8 MBq of strontium-89 per milligram of strontium.

*Strontium*: 6.0 mg/mL to 12.5 mg/mL.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of strontium-89*: see general chapter *5.7. Table of physical characteristics of radionuclides.*

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Result*: the gamma photon detected has an energy of 0.909 MeV and is due to the short-lived daughter product, yttrium-89m (formed in 0.01 per cent of the disintegrations), in equilibrium with the strontium-89.

B. To 0.1 mL of the preparation to be examined, add 1 mL of a freshly prepared 1 g/L solution of *sodium rhodizonate R*. Mix and allow to stand for 1 min. A reddish-brown precipitate is formed.

C. To 0.1 mL of *silver nitrate solution R2* add 50 µL of the preparation to be examined. A white precipitate is formed.

TESTS

**pH** (*2.2.3*): 4.0 to 7.5.

*Note: the following tests for aluminium, iron and lead may be carried out simultaneously with the test for strontium. If this is not the case, the reference solutions are prepared such that they contain strontium at approximately the same concentration as in the test solution.*

**Aluminium**: maximum 2 µg/mL.

Atomic emission spectrometry (plasma or arc method) (*2.2.22, Method I*).

*Test solution.* Dilute 0.2 mL of the preparation to be examined to a suitable volume with *dilute nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *aluminium standard solution (10 ppm Al) R* diluted as necessary with *dilute nitric acid R*.

**Iron**: maximum 5 µg/mL.

Atomic emission spectrometry (plasma or arc method) (*2.2.22, Method I*).

*Test solution.* Dilute 0.2 mL of the preparation to be examined to a suitable volume with *dilute nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R* diluted as necessary with *dilute nitric acid R*.

**Lead**: maximum 5 µg/mL.

Atomic emission spectrometry (plasma or arc method) (*2.2.22, Method I*).

*Test solution.* Dilute 0.2 mL of the preparation to be examined to a suitable volume with *dilute nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* diluted as necessary with *dilute nitric acid R*.

**Strontium**: 6.0 mg/mL to 12.5 mg/mL.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dilute 0.2 mL of the preparation to be examined to a suitable volume with *dilute nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *strontium standard solution (1.0 per cent Sr) R* diluted as necessary with *dilute nitric acid R*.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125).*

*RADIONUCLIDIC PURITY*

The total radioactivity due to radionuclides other than strontium-89 is not more than 0.6 per cent.

**Gamma emitters other than yttrium-89m**: maximum 0.4 per cent of the total radioactivity.

Gamma-ray and X-ray spectrometry.

**Beta emitters**. Evaporate to dryness 100 µL of the preparation to be examined under a radiant heat source. Dissolve the residue in 2 mL of *47 per cent hydrobromic acid R*, evaporate to dryness under the radiant heat source and dissolve the residue in 2 mL of *dilute hydrobromic acid R1*. Transfer the solution to the top of a column, 5-6 mm in diameter, packed with approximately 2 mL of *cation-exchange resin R1* (100-250 µm), previously conditioned with *dilute hydrobromic acid R1* and elute the column with the same solvent until 10 mL of eluate has been collected into a container containing 50 µL of a 15 g/L solution of *anhydrous sodium sulfate R* in *1 M hydrochloric acid*.

To a liquid scintillation cocktail vial add an appropriate volume of *liquid scintillation cocktail R* followed by 1 mL of *water R*, 0.1 mL of a 15 g/L solution of *anhydrous sodium sulfate R* in *1 M hydrochloric acid* and 100 µL of eluate. Shake to obtain a clear solution. Using suitable counting equipment determine the radioactivity due to impurities A and B in the sample.

Taking into account the recovery efficiency of the separation, counting efficiency and radioactive decay, determine the radioactive concentration of impurities A and B in the sample and hence the percentage of total beta emitting impurities in the injection to be examined.

*Result*:

– *impurities A and B*: maximum 0.2 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. sulfur-35,

B. phosphorus-32.

**07/2017:2123**

# TECHNETIUM ($^{99m}$Tc) BICISATE INJECTION

## Technetii ($^{99m}$Tc) bicisati solutio iniectabilis

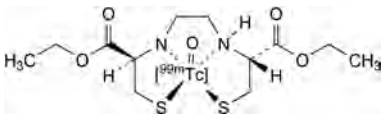

DEFINITION

Sterile solution of a complex of technetium-99m with diethyl *N*,*N*′-ethylenedi-L-cysteinate. It may contain stabilisers and inert additives such as *Mannitol (0559)* and *Disodium edetate (0232)*.

*Content*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label.

PRODUCTION

It is prepared from *N*,*N*′-(1,2-ethylenediyl)bis[(2*R*)-2-amino-3-sulfanylpropanoic acid] diethyl ester and *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* in the presence of reducing agents such as a stannous salt.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the test for radiochemical purity (see Tests).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retardation factor to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 6.5 to 7.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Impurities A, B, C, D, E, F, G**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. To vial B of *bicisate labelling kit CRS* in lead shielding add 2 mL of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) containing 400-800 MBq. Dissolve the contents of vial A of *bicisate labelling kit CRS* in 3 mL of a 9 g/L solution of *sodium chloride R*. Immediately transfer 1.0 mL of the solution contained in vial A to vial B. Mix and allow to stand for 30 min at room temperature.

*Reference solution (b)*. Sodium pertechnetate ($^{99m}$Tc) injection (fission or non fission).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*.

*Application*: 5 µL, allow the spots to dry for 5-10 min.

*Development*: over 4/5 of the plate.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a suitable detector.

*Retardation factors*: technetium-99m bicisate = more than 0.4; impurities A, B, C, D, E, F and G = less than 0.2.

*System suitability*: the retardation factor of the principal peak in the chromatogram obtained with reference solution (a) is clearly different from the retardation factor of the peak in the chromatogram obtained with reference solution (b).

*Limits*:

– *sum of impurities A, B, C, D, E, F and G*: not more than 10 per cent of the total radioactivity;

– *technetium-99m bicisate*: minimum 90 per cent of the total radioactivity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. technetium-99m in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion,

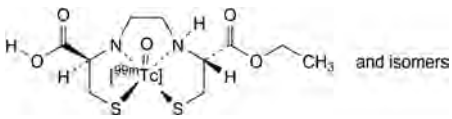


C. complex of technetium-99m with ethyl hydrogen *N*,*N*′-ethylenedi-L-cysteinate,

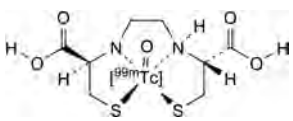

D. complex of technetium-99m with *N*,*N*′-ethylenedi-L-cysteine,

E. complex of technetium-99m with mannitol,

F. complex of technetium-99m with disodium edetate,

G. other complexes of technetium-99m with diethyl *N*,*N*′-ethylenedi-L-cysteinate.

**01/2009:0126**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) COLLOIDAL RHENIUM SULFIDE INJECTION

## Rhenii sulfidi colloidalis et technetii ($^{99m}$Tc) solutio iniectabilis

DEFINITION

Sterile colloidal dispersion of rhenium sulfide, the micelles of which are labelled with technetium-99m. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. It is stabilised with gelatin. The pH of the injection may be adjusted by the addition of a suitable buffer such as citrate buffer.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Rhenium*: maximum 0.22 mg/mL.

CHARACTERS

*Appearance*: light brown liquid.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is 0.0 to 0.1.

C. To 1 mL add 1 mL of a 200 g/L solution of *stannous chloride R* in *hydrochloric acid R*, 5 mL of *hydrochloric acid R* and 5 mL of a 50 g/L solution of *thiourea R*. A yellow colour develops.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

**Rhenium**: maximum 0.22 mg/mL.

*Test solution.* The preparation to be examined.

*Reference solutions.* Using a solution containing 100 µg of *potassium perrhenate R* (equivalent to 60 ppm of Re) and 240 µg of *sodium thiosulfate R* per millilitre, prepare a range of solutions and dilute to the same final volume with *water R*.

To 1 mL of the test solution and to 1 mL of each of the reference solutions add 1 mL of a 200 g/L solution of *stannous chloride R* in *hydrochloric acid R*, 5 mL of *hydrochloric acid R* and 5 mL of a 50 g/L solution of *thiourea R* and dilute to 25.0 mL with *water R*. Allow to stand for 40 min and measure the absorbance (*2.2.25*) of each solution at 400 nm, using a reagent blank as the compensation liquid. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the concentration of rhenium in the preparation to be examined.

**Physiological distribution**. Inject a volume not greater than 0.2 mL into a caudal vein of each of 3 mice each weighing 20-25 g. Euthanise the mice 20 min after the injection, remove the liver, spleen and lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs using the following expression:

$$\frac{A}{B} \times 100$$

$A$ = radioactivity of the organ concerned;

$B$ = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In each of the 3 mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in 1 of the 3 mice does not correspond to the prescribed proportions, repeat the test on a further 3 mice. The preparation complies with the test if the prescribed distribution of radioactivity is found in 5 of the 6 mice used. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**[$^{99m}$Tc]Technetium in colloidal form**. Ascending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Paper*: *paper for chromatography R*.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: 10 µL.

*Development*: immediately over a path of 10-15 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium in colloidal form = 0.0 to 0.1; impurity A = about 0.6; other impurities = 0.8 to 0.9.

*Limit*:

– *[$^{99m}$Tc]technetium in colloidal form*: minimum 92 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states the concentration of rhenium expressed in milligrams per millilitre.

IMPURITIES

A. [$^{99m}$Tc]pertechnetate ion.

**01/2008:0131**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) COLLOIDAL SULFUR INJECTION

## Sulfuris colloidalis et technetii ($^{99m}$Tc) solutio iniectabilis

DEFINITION

Sterile, apyrogenic colloidal dispersion of sulfur, the micelles of which are labelled with technetium-99m. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*.

It may be stabilised with a colloid-protecting substance based on gelatin. The pH of the injection may be adjusted by the addition of a suitable buffer, such as an acetate, citrate or phosphate buffer solution. The injection contains a variable quantity of colloidal sulfur, according to the method of preparation.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear or opalescent, colourless or yellowish liquid.

*Half-life and nature of radiation of technetium-99 m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is 0.0 to 0.1.

C. In a test-tube 100 mm long and 16 mm in internal diameter, evaporate 0.2 mL of the preparation to be examined to dryness. Dissolve the sulfur by shaking the residue with 0.2 mL of *pyridine R* and add about 20 mg of *benzoin R*. Cover the open end of the tube with a filter paper moistened with *lead acetate solution R*. Heat the test-tube in a bath containing glycerol at 150 °C. The paper slowly becomes brown.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

**Physiological distribution**. Inject a volume not greater than 0.2 mL into the caudal vein of each of 3 mice, each weighing 20-25 g. Euthanise the mice 20 min after the injection, remove the liver, spleen and lungs and measure the radioactivity in these organs using a suitable instrument. Measure the radioactivity in the rest of the body of each animal after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs using the following expression:

$$\frac{A}{B} \times 100$$

$A$ = radioactivity of the organ concerned;

$B$ = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In each of the 3 mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in 1 of the 3 mice does not correspond to the prescribed proportions, repeat the test on a further 3 mice. The preparation to be examined complies with the test if the prescribed distribution of radioactivity is found in 5 of the 6 mice used. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Pyrogens**. It complies with the test for pyrogens prescribed in the monograph *Radiopharmaceutical preparations (0125)*. Inject, per kilogram of the rabbit's mass, not less than 0.1 mL. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**[$^{99m}$Tc]Technetium in colloidal form**. Ascending paper chromatography (*2.2.26*).

*Test solution*. The preparation to be examined.

*Paper*: *paper for chromatography R*.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: 10 µL.

*Development*: immediately, over a path of 10-15 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium in colloidal form = 0.0 to 0.1; impurity A = about 0.6; other impurities = 0.8 to 0.9.

*Limit*:

– *[$^{99m}$Tc]technetium in colloidal form*: minimum 92 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]pertechnetate ion.

**01/2008:0689**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) COLLOIDAL TIN INJECTION

## Stanni colloidalis et technetii ($^{99m}$Tc) solutio iniectabilis

DEFINITION

Sterile, colloidal dispersion of tin labelled with technetium-99m. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. The injection contains a variable quantity of tin not exceeding 1 mg of Sn per millilitre; it contains fluoride ions, it may be stabilised with a suitable, apyrogenic colloid-protecting substance and it may contain a suitable buffer.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Tin*: maximum 1 mg/mL.

CHARACTERS

*Appearance*: clear or opalescent, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Mix 0.05 mL of *zirconyl nitrate solution R* with 0.05 mL of *alizarin S solution R*. Add 0.05 mL of the preparation to be examined. A yellow colour is produced.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

**Tin**: maximum 1 mg/mL.

*Test solution*. Dilute 3.0 mL of the preparation to be examined to 50.0 mL with a 103 g/L solution of *hydrochloric acid R*.

Radiopharmaceutical preparations

*Reference solution*. Dissolve 0.115 g of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

To 1.0 mL of each solution add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Physiological distribution**. Inject not more than 0.2 mL into a caudal vein of each of 3 mice, each weighing 20-25 g. Euthanise the mice 20 min after the injection and remove the liver, spleen and lungs. Measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body of each animal, after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs with respect to the total radioactivity of all organs and the rest of the body excluding the tail.

In each of the 3 mice at least 80 per cent of the radioactivity is found in the liver and spleen and not more than 5 per cent in the lungs. If the distribution of radioactivity in 1 of the 3 mice does not correspond to the prescribed proportions, repeat the test on a further 3 mice. The preparation to be examined complies with the test if the prescribed distribution of radioactivity is found in 5 of the 6 mice used.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**[$^{99m}$Tc]Technetium in colloidal form**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.

*Mobile phase*: 9 g/L solution of *sodium chloride R* purged with *nitrogen R*.

*Application*: 5-10 µL.

*Development*: over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium in colloidal form = 0.0 to 0.1; impurity A = 0.9 to 1.0.

*Limit*:

– *[$^{99m}$Tc]technetium in colloidal form*: minimum 95 per cent of the radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]pertechnetate ion.

**04/2015:0585**

# TECHNETIUM ($^{99m}$Tc) ETIFENIN INJECTION

## Technetii ($^{99m}$Tc) et etifenini solutio iniectabilis

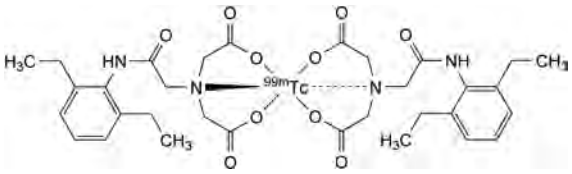

$C_{32}H_{40}N_4O_{10}{}^{99m}Tc$ $M_r$ 740

DEFINITION

Sterile solution of a complex of technetium-99m with etifenin. It is prepared by mixing *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* with solutions of [[[(2,6-diethylphenyl)carbamoyl]methyl]imino]diacetic acid (etifenin) and stannous chloride. It may contain stabilisers and inert additives.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution*. To 40 mg of *etifenin CRS* add 2 mL of *water R* and shake. While shaking, add dropwise a 20 g/L solution of *sodium hydroxide R* until complete dissolution. Adjust to pH 7 with a 51.5 g/L solution of *hydrochloric acid R* and dilute to 4 mL with *water R*. Introduce this solution into a closed vial filled with *nitrogen R*. Add 0.1 mL of a 5 g/L solution of *stannous chloride R* in a 5 g/L solution of *hydrochloric acid R*. Add 1 mL of a solution of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) containing 100-200 MBq. Allow to stand for 15 min.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 3.85 g/L solution of *ammonium acetate R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 70 | 30 |
| 20 - 25 | 70 → 0 | 30 → 100 |
| 25 - 30 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector.

*Injection*: 20 µL.

*Result*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

**Tin**: maximum 0.2 mg/mL.

*Test solution.* Dilute 1.0 mL of the preparation to be examined to 5.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution.* Dissolve 7.5 mg of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 100 mL with the same acid.

To 1.0 mL of each solution add successively 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R*, 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**A. Impurity A**. Ascending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Reference solution.* To 1 mL of a 1 g/L solution of *stannous chloride R* in a 5 g/L solution of *hydrochloric acid R* in a closed vial, add 2 mL of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) containing 100-200 MBq. Use within 30 min.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Application*: about 5 µL.

*Development*: immediately, over a path of 10 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0-0.1; impurity B and [$^{99m}$Tc]technetium etifenin = 0.9-1.0.

*System suitability*: the retardation factor of the principal peak in the chromatogram obtained with the reference solution is not more than 0.15; the retardation factor of the principal peak in the chromatogram obtained with the test solution is more than 0.7.

**B. Impurity B**. Ascending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Reference solution.* Sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission).

*Paper*: *paper for chromatography R* saturated with a 25 g/L solution of *sodium hydrogen carbonate R* and stored protected from moisture after drying at 80 °C.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: about 5 µL.

*Development*: immediately, over a path of 10 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A and [$^{99m}$Tc]technetium etifenin = 0.0-0.1; impurity B = 0.9-1.0.

*System suitability*: the retardation factor of the principal peak in the chromatogram obtained with the test solution is not more than 0.15; the retardation factor of the principal peak in the chromatogram obtained with the reference solution is more than 0.7.

*Limits*:

- *impurity A*: not more than 3.0 per cent of the total radioactivity due to technetium-99m in the chromatogram obtained in test A;
- *impurity B*: not more than 3.0 per cent of the total radioactivity due to technetium-99m in the chromatogram obtained in test B;
- *sum of impurities A and B*: maximum 5.0 per cent of the total radioactivity due to technetium-99m in the chromatograms obtained in tests A and B.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2008:1925**

# TECHNETIUM ($^{99m}$Tc) EXAMETAZIME INJECTION

## Technetii ($^{99m}$Tc) exametazimi solutio iniectabilis

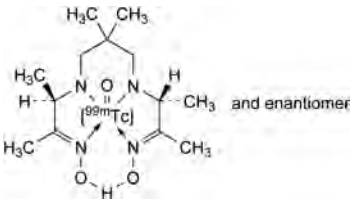


DEFINITION

Sterile solution of lipophilic technetium-99m exametazime which may be prepared by dissolving a racemic mixture of (3*RS*,9*RS*)-4,8-diaza-3,6,6,9-tetramethylundecane-2,10-dione bisoxime in the presence of a stannous salt in *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)*. It may contain stabilisers and inert additives.

*Content*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Purity*: minimum of 80 per cent of the total radioactivity corresponds to lipophilic technetium-99m exametazime and its *meso* isomer.

CHARACTERS

*Appearance*: clear solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Comparison*: standardised technetium-99m solution, or by using a calibrated instrument. Standardised technetium-99m solutions and/or standardisation services are available from the competent authority.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that obtained with a standardised technetium-99m solution. The most prominent gamma photon has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the test Impurity A under Radiochemical purity.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the peak due to lipophilic technetium-99m exametazime in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 5.0 to 10.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: about 5 µL.

*Development*: immediate, over 2/3 of the plate.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a suitable detector.

*Retardation factors*: impurity C = 0.8 to 1.0; lipophilic technetium-99m exametazime and impurities A, B, D and E do not migrate.

*Limits*:

– *impurity C*: maximum 10 per cent of the total radioactivity.

**Total of lipophilic technetium-99m exametazime and impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: about 5 µL.

*Development*: immediate, over 2/3 of the plate.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a suitable detector.

*Retardation factors*: lipophilic technetium-99m exametazime = 0.8 to 1.0, impurity A = 0.8 to 1.0, impurity C = 0.8 to 1.0; impurities B, D and E do not migrate.

*Limits*: calculate the percentage of radioactivity due to impurities B, D and E from test B (*B*) and the percentage of the radioactivity due to impurity C from test A (*A*). Calculate the total percentage of lipophilic technetium-99m exametazime and impurity A from the expression:

$$100 - A - B$$

– *total of lipophilic technetium-99m exametazime and impurity A*: minimum 80 per cent of the total radioactivity.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution*. Dissolve the contents of a vial of *meso-rich exametazime CRS* in 0.5 mL of a 9 g/L solution of *sodium chloride R* and transfer to a lead-shielded, nitrogen-filled vial. Add 6 µL of a freshly prepared 1 g/L solution of *stannous chloride R* in *0.05 M hydrochloric acid* and 2.5 mL of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) containing 370-740 MBq. Mix carefully and use within 30 min of preparation.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: spherical *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 13 nm and a carbon loading of 11 per cent.

*Mobile phase*: mix 33 volumes of *acetonitrile R* and 67 volumes of *0.1 M phosphate buffer solution pH 3.0 R*.

*Flow rate*: 1.5 mL/min.

*Detection*: radioactivity detector.

*Injection*: loop injector.

*Run time*: 20 min.

*Relative retention* with reference to lipophilic technetium-99m exametazime: impurity A = about 1.2.

*System suitability*: reference solution:

– chromatogram similar to the chromatogram provided with *meso-rich exametazime CRS*,

– *resolution*: minimum of 2 between the peaks due to lipophilic technetium-99m exametazime and to impurity A.

*Limits*:

– *impurity A*: maximum 5 per cent of the radioactivity due to lipophilic technetium-99m exametazime and impurity A.

RADIOACTIVITY

Measure the radioactivity using suitable equipment by comparison with a standardised technetium-99m solution or by using a calibrated instrument.

IMPURITIES

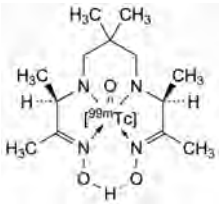


A. *meso* isomer of lipophilic technetium-99m exametazime,

B. technetium-99m in colloidal form,

C. [$^{99m}$Tc]pertechnetate ion,

D. non lipophilic technetium-99m exametazime complex,

E. *meso* isomer of non lipophilic technetium-99m exametazime complex.

**01/2008:1047**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) GLUCONATE INJECTION

## Technetii ($^{99m}$Tc) gluconatis solutio iniectabilis

DEFINITION

Sterile solution of a complex of technetium-99m with calcium gluconate. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection fission (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection non fission (0283)*.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: slightly opalescent solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.
*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. 5 µL of the preparation to be examined complies with identification A prescribed in the monograph *Calcium gluconate (0172)*.

C. Examine the chromatograms obtained in tests A and B for radiochemical purity (see Tests).
*Results*:
– the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in test A is 0.9 to 1.0;
– the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in test B is 0.0 to 0.1.

TESTS

**pH** (*2.2.3*): 6.0 to 8.5.

**Physiological distribution**. Inject a volume not greater than 0.2 mL into the caudal vein of each of 3 rats weighing 150-250 g. Measure the radioactivity of the syringe before and after injection. Euthanise the rats 30 min after the injection. Remove at least 1 g of blood by a suitable method and remove the kidneys, the liver, the bladder plus voided urine and the tail. Weigh the sample of blood.

Determine the radioactivity in the organs, the blood sample and the tail using a suitable instrument. Calculate the percentage of radioactivity in each organ and in 1 g of blood with respect to the total radioactivity calculated as the difference between the 2 measurements made on the syringe minus the activity in the tail. Correct the blood concentration by multiplying by a factor of $m/200$ where $m$ is the body mass of the rat in grams.

In not fewer than 2 of the 3 rats used, the radioactivity is:
– *in the kidneys*: minimum 15 per cent,
– *in the bladder plus voided urine*: minimum 20 per cent,
– *in the liver*: maximum 5 per cent.
– *in the blood*, after correction: maximum 0.50 per cent.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

A. Impurity A. Thin-layer chromatography (*2.2.27*).
*Test solution*. The preparation to be examined.
*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.
*Mobile phase*: 9 g/L solution of *sodium chloride R*.
*Application*: 5-10 µL.
*Development*: immediately over a path of 10-15 cm in about 10 min.
*Drying*: in air.
*Detection*: suitable detector to determine the distribution of radioactivity.
*Retardation factors*: impurity A = 0.0 to 0.1; [$^{99m}$Tc]technetium gluconate and impurity B = 0.9 to 1.0.

B. Impurity B. Thin-layer chromatography (*2.2.27*).
*Test solution*. The preparation to be examined.
*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.
*Mobile phase*: *methyl ethyl ketone R*.
*Application*: 5-10 µL and allow to dry.
*Development*: over a path of 10-15 cm in about 10 min.
*Drying*: in a current of warm air.
*Detection*: suitable detector to determine the distribution of radioactivity.
*Retardation factors*: [$^{99m}$Tc]technetium gluconate and impurity A = 0.0 to 0.1; impurity B = 0.9 to 1.0.

*Limit*:
– *sum of impurities A and B*: maximum 10 per cent of the radioactivity due to technetium-99m in the chromatograms obtained in tests A and B.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2008:0640**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) HUMAN ALBUMIN INJECTION

## Technetii ($^{99m}$Tc) humani albumini solutio iniectabilis

DEFINITION

Sterile, apyrogenic solution of human albumin labelled with technetium-99m. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. It contains a reducing substance, such as a tin salt in an amount not exceeding 1 mg of Sn per millilitre. Although, at present, no definite value for a maximum limit of tin can be fixed, available evidence tends to suggest the importance of keeping the ratio of tin to albumin as low as possible. It may contain a suitable buffer and an antimicrobial preservative. The human albumin used complies with the requirements of the monograph *Human albumin solution (0255)*.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Albumin*: 90.0 per cent to 110.0 per cent of the quantity of albumin stated on the label.

CHARACTERS

*Appearance*: clear, colourless or pale yellow solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.
*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. The test is to be carried out using antisera specific to the plasma proteins of each species of domestic animal currently used in the preparation of materials of biological origin in the country concerned. The preparation is shown to contain proteins of human origin and gives negative results with antisera specific to plasma proteins of other species.

C. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted if necessary. The main component of the

preparation to be examined corresponds to the main component of the normal human serum. The diluted preparation may show the presence of small quantities of other plasma proteins.

TESTS

**pH** (*2.2.3*): 2.0 to 6.5.

**Albumin**

*Test solution.* The preparation to be examined.

*Reference solution.* Dilute *human albumin solution R* with a 9 g/L solution of *sodium chloride R* to a concentration of 5 mg of albumin per millilitre.

To 1.0 mL of the test solution and to 1.0 mL of the reference solution add 4.0 mL of *biuret reagent R* and mix. After exactly 30 min, measure the absorbance (*2.2.25*) of each solution at 540 nm, using as the compensation liquid a 9 g/L solution of *sodium chloride R* treated in the same manner. From the absorbances measured, calculate the content of albumin in the preparation to be examined in milligrams per millilitre.

**Tin**: maximum 1 mg/mL.

*Test solution.* To 1.0 mL of the preparation to be examined add 1.0 mL of a 206 g/L solution of *hydrochloric acid R*. Heat in a water-bath at 100 °C for 30 min. Cool and centrifuge at 300 *g* for 10 min. Dilute 1.0 mL of the supernatant to 10 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution.* Dissolve 95 mg of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

To 1.0 mL of each solution add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Physiological distribution**. Inject a volume not greater than 0.5 mL and containing not more than 1.0 mg of albumin into a suitable vein such as a caudal vein or a saphenous vein of each of 3 male rats, each weighing 150-250 g. Measure the radioactivity in the syringe before and after the injection. Euthanise the rats 30 min after the injection. Take 1 ml of blood by a suitable method and remove the liver and, if a caudal vein has been used for the injection, the tail. Using a suitable instrument determine the radioactivity in these organs and blood. Determine the percentage of radioactivity in the liver and in 1 mL of blood with respect to the total radioactivity calculated as the difference between both measurements made on the syringe minus the activity in the tail (if a caudal vein has been used for the injection). Correct the blood radioactivity by multiplying by a factor of *m*/200 where *m* is the body mass of the rat in grams. In not fewer than 2 of the 3 rats used, the radioactivity in the liver is not more than 15 per cent and that in blood, after correction, is not less than 3.5 per cent.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet, heated at 110 °C for 10 min.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: 5-10 µL and allow to dry.

*Development*: over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium human albumin = 0.0 to 0.1; impurity A = 0.9 to 1.0.

*Limit*:

– *impurity A*: maximum 5.0 per cent of the total radioactivity due to technetium-99m.

**[$^{99m}$Tc]Technetium albumin fractions II to V**. Size-exclusion chromatography (*2.2.30*).

*Mobile phase (concentrated).* Dissolve 1.124 g of *potassium dihydrogen phosphate R*, 4.210 g of *disodium hydrogen phosphate dodecahydrate R*, 1.17 g of *sodium chloride R* and 0.10 g of *sodium azide R* in *water R* and dilute to 100 mL with the same solvent.

*Test solution.* Mix 0.25 mL of the preparation to be examined with 0.25 mL of the mobile phase (concentrated). *Use immediately after dilution.*

*Column*:

– *size*: *l* = 0.6 m, Ø = 7.5 mm;

– *stationary phase*: *silica gel for size-exclusion chromatography R*.

*Mobile phase*: mobile phase (concentrated), *water R* (50:50 *V*/*V*).

*Flow rate*: 0.6 mL/min.

*Detection*: radioactivity detector set for technetium-99m.

*Injection*: 200 µL.

*Run time*: at least 10 min after background level is reached.

Retention times of eluted peaks:

| | | |
|---|---|---|
| I | High molecular mass compound | 19-20 min |
| II | Poly III-albumin | 23-24 min |
| III | Poly II-albumin | 25-27 min |
| IV | Poly I-albumin | 28-29 min |
| V | Human serum albumin | 32-33 min |
| VI | Tin colloid | 40-47 min |
| VII | Pertechnetate | 48 min |

*Limit*:

– *[$^{99m}$Tc]technetium albumin fractions II to V*: minimum 80 per cent of the radioactivity due to technetium-99m applied to the column.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

– the amount of albumin;

– the amount of tin, if any.

IMPURITIES

A. [$^{99m}$Tc]pertechnetate ion.

**01/2009:0296**
**corrected 7.4**

# TECHNETIUM ($^{99m}$Tc) MACROSALB INJECTION

## Technetii ($^{99m}$Tc) macrosalbi suspensio iniectabilis

DEFINITION

Sterile suspension of human albumin in the form of irregular insoluble aggregates obtained by denaturing human albumin in aqueous solution. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. The particles are labelled with technetium-99m and have a typical diameter between 10 µm and 100 µm. The injection contains reducing substances, such as tin salts; it may contain a suitable buffer such as acetate, citrate or phosphate buffer and also non-denatured human albumin and an antimicrobial preservative such as benzyl alcohol.

The human albumin employed complies with the requirements prescribed in the monograph *Human albumin solution (0255)*.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Specific radioactivity*: minimum 37 MBq of technetium-99m per milligram of aggregated albumin at the date and time of administration.

CHARACTERS

*Appearance*: white suspension which may separate on standing.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. The tests for non-filterable radioactivity and particle size contribute to the identification of the preparation (see Tests).

C. Transfer 1 mL of the preparation to be examined to a centrifuge tube and centrifuge at 2500 *g* for 5-10 min. Decant the supernatant. To the residue add 5 mL of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. If necessary, heat to dissolve the particles and allow to cool. Add rapidly 0.5 mL of *dilute phosphomolybdotungstic reagent R*, mixing immediately. A blue colour develops.

TESTS

**pH** (*2.2.3*): 3.8 to 7.5.

**Non-filterable radioactivity**: minimum 90 per cent of the total radioactivity.

Use a polycarbonate membrane filter 13-25 mm in diameter, 10 µm thick and with circular pores 3 µm in diameter. Fit the membrane into a suitable holder. Place 0.2 mL of the preparation to be examined on the membrane and filter, adding 20 mL of a 9 g/L solution of *sodium chloride R* during the filtration. Determine the radioactivity remaining on the membrane.

**Particle size**: not more than 10 particles have a maximum dimension greater than 100 µm and no particle having a maximum dimension greater than 150 µm is present.

Examine using a microscope. Dilute the preparation to be examined if necessary so that the number of particles is just low enough for individual particles to be distinguished. Using a syringe fitted with a needle having a calibre not less than 0.35 mm, place a suitable volume in a suitable counting chamber such as a haemocytometer cell, taking care not to overfill the chamber. Allow the preparation to be examined to settle for 1 min and, carefully add a cover slide without squeezing the sample. Scan an area corresponding to at least 5000 particles.

**Aggregated albumin**

*Test solution*. Transfer a volume of the preparation to be examined containing about 1 mg of aggregated albumin to a centrifuge tube and centrifuge at about 2500 *g* for 5-10 min. Decant the supernatant. Resuspend the residue in 2.0 mL of a 9 g/L solution of *sodium chloride R*. Centrifuge at 2500 *g* for 5-10 min. Decant the supernatant. Resuspend the residue in 5.0 mL of *sodium carbonate solution R1*. Heat in a water-bath at 80-90 °C to dissolve the aggregated albumin. Allow to cool, transfer to a volumetric flask and dilute to 10.0 mL with *sodium carbonate solution R1*.

*Reference solutions*. Prepare a range of solutions containing 0.05-0.2 mg of human albumin per millilitre by diluting *human albumin solution R* with *sodium carbonate solution R1*.

Introduce 3.0 mL of each solution separately into 25 mL flasks. To each flask add 15.0 mL of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. Add rapidly 1.5 mL of *dilute phosphomolybdotungstic reagent R* and mix immediately. Allow to stand for 30 min and measure the absorbance (*2.2.25*) of each solution at 750 nm using *sodium carbonate solution R1* as the compensation liquid. Using the absorbances obtained with the reference solutions, draw a calibration curve and calculate the content of aggregated albumin in the preparation to be examined.

**Tin**: maximum 3 mg/mL.

*Test solution*. To 1.0 mL of the preparation to be examined, add 1.0 mL of a 206 g/L solution of *hydrochloric acid R*. Heat in a water-bath for 30 min. Cool and centrifuge for 10 min at 300 *g*. Dilute 1.0 mL of the supernatant to 25.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution*. Dissolve 0.115 g of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

To 1.0 mL of each solution, add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Physiological distribution**. Inject a volume not greater than 0.2 mL into a caudal vein of each of 3 rats weighing 150-250 g. Euthanise the rats 15 min after the injection, remove the liver, the spleen and the lungs and measure the radioactivity in the organs using a suitable instrument. Measure the radioactivity in the rest of the body, including the blood, after having removed the tail. Determine the percentage of radioactivity in the lungs, the liver and the spleen from the following expression:

$$\frac{A}{B} \times 100$$

$A$ = radioactivity of the organ concerned;

$B$ = total radioactivity in the liver, the spleen, the lungs and the rest of the body.

In not fewer than 2 of the 3 rats used, at least 80 per cent of the radioactivity is found in the lungs and not more than a total of 5 per cent in the liver and spleen. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- the concentration of tin expressed in milligrams per millilitre, if any;
- that the preparation is to be shaken before use;
- that the preparation is not to be used if after shaking, the suspension does not appear homogeneous.

**07/2019:2393**

# TECHNETIUM ($^{99m}$Tc) MEBROFENIN INJECTION

## Technetii ($^{99m}$Tc) mebrofenini solutio iniectabilis

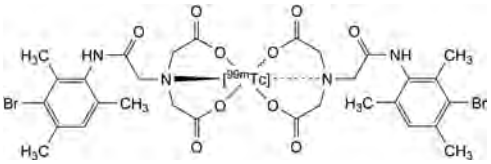


DEFINITION

Sterile solution of a complex of technetium-99m with mebrofenin. It may contain stabilisers and inert additives.

*Content*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

PRODUCTION

It is prepared by dissolving [[2-(3-bromo-2,4,6-trimethylanilino)-2-oxoethyl]azanediyl]diacetic acid (mebrofenin) in *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* in the presence of a reducing agent such as a stannous salt.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for other radiochemical impurities (see Tests).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the peak due to technetium-99m mebrofenin in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.0 to 7.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

*Technetium-99m mebrofenin*: minimum 90 per cent of the total radioactivity.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* To 1 mL of a 1 g/L solution of *stannous chloride R* in *0.05 M hydrochloric acid* in a closed vial, add 2 mL of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission). Use within 30 min after preparation.

*Reference solution (b).* Dissolve 40 mg of *mebrofenin CRS* in 2 mL of a 4 g/L solution of *sodium hydroxide R* and adjust to pH 6.5 with *1 M hydrochloric acid* or a 40 g/L solution of *sodium hydroxide R*. To this solution add 25 µL of a 20 mg/mL solution of *stannous chloride R* in *0.05 M hydrochloric acid* and 400 MBq of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) in a volume of 2 mL. Allow to stand for 30-60 min.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Application*: about 5 µL.

*Development*: immediately, over 4/5 of the plate.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a suitable detector.

*Retardation factor*: impurity A = 0.0 to 0.1.

*System suitability*: the retardation factor of the principal peak in the chromatogram obtained with reference solution (a) is not more than 0.1; the retardation factor of the principal peak in the chromatogram obtained with reference solution (b) is more than 0.7.

*Limit*:

- *impurity A*: maximum 5 per cent of the total radioactivity.

**Other radiochemical impurities**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution.* Use reference solution (b) of the test for impurity A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 3.85 g/L solution of *ammonium acetate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 70 | 30 |
| 20 - 25 | 70 → 0 | 30 → 100 |
| 25 - 30 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: radioactivity detector.

*Injection*: 20 µL.

*Relative retention* with reference to technetium-99m mebrofenin (retention time = about 20 min): impurity B = about 0.17.

*Limits*:

– *sum of the areas of the peaks eluted before the principal peak (corresponding to hydrophilic impurities, including impurity B)*: maximum 6 per cent of the total area of the peaks in the chromatogram obtained with the test solution.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. technetium-99m in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2016:0641**

# TECHNETIUM ($^{99m}$Tc) MEDRONATE INJECTION

## Technetii ($^{99m}$Tc) medronati solutio iniectabilis

DEFINITION

Sterile solution of a complex of technetium-99m with sodium methylenediphosphonate. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)*. It may contain antimicrobial preservatives, antioxidants, stabilisers and buffers.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the tests for Impurity A and Impurity B under Radiochemical purity (see Tests).

*Results*: the principal peak in the chromatogram obtained with the test solution is, in each of the tests, similar in retardation factor to the principal peak in the chromatogram obtained with reference solution (b).

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a solution containing about 0.1 mg/mL of medronic acid.

*Reference solution (a)*. Dissolve 3 mg of *sodium oxidronate R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 1 mg of *medronic acid R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: to 30 mL of *water R* add 35 mL of *ethanol (96 per cent) R*, 15 mL of *2-methylpropanol R*, 0.4 mL of *concentrated ammonia R*, 5 g of *trichloroacetic acid R* and 20 mL of *2-propanol R* and mix.

*Application*: about 5 µL, applied in 1 µL portions with drying after each application.

*Development*: over 2/3 of the plate.

*Drying*: in air for at least 14 h.

*Detection*: spray with a solution prepared as follows: to 25 mL of a 40 g/L solution of *ammonium molybdate R* add 63 mL of *water R*, 2 mL of *hydrochloric acid R*, 2 mL of *sulfuric acid R* and 8 mL of *perchloric acid R* and mix. Expose the plate to ultraviolet light at 254 nm for 15 min.

*System suitability*:

– the retardation factor of the spot in the chromatogram obtained with reference solution (b) is about 0.6 and clearly different from that obtained with reference solution (a), which is about 0.5.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (b).

The preparation may be released for use before completion of the test.

TESTS

**pH** (*2.2.3*): 3.5 to 7.5.

**Tin**: maximum 3 mg/*V*, *V* being the maximum recommended dose in millilitres.

Determine the content of tin using a *semi-quantitative tin test kit R*, following the instructions given by the test kit manufacturer. If necessary, dilute the preparation to be examined with *water R* to obtain a concentration of tin within the measurement range of the test kit.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. To 1 mL of a 1 g/L solution of *stannous chloride R* in a 5.15 g/L solution of *hydrochloric acid R* in a closed vial, add 2 mL of *sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* containing 100-400 MBq. Use within 30 min.

*Reference solution (b)*. To a vial of *medronate for radiochemical purity testing CRS* add 2 mL of *sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* containing 100-400 MBq. Allow to stand for 15 min.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 136 g/L solution of *sodium acetate R*.

*Application*: about 2 µL.

*Development*: immediately, over 4/5 of the plate.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0-0.1; impurity B and [$^{99m}$Tc]technetium medronate = 0.9-1.0.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Reference solution (a)*. *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)*.

*Reference solution (b).* Reference solution (b) of the test for impurity A.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: about 2 µL.

*Development*: immediately, over 4/5 of the plate.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A and [$^{99m}$Tc]technetium medronate = 0.0-0.1 and impurity B = 0.9-1.0.

*Limit*:

– [$^{99m}$Tc]*technetium medronate*: minimum 95 per cent of the total radioactivity due to technetium-99m.

Calculate the percentage of radioactivity due to [$^{99m}$Tc]technetium medronate using the following expression:

$$100 - (A + B)$$

*A* = percentage of radioactivity due to impurity A determined in the test for impurity A under Radiochemical purity;

*B* = percentage of radioactivity due to impurity B determined in the test for impurity B under Radiochemical purity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

*Specified impurities: A, B.*

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2008:1372**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) MERTIATIDE INJECTION

## Technetii ($^{99m}$Tc) mertiatidi solutio iniectabilis

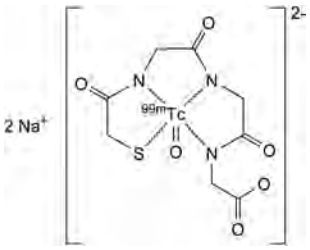


DEFINITION

Sterile solution of disodium oxo[*N*-[*N*-[*N*-(sulfanylacetyl)-glycyl]glycyl]glycynato(5-)-κ$^4$*N*,*N*′,*N*″,*S*][$^{99m}$Tc]technetate(V). It may be prepared by either heating a mixture containing *S*-benzoylmercaptoacetyltriglycine (betiatide), a weak chelating agent such as tartrate, a stannous salt and *Sodium pertechnetate ($^{99m}$Tc) injection fission (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection non-fission (0283)*, or by mixing solutions of mercaptoacetyltriglycine (mertiatide), a stannous salt and *Sodium pertechnetate ($^{99m}$Tc) injection fission (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection non-fission (0283)* at alkaline pH. It may contain stabilisers and a buffer.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.
*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the test of other radiochemical impurities in the section Radiochemical purity (see Tests).
*Result*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 5.0 to 7.5.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Impurity A**. Ascending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Paper*: *paper for chromatography R*.

*Mobile phase*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factor*: impurity A = 0.0-0.1.

*Limit*:

– *impurity A*: maximum 2.0 per cent of the total radioactivity.

**Other radiochemical impurities**. Liquid chromatography (*2.2.29*).

*Test solution.* The preparation to be examined.

*Reference solution.* Dissolve with heating on a water-bath 5 mg of *S-benzylmercaptoacetyltriglycine CRS* in 5 mL of *water R*. To 1 mL of this solution in a closed vial filled with *nitrogen R*, add 0.5 mL of a 40 g/L solution of *sodium potassium tartrate R*, 25 µL of a 4 g/L solution of *stannous chloride R* in a 5 g/L solution of *hydrochloric acid R* and 370-740 MBq of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission) in a volume not exceeding 3 mL. Heat the mixture on a water-bath for 10 min and allow to cool to room temperature.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase A*: mix 7 volumes of *anhydrous ethanol R* with 93 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R*, adjusted to pH 6.0 with a 4 g/L solution of *sodium hydroxide R*.

*Mobile phase B*: *water R*, *methanol R* (10:90 *V/V*).

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Equilibration*: with mobile phase A for 20 min.

*Injection*: 20 µL.

*Limits*:

– *sum of the areas preceding the principal peak (corresponding to hydrophilic impurities, including impurity B)*: maximum 3.0 per cent of the sum of the areas of all peaks in the chromatogram obtained with the test solution;
– *sum of the peaks following the principal peak (corresponding to lipophilic impurities)*: maximum 4.0 per cent of the sum of the area of all peaks in the chromatogram obtained with the test solution;
– *[$^{99m}$Tc]technetium mertiatide*: minimum 94 per cent of the radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2009:0570**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) MICROSPHERES INJECTION

## Technetii ($^{99m}$Tc) microsphaerarum suspensio iniectabilis

DEFINITION

Sterile suspension of human albumin which has been denatured to form spherical insoluble particles. The particles are labelled with technetium-99m and have a typical diameter of 10-50 µm. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. The injection contains reducing substances, such as tin salts. It may contain a suitable buffer such as acetate, citrate or phosphate and additives such as wetting agents.

The human albumin used complies with the requirements of the monograph *Human albumin solution (0255)*.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Radioactivity*: minimum 185 MBq of technetium-99m per million particles at the date and time of administration.

CHARACTERS

*Appearance*: suspension of white, yellow or artificially coloured particles which may separate on standing.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. The tests for non-filterable radioactivity and particle size (see Tests) contribute to the identification of the preparation.

C. Transfer 1 mL of the preparation to be examined to a centrifuge tube and centrifuge at 2500 *g* for 5-10 min. Decant the supernatant. To the residue add 5 mL of *cupri-tartaric solution R2*, mix and allow to stand for 10 min. If necessary, heat to dissolve the particles and allow to cool. Add rapidly 0.5 mL of *dilute phosphomolybdotungstic reagent R*, mix immediately. A blue colour develops.

TESTS

**pH** (*2.2.3*): 4.0 to 9.0.

**Non-filterable radioactivity**: minimum 95 per cent of the total radioactivity.

Use a polycarbonate membrane filter 13-25 mm in diameter, 10 µm thick and with circular pores 3 µm in diameter. Fit the membrane into a suitable holder. Place 0.2 mL of the preparation to be examined on the membrane and filter, adding 20 mL of a 9 g/L solution of *sodium chloride R* during the filtration. Determine the radioactivity remaining on the membrane.

**Particle size**: maximum 10 particles have a maximum dimension greater than 75 µm but no particle have a maximum dimension greater than 100 µm.

Examine using a microscope. Dilute the preparation if necessary so that the number of particles is just low enough for individual particles to be distinguished. Using a syringe fitted with a needle having a calibre not less than 0.35 mm, place a suitable volume in a suitable counting chamber such as a haemocytometer cell, taking care not to overfill the chamber. Allow the suspension to settle for 1 min and carefully add a cover slide without squeezing the sample. Scan an area corresponding to at least 5000 particles. The particles have a uniform spherical appearance.

**Number of particles**. Examine using a microscope. Fill a suitable counting chamber such as a haemocytometer cell with a suitable dilution of the preparation taking care that particles do not separate during the transfer. Count the number of particles in the chamber. Repeat this procedure twice and calculate the number of particles per millilitre of the preparation to be examined.

**Tin**: maximum 3 mg/mL.

*Test solution*. To 1.0 mL of the preparation to be examined add 0.5 mL of *sulfuric acid R* and 1.5 mL of *nitric acid R*. Heat and evaporate to approximately 1 mL. Add 2 mL of *water R* and evaporate again to approximately 1 mL. Repeat this procedure twice, cool and dilute to 25.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution*. Dissolve 0.115 g of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

To 1.0 mL of each solution add 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R*, 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Physiological distribution**. Inject a volume not greater than 0.2 mL into a caudal vein of each of 3 rats weighing 150-250 g. Euthanise the rats 15 min after the injection, remove the liver, the spleen and the lungs and measure the radioactivity in these organs using a suitable instrument. Measure the radioactivity in the rest of the body, including the blood and voided urine, after having removed the tail. Determine the percentage of radioactivity in the liver, the spleen and the lungs, using the following expression:

$$\frac{A}{B} \times 100$$

*A* = radioactivity of the organ concerned;

*B* = total radioactivity in the liver, the spleen, the lungs and the rest of the body, including voided urine.

In not fewer than 2 of the 3 rats used, not less than 80 per cent of the radioactivity is found in the lungs and not more than a total of 5 per cent in the liver and spleen. The preparation may be released for use before completion of the test.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- the concentration of tin expressed in milligrams per millilitre, if any,
- that the preparation is to be shaken before use.

**04/2016:2376**

# TECHNETIUM ($^{99m}$Tc) OXIDRONATE INJECTION

## Technetii ($^{99m}$Tc) oxidronati solutio iniectabilis

DEFINITION

Sterile solution of a complex of technetium-99m with sodium hydroxymethylenediphosphonate (sodium oxidronate). It is prepared by dissolving sodium oxidronate in *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* in the presence of a reducing agent. It may contain stabilisers and inert additives.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear or opalescent, colourless or yellowish solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the tests for Impurity A and Impurity B under Radiochemical purity (see Tests).

*Results*: the principal peak in the chromatogram obtained with the test solution is, in each of the tests, similar in retardation factor to the principal peak in the chromatogram obtained with reference solution (b).

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a solution containing about 0.3 mg/mL of sodium oxidronate.

*Reference solution (a).* Dissolve 3 mg of *sodium oxidronate R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 1 mg of *medronic acid R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: to 30 mL of *water R* add 35 mL of *ethanol (96 per cent) R*, 15 mL of *2-methylpropanol R*, 0.4 mL of *concentrated ammonia R*, 5 g of *trichloroacetic acid R* and 20 mL of *2-propanol R* and mix.

*Application*: about 5 µL, applied in 1 µL portions with drying after each application.

*Development*: over 2/3 of the plate.

*Drying*: in air for at least 14 h.

*Detection*: spray with a solution prepared as follows: to 25 mL of a 40 g/L solution of *ammonium molybdate R* add 63 mL of *water R*, 2 mL of *hydrochloric acid R*, 2 mL of *sulfuric acid R* and 8 mL of *perchloric acid R* and mix. Expose the plate to ultraviolet light at 254 nm for 15 min.

*System suitability*:

- the retardation factor of the spot in the chromatogram obtained with reference solution (b) is about 0.6 and clearly different from that obtained with reference solution (a), which is about 0.5.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (a).

The preparation may be released for use before completion of the test.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**Impurity A**. Ascending paper chromatography (*2.2.26*).

*Test solution.* The preparation to be examined.

*Reference solution (a).* To 1 mL of a 1 g/L solution of *stannous chloride R* in a 5.15 g/L solution of *hydrochloric acid R* in a closed vial, add 2 mL of *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* containing 100-400 MBq. Use within 30 min.

*Reference solution (b).* To a vial of *oxidronate for radiochemical purity testing CRS* add 2 mL of *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)* containing 100-400 MBq. Allow to stand for 10 min.

*Paper*: *paper for chromatography R*.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: about 5 µL.

*Development*: immediately, over a path of 15 cm.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0-0.1; impurity B = 0.6-0.8; [$^{99m}$Tc]technetium oxidronate = 0.9-1.0.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution (a). Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283).*

*Reference solution (b).* Reference solution (b) of the test for impurity A.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: about 5 µL.

*Development*: immediately, over 4/5 of the plate.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A and [$^{99m}$Tc]technetium oxidronate = 0.0-0.1; impurity B = 0.9-1.0.

*Limit*:

– *[$^{99m}$Tc]technetium oxidronate*: minimum 95 per cent of the total radioactivity due to technetium-99m.

Calculate the percentage of radioactivity due to [$^{99m}$Tc]technetium oxidronate using the following expression:

$$100 - (A + B)$$

*A* = percentage of radioactivity due to impurity A determined in the test for impurity A under Radiochemical purity;

*B* = percentage of radioactivity due to impurity B determined in the test for impurity B under Radiochemical purity.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

*Specified impurities: A, B.*

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2008:0642**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) PENTETATE INJECTION

## Technetii ($^{99m}$Tc) pentetatis solutio iniectabilis

DEFINITION

Sterile solution of a complex of technetium-99m with sodium pentetate or calcium trisodium pentetate. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. It may contain suitable antimicrobial preservatives, antioxidants, stabilisers and buffers.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in tests A and B for radiochemical purity (see Tests).

*Results*:

– the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in test A is 0.9 to 1.0;

– the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in test B is 0.0 to 0.1.

C. *Test solution.* In a clean, dry, 10 mL glass tube, place a volume of the preparation to be examined containing 2 mg of pentetate. Dilute, if necessary, to 1 mL with *water R*.

*Reference solution.* In a clean, dry, 10 mL glass tube, place 1 mL of *water R*.

To each tube add 0.1 mL of a 1 g/L solution of *nickel sulfate R*, 0.5 mL of a 50 per cent *V/V* solution of *glacial acetic acid R* and 0.75 mL of a 50 g/L solution of *sodium hydroxide R*. Mix and check that the pH is not above 5. To each tube add 0.1 mL of a 10 g/L solution of *dimethylglyoxime R* in *ethanol (96 per cent) R*. Mix and allow to stand for 2 min. Adjust the pH in each tube to not less than 12 by adding a 100 g/L solution of *sodium hydroxide R*. Mix and check that the pH is not below 12. Allow to stand for 2 min. Heat the tubes gently on a water-bath for 2 min.

*Results*:

– the test solution remains clear and colourless throughout;

– the reference solution becomes red on addition of dimethylglyoxime solution and a red precipitate is formed when the tube is heated on the water-bath.

TESTS

**pH** (*2.2.3*): 4.0 to 7.5.

**Tin**: maximum 1 mg/mL.

*Test solution.* Dilute 1.5 mL of the preparation to be examined to 25.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution.* Dissolve 0.115 g of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

To 1.0 mL of each solution add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**A. Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet, previously heated at 110 °C for 10 min.

*Mobile phase*: 9 g/L solution of *sodium chloride R*.

*Application*: 5-10 µL.

*Development*: immediately, over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0 to 0.1; [$^{99m}$Tc]technetium pentetate and impurity B = 0.9 to 1.0.

**B. Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel as the coating substance on a glass-fibre sheet, previously heated at 110 °C for 10 min.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: 5-10 µL; allow to dry.

*Development*: over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium pentetate and impurity A = 0.0 to 0.1; impurity B = 0.9 to 1.0.

*Limit*:

– *sum of impurities A and B*: maximum 5.0 per cent of the radioactivity due to technetium-99m in the chromatograms obtained in tests A and B.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**01/2008:1926**
**corrected 9.2**

# TECHNETIUM ($^{99m}$Tc) SESTAMIBI INJECTION

## Technetii ($^{99m}$Tc) sestamibi solutio iniectabilis

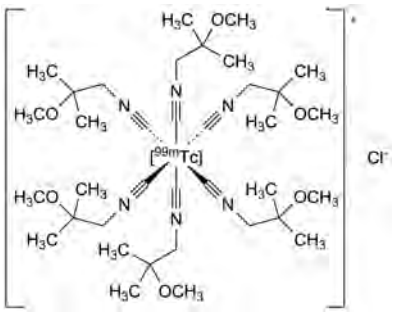

DEFINITION

Sterile solution of (*OC*-6-11)-hexakis[1-(isocyano-κ*C*)-2-methoxy-2-methylpropane][$^{99m}$Tc]technetium(I) chloride, which may be prepared by heating a mixture containing [tetrakis(2-methoxy-2-methylpropyl-1-isocyanide)copper (1+)] tetrafluoroborate, a weak chelating agent, a stannous salt and *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non-fission) (0283)*.

*Content*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and hour stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Results*: the spectrum obtained with the solution to be examined does not differ significantly from that of a standardised technetium-99m solution. The most prominent gamma photon has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the test for impurity C under Radiochemical purity.

*Results*: the principal peak in the radiochromatogram obtained with the test solution is similar in retention time to the principal peak in the radiochromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 5.0 to 6.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph on *Radiopharmaceutical preparations (0125)*. The injection may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Impurity A and other polar impurities**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: mix 10 volumes of *tetrahydrofuran R*, 20 volumes of a 38.5 g/L solution of *ammonium acetate R*, 30 volumes of *methanol R* and 40 volumes of *acetonitrile R*.

*Application*: about 5 µL.

*Development*: immediately over a path of 6 cm.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a radioactivity detector.

*Retardation factors*: impurity B and apolar impurities = 0 to 0.1; impurity C and technetium-99m sestamibi = 0.3 to 0.6; impurity A and other polar impurities = 0.9 to 1.0.

*Limit*: see test for impurity B.

**Impurity B**. Paper chromatography (*2.2.26*). *If no activity is found at retardation factor 0 to 0.1 in the test for impurity A and other polar impurities, impurity B is absent and the test for impurity B may be omitted.*

*Test solution*. The preparation to be examined.

*Paper*: *paper for chromatography R*.

*Mobile phase*: mix equal volumes of *acetonitrile R*, a 30 g/L solution of *glacial acetic acid R* and a 20 g/L solution of *sodium chloride R*.

*Application*: about 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: determine the distribution of radioactivity using a radioactivity detector.

*Retardation factors*: impurity B = 0 to 0.1; impurity A, impurity C and technetium-99m sestamibi = 0.8 to 1.0.

*Limit*:

– *sum of impurity A and other polar impurities, and impurity B*: maximum 5 per cent of the total radioactivity.

**Impurity C**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Reference solution*. To a vial of *sestamibi labelling kit CRS* add 3 mL of a 9 g/L solution of *sodium chloride R* containing 700 MBq to 900 MBq of sodium pertechnetate ($^{99m}$Tc) injection (fission or non-fission). Heat the mixture in a water-bath for 10 min and allow to cool to room temperature.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: spherical *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 35 volumes of a 6.6 g/L solution of *ammonium sulfate R* and 45 volumes of *methanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: radioactivity detector.

*Injection*: 25 µL.

*Run time*: 25 min.

*Relative retention* with reference to technetium-99m sestamibi: impurity C = about 1.3.

*System suitability*: reference solution:

- the chromatogram is similar to the chromatogram provided with *sestamibi labelling kit CRS*,
- *relative retention* with reference to technetium-99m sestamibi: impurity C = minimum 1.2.

*Limits*:

- *impurity C*: not more than 3 per cent of the total radioactivity,
- *technetium-99m sestamibi*: minimum 94 per cent of the total radioactivity.

Calculate the percentage of radioactivity due to technetium-99m sestamibi from the expression:

$$\frac{(100 - B) \times T}{100}$$

$B$ = percentage of radioactivity due to impurity B determined in the test for impurity B under Radiochemical purity,

$T$ = area of the peak due to technetium-99m sestamibi in the chromatogram obtained with the test solution.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. $[^{99m}Tc]O_4^-$: ($^{99m}$Tc)pertechnetate ion,

B. technetium-99m in colloidal form,

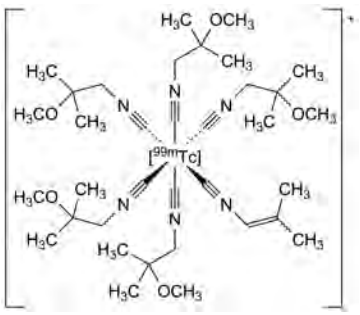

C. (*OC*-6-22)-pentakis[1-(isocyano-κ*C*)-2-methoxy-2-methylpropane][1-(isocyano-κ*C*)-2-methylprop-1-ene][$^{99m}$Tc]technetium (1+).

**01/2008:0643**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) SUCCIMER INJECTION

## Technetii ($^{99m}$Tc) succimeri solutio iniectabilis

DEFINITION

Sterile solution of a complex of technetium-99m with *meso*-2,3-dimercaptosuccinic acid. It is prepared using *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*. It contains a reducing substance, such as tin salt and may contain stabilisers, antioxidants such as ascorbic acid, and inert additives.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatogram obtained in the test for radiochemical purity (see Tests).

*Result*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is 0.0 to 0.1.

C. Place 1 mL of the preparation to be examined in a test-tube and add 0.1 mL of *glacial acetic acid R* and 1 mL of a 20 g/L solution of *sodium nitroprusside R*. Mix. Carefully place a layer of *concentrated ammonia R* at the top of the solution. A violet ring develops between the layers.

TESTS

**pH** (*2.2.3*): 2.3 to 3.5.

**Tin**: maximum 1 mg/mL.

*Test solution*. Dilute 1.5 mL of the preparation to be examined to 25.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Reference solution*. Dissolve 0.115 g of *stannous chloride R* in a 103 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same solution.

To 1.0 mL of each solution add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R* and 3.0 mL of a 21 g/L solution of *hydrochloric acid R*. Mix. Allow to stand for 60 min. Measure the absorbance (*2.2.25*) of each solution at 540 nm, using a 21 g/L solution of *hydrochloric acid R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Physiological distribution**. Inject a volume not greater than 0.2 mL and containing not more than 0.1 mg of dimercaptosuccinic acid into a suitable vein, such as a caudal vein or a saphenous vein, of each of 3 rats each weighing 150-250 g. Measure the radioactivity in the syringe before and after the injection. Euthanise the rats 1 h after the injection. Remove the kidneys, the liver, the stomach, the lungs and, if a caudal vein has been used for the injection, the tail. Using

a suitable instrument determine the radioactivity in these organs. Determine the percentage of radioactivity in each organ with respect to the total radioactivity calculated as the difference between the 2 measurements made on the syringe minus the activity in the tail (if a caudal vein has been used for the injection).

In not fewer than 2 of the 3 rats used, the radioactivity is:

- in the kidneys: minimum 40 per cent;
- in the liver: maximum 10.0 per cent;
- in the lungs: maximum 5.0 per cent;
- in the stomach: maximum 2.0 per cent.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIOCHEMICAL PURITY*

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel plate as the coating substance on a glass-fibre sheet, heated at 110 °C for 10 min.

*Mobile phase*: *methyl ethyl ketone R*.

*Application*: 5-10 µL.

*Development*: immediately, over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium succimer = 0.0 to 0.1; impurity A = 0.9 to 1.0.

*Limits*:

- *[$^{99m}$Tc]technetium succimer*: minimum 95.0 per cent of the total radioactivity due to technetium-99m;
- *impurity A*: maximum 2.0 per cent of the total radioactivity due to technetium-99m.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

STORAGE

Protected from light.

IMPURITIES

A. [$^{99m}$Tc]pertechnetate ion.

**01/2009:0129**
**corrected 7.0**

# TECHNETIUM ($^{99m}$Tc) TIN PYROPHOSPHATE INJECTION

## Stanni pyrophosphatis et technetii ($^{99m}$Tc) solutio iniectabilis

DEFINITION

Sterile solution which may be prepared by mixing solutions of sodium pyrophosphate and stannous chloride with *Sodium pertechnetate ($^{99m}$Tc) injection (fission) (0124)* or *Sodium pertechnetate ($^{99m}$Tc) injection (non fission) (0283)*.

*Technetium-99m*: 90 per cent to 110 per cent of the declared technetium-99m radioactivity at the date and time stated on the label.

*Sodium pyrophosphate* ($Na_4P_2O_7$,$10H_2O$): 1 mg/mL to 50 mg/mL.

*Tin*: maximum 3.0 mg/mL.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of technetium-99m*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.

*Result*: the most prominent gamma photon of technetium-99m has an energy of 0.141 MeV.

B. Examine the chromatograms obtained in the tests A and B for radiochemical purity (see Tests).

*Results*:

- the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in the test A is 0.9 to 1.0,
- the retardation factor of the principal peak in the radiochromatogram obtained with the test solution in the test B is 0.0 to 0.1.

C. To 1 mL add 1 mL of *acetic acid R*. Heat on a water-bath for 1 h. After cooling, add 10 mL of *nitro-molybdovanadic reagent R* and allow to stand for 30 min. A yellow colour develops.

D. To 1 mL add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R*, 1 mL of *hydrochloric acid R*, 2 mL of a 30 per cent *V/V* solution of *sulfuric acid R* and allow to stand for 30 min. A pink colour develops.

TESTS

**pH** (*2.2.3*): 6.0 to 7.0.

**Sodium pyrophosphate**: 1 mg/mL to 50 mg/mL.

*Test solution*. Use 1 mL of the preparation to be examined or a suitable dilution of it.

*Reference solutions*. Using a solution containing *sodium pyrophosphate R* and *stannous chloride R* in the same proportions as in the test solution, prepare a range of solutions and dilute to the same final volume with *water R*.

To the test solution and to 1 mL of each of the reference solutions add successively 10 mL of a 1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, 10 mL of *iron standard solution (8 ppm Fe) R*, 5 mL of *glacial acetic acid R* and 5 mL of a 1 g/L solution of *hydroxylamine hydrochloride R*. Dilute each solution to 40 mL with *water R* and heat in a water-bath at 40 °C for 1 h. To each solution, add 4 mL of a 1 g/L solution of *phenanthroline hydrochloride R* and dilute to 50.0 mL with *water R*. Measure the absorbance (*2.2.25*) of each solution at 515 nm using as the compensation liquid a reagent blank containing hydrochloric acid (1.1 g/L HCl) instead of the *iron standard solution (8 ppm Fe) R*. Using the absorbances obtained with each of the reference solutions, draw a calibration curve and calculate the concentration of sodium pyrophosphate in the preparation to be examined.

**Tin**: maximum 3.0 mg/mL.

*Test solution*. Use 1 mL of the preparation to be examined or a suitable dilution of it.

*Reference solutions*. Using a solution in hydrochloric acid (6.2 g/L HCl) containing *sodium pyrophosphate R* and *stannous chloride R* in the same proportions as in the test solution, prepare a range of solutions and dilute to the same final volume with hydrochloric acid (6.2 g/L HCl).

To the test solution and to 1 mL of each of the reference solutions add 0.05 mL of *thioglycollic acid R*, 0.1 mL of *dithiol reagent R*, 0.4 mL of a 20 g/L solution of *sodium laurilsulfate R*, 1 mL of *hydrochloric acid R* and 2 mL of a 300 g/L solution of *sulfuric acid R*, and dilute to 15 mL with hydrochloric acid (6.2 g/L HCl). Allow the solutions to stand for 30 min and measure the absorbance (*2.2.25*) of each solution at 530 nm, using as the compensation liquid a reagent blank containing

the same quantity of *sodium pyrophosphate R* as the test solution. Using the absorbances obtained with each of the reference solutions, draw a calibration curve and calculate the concentration of tin in the preparation to be examined.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres.

*RADIOCHEMICAL PURITY*

**A. Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel plate as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.

*Mobile phase*: 136 g/L solution of *sodium acetate R*.

*Application*: 5-10 µL.

*Development*: immediately, over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity A = 0.0 to 0.1; [$^{99m}$Tc]technetium tin pyrophosphate and impurity B = 0.9 to 1.0.

**B. Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution*. The preparation to be examined.

*Plate*: *TLC silica gel plate R*; use silica gel plate as the coating substance on a glass-fibre sheet heated at 110 °C for 10 min.

*Mobile phase*: *methyl ethyl ketone R* through which nitrogen has been bubbled in the chromatographic tank for 10 min immediately before the chromatography.

*Application*: 5-10 µL and dry in a steam of nitrogen.

*Development*: over a path of 10-15 cm in about 10 min.

*Drying*: in air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: [$^{99m}$Tc]technetium tin pyrophosphate = 0.0 to 0.1; impurity B = 0.95 to 1.0.

*Limit*:

– *sum of impurities A and B*: maximum 10 per cent of the total radioactivity due to technetium-99m in the chromatograms obtained in tests A and B.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

– the concentration of sodium pyrophosphate expressed in milligrams per millilitre;

– the concentration of tin expressed in milligrams per millilitre.

IMPURITIES

A. [$^{99m}$Tc]technetium in colloidal form,

B. [$^{99m}$Tc]pertechnetate ion.

**07/2017:2294**

# TETRA-*O*-ACETYL-MANNOSE TRIFLATE FOR RADIOPHARMACEUTICAL PREPARATIONS

## Tetra-*O*-acetylmannosi triflas ad radiopharmaceutica

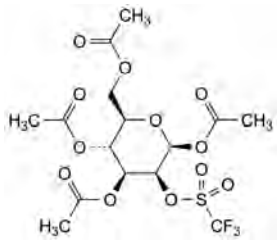

$C_{15}H_{19}F_3O_{12}S$ $M_r$ 480.4
[92051-23-5]

DEFINITION

1,3,4,6-Tetra-*O*-acetyl-2-*O*-trifluoromethanesulfonyl-β-D-mannopyranose.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, hygroscopic powder.

*Solubility*: practically insoluble in water, very soluble in acetonitrile, freely soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tetra-O-acetyl-mannose triflate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): – 12.0 to – 16.0 (dried substance), measured at 20 °C.

Dissolve 0.250 g in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

**Impurity B**. $^{19}$F Nuclear magnetic resonance spectrometry (*2.2.33*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 20.0 mg of the substance to be examined in *deuterated acetonitrile R* and dilute to 1.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20.0 mg of *tetra-O-acetyl-mannose triflate CRS* in *deuterated acetonitrile R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 4.0 mg of *lithium trifluoromethanesulfonate R* (lithium salt of impurity B) in *deuterated acetonitrile R* and dilute to 1.0 mL with the same solvent.

*Reference solution (c)*. Mix 1.0 mL of reference solution (a) and 10 µL of reference solution (b).

*Limit*: the peak area identified in the spectrum obtained with the test solution at about – 78 ppm is smaller than the peak area identified in the spectrum obtained with reference solution (c) at the same chemical shift (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 0.200 g of the substance to be examined in *acetonitrile R* and dilute to 2.0 mL with the same solvent.

*Test solution (b).* Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *tetra-O-acetyl-mannose triflate CRS* in *acetonitrile R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 100.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 10 mg of *1,3,4,6-tetra-O-acetyl-β-D-mannopyranose R* (impurity A) in 5 mL of *acetonitrile R*. Mix 1 mL of this solution and 1 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 80 | 20 |
| 1 - 20 | 80 → 55 | 20 → 45 |
| 20 - 35 | 55 | 45 |
| 35 - 45 | 55 → 0 | 45 → 100 |
| 45 - 50 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Relative retention* with reference to tetra-*O*-acetyl-mannose triflate (retention time = about 29 min): impurity A = about 0.2.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to impurity A and tetra-*O*-acetyl-mannose triflate.

*Limits*:

- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying**: maximum 0.6 per cent, determined on 25 mg by thermogravimetry (*2.2.34*). Heat to 80 °C at a rate of 2.5 °C/min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{15}H_{19}F_3O_{12}S$ from the declared content of *tetra-O-acetyl-mannose triflate CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities*: *A, B.*

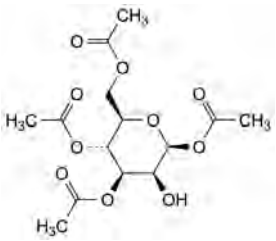

A. 1,3,4,6-tetra-*O*-acetyl-β-D-mannopyranose,

B. trifluoromethanesulfonic acid.

**01/2008:0571**
**corrected 7.0**

# THALLOUS ($^{201}$Tl) CHLORIDE INJECTION

## Thallosi ($^{201}$Tl) chloridi solutio iniectabilis

DEFINITION

Sterile solution of thallium-201 in the form of thallous chloride. It may be made isotonic by the addition of *Sodium chloride (0193)* and may contain a suitable antimicrobial preservative such as *Benzyl alcohol (0256)*.

*Thallium-201*: 90 per cent to 110 per cent of the declared thallium-201 radioactivity, at the date and time stated on the label.

*Specific radioactivity*: minimum 3.7 GBq per milligram of thallium.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of thallium-201*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray and X-ray spectrometry.

*Results*: the most prominent gamma photons of thallium-201 have energies of 0.135 MeV, 0.166 MeV and 0.167 MeV; the X-rays have energies of 0.069 MeV to 0.083 MeV.

B. Examine the electropherogram obtained in the test for radiochemical purity (see Tests). The distribution of radioactivity contributes to the identification of the preparation.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

**Thallium**.

*Test solution.* To 0.5 mL of the preparation to be examined add 0.5 mL of hydrochloric acid (220 g/L HCl) and 0.05 mL of *bromine water R*, and mix. Add 0.1 mL of a 30 g/L solution of *sulfosalicylic acid R*. After decolorisation add 1.0 mL of a 1 g/L solution of *rhodamine B R*. Add 4 mL of *toluene R* and shake for 60 s. Separate the toluene layer.

*Reference solution*. Prepare at the same time and in the same manner as the test solution, using 0.5 mL of *thallium standard solution (10 ppm Tl) R*.

The toluene layer of the test solution is not more intensely coloured than the toluene layer of the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

**Thallium-201**: minimum 97.0 per cent of the total radioactivity.

Gamma-ray and X-ray spectrometry.

Determine the relative amounts of thallium-200, thallium-201, thallium-202, lead-201, lead-203 and other radionuclidic impurities present.

*Result*: the total radioactivity due to thallium-202 is not more than 2.0 per cent.

*RADIOCHEMICAL PURITY*

**[$^{201}$Tl]Thallous ions**. Zone electrophoresis (*2.2.31*).

Use a suitable cellulose acetate strip as the supporting medium and a 18.6 g/L solution of *sodium edetate R* as the electrolyte solution. Soak the strip in the electrolyte solution for 45-60 min. Remove the strip with forceps taking care to handle the outer edges only. Place the strip between 2 absorbent pads and blot to remove excess solution.

*Test solution*. Mix equal volumes of the preparation to be examined and the electrolyte solution.

Apply not less than 5 µL of the test solution to the centre of the strip and mark the point of application. Apply an electric field of 17 V/cm for at least 10 min. Allow the strip to dry in air. Determine the distribution of radioactivity using suitable detector.

*Result*: minimum 95.0 per cent of the radioactivity migrates towards the cathode.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

IMPURITIES

A. lead-201,

B. lead-203,

C. thallium-200,

D. thallium-202,

E. [$^{201}$Tl]thallic(III) ion,

**01/2008:0112**
**corrected 7.0**

# TRITIATED ($^3$H) WATER INJECTION

## Aquae tritiatae ($^3$H) solutio iniectabilis

DEFINITION

Water for injections in which some of the water molecules contain tritium atoms in place of protium atoms. It may be made isotonic by the addition of sodium chloride.

*Tritium*: 90 per cent to 110 per cent of the declared tritium radioactivity at the date stated on the label.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of tritium*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

Beta-ray spectrometry as described in test A for radionuclidic purity (see Tests).

*Result*: the maximum energy of the beta radiation is 0.019 MeV.

TESTS

**pH** (*2.2.3*): 4.5 to 7.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*.

*RADIONUCLIDIC PURITY*

A. *Test solution*. Mix 100 µL of a suitable dilution of the preparation to be examined with 10 mL of *liquid scintillation cocktail R1*.

*Reference solution*. A standardised tritiated ($^3$H) water having approximately the same radioactivity as the test solution.

Measure the radioactivity of the test solution in a liquid scintillation counter fitted with a discriminator. The count should be about 5000 impulses per second at the lowest setting of the discriminator. Record the count at different discriminator settings. For each measurement, count at least 10 000 impulses over a period of at least 1 min. Immediately determine in the same conditions the count for the reference solution.

Plot the counts at each discriminator setting, correcting for background activity, on semi-logarithmic paper, the discriminator settings being in arbitrary units as the abscissae. The vertical distance between the 2 curves obtained is constant. They obey the following mathematical relationship:

$$\frac{\frac{A_1}{B_1} - \frac{A_2}{B_2}}{\frac{A_1}{B_1}} \times 100 < 20$$

$A_1$ = radioactivity recorded for the reference solution at the lowest discriminator setting;

$B_1$ = radioactivity recorded for the test solution at the lowest discriminator setting;

$A_2$ = radioactivity recorded for the reference solution at the discriminator setting such that $A_2 \approx A_1 \times 10^{-3}$;

$B_2$ = radioactivity recorded for the test solution at the latter discriminator setting.

B. Gamma-ray spectrometry. The instrument registers only background activity.

*RADIOCHEMICAL PURITY*

Place a quantity of the preparation to be examined equivalent to about 74 kBq, diluted to 50 mL with *water R*, in an all-glass distillation apparatus of the type used for the determination of distillation range (*2.2.11*). Determine the radioactive concentration. Distil until about 25 mL of distillate has been collected. Precautions must be taken to avoid contamination of the air. If the test is carried out in a fume cupboard, the equipment must be protected from draughts. Determine the radioactive concentration of the distillate and of the liquid remaining in the distillation flask. Neither of the radioactive concentrations determined after distillation differs by more than 5 per cent from the value determined before distillation.

RADIOACTIVITY

Determine the radioactivity using a liquid scintillation counter.

**01/2008:1582**
**corrected 7.0**

# WATER ($^{15}$O) INJECTION

## Aquae ($^{15}$O) solutio iniectabilis

DEFINITION

Sterile solution of [$^{15}$O]water for diagnostic use.

*Oxygen-15*: 90 per cent to 110 per cent of the declared oxygen-15 radioactivity at the date and time stated on the label.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Half-life and nature of radiation of oxygen-15*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Gamma-ray spectrometry.
*Results*: the only gamma photons of [$^{15}$O]water have an energy of 0.511 MeV and, depending on the measurement geometry, a sum peak of 1.022 MeV may be observed.

B. Radionuclidic purity (see Tests).

C. Examine the chromatogram obtained in the test for radiochemical purity (see Tests). The retention time of the 2nd peak is due to the radioactivity eluting in the void volume.

TESTS

**pH** (*2.2.3*): 5.5 to 8.5.

**Ammonium** (*2.4.1*): maximum 10 ppm, determined on 1 mL. The preparation may be released for use before completion of the test.

**Nitrates**: maximum 10 ppm. The preparation may be released for use before completion of the test.

*Test solution*. To 1 mL add 49 mL of *nitrate-free water R*. Place 5 mL of this solution in a test-tube immersed in iced water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *sulfuric acid R*. Transfer the tube to a water-bath at 50 °C.

*Reference solution*. Prepare at the same time in the same manner as the test solution, using a mixture of 4.5 mL of *nitrate-free water R* and 0.5 mL of *nitrate standard solution (2 ppm $NO_3$) R*.

After 15 min, any blue colour in the test solution is not more intense than that in the reference solution.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 175/*V* IU/mL, *V* being the maximum recommended dose in millilitres. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

The preparation may be released for use before completion of the test.

**Oxygen-15**: minimum 99 per cent of total radioactivity.

Gamma-ray spectrometry.

*Results*:

- the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised fluorine-18 solution;
- the half-life is between 1.9 min and 2.2 min.

*RADIOCHEMICAL PURITY*

The preparation may be released for use before completion of the test.

**[$^{15}$O]Water**. Liquid chromatography (*2.2.29*).

*Test solution*. The preparation to be examined.

*Column*:

- *size*: l = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (10 µm);
- *temperature*: constant, at 20-30 °C.

*Mobile phase*: 10 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: suitable detector to determine the distribution of radioactivity and internal recovery detection system, consisting of a loop of the chromatographic tubing between the injector and the column through the radioactivity detector, which has been calibrated for count recovery.

*Run time*: 10 min.

*Identification of peaks*: in the chromatogram obtained with the test solution, the 1st peak corresponds to the injected radioactivity of the test solution, the 2nd peak corresponds to the amount of radioactivity as [$^{15}$O]water.

*Limit*:

- *[$^{15}$O]water*: minimum 99 per cent of the total radioactivity due to oxygen-15.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

**01/2008:0133**
**corrected 7.0**

# XENON ($^{133}$Xe) INJECTION

## Xenoni ($^{133}$Xe) solutio iniectabilis

DEFINITION

Sterile solution of xenon-133 that may be made isotonic by the addition of sodium chloride.

*Xenon-133*: 80 per cent to 130 per cent of the declared xenon-133 radioactivity at the date and time stated on the label.

The injection is presented in a container that allows the contents to be removed without introducing air bubbles. The container is filled as completely as possible and any gas bubble present does not occupy more than 1 per cent of the volume of the injection as judged by visual comparison with a suitable standard.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of xenon-133*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

Gamma-ray and X-ray spectrometry.

*Comparison*: standardised xenon-133 solution in a 9 g/L solution of *sodium chloride R*.

*Results*: the most prominent gamma photon of xenon-133 has an energy of 0.081 MeV and there is an X-ray (resulting from internal conversion) of 0.030 MeV to 0.035 MeV.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

*RADIONUCLIDIC PURITY*

A. Gamma-ray and X-ray spectrometry.

*Comparison*: standardised xenon-133 solution in a 9 g/L solution of *sodium chloride R*.

*Result*: the spectrum obtained with the preparation to be examined does not differ significantly from that obtained with a standardised xenon-133 solution in a 9 g/L solution of *sodium chloride R*, apart from any differences attributable to the presence of xenon-131m and xenon-133m.

B. Transfer 2 mL of the preparation to be examined to an open flask and pass a current of air through the solution for 30 min, taking suitable precautions concerning the dispersion of radioactivity. Measure the residual beta and gamma activity of the solution. The activity does not differ significantly from the background activity detected by the instrument.

RADIOACTIVITY

Weigh the container with its contents. Determine its total radioactivity using suitable counting equipment by comparison with a standardised xenon-133 solution or by measurement in an instrument calibrated with the aid of such a solution, operating in strictly identical conditions. If an ionisation chamber is used its inner wall should be such that the radiation is not seriously attenuated. Remove at least half the contents and re-weigh the container. Measure the total residual radioactivity of the container and the remaining contents as described above. From the measurements, calculate the radioactive concentration of xenon-133 in the preparation to be examined.

*CAUTION*

*Significant amounts of xenon-133 may be present in the closures and on the walls of the container. This must be taken into account in applying the rules concerning the transport and storage of radioactive substances and in disposing of used containers*

IMPURITIES

A. xenon-131m.

**04/2019:2803**

# YTTRIUM ($^{90}$Y) CHLORIDE SOLUTION FOR RADIOLABELLING

## Yttrii ($^{90}$Y) chloridi solutio ad radio-signandum

$^{90}YCl_3$ $M_r$ 196.3

[39271-65-3]

DEFINITION

Sterile solution containing yttrium-90 derived from fission-produced strontium-90, in the form of yttrium chloride in dilute hydrochloric acid.

*Content*:

– *yttrium-90*: 90 per cent to 110 per cent of the declared yttrium-90 radioactivity at the date and time stated on the label.

PRODUCTION

**Alpha-emitting impurities**: maximum $1 \times 10^{-5}$ per cent of the total radioactivity, determined on the parent radionuclide strontium-90 by a suitable, validated method.

CHARACTERS

*Appearance*: clear, colourless solution.

*Half-life and nature of radiation of yttrium-90*: see general chapter *5.7. Table of physical characteristics of radionuclides*.

IDENTIFICATION

A. Beta-particle spectrometry.

*Result*: the beta-particle spectrum obtained with the preparation to be examined is qualitatively similar to the beta-particle spectrum obtained with a certified yttrium-90 reference material.

B. pH (see Tests).

C. To 100 µL of *silver nitrate solution R2* add 50 µL of the preparation to be examined. A white precipitate is formed.

D. Examine the chromatograms obtained in the test for radiochemical purity.

*Result*: the retardation factor of the principal peak in the chromatogram obtained with the test solution is 0.4 to 0.7.

TESTS

**pH** (*2.2.4*): 1 to 2.

**Cadmium** (*2.4.20*): maximum 0.6 µg/GBq.

**Copper** (*2.4.20*): maximum 0.6 µg/GBq.

**Iron** (*2.4.20*): maximum 6.0 µg/GBq.

**Lead** (*2.4.20*): maximum 3.0 µg/GBq.

**Zinc** (*2.4.20*): maximum 3.0 µg/GBq.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Radiopharmaceutical preparations (0125)*. The preparation may be released for use before completion of the test.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU/mL, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

*RADIONUCLIDIC PURITY*

*The preparation may be released for use before completion of test B for yttrium-90.*

**Strontium-90**. Beta-particle spectrometry (*2.2.66*).

*Limit*: maximum 0.002 per cent of the total yttrium-90 radioactivity.

Determine the relative amount of strontium-90 after its separation from the examined sample of the preparation to be examined.

Soak 100 mg of *extraction resin R* (50-100 µm) in 1 mL of a 94.7 g/L solution of *nitric acid R* for at least 24 h. Stir the resin suspension for 10 min using a magnetic stirrer at about 800 r/min. Into a polypropylene column with a suitable bed support at the bottom, introduce 1 mL of the resin suspension. Cover the resin in the column with another bed support. Keep the resin bed covered with a 94.7 g/L solution of *nitric acid R* and free from air bubbles.

*Test solution.* Add a volume of the preparation to be examined corresponding to 100-200 MBq to 4 mL of a 100 mg/mL solution of *strontium chloride hexahydrate R* in a 474 g/L solution of *nitric acid R*. Measure the radioactivity of this solution using a calibrated instrument. Add 50 µL of *strontium-85 spiking solution R* and mix.

*Reference solution.* Mix 50 µL of *strontium-85 spiking solution R* and 2.5 mL of a 9.5 g/L solution of *nitric acid R* in a vial for liquid scintillation counting and add 10 mL of *liquid scintillation cocktail R*.

Condition the extraction resin column with 2 mL of a 474 g/L solution of *nitric acid R*. Apply the test solution to the top of the column. Collect the eluate in a tared vial. Rinse the column with 4 mL of a 474 g/L solution of *nitric acid R* and collect the eluate in the tared vial. Perform the elutions of the column at a flow rate not exceeding 1 mL/min (0.6 mL/min is recommended). Combine the eluates and weigh the resulting mixture.

Transfer 1 mL of the eluate to a vial for liquid scintillation counting and determine the mass of the sample, then add 1.5 mL of *water R* and 10 mL of *liquid scintillation cocktail R* and mix (eluate mixture).

Within 20 min following collection of the eluate determine the total radioactivity due to strontium-90, yttrium-90 and strontium-85 in the eluate mixture and in the reference solution by liquid scintillation spectrometry. The recommended counting time is 900 s.

Calculate the recovery of strontium-85 in the eluate mixture.

Calculate the total radioactivity due to strontium-90 and yttrium-90 in the eluate, taking into account the recovery of strontium and the fraction of eluate in the eluate mixture.

*System suitability*:

- the radioactivity due to yttrium-90 in the eluate does not exceed 50 per cent of the radioactivity due to strontium-90.

**Yttrium-90**: minimum 99.9 per cent of the total radioactivity.

A. Gamma-ray-emitting impurities. Gamma-ray spectrometry. Preliminary test.

   Place a volume of the preparation to be examined corresponding to about 200 MBq in a glass vial and measure the radioactivity in an ionisation chamber.

   Place the vial in a lead container with 10 mm-thick walls. Place the lead container in a gamma-spectrometer chamber and perform the measurement with the acquisition time set to 3600 s.

   If any impurities are detected, calculate their radioactivities corrected to correspond to the time that the radioactivity of the sample of the preparation to be examined was measured. Calculate the relative impurity content in the sample of the preparation to be examined.

B. Gamma-ray-emitting impurities. Gamma-ray spectrometry.

   Determine the relative amounts of gamma-ray-emitting impurities present. For the detection and quantification of gamma-ray-emitting impurities, retain the preparation to be examined for a sufficient time to allow yttrium-90 to decay to a level that permits the detection of radionuclidic impurities.

   *Result*: the total radioactivity due to radionuclidic impurities is not greater than 0.1 per cent.

*RADIOCHEMICAL PURITY*

**Yttrium in the form of $Y^{3+}$ ion**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The preparation to be examined.

*Reference solution*. To 1.0 mL of the test solution add 0.4 mL of a 10 g/L solution of *pentetic acid R* in a 4 g/L solution of *sodium hydroxide R*.

*Plate*: *TLC silica gel plate R*; use a glass-fibre plate.

*Mobile phase*: 9.0 g/L solution of *sodium chloride R* adjusted to pH 2.3 ± 0.05 with *dilute hydrochloric acid R*.

*Application*: 5 µL.

*Development*: immediately, over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: suitable detector to determine the distribution of radioactivity.

*Retardation factors*: impurity B = less than 0.2; yttrium-90 in the form of $Y^{3+}$ ion = 0.4 to 0.7; yttrium-90 in the form of yttrium pentetate = greater than 0.9.

*System suitability*: the retardation factor of the principal peak in the radiochromatogram obtained with the test solution is clearly different from that of the principal peak in the chromatogram obtained with the reference solution.

*Limit*:

- *yttrium in the form of $Y^{3+}$ ion*: minimum 99 per cent of the total radioactivity due to yttrium-90.

RADIOACTIVITY

Determine the radioactivity using a calibrated instrument.

LABELLING

The label states:

- that the solution is not for direct administration to humans;
- the concentration of hydrochloric acid;
- the content of cadmium, copper, iron, lead, zinc and strontium-90;
- that it is the user's obligation to verify that the content of metal impurities and strontium-90 is sufficiently low for the intended application;
- that the solution is intended for use in the preparation of yttrium-90-labelled radiopharmaceuticals.

IMPURITIES

A. strontium-90,

B. yttrium-90 in colloidal form.

# Sutures for human use

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Catgut, sterile.. ..... 1269
Sutures, sterile non-absorbable.. ..... 1270
Sutures, sterile synthetic absorbable braided .. ..... 1274
Sutures, sterile synthetic absorbable monofilament .. ..... 1275

**01/2008:90004 corrected 10.0**

# SUTURES FOR HUMAN USE: INTRODUCTION

*The following monographs apply to sutures for human use: Catgut, sterile (0317), Sutures, sterile non-absorbable (0324), Sutures, sterile synthetic absorbable braided (0667) and Sutures, sterile synthetic absorbable monofilament (0666). They cover performance characteristics of sutures and may include methods of identification. Sutures are medical devices as defined in Directive 93/42/EEC.*

*These monographs can be applied to show compliance with essential requirements as defined in Article 3 of Directive 93/42/EEC covering the following:*

*Physical performance characteristics: diameter, breaking load, needle attachment, packaging, sterility, information supplied by the manufacturer (see Section 13 of Annex 1 of Directive 93/42/EEC), labelling.*

*To show compliance with other essential requirements, the application of appropriate harmonised standards as defined in Article 5 of Directive 93/42/EEC may be considered.*

**01/2008:0317**

# CATGUT, STERILE

## Chorda resorbilis sterilis

DEFINITION

Sterile catgut consists of sutures prepared from collagen taken from the intestinal membranes of mammals. After cleaning, the membranes are split longitudinally into strips of varying width, which, when assembled in small numbers, according to the diameter required, are twisted under tension, dried, polished, selected and sterilised. The sutures may be treated with chemical substances such as chromium salts to prolong absorption and glycerol to make them supple, provided such substances do not reduce tissue acceptability.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile catgut is a surgical wound-closure device. Being an absorbable suture it serves to approximate tissue during the healing period and is subsequently metabolised by proteolytic activity.

PRODUCTION

Production complies with relevant regulations on the use of animal tissues in medical devices notably concerning the risk of transmission of animal spongiform encephalopathy agents.

Appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of catgut that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements outlined below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of sterile catgut are suitable for wound closure according to usual surgical techniques.

TESTS

*If stored in a preserving liquid, remove the sutures from the sachet and measure promptly and in succession the length, diameter and breaking load. If stored in the dry state, immerse the sutures in alcohol R or a 90 per cent V/V solution of 2-propanol R for 24 h and proceed with the measurements as indicated below.*

**Length**. Measure the length without applying to the suture more tension than is necessary to keep it straight. The length of each suture is not less than 90 per cent of the length stated on the label and does not exceed 350 cm.

**Diameter**. Carry out the test on 5 sutures. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurement, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at 3 points approximately evenly spaced along the suture. The suture is not subjected to more tension than is necessary to keep it straight during measurement. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0317.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0317.-1 for the gauge number concerned.

Table 0317.-1. – *Diameters and Breaking Loads*

| Gauge number | Diameter (millimetres) | | | | Breaking load (newtons) | |
|---|---|---|---|---|---|---|
| | A | | B | | C | D |
| | min. | max. | min. | max. | | |
| 0.1 | 0.010 | 0.019 | 0.005 | 0.025 | - | - |
| 0.2 | 0.020 | 0.029 | 0.015 | 0.035 | - | - |
| 0.3 | 0.030 | 0.039 | 0.025 | 0.045 | 0.20 | 0.05 |
| 0.4 | 0.040 | 0.049 | 0.035 | 0.060 | 0.30 | 0.10 |
| 0.5 | 0.050 | 0.069 | 0.045 | 0.085 | 0.40 | 0.20 |
| 0.7 | 0.070 | 0.099 | 0.060 | 0.125 | 0.70 | 0.30 |
| 1 | 0.100 | 0.149 | 0.085 | 0.175 | 1.8 | 0.40 |
| 1.5 | 0.150 | 0.199 | 0.125 | 0.225 | 3.8 | 0.70 |
| 2 | 0.200 | 0.249 | 0.175 | 0.275 | 7.5 | 1.8 |
| 2.5 | 0.250 | 0.299 | 0.225 | 0.325 | 10 | 3.8 |
| 3 | 0.300 | 0.349 | 0.275 | 0.375 | 12.5 | 7.5 |
| 3.5 | 0.350 | 0.399 | 0.325 | 0.450 | 20 | 10 |
| 4 | 0.400 | 0.499 | 0.375 | 0.550 | 27.5 | 12.5 |
| 5 | 0.500 | 0.599 | 0.450 | 0.650 | 38.0 | 20.0 |
| 6 | 0.600 | 0.699 | 0.550 | 0.750 | 45.0 | 27.5 |
| 7 | 0.700 | 0.799 | 0.650 | 0.850 | 60.0 | 38.0 |
| 8 | 0.800 | 0.899 | 0.750 | 0.950 | 70.0 | 45.0 |

**Minimum breaking load**. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0317.-1) and pulling the knot tight. Carry out the test on 5 sutures. Submit sutures of length greater than 75 cm to 2 measurements and shorter sutures to one measurement. Determine the breaking load using a suitable tensilometer. The apparatus has 2 clamps for holding the suture, one of which is mobile and is driven at a constant rate of 30 cm/min. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value given in column C in Table 0317.-1 and no individual result is less than that given in column D for the gauge number concerned.

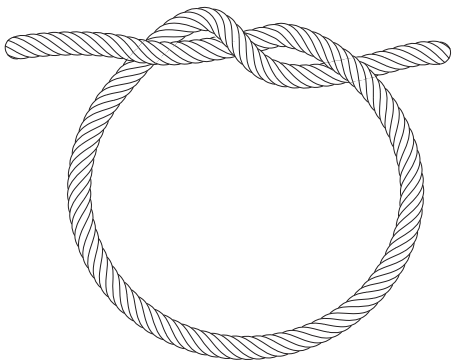

Figure 0317.-1. – *Simple knot*

**Soluble chromium compounds**. Place 0.25 g in a conical flask containing 1 mL of *water R* per 10 mg of catgut. Stopper the flask, allow to stand at 37 ± 0.5 °C for 24 h, cool and decant the liquid. Transfer 5 mL to a small test tube and add 2 mL of a 10 g/L solution of *diphenylcarbazide R* in *alcohol R* and 2 mL of *dilute sulfuric acid R*. The solution is not more intensely coloured than a standard prepared at the same time using 5 mL of a solution containing 2.83 µg of *potassium dichromate R* per millilitre, 2 mL of *dilute sulfuric acid R* and 2 mL of a 10 g/L solution of *diphenylcarbazide R* in *alcohol R* (1 ppm of Cr).

**Needle attachment**. If the catgut is supplied with an eyeless needle attached that is not stated to be detachable, it complies with the test for needle attachment. Carry out the test on 5 sutures. Use a suitable tensilometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the 5 determinations and all individual values are not less than the respective values given in Table 0317.-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional 10 sutures. The catgut complies with the test if none of these 10 values is less than the individual value in Table 0317.-2 for the gauge number concerned.

Table 0317.-2. – *Minimum Strengths of Needle Attachment*

| Gauge number | Mean value (newtons) | Individual values (newtons) |
|---|---|---|
| 0.5 | 0.50 | 0.25 |
| 0.7 | 0.80 | 0.40 |
| 1 | 1.7 | 0.80 |
| 1.5 | 2.3 | 1.1 |
| 2 | 4.5 | 2.3 |
| 2.5 | 5.6 | 2.8 |
| 3 | 6.8 | 3.4 |
| 3.5 | 11.0 | 4.5 |
| 4 | 15.0 | 4.5 |
| 5 | 18.0 | 6.0 |

STORAGE (PACKAGING)

Sterile catgut sutures are presented in individual sachets that maintain sterility and allow the withdrawal and use of the sutures in aseptic conditions. Sterile catgut may be stored dry or in a preserving liquid to which an antimicrobial agent but not an antibiotic may be added.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices shall be considered.

LABELLING

Reference may be made to the appropriate harmonised standards for labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical suture, absorbable).

**07/2018:0324**
**corrected 10.0**

# SUTURES, STERILE NON-ABSORBABLE

## Fila non resorbilia sterilia

DEFINITION

Sterile non-absorbable sutures are sutures that, when introduced into a living organism, are not metabolised by that organism. Sterile non-absorbable sutures vary in origin, which may be animal, vegetable, metallic or synthetic. They occur as cylindrical monofilaments or as multifilament sutures consisting of elementary fibres that are assembled by twisting, cabling or braiding; they may be sheathed; they may be treated to render them non-capillary; they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile non-absorbable surgical sutures serve to approximate tissue during the healing period and provide continuing wound support.

The following materials are frequently used, as well as blends thereof, as blends of synthetic materials are common.

**Silk (Filum bombycis)**

Sterile braided silk suture is obtained by braiding a number of threads, according to the diameter required, of degummed silk obtained from the cocoons of the silkworm *Bombyx mori* L.

**Linen (Filum lini)**

Sterile linen thread consists of the pericyclic fibres of the stem of *Linum usitatissimum* L. The elementary fibres, 2.5-5 cm long, are assembled in bundles 30-80 cm long and spun into continuous lengths of suitable diameter.

**Poly(ethylene terephthalate) (Filum ethyleni polyterephthalici)**

Sterile poly(ethylene terephthalate) suture is obtained by drawing poly(ethylene terephthalate) through a suitable die. The suture is prepared by braiding very fine filaments in suitable numbers, depending on the gauge required.

**Polyamide 6 (Filum polyamidicum-6)**

Sterile polyamide 6 suture is obtained by drawing through a suitable die a synthetic plastic material formed by the polymerisation of ε-caprolactam. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted sutures sheathed with the same material.

**Polyamide 6/6 (Filum polyamidicum-6/6)**

Sterile polyamide 6/6 suture is obtained by drawing through a suitable die a synthetic plastic material formed by the polycondensation of hexamethylenediamine and adipic acid. It consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted sutures sheathed with the same material.

**Polypropylene (Filum polypropylenicum)**

Polypropylene suture is obtained by drawing polypropylene through a suitable die. It consists of smooth cylindrical mono-filaments.

**Monofilament and multifilament stainless steel (Filum aciei irrubiginibilis monofilamentum/multifilamentum)**

Sterile stainless steel sutures have a chemical composition as specified in ISO 5832-1 - Metallic Materials for surgical implants - Part 1: Specification for wrought stainless steel, and comply with ISO 10334 - Implants for surgery - Malleable wires for use as sutures and other surgical applications.

Stainless steel sutures consist of smooth, cylindrical monofilaments or twisted filaments or braided filaments.

**Poly(vinylidene difluoride) (PVDF) (Filum poly(vinylideni difluoridum))**

Sterile PVDF suture is obtained by drawing through a suitable die a synthetic plastic material formed by polymerisation of 1,1-difluoroethylene. It consists of smooth, cylindrical monofilaments.

IDENTIFICATION

Synthetic and natural materials may be identified by infrared spectrophotometry (*2.2.24*) using attenuated total reflection (ATR) or by differential scanning calorimetry. Additives and coatings of materials may lead to additional peaks. Natural materials may also be identified by microscopic examination of the morphology of the fibres.

**Identification of silk**

A. Dissect the end of a suture, using a needle or fine tweezers, to isolate a few individual fibres. The fibres are sometimes marked with very fine longitudinal striations parallel to the axis of the suture. Examined under a microscope, a cross-section is more or less triangular to semi-circular, with rounded edges and without a lumen.

B. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3280 ± 5 $cm^{-1}$ (strong); 2923 ± 15 $cm^{-1}$ (medium); 1622 ± 15 $cm^{-1}$ (strong); 1512 ± 5 $cm^{-1}$ (strong); 1444 ± 7 $cm^{-1}$ (medium); 1226 ± 10 $cm^{-1}$ (medium).

**Identification of linen**

A. Dissect the end of a suture, using a needle or fine tweezers, to isolate a few individual fibres. Examined under a microscope, the fibres are seen to be 12-31 µm wide and, along the greater part of their length, have thick walls, sometimes marked with fine longitudinal striations, and a narrow lumen. The fibres gradually narrow to a long, fine point. Sometimes there are unilateral swellings with transverse lines.

B. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3326 ± 10 $cm^{-1}$ (medium); 2911 ± 12 $cm^{-1}$ (medium); 1645 ± 10 $cm^{-1}$ (weak); 1426 ± 5 $cm^{-1}$ (medium); 1315 ± 3 $cm^{-1}$ (medium); 1154 ± 7 $cm^{-1}$ (medium); 1104 ± 5 $cm^{-1}$ (medium); 1050 ± 5 $cm^{-1}$ (strong); 1026 ± 7 $cm^{-1}$ (strong).

**Identification of poly(ethylene terephthalate)**

It is attacked by strongly alkaline solutions. It is incompatible with phenols.

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 1712.5 ± 5 $cm^{-1}$ (strong); 1408 ± 5 $cm^{-1}$ (medium); 1338 ± 5 $cm^{-1}$ (medium); 1243 ± 12 $cm^{-1}$ (strong); 1093 ± 5 $cm^{-1}$ (strong); 1017 ± 5 $cm^{-1}$ (medium); 872 ± 5 $cm^{-1}$ (medium); 722 ± 2 $cm^{-1}$ (strong).

**Identification of polyamide 6, polyamide 6/6 and blends thereof**

They are not attacked by dilute alkaline solutions (for example a 100 g/L solution of *sodium hydroxide R*) but are attacked by dilute mineral acids (for example a 20 g/L solution of *sulfuric acid R*) and by hot *glacial acetic acid R*.

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3296 ± 10 $cm^{-1}$ (medium); 2930 ± 15 $cm^{-1}$ (medium); 2862 ± 15 $cm^{-1}$ (medium); 1635 ± 5 $cm^{-1}$ (strong); 1539 ± 12 $cm^{-1}$ (strong); 1462 ± 10 $cm^{-1}$ (medium); 1262 ± 12 $cm^{-1}$ (medium).

**Identification of polypropylene**

Polypropylene is soluble in decahydronaphthalene, in 1-chloronaphthalene and in trichloroethylene. It is insoluble in ethanol (96 per cent) and in cyclohexanone.

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 2950 ± 5 $cm^{-1}$ (strong); 2916 ± 5 $cm^{-1}$ (strong); 2870 ± 5 $cm^{-1}$ (medium); 2838 ± 5 $cm^{-1}$ (medium); 1456 ± 5 $cm^{-1}$ (medium); 1376 ± 5 $cm^{-1}$ (medium).

**Identification of stainless steel**

Stainless steel sutures are identified by confirming that the composition is in accordance with ISO 5832 Part 1.

**Identification of poly(vinylidene difluoride)**

It is soluble in warm dimethylformamide. It is insoluble in anhydrous ethanol, in hot and cold isopropyl alcohol, in ethyl acetate and in tetrachloroethylene.

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 1399 ± 5 $cm^{-1}$ (medium); 1275 ± 2 $cm^{-1}$ (medium); 1165 ± 10 $cm^{-1}$ (strong); 1070 ± 5 $cm^{-1}$ (medium); 873 ± 3 $cm^{-1}$ (strong); 838 ± 2 $cm^{-1}$ (strong).

PRODUCTION

The appropriate harmonised standards apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure in accordance with usual surgical techniques.

TESTS

*Remove the sutures from the sachet and measure promptly and in succession the length, diameter and minimum load.*

If linen is tested the sutures are conditioned as follows: if stored in the dry state, expose to an atmosphere with a relative humidity of 65 ± 5 per cent at 20 ± 2 °C for 4 h immediately before measuring the diameter and for the determination of minimum breaking load, immerse in *water R* at room temperature for 30 min immediately before carrying out the test.

**Length**. Measure the length without applying more tension than is necessary to keep them straight. The length of the suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

Table 0324.-1. – *Diameters and minimum breaking loads*

| Gauge number | Diameter (millimetres) | | | | Minimum breaking load (newtons) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | Linen thread | | All other non-absorbable strands | | Stainless steel | |
| | min. | max. | min. | max. | C | D | C | D | C | D |
| 0.05 | 0.005 | 0.009 | 0.003 | 0.012 | - | - | 0.01 | - | | |
| 0.1 | 0.010 | 0.019 | 0.005 | 0.025 | - | - | 0.03 | - | | |
| 0.15 | 0.015 | 0.019 | 0.012 | 0.025 | - | - | 0.06 | 0.01 | | |
| 0.2 | 0.020 | 0.029 | 0.015 | 0.035 | - | - | 0.1 | - | | |
| 0.3 | 0.030 | 0.039 | 0.025 | 0.045 | - | - | 0.35 | 0.06 | | |
| 0.4 | 0.040 | 0.049 | 0.035 | 0.060 | - | - | 0.60 | 0.15 | 1.1 | |
| 0.5 | 0.050 | 0.069 | 0.045 | 0.085 | - | - | 1.0 | 0.35 | 1.6 | |
| 0.7 | 0.070 | 0.099 | 0.060 | 0.125 | 1.0 | 0.3 | 1.5 | 0.60 | 2.7 | |
| 1 | 0.100 | 0.149 | 0.085 | 0.175 | 2.5 | 0.6 | 3.0 | 1.0 | 5.3 | 4.0 |
| 1.5 | 0.150 | 0.199 | 0.125 | 0.225 | 5.0 | 1.0 | 5.0 | 1.5 | 8.0 | 6.0 |
| 2 | 0.200 | 0.249 | 0.175 | 0.275 | 8.0 | 2.5 | 9.0 | 3.0 | 13.3 | 10.0 |
| 2.5 | 0.250 | 0.299 | 0.225 | 0.325 | 9.0 | 5.0 | 13.0 | 5.0 | 15.5 | 11.6 |
| 3 | 0.300 | 0.349 | 0.275 | 0.375 | 11.0 | 8.0 | 15.0 | 9.0 | 17.7 | 13.3 |
| 3.5 | 0.350 | 0.399 | 0.325 | 0.450 | 15.0 | 9.0 | 22.0 | 13.0 | 33.4 | 25.0 |
| 4 | 0.400 | 0.499 | 0.375 | 0.550 | 18,0 | 11.0 | 27.0 | 15.0 | 46.7 | 35.0 |
| 5 | 0.500 | 0.599 | 0.450 | 0.650 | 26.0 | 15.0 | 35.0 | 22.0 | 57.9 | 43.4 |
| 6 | 0.600 | 0.699 | 0,.550 | 0.750 | 37.0 | 18.0 | 50.0 | 27.0 | 89.4 | 67.0 |
| 7 | 0.700 | 0.799 | 0.650 | 0.850 | 50.0 | 26.0 | 62.0 | 35.0 | 111.8 | 83.9 |
| 8 | 0.800 | 0.899 | 0.750 | 0.950 | 65.0 | 37.0 | 73.0 | 50.0 | 133.4 | 100.1 |
| 9 | 0.900 | 0.999 | 0.850 | 1.050 | | | | | 156.0 | 117.0 |
| 10 | 1.000 | 1.099 | 0.950 | 1.150 | | | | | 178.5 | 133.9 |

**Diameter**. Unless otherwise prescribed, measure the diameter by the following method using 5 sutures. Use a suitable mechanical instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10-15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at 3 points approximately evenly spaced along the suture. During the measurement submit monofilament sutures to a tension not greater than that required to keep them straight. Submit multifilament sutures to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0324.-1 appropriate to the gauge number and type of material concerned or 10 N whichever is less. Stainless steel sutures do not require tension to be applied during the measurement of diameter. For multifilament sutures of gauge number above 1.5 make 2 measurements at each point, the second measurement being made after rotating the suture through 90°. The diameter of that point is the average of the 2 measurements. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the column under A in

Table 0324.-1 for the gauge number concerned. None of the measurements are outside the limits given in the columns under B in Table 0324.-1 for the gauge number concerned.

**Minimum breaking load**. Unless otherwise prescribed, determine the minimum breaking load by the following method using sutures in the condition in which they are presented. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0324.-1) and pulling the knot tight. For stainless steel sutures gauges 3.5 and above, the minimum breaking load is determined on a straight pull. Carry out the test on 5 sutures. Submit sutures of length greater than 75 cm to 2 measurements and shorter sutures to 1 measurement. Determine the breaking load using a suitable tensilometer. The apparatus has 2 clamps for holding the suture, 1 of which is mobile and is driven at a constant rate of 30 cm/min. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5-20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value given in column C in Table 0324.-1 and no value is less than that given in column D for the gauge number and type of material concerned.

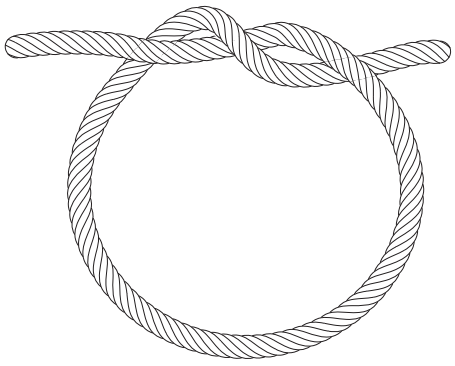

Figure 0324.-1 . – *Simple knot*

**Needle attachment**. If the sutures are supplied with an eyeless needle attached that is not stated to be detachable, they comply with the test for needle attachment. Carry out the test on 5 sutures. Use a suitable tensilometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the 5 determinations and all individual values are not less than the respective values given in Table 0324.-2 for the gauge number concerned. If not more than 1 individual value fails to meet the individual requirement, repeat the test on an additional 10 sutures. The attachment complies with the test if none of these 10 values is less than the individual value in Table 0324.-2 for the gauge number concerned.

Table 0324.-2. – *Minimum strengths of needle attachment*

| Gauge number | Mean value (newtons) | Individual value (newtons) |
|---|---|---|
| 0.4 | 0.50 | 0.25 |
| 0.5 | 0.80 | 0.40 |
| 0.7 | 1.7 | 0.80 |
| 1 | 2.3 | 1.1 |
| 1.5 | 4.5 | 2.3 |
| 2 | 6.8 | 3.4 |
| 2.5 | 9.0 | 4.5 |
| 3 | 11.0 | 4.5 |
| 3.5 | 15.0 | 4.5 |
| 4 | 18.0 | 6.0 |
| 5 | 18.0 | 7.0 |
| 6 | 25.0 | 12.5 |
| 7 | 25.0 | 12.5 |
| 8 | 50.0 | 25 |
| 9 | 50.0 | 25 |
| 10 | 75.0 | 37.5 |

**Extractable colour**. Sutures that are dyed and intended to remain so during use comply with the test for extractable colour. Place 0.25 g of the suture to be examined in a conical flask, add 25.0 mL of *water R* and cover the mouth of the flask with a short-stemmed funnel. Boil for 15 min, cool and adjust to the original volume with *water R*. Depending on the colour of the suture, prepare the appropriate reference solution as described in Table 0324.-3 using the primary colour solutions (*2.2.2*).

Table 0324.-3. – *Colour reference solutions*

| Colour of strand | Composition of reference solution (parts by volume) | | | |
|---|---|---|---|---|
| | Red primary solution | Yellow primary solution | Blue primary solution | *Water R* |
| Yellow-brown | 0.2 | 1.2 | - | 8.6 |
| Pink-red | 1.0 | - | - | 9.0 |
| Green-blue | - | - | 2.0 | 8.0 |
| Violet | 1.6 | - | 8.4 | - |

The test solution is not more intensely coloured than the appropriate reference solution.

**Monomer and oligomers**: maximum 2 per cent for the polyamide 6 suture.

In a continuous-extraction apparatus, treat 1.00 g with 30 mL of *methanol R* at a rate of at least 3 extractions per hour for 7 h. Evaporate the extract to dryness, dry the residue at 110 °C for 10 min, allow to cool in a desiccator and weigh. The residue weighs a maximum of 20 mg.

STORAGE (PACKAGING)

Sterile non-absorbable sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of a suture in aseptic conditions. They may be stored dry or in a preserving liquid to which an antimicrobial agent but no antibiotic may be added.

Sterile non-absorbable sutures are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices shall be considered in addition.

LABELLING

Reference may be made to the appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number;
- length, in centimetres or metres;
- if appropriate, that the needle is detachable;
- name of the product;
- intended use (surgical suture, non-absorbable);
- if appropriate, that the suture is coloured;
- if appropriate, the structure (braided, monofilament, sheathed).

**01/2008:0667**

# SUTURES, STERILE SYNTHETIC ABSORBABLE BRAIDED

## Fila resorbilia synthetica torta sterilia

DEFINITION

Sterile synthetic absorbable braided sutures consist of sutures prepared from a synthetic polymer, polymers or copolymers which, when introduced into a living organism, are absorbed by that organism and cause no undue tissue irritation. They consist of completely polymerised material. They occur as multifilament sutures consisting of elementary fibres which are assembled by braiding. The sutures may be treated to facilitate handling and they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile synthetic absorbable braided sutures are wound-closure devices. Being absorbable they serve to approximate tissue during the healing period and subsequently lose tensile strength by hydrolysis.

PRODUCTION

Appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure according to usual surgical techniques.

TESTS

*Carry out the following tests on the sutures in the state in which they are removed from the sachet.*

**Length**. Measure the length of the suture without applying more tension than is necessary to keep it straight. The length of each suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

**Diameter**. Unless otherwise prescribed, measure the diameter by the following method, using five sutures in the condition in which they are presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at three points approximately evenly spaced along the suture. During the measurement, submit the sutures to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0667.-1 appropriate to the gauge number and type of material or 10 N whichever is less. For sutures of gauge number above 1.5 make two measurements at each point, the second measurement being made after rotating the suture through 90°. The diameter of that point is the average of the two measurements. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0667.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0667.-1 for the gauge number concerned.

**Minimum breaking load**. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0667.-1) and pulling the knot tight.

Carry out the test on five sutures. Submit sutures of length greater than 75 cm to two measurements and shorter sutures to one measurement. Determine the breaking load using a suitable tensilometer. The apparatus has two clamps for holding the suture, one of which is mobile and is driven at a constant rate of 25 cm to 30 cm per minute. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results excluding those legitimately discarded is equal to or greater than the value given in column C in Table 0667.-1 and no individual result is less than that given in column D for the gauge number concerned.

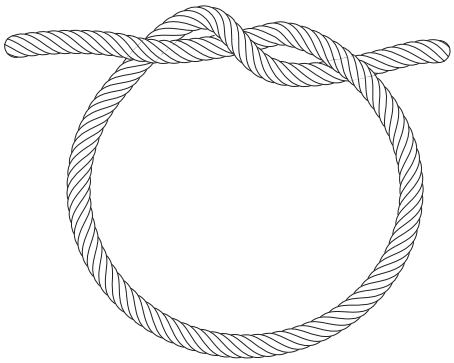

Figure 0667.-1. – *Simple knot*

Table 0667.-1. – *Diameters and breaking loads*

| Gauge number | Diameter (millimetres) | | | | Breaking load (newtons) | |
|---|---|---|---|---|---|---|
| | A | | B | | C | D |
| | min. | max. | min. | max. | | |
| 0.01 | 0.001 | 0.004 | 0.0008 | 0.005 | - | - |
| 0.05 | 0.005 | 0.009 | 0.003 | 0.012 | - | - |
| 0.1 | 0.010 | 0.019 | 0.005 | 0.025 | - | - |
| 0.2 | 0.020 | 0.029 | 0.015 | 0.035 | - | - |
| 0.3 | 0.030 | 0.039 | 0.025 | 0.045 | 0.45 | 0.23 |
| 0.4 | 0.040 | 0.049 | 0.035 | 0.060 | 0.70 | 0.35 |
| 0.5 | 0.050 | 0.069 | 0.045 | 0.085 | 1.4 | 0.7 |
| 0.7 | 0.070 | 0.099 | 0.060 | 0.125 | 2.5 | 1.3 |
| 1 | 0.100 | 0.149 | 0.085 | 0.175 | 6.8 | 3.4 |
| 1.5 | 0.150 | 0.199 | 0.125 | 0.225 | 9.5 | 4.8 |
| 2 | 0.200 | 0.249 | 0.175 | 0.275 | 17.7 | 8.9 |
| 2.5 | 0.250 | 0.299 | 0.225 | 0.325 | 21.0 | 10.5 |
| 3 | 0.300 | 0.349 | 0.275 | 0.375 | 26.8 | 13.4 |
| 3.5 | 0.350 | 0.399 | 0.325 | 0.450 | 39.0 | 18.5 |
| 4 | 0.400 | 0.499 | 0.375 | 0.550 | 50.8 | 25.4 |
| 5 | 0.500 | 0.599 | 0.450 | 0.650 | 63.5 | 31.8 |
| 6 | 0.600 | 0.699 | 0.550 | 0.750 | - | - |
| 7 | 0.700 | 0.799 | 0.650 | 0.850 | - | - |

**Needle attachment**. If the suture is supplied with an eyeless needle attached that is not stated to be detachable the attachment, it complies with the test for needle attachment. Carry out the test on five sutures. Use a suitable tensilometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the five deter-minations and all individual values are not less than the respective values given in Table 0667.-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional ten sutures. The attachment complies with the test if none of the ten values is less than the individual value in Table 0667.-2 for the gauge number concerned.

Table 0667.-2. – *Minimum strengths of needle attachment*

| Gauge number | Mean value (newtons) | Individual value (newtons) |
|---|---|---|
| 0.4 | 0.50 | 0.25 |
| 0.5 | 0.80 | 0.40 |
| 0.7 | 1.7 | 0.80 |
| 1 | 2.3 | 1.1 |
| 1.5 | 4.5 | 2.3 |
| 2 | 6.8 | 3.4 |
| 2.5 | 9.0 | 4.5 |
| 3 | 11.0 | 4.5 |
| 3.5 | 15.0 | 4.5 |
| 4 | 18.0 | 6.0 |
| 5 | 18.0 | 7.0 |

STORAGE (PACKAGING)

Sterile synthetic absorbable braided sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of the sutures in aseptic conditions. The sutures must be stored dry.

They are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices may be considered in addition.

LABELLING

Reference may be made to the appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical absorbable suture),
- if appropriate, that the suture is coloured,
- the structure (braided).

**01/2008:0666**

# SUTURES, STERILE SYNTHETIC ABSORBABLE MONOFILAMENT

## Fila resorbilia synthetica monofilamenta sterilia

DEFINITION

Sterile synthetic absorbable monofilament sutures consist of sutures prepared from a synthetic polymer, polymers or copolymers which, when introduced into a living organism, are absorbed by that organism and cause no undue tissue irritation. They consist of completely polymerised material. They occur as monofilament sutures. The sutures may be treated to facilitate handling and they may be coloured.

Appropriate harmonised standards may be considered when assessing compliance with respect to origin and processing of raw materials and with respect to biocompatibility.

Sterile synthetic absorbable monofilament sutures are wound-closure devices. Being absorbable they serve to approximate tissue during the healing period and subsequently lose tensile strength by hydrolysis.

PRODUCTION

The appropriate harmonised standards may apply with respect to appropriate validated methods of sterilisation, environmental control during manufacturing, labelling and packaging.

It is essential for the effectiveness and the performance characteristics during use and during the functional lifetime of these sutures that the following physical properties are specified: consistent diameter, sufficient initial strength and firm needle attachment.

The requirements below have been established, taking into account stresses which occur during normal conditions of use. These requirements can be used to demonstrate that individual production batches of these sutures are suitable for wound closure according to usual surgical techniques.

TESTS

*Carry out the following tests on the sutures in the state in which they are removed from the sachet.*

**Length**. Measure the length of the suture without applying more tension than is necessary to keep it straight. The length of each suture is not less than 95 per cent of the length stated on the label and does not exceed 400 cm.

**Diameter**. Unless otherwise prescribed, measure the diameter by the following method, using five sutures in the condition in which they are presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the suture being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the suture. Measure the diameter at intervals of 30 cm over the whole length of the suture. For a suture less than 90 cm in length, measure at three points approximately evenly spaced along the suture. During the measurement, submit the sutures to a tension not greater than that required to keep them straight. The average of the measurements carried out on the sutures being tested and not less than two-thirds of the measurements taken on each suture are within the limits given in the columns under A in Table 0666.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0666.-1 for the gauge number concerned.

Table 0666.-1. – *Diameters and breaking loads*

| Gauge number | Diameter (millimetres) | | | | Breaking load (newtons) | |
|---|---|---|---|---|---|---|
| | A | | B | | C | D |
| | min. | max. | min. | max. | | |
| 0.5 | 0.050 | 0.094 | 0.045 | 0.125 | 1.4 | 0.7 |
| 0.7 | 0.095 | 0.149 | 0.075 | 0.175 | 2.5 | 1.3 |
| 1 | 0.150 | 0.199 | 0.125 | 0.225 | 6.8 | 3.4 |
| 1.5 | 0.200 | 0.249 | 0.175 | 0.275 | 9.5 | 4.7 |
| 2 | 0.250 | 0.339 | 0.225 | 0.375 | 17.5 | 8.9 |
| 3 | 0.340 | 0.399 | 0.325 | 0.450 | 26.8 | 13.4 |
| 3.5 | 0.400 | 0.499 | 0.375 | 0.550 | 39.0 | 18.5 |
| 4 | 0.500 | 0.570 | 0.450 | 0.600 | 50.8 | 25.4 |
| 5 | 0.571 | 0.610 | 0.500 | 0.700 | 63.5 | 31.8 |

**Minimum breaking load**. The minimum breaking load is determined over a simple knot formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed (see Figure 0666.-1) and pulling the knot tight.

Carry out the test on five sutures. Submit sutures of length greater than 75 cm to two measurements and shorter sutures to one measurement. Determine the breaking load using a suitable tensilometer. The apparatus has two clamps for holding the suture, one of which is mobile and is driven at a constant rate of 25 cm to 30 cm per minute. The clamps are designed so that the suture being tested can be attached without any possibility of slipping. At the beginning of the test the length of suture between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the suture. If the suture breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another suture. The average of all the results excluding those legitimately discarded is equal to or greater than the value given in column C in Table 0666.-1 and no individual result is less than that given in column D for the gauge number concerned.

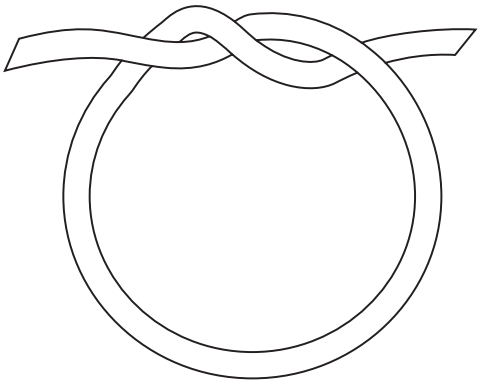

Figure 0666.-1. – *Simple knot*

**Needle attachment**. If the suture is supplied with an eyeless needle attached that is not stated to be detachable, the attachment complies with the test for needle attachment. Carry out the test on five sutures. Use a suitable tensilometer, such as that described for the determination of the minimum breaking load. Fix the needle and suture (without knot) in the clamps of the apparatus in such a way that the swaged part of the needle is completely free of the clamp and in line with the direction of pull on the suture. Set the mobile clamp in motion and note the force required to break the suture or to detach it from the needle. The average of the five determinations and all individual values are not less than the respective values given in Table 0666.-2 for the gauge number concerned. If not more than one individual value fails to meet the individual requirement, repeat the test on an additional ten sutures. The attachment complies with the test if none of the ten values is less than the individual value in Table 0666.-2 for the gauge number concerned.

Table 0666.-2. – *Minimum strengths of needle attachment*

| Gauge number | Mean value (newtons) | Individual value (newtons) |
|---|---|---|
| 0.5 | 0.80 | 0.40 |
| 0.7 | 1.7 | 0.80 |
| 1 | 2.3 | 1.1 |
| 1.5 | 4.5 | 2.3 |
| 2 | 6.8 | 3.4 |
| 2.5 | 9.0 | 4.5 |
| 3 | 11.0 | 4.5 |
| 3.5 | 15.0 | 4.5 |
| 4 | 18.0 | 6.0 |
| 5 | 18.0 | 7.0 |

STORAGE (PACKAGING)

Sterile synthetic absorbable monofilament sutures are presented in a suitable sachet that maintains sterility and allows the withdrawal and use of the sutures in aseptic conditions. The sutures must be stored dry.

They are intended to be used only on the occasion when the sachet is first opened.

Sutures in their individual sachets (primary packaging) are kept in a protective cover (box) which maintains the physical and mechanical properties until the time of use.

The application of appropriate harmonised standards for packaging of medical devices may be considered in addition.

LABELLING

Reference may be made to appropriate harmonised standards for the labelling of medical devices.

The details strictly necessary for the user to identify the product properly are indicated on or in each sachet (primary packaging) and on the protective cover (box) and include at least:

- gauge number,
- length in centimetres or metres,
- if appropriate, that the needle is detachable,
- name of the product,
- intended use (surgical absorbable suture),
- if appropriate, that the suture is coloured,
- the structure (monofilament).

# Sutures for veterinary use

Catgut, sterile, in distributor for veterinary use.. ..... 1281
Linen thread, sterile, in distributor for veterinary use .. ... 1282
Polyamide suture, sterile, in distributor for veterinary use..... 1282
Poly(ethylene terephthalate) suture, sterile, in distributor for veterinary use.. ..... 1283
Silk suture, sterile, braided, in distributor for veterinary use..... 1283
Strands, sterile non-absorbable, in distributor for veterinary use ..... 1283

**01/2008:0660**

# CATGUT, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

## Chorda resorbilis sterilis in fuso ad usum veterinarium

DEFINITION

Sterile catgut in distributor for veterinary use consists of strands prepared from collagen taken from the intestinal membranes of mammals. After cleaning, the membranes are split longitudinally into strips of varying width, which, when assembled in small numbers, according to the diameter required, are twisted under tension, dried, polished, selected and sterilised. The strands may be treated with chemical substances such as chromium salts to prolong absorption and glycerol to make them supple, provided such substances do not reduce tissue acceptability.

The strand is presented in a distributor that allows the withdrawal and use of all or part of it in aseptic conditions. The design of the distributor is such that with suitable handling the sterility of the content is maintained even when part of the strand has been withdrawn. It may be stored dry or in a preserving liquid to which an antimicrobial preservative but not an antibiotic may be added.

TESTS

*If stored in a preserving liquid, remove the strand from the distributor and measure promptly and in succession the length, diameter and breaking load. If stored in the dry state, immerse the strand in alcohol R* or a 90 per cent *V/V* solution of *2-propanol R* for 24 h and proceed with the measurements as indicated above.

**Length**. Measure the length without applying to the strand more tension than is necessary to keep it straight. The length is not less than 95 per cent of the length stated on the label. If the strand consists of several sections joined by knots, the length of each section is not less than 2.5 m.

**Diameter**. Carry out the test using a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the strand being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the strand. Make not fewer than one measurement per 2 m of length. If the strand consists of several sections joined by knots, make not fewer than three measurements per section. In any case make not fewer than twelve measurements. Make the measurements at points evenly spaced along the strand or along each section. The strand is not subjected to more tension than is necessary to keep it straight during measurement. The average of the measurements carried out on the strand being tested and not less than two-thirds of the individual measurements are within the limits given in the column under A in Table 0660.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0660.-1 for the gauge number concerned.

**Minimum breaking load**. The minimum breaking load is determined over a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed (see Figure 0660.-1) and pulling the knot tight.

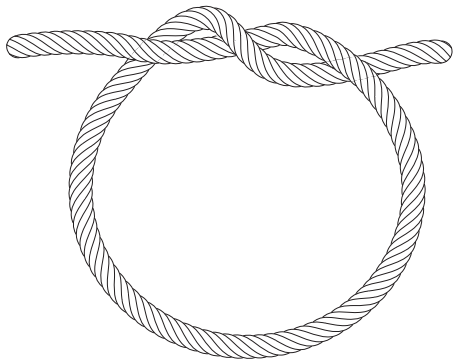

Figure 0660.-1. – *Simple knot*

Make not fewer than one measurement per 2 m of length. If the strand consists of several sections joined by knots, make not fewer than three measurements per section and, in any case, not fewer than one measurement per 2 m of length at points evenly spaced along the strand or along each section. Determine the breaking load using a suitable tensilometer. The apparatus has two clamps for holding the strand, one of which is mobile and is driven at a constant rate of 30 cm per minute. The clamps are designed so that the strand being tested can be attached without any possibility of slipping. At the beginning of the test the length of strand between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the strand. If the strand breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another part of the strand. The average of all the results, excluding those legitimately discarded, is equal to or greater than the value in column C and no value is less than that given in column D in Table 0660.-1 for the gauge number concerned.

Table 0660.-1. – *Diameters and breaking loads*

| Gauge number | Diameter (millimetres) | | | | Breaking load (newtons) | |
|---|---|---|---|---|---|---|
| | A | | B | | C | D |
| | min. | max. | min. | max. | | |
| 1 | 0.100 | 0.149 | 0.085 | 0.175 | 1.8 | 0.4 |
| 1.5 | 0.150 | 0.199 | 0.125 | 0.225 | 3.8 | 0.7 |
| 2 | 0.200 | 0.249 | 0.175 | 0.275 | 7.5 | 1.8 |
| 2.5 | 0.250 | 0.299 | 0.225 | 0.325 | 10 | 3.8 |
| 3 | 0.300 | 0.349 | 0.275 | 0.375 | 12.5 | 7.5 |
| 3.5 | 0.350 | 0.399 | 0.325 | 0.450 | 20 | 10 |
| 4 | 0.400 | 0.499 | 0.375 | 0.550 | 27.5 | 12.5 |
| 5 | 0.500 | 0.599 | 0.450 | 0.650 | 38.4 | 20.0 |
| 6 | 0.600 | 0.699 | 0.550 | 0.750 | 45.0 | 27.5 |
| 7 | 0.700 | 0.799 | 0.650 | 0.850 | 60.0 | 38.0 |
| 8 | 0.800 | 0.899 | 0.750 | 0.950 | 70.0 | 45.0 |

**Soluble chromium compounds**. Place 0.25 g in a conical flask containing 1 mL of *water R* per 10 mg of catgut. Stopper the flask, allow to stand at 37 ± 0.5 °C for 24 h, cool and decant the liquid. Transfer 5 mL to a small test tube and add 2 mL of a 10 g/L solution of *diphenylcarbazide R* in *alcohol R* and 2 mL of *dilute sulfuric acid R*. The solution is not more intensely coloured than a standard prepared at the same time using 5 mL of a solution containing 2.83 µg of *potassium dichromate R* per millilitre, 2 mL of *dilute sulfuric acid R* and 2 mL of a 10 g/L solution of *diphenylcarbazide R* in *alcohol R* (1 ppm of Cr).

**Sterility** (*2.6.1*). It complies with the test for sterility as applied to catgut and other surgical sutures. Carry out the test on three sections, each 30 cm long, cut off respectively from the beginning, the centre and the end of the strand.

STORAGE

Store protected from light and heat.

LABELLING

The label states:

– the gauge number,

– the length in centimetres or in metres.

07/2019:0608

# LINEN THREAD, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

## Filum lini sterile in fuso ad usum veterinarium

DEFINITION

Sterile linen thread in distributor for veterinary use consists of the pericyclic fibres of the stem of *Linum usitatissimum* L. The elementary fibres, 2.5-5 cm long, are assembled in bundles 30-80 cm long and spun into continuous lengths of suitable diameter. The thread may be creamy-white or may be coloured with colouring matter authorised by the competent authority. The thread is then sterilised.

IDENTIFICATION

A. Dissect the end of a thread, using a needle or fine tweezers, to isolate a few individual fibres. Examined under a microscope, the fibres are seen to be 12-31 µm wide and, along the greater part of their length, have thick walls, sometimes marked with fine longitudinal striations, and a narrow lumen. The fibres gradually narrow to a long, fine point. Sometimes there are unilateral swellings with transverse lines.

B. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3326 ± 10 $cm^{-1}$ (medium); 2911 ± 12 $cm^{-1}$ (medium); 1645 ± 10 $cm^{-1}$ (weak); 1426 ± 5 $cm^{-1}$ (medium); 1315 ± 3 $cm^{-1}$ (medium); 1154 ± 7 $cm^{-1}$ (medium); 1104 ± 5 $cm^{-1}$ (medium); 1050 ± 5 $cm^{-1}$ (strong); 1026 ± 7 $cm^{-1}$ (strong).

TESTS

It complies with the tests prescribed in the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

*If stored in a dry state, expose to an atmosphere with a relative humidity of 65 ± 5 per cent at 20 ± 2 °C for 4 h immediately before measuring the diameter and for the determination of minimum breaking load immerse in water R at room temperature for 30 min immediately before carrying out the test.*

STORAGE

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

07/2019:3083

# POLYAMIDE SUTURE, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

## Filum polyamidi sterile in fuso ad usum veterinarium

DEFINITION

Sterile polyamide 6 suture in distributor for veterinary use is obtained by drawing through a suitable die a synthetic plastic material formed by the polymerisation of ε-caprolactam (azepan-2-one). Sterile polyamide 6/6 suture in distributor for veterinary use is obtained by drawing through a suitable die a synthetic plastic material formed by the polycondensation of hexamethylenediamine (hexane-1,6-diamine) and adipic acid (hexanedioic acid).

Blends of polyamide 6 and polyamide 6/6 are common.

Polyamide suture consists of smooth, cylindrical monofilaments or braided filaments, or lightly twisted strands sheathed with the same material, according to the diameter required. It may be coloured with colouring matter or pigments authorised by the competent authority. The suture is then sterilised.

PRODUCTION

**ε-Caprolactam monomer and oligomers** (for sutures containing polyamide 6): maximum 2 per cent.

In a continuous-extraction apparatus, treat 1.00 g with 30 mL of *methanol R* at a rate of at least 3 extractions per hour for 7 h. Evaporate the extract to dryness, dry the residue at 110 °C for 10 min, allow to cool in a desiccator and weigh. The residue weighs a maximum of 20 mg.

CHARACTERS

It is not attacked by dilute alkaline solutions (for example a 100 g/L solution of sodium hydroxide) but is attacked by dilute mineral acids (for example 20 g/L sulfuric acid) and by hot glacial acetic acid.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3296 ± 10 $cm^{-1}$ (medium); 2930 ± 15 $cm^{-1}$ (medium); 2862 ± 15 $cm^{-1}$ (medium); 1635 ± 5 $cm^{-1}$ (strong); 1539 ± 12 $cm^{-1}$ (strong); 1462 ± 10 $cm^{-1}$ (medium); 1262 ± 12 $cm^{-1}$ (medium).

Additives and coatings of materials might lead to additional peaks.

TESTS

It complies with the tests prescribed in the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

The label states whether the suture is braided, monofilament or sheathed.

Sutures

**07/2018:0607**

# POLY(ETHYLENE TEREPHTHALATE) SUTURE, STERILE, IN DISTRIBUTOR FOR VETERINARY USE

## Filum ethyleni polyterephthalici sterile in fuso ad usum veterinarium

DEFINITION

Sterile poly(ethylene terephthalate) suture in distributor for veterinary use is obtained by drawing poly(ethylene terephthalate) through a suitable die. The suture is prepared by braiding very fine filaments in suitable numbers, depending on the gauge required. It may be whitish in colour, or may be coloured with authorised colouring matter or pigments authorised by the competent authority. The suture is sterilised.

CHARACTERS

It is attacked by strongly alkaline solutions and is incompatible with phenols.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 1712.5 ± 5 $cm^{-1}$ (strong); 1408 ± 5 $cm^{-1}$ (medium); 1338 ± 5 $cm^{-1}$ (medium); 1243 ± 12 $cm^{-1}$ (strong); 1093 ± 5 $cm^{-1}$ (strong); 1017 ± 5 $cm^{-1}$ (medium); 872 ± 5 $cm^{-1}$ (medium); 722 ± 2 $cm^{-1}$ (strong).

Additives and coatings of materials might lead to additional peaks.

TESTS

It complies with the tests prescribed in the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

**07/2019:0606**

# SILK SUTURE, STERILE, BRAIDED, IN DISTRIBUTOR FOR VETERINARY USE

## Filum bombycis tortum sterile in fuso ad usum veterinarium

DEFINITION

Sterile braided silk suture in distributor for veterinary use is obtained by braiding a variable number of threads, according to the diameter required, of degummed silk obtained from the cocoons of the silkworm *Bombyx mori* L. It may be coloured with colouring matter authorised by the competent authority. The suture is then sterilised.

IDENTIFICATION

A. Dissect the end of a strand, using a needle or fine tweezers, to isolate a few individual fibres. The fibres are sometimes marked with very fine longitudinal striations parallel to the axis of the strand. Examined under a microscope, a cross-section is more or less triangular or semi-circular, with rounded edges and without a lumen.

B. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflection (ATR).

*Absorption maxima and intensities*: 3280 ± 5 $cm^{-1}$ (strong); 2923 ± 15 $cm^{-1}$ (medium); 1622 ± 15 $cm^{-1}$ (strong); 1512 ± 5 $cm^{-1}$ (strong); 1444 ± 7 $cm^{-1}$ (medium); 1226 ± 10 $cm^{-1}$ (medium).

Additives and coatings of materials might lead to additional peaks.

TESTS

It complies with the tests prescribed in the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

STORAGE

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

LABELLING

See the monograph *Strands, sterile non-absorbable, in distributor for veterinary use (0605)*.

**01/2008:0605**

# STRANDS, STERILE NON-ABSORBABLE, IN DISTRIBUTOR FOR VETERINARY USE

## Fila non resorbilia sterilia in fuso ad usum veterinarium

DEFINITION

*The statements in this monograph are intended to be read in conjunction with the individual monographs on sterile non-absorbable strands in distributor for veterinary use in the Pharmacopoeia. The requirements do not necessarily apply to sterile non-absorbable strands which are not the subject of such monographs.*

Sterile non-absorbable strands in distributor for veterinary use are strands which, when introduced into a living organism, are not metabolised by that organism. Sterile non- absorbable strands vary in origin, which may be animal, vegetable or synthetic. They occur as cylindrical monofilaments or as multifilament strands. Multifilament strands consist of elementary fibres which are assembled by twisting, cabling or braiding. Such strands may be sheathed. Sterile non- absorbable strands may be treated to render them non-capillary, and they may be coloured with colouring matter or pigments authorised by the competent authority. The strands are sterilised.

They are presented in a suitable distributor that allows the withdrawal and use of all or part of the strand in aseptic conditions. The design of the distributor is such that with suitable handling the sterility of the content is maintained even when part of the strand has been removed. They may be stored dry or in a preserving liquid to which an antimicrobial preservative but not an antibiotic may be added.

TESTS

*Remove the strand from the distributor and measure promptly and in succession the length, diameter and minimum breaking load.*

**Length**. Measure the length in the condition in which the strand is presented and without applying more tension than is necessary to keep it straight. The length of the strand is not less than 95 per cent of the length stated on the label.

**Diameter**. Unless otherwise prescribed, measure the diameter by the following method using the strand in the condition in which it is presented. Use a suitable instrument capable of measuring with an accuracy of at least 0.002 mm and having a circular pressor foot 10 mm to 15 mm in diameter. The pressor foot and the moving parts attached to it are weighted so as to apply a total load of 100 ± 10 g to the strand being tested. When making the measurements, lower the pressor foot slowly to avoid crushing the strand. Make not fewer than one measurement per 2 m of length and in any case not fewer than 12 measurements at points evenly spaced along the strand. During the measurement submit monofilament strands to a tension not greater than that required to keep them straight. Submit multifilament strands to a tension not greater than one-fifth of the minimum breaking load shown in column C of Table 0605.-1 appropriate to the gauge number and type of material concerned or 10 N whichever is less. For multifilament strands of gauge number above 1.5 make two measurements at each point, the second measurement being made after rotating the strand through 90°. The diameter of that point is the average of the two measurements. The average of the measurements carried out on the strand being tested and not less than two-thirds of the individual measurements are within the limits given in the columns under A in Table 0605.-1 for the gauge number concerned. None of the measurements is outside the limits given in the columns under B in Table 0605.-1 for the gauge number concerned.

Table 0605.-1. – *Diameters and minimum breaking loads*

| Gauge number | Diameter (millimetres) | | | | Minimum breaking load (newtons) | | | |
|---|---|---|---|---|---|---|---|---|
| | A | | B | | Linen thread | | All other non-absorbable strands | |
| | min. | max. | min. | max. | C | D | C | D |
| 0.5 | 0.050 | 0.069 | 0.045 | 0.085 | - | - | 1.0 | 0.35 |
| 0.7 | 0.070 | 0.099 | 0.060 | 0.125 | 1.0 | 0.3 | 1.5 | 0.60 |
| 1 | 0.100 | 0.149 | 0.085 | 0.175 | 2.5 | 0.6 | 3.0 | 1.0 |
| 1.5 | 0.150 | 0.199 | 0.125 | 0.225 | 5.0 | 1.0 | 5.0 | 1.5 |
| 2 | 0.200 | 0.249 | 0.175 | 0.275 | 8.0 | 2.5 | 9.0 | 3.0 |
| 2.5 | 0.250 | 0.299 | 0.225 | 0.325 | 9.0 | 5.0 | 13.0 | 5.0 |
| 3 | 0.300 | 0.349 | 0.275 | 0.375 | 11.0 | 8.0 | 15.0 | 9.0 |
| 3.5 | 0.350 | 0.399 | 0.325 | 0.450 | 15.0 | 9.0 | 22.0 | 13.0 |
| 4 | 0.400 | 0.499 | 0.375 | 0.550 | 18.0 | 11.0 | 27.0 | 15.0 |
| 5 | 0.500 | 0.599 | 0.450 | 0.650 | 26.0 | 15.0 | 35.0 | 22.0 |
| 6 | 0.600 | 0.699 | 0.550 | 0.750 | 37.0 | 18.0 | 50.0 | 27.0 |
| 7 | 0.700 | 0.799 | 0.650 | 0.850 | 50.0 | 26.0 | 62.0 | 35.0 |
| 8 | 0.800 | 0.899 | 0.750 | 0.950 | 65.0 | 37.0 | 73.0 | 50.0 |

**Minimum breaking load**. Unless otherwise prescribed, determine the minimum breaking load by the following method using the strand in the condition in which it is presented. The minimum breaking load is determined over a simple knot formed by placing one end of a strand held in the right hand over the other end held in the left hand, passing one end over the strand and through the loop so formed (see Figure 0605.-1) and pulling the knot tight.

Make not fewer than one measurement per 2 m of length at points evenly spaced along the strand. Determine the breaking load using a suitable tensilometer. The apparatus has two clamps for holding the strand, one of which is mobile and is driven at a constant rate of 30 cm per minute. The clamps are designed so that the strand being tested can be attached with-out any possibility of slipping. At the beginning of the test the length of strand between the clamps is 12.5 cm to 20 cm and the knot is midway between the clamps. Set the mobile clamp in motion and note the force required to break the strand. If the strand breaks in a clamp or within 1 cm of it, the result is discarded and the test repeated on another part of the strand. The average of all the results, excluding those legitimately dis-carded, is equal to or greater than the value in column C and no value is less than that given in column D in Table 0605.-1 for the gauge number and type of material concerned.

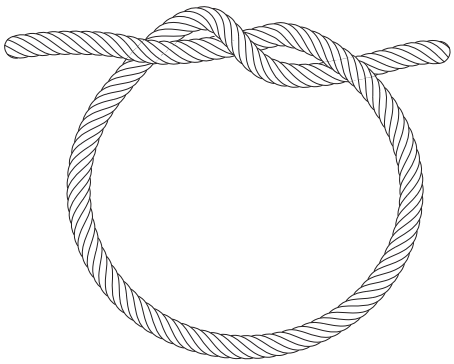

Figure 0605.-1. – *Simple knot*

**Sterility** (*2.6.1*). They comply with the test for sterility as applied to catgut and other surgical sutures. Carry out the test on three sections each 30 cm long, cut off respectively from the beginning, the centre and the end of the strand.

**Extractable colour**. Strands that are dyed and intended to remain so during use comply with the test for extractable colour. Place 0.25 g of the strand to be examined in a conical flask, add 25.0 mL of *water R* and cover the mouth of the flask with a short-stemmed funnel. Boil for 15 min, cool and adjust to the original volume with *water R*. Depending on the colour of the strand, prepare the appropriate reference solution as described in Table 0605.-2 using the primary colour solutions (*2.2.2*).

Table 0605.-2. – *Colour reference solutions*

| Colour of strand | Composition of reference solution (parts by volume) | | | |
|---|---|---|---|---|
| | Red primary solution | Yellow primary solution | Blue primary solution | Water |
| Yellow - brown | 0.2 | 1.2 | – | 8.6 |
| Pink - red | 1.0 | – | – | 9.0 |
| Green - blue | – | – | 2.0 | 8.0 |
| Violet | 1.6 | – | 8.4 | – |

The test solution is not more intensely coloured than the appropriate reference solution.

STORAGE

Store protected from light and heat.

LABELLING

The label states:

- the gauge number,
- the length in centimetres or in metres,
- where appropriate, that the strand is coloured and intended to remain so during use.

Sutures

# Herbal drugs and herbal drug preparations


Herbal drugs: introduction.. 1289
Abelmoschi corolla.. 1289
Acacia.. 1290
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Agnus castus fruit dry extract.. 1296
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Angelica archangelica root.. 1309
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Angelica pubescens root.. 1312
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Anise oil.. 1315
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Artichoke leaf dry extract.. 1323
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Atractylodes rhizome, largehead.. 1328
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Belladonna leaf dry extract, standardised.. 1337
Belladonna leaf tincture, standardised.. 1338
Belladonna, prepared.. 1339
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Benzoin tincture, Siam.. 1342
Benzoin tincture, Sumatra.. 1343
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Bilberry fruit, fresh.. 1344
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Bistort rhizome.. 1347
Bitter-fennel fruit oil.. 1348
Bitter-fennel herb oil.. 1350
Bitter-orange epicarp and mesocarp.. 1352
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Black cohosh.. 1355
Black horehound.. 1358
Blackcurrant leaf.. 1359
Bogbean leaf.. 1360
Boldo leaf.. 1361
Boldo leaf dry extract.. 1363
Buckwheat herb.. 1364
Bupleurum root.. 1365
Butcher's broom.. 1367
Calendula flower.. 1368
Cape jasmine fruit.. 1369
Capsicum.. 1371
Capsicum oleoresin, refined and standardised.. 1373
Capsicum soft extract, standardised.. 1374
Capsicum tincture, standardised.. 1375
Caraway fruit.. 1376
Caraway oil.. 1377
Cascara.. 1378
Cascara dry extract, standardised.. 1380
Cassia oil.. 1381
Centaury.. 1382
Centella.. 1383
Chamomile flower, Roman.. 1384
Chinese goldthread rhizome.. 1386
Cinchona bark.. 1387
Cinchona liquid extract, standardised.. 1389
Cinnamon.. 1390
Cinnamon bark oil, Ceylon.. 1391
Cinnamon leaf oil, Ceylon.. 1391
Citronella oil.. 1392
Clary sage oil.. 1393
Clematis armandii stem.. 1394
Clove.. 1396
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Coix seed.. 1398
Cola.. 1400
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Frangula bark.. 1439
Frangula bark dry extract, standardised.. 1441
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Gentian root.. 1448
Gentian tincture.. 1449
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Ginkgo dry extract, refined and quantified.. 1451
Ginkgo leaf.. 1454
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Ginseng dry extract.. 1457
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Goldenrod, European.. 1460
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Greater celandine.. 1463
Green tea.. 1464
Guar.. 1466
Guarana.. 1467
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Hamamelis leaf.. 1469
Hawthorn berries.. 1470
Hawthorn leaf and flower.. 1471
Hawthorn leaf and flower dry extract.. 1472
Hawthorn leaf and flower liquid extract, quantified.. 1473
Hop strobile.. 1474
Horse-chestnut.. 1475
Horse-chestnut dry extract, standardised.. 1477
Houttuynia herb.. 1478
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Indigo plant leaf.. 1481
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Ipecacuanha, prepared.. 1483
Ipecacuanha root.. 1485
Ipecacuanha tincture, standardised.. 1486
Isatis root.. 1487
Ispaghula husk.. 1488
Ispaghula seed.. 1489
Ivy leaf.. 1489
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Juniper.. 1492
Juniper oil.. 1493
Kelp.. 1494
Knotgrass.. 1495
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Lavender flower.. 1497
Lavender oil.. 1499
Lemon oil.. 1500
Lemon verbena leaf.. 1502
Lightyellow sophora root.. 1503
Ligusticum root and rhizome.. 1505
Lime flower.. 1506
Linseed.. 1507
Liquorice dry extract for flavouring purposes.. 1508
Liquorice root.. 1509
Long pepper.. 1510
Loosestrife.. 1511
Lovage root.. 1512
Lycopus lucidus herb.. 1514
Magnolia biondii flower bud.. 1515
Magnolia officinalis bark.. 1517
Magnolia officinalis flower.. 1519
Mallow flower.. 1520
Mallow leaf.. 1521
Mandarin epicarp and mesocarp.. 1522
Mandarin oil.. 1524
Marshmallow leaf.. 1525
Marshmallow root.. 1526
Mastic.. 1526
Mate leaf.. 1527
Matricaria flower.. 1529
Matricaria liquid extract.. 1530
Matricaria oil.. 1531
Meadowsweet.. 1533
Melilot.. 1534
Melissa leaf.. 1536
Melissa leaf dry extract.. 1537
Milk thistle dry extract, refined and standardised.. 1538
Milk thistle fruit.. 1539
Mint oil, partly dementholised.. 1541
Motherwort.. 1542
Moutan bark.. 1543
Mullein flower.. 1545
Myrrh.. 1546
Myrrh tincture.. 1547
Narrow-leaved coneflower root.. 1548
Neroli oil.. 1550
Nettle leaf.. 1551
Nettle root.. 1553
Niaouli oil, cineole type.. 1554
Notoginseng root.. 1555
Nutmeg oil.. 1556
Oak bark.. 1557
Olive leaf.. 1557
Olive leaf dry extract.. 1559
Opium dry extract, standardised.. 1560
Opium, prepared.. 1561
Opium, raw.. 1562
Opium tincture, standardised.. 1564
Oregano.. 1565
Orientvine stem.. 1567
Pale coneflower root.. 1568
Passion flower.. 1570
Passion flower dry extract.. 1571
Pelargonium root.. 1571
Peony root, red.. 1572
Peony root, white.. 1573
Pepper.. 1575
Peppermint leaf.. 1577
Peppermint leaf dry extract.. 1578
Peppermint oil.. 1579
Peru balsam.. 1580
Pine sylvestris oil.. 1581
Platycodon root.. 1582
Polygonum cuspidatum rhizome and root.. 1583
Polygonum orientale fruit.. 1585
Poria.. 1587
Primula root.. 1588
Psyllium seed.. 1588
Purple coneflower herb.. 1589
Purple coneflower root.. 1591
Pygeum africanum bark.. 1593
Quillaia bark.. 1593
Red poppy petals.. 1595
Restharrow root.. 1596
Rhatany root.. 1597
Rhatany tincture.. 1598
Rhubarb.. 1598
Ribwort plantain.. 1599
Roselle.. 1600
Rosemary leaf.. 1601
Rosemary oil.. 1603
Round amomum fruit.. 1604
Safflower flower.. 1606
Sage leaf (Salvia officinalis).. 1607
Sage leaf, three-lobed.. 1608
Sage tincture.. 1609
Salvia miltiorrhiza root and rhizome.. 1610
Sanguisorba root.. 1611
Saw palmetto extract.. 1612
Saw palmetto fruit.. 1615
Schisandra fruit.. 1617
Senega root.. 1618
Senna leaf.. 1620
Senna leaf dry extract, standardised.. 1621
Senna pods, Alexandrian.. 1622
Senna pods, Tinnevelly.. 1623
Serratula coronata herb.. 1624
Sophora flower.. 1626

Sophora flower-bud.. 1628
Spanish sage oil.. 1629
Spike lavender oil.. 1630
St. John's wort.. 1631
St. John's wort dry extract, quantified.. 1633
Star anise.. 1635
Star anise oil.. 1636
Stramonium leaf.. 1638
Stramonium, prepared.. 1640
Sweet orange oil.. 1641
Szechwan lovage rhizome.. 1642
Tea tree oil.. 1643
Thomson kudzuvine root.. 1644
Thyme.. 1646
Thyme oil, thymol type.. 1648
Tolu balsam.. 1649
Tormentil.. 1650
Tormentil tincture.. 1650
Tragacanth.. 1651
Turmeric, Javanese.. 1652
Turmeric rhizome.. 1653
Turpentine oil.. 1655
Typhae pollen.. 1656
Uncaria stem with hooks.. 1657
Valerian dry aqueous extract .. 1659
Valerian dry hydroalcoholic extract.. 1660
Valerian root.. 1660
Valerian root, cut.. 1662
Valerian tincture.. 1664
Verbena herb.. 1665
White horehound.. 1667
Wild pansy (flowering aerial parts).. 1668
Wild thyme.. 1669
Willow bark.. 1671
Willow bark dry extract.. 1672
Wormwood.. 1673
Yarrow.. 1674
Zanthoxylum bungeanum pericarp.. 1675

**01/2011:90029**
**corrected 10.0**

# HERBAL DRUGS: INTRODUCTION

*For practical purposes and for the convenience of users of the European Pharmacopoeia, monographs on herbal drugs and herbal drug preparations are brought together in the following section.*

*This is not an exhaustive list and it is not intended to provide a regulatory definition of herbal materials. It is based on the definitions in the general monographs Herbal drugs (1433) and Herbal drug preparations (1434) and is intended solely for the convenience of users.*

**01/2020:2827**

# ABELMOSCHI COROLLA

## Abelmoschi corolla

DEFINITION

Dried corolla of *Abelmoschus manihot* (L.) Medik.

*Content*: minimum 1.0 per cent of hibifolin ($C_{21}H_{18}O_{14}$; $M_r$ 494.4) (dried drug).

IDENTIFICATION

A. The corolla is often crumpled and fragmented, and when whole, consists of 5 petals slightly fused at their base. Each petal is triangular and broadly obovate, with slightly sinuous margins, about 7-20 cm long and 7-12 cm wide. They are yellow or pale yellow, with a dark violet or brown unguis at the base. The outer surface shows numerous pale green radial longitudinal striations, and stamens united by their filaments into a tube about 1.5-2.5 cm long bearing numerous sessile anthers. At the centre of the stamen tube is the style, ending in a dark violet or black stigma, with 5 lobes rolled outwards.

B. Microscopic examination (*2.8.23*). The powder is yellow or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2827.-1): fragments of the epidermis of the distal part of the corolla (surface view [B], transverse section [F]) with papillose cells [Ba, Fa] and long glandular trichomes, up to 800 µm long, with a stalk composed of 1 [Bc] to 4 cells [H] and a multicellular head whose diameter does not exceed that of the stalk, composed of cells with slightly granular contents [Bb]; epidermal cells surrounding each glandular trichome that are not papillose [Bd]; fragments of the epidermis of the base of the corolla [A], often pink or violet, with elongated epidermal cells [Aa], unicellular covering trichomes either isolated [Ab] or paired [Ac], up to 200 µm long, and glandular trichomes with a club-shaped secretory head [E]; fragments of the characteristic layer of the pollen sac of the anthers with polyhedral cells, whose walls show thickened parallel striations [G], accompanied by oil droplets [Ga]; spherical pollen grains [D] with finely pitted walls, exceeding 100 µm in diameter, spiny and with numerous germinal pores [Da]; numerous annular or spiral vascular bundles [C]; fragments of parenchyma consisting of polyhedral cells, some of which contain cluster crystals of calcium oxalate about 20 µm in diameter [J].

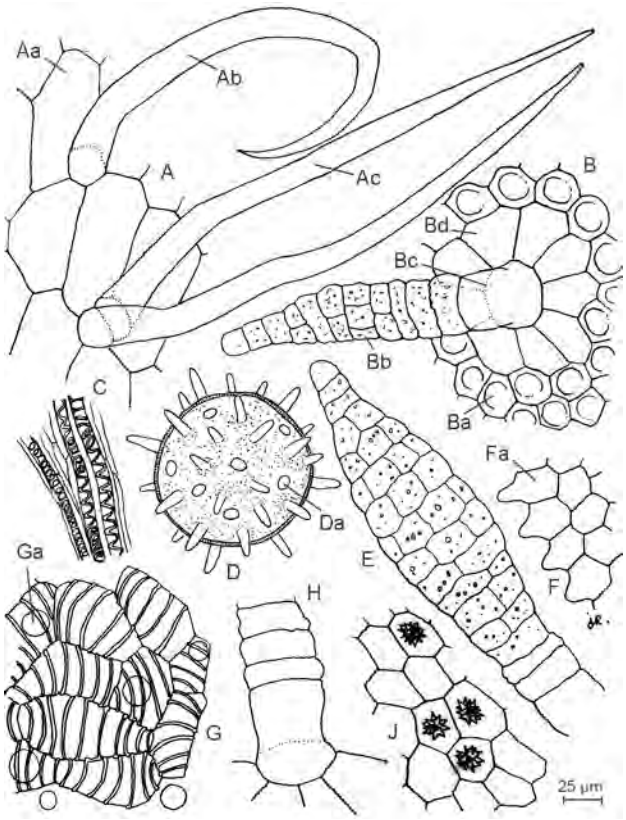


Figure 2827.-1. – *Illustration for identification test B of powdered herbal drug of abelmoschi corolla*

C. High-performance thin-layer chromatography (*2.8.25*). *Prepare the solutions immediately before use.*

*Test solution.* To 0.25 g of the powdered herbal drug (1000) (*2.9.12*) add 10 mL of a mixture of 4 volumes of *water R* and 6 volumes of *acetonitrile R*, and sonicate for 15 min. Centrifuge the solution for 15 min and filter the supernatant through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 4.0 mg of *rutoside trihydrate R* and 5.0 mg of *hibifolin R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 2.5 mg of *hibifolin R* and 2.5 mg of *hyperoside R* in *methanol R* and dilute to 5 mL with the same solvent.

*Intensity marker*: hibifolin.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm) [or *TLC silica gel $F_{254}$ plate R* (5-40 µm)].

*Mobile phase*: *anhydrous formic acid R*, *acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 3 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate (or 120 mm).

*Drying*: in air.

*Detection*: heat at 100-105 °C for 3-4 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the middle third; both the lower zone (hibifolin) and the upper zone (hyperoside) show a yellow fluorescence.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint fluorescent zones may be present.

| Top of the plate | |
|---|---|
| | |
| _____ | _____ |
| | A blue zone, equivalent |
| | A yellow zone, equivalent |
| | A yellow zone, equivalent (hyperoside) |
| Hibifolin: a yellow zone | A yellow zone, equivalent (hibifolin) |
| Rutoside: a yellow zone | A yellowish zone, faint (rutoside) |
| _____ | _____ |
| | A yellowish zone, very faint |
| | A blue zone, equivalent |
| **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (1000) (*2.9.12*) by drying in an oven at 105 °C for 4 h.

**Total ash** (*2.4.16*): maximum 8 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture A*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Solvent mixture B*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Test solution.* Reduce the herbal drug to a powder (1000) (*2.9.12*) using a refrigerated blade grinder. To 0.500 g add 20 mL of solvent mixture A and homogenise using a shaker. Sonicate for 30 min. Centrifuge for 15 min and transfer the supernatant to a 50 mL volumetric flask. Change the water in the ultrasonic bath every 30 min to prevent heating and repeat the extraction twice, adding to the residue 15 mL of solvent mixture A. Combine the supernatants, cool, and dilute to 50.0 mL with solvent mixture A. Dilute 2.0 mL of the solution to 5.0 mL with solvent mixture B. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 6.0 mg of *hibifolin CRS* in solvent mixture A and dilute to 10.0 mL with solvent mixture A.

*Reference solution (b).* Dissolve 2.5 mg of *quercitrin R* in solvent mixture A and dilute to 5 mL with solvent mixture A.

*Reference solution (c).* Mix 0.4 mL of reference solution (b) and 0.5 mL of reference solution (a), and dilute to 5 mL with solvent mixture B.

*Reference solution (d).* Dilute 0.70 mL of reference solution (a) to 5.0 mL with solvent mixture B.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, 0.07 per cent *V/V* solution of *phosphoric acid R* (5:95 *V/V*);
– *mobile phase B*: 0.07 per cent *V/V* solution of *phosphoric acid R*, *acetonitrile R* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 89 | 11 |
| 10 - 15 | 89 → 87 | 11 → 13 |
| 15 - 25 | 87 → 75 | 13 → 25 |
| 25 - 30 | 75 → 60 | 25 → 40 |
| 30 - 33 | 60 | 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 360 nm.

*Autosampler*: set at 10 °C.

*Injection*: 10 µL of the test solution and reference solutions (c) and (d).

*Retention time*: hibifolin = about 22 min; quercitrin = about 23 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to hibifolin and quercitrin.

Calculate the percentage content of hibifolin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 1.75}{A_2 \times m_1}$$

$A_1$ = area of the peak due to hibifolin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hibifolin in the chromatogram obtained with reference solution (d);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *hibifolin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of hibifolin in *hibifolin CRS*.

**07/2019:0307**

# ACACIA

## Acaciae gummi

DEFINITION

Air-hardened, gummy exudate flowing naturally from or obtained by incision of the trunk and branches of *Acacia senegal* L. Willd. (syn. *Senegalia senegal* (L.) Britton), other species of *Acacia* of African origin and *Acacia seyal* Delile.

CHARACTERS

It is almost completely but very slowly soluble, after about 2 h, in twice its mass of water leaving only a very small residue of vegetable particles; the liquid obtained is colourless or

yellowish, dense, viscous, adhesive, translucent and weakly acid to blue litmus paper. It is practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It occurs as yellowish-white, yellow or pale amber, sometimes with a pinkish tint, friable, opaque, spheroidal, oval or reniform pieces (tears) of a diameter from about 1-3 cm, frequently with a cracked surface, easily broken into irregular, whitish or slightly yellowish angular fragments with a conchoidal fracture and a glassy and transparent appearance. In the centre of an unbroken tear there is sometimes a small cavity.

B. Microscopic examination (*2.8.23*). The powder is white or yellowish-white. Examine under a microscope using *ethanol (96 per cent) R*. The powder shows the following diagnostic characters: angular, irregular, colourless, transparent fragments. Only traces of starch or plant tissues are visible. No stratified membrane is apparent.

C. Examine the chromatograms obtained in the test for glucose and fructose.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution.

| **Top of the plate** | |
|---|---|
| | 3 blue zones, very faint |
| Rhamnose: a greenish-brown zone | A greenish-brown zone, very faint to equivalent (rhamnose) |
| Xylose: a brownish-grey zone | |
| _____ | _____ |
| Arabinose: a brownish-grey zone | A brownish-grey zone, intense (arabinose) |
| Glucose: a greyish-blue zone | |
| Galactose: a greyish-blue zone | A greyish-blue zone, intense (galactose) |
| _____ | _____ |
| | 1 or 2 brownish-grey zones, very faint to equivalent |
| | 1 or 2 blue zones, faint to equivalent |
| **Reference solution (a)** | **Test solution** |

D. Dissolve 1 g of the powdered herbal drug (355) (*2.9.12*) in 2 mL of *water R* by stirring frequently for 2 h. Add 2 mL of *ethanol (96 per cent) R*. After shaking, a white gelatinous mucilage is formed that becomes fluid upon addition of 10 mL of *water R*.

TESTS

**Solution S**. Dissolve 3.0 g of the powdered herbal drug (355) (*2.9.12*) in 25 mL of *water R* by stirring for 30 min. Allow to stand for 30 min and dilute to 30 mL with *water R*.

**Insoluble matter**: maximum 0.5 per cent.

To 5.0 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of *water R* and 14 mL of *dilute hydrochloric acid R*, boil gently for 15 min, shaking frequently and filter while hot through a tared sintered-glass filter (*2.1.2*). Wash with hot *water R* and dry at 100-105 °C. The residue weighs a maximum of 25 mg.

**Glucose and fructose**. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.1 g of the powdered herbal drug (355) (*2.9.12*) in a thick-walled centrifuge tube, add 2 mL of a 100 g/L solution of *trifluoroacetic acid R* and shake vigorously. Stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge, transfer 1 mL of the clear supernatant into a 10 mL flask and add 5 mL of *methanol R*.

*Reference solution (a)*. Dissolve 5 mg of *arabinose R*, 5 mg of *galactose R*, 5 mg of *glucose R*, 5 mg of *rhamnose R* and 5 mg of *xylose R* in 1 mL of *water R* and dilute to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 5 mg of *galactose R* and 5 mg of *glucose R* in 1 mL of *water R* and dilute to 10 mL with *methanol R*.

*Intensity marker*: galactose.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 4 µL of the test solution and reference solutions (a) and (b), and 2 µL of reference solution (c), as bands of 8 mm.

*Development A*: 70 mm from the lower edge of the plate, in an unsaturated tank.

*Drying A*: in air.

*Development B*: 70 mm from the lower edge of the plate, in an unsaturated tank, using freshly prepared mobile phase.

*Drying B*: in air.

*Detection*: treat with a solution prepared as follows: dissolve 4 g of *diphenylamine R* and 4 mL of *aniline R* in 160 mL of *acetone R* and add *phosphoric acid R* until the precipitate formed dissolves again (about 30 mL). Heat at 120 °C for 5-10 min and examine in daylight.

*System suitability*: reference solution (c):

– the chromatogram shows in the middle third 2 distinct zones, which may be touching; the lower zone (galactose) and the upper zone (glucose) are greyish-blue.

*Results*: the chromatogram obtained with the test solution shows no greyish-blue zone and no reddish zone between the zones due to galactose and arabinose in the chromatogram obtained with reference solution (a).

**Starch, dextrin and agar**. To 10 mL of solution S, previously boiled and cooled, add 0.1 mL of *0.05 M iodine*. No blue or reddish-brown colour develops.

**Sterculia gum**

A. Place 0.2 g of the powdered herbal drug (355) (*2.9.12*) in a 10 mL ground-glass-stoppered cylinder graduated in 0.1 mL. Add 10 mL of *ethanol (60 per cent V/V) R* and shake. Any gel formed occupies a maximum of 1.5 mL.

B. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of *water R* and shake. Add 0.1 mL of *methyl red solution R*. Not more than 5.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Tannins**. To 10 mL of solution S add 0.1 mL of *ferric chloride solution R1*. A gelatinous precipitate is formed, but neither the precipitate nor the liquid is dark blue.

**Tragacanth**. Examine the chromatograms obtained in the test for glucose and fructose.

*Results*: the chromatogram obtained with the test solution shows no faint to intense brownish-grey zone corresponding to the zone due to xylose in the chromatogram obtained with reference solution (a).

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for acacia used as a viscosity-increasing agent and/or suspending agent in aqueous preparations.*

**Apparent viscosity**. Determine the dynamic viscosity using a capillary viscometer (*2.2.9*) or a rotating viscometer (*2.2.10*) on a 100 g/L solution of acacia (dried substance).

**07/2017:2432**

# ACANTHOPANAX BARK

## Acanthopanacis gracilistyli cortex

DEFINITION

Dried root bark of *Eleutherococcus nodiflorus* (Dunn) S.Y.Hu (syn. *Acanthopanax gracilistylus* W.W.Sm.) collected in summer and autumn.

IDENTIFICATION

A. The bark occurs in irregular quills, 5-15 cm long, 0.4-1.4 cm in diameter, about 2 mm thick. The outer surface is greyish-brown, with slightly twisted longitudinal wrinkles and transverse lenticel-like scars. The inner surface is pale yellow or greyish-yellow, with fine longitudinal striations. The texture is light, fragile, easily broken. The fracture is irregular, greyish-white.

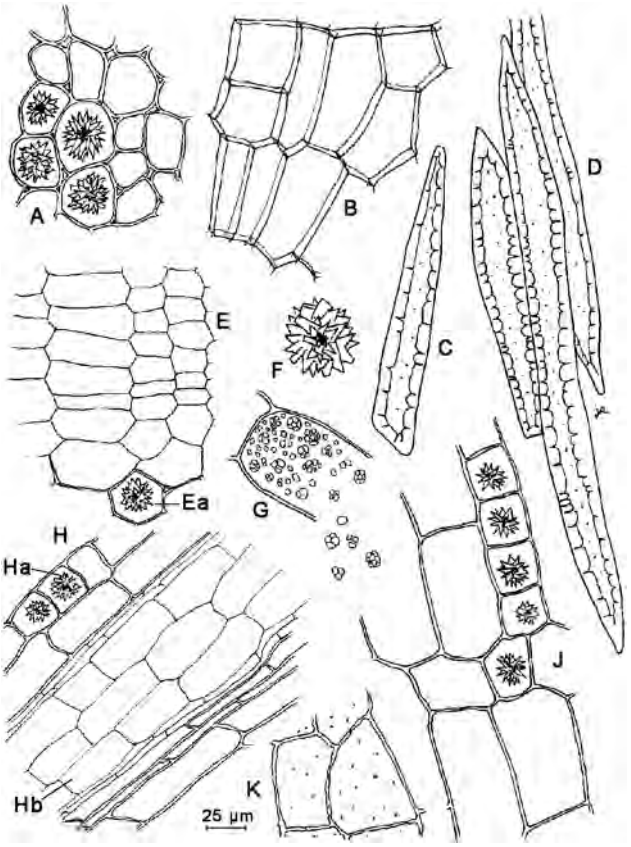


Figure 2432.-1. – *Illustration for identification test B of powdered herbal drug of acanthopanax bark*

B. Microscopic examination (*2.8.23*). The powder is greyish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2432.-1): cluster crystals of calcium oxalate, 8-64 µm in diameter, free [F] or included in parenchyma cells [A, Ea, Ha] sometimes forming crystal sheaths [J]; cork cells, rectangular or polygonal, thin-walled (surface view [B], transverse section [E]), sometimes walls of cork cells of older barks unevenly thickened, slightly pitted [K]; fragments of secretory canals [H] consisting of thin-walled cells [Hb] containing colourless or pale yellow secretions; phloem fibres, free [C] or in bundles of 2-4 fibres [D] present in older barks. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules, simple, polygonal or subspherical, 2-8 µm in diameter, or 2-10 compound, free or included in cells [G].

C. Examine the chromatogram obtained in the test for *Periploca sepium*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| ——— | ——— |
| Thymol: an orange zone | |
| ——— | ——— |
| Borneol: a brown zone | |
| | A broad pink zone |
| **Reference solution** | **Test solution** |



Herbal drugs

TESTS

***Periploca sepium***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.3 g of the powdered herbal drug (355) (*2.9.12*) add 3 mL of *methanol R*, heat in a water-bath at 60 °C for 1 min and filter.

*Reference solution*. Dissolve 5 mg of *thymol R* and 8 mg of *borneol R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *methylene chloride R* (2:98 *V/V*).

*Application*: 20 µL [or 1 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 105 °C for 5 min and examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no intense coloured zones above the zone due to borneol in the chromatogram obtained with the reference solution.

***Acanthopanax giraldii***. The presence of scaly covering trichomes on the outer surface of the root bark indicates adulteration by *Acanthopanax giraldii*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

**Extractable matter**: minimum 16.0 per cent.

To 2.00 g of the powdered herbal drug (250) (*2.9.12*) add a mixture of 8 g of *water R* and 12 g of *ethanol (96 per cent) R* and allow to macerate for 2 h, shaking frequently. Filter, evaporate the filtrate to dryness on a water-bath *in vacuo* and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 320 mg.

**04/2019:2999**
**corrected 10.0**

# ACHYRANTHES BIDENTATA ROOT

## Achyranthis bidentatae radix

DEFINITION

Whole or fragmented, dried principal root of *Achyranthes bidentata* Blume with secondary roots removed.

*Content*: minimum 0.1 per cent of total sterones, expressed as β-ecdysterone ($C_{27}H_{44}O_7$; $M_r$ 480.6) (dried drug).

IDENTIFICATION

A. *Whole drug*. The whole root is slender, cylindrical, straight or slightly curved, 13-90 cm long and 4-10 mm in diameter. The outer surface is greyish-yellow to greyish-brown or pale brown to pale yellowish-brown with fine longitudinal wrinkles, slightly twisted; transverse lenticels and sparse protruding rootlet scars are present. The root is easily broken and has a hard, fragile texture. The fracture is pale yellowish-brown, slightly horny. Numerous small vascular bundles appear as dots arranged in 2-4 whorls surrounding 2-3 larger central bundles; the xylem vessels are yellowish-white.

*Fragmented drug*. The fragmented drug occurs as short cylindrical pieces or as oblique slices, 3-10 mm in diameter. The outer surface is greyish-yellow to greyish-brown or pale brown to pale yellowish-brown with fine longitudinal wrinkles, slightly twisted; transverse lenticels and sparse protruding rootlet scars may be visible. The texture is hard. The cut surface is pale yellowish-brown, yellowish-brown or brown, slightly horny. Numerous small vascular bundles appear as dots arranged in 2-4 whorls surrounding 2-3 larger central bundles; the xylem vessels are yellowish-white.

B. Microscopic examination (*2.8.23*). The powder is pale brown or whitish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2999.-1): numerous fragments of parenchyma [B, E] consisting of thin-walled, rounded, ovoid or subrectangular cells, some of which contain triangular, pointed, subsquare or irregularly shaped sandy calcium oxalate microcrystals [Ba, Ea]; bundles of vessels [C, F], reticulate [Fa] or bordered pitted (8-93 µm in diameter) [Ca, Fb], sometimes accompanied by fibres [Cb], with slightly thickened walls and sparse, oblique-slit-shaped, cross-shaped or V-shaped pits; numerous fragments of isolated vessels [H]; cork fragments with more or less square, rectangular, rounded or polygonal cells (surface view [A]); the cork layers (transverse section [G]) are superimposed and associated with the 1st layers of parenchyma [Ga]; numerous scattered microcrystals of calcium oxalate [D], clearly visible in polarised light.

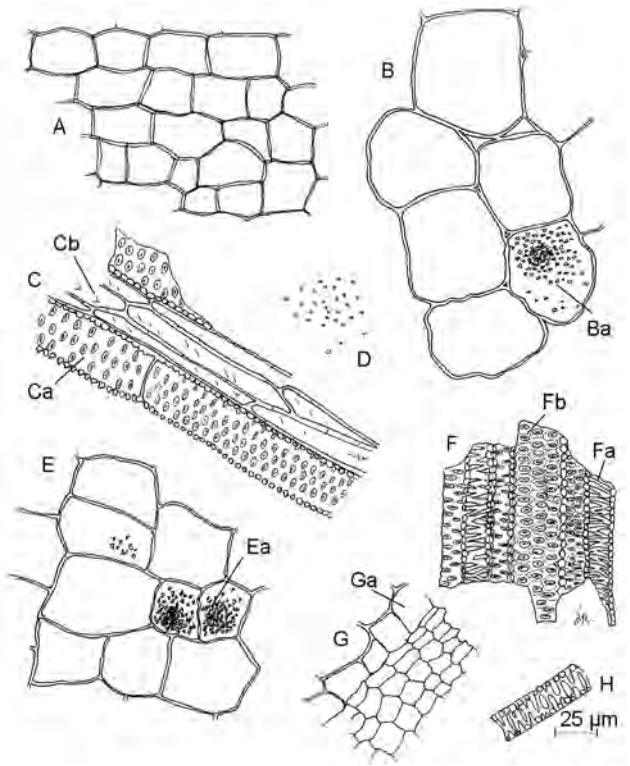


Figure 2999.-1. – *Illustration for identification test B of powdered herbal drug of Achyranthes bidentata root*

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of a mixture of 2 volumes of *water R* and 8 volumes of *methanol R*. Sonicate for 30 min and centrifuge. Take up the residue with 8 mL of *water R*. Sonicate for 1 min (or shake for 1 min), centrifuge and combine the supernatants. Condition a solid phase extraction (SPE) column containing 0.200 g of *octadecylsilyl silica gel for chromatography R* with 5 mL of *methanol R* and then with 3 mL of *water R* at a rate of 1 drop per second, ensuring that the column does not dry out. Transfer the solution to the SPE column. Allow to drain, ensuring that the column does not dry out, and discard the eluate. Wash the column with 2 mL of *water R*. Elute the column using 1 mL of *methanol R*. Collect the eluate.

*Reference solution (a).* Dissolve 1.0 mg of *β-ecdysterone R* and 1.0 mg of *ginsenoside Ro R* in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution (b).* Mix 1.0 mL of reference solution (a) and 3.0 mL of *methanol R*.

*Reference solution (c).* Dissolve 1 mg of *β-ecdysterone R* and 1 mg of *cyasterone R* in *methanol R* and dilute to 2 mL with the same solvent.

*Intensity marker*: β-ecdysterone.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *methylene chloride R* (1:1:5:14 *V/V/V/V*).

*Application*: 3 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of cold air for 5 min.

*Detection*: treat with a 100 g/L solution of *sulfuric acid R* in *ethanol (96 per cent) R* and heat at 100 °C for 5 min; examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows in the upper third 2 distinct zones, which may be touching; the lower zone (β-ecdysterone) shows a blue fluorescence and the upper zone (cyasterone) shows a bright blue fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint fluorescent zones may be present.

| Top of the plate | |
|---|---|
| | 3 reddish zones, faint |
| β-Ecdysterone: a blue fluorescent zone | A blue fluorescent zone (β-ecdysterone) |
| | A green zone, faint to intense |
| | A green zone, faint to equivalent |
| | A green zone |
| Ginsenoside Ro: a green zone | A green zone, faint to intense (ginsenoside Ro) |
| | A reddish-zone, faint to equivalent (maybe overlapping with the zone below) |
| | A reddish-violet zone, intense |
| | A reddish-violet zone, intense |
| **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R* (20:80 *V/V*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 45 mL of the solvent mixture and 5 mL of *butanol R* saturated with *water R*. Sonicate for 30 min and filter. Evaporate the filtrate to dryness under reduced pressure. Dissolve the residue in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 6.0 mg of *β-ecdysterone CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 2.0 mL of the solution to 5.0 mL with the solvent mixture.

*Reference solution (b).* To 5 mg of *Achyranthes bidentata root dry extract for system suitability HRS* add 5 mL of the solvent mixture. Sonicate for 10 min and shake well. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *solid core octadecylsilyl silica gel for chromatography R* (2.7 µm);
– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;
– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 85 | 15 |
| 3 - 18 | 85 → 81 | 15 → 19 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *Achyranthes bidentata root dry extract for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to sterones 2 and 3 and peak 4; use the chromatogram obtained with reference solution (a) to identify the peak due to β-ecdysterone (sterone 1).

*Relative retention* with reference to β-ecdysterone (retention time = about 12.5 min): peak 4 = about 1.04; sterone 2 = about 1.11; sterone 3 = about 1.19.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to β-ecdysterone and peak 4.

Calculate the percentage content of total sterones expressed as β-ecdysterone using the following expression:

$$\frac{A_1 \times m_2 \times 4 \times p}{A_2 \times m_1 \times 25}$$

$A_1$ = sum of the peak areas due to sterones 2 and 3 and β-ecdysterone in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to β-ecdysterone in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *β-ecdysterone CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of β-ecdysterone in *β-ecdysterone CRS*.

Herbal drugs

**01/2009:0310**

# AGAR

## Agar

DEFINITION

Polysaccharides from various species of Rhodophyceae mainly belonging to the genus *Gelidium*. It is prepared by treating the algae with boiling water; the extract is filtered whilst hot, concentrated and dried.

CHARACTERS

*Appearance*: powder or crumpled strips 2-5 mm wide or sometimes flakes, colourless or pale yellow, translucent, somewhat tough and difficult to break, becoming more brittle on drying.

Mucilaginous taste.

IDENTIFICATION

A. Examine under a microscope. When mounted in *0.005 M iodine*, the strips or flakes are partly stained brownish-violet. Magnified 100 times, they show the following diagnostic characters: numerous minute, colourless, ovoid or rounded grains on an amorphous background; occasional brown, round or ovoid spores with a reticulated surface, measuring up to 60 µm, may be present. Reduce to a powder, if necessary. The powder is yellowish-white. Examine under a microscope using *0.005 M iodine*. The powder presents angular fragments with numerous grains similar to those seen in the strips and flakes; some of the fragments are stained brownish-violet.

B. Dissolve 0.1 g with heating in 50 mL of *water R*. Cool. To 1 mL of the mucilage carefully add 3 mL of *water R* so as to form 2 separate layers. Add 0.1 mL of *0.05 M iodine*. A dark brownish-violet colour appears at the interface. Mix. The liquid becomes pale yellow.

C. Heat 5 mL of the mucilage prepared for identification test B on a water-bath with 0.5 mL of *hydrochloric acid R* for 30 min. Add 1 mL of *barium chloride solution R1*. A white turbidity develops within 30 min.

D. Heat 0.5 g with 50 mL of *water R* on a water-bath until dissolved. Only a few fragments remain insoluble. During cooling, the solution gels between 35 °C and 30 °C. Heat the gel thus obtained on a water-bath; it does not liquefy below 80 °C.

TESTS

**Swelling index** (*2.8.4*): minimum 10 and within 10 per cent of the value stated on the label, determined on the powdered herbal drug (355) (*2.9.12*).

**Insoluble matter**: maximum 1.0 per cent.

To 5.00 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of *water R* and 14 mL of *dilute hydrochloric acid R*. Boil gently for 15 min with frequent stirring. Filter the hot liquid through a tared, sintered-glass filter (160) (*2.1.2*), rinse the filter with hot *water R* and dry at 100-105 °C. The residue weighs a maximum of 50 mg.

**Gelatin**. To 1.00 g add 100 mL of *water R* and heat on a water-bath until dissolved. Allow to cool to 50 °C. To 5 mL of this solution add 5 mL of *picric acid solution R*. No turbidity appears within 10 min.

**Loss on drying** (*2.2.32*): maximum 20.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the swelling index.

**01/2015:2147**

# AGNUS CASTUS FRUIT

## Agni casti fructus

DEFINITION

Whole, ripe, dried fruit of *Vitex agnus-castus* L.

*Content*: minimum 0.08 per cent of casticin ($C_{19}H_{18}O_8$; $M_r$ 374.3) (dried drug).

IDENTIFICATION

A. Agnus castus fruit is oval or almost globular, with a diameter of up to 5 mm. The persistent calyx is greenish-grey, finely pubescent, ends in 4-5 short teeth and envelops 2/3 to 3/4 of the surface of the fruit. The blackish-brown fruit consists of a pericarp that becomes progressively sclerous up to the endocarp. The style scar is often visible. Some of the fruits may retain a stalk, about 1 mm long. A transverse section of the fruit shows 4 locules, each containing an elongated seed.

B. Microscopic examination (*2.8.23*). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the outer epidermis of the calyx composed of polygonal cells densely covered with short, bent or undulate, uni-, bi- or tri-cellular uniseriate covering trichomes; cells of the epicarp with thick walls and well-marked, large pits; isolated glandular trichomes with a unicellular stalk and a uni- or multi-cellular head; layers of parenchyma from the outer part of the mesocarp, some containing brown pigment, others extending into septa; fragments from the inner part of the mesocarp composed of thin-walled, pitted, sclerenchymatous cells and of typical isodiametric sclerous cells with very thick, deeply grooved walls and a narrow, stellate lumen; small brown cells of the endocarp; fragments of the testa containing areas of fairly large, thin-walled lignified cells with reticulate bands of thickening; numerous fragments of the endosperm composed of thin-walled parenchymatous cells containing aleurone grains and oil droplets.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min. Allow to cool and filter.

*Reference solution*. Dissolve 0.5 mg of *aucubin R* and 1 mg of *agnuside R* in *methanol R* and dilute to 1.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 10 µL [or 8 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 8 cm [or 5 cm].

*Drying*: in air.

*Detection*: treat with *anhydrous formic acid R* and heat at 120 °C for 10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Agnuside: a blue zone | A blue zone (agnuside) |
| ——— | ——— |
| Aucubin: a blue zone | A blue zone (aucubin) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3.0 per cent.

**Other species of *Vitex*, in particular *Vitex negundo* L.** No fruit of other species with a much greater diameter is present.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Extract 1.000 g of the powdered herbal drug (355) (*2.9.12*) with 40 mL of *methanol R* for 2 min using a suitable-speed homogeniser. Collect the supernatant liquid and filter into a 250 mL flask. Repeat the extraction with a further 40 mL of *methanol R*, collecting the supernatant liquid and filtering as before. Rinse the residue carefully with a small quantity of *methanol R*. Combine the methanol extracts and rinsings and evaporate to dryness *in vacuo* in a water-bath at not more than 30 °C. With the aid of ultrasound, dissolve the residue obtained in *methanol R* and dilute to 20.0 mL with the same solvent. Filter the solution through a membrane filter (nominal pore size 0.45 µm). Dilute 2.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution.* Suspend a quantity of *agnus castus fruit dry extract HRS* corresponding to 0.10 mg of casticin in 7.5 mL of *methanol R*, sonicate for 5 min and dilute to 10.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 5.88 g/L solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 70 → 45 | 30 → 55 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 348 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *agnus castus fruit dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to penduletin and casticin.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to penduletin and casticin.

Calculate the percentage content of casticin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 10}{A_2 \times m_1}$$

$A_1$ = area of the peak due to casticin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to casticin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug used to prepare the test solution, in grams;

$m_2$ = mass of *agnus castus fruit dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of casticin in *agnus castus fruit dry extract HRS*.

**07/2019:2309**

# AGNUS CASTUS FRUIT DRY EXTRACT

## Agni casti fructus extractum siccum

DEFINITION

Dry extract produced from *Agnus castus fruit (2147)*.

*Content*: minimum 0.1 per cent of casticin ($C_{19}H_{18}O_8$; $M_r$ 374.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (40-80 per cent *V/V*).

CHARACTERS

*Appearance*: brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 0.5 g of the extract to be examined in 5 mL of *methanol R* and sonicate for 15 min. Shake the mixture vigorously 3 or 4 times during the procedure. Filter.

*Reference solution.* Dissolve 1 mg of *caffeic acid R* and 4 mg of *homoorientin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *anhydrous formic acid R*, *toluene R*, *tetrahydrofuran R* (1:2:8:16 *V/V/V/V*).

*Application*: 12 µL [or 3 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 11 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and dry in a current of cold air, then treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*, allow to dry and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a light blue fluorescent zone | An orange fluorescent zone and a white or pale yellow fluorescent zone, partly superimposed |
| ——— | ——— |
| | Several light blue fluorescent zones |
| | 1 or 2 yellow fluorescent zones |
| ——— | ——— |
| | A light blue fluorescent zone |
| Homoorientin: a yellow fluorescent zone | A yellow fluorescent zone (homoorientin) |
| | A yellow fluorescent zone |
| **Reference solution** | **Test solution** |

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Suspend 0.200 g of the extract to be examined in 15 mL of *methanol R*, sonicate for 5 min and dilute to 20.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Suspend a quantity of *agnus castus fruit dry extract HRS* corresponding to 0.10 mg of casticin in 7.5 mL of *methanol R*, sonicate for 5 min and dilute to 10.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 5.88 g/L solution of *phosphoric acid R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 70 → 45 | 30 → 55 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 348 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *agnus castus fruit dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to penduletin and casticin.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to penduletin and casticin.

Calculate the percentage content of casticin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to casticin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to casticin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *agnus castus fruit dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of casticin in *agnus castus fruit dry extract HRS*.

**01/2011:1587**

# AGRIMONY

## Agrimoniae herba

DEFINITION

Dried flowering tops of *Agrimonia eupatoria* L.

*Content*: minimum 2.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The stem is green or, more usually, reddish, cylindrical and infrequently branched. It is covered with long, erect or tangled hairs. The leaves are compound imparipennate with 3 or 6 opposite pairs of leaflets, with 2 or 3 smaller leaflets between. The leaflets are deeply dentate to serrate, dark green on the upper surface, greyish and densely tomentose on the lower face. The flowers are small and form a terminal spike. They are pentamerous and borne in the axils of hairy bracts, the calyces closely surrounded by numerous terminal hooked spires, which occur on the rim of the hairy receptacle. The petals are free, yellow and deciduous. Fruit-bearing obconical receptacles, with deep furrows and hooked bristles, are usually present at the base of the inflorescence.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green or grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1587.-1): numerous straight or bent, unicellular, long, thick-walled (about 500 µm) covering trichomes [Ab, Ca, F], finely warty, and sometimes spirally marked, often fragmented [F]; fragments of the epidermis of the stems [A] with stomata [Aa], covering trichomes [Ab] and glandular trichomes [Ac]; fragments of upper leaf epidermis (surface view [C]) with straight walls bearing covering trichomes [Ca], accompanied by palisade parenchyma [Cb], with some of the cells containing calcium oxalate prisms [Cc]; fragments of lower leaf epidermis in surface view [J] with sinuous walls and abundant stomata [Ja], mostly anomocytic (*2.8.3*) but occasionally anisocytic, and glandular trichomes [Jb]; ovoid to subspherical pollen grains, with 3 pores and a smooth exine [D]; glandular trichomes with a multicellular, uniseriate stalk and a unicellular to quadricellular head [B, Jb]; fragments of the stems [H] with groups of fibres [Ha] and parenchymatous cells, some of which contain cluster crystals of calcium oxalate [Hb]; small spiral vessels from the leaflets [G]; fragments of large, spiral or bordered-pitted vessels from the stem [E].

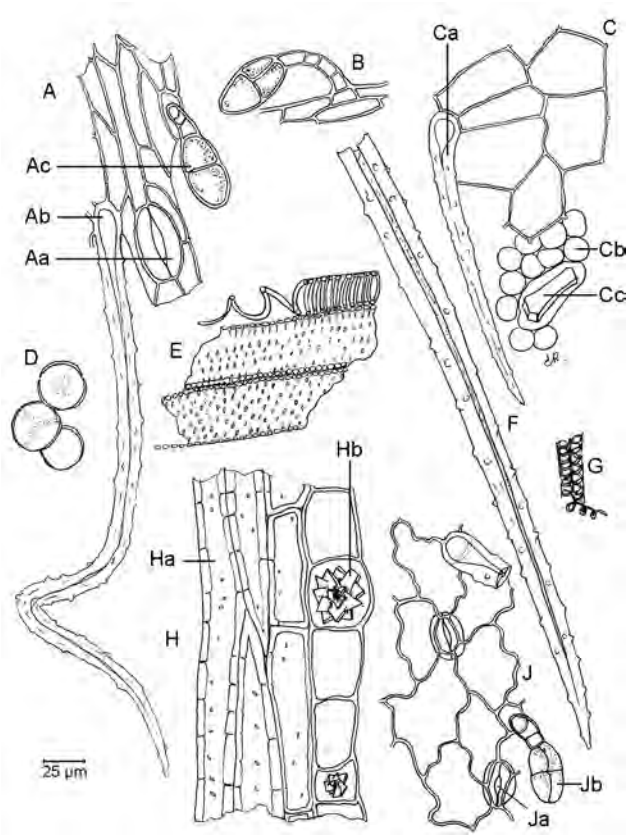


Figure 1587.-1. – *Illustration for identification test B of powdered herbal drug of agrimony*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *methanol R*. Heat with shaking at 40 °C for 10 min. Filter.

*Reference solution.* Dissolve 1.0 mg of *isoquercitroside R* and 1.0 mg of *rutoside trihydrate R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 12 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| **Top of the plate** | |
|---|---|
| | An orange fluorescent zone may be present (quercitrin) |
| Isoquercitroside: an orange fluorescent zone | An orange fluorescent zone (isoquercitroside) |
| | An orange fluorescent zone (hyperoside) |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 1.000 g of the powdered herbal drug (180) (*2.9.12*).

**01/2019:2472**
**corrected 10.0**

# AKEBIA STEM

## Akebiae caulis

DEFINITION

Whole or fragmented, dried stem of *Akebia quinata* (Houtt.) Decne. or *Akebia trifoliata* (Thunb.) Koidz., or a mixture of the 2 species.

*Content*: minimum 0.15 per cent of oleanolic acid ($C_{30}H_{48}O_3$; $M_r$ 456.7) (dried drug).

IDENTIFICATION

A. The whole drug is cylindrical, often slightly twisted, 30-70 cm long and 0.5-2 cm in diameter. Externally light grey or greyish-brown, rough, with irregular longitudinal and horizontal cracks and irregular longitudinal furrows. The outer bark is sometimes easily removed; the exposed inner surface is yellowish-brown or reddish-brown. Lenticels are distinct. Nodes are sometimes swollen, with scars of lateral branches. The texture is light and compact. The fracture is uneven; the bark is relatively thick and easily distinguished from the wood, sometimes with arched hollows around the outside of the wood; it is yellowish-brown or reddish-brown, with pale yellow, granular dots visible; the wood is yellowish-white, with distinct radial striations, the pith is small, occasionally hollowed, yellowish-white or yellowish-brown.

The fragmented drug occurs as rounded, elliptical or irregular slices, 0.5-2 cm in diameter. Externally light grey or greyish-brown, rough, with irregular longitudinal and horizontal cracks and occasional lenticels; the outer bark is easily removed, the inner exposed surface is yellowish-brown or reddish-brown. The texture is light, compact and easily broken. The cut surface has a relatively thick, yellowish-brown, greyish-brown or reddish-brown bark that is easily distinguished from the wood and appears granular and sometimes lamellar in transverse section. Hollows appearing as arches may be present around the outside of the wood. The wood is yellowish-white, with distinct radial striations and distinct xylem vessels. The pith is small, occasionally hollowed, yellowish-white or yellowish-brown.

B. Microscopic examination (*2.8.23*). The powder is pale yellow to light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2472.-1): groups of fibres, frequently broken [C], whose narrow lumens contain small prisms of calcium oxalate [Ca]; these groups are usually surrounded by crystal sheaths of calcium oxalate [Cb]; groups of parenchymatous cells with thick, pitted walls [F], some containing compact masses of large polyhedral or prismatic crystals of calcium oxalate [Fa]; some of these cells are isolated [G]; cork fragments consisting of reddish-brown polygonal cells with brown contents (surface view [A]); fragments of parenchyma consisting of cells with thin colourless walls (transverse section [K], longitudinal section [L]); isolated cubic or prismatic crystals of calcium oxalate [D]; rare tetragonal, polygonal, elongated or rounded sclereids with very thick walls and granular contents [B, M]; abundant single or

grouped narrow tracheids with oblique pits and very fine oblique or criss-cross lines [E]; vessels often fragmented, mostly bordered pitted, up to 120-160 µm in diameter [J]; ligneous, more or less rectangular parenchyma cells of the medullary rays with very thick, pitted walls [H].

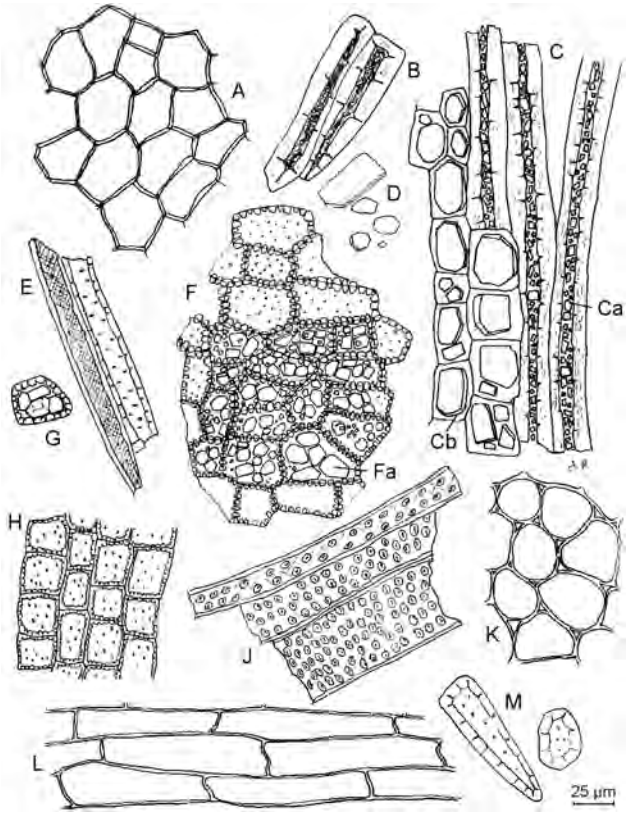


Figure 2472.-1. – *Illustration for identification test B of powdered herbal drug of akebia stem*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (1400) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min and filter or centrifuge. Use the filtrate or the supernatant as the test solution.

*Reference solution.* Dissolve 1 mg of *hederagenin R* and 1 mg of *oleanolic acid R* in 8 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *toluene R* (5:20:80 *V/V/V*).

*Application*: 15 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *ethanol (96 per cent) R*, heat at 105 °C until the spots appear and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other coloured zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint violet-blue zone |
| _____ | _____ |
| | A violet-blue zone |
| Oleanolic acid: a pink zone | A faint pink zone (oleanolic acid) |
| | Sequence of narrow violet-blue zones |
| Hederagenin: a pinkish-blue zone | |
| | A prominent violet-blue zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Total ash** (*2.4.16*): maximum 6.5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 3 h.

**Aristolochic acids** (*2.8.21, Method A*). It complies with the test.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 2.00 g of the powdered herbal drug (1400) (*2.9.12*) add 50 mL of *methanol R*, sonicate for 30 min and filter. Wash the residue with an appropriate quantity of *methanol R*, combine the filtrate and washings and evaporate to dryness. Dissolve the residue in 10 mL of *water R*, extract with 3 quantities, each of 20 mL, of *butanol R* saturated with *water R*, combine the extracts and evaporate to dryness. Take up the residue with a mixture of 2 mL of *hydrochloric acid R* and 20 mL of *methanol R* and heat under a reflux condenser for 2 h. Add 10 mL of *water R*, extract with 2 quantities, each of 20 mL, of *methylene chloride R*, combine the extracts and evaporate to dryness. Dissolve the residue in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 20.0 mg of *oleanolic acid CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *ursolic acid R* in reference solution (a) and dilute to 10.0 mL with the same solution.

*Reference solutions (c), (d), (e), (f), (g), (h).* Dilute reference solution (a) with *methanol R* to obtain 6 reference solutions of oleanolic acid, the concentrations of which span the expected value in the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *triethylamine R*, *glacial acetic acid R*, *water for chromatography R*, *methanol R* (0.02:0.04:13:87 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector: the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion for signal-to-noise ratio:

– *carrier gas*: *nitrogen R*;
– *pressure*: 350 kPa;
– *evaporator temperature*: 60 °C.

*Injection*: 10 µL.

*Run time*: 24 min.

*Retention time*: oleanic acid = about 19 min; ursolic acid = about 20 min.

*System suitability*:

- *resolution*: minimum 1.7 between the peaks due to oleanolic acid and ursolic acid in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 40 for the peak due to oleanolic acid in the chromatogram obtained with reference solution (a).

Establish a calibration curve with the logarithm of the concentration (in milligrams per 10 mL) of reference solutions (c), (d), (e), (f), (g) and (h) (corrected by the declared percentage content of *oleanolic acid CRS*) as the abscissa and the logarithm of the corresponding peak area as the ordinate.

Calculate the percentage content of oleanolic acid using the following expression:

$$\frac{10^A}{m \times 10}$$

$A$ = logarithm of the concentration of oleanolic acid in the test solution, determined from the calibration curve;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams.

**07/2019:1387**

# ALCHEMILLA

## Alchemillae herba

DEFINITION

Whole or cut, dried, flowering, aerial parts of *Alchemilla vulgaris* L. *s.l.*

*Content*: minimum 6.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The greyish-green, partly brownish-green, radical leaves, which are the main part of the drug, are reniform or slightly semicircular with a diameter generally up to 8 cm, seldom up to 11 cm and have 7-9 or 11 lobes and a long petiole. The smaller, cauline leaves, which have a pair of large stipules at the base, have 5-9 lobes and a shorter petiole or they are sessile. The leaves are densely pubescent especially on the lower surface and have a coarsely serrated margin. Young leaves are folded with a whitish-silvery pubescence; older leaves are slightly pubescent and have a finely meshed venation, prominent on the lower surface. The greyish-green or yellowish-green petiole is pubescent, about 1 mm in diameter, with an adaxial groove. The apetalous flowers are yellowish-green or light green and about 3 mm in diameter. The calyx is double with 4 small segments of the epicalyx alternating with 4 larger sepals, subacute or triangular. They are 4 short stamens and a single carpel with a capitate stigma. The greyish-green or yellowish-green stem is pubescent, more or less longitudinally wrinkled and hollow.

B. Microscopic examination (*2.8.23*). The powder is yellow to yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1387.-1): narrow, unicellular covering trichomes, isolated or included in an epidermis, whole [Ba] or sheared off [Ee], up to 1 mm long, with thick, lignified, spirally striated walls, bluntly pointed at the apex and pitted at the base; fragments of leaves (transverse section [E]) consisting of a slightly cuticularised and stomatiferous upper epidermis [Ea], 2 layers of palisade parenchyma with the cells of the upper layer [Eb] being 2-3 times longer than those of the lower layer [Ec] (which contain cluster crystals of calcium oxalate, up to 25 µm in diameter), and spongy parenchyma [Ed]; fragments of the upper epidermis of the leaf (surface view [C]) consisting of somewhat sinuous cells with beaded anticlinal walls [Ca], a few anomocytic stomata (*2.8.3*) [Cb], and accompanied by palisade parenchyma [Cc]; fragments of the lower epidermis of the leaf (surface view [A]) composed of sinuous cells with unevenly thickened anticlinal walls [Aa], anomocytic stomata [Ab] and covering trichomes or their scars [Ac], rounded, thick-walled and channelled; fragments from the petioles and stems [K], consisting of spiral or bordered-pitted vessels [Ka] and lignified fibres [Kb]; a few straight, conical hairs [D] from the corolla, about 300 µm long, with regularly thickened walls; parenchymatous cells of the mesophyll [H], with thin walls, containing cluster crystals of calcium oxalate [Ha], and spiral xylem vessels [Hb] accompanied by short fibres [Hc]; fragments of the epidermis of the stems [B] consisting of polygonal cells with straight, thin walls; spherical pollen grains [F], about 15 µm in diameter, with 3 distinct germinal pores and a granular exine; fragments of the corolla [J] with rectangular epidermal cells with sinuous walls [Ja] and parenchyma with polygonal cells, each containing a small cluster crystal of calcium oxalate [Jb]; occasional fragments of the ovary [G] with cells each containing a prism crystal of calcium oxalate.

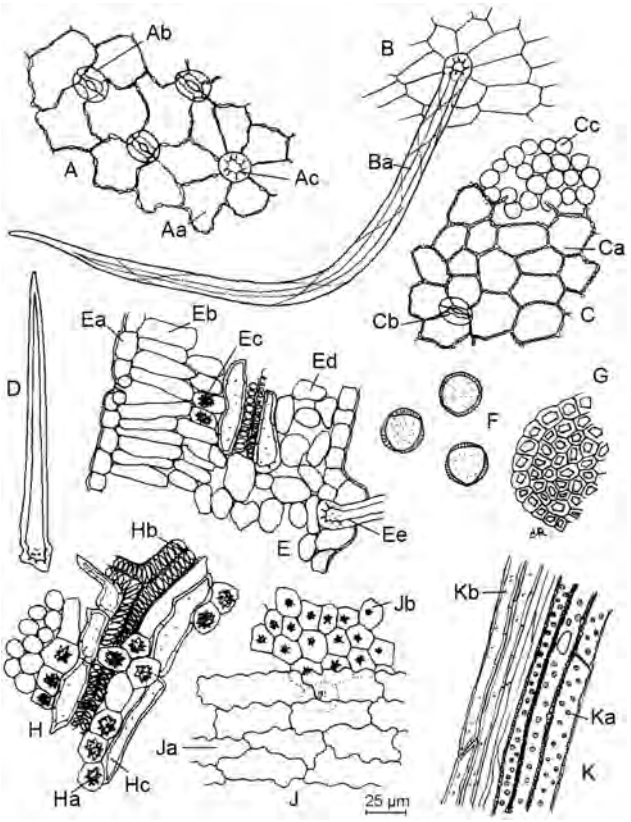


Figure 1387.-1. – *Illustration for identification test B of powdered herbal drug of alchemilla*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and heat in a water-bath at 70 °C under a reflux condenser for 5 min. Cool and filter.

*Reference solution.* Dissolve 1 mg of *caffeic acid R* and 1 mg of *chlorogenic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (8:8:84 *V/V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 5 min.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 red fluorescent zones (chlorophyll) |
| Caffeic acid: a light blue florescent zone | 1 or 2 intense light blue fluorescent zones |
| | 1 or several intense green or greenish-yellow fluorescent zones |
| Chlorogenic acid: a light blue fluorescent zone | An intense yellow or orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.50 g of the powdered herbal drug (355) (*2.9.12*).

**01/2015:0257**

# ALOES, BARBADOS

## Aloe barbadensis

DEFINITION

Concentrated and dried juice of the leaves of *Aloe barbadensis* Mill.

*Content*: minimum 28.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin ($C_{21}H_{22}O_9$; $M_r$ 418.4) (dried drug).

CHARACTERS

*Appearance*: dark brown masses, slightly shiny or opaque with a conchoidal fracture, or brown powder.

*Solubility*: partly soluble in boiling water, soluble in hot ethanol (96 per cent).

IDENTIFICATION

Examine the chromatograms obtained in the test for Cape aloes.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Aloe emodin: a yellow fluorescent zone | |
| | |
| Barbaloin: an orange fluorescent zone | An orange fluorescent zone (barbaloin) |
| | A bluish-white fluorescent zone |
| | A bluish-green fluorescent zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Aloe emodin: a violet zone | |
| | |
| Barbaloin: a brown zone | A brown zone (barbaloin) |
| | A violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

**Cape aloes**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.25 g of the powdered herbal drug add 20 mL of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

*Reference solution*. Dissolve 2 mg of *aloe emodin R* and 2 mg of *barbaloin R* in *methanol R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 20 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no blue fluorescent zones above the orange fluorescent zone due to barbaloin.

*Detection B*: treat with a 100 g/L solution of *potassium hydroxide R* in *methanol R*, heat at 110 °C for 5 min and examine in daylight.

ASSAY

*Carry out the assay protected from bright light.*

Introduce 0.300 g of the powdered herbal drug (180) (*2.9.12*) into a 250 mL conical flask. Moisten with 2 mL of *methanol R*, add 5 mL of *water R* warmed to about 60 °C, mix, then add a further 75 mL of *water R* at about 60 °C and shake for 30 min. Cool, filter into a volumetric flask, rinse the conical flask and filter with 20 mL of *water R*, add the rinsings to the volumetric flask and dilute to 1.0 L with *water R*. Transfer 10.0 mL of this solution to a 100 mL round-bottomed flask containing 1 mL of a 600 g/L solution of *ferric chloride R* and 6 mL of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 mL of *water R*, 4 mL of *1 M sodium hydroxide* and 4 mL of *water R* and add the rinsings to the separating funnel. Shake the contents of the separating funnel with 3 quantities, each of 20 mL, of *ether R*. Wash the combined ether layers with 2 quantities, each of 10 mL, of *water R*. Discard the washings and dilute the organic phase to 100.0 mL with *ether R*. Evaporate 20.0 mL of the solution carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene derivatives, expressed as barbaloin, using the following expression:

$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of barbaloin to be 255.

$A$ = absorbance at 512 nm;

$m$ = mass of the substance to be examined, in grams.

STORAGE

In an airtight container.

**01/2017:0258**

# ALOES, CAPE

## Aloe capensis

DEFINITION

Concentrated and dried juice of the leaves of *Aloe ferox* Mill.

*Content*: minimum 18.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin ($C_{21}H_{22}O_9$; $M_r$ 418.4) (dried drug).

CHARACTERS

*Appearance*: dark brown masses tinged with green and having a shiny conchoidal fracture, or greenish-brown powder.

*Solubility*: partly soluble in boiling water, soluble in hot ethanol (96 per cent).

IDENTIFICATION

Examine the chromatograms obtained in the test for Barbados aloes.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Aloe emodin: a yellow fluorescent zone | |
| | A blue fluorescent zone |
| | A blue fluorescent zone |
| Barbaloin: an orange fluorescent zone | An orange fluorescent zone (barbaloin) |
| | A bluish-white fluorescent zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Aloe emodin: a violet zone | |
| Barbaloin: a brown zone | A brown zone (barbaloin) |
| **Reference solution** | **Test solution** |

TESTS

**Barbados aloes**. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the powdered herbal drug add 20 mL of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

*Reference solution.* Dissolve 2 mg of *aloe emodin R* and 2 mg of *barbaloin R* in *methanol R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 20 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no prominent bluish-green fluorescent zone below the bluish-white fluorescent zone in the lower third.

*Detection B*: treat with a 100 g/L solution of *potassium hydroxide R* in *methanol R*, heat at 110 °C for 5 min and examine in daylight.

*Results B*: the chromatogram obtained with the test solution shows no violet zone just below the zone due to barbaloin.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Introduce 0.400 g of the powdered herbal drug (180) (*2.9.12*) into a 250 mL conical flask. Moisten with 2 mL of *methanol R*, add 5 mL of *water R* warmed to about 60 °C, mix, then add a further 75 mL of *water R* at about 60 °C and shake for 30 min.

Cool, filter into a volumetric flask, rinse the conical flask and filter with 20 mL of *water R*, add the rinsings to the volumetric flask and dilute to 1000.0 mL with *water R*. Transfer 10.0 mL of this solution to a 100 mL round-bottomed flask containing 1 mL of a 600 g/L solution of *ferric chloride R* and 6 mL of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 mL of *water R*, 4 mL of *1 M sodium hydroxide* and 4 mL of *water R* and add the rinsings to the separating funnel. Shake the contents of the separating funnel with 3 quantities, each of 20 mL, of *ether R*. Wash the combined ether layers with 2 quantities, each of 10 mL, of *water R*. Discard the washings and dilute the organic phase to 100.0 mL with *ether R*. Evaporate 20.0 mL of the solution carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene derivatives, expressed as barbaloin, using the following expression:

$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of barbaloin to be 255.

$A$ = absorbance at 512 nm;

$m$ = mass of the substance to be examined in grams.

STORAGE

In an airtight container.

**01/2015:0259**
**corrected 10.0**

# ALOES DRY EXTRACT, STANDARDISED

## Aloes extractum siccum normatum

DEFINITION

Standardised dry extract prepared from *Barbados aloes (0257)* or *Cape aloes (0258)*, or a mixture of both.

*Content*: 19.0 per cent to 21.0 per cent of hydroxyanthracene derivatives, expressed as barbaloin ($C_{21}H_{22}O_9$; $M_r$ 418.4), adjusted if necessary (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using boiling water.

CHARACTERS

*Appearance*: brown or yellowish-brown powder.

*Solubility*: sparingly soluble in boiling water.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the extract to be examined add 20 mL of *methanol R* and heat to boiling in a water-bath. Shake for a few minutes and decant the solution. Store at about 4 °C and use within 24 h.

*Reference solution.* Dissolve 2 mg of *aloe emodin R* and 2 mg of *barbaloin R* in *methanol R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 20 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| Aloe emodin: a yellow fluorescent zone | | |
| | | A blue fluorescent zone |
| | | A blue fluorescent zone |
| Barbaloin: an orange fluorescent zone | An orange fluorescent zone (barbaloin) | An orange fluorescent zone (barbaloin) |
| | A bluish-white fluorescent zone | A bluish-white fluorescent zone |
| | A bluish-green fluorescent zone | |
| **Reference solution** | **Test solution (*Barbados aloes*)** | **Test solution (*Cape aloes*)** |

*Detection B*: treat with a 100 g/L solution of *potassium hydroxide R* in *methanol R*, heat at 110 °C for 5 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| Aloe emodin: a violet zone | | |
| Barbaloin: a brown zone | A brown zone (barbaloin) | A brown zone (barbaloin) |
| | A violet zone | |
| **Reference solution** | **Test solution (*Barbados aloes*)** | **Test solution (*Cape aloes*)** |

TESTS

**Loss on drying** (*2.8.17*): maximum 4.0 per cent.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Introduce 0.400 g of the extract to be examined into a 250 mL conical flask. Moisten with 2 mL of *methanol R*, add 5 mL of *water R* warmed to about 60 °C, mix, add a further 75 mL of *water R* at about 60 °C and shake for 30 min. Cool, filter into a volumetric flask, rinse the conical flask and the filter with 20 mL of *water R*, add the rinsings to the volumetric flask and

dilute to 1.0 L with *water R*. Transfer 10.0 mL of this solution to a 100 mL round-bottomed flask containing 1 mL of a 600 g/L solution of *ferric chloride R* and 6 mL of *hydrochloric acid R*. Heat in a water-bath under a reflux condenser for 4 h, with the water level above that of the liquid in the flask. Allow to cool, transfer the solution to a separating funnel, rinse the flask successively with 4 mL of *water R*, 4 mL of *1 M sodium hydroxide* and 4 mL of *water R*, and add the rinsings to the separating funnel. Shake the contents of the separating funnel with 3 quantities, each of 20 mL, of *ether R*. Wash the combined ether layers with 2 quantities, each of 10 mL, of *water R*. Discard the washings and dilute the organic layer to 100.0 mL with *ether R*. Evaporate 20.0 mL carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 512 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene derivatives, expressed as barbaloin, using the following expression:

$$\frac{A \times 19.6}{m}$$

i.e. taking the specific absorbance of barbaloin to be 255.

$A$ = absorbance at 512 nm;

$m$ = mass of the substance to be examined, in grams.

**04/2014:2554**
**corrected 10.0**

# AMOMUM FRUIT

## Amomi fructus

DEFINITION

Dried, whole or fragmented, peeled or unpeeled ripe fruit of *Amomum villosum* Lour. or *Amomum longiligulare* T.L.Wu.

*Content*:

- *essential oil*: minimum 30 mL/kg for *A. villosum* (anhydrous drug) and minimum 10 mL/kg for *A. longiligulare* (anhydrous drug);
- *bornyl acetate* ($C_{12}H_{20}O_2$; $M_r$ 196.3): minimum 30.0 per cent of the essential oil.

IDENTIFICATION

A. *A. villosum*. The fruit is an indehiscent capsule, ovoid or ellipsoidal, indistinctly 3-ridged, up to 2 cm long and 1.5 cm in diameter. The outer surface is brown and covered with soft spiny protuberances. The apex bears the remains of the perianth and the base usually bears a stalk. The pericarp is thin and soft. The seeds are agglomerated into 3 masses, each consisting of 5-25 seeds, separated by whitish septa. The seeds are hard, irregularly polyhedral, 2-3 mm in diameter, reddish-brown or blackish-brown on the surface, finely wrinkled and covered with a pale brown, transparent, membranous aril; the endosperm is whitish-grey.

*A. longiligulare*. The fruit is an indehiscent capsule, long, ovoid or ellipsoidal, distinctly 3-ridged, up to 2 cm long and 1.2 cm in diameter. The outer surface is brown, longitudinally channelled and scaly, and bears numerous soft spiny protuberances, sometimes branched. The apex bears a prominent stylopodium and the base shows the scar of the stalk. The pericarp is thick and hard. The seeds are agglomerated into 3 relatively small masses, each consisting of about 2-24 seeds, separated by incomplete septa. The seeds are small, 1.5-2 mm in diameter, polyhedral with rounded edges, reddish-brown or blackish-brown on the surface, finely wrinkled and covered with a pale brown, transparent, membranous aril; the endosperm is whitish.

B. *A. villosum*. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of epicarp consisting of brownish-orange polyhedral cells containing microcrystals and prisms of calcium oxalate, clearly visible in polarised light and sometimes long covering trichomes, about 250 µm long, usually unicellular, straight or bent, with slightly and regularly thickened walls; fragments of mesocarp composed of thin-walled polygonal cells and round oil cells, with orange to dark brown contents; vascular bundles consisting of vessels, mainly spiral, and fibres with thick and pitted walls; fragments of the outer testa consisting of a layer of cells, fusiform in surface view, with slightly and regularly thickened walls, usually accompanied by a layer of rectangular or polyhedral cells, perpendicular to the previous layers, and sometimes by underlying oil cells; brownish-red fragments of the inner testa, in surface view, composed of very regularly polyhedral cells with heavily thickened walls and a punctiform lumen; brownish-red fragments of the inner testa, in transverse section, composed of palisade cells with strongly thickened inner and lateral walls. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous fragments composed of sub-rectangular or irregular cells, filled with aggregates of small starch granules; some of these cells also contain small prisms of calcium oxalate; a few aggregates of small starch granules are also present.

*A. longiligulare*. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of epicarp consisting of brownish-orange, polyhedral or elongated cells containing microcrystals and prisms of calcium oxalate, clearly visible in polarised light; fragments of mesocarp composed of thin-walled polygonal cells and occasional round oil cells with orange to dark brown contents; vascular bundles composed of spiral or reticulate vessels and fibres with thick and distinctly pitted walls; fragments of the aril consisting of elongated, very thin-walled cells, some of which contain prisms and microcrystals that are clearly visible in polarised light; fragments of the outer testa consisting of a layer of cells, fusiform in surface view, with slightly and regularly thickened walls, usually accompanied by a layer of rectangular or polyhedral cells with brown contents, perpendicular to the previous layers; brownish-red fragments of the inner testa, composed of very thick-walled and very regularly polyhedral cells, in surface view; brownish-red fragments of the inner testa, in transverse section, composed of rectangular or palisade cells with heavily thickened inner and lateral walls. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous fragments of the endosperm with sub-rectangular or polyhedral cells, filled with small starch granules aggregated into masses and free aggregates of starch granules.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of a mixture of equal volumes of *toluene R* and *xylene R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 10 µL of *bornyl acetate R*, 10 µL of *cineole R* and 10 mg of *borneol R* in 1 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *ethyl acetate R*, *toluene R* (7:93 *V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 366 nm.

*Results A*: see below the sequence of zones present in the chromatogram obtained with the test solution. The reference solution shows no spots at 366 nm. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint blue fluorescent zone |
| _____ | _____ |
| | 1 or 2 red fluorescent zones |
| _____ | _____ |
| | A red fluorescent zone |
| | 1 or 2 red fluorescent zones |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 3 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish-brown zone |
| _____ | _____ |
| | A bluish-violet zone |
| Bornyl acetate: a greyish-brown zone | A greyish-brown zone (bornyl acetate) |
| | A pink zone |
| 1,8-Cineole: a bluish-violet zone | |
| | A greyish-brown zone |
| Borneol: a greyish-brown zone at the border between the middle and lower thirds | A greyish-brown zone (borneol) at the border between the middle and lower thirds |
| _____ | _____ |
| | A bluish-violet zone |
| | 1 or 2 bluish-violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.2.13*): maximum 120 mL/kg, determined by distillation on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the herbal drug reduced to a powder (1400) (*2.9.12*) immediately before the assay, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.5 mL of *trimethylpentane R* in the graduated tube. Distil at a rate of 3-3.5 mL/min for 5 h.

**Bornyl acetate**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dilute a volume of the essential oil-trimethylpentane mixture obtained in the assay of essential oil corresponding to 150 µL of the essential oil in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 25 mg of *camphor R* in *heptane R*, add 25 µL of *bornyl acetate R* and dilute to 5.0 mL with *heptane R*.

*Reference solution (b)*. Dilute 5 µL of *bornyl acetate R* to 100.0 mL with *heptane R*. Dilute 1.5 mL of the solution to 10.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 60 | 60 → 210 |
| Injection port | | 230 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to camphor and bornyl acetate.

*Relative retention* with reference to camphor (retention time = about 26 min): bornyl acetate = about 1.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 5 between the peaks due to camphor and bornyl acetate.

Calculate the percentage content of bornyl acetate. Disregard any peak due to the solvent or with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

LABELLING

The label states the species present.

**04/2017:2712**
**corrected 9.3**

# ANDROGRAPHIS HERB

## Andrographidis herba

DEFINITION

Dried, whole or fragmented flowering and/or fruit-bearing aerial parts of *Andrographis paniculata* (Burm.f.) Nees.

*Content*: minimum 0.80 per cent of the sum of andrographolide ($C_{20}H_{30}O_5$; $M_r$ 350.4) and 14-deoxy-11,12-didehydroandrographolide ($C_{20}H_{28}O_4$; $M_r$ 332.4) (dried drug).

IDENTIFICATION

A. The whole aerial parts consist of the following: the green, brown, or yellowish-green stems are mostly square in cross-section with a white, central pith, about 50-70 cm long, 1.2-5 mm (sometimes up to 9 mm) in diameter, frequently branched, slightly swollen at the nodes, with longitudinal wrinkles on the outer surface; the leaves are opposite, simple, glabrous, shortly petiolate or nearly sessile with a lamina that is crumpled and easily broken; when whole, the lamina is lanceolate to ovate-lanceolate, 2-15 cm long and 1-5 cm wide; the apex is acuminate, the base cuneate and decurrent, the margin is entire or undulate, the upper surface is green to dark almost blackish-green and the lower surface is greyish-green or light green. Cystoliths may be visible as short, lighter-coloured markings on the upper surface of the leaf and stem. The texture is fragile. The fragmented aerial parts occur as small pieces of flattened green, brown, or yellowish-green stems, mostly square in cross-section with a white, central pith, 1.2-5 mm (sometimes up to 9 mm) in diameter and 0.5-5.0 cm in length, with longitudinal wrinkles on the outer surface, and fragments of crumpled lamina, green to dark almost blackish-green on the upper surface and greyish-green or light green on the lower surface. Cystoliths may be visible as short, lighter-coloured markings on the upper surface of the leaf and stem. The texture is fragile. Partial remains of flowers are sometimes present consisting of fine and delicate pedicels and sepals with a pointed apex bearing glandular hairs with a long stalk and dark head. The light yellowish-brown or greenish-yellow fruits are narrow linear-lanceolate or oblong capsules, with 2 locules, widely opening from the apex to form a V-shaped fruit, with 2 valves that mostly no longer contain seeds; when seeds are present, they are dark brown or brownish-yellow and sub-polyhedral to square; funicle remains are often present.

B. Microscopic examination (*2.8.23*). The powder is dark green or greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2712.-1): fragments of stem and leaf tissue with cystolithic cells, diacytic stomata, and short glandular trichomes. Ovoid, rounded or elongated cystolithic cells up to 250 µm long [leaf: Ab, Bd, Dc] and up to 39 µm wide, with concentric striations. Short glandular trichomes with a unicellular stalk and a rounded, multicellular head, 27-37 µm in diameter, consisting of 6-8 (sometimes 5) cells [leaf: Ad, Bc]; green fragments of leaf, fragments of the upper epidermis of the leaves (surface view [B]) with rigid-walled polygonal cells [Ba], cystolithic cells [Bb] and rare diacytic stomata (*2.8.3*); fragments of the lower epidermis of the leaves (surface view [A]) with slightly sinuous to very sinuous cells [Aa], cystolithic cells [Ab] and numerous diacytic stomata [Ac]; rare glandular trichomes [Ad, Bc] and very rare, unicellular or multicellular (2-4 cells) conical covering trichomes with a pointed or rounded apex, thick, sometimes lignified cell walls and a striated cuticle, up to 190 µm long and 40 µm wide at the base (transverse section [Da]), which may be present on both epidermises; fragments of leaf mesophyll [D] consisting of 1 or 2 layers of palisade parenchyma, one of which, under the upper epidermis [Db], is composed of long narrow cells (up to 150 µm long and 8-17 µm wide), cells containing cystoliths, (surface view [Ab, Bd], transverse section [Dc]) and loosely arranged spongy parenchyma [Dd]; yellowish-brown or colourless fragments of stem with polygonal epidermal cells, cystolithic cells, diacytic stomata and short glandular hairs; fragments of pith from the stem with polygonal cells with pitted walls; numerous fragments of vascular tissue [E] from the stems, with reticulate or pitted [Ea] vessels and long, thick-walled and pitted fibres [Eb]. If flowers are present, the powder also shows glandular trichomes (which may exceed 500 µm in length) with a multicellular, uniseriate stalk and a multicellular cup-shaped head [C], short glandular trichomes and fragmented or whole unicellular or multicellular covering trichomes [F] from the outer surface of the flower (sepals) and pedicel. If ripe fruits are present, the powder shows fragments of epicarp [G] consisting of elongated cells [Ga], covered by a thin striated cuticle, and diacytic stomata [Gb] and also shows groups of short, very thick-walled fibres arranged in perpendicular layers [H], from the mesocarp. Tricolpate pollen with a fine spiculate exine may be present.

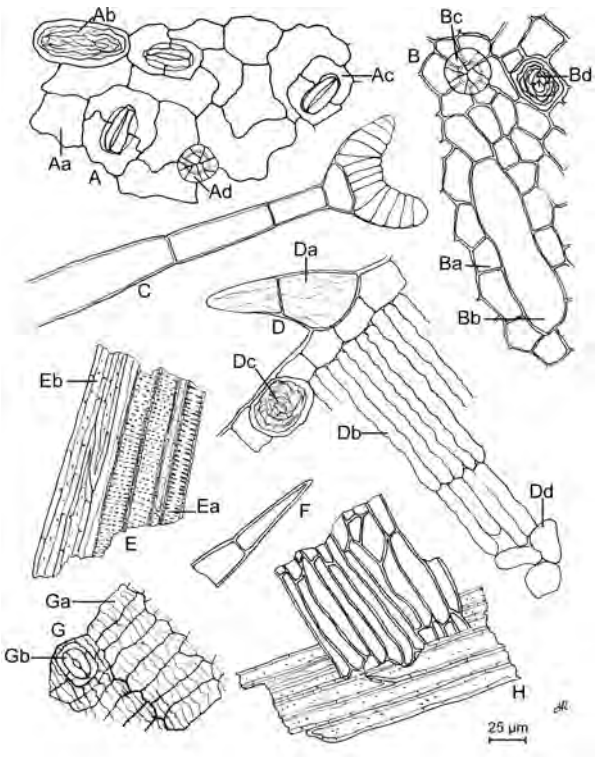


Figure 2712.-1. – *Illustration for identification test B of powdered herbal drug of andrographis herb*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge or filter; use the supernatant or filtrate.

*Reference solution.* Dissolve 1 mg of *14-deoxy-11,12-didehydroandrographolide R* and 2 mg of *andrographolide R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *methanol R*, *ethyl acetate R*, *methylene chloride R* (4:30:40 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 100 °C for 5 min; examine in ultraviolet light at 366 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A red fluorescent zone |
| | 2 violet-brown fluorescent zones |
| _____ | _____ |
| 14-deoxy-11,12-didehydroandrographolide: a pale violet fluorescent zone | A pale violet fluorescent zone (14-deoxy-11,12-didehydroandrographolide) |
| _____ | _____ |
| Andrographolide: a pale violet fluorescent zone | A pale violet fluorescent zone (andrographolide) |
| | A violet-brown fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 2.000 g of the powdered herbal drug (355) (*2.9.12*) in 50 mL of *methanol R*. Heat on a water-bath under a reflux condenser for 15 min. Cool. Collect the methanolic extract. Repeat the extraction with 3 additional quantities, each of 50 mL, of *methanol R*. Combine the extracts. Filter. Evaporate under vacuum. Dilute to 50.0 mL with *methanol R*. Dilute 25.0 mL of the solution to 50.0 mL with *acetonitrile for chromatography R*.

*Reference solution (a).* Dissolve 5.0 mg of *andrographolide CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *acetonitrile for chromatography R*.

*Reference solution (b).* Disperse 2 g of *andrographis herb HRS* in 50 mL of *methanol R*. Heat on a water-bath under a reflux condenser for 15 min. Cool. Collect the methanolic extract. Repeat the extraction with 3 additional quantities, each of 50 mL, of *methanol R*. Combine the extracts. Filter. Evaporate under vacuum. Dilute to 50.0 mL with *methanol R*. Dilute 25.0 mL of the solution to 50.0 mL with *acetonitrile for chromatography R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with embedded polar groups R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*, adjusted to pH 3.2 with *anhydrous formic acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 90 | 10 |
| 3 - 11 | 90 → 62 | 10 → 38 |
| 11 - 25 | 62 | 38 |
| 25 - 30 | 62 → 50 | 38 → 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 223 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *andrographis herb HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrographolide.

*Relative retention* with reference to andrographolide (retention time = about 16 min): 14-deoxyandrographolide = about 1.5; 14-deoxy-11,12-didehydroandrographolide = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to 14-deoxyandrographolide and 14-deoxy-11,12-didehydroandrographolide.

Calculate the percentage content of the sum of andrographolide and 14-deoxy-11,12-didehydroandrographolide using the following expression:

$$\frac{(A_1 + (A_3 \times 1.45)) \times m_2 \times p}{A_2 \times m_1 \times 5}$$

$A_1$ = area of the peak due to andrographolide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to andrographolide in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to 14-deoxy-11,12-didehydroandrographolide in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *andrographolide CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of andrographolide in *andrographolide CRS*;

1.45 = peak correlation factor between andrographolide and 14-deoxy-11,12-didehydroandrographolide.

**01/2016:2661**
**corrected 8.7**

# ANEMARRHENA ASPHODELOIDES RHIZOME

## Anemarrhenae asphodeloides rhizoma

DEFINITION

Dried, whole or fragmented, peeled or unpeeled, rhizome of *Anemarrhena asphodeloides* Bunge, with roots removed, collected in spring or autumn.

*Content*: minimum 0.5 per cent of mangiferin ($C_{19}H_{18}O_{11}$; $M_r$ 422.3) (dried drug).

IDENTIFICATION

A. The rhizome with cork intact (called maozhimu) is somewhat flattened, slightly curved, occasionally branched, 2-15 cm long and 0.8-2 cm in diameter. The outer surface is brownish-yellow, reddish-brown or brown, with the upper surface generally showing a concave groove. It consists of many annular nodes with dense brownish-yellow fibrous remains of the vasculature of the leaf bases growing upwards on both sides of the groove; at the branched end of the rhizome, whitish-yellow or light brown leaf blade bases may also be present; the lower surface is coarsely longitudinally wrinkled, showing depressed or

protruding dotted root scars; the surface of the root scars is whitish-yellow or light brown and may show a circular ring of xylem tissue protruding from its centre. The rhizome with outer cork removed (called guangzhimu) is 5-15 cm in diameter. The outer surface is yellowish-white, with twisted grooves, sometimes with leaf scars and root scars.

The fragmented rhizome occurs in slices about 1.1-4 mm thick and 0.8-3.4 cm in diameter, often with a furrowed outline. It can be cut either longitudinally or transversely or obliquely. The outer surface is yellowish-white (guangzhimu) or brownish-yellow, reddish-brown or brown (maozhimu); it shows fibrous (vascular bundles) remains of leaves as well as annular striations and round root scars or short remnants of the root bases. The section is pale yellow or brownish-yellow, marked with numerous whitish-yellow pits or lines corresponding to vascular bundles.

B. Microscopic examination (*2.8.23*). The powder is pale yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2661.-1): very numerous, whole or fragmented raphides of calcium oxalate up to 150 µm long, free [D] or included in oval or rounded parenchyma cells of various sizes [A, E]; fragments of parenchyma, with thin walls [B] or with slightly thickened and pitted walls [H]; fragments of vessels [F] sometimes spiral [Fa], more often reticulate [Fb] or pitted [Fc], often accompanied by cells containing large acicular crystals of calcium oxalate, in bundles, whole or broken, up to 300 µm long and 7 µm wide [Fd]; free bundles of large acicular crystals may also be observed [J]; a few fragments of cork, orange-brown, present only in the unpeeled herbal drug [K]; a few fragments of parenchyma with elongated or polygonal cells with heavily lignified and pitted walls from the remains of the aerial parts [C]; a few fibres in bundles, with slightly thickened walls and rounded ends, from the remains of the vascular bundles of the leaves [G].

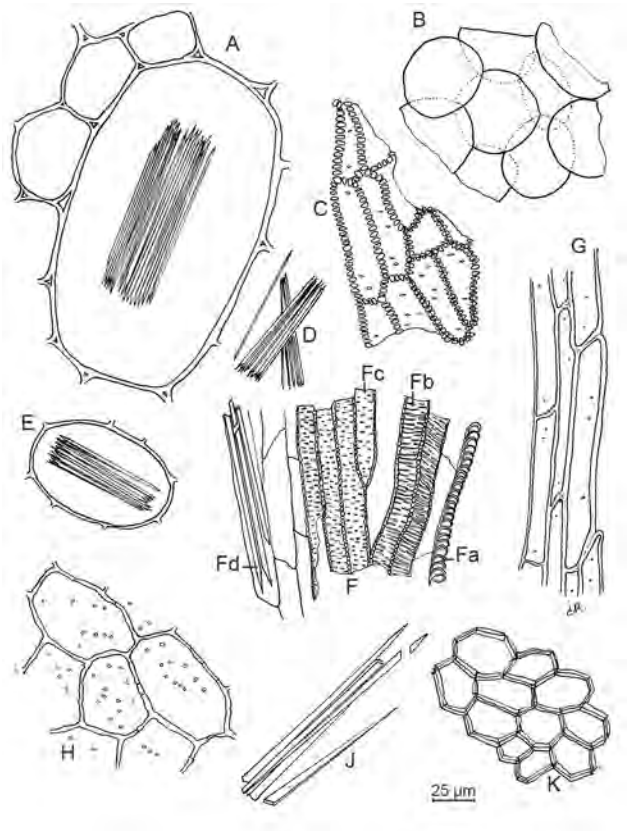


Figure 2661.-1. – *Illustration for identification test B of powdered herbal drug of Anemarrhena asphodeloides rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of a mixture of *glacial acetic acid R* and *methanol R* (10:90 *V/V*). Sonicate for 10 min. Centrifuge or filter; use the supernatant or filtrate.

*Reference solution.* Dissolve 1 mg of *arbutin R* and 1 mg of *aescin R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (4:30:70 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 3 min; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| | A brown zone |
| | A green zone |
| ——— | ——— |
| | A brown zone |
| Aescin: a brownish zone | |
| | A pale green zone |
| | A pale green zone |
| ——— | ——— |
| Arbutin: a greenish zone | A brown zone |
| | A brownish-green zone |
| | A brown zone |
| | A very broad light blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.100 g of the powdered herbal drug (355) (*2.9.12*) in 25.0 mL of a 50 per cent *V/V* solution of *methanol R*, weigh. Sonicate for 30 min. Allow to cool and weigh again. Compensate for the loss of solvent with a 50 per cent *V/V* solution of *methanol R*, mix and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *mangiferin CRS* in a 50 per cent *V/V* solution of *methanol R* and dilute to 100.0 mL with the same solution.

*Reference solution (b).* Disperse 0.1 g of *Anemarrhena rhizome for system suitability HRS* in 25.0 mL of a 50 per cent *V/V* solution of *methanol R*, weigh. Sonicate for 30 min. Allow

to cool and weigh again. Compensate for the loss of solvent with a 50 per cent *V/V* solution of *methanol R*, mix and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 0.2 per cent *V/V* solution of *glacial acetic acid R* (15:85 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 258 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of mangiferin.

*Identification of peaks*: use the chromatogram supplied with *Anemarrhena rhizome for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to isomangiferin.

*Retention time*: mangiferin = about 4.0 min; isomangiferin = about 4.9 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to mangiferin and isomangiferin.

Calculate the percentage content of mangiferin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.25}{A_2 \times m_1}$$

$A_1$ = area of the peak due to mangiferin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to mangiferin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *mangiferin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of mangiferin in *mangiferin CRS*.

**01/2013:1857**

# ANGELICA ARCHANGELICA ROOT

## Angelicae archangelicae radix

DEFINITION

Whole or cut, carefully dried rhizome and root of *Angelica archangelica* L. (syn. *A. officinalis* Hoffm.).

*Content*: minimum 2.0 mL/kg of essential oil (dried drug).

CHARACTERS

Bitter taste.

IDENTIFICATION

A. The rhizome is greyish-brown or reddish-brown, with transversely annulated thickenings. The base bears greyish-brown or reddish-brown, cylindrical, longitudinally furrowed, occasionally branched roots often with incompletely encircling, transverse ridges. The apex sometimes shows remnants of stem and leaf bases. The fracture is uneven. The transversely cut surface shows a greyish-white, spongy, distinctly radiate bark, in which the secretory channels are visible as brown spots, and a bright yellow or greyish-yellow wood which, in the rhizome, surrounds the greyish or brownish-white pith.

B. Microscopic examination (*2.8.23*). The powder is brownish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1857.-1): fragments of cork consisting of several layers of thin-walled, greyish-brown or reddish-brown cells (surface view [C], transverse section [E]); large, yellowish-brown secretory channels, whole or fragmented (transverse section [A], longitudinal section [F]); fragments of medullary rays, 2 or 4 cells wide [G]; fragments of xylem [B] consisting of lignified vessels with reticulate thickening [Ba] occurring singly or in small groups, and unlignified parenchyma in which some of the cells associated with the vessels are collenchymatously thickened. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous, simple starch granules 2-4 µm in diameter, free or included in parenchyma cells [D].

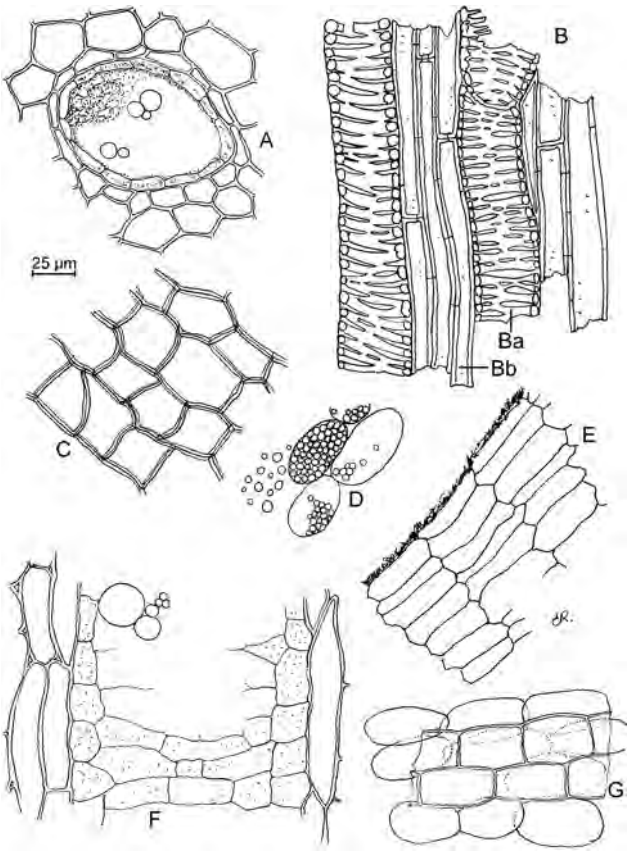


Figure 1857.-1. – *Illustration for identification test B of powdered herbal drug of angelica archangelica root*

C. Examine the chromatograms obtained in the test for other species of *Angelica*, *Levisticum* and *Ligusticum* described in the European Pharmacopoeia.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish-white fluorescent zone | |
| _____ | _____ |
| Osthole: a blue fluorescent zone | A blue fluorescent zone |
| Imperatorin: a whitish fluorescent zone | A whitish fluorescent zone |
| | A blue fluorescent zone |
| _____ | _____ |
| | 3 blue fluorescent zones |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a blue fluorescent zone | |
| _____ | _____ |
| Osthole: a quenching zone | A quenching zone |
| Imperatorin: a quenching zone | A quenching zone |
| | A quenching zone |
| _____ | _____ |
| | Several quenching zones |
| **Reference solution** | **Test solution** |

TESTS

**Other species of *Angelica, Levisticum* and *Ligusticum* described in the European Pharmacopoeia**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the freshly powdered herbal drug (355) (*2.9.12*) add 4 mL of *heptane R*, close and sonicate for 5 min. Centrifuge the mixture and use the supernatant.

*Reference solution*. Dissolve 1 mg of *imperatorin R*, 1 mg of *(Z)-ligustilide R* and 1 mg of *osthole R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no zone at the position of (*Z*)-ligustilide in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no zone at or just below the position of (*Z*)-ligustilide in the chromatogram obtained with the reference solution.

**Foreign matter** (*2.8.2*): maximum 5 per cent of leaf bases and stem bases, maximum 5 per cent of discoloured pieces and maximum 1 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Reduce the herbal drug to a powder (500) (*2.9.12*) and immediately use 40.0 g for the determination. Use a 2 L round-bottomed flask, 10 drops of *liquid paraffin R*, 500 mL of *water R* as distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 4 h.

**01/2018:2556**

# ANGELICA DAHURICA ROOT

## Angelicae dahuricae radix

DEFINITION

Dried, whole or fragmented root, with rootlets removed, of *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. collected in summer or autumn.

*Content*: minimum 0.08 per cent of imperatorin ($C_{16}H_{14}O_4$; $M_r$ 270.3) (dried drug).

IDENTIFICATION

A. The non-fragmented drug consists of conical roots, about 10-25 cm long and 1.2-3.1 cm in diameter. The root crown is sub-quadrangular and obtuse and shows stem scars on prominences. It tapers to the tip. The outer surface is brownish-grey or yellowish-brown and clearly striated longitudinally, showing scars of the rootlets and lenticel-like transverse protuberances, some of them arranged in 4 longitudinal rows. The texture is compact, hard and heavy. The fracture is white or whitish-grey and mealy. The cambium occurs as a fine-lined, distinct brown ring; it is subrounded, subsquare or subrectangular. Numerous brown to yellowish dots, corresponding to secretory canals, are visible in the cortex.

The fragmented drug occurs as subrounded or subsquare slices with an irregular margin, 1.2-3.1 cm in diameter and 0.1-0.4 cm thick. The cut surface is white, yellowish-white or greyish-white and mealy. The cambium occurs as a fine-lined, distinct brown ring; it is subrounded, subsquare or subrectangular. Numerous brown to yellowish dots, corresponding to secretory canals, are visible in the cortex.

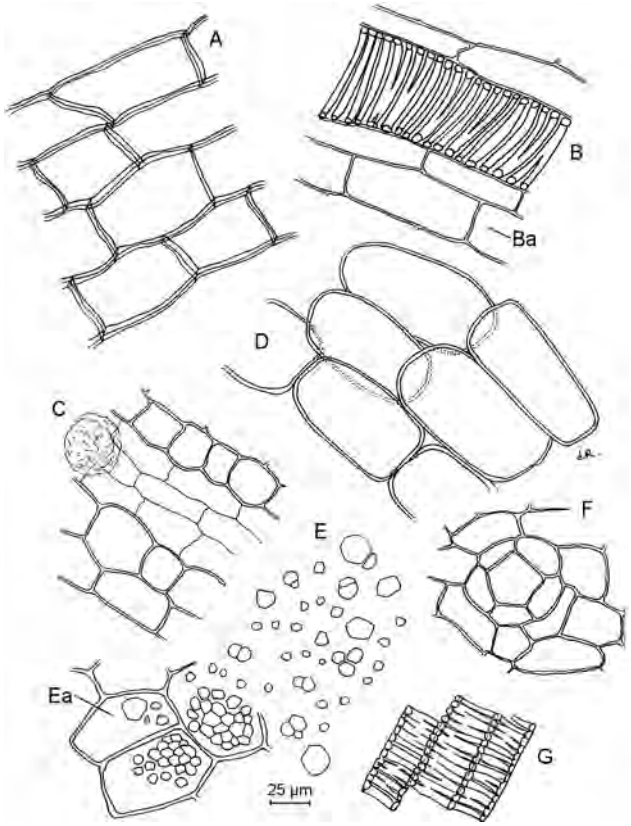


Figure 2556.-1. – *Illustration for identification test B of powdered herbal drug of Angelica dahurica root*

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2556.-1): reticulate vessels [B, G], free or in groups of 2 or 3 [G], and accompanied by xylem parenchyma cells with thin cellulose walls [Ba]; numerous fragments of parenchyma with ovoid cells [D]; a few orange cork fragments, consisting of several layers of superimposed cells (surface view [A]); secretory canals (longitudinal section [C], transverse section [F]), usually broken, with yellow or pale brown contents and droplets of essential oil. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous starch granules varying in size from 5 µm to 25 µm, simple or 2-8 compound; most are polyhedral, either due to compound granules breaking up or to compression in the cells; they are either isolated [E] or included in parenchyma cells [Ea].

C. Examine the chromatograms obtained in the test for other officinal species of *Angelica*, *Levisticum* and *Ligusticum*.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish-white fluorescent zone | |
| _____ | _____ |
| | A whitish fluorescent zone |
| Osthole: a blue fluorescent zone | A blue fluorescent zone |
| Imperatorin: a whitish fluorescent zone | A whitish fluorescent zone (imperatorin) |
| _____ | _____ |
| | A whitish fluorescent zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution, in particular in the lower third of the chromatogram, below the zone due to imperatorin.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a blue fluorescent zone | |
| _____ | _____ |
| | A quenching zone |
| Osthole: a quenching zone | |
| Imperatorin: a quenching zone | A quenching zone (imperatorin) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Results C*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 prominent reddish zones |
| (*Z*)-Ligustilide: a grey zone | |
| _____ | _____ |
| | A faint blue zone |
| Osthole: a violet zone | |
| Imperatorin: a reddish-grey zone | A yellow and violet double-zone |
| _____ | _____ |
| | A prominent violet zone |
| | A yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Other officinal species of *Angelica*, *Levisticum* and *Ligusticum*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 4 mL of *heptane R*, close and sonicate for 5 min. Centrifuge the mixture and use the supernatant.

*Reference solution.* Dissolve 1 mg of *imperatorin R*, 1 mg of *(Z)-ligustilide R* and 1 mg of *osthole R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no bluish-white fluorescent zone corresponding to the zone due to (*Z*)-ligustilide in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no blue fluorescent zone corresponding to the zone due to (*Z*)-ligustilide in the chromatogram obtained with the reference solution; the chromatogram obtained with the test solution shows no quenching zone corresponding to the zone due to osthole in the chromatogram obtained with the reference solution.

*Detection C*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 100 °C for 5 min and examine in daylight.

*Results C*: the chromatogram obtained with the test solution shows no violet zone corresponding to the zone due to osthole in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.400 g of the powdered herbal drug (355) (*2.9.12*) in 45 mL of *methanol R* and sonicate for 1 h. Cool and dilute to 50.0 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *imperatorin CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Disperse 80 mg of *Angelica dahurica root HRS* in 9 mL of *methanol R* and sonicate for 1 h. Cool and dilute to 10 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Precolumn*:

- *size*: *l* = 4 mm, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 45 | 55 |
| 15 - 33 | 45 → 5 | 55 → 95 |
| 33 - 35 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *Angelica dahurica root HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to phellopterin.

*Relative retention* with reference to imperatorin (retention time = about 5 min): phellopterin = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to imperatorin and phellopterin.

Calculate the percentage content of imperatorin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to imperatorin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to imperatorin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *imperatorin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of imperatorin in *imperatorin CRS*.

**01/2018:2557**

# ANGELICA PUBESCENS ROOT

## Angelicae pubescentis radix

DEFINITION

Whole or fragmented dried root, without rootlets, of *Angelica biserrata* (R.H.Shan & C.Q.Yuan) C.Q.Yuan & R.H.Shan (syn. *Angelica pubescens* Maxim. f. *biserrata* R.H.Shan et C.Q.Yuan), collected in early spring before sprouting, or in the end of autumn when stem and leaves wither.

*Content*: minimum 0.50 per cent of osthole ($C_{15}H_{16}O_3$; $M_r$ 244.3) (dried drug).

IDENTIFICATION

A. The taproot is more or less cylindrical, branching rapidly into 2-3 or more principal roots at the lower part; the whole is about 5-30 cm long. The root crown is enlarged, with transverse, annulated wrinkles and measures about 0.5-4.5 cm in diameter; it shows the remains of stems, leaves or buds. The greyish-brown or dark brown outer surface is longitudinally wrinkled and shows slightly prominent rootlet scars and transverse lenticel-like protuberances. The fracture shows greyish-yellow bark, with abundant distinct brown dots due to secretory canals that may be radially arranged; the cambium ring is brown and the wood is greyish-yellow or yellowish-brown; fine rod-like crystals may be present on the fractured surface.

The fragmented drug occurs as subrounded or irregular slices, 0.4-3.9 cm in diameter and 0.2-0.6 cm thick. Externally it is greyish-brown or brown, with wrinkles. Fine rod-like crystals may be present on the cut surface. The bark is greyish-white or greyish-brown, with abundant, distinct brown or yellowish-brown dots due to secretory canals that may be radially arranged. The cambium ring is brown.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown or brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2557.-1): fragments of vessels up to 90 µm in diameter with spiral or reticulate thickenings, free or in groups of 2 or 3 [E]; fragments of parenchyma with polyhedral cells [B]; fragments of phloem parenchyma [C] with fine, sinuous, fusiform cells, about 7-38 µm in diameter, with slightly thickened walls and fine,

oblique criss-cross striations [Ca], sometimes accompanied by medullary rays [Cb]; orange-brown cork fragments, consisting of several layers of superimposed, somewhat polyhedral cells (surface view [A]); secretory canals, usually broken, with yellow or pale brown contents and droplets of essential oil (transverse section [D], longitudinal section [F]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous small, rounded or ovoid, simple starch granules, up to 10 µm in diameter, with a punctiform hilum that is visible on the largest granules; a few starch granules consisting of 2-10 components are also present [G].

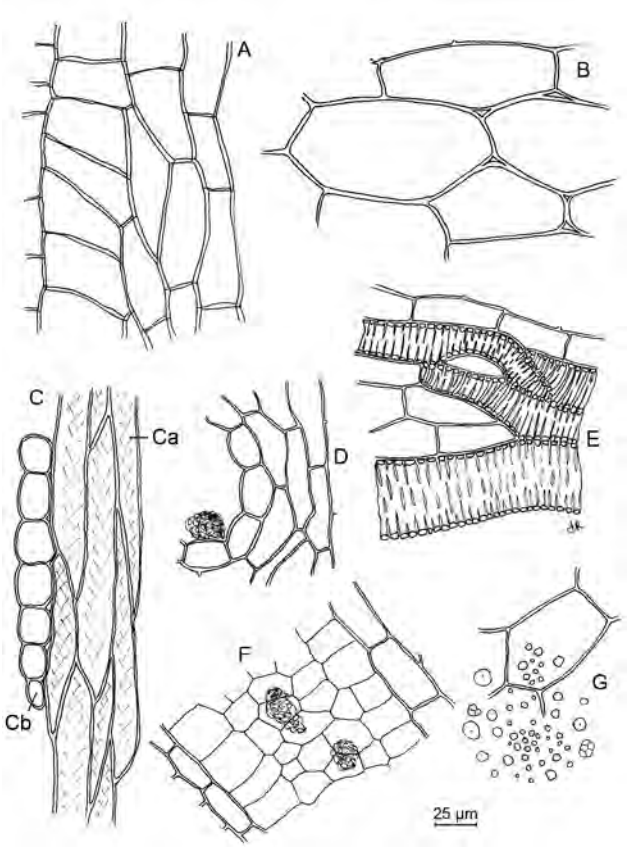


Figure 2557.-1. – *Illustration for identification test B of powdered herbal drug of Angelica pubescens root*

C. Examine the chromatograms obtained in the test for other officinal species of *Angelica*, *Levisticum* and *Ligusticum*.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish-white fluorescent zone | A bluish-white fluorescent zone |
| | A very faint whitish zone |
| Osthole: a blue fluorescent zone | A prominent blue fluorescent zone (osthole) |
| Imperatorin: a whitish fluorescent zone | A whitish fluorescent zone (may be missing) |
| | A blue fluorescent zone |
| | 3 blue fluorescent zones |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a blue fluorescent zone | A faint quenching zone |
| Osthole: a quenching zone | A quenching zone (osthole) |
| | A blue fluorescent zone |
| Imperatorin: a quenching zone | A quenching zone (may be missing) |
| | 2 or 3 quenching zones |
| **Reference solution** | **Test solution** |

*Results C*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A prominent reddish zone |
| (*Z*)-Ligustilide: a grey zone | |
| Osthole: a violet zone | A violet zone (osthole) |
| Imperatorin: a grey zone | A violet zone (may be missing) |
| | A prominent violet zone |
| | A yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Other officinal species of *Angelica*, *Levisticum* and *Ligusticum*.** Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 4 mL of *heptane R*, close and sonicate for 5 min. Centrifuge the mixture and use the supernatant.

*Reference solution*. Dissolve 1 mg of *imperatorin R*, 1 mg of *(Z)-ligustilide R* and 1 mg of *osthole R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no intense whitish fluorescent zone directly above the position of osthole and no blue fluorescent zone just below the position of imperatorin in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no blue fluorescent zone corresponding to the zone due to (*Z*)-ligustilide in the chromatogram obtained with the reference solution.

*Detection C*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 100 °C for 5 min and examine in daylight.

*Results C*: the chromatogram obtained with the test solution shows no zone corresponding to the zone due to (*Z*)-ligustilide in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.500 g of the powdered herbal drug (355) (*2.9.12*) in 18 mL of *methanol R* and sonicate for 30 min. Cool and dilute to 20.0 mL with *methanol R*. Mix and filter. Dilute 5.0 mL of the filtrate to 20.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 5.0 mg of *osthole CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Disperse 0.250 g of *Angelica pubescens root HRS* in 9 mL of *methanol R* and sonicate for 30 min. Cool and dilute to 10.0 mL with *methanol R*. Mix and filter. Dilute 5.0 mL of the filtrate to 20.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 2.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*: *water for chromatography R, acetonitrile R* (40:60 *V/V*).

*Flow rate*: 0.23 mL/min.

*Detection*: spectrophotometer at 322 nm.

*Injection*: 10 µL.

*Retention time*: osthole = about 8 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peak due to osthole and peak 2; use the chromatogram supplied with *Angelica pubescens root HRS* to identify peak 2.

Calculate the percentage content of osthole using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.8}{A_2 \times m_1}$$

$A_1$ = area of the peak due to osthole in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to osthole in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *osthole CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of osthole in *osthole CRS*.

**04/2017:2558**

# ANGELICA SINENSIS ROOT

## Angelicae sinensis radix

DEFINITION

Smoke-dried, whole or fragmented root, with rootlets removed, of *Angelica sinensis* (Oliv.) Diels collected in late autumn.

*Content*: minimum 0.050 per cent of *trans*-ferulic acid ($C_{10}H_{10}O_4$; $M_r$ 194.2) (dried drug).

IDENTIFICATION

A. Taproot branching rapidly into 10 or more conical principal roots; the whole is about 15-25 cm long. The annulated root crown is about 1.5-4 cm in diameter; its blunt, rounded tip shows the yellowish-green remains of stems and petioles of leaves. The outer surface is light brownish-yellow or dark brown, lumpy, irregularly striated longitudinally and shows scars of secondary roots and transversal lenticel-like markings. The branching roots have a thick upper part (0.3-1 cm in diameter) and a thin lower part. They are frequently twisted and show few scars of secondary roots. The texture is friable. The fracture, yellowish-white or yellowish-brown, shows a thick bark with some clefts and numerous brown dots due to secretory canals. The cambium occurs as a yellowish-brown ring. The wood is light coloured.

   The fragmented roots occur as long strips about 1.5-2 mm thick, 1.5-4 cm wide at the root crown and 10-15 cm long.

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2558.-1): reticulate or scalariform vessels [D] up to 80 µm in diameter, free or in groups of 2 or 3 and accompanied by xylem parenchyma cells [Da] and medullary rays [Db]; numerous fragments of parenchyma with ovoid cells [C]; orange cork fragments, consisting of several layers of superimposed cells, more or less rectangular (surface view [A], transverse section [B]); very small calcium oxalate prisms in the cork [Aa], visible in polarised light; secretory canals, often broken, up to 170 µm in diameter (transverse section [G], longitudinal section [F]) with orange-yellow contents in droplets [Fa, Ga]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows small (less than 10 µm), simple, rounded or ovoid starch granules, usually included in parenchyma cells [E].

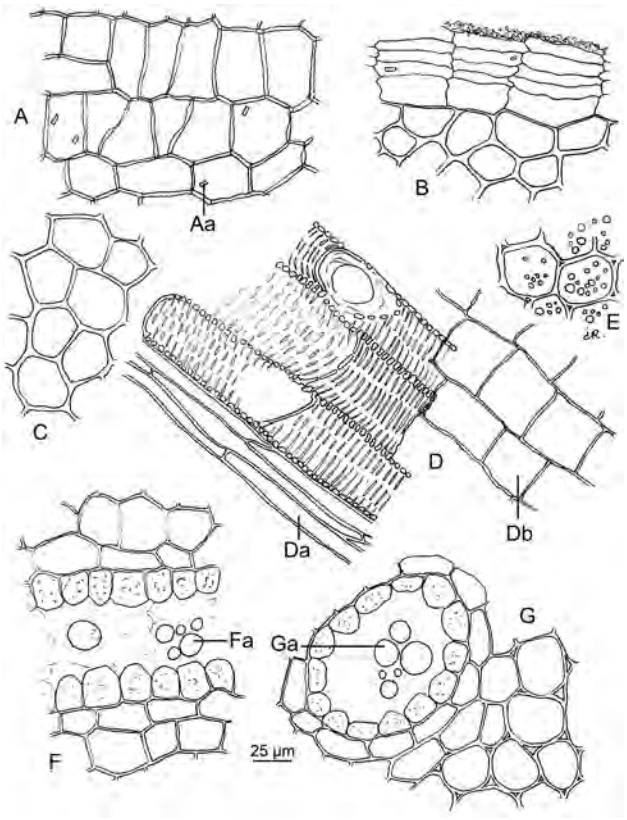


Figure 2558.-1. – *Illustration for identification test B of powdered herbal drug of Angelica sinensis root*

C. Examine the chromatograms obtained in the test for other officinal species of *Angelica*, *Levisticum* and *Ligusticum*.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish-white fluorescent zone<br>———<br>Osthole: a blue fluorescent zone<br>Imperatorin: a whitish fluorescent zone<br>——— | A prominent bluish-white fluorescent zone ((*Z*)-ligustilide)<br>——— |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a blue fluorescent zone<br>———<br>Osthole: a quenching zone<br>Imperatorin: a quenching zone<br>——— | A prominent blue fluorescent zone ((*Z*)-ligustilide)<br>A faint quenching zone<br>———<br>A faint quenching zone<br>——— |
| **Reference solution** | **Test solution** |

TESTS

**Other officinal species of *Angelica, Levisticum* and *Ligusticum*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 4 mL of *heptane R*, close and sonicate for 5 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *(Z)-ligustilide R*, 1 mg of *imperatorin R* and 1 mg of *osthole R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no intense blue fluorescent zone at or below the position of osthole in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no quenching zone at or below the position of imperatorin in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.200 g of the powdered herbal drug (355) (*2.9.12*) in 20.0 mL of a 70 per cent *V/V* solution of *methanol R* in a conical flask, stopper tightly and weigh. Heat under a reflux condenser for 30 min, cool and weigh again. Compensate the loss of solvent with a 70 per cent *V/V* solution of *methanol R*, mix well and allow to stand. Filter the supernatant through a membrane filter (nominal pore size 0.45 µm); use the filtrate.

*Reference solution (a).* In a brown-glass volumetric flask, dissolve 10.0 mg of *ferulic acid CRS* in a 70 per cent *V/V* solution of *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* In order to prepare *cis*-ferulic acid *in situ*, introduce 2 mL of reference solution (a) into a transparent vial and expose to ultraviolet light at 254 nm for about 60 min.

*Column*:

- *size*: *l* = 0.150 m, Ø = 2.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 35 °C.

*Mobile phase*: *acetonitrile R*, 0.085 per cent *V/V* solution of *phosphoric acid R* (17:83 *V/V*).

*Flow rate*: 0.23 mL/min.

*Detection*: spectrophotometer at 316 nm.

*Injection*: 10 µL.

*Retention time*: *trans*-ferulic acid = about 13 min; *cis*-ferulic acid = about 14 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.3 between the peaks due to *trans*-ferulic acid and *cis*-ferulic acid.

Calculate the percentage content of *trans*-ferulic acid using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 5}$$

$A_1$ = area of the peak due to *trans*-ferulic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to *trans*-ferulic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ferulic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of *trans*-ferulic acid in *ferulic acid CRS*.

**01/2008:0804**
**corrected 7.0**

# ANISE OIL

## Anisi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the dry ripe fruits of *Pimpinella anisum* L.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

Herbal drugs

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1 g of the substance to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 µL of *linalol R*, 30 µL of *anisaldehyde R* and 200 µL of *anethole R* in *toluene R* and dilute to 15 mL with the same solvent. Dilute 1 mL of this solution to 5 mL with *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (7:93 *V/V*).

*Application*: 5 µL as bands of 10 mm (for normal TLC plates) or 2 µL as bands of 10 mm (for fine particle size plates).

*Development*: over a path of 15 cm (for normal TLC plates) or over a path of 6 cm (for fine particle size plates).

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Anethole: a quenching zone | A very strong quenching zone (anethole) |
| _____ | _____ |
| | A quenching zone |
| Anisaldehyde: a quenching zone | A quenching zone (anisaldehyde) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *methyl 4-acetylbenzoate reagent R* and heat at 100-105 °C for 10 min; examine the still hot plate in daylight within 5 min.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet-brown zone (monoterpene hydrocarbons) (solvent front) |
| Anethole: a brown zone | A very strong brown zone (anethole), distinctly separated |
| _____ | _____ |
| | A grey zone |
| Anisaldehyde: a yellow zone | A yellow zone (anisaldehyde) |
| _____ | _____ |
| Linalol: a grey zone | A grey zone (linalol) |
| | A grey zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.980 to 0.990.

**Refractive index** (*2.2.6*): 1.552 to 1.561.

**Freezing point** (*2.2.18*): 15 °C to 19 °C.

**Fenchone**. Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

*Test solution*. Dissolve 400 µL of the substance to be examined in 2.0 mL of *hexane R*.

*Reference solution (a)*. Dilute 10 µL of *fenchone R* to 1.2 g with *hexane R*.

*Reference solution (b)*. Dilute 100 µL of reference solution (a) to 100 mL with *hexane R*.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limit*:

– *fenchone*: maximum 0.01 per cent.

**Foeniculin**. Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

*Test solution.* The substance to be examined.

*Reference solution (a).* Dilute 10 mg of the test solution to 1.000 g with *hexane R*. Dilute 0.5 mL of this solution to 100 mL with *hexane R*.

*Reference solution (b). Foeniculin for peak identification CRS.*

*System suitability*:

– the chromatogram obtained with reference solution (b) is similar to the chromatogram provided with *foeniculin for peak identification CRS*,

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

*Limit*: locate the peak due to foeniculin by comparison with the chromatogram provided with *foeniculin for peak identification CRS*.

– *foeniculin*: maximum 0.01 per cent.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test for fatty oils and resinified essential oils.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 200 µL of the substance to be examined in 1.0 mL of *hexane R*.

*Reference solution.* To 1.0 mL of *hexane R*, add 20 µL of *linalol R*, 20 µL of *estragole R*, 20 µL of *α-terpineol R*, 60 µL of *anethole R* and 30 µL of *anisaldehyde R*.

*Column*:

– *material*: fused silica,

– *size*: *l* = 30 m, Ø = 0.25 mm,

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 – 5 | 60 |
| | 5 – 80 | 60 → 210 |
| | 80 – 95 | 210 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

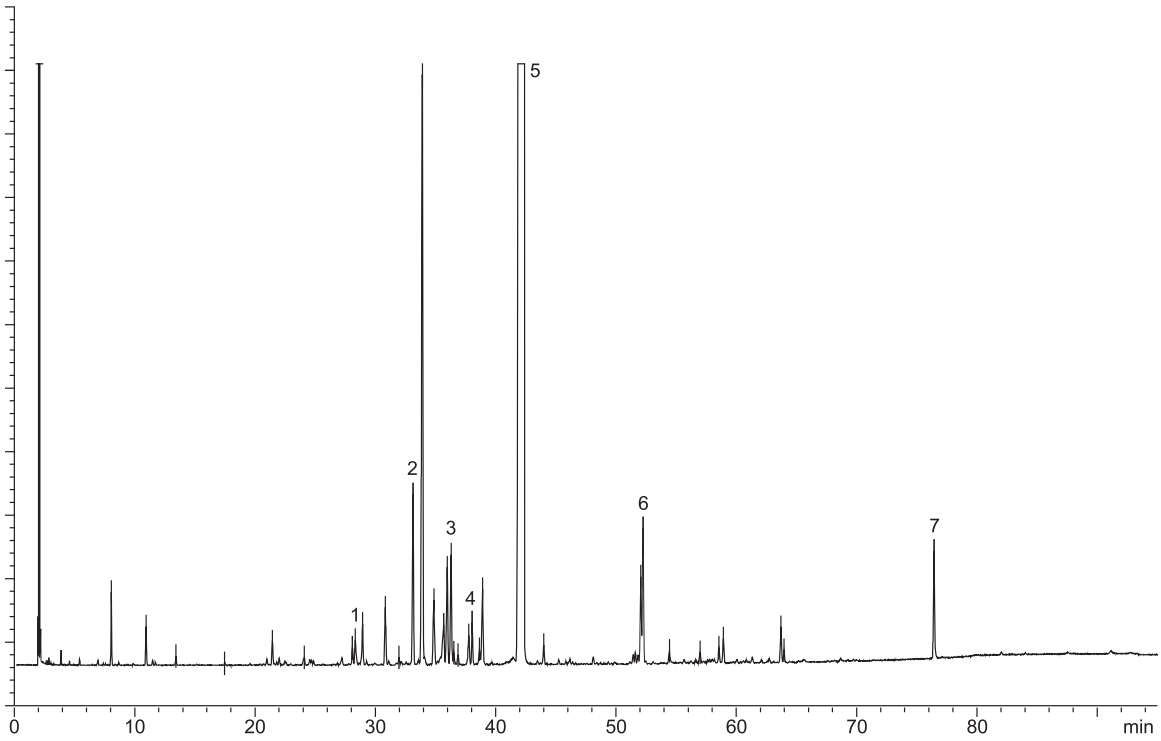


Figure 0804.-1. – *Chromatogram for the test for chromatographic profile of anise oil*

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to estragole and α-terpineol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution and locate *cis*-anethole and pseudoisoeugenyl 2-methylbutyrate using the chromatogram shown in Figure 0804.-1 (disregard any peak due to hexane).

Determine the percentage content of these components. The percentages are within the following ranges:

– *linalol*: maximum 1.5 per cent,
– *estragole*: 0.5 per cent to 5.0 per cent,
– *α-terpineol*: maximum 1.2 per cent,
– *cis-anethole*: 0.1 per cent to 0.4 per cent,
– *trans-anethole*: 87 per cent to 94 per cent,
– *anisaldehyde*: 0.1 per cent to 1.4 per cent,
– *pseudoisoeugenyl 2-methylbutyrate*: 0.3 per cent to 2.0 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**07/2017:0262**

# ANISEED

## Anisi fructus

DEFINITION

Whole, dry cremocarp of *Pimpinella anisum* L.

*Content*: minimum 20 mL/kg of essential oil (anhydrous drug).

CHARACTERS

Reminiscent odour of anethole.

The fruit is a cremocarp and generally entire; a small fragment of the thin, rigid, slightly curved pedicel is frequently attached.

IDENTIFICATION

A. The cremocarp is ovoid or pyriform and slightly compressed laterally, yellowish-green or greenish-grey, 3-5 mm long and up to 3 mm wide, surmounted by a stylopod with 2 short, reflexed stylar points. The mericarps are attached by their tops to the carpophore with a plane commissural surface and a convex dorsal surface, the latter being covered with short, warty trichomes visible using a lens; each mericarp shows 5 primary ridges, running longitudinally, comprising 3 dorsal ridges and 2 lateral ridges, non-prominent, and lighter in colour.

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow or brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0262.-1): fragments of epicarp in surface view [D] with a striated cuticle, occasional anomocytic stomata (*2.8.3*) [Da], bases of covering trichomes [Dc] and whole covering trichomes [Db], mostly unicellular, sometimes curved, with a blunt apex and a warty cuticle; isolated fragments of covering trichomes [E]; fragments [H] of numerous narrow, branched vittae [Ha], often accompanied by elongated cells of the commissural surface [Hb]; fragments of testa [B] consisting of a layer of brown, polyhedral, thin-walled cells; fragments of endosperm [G] containing oil droplets [Ga], aleurone grains and small cluster crystals of calcium oxalate [Gb]; oblong sclereids from the mesocarp [C] or the commissural surface of the fruit; bundles of short sclerenchymatous fibres [A] from the carpophore and the pedicel [Ab], accompanied by vessels with spiral or annular thickening [Aa, F].

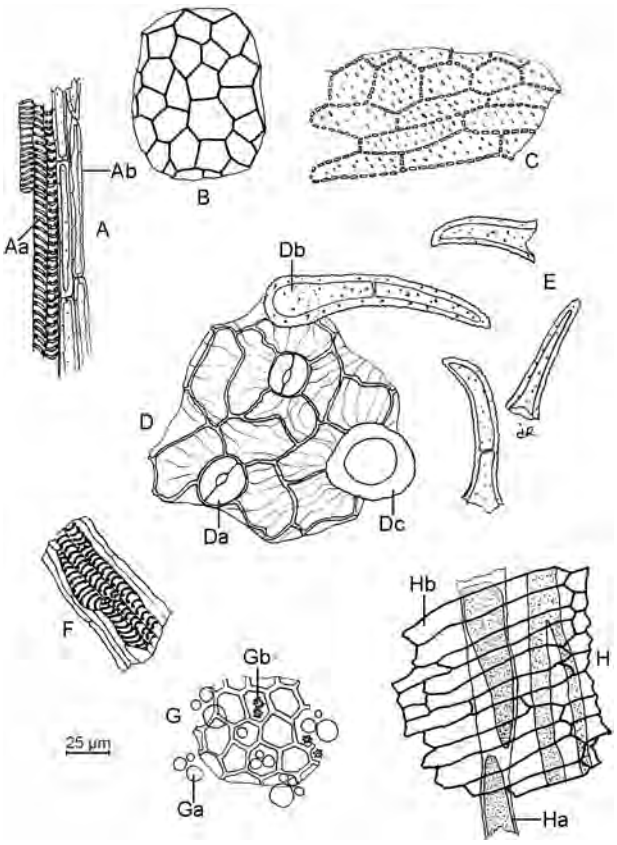


Figure 0262.-1. – *Illustration for identification test B of powdered herbal drug of aniseed*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.10 g of the powdered herbal drug (1400) (*2.9.12*) with 2 mL of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 mL of *toluene R*.

*Reference solution.* Dissolve 3 µL of *anethole R* and 40 µL of *olive oil R* in 1 mL of *toluene R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *toluene R*.

*Application*: 2 µL and 3 µL of the test solution, then 1 µL, 2 µL and 3 µL of the reference solution, at 2 cm intervals.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms show a quenching zone (anethole) in the central part against a light background.

*Detection B*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*, using 10 mL for a 200 mm square plate, and heat at 120 °C for 5 min.

*Results B*: the spots due to anethole appear blue against a yellow background. In the chromatogram obtained with 2 µL of the test solution, the spot due to anethole is intermediate in size between the corresponding spots in the chromatograms obtained with 1 µL and 3 µL of the reference solution. The chromatograms obtained with the test solution show in the lower third a blue spot (triglycerides) similar in position to the spot in the lower third of the chromatograms obtained with the reference solution (triglycerides of olive oil).

TESTS

**Water** (*2.2.13*): maximum 90 mL/kg, determined on 20.0 g of the herbal drug reduced to a coarse powder immediately before use.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.5 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the herbal drug reduced to a coarse powder immediately before the determination, a 250 mL round-bottomed flask, and 100 mL of *water R* as the distillation liquid. Place 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2.5-3.5 mL/min for 2 h.

**01/2012:1391**

# ARNICA FLOWER

## Arnicae flos

DEFINITION

Whole or partially broken, dried flower-heads of *Arnica montana* L.

*Content*: minimum 0.40 per cent *m/m* of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate (dried drug).

CHARACTERS

Aromatic odour.

The capitulum, when spread out, is about 20 mm in diameter and about 15 mm deep, and has a peduncle 2-3 cm long. The involucre consists of 18-24 elongated lanceolate bracts, with acute apices, arranged in 1-2 rows: the bracts, about 8-10 mm long, are green with yellowish-green external hairs visible under a lens. The receptacle, about 6 mm in diameter, is convex, alveolate and covered with hairs. Its periphery bears about 20 ligulate florets 20-30 mm long; the disc bears a greater number of tubular florets about 15 mm long. The ovary, 4-8 mm long, is crowned by a pappus of whitish bristles 4-8 mm long. Some brown achenes, crowned or not by a pappus, may be present.

IDENTIFICATION

A. The involucre consists of elongated oval bracts with acute apices; the margin is ciliated. The ligulate floret has a reduced calyx crowned by fine, shiny, whitish bristles, bearing small coarse trichomes. The orange-yellow corolla bears 7-10 parallel veins and ends in 3 small lobes. The stamens, with free anthers, are incompletely developed. The narrow, brown ovary bears a stigma divided into 2 branches curving outwards. The tubular floret is actinomorphic. The ovary and the calyx are similar to those of the ligulate floret. The short corolla has 5 reflexed triangular lobes; the 5 fertile stamens are fused at the anthers.

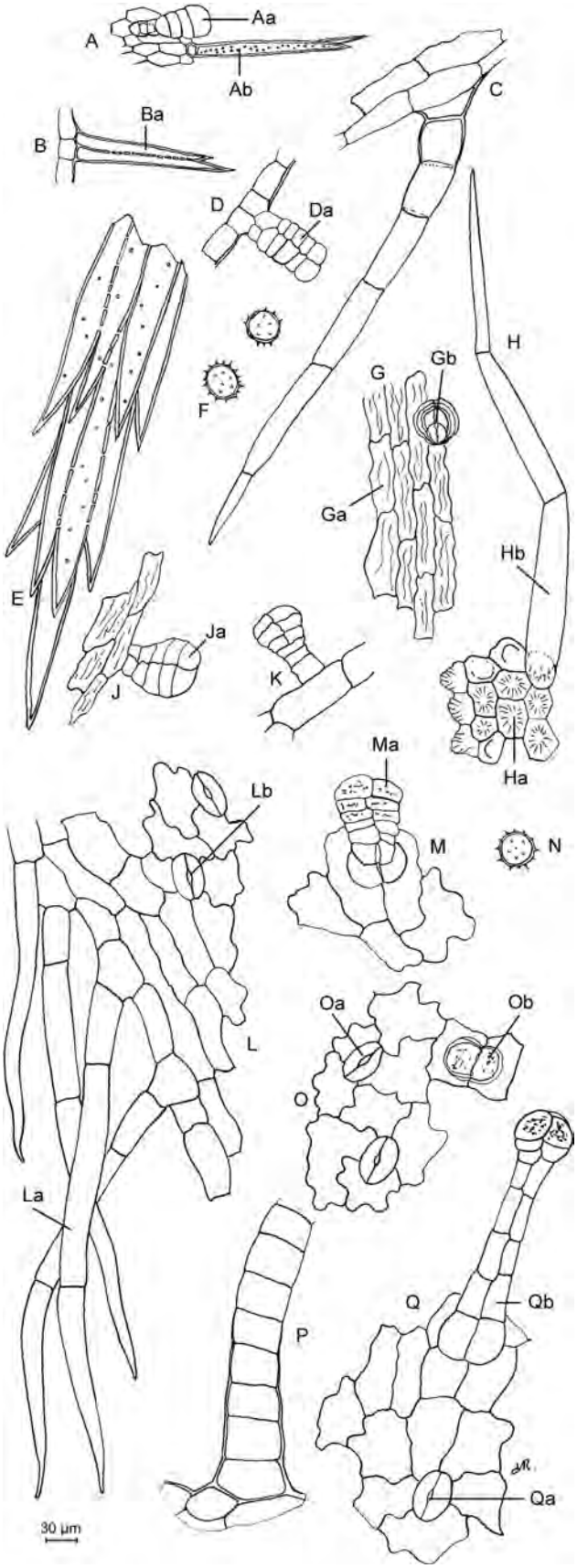


Figure 1391.-1. – *Illustration for identification test B of powdered herbal drug of arnica flower*

B. Microscopic examination (*2.8.23*). Separate the capitulum into its different parts. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1391.-1): the epidermises of the bracts of the involucre [L, M, O, Q] have stomata [Lb, Oa, Qa] and trichomes, more abundant on the outer (abaxial) surface. There are several different types of trichomes: uniseriate multicellular covering trichomes, varying in length from 50-500 µm, particularly abundant on the margins of the bract, whole [La] or fragmented [P]; secretory trichomes with uni- or biseriate multicellular stalks and with multicellular, globular heads, about 300 µm long, abundant on the outer surface of the bract [Qb]; secretory trichomes with multicellular stalks and with multicellular, globular heads, about 80 µm long, abundant on the inner surface of the bract (surface view [Ob], side view [Ma]). The epidermis of the ligulate corolla [C, G, H, J] consists of lobed or elongated cells covered by a striated cuticle [Ga], a few stomata and trichomes of different types: covering trichomes, with very sharp ends, whose length may exceed 500 µm, consisting of 1-3 proximal, thick-walled cells and 2-4 distal, thin-walled cells [C, Hb]; secretory trichomes with biseriate multicellular heads (surface view [Gb], side view [Ja]); secretory trichomes with multicellular stalks and multicellular globular heads [K]. The ligule ends in rounded papillose cells [Ha]. Fragments of the epidermis of the ovary [A, B, D] are covered with trichomes of 2 types: secretory trichomes with short stalks and multicellular globular heads (surface view [Aa], side view [Da]); twinned covering trichomes usually consisting of 2 longitudinally united cells, with common pitted walls (surface view [Ab], side view [Ba]); their ends are sharp and sometimes bifid. The epidermises of the calyx consist of elongated cells bearing short, unicellular, covering trichomes pointing towards the upper end of the bristle [E]. The pollen grains have a diameter of about 30 µm, are rounded, with a spiny exine, and have 3 germinal pores [F, N].

C. Examine the chromatograms obtained in the test for *Calendula officinalis* L. - *Heterotheca inuloides* Cass.

*Results*: the chromatogram obtained with the test solution shows, in the middle, a fluorescent blue zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution; it shows, above this zone, 3 fluorescent yellowish-brown or orange-yellow zones, and above these 3 zones a fluorescent greenish-yellow zone due to astragalin; the zone located below the astragalin zone is due to isoquercitroside; the zone located just below this zone is due to luteolin-7-glucoside; it also shows a fluorescent greenish-blue zone below the zone due to caffeic acid in the chromatogram obtained with the reference solution.

TESTS

**Foreign matter** (*2.8.2*): maximum 5.0 per cent.

***Calendula officinalis* L. - *Heterotheca inuloides* Cass.**
Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.00 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C for 5 min with shaking. Cool and filter.

*Reference solution.* Dissolve 2.0 mg of *caffeic acid R*, 2.0 mg of *chlorogenic acid R* and 5.0 mg of *rutoside trihydrate R* in *methanol R* and dilute to 30 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 15 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air for a few minutes.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; heat at 100-105 °C for 5 min, allow to dry in air and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower part an orange-yellow fluorescent zone due to rutoside, in the middle part a fluorescent zone due to chlorogenic acid and in the upper part a light bluish fluorescent zone due to caffeic acid; the chromatogram obtained with the test solution does not show a fluorescent orange-yellow zone corresponding to the zone due to rutoside in the chromatogram obtained with the reference solution, nor does it show a zone below this.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Internal standard solution.* Dissolve immediately before use 0.010 g of *santonin CRS*, accurately weighed, in 10.0 mL of *methanol R*.

*Test solution.* Introduce 1.00 g of the powdered herbal drug (355) (*2.9.12*) into a 250 mL round-bottomed flask, add 50 mL of a mixture of equal volumes of *methanol R* and *water R* and heat under a reflux condenser in a water-bath at 50-60 °C for 30 min, shaking frequently. Allow to cool and filter through a paper filter. Add the paper filter, cut into pieces, to the residue in the round-bottomed flask, add 50 mL of a mixture of equal volumes of *methanol R* and *water R* and heat under a reflux condenser in a water-bath at 50-60 °C for 30 min, shaking frequently. Repeat this procedure twice. To the combined filtrates add 3.00 mL of the internal standard solution and evaporate to 18 mL under reduced pressure. Rinse the round-bottomed flask with *water R* and dilute, with the washings, to 20.0 mL. Transfer the solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 20 min. Elute with 200 mL of a mixture of equal volumes of *ethyl acetate R* and *methylene chloride R*. Evaporate the eluate to dryness in a 250 mL round-bottomed flask. Dissolve the residue in 10.0 mL of *methanol R* and add 10.0 mL of *water R*. Add 7.0 g of *neutral aluminium oxide R*, shake for 120 s, centrifuge at 5000 *g* for 10 min and filter through a paper filter. Evaporate 10.0 mL of the filtrate to dryness. Dissolve the residue in 3.0 mL of a mixture of equal volumes of *methanol R* and *water R* and filter.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 62 | 38 |
| 3 - 20 | 62 → 55 | 38 → 45 |
| 20 - 30 | 55 | 45 |
| 30 - 55 | 55 → 45 | 45 → 55 |
| 55 - 57 | 45 → 0 | 55 → 100 |
| 57 - 70 | 0 | 100 |
| 70 - 90 | 62 | 38 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL loop injector.

Calculate the percentage content of total sesquiterpene lactones, expressed as dihydrohelenalin tiglate, using the following expression:

$$\frac{S_{LS} \times C \times V \times 1.187 \times 100}{S_S \times m \times 1000}$$

$S_{LS}$ = area of all peaks due to sesquiterpene lactones appearing after the santonin peak in the chromatogram obtained with the test solution;

$S_S$ = area of the peak due to santonin in the chromatogram obtained with the test solution;

$m$ = mass of the herbal drug to be examined, in grams;

$C$ = concentration of santonin in the internal standard solution used for the test solution, in milligrams per millilitre;

$V$ = volume of the internal standard solution used for the test solution, in millilitres;

1.187 = peak correlation factor between dihydrohelenalin tiglate and santonin.

**01/2008:1809 corrected 6.3**

# ARNICA TINCTURE

## Arnicae tinctura

DEFINITION

Tincture produced from *Arnica flower (1391)*.

*Content*: minimum 0.04 per cent of sesquiterpene lactones expressed as dihydrohelenalin tiglate ($C_{20}H_{26}O_5$; $M_r$ 346.42).

PRODUCTION

The tincture is produced from the herbal drug by a suitable procedure using 10 parts of ethanol (60-70 per cent *V/V*) for 1 part of drug.

CHARACTERS

*Appearance*: yellowish-brown liquid.

IDENTIFICATION

Examine the chromatograms obtained in the test for *Calendula officinalis - Heterotheca inuloides*.

*Chromatogram obtained with the test solution*:

- in the middle, a fluorescent blue zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution;
- above this zone, 3 fluorescent yellowish-brown to orange-yellow zones, and above these 3 zones a fluorescent greenish-yellow zone corresponding to astragalin; the zone located below the astragalin zone corresponds to isoquercitrin; the zone located just below this zone corresponds to luteolin-7-glucoside;
- a fluorescent greenish-blue zone below the zone due to caffeic acid in the chromatogram obtained with the reference solution.

TESTS

***Calendula officinalis - Heterotheca inuloides.*** Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution.* Dissolve 2.0 mg of *caffeic acid R*, 2.0 mg of *chlorogenic acid R* and 5.0 mg of *rutoside trihydrate R* in *methanol R* and dilute to 30.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

Herbal drugs

*Application*: 30 µL [or 8 µL] as bands.

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: at 80-105 °C.

*Detection*: spray the plate whilst still hot with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; heat 5 min at 100-105 °C, allow the plate to dry in air and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower part an orange-yellow fluorescent zone (rutoside), in the middle part a fluorescent zone due to chlorogenic acid and in the upper part a light bluish fluorescent zone (caffeic acid). The chromatogram obtained with the test solution does not show any fluorescent orange-yellow zone corresponding to rutoside in the chromatogram obtained with the reference solution and no zone below the zone corresponding to rutoside.

**Ethanol** (*2.9.10*): the final ethanol concentration is not less than 90 per cent of that of the initial extraction solvent.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

**Dry residue** (*2.8.16*): minimum 1.7 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Internal standard solution.* Dissolve immediately before use 0.010 g accurately weighed of *santonin CRS* and 0.02 g of *butyl 4-hydroxybenzoate R* in 10.0 mL of *methanol R*.

*Test solution.* In a round-bottomed flask introduce 5.00 g of the tincture to be examined, add 2.00 mL of the internal standard solution and 3 g of *anhydrous aluminium oxide R*, shake for 120 s and filter through a filter paper. Rinse the round-bottomed flask and filter with 5 mL of a mixture of equal volumes of *methanol R* and *water R* and filter. Evaporate the filtrate to dryness. Dissolve the residue in 2.0 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 0.02 g of *methyl 4-hydroxybenzoate R* and 0.02 g of *ethyl 4-hydroxybenzoate R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 62 | 38 |
| 3 - 20 | 62 → 55 | 38 → 45 |
| 20 - 30 | 55 | 45 |
| 30 - 55 | 55 → 45 | 45 → 55 |

*Flow rate*: 1.2 mL/min.

*Detector*: spectrophotometer at 225 nm.

*Injection*: 20 µL.

*Relative retention* with reference to santonin (retention time = about 9.5 min): butyl 4-hydroxybenzoate = about 4.6.

*System suitability*: reference solution:

- *resolution*: minimum 5 between the peaks due to methyl 4-hydroxybenzoate and ethyl 4-hydroxybenzoate.

Calculate the percentage of lactone sesquiterpenes, expressed as dihydrohelenalin tiglate, using the following expression:

$$\frac{F_1 \times C \times V \times 1.187}{F_2 \times m \times 10}$$

$F_1$ = area of all peaks appearing between the peaks due to santonin and butyl 4-hydroxybenzoate in the chromatogram obtained with the test solution;

$F_2$ = area of the peak due to santonin in the chromatogram obtained with the test solution;

$m$ = mass of the tincture to be examined, in grams;

$C$ = concentration of santonin in the internal standard solution used to prepare the test solution, in milligrams per millilitre;

$V$ = volume of the internal standard solution used to prepare the test solution, in millilitres;

1.187 = peak correlation factor between dihydrohelenalin tiglate and santonin.

**04/2018:1866**
**corrected 10.0**

# ARTICHOKE LEAF

## Cynarae folium

DEFINITION

Whole or cut, dried leaf of *Cynara cardunculus* L. (syn. *C. scolymus* L.).

*Content*: minimum 0.7 per cent of chlorogenic acid ($C_{16}H_{18}O_9$; $M_r$ 354.3) (dried drug).

IDENTIFICATION

A. The entire leaf may be up to 70 cm long and 30 cm wide. The lamina is deeply lobed in the upper part to within 1-2 cm of the petiole on either side, in the lower part the leaf becomes pinnate; all the segments have markedly dentate margins and taper at the apex. Spines are absent. The upper surface of the lamina is green with a fine covering of whitish hairs, the lower surface is pale green or white and densely tomentose with long, tangled hairs. The petiole and main veins are flat on the upper surface, prominently raised and longitudinally ridged on the lower surface, with conspicuous hairs on both surfaces.

Herbal drugs

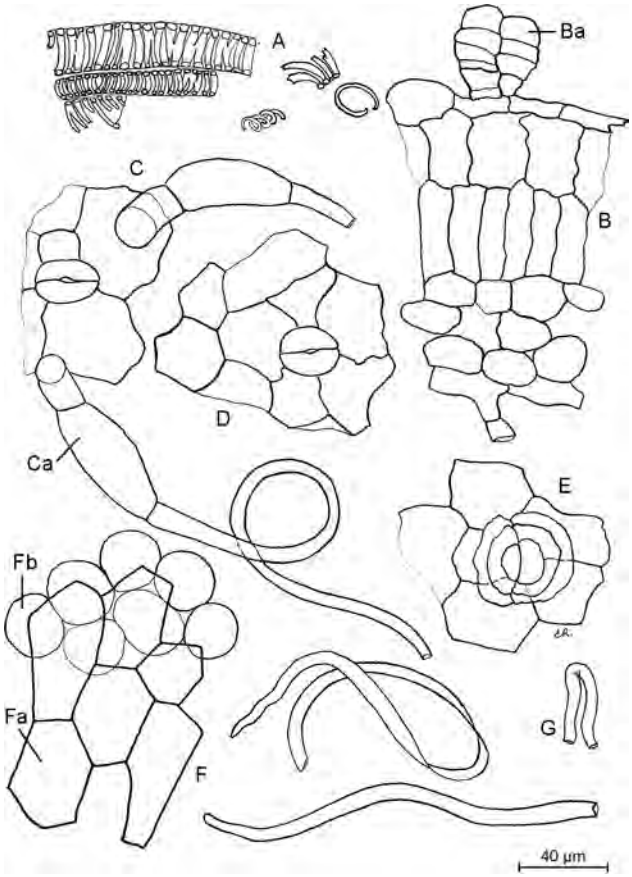


Figure 1866.-1. – *Illustration for identification test B of powdered herbal drug of artichoke leaf*

B. Microscopic examination (*2.8.23*). Reduce to a powder (1000) (*2.9.12*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1866.-1): fragments of the epidermises of the lamina, in surface view; the upper epidermis [F] is composed of cells with straight or slightly sinuous walls [Fa], accompanied by palisade parenchyma [Fb]; the lower epidermis [C] is composed of more sinuous-walled cells; abundant anomocytic stomata (*2.8.3*) on both surfaces [D] and multicellular, uniseriate covering trichomes in felted masses, the majority fragmented [Ca] with a short stalk composed of several cells and a very long, narrow and frequently curled terminal cell, others consisting of 4-6 cylindrical cells; very occasional glandular trichomes with a short stalk and a uniseriate or biseriate head (surface view [E], transverse section [Ba]); abundant fragments of covering trichomes [G]; fragments of the lamina (transverse section [B]); abundant fragments of vascular tissue from the petiole and veins [A].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (1000) (*2.9.12*) add 20 mL of *ethanol (60 per cent V/V) R*. Allow to stand for 2 h with occasional stirring. Filter.

*Reference solution.* Dissolve 5 mg of *luteolin-7-glucoside R* and 5 mg of *chlorogenic acid CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 13 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min; treat the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* followed by a 50 g/L solution of *macrogol 400 R* in *methanol R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue fluorescent zone |
| Luteolin-7-glucoside: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone (luteolin-7-glucoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| **Reference solution** | **Test solution** |

TESTS

**Total ash** (*2.4.16*): maximum 20.0 per cent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (30:70 *V/V*).

*Test solution.* To 0.500 g of the powdered herbal drug (1000) (*2.9.12*) add 50.0 mL of *methanol R* and heat under a reflux condenser on a water-bath at 70 °C for 1 h. Centrifuge and transfer the supernatant to a 200 mL volumetric flask. Repeat the procedure and dilute to 200.0 mL with *water R*.

*Reference solution (a).* Dissolve 5.0 mg of *chlorogenic acid CRS* in 50.0 mL of *methanol R*. Transfer 5.0 mL of this solution to a volumetric flask, add 5 mL of *methanol R* and dilute to 20.0 mL with *water R*.

*Reference solution (b).* Dissolve 30 mg of *artichoke leaf dry extract HRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *water for chromatography R* (0.5:99.5 *V/V*);
- *mobile phase B*: *phosphoric acid R*, *acetonitrile R* (0.5:99.5 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 92 | 8 |
| 1 - 20 | 92 → 75 | 8 → 25 |
| 20 - 33 | 75 | 25 |
| 33 - 35 | 75 → 0 | 25 → 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 25 µL.

*System suitability*: reference solution (b):
- the chromatogram obtained is similar to the chromatogram supplied with the *artichoke leaf dry extract HRS*;

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to cryptochlorogenic acid and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to chlorogenic acid.

Calculate the percentage content of chlorogenic acid using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *chlorogenic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of chlorogenic acid in *chlorogenic acid CRS*.

**04/2018:2389**

# ARTICHOKE LEAF DRY EXTRACT

## Cynarae folii extractum siccum

DEFINITION

Dry extract produced from *Artichoke leaf (1866)*.

*Content*: minimum 0.6 per cent of chlorogenic acid ($C_{16}H_{18}O_9$; $M_r$ 354.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using water of minimum 80 °C.

CHARACTERS

*Appearance*: light brown or brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the extract to be examined in 10 mL of *ethanol (60 per cent V/V) R*. Sonicate for 5 min and filter.

*Reference solution.* Dissolve 5 mg of *luteolin-7-glucoside R* and 5 mg of *chlorogenic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 13 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* followed by a 50 g/L solution of *macrogol 400 R* in *methanol R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A light blue fluorescent zone |
| ——— | ——— |
| Luteolin-7-glucoside: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone (luteolin-7-glucoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 6.0 per cent.

**Total ash** (*2.4.16*): maximum 30.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (30:70 *V/V*).

*Test solution.* Dissolve 30.0 mg of the extract to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5.0 mg of *chlorogenic acid CRS* in 50.0 mL of *methanol R*. Transfer 5.0 mL of this solution to a volumetric flask, add 5 mL of *methanol R* and dilute to 20.0 mL with *water R*.

*Reference solution (b).* Dissolve 30 mg of the *artichoke leaf dry extract HRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *water for chromatography R* (0.5:99.5 *V/V*);

– *mobile phase B*: *phosphoric acid R*, *acetonitrile R* (0.5:99.5 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 92 | 8 |
| 1 - 20 | 92 → 75 | 8 → 25 |
| 20 - 33 | 75 | 25 |
| 33 - 35 | 75 → 0 | 25 → 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 25 µL.

*System suitability*: reference solution (b):

– the chromatogram obtained is similar to the chromatogram supplied with the *artichoke leaf dry extract HRS*;

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to cryptochlorogenic acid and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to chlorogenic acid.

Herbal drugs

Calculate the percentage content of chlorogenic acid using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.125}{A_2 \times m_1}$$

$A_1$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in milligrams;

$m_2$ = mass of *chlorogenic acid CRS* used to prepare reference solution (a), in milligrams;

$p$ = percentage content of chlorogenic acid in *chlorogenic acid CRS*.

**07/2012:1600**

# ASH LEAF

## Fraxini folium

DEFINITION

Dried leaf of *Fraxinus excelsior* L. or *Fraxinus angustifolia* Vahl (syn. *Fraxinus oxyphylla* M. Bieb) or of hybrids of these 2 species or of a mixture.

*Content*: minimum 2.5 per cent of total hydroxycinnamic acid derivatives, expressed as chlorogenic acid ($C_{16}H_{18}O_9$; $M_r$ 354.3) (dried drug).

IDENTIFICATION

A. The leaf consists of leaflets that are sometimes detached and separated from the rachis. The leaflet is about 6 cm long and 3 cm wide. Each leaflet is subsessile or shortly petiolate, oblong, lanceolate, somewhat unequal at the base, acuminate at the apex, with fine, acute teeth on the margins; the upper surface is dark green and the lower surface is greyish-green. The midrib and secondary veins are whitish and prominent on the lower surface.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1600.-1): fragments of the upper epidermis of the lamina (surface view [B]), with some of the cells showing cuticular striations, accompanied by underlying palisade parenchyma [Ba]; fragments of the lower epidermis in surface view [A] consisting of cells covered by fine cuticular striations [Aa], numerous anomocytic stomata (*2.8.3*) [Ab] and rare peltate glandular trichomes with a unicellular stalk and a glandular head composed of radiating cells [Ac]; fragments of lamina in transverse section [F] with 2 layers of palisade parenchyma [Fa], spongy parenchyma [Fb] and, occasionally, glandular trichomes embedded in the epidermis [Fc]; occasional multicellular, uniseriate, conical covering trichomes composed of cells with thick striated walls, either on an epidermis [C] or fragmented [D]; fragments of vascular tissue from the leaflets [E] composed of spiral vessels [Ea], short fibres [Eb] and sometimes palisade parenchyma [Ec]; fragments of vascular tissue from the veins [G] composed of fibres [Ga], sometimes accompanied by cells with thick, pitted walls from the medullary rays [Gb].

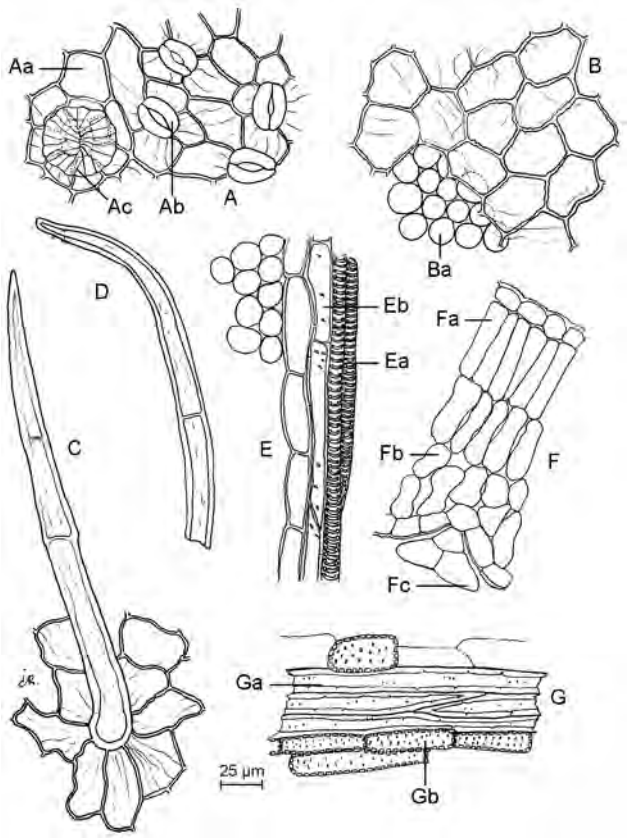


Figure 1600.-1. – *Illustration for identification test B of powdered herbal drug of ash leaf*

C. Examine the chromatograms obtained in the test for *Fraxinus ornus*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. The intensity of the zones present in the chromatogram obtained with the test solution may vary depending on the presence of *F. excelsior*, *F. angustifolia*, their hybrids or their concentration in a mixture. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| ——— | ——— |
| | A light blue fluorescent zone (acteoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone may be present (chlorogenic acid) |
| ——— | ——— |
| | A light blue fluorescent zone |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3.0 per cent of stems and maximum 2.0 per cent of other foreign matter.

***Fraxinus ornus***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *methanol R*. Stir with a magnetic stirrer for 10 min. Filter.

*Reference solution*. Dissolve 5 mg of *rutoside trihydrate R* and 5 mg of *chlorogenic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min; treat the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; dry in air; treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*; dry in air; examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution does not show any intense light blue fluorescent zones in the upper third of the chromatogram.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

*Test solution (a).* To 0.300 g of the powdered herbal drug (355) (*2.9.12*) add 95 mL of *ethanol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Rinse the filter with 5 mL of *ethanol (50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Test solution (b).* To 1.0 mL of test solution (a) in a test tube, add 2 mL of *0.5 M hydrochloric acid*, 2 mL of a solution prepared by dissolving 10 g of *sodium nitrite R* and 10 g of *sodium molybdate R* in 100 mL of *water R*, then add 2 mL of *dilute sodium hydroxide solution R* and dilute to 10.0 mL with *water R*; mix.

Immediately measure the absorbance (*2.2.25*) of test solution (b) at 525 nm, using as compensation liquid a solution prepared as follows: mix 1.0 mL of test solution (a), 2 mL of *0.5 M hydrochloric acid*, 2 mL of *dilute sodium hydroxide solution R* and dilute to 10.0 mL with *water R*.

Calculate the percentage content of total hydroxycinnamic acid derivatives, expressed as chlorogenic acid, using the following expression:

$$\frac{A \times 5.3}{m}$$

taking the specific absorbance of chlorogenic acid to be 188.

$A$ = absorbance at 525 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**07/2017:2435**

# ASTRAGALUS MONGHOLICUS ROOT

## Astragali mongholici radix

DEFINITION

Whole or fragmented, dried root of *Astragalus mongholicus* Bunge with secondary roots and root crown removed.

*Content*: minimum 0.040 per cent of astragaloside IV ($C_{41}H_{68}O_{14}$; $M_r$ 785) (dried drug).

IDENTIFICATION

A. Cylindrical, often with branches, upper part relatively thick, 30-90 cm long and 1-3.5 cm in diameter. Externally pale brownish-yellow or pale brown, with irregular, longitudinal wrinkles or furrows. Texture hard and tenacious; uneasily broken, fracture highly fibrous and weakly (cultivated origin) or strongly starchy (wild origin), bark yellowish-white, wood pale yellow, with radiate striations and fissures; the central region is dark brown and in older roots may be broken down to form a hollow surrounded by fragments of disintegrating tissue.

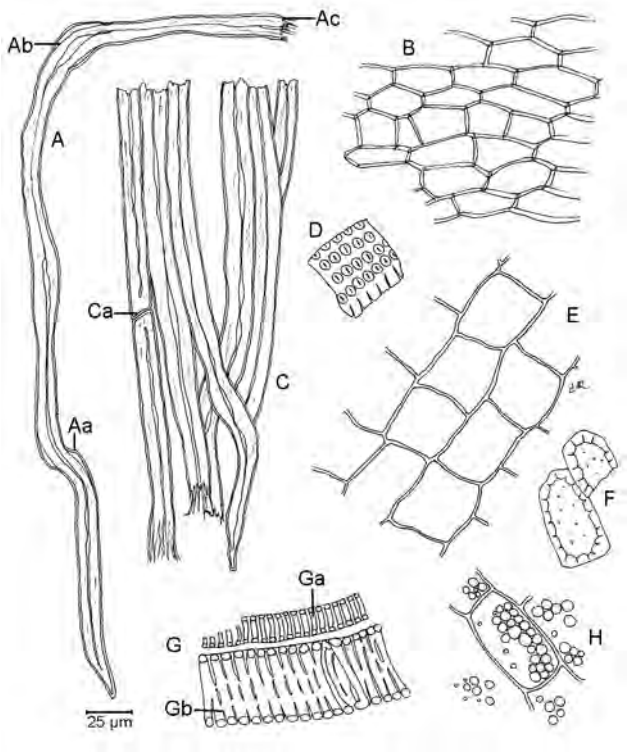


Figure 2435.-1. – *Illustration for identification test B of powdered herbal drug of astragalus mongholicus root*

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2435.-1): fibres, isolated [A] or in groups [C], 8-30 µm in diameter, with thick walls having longitudinal striations on the surface, the primary walls [Aa] sometimes separated from the secondary walls [Ab] with the ends of the fibres often broken and fibrous [Ac], rounded or slightly truncated [Ca]; vessels [G] are annular [Ga], reticulate [Gb] or bordered-pitted with closely spaced pits [D], colourless or orange; rare sclereids [F], rounded, oblong or irregular, with slightly thickened walls; cork fragments with polygonal cells (surface view [B]); numerous medullary rays with thin-walled cells [E]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows small (less than 10 µm), simple, rounded or ovoid starch granules, usually included in parenchyma cells [H].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Heat 3 g of the powdered herbal drug (355) (*2.9.12*) with 50 mL of *methanol R* for 50 min under reflux and then filter. Evaporate the filtrate under reduced pressure to dryness and take up the residue in 1 mL of *water R*. Apply the solution to a 6 mL solid phase extraction column containing *octadecylsilyl silica gel for chromatography R* previously conditioned with 3 mL of *methanol R* and then with 3 mL of *water R*. Wash the column with 15 mL of *water R* followed by 15 mL of a 30 per cent *V/V* solution of *methanol R*. Discard the washings. Elute with 20 mL of *methanol R* and collect the eluate. Evaporate the eluate under reduced pressure to dryness and take up the residue with 2 mL of *methanol R*.

*Reference solution.* Dissolve 10.0 mg of *daidzin R* and 5.0 mg of *daidzein R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (10:13.5:100 *V/V/V*).

*Application*: 3 µL as bands of 8 mm.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue fluorescent zone |
| Daidzein: a quenching zone | |
| | A quenching zone |
| ——— | ——— |
| | A quenching zone |
| Daidzin: a quenching zone | A quenching zone |
| ——— | ——— |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R*. Heat at 100 °C for 3 min. Examine in ultraviolet light at 366 nm.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| Daidzein: a pale blue zone | |
| | A violet zone |
| ——— | ——— |
| | A violet zone |
| Daidzin: a pale blue zone | A brown zone |
| | 5 brown zones |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 3 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 4.000 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *methanol R* and sonicate at 80 °C for 30 min. Centrifuge for 7 min and transfer the supernatant to a round-bottomed flask. Add to the residue 25 mL of *methanol R* and sonicate at 80 °C for 30 min. Centrifuge for 7 min and add the supernatant to the same round-bottomed flask. Repeat this operation once. Combine the 3 fractions of supernatant and evaporate to dryness. Dissolve the residue in 10 mL of *water R* using sonication if necessary. Transfer the solution to a separating funnel and shake with 4 quantities, each of 40 mL, of *butanol R* saturated with *water R*. Combine the butanol extracts and wash with 2 quantities, each of 40 mL, of *ammonia R*. Discard the ammonia layers and evaporate the butanol layers to dryness. Dissolve the residue in 5.0 mL of *methanol R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *astragaloside IV CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solutions (b), (c), (d).* Dilute reference solution (a) to obtain 3 reference solutions of astragaloside IV, the concentrations of which span the expected value in the test solution.

*Reference solution (e).* Dissolve 5.0 mg of *ginsenoside Rb1 R* in 5 mL of *methanol R* and dilute to 10.0 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.2 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 10 | 90 → 80 | 10 → 20 |
| 10 - 20 | 80 → 75 | 20 → 25 |
| 20 - 30 | 75 → 67 | 25 → 33 |
| 30 - 40 | 67 → 65 | 33 → 35 |
| 40 - 50 | 65 → 40 | 35 → 60 |
| 50 - 55 | 40 | 60 |

*Flow rate*: 0.5 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:

- *carrier gas*: air;
- *flow rate*: 1.5 mL/min;
- *evaporator temperature*: 50 °C.

*Injection*: 20 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Relative retention* with reference to ginsenoside Rb1 (retention time = about 33.6 min): astragaloside IV = about 1.05.

*System suitability*:

- *resolution*: minimum 4.0 between the peaks due to astragaloside IV and ginsenoside Rb1 in the chromatogram obtained with reference solution (e).

Establish a calibration curve with the logarithm of the concentration (mg/mL) of reference solutions (b), (c) and (d) (corrected by the declared percentage content of *astragaloside IV CRS*) as the abscissa and the logarithm of the corresponding peak area as the ordinate. Calculate the percentage content of astragaloside IV using the following expression:

$$\frac{10^{A} \times 0.5}{m}$$

$A$ = logarithm of the concentration corresponding to the astragaloside IV peak in the chromatogram obtained with the test solution, determined from the calibration curve;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams.

Herbal drugs

**04/2019:2559**

# ATRACTYLODES LANCEA RHIZOME

## Atractylodis lanceae rhizoma

### DEFINITION

Dried, whole or fragmented rhizome of *Atractylodes lancea* (Thunb.) DC. (syn. *Atractylodes chinensis* (Bunge) Koidz.; *Atractylodes Japonica* Koidz.) with the roots removed, collected in spring and autumn.

*Content*: minimum 14 mL/kg of essential oil (anhydrous drug).

### IDENTIFICATION

A. *Whole drug.* The whole rhizome is somewhat curved, irregularly moniliform or nodular-cylindrical, occasionally branched, 3-10 cm long and 1-3 cm in diameter. The external surface is wrinkled and irregularly transversely segmented, dark greyish-brown or yellowish-brown; it shows numerous rounded protuberances and large circular stem scars and smaller root scars. The texture is hard, easily broken; the fractured surface is yellowish-white or brownish-yellow, uneven, with many glistening yellowish-orange or reddish-brown oil cavities appearing as dots scattered throughout the tissue.

*Fragmented drug.* The fragmented rhizome occurs in transverse or longitudinal slices with a highly variable diameter (1-4 cm) and a thickness up to 0.5 cm. The external surface is wrinkled, dark greyish-brown or yellowish-brown and shows numerous small rootlet scars. The cut surface is whitish-yellow or brownish-yellow, with particularly abundant yellowish-orange or reddish-brown oil cavities appearing as dots scattered throughout the tissue. Occasionally, some cavities crystallise out as fine white needle- or hair-like crystals.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2559.-1): fragments of orange cork with polyhedral cells (surface view [K]); fragments of dermal tissue (transverse section [H]), consisting of several layers of cork [Ha] often accompanied by subrectangular or ovoid sclereids from the phelloderm [Hb] with very thick, channelled walls and a narrow lumen; fragments of phelloderm containing groups of sclereids (surface view [J]); isolated sclereids, variable in shape (20-51 µm in diameter) [B, G]; fragments of parenchyma with polyhedral, subrectangular or subrounded cells containing small needle-shaped crystals of calcium oxalate (5-30 µm in length) clearly visible in polarised light [D]; fragments of fibres in bundles, with heavily thickened and slightly pitted walls (up to 40 µm in diameter) and a narrow lumen, very often associated with xylem vessels [A]; fragments of short, reticulate or pitted vessels, usually included in parenchyma with thin-walled cells [F]; fragments of oil glands with thin-walled cells and orange-brown contents [C] accompanied by parenchyma cells containing small needle-shaped crystals of calcium oxalate [Ca]. Examine under a microscope, without heating, using *glycerol R*. The powder shows pieces of inulin, free [E] or included in parenchyma cells.

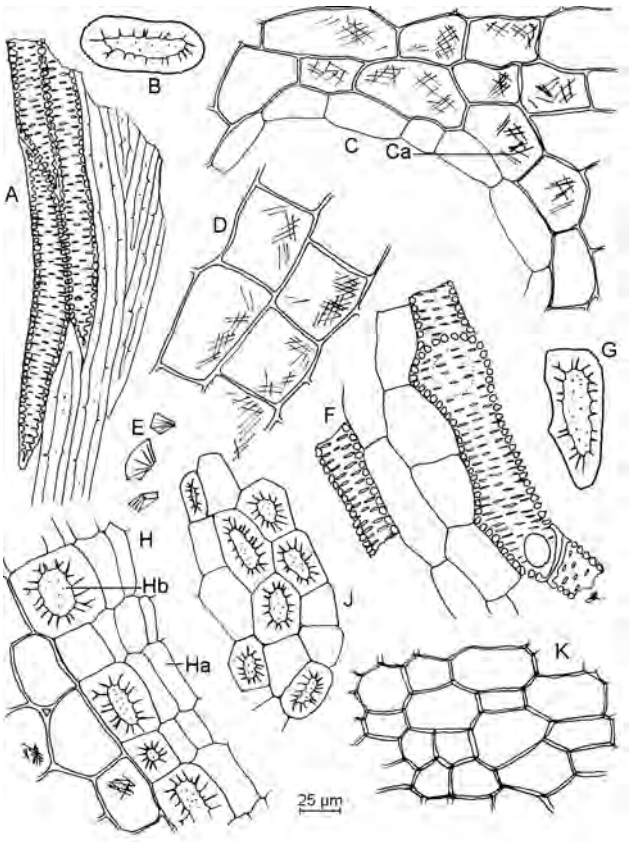


Figure 2559.-1. – *Illustration for identification test B of powdered herbal drug of Atractylodes lancea rhizome*

C. Examine the chromatograms obtained in the test for *Atractylodes macrocephala*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| β-Caryophyllene: a pink zone | A pink or violet zone |
| | An orange zone may be present |
| _____ | _____ |
| | An intense greyish-green zone |
| | A very faint violet zone |
| _____ | _____ |
| Bornyl acetate: a brown zone | A violet zone |
| | Several violet zones |
| **Reference solution** | **Test solution** |

### TESTS

***Atractylodes macrocephala.*** Thin-layer chromatography (*2.2.27*).

*Test solution.* Introduce 0.5 g of the powdered herbal drug (355) (*2.9.12*) into a centrifuge tube, add 2 mL of *methanol R* and stopper the tube. Sonicate at 25 °C for 15 min and centrifuge.

*Reference solution.* Dissolve 10 mg of *β-caryophyllene R* and 10 mg of *bornyl acetate R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *heptane R* (5:95 *V/V*).

*Application*: 5 µL [or 3 µL] as bands of 10 mm [or 6 mm].

*Development*: in an unsaturated tank, over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows an intense greyish-green zone in the middle third. In the case of a substitution by *Atractylodes macrocephala*, no intense greyish-green zone is present in the middle third.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 15.0 g of freshly powdered herbal drug (710) (*2.9.12*), a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**04/2019:2560**

# ATRACTYLODES RHIZOME, LARGEHEAD

## Atractylodis macrocephalae rhizoma

DEFINITION

Dried, whole or fragmented rhizome of *Atractylodes macrocephala* Koidz. with the roots removed, collected in winter when the lower leaves of the plant turn yellow and the upper leaves become fragile.

*Content*: minimum 9 mL/kg of essential oil (anhydrous drug).

IDENTIFICATION

A. *Whole drug.* The whole rhizome is irregularly shaped, 3-13 cm long and 1.5-7 cm in diameter, externally yellowish greyish-brown or dark brown. Small knob-like protrusions are present, concentrated at the rhizome apex and interspersed with straighter narrower regions, whose outer surface is covered with longitudinal wrinkles and grooves. The texture is hard, difficult to break, and the fracture is uneven with wide spaces between the tissues, especially in the central region. The fracture is whitish-yellow to brownish, with yellowish-brown to orange oil cavities scattered throughout, particularly abundant in the external tissues. A greyish-brown cambium is occasionally visible.

*Fragmented drug.* The fragmented rhizome mostly occurs as longitudinal slices with a highly variable diameter (1-7 cm) and a thickness of about 0.5 cm. The external surface is wrinkled or grooved, more or less dark yellowish greyish-brown with root scars and knob-like protrusions. The texture is hard. The cut surface is whitish-yellow to brownish, consisting of tissues with wide spaces between them and scattered with many yellowish-brown to orange oil cavities appearing as dots that are particularly abundant in the external tissues.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2560.-1): fragments of orange cork (surface view [A], transverse section [B]), with superimposed cells [Aa, Ba] sometimes accompanied by parenchyma cells containing fine needles of calcium oxalate [Ab, Bb]; fragments of parenchyma with polyhedral, subrectangular or subrounded cells, many of which contain small needle-shaped crystals of calcium oxalate (10-32 µm in length) clearly visible in polarised light [E]; sclereids, isolated or in small groups, with very thick, channelled walls, variable in shape (35-65 µm in diameter) [D, F]; larger sclereids often with thinner walls and a large lumen; rare fragments of fibres, isolated or in bundles, with moderately thickened and slightly pitted walls (up to 40 µm in diameter) [C]; fragments of xylem [J] consisting of short, reticulate or pitted vessels [Ja], usually included in parenchyma with thin-walled cells [Jb]; oil glands [G] or fragments of oil glands with thin-walled cells [Ga] and orange-brown contents, accompanied by parenchyma cells containing small, needle-shaped crystals of calcium oxalate [Gb]. Examine under a microscope, without heating, using *glycerol R*. The powder shows abundant pieces of inulin, free [H] or included in parenchyma cells.

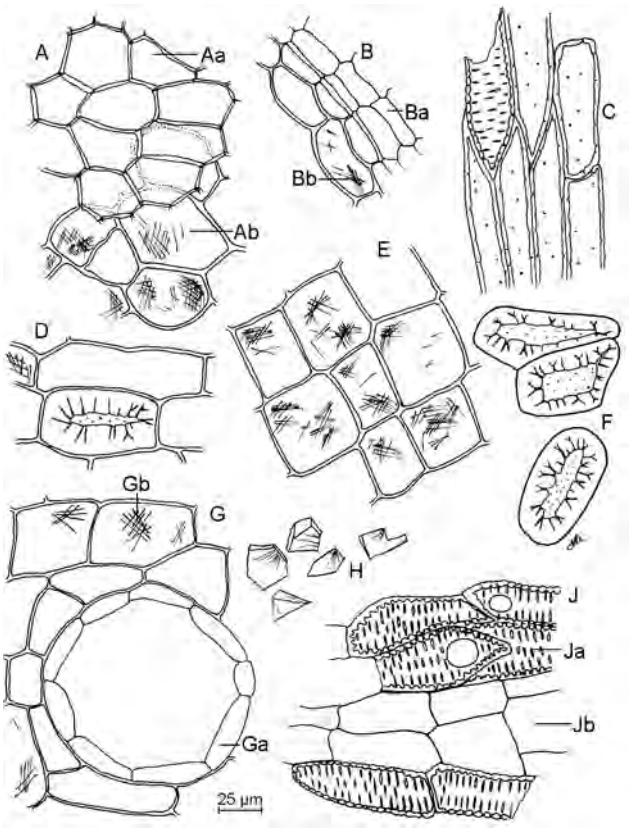


Figure 2560.-1. – *Illustration for identification test B of powdered herbal drug of Atractylodes rhizome, largehead*

C. Examine the chromatograms obtained in the test for *Atractylodes lancea*.



Herbal drugs

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| β-Caryophyllene: a pink zone | A pink or violet zone |
| | An orange zone |
| ——— | ——— |
| | A very faint violet zone |
| ——— | ——— |
| Bornyl acetate: a brown zone | |
| | A very faint violet zone |
| | Several faint violet zones |
| **Reference solution** | **Test solution** |

D. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *ethanol (96 per cent) R*, heat in a water-bath at 60 °C for 2 min and filter. To 1 mL of the filtrate add 0.25 mL of a solution freshly prepared as follows: dissolve 5 mg of *vanillin R* in 0.5 mL of *ethanol (96 per cent) R*, to this solution add 0.5 mL of *water R* and 3 mL of *hydrochloric acid R*. Shake immediately; a red or reddish-purple colour develops and persists.

TESTS

***Atractylodes lancea***. Thin-layer chromatography (*2.2.27*).

*Test solution*. Introduce 0.5 g of the powdered herbal drug (355) (*2.9.12*) into a centrifuge tube, add 2 mL of *methanol R* and stopper the tube. Sonicate at 25 °C for 15 min and centrifuge.

*Reference solution*. Dissolve 10 mg of *β-caryophyllene R* and 10 mg of *bornyl acetate R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *heptane R* (5:95 *V/V*).

*Application*: 5 µL [or 3 µL] as bands of 10 mm [or 6 mm].

*Development*: in an unsaturated tank, over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no greyish-green zone in the middle third, above the very faint violet zone.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (710) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 15.0 g of freshly powdered herbal drug (710) (*2.9.12*), a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**07/2017:1797**

# AUCKLANDIA ROOT

## Aucklandiae radix

DEFINITION

Whole or fragmented dried root of *Saussurea costus* (Falc.) Lipsch. (syn. *Saussurea lappa* C.B.Clarke, *Aucklandia lappa* Decne., *Aucklandia costus* Falc.). It is collected in winter and spring, and the rootlets removed.

*Content*: minimum 0.6 per cent of costunolide ($C_{15}H_{20}O_2$; $M_r$ 232.3) and minimum 1.8 per cent for the sum of costunolide and dehydrocostus lactone ($C_{15}H_{18}O_2$; $M_r$ 230.3) (dried drug).

IDENTIFICATION

A. *Whole drug*. It consists of long, sub-cylindrical or sub-conical, slightly flattened, occasionally branched pieces, 4-12 cm in length, 0.5-5 cm in diameter, with some pieces slightly bent, and sometimes longitudinally cut. The stem scar forms a dent at the crown. The outer surface is yellowish-brown or greyish-brown, with coarse, longitudinal wrinkles and furrows, and also additional fine, reticulate furrows. The remains and scars of lateral roots are also present. The bark is tightly adhering; occasional roots are without bark, and have an exposed surface without a distinct network of vessels. The fractured surface has brownish-yellow, reddish-brown or dark brown oil dots and cavities scattered throughout, radially striated xylem, and a brown or greyish-brown cambium. The texture is hard, dense and difficult to break.

*Fragmented drug*. It occurs as subrounded or irregular transverse slices, oblique or longitudinal slices or as small, irregular pieces. The slices are mostly about 5 mm thick, 6-33 mm in diameter, externally greyish-brown or brownish-yellow, with longitudinal wrinkles and furrows and occasional lateral root scars. The cut surface has narrow, radially striated xylem with fine clefts occasionally present, which do not extend into the centre; the brownish cambium is mostly distinct; dark brown or greyish-brown annulations are occasionally visible at intervals. There are scattered dots and oil cavities, that are brownish-yellow, reddish-brown or dark brown in colour. The peripheral outer layer is mostly yellowish-brown or greyish-brown. The older roots have a broad pith sometimes forming a hollow. The texture is hard and dense.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green or yellow-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1797.-1): yellowish-brown cork fragments consisting of several layers of thin-walled cells (surface view [B], transverse section [E]); fragments of oil canals with orange-brown granular contents, some about 50 µm in diameter; numerous fragments of parenchyma with ovoid cells [F, D], some associated with oil canal fragments (transverse section [Da]); fragments of xylem [A, G] with dense, cellulosic xylem parenchyma [Aa, Gb], and mostly reticulate vessels [Ac, Ga] though occasionally pitted, lignified or slightly lignified, and sometimes associated with oil canal fragments (longitudinal section [Ab]); fragments of medullary ray, composed of aligned rectangular cells (longitudinal section [J]); occasional bundles of long, fusiform fibres, usually fragmented, about 20 µm in diameter, with walls showing slit-, V- or Y-shaped pits (surface view [C]). Examine under a microscope using a solution of *glycerol R*. The powder shows abundant inulin fragments, irregular, mostly angular, some wing-like, either free [H] or included in parenchymatous cells [K].

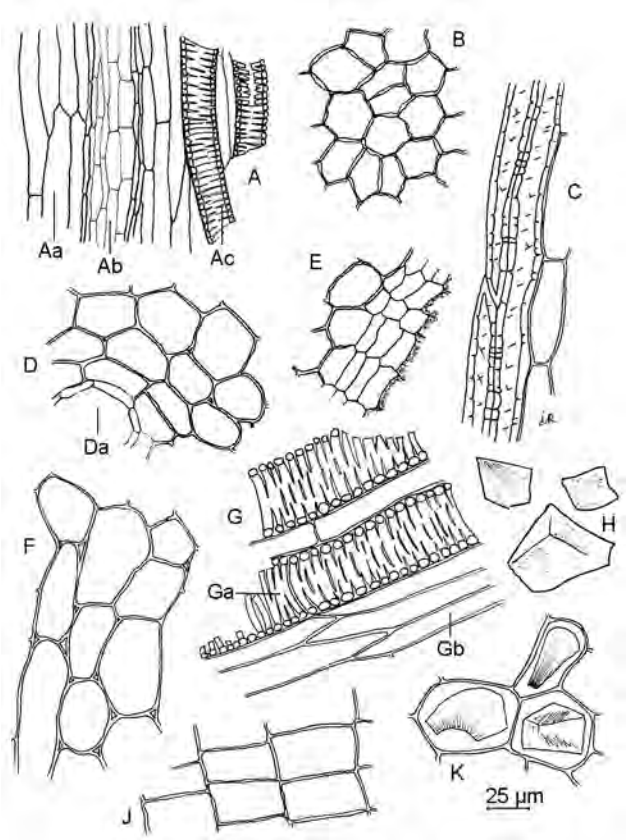


Figure 1797.-1. – *Illustration for identification test B of powdered herbal drug of aucklandia root*

C. Examine the chromatograms obtained in the test for *Vladimiria souliei* (Franch.) Y. Ling and *Inula helenium* L.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint pink zones may be present below the zone due to costunolide in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint pink zone |
| _____ | _____ |
| _____ | _____ |
| Dehydrocostus lactone: a violet zone | A violet zone (dehydrocostus lactone) |
| Costunolide: a greyish-violet zone | A greyish-violet zone (costunolide) |
| **Reference solution** | **Test solution** |

TESTS

***Vladimiria souliei* (Franch.) Y. Ling and *Inula helenium* L.**
Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methylene chloride R* and sonicate for 30 min; filter.

*Reference solution.* Dissolve 5 mg of *dehydrocostus lactone R* and 5 mg of *costunolide R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *cyclohexane R*, *methylene chloride R* (1:5 *V/V*).

*Application*: 2 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no zone in the middle third, and no violet zone directly above the zone due to dehydrocostus lactone.

**Aristolochic acids** (*2.8.21, Method A*). It complies with the test.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.300 g of the freshly powdered herbal drug (355) (*2.9.12*) in 50.0 mL of *methanol R* in a conical flask, stopper tightly and weigh. Sonicate for 30 min and shake for 1 h. Allow to cool and weigh again. Compensate the loss of solvent with *methanol R* and mix thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *costunolide CRS* in 5 mL of *methanol R*, shake vigorously, dilute to 50.0 mL with the same solvent and mix thoroughly.

*Reference solution (b).* Dissolve 2.5 mg of *dehydrocostus lactone R* in 5 mL of reference solution (a), shake vigorously, dilute to 25.0 mL with reference solution (a) and mix thoroughly.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 20 min.

*Retention time*: costunolide = about 8 min; dehydrocostus lactone = about 10 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to costunolide and dehydrocostus lactone.

Calculate the percentage content of costunolide using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to costunolide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to costunolide in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *costunolide CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of costunolide in *costunolide CRS*.

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Calculate the percentage content of the sum of costunolide and dehydrocostus lactone using the following expression:

$$\frac{(A_1 + (1.52 \times A_3)) \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to costunolide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to costunolide in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to dehydrocostus lactone in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *costunolide CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of costunolide in *costunolide CRS*;

1.52 = correlation factor between dehydrocostus lactone and costunolide.

**04/2011:2438**

# BAICAL SKULLCAP ROOT

## Scutellariae baicalensis radix

DEFINITION

Dried, peeled, usually fragmented root of *Scutellaria baicalensis* Georgi without rootlets. It is collected in spring or autumn.

*Content*: not less than 9.0 per cent of baicalin ($C_{21}H_{18}O_{11}$; $M_r$ 446.4) (dried drug).

IDENTIFICATION

A. The root is conical, twisted and, if not reduced in size, 8-25 cm long and 1-3 cm in diameter. The outer surface is brownish-yellow or dark yellow, bearing sparse, warty traces of rootlets, the upper part rough, with twisted longitudinal wrinkles or irregular reticula, the lower part with longitudinal striations and fine wrinkles. Texture hard and fragile, easily broken, fracture yellow, reddish-brown in the centre; the central part of an old root dark brown or brownish-black, withered or hollowed.

B. Microscopic examination (*2.8.23*). The powder is yellow or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: phloem fibres, single or in bundles, fusiform, 60-250 µm long, 9-33 µm in diameter, with thick, channelled walls; stone cells sub-spherical, square or rectangular with rounded edges, with thickened walls, sometimes heavily; cork cells polygonal and brownish-yellow; numerous reticulated vessels, 24-72 µm in diameter; lignified fibres frequently broken, about 12 µm in diameter, with sparse, oblique pits. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant starch granules, simple, spheroidal, 2-10 µm in diameter, with a distinct hilum, or compound with 2-3 components.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 1 mg of *baicalin R* and 1 mg of *acteoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (1:1:2:15 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100-105 °C for 3 min, treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*, allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint blue fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 3-4 fluorescent zones |
| _____ | _____ |
| | 2 fluorescent zones |
| _____ | _____ |
| Verbascoside: a blue fluorescent zone | A strong blue fluorescent zone |
| | A blue fluorescent zone |
| Baicalin: a black zone | A black zone |
| | A weak yellow fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 0.300 g of the powdered herbal drug (355) (*2.9.12*) add 40 mL of *ethanol (70 per cent V/V) R*, heat under a reflux condenser on a water bath for 3 h, cool and filter. Transfer the filtrate to a 100 mL volumetric flask. Wash both the container and the residue several times with a small volume of *ethanol (70 per cent V/V) R* and filter the washings into the same flask. Dilute to 100.0 mL with *ethanol (70 per cent V/V) R*. Mix well. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*. Mix well.

*Reference solution (a)*. Dissolve 5.0 mg of *baicalin CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 2 mg of *methyl parahydroxybenzoate R* in *methanol R*, add 20 mL of reference solution (a) and dilute to 100 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

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| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 90 → 60 | 10 → 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Retention time*: methyl parahydroxybenzoate = about 15 min; baicalin = about 16 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 3 between the peaks due to methyl parahydroxybenzoate and baicalin.

Calculate the percentage content of baicalin using the following expression:

$$\frac{m_2 \times S_1 \times 10 \times p}{S_2 \times m_1}$$

$m_1$ = mass of the herbal drug, in grams;

$m_2$ = mass of baicalin used to prepare reference solution (a), in grams;

$S_1$ = area of the peak due to baicalin in the chromatogram obtained with the test solution;

$S_2$ = area of the peak due to baicalin in the chromatogram obtained with reference solution (a);

$p$ = percentage content of baicalin in *baicalin CRS*.

STORAGE

Protected from moisture.

**01/2018:2612**

# BARBARY WOLFBERRY FRUIT

## Lycii fructus

DEFINITION

Dried, whole, ripe fruit of *Lycium barbarum* L.

IDENTIFICATION

A. The berry is elliptical, fusiform or ovoid and frequently flattened, about 6-20 mm long and 3-10 mm in diameter. The apex of the fruit shows a ring-shaped scar of the nectar-bearing base of the style and the base of the fruit bears the whitish to light brown remnants of the cut stalk. The external surface is orange-red or dark red. The pericarp is fleshy, wrinkled, soft and viscous. It contains 20-50 hard, flat, subreniform seeds, bent upwards. Each pale yellow or yellowish-brown seed is about 1.7 mm long and 1.5 mm wide.

B. Microscopic examination (*2.8.23*). The powder is orange-red or reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2612.-1): fragments of the epicarp (surface view [A]) with polygonal or elongated cells, about 60 µm in diameter, with straight or slightly wavy walls, covered with a thick cuticle, with distinct, more or less parallel striations; fragments of the epicarp (transverse section [F]) covered by a crenate cuticle [Fa], consisting of 1 or 2 layers of cells [Fb] associated with the mesocarp [Fc]; fragments of the mesocarp [C] with thin-walled subpolygonal cells containing reddish-orange or brownish-red spherical granules [Ca, Fd] or microsphenoidal crystals of calcium oxalate [Cb]; fragments of the seeds (surface view [B]) with a testa consisting of greenish-yellow sclereids with heavily thickened, striated and lobed walls and (transverse section [E]) with a testa having thin external walls [Ea] and deeply lobed, irregularly thickened, striated, radial internal walls [Eb]; fragments of endosperm containing oil droplets [D]; fragments of vascular tissue with narrow, spiral or annular vessels [G].

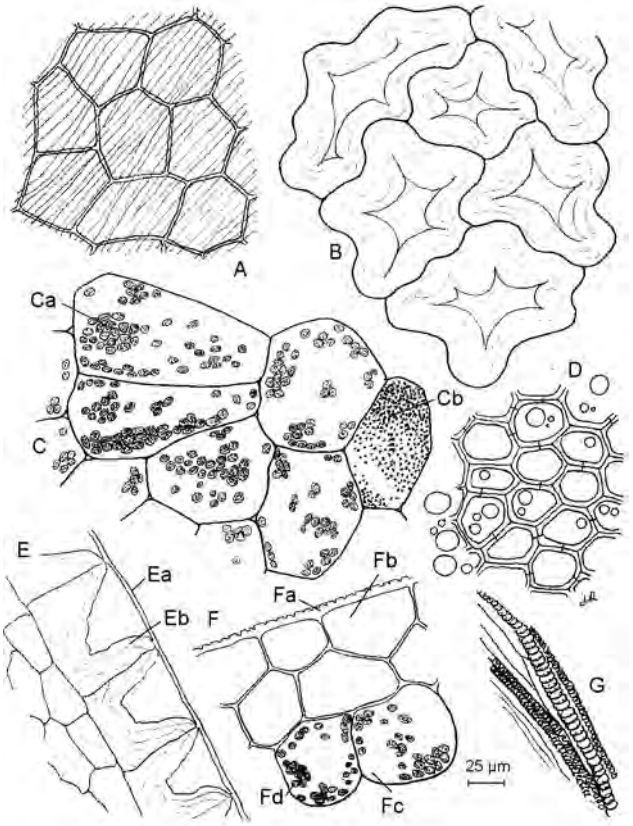


Figure 2612.-1. – *Illustration for identification test B of powdered herbal drug of barbary wolfberry fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.1 g of the powdered herbal drug (355) (*2.9.12*) add 7 mL of *water R*. Sonicate for 10 min and centrifuge. Prepare a ready-to-use sample preparation cartridge containing 0.50 g of octadecylsilyl silica gel (50 µm) using 3 mL of *methanol R*, drying with a stream of air, then using 3 mL of *water R*. The flow rate does not exceed 6 mL/min. Apply 4 mL of the supernatant to the top of the cartridge. Wash the cartridge twice with 1 mL of a mixture of 1 volume of *methanol R* and 9 volumes of *water R*. Elute the cartridge with 1 mL of *methanol R*; collect the eluate and use it as the test solution.

*Reference solution.* Dissolve 1 mg of *scopoletin R* in 10 mL of *methanol R*. Dilute 1 mL of the solution to 10 mL with *methanol R* to obtain solution (a). Dissolve 1 mg of *rutoside trihydrate R* in 5 mL of solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 2 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min; treat the still-warm plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; examine in ultraviolet light at 365 nm after 5 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Scopoletin: a bright blue fluorescent zone | A blue fluorescent zone (scopoletin) |
| | A blue fluorescent zone |
| _____ | _____ |
| | 2 blue fluorescent zones |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone (rutoside) |
| | 3-4 blue fluorescent zones |
| _____ | _____ |
| | An orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 2.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Extractable matter**: minimum 55.0 per cent.

To 2.00 g of the powdered herbal drug (355) (*2.9.12*) add 50.0 g of *water R*, boil under reflux for 1 h, compensate the loss of water and filter. Evaporate 25.0 g of the filtrate to dryness on a water-bath and dry in an oven at 105 °C for 3 h. The residue weighs a minimum of 0.55 g.

**07/2013:1054**

# BEARBERRY LEAF

## Uvae ursi folium

DEFINITION

Whole or fragmented, dried leaf of *Arctostaphylos uva-ursi* (L.) Spreng.

*Content*: minimum 7.0 per cent of anhydrous arbutin ($C_{12}H_{16}O_7$; $M_r$ 272.3) (dried drug).

IDENTIFICATION

A. The leaf, shiny and dark green on the adaxial surface, lighter on the abaxial surface, is generally 7-30 mm long and 5-12 mm wide. The entire leaf is obovate with smooth margins, somewhat reflexed downwards, narrowing at the base into a short petiole. The leaf is obtuse or retuse at its apex. The lamina is thick and coriaceous. The venation, pinnate and finely reticulate, is clearly visible on both surfaces. The adaxial surface is marked with sunken veinlets, giving it a characteristic grainy appearance. Only the young leaf has ciliated margins. Old leaves are glabrous.

B. Microscopic examination (*2.8.23*). The powder is green, greenish-grey or yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1054.-1): fragments of adaxial epidermis (surface view [A]) showing thick and irregularly pitted polygonal cells [Aa] usually accompanied by palisade parenchyma [Ab]; fragments of adaxial epidermis (transverse section [G]), showing straight-walled cells [Ga] covered by a thick smooth cuticle [Gb], and accompanied by palisade parenchyma [Gc] consisting of 3 or 4 layers of cells of unequal lengths, some of which contain numerous prisms of calcium oxalate [Gd]; fragments of abaxial epidermis (surface view [B, E]), showing anomocytic stomata (*2.8.3*) [Ba] surrounded by 5-11 subsidiary cells, scars of hair bases [Ea], and accompanied by spongy parenchyma [Eb]; groups of lignified fibres from the pericycle [D]; fragments of the vascular system [F] consisting of pitted vessels [Fa] and fibres [Fb] accompanied by rows of cells containing prisms of calcium oxalate [Fc]; oil droplets are present in the parenchymatous cells; occasional fragments of conical, unicellular covering trichomes [C].

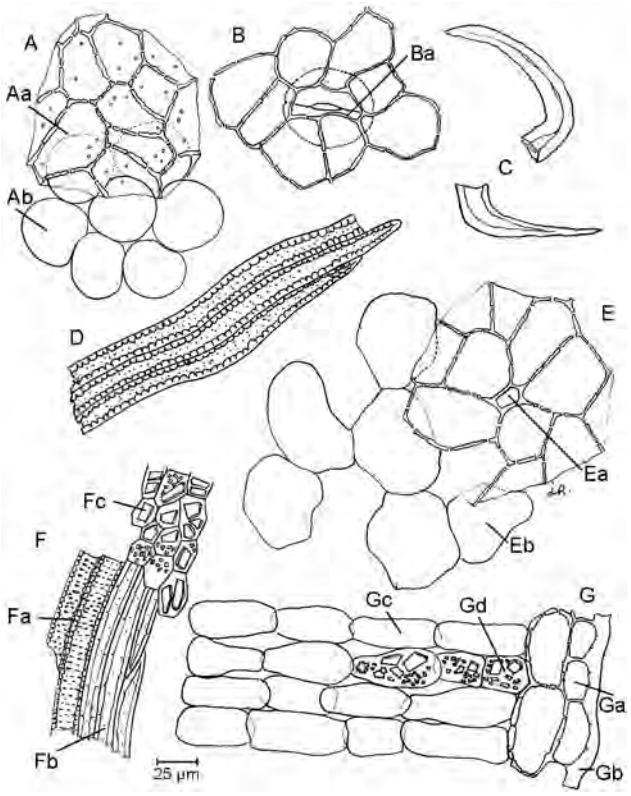


Figure 1054.-1. – *Illustration for identification test B of powdered herbal drug of bearberry leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of a mixture of equal volumes of *methanol R* and *water R*, and heat under a reflux condenser for 10 min. Filter whilst hot. Wash the flask and the filter with a mixture of equal volumes of *methanol R* and *water R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution.* Dissolve 50 mg of *arbutin R* and 25 mg of *gallic acid R* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:6:88 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: at 105-110 °C until the mobile phase has evaporated.

*Detection*: treat with a 10 g/L solution of *dichloroquinonechlorimide R* in *methanol R*, then treat with a 20 g/L solution of *anhydrous sodium carbonate R*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other blue or brown zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Gallic acid: a brownish zone | A brownish zone |
| Arbutin: a blue zone | A brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems and maximum 3 per cent of other foreign matter.

**Leaves of different colour**: maximum 10 per cent, determined in the same manner as foreign matter (*2.8.2*).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 100 mL flask with a ground-glass neck, place 0.800 g of the powdered herbal drug (250) (*2.9.12*). Add 20 mL of *water R* and heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter the liquid through a plug of absorbent cotton. Add the absorbent cotton to the residue in the 100 mL flask and extract with 20 mL of *water R* under a reflux condenser on a water-bath for 30 min. Allow to cool and filter through a paper filter. Combine the filtrates and dilute to 50.0 mL with *water R*. Filter through a paper filter. Discard the first 10 mL of the filtrate.

*Reference solution (a).* Dissolve 50.0 mg of *arbutin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.5 mg of *hydroquinone R* in the mobile phase and dilute to 10.0 mL with the mobile phase. To 5.0 mL of the solution, add 2.5 mL of reference solution (a) and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water R* (10:90 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to arbutin and hydroquinone.

Calculate the percentage content of arbutin using the following expression:

$$\frac{F_1 \times m_2 \times p}{F_2 \times m_1}$$

$F_1$ = area of the peak due to arbutin in the chromatogram obtained with the test solution;

$F_2$ = area of the peak due to arbutin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *arbutin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of arbutin in *arbutin CRS*.

**01/2014:2561**

# BELAMCANDA CHINENSIS RHIZOME

## Belamcandae chinensis rhizoma

DEFINITION

Dried, whole or fragmented rhizome of *Iris domestica* (L.) Goldblatt et Mabb. (syn. *Belamcanda chinensis* (L.) DC.), collected in early spring while the plant is budding or in late autumn while the aerial part is withering, with roots removed.

*Content*: minimum 0.10 per cent of irisflorentin ($C_{20}H_{18}O_8$; $M_r$ 386.4) (dried drug).

IDENTIFICATION

A. The whole rhizome is nodular, rounded, about 3-10 cm long and 1-2 cm in diameter, irregular, more or less branched, with numerous annular striations; there are crateriform, annular stem scars on the upper surface and small roots about 2-3 mm in diameter on the lower surface. The longitudinally fragmented rhizome is found as pieces about 2-8 cm long, 2 cm wide and 1 cm thick; stem scars and root fragments are present. The orange-brown or dark brown outer surface is the same colour as the fracture. The central parenchyma has a pitted appearance due to the numerous primary vascular bundles. The texture is hard. The fracture is granular.

B. Microscopic examination (*2.8.23*). The powder is orange-brown or dark brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: rare fragments of brown cork with superimposed polyhedral cells; numerous, somewhat rounded parenchyma cells with irregularly thickened and pitted walls, granular and oily contents, with some of the cells containing a very large calcium oxalate prism up to 250 µm long and about 50 µm in diameter; very numerous free calcium oxalate prisms, usually broken; reticulate or pitted lignified vessels. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous rounded or ovoid starch granules, 3-15 µm in diameter, simple or rarely compound with 2-5 components. The punctiform hilum is sometimes visible. The starch granules are free and very often included in the parenchymatous cells.

C. Examine the chromatograms obtained in the test for *Iris tectorum* Maxim.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Coumarin: a quenching zone | A quenching zone |
| Irisflorentin: a quenching zone | A quenching zone (irisflorentin) |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Coumarin: a faint dark blue fluorescent zone | A faint blue fluorescent zone |
| | A black zone |
| | A broad blue fluorescent zone |
| Irisflorentin: a blue fluorescent zone | A blue fluorescent zone (irisflorentin) |
| **Reference solution** | **Test solution** |

TESTS

***Iris tectorum* Maxim**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate for 10 min. Centrifuge and filter.

*Reference solution*. Dissolve 1 mg of *irisflorentin R* and 1 mg of *coumarin R* in 4 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *cyclohexane R*, *ethyl acetate R* (1:20:80 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution shows no quenching zone between the zones due to coumarin and irisflorentin in the chromatogram obtained with the reference solution. No quenching zones are present in the lower third of the chromatogram obtained with the test solution.

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: the chromatogram obtained with the test solution shows no pale blue fluorescent zone above the zone due to coumarin in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Disperse 0.100 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *ethanol (70 per cent V/V) R* in a 50 mL centrifuge tube. Sonicate for 30 min. Mix and centrifuge for 5 min. Transfer the supernatant to a 25 mL volumetric flask. Add to the residue 10 mL of *ethanol (70 per cent V/V) R* and sonicate for 30 min. Filter and add the filtrate to the same volumetric flask. Dilute to 25.0 mL with *ethanol (70 per cent V/V) R* and mix. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 5.0 mg of *irisflorentin CRS* in *ethanol (70 per cent V/V) R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (70 per cent V/V) R*.

*Reference solution (b)*. Disperse 0.10 g of *belamcanda chinensis rhizome HRS* in 10 mL of *ethanol (70 per cent V/V) R* in a 50 mL centrifuge tube. Sonicate for 30 min. Mix and centrifuge for 5 min. Transfer the supernatant to a 25 mL volumetric flask. Add to the residue 10 mL of *ethanol (70 per cent V/V) R* and sonicate for 30 min. Filter and add the filtrate to the same volumetric flask. Dilute to 25.0 mL with *ethanol (70 per cent V/V) R* and mix. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 0.05 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 82 | 18 |
| 5 - 20 | 82 → 80 | 18 → 20 |
| 20 - 30 | 80 → 67 | 20 → 33 |
| 30 - 50 | 67 → 60 | 33 → 40 |
| 50 - 65 | 60 → 47 | 40 → 53 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to irisflorentin; use the chromatogram supplied with *belamcanda chinensis rhizome HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to tectoridin and peak 2 (unknown).

*Retention time*: irisflorentin = about 54 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peak due to tectoridin and peak 2.

Calculate the percentage content of irisflorentin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 20}$$

$A_1$ = area of the peak due to irisflorentin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to irisflorentin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *irisflorentin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of irisflorentin in *irisflorentin CRS*.

**01/2012:0221**
**corrected 9.2**

# BELLADONNA LEAF

## Belladonnae folium

DEFINITION

Dried leaf or dried leaf and flowering, and occasionally fruit-bearing, tops of *Atropa belladonna* L.

*Content*: minimum 0.30 per cent of total alkaloids, expressed as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4) (dried drug). The alkaloids consist mainly of hyoscyamine together with small quantities of hyoscine (scopolamine).

CHARACTERS

Slightly nauseous odour.

IDENTIFICATION

A. The leaves are green or brownish-green, slightly darker on the upper surface, often crumpled and rolled and partly matted together in the drug. The leaf is petiolate and the lamina is acute and decurrent. The margin is entire. The flowering stems are flattened and bear at each node a pair of leaves unequal in size, in the axils of which occur singly the flowers or occasionally fruits. The flowers have a gamosepalous calyx and campanulate corolla. The drug may contain fruits, as globular berries, green or brownish-black and surrounded by the persistent calyx with widely spread lobes.

B. Microscopic examination (*2.8.23*). The powder is dark green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0221.-1): fragments of the lamina showing sinuous-walled epidermal cells with striated cuticle [A, C] and part of the underlying palisade parenchyma [Aa] associated with the upper epidermis [A]; numerous stomata [Ca] more frequent on the lower epidermis [C], anisocytic and also some anomocytic (*2.8.3*); multicellular, uniseriate covering trichomes with a smooth cuticle [F], glandular trichomes with unicellular heads and multicellular, uniseriate stalks [D] or with multicellular heads and unicellular stalks [B]; parenchyma cells including rounded cells, some of which contain microsphenoidal crystals of calcium oxalate [E]; annularly and spirally thickened vessels [K]. The powdered herbal drug may also show: fibres and reticulately thickened vessels from the stems; subspherical pollen grains, 40-50 µm in diameter, with 3 germinal pores, 3 furrows and an extensively pitted exine [H]; fragments of the corolla with a papillose epidermis [J] or bearing numerous covering or glandular trichomes of the types previously described [L]; fragments of the brownish-yellow testa consisting of irregularly sclerified cells [G].

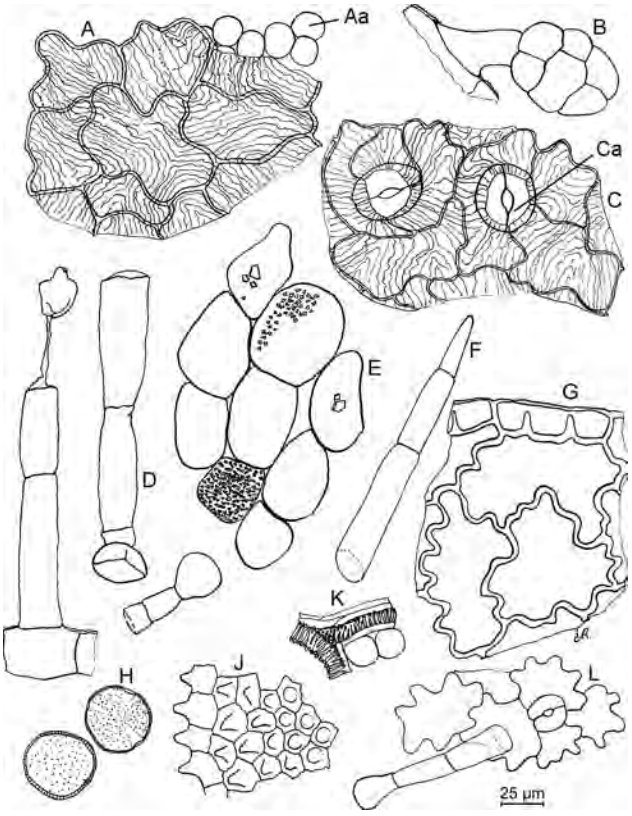


Figure 0221.-1. – *Illustration for identification test B of powdered herbal drug of belladonna leaf*

C. Shake 1 g of the powdered herbal drug (180) (*2.9.12*) with 10 mL of *dilute sulfuric acid R1* for 2 min. Filter and add to the filtrate 1 mL of *concentrated ammonia R* and 5 mL of *water R*. Shake cautiously with 15 mL of *ether R*, avoiding formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulfate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 mL of *fuming nitric acid R* and evaporate to dryness on a water-bath. Add 10 mL of *acetone R* and, dropwise, a 30 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. A deep violet colour develops.

D. Examine the chromatograms obtained in the chromatography test.

*Results*: the principal zones in the chromatograms obtained with the test solution are similar in position, colour and size to the principal zones in the chromatograms obtained with the same volume of the reference solution.

TESTS

**Chromatography**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.6 g of the powdered herbal drug (180) (*2.9.12*) add 15 mL of *dilute sulfuric acid R1*, shake for 15 min and filter. Wash the filter with *dilute sulfuric acid R1* until 20 mL of filtrate is obtained. To the filtrate add 1 mL of *concentrated ammonia R* and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution*. Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 1.8 mL of the hyoscine hydrobromide solution and 8 mL of the hyoscyamine sulfate solution.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 10 µL and 20 µL, as bands of 20 mm by 3 mm, leaving 1 cm between the bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min; allow to cool.

*Detection A*: spray with *potassium iodobismuthate solution R2*, using about 10 mL for a plate 200 mm square, until the orange or brown zones become visible against a yellow background.

*Results A*: the zones in the chromatograms obtained with the test solution are similar in position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatograms) and colour to the bands in the chromatograms obtained with the reference solution. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µL of the test solution or near the starting point in the chromatogram obtained with 10 µL of the test solution.

*Detection B*: spray with *sodium nitrite solution R* until the coating is transparent; examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the reference solution and the test solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

**Foreign matter** (*2.8.2*): maximum 3 per cent of stems with a diameter greater than 5 mm.

**Total ash** (*2.4.16*): maximum 16.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

a) Determine the loss on drying (*2.2.32*) on 2.000 g of the powdered herbal drug (180) (*2.9.12*), by drying in an oven at 105 °C.

b) Moisten 10.00 g of the powdered herbal drug (180) (*2.9.12*) with a mixture of 5 mL of *ammonia R*, 10 mL of *ethanol (96 per cent) R* and 30 mL of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in *0.25 M sulfuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 mL by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than 3 quantities, each of 20 mL, of *0.25 M sulfuric acid*, separate the 2 layers by centrifugation if necessary and transfer the acid layers to a 2nd separating funnel. Make the acid layer alkaline with *ammonia R* and shake with 3 quantities, each of 30 mL, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulfate with 3 quantities, each of 10 mL, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 mL of *0.01 M sulfuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88 \times (20 - n)}{(100 - d) \times m}$$

$d$ = loss on drying, as a percentage;

$n$ = volume of *0.02 M sodium hydroxide*, in millilitres;

$m$ = mass of the powdered herbal drug, in grams.

**07/2015:1294**
**corrected 9.2**

# BELLADONNA LEAF DRY EXTRACT, STANDARDISED

## Belladonnae folii extractum siccum normatum

DEFINITION

Standardised dry extract obtained from *Belladonna leaf (0221)*.

*Content*: 0.95 per cent to 1.05 per cent of total alkaloids, expressed as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (70 per cent *V/V*).

CHARACTERS

*Appearance*: brown or greenish, hygroscopic powder.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the extract to be examined add 5.0 mL of *methanol R*. Shake for 2 min and filter.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: treat the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatograms obtained with the reference solution and the test solution show in the central part a light blue fluorescent zone (chlorogenic acid) and in the lower part a yellowish-brown fluorescent zone (rutoside); furthermore, the chromatogram obtained with the test solution shows a little above the start a yellowish-brown fluorescent zone and directly above that a yellow fluorescent zone, and a yellow or yellowish-brown fluorescent zone between the zone due to rutoside and the zone due to chlorogenic acid. Further zones may be present.

B. Examine the chromatograms obtained in the test for atropine.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position and colour to the principal zones in the chromatogram obtained with the reference solution.

TESTS

**Atropine**. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.20 g of the extract to be examined add 10.0 mL of *dilute sulfuric acid R1*, shake for 2 min and filter. Add 1.0 mL of *concentrated ammonia R* and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over about 2 g of *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution.* Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 1.8 mL of the hyoscine hydrobromide solution and 8 mL of the hyoscyamine sulfate solution.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min; allow to cool.

*Detection A*: treat with *potassium iodobismuthate solution R2*, until orange or brown zones become visible against a yellow background.

*Results A*: the zones in the chromatogram obtained with the test solution are similar in position (hyoscyamine in the lower third, hyoscine in the upper third) and colour to those in the chromatogram obtained with the reference solution. Other faint zones may be present in the chromatogram obtained with the test solution.

*Detection B*: treat with *sodium nitrite solution R* until the coating is transparent and examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from orange or brown to reddish-brown but not to greyish-blue (atropine).

**Loss on drying** (*2.8.17*): maximum 5.0 per cent.

ASSAY

At each extraction stage it is necessary to check that the alkaloids have been completely extracted. If the extraction is into the organic phase this is done by evaporating to dryness a few millilitres of the last organic layer, dissolving the residue in *0.25 M sulfuric acid* and verifying the absence of alkaloids using *potassium tetraiodomercurate solution R*. If the extraction is into the acid aqueous phase, this is done by taking a few millilitres of the last acid aqueous phase and verifying the absence of alkaloids using *potassium tetraiodomercurate solution R*.

Disperse 3.00 g in a mixture of 5 mL of *ammonia R* and 15 mL of *water R*. Shake with no fewer than 3 quantities, each of 40 mL, of a mixture of 1 volume of *methylene chloride R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Concentrate the combined organic layers to about 50 mL by distilling on a water-bath and transfer the resulting liquid to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the liquid to produce a layer having a density well below that of water. Shake the resulting solution with no fewer than 3 quantities, each of 20 mL, of *0.25 M sulfuric acid* until the alkaloids are completely extracted. Separate the layers by centrifugation, if necessary, and transfer the acid layers to a 2nd separating funnel. Make the combined acid layers alkaline with *ammonia R* and shake with no fewer than 3 quantities, each of 30 mL, of *methylene chloride R* until the alkaloids are completely extracted. Combine the organic layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the methylene chloride and wash the sodium sulfate with 3 quantities, each of 10 mL, of *methylene chloride R*. Combine the organic extracts and evaporate to dryness on a water-bath. Heat the residue in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, evaporate to dryness on a water-bath and again heat the residue in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, add 20.0 mL of *0.01 M sulfuric acid* and remove the methylene chloride by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88 \times (20 - n)}{100 \times m}$$

$n$ = volume of *0.02 M sodium hydroxide* used, in millilitres;

$m$ = mass of the extract to be examined, in grams.

**01/2008:1812**
**corrected 9.2**

# BELLADONNA LEAF TINCTURE, STANDARDISED

## Belladonnae folii tinctura normata

DEFINITION

Tincture produced from *Belladonna leaf (0221)*.

*Content*: 0.027 per cent to 0.033 per cent of total alkaloids, calculated as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4). The alkaloids consist mainly of hyoscyamine together with small quantities of hyoscine.

PRODUCTION

The tincture is produced from 1 part of the powdered herbal drug (355) (*2.9.12*) and 10 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Evaporate to dryness 10.0 mL of the tincture to be examined in a water-bath at 40 °C under reduced pressure. Dissolve the residue in 1.0 mL of *methanol R*.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 40 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently spray the plate with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow the plate to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid)<br>A yellow or yellowish-brown fluorescent zone |
| ——— | ——— |
| Rutoside: a yellowish-brown fluorescent zone | A bluish-grey fluorescent zone<br>A yellow fluorescent zone<br>A yellowish-brown fluorescent zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for atropine, detection A.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 40 µL of the test solution or near the point of application in the chromatogram obtained with 20 µL of the test solution.

| Top of the plate | |
|---|---|
| Hyoscine: a brownish-orange zone | A brownish-orange zone (hyoscine) |
| ——— | ——— |
| | Faint secondary zones |
| ——— | ——— |
| Hyoscyamine: a brownish-orange zone | A brownish-orange zone (hyoscyamine)<br>Faint secondary zones |
| **Reference solution** | **Test solution** |

TESTS

**Atropine**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 15.0 mL of the tincture to be examined add 15 mL of *dilute sulfuric acid R1*. Filter. Add 1 mL of *concentrated ammonia R* to the filtrate and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. Separate by centrifugation if necessary. Dry the combined ether layers over *anhydrous sodium sulfate R*. Filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution*. Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 1.8 mL of the hyoscine hydrobromide solution and 8 mL of the hyoscyamine sulfate solution.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 20 µL and 40 µL of each solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min.

*Detection A*: spray with *potassium iodobismuthate solution R2*.

*Detection B*: spray with *sodium nitrite solution R* until the plate is transparent. Examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brownish-orange to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

**Ethanol** (*2.9.10*): 64 per cent *V/V* to 69 per cent *V/V*.

ASSAY

Evaporate 50.0 g of the tincture to be examined to a volume of about 10 mL. Transfer quantitatively to a separating funnel, with the minimum volume of *alcohol (70 per cent V/V) R*. Add 5 mL of *ammonia R* and 15 mL of *water R*. Shake with not fewer than 3 quantities each of 40 mL of a mixture of 1 volume of *methylene chloride R* and 3 volumes of *peroxide-free ether R*, carefully to avoid emulsion, until the alkaloids are completely extracted. Combine the organic layers and concentrate the solution to a volume of about 50 mL by distilling on a water-bath. Transfer the resulting solution quantitatively to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the solution to produce a layer having a density well below that of water. Shake the resulting solution with not fewer than 3 quantities each of 20 mL of *0.25 M sulfuric acid* until the alkaloids are completely extracted. Separate the layers by centrifugation if necessary and transfer the layers to a separating funnel. Make the combined layers alkaline with *ammonia R* and shake with not fewer than 3 quantities each of 30 mL of *methylene chloride R* until the alkaloids are completely extracted. Combine the organic layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the methylene chloride and filter. Wash the sodium sulfate with 3 quantities each of 10 mL of *methylene chloride R*. Combine the organic extracts, evaporate to dryness on a water-bath. Heat the residue in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *methylene chloride R*, evaporate to dryness on a water-bath and heat the residue in an oven at 100-105 °C for 15 min again. Dissolve the residue in a few millilitres of *methylene chloride R*. Add 20.0 mL of *0.01 M sulfuric acid* and remove the methylene chloride by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88 \times (20 - n)}{100 \times m}$$

$n$ = volume of *0.02 M sodium hydroxide* used, in millilitres,

$m$ = mass of the herbal drug used, in grams.

**01/2008:0222**
**corrected 10.0**

# BELLADONNA, PREPARED

## Belladonnae pulvis normatus

DEFINITION

Belladonna leaf powder (180) (*2.9.12*) adjusted, if necessary, by adding powdered lactose or belladonna leaf powder with a lower alkaloidal content.

*Content*: 0.28 per cent to 0.32 per cent of total alkaloids, expressed as hyoscyamine ($M_r$ 289.4) (dried drug).

CHARACTERS

Slightly nauseous odour.

IDENTIFICATION

A. Microscopic examination (*2.8.23*) of the powder (180) (*2.9.12*). The powder is dark green. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing sinuous-walled epidermal cells, a striated cuticle and numerous stomata predominantly

present on the lower epidermis (anisocytic and also some anomocytic) (*2.8.3*); multicellular uniseriate covering trichomes with smooth cuticle, glandular trichomes with unicellular heads and multicellular, uniseriate stalks or with multicellular heads and unicellular stalks; parenchyma cells including rounded cells containing microsphenoidal crystals of calcium oxalate; annular and spirally thickened vessels. The powdered herbal drug may also show the following: fibres and reticulately thickened vessels from the stems; subspherical pollen grains, 40-50 µm in diameter, with 3 germinal pores, 3 furrows and an extensively pitted exine; fragments of the corolla, with a papillose epidermis or bearing numerous covering or glandular trichomes of the types previously described; brownish-yellow seed fragments containing irregularly sclerified and pitted cells of the testa. Examined in *glycerol (85 per cent) R*, the powder may be seen to contain lactose crystals.

B. Shake 1 g with 10 mL of *dilute sulfuric acid R1* for 2 min. Filter and add to the filtrate 1 mL of *concentrated ammonia R* and 5 mL of *water R*. Shake cautiously with 15 mL of *ether R*, avoiding formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulfate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 mL of *fuming nitric acid R* and evaporate to dryness on a water-bath. Add 10 mL of *acetone R* and, dropwise, a 30 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. A deep violet colour develops.

C. Examine the chromatograms obtained in the test Chromatography.

*Results*: the principal zones in the chromatograms obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.

TESTS

**Chromatography**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.6 g of the drug to be examined add 15 mL of *dilute sulfuric acid R1*, shake for 15 min and filter. Wash the filter with *dilute sulfuric acid R1* until 20 mL of filtrate is obtained. To the filtrate add 1 mL of *concentrated ammonia R* and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulfate R*, filter, and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution*. Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 1.8 mL of the hyoscine hydrobromide solution and 8 mL of the hyoscyamine sulfate solution.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 10 µL and 20 µL of each solution, as bands of 20 mm by 3 mm, leaving 1 cm between each band.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min; allow to cool.

*Detection A*: spray with *potassium iodobismuthate solution R2*, using about 10 mL for a plate 200 mm square, until orange or brown zones become visible against a yellow background.

*Results A*: the zones in the chromatograms obtained with the test solution are similar in position (hyoscyamine in the lower third, hyoscine in the upper third) and colour to those in the chromatograms obtained with the reference solution; the zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution; faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µL of the test solution or near the point of application in the chromatogram obtained with 10 µL of the test solution.

*Detection B*: spray with *sodium nitrite solution R* until the coating is transparent and examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine), and any secondary zones disappear.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 16.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

a) Determine the loss on drying (*2.2.32*) on 2.000 g by drying in an oven at 105 °C.

b) Moisten 10.00 g with a mixture of 5 mL of *ammonia R*, 10 mL of *ethanol (96 per cent) R* and 30 mL of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in *0.25 M sulfuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 mL by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than 3 quantities, each of 20 mL, of *0.25 M sulfuric acid*, separate the 2 layers by centrifugation if necessary and transfer the acid layers to a 2nd separating funnel. Make the acid layer alkaline with *ammonia R* and shake with 3 quantities, each of 30 mL, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulfate with 3 quantities, each of 10 mL, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 mL of *0.01 M sulfuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88 \times (20 - n)}{(100 - d) \times m}$$

$d$ = loss on drying as a percentage;

$n$ = volume of *0.02 M sodium hydroxide* used, in millilitres;

$m$ = mass of the herbal drug used, in grams.

STORAGE

In an airtight container.

**01/2016:2158**

# BENZOIN, SIAM

## Benzoe tonkinensis

DEFINITION

Resin obtained by incising the trunk of *Styrax tonkinensis* (Pierre) Craib ex Hartwich.

*Content*: 35.0 per cent to 55.0 per cent of total acids, expressed as benzoic acid ($C_7H_6O_2$; $M_r$ 122.1) (dried drug).

CHARACTERS

Reminiscent odour of vanillin.

IDENTIFICATION

A. Siam benzoin occurs as opaque, often shiny, rounded, ovoid or angular masses (tears or almonds), usually broken and varying in size from a few millimetres up to about 3 cm. Individual tears are yellowish-white or reddish externally, often with brown or grey patches of discolouration, and have a waxy, whitish fracture which becomes reddish on prolonged exposure to air.

B. Examine the chromatograms obtained in test B for Sumatra benzoin.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Methyl cinnamate: a quenching zone | |
| Cinnamic acid: a quenching zone | |
| | A quenching zone |
| | A very prominent quenching zone |
| ——— | ——— |
| Vanillin: a quenching zone | |
| | A quenching zone |
| | 3 quenching zones |
| **Reference solution** | **Test solution** |

TESTS

**Sumatra benzoin**

A. To 0.2 g of the finely powdered herbal drug add 10 mL of *ethanol (96 per cent) R*. Shake vigorously until almost completely dissolved and filter. Place 5 mL of the filtrate in a test-tube and add 0.5 mL of a 50 g/L solution of *ferric chloride R* in *ethanol (96 per cent) R*. A green colour is produced. No yellow colour is produced.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Sonicate 0.2 g of the finely powdered herbal drug in 5 mL of *ethanol (96 per cent) R* and centrifuge. Use the supernatant.

*Reference solution*. Dissolve 4 mg of *vanillin R*, 10 mg of *trans-cinnamic acid R* and 10 mg of *methyl cinnamate R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *cyclohexane R* (10:40:60 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution shows no zone in the same position as the zone due to cinnamic acid in the chromatogram obtained with the reference solution.

**Matter insoluble in ethanol**: maximum 5 per cent.

To 2.0 g of the powdered herbal drug add 25 mL of *ethanol (90 per cent V/V) R*. Boil until almost completely dissolved. Filter through a previously tared sintered-glass filter (16) (*2.1.2*) and wash with 3 quantities, each of 5 mL, of boiling *ethanol (90 per cent V/V) R*. Heat the glass filter and its contents in an oven at 100-105 °C for 2 h. Weigh after cooling.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 2.000 g of the coarsely powdered herbal drug by drying *in vacuo* for 4 h.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

ASSAY

Place 0.750 g of the finely powdered herbal drug in a 250 mL borosilicate glass flask and add 15.0 mL of *0.5 M alcoholic potassium hydroxide*. Boil under a reflux condenser on a water-bath for 30 min. Allow to cool and rinse the condenser with 20 mL of *ethanol (96 per cent) R*. Titrate the excess of potassium hydroxide with *0.5 M hydrochloric acid*. Determine the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.05 mg of benzoic acid ($C_7H_6O_2$).

**01/2008:1814**

# BENZOIN, SUMATRA

## Benzoe sumatranus

DEFINITION

Resin obtained by incising the trunk of *Styrax benzoin* Dryander.

*Content*: 25.0 per cent to 50.0 per cent of total acids, calculated as benzoic acid ($C_7H_6O_2$; $M_r$ 122.1) (dried drug).

IDENTIFICATION

A. Sumatra benzoin occurs as creamy white, rounded to ovoid tears, which may be embedded in a dull greyish-brown or reddish-brown matrix. It is hard and brittle and the fractured surface is dull and uneven.

B. Examine the chromatograms obtained in test B for *Styrax tonkinensis*.

*Results*: see below the sequence of quenching zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A very intense dark zone |
| _____ | _____ |
| Methyl cinnamate: a very intense dark zone | A dark zone |
| Benzoic acid: a dark zone | A very weak dark zone (benzoic acid) |
| Cinnamic acid: an intense dark zone | A very intense dark zone (cinnamic acid) |
| _____ | _____ |
| | A dark zone |
| | A very intense dark zone |
| | A dark zone |
| Vanillin: a dark zone | A very weak dark zone (vanillin) |
| | Series of unresolved zones including 2 dark zones |
| **Reference solution** | **Test solution** |

TESTS

**Dammar gum**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.2 g of the drug to be examined with gentle heating in 10 mL of *ethanol (90 per cent V/V) R* and centrifuge.

*Plate*: *TLC aluminium oxide G plate R*.

*Mobile phase*: *light petroleum R4*, *ether R* (40:60 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5 min.

*Results*: the chromatogram obtained does not show any prominent spot with an $R_F$ between 0.4 and 1.0.

***Styrax tonkinensis***

A. To 0.2 g of the finely powdered herbal drug add 10 mL of *ethanol (96 per cent) R*. Shake vigorously until almost completely dissolved and filter. Place 5 mL of the filtrate in a test-tube and add 0.5 mL of a 50 g/L solution of *ferric chloride R* in *ethanol (96 per cent) R*. A yellowish, slightly green colour is produced.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Sonicate 0.2 g of the finely powdered herbal drug in 5 mL of *ethanol (96 per cent) R* and filter. Collect the filtrate.

*Reference solution*. Dissolve 20 mg of *benzoic acid R*, 10 mg of *trans-cinnamic acid R*, 4 mg of *vanillin R* and 20 mg of *methyl cinnamate R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *hexane R* (10:40:60 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 12 cm [or 5 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution shows 2 faint zones in the same positions as the dark zones due to benzoic acid and vanillin in the chromatogram obtained with the reference solution.

**Matter insoluble in ethanol**: maximum 20.0 per cent.

To 2.0 g of the powdered herbal drug add 25 mL of *ethanol (90 per cent V/V) R*. Boil until almost completely dissolved. Filter through a tared sintered-glass filter (16) (*2.1.2*) and wash with 3 quantities, each of 5 mL, of boiling *ethanol (90 per cent V/V) R*. Heat the glass filter and its contents in an oven at 100-105 °C for 2 h. Allow to cool and weigh.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 2.000 g of the coarsely powdered herbal drug by drying *in vacuo* for 4 h.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

ASSAY

Place 0.750 g of the finely powdered herbal drug in a 250 mL borosilicate glass flask and add 15.0 mL of *0.5 M alcoholic potassium hydroxide*. Boil under a reflux condenser on a water-bath for 30 min. Allow to cool and rinse the condenser with 20 mL of *ethanol (96 per cent) R*. Titrate the excess of potassium hydroxide with *0.5 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.05 mg of benzoic acid ($C_7H_6O_2$).

**01/2008:2157**

# BENZOIN TINCTURE, SIAM

## Benzois tonkinensis tinctura

DEFINITION

Tincture produced from *Siam benzoin (2158)*.

*Content*: minimum 5.0 per cent *m/m* of total acids, calculated as benzoic acid ($C_7H_6O_2$; $M_r$ 122.1).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (75 per cent *V/V* to 96 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: orange-yellow liquid.

It has a characteristic odour of vanillin.

IDENTIFICATION

A. Place 10 mL in a test tube; add 0.5 mL of a 50 g/L solution of *ferric chloride R* in *ethanol (96 per cent) R*. A green colour is produced.

B. Examine the chromatograms obtained in the test for Sumatra benzoin tincture.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Methyl cinnamate: a very prominent quenching zone | |
| Benzoic acid: a quenching zone | A quenching zone (benzoic acid) |
| Cinnamic acid: a prominent quenching zone | |
| _____ | _____ |
| | A quenching zone |
| | A very prominent quenching zone |
| Vanillin: a quenching zone | A quenching zone (vanillin) |
| | Series of unresolved zones including a quenching zone |
| **Reference solution** | **Test solution** |

TESTS

**Sumatra benzoin tincture**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution*. Dissolve 20 mg of *benzoic acid R*, 10 mg of *trans-cinnamic acid R*, 4 mg of *vanillin R* and 20 mg of *methyl cinnamate R* in 20 mL of ethanol of the same concentration as that used for the production of the tincture.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *hexane R* (10:40:60 *V/V/V*).

*Application*: 20 µL [or 8 µL] as bands.

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution does not show any zone in the same position as the zones due to cinnamic acid and methyl cinnamate in the chromatogram obtained with the reference solution.

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the content stated on the label.

ASSAY

Place 3.50 g in a 250 mL borosilicate glass flask and add 15.0 mL of *0.5 M alcoholic potassium hydroxide*. Boil under a reflux condenser on a water-bath for 30 min. Allow to cool and rinse the condenser with 20 mL of *ethanol (96 per cent) R*. Titrate the excess of potassium hydroxide with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.05 mg of benzoic acid ($C_7H_6O_2$).

**01/2008:1813**

# BENZOIN TINCTURE, SUMATRA

## Benzois sumatrani tinctura

DEFINITION

Tincture produced from *Sumatra benzoin (1814)*.

*Content*: minimum 4.0 per cent *m/m* of total acids, calculated as benzoic acid ($C_7H_6O_2$; $M_r$ 122.1).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (75 per cent *V/V* to 96 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: orange-yellow liquid.

IDENTIFICATION

Examine the chromatograms obtained in the test for Siam benzoin tincture.

*Results*: see below the sequence of quenching zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A very intense dark zone |
| _____ | _____ |
| Methyl cinnamate: a very intense dark zone | A dark zone |
| Benzoic acid: a dark zone | A very weak dark zone (benzoic acid) |
| Cinnamic acid: an intense dark zone | A very intense dark zone (cinnamic acid) |
| _____ | _____ |
| | A dark zone |
| | A very intense dark zone |
| | A dark zone |
| Vanillin: a dark zone | A very weak dark zone (vanillin) |
| | Series of unresolved dark zones |
| **Reference solution** | **Test solution** |

TESTS

**Siam benzoin tincture**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution*. Dissolve 20 mg of *benzoic acid R*, 10 mg of *trans-cinnamic acid R*, 4 mg of *vanillin R* and 20 mg of *methyl cinnamate R* in 20 mL of ethanol of the same concentration as that used for the production of the tincture.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *hexane R* (10:40:60 *V/V/V*).

*Application*: 20 µL [or 8 µL] as bands.

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution does not show zones due to benzoic acid and vanillin that are more intense than the corresponding zones in the chromatogram obtained with the reference solution.

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the content stated on the label.

ASSAY

Place 3.50 g in a 250 mL borosilicate glass flask and add 15.0 mL of *0.5 M alcoholic potassium hydroxide*. Boil under a reflux condenser on a water-bath for 30 min. Allow to cool and rinse the condenser with 20 mL of *ethanol (96 per cent) R*. Titrate the excess of potassium hydroxide with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.05 mg of benzoic acid ($C_7H_6O_2$).

**01/2019:1588**

# BILBERRY FRUIT, DRIED

## Myrtilli fructus siccus

DEFINITION

Dried ripe fruit of *Vaccinium myrtillus* L.

*Content*: minimum 1.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. Dried bilberry is a blackish-blue, subglobular, shrunken berry about 5 mm in diameter covered in a whitish bloom. Its lower end shows a scar or, rarely, a fragment of the pedicel. The upper end is slightly depressed and is surmounted by the remains of the style and of the persistent calyx, which appears as a circular fold. The deep violet, fleshy mesocarp contains numerous (more than 10) small, brown, ovoid seeds.

B. Microscopic examination (*2.8.23*). The powder is violet-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1588.-1): fragments of dark red epicarp [A] consisting of small groups of 2-4 polygonal, rectangular or quadrangular cells; in each group, the inner walls are thin [Aa] whereas the outer walls are regularly thickened [Ab]; fragments of mesocarp [B] consisting of large, rounded, thin-walled cells associated with fine spiral vessels [Ba]; numerous sclereids, isolated or in small groups, from the mesocarp [C]; groups of sclereids from the endocarp [D]; yellowish-brown fragments from the testa, with large sclereids having walls that are extensively channelled and pitted (surface view [E]) and with U-shaped thickenings (transverse section [F]); irregular cluster crystals of calcium oxalate [G] and isolated prisms of calcium oxalate [H] from the mesocarp.

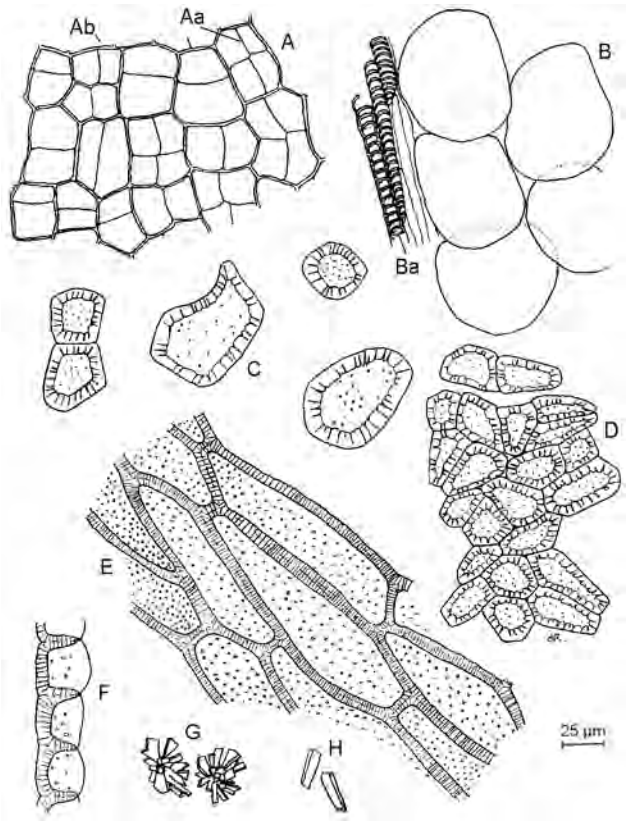


Figure 1588.-1. – *Illustration for identification test B of powdered herbal drug of dried bilberry fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *methanol R*. Shake for 15 min and filter.

*Reference solution*. Dissolve 0.10 g of *bilberry dry extract HRS* in 25 mL of *methanol R*. Stir for 15 min and filter.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *butanol R* (16:19:65 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| A violet-red zone | A violet-red zone |
| A violet zone | A violet zone |
| A violet zone | A violet zone |
| A broad unresolved bluish-violet zone | A broad unresolved bluish-violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent, with no fruit of *Sambucus nigra* present.

Adulteration with *S. nigra* L. is indicated by the presence of glossy, violet-black, ovoid berries without the circular fold due to the calyx, and containing not more than 4 seeds.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 1.500 g of the powdered herbal drug (355) (*2.9.12*).

**01/2019:1602**

# BILBERRY FRUIT, FRESH

## Myrtilli fructus recens

DEFINITION

Fresh or frozen, ripe fruit of *Vaccinium myrtillus* L.

*Content*: minimum 0.30 per cent of anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride (chrysanthemin, $C_{21}H_{21}ClO_{11}$; $M_r$ 484.8) (dried drug).

IDENTIFICATION

A. The fresh fruit is a blackish-blue globular berry about 5 mm in diameter, covered in a whitish bloom. Its lower end shows a scar or, rarely, a fragment of the pedicel. The upper end is slightly depressed and is surmounted by the remains of the style and of the persistent calyx, which appears as a circular fold. The violet, fleshy mesocarp includes 4 to 5 locules containing numerous (more than 10) small, brown, ovoid seeds.

B. The crushed fresh fruit is violet-red. Examine small fragments under a microscope using *chloral hydrate solution R*. They show the following diagnostic characters (Figure 1602.-1): fragments of dark red epicarp [A] consisting of small groups of 2-4 polygonal, rectangular or quadrangular cells; in each group, the inner walls

Herbal drugs

are thin [Aa] whereas the outer walls are regularly thickened [Ab]; fragments of mesocarp [B] consisting of large, rounded, thin-walled cells associated with fine spiral vessels [Ba]; numerous sclereids, isolated or in small groups, from the mesocarp [C]; groups of sclereids from the endocarp [D]; yellowish-brown fragments from the testa, with large sclereids having walls that are extensively channelled and pitted (surface view [E]) and with U-shaped thickenings (transverse section [F]); irregular cluster crystals of calcium oxalate [G] and isolated prisms of calcium oxalate [H] from the mesocarp.

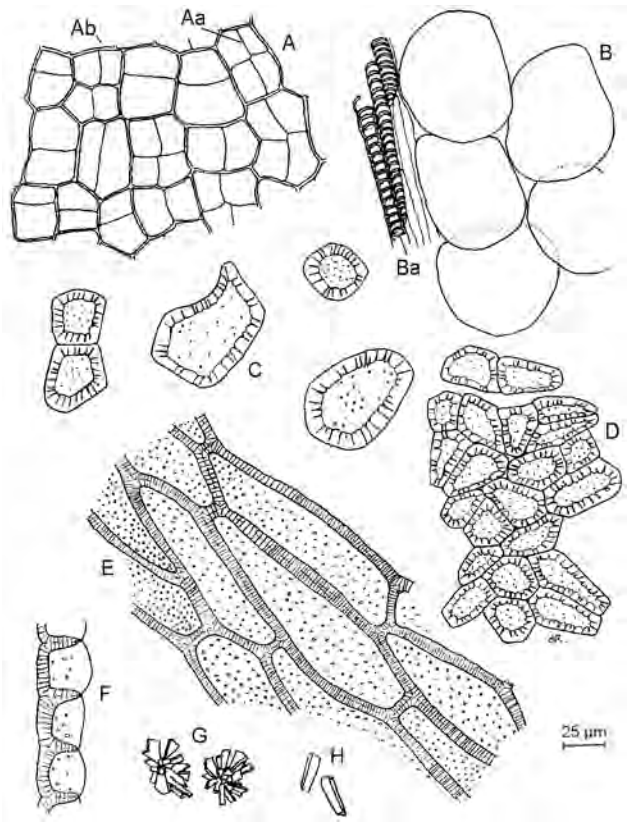


Figure 1602.-1. – *Illustration for identification test B of the herbal drug of fresh bilberry fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 5 g of the freshly crushed drug, add 20 mL of *methanol R*. Stir for 15 min and filter.

*Reference solution.* Dissolve 0.10 g of *bilberry dry extract HRS* in 25 mL of *methanol R*. Stir for 15 min and filter.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *butanol R* (16:19:65 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| A violet-red zone | A violet-red zone |
| A violet zone | A violet zone |
| A violet zone | A violet zone |
| A broad unresolved bluish-violet zone | A broad unresolved bluish-violet zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent, with no fruit of *Sambucus nigra* present.

Adulteration with *S. nigra* L. is indicated by the presence of glossy, violet-black, ovoid berries without the circular fold due to the calyx, and containing not more than 4 seeds.

**Loss on drying** (*2.2.32*): 80.0 per cent to 90.0 per cent, determined on 5.000 g of the freshly crushed drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 0.6 per cent.

ASSAY

Crush 50 g immediately before use. To about 5.00 g of the crushed, accurately weighed drug, add 95 mL of *methanol R*. Stir mechanically for 30 min. Filter into a 100.0 mL volumetric flask. Rinse the filter and dilute to 100.0 mL with *methanol R*. Prepare a 50-fold dilution of this solution in a 0.1 per cent *V/V* solution of *hydrochloric acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the solution at 528 nm, using a 0.1 per cent *V/V* solution of *hydrochloric acid R* in *methanol R* as the compensation liquid.

Calculate the percentage content of anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride, using the following expression:

$$\frac{A \times 5000}{718 \times m}$$

718 = specific absorbance of cyanidin 3-*O*-glucoside chloride at 528 nm;

$A$ = absorbance at 528 nm;

$m$ = mass of the substance to be examined in grams.

STORAGE

When frozen, store at or below − 18 °C.

**01/2017:1174**

# BIRCH LEAF

## Betulae folium

DEFINITION

Whole or fragmented, dried leaves of *Betula pendula* Roth and/or *Betula pubescens* Ehrh. as well as hybrids of both species.

*Content*: minimum 1.5 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The leaves of both species are dark green on the adaxial surface and lighter greenish-grey on the abaxial surface; they show a characteristic dense reticulate venation. The veins are light brown or almost white.

The leaves of *B. pendula* are glabrous and show closely spaced glandular pits on both surfaces. The leaves of *B. pendula* are 3-7 cm long and 2-5 cm wide; the petiole is long and the doubly dentate lamina is triangular or rhomboid and broadly cuneate or truncate at the base. The angle on each side is unrounded or slightly rounded, and the apex is long and acuminate.

The leaves of *B. pubescens* show few glandular trichomes and are slightly pubescent on both surfaces. The abaxial surface shows small bundles of yellowish-grey trichomes at the branch points of the veins. The leaves of *B. pubescens* are slightly smaller, oval or rhomboid and more rounded. They are more roughly and more regularly dentate. The apex is acute rather than acuminate.

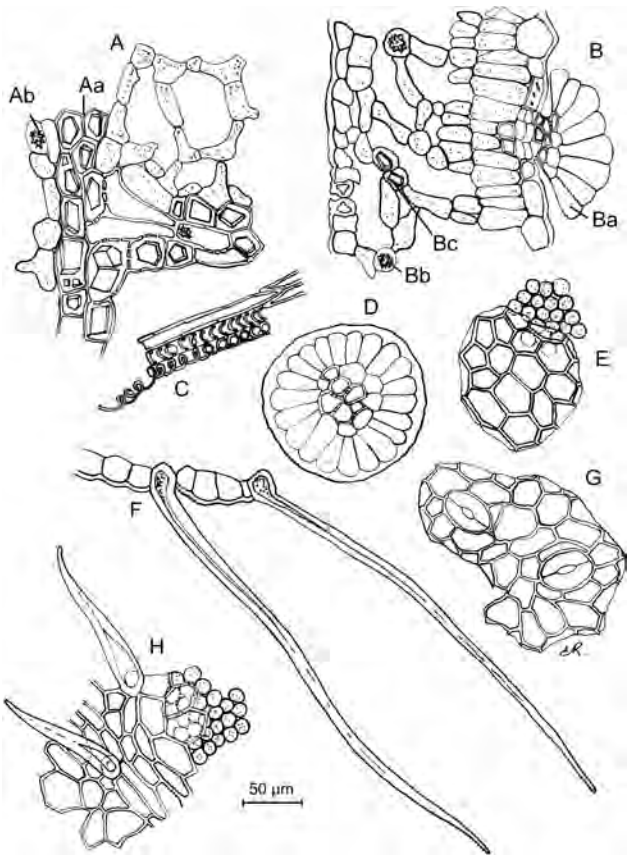


Figure 1174.-1. – *Illustration for identification test B of powdered herbal drug of birch leaf*

B. Microscopic examination (*2.8.23*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1174.-1): numerous fragments of the lamina, in surface view, with straight-walled, adaxial epidermal cells accompanied by underlying palisade parenchyma [E] and cells of the abaxial epidermis surrounding anomocytic stomata (*2.8.3*) [G]; large, free, glandular trichomes usually measuring 100-120 µm [D]; fragments of the lamina (transverse section [B]), showing glandular trichomes on the epidermises [Ba], heterogeneous, asymmetrical mesophyll containing cluster crystals [Bb] and prisms [Bc] of calcium oxalate; fragments of spongy parenchyma [A] accompanied by crystal sheaths [Aa] and cells containing cluster crystals of calcium oxalate [Ab]; fragments of vessels and sclerenchyma fibres [C]. If *B. pubescens* is present, the powder also contains unicellular covering trichomes with very thick walls, about 80-600 µm long, usually 100-200 µm, numerous on the margin of the lamina [F] or on the epidermises (surface view [H]).

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*. Sonicate for 15 min, then filter or centrifuge the solution and use the filtrate or supernatant.

*Reference solution (a).* Dissolve 2.5 mg of *hyperoside R* and 3.5 mg of *quercitrin R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 2.5 mg of *hyperoside R* and 3 mg of *chlorogenic acid R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Intensity marker*: hyperoside.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 5 min. Spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*. Allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the middle third which may, however, be touching. The lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow or orange fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint fluorescent zones, which may be yellow or orange, or light blue, may be present and a light blue fluorescent zone may especially be present below the zone due to hyperoside.

| Top of the plate | |
|---|---|
| | 2 red fluorescent zones, the lower may be overlapped with an orange fluorescent zone |
| | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone |
| Quercitrin: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone (quercitrin) |
| _____ | _____ |
| | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone |
| Hyperoside: a yellow or orange fluorescent zone | An intense yellow or orange fluorescent zone (hyperoside) |
| | A very faint to intense yellow or orange fluorescent zone |
| _____ | _____ |
| **Reference solution (a)** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of fragments of female catkins and maximum 3 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask introduce 0.200 g of the powdered herbal drug (355) (*2.9.12*), 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a 100 mL flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool to room temperature, filter the liquid through a plug of absorbent cotton then through a filter paper into the volumetric flask, and dilute to 100.0 mL with *acetone R* by rinsing the flask and filter. Introduce 20.0 mL of the solution into a separating funnel, add 20 mL of *water R* and extract the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 mL, of *water R*, and filter the extract over 10 g of *anhydrous sodium sulfate R* into a 50 mL volumetric flask and dilute to 50.0 mL with *ethyl acetate R*.

*Test solution.* To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min, by comparison with the compensation liquid at 425 nm.

Calculate the percentage content of flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

$A$ = absorbance at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2014:2384**

# BISTORT RHIZOME

## Bistortae rhizoma

DEFINITION

Whole or fragmented, dried rhizome of *Persicaria bistorta* (L.) Samp. (syn. *Polygonum bistorta* L.) without adventitious roots.

*Content*: minimum 3.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

CHARACTERS

The whole rhizome is up to 13 cm long and 2.5 cm in diameter. The remnants of the roots are not longer than 1 cm and are about 1 mm in diameter.

IDENTIFICATION

A. The whole rhizome, reddish-brown or blackish-brown, is thick, twisted, and turned back on itself. Its outer surface shows transverse striations and blackish spots. It is flattened and somewhat depressed on the upper surface, convex on the lower surface. It shows adventitious root scars on the surface. The fracture, pinkish-beige, shows an elliptical zone of whitish pits corresponding to the vessels. The drug may also be obtained as more or less cylindrical fragments about 0.3 cm in diameter and up to 1 cm long, with a reddish-brown outer surface, marked by adventitious root scars and a pinkish-beige fracture.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2384.-1): very numerous cluster crystals of calcium oxalate, 15-65 µm in diameter, either free [G] or included in parenchyma cells [Da]; rare cork fragments (side view [B], surface view [H]); vascular bundles (longitudinal section [E], transverse section [J]) including small pitted vessels [Ea, Ja] accompanied by finely pitted, thick-walled fibres [Eb, Jb]; free fragments of vessels [C]; free fibres [F]; fragments of parenchyma [D] with rounded cells with slightly thickened walls; fragments of collenchyma [K]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rounded or ovoid starch granules, simple, about 5-12 µm in diameter, free or included in parenchyma cells [A].

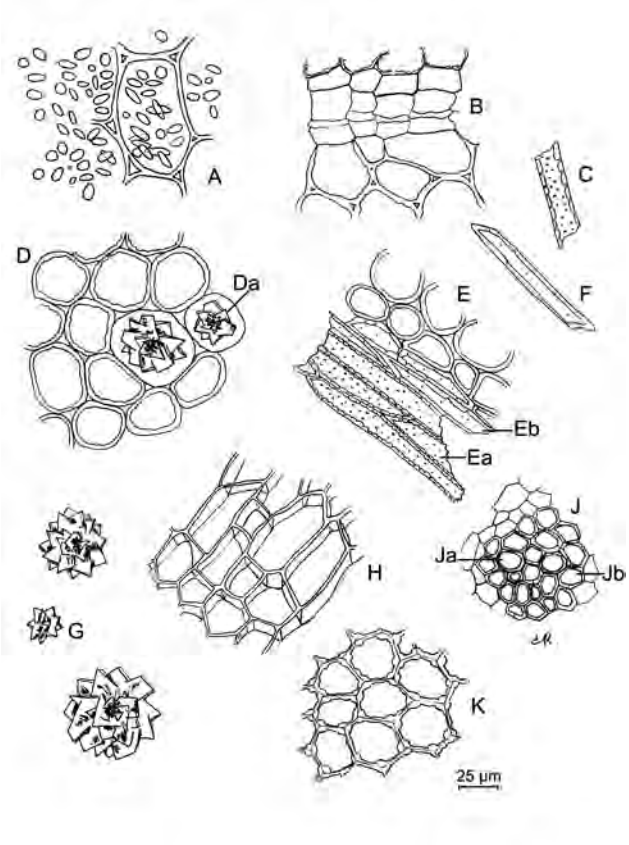


Figure 2384.-1. – *Illustration for identification test B of powdered herbal drug of bistort rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of a mixture of equal volumes of *methanol R* and *water R*, heat on a water-bath at about 65 °C for 30 min and filter.

*Reference solution.* Dissolve 5 mg of *fructose R* and 5 mg of *catechin R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (5:10:85 *V/V/V*).

*Application*: 2 µL as bands.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Catechin: a brown zone | A brown zone (catechin) |
| _____ | _____ |
| | A brown zone |
| | A violet zone |
| | A brown zone |
| | An orange zone |
| _____ | _____ |
| Fructose: a green zone | A green zone (fructose) |
| **Reference solution** | **Test solution** |

TESTS

***Paris polyphylla* Sm. or *Paris quadrifolia* L.** Microscopic examination (*2.8.23*). Examine under a microscope using *chloral hydrate solution R*. The presence of raphides of calcium oxalate, free or in bundles, indicates adulteration by the rhizome of *P. polyphylla* Sm. var. *yunnanensis* (Franch.) Hand.-Mazz. or *P. polyphylla* Sm. var. *chinensis* (Franch.) H.Hara or *P. quadrifolia* L.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 1.000 g of the powdered herbal drug (180) (*2.9.12*).

**01/2008:1826**

# BITTER-FENNEL FRUIT OIL

## Foeniculi amari fructus aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the ripe fruits of *Foeniculum vulgare* Miller, ssp. *vulgare* var. *vulgare*.

*Content*:

– fenchone: 12.0 per cent to 25.0 per cent,

– *trans*-anethole: 55.0 per cent to 75.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 mL of the essential oil to be examined in 5 mL of *toluene R*.

*Reference solution.* Dissolve 10 µL of *fenchone R* and 80 µL of *anethole R* in 5 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 150 °C for 15 min; examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Anethole: a dark blue to dark violet zone | A dark blue to dark violet zone (anethole) |
| Fenchone: a blue or bluish-grey zone | A blue or bluish-grey zone (fenchone) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.961 to 0.975.

**Refractive index** (*2.2.6*): 1.528 to 1.539.

**Optical rotation** (*2.2.7*): + 10.0° to + 24.0°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 0.20 mL of the essential oil to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 20 µL of *α-pinene R*, 20 µL of *limonene R*, 50 µL of *fenchone R*, 20 µL of *estragole R*, 100 µL of *anethole R* and 20 µL of *anisaldehyde R* in *heptane R* and dilute to 10.0 mL with the same solvent.

*Column*:
- *material*: fused silica,
- *size*: *l* = 60 m, Ø = 0.25 mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 – 4 | 60 |
| | 4 – 26 | 60 → 170 |
| | 26 – 41 | 170 |
| Injection port | | 220 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:
- *resolution*: minimum 5.0 between the peaks due to estragole and *trans*-anethole.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution and locate *cis*-anethole using Figure 1826.-1. (Disregard the peak due to heptane).

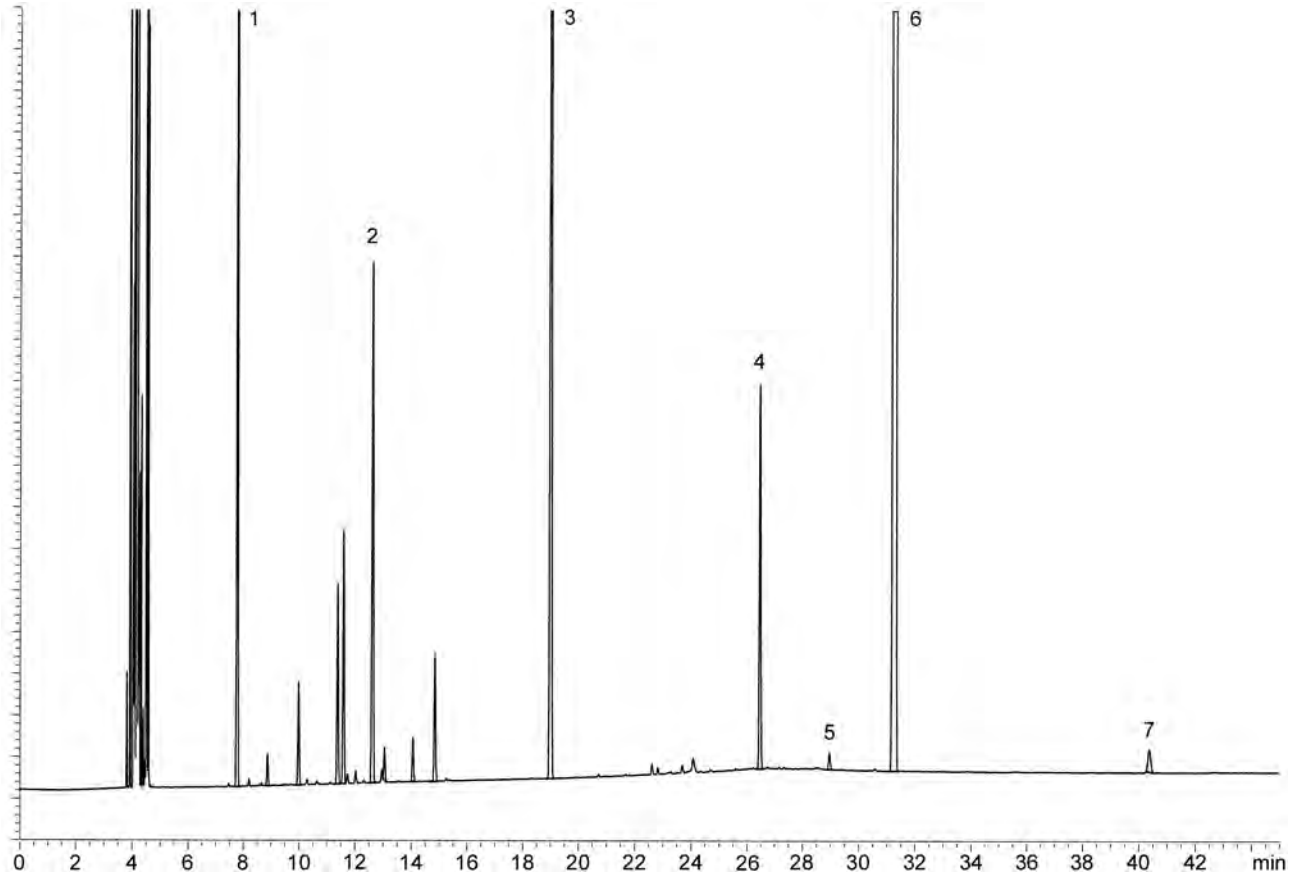


Figure 1826.-1. – *Chromatogram for the test for chromatographic profile of bitter-fennel fruit oil*

Determine the percentage content of each of these components. The percentages are within the following ranges:
- *α-pinene*: 1.0 per cent to 10.0 per cent,
- *limonene*: 0.9 per cent to 5.0 per cent,
- *fenchone*: 12.0 per cent to 25.0 per cent,
- *estragole*: maximum 6.0 per cent,
- *cis-anethole*: maximum 0.5 per cent,
- *trans-anethole*: 55.0 per cent to 75.0 per cent,
- *anisaldehyde*: maximum 2.0 per cent.

The ratio of α-pinene content to limonene content is greater than 1.0.

STORAGE

At a temperature not exceeding 25 °C.

**07/2009:2380**
**corrected 7.0**

# BITTER-FENNEL HERB OIL

## Foeniculi amari herbae aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the aerial parts of *Foeniculum vulgare* Mill. ssp. *vulgare*, var. *vulgare* collected during fruiting.

CHARACTERS

*Appearance*: clear, pale or intense yellow liquid.

Anise-like odour.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.1 mL of the oil to be examined in 5 mL of *toluene R*.

*Reference solution*. Dissolve 10 µL of *fenchone R* and 40 µL of *anethole R* in 5 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 8 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 150 °C for 15 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Anethole: a dark blue or dark violet zone | A dark blue or dark violet zone (anethole) |
| Fenchone: a blue or bluish-grey zone | A sometimes faint blue or bluish-grey zone (fenchone) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*:
- *Spanish type*: the characteristic peaks due to α-pinene, β-pinene, β-myrcene, α-phellandrene, limonene, fenchone, estragole and *trans*-anethole in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a);
- *Tasmanian type*: the characteristic peaks due to α-pinene, α-phellandrene, limonene, fenchone, estragole and *trans*-anethole in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*):
- *Spanish type*: 0.877 to 0.921;
- *Tasmanian type*: 0.940 to 0.973.

**Refractive index** (*2.2.6*):
- *Spanish type*: 1.487 to 1.501;
- *Tasmanian type*: 1.512 to 1.538.

**Optical rotation** (*2.2.7*):
- *Spanish type*: + 42° to + 68°;
- *Tasmanian type*: + 11° to + 35°.

**Solubility in alcohol** (*2.8.10*):
- *Spanish type*: 1 volume is soluble in 2 volumes and more of *ethanol (90 per cent V/V) R*;
- *Tasmanian type*: 1 volume is soluble in 10 volumes and more of *ethanol (85 per cent V/V) R*.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.20 mL of the oil to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 µL of *α-pinene R*, 10 µL of *β-pinene R*, 20 µL of *β-myrcene R*, 20 µL of *α-phellandrene R*, 20 µL of *limonene R*, 40 µL of *fenchone R*, 10 µL of *estragole R*, 40 µL of *anethole R*, 10 µL of *anisaldehyde R* and 10 µL of *anise ketone R* in *acetone R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 µL of *anethole R* in 25.0 mL of *acetone R*. Dilute 0.5 mL of this solution to 20.0 mL with *acetone R*.

*Column*:
- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 35 | 70 → 210 |
| | 35 - 42 | 210 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of these substances.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to β-myrcene and α-phellandrene.

Herbal drugs

Using the chromatogram obtained with the reference solution, locate the relevant components for the type of the essential oil to be examined in the chromatogram obtained with the test solution, and locate *cis*-anethole using Figures 2380.-1 and 2380.-2.

Determine the percentage content of each of these components.

For Spanish-type bitter-fennel herb oil, the percentages are within the following ranges:

- *α-pinene*: 2.0 to 8.0 per cent;
- *β-pinene*: 1.0 to 4.0 per cent;
- *β-myrcene*: 1.0 to 12.0 per cent;
- *α-phellandrene*: 1.0 to 25.0 per cent;
- *limonene*: 8.0 to 30.0 per cent;
- *fenchone*: 7.0 to 16.0 per cent;
- *estragole*: 2.0 to 7.0 per cent;
- *cis-anethole*: maximum 0.5 per cent;
- *trans-anethole*: 15.0 to 40.0 per cent;
- *anisaldehyde*: maximum 1.0 per cent;
- *anise ketone*: maximum 0.05 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.025 per cent).

For Tasmanian-type bitter-fennel herb oil, the percentages are within the following ranges:

- *α-pinene*: 2.0 to 11.0 per cent;
- *α-phellandrene*: 1.0 to 8.5 per cent;
- *limonene*: 1.0 to 6.0 per cent;
- *fenchone*: 10.0 to 25.0 per cent;
- *estragole*: 1.5 to 6.0 per cent;
- *cis-anethole*: maximum 0.5 per cent;
- *trans-anethole*: 45.0 to 78.0 per cent;
- *anisaldehyde*: maximum 1.0 per cent;
- *anise ketone*: maximum 0.05 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.025 per cent).

STORAGE

At a temperature not exceeding 25 °C.

LABELLING

The label states that the content is Spanish-type or Tasmanian-type.

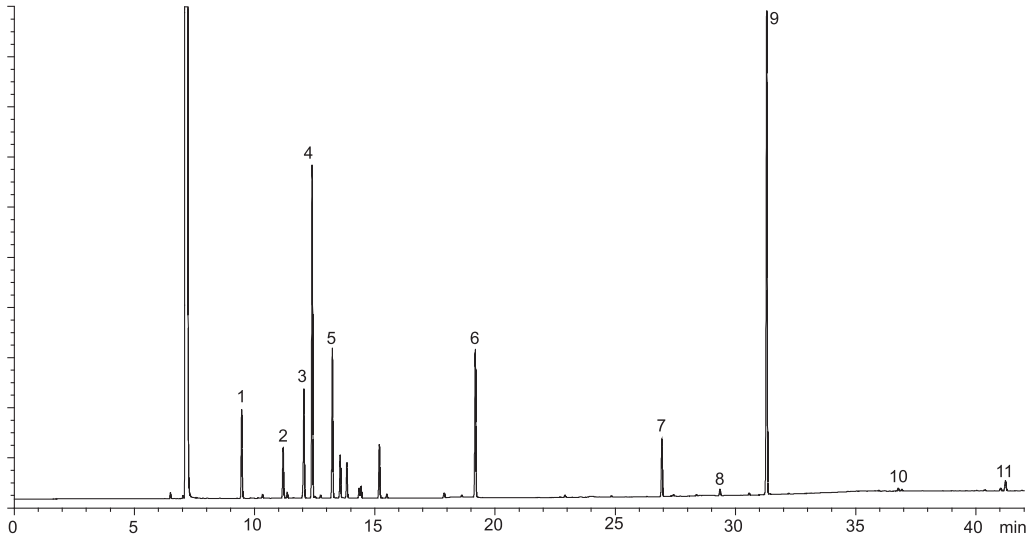


1. α-pinene
2. β-pinene
3. β-myrcene
4. α-phellandrene
5. limonene
6. fenchone
7. estragole
8. *cis*-anethole
9. *trans*-anethole
10. anisaldehyde
11. anise ketone

Figure 2380.-1. – *Chromatogram for the test for chromatographic profile of Spanish-type bitter-fennel herb oil*

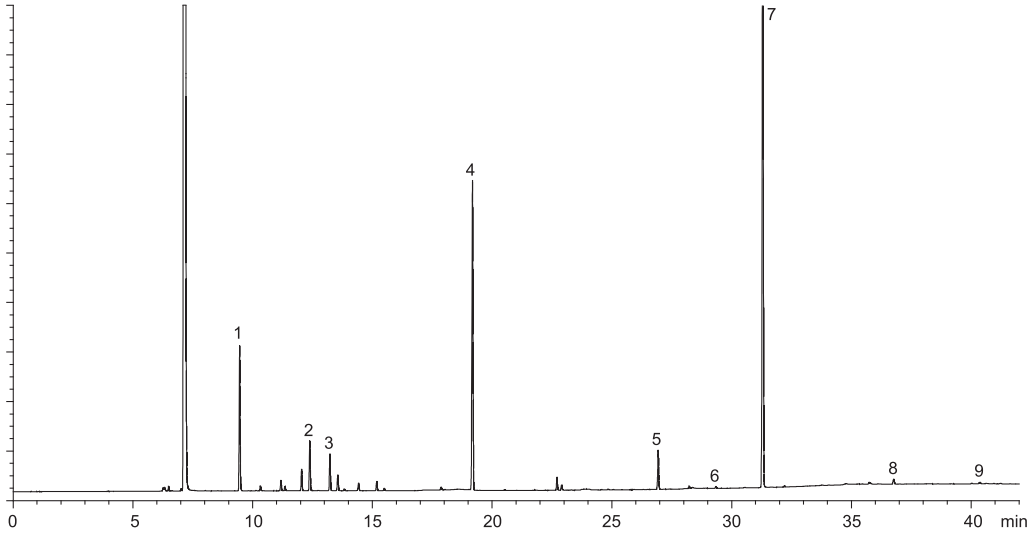


1. α-pinene
2. α-phellandrene
3. limonene
4. fenchone
5. estragole
6. *cis*-anethole
7. *trans*-anethole
8. anisaldehyde
9. anise ketone

Figure 2380.-2. – *Chromatogram for the test for chromatographic profile of Tasmanian-type bitter-fennel herb oil*

**07/2015:1603**

# BITTER-ORANGE EPICARP AND MESOCARP

## Aurantii amari epicarpium et mesocarpium

DEFINITION

Dried epicarp and mesocarp of the ripe fruit of *Citrus aurantium* L. ssp. *aurantium* (*C. aurantium* L. ssp. *amara* Engl.) partly freed from the white spongy tissue of the mesocarp and endocarp.

*Content*: minimum 20 mL/kg of essential oil (anhydrous drug).

IDENTIFICATION

A. The herbal drug consists of elliptical or irregular pieces 5-8 cm long, 3-5 cm broad and about 3 mm thick. The outer surface is yellowish or reddish-brown and distinctly punctate, the inner surface is yellowish or brownish-white.

B. Microscopic examination (*2.8.23*). The powder is light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1603.-1): fragments of epicarp (surface view [B]) composed of small polygonal cells with slightly thickened anticlinal walls, filled with chromatophores and some anomocytic stomata (*2.8.3*); fragments of epicarp showing the thickened cuticle (side view [N]); fragments of pericarp (transverse section [A]) showing the cuticularised epicarp [Aa], the sub-epicarpal layers with cells with collenchymatous thickening [Ab], some of which contain a prism of calcium oxalate [Ac], and fragments of schizolysigenous oil glands [Ad]; fragments of the collenchymatous sub-epicarpal layers (surface view [G]); groups of parenchyma cells [L], some of which contain a prism of calcium oxalate [C]; numerous fragments of mesocarp [D, E, F, H, K, M]; free prisms of calcium oxalate [J]. Examine under a microscope using a 20 g/L solution of *potassium hydroxide R*. The mounting medium becomes yellow due to the presence of hesperidin in the herbal drug.

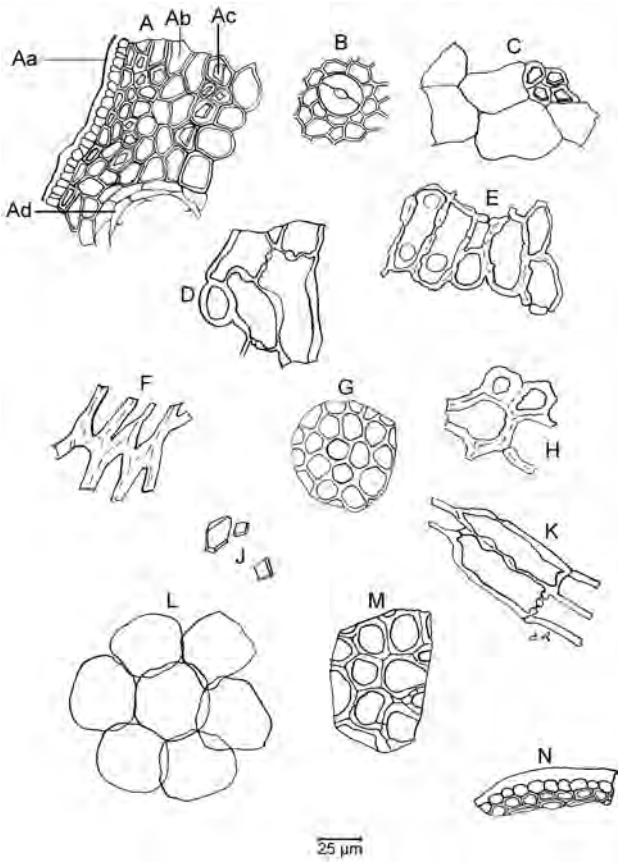


Figure 1603.-1. – *Illustration for identification test B of powdered herbal drug of bitter-orange epicarp and mesocarp*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *methanol R* and heat in a water-bath at 65 °C for 5 min with shaking. Allow to cool and filter.

*Reference solution*. Dissolve 1.0 mg of *naringin R* and 1.0 mg of *caffeic acid R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (10:15:75 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air, then in an oven at 110-120 °C for 5 min.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. After at least 1 h, examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue fluorescent zone |
| | A light blue fluorescent zone |
| Caffeic acid: a light blue fluorescent zone | |
| | A light blue fluorescent zone |
| | A light blue fluorescent zone |
| Naringin: a dark green fluorescent zone | A dark green fluorescent zone (naringin) |
| | A red fluorescent zone (neoeriocitrin) |
| | An orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.2.13*): maximum 10.0 per cent, determined by distillation on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Extractable matter**: minimum 25.0 per cent.

To 2.000 g of the powdered herbal drug (250) (*2.9.12*) add 10.0 mL of a mixture of 30 volumes of *water R* and 70 volumes of *ethanol (96 per cent) R* and extract for 2 h, shaking frequently. Filter, evaporate 2.000 mL of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* and weigh. The residue weighs a minimum of 0.100 g.

ASSAY

**Essential oil** (*2.8.12*). Use a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Reduce the drug to a powder (710) (*2.9.12*) and immediately use 15.0 g for the determination. Distil at a rate of 2-3 mL/min for 90 min.

**01/2008:1604**

# BITTER-ORANGE-EPICARP AND MESOCARP TINCTURE

## Aurantii amari epicarpii et mesocarpii tinctura

DEFINITION

Tincture produced from *Bitter-orange epicarp and mesocarp (1603)*.

PRODUCTION

The tincture is produced from 1 part of the freshly powdered herbal drug (2000) (*2.9.12*) and 5 parts of alcohol (70 per cent *V/V*) by an appropriate procedure.

CHARACTERS

Liquid with a bitter taste.

IDENTIFICATION

Examine by thin-layer chromatography (*2.2.27*).

*Test solution*. The tincture to be examined.

*Reference solution*. Dissolve 1.0 mg of *naringin R* and 1.0 mg of *caffeic acid R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (10:15:75 *V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air, and heat in an oven at 110-120 °C for 5 min.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. After 1 h, examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue fluorescent zone |
| | A light blue fluorescent zone |
| Caffeic acid: a light blue fluorescent zone | |
| | A light blue fluorescent zone |
| | A light blue fluorescent zone |
| Naringin: a dark green fluorescent zone | A dark green fluorescent zone (naringin) |
| | A red fluorescent zone (neoeriocitrin) |
| | An orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol content** (*2.9.10*): 63 per cent to 67 per cent *V/V*.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

**Dry residue**: minimum 6.0 per cent *m/m*, determined on 2.00 g of tincture to be examined.

**01/2012:1810**

# BITTER-ORANGE FLOWER

## Aurantii amari flos

DEFINITION

Whole, dried, unopened flower of *Citrus aurantium* L. ssp. *aurantium* (*C. aurantium* L. ssp. *amara* Engl.).

*Content*: minimum 8.0 per cent of total flavonoids, expressed as naringin ($C_{27}H_{32}O_{14}$; $M_r$ 580.5) (dried drug).

Herbal drugs

IDENTIFICATION

A. The flower buds are white or yellowish-white and may reach up to 25 mm in length. The dialypetalous corolla is composed of 5 thick, oblong and concave petals dotted with oil glands visible under a hand lens; the short, yellowish-green persistent gamosepalous calyx has 5 spreading sepals, connate at the base and forming a star-shaped structure attached to the yellowish-green peduncle, which is about 5-10 mm long. The flower buds contain at least 20 stamens with yellow anthers and with filaments fused at the base into groups of 4 or 5; the ovary is superior, brownish-black and spherical, consists of 8-10 multi-ovular loculi and is surrounded at the base by an annular granular hypogynous disc; the thick, cylindrical style ends in a capitate stigma.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1810.-1): very numerous spherical pollen grains, with a finely pitted exine and 3-5 germinal pores [H, K]; fragments of the epidermis of the sepals (surface view [D], transverse section [A, C]) accompanied by underlying mesophyll [B], some cells of which contain prisms of calcium oxalate [Aa, Ba, Db], unicellular covering trichomes [Ca] and numerous anomocytic stomata (*2.8.3*) [Da]; fragments of the epidermis of the petals (surface view [F, G, J]), with a distinctly striated cuticle; fragments of large schizolysigenous oil glands in transverse section [E], which measure up to 100 µm in diameter. Examine under a microscope using a 20 g/L solution of *potassium hydroxide R*. The mounting medium becomes yellow because of the presence of hesperidin in the drug.

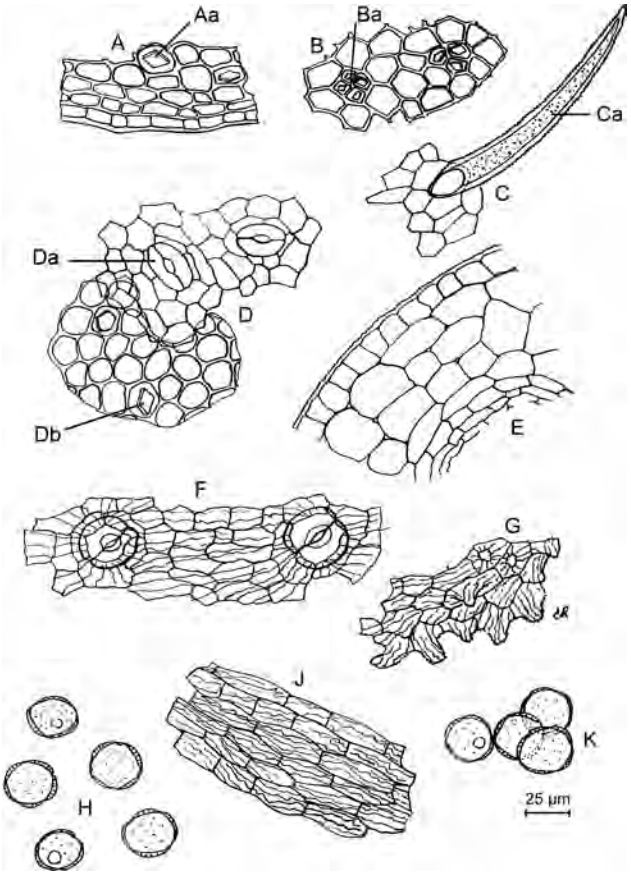


Figure 1810.-1. – *Illustration for identification test B of powdered herbal drug of bitter-orange flower*

C. Examine the chromatograms obtained in the test for sweet-orange flower.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A weak yellow fluorescent zone |
| | A weak yellow fluorescent zone |
| Hesperidin: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (hesperidin) |
| Naringin: a yellow fluorescent zone | A yellow fluorescent zone (naringin) |
| | A red fluorescent zone (neoeriocitrin) |
| | A yellow fluorescent zone (diosmin and neodiosmin) |
| **Reference solution** | **Test solution** |

TESTS

**Sweet-orange flower**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Heat with stirring at 40 °C for 10 min. Filter.

*Reference solution*. Dissolve 3.0 mg of *naringin R* and 3.0 mg of *hesperidin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (10:15:75 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air; heat in an oven at 110-120 °C for 5 min.

*Detection*: spray the hot plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; after at least 1 h, examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows a yellow zone similar in position to the zone of naringin in the chromatogram obtained with the reference solution, and immediately below it a red zone (neoeriocitrin).

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

*Stock solution*. To 0.175 g of the powdered herbal drug (355) (*2.9.12*) add 95 mL of *ethanol (50 per cent V/V) R*. Heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter through a sintered-glass filter (*2.1.2*). Rinse the filter with 5 mL of *ethanol (50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Test solution*. Into a test tube (10 mm × 180 mm) introduce 0.150 g of powdered *magnesium R* (250) (*2.9.12*), a magnetic stirring bar 25 mm long and 2.00 mL of the stock solution. Maintain the test tube upright, centrifuge at 125 *g* and carefully add dropwise, especially at the beginning, 2.0 mL of *hydrochloric acid R*, and then 6.0 mL of *ethanol (50 per cent V/V) R*. Stopper the tube and mix by inverting.

*Compensation solution*. Into a 2nd test tube, introduce 2.00 mL of the stock solution and carefully add dropwise, especially at the beginning, 2.0 mL of *hydrochloric acid R* and then 6.0 mL of *ethanol (50 per cent V/V) R*.

After 10 min, measure the absorbance (*2.2.25*) of the test solution at 530 nm.

Calculate the percentage content of total flavonoids, expressed as naringin, using the following expression:

$$\frac{A \times 9.62}{m}$$

i.e. taking the specific absorbance of the reaction product of naringin to be 52.

$A$ = absorbance at 530 nm;

$m$ = mass of the substance to be examined, in grams.

**04/2014:2069**

# BLACK COHOSH

## Cimicifugae rhizoma

DEFINITION

Dried, whole or fragmented rhizome and root of *Actaea racemosa* L. (syn. *Cimicifuga racemosa* (L.) Nutt.).

*Content*: minimum 1.0 per cent of triterpene glycosides, expressed as monoammonium glycyrrhizate ($C_{42}H_{65}NO_{16}$; $M_r$ 840) (dried drug).

IDENTIFICATION

A. *Whole drug.* The rhizome is dark brown, hard, subcylindrical and somewhat knotted; 1.5-2.5 cm in diameter and 2-15 cm long; it shows numerous closely arranged, upright or curved branches each terminating in the remains of a bud or in a circular, cup-shaped scar. The fracture is horny, the transverse section shows a thin outer bark surrounding a ring of numerous pale, narrow wedges of vascular tissue alternating with darker medullary rays and a large central pith. Roots attached to the lower surface of the rhizome are usually broken off, leaving circular scars. The roots are dark brown, 1-3 mm in diameter, brittle, nearly cylindrical or obtusely quadrangular and longitudinally wrinkled; the fracture is short; the transverse section shows a wide outer bark, a dark brown cylinder, in which the central region is composed of 3-6 lighter wedges of vascular tissue united at the centre and separated by broad, non-lignified medullary rays.

*Fragmented drug.* More or less angular, irregular pieces of the rhizome and cylindrical pieces of the roots. The hard, horny rhizome fragments usually show a dark brown surface corresponding to the outer surface and several frequently striated, light brown surfaces corresponding to the section. The dark brown, more or less cylindrical root fragments are wrinkled longitudinally. The lighter coloured transverse section shows a distinct cambium line separating a thick outer bark from a central region composed of 3-6 wedges of vascular tissue united at the centre and separated by broad medullary rays.

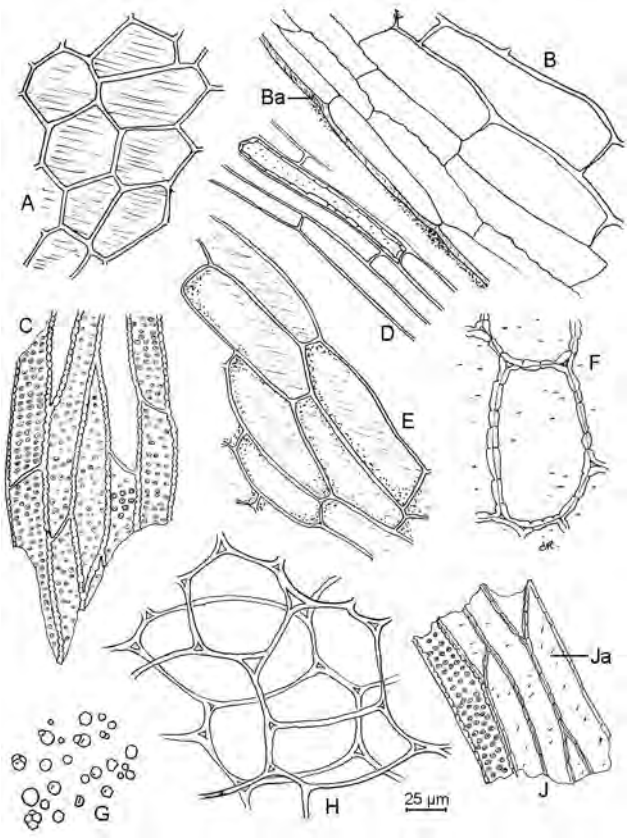


Figure 2069.-1. – *Illustration for identification test B of powdered herbal drug of black cohosh*

B. Microscopic examination (*2.8.23*). The powder is light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2069.-1): fragments of the epidermis of the rhizome, with brown polygonal cells [A]; numerous fragments of parenchyma consisting of rounded cells, with slightly and regularly thickened walls with small triangular spaces between them [H]; groups of short vessels with closely arranged bordered pits [C, J] sometimes accompanied by finely pitted fibres [Ja]; fragments of the parenchyma of the pith of the rhizome with thick-walled and channelled ovoid cells [F]; a few fragments of the phloem containing long isolated sclereids [D]; fragments of the dermal tissue of the roots (surface view [E], longitudinal section [B]), consisting of brown cells covered by a dark brown cuticle [Ba]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant starch granules, spherical or polygonal, simple, 5-10 µm in diameter, or 2 or 3 (rarely up to 6) compound; some granules have a slit-shaped hilum [G].

C. Examine the chromatograms obtained in the test for substitution by *Cimicifuga americana* Michx., *C. foetida* L., *C. dahurica* (Turcz.) Maxim. or *C. heracleifolia* Kom.

*Results B*: use the chromatograms supplied with *Actaea racemosa HRS* and the chromatogram obtained with reference solution (a) to identify the bands corresponding to *A. racemosa*.

See below the sequence of zones present in the chromatograms obtained with reference solutions (a) and (b) and the test solution. Furthermore, other faint zones may be present in the chromatograms obtained with reference solution (a) and the test solution.

| Top of the plate | | |
|---|---|---|
| ——— | ——— | ——— |
| Actein: a brown zone | Actein: a brown zone | A brown zone (actein) |
| 23-Epi-26-deoxyactein: a brown zone | | A brown zone (23-epi-26-deoxyactein) |
| ——— | ——— | ——— |
| A violet zone | | A violet zone |
| A violet zone | | A violet zone |
| A brown zone | | A brown zone |
| **Reference solution (a)** | **Reference solution (b)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Total ash** (*2.4.16*): maximum 10 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5 per cent.

**Substitution by *Cimicifuga americana* Michx., *C. foetida* L., *C. dahurica* (Turcz.) Maxim. or *C. heracleifolia* Kom.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.50 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (50 per cent V/V) R* and shake well. Sonicate for 10 min and centrifuge. Use the supernatant.

*Reference solution (a).* To 0.50 g of *Actaea racemosa HRS* add 10 mL of *ethanol (50 per cent V/V) R* and shake well. Sonicate for 10 min and centrifuge. Use the supernatant.

*Reference solution (b).* Dissolve 2 mg of *actein R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *ethyl formate R*, *toluene R* (20:30:50 *V/V/V*).

*Application*: 2 µL as bands of 8 mm (see Table 2069.-1).

*Development*: over a path of 6 cm.

*Drying*: in air.

*System suitability*: reference solution (b):

– the $R_F$ value of the zone due to actein is between 0.35 and 0.40 (detection B).

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution does not show any quenching zones more intense than those in the chromatogram obtained with reference solution (a) between $R_F$ value 0.2 and $R_F$ value 0.35.

*Detection B*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*; heat at 100 °C for 5 min; allow to cool to room temperature and examine in daylight.

**Adulteration with *Cimicifuga americana* Michx., *C. foetida* L., *C. dahurica* (Turcz.) Maxim. and/or *C. heracleifolia* Kom.** Thin-layer chromatography (*2.2.27*) as described in the test for substitution by *Cimicifuga americana* Michx., *C. foetida* L., *C. dahurica* (Turcz.) Maxim. or *C. heracleifolia* Kom., with the following modifications.

*Reference solution (c).* Dissolve 2 mg of *cimifugin R* in *methanol R* and dilute to 10 mL with the same solvent.

*Application*: 2 µL of reference solutions (b) and (c), 20 µL of the test solution and reference solution (a), as bands of 8 mm (see Table 2069.-2).

*System suitability*: reference solution (b):

– the $R_F$ value of the zone due to actein is between 0.35 and 0.40 (detections B and C).

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: absence of more than 10 per cent of *C. americana*.

Compare the chromatogram supplied with *Actaea racemosa HRS* for *C. americana* and the chromatograms obtained with the test solution and reference solution (a). The chromatogram obtained with the test solution does not show any quenching zone at $R_F$ value 0.3 (zone presented in capitals in the chromatogram of *C. americana*, see below). The presence of this zone in the chromatogram obtained with the test solution indicates adulteration with *C. americana* at a level greater than 10 per cent.

Table 2069.-1. – *Application scheme*

| Track | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Application volume (µL) | 2 | 2 | 2 | - | 2 | 2 | 2 |
| Solution | Reference solution (a) | Reference solution (b) | Test solution | Blank | Reference solution (a) | Reference solution (b) | Test solution |
| After development, the plate is cut along track 4 (blank). Tracks 1-3 are used for detection of a substitution by *C. americana*, *C. foetida*, *C. dahurica* or *C. heracleifolia* (detection A), tracks 5-7 for Identification C (detection B). | | | | | | | |

Table 2069.-2. – *Application scheme*

| Track | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Application volume (µL) | 20 | 2 | 2 | 20 | - | 20 | 2 | 2 | 20 |
| Solution | Reference solution (a) | Reference solution (b) | Reference solution (c) | Test solution | Blank | Reference solution (a) | Reference solution (b) | Reference solution (c) | Test solution |
| After development and examination for detection of *C. americana* (detection A), the plate is cut along track 5 (blank). Tracks 1-4 are used for detection of adulteration with *C. foetida* (detection B), tracks 6-9 for detection of adulteration with *C. heracleifolia* and/or *C. dahurica* (detection C). | | | | | | | | | |

| Top of the plate | |
|---|---|
| _____ | _____ |
| A weak zone | A weak zone |
| 2 weak zones | 2 weak zones |
| A weak zone | A weak zone |
| _____ | _____A DARK ZONE |
| A weak zone | A weak zone |
| A dark zone | A dark zone |
| A dark zone | A dark zone |
| **Reference solution (a)** | ***C. americana* (10 per cent)** |

*Detection B*: dissolve 4.5 g of *boric acid R* in 150 mL of *anhydrous ethanol R* (solution A); dissolve 5 g of *oxalic acid R* in 50 mL of *anhydrous ethanol R* (solution B); combine solutions A and B and mix well; treat the plate with this freshly prepared solution and heat at 120 °C for 5 min; examine in ultraviolet light at 365 nm.

*Results B*: absence of more than 5 per cent of *C. foetida*.

Compare the chromatogram supplied with *Actaea racemosa HRS* for *C. foetida* and the chromatograms obtained with the test solution and reference solutions (a), (b) and (c). The chromatogram obtained with the test solution does not show any intense fluorescent zone between $R_F$ value 0.03 and $R_F$ value 0.06 or at the same position as the bright fluorescent zone in the chromatogram obtained with reference solution (c) (zones presented in capitals in the chromatogram of *C. foetida*, see below). The presence of 1 or both zones in the chromatogram obtained with the test solution indicates adulteration with *C. foetida* at a level greater than 5 per cent.

| Top of the plate | | | |
|---|---|---|---|
| _____ | _____ | _____ | _____ |
| Actein: a weak whitish zone | Actein: a weak whitish zone | | A weak whitish zone (actein) |
| _____ | _____ | _____ | _____ |
| A bluish zone | | | A bluish zone |
| | | Cimifugin: a bright fluorescent zone | A BRIGHT FLUORESCENT ZONE (CIMIFUGIN) |
| A brownish zone | | | A brownish zone |
| A bluish zone | | | A bluish zone |
| | | | A FLUORESCENT ZONE |
| **Reference solution (a)** | **Reference solution (b)** | **Reference solution (c)** | ***C. foetida* (5 per cent)** |

*Detection C*: dissolve 8 g of *antimony trichloride R* in 200 mL of *methylene chloride R*; treat with this solution and heat at 120 °C for 10 min; examine in ultraviolet light at 365 nm.

*Results C*: absence of more than 5 per cent of *C. heracleifolia* and/or *C. dahurica*.

Compare the chromatogram supplied with *Actaea racemosa HRS* for *C. heracleifolia* and *C. dahurica* and the chromatograms obtained with the test solution and reference solutions (a) and (b). The chromatogram obtained with the test solution does not show any bright fluorescent zone just above the zone due to actein (zone presented in capitals in the chromatogram of *C. heracleifolia* or *C. dahurica*, see below).

The presence of this zone in the chromatogram obtained with the test solution indicates adulteration with *C. heracleifolia* and/or *C. dahurica* at a level greater than 5 per cent.

| Top of the plate | | |
|---|---|---|
| _____ | _____ | _____ |
| | | A BRIGHT FLUORESCENT ZONE |
| Actein: a weak brownish zone | Actein: a weak brownish zone | A weak brownish zone (actein) |
| _____ | _____ | _____ |
| A brownish zone | | A brownish zone |
| A bluish zone | | A bluish zone |
| **Reference solution (a)** | **Reference solution (b)** | ***C. heracleifolia* (5 per cent) and/or *C. dahurica* (5 per cent)** |

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Introduce 4.00 g of the powdered herbal drug (355) (*2.9.12*) into a 200 mL screw-cap bottle. Add 50.0 mL of a mixture of equal volumes of *methanol R* and *water R*. Sonicate for 45 min and shake for 15 min. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 10.0 mg of *Actaea racemosa for assay CRS* (containing monoammonium glycyrrhizate) in *methanol R* with the aid of ultrasound and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with *methanol R*.

*Reference solution (e)*. Dissolve 500 mg of *Actaea racemosa dry extract for system suitability HRS* in *methanol R* and dilute to 10.0 mL with the same solvent; sonicate and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *anhydrous formic acid R* in *water R*;
- *mobile phase B*: 0.1 per cent *V/V* solution of *anhydrous formic acid R* in a mixture of equal volumes of *acetonitrile R* and *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 50 → 20 | 50 → 80 |
| 40 - 41 | 20 → 5 | 80 → 95 |
| 41 - 44 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion for the signal-to-noise ratio:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 0.8 mL/min;
- *evaporator temperature*: 100 °C;
- *nebuliser temperature*: 60 °C.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *Actaea racemosa dry extract for system suitability HRS* and the chromatogram obtained with reference solution (e) to identify the peaks to be quantified.

*System suitability*:

- *signal-to-noise ratio*: minimum 4.0 for the peak due to monoammonium glycyrrhizate in the chromatogram obtained with reference solution (d);
- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of peak 4 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from peak 5 in the chromatogram obtained with reference solution (e).

Establish a calibration curve with the logarithm to base 10 of the concentration (in milligrams per millilitre) of reference solutions (a), (b), (c) and (d) (corrected by the assigned percentage content of monoammonium glycyrrhizate in *Actaea racemosa for assay CRS*) as the abscissa and the logarithm to base 10 of the corresponding peak area as the ordinate.

Calculate the percentage content of each peak using the following expression:

$$\frac{10^A \times 5}{m}$$

$A$ = logarithm to base 10 of the concentration of each peak in the chromatogram obtained with the test solution, determined from the calibration curve;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams.

Calculate the percentage content of triterpene glycosides by taking the sum of the percentage contents of peaks 1 to 12.

**07/2011:1858**

# BLACK HOREHOUND

## Ballotae nigrae herba

DEFINITION

Dried flowering tops of *Ballota nigra* L.

*Content*: minimum 1.5 per cent of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside ($C_{29}H_{36}O_{15}$; $M_r$ 625) (dried drug).

IDENTIFICATION

A. The stems are conspicuously 4-angled, longitudinally striated, dark green or reddish-brown and more or less pubescent. The leaves are greyish-green, petiolate, the lamina ovate or orbicular, 2-4 cm wide, the margin irregularly crenate, and cuneate or cordate at the base; both surfaces are covered with abundant whitish hairs; the venation is pinnate, prominent on the lower surface, slightly depressed on the upper. The flowers are sessile or very shortly pedicellate, the calyx is infundibuliform, densely pubescent, with 10 prominent ribs and 5 subequal, broadly ovate teeth; the corolla, with a tube slightly shorter than the calyx tube, is purple and bilabiate, the upper lip pubescent on the outer surface and the lower lip with 3 lobes, the middle of which is notched.

B. Microscopic examination (*2.8.23*). The powder is greyish-green and slightly flocculent. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1858.-1): numerous long, uniseriate, multicellular covering trichomes consisting of 4 or more cells, thickened and swollen at the junctions, with slightly lignified and pitted walls, free [C] or on an epidermis (transverse section [Ea]); fewer glandular trichomes, usually on epidermises (transverse section [E, F, G]): some with a unicellular or multicellular stalk and a globose, uni- or bicellular head [Ga], others with a unicellular stalk and a multicellular head (surface view [Ac], transverse section [Eb]), others with a unicellular stalk and an 8-celled head of lamiaceous type (surface view [Ad], transverse section [Fa]); fragments of the adaxial leaf epidermis [B] with cells with sinuous walls, accompanied by cells of the palisade parenchyma, most containing fine, needle-shaped crystals [Ba]; fragments of the abaxial leaf epidermis [A] bearing numerous stomata, the majority anomocytic (*2.8.3*) [Aa] but some diacytic [Ab]; fragments of the epidermis of the corolla composed of polygonal cells, those of the inner epidermis of the lips papillose [H] and those of the inner epidermis of the tube bearing uni- or bicellular covering trichomes in a stellate arrangement [K]; pollen grains subspherical with 3 pores and a smooth exine [D]; fragments from the stem (G) with groups of collenchymatous cells [Gb] and lignified vessels, with annular or spiral thickenings [J].

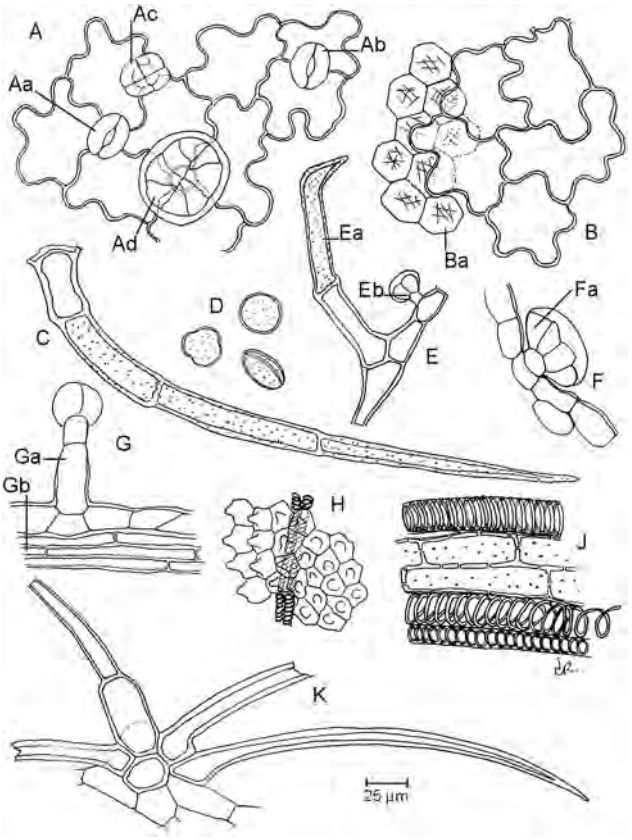


Figure 1858.-1. – *Illustration for identification test B of powdered herbal drug of black horehound*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of *methanol R*. Heat on a water-bath under a reflux condenser for 30 min. Allow to cool. Filter. Evaporate the filtrate under reduced pressure until a volume of about 10 mL is obtained.

*Reference solution.* Dissolve 1 mg of *chlorogenic acid R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a solution containing 10 g/L of *diphenylboric acid aminoethyl ester R* and 50 g/L of *macrogol 400 R* in *methanol R*; allow to dry in a current of warm air; examine in ultraviolet light at 365 nm after 30 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A reddish fluorescent zone |
| | A faint yellow fluorescent zone |
| | A light blue fluorescent zone (caffeoylmalic acid) |
| | A greenish-blue fluorescent zone (acteoside) |
| | A yellowish-brown fluorescent zone (luteolin 7-lactate) |
| Chlorogenic acid: a light blue fluorescent zone | |
| | A greenish-blue fluorescent zone (forsythoside B) |
| Rutoside: an orange-yellow fluorescent zone | 2 greenish-blue fluorescent zones (arenarioside) |
| | A yellow fluorescent zone (luteolin 7-lactate glucoside). |
| | A faint greenish-blue fluorescent zone (ballotetroside). |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 13.0 per cent.

ASSAY

*Stock solution.* Place 1.000 g of the powdered herbal drug (355) (*2.9.12*) in a flask. Add 90 mL of *ethanol (50 per cent V/V) R*. Heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter, collecting the filtrate in a 100 mL volumetric flask. Rinse the flask and the filter with 10 mL of *ethanol (50 per cent V/V) R*. Add the rinsings to the filtrate and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Test solution.* Into a 10 mL volumetric flask, introduce successively, with shaking after each addition, 1.0 mL of the stock solution, 2 mL of *0.5 M hydrochloric acid*, 2 mL of a solution containing 100 g/L of *sodium nitrite R* and 100 g/L of *sodium molybdate R*, and 2 mL of *dilute sodium hydroxide solution R*, and dilute to 10.0 mL with *water R*.

*Compensation liquid.* Into a 10 mL volumetric flask, introduce 1.0 mL of the stock solution, 2 mL of *0.5 M hydrochloric acid* and 2 mL of *dilute sodium hydroxide solution R*, and dilute to 10.0 mL with *water R*.

Measure immediately the absorbance (*2.2.25*) of the test solution at 525 nm, by comparison with the compensation liquid.

Calculate the percentage content of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside, using the following expression:

$$\frac{A \times 1000}{185 \times m}$$

i.e. taking the specific absorbance of acteoside to be 185.

$A$ = absorbance at 525 nm;

$m$ = mass of the substance to be examined, in grams.

**07/2013:2528**

# BLACKCURRANT LEAF

## Ribis nigri folium

DEFINITION

Dried leaf of *Ribes nigrum* L.

*Content*: minimum 1.0 per cent of flavonoids, expressed as isoquercitroside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The leaf is simple. The lamina may be up to 10 cm long and 12 cm wide and shows 3 (rarely 5) rounded triangular lobes, dentate or crenate on the margins, with the median lobe being the largest. The light-brown midrib and secondary veins are very visible on the lower surface, and form a characteristic network through numerous anastomoses. The rigid, light-brown petiole shows a very distinct gutter on the upper part and its length is equal to half the length of the lamina.

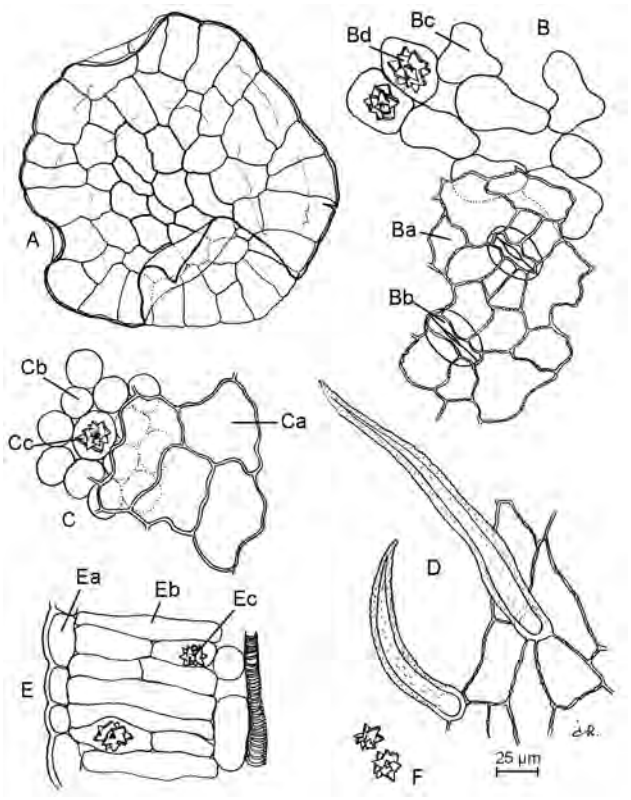


Figure 2528.-1. – *Illustration for identification test B of powdered herbal drug of blackcurrant leaf*

B. Microscopic examination (*2.8.23*). The powder is brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2528.-1): curved, unicellular covering trichomes, with moderately thickened, slightly verrucose walls [D]; orange-yellow, globular or ovoid glandular trichomes, lacking a visible stalk, with a multicellular head up to 200 µm in diameter (surface view [A]); fragments of the lower epidermis (surface view [B]) composed of cells with irregularly thickened walls [Ba], numerous anomocytic stomata (*2.8.3*) [Bb] accompanied by spongy parenchyma [Bc]; fragments (surface view [C], transverse section [E]) of the upper epidermis [Ca, Ea], accompanied by palisade

Herbal drugs

parenchyma [Cb, Eb]; cluster crystals of calcium oxalate up to 30 µm in diameter, isolated [F] or included in parenchymatous cells [Bd, Cc, Ec].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min with occasional stirring. Allow to cool. Filter.

*Reference solution.* Dissolve 5 mg of *isoquercitroside R* and 5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: at 100-105 °C for 10 min.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min, then examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | A green zone |
| Isoquercitroside: an orange zone | An orange zone (mainly isoquercitroside) |
| | A light blue zone |
| ——— | ——— |
| Rutoside: an orange-yellow zone | An orange-yellow zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.200 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of an 80 per cent *V/V* solution of *methanol R*. Heat under a reflux condenser in a water-bath at 60 °C for 30 min. Sonicate for 15 min. Allow to cool, dilute to 20.0 mL with an 80 per cent *V/V* solution of *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *isoquercitroside CRS* in an 80 per cent *V/V* solution of *methanol R* and dilute to 100.0 mL with the same solution.

*Reference solution (b).* Dissolve 5.0 mg of *rutoside trihydrate R* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dilute 10.0 mL of reference solution (a) to 20.0 mL with reference solution (b).

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with an 80 per cent *V/V* solution of *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 45 | 97 → 60 | 3 → 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 350 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to isoquercitroside and the chromatogram obtained with reference solution (b) to identify the peak due to rutoside.

*Retention time*: rutoside = about 28 min; isoquercitroside = about 29 min.

*System suitability*: reference solution (c):
- *resolution*: minimum 3.0 between the peaks due to rutoside and isoquercitroside.

*Disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d).

Calculate the percentage content of total flavonoids, expressed as isoquercitroside, using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 5}$$

$A_1$ = sum of the areas of the peak due to rutoside and all peaks eluting after the peak due to rutoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to isoquercitroside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *isoquercitroside CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of isoquercitroside in *isoquercitroside CRS*.

**01/2008:1605**
**corrected 6.0**

# BOGBEAN LEAF

## Menyanthidis trifoliatae folium

DEFINITION

Dried, entire or fragmented leaf of *Menyanthes trifoliata* L.

CHARACTERS

Very bitter and persistant taste.

IDENTIFICATION

A. The leaf is long-petiolated, trifoliate, with long sheaths from the base; the petiole is up to 5 mm in diameter and strongly striated longitudinally. The lamina is divided into equal leaflets, sessile, obovate up to 10 cm long and up to 5 cm wide, with an entire, occasionally sinuous margin

with brownish or reddish hydathodes and a spathulate base; it is glabrous, dark green on the upper surface and paler green on the lower surface, with a wide, whitish, finely striated prominent midrib.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of upper epidermis with polyhedral cells and thin wavy walls; fragments of lower epidermis with sinuous walls; anomocytic stomata (*2.8.3*), on both surfaces, with the subsidiary cells showing radiating striations; epidermal cells from the veins straight walled and papillose; fragments of mesophyll parenchyma with large intercellular spaces (aerenchyma); irregular cells with rare sclereids; fragments of spiral or annular vessels.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat, with stirring, in a water-bath at 60 °C for 5 min. Allow to cool and filter. Evaporate to dryness under reduced pressure in a water-bath at 60 °C. Dissolve the residue in 2.0 mL of *methanol R*.

*Reference solution.* Dissolve 5 mg of *loganin R* in 15 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 30 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R*. Heat in an oven at 100-105 °C for 10 min. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Loganine: a greyish-violet zone | A violet zone |
| | An intense blue zone |
| | A violet to greyish-violet zone |
| | A grey to greyish-blue zone |
| | A brownish zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Bitterness value** (*2.8.15*): minimum 3000.

*Content*: minimum 0.1 per cent of total alkaloids, expressed as boldine ($C_{19}H_{21}NO_4$; $M_r$ 327.4) (anhydrous drug).

IDENTIFICATION

A. The leaf is oval or elliptical usually 5 cm long with a short petiole, an obtuse or slightly emarginate or mucronate apex and an equal and rounded base; the margin is entire and slightly undulate and the thickened edges are more or less revolute. The lamina is greyish-green, thick, tough and brittle. The upper surface is rough with numerous prominent small protuberances and a depressed venation. The lower surface is finely pubescent, with the protuberances less well-marked, and a prominent, pinnate venation.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1396.-1): fragments of the lamina of the leaf (surface view [C], transverse section [G]) consisting of the upper epidermis with rigid, thick-walled cells [Ca, Ga], the hypodermis with cells with straight or slightly sinuous, thickened and beaded walls [Cb, Gb], and palisade parenchyma [Cc] with 2 layers of cells [Gc]; fragments of the lower epidermis with numerous stomata surrounded by 4-7 subsidiary cells (surface view [A, J]); solitary [F], bifurcated or stellate clustered [D, H] unicellular covering trichomes with more or less thickened and lignified walls; debris of the spongy mesophyll (surface view [B]) including numerous large, rounded oil cells [Ba, Ge]; fragments of parenchyma containing fine needle-shaped crystals [Bb, Gd], thick-walled fibres and lignified, pitted parenchymatous cells associated with vascular tissue from the veins [E].

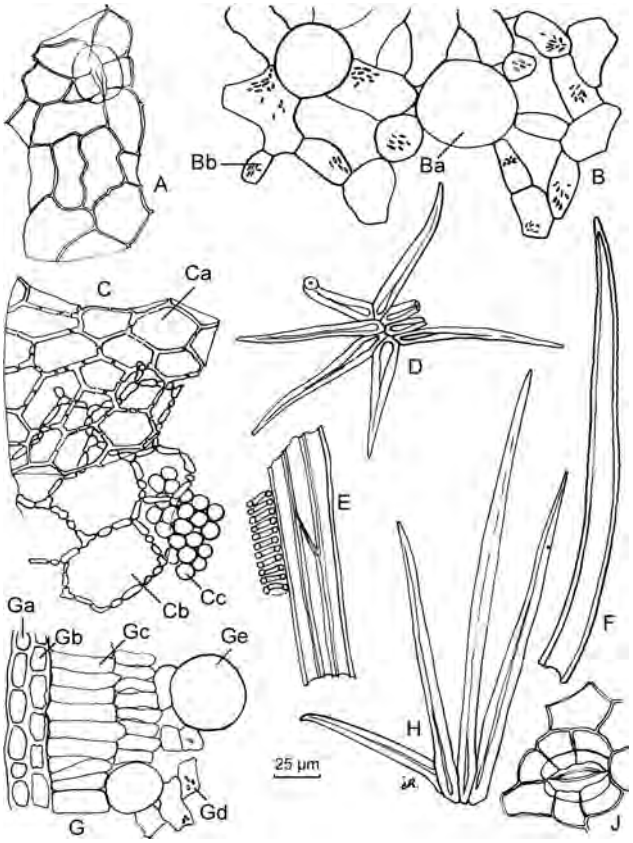


Figure 1396.-1. – *Illustration for identification test B of powdered herbal drug of boldo leaf*

**04/2016:1396**

# BOLDO LEAF

## Boldi folium

DEFINITION

Whole or fragmented dried leaf of *Peumus boldus* Molina.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Mix 1.5 g of the powdered herbal drug (355) (*2.9.12*) and 5 mL of *methanol R* and sonicate for 10 min. Filter the supernatant through a 3 cm × 0.5 cm column of *cellulose for chromatography R1*. Use the first 1 mL of the eluate.

*Reference solution.* Dissolve 2 mg of *boldine R* and 10 mg of *hyoscine hydrobromide R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *diethylamine R*, *methanol R*, *toluene R* (10:10:80 *V/V/V*).

*Application*: 40 µL [or 6 µL] of the test solution and 20 µL [or 2 µL] of the reference solution as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *potassium iodobismuthate solution R2*, allow to dry in air for 5 min and treat with *sodium nitrite solution R*; examine in daylight after 30 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A yellowish-brown zone |
| Hyoscine: a pale brown zone | |
| | A yellow zone |
| | A brown zone |
| | A brown zone |
| Boldine: a brown zone | A brown zone (boldine) |
| | Several zones |
| **Reference solution** | **Test solution** |

TESTS

**Essential oil** (*2.8.12*): maximum 40 mL/kg (anhydrous drug).

Use 10.0 g of the freshly fragmented herbal drug, a 1000 mL flask and 300 mL of *water R* as the distillation liquid. Distil at a rate of 2-3 mL/min for 3 h.

**Foreign matter** (*2.8.2*): maximum 4 per cent of twigs and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined by distillation of 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 13.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 1.000 g of the powdered herbal drug (355) (*2.9.12*) in 50 mL of *dilute hydrochloric acid R* and shake in a water-bath at 80 °C for 30 min. Filter, take up the residue with 50 mL of *dilute hydrochloric acid R* and shake in a water-bath at 80 °C for 30 min. Filter and repeat the operation once on the residue obtained. Filter. Combine the cooled filtrates and shake with 100 mL of a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Discard the organic layer. Adjust the aqueous layer to pH 9.5 with *concentrated ammonia R*. Shake successively with 100 mL, 50 mL and a further 50 mL of *methylene chloride R*, taking care not to form an emulsion. If necessary, centrifuge at 1200 *g* for 10 min. Combine the lower layers and evaporate to dryness under reduced pressure. Dissolve the residue in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 12.0 mg of *boldine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Disperse 0.5 g of *boldo leaf dry extract HRS* in 50 mL of *dilute hydrochloric acid R* and sonicate for 10 min. Transfer to a separating funnel and wash with 10 mL of a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Discard the organic layer. Adjust the aqueous phase to pH 9.5 with *concentrated ammonia R*. After cooling, shake successively with 100 mL, 50 mL and a further 50 mL of *methylene chloride R*, taking care not to form an emulsion. If necessary, centrifuge at 1200 *g* for 10 min. Combine the lower layers and evaporate to dryness under reduced pressure. Dissolve the residue in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Solution A*. Mix 0.2 mL of *diethylamine R* and 99.8 mL of *acetonitrile R*.

*Solution B*. Mix 0.2 mL of *diethylamine R* and 99.8 mL of *water R* and adjust to pH 3 with *anhydrous formic acid R*.

*Mobile phase*: solution A, solution B (16:84 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 304 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *boldo leaf dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to alkaloids 1, 3, 4, 5 and 6; use the chromatogram obtained with reference solution (a) to identify the peak due to boldine.

*Relative retention* with reference to boldine (retention time = about 6 min): alkaloid 1 = about 0.9; alkaloid 3 = about 1.8; alkaloid 4 = about 2.0; alkaloid 5 = about 2.9; alkaloid 6 = about 3.1. Additional peaks may be present.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to alkaloid 1 and boldine.

Calculate the percentage content of total alkaloids, expressed as boldine, using the following expression:

$$\frac{(\sum A_1) \times m_2 \times p}{A_2 \times m_1 \times 100}$$

$\Sigma A_1$ = sum of the areas of the peaks due to alkaloids 1, 3, 4, 5 and 6 and the peak due to boldine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to boldine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *boldine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of boldine in *boldine CRS*.

**04/2016:1816**

# BOLDO LEAF DRY EXTRACT

## Boldi folii extractum siccum

DEFINITION

Extract produced from *Boldo leaf (1396)*.

*Content*:

– *for aqueous extracts*: minimum 0.1 per cent of total alkaloids, expressed as boldine ($C_{19}H_{21}NO_4$; $M_r$ 327.4) (anhydrous extract);

– *for hydroalcoholic extracts*: minimum 0.2 per cent of total alkaloids, expressed as boldine ($C_{19}H_{21}NO_4$; $M_r$ 327.4) (anhydrous extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using either hot water at not less than 65 °C or a hydroalcoholic solvent equivalent in strength to ethanol (45-75 per cent *V/V*).

CHARACTERS

*Appearance*: brown or greenish-brown, hygroscopic powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the extract to be examined add 1 mL of *hydrochloric acid R* and 20 mL of *water R*. Sonicate for 10 min. Transfer the liquid to a separating funnel and make alkaline with 2 mL of *dilute ammonia R1*. Shake with 2 quantities, each of 20 mL, of *methylene chloride R*. Evaporate the combined organic layers to dryness. Dissolve the residue in 1 mL of *methanol R*.

*Reference solution.* Dissolve 2 mg of *boldine R* and 10 mg of *hyoscine hydrobromide R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *diethylamine R*, *methanol R*, *toluene R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 3 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *potassium iodobismuthate solution R2*, allow to dry in air for 5 min and treat with *sodium nitrite solution R*; examine in daylight after 30 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A yellowish-brown zone |
| | An orange-yellow zone |
| Hyoscine: a pale brown zone | |
| | An orange zone |
| | An orange zone |
| _____ | _____ |
| Boldine: a brown zone | A brown zone (boldine) |
| | Several orange zones |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 1.000 g of the extract to be examined in 50 mL of *dilute hydrochloric acid R* and sonicate for 10 min. Transfer to a separating funnel and wash with 10 mL of a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Discard the organic layer. Adjust the aqueous phase to pH 9.5 with *concentrated ammonia R*. After cooling, shake successively with 100 mL, 50 mL, and a further 50 mL of *methylene chloride R*, taking care not to form an emulsion. If necessary, centrifuge at 1200 *g* for 10 min. Combine the lower layers and evaporate to dryness under reduced pressure. Dissolve the residue in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 12.0 mg of *boldine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Disperse 0.5 g of *boldo leaf dry extract HRS* in 50 mL of *dilute hydrochloric acid R* and sonicate for 10 min. Transfer to a separating funnel and wash with 10 mL of a mixture of equal volumes of *ethyl acetate R* and *hexane R*. Discard the organic layer. Adjust the aqueous phase to pH 9.5 with *concentrated ammonia R*. After cooling, shake successively with 100 mL, 50 mL, and a further 50 mL of *methylene chloride R*, taking care not to form an emulsion. If necessary, centrifuge at 1200 *g* for 10 min. Combine the lower layers and evaporate to dryness under reduced pressure. Dissolve the residue in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Solution A*. Mix 0.2 mL of *diethylamine R* and 99.8 mL of *acetonitrile R*.

*Solution B*. Mix 0.2 mL of *diethylamine R* and 99.8 mL of *water R* and adjust to pH 3 with *anhydrous formic acid R*.

*Mobile phase*: solution A, solution B (16:84 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 304 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *boldo leaf dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to alkaloids 1, 3, 4, 5 and 6; use the chromatogram obtained with reference solution (a) to identify the peak due to boldine.

*Relative retention* with reference to boldine (retention time = about 6 min): alkaloid 1 = about 0.9; alkaloid 3 = about 1.8; alkaloid 4 = about 2.0; alkaloid 5 = about 2.9; alkaloid 6 = about 3.1. Additional peaks may be present.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to alkaloid 1 and boldine.

Calculate the percentage content of total alkaloids, expressed as boldine, using the following expression:

$$\frac{(\sum A_1) \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$\Sigma A_1$ = sum of the areas of the peaks due to alkaloids 1, 3, 4, 5 and 6 and the peak due to boldine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to boldine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *boldine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of boldine in *boldine CRS*.

**07/2013:2184**

# BUCKWHEAT HERB

## Fagopyri herba

DEFINITION

Whole or fragmented aerial parts of *Fagopyrum esculentum* Moench, collected in the early flowering period prior to fruiting and dried immediately.

*Content*: minimum 3.0 per cent of rutoside ($C_{27}H_{30}O_{16}$; $M_r$ 611) (dried drug).

IDENTIFICATION

A. The stem is cylindrical, hollow, finely ridged longitudinally, about 2-6 mm in diameter, brownish-green or reddish, with few branches and thickened at the internodes; the leaves are arranged spirally and have membranous, sheathing stipules; the surface is glabrous except in the region of the stipules, where short, white hairs may occur. The leaves are dark green, paler on the lower surface, up to 7 cm wide and 11 cm long, saggitate or cordate, almost pentagonal with 2 widely rounded lobes; the lower leaves are petiolate, the upper leaves sessile or amplexicaul; the lamina is glabrous and the margin finely sinuate and fringed with minute, reddish-brown projections; similar projections occur on the veins on the upper surface. The inflorescence is a cymose panicle, the individual flowers 1-2 mm long and 6 mm in diameter with 5 free, white or reddish petals.

B. Microscopic examination (*2.8.23*). The powder is dark green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2184.-1): fragments of the epidermis of the stem (surface view [D]) composed of elongated cells showing striations on the outer walls [Da] and anomocytic stomata (*2.8.3*) [Db]; fragments of the upper epidermis of the lamina (surface view [B]), consisting of polygonal cells covered by a striated cuticle [Ba] and anomocytic stomata [Bb], often accompanied by palisade parenchyma [Bc]; fragments of the epidermis of the leaf margins [A] and of the epidermis covering the veins, often showing ovoid or rounded papilla-like projections, often reddish, with thickened and striated walls; fragments of the lower epidermis of the lamina [C] with thin-walled polygonal cells, numerous stomata [Ca] and rare glandular trichomes with a biseriate stalk and a globular head usually composed of 8 cells [Cb]; fragments of mesophyll [F] with narrow, annular or spiral vessels [Fa] and of spongy parenchyma, numerous cells of which contain cluster crystals of calcium oxalate, varying in diameter (25-100 µm) [Fb], smaller prismatic crystals of calcium oxalate [Fc], occurring scattered in the mesophyll and also in the parenchyma of the stem; fragments of lignified tissue [H] with bordered-pitted [Ha], reticulate or annular [Hb] vessels and thin-walled, pitted fibres [Hc]; occasional fragments of the corolla with a papillose epidermis [E]; spherical or ovoid pollen grains, about 50 µm in diameter, with a pitted exine and 3 furrows [G].

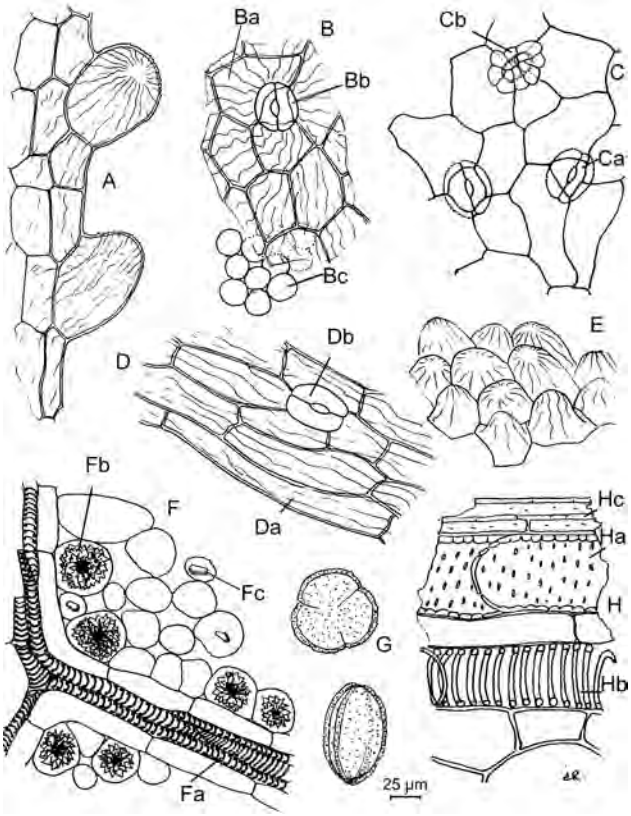


Figure 2184.-1. – *Illustration for identification test B of powdered herbal drug of buckwheat herb*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R* and heat in a water-bath at 60 °C under a reflux condenser for 10 min. Cool and filter.

*Reference solution.* Dissolve 10 mg of *hyperoside R* and 10 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: at 100-105 °C.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, subsequently treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 red zones |
| | 1-2 light blue zones |
| ——— | ——— |
| | An orange zone |
| | An orange zone |
| Hyperoside: an orange zone | 2 blue zones |
| ——— | ——— |
| Rutoside: an orange-yellow zone | An orange-yellow zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*), add 30 mL of an 80 per cent *V/V* solution of *methanol R*. Heat the mixture under a reflux condenser in a water-bath at 60 °C for 30 min, then extract the mixture in an ultrasonic bath for 15 min. Allow to cool, dilute to 50.0 mL with an 80 per cent *V/V* solution of *methanol R* and filter.

*Reference solution (a).* Dissolve 25.0 mg of *rutoside trihydrate CRS* in an 80 per cent *V/V* solution of *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20.0 mg of *troxerutin R* and 5.0 mg of *quercitrin R* in an 80 per cent *V/V* solution of *methanol R* and dilute to 50.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 50 volumes of *acetonitrile R* and 950 volumes of *water R* adjusted to pH 2 with *phosphoric acid R*;
- *mobile phase B*: mix 95 volumes of *water R* adjusted to pH 2 with *phosphoric acid R* and 905 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 94 | 6 |
| 6 - 16.5 | 94 → 85 | 6 → 15 |
| 16.5 - 22 | 85 → 76 | 15 → 24 |
| 22 - 25 | 76 → 59 | 24 → 41 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 350 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (b):

- *elution order*: order indicated in the composition of reference solution (b), when the chromatogram is recorded in the prescribed conditions;
- *resolution*: minimum 3 between the peaks due to troxerutin and quercitrin.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the peak due to rutoside in the chromatogram obtained with the test solution.

Calculate the percentage content of rutoside using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rutoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rutoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rutoside trihydrate CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rutoside in *rutoside trihydrate CRS*.

**04/2018:2562**

# BUPLEURUM ROOT

## Bupleuri radix

DEFINITION

Dried, whole or fragmented root of *Bupleurum chinense* DC. or *Bupleurum scorzonerifolium* Willd.

*Content*: minimum 0.16 per cent of saikosaponin A ($C_{42}H_{68}O_{13}$; $M_r$ 781) (dried drug).

IDENTIFICATION

A. *B. chinense*. The whole root is cylindrical or elongated conical, branched in the lower part, up to 23 cm long and 1.2 cm in diameter. The upper part consists of a bulgy root crown, usually composed of 3-15 stem bases as well as the fibrous remnants of the leaf bases. The fragmented root consists of irregular pieces, 1-5 cm long, 0.2-0.3 cm in diameter. The outer surface is blackish-brown or light brown, marked with longitudinal wrinkles and showing rootlet scars and lenticel-like protuberances. The texture is hard and compact, difficult to break. The fracture shows concentric fibrous rings in the wood; the bark is thin, light brown or orange-brown, while the wood is whitish-yellow.

*B. scorzonerifolium*. The whole root is thinner than that of *B. chinense*, elongated conical, usually unbranched or very slightly branched in the lower part, up to 15 cm long and 0.3-0.5 cm in diameter. The root crown bears numerous fibres from the bases of wilted leaves in a brush-like shape. The fragmented root consists of irregular pieces, 1-5 cm long. The outer surface is blackish-brown or reddish-brown and shows numerous annular striations near the root crown. The texture is slightly soft and the root breaks easily. The fracture is even and non-fibrous.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2562.-1): fragments of yellowish-brown to reddish-brown cork (surface view [A]) with rectangular and flattened cells (transverse section [H]), some cork cells containing small prism crystals visible mainly in polarised light [Ha]; fragments of vessels (5-80 µm in diameter) with various thickenings [G], most of which are reticulate, accompanied by xylem parenchyma with elongated, thin-walled cells or by fibres with thick (2-8 µm) and channelled walls [C]; xylem fibres, isolated [F] or in bundles [E], long, fusiform, about 7-25 µm in diameter; fragments of secretory canals

(10-15 µm up to 40 µm in diameter) with inconspicuous secretory cells and yellowish to orange contents [D]; fragments of parenchyma with conspicuous secretory canals visible as yellowish to orange-red ribbon-like secretions [B].

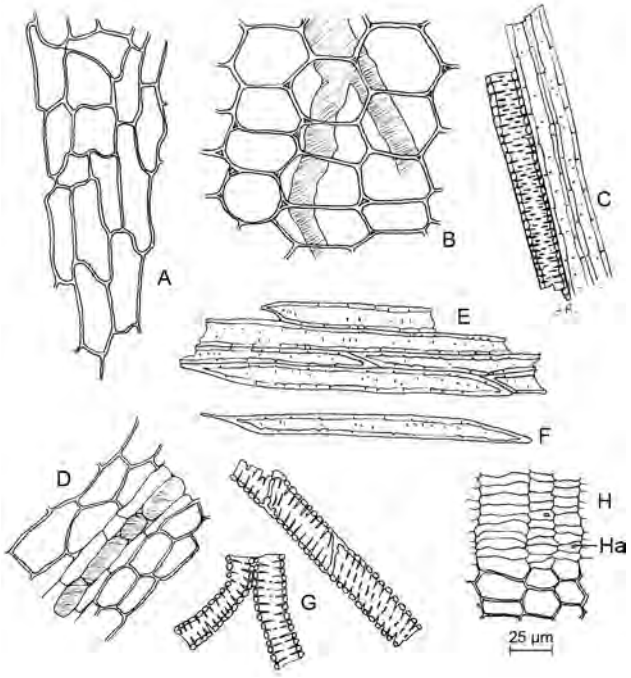


Figure 2562.-1. – *Illustration for identification test B of powdered herbal drug of bupleurum root*

C. High-performance thin-layer chromatography (*2.8.25*).

Examine the chromatograms obtained in the test for *Bupleurum longiradiatum* Turcz.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| ——— | ——— |
| | An orange or faint orange zone |
| Saikosaponin D: an orange-brown zone | An orange-brown zone (saikosaponin D) |
| Saikosaponin A: a grey zone | A grey zone (saikosaponin A) |
| ——— | ——— |
| | A reddish-violet zone (saikosaponins C, H) |
| **Reference solution (a)** | **Test solution** |

TESTS

***Bupleurum longiradiatum* Turcz**. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10.0 mL of *methanol R* and sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution (a)*. Dissolve 1.0 mg of *saikosaponin A R* and 1.0 mg of *saikosaponin D R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Intensity marker*: saikosaponin D.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *2-propanol R*, *ethyl acetate R* (10:30:80 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 3 min; examine in daylight.

*System suitability*: reference solution (a):

– the chromatogram shows 2 distinct zones in the lower part of the middle third; the lower zone (saikosaponin A) is grey and the upper zone (saikosaponin D) is orange-brown.

*Results*: in the chromatogram obtained with the test solution, the presence of a greyish zone directly above the zone due to saikosaponin H (which co-migrates with saikosaponin C) indicates adulteration by *B. longiradiatum*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Disperse 0.250 g of the powdered herbal drug (355) (*2.9.12*) in a mixture of 3 mL of *concentrated ammonia R* and 12 mL of *methanol R1*. Sonicate for 30 min and centrifuge for 10 min. Repeat the extraction twice, combine the supernatants and evaporate to dryness under reduced pressure. Dissolve the residue in *methanol R1* and dilute to 5.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 10.0 mg of *saikosaponin A CRS* in *methanol R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 1 mg of *propyl parahydroxybenzoate R* in 1 mL of reference solution (a) and dilute to 10.0 mL with *methanol R1*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, *water for chromatography R* (36:64 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of saikosaponin A.

*Relative retention* with reference to saikosaponin A (retention time = about 16 min): propyl parahydroxybenzoate = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to propyl parahydroxybenzoate and saikosaponin A.

Calculate the percentage content of saikosaponin A using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.5}{A_2 \times m_1}$$

$A_1$ = area of the peak due to saikosaponin A in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to saikosaponin A in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *saikosaponin A CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of saikosaponin A in *saikosaponin A CRS*.

**01/2017:1847**

# BUTCHER'S BROOM

## Rusci rhizoma

DEFINITION

Dried, whole or fragmented underground parts of *Ruscus aculeatus* L.

*Content*: minimum 1.0 per cent of total sapogenins, expressed as ruscogenins [mixture of neoruscogenin ($C_{27}H_{40}O_4$; $M_r$ 428.6) and ruscogenin ($C_{27}H_{42}O_4$; $M_r$ 430.6)] (dried drug).

IDENTIFICATION

A. The rhizome consists of yellowish, branched, articulated, somewhat knotty pieces, cylindrical or subconical, about 5-10 cm long and about 5 mm thick. The surface is marked with thin annulations about 1-3 mm wide, separated from one another; rounded scars of the aerial stems are present on the upper surface. On the lower surface numerous roots, or their scars, occur; the roots are about 2 mm in diameter and similar in colour to the rhizome. The outer layer is easily detached, revealing a yellowish-white, very hard central cylinder.

B. Microscopic examination (*2.8.23*). The powder is yellowish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1847.-1): groups of sclereids of the rhizome, with variously-shaped cells, rounded, elongated or rectangular; the walls are moderately thickened and distinctly beaded, with large, rounded or oval pits [F, G, L, P, Q]; fragments of the endodermis composed of a single layer of irregularly thickened cells [K]; groups of rounded parenchymatous cells, thickened at the corners, with small, triangular intercellular spaces [D, E, N]; thin-walled parenchyma [J] with some cells containing raphides of calcium oxalate [C]; groups [H] of thick-walled fibres [Ha] and small vessels, up to about 50 µm in diameter, the walls showing numerous small, slit-shaped pits [A, Hb]; rare fragments of dermal tissue of the root [B]; raphides of calcium oxalate, isolated [M].

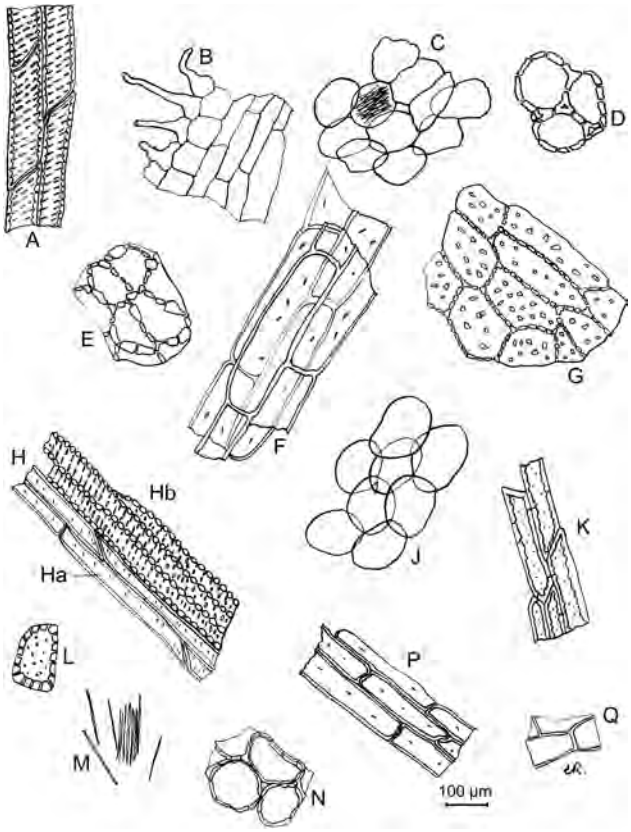


Figure 1847.-1. – *Illustration for identification test B of powdered herbal drug of butcher's broom*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Introduce 1.0 g of the powdered herbal drug (355) (*2.9.12*) and 50 mL of *dilute hydrochloric acid R* into a 100 mL flask with a ground-glass neck. Heat on a water-bath under a reflux condenser for 40 min. Allow to cool and extract the unfiltered mixture with 3 quantities, each of 25 mL, of *methylene chloride R*. Combine the organic solutions and dry over *anhydrous sodium sulfate R*. Filter and evaporate to dryness. Dissolve the residue in 5 mL of *methanol R*.

*Reference solution*. Dissolve 1 mg of *ruscogenins CRS* and 1 mg of *stigmasterol R* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methanol R*, *methylene chloride R* (7:93 *V/V*).

*Application*: 10 µL [or 4 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *vanillin reagent R*, dry in an oven at 100-105 °C for 1 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | Several zones of various colours |
| Stigmasterol: a violet zone | A violet zone |
| _____ | _____ |
| | A violet zone |
| Ruscogenins: a yellow zone | A yellow zone (ruscogenins) |
| | Several zones of various colours |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 2.000 g of the powdered herbal drug (355) (*2.9.12*) add 60 mL of *anhydrous ethanol R*, 15 mL of *water R* and 0.2 g of *potassium hydroxide R*. Extract on a water-bath under a reflux condenser for 4 h. Allow to cool and filter into a 100 mL volumetric flask. Rinse the extraction flask and the residue in the filter with 3 quantities, each of 10 mL, of *anhydrous ethanol R* and add the rinsings to the volumetric flask. Dilute to 100.0 mL with *anhydrous ethanol R*. Evaporate 25.0 mL of this solution to dryness under reduced pressure. Dissolve the residue in 10 mL of *butanol R* and add 3 mL of *hydrochloric acid R1* and 8 mL of *water R*. Heat on a water-bath under a reflux condenser for 1 h. Allow to cool and transfer to a 100 mL volumetric flask. Rinse the round-bottomed flask with 3 quantities, each of 20 mL, of *methanol R*. Add the rinsings to the volumetric flask and dilute to 100.0 mL with *methanol R*.

*Reference solution.* Dissolve 5.0 mg of *ruscogenins CRS* in 100 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 40 | 60 |
| 25 - 27 | 40 → 0 | 60 → 100 |
| 27 - 37 | 0 | 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 203 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *ruscogenins CRS* and the chromatogram obtained with the reference solution to identify the peaks due to neoruscogenin and ruscogenin.

*Relative retention* with reference to neoruscogenin (retention time = about 16 min): ruscogenin = about 1.2.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to neoruscogenin and ruscogenin.

Calculate the percentage content of sapogenins, expressed as ruscogenins (neoruscogenin and ruscogenin), using the following expression:

$$\frac{A_1 \times m_2 \times 4 \times p_1}{A_2 \times m_1} + \frac{A_3 \times m_2 \times 4 \times p_2}{A_4 \times m_1}$$

$A_1$ = area of the peak due to ruscogenin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ruscogenin in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to neoruscogenin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to neoruscogenin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ruscogenins CRS* used to prepare the reference solution, in grams;

$p_1$ = percentage content of ruscogenin in *ruscogenins CRS*;

$p_2$ = percentage content of neoruscogenin in *ruscogenins CRS*.

**01/2011:1297**

# CALENDULA FLOWER

## Calendulae flos

DEFINITION

Whole or cut, dried, and fully opened flowers that have been detached from the receptacle of the cultivated, double-flowered varieties of *Calendula officinalis* L.

*Content*: minimum 0.4 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The ligulate florets consist of a yellow or orange-yellow ligule, about 3-5 mm wide and about 7 mm in the middle part, with a 3-toothed apex and a hairy, partly sickle-shaped, yellowish-brown or orange-brown tube with a projecting style and a bifid stigma occasionally with a partly bent yellowish-brown or orange-brown ovary. The tubular florets, about 5 mm long, are present and consist of the yellow, orange-red or reddish-violet 5-lobed corolla and the yellowish-brown or orange-brown tube, hairy in its lower part, mostly with a partly bent yellowish-brown or orange-brown ovary.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1297.-1): fragments of epidermises of the corolla [C, F, K] containing light yellow oil droplets, some with fairly large anomocytic stomata (*2.8.3*) [Fa, Ka]; covering trichomes biseriate, multicellular and conical [G], usually fragmented, and glandular trichomes with a multicellular stalk [E], very abundant on the base of the corolla [D]; fragments of parenchyma of the corolla [B] containing prisms and very small cluster crystals of calcium oxalate [Ba, Da] and small

vessels [Bb]; spherical pollen grains up to about 40 µm in diameter with a sharply spiny exine and 3 germinal pores [A, J]; occasional fragments of the stigmas with short, bulbous papillae [H].

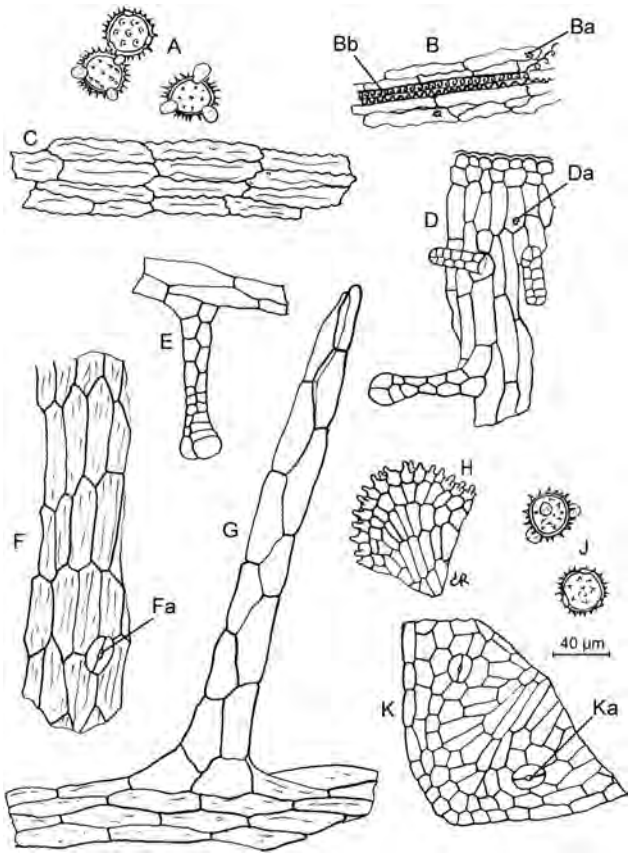


Figure 1297.-1. – *Illustration for identification test B of powdered herbal drug of calendula flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Mix 1.0 g of the powdered herbal drug (500) (*2.9.12*) and 10 mL of *methanol R* and heat on a water-bath under a reflux condenser for 10 min. Cool and filter.

*Reference solution.* Dissolve 1.0 mg of *caffeic acid R*, 1.0 mg of *chlorogenic acid R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower part a yellowish-brown fluorescent zone (rutoside), in the middle part a light bluish fluorescent zone (chlorogenic acid) and in the upper part a light bluish fluorescent zone (caffeic acid). The chromatogram obtained with the test solution shows a yellowish-brown fluorescent zone corresponding in position to the zone due to rutoside in the chromatogram obtained with the reference solution, below and directly above it, it shows a yellowish-green fluorescent zone and a light bluish fluorescent zone corresponding to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution, a yellowish-green fluorescent zone above it and a light bluish fluorescent zone shortly below the zone due to caffeic acid in the chromatogram obtained with the reference solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of bracts and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (500) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

*Stock solution.* Into a 100 mL round-bottomed flask introduce 0.800 g of the powdered herbal drug (500) (*2.9.12*), 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 7 mL of *hydrochloric acid R1* and 20 mL of *acetone R*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a 100 mL volumetric flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool to room temperature, filter the liquid through a plug of absorbent cotton, then filter the combined acetone solution through a filter-paper into the volumetric flask, and dilute to 100.0 mL with *acetone R* by rinsing the flask and filter. Introduce 20.0 mL of this solution into a separating funnel, add 20 mL of *water R* and extract the mixture with 1 quantity of 15 mL and then with 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, rinse with 2 quantities, each of 50 mL, of *water R*, filter the extract over 10 g of *anhydrous sodium sulfate R* into a 50 mL volumetric flask and dilute to 50.0 mL with *ethyl acetate R*.

*Test solution.* To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min, by comparison with the compensation liquid at 425 nm.

Calculate the percentage content of flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

$A$ = absorbance at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2017:2565**

# CAPE JASMINE FRUIT

## Gardeniae fructus

DEFINITION

Whole or fragmented, ripe fruit of *Gardenia jasminoides* J.Ellis, with stalk removed, steamed or treated with boiling water, then dried.

*Content*: minimum 2.0 per cent of geniposide ($C_{17}H_{24}O_{10}$; $M_r$ 388.4) (dried drug).

IDENTIFICATION

A. *Whole drug.* The fruit is obovate or elliptical and may be elongated or more rounded; it is about 1-5 cm long (including the sepal remains) and 0.8-1.7 cm in diameter. The smooth or slightly rough outer surface is yellowish, reddish-yellow or brownish-red and usually has 6 antesepalous raised, longitudinal winged ribs; between the winged ribs, a well-marked, longitudinal and sometimes branched vein is clearly visible; raised, yellowish-brown, dark brown or black papillae are also commonly present on the fruit surface between the winged ribs. The remains of the basally gamosepalous calyx are visible at the upper end of the fruit (inferior ovary); the tips of the sepals are free and are either tapering and pointed or broken. At the lower end of the fruit, the 6 winged ribs join together at the stalk. In the centre of the fused sepals, a stylar scar is visible, surrounded by a disc-shaped nectary. A transverse section of the fruit shows a single locule resulting from the incomplete fusion of both carpels; remnants of the incomplete septa are present on the inner surface of the fruit wall; the locule contains numerous seeds aggregated into a mass in a yellowish, reddish-yellow or, more rarely, dark red placenta, which protrudes between the seeds; the seed shape is variable and may be rounded, triangular or irregularly angular, and the seeds themselves are flat, brownish-red or yellowish-red, measuring up to 0.5 cm in length; the outer surface is densely and finely verrucose. The pericarp is brittle with a smooth, lustrous pale yellow or brownish-yellow inner surface.

*Fragmented drug.* The fruit fragments consist of thin, brittle pieces of pericarp, with a smooth or slightly rough outer surface that is yellowish, reddish-yellow or brownish-red, sometimes with raised yellowish-brown, dark brown or black papillae or crossed by a fragment of winged rib and/or vein; the inner surface is smooth, lustrous, and pale yellow or brownish-yellow. The remains of the calyx or the stalk may be present. Numerous seeds, often aggregated, are visible, they are near circular, triangular or irregularly angular in shape, flat and measure to 0.5 cm in the long axis; their brownish-red or yellowish-red outer surface is densely and finely verrucose. The remains of the yellowish, yellowish-red or, more rarely, dark red placenta are present together with the seeds.

B. Microscopic examination (*2.8.23*). The powder is orange-brown or orange-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2565.-1): fragments of the epicarp (surface view [G]) consisting of polyhedral cells; fragments of pericarp (transverse section [D]) consisting of thin-walled parenchymatous cells [Da]; fragments of epicarp from the winged ribs or the calyx (surface view [F, J]) consisting of polyhedral cells, scars of covering trichomes [Ja] and rare, pointed, unicellular trichomes either short (up to 380 µm) or more rarely longer, with smooth, thick walls [Fa]; fragments of mesocarp [A, B] consisting of thin-walled parenchymatous cells [Aa, Ba] associated with fibres and yellowish spiral or annular xylem vessels, up to 12 µm in diameter [Bb]; parenchyma, sometimes containing oil droplets [Dc] or cluster crystals [Ab, Db, Bf] or prisms [Ac, Be] of calcium oxalate; fibres, fusiform [Bd] or rectangular [Bc], with thickened, pitted walls about 10 µm in diameter and up to 100 µm long; parenchyma may also be associated with small groups of subrounded, polygonal or subrectangular sclereids with regularly thickened, pitted walls, 15-57 µm long and 11-30 µm in diameter [Ad]; fragments of endocarp [C] consisting of compact masses of mosaic-arranged fibres overlaid or cross-embedded with sclereids containing calcium oxalate prisms, 4-14 µm long [Ca, Cb], especially visible in polarised light; endocarp fibres, long and thin, about 10 µm in diameter and up to 100 µm long [Cc]; fragments of testa [H] consisting of large irregularly shaped sclereids, 58-150 µm in diameter and up to 260 µm long, with irregularly thickened yellowish walls, wide pits and containing a reddish-brown substance; fragments of endosperm with cells containing aleurone grains [K]; prisms or scattered clusters of calcium oxalate [E].

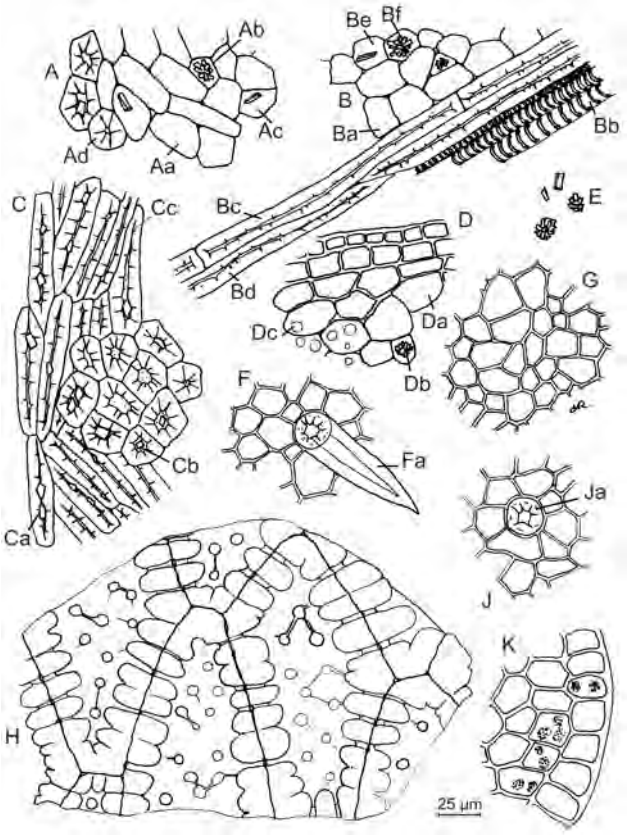


Figure 2565.-1. – *Illustration for identification test B of powdered herbal drug of cape jasmine fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of a 50 per cent *V/V* solution of *methanol R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 6 mg of *aescin R* and 5 mg of *geniposide R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *acetone R*, *ethyl acetate R* (8:8:42:42 *V/V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 2 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Geniposide: a prominent brown zone | A prominent brown zone (geniposide)<br>A blue zone<br>A reddish zone |
| ——— | ——— |
| Aescin: a violet zone | A brown zone<br>A blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.5 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.500 g of the powdered herbal drug (355) (*2.9.12*) in 50 mL of a 50 per cent *V/V* solution of *methanol R* and sonicate for 40 min. Centrifuge and transfer the supernatant to a 200 mL volumetric flask. Repeat the procedure and dilute to 200.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Reference solution (a).* Dissolve 5.0 mg of *geniposide CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Disperse 0.250 g of *cape jasmine fruit for system suitability HRS* in 25 mL of a 50 per cent *V/V* solution of *methanol R* and sonicate for 40 min. Centrifuge and transfer the supernatant to a 100 mL volumetric flask. Repeat the procedure and dilute to 100.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R* adjusted to pH 3.2 with *anhydrous formic acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 80 | 20 |
| 4 - 40 | 80 → 20 | 20 → 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to geniposide; use the chromatogram supplied with *cape jasmine fruit for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to genipin gentiobioside.

*Retention time*: genipin gentiobioside = about 14 min; geniposide = about 16 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to genipin gentiobioside and geniposide.

Calculate the percentage content of geniposide using the following expression:

$$\frac{A_1 \times m_2 \times p \times 8}{A_2 \times m_1}$$

$A_1$ = area of the peak due to geniposide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to geniposide in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *geniposide CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of geniposide in *geniposide CRS*.

**01/2014:1859**

# CAPSICUM

## Capsici fructus

DEFINITION

Dried ripe fruits of *Capsicum annuum* L. var. *minimum* (Miller) Heiser and small-fruited varieties of *Capsicum frutescens* L.

*Content*: minimum 0.4 per cent of total capsaicinoids, expressed as capsaicin ($C_{18}H_{27}NO_3$; $M_r$ 305.4) (dried drug).

CHARACTERS

Extremely pungent taste.

IDENTIFICATION

A. The fruit is yellowish-orange or reddish-brown, oblong conical with an obtuse apex, about 1-3 cm long and up to 1 cm in diameter at the widest part, occasionally attached to a 5-toothed inferior calyx and a straight peduncle. Pericarp somewhat shrivelled, glabrous, enclosing about 10-20 flat, reniform seeds 3-4 mm long, either loose or attached to a reddish dissepiment.

B. Microscopic examination (*2.8.23*). The powder is orange. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1859.-1): fragments of the epicarp, in surface view, with cells often arranged in rows of 5 to 7 [E], thick-walled when close to the peduncle [B] and with a cuticle uniformly striated [A]; fragments of the pericarp (transverse section [D]) showing the epicarp covered by a thick cuticle [Da] and parenchymatous cells frequently containing droplets of red oil, occasionally containing microsphenoidal crystals of calcium oxalate [Db]; fragments of endocarp [C] with characteristic island groups of sclerenchymatous cells [Ca], the groups being separated by thin-walled parenchymatous cells [Cb]; fragments of the seeds having an episperm composed of large, greenish-yellow, sinuous-walled sclereids with thin outer walls and strongly and unevenly thickened radial and inner walls which are conspicuously pitted [G]; endosperm parenchymatous cells with drops of oil and aleurone grains, 3-6 µm in diameter [H]; occasional fragments from the calyx having an outer epidermis with anisocytic stomata (*2.8.3*) [J], an inner epidermis with no stomata and many glandular trichomes with uniseriate stalks and

multicellular heads [N], and a mesophyll [L] with many idioblasts containing prisms of calcium oxalate [La] or microsphenoidal crystals of calcium oxalate [Lb]; prisms [K] or clusters [M] of calcium oxalate, isolated; annularly and spirally thickened vessels [F].

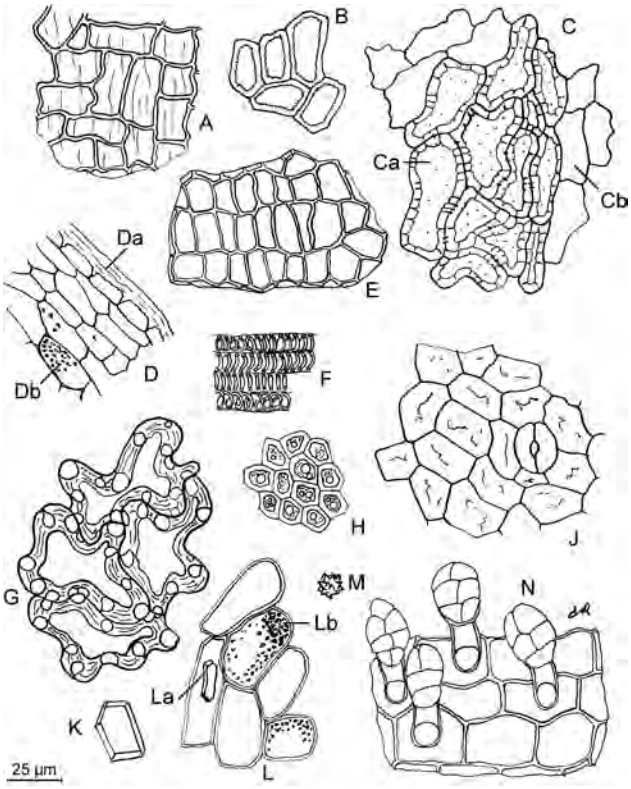


Figure 1859.-1. – *Illustration for identification test B of powdered herbal drug of capsicum*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.50 g of the powdered herbal drug (500) (*2.9.12*) add 5.0 mL of *ether R*, shake for 5 min and filter.

*Reference solution.* Dissolve 2 mg of *capsaicin R* and 2 mg of *dihydrocapsaicin R* in 5.0 mL of *ether R*.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *water R*, *methanol R* (20:80 *V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: treat with a 5 g/L solution of *dichloroquinonechlorimide R* in *methanol R*, and expose to ammonia vapour until blue zones appear. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Capsaicin: a blue zone | A blue zone (capsaicin) |
| Dihydrocapsaicin: a blue zone | A blue zone (dihydrocapsaicin) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Nonivamide**. Liquid chromatography (*2.2.29*).

*Test solution.* To 2.5 g of the powdered herbal drug (500) (*2.9.12*) add 100 mL of *methanol R*. Allow to macerate for 30 min. Place in an ultrasonic bath for 15 min. Filter into a 100 mL volumetric flask, rinse the flask and filter with *methanol R*, then dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *capsaicin CRS* and 2.0 mg of *nonivamide CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 4.0 mg of *nonivamide CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of dihydrocapsaicin.

*Elution order*: nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to nonivamide and capsaicin.

Calculate the percentage content of nonivamide with reference to the total capsaicinoid content, using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 100}{A_2 \times m_1 \times C}$$

$A_1$ = area of the peak due to nonivamide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to nonivamide in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *nonivamide CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of nonivamide in *nonivamide CRS*;

$C$ = percentage content of total capsaicinoids, as determined in the assay.

*Limit*:

- *nonivamide*: maximum 5.0 per cent of the total capsaicinoid content.

**Foreign matter** (*2.8.2*). Fruits of *C. annuum* L. var. *longum* (Sendtn.) are absent.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (500) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for nonivamide.

Calculate the percentage content of total capsaicinoids (C), expressed as capsaicin, using the following expression:

$$\frac{(A_3 + A_5 + A_6) \times m_4 \times p_2 \times 2}{A_4 \times m_3}$$

$A_3$ = area of the peak due to capsaicin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to capsaicin in the chromatogram obtained with reference solution (a);

$A_5$ = area of the peak due to dihydrocapsaicin in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to nordihydrocapsaicin in the chromatogram obtained with the test solution;

$m_3$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_4$ = mass of *capsaicin CRS* used to prepare reference solution (a), in grams;

$p_2$ = percentage content of capsaicin in *capsaicin CRS*.

**01/2014:2336**

# CAPSICUM OLEORESIN, REFINED AND STANDARDISED

## Capsici oleoresina raffinata et normata

DEFINITION

Refined and standardised oleoresin produced from *Capsicum (1859)*.

*Content*: 12.0 per cent to 18.0 per cent *m/m* of total capsaicinoids, expressed as capsaicin ($C_{18}H_{27}NO_3$; $M_r$ 305.4).

PRODUCTION

The oleoresin is produced from the herbal drug by an appropriate procedure, using ethanol (minimum 90 per cent *V/V*).

CHARACTERS

*Appearance*: red or brown mobile extract.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the oleoresin to be examined in 5 mL of *ether R*.

*Reference solution*. Dissolve 2 mg of *capsaicin R* and 2 mg of *dihydrocapsaicin R* in 5 mL of *ether R*.

*Plate*: *TLC octadecylsilyl silica gel plate R* (5-40 µm) [or *TLC octadecylsilyl silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R, methanol R* (20:80 *V/V*).

*Application*: 20 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 0.25 g/L solution of *dichloroquinonechlorimide R* in *ethyl acetate R*, expose to ammonia vapour until blue zones appear. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Capsaicin: a blue zone | A blue zone (capsaicin) |
| Dihydrocapsaicin: a blue zone | A faint blue zone (dihydrocapsaicin) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Nonivamide**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.300 g of the oleoresin to be examined in 60 mL of *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10.0 mg of *capsaicin CRS* and 2.0 mg of *nonivamide CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 4.0 mg of *nonivamide CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of dihydrocapsaicin.

*Elution order*: nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin.

*Relative retention* with reference to capsaicin (retention time = about 19 min): nordihydrocapsaicin = about 0.9; nonivamide = about 0.95; dihydrocapsaicin = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to nonivamide and capsaicin.

Calculate the percentage content of nonivamide with reference to the total capsaicinoid content, using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 100}{A_2 \times m_1 \times C}$$

$A_1$ = area of the peak due to nonivamide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to nonivamide in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the oleoresin to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *nonivamide CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of nonivamide in *nonivamide CRS*;

$C$ = percentage content of total capsaicinoids, as determined in the assay.

*Limit*:

- *nonivamide*: maximum 5.0 per cent of the total capsaicinoid content.

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 5.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for nonivamide.

Calculate the percentage content of total capsaicinoids (C), expressed as capsaicin, using the following expression:

$$\frac{(A_3 + A_5 + A_6) \times m_3 \times p_2 \times 2}{A_4 \times m_1}$$

$A_3$ = area of the peak due to capsaicin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to capsaicin in the chromatogram obtained with reference solution (a);

$A_5$ = area of the peak due to dihydrocapsaicin in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to nordihydrocapsaicin in the chromatogram obtained with the test solution;

$m_1$ = mass of the oleoresin to be examined used to prepare the test solution, in grams;

$m_3$ = mass of *capsaicin CRS* used to prepare reference solution (a), in grams;

$p_2$ = percentage content of capsaicin in *capsaicin CRS*.

**01/2014:2529**
**corrected 10.0**

# CAPSICUM SOFT EXTRACT, STANDARDISED

## Capsici extractum spissum normatum

DEFINITION

Standardised soft extract produced from *Capsicum (1859)*.

*Content*: 2.0 per cent to 2.4 per cent of total capsaicinoids, expressed as capsaicin ($C_{18}H_{27}NO_3$; $M_r$ 305.4).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (80 per cent *V/V*).

The content of total capsaicinoids in the extract is determined and adjusted, if necessary, to the value specified by adding a suitable inert excipient, for example liquid glucose.

CHARACTERS

*Appearance*: reddish-brown, glutinous matter.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the extract to be examined add 10 mL of a mixture of *water R* and *propanol R* (40:60 *V/V*). Shake for 5 min. Filter, if necessary.

*Reference solution.* Dissolve 2 mg of *capsaicin R* and 1 mg of *dihydrocapsaicin R* in 5 mL of *methanol R*.

*Plate*: *TLC octadecylsilyl silica gel plate R* (5-40 µm) [or *TLC octadecylsilyl silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R, methanol R* (20:80 *V/V*).

*Application*: 20 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 0.25 g/L solution of *dichloroquinonechlorimide R* in *ethyl acetate R*, expose to ammonia vapour until blue zones appear. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Capsaicin: a blue zone | A blue zone (capsaicin) |
| Dihydrocapsaicin: a blue zone | A blue zone (dihydrocapsaicin) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Nonivamide**. Liquid chromatography (*2.2.29*).

*Test solution.* Stir the extract to be examined until homogeneous, heating, if necessary, to not more than 60 °C. Disperse 0.350 g of the homogeneous extract in 35 mL of a mixture of *water R* and *propanol R* (40:60 *V/V*). Shake for 30 min and dilute to 50.0 mL with *propanol R*. Dilute 25.0 mL of the solution to 50.0 mL with the mobile phase and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 2.0 mg of *nonivamide CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase (solution A). Dissolve 8.0 mg of *capsaicin CRS* in a mixture of 5.0 mL of solution A and 45 mL of the mobile phase. Dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 8.0 mg of *nonivamide CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of dihydrocapsaicin.

*Elution order*: nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin.

*Relative retention* with reference to capsaicin (retention time = about 19 min): nordihydrocapsaicin = about 0.9; nonivamide = about 0.95; dihydrocapsaicin = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to nonivamide and capsaicin.

Calculate the percentage content of nonivamide with reference to the total capsaicinoid content, using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 5}{A_2 \times m_1 \times C}$$

$A_1$ = area of the peak due to nonivamide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to nonivamide in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *nonivamide CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of nonivamide in *nonivamide CRS*;

$C$ = percentage content of total capsaicinoids, as determined in the assay.

*Limit*:

– *nonivamide*: maximum 5.0 per cent of the total capsaicinoid content.

**Dry residue** (*2.8.16*): minimum 70.0 per cent, determined on 2.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for nonivamide.

Calculate the percentage content of total capsaicinoids (C), expressed as capsaicin, using the following expression:

$$\frac{(A_3 + A_5 + A_6) \times m_3 \times p_2}{A_4 \times m_1}$$

$A_3$ = area of the peak due to capsaicin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to capsaicin in the chromatogram obtained with reference solution (a);

$A_5$ = area of the peak due to dihydrocapsaicin in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to nordihydrocapsaicin in the chromatogram obtained with the test solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_3$ = mass of *capsaicin CRS* used to prepare reference solution (a), in grams;

$p_2$ = percentage content of capsaicin in *capsaicin CRS*.

**01/2014:2337**

# CAPSICUM TINCTURE, STANDARDISED

## Capsici tinctura normata

DEFINITION

Standardised tincture produced from *Capsicum (1859)* or *Refined and standardised capsicum oleoresin (2336)*.

*Content*: 90 per cent to 110 per cent of the nominal content of total capsaicinoids, expressed as capsaicin ($C_{18}H_{27}NO_3$; $M_r$ 305.4), stated on the label, which is between 0.020 per cent *m/m* and 0.060 per cent *m/m*.

PRODUCTION

The tincture is produced from the herbal drug or oleoresin and ethanol (70 per cent *V/V* to 85 per cent *V/V*) by an appropriate procedure.

CHARACTERS

*Appearance*: yellowish-orange or reddish-orange liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 10 mL of the tincture to be examined with 10 mL of *hexane R*. Allow to separate and use the lower layer.

*Reference solution.* Dissolve 1 mg of *capsaicin R* and 1 mg of *dihydrocapsaicin R* in 5 mL of *ether R*.

*Plate*: *TLC octadecylsilyl silica gel plate R* (5-40 µm) [or *TLC octadecylsilyl silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R* (20:80 *V/V*).

*Application*: 20 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 0.25 g/L solution of *dichloroquinonechlorimide R* in *ethyl acetate R*, expose to ammonia vapour until blue zones appear. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Capsaicin: a blue zone | A blue zone (capsaicin) |
| Dihydrocapsaicin: a blue zone | A faint blue zone (dihydrocapsaicin) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Nonivamide**. Liquid chromatography (*2.2.29*).

*Test solution.* Dilute 50.0 g of the tincture to be examined to 100.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 10.0 mg of *capsaicin CRS* and 2.0 mg of *nonivamide CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 4.0 mg of *nonivamide CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the rentention time of dihydrocapsaicin.

*Elution order*: nordihydrocapsaicin, nonivamide, capsaicin, dihydrocapsaicin.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to nonivamide and capsaicin.

Calculate the percentage content of nonivamide with reference to the total capsaicinoid content, using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 100}{A_2 \times m_1 \times C}$$

$A_1$ = area of the peak due to nonivamide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to nonivamide in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *nonivamide CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of nonivamide in *nonivamide CRS*;

$C$ = percentage content of total capsaicinoids, as determined in the assay.

*Limit*:

– *nonivamide*: maximum 5.0 per cent of the total capsaicinoid content.

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the content stated on the label.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for nonivamide.

Calculate the percentage content of total capsaicinoids (C), expressed as capsaicin, using the following expression:

$$\frac{(A_3 + A_5 + A_6) \times m_4 \times p_2 \times 2}{A_4 \times m_3}$$

$A_3$ = area of the peak due to capsaicin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to capsaicin in the chromatogram obtained with reference solution (a);

$A_5$ = area of the peak due to dihydrocapsaicin in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to nordihydrocapsaicin in the chromatogram obtained with the test solution;

$m_3$ = mass of the tincture to be examined used to prepare the test solution, in grams;

$m_4$ = mass of *capsaicin CRS* used to prepare reference solution (a), in grams;

$p_2$ = percentage content of capsaicin in *capsaicin CRS*.

**01/2008:1080**

# CARAWAY FRUIT

## Carvi fructus

DEFINITION

Whole, dry mericarp of *Carum carvi* L.

*Content*: minimum 30 mL/kg of essential oil (anhydrous drug).

CHARACTERS

Odour reminiscent of carvone.

IDENTIFICATION

A. The fruit is a cremocarp of almost cylindrical shape. It is generally 3-6.5 mm long and 1-1.5 mm wide. The mericarps, usually free, are greyish-brown or brown, glabrous, mostly sickle-shaped, with both ends sharply terminated. Each bears 5 prominent narrow ridges. When cut transversely the profile shows an almost regular pentagon and 4 vittae on the dorsal surface and 2 on the commissural surface may be seen with a lens.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the secretory cells composed of yellowish-brown or brown, thin-walled, polygonal secretory cells, frequently associated with a layer of thin-walled, transversely elongated cells, 8-12 µm wide; fragments of the epicarp with thick-walled cells and occasional anomocytic stomata (*2.8.3*); numerous endosperm fragments containing aleurone grains, droplets of fatty oil and microcrystals of calcium oxalate in rosette formation; spiral vessels accompanied by sclerenchymatous fibres; rarely some fibre bundles from the carpophore; groups of rectangular to sub-rectangular sclereids from the mesocarp with moderately thickened and pitted walls may be present.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Shake 0.5 g of the powdered herbal drug (710) (*2.9.12*) with 5.0 mL of *ethyl acetate R* for 2-3 min. Filter over 2 g of *anhydrous sodium sulfate R*.

*Reference solution*. Dissolve 2 µL of *carvone R* and 5 µL of *olive oil R* in 1.0 mL of *ethyl acetate R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms obtained with the test solution and with the reference solution show a quenching zone (carvone) in the central part against a light background.

*Detection B*: spray with *anisaldehyde solution R* and, while observing, heat at 100-105 °C for 2-4 min; examine in daylight.

*Results B*: the zones due to carvone are dark orange-brown; the chromatogram obtained with the test solution shows above the zone due to carvone a violet zone similar in position and colour to the zone due to triglycerides of olive oil in the chromatogram obtained with the reference solution; the chromatogram obtained with the test solution shows close to the solvent front a weak violet zone due to terpene hydrocarbons and in the lower part some weak, mostly violet-greyish and brownish zones.

TESTS

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 10.0 g of the powdered herbal drug.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of drug reduced to a powder (710) (*2.9.12*) immediately before the determination, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid, and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 90 min.

**01/2008:1817**

# CARAWAY OIL

## Carvi aetheroleum

DEFINITION

Oil obtained by steam distillation from the dry fruits of *Carum carvi* L.

CHARACTERS

*Appearance*: clear, colourless or yellow liquid.

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A*.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 40 µL of the substance to be examined in 1.0 mL of *toluene R*.

*Reference solution*. Dissolve 10 µL of *carvone R* and 5 µL of *carveol R* in 1.0 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 10 cm [or 5 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Carvone: a quenching zone | A quenching zone (carvone) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, several zones of weak intensity are present, particularly in the lower third, in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish-violet zone |
| ——— | ——— |
| | A reddish-violet zone |
| Carvone: a red to orange-brown zone | An intense red to orange-brown zone (carvone) |
| ——— | ——— |
| Carveol: a reddish-violet zone | A reddish-violet zone (carveol) |
| | A violet-blue zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.904 to 0.920.

**Refractive index** (*2.2.6*): 1.484 to 1.490.

**Optical rotation** (*2.2.7*): + 65° to + 81°.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.00 g.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.200 g of the substance to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 µL of *β-myrcene R*, 80 µL of *limonene R*, 5 µL of *dihydrocarvone R*, 100 µL of *carvone R* and 5 µL of *carveol R* in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 µL of *carvone R* in *heptane R* and dilute to 10 mL with the same solvent. Dilute 0.1 mL of this solution to 10 mL with *heptane R*.

*Column*:

– *material*: fused silica,

– *size*: *l* = 30 m, Ø = 0.53 mm,

– *stationary phase*: *macrogol 20 000 R*, (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 60 |
| | 5 - 68 | 60 → 250 |
| | 68 - 75 | 250 |
| Injection port | | 250 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of reference solution (a). Record the retention times of these substances.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.5 between the peaks due to β-myrcene and limonene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Herbal drugs

*Limits*:

- *β-myrcene*: 0.1 per cent to 1.0 per cent,
- *limonene*: 30.0 per cent to 45.0 per cent,
- *trans-dihydrocarvone*: maximum 2.5 per cent,
- *carvone*: 50.0 per cent to 65.0 per cent,
- *trans-carveol*: maximum 2.5 per cent.
- *disregard limit*: the area of the peak in the chromatogram obtained with reference solution (b).

**Chiral purity**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *(–)-carvone R* and 10 mg of *carvone R1* in *heptane R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.25 mm,
- *stationary phase*: *modified β-cyclodextrin for chiral chromatography R1* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.0 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 80 | 50 → 170 |
| Injection port | | 230 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

- *resolution*: minimum 2.4 between the peaks due to (–)-carvone (1st peak) and carvone R1 (2nd peak).

Calculate the percentage content of the (–)-carvone from the following expression:

$$\frac{A_1}{A_1 + A_2} \times 100$$

$A_1$ = area of the peak due to (–)-carvone,

$A_2$ = area of the peak due to carvone R1.

*Limit*:

- *(–)-carvone*: maximum 1 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**04/2011:0105**

# CASCARA

## Rhamni purshianae cortex

DEFINITION

Dried, whole or fragmented bark of *Rhamnus purshiana* DC. (syn. *Frangula purshiana* (DC.) A.Gray).

*Content*: minimum 8.0 per cent of hydroxyanthracene glycosides of which minimum 60 per cent consists of cascarosides, both expressed as cascaroside A ($C_{27}H_{32}O_{14}$; $M_r$ 580.5) (dried drug).

IDENTIFICATION

A. The bark occurs in slightly channelled or nearly flat pieces, usually 1-5 mm in thickness, usually varying greatly in length and width. The outer surface is grey or dark greyish-brown and shows occasional lenticels that are orientated transversally. It is usually more or less completely covered by a whitish coat of lichens, epiphytic moss and foliaceous liverwort. The inner surface is yellow or reddish-brown or almost black with fine longitudinal striations; it turns red when treated with alkali. The yellow fracture is short and granular in the outer part and somewhat fibrous in the inner part.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0105.-1): bundles [A] of partly lignified phloem fibres [Aa], accompanied by crystal sheaths containing prisms of calcium oxalate [Ab] and sometimes including medullary rays [Ac]; isolated sclereids [G] or groups of sclereids [B] accompanied by crystal sheaths [Ba]; isolated cluster crystals [C] or prisms [E] of calcium oxalate; parenchymatous cells [F, H] containing a yellow substance that becomes deep red when treated with alkali, sometimes accompanied by cells containing cluster crystals of calcium oxalate [Ha]; cork cells (surface view [D], transverse section [J]), associated with parenchyma, some cells of which contain cluster crystals of calcium oxalate [Ja]; frequently epiphytes [K], which may be liverworts, entire or in fragments, having a lamina 1 cell thick without a midrib and composed of isodiametric cells, or leaves of mosses, having a lamina 1 cell thick composed of elongated cells and possessing a midrib several cells thick.

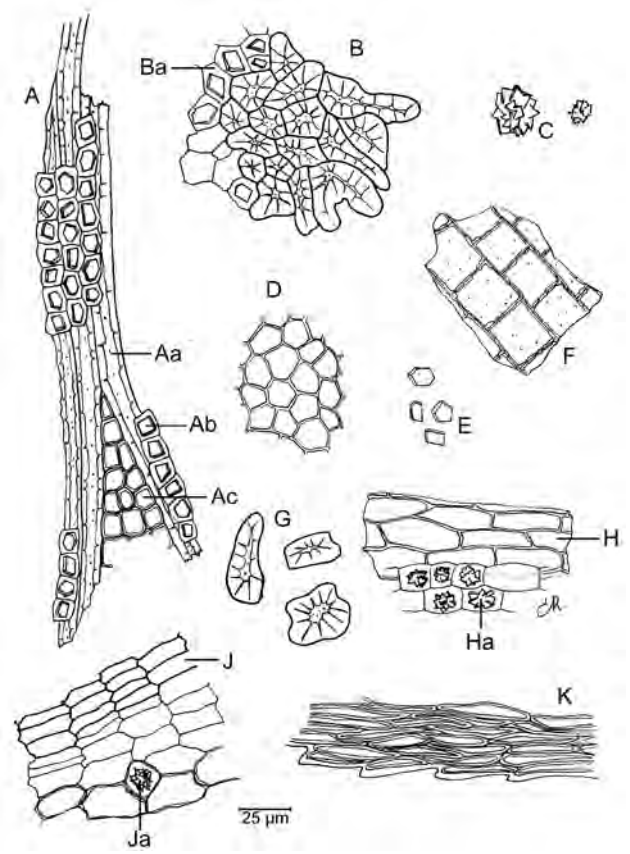


Figure 0105.-1. – *Illustration for identification test B of powdered herbal drug of cascara*

C. Examine the chromatograms obtained in test A for Other species of *Rhamnus*; anthrones.

*Results*: the chromatogram obtained with the test solution shows several reddish-brown zones with different intensities: there are 4 faint zones, 3 being situated at about the mid-point of the chromatogram and 1 in the lower third and there is a strong zone in the upper third of the chromatogram. Examine in ultraviolet light at 365 nm. The chromatogram obtained with the test solution shows several zones with the same fluorescence, situated above and particularly below (cascarosides) that due to barbaloin in the chromatogram obtained with the reference solution.

D. Heat 0.2 g of the powdered herbal drug (180) (*2.9.12*) with 50 mL of *water R* on a water-bath for 15 min. Allow to cool and filter. To 10 mL of the filtrate add 20 mL of *hydrochloric acid R1* and heat on a water-bath for 15 min. Allow to cool, transfer to a separating funnel and shake with 3 quantities, each of 20 mL, of *ether R*. Reserve the aqueous layer (solution A). Combine the 3 ether extracts and shake with 10 mL of *dilute ammonia R2*. The aqueous layer becomes reddish-violet. Transfer solution A to a small flask, add 5 g of *ferric chloride R* and heat on a water-bath for 30 min. Allow to cool, transfer to a separating funnel and shake with 15 mL of *ether R*. Wash the ether layer with 10 mL of *water R*, discard the aqueous layer and shake the ether layer with 5 mL of *dilute ammonia R2*. A red colour develops in the aqueous layer.

TESTS

**Other species of *Rhamnus*; anthrones**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (180) (*2.9.12*) add 5 mL of *ethanol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant immediately and use within 30 min.

*Reference solution*. Dissolve 20 mg of *barbaloin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

*Plates*: *TLC silica gel plate R* (2 plates).

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

A. *Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 5 min.

*Detection*: spray with about 10 mL of a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* and heat at 100-105 °C for 15 min; examine immediately after heating.

*Results*: the chromatogram obtained with the reference solution shows, in the central part, a reddish-brown zone due to barbaloin; examine in ultraviolet light at 365 nm; the zone due to barbaloin shows intense yellowish-brown fluorescence; in the chromatogram with the test solution, no zone with orange-brown fluorescence is seen between the zone due to barbaloin and the zones due to cascarosides.

B. *Application*: 10 µL of the test solution, as a band.

*Development*: over a path of 10 cm.

*Drying*: in air for not more than 5 min.

*Detection*: spray immediately with a 5 g/L solution of *nitrotetrazolium blue R* in *methanol R* and examine immediately.

*Results*: no violet or greyish-blue zones appear.

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (180) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

*Carry out the assay in 24 h, protected from bright light.*

Stir 1.00 g of the powdered herbal drug (180) (*2.9.12*) into 100 mL of boiling *water R* and continue boiling and stirring for 5 min. Allow to cool, dilute to 100.0 mL with *water R*, shake, filter and discard the first 20 mL of filtrate. Transfer 10.0 mL of the filtrate to a separating funnel, add 0.1 mL of *1 M hydrochloric acid* and shake with 2 quantities, each of 20 mL, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic extracts with 5 mL of *water R*, discard the organic layer and return the rinsings to the aqueous layer. Shake the combined aqueous layers with 4 quantities, each of 30 mL, of *ethyl acetate R* freshly saturated with *water R* (to 150 mL of *ethyl acetate R* add 15 mL of *water R*, shake for 3 min and allow to stand) on each occasion allowing separation to take place until the organic layer is clear. Combine the ethyl acetate extracts. Use the aqueous layer for the assay for cascarosides and the organic layer for the assay for hydroxyanthracene glycosides other than cascarosides.

**Hydroxyanthracene glycosides other than cascarosides**. Transfer the organic layer to a suitable flask and remove the solvent by distillation, evaporating almost to dryness. Dissolve the residue in 0.3-0.5 mL of *methanol R* and transfer to a volumetric flask, rinsing the 1st flask with warm *water R* and adding the rinsings to the methanolic solution. Allow to cool and dilute to 50.0 mL with *water R*. Transfer 20.0 mL of this solution to a 100 mL round-bottomed flask with a ground-glass neck and containing 2 g of *ferric chloride R* and 12 mL of *hydrochloric acid R*. Attach a reflux condenser and place the flask in a water-bath so that the level of the water is above that of the liquid in the flask and heat for 4 h. Allow to cool, transfer the solution to a separating funnel and rinse the flask successively with 3-4 mL of *1 M sodium hydroxide* and 3-4 mL of *water R*, adding the rinsings to the separating funnel. Shake the contents of the separating funnel with 3 quantities, each of 30 mL, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic layers with 2 quantities, each of 10 mL, of *water R* and discard the rinsings. Dilute the organic layer to 100.0 mL with the mixture of ether and hexane. Take 20.0 mL, evaporate carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 440 nm and 515 nm using *methanol R* as the compensation liquid. If the ratio of the absorbance at 515 nm to that at 440 nm is less than 2.4, the assay is invalid.

Calculate the percentage content of hydroxyanthracene glycosides other than cascarosides, expressed as cascaroside A, using the following expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

**Cascarosides**. Dilute the aqueous layer to 50.0 mL with *water R*. Treat 20.0 mL of this solution as described above in the assay of hydroxyanthracene glycosides other than cascarosides. Measure the absorbance (*2.2.25*) of the test solution at 440 nm and 515 nm. If the ratio of the absorbance at 515 nm to that at 440 nm is less than 2.7, the assay is invalid.

Calculate the percentage content of cascarosides, expressed as cascaroside A, using the following expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

01/2017:1844

# CASCARA DRY EXTRACT, STANDARDISED

## Rhamni purshianae extractum siccum normatum

DEFINITION

Standardised dry extract obtained from *Cascara (0105)*.

*Content*:

– *hydroxyanthracene glycosides expressed as cascaroside A* ($C_{27}H_{32}O_{14}$; $M_r$ 580.5): 8.0 per cent to 25.0 per cent *m/m* (dried extract) and 90 per cent to 110 per cent of the nominal content stated on the label;

– *cascarosides expressed as cascaroside A* ($C_{27}H_{32}O_{14}$; $M_r$ 580.5): minimum 60 per cent of the hydroxyanthracene glycosides.

PRODUCTION

The extract is produced from the herbal drug by an appropriate procedure using either boiling water or a hydroalcoholic solvent at least equivalent in strength to ethanol (60 per cent *V/V*).

CHARACTERS

*Appearance*: brown, free-flowing powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.2 g of the extract to be examined add 5 mL of *ethanol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant solution immediately and use within 30 min.

*Reference solution*. Dissolve 20 mg of *barbaloin R* and 2 mg of *emodin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air for 5 min.

*Detection*: treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* and heat to 100-105 °C for 15 min; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emodin: a red fluorescent zone | A faint red fluorescent zone |
| Barbaloin: a yellowish-brown fluorescent zone | A yellowish-brown fluorescent zone<br>A blue fluorescent zone |
| | An intense yellowish-brown fluorescent zone<br>3 yellowish-brown fluorescent zones |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 5.0 per cent.

ASSAY

*Carry out the assay within 24 h, protected from bright light.*

To 0.500 g of the extract to be examined add 90 mL of *water R* and sonicate at 40 °C for 15 min. Shake, cool and dilute to 100.0 mL with *water R*. Shake and filter, discarding the first 20 mL of filtrate. Transfer 10.0 mL of the filtrate to a separating funnel, add 0.1 mL of *1 M hydrochloric acid* and shake with 2 quantities, each of 20 mL, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic extracts with 5 mL of *water R*. Discard the organic layer and return the rinsings to the hydroalcoholic layer. Shake with 4 quantities, each of 30 mL, of *ethyl acetate R* freshly saturated with *water R* (prepared as follows: to 150 mL of *ethyl acetate R* add 15 mL of *water R*, shake for 3 min and allow to stand), on each occasion allowing the layers to separate until the organic layer is clear. Combine the ethyl acetate extracts. Use the aqueous layer for the assay of cascarosides and the organic layer for the assay of hydroxyanthracene glycosides other than cascarosides.

**Hydroxyanthracene glycosides other than cascarosides**. Transfer the organic layer to a round-bottomed flask and remove the solvent by distillation, evaporating almost to dryness. Dissolve the residue in 0.5 mL of *methanol R*, add 10 mL of *water R* at 40 °C and transfer to a 50 mL volumetric flask, rinsing the round-bottomed flask with *water R* at 40 °C and adding the rinsings to the hydromethanolic solution. Allow to cool and dilute to 50.0 mL with *water R*. Transfer 20.0 mL of the solution to a 100 mL round-bottomed flask with a ground-glass neck containing 2 g of *ferric chloride R* and 12 mL of *hydrochloric acid R*. Attach a reflux condenser and place the flask in a water-bath so that the level of the water is above that of the liquid in the flask and heat for 4 h. Allow to cool, transfer the solution to a separating funnel and rinse the flask successively with 4 mL of *1 M sodium hydroxide* and 4 mL of *water R*, adding the rinsings to the separating funnel. Shake the contents of the separating funnel with 3 quantities, each of 30 mL, of a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Wash the combined organic layers with 2 quantities, each of 10 mL, of *water R* and discard the rinsings. Dilute the organic layer to 100.0 mL with a mixture of 1 volume of *ether R* and 3 volumes of *hexane R*. Take 20.0 mL of the solution, evaporate carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 440 nm and 515 nm, using *methanol R* as the compensation liquid.

*System suitability*:

– *absorbance ratio*: $A_{515}/A_{440}$ is not less than 2.4.

Calculate the percentage content of hydroxyanthracene glycosides other than cascarosides, expressed as cascaroside A, using the following expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

**Cascarosides**. Dilute the aqueous layer to 50.0 mL with *water R*. Treat 20.0 mL of this solution as described above in the assay of hydroxyanthracene glycosides other than cascarosides. Measure the absorbance (*2.2.25*) at 440 nm and 515 nm.

*System suitability*:

– *absorbance ratio*: $A_{515}/A_{440}$ is not less than 2.7.

Calculate the percentage content of cascarosides, expressed as cascaroside A, using the following expression:

$$\frac{A \times 6.95}{m}$$

i.e. taking the specific absorbance to be 180.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

LABELLING

The label states the nominal content of hydroxyanthracene glycosides, expressed as cascaroside A.

**01/2008:1496**
**corrected 10.0**

# CASSIA OIL

## Cinnamomi cassiae aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the leaves and young branches of *Cinnamomum cassia* (L.) J.Presl (syn. *Cinnamomum aromaticum* Nees).

CHARACTERS

*Appearance*: clear, mobile, yellow or reddish-brown liquid.

Characteristic odour reminiscent of cinnamic aldehyde.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.5 mL of the essential essential oil to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 50 µL of *trans-cinnamic aldehyde R*, 10 µL of *eugenol R* and 50 mg of *coumarin R* in *acetone R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *toluene R* (10:90 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the zone of blue fluorescence in the chromatogram obtained with the test solution is similar in position and colour to the zone in the chromatogram obtained with the reference solution (coumarin).

*Detection B*: spray with *anisaldehyde solution R*; examine in daylight while heating at 100-105 °C for 5-10 min.

*Results B*: the chromatogram obtained with the reference solution shows in its upper part a violet zone (eugenol) and above this zone a greenish-blue zone (*trans*-cinnamic aldehyde). The chromatogram obtained with the test solution shows a zone similar in position and colour to the zone due to *trans*-cinnamic aldehyde in the chromatogram obtained with the reference solution and may show a very faint zone due to eugenol. Other faint zones are present.

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. Eugenol may be absent from the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 1.052 to 1.070.

**Refractive index** (*2.2.6*): 1.600 to 1.614.

**Optical rotation** (*2.2.7*): – 1° to + 1°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The essential oil to be examined.

*Reference solution*. Dissolve 100 µL of *trans-cinnamic aldehyde R*, 10 µL of *cinnamyl acetate R*, 10 µL of *eugenol R*, 10 µL of *trans-2-methoxycinnamaldehyde R* and 20 mg of *coumarin R* in 1 mL of *acetone R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 60 m, Ø = about 0.25 mm;

– *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 75 | 60 → 190 |
| | 75 - 160 | 190 |
| Injection port | | 200 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution, depending on the operating conditions and the state of the column, coumarin may elute before or after *trans*-2-methoxycinnamaldehyde; record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to *trans*-2-methoxycinnamaldehyde and coumarin.

*Identification of components*: using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *trans-cinnamic aldehyde*: 70 per cent to 90 per cent;
- *cinnamyl acetate*: 1.0 per cent to 6.0 per cent;
- *eugenol*: maximum 0.5 per cent;
- *trans-2-methoxycinnamaldehyde*: 3.0 per cent to 15 per cent;
- *coumarin*: 1.5 per cent to 4.0 per cent.

STORAGE

Protected from heat.

**01/2008:1301**
**corrected 6.0**

# CENTAURY

## Centaurii herba

DEFINITION

Whole or fragmented dried flowering aerial parts of *Centaurium erythraea* Rafn s. l. including *C. majus* (H. et L.) Zeltner and *C. suffruticosum* (Griseb.) Ronn. (syn.: *Erythraea centaurium* Persoon; *C. umbellatum* Gilibert; *C. minus* Gars.).

CHARACTERS

Bitter taste.

IDENTIFICATION

A. The hollow cylindrical, light green to dark brown stem has longitudinal ridges, and is branched only in its upper part. The sessile leaves are entire, decussately arranged, and have an ovate to lanceolate lamina, up to about 3 cm long. Both surfaces are glabrous and green to brownish-green. The inflorescence is diaxially branched. The tubular calyx is green and has 5 lanceolate, acuminate teeth. The corolla consists of a whitish tube divided into 5 elongated lanceolate pink to reddish lobes, about 5-8 mm long. 5 stamens are present attached to the top of the corolla tube. The ovary is superior and has a short style, a broad bifid stigma and numerous ovules. Cylindrical capsules, about 7-10 mm long, with small brown markedly rough seeds are frequently present.

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow or brownish. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments from the stem with lignified groups of fibres associated with narrow vessels, tracheidal vessels occasional vessels with spiral thickening; pitted parenchyma of the pith and medullary rays; fragments of leaf lamina with sinuous epidermal cells and striated cuticle, especially over the margins and surrounding the stomata; numerous stomata, mainly anisocytic (*2.8.3*); fragments of the palisade mesophyll, each cell containing a single prism crystal or, less frequently, a cluster crystal of calcium oxalate; fragments of calyx and corolla, those of the calyx with straight-walled epidermal cells, those of the inner epidermis of the corolla with obtuse papillae and radially striated cuticle; parts of the endothecium with reticulate or ridge-shaped wall thickenings; triangularly rounded or elliptical, yellow pollen grains, about 30 µm in diameter, with a distinctly pitted exine and 3 germinal pores; fragments of the wall of the fruit capsule composed of crossed layers of fusiform cells; oil droplets from the seeds, fragments of the epidermis of the testa showing large, brown reticulations and a pitted surface.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *methanol R*, shake for 15 min and filter. Evaporate the filtrate to dryness under reduced pressure and at a temperature not exceeding 50 °C. Take up the residue with small quantities of *methanol R* so as to obtain 5 mL of solution, which may contain a sediment.

*Reference solution.* Dissolve 1 mg of *rutoside trihydrate R* and 1 mg of *swertiamarin R* in *methanol R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl formate R* (4:8:88 *V/V/V*).

*Application*: 10 µL [or 5 µL] as bands.

*Development*: in an unsaturated tank over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other less intense quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| ——— | ——— |
| Swertiamarin: a quenching zone | A prominent quenching zone (swertiamarin) |
| Rutoside: a quenching zone | |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine in daylight.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other less intense coloured zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| ——— | ——— |
| Swertiamarin: a brown zone | A brown zone (swertiamarin) |
| Rutoside: a yellow zone | |
| | A brownish-grey zone |
| | A yellow zone |
| | A grey zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Bitterness value** (*2.8.15*): minimum 2000.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**01/2016:1498**

# CENTELLA

## Centellae asiaticae herba

DEFINITION

Dried, fragmented aerial parts of *Centella asiatica* (L.) Urb.

*Content*: minimum 6.0 per cent of total triterpenoid derivatives, expressed as asiaticoside ($C_{48}H_{78}O_{19}$; $M_r$ 959) (dried drug).

IDENTIFICATION

A. The leaves are alternate, sometimes grouped together at the nodes, reniform or orbicular or oblong-elliptic and have palmate nervation, usually with 7 veins, and a crenate margin. The leaves are very variable in size; the petiole is usually 5-10, sometimes 15, times longer than the lamina, which is 10-40 mm long and 20-40 mm, sometimes up to 70 mm, wide. Young leaves show a few trichomes on the lower surface while adult leaves are glabrous. The inflorescence, if present, is a single umbel which usually consists of 3 flowers, rarely 2 or 4; the flowers are very small (about 2 mm), pentamerous and have an inferior ovary; the fruit, a brownish-grey, orbicular cremocarp, up to 5 mm long, is very flattened laterally and has 7-9 prominent curved ridges per mericarp.

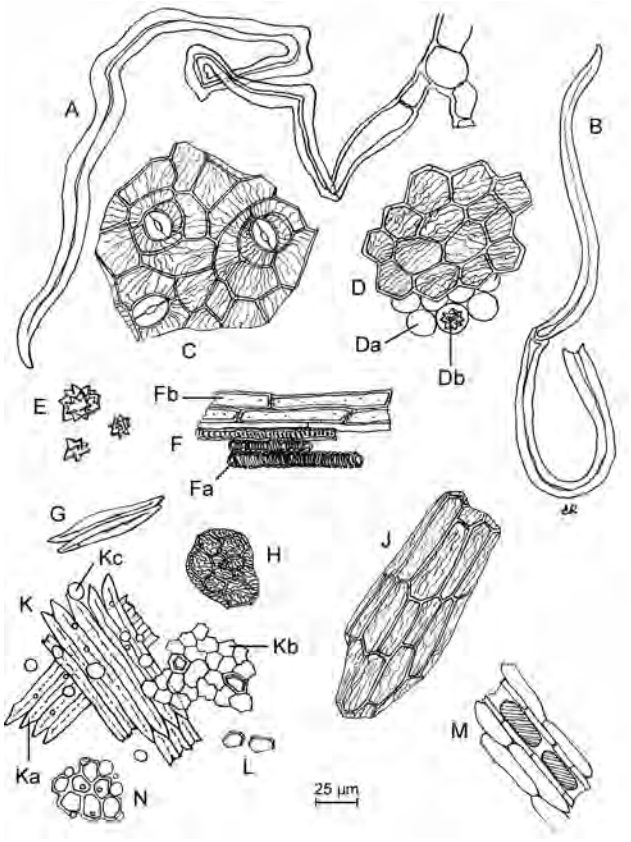


Figure 1498.-1. – *Illustration for identification test B of powdered herbal drug of centella*

B. Microscopic examination (*2.8.23*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1498.-1): numerous fragments of upper epidermis of the leaf [D] composed of polygonal cells having an irregularly striated cuticle, accompanied by underlying palisade parenchyma [Da], with some cells containing cluster crystals of calcium oxalate [Db]; fragments of the lower epidermis of the leaf with usually paracytic stomata (*2.8.3*) [C]; fragments of petiole epidermis with elongated cells covered by a striated cuticle [J]; whole [A] or, usually, fragmented [B], uniseriate, long, flexuous, unicellular or occasionally multicellular covering trichomes of young leaves; secretory canals [M]; small prisms [L] and cluster crystals [E] of calcium oxalate up to 40 µm in diameter; vascular bundles from the stem [F] containing vessels [Fa] and narrow septate fibres [Fb]; fragments of the epicarp of the fruit [H] with polygonal cells having a markedly striated cuticle; fragments of the fruit [K] usually containing 2 layers of short fibres in a perpendicular parquetry arrangement [Ka], annular vessels, parenchymatous cells containing prisms of calcium oxalate [Kb] and numerous oil droplets [Kc]; short fibres, free or in small groups [G]; fragments of the endosperm with oily contents [N].

C. Examine the chromatograms obtained in the test for *Bacopa monnieri* L.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, several brown zones may be present in the lower third of the chromatograms obtained with the test solution and the reference solution.

| Top of the plate | |
|---|---|
| A violet zone | A violet zone |
| A violet zone | A violet zone |
| _____ | _____ |
| _____ | _____ |
| A violet zone | A violet zone |
| A violet zone | A violet zone |
| **Reference solution** | **Test solution** |

TESTS

***Bacopa monnieri* L.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *ethanol (96 per cent) R*, sonicate for 10 min and centrifuge. Use the supernatant.

*Reference solution.* Dissolve 50 mg of *centella dry extract HRS* in 2 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (1:6:14 *V/V/V*).

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 120 °C for 3 min and examine in daylight.

*Results*: no brown zone is present in the middle third of the chromatogram obtained with the test solution.

**Foreign matter** (*2.8.2*): maximum 5 per cent of underground organs and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Place 1.000 g of the powdered herbal drug (355) (*2.9.12*) in a cellulose fingerstall in a continuous extraction apparatus (Soxhlet type). Add 100 mL of *methanol R* and heat for 8 h. Cool and dilute the extract to 100.0 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm). Dilute 5.0 mL of the filtrate to 10.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 6.0 mg of *asiaticoside CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 50.0 mg of *centella dry extract HRS* in *methanol R*, using sonication if necessary, and dilute to 10.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.3 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 65 | 78 → 45 | 22 → 55 |
| 65 - 66 | 45 → 5 | 55 → 95 |
| 66 - 75 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *centella dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to asiaticoside B, madecassoside, asiaticoside, madecassic acid, terminolic acid and asiatic acid.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to terminolic acid and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to madecassic acid.

Calculate the percentage content of total triterpenoid derivatives, expressed as asiaticoside, using the following expression:

$$\frac{[A_1 + (A_2 \times 1.017) + (A_3 \times 0.526) + (A_4 \times 0.509)] \times m_2 \times p \times 10}{A_5 \times m_1}$$

$A_1$ = area of the peak due to asiaticoside in the chromatogram obtained with the test solution;

$A_2$ = sum of the areas of the peaks due to asiaticoside B and madecassoside in the chromatogram obtained with the test solution;

$A_3$ = sum of the areas of the peaks due to madecassic acid and terminolic acid in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to asiatic acid in the chromatogram obtained with the test solution;

$A_5$ = area of the peak due to asiaticoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *asiaticoside CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of asiaticoside in *asiaticoside CRS*;

1.017 = molecular mass conversion factor for asiaticoside B and madecassoside;

0.526 = molecular mass conversion factor for madecassic acid and terminolic acid;

0.509 = molecular mass conversion factor for asiatic acid.

**01/2017:0380**

# CHAMOMILE FLOWER, ROMAN

## Chamomillae romanae flos

DEFINITION

Dried flower-head of the cultivated double variety of *Chamaemelum nobile* (L.) All. (*Anthemis nobilis* L.).

*Content*: minimum 7 mL/kg of essential oil (dried drug).

CHARACTERS

The flower-heads are white or yellowish-grey, composed of solitary hemispherical capitula, made up of a solid conical receptacle bearing the florets, each subtended by a transparent small palea.

IDENTIFICATION

A. The capitula have a diameter of 8-20 mm; the receptacle is solid; the base of the receptacle is surrounded by an involucre consisting of 2-3 rows of compact and imbricated bracts with scarious margins. Most florets are ligulate, but a few pale yellow tubular florets occur in the central region. Ligulate florets are white, dull, lanceolate and reflexed with a dark brown, inferior ovary, a filiform style and a bifid stigma; tubular florets have a five-toothed corolla tube, 5 syngenesious, epipetalous stamens and a gynoecium similar to that of the ligulate florets.

B. Microscopic examination (*2.8.23*). The powder is pale yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0380.-1): numerous glandular trichomes, free (side view [D, G]) or on an epidermis (surface view [Ha]), short, biseriate, with a stalk consisting of 2-4 cells and a head usually consisting of 2 cells covered by a swollen cuticle; numerous conical covering trichomes, free or on an epidermis [M], up to 900 µm long, each consisting of 3-4 very short basal cells and a long, thin-walled, terminal cell, about 20 µm wide; all epidermises bear glandular trichomes and whole or fragmented covering trichomes [Ka]; fragments of the involucral bracts [F] with fine spiral vessels [Fb] and cells, some of which are thick-walled, in longitudinal rows [Fa]; bases of the bracts [K] show anomocytic stomata; fragments of paleae [B], with elongated cells and finely pitted sclereids at the base [Ba], the margins of the paleae consist of a single layer of elongated thin-walled cells [E]; fragments of the corolla of the ligulate flowers whose epidermis is covered by a striated cuticle (surface view [H, M]); papillose cells [J] at the apex of the florets; fragments of the ovary [A] which have at their base a sclerous ring [Ab]; cells of the parenchyma of the ovary and the involucre containing small cluster crystals of calcium oxalate [Aa, Fc];

Herbal drugs

fragments of papillose stigmas [L]; rare rounded or triangular pollen grains about 35 µm in diameter, with 3 germinal pores and a spiny exine [C].

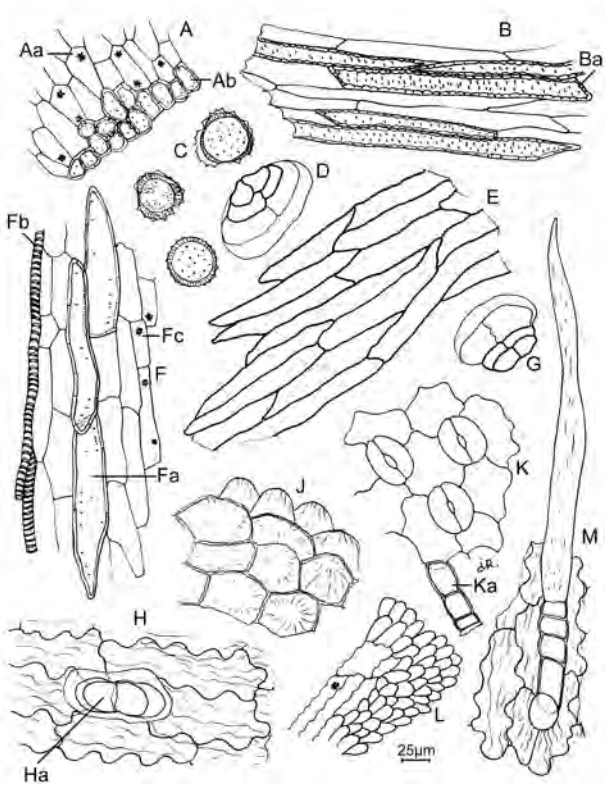


Figure 0380.-1. – *Illustration for identification test B of powdered herbal drug of Roman chamomile flower*

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*. Sonicate for 15 min, then filter or centrifuge the solution and use the filtrate or supernatant.

*Reference solution (a).* Dissolve 3 mg of *chlorogenic acid R* and 5 mg of *apigenin 7-glucoside R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 2.5 mg of *hyperoside R* and 3 mg of *chlorogenic acid R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Intensity marker*: chlorogenic acid.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 5 min. Spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*. Allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the middle third which may, however, be touching. The lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow or orange fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint blue and greenish-blue fluorescent zones may be present, and the greenish-blue fluorescent zone and the brownish-yellow or orange fluorescent to faint fluorescent zone near the solvent front may be overlapped.

| **Top of the plate** | |
|---|---|
| | A greenish-blue fluorescent zone or a faint greenish-blue fluorescent zone |
| | A brownish-yellow or orange fluorescent zone or a faint brownish-yellow or orange fluorescent zone |
| | 2 light blue fluorescent zones or 2 faint light blue fluorescent zones |
| | A usually intense light blue fluorescent zone |
| Apigenin-7-glucoside: a greenish-blue fluorescent zone | A greenish-blue fluorescent zone or an intense greenish-blue fluorescent zone (apigenin-7-glucoside) |
| | A brownish-yellow or orange fluorescent zone or a faint to very faint brownish-yellow or orange fluorescent zone |
| | A light blue fluorescent zone or a faint light blue fluorescent zone |
| Chlorogenic acid: a light blue fluorescent zone | |
| **Reference solution (a)** | **Test solution** |

TESTS

**Diameter of the flower-heads**: maximum 3 per cent of flower-heads have a diameter smaller than 8 mm.

**Deteriorated flower-heads**: brown or darkened flower-heads are absent.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of whole herbal drug, a 500 mL round-bottomed flask, 250 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 3-3.5 mL/min for 3 h.

**04/2017:2715**

# CHINESE GOLDTHREAD RHIZOME

## Coptidis rhizoma

DEFINITION

Whole or fragmented, dried rhizome of *Coptis chinensis* Franch., *Coptis deltoidea* C.Y. Cheng & P.K. Hsiao and/or *Coptis teeta* Wall., with roots removed, collected in autumn.

*Content*: minimum 5.0 per cent of berberine ($C_{20}H_{18}NO_4^+$; $M_r$ 336.4) (dried drug).

IDENTIFICATION

A. *Whole drug.* Whole rhizome with roots and rootlets removed. The outer surface, yellowish-grey or yellowish-brown, is rough, with irregular nodular protuberances, sometimes with fine, smooth internodes; it is shiny in some parts or covered with a fine powder. Rootlet remains are sometimes present. The apex bears the remains of brown scale leaves and sometimes the remains of stems and petioles. The texture is hard, the fracture uneven with reddish-orange, dark reddish-brown or dark brown bark in transverse section; the wood is bright yellow or orange-yellow, radially arranged and clefts may be present. The pith is often of similar colour to the bark and sometimes hollowed. The rhizome of *C. chinensis*, 3-6 cm long and up to 0.8 cm in diameter (below the crown), may be curved, usually with a few to several rhizomes coming together at the crown; that of *C. deltoidea* is usually singular, somewhat cylindrical and curved with smooth and relatively long internodes, about 4-8 cm long and up to 1 cm in diameter; that of *C. teeta* is singular, distinctly hook-shaped and narrow.

*Fragmented drug.* The rhizome is fragmented in transverse or longitudinal slices, 1.2-4.2 cm long, up to 1.1 cm in diameter and up to 4 mm thick. The slices show reddish-orange, dark reddish-brown or dark brown bark, with bright yellow or orange-yellow wood. The wood is radially arranged in transverse section and clefts may be present. The pith is often of similar colour to the bark and sometimes hollowed. The outer surface is yellowish-grey or yellowish-brown, rough, with irregular nodular protuberances; rootlet remains are sometimes present. The slices are easily broken.

B. Microscopic examination (*2.8.23*). The powder is orange or orange-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2715.-1): yellowish-brown or reddish-brown fragments of cork (surface view [B], transverse section [F]); groups of long, often narrow-elliptical, slightly lignified pericyclic fibres with thick channelled walls [C] and a narrow lumen, fibres up to 26 µm in diameter; numerous sclereids (*C. chinensis* or *C. deltoidea*) isolated [D, J] or in groups [E], or also included in parenchyma; sclereids, up to 90 µm in diameter, with thick, yellow lignified walls with pits, channels and striations, cell lumen sometimes with reddish-brown contents; occasional xylem bundles [G]; groups of lignified xylem fibres [A] with slightly thickened walls, 7-28 µm in diameter; the remains of leaves [H] showing an epidermis consisting of cells with beaded walls [Ha] and parenchyma cells with smooth walls [Hb].

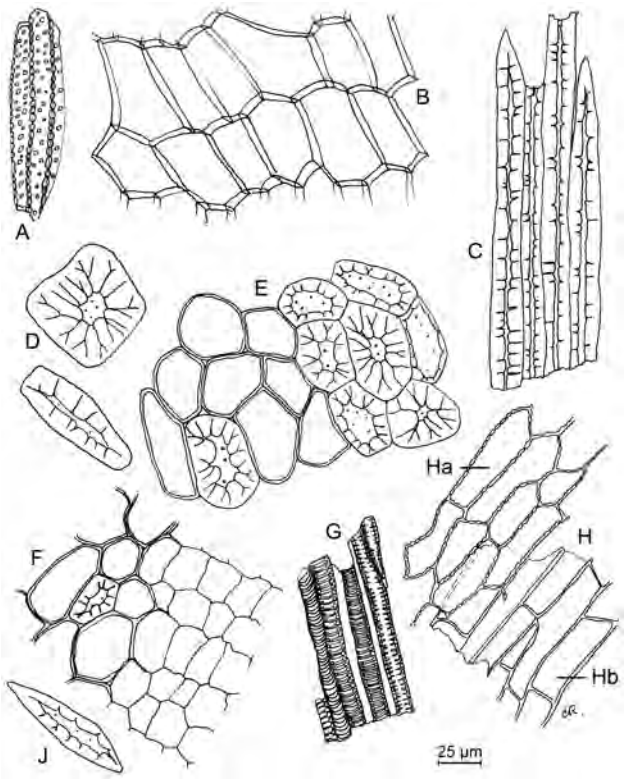


Figure 2715.-1. – *Illustration for identification test B of powdered herbal drug of chinese goldthread rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 15 min. Centrifuge the solution and use the supernatant.

*Reference solution.* Dissolve 5 mg of *berberine chloride R* and 5 mg of *palmatine R* in *methanol R* and dilute to 100 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *2-propanol R*, *ethyl acetate R*, *toluene R* (1:5:5:10:20 *V/V/V/V/V*).

*Application*: 1 µL as bands of 8 mm.

*Development*: in a twin trough tank previously saturated for 20 min without filter paper, over a path of 6 cm.

For saturation, add mobile phase to one trough and *concentrated ammonia R* to the other.

*Drying*: in a current of cold air for 10 min.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A yellow fluorescent zone |
| | A yellow fluorescent zone (may be missing) |
| Berberine: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (berberine) |
| Palmatine: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (palmatine) |
| **Reference solution** | **Test solution** |

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TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of fragments of roots and leaves and maximum 3 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.100 g of the powdered herbal drug (355) (*2.9.12*) in 100.0 mL of a mixture of *hydrochloric acid R* and *methanol R* (1:100 *V/V*). Sonicate for 20 min. Filter 1.5 mL of the solution through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *berberine chloride CRS* in a mixture of *hydrochloric acid R* and *methanol R* (1:100 *V/V*) and dilute to 100.0 mL with the same mixture.

*Reference solution (b).* Dissolve 1 mg of *palmatine R* in reference solution (a) and dilute to 10.0 mL with reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 0.5 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: 0.5 per cent *V/V* solution of *phosphoric acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 | 20 |
| 10 - 20 | 80 → 79 | 20 → 21 |
| 20 - 35 | 79 → 65 | 21 → 35 |
| 35 - 40 | 65 | 35 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 344 nm.

*Injection*: 10 µL.

*Retention time*: palmatine = about 25 min; berberine = about 28 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to palmatine and berberine.

Calculate the percentage content of berberine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to berberine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to berberine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *berberine chloride CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of berberine in *berberine chloride CRS*.

**01/2011:0174**

# CINCHONA BARK

## Cinchonae cortex

DEFINITION

Whole or cut, dried bark of *Cinchona pubescens* Vahl (*Cinchona succirubra* Pav.), of *Cinchona calisaya* Wedd., of *Cinchona ledgeriana* Moens ex Trimen, or of their varieties or hybrids.

*Content*: minimum 6.5 per cent of total alkaloids, of which 30 per cent to 60 per cent consists of quinine-type alkaloids (dried drug).

CHARACTERS

Intense bitter, somewhat astringent taste.

IDENTIFICATION

A. The stem and branch bark is supplied in quilled or curved pieces 2-6 mm thick. The outer surface is dull brownish-grey or grey and frequently bears lichens; it is usually rough, marked with transverse fissures and longitudinally furrowed or wrinkled; exfoliation of the outer surface occurs in some varieties. The inner surface is striated and deep reddish-brown; the fracture is short in the outer part and fibrous in the inner part.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0174.-1): thin-walled cork cells filled with reddish-brown contents (surface view [K], transverse section [H]); yellow, spindle-shaped striated phloem fibres up to 90 µm in diameter and up to 1300 µm in length, very thick-walled with an uneven lumen and with conspicuous, funnel-shaped pits, whole [A] or fragmented [F, J]; parenchymatous idioblasts filled with microprisms of calcium oxalate [E, G]; clusters of thin-walled phloem parenchyma cells [L] accompanied by medullary rays (tangential section [D]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows a few starch granules 6-10 µm in diameter, mostly simple but occasionally with 2 or 3 components, free [B] or included in parenchymatous cells [C].

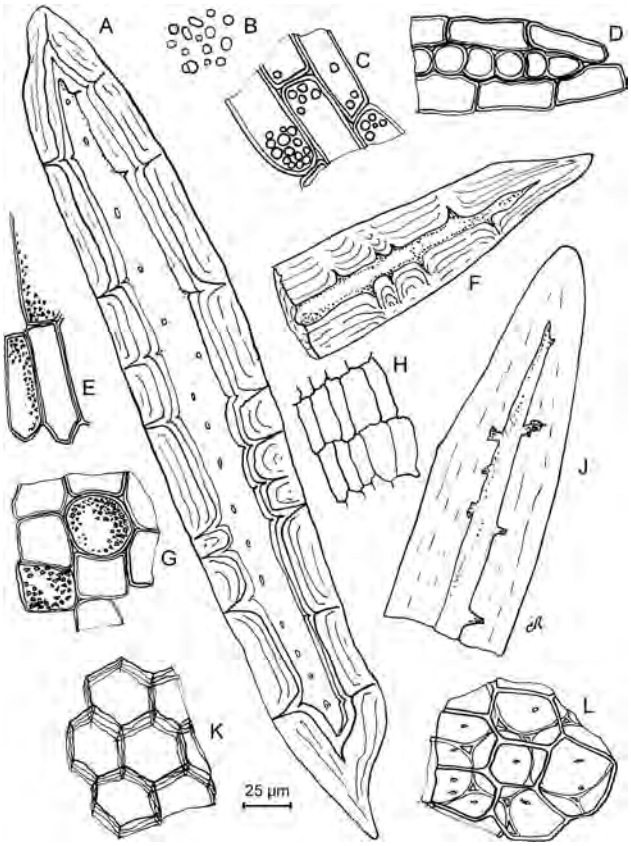


Figure 0174.-1. – *Illustration for identification test B of powdered herbal drug of cinchona bark*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.10 g of the powdered herbal drug (180) (*2.9.12*) in a test-tube add 0.1 mL of *concentrated ammonia R* and 5 mL of *methylene chloride R*. Shake vigorously occasionally during 30 min and filter. Evaporate the filtrate to dryness on a water-bath and dissolve the residue in 1 mL of *anhydrous ethanol R*.

*Reference solution*. Dissolve 17.5 mg of *quinine R*, 2.5 mg of *quinidine R*, 10 mg of *cinchonine R* and 10 mg of *cinchonidine R* in 5 mL of *anhydrous ethanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *diethylamine R*, *ethyl acetate R*, *toluene R* (10:20:70 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: twice over a path of 15 cm.

*Drying*: at 100-105 °C, then allow to cool.

*Detection A*: spray with *anhydrous formic acid R* and allow to dry in air; examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Quinidine: a distinct blue fluorescent zone | A distinct blue fluorescent zone (quinidine) |
| ——— | ——— |
| Quinine: a distinct blue fluorescent zone | A distinct blue fluorescent zone (quinine) |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *iodoplatinate reagent R*.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Cinchonine: a violet zone that becomes violet-grey | A violet zone that becomes violet-grey (cinchonine) |
| Quinidine: a violet zone that becomes violet-grey | A violet zone that becomes violet-grey (quinidine) |
| Cinchonidine: an intense dark blue zone | An intense dark blue zone (cinchonidine) |
| ——— | ——— |
| Quinine: a violet zone that becomes violet-grey | A violet zone that becomes violet-grey (quinine) |
| **Reference solution** | **Test solution** |

TESTS

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Loss on drying** (*2.2.32*): maximum 10 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

ASSAY

*Test solution*. In a 250 mL conical flask mix 1.000 g of the powdered herbal drug (180) (*2.9.12*) with 10 mL of *water R* and 7 mL of *dilute hydrochloric acid R*. Heat in a water-bath for 30 min, allow to cool and add 25 mL of *methylene chloride R*, 50 mL of *ether R* and 5 mL of a 200 g/L solution of *sodium hydroxide R*. Shake the mixture repeatedly for 30 min, add 3 g of powdered *tragacanth R* and shake until the mixture becomes clear. Filter through a plug of absorbent cotton and rinse the flask and the cotton with 5 quantities, each of 20 mL, of a mixture of 1 volume of *methylene chloride R* and 2 volumes of *ether R*. Combine the filtrate and washings, evaporate to dryness and dissolve the residue in 10.0 mL of *anhydrous ethanol R*. Evaporate 5.0 mL of this solution to dryness, dissolve the residue in *0.1 M hydrochloric acid* and dilute to 1000.0 mL with the same acid.

*Reference solutions*. Dissolve separately 30.0 mg of *quinine R* and 30.0 mg of *cinchonine R* in *0.1 M hydrochloric acid* and dilute each solution to 1000.0 mL with the same acid.

Measure the absorbances (*2.2.25*) of the 3 solutions at 316 nm and 348 nm using *0.1 M hydrochloric acid* as the compensation liquid.

Calculate the percentage content of alkaloids using the following equations:

$$x = \frac{[A_{316} \times A_{348c}] - [A_{316c} \times A_{348}]}{[A_{316q} \times A_{348c}] - [A_{316c} \times A_{348q}]} \times \frac{100}{m} \times \frac{2}{1000}$$

$$y = \frac{[A_{316} \times A_{348q}] - [A_{316q} \times A_{348}]}{[A_{316c} \times A_{348q}] - [A_{316q} \times A_{348c}]} \times \frac{100}{m} \times \frac{2}{1000}$$

$m$ = mass of the herbal drug used, in grams;

$x$ = percentage content of quinine-type alkaloids;

$y$ = percentage content of cinchonine-type alkaloids;

$A_{316}$ = absorbance of the test solution at 316 nm;

$A_{348}$ = absorbance of the test solution at 348 nm;

$A_{316c}$ = absorbance of the reference solution containing cinchonine at 316 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{316q}$ = absorbance of the reference solution containing quinine at 316 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{348c}$ = absorbance of the reference solution containing cinchonine at 348 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{348q}$ = absorbance of the reference solution containing quinine at 348 nm, corrected to a concentration of 1 mg/1000 mL.

Calculate the content of total alkaloids ($x + y$), and calculate the relative content of quinine-type alkaloids using the following expression:

$$\frac{100x}{x+y}$$

**01/2008:1818**

# CINCHONA LIQUID EXTRACT, STANDARDISED

## Cinchonae extractum fluidum normatum

DEFINITION

Liquid extract produced from *Cinchona bark (0174)*.

*Content*: minimum 4.0 per cent and maximum 5.0 per cent of total alkaloids, of which 30 per cent to 60 per cent are alkaloids of the quinine type ($C_{20}H_{24}N_2O_2$; $M_r$ 324.4).

PRODUCTION

Standardised cinchona liquid extract is produced from the herbal drug by an appropriate procedure using:

- ethanol (30 per cent *V/V* to 90 per cent *V/V*), or;
- a mixture of diluted hydrochloric acid, ethanol (96 per cent *V/V*), glycerol, water (1:2:5:20 *V/V*).

CHARACTERS

*Appearance*: brownish-red liquid.

It has a bitter, astringent taste.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1 mL of the extract to be examined in 1 mL of *anhydrous ethanol R*.

*Reference solution.* Dissolve 2.5 mg of *quinidine R*, 10 mg of *cinchonidine R*, 10 mg of *cinchonine R* and 17.5 mg of *quinine R* in 5 mL of *anhydrous ethanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *diethylamine R*, *ethyl acetate R*, *toluene R* (10:20:70 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: twice over a path of 15 cm [or 6 cm].

*Drying*: at 100-105 °C then allow to cool.

*Detection A*: spray with a 50 g/L solution of *anhydrous formic acid R* and allow to dry in air; examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Quinidine: a distinct blue fluorescent zone | A distinct blue fluorescent zone (quinidine) |
| ——— | ——— |
| Quinine: a distinct blue fluorescent zone | A distinct blue fluorescent zone (quinine) |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *iodoplatinate reagent R*.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Cinchonine: a violet-grey zone | A violet-grey zone (cinchonine) |
| Quinidine: a violet-grey zone | A violet-grey zone (quinidine) |
| Cinchonidine: an intense dark blue zone | An intense dark blue zone (cinchonidine) |
| ——— | ——— |
| Quinine: a violet-grey zone | A violet-grey zone (quinine) |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the content stated on the label.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

**Dry residue** (*2.8.16*): minimum 12.0 per cent for glycerol-free standardised cinchona liquid extract and minimum 30.0 per cent for glycerol-containing standardised cinchona extract, determined on 2.0 g.

ASSAY

*Test solution.* In a 250 mL conical flask, mix about 1.000 g of the extract to be examined with 10 mL of *water R* and 7 mL of *dilute hydrochloric acid R*. Heat in a water-bath for 30 min, allow to cool and add 25 mL of *methylene chloride R*, 50 mL of *ether R* and 5 mL of a 200 g/L solution of *sodium hydroxide R*. Shake the mixture frequently for 30 min, add 3 g of powdered *tragacanth R* and shake until the mixture becomes clear. Filter through a plug of absorbent cotton, rinse the flask and the cotton with 5 quantities, each of 20 mL, of a mixture of 1 volume of *methylene chloride R* and 2 volumes of *ether R*. Combine the filtrate and washings, evaporate to dryness and dissolve the residue in 10.0 mL of *ethanol (96 per cent) R*. Evaporate 5.0 mL of this solution to dryness, dissolve the residue in *0.1 M hydrochloric acid* and dilute to 1000.0 mL with the same acid.

*Reference solution (a).* Dissolve 30.0 mg of *cinchonine R* in *0.1 M hydrochloric acid* and dilute to 1000.0 mL with the same acid.

*Reference solution (b).* Dissolve 30.0 mg of *quinine R* in *0.1 M hydrochloric acid* and dilute to 1000.0 mL with the same acid.

Measure the absorbances (*2.2.25*) of the 3 solutions at 316 nm and 348 nm, using *0.1 M hydrochloric acid* as the compensation liquid.

Calculate the percentage content of alkaloids from the following equations:

$$n_1 = \frac{[A_1 \times A_{2a}] - [A_{1a} \times A_2]}{[A_{1b} \times A_{2a}] - [A_{1a} \times A_{2b}]} \times \frac{100}{m} \times \frac{2}{1000}$$

$$n_2 = \frac{[A_1 \times A_{2b}] - [A_{1b} \times A_2]}{[A_{1a} \times A_{2b}] - [A_{1b} \times A_{2a}]} \times \frac{100}{m} \times \frac{2}{1000}$$

$m$ = mass of the liquid extract to be examined in grams;

$n_1$ = percentage content of quinine-type alkaloids;

$n_2$ = percentage content of cinchonine-type alkaloids;

$A_1$ = absorbance of the test solution at 316 nm;

$A_2$ = absorbance of the test solution at 348 nm;

$A_{1a}$ = absorbance of reference solution (a) at 316 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{1b}$ = absorbance of reference solution (b) at 316 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{2a}$ = absorbance of reference solution (a) at 348 nm, corrected to a concentration of 1 mg/1000 mL;

$A_{2b}$ = absorbance of reference solution (b) at 348 nm, corrected to a concentration of 1 mg/1000 mL.

Calculate the content of total alkaloids ($n_1 + n_2$), and the relative content of quinine-type alkaloids, from the following expression:

$$\frac{n_1 \times 100}{n_1 + n_2}$$

LABELLING

The label states the solvent composition used for the production.

**04/2011:0387**

# CINNAMON

## Cinnamomi cortex

DEFINITION

Dried bark, freed from the outer cork and the underlying parenchyma, of the shoots grown on cut stock of *Cinnamomum verum* J.Presl.

*Content*: minimum 12 mL/kg of essential oil.

CHARACTERS

Characteristic, aromatic odour.

IDENTIFICATION

A. The bark is about 0.2-0.8 mm thick and occurs in closely packed compound quills made up of single or double quills. The outer surface is smooth, yellowish-brown with faint scars marking the position of leaves and axillary buds and has fine, whitish and wavy longitudinal striations. The inner surface is slightly darker and longitudinally striated. The fracture is short and fibrous.

B. Microscopic examination (*2.8.23*). The powder is yellowish or reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0387.-1): rounded sclereids with pitted, channelled and moderately thickened walls, single [E, F] or in groups [C]; numerous colourless, single fibres, often whole [A], or fragmented [D], with a narrow lumen, thickened, lignified walls and few pits; small acicular crystals of calcium oxalate in parenchymatous cells [J]; very numerous oil droplets [B]. Cork fragments [G] are absent or very rare. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant starch granules [H].

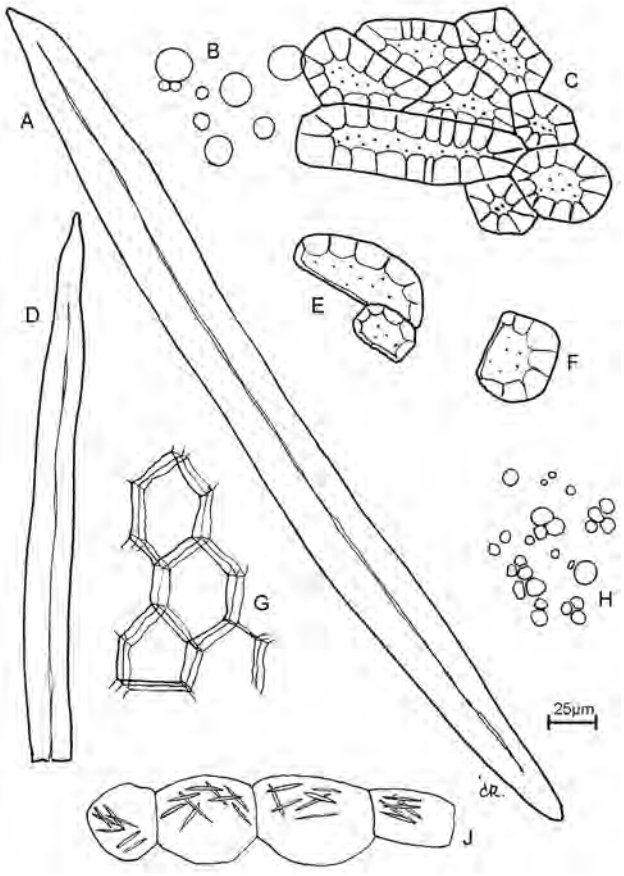


Figure 0387.-1. – *Illustration for identification test B of powdered herbal drug of cinnamon*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.1 g of the powdered herbal drug (500) (*2.9.12*) with 2 mL of *methylene chloride R* for 15 min. Filter and evaporate the filtrate carefully almost to dryness on a water-bath. Dissolve the residue in 0.4 mL of *toluene R*.

*Reference solution.* Dissolve 50 µL of *cinnamic aldehyde R* and 10 µL of *eugenol R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *methylene chloride R*.

*Application*: 10 µL as bands of 20 mm by 3 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm and mark the quenching zones, then examine in ultraviolet light at 365 nm and mark the fluorescent zones.

*Results A*: examined in ultraviolet light at 254 nm, the chromatograms obtained with the test solution and the reference solution show a quenching zone due to cinnamaldehyde in the median part and, just above it, a weaker quenching zone due to eugenol; examined in ultraviolet light at 365 nm, the chromatogram obtained with the test solution shows a fluorescent light blue zone due to *o*-methoxycinnamaldehyde just below the zone due to cinnamaldehyde.

*Detection B*: spray with *phloroglucinol solution R*.

*Results B*: the zone due to cinnamaldehyde is yellowish-brown and the zone due to *o*-methoxycinnamaldehyde is violet.

TESTS

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of drug reduced to a powder (710) (*2.9.12*) immediately before the determination, a 500 mL flask, 200 mL of *0.1 M hydrochloric acid* as the distillation liquid, and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2.5-3.5 mL/min for 3 h.

**04/2011:1501**
**corrected 10.0**

# CINNAMON BARK OIL, CEYLON

## Cinnamomi zeylanici corticis aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the bark of the shoots of *Cinnamomum verum* J.Presl.

CHARACTERS

*Appearance*: clear, mobile, light yellow liquid becoming reddish over time.

Characteristic odour reminiscent of cinnamic aldehyde.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1 mL of the essential oil to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 µL of *trans-cinnamic aldehyde R*, 10 µL of *eugenol R*, 10 µL of *linalol R* and 10 µL of *β-caryophyllene R* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *methanol R*, *toluene R* (10:90 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*; heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the zones in the chromatogram obtained with the test solution are similar in position and colour to those in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. Safrole, coumarin and cineole may be absent from the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 1.000 to 1.030.

**Refractive index** (*2.2.6*): 1.572 to 1.591.

**Optical rotation** (*2.2.7*): − 2° to + 1°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The essential oil to be examined.

*Reference solution.* Dissolve 10 µL of *cineole R*, 10 µL of *linalol R*, 10 µL of *β-caryophyllene R*, 10 µL of *safrole R*, 100 µL of *trans-cinnamic aldehyde R*, 10 µL of *eugenol R*, 20 mg of *coumarin R*, 10 µL of *trans-2-methoxycinnamaldehyde R* and 10 µL of *benzyl benzoate R* in 1 mL of *acetone R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 75 | 60 → 190 |
| | 75 - 200 | 190 |
| Injection port | | 200 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution; depending on the operating conditions and the state of the column, coumarin may elute before or after *trans*-2-methoxycinnamaldehyde; record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to linalol and β-caryophyllene.

*Identification of components*: using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *cineole*: maximum 3.0 per cent;
- *linalol*: 1.0 per cent to 6.0 per cent;
- *β-caryophyllene*: 1.0 per cent to 4.0 per cent;
- *safrole*: maximum 0.5 per cent;
- *trans-cinnamic aldehyde*: 55 per cent to 75 per cent;
- *eugenol*: maximum 7.5 per cent;
- *coumarin*: maximum 0.5 per cent;
- *trans-2-methoxycinnamaldehyde*: 0.1 per cent to 1.0 per cent;
- *benzyl benzoate*: maximum 1.0 per cent.

STORAGE

Protected from heat.

**01/2008:1608**
**corrected 7.0**

# CINNAMON LEAF OIL, CEYLON

## Cinnamomi zeylanici folii aetheroleum

DEFINITION

Oil obtained by steam distillation of the leaves of *Cinnamomum verum* J.S. Presl.

Herbal drugs

CHARACTERS

*Appearance*: clear, mobile, reddish-brown or dark brown liquid.

Characteristic odour reminiscent of eugenol.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1 mL of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dilute about 50 µL of *trans-cinnamic aldehyde R*, 10 µL of *eugenol R*, 10 µL of *linalol R* and 10 µL of *β-caryophyllene R* in *alcohol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *methanol R*, *toluene R* (10:90 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*. Examine in day light while heating at 100-105 °C for 5-10 min.

*Results*: the zones in the chromatogram obtained with the test solution are similar in position and colour to those in the chromatogram obtained with the reference solution. The zone due to *trans*-cinnamic aldehyde may be very faint or absent.

B. Examine the chromatogram obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. The peaks corresponding to cineole, safrole, *trans*-cinnamic aldehyde, cinnamyl acetate and coumarin may be absent in the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 1.030 to 1.059.

**Refractive index** (*2.2.6*): 1.527 to 1.540.

**Optical rotation** (*2.2.7*): − 2.5° to + 2.0°.

**Chromatographic profile.** Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution.* Dissolve 10 µL of *cineole R*, 10 µL of *linalol R*, 10 µL of *β-caryophyllene R*, 10 µL of *safrole R*, 10 µL of *trans-cinnamic aldehyde R*, 10 µL of *cinnamyl acetate R*, 100 µL of *eugenol R* and 10 mg of *coumarin R* in 1 mL of *acetone R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 60 m, Ø = 0.25 mm,
- *stationary phase*: *macrogol 20 000 R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1/100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 45 |
| | 10 - 78 | 45 → 180 |
| | 78 - 88 | 180 |
| Injection port | | 200 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: the order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum of 1.5 between the peaks due to linalol and β-caryophyllene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components.

The percentages are within the following ranges:

- *cineole*: maximum 1.0 per cent,
- *linalol*: 1.5 per cent to 3.5 per cent,
- *β-caryophyllene*: 1.5 per cent to 7.0 per cent,
- *safrole*: maximum 3.0 per cent,
- *trans-cinnamic aldehyde*: maximum 3.0 per cent,
- *cinnamyl acetate*: maximum 2.0 per cent,
- *eugenol*: 70 per cent to 85 per cent,
- *coumarin*: maximum 1.0 per cent.

STORAGE

Protected from heat.

**01/2008:1609**
**corrected 7.0**

# CITRONELLA OIL

## Citronellae aetheroleum

DEFINITION

Oil obtained by steam distillation from the fresh or partially dried aerial parts of *Cymbopogon winterianus* Jowitt.

CHARACTERS

*Appearance*: pale yellow or brown-yellow liquid.

Very strong odour of citronellal.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 0.1 g of citronella oil in 10.0 mL of *alcohol R.*

*Reference solution.* Dilute 20 µL of *citronellal R* in 10.0 mL of *alcohol R.*

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 5 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min. Examine in ultraviolet light at 365 nm.

*Result*: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Citronellal: a violet zone | A zone similar in colour to the citronellal zone<br>An orange zone (citronellol-geraniol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution. Neral and geranial may be absent in the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 0.881 to 0.895.

**Refractive index** (*2.2.6*): 1.463 to 1.475.

**Optical rotation** (*2.2.7*): – 4° to + 1.5°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution*. Dilute 25 µL of *limonene R*, 100 µL of *citronellal R*, 25 µL of *citronellyl acetate R*, 25 µL of *citral R*, 25 µL of *geranyl acetate R*, 25 µL of *citronellol R* and 100 µL of *geraniol R* in 5 mL of *hexane R*.

*Column*:

– *material*: fused silica,
– *size*: *l* = 60 m, Ø = 0.25 mm,
– *stationary phase*: *macrogol 20 000 R* (0.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 80 |
| | 2 - 26 | 80 → 150 |
| | 26 - 42 | 150 → 185 |
| | 42 - 49 | 185 → 250 |
| Injection port | | 260 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the reference solution, 0.2 µL of the test solution.

*Elution order*: the order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum of 1.2 between the peaks due to geranyl acetate and citronellol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components.

The percentages are within the following values:

– *limonene*: 1.0 per cent to 5.0 per cent,
– *citronellal*: 30.0 per cent to 45.0 per cent,
– *citronellyl acetate*: 2.0 per cent to 4.0 per cent,
– *neral*: maximum 2.0 per cent,
– *geranial*: maximum 2.0 per cent,
– *geranyl acetate*: 3.0 per cent to 8.0 per cent,
– *citronellol*: 9.0 per cent to 15.0 per cent,
– *geraniol*: 20.0 per cent to 25.0 per cent.

**01/2008:1850**
**corrected 7.0**

# CLARY SAGE OIL

## Salviae sclareae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh or dried flowering stems of *Salvia sclarea* L.

CHARACTERS

*Appearance*: colourless or brownish-yellow liquid, usually pale yellow.

Characteristic odour.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1 mL of the substance to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 60 µL of *linalol R*, 200 µL of *linalyl acetate R* and 60 µL of *α-terpineol R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 5 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R* and heat at 100-105 °C for 5-10 min; examine in daylight within 5 min.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| α-Terpineol: a dark violet zone | A dark violet zone |
| Linalyl acetate: a dark violet zone | A dark violet zone |
| Linalol: a dark violet zone | A dark violet zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the chromatogram obtained with the test solution shows 5 peaks similar in position to the 5 peaks in the chromatogram obtained with the reference solution. The 2 peaks corresponding to α- and β-thujone may be absent.

TESTS

**Relative density** (*2.2.5*): 0.890 to 0.908.

**Refractive index** (*2.2.6*): 1.456 to 1.466.

**Optical rotation** (*2.2.7*): – 26° to – 10°.

**Acid value** (*2.5.1*): maximum 1.0.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution.* To 1 g of *hexane R*, add 5 µL of *thujone R*, 5 µL of *linalol R*, 100 µL of *linalyl acetate R*, 10 µL of *α-terpineol R* and 25 mg (± 20 per cent) of *sclareol R*. Mix thoroughly by stirring.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 75 | 60 → 190 |
| | 75 - 120 | 190 |
| Injection port | | 220 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to linalol and linalyl acetate,

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution (disregard any peak due to hexane). *Thujone R* is a mixture of α- and β-thujone. α-Thujone elutes before β-thujone under the described conditions.

Determine the percentage content of each of these components.

Also determine the percentage content of germacrene-D. The germacrene-D peak can be identified in the chromatogram obtained with the test solution by its relative retention of 1.23 with reference to linalol under the described operating conditions.

The percentages are within the following ranges:

- *α- and β-thujone*: maximum 0.2 per cent,
- *linalol*: 6.5 per cent to 24 per cent,
- *linalyl acetate*: 56 per cent to 78 per cent,
- *α-terpineol*: maximum 5.0 per cent,
- *germacrene-D*: 1.0 per cent to 12 per cent,
- *sclareol*: 0.4 per cent to 2.6 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**01/2018:2463**

# CLEMATIS ARMANDII STEM

## Clematidis armandii caulis

DEFINITION

Whole or fragmented, dried stem of *Clematis armandii* Franch., with cork removed, collected in spring or autumn.

*Content*: minimum 0.30 per cent of oleanolic acid ($C_{30}H_{48}O_3$; $M_r$ 456.7) (dried drug).

IDENTIFICATION

A. The whole stem is long and cylindrical, slightly twisted on itself, about 1-6.5 cm in diameter. It shows nodes, usually swollen, with leaf and branch scars. The outer surface is brownish-yellow or dull brownish-yellow, showing longitudinal grooves and striations corresponding to the ends of the medullary rays. Rare cork remnants are easily removed as longitudinal strips. The texture is hard. The fracture is difficult.

The fragmented stem occurs in thick or thin slices, about 1-5 mm thick and 1.2-4.6 cm in diameter, with uneven margins; most of the transverse section consists of the pale yellow or slightly brownish-yellow wood and shows numerous radial striations and cracks corresponding to the medullary rays; the vessels are clearly visible in transverse section arranged in groups in more or less discontinuous concentric rings. Narrow, convex-shaped bark remnants are sometimes present with an enclosed lacuna between the bark and wood parts. The bark is easily removed. The pale yellow or whitish, rarely blackened, reduced pith, is sometimes replaced by a hollow.

B. Microscopic examination (*2.8.23*). The powder is whitish-yellow to brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2463.-1): very numerous fragments of pitted vessels [B, G] up to 250 µm in diameter, isolated or associated with elongated tracheids about 15-25 µm in diameter with lignified, thickened and pitted walls [Ba], some groups of vessels [F] show oblique striations [Fa], sometimes associated with xylem parenchyma cells with lignified and pitted walls [Fb]; groups of fusiform pericyclic fibres 25-30 µm in diameter, with thick, pitted walls [J]; thin-walled parenchymatous cells of the phloem and of outer parts of the medullary rays [E]; parenchymatous cells of the secondary xylem and the inner parts of the medullary rays and pith, with slightly thickened, pitted and lignified walls [H]; sub-rectangular or fusiform sclereids, about 100 µm long and 35 µm wide, with thick and pitted walls [C]; rare fragments of orange-brown cork covered by a cuticle showing striations [A]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rare starch granules, simple

Herbal drugs

or 2-3 compound, spherical or ovate; individual granules up to 17 µm in diameter, with a punctiform or slit-shaped hilum [D].

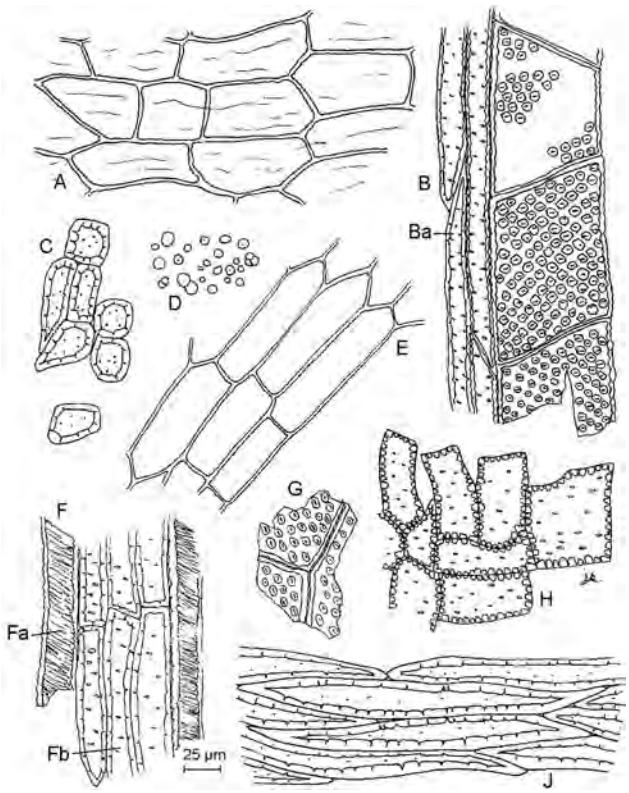


Figure 2463.-1. – *Illustration for identification test B of powdered herbal drug of Clematis armandii stem*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (1400) (*2.9.12*) add 5 mL of *methanol R* and heat on a water-bath at 60 °C for 5 min. Filter.

*Reference solution.* Dissolve 4 mg of *hederagenin R* and 4 mg of *oleanolic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *acetone R*, *toluene R* (2:8:32 *V/V/V*).

*Application*: 40 µL [or 10 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 13 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *vanillin reagent R*, heat at 100 °C for 5 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other mainly grey zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A bluish-violet zone |
| Oleanolic acid: a reddish-violet zone | A reddish-violet zone |
| | A weak light blue or grey zone |
| _____ | _____ |
| Hederagenin: a greenish-brown zone | |
| | An orange zone |
| **Reference solution** | **Test solution** |

TESTS

***Aristolochia manshuriensis* Kom. and other species of *Aristolochia*.** Examine the powdered herbal drug (355) (*2.9.12*) under a microscope using *chloral hydrate solution R*; no cluster crystals are visible.

**Aristolochic acids** (*2.8.21, Method A*). It complies with the test.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (1400) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 100 mL flask, disperse 1.00 g of the powdered herbal drug (355) (*2.9.12*) in *methanol R*, add 3 mL of *6 M hydrochloric acid R* and dilute to 30 mL with *methanol R*. Shake for 2 h. Filter, add to the filtrate 10 mL of *water R* by rinsing the flask and the filter, and extract with 3 quantities, each of 30 mL, of *methylene chloride R*. Combine the methylene chloride extracts and evaporate to dryness. Dissolve the residue in 10.0 mL of *methanol R*, shake and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *oleanolic acid CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *ursolic acid R* in reference solution (a) and dilute to 10.0 mL with the same solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: 0.4 per cent *V/V* solution of *acetic acid R*, *methanol R* (15:85 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 1.2 times the retention time of ursolic acid.

*Retention time*: oleanolic acid = about 21 min; ursolic acid = about 22 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.3 between the peaks due to oleanolic acid and ursolic acid.

Calculate the percentage content of oleanolic acid using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to oleanolic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to oleanolic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *oleanolic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of oleanolic acid in *oleanolic acid CRS*.

**01/2008:0376**
**corrected 7.6**

# CLOVE

## Caryophylli flos

DEFINITION

Whole flower buds of *Syzygium aromaticum* (L.) Merr. et L.M.Perry (syn. *Eugenia caryophyllus* (Spreng.) Bullock et S.G.Harrison) dried until they become reddish-brown.

*Content*: minimum 150 mL/kg of essential oil.

CHARACTERS

Characteristic, aromatic odour.

IDENTIFICATION

A. The flower bud is reddish-brown and consists of a quadrangular stalked portion, the hypanthium, 10-12 mm long and 2-3 mm in diameter, surmounted by 4 divergent lobes of sepals which surround a globular head 4-6 mm in diameter. A bilocular ovary containing numerous ovules is situated in the upper part of the hypanthium. The head is globular and dome-shaped, composed of 4 imbricated petals that enclose numerous incurved stamens and a short, erect style with a nectary disc at the base. The hypanthium exudes essential oil when indented with the finger-nail.

B. Microscopic examination (*2.8.23*). The powder is dark brown and has the odour and taste of the unground drug. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the hypanthium showing the epidermis and underlying parenchyma containing large oil glands; short fibres occurring singly or in small groups, with thickened, lignified walls and few pits; abundant fragments of parenchyma containing cluster crystals of calcium oxalate; numerous triangular pollen grains about 15 µm in diameter with 3 pores in the angles. Starch granules are absent.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.1 g of the powdered herbal drug (500) (*2.9.12*) with 2 mL of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 2 mL of *toluene R*.

*Reference solution.* Dissolve 20 µL of *eugenol R* in 2 mL of *toluene R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *toluene R*.

*Application*: 10 µL of the reference solution and 20 µL of the test solution, as bands of 20 mm by 3 mm.

*Development*: twice, in an unsaturated tank over a path of 10 cm; allow the plate to stand for 5 min between the 2 developments.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm and mark the quenching zones.

*Results A*: in the chromatogram obtained with the test solution there is in the median part a quenching zone due to eugenol similar in position to the quenching zone in the chromatogram obtained with the reference solution and there may be a weak quenching zone due to acetyleugenol just below the zone due to eugenol.

*Detection B*: spray with *anisaldehyde solution R* using 10 mL for a plate 200 mm square and heat at 100-105 °C for 5-10 min. Examine in daylight.

*Results B*: the zones due to eugenol in the chromatograms obtained with the test and reference solutions are strong brownish-violet and the zone due to acetyleugenol in the chromatogram obtained with the test solution is faint violet-blue. In the chromatogram obtained with the test solution there are other coloured zones, particularly a faint red zone in the lower part and a reddish-violet zone due to caryophyllene in the upper part.

TESTS

**Foreign matter** (*2.8.2*): maximum 6 per cent of peduncles, petioles and fruits, maximum 2 per cent of deteriorated cloves and maximum 0.5 per cent of other foreign matter.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use a 250 mL flask, 100 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Grind 5.0 g of the drug with 5.0 g of *diatomaceous earth R* to form a fine, homogeneous powder and proceed immediately with the determination using 4.0 g of the mixture. Distil at a rate of 2.5-3.5 mL/min for 2 h.

**01/2008:1091**
**corrected 7.6**

# CLOVE OIL

## Caryophylli floris aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the dried flower buds of *Syzygium aromaticum* (L.) Merr. et L.M.Perry (syn. *Eugenia caryophyllus* (Spreng.) Bullock et S.G.Harrison).

CHARACTERS

*Appearance*: clear, yellow liquid, which becomes brown when exposed to air.

*Solubility*: miscible with methylene chloride, with toluene and with fatty oils.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 µL of the substance to be examined in 2.0 mL of *toluene R*.

*Reference solution.* Dissolve 15 µL of *eugenol R* and 15 µL of *acetyleugenol R* in 2.0 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *toluene R*.

*Application*: 20 µL of the test solution and 15 µL of the reference solution, as bands.

*Development*: twice in an unsaturated tank over a path of 10 cm; allow to stand for 5 min between the 2 developments.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm and mark the quenching zones.

*Results A*: the chromatogram obtained with the test solution shows in the middle part a quenching zone (eugenol) that is similar in position to the quenching zone in the chromatogram obtained with the reference solution; just below, there is a weak quenching zone (acetyleugenol) that is similar in position to the zone of acetyleugenol in the chromatogram obtained with the reference solution.

*Detection B*: spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

*Results B*: the zone due to eugenol in the chromatograms obtained with the test and reference solutions is strong brownish-violet and the zone due to acetyleugenol in the chromatogram obtained with the test solution is faint violet-blue; in the chromatogram obtained with the test solution there are other coloured zones, particularly a faint red zone in the lower part and a reddish-violet zone (β-caryophyllene) in the upper part.

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the 3 principal peaks in chromatogram obtained with the test solution are similar in retention time to the 3 principal peaks in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 1.030 to 1.063.

**Refractive index** (*2.2.6*): 1.528 to 1.537.

**Optical rotation** (*2.2.7*): – 2° to 0°.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test.

**Solubility in alcohol** (*2.8.10*). 1.0 mL is soluble in 2.0 mL and more of *ethanol (70 per cent V/V) R*.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.2 g of the substance to be examined in 10 g of *hexane R*.

*Reference solution*. Dissolve 7 mg of *β-caryophyllene R*, 80 mg of *eugenol R* and 4 mg of *acetyleugenol R* in 10 g of *hexane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = about 0.25 mm;
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 8 | 60 |
| | 8 - 48 | 60 → 180 |
| | 48 - 53 | 180 |
| Injection port | | 270 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to eugenol and acetyleugenol;
- *number of theoretical plates*: minimum 30 000, calculated for the peak due to β-caryophyllene at 110 °C.

*Identification of components*: using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution on the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The limits are within the following ranges:

- *β-caryophyllene*: 5.0 per cent to 14.0 per cent;
- *eugenol*: 75.0 per cent to 88.0 per cent;
- *acetyleugenol*: 4.0 per cent to 15.0 per cent.

STORAGE

Protected from heat.

**01/2017:2714**
**corrected 9.3**

# CODONOPSIS ROOT

## Codonopsidis radix

DEFINITION

Whole or fragmented, dried root of *Codonopsis pilosula* (Franch.) Nannf., collected in autumn.

IDENTIFICATION

A. The whole root is cylindrical, slightly curved and may be up to 35 cm long and 2 cm in diameter, whereas the fragmented root occurs in pieces about 1-2 cm long. The root crown, which is broader than the root, forms an irregular head with a convex centre and consisting of buds and numerous, prominent, rounded verrucose stem scars. The root shows numerous transverse annulations below the root crown, occurring further and further apart until half the length of the root is reached. These transverse annulations may be absent in the cultivated drug. The root shows deep longitudinal wrinkles and prominent scattered protuberances, resembling lenticels, over its entire length; the fracture of the secondary roots is sometimes covered by a blackish gelatinous secretion. The outer surface is brownish-yellow or brownish-grey. The bark adheres tenaciously to the wood and is difficult to remove. The texture is compact and the fracture is relatively short.

B. Microscopic examination (*2.8.23*). The powder is pale yellow or light brown. Examine under a microscope using *lactic reagent R*. The powder shows the following diagnostic characters (Figure 2714.-1): cork fragments (surface view [F]), consisting of numerous layers of rectangular or polyhedral cells; very numerous fragments of parenchyma [A, H, J] containing ramified laticiferous vessels (longitudinal section [Aa], transverse section [Ha]), with brown granular contents; numerous rectangular sclereids, with distinctly thickened walls and conspicuous channels, isolated [C] or in groups [B]; granular secretions of the laticiferous vessels, as more or less coiled strands [E]; reticulate or scalariform vessels about 100 µm in diameter [D, G]; violet-blue rounded or ovoid starch granules, simple, with a diameter of about 2-10 µm, or 2- to 3-compound, either free or included in colourless

parenchyma cells [Jb]; colourless pieces of inulin in parenchyma cells [Ja], either fan-shaped or as angular, polyhedral fragments.

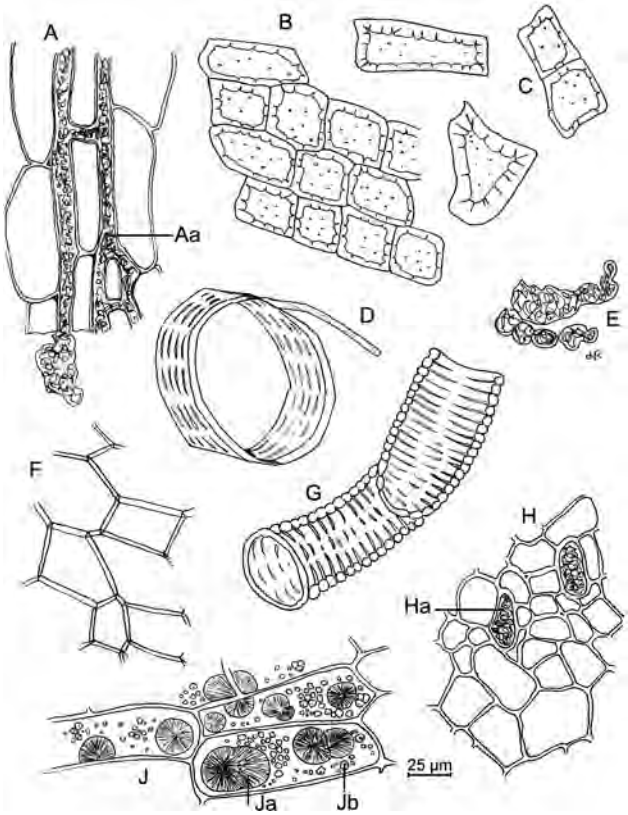


Figure 2714.-1. – *Illustration for identification test B of powdered herbal drug of codonopsis root*

C. Examine the chromatograms obtained in the test for *Platycodon grandiflorus*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A brownish zone |
| _____ | _____ |
| Xylose: a yellowish-brown zone | A weak violet zone |
| _____ | A yellowish-brown zone _____ |
| Glucose: a yellowish-brown zone | |
| **Reference solution** | **Test solution** |

TESTS

***Platycodon grandiflorus***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.500 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *ethanol (70 per cent V/V) R*. Sonicate for 10 min and centrifuge or filter. Evaporate the supernatant or filtrate to dryness under reduced pressure. Dissolve the residue in 1.0 mL of *water R*. Prepare a ready-to-use sample preparation cartridge containing 50 mg of octadecylsilyl silica gel (55 µm) using 3 mL of *methanol R* followed by 3 mL of *water R*. Apply 1.0 mL of the solution to be analysed to the top of the cartridge. Elute the cartridge with 3 mL of *water R*; collect the eluate. Under reduced pressure, evaporate the eluate to dryness. Dissolve the residue in 1 mL of *methanol R*.

*Reference solution*. Dissolve 1 mg of *glucose R* and 1 mg of *xylose R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *glacial acetic acid R*, *methylene chloride R* (2:3:8:15 *V/V/V/V*).

*Application*: 10 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 3 min; examine in daylight.

*Results*: in the chromatogram obtained with the test solution, the presence of a yellowish-brown zone at, directly above, or directly below the zone due to glucose in the chromatogram obtained with the reference solution indicates adulteration with *Platycodon grandiflorus*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.5 per cent.

**Extractable matter**: minimum 21.0 per cent.

To 4.00 g of the powdered herbal drug (710) (*2.9.12*) add 100 g of *ethanol (70 per cent V/V) R* and allow to macerate for 6 h, shaking frequently. Allow to stand for 18 h. Filter, evaporate 20 g of the filtrate to dryness on a water-bath and dry the residue in an oven at 105 °C for 3 h. The residue weighs a minimum of 0.168 g.

**07/2017:2454**

# COIX SEED

## Coicis semen

DEFINITION

Dried, ripe, caryopsis, freed from the shell, of *Coix lacryma-jobi* L. subsp. *ma-yuen* (Rom. Caill.) T.Koyama.

*Content*: minimum 0.50 per cent of triolein ($C_{57}H_{104}O_6$; $M_r$ 885) (dried drug).

IDENTIFICATION

A. The white or pale yellow caryopsis freed from the shell is roughly ovoid or elongated-elliptical, about 4-8 mm long and 3-6 mm wide. The dorsal surface is rounded, milky white and smooth; the ventral surface shows a deep longitudinal furrow; yellowish-brown remnants of the membranous floral parts may be present. One end is obtusely rounded, the other end is relatively flat and slightly dented with an indistinct, pale brown hilum.

B. Microscopic examination (*2.8.23*). The powder is light grey or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2454.-1): fragments of the endosperm with oval to elongated [B] or rounded [E] thin-walled cells and fragments consisting of elongated cells with thicker walls [F]; fragments of tissue, with light yellow walls, with large, elongated, slightly sinuous cells [A]; cells of the middle layer of the mesocarp are brownish-yellow, irregularly tube-like, slightly curved and irregularly crossed [D]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules, simple or 2-3 compound, spherical or slightly polyhedral, 3-20 µm in diameter, with

a stellate, Y-shaped, cleft-like or point-like hilum [C] and fragments of parenchyma containing starch granules [G].

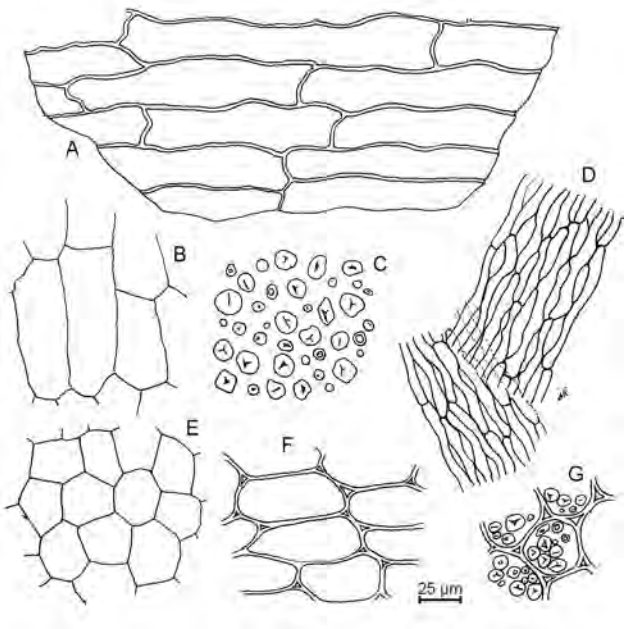


Figure 2454.-1. – *Illustration for identification test B of powdered herbal drug of coix seed*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *light petroleum R1* and sonicate for 30 min. Filter and reduce *in vacuo* to 1 mL.

*Reference solution*. Dissolve 2 mg of *oleic acid R* and 2 mg of *triolein R* in *methanol R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R* (5-40 µm) [or *TLC octadecylsilyl silica gel plate R* (2-10 µm)].

*Mobile phase*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (20:40:50 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: treat with a 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*, heat at 120 °C for about 3 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A purple zone |
| Oleic acid: a purple zone | A purple zone (oleic acid) |
| _____ | _____ |
| | A faint purple zone |
| | A purple zone |
| | A purple zone |
| _____ | _____ |
| Triolein: a purple zone | A purple zone (triolein) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 0.600 g of the powdered herbal drug (355) (*2.9.12*) add 50 mL of the mobile phase and stir with a magnetic stirrer for 2 h. Sonicate for 30 min. Allow to cool, dilute to 50.0 mL with the mobile phase and filter.

*Reference solution (a)*. Dissolve 10.0 mg of *triolein CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. To 0.600 g of *coix seed HRS* add 50 mL of the mobile phase and stir with a magnetic stirrer for 2 h. Sonicate for 30 min. Allow to cool, dilute to 50.0 mL with the mobile phase and filter.

*Reference solutions (c), (d), (e), (f), (g), (h)*. Dilute reference solution (a) to obtain 6 reference solutions of triolein, the concentrations of which span the expected value in the test solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methylene chloride R*, *acetonitrile R* (35:65 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion for signal-to-noise ratio:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 0.8 mL/min;
- *evaporator temperature*: 100 °C.

*Injection*: 10 µL.

*Run time*: 30 min.

*Retention time*: triolein = about 18 min; peak 2 = about 19 min.

*System suitability*:

- *resolution*: minimum 1.5 between the peak due to triolein and peak 2 in the chromatogram obtained with reference solution (b); use the chromatogram supplied with *coix seed HRS* to identify peak 2;
- *signal-to-noise ratio*: minimum 30 for the peak due to triolein in the chromatogram obtained with reference solution (a).

Establish a calibration curve with the logarithm of the mass of triolein (in milligrams) per 50 mL of reference solutions (c), (d), (e), (f), (g) and (h) (corrected by the assigned percentage content of *triolein CRS*) as the abscissa and the logarithm of the corresponding peak area as the ordinate.

Calculate the percentage content of triolein using the following expression:

$$\frac{10^{A}}{m \times 10}$$

$A$ = logarithm of the mass of triolein in the test solution, determined from the calibration curve and the area of the corresponding peak in the chromatogram obtained with the test solution;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams.

**01/2008:1504 corrected 6.0**

# COLA

## Colae semen

DEFINITION

Whole or fragmented dried seeds, freed from the testa, of *Cola nitida* (Vent.) Schott et Endl. (*C. vera* K. Schum.) and its varieties, as well as of *Cola acuminata* (P. Beauv.) Schott et Endl. (*Sterculia acuminata* P. Beauv.).

*Content*: minimum 1.5 per cent of caffeine ($M_r$ 194.2) (dried drug).

IDENTIFICATION

A. The kernels have an oblong, somewhat obtuse, sub-tetragonal shape, with deformations resulting from mutual pressure inside the fruit; they vary in size and mass, ranging from 5-15 g; the outside is hard, smooth and very dark brown, the inside is more reddish-brown. In *C. nitida* and its varieties, the kernels are divided in 2 parts, almost plano-convex, corresponding to the cotyledons and usually occurring separated in the commercial drug; the cotyledons are 3-4 cm long, 2-2.5 cm wide and 1-2 cm thick. In *C. acuminata*, the cotyledons are smaller and divided into 4-6 irregular parts.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows the following diagnostic characters: numerous ovoid or reniform starch granules, 5-25 µm in size, with concentric striations and a stellate, slightly eccentric hilum; fragments of cotyledon tissue showing large, thick-walled, reddish polygonal cells filled with starch granules; occasional fragments of the external epidermis of the cotyledons.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *ethanol (60 per cent V/V) R*. Shake mechanically at 40 °C for 30 min and filter.

*Reference solution (a).* Dissolve 25 mg of *caffeine R* in 10 mL of *ethanol (60 per cent V/V) R*.

*Reference solution (b).* Dissolve 50 mg of *theobromine R* in 10 mL of the mobile phase. Filter.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (10:13:77 *V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 5 min.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution shows 2 principal quenching zones which are similar in position to the zones in the chromatograms obtained with reference solutions (a) and (b).

*Detection B*: spray with a mixture of equal volumes of *ethanol (96 per cent) R* and *hydrochloric acid R* and then with a solution prepared immediately before use by dissolving 1 g of *iodine R* and 1 g of *potassium iodide R* in 100 mL of *ethanol (96 per cent) R*.

*Results B*: the chromatogram obtained with the test solution shows a reddish-brown principal zone similar in position and colour to the zone in the chromatogram obtained with reference solution (a).

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 2.00 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.00 g ($m_1$) of the powdered herbal drug (355) (*2.9.12*), add 50 mL of *methanol R*. Heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter. Rinse the filter with 10 mL of *methanol R*. Take up the residue with 50 mL of *methanol R*. Proceed as before. Combine the filtrates and the washings in a 200.0 mL volumetric flask and dilute to 200.0 mL with *methanol R*. Transfer 20.0 mL of this solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with the mobile phase, transfer to a 50.0 mL volumetric flask and dilute to 50.0 mL with the mobile phase.

*Reference solution.* In a 100.0 mL volumetric flask, dissolve 30.0 mg ($m_2$) of *caffeine CRS* and 15.0 mg of *theobromine R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Transfer 10.0 mL of this solution to a 100.0 mL volumetric flask and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water R* (25:75 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: the chosen volume of each solution; loop injector.

*System suitability*: reference solution:

- *resolution*: minimum 2.5 between the peaks due to caffeine and theobromine. If necessary, adjust the volume of *water R* in the mobile phase.

Calculate the caffeine content using the following expression:

$$\frac{m_2 \times A_1 \times 50}{m_1 \times A_2}$$

$A_1$ = area of the peak due to caffeine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to caffeine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined in the test solution, in grams;

$m_2$ = mass of *caffeine CRS* in the reference solution, in grams.

**01/2008:1862**

# COLOPHONY

## Colophonium

DEFINITION

Residue remaining after distillation of the volatile oil from the oleoresin obtained from various species of *Pinus*.

IDENTIFICATION

A. Translucent, pale yellow to brownish-yellow, angular, irregularly-shaped, brittle, glassy pieces of different sizes the surfaces of which bear conchoidal markings.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1 g in 10 mL of *methanol R* by gently warming.

*Reference solution*. Dissolve 10 mg of *thymol R* and 10 mg of *linalol R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*.

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min; examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other coloured zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A purple band |
| | A purple band |
| ——— | ——— |
| | 2 purple bands |
| Thymol: an orange band | |
| ——— | ——— |
| Linalol: a purple band | Sequence of narrow purple bands |
| | Purple extended baseline band |
| **Reference solution** | **Test solution** |

TESTS

**Acid value** (*2.5.1*): 145 to 180, determined on 1.0 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent.

STORAGE

Do not reduce to a powder.

**01/2017:2439**

# COMMON SELFHEAL FRUIT-SPIKE

## Prunellae spica

DEFINITION

Dried fruit-spike of *Prunella vulgaris* L.

*Content*: minimum 0.12 per cent of the sum of oleanolic acid ($C_{30}H_{48}O_3$; $M_r$ 456.7) and ursolic acid ($C_{30}H_{48}O_3$; $M_r$ 456.7), expressed as ursolic acid, of which not less than 70.0 per cent consists of ursolic acid (dried drug).

IDENTIFICATION

A. Cylindrical, somewhat flattened, 1.5-8 cm long, 0.8-1.5 cm in diameter, accompanied by remains of the stem up to 15 cm long, pale brown or brownish-red. The whole spike is composed of up to 10 or more whorls of persistent calyices and bracts, each whorl with 2 opposite bracts, fan-shaped, apex acuminate, striations of vein distinct, the outer surface with white hairs. Each bract is accompanied by 3 flowers, each with a persistent bilabiate calyx but often with the corolla missing, and by 4 small brown ovoid nutlets, each white and convex at the acute end. The calyx is closed at the fruiting stage.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown or brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2439.-1): very numerous covering trichomes, multicellular, scattered or on the epidermises, usually broken [E, Da, Dc], sometimes exceeding 1 mm long and 125 µm wide at the base, with spiny walls, upper cell usually short and acuminate, fine needle-shaped crystals may be visible in the cells [Ea, Db]; fragments of the bracts (surface view [A, C]), with lobed cells on the upper surface [Aa] and slightly sinuous cells covered by a thin, striated cuticle on the lower surface [Ca], with trichomes, mostly unicellular [Cb], sometimes bi- [Cc] or tricellular, conical, acuminate, short and serrate; diacytic stomata (*2.8.3*) usually accompanied by 2 subsidiary cells very unequal in size [Ab, Cd] and rare glandular trichomes with a unicellular stalk and a bicellular head [Ac, G] or a bicellular stalk and a unicellular head [B]; fragments of the bracts [D] and/or calyx margins with numerous serrate trichomes pointing in the same direction [Dd]; fragments of the calyx (surface view [L]), composed of lobed cells strongly thickened and deeply grooved; fragments of reticulate or bordered-pitted vessels from the stems; rare fragments of the nutlets (surface view [H], transverse section [J]) consisting of the epicarp [Ja], 3 layers of mesocarp, 2 of which have strongly thickened walls [Jb, Jc, Ha] and are positioned on either side of the middle layer whose cells contain microcrystals of calcium oxalate [Jd], and the endocarp [Je, Hb] consisting of cells with finely striated walls; fragments of the seed (surface view [K]) consisting of testa cells with finely reticulate walls [Ka], associated with cells of the endosperm [Kb] with oily contents; very numerous oil droplets [Kc]; rare fragments of the cauline leaves [F] may be present, with an epidermis covered with a striated cuticle [Fa] bearing glandular trichomes of lamiaceous type with 4 secretory cells [Fb] and diacytic stomata [Fc].

Herbal drugs

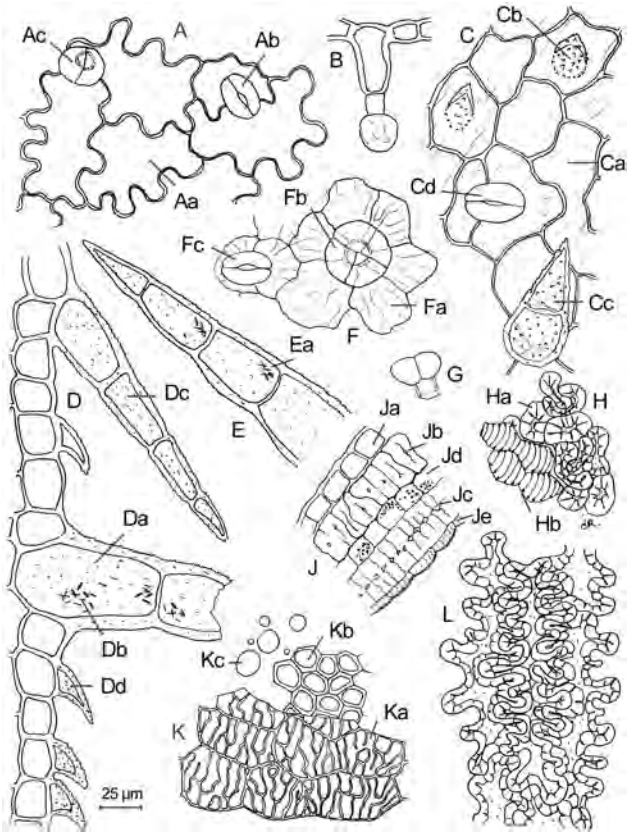


Figure 2439.-1. – *Illustration for identification test B of powdered herbal drug of common selfheal fruit-spike*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*, sonicate for 10 min and centrifuge; use the supernatant.

*Reference solution.* Dissolve 1 mg of *β-sitosterol R* and 1 mg of *ursolic acid R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (1:16:40 *V/V/V*).

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *anhydrous ethanol R* and heat at 100 °C for 3 min; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A pale violet fluorescent zone |
| _____ | _____ |
| | 2 faint yellow fluorescent zones |
| β-sitosterol: a violet fluorescent zone | |
| Ursolic acid: a yellowish-orange fluorescent zone | A yellowish-orange fluorescent zone (ursolic acid) |
| _____ | _____ |
| | 2 faint green fluorescent zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems longer than 15 cm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *1,1-dimethylethyl methyl ether R* (20:80 *V/V*).

*Test solution.* Disperse 2.000 g of the powdered herbal drug (355) (*2.9.12*) in 20 mL of the solvent mixture, heat under reflux at 80 °C for 30 min and filter. Repeat the extraction twice. Combine the filtrates and dilute to 100.0 mL with the solvent mixture. Evaporate 50.0 mL of this solution to dryness at 40 °C. Dissolve the residue in 1.0 mL of *1,1-dimethylethyl methyl ether R*. Rinse the flask 4 times with 1.0 mL of *1,1-dimethylethyl methyl ether R*. Pre-condition a 3 mL solid phase extraction column, containing 500 mg of *aminopropylsilyl silica gel for chromatography R1*, using 2 mL of *methanol R* followed by 2 mL of *1,1-dimethylethyl methyl ether R*. Subsequently apply the solution and the washings to the pre-conditioned column. Wash the column with 1.0 mL of *1,1-dimethylethyl methyl ether R* followed by 5 quantities, each of 1.0 mL, of *methanol R*. Apply 1.0 mL of a 2 per cent *V/V* solution of *anhydrous formic acid R* in *methanol R* and elute after 5 min. Repeat the elution 3 times and dilute the eluates to 5.0 mL with a 2 per cent *V/V* solution of *anhydrous formic acid R* in *methanol R*.

*Solution A*. Dissolve 10.0 mg of *ursolic acid CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of solution A to 10.0 mL with a 2 per cent *V/V* solution of *anhydrous formic acid R* in *methanol R*.

*Reference solution (b).* Dissolve 10.0 mg of *oleanolic acid R* in *methanol R* and dilute to 10.0 mL with the same solvent. Mix 1.0 mL of the solution and 1.0 mL of solution A and dilute to 10.0 mL with a 2 per cent *V/V* solution of *anhydrous formic acid R* in *methanol R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of a 4.6 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 6.0 with *strong sodium hydroxide solution R*, 35 volumes of *methanol R1* and 40 volumes of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Run time*: 1.1 times the retention time of ursolic acid.

*Elution order*: oleanolic acid, ursolic acid.

*Relative retention* with reference to ursolic acid (retention time = about 28 min): oleanolic acid = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to oleanolic acid and ursolic acid.

Calculate the percentage contents of ursolic acid and oleanolic acid, expressed as ursolic acid, using the following equations:

$$n_1 = \frac{A_1 \times m_2 \times p \times 0.1}{A_2 \times m_1}$$

$$n_2 = \frac{A_3 \times m_2 \times p \times 0.1}{A_2 \times m_1}$$

$n_1$ = percentage content of ursolic acid;

$n_2$ = percentage content of oleanolic acid;

$A_1$ = area of the peak due to ursolic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ursolic acid in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to oleanolic acid in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ursolic acid CRS* used to prepare solution A, in grams;

$p$ = assigned percentage content of ursolic acid in *ursolic acid CRS*.

Calculate the sum of the percentage contents of ursolic acid and oleanolic acid ($n_1 + n_2$) and the relative content of ursolic acid using the following expression:

$$\frac{n_1 \times 100}{(n_1 + n_2)}$$

**07/2014:1304**

# CORIANDER

## Coriandri fructus

DEFINITION

Dried cremocarp of *Coriandrum sativum* L.

*Content*: minimum 3 mL/kg of essential oil (dried drug).

IDENTIFICATION

A. The fruit is brown or light brown, more or less spherical, about 1.5-5 mm in diameter, or oval and 2-6 mm long. It consists of the entire cremocarp, with the mericarps usually tightly connected. The fruit is glabrous and has 10 wavy, slightly raised primary ridges and 8 straight, more prominent secondary ridges. The mericarps are concave on the internal surface. The stylopod crowns the apex and a small fragment of the pedicel may be present.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1304.-1): numerous oil droplets [B]; fragments of endosperm [A] with small, thick-walled, regular cells containing microrosettes [Aa] and microcrystals of calcium oxalate and oil droplets [Ab]; fragments of endocarp (surface view [C, J], transverse section [H]), with very narrow cells having a parquetry arrangement [Ca, Ha] and usually associated with a layer of thin-walled [Cb, Hb] or thicker-walled [Ja] rectangular sclereids of the mesocarp; fragments from the sclerenchymatous layer of the mesocarp [G] with short, strongly thickened, pitted, fusiform cells occurring in layers with the cells of adjacent layers approximately at right angles to one another; fragments of parenchyma of the mesocarp (transverse section [E]) with small cells with slightly thickened walls [Ea], the remains of secretory canals [Eb] and sclereids [Ec]; fragments of epicarp (surface view [F]) with thin-walled polyhedral cells, some of which contain small prisms of calcium oxalate [Fa]; rare fragments of secretory canals with brown cells, (surface view [D]); occasional fragments of vascular bundles [K].

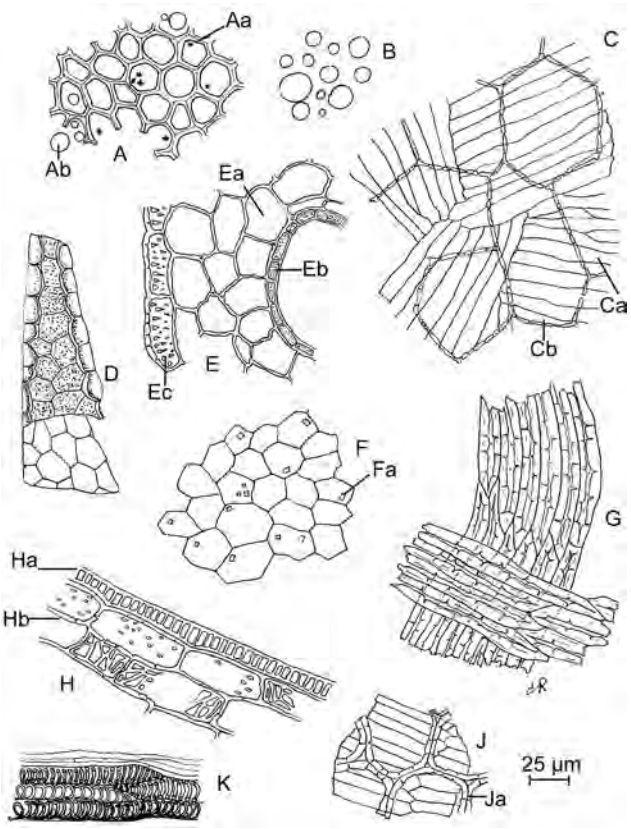


Figure 1304.-1. – *Illustration for identification test B of powdered herbal drug of coriander*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the freshly powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min and centrifuge or filter; use the supernatant or filtrate.

*Reference solution.* Dissolve 10 µL of *linalol R* and 2 µL of *geranyl acetate R* in 1.0 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air for 5 min.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

Herbal drugs

| Top of the plate | |
|---|---|
| | A bluish-violet zone |
| Geranyl acetate: a violet-blue zone | |
| Linalol: an intense violet zone | A violet zone (linalol) |
| | A violet-blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*). It complies with the test. None of the cremocarps show perforations due to insects.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Reduce the herbal drug to a coarse powder and immediately use 30.0 g for the determination. Distil at a rate of 2-3 mL/min for 2 h.

**07/2014:1820**

# CORIANDER OIL

## Coriandri aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fruits of *Coriandrum sativum* L.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Examine by thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 µL of the substance to be examined in 1.0 mL of *toluene R*.

*Reference solution.* Dissolve 10 µL of *linalol R* and 2 µL of *geranyl acetate R* in 1.0 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air for 5 min.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A bluish-violet zone |
| Geranyl acetate: a violet-blue zone | |
| Linalol: an intense violet zone | An intense violet zone (linalol) |
| | A violet-blue zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.860 to 0.880.

**Refractive index** (*2.2.6*): 1.462 to 1.470.

**Optical rotation** (*2.2.7*): + 7° to + 13°.

**Acid value** (*2.5.1*): maximum 3.0, determined on 5.00 g of the substance to be examined.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution (a).* Dissolve 10 µL of *α-pinene R*, 10 µL of *limonene R*, 10 µL of *γ-terpinene R*, 10 µL of *p-cymene R*, 10 mg of *camphor R*, 20 µL of *linalol R*, 10 µL of *α-terpineol R*, 10 µL of *geranyl acetate R* and 10 µL of *geraniol R* in 1 mL of *heptane R*.

*Reference solution (b).* Dissolve 5 µL of *geraniol R* in *heptane R* and dilute to 10 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:65.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 75 | 60 → 190 |
| | 75 - 120 | 190 |
| Injection port | | 220 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of reference solution (a). Record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to linalol and camphor.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *α-pinene*: 3.0 per cent to 7.0 per cent;
- *limonene*: 1.5 per cent to 5.0 per cent;
- *γ-terpinene*: 1.5 per cent to 8.0 per cent;
- *p-cymene*: 0.5 per cent to 4.0 per cent;
- *camphor*: 3.0 per cent to 6.0 per cent;
- *linalol*: 65.0 per cent to 78.0 per cent;
- *α-terpineol*: 0.1 per cent to 1.5 per cent;
- *geranyl acetate*: 0.5 per cent to 4.0 per cent;
- *geraniol*: 0.5 per cent to 3.0 per cent;
- *disregard limit*: area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chiral purity**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.02 g of the substance to be examined in *pentane R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 µL of *linalol R* and 5 mg of *borneol R* in *pentane R* and dilute to 10 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *modified β-cyclodextrin for chiral chromatography R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 65 | 50 → 180 |
| Injection port | | 230 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

- *resolution*: minimum 5.5 between the peaks due to (*R*)-linalol (1st peak) and (*S*)-linalol (2nd peak) and minimum 2.9 between the peaks due to (*S*)-linalol and borneol (3rd peak).

*Limit*: calculate the percentage content of (*R*)-linalol using the expression:

$$\frac{A_R}{A_S + A_R} \times 100$$

$A_S$ = area of the peak due to (*S*)-linalol;

$A_R$ = area of the peak due to (*R*)-linalol.

- *(R)-linalol*: maximum 14 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**04/2019:2976**

# CORYDALIS RHIZOME

## Corydalis rhizoma

DEFINITION

Whole or fragmented tuber of *Corydalis yanhusuo* (Y.H.Chou & Chun C.Hsu) W.T.Wang ex Z.Y.Su & C.Y.Wu, with roots removed, treated with boiling water until no white core is visible and then dried.

*Content*: minimum 0.20 per cent for the sum of tetrahydropalmatine and corydaline, expressed as tetrahydropalmatine ($C_{21}H_{25}NO_4$; $M_r$ 355.4) (dried drug).

IDENTIFICATION

A. *Whole drug*. The whole tuber is irregularly oblate, 0.5-1.8 cm in diameter and 0.5-1.3 cm (rarely 1.7 cm) thick. Occasionally, 2-3 tubers are grouped together. The apex usually shows a slightly depressed stem scar and occasional remains of aerial parts. The base is tuberculate or with a slight navel-like depression from which shallow furrows may radiate. The external surface is yellow or yellowish-brown to greenish-yellow with coarse or fine reticulate wrinkles. The underlying cortex, when exposed, is blackish-green or brownish-yellow. The texture is hard, mostly difficult to break. The fracture is horny, slightly shiny and translucent with a yellow, yellowish-brown or dark brown surface. The pith, if present, is whitish-yellow.

*Fragmented drug*. The fragmented tuber occurs as thick or thin, sub-rounded or sub-oval transversal or longitudinal slices or as irregular pieces. The slices are 0.5-1.9 cm in diameter and 0.1-0.5 cm thick. Occasionally, slices consist of 2-3 tubers grouped together. Rare slices from the tuber apex or base are present, with those from the apex having a slightly depressed stem scar and occasional remains of aerial parts, while those from the base have tuberculate protrusions or have a slight navel-like depression from which shallow furrows may radiate. The external surface is yellow or yellowish-brown with coarse or fine wrinkles.

The underlying cortex, when exposed, is blackish-green or brownish-yellow. The texture is hard and fragile. The fracture is horny and slightly shiny. The cut surface is horny, slightly shiny and translucent with occasional fissures; the surface is yellow, yellowish-brown, reddish-brown or dark blackish-brown and occasionally shows a gradient of colours. The pith, if present, is a lighter colour than the surrounding tissue, or if absent, it is replaced by a central cavity.

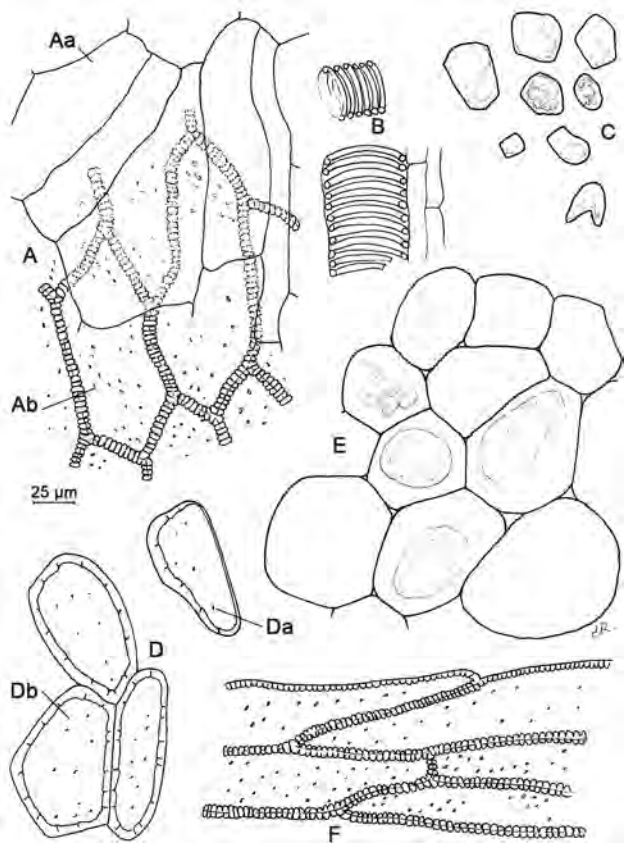


Figure 2976.-1. – *Illustration for identification test B of powdered herbal drug of corydalis rhizome*

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow to brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2976.-1): greenish-yellow to brownish-yellow fragments of cortical sclerenchyma [F], consisting of elongated, polygonal, sub-square or irregularly shaped cells 35-321 µm long and 15-127 µm in diameter with lignified, often sinuous and beaded cell walls having fine, sparse pits and large cell lumens; some fragments [A] of cortical parenchyma [Ab] are covered by dermal tissue consisting of a layer of rectangular or polygonal thin-walled cells [Aa]; sclereids [D] isolated or in groups [Db], sub-square to sub-rounded, sub-polygonal, sub-triangular or irregular in shape, 32-290 µm long and 22-103 µm in diameter, with yellowish-green to brownish-yellow cell walls; sclereids with lignified cell walls having dense, fine pits and occasional striations; some sclereids have irregularly thickened cell walls, with one thinner wall [Da]; rare, inconspicuous fragments of xylem vessels, often embedded in parenchyma tissue, colourless or pale yellow, irregularly spiral, annular or reticulate and 10-43 µm in diameter [B]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows the following diagnostic characters: abundant large and small colourless or pale yellow masses of gelatinised starch granules [C], varying in shape, but often polygonal, elongate oval or rectangular (up to 530 µm long and 332 µm in diameter) and surrounded by colourless parenchyma [E]; rare free starch granules, single or 2-5 compound, with single granules being sub-rounded or irregular with a distinct hilum.

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 10.0 mL of *ethanol (50 per cent V/V) R* and sonicate for 15 min. Centrifuge or filter the solution and use the supernatant or filtrate.

*Reference solution (a)*. Dissolve 2.5 mg of *corydaline R* and 2.5 mg of *tetrahydropalmatine R* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Intensity marker*: tetrahydropalmatine.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R*, *ethyl acetate R* (10:10:50:50 *V/V/V/V*).

*Application*: 2 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of cold air for 5 min.

*Detection*: expose the plate for 3 min to iodine vapour using a chromatographic tank previously saturated with iodine for 30 min; remove the excess of absorbed iodine in air for 10 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (a):

– the chromatogram shows near the border between the lower and middle thirds 2 distinct zones, which may be touching; the lower zone (tetrahydropalmatine) shows a green fluorescence and the upper zone (corydaline) shows a light blue fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, other faint blue, yellow or green fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| ——— | ——— |
| | A green fluorescent zone, faint to equivalent |
| | A green fluorescent zone, faint |
| Corydaline: a light blue fluorescent zone | A light blue fluorescent zone (corydaline) |
| ——— | ——— |
| Tetrahydropalmatine: a green fluorescent zone | A green fluorescent zone (tetrahydropalmatine) |
| | A green fluorescent zone, faint |
| | A green or yellow fluorescent zone, equivalent to intense |
| **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 5 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 0.5 per cent.

**Aflatoxins** (*2.8.18*): maximum 2 µg/kg (aflatoxin $B_1$) and maximum 4 µg/kg (sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$).

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.00 g of the powdered herbal drug (355) (*2.9.12*) add 25.0 mL of *ethanol (50 per cent V/V) R*. Weigh and sonicate for 30 min. Allow to cool, weigh and compensate for the loss of solvent with *ethanol (50 per cent V/V) R*; shake thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *tetrahydropalmatine CRS* in *ethanol (50 per cent V/V) R* and dilute to 25.0 mL with the same solvent. Sonicate for 1 h. Dilute 2.0 mL of the solution to 10.0 mL with *ethanol (50 per cent V/V) R*.

*Reference solution (b).* To 0.10 g of *corydalis rhizome dry extract HRS* add 5.0 mL of *ethanol (50 per cent V/V) R*. Weigh and sonicate for 10 min. Allow to cool, weigh and compensate for the loss of solvent with *ethanol (50 per cent V/V) R*; shake thoroughly. Centrifuge and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: mix 0.05 volumes of *anhydrous acetic acid R* and 99.95 volumes of *water for chromatography R* and adjust to pH 6 with *triethylamine R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 60 | 40 |
| 3 - 31 | 60 → 38 | 40 → 62 |
| 31 - 32 | 38 → 5 | 62 → 95 |
| 32 - 37 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *corydalis rhizome dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to corydaline and peak 2; use the chromatogram obtained with reference solution (a) to identify the peak due to tetrahydropalmatine.

*Relative retention* with reference to tetrahydropalmatine (retention time = about 16.4 min): peak 2 (unknown) = about 1.54; corydaline = about 1.65.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between peak 2 and the peak due to corydaline.

Calculate the percentage content of the sum of tetrahydropalmatine and corydaline, expressed as tetrahydropalmatine, using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 5}$$

$A_1$ = sum of the areas of the peaks due to tetrahydropalmatine and corydaline in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to tetrahydropalmatine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *tetrahydropalmatine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of tetrahydropalmatine in *tetrahydropalmatine CRS*.

**04/2011:1306**

# COUCH GRASS RHIZOME

## Graminis rhizoma

DEFINITION

Whole or cut, washed and dried rhizome of *Agropyron repens* (L.) P.Beauv. (*Elymus repens* (L.) Gould); the adventitious roots are removed.

IDENTIFICATION

A. The shiny yellowish, light brown or yellowish-brown pieces of the rhizome are 2-3 mm thick and longitudinally furrowed. At the nodes are the remains of very thin, more or less branched roots and whitish or brownish scale-like leaves; the internodes, up to 6 cm long, are furrowed and hollow inside. The transverse section of the nodes shows a yellowish medulla.

B. Microscopic examination (*2.8.23*). The powder is whitish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1306.-1): fragments of the epidermis (surface view [A]) covered with a thick cuticle and composed of rectangular and elongated, thick-walled cells with pitted, slightly wavy walls, which usually alternate with small, thin-walled, rounded or almost square twin cells; fragments (transverse section [B]) showing the epidermis [Ba] associated with thick-walled cells of the hypodermis; fragments in transverse section [F] consisting of endodermic cells with U-shaped thickening of the walls [Fa] accompanied by pericyclic fibres [Fb]; numerous fragments of moderately thickened fibres [C]; groups of vessels [D, G] with slit-shaped pits [Da] or with spiral and annular thickening [Ga], accompanied by fibres [Db, Gb]; numerous fragments of the cortical parenchyma and the pith with slightly thickened and pitted cells [E].

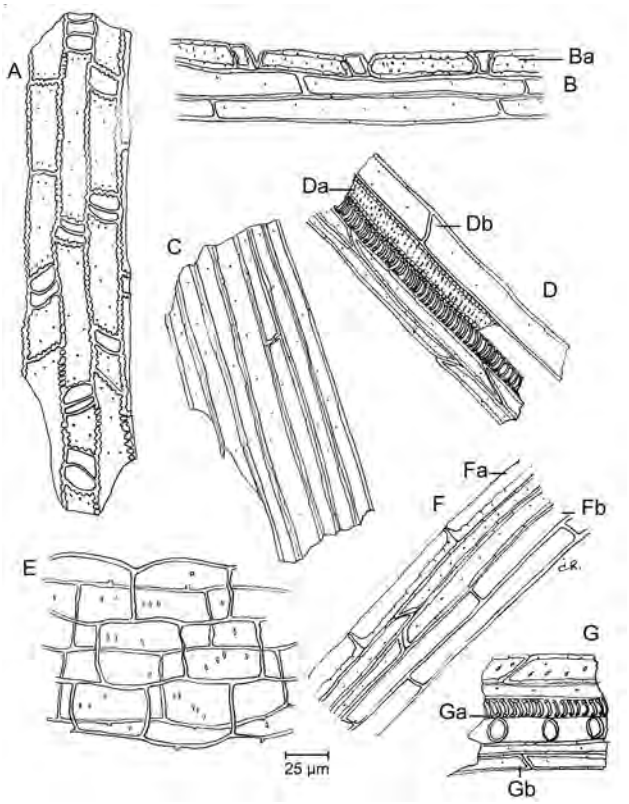


Figure 1306.-1. – *Illustration for identification test B of powdered herbal drug of couch grass rhizome*

TESTS

***Cynodon dactylon, Imperata cylindrica.*** Examine under a microscope using *iodine solution R1*. No blue starch grains are visible.

**Foreign matter** (*2.8.2*): maximum 15 per cent of blackish-grey pieces of rhizome in the cut herbal drug.

**Water-soluble extractive**: minimum 25 per cent.

To 5.0 g of the powdered herbal drug (355) (*2.9.12*) add 200 mL of boiling *water R*. Allow to stand for 10 min, shaking occasionally. Allow to cool, dilute to 200.0 mL with *water R* and filter. Evaporate 20.0 mL of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100-105 °C. The residue weighs a minimum of 0.125 g.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

**07/2012:1851**

# DANDELION HERB WITH ROOT

## Taraxaci officinalis herba cum radice

DEFINITION

Mixture of whole or fragmented, dried aerial and underground parts of *Taraxacum officinale* F.H. Wigg.

CHARACTERS

Bitter taste.

IDENTIFICATION

A. The underground parts consist of dark brown or blackish fragments 2-3 cm long, deeply wrinkled longitudinally on the outer surface. The thickened crown shows many scars left by the rosette of leaves. The fracture is short. A transverse section shows a greyish-white or brownish cortex containing concentric layers of brownish laticiferous vessels and a porous, pale yellow, non-radiate wood. Leaf fragments are green, glabrous or densely pilose. They are crumpled and usually show a clearly visible midrib on the inner surface. The lamina, with deeply dentate margins, is crumpled. The solitary flower heads, on hollow stems, consist of an involucre of green, foliaceous bracts surrounding the yellow florets, all of which are ligulate; a few achenes bearing a white, silky, outspread pappus may be present.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1851.-1): fragments of cork [G] with flattened, thin-walled cells; reticulate lignified vessels [H] from the roots; fragments of parenchyma containing branched laticiferous vessels [F]; fragments of leaves, in surface view, showing upper [E] and lower [C] epidermises consisting of interlocking lobed cells and anomocytic stomata (*2.8.3*) [Ca, Ea]; elongated, multicellular covering trichomes with constrictions, which are more or less abundant depending on the variety or sub-variety [B, D]; fragments of the upper [E] epidermis usually accompanied by underlying palisade parenchyma [Eb] and fragments of the lower [C] epidermis accompanied by underlying spongy parenchyma [Cb]; lignified, spirally or annularly thickened vessels; fragments of flower-stem epidermis with stomata and rigid-walled, elongated cells [A]; pollen grains with a pitted exine [J]. Examine under a microscope using *glycerol R*. The powder shows angular, irregular inulin fragments, free or included in the parenchyma cells.

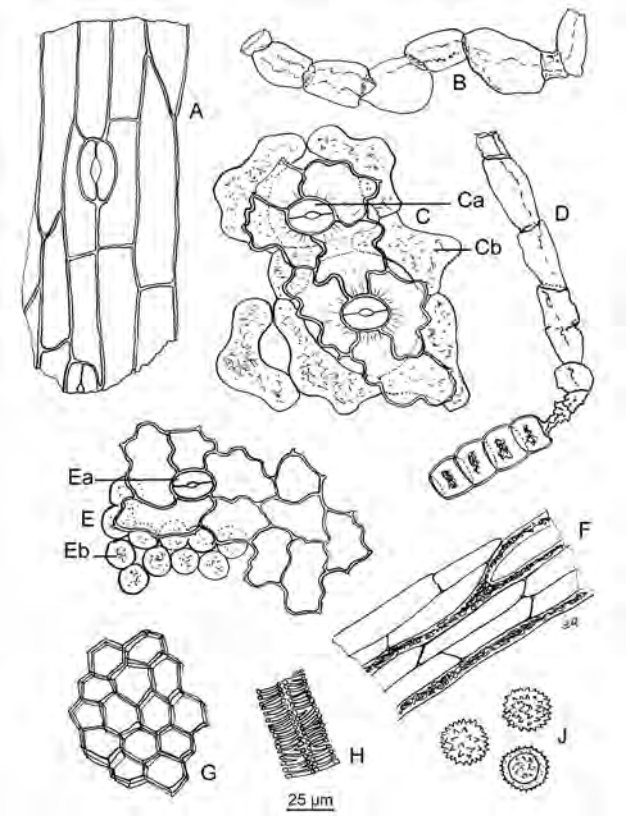


Figure 1851.-1. – *Illustration for identification test B of powdered herbal drug of dandelion herb with root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C or sonicate for 10 min. Cool and filter.

*Reference solution.* Dissolve 2 mg of *chlorogenic acid R* and 2 mg of *rutoside trihydrate R* in *methanol R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 7 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min; spray with or dip briefly into a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and dry at 100 °C for 5 min; spray with or dip briefly into a 50 g/L solution of *macrogol 400 R* in *methanol R*; heat at 100 °C for 5 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint red zone |
| | A faint yellow zone |
| _____ | _____ |
| Chlorogenic acid: a blue zone | 2 light blue zones |
| _____ | _____ |
| Rutoside: a yellowish-brown zone | |
| | A light blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 17.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5.0 per cent.

**Extractable matter**: minimum 30.0 per cent.

To 2.000 g of the powdered herbal drug (250) (*2.9.12*) add 40 g of *water R*. Stir for 1 h and filter. Evaporate 10 g of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 0.15 g.

**Bitterness value** (*2.8.15*): minimum 100.

**01/2011:1852**

# DANDELION ROOT

## Taraxaci officinalis radix

DEFINITION

Whole or cut, dried underground parts of *Taraxacum officinale* F.H.Wigg.

CHARACTERS

Bitter taste.

IDENTIFICATION

A. The dark brown or blackish taproot shows little branching and is deeply wrinkled longitudinally on the outer surface. The thickened crown shows many scars left by the rosette of leaves. The fracture is short. A transverse section shows a greyish-white or brownish cortex containing concentric layers of brownish laticiferous vessels and a porous, pale yellow, non-radiate wood.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1852.-1): fragments of brown or reddish-brown cork (surface view [G], transverse section [C]) with flattened, thin-walled cells [Ca], sometimes accompanied by parenchyma [Cb]; reticulate lignified vessels [E, J, M]; fragments of parenchyma [A, D, K, L], some containing branched laticiferous vessels (longitudinal section [Ka], transverse section [Da]); granular contents of laticiferous vessels [B, H]. Examine under a microscope using *glycerol R*. The powder shows numerous irregular, angular inulin fragments, free [F] or included in the parenchyma cells [La].

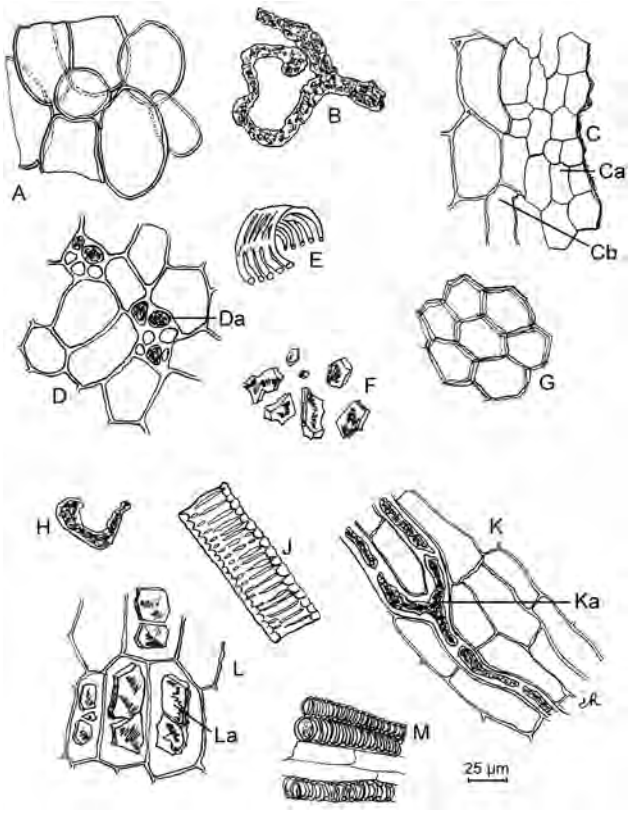


Figure 1852.-1. – *Illustration for identification test B of powdered herbal drug of dandelion root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C or sonicate for 10 min. Cool and filter.

*Reference solution.* Dissolve 2 mg of *chlorogenic acid R* and 2 mg of *rutoside trihydrate R* in *methanol R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 7 cm].

*Drying*: in air.

Herbal drugs

*Detection*: heat at 100 °C for 5 min; spray with or dip briefly into a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and dry at 100 °C for 5 min; spray with or dip briefly into a 50 g/L solution of *macrogol 400 R* in *methanol R*; heat at 100 °C for 5 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue zone |
| Chlorogenic acid: a blue zone | A blue zone (chlorogenic acid) |
| Rutoside: a yellowish-brown zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

**Extractable matter**: minimum 20.0 per cent.

To 2.000 g of the powdered herbal drug (250) (*2.9.12*) add 40 g of *water R*. Stir for 1 h and filter. Evaporate 10 g of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 0.10 g.

**Bitterness value** (*2.8.15*): minimum 100.

**01/2008:1871**

# DEVIL'S CLAW DRY EXTRACT

## Harpagophyti extractum siccum

DEFINITION

Dry extract obtained from *Devil's claw root (1095)*.

*Content*: minimum 1.5 per cent of harpagoside ($C_{24}H_{30}O_{11}$; $M_r$ 494.5) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by an appropriate procedure using either water or a hydroalcoholic solvent that is at most equivalent in strength to ethanol (95 per cent *V/V*).

CHARACTERS

*Appearance*: light brown powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the extract to be examined add 10 mL of *methanol R* and heat in a water-bath at 60 °C for 10 min. Cool and filter.

*Reference solution*. Dissolve 1.0 mg of *harpagoside R* and 2.5 mg of *fructose R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a 10 g/L solution of *phloroglucinol R* in *ethanol (96 per cent) R* and then with *hydrochloric acid R*; heat at 80 °C for 5-10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Harpagoside: a green zone | A green zone (harpagoside) |
| | A yellow zone |
| | A light green zone |
| Fructose: a yellowish-grey zone | A yellowish-grey zone may be present (fructose) |
| | A brown zone |
| **Reference solution** | **Test solution** |

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Introduce 0.350 g of the extract to be examined into a 100 mL volumetric flask, add 90 mL of *methanol R* and sonicate for 20 min. Cool to room temperature, dilute to 100.0 mL with *methanol R* and filter through a membrane filter (nominal pore size 0.2 µm).

*Reference solution*. Dissolve the contents of 1 vial of *harpagoside CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water R* (50:50 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of harpagoside.

*Retention time*: harpagoside = about 7 min.

Calculate the percentage content of harpagoside using the following expression:

$$\frac{A_1 \times m_2 \times 1000}{A_2 \times m_1}$$

$A_1$ = area of the peak due to harpagoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to harpagoside in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of harpagoside contained in 1 vial of *harpagoside CRS*, in grams.

**01/2011:1095**
**corrected 9.6**

# DEVIL'S CLAW ROOT

## Harpagophyti radix

DEFINITION

Cut and dried, tuberous secondary roots of *Harpagophytum procumbens* DC. and/or *Harpagophytum zeyheri* Decne.

*Content*: minimum 1.2 per cent of harpagoside ($C_{24}H_{30}O_{11}$; $M_r$ 494.5) (dried drug).

CHARACTERS

The root is greyish-brown or dark brown.

IDENTIFICATION

A. It consists of thick, fan-shaped or rounded slices or of roughly crushed discs. The darker outer surface is traversed by tortuous longitudinal wrinkles. The paler cut surface shows a dark cambial zone and xylem bundles distinctly aligned in radial rows. The central cylinder shows fine concentric striations. Seen under a lens, the cut surface presents yellow or brownish-red granules.

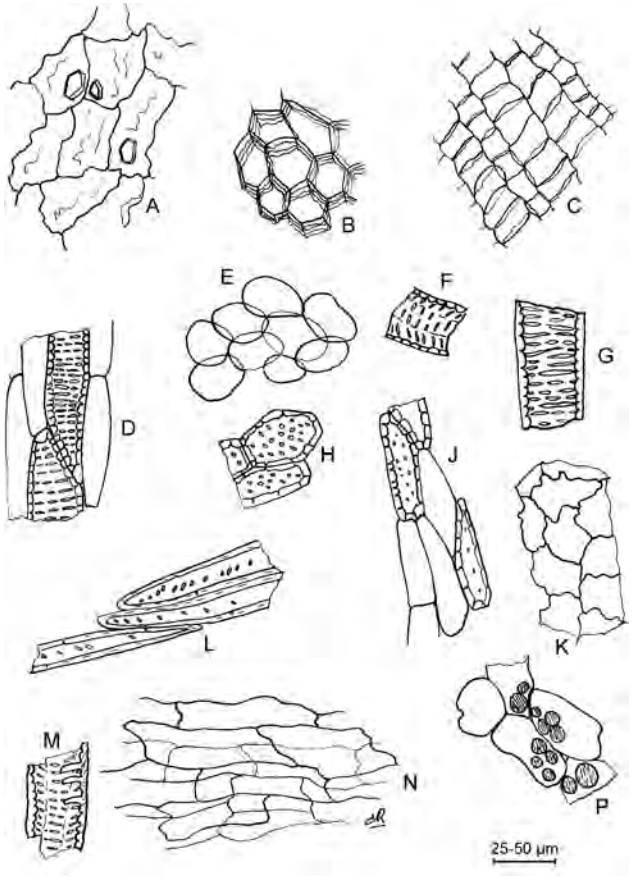


Figure 1095.-1. – *Illustration for identification test B of powdered herbal drug of devil's claw root*

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1095.-1): fragments of cork consisting of yellowish-brown, thin-walled cells (surface view [B], transverse section [C]); fragments of cortical parenchyma consisting of large, thin-walled cells [E, K, N, P], sometimes containing reddish-brown granular inclusions and isolated yellow droplets [P]; fragments of reticulately thickened or pitted vessels [D, F, G, M] and fragments of lignified parenchyma [L], sometimes associated with vessels, from the central cylinder; prism crystals [A] and rare small needles of calcium oxalate in the parenchyma. The powder may also show rectangular or polygonal sclereids with dark reddish-brown contents [H, J]. With a solution of phloroglucinol in hydrochloric acid, the parenchyma turns green.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Heat 1.0 g of the powdered herbal drug (355) (*2.9.12*) with 10 mL of *methanol R* on a water-bath at 60 °C for 10 min. Filter and reduce the filtrate to about 2 mL under reduced pressure at a temperature not exceeding 40 °C.

*Reference solution*. Dissolve 1 mg of *harpagoside R* and 2.5 mg of *fructose R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands.

*Development*: over a path of 10 cm [or 7.5 cm].

*Drying*: in a current of warm air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution; the chromatogram obtained with the test solution shows other distinct zones, mainly above the zone due to harpagoside. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Harpagoside: a quenching zone | A quenching zone: harpagoside |
| **Reference solution** | **Test solution** |

*Detection B*: spray with a 10 g/L solution of *phloroglucinol R* in *ethanol (96 per cent) R* and then with *hydrochloric acid R*; heat at 80 °C for 5-10 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution; the chromatogram obtained with the test solution also shows several yellow or brown zones above the zone due to harpagoside. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Harpagoside: a green zone | A green zone (harpagoside) |
| | A yellow zone |
| | A light green zone |
| Fructose: a yellowish-grey zone | A yellowish-grey zone may be present (fructose) |
| | A brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Starch**. Examine the powdered herbal drug (355) (*2.9.12*) under a microscope using *water R*. Add *iodine solution R1*. No blue colour develops.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*) add 100.0 mL of *methanol R*. Shake for 4 h and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve the contents of a vial of *harpagoside CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R, water for chromatography R* (50:50 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of harpagoside.

*Retention time*: harpagoside = about 7 min.

Calculate the percentage content of harpagoside using the following expression:

$$\frac{m_2 \times A_1 \times 1000}{A_2 \times m_1}$$

$A_1$ = area of the peak due to harpagoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to harpagoside in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *harpagoside CRS* in the reference solution, in grams.

**07/2013:0117**

# DIGITALIS LEAF

## Digitalis purpureae folium

DEFINITION

Dried leaf of *Digitalis purpurea* L.

*Content*: minimum 0.3 per cent of cardenolic glycosides, expressed as digitoxin ($M_r$ 765) (dried drug).

CHARACTERS

Faint but characteristic odour.

The whole leaf is about 10-40 cm long and 4-15 cm wide. The lamina is ovate lanceolate or broadly ovate. The winged petiole is from 1/4 as long as to equal in length to the lamina.

IDENTIFICATION

A. The leaf is brittle and often occurs broken. The upper surface is green and the lower surface is greyish-green. The apex is subacute and the margin is irregularly crenate, dentate or serrate. The base is decurrent. The venation is pinnate, the lateral veins being prominent especially on the lower surface, leaving the midrib at about 45° and anastomosing near the margin; a veinlet terminates in each tooth of the margin and the lower veins run down the winged petiole. The upper surface is rugose and pubescent; the lower surface shows a network of raised veinlets and is densely pubescent.

B. Microscopic examination (*2.8.23*). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0117.-1): fragments of the upper epidermis (surface view [K, L]), with cells with a smooth cuticle and anticlinal walls that are slightly thickened, are straight or slightly sinuous, and may show slight beading and pitting [La] and sometimes scars of covering trichomes [Ka], accompanied by underlying palisade parenchyma [Lb]; fragments of the lower epidermis (surface view [G]), with markedly sinuous cells and anomocytic stomata (*2.8.3*) [Ga]; trichomes are of 2 types: a) uniseriate covering trichomes with blunt apex, usually consisting of 3-5 cells [H, J], often with 1 or more collapsed cells [Ja], walls mostly finely warty or faintly striated; b) glandular trichomes usually with a unicellular [C, D], sometimes a multicellular, uniseriate [A, B, E] stalk and a unicellular head [A, B, C, E] or bicellular head (side view [D], surface view [F]) or exceptionally a tetracellular head.

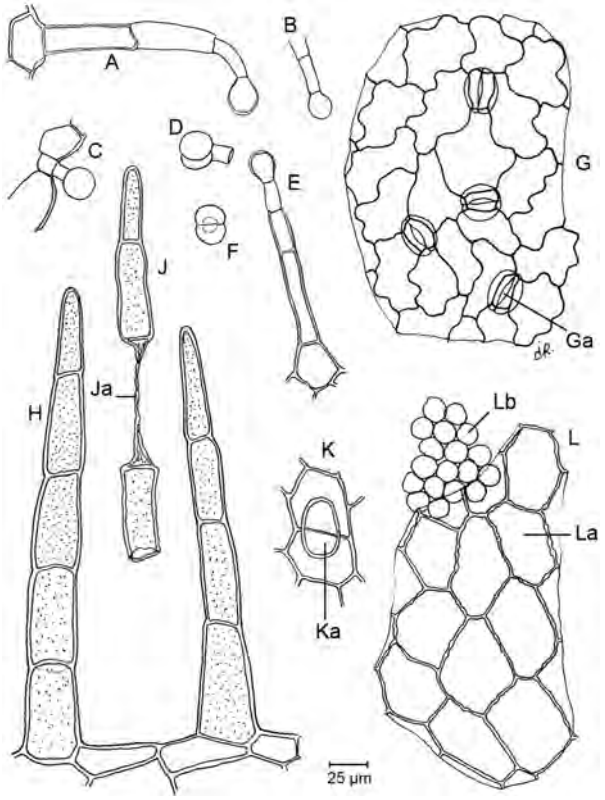


Figure 0117.-1. – *Illustration for identification test B of powdered herbal drug of digitalis leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (180) (*2.9.12*) add a mixture of 20 mL of *ethanol (50 per cent V/V) R* and 10 mL of *lead acetate solution R*. Boil for 2 min, allow to cool and centrifuge. Shake the supernatant solution with 2 quantities, each of 15 mL, of *chloroform R*; separate the 2 layers by centrifugation if necessary. Dry the chloroform layers over *anhydrous sodium sulfate R* and filter. Evaporate 10 mL of the solution to dryness on a water-bath and dissolve the residue in 1 mL of a mixture of equal volumes of *chloroform R* and *methanol R*.

*Reference solution.* Dissolve 5 mg of *purpureaglycoside A CRS*, 2 mg of *purpureaglycoside B CRS*, 5 mg of *digitoxin R* and 2 mg of *gitoxin R* in a mixture of equal volumes of *chloroform R* and *methanol R*, then dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (7.5:10:75 *V/V/V*).

*Application*: 20 µL as bands of 2 cm by 0.3 cm.

*Development*: over a path of 10 cm.

*Drying*: until the solvents have evaporated.

*Detection*: treat with a mixture of 2 volumes of a 10 g/L solution of *chloramine R* and 8 volumes of a 250 g/L solution of *trichloroacetic acid R* in *ethanol (96 per cent) R*, then heat at 100-105 °C for 10 min; examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows a zone of light blue fluorescence in the lower part of the chromatogram, due to purpureaglycoside B, and, just above it, a zone of brownish-yellow fluorescence due to purpureaglycoside A; a zone of light blue fluorescence, due to gitoxin, appears in the middle of the chromatogram and above it a zone of brownish-yellow fluorescence, due to digitoxin; the zones in the chromatogram obtained with the test solution are similar in position, colour and size to the zones in the chromatogram obtained with the reference solution. Other zones of fluorescence may also appear in the chromatogram obtained with the test solution.

D. Evaporate 5 mL of the chloroformic solution obtained in identification test C to dryness on a water-bath. To the residue add 2 mL of *dinitrobenzoic acid solution R* and 1 mL of *1 M sodium hydroxide*. A reddish-violet colour develops within 5 min.

E. Evaporate 5 mL of the chloroformic solution obtained in identification test C to dryness on a water-bath. To the residue add 3 mL of *xanthydrol solution R* and heat on a water-bath for 3 min. A red colour develops.

TESTS

***Digitalis lanata* Ehrh.** The presence of leaves with few or no trichomes and with parallel venation or the presence of cells of the abaxial epidermis with beaded anticlinal walls and of cells of the adaxial epidermis with numerous stomata indicates adulteration by *Digitalis lanata* Ehrh.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5.0 per cent.

ASSAY

Shake 0.250 g of the powdered herbal drug (180) (*2.9.12*) with 50.0 mL of *water R* for 1 h. Add 5.0 mL of a 150 g/L solution of *lead acetate R*, shake, and after a few minutes add 7.5 mL of a 40 g/L solution of *disodium hydrogen phosphate dodecahydrate R*. Filter through a pleated paper filter. Heat 50.0 mL of the filtrate with 5 mL of hydrochloric acid (150 g/L HCl) under a reflux condenser on a water-bath for 1 h. Transfer to a separating funnel, rinse the flask with 2 quantities, each of 5 mL, of *water R* and shake with 3 quantities, each of 25 mL, of *chloroform R*. Dry the combined chloroform layers over *anhydrous sodium sulfate R* and dilute to 100.0 mL with *chloroform R*. Evaporate 40.0 mL of the chloroformic solution to dryness, dissolve the residue in 7 mL of *ethanol (50 per cent V/V) R*, add 2 mL of *dinitrobenzoic acid solution R* and 1 mL of *1 M sodium hydroxide*. At the same time prepare a reference solution as follows. Dissolve 50.0 mg of *digitoxin CRS* in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*. To 5.0 mL of the resulting solution add 25 mL of *water R* and 3 mL of hydrochloric acid (150 g/L HCl). Heat the solution under a reflux condenser on a water-bath for 1 h and complete the preparation as described above. Measure the absorbance (*2.2.25*) of the 2 solutions at 540 nm several times during the first 12 min until the maximum is reached, using as the compensation liquid a mixture of 7 mL of *ethanol (50 per cent V/V) R*, 2 mL of *dinitrobenzoic acid solution R* and 1 mL of *1 M sodium hydroxide*.

From the absorbances measured and the concentrations of the solutions, calculate the content of cardenolic glycosides, expressed as digitoxin.

STORAGE

Protected from moisture.

**04/2017:2890**
**corrected 9.5**

# DIOSCOREA NIPPONICA RHIZOME

## Dioscoreae nipponicae rhizoma

DEFINITION

Dried, whole or fragmented, scraped rhizome of *Dioscorea nipponica* Makino, with roots removed.

*Content*: minimum 1.0 per cent of diosgenin ($C_{27}H_{42}O_3$; $M_r$ 414.6) (dried drug).

IDENTIFICATION

A. *Whole drug*. The rhizome is subcylindrical, slightly curved, up to 15-20 cm long and 1.0-1.5 cm in diameter. The outer surface is yellowish-white or brownish-yellow, irregularly and longitudinally furrowed, bearing spinous remains of roots and protuberant stem scars.

*Fragmented drug*. Transverse slices of the rhizome, oblique or more or less longitudinal, circular, oval or elongated, whole or broken, up to 0.7 cm thick. The outer surface is light brown or yellowish-brown, irregularly and longitudinally furrowed; spinous remains of roots and protuberant stem scars may be visible. A transverse section is yellowish-white, can easily be imprinted with one's nail due to an abundance of starch, and shows numerous light yellowish-brown pits corresponding to the vascular bundles.

B. Microscopic examination (*2.8.23*). The powder is whitish or light yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2890.-1): vessels up to 50 µm in diameter with numerous fine, dense and elliptical pits [G]; numerous fragments of parenchyma with polyhedral or ovoid cells with slightly thickened and pitted walls (transverse section [B], longitudinal section [C]); cells containing raphides of calcium oxalate up to 110 µm long [D]; free needles of calcium oxalate [A]; rare cork fragments with polyhedral cells (surface view [F]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows extremely abundant starch granules, simple, irregular, ovoid, oblong, sub-triangular, with a maximum dimension of up to 30 µm; the hilum is usually eccentric and slit-shaped [E].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 1 mg of *aescin R* and 1 mg of *glucose R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (10:50:64 *V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100 °C for 3 min, allow to cool for 10 min and examine in daylight.

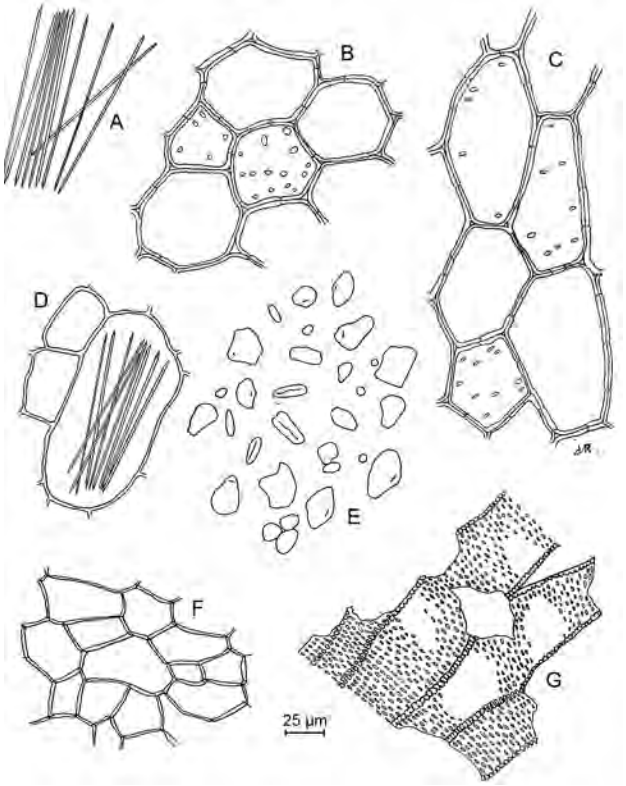


Figure 2890.-1. – *Illustration for identification test B of powdered herbal drug of Dioscorea nipponica rhizome*

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 3 olive-green zones |
| _____ | _____ |
| Aescin: a violet zone | |
| | A green zone |
| | A faint to strong green zone |
| Glucose: a yellow or brown zone | A yellowish-brown zone |
| | A faint brown zone |
| _____ | _____ |
| | A yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 2.000 g of the powdered herbal drug (355) (*2.9.12*) in a round-bottomed flask add 40 mL of a 15 per cent *V/V* solution of *sulfuric acid R*. Heat in a water-bath under a reflux condenser for 3 h. Allow to cool and filter. Wash the residue with *water R* until the filtrate is neutral. Sonicate the residue for 30 min in 80 mL of *methanol R* and filter. Wash the residue with 20 mL of *methanol R*, combine the filtrate and the washing and dilute to 100.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 5.0 mg of *diosgenin CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 2 mg of *(25R)-spirost-5-en-3-one CRS* in reference solution (a) and dilute to 10.0 mL with the same solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile for chromatography R* (15:85 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 5 µL.

*Run time*: 20 min.

*Retention time*: diosgenin = about 8 min; (25*R*)-spirost-5-en-3-one = about 10 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.9 between the peaks due to diosgenin and (25*R*)-spirost-5-en-3-one.

Calculate the percentage content of diosgenin using the following expression:

$$\frac{A_1 \times m_2 \times 4 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to diosgenin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to diosgenin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *diosgenin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of diosgenin in *diosgenin CRS*.

**07/2016:2473**

# DIOSCOREA OPPOSITIFOLIA RHIZOME

## Dioscoreae oppositifoliae rhizoma

DEFINITION

Dried, scraped, whole or fragmented rhizome of *Dioscorea oppositifolia* L. (syn. *Dioscorea opposita* Thunb.), with roots removed, collected in winter when the stem and leaves are withered.

IDENTIFICATION

A. The rhizome occurs in subcylindrical pieces, sometimes flattened, about 15-30 cm long and 1.5-6 cm thick; the outer surface is yellowish-white or pale yellow, longitudinally furrowed and wrinkled, and bearing slit-shaped root scars with occasional patches of brownish cork. The fragmented rhizome occurs in whole or fragmented slices, about 0.2-0.5 cm thick; some slices still bear slit-shaped root scars. The texture is heavy, compact and tough; the fracture is white and starchy.

B. Microscopic examination (*2.8.23*). The powder is whitish or yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2473.-1): reticulate or pitted vessels (longitudinal section [C, E]); fragments of xylem (transverse section [G]) consisting of vessels up to 80 µm in diameter [Ga] and ligneous parenchyma with distinctly pitted cells [Gb]; numerous fragments of parenchyma consisting of polyhedral or ovoid cells with thin walls [B]; cells containing calcium oxalate raphides up to 250 µm long and up to 5 µm in diameter [F]; free needles of calcium oxalate [A]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows extremely abundant starch granules [D], simple, ovoid or oblong, flattened with rounded extremities, with a maximum dimension of up to 50 µm; the hilum is usually eccentric and punctiform; rare 2- to 4-compound starch granules may be present.

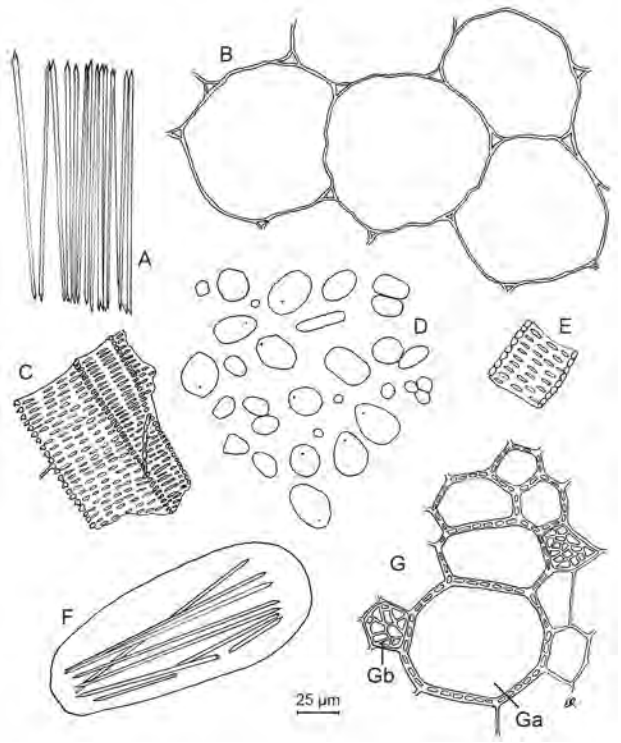


Figure 2473.-1. – *Illustration for identification test B of powdered herbal drug of Dioscorea oppositifolia rhizome*

C. Examine the chromatograms obtained in the test for *Dioscorea bulbifera* L.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 3-5 violet zones |
| _____ | _____ |
| Aescin: a violet zone | A violet zone |
| Glucose: a yellow or green zone | A yellow or brown zone |
| _____ | _____ |
| | A violet zone |
| **Reference solution** | **Test solution** |

TESTS

***Dioscorea bulbifera* L**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 1 mg of *aescin R* and 3 mg of *glucose R* in 7 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (8:40:52 *V/V/V*).

*Application*: 15 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100 °C for 3 min, allow to cool for 10 min and examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no brownish-green or orange-brown zones in the upper third of the chromatogram.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 0.5 per cent.

**Extractable matter**: minimum 7.0 per cent (dried drug).

To 1.00 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *water R*, shake for 6 h and allow to macerate for 18 h. Filter, evaporate the filtrate to dryness on a water-bath under reduced pressure and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 70 mg.

**07/2019:1510**

# DOG ROSE

## Rosae pseudo-fructus

DEFINITION

Rose hips made up of the receptacle and the remains of the dried sepals of *Rosa canina* L., *Rosa pendulina* L. and other *Rosa* species, with the achenes removed.

*Content*: minimum 0.3 per cent of ascorbic acid ($C_6H_8O_6$; $M_r$ 176.1) (dried drug).

IDENTIFICATION

A. It consists of fragments of the fleshy, hollow, urceolate receptacle, bearing the remains of the reduced sepals, light pink or orange-pink, the convex outer surface shiny and strongly wrinkled; bearing on its lighter inner surface abundant bristle-like hairs.

B. Microscopic examination (*2.8.23*). The powder is orange-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1510.-1): numerous fragments of the outer surface of the receptacle (transverse section [B], surface view [D]) consisting of the outer epidermis with irregularly thickened polyhedral cells [Ba] and a thick cuticle, sometimes accompanied by parenchyma [Bb]; fragments of the inner epidermis of the receptacle (transverse section [C], surface view [G]) with slightly wavy cells [Ca, Ga] and lignified bases of the covering trichomes [Cc, Gb], covered in a finely wrinkled cuticle around the basal cell of the trichomes, usually accompanied by the inner layers of the parenchyma [Cd, Gc]; fragments of the inner layers of the parenchyma [H], whose cells almost all contain crystals of calcium oxalate (mostly

Herbal drugs

cluster crystals [Ce, Gd, Ha] but also prisms [Cb, Ge, Hb]); scattered lignified cells, isodiametric or oval, with thickened and pitted walls corresponding to the trichome bases [E]; abundant unicellular trichomes [A], up to 2 mm long and 30-45 µm thick, tapering towards each end (apical end [Aa], distal end [Ab, K]), with walls heavily thickened and with a waxy cuticle that may show fissures in a spiral arrangement [Ac, Ka]; fragments of parenchyma [J] consisting of large ovoid cells with irregularly thickened walls and granular contents with small oil droplets [Ja], and small cells each containing a cluster crystal of calcium oxalate [Jb]; scattered cluster crystals of calcium oxalate [F].

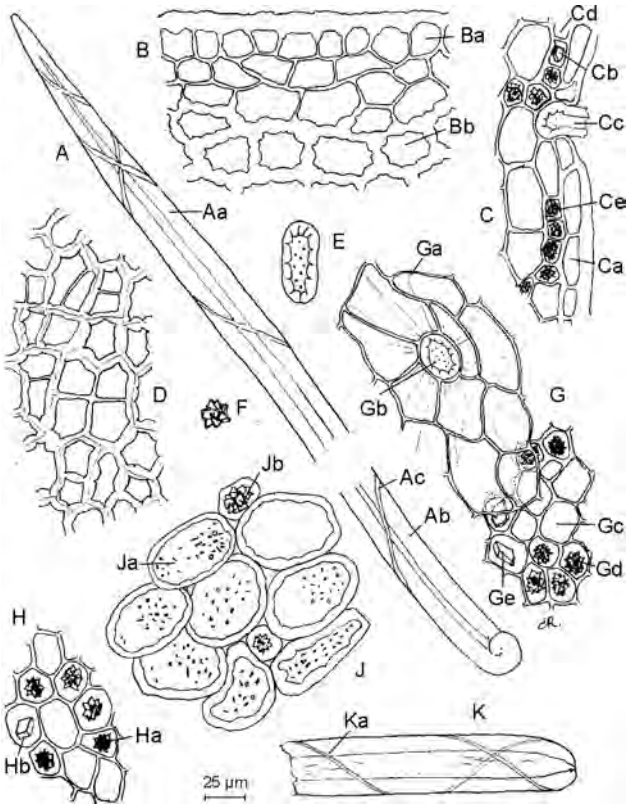


Figure 1510.-1. – *Illustration for identification test B of powdered herbal drug of dog rose*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 5 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *ethanol (96 per cent) R*, shake for 30 min and filter.

*Reference solution*. Dissolve 10 mg of *ascorbic acid R* in 5 mL of *ethanol (60 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetone R*, *glacial acetic acid R*, *methanol R*, *toluene R* (5:5:20:70 *V/V/V/V*).

*Application*: 20 µL of the test solution and 2 µL of the reference solution.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution shows a quenching zone similar in position to the principal zone in the chromatogram obtained with the reference solution.

*Detection B*: spray with a 0.2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *ethanol (96 per cent) R* and examine in daylight.

*Results B*: the chromatogram obtained with the test solution shows a white zone on a pink background (ascorbic acid) similar in position and colour to the principal zone in the chromatogram obtained with the reference solution; the chromatogram also shows an intense orange-yellow zone near the solvent front and a yellow zone in the upper third (carotenoids).

TESTS

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

*Test solution*. In a round-bottomed flask, weigh 0.500 g of the freshly powdered herbal drug (710) (*2.9.12*). Add a solution of 1.0 g of *oxalic acid R* in 50.0 mL of *methanol R*. Boil under a reflux condenser for 10 min, and cool in iced water until the temperature reaches 15-20 °C. Filter. Transfer 2.0 mL of the filtrate to a 50 mL conical flask. Add successively, with gentle shaking after each addition, 2.0 mL of *dichlorophenolindophenol standard solution R* and then, exactly 60 s later, 0.5 mL of a 100 g/L solution of *thiourea R* in *ethanol (50 per cent V/V) R* and 0.7 mL of *dinitrophenylhydrazine-sulfuric acid solution R*. Heat under a reflux condenser at 50 °C for 75 min, and place immediately in iced water for 5 min. Add dropwise 5.0 mL of a mixture of 12 mL of *water R* and 50 mL of *sulfuric acid R*, taking care to carry out the addition over a period of minimum 90 s and maximum 120 s while maintaining vigorous stirring in iced water. Allow to stand for 30 min at room temperature and measure the absorbance (*2.2.25*) at 520 nm using solution A as compensation liquid.

*Solution A*. Treat 2.0 mL of the filtrate obtained during the preparation of the test solution as described but adding the *dinitrophenylhydrazine-sulfuric acid solution R* just before the absorbance is measured.

*Reference solution*. Dissolve 40.0 mg of *ascorbic acid R* in a freshly prepared 20 g/L solution of *oxalic acid R* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with a freshly prepared 20 g/L solution of *oxalic acid R* in *methanol R*. Treat 2.0 mL of the solution as described above for the filtrate obtained during the preparation of the test solution. Measure the absorbance (*2.2.25*) at 520 nm using solution B as the compensation liquid.

*Solution B*. Treat 2.0 mL of the reference solution as described above for solution A.

Calculate the percentage content of ascorbic acid from the following expression:

$$\frac{2.5 \times A_1 \times m_2}{A_2 \times m_1}$$

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the reference solution;

$m_1$ = mass of the substance to be examined, in grams;

$m_2$ = mass of ascorbic acid used, in grams.

**07/2017:2563**

# DRYNARIA RHIZOME

## Drynariae rhizoma

DEFINITION

Dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. The ramenta may be removed.

*Content*: minimum 0.5 per cent of naringin ($C_{27}H_{32}O_{14}$; $M_r$ 580.5) (dried drug).

IDENTIFICATION

A. Long, flattened, slat-shaped rhizome, often curved and branched, 5-15 cm long and 1-1.5 cm thick. The surface is either completely covered in scaly, dark brown hairs (rhizome with ramenta) or glabrous with dark brown dots (rhizome without ramenta). The upper surface and both sides show circular frond scars, rarely the frond bases. The lower surface shows scars or the remains of fibrous roots. The texture is light, fragile, easily broken. The section is reddish-brown; the steles form a ring of small yellow dots.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2563.-1): numerous polyhedral parenchymatous cells [A] or fragments of parenchymatous cells [E, F, G] with slightly and regularly thickened pitted walls; scalariform vessels of variable diameter up to 60 µm [D]; fragments of reddish-brown scaly hairs forming a tissue consisting of cells with thickened, somewhat sinuous walls [B]; the margins of the scaly hairs [C] have elongated cells with rigid walls [Ca] and expansions, usually bicellular [Cb].

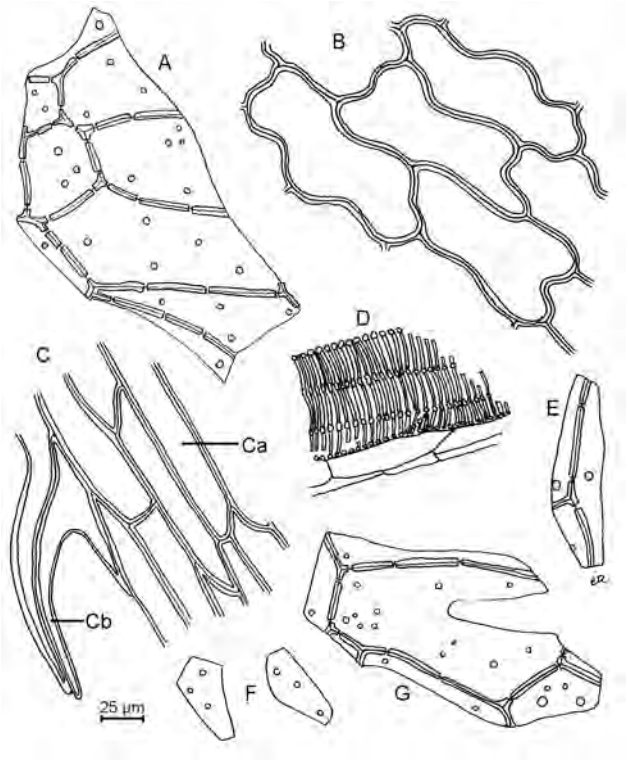


Figure 2563.-1. – *Illustration for identification test B of powdered herbal drug of drynaria rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate for 10 min. Cool, centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *naringin R* and 1 mg of *hyperoside R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (11:11:26:100 *V/V/V/V*).

*Application*: 10 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *aluminium chloride reagent R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Hyperoside: a yellow zone<br>Naringin: a bluish-white zone | A bluish-white zone (naringin)<br>A bluish-white zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 13.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.100 g of the powdered herbal drug (355) (*2.9.12*) in a 50 per cent *V/V* solution of *methanol R* and dilute to 10.0 mL with the same solvent. Weigh, sonicate for 45 min. Allow to cool, weigh and compensate the loss of solvent with a 50 per cent *V/V* solution of *methanol R*, shake well. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *naringin CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *neohesperidin R* in reference solution (a) and dilute to 10.0 mL with reference solution (a).

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 0.4 per cent *V/V* solution of *acetic acid R* (18:82 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of naringin.

*Retention time*: naringin = about 9 min; neohesperidin = about 12 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to naringin and neohesperidin.

Calculate the percentage content of naringin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 20}$$

$A_1$ = area of the peak due to naringin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to naringin in the chromatogram obtained with reference solution (c);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *naringin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of naringin in *naringin CRS*.

**07/2019:3000**

# DWARF LILYTURF TUBER

## Ophiopogonis radix

DEFINITION

Dried root tuber of *Ophiopogon japonicus* (Thunb.) Ker Gawl., with the spindly roots removed.

*Content*: minimum 0.12 per cent of total saponins, expressed as ruscogenin ($C_{27}H_{42}O_4$; $M_r$ 430.6) (dried drug).

IDENTIFICATION

A. It is fusiform with both ends slightly tapered, 1.5-3 cm long and 3-6 mm in diameter. The outer surface is whitish-yellow, fine and longitudinally wrinkled. The texture is hard, the fracture is yellowish-white and translucent, and the vascular system appears as a small ring in the centre.

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 3000.-1): numerous fragments of parenchyma consisting of suborbicular or elliptical cells [A]; different sized raphides of calcium oxalate, free [F] or included in parenchymatous cells [Aa], either fine, 12-65 µm long [Aa], or thicker, up to 130 µm long [F]; sclereids, usually in groups (surface view [E]), subsquare, rectangular or polygonal (20-57 µm in diameter) with distinct, dense channels; fragments of the endodermis with rectangular or elongated cells, with channelled and pitted walls (surface view [C]); fragments (transverse section [B]) showing the endodermis [Ba], sclereids with unevenly thickened walls [Bb] and parenchymatous cells [Bc]; thin xylem fibres [G], with oblique or square ends, slightly thickened walls, and oblique, cross-shaped or V-shaped pits; fragments of xylem [D] consisting of spiral or reticulate vessels [Da] and fibres [Db, Dc].

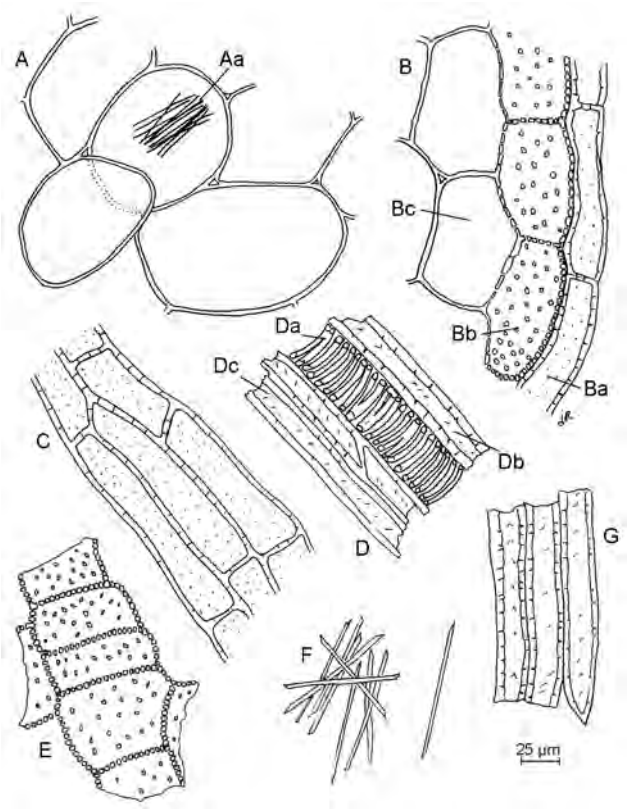


Figure 3000.-1. – *Illustration for identification test B of powdered herbal drug of dwarf lilyturf tuber*

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *anhydrous ethanol R*. Sonicate for 30 min, filter and evaporate to dryness. Dissolve the residue in 0.5 mL of *anhydrous ethanol R*, centrifuge and use the supernatant.

*Reference solution (a)*. Dissolve 1 mg of *β-sitosterol R* and 1 mg of *methylophiopogonanone A R* in 2.0 mL of *anhydrous ethanol R*.

*Reference solution (b)*. Mix 1.0 mL of reference solution (a) and 3.0 mL of *anhydrous ethanol R*.

*Reference solution (c)*. Dilute 3 µL of *isoeugenol R* and 6 µL of *methyleugenol R* in 20 mL of *toluene R*.

*Intensity markers*: β-sitosterol and methylophiopogonanone A.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of cold air for 5 min.

*Detection*: dip the plate in a 10 per cent *V/V* solution of *sulfuric acid R* in *anhydrous ethanol R*; heat the plate at 105 °C for 10 min and examine under white light.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the middle third; the lower zone (isoeugenol) and the upper zone (methyleugenol) show a violet-red fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint fluorescent zones may be present.

| Top of the plate | |
|---|---|
| | 2 violet-red zones |
| ——— | ——— |
| Methylophiopogonanone A: a brownish-yellow zone | A brownish-yellow zone, faint to equivalent |
| ——— | ——— |
| | 1 or 2 violet-red or reddish zones, faint to equivalent (possibly overlapping) |
| β-sitosterol: a violet-red zone | A violet-red zone |
| | A broad, intense violet-red zone |
| **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 2.5 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 0.5 per cent.

ASSAY

*Test solution.* Introduce 1.20 g of the powdered herbal drug (355) (*2.9.12*) into a 50 mL round-bottomed flask, add 20.0 mL of *methanol R* and weigh. Boil under a reflux condenser for 2 h. Allow to cool to room temperature, weigh again and adjust to the original mass with *methanol R*. Mix thoroughly and filter through a membrane filter (nominal pore size 0.22 µm). Evaporate 12.5 mL of the filtrate to dryness under a current of *nitrogen R*. Dissolve the residue in 5 mL of *water R* and extract with 5 quantities, each of 5 mL, of *butanol R* saturated with *water R* using a separating funnel. Combine the butanol extracts in a separating funnel and wash with 2 quantities, each of 2.5 mL, of *dilute ammonia R1*. Evaporate to dryness under a current of *nitrogen R*. Dissolve the residue in a mixture of 20 volumes of *water R* and 80 volumes of *methanol R* and dilute to 25.0 mL with the same mixture of solvents. Evaporate 2.5 mL of the solution to dryness under a current of *nitrogen R*. Dissolve the residue in *perchloric acid R* and dilute to 10.0 mL with the same solvent. Transfer the solution to a test tube, stopper and heat at 80 °C in a water-bath for 15 min. Cool to room temperature.

Measure the absorbance (*2.2.25*) of the test solution at 397 nm using *perchloric acid R* as the compensation liquid.

Calculate the percentage content of total saponins, expressed as ruscogenin, using the following expression:

$$\frac{A \times 0.16}{m \times 19.6}$$

i.e. taking the specific absorbance of ruscogenin to be 19.6.

$A$ = absorbance at 397 nm;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams.

**07/2019:2377**

# DWARF PINE OIL

## Pini pumilionis aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the fresh leaves and twigs of *Pinus mugo* Turra. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1 mL of the substance to be examined to 10 mL with *toluene R*.

*Reference solution.* Dissolve 10 mg of *borneol R* and 10 µL of *bornyl acetate R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL], as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A pink zone |
| ——— | ——— |
| Bornyl acetate: a brown or greyish-brown zone | A brown or greyish-brown zone (bornyl acetate) |
| | A pink zone |
| ——— | ——— |
| Borneol: a brown or greyish-brown zone | A cluster of violet zones |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.857 to 0.870.

**Refractive index** (*2.2.6*): 1.474 to 1.480.

**Optical rotation** (*2.2.7*): – 15° to – 6°.

**Acid value** (*2.5.1*): maximum 1.0.

**Peroxide value** (*2.5.5*): maximum 20.

**Fatty oils and resinified oils** (*2.8.7*). It complies with the test.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dilute 200 µL of the substance to be examined to 10.0 mL with *heptane R*.

*Reference solution (a)*. Dilute 30 µL of *α-pinene R*, 5 mg of *camphene R*, 10 µL of *β-pinene R*, 20 µL of *car-3-ene R*, 5 µL of *β-myrcene R*, 10 µL of *limonene R*, 5 µL of *p-cymene R*, 10 µL of *terpinolene R*, 5 µL of *bornyl acetate R* and 5 µL of *β-caryophyllene R* in *heptane R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *camphene R* in *heptane R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 65 |
| | 10 - 41 | 65 → 220 |
| | 41 - 50 | 220 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to car-3-ene and β-myrcene.

*Identification of components*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution; the peak due to β-phellandrene is eluted after the peak due to limonene with a relative retention of about 1.03 with reference to limonene.

Determine the percentage content of each of these components. The limits are within the following ranges:

- *α-pinene*: 10.0 per cent to 30.0 per cent;
- *camphene*: maximum 2.0 per cent;
- *β-pinene*: 3.0 per cent to 14.0 per cent;
- *car-3-ene*: 10.0 per cent to 40.0 per cent;
- *β-myrcene*: 3.0 per cent to 12.0 per cent;
- *limonene*: 5.0 per cent to 14.0 per cent;
- *β-phellandrene*: 9.0 per cent to 19.0 per cent;
- *p-cymene*: maximum 2.5 per cent;
- *terpinolene*: maximum 8.0 per cent;
- *bornyl acetate*: 0.5 per cent to 5.0 per cent;
- *β-caryophyllene*: 0.5 per cent to 5.0 per cent;
- *reporting threshold*: 0.05 per cent (principal peak in the chromatogram obtained with reference solution (b)).

STORAGE

In an inert container and at a temperature not exceeding 25 °C.

**07/2017:2564**

# ECLIPTA HERB

## Ecliptae herba

DEFINITION

Dried, whole or fragmented, flowering aerial parts of *Eclipta prostrata* (L.) L.

*Content*: minimum 0.04 per cent of wedelolactone ($C_{16}H_{10}O_7$; $M_r$ 314.2) (dried drug).

IDENTIFICATION

A. The cylindrical stems are striated longitudinally and are 2-5 mm in diameter. The external surface is brownish-green or dark green and bears covering trichomes, flattened against the stem and all pointing upwards. The hairy, dark green leaves are opposite, always sessile, elongate, lanceolate, with entire or slightly dentate margins. The capitula are 2-6 mm in diameter, with whitish flowers that do not extend beyond the bracts of the involucre. The fruits are elliptical, flattened achenes, brown or pale brown, 2-3 mm long.

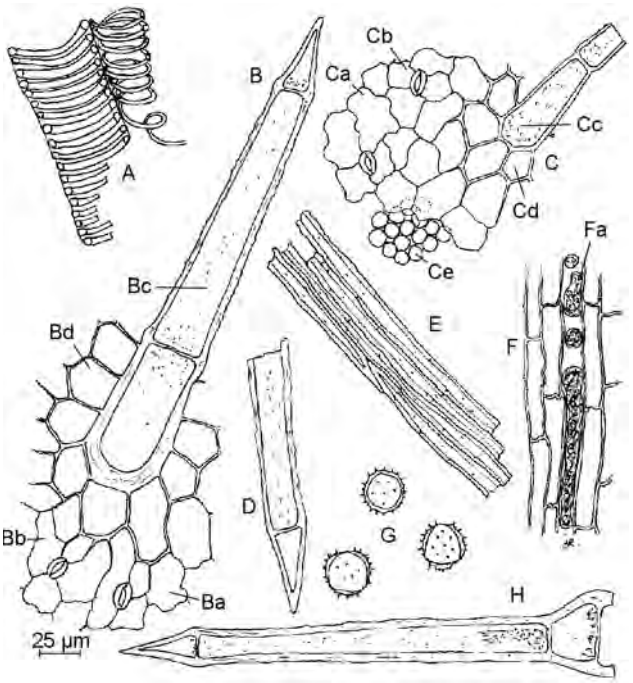


Figure 2564.-1. – *Illustration for identification test B of powdered herbal drug of eclipta herb*

B. Microscopic examination (*2.8.23*). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2564.-1): numerous isolated, whole [H] or broken [D] covering trichomes, usually tricellular, up to 700 µm long, with a broad basal cell, a relatively long median cell with thick warty walls, and a very short, pointed, sub-triangular distal cell; fragments of lamina [B, C] with sinuous epidermal cells [Ca, Ba] and anomocytic stomata (*2.8.3*) with 3-4 subsidiary cells [Cb, Bb]; some fragments of epidermis showing covering trichomes similar to those previously described, whole [Bc] or broken [Cc], and subsidiary cells with slightly thickened walls [Bd, Cd]; fragments of the upper epidermis usually accompanied by palisade parenchyma [Ce]; fragments of stem with different types of vascular bundles [A]; rare fragments of parenchyma (longitudinal

section [F]) containing secretory canals with orange-brown contents [Fa]; bundles of fibres with thickened walls [E]; pollen grains [G] about 20-25 µm in diameter, with 3 pores and a spiny exine.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate at 60 °C for 10 min. Allow to cool, centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *wedelolactone R* and 1 mg of *rosmarinic acid R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R, acetone R, toluene R* (1:6:11 *V/V/V*).

*Application*: 10 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min and treat the plate whilst still hot with a 0.5 per cent *V/V* solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A red fluorescent zone |
| | A diffuse pale blue fluorescent zone |
| | A greenish-white fluorescent zone |
| _____ | _____ |
| | A pale blue fluorescent zone |
| Wedelolactone: a bluish-white fluorescent zone | A bluish-white fluorescent zone (wedelolactone) |
| | A bluish-white fluorescent zone |
| _____ | _____ |
| Rosmarinic acid: a bluish-white fluorescent zone | |
| | A bluish-white fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 13.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.300 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *ethanol (70 per cent V/V) R* in a conical flask and weigh. Heat under a reflux condenser for 1 h, cool and weigh again. Compensate the loss of solvent with *ethanol (70 per cent V/V) R*, mix well and allow to stand. Filter through a membrane filter (nominal pore size 0.22 µm).

*Reference solution (a).* Dissolve 4.0 mg of *wedelolactone CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 2 mg of *ethyl parahydroxybenzoate R* in reference solution (a) and dilute to 50 mL with reference solution (a).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 0.2 per cent *V/V* solution of *phosphoric acid R* (24:76 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 249 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of wedelolactone.

*Relative retention* with reference to wedelolactone (retention time = about 17 min): ethyl parahydroxybenzoate = about 1.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to wedelolactone and ethyl parahydroxybenzoate.

Calculate the percentage content of wedelolactone using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to wedelolactone in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to wedelolactone in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *wedelolactone CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of wedelolactone in *wedelolactone CRS*.

**01/2013:1217**

# ELDER FLOWER

## Sambuci flos

DEFINITION

Dried flowers of *Sambucus nigra* L.

*Content*: minimum 0.80 per cent of flavonoids, expressed as isoquercitroside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The flower, about 5 mm in diameter, has 3 small bracts, visible under a lens, and may have a peduncle. The 5-toothed calyx is small; the corolla is light yellow, with 5 broadly oval petals fused at their bases into a tube. The filaments of the 5 yellow stamens alternate with the petals. The corolla is often isolated or attached to the stamens, to which it is fused at the base. The ovary is inferior and it bears a short style with 3 obtuse stigmata.

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1217.-1): numerous spherical, sometimes ellipsoidal, pollen grains about 30 µm in diameter, with 3 germinal pores and very finely pitted exine [G]; cells of the lower epidermis of the sepals often containing oil globules and covered by a striated cuticle (surface view [A]); rare fragments of the rim of the sepals showing unicellular marginal teeth (transverse section [E]);

petal fragments with numerous small globules of essential oil [H]; fragments of upper epidermis of the sepals [B] or petals [F], in surface view, with slightly and irregularly thickened walls [Ba, Fa], anomocytic stomata (*2.8.3*) [Bb, Fb] and a striated cuticle; mesophyll cells of petals and sepals with idioblasts containing numerous microsphenoid crystals of calcium oxalate [Bc]; fragments of anthers (transverse section [C], surface view [D]) showing the outer layer [Ca] and the cells of the fibrous layer [Cb, Cc, D].

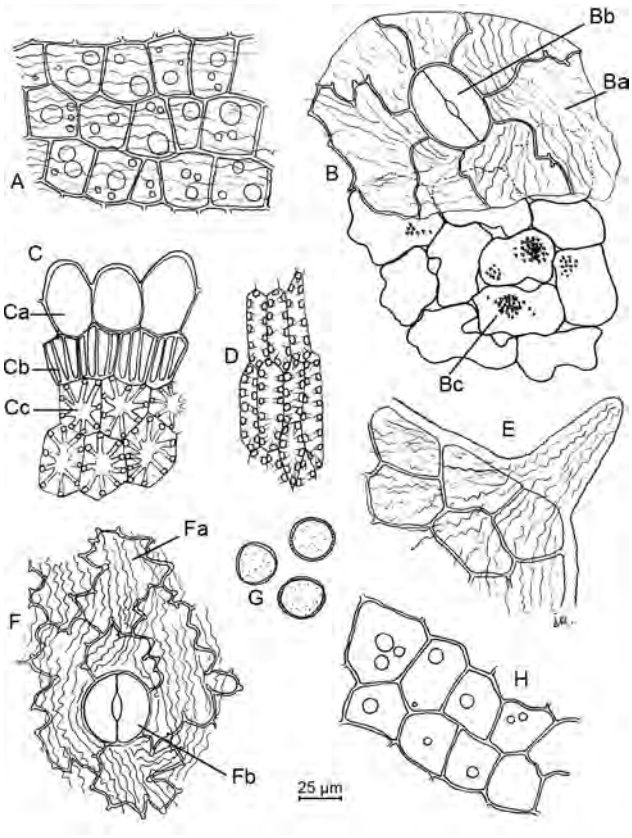


Figure 1217.-1. – *Illustration for identification B of powdered herbal drug of elder flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate for 10 min. Centrifuge for 5 min.

*Reference solution.* Dissolve 1 mg of *caffeic acid R*, 1 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat the plate for 5 min at 100 °C and treat with a 1 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then treat with a 5 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow to dry in air for 30 min. Examine in daylight (results A) and in ultraviolet light at 365 nm (results B).

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | An orange zone |
| Hyperoside: a dark yellow zone | |
| ——— | ——— |
| Rutoside: a dark yellow zone | A dark yellow zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a blue fluorescent zone | |
| | An intense, light blue fluorescent zone |
| | 2 light blue fluorescent zones |
| ——— | ——— |
| | An orange fluorescent zone |
| Hyperoside: an orange fluorescent zone | |
| Chlorogenic acid: a light blue fluorescent zone | An intense, light blue fluorescent zone |
| ——— | ——— |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 8 per cent of fragments of coarse pedicels and other foreign matter and maximum 15 per cent of discoloured, brown flowers. Carry out the determination on 10 g.

***Sambucus ebulus* L.** Examine the chromatograms obtained in identification C.

*Results B*: the chromatogram obtained with the test solution does not show a greenish-white zone above the zone due to caffeic acid in the chromatogram obtained with the reference solution; in the chromatogram obtained with the test solution, no green fluorescent zone is seen just below the orange fluorescent zone due to rutoside in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask, introduce 0.600 g of the powdered herbal drug (355) (*2.9.12*), add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the mixture

through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool, filter each extract through the plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a filter paper into a volumetric flask and dilute to 100.0 mL with *acetone R* by rinsing the flask and the filter paper. Introduce 20.0 mL of this solution into a separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 mL, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask and dilute to 50.0 mL with *ethyl acetate R*.

*Test solution.* To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 425 nm, by comparison with the compensation liquid.

Calculate the percentage content of flavonoids, expressed as isoquercitroside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of isoquercitroside to be 500.

$A$ = absorbance at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2008:1419**
**corrected 7.0**

# ELEUTHEROCOCCUS

## Eleutherococci radix

DEFINITION

Dried, whole or cut underground organs of *Eleutherococcus senticosus* (Rupr. et Maxim.) Maxim.

*Content*: minimum 0.08 per cent for the sum of eleutheroside B ($M_r$ 372.4) and eleutheroside E ($M_r$ 742.7).

IDENTIFICATION

A. The rhizome is knotty, of irregular cylindrical shape, 1.5 cm to 4.0 cm in diameter; the surface is rugged, longitudinally wrinkled and greyish-brown to blackish-brown; the bark, about 2 mm thick, closely adheres to the xylem; the heartwood is light brown and the sapwood is pale yellow; the fracture shows short thin fibres in the bark and is coarsely fibrous, especially in the internal part of the xylem. The lower surface bears numerous cylindrical and knotty roots, 3.5 cm to 15 cm long and 0.3 cm to 1.5 cm in diameter; with a smooth, greyish-brown to blackish-brown surface; the bark is about 0.5 mm thick, closely adhering to the pale yellow xylem; the fracture is slightly fibrous; in places where the outer layer has been removed, the outer surface is yellowish-brown.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows numerous groups of thick-walled, lignified fibres; fragments of reticulate and bordered pitted vessels with a wide lumen; groups of secretory canals, up to 20 µm in diameter with brown contents; parenchymatous cells containing cluster crystals of calcium oxalate 10 µm to 50 µm in diameter. Examine under a microscope, using a 50 per cent *V/V* solution of *glycerol R*. The powder shows small starch granules, rounded to slightly angular in outline, single compounds or with 2 or 3 components.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *alcohol (50 per cent V/V) R* and boil under reflux for 1 h. Cool and filter. Evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 2.5 mL of a mixture of 5 volumes of *water R* and 20 volumes of *alcohol (50 per cent V/V) R* and filter.

*Reference solution.* Dissolve 2.0 mg of *esculin R* and 2.0 mg of *catalpol R* in 20 mL of a mixture of 2 volumes of *water R* and 8 volumes of *alcohol (50 per cent V/V) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (4:30:70 *V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the reference solution shows in the upper half a blue fluorescent zone (esculin).

*Detection B*: spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A brown zone (eleutheroside B) |
| Esculin: a blue fluorescent zone (marked at 365 nm) | |
| | A reddish-brown zone (eleutheroside E) |
| _____ | _____ |
| Catalpol: a violet-brown zone | |
| _____ | _____ |
| | 2 brown zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*) in a 100 mL round-bottomed flask, add 30 mL of a mixture of equal volumes of *alcohol R* and *water R*. Heat in a water-bath at 60 °C for 30 min. Allow to cool and filter through a sintered-glass filter (*2.1.2*). Collect the liquid in a 250 mL round-bottomed flask. Repeat this operation twice, using the residue obtained in the filtration step instead of the powdered herbal drug. Add both fractions of supernatant

to the 250 mL round-bottomed flask. Evaporate under reduced pressure until about 10 mL of supernatant is left in the flask. Transfer the supernatant quantitatively to a 20.0 mL volumetric flask and dilute to 20.0 mL with a mixture of equal volumes of *alcohol R* and *water R*. Filter through a nylon filter (pore size 0.45 µm).

*Reference solution (a).* Dissolve 10 mg of *ferulic acid R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 10 mg of *caffeic acid R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solution (c).* Transfer 1 mL of reference solution (a) to a 25 mL volumetric flask and dilute to 25.0 mL with a mixture of equal volumes of *methanol R* and *water R*. Filter through a nylon filter (pore size 0.45 µm).

*Reference solution (d).* Transfer 1 mL of reference solution (a) and 1 mL of reference solution (b) in a mixture of equal volumes of *methanol R* and *water R* and dilute to 25.0 mL with the same mixture of solvents. Filter through a nylon filter (pore size 0.45 µm).

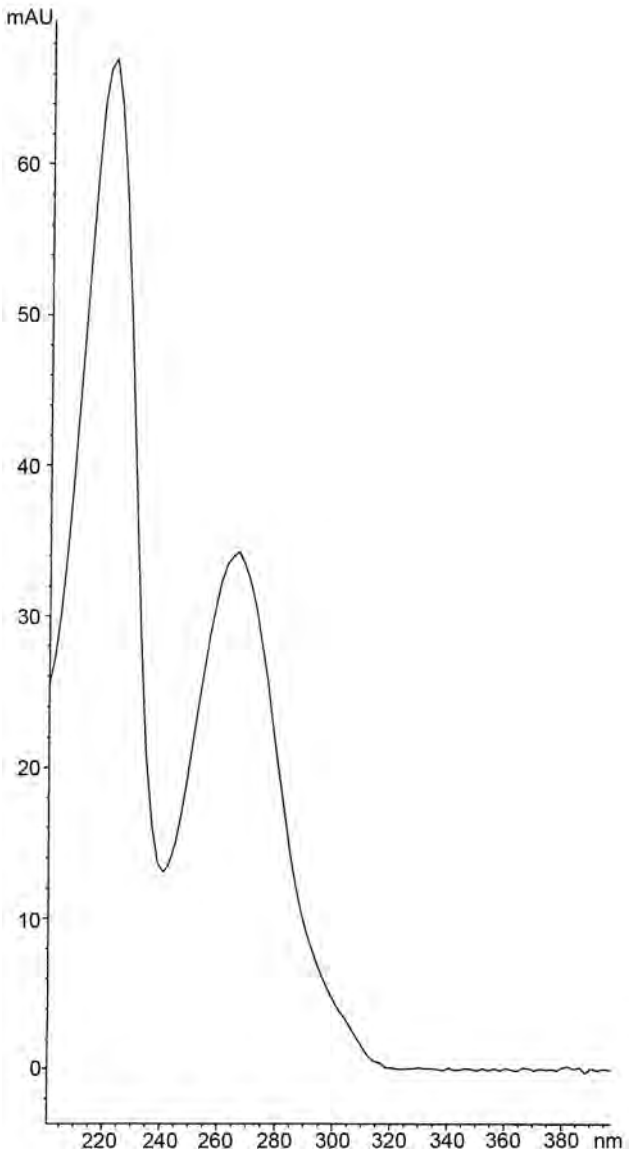


Figure 1419.-1. – *UV spectrum of eleutheroside B for the assay of eleutherococcus*

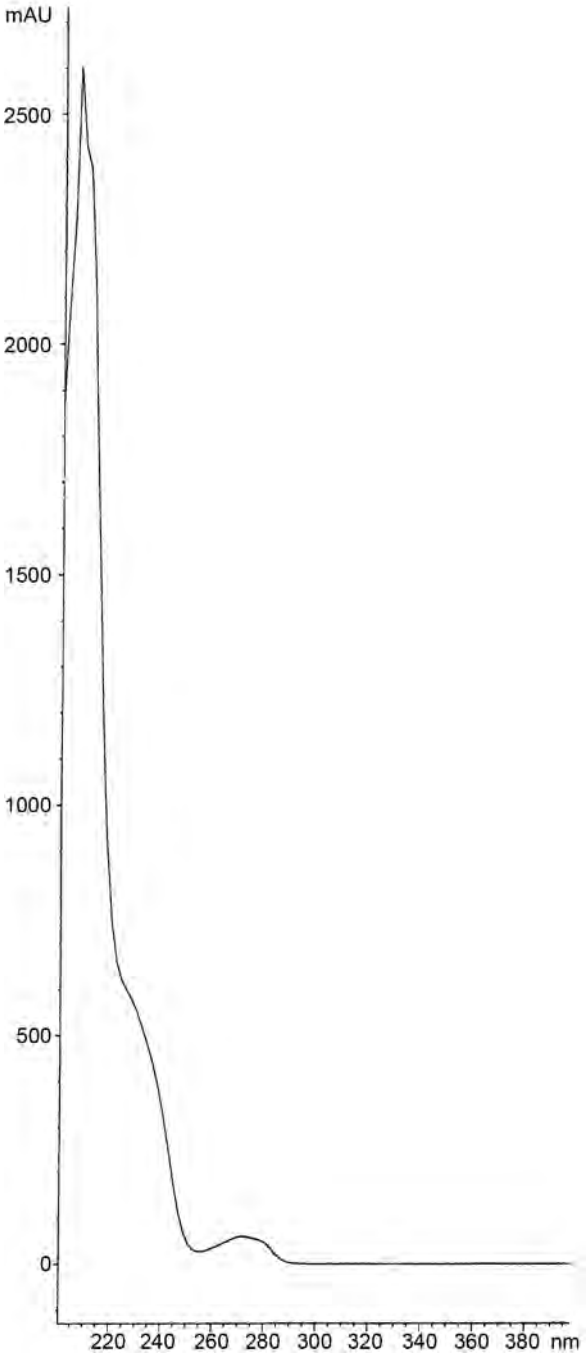


Figure 1419.-2. – *UV spectrum of eleutheroside E for the assay of eleutherococcus*

*Precolumn*:

– *size*: *l* = 4 mm, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *water R* (0.5:99.5 *V/V*),

– *mobile phase B*: *acetonitrile for chromatography R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 27 | 90 → 80 | 10 → 20 |
| 27 - 30 | 80 → 50 | 20 → 50 |
| 30 - 35 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*Retention time*: eleutheroside B = about 10 min; eleutheroside E = about 22 min.

Locate the peaks due to eleutheroside B and eleutheroside E using the UV spectra shown in Figures 1419.-1 and 1419.-2.

*System suitability*: reference solution (d):

– *resolution*: minimum 15 between the peaks due to caffeic acid and ferulic acid.

Calculate the total percentage content of eleutheroside B and eleutheroside E from the expression:

$$\frac{(A_B \times C \times 0.73 \times 2)}{(A_R \times m)} + \frac{(A_E \times C \times 1.90 \times 2)}{(A_R \times m)}$$

$A_B$ = area of the peak due to eleutheroside B in the chromatogram obtained with the test solution,

$A_E$ = area of the peak due to eleutheroside E in the chromatogram obtained with the test solution,

$A_R$ = area of the peak due to ferulic acid in the chromatogram obtained with reference solution (c),

$C$ = concentration of ferulic acid in reference solution (c), in micrograms per millilitre,

$m$ = mass of the herbal drug to be examined, in milligrams.

**07/2016:2451**

# EPHEDRA HERB

## Ephedrae herba

DEFINITION

Whole or fragmented, dried sterile aerial part of *Ephedra sinica* Stapf, *Ephedra intermedia* Schrenk and C.A.Mey. or *Ephedra equisetina* Bunge or a mixture of these.

*Content*: minimum 1.0 per cent of ephedrine ($C_{10}H_{15}NO$; $M_r$ 165.2) (dried drug).

IDENTIFICATION

A. The stems are pale green, yellowish green or light brown in colour, thin, cylindrical, branched or unbranched, and up to 30 cm long (whole drug) or in fragments up to 5 cm long (fragmented drug) and 1-3 mm in diameter; they are longitudinally striated and slightly rough, with internodes varying in length from 1-6 cm. The leaves, opposite and decussate, are reduced to sheaths surrounding the stem at the nodes, carrying diminutive laminae 1.5-4 mm long with 2 (rarely 3) acutely triangular lobes with a greyish-white apex and a reddish-brown or blackish-brown tubular base (laminae can also be shorter, with a more rounded apex). The fracture is fibrous with a light or dark reddish-brown pith.

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow, greenish-brown or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2451.-1): fragments of the epidermis (surface view [A, F]) consisting of elongated, more or less thick-walled cells [Aa] and stomata partially covered by an occasionally speckle-patterned cuticle [Ab] so that only the pore is visible; anomocytic stomata clearly visible below the cuticle (surface view [F]), with guard cells with thickened end walls [Fa]; fragments of the stem (transverse section [D]) showing the epidermis covered by a thick cuticle [Da] with papillose thickenings above some cells [Db] and composed of thick-walled epidermal cells [Dc] and sunken stomata [Dd] sometimes accompanied by cortical parenchyma with palisade cells containing small prisms of calcium oxalate [Df] and groups of fibres [De]; fibres, isolated or in groups [B], long, thick-walled and with a reduced lumen; fragments of vascular tissue [C, E] composed of pitted tracheids [Ca, Ea] and annular, spiral [Cb] or rarely reticulate vessels; groups of more or less rectangular parenchymatous cells from the pith with slightly thickened walls and often with orange or brown contents [Eb]; numerous fragments of the orange or brown contents of parenchymatous cells [G]; numerous small crystals of calcium oxalate occurring as prisms or cluster crystals [Ba], frequently agglutinated along fibres or in parenchymatous cells [H] or isolated.

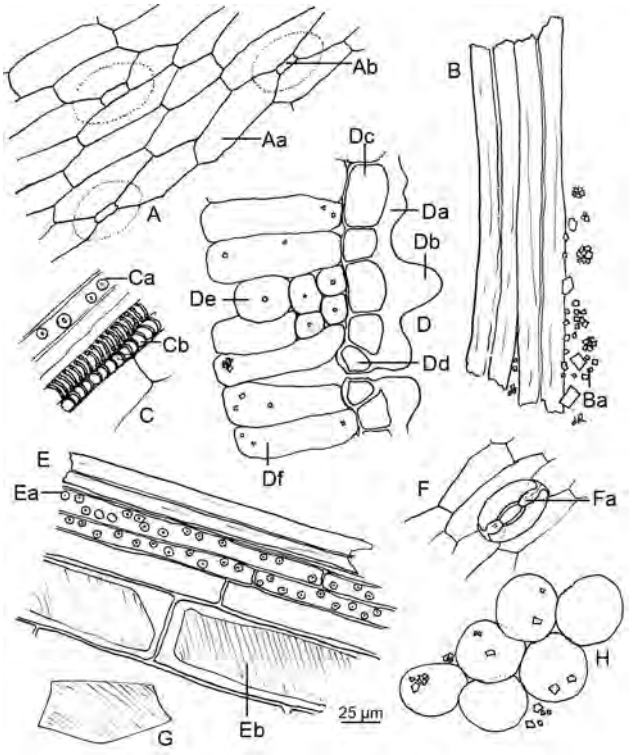


Figure 2451.-1. – *Illustration for identification test B of powdered herbal drug of ephedra herb*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.2 g of the powdered herbal drug (355) (*2.9.12*) add 0.5 mL of *concentrated ammonia R* and 10 mL of *methylene chloride R*. Boil in a water-bath under a reflux condenser for 1 h. Allow to cool, filter and evaporate the filtrate to dryness; dissolve the residue in 2 mL of *methanol R*.

*Reference solution*. Dissolve 1 mg of *ephedrine hydrochloride CRS* and 1 mg of *2-indanamine hydrochloride R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

Herbal drugs

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:10:40 *V/V/V*).

*Application*: 10 µL [or 1 µL] as spots with a diameter of 5 mm [or 2 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *ninhydrin R* in *ethanol (96 per cent) R*; heat at 110 °C for 10 min and examine immediately in daylight.

*Results*: see below the sequence of spots present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint spots may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| 2-Indanamine: a purple spot | A purple spot may be present |
| Ephedrine: a purple spot at the border between the middle and lower thirds | A purple spot (ephedrine) at the border between the middle and lower thirds |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 0.200 g of the powdered herbal drug (355) (*2.9.12*) add 25.0 mL of *methanol R*, weigh and sonicate for 45 min. Allow to cool, weigh and adjust to the original mass with *methanol R*, shake well and filter. Transfer 1.0 mL of the filtrate to a small column (1 cm in diameter) packed with 1.50 g of *neutral aluminium oxide R* (60-210 µm). Elute with a mixture of equal volumes of *methanol R* and *water R*. Collect about 9 mL of the eluate, add 0.5 mL of *phosphoric acid R* and dilute to 10.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution (a)*. Dissolve 10.0 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 25.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 1 mg of *ephedrine hydrochloride CRS* and 1 mg of *terbutaline sulfate CRS* in *methanol R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 25 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, 0.1 per cent *V/V* solution of *phosphoric acid R* (15:85 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 207 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of ephedrine.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.5 between the peaks due to terbutaline and ephedrine.

Calculate the percentage content of ephedrine using the following expression:

$$\frac{A_1 \times m_2 \times p \times 165.2}{A_2 \times m_1 \times 5 \times 201.7}$$

$A_1$ = area of the peak due to ephedrine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ephedrine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ephedrine hydrochloride CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of ephedrine hydrochloride in *ephedrine hydrochloride CRS*.

**04/2012:1825**

# EQUISETUM STEM

## Equiseti herba

DEFINITION

Whole or cut, dried sterile aerial parts of *Equisetum arvense* L.

*Content*: minimum 0.3 per cent of total flavonoids, expressed as isoquercitroside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. It consists of fragments of grooved main stems, branches with longitudinal sharp ridges and leaves in whorls, united at the base into a sheath, light green or greenish-grey. The fragments are rough to the touch, brittle and crunchy when crushed. The main stems are about 1-4.5 mm in diameter, hollow, jointed at the nodes, which occur at intervals of about 1.5-4.5 cm; distinct vertical grooves are present on the internodes, ranging in number from 4 to 14 or more. The central hollow is less than 50 per cent but more than 25 per cent of the diameter of the main stem. Verticils of widely spaced and erect branches, usually simple, each about 1 mm thick with 3-5 longitudinal, sharp ridges, occur at the nodes; at the end of each ridge is a protruding, distinct collenchymatic bundle under the epidermis. The branches are not hollow. The leaves are small, linear, verticillate at each node, concrescent at the base; they form a toothed sheath around the stem with the number of teeth corresponding to the number of grooves on the stem. Each tooth, often brown, is lanceolate-triangular. The lowest internode of each branch is longer than the sheath of the stem to which it belongs.

B. Microscopic examination (*2.8.23*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1825.-1): fragments of the epidermis (surface view [B, C]) composed of rectangular cells with wavy walls and paracytic stomata (*2.8.3*) in 2-4 rows, the 2 subsidiary cells are in the same plane as the epidermis, cover the guard cells and show radial ridges; small silica pilulae are scattered on the surface of the subsidiary cells and appear more frequent at the margin forming a distinct ring surrounding the subsidiary cells [C]; 2-celled papillae on the ridges, less distinct on the main stem [A] but large and rectangular on the branches, oriented longitudinally [F]; in surface view, the epidermis of the main stems consists of elongated cells [G], the epidermis of the secondary branches shows the 2-celled papillae which resemble pairs of small cells separated by a

larger cell [D]; fragments of large-celled parenchyma [H] and groups of long unlignified fibres with narrow lumens; small vessels with spiral or annular thickening [E].

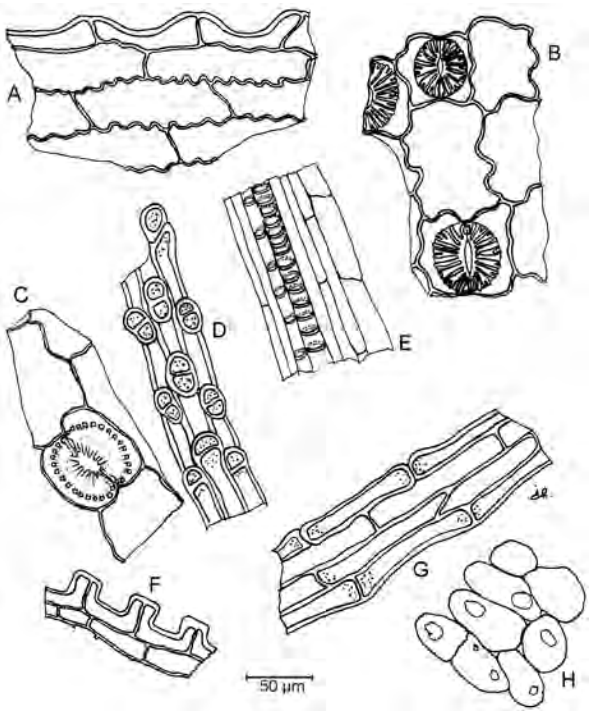


Figure 1825.-1. – *Illustration for identification test B of powdered herbal drug of equisetum stem*

C. Examine the chromatograms obtained in the test for *Equisetum palustre*.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (b) and the test solution. Furthermore, other weak fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 red fluorescent zones |
| Caffeic acid: a greenish-blue fluorescent zone | |
| | 2 greenish-blue fluorescent zones |
| ——— | ——— |
| | An orange fluorescent zone |
| Hyperoside: an orange fluorescent zone | |
| | 2 greenish-blue fluorescent zones |
| ——— | ——— |
| Rutoside: an orange fluorescent zone | |
| **Reference solution (b)** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

***Equisetum palustre***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min with occasional shaking. Allow to cool. Filter.

*Reference solution (a)*. To 100.0 mg of *Equisetum palustre HRS* add 10 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min with occasional shaking. Allow to cool. Filter.

*Reference solution (b)*. Dissolve 1.0 mg of *caffeic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutoside trihydrate R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 *V/V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in a current of cold air for 5 min.

*Detection*: heat at 100 °C for 3 min and treat the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in a current of cold air and examine after 10 min in ultraviolet light at 365 nm.

*System suitability*: the chromatogram obtained with reference solution (a) shows 2 greenish fluorescent zones just above the line of application.

*Results*: in the chromatogram obtained with the test solution, any greenish fluorescent zones just above the line of application are not more intense than the corresponding zones (characteristic of *E. palustre* L.) in the chromatogram obtained with reference solution (a).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Ash insoluble in hydrochloric acid** (*2.8.1*): minimum 3.0 per cent and maximum 15.0 per cent.

**Total ash** (*2.4.16*): minimum 12.0 per cent and maximum 27.0 per cent.

ASSAY

*Stock solution*. In a 100 mL round-bottomed flask, introduce 0.800 g of the powdered herbal drug (355) (*2.9.12*) and add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool and filter each extract through a plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a filter paper into a volumetric flask and dilute to 100.0 mL with *acetone R* by rinsing the flask and the filter paper. Introduce 20.0 mL of the solution into a separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 mL, of *water R*, and filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask. Dilute to 50.0 mL with *ethyl acetate R*.

*Test solution*. To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation solution*. Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min, by comparison with the compensation solution at 425 nm. Calculate the percentage content of flavonoids, expressed as isoquercitroside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of isoquercitroside to be 500.

$A$ = absorbance at 425 nm;

$m$ = mass of the substance to be examined, in grams.

**07/2014:1320**

# EUCALYPTUS LEAF

## Eucalypti folium

DEFINITION

Whole or cut, dried leaves of older branches of *Eucalyptus globulus* Labill.

*Essential oil content*:

– for the whole drug, minimum 20 mL/kg (anhydrous drug);

– for the cut drug, minimum 15 mL/kg (anhydrous drug).

CHARACTERS

Aromatic odour of cineole.

IDENTIFICATION

A. The leaves, which are mainly greyish-green and relatively thick, are elongated, elliptical and slightly sickle-shaped and usually up to 25 cm in length and up to 5 cm in width. The petiole is twisted, strongly wrinkled and is 2-3 cm, rarely 5 cm, in length. The coriaceous, stiff leaves are entire and glabrous and have a yellowish-green midrib. Lateral veins anastomose near the margin to a continuous line. The margin is even and somewhat thickened. On both surfaces there are minute, irregularly distributed, warty, dark brown spots. Small oil glands may be seen in transmitted light.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1320.-1): fragments of glabrous lamina (surface view [A, L], transverse section [F, H]), with small, thick-walled epidermal cells bearing a thick cuticle [Fa, Ha], numerous anomocytic stomata (*2.8.3*) greater than 80 µm in diameter [Aa, La] with occasional groups of brown cork cells, 300 µm in diameter and brownish-black in their centre, and underlying palisade parenchyma [Ab, Fb]; fragments of bilateral mesophyll (side view [G]), with 2-3 layers of palisade parenchyma [Ga] on each side and in the centre several layers of spongy mesophyll [Gb] with elongated cells having the same orientation as the palisade cells and containing prisms [B, Gd] and cluster crystals of calcium oxalate [Gc, K]; large schizogenous oil glands, whole [E] or usually broken, accompanied by palisade parenchyma [Ea]; fragments of vessels [J] and thick-walled and slightly channelled fibres [C] accompanied by crystal sheaths [Ca, Ja]; crystal sheaths containing prisms of calcium oxalate [D].

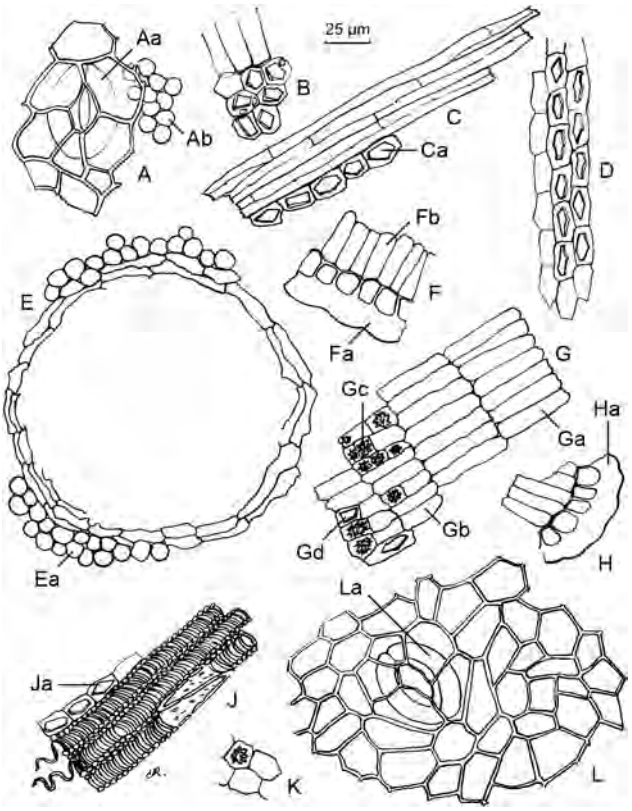


Figure 1320.-1. – *Illustration for identification test B of powdered herbal drug of eucalyptus leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.5 g of the freshly powdered herbal drug (355) (*2.9.12*) with 5 mL of *toluene R* for 2-3 min and filter over about 2 g of *anhydrous sodium sulfate R*.

*Reference solution.* Dissolve 50 µL of *cineole R* in *toluene R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 10-15 min; examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows in the middle a zone due to cineole. The chromatogram obtained with the test solution shows a principal zone similar in position and colour to the zone due to cineole in the chromatogram obtained with the reference solution, it also shows an intense violet zone (hydrocarbons) near the solvent front and there may also be other fainter zones.

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of dark and brown leaves, maximum 5 per cent of stems and maximum 2 per cent of other foreign matter. Cordate or ovate sessile leaves of young branches, with numerous glands on both sides, visible as points in transmitted light, are not present. Carry out the determination using 30 g of the herbal drug to be examined.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the herbal drug, cut immediately before determination, a 500 mL round-bottomed flask, 200 mL of *water R* and 100 mL of *glycerol R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**07/2010:0390**

# EUCALYPTUS OIL

## Eucalypti aetheroleum

DEFINITION

Essential oil obtained by steam distillation and rectification from the fresh leaves or the fresh terminal branchlets of various species of *Eucalyptus* rich in 1,8-cineole. The species mainly used are *Eucalyptus globulus* Labill., *Eucalyptus polybractea* R.T.Baker and *Eucalyptus smithii* R.T.Baker.

CHARACTERS

*Appearance*: colourless or pale yellow liquid.

*Odour*: reminiscent of 1,8-cineole.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 g of the essential oil to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 µL of *α-terpineol R* and 50 µL of *cineole R* in *toluene R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 6 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution, near the solvent front and at the level of α-terpineol.

| Top of the plate | |
|---|---|
| — | — |
| 1,8-Cineole: a violet-brown zone | An intense violet-brown zone (1,8-cineole) |
| — | — |
| α-Terpineol: a violet-brown zone | |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks due to α-pinene, β-pinene, α-phellandrene, limonene and 1,8-cineole in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a). Sabinene and camphor may be present in the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 0.906 to 0.927.

**Refractive index** (*2.2.6*): 1.458 to 1.470.

**Optical rotation** (*2.2.7*): 0° to + 10°.

**Solubility in alcohol** (*2.8.10*). It is soluble in 5 volumes of *ethanol (70 per cent V/V) R*.

**Aldehydes**. To 10 mL in a ground-glass-stoppered tube 25 mm in diameter and 150 mm long, add 5 mL of *toluene R* and 4 mL of *alcoholic hydroxylamine solution R*. Shake vigorously and titrate immediately with *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* until the red colour changes to yellow. Continue the titration with shaking; the end-point is reached when the pure yellow colour of the indicator is permanent in the lower layer after shaking vigorously for 2 min and allowing separation to take place. The reaction is complete in about 15 min. Repeat the titration using a further 10 mL of the substance to be examined and, as a reference solution for the end-point, the titrated liquid from the 1st determination to which has been added 0.5 mL of *0.5 M potassium hydroxide in alcohol (60 per cent V/V)*. Not more than 2.0 mL of *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* is required in the 2nd titration.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 200 µL of the essential oil to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 µL of *α-pinene R*, 5 µL of *β-pinene R*, 5 µL of *sabinene R*, 5 µL of *α-phellandrene R*, 10 µL of *limonene R*, 50 µL of *cineole R* and 5 mg of *camphor R* in *heptane R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 µL of *limonene R* in *heptane R* and dilute to 50.0 mL with the same solvent. Dilute 0.5 mL of the solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = about 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 60 |
| | 5 - 33 | 60 → 200 |
| | 33 - 38 | 200 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a). Record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to limonene and cineole.

*Identification of components*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *α-pinene*: 0.05 per cent to 10.0 per cent;

Herbal drugs

- *β-pinene*: 0.05 per cent to 1.5 per cent;
- *sabinene*: maximum 0.3 per cent;
- *α-phellandrene*: 0.05 per cent to 1.5 per cent;
- *limonene*: 0.05 per cent to 15.0 per cent;
- *1,8-cineole*: minimum 70.0 per cent;
- *camphor*: maximum 0.1 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

STORAGE

At a temperature not exceeding 25 °C.

**07/2017:2412**

# EUCOMMIA BARK

## Eucommiae cortex

DEFINITION

Whole or fragmented, scraped, dried bark of the stem of *Eucommia ulmoides* Oliv.

*Content*: minimum 0.10 per cent of pinoresinol diglucoside ($C_{32}H_{42}O_{16}$; $M_r$ 683) (dried drug).

IDENTIFICATION

A. Pieces are flat, curved or channelled, varying in size, about 3-7 mm thick. The outer surface is pale brown or greenish-brown, markedly wrinkled or fissured, sometimes with intentional scarring in a rhombus shape; some barks show lenticels. The inner surface is dark reddish-brown or dark purplish-brown, smooth to the touch. The texture is fragile, easily broken, with the edges of the fracture connected by fine, dense, silvery and elastic rubber threads.

B. Microscopic examination (*2.8.23*). The powder is brownish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2412.-1): many ribbon-shaped latex fragments with a granular surface, twisted or folded back on themselves [B]; numerous sclereids up to 180 µm long and 20-80 µm in diameter, isolated [F] or mostly in groups [C], with very thick and markedly channelled walls, some sclereids having masses of latex in their lumen [Ca]; fibres with very narrow lumens, usually in groups associated with sclereids [D]; fragments of cork (transverse section [A]) consisting of layers of cells with slightly and regularly thickened walls [Aa] and layers of hard cork with rectangular cells whose walls are irregularly thickened with fine pits on 3 sides and thin on the upper side [Ab]; fragments of cork consisting of polygonal cells with slightly thickened walls (surface view [G]) and fragments of hard cork consisting of polygonal cells 15-40 µm in diameter (surface view [E]); numerous isolated hard cork cells ([K], surface view [Ka], transverse section [Kb]); ovoid parenchyma cells [J]; rare fragments of phloem parenchyma (transverse section [H]) consisting of medullary rays [Ha], phloem parenchyma [Hb] and laticiferous vessels [Hc].

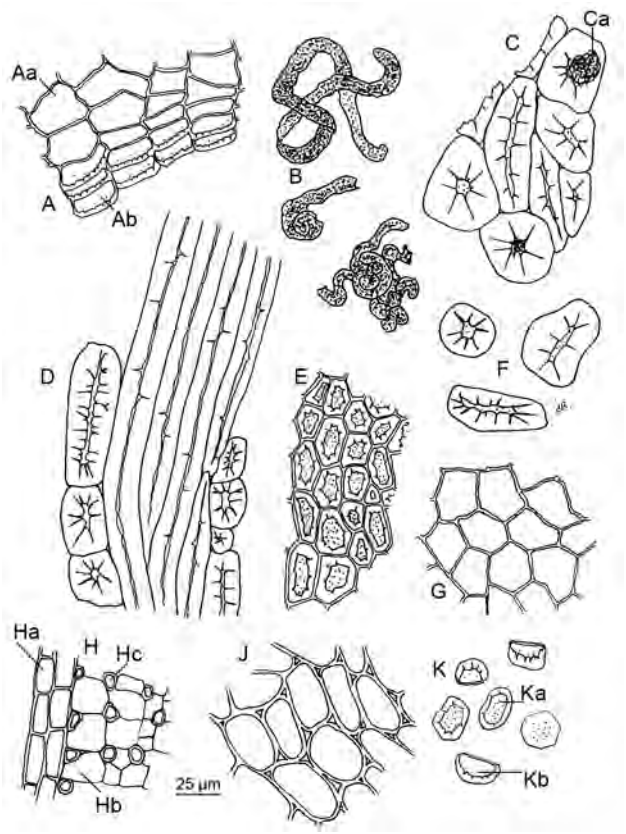


Figure 2412.-1. – *Illustration for identification test B of powdered herbal drug of eucommia bark*

C. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methylene chloride R* and allow to stand for 2 h. Filter and evaporate the filtrate to dryness. Take up the residue with 1.0 mL of *anhydrous ethanol R*; an elastic film is formed.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methylene chloride R*. Sonicate for 10 min. Discard the liquid phase and repeat the extraction with another 10 mL of *methylene chloride R*. Discard the liquid phase again. Dry the residue in air. Add 7 mL of *methanol R*. Sonicate in a centrifuge tube at 60 °C for 20 min. Centrifuge; use the supernatant.

*Reference solution.* Dissolve 2 mg of *5,7-dihydroxy-4-methylcoumarin R* and 20 mg of *β-sitosterol R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *toluene R* (1:35:65 *V/V/V*).

*Application*: 20 µL [or 10 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| _____ | _____ |
| | A violet zone |
| β-Sitosterol: a blue zone | A violet zone |
| _____ | _____ |
| | A violet zone |
| 5,7-Dihydroxy-4-methylcoumarin: an orange zone | |
| | Several zones |
| | Several zones |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Treat 2.00 g of the freshly powdered herbal drug (355) (*2.9.12*) with 75 mL of *methylene chloride R* in a continuous extraction apparatus (Soxhlet type) for 1 h. Cool. Discard the organic solution and replace with 75 mL of *methanol R*. Extract for 6 h in the same apparatus. Filter and evaporate the filtrate to dryness. Take up the residue with 10.0 mL of a mixture of *methanol R* and *water R* (30:70 *V/V*). Centrifuge. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Treat 2.00 g of *eucommia bark HRS* with 75 mL of *methylene chloride R* in a continuous extraction apparatus (Soxhlet type) for 1 h. Cool. Discard the organic solution and replace with 75 mL of *methanol R*. Extract for 6 h in the same apparatus. Filter and evaporate the filtrate to dryness. Take up the residue with 10.0 mL of a mixture of *methanol R* and *water R* (30:70 *V/V*). Centrifuge. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (b).* Dissolve 20.0 mg of *caffeine CRS* in mobile phase A and dilute to 20.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 25.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R*.

*Mobile phase*:

- *mobile phase A*: 1.0 g/L solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 87 → 75 | 13 → 25 |
| 35 - 40 | 75 → 0 | 25 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *eucommia bark HRS* and the chromatogram obtained with reference solution (a) to identify the peak due to pinoresinol diglucoside and peak 2 (unknown).

*Retention time*: caffeine = about 8 min; pinoresinol diglucoside = about 10 min; peak 2 = about 11 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peak due to pinoresinol diglucoside and peak 2.

Calculate the percentage content of pinoresinol diglucoside using the following expression:

$$\frac{A_1 \times m_2 \times 5.6 \times p}{A_2 \times m_1 \times 50}$$

$A_1$ = area of the peak due to pinoresinol diglucoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to caffeine in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *caffeine CRS* used to prepare reference solution (b), in grams;

$p$ = percentage content of caffeine in *caffeine CRS*;

5.6 = correction factor for caffeine with respect to pinoresinol diglucoside.

**07/2017:2718**
**corrected 9.6**

# EVODIA FRUIT

## Evodiae fructus

DEFINITION

Dried, whole unopened fruit of *Tetradium ruticarpum* (A.Juss.) T.G.Hartley (syn. *Evodia ruticarpa* (A.Juss.) Hook.f. & Thomson) collected just before ripening.

*Content*: minimum 0.15 per cent for the sum of evodiamine and rutecarpine, expressed as evodiamine ($C_{19}H_{17}N_3O$; $M_r$ 303.4) (dried drug).

IDENTIFICATION

A. The whole fruit is pentagonal to spheroidal, flattened at the apex, about 2-5 mm in diameter. It consists of 5 follicles more or less fused together, with a dark greenish-brown or brown external surface, rough, with numerous protuberances due to the presence of oil cavities; the flattened upper part is more or less regularly stellate whereas the conical lower surface bears the hairy remnants of the sepals and peduncle. The texture is tough. A transverse section of the fruit shows 5 loculi, each containing a tiny yellowish seed.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2718.-1): fragments of epicarp (surface view [A, B]) with cells whose walls are usually rigid and covered in a finely grained cuticle [Aa, Ba], bearing unicellular [Ac] or multicellular [Ab] covering trichomes (with fine internal separations between the cells) with spiny outer walls, occasionally remaining only as circular scars [Ae], and glandular trichomes with a multicellular stalk (2-6 cells) with slightly thickened walls and a globular, ovoid, multicellular head [Ad]; anomocytic stomata (*2.8.3*) [Bb] and acicular crystals in dark brown bundles [Bc] are present; isolated covering trichomes, often fragmented, with a sharp [C, L] or sometimes rounded [E] tip; numerous isolated glandular trichomes [H]; fragments of the outer part of the pericarp (transverse section [F]), consisting of the epicarp [Fa] with small cells, the mesocarp [Fc] and fragments of schizolysigenous oil glands [Fb]; fragments from the inner part of the mesocarp

(surface view [G], transverse section [K]) consisting of small vascular bundles with spiral vessels [Kc] and parenchyma containing very numerous cluster crystals of calcium oxalate [Ga, Kb], sometimes associated with the endocarp composed of fine elongated cells [Gb, Ka]; fragments of parenchyma from the base of the fruit [D] containing prisms of calcium oxalate [Db] and a few sclereids [Da]; sclereids, isolated or in small groups [J].

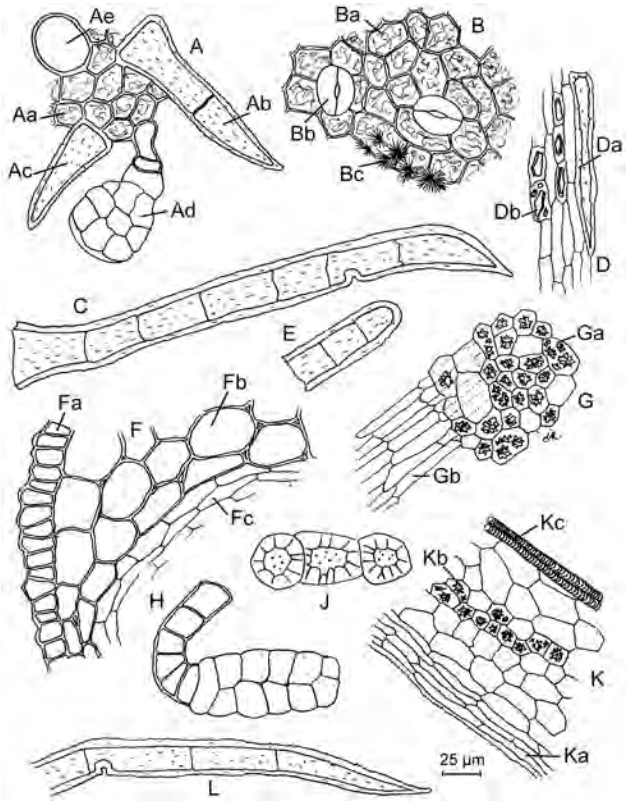


Figure 2718.-1. – *Illustration for identification test B of powdered herbal drug of evodia fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (96 per cent) R*, sonicate for 10 min, centrifuge and use the supernatant.

*Reference solution*. Dissolve 1 mg of *evodiamine R* and 1 mg of *rutecarpine R* in 1 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *ethanol (96 per cent) R*, *triethylamine R*, *ethyl acetate R*, *cyclohexane R* (1:1:5:19 *V/V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Rutecarpine: a quenching zone | A quenching zone (rutecarpine) |
| Evodiamine: a quenching zone | A quenching zone (evodiamine) |
| _____ | _____ |
| | A quenching zone |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a mixture of 10 volumes of *sulfuric acid R* and 90 volumes of *ethanol (96 per cent) R*, heat at 100 °C for 3 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 3 faint brown to violet zones |
| | A faint brownish zone |
| _____ | _____ |
| Rutecarpine: a very faint brownish zone | A faint brownish zone (rutecarpine) |
| Evodiamine: a brownish zone | A brownish zone (evodiamine) |
| _____ | _____ |
| | A brownish zone |
| | A brownish-grey zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 0.200 g of the powdered herbal drug (355) (*2.9.12*) add 10.0 mL of *ethanol (50 per cent V/V) R* and allow to macerate for 1 h. Sonicate for 30 min, centrifuge for 5 min and transfer the supernatant to a 50.0 mL volumetric flask. Add 10.0 mL of *ethanol (50 per cent V/V) R* to the residue and sonicate for 30 min. Centrifuge for 5 min and transfer the supernatant to the same volumetric flask. Repeat this operation once more. Dilute the 3 fractions of supernatant in the volumetric flask to 50.0 mL with *ethanol (50 per cent V/V) R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 5.0 mg of *evodiamine CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dilute 10.0 mL of reference solution (a) to 100.0 mL with *anhydrous ethanol R*.

*Reference solution (c).* Dissolve 2.5 mg of *rutecarpine R* in 50.0 mL of reference solution (a) and dilute to 100.0 mL with *anhydrous ethanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 45 | 55 |
| 15 - 17 | 45 → 10 | 55 → 90 |
| 17 - 30 | 10 | 90 |
| 30 - 32 | 10 → 45 | 90 → 55 |
| 32 - 45 | 45 | 55 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Retention time*: evodiamine = about 8 min; rutecarpine = about 10 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 5.0 between the peaks due to evodiamine and rutecarpine.

Calculate the percentage content of evodiamine and rutecarpine, expressed as evodiamine, using the following expression:

$$\frac{[A_1 + (A_3 \times 4)] \times m_2 \times p}{A_2 \times m_1 \times 20}$$

$A_1$ = area of the peak due to evodiamine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to evodiamine in the chromatogram obtained with reference solution (b);

$A_3$ = area of the peak due to rutecarpine in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *evodiamine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of evodiamine in *evodiamine CRS*.

**04/2013:0824 corrected 10.0**

# FENNEL, BITTER

## Foeniculi amari fructus

DEFINITION

Dry cremocarps and mericarps of *Foeniculum vulgare* Mill. ssp. *vulgare* var. *vulgare*.

*Content*:

– *essential oil*: minimum 40 mL/kg (anhydrous drug);

– *anethole*: minimum 60.0 per cent in the essential oil;

– *fenchone*: minimum 15.0 per cent in the essential oil.

CHARACTERS

Bitter fennel is greenish-brown, brown or green.

IDENTIFICATION

A. The fruit of bitter fennel is a cremocarp, of almost cylindrical shape with a rounded base and a narrower summit crowned with a large stylopod. It is generally 3-12 mm long and 3-4 mm wide. The mericarps, usually free, are glabrous. Each bears 5 prominent, slightly carenated ridges. When cut transversely, 4 vittae on the dorsal surface and 2 on the commissural surface may be seen with a lens.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown or greyish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0824.-1): yellow fragments of wide secretory canals, often made up of yellowish-brown-walled polygonal secretory cells [D, H]; reticulate parenchyma of the mesocarp [B]; numerous fibre bundles [G] from the ridges [Ga], often accompanied by narrow spiral vessels [Gb]; very numerous endosperm fragments [F] containing aleurone grains [Fb] and very small cluster crystals of calcium oxalate [Fa]; some fibre bundles from the carpophore [E]; fragments of the endocarp (surface view [A, K]) consisting of thin-walled, transversely elongated cells, 2-9 µm wide, having a parquetry arrangement, sometimes accompanied by the inner layer of the mesocarp [Aa]; fragments of the epicarp with stomata accompanied by oil droplets [C]; very numerous oil droplets [J].

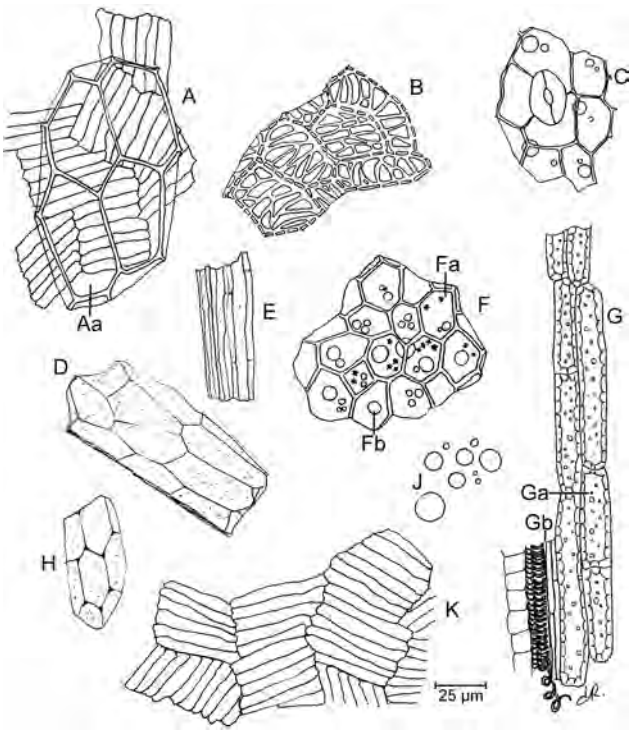


Figure 0824.-1. – *Illustration for identification test B of powdered herbal drug of bitter fennel*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.3 g of the freshly powdered herbal drug (1400) (*2.9.12*) with 5.0 mL of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 mL of *toluene R*.

*Reference solution.* Dissolve 50 µL of *anethole R* and 10 µL of *fenchone R* in 5.0 mL of *hexane R*.

*Plate*: *TLC silica gel GF$_{254}$ plate R*.

*Mobile phase*: *hexane R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL as bands of 20 mm by 3 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms show in the central part a quenching zone due to anethole.

*Detection B*: treat with *sulfuric acid R* and heat at 140 °C for 5-10 min until a yellow zone due to fenchone appears in the lower third of the chromatograms.

*Results B*: anethole appears as a violet band in the central part; the chromatogram obtained with the test solution also shows a reddish-brown zone in its upper third (terpenes).

TESTS

**Estragole**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute the mixture of essential oil and *xylene R* obtained in the determination of essential oil to 5.0 mL with *xylene R*, by rinsing the apparatus.

*Reference solution.* Dissolve 5 mg of *estragole R* in 0.5 mL of *xylene R*.

*Column*:

– *size*: *l* = 30-60 m, Ø = 0.3 mm;

– *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 0.40 mL/min.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 60 |
| | 4 - 26 | 60 → 170 |
| | 26 - 41 | 170 |
| Injection port | | 220 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Limit*:

– *estragole*: maximum 5.0 per cent in the essential oil obtained in the assay.

**Foreign matter** (*2.8.2*): maximum 1.5 per cent of peduncles and maximum 1.5 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (710) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 5.0 g of the herbal drug reduced to a powder (1400) (*2.9.12*) immediately before the assay, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid, and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**Anethole and fenchone**. Gas chromatography (*2.2.28*) as described in the test for estragole with the following modifications.

*Reference solution.* Dissolve 5 mg of *fenchone R* and 5 mg of *anethole R* in 0.5 mL of *xylene R*.

*Elution order*: the order indicated in the composition of the reference solution; record the retention times of these substances.

STORAGE

Protected from moisture.

**04/2011:0825**
**corrected 10.0**

# FENNEL, SWEET

## Foeniculi dulcis fructus

DEFINITION

Dry cremocarps and mericarps of *Foeniculum vulgare* Mill. subsp. *vulgare* var. *dulce* (Mill.) Batt. & Trab.

*Content*:

– *essential oil*: minimum 20 mL/kg (anhydrous drug);

– *anethole*: minimum 80.0 per cent in the essential oil.

CHARACTERS

Sweet fennel is pale green or pale yellowish-brown.

IDENTIFICATION

A. The fruit of sweet fennel is a cremocarp of almost cylindrical shape with a rounded base and a narrowed summit crowned with a large stylopod. It is generally 3-12 mm long and 3-4 mm wide. The mericarps, usually free, are glabrous. Each bears 5 prominent, slightly carenated ridges. When cut transversely, 4 vittae on the dorsal surface and 2 on the commissural surface may be seen with a lens.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown or greyish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0825.-1.): yellow fragments of wide secretory canals, often made up of yellowish-brown-walled polygonal secretory cells [D, H]; reticulate parenchyma of the mesocarp [B]; numerous fibre bundles [G] from the ridges [Ga], often accompanied by narrow spiral vessels [Gb]; very numerous endosperm fragments [F] containing aleurone grains [Fb] and very small calcium oxalate cluster crystals [Fa]; some fibre bundles from the carpophore [E]; fragments of the endocarp (surface view [K, A]) consisting of thin-walled, transversely elongated cells 2-9 µm wide, having a parquetry arrangement, sometimes accompanied by the inner layer of the mesocarp [Aa]; fragments of the epicarp with stomata accompanied by oil droplets [C]; very numerous oil droplets [J].

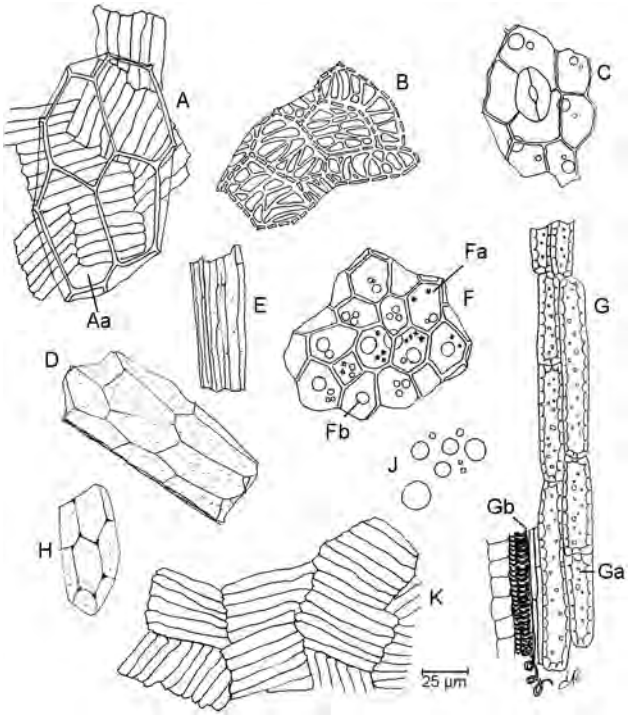


Figure 0825.-1. – *Illustration for identification test B of powdered herbal drug of sweet fennel*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.3 g of the freshly powdered herbal drug (1400) (*2.9.12*) with 5.0 mL of *methylene chloride R* for 15 min. Filter and carefully evaporate the filtrate to dryness on a water-bath at 60 °C. Dissolve the residue in 0.5 mL of *toluene R*.

*Reference solution.* Dissolve 60 µL of *anethole R* in 5.0 mL of *hexane R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *hexane R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL as bands of 20 mm by 3 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms show in the central part a quenching zone due to anethole.

*Detection B*: spray with *sulfuric acid R* and heat at 140 °C for 5 min; examine in daylight.

*Results B*: the chromatograms show in the central part a violet band due to anethole; the chromatogram obtained with the test solution also shows a reddish-brown zone in the upper third (terpenes).

TESTS

**Estragole and fenchone**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute the mixture of essential oil and *xylene R* obtained in the assay of essential oil to 5.0 mL with *xylene R*, by rinsing the apparatus.

*Reference solution.* Dissolve 5 mg of *estragole R* and 5 mg of *fenchone R* in 0.5 mL of *xylene R*.

*Column*:

– *size*: *l* = 30-60 m, Ø = 0.3 mm;

– *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 0.40 mL/min.

*Split ratio*: 1:200.

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 60 |
| | 4 - 26 | 60 → 170 |
| | 26 - 41 | 170 |
| Injection port | | 220 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Limits*:

– *estragole*: maximum 10.0 per cent in the essential oil;

– *fenchone*: maximum 7.5 per cent in the essential oil.

**Foreign matter** (*2.8.2*): maximum 1.5 per cent of peduncles and maximum 1.5 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 80 mL/kg, determined on 20.0 g of the powdered herbal drug (710) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the herbal drug reduced to a powder (1400) (*2.9.12*) immediately before the assay, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid, and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**Anethole**. Gas chromatography (*2.2.28*) as described in the test for estragole and fenchone with the following modification.

*Reference solution.* Dissolve 5 mg of *anethole R* in 0.5 mL of *xylene R*.

STORAGE

Protected from moisture.

**01/2019:1323**

# FENUGREEK

## Trigonellae foenugraeci semen

DEFINITION

Dried, ripe seeds of *Trigonella foenum-graecum* L.

IDENTIFICATION

A. The seed is hard, flattened, brown or reddish-brown and more or less rhomboidal with rounded edges. It is 3-5 mm long, 2-3 mm wide and 1.5-2 mm thick. The widest surfaces are marked by a groove that divides the seed into 2 unequal parts. The smaller part contains the radicle; the larger part contains the cotyledons.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1323.-1): fragments of the testa (transverse section [B]) with lageniform epidermal cells [Bb] covered by a thick cuticle [Ba], a hypodermis consisting of large cells, narrower at the upper end and constricted in the middle, with bar-like thickenings of the radial walls [Bc] and parenchyma with flattened cells [Bd]; fragments of the epidermis in surface view, yellowish-brown, consisting of small polygonal cells, either with thick, channelled walls and a narrow lumen (view from above [G]) or with smooth walls and a larger lumen (view from below [C]); fragments of the hypodermis in surface view, with cells having either a circular outline and

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thickened walls, closely beaded (view from above [H]), or having a polyhedral outline whose bar-like thickenings extend from the lower to the upper walls (view from below [A]); parenchyma of the testa consisting of loosely arranged cells leaving numerous spaces [E]; fragments of endosperm with cells that are rounded [F] or elongated [D] depending on the orientation, associated with mucilage [Fa] and sometimes with small spiral or annular vessels [J].

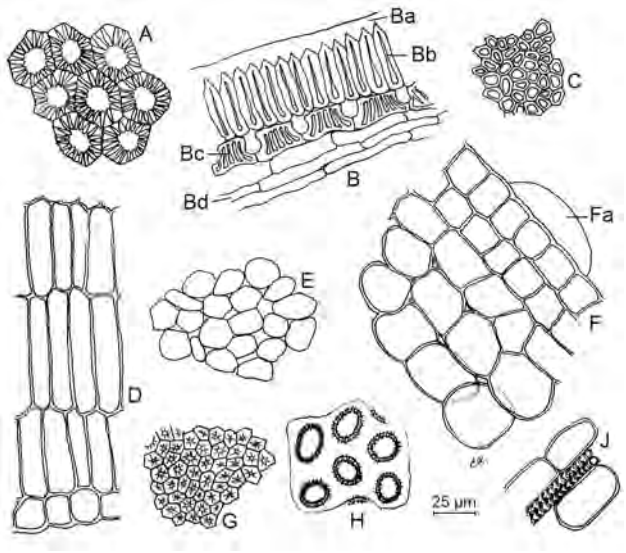


Figure 1323.-1. – *Illustration for identification test B of powdered herbal drug of fenugreek*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Place 1.0 g of the powdered herbal drug (710) (*2.9.12*) in a 25 mL conical flask and add 5.0 mL of *methanol R*. Heat in a water-bath at 65 °C for 5 min. Cool and filter.

*Reference solution.* Dissolve 3.0 mg of *trigonelline hydrochloride R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *methanol R* (30:70 *V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution shows in its lower half a quenching zone similar in position and fluorescence to the zone in the chromatogram obtained with the reference solution.

*Detection B*: spray with *potassium iodobismuthate solution R2*.

*Results B*: the chromatogram obtained with the test solution shows an intense orange-red zone similar in position and colour to the zone in the chromatogram obtained with the reference solution. It also shows in its upper half, a broad light brownish-yellow zone (triglycerides).

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Swelling index** (*2.8.4*): minimum 6, determined on the powdered herbal drug (710) (*2.9.12*).

**01/2008:1516**
**corrected 6.0**

# FEVERFEW

## Tanaceti parthenii herba

DEFINITION

Dried, whole or fragmented aerial parts of *Tanacetum parthenium* (L.) Schultz Bip.

*Content*: minimum 0.20 per cent of parthenolide ($C_{15}H_{20}O_3$; $M_r$ 248.3) (dried drug).

CHARACTERS

Camphoraceous odour.

IDENTIFICATION

A. The leafy, more or less branched stem has a diameter of up to 5 mm; it is almost quadrangular, channelled longitudinally and slightly pubescent. The leaves are ovate, 2-5 cm long, sometimes up to 10 cm, yellowish-green, petiolate and alternate. They are pinnate or bipinnate, deeply divided into 5-9 segments, each with a coarsely crenate margin and an obtuse apex. Both surfaces are somewhat pubescent and the midrib is prominent on the lower surface. When present, the flowering heads are 12-22 mm in diameter with long pedicels; they are clustered into broad corymbs consisting of 5-30 flower-heads. The hemispherical involucre is 6-8 mm wide and consists of many overlapping bracts, which are rather narrow, obtuse and scarious and have membranous margins. The central flowers are yellow, hermaphrodite, tube-shaped with 5 teeth and have 5 stamens inserted in the corolla; the filaments of the stamens are separate from each other but the anthers are fused into a tube through which passes the style, bearing 2 stigmatic branches. The peripheral flowers are female and have a white, three-toothed ligule, 2-7 mm long. The fruit is an achene, 1.2-1.5 mm long, brown when ripe, with 5-10 white longitudinal ribs. It is glandular and bears a short, crenate, membranous crown.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous large, multicellular, uniseriate covering trichomes consisting of a rhomboidal basal cell, 3-5 smaller, thick-walled rectangular cells and a very long, flat, slender terminal cell, often curved at a right angle to the axis of the basal cell; glandular trichomes with a short, biseriate, 2-4 celled stalk and a biseriate head of 4 cells around which the cuticle forms a bladder-like covering; epidermal cells with very sinuous, anticlinal walls, a striated cuticle and anomocytic stomata (*2.8.3*); numerous spirally and annularly thickened vessels; stratified parenchyma and collenchyma. Fragments of disc florets containing pale yellow amorphous masses and small rosette crystals of calcium oxalate may be present; spherical pollen grains about 25 µm in diameter, with 3 pores and a spiny exine may be present.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *methanol R*. Heat in a water-bath at 60 °C for 15 min. Allow to cool and filter. Evaporate to dryness under reduced pressure and dissolve the residue in 2 mL of *methanol R*.

*Reference solution.* Dissolve 5 mg of *parthenolide R* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetone R*, *toluene R* (15:85 *V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 5 g/L solution of *vanillin R* in a mixture of 20 volumes of *anhydrous ethanol R* and 80 volumes of *sulfuric acid R*. Examine in daylight after 5 min.

*Results*: the chromatogram obtained with the test solution shows in its central part a blue principal zone that is similar in position, colour and size to the principal zone in the chromatogram obtained with the reference solution, and somewhat below the principal zone a 2nd blue zone may be present; 1 or 2 blue zones are also present in its lower third; other violet zones may be present.

TESTS

**Foreign matter** (*2.8.2*): maximum 10.0 per cent of stem with a diameter greater than 5 mm and maximum 2.0 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Completely reduce about 50 g of the drug to be examined to a powder (355) (*2.9.12*). After homogenisation, introduce 1.00 g of the powdered herbal drug into a flask and add 40 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min. Allow to cool and filter. Rinse the filter with 15 mL of *methanol R*. Take up the residue with 40 mL of *methanol R*. Repeat the operation. Collect the filtrates and rinsings and evaporate to dryness under reduced pressure. Take up the residue with *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase. Filter (0.45 µm).

*Reference solution.* Dissolve 5.0 mg of *parthenolide R* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 2.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Retention time*: parthenolide = about 11.5 min.

Calculate the percentage content of parthenolide using the following expression:

$$\frac{A_1 \times m_2 \times 40}{A_2 \times m_1}$$

$A_1$ = area of the peak due to parthenolide in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to parthenolide in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined in the test solution, in grams;

$m_2$ = mass of parthenolide in the reference solution, in grams.

**01/2014:2433**

# FLEECEFLOWER ROOT

## Polygoni multiflori radix

DEFINITION

Whole or fragmented dried tuberous root of *Fallopia multiflora* (Thunb.) Haraldson (syn. *Polygonum multiflorum* Thunb.).

*Content*: minimum 1.0 per cent of 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside ($C_{20}H_{22}O_9$; $M_r$ 406.4) (dried drug).

IDENTIFICATION

A. The whole drug consists of an irregular, fusiform, tuberous, root 6-15 cm long and 4-12 cm in diameter; the fragmented drug consists of slices or irregular pieces.

The external surface of the root is reddish-brown with irregular wrinkles, resembling transversely elongated lenticels, and with fine rootlet scars. The texture is dense, compact and granular. The fracture is pale yellowish-brown or reddish-brown. The drug is powdery when it is fractured. In the cortex there are 4-11 bundles giving rise to a cloud-like appearance. The central xylem is large, sometimes distinguishable as a central lignified part.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: cluster crystals of calcium oxalate 10-80 µm, sometimes up to 160 µm, in diameter, with obtuse angles; rare, relatively large, isolated tetragonal prism crystals; fragments of parenchyma consisting of thin-walled, sub-rounded or rectangular cells, sometimes containing brown, yellowish-brown or reddish-brown inclusions; rare fragments of cork consisting of several layers of regular cells filled with brown contents; fragments of pitted vessels 15-180 µm in diameter; few groups of xylem fibres; scattered brown masses, varying in shape, size and colour. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple or 2-9 compound starch granules, the simple granules are sub-rounded, 4-50 µm in diameter, with a V-shaped, stellate or Y-shaped hilum, the large granules show clearly visible layers.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Heat in a water-bath at 60 °C for 15 min and filter.

*Reference solution.* Dissolve 1 mg of *emodin R* and 1 mg of *resveratrol R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *anhydrous ethanol R*, *toluene R* (1:4:16 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

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| Top of the plate | |
|---|---|
| | A yellow fluorescent zone |
| Emodin: a yellow fluorescent zone | A yellow fluorescent zone (emodin) |
| ——— | ——— |
| Resveratrol: a light blue fluorescent zone | |
| ——— | ——— |
| | A light blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Weigh 0.250 g of the powdered herbal drug (355) (*2.9.12*) in a 100 mL glass vial with a screw cap. Add 50.0 mL of a 50 per cent *V/V* solution of *methanol R*, close and extract for 1 h using ultrasound. Filter the solution through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *resveratrol CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Weigh 0.250 g of *fleeceflower root HRS* in a 100 mL glass vial with a screw cap. Add 50.0 mL of a 50 per cent *V/V* solution of *methanol R*, close and extract for 1 h using ultrasound. Filter the solution through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *anhydrous formic acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 70 | 10 → 30 |
| 15 - 16 | 70 → 20 | 30 → 80 |
| 16 - 21 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 320 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *fleeceflower root HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside and peak 2 (unknown).

*Retention time*: 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside = about 12 min; peak 2 = about 13 min; resveratrol = about 17 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peak due to 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside and peak 2.

Calculate the percentage content of 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 4 \times 0.5}$$

$A_1$ = area of the peak due to 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to resveratrol in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *resveratrol CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of resveratrol in *resveratrol CRS*.

0.5 = correction factor for resveratrol with respect to 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside.

**01/2013:2478**

# FOURSTAMEN STEPHANIA ROOT

## Stephaniae tetrandrae radix

DEFINITION

Scraped, cut and dried root of *Stephania tetrandra* S.Moore.

*Content*: minimum 1.6 per cent for the sum of tetrandrine and fangchinoline, expressed as tetrandrine ($C_{38}H_{42}N_2O_6$; $M_r$ 623) (dried drug).

IDENTIFICATION

A. The root is found as slices or irregularly cylindrical or semi-cylindrical pieces, mostly tortuous, about 0.5-1 cm thick and 1-5 cm in diameter. The greyish-yellow outer surface usually shows deep and sinuous transversal striations; the curved parts are knotty and bumpy. The texture is dense and compact. The cut surface is greyish-white and shows radial striations.

B. Microscopic examination (*2.8.23*). The powder is whitish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous fragments of parenchyma with cells having slightly thickened and moniliform walls; reticulate or pitted xylem vessels accompanied by fibres; fragments of phelloderm containing sclereids; rare cork fragments; rare, fine, rod-shaped calcium oxalate crystals. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very many round or truncated, simple or 2- or 3-compound starch granules, 10-20 µm in diameter, with a punctiform hilum.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.4 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of a mixture of 1 volume of *anhydrous formic acid R*, 9 volumes of *water R* and 40 volumes of *methanol R*. Sonicate at 25 °C for 10 min and filter.

*Reference solution.* Dissolve 10 mg of *protopine hydrochloride R* and 10 mg of *tetrandrine R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (0.3:5:10:10 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in a current of warm air for 5 min.

*Detection*: treat with a 5 g/L solution of *iodine R* in *ethanol (96 per cent) R* until the background becomes yellow; examine in daylight after the yellow colour has disappeared.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Protopine: an orange zone | |
| _____ | _____ |
| Tetrandrine: an orange zone | An orange zone (tetrandrine) |
| | An orange zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

***Aristolochia fangchi***. Test for aristolochic acids in herbal drugs (*2.8.21*). The drug to be examined complies with method A.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Tetrandrine and fangchinoline**. Liquid chromatography (*2.2.29*).

*Test solution*. In a 50 mL round-bottomed flask, weigh 0.500 g of the powdered herbal drug (355) (*2.9.12*). Add 25 mL of a 2 per cent *V/V* solution of *hydrochloric acid R* in *methanol R*. Weigh. Heat under a reflux condenser on a water-bath at 60 °C for 30 min. Cool and weigh. Adjust to the initial weight using a 2 per cent *V/V* solution of *hydrochloric acid R* in *methanol R*. Filter. Dilute 5.0 mL of the filtrate to 10.0 mL with the mobile phase.

*Reference solution*. Dissolve 10.0 mg of *tetrandrine CRS* in 5 mL of *methanol R* and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 4.1 g/L solution of *sodium laurylsulfonate for chromatography R* in a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *methanol R*, 30 volumes of *water R* and 40 volumes of *acetonitrile R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 30 min.

*Relative retention* with reference to tetrandrine (retention time = about 18 min): fangchinoline = about 0.7.

*System suitability*: test solution:

- *resolution*: minimum 3.0 between the peaks due to fangchinoline and tetrandrine.

Calculate the percentage content of tetrandrine and fangchinoline, expressed as tetrandrine, using the following expression:

$$\frac{(A_1 + A_3) \times m_2 \times p \times 5}{A_2 \times m_1}$$

$A_1$ = area of the peak due to tetrandrine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to tetrandrine in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to fangchinoline in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *tetrandrine CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of tetrandrine in *tetrandrine CRS*.

**04/2011:0025**

# FRANGULA BARK

## Frangulae cortex

DEFINITION

Dried, whole or fragmented bark of the stems and branches of *Rhamnus frangula* L. (*Frangula alnus* Miller).

*Content*: minimum 7.0 per cent of glucofrangulins, expressed as glucofrangulin A ($C_{27}H_{30}O_{14}$; $M_r$ 578.5) (dried drug).

IDENTIFICATION

A. The bark occurs in curved, almost flat or rolled fragments or in single or double quilled pieces usually 0.5-2 mm thick and variable in length and width. The greyish-brown or dark brown outer surface is wrinkled longitudinally and covered with numerous greyish, transversely elongated lenticels; when the outer layers are removed, a dark red layer is exposed. The orange-brown or reddish-brown inner surface is smooth and bears fine longitudinal striations; it becomes red when treated with alkali. The fracture is short, fibrous in the inner part.

B. Microscopic examination (*2.8.23*). The powder is yellowish or reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0025.-1): numerous phloem fibres (tangential section [D], longitudinal section [K]), partially lignified, in groups [Da, Ka] with crystal sheaths containing calcium oxalate prisms [Db, Kb], sometimes including medullary rays [Dc]; reddish-brown fragments of cork [H]; fragments of phloem parenchyma, in longitudinal section [G] containing calcium oxalate cluster crystals [A, E] or in tangential section [C] including medullary rays [Ca] and cells containing calcium oxalate cluster crystals [Cb]; a few fragments of collenchyma [F]; isolated calcium oxalate cluster crystals [B] and prisms [J].

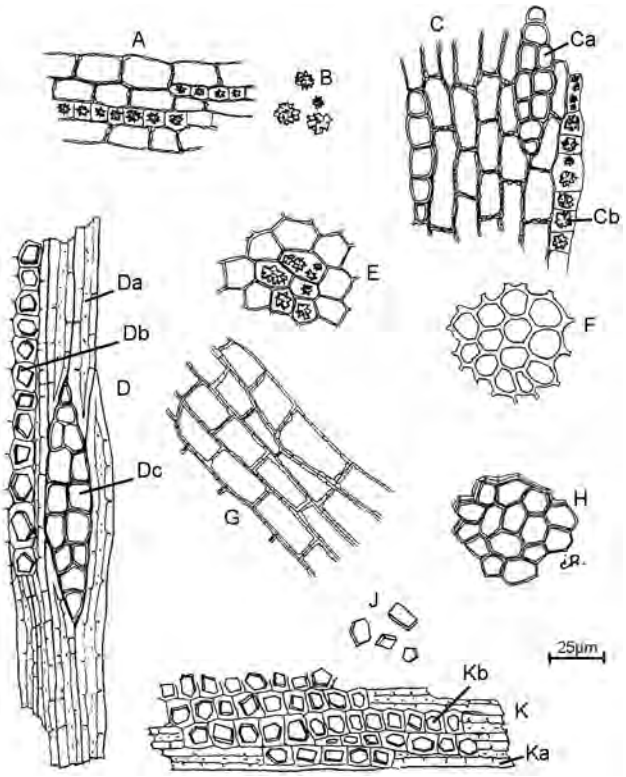


Figure 0025.-1. – *Illustration for identification test B of powdered herbal drug of frangula bark*

C. Examine the chromatogram obtained in test A for other species of *Rhamnus*; anthrones in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows 2 orange-brown zones (glucofrangulins) in the lower third and 2-4 red zones (frangulins, not always clearly separated, and above them frangula-emodin) in the upper third.

D. To about 50 mg of the powdered herbal drug (180) (*2.9.12*) add 25 mL of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 mL of *ether R* and discard the aqueous layer. Shake the ether layer with 10 mL of *dilute ammonia R1*. The aqueous layer becomes reddish-violet.

TESTS

**Other species of *Rhamnus*; anthrones**. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (180) (*2.9.12*) add 5 mL of *ethanol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant immediately and use within 30 min.

*Reference solution.* Dissolve 20 mg of *barbaloin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

*Plates*: *TLC silica gel plate R* (2 plates).

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

A. *Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 5 min.

*Detection*: spray with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R*, and heat at 100-105 °C for 15 min; examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows a brownish-yellow zone due to barbaloin in the central part; the chromatogram obtained with the test solution shows no zones of intense yellow fluorescence and no zone of orange or reddish fluorescence similar in position to the zone due to barbaloin in the chromatogram obtained with the reference solution.

B. *Application*: 10 µL of the test solution as a band.

*Development*: over a path of 10 cm.

*Drying*: in air for maximum 5 min.

*Detection*: spray immediately with a 5 g/L solution of *nitrotetrazolium blue R* in *methanol R*; examine immediately.

*Results*: no violet or greyish-blue zones appear.

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

In a tared, round-bottomed flask with a ground-glass neck, weigh 0.250 g of the powdered herbal drug (180) (*2.9.12*). Add 25.0 mL of a 70 per cent *V/V* solution of *methanol R*; mix and weigh. Heat in a water-bath under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with a 70 per cent *V/V* solution of *methanol R*. Filter and transfer 5.0 mL of the filtrate to a separating funnel. Add 50 mL of *water R* and 0.1 mL of *hydrochloric acid R*. Shake with 5 quantities, each of 20 mL, of *light petroleum R*. Allow the layers to separate and transfer the aqueous layer to a 100 mL volumetric flask. Combine the light petroleum layers and wash with 2 quantities, each of 15 mL, of *water R*. Use this water for washing the separating funnel and add it to the aqueous solution in the volumetric flask. Add 5 mL of a 50 g/L solution of *sodium carbonate R* and dilute to 100.0 mL with *water R*. Discard the light petroleum layer. Transfer 40.0 mL of the aqueous solution to a 200 mL round-bottomed flask with a ground-glass neck. Add 20 mL of a 200 g/L solution of *ferric chloride R* and heat under a reflux condenser for 20 min in a water-bath with the water level above that of the liquid in the flask. Add 2 mL of *hydrochloric acid R* and continue heating for 20 min, shaking frequently, until the precipitate is dissolved. Allow to cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R*, previously used to rinse the flask. Combine the ether extracts and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the ether layer to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 20.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of glucofrangulins, expressed as glucofrangulin A, using the following expression:

$$\frac{A \times 3.06}{m}$$

i.e. taking the specific absorbance of glucofrangulin A to be 204.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

**07/2015:1214**

# FRANGULA BARK DRY EXTRACT, STANDARDISED

## Frangulae corticis extractum siccum normatum

DEFINITION

Standardised dry extract obtained from *Frangula bark (0025)*.

*Content*: 15.0 per cent to 30.0 per cent of glucofrangulins, expressed as glucofrangulin A ($C_{27}H_{30}O_{14}$; $M_r$ 578.5) (dried extract). The measured content does not deviate from that stated on the label by more than ± 10 per cent.

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (50-90 per cent *V/V*).

CHARACTERS

*Appearance*: yellowish-brown, fine powder.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.05 g of the extract to be examined add 5 mL of *ethanol (70 per cent V/V) R* and heat to boiling. Cool and centrifuge. Decant the supernatant immediately and use within 30 min.

*Reference solution.* Dissolve 20 mg of *barbaloin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (13:17:100 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 5 min.

*Detection*: treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* and heat at 100-105 °C for 15 min; examine immediately after heating.

*Results*: the chromatogram obtained with the reference solution shows in the middle third a reddish-brown zone due to barbaloin. The chromatogram obtained with the test solution shows 2 orange-brown zones (glucofrangulins) in the lower third and 2-4 red zones (frangulins, not always clearly separated, and above them frangula-emodin) in the upper third.

B. To about 25 mg add 25 mL of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 mL of *ether R* and discard the aqueous layer. Shake the ether layer with 10 mL of *dilute ammonia R1*. The aqueous layer becomes reddish-violet.

TESTS

**Loss on drying** (*2.8.17*): maximum 5.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Into a tared round-bottomed flask with a ground-glass neck, weigh 0.100 g. Add 25.0 mL of a 70 per cent *V/V* solution of *methanol R*, mix and weigh again. Heat the flask in a water-bath under a reflux condenser at 70 °C for 15 min. Allow to cool, weigh and adjust to the original mass with a 70 per cent *V/V* solution of *methanol R*. Filter and transfer 5.0 mL of the filtrate to a separating funnel. Add 50 mL of *water R* and 0.1 mL of *hydrochloric acid R*. Shake with 5 quantities, each of 20 mL, of *light petroleum R1*. Allow the layers to separate and transfer the aqueous layer to a 100 mL volumetric flask. Combine the light petroleum layers and wash with 2 quantities, each of 15 mL, of *water R*. Use this water for washing the separating funnel and add it to the aqueous solution in the volumetric flask. Add 5 mL of a 50 g/L solution of *sodium carbonate R* and dilute to 100.0 mL with *water R*. Discard the light petroleum layer. Transfer 40.0 mL of the aqueous solution to a 200 mL round-bottomed flask with a ground-glass neck. Add 20 mL of a 200 g/L solution of *ferric chloride R* and heat under a reflux condenser for 20 min in a water-bath with the water level above that of the liquid in the flask. Add 2 mL of *hydrochloric acid R* and continue heating for 20 min, shaking frequently, until the precipitate is dissolved. Allow to cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R*, previously used to rinse the flask. Combine the ether extracts and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the ether layer to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 20.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of glucofrangulins, expressed as glucofrangulin A, using the following expression:

$$\frac{A \times 3.06}{m}$$

i.e. taking the specific absorbance of glucofrangulin A to be 204, calculated on the basis of the specific absorbance of barbaloin.

$A$ = absorbance at 515 nm;

$m$ = mass of the extract to be examined, in grams.

LABELLING

The label states the content of glucofrangulins.

**01/2015:2452**

# FRAXINUS RHYNCHOPHYLLA BARK

## Fraxini rhynchophyllae cortex

DEFINITION

Whole or fragmented, dried branch or trunk bark of *Fraxinus rhynchophylla* Hance, collected in spring or autumn.

*Content*: minimum 1.0 per cent for the sum of esculin ($C_{15}H_{16}O_9$; $M_r$ 340.3) and esculetin ($C_9H_6O_4$; $M_r$ 178.1) (dried drug).

IDENTIFICATION

A. The branch bark occurs as flexible, curved or channelled, rolled or folded pieces up to 60 cm long and 3 mm thick; the outer surface is whitish-grey to dark brownish-grey, sometimes in patches, and is smooth or slightly rough, dotted with whitish-grey, rounded lenticels; the inner surface is smooth, soft to the touch, yellowish-white or brown. The fracture is fibrous.

The trunk bark occurs as compact, rigid, slat-shaped pieces, up to 6 mm thick; the outer surface is brownish-grey, with fine longitudinal furrows and many reddish-brown lenticels, rounded or slightly split transversally; the inner surface is smooth, orange-brown. The fracture is fibrous.

B. Microscopic examination (*2.8.23*). The powder is brownish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: large sclereids up to 300 µm in diameter, single or in groups, with a very narrow lumen; fragments of brownish cork; ovoid parenchymatous cells.

C. To 0.1 g of the powdered herbal drug (355) (*2.9.12*), add 10 mL of *water R* previously heated to 60 °C. Allow to stand for 2 min and filter. Examined in ultraviolet light at 365 nm, the solution shows an intense blue fluorescence that fades considerably after the addition of 2 mL of *hydrochloric acid R*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Heat in a water-bath at 60 °C for 1 min. Centrifuge and use the supernatant; filter, if necessary.

*Reference solution.* Dissolve 1 mg of *esculin R* and 1 mg of *esculetin R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a solution containing 10 g/L of *diphenylboric acid aminoethyl ester R* and 50 g/L of *macrogol 400 R* in *methanol R*. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Esculetin: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (esculetin) |
| | A green fluorescent zone may be present |
| | A blue fluorescent zone may be present |
| Esculin: an intense blue fluorescent zone | An intense blue fluorescent zone (esculin) |
| | A whitish-blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*), add 50.0 mL of *methanol R* and weigh. Heat on a water-bath under a reflux condenser for 1 h. Cool and weigh again. Compensate for the loss of solvent with *methanol R* and mix. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *esculin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *esculetin CRS* in 10 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Mix 5.0 mL of reference solution (a) with 3.0 mL of reference solution (b) and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 0.1 per cent *V/V* solution of *phosphoric acid R* (12:88 *V/V*).

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 334 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of esculetin.

*Retention time*: esculin = about 4.5 min; esculetin = about 8.5 min.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to esculin and the chromatogram obtained with reference solution (b) to identify the peak due to esculetin.

*System suitability*: reference solution (c):

– *resolution*: minimum 5.0 between the peaks due to esculin and esculetin.

Calculate the percentage content of the sum of esculetin and esculin using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 0.5}{A_2 \times m_1} + \frac{A_3 \times m_3 \times p_2 \times 0.3}{A_4 \times m_1}$$

$A_1$ = area of the peak due to esculin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to esculin in the chromatogram obtained with reference solution (c);

$A_3$ = area of the peak due to esculetin in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to esculetin in the chromatogram obtained with reference solution (c);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *esculin CRS* used to prepare reference solution (a), in grams;

$m_3$ = mass of *esculetin CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of esculin in *esculin CRS*;

$p_2$ = percentage content of esculetin in *esculetin CRS*.

**04/2019:2394**

# FRESH BILBERRY FRUIT DRY EXTRACT, REFINED AND STANDARDISED

## Myrtilli fructus recentis extractum siccum raffinatum et normatum

DEFINITION

Refined and standardised dry extract produced from *Fresh bilberry fruit (1602)*.

*Content*: 32.4 per cent to 39.6 per cent of anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride [chrysanthemin ($C_{21}H_{21}ClO_{11}$; $M_r$ 484.8)] (dried extract).

Herbal drugs

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (70-96 per cent *V/V*) or methanol (minimum 60 per cent *V/V*). Refinement may be performed by ion-exchange chromatography.

CHARACTERS

*Appearance*: dark reddish-violet, amorphous, hygroscopic powder.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the extract to be examined in 25 mL of *methanol R*. Stir for 15 min and filter.

*Reference solution.* Dissolve 0.10 g of *bilberry dry extract HRS* in 25 mL of *methanol R*. Stir for 15 min and filter.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *butanol R* (16:19:65 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| A violet-red zone | A violet-red zone |
| A violet zone | A violet zone |
| A violet zone | A violet zone |
| A broad unresolved bluish-violet zone | A broad unresolved bluish-violet zone |
| ——— | ——— |
| **Reference solution** | **Test solution** |

B. Liquid chromatography (*2.2.29*) as described in the test for total anthocyanidins.

The characteristic anthocyanin peaks (peaks 1-8, 10-15 and 17) in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (b).

TESTS

**Total anthocyanidins**. Liquid chromatography (*2.2.29*). *Maintain the solutions at 4 °C.*

*Solvent mixture*: *hydrochloric acid R*, *methanol R* (2:98 *V/V*).

*Test solution.* Dissolve 0.1250 g of the extract to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 20.0 mL with *dilute phosphoric acid R*.

*Reference solution (a).* Dissolve 10.0 mg of *cyanidin chloride CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 2.0 mL of the solution to 100.0 mL with *dilute phosphoric acid R*.

*Reference solution (b).* Dissolve 0.1250 g of *bilberry dry extract HRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 20.0 mL with *dilute phosphoric acid R*.

*Column*:

- *size*: *l* = 0.250 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with extended pH range R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *anhydrous formic acid R*, *water for chromatography R* (8.5:91.5 *V/V*);
- *mobile phase B*: *anhydrous formic acid R*, *acetonitrile R*, *methanol R*, *water for chromatography R* (8.5:22.5:22.5:41.5 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 93 → 75 | 7 → 25 |
| 35 - 45 | 75 → 35 | 25 → 65 |
| 45 - 46 | 35 → 0 | 65 → 100 |
| 46 - 50 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 535 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *bilberry dry extract HRS* and the chromatograms obtained with reference solutions (a) and (b) to identify the peaks due to the anthocyanins and the anthocyanidins.

*Retention times*: the retention times and the elution order of the peaks are similar to those shown in the chromatogram (Figure 2394.-1).

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to cyanidin 3-*O*-galactoside (peak 3) and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to delphinidin 3-*O*-arabinoside (peak 4).

Calculate the percentage content of total anthocyanidins, expressed as cyanidin chloride, using the following expression:

$$\frac{A_1 \times m_2 \times 100 \times p}{m_1 \times A_2 \times 1250}$$

$A_1$ = sum of the areas of the peaks due to the anthocyanidins (peaks 9, 16, 18-20) in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to cyanidin chloride (peak 16) in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *cyanidin chloride CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of cyanidin chloride in *cyanidin chloride CRS*.

*Limit*: not more than 1.0 per cent of total anthocyanidins, expressed as cyanidin chloride.

**Loss on drying** (*2.8.17*): maximum 4.5 per cent.

**Total ash** (*2.4.16*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for total anthocyanidins with the following modification.

*Injection*: test solution and reference solution (b).

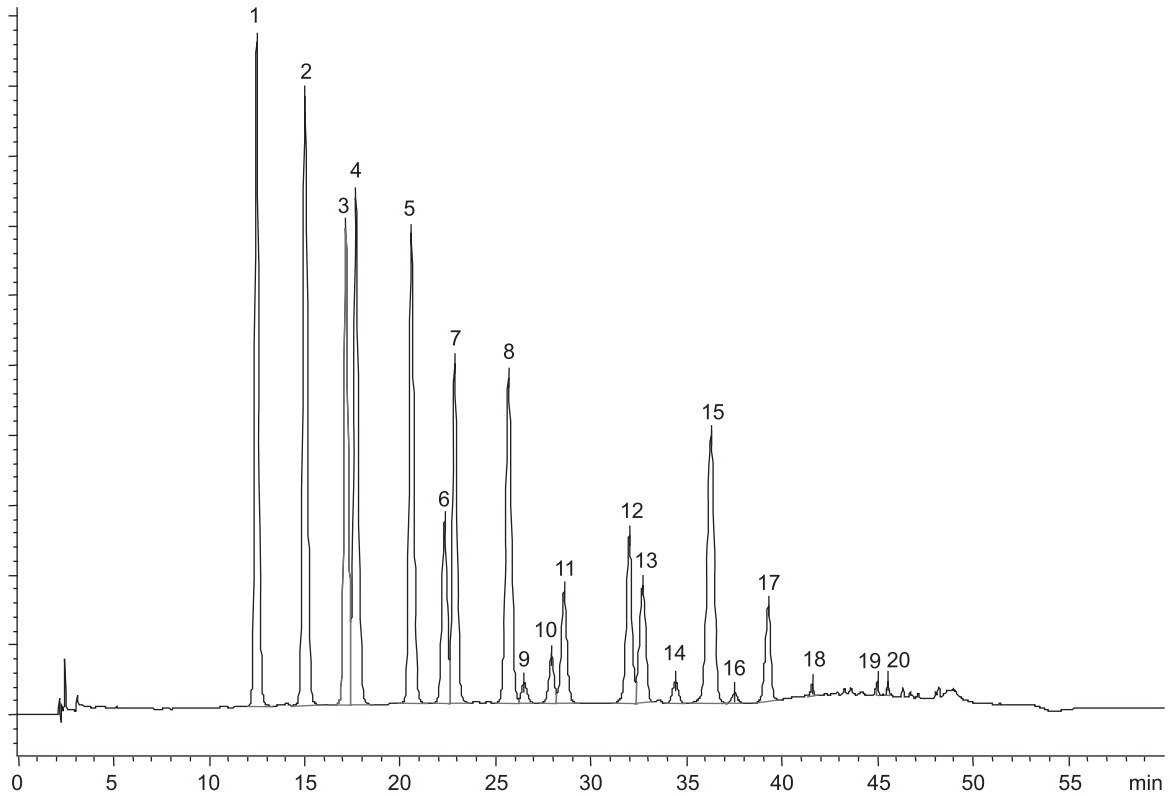


1. delphinidin 3-*O*-galactoside chloride
2. myrtillin (delphinidin 3-*O*-glucoside chloride)
3. cyanidin 3-*O*-galactoside chloride
4. delphinidin 3-*O*-arabinoside chloride
5. chrysanthemin (cyanidin 3-*O*-glucoside chloride)
6. petunidin 3-*O*-galactoside chloride
7. cyanidin 3-*O*-arabinoside chloride
8. petunidin 3-*O*-glucoside chloride
9. delphinidin chloride
10. peonidin 3-*O*-galactoside chloride
11. petunidin 3-*O*-arabinoside chloride
12. peonidin 3-*O*-glucoside chloride
13. malvidin 3-*O*-galactoside chloride
14. peonidin 3-*O*-arabinoside chloride
15. malvidin 3-*O*-glucoside chloride
16. cyanidin chloride
17. malvidin 3-*O*-arabinoside chloride
18. petunidin chloride
19. peonidin chloride
20. malvidin chloride

Figure 2394.-1. – *Chromatogram for the assay of refined and standardised fresh bilberry fruit dry extract*

Calculate the percentage content of total anthocyanins, expressed as cyanidin 3-*O*-glucoside chloride, using the following expression:

$$\frac{A_1 \times m_2 \times p}{m_1 \times A_2}$$

$A_1$ = sum of the areas of the peaks due to the anthocyanins (peaks 1-8, 10-15 and 17) in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to cyanidin 3-*O*-glucoside chloride (peak 5) in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *bilberry dry extract HRS* used to prepare reference solution (b), in grams;

$p$ = percentage content of cyanidin 3-*O*-glucoside chloride in *bilberry dry extract HRS*.

**07/2015:1869 corrected 9.2**

# FUMITORY

## Fumariae herba

DEFINITION

Whole or fragmented, dried aerial parts of *Fumaria officinalis* L. harvested in full bloom.

*Content*: minimum 0.40 per cent of total alkaloids, expressed as protopine ($C_{20}H_{19}NO_5$; $M_r$ 353.4) (dried drug).

IDENTIFICATION

A. The hollow, angular stem is light green or greenish-brown. The leaves are alternate, bipinnatisect with 2 or 3 leaf segments, the ultimate lobes lanceolate or obovate; they are greenish-blue and glabrous on both surfaces. The flowers are small and occur in loose racemes; each has a short pedicel and is subtended by a leafy bract; they are pink or purplish-red, dark purple or brown at the apex; the calyx is short, composed of 2 petalloid sepals and the corolla

Herbal drugs

is tubular with 4 petals, the upper petal slightly spurred; there are 6 stamens united by their filaments into 2 groups of 3. The greenish-brown, indehiscent fruits are globular or keel-shaped, truncated or slightly emarginate at the apex, and each contains a small brown seed.

B. Microscopic examination (*2.8.23*). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1869.-1): fragments of the leaf lamina (surface view [D]) with the upper epidermis composed of irregularly polygonal cells [Da], some of which contain microcrystals of calcium oxalate [Db], and underlying palisade parenchyma [Dc]; marginal cells at the apex of the lamina elongated to form blunt papillae [Dd], and with the lower epidermis [A] composed of cells having wavier walls [Aa] and underlying spongy parenchyma [Ac]; anomocytic stomata (*2.8.3*) [Ab, De] on both surfaces; groups [G] of lignified fibres [Ga] and spiral [Gb], reticulate or bordered-pitted [B] vessels from the stem; fragments of the epidermis of the petals [F] composed of polygonal cells with sinuous or wavy anticlinal walls and no papillae; spherical pollen grains [E], about 30 µm in diameter, with a pitted exine and 6 large pores; fragments of the fruit with polygonal cells with a thick, warty cuticle, from the epicarp [H], and sinuous sclereids with thick and channelled walls, from the endocarp [C].

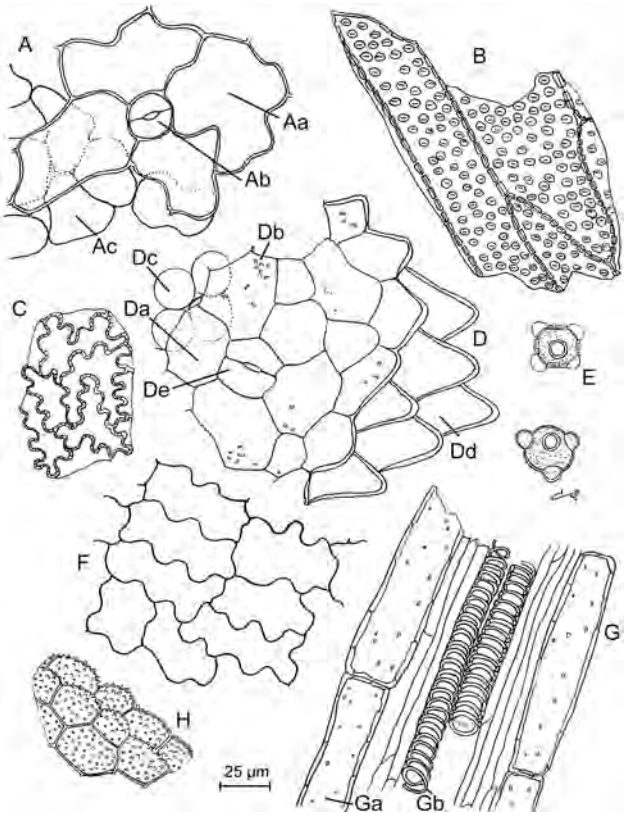


Figure 1869.-1. – *Illustration for identification test B of powdered herbal drug of fumitory*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2 g of the powdered herbal drug (355) (*2.9.12*) add 15 mL of *dilute sulfuric acid R1* and stir for 15 min. Filter. Dilute the filtrate to 20 mL with *dilute sulfuric acid R1*. Add 1 mL of *concentrated ammonia R* and 10 mL of *ethyl acetate R*. Stir and centrifuge. Collect the upper organic layer. Repeat the extraction in the same manner. Collect the organic layers and dry over *anhydrous sodium sulfate R*. Evaporate to dryness under reduced pressure. Take up the residue with 0.5 mL of *methanol R*.

*Reference solution*. Dissolve 5 mg of *protopine hydrochloride R* and 5 mg of *quinine R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (2:6:40:52 *V/V/V/V*).

*Application*: 30 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other blue fluorescent zones are present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | 4 blue fluorescent zones |
| Quinine: a blue fluorescent zone | |
| | A greenish-blue fluorescent zone |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a mixture of *potassium iodobismuthate solution R2*, *acetic acid R* and *water R* (1:2:10 *V/V/V*) until orange zones appear against a yellow background.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other less intense orange zones are present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Protopine: an orange zone | An orange zone (protopine) |
| | 2 orange zones |
| Quinine: an orange zone | A faint orange zone |
| **Reference solution** | **Test solution** |

TESTS

**Cadmium** (*2.4.27*): maximum 1.5 ppm.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

ASSAY

To 5.000 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *dilute ammonia R1* and 50 mL of *ethyl acetate R*. Shake for 15 min. Filter. Repeat the procedure in the same manner and combine the filtrates. Evaporate the filtrates to dryness under reduced pressure. Dissolve the residue by sonication for 10 min in 50 mL of *dilute sulfuric acid R1*. Filter. Dilute the filtrate to 100 mL with *dilute sulfuric acid R1*. Adjust to pH 9-10 with *concentrated ammonia R* and then add 50 mL of *ethyl acetate R*. Shake gently. Collect the upper organic layer, after centrifugation if necessary. Repeat the procedure in the same manner. Combine the organic layers and dry over *anhydrous sodium sulfate R*. Evaporate to dryness

under reduced pressure. Take up the residue with 100 mL of *anhydrous acetic acid R*. Titrate with *0.02 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.02 M perchloric acid* is equivalent to 7.068 mg of protopine.

Calculate the percentage content of total alkaloids, expressed as protopine, using the following expression:

$$\frac{n \times 706.8}{m}$$

$n$ = volume of *0.02 M perchloric acid* used, in millilitres;

$m$ = mass of the herbal drug to be examined, in milligrams.

**01/2019:1216**

# GARLIC POWDER

## Allii sativi bulbi pulvis

DEFINITION

Bulb of *Allium sativum* L., with the outer corneous layer removed, cut, freeze-dried or dried at a temperature not exceeding 65 °C and powdered.

*Content*: minimum 0.45 per cent of allicin ($C_6H_{10}OS_2$; $M_r$ 162.3) (dried drug).

CHARACTERS

*Appearance*: light yellowish powder.

IDENTIFICATION

A. Microscopic examination (*2.8.23*). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1216.-1): numerous fragments of parenchyma (surface view [A], transverse section [B]) and groups of spiral or annular vessels, sometimes with a large diameter [C, D], accompanied by thin-walled parenchymatous cells.

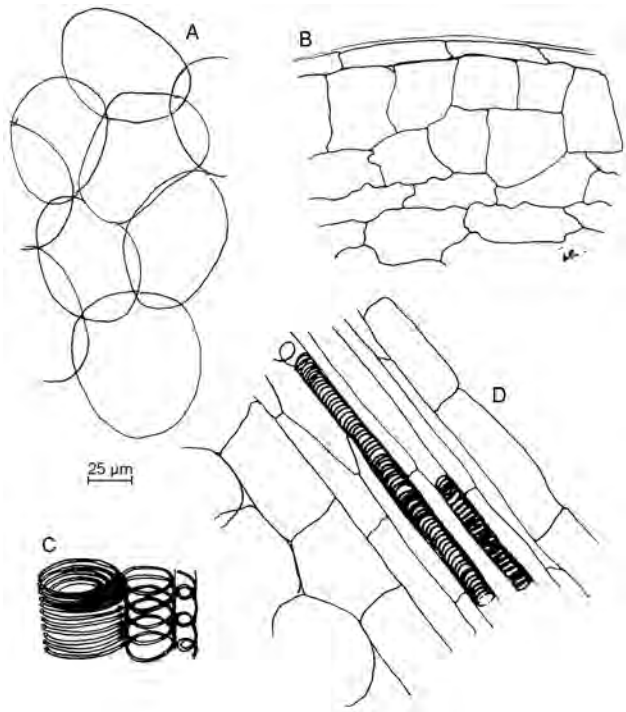


Figure 1216.-1. – *Illustration for identification test A of garlic powder*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of garlic powder add 5.0 mL of *methanol R*, shake for 60 s and filter.

*Reference solution.* Dissolve 5 mg of *alanine R* in 10 mL of *water R* and dilute to 20 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *propanol R*, *water R*, *anhydrous ethanol R* (20:20:20:40 *V/V/V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *ninhydrin R* in a mixture of 5 volumes of *glacial acetic acid R* and 95 volumes of *butanol R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows a violet zone (alanine) in the middle third. The chromatogram obtained with the test solution shows a violet or brownish-red zone similar in position to that in the chromatogram obtained with the reference solution and corresponding to alliin; above and below this zone are other, generally fainter, violet zones.

TESTS

**Starch**. Examine the powdered herbal drug under a microscope using *water R*. Add *iodine solution R1*. No blue colour develops.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Carry out the assay as quickly as possible.*

*Internal standard solution.* Dissolve 20.0 mg of *butyl parahydroxybenzoate CRS* in 100.0 mL of a mixture of equal volumes of *methanol R* and *water R*.

*Test solution.* To 0.800 g of garlic powder add 20.0 mL of *water R* and homogenise the mixture in an ultrasonic bath at 4 °C for 5 min. Allow to stand at room temperature for 30 min, then centrifuge for 30 min. Dilute 10.0 mL of the supernatant to 25.0 mL with a mixture of 40 volumes of a 1 per cent *V/V* solution of *anhydrous formic acid R* and 60 volumes of *methanol R* (stock solution). Shake and centrifuge for 5 min. Place 0.50 mL of the internal standard solution in a volumetric flask and dilute to 10.0 mL with the stock solution.

*Precolumn*:

- *size*: *l* = 20 mm, Ø = 4 mm;
- *stationary phase*: silanised *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: silanised *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 40 volumes of a 1 per cent *V/V* solution of *anhydrous formic acid R* and 60 volumes of *methanol R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 1 µL of the internal standard solution and 10 µL of the test solution.

Calculate the percentage content of allicin using the following expression:

$$\frac{S_1 \times m_2 \times 22.75}{S_2 \times m_1}$$

$S_1$ = area of the peak due to allicin (principal peak) in the chromatogram obtained with the test solution;

$S_2$ = area of the peak due to butyl parahydroxybenzoate in the chromatogram obtained with the test solution;

$m_1$ = mass of the herbal drug to be examined, in grams;

$m_2$ = mass of butyl parahydroxybenzoate in 100.0 mL of the internal standard solution, in grams. 1 mg of butylparahydroxybenzoate corresponds to 8.65 mg of allicin.

**01/2019:2721**

# GASTRODIA RHIZOME

## Gastrodiae rhizoma

DEFINITION

Steamed, fragmented and dried tuber of *Gastrodia elata* Blume.

*Content*: minimum 0.20 per cent of gastrodin ($C_{13}H_{18}O_7$; $M_r$ 286.3) (dried drug).

IDENTIFICATION

A. Whole or fragmented slices up to 3 cm in diameter and 0.1-0.2 cm thick, uniformly yellow or brownish-yellow, translucent and vitreous. The texture is corneous. The fracture is hard.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2721.-1): fragments of internal parenchyma consisting of large spheroidal cells, up to 350 µm in diameter, with slightly thickened and pitted walls [E]; numerous fragments of parenchyma with shrivelled and often ripped cells caused by the treatment of the herbal drug [B]; rare fragments of dermal tissue [A, C] with cells that are polyhedral (surface view [A]) and elongated (transverse section [Ca]) accompanied by a few layers of parenchyma cells of the same shape [Cb]; rare fragments of parenchyma containing idioblasts with raphides of calcium oxalate, the needles of which are 50-70 µm long [D]; fragments of annular or scalariform vessels up to 35 µm in diameter [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder mainly shows colourless or pale yellow, gelatinised masses, that turn uniformly violet or brownish-violet upon addition of *iodine solution R1*.

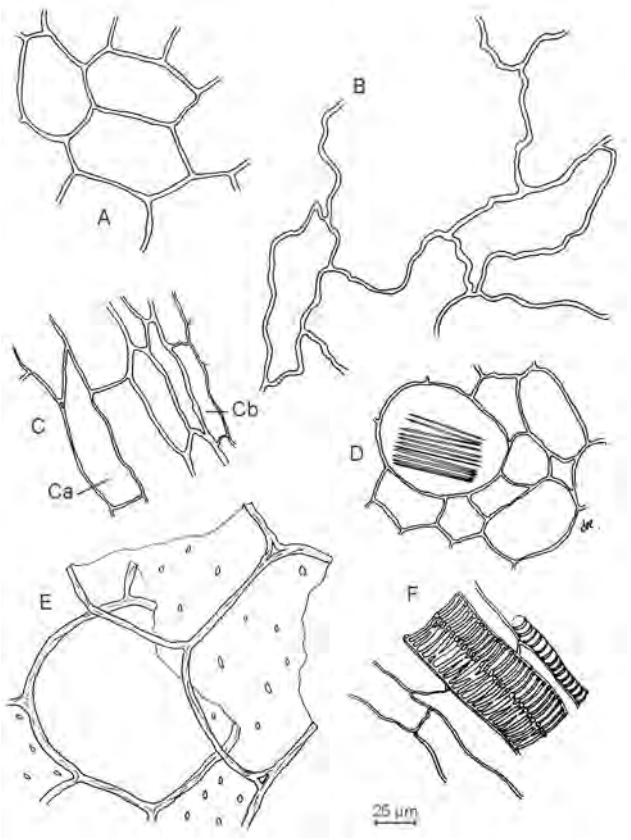


Figure 2721.-1. – *Illustration for identification test B of powdered herbal drug of gastrodia rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 1.5 mg of *gastrodin R* and 1.5 mg of *β-sitosterol R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *ethyl acetate R*, *methanol R*, *methylene chloride R* (2:20:20:58 *V/V/V/V*).

*Application*: 15 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 120 °C for 3 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| β-Sitosterol: a reddish-violet zone | A faint reddish-violet zone<br>A prominent brown zone |
| ——— | ——— |
| | A faint reddish-violet zone |
| ——— | ——— |
| Gastrodin: a brown zone | A brown zone (gastrodin)<br>A prominent dark brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 2.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (3:97 *V/V*).

*Test solution.* To 2.000 g of the powdered herbal drug (355) (*2.9.12*) in a round-bottomed flask add 30 mL of *ethanol (50 per cent V/V) R*. Heat under a reflux condenser in a water-bath at 90 °C for 3 h. Allow to cool. Filter through a paper filter and rinse the round-bottomed flask and the filter with *ethanol (50 per cent V/V) R*. Combine the filtrate and the rinsings and dilute to 50.0 mL with *ethanol (50 per cent V/V) R*. Evaporate 10.0 mL of the solution to dryness. Dissolve the residue in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *gastrodin CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 1 mg of *arbutin R* in 2 mL of reference solution (a) and dilute to 20 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 18 | 97 | 3 |
| 18 - 22 | 97 → 0 | 3 → 100 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Retention time*: arbutin = about 9 min; gastrodin = about 14 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to arbutin and gastrodin.

Calculate the percentage content of gastrodin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 1.25}{A_2 \times m_1}$$

$A_1$ = area of the peak due to gastrodin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to gastrodin in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *gastrodin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of gastrodin in *gastrodin CRS*.

**07/2015:0392**

# GENTIAN ROOT

## Gentianae radix

DEFINITION

Dried, fragmented underground organs of *Gentiana lutea* L.

CHARACTERS

Strong and persistent bitter taste.

IDENTIFICATION

A. Gentian root occurs as single or branched subcylindrical pieces of various lengths (typically 5-15 cm) and usually 5-40 mm in diameter. The surface is yellowish-brown or greyish-brown, and the colour of a transverse section is yellowish or reddish-yellow, but not reddish-brown. The root is longitudinally wrinkled and bears occasional rootlet scars. The branches of the rhizome frequently bear a terminal bud and are always encircled by closely arranged leaf scars. The rhizome and root are brittle when dry and break with a short fracture but they absorb moisture readily to become flexible. The smoothed, transversely cut surface shows a bark, occupying about one-quarter of the radius, separated by the well-marked cambium from an indistinctly radiate and mainly parenchymatous xylem.

B. Microscopic examination (*2.8.23*). The powder is light brown or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0392.-1): fragments of cork with polyhedral, thin-walled, yellowish-brown cells (surface view [E]); fragments of dermal tissue (transverse section [C]) consisting of thin-walled, yellowish-brown cork cells [Ca] and thick-walled collenchymatous cells (phelloderm) [Cb]; fragments of parenchyma (longitudinal section [B], transverse section [D]) with moderately thick-walled cells containing droplets of oil [Ba, Da], small prisms [Bb, Db] and minute needles of calcium oxalate [Bc, Dc]; isolated fragments of lignified vessels with spiral [H] or reticulate [G] thickening and up to 80 µm in diameter; fragments of xylem (longitudinal section [A], transverse section [F]) consisting of vessels [Aa, Fa] and of moderately thick-walled parenchymatous cells containing droplets of oil [Ab, Fb].

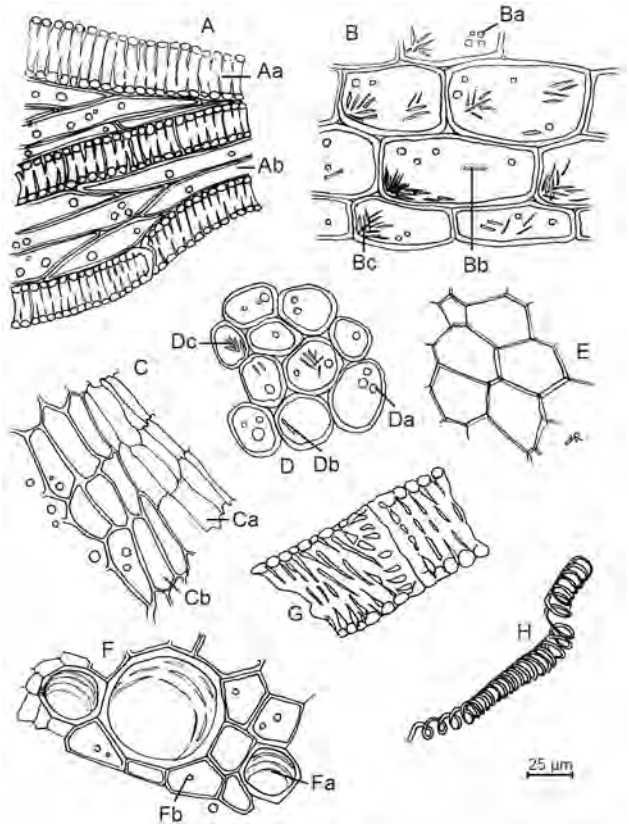


Figure 0392.-1. – *Illustration for identification test B of powdered herbal drug of gentian root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *methanol R*, shake for 15 min and filter. Evaporate the filtrate to dryness under reduced pressure, at a temperature not exceeding 50 °C. Take up the residue with small quantities of *methanol R* so as to obtain 5 mL of a solution, which may contain a sediment.

*Reference solution.* Dissolve 5 mg of *hyperoside R* and 5 mg of *phenazone R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl formate R* (4:8:88 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: in an unsaturated tank, over a path of 8 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Phenazone: a quenching zone | A prominent quenching zone |
| | A weak quenching zone (amarogentin) |
| _____ | _____ |
| _____ | _____ |
| Hyperoside: a quenching zone | A prominent quenching zone (gentiopicroside) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 100 g/L solution of *potassium hydroxide R* in *methanol R* and then with a freshly prepared 2 g/L solution of *fast blue B salt R* in a mixture of 50 volumes of *anhydrous ethanol R* and 50 volumes of *water R*; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A prominent dark violet zone |
| | A violet-red zone (amarogentin) |
| _____ | _____ |
| _____ | _____ |
| Hyperoside: a brownish-red zone | A weak light brown zone (gentiopicroside) |
| **Reference solution** | **Test solution** |

TESTS

**Other species of *Gentiana*.** Examine the chromatograms obtained in identification test C, detection B.

*Results*: the chromatogram obtained with the test solution does not show violet zones immediately above the zone due to amarogentin.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Bitterness value** (*2.8.15*): minimum 10 000.

**Water-soluble extractive**: minimum 33 per cent.

To 5.0 g of the powdered herbal drug (710) (*2.9.12*) add 200 mL of boiling *water R*. Allow to stand for 10 min, shaking occasionally. Allow to cool, dilute to 200.0 mL with *water R* and filter. Evaporate 20.0 mL of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100-105 °C. The residue weighs a minimum of 0.165 g.

**01/2008:1870**
**corrected 10.0**

# GENTIAN TINCTURE

## Gentianae tinctura

DEFINITION

Tincture produced from *Gentian root (0392)*.

PRODUCTION

The tincture is produced from 1 part of the comminuted drug and 5 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: yellowish-brown or reddish-brown liquid.

It has a strong bitter taste.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution.* Dissolve 5 mg of *phenazone R* and 5 mg of *hyperoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl formate R* (4:8:88 *V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 8 cm, in an unsaturated tank.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A prominent quenching zone |
| Phenazone: a quenching zone | |
| | A weak quenching zone (amarogentin) |
| _____ | _____ |
| _____ | _____ |
| Hyperoside: a quenching zone | A prominent quenching zone (gentiopicroside) |
| **Reference solution** | **Test solution** |

*Detection B*: spray with a 10 per cent *V/V* solution of *potassium hydroxide R* in *methanol R* and then with a freshly prepared 2 g/L solution of *fast blue B salt R* in a mixture of *ethanol R* and *water R* (50:50 *V/V*). Examine in daylight.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A prominent dark violet zone |
| | A violet-red zone (amarogentin) |
| _____ | _____ |
| _____ | _____ |
| Hyperoside: a brownish-red zone | A weak light brown zone (gentiopicroside) |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol content** (*2.9.10*): 62 per cent *V/V* to 67 per cent *V/V*.

**Bitterness value** (*2.8.15*): minimum 1000.

**Dry residue** (*2.8.16*): minimum 5.0 per cent, determined on 3.00 g.

**01/2011:1522**
**corrected 10.0**

# GINGER

## Zingiberis rhizoma

DEFINITION

Dried, whole or cut rhizome of *Zingiber officinale* Roscoe, with the cork removed, either completely or from the wide, flat surfaces only.

*Content*: minimum 15 mL/kg of essential oil (anhydrous drug).

CHARACTERS

Characteristic aromatic odour.

Spicy and burning taste.

IDENTIFICATION

A. The rhizome is laterally compressed, bearing short, flattened, obovate oblique branches on the upper side, each sometimes having a depressed scar at the apex; the whole rhizomes are about 5-10 cm long, 1.5-3 cm or 4 cm wide and 1-1.5 cm thick, sometimes split longitudinally. The scraped rhizome with a light-brown external surface shows longitudinal striations and occasional loose fibres; the outer surface of the unscraped rhizome varies from pale to dark brown and is more or less covered with cork that shows conspicuous, narrow, longitudinal and transverse ridges; the cork readily exfoliates from the lateral surfaces but persists between the branches. The fracture is short and starchy with projecting fibres. The smoothed transversely cut surface exhibits a narrow cortex separated by an endodermis from a much wider stele; it shows numerous, scattered, fibrovascular bundles and abundant scattered oleoresin cells with yellow contents. The unscraped rhizome shows, in addition, an outer layer of dark brown cork.

B. Microscopic examination (*2.8.23*). The powder is pale yellow or brownish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1522.-1): groups of large, thin-walled, septate fibres, with one wall frequently dentate [C, D, G]; fragments [K] containing vessels with reticulate thickening [Ka] often accompanied by narrow, thin-walled cells containing brown pigment [Kb] and amyliferous parenchyma [Kc]; abundant reticulate vessels, fairly large, isolated [H, L]; abundant thin-walled parenchyma of the ground tissue [J, M], some cells containing brown oleoresin [Ja]; fragments of brown cork, usually seen in surface view [F] but sometimes in transverse section [E]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant starch granules, simple, flattened, oblong or oval or irregular, up to about 50 µm long and 25 µm wide, with a small point hilum situated at the narrower end; sometimes, granules show faint, transverse striations, and may be free [A], agglomerated [B] or included in parenchymatous cells (Kc).

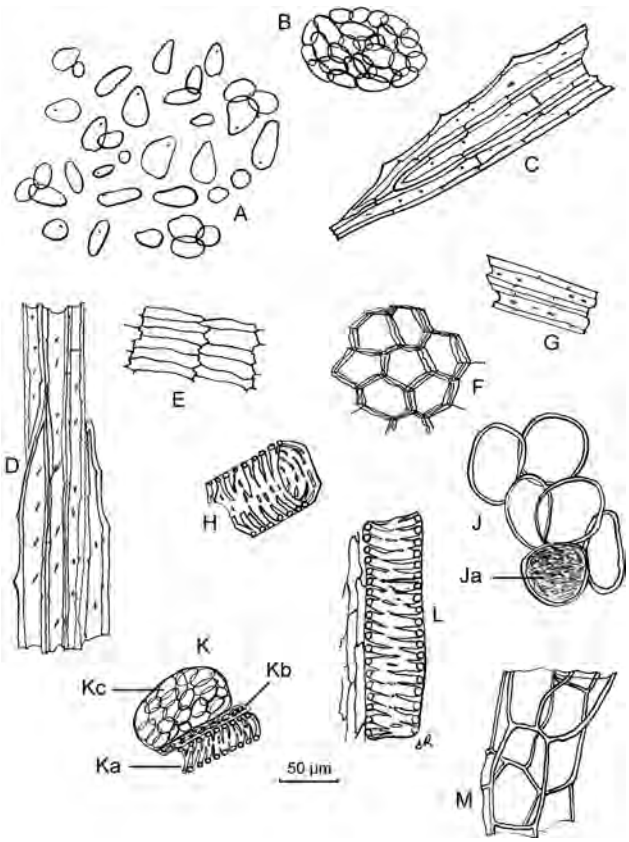


Figure 1522.-1. – *Illustration for identification test B of powdered herbal drug of ginger*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 5 mL of *methanol R*. Shake for 15 min and filter.

*Reference solution.* Dissolve 10 µL of *citral R* and 10 mg of *resorcinol R* in 10 mL of *methanol R*. Prepare the solution immediately before use.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (40:60 *V/V*).

*Application*: 20 µL as bands.

*Development*: in an unsaturated tank, over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 10 g/L solution of *vanillin R* in *sulfuric acid R* and examine in daylight while heating at 100-105 °C for 10 min.

*Results*: the chromatogram obtained with the reference solution shows in the lower half an intense red zone (resorcinol) and in the upper half 2 violet zones (citral); the chromatogram obtained with the test solution shows below the zone due to resorcinol in the chromatogram obtained with the reference solution 2 intense violet zones (gingerols) and in the middle, between the zones due to resorcinol and citral in the chromatogram obtained with the reference solution, 2 other less intense violet zones (shogaols); other zones may be present.

TESTS

**Water** (*2.2.13*): maximum 100 mL/kg, determined by distillation on 20.0 g of the powdered herbal drug (710) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the freshly, coarsely powdered herbal drug, a 1000 mL round-bottomed flask, 10 drops of *liquid paraffin R* or other antifoam, 500 mL of *water R* as distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 4 h.

**04/2008:1827**

# GINKGO DRY EXTRACT, REFINED AND QUANTIFIED

## Ginkgonis extractum siccum raffinatum et quantificatum

DEFINITION

Refined and quantified dry extract produced from *Ginkgo leaf (1828)*.

*Content*:

- *flavonoids, expressed as flavone glycosides* ($M_r$ 756.7): 22.0 per cent to 27.0 per cent (dried extract);
- *bilobalide*: 2.6 per cent to 3.2 per cent (dried extract);
- *ginkgolides A, B and C*: 2.8 per cent to 3.4 per cent (dried extract);
- *ginkgolic acids*: maximum 5 ppm (dried extract).

PRODUCTION

The extract is produced from the herbal drug by an appropriate procedure using organic solvents and their mixtures with water, physical separation steps as well as other suitable processes.

CHARACTERS

*Appearance*: bright yellow-brown, powder or friable mass.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20.0 mg of the extract to be examined in 10 mL of a mixture of 2 volumes of *water R* and 8 volumes of *methanol R*.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R* and 3.0 mg of *rutoside trihydrate R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) or [*TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:17.5:67.5 *V/V/V/V*).

*Application*: 20 µL [or 5 µL], as bands.

*Development*: over a path of 17 cm [or 6 cm].

*Drying*: at 100-105 °C.

*Detection*: spray the plate whilst still hot with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other, weaker fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A blue fluorescent zone |
| | Several faint coloured zones |
| _____ | _____ |
| | A brown fluorescent zone |
| | A green fluorescent zone |
| | An intense light blue fluorescent zone sometimes overlapped by a greenish-brown fluorescent zone |
| Chlorogenic acid: a light blue fluorescent zone | |
| | One or two green fluorescent zones |
| Rutoside: a yellowish-brown fluorescent zone | One or two yellowish-brown fluorescent zones |
| _____ | _____ |
| | Several green and yellowish-brown fluorescent zones |
| **Reference solution** | **Test solution** |

ASSAY

**Flavonoids**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.200 g of the extract to be examined in 20 mL of *methanol R*. Add 15.0 mL of *dilute hydrochloric acid R* and 5 mL of *water R* and dilute to 50.0 mL with *methanol R*. Transfer 10.0 mL of this solution into a 10 mL brown-glass vial. Close the vial with a tight rubber membrane stopper and secure with an aluminium crimped cap. Heat on a water-bath for 25 min. Allow to cool to 20 °C.

*Reference solution.* Dissolve 10.0 mg of *quercetin dihydrate CRS* in 20 mL of *methanol R*. Add 15.0 mL of *dilute hydrochloric acid R* and 5 mL of *water R* and dilute to 50.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

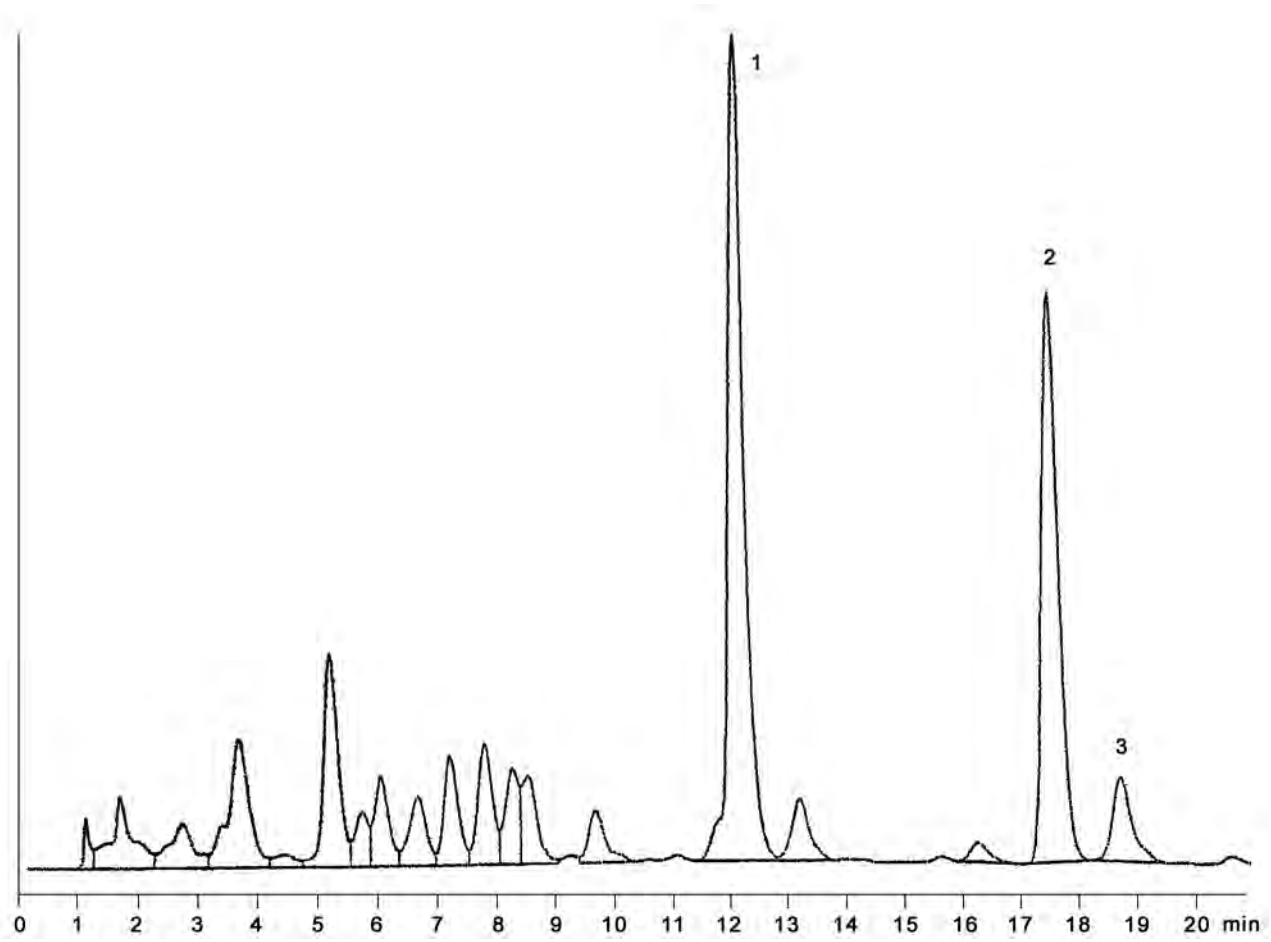


1. quercetin 2. kaempferol 3. isorhamnetin

Figure 1827.-1. – *Chromatogram for the assay of flavonoids in refined and quantified ginkgo dry extract*

*Mobile phase*:

– *mobile phase A*: 0.3 g/L solution of *phosphoric acid R* adjusted to pH 2.0;

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 60 | 40 |
| 1 - 20 | 60 → 45 | 40 → 55 |
| 20 - 21 | 45 → 0 | 55 → 100 |
| 21 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detector*: spectrophotometer at 370 nm.

*Injection*: 10 µL.

*Relative retention* with reference to quercetin (retention time = about 12.5 min): kaempferol = about 1.4; isorhamnetin = about 1.5.

*System suitability*: test solution:

– *resolution*: minimum 1.5 between the peaks due to kaempferol and isorhamnetin.

Determine the sum of the areas including all the peaks from the peak due to quercetin to the peak due to isorhamnetin in the chromatogram obtained with the test solution (see Figure 1827.-1).

Calculate the percentage content of flavonoids, expressed as flavone glycosides, using the following expression:

$$\frac{F_1 \times m_1 \times 2.514 \times p}{F_2 \times m_2}$$

$F_1$ = sum of the areas of all the peaks from the peak due to quercetin to the peak due to isorhamnetin in the chromatogram obtained with the test solution;

$F_2$ = area of the peak due to quercetin in the chromatogram obtained with the reference solution;

$m_1$ = mass of *quercetin dihydrate CRS* in the reference solution, in grams;

$m_2$ = mass of the extract to be examined used to prepare the test solution, in grams;

$p$ = percentage content of anhydrous quercetin in *quercetin dihydrate CRS*.

**Terpene lactones**. Liquid chromatography (*2.2.29*).

*Test solution.* Place 0.120 g of the extract to be examined in a 25 mL beaker and dissolve it in 10 mL of *phosphate buffer solution pH 5.8 R* by stirring. Transfer the solution into a chromatography column, about 0.15 m long and about 30 mm in internal diameter, containing 15 g of *kieselguhr for chromatography R*. Wash the beaker with 2 quantities, each of 5 mL, of *phosphate buffer solution pH 5.8 R* and transfer the washings to the chromatography column. Allow to stand for 15 min. Elute with 100 mL of *ethyl acetate R*. Evaporate the eluate to dryness at a pressure not exceeding 4 kPa in a water-bath at 50 °C. The residue of solvent is eliminated by an air-current. Take up the residue in 2.5 mL of the mobile phase.

*Reference solution (a).* Dissolve 30.0 mg of *benzyl alcohol CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Place 0.120 g of the *ginkgo dry extract for peak identification CRS* in a 25 mL beaker and dissolve it in 10 mL of *phosphate buffer solution pH 5.8 R* by stirring, then proceed as described for the test solution.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *tetrahydrofuran R*, *methanol R*, *water R* (10:20:75 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: refractometer maintained at 35 °C.

*Injection*: 100 µL.

*Identification of peaks*: use the chromatogram supplied with *ginkgo dry extract for peak identification CRS* and the chromatogram obtained with the reference solution (b) to identify the peaks due to bilobalide and ginkgolides A, B and C.

*System suitability*:

- the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *ginkgo dry extract for peak identification CRS*.

Calculate the percentage content of bilobalide, using the following expression:

$$\frac{F_1 \times m_1 \times p \times 0.025 \times 1.20}{F_5 \times m_2}$$

Calculate the percentage content of ginkgolide A, using the following expression:

$$\frac{F_2 \times m_1 \times p \times 0.025 \times 1.22}{F_5 \times m_2}$$

Calculate the percentage content of ginkgolide B, using the following expression:

$$\frac{F_3 \times m_1 \times p \times 0.025 \times 1.19}{F_5 \times m_2}$$

Calculate the percentage content of ginkgolide C, using the following expression:

$$\frac{F_4 \times m_1 \times p \times 0.025 \times 1.27}{F_5 \times m_2}$$

$F_1$ = area of the peak due to bilobalide in the chromatogram obtained with the test solution;

$F_2$ = area of the peak due to ginkgolide A in the chromatogram obtained with the test solution;

$F_3$ = area of the peak due to ginkgolide B in the chromatogram obtained with the test solution;

$F_4$ = area of the peak due to ginkgolide C in the chromatogram obtained with the test solution;

$F_5$ = area of the peak due to benzyl alcohol in the chromatogram obtained with reference solution (a);

$m_1$ = mass of *benzyl alcohol CRS* in reference solution (a), in grams;

$m_2$ = mass of the extract to be examined used to prepare the test solution, in grams;

$p$ = percentage content of benzyl alcohol in *benzyl alcohol CRS*.

Calculate the percentage content of the sum of ginkgolides A, B and C, using the following expression:

$$G_A + G_B + G_C$$

$G_A$ = percentage content of ginkgolide A;

$G_B$ = percentage content of ginkgolide B;

$G_C$ = percentage content of ginkgolide C.

**Ginkgolic acids**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.500 g of the powdered extract to be examined in 8 mL of *methanol R*, sonicating if necessary, and dilute to 10.0 mL with the same solvent. Centrifuge if necessary.

*Reference solution.* Dissolve 10.0 mg of *ginkgolic acids CRS* in 8 mL of *methanol R*, sonicating if necessary, and dilute to 10.0 mL with the same solvent. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dilute 0.1 mL of *trifluoroacetic acid R* to 1000 mL with *water R*;
- *mobile phase B*: dilute 0.1 mL of *trifluoroacetic acid R* to 1000 mL with *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 25 → 10 | 75 → 90 |
| 30 - 35 | 10 | 90 |
| 35 - 36 | 10 → 25 | 90 → 75 |
| 36 - 45 | 25 | 75 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Identification of components*: use the chromatogram supplied with *ginkgolic acids CRS* and the chromatogram obtained with the test solution to identify the peaks due to ginkgolic acids C13, C15 and C17.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to ginkgolic acids C13 and C15;
- *symmetry factor*: 0.8 to 2.0 for the peaks due to ginkgolic acids C13, C15 and C17.

Calculate the content in parts per million of ginkgolic acids expressed as ginkgolic acid C17, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2000}{A_2 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to the ginkgolic acids C13, C15 and C17 in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ginkgolic acid C17 in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ginkgolic acids CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of ginkgolic acid C17 in *ginkgolic acids CRS*.

01/2011:1828

# GINKGO LEAF

## Ginkgonis folium

DEFINITION

Whole or fragmented, dried leaf of *Ginkgo biloba* L.

*Content*: not less than 0.5 per cent of flavonoids, expressed as flavone glycosides ($M_r$ 757) (dried drug).

IDENTIFICATION

A. The leaf is greyish or yellowish-green or yellowish-brown. The upper surface is slightly darker than the lower surface. The petioles are about 4-9 cm long. The lamina is about 4-10 cm wide, fan-shaped, usually bilobate or sometimes undivided. Both surfaces are smooth, and the venation dichotomous, the veins appearing to radiate from the base; they are equally prominent on both surfaces. The distal margin is incised, irregularly and to different degrees, and irregularly lobate or emarginate. The lateral margins are entire and taper towards the base.

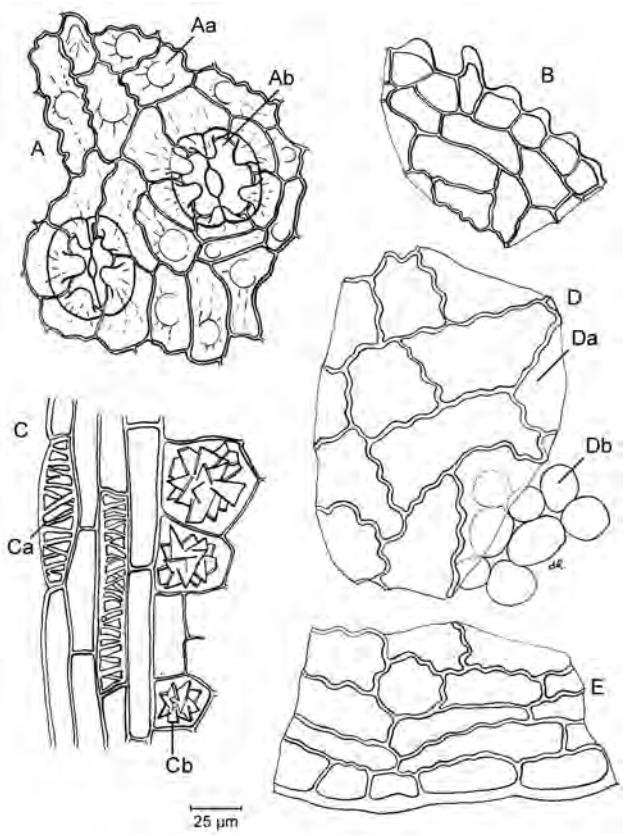


Figure 1828.-1. – *Illustration for identification test B of powdered herbal drug of ginkgo leaf*

B. Microscopic examination (*2.8.23*). The powder is greyish or yellowish-green or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1828.-1): irregularly-shaped fragments of the lamina[A, B, D, E], with the upper epidermis (surface view [D], transverse section [E]) consisting of elongated cells with irregularly sinuous walls [Da], often accompanied by palisade parenchyma [Db], and the lower epidermis (surface view [A], transverse section [B]), consisting of small cells, with a finely striated cuticle and each cell shortly papillose [Aa], and stomata [Ab] about 60 µm, wide, deeply sunken with 6-8 subsidiary cells; fragments of vascular tissue from the petiole and veins [C] with xylem [Ca] and parenchyma, some cells containing abundant cluster crystals of calcium oxalate of various sizes [Cb].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2.0 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath at 65 °C for 10 min. Shake frequently. Allow to cool to room temperature and filter.

*Reference solution*. Dissolve 1.0 mg of *chlorogenic acid R* and 3.0 mg of *rutoside trihydrate R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:17.5:67.5 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 17 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with the same volume of a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A yellowish-brown fluorescent zone |
| | A green fluorescent zone |
| | 2 yellowish-brown fluorescent zones |
| | An intense light blue fluorescent zone sometimes overlapped by a greenish-brown fluorescent zone |
| Chlorogenic acid: a light blue fluorescent zone | |
| | A green fluorescent zone |
| Rutoside: a yellowish-brown fluorescent zone | 2 yellowish-brown fluorescent zones |
| | A green fluorescent zone |
| | A yellowish-brown fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems and 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 11.0 per cent.

ASSAY

**Flavonoids**. Liquid chromatography (*2.2.29*).

*Test solution*. Heat 2.500 g of the powdered herbal drug (710) (*2.9.12*) in 50 mL of a 60 per cent *V/V* solution of *acetone R* under a reflux condenser for 30 min. Filter and collect the filtrate. Extract the drug residue a 2nd time in the same manner, using 40 mL of a 60 per cent *V/V* solution of *acetone R* and filter. Collect the filtrates and dilute to 100.0 mL with a 60 per cent *V/V* solution of *acetone R*. Evaporate 50.0 mL of the solution to eliminate the acetone and transfer to a 50.0 mL vial, rinsing with 30 mL of *methanol R*. Add 4.4 mL of *hydrochloric acid R1*, dilute to 50.0 mL with *water R* and centrifuge. Place 10 mL of the supernatant in a 10 mL brown-glass vial. Close

with a rubber seal and an aluminium cap and heat on a water-bath for 25 min. Allow to cool to room temperature.

*Reference solution*. Dissolve 10.0 mg of *quercetin dihydrate R* in 20 mL of *methanol R*. Add 15.0 mL of *dilute hydrochloric acid R* and 5 mL of *water R* and dilute to 50.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 0.3 g/L solution of *phosphoric acid R* adjusted to pH 2.0;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 60 | 40 |
| 1 - 20 | 60 → 45 | 40 → 55 |
| 20 - 21 | 45 → 0 | 55 → 100 |
| 21 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 370 nm.

*Injection*: 10 µL.

*Relative retention* with reference to quercetin (retention time = about 12.5 min): kaempferol = about 1.4; isorhamnetin = about 1.5.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to kaempferol and isorhamnetin.

Do not take into account peaks eluting before the quercetin peak or after the isorhamnetin peak in the chromatogram obtained with the test solution.

Calculate the percentage content of flavonoids, expressed as flavone glycosides, using the following expression:

$$2 \times \frac{F_1 \times m_1 \times 2.514 \times p}{F_2 \times m_2}$$

$F_1$ = sum of the areas of all the considered peaks in the chromatogram obtained with the test solution;

$F_2$ = area of the peak due to quercetin in the chromatogram obtained with the reference solution;

$m_1$ = mass of quercetin used to prepare the reference solution, in grams;

$m_2$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$p$ = percentage content of anhydrous quercetin in *quercetin dihydrate R*.

**07/2019:1523**

# GINSENG

## Ginseng radix

DEFINITION

Whole or cut dried root, designated white ginseng; treated with steam and then dried, designated red ginseng, of *Panax ginseng* C.A.Mey.

*Content*: minimum 0.40 per cent for the sum of ginsenosides Rg1 ($C_{42}H_{72}O_{14}$,$2H_2O$; $M_r$ 837) and Rb1 ($C_{54}H_{92}O_{23}$,$3H_2O$; $M_r$ 1163) (dried drug).

IDENTIFICATION

A. The principal root is fusiform or cylindrical, sometimes branched, up to about 20 cm long and 2.5 cm in diameter, and may be curved or markedly re-curved. The surface is pale yellow to cream in white ginseng, brownish-red in red ginseng and shows longitudinal ridges. Stem scars may be seen at the crown. The fracture is short. The transversely cut surface shows a wide outer zone with scattered orange-red resin canals and a finely radiate inner region. The rootlets, numerous in the lower part of white ginseng, are normally absent in red ginseng.

B. Microscopic examination (*2.8.23*). The powder is pale yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1523.-1): abundant fragments of parenchymatous cells [A, E] with thin [Ea] or slightly thickened [Aa] walls, some of which contain cluster crystals of calcium oxalate [Ab]; fragments of large secretory canals [Eb] containing yellowish-brown resin in granular masses [Ec]; non-lignified tracheids and partially lignified vessels with spiral or reticulate thickening, isolated [J]; fragments of xylem (longitudinal section [C], transverse section [F]) consisting of vessels [Ca, Fa] and thin-walled parenchymatous cells [Cb, Fb]; isolated cluster crystals of calcium oxalate [D, G]; fragments of cork (surface view [B], transverse section [H]) often associated with phelloderm having slightly thickened cells [Ha] and with the outer layers of the cortical parenchyma [Hb]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The starch granules [K] are very abundant, simple or compound, and range from 1-10 µm in diameter. In red ginseng, the starch granules are deformed and often destroyed by treating with steam, or may be absent.

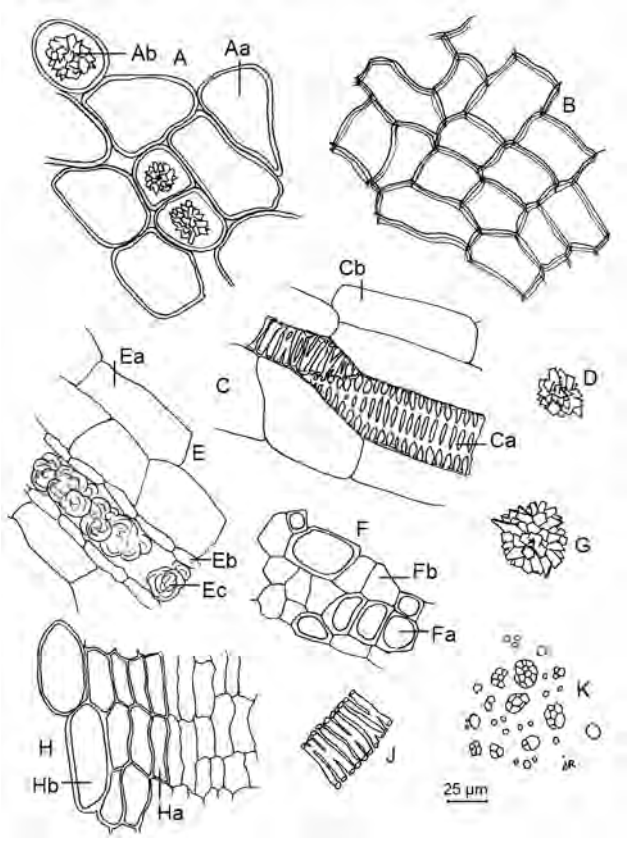


Figure 1523.-1. – *Illustration for identification test B of powdered herbal drug of ginseng*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Boil 1.0 g of the powdered herbal drug (355) (*2.9.12*) under a reflux condenser with 10 mL of a 70 per cent *V/V* solution of *methanol R* for 15 min. Filter after cooling and dilute to 10.0 mL with *methanol R*.

*Reference solution.* Dissolve 5.0 mg of *aescin R* and 5.0 mg of *arbutin R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *water R*, *butanol R* (25:50:100 *V/V/V*); allow the mixture to separate for 10 min and use the upper layer.

*Application*: 20 µL [or 4 µL] as bands.

*Development*: over 10 cm [or 5 cm] in an unsaturated tank.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| Arbutin: a brown zone | |
| ——— | ——— |
| | A violet zone (ginsenosides Rg1 + Rg2) |
| | A faint violet zone (ginsenoside Rf) |
| | A violet zone (ginsenoside Re) |
| | A violet zone (ginsenoside Rd) |
| | A faint violet zone |
| ——— | ——— |
| | A violet zone (ginsenoside Rc) |
| Aescin: a grey zone | A violet zone (ginsenosides Rb1 + Rb2) |
| **Reference solution** | **Test solution** |

TESTS

***Panax quinquefolium***. Examine the chromatograms obtained in the assay. The chromatogram obtained with the test solution shows a peak due to ginsenoside Rf (see Figure 1523.-2). In the case of a substitution by *Panax quinquefolium*, no peak due to ginsenoside Rf is present.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Reduce about 50 g to a powder (355) (*2.9.12*). Place 1.00 g of the powdered herbal drug and 70 mL of a 50 per cent *V/V* solution of *methanol R* in a 250 mL round-bottomed flask. After adding a few grains of pumice, boil on a water-bath under a reflux condenser for 1 h. After cooling, centrifuge and collect the supernatant. Treat the residue as described above. Mix the collected liquids and evaporate to dryness under reduced pressure at a temperature not exceeding 60 °C. Take up the residue with 20.0 mL of a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Dilute 2.0 mL of the solution to 10.0 mL with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Filter through a suitable membrane filter (nominal pore size 0.45 µm) before injection.

*Reference solution.* Dissolve 3.0 mg of *ginsenoside Rg1 CRS*, 3.0 mg of *ginsenoside Re R*, 3.0 mg of *ginsenoside Rf R* and 3.0 mg of *ginsenoside Rb1 CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R* adjusted to pH 2 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 80 | 20 |
| 8 - 40 | 80 → 60 | 20 → 40 |
| 40 - 45 | 60 → 40 | 40 → 60 |
| 45 - 47 | 40 → 0 | 60 → 100 |
| 47 - 52 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 203 nm.

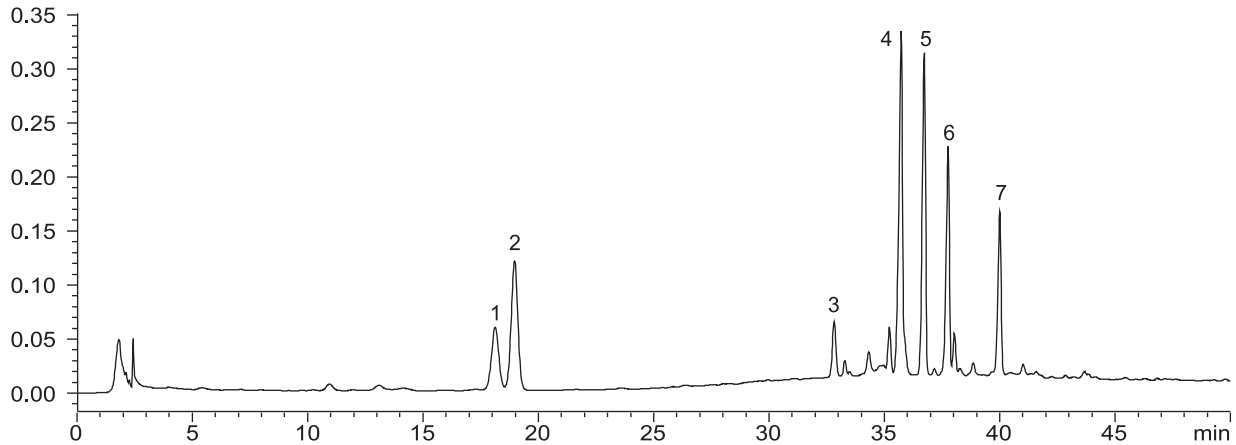


1. ginsenoside Rg1
2. ginsenoside Re
3. ginsenoside Rf
4. ginsenoside Rb1
5. ginsenoside Rc
6. ginsenoside Rb2
7. ginsenoside Rd

Figure 1523.-2. – *Chromatogram for the assay of ginseng: test solution*

*Equilibration*: 20 min.

*Injection*: 20 µL.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.0 between the peaks due to ginsenoside Rg1 and ginsenoside Re.

Locate the peaks due to ginsenoside Rb1 and ginsenoside Rg1 in the chromatogram obtained with the test solution.

Calculate the percentage content of ginsenosides Rb1 and Rg1 using the following expression:

$$\frac{A_1 \times m_2 \times p_1}{A_3 \times m_1 \times 100} + \frac{A_2 \times m_3 \times p_2}{A_4 \times m_1 \times 100}$$

$A_1$ = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the reference solution;

$A_4$ = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ginsenoside Rb1 CRS* used to prepare the reference solution, in milligrams;

$m_3$ = mass of *ginsenoside Rg1 CRS* used to prepare the reference solution, in milligrams;

$p_1$ = percentage content of ginsenoside Rb1 in *ginsenoside Rb1 CRS*;

$p_2$ = percentage content of ginsenoside Rg1 in *ginsenoside Rg1 CRS*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *water R*, *butanol R* (25:50:100 *V/V/V*); allow the phases to separate for 10 min and use the upper layer.

*Application*: 20 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 5 cm] in an unsaturated tank.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatograms obtained with the test solution and the reference solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| A violet zone (ginsenosides Rg1 + Rg2) | A violet zone (ginsenosides Rg1 + Rg2) |
| A faint violet zone (ginsenoside Rf) | A faint violet zone (ginsenoside Rf) |
| A violet zone (ginsenoside Re) | A violet zone (ginsenoside Re) |
| A violet zone (ginsenoside Rd) | A violet zone (ginsenoside Rd) |
| A faint violet zone | A faint violet zone |
| A violet zone (ginsenoside Rc) | A violet zone (ginsenoside Rc) |
| A faint violet zone | A faint violet zone |
| _____ | _____ |
| A violet zone (ginsenosides Rb1 + Rb2) | A violet zone (ginsenosides Rb1 + Rb2) |
| Several unresolved violet and greenish zones | Several unresolved violet and greenish zones |
| **Reference solution** | **Test solution** |

**01/2013:2356**

# GINSENG DRY EXTRACT

## Ginseng extractum siccum

DEFINITION

Dry extract produced from *Ginseng (1523)*.

*Content*: minimum 4.0 per cent of the sum of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2, expressed as ginsenoside Rb1 ($C_{54}H_{92}O_{23}$; $M_r$ 1109) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using a hydroalcoholic solvent equivalent in strength to ethanol (35-90 per cent *V/V*).

CHARACTERS

*Appearance*: light brownish-yellow, hygroscopic powder or brittle mass.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.15 g of the extract to be examined in 10 mL of a 70 per cent *V/V* solution of *methanol R*.

*Reference solution.* Dissolve 0.15 g of *ginseng dry extract HRS* in 10 mL of a 70 per cent *V/V* solution of *methanol R*.

TESTS

**Loss on drying** (*2.8.17*): maximum 7.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 3.5 g of *disodium hydrogen phosphate dihydrate R* and 7.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

*Test solution.* Dissolve 0.100 g of the extract to be examined in the buffer solution and dilute to 10.0 mL with the buffer solution. Prepare a ready-to-use sample-preparation cartridge containing 0.50 g of octadecylsilyl silica gel (45 µm), using 5 mL of *methanol R* followed by 20 mL of *water R*. Apply 5.0 mL of the solution to be analysed to the top of the cartridge. Wash the cartridge with 20 mL of *water R* followed by 15 mL of a 30 per cent *V/V* solution of *methanol R*. Discard the eluates after confirming that no ginsenosides are present, otherwise repeat the preparation of the solution with another brand of cartridge where no ginsenosides are eluted with a 30 per cent *V/V* solution of *methanol R*. Elute the cartridge with 20 mL of *methanol R*; collect the eluate. Under reduced pressure, evaporate the eluate to dryness. Dissolve the residue in 2.0 mL of *methanol R*. Filter through a suitable membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 0.100 g of *ginseng dry extract HRS* in the buffer solution and dilute to 10.0 mL with the buffer solution. Prepare a ready-to-use sample-preparation cartridge containing 0.50 g of octadecylsilyl silica gel (45 µm), using 5 mL of *methanol R* followed by 20 mL of *water R*. Apply 5.0 mL of the solution to be analysed to the top of the

cartridge. Wash the cartridge with 20 mL of *water R* followed by 15 mL of a 30 per cent *V/V* solution of *methanol R*. Discard the eluates after confirming that no ginsenosides are present, otherwise repeat the preparation of the solution with another brand of cartridge where no ginsenosides are eluted with a 30 per cent *V/V* solution of *methanol R*. Elute the cartridge with 20 mL of *methanol R*; collect the eluate. Under reduced pressure, evaporate the eluate to dryness. Dissolve the residue in 2.0 mL of *methanol R*. Filter through a suitable membrane filter (nominal pore size 0.45 µm).

*Reference solution (b).* Dissolve 3.0 mg of *ginsenoside Rb1 CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (c).* Dissolve 3.0 mg of *ginsenoside Rg2 R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 2.0 mL with reference solution (c).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *water R* adjusted to pH 2 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 80 | 20 |
| 8 - 40 | 80 → 60 | 20 → 40 |
| 40 - 45 | 60 → 40 | 40 → 60 |
| 45 - 47 | 40 → 0 | 60 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 203 nm.

*Injection*: 20 µL.

*Elution order*: ginsenoside Rg1, ginsenoside Re, ginsenoside Rf, ginsenoside Rb1, ginsenoside Rg2, ginsenoside Rc, ginsenoside Rb2, ginsenoside Rd; depending on the operating conditions and the state of the column, ginsenoside Rb1 may elute before or after ginsenoside Rg2.

*Identification of peaks*: use the chromatogram supplied with *ginseng dry extract HRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to ginsenosides Rg1, Re, Rf, Rc, Rb2 and Rd; use the chromatogram obtained with reference solution (b) to identify the peak due to ginsenoside Rb1; use the chromatogram obtained with reference solution (c) to identify the peak due to ginsenoside Rg2.

*Relative retention* with reference to ginsenoside Rb1 (retention time = about 33 min): ginsenoside Rg1 = about 0.53; ginsenoside Re = about 0.54; ginsenoside Rf = about 0.88; ginsenoside Rg2 = about 0.98; ginsenoside Rc = about 1.04; ginsenoside Rb2 = about 1.08; ginsenoside Rd = about 1.17.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to ginsenosides Rg2 and Rb1.

Calculate the percentage content of the sum of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2, expressed as ginsenoside Rb1, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.8}{A_2 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ginsenoside Rb1 CRS* used to prepare reference solution (b), in grams;

$p$ = percentage content of ginsenoside Rb1 in *ginsenoside Rb1 CRS*.

**04/2014:1892**

# GOLDENROD

## Solidaginis herba

DEFINITION

Whole or cut, dried, flowering aerial parts of *Solidago gigantea* Aiton or *Solidago canadensis* L., their varieties or hybrids and/or mixtures of these.

*Content*: minimum 2.5 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The stems are greenish-yellow or greenish-brown, partly tinted reddish, roundish, more or less conspicuously grooved, glabrous and smooth in the lower part, slightly or densely pubescent in the upper part. They are solid with a whitish pith.

The leaves are green, sessile, lanceolate, with a serrate margin, 8-12 cm long and about 1-3 cm wide, the upper surface is green and more or less glabrous, the lower surface is greyish-green and pubescent, especially on the veins. The inflorescence consists of a number of unilateral, curved racemes which together form a pyramidal panicle at the end of the stems.

Each capitulum has an involucre composed of linear-lanceolate, imbricated yellowish-green bracts, surrounding a single row of yellow ligulate florets about the same length as the involucre; yellow, radially arranged tubular florets, as long as, or longer, than the ligulate florets; a brownish inferior ovary surmounted by a white pappus of silky hairs.

B. Microscopic examination (*2.8.23*). The powder is green or greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1892.-1): pappus bristles, usually broken, consisting of multiseriate trichomes composed of elongated cells with the tips free from the surface and forming pointed projections over the entire length [J]; fragments of the mesophyll [F] with vascular bundles [Fa], oil glands [Fb] and palisade parenchyma [Fc]; fragments of the leaf epidermises [E, H] with sinuous or wavy-walled cells accompanied by palisade parenchyma [Ea] (upper epidermis), with whip-like, multicellular covering trichomes [Eb] and anomocytic stomata (mainly on the lower epidermis) (*2.8.3*) [Ha]; multicellular uniseriate covering trichomes from the stems and the margins of the leaves with up to 5 or 6 thick-walled cells [C]; in *Solidago canadensis* L., covering trichomes with a terminal cell that may be bent at a right angle [G]; fragments of the style [A] with short papillae [Aa] and long,

slender papillae with wrinkled walls [Ab]; pollen grains with 3 germinal pores and a spiny exine [B]; a few rare fragments of the ovary with twin covering trichomes [D].

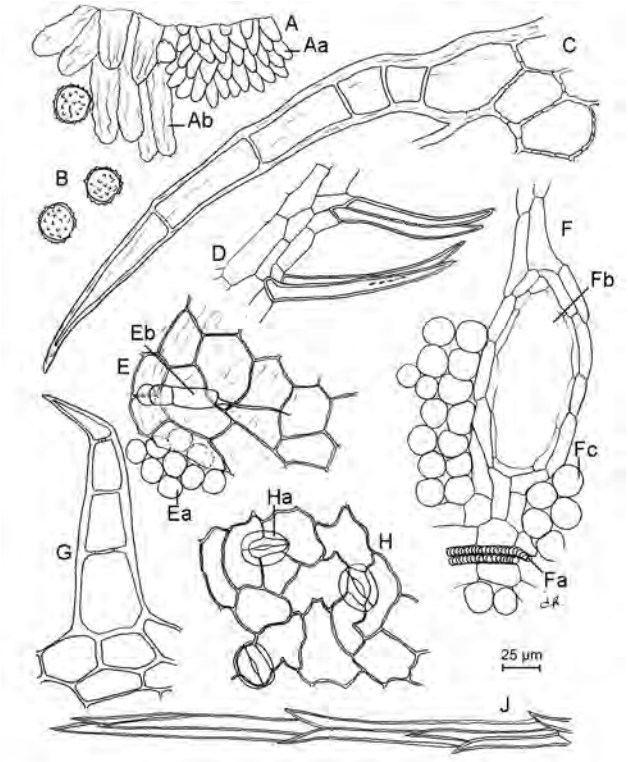


Figure 1892.-1. – *Illustration for identification test B of powdered herbal drug of goldenrod*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.75 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and boil in a water-bath under a reflux condenser for 10 min. Cool and filter.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R*, 2.5 mg of *quercitrin R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (6:6:18:30 *V/V/V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to stand for 30 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A bluish-green fluorescent zone |
| Quercitrin: a yellowish-brown fluorescent zone | A faint to intense yellowish-brown fluorescent zone (quercitrin) |
| | A more or less intense yellowish-brown zone |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) and/or a yellow fluorescent zone |
| Rutoside: an orange fluorescent zone | A faint to intense yellowish-brown fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of brownish parts and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10 per cent, determined on 0.500 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask, introduce 0.200 g of the powdered herbal drug (250) (*2.9.12*), add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a small plug of absorbent cotton into a 100 mL flask. Add the absorbent cotton to the residue in the round-bottomed flask, extract with 2 quantities, each of 20 mL of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool. Filter the combined acetone extracts through a filter paper into a volumetric flask. Rinse the flask and the filter paper and dilute to 100.0 mL with *acetone R*. Introduce 20.0 mL of the solution into a separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then with 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash twice with 50 mL of *water R* and filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask. Dilute to 50.0 mL with *ethyl acetate R*, rinsing the separating funnel and the sodium sulfate.

*Test solution.* To 10.0 mL of the stock solution add 1.0 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation solution.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance of the test solution (*2.2.25*) at 425 nm after 30 min by comparison with the compensation solution.

Calculate the percentage content of flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the value of the specific absorbance of hyperoside to be 500.

*A* = absorbance measured at 425 nm;

*m* = mass of the herbal drug to be examined, in grams.

01/2013:1893

# GOLDENROD, EUROPEAN

## Solidaginis virgaureae herba

DEFINITION

Whole or fragmented, dried, flowering aerial parts of *Solidago virgaurea* L.

*Content*: minimum 0.5 per cent and maximum 1.5 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The stem is cylindrical, striated, the lower part often reddish-violet, sometimes entirely glabrous or pubescent with short, bent, apically directed hairs. The basal leaves are obovate or oblanceolate, with a serrate margin, and taper at the base into a long, winged petiole; the cauline leaves are alternate, smaller than the basal leaves and more elliptical in outline, with an entire or slightly toothed margin; they are sessile or with only a short petiole. Both surfaces of the leaves are glabrous or only slightly pubescent with a prominent reticulate venation on the lower surface. The capitula form a tightly packed panicle. At the base of the pedicels there are 2 small, linear bracts with scarious margins. The involucre consists of 2-4 rows of loosely arranged, imbricate bracts, each bract greenish-yellow with a smooth and shiny inner surface, the outer surface hairy or glabrous, with a scarious margin. Each capitulum contains 6-12 widely separated female ray florets, about twice as long as the bracts, and about 10-30 hermaphrodite, tubular florets. All florets are yellow. The brown, inferior ovary tapers towards the base and has a ribbed surface, covered with scattered hairs; it is surmounted by a whitish pappus composed of smooth or rough, bristly hairs.

B. Microscopic examination (*2.8.23*). The powder is light green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figures 1893.-1 and 1893.-2): fragments of the upper epidermis of the leaf (surface view [B, H, M]), covered by a distinctly striated cuticle, composed of polygonal cells with straight, beaded, thickened walls [Ba, Ma], uniseriate, multicellular covering trichomes [Ha] or rounded, thick-walled, covering trichome scars with a pitted lumen [Mb], and a few anomocytic stomata (*2.8.3*) [Bb] sometimes accompanied by underlying palisade parenchyma [Bc]; fragments of the lower epidermis of the leaf (surface view [A, K, N]) covered by a slightly striated cuticle composed of cells with sinuous walls in the area of the lamina [Aa] or with more rigid walls near the veins [N], numerous anomocytic stomata (*2.8.3*) [Ab], occasional glandular trichomes with a unicellular stalk and a unicellular head [Ka, Na], covering trichomes some of which are pennant-like [Ac, F], uniseriate, multicellular, with 1-3 thin-walled basal cells [Fa], a flagella-like distal cell [Fb], and an enlarged, more or less rounded cell [Fc] between them, others are uniseriate, multicellular (up to about 10 cells), with thick, finely wrinkled walls and a rigid conical distal cell (side view [E]); rare fragments from the ovary [G] bearing paired, covering trichomes with a distinctly pitted central wall and a bifid apex (surface view [Ga], side view [Gb]); vascular tissue from the stems [L] composed of vessels [La] and groups of fibres [Lb]; fragments of the epidermis of the petals with a striated cuticle, through which run fine spiral vessels [S], and bearing biseriate glandular trichomes (side view [P]); spherical pollen grains, with 3 germinal pores and a spiny exine [J]; abundant pappus hairs and their fragments [C, D], multiseriate with the marginal cells overlapping outwards; fragments of parenchyma [Q], some showing cells containing small, isolated cluster crystals of calcium oxalate [Qa]; fragments of bracts [R] with a finely striated cuticle, polygonal cells [Ra], bearing pennant-like covering trichomes [Rb] and whose margin bears uniseriate, multicellular covering trichomes [Rc].

C. Thin-layer chromatography (*2.2.27*) as described in the test for *Solidago gigantea* Aiton and *Solidago canadensis* L.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue fluorescent zone |
| Quercitrin: an orange fluorescent zone | |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

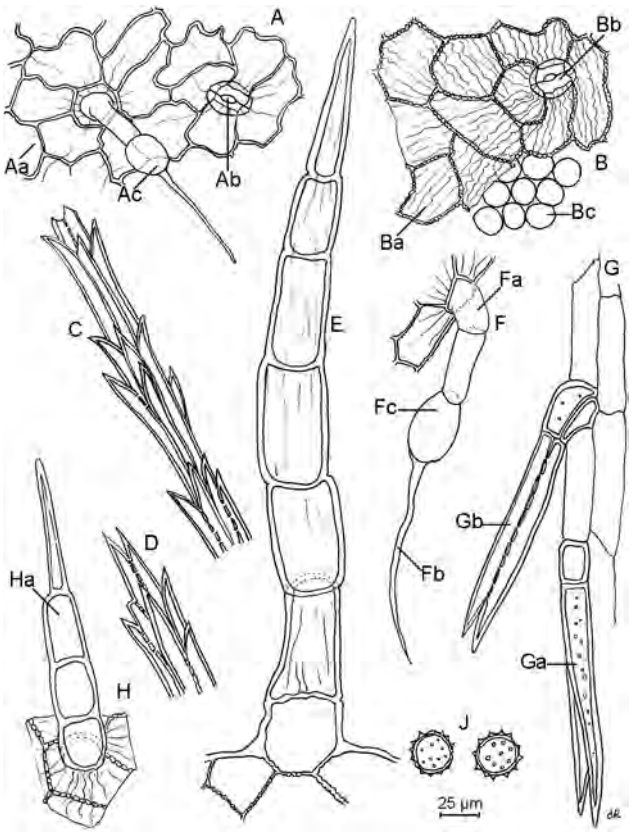


Figure 1893.-1. – *Illustration for identification test B of powdered herbal drug of European goldenrod*

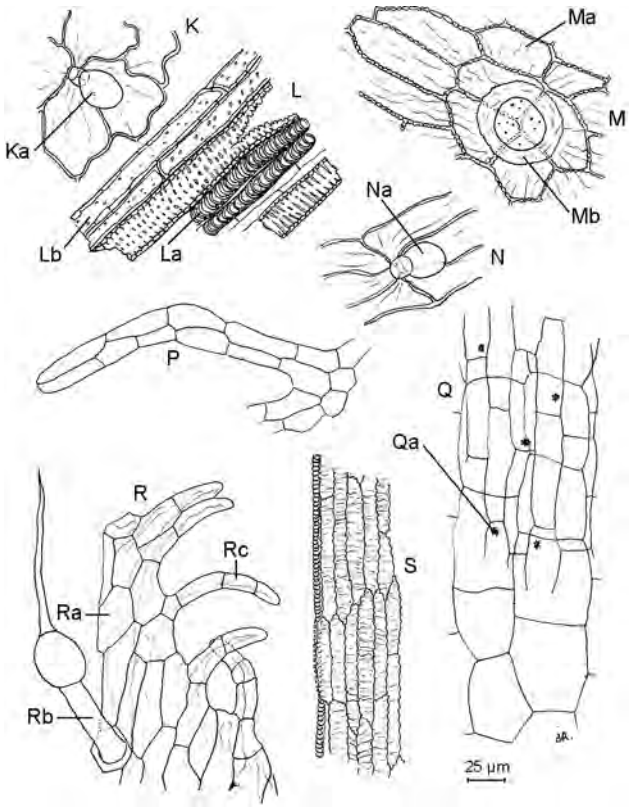


Figure 1893.-2. – *Illustration for identification test B of powdered herbal drug of European goldenrod*

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of brown coloured matter and maximum 5 per cent of other foreign matter.

***Solidago gigantea* Aiton and *Solidago canadensis* L.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.75 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and heat on a water-bath under a reflux condenser for 10 min. Cool and filter.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R*, 2.5 mg of *quercitrin R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (6:6:18:30 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat the plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Examine in ultraviolet light at 365 nm after 30 min.

*Results*: the chromatogram obtained with the test solution shows no strong orange fluorescent zone similar in position to the zone of quercitrin in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask, place 0.200 g of the powdered herbal drug (355) (*2.9.12*), add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture in a water-bath under a reflux condenser for 30 min. Filter the liquid through a small plug of absorbent cotton into a 100 mL flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool. Filter the combined acetone extracts through filter paper, dilute to 100.0 mL with *acetone R*, rinsing the volumetric flask and the filter paper with acetone. Introduce 20.0 mL of the solution into a suitable separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then with 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash twice with 50 mL of *water R* and filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask. Dilute to 50.0 mL with *ethyl acetate R*, rinsing the separating funnel and the sodium sulfate.

*Test solution.* To 10.0 mL of the stock solution add 1.0 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 425 nm by comparison with the compensation liquid.

Calculate the percentage content of flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

$A$ = measured absorbance at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2011:1831**
**corrected 10.0**

# GOLDENSEAL RHIZOME

## Hydrastis rhizoma

DEFINITION

Whole or cut, dried rhizome and root of *Hydrastis canadensis* L.

*Content*:

- *hydrastine* ($C_{21}H_{21}NO_6$; $M_r$ 383.4): minimum 2.5 per cent (dried drug);
- *berberine* ($C_{20}H_{18}NO_4$; $M_r$ 336.4): minimum 3.0 per cent (dried drug).

IDENTIFICATION

A. The rhizome is tortuous and knotty, about 5 cm long and 5-10 mm thick. The surface is yellowish or brownish-grey, irregularly wrinkled, and bears the remains of numerous slender, wiry roots; stem bases and scale leaves occur on the upper surface. The fracture is short and resinous. The transversely cut surface is yellowish-brown and shows a fairly wide bark, a ring of 12-20 widely separated xylem bundles and a large, central pith.

B. Microscopic examination (*2.8.23*). Reduce to a powder (180) (*2.9.12*). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1831.-1): abundant thin-walled fragments of parenchyma [A, G, K]; occasional fragments of yellowish-brown cork from the rhizome and roots (surface view [J], transverse section [F]); groups of small vessels with conspicuous

perforations in the oblique end walls [L] and with simple or bordered, slit-shaped pits [B, D, E]; infrequent groups of thin-walled, pitted fibres [H], usually found associated with the vessels; numerous ovoid or spherical, orange-brown granular masses. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant starch granules [C], mostly simple but sometimes compound with up to 4 components; the granules are small, spherical or ovoid, up to about 10 µm in diameter, occasionally with a small, rounded or slit-shaped hilum.

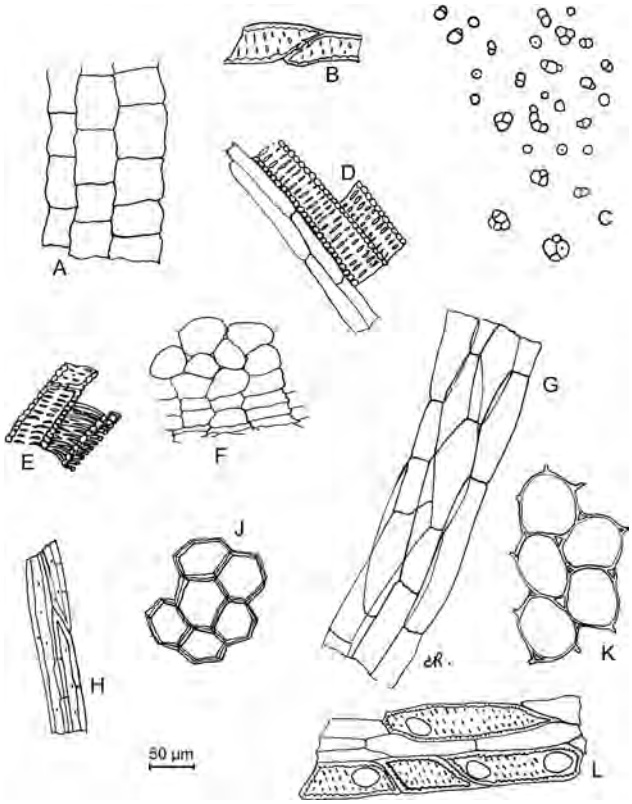


Figure 1831.-1. – *Illustration for identification test B of powdered herbal drug of goldenseal rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 250 mg of the powdered herbal drug (180) (*2.9.12*) add 4 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*. Sonicate for 10 min and filter. Wash the residue with 2 quantities, each of 2 mL, of *methanol R*. Combine the solutions and dilute to 20 mL with *methanol R*.

*Reference solution.* Immediately before use, dissolve 5 mg of *hydrastine hydrochloride R* and 5 mg of *berberine chloride R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Berberine: a bright yellow fluorescent zone | A bright yellow fluorescent zone (berberine) |
| Hydrastine: a deep blue fluorescent zone | A deep blue fluorescent zone (hydrastine) |
| ——— | ——— |
| | A bright light blue fluorescent zone (hydrastinine) |
| | A deep blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (180) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (355) (*2.9.12*) in a 100 mL round-bottomed flask, add 50 mL of a 1 per cent *V/V* solution of *concentrated ammonia R* in *ethanol (96 per cent) R* and boil the mixture under a reflux condenser for 30 min. Allow to cool to room temperature and filter the liquid through a plug of absorbent cotton into a flask. Add the plug of absorbent cotton to the residue in the round-bottomed flask and repeat the extraction with a further 2 quantities, each of 30 mL, of a 1 per cent *V/V* solution of *concentrated ammonia R* in *ethanol (96 per cent) R*, each time boiling under a reflux condenser for 10 min and filtering through a plug of absorbent cotton in the same flask as previously. Filter the combined filtrates through a filter paper into a 250 mL round-bottomed flask, and rinse the flask and the filter with 20 mL of a 1 per cent *V/V* solution of *concentrated ammonia R* in *ethanol (96 per cent) R*. Evaporate the filtrate to dryness *in vacuo* in a water-bath at 55 °C. Dissolve the residue in 50.0 mL of the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution.* Immediately before use, dissolve 10.0 mg of *hydrastine hydrochloride CRS* and 10.0 mg of *berberine chloride CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 9.93 g of *potassium dihydrogen phosphate R* in 730 mL of *water R*, add 270 mL of *acetonitrile R* and mix.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*System suitability*: reference solution:

- *elution order*: order indicated in the composition of the reference solution; record the retention times of these substances;
- *resolution*: minimum 1.5 between the peaks due to hydrastine and berberine.

Using the retention times determined from the chromatogram obtained with the reference solution, locate in the chromatogram obtained with the test solution the components of the reference solution.

Calculate the percentage content of each alkaloid (hydrastine and berberine) using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1} \times 2.5$$

$A_1$ = area of the peak due to hydrastine or berberine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hydrastine or berberine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *hydrastine hydrochloride CRS* or *berberine chloride CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of hydrastine in *hydrastine hydrochloride CRS* or berberine in *berberine chloride CRS*.

**07/2012:1861**

# GREATER CELANDINE

## Chelidonii herba

DEFINITION

Dried, whole or cut aerial parts of *Chelidonium majus* L. collected during flowering.

*Content*: minimum 0.6 per cent of total alkaloids, expressed as chelidonine ($C_{20}H_{19}NO_5$; $M_r$ 353.4) (dried drug).

IDENTIFICATION

A. The stems are rounded, ribbed, yellowish or greenish-brown, somewhat pubescent, about 3-7 mm in diameter, hollow and mostly collapsed. The leaves are thin, irregularly pinnate, the leaflets ovate to oblong with coarsely dentate margins, the terminal leaflet often 3-lobed; the adaxial surface is bluish-green and glabrous, the abaxial surface paler and pubescent, especially on the veins. The flowers have 2 deeply concavo-convex sepals, readily removed, and 4 yellow, broadly ovate, spreading petals about 8-10 mm long; the stamens are numerous, yellow, and a short style arises from a superior ovary; long, capsular, immature fruits are rarely present.

B. Microscopic examination (*2.8.23*). The powder is dark greyish-green or brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1861.-1): numerous fragments of upper epidermis, composed of cells with sinuous walls (surface view [B]), accompanied by underlying palisade parenchyma [Ba]; numerous fragments of lower epidermis in surface view [A, E] bearing anomocytic stomata (*2.8.3*) [Aa] and bases of covering trichomes [Ab], sometimes accompanied by underlying spongy parenchyma [Ea]; long, uniseriate, multicellular covering trichomes, usually fragmented, with thin-walled cells, sometimes collapsed [G]; vascular tissue from the leaves and stems consisting of pitted and spirally thickened vessels [D]; groups of fibres [C]; articulated latex tubes with yellowish-brown contents [F]; occasional fragments of the corolla [H] consisting of thin-walled cells containing numerous pale yellow droplets of oil [Ha]; spherical pollen grains about 30-40 µm in diameter with 3 pores and a finely pitted exine [J].

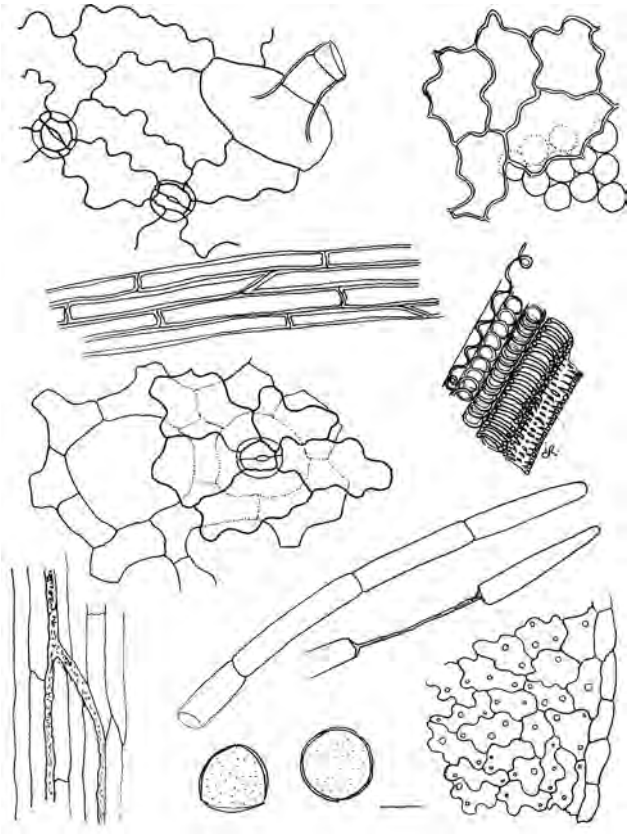

Figure 1861.-1. – *Illustration for identification test B of powdered herbal drug of greater celandine*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.4 g of the powdered herbal drug (710) (*2.9.12*) add 50 mL of *dilute acetic acid R*. Boil in a water-bath under a reflux condenser for 30 min. Cool and filter. To the filtrate add *concentrated ammonia R* until a strong alkaline reaction is produced. Shake with 30 mL of *methylene chloride R*. Dry the organic layer over *anhydrous sodium sulfate R*, filter and evaporate *in vacuo* to dryness. Dissolve the residue in 1.0 mL of *methanol R*.

*Reference solution.* Dissolve 2 mg of *methyl red R* and 2 mg of *papaverine hydrochloride R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *propanol R* (1:9:90 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R* and dry in air; spray with *sodium nitrite solution R* and allow to dry in air; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weaker zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Methyl red: a red zone | A brown zone |
| | A brown zone |
| Papaverine: a greyish-brown zone | A greyish-brown zone |
| ——— | ——— |
| | 2 brown zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 10.0 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 13.0 per cent.

ASSAY

*Test solution.* To 0.750 g of the powdered herbal drug (710) (*2.9.12*) add 200 mL of *dilute acetic acid R* and heat on a water-bath for 30 min, shaking frequently. Cool and dilute to 250.0 mL with *dilute acetic acid R*. Filter. Discard the first 20 mL of the filtrate. To 30.0 mL of the filtrate add 6.0 mL of *concentrated ammonia R* and 100.0 mL of *methylene chloride R*. Shake for 30 min. Separate the organic layer, place 50.0 mL in a 100 mL round-bottomed flask and evaporate to dryness *in vacuo* at a temperature not exceeding 40 °C. Dissolve the residue in about 2-3 mL of *ethanol (96 per cent) R*, warming slightly. Transfer the solution to a 25 mL volumetric flask by rinsing the round-bottomed flask with *dilute sulfuric acid R* and dilute to 25.0 mL with the same solvent. To 5.0 mL of the solution add 5.0 mL of a 10 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* in a 25 mL volumetric flask, stopper the flask and mix carefully. Dilute to 25.0 mL with *sulfuric acid R* and stopper the flask.

*Compensation liquid.* Prepare at the same time and in the same manner as for the test solution: place in a 25 mL volumetric flask 5.0 mL of *dilute sulfuric acid R* and 5.0 mL of a 10 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R*, stopper the flask and mix carefully. Dilute to 25.0 mL with *sulfuric acid R* and stopper the flask.

Place both solutions on a water-bath for 10 min. Cool to about 20 °C and dilute if necessary to 25.0 mL with *sulfuric acid R*. Measure the absorbance (*2.2.25*) of the test solution at 570 nm by comparison with the compensation liquid.

Calculate the percentage content of total alkaloids, expressed as chelidonine, using the following expression:

$$\frac{A \times 2.23}{m}$$

i.e. taking the specific absorbance of chelidonine to be 933.

$A$ = absorbance at 570 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**04/2018:2668**

# GREEN TEA

## Camelliae sinensis non fermentata folia

DEFINITION

Young, unfermented leaf of *Camellia sinensis* (L.) Kuntze rapidly stabilised by short time heating and then dried.

*Content*:

– *caffeine* ($C_8H_{10}N_4O_2$; $M_r$ 194.2): minimum 1.5 per cent (dried drug);

– *total catechins, expressed as (–)-epigallocatechin-3-O-gallate* ($C_{22}H_{18}O_{11}$; $M_r$ 458.4): minimum 8.0 per cent (dried drug).

CHARACTERS

Greyish-green leaf with petiole removed, rolled and often folded or twisted, whole or cut before being rolled.

IDENTIFICATION

A. Examine after allowing the leaf to unroll for a few minutes in warm water. The leaf is oval, elongated and acuminate. Starting at a quarter of the distance from the base, the margins are serrate with peculiarly shaped teeth bearing a small blackish point curved in a claw shape. The secondary veins branch from the midrib, curving near the margins of the lamina so that they anastomose in an arc. Flexuous, conical, covering trichomes are abundant on the lower surface of the leaves.

B. Microscopic examination (*2.8.23*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2668.-1): numerous fragments of unicellular, flexuous, thick-walled covering trichomes [E]; fragments of abaxial epidermis (surface view [A]) consisting of cells with slightly wavy walls [Aa], numerous anomocytic stomata (*2.8.3*) surrounded by 3 or 4 narrow subsidiary cells [Ab] and long, flexuous, thick-walled, unicellular covering trichomes, pointed at the apex [Ac]; fragments of adaxial epidermis (surface view [F]) consisting of cells with rigid, slightly thickened walls [Fa], accompanied by cells of the underlying palisade parenchyma [Fb] and sometimes by the end of a sclereid [Fc]; numerous isolated sclereids, often very ramified, with thick-walls and distinct channels [C]; fragments of lamina (transverse section [B]), showing the adaxial epidermis covered by a smooth cuticle [Ba], palisade parenchyma usually arranged in 2 layers [Bb], spongy parenchyma [Bc] including cells containing cluster crystals of calcium oxalate [Bd], and the abaxial epidermis [Be] with stomata; fragments of spiral vessels (longitudinal section [D]) accompanied by spongy parenchyma [Db] with some cells containing cluster crystals of calcium oxalate [Da]; vessels [G] accompanied by lignified fibres [Ga] from the principal veins.

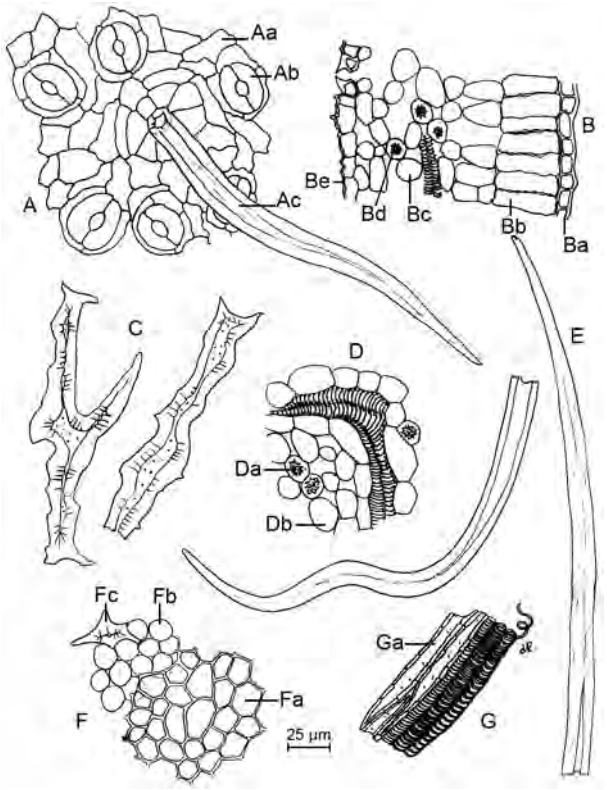


Figure 2668.-1. – *Illustration for identification test B of powdered herbal drug of green tea*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of a mixture of *water R* and *ethanol (96 per cent) R* (20:80 *V/V*). Sonicate for 10 min and filter.

*Reference solution.* Dissolve 2 mg of *(–)-epicatechin R* and 5 mg of *(–)-epigallocatechin-3-O-gallate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R, acetone R, toluene R* (10:45:45 *V/V/V*).

*Application*: 30 µL [or 5 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a freshly prepared 5 g/L solution of *fast blue B salt R*. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| _____ | _____ |
| (–)-Epicatechin: a reddish-brown zone | A reddish-brown zone ((–)-epicatechin) |
| | 1 or 2 reddish-brown zone(s) |
| (–)-Epigallocatechin-3-*O*-gallate: a reddish-brown zone | A reddish-brown zone ((–)-epigallocatechin-3-*O*-gallate) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Stabilising solution.* Dissolve 250 mg of *(ethylenedinitrilo)tetraacetic acid R* and 250 mg of *ascorbic acid R* in 500 mL of *water R*, add 100 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Extraction solution.* Place a sealed container filled with a mixture of *water R* and *methanol R* (30:70 *V/V*) in a water-bath at 70 °C for at least 30 min in order to reach the temperature.

*Test solution.* Introduce 0.200 g of the powdered herbal drug (355) (*2.9.12*) into a test tube, place in a water-bath at 70 °C and add 5 mL of the extraction solution (pre-warmed to 70 °C). Stopper the tube and carefully mix using a vortex mixer. Continue heating the test tube in the water-bath for 10 min, mixing on the vortex mixer after 5 min and 10 min. Remove the test tube from the water-bath, allow to cool and centrifuge at 2350 *g* for 10 min. Decant the supernatant liquid into a 10 mL volumetric flask. Repeat the extraction procedure again with 5 mL of extraction solution, combine the extracts in the same volumetric flask and dilute to 10.0 mL with a cold mixture of *water R* and *methanol R* (30:70 *V/V*). Dilute 1.0 mL of this solution to 20.0 mL with the stabilising solution. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 20.0 mg of *(–)-epigallocatechin-3-O-gallate CRS* and 20.0 mg of *caffeine CRS* in *water R* and dilute to 25.0 mL with the same solvent. Dilute 2.0 mL of the solution to 50.0 mL with the stabilising solution. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (b).* Dissolve 0.100 g of *green tea dry extract HRS* in 5 mL of *water R* using sonication, add 1.0 mL of *acetonitrile R* and dilute to 10.0 mL with *water R*. Dilute 2.0 mL of the solution to 50.0 mL with the stabilising solution. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 0.05 per cent *V/V* solution of *anhydrous formic acid R*;
– *mobile phase B*: 0.05 per cent *V/V* solution of *anhydrous formic acid R* in *methanol R2*;
– *mobile phase C*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 5 | 97 | 0 | 3 |
| 5 - 23 | 97 → 67 | 0 → 30 | 3 |
| 23 - 29 | 67 | 30 | 3 |
| 29 - 30 | 67 → 30 | 30 → 67 | 3 |
| 30 - 31 | 30 | 67 | 3 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *green tea dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to (+)-gallocatechin, (–)-epigallocatechin, (+)-catechin, (–)-epigallocatechin-3-*O*-gallate, (–)- epicatechin, (–)-gallocatechin-3-*O*-gallate and (–)-epicatechin-3-*O*-gallate.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to (−)-epigallocatechin-3-*O*-gallate and (−)-epicatechin.

Calculate the percentage content of total catechins, expressed as (−)-epigallocatechin-3-*O*-gallate, using the following expression:

$$\frac{A_1 \times m_2 \times 0.32 \times p}{A_2 \times m_1}$$

$A_1$ = sum of the area of the peaks due to catechins [(+)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epigallocatechin-3-*O*-gallate, (−)-epicatechin, (−)-gallocatechin-3-*O*-gallate and (−)-epicatechin-3-*O*-gallate] in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to (−)-epigallocatechin-3-*O*-gallate in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *(−)-epigallocatechin-3-O-gallate CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of (−)-epigallocatechin-3-*O*-gallate in *(−)-epigallocatechin-3-O-gallate CRS*.

Calculate the percentage content of caffeine using the following expression:

$$\frac{A_1 \times m_2 \times 0.32 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to caffeine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to caffeine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *caffeine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of caffeine in *caffeine CRS*.

STORAGE

Protected from moisture.

**01/2010:1218**
**corrected 9.0**

# GUAR

## Cyamopsidis seminis pulvis

DEFINITION

Guar is obtained by grinding the endosperms of seeds of *Cyamopsis tetragonolobus* (L.) Taub. It consists mainly of guar galactomannan.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: it yields a mucilage of variable viscosity when dissolved in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Examined under a microscope in *glycerol R*, the substance to be examined (125) (*2.9.12*) shows pyriform or ovoid cells, usually isolated, having very thick walls around a central somewhat elongated lumen with granular contents, and smaller polyhedral cells, isolated or in clusters, with thinner walls.

B. In a conical flask place 2 g, add rapidly 45 mL of *water R* and stir vigorously for 30 s. After 5-10 min a stiff gel forms which does not flow when the flask is inverted.

C. Mix a suspension of 0.1 g in 10 mL of *water R* with 1 mL of a 10 g/L solution of *disodium tetraborate R*; the mixture soon gels.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 10 mg of the substance to be examined in a thick-walled centrifuge tube add 2 mL of a 100 g/L solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant carefully into a 50 mL flask, add 10 mL of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 mL of *water R* and 0.9 mL of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant, if necessary, to 1 mL with *methanol R*.

*Reference solution.* Dissolve 10 mg of *galactose R* and 10 mg of *mannose R* in 2 mL of *water R*, then dilute to 20 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 5 µL, as bands.

*Development*: over a path of 15 cm.

*Detection*: spray with *aminohippuric acid reagent R* and dry at 120 °C for 5 min.

*Results*: the chromatogram obtained with the reference solution shows in the lower part 2 clearly separated brownish zones due to galactose and mannose in order of increasing $R_F$ value; the chromatogram obtained with the test solution shows 2 zones due to galactose and mannose.

TESTS

**Tragacanth, sterculia gum, agar, alginates, carrageenan**. To a small amount of the substance to be examined add 0.2 mL of freshly prepared *ruthenium red solution R*. Examined under a microscope the cell walls do not stain red.

**Protein**: maximum 8.0 per cent.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.170 g. Multiply the result by 6.25.

**Apparent viscosity** (*2.2.10*): 85 per cent to 115 per cent of the value stated on the label.

Moisten a quantity equivalent to 1.00 g of the dried substance with 2.5 mL of *2-propanol R*. While stirring, dilute to 100.0 mL with *water R*. After 1 h, determine the viscosity at 20 °C using a rotating viscometer and a shear rate of 100 $s^{-1}$.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Total ash** (*2.4.16*): maximum 1.8 per cent.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the apparent viscosity in millipascal seconds for a 10 g/L solution.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in*

Herbal drugs

*the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for guar used as viscosity-increasing agent or binder.*

**Apparent viscosity**: see Tests.

**04/2018:2669**

# GUARANA

## Guaranae semen

DEFINITION

Dried seed of *Paullinia cupana* Kunth (syn. *Paullinia sorbilis* Mart.).

*Content*: minimum 3.5 per cent of caffeine ($C_8H_{10}N_4O_2$; $M_r$ 194.2) (dried drug).

IDENTIFICATION

A. The seed is more or less spherical, about 12 mm in diameter, shiny, glabrous, dark brown with a large, light-coloured spot corresponding to the hilum.

B. Microscopic examination (*2.8.23*). Reduce to a powder without sieving. The powder is brownish and oily. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2669.-1): polyhedral endosperm cells with thick cellulose walls; fragments of episperm cells with lobed and regularly thickened walls (surface view [D]) and forming a palisade layer (transverse section [B, E]); numerous fragments of cotyledons with rounded to ovoid cells, with spaces between them [H] and filled with starch; more or less polyhedral sclereids with channelled walls, free [F] or in groups [A]; rare spiral vessels [G]; fragments of the embryo with thin-walled elongated cells (surface view [C]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous rounded or oval starch granules [J], up to 30 µm long and 18 µm wide, simple or sometimes 2-3 compound, either free or included in the cells of the cotyledons.

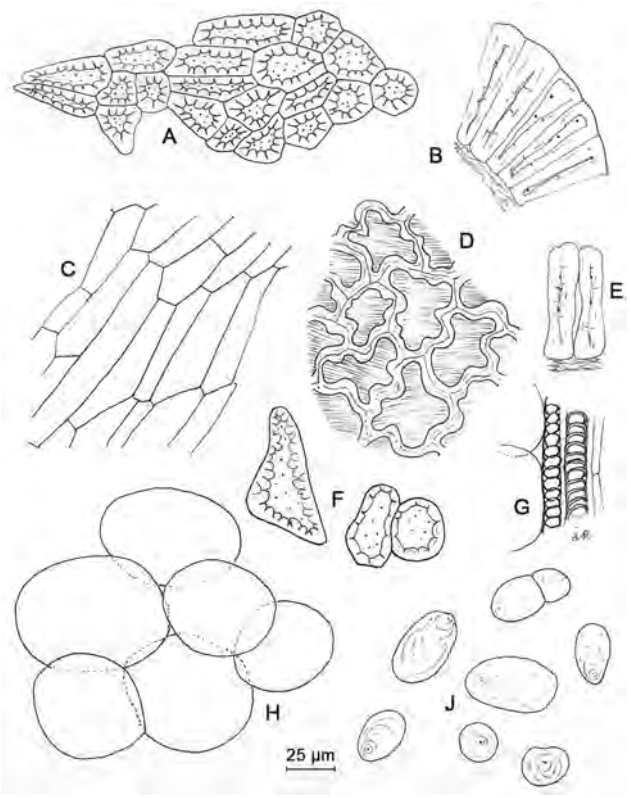


Figure 2669.-1. – *Illustration for identification test B of powdered herbal drug of guarana*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*, sonicate for 10 min, then centrifuge for 10 min and use the supernatant.

*Reference solution.* Dissolve 2.0 mg of *caffeine R* and 1.0 mg of *catechin R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *toluene R*, *acetone R* (10:45:45 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: in an unsaturated tank over a path of 6 cm.

*Drying*: in a current of air at room temperature.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeine: an intense quenching zone | An intense quenching zone (caffeine) |
| **Reference solution** | **Test solution** |

*Detection B*: heat at 100 °C for 2 min, then treat the still-hot plate with *fast blue B salt solution R* and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Catechin: a reddish-brown zone | An intense reddish-brown zone (catechin)<br>2 red or brown zones of varying intensity |
| ——— | ——— |
| | 1 or 2 reddish-brown zones |
| | A reddish-brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 30.0 mg of *caffeine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution*. Introduce 0.500 g of the powdered herbal drug (355) (*2.9.12*) into a 100 mL round-bottomed flask and add 40 mL of *methanol R*. Heat in a water-bath at 70 °C under a reflux condenser for 30 min. Allow to cool and filter through a plug of absorbent cotton into a 100 mL volumetric flask. Transfer the absorbent cotton with the drug residue into the round-bottomed flask. Add 40 mL of *methanol R*, proceed as before and filter into the same volumetric flask. Rinse the round-bottomed flask and the filter with *methanol R*, add the rinsings to the volumetric flask and dilute to 100.0 mL with *methanol R*. Shake to homogenise the solution. Transfer 10.0 mL of this solution into a volumetric flask, add 30 mL of *water R* and dilute to 50.0 mL with the mobile phase. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dilute 5.0 mL of solution A to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 15.0 mg of *(−)-epicatechin R* in the mobile phase and dilute to 25.0 mL with the mobile phase. To 10.0 mL of the solution add 5 mL of solution A and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water for chromatography R* (25:75 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of caffeine.

*Retention time*: caffeine = about 11 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to caffeine and (−)-epicatechin.

Calculate the percentage content of caffeine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to caffeine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to caffeine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *caffeine CRS* used to prepare solution A, in grams;

$p$ = percentage content of caffeine in *caffeine CRS*.

**01/2016:2532**

# HAMAMELIS BARK

## Hamamelidis cortex

DEFINITION

Cut, dried bark from the trunk and branches of *Hamamelis virginiana* L.

*Content*: minimum 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. Pieces of different lengths, channelled, sometimes quilled or in strips, up to 2 cm long and 3 mm thick. The outer surface is ochre-brown or reddish-brown and has a thin, whitish or greyish-brown cork with numerous lenticels. The inner surface is yellowish-brown or reddish-brown and is longitudinally striated. The fracture is splintery and fibrous.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2532.-1): very abundant sclereids of 2 types: one type (more abundant) rounded, oval or subrectangular, isolated [C] but more often in groups [A], with heavily thickened and striated walls and numerous and branched channels, sometimes accompanied by cells containing prisms of calcium oxalate [Aa]; the other type much more regular in size and shape, polygonal, with walls only moderately thickened and with simple channels, occurring in dense groups with no intercellular spaces [E], sometimes accompanied by cells containing prisms of calcium oxalate [Ea]; abundant groups of fibres [G], usually surrounded by a sheath of prisms of calcium oxalate [Gb]; fibres, very thick-walled and lignified, with an indistinct lumen [Ga]; thin-walled, parenchymatous cells of the phloem [D], sometimes filled with dark brown contents [Db]; uniseriate medullary rays, composed of cells with slightly thickened walls, somewhat rectangular (longitudinal section [Gc]) and rounded (tangential section [Da]); fragments of cork [H] consisting of thin-walled cells, polyhedral (surface view [B, Ha]), sometimes accompanied by an underlying layer of sclereids with slightly thickened and channelled walls [Hb]; abundant isolated prisms of calcium oxalate [F].

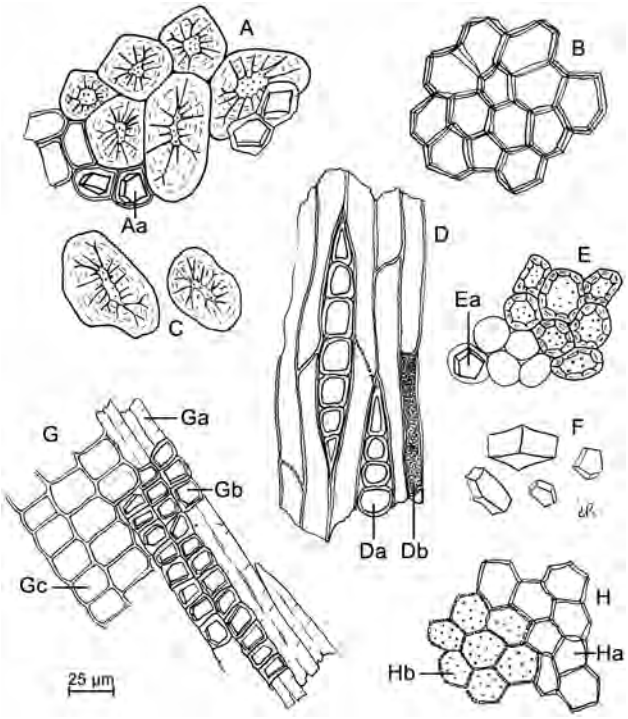


Figure 2532.-1. – *Illustration for identification test B of powdered herbal drug of hamamelis bark*

C. Examine the chromatogram obtained in the test for *Corylus avellana* L. bark and twigs and *Hamamelis virginiana* L. twigs.

*Results*: see below the sequence of zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the upper third of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Gallic acid: a blue fluorescent zone | |
| Hamamelitannin: a blue fluorescent zone | An intense blue fluorescent zone (hamamelitannin) |
| | A blue fluorescent zone |
| | 2 or 3 blue fluorescent zones |
| **Reference solution** | **Test solution** |

TESTS

***Corylus avellana* L. bark and twigs and *Hamamelis virginiana* L. twigs**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10.0 mL of *methanol R*, shake for 15 min and filter.

*Reference solution*. Dissolve 5.0 mg of *gallic acid R* and 8.0 mg of *hamamelitannin R* in 5.0 mL of *ethanol (60 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl formate R* (10:10:80 *V/V/V*).

*Application*: 6 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min; treat the plate whilst still hot with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, allow to dry in air and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows no yellow or orange zones.

**Foreign matter** (*2.8.2*): maximum 3 per cent of branches and twigs and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Carry out the determination with the following modifications.

*Preparation of the test solution*. Introduce 0.150 g of the powdered herbal drug (355) (*2.9.12*) into a 250 mL round-bottomed flask, add 150 mL of *water R* and heat on a water-bath for 30 min. Cool under running water and transfer quantitatively to a 250 mL volumetric flask. Rinse the round-bottomed flask and collect the washings in the volumetric flask, dilute to 250.0 mL with *water R* and shake. Centrifuge 25 mL at 1000 *g* for 10 min.

*Total polyphenols*. Use the supernatant instead of the filtrate described in the general method.

*Polyphenols not adsorbed by hide powder*. Use the supernatant instead of the filtrate described in the general method.

**01/2011:0909**

# HAMAMELIS LEAF

## Hamamelidis folium

DEFINITION

Whole or cut, dried leaf of *Hamamelis virginiana* L.

*Content*: minimum 3 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The leaf is green or greenish-brown, often broken, crumpled and compressed into more or less compact masses. The lamina is broadly ovate or obovate; the base is oblique and asymmetric and the apex is acute or, rarely, obtuse. The margins of the lamina are roughly crenate or dentate. The venation is pinnate and prominent on the abaxial surface. Usually, 4-6 pairs of secondary veins are attached to the main vein, emerging at an acute angle and curving gently to the marginal points where there are fine veins often at right angles to the secondary veins.

B. Microscopic examination (*2.8.23*). The powder is brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0909.-1): fragments of adaxial epidermis with wavy anticlinal walls (surface view [C, J]), often accompanied by small, cylindrical cells of the palisade parenchyma (surface view [Ja]), or elongated (transverse section [F]); fragments of abaxial epidermis with stomata mainly paracytic (*2.8.3*) (surface view [B]), which may be accompanied by irregular-shaped cells of spongy mesophyll [K, L]; star-shaped covering trichomes, either entire or broken [A, D, M], composed of 4-12 unicellular branches that are united by their bases, elongated, conical and curved, usually up to 250 µm long, thick-walled and with a clearly visible lumen whose contents are often brown; fibres are lignified and thick-walled, isolated or in groups, and accompanied by a sheath of prismatic calcium oxalate crystals [N, P]; sclereids, frequently enlarged at 1 or both ends, 150-180 µm long, whole or fragmented [H]; fragments of annular or spiral vessels [E]; isolated prisms of calcium oxalate [G].

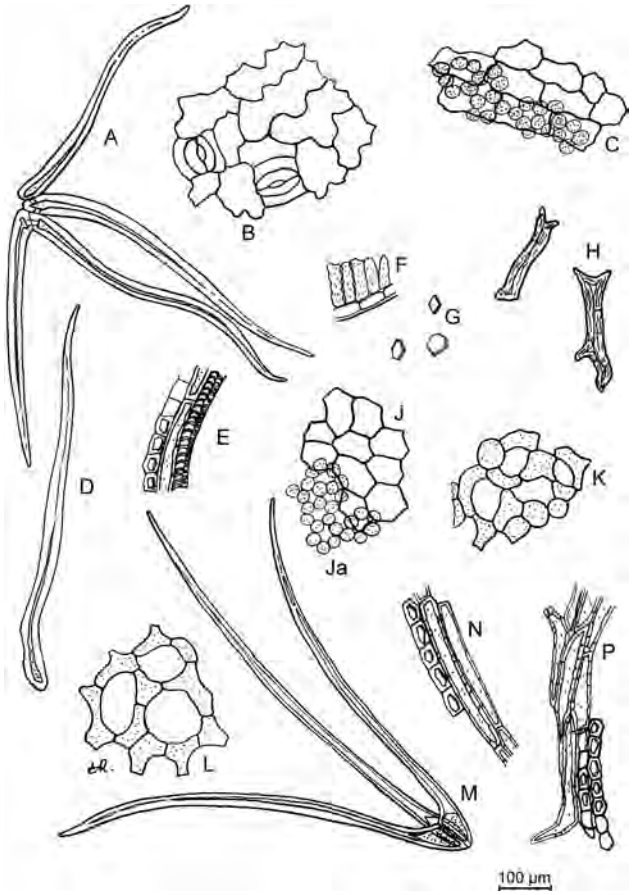


Figure 0909.-1. – *Illustration for identification test B of powdered herbal drug of hamamelis leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (60 per cent V/V) R*, shake for 15 min and filter.

*Reference solution (a).* Dissolve 30 mg of *tannic acid R* in 5 mL of *ethanol (60 per cent V/V) R*.

*Reference solution (b).* Dissolve 5 mg of *gallic acid R* in 5 mL of *ethanol (60 per cent V/V) R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *anhydrous formic acid R, water R, ethyl formate R* (10:10:80 *V/V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 10 min, then allow to cool.

*Detection*: spray with *ferric chloride solution R2* until bluish-grey zones (phenolic compounds) appear.

*Results*: the chromatogram obtained with the test solution shows in its lower third a principal zone similar in position to the principal zone in the chromatogram obtained with reference solution (a) and, in its upper part, a narrow zone similar in position to the principal zone in the chromatogram obtained with reference solution (b); the chromatogram obtained with the test solution shows, in addition, several slightly coloured zones in the central part.

TESTS

**Foreign matter** (*2.8.2*): maximum 7 per cent of stems and maximum 2 per cent of other foreign matter, determined on 50 g.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 2.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 4 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.750 g of the powdered herbal drug (180) (*2.9.12*).

**04/2013:1220**

# HAWTHORN BERRIES

## Crataegi fructus

DEFINITION

Dried false fruits of *Crataegus monogyna* Jacq. (Lindm.) or *C. laevigata* (Poir.) DC. (syn. *C. oxyacantha* L.) or their hybrids or a mixture of these false fruits.

*Content*: minimum 0.06 per cent of procyanidins, expressed as cyanidin chloride ($C_{15}H_{11}ClO_6$; $M_r$ 322.7) (dried drug).

IDENTIFICATION

A. The false fruit of *C. monogyna* is obovate or globular, generally 6-10 mm long and 4-8 mm wide, reddish-brown or dark red. The surface is pitted or, more rarely, reticulated. The upper end of the fruit is crowned by the remains of 5 reflexed sepals surrounding a small, sunken disc with a shallow, raised rim. The remains of the style occur in the centre of the disc with tufts of stiff, colourless hairs at the base. At the lower end of the fruit is a short length of pedicel or, more frequently, a small, pale, circular scar where the pedicel was attached. The receptacle is fleshy and encloses a yellowish-brown, ovoid fruit with a hard, thick wall containing a single, elongated, pale brown, smooth and shiny seed.

The false fruit of *C. laevigata* is up to 13 mm long. It contains 2-3 stony fruits, ventrally flattened, with short hairs at the top. Frequently, in the centre of the disc of the false fruit occur the remains of the 2 styles.

B. Microscopic examination (*2.8.23*). The powder is greyish-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1220.-1): covering trichomes [F] from inside the disc that are long, unicellular, frequently bent, tapering to a point, with much thickened and lignified walls; fragments of the red outer layer of the receptacle (surface view [G]); fragments of the inner layers of the receptacle [A], some cells containing cluster crystals [Aa] or prisms [Ab] of calcium oxalate; occasional fragments [J, K] including groups of sclereids [Ka] and vascular bundles [Ja, Kb] associated with rows of cells containing prisms of calcium oxalate [Jb, Kc]; fragments of the pericarp [B] consisting of parenchyma including some cells containing cluster crystals of calcium oxalate [Ba] and groups of sclereids of various sizes with numerous pits [Bb]; thick-walled sclereids [E, H], some channelled (E), some with conspicuously branched channels (H); a few fragments of the testa [C] having an outer layer composed of hexagonal, mucilaginous cells [Ca] beneath which is a yellowish-brown pigment layer containing numerous prisms of calcium oxalate [Cb]; parenchyma of the endosperm and cotyledons consisting of cells containing aleurone grains and globules of fixed oil [D].

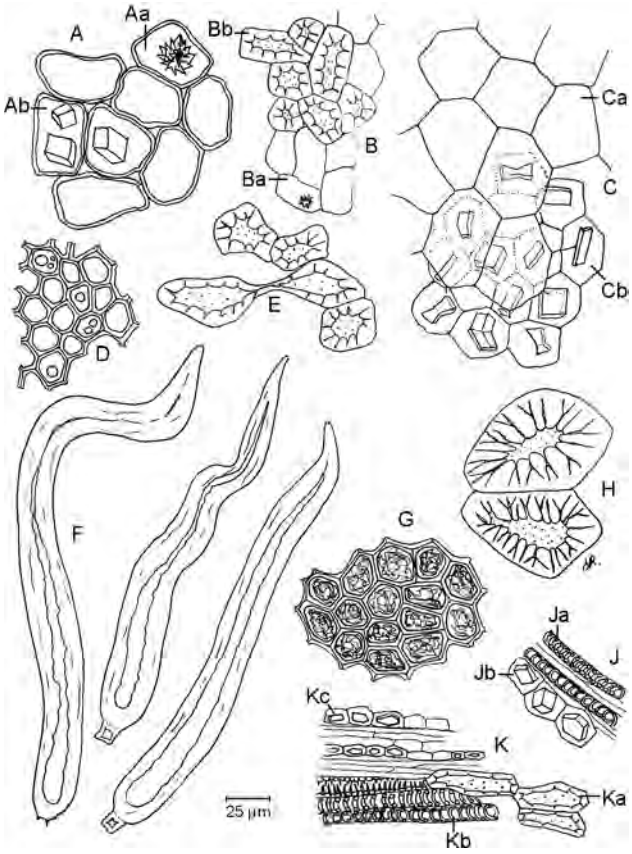


Figure 1220.-1. – *Illustration for identification test B of powdered herbal drug of hawthorn berries*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and heat on a water bath at 65 °C for 5 min, shaking frequently. Allow to cool to room temperature and filter. Dilute the filtrate to 10 mL with *methanol R*.

*Reference solution.* Dissolve 2 mg of *chlorogenic acid R*, 2 mg of *caffeic acid R*, 5 mg of *hyperoside R* and 5 mg of *rutoside trihydrate R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 30 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray whilst hot with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower half, in order of increasing $R_F$ values, a yellowish-brown fluorescent zone (rutoside), a light blue fluorescent zone (chlorogenic acid) and a yellowish-brown fluorescent zone (hyperoside); in the upper third appears a light blue fluorescent zone (caffeic acid). The chromatogram obtained with the test solution shows 3 zones similar in position and fluorescence to the zones due to chlorogenic acid, hyperoside and caffeic acid in the chromatogram obtained with the reference solution, and 3 weak reddish fluorescent zones, one corresponding to the zone due to rutoside in the chromatogram obtained with the reference solution and both of the others located above the zone due to hyperoside; below and above the zone due to caffeic acid some light blue zones appear.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of deteriorated false fruit and maximum 2 per cent of other foreign matter. It does not contain false fruits of other *Crataegus* species (*C. nigra* Waldst. et Kit., *C. pentagyna* Waldst. et Kit. ex Willd. and *C. azarolus* L.), which are characterised by the presence of more than 3 hard stones.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

To 2.50 g of the powdered herbal drug (355) (*2.9.12*) add 30 mL of *ethanol (70 per cent V/V) R*. Heat under a reflux condenser for 30 min and filter. Wash the residue with 10.0 mL of *ethanol (70 per cent V/V) R*. Add to the filtrate 15.0 mL of *hydrochloric acid R1* and 10.0 mL of *water R*. Heat under a reflux condenser for 80 min. Allow to cool, filter and wash the residue with *ethanol (70 per cent V/V) R* until the filtrate is colourless. Dilute the filtrate to 250.0 mL with *ethanol (70 per cent V/V) R*. Evaporate 50.0 mL of this solution in a round-bottomed flask to about 3 mL and transfer to a separating funnel. Rinse the round-bottomed flask sequentially with 10 mL and 5 mL of *water R* and transfer to the separating funnel. Shake the combined solution with 3 quantities, each of 15 mL, of *butanol R*. Combine the organic layers and dilute to 100.0 mL with *butanol R*.

Measure the absorbance (*2.2.25*) of the solution at 555 nm.

Calculate the percentage content of procyanidins, expressed as cyanidin chloride, using the following expression:

$$\frac{A \times 500}{1200 \times m}$$

i.e. taking the specific absorbance of cyanidin chloride to be 1200.

$A$ = absorbance at 555 nm;

$m$ = mass of the substance to be examined, in grams.

**01/2010:1432**

# HAWTHORN LEAF AND FLOWER

## Crataegi folium cum flore

DEFINITION

Whole or cut, dried flower-bearing branches of *Crataegus monogyna* Jacq. (Lindm.), *C. laevigata* (Poir.) DC. (syn. *C. oxyacanthoides* Thuill.; *C. oxyacantha* auct.) or their hybrids or, more rarely, other European *Crataegus* species including *C. pentagyna* Waldst. et Kit. ex Willd., *C. nigra* Waldst. et Kit. and *C. azarolus* L.

*Content*: minimum 1.5 per cent of total flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The stems are dark brown, woody, 1-2.5 mm in diameter, bearing alternate, petiolate leaves with small, often deciduous stipules and corymbs of numerous small white flowers. The leaves are more or less deeply lobed with slightly serrate or almost entire margins; those of *C. laevigata* are pinnately lobed or pinnatifid with 3, 5 or 7 obtuse lobes, those of *C. monogyna* pinnatisect with 3 or 5 acute lobes; the adaxial surface is dark green or brownish-green, the abaxial surface is lighter greyish-green and shows a prominent, dense, reticulate venation. The

leaves of *C. laevigata*, *C. monogyna* and *C. pentagyna* are glabrous or bear only isolated trichomes, those of *C. azarolus* and *C. nigra* are densely pubescent. The flowers have a brownish-green tubular calyx composed of 5 free, reflexed sepals, a corolla composed of 5 free, yellowish-white or brownish, rounded or broadly ovate and shortly unguiculate petals and numerous stamens. The ovary is fused to the calyx and consists of 1-5 carpels, each with a long style and containing a single ovule; in *C. monogyna* there is 1 carpel, in *C. laevigata* 2 or 3, in *C. azarolus* 2 or 3, or sometimes only 1, in *C. pentagyna* 5 or, rarely, 4.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: unicellular covering trichomes, usually with a thick wall and wide lumen, almost straight or slightly curved, pitted at the base; fragments of leaf epidermis with cells which have sinuous or polygonal anticlinal walls and with large anomocytic stomata (*2.8.3*) surrounded by 4-7 subsidiary cells; parenchymatous cells of the mesophyll containing calcium oxalate clusters, usually measuring 10-20 µm, those associated with the veins containing groups of small prism crystals; fragments of petals showing rounded polygonal epidermal cells, strongly papillose, with thick walls, the cuticle of which clearly shows wavy striations; fragments of anthers showing endothecium with an arched and regularly thickened margin; fragments of stems containing collenchymatous cells, bordered pitted vessels and groups of lignified sclerenchymatous fibres with narrow lumina; numerous spherical to elliptical or triangular pollen grains up to 45 µm in diameter, with 3 germinal pores and a faintly granular exine.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and heat in a water-bath at 65 °C under a reflux condenser for 5 min. Cool and filter.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *hyperoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A yellowish-green fluorescent zone (vitexin) |
| Hyperoside: a yellowish-orange fluorescent zone | A yellowish-orange fluorescent zone (hyperoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| | A yellowish-green fluorescent zone (vitexin-2″-rhamnoside) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 8 per cent of lignified branches with a diameter greater than 2.5 mm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

*Stock solution.* Into a 200 mL flask introduce 0.400 g of the powdered herbal drug (250) (*2.9.12*) and 40 mL of *ethanol (60 per cent V/V) R*. Heat in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter through a plug of absorbent cotton into a 100 mL volumetric flask. Transfer the absorbent cotton with the drug residue back to the 200 mL flask, add 40 mL of *ethanol (60 per cent V/V) R* and heat again in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter into the same 100 mL volumetric flask. Rinse the 200 mL flask with a further quantity of *ethanol (60 per cent V/V) R*, filter and transfer to the same 100 mL volumetric flask. Dilute to 100.0 mL with *ethanol (60 per cent V/V) R* and filter.

*Test solution.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to the same 25 mL volumetric flask. Add 10.0 mL of a solution containing 25.0 g/L of *boric acid R* and 20.0 g/L of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to the same 25 mL volumetric flask. Add 10.0 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 410 nm, by comparison with the compensation liquid.

Calculate the percentage content of total flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.235}{m}$$

i.e. taking the specific absorbance of hyperoside to be 405.

$A$ = absorbance at 410 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2010:1865**

# HAWTHORN LEAF AND FLOWER DRY EXTRACT

## Crataegi folii cum flore extractum siccum

DEFINITION

Dry extract produced from *Hawthorn leaf and flower (1432)*.

*Content*:
- for aqueous extracts: minimum 2.5 per cent of total flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried extract);
- for hydroalcoholic extracts: minimum 6.0 per cent of total flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using either water or a hydroalcoholic solvent at least equivalent in strength to ethanol (45 per cent *V/V*).

CHARACTERS

*Appearance*: light brown or greenish-brown powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 0.2 g of the extract to be examined in 20 mL of *ethanol (70 per cent V/V) R* and filter.

*Reference solution.* Dissolve 1 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light yellow fluorescent zone |
| Hyperoside: a yellowish-orange fluorescent zone | A yellowish-orange fluorescent zone (hyperoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| | A yellowish-green fluorescent zone (vitexin 2″-rhamnoside) |
| Rutoside: a yellowish-orange fluorescent zone | A yellowish-orange fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 0.500 g of the extract to be examined by drying in an oven at 105 °C for 2 h.

ASSAY

*Stock solution.* Dissolve 0.100 g of the extract to be examined in *ethanol (60 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue in 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to the same 25 mL volumetric flask. Add 10.0 mL of a solution containing 25.0 g/L of *boric acid R* and 20.0 g/L of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue in 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *anhydrous acetic acid R* and transfer to the same 25 mL volumetric flask. Add 10.0 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 410 nm, by comparison with the compensation liquid.

Calculate the percentage content of total flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A \times 1.235}{m}$$

i.e. taking the specific absorbance of hyperoside to be 405.

$A$ = absorbance at 410 nm;

$m$ = mass of the extract to be examined, in grams.

**01/2008:1864**

# HAWTHORN LEAF AND FLOWER LIQUID EXTRACT, QUANTIFIED

## Crataegi folii cum flore extractum fluidum quantificatum

DEFINITION

Quantified liquid extract produced from *Hawthorn leaf with flower (1432)*.

*Content*: 0.8 per cent to 3.0 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4).

PRODUCTION

The extract is produced from the herbal drug and ethanol (30 per cent *V/V* to 70 per cent *V/V*) by an appropriate procedure.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1.0 g in *methanol R* and dilute to 5 mL with the same solvent. Shake and filter.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R* and 2.5 mg of *hyperoside R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 20 µL [or 5 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: at 100-105 °C.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A yellowish-green fluorescent zone |
| Hyperoside: a yellowish-orange fluorescent zone | A yellowish-orange fluorescent zone (hyperoside) |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| | A yellowish-green fluorescent zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 95 per cent *V/V* to 105 per cent *V/V* of the quantity stated on the label.

ASSAY

*Stock solution.* Dilute about 0.400 g, accurately weighed, in *ethanol (60 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 mL volumetric flask. Add 10.0 mL of a solution containing 25.0 g/L of *boric acid R* and 20.0 g/L of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 mL volumetric flask. Add 10.0 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

After 30 min measure the absorbance (*2.2.25*) of the test solution at 410 nm.

Calculate the percentage content of total flavonoids, expressed as hyperoside, from the following expression:

$$\frac{A \times 1.235}{m}$$

i.e. taking the value of the specific absorbance of hyperoside to be 405.

$A$ = absorbance at 410 nm,

$m$ = mass of the extract to be examined, in grams.

**01/2011:1222**

# HOP STROBILE

## Lupuli flos

DEFINITION

Dried, generally whole, female inflorescence of *Humulus lupulus* L.

CHARACTERS

Characteristic, aromatic odour.

IDENTIFICATION

A. Hop strobiles are generally isolated and 2-5 cm long, petiolate, ovoid, made up of many oval, greenish-yellow, sessile, membranous, overlapping bracts. The external bracts are flattened and symmetrical. The internal bracts are longer and asymmetrical at the base because of a fold generally encircling an induviate fruit (achene). The ovary or rarely the fruit, the base of the bracts and especially the induvial fold, are covered with small orange-yellow glands.

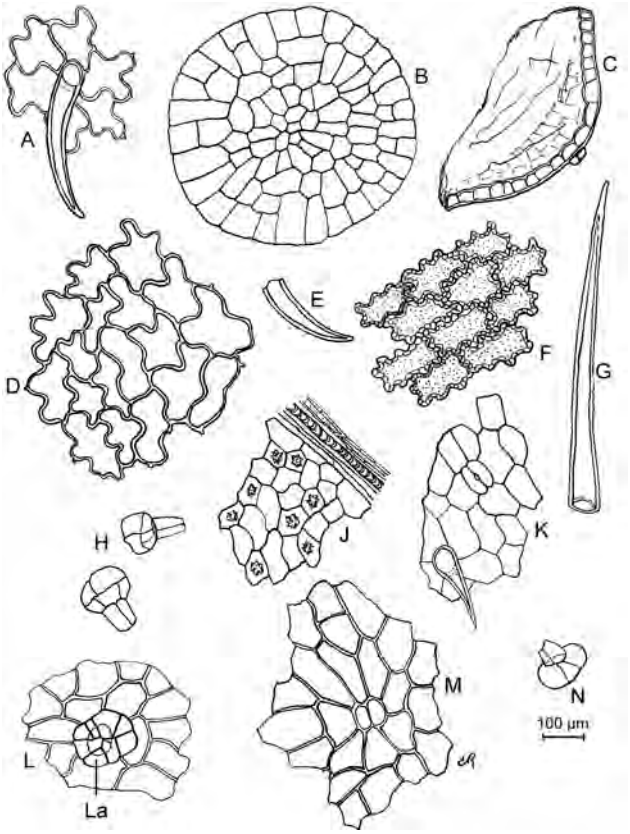


Figure 1222.-1. – *Illustration for identification test B of powdered herbal drug of hop strobile*

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1222.-1): fragments of bracts and bracteoles covered by polygonal, irregular or wavy-walled epidermal cells [D, L, M]; unicellular, conical, straight or curved covering trichomes with thin, smooth walls, fragmented [E, G] or attached to an epidermis [A]; rare anomocytic stomata (*2.8.3*) [K]; glandular trichomes, usually free, with bicellular biseriate stalks and heads consisting of 8 small cells [H, N], rarely attached to an epidermis [La]; fragments of mesophyll containing small calcium oxalate cluster crystals [J]; many characteristic orange-yellow glandular trichomes with short, bicellular biseriate stalks, bearing a part widening into a cup,

150-250 µm in diameter, made up of a hemispherical layer of secretory cells with a cuticle that has been detached and distended by the accumulation of oleoresinous secretions (surface view [B], side view [C]); fragments of elongated sclerenchymatous cells of the testa with thick walls showing striations and numerous pits [F].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the freshly powdered herbal drug (355) (*2.9.12*) add 10 mL of a mixture of 3 volumes of *water R* and 7 volumes of *methanol R*; shake for 15 min and filter.

*Reference solution.* Dissolve 1.0 mg of *Sudan orange R*, 2.0 mg of *curcumin R* and 2.0 mg of *dimethylaminobenzaldehyde R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the reference solution shows 3 quenching zones; in the lower quarter is the faint zone due to curcumin, somewhat below the middle is the zone due to dimethylaminobenzaldehyde, and above, the zone due to Sudan orange. The chromatogram obtained with the test solution shows a number of quenching zones similar in position to the zones in the chromatogram obtained with the reference solution: at about the level of the zone due to curcumin is a faint zone due to xanthohumol, near the level of the zone due to dimethylaminobenzaldehyde are zones due to humulones, and near the level of the zone due to Sudan orange are zones due to lupulones.

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: in the chromatogram obtained with the test solution the zones due to lupulones show blue fluorescence, the zones due to humulones show brown fluorescence and the zone due to xanthohumol shows dark brown fluorescence.

*Detection C*: spray with *dilute phosphomolybdotungstic reagent R*; expose to ammonia vapour and examine in daylight.

*Results C*: in the chromatogram obtained with the test solution the zones due to humulones and to lupulones are bluish-grey and the zone due to xanthohumol is greenish-grey; in the chromatogram obtained with the reference solution the zones are bluish-grey or brownish-grey.

TESTS

**Matter extractable by ethanol (70 per cent *V/V*)**: minimum 25.0 per cent.

To 10.0 g of the powdered herbal drug (355) (*2.9.12*) add 300 mL of *ethanol (70 per cent V/V) R* and heat for 10 min on a water-bath under a reflux condenser. Allow to cool, filter, and discard the first 10 mL of the filtrate. Evaporate 30.0 mL of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 0.250 g.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**01/2017:1830**

# HORSE-CHESTNUT

## Hippocastani semen

DEFINITION

Whole or fragmented, dried, ripe seeds of *Aesculus hippocastanum* L.

*Content*: minimum 1.5 per cent of triterpene glycosides, expressed as protoaescigenin ($C_{30}H_{50}O_6$; $M_r$ 506.7) (dried drug).

IDENTIFICATION

A. The whole, spherical or oval, slightly flattened seed is 2-4 cm in diameter. It has a shiny, dark-brown testa with a broad, round, matt, light-brown spot (hilum); particularly on larger seeds, a short, narrow v-shaped ridge marks the position of the radicle, with the point terminating close to the hilum.

The fragmented seed occurs as more or less polyhedral pieces, about 1-2 cm in diameter, or as slices. The surfaces corresponding to the cotyledons are matt, light brown, with a clean fracture. Those corresponding to the testa are shiny, dark brown, except at the hilum, where they are matt, light brown. The testa is weakly bound to the cotyledons and is often detached.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1830.-1): numerous different-sized droplets of fatty oil that are either free [D] or inside the thin-walled colourless cells of the cotyledons [E, G]; yellowish-brown fragments of the outer testa consisting of sclerenchymatous cells with thick walls (surface view [C], transverse section [A]); fragments from the inner testa consisting of thick-walled, colourless parenchymatous cells varying in shape [B, H, J] with poorly visible pits and occasional annular or spiral vessels [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. Starch [K] is present in 3 forms: pyriform or reniform simple granules, often with verruciform excrescences, about 15-25 µm in size, sometimes up to 30 µm;

roundish simple granules 5-10 µm in diameter; and a few granules that form rows consisting of 2-4 simple granules that are up to about 35 µm in length and occasionally up to about 45 µm; most of the granules have either a stellate (2 or more rays) or more rarely a punctiform hilum.

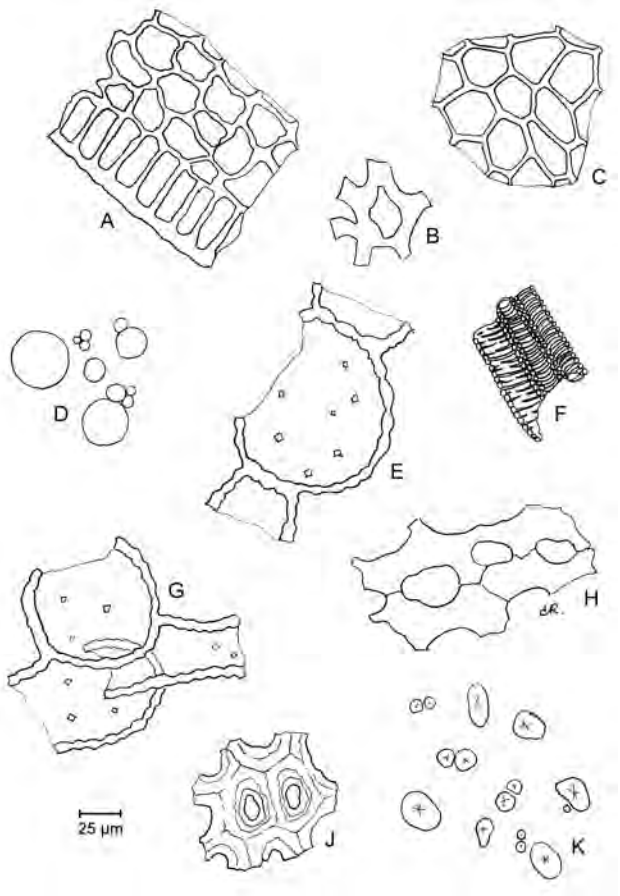


Figure 1830.-1. – *Illustration for identification test B of powdered herbal drug of horse-chestnut*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (70 per cent V/V) R* and heat under a reflux condenser for 15 min. Cool and filter.

*Reference solution.* Dissolve 5 mg of *aescin for LC assay HRS* and 5 mg of *sucrose R* in 1.0 mL of *ethanol (70 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *ethyl acetate R*, *water R*, *propanol R* (1.5:30:30:40 *V/V/V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 6 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A yellowish-violet zone |
| Aescin: a violet-blue zone | An intense violet-blue zone (aescin) |
| Sucrose: a brownish-green zone | A brownish-green zone (sucrose) |
| | 2 brownish-green zones |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, 0.05 per cent *V/V* solution of *trifluoroacetic acid R* (40:60 *V/V*).

*Internal marker solution.* Dissolve 25.0 mg of *methyl salicylate R* and 75.0 mg of *ibuprofen R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Test solution.* To 2.00 g of the powdered herbal drug (355) (*2.9.12*) add 10.0 mL of the internal marker solution and 10.0 mL of the solvent mixture and sonicate for 10 min. Centrifuge and filter 2.0 mL of the supernatant through a membrane filter (0.45 µm). Use the filtrate as the test solution.

*Reference solution (a).* Dissolve 50.0 mg of *aescin for LC assay HRS* in the internal marker solution and dilute to 10.0 mL with the internal marker solution. Sonicate for 10 min and filter through a membrane filter (0.45 µm).

*Reference solution (b).* Dilute 1.0 mL of the internal marker solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 70 | 30 |
| 15 - 25 | 70 → 65 | 30 → 35 |
| 25 - 35 | 65 | 35 |
| 35 - 65 | 65 → 50 | 35 → 50 |
| 65 - 70 | 50 → 10 | 50 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *aescin for LC assay HRS* and the chromatogram obtained with reference solution (a) to identify peaks A and B due to aescin.

*System suitability*:

- the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *aescin for LC assay HRS* regarding the peaks between those due to methyl salicylate and ibuprofen;
- *retention time*: methyl salicylate = 11.5 min to 15.5 min; ibuprofen = 34.0 min to 46.0 min;
- *resolution*: minimum 2.0 between peaks A and B due to aescin in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peak due to methyl salicylate in the chromatogram obtained with reference solution (b).

*Integration*:

- *test solution*: integrate the peaks eluting between the peak due to methyl salicylate and the peak due to ibuprofen; integrate the area of the individual peaks or integrate groups of peaks in case of incomplete separation of the individual peaks;
- *reference solution (a)*: for quantification, integrate as 1 integral, valley-to-valley, starting from the 1st peak after the peak due to methyl salicylate and ending with the last peak before the peak due to ibuprofen; if a peak is poorly separated from the peaks due to methyl salicylate or ibuprofen, integrate it separately; use the sum of the peak areas for the calculation.

Calculate the percentage content of triterpene glycosides, expressed as protoaescigenin, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = total area (integrated as described above) of the peaks eluting between the peaks due to methyl salicylate and ibuprofen in the chromatogram obtained with the test solution;

$A_2$ = total area (integrated as described above) of the peaks eluting between the peaks due to methyl salicylate and ibuprofen in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *aescin for LC assay HRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of aescin, expressed as protoaescigenin, in *aescin for LC assay HRS*.

STORAGE

In an airtight container.

**01/2017:1829**

# HORSE-CHESTNUT DRY EXTRACT, STANDARDISED

## Hippocastani seminis extractum siccum normatum

DEFINITION

Standardised dry extract produced from *Horse-chestnut (1830)*.

*Content*: 6.5 per cent to 10.0 per cent of total triterpene glycosides, expressed as protoaescigenin ($C_{30}H_{50}O_6$; $M_r$ 506.7) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using a hydroalcoholic solvent equivalent in strength to ethanol (40-80 per cent *V/V*).

CHARACTERS

*Appearance*: yellowish or yellowish-brown, hygroscopic powder or agglomerate.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the extract to be examined add 10 mL of *ethanol (70 per cent V/V) R*, shake for 2 min and filter.

*Reference solution.* Dissolve 5 mg of *aescin for LC assay HRS* and 5 mg of *sucrose R* in 1.0 mL of *ethanol (70 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *ethyl acetate R*, *water R*, *propanol R* (1.5:30:30:40 *V/V/V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 6 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 1 or 2 yellowish-violet zones |
| Aescin: an intense violet-blue zone | An intense violet-blue zone (aescin) |
| Sucrose: a brownish-green zone | A brownish-green zone (sucrose) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, 0.05 per cent *V/V* solution of *trifluoroacetic acid R* (40:60 *V/V*).

*Internal marker solution.* Dissolve 25.0 mg of *methyl salicylate R* and 75.0 mg of *ibuprofen R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Test solution.* Dissolve 0.1000 g of the extract to be examined in the internal marker solution and dilute to 10.0 mL with the internal marker solution. Sonicate for 10 min and filter through a membrane filter (0.45 µm).

*Reference solution (a).* Dissolve 20.0 mg of *aescin for LC assay HRS* in the internal marker solution and dilute to 10.0 mL with the internal marker solution. Sonicate for 10 min and filter through a membrane filter (0.45 µm).

*Reference solution (b).* Dilute 1.0 mL of the internal marker solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: 0.05 per cent *V*/*V* solution of *trifluoroacetic acid R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V*/*V*) | Mobile phase B (per cent *V*/*V*) |
|---|---|---|
| 0 - 15 | 70 | 30 |
| 15 - 25 | 70 → 65 | 30 → 35 |
| 25 - 35 | 65 | 35 |
| 35 - 65 | 65 → 50 | 35 → 50 |
| 65 - 70 | 50 → 10 | 50 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *aescin for LC assay HRS* and the chromatogram obtained with reference solution (a) to identify peaks A and B due to aescin.

*System suitability*:

– the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *aescin for LC assay HRS* regarding the peaks between those due to methyl salicylate and ibuprofen;

– *retention time*: methyl salicylate = 11.5 min to 15.5 min; ibuprofen = 34.0 min to 46.0 min;

– *resolution*: minimum 2.0 between peaks A and B due to aescin in the chromatogram obtained with reference solution (a);

– *signal-to-noise ratio*: minimum 10 for the peak due to methyl salicylate in the chromatogram obtained with reference solution (b).

*Integration*:

– *test solution*: integrate the peaks eluting between the peak due to methyl salicylate and the peak due to ibuprofen; integrate the area of the individual peaks or integrate groups of peaks in case of incomplete separation of the individual peaks;

– *reference solution (a)*: for quantification, integrate as 1 integral, valley-to-valley, starting from the 1st peak after the peak due to methyl salicylate and ending with the last peak before the peak due to ibuprofen; if a peak is poorly separated from the peaks due to methyl salicylate or ibuprofen, integrate it separately; use the sum of the peak areas for the calculation.

Calculate the percentage content of triterpene glycosides, expressed as protoaescigenin, using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = total area (integrated as described above) of the peaks eluting between the peaks due to methyl salicylate and ibuprofen in the chromatogram obtained with the test solution;

$A_2$ = total area (integrated as described above) of the peaks eluting between the peaks due to methyl salicylate and ibuprofen in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *aescin for LC assay HRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of aescin, expressed as protoaescigenin, in *aescin for LC assay HRS*.

**04/2018:2722**

# HOUTTUYNIA HERB

## Houttuyniae herba

DEFINITION

Whole or fragmented, dried, flowering aerial parts of *Houttuynia cordata* Thunb.

*Content*: minimum 0.10 per cent of quercitrin ($C_{21}H_{20}O_{11}$; $M_r$ 448.4) (dried drug).

IDENTIFICATION

A. *Whole drug.* It comprises stems, abundant leaves and flowering spikes. Stems are compressed-cylindrical, externally yellowish-brown, with several deep longitudinal furrows and distinctly jointed nodes. The leaves (if still attached to stems) are alternate, with petioles shorter than the leaf blade and enclosed by a downy membranous sheath (stipule) about 1/4 to 1/2 the length of the petiole; leaf blades are rolled or crumpled and densely gland-dotted; when intact and expanded, the leaf blades are broadly ovate (3-10 cm long, 3-5 cm wide) with a cordate base and a narrowly pointed tip and entire margins; the upper surface is dark yellowish-green or dark brown, the lower surface greyish-green or greyish-brown. The flowers are densely packed in yellowish-brown cylindrical heads (about 1 cm long, 0.3 cm in diameter), occasionally with 4 (rarely 6 or 8) shrivelled brown bracts (about 1 cm long), persistent at the base. Individual flowers are very small (about 1 mm in diameter), always petal-less, comprising of 3 stamens and 3 carpels, each with a recurved style and a semi-inferior ovary. A few fruiting spikes may be present. Individual fruits are very small (about 2-3 mm long, 1 mm in diameter), dry, subglobose capsules with persistent styles, and are apically dehiscent. Once dehisced, fruits resemble tiny goblets with a 3-toothed lip.

*Fragmented drug.* It consists of fragments (up to about 4 cm long, 2.5-5 mm in diameter) of stems, leaves and the spike, similar to those described above.

B. Microscopic examination (*2.8.23*). The powder is dark brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2722.-1): fragments of the upper epidermis of the leaf (surface view [B]), covered by a strongly striated cuticle, composed of polygonal cells [Ba] usually associated with a hypodermis consisting of a layer of large polygonal cells with irregularly thickened walls [Bb]; fragments of the lower epidermis of the leaf (surface view [A]), covered by a striated cuticle, with polygonal cells [Aa], anomocytic stomata [Ab] (*2.8.3*) and uniseriate glandular trichomes, with a multicellular stalk and a unicellular head, whole (rare) [Ac] or broken (frequent) [Cb]; epidermal cells located above the oil cells are arranged in a rosette and the cuticle shows radial striations [Ad]; fragments of palisade parenchyma (surface view [E]) with each cell containing a small cluster crystal of calcium oxalate [Ea], sometimes associated with spiral vessels of the veins [Eb]; fragments of the epidermis of the veins with elongated cells [C], uniseriate and multicellular covering trichomes, with rounded ends and strongly striated walls [Ca], and glandular trichomes;

scattered covering trichomes of the veins [D]; fragments of parenchyma [G] consisting of oil cells with orange-yellow contents [Ga]; fragments of vascular tissue from the stems [F] with large vessels [Fb] and pitted fibres [Fa].

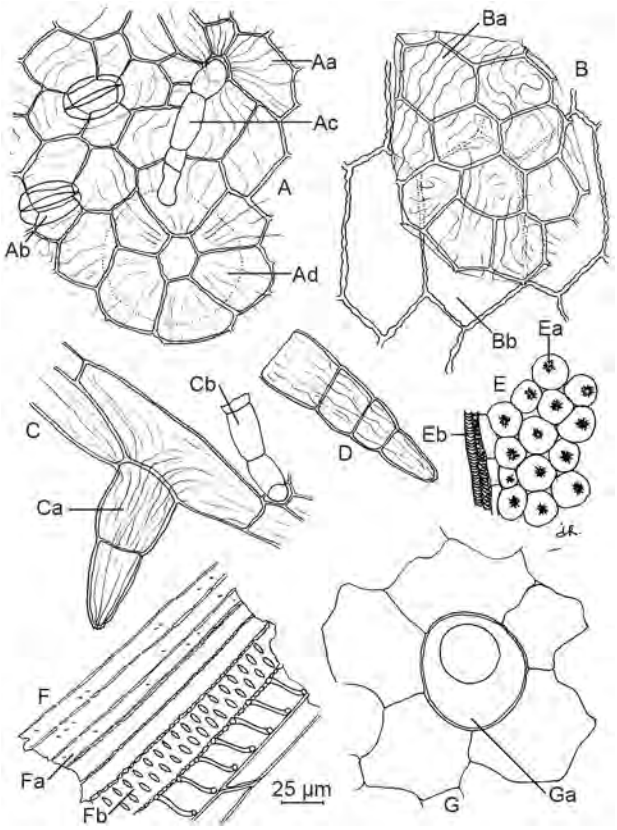


Figure 2722.-1. – *Illustration for identification test B of powdered herbal drug of houttuynia herb*

C. High performance thin-layer chromatography (*2.8.25*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate for 10 min. Centrifuge and use the supernatant. If necessary, filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 2.0 mg of *hyperoside R* and 5.0 mg of *quercitrin R* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 1 mg of *chlorogenic acid R* and 2 mg of *hyperoside R* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Intensity marker*: quercitrin.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min. Spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*. Allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the middle third; the lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| | | 2 red zones |
| | Quercitrin: a yellow zone | A yellow zone (quercitrin) |
| | | A faint yellow zone |
| Hyperoside: a yellow zone | Hyperoside: a yellow zone | A yellow zone (hyperoside) |
| | | A blue zone |
| Chlorogenic acid: a bluish zone | | A blue zone |
| | | A faint yellow zone |
| **Reference solution (c)** | **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (355) (*2.9.12*) add 50.0 mL of a mixture of equal volumes of *methanol R* and *water R*. Weigh and sonicate for 1 h. Allow to cool and weigh again. Compensate for the loss of solvent with a mixture of equal volumes of *methanol R* and *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *quercitrin CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 2.5 mg of *isoquercitrin R* in reference solution (a) and dilute to 5.0 mL with reference solution (a). Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 → 70 | 20 → 30 |
| 10 - 10.1 | 70 → 5 | 30 → 95 |
| 10.1 - 20 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 258 nm.

*Injection*: 20 µL.

*Retention time*: isoquercitrin = about 6.5 min; quercitrin = about 8 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 5 between the peaks due to isoquercitrin and quercitrin.

Calculate the percentage content of quercitrin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to quercitrin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to quercitrin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *quercitrin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of quercitrin in *quercitrin CRS*.

**07/2010:1439**

# ICELAND MOSS

## Lichen islandicus

DEFINITION

Whole or cut, dried thallus of *Cetraria islandica* (L.) Acharius s.l.

IDENTIFICATION

A. The thallus, up to 15 cm long, is irregularly dichotomous and consists of glabrous, groove-shaped or almost flat, stiff, brittle bands, 0.3-1.5 cm wide and about 0.5 mm thick, sometimes serrated with the margin appearing ciliated (pycnidia). The upper surface is greenish or greenish-brown, the lower surface is greyish-white or light brownish and shows whitish, depressed spots (so-called respiratory cavities). On the apices of the terminal lobes, very rarely, there are brown, discoid apothecia.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous fragments of the pseudoparenchyma consisting of narrow-lumened, thick-walled hyphae from the marginal layer and wide-lumened hyphae from the adjacent layer consisting of loosely entwined hyphae, in which, in the medullary zone, greenish or brownish algae cells up to 15 µm in diameter, are embedded; occasionally marginal fragments of the thallus with tube-like or cylindrical spermogonia, up to about 160 µm wide and up to about 400 µm long.

C. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *water R* and boil for 2-3 min. The greyish-brown solution forms a gel after cooling which gives a blue colour with *iodine solution R1*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *acetone R* and heat in a water-bath under a reflux condenser for 2-3 min. Cool and filter.

*Reference solution.* Dissolve 5 mg of *anethole R* and 5 mg of *caffeic acid R* in 2 mL of *acetone R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *acetone R*, *methanol R*, *anhydrous formic acid R*, *toluene R* (5:5:10:80 *V/V/V/V*).

*Application*: 20 µL [or 4 µL] of the test solution and 10 µL [or 2 µL] of the reference solution, as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A greyish-blue zone |
| Anethole: a blue or bluish-violet zone | |
| _____ | _____ |
| | 2 weak greyish-blue zones |
| | A weak greyish-brown or grey zone |
| _____ | _____ |
| | A greyish-violet zone |
| Caffeic acid: a greyish-blue zone | |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Lead** (*2.4.27*): maximum 10.0 ppm.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

**Swelling index** (*2.8.4*): minimum 4.5, determined on the powdered herbal drug (355) (*2.9.12*).

**01/2013:2310**

# INDIAN FRANKINCENSE

## Olibanum indicum

DEFINITION

Air-dried gum-resin exudate, obtained by incision in the stem or branches of *Boswellia serrata* Roxb. ex Colebr.

*Content*:

– *11-keto-β-boswellic acid* ($C_{30}H_{46}O_4$; $M_r$ 470.7): minimum 1.0 per cent (dried drug);

– *acetyl-11-keto-β-boswellic acid* ($C_{32}H_{48}O_5$; $M_r$ 512.7): minimum 1.0 per cent (dried drug).

IDENTIFICATION

A. Indian frankincense consists of translucent, roundish or irregularly shaped, variable size pieces of up to 3 cm. They are yellowish or reddish-brown. Their surface is covered with grey dust. The fracture is dull or slightly glossy.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 90 mL of *methanol R* and sonicate for 10 min. Shake the mixture vigorously 3 or 4 times during this procedure. Dilute to 100 mL with *methanol R*. Centrifuge and use the clear supernatant solution.

*Reference solution.* Dissolve 2 mg of *11-keto-β-boswellic acid R* and 2 mg of *acetyl-11-keto-β-boswellic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 μm) [or *TLC silica gel $F_{254}$ plate R* (2-10 μm)].

*Mobile phase*: *anhydrous formic acid R, heptane R, ethyl acetate R, toluene R* (3:10:20:80 *V/V/V/V*).

*Application*: 10 μL [or 3 μL] as bands.

*Development*: over a path of 8 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. The zones due to 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid in the test solution are of approximately equivalent intensity. Furthermore, other weak quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| ——— | ——— |
| Acetyl-11-keto-β-boswellic acid: a quenching zone | A quenching zone (acetyl-11-keto-β-boswellic acid) |
| 11-Keto-β-boswellic acid: a quenching zone | A quenching zone (11-keto-β-boswellic acid) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 3 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 90 mL of *methanol R* and sonicate for 10 min. Shake the mixture vigorously 3 or 4 times during this procedure. Dilute to 100.0 mL with *methanol R*. Centrifuge for 5 min. Dilute 1.0 mL of the clear solution to 10.0 mL with a mixture of 16 volumes of mobile phase A and 84 volumes of mobile phase B.

*Reference solution.* Dissolve 1.0 mg of *11-keto-β-boswellic acid R* and 1.0 mg of *acetyl-11-keto-β-boswellic acid R* in 20.0 mL of *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 16 volumes of mobile phase A and 84 volumes of mobile phase B.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 μm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R, water R* (0.1:99.9 *V/V*);
- *mobile phase B*: *phosphoric acid R, acetonitrile R* (0.1:99.9 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12.5 | 16 → 6 | 84 → 94 |
| 12.5 - 13.5 | 6 → 0 | 94 → 100 |
| 13.5 - 28 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 20 μL.

*Retention time*: 11-keto-β-boswellic acid = about 8 min; acetyl-11-keto-β-boswellic acid = about 12 min.

*System suitability*: reference solution:

- *resolution*: minimum 6.0 between the peaks due to 11-keto-β-boswellic acid and acetyl-11-keto-β-boswellic acid.

Calculate the percentage content of 11-keto-β-boswellic acid using the following expression:

$$\frac{A_1 \times m_1 \times 5 \times p_1}{A_2 \times m}$$

$A_1$ = area of the peak due to 11-keto-β-boswellic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to 11-keto-β-boswellic acid in the chromatogram obtained with the reference solution;

$m$ = mass of the substance to be examined, in grams;

$m_1$ = mass of *11-keto-β-boswellic acid R* in the reference solution, in grams;

$p_1$ = percentage content of 11-keto-β-boswellic acid in *11-keto-β-boswellic acid R*.

Calculate the percentage content of acetyl-11-keto-β-boswellic acid using the following expression:

$$\frac{A_3 \times m_2 \times 5 \times p_2}{A_4 \times m}$$

$A_3$ = area of the peak due to acetyl-11-keto-β-boswellic acid in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to acetyl-11-keto-β-boswellic acid in the chromatogram obtained with the reference solution;

$m$ = mass of the substance to be examined, in grams;

$m_2$ = mass of *acetyl-11-keto-β-boswellic acid R* in the reference solution, in grams;

$p_2$ = percentage content of acetyl-11-keto-β-boswellic acid in *acetyl-11-keto-β-boswellic acid R*.

**01/2020:2727**

# INDIGO PLANT LEAF

## Persicariae tinctoriae folium

DEFINITION

Whole or fragmented, dried leaf of *Persicaria tinctoria* (Aiton) Spach (syn. *Polygonum tinctorium* Aiton) collected in summer and autumn.

*Content*: minimum 0.55 per cent of indigo ($C_{16}H_{10}N_2O_2$; $M_r$ 262.3) (dried drug).

IDENTIFICATION

A. The leaf is usually fragmented into small crumpled pieces. The whole leaf is elliptical, about 3-8 cm long and 2-5 cm wide, bluish-green or dark bluish-green, with an obtuse or slightly acute apex and attenuate base, and with entire margins, which are slightly ciliated. The adaxial surface is glabrous and the abaxial surface is slightly pubescent along the veins. The veins, slightly prominent on the abaxial surface, are yellow or pale brown. The petiole is short, about 5-10 mm long, flattened, occasionally with membranous ochrea with long ciliate extensions.

B. Microscopic examination (*2.8.23*). The powder is dark bluish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2727.-1): fragments of epidermis covered by a fine striated cuticle (surface view [C, E, F]) consisting of polygonal cells with straight [Ea, Fa] or slightly sinuous [Ca] anticlinal walls, glandular trichomes with a bi- or multicellular stalk and a 2- to 8-celled head [Fc], or their scars [Cb], paracytic stomata (*2.8.3*), rare on the adaxial surface [Fd] and frequent on the abaxial surface [Cc]; fragments of mesophyll [A] consisting of palisade parenchyma (surface view [Fb]) and spongy parenchyma (surface view [Ab]) with granular bluish or bluish-black pigmented contents including some idioblastic cells containing calcium oxalate cluster crystals [Aa] or calcium oxalate spherocrystals [Ac] of various sizes (10-80 µm); cluster crystals [B] or spherocrystals, whole [H] or fragmented [D], isolated; multicellular, multiseriate covering trichomes, 500 µm long or more, sometimes whole [Eb] or, most often, occurring as isolated fragments [G], with cells with lignified walls; fragments of the vascular bundles of the petioles or veins [J] with spiral or annular vessels [Ja] accompanied by fibres [Jb].

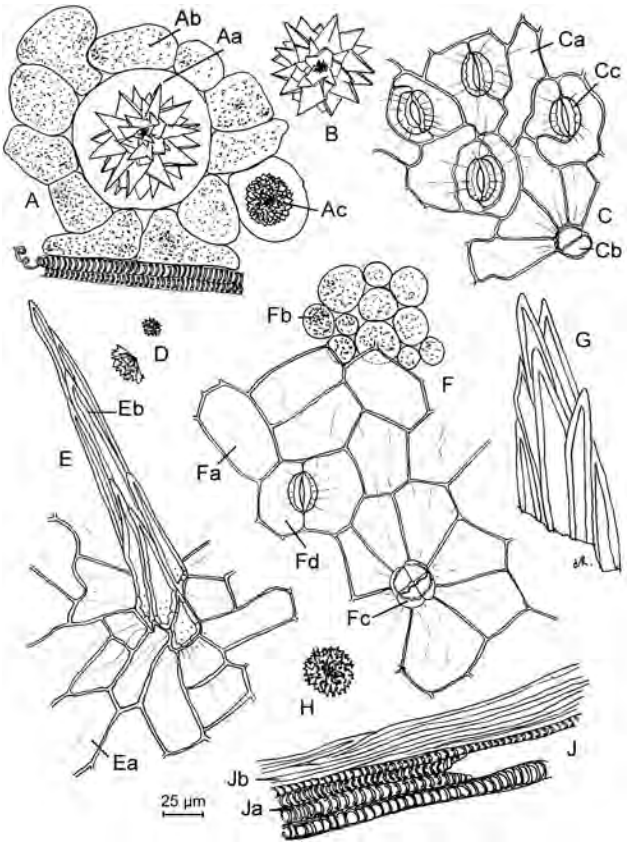


Figure 2727.-1. – *Illustration for identification test B of powdered herbal drug of indigo plant leaf*

C. Thin-layer chromatography (*2.2.27*).

*Solution A*: 20 g/L solution of *chloral hydrate R* in *methylene chloride R*.

*Test solution*. To 0.1 g of the powdered herbal drug (355) (*2.9.12*) add 45 mL of solution A and sonicate at 30 °C for 30 min. Allow to cool, centrifuge, dilute the supernatant to 50.0 mL with solution A and mix thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution*. To 1 mg of *indigo R* and 1 mg of *indirubin R* add 9.5 mL of solution A and sonicate at 30 °C for 30 min. Allow to cool, dilute to 10.0 mL with solution A and mix thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *acetone R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 15 µL as bands of 8 mm.

*Development*: immediately, over a path of 6 cm.

*Drying*: immediately, in air.

*Detection*: examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A light blue zone |
| Indigo: a blue zone | A blue zone (indigo) |
| Indirubin: a red zone | A red zone (indirubin) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 7.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A*: 20 g/L solution of *chloral hydrate R* in *methylene chloride R*.

*Test solution*. To 50.0 mg of the powdered herbal drug (355) (*2.9.12*) add 45 mL of solution A and sonicate at 30 °C for 30 min. Allow to cool, centrifuge, dilute the supernatant to 50.0 mL with solution A and mix thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution*. To 5.0 mg of *indigo CRS* and 5.0 mg of *indirubin R* add 950 mL of solution A and sonicate at 30 °C for 30 min. Allow to cool, dilute to 1000.0 mL with solution A and mix thoroughly. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 70 per cent *V/V* solution of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 5 µL.

*Run time*: 2.5 times the retention time of indigo.

*Relative retention* with reference to indigo (retention time = about 10 min): indirubin = about 1.7.

*System suitability*: reference solution:

– *resolution*: minimum 4.0 between the peaks due to indigo and indirubin.

Calculate the percentage content of indigo using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.05}{A_2 \times m_1}$$

$A_1$ = area of the peak due to indigo in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to indigo in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *indigo CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of indigo in *indigo CRS*.

**04/2016:1875**

# IPECACUANHA LIQUID EXTRACT, STANDARDISED

## Ipecacuanhae extractum fluidum normatum

DEFINITION

Standardised liquid extract produced from *Ipecacuanha root (0094)*.

*Content*: 1.80 per cent to 2.20 per cent of total alkaloids, expressed as emetine ($C_{29}H_{40}N_2O_4$; $M_r$ 480.6).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (60-80 per cent *V/V*).

CHARACTERS

*Appearance*: dark brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 5.0 mL of the extract to be examined to 50 mL with *ethanol (70 per cent V/V) R*. To 2.0 mL of this solution add 2 mL of *water R* and 0.1 mL of *concentrated ammonia R*. Add 10 mL of *ether R* and shake. Separate the upper layer, dry it over about 2 g of *anhydrous sodium sulfate R* and filter.

*Reference solution*. Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Emetine: a blue fluorescent zone | A blue fluorescent zone (emetine) |
| Cephaeline: a blue fluorescent zone | A blue or faint blue fluorescent zone (cephaeline) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 5 g/L solution of *iodine R* in *ethanol (96 per cent) R*, heat at 60 °C for 10 min and examine in ultraviolet light at 365 nm.

*System suitability*: reference solution:

– the blue fluorescent zone due to cephaeline and the yellow fluorescent zone due to emetine are clearly separated.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Emetine: a yellow fluorescent zone | A yellow fluorescent zone (emetine) |
| Cephaeline: a blue fluorescent zone | A blue fluorescent zone (cephaeline) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the quantity stated on the label.

ASSAY

Dilute 1.00 g of the extract to be examined to 10 mL with *ethanol (70 per cent V/V) R* and transfer to a chromatography column about 0.2 m long and about 15 mm in internal diameter, containing 8 g of *basic aluminium oxide R*. After infiltration into the aluminium oxide layer, rinse the internal wall of the column with 3 quantities, each of 2 mL, of *ethanol (70 per cent V/V) R*. Elute in portions, with 40 mL of *ethanol (70 per cent V/V) R*. Avoid disturbance or drying of the surface of the aluminium oxide layer. Collect the whole of the eluate. Evaporate the eluate on a water-bath to about 10 mL. Allow to cool. Add 10.0 mL of *0.02 M hydrochloric acid* and 20 mL of *carbon dioxide-free water R*. Titrate the excess acid with *0.02 M sodium hydroxide* using 0.15 mL of *methyl red mixed solution R* as indicator.

Perform a blank assay replacing the extract to be examined with 10.0 mL of ethanol of the strength stated on the label.

1 mL of *0.02 M hydrochloric acid* is equivalent to 4.807 mg of total alkaloids, expressed as emetine.

**04/2016:0093**

# IPECACUANHA, PREPARED

## Ipecacuanhae pulvis normatus

DEFINITION

*Ipecacuanha root (0094)* powdered (180) (*2.9.12*) and adjusted, if necessary, by the addition of powdered lactose or ipecacuanha root powder with a lower alkaloidal content.

*Content*: 1.9 per cent to 2.1 per cent of total alkaloids, expressed as emetine ($C_{29}H_{40}N_2O_4$; $M_r$ 480.6) (dried drug).

CHARACTERS

*Appearance*: light grey or yellowish-brown powder.

IDENTIFICATION

A. Microscopic examination (*2.8.23*). The powder is light grey or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0093.-1): fragments of parenchyma [G] with ovoid cells [Ga]; raphides of calcium oxalate up to 80 µm in length either in bundles [Bc] or scattered throughout the powder [Gb]; fragments [E] of tracheids and vessels usually 10-20 µm in diameter, with bordered pits [Eb] accompanied by ligneous parenchyma with longitudinally elongated rectangular cells [Ea]; larger vessels and sclereids from the rhizome [D]; fragments of dermal tissue (transverse section [B]) with reddish-brown cork [Ba] and phelloderm [Bb], with some cells containing raphides of calcium oxalate [Bc]; large parenchymatous cells with slightly thickened and pitted walls, from the pith of the rhizome [C]; a few fragments of fibres with moderately thickened and slightly pitted walls from the xylem, isolated [H] or associated with vessels [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple [Aa] or 2- to 10-compound [Ab] starch granules contained in parenchymatous cells [A], the simple granules being up to 15 µm in diameter in Costa Rica ipecacuanha and up to 22 µm in diameter in Mato Grosso ipecacuanha.

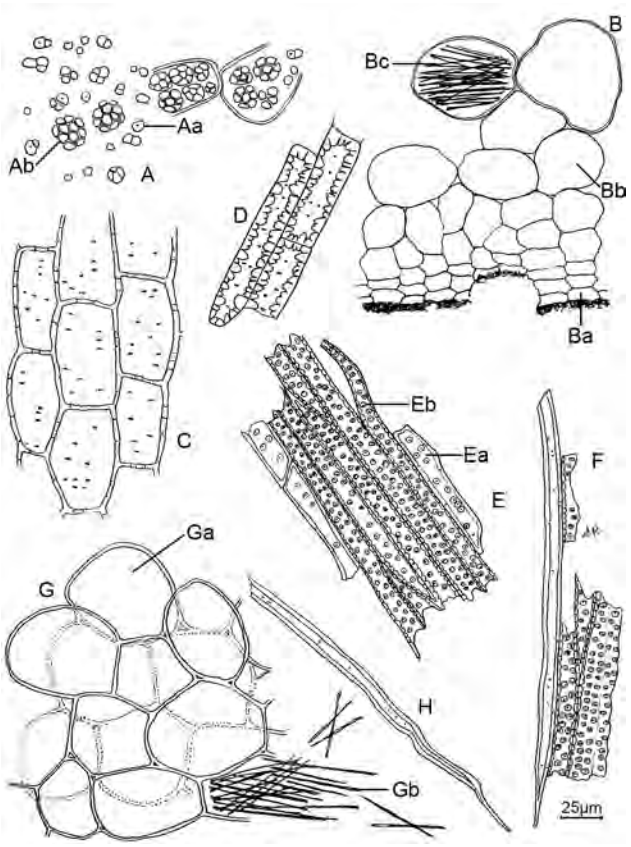


Figure 0093.-1. – *Illustration for identification test A of prepared ipecacuanha*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.1 g of the herbal drug to be examined in a test-tube add 0.05 mL of *concentrated ammonia R* and 5 mL of *ether R* and stir the mixture vigorously with a glass rod. Allow to stand for 30 min and filter.

*Reference solution.* Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emetine: a blue fluorescent zone<br>Cephaeline: a blue fluorescent zone | A blue fluorescent zone (emetine)<br>A blue or faint blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 5 g/L solution of *iodine R* in *ethanol (96 per cent) R*, heat at 60 °C for 10 min and examine in ultraviolet light at 365 nm.

*System suitability*: reference solution:

– the blue fluorescent zone due to cephaeline and the yellow fluorescent zone due to emetine are clearly separated.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emetine: a yellow fluorescent zone<br>Cephaeline: a blue fluorescent zone | A yellow fluorescent zone (emetine)<br>A blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g of the herbal drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Place 7.5 g of the herbal drug in a dry flask, add 100 mL of *ether R* and shake for 5 min. Add 5 mL of *dilute ammonia R1*, shake for 1 h, add 5 mL of *water R* and shake vigorously. Decant the ether layer into a flask through a plug of cotton. Wash the residue in the flask with 2 quantities, each of 25 mL, of *ether R*, decanting each portion through the same plug of cotton. Combine the ether solutions and eliminate the ether by distillation. Dissolve the residue in 2 mL of *ethanol (90 per cent V/V) R*, evaporate to dryness and heat at 100 °C for 5 min. Dissolve the residue in 5 mL of previously neutralised *ethanol (90 per cent V/V) R*, warming on a water-bath. Add 15.0 mL of *0.1 M hydrochloric acid* and titrate the excess acid with *0.1 M sodium hydroxide* using 0.5 mL of *methyl red mixed solution R* as indicator.

1 mL of *0.1 M hydrochloric acid* is equivalent to 24.03 mg of total alkaloids, expressed as emetine.

STORAGE

In an airtight container.

**04/2016:0094**

# IPECACUANHA ROOT

## Ipecacuanhae radix

DEFINITION

Fragmented and dried underground organs of *Carapichea ipecacuanha* (Brot.) L. Andersson (syn. *Cephaëlis ipecacuanha* (Brot.) A. Rich.; *Cephaëlis acuminata* H. Karst.) from Mato Grosso or Costa Rica. The principal alkaloids are emetine and cephaeline.

*Content*: minimum 2.0 per cent of total alkaloids, expressed as emetine ($C_{29}H_{40}N_2O_4$; $M_r$ 480.6) (dried drug).

IDENTIFICATION

A. *Mato Grosso ipecacuanha*: the root occurs as somewhat tortuous pieces, dark reddish-brown or very dark brown, seldom more than 15 cm long or 6 mm thick, closely annulated externally, having rounded ridges completely encircling the root; the fracture is short in the bark and splintery in the wood. The transversely cut surface shows a wide greyish bark and a small uniformly dense wood. The rhizome occurs as short lengths usually attached to roots, cylindrical, up to 2 mm in diameter, finely wrinkled longitudinally and with pith occupying approximately one-sixth of the whole diameter.

*Costa Rica ipecacuanha:* the root in general resembles the root of *Mato Grosso ipecacuanha*, but differs in the following particulars: it is often up to 9 mm thick; the external surface is greyish-brown or reddish-brown with transverse ridges at intervals of usually 1-3 mm, the ridges being about 0.5-1 mm wide, extending about half-way round the circumference and fading at the extremities into the general surface level.

B. Microscopic examination (*2.8.23*). The powder is light grey or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0094.-1): fragments of parenchyma [G] with ovoid cells [Ga]; raphides of calcium oxalate up to 80 µm in length either in bundles [Bc] or scattered throughout the powder [Gb]; fragments [E] of tracheids and vessels usually 10-20 µm in diameter, with bordered pits [Eb] accompanied by ligneous parenchyma with longitudinally elongated rectangular cells [Ea]; larger vessels and sclereids from the rhizome [D]; fragments of dermal tissue (transverse section [B]) with reddish-brown cork [Ba] and phelloderm [Bb], with some cells containing raphides of calcium oxalate [Bc]; large parenchymatous cells with slightly thickened and pitted walls, from the pith of the rhizome [C]; a few fragments of fibres with moderately thickened and slightly pitted walls from the xylem, isolated [H] or associated with vessels [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple [Aa] or 2- to 10-compound [Ab] starch granules contained in parenchymatous cells [A], the simple granules being up to 15 µm in diameter in Costa Rica ipecacuanha and up to 22 µm in diameter in Mato Grosso ipecacuanha.

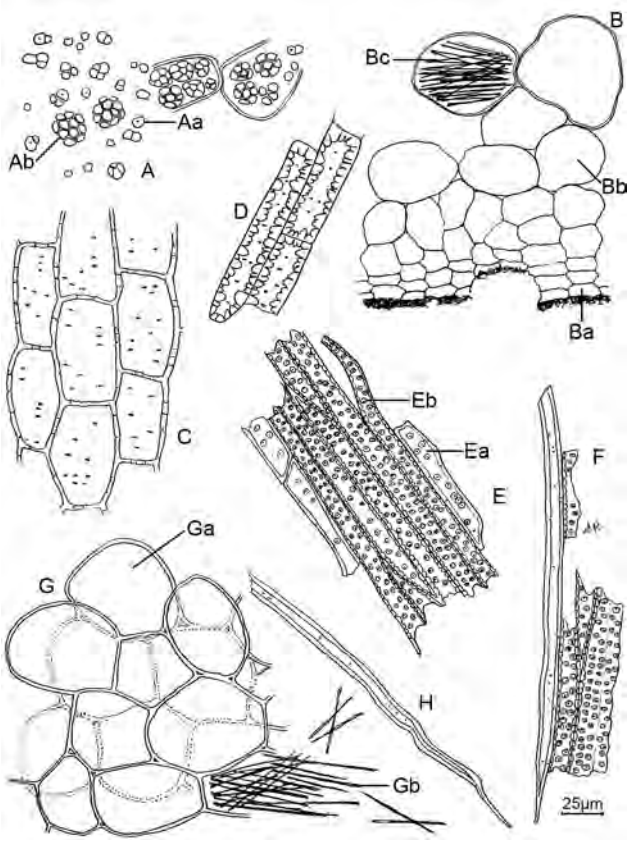


Figure 0094.-1. – *Illustration for identification test B of powdered herbal drug of ipecacuanha root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.1 g of the powdered herbal drug (180) (*2.9.12*) in a test-tube add 0.05 mL of *concentrated ammonia R* and 5 mL of *ether R* and stir the mixture vigorously with a glass rod. Allow to stand for 30 min and filter.

*Reference solution.* Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| Emetine: a blue fluorescent zone<br>Cephaeline: a blue fluorescent zone | A blue fluorescent zone (emetine)<br>A blue fluorescent zone (cephaeline) | A blue fluorescent zone (emetine)<br>A faint blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution (Costa Rica ipecacuanha)** | **Test solution (Mato Grosso ipecacuanha)** |

Herbal drugs

*Detection B*: treat with a 5 g/L solution of *iodine R* in *ethanol (96 per cent) R*, heat at 60 °C for 10 min and examine in ultraviolet light at 365 nm.

*System suitability*: reference solution:

– the blue fluorescent zone due to cephaeline and the yellow fluorescent zone due to emetine are clearly separated.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| Emetine: a yellow fluorescent zone<br>Cephaeline: a blue fluorescent zone | A yellow fluorescent zone (emetine)<br>A blue fluorescent zone (cephaeline) | A yellow fluorescent zone (emetine)<br>A blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution (Costa Rica ipecacuanha)** | **Test solution (Mato Grosso ipecacuanha)** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (180) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Place 7.5 g of the powdered herbal drug (180) (*2.9.12*) in a dry flask, add 100 mL of *ether R* and shake for 5 min. Add 5 mL of *dilute ammonia R1*, shake for 1 h, add 5 mL of *water R* and shake vigorously. Decant the ether layer into a flask through a plug of cotton. Wash the residue in the flask with 2 quantities, each of 25 mL, of *ether R*, decanting each portion through the same plug of cotton. Combine the ether solutions and eliminate the ether by distillation. Dissolve the residue in 2 mL of *ethanol (90 per cent V/V) R*, evaporate to dryness and heat at 100 °C for 5 min. Dissolve the residue in 5 mL of previously neutralised *ethanol (90 per cent V/V) R*, warming on a water-bath. Add 15.0 mL of *0.1 M hydrochloric acid* and titrate the excess acid with *0.1 M sodium hydroxide* using 0.5 mL of *methyl red mixed solution R* as indicator.

1 mL of *0.1 M hydrochloric acid* is equivalent to 24.03 mg of total alkaloids, expressed as emetine.

STORAGE

In an airtight container.

**04/2016:1530**

# IPECACUANHA TINCTURE, STANDARDISED

## Ipecacuanhae tinctura normata

DEFINITION

Tincture produced from *Ipecacuanha root (0094)*.

*Content*: 0.18 per cent *m/m* to 0.22 per cent *m/m* of total alkaloids, expressed as emetine ($C_{29}H_{40}N_2O_4$; $M_r$ 480.6).

PRODUCTION

The tincture is produced from the herbal drug by a suitable procedure using ethanol (70 per cent *V/V*).

CHARACTERS

*Appearance*: yellowish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2.0 mL of the tincture to be examined add 2 mL of *water R* and 0.1 mL of *concentrated ammonia R*. Add 10 mL of *ether R* and shake. Separate the ether layer, dry it over about 2 g of *anhydrous sodium sulfate R* and filter.

*Reference solution*. Dissolve 2.5 mg of *emetine hydrochloride CRS* and 3 mg of *cephaeline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:15:18:65 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emetine: a blue fluorescent zone<br>Cephaeline: a blue fluorescent zone | A blue fluorescent zone (emetine)<br>A blue or faint blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 5 g/L solution of *iodine R* in *ethanol (96 per cent) R*, heat at 60 °C for 10 min and examine in ultraviolet light at 365 nm.

*System suitability*: reference solution:

– the blue fluorescent zone due to cephaeline and the yellow fluorescent zone due to emetine are clearly separated.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emetine: a yellow fluorescent zone<br>Cephaeline: a blue fluorescent zone | A yellow fluorescent zone (emetine)<br>A blue fluorescent zone (cephaeline) |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the quantity stated on the label.

ASSAY

Transfer 10.00 g of the tincture to be examined to a chromatography column about 0.2 m long and about 15 mm in internal diameter, containing 8 g of *basic aluminium oxide R*. After infiltration into the aluminium oxide layer, rinse the

internal wall of the column with 3 quantities, each of 2 mL, of *ethanol (70 per cent V/V) R*. Elute in portions, with 40 mL of *ethanol (70 per cent V/V) R*. Avoid disturbance or drying of the surface of the aluminium oxide layer. Collect the whole of the eluate. Evaporate the eluate on a water-bath to about 10 mL. Allow to cool. Add 10.0 mL of *0.02 M hydrochloric acid* and 20 mL of *carbon dioxide-free water R*. Titrate the excess acid with *0.02 M sodium hydroxide* using 0.15 mL of *methyl red mixed solution R* as indicator.

Perform a blank assay replacing the tincture to be examined with 10.0 mL of ethanol of the strength stated on the label.

1 mL of *0.02 M hydrochloric acid* is equivalent to 4.807 mg of total alkaloids, expressed as emetine.

**01/2019:2566**

# ISATIS ROOT

## Isatidis radix

DEFINITION

Whole or fragmented, dried root of *Isatis tinctoria* L. (*I. indigotica* Fortune ex Lindl.) collected in autumn.

*Content*: minimum 1.0 per cent of arginine ($C_6H_{14}N_4O_2$; $M_r$ 174.2) (dried drug).

IDENTIFICATION

A. *Whole drug*. It is cylindrical, slightly tortuous, 8-22 cm long, 1.5 cm in diameter, externally greyish-yellow or brownish-yellow, wrinkled longitudinally and lenticellate transversally, with rootlets or rootlet scars. The root crown is slightly expanded, exhibiting dark green or dark brown petiole bases arranged in whorls, and dense tubercles. The texture is compact and easily broken. The transversely cut surface shows a yellowish-white, brown or dark brown bark, darkest near the cambium, sometimes appearing as a thin dark line, and a yellow or brown wood.

*Fragmented drug*. It occurs as transverse or oblique slices, rounded or elliptical, or as short, thin, cylindrical pieces, 0.3-1.3 cm in diameter. It is externally greyish-yellow or brownish-yellow, wrinkled longitudinally, and transverse lenticels, rootlets and rootlet scars are sometimes visible. The transversely cut surface shows a yellowish-white, brown or dark brown bark, darkest near the cambium, sometimes appearing as a thin dark line, and a yellow or brown wood.

B. Microscopic examination (*2.8.23*). The powder is whitish-yellow, yellow or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2566.-1): fragments of cork consisting of thin-walled cells (surface view [A], transverse section [B]); fragments of xylem [D] consisting of reticulate, pitted or, more rarely, spiral vessels [Da] included in thin-walled parenchyma cells [Db]; groups of lignified xylem fibres, occasionally accompanying vessels, with sparse irregularly-positioned pits may be present; thin-walled parenchyma cells (longitudinal section [E], transverse section [F]); groups of greenish-yellow sclereids with thick, pitted walls embedded in parenchyma tissue may be present. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows abundant, single or compound (mostly 2, or less frequently, 3 or 4) starch granules, 1.5-18 µm in diameter, free [C] or included in parenchyma [G], with a punctiform, slit-shaped or V-shaped hilum.

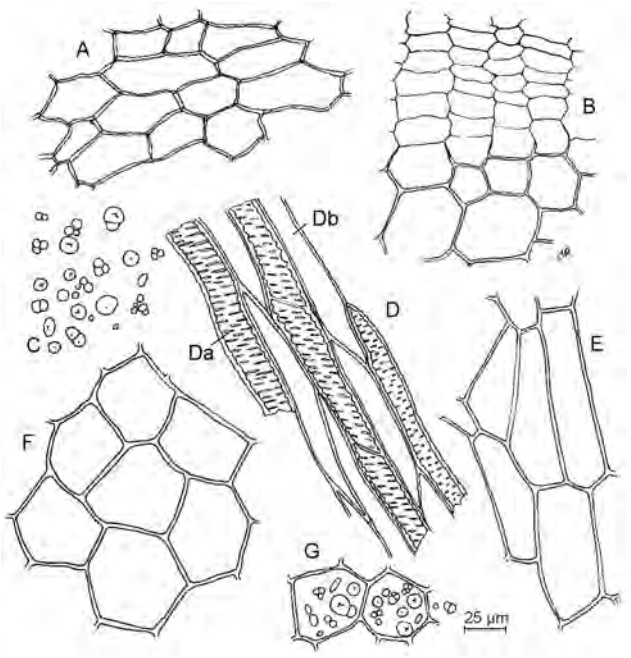


Figure 2566.-1. – *Illustration for identification test B of powdered herbal drug of isatis root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *ethanol (70 per cent V/V) R* and sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution*. Dissolve 4 mg of *arginine R* and 4 mg of *cysteine hydrochloride R* in 1 mL of *ethanol (70 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *acetonitrile R* (2:8:30 *V/V/V*).

*Application*: 4 µL as bands of 10 mm [or 8 mm].

*Development*: over a path of 8.5 cm [or 6 cm].

*Drying*: in air.

*Detection*: expose to *concentrated ammonia R* vapour for 5 min, treat with *ninhydrin solution R4*, then heat at 120 °C for 3 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint coloured zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| _____ | _____ |
| | A prominent brown zone |
| Cysteine: a brown zone | |
| | A brown zone |
| Arginine: a brown zone | A brown zone (arginine) |
| | A faint brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 9.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.100 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *ethanol (70 per cent V/V) R*, sonicate for 20 min, filter, and evaporate the filtrate to dryness. Dissolve the residue in *ethanol (70 per cent V/V) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 25.0 mg of *arginine CRS* in *ethanol (70 per cent V/V) R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 3.0 mg of *cysteine hydrochloride R* in 6.0 mL of reference solution (a) and dilute to 10.0 mL with *ethanol (70 per cent V/V) R*.

*Reference solutions (c), (d), (e), (f), (g), (h).* Dilute reference solution (a) to obtain 6 reference solutions of arginine, the concentrations of which span the expected value in the test solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 30 °C.

*Mobile phase*: *trifluoroacetic acid R*, *water for chromatography R* (0.2:99.8 *V/V*).

*Flow rate*: 0.2 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion for the signal-to-noise ratio:

– *carrier gas*: *nitrogen R*;

– *pressure*: 330 kPa;

– *evaporator temperature*: 80 °C.

*Injection*: 10 µL.

*Run time*: 25 min.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to cysteine and arginine in the chromatogram obtained with reference solution (b);

– *signal-to-noise ratio*: minimum 50 for the peak due to arginine in the chromatogram obtained with reference solution (a).

Establish a calibration curve with the logarithm of the concentration (in milligrams per 10 mL) of reference solutions (c), (d), (e), (f), (g) and (h) (corrected by the assigned percentage content of *arginine CRS*) as the abscissa and the logarithm of the corresponding peak areas as the ordinate.

Calculate the percentage content of arginine using the following expression:

$$\frac{10^{A}}{m \times 10}$$

*A* = logarithm of the concentration of arginine in the test solution, determined from the calibration curve;

*m* = mass of the herbal drug to be examined used to prepare the test solution, in grams.

**01/2008:1334**
**corrected 6.0**

# ISPAGHULA HUSK

## Plantaginis ovatae seminis tegumentum

DEFINITION

Episperm and collapsed adjacent layers removed from the seeds of *Plantago ovata* Forssk. (*P. ispaghula* Roxb.).

IDENTIFICATION

A. The husk consists of pinkish-beige fragments or flakes up to about 2 mm long and 1 mm wide, some showing a light brown spot corresponding to the location of the embryo before it was removed from the seed.

B. Microscopic examination (*2.8.23*). The powder is pale yellow. Examine under a microscope using *lactic reagent R*. The powder shows the following diagnostic characters: mainly fragments of the episperm with polygonal cells filled with mucilage; fragments of the inner layers of the testa with brownish thin-walled cells often associated with the outer layers of the endosperm. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows occasional starch granules, single or in groups of 2-4, measuring 3-25 µm in diameter.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 10 mg of the powdered herbal drug (355) (*2.9.12*) in a thick-walled centrifuge tube, add 2 mL of a 230 g/L solution of *trifluoroacetic acid R* and shake vigorously. Stopper the test tube and heat at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant into a 50 mL flask, add 10 mL of *water R* and evaporate to dryness under reduced pressure. Take up the residue in 10 mL of *water R* and evaporate again to dryness under reduced pressure. Take up the residue with 2 mL of *methanol R*.

*Reference solution (a).* Dissolve 10 mg of *arabinose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution (b).* Dissolve 10 mg of *xylose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution (c).* Dissolve 10 mg of *galactose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Detection*: spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows 2 orange-pink zones (arabinose and xylose) and a yellow zone (galactose) similar in position and colour to the zones in the chromatograms obtained with the reference solutions.

TESTS

**Foreign matter** (*2.8.2*). Carry out the determination using 5.0 g.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Swelling index** (*2.8.4*): minimum 40, determined on 0.1 g of the powdered herbal drug (355) (*2.9.12*).

**01/2008:1333**
**corrected 6.0**

## ISPAGHULA SEED

### Plantaginis ovatae semen

DEFINITION

Dried ripe seeds of *Plantago ovata* Forssk. (*P. ispaghula* Roxb.).

IDENTIFICATION

A. Ispaghula seed is pinkish-beige, smooth, boat-shaped and curved. It is 1.5 mm to 3.5 mm long, 1.5 mm to 2 mm wide and 1 mm to 1.5 mm thick. The concave surface shows in the centre a light coloured spot corresponding to the hilum. The convex surface shows a light brown spot corresponding to the location of the embryo and takes up about one quarter of the length of the seed.

B. Microscopic examination (*2.8.23*). The powder is pale brown. Examine under a microscope using *lactic reagent R*. The powder shows mainly fragments of the episperm with polygonal cells filled with mucilage; fragments of the inner layers of the testa with brownish thin-walled cells often associated with the outer layers of the endosperm; fragments of the endosperm with cells with thick cellulose walls containing aleurone grains and oil droplets; a few fragments of embryo with thin-walled cells. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows starch granules, single or in groups of 2 to 4 and measuring 3 µm to 25 µm in diameter.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 50 mg of the powdered herbal drug (355) (*2.9.12*) in a thick-walled centrifuge tube add 2 mL of a 230 g/L solution of *trifluoroacetic acid R*, and shake vigorously. Stopper the test tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant into a 50 mL flask, add 10 mL of *water R* and evaporate the solution to dryness under reduced pressure. Take up the residue in 10 mL of *water R* and evaporate again to dryness under reduced pressure. Take up the residue in 2 mL of *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *arabinose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution (b)*. Dissolve 10 mg of *xylose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution (c)*. Dissolve 10 mg of *galactose R* in a small quantity of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Detection*: Spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference and the test solutions.

| Top of the plate | |
|---|---|
| Xylose: an orange-pink zone | An orange-pink zone (xylose) |
| Arabinose: an orange-pink zone | An orange-pink zone (arabinose) |
| Galactose: a yellow zone | A yellow zone (galactose) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*). Carry out the determination using 10.0 g.

**Swelling index** (*2.8.4*): minimum 9.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**04/2014:2148**

## IVY LEAF

### Hederae folium

DEFINITION

Whole or cut, dried leaves of *Hedera helix* L., collected in spring and summer.

*Content*: minimum 3.0 per cent of hederacoside C ($C_{59}H_{96}O_{26}$; $M_r$ 1221) (dried drug).

IDENTIFICATION

A. Whole leaves are coriaceous, 4-10 cm in length and width, cordate at the base. The lamina is palmately 3-5 lobed, the lobes more or less triangular with entire margins. The upper surface is dark green with a paler, radiate venation, the lower surface more greyish-green and the venation is distinctly raised. The petioles are long, cylindrical, about 2 mm in diameter and grooved longitudinally. Scattered white hairs occur on the petioles and on the surfaces of younger leaves, the older leaves are glabrous. Occasional entire, ovate-rhombic to lanceolate leaves 3-8 cm long from the flowering stems may be present.

B. Microscopic examination (*2.8.23*). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2148.-1): fragments of the upper epidermis (surface view [F]), showing cells with thickened, rather sinuous, finely pitted anticlinal walls [Fa] usually accompanied by underlying palisade parenchyma [Fb] including some cells containing cluster crystals of calcium oxalate [Fc]; fragments of the lower epidermis (surface view [E]), showing cells with sinuous, irregularly thickened and pitted walls [Ea], stomata that are mostly

anomocytic [Eb] but occasionally anisocytic (*2.8.3*), surrounded by cells including some that show faint cuticular striations; the lower epidermis is accompanied by underlying spongy parenchyma [Ec] including some cells containing cluster crystals of calcium oxalate [Ed]; scattered stellate covering trichomes may be present, composed of 4-8 branches joined at the base on a multicellular, biseriate stalk (surface view [B], side view [A]); cluster crystals of calcium oxalate, about 40 µm in diameter, scattered [C] or occurring throughout the parenchyma [Ed, Fc]; groups of lignified fibro-vascular tissue from the veins [D].

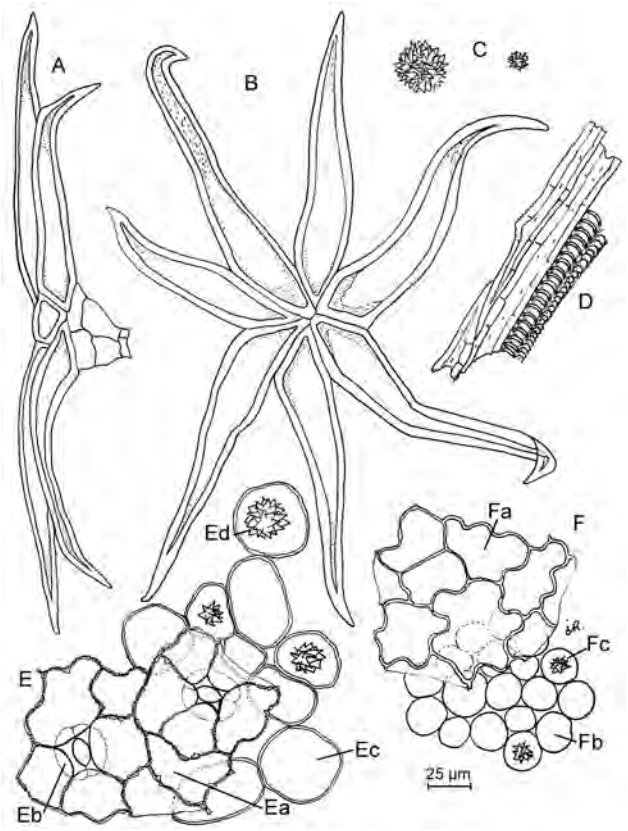


Figure 2148.-1. – *Illustration for identification test B of powdered herbal drug of ivy leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Extract 0.50 g of the powdered herbal drug (355) (*2.9.12*) under a reflux condenser in a water-bath at 60 °C with 5 mL of *methanol R* for 30 min. Cool and filter.

*Reference solution.* Dissolve 1.0 mg of *hederacoside C R* and 1.0 mg of *α-hederin R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *acetone R*, *methanol R*, *ethyl acetate R* (4:20:20:30 *V/V/V/V*).

*Application*: 20 µL as bands of 15 mm.

*Development*: over a path of 12 cm.

*Drying*: at 100-105 °C.

*Detection*: treat with *alcoholic solution of sulfuric acid R*, heat at 110 °C for 10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A green zone |
| α-Hederin: a purple zone | A very faint purple zone (α-hederin) |
| | A broad yellow zone |
| | 2-3 purple or green zones |
| Hederacoside C: a purple zone | A purple zone (hederacoside C) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 10 per cent of discoloured leaves, maximum 10 per cent of stems, and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R* (20:80 *V/V*).

*Test solution.* To 1.00 g of the powdered herbal drug (355) (*2.9.12*) in a 250 mL round-bottomed flask add 50 mL of the solvent mixture and heat under a reflux condenser in a water-bath at 80 °C for 1 h. Cool and filter through a plug of absorbent cotton into a 100 mL volumetric flask. The plug of absorbent cotton together with the residue is again extracted with 30 mL of the solvent mixture under reflux for 30 min. Filter and combine the filtrates. Rinse the round-bottomed flask and the plug of absorbent cotton with the solvent mixture and use the solvent mixture to dilute the contents of the volumetric flask to exactly 100.0 mL. Filter through a suitable membrane before use.

*Reference solution.* To 20.0 mg of *ivy leaf dry extract HRS* add 5.0 mL of *methanol R* and sonicate for 10 min. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 14 volumes of *acetonitrile R* with 88 volumes of *water R* and adjust to pH 2.0 with *phosphoric acid R*;
– *mobile phase B*: *phosphoric acid R*, *acetonitrile R* (0.2:99.8 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 6 | 100 → 94 | 0 → 6 |
| 6 - 40 | 94 → 60 | 6 → 40 |
| 40 - 41 | 60 → 0 | 40 → 100 |
| 41 - 55 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*System suitability*: reference solution:

– *retention time*: hederacoside C = about 20 min; if necessary, adjust the time intervals of the gradient.

Calculate the percentage content of hederacoside C with reference to the dried drug using the following expression:

$$\frac{A_1 \times m_2 \times 20 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to hederacoside C in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hederacoside C in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ivy leaf dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of hederacoside C in *ivy leaf dry extract HRS*.

**01/2016:1229**

# JAVA TEA

## Orthosiphonis folium

DEFINITION

Whole or fragmented, dried leaf and top of stem of *Orthosiphon aristatus* (Blume) Miq. var. *aristatus* (syn. *Orthosiphon stamineus* Benth.).

*Content*: minimum 0.3 per cent of rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (dried drug).

IDENTIFICATION

A. The leaf is friable, whole or fragmented, the entire leaf measuring on average 7.5 cm in length and 2.5 cm in width. The petiole is short. The lamina is oval or lanceolate, the apex acuminate and the base cuneate. The abaxial surface of the leaves is light greyish-green and the adaxial surface is green or dark green. The venation is pinnate with few secondary veins. Examined under a lens (× 10), the secondary veins, after running parallel to the midrib, diverge at an acute angle. The margin is irregularly and roughly dentate, sometimes crenate, and the abaxial surface is slightly curved. The petioles are thin, quadrangular, 4-8 mm long and, like the primary venation, usually violet-coloured. Occasionally, inflorescences in clusters of bluish-white or violet flowers, not yet opened, are found.

B. Microscopic examination (*2.8.23*). The powder is green or dark green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1229.-1): articulated uniseriate covering trichomes up to 450 µm long, consisting of 3-8 cells with thick pitted walls, usually broken [C, E] but sometimes attached to epidermis (surface view [Hc], side view [J]); unicellular or bicellular conical covering trichomes [Fa], mainly present on the margins of the lamina [F]; secretory trichomes with unicellular stalks and tetracellular heads, isolated (surface view [G]) or attached to epidermis (surface view [Db], side view [Aa]); secretory trichomes with unicellular stalks and unicellular (surface view [Hb], side view [Ab]) or bicellular heads (surface view [Ba]); fragments of upper epidermis (surface view [B]) with cells with sinuous outlines, and with underlying palisade parenchyma [Bb]; fragments of lower epidermis [D, H] with diacytic stomata [Da, Ha] (*2.8.3*); fragments of lamina (transverse section [A]) showing usually 2 layers of palisade parenchyma [Ac].

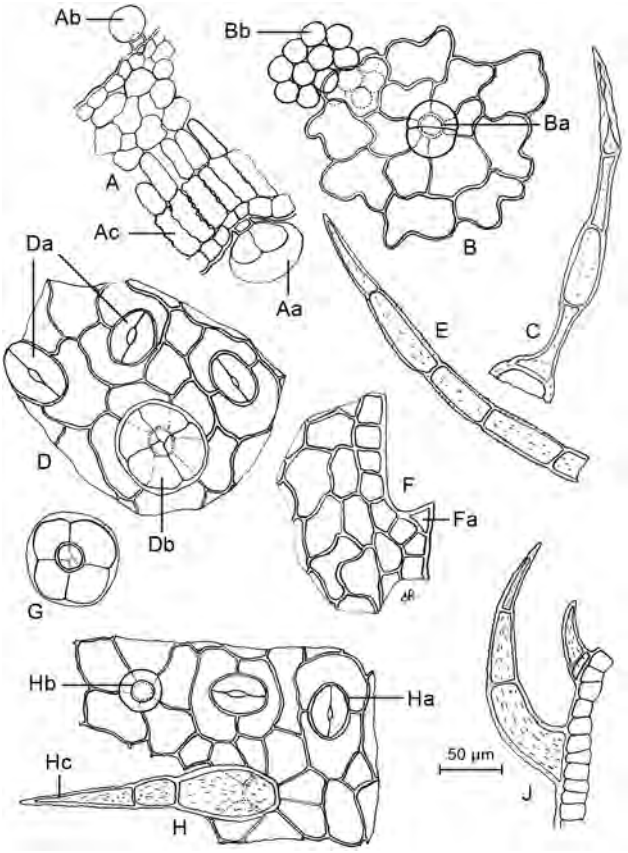


Figure 1229.-1. – *Illustration for identification test B of powdered herbal drug of Java tea*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 1 g of the powdered herbal drug (355) (*2.9.12*) with 10 mL of *methanol R* in a water-bath at 60 °C for 5 min and filter the cooled solution.

*Reference solution.* Dissolve 1 mg of *sinensetin R* and 1 mg of *scopoletin R* in *methanol R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methanol R, ethyl acetate R, toluene R* (5:40:55 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*System suitability*: reference solution:

– the zones due to sinensetin and scopoletin are clearly separated.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, red fluorescent zones are present in the lower third and near the solvent front of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | A blue fluorescent zone |
| Scopoletin: a blue fluorescent zone<br>Sinensetin: a blue fluorescent zone | A prominent blue fluorescent zone (sinensetin) |
| ——— | ——— |
| | 1 or 2 bluish fluorescent zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems with a diameter greater than 1 mm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Carry out the assay protected from light.*

*Test solution.* Disperse 0.500 g of the powdered herbal drug (355) (*2.9.12*) in 90 mL of *ethanol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min, cool, and filter into a 100 mL volumetric flask. Rinse the flask and the filter with 10 mL of *ethanol (50 per cent V/V) R* and dilute to 100.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *rosmarinic acid CRS* in *ethanol (50 per cent V/V) R* and dilute to 50.0 mL with the same solvent (solution A). Dilute 4.0 mL of solution A to 20.0 mL with *ethanol (50 per cent V/V) R*.

*Reference solution (b).* Dissolve 5.0 mg of *ferulic acid R* in *ethanol (50 per cent V/V) R* and dilute to 50.0 mL with the same solvent. To 5.0 mL of the solution add 5.0 mL of solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *acetonitrile R*, *water R* (1:19:80 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *methanol R*, *acetonitrile R* (1:40:59 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 20 | 100 → 55 | 0 → 45 |
| 20 - 25 | 55 → 0 | 45 → 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rosmarinic acid (retention time = about 11 min): ferulic acid = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to ferulic acid and rosmarinic acid.

Calculate the percentage content of rosmarinic acid using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.4}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rosmarinic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rosmarinic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rosmarinic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rosmarinic acid in *rosmarinic acid CRS*.

**07/2019:1532**

# JUNIPER

## Juniperi galbulus

DEFINITION

Dried ripe cone berry of *Juniperus communis* L.

*Content*: minimum 10 mL/kg of essential oil (anhydrous drug).

CHARACTERS

Strongly aromatic odour reminiscent of terpinen-4-ol, especially if crushed.

IDENTIFICATION

A. The berry-shaped cone is globular, up to 10 mm in diameter, and violet-brown or blackish-brown, frequently with a bluish bloom. It consists of 3 fleshy scales. The apex has a 3-rayed closed cleft and 3 not-very-clearly defined projections. A remnant of peduncle is frequently attached at the base. The fleshy part is crumbly and brownish. It contains 3 or, more rarely, 2 small, elongated, extremely hard seeds that have 3 sharp edges and are slightly rounded at the back, acuminate at the apex. The seeds are fused with the fleshy part of the cone berry in the lower part on the outside of their bases. Very large, oval oil glands containing sticky resin lie at the outer surface of the seeds.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1532.-1): fragments of epidermis of the cone berry wall (surface view [C], transverse section [D]) consisting of cells with thick, pitted, colourless walls and brown granular contents [Ca, Da], occasionally with anomocytic stomata (*2.8.3*) [Cb]; fragments of the 3-rayed apical cleft of the cone berry with epidermal cells interlocked by papillous outgrowths (transverse section [G]); fragments of the hypodermis with thickened collenchymatous cells [J]; fragments of the mesocarp consisting of large, thin-walled parenchymatous cells, usually rounded, with large intercellular spaces and irregular, large, usually scarcely pitted, yellow idioblasts [F]; fragments of secretory canals (transverse section [B]) with schizogenous oil cells; fragments of the testa with thick-walled, pitted, colourless sclereids [E] containing 1 or more prism crystals of calcium oxalate [Ea]; fragments of the seed (surface view [A], transverse section [H]) with a finely pitted testa [Aa, Ha] and thin-walled endosperm cells containing droplets of fatty oil and aleurone grains [Ab, Hb].

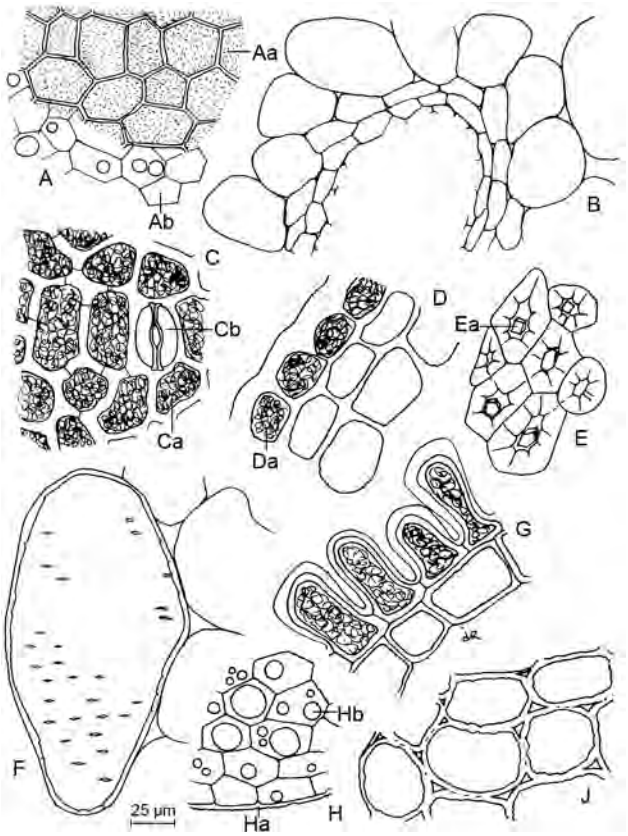


Figure 1532.-1. – *Illustration for identification test B of powdered herbal drug of juniper*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute the oil-xylene mixture obtained in the assay to 5.0 mL with *hexane R*.

*Reference solution.* Dissolve 4.0 mg of *guaiazulene R* and 50 µL of *cineole R* in 10 mL of *hexane R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows a red zone (guaiazulene) in the upper half and a brownish-violet or greyish-violet zone (cineole) in the lower half; the chromatogram obtained with the test solution shows a strong violet zone (mono- and sesquiterpenes) similar in position to the zone due to guaiazulene in the chromatogram obtained with the reference solution, a reddish-violet zone a little above the zone due to cineole in the chromatogram obtained with the reference solution, a greyish-violet zone (terpinen-4-ol) a little below the zone due to cineole in the chromatogram obtained with the reference solution, and just below that a blue zone; a faint violet zone may be present in a similar position to the zone due to cineole; further zones are present.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of unripe or discoloured cone berries and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 120 mL/kg, determined on 20.0 g of the crushed drug.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the herbal drug reduced to a coarse powder using a suitable mill immediately before the assay, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 90 min.

**07/2013:1832**
**corrected 10.0**

# JUNIPER OIL

## Juniperi aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the ripe, non-fermented berry cones of *Juniperus communis* L. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: mobile, colourless or yellowish liquid.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.2 mL of the substance to be examined in 5 mL of *heptane R*.

*Reference solution.* Dissolve 20 mg of *α-terpineol R* and 20 µL of *terpinen-4-ol R* in 25 mL of *heptane R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C until the zones appear; examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| **Top of the plate** | |
|---|---|
| | An intense brownish-violet zone |
| | A brown zone |
| | A violet-pink zone |
| Terpinen-4-ol: a brownish-violet zone | A brownish-violet zone (terpinen-4-ol) |
| | A violet zone |
| α-Terpineol: a violet or brownish-violet zone | A violet or brownish-violet zone (α-terpineol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.857 to 0.876.

**Refractive index** (*2.2.6*): 1.471 to 1.483.

**Optical rotation** (*2.2.7*): – 15° to – 0.5°.

Herbal drugs

**Peroxide value** (*2.5.5*): maximum 20.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test for fatty oils and resinified essential oils.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 60 mg of the substance to be examined in *trimethylpentane R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Mix 25 µL each of *α-pinene R*, *sabinene R*, *β-pinene R*, *β-myrcene R*, *α-phellandrene R*, *limonene R*, *terpinen-4-ol R*, *bornyl acetate R* and *β-caryophyllene R* and dilute to 25.0 mL with *trimethylpentane R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.0 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 60 |
| | 1 - 58 | 60→230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to sabinene and β-pinene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of the components. Disregard the peak due to trimethylpentane and peaks comprising less than 0.01 per cent of the total surface area. The percentages are within the following ranges:

– *α-pinene*: 20 per cent to 50 per cent;

– *sabinene*: maximum 20 per cent;

– *β-pinene*: 1.0 per cent to 12 per cent;

– *β-myrcene*: 1.0 per cent to 35 per cent;

– *α-phellandrene*: maximum 1.0 per cent;

– *limonene*: 2.0 per cent to 12 per cent;

– *terpinen-4-ol*: 0.5 per cent to 10 per cent;

– *bornyl acetate*: maximum 2.0 per cent;

– *β-caryophyllene*: maximum 7.0 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**01/2008:1426**
**corrected 6.0**

# KELP

## Fucus vel Ascophyllum

DEFINITION

Fragmented dried thallus of *Fucus vesiculosus* L. or *F. serratus* L. or *Ascophyllum nodosum* Le Jolis.

*Content*: minimum 0.03 per cent and maximum 0.2 per cent of total iodine ($A_r$ 126.9) (dried drug).

CHARACTERS

Salty and mucilaginous taste.

Unpleasant marine odour.

IDENTIFICATION

A. The drug consists of fragments with a corneous consistency, blackish-brown to greenish-brown, sometimes covered with whitish efflorescence. The thallus consists of a ribbon-like blade, branching dichotomously with prominent central ribs (pseudoveins). *F. vesiculosus* typically shows a foliose blade with smooth edges and bears occasional ovoid, single or paired, air vesicles. The ends of certain branches are of ovoid shape and a little widened. They bear numerous reproductive organs (conceptacles). *F. serratus* has a foliose blade with a serrate margin and no vesicles, the branches bearing conceptacles are less swollen. The thallus of *A. nodosum* is irregularly branched, without pseudo-midrib. It shows single ovoid air vesicles; the falciform conceptacles are located at the end of small branches.

B. Microscopic examination (*2.8.23*). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows fragments of surface tissue with regular isodiametric cells with brown contents, and fragments of deep tissue with colourless, elongated cells arranged in long filaments with large mucilaginous spaces between them. Thick-walled cells in files and in closely packed groups, from the pseudovein, are sometimes visible.

C. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of a 2 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously and filter. Wash the residue with 10 mL of *water R* and filter. To the residue add 10 mL of a 200 g/L solution of *sodium carbonate R*. Shake and centrifuge. Collect the supernatant. Adjust to pH 1.5 using *sulfuric acid R*. A white, flocculent precipitate is slowly formed.

TESTS

**Arsenic** (*2.4.27*) : maximum 90 ppm.

**Cadmium** (*2.4.27*) : maximum 4 ppm.

**Lead** (*2.4.27*) : maximum 5 ppm.

**Mercury** (*2.4.27*) : maximum 0.1 ppm.

**Swelling index** (*2.8.4*): minimum 6.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 105 °C, for 2 h.

**Total ash** (*2.4.16*): maximum 24 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

**Total iodine**. To 1.000 g of the powdered herbal drug, in a tall silica crucible, add 5 mL of *water R* and 5 g of *potassium hydroxide R*. Stir with a magnesium rod. Heat on a water bath. Add 1 g of *potassium carbonate R*. Mix, add the tip of the

magnesium rod with the residues of the drug and dry, first on a water-bath then over an open flame. Incinerate raising the temperature progressively to not more than 600 °C. Allow to cool. Add 20 mL of *water R* and heat gently to boiling, stirring with a glass rod. Filter the hot mixture through an unpleated filter, into a conical flask. Rinse the residue with 4 quantities, each of 20 mL, of hot *water R*. Rinse the filter and the crucible with 50 mL of hot *water R*. Combine the solutions. Allow to cool. Neutralise with *dilute sulfuric acid R* in the presence of *methyl orange solution R*. Add 3 mL of *dilute sulfuric acid R* and 1 mL of *bromine water R*. The solution is yellow. After 5 min add 0.6 mL of a 50 g/L solution of *phenol R*. The solution is clear. Acidify with 5 mL of *phosphoric acid R* and add 0.2 g of *potassium iodide R*. Allow to stand for 5 min protected from light. Add 1 mL of *starch solution R* and titrate with *0.01 M sodium thiosulfate*.

1 mL of *0.01 M sodium thiosulfate* is equivalent to 0.2115 mg of iodine.

LABELLING

The label states the species of kelp present.

**07/2013:1885**

# KNOTGRASS

## Polygoni avicularis herba

DEFINITION

Whole or fragmented, dried flowering aerial parts of *Polygonum aviculare* L. *s.l.*

*Content*: minimum 0.30 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The stem is 0.5-2 mm thick, branched, with nodes, cylindrical or slightly angular, and longitudinally striated. It bears sessile or shortly petiolate, glabrous, entire leaves, which differ widely in shape and size. The sheath-like stipules (ochrea) are lacerate and silvery. The small, axillary flowers have 5 greenish-white perianth segments, the tips of which are often red. The dry, indehiscent fruits are 2-4 mm, brown or black, triangular, usually punctate or striate.

B. Microscopic examination (*2.8.23*). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1885.-1): fragments of lower [A] and upper [D] leaf epidermises with a striated cuticle and anisocytic stomata (*2.8.3*) [Aa, Da]; polygonal cells of the upper epidermis [D] with slightly thickened beaded walls, often associated with palisade parenchyma [Db]; cells of the lower epidermis [A], with thin, sinuous walls; fragments of the margin of the lamina of the leaf with irregular cells [J]; fragments of parenchyma [G] with numerous cells containing cluster crystals of calcium oxalate, some of which are very large [Ga], often associated with vessels [Gb]; groups of fibres [B, C] with thick walls [Ba, Cb] from the hypodermis of the stem associated either with the epidermis [Ca] or with parenchyma consisting of cells containing cluster crystals of calcium oxalate [Bb]; fragments of the ochrea [E] with elongated, thin-walled cells [Ea], along which run very elongated fibres [Eb]; globular pollen grains with a smooth exine and 3 germinal pores [H]; occasional brown fragments of the exocarp composed of cells with thick, sinuous walls [F].

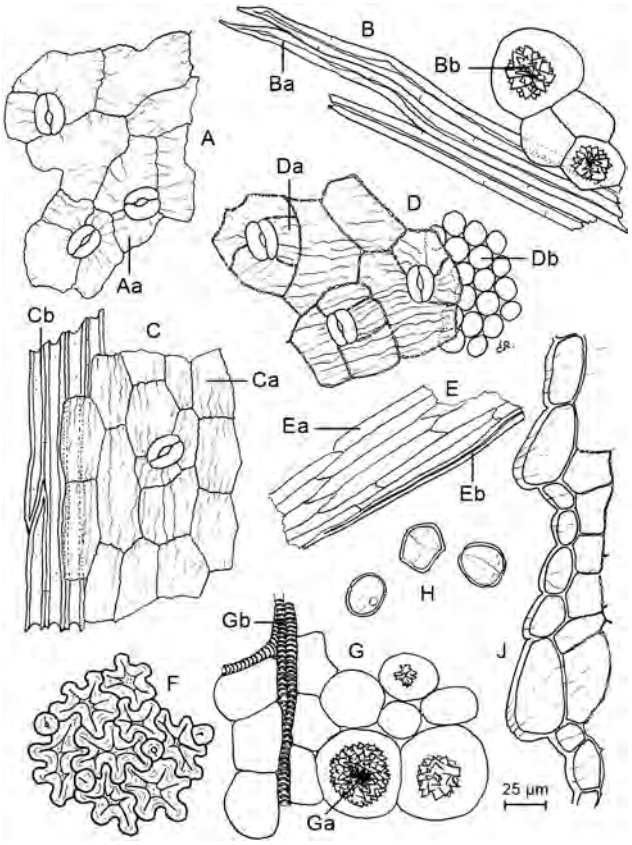


Figure 1885.-1. – *Illustration for identification test B of powdered herbal drug of knotgrass*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat the mixture in a water-bath under a reflux condenser for 10 min. Cool and filter.

*Reference solution*. Dissolve 1 mg of *caffeic acid R*, 1 mg of *chlorogenic acid R* and 2.5 mg of *hyperoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7:7:14:72 *V/V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a light blue fluorescent zone | 1 or 2 blue fluorescent zones (caffeic acid) |
| _____ | _____ |
| | 1 or 2 yellowish-green fluorescent zones |
| | A yellow fluorescent zone |
| Hyperoside: a yellowish-brown fluorescent zone | |
| | A yellowish-brown fluorescent zone |
| Chlorogenic acid: a light blue fluorescent zone | A light blue fluorescent zone (chlorogenic acid) |
| _____ | _____ |
| | A yellowish-brown fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of roots and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask, place 0.800 g of the powdered herbal drug (355) (*2.9.12*), and add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool, filter each extract through the plug of absorbent cotton into the flask. Filter the combined acetone extracts through a filter paper into a volumetric flask and dilute to 100.0 mL with *acetone R*, rinsing the flask and the filter paper. Introduce 20.0 mL of the solution into a separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel and wash with 2 quantities, each of 50 mL, of *water R*. Dry the extracts over 10 g of *anhydrous sodium sulfate R*, filter into a 50 mL volumetric flask and dilute to volume with *ethyl acetate R*.

*Test solution.* To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min by comparison with the compensation liquid at 425 nm. Calculate the percentage content of flavonoids, calculated as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

*A* = absorbance at 425 nm;

*m* = mass of the herbal drug to be examined, in grams.

**04/2019:2434**

# KUDZUVINE ROOT

## Puerariae lobatae radix

DEFINITION

Fragmented, dried root of *Pueraria montana* (Lour.) Merr. var. *lobata* (Willd.) Maesen & S.M.Almeida ex Sanjappa & Predeep (syn. *Pueraria lobata* (Willd.) Ohwi).

*Content*: minimum 6.5 per cent of total isoflavonoids, expressed as puerarin ($C_{21}H_{20}O_9$; $M_r$ 416.4) (dried drug), of which minimum 45 per cent consists of puerarin.

IDENTIFICATION

A. Small, square pieces or thick, rectangular slices, 5-35 cm long and 0.5-1 cm thick. The outer bark is pale brown, with longitudinal wrinkles and rough; the section is yellowish-white and shows indistinct striations. The texture is strongly fibrous.

B. Microscopic examination (*2.8.23*). The powder is pale brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2434.-1): thick-walled lignified fibres, which occur in bundles, surrounded by a calcium oxalate prism sheath [E]; cork fragments with polygonal cells (surface view [D], transverse section [B]); fragments of xylem [C] consisting of relatively large vessels with hexagonal or elliptical bordered-pits, arranged very densely [Ca] and xylem parenchyma cells with slightly thickened and pitted walls [Cb]; rare sclereids, subrounded or elliptical, about 50 µm in diameter [F]; fragments of parenchyma [A]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules [G], simple or 2-20 compound; the individual starch granules, 15-30 µm in diameter, are spheroidal, semi-rounded or polygonal, with a punctiform, slit-shaped or stellate hilum.

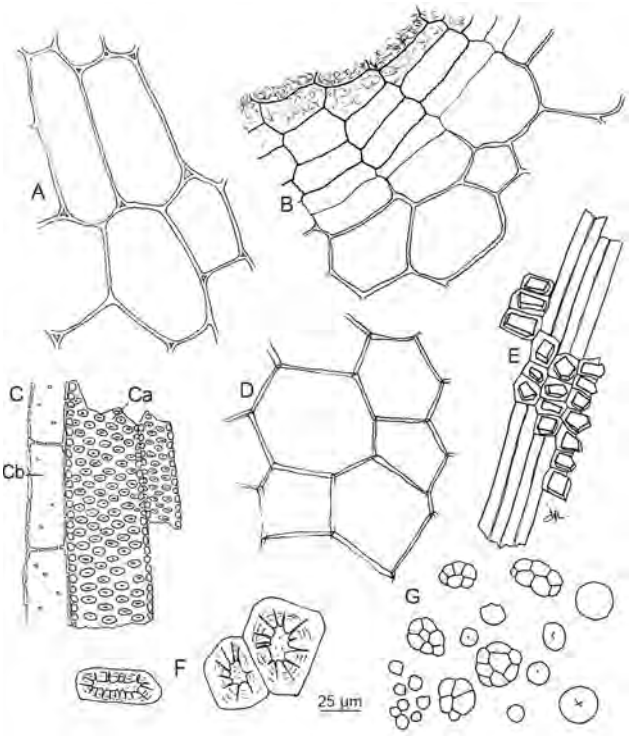


Figure 2434.-1. – *Illustration for identification test B of powdered herbal drug of kudzuvine root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Sonicate 0.5 g of the powdered herbal drug (355) (*2.9.12*) with 5 mL of *methanol R*, then centrifuge; use the supernatant.

*Reference solution.* Dissolve 5 mg of *puerarin R* and 5 mg of *daidzin R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *methylene chloride R*, *methanol R*, *ethyl acetate R* (10:20:22:40 *V/V/V/V*); use the lower layer.

*Application*: 7 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A weak quenching zone |
| ——— | ——— |
| | A quenching zone |
| Daidzin: a quenching zone | A quenching zone |
| Puerarin: a quenching zone | A quenching zone |
| ——— | ——— |
| | At least 5 quenching zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 0.100 g of the powdered herbal drug (355) (*2.9.12*) into a 250 mL conical flask, add 50.0 mL of *ethanol (30 per cent V/V) R* and weigh. Heat under a reflux condenser for 30 min. Allow to cool and weigh again. Adjust to the initial mass with *ethanol (30 per cent V/V) R*, mix well and filter.

*Reference solution.* Introduce an amount of *kudzuvine root dry extract HRS* corresponding to 3.0 mg of puerarin into a 250 mL conical flask, add 50.0 mL of *ethanol (30 per cent V/V) R* and weigh. Heat under a reflux condenser for 30 min. Allow to cool and weigh again. Adjust to the initial mass with *ethanol (30 per cent V/V) R*, mix well and filter.

*Column*: 2 columns coupled in series:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R.*

*Mobile phase*:

- *mobile phase A*: *glacial acetic acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16.5 | 90 → 71 | 10 → 29 |

*Flow rate*: 3.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *kudzuvine root dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to the isoflavonoids (3-hydroxypuerarin, puerarin, 3-methoxypuerarin, 6-*O*″-D-xylosylpuerarin and daidzin).

*Relative retention* with reference to puerarin (retention time = about 3.4 min): 3-hydroxypuerarin = about 0.7; 3-methoxypuerarin = about 1.09; 6-*O*″-D-xylosylpuerarin = about 1.15; daidzin = about 1.4.

*System suitability*: reference solution:

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to 3-methoxypuerarin and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to puerarin.

Calculate the percentage content of puerarin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to puerarin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to puerarin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *kudzuvine root dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of puerarin in *kudzuvine root dry extract HRS*.

Calculate the percentage content of total isoflavonoids (3-hydroxypuerarin, puerarin, 3-methoxypuerarin, 6-*O*″-D-xylosylpuerarin and daidzin) using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to the isoflavonoids (3-hydroxypuerarin, puerarin, 3-methoxypuerarin, 6-*O*″-D-xylosylpuerarin and daidzin) in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to puerarin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *kudzuvine root dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of puerarin in *kudzuvine root dry extract HRS*.

**07/2018:1534**

# LAVENDER FLOWER

## Lavandulae flos

DEFINITION

Dried flower of *Lavandula angustifolia* Mill. (*L. officinalis* Chaix).

*Content*: minimum 13 mL/kg of essential oil (anhydrous drug).

IDENTIFICATION

*First identification*: *A, B, D.*

*Second identification*: *A, B, C.*

A. The flower has a short peduncle and consists of a bluish-grey tubular calyx divided distally into 4 very short teeth and a small rounded lobe, a blue bilabial corolla with the upper lip bifid and the lower lip trilobate and 4 didynamous stamens with ovoid anthers.

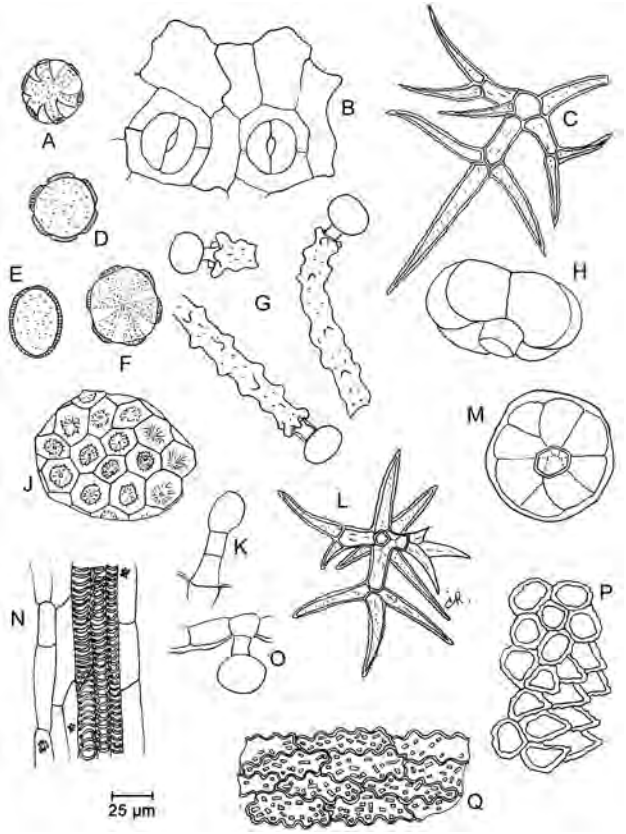


Figure 1534.-1. – *Illustration for identification test B of powdered herbal drug of lavender flower*

B. Microscopic examination (*2.8.23*). The powder is bluish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1534.-1): covering trichomes bifurcating at one or more levels [C, L]; secretory trichomes with short stalks and 8-celled heads of the *Lamiaceae* type (side view [H], surface view [M]); glandular trichomes with unicellular [O] or multicellular [K] stalks and unicellular heads; glandular trichomes with long uneven stalks and unicellular heads, separated from the stalk by an intermediary cell with a smooth cuticle, certain trichomes show a crown of small spheroid protuberances just below the insertion point of the intermediary cell on the stalk [G]; fragments of papillose epidermis from the inner surface of the petals (surface view [J], side view [P]); fragments of calyx epidermis with sinuous-walled cells and containing prismatic crystals of calcium oxalate [Q]; spherical pollen grains which have a diameter of about 45 µm and an exine with 6 slit-like germinal pores and 6 ribbon-like groins radiating from the poles [A, D, E, F]; rare fragments of leaf epidermis with stomata, mostly of the diacytic type (*2.8.3*) [B]; fragments of vascular tissue with spiral vessels included in parenchyma with some cells containing small cluster crystals of calcium oxalate [N].

C. Examine the chromatograms obtained in the test for lavandin flower.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A violet zone |
| Linalyl acetate: a violet zone | A violet zone |
| | A pink zone |
| 1,8-Cineole: a violet or brown zone | |
| Linalol: a violet zone | A violet zone (linalol) |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the test for other species and varieties of lavender.

*Results*: the 5 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with the reference solution; the 2 main peaks are due to linalol and linalyl acetate.

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of stems and maximum 2 per cent of other foreign matter.

**Lavandin flower *(Lavandula x intermedia Emeric ex Loisel)***. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *toluene R*, sonicate for 5 min and filter.

*Reference solution.* Dilute 10 µL of *cineole R*, 5 µL of *linalol R* and 5 µL of *linalyl acetate R* to 1 mL with *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine immediately in daylight.

*Results*: the chromatogram obtained with the test solution shows no zone between the zones due to 1,8-cineole and linalol in the chromatogram obtained with the reference solution.

**Other species and varieties of lavender**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 0.2 mL of the essential oil-xylene mixture obtained in the assay to 5 mL with *heptane R*, add 1 g of *anhydrous sodium sulfate R*, shake and use the supernatant.

*Reference solution.* Dissolve 0.05 g of *camphor R* and 0.2 g of *α-terpineol R* in *heptane R*, add 0.1 g of *limonene R*, 0.2 g of *cineole R*, 0.4 g of *linalol R* and 0.6 g of *linalyl acetate R*, then dilute to 100 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 70 |
| | 15 - 70 | 70 → 180 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: the same volume of each solution.

*Elution order*: limonene, 1,8-cineole, camphor, linalol, linalyl acetate, α-terpineol.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to limonene and 1,8-cineole;
- *number of theoretical plates*: minimum 30 000, calculated for the peak due to limonene at 110 °C.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the 6 components of the reference solution in the chromatogram obtained with the test solution. Disregard the peaks due to heptane and xylene.

*Limit*:

- *camphor*: maximum 1 per cent.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the herbal drug, a 1000 mL round-bottomed flask, 500 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**07/2018:1338**

# LAVENDER OIL

## Lavandulae aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the flowering tops of *Lavandula angustifolia* Mill. (*Lavandula officinalis* Chaix).

CHARACTERS

*Appearance*: colourless or pale yellow, clear liquid.

*Odour*: complex, reminiscent of linalyl acetate.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 20 µL of the essential oil to be examined to 1 mL with *toluene R*.

*Reference solution.* Dilute 10 µL of *cineole R*, 5 µL of *linalol R* and 5 µL of *linalyl acetate R* to 1 mL with *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 6 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| Linalyl acetate: a violet zone | An intense violet zone (linalyl acetate)<br>A pink zone |
| 1,8-Cineole: a violet or brown zone | Possibly a weak violet-brown zone (1,8-cineole) |
| Linalol: a violet zone | An intense violet zone (linalol)<br>A greyish or brownish zone |
| **Reference solution** | **Test solution** |

B. The essential oil to be examined complies with the limits of the test for chromatographic profile.

TESTS

**Relative density** (*2.2.5*): 0.878 to 0.892.

**Refractive index** (*2.2.6*): 1.455 to 1.466.

**Optical rotation** (*2.2.7*): − 12.5° to − 6.0°.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.00 g of the essential oil to be examined dissolved in 50 mL of the prescribed mixture of solvents.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 200 µL of the essential oil to be examined to 10.0 mL with *heptane R*.

*Reference solution (a).* Dilute 200 µL of *lavender oil for peak identification HRS* to 10.0 mL with *heptane R*.

*Reference solution (b).* Dilute 5 µL of *limonene R* to 50.0 mL with *heptane R*. Dilute 0.5 mL of this solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 70 |
| | 15 - 70 | 70 → 180 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: limonene, 1,8-cineole, 3-octanone, camphor, linalol, linalyl acetate, terpinen-4-ol, lavandulyl acetate, lavandulol, α-terpineol.

*Identification of peaks*: use the chromatogram supplied with *lavender oil for peak identification HRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to limonene, 1,8-cineole, 3-octanone, camphor, linalol, linalyl acetate, terpinen-4-ol, lavandulyl acetate, lavandulol and α-terpineol.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.4 between the peaks due to terpinen-4-ol and lavandulyl acetate.

Determine the percentage content of each of the following components. The percentages are within the following ranges:

– *limonene*: maximum 1.0 per cent;
– *1,8-cineole*: maximum 2.5 per cent;
– *3-octanone*: 0.1 per cent to 5.0 per cent;
– *camphor*: maximum 1.2 per cent;
– *linalol*: 20.0 per cent to 45.0 per cent;
– *linalyl acetate*: 25.0 per cent to 47.0 per cent;
– *terpinen-4-ol*: 0.1 per cent to 8.0 per cent;
– *lavandulyl acetate*: minimum 0.2 per cent;
– *lavandulol*: minimum 0.1 per cent;
– *α-terpineol*: maximum 2.0 per cent;
– *reporting threshold*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chiral purity**. Gas chromatography (*2.2.28*).

*Test solution.* Dilute 0.02 g of the essential oil to be examined to 10 mL with *pentane R*.

*Reference solution.* Dissolve 5 mg of *borneol R* in *pentane R*, add 10 µL of *linalol R* (mixture of (*R*)-linalol and (*S*)-linalol) and 10 µL of *linalyl acetate R* (mixture of (*R*)-linalyl acetate and (*S*)-linalyl acetate) and dilute to 10 mL with *pentane R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.25 mm;
– *stationary phase*: *modified β-cyclodextrin for chiral chromatography R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 65 | 50 → 180 |
| Injection port | | 230 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: (*R*)-linalol, (*S*)-linalol, borneol, (*R*)-linalyl acetate, (*S*)-linalyl acetate; depending on the operating conditions and the state of the column, borneol may elute before or after (*S*)-linalol.

*System suitability*: reference solution:

– *resolution*: minimum 5.5 between the peaks due to (*R*)-linalol and (*S*)-linalol; minimum 2.9 between the peaks due to (*S*)-linalol and borneol; minimum 2.0 between the peaks due to (*R*)-linalyl acetate and (*S*)-linalyl acetate.

Calculate the percentage content of the specified (*S*)-enantiomers using the following expression:

$$\frac{A_S}{A_S + A_R} \times 100$$

$A_S$ = area of the peak due to the corresponding (*S*)-enantiomer;

$A_R$ = area of the peak due to the corresponding (*R*)-enantiomer.

*Limits*:

– *(S)-linalol*: maximum 12 per cent;
– *(S)-linalyl acetate*: maximum 1 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**01/2008:0620**

# LEMON OIL

## Limonis aetheroleum

DEFINITION

Essential oil obtained by suitable mechanical means, without the aid of heat, from the fresh peel of *Citrus limon* (L.) Burman fil.

CHARACTERS

*Appearance*: clear, mobile, pale yellow or greenish-yellow liquid. It may become cloudy at low temperatures.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Mix 1 mL of the substance to be examined in 1 mL of *toluene R*.

*Reference solution.* Dissolve 10 mg of *citropten R* and 50 µL of *citral R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (15:85 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A quenching zone (bergamotin) |
| Citral: a quenching zone | A quenching zone (citral) |
| | A dark blue zone (5-geranyloxy-7-methoxycoumarin) |
| Citropten: a light blue fluorescent zone | A light blue fluorescent zone (citropten) |
| | A quenching zone (psoralen derivative) |
| | A quenching zone (biakangelicin) |
| **Reference solution** | **Test solution** |

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A yellow fluorescent zone (bergamotin) |
| Citral: a quenching zone | A quenching zone (citral) |
| | A bright blue fluorescent zone (5-geranyloxy-7-methoxycoumarin) |
| Citropten: a bright blue fluorescent zone | A bright violet-blue fluorescent zone (citropten) |
| | A yellow fluorescent zone (psoralen derivative) |
| | An orange zone (biakangelicin) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.850 to 0.858.

**Refractive index** (*2.2.6*): 1.473 to 1.476.

**Optical rotation** (*2.2.7*): + 57° to + 70°.

**Absorbance** (*2.2.25*). Dissolve 0.250 g of the substance to be examined in *alcohol R*, mix and dilute to 100.0 mL with the same solvent. Measure the absorbance over the range 260 nm to 400 nm. If a manual instrument is used, measure the absorbance at 5 nm intervals from 260 nm to about 12 nm before the expected absorption maximum, then at 3 nm intervals for 3 readings and at 1 nm intervals to about 5 nm beyond the maximum and finally at 10 nm intervals to 400 nm. Plot a curve representing the absorption spectrum with the absorbances as ordinates and the wavelengths as abscissae. Draw as a baseline the tangent between *A* and *B* (Figure 0620.-1). The absorption maximum *C* is situated at 315 ± 3 nm. From *C* draw a line perpendicular to the axis of abscissae and intersecting *AB* at *D*. Deduct the absorbance corresponding to point *D* from that corresponding to point *C*. The value *C* – *D* is 0.20 to 0.96 and for Italian-type lemon oil it is not less than 0.45.

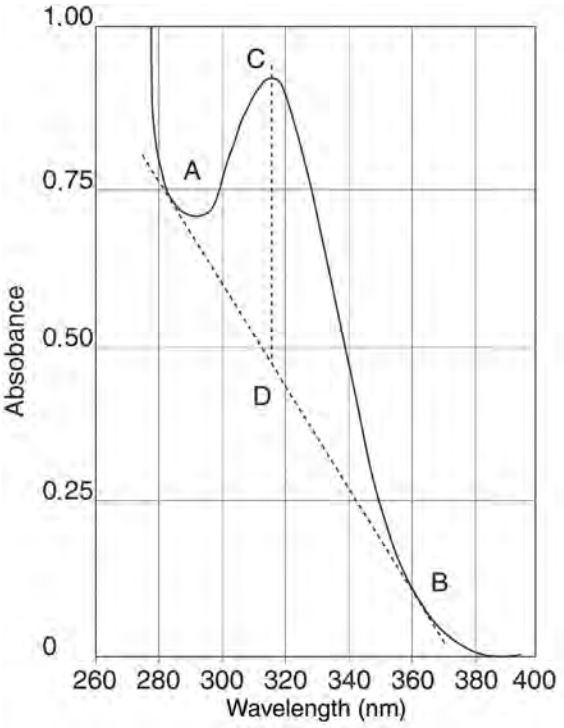


Figure 0620.-1. – *Typical spectrum of lemon oil for the test for absorbance*

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test for fatty oils and resinified essential oils.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution.* Dissolve 20 µL of *β-pinene R*, 10 µL of *sabinene R*, 100 µL of *limonene R*, 10 µL of *γ-terpinene R*, 5 µL of *β-caryophyllene R*, 20 µL of *citral R*, 5 µL of *α-terpineol R*, 5 µL of *neryl acetate R* and 5 µL of *geranyl acetate R* in 1 mL of *acetone R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 45 |
| | 6 - 21 | 45 → 90 |
| | 21 - 39 | 90 → 180 |
| | 39 - 55 | 180 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL of the reference solution and 0.2 µL of the test solution.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to β-pinene and sabinene and minimum 1.5 between the peaks due to geranial and geranyl acetate.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The percentages are within the following ranges:

– *β-pinene*: 7.0 per cent to 17.0 per cent,
– *sabinene*: 1.0 per cent to 3.0 per cent,
– *limonene*: 56.0 per cent to 78.0 per cent,
– *γ-terpinene*: 6.0 per cent to 12.0 per cent,
– *β-caryophyllene*: maximum 0.5 per cent,
– *neral*: 0.3 per cent to 1.5 per cent,
– *α-terpineol*: maximum 0.6 per cent,
– *neryl acetate*: 0.2 per cent to 0.9 per cent,
– *geranial*: 0.5 per cent to 2.3 per cent,
– *geranyl acetate*: 0.1 per cent to 0.8 per cent.

**Residue on evaporation** (*2.8.9*): 1.8 per cent to 3.6 per cent after heating on the water-bath for 4 h.

STORAGE

At a temperature not exceeding 25 °C.

LABELLING

The label states, where applicable, that the contents are Italian-type lemon oil.

**01/2012:1834**

# LEMON VERBENA LEAF

## Verbenae citriodorae folium

DEFINITION

Whole or fragmented, dried leaves of *Aloysia citrodora* Paláu (syn. *Aloysia triphylla* (L'Hér.) Kuntze; *Verbena triphylla* L'Hér.; *Lippia citriodora* Kunth).

*Content*:

– *acteoside* ($C_{29}H_{36}O_{15}$; $M_r$ 625): minimum 2.5 per cent, expressed as ferulic acid (dried drug);
– *essential oil*: minimum 3.0 mL/kg for the whole drug and minimum 2.0 mL/kg for the fragmented drug (dried drug).

CHARACTERS

After grinding, it has a characteristic odour reminiscent of lemon.

IDENTIFICATION

A. The leaves are simple with short petioles. They are narrow, lanceolate, and about 4 times longer than they are wide. The entire, slightly undulating margins are curled towards the upper surface. The upper surface is dark green and rough to the touch; the lower surface is paler green and shows a prominent midrib with secondary veins running to the margins.

B. Microscopic examination (*2.8.23*). The powder is light green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1834.-1): fragments of the upper epidermis of the lamina (surface view [A, B, H]), composed of polygonal cells with numerous short, unicellular, thick-walled cystolithic trichomes, each arising from a rosette of cells at the base and containing calcium concretions [B], glandular trichomes with a unicellular stalk and a unicellular, globular head of variable size (surface view [Ha], transverse section [D, F]); these fragments are usually accompanied by palisade parenchyma [Aa, Hb]; fragments of the lower epidermis of the lamina (surface view [E]) covered by a striated cuticle and composed of cells more irregular and somewhat sinuous in outline, with abundant anomocytic stomata (*2.8.3*) [Ea] and numerous glandular trichomes in surface view [Eb] and/or their scars [Ec]; fragments of the lamina (transverse section [G]) with 2 layers of palisade parenchyma [Ga] and spongy parenchyma [Gb]; lignified tissue from the veins [C].

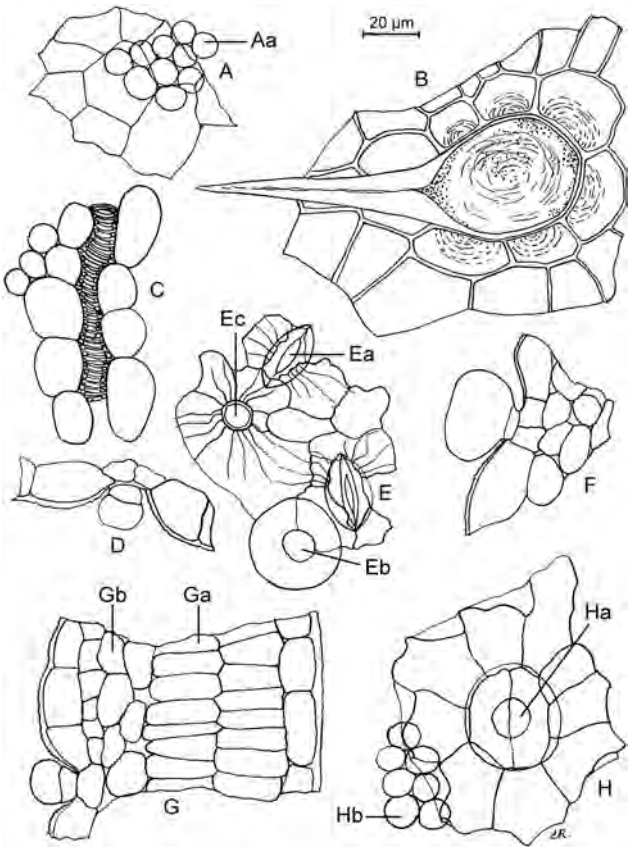


Figure 1834.-1. – *Illustration for identification test B of powdered herbal drug of lemon verbena leaf*

C. Examine the chromatograms obtained in the test for *Verbena officinalis*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution. Zones may be present in the chromatogram obtained with the test solution below the zone due to rutoside in the chromatogram obtained with the reference solution.

| **Top of the plate** | |
|---|---|
| _____ | _____ |
| | An intense greyish-green zone |
| Arbutin: a blue or brown zone | |
| Rutoside: a dark brownish-yellow zone | A blue or violet zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

***Verbena officinalis***. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.50 g of the powdered herbal drug (710) (*2.9.12*) add 5 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min. Cool and filter.

*Reference solution.* Dissolve 10 mg of *arbutin R* and 10 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 20 µL [or 5 µL] as bands.

*Development*: over a path of about 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and dry at 100-105 °C for about 10 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no brownish-grey zone at a position between that of arbutin and rutoside in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 13.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.5 per cent.

ASSAY

**Acteoside**. Liquid chromatography (*2.2.29*).

*Test solution.* To 1.00 g of the powdered herbal drug (710) (*2.9.12*) add 50.0 mL of the reference solution and stir for 2 h with a magnetic stirrer. Centrifuge for 15 min and pass the supernatant through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 10.0 mg of *ferulic acid CRS* in *ethanol (60 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

*Precolumn*:

- *size*: *l* = 0.01 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*:

- *mobile phase A*: 0.3 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 93 → 83 | 7 → 17 |
| 20 - 30 | 83 | 17 |
| 30 - 35 | 83 → 75 | 17 → 25 |
| 35 - 40 | 75 → 20 | 25 → 80 |
| 40 - 45 | 20 → 93 | 80 → 7 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*System suitability*: test solution:

- *resolution*: minimum 3.5 between the peaks due to ferulic acid and acteoside.

Calculate the percentage content of acteoside, expressed as ferulic acid, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.5 \times 3.1}{A_2 \times m_1}$$

$A_1$ = area of the peak due to acteoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ferulic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug in the test solution, in grams;

$m_2$ = mass of *ferulic acid CRS* in the reference solution, in grams;

$p$ = percentage content of ferulic acid in *ferulic acid CRS*;

3.1 = correlation factor between acteoside and ferulic acid.

**Essential oil** (*2.8.12*). Introduce 25.0 g of the freshly crushed herbal drug into a 1000 mL flask and add 500 mL of a 10 g/L solution of *sodium chloride R* as the distillation liquid. Use 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 3.0-3.5 mL/min for 3 h.

**01/2019:2440**

# LIGHTYELLOW SOPHORA ROOT

## Sophorae flavescentis radix

DEFINITION

Whole or fragmented, dried root of *Sophora flavescens* Aiton, with root crown and rootlets removed, collected in spring or autumn.

*Content*: minimum 1.2 per cent for the sum of matrine ($C_{15}H_{24}N_2O$; $M_r$ 248.4) and oxymatrine ($C_{15}H_{24}N_2O_2$; $M_r$ 264.4) (dried drug).

IDENTIFICATION

A. The root is cylindrical, usually branched in the lower part, 10-30 cm long, 1-6.5 cm in diameter, externally greyish-brown or brownish-yellow, exhibiting longitudinal wrinkles and transverse elongated lenticel-like protuberances. The outer bark is thin, mostly broken and recurved, easily exfoliated, with the exposed surface appearing yellow and smooth. The texture is hard and difficult to break. The fracture is fibrous. Slices are 3-6 mm thick; the transversely cut surface is yellowish-white with radial lines and cracks, sometimes exhibiting abnormal vascular bundles arranged in concentric rings or scattered irregularly.

B. Microscopic examination (*2.8.23*). The powder is pale brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2440.-1): very numerous unlignified fibres, single or in groups [C, G], usually surrounded by crystal sheaths of calcium oxalate [Ca]; some fibres accompanied by medullary rays with cells having slightly thickened and pitted walls [Ga]; xylem vessels, pitted or reticulate [K, L], 50-200 µm in diameter; calcium oxalate prisms, 10-25 µm in diameter, either isolated [B] or inside parenchymatous cells [Da], sometimes several prisms in the same cell, or forming crystal sheaths

around the fibres [Ca]; numerous fragments of parenchyma with rounded to ovoid, slightly pitted cells [D]; fragments of cork (surface view [A]) consisting of polygonal cells, finely reticulate inside; rare sclereids, either in groups [F] or, more often, isolated [E]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very abundant starch granules, either included in parenchyma cells [J] or isolated [H]; some are simple, most are 2-8 compound of variable size; the hilum is usually visible.

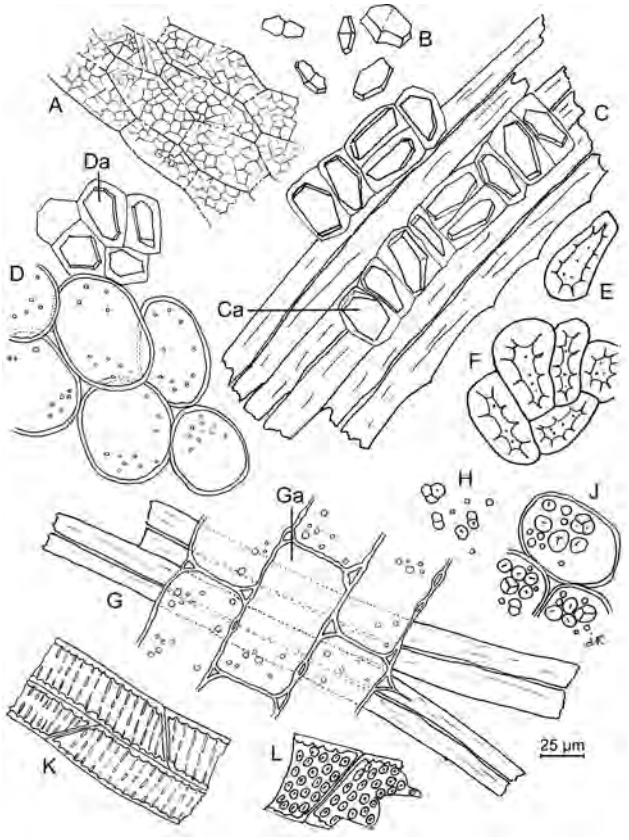


Figure 2440.-1. – *Illustration for identification test B of powdered herbal drug of lightyellow sophora root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 20 mL of *water R*. Heat on a water-bath under a reflux condenser at 90 °C for 30 min. Allow to cool and centrifuge for 5 min. Dilute 5 mL of the supernatant to 10 mL with *methanol R*. A white precipitate is formed. Centrifuge for 5 min and use the clear, slightly yellow supernatant.

*Reference solution.* Dissolve 1 mg of *brucine R* and 1 mg of *quinine R* in 5 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (5:10:85 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *potassium iodobismuthate solution R* and then with *dilute hydrogen peroxide solution R* until the orange or brown zones become visible against a yellow background; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint brown or orange-red zone |
| | A brown or orange-red zone |
| _____ | _____ |
| An orange-red zone (brucine) | |
| A brown or orange-red zone (quinine) | |
| _____ | _____ |
| | 2 or 3 prominent brown or orange-red zones |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.300 g of the powdered herbal drug (355) (*2.9.12*) add 0.5 mL of *concentrated ammonia R* and 25.0 mL of *methylene chloride R*, weigh and sonicate for 30 min. Allow to cool, weigh and adjust to the original mass with *methylene chloride R*, shake well and filter. Transfer 5.0 mL of the filtrate to a chromatographic column about 0.2 m long and 10 mm in internal diameter packed with 5 g of *neutral aluminium oxide R* (60-210 µm). Elute with 20 mL of a mixture of 3 volumes of *methanol R* and 7 volumes of *methylene chloride R*. Evaporate the eluate to dryness. Dissolve the residue in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 5.0 mg of *matrine CRS* and 5.0 mg of *oxymatrine CRS* in a mixture of 2 volumes of *anhydrous ethanol R* and 8 volumes of *acetonitrile R*, and dilute to 5.0 mL with the same mixture of solvents. Dilute 1.0 mL of the solution to 20.0 mL with a mixture of 2 volumes of *anhydrous ethanol R* and 8 volumes of *acetonitrile R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 1 per cent *V/V* solution of *phosphoric acid R* in *ethanol (96 per cent) R*, *acetonitrile for chromatography R* (20:80 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Retention time*: matrine = about 14 min; oxymatrine = about 24 min.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to matrine and oxymatrine.

Calculate the percentage content of the sum of matrine and oxymatrine using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 0.50}{A_2 \times m_1} + \frac{A_3 \times m_3 \times p_2 \times 0.50}{A_4 \times m_1}$$

$A_1$ = area of the peak due to matrine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to matrine in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to oxymatrine in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to oxymatrine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *matrine CRS* used to prepare the reference solution, in grams;

$m_3$ = mass of *oxymatrine CRS* used to prepare the reference solution, in grams;

$p_1$ = percentage content of matrine in *matrine CRS*;

$p_2$ = percentage content of oxymatrine in *oxymatrine CRS*.

**04/2019:2431**

# LIGUSTICUM ROOT AND RHIZOME

## Ligustici radix et rhizoma

DEFINITION

Whole or fragmented, dried rhizome and root of *Ligusticum sinense* Oliv. or *Ligusticum jeholense* (Nakai & Kitag.) Nakai & Kitag.

*Content*: minimum 5.0 mL/kg of essential oil (dried drug).

IDENTIFICATION

A. The rhizome is short, up to 3 cm in diameter, brown or yellowish-brown, simple or somewhat twisted. The roots are usually up to 1.5 mm thick, show no ramification, and are the same colour as the rhizome; the fracture is usually fibrous; the remaining stem bases are cylindrical, 5-6 mm thick and their longitudinal fracture shows a yellowish-white medulla. The fragmented rhizome occurs as more or less thick slices.

The rhizome of *Ligusticum sinense* shows an irregular brown or blackish-brown outer surface; the yellowish-white or pale yellowish-brown transverse section is fibrous, porous and cracked.

The rhizome of *Ligusticum jeholense* shows root scars and the spiny remains of roots on its outer surface; a transverse section shows radial striations and cracks in the xylem.

B. Microscopic examination (*2.8.23*). The powder is brownish-beige. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2431.-1): cork fragments made up of cells with brown contents which are polygonal in surface view [C]; numerous fragments of parenchyma consisting of rounded or ovoid cells, with thin or slightly thickened walls [H]; fragments of brownish-yellow secretory canals up to 170 µm wide (transverse section [A], longitudinal section [E]); fragments of reticulate vessels about 80 µm in diameter, isolated or in groups of 2 or 3 [B]; groups of smaller reticulate vessels surrounded by either xylem parenchyma [G] or by lignified fibres with thick, moderately pitted walls [J]; fibres are isolated [L] or in groups of 2 or 3, colourless, often whole with pitted walls, sometimes reaching 700 µm in length and about 20 µm wide; round or rectangular sclereids, isolated [F] or grouped in clusters [D], with thickened and channelled walls (some of the larger sclereids may be up to 100 µm long). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows many isolated, small (5-10 µm), round or ovoid starch granules [K].

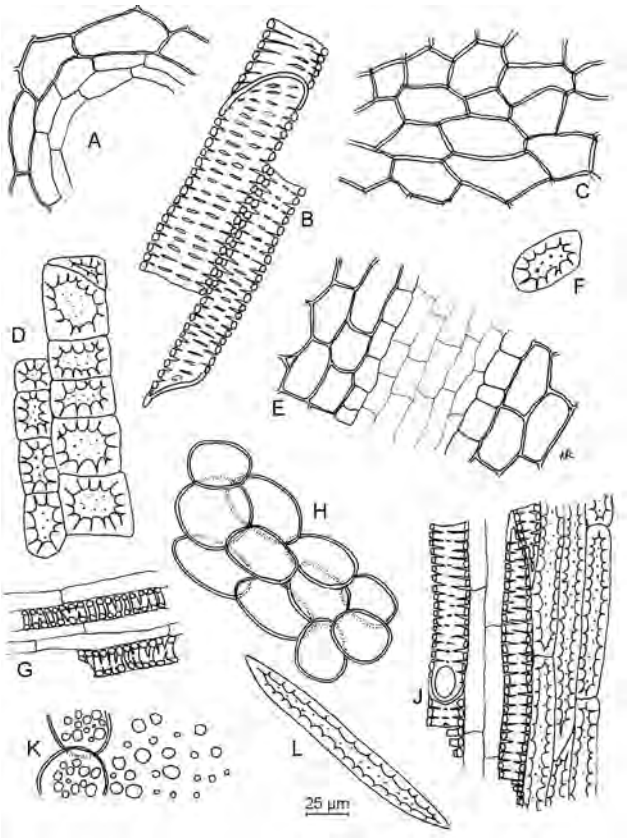


Figure 2431.-1. – *Illustration for identification test B of powdered herbal drug of ligusticum root and rhizome*

C. Thin-layer chromatography (*2.2.27*).

Examine the chromatograms obtained in the test for officinal species of *Angelica* and *Levisticum*.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (Z)-Ligustilide: a bluish-white fluorescent zone | A bluish-white fluorescent zone ((Z)-ligustilide) |
| | A faint bluish-white fluorescent zone |
| _____ | _____ |
| Osthole: a blue fluorescent zone | |
| Imperatorin: a whitish fluorescent zone | A faint blue fluorescent zone |
| _____ | _____ |
| | A faint bluish-white fluorescent zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A quenching zone (*L. jeholense*) |
| (*Z*)-Ligustilide: a blue fluorescent zone | A blue fluorescent zone ((*Z*)-ligustilide) |
| _____ | _____ |
| | 2 quenching zones |
| Osthole: a quenching zone | |
| Imperatorin: a quenching zone | |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Results C*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 prominent reddish zones |
| | A brown zone (*L. jeholense*) |
| (*Z*)-Ligustilide: a grey zone | |
| | A violet zone |
| _____ | _____ |
| Osthole: a violet zone | |
| Imperatorin: a reddish-grey zone | A broad reddish zone |
| | A violet zone |
| _____ | _____ |
| | Several faint violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Officinal species of *Angelica* and *Levisticum*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the freshly powdered herbal drug (355) (*2.9.12*) add 4 mL of *methanol R* and sonicate for 5 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *imperatorin R*, 1 mg of *(Z)-ligustilide R* and 1 mg of *osthole R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no prominent blue fluorescent zone directly below or above the zone due to imperatorin in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no zone corresponding in position to, or directly below, the zone due to imperatorin in the chromatogram obtained with the reference solution.

*Detection C*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*; heat at 100 °C for 5 min and examine in daylight.

*Results C*: the chromatogram obtained with the test solution shows no distinct greenish zone below the zone due to imperatorin in the chromatogram obtained with the reference solution.

**Foreign matter** (*2.8.2*): maximum 3 per cent, determined on 50 g.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 25.0 g of the powdered herbal drug (500) (*2.9.12*) immediately before the assay using a blade grinder refrigerated at 5 °C, a 1000 mL round-bottomed flask, 500 mL of *water R* as the distillation liquid, 10 drops of *liquid paraffin R*, a few grains of pumice and 0.50 mL of *o-xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 3 h.

**01/2008:0957**
**corrected 6.0**

# LIME FLOWER

## Tiliae flos

DEFINITION

Whole, dried inflorescence of *Tilia cordata* Miller, of *Tilia platyphyllos* Scop., of *Tilia ×vulgaris* Heyne or a mixture of these.

CHARACTERS

Faint aromatic odour.

Faint, sweet and mucilaginous taste.

IDENTIFICATION

A. The inflorescence is yellowish-green. The main axis of the inflorescence bears a linguiform bract, membranous, yellowish-green, practically glabrous, the central vein of which is joined for up to about half of its length with the peduncle. The inflorescence usually consists of 2-7 flowers, occasionally up to 16. The sepals are detached easily from the perianth; they are up to 6 mm long, their abaxial surface is usually glabrous, their adaxial surface and their borders are strongly pubescent. The 5 spatulate, thin petals are yellowish-white, up to 8 mm long. They show fine venation and their borders only are sometimes covered with isolated trichomes. The numerous stamens are free and usually constitute 5 groups. The superior ovary has a pistil with a somewhat 5-lobate stigma.

B. Separate the inflorescence into its different parts. Examine under a microscope using *chloral hydrate solution R*. The adaxial epidermis of the bract shows cells with straight or slightly sinuous anticlinal walls; the abaxial epidermis shows cells with wavy-sinuous anticlinal walls and anomocytic stomata (*2.8.3*). Isolated cells in the mesophyll contain small calcium oxalate cluster crystals. The parenchyma of the sepals shows, particularly near the veins, numerous mucilaginous cells and cells containing small calcium oxalate clusters. The adaxial epidermis of sepals bears bent, thick-walled covering trichomes, unicellular or stellate with up to 5 cells. The epidermal cells of the petals show straight anticlinal walls with a striated cuticle

Herbal drugs

without stomata. The parenchyma of the petals shows small calcium oxalate clusters and especially in its acuminate part mucilaginous cells. The pollen grains have a diameter of about 30-40 µm and are oval or slightly triangular with 3 germinal pores and a finely granulated exine. The ovary is glabrous or densely covered with trichomes, often very twisted, unicellular or stellate with 2-4 branches.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 1.0 g of the powdered herbal drug (355) (*2.9.12*) with 10 mL of *methanol R* in a water-bath at 65 °C for 5 min. Allow to cool and filter.

*Reference solution.* Dissolve 2.0 mg of *caffeic acid R*, 5 mg of *hyperoside R* and 5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Then spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to dry for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in order of increasing $R_F$ value yellowish-orange or brownish-orange fluorescent zones due to rutoside and hyperoside and a greenish-blue fluorescent zone due to caffeic acid. In the chromatogram obtained with the test solution, the main zone shows brownish-yellow or orange fluorescence. This zone is situated just above the zone due to hyperoside in the chromatogram obtained with the reference solution. In daylight, this zone stands out from the other zones as the main zone. At the $R_F$ level of rutoside there is also a brownish-yellow fluorescent zone. Below this zone, 2 yellow fluorescent zones may be present. Between the zones due to rutoside and hyperoside, orange and yellow fluorescent zones are visible. Between the zones due to hyperoside and caffeic acid, up to 5 yellow or orange fluorescent zones are present. Immediately below the zone due to caffeic acid is a a blue fluorescent zone.

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent, determined on 30 g. There are no inflorescences with a bract bearing at the abaxial face stellate, five- to eight-rayed trichomes and flowers having an apparent double corolla by transformation of five stamens into petal-like staminoids and having a pistil which is not lobular nor indented. Hexamerous flowers occur only occasionally (*Tilia americana* L., *Tilia tomentosa* Moench).

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**04/2011:0095**

# LINSEED

## Lini semen

DEFINITION

Dried, ripe seeds of *Linum usitatissimum* L.

IDENTIFICATION

A. The seed has a flattened, elongated ovoid shape. The testa is dark reddish-brown or yellow, smooth and shiny. The seeds are 4-6 mm long, 2-3 mm wide and 1.5-2 mm thick; one end is rounded and the other end forms an oblique point near which the hilum appears as a slight depression. When viewed with a lens, the surface of the seed-coat is seen to be minutely pitted. Inside the testa a narrow, whitish endosperm and an embryo composed of 2 large, flattened, yellowish and oily cotyledons are present; the radicle points towards the hilum.

B. Microscopic examination (*2.8.23*). The powder is greasy to the touch. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0095.-1): fragments of the outer testa [A, B] with cells that are polygonal (surface view [Aa]) or narrow (transverse section [Ba]) and filled with mucilage [Bb]; fragments of the collenchymatously thickened sub-epidermal layer (transverse section [Bc], surface view [Ab]) with rounded cells with triangular intercellular spaces often attached to the sclerenchymatous layer composed of elongated cells, with thickened and pitted walls [Ca], some with strongly thickened and pitted walls [G]; fragments (surface view [C]) consisting of the hyaline layer with thin-walled cells [Cb] often remaining attached to the layer of elongated sclereids and crossing them at approximately right angles [Ca]; fragments of the inner testa (surface view [D]), composed of moderately thickened polygonal cells filled with brown-orange pigment; small polyhedral masses of pigment [H]; numerous fragments of parenchyma from the testae, with large, slightly and regularly thickened cells (surface view [J, L]); parenchyma of the endosperm [K] and cotyledons [E] containing aleurone grains and oil droplets; very numerous isolated oil droplets [F].

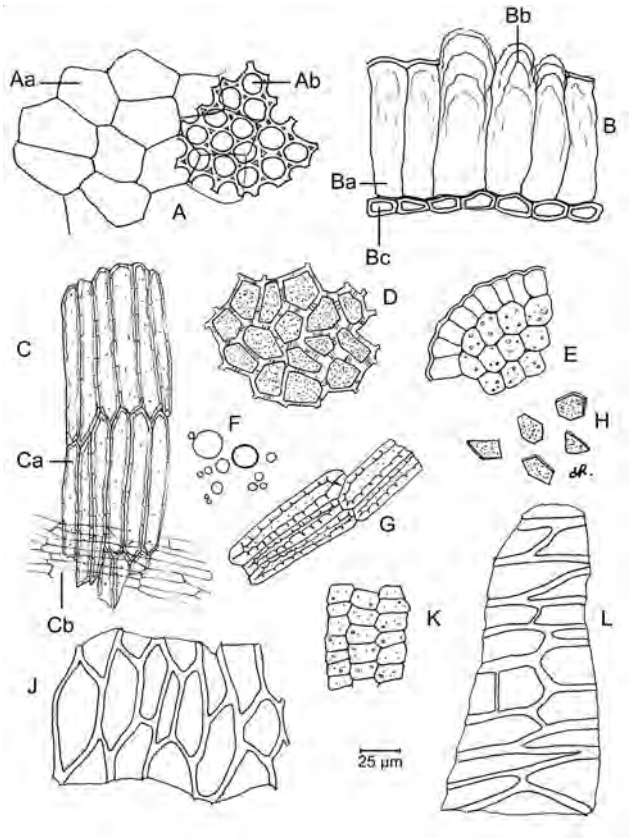


Figure 0095.-1. – *Illustration for identification test B of powdered herbal drug of linseed*

TESTS

**Foreign matter** (*2.8.2*): maximum 10 per cent of seeds with a dull coat and maximum 1.5 per cent of other foreign matter.

**Swelling index** (*2.8.4*): minimum 4.

**Cadmium** (*2.4.27*): maximum 0.5 ppm.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**01/2012:2378**

# LIQUORICE DRY EXTRACT FOR FLAVOURING PURPOSES

## Liquiritiae extractum siccum ad saporandum

DEFINITION

Dry extract produced from *Liquorice root (0277)*.

*Content*: 5.0 per cent to 7.0 per cent of 18β-glycyrrhizic acid ($C_{42}H_{62}O_{16}$; $M_r$ 823) (dried extract).

PRODUCTION

The extract is produced from the cut herbal drug by a suitable procedure using water.

CHARACTERS

*Appearance*: yellowish-brown or brown powder.

Very sweet taste.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *ethyl acetate R*, *methanol R* (50:50 *V/V*).

*Test solution.* To 0.30 g of the extract to be examined add 30 mL of *hydrochloric acid R1* and boil on a water-bath under a reflux condenser for 60 min. After cooling, extract the mixture with 2 quantities, each of 20 mL, of *ethyl acetate R*. Combine the organic layers and filter through a filter covered with *anhydrous sodium sulfate R*. Evaporate the filtrate to dryness *in vacuo* and dissolve the residue in 2.0 mL of the solvent mixture.

*Reference solution.* Dissolve 5.0 mg of *glycyrrhetic acid R* and 5.0 mg of *thymol R* in 5.0 mL of the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethanol (96 per cent) R*, *ethyl acetate R* (1:9:25:65 *V/V/V/V*).

*Application*: 20 µL [or 10 µL] as bands.

*Development*: over a path of 15 cm [or 7 cm].

*Drying*: in air for 5 min.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Thymol: a red zone | A yellow zone |
| _____ | _____ |
| _____ | _____ |
| Glycyrrhetic acid: a violet zone | A violet zone (glycyrrhetic acid) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 7.0 per cent.

**Ochratoxin A** (*2.8.22*): maximum 80 µg per kilogram of extract.

The maximum content applies to the pure undiluted extract. Where excipients are added to reduce the strength of the extract, the maximum content should be reduced proportionally.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R* (20:80 *V/V*).

*Test solution.* Place 0.200 g of the extract to be examined in a 150 mL ground-glass conical flask. Add 100.0 mL of the solvent mixture and sonicate for 2 min. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 50.0 mg of *monoammonium glycyrrhizate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (6:30:64 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of 18β-glycyrrhizic acid.

*Retention time*: 18β-glycyrrhizic acid = about 9 min.

*Identification of peaks*: use the chromatogram supplied with *monoammonium glycyrrhizate CRS* and the chromatogram obtained with the reference solution to identify the peaks due to 18β-glycyrrhizic acid and 18α-glycyrrhizic acid.

*System suitability*: reference solution:

- the chromatogram obtained with the reference solution is similar to the chromatogram supplied with *monoammonium glycyrrhizate CRS*;
- *resolution*: minimum 2.0 between the peaks due to 18β-glycyrrhizic acid and 18α-glycyrrhizic acid.

Calculate the percentage content of 18β-glycyrrhizic acid, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.979}{A_2 \times m_1 \times 5}$$

$A_1$ = area of the peak due to 18β-glycyrrhizic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to 18β-glycyrrhizic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *monoammonium glycyrrhizate CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of 18β-glycyrrhizic acid in *monoammonium glycyrrhizate CRS*;

0.979 = peak correlation factor between 18β-glycyrrhizic acid and monoammonium glycyrrhizate.

**01/2012:0277**

# LIQUORICE ROOT

## Liquiritiae radix

DEFINITION

Dried, unpeeled or peeled, whole or cut root and stolons of *Glycyrrhiza glabra* L. and/or of *Glycyrrhiza inflata* Bat. and/or *Glycyrrhiza uralensis* Fisch.

*Content*: minimum 4.0 per cent of 18β-glycyrrhizic acid ($C_{42}H_{62}O_{16}$; $M_r$ 823) (dried drug).

IDENTIFICATION

A. The root has few branches. Its bark is brown or brownish-grey with longitudinal striations and bears traces of lateral roots. The cylindrical stolons are 1-2 cm in diameter; their external appearance is similar to that of the root but there are occasional small buds. The fracture of the root and the stolon is granular and fibrous. The cork layer is thin; the secondary phloem region is thick and light yellow with radial striations. The yellow xylem cylinder is compact, with a radiate structure. The stolon has a central pith, which is absent from the root. The external part of the bark is absent from the peeled root.

B. Microscopic examination (*2.8.23*). The powder is light yellow or faintly greyish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of yellow thick-walled fibres, 700-1200 µm long and 10-20 µm wide with a punctiform lumen, often accompanied by crystal sheaths containing prisms of calcium oxalate 10-35 µm long and 2-5 µm wide. The walls of the vessels are yellow, 5-10 µm thick, lignified and have numerous bordered pits with a slit-shaped aperture; fragments of cork consisting of thin-walled cells and isolated prisms of calcium oxalate occur as well as fragments of parenchymatous tissue. Fragments of cork are absent from the peeled root. Examine under a microscope using a mixture of equal volumes of *glycerol R* and *water R*. The powder shows the following diagnostic characters: simple, round or oval starch granules, 2-20 µm in diameter.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.50 g of the powdered herbal drug (180) (*2.9.12*) in a 50 mL round-bottomed flask add 16.0 mL of *water R* and 4.0 mL of *hydrochloric acid R1* and heat on a water-bath under a reflux condenser for 30 min. Cool and filter. Dry the filter and the round-bottomed flask at 105 °C for 60 min. Place the filter in the round-bottomed flask, add 20.0 mL of *ether R* and heat in a water-bath at 40 °C under a reflux condenser for 5 min. Cool and filter. Evaporate the filtrate to dryness. Dissolve the residue in 5.0 mL of *ether R*.

*Reference solution.* Dissolve 5.0 mg of *glycyrrhetic acid R* and 5.0 mg of *thymol R* in 5.0 mL of *ether R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethanol (96 per cent) R*, *ethyl acetate R* (1:9:25:65 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air for 5 min.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms obtained with the test solution and the reference solution show in the lower half a quenching zone due to glycyrrhetic acid.

*Detection B*: treat with *anisaldehyde solution R*, and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results B*: the chromatogram obtained with the reference solution shows in the lower half a violet zone due to glycyrrhetic acid and in the upper third a red zone due to thymol. The chromatogram obtained with the test solution shows in the lower half a violet zone corresponding to the zone of glycyrrhetic acid in the chromatogram obtained with the reference solution and a yellow zone (isoliquiridigenine) in the upper third under the zone of thymol in the chromatogram obtained with the reference solution. Further zones may be present.

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent for the unpeeled drug and maximum 6.0 per cent for the peeled drug.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent for the unpeeled drug and maximum 0.5 per cent for the peeled drug.

**Ochratoxin A** (*2.8.22*): maximum 20 µg per kilogram of herbal drug.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Place 1.000 g of the powdered herbal drug (180) (*2.9.12*) in a 150 mL ground-glass conical flask. Add 100.0 mL of an 8 g/L solution of *ammonia R* and treat in an ultrasonic bath for 30 min. Centrifuge a part of the solution and dilute 1.0 mL of the supernatant layer to 5.0 mL with an 8 g/L solution of *ammonia R*. Filter through a membrane filter (nominal pore size 0.45 µm); use the filtrate as the test solution.

*Solution A.* Dissolve 0.130 g of *monoammonium glycyrrhizate CRS* in an 8 g/L solution of *ammonia R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 5.0 mL of solution A to 100.0 mL with an 8 g/L solution of *ammonia R*.

*Reference solution (b).* Dilute 10.0 mL of solution A to 100.0 mL with an 8 g/L solution of *ammonia R*.

*Reference solution (c).* Dilute 15.0 mL of solution A to 100.0 mL with an 8 g/L solution of *ammonia R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (6:30:64 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

Establish a calibration curve with the mass of monoammonium glycyrrhizate in the reference solutions, in grams, as the abscissa and the corresponding peak areas as the ordinate.

Using the retention times and the peak areas determined from the chromatograms obtained with the reference solutions, locate and integrate the peak due to 18β-glycyrrhizic acid in the chromatogram obtained with the test solution.

Calculate the percentage content of 18β-glycyrrhizic acid using the following expression:

$$A \times \frac{5}{m} \times B \times \frac{823}{840}$$

$A$ = mass equivalent of monoammonium glycyrrhizate in the test solution, determined from the calibration curve, in grams;

$B$ = declared percentage content of *monoammonium glycyrrhizate CRS*;

$m$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

823 = molecular mass of 18β-glycyrrhizic acid;

840 = molecular mass of monoammonium glycyrrhizate (without any water of crystallisation).

LABELLING

The label states whether the drug is peeled or unpeeled.

**07/2014:2453**
**corrected 10.0**

# LONG PEPPER

## Piperis longi fructus

DEFINITION

Dried, ripe or nearly ripe fruiting spikes of *Piper longum* L. or *Piper retrofractum* Vahl (syn. *P. chaba* Hunter and *P. officinarum* (Miq.) C. DC.) or a mixture of both species.

*Content*:

– *essential oil*: minimum 6.0 mL/kg (dried drug);

– *piperine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): minimum 3.0 per cent (dried drug).

IDENTIFICATION

A. *P. longum*. The fruiting spikes are cylindrical or irregularly cylindrical, 1-2.5 cm long (rarely longer than 2.5 cm), 3-5 mm in diameter, blackish-brown or almost black. The spikes are quite compact, tough, composed of small fruits firmly fixed on the receptacle in regular or oblique rows. The berries are spherical, about 1 mm in diameter. The bracts are black, small, punctiform, confined to depressions between adjacent berries. The remains of the peduncle may be present at the base of the cylinder. Spikes can be easily broken; the fracture is irregular and granular.

*P. retrofractum*. The fruiting spikes are similar to those of *P. longum* but clearly more robust, straight and cylindrical, 2.5-4 cm long (rarely smaller than 2.5 cm), 5-8 mm in diameter, brown or reddish-brown. The berries are also firmly fixed on the receptacle but, in contrast to those of *P. longum*, arranged more obviously in spiral rows. The bracts are more prominent than those of *P. longum*.

B. Microscopic examination (*2.8.23*). The powder is grey or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2453.-1): fragments of the endocarp (surface view [A]), consisting of sclereids, more or less elongated, about 75 µm long, pitted, with irregularly thickened walls and wide channels [Aa], associated with the reddish-brown pigmented layer of the testa with indistinct cells [Ab] and also associated with the inner testa with rectangular, brown, thin-walled cells [Ac]; fragments of the endocarp (transverse section [D]), showing sclereids with irregularly thickened inner walls on the 3 lower sides [Da], usually associated with the testa (namely the pigmented layer with indistinct cells [Db] and the layer of the inner testa [Dc]); fragments of the parenchyma of the mesocarp [B, C] containing more or less polygonal sclereids, isolated [Ba] or in groups [Bb], and oil cells about 50 µm in diameter [Ca]; numerous ovoid or polygonal cells of the parenchyma of the seed [Dd]; fragments of the epicarp [F] with thin-walled cells; rare fragments from the centre of the spike [E], consisting of parenchymatous cells [Eb] and elongated sclereids up to 400 µm long [Ea]; a few fragments of vascular tissue [H] with spiral or striated vessels [Ha] and fibres [Hb]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. Starch is visible as rounded, compound granules, each about 20 µm in diameter [Ga], made up of tiny individual granules, ovoid or polyhedral by compression, free [Gb] or included in the parenchymatous cells of the seed [G].

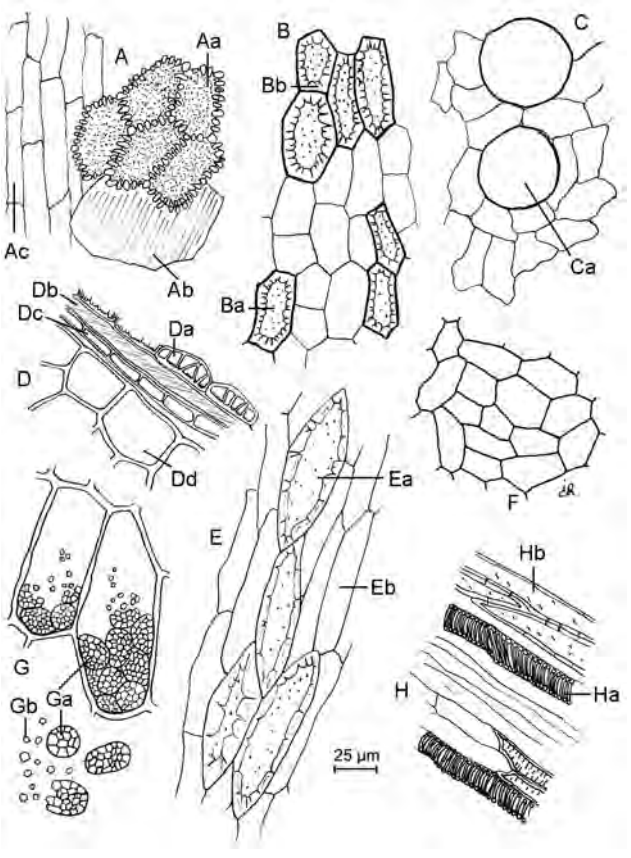


Figure 2453.-1. – *Illustration for identification test B of powdered herbal drug of long pepper*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min, centrifuge and use the supernatant.

*Reference solution*. Dissolve 10 mg of *borneol R* and 15 mg of *piperine R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

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*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (30:50 *V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | 2 strong quenching zones |
| | A quenching zone |
| ——— | ——— |
| | A quenching zone |
| Piperine: a quenching zone | A strong quenching zone (piperine) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A purple-grey zone |
| ——— | ——— |
| | A purple zone |
| Borneol: a yellowish-brown zone | A violet zone |
| | A purple-grey zone |
| ——— | ——— |
| Piperine: a green or brownish zone | A green or brownish zone (piperine) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the freshly powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 25.0 g of the freshly powdered herbal drug (1400) (*2.9.12*), a 1000 mL round-bottomed flask, 400 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 3 h.

**Piperine**. Liquid chromatography (*2.2.29*). *Carry out the assay protected from light.*

*Test solution.* Disperse 0.250 g of the powdered herbal drug (355) (*2.9.12*) in 40 mL of *ethanol (96 per cent) R*. Sonicate for 20 min and filter. Rinse the flask and the filter with 5 mL of *ethanol (96 per cent) R*, combine the filtrate and washings and dilute to 50.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 15.0 mg of *piperine CRS* in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Disperse 0.250 g of *long pepper for system suitability HRS* (355) (*2.9.12*) in 40 mL of *ethanol (96 per cent) R*. Sonicate for 20 min and filter. Rinse the flask and the filter with 5 mL of *ethanol (96 per cent) R*, combine the filtrate and washings and dilute to 50.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 50 | 50 |
| 5 - 20 | 50 → 5 | 50 → 95 |
| 20 - 22 | 5 → 0 | 95 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 343 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *long pepper for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to piperine and peak 2.

*Retention time*: piperine = about 10 min.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of peak 2 and $H_v$ = height above the baseline of the lowest point of the curve separating the peak due to piperine from peak 2.

Calculate the percentage content of piperine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to piperine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to piperine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *piperine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of piperine in *piperine CRS*.

**04/2013:1537**

# LOOSESTRIFE

## Lythri herba

DEFINITION

Dried flowering tops, whole or cut, of *Lythrum salicaria* L.

*Content*: minimum 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The stems are rigid, 4-angled, branching at the top, brownish-green, longitudinally wrinkled and pubescent. The leaves are opposite, decussate, rarely verticillate in threes and sometimes alternate at the inflorescence which forms a long terminal spike. The leaves are sessile, lanceolate and cordate at the base, 5-15 cm long and 1-2.5 cm wide, pubescent on the lower surface; the subsidiary veins form arcs that anastomose near the leaf margin. The flowers have a pubescent, tubular, persistent gamosepalous calyx, 4-8 mm long, consisting of 6 sepals bearing 6 small, triangular teeth alternating with 6 large, acute teeth at least half as long as the tube; a polypetalous corolla consisting of 6 violet-pink petals, each expanded at the top with a wavy outline and narrowing at the base. The androecium consists of 2 verticils of 6 stamens (1 verticil with short, barely emerging stamens, the other with long stamens extending well out of the corolla). The fruit, if formed, is a small capsule included in the persistent calyx.

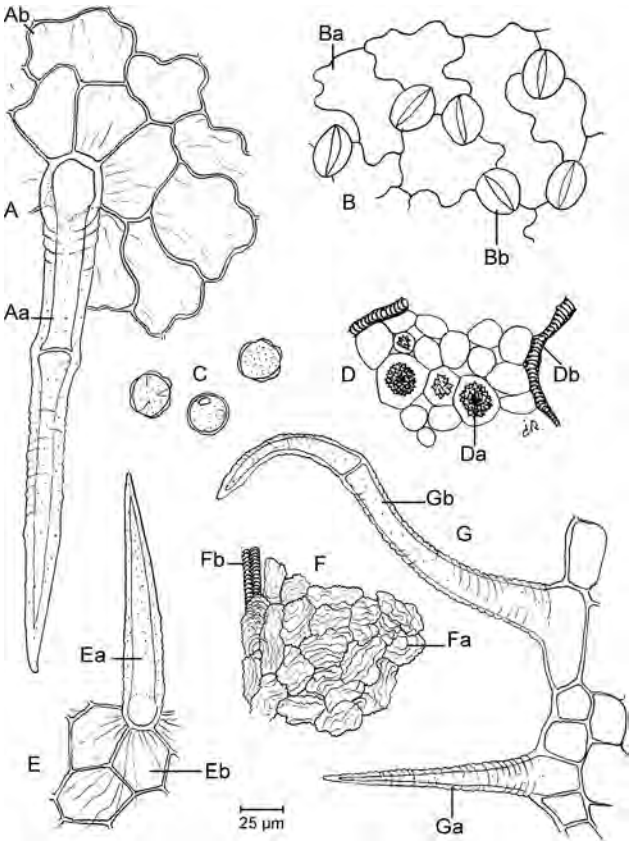


Figure 1537.-1. – *Illustration for identification test B of powdered herbal drug of loosestrife*

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1537.-1): unicellular [Ea] or bicellular [Aa], uniseriate, thick-walled, finely pitted covering trichomes from the epidermis of the leaf [A] and stem [E]; numerous uniseriate, unicellular [Ga] or bicellular [Gb], thin-walled, finely pitted, annularly striated covering trichomes from the calyx (side view [G]); transparent violet-pink fragments from the petals [F] consisting of epidermal cells with sinuous walls and a grainy cuticle [Fa], covering fine spiral vessels [Fb]; fragments of parenchyma from the leaf [D] with numerous cells containing cluster crystals of calcium oxalate [Da], associated with spiral vessels [Db]; pollen grains with 3 pores and a thin and slightly granular exine [C]; fragments of the upper epidermis of the leaf [A] with large polygonal cells and sinuous walls, covered by a finely striated cuticle [Ab]; fragments of the lower epidermis of the leaf [B] with smaller polygonal cells [Ba] and anomocytic stomata [Bb] (*2.8.3*); fragments of the stem [E] consisting of polygonal cells with straight anticlinal walls and a striated cuticle [Eb].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and heat in a water-bath at 65 °C for 5 min with frequent shaking. Cool and filter. Dilute the filtrate to 10 mL with *methanol R*.

*Reference solution*. Dissolve 0.5 mg of *chlorogenic acid R*, 1 mg of *hyperoside R*, 1 mg of *rutoside trihydrate R* and 1 mg of *vitexin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: treat the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower third a yellowish-brown fluorescent zone due to rutoside and in the middle third a light blue fluorescent zone due to chlorogenic acid, above it a yellowish-brown fluorescent zone due to hyperoside and a green fluorescent zone due to vitexin. The chromatogram obtained with the test solution shows a bright green fluorescent zone slightly above the zone due to rutoside in the chromatogram obtained with the reference solution, a yellow fluorescent zone similar in position to the zone due to chlorogenic acid in the chromatogram obtained with the reference solution, a yellow fluorescent zone similar in position to the zone due to hyperoside in the chromatogram obtained with the reference solution, and a bright green fluorescent zone corresponding to the zone due to vitexin in the chromatogram obtained with the reference solution.

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.750 g of the powdered herbal drug (180) (*2.9.12*).

**01/2013:1233**

# LOVAGE ROOT

## Levistici radix

DEFINITION

Whole or cut, dried rhizome and root of *Levisticum officinale* Koch.

*Content*: minimum 4.0 mL/kg of essential oil for the whole drug and minimum 3.0 mL/kg of essential oil for the cut drug (dried drug).

IDENTIFICATION

A. The rhizome and the large roots are often split longitudinally. The rhizome is short, up to 5 cm in diameter, light greyish-brown or yellowish-brown, simple or with several protuberances; the roots, showing little ramification, are the same colour as the rhizome; they are usually up to 1.5 cm thick and up to about 25 cm long; the fracture is usually smooth and shows a very wide yellowish-white bark and a narrow brownish-yellow wood.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: cork cells, polygonal or rounded in surface view, with brown contents; abundant parenchyma, mostly thin-walled and rounded but some with thicker walls; groups of small, reticulately thickened vessels embedded in small-celled, unlignified parenchyma; fragments of larger vessels with reticulate thickening, up to 125 µm in diameter; fragments of secretory canals up to 180 µm wide. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows starch granules, simple, rounded or ovoid, up to about 12 µm, and numerous larger, compound granules, many with several components.

C. Examine the chromatograms obtained in the test for species of *Angelica* and *Ligusticum* described in the European Pharmacopoeia.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish-white fluorescent zone | A bluish-white fluorescent zone |
| _____ | _____ |
| Osthole: a blue fluorescent zone | |
| Imperatorin: a whitish fluorescent zone | A weak whitish fluorescent zone |
| _____ | _____ |
| | A weak whitish fluorescent zone |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| (*Z*)-Ligustilide: a bluish fluorescent zone | A bluish fluorescent zone |
| _____ | _____ |
| Osthole: a quenching zone | A weak quenching zone |
| Imperatorin: a quenching zone | |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Results C*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 prominent reddish zones |
| (*Z*)-Ligustilide: a grey zone | A grey zone |
| _____ | _____ |
| Osthole: a violet zone | |
| Imperatorin: a grey zone | |
| _____ | _____ |
| | 2 purple zones |
| | A distinct brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Species of *Angelica* and *Ligusticum* described in the European Pharmacopoeia**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the freshly powdered herbal drug (355) (*2.9.12*) add 4 mL of *heptane R* and sonicate for 5 min. Centrifuge the mixture and use the supernatant.

*Reference solution*. Dissolve 1 mg of *imperatorin R*, 1 mg of *(Z)-ligustilide R* and 1 mg of *osthole R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL, as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no blue fluorescent zone just below or above the zone due to imperatorin in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm.

*Results B*: the chromatogram obtained with the test solution shows no zone at or just below the zone due to imperatorin in the chromatogram obtained with the reference solution.

*Detection C*: treat the plate with a solution of 20 mL of *sulfuric acid R* in 180 mL of ice-cooled *methanol R*; heat at 100-105 °C for 5 min and examine in daylight.

*Results C*: the chromatogram obtained with the test solution shows no purple zone between the 2 reddish zones at the top of the chromatogram and the zone due to (*Z*)-ligustilide in the chromatogram obtained with the reference solution; the chromatogram obtained with the test solution shows no purple zone between the zones due to (*Z*)-ligustilide and osthole in the chromatogram obtained with the reference solution.

**Foreign matter** (*2.8.2*): maximum 3 per cent, determined on 50 g.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use a 2 L flask, 10 drops of *liquid paraffin R*, 500 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Reduce the herbal drug to a powder (500) (*2.9.12*) and immediately use 40.0 g for the determination. Distil at a rate of 2-3 mL/min for 4 h.

04/2017:2723

# LYCOPUS LUCIDUS HERB

## Lycopi herba

DEFINITION

Whole or fragmented, dried aerial parts of *Lycopus lucidus* var. *hirtus* (Regel) Makino & Nemoto.

*Content*: minimum 0.15 per cent of rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (dried drug).

IDENTIFICATION

A. *Whole drug.* The stems are square in cross-section with a hollowed pith and few branches, 50-100 cm long and 2-6 mm in diameter; the greenish-yellow or reddish outer surface shows, on each side, shallow longitudinal furrows and reddish nodes covered in white hairs. The leaves are opposite, with short petioles or almost sessile; when whole, the lamina, usually crumpled, is lanceolate or oblong, 5-10 cm long, with dentate margins, an acute apex and a slightly decurrent base, and is pubescent on both surfaces; the upper surface is dark green or blackish-green while the lower surface is greyish-green, and densely dotted with glandular trichomes; the inflorescence is grouped in axillary whorls and consists only of persistent calyces and leaf-like bracts, as the corollas have usually fallen off. The bracts are lanceolate with hairs on the margins. The calyx is hairy, and 2-lipped with 5 teeth. The texture is fragile.

*Fragmented drug.* It occurs in various sizes 1-3 cm long. The fragments of the stems are flattened, angular, longitudinally striated and have a hollowed whitish pith; some fragments have nodes covered in white hairs; the fragments of the leaves often show the dentate margin of the lamina, and prominent veins on the lower surface; some fragments have inflorescences in axillary whorls, reduced to brown 2-lipped calyces.

B. Microscopic examination (*2.8.23*). The powder is dark green or brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2723.-1): fragments of the upper epidermis of the leaves (surface view [B]) consisting of cells usually with rigid walls [Ba] bearing covering trichomes and glandular trichomes; the covering trichomes have spiny walls, some are unicellular, short and conical [Bb], others are uni- [C, E] or multicellular (2-5 cells) [G], occasionally remaining only as circular, thickened scars on the epidermis [F]; the glandular trichomes, rarer, have a unicellular stalk and an ovoid head, either uni- or bicellular [Bc], sometimes associated with loosely arranged palisade parenchyma [Bd]; fragments of the lower epidermis of the lamina (surface view [A]) covered by a striated cuticle composed of cells with sinuous walls [Aa], diacytic stomata [Ab], uni- or multicellular covering trichomes similar to those of the upper epidermis, mainly visible on the veins, and numerous glandular trichomes, sometimes with a unicellular stalk and a unicellular head ([Ac], isolated side view [D]), or a unicellular stalk and a bicellular head [Ad], or a unicellular stalk and an octocellular head of laminaceous type [Ae] about 60-80 µm in diameter; bundles of long fibres [H, K] from the stems, with thick walls [Ha, Ka], sometimes associated with bordered-pitted vessels [Hb] or parenchymatous cells of the pith [Kb]; frequent sclereids [J], usually isolated [Ja, Jb] or in small groups [Jc], ovoid to rectangular, with varying thickened and channelled walls, from the pith near the nodes.

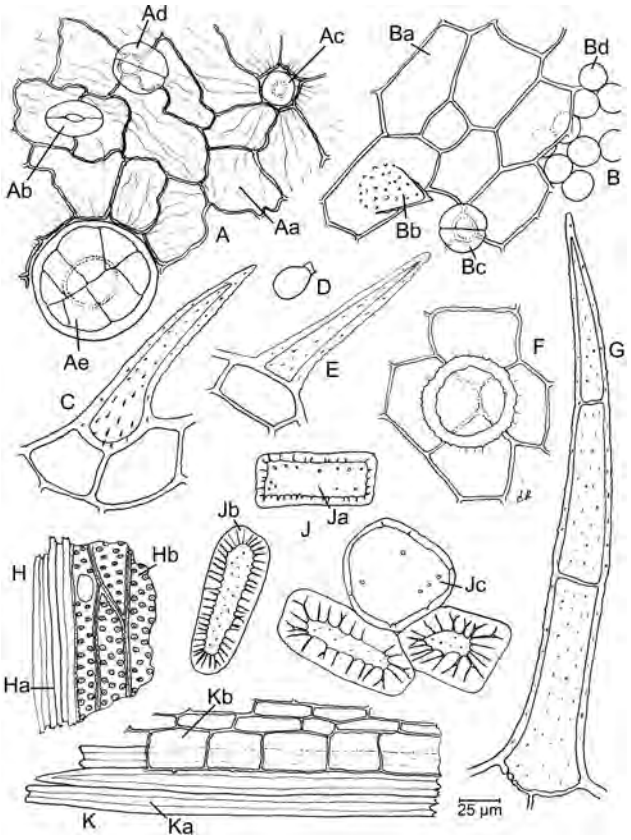


Figure 2723.-1. – *Illustration for identification test B of powdered herbal drug of lycopus lucidus herb*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *ethyl acetate R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1.0 mg of *luteolin R* and 1.0 mg of *ursolic acid R* in 2.0 mL of *ethyl acetate R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *methylene chloride R*, *ethyl acetate R*, *cyclohexane R* (1:5:8:20 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *2.5 M alcoholic sulfuric acid R* and heat at 110 °C for 3 min. Examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A pale violet zone |
| _____ | _____ |
| | A reddish-violet zone |
| Ursolic acid: a violet zone | A violet zone (ursolic acid) |
| _____ | _____ |
| | 2 dark violet zones |
| Luteolin: a yellow zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (355) (*2.9.12*) add 25.0 mL of a 70 per cent *V/V* solution of *methanol R* and weigh. Boil on a water-bath under a reflux condenser for 30 min. Allow to cool and weigh again. Compensate for the loss of solvent with a 70 per cent *V/V* solution of *methanol R* and mix. Filter the solution and dilute 2.0 mL of the filtrate to 10.0 mL with a 70 per cent *V/V* solution of *methanol R*. Filter this solution through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *rosmarinic acid CRS* in 20.0 mL of a 70 per cent *V/V* solution of *methanol R*. Dilute 1.0 mL of this solution to 25.0 mL with a 70 per cent *V/V* solution of *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *ferulic acid R* in 25 mL of a 70 per cent *V/V* solution of *methanol R*. Dilute 1 mL of this solution to 10.0 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *acetonitrile R*, *water for chromatography R* (1:19:80 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *methanol R*, *acetonitrile R* (1:40:59 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 70 | 0 → 30 |
| 20 - 25 | 70 → 0 | 30 → 100 |
| 25 - 30 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to rosmarinic acid.

*Retention time*: ferulic acid = about 10 min; rosmarinic acid = about 14 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to ferulic acid and rosmarinic acid.

Calculate the percentage content of rosmarinic acid using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 4}$$

$A_1$ = area of the peak due to rosmarinic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rosmarinic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rosmarinic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rosmarinic acid in *rosmarinic acid CRS*.

**01/2018:2742**

# MAGNOLIA BIONDII FLOWER BUD

## Magnoliae biondii flos immaturus

DEFINITION

Whole, dried flower bud of *Magnolia biondii* Pamp. (syn. *Yulania biondii* (Pamp.) D.L.Fu).

*Content*:

- *essential oil*: minimum 14.0 mL/kg (anhydrous drug);
- *magnolin* ($C_{23}H_{28}O_7$; $M_r$ 416.5): minimum 3.0 per cent (anhydrous drug).

IDENTIFICATION

A. The flower bud, whose texture is light and fragile, is ovoid to elongated-ovoid, whitish-grey, yellowish-grey or greenish-grey, silky, pubescent, with a pointed apex; 1.2-3.0 cm long and 8-16 mm in diameter. The ligneous, short peduncle, covered in whitish lenticels, is usually present at the base of the flower bud. The flower bud is surrounded by 2-3 whorls of bracts: each bract has an outer surface densely covered in silky hairs, and a glabrous, brownish inner surface. The perianth consists of 9 light yellow or brownish tepals arranged in 3 whorls; the outer whorl consists of 3 sepaloid tepals, linear, whose length is about a quarter of that of the other tepals; the petaloid tepals are arranged in 2 inner whorls, each consisting of 3 tepals. The stamens and carpels are numerous, arranged in a spiral.

B. Microscopic examination (*2.8.23*). The unsieved powder is greenish to brownish-grey, dark-brown or slightly greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2742.-1): isolated and fragmented, multicellular (2-6 cells when whole) covering trichomes [B, C, D], whose cells have thick walls, often with spiral striations [Ba]; their bases are enlarged and consist of cells with very thick walls like sclereids, with granular orange-brown contents [Bb]; numerous sclereids, more or less branched, isolated [F] or in groups [E], with very thick, striated, channelled walls, up to 60 µm in diameter; fragments of perianth segments (surface view [A]; sectional view [G]) covered by a cuticle [Ga] and composed of polyhedral epidermal cells [Aa, Gb], paracytic stomata

Herbal drugs

(*2.8.3*) [Ab] and parenchyma consisting of rounded cells [Ad, Gc] and including rounded oil cells (up to 100 µm in diameter) with orange-yellow contents [Ac, Gd].

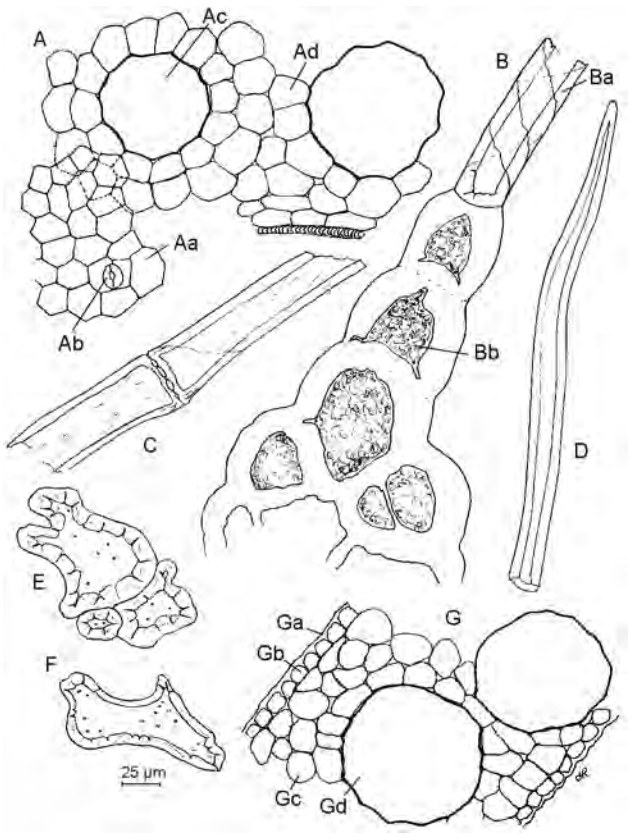


Figure 2742.-1. – *Illustration for identification test B of powdered herbal drug of Magnolia biondii flower bud*

C. Thin-layer chromatography (*2.2.27*) as described in the test for *Magnolia officinalis* Rehder & E.H.Wilson with the following modifications.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Fargesin: a dark blue fluorescent zone | A dark blue fluorescent zone (fargesin) |
| _____ | _____ |
| Magnolin: a quenching zone | A quenching zone (magnolin) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *vanillin reagent R*, heat at 100-105 °C for 5-7 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint violet zone |
| _____ | _____ |
| Fargesin: a brown zone | A brown zone (fargesin) |
| | A dark blue zone |
| _____ | _____ |
| | A faint pinkish-violet zone |
| Magnolin: a pinkish-violet zone | A pinkish-violet zone (magnolin) |
| **Reference solution** | **Test solution** |

TESTS

***Magnolia officinalis* Rehder & E.H.Wilson**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Reduce the herbal drug to a powder using a blade grinder to prevent heating. To 0.5 g of the powdered herbal drug add 3.0 mL of *methanol R*. Sonicate for 15 min and centrifuge for 15 min. Transfer the supernatant to a 5 mL flask. Add 2 mL of *methanol R* to the residue, sonicate for 15 min and centrifuge. Transfer the supernatant into the same 5 mL flask. Dilute the combined supernatants to 5.0 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm) if necessary.

*Reference solution*. Dissolve 2.0 mg of *fargesin R* and 2.0 mg of *magnolin R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *methanol R*, *ethyl acetate R*, *toluene R* (1:5:30 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: treat with *vanillin reagent R*, heat at 100-105 °C for 5-7 min and examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no pinkish zone (due to magnolol) just below the zone due to fargesin in the chromatogram obtained with the reference solution.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 25.0 g of the finely cut herbal drug.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 15.0 g of freshly, finely cut herbal drug, a 1 L round-bottomed flask, 300 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 3 h.

**Magnolin**. Liquid chromatography (*2.2.29*).

*Test solution*. Reduce the herbal drug to a powder using a blade grinder to prevent heating. To 0.250 g of the powdered herbal drug add 35 mL of *methanol R*. Sonicate for 1 h, changing the water of the ultrasonic bath after 30 min of sonication to prevent heating. Centrifuge for 15 min. Transfer the supernatant to a 100 mL volumetric flask. Repeat the extraction twice, adding to the residue 35 mL of *methanol R* the first time and 20 mL of *methanol R* the second time. Combine the supernatants, cool, and dilute to 100.0 mL with *methanol R*. Dilute 1.0 mL of the solution to 2.0 mL with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water for chromatography R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *magnolin CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 5.0 mL with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water for chromatography R*.

*Reference solution (b).* To 0.250 g of *magnolia biondii flower for system suitability HRS* add 35 mL of *methanol R*. Sonicate for 1 h, changing the water of the ultrasonic bath after 30 min of sonication to prevent heating. Centrifuge for 15 min. Transfer the supernatant to a 100 mL volumetric flask. Repeat the extraction twice, adding to the residue 35 mL of *methanol R* the first time and 20 mL of *methanol R* the second time. Combine the supernatants, cool, and dilute to 100.0 mL with *methanol R*. Dilute 1.0 mL of the solution to 2.0 mL with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water for chromatography R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *anhydrous acetic acid R*, *acetonitrile R*, *water for chromatography R* (0.1:14:85.9 *V/V/V*);
- *mobile phase B*: *anhydrous acetic acid R*, *water for chromatography R*, *acetonitrile R* (0.1:4.9:95 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 68 | 32 |
| 12 - 20 | 68 → 0 | 32 → 100 |
| 20 - 28 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *magnolia biondii flower for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to eudesmin; use the chromatogram obtained with reference solution (a) to identify the peak due to magnolin.

*Retention time*: eudesmin = about 12 min; magnolin = about 13 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to eudesmin and magnolin.

If necessary, dilute the test solution with a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water for chromatography R* to obtain a peak due to magnolin with a height that is similar to or smaller than the height of the corresponding peak in the chromatogram obtained with reference solution (a). Take into account the additional dilution factor *d*.

Calculate the percentage content of magnolin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 4 \times d}{A_2 \times m_1}$$

$A_1$ = area of the peak due to magnolin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to magnolin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *magnolin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of magnolin in *magnolin CRS*;

$d$ = additional dilution factor.

**04/2014:2567**
**corrected 9.2**

# MAGNOLIA OFFICINALIS BARK

## Magnoliae officinalis cortex

DEFINITION

Dried bark from the stem and branch of *Magnolia officinalis* Rehder et E.H. Wilson.

*Content*: minimum 2.0 per cent for the sum of magnolol ($C_{18}H_{18}O_2$; $M_r$ 266.3) and honokiol ($C_{18}H_{18}O_2$; $M_r$ 266.3) (dried drug).

IDENTIFICATION

A. Fragments of stem and branch bark, quilled singly or double quilled, about 30 cm long and 2-7 mm thick. The outer surface is brownish-grey, rough, sometimes scaly, easily exfoliated, with distinct lenticels and longitudinal striations. The inner surface is reddish-brown or dark brown, smooth, with numerous fine, longitudinal striations. The texture is hard and difficult to break. The fracture is granular, brownish-grey in the outer layers and reddish-brown or dark brown in the inner layers.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2567.-1): numerous sclereids [D] of various shapes and sizes, free or in groups, often branched, up to 100 µm long, with very thick, striated walls and conspicuous pit canals; oval [F] or rounded [G] oil cells, up to 100 µm in diameter, with orange-yellow contents; narrow, thick-walled fibres, often in bundles [C, H], accompanied by fusiform medullary rays (tangential section [Ca]) or consisting of files of rectangular cells (longitudinal section [Ha]); brown cork fragments with regularly and finely thickened walls [B]; fragments of phloem parenchyma [A] consisting of cells with irregular walls [Aa], accompanied by fusiform medullary rays (tangential section [Ab]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rounded, ovoid or polyhedral starch granules, about 2-12 µm in diameter, simple or 2-6 compound, free [J] or included in parenchyma cells [E].

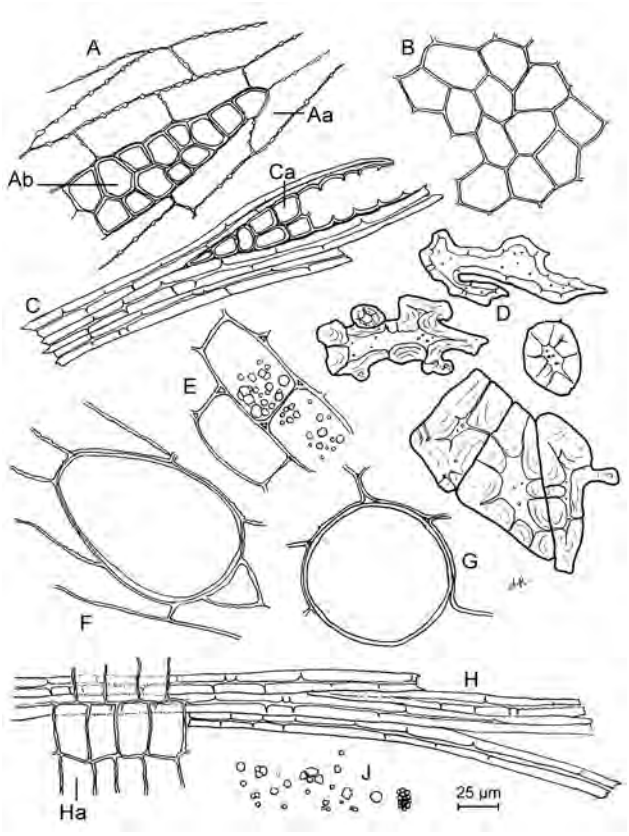


Figure 2567.-1. – *Illustration for identification test B of powdered herbal drug of Magnolia officinalis bark*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Reduce to a powder (355) (*2.9.12*), avoiding heating. To 0.5 g of the powdered herbal drug add 5 mL of *methanol R*, sonicate for 5 min, centrifuge, and use the supernatant. Filter through a membrane filter (nominal pore size 0.45 µm) if necessary.

*Reference solution.* Dissolve 1 mg of *honokiol R*, 1 mg of *magnolol R* and 2 mg of *eugenol R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *methanol R*, *ethyl acetate R*, *toluene R* (4:8:120 *V/V/V*).

*Application*: 5 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 7 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Eugenol: a faint quenching zone | |
| Magnolol: a dark blue fluorescent zone | A dark blue fluorescent zone (magnolol) |
| Honokiol: a quenching zone | A quenching zone (honokiol) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *vanillin reagent R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A bluish-violet zone |
| _____ | _____ |
| Eugenol: a brown zone | |
| Magnolol: a pinkish-violet zone | A pinkish-violet zone (magnolol) |
| Honokiol: a dark violet zone | A dark violet zone (honokiol) |
| | A bluish-violet zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*) add 80 mL of *methanol R* and heat in a water-bath under a reflux condenser for 30 min. Cool, then dilute to 100.0 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 4.0 mg of *honokiol CRS* and 4.0 mg of *magnolol CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 2.0 mg of *honokiol CRS* in 2.0 mL of *acetonitrile R*. To 1.0 mL of the solution add 15 µL of *acetic anhydride R* and mix. Heat at 50 °C for 60 min. Cool. Add successively, mixing after each addition, 16 µL of *concentrated ammonia R*, 1.0 mL of *acetonitrile R* and 2.0 mL of *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: 0.5 per cent *V/V* solution of *acetic acid R*, *acetonitrile for chromatography R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of honokiol for the test solution and reference solution (a); 3 times the retention time of honokiol for reference solution (b).

*Relative retention* with reference to honokiol (retention time = about 8 min): magnolol = about 1.4; honokiol monoacetate isomer 1 = about 1.5; honokiol monoacetate isomer 2 = about 1.6; honokiol diacetate = about 2.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to honokiol monoacetate isomers 1 and 2.

Calculate the sum of the percentage contents of honokiol and magnolol using the following expression:

$$\frac{A_1 \times m_2 \times p_1 \times 5}{A_2 \times m_1} + \frac{A_3 \times m_3 \times p_2 \times 5}{A_4 \times m_1}$$

$A_1$ = area of the peak due to honokiol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to honokiol in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to magnolol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to magnolol in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *honokiol CRS* used to prepare reference solution (a), in grams;

$m_3$ = mass of *magnolol CRS* used to prepare reference solution (a), in grams;

$p_1$ = percentage content of honokiol in *honokiol CRS*;

$p_2$ = percentage content of magnolol in *magnolol CRS*.

**04/2013:2568**
**corrected 9.3**

# MAGNOLIA OFFICINALIS FLOWER

## Magnoliae officinalis flos

DEFINITION

Steamed and dried, unopened flower of *Magnolia officinalis* Rehder et E.H. Wilson.

*Content*: minimum 0.20 per cent of the sum of magnolol ($C_{18}H_{18}O_2$; $M_r$ 266.3) and honokiol ($C_{18}H_{18}O_2$; $M_r$ 266.3) (dried drug).

IDENTIFICATION

A. The greyish-yellow pedicel is short (0.5-2 cm) and densely tomentose. The brown or reddish-brown flower bud is elongated, conical, 4-7 cm long and 1.5-2.5 cm in diameter at the base; it usually consists of 12 perianth segments in several whorls. The stamens are numerous with a fine, short filament and a linear, yellowish-brown anther. The carpels are free and numerous, spirally arranged on a conical receptacle.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the perianth segments with polyhedral or elliptical epidermal cells, with irregularly thickened walls and anomocytic stomata (4-6 subsidiary cells) (*2.8.3*), accompanied by parenchyma that includes oval or rounded oil cells about 50 µm in diameter with orange-yellow contents; certain fragments contain epidermal cells with rounded papillae; numerous, branched sclereids, with channelled walls and a large lumen, about 15 µm in diameter; numerous elliptical pollen grains about 50 µm long and 40 µm wide, with a smooth exine.

C. Examine the chromatograms obtained in the test for other *Magnolia* species.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Eugenol: a faint quenching zone | |
| Magnolol: a dark blue fluorescent zone | A dark blue fluorescent zone (magnolol) |
| Honokiol: a quenching zone | A quenching zone (honokiol) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *vanillin reagent R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A bluish-violet zone |
| Eugenol: a brown zone | |
| Magnolol: a pinkish-violet zone | A pinkish-violet zone (magnolol) |
| Honokiol: a dark violet zone | A dark violet zone (honokiol) |
| | A bluish-violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Other *Magnolia* species**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Reduce the herbal drug to a powder (710) (*2.9.12*), avoiding heating. To 0.5 g of the powdered herbal drug add 2.5 mL of *methanol R*. Sonicate for 15 min at a power of 80 W and a frequency of 37 kHz (sonication time may be adapted according to the power and frequency used), then centrifuge at 1500-2000 *g* for 10 min and transfer the supernatant to a 5 mL flask. Add 2 mL of *methanol R* to the residue, sonicate for 15 min and centrifuge. Transfer the supernatant into the same 5 mL flask. Dilute to 5 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm) if necessary.

*Reference solution*. Dissolve 1 mg of *honokiol R*, 1 mg of *magnolol R* and 2 mg of *eugenol R* in 4 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *methanol R*, *ethyl acetate R*, *toluene R* (1:5:30 *V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 7 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows no blue fluorescent zone in the lower part of the plate and no green fluorescent zone in the upper part, nor any other fluorescent zone.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Reduce the herbal drug to a powder (710) (*2.9.12*) using a blade grinder equipped with a double-walled grinding chamber cooled to a temperature of about 10 °C. To 0.500 g of the powdered herbal drug add 10 mL of *methanol R*. Sonicate for 1 h at a power of 80 W and a frequency of 37 kHz (sonication time may be adapted according to the power and frequency used). Change the water of the ultrasonic bath after 30 min of sonication to prevent heating. Centrifuge at 1500-2000 *g* for 15 min. Transfer the supernatant to a 20.0 mL flask. Add 9.5 mL of *methanol R* to the residue. Repeat the sonication for 1 h. Change the water of the ultrasonic bath after 30 min of sonication to prevent heating. Centrifuge. Transfer the supernatant to the same 20.0 mL flask. Cool, then dilute to 20.0 mL with *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *honokiol CRS* in *methanol R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 6.0 mg of *magnolol CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (c).* Dissolve 2.0 mg of *honokiol R* in 2.0 mL of *acetonitrile R*. Add 30 µL of *acetic anhydride R* and mix. Heat at 50 °C for 60 min. Cool. Add successively, mixing after each addition, 32 µL of *concentrated ammonia R*, 2.0 mL of *acetonitrile R* and 4.0 mL of *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 25 ± 2 °C.

*Mobile phase*:

- *mobile phase A*: *anhydrous formic acid R*, *water R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 47 | 53 |
| 20 - 22 | 47 → 5 | 53 → 95 |
| 22 - 27 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 292 nm.

*Injection*: 20 µL.

*Relative retention* with reference to honokiol (retention time = about 10 min): magnolol = about 1.3; honokiol monoacetate isomer 1 = about 1.4; honokiol monoacetate isomer 2 = about 1.5; honokiol diacetate = about 1.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to honokiol monoacetate isomers 1 and 2.

If necessary, dilute the test solution to obtain peaks of honokiol and magnolol that are similar in height to the corresponding peaks in reference solutions (a) and (b).

Calculate the sum of the percentage contents of honokiol and magnolol using the following expression:

$$\frac{A_1 \times m_2 \times 0.16 \times p_1 \times d}{A_2 \times m_1} + \frac{A_3 \times m_3 \times p_2 \times d}{A_4 \times m_1}$$

$A_1$ = area of the peak due to honokiol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to honokiol in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to magnolol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to magnolol in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *honokiol CRS* used to prepare reference solution (a), in grams;

$m_3$ = mass of *magnolol CRS* used to prepare reference solution (b), in grams;

$p_1$ = percentage content of honokiol in *honokiol CRS*;

$p_2$ = percentage content of magnolol in *magnolol CRS*;

$d$ = dilution factor of the test solution.

**01/2011:1541**

# MALLOW FLOWER

## Malvae sylvestris flos

DEFINITION

Whole or fragmented dried flower of *Malva sylvestris* L. or its cultivated varieties.

IDENTIFICATION

A. The flower consists of an epicalyx with 3 oblong or elliptical-lanceolate parts that are shorter than those of the calyx and situated immediately below it; a calyx with 5 pubescent triangular lobes, gamosepalous at the base; a corolla 3-4 times longer than the calyx with 5 wedge-shaped, notched petals fused to the staminal tube at their base; numerous stamens, the filaments of which fuse into a staminal tube covered by small star-shaped trichomes and occasional simple trichomes visible using a lens; numerous wrinkled carpels, glabrous or sometimes pubescent, enclosed in the staminal tube and arranged into a circle around a central style ending with numerous filiform stigmas. In cultivated varieties, the epicalyx is 3-7 partite, the calyx 5-8 partite and the corolla 5-10 partite.

B. Microscopic examination (*2.8.23*). The powder is bluish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1541.-1): unicellular, thick-walled, flexuous covering trichomes, from the calyx and the epicalyx, up to 2 mm in length, whole [L] or, most often, fragmented [Q]; fragments of the epidermis of the sepals (surface view [D, J]) with anomocytic stomata (*2.8.3*) [Dc]; club-shaped glandular trichomes with multicellular heads [Db] and short unicellular covering trichomes, somewhat curved, either isolated [J] or in star-shaped groups of 2-6 [Da]; fragments of covering trichomes [N]; isolated glandular trichomes (surface view [F], transverse section [G]); fragments of the mesophyll of the calyx and the epicalyx whose cells contain small cluster crystals of calcium oxalate [K]; veins of the sepals [P] with vessels [Pa] accompanied by cells with cluster crystals of calcium oxalate [Pb]; fragments of petal epidermis, with elongated cells and sinuous margins, narrow in the wild plant [A], shorter and broader in the cultivated varieties [B], bearing

sessile glandular trichomes with multicellular club-shaped heads [Ba, C, E]; fragments of petal mesophyll [H] consisting of large mucilage cells [Hc], sometimes cells with small cluster crystals of calcium oxalate [Hb] and spiral vessels [Ha]; spherical pollen grains, about 150 µm in diameter, with a roughly spiny exine [M].

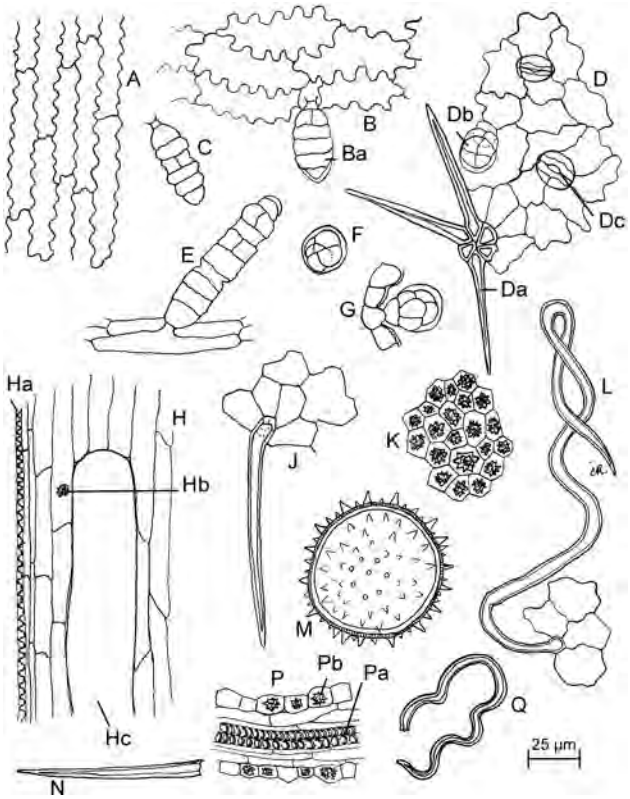


Figure 1541.-1. – *Illustration for identification test B of powdered herbal drug of mallow flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (60 per cent V/V) R*. Stir for 15 min and filter.

*Reference solution*. 0.5 g/L solution of *quinaldine red R* in *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (15:30:60 *V/V/V*).

*Application*: 10 µL of the test solution and 5 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows an orange-red zone in the upper part of the middle third ; the chromatogram obtained with the test solution shows, below the zone in the chromatogram obtained with the reference solution, 2 violet zones in the middle third, with the principal zone (6″-malonyl malvin) situated just below the other violet zone (malvin).

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 14.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

**Swelling index** (*2.8.4*): minimum 15, determined on 0.2 g of the powdered herbal drug (710) (*2.9.12*) moistened with 0.5 mL of *anhydrous ethanol R*.

**07/2011:2391**

# MALLOW LEAF

## Malvae folium

DEFINITION

Whole or fragmented, dried leaf of *Malva sylvestris* L., *Malva neglecta* Wallr. or a mixture of both species.

IDENTIFICATION

A. The leaves of *M. sylvestris* are up to 12 cm long and up to 15 cm wide with 3, 5 or 7 lobes and sinuate at the base; the leaves of *M. neglecta* are up to 9 cm long and wide, round or kidney-shaped with 5-7 indistinct lobes. The leaves of both species have irregular dentate margins and are green or brownish-green. The abaxial surface of the lamina bears more hairs and shows a more prominent venation than the adaxial surface. The major veins on the upper surface of the leaves and the petioles may be violet. The petioles are as long as the leaves, up to 2 mm wide, rounded and somewhat flattened, longitudinally slightly grooved, green or brownish-green or violet. The fragmented drug consists of occasionally agglomerated, crumpled pieces of leaves showing prominent veins.

B. Microscopic examination (*2.8.23*). The powder is green or yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2391.-1): fragments of the lamina (transverse section [F]), consisting of the lower epidermis (surface view [C]) and the upper epidermis (surface view [D], transverse section [Fb]), with cells that show straight, or more or less sinuous anticlinal walls; stomata mostly anisocytic (*2.8.3*) on both surfaces [Ca, Da]; long covering trichomes with thickened walls and tapering to a point at the apex, usually unicellular, whole [A, Fa] or fragmented [Db], but in *M. Sylvestris* they may be stellate with 2-8 components [H], each strongly pitted at the base; club-shaped glandular trichomes composed of 2-6 cells [E] occur in both species; fragments of the mesophyll consisting of palisade parenchyma (surface view [Dc], transverse section [Fc]) and spongy mesophyll cells containing mucilage, cells containing cluster crystals of calcium oxalate, often associated with vessels [B]; occasional spherical pollen grains, 110-170 µm in diameter, with a spiny exine [G].

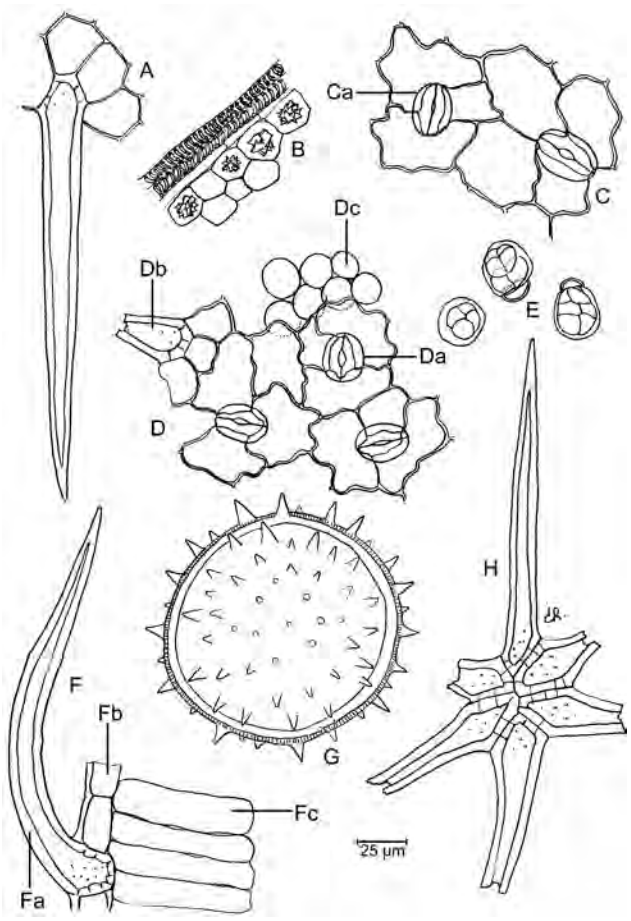


Figure 2391.-1. – *Illustration for identification test B of powdered herbal drug of mallow leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (710) (*2.9.12*) add 20 mL of an 80 per cent *V/V* solution of *tetrahydrofuran R*; extract for 10 min using sonication and filter.

*Reference solution.* Dissolve 3 mg of *hyperoside R* and 3 mg of *rutoside trihydrate R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *anhydrous acetic acid R*, *water R*, *ethyl formate R*, *3-pentanone R* (4:11:14:20:50 *V/V/V/V/V*).

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10-12 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 10 min; spray or dip the warm plate in a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; remove the solvent with cold air; spray or dip the plate in a 50 g/L solution of *macrogol 400 R* in *methanol R*, dry in air and examine after 15 min in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Hyperoside: a yellow fluorescent zone | A yellow fluorescent zone |
| ——— | ——— |
| Rutoside: a yellow fluorescent zone | A yellow fluorescent zone |
| | A light blue fluorescent zone |
| | An orange fluorescent zone |
| | An orange fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of foreign organs, maximum 5 per cent of leaves with blisters of spores of *Puccinia malvacearum* and maximum 2 per cent of foreign elements.

Foreign organs can be flowers, fruits and parts of the stem. The blisters of spores on the leaves are mostly 1 mm wide, and red or brown. Examine under a microscope using *chloral hydrate solution R*. The spores of *Puccinia malvacearum* are oblong or oval with brownish walls and a small appendage.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 17.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

**Swelling index** (*2.8.4*): minimum 7, determined on 1.0 g of the powdered herbal drug (710) (*2.9.12*).

**04/2016:2430**

# MANDARIN EPICARP AND MESOCARP

## Citri reticulatae epicarpium et mesocarpium

DEFINITION

Dried epicarp and mesocarp of the ripe fruit of *Citrus reticulata* Blanco or its cultivars, partly freed from the white spongy tissue of the mesocarp.

*Content*: minimum 3.5 per cent of hesperidin ($C_{28}H_{34}O_{15}$; $M_r$ 611) (dried drug).

IDENTIFICATION

A. The pericarp consists of variously sized irregular pieces, or it is cut into strips, which may be folded or form compressed loops. The larger pieces are mostly lobed, the lobes attached at the base of the pericarp where the remains of a fruit stalk or stalk scar may be present. Isolated lobes of pericarp may also be present. Irregular pieces 0.5-7.6 cm long, and 0.4-4.6 cm wide; strips up to 7.6 cm long and up to 2 cm wide; both forms 1-4.5 mm thick. The outer surface is yellowish-brown or orange, reddish-brown or more rarely, blackish- or greenish-brown; often finely wrinkled and slightly shiny. Scattered, concave or raised oil glands are dense on the outer surface; when visible in transverse section oil glands appear as irregularly shaped cavities beneath the epicarp. The inner surface of the pericarp appears whitish or whitish-yellowish, rough

and often fibrous in texture, bearing yellowish-white or yellowish-brown raised, often branched vascular bundles. The texture is mostly hard, stiff and fragile, easily broken.

B. Microscopic examination (*2.8.23*). The powder is light yellowish or orange-brown, more rarely reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2430.-1): fragments of epicarp (surface view [H]) consisting of small polygonal cells, sub-square or rectangular, with slightly thickened anticlinal walls [Ha] and occasional rounded stomata [Hb] (18-27 µm in diameter) encircled by a combination of small polygonal plus narrow, elongate subsidiary cells; epicarp usually accompanied by sub-epicarpal layers of thin-walled cells containing prisms of calcium oxalate [Hc]; prisms commonly biconical, also rhomboidal or polyhedral prisms present, 5-30 µm long and 3-18 µm in diameter; fragments of pericarp (transverse section [E]) showing the epicarp covered by a thick cuticle [Ea] and sub-epicarpal layers composed of cells, some of which contain prisms of calcium oxalate [Eb]; sometimes more than 1 prism crystal is found in the same cell [Aa, Hd]; fragments of schizolysigenous oil glands [C]; numerous groups of mesocarp cells (transverse section [A], longitudinal section [D]) with irregularly thickened, weakly lignified cell walls; free prism crystals of calcium oxalate or single crystals within isolated cells [B]; yellow hesperidin crystals sometimes abundant in cells; small droplets of orange-yellow essential oil occasionally present; numerous small vascular bundles [F] with narrow reticulate or spiral xylem [Fa] (4-23 µm in diameter), and occasional pitted tracheids; vasculature, sometimes accompanied by crystal sheaths [Fb]; occasional isolated, short vessels [G]. Examine under a microscope using a 20 g/L solution of *potassium hydroxide R*. The mounting medium becomes yellow because of the presence of hesperidin.

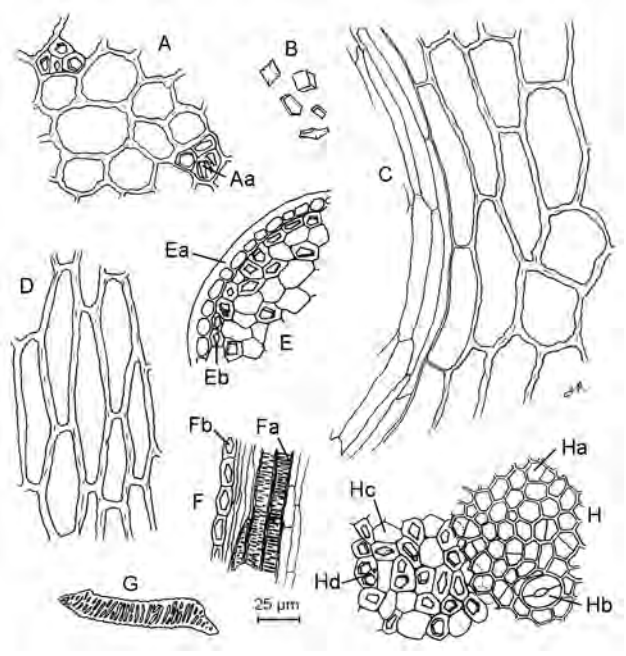


Figure 2430.-1. – *Illustration for identification test B of powdered herbal drug of mandarin epicarp and mesocarp*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and heat in a water-bath at 65 °C for 5 min, shaking frequently. Allow to cool and filter.

*Reference solution.* Dissolve 1 mg of *caffeic acid R* and 2 mg of *hesperidin R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (10:15:75 *V/V/V*).

*Application*: 5 µL of the test solution and 10 µL of the reference solution as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air, then heat at 110-120 °C for 5 min.

*Detection*: treat with a 10 g/L solution of *aluminium chloride R* in *ethanol (96 per cent) R* and heat at 110-120 °C for 5 min; treat the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. After 60 min examine the chromatograms in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Caffeic acid: a light blue fluorescent zone | |
| | A blue fluorescent zone |
| Hesperidin: a greenish-brown fluorescent zone | A greenish-brown fluorescent zone (hesperidin) |
| | A yellowish-green fluorescent zone |
| | A greenish fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Bitter-orange epicarp and mesocarp**. Examine the chromatograms obtained in the assay.

*Results*: the chromatogram obtained with the test solution shows no peak at the retention time of naringin with an area of more than 1 per cent of the area of the peak due to hesperidin.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Place 0.125 g of the powdered herbal drug (355) (*2.9.12*) in a 100 mL round-bottomed flask. Add 50.0 mL of *methanol R*, stir for 2 h and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *hesperidin CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *naringin R* in reference solution (a) and dilute to 25.0 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *glacial acetic acid R*, *methanol R*, *water R* (2.7:3.7:22:71.6 *V/V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of hesperidin.

*Retention time*: naringin = about 15 min; hesperidin = about 20 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to naringin and hesperidin.

Calculate the percentage content of hesperidin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to hesperidin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hesperidin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *hesperidin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of hesperidin in *hesperidin CRS*.

**01/2008:2355**
**corrected 10.0**

# MANDARIN OIL

## Citri reticulatae aetheroleum

DEFINITION

Essential oil obtained without heating, by suitable mechanical treatment, from the peel of the fresh fruit of *Citrus reticulata* Blanco.

CHARACTERS

*Appearance*: greenish, yellow or reddish orange liquid showing blue fluorescence.

Characteristic odour.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 0.1 mL of the substance to be examined to 1 mL with *toluene R*.

*Reference solution.* Dissolve 2 µL of *methyl N-methylanthranilate R*, 4 mg of *guaiazulene R* and 10 mg of *α-terpineol R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (15:85 *V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the intense blue fluorescent zone in the chromatogram obtained with the test solution is similar in position and fluorescence to the zone due to methyl *N*-methylanthranilate in the chromatogram obtained with the reference solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

*Detection B*: spray with a 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 100 °C for 10 min; examine in daylight.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue zone |
| Guaiazulene: a blue zone | A blue zone |
| | A blue zone |
| _____ | _____ |
| | A blue zone |
| _____ | _____ |
| α-Terpineol: a blue zone | A blue zone (α-terpineol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.848 to 0.855.

**Refractive index** (*2.2.6*): 1.474 to 1.478.

**Optical rotation** (*2.2.7*): + 64° to + 75°.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 0.20 g of the substance to be examined to 10.0 mL with *heptane R*.

*Reference solution (a).* Dilute 5 µL of *α-pinene R*, 5 µL of *sabinene R*, 5 µL of *β-pinene R*, 5 µL of *β-myrcene R*, 5 µL of *p-cymene R*, 70 µL of *limonene R*, 20 µL of *γ-terpinene R* and 5 µL of *methyl N-methylanthranilate R* to 5.0 mL with *heptane R*.

*Reference solution (b).* Dissolve 5 µL of *limonene R* in 50 mL of *heptane R*. Dilute 0.5 mL of this solution to 5.0 mL with *heptane R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 60 m, Ø = 0.25 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.4 mL/min.

*Split ratio*: 1:70.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 90 | 50 → 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to sabinene and β-pinene and minimum 1.5 between the peaks due to *p*-cymene and limonene.

*Identification of components*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution. Disregard the peak due to heptane.

Determine the percentage content of each of these components. The limits are within the following ranges:

– *α-pinene*: 1.6 per cent to 3.0 per cent;
– *sabinene*: maximum 0.3 per cent;
– *β-pinene*: 1.2 per cent to 2.0 per cent;
– *β-myrcene*: 1.5 per cent to 2.0 per cent;
– *p-cymene*: maximum 1.0 per cent;
– *limonene*: 65.0 per cent to 75.0 per cent;
– *γ-terpinene*: 16.0 per cent to 22.0 per cent;
– *methyl N-methylanthranilate*: 0.30 per cent to 0.60 per cent;
– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (b).

**Residue on evaporation** (*2.8.9*): 1.6 per cent to 4.0 per cent, determined after heating on a water-bath for 4 h.

STORAGE

At a temperature not exceeding 25 °C.

**01/2012:1856**

# MARSHMALLOW LEAF

## Althaeae folium

DEFINITION

Whole or cut, dried leaf of *Althaea officinalis* L.

IDENTIFICATION

A. The leaves have long petioles and are about 7-10 cm long; the lamina is cordate or ovate with 3-5 shallow lobes and crenate or dentate margins; the venation is palmate. The petioles and both surfaces of the lamina are greyish-green and densely pubescent. Rarely, fragments of the inflorescence or immature fruits may be present.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1856.-1): numerous long, rigid, unicellular covering trichomes with thick walls, pointed at the apex, often fragmented [C], angular and pitted at the base where they are sometimes still united to form stellate structures with up to 8 components (surface view [B], transverse section [E]); few secretory trichomes, isolated, with unicellular stalks and globular, multicellular heads [F]; fragments of the lower [A] and upper [D] leaf epidermises in surface view with anomocytic [Aa] or paracytic [Da] stomata (*2.8.3*), glandular trichomes [Ab] and basal cells of covering trichomes [Ac], often accompanied by palisade parenchyma [Db]; cluster crystals of calcium oxalate, isolated [H] or included in the parenchyma of the mesophyll [Gc, Kb]; fragments of veins [G] with small, spiral [Gb] or annular [Ga] vessels, often accompanied by sheaths containing cluster crystals of calcium oxalate [Gc]; fragments of the lamina, in transverse section [K], showing the epidermises bearing broken covering trichomes [Ka], a symmetrical, heterogeneous mesophyll with some cells containing cluster crystals of calcium oxalate [Kb]; occasional pollen grains, spherical, with a roughly spiny exine, about 150 µm in diameter [J]. Examine under a microscope using *ruthenium red solution R*. The powder shows groups of parenchyma containing mucilage, which stains orange-red.

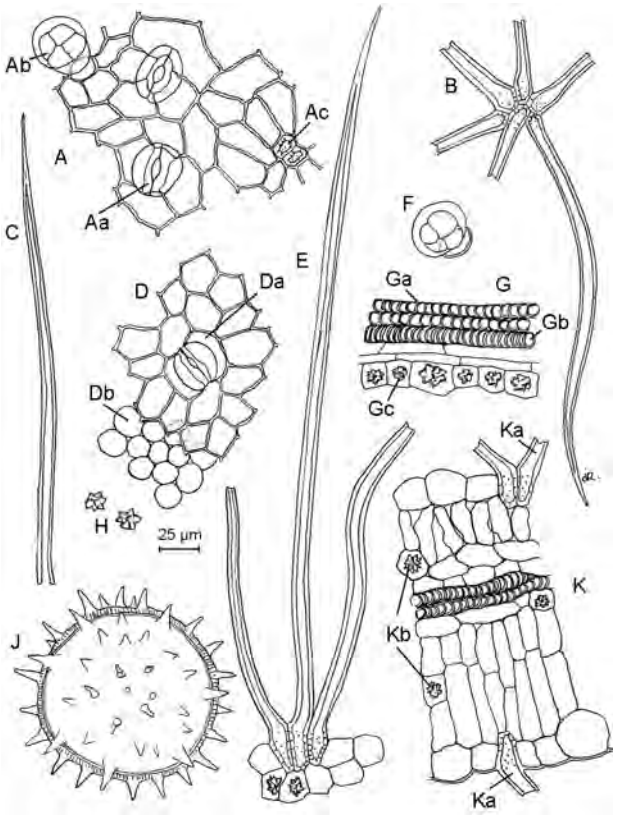


Figure 1856.-1. – *Illustration for identification test B of powdered herbal drug of marshmallow leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat in a water-bath under a reflux condenser for 5 min. Allow to cool and filter. Distil the filtrate under reduced pressure until the total volume is about 2 mL.

*Reference solution.* Dissolve 2.5 mg of *chlorogenic acid R* and 2.5 mg of *quercitrin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue fluorescent zone |
| | A yellow fluorescent zone |
| Quercitrin: an orange zone | |
| ——— | ——— |
| | An orange fluorescent zone |
| | An orange fluorescent zone |
| ——— | ——— |
| Chlorogenic acid: a blue fluorescent zone | |
| | A blue fluorescent zone |
| | An orange fluorescent zone |
| | An intense yellow fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 4 per cent of leaves infected by *Puccinia malvacearum*, showing red spots, and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 18.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

**Swelling index** (*2.8.4*): minimum 12, determined on 0.2 g of the powdered herbal drug (355) (*2.9.12*).

**01/2012:1126**

# MARSHMALLOW ROOT

## Althaeae radix

DEFINITION

Peeled or unpeeled, whole or cut, dried root of *Althaea officinalis* L.

IDENTIFICATION

A. The unpeeled, non-fragmented drug consists of cylindrical, slightly twisted roots, up to 2 cm thick, with deep longitudinal furrows. The outer surface is greyish-brown and bears numerous rootlet scars. The fracture is fibrous externally, rugged and granular internally. The section shows a more or less thick, whitish bark with brownish periderm, separated by the well-marked, brownish cambium from a white xylem. The stratified structure of the bark and the radiate structure of xylem become more distinct when moistened.

   The peeled drug has a greyish-white, finely fibrous outer surface. Cork and external cortical parenchyma are absent.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown (unpeeled root) or whitish (peeled root). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1126.-1): fragments of colourless, mainly unlignified, thick-walled fibres [C, D, M] with split or pointed ends [D], sometimes accompanied by parenchymatous cells of the medullary rays [M], or grouped [C]; fragments of vessels, bordered-pitted or with reticulate or scalariform thickenings [G, H]; cluster crystals of calcium oxalate about 20-35 µm, mostly 25-30 µm in size, isolated [K] or included in parenchymatous cells [B]; fragments of parenchyma [E] with cells containing mucilage [Ea, F]; fragments of cork with thin-walled, tabular cells (surface view [A], transverse section [L]) (unpeeled root). Examine under a microscope using *ruthenium red solution R*. The powder shows groups of parenchyma containing mucilage, which stains orange-red. Examine under a microscope using *water R*. The powder shows numerous starch granules [J], about 3-25 µm in size, occasionally with a longitudinal hilum. The starch granules are mostly simple [Ja], a few being 2-4 compound [Jb].

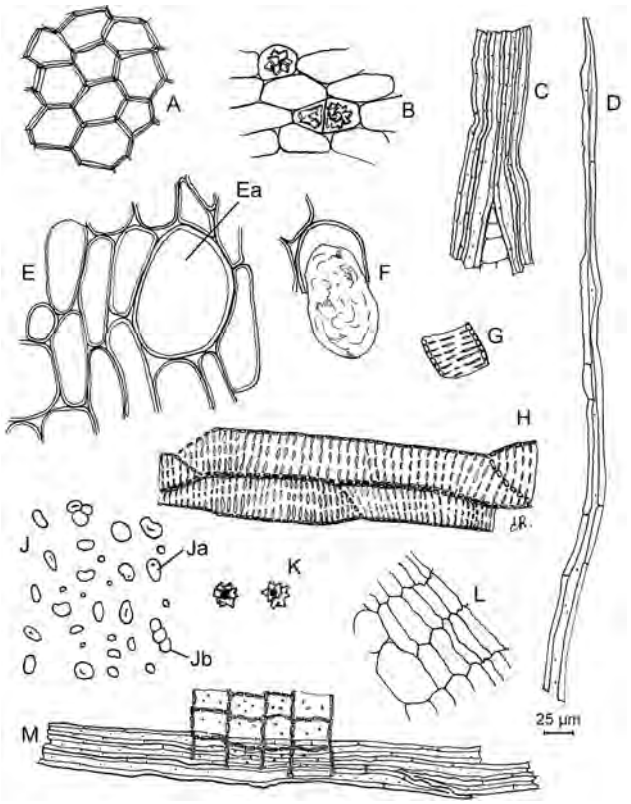


Figure 1126.-1. – *Illustration for identification test B of powdered herbal drug of marshmallow root*

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of brown deteriorated drug.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent for the peeled root and maximum 8.0 per cent for the unpeeled root.

**Swelling index** (*2.8.4*): minimum 10, determined on the powdered herbal drug (710) (*2.9.12*).

**01/2017:1876**
**corrected 10.0**

# MASTIC

## Mastix

DEFINITION

Dried resinous exudate obtained from stems and branches of *Pistacia lentiscus* L.

*Content*: minimum 10 mL/kg of essential oil (anhydrous drug).

IDENTIFICATION

A. Small light yellow to greenish-yellow, non-uniform, spherical or pyriform, clear or opaque, hard glassy fragments.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1 g of the substance to be examined in 10 mL of *methylene chloride R* and filter after 1-2 min.

*Reference solution.* Dissolve 25 mg of *eugenol R* and 25 mg of *borneol R* in 3 mL of *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *light petroleum R*, *toluene R* (5:95 *V/V*).

*Application*: 1 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R* and heat at 100-105 °C for 5 min.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| _____ | _____ |
| | A pale violet zone |
| | A very pale violet zone |
| _____ | _____ |
| Eugenol: a brown zone | A blue zone |
| Borneol: a greenish-blue zone | A bluish-violet zone |
| | A dark violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Acid value** (*2.5.1*): 50 to 70, determined on 1.0 g.

**Water** (*2.2.13*): maximum 10 mL/kg, determined on 25.0 g of the drug reduced to a powder (1400) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 0.5 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use a 500 mL round-bottomed flask and 200 mL of *water R* as the distillation liquid. Reduce the drug to a powder (1400) (*2.9.12*) and immediately use 20.0 g for the determination. Introduce 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

STORAGE

Do not powder.

**04/2018:2678**

# MATE LEAF

## Mate folium

DEFINITION

Leaf of *Ilex paraguariensis* A.St.-Hil., rapidly desiccated by heating and cut.

*Content*: minimum 1.0 per cent of caffeine ($C_8H_{10}N_4O_2$; $M_r$ 194.2) (dried drug).

IDENTIFICATION

A. The yellowish-green or brownish-green leaf is tough and brittle with a short petiole. It is 8-12 cm long and 5-6 cm wide. The lamina is elliptic oval and glabrous, with dentate margins. The venation is pinnate and prominent on the lower surface. The cut herbal drug occurs as glabrous, yellowish-green, irregular, angular pieces ranging in length from 2-4 mm.

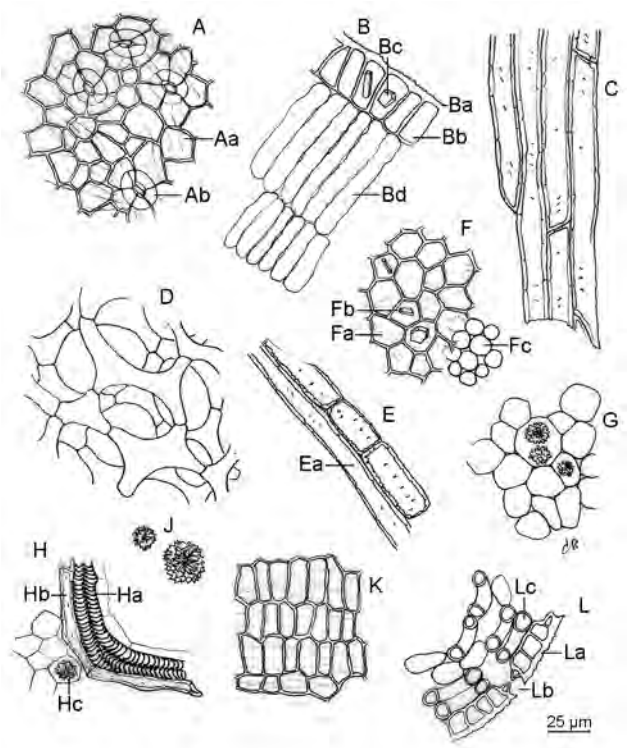


Figure 2678.-1. – *Illustration for identification test B of powdered herbal drug of mate leaf*

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2678.-1): fragments of the upper epidermis of the lamina (surface view [F], transverse section [B]), covered with an irregularly striated cuticle [Fa, Ba] and consisting of cells with slightly and regularly thickened walls [Fa, Bb], with some cells containing a prism of calcium oxalate [Fb, Bc], and accompanied by underlying palisade parenchyma [Fc, Bd]; fragments of the upper epidermis of the lamina from the area near a vein (surface view [K]), consisting of nearly rectangular cells in a regular arrangement; fragments of the lower epidermis of the lamina (surface view [A], transverse section [L]), covered by a striated cuticle [Aa, La] consisting of polygonal cells [Aa] and anomocytic stomata (*2.8.3*) [Lb, Ab]; fragments of spongy parenchyma (surface view [D]), sometimes accompanying the lower epidermis (transverse section [Lc]); cluster crystals of calcium oxalate of various sizes that may exceed 30 µm in diameter, isolated [J] or included in parenchyma cells [G]; groups of pericyclic fibres [C]; rectangular, thick-walled and channelled cells from the endodermis [E], often accompanying pericyclic fibres [Ea]; vascular bundles [H] consisting of spiral vessels with a small diameter [Ha], accompanied by fibres [Hb] and sometimes by parenchyma, with some cells containing a cluster crystal of calcium oxalate [Hc].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and sonicate for 10 min. Centrifuge or filter.

*Reference solution.* Dissolve 7 mg of *caffeine R*, 1 mg of *chlorogenic acid R* and 1 mg of *rutoside trihydrate R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *toluene R*, *water R*, *anhydrous formic acid R*, *ethyl formate R* (3:6:8:60 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in a current of air at room temperature.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | A quenching zone |
| Caffeine: a quenching zone | A quenching zone (caffeine) |
| | A quenching zone |
| Chlorogenic acid: a quenching zone | A quenching zone (chlorogenic acid) |
| ——— | ——— |
| Rutoside: a quenching zone | A quenching zone (rutoside) |
| **Reference solution** | **Test solution** |

*Detection B*: heat at 100-105 °C for 5 min. Treat the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air. Examine in ultraviolet light at 366 nm.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. 1 or 2 faint blue fluorescent zones may be present in the upper third of the chromatogram obtained with the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 1 or 2 red fluorescent zones |
| ——— | ——— |
| | An intense blue fluorescent zone |
| | A blue fluorescent zone |
| Chlorogenic acid: a blue fluorescent zone | An intense blue fluorescent zone (chlorogenic acid) |
| ——— | ——— |
| Rutoside: an orange fluorescent zone | An orange fluorescent zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).
*Solution A*. Dissolve 30.0 mg of *caffeine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution.* Introduce 1.500 g of the powdered herbal drug (355) (*2.9.12*) into a 100 mL round-bottomed flask and add 40 mL of *methanol R*. Heat in a water-bath at 70 °C under a reflux condenser for 30 min. Allow to cool and filter through a plug of absorbent cotton into a 100 mL volumetric flask. Transfer the absorbent cotton with the drug residue into the round-bottomed flask. Add 40 mL of *methanol R*, proceed as before and filter into the same volumetric flask. Rinse the round-bottomed flask and the filter with *methanol R*, add the rinsings to the volumetric flask and dilute to 100.0 mL with *methanol R*. Shake to obtain a homogeneous solution. Transfer 10.0 mL of this solution into a volumetric flask, add 30 mL of *water R* and dilute to 50.0 mL with the mobile phase. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dilute 5.0 mL of solution A to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 15.0 mg of *(−)-epicatechin R* in the mobile phase and dilute to 25.0 mL with the mobile phase. To 10.0 mL of the solution add 5.0 mL of solution A and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water for chromatography R* (25:75 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of caffeine.

*Retention time*: caffeine = about 11 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to caffeine and (−)-epicatechin.

Calculate the percentage content of caffeine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to caffeine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to caffeine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *caffeine CRS* used to prepare solution A, in grams;

$p$ = percentage content of caffeine in *caffeine CRS*.

**07/2019:0404**

# MATRICARIA FLOWER

## Matricariae flos

DEFINITION

Dried capitula of *Matricaria recutita* L. (*Chamomilla recutita* (L.) Rauschert).

*Content*:

– *blue essential oil*: minimum 4 mL/kg (dried drug);

– *total apigenin 7-glucoside* ($C_{21}H_{20}O_{10}$; $M_r$ 432.4): minimum 0.25 per cent (dried drug).

IDENTIFICATION

A. Capitula, when spread out, consisting of an involucre made up of many bracts arranged in 1-3 rows; an elongated-conical receptacle, occasionally hemispherical (young capitula); 12-20 marginal ligulate florets with a white ligule; several dozen yellow central tubular florets. The involucre bracts are ovate or lanceolate, with a brownish-grey scarious margin. The receptacle is hollow, without paleae. The corolla of the ligulate florets has a brownish-yellow tube at the base extending to form a white, elongated-oval ligule. The inferior ovary is dark brown, ovoid or spherical, and has a long style and bifid stigma. The tubular florets are yellow and have a five-toothed corolla tube, 5 syngenesious, epipetalous stamens and a gynoecium similar to that of the ligulate florets.

B. Microscopic examination (*2.8.23*). The powder is light yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0404.-1): fragments of the outer epidermis of the bracts of the involucre (surface view [K]), consisting of, on the margin, thin-walled cells covered by a finely striated cuticle [Ka], anomocytic stomata (*2.8.3*) [Kb] and occasional biseriate glandular trichomes [Kc], and a central region [J] composed of elongated sclereids with moderately thickened and channelled walls [Ja]; fragments of the inner epidermis of the corolla of the ligulate florets consisting of thin-walled, polygonal cells, slightly papillose [B]; fragments of the outer epidermis of the ligulate florets consisting of sinuous cells, covered by a striated cuticle [D], often accompanied by underlying narrow vessels [Da]; fragments from the apex of the lobes of the corolla of the tubular florets [A] with elongated cells on the margin [Aa] and slightly papillose cells [Ab]; biseriate glandular trichomes with a short stalk (1 or 2 tiers of 2 cells) and a head of 2-3 tiers of 2 cells, on the epidermises of the corollas of both types of floret and of the bracts of the involucre and on the ovary (surface view [Eb, G, Kc], side view [Ha]); fragments of the base of the flower where the ovary is located, with a ring of thick-walled sclerous cells [C]; fragments of the epidermis surrounding the ovary (surface view [E]) consisting of thin, longitudinally elongated cells [Ea], with numerous glandular trichomes [Eb], alternating with large elongated cells filled with mucilage in transversally folded layers [Ec]; fragments of the epidermis surrounding the ovary (side view [H]) bearing glandular trichomes [Ha] and underlying parenchymatous cells containing small cluster crystals of calcium oxalate [Hb]; groups of cells at the apex of the stigmas forming elongated papillae [L]; pollen grains spherical or triangular, about 25 µm in diameter, with 3 pores and a spiny exine [F].

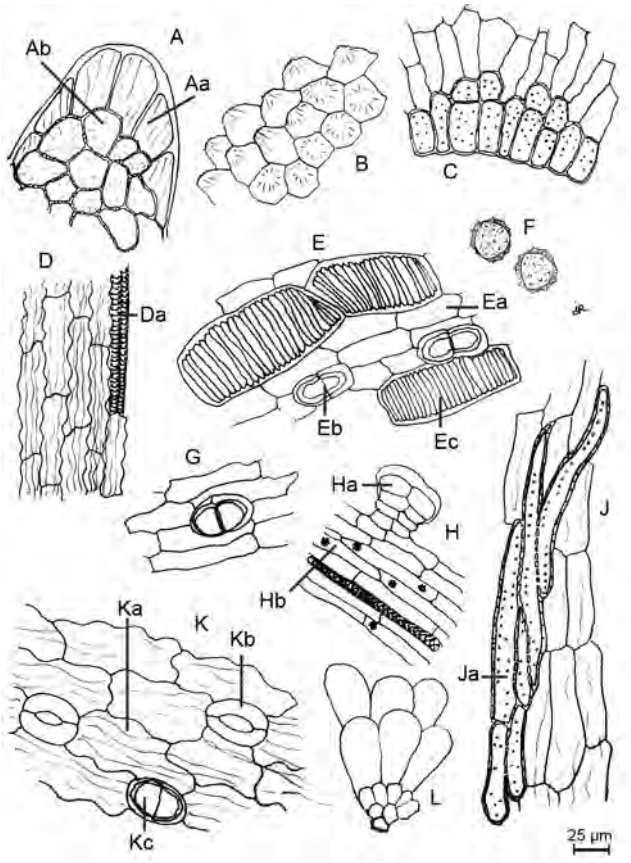


Figure 0404.-1. – *Illustration for identification test B of powdered herbal drug of matricaria flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 50 µL of essential oil obtained in the assay of essential oil in 1 mL of *xylene R*.

*Reference solution.* Dissolve 2 µL of *chamazulene R*, 5 µL of *(–)-α-bisabolol R* and 10 mg of *bornyl acetate R* in 5 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | 1 or 2 blue or bluish-violet zones |
| Chamazulene: a red or reddish-violet zone | A red or reddish-violet zone (chamazulene) |
| Bornyl acetate: a yellowish-brown zone | A brown zone (en-yne-dicycloether) |
| (−)-α-Bisabolol: a reddish-violet or bluish-violet zone | A reddish-violet or bluish-violet zone ((−)-α-bisabolol) |
| **Reference solution** | **Test solution** |

TESTS

**Broken drug**: maximum 25 per cent, determined on 20.0 g, passes through a sieve (710) (*2.9.12*).

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 13.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 30 g of whole herbal drug, a 1000 mL flask, 500 mL of *water R* as distillation liquid and 0.50 mL of *1,2,4-trimethylbenzene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 4 h. Towards the end of this period, stop the flow of water to the condenser assembly but continue distilling until the blue, steam-volatile components have reached the lower end of the condenser. Immediately re-start the flow of water to the condenser assembly to avoid warming the separation space. Stop the distillation after a further 10 min.

**Total apigenin 7-glucoside**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (25:75 *V/V*).

*Test solution*. Reduce 40 g of the herbal drug to a powder (500) (*2.9.12*). Place 2.00 g of the powdered herbal drug in a 500 mL round-bottomed flask. Add 200 mL of *ethanol (96 per cent) R*. Heat the mixture under a reflux condenser on a water-bath for 15 min. Cool and filter. Rinse the filter and the residue with a few millilitres of *ethanol (96 per cent) R*. To the filtrate add 10 mL of freshly prepared *dilute sodium hydroxide solution R* and heat the mixture under a reflux condenser on a water-bath for about 1 h. Cool. Dilute to 250.0 mL with *ethanol (96 per cent) R*. To 50.0 mL of the solution add 0.5 g of *citric acid monohydrate R*. Shake for 5 min and filter. Dilute 5.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10.0 mg of *apigenin 7-glucoside CRS* in 100.0 mL of *methanol R*. Dilute 25.0 mL of this solution to 200.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10.0 mg of *5,7-dihydroxy-4-methylcoumarin R* in 100.0 mL of *methanol R*. Dilute 25.0 mL of this solution to 100.0 mL with the solvent mixture. To 4.0 mL of this solution add 4.0 mL of reference solution (a) and dilute to 10.0 mL with the solvent mixture.

*Precolumn*:

- *size*: *l* = 8 mm, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.5 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *phosphoric acid R*, *acetonitrile R* (0.5:99.5 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 75 | 25 |
| 9 - 19 | 75 → 25 | 25 → 75 |
| 19 - 24 | 25 | 75 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 340 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to apigenin 7-glucoside and 5,7-dihydroxy-4-methylcoumarin.

Calculate the percentage content of total apigenin 7-glucoside using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.625}{A_2 \times m_1}$$

$A_1$ = area of the peak due to apigenin 7-glucoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to apigenin 7-glucoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *apigenin 7-glucoside CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of apigenin 7-glucoside in *apigenin 7-glucoside CRS*.

**01/2008:1544**

# MATRICARIA LIQUID EXTRACT

## Matricariae extractum fluidum

DEFINITION

Liquid extract produced from *Matricaria flower (0404)*.

*Content*: minimum 0.30 per cent of blue residual oil.

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure for liquid extracts using a mixture of 2.5 volumes of a 10 per cent *m/m* solution of ammonia ($NH_3$), 47.5 volumes of water and 50 volumes of ethanol (96 per cent).

CHARACTERS

*Appearance*: brownish, clear liquid.

Intense characteristic odour and characteristic bitter taste.

*Solubility*: miscible with water and with ethanol (96 per cent) with development of turbidity, soluble in ethanol (50 per cent *V/V*).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Place 10 mL of the extract to be examined in a separating funnel and shake with 2 quantities, each of 10 mL, of *pentane R*. Combine the pentane layers, dry over 2 g of *anhydrous sodium sulfate R* and filter. Evaporate the filtrate to dryness on a water-bath and dissolve the residue in 0.5 mL of *toluene R*.

*Reference solution*. Dissolve 4 mg of *guaiazulene R*, 20 mg of *(–)-α-bisabolol R* and 20 mg of *bornyl acetate R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatogram obtained with the test solution shows several quenching zones, of which 2 main zones are in the middle third (en-yne-dicycloether).

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: the chromatogram obtained with the test solution shows in the middle part an intense blue fluorescent zone (herniarin).

*Detection C*: spray with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

*Results C*: the chromatogram obtained with the reference solution shows in the lower third a reddish-violet or bluish-violet zone ((-)-α-bisabolol), in the middle third a yellowish-brown or greyish-green zone (bornyl acetate) and in the upper third a red or reddish-violet zone (guaiazulene). The chromatogram obtained with the test solution shows in the lower third yellowish-brown or greenish-yellow and violet zones and a reddish-violet or bluish-violet zone due to (-)-α-bisabolol in the chromatogram obtained with the reference solution; a brownish zone (en-yne-dicycloether) similar in position to the zone due to bornyl acetate in the chromatogram obtained with the reference solution; a red or reddish-violet zone (chamazulene) corresponding to guaiazulene in the chromatogram obtained with the reference solution and immediately above it 1 or 2 blue or bluish-violet zones; further weak zones may be present in the chromatogram obtained with the test solution.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* The extract to be examined.

*Reference solution.* Dissolve 1.0 mg of *chlorogenic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (7.5:7.5:18:67 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; subsequently spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the middle part a light blue fluorescent zone (chlorogenic acid), below it a yellowish-brown fluorescent zone (rutoside) and above it a yellowish-brown fluorescent zone (hyperoside). The chromatogram obtained with the test solution shows a yellowish-brown fluorescent zone corresponding to the zone of rutoside in the chromatogram obtained with the reference solution, a light blue fluorescent zone corresponding to the zone of chlorogenic acid in the chromatogram obtained with the reference solution, a yellowish-brown fluorescent zone similar in position to the zone of hyperoside in the chromatogram obtained with the reference solution; it also shows above the yellowish-brown fluorescent zone a green fluorescent zone, then several bluish or greenish fluorescent zones and near the solvent front a yellowish fluorescent zone.

TESTS

**Ethanol** (*2.9.10*): 38 per cent *V/V* to 53 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 12.0 per cent.

ASSAY

Place 20.0 g in a 1000 mL round-bottomed flask, add 300 mL of *water R* and distil until 200 mL has been collected in a flask. Transfer the distillate into a separating funnel. Dissolve 65 g of *sodium chloride R* in the distillate and shake with 3 quantities, each of 30 mL, of *pentane R* previously used to rinse the reflux condenser and the flask. Combine the pentane layers, dry over 2 g of *anhydrous sodium sulfate R* and filter into a tared 100 mL round-bottomed flask which has been dried in a desiccator for 3 h. Rinse the anhydrous sodium sulfate and the filter with 2 quantities, each of 20 mL, of *pentane R*. Evaporate the pentane in a water-bath at 45 °C. The residue of pentane is eliminated in a current of air for 3 min. Dry the flask in a desiccator for 3 h and weigh. The residual oil is blue (chamazulene).

**01/2008:1836**

# MATRICARIA OIL

## Matricariae aetheroleum

DEFINITION

Blue essential oil obtained by steam distillation from the fresh or dried flower-heads or flowering tops of *Matricaria recutita* L. (*Chamomilla recutita* L. Rauschert). There are 2 types of matricaria oil which are characterised as rich in bisabolol oxides, or rich in (−)-α-bisabolol.

CHARACTERS

*Appearance*: clear, intensely blue, viscous liquid.

Intense characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 µL of the substance to be examined in 1.0 mL of *toluene R*.

*Reference solution.* Dissolve 2 mg of *guaiazulene R*, 5 µL of *(−)-α-bisabolol R* and 10 mg of *bornyl acetate R* in 5.0 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in daylight.

*Results A*: see below for the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

| **Top of the plate** | |
|---|---|
| Guaiazulene: a blue zone | A blue zone (chamazulene) |
| _____ | _____ |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

*Results B*: see below for the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, yellowish-brown to greenish-yellow zones (lower third), violet zones (lower third) and further weak zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 1 or 2 blue to bluish-violet zones |
| Guaiazulene: a red to reddish-violet zone | A red to reddish-violet zone (chamazulene) |
| ——— | ——— |
| Bornyl acetate: a yellowish-brown to greyish-green zone | A brown zone (en-yne-dicycloether) |
| ——— | ——— |
| (−)-α-Bisabolol: a reddish-violet to bluish-violet zone | A reddish-violet to bluish-violet zone ((−)-α-bisabolol)<br>A brownish zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks due to (−)-α-bisabolol and chamazulene in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 20 µL of the essential oil to be examined in *cyclohexane R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Dissolve 20 µL of *(−)-α-bisabolol R*, 5 mg of *chamazulene R* and 6 mg of *guaiazulene R* in *cyclohexane R* and dilute to 5.0 mL with the same solvent.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm, when using a column longer than 30 m, an adjustment of the temperature programme may be necessary,
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1-2 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 40 | 70 → 230 |
| | 40 - 50 | 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*Relative retention* with reference to chamazulene (retention time = about 34.4 min): β-farnesene = about 0.5; bisabolol oxide B = about 0.8; bisabolone = about 0.87; (−)-α-bisabolol = about 0.9; bisabolol oxide A = about 1.02.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to chamazulene and guaiazulene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate (−)-α-bisabolol and chamazulene in the chromatogram obtained with the test solution; locate bisabolol oxides (bisabolol oxide B, bisabolone and bisabolol oxide A) using Figures 1836.-1 and 1836.-2 (disregard the peak due to cyclohexane). The chromatogram obtained with the test solution does not show a peak with the retention time of guaiazulene.

Determine the percentage content of the components. The limits are within the following ranges.

| | Matricaria oil rich in bisabolol oxides (per cent) | Matricaria oil rich in (−)-α-bisabolol (per cent) |
|---|---|---|
| Bisabolol oxides | 29 - 81 | |
| (−)-α-Bisabolol | | 10 - 65 |
| Chamazulene | ≥ 1.0 | ≥ 1.0 |
| Total of bisabolol oxides and (−)-α-Bisabolol | | ≥ 20 |

STORAGE

At a temperature not exceeding 25 °C.

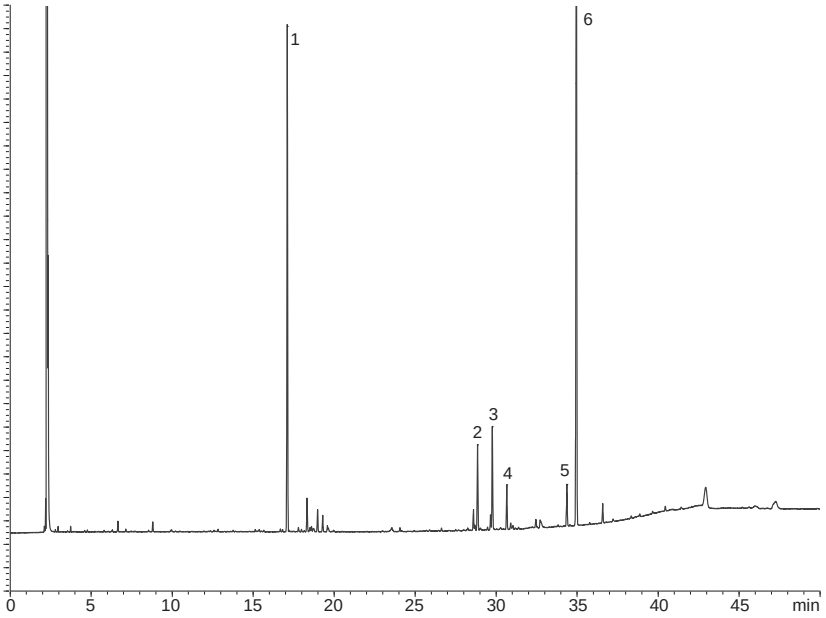


1. β-farnesene
2. bisabolol oxide B
3. bisabolone
4. (−)-α-bisabolol
5. chamazulene
6. bisabolol oxide A

Figure 1836.-1. – *Chromatogram of matricaria oil rich in bisabolol oxides*

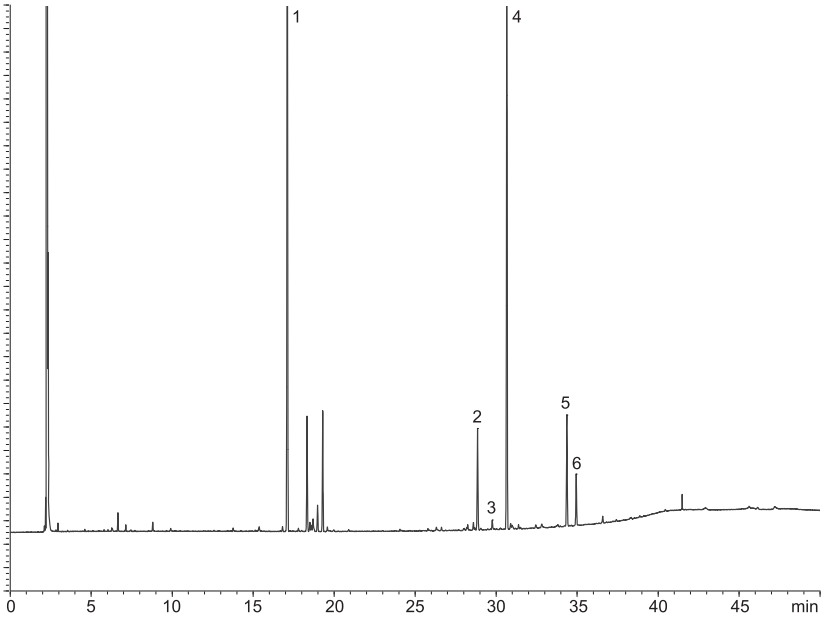


1. β-farnesene
2. bisabolol oxide B
3. bisabolone
4. (−)-α-bisabolol
5. chamazulene
6. bisabolol oxide A

Figure 1836.-2. – *Chromatogram of matricaria oil rich in (−)-α-bisabolol*

**04/2013:1868**

# MEADOWSWEET

## Filipendulae ulmariae herba

DEFINITION

Whole or cut, dried flowering tops of *Filipendula ulmaria* (L.) Maxim. (syn. *Spiraea ulmaria* L.).

*Content*: minimum 1 mL/kg of essential oil (dried drug).

CHARACTERS

Aromatic odour of methyl salicylate, after crushing.

IDENTIFICATION

A. The stem, up to 5 mm in diameter, is greenish-brown, stiff, angular, hollow except at the apex, and has regular, straight, longitudinal furrows. The petiolate leaf, compound imparipinnate, has 2 reddish-brown angular stipules. It consists of 3-9 pairs of leaflets, unevenly dentate, some of which are small and fan-shaped. The leaflets are dark green and glabrous on the upper surface, tomentose and lighter, sometimes silvery on the lower surface. The terminal leaflet, the largest, is divided into 3 segments. The veins are prominent and brown on the lower surface. The inflorescence is complex and composed of very numerous flowers arranged in irregular cymose panicles. The flowers are creamish-white and about 3-6 mm in diameter; the calyx consists of 5 dark green, reflexed and hairy sepals fused at the base to a concave receptacle; the 5 free petals, which are readily detached, are pale yellow, obovate and distinctly narrowed at the base; the stamens are numerous with rounded anthers and they extend beyond the petals; the gynoecium consists of about 4-6 carpels, each with a short style and a globular stigma; the carpels become twisted together spirally to form yellowish-brown fruits with a helicoidal twist. Unopened flower buds are frequently present. If the fruit is present, it has a helicoidal twist and contains brownish seeds.

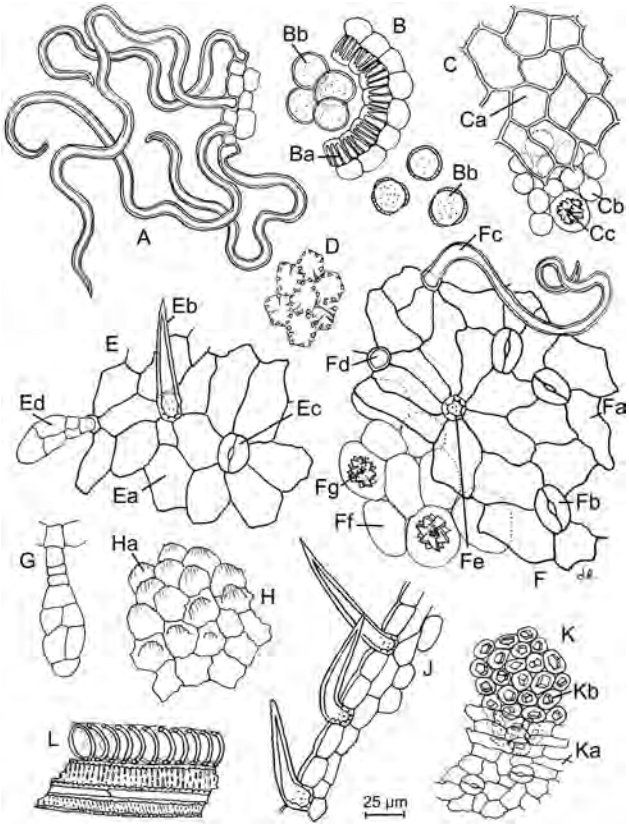


Figure 1868.-1. – *Illustration for identification test B of powdered herbal drug of meadowsweet*

B. Microscopic examination (*2.8.23*). The powder is green or yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1868.-1): fragments of the epidermises of the leaves and sepals [C, E, F] with sinuous or wavy cells [Ca, Ea, Fa], short, thick-walled, conical covering trichomes thickened at the base (surface view [Eb], side view [J]), unicellular covering trichomes, thin-walled, very long and flexuous, with pointed ends (surface view [Fc], side view [A]) or their scars (flexuous

Herbal drugs

trichome [Fd], conical trichome [Fe]) and occasional clavate glandular trichomes with a 1- to 3-celled ([Ed] and [G], respectively), uniseriate stalk, a multicellular head and dense brown contents; fragments of the upper epidermis often accompanied by palisade parenchyma [Cb] including some hypertrophied cells containing a cluster crystal of calcium oxalate [Cc]; fragments of the lower epidermis with anomocytic stomata (*2.8.3*) [Ec, Fb], sometimes accompanied by spongy parenchyma [Ff] with some cells containing cluster crystals of calcium oxalate [Fg]; fragments of the petals [H] with thin-walled epidermal cells, some showing rounded papillae [Ha]; numerous spherical pollen grains with 3 pores and a faintly pitted exine [Bb]; fragments of the anther [B, D] whose fibrous layer shows specific thickenings (surface view [D], side view [Ba]); fragments of the ovary [K] with an epidermis bearing stomata [Ka] and with parenchyma containing prism crystals of calcium oxalate [Kb]; fragments of vascular tissue [L] with annular, spiral or pitted vessels from the leaves and stems.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Xylene solution obtained in the assay.

*Reference solution.* Dissolve 0.1 mL of *methyl salicylate R* and 0.1 mL of *salicylaldehyde R* in *xylene R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *hexane R, toluene R* (50:50 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with 3 mL of *ferric chloride solution R3* and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Methyl salicylate: a violet-brown zone | A violet-brown zone (methyl salicylate) |
| Salicylaldehyde: a violet-brown zone | A violet-brown zone (salicylaldehyde) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5.0 per cent of stems with a diameter greater than 5 mm and maximum 2.0 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 50.0 g of the cut herbal drug, a 1000 mL flask, 300 mL of *dilute hydrochloric acid R* as the distillation liquid, and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**07/2012:2120**

# MELILOT

## Meliloti herba

DEFINITION

Whole or cut, dried aerial parts of *Melilotus officinalis* (L.) Lam.

*Content*: minimum 0.3 per cent of coumarin ($C_9H_6O_2$; $M_r$ 146.1) (dried drug).

IDENTIFICATION

A. The stem is green, cylindrical, glabrous and finely ridged. The leaves are alternate, petiolate and trifoliate with 2 lanceolate stipules; the leaflets are up to about 3 cm long and 20 mm wide, elongated or ovate with a finely dentate margin, acute at the apex and base; the upper surface is dark green and glabrous, the lower surface paler green with short, fine hairs, especially at the base. The inflorescence is racemose with numerous pale yellow flowers, about 7 mm long, each having a hairy calyx with 5 deeply-divided, unequal teeth, and a papilionate corolla. The fruit is an indehiscent pod, often persistent within the calyx, yellowish-brown, short and tapering at the apex; the surface is glabrous and transversely wrinkled.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2120.-1): fragments of the leaf lamina (surface view [D]) showing unevenly thickened, slightly sinuous epidermal cells; numerous stomata [Db], mostly anomocytic (*2.8.3*) with 3-6 subsidiary cells [Da] and frequently, underlying palisade parenchyma [Dc]; uniseriate covering trichomes with 2 short, smooth-walled basal cells and a long terminal cell, bent at right angles, with a thick wall and a warty cuticle [A, B]; occasional glandular trichomes with a short, 2- or 3- celled stalk and ovoid, biseriate head with 4 indistinct cells [H]; fragments of the petals composed of cells with wavy walls [M]; fragments of vascular tissue from the stem [F, G], including large vessels [G], sometimes associated with unlignified septate fibres [Fa] and a sheath of parenchymatous cells containing prisms of calcium oxalate [Fb]; fragments of mesophyll [J] including some cells which may occasionally contain cluster crystals of calcium oxalate [Ja];

fragments of the stem epidermis with elongated, straight-walled cells and anomocytic (*2.8.3*) stomata [L]; fragments of the fibrous layer of the anthers (surface view [E], transverse section [K]); spherical or ovoid pollen grains about 25 µm long with 3 germinal pores and a smooth exine [C].

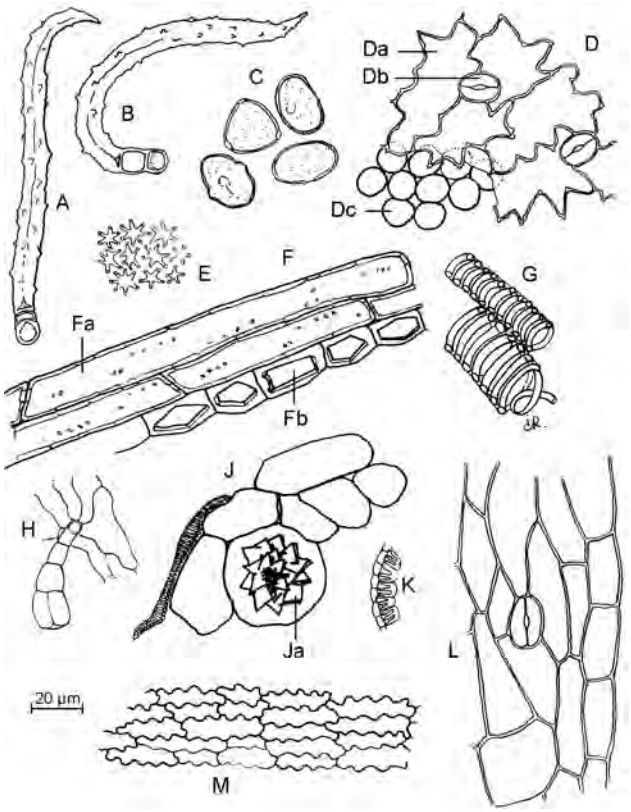


Figure 2120.-1. – *Illustration for identification test B of powdered herbal drug of melilot*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.3 g of the powdered herbal drug (355) (*2.9.12*) add 3 mL of *methanol R*. Heat on a water-bath at 100 °C for 1 min and filter.

*Reference solution.* Dissolve 50 mg of *coumarin CRS* and 20 mg of *o-coumaric acid R* in 50 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *dilute acetic acid R*, *ether R*, *toluene R* (10:50:50 *V/V/V*); use the upper layer.

*Application*: 25 µL [or 3 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *2 M alcoholic potassium hydroxide R* and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones of various colours may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Coumarin: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (coumarin) |
| | A blue fluorescent zone |
| *o*-Coumaric acid: a greenish-yellow fluorescent zone | A greenish-yellow fluorescent zone (*o*-coumaric acid) may be present |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of stems with a diameter greater than 3 mm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Completely reduce about 50 g of the herbal drug to a powder (500) (*2.9.12*). To 5.00 g of the powdered herbal drug add 90 mL of *methanol R* and boil under a reflux condenser for 30 min. Allow to cool. Filter under vacuum through a fibre-glass filter. Take up the residue and the fragmented filter with 90 mL of *methanol R*. Treat in the same manner as before. Combine the filtrates and dilute to 250.0 mL with *methanol R*.

*Reference solution.* Dissolve 25.0 mg of *coumarin CRS* in *methanol R* and dilute to 250.0 mL with the same solvent.

*Column*:

– *size*: $l$ = 0.25 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 5 g/L solution of *phosphoric acid R* (22:78 *V/V*).

*Flow rate*: 1.7 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*System suitability*:

– *retention time*: coumarin = about 7.8 min.

Calculate the percentage content of coumarin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to coumarin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to coumarin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *coumarin CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of coumarin in *coumarin CRS*.

01/2011:1447

# MELISSA LEAF

## Melissae folium

DEFINITION

Dried leaf of *Melissa officinalis* L.

*Content*: minimum 1.0 per cent of rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (dried drug).

CHARACTERS

Odour reminiscent of lemon.

IDENTIFICATION

A. The leaves have a petiole of varying length; the lamina is broadly ovate, up to about 8 cm long and 5 cm wide, acute at the apex and rounded to cordate at the base; the margins are crenate to dentate. The upper surface is intense green, the lower surface is paler green and shows a conspicuous midrib and a raised, reticulate venation; scattered hairs occur on the upper surface and along the veins on the lower surface, which is also finely punctuate.

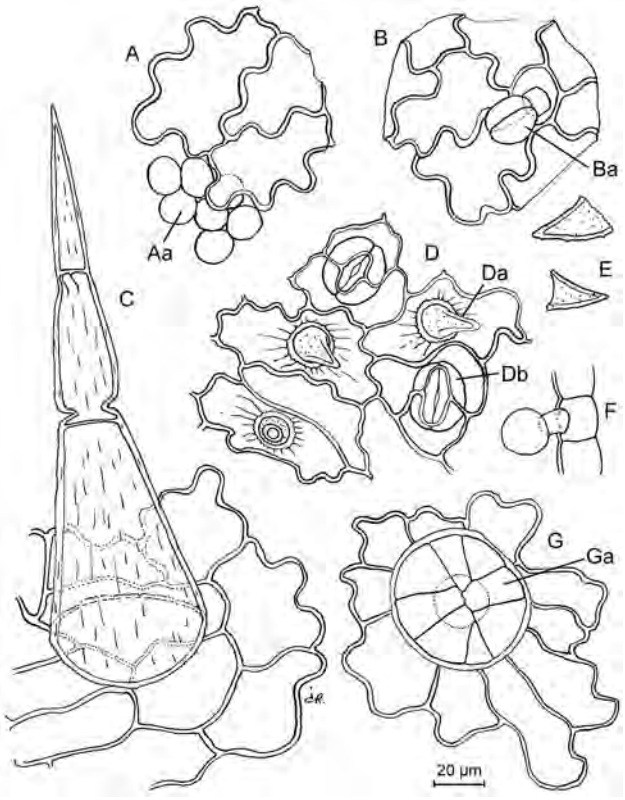


Figure 1447.-1.– *Illustration for identification test B of powdered herbal drug of melissa leaf*

B. Microscopic examination (*2.8.23*). The powder is greenish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1447.-1): fragments of the upper epidermis, in surface view, with sinuous walls [A, B, G], sometimes accompanied by palisade parenchyma [Aa]; fragments of the lower epidermis [D] with diacytic stomata (*2.8.3*) [Db]; short, straight, unicellular, conical covering trichomes with a finely striated cuticle, free [E] or attached to an epidermis [Da]; multicellular, uniseriate covering trichomes with pointed ends and thick, warty cuticles [C]; eight-celled secretory trichomes of lamiaceous type (surface view [Ga]); secretory trichomes with unicellular to tricellular stalks and unicellular or, more rarely, bicellular heads (surface view [Ba], transverse section [F]).

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Place 2.0 g of the powdered herbal drug (355) (*2.9.12*) in a 250 mL round-bottomed flask and add 100 mL of *water R*. Distil for 1 h using the apparatus for the determination of essential oils in herbal drugs (*2.8.12*) and 0.5 mL of *xylene R* in the graduated tube. After distillation transfer the organic phase to a 1 mL volumetric flask, rinsing the graduated tube of the apparatus with the aid of a small portion of *xylene R*, and dilute to 1.0 mL with the same solvent.

*Reference solution.* Dissolve 1.0 µL of *citronellal R* and 10.0 µL of *citral R* (composed of neral and geranial) in 25 mL of *xylene R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *hexane R* (10:90 *V/V*).

*Application*: 20 µL [or 4 µL] as bands.

*Development*: in an unsaturated tank over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10-15 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Citronellal: a grey or greyish-violet zone at the border between the upper and middle thirds | A grey or greyish-violet zone (citronellal) at the border between the upper and middle thirds<br>A reddish-violet zone |
| Citral: 2 greyish-violet or bluish-violet zones at the border between the middle and lower thirds | 2 greyish-violet or bluish-violet zones (citral) at the border between the middle and lower thirds |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 10 per cent of stems with a diameter greater than 1 mm and maximum 2 per cent of other foreign matter, determined on 20 g.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Use brown-glass flasks. Disperse 0.100 g of the powdered herbal drug (355) (*2.9.12*) in 90 mL of *ethanol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min, cool, and filter into a 100 mL volumetric flask. Rinse the flask and the filter with 10 mL of *ethanol (50 per cent V/V) R* and dilute to 100.0 mL with the same solvent. Filter through a 0.45 µm filter.

*Reference solution (a).* Dissolve 20.0 mg of *rosmarinic acid CRS* in *ethanol (50 per cent V/V) R* and dilute to 100.0 mL with the same solvent. Dilute 20.0 mL of this solution to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Reference solution (b).* Dissolve 5.0 mg of *ferulic acid R* in reference solution (a) and dilute to 50.0 mL with the same solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *acetonitrile R*, *water R* (1:19:80 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *methanol R*, *acetonitrile R* (1:40:59 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 55 | 0 → 45 |
| 20 - 25 | 55 → 0 | 45 → 100 |
| 25 - 30 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rosmarinic acid (retention time = about 11 min): ferulic acid = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to ferulic acid and rosmarinic acid.

Calculate the percentage content of rosmarinic acid using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rosmarinic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rosmarinic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rosmarinic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rosmarinic acid in *rosmarinic acid CRS*.

**01/2010:2524**

# MELISSA LEAF DRY EXTRACT

## Melissae folii extractum siccum

DEFINITION

Dry extract produced from *Melissa leaf (1447)*.

*Content*: minimum 2.0 per cent of rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using either hot water (not less than 70 °C) or a hydroalcoholic solvent that is at most equivalent in strength to ethanol (70 per cent *V/V*).

CHARACTERS

*Appearance*: brown or greenish-brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.2 g of the extract to be examined add 5 mL of *methanol R*. Sonicate for 5 min and filter.

*Reference solution.* Dissolve 1.0 mg of *hyperoside R*, 1.0 mg of *rutoside trihydrate R* and 5.0 mg of *rosmarinic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:6:90 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 8 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min, spray the plate whilst still hot with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weaker fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Rosmarinic acid: a light blue fluorescent zone | An intense light blue fluorescent zone (rosmarinic acid) |
| | A blue fluorescent zone |
| _____ | _____ |
| | A blue fluorescent zone |
| _____ | _____ |
| Hyperoside: an orange or greenish-yellow fluorescent zone | |
| | A light blue fluorescent zone |
| Rutoside: an orange or greenish-yellow fluorescent zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Use brown glass flasks. To 0.200 g of the extract to be examined add 50 mL of *ethanol (50 per cent V/V) R*. Sonicate for 10 min and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 20.0 mg of *rosmarinic acid CRS* in *ethanol (50 per cent V/V) R* and dilute to 100.0 mL with the same solvent. Dilute 20.0 mL of this solution to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Reference solution (b).* Dissolve 5 mg of *ferulic acid R* in reference solution (a) and dilute to 50 mL with reference solution (a).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *acetonitrile R*, *water R* (1:19:80 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *methanol R*, *acetonitrile R* (1:40:59 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 55 | 0 → 45 |
| 20 - 25 | 55 → 0 | 45 → 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rosmarinic acid (retention time = about 11 min): ferulic acid = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to ferulic acid and rosmarinic acid.

Calculate the percentage content of rosmarinic acid using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rosmarinic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rosmarinic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rosmarinic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rosmarinic acid in *rosmarinic acid CRS*.

**01/2019:2071**

# MILK THISTLE DRY EXTRACT, REFINED AND STANDARDISED

## Silybi mariani extractum siccum raffinatum et normatum

DEFINITION

Dry extract, refined and standardised, produced from *Milk thistle fruit (1860)*.

*Content*: 90 per cent to 110 per cent of the nominal content of silymarin, expressed as silibinin ($C_{25}H_{22}O_{10}$; $M_r$ 482.4), stated on the label. The nominal content of silymarin is within the range 30 per cent *m/m* to 65 per cent *m/m* (anhydrous extract).

The content of silymarin corresponds to:

– *sum of the contents of silicristin and silidianin* (both $C_{25}H_{22}O_{10}$; $M_r$ 482.4): 20 per cent to 45 per cent, calculated with reference to total silymarin;

– *sum of the contents of silibinin A and silibinin B* (both $C_{25}H_{22}O_{10}$; $M_r$ 482.4): 40 per cent to 65 per cent, calculated with reference to total silymarin;

– *sum of the contents of isosilibinin A and isosilibinin B* (both $C_{25}H_{22}O_{10}$; $M_r$ 482.4): 10 per cent to 20 per cent, calculated with reference to total silymarin.

PRODUCTION

The extract is produced from the herbal drug by an appropriate procedure, using one or more of the following solvents:

– ethyl acetate;

– acetone or mixture of acetone and water;

– ethanol or mixture of ethanol and water;

– methanol or mixture of methanol and water.

CHARACTERS

*Appearance*: yellowish-brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.250 g of the extract to be examined in 5 mL of *methanol R*.

*Reference solution.* Dissolve 2 mg of *silibinin R* and 5 mg of *taxifolin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (8.5:16.5:75 *V/V/V*).

*Application*: 10 µL [or 8 µL] of the test solution and 10 µL [or 2 µL] of the reference solution, as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: at 100-105 °C.

*Detection*: treat the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and subsequently treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other yellowish-green fluorescent zones may be present between the zones due to silibinin and taxifolin in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Silibinin: a yellowish-green fluorescent zone | A yellowish-green fluorescent zone (silibinin) |
| Taxifolin: an orange fluorescent zone | An orange fluorescent zone (taxifolin) |
| | A yellowish-green fluorescent zone (silicristin) |
| | A fluorescent zone (line of application) |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 60.0 mg of the extract to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution.* Dissolve a quantity of *milk thistle dry extract HRS* corresponding to 10.0 mg of silibinin in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *methanol R*, *water for chromatography R* (0.5:35:65 *V/V/V*);

– *mobile phase B*: *phosphoric acid R*, *methanol R*, *water for chromatography R* (0.5:50:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 28 | 100 → 0 | 0 → 100 |
| 28 - 35 | 0 | 100 |
| 35 - 36 | 0 → 100 | 100 → 0 |
| 36 - 51 | 100 | 0 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *milk thistle dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B. In the chromatogram obtained with the test solution the peak due to silidianin may vary in size or be absent.

*Retention time*: silibinin B = about 30 min; if necessary, adjust the time periods of the gradient.

*System suitability*: reference solution:

- *resolution*: minimum 1.8 between the peaks due to silibinin A and silibinin B;
- the chromatogram is similar to the chromatogram supplied with *milk thistle dry extract HRS*.

Calculate the percentage content of total silymarin, expressed as silibinin, using the following expression:

$$\frac{(A_1 + A_2 + A_3 + A_4 + A_5 + A_6) \times m_1 \times p}{(A_7 + A_8) \times m_2}$$

Calculate the percentage content of the sum of silicristin and silidianin, with reference to total silymarin, using the following expression:

$$\frac{(A_1 + A_2) \times 100}{A_1 + A_2 + A_3 + A_4 + A_5 + A_6}$$

Calculate the percentage content of the sum of silibinin A and silibinin B, with reference to total silymarin, using the following expression:

$$\frac{(A_3 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4 + A_5 + A_6}$$

Calculate the percentage content of the sum of isosilibinin A and isosilibinin B, with reference to total silymarin, using the following expression:

$$\frac{(A_5 + A_6) \times 100}{A_1 + A_2 + A_3 + A_4 + A_5 + A_6}$$

$A_1$ = area of the peak due to silicristin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to silidianin in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to silibinin A in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to silibinin B in the chromatogram obtained with the test solution;

$A_5$ = area of the peak due to isosilibinin A in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to isosilibinin B in the chromatogram obtained with the test solution;

$A_7$ = area of the peak due to silibinin A in the chromatogram obtained with the reference solution;

$A_8$ = area of the peak due to silibinin B in the chromatogram obtained with the reference solution;

$m_1$ = mass of *milk thistle dry extract HRS* used to prepare the reference solution, in grams;

$m_2$ = mass of the extract to be examined used to prepare the test solution, in grams;

$p$ = combined percentage content of silibinin A and silibinin B in *milk thistle dry extract HRS*.

**01/2014:1860**

# MILK THISTLE FRUIT

## Silybi mariani fructus

DEFINITION

Mature fruit, devoid of the pappus, of *Silybum marianum* L. Gaertn.

*Content*: minimum 1.5 per cent of silymarin, expressed as silibinin ($C_{25}H_{22}O_{10}$; $M_r$ 482.4) (dried drug).

CHARACTERS

No rancid odour.

IDENTIFICATION

A. The achene is strongly compressed, elongate-obovate, about 6-8 mm long, 3 mm broad and 1.5 mm thick; the outer surface is smooth and shiny with a grey or pale brown ground colour variably streaked dark brown longitudinally to give an overall pale greyish or brown colour; the fruit is tapering at the base and crowned at the apex with a glistening, pale yellow extension forming a collar about 1 mm high surrounding the remains of the style. Cut transversely, the fruit shows a narrow, brown outer area and 2 large, dense, white oily cotyledons.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow with darker specks. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epicarp composed of colourless cells, polygonal in surface view, the lumen appearing fairly large or as a small slit, depending on the orientation; groups of parenchymatous cells from the pigment layer, some of them containing colouring matter which appears bright red; very abundant groups of large sclereids from the testa with bright yellow pitted walls and a narrow lumen; occasionally fragments of small-celled parenchyma with pitted and beaded walls; abundant thin-walled parenchymatous cells from the cotyledons containing oil globules and scattered cluster crystals of calcium oxalate; a few larger, prismatic crystals of calcium oxalate.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (500) (*2.9.12*) add 10 mL of *methanol R*. Heat under reflux in a water-bath at 70 °C for 5 min. Cool and filter. Evaporate the filtrate to dryness and dissolve the residue in 1.0 mL of *methanol R*.

*Reference solution*. Dissolve 2 mg of *silibinin R* and 5 mg of *taxifolin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (8.5:16.5:75 *V/V/V*).

*Application*: 30 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C.

*Detection*: treat the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and subsequently treat with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to dry for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other orange and yellowish-green fluorescent zones are present between the zones due to silibinin and taxifolin in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Silibinin: a yellowish-green fluorescent zone | A yellowish-green fluorescent zone (silibinin) |
| Taxifolin: an orange fluorescent zone | An orange fluorescent zone (taxifolin)<br>A yellowish-green fluorescent zone (silicristin) |
| | A light blue fluorescent zone (line of application) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (500) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Place 5.00 g of the powdered herbal drug (500) (*2.9.12*) in a continuous-extraction apparatus. Add 100 mL of *light petroleum R* and heat in a water-bath for 8 h. Allow the defatted drug to dry at room temperature. In a continuous-extraction apparatus, extract the latter with 100 mL of *methanol R* in a water-bath for 5 h. Evaporate the methanolic extract *in vacuo* to a volume of about 30 mL. Filter into a 50 mL volumetric flask, rinsing the extraction flask and the filter, and diluting to 50.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 50.0 mL with *methanol R*.

*Reference solution*. Dissolve a quantity of *milk thistle dry extract HRS* corresponding to 10.0 mg of silibinin in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*; *methanol R*, *water R* (0.5:35:65 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*; *methanol R*, *water R* (0.5:50:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 28 | 100 → 0 | 0 → 100 |
| 28 - 35 | 0 | 100 |
| 35 - 36 | 0 → 100 | 100 → 0 |
| 36 - 51 | 100 | 0 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *milk thistle dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B. In the chromatogram obtained with the test solution the peak due to silidianin may vary in size, be absent or be present as the principal peak.

*Retention time*: silibinin B = about 30 min; if necessary, adjust the time periods of the gradient.

*System suitability*: reference solution:

- *resolution*: minimum 1.8 between the peaks due to silibinin A and silibinin B;
- the chromatogram obtained is similar to the chromatogram supplied with *milk thistle dry extract HRS*.

Calculate the percentage content of total silymarin, expressed as silibinin, using the following expression:

$$\frac{(A_1 + A_2 + A_3 + A_4 + A_5 + A_6) \times m_1 \times p \times 5}{(A_7 + A_8) \times m_2}$$

$A_1$ = area of the peak due to silicristin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to silidianin in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to silibinin A in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to silibinin B in the chromatogram obtained with the test solution;

$A_5$ = area of the peak due to isosilibinin A in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to isosilibinin B in the chromatogram obtained with the test solution;

$A_7$ = area of the peak due to silibinin A in the chromatogram obtained with the reference solution;

$A_8$ = area of the peak due to silibinin B in the chromatogram obtained with the reference solution;

$m_1$ = mass of *milk thistle dry extract HRS* used to prepare the reference solution, in grams;

$m_2$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$p$ = combined percentage content of silibinin A and silibinin B in *milk thistle dry extract HRS*.

**01/2008:1838**

# MINT OIL, PARTLY DEMENTHOLISED

## Menthae arvensis aetheroleum partim mentholum depletum

DEFINITION

Essential oil obtained by steam distillation from the fresh, flowering aerial parts, recently gathered from *Mentha canadensis* L. (syn. *M. arvensis* L. var. *glabrata* (Benth) Fern., *M. arvensis* var. *piperascens* Malinv. ex Holmes), followed by partial separation of menthol by crystallisation.

CHARACTERS

*Appearance*: colourless, pale yellow or greenish-yellow liquid.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 mL of the substance to be examined in 1.0 mL of *toluene R*.

*Reference solution.* Dissolve 4 µL of *carvone R*, 4 µL of *pulegone R*, 10 µL of *menthyl acetate R*, 20 µL of *cineole R* and 50 mg of *menthol R* in 5 mL of *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, a quenching zone may be present in the upper third of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Carvone and pulegone: a quenching zone | A quenching zone<br>A quenching zone |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min. Examine immediately in daylight.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, the zone due to cineole in the reference solution is absent in the chromatogram obtained with the test solution. No yellowish-brown zone below the intense reddish-violet zone is present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An intense reddish-violet zone (near the solvent front) |
| Menthyl acetate: a bluish-violet zone | A bluish-violet zone (menthyl acetate) |
| | A strongly greenish zone |
| | A greenish zone |
| Carvone and pulegone: a reddish zone | A reddish zone |
| Cineole: a violet zone | |
| | A distinctly violet zone |
| Menthol: an intense blue zone | A very intense blue zone (menthol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are approximately similar in retention time to those in the chromatogram obtained with the reference solution. Carvone may be absent from the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 0.888 to 0.910.

**Refractive index** (*2.2.6*): 1.456 to 1.470.

**Optical rotation** (*2.2.7*): − 16.0° to − 34.0°.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.00 g of the substance to be examined dissolved in 50 mL of the prescribed mixture of solvents.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 0.20 g of the substance to be examined in *hexane R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *limonene R*, 20 mg of *cineole R*, 40 mg of *menthone R*, 10 mg of *(+)-isomenthone R*, 40 mg of *menthyl acetate R*, 20 mg of *isopulegol R*, 60 mg of *menthol R*, 20 mg of *pulegone R* and 10 mg of *carvone R* in *hexane R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- *stationary phase*: *macrogol 20 000 R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 70 | 60 → 180 |
| | 70 - 75 | 180 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to limonene and cineole.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The percentages are within the following ranges:

– *limonene*: 1.5 per cent to 7.0 per cent,
– *cineole*: maximum 1.5 per cent,
– *menthone*: 17.0 per cent to 35.0 per cent,
– *isomenthone*: 5.0 per cent to 13.0 per cent,
– *menthyl acetate*: 1.5 per cent to 7.0 per cent,
– *isopulegol*: 1.0 per cent to 3.0 per cent,
– *menthol*: 30.0 per cent to 50.0 per cent,
– *pulegone*: maximum 2.5 per cent,
– *carvone*: maximum 2.0 per cent.

The ratio of cineole content to limonene content is less than 1.

STORAGE

At a temperature not exceeding 25 °C.

**01/2013:1833**

# MOTHERWORT

## Leonuri cardiacae herba

DEFINITION

Whole or cut, dried flowering aerial parts of *Leonurus cardiaca* L.

*Content*: minimum 0.2 per cent of flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The stem pieces are hairy, longitudinally striated, quadrangular, hollow, and up to about 10 mm wide; they bear opposite and decussate, petiolate leaves and, in the axils of the upper leaves, about 6-12 small flowers, arranged in sessile whorls forming a long, leafy spike. The lower leaves are ovate-orbicular, palmately 3- to 5-lobed, rarely 7-lobed, the lobes irregularly dentate. The upper leaves are entire or slightly trifid, lanceolate with a serrate margin and cuneate at the base. The upper surface of the leaves is green with scattered hairs, the lower surface is paler green, densely pubescent and shows a prominent palmate and reticulate venation. The flowers have a funnel-shaped calyx, 3 mm to 5 mm long with 5 stiff, recurved teeth; the corolla is 2-lipped, the upper lip pink and pubescent on the outer surface, the lower lip white with purplish spots; stamens 4, densely pubescent.

B. Microscopic examination (*2.8.23*). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1833.-1): numerous covering trichomes [A], whole [Aa] or fragmented [Ab], uniseriate, with warty walls, composed of 2-8 cells with slight swellings at the junctions, up to 1500 µm long; fragments of the upper epidermis (surface view [B]) with cells with straight or sinuous anticlinal walls [Ba], often accompanied by palisade parenchyma [Bb]; fragments of the lower epidermis (surface view [C]) with cells with sinuous anticlinal walls [Ca], diacytic stomata (*2.8.3*) [Cb], bearing glandular trichomes with a short unicellular stalk and a globular head composed of 8-16 cells [Cc], glandular trichomes with a uni- or bicellular stalk and a bi- or tetracellular head [Cd] and sometimes covering trichomes; fragments of the lamina, in transverse section [D], composed of epidermises bearing glandular trichomes with a globular head consisting of 8-16 cells [Da] or a bi- or tetracellular head [Db], a 1-layered palisade mesophyll extending almost halfway across the section [Dc], and a loosely arranged spongy parenchyma [Dd]; fragments of the calyx [G] with an epidermis consisting of polygonal cells bearing uni- or bicellular conical covering trichomes, with spiny walls [Ga], often associated with fusiform mesophyll cells with thick walls and containing small prism crystals of calcium oxalate [Gb]; isolated glandular trichomes [H], either with a multicellular stalk and a unicellular head from the anthers [Ha] or a uni- or multicellular stalk and bi- to tetracellular head [Hb]; spherical pollen grains, about 25-30 µm in diameter, with 3 pores and 3 furrows and a smooth exine [E]; thick-walled, lignified fibres [F]; fragments from the stem with spirally and annularly thickened vessels [K]; occasional fragments of pericarp [J] consisting of lobed cells with thick, pitted walls, each containing a single prism crystal of calcium oxalate [Ja].

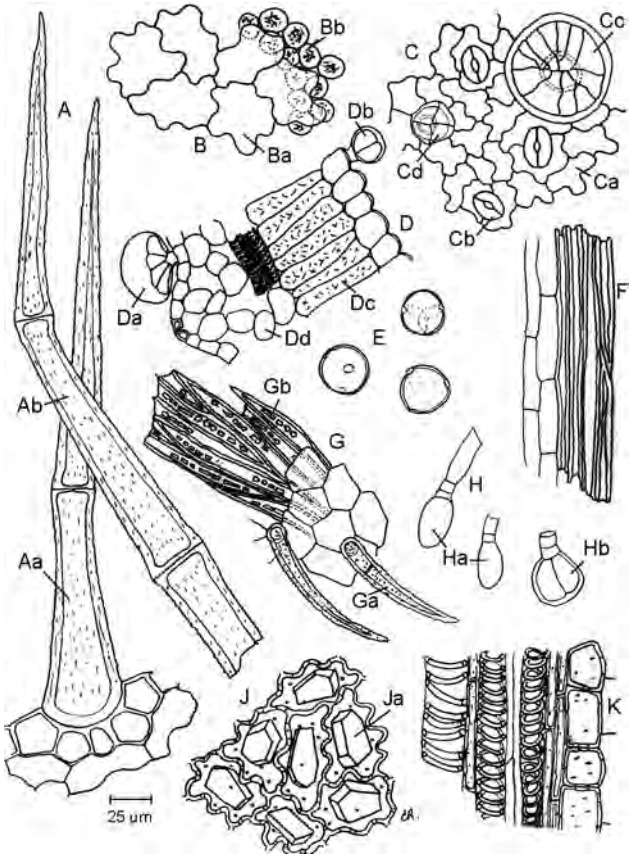


Figure 1833.-1. – *Illustration for identification test B of powdered herbal drug of motherwort*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Heat on a water-bath at 65 °C for 5 min with shaking. Cool and filter.

*Reference solution.* Dissolve 5 mg of *naphthol yellow S R* and 2.0 mg of *catalpol R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R* (20:20:60 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with *dimethylaminobenzaldehyde solution R2*, using about 5 mL for a plate 200 mm square; heat at 100-105 °C for 10 min until the spots appear; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak greyish-blue zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A wide white zone |
| | A greyish-blue zone (iridoid) |
| ——— | ——— |
| Naphthol yellow S: an intense yellow zone<br>Catalpol: a greyish-blue zone | 1 or 2 greyish-blue zones (iridoid) |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of brown or yellow leaves and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask place 1.00 g of the powdered herbal drug (355) (*2.9.12*), add 1 mL of a 5 g/L solution of *hexamethylenetetramine R*, 20 mL of *acetone R* and 2 mL of *hydrochloric acid R1*. Boil the mixture under a reflux condenser for 30 min. Filter the liquid through a plug of absorbent cotton into a flask. Add the absorbent cotton to the residue in the round-bottomed flask and extract with 2 quantities, each of 20 mL, of *acetone R*, each time boiling under a reflux condenser for 10 min. Allow to cool and filter each extract through the plug of absorbent cotton into the flask. After cooling, filter the combined acetone extracts through a paper filter into a volumetric flask and dilute to 100.0 mL with *acetone R* by rinsing the flask and the paper filter. Introduce 20.0 mL of the solution into a separating funnel, add 20 mL of *water R* and shake the mixture with 1 quantity of 15 mL and then 3 quantities, each of 10 mL, of *ethyl acetate R*. Combine the ethyl acetate extracts in a separating funnel, wash with 2 quantities, each of 50 mL, of *water R*, filter the extracts over 10 g of *anhydrous sodium sulfate R* into a volumetric flask and dilute to 50.0 mL with *ethyl acetate R*.

*Test solution.* To 10.0 mL of the stock solution add 1 mL of *aluminium chloride reagent R* and dilute to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

*Compensation liquid.* Dilute 10.0 mL of the stock solution to 25.0 mL with a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 30 min, by comparison with the compensation liquid at 425 nm. Calculate the percentage content of flavonoids, calculated as hyperoside, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of hyperoside to be 500.

$A$ = absorbance at 425 nm;

$m$ = mass of the substance to be examined, in grams.

**04/2018:2474**

# MOUTAN BARK

## Moutan cortex

DEFINITION

Dried, whole or fragmented, scraped or unscraped root bark of *Paeonia* ×*suffruticosa* Andrews, collected in autumn.

*Content*:

– *paeonol* ($C_9H_{10}O_3$; $M_r$ 166.2): minimum 2.2 per cent (dried drug);

– *paeoniflorin* ($C_{23}H_{28}O_{11}$; $M_r$ 480.5): minimum 1.1 per cent (dried drug).

IDENTIFICATION

A. The whole bark is quilled or crescent-shaped, about 2-4 mm thick, 0.6-1.4 cm in diameter and up to 20 cm long. The fragmented bark occurs as cleft rings, about 2-4 mm thick and 0.5-1 cm in diameter. The greyish-brown or yellowish-brown outer surface of the unscraped bark shows long transverse lenticels and rootlet scars; where the dermal tissue is absent, the exposed surface is pink. The outer surface of the scraped bark is pinkish to greyish-white; transverse lenticels and rootlet scars are present; sometimes remains of the outer bark are visible. The pale greyish-yellow or pale brown inner surface shows fine longitudinal striations. The texture is hard and fragile, easily broken. The fracture is greyish-white to pinkish or light brown and finely granular.

B. Microscopic examination (*2.8.23*). The powder is pale brown to pinkish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2474.-1): numerous fragments of parenchyma consisting of cells of various shapes and sizes: some rounded with slightly and regularly thickened and pitted walls [D], some with irregularly thickened beaded walls [J] and some [H] containing one [Ha] or more [Hb] cluster crystals of calcium oxalate up to 45 µm in diameter; files of cells containing cluster crystals of calcium oxalate may be present [A];

very numerous free cluster crystals of calcium oxalate [C]; more or less abundant fragments of cork with superimposed polyhedral cells (surface view [B], longitudinal section [E]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous rounded or polygonal starch granules about 3-15 µm in diameter, simple or 2-6 compound, free [G] or included in parenchyma cells [F]; the punctiform, cleft, V-, Y-, or X-shaped hilum is clearly visible.

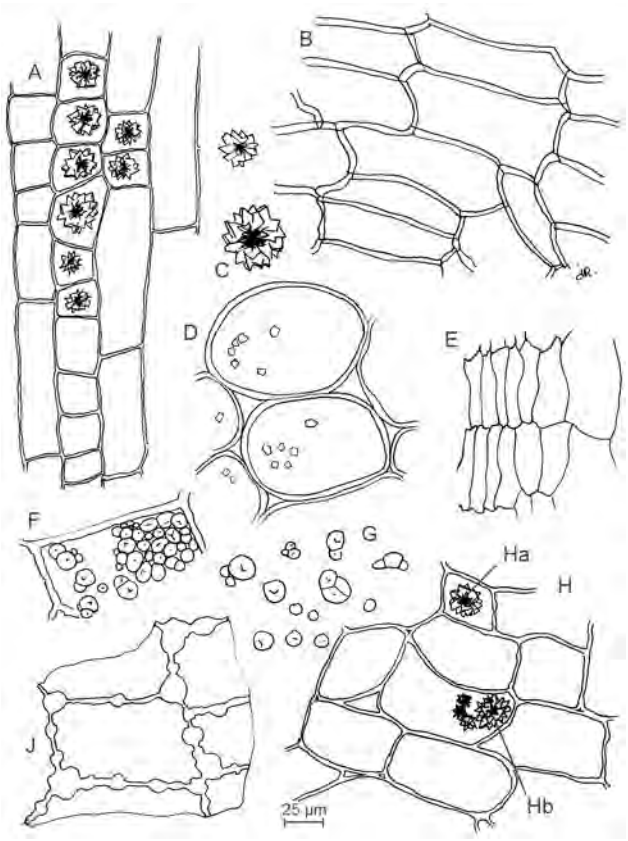


Figure 2474.-1. – *Illustration for identification test B of powdered herbal drug of moutan bark*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and heat in a water-bath at 60 °C for 1 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *paeoniflorin R* and 1 mg of *paeonol R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *methanol R*, *methylene chloride R* (3:5:5:35 *V/V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a quenching zone | A quenching zone (paeonol) |
| | A prominent quenching zone |
| _____ | _____ |
| | A prominent quenching zone |
| | A quenching zone |
| | A quenching zone |
| _____ | _____ |
| Paeoniflorin: a quenching zone | A quenching zone (paeoniflorin) |
| | A quenching zone |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *ethanol (96 per cent) R* and heat at 100 °C for 5 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a yellow zone | A yellow zone (paeonol) |
| | 2 violet zones |
| _____ | _____ |
| | A sharp, prominent violet zone |
| | 2 violet zones |
| | A diffuse yellowish-brown zone |
| | A yellowish-brown zone |
| _____ | _____ |
| Paeoniflorin: a brown zone | A brown zone (paeoniflorin) |
| | A brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 1.0 per cent.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.200 g of the powdered herbal drug (355) (*2.9.12*) add 3.0 mL of *methanol R1* and sonicate for 30 min. Dilute to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *paeoniflorin CRS* in *methanol R1* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dissolve 7.0 mg of *paeonol CRS* in *methanol R1* and dilute to 10.0 mL with the same solvent. Dilute 3.0 mL of the solution to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Reference solution (c).* Dissolve 2.5 mg of *4′-hydroxyacetophenone R* in 2.5 mL of reference solution (a), add 12.5 mL of *methanol R1* and dilute to 50.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Reference solution (d).* Dilute 2.5 mL of reference solution (a) to 7.5 mL with *methanol R1*. Dilute this solution to 25.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Column:*

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase:*

- *mobile phase A*: 6.8 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 70 | 30 |
| 15 - 18 | 70 → 40 | 30 → 60 |
| 18 - 25 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Retention time*: paeoniflorin = about 9.5 min; 4′-hydroxyacetophenone = about 10.5 min; paeonol = about 23 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to paeoniflorin and 4′-hydroxyacetophenone.

Calculate the percentage content of paeoniflorin using the following expression:

$$\frac{A_1 \times m_2 \times p_1}{A_2 \times m_1 \times 5}$$

$A_1$ = area of the peak due to paeoniflorin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to paeoniflorin in the chromatogram obtained with reference solution (d);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *paeoniflorin CRS* used to prepare reference solution (a), in grams;

$p_1$ = percentage content of paeoniflorin in *paeoniflorin CRS*.

Calculate the percentage content of paeonol using the following expression:

$$\frac{A_3 \times m_3 \times p_2 \times 0.3}{A_4 \times m_1}$$

$A_3$ = area of the peak due to paeonol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to paeonol in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_3$ = mass of *paeonol CRS* used to prepare reference solution (b), in grams;

$p_2$ = percentage content of paeonol in *paeonol CRS*.

**01/2011:1853**

# MULLEIN FLOWER

## Verbasci flos

DEFINITION

Dried flower, reduced to the corolla and the androecium, of *Verbascum thapsus* L., *V. densiflorum* Bertol. (*V. thapsiforme* Schrad), and *V. phlomoides* L.

IDENTIFICATION

A. The corolla of *V. thapsus* is pale yellow, yellow or brown, funnel-shaped, about 20 mm in diameter, with 5 slightly unequal and spreading lobes. The corolla lobes are densely hairy on the outer surface, glabrous on the inner surface, with a fine network of light brown veins. There are 5 stamens, alternating with the petal lobes; 2 of these are long, with glabrous filaments, the other 3 shorter, with densely tomentose filaments. The anthers are attached transversely. In *V. phlomoides* the corolla is up to about 30 mm in diameter, bright yellow or orange, and the anthers are obliquely attached to the filaments. The corolla of *V. densiflorum*, about 30 mm in diameter, is almost flat and deeply divided into 5 slightly unequal lobes, with rounded apices.

B. Microscopic examination (*2.8.23*). The powder is yellow or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1853.-1): many covering trichomes from the corolla, whole and fragmented, pluricellular, of the candelabra type, with a central uniseriate axis from which whorls of branch cells arise at the position of the cross walls and at the apex (side view [A, B], surface view [F]); the covering trichomes from the stamen filaments [G] are unicellular, long, thin-walled and tubular, have a distinctly granular or striated surface with a sharp tip [Ga] or sometimes with a club-shaped tip [Gb, Gc]; numerous pollen grains, ovoid with a finely granular exine with 3 pores [D]; fragments of the fibrous layer of the anther with thickened walls giving a characteristic star-shaped appearance [C]; yellow fragments of the petals (surface view [E]) with polygonal and isodiametric epidermal cells [Ea]; fragments of the underlying mesophyll consisting of irregular parenchymatous cells [Eb] sometimes accompanied by spiral vessels [Ec].

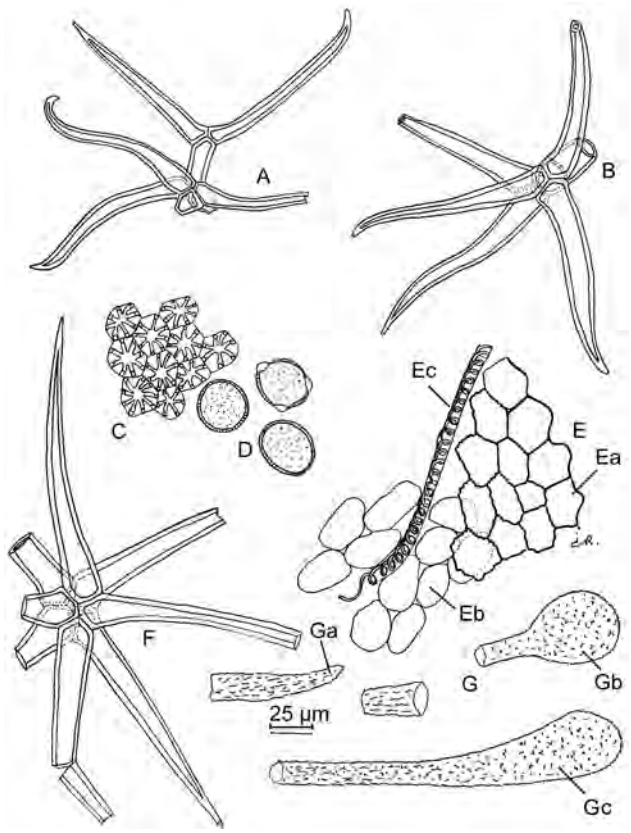


Figure 1853.-1. – *Illustration for identification test B of powdered herbal drug of mullein flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Heat 1.0 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *methanol R* in a water-bath at 60 °C for 5 min, with stirring. Cool and filter.

*Reference solution*. Dissolve 1 mg of *caffeic acid R*, 2.5 mg of *hyperoside R* and 2.5 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL of the reference solution and 30 µL of the test solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C.

*Detection*: spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for 30 min and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A yellow or yellowish-green fluorescent zone |
| Caffeic acid: a greenish-blue fluorescent zone | |
| | A bluish fluorescent zone |
| | A greenish fluorescent zone |
| | A yellowish-green fluorescent zone |
| | A bluish fluorescent zone |
| Hyperoside: a yellowish-brown fluorescent zone | |
| | A greenish fluorescent zone |
| Rutoside: a yellowish-brown fluorescent zone | |
| **Reference solution** | **Test solution** |

D. Boil 1.0 g of the powdered herbal drug (355) (*2.9.12*) with 15 mL of *water R* for 1 min. Filter. Add 1 mL of *hydrochloric acid R* and boil for 1 min. A greenish-blue colour develops and, after a few minutes, cloudiness appears and then a blackish precipitate (iridoids).

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of brown petals and maximum 2 per cent of fragments of the calyx and other foreign matter, determined on 20 g.

**Swelling index** (*2.8.4*): minimum 9, determined on the powdered herbal drug (710) (*2.9.12*), moistened with 2 mL of *ethanol (96 per cent) R*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

STORAGE

In an airtight container.

**07/2017:1349**

# MYRRH

## Myrrha

DEFINITION

Gum-resin, hardened in air, obtained by incision or produced by spontaneous exudation from the stem and branches of *Commiphora myrrha* (Nees) Engl. (syn. *Commiphora molmol* (Engl.) Engl. ex Tschirch) and/or other species of *Commiphora*.

CHARACTERS

Bitter taste.

IDENTIFICATION

A. The light or dark orange-brown, irregular or roundish grains or pieces of different size show components of various colours. Their surface is mostly covered with grey or yellowish-brown dust.

B. Microscopic examination (*2.8.23*). The powder is brownish-yellow or reddish-brown. Examine under a microscope, using *chloral hydrate solution R*. The powder shows the following diagnostic characters: a few tissue fragments from the original plants including: reddish-brown cork fragments; single or grouped

polyhedral or elongated stone cells with partly strongly thickened, pitted and lignified walls with a brownish content; fragments of thin-walled parenchyma and sclerenchymatous fibres; irregular prismatic or polyhedral crystals of calcium oxalate, about 10-25 µm in size.

C. Examine the chromatograms obtained in the test for *Commiphora mukul*.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish-violet zone |
| Anethole: a violet zone | |
| _____ | _____ |
| | A red zone |
| Thymol: an orange zone | |
| | A violet zone |
| | A brown zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

TESTS

**Commiphora mukul**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.2 g of the powdered herbal drug (355) (*2.9.12*) add 4.0 mL of *ethanol (96 per cent) R*, sonicate for 10 min and filter.

*Reference solution*. Dissolve 10 mg of *thymol R* and 40 µL of *anethole R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *cyclohexane R* (10:40:60 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 366 nm.

*Results A*: the chromatogram obtained with the test solution shows no blue or violet fluorescent zones in the lower third of the chromatogram.

*Detection B*: treat with *anisaldehyde solution R*, heat at 100 °C for 2 min and examine in daylight.

**Matter insoluble in ethanol**: maximum 75 per cent.

Place 1.00 g of the powdered herbal drug (250) (*2.9.12*) in a flask. Add 30 mL of *ethanol (96 per cent) R* and shake vigorously for 10 min. Filter the supernatant through a tared sintered-glass filter (16) (*2.1.2*), avoiding the transfer of sediment from the flask. Repeat the extraction with 2 quantities, each of 20 mL, of *ethanol (96 per cent) R*. Quantitatively transfer the sediment to the filter by rinsing the flask with *ethanol (96 per cent) R*. Dry the filter and the residue in an oven at 100-105 °C and weigh.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**07/2017:1877**
**corrected 10.0**

# MYRRH TINCTURE

## Myrrhae tinctura

DEFINITION

Tincture produced from *Myrrh (1349)*.

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (90 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: clear, yellowish-brown or orange-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 5 mL of the tincture to be examined to 10 mL with *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 10 mg of *thymol R* and 40 µL of *anethole R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *di-isopropyl ether R*, *cyclohexane R* (10:40:60 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100 °C for 2 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish-violet zone |
| Anethole: a violet zone | |
| _____ | _____ |
| | A red zone |
| Thymol: an orange zone | |
| | A violet zone |
| | A brown zone |
| _____ | _____ |
| **Reference solution** | **Test solution** |

Herbal drugs

TESTS

**Ethanol** (*2.9.10*): 82 per cent *V/V* to 88 per cent *V/V*.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

**Dry residue** (*2.8.16*): minimum 4.0 per cent.

STORAGE

Plastic containers are not recommended.

**01/2008:1821 corrected 6.0**

# NARROW-LEAVED CONEFLOWER ROOT

## Echinaceae angustifoliae radix

DEFINITION

Dried, whole or cut underground parts of *Echinacea angustifolia* (D.C.).

*Content*: minimum 0.5 per cent of echinacoside ($C_{35}H_{46}O_{20}$; $M_r$ 786.5) (dried drug).

IDENTIFICATION

*First identification*: *A, B, C.*

*Second identification*: *A, B, D.*

A. The root crown is up to about 30 mm in diameter and shows only a few stem bases. The roots are not very numerous, up to about 15 mm in diameter, cylindrical or slightly tapering and sometimes spirally twisted, the outer surface is pale brown to yellowish-brown. The fracture is short, dark brown with a radiate structure.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: narrow lignified fibres (up to about 800 µm in length and 50 µm in diameter) joined together in long bundles surrounded by phytomelanin deposits; lignified reticulately or scalariformly thickened vessels (up to about 60 µm in diameter); abundant sclereids occuring singly or, more usually, in groups of 2 to 10, mostly elongated to rectangular, (up to about 150 µm in length and 40 µm wide), with intercellular spaces filled with phytomelanin deposit; fragments of oleoresin canal (80-150 µm in diameter) with yellowish-orange to reddish-brown content; groups of squarish to rectangular cells, about 30-45 µm from the outer layers of the roots; abundant fine-walled pitted parenchyma with sphaerocrystalline masses of inulin.

C. Examine the chromatograms obtained in the test for *Echinacea purpurea*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint dark blue fluorescent zones may be present between the zones of echinacoside and cynarin in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a strong blue fluorescent zone | |
| Cynarin: a strong greenish fluorescent zone | A greenish fluorescent zone (cynarin) |
| Echinacoside: a strong greenish fluorescent zone | A strong greenish fluorescent zone (echinacoside) |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay.

*Results*: the chromatogram obtained with the test solution shows 1 major peak due to echinacoside and a minor peak due to cynarin. Peaks due to caffeic acid, caftaric acid and chlorogenic acid are minor peaks or may be absent.

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

***Echinacea purpurea***. Thin layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*, treat in an ultrasonic bath for 5 min. Centrifuge and use the supernatant solution.

*Reference solution*. Dissolve 1 mg of *echinacoside R*, 1 mg of *cynarin R* and 0.5 mg of *caffeic acid R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (3:3:9:15 *V/V/V/V*).

*Application*: 25 µL [or 5 µL] of the test solution and 10 µL [or 2 µL] of the reference solution as bands.

*Development*: over a path of 15 cm [or 5 cm].

*Drying*: in a stream of cold air for about 10 min followed by 2 min at 100-105 °C.

*Detection*: treat the hot plate using a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*; examine in ultraviolet light at 365 nm after 30 min.

*Results*: the chromatogram obtained with the test solution shows no greenish fluorescent zone just below the zone due to caffeic acid in the chromatogram obtained with the reference solution and no greenish fluorescent zone below the zone due to cynarin in the chromatogram obtained with the reference solution. In the chromatogram obtained with the test solution no zones apart from faint dark blue fluorescent zones are visible between the zones due to echinacoside and cynarin.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. In a 100 mL volumetric flask place 0.500 g of the powdered herbal drug (355) (*2.9.12*) and add 80 mL of *ethanol (70 per cent V/V) R*. Treat in an ultrasonic bath for 15 min and dilute to 100.0 mL with *ethanol (70 per cent V/V) R*. Mix the suspension and allow to stand for a few minutes so that visible solids settle. Filter a suitable proportion of the solution through a membrane filter (nominal pore size 0.45 µm) before injection.

*Reference solution*. Dissolve 10.0 mg of *chlorogenic acid CRS* and 10.0 mg of *caffeic acid R* in *ethanol (70 per cent V/V) R*, sonicate for 15 min and dilute to 10.0 mL with the same solvent. Dilute 4.0 mL of this solution to 100.0 mL with *ethanol (70 per cent V/V) R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water R* (1:999 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 90 | 10 |
| 0 - 13 | 90 → 78 | 10 → 22 |
| 13 - 14 | 78 → 60 | 22 → 40 |
| 14 - 14.5 | 60 | 40 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 10 µL.

*Relative retention* with reference to chlorogenic acid: caftaric acid = about 0.8; caffeic acid = about 1.5; cynarin = about 1.6; echinacoside = about 1.7; cichoric acid = about 2.3.

*System suitability*: reference solution:

- *resolution*: minimum 10 between the peaks due to caffeic acid and chlorogenic acid.

Locate the peaks due to caffeic acid and chlorogenic acid using the chromatogram obtained with the reference solution. Locate the peaks due to echinacoside and cynarin using the chromatogram in Figure 1821.-1.

Calculate the percentage content of echinacoside from the following expression:

$$\frac{A_1 \times C_2 \times 100 \times 2.221}{A_2 \times C_1}$$

$A_1$ = area of the peak due to echinacoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the reference solution;

$C_1$ = concentration of the test solution, in milligrams per millilitre;

$C_2$ = concentration of chlorogenic acid in the reference solution, in milligrams per millilitre;

2.221 = peak correlation factor between chlorogenic acid and echinacoside.

STORAGE

Store uncomminuted.

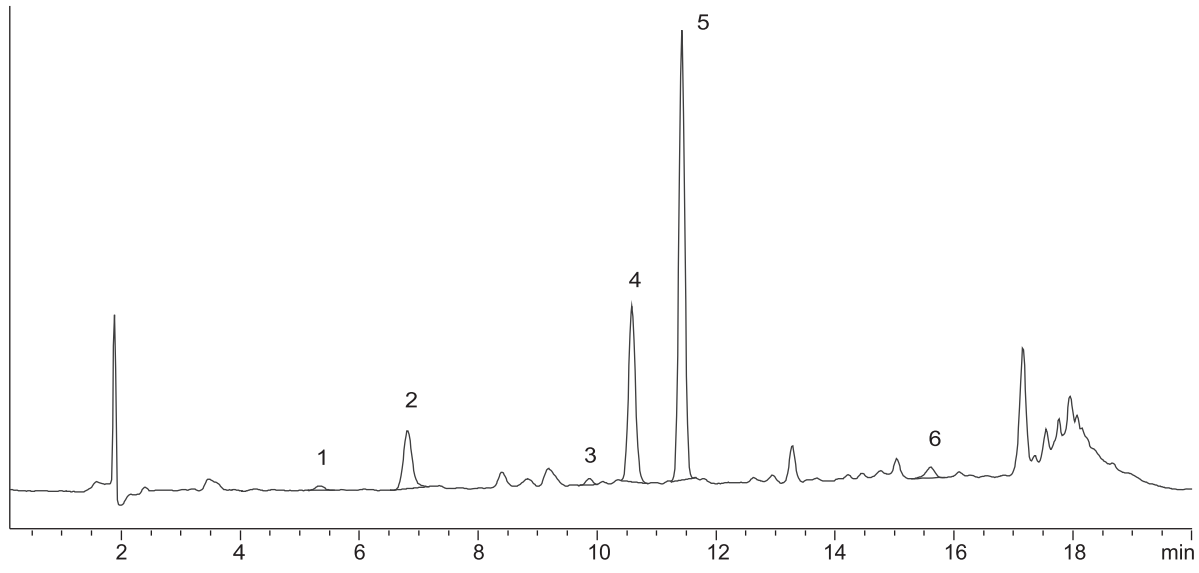


1. caftaric acid
2. chlorogenic acid
3. caffeic acid
4. cynarin
5. echinacoside
6. cichoric acid

Figure 1821.-1. – *Chromatogram for the assay of echinacoside in narrow-leaved coneflower root*

01/2008:1175

# NEROLI OIL

## Neroli aetheroleum

DEFINITION

Neroli oil is obtained by steam distillation from the fresh flowers of *Citrus aurantium* L. subsp. *aurantium* L. (*C. aurantium* L. subsp. *amara* Engl.).

CHARACTERS

*Appearance*: clear, pale-yellow or dark-yellow liquid.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Examine the chromatograms obtained in the test for bergapten.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore other zones may be present in the chromatograms obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Methyl anthranilate: a blue fluorescent zone | A faint blue fluorescent zone (methyl anthranilate) |
| Bergapten: a greenish-yellow fluorescent zone | |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *anisaldehyde solution R*; heat at 100-105 °C for 10 min; examine the chromatograms in ultraviolet light at 365 nm.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. In the chromatogram obtained with the test solution the zone due to linalol is more intense than the zone due to linalyl acetate.

| Top of the plate | |
|---|---|
| | A brown fluorescent zone |
| Linalyl acetate: a brownish-red fluorescent zone | An intense brownish-red fluorescent zone (linalyl acetate) |
| _____ | _____ |
| Methyl anthranilate: a blue fluorescent zone | A faint blue fluorescent zone (methyl anthranilate) |
| | A faint brownish-red fluorescent zone |
| Linalol: a brownish-red fluorescent zone | A brownish-red fluorescent zone (linalol) |
| Bergapten: a greenish-yellow fluorescent zone | |
| _____ | _____ |
| | Several blue and brownish-red fluorescent zones |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to the principal peaks in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.863 to 0.880.

**Refractive index** (*2.2.6*): 1.464 to 1.474.

**Optical rotation** (*2.2.7*): + 1.5° to + 11.5°.

**Acid value** (*2.5.1*): maximum 2.0.

**Bergapten**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Dissolve 2 µL of *methyl anthranilate R*, 10 µL of *linalyl acetate R*, 20 µL of *linalol R* and 5 mg of *bergapten R* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (15:85 *V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution does not show a zone corresponding to the zone due to bergapten in the chromatogram obtained with the reference solution.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution (a).* Dissolve 20 µL of *β-pinene R*, 5 mg of *sabinene R*, 40 µL of *limonene R*, 40 µL of *linalol R*, 20 µL of *linalyl acetate R*, 5 mg of *α-terpineol R*, 5 µL of *neryl acetate R*, 5 µL of *geranyl acetate R*, 5 µL of *trans-nerolidol R*, 5 µL of *methyl anthranilate R* and 5 µL *(E,E)-farnesol R* in 2 mL of *heptane R*.

*Reference solution (b).* Dissolve 5 µL of *methyl anthranilate R* in *heptane R* and dilute to 10 mL with the same solvent.

*Column*:

- *material*: fused silica,
- *size*: *l* = 60 m, Ø = 0.25 mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 75 |
| | 4 - 42.8 | 75 → 230 |
| | 42.8 - 63 | 230 |
| Injection port | | 270 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of reference solution (a). Record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to β-pinene and sabinene.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

*Limits*:

- *β-pinene*: 7.0 per cent to 17.0 per cent,
- *limonene*: 9.0 per cent to 18.0 per cent,
- *linalol*: 28.0 per cent to 44.0 per cent,
- *linalyl acetate*: 2.0 per cent to 15.0 per cent,
- *α-terpineol*: 2.0 per cent to 5.5 per cent,
- *neryl acetate*: maximum 2.5 per cent,
- *geranyl acetate*: 1.0 per cent to 5.0 per cent,
- *trans-nerolidol*: 1.0 per cent to 5.0 per cent,
- *methyl anthranilate*: 0.1 per cent to 1.0 per cent,
- *(E,E)-farnesol*: 0.8 per cent to 4.0 per cent,
- *disregard limit*: area of the peak in the chromatogram obtained with reference solution (b).

**Chiral purity**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *pentane R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. To 10 µL of *linalol R* add 10 µL of *linalyl acetate R*. Dilute to 10.0 mL with *pentane R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 25 m, Ø = 0.25 mm,
- *stationary phase*: *modified β-cyclodextrin for chiral chromatography R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 65 | 50 → 180 |
| Injection port | | 230 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

- *resolution*: minimum 5.5 between the peaks due to (*R*)(–)-linalol (1st peak) and (*S*)(+)-linalol (2nd peak); minimum 2.7 between the peaks due to (*R*)(–)-linalyl acetate (3rd peak) and (*S*)(+)-linalyl acetate 4th peak).

Calculate the percentage content of the specified (*S*)-enantiomers from the following expression:

$$\frac{A_1}{A_1 + A_2} \times 100$$

$A_1$ = area of the corresponding (*S*)-enantiomer,

$A_2$ = area of the corresponding (*R*)-enantiomer.

*Limits*:

- (*S*)(+)-linalol: maximum 30 per cent,
- (*S*)(+)-linalyl acetate: maximum 5 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**01/2011:1897**

# NETTLE LEAF

## Urticae folium

DEFINITION

Whole or cut dried leaves of *Urtica dioica* L., *Urtica urens* L., or a mixture of the 2 species.

*Content*: minimum 0.3 per cent for the sum of caffeoylmalic acid and chlorogenic acid, expressed as chlorogenic acid ($C_{16}H_{18}O_9$; $M_r$ 354.3) (dried drug).

IDENTIFICATION

A. The leaves are dark green, dark greyish-green or brownish-green on the upper surface, paler on the lower surface; scattered stinging hairs occur on both surfaces, also small covering trichomes that are more numerous along the margins and on the veins on the lower surface. The lamina is strongly shrunken, ovate or oblong, up to 100 mm long and 50 mm wide, with a coarsely serrate margin and a cordate or rounded base. The venation is reticulate and distinctly prominent on the lower surface. The petiole is green or brownish-green, rounded or flattened, about 1 mm wide, longitudinally furrowed and twisted; it bears stinging hairs and covering trichomes.

B. Microscopic examination (*2.8.23*). The powder is green or greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1897.-1): fragments of unicellular stinging hairs [A, B, C], up to 2 mm long, composed of an elongated tapering cell with a slightly swollen stinging tip that readily breaks off, arising from a raised, multicellular base [Ca]; small glandular trichomes [F] (35-65 µm), with a uni- or bicellular stalk and a bi- or quadricellular head, isolated [Fa], or on fragments of the epidermis [Fb]; fragments of the upper epidermis of the leaves (surface view [G], transverse section [D]) showing slightly sinuous cells [Da, Gc], unicellular, straight or slightly curved covering trichomes, enlarged at the base, up to 700 µm long [Dc, Ga] and abundant large cystoliths [Db, Ea, Gb], empty or containing dense, granular masses of calcium carbonate; palisade parenchyma (surface view [E]) with rounded cells [Eb] surrounding cystoliths [Ea] (transverse section [Dd]); fragments of lower epidermis of leaves showing sinuous or wavy-walled cells [H], anomocytic [Ha] or anisocytic stomata [Hb] (*2.8.3*) accompanied by spongy mesophyll (surface view [Hc],

transverse section [De]) containing small cluster crystals of calcium oxalate (surface view [Hd], transverse section [Df]); occasional small groups of vessels, accompanied by parenchyma containing cluster crystals of calcium oxalate [J].

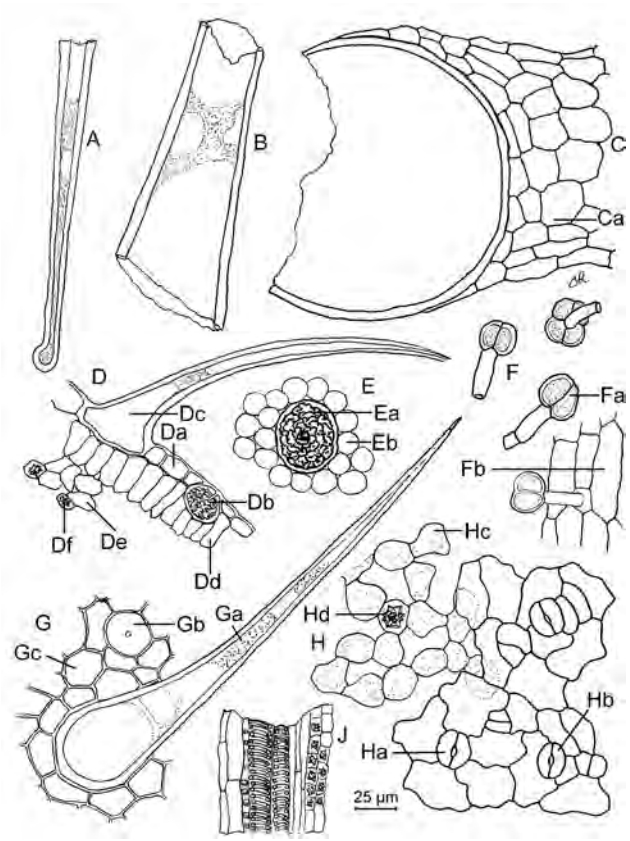


Figure 1897.-1. – *Illustration for identification test B of powdered herbal drug of nettle leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Boil under a reflux condenser for 15 min. Cool and filter. Evaporate to dryness *in vacuo* at 40 °C. Dissolve the residue in 2 mL of *methanol R*.

*Reference solution.* Dissolve 1 mg of *scopoletin R* and 2 mg of *chlorogenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *methanol R*, *water R*, *ethyl acetate R* (2.5:4:4:50 *V/V/V/V*).

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 8 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 5 min; spray the still-warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint blue or yellow fluorescent zones may be present in the lower half of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 red zones |
| Scopoletin: an intense blue fluorescent zone | A blue fluorescent zone (scopoletin) |
| | A blue fluorescent zone |
| _____ | _____ |
| _____ | _____ |
| Chlorogenic acid: a blue fluorescent zone | A blue fluorescent zone (chlorogenic acid) |
| | A brownish-yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems and maximum 5 per cent of other foreign matter (including inflorescences).

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 20.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.200 g of the powdered herbal drug (355) (*2.9.12*) add 25.0 mL of a 40 per cent *V/V* solution of *methanol R*. Extract for 30 min in an ultrasonic bath at 40 °C and filter.

*Reference solution.* Dissolve 10.0 mg of *chlorogenic acid CRS* in 100.0 mL of a 40 per cent *V/V* solution of *methanol R*. Dilute 5.0 mL of this solution to 25.0 mL with a 40 per cent *V/V* solution of *methanol R*.

*Precolumn*:

- *size*: *l* = 4 mm, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: mix 15 volumes of *methanol R* and 85 volumes of *water R* and adjust to pH 2.0 with *dilute phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 1 - 25 | 100 → 85 | 0 → 15 |
| 25 - 35 | 85 | 15 |
| 35 - 36 | 85 → 0 | 15 → 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Relative retention* with reference to chlorogenic acid (retention time = about 13 min): caffeoylmalic acid = about 2.2.

Herbal drugs

Calculate the percentage content of caffeoylmalic acid and chlorogenic acid, expressed as chlorogenic acid, using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 20}$$

$A_1$ = sum of the areas of the peaks due to caffeoylmalic acid and chlorogenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *chlorogenic acid CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of chlorogenic acid in *chlorogenic acid CRS*.

**01/2015:2538**
**corrected 8.7**

# NETTLE ROOT

## Urticae radix

DEFINITION

Dried, whole or fragmented underground parts of *Urtica dioica* L. or *Urtica urens* L., their hybrids or their mixtures.

IDENTIFICATION

A. Irregular cylindrical rhizome, greyish-brown, sporadically purple or greenish, 3-10 mm in diameter, the internodes are up to 2-3 cm long, with deep longitudinal furrows, alternating with short, slightly swollen nodes; the nodes bear numerous roots that are externally greyish-brown, 0.5-2 mm in diameter and up to about 10 cm long. The transverse section shows a thin, dark bark, the inner part is creamish-white with a hollow centre. The fracture of the rhizome is fibrous and uneven, especially in the inner part.

B. Microscopic examination (*2.8.23*). The powder is pale yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of brownish cork with thin-walled cells; fragments of vessels with bordered pits, 50-150 µm in diameter; fragments of fibres with thick lignified walls, occurring singly or in groups; abundant fragments of parenchyma with thin-walled cells, some containing large cluster crystals of calcium oxalate; rare, scattered, simple crystals of calcium oxalate; fragments of the medullary rays with moderately thick-walled, pitted cells.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Sonicate for 10 min and filter. Evaporate the filtrate to dryness and dissolve the residue in 2 mL of *methanol R*.

*Reference solution*. Dissolve 1 mg of *scopoletin R* in 10 mL of *methanol R* (solution A). Dissolve 10 mg of *arbutin R* and 20 mg of *β-sitosterol R* in *methanol R*, add 1 mL of solution A and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *methanol R*, *water R*, *ethyl acetate R* (2.5:4:4:50 *V/V/V/V*).

*Application*: 20 µL [or 10 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 8 cm [or 6 cm].

*Drying*: in air for 5 min.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Futhermore, other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue or greenish-blue fluorescent zone |
| Scopoletin: a blue fluorescent zone | A blue fluorescent zone (scopoletin) |
| _____ | _____ |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A purple zone |
| β-sitosterol: a purple zone | A purple zone (β-sitosterol) |
| _____ | _____ |
| | A faint purple zone |
| _____ | _____ |
| Arbutin: a greenish-brown zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Lead** (*2.4.27*): maximum 7.0 ppm.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

**Extractable matter**: minimum 7.0 per cent.

To 2.000 g of the powdered herbal drug (250) (*2.9.12*) add a mixture of 6 g of *water R* and 14 g of *ethanol (96 per cent) R* and extract for 2 h, shaking frequently. Filter, evaporate 5.000 g of the filtrate to dryness on a water-bath and dry in an oven at 105 °C for 2 h. The residue weighs a minimum of 35 mg.

07/2012:2468

# NIAOULI OIL, CINEOLE TYPE

## Niaouli typo cineolo aetheroleum

DEFINITION

Essential oil obtained by steam distillation from young leafy branches of *Melaleuca quinquenervia* (Cav.) S.T.Blake.

CHARACTERS

*Appearance*: colourless or pale yellow liquid.

Aromatic odour of cineole.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 100 µL of the essential oil to be examined in *toluene R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 25 µL of *trans-nerolidol R* and 50 µL of *cineole R* in *toluene R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 3 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint grey zone |
| | A purple zone |
| 1,8-Cineole: a violet-brown zone | An intense violet-brown zone (1,8-cineole) |
| *trans*-Nerolidol: a dark violet zone | |
| | An intense violet-brown zone |
| | A violet-brown zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.904 to 0.925.

**Refractive index** (*2.2.6*): 1.463 to 1.472.

**Optical rotation** (*2.2.7*): – 4° to + 1°.

**Methyleugenol and isomethyleugenol**. Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

*Reference solution.* Dissolve 5 µL of *methyleugenol R* and 5 µL of *isomethyleugenol R* in *heptane R* and dilute to 50.0 mL with the same solvent. Dilute 0.5 mL of the solution to 5.0 mL with *heptane R*.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of methyleugenol and isomethyleugenol.

*Identification of peaks*: using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

*Limits*:

- *methyleugenol*: maximum 0.05 per cent;
- *isomethyleugenol*: maximum 0.05 per cent.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 0.2 mL of the essential oil to be examined to 10.0 mL with *heptane R*.

*Reference solution (a).* Dilute 10 µL of *α-pinene R*, 5 µL of *β-pinene R*, 10 µL of *limonene R*, 50 µL of *cineole R*, 5 µL of *p-cymene R*, 5 µL of *benzaldehyde R*, 5 mg of *α-terpineol R* and 5 µL of *trans-nerolidol R* in *heptane R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 µL of *limonene R* in *heptane R* and dilute to 50.0 mL with the same solvent. Dilute 0.5 mL of the solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 65 |
| | 5 - 65 | 65 → 185 |
| | 65 - 80 | 185 → 230 |
| Injection port | | 230 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*Identification of peaks*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution; the peak due to viridiflorol elutes with a relative retention of about 1.02 with reference to *trans*-nerolidol.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to limonene and 1,8-cineole.

Determine the percentage content of each of the following components. The limits are within the following ranges:

- *α-pinene*: 5.0 per cent to 15.0 per cent;
- *β-pinene*: 1.0 per cent to 4.0 per cent;
- *limonene*: 5.0 per cent to 10.0 per cent;

- *1,8-cineole*: 45.0 per cent to 65.0 per cent;
- *p-cymene*: 0.05 per cent to 4.0 per cent;
- *benzaldehyde*: 0.05 per cent to 0.5 per cent;
- *α-terpineol*: 3.0 per cent to 8.0 per cent;
- *trans-nerolidol*: 0.05 per cent to 1.5 per cent;
- *viridiflorol*: 2.5 per cent to 9.0 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

STORAGE

At a temperature not exceeding 25 °C.

**07/2019:2383**

# NOTOGINSENG ROOT

## Notoginseng radix

DEFINITION

Whole or fragmented taproot, without secondary roots, of *Panax notoginseng* (Burkill) F.H.Chen [*Panax pseudoginseng* var. *notoginseng* (Burkill) G.Hoo & C.L.Tseng] treated with steam and dried.

*Content*: minimum 3.8 per cent for the sum of ginsenosides Rg1 ($C_{42}H_{72}O_{14}$,$2H_2O$; $M_r$ 837) and Rb1 ($C_{54}H_{92}O_{23}$,$3H_2O$; $M_r$ 1163) (dried drug).

IDENTIFICATION

A. The primary root is conical, subconical or cylindrical, up to 6 cm long and 4 cm in diameter. The outer surface, showing shallow transverse striations and secondary root scars, is brownish-grey or yellowish-grey. The aerial stem scar is surrounded by warty protuberances at the crown. The texture of the root is compact. The fracture is smooth, shiny, brownish-grey and shows a yellowish-grey ring (cambial zone) and many radial striations.

B. Microscopic examination (*2.8.23*). The powder is light yellowish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2383.-1): abundant fragments of parenchymatous cells with rounded or ovoid cells [D]; fragments of secretory canals (transverse section [G], longitudinal section [E]) with thin-walled cells [Ea, Ga] and containing a granular yellowish-brown resin diffused in the cells [Eb, Gb]; rare vessels, reticulate [B] or pitted, ranging from 20-50 µm in diameter; rare fragments of cork (surface view [A], transverse section [F]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The starch granules, sometimes deformed, single, with a diameter of 2-20 µm, or in groups of 2-9, are very abundant [C].

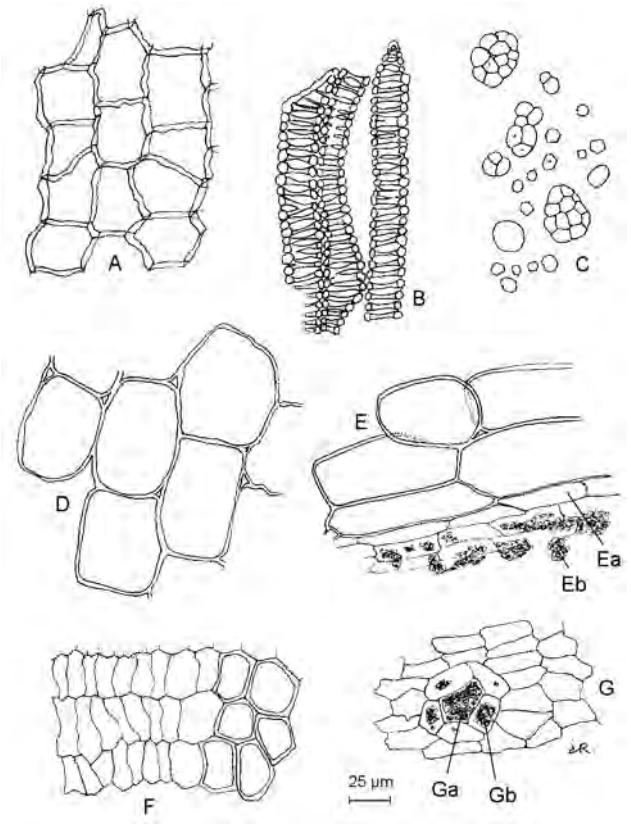


Figure 2383.-1. – *Illustration for identification test B of powdered herbal drug of notoginseng root*

C. Examine the chromatograms obtained in the test for *Panax ginseng* or *Panax quinquefolium*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone (at the solvent front) |
| | A violet zone |
| Arbutin: a brown zone | |
| ——— | ——— |
| | A violet zone (ginsenosides Rg1 + Rg2) |
| | 2 violet zones |
| | 2 faint violet zones |
| ——— | ——— |
| Aescin: a grey zone | A violet zone |
| | Several violet and greenish zones |
| **Reference solution** | **Test solution** |

TESTS

***Panax ginseng* or *Panax quinquefolium***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of a 70 per cent *V/V* solution of *methanol R* and boil under a reflux condenser for 15 min. Filter after cooling and dilute to 10.0 mL with *methanol R*.

*Reference solution*. Dissolve 5.0 mg of *aescin R* and 5.0 mg of *arbutin R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *water R*, *butanol R* (25:50:100 *V/V/V*); allow to stand for 10 min and use the upper layer.

*Application*: 20 µL, as bands of 15 mm [or 4 µL of the test solution and 2 µL of the reference solution, as bands of 8 mm].

*Development*: in an unsaturated tank, over a path of 10 cm [or 5 cm].

*Drying*: in air for 30 min.

*Detection*: treat with *anisaldehyde solution R* and heat at 105-110 °C for 5-10 min; examine in daylight.

*Results*: in the chromatogram obtained with the test solution, the absence of a violet zone immediately above the zone due to arbutin in the chromatogram obtained with the reference solution indicates the presence of *Panax ginseng*; in the chromatogram obtained with the test solution, the presence of a brown zone immediately below the violet zone due to ginsenosides Rg1 + Rg2 indicates adulteration with *Panax quinquefolium*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Reduce about 50 g to a powder (355) (*2.9.12*). Place 0.250 g of the powdered herbal drug and 70 mL of a 50 per cent *V/V* solution of *methanol R* in a 250 mL round-bottomed flask. After adding a few grains of pumice, boil on a water-bath under a reflux condenser for 1 h. After cooling, centrifuge and collect the supernatant. Treat the residue as described above. Mix the collected liquids and evaporate to dryness under reduced pressure at a temperature not exceeding 60 °C. Take up the residue with 10.0 mL of a buffer solution, adjusted to pH 4.5, containing 3.5 g of *sodium dihydrogen phosphate R* and 7.2 g of *potassium dihydrogen phosphate R* in 1000 mL of *water R* (solution A). Wash a cartridge containing about 0.36 g of *octadecylsilyl silica gel for chromatography R* with 5 mL of *methanol R* followed by 20 mL of *water for chromatography R*. Apply 5.0 mL of solution A to the cartridge. Elute with 20 mL of *water for chromatography R*, followed by 15 mL of a 30 per cent *V/V* solution of *methanol R*. Discard the eluates after confirming that no ginsenosides are present, otherwise repeat the assay with another type of cartridge. Elute the cartridge with 20 mL of *methanol R* and evaporate the eluate to dryness. Take up the residue with 5.0 mL of *methanol R*.

*Reference solution.* Dissolve 3.0 mg of *ginsenoside Rb1 CRS*, 3.0 mg of *ginsenoside Rf R* and 3.0 mg of *ginsenoside Rg1 CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*;
- *mobile phase B*: *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 90 | 10 |
| 14 - 18 | 90 → 80 | 10 → 20 |
| 18 - 55 | 80 | 20 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 203 nm.

*Injection*: 20 µL.

*System suitability*: reference solution:

- *resolution*: minimum 3.0 between the peaks due to ginsenosides Rg1 and Rf.

Calculate the sum of the percentage contents of ginsenosides Rb1 and Rg1 using the following expression:

$$\frac{A_1 \times m_2 \times 2 \times p_1}{m_1 \times A_3} + \frac{A_2 \times m_3 \times 2 \times p_2}{m_1 \times A_4}$$

$A_1$ = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to ginsenoside Rb1 in the chromatogram obtained with the reference solution;

$A_4$ = area of the peak due to ginsenoside Rg1 in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *ginsenoside Rb1 CRS* used to prepare the reference solution, in grams;

$m_3$ = mass of *ginsenoside Rg1 CRS* used to prepare the reference solution, in grams;

$p_1$ = percentage content of ginsenoside Rb1 in *ginsenoside Rb1 CRS*;

$p_2$ = percentage content of ginsenoside Rg1 in *ginsenoside Rg1 CRS*.

**01/2008:1552**
**corrected 10.0**

# NUTMEG OIL

## Myristicae fragrantis aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the dried and crushed kernels of *Myristica fragrans* Houtt.

CHARACTERS

*Appearance*: colourless or pale yellow liquid.

Spicy odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1 mL of the substance to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 µL of *myristicine R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R*, heat at 100-105 °C for 10 min and examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows in the upper third a pink or reddish-brown zone (myristicine); the chromatogram obtained with the

test solution shows a series of zones of which 1 is similar in position and colour to the zone in the chromatogram obtained with the reference solution; above this zone a brownish zone (safrole) and a violet zone (hydrocarbons) are present; below the myristicine zone, 5 blue zones of variable intensity are present.

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.885 to 0.905.

**Refractive index** (*2.2.6*): 1.475 to 1.485.

**Optical rotation** (*2.2.7*): + 8° to + 18°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution*. Dissolve 15 µL of *α-pinene R*, 15 µL of *β-pinene R*, 15 µL of *sabinene R*, 5 µL of *car-3-ene R*, 5 µL of *limonene R*, 5 µL of *γ-terpinene R*, 5 µL of *terpinen-4-ol R*, 5 µL of *safrole R* and 10 µL of *myristicine R* in 1 mL of *hexane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25-60 m, Ø = about 0.3 mm;
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 50 |
| | 10 - 75 | 50 → 180 |
| | 75 - 130 | 180 |
| Injection port | | 200 - 220 |
| Detector | | 240 - 250 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to β-pinene and sabinene.

*Identification of components*: using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *α-pinene*: 15 per cent to 28 per cent;
- *β-pinene*: 13 per cent to 18 per cent;
- *sabinene*: 14 per cent to 29 per cent;
- *car-3-ene*: 0.5 per cent to 2.0 per cent;
- *limonene*: 2.0 per cent to 7.0 per cent;
- *γ-terpinene*: 2.0 per cent to 6.0 per cent;
- *terpinen-4-ol*: 2.0 per cent to 6.0 per cent;
- *safrole*: maximum 2.5 per cent;
- *myristicine*: 5.0 per cent to 12.0 per cent.

STORAGE

Protected from heat.

**01/2008:1887**
**corrected 6.0**

# OAK BARK

## Quercus cortex

DEFINITION

Cut and dried bark from the fresh young branches of *Quercus robur* L., *Q. petraea* (Matt.) Liebl. and *Q. pubescens* Willd.

*Content*: minimum 3.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The bark occurs in channelled or quilled pieces, not more than 3 mm thick. The outer surface is light grey or greenish-grey, rather smooth, with occasional lenticels. The inner surface is dull brown or reddish-brown and has slightly raised longitudinal striations about 0.5-1 mm wide. The fracture is splintery and fibrous.

B. Microscopic examination (*2.8.23*). The powder is light brown or reddish-brown and fibrous. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: groups of thick-walled fibres surrounded by a moderately thickened parenchymatous sheath containing prism crystals of calcium oxalate; fragments of cork composed of thin-walled tabular cells filled with brownish or reddish contents; abundant sclereids, isolated and in groups, some large with thick, stratified walls and branching pits, others smaller and thinner-walled with simple pits, often with dense brown contents; fragments of parenchyma containing cluster crystals of calcium oxalate; occasional fragments of sieve tissue, thin-walled, some showing sieve areas on the oblique end-walls.

C. To 1 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *ethanol (30 per cent V/V) R* and heat the mixture under a reflux condenser on a water-bath for 30 min. Cool and filter. To 1 mL of this solution add 2 mL of a 10 g/L solution of *vanillin R* in *hydrochloric acid R*. A red colour develops.

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.700 g of the powdered herbal drug (710) (*2.9.12*).

**01/2009:1878**

# OLIVE LEAF

## Oleae folium

DEFINITION

Dried leaf of *Olea europaea* L.

*Content*: minimum 5.0 per cent of oleuropein ($C_{25}H_{32}O_{13}$; $M_r$ 540.5) (dried drug).

IDENTIFICATION

A. The leaf is simple, thick and coriaceous, lanceolate to obovate, 30-50 mm long and 10-15 mm wide, with a mucronate apex and tapering at the base to a short petiole; the margins are entire and reflexed abaxially. The upper surface is greyish-green, smooth and shiny, the lower surface paler and pubescent, particularly along the midrib and main lateral veins.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis in surface view with small, thick-walled polygonal cells and, in the lower epidermis only, small anomocytic stomata (*2.8.3*); fragments of the lamina in sectional view showing a thick cuticle, a palisade composed of 3 layers of cells and a small-celled spongy parenchyma; numerous sclereids, very thick-walled and mostly fibre-like with blunt or, occasionally, forked ends, isolated or associated with the parenchyma of the mesophyll; abundant, very large peltate trichomes, with a central unicellular stalk from which radiate some 10-30 thin-walled cells that become free from the adjoining cells at the margin of the shield, giving an uneven, jagged appearance.

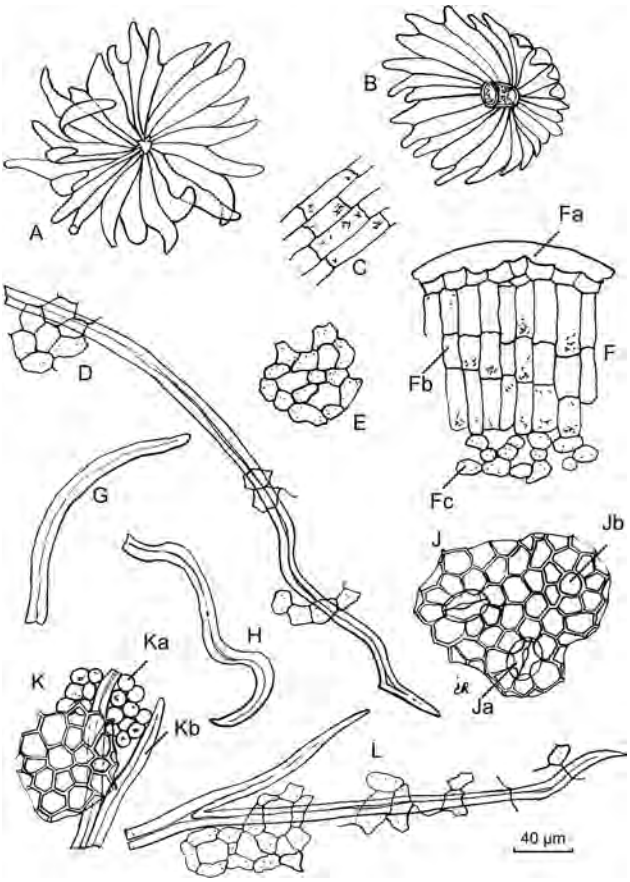


A. Peltate trichome, seen from above

B. Peltate trichome, seen from below

C. Palisade parenchyma

D, G, H and L. Fibre-like sclereids, some accompanied by parenchymatous fragments of the spongy mesophyll

E. Spongy parenchyma

F. Fragment of the lamina, in transverse section, showing a thick cuticle (Fa), palisade parenchyma composed of 3 layers of cells (Fb), and spongy parenchyma (Fc)

J. Fragment of lower epidermis with anomocytic stomata (Ja) and cicatrix of peltate trichome (Jb)

K. Fragment of upper epidermis, in surface view, with underlying palisade parenchyma (Ka) and sclereids of the spongy mesophyll (Kb)

Figure 1878.-1. – *Illustration of powdered herbal drug of olive leaf (see Identification B)*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Boil under a reflux condenser for 15 min. Cool and filter.

*Reference solution.* Dissolve 10 mg of *oleuropein R* and 1 mg of *rutoside trihydrate R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (1.5:15:85 *V/V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A dark violet-blue zone (solvent front) |
| | A dark violet-blue zone |
| _____ | _____ |
| Oleuropein: a brownish-green zone | A brownish-green zone (oleuropein) |
| _____ | _____ |
| Rutoside: a brownish-yellow zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a flask, place 1.000 g of the powdered herbal drug (355) (*2.9.12*) and add 50 mL of *methanol R*. Heat in a water-bath at 60 °C for 30 min with shaking. Allow to cool and filter into a 100 mL volumetric flask. Rinse the flask and the filter with *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.5 mL of this solution to 25.0 mL with *water R*.

*Reference solution.* Dissolve 5.0 mg of *oleuropein CRS* in 5.0 mL of *methanol R*. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: dilute 1.0 mL of *glacial acetic acid R* to 100 mL with *water R*;
– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 85 → 40 | 15 → 60 |
| 5 - 12 | 40 → 20 | 60 → 80 |
| 12 - 15 | 20 → 85 | 80 → 15 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Retention time*: oleuropein = about 9 min.

Calculate the percentage content of oleuropein using the following expression:

$$\frac{A_1 \times m_2 \times p \times 8}{A_2 \times m_1}$$

$A_1$ = area of the peak due to oleuropein in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to oleuropein in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined in the test solution, in grams;

$m_2$ = mass of *oleuropein CRS* in the reference solution, in grams;

$p$ = percentage content of oleuropein in *oleuropein CRS*.

**04/2009:2313**

# OLIVE LEAF DRY EXTRACT

## Oleae folii extractum siccum

DEFINITION

Dry extract produced from *Olive leaf (1878)*.

*Content*: minimum 16.0 per cent of oleuropein ($C_{25}H_{32}O_{13}$; $M_r$ 540.5) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (65-96 per cent *V/V*).

CHARACTERS

*Appearance*: greenish-brown or brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the extract to be examined add 10 mL of *methanol R*. Sonicate for 15 min and filter.

*Reference solution.* Dissolve 5 mg of *oleuropein R* and 1 mg of *rutoside trihydrate R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *anhydrous formic acid R*, *ethyl acetate R* (7:13:80 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A dark violet-blue zone |
| _____ | _____ |
| Oleuropein: a brownish-green zone | A brownish-green zone (oleuropein) |
| _____ | _____ |
| Rutoside: a yellow zone | |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 8.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* To 0.250 g of the extract to be examined add 50 mL of *methanol R*. Sonicate for 15 min and filter into a 100 mL volumetric flask. Rinse the flask and the filter with 2 mL of *methanol R* and dilute to 100.0 mL with *water R*.

*Reference solution (a).* Dissolve 10.0 mg of *oleuropein CRS* in 10.0 mL of *methanol R* and dilute to 25.0 mL with *water R*.

*Reference solution (b).* Dissolve 4 mg of *rutoside trihydrate R* in 10 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *trifluoroacetic acid R*, *methanol R*, *water R* (1:400:600 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 233 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of oleuropein.

*Relative retention* with reference to oleuropein (retention time = about 11 min): rutoside = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to rutoside and oleuropein.

Calculate the percentage content of oleuropein using the following expression:

$$\frac{A_1 \times m_2 \times p \times 4}{A_2 \times m_1}$$

$A_1$ = area of the peak due to oleuropein in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to oleuropein in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *oleuropein CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of oleuropein in *oleuropein CRS*.

**01/2015:1839**
**corrected 8.5**

# OPIUM DRY EXTRACT, STANDARDISED

## Opii extractum siccum normatum

DEFINITION

Standardised dry extract produced from *Raw opium (0777)*.

*Content*:

– *morphine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): 19.6 per cent to 20.4 per cent (dried extract);

– *codeine* ($C_{18}H_{21}NO_3$; $M_r$ 299.4): minimum 2.0 per cent (dried extract).

Content adjusted if necessary by adding a suitable excipient (e.g. lactose, dextrin).

PRODUCTION

The extract is produced from the drug by a suitable procedure using water.

CHARACTERS

*Appearance*: brown, amorphous powder.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Triturate 0.05 g of the extract to be examined with 5 mL of *ethanol (70 per cent V/V) R*. Transfer to a 25 mL conical flask. Rinse with 3 mL of *ethanol (70 per cent V/V) R* and transfer the rinsings to the same 25 mL conical flask. Heat in a water-bath at 50-60 °C, while stirring, for 30 min. Cool, filter, wash the filter with *ethanol (70 per cent V/V) R* and dilute the combined filtrates to 10 mL with the same solvent.

*Reference solution.* Dissolve 5 mg of *morphine hydrochloride R* in a solution prepared as follows and dilute to 5 mL with the same solution: dissolve 2 mg of *papaverine hydrochloride R*, 12 mg of *codeine phosphate R* and 12 mg of *noscapine hydrochloride R* in *ethanol (70 per cent V/V) R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (2:6:40:40 *V/V/V/V*); use a freshly prepared mixture.

*Application*: 20 µL [or 6 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: at 100-105 °C for 15 min.

*Detection*: allow to cool and treat with *potassium iodobismuthate solution R2* and then with a 4 g/L solution of *sulfuric acid R*; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. A dark red zone (thebaine) situated between the zone due to codeine and the zone due to papaverine may be present in the chromatogram obtained with the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Noscapine: an orange-red or red zone | An orange-red or red zone (noscapine) |
| Papaverine: an orange-red or red zone | An orange-red or red zone (papaverine) |
| Codeine: an orange-red or red zone | An orange-red or red zone (codeine) |
| Morphine: an orange-red or red zone | An orange-red or red zone (morphine) |
| **Reference solution** | **Test solution** |

B. To 0.5 g of the extract to be examined add 5 mL of *water R*, shake for 5 min and filter. To the filtrate add 0.25 mL of *ferric chloride solution R2*. A red colour develops which does not disappear upon the addition of 0.5 mL of *dilute hydrochloric acid R*.

TESTS

**Thebaine**. Liquid chromatography (*2.2.29*).

*Test solution.* Suspend 0.500 g of the extract to be examined in 50 mL of *ethanol (50 per cent V/V) R*, mix using sonication for 1 h, allow to cool and dilute to 100.0 mL with the same solvent. Allow to stand. To 10.0 mL of the supernatant add 5 mL of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 mL with *water R* and mix. Transfer 20.0 mL of this solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with 2 quantities, each of 40 mL, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the combined eluates to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask using the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *thebaine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 12.0 mg of *morphine hydrochloride trihydrate CRS* in the mobile phase and dilute to 15.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10.0 mg of *codeine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10.0 mL of the solution add 10.0 mL of reference solution (b).

*Precolumn*:

– *size*: *l* = 4 mm, Ø = 4.0 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium heptanesulfonate monohydrate R* in 420 mL of *water R*, adjust to pH 3.2 with a 4.9 g/L solution of *phosphoric acid R* and add 180 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of thebaine.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to morphine and codeine.

Calculate the percentage content of the relevant alkaloid using the following expression:

$$\frac{A_1 \times m_2 \times F \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with reference solution (a) for thebaine or reference solution (c) for morphine and codeine;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of the relevant alkaloid used to prepare reference solution (a) for thebaine, reference solution (b) for morphine or reference solution (c) for codeine, in grams;

$F$ = 6.250 for the determination of thebaine;

$p$ = percentage content of the relevant alkaloid in the corresponding CRS.

*Limit*:

– *thebaine*: maximum 6.0 per cent (dried extract).

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g of the extract to be examined by drying in an oven at 105 °C for 4 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for thebaine with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

– *repeatability*: maximum relative standard deviation of 1.0 per cent for the area of the peak due to morphine after 6 injections.

Calculate the percentage content of morphine and the percentage content of codeine using the expression given in the test for thebaine, with $F$ = 10.417 for morphine and $F$ = 3.125 for codeine.

To obtain the $p$ value to be used for the calculation of the morphine content, multiply the percentage content of morphine hydrochloride in *morphine hydrochloride trihydrate CRS* by 0.887.

**01/2015:1840**
**corrected 8.5**

# OPIUM, PREPARED

## Opii pulvis normatus

DEFINITION

*Raw opium (0777)* powdered (180) (*2.9.12*) and dried at a temperature not exceeding 70 °C, with a morphine content adjusted, if necessary, by adding a suitable excipient or raw opium powder with a lower alkaloidal content.

*Content*:

– *morphine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): 9.5 per cent to 10.5 per cent (dried preparation);

– *codeine* ($C_{18}H_{21}NO_3$; $M_r$ 299.4): minimum 1.0 per cent (dried preparation).

CHARACTERS

*Appearance*: yellowish-brown or dark brown powder.

IDENTIFICATION

A. Microscopic examination (*2.8.23*). The powder (500) (*2.9.12*) is light brown. Examine under a microscope using *chloral hydrate solution R*. Before heating, the powder shows the following diagnostic characters (Figure 1840.-1): granules of latex agglomerated in irregular masses [A] and light brown elongated filaments. After heating, some fragments of vessels [J, K] and rather elongated, refringent crystals [F] are also visible, as well as a smaller number of round pollen grains with 3 pores and a very finely pitted exine [E] and fragments of elongated fibres [D]. Fragments of epicarp (surface view [B, C, G], transverse section [H]), consisting of polygonal cells with thick walls defining a stellate lumen, and sometimes anomocytic stomata (*2.8.3*) [Ba] may also be present. Some elements of various origin introduced during handling of the latex may also be present in small quantities (fragments of covering trichomes and starch granules).

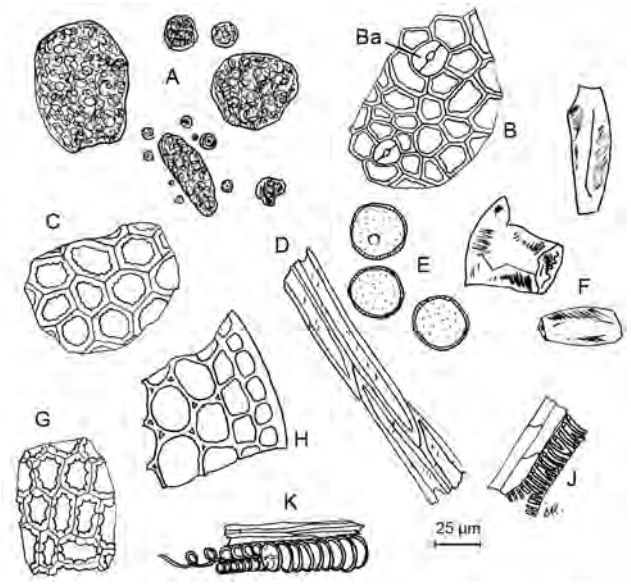


Figure 1840.-1. – *Illustration for identification test A of prepared opium*

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Triturate 0.10 g of the preparation to be examined with 5 mL of *ethanol (70 per cent V/V) R*. Transfer to a 25 mL conical flask. Rinse with 3 mL of *ethanol (70 per cent V/V) R* and transfer the rinsings to the same 25 mL conical flask. Heat in a water-bath at 50-60 °C, while stirring, for 30 min. Cool, filter, wash the filter with *ethanol (70 per cent V/V) R* and dilute the combined filtrates to 10 mL with the same solvent.

*Reference solution*. Dissolve 5 mg of *morphine hydrochloride R* in a solution prepared as follows and dilute to 5 mL with the same solution: dissolve 2 mg of *papaverine hydrochloride R*, 12 mg of *codeine phosphate R* and 12 mg of *noscapine hydrochloride R* in *ethanol (70 per cent V/V) R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (2:6:40:40 *V/V/V/V*); use a freshly prepared mixture.

*Application*: 20 µL [or 6 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: at 100-105 °C for 15 min.

*Detection*: allow to cool and treat with *potassium iodobismuthate solution R2* and then with a 4 g/L solution of *sulfuric acid R*; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. A dark red zone (thebaine) situated between the zone due to codeine and the zone due to papaverine may be present in the chromatogram obtained with the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Noscapine: an orange-red or red zone | An orange-red or red zone (noscapine) |
| Papaverine: an orange-red or red zone | An orange-red or red zone (papaverine) |
| Codeine: an orange-red or red zone | An orange-red or red zone (codeine) |
| Morphine: an orange-red or red zone | An orange-red or red zone (morphine) |
| **Reference solution** | **Test solution** |

C. To 1.0 g of the preparation to be examined add 5 mL of *water R*, shake for 5 min and filter. To the filtrate add 0.25 mL of *ferric chloride solution R2*. A red colour develops which does not disappear upon the addition of 0.5 mL of *dilute hydrochloric acid R*.

TESTS

**Thebaine**. Liquid chromatography (*2.2.29*).

*Test solution.* Suspend 1.000 g of the preparation to be examined in 50 mL of *ethanol (50 per cent V/V) R*, mix using sonication for 1 h, allow to cool and dilute to 100.0 mL with the same solvent. Allow to stand. To 10.0 mL of the supernatant add 5 mL of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 mL with *water R* and mix. Transfer 20.0 mL of this solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with 2 quantities, each of 40 mL, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the combined eluates to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask using the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *thebaine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 12.0 mg of *morphine hydrochloride trihydrate CRS* in the mobile phase and dilute to 15.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10.0 mg of *codeine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10.0 mL of the solution add 10.0 mL of reference solution (b).

*Precolumn*:

- *size*: $l$ = 4 mm, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium heptanesulfonate monohydrate R* in 420 mL of *water R*, adjust to pH 3.2 with a 4.9 g/L solution of *phosphoric acid R* and add 180 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of thebaine.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to morphine and codeine.

Calculate the percentage content of the relevant alkaloid using the following expression:

$$\frac{A_1 \times m_2 \times F \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with reference solution (a) for thebaine or reference solution (c) for morphine and codeine;

$m_1$ = mass of the preparation to be examined used to prepare the test solution, in grams;

$m_2$ = mass of the relevant alkaloid used to prepare reference solution (a) for thebaine, reference solution (b) for morphine or reference solution (c) for codeine, in grams;

$F$ = 6.250 for the determination of thebaine;

$p$ = percentage content of the relevant alkaloid in the corresponding CRS.

*Limit*:

- *thebaine*: maximum 3.0 per cent (dried preparation).

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the preparation to be examined by drying in an oven at 105 °C for 4 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for thebaine with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

- *repeatability*: maximum relative standard deviation of 1.0 per cent for the area of the peak due to morphine after 6 injections.

Calculate the percentage content of morphine and the percentage content of codeine using the expression given in the test for thebaine, with $F$ = 10.417 for morphine and $F$ = 3.125 for codeine.

To obtain the $p$ value to be used for the calculation of the morphine content, multiply the percentage content of morphine hydrochloride in *morphine hydrochloride trihydrate CRS* by 0.887.

**01/2015:0777**
**corrected 8.5**

# OPIUM, RAW

## Opium crudum

*Raw opium is intended only as starting material for the manufacture of galenical preparations. It is not dispensed as such.*

DEFINITION

Air-dried latex obtained by incision from the unripe capsules of *Papaver somniferum* L.

*Content*:

- *morphine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): minimum 10.0 per cent (dried drug);

Herbal drugs

– *codeine* ($C_{18}H_{21}NO_3$; $M_r$ 299.4): minimum 2.0 per cent (dried drug).

CHARACTERS

*Appearance*: blackish-brown masses of various sizes, which tend to be soft and shiny and, after drying, become hard and brittle.

IDENTIFICATION

*Strip off any covering, cut the substance to be examined into thin slices, dry at about 60 °C for 48 h, if necessary, and reduce to a powder (500) (2.9.12).*

A. Microscopic examination (*2.8.23*). The powder (500) (*2.9.12*) is light brown. Examine under a microscope using *chloral hydrate solution R*. Before heating, the powder shows the following diagnostic characters (Figure 0777.-1): granules of latex agglomerated in irregular masses [A] and light brown elongated filaments. After heating, some fragments of vessels [J, K] and rather elongated, refringent crystals [F] are also visible, as well as a smaller number of round pollen grains with 3 pores and a very finely pitted exine [E] and fragments of elongated fibres [D]. Fragments of epicarp (surface view [B, C, G], transverse section [H]), consisting of polygonal cells with thick walls defining a stellate lumen, and sometimes anomocytic stomata (*2.8.3*) [Ba] may also be present. Some elements of various origin introduced during handling of the latex may also be present in small quantities (fragments of covering trichomes and starch granules).

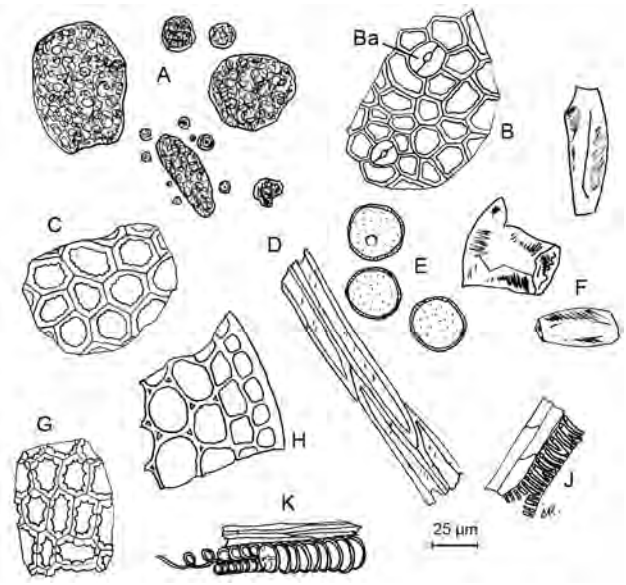


Figure 0777.-1. – *Illustration for identification test A of powdered raw opium*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Triturate 0.10 g of the powdered substance to be examined (500) (*2.9.12*) with 5 mL of *ethanol (70 per cent V/V) R*. Transfer to a 25 mL conical flask. Rinse with 3 mL of *ethanol (70 per cent V/V) R* and transfer the rinsings to the same 25 mL conical flask. Heat in a water-bath at 50-60 °C, while stirring, for 30 min. Cool, filter, wash the filter with *ethanol (70 per cent V/V) R* and dilute the combined filtrates to 10 mL with the same solvent.

*Reference solution.* Dissolve 5 mg of *morphine hydrochloride R* in a solution prepared as follows and dilute to 5 mL with the same solution: dissolve 2 mg of *papaverine hydrochloride R*, 12 mg of *codeine phosphate R* and 12 mg of *noscapine hydrochloride R* in *ethanol (70 per cent V/V) R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (2:6:40:40 *V/V/V/V*); use a freshly prepared mixture.

*Application*: 20 µL [or 6 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: at 100-105 °C for 15 min.

*Detection*: allow to cool and treat with *potassium iodobismuthate solution R2* and then with a 4 g/L solution of *sulfuric acid R*; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. A dark red zone (thebaine) situated between the zone due to codeine and the zone due to papaverine may be present in the chromatogram obtained with the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Noscapine: an orange-red or red zone | An orange-red or red zone (noscapine) |
| Papaverine: an orange-red or red zone | An orange-red or red zone (papaverine) |
| Codeine: an orange-red or red zone | An orange-red or red zone (codeine) |
| Morphine: an orange-red or red zone | An orange-red or red zone (morphine) |
| **Reference solution** | **Test solution** |

C. To 1.0 g of the powdered substance (500) (*2.9.12*) add 5 mL of *water R*, shake for 5 min and filter. To the filtrate add 0.25 mL of *ferric chloride solution R2*. A red colour develops which does not disappear upon the addition of 0.5 mL of *dilute hydrochloric acid R*.

TESTS

**Thebaine**. Liquid chromatography (*2.2.29*).

*Test solution.* Suspend 1.000 g of the substance to be examined, cut into thin slices, in 50 mL of *ethanol (50 per cent V/V) R*, mix using sonication for 1 h, allow to cool and dilute to 100.0 mL with the same solvent. Allow to stand. To 10.0 mL of the supernatant add 5 mL of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 mL with *water R* and mix. Transfer 20.0 mL of this solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with 2 quantities, each of 40 mL, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the combined eluates to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask using the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution* (a). Dissolve 5.0 mg of *thebaine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 12.0 mg of *morphine hydrochloride trihydrate CRS* in the mobile phase and dilute to 15.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10.0 mg of *codeine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10.0 mL of the solution add 10.0 mL of reference solution (b).

*Precolumn*:

– *size*: *l* = 4 mm, Ø = 4.0 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium heptanesulfonate monohydrate R* in 420 mL of *water R*, adjust to pH 3.2 with a 4.9 g/L solution of *phosphoric acid R* and add 180 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of thebaine.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to morphine and codeine.

Calculate the percentage content of the relevant alkaloid using the following expression:

$$\frac{A_1 \times m_2 \times F \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with reference solution (a) for thebaine or reference solution (c) for morphine and codeine;

$m_1$ = mass of the substance to be examined used to prepare the test solution, in grams;

$m_2$ = mass of the relevant alkaloid used to prepare reference solution (a) for thebaine, reference solution (b) for morphine or reference solution (c) for codeine, in grams;

$F$ = 6.250 for the determination of thebaine;

$p$ = percentage content of the relevant alkaloid in the corresponding CRS.

*Limit*:

– *thebaine*: maximum 3.0 per cent (dried drug).

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g of the substance to be examined, cut into thin slices, by drying in an oven at 105 °C for 4 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for thebaine with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

– *repeatability*: maximum relative standard deviation of 1.0 per cent for the area of the peak due to morphine after 6 injections.

Calculate the percentage content of morphine and the percentage content of codeine using the expression given in the test for thebaine, with $F$ = 10.417 for morphine and $F$ = 3.125 for codeine.

To obtain the $p$ value to be used for the calculation of the morphine content, multiply the percentage content of morphine hydrochloride in *morphine hydrochloride trihydrate CRS* by 0.887.

**01/2015:1841**
**corrected 10.0**

# OPIUM TINCTURE, STANDARDISED

## Opii tinctura normata

DEFINITION

Standardised tincture produced from *Raw opium (0777)*.

*Content*:

– *morphine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): 0.95 per cent to 1.05 per cent;

– *codeine* ($C_{18}H_{21}NO_3$; $M_r$ 299.4): minimum 0.1 per cent.

PRODUCTION

The tincture is produced from the drug by an appropriate procedure using equal volumes of ethanol (70 per cent *V/V*) and water.

CHARACTERS

*Appearance*: reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1.0 mL of the tincture to be examined to 10 mL with *ethanol (70 per cent V/V) R*.

*Reference solution.* Dissolve 5 mg of *morphine hydrochloride R* in a solution prepared as follows and dilute to 5 mL with the same solution: dissolve 2 mg of *papaverine hydrochloride R*, 12 mg of *codeine phosphate R* and 12 mg of *noscapine hydrochloride R* in *ethanol (70 per cent V/V) R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (2:6:40:40 *V/V/V/V*); use a freshly prepared mixture.

*Application*: 20 µL [or 6 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 8 cm].

*Drying*: at 100-105 °C for 15 min.

*Detection*: allow to cool and treat with *potassium iodobismuthate solution R2* and then with a 4 g/L solution of *sulfuric acid R*; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. A dark red zone (thebaine) situated between the zone due to codeine and the zone due to papaverine may be present in the chromatogram obtained with the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Noscapine: an orange-red or red zone | An orange-red or red zone (noscapine) |
| Papaverine: an orange-red or red zone | An orange-red or red zone (papaverine) |
| Codeine: an orange-red or red zone | An orange-red or red zone (codeine) |
| Morphine: an orange-red or red zone | An orange-red or red zone (morphine) |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 31 per cent *V/V* to 34 per cent *V/V*.

Herbal drugs

**Thebaine**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute 2.000 g of the tincture to be examined to 25.0 mL with *ethanol (50 per cent V/V) R*. To 10.0 mL of the solution add 5 mL of *ammonium chloride buffer solution pH 9.5 R*, dilute to 25.0 mL with *water R* and mix. Transfer 20.0 mL of this solution to a chromatography column about 0.15 m long and about 30 mm in internal diameter containing 15 g of *kieselguhr for chromatography R*. Allow to stand for 15 min. Elute with 2 quantities, each of 40 mL, of a mixture of 15 volumes of *2-propanol R* and 85 volumes of *methylene chloride R*. Evaporate the combinated eluates to dryness *in vacuo* at 40 °C. Transfer the residue to a volumetric flask using the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *thebaine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 12.0 mg of *morphine hydrochloride trihydrate CRS* in the mobile phase and dilute to 15.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 10.0 mg of *codeine CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10.0 mL of the solution add 10.0 mL of reference solution (b).

*Precolumn*:

- *size*: *l* = 4 mm, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium heptanesulfonate monohydrate R* in 420 mL of *water R*, adjust to pH 3.2 with a 4.9 g/L solution of *phosphoric acid R* and add 180 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of thebaine.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to morphine and codeine.

Calculate the percentage content of the relevant alkaloid using the following expression:

$$\frac{A_1 \times m_2 \times F \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the relevant alkaloid in the chromatogram obtained with reference solution (a) for thebaine or reference solution (c) for morphine and codeine;

$m_1$ = mass of the tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of the relevant alkaloid used to prepare reference solution (a) for thebaine, reference solution (b) for morphine or reference solution (c) for codeine, in grams;

$F$ = 1.563 for the determination of thebaine;

$p$ = percentage content of the relevant alkaloid in the corresponding CRS.

*Limit*:

- *thebaine*: maximum 0.3 per cent.

**Dry residue** (*2.8.16*): minimum 4.0 per cent, determined on 3.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for thebaine with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

- *repeatability*: maximum relative standard deviation of 1.0 per cent for the area of the peak due to morphine after 6 injections.

Calculate the percentage content of morphine and the percentage content of codeine using the expression given in the test for thebaine, with $F$ = 2.604 for morphine and $F$ = 0.781 for codeine.

To obtain the *p* value to be used for the calculation of the morphine content, multiply the percentage content of morphine hydrochloride in *morphine hydrochloride trihydrate CRS* by 0.887.

**01/2011:1880**
**corrected 10.0**

# OREGANO

## Origani herba

DEFINITION

Dried leaves and flowers separated from the stems of *Origanum onites* L. or *Origanum vulgare* L. subsp. *hirtum* (Link) Ietsw., or a mixture of both species.

*Content*:

- *essential oil*: minimum 25 mL/kg (anhydrous drug);
- *sum of the contents of carvacrol and thymol* (both $C_{10}H_{14}O$; $M_r$ 150.2): minimum 60 per cent in the essential oil.

IDENTIFICATION

A. *O. onites*. The leaf is yellowish-green, usually 4-22 mm long and 3-14 mm wide. It has a long or short petiole or is sessile. The lamina is ovate, elliptic or ovate-lanceolate. Margins are entire or serrate, the apex is acute or obtuse. The veins are yellowish and conspicuous on the adaxial surface. Flowers are solitary or seen as broken parts of the corymb. The calyx is bract-like and inconspicuous. The corolla is white, on top of inflorescences or single flowers, or inconspicuous. The bracts are imbricate and green like the leaves. The drug contains yellowish or yellowish-brown stem parts.

*O. vulgare* (subsp. *hirtum*). The leaf is green and usually 3-28 mm long and 2.5-19 mm wide. It is petiolate or sessile. The lamina is ovate or ovate-eliptic. The margins are entire or serrate, the apex is acute or obtuse. Flowers are rare, found as broken parts of the corymbs. Bracts are greenish-yellow and imbricate. The calyx is corolla-like and inconspicuous. The corolla is white, on top of inflorescences, slightly conspicuous or inconspicuous.

B. Microscopic examination (*2.8.23*). Reduce to a powder (710) (*2.9.12*). The powder is green (*O. vulgare*) or yellowish-green (*O. onites*). Examine under a microscope using *chloral hydrate solution R* (Figure 1880.-1).

*O. onites* powder shows fragments of leaf epidermis [A, D, G] composed of cells with sinuous walls, diacytic stomata (*2.8.3*) [Ga], covering trichomes and glandular trichomes; there are 2 types of glandular trichomes: some of lamiaceous type with 8-16 cells (surface view [Da]) and a very common type with a unicellular head and uni- [Gc], bi- [H] or tricellular stalk; the covering trichomes have smooth, thick walls; some are multicellular [B, Gb], often broken [Aa], and contain prisms of calcium oxalate, while others, which are rare, are unicellular and conical [C]; scars

from covering and glandular trichomes are visible on the epidermises [Gd, Ge]; pollen grains, with smooth exine, are frequent [E, F].

*O. vulgare* subsp. *hirtum* powder shows fragments of the upper epidermis with cells with sinuous, beaded walls, accompanied by palisade parenchyma [J]; fragments of the lower epidermis [N] composed of cells with finely and irregularly thickened walls, diacytic stomata (*2.8.3*) [Na], covering trichomes and glandular trichomes; there are 2 types of glandular trichomes: some of lamiaceous type with 12 cells, in surface view [Nb], and a rare type with a unicellular head [Nc] and bi- or tricellular stalk; the covering trichomes have thick, warty walls and contain fine needles of calcium oxalate; some are conical, multicellular and serrate [L, M], while others, which are rare, are unicellular [K]; there are occasional pollen grains, with smooth exine [E, F].

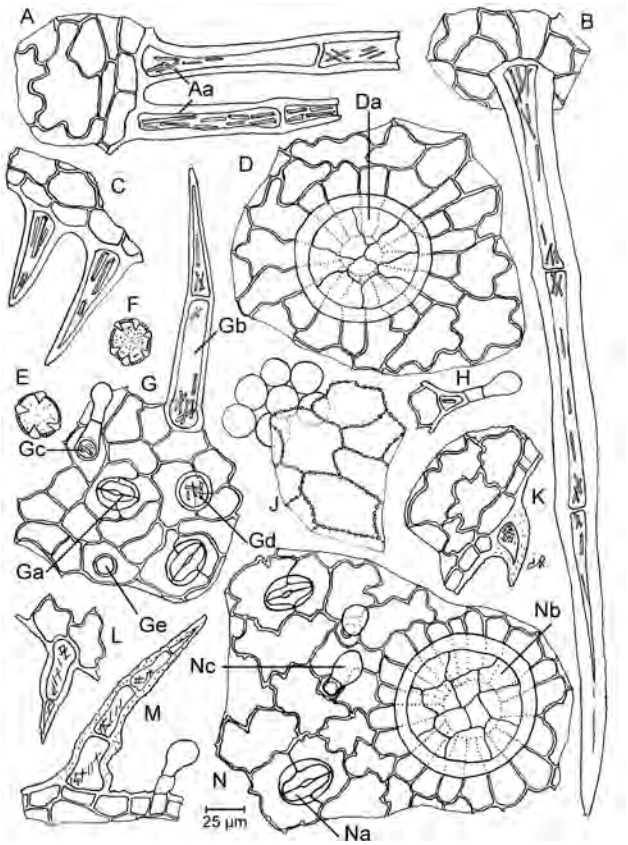


Figure 1880.-1. – *Illustration for identification test B of powdered herbal drug of oregano*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methylene chloride R* and shake for 3 min, then filter through about 2 g of *anhydrous sodium sulfate R*.

*Reference solution.* Dissolve 1 mg of *thymol R* and 10 µL of *carvacrol R* in 10 mL of *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*.

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* using 10 mL for a plate 200 mm square and heat at 100-105 °C for 10 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the lower third and upper part of the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A bluish-purple zone |
| ——— | ——— |
| | A pale green zone |
| Thymol: a pink zone | A pink zone (thymol) |
| Carvacrol: a pale violet zone | A pale violet zone (carvacrol) |
| ——— | ——— |
| | A pale purple zone |
| | A grey zone |
| | A pale green zone |
| | A bluish-purple zone |
| | An intense brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.2.13*): maximum 120 mL/kg, determined on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 30.0 g of the drug to be examined, a 1000 mL round-bottomed flask and 400 mL of *water R* as the distillation liquid. Distil at a rate of 2-3 mL/min for 2 h without *xylene R* in the graduated tube.

**Carvacrol and thymol**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Filter the essential oil obtained in the assay of essential oil over a small amount of *anhydrous sodium sulfate R* and dilute to 5.0 mL with *heptane R* by rinsing the apparatus and the anhydrous sodium sulfate.

*Reference solution.* Dissolve 0.20 g of *thymol R* and 50 mg of *carvacrol R* in *heptane R* and dilute to 5.0 mL with the same solvent.

*Column*:
- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *nitrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | **Time (min)** | **Temperature (°C)** |
|---|---|---|
| Column | 0 - 45 | 40 → 250 |
| Injection port | | 190 |
| Detector | | 210 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to thymol and carvacrol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of the sum of carvacrol and thymol.

**01/2013:2450**
**corrected 7.8**

# ORIENTVINE STEM

## Sinomenii caulis

DEFINITION

Dried, whole or fragmented stem of *Sinomenium acutum* (Thunb.) Rehder et E.H.Wilson, collected in late autumn and early winter.

*Content*: minimum 0.5 per cent of sinomenine ($C_{19}H_{23}NO_4$; $M_r$ 329.4) (dried drug).

IDENTIFICATION

A. *Whole drug.* Long cylindrical stem, somewhat curved, 60 cm long or more, 0.5-2 cm in diameter. The outer bark is greenish-brown or brown, sometimes greyish-brown, with relatively wide longitudinal striations and prominent verrucose lenticels; the nodes are slightly swollen and branched. The texture is light, hard and difficult to break; the fracture is uneven, greyish-yellow or pale greyish-brown; the bark is thin (about 1/10 of the diameter); the medullary rays are very conspicuous; the pith is yellowish-white or pale yellowish-brown.

*Fragmented drug.* Fragments of stems, in discs, about 1.5 cm in diameter and 0.3 cm thick, with greenish-brown, brown or greyish-brown outer surface; a transverse section shows a narrow, pale yellow cortical zone, it is mainly occupied by the vascular system (about 3/4 of the section) consisting of very numerous vascular bundles (about 15-20) in a circle around the yellowish-white or pale yellowish-brown, small, circular pith; each bundle is delimited on the outside by a narrow and continuous, wavy, light brown zone and is separated from the next bundle by a narrow, light brown medullary ray; the xylem vessels with a relatively wide interior lumen are clearly visible.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown or greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: rare fragments of epidermis with polyhedral cells, in surface view, covered with a thick, pale yellow cuticle about 50 µm in diameter; sclereids isolated or in groups, of various sizes and shapes (subsquare, fusiform, elliptical or irregular), with thickened, pitted walls with conspicuous pit canals, free or included in fragments of parenchyma; pale yellow or yellow fibres, 30-70 µm in diameter with thick, distinctly channelled walls and a very narrow lumen; fragments of parenchyma with thin-walled cells containing fine, needle-shaped crystals of calcium oxalate; fragments of xylem consisting of reticulate or pitted vessels, up to 200 µm in diameter, accompanied by ligneous parenchyma with slightly and regularly thickened and pitted cells. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple, spherical starch granules about 10 µm in diameter, free or contained in parenchymatous cells.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2 g of the powdered herbal drug (355) (*2.9.12*) add 25 mL of *ethanol (96 per cent) R* and heat under reflux for 1 h. Filter and evaporate the filtrate to dryness. Dissolve the residue in 2 mL of *ethanol (96 per cent) R*.

*Reference solution.* Dissolve 5 mg of *sinomenine R* and 5 mg of *papaverine hydrochloride R* in 5 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *concentrated ammonia R*, *water R*, *toluene R*, *methanol R*, *ethyl acetate R* (2:10:20:30:40 *V/V/V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Papaverine: a quenching zone | A quenching zone |
| Sinomenine: a quenching zone | A quenching zone (sinomenine) |
| | A dark blue fluorescent zone |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 10 g/L solution of *sodium nitrite R* in *potassium iodobismuthate solution R5* and allow to dry in air. Examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Papaverine: an orange zone | An orange zone |
| | 3 light orange zones |
| Sinomenine: an orange zone | An orange zone (sinomenine) |
| | 2 orange zones |
| | A light orange zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 3 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Aristolochic acids** (*2.8.21, Method A*). It complies with the test.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 0.500 g of the powdered herbal drug (355) (*2.9.12*) in 20.0 mL of *ethanol (70 per cent V/V) R* in a conical flask and weigh. Sonicate for 20 min. Cool and weigh again. Compensate the loss of solvent with *ethanol (70 per cent V/V) R* and stopper the flask. Shake thoroughly and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 3.0 mg of *sinomenine CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Disperse 0.250 g of *orientvine stem HRS* in 10.0 mL of *ethanol (70 per cent V/V) R* in a conical flask and weigh. Sonicate for 20 min. Cool and weigh again. Compensate the loss of solvent with *ethanol (70 per cent V/V) R* and stopper the flask. Shake thoroughly and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Adjust a 1.8 g/L solution of *disodium hydrogen phosphate dodecahydrate R* to pH 8.0 with a 0.8 g/L solution of *sodium dihydrogen phosphate R*, then adjust to pH 9.0 with a 10 g/L solution of *triethylamine R*. Mix 60 volumes of this solution with 40 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 262 nm.

*Injection*: 20 µL.

*Retention time*: sinomenine = about 3 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between peak 1 and the peak due to sinomenine; identify peak 1 using the chromatogram supplied with *orientvine stem HRS*.

Calculate the percentage content of sinomenine using the following expression:

$$\frac{A_1 \times m_2 \times 2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to sinomenine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to sinomenine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *sinomenine CRS* used to prepare reference solution (a), in grams;

$p$ = assigned percentage content of sinomenine in *sinomenine CRS*.

**01/2008:1822**
**corrected 6.0**

# PALE CONEFLOWER ROOT

## Echinaceae pallidae radix

DEFINITION

Dried, whole or cut underground parts of *Echinacea pallida* Nutt.

*Content*: minimum 0.2 per cent of echinacoside ($C_{35}H_{46}O_{20}$; $M_r$ 786.5) (dried drug).

IDENTIFICATION

A. The rhizome and roots are 4-20 mm in diameter, cylindrical and sometimes spirally twisted, longitudinally wrinkled or deeply furrowed; the outer surface is reddish-brown to greyish-brown.

B. Microscopic examination (*2.8.23*). The powder is greyish-brown to light yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: short lignified fibres (100-300 µm in length, up to about 80 µm in diameter) occurring singly or joined together in long bundles, sometimes with phytomelanin deposits; lignified reticulately or scalariformly thickened vessels (up to about 70 µm in diameter); abundant sclereids, occurring singly or in small groups of less than 10, varying considerably in shape from rounded to rectangular or irregular, sometimes much elongated and fibre-like and measuring up to 400 µm in length; all the sclereids have associated black, phytomelanin deposits; fragments of oleoresin canals (up to 240 µm in diameter) with yellowish-orange content; groups of squarish to rectangular cells of the outer layers (about 40 × 80 µm); abundant thin-walled pitted parenchyma with sphaerocrystalline masses of inulin.

C. Examine the chromatograms obtained in the test for other *Echinacea* species and *Parthenium integrifolium*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. The chromatogram obtained with the test solution may also show a weak zone close to the solvent front.

| Top of the plate | |
|---|---|
| | A greenish-brown to brown zone |
| | A yellow zone |
| | A violet zone |
| *β*-Sitosterol: a violet to pink zone | A violet to pink zone (*β*-sitosterol) |
| *N*-Isobutyldodecatetraenamide: a greyish-blue zone | |
| | A dark grey-blue zone |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay.

*Results*: the major peak in the chromatogram obtained with the test solution is due to echinacoside. Peaks due to caftaric acid, caffeic acid, cynarin, chlorogenic acid and cichoric acid are minor peaks or may be absent.

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Other *Echinacea* species and *Parthenium integrifolium*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methylene chloride R* and sonicate for 5 min. Centrifuge and use the supernatant solution.

*Reference solution.* Dissolve 1 mg of *β-sitosterol R* and a volume of *N-isobutyldodecatetraenamide solution R* corresponding to 1 mg of *N-isobutyldodecatetraenamide R* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *cyclohexane R*, *ethyl acetate R*, *toluene R* (0.9:3:6:24 *V/V/V/V*).

Herbal drugs

*Application*: 25 µL [or 5 µL] of the test solution and 10 µL [or 4 µL] of the reference solution as bands.

*Development*: over a path of 15 cm [or 5 cm].

*Drying*: in a stream of cold air for about 10 min.

*Detection*: treat the plate using *anisaldehyde solution R* and heat at 105 °C for 3 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no greyish-blue zone at the position of *N*-isobutyldodecatetraenamide in the chromatogram obtained with the reference solution, and no blue zone at the position of the violet zone due to *β*-sitosterol in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 100 mL volumetric flask place 0.500 g of the powdered herbal drug (355) (*2.9.12*) and add 80 mL of *ethanol (70 per cent V/V) R*. Sonicate for 15 min and dilute to 100.0 mL with *ethanol (70 per cent V/V) R*. Mix the suspension and allow to stand for a few minutes to allow visible solids to settle. Filter a suitable proportion of the solution through a membrane filter (nominal pore size 0.45 µm) before injection.

*Reference solution.* Dissolve 10.0 mg of *chlorogenic acid CRS* and 10.0 mg of *caffeic acid R* in *ethanol (70 per cent V/V) R*, sonicate for 15 min and dilute to 10.0 mL with the same solvent. Dilute 4.0 mL of this solution to 100.0 mL with *ethanol (70 per cent V/V) R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water R* (1:999 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 90 | 10 |
| 0 - 13 | 90 → 78 | 10 → 22 |
| 13 - 14 | 78 → 60 | 22 → 40 |
| 14 - 20 | 60 | 40 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 10 µL.

*Relative retention* with reference to chlorogenic acid (retention time = about 7 min): caftaric acid = about 0.8; caffeic acid = about 1.5; cynarin = about 1.6; echinacoside = about 1.7; cichoric acid = about 2.3.

*System suitability*: reference solution:

- *resolution*: minimum 10 between the peaks due to caffeic acid and chlorogenic acid.

Locate the peaks due to caffeic acid and chlorogenic acid using the chromatogram obtained with the reference solution. Locate the peaks due to echinacoside, caftaric acid and cichoric acid using the chromatogram in Figure 1822.-1.

Calculate the percentage content of echinacoside using the following expression:

$$\frac{A_1 \times C_2 \times 100 \times 2.221}{A_2 \times C_1}$$

$A_1$ = area of the peak due to echinacoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the reference solution;

$C_1$ = concentration of the test solution, in milligrams per millilitre;

$C_2$ = concentration of chlorogenic acid in the reference solution, in milligrams per millilitre;

2.221 = peak correlation factor between chlorogenic acid and echinacoside.

STORAGE

Uncomminuted.

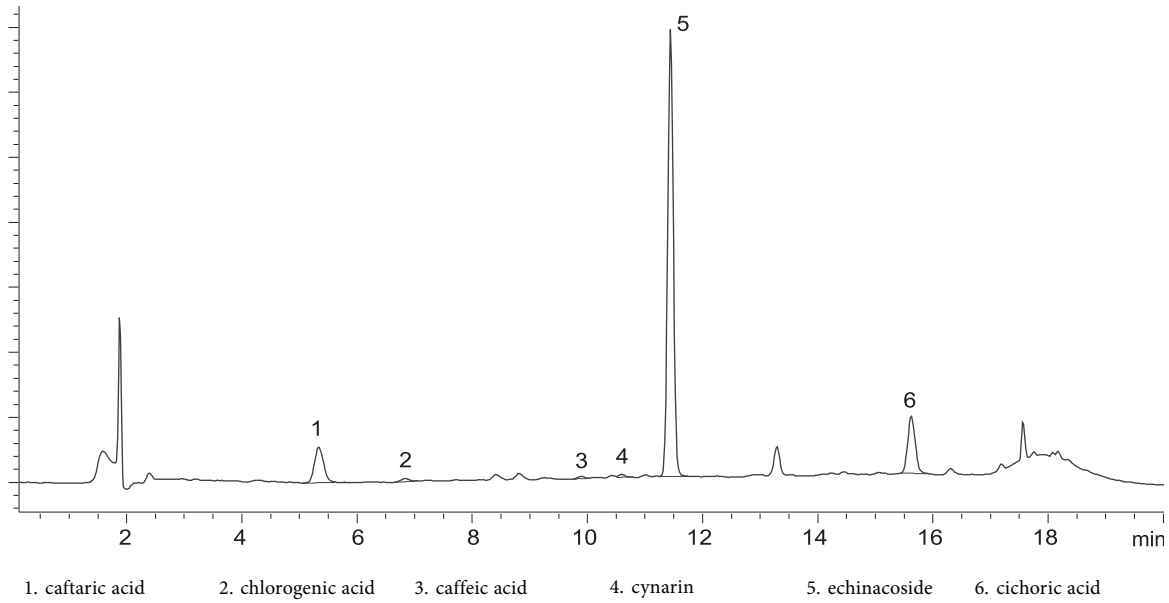


Figure 1822.-1. – *Chromatogram for the assay of echinacoside in pale coneflower root*

**01/2008:1459**
**corrected 6.0**

# PASSION FLOWER

## Passiflorae herba

DEFINITION

Fragmented or cut, dried aerial parts of *Passiflora incarnata* L. It may also contain flowers and/or fruits.
*Content*: minimum 1.5 per cent of total flavonoids, expressed as vitexin ($C_{21}H_{20}O_{10}$; $M_r$ 432.4) (dried drug).

IDENTIFICATION

A. The green or greenish-grey or brownish stem is ligneous, hollow, longitudinally striated, glabrous or very slightly pubescent, with a diameter that is generally less than 8 mm. The green or greenish-brown leaves are alternate, finely dentate and pubescent, deeply divided into 3 acute lobes of which the central lobe is the largest. The midrib is much more prominent on the lower surface. The petiole is pubescent and bears 2 dark nectaries near the lamina. The tendrils are very numerous and grow from the axils of the leaves; they are fine, smooth, round and terminated in cylindrical spirals. The radiate flowers, if present, have 3 small bracts and a corolla consisting of 5 white, elongated petals with several rows of filiform, petaloid appendices. If present, the greenish or brownish fruit is flattened and oval; it contains several flattened, brownish-yellow, pitted seeds.

B. Microscopic examination (*2.8.23*). The powder is light green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the leaf epidermis with sinuous walls and anomocytic stomata (*2.8.3*); numerous cluster crystals of calcium oxalate isolated or aligned along the veins; many isolated or grouped fibres from the stems associated with pitted vessels and tracheids; uniseriate trichomes with 1-3 thin-walled cells, straight or slightly curved, ending in a point or sometimes a hook. In addition, the powder shows, if flowers are present, papillose epidermises of the petals and appendages and pollen grains with a reticulate exine; and if mature fruits are present, scattered brown tannin cells and brownish-yellow, pitted fragments of the testa.

C. Examine the chromatograms obtained in the test for other species of *Passiflora*.

*Results*: the chromatogram obtained with the test solution shows below the zone due to rutoside in the chromatogram obtained with the reference solution a zone of intense yellow fluorescence, above it a zone of green fluorescence (diglycosylflavone), below the zone due to hyperoside in the chromatogram obtained with the reference solution a zone of yellow fluorescence (iso-orientin) and above a zone of green fluorescence (isovitexin), above the zone due to hyperoside in the chromatogram obtained with the reference solution a zone of brownish-yellow fluorescence (orientin) and above it a zone of green fluorescence (vitexin). These latter 2 zones may be absent. Further zones may be present.

TESTS

**Other species of *Passiflora*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Heat to boiling under a reflux condenser for 10 min. Cool and filter.

*Reference solution.* Dissolve with heating 2.0 mg of *rutoside trihydrate R* and 2.0 mg of *hyperoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow to dry in air for 30 min. Examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the reference solution shows in the lower third a zone of yellowish-brown fluorescence due to rutoside and in the middle third a zone of yellowish-brown fluorescence due to hyperoside. The chromatogram obtained with the test solution shows no intense zones of greenish-yellow or orange-yellow fluorescence between the zone due to diglycosylflavones and that due to iso-orientin (*P. coerulea* and *P. edulis*).

**Total ash** (*2.4.16*): maximum 13.0 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

ASSAY

*Stock solution.* In a 100 mL round-bottomed flask, introduce 0.200 g of the powdered herbal drug (250) (*2.9.12*) and add 40 mL of *ethanol (60 per cent V/V) R*. Heat in a water-bath at 60 °C under a reflux condenser for 30 min while shaking frequently. Allow to cool and filter the mixture through a plug of absorbent cotton in a 100 mL flask. Transfer the absorbent cotton with the drug residue into the round-bottomed flask. Add 40 mL of *ethanol (60 per cent V/V) R* and heat again in a water-bath at 60 °C under reflux for 10 min. Allow to cool and filter the mixture and the first filtrate from the 100 mL flask through a filter paper into the 100 mL volumetric flask. Dilute to 100 mL with the same solvent, while rinsing the flask, round-bottomed flask and filter.

*Test solution.* Introduce 5.0 mL of stock solution into a flask. Evaporate to dryness under reduced pressure and take up the residue with 10 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R*. Add 10 mL of a solution consisting of 25 g/L of *boric acid R* and 20 g/L of oxalic acid in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a second flask. Evaporate to dryness under reduced pressure and take up the residue with 10 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R*. Add 10 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 401 nm, by comparison with the compensation liquid.

Calculate the percentage content of total flavonoids, expressed as vitexin, using the following expression:

$$\frac{A \times 0.8}{m}$$

i.e. taking the specific absorbance of vitexin to be 628.

$A$ = absorbance at 401 nm,

$m$ = mass of the herbal drug to be examined, in grams.

**01/2008:1882**

# PASSION FLOWER DRY EXTRACT

## Passiflorae herbae extractum siccum

DEFINITION

Dry extract produced from *Passion flower (1459)*.

*Content*: minimum 2.0 per cent of flavonoids, expressed as vitexin ($C_{21}H_{20}O_{10}$; $M_r$ 432.4) (dried extract).

PRODUCTION

The extract is produced from the herbal drug and ethanol (40 per cent *V/V* to 90 per cent *V/V*), methanol (60 per cent *V/V*) or acetone (40 per cent *V/V*) by an appropriate procedure.

CHARACTERS

*Appearance*: greenish-brown amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.25 g of the extract to be examined add *methanol R*. Shake, filter and dilute to 5 mL with *methanol R*.

*Reference solution.* Dissolve 2.0 mg of *hyperoside R* and 2.0 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] as bands.

*Development*: over a path of 15 cm [or 5 cm].

*Drying*: at 100-105 °C.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Subsequently spray with a 50 g/L solution of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for about 30 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Other fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Hyperoside: a yellowish-orange fluorescent zone | A green fluorescent zone |
| | A yellow fluorescent zone |
| Rutoside: a yellowish-orange fluorescent zone | |
| ——— | ——— |
| | A green fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 5.0 per cent, determined on 0.500 g.

ASSAY

*Stock solution.* To 50 mg of the extract to be examined add *ethanol (60 per cent V/V) R*. Shake, filter and dilute to 100.0 mL with *ethanol (60 per cent V/V) R*.

*Test solution.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 mL volumetric flask. Add 10.0 mL of a solution containing 25.0 g/L of *boric acid R* and 20.0 g/L of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the 25 mL volumetric flask. Add 10.0 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

After 30 min, measure the absorbance (*2.2.25*) of the test solution at 401 nm.

Calculate the percentage content of total flavonoids, expressed as vitexin, from the following expression:

$$\frac{A \times 0.8}{m}$$

i.e. taking the specific absorbance of vitexin to be 628.

$A$ = absorbance at 401 nm,

$m$ = mass of the extract to be examined, in grams.

**01/2008:2264**
**corrected 6.0**

# PELARGONIUM ROOT

## Pelargonii radix

DEFINITION

Dried, usually fragmented, underground organs of *Pelargonium sidoides* DC and/or *Pelargonium reniforme* Curt.

*Content*: minimum 2.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The root is covered with dark, partly reddish-brown, longitudinally fissured bark. The transverse section shows, underneath the cork layer, yellow or white wood, which clearly shows partly brownish medullary rays.

B. Microscopic examination (*2.8.23*). The powder is brownish-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: multilayer cork cells consisting of almost uniform, rectangular cells; fragments of parenchyma underneath the cork containing sclereids with a wide lumen; numerous calcium oxalate cluster crystals. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple starch granules without striations or cracks.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*, shake for 15 min and filter.

*Reference solution.* Dissolve 1 mg of *scopoletin R* and 2 mg of *esculin R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (10:14:76 *V/V/V*).

*Application*: 10 µL [or 5 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *alcoholic potassium hydroxide solution R*. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other blue fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue fluorescent zone |
| Scopoletin: a very bright blue fluorescent zone | A weak blue fluorescent zone (scopoletin) |
| ——— | ——— |
| | One or two bright blue fluorescent zones |
| Esculin: a very bright blue fluorescent zone | |
| | A blue fluorescent zone |
| | A weak blue fluorescent zone |
| ——— | ——— |
| | A blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.750 g of the powdered herbal drug (180) (*2.9.12*).

**01/2017:2425**

# PEONY ROOT, RED

## Paeoniae radix rubra

DEFINITION

Dried, whole or fragmented root of *Paeonia lactiflora* Pall. or *Paeonia veitchii* Lynch or a mixture of the two, with rhizome and rootlets removed.

*Content*: minimum 1.8 per cent of paeoniflorin ($C_{23}H_{28}O_{11}$; $M_r$ 480.5) (dried drug).

IDENTIFICATION

A. *Whole drug.* The whole root is cylindrical, slightly curved. Truncated at both ends, 4-40 cm long, 0.5-3 cm in diameter, externally brown or reddish-brown, rough, longitudinally furrowed and wrinkled, showing rootlet scars and transversely prominent lenticels. The outer bark is sometimes easily exfoliated. The texture is hard and fragile, easily broken.

*Fragmented drug.* The fragmented root usually occurs as longitudinal slices, 2-8 cm long, about 1-5 mm thick and 0.5-3 cm in diameter, or as transversal slices, about 2-4 mm thick and 0.5-3 cm in diameter. The edges are brown, the cut surface is chalk-white or pinkish, the bark is narrow and the wood shows distinct radial striations, sometimes with clefts.

B. Microscopic examination (*2.8.23*). The powder is greyish-white or pale brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2425.-1): reticulate or bordered pitted vessels [G] up to 65 µm in diameter; cluster crystals of calcium oxalate, 10-35 µm in diameter, isolated [C] or included in parenchymatous cells [B], sometimes several in the same cell [Ba], sometimes arranged in rows [Ga]; long, fusiform, ligneous fibres, with thickened and slightly lignified walls with large oblique or cross pits [E]; brown cork fragments (surface view [A], transverse section [D]). Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple, spheroidal starch granules up to 50 µm in diameter [F].

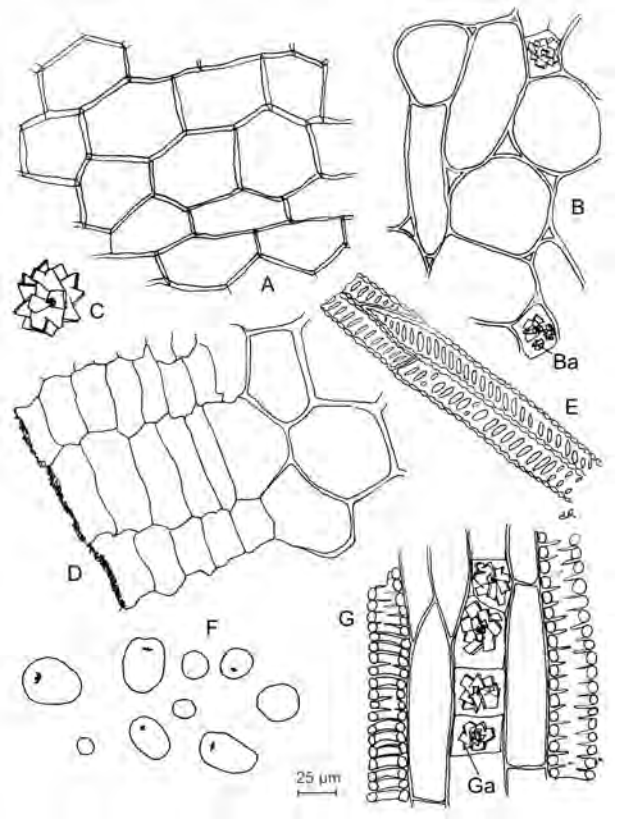


Figure 2425.-1. – *Illustration for identification test B of powdered herbal drug of peony root, red*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and heat in a water-bath at 60 °C for 1 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *paeoniflorin R* and 1 mg of *paeonol R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *methanol R*, *methylene chloride R* (3:5:5:35 *V/V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a quenching zone | |
| | A diffuse quenching zone |
| ——— | ——— |
| | A diffuse quenching zone |
| | A quenching zone |
| ——— | ——— |
| Paeoniflorin: a quenching zone | A quenching zone (paeoniflorin) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *ethanol (96 per cent) R*, heat at 100 °C for 5 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a yellow zone | |
| | 2 violet zones |
| ——— | ——— |
| | 2 violet zones |
| | 2 violet zones |
| | A diffuse yellowish-brown zone |
| ——— | ——— |
| Paeoniflorin: a brown zone | A brown zone (paeoniflorin) |
| | A brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 30.0 mg of the powdered herbal drug (355) (*2.9.12*) add 3.0 mL of *methanol R* and sonicate for 30 min. Dilute to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 5.0 mg of *paeoniflorin CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 1.0 mg of *4′-hydroxyacetophenone R* in 6.0 mL of reference solution (a). Dilute 3.0 mL of this solution to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Reference solution (c)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 6.8 g/L solution of *potassium dihydrogen phosphate R* (30:70 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 18 min.

*Retention time*: paeoniflorin = about 8 min; 4′-hydroxyacetophenone = about 9 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to paeoniflorin and 4′-hydroxyacetophenone.

Calculate the percentage content of paeoniflorin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to paeoniflorin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to paeoniflorin in the chromatogram obtained with reference solution (c);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *paeoniflorin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of paeoniflorin in *paeoniflorin CRS*.

**01/2017:2424**

# PEONY ROOT, WHITE

## Paeoniae radix alba

DEFINITION

Whole or fragmented, peeled root, of *Paeonia lactiflora* Pall. with rhizome and rootlets removed, treated with boiling water and dried.

*Content*: minimum 1.6 per cent of paeoniflorin ($C_{23}H_{28}O_{11}$; $M_r$ 480.5) (dried drug).

IDENTIFICATION

A. *Whole drug.* The whole root is cylindrical, straight or slightly curved, truncated at both ends, 5-18 cm long, 1-2.5 cm in diameter, externally whitish or pale reddish-brown, glossy or with longitudinal wrinkles and rootlet scars. Blackish-brown zones due to lesions, especially those around the rootlet scars, and remains of brown cork are occasionally present. The texture is compact, relatively even and not easily broken.

*Fragmented drug.* The fragmented root usually occurs as longitudinal slices, 2-8 cm long, about 1-5 mm thick and 1-2.5 cm in diameter, or as transversal slices, about 2-4 mm thick and 1-2.5 cm in diameter. The edges are whitish or pale reddish-brown and the cut surface is whitish, often with a pinkish tone. The cambium ring and radial striations are distinct.

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2424.-1): bordered-pitted or reticulate vessels [A, E], 20-65 µm in diameter; cluster crystals of calcium oxalate, 10-35 µm in diameter, isolated [C, D] or included in parenchymatous cells [B, F], sometimes several in the same cell [Fa], often arranged in rows [Ba]; ligneous fibres with thickened walls with large pits [Ea]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous masses of gelatinised starch, either isolated [H] or associated with the remains of cells [G].

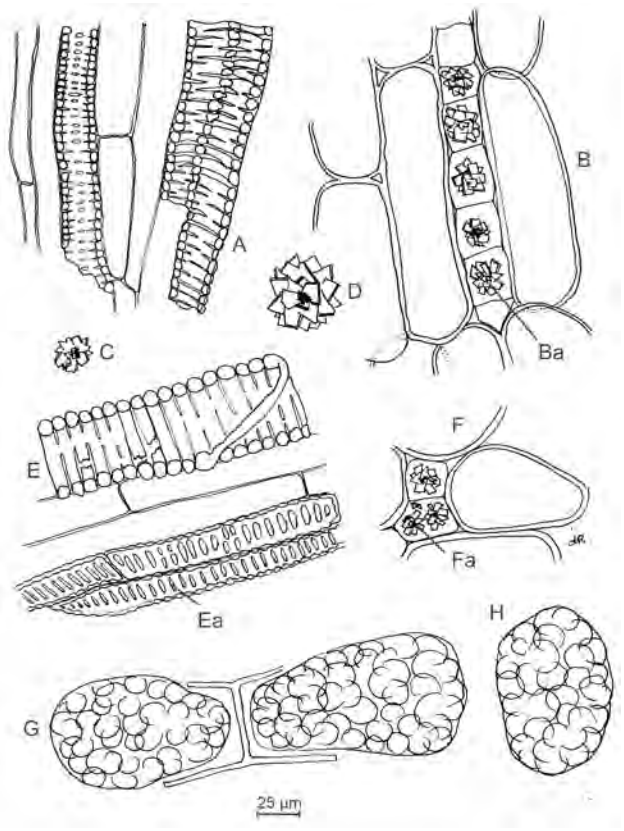


Figure 2424.-1. – *Illustration for identification test B of powdered herbal drug of peony root, white*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and heat in a water-bath at 60 °C for 1 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *paeoniflorin R* and 1 mg of *paeonol R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *methanol R*, *methylene chloride R* (3:5:5:35 *V/V/V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a quenching zone | |
| _____ | _____ |
| _____ | _____ |
| Paeoniflorin: a quenching zone | A faint quenching zone (paeoniflorin) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *ethanol (96 per cent) R*, heat at 100 °C for 5 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Paeonol: a yellow zone | |
| | 2 violet zones |
| _____ | _____ |
| | A sharp violet zone |
| | A violet zone |
| | A violet zone |
| | A yellowish-brown zone |
| _____ | _____ |
| Paeoniflorin: a brown zone | A brown zone (paeoniflorin) |
| | A brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 0.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 30.0 mg of the powdered herbal drug (355) (*2.9.12*) add 3.0 mL of *methanol R* and sonicate for 30 min. Dilute to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *paeoniflorin CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 1.0 mg of *4′-hydroxyacetophenone R* in 6.0 mL of reference solution (a). Dilute 3.0 mL of this solution to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Reference solution (c).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with a 6.8 g/L solution of *potassium dihydrogen phosphate R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 6.8 g/L solution of *potassium dihydrogen phosphate R* (30:70 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 18 min.

*Retention time*: paeoniflorin = about 8 min; 4′-hydroxyacetophenone = about 9 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to paeoniflorin and 4′-hydroxyacetophenone.

Calculate the percentage content of paeoniflorin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to paeoniflorin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to paeoniflorin in the chromatogram obtained with reference solution (c);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *paeoniflorin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of paeoniflorin in *paeoniflorin CRS*.

**01/2013:2477**
**corrected 10.0**

# PEPPER

## Piperis fructus

DEFINITION

Dried, ripe or nearly ripe fruit of *Piper nigrum* L. with an unbroken pericarp (black pepper) or with the outer layers of the pericarp removed (white pepper).

*Content*:

– *essential oil*: minimum 25 mL/kg (anhydrous drug);
– *piperine* ($C_{17}H_{19}NO_3$; $M_r$ 285.3): minimum 3.0 per cent (anhydrous drug).

IDENTIFICATION

A. *White pepper.* Spheroid berries, 3-5 mm in diameter, slightly flattened at one pole and with a small protuberance at the other, with smooth, externally matt, brownish-grey, greyish-white or pale yellowish-white surface, with numerous pale, linear striations between apex and base.

*Black pepper.* Spheroid berries, 3-6 mm in diameter, externally blackish-brown, with raised reticular wrinkles, bearing fine remains of the style at the apex and a scar of the peduncle at the base. The texture is hard, the epicarp can be stripped, the endocarp is greyish-white or pale yellow. The fracture is greyish-white, starchy, possessing a small space at the centre.

B. Microscopic examination (*2.8.23*).

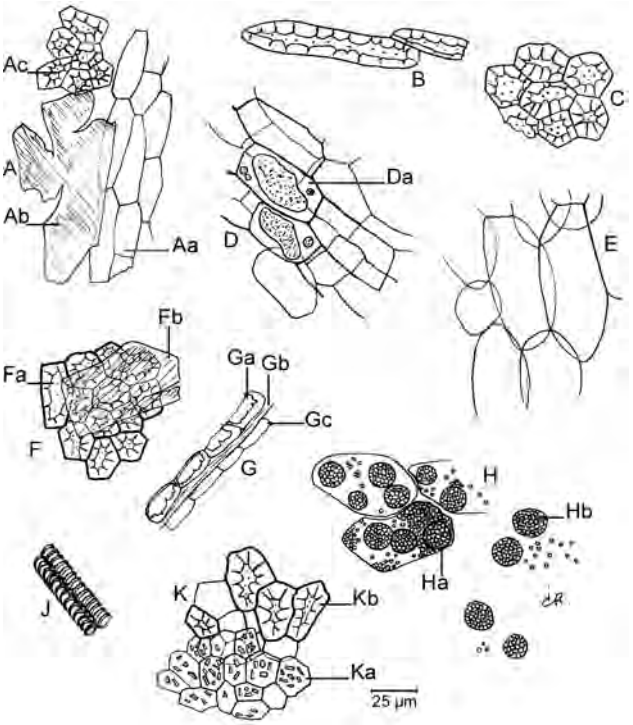


Figure 2477.-1. – *Illustration for identification test B of powdered herbal drug of pepper*

*White pepper.* The powder is light grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2477.-1): fragments of the endocarp in surface view, consisting of more or less polygonal sclereids about 20-30 µm in diameter, which have irregularly thickened walls [Ac, C, Fa] and which may or may not be associated with the testa [A, F], consisting of a layer of indistinct, reddish-brown pigmented cells constituting the 'pigmented layer' [Ab, Fb] and a layer of very thin-walled polygonal cells constituting the 'hyaline layer' [Aa]; fragments of the endocarp, in transverse section [G], showing sclereids with thickened inner walls on the 3 lower sides [Ga], usually associated with the testa (pigmented layer [Gb] and hyaline layer [Gc]); fragments of the parenchyma of the mesocarp [D] containing large oil cells 50-75 µm in diameter [Da]; numerous thin-walled, ovoid or polygonal cells of the parenchyma of the seed [E]; rare, elongated sclereids, with thickened walls, from the fruit peduncle [B]; a few fragments of vascular tissue with narrow spiral vessels [J]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. Rounded, compound starch granules [H], about 30 µm in diameter, made up of tiny individual granules, ovoid or polyhedral by compression, free [Hb] or included in the parenchymatous cells of the seed [Ha].

*Black pepper*. The powder is grey. Examine under a microscope using *chloral hydrate solution R*. In addition to the diagnostic characters described for white pepper, the powdered black pepper shows the following diagnostic characters (Figure 2477.-1): fragments of the epicarp [K] with extremely thin-walled, brownish-red pigmented, polygonal or ovoid cells, which contain small prisms of calcium oxalate [Ka], and which are associated with the outer layers of the mesocarp consisting of groups of sclereids with strongly thickened walls [Kb].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min, centrifuge and use the supernatant.

*Reference solution*. Dissolve 10 mg of *borneol R* and 15 mg of *piperine R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (30:50 *V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | 3 quenching zones |
| ——— | ——— |
| | A quenching zone |
| Piperine: a quenching zone | A strong quenching zone (piperine) |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A strong purple zone |
| | A purple zone |
| ——— | ——— |
| Borneol: a yellowish-brown zone | |
| | A purple-grey zone |
| ——— | ——— |
| | A violet-grey zone |
| | A grey zone |
| Piperine: a green or brownish zone | A green or brownish zone (piperine) |
| | A grey zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Water** (*2.2.13*): maximum 120 mL/kg, determined on 20.0 g of the freshly powdered herbal drug (1400) (*2.9.12*) reduced using a knife mill.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the freshly powdered herbal drug (1400) (*2.9.12*), a 1000 mL round-bottomed flask, 400 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 3 h.

**Piperine**. Liquid chromatography (*2.2.29*). *Carry out the assay protected from light.*

*Test solution*. Disperse 0.250 g of the powdered herbal drug (355) (*2.9.12*) in 40 mL of *ethanol (96 per cent) R*. Sonicate for 20 min and filter. Rinse the flask and the filter with 5 mL of *ethanol (96 per cent) R*, combine the filtrate and washings and dilute to 50.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 15.0 mg of *piperine CRS* in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Disperse 0.250 g of *long pepper for system suitability HRS* (355) (*2.9.12*) in 40 mL of *ethanol (96 per cent) R*. Sonicate for 20 min and filter. Rinse the flask and the filter with 5 mL of *ethanol (96 per cent) R*, combine the filtrate and washings and dilute to 50.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 50 | 50 |
| 5 - 20 | 50 → 5 | 50 → 95 |
| 20 - 22 | 5 → 0 | 95 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 343 nm.

*Injection*: 10 µL.

*Retention time*: piperine = about 10 min.

*Identification of peaks*: use the chromatogram supplied with *long pepper for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to piperine and peak 2.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of peak 2 and $H_v$ = height above the baseline of the lowest point of the curve separating the peak due to piperine from peak 2.

Calculate the percentage content of piperine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to piperine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to piperine in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *piperine CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of piperine in *piperine CRS*.

**07/2017:0406**

# PEPPERMINT LEAF

## Menthae piperitae folium

DEFINITION

Whole or cut, dried leaf of *Mentha ×piperita* L.

*Essential oil content*:

– for the whole drug, minimum 12 mL/kg (anhydrous drug);

– for the cut drug, minimum 9 mL/kg (anhydrous drug).

CHARACTERS

Penetrating odour reminiscent of menthol.

Peppermint leaf is green or brownish-green, with brownish-violet veins in some varieties. The petioles are green or brownish-violet.

IDENTIFICATION

A. The leaf is entire, broken or cut, thin, fragile and often crumpled; the entire leaf is 3-9 cm long and 1-3 cm wide. The lamina is oval or lanceolate, the apex acuminate, the margin sharply dentate and the base asymmetrical. Venation is pinnate, prominent on the lower surface, with lateral veins leaving the midrib at about 45°. The lower surface is slightly pubescent and secretory trichomes are visible under a lens (6×) as bright yellowish points. The petiole is grooved, usually up to 1 mm in diameter and 0.5-1 cm long.

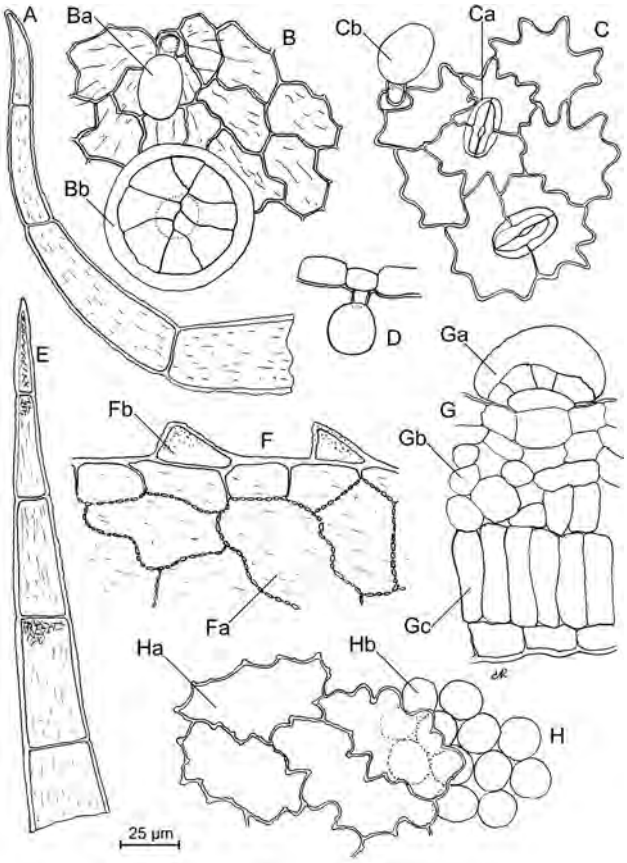


Figure 0406.-1. – *Illustration for identification test B of powdered herbal drug of peppermint leaf*

B. Microscopic examination (*2.8.23*). The powder is brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0406.-1): fragments of epidermises bearing covering and glandular trichomes; adaxial epidermis (surface view [B, H]) having cells with sinuous-wavy walls [Ha] and cuticle striated over the veins [B] associated with palisade parenchyma [Hb]; abaxial epidermis [C] with diacytic stomata (*2.8.3*) [Ca]; covering trichomes are usually fragmented, elongated, uniseriate with 3-8 cells with striated cuticle [A, E]; glandular trichomes of 2 types: a) unicellular stalk with small, rounded unicellular head 15-25 µm in diameter (surface view [Ba, Cb], transverse section [D]), b) unicellular stalk with enlarged oval head 55-70 µm in diameter composed of 8 radiating cells (surface view [Bb], transverse section [Ga]); fragments from near the leaf margin [F] with isodiametric cells whose anticlinal walls are more-or-less straight and beaded [Fa] and short, conical, unicellular or bicellular covering trichomes [Fb]; dorsiventral mesophyll fragments (transverse section [G]), with a single palisade layer [Gc] and 4-6 layers of spongy parenchyma [Gb]. Yellowish crystals of menthol under the cuticle of secretory cells may be present.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 2.5 mL of *methanol R* and 2.5 mL of *water R*, sonicate for 5 min and filter.

*Reference solution.* Dissolve 2 mg of *luteolin-7-glucoside R*, 2 mg of *rutoside trihydrate R* and 5 mg of *rosmarinic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (7:7:18:68 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

Herbal drugs

*Drying*: in air.

*Detection*: heat at 100-105 °C for 5 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Rosmarinic acid: a light blue fluorescent zone | A light blue fluorescent zone (rosmarinic acid) |
| Luteolin-7-glucoside: an orange fluorescent zone | An orange-yellow fluorescent zone<br>A red fluorescent zone |
| Rutoside: an orange fluorescent zone | An orange-yellow fluorescent zone<br>An orange-yellow fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems, whose diameter is not greater than 1.5 mm; maximum 2 per cent of foreign elements; not more than 8 per cent of the leaves show brown stains due to *Puccinia menthae*.

Carry out the determination using 10 g of the drug.

**Water** (*2.2.13*): maximum 110 mL/kg, determined on 20.0 g.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the crushed herbal drug, a 500 mL flask, 200 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 2 h.

**07/2017:2382**

# PEPPERMINT LEAF DRY EXTRACT

## Menthae piperitae folii extractum siccum

DEFINITION

Dry extract produced from *Peppermint leaf (0406)*.

*Content*: minimum 0.5 per cent of rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (30-50 per cent *V/V*) or water at minimum 60 °C.

CHARACTERS

*Appearance*: brown, amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.2 g of the extract to be examined add 2.5 mL of *methanol R* and 2.5 mL of *water R*, sonicate for 5 min and filter.

*Reference solution.* Dissolve 2 mg of *luteolin-7-glucoside R*, 2 mg of *rutoside trihydrate R* and 5 mg of *rosmarinic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (7:7:18:68 *V/V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100-105 °C for 5 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Rosmarinic acid: a light blue fluorescent zone | A light blue fluorescent zone (rosmarinic acid) |
| Luteolin-7-glucoside: an orange fluorescent zone | A faint orange fluorescent zone<br>An orange-yellow fluorescent zone<br>A red fluorescent zone |
| Rutoside: an orange fluorescent zone | An orange-yellow fluorescent zone<br>An orange-yellow fluorescent zone |
| **Reference solution** | **Test solution** |

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Use brown glass flasks. To 0.400 g of the extract to be examined add 15 mL of *ethanol (50 per cent V/V) R*, sonicate for 10 min and filter into a 20 mL volumetric flask. Rinse the flask and the filter with *ethanol (50 per cent V/V) R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *rosmarinic acid CRS* in *ethanol (50 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *ferulic acid R* in reference solution (a) and dilute to 50 mL with the same solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *acetonitrile R*, *water for chromatography R* (1:19:80 *V/V/V*);

– *mobile phase B*: *phosphoric acid R*, *methanol R*, *acetonitrile R* (1:40:59 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 55 | 0 → 45 |
| 20 - 25 | 55 → 0 | 45 → 100 |
| 25 - 30 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to rosmarinic acid.

*Relative retention* with reference to rosmarinic acid (retention time = about 11 min): ferulic acid = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to ferulic acid and rosmarinic acid.

Calculate the percentage content of rosmarinic acid using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rosmarinic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rosmarinic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rosmarinic acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rosmarinic acid in *rosmarinic acid CRS*.

**04/2019:0405**

# PEPPERMINT OIL

## Menthae piperitae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh aerial parts of *Mentha* × *piperita* L.

CHARACTERS

*Appearance:* colourless, pale yellow or pale greenish-yellow liquid.

Characteristic odour and taste followed by a sensation of cold.

*Solubility:* miscible with ethanol (96 per cent) and with methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Mix 0.1 g of the substance to be examined with *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *menthol R*, 20 µL of *cineole R*, 10 mg of *thymol R* and 10 µL of *menthyl acetate R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 1 µL] of the reference solution and 20 µL [or 2 µL] of the test solution, as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Thymol: a quenching zone | |
| | Quenching zones may be present (carvone, pulegone) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine immediately in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other less intensely coloured zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An intense violet-red zone (near the solvent front) (hydrocarbons) |
| | A brownish-yellow zone (menthofuran) |
| _____ | _____ |
| Menthyl acetate: a violet-blue zone | A violet-blue zone (menthyl acetate) |
| | A greenish-blue zone (menthone) |
| Thymol: a pink zone | |
| | Light pink or greyish-blue or greyish-green zones may be present (carvone, pulegone, isomenthone) |
| 1,8-Cineole: a violet-blue or brown zone | A faint violet-blue or brown zone (1,8-cineole) |
| _____ | _____ |
| Menthol: an intense blue or violet zone | An intense blue or violet zone (menthol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks due to limonene, 1,8-cineole, menthone, menthofuran, isomenthone, menthyl acetate and menthol in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a). Isopulegol, pulegone and carvone may be present in the chromatogram obtained with the test solution.

TESTS

**Relative density** (*2.2.5*): 0.900 to 0.916.

**Refractive index** (*2.2.6*): 1.457 to 1.467.

**Optical rotation** (*2.2.7*): – 30° to – 10°.

**Acid value** (*2.5.1*): maximum 1.4, determined on 5.0 g diluted in 50 mL of the prescribed mixture of solvents.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test for fatty oils and resinified essential oils.

**Mint oil**. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the chromatogram obtained with the test solution does not show a peak with the retention time of isopulegol that has an area of more than 0.2 per cent of the total area.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Mix 0.20 mL of the substance to be examined with *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 µL of *limonene R*, 20 µL of *cineole R*, 40 µL of *menthone R*, 10 µL of *menthofuran R*, 10 µL of *(+)-isomenthone R*, 40 µL of *menthyl acetate R*, 20 µL of *isopulegol R*, 60 mg of *menthol R*, 20 µL of *pulegone R*, 10 µL of *piperitone R* and 10 µL of *carvone R* in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5 µL of *isopulegol R* in *heptane R* and dilute to 10.0 mL with the same solvent. Dilute 0.1 mL of the solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 70 | 60 → 180 |
| | 70 - 75 | 180 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*Identification of peaks*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to limonene and 1,8-cineole; minimum 1.5 between the peaks due to piperitone and carvone.

Determine the percentage content of each of the following components. The limits are within the following ranges:

- *limonene*: 1.0 per cent to 3.5 per cent;
- *1,8-cineole*: 3.5 per cent to 8.0 per cent;
- *menthone*: 14.0 per cent to 32.0 per cent;
- *menthofuran*: 1.0 per cent to 8.0 per cent;
- *isomenthone*: 1.5 per cent to 10.0 per cent;
- *menthyl acetate*: 2.8 per cent to 10.0 per cent;
- *isopulegol*: maximum 0.2 per cent;
- *menthol*: 30.0 per cent to 55.0 per cent;
- *pulegone*: maximum 3.0 per cent;
- *carvone*: maximum 1.0 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

The ratio of 1,8-cineole content to limonene content is a minimum of 2.

STORAGE

At a temperature not exceeding 25 °C.

**01/2008:0754**

# PERU BALSAM

## Balsamum peruvianum

DEFINITION

Balsam obtained from the scorched and wounded trunk of *Myroxylon balsamum* (L.) Harms var. *pereirae* (Royle) Harms.

*Content*: 45.0 per cent *m/m* to 70.0 per cent *m/m* of esters, mainly benzyl benzoate and benzyl cinnamate.

CHARACTERS

*Appearance*: dark brown, viscous liquid which is transparent and yellowish-brown when viewed in a thin layer; it is not sticky, non-drying and does not form threads.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, not miscible with fatty oils, except for castor oil.

IDENTIFICATION

A. Dissolve 0.20 g in 10 mL of *ethanol (96 per cent) R*. Add 0.2 mL of *ferric chloride solution R1*. A green or yellowish-green colour develops.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.5 g of the substance to be examined in 10 mL of *ethyl acetate R*.

*Reference solution.* Dissolve 4 mg of *thymol R*, 30 mg of *benzyl cinnamate R* and 80 µL of *benzyl benzoate R* in 5 mL of *ethyl acetate R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *hexane R* (0.5:10:90 *V/V/V*).

*Application*: 10 µL, as bands of 20 mm by 3 mm.

*Development*: twice over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm and mark the quenching zones.

*Results A*: the chromatogram obtained with the reference solution shows in the upper third 2 quenching zones, the higher one due to benzyl benzoate and the lower one due to benzyl cinnamate. The chromatogram obtained with the test solution shows 2 quenching zones at the same levels and of approximately the same size.

*Detection B*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*, using 10 mL for a plate 200 mm square and examine in daylight while heating at 100-105 °C for 5-10 min.

*Results B*: the zones due to benzyl benzoate and benzyl cinnamate are blue against a yellow background. The chromatogram obtained with the reference solution shows at about the middle a violet-grey zone (thymol). In the chromatogram obtained with the test solution, a blue zone

(nerolidol) is seen just below the level of the zone due to thymol in the chromatogram obtained with the reference solution. Just below the zone due to nerolidol, no blue zone is seen corresponding to a quenching zone seen when examined in ultraviolet light at 254 nm (colophony). In the upper and lower part of the chromatogram obtained with the test solution, other faint blue zones may be seen.

TESTS

**Relative density** (*2.2.5*): 1.14 to 1.17.

**Saponification value** (*2.5.6*): 230 to 255, determined on the residue obtained in the assay.

**Artificial balsams**. Shake 0.20 g with 6 mL of *light petroleum R1*. The light petroleum solution is clear and colourless and the whole of the insoluble parts of the balsam stick to the wall of the test-tube.

**Fatty oils**. Shake 1 g with 3 mL of a 1000 g/L solution of *chloral hydrate R*. The resulting solution is as clear as the 1000 g/L solution of *chloral hydrate R*.

**Turpentine**. Evaporate to dryness 4 mL of the solution obtained in the test for artificial balsams. The residue has no odour of turpentine.

ASSAY

To 2.50 g in a separating funnel add 7.5 mL of *dilute sodium hydroxide solution R* and 40 mL of *peroxide-free ether R* and shake vigorously for 10 min. Separate the lower layer and shake it with 3 quantities, each of 15 mL, of *peroxide-free ether R*. Combine the ether layers, dry over 10 g of *anhydrous sodium sulfate R* and filter. Wash the sodium sulfate with 2 quantities, each of 10 mL, of *peroxide-free ether R*. Combine the ether layers and evaporate to dryness. Dry the residue (esters) at 100-105 °C for 30 min and weigh.

STORAGE

Protected from light.

**01/2008:1842**

# PINE SYLVESTRIS OIL

## Pini sylvestris aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the fresh leaves and branches of *Pinus sylvestris* L. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

Characteristic odour.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 1 mL of the substance to be examined to 10 mL with *toluene R*.

*Reference solution*. Dissolve 10 mg of *borneol R* and 10 µL of *bornyl acetate R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A pink zone (hydrocarbons) |
| _____ | _____ |
| Bornyl acetate: a brown or grey-brown zone | A brown or grey-brown zone (bornyl acetate) |
| | A pink zone |
| _____ | _____ |
| Borneol: a brown or grey-brown zone | A cluster of violet zones |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.855 to 0.875.

**Refractive index** (*2.2.6*): 1.465 to 1.480.

**Optical rotation** (*2.2.7*): – 9° to – 30°.

**Acid value** (*2.5.1*): maximum 1.0.

**Peroxide value** (*2.5.5*): maximum 20.

**Fatty oils and resinified oils** (*2.8.7*). It complies with the test.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution (a)*. Dissolve 30 µL of *α-pinene R*, 10 mg of *camphene R*, 20 µL of *β-pinene R*, 10 µL of *car-3-ene R*, 10 µL of *β-myrcene R*, 20 µL of *limonene R*, 10 µL of *p-cymene R*, 10 µL of *terpinolene R*, 10 µL of *bornyl acetate R* and 10 µL of *β-caryophyllene R* in 1 mL of *heptane R*.

*Reference solution (b)*. Dissolve 10 mg of *camphene R* in *heptane R* and dilute to 2 mL with the same solvent. Dilute 0.1 mL of the solution to 1 mL with *heptane R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 60 m, Ø = 0.22 mm,
- *stationary phase*: *macrogol 20 000 R* (0.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 65 |
| | 10 - 41 | 65 → 220 |
| | 41 - 50 | 220 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the preparation of reference solution (a). Record the retention times of these substances.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to car-3-ene and β-myrcene.

*Identification of components*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution. The peak due to β-phellandrene is eluted after the peak due to limonene with a relative retention of about 1.03 with reference to limonene.

Determine the percentage content of these components. The limits are within the following ranges:

– *α-pinene*: 32.0 per cent to 60.0 per cent,
– *camphene*: 0.5 per cent to 2.0 per cent,
– *β-pinene*: 5.0 per cent to 22.0 per cent,
– *car-3-ene*: 6.0 per cent to 18.0 per cent,
– *β-myrcene*: 1.5 per cent to 10.0 per cent,
– *limonene*: 7.0 per cent to 12.0 per cent,
– *β-phellandrene*: maximum 2.5 per cent,
– *p-cymene*: maximum 2.0 per cent,
– *terpinolene*: maximum 4.0 per cent,
– *bornyl acetate*: 1.0 per cent to 4.0 per cent,
– *β-caryophyllene*: 1.0 per cent to 6.0 per cent,
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b).

STORAGE

At a temperature not exceeding 25 °C.

**04/2018:2660**
**corrected 9.6**

# PLATYCODON ROOT

## Platycodonis radix

DEFINITION

Dried whole or fragmented root of *Platycodon grandiflorus* (Jacq.) A.DC., peeled or unpeeled, with rootlets removed, collected in spring or autumn.

*Content*: minimum 0.3 per cent of total saponins, expressed as platycodin D ($C_{57}H_{92}O_{28}$; $M_r$ 1225) (dried drug).

IDENTIFICATION

A. *Whole drug.* The whole root is cylindrical or slightly fusiform, gradually tapering downwards, sometimes branched, slightly twisted, about 5-20 cm long and 0.3-2 cm in diameter. It shows deep, longitudinally twisted furrows and rootlet scars or transverse lenticel-like scars; the margins of the rootlet scars may appear shiny and slightly translucent; the upper part of the root also shows transverse striations. The outer surface of the peeled root is white or brownish-yellow, whilst the outer surface of the unpeeled root is brownish-yellow or brownish-grey. Sometimes the upper part shows a relatively short or inconspicuous rhizome, marked by several crescent-shaped or irregularly round stem scars. The texture is fragile and the fracture uneven.

*Fragmented drug.* The fragmented root occurs as thin discs, about 1.5-4 mm thick, sometimes oblique or irregular; most come from peeled roots, but sometimes showing patches of bark still adhering. The outer surface may sometimes also be cracked. A transverse section shows a distinctly crenate outline, a yellow or pale brown cortex, a distinct, more or less dark brown cambium ring and a well-developed yellow or pale brown xylem showing radial striations and occasional clefts.

B. Microscopic examination (*2.8.23*). The powder is yellowish-white or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2660.-1): reticulate or pitted xylem vessels (longitudinal section [B]) up to 56 µm in diameter, free or in groups of 2, 3 or more, accompanied by thin-walled parenchyma cells [Ba]; the sieve plates of the vessels have a thickened outline giving the appearance of a hole; numerous fragments of parenchyma consisting of ovoid cells [A] with slightly thickened and lignified walls; fragments of phloem containing articulated laticiferous tubes, with thick non-lignified walls and granular yellowish-grey or brown contents (longitudinal section [F], transverse section [D]); fragments of orange cork, consisting of several layers of superimposed cells (surface view [C], transverse section [E]), frequent in unpeeled roots and relatively rare in peeled roots; small calcium oxalate prism crystals may be present in the parenchymatous cells. Examine under a microscope using *glycerol R*. The powder shows numerous pieces of inulin, angular, irregular [H] or crystallised in a fan shape, free or included in parenchyma cells [G].

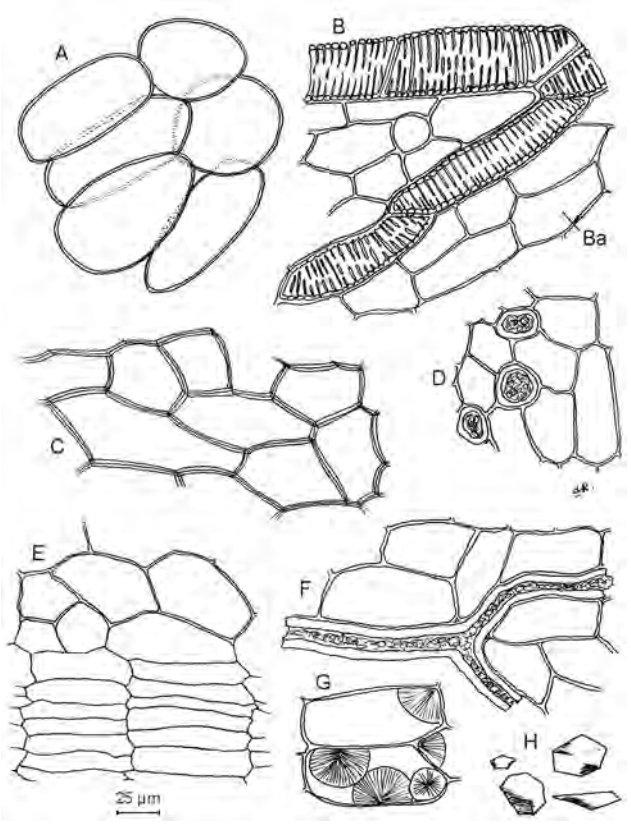


Figure 2660.-1. – *Illustration for identification test B of powdered herbal drug of platycodon root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.500 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *ethanol (70 per cent V/V) R*. Sonicate for 10 min and centrifuge or filter. Evaporate the supernatant or filtrate to dryness under reduced pressure. Dissolve the residue in 1.0 mL of *water R*. Prepare a ready-to-use sample preparation cartridge containing 50 mg of octadecylsilyl silica gel (55 µm), using 3 mL of *methanol R* followed by 3 mL of *water R*. Apply 1.0 mL of the solution to be analysed to the top of the cartridge. Elute the cartridge with 3 mL of *water R* and collect the eluate. Evaporate the eluate to dryness under reduced pressure. Dissolve the residue in 1.0 mL of *methanol R*.

*Reference solution.* Dissolve 1.0 mg of *glucose R* and 1.0 mg of *xylose R* in 2.0 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *glacial acetic acid R*, *methylene chloride R* (2:3:8:15 *V/V/V/V*).

*Application*: 10 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 3 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Xylose: a yellowish-brown zone | A yellowish-brown zone<br>A yellowish-brown zone |
| Glucose: a yellowish-brown zone | 2 yellowish-brown zones |
| **Reference solution** | **Test solution** |

TESTS

***Codonopsis pilosula* (Franch.) Nannf**. Examine the herbal drug as described in identification test B. The presence of sclereids, isolated or in groups, and starch granules indicates adulteration by *Codonopsis pilosula* (Franch.) Nannf.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *anhydrous ethanol R* (30:70 *V/V*).

*Test solution.* To 2.00 g of the powdered herbal drug (355) (*2.9.12*) add 50.0 mL of the solvent mixture and sonicate for 45 min. Allow to cool and filter. Rinse the filter with 10 mL of solvent mixture. Combine the filtrate and washings, and evaporate to dryness under reduced pressure. Take up the residue with the solvent mixture, transfer to a volumetric flask and dilute to 10.0 mL with the solvent mixture. Shake well and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 8.0 mg of *platycodin D CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions (b), (c), (d), (e), (f), (g).* Dilute reference solution (a) to obtain 6 reference solutions of platycodin D, the concentrations of which span the expected value in the test solution.

*Reference solution (h).* Dissolve 5.0 mg of *platycodon root dry extract for system suitability HRS* in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.7 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 85 → 75 | 15 → 25 |
| 10 - 30 | 75 | 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria:

- *carrier gas*: *nitrogen R*;
- *pressure*: 350 kPa;
- *evaporator temperature*: 45 °C.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *platycodon root dry extract for system suitability HRS* and the chromatogram obtained with reference solution (h) to identify the peaks due to saponins 1, 2 (platycodin D), 3, 4 and 5; use the chromatogram obtained with reference solution (a) to identify the peak due to platycodin D.

*Relative retention* with reference to platycodin D (retention time = about 21 min): saponin 1 = about 0.98; saponin 3 = about 1.03; saponin 4 = about 1.06; saponin 5 = about 1.08. Additional peaks may be present.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to saponin 1 and platycodin D in the chromatogram obtained with reference solution (h);
- *signal-to-noise ratio*: minimum 50 for the principal peak in the chromatogram obtained with reference solution (a).

Establish a calibration curve with the common logarithm of the concentration (in milligrams per millilitre) of reference solutions (a), (b), (c), (d), (e), (f) and (g) (corrected by the assigned percentage content of *platycodin D CRS*) as the abscissa and the common logarithm of the corresponding peak area as the ordinate.

Calculate the percentage content of saponins 1, 2, 3, 4 and 5 using the following expression:

$$\frac{10^A}{m}$$

*A* = common logarithm of the concentration of the peak due to each saponin in the chromatogram obtained with the test solution, determined from the calibration curve;

*m* = mass of the herbal drug to be examined used to prepare the test solution, in grams.

Calculate the percentage content of total saponins, expressed as platycodin D, by taking the sum of the percentage contents of saponins 1, 2, 3, 4 and 5.

**01/2017:2724**

# POLYGONUM CUSPIDATUM RHIZOME AND ROOT

## Polygoni cuspidati rhizoma et radix

DEFINITION

Dried, fragmented rhizome and root, with rootlets removed, of *Reynoutria japonica* Houtt. (syn. *Polygonum cuspidatum* Sieb. et Zucc.) collected in spring or autumn.

*Content*:

– *emodin* ($C_{15}H_{10}O_5$; $M_r$ 270.2): minimum 1.0 per cent (dried drug),

– *polydatin* ($C_{20}H_{22}O_8$; $M_r$ 390.4): minimum 1.5 per cent (dried drug).

IDENTIFICATION

A. Irregular thick slices or sections, about 2-9 cm long and 5-27 mm in diameter or sometimes cylindrical pieces. The outer surface is yellowish-brown, with longitudinal striations and rootlet scars. In transverse section, the brown or reddish-brown bark is relatively thin, separating easily from the wood. The wood is thick, yellowish-brown, with numerous radial striations. The pith of the rhizome is large, hollowed and transversally septate.

B. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2724.-1): very numerous cluster crystals of calcium oxalate, up to 100 µm in diameter, usually free [H], or sometimes inside parenchyma cells [Ca]; brownish-yellow fragments of cork (surface view [A]), with wavy striations in the cells; sclereids, elongated and ramified at one end, with clearly channelled walls, isolated or in small groups [G]; bundles of phloem fibres [E], thick-walled and channelled; fragments of the medullary rays [B], with rectangular or sub-rectangular cells with slightly thickened, beaded and pitted walls; fragments of large vessels, usually with bordered pits [D, F]; fragments of parenchyma with large ovoid cells having slightly thickened and pitted walls [C]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules, rounded or elliptical, on average 10 µm in diameter and with a punctiform or slightly eccentric slit-shaped hilum, simple or 2-10 compound [J].

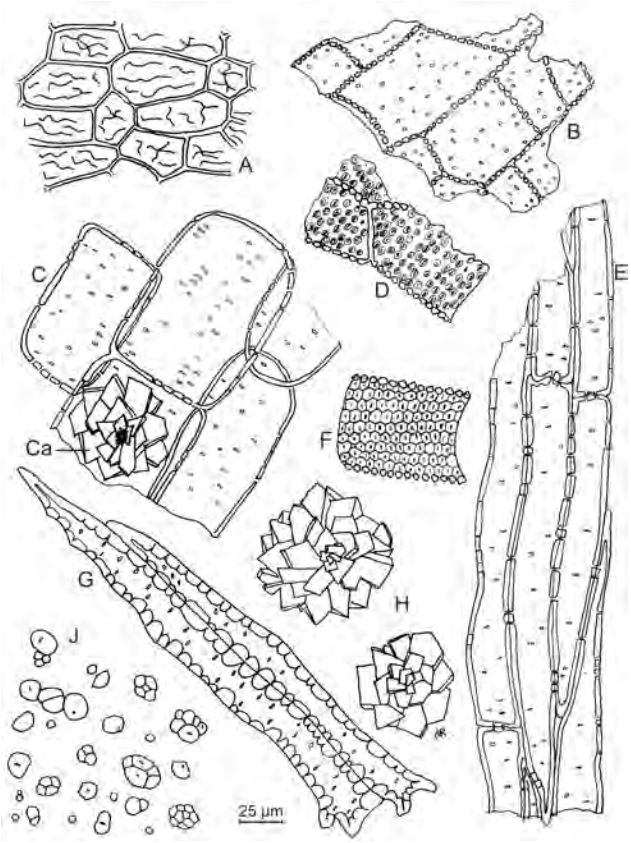


Figure 2724.-1. – *Illustration for identification test B of powdered herbal drug of Polygonum cuspidatum rhizome and root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and sonicate for 10 min. Centrifuge or filter, and use the supernatant or the filtrate as the test solution.

*Reference solution.* Dissolve 1 mg of *polydatin R* and 1 mg of *resveratrol R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *methanol R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 8 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | 2 yellow fluorescent zones |
| Resveratrol: a blue fluorescent zone | A blue fluorescent zone (resveratrol) |
| | An orange fluorescent zone |
| _____ | _____ |
| | A blue fluorescent zone |
| | A broad orange fluorescent zone |
| _____ | _____ |
| Polydatin: a blue fluorescent zone | A blue fluorescent zone (polydatin) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 4 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Emodin**. Liquid chromatography (*2.2.29*).

*Test solution.* To 0.150 g of the powdered herbal drug (355) (*2.9.12*) in a round-bottomed flask add 150.0 mL of a 14 per cent *V/V* solution of *sulfuric acid R* and 150.0 mL of *methylene chloride R*. Heat under a reflux condenser in a water-bath at 60 °C for 2 h. Allow to cool and transfer the contents to a separating funnel. Separate the organic layer and shake the acid layer with 3 quantities, each of 15 mL, of *methylene chloride R*. Combine the organic extracts and evaporate to dryness. Dissolve the residue in *methanol R* and dilute to 100.0 mL with the same solvent. Filter the solution through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *emodin CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 20.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 2.5 mg of *rhein R* in *methanol R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R, water for chromatography R* (0.1:99.9 *V/V*);

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 11 | 15 | 85 |
| 11 - 11.1 | 15 → 5 | 85 → 95 |
| 11.1 - 22 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Retention time*: rhein = about 4 min; emodin = about 6 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 5 between the peaks due to rhein and emodin.

Calculate the percentage content of emodin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 2}$$

$A_1$ = area of the peak due to emodin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to emodin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *emodin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of emodin in *emodin CRS*.

**Polydatin**. Liquid chromatography (*2.2.29*). *Carry out the assay protected from bright light.*

*Test solution.* To 0.200 g of the powdered herbal drug (355) (*2.9.12*) in a round-bottomed flask add 50.0 mL of *ethanol (50 per cent V/V) R* and weigh. Heat under a reflux condenser in a water-bath at 95 °C for 30 min. Allow to cool, weigh and adjust to the original mass with *ethanol (50 per cent V/V) R* and shake well. Filter and dilute 3.0 mL of the filtrate to 25.0 mL with a 60 per cent *V/V* solution of *methanol R*.

*Reference solution (a).* Dissolve 5.0 mg of *polydatin CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with a 60 per cent *V/V* solution of *methanol R*.

*Reference solution (b).* Dissolve 5.0 mg of *resveratrol R* in a 60 per cent *V/V* solution of *methanol R* and dilute to 50.0 mL with the same solution. Dilute 3.0 mL of the solution to 25.0 mL with reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 82 | 18 |
| 12 - 18 | 82 → 5 | 18 → 95 |
| 18 - 25 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 306 nm.

*Injection*: 20 µL.

*Retention time*: polydatin = about 10 min; resveratrol = about 17 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 5 between the peaks due to polydatin and resveratrol.

Calculate the percentage content of polydatin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 1.67}{A_2 \times m_1}$$

$A_1$ = area of the peak due to polydatin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to polydatin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *polydatin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of polydatin in *polydatin CRS*.

**01/2017:2726**
**corrected 9.2**

# POLYGONUM ORIENTALE FRUIT

## Polygoni orientalis fructus

DEFINITION

Dried ripe fruit of *Persicaria orientalis* (L.) Spach (syn. *Polygonum orientale* L.).

*Content*: minimum 0.15 per cent of taxifolin ($C_{15}H_{12}O_7$; $M_r$ 304.3) (dried drug).

IDENTIFICATION

A. The fruit is roundish and oblate, the flattened sides slightly concave, about 2-3.5 mm in diameter and about 1-1.5 mm thick. The glossy surface is brownish-black or reddish-brown. There is a light brown and slightly protruding pedicel at the base with the remains of the membranous perianth. The texture is hard.

Herbal drugs

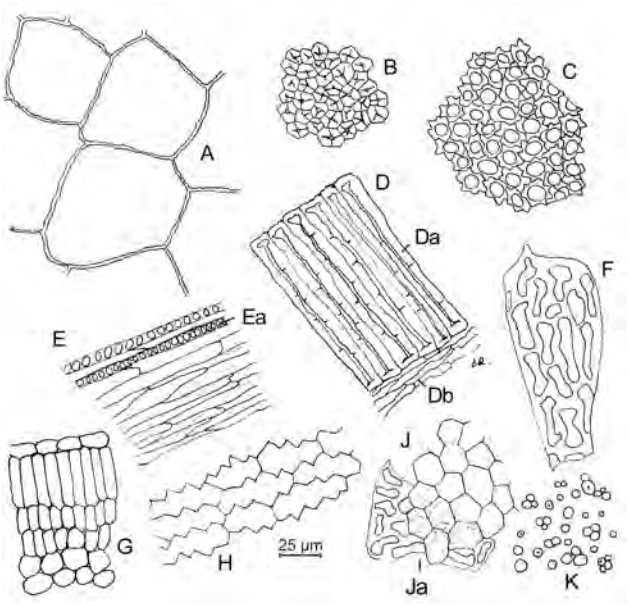


Figure 2726.-1. – *Illustration for identification test B of powdered herbal drug of Polygonum orientale fruit*

B. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2726.-1): fragments of the pericarp consisting of groups of yellowish-brown or reddish-brown palisade cells, 100-190 µm long and 15-30 µm wide, with thickened walls [Da], arranged as a single layer (side view [D]), which exhibit, on the outer surface, a polygonal or near-circular shape, narrow lumina and pitted walls (surface view [B]), and on the inner surface, a sinuous, anticlinal shape and circular lumina (surface view [C]); fragments of the inner part of the pericarp composed of brown rectangular cells [Db]; fragments of the parenchyma of the mesocarp, with colourless thin-walled cells [E], sometimes accompanied by fine annular or spiral vessels [Ea]; fragments of the testa consisting of a layer of cells with a barely visible zigzag [H] or polygonal [J] pattern mostly attached to a layer with a sinuous wall structure [Ja, F]; fragments of the cotyledons with thin-walled, small parenchyma cells (transverse section [G]); fragments of the endosperm with thin-walled cells [A]; possibly fragments of the perianth with cluster crystals of calcium oxalate up to 50 µm in diameter, small vascular bundles and a partly papillous epidermis. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous simple starch granules 2-9 µm in diameter, sometimes with a cleft hilum [K].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate at 60 °C for 10 min. Allow to cool and filter; use the filtrate.

*Reference solution*. Dissolve 2.0 mg of *taxifolin R* and 3.0 mg of *kaempferol R* in 6 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *acetone R*, *glacial acetic acid R*, *anhydrous formic acid R*, *methylene chloride R* (4:5:5:50 *V/V/V/V*).

*Application*: 7 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min; treat the still-warm plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, and then with a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow to dry in air for 60 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A red fluorescent zone |
| _____ | _____ |
| | A pale blue zone |
| Kaempferol: a pale blue zone | |
| | A pale blue zone |
| | 3 bluish-green zones |
| _____ | _____ |
| Taxifolin: an orangish-brown zone | An orangish-brown zone (taxifolin) |
| | A blue zone |
| | An orangish-brown zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. 0.500 g of the powdered herbal drug (355) (*2.9.12*) in a round-bottomed flask, add 25 mL of a 55 per cent *V/V* solution of *methanol R*. Heat under a reflux condenser in a water-bath at 90 °C for 1 h. Allow to cool and filter. Rinse the filter with 5.0 mL of *hydrochloric acid R1*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 50.0 mL with a 55 per cent *V/V* solution of *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 5.0 mg of *taxifolin CRS* in a 55 per cent *V/V* solution of *methanol R*. Add 2.5 mL of *hydrochloric acid R1* and dilute to 25.0 mL with a 55 per cent *V/V* solution of *methanol R*. To 10.0 mL of this solution add 4.0 mL of *hydrochloric acid R1* and dilute to 50.0 mL with a 55 per cent *V/V* solution of *methanol R*.

*Reference solution (b)*. 0.500 g of *polygonum orientale fruit for system suitability HRS* in a round-bottomed flask, add 25.0 mL of a 55 per cent *V/V* solution of *methanol R*. Heat under a reflux condenser in a water-bath at 90 °C for 1 h. Allow to cool and filter. Rinse the filter with 5.0 mL of *hydrochloric acid R1*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 50.0 mL with a 55 per cent *V/V* solution of *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.150 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 85 | 15 |
| 20 - 21 | 85 → 0 | 15 → 100 |
| 21 - 30 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *polygonum orientale fruit for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peak due to epitaxifolin.

*Retention time*: taxifolin = about 14 min; epitaxifolin = about 16 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to taxifolin and epitaxifolin.

Calculate the percentage content of taxifolin using the following expression:

$$\frac{A_1 \times m_2 \times 0.4 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to taxifolin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to taxifolin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *taxifolin CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of taxifolin in *taxifolin CRS*.

**07/2012:2475**

# PORIA

## Poria

DEFINITION

Dried sclerotium without skin of *Wolfiporia extensa* (Peck) Ginns (syn. *Poria cocos* (Schw.) Wolf; *Wolfiporia cocos* (F.A. Wolf) Ryvarden & Gilb.).

IDENTIFICATION

A. Square, rectangular or polyhedral pieces, or slices, varying in length and thickness; whitish with a pale brown hue, flat and smooth, square, rectangular or polyhedral pieces, with no brown skin, difficult to break; slices easily broken, rough fracture with granular or farinaceous texture.

B. Microscopic examination (*2.8.23*). The powder is whitish with a pale brown hue. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: irregularly shaped and occasionally branched colourless particles, which dissolve gradually in *chloral hydrate solution R*. Examine under a microscope using a 50 g/L solution of *potassium hydroxide R*. The powder shows the following diagnostic characters: fragments of hyphae, colourless, slender, slightly curved, sometimes with septa, branched, 3-16 µm in diameter.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (250) (*2.9.12*) add a mixture of 2 mL of *ethyl acetate R* and 3 mL of *methanol R*. Sonicate for 10 min, centrifuge and use the supernatant.

*Reference solution.* Dissolve 10 mg of *4-aminobenzoic acid R*, 10 mg of *coumarin R* and 10 mg of *thymol R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *2-propanol R*, *cyclohexane R* (10:10:80 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution between the zones due to 4-aminobenzoic acid and coumarin in the chromatogram obtained with the reference solution.

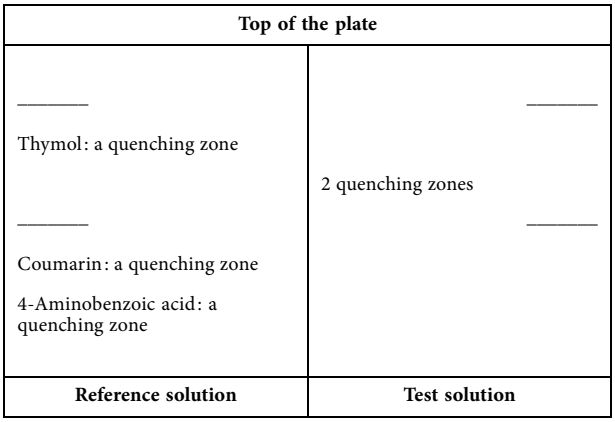


D. The herbal drug sticks to the pestle when moistened with *water R* and pressed into a mortar.

E. To a small piece of the herbal drug add 1 drop of *iodinated potassium iodide solution R1*. A deep red colour is produced.

TESTS

**Foreign matter** (*2.8.2*): maximum 0.1 per cent of brown skins and roots of conifer and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 13.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 1.0 per cent.

**Water-soluble extractive**: minimum 1.5 per cent.

To 5.00 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of boiling *water R*. Allow to stand for 10 min, shaking occasionally. Allow to cool, dilute to 100.0 mL with *water R* and filter. Evaporate 25.0 mL of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100-105 °C. The residue weighs a minimum of 18.75 mg.

**04/2013:1364**

# PRIMULA ROOT

## Primulae radix

DEFINITION

Whole or cut, dried rhizome and root of *Primula veris* L. or *Primula elatior* Hill.

IDENTIFICATION

A. The coarsely torose, greyish-brown rhizome is straight or slightly curved, about 1-5 cm long and about 2-4 mm thick. The rhizome crown often bears the remains of stems and leaves. Attached to the rhizome are numerous brittle roots, about 1 mm thick and usually 6-8 cm long. The root of *P. elatior* is light brown or reddish-brown, that of *P. veris* light yellow or yellowish-white. The fracture is smooth.

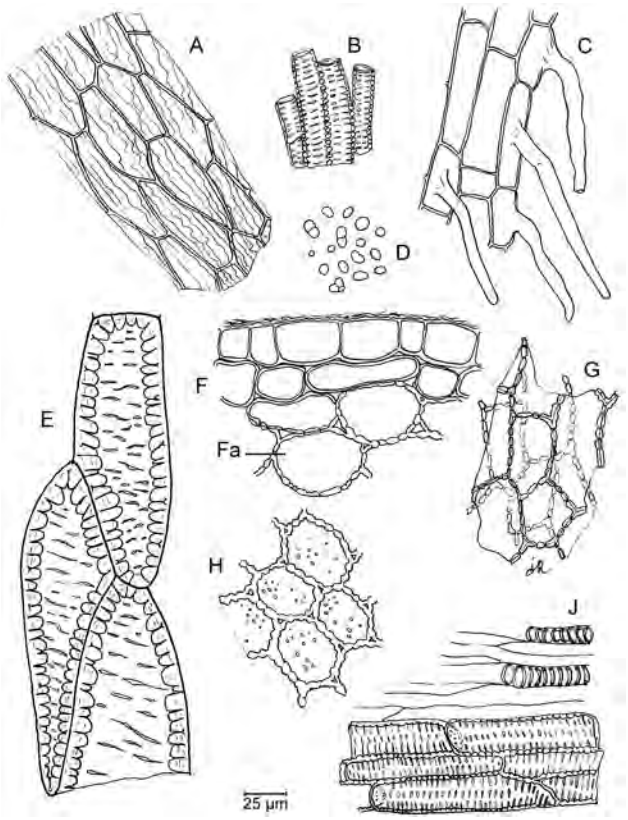


Figure 1364.-1. – *Illustration for identification test B of powdered herbal drug of primula root*

B. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1364.-1): fragments of parenchyma from the bark of the root or the rhizome and from the medulla of the rhizome [G, H], consisting of rounded or ovoid cells with irregularly thickened and pitted walls; brownish fragments from the dermal tissue of the root showing absorbent hairs [C]; yellow or brownish fragments of the epidermis of the rhizome covered by a striated cuticle (surface view [A], transverse section [F]) accompanied by parenchyma from the bark [Fa]; reticulate vessels [B] sometimes accompanied by spiral vessels [J]; groups of large, strongly pitted, yellowish-green sclereids from the medullary parenchyma of the rhizome [E], which are characteristic of *P. elatior*. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple or compound starch granules of various shapes and sizes [D].

C. Thin-layer chromatography (*2.2.27*) as described in the test for *Vincetoxicum hirundinaria* Medik. root with the following modifications.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the main zone (aescin) in the chromatogram obtained with the reference solution is bluish-violet and is situated near the boundary between the lower and middle thirds. The chromatogram obtained with the test solution shows 1-2 strong dark violet zones a little below the zone due to aescin in the chromatogram obtained with the reference solution; further pale violet, yellowish or brownish-green zones may be visible.

TESTS

***Vincetoxicum hirundinaria* Medik. root**. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (500) (*2.9.12*) add 10 mL of *ethanol (70 per cent V/V) R* and heat under a reflux condenser for 15 min. Cool and filter.

*Reference solution.* Dissolve 10 mg of *aescin R* in 1.0 mL of *ethanol (70 per cent V/V) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (10:40:50 *V/V/V*); use the upper layer.

*Application*: 20 µL as bands.

*Development*: over a path of 12 cm.

*Drying*: in an oven at 100-105 °C.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the chromatograms obtained with the reference solution and the test solution show a quenching zone (aescin) near the boundary between the lower and the middle thirds. Mark this zone.

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: in the chromatogram obtained with the test solution no zones of light-blue or greenish fluorescence occur below the main zone due to aescin in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

**01/2008:0858**
**corrected 6.0**

# PSYLLIUM SEED

## Psyllii semen

DEFINITION

Ripe, whole, dry seeds of *Plantago afra* L. (*Plantago psyllium* L.) or *Plantago indica* L. (*Plantago arenaria* Waldstein and Kitaibel).

CHARACTERS

Sweet taste.

IDENTIFICATION

*P. afra* seeds are light brown to very dark brown but never black, smooth and shiny having an elliptical oblong shape. They are 2-3 mm long and 0.8-1.0 mm wide, one end being wider than the other. Towards the middle of the dorsal surface there is a fairly marked transverse constriction of light colour. On the ventral surface, there is a linear lighter-coloured groove in the middle of which is a clear spot corresponding to the hilum and bounded by swollen edges.

*P. indica* seeds are almost identical to the seeds of *P. afra*, but a little less shiny; they are 2-3 mm long and have a maximum diameter of 1.5 mm.

TESTS

**Swelling index** (*2.8.4*): minimum 10.

**Foreign matter** (*2.8.2*): maximum 1.0 per cent, determined on 10.0 g of the drug, including greenish unripe seeds. Psyllium seed does not contain seeds having a dark central spot on the groove (*Plantago lanceolata* L. and *P. major* L.) or seeds with brownish-grey or pinkish outer coats (*P. ovata* Forssk. and *P. sempervirens* Crantz).

**Loss on drying** (*2.2.32*): maximum 14.0 per cent, determined on 1.000 g of drug by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

STORAGE

Store protected from moisture.

**01/2008:1823**
**corrected 6.0**

# PURPLE CONEFLOWER HERB

## Echinaceae purpureae herba

DEFINITION

Dried, whole or cut flowering aerial parts of *Echinacea purpurea* (L.) Moench.

*Content*: minimum 0.1 per cent for the sum of caftaric acid ($C_{13}H_{12}O_9$; $M_r$ 312.2) and cichoric acid ($C_{22}H_{18}O_{12}$; $M_r$ 474.3) (dried drug).

IDENTIFICATION

*First identification: A, B, C.*

*Second identification: A, B, D.*

A. The herbaceous perennial plant is 60-150 cm, rarely up to 180 cm high. The stem is green to red, upright and slightly branched. The leaves are alternate, ovate to ovate-lanceolate, irregularly serrate, rugose on both surfaces, dark green with prominent light green veins; the lamina is thick and shiny. The involucral bracts of the large capitulum are arranged in 2 or 3 rows. The solid receptacle is slightly convex. Each of the outer violet ligulate florets (4-6 cm) and of the inner violet-pink tubular florets is attached to a reddish acute and coriaceous bract, which overtops the tubular florets. The calyx is reduced to a very short crown, one of the sepals is up to 1 mm long.

B. Microscopic examination (*2.8.23*). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: whitish-green groups of fibres, 150-200 µm in length, 10-15 µm in diameter, sometimes with black deposits; fragments of leaves in surface view showing anomocytic or anisocytic stomata (*2.8.3*) (about 35-40 µm in length); uniseriate covering trichomes or fragments thereof consisting mainly of 3 or 4 thick-walled cells of which the apical cell is markedly longer than the others; fragments of leaves with rosette-like arranged epidermal cells around the base of the covering trichomes; uniseriate glandular trichomes composed of very thin-walled cells; pitted parenchymatous cells from the pith of the stem as well as pitted elongated cells from the mesocarp of the achenes; fragments of parenchyma from the seeds with oil droplets; fragments of the epidermis of ligulate florets composed of red to violet papillous cells; spheroidal pollen grains, 30-40 µm in diameter, with a spiny exine.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and sonicate for 5 min. Centrifuge and use the supernatant solution.

*Reference solution.* Dissolve 0.5 mg of *caffeic acid R* and 0.5 mg of *chlorogenic acid R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (3:3:9:15 *V/V/V/V*).

*Application*: 25 µL [or 5 µL] of the test solution and 10 µL [or 2 µL] of the reference solution, as bands.

*Development*: over a path of 15 cm [or 5 cm].

*Drying*: in a stream of cold air for about 10 min, then at 100 °C for 2 min.

*Detection*: spray the still-warm plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*; after 30 min, examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint blue fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An intense red fluorescent zone |
| Caffeic acid: a strong blue fluorescent zone | A blue fluorescent zone |
| | A blue fluorescent zone |
| Chlorogenic acid: a strong blue fluorescent zone | A faint yellow-orange fluorescent zone |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is due to cichoric acid and a smaller peak is due to caftaric acid. Peaks due to caffeic acid and chlorogenic acid are minor or may be absent.

Herbal drugs

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 100 mL volumetric flask place 0.500 g of the powdered herbal drug (355) (*2.9.12*) and add 80 mL of *ethanol (70 per cent V/V) R*. Sonicate for 15 min and dilute to 100.0 mL with *ethanol (70 per cent V/V) R*. Mix the suspension and allow to stand for a few minutes to allow visible solids to settle.

*Reference solution.* Dissolve 10.0 mg of *chlorogenic acid CRS* and 10.0 mg of *caffeic acid R* in *ethanol (70 per cent V/V) R*, sonicate for 15 min and dilute to 10.0 mL with the same solvent. Dilute 4.0 mL of this solution to 100.0 mL with *ethanol (70 per cent V/V) R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water R* (1:999 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 90 | 10 |
| 0 - 13 | 90 → 78 | 10 → 22 |
| 13 - 14 | 78 → 60 | 22 → 40 |
| 14 - 20 | 60 | 40 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 10 µL.

*Relative retention* with reference to chlorogenic acid (retention time = about 7 min): caftaric acid = about 0.8; caffeic acid = about 1.5; cynarin = about 1.6; echinacoside = about 1.7; cichoric acid = about 2.3.

*System suitability*: reference solution:

- *resolution*: minimum 5 between the peaks due to caffeic acid and chlorogenic acid.

Locate the peaks due to caffeic acid and chlorogenic acid using the chromatogram obtained with the reference solution. Locate the peaks due to caftaric acid and cichoric acid using the chromatogram in Figure 1823.-1.

Calculate the percentage content of caftaric acid using the following expression:

$$\frac{A_1 \times C_2 \times 100 \times 0.881}{A_2 \times C_1}$$

Calculate the percentage content of cichoric acid using the following expression:

$$\frac{A_3 \times C_2 \times 100 \times 0.695}{A_2 \times C_1}$$

$A_1$ = area of the peak due to caftaric acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to cichoric acid in the chromatogram obtained with the test solution;

$C_1$ = concentration of the test solution, in milligrams per millilitre;

$C_2$ = concentration of chlorogenic acid in the reference solution, in milligrams per millilitre;

0.695 = peak correlation factor based upon the liquid chromatography response observed;

0.881 = peak correlation factor between caftaric acid and chlorogenic acid.

STORAGE

Uncomminuted.

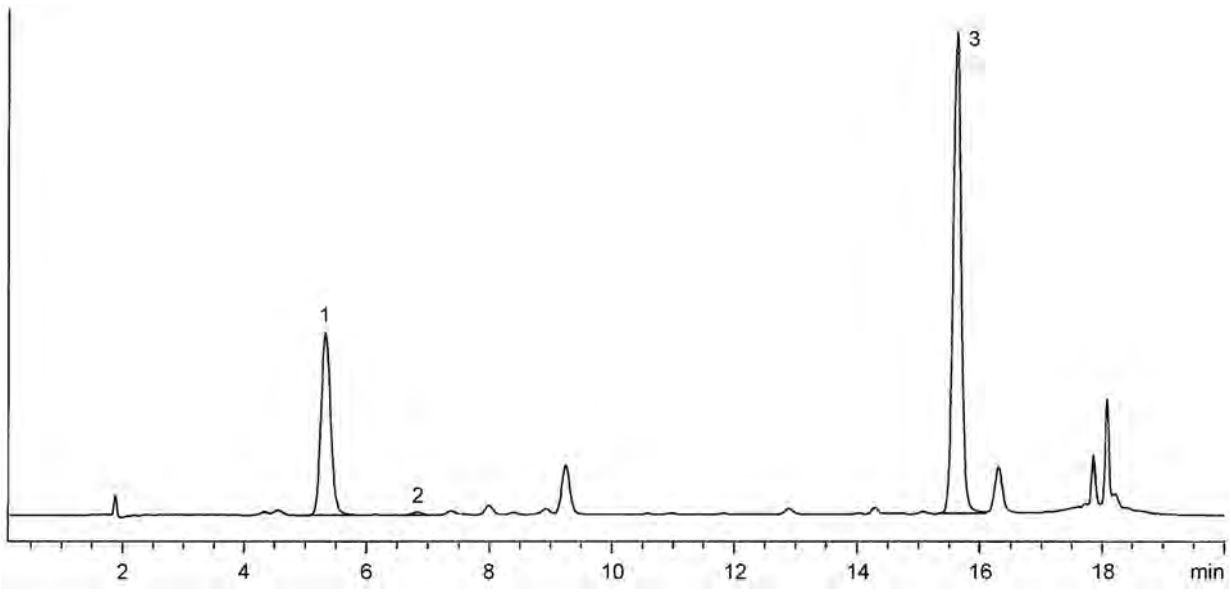


1. caftaric acid 2. chlorogenic acid 3. cichoric acid

Figure 1823.-1. – *Chromatogram for the assay of caftaric acid and cichoric acid in purple coneflower herb*

**01/2008:1824**
**corrected 6.0**

# PURPLE CONEFLOWER ROOT

## Echinaceae purpureae radix

DEFINITION

Dried, whole or cut underground parts of *Echinacea purpurea* (L.) Moench.

*Content*: minimum 0.5 per cent for the sum of caftaric acid ($C_{13}H_{12}O_9$; $M_r$ 312.2) and cichoric acid ($C_{22}H_{18}O_{12}$; $M_r$ 474.3) (dried drug).

IDENTIFICATION

*First identification: A, B, C, E.*

*Second identification: A, B, D, E.*

A. The rhizome is up to 15 cm long, branched, reddish-brown to dark brown on the surface and carries many stem bases; the inside is fibrous and white. The numerous roots are spirally twisted, light to dark brown and show a fine cross structuring on the surface.

B. Microscopic examination (*2.8.23*). The powder is light yellow to pinkish-beige. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous light-brown spindle-shaped fibres that are joined together in long bundles without black deposits; rare sclereids from the rhizomes and roots, usually occuring singly, those from the rhizomes being isodiametric, about 60 µm in diameter, with black deposits, those from the roots being 50-120 µm in length with no black deposits; secretory cavities up to 180 µm in diameter with yellow oil droplets; squarish to rectangular cells of the outer layers, some with reddish walls; bordered-pitted vessels from the rhizome, 30-40 µm in diameter.

C. Examine the chromatogram obtained in the test for other *Echinacea* species and *Parthenium integrifolium*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, faint greenish fluorescent zones may be present just below the zone situated in the middle of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a strong blue fluorescent zone | A strong blue fluorescent zone |
| _____ | _____ |
| Cynarin: a strong greenish fluorescent zone | A blue fluorescent zone |
| _____ | _____ |
| Echinacoside: a strong greenish fluorescent zone | |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is due to cichoric acid and a smaller peak is due to caftaric acid. Peaks due to caffeic acid and chlorogenic acid are minor or may be absent.

E. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methylene chloride R* and sonicate for 5 min. Centrifuge and use the supernatant solution.

*Reference solution.* Dissolve 1 mg of *β-sitosterol R* and a volume of *N-isobutyldodecatetraenamide solution R* corresponding to 1 mg of *N-isobutyldodecatetraenamide R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *cyclohexane R*, *ethyl acetate R*, *toluene R* (0.9:3:6:24 *V/V/V/V*).

*Application*: 25 µL [or 5 µL], as bands.

*Development*: over a path of about 15 cm [or 5 cm].

*Drying*: in a stream of cold air for about 10 min.

*Detection*: dip the plate into *anisaldehyde solution R* for 1 s and heat at 100-105 °C for 3 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A bluish-violet zone |
| β-Sitosterol: a violet or pink zone | A violet or pink zone (β-sitosterol) |
| *N*-Isobutyldodecatetraenamide: a greyish-blue zone | A greyish-blue zone (*N*-isobutyldodecatetraenamide) |
| _____ | _____ |
| | A dark greyish-blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Other *Echinacea* species and *Parthenium integrifolium*.** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and sonicate for 5 min. Centrifuge and use the supernatant solution.

*Reference solution.* Dissolve 1 mg of *echinacoside R*, 1 mg of *cynarin R* and 0.5 mg of *caffeic acid R* in 5.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methyl ethyl ketone R*, *ethyl acetate R* (3:3:9:15 *V/V/V/V*).

*Application*: 10 µL [or 5 µL] of the test solution and 5 µL [or 2 µL] of the reference solution, as bands.

*Development*: over a path of 10 cm [or 5 cm].

*Drying*: in a stream of cold air for about 10 min, then at 105 °C for 2 min.

*Detection*: spray the still-warm plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*; after 30 min, examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows no greenish fluorescent zone corresponding to the zone due to echinacoside in the chromatogram obtained with the reference solution, and no greenish fluorescent zone corresponding to the zone due to cynarin in the chromatogram obtained with the reference solution. No other zones apart from very faint dark blue fluorescent zones are seen in the lower half of the chromatogram of the test solution.

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 100 mL volumetric flask place 0.500 g of the powdered herbal drug (355) (*2.9.12*) and add 80 mL of *ethanol (70 per cent V/V) R*. Sonicate for 15 min and dilute to 100.0 mL with *ethanol (70 per cent V/V) R*. Mix the suspension and allow to stand for a few minutes to allow visible solids to settle.

*Reference solution.* Dissolve 10.0 mg of *chlorogenic acid CRS* and 10.0 mg of *caffeic acid R* in *ethanol (70 per cent V/V) R*, sonicate for 15 min and dilute to 10.0 mL with the same solvent. Dilute 4.0 mL of this solution to 100.0 mL with *ethanol (70 per cent V/V) R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R, water R* (1:999 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 90 | 10 |
| 0 - 13 | 90 → 78 | 10 → 22 |
| 13 - 14 | 78 → 60 | 22 → 40 |
| 14 - 20 | 60 | 40 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 10 µL.

*Relative retention* with reference to chlorogenic acid (retention time = about 7 min): caftaric acid = about 0.8; caffeic acid = about 1.5; cynarin = about 1.6; echinacoside = about 1.7; cichoric acid = about 2.3.

*System suitability*: reference solution:

- *resolution*: minimum 5 between the peaks due to caffeic acid and chlorogenic acid.

Locate the peaks due to caffeic acid and chlorogenic acid using the chromatogram obtained with the reference solution. Locate the peaks due to caftaric acid and cichoric acid using the chromatogram in Figure 1824.-1.

Calculate the percentage content of caftaric acid using the following expression:

$$\frac{A_1 \times C_2 \times 100 \times 0.881}{A_2 \times C_1}$$

Calculate the percentage content of cichoric acid using the following expression:

$$\frac{A_3 \times C_2 \times 100 \times 0.695}{A_2 \times C_1}$$

$A_1$ = area of the peak due to caftaric acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to chlorogenic acid in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to cichoric acid in the chromatogram obtained with the test solution;

$C_1$ = concentration of the dried drug in the test solution, in milligrams per millilitre;

$C_2$ = concentration of chlorogenic acid in the reference solution, in milligrams per millilitre;

0.695 = peak correlation factor based upon the liquid chromatography response observed;

0.881 = peak correlation factor between caftaric acid and chlorogenic acid.

STORAGE

Uncomminuted.

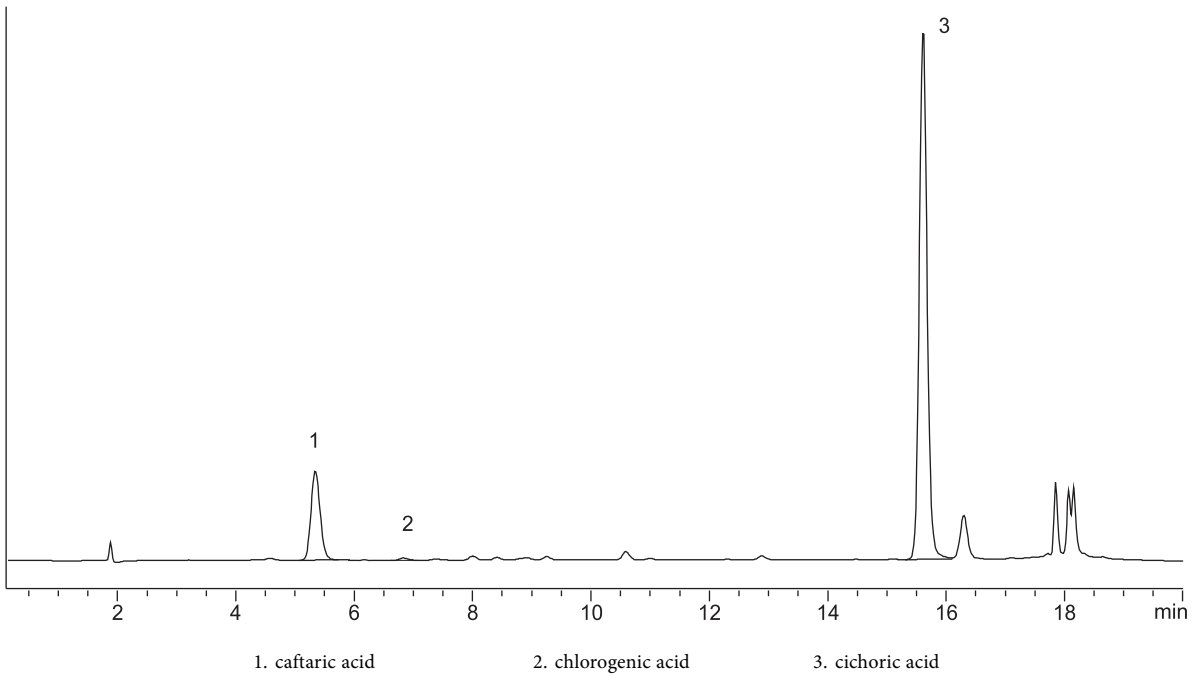


Figure 1824.-1. – *Chromatogram for the assay of caftaric acid and cichoric acid in purple coneflower root*

**01/2015:1886**

# PYGEUM AFRICANUM BARK

## Pruni africanae cortex

DEFINITION

Whole or fragmented, dried bark of the stems and branches of *Prunus africana* (Hook.f.) Kalkman (syn. *Pygeum africanum* Hook.f.).

IDENTIFICATION

A. The dark brown or reddish-brown bark occurs in curved, hard, irregular pieces. The outer surface has a wrinkled dark reddish-brown cork with areas of adhering lichen. The reddish-brown or dark brown inner surface bears longitudinal striations. It may also occur in rolled fragments with a fibrous fracture.

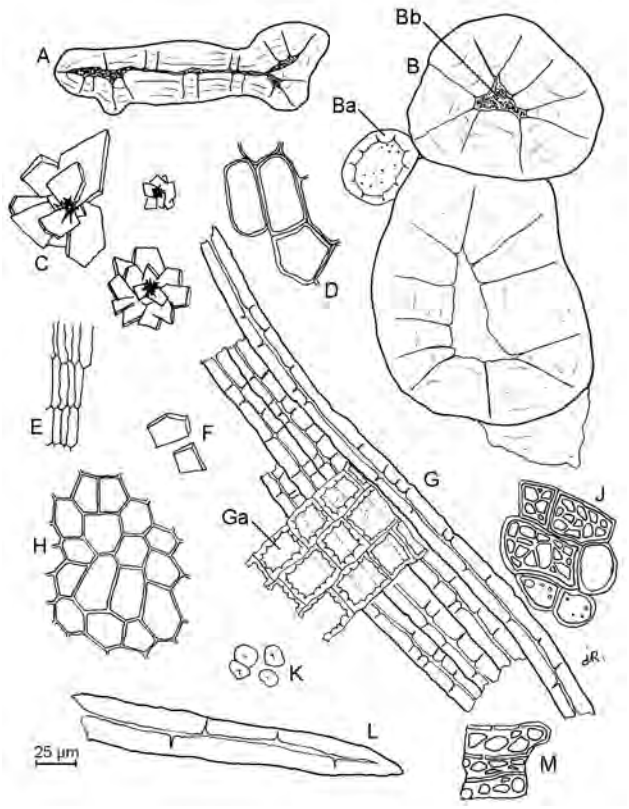


Figure 1886.-1. – *Illustration for identification test B of powdered herbal drug of pygeum africanum bark*

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1886.-1): numerous sclereids, varying greatly in size, up to more than 500 µm in diameter, with very thick walls showing concentric striations and a reduced lumen, isolated [A] or in groups [B] sometimes accompanied by sclereids about 50 µm in diameter [Ba], some with granular reddish-brown contents [Bb]; isolated cluster crystals of calcium oxalate of various sizes [C] and a few calcium oxalate prisms [F]; numerous lignified fibres, usually broken, thick-walled and channelled with a narrow lumen, sometimes isolated [L], but usually in groups [G] accompanied by rectangular cells of the medullary rays [Ga]; fragments of parenchyma with reddish-brown, polygonal or ovoid cells [D], including some with reticulate walls [J, M]; fragments of cork (surface view [H], side view [E]). Examine under a microscope using *lactic reagent R*. The powder shows a few simple starch granules that stain violet-blue, rounded, 10-20 µm in diameter, with a punctiform or Y-shaped hilum [K].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Extract 15.0 g of the powdered herbal drug (250) (*2.9.12*) with *methylene chloride R* for 30 min in a continuous extraction apparatus (Soxhlet type). Filter. Evaporate the solvent to dryness under reduced pressure. Dissolve the residue in 1 mL of *methylene chloride R*.

*Reference solution*. Dissolve 20 mg of *β-sitosterol R* and 20 mg of *ursolic acid R* in 10 mL of a mixture of equal volumes of *methanol R* and *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Application*: 10 µL as bands of 10 mm.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with *vanillin reagent R*, heat at 100-105 °C for 10 min and allow to cool; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| | Several weak violet, blue or grey zones |
| _____ | _____ |
| β-Sitosterol: a violet zone | A violet zone (β-sitosterol) |
| Ursolic acid: a blue zone | A blue zone (ursolic acid) |
| | Several weak violet, blue or grey zones |
| _____ | _____ |
| | A violet zone (β-sitosterol glucoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3.0 per cent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Extractable matter**: minimum 0.5 per cent.

Extract 20.0 g of the powdered herbal drug (250) (*2.9.12*) with *methylene chloride R* for 4 h in a continuous extraction apparatus (Soxhlet type). Evaporate the solution to dryness on a water-bath *in vacuo* and then dry the residue at 80 °C for 2 h. The residue weighs a minimum of 0.10 g.

**04/2013:1843**

# QUILLAIA BARK

## Quillajae cortex

DEFINITION

Whole or fragmented, dried bark, with the cork and underlying parenchyma removed, of *Quillaja saponaria* Molina *s.l.*

*Content*: minimum 6.5 per cent of triterpene glycosides, expressed as quillaia saponin III ($C_{104}H_{168}O_{55}$; $M_r$ 2298) (dried drug).

IDENTIFICATION

A. Large, flat pieces of variable length and width, 3-10 mm thick, or smaller, splintered pieces. The outer surface is brownish-white or pale reddish-brown, longitudinally striated or coarsely reticulated, with occasional blackish-brown patches of incompletely removed outer bark. The inner surface is yellowish-white and smooth. The fracture is splintery and laminated, the surface often glistening due to the presence of numerous large prisms of calcium oxalate.

B. Microscopic examination (*2.8.23*). The powder is pale pinkish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1843.-1): abundant phloem fibres [E, F], up to 1 mm long, isolated or, more usually, in groups, each fibre irregular in outline with lignified walls of varying thickness and an uneven lumen; numerous, multiseriate medullary rays, spindle-shaped (tangential section [Ca, Fb]), accompanied by either phloem fibres [Fa] or phloem parenchyma [Cb]; very numerous prisms of calcium oxalate, up to 200 µm long, free, whole or, more usually, fragmented [A] or included in phloem parenchyma cells [Cc, Cd]; occasional sclereids of 2 types: the 1st type is sub-rectangular with pitted, slightly thickened walls, isolated [G] or included in phloem parenchyma cells [H], while the 2nd type has an irregularly shaped outline and very thick walls [J], sometimes adjacent to the bundles of phloem fibres; occasional dark brown or reddish-brown fragments of cork [D]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous, small (5-20 µm), mainly simple, spherical starch granules, either scattered or as compacted masses in parenchyma cells [B].

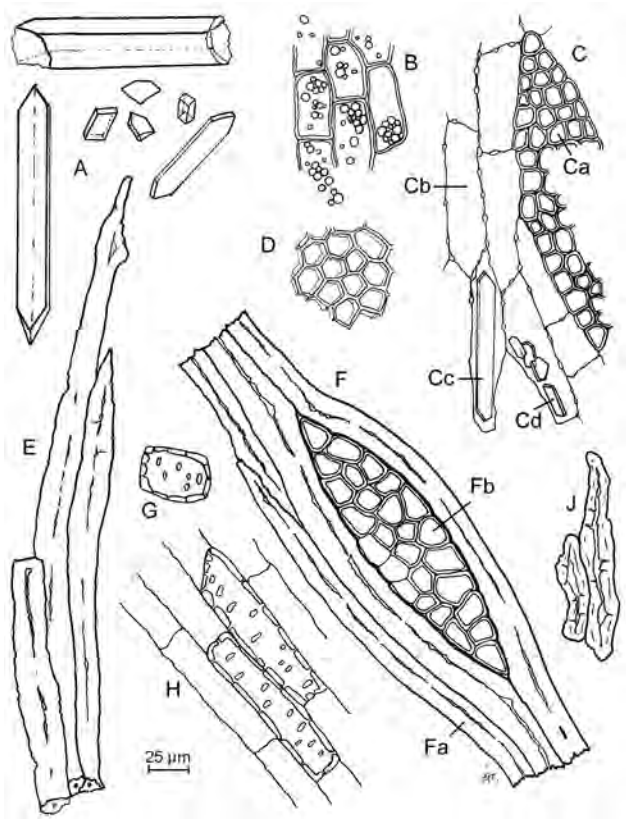


Figure 1843.-1. – *Illustration for identification test B of powdered herbal drug of quillaia bark*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R* and 5 mL of *water R*. Sonicate for 10 min and filter.

*Reference solution.* Dissolve 10 mg of *purified quillaia saponins R* and 2 mg of *sucrose R* in 1 mL of *water R* and mix with 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *water R*, *propanol R* (1.5:30:30:40 *V/V/V/V*).

*Application*: 5 µL as bands of 6 mm.

*Development*: over a path of 6 cm.

*Drying*: in hot air.

*Detection*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*; heat at 120 °C for 5 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Quillaia saponins: 3 or more green or brown zones | 3 or more green or brown zones (quillaia saponins)<br>A blue zone |
| Sucrose: a brown or blue zone | A brown or blue zone (sucrose) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 0.500 g of the powdered herbal drug (355) (*2.9.12*) into a round-bottomed flask, add 20 mL of a 20 g/L solution of *potassium hydroxide R* and heat under a reflux condenser in a water-bath for 2 h. After cooling, add 2 mL of *phosphoric acid R* and filter through a plug of absorbent cotton. Add the absorbent cotton to the residue, add 25 mL of *ethanol (96 per cent) R* and shake thoroughly. Filter. Combine the filtrates and dilute to 50.0 mL with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 12.0 mg of *quillaia saponin for assay CRS* (containing monoammonium glycyrrhizate) in a mixture of equal volumes of *ethanol (96 per cent) R* and a 10 g/L solution of *phosphoric acid R*, and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (b).* Introduce 12 mg of *purified quillaia saponins HRS* into a 50 mL round-bottomed flask, add 20 mL of a 20 g/L solution of *potassium hydroxide R* and heat under a reflux condenser in a water-bath for 2 h. After cooling, add 2 mL of *phosphoric acid R*. Add 25 mL of *ethanol (96 per cent) R* and shake thoroughly. Dilute to 50.0 mL with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 ± 2 °C.

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (35:65 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Run time*: 1.2 times the retention time of glycyrrhizic acid.

*Identification of peaks*: use the chromatogram supplied with *purified quillaia saponins HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to monodesmosidic quillaia saponins 1 and 3; a minor peak due to monodesmosidic quillaia saponin 2 may be present between the peaks due to monodesmosidic quillaia saponins 1 and 3.

*Retention time*: monodesmosidic quillaia saponin 1 = about 9 min; monodesmosidic quillaia saponin 3 = about 10 min; glycyrrhizic acid = about 13 min.

Calculate the percentage content of triterpene glycosides, expressed as quillaia saponin III, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2298 \times 0.6}{A_2 \times m_1 \times 957}$$

$A_1$ = sum of the areas of the peaks due to monodesmosidic quillaia saponins (1, 2 and 3) in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to glycyrrhizic acid derived from monoammonium glycyrrhizate in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *quillaia saponin for assay CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of monoammonium glycyrrhizate in *quillaia saponin for assay CRS*;

0.6 = response factor between monoammonium glycyrrhizate and monodesmosidic quillaia saponin 3;

2298 = molecular mass of quillaia saponin III;

957 = molecular mass of monodesmosidic quillaia saponin 3.

**01/2011:1881**

# RED POPPY PETALS

## Papaveris rhoeados flos

DEFINITION

Dried, whole or fragmented petals of *Papaver rhoeas* L.

IDENTIFICATION

A. The petal is dark red or dark violet-brown, very thin, floppy, wrinkled, often crumpled into a ball and velvety to the touch. It is broadly ovate with an entire margin, about 6 cm long and 4-6 cm wide, narrowing at the base where there is a black spot. The vascular bundles radiate from the base and they anastomose in a continuous arc, all at the same short distance from the margin.

B. Microscopic examination (*2.8.23*). Examine under a microscope using *chloral hydrate solution R*. The powder has an intense reddish-pink colour and shows the following diagnostic characters (Figure 1881.-1): fragments of epidermis composed of elongated, sinuous-walled cells [B, D, G] with small, rounded, anomocytic stomata (*2.8.3*) [Ba]; numerous vascular bundles with spiral vessels [E] embedded in the parenchyma; occasional fragments of the fibrous layer of the anthers [F]; rounded pollen grains, about 30 µm in diameter, with 3 pores and a finely verrucose exine [A, C, H].

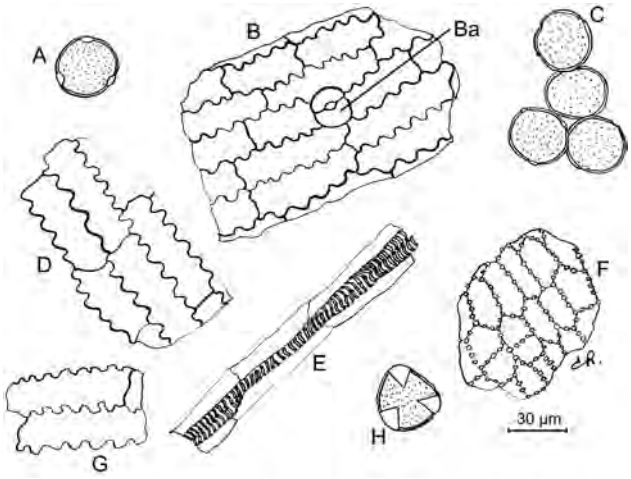


Figure 1881.-1. – *Illustration for identification test B of powdered herbal drug of red poppy petals*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (60 per cent V/V) R*. Stir for 15 min. Filter through a filter paper.

*Reference solution.* Dissolve 1 mg of *quinaldine red R* and 1 mg of *sulfan blue R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *butanol R* (10:12:40 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 yellow zones |
| Quinaldine red: an orange-red zone | |
| | A violet principal zone |
| | A violet zone |
| | A yellow zone |
| Sulfan blue: a blue zone | |
| | A compact group of violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2.0 per cent of capsules and maximum 1.0 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 11.0 per cent.

**Colouring intensity**. Place 1.0 g of the powdered herbal drug (355) (*2.9.12*) in a 250 mL flask and add 100 mL of *ethanol (30 per cent V/V) R*. Allow to macerate for 4 h with frequent stirring. Filter and discard the first 10 mL. To 10.0 mL of the

Herbal drugs

filtrate add 2 mL of *hydrochloric acid R* and dilute to 100.0 mL with *ethanol (30 per cent V/V) R*. Allow to stand for 10 min. The absorbance (*2.2.25*) measured at 523 nm using *ethanol (30 per cent V/V) R* as the compensation liquid is not less than 0.6.

**07/2014:1879**

# RESTHARROW ROOT

## Ononidis radix

DEFINITION

Whole or cut, dried root of *Ononis spinosa* L.

IDENTIFICATION

A. The root is more or less flattened, twisted and branched, deeply wrinkled, brown and grooved longitudinally. The transversely cut surface shows a thin bark and a xylem cylinder with a conspicuously radiate structure. The fracture of the root is short and fibrous.

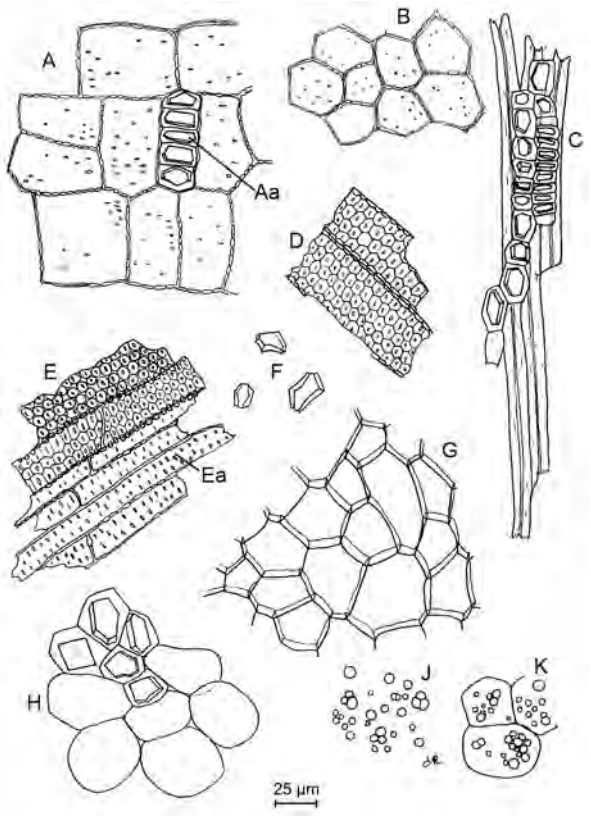


Figure 1879.-1. – *Illustration for identification test B of powdered herbal drug of restharrow root*

B. Microscopic examination (*2.8.23*). The powder is light brown or brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1879.-1): brown fragments of cork composed of thin-walled, polygonal cells (surface view [G]); groups of thick-walled, narrow fibres, often accompanied by a parenchymatous crystal sheath containing prisms of calcium oxalate [C]; vascular fragments [D, E] consisting of vessels with numerous bordered pits, often accompanied by lignified fibres with pitted walls [Ea]; thin-walled parenchymatous cells from the bark, some containing a single prism of calcium oxalate [H]; ligneous parenchyma cells with slightly thickened and pitted walls [A, B], some of which contain prisms of calcium oxalate [Aa]; numerous free prisms of calcium oxalate [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous, rounded starch granules, 5-10 µm in diameter, simple or sometimes 2-4 compound, free [J] or inside parenchymatous cells [K].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (180) (*2.9.12*) add 15.0 mL of *methanol R* and boil under a reflux condenser for 30 min. Cool and filter.

*Reference solution.* Dissolve 10 mg of *resorcinol R* and 50 mg of *vanillin R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethanol (96 per cent) R*, *methylene chloride R*, *toluene R* (10:45:45 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm and 365 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fluorescent zones are present in the middle third of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Vanillin: a zone visible at 254 nm | |
| Resorcinol: a zone visible at 254 nm | An intense blue fluorescent zone visible at 365 nm |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min. Examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| Vanillin: a greyish-violet zone | |
| Resorcinol: a red zone | A violet zone (onocol) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Extractable matter**: minimum 15.0 per cent.

To 2.00 g of the powdered herbal drug (250) (*2.9.12*) add a mixture of 8 g of *water R* and 12 g of *ethanol (96 per cent) R* and allow to macerate for 2 h, shaking frequently. Filter, evaporate 5 g of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 75 mg.

**01/2015:0289**

# RHATANY ROOT

## Ratanhiae radix

DEFINITION

Dried, usually fragmented, underground organs of *Krameria triandra* Ruiz et Pav., known as Peruvian rhatany.

*Content*: minimum 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The taproot is dark reddish-brown and has a thick, knotty crown. The secondary roots are the same colour and nearly straight or somewhat tortuous. The bark is rugged or scaly in the older pieces and smooth with sharp, transverse fissures in the younger pieces; it separates readily from the wood. The fracture is fibrous in the bark and splintery in the wood. The smooth, transversely cut surface shows a dark brownish-red bark about one third of the radius in thickness; a dense, pale reddish-brown and finely porous wood is present with numerous fine medullary rays; the central heartwood is often darker.

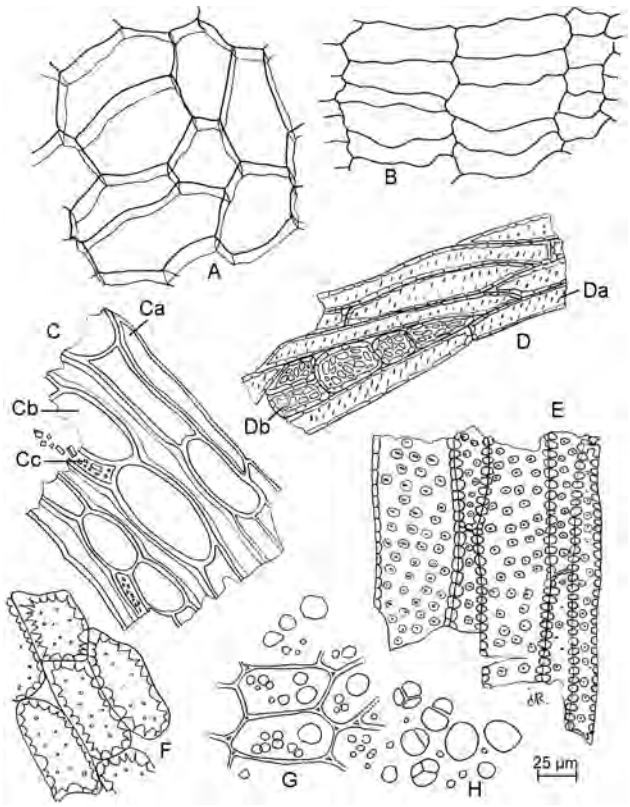


Figure 0289.-1. – *Illustration for identification test B of powdered herbal drug of rhatany root*

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0289.-1): cork cells containing dark brown phlobaphenes (surface view [A], side view [B]); fragments of phloem [C] consisting of unlignified fibres, usually 12-30 µm in diameter with moderately thickened walls [Ca], parenchyma cells some containing prisms and microcrystals of calcium oxalate [Cc], and cells of the medullary rays [Cb]; fragments of vessels usually 20-60 µm in diameter with bordered pits [E]; fragments of tracheids [D] up to 20 µm wide with slit-shaped pits [Da] and cells of the medullary rays [Db]; lignified parenchymatous cells with thick and channelled walls [F]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rounded, simple or 2- to 4-compound starch granules, an individual granule measuring up to 30 µm in diameter [H] and some granules being found in the cells of the medullary rays and in the parenchyma [G].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and sonicate for 10 min. Centrifuge or filter. Use the supernatant or filtrate.

*Reference solution.* Dissolve 5 mg of *thymol R* and 20 mg of *dichlorophenolindophenol, sodium salt R* in 20 mL of *ethanol (60 per cent V/V) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methylene chloride R*.

*Application*: 10 µL [or 4 µL] as bands of 8 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 5 g/L solution of *fast blue B salt R*, allow to dry in air and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Thymol: a brownish-yellow zone | A faint violet zone |
| | An orange zone |
| | A bluish-grey zone |
| Dichlorophenolindophenol: a greyish-blue zone | An intense violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of foreign matter and maximum 5 per cent of fragments of crown or root exceeding 25 mm in diameter. Root without bark may be present in very small quantities.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 5.5 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.750 g of the powdered herbal drug (180) (*2.9.12*).

Herbal drugs

01/2015:1888

# RHATANY TINCTURE

## Ratanhiae tinctura

DEFINITION

Tincture produced from *Rhatany root (0289)*.

*Content*: minimum 1.0 per cent *m/m* of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1).

PRODUCTION

The tincture is produced from 1 part of the herbal drug and 5 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution.* Dissolve 5 mg of *thymol R* and 20 mg of *dichlorophenolindophenol, sodium salt R* in 20 mL of *ethanol (60 per cent V/V) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methylene chloride R*.

*Application*: 10 µL [or 4 µL] as bands of 8 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 5 g/L solution of *fast blue B salt R*, allow to dry in air and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Thymol: a brownish-yellow zone | A violet zone |
| | An orange zone |
| | A bluish-grey zone |
| Dichlorophenolindophenol: a greyish-blue zone | |
| | An intense violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 63 per cent *V/V* to 67 per cent *V/V*.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

ASSAY

**Tannins** (*2.8.14*). Use 2.500 g of the tincture to be examined.

01/2008:0291
corrected 6.0

# RHUBARB

## Rhei radix

DEFINITION

Rhubarb consists of the whole or cut, dried underground parts of *Rheum palmatum* L. or of *Rheum officinale* Baillon or of hybrids of these two species or of a mixture. The underground parts are often divided; the stem and most of the bark with the rootlets are removed. It contains not less than 2.2 per cent of hydroxyanthracene derivatives, expressed as rhein ($C_{15}H_8O_6$, $M_r$ 284.2), calculated with reference to the dried drug.

CHARACTERS

Characteristic, aromatic odour.

IDENTIFICATION

A. The appearance is variable: disc-shaped pieces up to 10 cm in diameter and 1 cm to 5 cm in thickness; cylindrical pieces; oval or planoconvex pieces. The surface has a pinkish tinge and is usually covered with a layer of brownish-yellow powder. It shows, especially after moistening, a reticulum of darker lines. This structure causes the marbled appearance of the drug. The fracture is granular. The transverse section of the rhizome shows a narrow outer zone of radiating brownish-red lines. These medullary rays are crossed perpendicularly by a dark cambial ring. Inside this zone is a ring of small star-spot formations of anomalous vascular bundles. The root shows a more radiate structure.

B. Microscopic examination (*2.8.23*). The powder is orange to brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: large calcium oxalate cluster crystals, which may measure more than 100 µm, and their fragments; reticulately thickened non-lignified vessels measuring up to 175 µm. Numerous groups of rounded or polygonal, thin-walled parenchyma cells. Sclereids and fibres are absent. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows simple, rounded or compound (2 to 4) starch granules with a star-shaped hilum.

C. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution.* Heat 50 mg of the powdered herbal drug (180) (*2.9.12*) in a water-bath for 15 min with a mixture of 1 mL of *hydrochloric acid R* and 30 mL of *water R*. Allow to cool and shake the liquid with 25 mL of *ether R*. Dry the ether layer over *anhydrous sodium sulfate R* and filter. Evaporate the ether layer to dryness and dissolve the residue in 0.5 mL of *ether R*.

*Reference solution.* Dissolve 5 mg of *emodin R* in 5 mL of *ether R*.

Apply separately to the plate as bands 20 µL of each solution. Develop over a path of 10 cm using a mixture of 1 volume of *anhydrous formic acid R*, 25 volumes of *ethyl acetate R* and 75 volumes of *light petroleum R*. Allow the plate to dry in air and examine in ultraviolet light at 365 nm. The chromatogram obtained with the reference solution shows in its central part a zone of orange fluorescence (emodin). The chromatogram obtained with the test solution shows: a zone due to emodin; above the emodin zone, two zones of similar fluorescence (physcione and chrysophanol, in order of increasing $R_F$ value); below the emodin zone, also two zones of similar fluorescence

Herbal drugs

(rhein and aloe-emodin, in order of decreasing $R_F$ value). Spray with a 100 g/L solution of *potassium hydroxide R* in *methanol R*. All the zones become red to violet.

D. To about 50 mg of the powdered herbal drug (180) (*2.9.12*) add 25 mL of *dilute hydrochloric acid R* and heat the mixture on a water-bath for 15 min. Allow to cool, shake with 20 mL of *ether R* and discard the aqueous layer. Shake the ether layer with 10 mL of *dilute ammonia R1*. The aqueous layer becomes red to violet.

TESTS

**Rheum rhaponticum**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. To 0.2 g of the powdered herbal drug (180) (*2.9.12*) add 2 mL of *methanol R* and boil for 5 min under a reflux condenser. Allow to cool and filter. Use the filtrate as the test solution.

*Reference solution*. Dissolve 10 mg of *rhaponticin R* in 10 mL of *methanol R*.

Apply separately to the plate, as bands not more than 20 mm by 3 mm, 20 µL of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air and spray with *phosphomolybdic acid solution R*. The chromatogram obtained with the test solution does not show a blue zone near the line of application (rhaponticin) corresponding to the zone in the chromatogram obtained with the reference solution.

**Loss on drying** (*2.2.32*). Not more than 12.0 per cent, determined on 1.000 g of the powdered herbal drug (180) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*). Not more than 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*). Not more than 2.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Introduce 0.100 g of the powdered herbal drug (180) (*2.9.12*) into a 100 mL flask. Add 30.0 mL of *water R*, mix and weigh. Heat in a water-bath under a reflux condenser for 15 min. Allow to cool, add 50 mg of *sodium hydrogen carbonate R*, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 10.0 mL of the liquid to a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *ferric chloride solution R1* and mix. Heat under a reflux condenser on a water-bath for 20 min, add 1 mL of *hydrochloric acid R* and heat for a further 20 min, shaking frequently. Cool, transfer to a separating funnel and shake with three quantities, each of 25 mL, of *ether R* previously used to rinse the flask. Combine the ether extracts and wash with two quantities, each of 15 mL, of *water R*. Filter the ether extracts through a plug of absorbent cotton into a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 10.0 mL carefully to dryness on a water-bath and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm, using *methanol R* as the compensation liquid.

Calculate the percentage content of rhein from the expression:

$$\frac{A \times 0.64}{m}$$

i.e. taking the specific absorbance of rhein to be 468, calculated on the basis of the specific absorbance of barbaloin.

$A$ = absorbance at 515 nm,

$m$ = mass of the herbal drug used, in grams.

**01/2012:1884**

# RIBWORT PLANTAIN

## Plantaginis lanceolatae folium

DEFINITION

Whole or fragmented, dried leaf and scape of *Plantago lanceolata* L. *s.l.*

*Content*: minimum 1.5 per cent of total *ortho*-dihydroxycinnamic acid derivatives expressed as acteoside ($C_{29}H_{36}O_{15}$; $M_r$ 624.6) (dried drug).

IDENTIFICATION

A. The leaf is up to 30 cm long and 4 cm wide, yellowish-green to brownish-green, with a prominent, whitish-green, almost parallel venation on the abaxial surface. It consists of a lanceolate lamina narrowing at the base into a channelled petiole. The margin is indistinctly dentate and often undulate. It has 3, 5 or 7 primary veins, nearly equal in length and running almost parallel. Hairs may be almost absent, sparsely scattered or sometimes abundant, especially on the lower surface and over the veins. The scape is brownish-green, longer than the leaves, 3-4 mm in diameter and is deeply grooved longitudinally, with 5-7 conspicuous ribs. The surface is usually covered with fine hairs.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1884.-1): fragments of epidermis, composed of cells with irregularly sinuous anticlinal walls, the fragments of the upper epidermis of the lamina (surface view [H], transverse section [D]) are accompanied by palisade parenchyma [Da, Ha], and those of the lower epidermis (surface view [G]) show stomata (*2.8.3*) mostly of the diacytic type [Ga] and sometimes of the anomocytic type [Gb]; the multicellular, uniseriate, conical covering trichomes are highly characteristic, whole [C] or mostly fragmented [A], with a basal cell larger than the other epidermal cells followed by a short cell supporting 2 or more elongated cells with the lumen narrow and variable, occluded at intervals corresponding to slight swellings in the trichome and giving a jointed appearance, the terminal cell has an acute apex and a filiform lumen; the glandular trichomes have a unicellular, cylindrical stalk and a multicellular, elongated, conical head consisting of several rows of small cells and a single terminal cell [B, Gc]; dense groups of lignified fibro-vascular tissue with narrow, spirally and annularly thickened vessels and slender, moderately thickened fibres [F]; fragments of the scape [E] with cells with thickened walls and a coarsely ridged cuticle, stomata [Ec], multicellular, uniseriate covering trichomes [Eb] and glandular trichomes [Ea] of the type previously described.

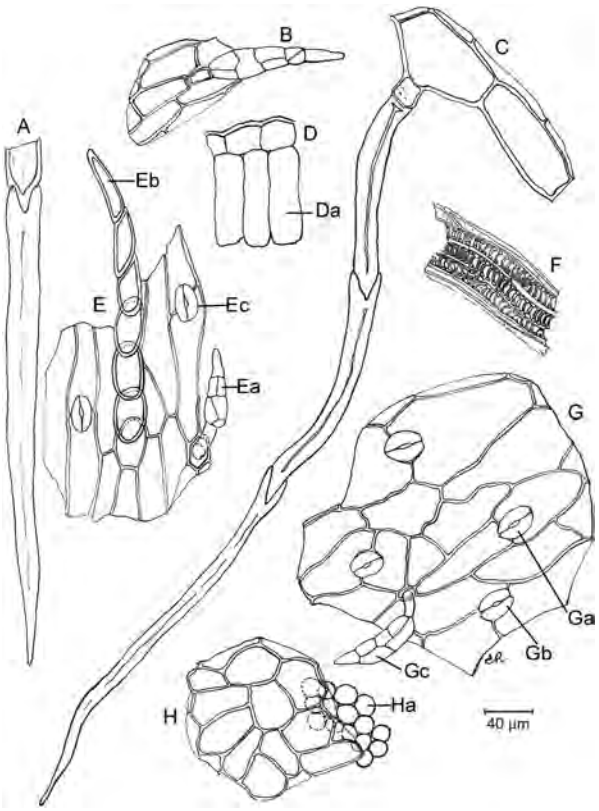


Figure 1884.-1. – *Illustration for identification test B of powdered herbal drug of ribwort plantain*

C. Examine the chromatograms obtained in the test for *Digitalis lanata* leaves.

*Results A*: see below the sequence of zones present in the chromatogram obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Acteoside: a yellow zone | A yellow zone (acteoside) |
| ——— | ——— |
| Aucubin: a blue zone | A blue zone (aucubin) |
| **Reference solution** | **Test solution** |

TESTS

***Digitalis lanata* leaves**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (30:70 *V/V*).

*Test solution.* Use a freshly prepared solution. To 1 g of the powdered herbal drug (355) (*2.9.12*) in a 25 mL flask, add 10 mL of the solvent mixture and shake for 30 min. Filter, rinse the flask and the filter with 2 quantities, each of 5 mL, of the solvent mixture. Dilute to 25 mL with the solvent mixture.

*Reference solution.* Dissolve 1 mg of *acteoside R* and 1 mg of *aucubin R* in 1 mL of the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 8 cm; heat immediately after development at about 120 °C for 5-10 min.

*Detection A*: examine in daylight.

*Detection B*: examine in ultraviolet light at 365 nm.

*Results B*: the chromatogram obtained with the test solution shows no bright blue fluorescent zone just below the reddish-brown fluorescent zone corresponding to aucubin in the chromatogram obtained with the reference solution.

**Foreign matter** (*2.8.2*): maximum 5 per cent of leaves of different colour and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 14.0 per cent.

ASSAY

*Stock solution.* In a flask, place 1.000 g of the powdered herbal drug (355) (*2.9.12*) and add 90 mL of *ethanol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter into a 100 mL volumetric flask. Rinse the flask and the filter with 10 mL of *ethanol (50 per cent V/V) R*. Combine the filtrate and the rinsings and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Test solution.* To a 10 mL volumetric flask add, mixing after each addition, 1.0 mL of the stock solution, 2 mL of *0.5 M hydrochloric acid*, 2 mL of a solution prepared by dissolving 10 g of *sodium nitrite R* and 10 g of *sodium molybdate R* in 100 mL of *water R*, and 2 mL of *dilute sodium hydroxide solution R*. Dilute to 10.0 mL with *water R*.

Immediately measure the absorbance (*2.2.25*) of the test solution at 525 nm using as compensation liquid a solution prepared as follows: to a 10 mL volumetric flask add 1.0 mL of the stock solution, 2 mL of *0.5 M hydrochloric acid* and 2 mL of *dilute sodium hydroxide solution R*, and dilute to 10.0 mL with *water R*.

Calculate the percentage content of total *ortho*-dihydroxycinnamic acid derivatives, expressed as acteoside, using the following expression:

$$\frac{A \times 1000}{185 \times m}$$

i.e. taking the specific absorbance to be 185 for acteoside at 525 nm.

$A$ = absorbance of the test solution at 525 nm;

$m$ = mass of the substance to be examined, in grams.

**01/2013:1623**
**corrected 10.0**

# ROSELLE

## Hibisci sabdariffae flos

DEFINITION

Whole or cut dried calyces and epicalyces of *Hibiscus sabdariffa* L. collected during fruiting.

*Content*: minimum 13.5 per cent of acids, expressed as citric acid ($C_6H_8O_7$; $M_r$ 192.1) (dried drug).

CHARACTERS

Acidic taste.

IDENTIFICATION

A. The calyx is joined in the lower half to form an urceolate structure, the upper half dividing to form 5 long acuminate recurved tips. The tips have a prominent, slightly protruding midrib and a large, thick nectary gland about 1 mm in diameter. The epicalyx consists of 8-12 small, obovate leaflets, which are adnate to the base of the calyx.

The calyx and epicalyx are fleshy, dry, easily fragmented and bright red or deep purple, somewhat lighter at the base of the inner side.

B. Microscopic examination (*2.8.23*). The powder is red or violet-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1623.-1): predominantly red fragments [A, F] consisting of polygonal epidermal cells with very irregularly thickened walls (surface view [Ac, Fa]), some containing cluster crystals of calcium oxalate [Fb], with underlying parenchyma consisting of ovoid cells with slightly thickened walls [Aa], some containing cluster crystals of calcium oxalate [Ab] whilst others are filled with mucilage, unicellular, long, flexuous, twisted covering trichomes [Ad], rigid, straight, unicellular covering trichomes, simple or in groups of 2-4 [Fd], glandular trichomes with a unicellular stalk and a globular or oval, multicellular and biseriate head [Fe] and stomata usually of the anisocytic type (*2.8.3*) [Fc]; numerous fragments of vascular bundles [D] with spiral or reticulate vessels [Da], sometimes accompanied by sclerenchymatous fibres with a wide lumen [Db], and parenchyma [Dc], of which some cells contain cluster crystals of calcium oxalate [Dd], whilst others are mucilage-filled [De]; rare, rectangular, parenchymatous sclereids [H]; numerous fragments of rigid [C, G] or flexuous [J] covering trichomes; free cluster crystals of calcium oxalate [B] and glandular trichomes [E]; exceptionally, spherical pollen grains, about 200 µm in diameter, with a spiny exine.

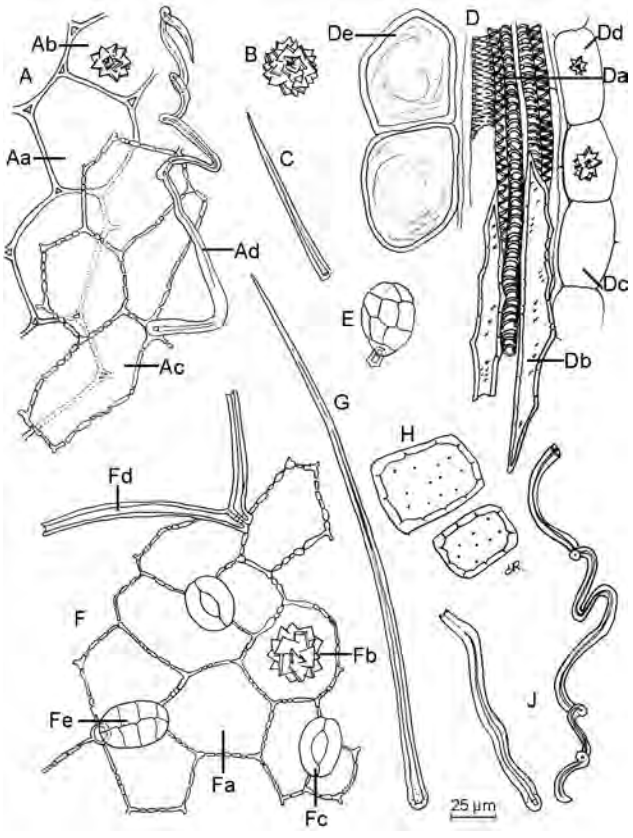


Figure 1623.-1. – *Illustration for identification test B of powdered herbal drug of roselle*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (60 per cent V/V) R*. Shake for 15 min and filter.

*Reference solution*. Dissolve 2.5 mg of *quinaldine red R* and 2.5 mg of *sulfan blue R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *butanol R* (10:12:40 *V/V/V*).

*Application*: 5 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Quinaldine red: an orange-red zone | |
| | An intense violet zone |
| Sulfan blue: a blue zone | |
| | An intense violet-blue zone |
| ——— | ——— |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 2 per cent of fragments of fruits (red funicles and parts of the 5-caverned capsule with yellowish-grey pericarp, whose thin walls consist of several layers of differently directed fibres; flattened, reniform seeds with a dotted surface) and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Colouring intensity**. Reduce 100 g to a powder (1400) (*2.9.12*) and homogenise. Reduce about 10 g of this mixture to a very fine powder (355) (*2.9.12*). To 1.0 g of this powder in a 100 mL flask add 25 mL of boiling *water R* and heat for 15 min on a water-bath with frequent shaking. Filter the hot mixture into a 50 mL graduated flask; rinse successively the 100 mL flask and the filter with 3 quantities, each of 5 mL, of warm *water R*. After cooling, dilute to 50 mL with *water R*. Dilute 5 mL of this solution to 50 mL with *water R*. Measure the absorbance (*2.2.25*) at 520 nm using *water R* as the compensation liquid. The absorbance is not less than 0.350 for the whole drug and not less than 0.250 for the cut drug.

ASSAY

Shake 1.00 g of the powdered herbal drug (355) (*2.9.12*) with 100.0 mL of *carbon dioxide-free water R* for 15 min. Filter. To 50.0 mL of the filtrate add 100 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide* to pH 7.0, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 6.4 mg of citric acid.

**01/2013:1560**

# ROSEMARY LEAF

## Rosmarini folium

DEFINITION

Whole, dried leaf of *Rosmarinus officinalis* L.

Herbal drugs

*Content*:

– minimum 12 mL/kg of essential oil (anhydrous drug);

– minimum 3 per cent of total hydroxycinnamic derivatives, expressed as rosmarinic acid ($C_{18}H_{16}O_8$; $M_r$ 360.3) (anhydrous drug).

CHARACTERS

Strongly aromatic odour.

IDENTIFICATION

A. The leaves are sessile, tough, linear or linear-lanceolate, 1-4 cm long and 2-4 mm wide, with recurved edges. The upper surface is dark green, glabrous and grainy, the lower surface is greyish-green and densely tomentose with a prominent midrib.

B. Microscopic examination (*2.8.23*). The powder is greyish-green or yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1560.-1): fragments of the lower epidermis (surface view [B, J]) with straight or sinuous-walled cells [Ba] and numerous diacytic stomata (*2.8.3*) [Bb] and glandular trichomes [Ja] or covering trichomes or their scars [Bc, Bd]; numerous multicellular, mostly branched, covering trichomes of the lower epidermis, usually fragmented [A, C, D]; fragments of the upper epidermis (surface view [F]) with cells with straight, thickened and pitted walls [Fa], and an underlying hypodermis composed of large, irregular cells with thickened and beaded anticlinal walls [Fb]; fragments of the lamina (transverse section [G]), showing the epidermis covered by a very thick cuticle [Ga], hypodermal cells extending across the mesophyll [Gb] at intervals, separating 1 or 2 layers of palisade parenchyma into large, crescent-shaped areas [Gc]; glandular trichomes of 2 types, the majority with a short, unicellular stalk and a radiate head composed of 8 cells (surface view [E], side view [H]), others, less abundant, with a uni- or bicellular stalk and a spherical, unicellular head [Ja, K].

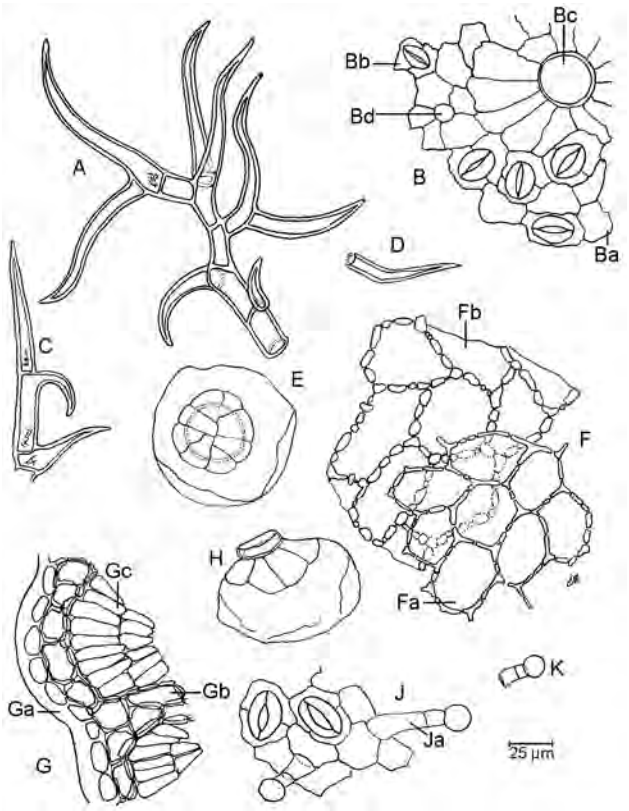


Figure 1560.-1. – *Illustration for identification test B of powdered herbal drug of rosemary leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 µL of the oil obtained in the assay in 1 mL of *hexane R*.

*Reference solution.* Dissolve 5 mg of *borneol R*, 5 mg of *bornyl acetate R* and 10 µL of *cineole R* in 1 mL of *hexane R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A red zone |
| Bornyl acetate: a yellowish-brown zone | A yellowish-brown zone of low intensity |
| | A coloured zone of low intensity |
| Cineole: a violet zone | A violet zone |
| | Coloured zones of low intensity |
| Borneol: a violet-brown zone | A violet-brown zone |
| | A coloured zone of low intensity |
| **Reference solution** | **Test solution** |

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Grind 1.0 g of the herbal drug in 10 mL of *methanol R* and filter.

*Reference solution.* Dissolve 1.0 mg of *caffeic acid R* and 5.0 mg of *rosmarinic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (8.5:25:85 *V/V/V*).

*Application*: 10 µL of the test solution and 20 µL of the reference solution, as bands.

*Development*: over a path of 8 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A pink fluorescent zone |
| Caffeic acid: a light blue fluorescent zone | A blue fluorescent zone of low intensity |
| Rosmarinic acid: a light blue fluorescent zone | An intense light blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

**Total hydroxycinnamic derivatives**

*Stock solution.* To 0.200 g of the powdered herbal drug (355) (*2.9.12*) add 80 mL of *ethanol (50 per cent V/V) R*. Boil in a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Rinse the filter with 10 mL of *ethanol*

Herbal drugs

*(50 per cent V/V) R*. Combine the filtrate and the rinsings in a volumetric flask and dilute to 100.0 mL with *ethanol (50 per cent V/V) R*.

*Test solution*. To 1.0 mL of the stock solution add 2 mL of *0.5 M hydrochloric acid*, 2 mL of a solution prepared by dissolving 10 g of *sodium nitrite R* and 10 g of *sodium molybdate R* in 100 mL of *water R*, and then add 2 mL of *dilute sodium hydroxide solution R* and dilute to 10.0 mL with *water R*; mix.

*Compensation solution*. Dilute 1.0 mL of the stock solution to 10.0 mL with *water R*.

Measure immediately the absorbance (*2.2.25*) of the test solution at 505 nm.

Calculate the percentage content of total hydroxycinnamic derivatives, expressed as rosmarinic acid, using the following expression:

$$\frac{A \times 2.5}{m}$$

i.e. taking the specific absorbance of rosmarinic acid to be 400.

$A$ = absorbance of the test solution at 505 nm;

$m$ = mass of the substance to be examined, in grams.

**Essential oil** (*2.8.12*). Use 25.0 g of the crushed herbal drug, a 1000 mL flask and 300 mL of *water R* as the distillation liquid. Distil at a rate of 2-3 mL/min for 3 h.

**01/2008:1846**

# ROSEMARY OIL

## Rosmarini aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the flowering aerial parts of *Rosmarinus officinalis* L.

CHARACTERS

*Appearance*: clear, mobile, colourless or pale yellow liquid.

Characteristic odour.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.5 mL of the substance to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 50 mg of *borneol R*, 50 mg of *bornyl acetate R* and 100 µL of *cineole R* in *toluene R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray the plate with *vanillin reagent R* and heat the plate at 100-105 °C for 10 min. Examine immediately in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, several violet-blue to violet-grey zones of medium intensity (terpene alcohols) are present in the lower third of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An intense violet zone |
| | A violet-grey zone |
| _____ | _____ |
| Bornyl acetate: a bluish-grey zone of low intensity | A bluish-grey zone of low intensity (bornyl acetate) |
| | A violet-pink zone |
| _____ | _____ |
| Cineole: an intense blue zone | An intense blue zone (cineole) |
| Borneol: a violet-blue zone of medium intensity | A violet -blue zone of medium intensity (borneol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.895 to 0.920.

**Refractive index** (*2.2.6*): 1.464 to 1.473.

**Optical rotation** (*2.2.7*): − 5° to + 8°.

**Acid value** (*2.5.1*): maximum 1.0.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.20 mL of the substance to be examined in *hexane R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 20 µL of *α-pinene R*, 10 mg of *camphene R*, 20 µL of *β-pinene R*, 10 µL of *β-myrcene R*, 20 µL of *limonene R*, 50 µL of *cineole R*, 10 µL of *p-cymene R*, 50 mg of *camphor R*, 30 mg of *bornyl acetate R*, 10 mg of *α-terpineol R*, 10 mg of *borneol R* and 10 µL of *verbenone R* in *hexane R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 50 |
| | 10 - 85 | 50 → 200 |
| | 85 - 110 | 200 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to limonene and cineole and minimum 1.5 between the peaks due to α-terpineol and borneol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of these components.

For rosemary oil, Spanish type, the percentages are within the following ranges:

– *α-pinene*: 18 per cent to 26 per cent,
– *camphene*: 8.0 per cent to 12.0 per cent,
– *β-pinene*: 2.0 per cent to 6.0 per cent,
– *β-myrcene*: 1.5 per cent to 5.0 per cent,
– *limonene*: 2.5 per cent to 5.0 per cent,
– *cineole*: 16.0 per cent to 25.0 per cent,
– *p-cymene*: 1.0 per cent to 2.2 per cent,
– *camphor*: 13.0 per cent to 21.0 per cent,
– *bornyl acetate*: 0.5 per cent to 2.5 per cent,
– *α-terpineol*: 1.0 per cent to 3.5 per cent,
– *borneol*: 2.0 per cent to 4.5 per cent,
– *verbenone*: 0.7 per cent to 2.5 per cent.

For rosemary oil, Moroccan and Tunisian type, the percentages are within the following ranges:

– *α-pinene*: 9.0 per cent to 14.0 per cent,
– *camphene*: 2.5 per cent to 6.0 per cent,
– *β-pinene*: 4.0 per cent to 9.0 per cent,
– *β-myrcene*: 1.0 per cent to 2.0 per cent,
– *limonene*: 1.5 per cent to 4.0 per cent,
– *cineole*: 38.0 per cent to 55.0 per cent,
– *p-cymene*: 0.8 per cent to 2.5 per cent,
– *camphor*: 5.0 per cent to 15.0 per cent,
– *bornyl acetate*: 0.1 per cent to 1.5 per cent,
– *α-terpineol*: 1.0 per cent to 2.6 per cent,
– *borneol*: 1.5 per cent to 5.0 per cent,
– *verbenone*: maximum 0.4 per cent.

STORAGE

At a temperature not exceeding 25 °C.

LABELLING

The label states that the content is Spanish type or Moroccan and Tunisian type.

**04/2014:2555**
**corrected 10.0**

# ROUND AMOMUM FRUIT

## Amomi fructus rotundus

DEFINITION

Dried, whole, peeled or unpeeled ripe fruit of *Amomum krervanh* Pierre ex Gagnep. or *Amomum compactum* Sol. ex Maton.

*Content*:

– *essential oil*: minimum 50 mL/kg for *A. krervanh* (anhydrous drug) and minimum 40 mL/kg for *A. compactum* (anhydrous drug);

– *1,8-cineole* ($C_{10}H_{18}O$; $M_r$ 154.3): minimum 65.0 per cent of the essential oil.

IDENTIFICATION

A. *A. krervanh*. The fruit is a trilocular, indehiscent capsule, subspherical, about 1.5-2 cm in diameter. The outer surface is whitish-yellow or pale brownish-yellow, smooth, and shows 3 deep longitudinal furrows. The apex bears a prominent stylopodium; the base shows the dentate scar of the stalk. Both ends and the hollows of the furrows are covered by a pale brown pubescence. The thin, brittle pericarp is easily broken, showing 3 locules each containing 4-10 seeds. The seeds are hard, irregularly polyhedral, with a slightly raised dorsal surface, 3-4 mm in diameter, brown on the surface, finely wrinkled, bearing the remains of the fine, membranous aril.

*A. compactum*. The fruit is a trilocular, indehiscent capsule, subspherical, about 1-2 cm in diameter. The outer surface is whitish-yellow or pale brownish-yellow, sometimes slightly reddish, and shows about 15 longitudinal furrows, 3 of which are deep. The apex bears a prominent stylopodium; the stalk is usually fragmented. Both ends and the hollows of the furrows are covered by a brownish-yellow pubescence. The thin, brittle pericarp is easily broken, showing 3 locules each containing 6-12 seeds. The seeds are hard, irregularly polyhedral, 2-3 mm in diameter, blackish-brown on the surface, wrinkled, bearing the remains of the transparent, membranous aril.

B. *A. krervanh*. Microscopic examination (*2.8.23*). The powder is greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of epicarp consisting of polyhedral cells, numerous scars of covering trichomes with thick, channelled walls and rare stomata, paracytic or anomocytic (*2.8.3*); covering trichomes, mostly unicellular and usually fragmented, with regularly thickened walls, up to 800 µm in length; fragments of mesocarp composed of round cells with spaces between them, containing fine acicular crystals or prisms of calcium oxalate; groups of ovoid sclereids with thick, channelled walls, about 50 µm in diameter, from the inner layers of the mesocarp; vascular bundles composed of spiral or reticulate vessels accompanied by fibres with thick, channelled walls and sclereids; fragments of the aril consisting of very fine cells, some of which contain small crystals; fragments of the outer testa consisting of elongated cells with distinct, yellow, finely and regularly thickened walls and rounded ends, accompanied by an underlying layer consisting of rectangular or polyhedral cells with orange-yellow contents, perpendicular to the previous layer; fragments of the reddish-brown inner testa composed of very thick-walled cells, regularly polyhedral in surface view and U-shaped in side view; fragments of the endosperm with round cells. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous round cells of the endosperm, filled with small starch granules aggregated into masses and free aggregates of starch granules.

*A. compactum*. Microscopic examination (*2.8.23*). The powder is yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of epicarp consisting of polyhedral cells, numerous scars of covering trichomes with thick, channelled walls and rare stomata,

usually paracytic (*2.8.3*); unicellular covering trichomes, usually fragmented, with regularly thickened walls, up to 800 µm in length; fragments of mesocarp composed of round cells with spaces between them, containing fine acicular crystals or prisms of calcium oxalate, and oil cells; groups of ovoid sclereids with thick, channelled walls, about 50 µm in diameter, from the inner layers of the mesocarp; vascular bundles composed of spiral or reticulate vessels accompanied by fibres with thick, channelled walls and sclereids; fragments of the aril consisting of very fine cells, some of which contain small crystals; fragments of the pale yellow outer testa consisting of elongated cells with indistinct walls and rounded ends, accompanied by an underlying layer consisting of rectangular or polyhedral cells with dark orange-red contents, perpendicular to the previous layer; fragments of the reddish-brown inner testa composed of thick-walled cells, regularly polyhedral in surface view and U-shaped in side view; fragments of the endosperm, with polyhedral cells. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous fragments of the endosperm with polyhedral cells, filled with small starch granules aggregated into masses and free aggregates of starch granules.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methylene chloride R*. Sonicate for 10 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 10 µL of *bornyl acetate R*, 10 µL of *cineole R* and 10 mg of *borneol R* in 1 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *ethyl acetate R*, *toluene R* (7:93 *V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 366 nm.

*Results A*: see below the sequence of zones present in the chromatogram obtained with the test solution. The reference solution shows no spots at 366 nm. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A blue fluorescent zone |
| _____ | _____ |
| | A blue fluorescent zone |
| **Reference solution** | **Test solution** |

*Detection B*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 3 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish-brown zone |
| _____ | _____ |
| | A bluish-violet zone |
| Bornyl acetate: a greyish-brown zone | |
| 1,8-Cineole: a bluish-violet zone | A bluish-violet zone (1,8-cineole) |
| | A greyish-brown zone |
| Borneol: a greyish-brown zone at the border between the middle and lower thirds | |
| _____ | _____ |
| | A bluish-violet zone |
| | 1 or 2 bluish-violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.2.13*): maximum 120 mL/kg, determined by distillation on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 10.0 g of the herbal drug reduced to a powder (1400) (*2.9.12*) immediately before the assay, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.5 mL of *trimethylpentane R* in the graduated tube. Distil at a rate of 3-3.5 mL/min for 5 h.

**1,8-Cineole**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute a volume of the essential oil-trimethylpentane mixture obtained in the assay of essential oil corresponding to 150 µL of the essential oil in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 10 µL of *β-pinene R* and 15 µL of *cineole R* in *heptane R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 5 µL of *cineole R* to 100.0 mL with *heptane R*. Dilute 1.5 mL of the solution to 10.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 60 | 60 → 210 |
| Injection port | | 230 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to β-pinene and 1,8-cineole.

*Relative retention* with reference to β-pinene (retention time = about 11 min): 1,8-cineole = about 1.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 5 between the peaks due to β-pinene and 1,8-cineole.

Calculate the percentage content of 1,8-cineole. Disregard any peak due to the solvent or with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

LABELLING

The label states the species present.

**07/2014:2386**

# SAFFLOWER FLOWER

## Carthami flos

DEFINITION

Dried flower of *Carthamus tinctorius* L.

*Content*: minimum 1.0 per cent of total flavonoids, expressed as hyperoside ($C_{21}H_{20}O_{12}$; $M_r$ 464.4) (dried drug).

IDENTIFICATION

A. The orange-yellow or reddish-orange, tubular, gamopetalous, actinomorphic florets are separate from the capitulum. Each floret consists of a long, filiform tube, about 1 cm long divided into 5 equal, narrow, lanceolate lobes, about 0.5 cm long. From the opening of the tube emerges the hollow cylinder formed by the fused yellow anthers, in which the filiform style persists, thickened near the apex.

B. Microscopic examination (*2.8.23*). The powder is orange-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2386.-1): fragments of the corolla tube [E] with epidermis consisting of elongated, thin-walled, finely striated cells whose margins are lobed [B, Ea, J], and with parenchyma consisting of small polygonal cells containing prisms of calcium oxalate [Eb]; outer epidermis bearing glandular trichomes, usually sheared off, with only the base [Ja] persisting on the epidermis; these trichomes are isolated, biseriate with a multicellular stalk and a bicellular head [C]; fragments of the lobes of the corolla showing at their apices a large number of small, rounded, very prominent papillae [G]; fragments of parenchyma containing vascular bundles [Ed] surrounded by secretory canals with reddish-brown contents [Ec]; fragments of the filaments of the anthers consisting of elongated, thick-walled, pitted cells [K] and fragments of the characteristic layer of the anther whose walls show thickenings in bands [H]; fragments of the stigma, covered with rather long, conical papillae [D], usually accompanied by pollen grains; rounded or elliptical pollen grains up to 60 µm in diameter with 3 pores and an echinulate exine [A]; isolated prisms of calcium oxalate [F].

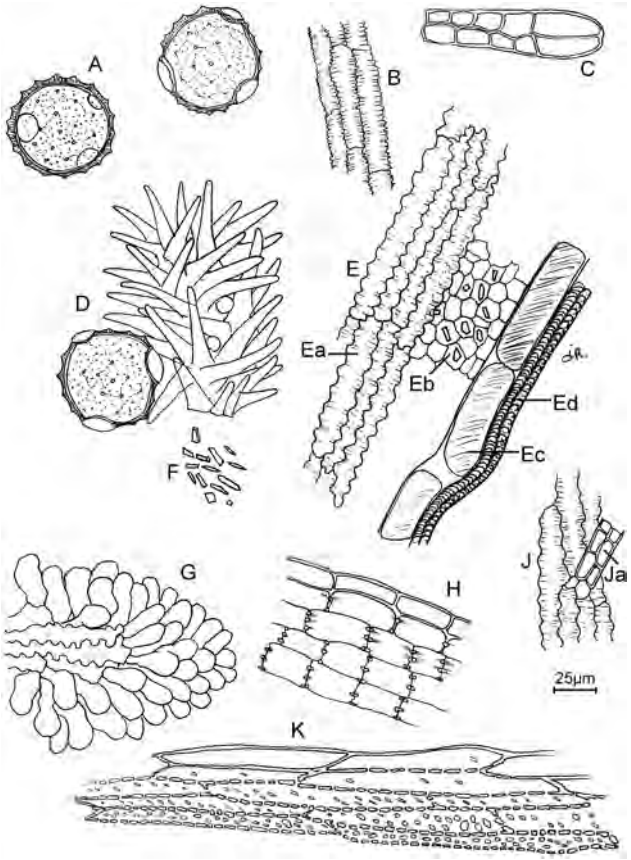


Figure 2386.-1. – *Illustration for identification test B of powdered herbal drug of safflower flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Sonicate for 10 min and centrifuge.

*Reference solution*. Dissolve 1 mg of *rutoside trihydrate R* and 5 mg of *quercetin dihydrate R* in 50 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *anhydrous formic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 25 µL [or 10 µL] as bands of 15 mm [or 8 mm].

*Development*: over a path of 12 cm [or 7 cm].

*Drying*: in air.

*Detection A*: examine in daylight.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Quercetin: a light yellow zone | |
| ——— | ——— |
| ——— | ——— |
| Rutoside: a light yellow zone | |
| | A red zone |
| | A yellow zone |
| | A yellow zone |
| **Reference solution** | **Test solution** |

*Detection B*: heat at 100 °C for 3 min; treat the plate whilst still hot with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; allow to dry in air for about 30 min; examine in ultraviolet light at 365 nm.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Quercetin: an orange fluorescent zone | |
| | A blue fluorescent zone |
| ——— | ——— |
| | A green fluorescent zone |
| | A brown fluorescent zone |
| | A green fluorescent zone |
| ——— | ——— |
| Rutoside: a yellow fluorescent zone | |
| | A yellow fluorescent zone |
| | A green fluorescent zone |
| | A brown fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Absorbance** (*2.2.25*).

A. *Yellow pigment*: macerate 0.1 g of the powdered herbal drug (355) (*2.9.12*) in 150 mL of *water R*, stir for 1 h, filter through a sintered-glass filter (40) (*2.1.2*) and dilute to 500.0 mL, washing the residue, with *water R*. The absorbance is not less than 0.40 at 401 nm.

B. *Red pigment*: to 0.25 g of the powdered herbal drug (355) (*2.9.12*) add 50 mL of a mixture of 20 volumes of *water R* and 80 volumes of *acetone R*. Heat on a water-bath at 50 °C for 90 min. Allow to cool, filter through a sintered-glass filter (40) (*2.1.2*) and dilute to 100.0 mL, washing the residue with a mixture of 20 volumes of *water R* and 80 volumes of *acetone R*. The absorbance is not less than 0.40 at 518 nm.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

*Solution A*. Place 0.250 g of the powdered herbal drug (180) (*2.9.12*) in a 250 mL flask and add 95 mL of *methanol R*. Heat under a reflux condenser on a water-bath for 30 min. Allow to cool and filter. Rinse the filter with 5 mL of *methanol R*. Combine the filtrate and the rinsing solution in a volumetric flask and dilute to 100.0 mL with *methanol R*.

*Test solution*. Place 5.0 mL of solution A in a volumetric flask and dilute to 20.0 mL with a 20 g/L solution of *aluminium chloride R* in *methanol R*.

*Compensation solution*. Place 5.0 mL of solution A in a volumetric flask and dilute to 20.0 mL with *methanol R*.

After exactly 15 min, measure the absorbance (*2.2.25*) of the test solution at 420 nm by comparison with the compensation solution. Calculate the percentage content of total flavonoids, expressed as hyperoside, using the following expression:

$$\frac{A}{m}$$

taking the specific absorbance of hyperoside at 420 nm to be 400.

$A$ = absorbance of the test solution at 420 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2015:1370**

# SAGE LEAF (SALVIA OFFICINALIS)

## Salviae officinalis folium

DEFINITION

Whole or cut, dried leaves of *Salvia officinalis* L.

*Essential oil content*:

- for the whole drug, minimum 12 mL/kg (anhydrous drug);
- for the cut drug, minimum 10 mL/kg (anhydrous drug).

IDENTIFICATION

A. The lamina of whole sage leaf (Salvia officinalis) is about 2-10 cm long and 1-2 cm wide, oblong-ovate, elliptical. The margin is finely crenate to smooth. The apex is rounded or subacute and the base is shrunken at the petiole and rounded or cordate. The upper surface is greenish-grey and finely granular; the lower surface is white and pubescent and shows a dense network of raised veinlets.

B. Microscopic examination (*2.8.23*). The powder is light grey or brownish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1370.-1): very numerous articulated and bent covering trichomes with narrow elongated cells and a base cell with very thick walls, whole [Bc] or fragmented, either isolated [C, G, H] or on an epidermis (surface view [Be], transverse section [Ab]); glandular trichomes of lamiaceous type, with a unicellular stalk and an 8- to 12-celled head covered by a common cuticle, isolated (side view [D]) or on an epidermis (surface view [Fa]); small glandular trichomes with a unicellular [Aa, Bd] or multicellular [Fb] stalk and a unicellular head, usually on an epidermis; more rarely, glandular trichomes (surface view [Eb, Ec], side view [Ed]) with a unicellular stalk [Ec] and a bicellular head [Eb, Ed]; fragments of the upper epidermis (surface view [E], transverse section [A]) with pitted, somewhat polygonal cells [Ea], covering trichomes and glandular trichomes, sometimes accompanied by 1 or 2 layers of palisade parenchyma [Ac, Ee]; some diacytic stomata (*2.8.3*) may be present; fragments of the lower epidermis [B, F] with sinuous cells [Ba] and numerous diacytic stomata (*2.8.3*) [Bb].

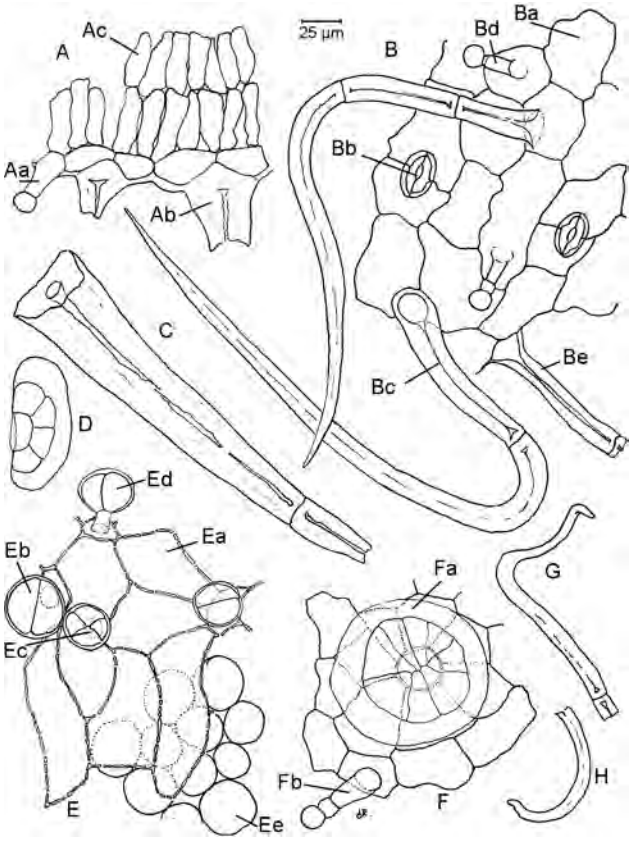


Figure 1370.-1. – *Illustration for identification test B of powdered herbal drug of sage leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.5 g of the freshly powdered herbal drug (355) (*2.9.12*) with 5 mL of *ethanol R* for 5 min.

*Reference solution.* Dissolve 20 µL of *thujone R* and 25 µL of *cineole R* in 20 mL of *ethanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with a 200 g/L solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100-105 °C for 10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue zone (near the solvent front) |
| _____ | _____ |
| α-Thujone and β-thujone: 2 pinkish-violet zones | 2 pinkish-violet zones (α-thujone and β-thujone) |
| Cineole: a blue zone | A blue zone (cineole) |
| _____ | _____ |
| | Blue zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of stems and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the herbal drug, cut, if necessary, immediately before the assay, a 500 mL flask and 250 mL of *water R* as the distillation liquid. Add 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**07/2014:1561**

# SAGE LEAF, THREE-LOBED

## Salviae trilobae folium

DEFINITION

Whole or cut, dried leaves of *Salvia fructicosa* Mill. (syn. *Salvia triloba* L. fil).

*Essential oil content*:

– for the whole drug, minimum 18 mL/kg (anhydrous drug);

– for the cut drug, minimum 12 mL/kg (anhydrous drug).

CHARACTERS

Spicy odour when ground, similar to eucalyptus oil.

IDENTIFICATION

A. The lamina of whole three-lobed sage leaf is about 8-50 mm long and about 4-20 mm wide, and oblong-ovate or lanceolate. The margin is finely crenate and undulate but indistinct owing to the dense, hairy covering on both surfaces. The base is obtuse and sometimes bears 1 or 2 more or less developed lobes. The upper surface is grey-tomentose pubescent, the lower surface is densely white-tomentose pubescent; the venation is indistinct. The densely white-tomentose pubescent petiole is about 1 mm in diameter.

B. Microscopic examination (*2.8.23*). The powder is greyish-green and tomentose. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1561.-1): very numerous covering and glandular trichomes, whole and attached to fragments of the epidermises [A, D, G, H] or fragmented and free [B, C, E, F]; uniseriate covering trichomes, either unicellular [Ab] or multicellular articulated and thick-walled [Ad]; those on the upper epidermis are straight [Ga], those on the lower epidermis are tortuous [Da]; glandular trichomes of 2 types: some with a unicellular [Hd] or multicellular [Ca, Gb, He] stalk and a unicellular [Cb, Hd] or bicellular [Cc] head; others of lamiaceous type, with a unicellular stalk and a head composed of 8-12 radiating cells with a raised common cuticle [Ae, B]; the upper epidermis (surface view [A], transverse section [G]) with pitted and beaded cells [Aa],

somewhat polygonal, with a few diacytic stomata (*2.8.3*), covering trichomes [Ab, Ad, Ga] or their scars [Ac] and glandular trichomes [Ae, Af, Gb]; the lower epidermis (surface view [H], transverse section [D]) with sinuous or wavy-walled cells [Ha] and numerous diacytic stomata (*2.8.3*) [Hb], glandular trichomes [Db, Hd, He] and covering trichomes [Da, Hc], some of which are unicellular and short, with finely pitted walls [Dc].

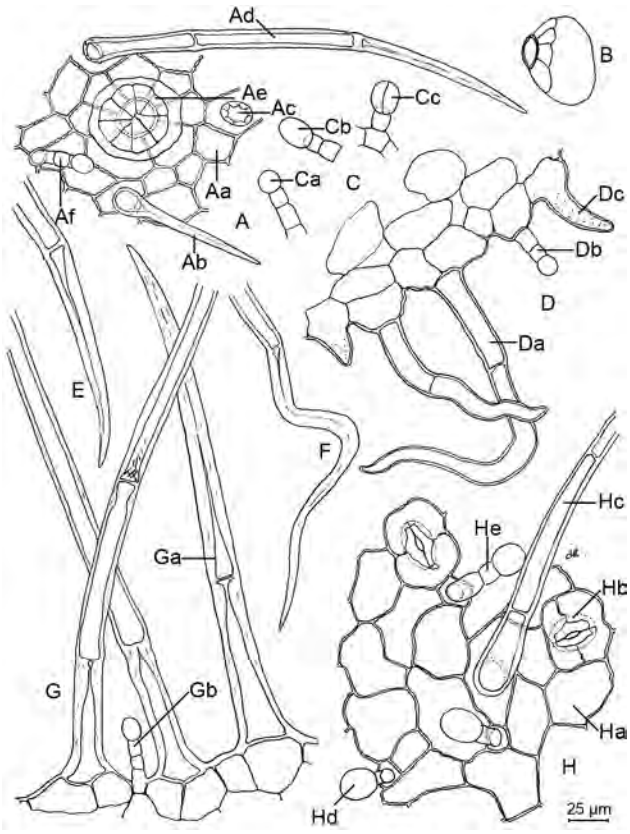


Figure 1561.-1. – *Illustration for identification test B of powdered herbal drug of three-lobed sage leaf*

C. Examine the chromatograms obtained in the test for thujone.

*Results*: the chromatogram obtained with the test solution shows a blue zone due to cineole, equal or greater in size and intensity to the zone in the chromatogram obtained with the reference solution. Further zones are present.

TESTS

**Thujone**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake 0.3 g of the freshly powdered herbal drug (355) (*2.9.12*) with 5.0 mL of *anhydrous ethanol R* for 5 min.

*Reference solution.* Dilute 20 µL of *thujone R* and 25 µL of *cineole R* in 20 mL of *anhydrous ethanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: treat with a 200 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R* and heat at 100-105 °C for 10 min. Examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows in the middle part a blue zone (cineole) and in the upper part a pink-blue zone (thujone). The chromatogram obtained with the test solution shows no zone or a very faint pink-blue zone due to thujone.

**Foreign matter** (*2.8.2*): maximum 8 per cent of stems and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of the herbal drug, cut, if necessary, immediately before the assay, a 500 mL flask and 250 mL of *water R* as the distillation liquid. Add 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

**01/2008:1889**
**corrected 10.0**

# SAGE TINCTURE

## Salviae tinctura

DEFINITION

Tincture produced from *Sage leaf (Salvia officinalis) (1370)*.

*Content*: minimum 0.1 per cent *m/m* of essential oil.

PRODUCTION

The tincture is produced from 1 part of comminuted drug and 10 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

*Appearance*: brownish liquid with a characteristic odour.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* The tincture to be examined.

*Reference solution.* Dissolve 20 µL of *thujone R* and 25 µL of *cineole R* in 20 mL of *ethanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 200 g/L solution of *phosphomolybdic acid R* in *ethanol R* and heat at 100-105 °C for 10 min. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue zone (near the solvent front) |
| ——— | ——— |
| α-Thujone and β-thujone: 2 pinkish-violet zones | 2 pinkish-violet zones (α-thujone and β-thujone) |
| Cineole: a blue zone | A blue zone (cineole) |
| ——— | ——— |
| | Blue zones |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol content** (*2.9.10*): 64 per cent *V/V* to 69 per cent *V/V*.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent of 2-propanol.

**Dry residue** (*2.8.16*): minimum 2.0 per cent, determined on 3.00 g.

Herbal drugs

ASSAY

In a 500 mL round-bottomed flask, place 30.0 g of the tincture and add 100 mL of *water R*. Distil, using a descending condenser, into a separating funnel which has been marked beforehand at 50 mL. Stop the distillation process as soon as the distillate reaches the 50 mL mark. Rinse the condenser with 10 mL of *pentane R*. Dissolve in the distillate sufficient *sodium chloride R* to produce a saturated solution. Shake with 3 quantities, each of 20 mL, of *pentane R*. Dry the combined pentane layers, including the pentane from rinsing the condenser, over *anhydrous sodium sulfate R* and filter through a plug of absorbent cotton into a weighed 100 mL round-bottomed flask. Wash the sodium sulfate several times with small quantities of *pentane R*. Remove the pentane carefully at a temperature not exceeding 40 °C. Dry the residue in a desiccator over *diphosphorus pentoxide R* and hard paraffin at atmospheric pressure and at room temperature for 2 h. Weigh the residue (essential oil).

**04/2017:2663**

# SALVIA MILTIORRHIZA ROOT AND RHIZOME

## Salviae miltiorrhizae radix et rhizoma

DEFINITION

Dried, whole or fragmented rhizome and root of *Salvia miltiorrhiza* Bunge, collected in spring or autumn.

*Content*:

- *salvianolic acid B* ($C_{36}H_{30}O_{16}$; $M_r$ 719): minimum 3.0 per cent (dried drug);
- *tanshinone $II_A$* ($C_{19}H_{18}O_3$; $M_r$ 294.3): minimum 0.12 per cent (dried drug).

IDENTIFICATION

A. The rhizome is short and thick, sometimes with stem remnants at the apex. The roots are numerous, about 10-20 cm long and 0.3-1 cm in diameter, cylindrical and slightly curved; some are branched, with secondary roots and rootlets. The outer surface is reddish-brown or dark reddish-brown, marked with longitudinal striations. The bark of old roots comes off usually as purplish-brown scales. The texture is hard and fragile. The fracture is soft, fissured or slightly even and dense, with a reddish-brown outer part and a greyish-yellow or purplish-brown wood, showing bundles of yellowish-white vessels, arranged radially.

Cultivars are relatively stout, about 0.5-1.5 cm in diameter. The outer surface is brownish-red, longitudinally wrinkled. The bark adheres closely to the wood and is difficult to remove. The texture is compact; the fracture is relatively even.

B. Microscopic examination (*2.8.23*). The powder is brownish-red. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2663.-1): fragments of cork consisting of subrectangular or polygonal cells, up to 150 µm in diameter, containing yellowish-brown pigment (surface view [A], transverse section [E]); fragments of parenchyma (longitudinal section [C]) consisting of polygonal or elongated cells, often septate [Ca]; fragments of parenchyma (transverse section [B]) with rounded cells; xylem fibres usually in bundles [F], long and fusiform, with walls showing pits shaped as crosses or oblique slits; very numerous reticulate or pitted vessels, 3-120 µm in diameter, free, in bundles (longitudinal section [D], transverse section [G]) or sometimes accompanying the fibres [Fa].

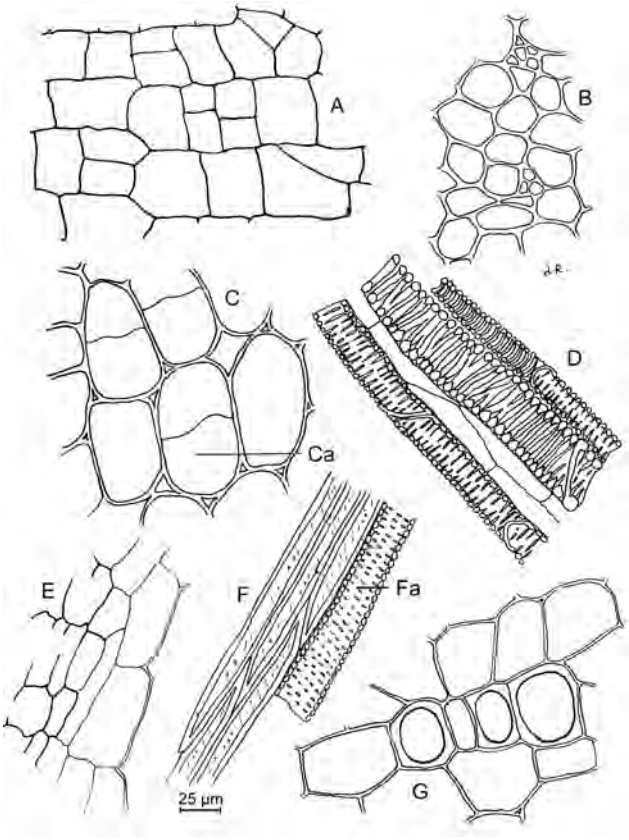


Figure 2663.-1. – *Illustration for identification test B of powdered herbal drug of salvia miltiorrhiza root and rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 40 mL of *methanol R*. Sonicate for 15 min. Filter. Evaporate the filtrate to 1 mL.

*Reference solution.* Dissolve 2 mg of *salvianolic acid B R* and 2 mg of *tanshinone $II_A$ R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *methanol R*, *anhydrous formic acid R*, *toluene R*, *methylene chloride R*, *ethyl acetate R* (5:20:20:30:40 *V/V/V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in daylight.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the upper third and middle part of the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Tanshinone $II_A$: a prominent red zone | A prominent red zone (tanshinone $II_A$) |
| | An orange zone |
| _____ | _____ |
| | A faint brownish-green zone |
| Salvianolic acid B: a faint grey zone | A faint grey zone (salvianolic acid B) |
| _____ | _____ |
| **Reference solution** | **Test solution** |

*Detection B*: examine in ultraviolet light at 254 nm.



Herbal drugs

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the upper third and middle part of the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Tanshinone $II_A$: a prominent quenching zone | A prominent quenching zone (tanshinone $II_A$) |
| | A quenching zone |
| | A quenching zone |
| Salvianolic acid B: a prominent quenching zone | A prominent quenching zone (salvianolic acid B) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Disperse 0.30 g of the powdered herbal drug (355) (*2.9.12*) in 50.0 mL of a 70 per cent *V/V* solution of *methanol R*. Sonicate for 1 h. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 5.0 mg of *tanshinone $II_A$ CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5.0 mg of *salvianolic acid B CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (c).* Dissolve 1 mg of *rosmarinic acid R* in *methanol R*, add 5 mL of reference solution (b) and dilute to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *anhydrous formic acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 79 → 71 | 21 → 29 |
| 10 - 15 | 71 → 65 | 29 → 35 |
| 15 - 25 | 65 → 28 | 35 → 72 |
| 25 - 37 | 28 → 0 | 72 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to tanshinone $II_A$ and the chromatogram obtained with reference solution (b) to identify the peak due to salvianolic acid B.

*Relative retention* with reference to tanshinone $II_A$ (retention time = about 33 min): rosmarinic acid = about 0.3; salvianolic acid B = about 0.4.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to rosmarinic acid and salvianolic acid B in the chromatogram obtained with reference solution (c);
- *symmetry factor*: maximum 2.0 for the peak due to salvianolic acid B in the chromatogram obtained with reference solution (b).

Calculate the percentage content of tanshinone $II_A$ using the following expression:

$$\frac{A_1 \times m_2 \times p_1}{A_2 \times m_1 \times 5}$$

$A_1$ = area of the peak due to tanshinone $II_A$ in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to tanshinone $II_A$ in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *tanshinone $II_A$ CRS* used to prepare reference solution (a), in grams;

$p_1$ = percentage content of tanshinone $II_A$ in *tanshinone $II_A$ CRS*.

Calculate the percentage content of salvianolic acid B using the following expression:

$$\frac{A_3 \times m_3 \times p_2 \times 2}{A_4 \times m_1}$$

$A_3$ = area of the peak due to salvianolic acid B in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to salvianolic acid B in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_3$ = mass of *salvianolic acid B CRS* used to prepare reference solution (b), in grams;

$p_2$ = percentage content of salvianolic acid B in *salvianolic acid B CRS*.

**04/2008:2385**

# SANGUISORBA ROOT

## Sanguisorbae radix

DEFINITION

Whole or fragmented, dried underground parts of *Sanguisorba officinalis* L. without rootlets.

*Content*: minimum 5.0 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

CHARACTERS

The adventitious roots are about 5-25 cm long and up to 2 cm in diameter.

Herbal drugs

IDENTIFICATION

A. The whole drug consists of the rhizome, often ramified, thick, short, fusiform or cylindrical and the adventitious roots whose surface is reddish-brown or blackish-brown, with longitudinal striations, sometimes with transverse fissures, and showing rootlet scars.

It may also be found as more or less cylindrical fragments up to 2 cm long or elliptical or irregular discs. The fracture is light-coloured and very fibrous.

B. Microscopic examination (*2.8.23*). The powder is light yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: numerous, whole or fragmented phloem fibres, usually isolated, narrow, sometimes more than 500 µm long and often rough-walled; calcium oxalate cluster crystals, free or inside parenchyma cells; a few reticulate lignified vessels; rare cork fragments. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows rounded or ovoid starch granules, single or in groups of 2-4; the diameter of a component granule may reach 30 µm. Some starch granules are found in the parenchyma cells or in cells of the medullary rays.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 50 mL of *water R* and boil under a reflux condenser for 30 min. Cool the solution and centrifuge for 10 min. Shake the supernatant with 2 quantities, each of 15 mL, of *di-isopropyl ether R* saturated with *hydrochloric acid R*. Combine the ether layers. Evaporate to dryness and dissolve the residue in 1.0 mL of *methanol R*. Filter through a polypropylene syringe filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 5 mg of *gallic acid R* and 20 mg of *resorcinol R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *toluene R* (10:30:60 *V/V/V*).

*Application*: 10 µL [or 4 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of quenching zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A quenching zone |
| ——— | ——— |
| Resorcinol: a quenching zone | |
| | A quenching zone |
| ——— | ——— |
| Gallic acid: a quenching zone | A quenching zone (gallic acid) |
| | A quenching zone |
| | A quenching zone |
| **Reference solution** | **Test solution** |

*Detection B*: spray with a 10 g/L solution of *ferric chloride R* in *anhydrous ethanol R* and heat at 100-105 °C for 15 min; examine in daylight.

*Results B*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Resorcinol: a brown zone | |
| | A blackish-blue zone |
| ——— | ——— |
| Gallic acid: a blackish-blue zone | A blackish-blue zone (gallic acid) |
| | A blackish-blue zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.500 g of the powdered herbal drug (180) (*2.9.12*).

**07/2019:2579**
**corrected 10.0**

# SAW PALMETTO EXTRACT

## Sabalis serrulatae extractum

DEFINITION

Extract produced from *Saw palmetto fruit (1848)*.

*Content*:

- *total fatty acids*: minimum 80.0 per cent (anhydrous extract);
- *lauric acid* ($C_{12}H_{24}O_2$; $M_r$ 200.3): minimum 23.0 per cent (anhydrous extract);
- *total sterols, expressed as β-sitosterol* ($C_{29}H_{50}O$; $M_r$ 414.7): minimum 0.20 per cent (anhydrous extract);
- *β-sitosterol* ($C_{29}H_{50}O$; $M_r$ 414.7): minimum 0.10 per cent (anhydrous extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (minimum 90 per cent *V/V*), or supercritical carbon dioxide or a mixture of mainly *n*-hexane and methylpentanes (bp: 65-70 °C).

CHARACTERS

*Appearance*: the ethanol extract is a dark greenish-brown, oily liquid; the supercritical carbon dioxide extract is a yellowish-brown or orange-brown, oily liquid; the hexane extract is a yellowish-green or orange-yellow, oily liquid.

*Odour*: strong but not rancid.

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A*.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.25 g of the extract to be examined in 20 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 4 mg of *β-amyrin R* and 10 mg of *β-sitosterol R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *toluene R* (1:30:70 *V/V/V*).

*Application*: 2 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present, especially in the lower third, in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue zone |
| | A blue zone |
| _____ | _____ |
| β-Amyrin: a blue zone | A strong bluish-violet zone |
| β-Sitosterol: a blue zone | |
| _____ | _____ |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the assay of total fatty acids.

*Results*: the peaks due to methyl caproate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitoleate, methyl palmitate, methyl linoleate, methyl linolenate, methyl oleate and methyl stearate in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (b); the principal peaks are due to methyl laurate and methyl oleate.

TESTS

**Water** (*2.5.12, Method A*): maximum 3.0 per cent, determined on 0.5 g.

**Relative density** (*2.2.5*): 0.850 to 0.950.

**Refractive index** (*2.2.6*): 1.40 to 1.50.

**Acid value** (*2.5.1*): 150.0 to 220.0, determined on 0.500 g.

**Iodine value** (*2.5.4, Method A*): 30.0 to 60.0.

**Peroxide value** (*2.5.5*): maximum 5.0.

**Saponification value** (*2.5.6*): 220.0 to 250.0.

**Solvents**. Residual solvents are controlled as described in chapter *5.4*, unless otherwise justified and authorised.

ASSAY

**Total fatty acids**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 0.47 g of *methyl margarate R* in 20 mL of *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Test solution*. Disperse 0.25 g of the extract to be examined in 10 mL of *dimethylformamide R*. Add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of a 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Reference solution (a)*. Dissolve 0.699 g of *lauric acid CRS* and 0.870 g of *oleic acid CRS* in *dimethylformamide R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of a 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Reference solution (b)*. Disperse 0.25 g of *saw palmetto extract HRS* in 10 mL of *dimethylformamide R*. Add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of a 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.20 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.33 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 150 |
| | 2 - 7 | 150 → 190 |
| | 7 - 12 | 190 |
| | 12 - 22 | 190 → 220 |
| | 22 - 32 | 220 |
| Injection port | | 300 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *saw palmetto extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to methyl caproate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitoleate, methyl palmitate, methyl linoleate, methyl linolenate, methyl oleate, methyl stearate and methyl margarate.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to methyl linolenate and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to methyl linoleate.

Calculate the percentage content of total fatty acids, where caproic, caprylic, capric, lauric, myristic, palmitoleic, palmitic and stearic acids are expressed as lauric acid ($C_{12}H_{24}O_2$; $M_r$ 200.3) and linoleic, linolenic and oleic acids are expressed as oleic acid ($C_{18}H_{34}O_2$; $M_r$ 282.5), using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times p_1 \times 0.1}{A_2 \times A_3 \times m_1} + \frac{A_5 \times A_4 \times m_3 \times p_2 \times 0.1}{A_6 \times A_3 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to methyl caproate, methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitoleate, methyl palmitate and methyl stearate in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to methyl laurate in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to methyl margarate in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to methyl margarate in the chromatogram obtained with reference solution (a);

$A_5$ = sum of the areas of the peaks due to methyl linoleate, methyl linolenate and methyl oleate in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to methyl oleate in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *lauric acid CRS* used to prepare reference solution (a), in grams;

$m_3$ = mass of *oleic acid CRS* used to prepare reference solution (a), in grams;

$p_1$ = percentage content of lauric acid in *lauric acid CRS*;

$p_2$ = percentage content of oleic acid in *oleic acid CRS*.

Calculate the percentage content of lauric acid using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times p \times 0.1}{A_2 \times A_3 \times m_1}$$

$A_1$ = area of the peak due to methyl laurate in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to methyl laurate in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to methyl margarate in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to methyl margarate in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *lauric acid CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of lauric acid in *lauric acid CRS*.

**Total sterols**. Gas chromatography (*2.2.28*).

*Derivatisation solution (a)*: *chlorotrimethylsilane R*, *N,O-bis(trimethylsilyl)acetamide R*, *N-trimethylsilylimidazole R* (2:3:3 *V/V/V*).

*Derivatisation solution (b)*: derivatisation solution (a), *N,O-bis(trimethylsilyl)trifluoroacetamide R*, *pyridine R* (1:1:1 *V/V/V*).

*Internal standard solution.* Dissolve 0.25 g of *cholesterol R* in 25.0 mL of *methylene chloride R*.

*Test solution.* Introduce 1.0 mL of the internal standard solution into a 50 mL round-bottomed flask and evaporate to dryness. Place 3.35 g of the extract to be examined, accurately weighed, into the round-bottomed flask and add 20 mL of a solution prepared as follows: dissolve 130 g of *potassium hydroxide R* in 200 mL of *water R* and dilute to 1000 mL with *methanol R*. Heat under reflux for 2 h, transfer quantitatively to a flask and dilute to 25.0 mL with *water R*. Apply 3.0 mL of this solution to a cartridge containing *diatomaceous earth R* capable of holding 3 mL of aqueous phase. Absorb the solution into the column by applying vacuum. Maintain the vacuum for at least 20 min, until the column returns to room temperature, indicating that the methanol is completely evaporated. Rinse the column with 90 mL of *methylene chloride R* and evaporate the eluate to dryness. Dissolve the residue in 1.0 mL of derivatisation solution (b).

*Reference solution (a).* To 9.0 mg of *β-sitosterol CRS* add 1.0 mL of the internal standard solution and dilute to 5.0 mL with *methylene chloride R*. Evaporate 0.6 mL of this solution to dryness under a stream of *nitrogen R*. Dissolve the residue in 1.0 mL of derivatisation solution (b).

*Reference solution (b).* Introduce 1.0 mL of the internal standard solution into a 50 mL round-bottomed flask and evaporate to dryness. Place 3.35 g of *saw palmetto extract HRS*, accurately weighed, into the round-bottomed flask and add 20 mL of a solution prepared as follows: dissolve 130 g of *potassium hydroxide R* in 200 mL of *water R* and dilute to 1000 mL with *methanol R*. Heat under reflux for 2 h, transfer quantitatively to a flask and dilute to 25.0 mL with *water R*. Apply 3.0 mL of this solution to a cartridge containing *diatomaceous earth R* capable of holding 3 mL of aqueous phase. Absorb the solution into the column by applying vacuum. Maintain the vacuum for at least 20 min, until the column returns to room temperature, indicating that the methanol is completely evaporated. Rinse the column with 90 mL of *methylene chloride R* and evaporate the eluate to dryness. Dissolve the residue in 1.0 mL of derivatisation solution (b).

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.20 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.33 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:40.

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 200 |
| | 3 - 13 | 200 → 300 |
| | 13 - 35 | 300 |
| Injection port | | 325 |
| Detector | | 325 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *saw palmetto extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to the trimethylsilyl derivatives of cholesterol, campesterol, stigmasterol, β-sitosterol and stigmastanol.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.6 between the peaks due to the trimethylsilyl derivatives of β-sitosterol and stigmastanol.

Calculate the percentage content of total sterols (campesterol, stigmasterol, β-sitosterol and stigmastanol), expressed as β-sitosterol, using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times p}{A_2 \times A_3 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to the trimethylsilyl derivatives of campesterol, stigmasterol, β-sitosterol and stigmastanol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the trimethylsilyl derivative of β-sitosterol in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to the trimethylsilyl derivative of cholesterol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to the trimethylsilyl derivative of cholesterol in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *β-sitosterol CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of β-sitosterol in *β-sitosterol CRS*.

Calculate the percentage content of β-sitosterol using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times p}{A_2 \times A_3 \times m_1}$$

$A_1$ = area of the peak due to the trimethylsilyl derivative of β-sitosterol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the trimethylsilyl derivative of β-sitosterol in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to the trimethylsilyl derivative of cholesterol in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to the trimethylsilyl derivative of cholesterol in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *β-sitosterol CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of β-sitosterol in *β-sitosterol CRS*.

**04/2019:1848**
**corrected 10.0**

# SAW PALMETTO FRUIT

## Sabalis serrulatae fructus

DEFINITION

Dried ripe fruit of *Serenoa repens* (W.Bartram) Small (syn. *Sabal serrulata* (Michx.) Schult.f.).

*Content*: minimum 11.0 per cent of total fatty acids (dried drug).

CHARACTERS

*Odour*: strong but not rancid.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, B, C.*

A. The fruit is an ovoid or subspherical drupe, with a dark brown or blackish, roughly wrinkled surface and more or less coppery sheen, up to 2.5 cm long and 1.5 cm in diameter. The apex sometimes bears the remains of the style and tubular calyx, with 3 teeth, and the base bears a small depression with the scar of the stalk. The epicarp and underlying mesocarp form a thin fragile layer, which partially peels off, revealing the thin, hard, pale brown endocarp, which is fibrous and easily separable. The seed is irregularly spherical or ovoid, up to 12 mm long and 8 mm in diameter, with a hard, smooth or finely pitted surface which is reddish-brown with a paler, raised and membranous area over the raphe and micropyle; cut transversely, the seed has a thin testa, narrow perisperm and a large area of dense, horny, greyish-white endosperm, with the embryo positioned to one side.

B. Microscopic examination (*2.8.23*). Reduce to a powder (1000) (*2.9.12*). The powder is reddish or blackish-brown and oily. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1848.-1): fragments of epicarp [A] consisting of polyhedral cells (10-40 µm), reddish-brown, pigmented and highly cutinised, in small groups separated by thin walls [Aa] and accompanied by considerably larger cells of the underlying layers [Ab]; groups of parenchyma cells of the mesocarp [E]; groups of xylem tissue of the mesocarp showing small, lignified, annular or spirally thickened vessels [F], sometimes accompanied by parenchyma containing small sclereids [Fa]; sclereids of the mesocarp (20-200 µm), usually occurring singly [J] but sometimes in small groups [G], with walls that are moderately thickened, distinctly striated and finely pitted; fragments of endocarp containing groups of sclereids about 300 µm long, with strongly thickened walls and numerous pits [B]; isolated sclereids of the endocarp [K]; fragments of the testa [C] consisting of small, thin-walled cells with brown contents [Ca] and underlying sclereids [Cb]; thick-walled endosperm cells [D] with large conspicuous pits, and containing aleurone grains and oil; very numerous fragments of the brown cuticle [H].

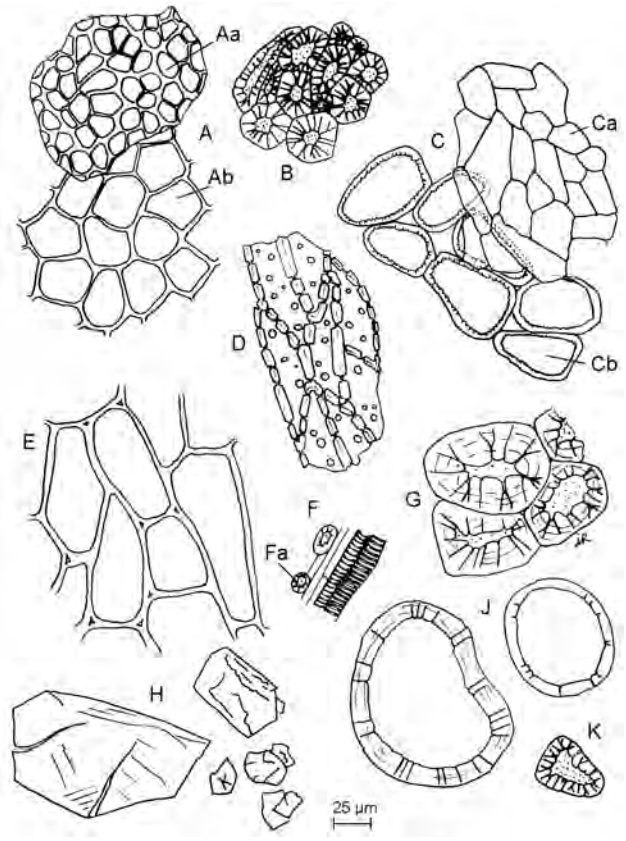


Figure 1848.-1. – *Illustration for identification test B of powdered herbal drug of saw palmetto fruit*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.5 g of the powdered herbal drug (710) (*2.9.12*), add 20 mL of *ethanol (96 per cent) R* and stir for 15 min. Filter.

*Reference solution.* Dissolve 4 mg of *β-amyrin R* and 10 mg of *β-sitosterol R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *ethyl acetate R*, *toluene R* (1:30:70 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*; heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present, especially in the lower third, in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A strong blue zone |
| ——— | ——— |
| | 2 faint blue zones |
| β-Amyrin: a blue zone | |
| | A strong bluish-violet zone |
| β-Sitosterol: a blue zone | A faint blue zone |
| ——— | ——— |
| | A faint blue zone |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay of total fatty acids.

*Results*: the peaks due to caproic, caprylic, capric, lauric, myristic, palmitoleic, palmitic, linoleic, linolenic, oleic and stearic acids in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (b); the principal peaks are due to lauric acid and oleic acid.

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

**Total fatty acids**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.47 g of *methyl margarate R* in 20.0 mL of *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Reduce 50 g of the herbal drug to a powder (200) (*2.9.12*). Disperse 4.00 g of the powdered herbal drug in 60 mL of *dimethylformamide R*. Sonicate for 15 min and then shake for 30 min. Dilute to 100.0 mL with *dimethylformamide R*. Allow to stand for a few minutes and filter. To 20.0 mL of this solution add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of an 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Reference solution (a).* Dissolve 0.699 g of *lauric acid CRS* and 0.870 g of *oleic acid CRS* in *dimethylformamide R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of an 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Reference solution (b).* Disperse 0.25 g of *saw palmetto extract HRS* in 10 mL of *dimethylformamide R*. Add 4.0 mL of the internal standard solution and dilute to 25.0 mL with *dimethylformamide R*. Mix 0.4 mL of this solution and 0.6 mL of an 18.84 g/L solution of *trimethylsulfonium hydroxide R* in *methanol R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.20 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.33 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | **Time (min)** | **Temperature (°C)** |
|---|---|---|
| Column | 0 - 2 | 150 |
| | 2 - 7 | 150 → 190 |
| | 7 - 12 | 190 |
| | 12 - 22 | 190 → 220 |
| | 22 - 32 | 220 |
| Injection port | | 300 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *saw palmetto extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to caproic, caprylic, capric, lauric, myristic, palmitoleic, palmitic, linoleic, linolenic, oleic and stearic acids and methyl margarate.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to linolenic acid and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to linoleic acid.

Calculate the percentage content of total fatty acids, where caproic, caprylic, capric, lauric, myristic, palmitoleic, palmitic and stearic acids are expressed as lauric acid ($C_{12}H_{24}O_2$; $M_r$ 200.3) and linoleic, linolenic and oleic acids are expressed as oleic acid ($C_{18}H_{34}O_2$; $M_r$ 282.5), using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times p_1 \times 0.5}{A_2 \times A_3 \times m_1} + \frac{A_5 \times A_4 \times m_3 \times p_2 \times 0.5}{A_6 \times A_3 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to caproic, caprylic, capric, lauric, myristic, palmitoleic, palmitic and stearic acids in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to lauric acid in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to methyl margarate in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to methyl margarate in the chromatogram obtained with reference solution (a);

$A_5$ = sum of the areas of the peaks due to linoleic, linolenic and oleic acids in the chromatogram obtained with the test solution;

$A_6$ = area of the peak due to oleic acid in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *lauric acid CRS* used to prepare reference solution (a), in grams;

$m_3$ = mass of *oleic acid CRS* used to prepare reference solution (a), in grams;

$p_1$ = percentage content of lauric acid in *lauric acid CRS*;

$p_2$ = percentage content of oleic acid in *oleic acid CRS*.

**07/2016:2428**
**corrected 9.1**

# SCHISANDRA FRUIT

## Schisandrae chinensis fructus

DEFINITION

Whole, dried or steamed and dried, ripe fruit of *Schisandra chinensis* (Turcz.) Baill.

*Content*: minimum 0.40 per cent of schisandrin ($C_{24}H_{32}O_7$; $M_r$ 432.5) (dried drug).

IDENTIFICATION

A. The berry is more or less spherical, up to 8 mm in diameter; red, reddish-brown or blackish outer surface, sometimes covered in a whitish frost; strongly shrivelled pericarp; presence of 1-2 reniform, yellowish-brown, lustrous seeds, with thin seed-coat.

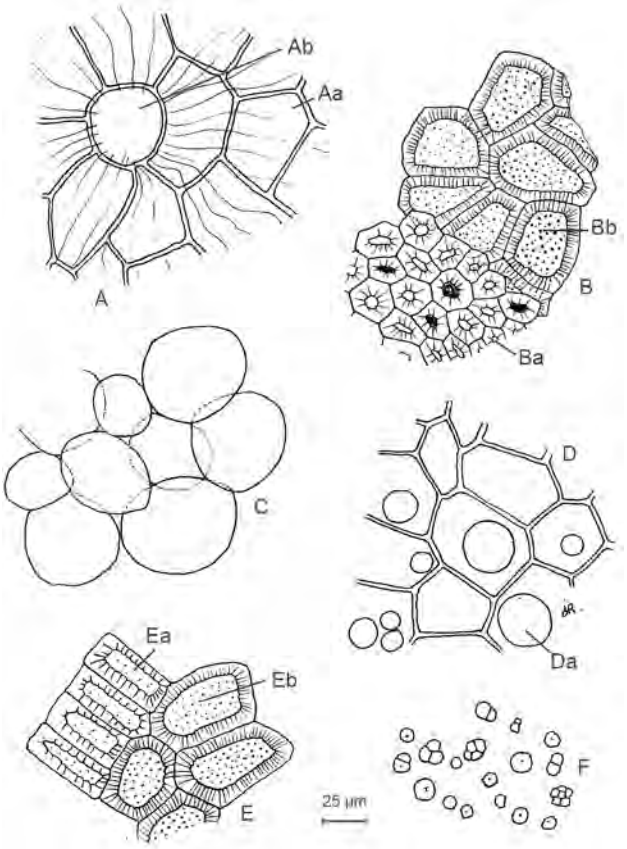


Figure 2428.-1. – *Illustration for identification test B of powdered herbal drug of schisandra fruit*

B. Microscopic examination (*2.8.23*). The powder is dark reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2428.-1): reddish-brown fragments of pericarp consisting of 1 layer of thin-walled epicarp cells (surface view [A]), covered by a striated cuticle [Aa] and accompanied by sparse essential oil cells [Ab], the cuticle is not striated above the essential oil cells; fragments of mesocarp [C] composed of several layers of ovoid, more or less flattened cells; fragments of the testa [B, E] with the outer layer consisting of polygonal sclereids, about 11-30 µm in diameter (surface view [Ba]) or in palisade arrangement (side view [Ea]), with thick, lignified, finely channelled walls and small lumen with reddish-brown or blackish contents with the inner layers of the testa consisting of sclereids with yellowish or slightly reddish walls, isolated or in small groups or together with the outer sclereids, about 80 µm in diameter, with slightly lignified, thickened and markedly channelled, pitted walls and a large lumen [Bb, Eb]; fragments of mostly colourless endosperm [D] consisting of polyhedral cells containing oil droplets [Da] and aleurone grains. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows parenchymatous cells of the mesocarp containing numerous small, round or slightly polyhedral starch granules and rare free starch granules, rounded or slightly polyhedral, simple or 2-5 compound [F]; the hilum is often visible.

C. Examine the chromatograms obtained in the test for *Schisandra sphenanthera*.

*Results A*: see below the sequence of quenching zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weak quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| γ-Schisandrin: a quenching zone | A quenching zone (γ-schisandrin) |
| _____ | _____ |
| | A weak quenching zone |
| _____ | _____ |
| Schisandrin: a quenching zone | A quenching zone (schisandrin) |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| γ-Schisandrin: a brown zone | A brown zone (γ-schisandrin) |
| _____ | _____ |
| _____ | _____ |
| Schisandrin: an intense, brownish-green zone | An intense, brownish-green zone (schisandrin) |
| **Reference solution** | **Test solution** |

TESTS

***Schisandra sphenanthera***. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 2.5 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Extract at 25 °C in an ultrasonic bath for 5 min and centrifuge.

*Reference solution*. Dissolve 5 mg of *schisandrin R* and 5 mg of *γ-schisandrin R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *acetic acid R*, *ethyl acetate R*, *toluene R* (2:22:46 *V/V/V*).

*Application*: 5 µL [or 2 µL] as bands of 10 mm [or 6 mm].

*Development*: over a path of 10 cm [or 7 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Detection B*: spray with a 100 g/L solution of *sulfuric acid R* in *methanol R* and heat in an oven at 120 °C for 7 min; examine in daylight.

*Results B*: the chromatogram obtained with the test solution shows a zone due to schisandrin and a zone due to γ-schisandrin; the chromatogram shows no intense violet-pink zone in the middle third.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Weigh 1.250 g of the powdered herbal drug (355) (*2.9.12*) into a 250 mL conical flask, add 90 mL of *methanol R* and sonicate for 30 min. Filter the solution into a volumetric flask, add 10 mL of *methanol R* whilst rinsing the filter and dilute to 100.0 mL with the same solvent.

*Reference solution*. Dissolve 5.0 mg of *schisandrin CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: *water R*, *methanol R* (35:65 *V/V*);
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 16 | 100 → 58 | 0 → 42 |
| 16 - 26 | 58 | 42 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Retention time*: schisandrin = about 8 min.

*System suitability*:
- *number of theoretical plates*: minimum 5000, calculated for the peak due to schisandrin in the chromatogram obtained with the reference solution.

Calculate the percentage content of schisandrin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to schisandrin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to schisandrin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *schisandrin CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of schisandrin in *schisandrin CRS*.

**07/2019:0202**

# SENEGA ROOT

## Polygalae radix

DEFINITION

Dried, whole or fragmented root and root crown of *Polygala senega* L. or root of *Polygala tenuifolia* Willd., with rootlets removed.

IDENTIFICATION

A. *P. senega*. The root crown of the whole drug is greyish-brown and distinctly wider than the root; it forms an irregular head up to 3 cm in diameter consisting of numerous, tightly packed remains or scars of stems and violet-brown buds. The taproot is flexuous and tortuous, brown or yellow, usually single but occasionally branched into 2 or 3 roots, usually 1-8 mm in diameter at the crown, gradually tapering to the tip; the external surface is transversely and longitudinally striated and often shows a more or less distinct, decurrent, elongated spiral ridge. The transverse section of the root is more or less triangular. The fracture is short and shows a yellowish cortex of

varying thickness surrounding a paler, central, woody area irregular in shape, especially at the ridge. The fragmented drug consists of pieces of varying shape; fragments of the root crown comprise the tightly packed remains or scars of stems and violet-brown buds; fragments of the root have a brown or yellow, transversely and longitudinally striated external surface with a paler internal surface.

*P. tenuifolia*. The whole drug consists of the root, light yellowish-brown or brown, straight or curved, 3-15 cm in length and 3-8 mm in diameter, showing numerous transverse wrinkles and fissures and, to a lesser extent, longitudinal striations; round rootlet scars are often visible. The xylem is light yellow and readily detached, with some fragments consisting only of rolls of bark with their edges folded inwards. The fracture shows a yellowish cortex and a pale yellow, usually circular, central woody area. The fragmented drug consists of more or less cylindrical slices, solid or hollow in the central part where the xylem has detached. The external surface is yellowish-grey to brownish-grey and transversely striated; the transverse section is yellowish-brown and shows a rounded, pale yellow, central woody area.

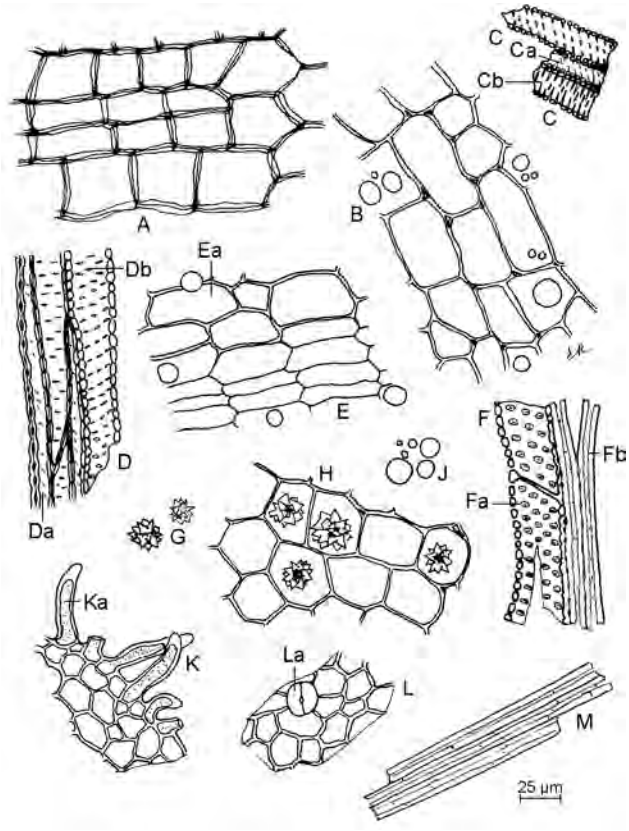


Figure 0202.-1. – *Illustration for identification test B of powdered herbal drug of senega root*

B. Microscopic examination (*2.8.23*). The powder is light yellow to brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0202.-1): fragments of lignified tissue [C, D, F] made up of numerous pitted tracheids [Ca, Da] and slightly larger, reticulate [Cb], pitted [Db] or bordered-pitted vessels [Fa]; yellowish parenchyma cells containing oil droplets [B]; fragments of cork (surface view [A], transverse section [E]), sometimes accompanied by phelloderm and parenchyma, some cells of which contain oil droplets [Ea]; numerous isolated oil droplets [J]. *P. tenuifolia* powder also contains calcium oxalate cluster crystals, isolated [G] or included in parenchymatous cells [H], and long, fine, thick-walled lignified fibres, most often fragmented, in clusters [M] or associated with vessels [Fb]. *P. senega* powder may show occasional fragments of epidermal tissue from bud scales [K, L] with stomata [La] and unicellular trichomes [Ka].

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *ethanol (70 per cent V/V) R*. Sonicate at 80 °C for 15 min, filter or centrifuge and use the filtrate or supernatant.

*Reference solution (a)*. Dissolve 1 mg of *rutoside trihydrate R* and 30 mg of *puerarin R* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 2 mg of *chlorogenic acid R* and 30 mg of *puerarin R* in *methanol R* and dilute to 25 mL with the same solvent.

*Intensity marker*: puerarin.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:26:100 *V/V/V/V*).

*Application*: 3 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 3 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows in the middle third 2 distinct zones; the lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (puerarin) shows a blue fluorescence.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint fluorescent zones may be present.

| **Top of the plate** | | |
|---|---|---|
| ——— | ——— | ——— |
| | | A blue zone |
| Puerarin: a blue zone | A blue zone | A blue zone |
| | A blue zone | A blue zone |
| | | A greyish zone, faint |
| Rutoside: an orange zone | An orange zone, faint | |
| | | A greenish-blue zone (at the border between the middle and lower third) |
| ——— | ——— | ——— |
| | | A bluish zone |
| | A whitish-blue zone | |
| | 3 bluish zones | |
| **Reference solution (a)** | **Test solution (*Polygala senega*)** | **Test solution (*Polygala tenuifolia*)** |

TESTS

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

**Extractable matter**: minimum 20.0 per cent.

To 1.00 g of the powdered herbal drug (355) (*2.9.12*) add a mixture of 4.5 g of *ethanol (96 per cent) R* and 15.5 g of *water R* and allow to macerate for 3 h, shaking frequently. Filter. Evaporate 5.0 g of the filtrate to dryness on a water-bath and dry in an oven at 100-105 °C for 2 h. The residue weighs a minimum of 50 mg.

**Foam index** (*2.8.24*): minimum 3.5, determined on 1.00 g of the powdered herbal drug (355) (*2.9.12*).

STORAGE

Protected from humidity.

LABELLING

The label states the species present.

**01/2015:0206**

# SENNA LEAF

## Sennae folium

DEFINITION

Dried leaflets of *Cassia senna* L. (syn. *Cassia acutifolia* Delile), known as Alexandrian or Khartoum senna, or *Cassia angustifolia* Vahl, known as Tinnevelly senna, or a mixture of the 2 species.

*Content*: minimum 2.5 per cent of hydroxyanthracene glycosides, expressed as sennoside B ($C_{42}H_{38}O_{20}$; $M_r$ 863) (dried drug).

IDENTIFICATION

A. *C. senna* occurs as greyish-green or brownish-green, thin, fragile leaflets, lanceolate, mucronate, asymmetrical at the base, usually 15-40 mm long and 5-15 mm wide, the maximum width being at a point slightly below the centre; the lamina is slightly undulant with both surfaces covered with fine, short trichomes. Pinnate venation is visible mainly on the lower surface, with lateral veins leaving the midrib at an angle of about 60° and anastomosing to form a ridge near the margin.

*Stomatal index* (*2.8.3*): 10-12.5-15.

*C. angustifolia* occurs as yellowish-green or brownish-green leaflets, elongated and lanceolate, slightly asymmetrical at the base, usually 20-50 mm long and 7-20 mm wide at the centre. Both surfaces are smooth with a very small number of short trichomes and are frequently marked with transverse or oblique lines.

*Stomatal index* (*2.8.3*): 14-17.5-20.

B. Microscopic examination (*2.8.23*). The powder is light green or greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0206.-1): fragments of epidermis (*C. angustifolia*: [A, B], *C. senna*: [J, K]) with polygonal cells [Aa, Ka], paracytic stomata (*2.8.3*) [Ab, Ac, Ba, Ja, Kb] and unicellular covering trichomes, conical in shape, with warty walls (surface view [Ad], side view (*C. senna*: [G])), or their scars [Bb, Jb], frequently accompanied by palisade parenchyma [Ae, Jc]; isolated, fragmented covering trichomes [E]; fibres [F] with a crystal sheath of prismatic crystals of calcium oxalate [Fa]; isolated prisms of calcium oxalate [D]; isolated cluster crystals of calcium oxalate [H]; fragments of median parenchyma from the lamina [C] with some cells containing cluster crystals of calcium oxalate [Ca], often accompanied by palisade parenchyma [Cb] and annular vessels [Cc].

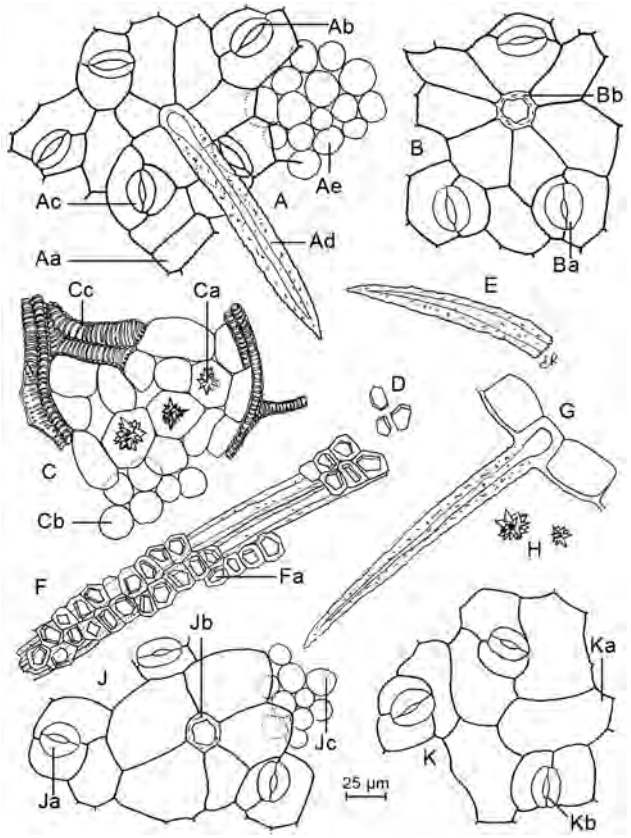


Figure 0206.-1. – *Illustration for identification test B of powdered herbal drug of senna leaf*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (180) (*2.9.12*) add 5 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.

*Reference solution.* Dissolve 10 mg of *senna extract CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* (a slight residue remains).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R*, *propanol R* (1:30:40:40 *V/V/V/V*).

*Application*: 10 µL as bands of 20 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with a 20 per cent *V/V* solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* until the zones appear.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in the order of increasing $R_F$ value), colour and size to the principal zones in the chromatogram obtained with the reference solution; between the zones due to sennosides D and C a red zone due to rhein-8-glucoside may be visible.

D. Place about 25 mg of the powdered herbal drug (180) (*2.9.12*) in a conical flask and add 50 mL of *water R* and 2 mL of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 mL of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulfate R*, evaporate

5 mL to dryness and to the cooled residue add 5 mL of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of foreign organs and maximum 1 per cent of foreign elements.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.5 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Place 0.150 g of the powdered herbal drug (180) (*2.9.12*) in a 100 mL flask. Add 30.0 mL of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 mL of the supernatant liquid to a 150 mL separating funnel. Add 0.1 mL of *dilute hydrochloric acid R* and shake with 3 quantities, each of 15 mL, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 mL of the supernatant liquid to a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *ferric chloride solution R1* and mix. Place the flask in a water-bath so that the water level is above that of the liquid in the flask, and heat under a reflux condenser for 20 min. Add 3 mL of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R* previously used to rinse the flask. Combine the 3 ether layers and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the combined ether layers to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 10.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene glycosides, expressed as sennoside B, using the following expression:

$$\frac{A \times 1.25}{m}$$

i. e. taking the specific absorbance of sennoside B to be 240.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

STORAGE

Protected from moisture.

**07/2015:1261**

# SENNA LEAF DRY EXTRACT, STANDARDISED

## Sennae folii extractum siccum normatum

DEFINITION

Standardised dry extract produced from *Senna leaf (0206)*.

*Content*: 5.5 per cent to 8.0 per cent of hydroxyanthracene glycosides, expressed as sennoside B ($C_{42}H_{38}O_{20}$; $M_r$ 863) (dried extract). The measured content does not deviate from the value stated on the label by more than ± 10 per cent.

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (50-80 per cent *V/V*).

CHARACTERS

*Appearance*: brownish or brown powder.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *ethanol (96 per cent) R*, *water R* (50:50 *V/V*).

*Test solution*. To 0.1 g of the extract to be examined add 5 mL of the solvent mixture and heat to boiling. Cool and centrifuge. Use the supernatant.

*Reference solution*. Dissolve 10 mg of *senna extract CRS* in 1 mL of the solvent mixture (a slight residue remains).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R*, *propanol R* (1:30:40:40 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with a 20 per cent *V/V* solution of *nitric acid R* and heat at 120 °C for 10 min; allow to cool and treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* until the zones appear.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the reference solution. The chromatograms show in the lower third a prominent brown zone due to sennoside B and above it a yellow zone followed by another prominent brown zone due to sennoside A. In the upper half of the chromatograms are visible, in order of increasing $R_F$ value, a prominent reddish-brown zone and an orange-brown zone followed by a faint pink zone and 2 yellow zones. Close to the solvent front a dark pink zone appears, which may be followed by several faint zones.

B. Place about 25 mg of the extract to be examined in a conical flask and add 50 mL of *water R* and 2 mL of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 mL of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulfate R*, evaporate 5 mL to dryness and to the cooled residue add 5 mL of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

**Loss on drying** (*2.8.17*): maximum 5.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Place 0.150 g of the extract to be examined in a 100 mL flask, disperse in *water R* and dilute to 100.0 mL with the same solvent. Filter the solution, discarding the first 10 mL of the filtrate. Transfer 20.0 mL of the filtrate to a 150 mL separating funnel. Add 0.1 mL of *dilute hydrochloric acid R* and shake with 3 quantities, each of 15 mL, of *ether R*. Allow the layers to separate and discard the ether layer. Add 0.10 g of *sodium hydrogen carbonate R* to the aqueous layer and shake for 3 min. Centrifuge and transfer 10.0 mL of the supernatant to a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *ferric chloride solution R1* and mix. Heat for 20 min under a reflux condenser in a water-bath with the water level above that of the liquid in the flask; add 3 mL of *hydrochloric acid R* and heat for a further 30 min with

frequent shaking to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R* previously used to rinse the flask. Combine the ether layers and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the ether layers to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 10.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5.0 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene glycosides expressed as sennoside B using the following expression:

$$\frac{A \times 4.167}{m}$$

i.e. taking the specific absorbance of sennoside B to be 240.

$A$ = absorbance at 515 nm;

$m$ = mass of the extract to be examined, in grams.

LABELLING

The label states the content of hydroxyanthracene glycosides.

**01/2015:0207**

# SENNA PODS, ALEXANDRIAN

## Sennae fructus acutifoliae

DEFINITION

Dried fruit of *Cassia senna* L. (syn. *Cassia acutifolia* Delile).

*Content*: minimum 3.4 per cent of hydroxyanthracene glycosides, expressed as sennoside B ($C_{42}H_{38}O_{20}$; $M_r$ 863) (dried drug).

IDENTIFICATION

A. Flattened reniform pods, green or greenish-brown with brown patches at the positions corresponding to the seeds, usually 40-50 mm long and at least 20 mm wide. At one end is a stylar point and at the other a short stalk. The pods contain 6-7 flattened and obovate seeds, green or pale brown, with a continuous network of prominent ridges on the testa.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0207.-1): fragments of the epicarp (surface view [B, K]) with polygonal cells, anomocytic [Ba] or paracytic [Bb, Ka] stomata (*2.8.3*) and covering trichomes [Kb] or their scars [Bc]; isolated conical warty covering trichomes, usually bent [C]; fragments of the mesocarp (surface view [D]) with fibres [Da] in 2 crossed layers accompanied by a layer of cells containing calcium oxalate prisms [Db] and sometimes cells of the underlying endocarp [Dc]; fragments of the outer layers of the testa (transverse section [J]) covered by a thick cuticle [Ja] with palisade cells about 50 µm long, with thick walls and a reduced lumen [Jb], accompanied by the hypodermis with pillar-like cells [Jc]; palisade cells of the testa (surface view [N]) and fragments of the hypodermis of the seed forming rings (surface view [A]); fragments of cotyledons (surface view [F], transverse section [E]) consisting of small cells of the epidermis [Ea, Fa] and of the palisade tissue [Eb, Fb]; prisms and cluster crystals of calcium oxalate, free [De, Ga] or included in parenchyma [G]; fragments of vascular bundles [L] with spiral vessels [La] and fibres with moderately thickened and pitted walls [Lb]; sclereids [M, O] and fibres [H] accompanied by crystal sheaths of calcium oxalate [Ha] from fruit stalks.

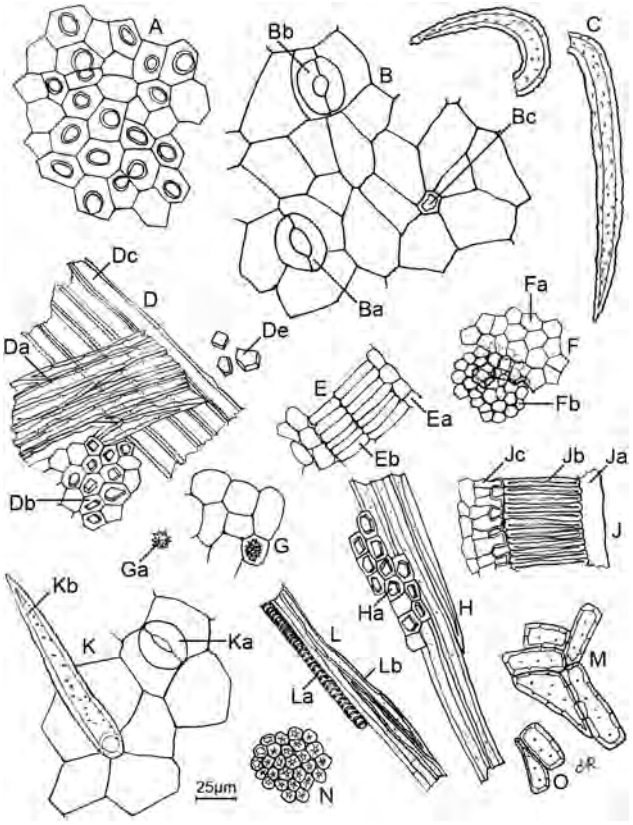


Figure 0207.-1. – *Illustration for identification test B of powdered herbal drug of Alexandrian senna pods*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (180) (*2.9.12*) add 5 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.

*Reference solution.* Dissolve 10 mg of *senna extract CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* (a slight residue remains).

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R*, *propanol R* (1:30:40:40 *V/V/V/V*).

*Application*: 10 µL as bands of 20 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with a 20 per cent *V/V* solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* until the zones appear.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in order of increasing $R_F$ value), colour and size to the principal zones in the chromatogram obtained with the reference solution; between the zones due to sennosides D and C a red zone due to rhein-8-glucoside may be visible; the zones due to sennosides D and C are faint in the chromatogram obtained with the test solution.

D. Place about 25 mg of the powdered herbal drug (180) (*2.9.12*) in a conical flask and add 50 mL of *water R* and 2 mL of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 mL of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulfate R*, evaporate 5 mL to dryness and to the cooled residue add 5 mL of

*dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Place 0.150 g of the powdered herbal drug (180) (*2.9.12*) in a 100 mL flask. Add 30.0 mL of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 mL of the supernatant liquid to a 150 mL separating funnel. Add 0.1 mL of *dilute hydrochloric acid R* and shake with 3 quantities, each of 15 mL, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 mL of the supernatant liquid to a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *ferric chloride solution R1* and mix. Place the flask in a water-bath so that the water level is above that of the liquid in the flask, and heat under a reflux condenser for 20 min. Add 3 mL of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R* previously used to rinse the flask. Combine the 3 ether layers and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the combined ether layers to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 10.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene glycosides, expressed as sennoside B, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of sennoside B to be 240.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

STORAGE

Protected from moisture.

**01/2015:0208**

# SENNA PODS, TINNEVELLY

## Sennae fructus angustifoliae

DEFINITION

Dried fruit of *Cassia angustifolia* Vahl.

*Content*: minimum 2.2 per cent of hydroxyanthracene glycosides, expressed as sennoside B ($C_{42}H_{38}O_{20}$; $M_r$ 863) (dried drug).

IDENTIFICATION

A. Flattened, slightly reniform pods, yellowish-brown or brown with dark brown patches at the positions corresponding to the seeds, usually 35-60 mm long and 14-18 mm wide. At one end is a stylar point and at the other a short stalk. The pods contain 5-8 flattened and obovate seeds, green or pale brown, with incomplete, wavy, transverse ridges on the testa.

B. Microscopic examination (*2.8.23*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0208.-1): fragments of the epicarp (surface view [B, K]) with polygonal cells, anomocytic [Ba] or paracytic [Bb, Ka] stomata (*2.8.3*) and covering trichomes [Kb] or their scars [Bc]; isolated conical warty covering trichomes, usually bent [C]; fragments of the mesocarp (surface view [D]) with fibres [Da] in 2 crossed layers accompanied by a layer of cells containing calcium oxalate prisms [Db] and sometimes cells of the underlying endocarp [Dc]; fragments of the outer layers of the testa (transverse section [J]) covered by a thick cuticle [Ja] with palisade cells about 50 µm long, with thick walls and a reduced lumen [Jb], accompanied by the hypodermis with pillar-like cells [Jc]; palisade cells of the testa (surface view [N]) and fragments of the hypodermis of the seed forming rings (surface view [A]); fragments of cotyledons (surface view [F], transverse section [E]) consisting of small cells of the epidermis [Ea, Fa] and of the palisade tissue [Eb, Fb]; prisms and cluster crystals of calcium oxalate, free [De, Ga] or included in parenchyma [G]; fragments of vascular bundles [L] with spiral vessels [La] and fibres with moderately thickened and pitted walls [Lb]; sclereids [M, O] and fibres [H] accompanied by crystal sheaths of calcium oxalate [Ha] from fruit stalks.

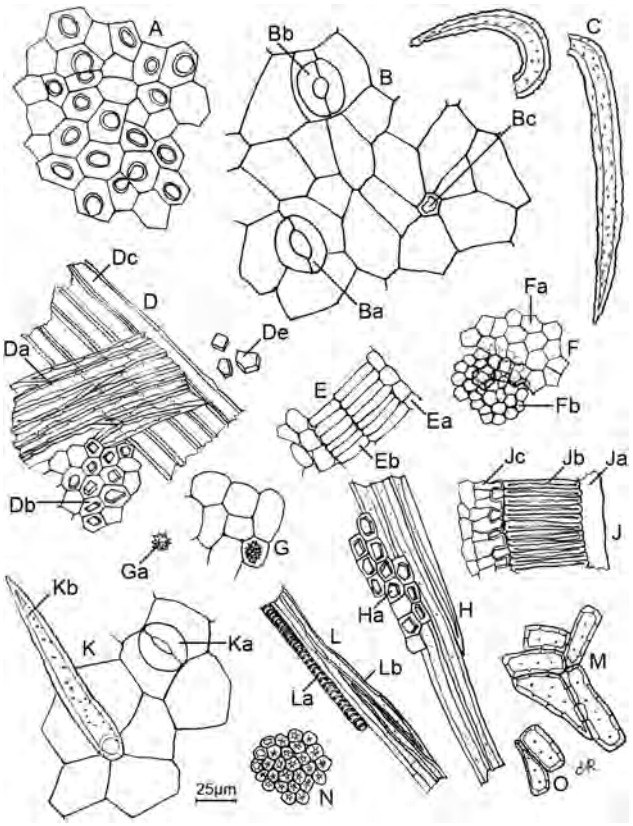


Figure 0208.-1. – *Illustration for identification test B of powdered herbal drug of Tinnevelly senna pods*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (180) (*2.9.12*) add 5 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and heat to boiling. Centrifuge and use the supernatant liquid.

*Reference solution.* Dissolve 10 mg of *senna extract CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* (a slight residue remains).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R*, *propanol R* (1:30:40:40 *V/V/V/V*).

*Application*: 10 µL as bands of 20 mm.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with a 20 per cent *V/V* solution of *nitric acid R* and heat at 120 °C for 10 min. Allow to cool and treat with a 50 g/L solution of *potassium hydroxide R* in *ethanol (50 per cent V/V) R* until the zones appear.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position (sennosides B, A, D and C in order of increasing $R_F$ value), colour and size to the principal zones in the chromatogram obtained with the reference solution; between the zones due to sennosides D and C a red zone due to rhein-8-glucoside may be visible; the zones due to sennosides D and C are faint in the chromatogram obtained with the test solution.

D. Place about 25 mg of the powdered herbal drug (180) (*2.9.12*) in a conical flask and add 50 mL of *water R* and 2 mL of *hydrochloric acid R*. Heat in a water-bath for 15 min, cool and shake with 40 mL of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulfate R*, evaporate 5 mL to dryness and to the cooled residue add 5 mL of *dilute ammonia R1*. A yellow or orange colour develops. Heat on a water-bath for 2 min. A reddish-violet colour develops.

TESTS

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

*Carry out the assay protected from bright light.*

Place 0.150 g of the powdered herbal drug (180) (*2.9.12*) in a 100 mL flask. Add 30.0 mL of *water R*, mix, weigh and place in a water-bath. Heat under a reflux condenser for 15 min. Allow to cool, weigh and adjust to the original mass with *water R*. Centrifuge and transfer 20.0 mL of the supernatant liquid to a 150 mL separating funnel. Add 0.1 mL of *dilute hydrochloric acid R* and shake with 3 quantities, each of 15 mL, of *chloroform R*. Allow to separate and discard the chloroform layer. Add 0.10 g of *sodium hydrogen carbonate R* and shake for 3 min. Centrifuge and transfer 10.0 mL of the supernatant liquid to a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *ferric chloride solution R1* and mix. Place the flask in a water-bath so that the water level is above that of the liquid in the flask, and heat under a reflux condenser for 20 min. Add 3 mL of *hydrochloric acid R* and heat for a further 20 min, with frequent shaking, to dissolve the precipitate. Cool, transfer the mixture to a separating funnel and shake with 3 quantities, each of 25 mL, of *ether R* previously used to rinse the flask. Combine the 3 ether layers and wash with 2 quantities, each of 15 mL, of *water R*. Transfer the combined ether layers to a volumetric flask and dilute to 100.0 mL with *ether R*. Evaporate 10.0 mL carefully to dryness and dissolve the residue in 10.0 mL of a 5 g/L solution of *magnesium acetate R* in *methanol R*. Measure the absorbance (*2.2.25*) at 515 nm using *methanol R* as the compensation liquid.

Calculate the percentage content of hydroxyanthracene glycosides, expressed as sennoside B, using the following expression:

$$\frac{A \times 1.25}{m}$$

i.e. taking the specific absorbance of sennoside B to be 240.

$A$ = absorbance at 515 nm;

$m$ = mass of the substance to be examined, in grams.

STORAGE

Protected from moisture.

**01/2020:2754**

# SERRATULA CORONATA HERB

## Serratulae coronatae herba

DEFINITION

Dried, whole or fragmented aerial part of *Serratula coronata* L. collected during flowering.

*Content*: minimum 5.0 per cent of β-ecdysterone ($C_{27}H_{44}O_7$; $M_r$ 480.6) (dried drug).

IDENTIFICATION

A. The stem is sulcate, erect, glabrous, usually terminally branched, rarely unbranched. Basal leaves are glabrescent, varying from pinnately lobed, or sometimes irregularly pinnatifid, to pinnatisect or pinnate; segments are elliptical to lanceolate, with hairy, irregularly serrate or dentate margins; main veins are sunken on the upper surface and prominent on the lower surface. Cauline leaves are similar to the basal leaves, but are smaller towards the top of the stem. The capitula, 15–25 mm in diameter, are campanulate and arranged as a loose, irregular panicle; they consist of an involucre, a receptacle with paleae up to 10 mm in length and numerous florets. Involucral bracts are yellowish-green tinged with reddish-brown to violet, rigid, acute and distinctly velutinous. Inner bracts are more elongated, sometimes hooked at the apex. Florets are pinkish-violet, tubular and 20–28 mm in length. Achenes, if present, are oblanceolate-ellipsoid and glabrous, sometimes still bearing a pappus of multiseriate, acute, unbranched hairs.

B. Microscopic examination (*2.8.23*). The powder is green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2754.-1): fragments of the upper epidermis of the leaf (surface view [D]), consisting of polygonal cells with slightly sinuous anticlinal walls [Da] and anomocytic stomata (*2.8.3*) with 3-5 subsidiary cells [Db], usually accompanied by palisade parenchyma [Dc]; fragments of the lower epidermis of the leaf (surface view [C]), composed of irregularly shaped cells [Ca] and anomocytic stomata with 3-5 subsidiary cells [Cb] often accompanied by spongy parenchyma cells [Cc]; fragments of the margin of the leaf lamina showing the remains of the bases of numerous uniseriate covering trichomes [B, H] composed of 3-5 cells with thickened walls and a striated cuticle [Ba], occasionally bearing very thin-walled cells [Bb]; fragments of the abaxial epidermis of the bracts of the involucre [A, G] with slightly thickened, elongated cells [Aa] bearing short, unicellular, lignified covering trichomes pointing in the same direction, fusiform in surface view [Ab] and spiny

in transverse section [Ga], and rare biseriate glandular trichomes [Ac]; fragments of the bristles of the calyx [J]; fragments of the style [E] composed of fine irregular cells [Ea], 1 or 2 secretory canals with orange contents [Eb] lying parallel to the annular vessels [Ec]; pollen grains, 60-70 µm in diameter, with 3 pores and a thick, spiny exine [F]; fragments of the ovary with long styloid calcium oxalate crystals and fragments of the corolla containing small prismatic crystals of calcium oxalate may also be present.

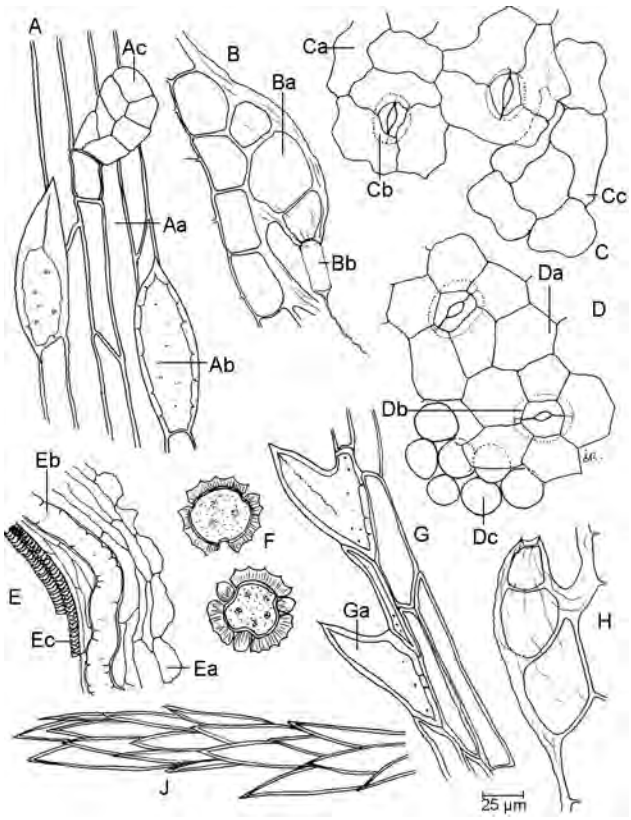


Figure 2754.-1. – *Illustration for identification test B of powdered herbal drug of Serratula coronata herb*

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution.* To 0.25 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*. Sonicate for 10 min, centrifuge and use the supernatant.

*Reference solution (a).* Dissolve 1.5 mg of *rutoside trihydrate R* and 2.5 mg of *isoquercitroside R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 1.5 mg of *chlorogenic acid R* and 1.5 mg of *hyperoside R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Intensity marker*: isoquercitroside.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 2 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 3 min; spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

- the chromatogram shows in the middle third 2 distinct zones, which may be touching; the lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow or orange fluorescence.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint fluorescent zones may be present.

| Top of the plate | |
|---|---|
| | A red zone, very faint to equivalent |
| | A yellow zone, very faint to intense |
| _____ | _____ |
| | 2 greenish-blue zones, very faint to equivalent (possibly overlapping with the yellow or orange zone below) |
| Isoquercitroside: a yellow or orange zone | A yellow or orange zone, very faint to faint |
| | A greenish-blue zone, equivalent |
| | A bluish zone, very faint to faint |
| _____ | _____ |
| Rutoside: a yellow or orange zone | A yellow or orange zone, very faint to faint |
| **Reference solution (a)** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Place 4.0 g of the powdered herbal drug (355) (*2.9.12*) in the cartridge of a continuous extraction apparatus (Soxhlet type). Place 150 mL of *methanol R* and a magnetic stirrer in a 500 mL round-bottomed flask and heat under reflux for 8 h. Transfer the resulting extract to a volumetric flask and dilute to 200.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 10.0 mg of *β-ecdysterone CRS* in *methanol R* using sonication and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 2 mg of *makisterone A R* in reference solution (a) using sonication and dilute to 10 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl diol silica gel for chromatography compatible with highly aqueous mobile phases R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: 0.125 per cent *V/V* solution of *anhydrous formic acid R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 17 | 85 → 72.5 | 15 → 27.5 |
| 17 - 23 | 72.5 → 10 | 27.5 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to β-ecdysterone; use the chromatogram obtained with reference solution (b) to identify the peak due to makisterone A.

*Retention time*: β-ecdysterone = about 11 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to β-ecdysterone and makisterone A.

Calculate the percentage content of β-ecdysterone using the following expression:

$$\frac{A_1 \times m_2 \times p \times 4}{A_2 \times m_1}$$

$A_1$ = area of the peak due to β-ecdysterone in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to β-ecdysterone in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *β-ecdysterone CRS* used to prepare reference solution (a), in grams;

$p$ = assigned percentage content of β-ecdysterone in *β-ecdysterone CRS*.

**01/2015:2639**

# SOPHORA FLOWER

## Sophorae japonicae flos

DEFINITION

Dried, opened flower of *Styphnolobium japonicum* (L.) Schott (syn. *Sophora japonica* L.).

*Content*:

– minimum 8.0 per cent of total flavonoids, expressed as rutoside ($C_{27}H_{30}O_{16}$; $M_r$ 611) (dried drug);

– minimum 6.0 per cent of rutoside ($C_{27}H_{30}O_{16}$; $M_r$ 611) (dried drug).

IDENTIFICATION

A. The opened flower is crumpled, rolled, and has a very thin and short pedicel. The dark green or brown, campanulate calyx is about 3-4 mm long and consists of 5 fused sepals with longitudinal striations at the base, divided at the apex into 5 slightly bilabiate lobes. The pale yellow or light yellowish-brown, papilionaceous type corolla is often broken and measures about 10-15 mm; the upper petal is the largest, subrounded, with a reflexed apex and a bright yellow unguis at its internal base. The other 4 petals are oblong. There are 10 free stamens surrounding a cylindrical and curved central style.

B. Microscopic examination (*2.8.23*). The powder is yellowish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2639.-1): roundish [La] or triangular [Lb] pollen grains [L] with 3 pores and a smooth exine, about 18 µm in diameter; isolated covering trichomes [A, B, F, O] of varying lengths (60-660 µm), slightly flexed, usually consisting of 1 or 2 basal cells and a long pointed distal cell, with smooth or slightly warty walls; fragments of sepals [C] composed of anomocytic stomata (*2.8.3*) with 4-8 subsidiary cells [Ca], covering trichomes [Cb] or their scars [Cc]; fragments of petals [G, H] with cells covered by a finely striated cuticle [Ga, Ha], sometimes accompanied by fine annular or spiral vessels [Hb] and parenchyma with some cells containing crystalline masses of rutoside [Hc]; fragments of parenchyma [E] from the sepals containing prisms of calcium oxalate [Ea] and crystalline masses of rutoside [Eb]; fragments of anthers [N] showing the characteristic fibrous layer (transverse section [Na], surface view [K]) and immature pollen grains [Nb]; free prisms of calcium oxalate [M]. Examine under a microscope using *chloral hydrate solution R*, without heating the preparation: brownish-yellow rutoside crystals are visible, free or included in cells, as crystalline masses [Eb, Hc, J] or in fan-shaped aggregates of very fine needles [D, Ec, Gb].

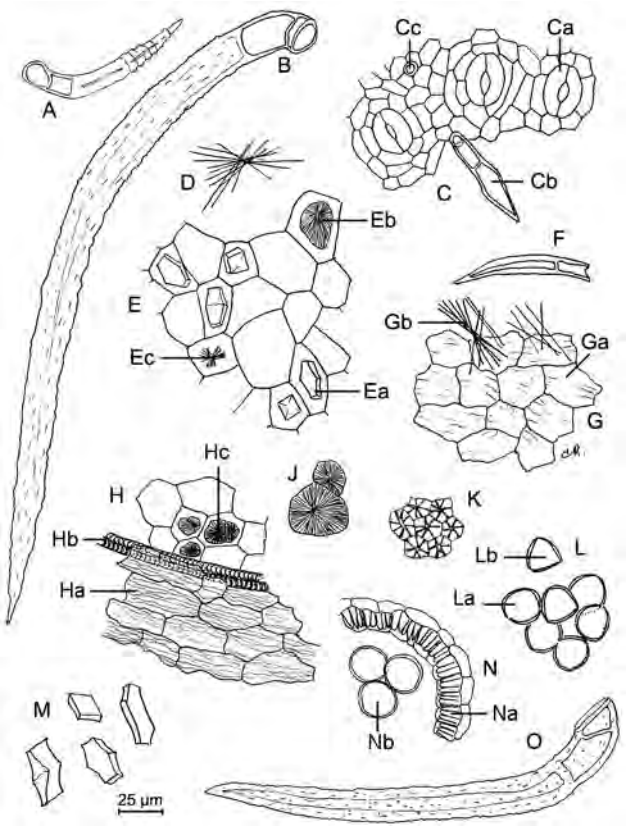


Figure 2639.-1. – *Illustration for identification test B of powdered herbal drug of sophora flower*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*, sonicate for 10 min and filter.

*Reference solution.* Dissolve 10 mg of *hyperoside R* and 10 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*, allow to dry in air for about 30 min, and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An orange-yellow zone |
| | A brown zone |
| Hyperoside: a yellowish-orange zone | |
| | 2 green zones |
| Rutoside: an orange-yellow zone | A very intense orange-yellow zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of flower buds and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

**Total flavonoids**

*Stock solution*. Place 2.000 g of the powdered herbal drug (355) (*2.9.12*) in the cartridge of a continuous-extraction apparatus (Soxhlet type). Add 100 mL of *heptane R* and heat under a reflux condenser until the extraction liquid is colourless. Allow to cool and discard the heptane. Add 90 mL of *methanol R* and continue the extraction with heating under a reflux condenser until the extraction liquid is colourless. Allow to cool. Transfer the methanolic solution to a 100 mL volumetric flask. Rinse the extraction flask with a few millilitres of *methanol R*. Combine the methanolic solutions and dilute to 100.0 mL with *methanol R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R* and shake vigorously.

*Test solution*. Dilute 10.0 mL of the stock solution to 100.0 mL with a 20 g/L solution of *aluminium chloride R* in *methanol R*.

*Compensation solution*. Dilute 10.0 mL of the stock solution to 100.0 mL with *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 15 min by comparison with the compensation solution at 425 nm.

Calculate the percentage content of total flavonoids, expressed as rutoside, using the following expression:

$$\frac{A \times 1000}{m \times 37}$$

i.e. taking the specific absorbance of rutoside to be 370.

$A$ = absorbance of the test solution at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**Rutoside**. Liquid chromatography (*2.2.29*).

*Test solution*. Place 0.500 g of the powdered herbal drug (355) (*2.9.12*) in a conical flask and add 50.0 mL of *methanol R*. Weigh, sonicate for 30 min and allow to cool. Weigh and compensate for the loss of solvent with *methanol R*. Shake vigorously, filter, and dilute 2.0 mL of the filtrate to 10.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 10.0 mg of *rutoside trihydrate CRS* in 2 mL of *methanol R* and dilute to 10.0 mL with a 50 per cent *V/V* solution of *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Reference solution (b)*. Dissolve 10.0 mg of *apigenin 7-glucoside R* and 10.0 mg of *rutoside trihydrate R* in 30 mL of *methanol R* and dilute to 50.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 1 per cent *V/V* solution of *glacial acetic acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 68 | 32 |
| 5 - 20 | 68 → 50 | 32 → 50 |
| 20 - 30 | 50 → 0 | 50 → 100 |
| 30 - 35 | 0 | 100 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 350 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rutoside (retention time = about 17 min): apigenin 7-glucoside = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to rutoside and apigenin 7-glucoside.

Calculate the percentage content of rutoside using the following expression:

$$\frac{A_1 \times m_2 \times p \times 5}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rutoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rutoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rutoside trihydrate CRS* used to prepare reference solution (a), in grams;

$p$ = assigned percentage content of rutoside in *rutoside trihydrate CRS*.

**01/2015:2427**

# SOPHORA FLOWER-BUD

## Sophorae japonicae flos immaturus

DEFINITION

Whole, dried flower bud of *Styphnolobium japonicum* (L.) Schott (syn. *Sophora japonica* L.).

*Content*:

– minimum 20.0 per cent of total flavonoids, expressed as rutoside ($C_{27}H_{30}O_{16}$; $M_r$ 611) (dried drug);

– minimum 15.0 per cent of rutoside ($C_{27}H_{30}O_{16}$; $M_r$ 611) (dried drug).

IDENTIFICATION

A. The flat flower bud, ovoid or ellipsoid, has a very thin and short pedicel and is about 7-10 mm long and 3-4 mm thick. The dark green or brown calyx, forming the lower part of the bud, is about 3-4 mm long and consists of 5 fused sepals with longitudinal striations at the base. The pale yellow or brownish-yellow corolla, unopened, delicate, extends beyond the calyx and contains 10 free stamens surrounding a central style.

B. Microscopic examination (*2.8.23*). The powder is pale yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2427.-1): roundish [La] or triangular [Lb] pollen grains [L] with 3 pores and a smooth exine, about 18 µm in diameter; isolated covering trichomes [A, B, F, O] of varying lengths (60-660 µm), slightly flexed, usually consisting of 1 or 2 basal cells and a long pointed distal cell, with smooth or slightly warty walls; fragments of sepals [C] composed of anomocytic stomata (*2.8.3*) with 4-8 subsidiary cells [Ca], covering trichomes [Cb] or their scars [Cc]; fragments of petals [G, H] with cells covered by a finely striated cuticle [Ga, Ha], sometimes accompanied by fine annular or spiral vessels [Hb] and parenchyma with some cells containing crystalline masses of rutoside [Hc]; fragments of parenchyma [E] from the sepals containing prisms of calcium oxalate [Ea] and crystalline masses of rutoside [Eb]; fragments of anthers [N] showing the characteristic fibrous layer (transverse section [Na], surface view [K]) and immature pollen grains [Nb]; free prisms of calcium oxalate [M]. Examine under a microscope using *chloral hydrate solution R*, without heating the preparation: brownish-yellow rutoside crystals are visible, free or included in cells, as crystalline masses [Eb, Hc, J] or in fan-shaped aggregates of very fine needles [D, Ec, Gb].

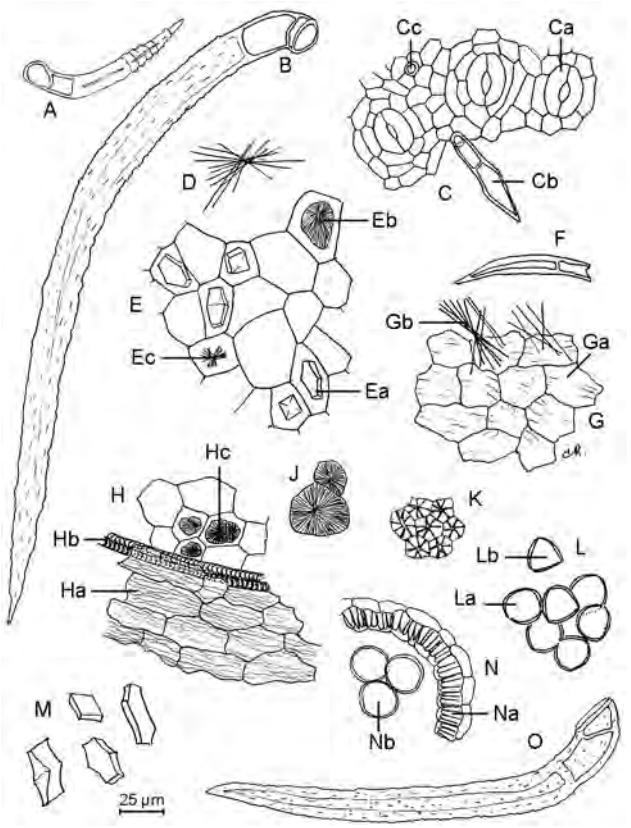


Figure 2427.-1. – *Illustration for identification test B of powdered herbal drug of sophora flower-bud*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.2 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*, sonicate for 10 min and filter.

*Reference solution.* Dissolve 10 mg of *hyperoside R* and 10 mg of *rutoside trihydrate R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 10 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*, allow to dry in air for about 30 min, and examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An orange-yellow zone |
| ——— | ——— |
| | A brown zone |
| Hyperoside: a yellowish-orange zone | |
| ——— | ——— |
| | 2 green zones |
| Rutoside: an orange-yellow zone | A very intense orange-yellow zone (rutoside) |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of opened flowers and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 9.0 per cent.

ASSAY

**Total flavonoids**

*Stock solution*. Place 1.00 g of the powdered herbal drug (355) (*2.9.12*) in the cartridge of a continuous-extraction apparatus (Soxhlet type). Add 100 mL of *heptane R* and heat under a reflux condenser until the extraction liquid is colourless. Allow to cool and discard the heptane. Add 90 mL of *methanol R* and continue the extraction with heating under a reflux condenser until the extraction liquid is colourless. Allow to cool. Transfer the methanolic solution to a 100 mL volumetric flask. Rinse the extraction flask with a few millilitres of *methanol R*. Combine the methanolic solutions and dilute to 100.0 mL with *methanol R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R* and shake vigorously.

*Test solution*. Dilute 10.0 mL of the stock solution to 100.0 mL with a 20 g/L solution of *aluminium chloride R* in *methanol R*.

*Compensation solution*. Dilute 10.0 mL of the stock solution to 100.0 mL with *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution after 15 min by comparison with the compensation solution at 425 nm.

Calculate the percentage content of total flavonoids, expressed as rutoside, using the following expression:

$$\frac{A \times 1000}{m \times 37}$$

i.e. taking the specific absorbance of rutoside to be 370.

$A$ = absorbance of the test solution at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**Rutoside**. Liquid chromatography (*2.2.29*).

*Test solution*. Place 0.200 g of the powdered herbal drug (355) (*2.9.12*) in a conical flask and add 50.0 mL of *methanol R*. Weigh, sonicate for 30 min and allow to cool. Weigh and compensate for the loss of solvent with *methanol R*. Shake vigorously, filter, and dilute 2.0 mL of the filtrate to 10.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 10.0 mg of *rutoside trihydrate CRS* in 2 mL of *methanol R* and dilute to 10.0 mL with a 50 per cent *V/V* solution of *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Reference solution (b)*. Dissolve 10.0 mg of *apigenin 7-glucoside R* and 10.0 mg of *rutoside trihydrate R* in 30 mL of *methanol R* and dilute to 50.0 mL with a 50 per cent *V/V* solution of *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1 per cent *V/V* solution of *glacial acetic acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 68 | 32 |
| 5 - 20 | 68 → 50 | 32 → 50 |
| 20 - 30 | 50 → 0 | 50 → 100 |
| 30 - 35 | 0 | 100 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 350 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rutoside (retention time = about 17 min): apigenin 7-glucoside = about 1.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to rutoside and apigenin 7-glucoside.

Calculate the percentage content of rutoside using the following expression:

$$\frac{A_1 \times m_2 \times p \times 5}{A_2 \times m_1}$$

$A_1$ = area of the peak due to rutoside in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to rutoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *rutoside trihydrate CRS* used to prepare reference solution (a), in grams;

$p$ = assigned percentage content of rutoside in *rutoside trihydrate CRS*.

**07/2008:1849**
**corrected 7.0**

# SPANISH SAGE OIL

## Salviae lavandulifoliae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the aerial parts of *Salvia lavandulifolia* Vahl, collected at the flowering stage.

CHARACTERS

*Appearance*: clear, colourless or pale yellow, mobile liquid.

Camphor-like odour.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.1 mL of the essential oil to be examined in 10 mL of *toluene R*.

*Reference solution*. Dissolve 20 µL of *thujone R* and 30 µL of *cineole R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 6 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 105 °C for 10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A blue zone |
| _____ | _____ |
| Thujone: 2 pinkish-violet zones | |
| Cineole: a blue zone | A blue zone (cineole) |
| _____ | _____ |
| | 3 blue zones |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.907 to 0.932.

**Refractive index** (*2.2.6*): 1.465 to 1.473.

**Optical rotation** (*2.2.7*): + 7° to + 17°.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.00 g.

**Solubility in alcohol** (*2.8.10*): 1 volume is soluble in 2 volumes and more of *ethanol (80 per cent V/V) R*.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.200 g of the essential oil to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.200 g of *Spanish sage oil for peak identification CRS* in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 µL of *limonene R* in *heptane R* and dilute to 50.0 mL with the same solvent. Dilute 0.5 mL of this solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 43 | 60 → 232 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *Spanish sage oil for peak identification CRS*;
- *resolution*: minimum 1.5 between the peaks due to limonene and 1,8-cineole and minimum 1.5 between the peaks due to α-terpinyl acetate and borneol.

Use the chromatogram supplied with *Spanish sage oil for peak identification CRS* and the chromatogram obtained with reference solution (a) to locate the peaks due to α-pinene, sabinene, limonene, 1,8-cineole, thujone, camphor, linalol, linalyl acetate, terpinen-4-ol, sabinyl acetate, α-terpinyl acetate and borneol.

Determine the percentage content of each of these components. The percentages are within the following ranges:

- *α-pinene*: 4.0 per cent to 11.0 per cent;
- *sabinene*: 0.1 per cent to 3.5 per cent;
- *limonene*: 2.0 per cent to 6.5 per cent;
- *1,8-cineole*: 10.0 per cent to 30.5 per cent;
- *thujone*: maximum 0.5 per cent;
- *camphor*: 11.0 per cent to 36.0 per cent;
- *linalol*: 0.3 per cent to 4.0 per cent;
- *linalyl acetate*: maximum 5.0 per cent;
- *terpinen-4-ol*: maximum 2.0 per cent;
- *sabinyl acetate*: 0.5 per cent to 9.0 per cent;
- *α-terpinyl acetate*: 0.5 per cent to 9.0 per cent;
- *borneol*: 1.0 per cent to 7.0 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

STORAGE

At a temperature not exceeding 25 °C.

**07/2018:2419**

# SPIKE LAVENDER OIL

## Spicae aetheroleum

DEFINITION

Essential oil obtained by steam distillation of the flowering tops of *Lavandula latifolia* Medik.

CHARACTERS

*Appearance*: clear, mobile, light yellow or greenish-yellow liquid.

*Odour*: reminiscent of cineole and camphor.

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A*.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 20 µL of the essential oil to be examined to 1 mL with *toluene R*.

*Reference solution.* Dilute 10 µL of *cineole R*, 5 µL of *linalol R* and 5 µL of *linalyl acetate R* to 1 mL with *toluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 6 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100 °C for 5 min; examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet zone |
| _____ | _____ |
| Linalyl acetate: a violet zone | A faint violet or brown zone may be present (linalyl acetate)<br>A pink zone |
| _____ | _____ |
| 1,8-Cineole: a violet or brown zone | A violet or brown zone (1,8-cineole) |
| Linalol: a violet zone | An intense violet zone (linalol)<br>A greyish or brownish zone |
| **Reference solution** | **Test solution** |

B. The essential oil to be examined complies with the limits of the test for chromatographic profile.

TESTS

**Relative density** (*2.2.5*): 0.894 to 0.907.

**Refractive index** (*2.2.6*): 1.461 to 1.468.

**Optical rotation** (*2.2.7*): − 7° to + 2°.

**Acid value** (*2.5.1*): maximum 1.5, determined on 5.00 g of the essential oil to be examined.

**Solubility in alcohol** (*2.8.10*): 1.0 mL of the essential oil to be examined is soluble, sometimes with opalescence, in 3.0 mL of *ethanol (70 per cent V/V) R*.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 200 µL of the essential oil to be examined to 10.0 mL with *heptane R*.

*Reference solution (a).* Dilute 200 µL of *spike lavender oil CRS* to 10.0 mL with *heptane R*.

*Reference solution (b).* Dilute 5 µL of *limonene R* to 50.0 mL with *heptane R*. Dilute 0.5 mL of this solution to 5.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 70 |
| | 15 - 70 | 70 → 180 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: limonene, 1,8-cineole, camphor, linalol, linalyl acetate, α-terpineol, *trans*-α-bisabolene.

*Identification of peaks*: use the chromatogram supplied with *spike lavender oil CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to limonene, 1,8-cineole, camphor, linalol, linalyl acetate, α-terpineol and *trans*-α-bisabolene.

*System suitability*: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *spike lavender oil CRS*;
- *resolution*: minimum 1.5 between the peaks due to limonene and 1,8-cineole.

Determine the percentage content of each of the following components. The percentages are within the following ranges:

- *limonene*: 0.5 per cent to 3.0 per cent;
- *1,8-cineole*: 16.0 per cent to 39.0 per cent;
- *camphor*: 8.0 per cent to 16.0 per cent;
- *linalol*: 34.0 per cent to 50.0 per cent;
- *linalyl acetate*: maximum 1.6 per cent;
- *α-terpineol*: 0.2 per cent to 2.0 per cent;
- *trans-α-bisabolene*: 0.4 per cent to 2.5 per cent;
- *reporting threshold*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

STORAGE

At a temperature not exceeding 25 °C.

**01/2017:1438**

# ST. JOHN'S WORT

## Hyperici herba

DEFINITION

Whole or fragmented, dried flowering tops of *Hypericum perforatum* L., harvested during flowering time.

*Content*: minimum 0.08 per cent of total hypericins, expressed as hypericin ($C_{30}H_{16}O_8$; $M_r$ 504.4) (dried drug).

Herbal drugs

IDENTIFICATION

A. The branched and bare stem shows 2 more or less prominent longitudinal ridges. The leaves are opposite, sessile, exstipulate, oblong-oval and 15-30 mm long; present on the leaf margins are glands which appear as black dots and over all the surface of the leaves many small, strongly translucent excretory glands which are visible in transmitted light. The flowers are regular and form corymbose clusters at the apex of the stem. They have 5 green, acute sepals, with black secretory glands on the margins; 5 orange-yellow petals, also with black secretory glands on the margins; 3 staminal blades, each divided into many orange-yellow stamens and 3 carpels surmounted by red styles.

The drug may also show the following: immature and ripe fruits and seeds. Immature fruits are green or yellowish, seeds are whitish. Occasional ripe fruits may be present; these are dry trilocular capsules containing numerous seeds, brown, broad or small-ovate, 5-10 mm long, with broad linear or punctiform glands, irregularly striated ducts, conducting secretions. Ripe seeds are 1-1.3 mm long, cylindrical or trigonous, shortly pointed at both ends, brown or almost black, minutely pitted longitudinally.

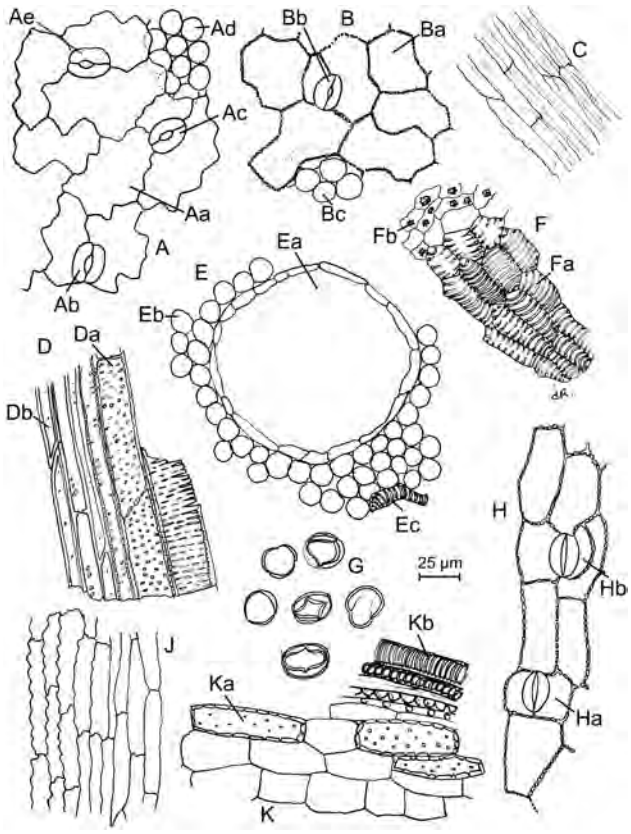


Figure 1438.-1. – *Illustration for identification test B of powdered herbal drug of St. John's wort*

B. Microscopic examination (*2.8.23*). The powder is greenish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1438.-1): fragments of the leaf epidermis [A, B] or stems [H] with paracytic [Ab, Ha], anisocytic [Ac, Bb, Hb] or anomocytic [Ae] stomata (*2.8.3*); fragments of the leaf epidermis often accompanied by palisade parenchyma [Ad, Bc]; polygonal cells of the upper epidermis with thickened and beaded walls [Ba]; more or less sinuous, thin-walled cells of the lower epidermis [Aa]; fragments of the leaf and sepal [E] with large, red-pigmented oil glands [Ea] associated with palisade parenchyma [Eb] and small vessels [Ec]; elongated cells of fragments of the petal epidermis with straight or wavy anticlinal walls [J]; vessels [D] with reticulate or pitted walls [Da] and groups of thick-walled fibres [Db]; fragments of the central parenchyma of the stems [K] with lignified and pitted rectangular cells [Ka] sometimes associated with vessels [Kb]; fragments of the anthers [F] showing the central part consisting of small cells containing cluster crystals of calcium oxalate [Fb] and cells from the fibrous layer [Fa]; fragments of the staminal filament with elongated, thin-walled cells with a striated cuticle [C]; numerous pollen grains with 3 germinal pores and a smooth exine, occurring singly [G] or in dense groups.

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R*. Sonicate for 15 min, then filter or centrifuge the solution and use the filtrate or supernatant.

*Reference solution (a)*. Dissolve 2.5 mg of *hyperoside R* and 3.5 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 2.5 mg of *hyperoside R* and 3 mg of *chlorogenic acid R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Intensity marker*: hyperoside.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:9:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 5 min. Spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*. Allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 distinct zones in the lower third which may, however, be partially overlapping. The lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow or orange fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint fluorescent zones, which may be blue, red or orange-yellow, may be present, especially above the red zones due to hypericin and pseudohypericin; the light blue fluorescent zone due to chlorogenic acid may be overlapped by the yellow or orange fluorescent zone due to hyperoside.

| Top of the plate | |
|---|---|
| | A red fluorescent zone or a faint red fluorescent zone |
| _____ | _____ |
| | 2 red fluorescent zones (hypericin and pseudohypericin) |
| | 2 yellow or orange fluorescent zones or 2 faint yellow or orange fluorescent zones |
| _____ | _____ |
| | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone |
| Hyperoside: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone or an intense yellow or orange fluorescent zone (hyperoside) |
| | A light blue fluorescent zone or a faint light blue fluorescent zone (chlorogenic acid) |
| Rutoside: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone (rutoside) |
| **Reference solution (a)** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of stems with a diameter greater than 5 mm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (500) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

*Test solution.* In a 100 mL round-bottomed flask, introduce 0.800 g of the powdered herbal drug (500) (*2.9.12*), 60 mL of a mixture of 20 volumes of *water R* and 80 volumes of *tetrahydrofuran R* and a magnetic stirrer. Boil the mixture in a water-bath at 70 °C under a reflux condenser for 30 min. Centrifuge (2 min at 700 *g*) and decant the supernatant into a 250 mL flask. Take up the residue with 60 mL of a mixture of 20 volumes of *water R* and 80 volumes of *tetrahydrofuran R*. Heat again under a reflux condenser for 30 min. Centrifuge (2 min at 700 *g*) and decant the supernatant. Combine the extracts and evaporate to dryness. Take up the residue with 15 mL of *methanol R* using ultrasound and transfer to a 25 mL measuring flask. Rinse the 250 mL flask with *methanol R* and dilute to 25.0 mL with the same solvent. Centrifuge again, filter 10 mL through a syringe filter (0.2 µm). Discard the first 2 millilitres of the filtrate. Introduce 5.0 mL of the filtrate into a measuring flask and dilute to 25.0 mL with *methanol R*.

*Compensation liquid*: *methanol R*.

Measure the absorbance (*2.2.25*) of the test solution at 590 nm, by comparison with the compensation liquid.

Calculate the percentage content of total hypericins, expressed as hypericin, using the following expression:

$$\frac{A \times 125}{m \times 870}$$

i.e. taking the specific absorbance of hypericin to be 870.

$A$ = absorbance at 590 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2017:1874**

# ST. JOHN'S WORT DRY EXTRACT, QUANTIFIED

## Hyperici herbae extractum siccum quantificatum

DEFINITION

Quantified dry extract obtained from *St. John's wort (1438)*.

*Content*:

- *total hypericins, expressed as hypericin* ($C_{30}H_{16}O_8$; $M_r$ 504.4): 0.10 per cent to 0.30 per cent (anhydrous extract);
- *flavonoids, expressed as rutoside* ($C_{27}H_{30}O_{16}$; $M_r$ 610.5): minimum 6.0 per cent (anhydrous extract);
- *hyperforin* ($C_{35}H_{52}O_4$; $M_r$ 536.8): maximum 6.0 per cent (anhydrous extract) and not more than the content stated on the label.

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (50-80 per cent *V/V*) or methanol (50-80 per cent *V/V*).

CHARACTERS

*Appearance*: brownish-grey powder.

IDENTIFICATION

High-performance thin-layer chromatography (*2.8.25*).

*Test solution.* To 0.1 g of the extract to be examined add 5.0 mL of *methanol R*. Sonicate for 15 min, then filter or centrifuge the solution and use the filtrate or supernatant.

*Reference solution (a).* Dissolve 2.5 mg of *hyperoside R* and 3.5 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 2.5 mg of *hyperoside R* and 3 mg of *chlorogenic acid R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Intensity marker*: hyperoside.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:9:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: 70 mm from the lower edge of the plate.

*Drying*: in a current of air at room temperature for 5 min.

*Detection*: heat at 100-105 °C for 5 min. Spray the warm plate with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*, then with a 50 g/L solution of *macrogol 400 R* in *methanol R* or, alternatively, dip the warm plate in a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then in a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*. Allow the plate to dry in air for about 1 min and examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

- the chromatogram shows 2 distinct zones in the lower third which may, however, be partially overlapping. The lower zone (chlorogenic acid) shows a light blue fluorescence and the upper zone (hyperoside) shows a yellow or orange fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint fluorescent

zones, which may be blue, red or orange-yellow, may be present, especially above the red zones due to hypericin and pseudohypericin; the light blue fluorescent zone due to chlorogenic acid may be overlapped by the yellow or orange fluorescent zone due to hyperoside.

| Top of the plate | |
|---|---|
| | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone |
| ——— | ——— |
| | 2 red fluorescent zones (hypericin and pseudohypericin) |
| | 2 yellow or orange fluorescent zones or 2 faint yellow or orange fluorescent zones |
| ——— | ——— |
| | A yellow or orange fluorescent zone or a faint yellow or orange fluorescent zone |
| Hyperoside: a yellow or orange fluorescent zone | An intense yellow or orange fluorescent zone (hyperoside) |
| | A light blue fluorescent zone or a faint light blue fluorescent zone (chlorogenic acid) |
| Rutoside: a yellow or orange fluorescent zone | A yellow or orange fluorescent zone (rutoside) |
| **Reference solution (a)** | **Test solution** |

TESTS

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

**Total hypericins**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 70.0 mg of the extract to be examined in 25.0 mL of *methanol R*. Sonicate and centrifuge the solution. Expose the solution to a xenon lamp at about 765 W/m$^2$ for 8 min.

*Reference solution.* Dissolve a quantity of *St. John's wort dry extract HRS* corresponding to 0.15 mg of hypericin in 25.0 mL of *methanol R*. Sonicate and centrifuge. Expose the solution to a xenon lamp at about 765 W/m$^2$ for 8 min.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 39 volumes of *ethyl acetate R*, 41 volumes of a 15.6 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 2 with *phosphoric acid R* and 160 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 590 nm.

*Injection*: 20 µL.

*Run time*: 15 min.

*Identification of peaks*: use the chromatogram supplied with *St. John's wort dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to pseudohypericin and hypericin.

*System suitability*: reference solution:

– the chromatogram obtained is similar to the chromatogram supplied with *St. John's wort dry extract HRS*;
– *resolution*: minimum 2 between the peaks due to pseudohypericin and hypericin.

Calculate the percentage content of total hypericins, expressed as hypericin, using the following expression:

$$\frac{(A_1 + A_2) \times m_2 \times p}{A_3 \times m_1}$$

$A_1$ = area of the peak due to pseudohypericin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hypericin in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to hypericin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *St. John's wort dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of hypericin in *St. John's wort dry extract HRS*.

**Hyperforin and flavonoids**. Liquid chromatography (*2.2.29*). *Carry out the assay protected from light.*

*Solvent mixture*: *water R*, *methanol R* (20:80 *V/V*).

*Test solution.* Dissolve 75.0 mg of the extract to be examined in 20.0 mL of the solvent mixture. Sonicate and centrifuge.

*Reference solution (a).* Dissolve 20.0 mg of *rutoside trihydrate CRS* in 200.0 mL of the solvent mixture.

*Reference solution (b).* Dissolve 75.0 mg of *St. John's wort dry extract HRS* in 20.0 mL of the solvent mixture. Sonicate and centrifuge.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *water R* (3:1000 *V/V*);
– *mobile phase B*: *phosphoric acid R*, *acetonitrile R* (3:1000 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 8 | 82 | 18 | 0.8 |
| 8 - 18 | 82 → 47 | 18 → 53 | 0.8 |
| 18 - 18.1 | 47 → 3 | 53 → 97 | 0.8 |
| 18.1 - 19 | 3 | 97 | 0.8 → 1.2 |
| 19 - 31 | 3 | 97 | 1.2 |

*Detection*: spectrophotometer at 360 nm, then at 275 nm after the elution of biapigenin (about 22 min).

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *St. John's wort dry extract HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to rutoside, hyperoside, isoquercitroside, quercitrin, quercetin, biapigenin, hyperforin and adhyperforin.

*System suitability*: reference solution (b):

– the chromatogram obtained is similar to the chromatogram supplied with *St. John's wort dry extract HRS*;
– *resolution*: minimum 2.0 between the peaks due to rutoside and hyperoside, and minimum 2.0 between the peaks due to hyperforin and adhyperforin.

Calculate the percentage content of hyperforin using the following expression:

$$\frac{A_4 \times m_4 \times p \times 2.3}{A_5 \times m_3 \times 10}$$

$A_4$ = area of the peak due to hyperforin in the chromatogram obtained with the test solution;

$A_5$ = area of the peak due to rutoside in the chromatogram obtained with reference solution (a);

$m_3$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_4$ = mass of *rutoside trihydrate CRS* used to prepare reference solution (a), in grams;

2.3 = correction factor for hyperforin with respect to rutoside;

$p$ = percentage content of rutoside in *rutoside trihydrate CRS*.

Calculate the percentage content of flavonoids, expressed as rutoside, using the following expression:

$$\frac{m_4 \times p \times (A_6 + A_7 + A_8 + A_9 + A_{10} + A_{11})}{m_3 \times A_5 \times 10}$$

$A_5$ = area of the peak due to rutoside in the chromatogram obtained with reference solution (a);

$A_6$ = area of the peak due to rutoside in the chromatogram obtained with the test solution;

$A_7$ = area of the peak due to hyperoside in the chromatogram obtained with the test solution;

$A_8$ = area of the peak due to isoquercitroside in the chromatogram obtained with the test solution;

$A_9$ = area of the peak due to quercitrin in the chromatogram obtained with the test solution;

$A_{10}$ = area of the peak due to quercetin in the chromatogram obtained with the test solution;

$A_{11}$ = area of the peak due to biapigenin in the chromatogram obtained with the test solution;

$m_3$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_4$ = mass of *rutoside trihydrate CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of rutoside in *rutoside trihydrate CRS*.

LABELLING

The label states the content of hyperforin.

**01/2008:1153**
**corrected 10.0**

# STAR ANISE

## Anisi stellati fructus

DEFINITION

Dried composite fruit of *Illicium verum* Hook.f.

*Content*:

- minimum 70 mL/kg of essential oil (anhydrous drug),
- minimum 86.0 per cent of *trans*-anethole in the essential oil.

CHARACTERS

The fruit carpels are brown.

Odour of anethole.

IDENTIFICATION

A. The fruit generally consists of 8 developed, one-seeded follicles, each 12-22 mm long and 6-12 mm high, radially arranged around a short, central, blunt-ending columella. In some fruits 1 or 2 follicles may be missing, but their position is clearly visible. Each follicle is boat-shaped or boot-shaped, with a greyish-brown dorsal surface showing rough markings and lateral surfaces bearing scars from the neighbouring follicles. One or more follicles are split open along the ventral suture, exposing a single, lenticular, shiny, reddish-brown seed about 8 mm in diameter. The markings on the dorsal surface are not visible from the ventral surface. Some of the follicles (1-3) may be imperfectly developed. Isolated follicles, pedicels and seeds may be present.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: brown epicarpal cells, polygonal in surface view, with a strongly striated cuticle and occasional anomocytic stomata (*2.8.3*); fragments of the endocarp with long palisade-like cells; fragments of the mesocarp with large parenchymatous cells, vessels, oil-containing cells and groups of stone cells; fragments of the seed testa with palisade-like, sclerified, strongly pitted, yellow cells up to 200 µm long; fragments of the columella and the fruit stalk with strongly and irregularly thickened, star-shaped stone cells about 400 µm long and 150 µm wide; rhomboidal or rectangular crystals of calcium oxalate.

C. Examine the chromatograms obtained in test B for *Illicium anisatum* (= *I. religiosum*) and certain other *Illicium* spp.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other weaker zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Caffeic acid: a light blue fluorescent zone | |
| Quercitrin: a brownish-yellow fluorescent zone | |
| | A brownish-yellow fluorescent zone |
| _____ | _____ |
| | A greenish fluorescent zone |
| Hyperoside: a brownish-yellow fluorescent zone | A brownish-yellow fluorescent zone |
| Chlorogenic acid: a light blue fluorescent zone | |
| | A green fluorescent zone |
| Rutoside: a brownish-yellow fluorescent zone | A brownish-yellow fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

***Illicium anisatum* (= *I. religiosum*) and certain other *Illicium* spp.**

A. Adulteration with *Illicium anisatum* or certain other *Illicium* spp. is indicated by the presence of fruits mainly consisting of more than 8 follicles; fruits either smaller than 2.5 cm or greater than 3.5 cm; follicles with the suture edged with a thickening extending to the neighbouring follicle, or with dorsal markings visible from the ventral surface; follicles somewhat undulate and ending in a fine beak or a

Herbal drugs

small, ventrally turned hook; follicles with a profile fitting into a rectangle; pedicels more than 5 cm long; seedless fruits; seeds either very flat or almost spherical.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R* and heat under a reflux condenser in a water-bath at 60 °C for 5 min. Allow to cool and filter.

*Reference solution.* Dissolve 1 mg of *caffeic acid R*, 1 mg of *chlorogenic acid R*, 2.5 mg of *quercitrin R*, 2.5 mg of *rutoside trihydrate R* and 2.5 mg of *hyperoside R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:26:100 *V/V/V/V*).

*Application*: 5 µL as bands.

*Development*: over a path of 6 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then with a 50 g/L solution of *macrogol 400 R* in *methanol R*; after 30 min, examine in ultraviolet light at 365 nm.

*Results*: the chromatogram obtained with the test solution shows no brownish-yellow fluorescent zone at or above the position of the zone due to quercitrin in the chromatogram obtained with the reference solution. No yellow fluorescent zone is seen at or above the position of the zone due to caffeic acid in the chromatogram obtained with the reference solution. No brownish-yellow fluorescent zone is seen directly above the zone due to hyperoside in the chromatogram obtained with the reference solution.

**Water** (*2.2.13*): maximum 100 mL/kg, determined by distillation on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 4.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use a 250 mL round-bottomed flask and 100 mL of *water R* as the distillation liquid. Immediately before the determination, reduce 50.0 g of the drug to a powder (1400) (*2.9.12*) and mix. Further reduce about 10.0 g of this mixture to a finer powder (710) (*2.9.12*). Use 2.50 g of the powder for the determination. Introduce 0.50 mL of *xylene R* into the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

***trans*-Anethole**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute the mixture of essential oil and *xylene R* obtained in the assay of essential oil to 5.0 mL with *xylene R* by rinsing the apparatus.

*Reference solution.* To 1.0 mL of *xylene R* add 20 µL of *estragole R*, 20 mg of *α-terpineol R* and 60 µL of *anethole R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.25 mm;

– *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 60 |
| | 5 - 80 | 60 → 210 |
| | 80 - 95 | 210 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the preparation of the reference solution.

*System suitability*: reference solution:

– *resolution*: minimum 5 between the peaks due to estragole and α-terpineol.

Use the retention times from the chromatogram obtained with the reference solution locate the components of the reference solution in the chromatogram obtained with the test solution.

Calculate the percentage content of *trans*-anethole. Disregard any peak due to the solvent or with an area less than 0.05 per cent of the area of the principal peak in the chromatogram obtained with the test solution.

**01/2008:2108**
**corrected 7.0**

# STAR ANISE OIL

## Anisi stellati aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the dry ripe fruits of *Illicium verum* Hook.f.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1 g of the substance to be examined in *toluene R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 µL of *linalol R*, 30 µL of *anisaldehyde R* and 200 µL of *anethole R* and in *toluene R* and dilute to 15 mL with the same solvent. Dilute 1 mL of this solution to 5 mL with *toluene R*.

*Plate*: *TLC silica gel* $F_{254}$ *plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (7:93 *V/V*).

*Application*: 5 µL as bands of 10 mm (for normal TLC plates) or 2 µL as bands of 10 mm (for fine particle TLC plates).

*Development*: over a path of 15 cm (for normal TLC plates) or over a path of 6 cm (for fine particle size plates).

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A quenching zone, partly separated |
| Anethole: a quenching zone | A very strong quenching zone (anethole) |
| Anisaldehyde: a quenching zone | A quenching zone (anisaldehyde) |
| **Reference solution** | **Test solution** |

*Detection B*: spray with *methyl 4-acetylbenzoate reagent R* and heat at 100-105 °C for 10 min; examine the still hot plate in daylight within 10 min.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A violet-brown zone, not fully separated |
| Anethole: a brown zone | A very strong brown zone (anethole) |
| Anisaldehyde: a yellow zone | A yellow zone (anisaldehyde) |
| Linalol: a grey zone | A grey zone (linalol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.979 to 0.985.

**Refractive index** (*2.2.6*): 1.553 to 1.556.

**Freezing point** (*2.2.18*): 15 °C to 19 °C.

**Fenchone**. Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

*Test solution.* Dissolve 400 µL of the substance to be examined in 2.0 mL of *hexane R*.

*Reference solution (a).* Dilute 10 µL of *fenchone R* to 1.2 g with *hexane R*.

*Reference solution (b).* Dilute 100 µL of reference solution (a) to 100 mL with *hexane R*.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limit*:

– *fenchone*: maximum 0.01 per cent.

**Pseudoisoeugenyl 2-methylbutyrate**. Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

*Test solution.* The substance to be examined.

*Reference solution (a).* Dilute 10 mg of the test solution to 1.000 g with *hexane R*. Dilute 0.5 mL of this solution to 100 mL with *hexane R*.

*Reference solution (b). Pseudoisoeugenyl 2-methylbutyrate for peak identification CRS.*

*System suitability*:

– the chromatogram obtained with reference solution (b) is similar to the chromatogram provided with *pseudoisoeugenyl 2-methylbutyrate for peak identification CRS.*

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

*Limit*: locate the peak due to pseudoisoeugenyl 2-methylbutyrate by comparison with the chromatogram provided with *pseudoisoeugenyl 2-methylbutyrate for peak identification CRS.*

– *pseudoisoeugenyl 2-methylbutyrate*: maximum 0.01 per cent.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test for fatty oils and resinified essential oils.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 200 µL of the substance to be examined in 1.0 mL of *hexane R*.

*Reference solution.* To 1.0 mL of *hexane R*, add 20 µL of *linalol R*, 20 µL of *estragole R*, 20 µL of *α-terpineol R*, 60 µL of *anethole R* and 30 µL of *anisaldehyde R*.

*Column*:

– *material*: fused silica,

– *size*: *l* = 30 m, Ø = 0.25 mm,

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 60 |
| | 5 - 80 | 60 → 210 |
| | 80 - 95 | 210 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of the reference solution; record the retention times of these substances.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to estragole and α-terpineol.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution and locate *cis*-anethole and foeniculin using the chromatogram shown in Figure 2108.-1 (disregard any peak due to hexane).

Determine the percentage content of these components. The percentages are within the following ranges:

– *linalol*: 0.2 per cent to 2.5 per cent,

– *estragole*: 0.5 per cent to 6.0 per cent,

– *α-terpineol*: maximum 0.3 per cent,

– *cis-anethole*: 0.1 per cent to 0.5 per cent,

– *trans-anethole*: 86 per cent to 93 per cent,

– *anisaldehyde*: 0.1 per cent to 0.5 per cent,

– *foeniculin*: 0.1 per cent to 3.0 per cent.

STORAGE

At a temperature not exceeding 25 °C.

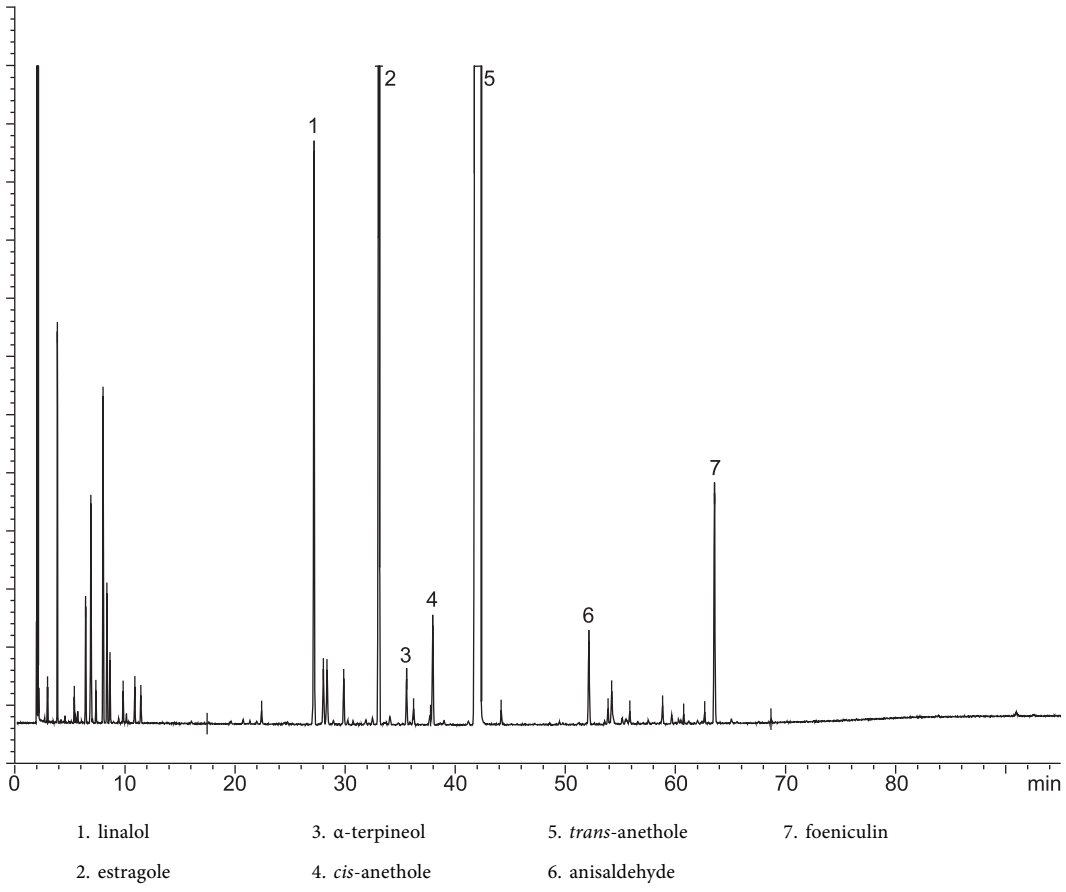


Figure 2108.-1. – *Chromatogram for the test for chromatographic profile of star anise oil*

**01/2012:0246**
**corrected 9.2**

# STRAMONIUM LEAF

## Stramonii folium

DEFINITION

Dried leaf or dried leaf and flowering, and occasionally fruit-bearing, tops of *Datura stramonium* L. and its varieties.

*Content*: minimum 0.25 per cent of total alkaloids, expressed as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4) (dried drug). The alkaloids consist mainly of hyoscyamine with varying proportions of hyoscine (scopolamine).

CHARACTERS

Unpleasant odour.

IDENTIFICATION

A. The leaves are dark brownish-green or dark greyish-green with a short petiole, often much twisted and shrunken during drying, thin and brittle, ovate or triangular-ovate, dentately lobed with an acuminate apex and often unequal at the base. Young leaves are pubescent on the veins, older leaves are nearly glabrous. Stems are green or purplish-green, slender, curved and twisted, wrinkled longitudinally and sometimes wrinkled transversely, branched dichasially, with a single flower or an immature fruit in the fork. Flowers, on short pedicels, have a gamosepalous calyx with 5 lobes and trumpet-shaped brownish-white or purplish corolla. The fruit is a capsule, usually covered with numerous short, stiff emergences; seeds are brown or black with a minutely pitted testa.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0246.-1): fragments of upper [A] and lower [C] epidermises of the lamina, in surface view, showing cells with slightly wavy anticlinal walls and a smooth cuticle accompanied by palisade [Aa] and spongy [Ca] parenchyma; anisocytic [Ac, Cb] and anomocytic [Ab] stomata (*2.8.3*), more frequent on the lower epidermis; fragments of covering trichomes, conical [E], uniseriate with 3-5 cells with warty walls, some of them collapsed [Ea]; glandular trichomes, short and clavate (side view [B]) with heads formed by 2-7 cells; dorsiventral mesophyll (transverse section [F]), with a single layer of palisade cells [Fa] and a spongy parenchyma [Fb] containing cluster crystals of calcium oxalate [Fc]; fragments of spongy parenchyma [D] with some cells containing small cluster crystals of calcium oxalate [Db], associated with annularly and spirally thickened vessels [Da], in surface view. The powdered herbal drug may also show: fibres and reticulately thickened vessels from the stems; subspherical pollen grains about 60-80 µm in diameter with 3 germinal pores and a nearly smooth exine [G]; fragments of the corolla [H] with wavy-walled cells [Ha] and underlying mesophyll [Hb] with some cells containing prisms [Hc] or cluster crystals [Hd] of calcium oxalate; seed fragments containing yellowish-brown, sinuous, thick-walled sclereids of the testa [J], and occasional prisms and microsphenoidal crystals of calcium oxalate.

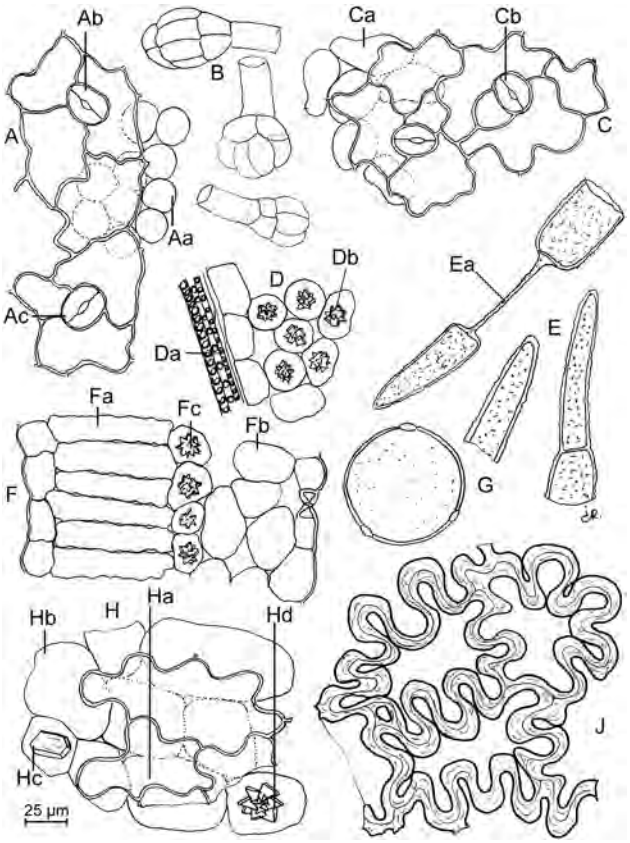


Figure 0246.-1. – *Illustration for identification test B of powdered herbal drug of stramonium leaf*

C. Examine the chromatograms obtained in the chromatography test.

*Results*: the principal zones in the chromatograms obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.

D. Shake 1 g of the powdered herbal drug (180) (*2.9.12*) with 10 mL of *dilute sulfuric acid R1* for 2 min. Filter and add to the filtrate 1 mL of *concentrated ammonia R* and 5 mL of *water R*. Shake cautiously with 15 mL of *peroxide-free ether R*, avoiding the formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulfate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 mL of *nitric acid R* and evaporate to dryness on a water-bath. Add 10 mL of *acetone R* and, dropwise, a 30 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. A deep violet colour develops.

TESTS

**Chromatography**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (180) (*2.9.12*) add 10 mL of *dilute sulfuric acid R1*, shake for 15 min and filter. Wash the filter with *dilute sulfuric acid R1* until 25 mL of filtrate is obtained. To the filtrate add 1 mL of *concentrated ammonia R* and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution*. Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 3.8 mL of the hyoscyamine sulfate solution and 4.2 mL of the hyoscine hydrobromide solution and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 10 µL and 20 µL of each solution, as bands of 20 mm by 3 mm, leaving 1 cm between the bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min; allow to cool.

*Detection A*: spray with *potassium iodobismuthate solution R2*, using about 10 mL for a plate 200 mm square, until the orange or brown zones become visible against a yellow background.

*Results A*: the zones in the chromatograms obtained with the test solution are similar in position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatograms) and colour to those in the chromatograms obtained with the reference solution. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µL of the test solution or near the point of application in the chromatogram obtained with 10 µL of the test solution.

*Detection B*: spray with *sodium nitrite solution R* until the coating is transparent; examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the reference solution and the test solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

**Foreign matter** (*2.8.2*): maximum 3 per cent of stems with a diameter greater than 5 mm.

**Total ash** (*2.4.16*): maximum 20.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

a) Determine the loss on drying (*2.2.32*) on 2.000 g of the powdered herbal drug (180) (*2.9.12*) by drying in an oven at 105 °C.

b) Moisten 10.0 g of the powdered herbal drug (180) (*2.9.12*) with a mixture of 5 mL of *ammonia R*, 10 mL of *ethanol (96 per cent) R* and 30 mL of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in *0.25 M sulfuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 mL by distilling on a water-bath and transfer it to a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than 3 quantities, each of 20 mL, of *0.25 M sulfuric acid*, separate the 2 layers by centrifugation if necessary and transfer the acid layers to a 2nd separating funnel. Make the acid layer alkaline with *ammonia R* and shake with 3 quantities, each of 30 mL, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the anhydrous sodium sulfate with 3 quantities, each of 10 mL, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 mL of *0.01 M sulfuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88 \times (20 - n)}{(100 - d) \times m}$$

$d$ = loss on drying, as a percentage;

$n$ = volume of *0.02 M sodium hydroxide*, in millilitres;

$m$ = mass of the powdered herbal drug, in grams.

STORAGE

Protected from moisture.

**01/2008:0247**
**corrected 10.0**

# STRAMONIUM, PREPARED

## Stramonii pulvis normatus

DEFINITION

Stramonium leaf powder (180) (*2.9.12*) adjusted, if necessary, by the addition of powdered lactose or stramonium leaf of lower content of total alkaloids.

*Content*: 0.23 per cent to 0.27 per cent of total alkaloids, expressed as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4) (dried drug).

CHARACTERS

*Appearance*: greyish-green powder.

Unpleasant odour.

IDENTIFICATION

A. Microscopic examination (*2.8.23*) of the powder (180) (*2.9.12*). Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of leaf lamina showing epidermal cells with slightly wavy anticlinal walls and smooth cuticle; stomata are more frequent on the lower epidermis (anisocytic and anomocytic) (*2.8.3*); covering trichomes are conical, uniseriate with 3-5 cells and warty walls; glandular trichomes are short and clavate with heads formed by 2-7 cells; dorsiventral mesophyll, with a single layer of palisade cells and a spongy parenchyma containing cluster crystals of calcium oxalate; annularly and spirally thickened vessels. The powdered herbal drug may also show the following diagnostic characters: fibres and reticulately thickened vessels from the stems; subspherical pollen grains usually about 60-80 µm in diameter with 3 germinal pores and nearly smooth exine; fragments of the corolla with papillose epidermis; seed fragments containing yellowish-brown, sinuous, thick-walled sclereids of testa; occasional prisms and microsphenoidal crystals of calcium oxalate. Examined in *glycerol (85 per cent) R*, it may be seen to contain lactose crystals.

B. Examine the chromatograms obtained in the Chromatography test.

*Results*: the principal zones in the chromatogram obtained with the test solution are similar in position, colour and size to the principal zones in the chromatogram obtained with the same volume of the reference solution.

C. Shake 1 g with 10 mL of *dilute sulfuric acid R1* for 2 min. Filter and add to the filtrate 1 mL of *concentrated ammonia R* and 5 mL of *water R*. Shake cautiously with 15 mL of *peroxide-free ether R*, avoiding the formation of an emulsion. Separate the ether layer and dry over *anhydrous sodium sulfate R*. Filter and evaporate the ether in a porcelain dish. Add 0.5 mL of *nitric acid R* and evaporate to dryness on a water-bath. Add 10 mL of *acetone R* and, dropwise, a 30 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. A deep violet colour develops.

TESTS

**Chromatography**. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the drug to be examined add 10 mL of *dilute sulfuric acid R1*, shake for 15 min and filter. Wash the filter with *dilute sulfuric acid R1* until 25 mL of filtrate is obtained. To the filtrate add 1 mL of *concentrated ammonia R* and shake with 2 quantities, each of 10 mL, of *peroxide-free ether R*. If necessary, separate by centrifugation. Dry the combined ether layers over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *methanol R*.

*Reference solution.* Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R*. Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R*. Mix 3.8 mL of the hyoscyamine sulfate solution and 4.2 mL of the hyoscine hydrobromide solution and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 10 µL and 20 µL of each solution as bands of 20 mm by 3 mm, leaving 1 cm between the bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min and allow to cool.

*Detection A*: spray with *potassium iodobismuthate solution R2*, using about 10 mL for a plate 200 mm square, until the orange or brown zones become visible against a yellow background.

*Results A*: the zones in the chromatograms obtained with the test solution are similar in position (hyoscyamine in the lower third, hyoscine in the upper third of the chromatogram) and colour to those in the chromatograms obtained with the reference solution. The zones in the chromatograms obtained with the test solution are at least equal in size to the corresponding zones in the chromatogram obtained with the same volume of the reference solution. Faint secondary zones may appear, particularly in the middle of the chromatogram obtained with 20 µL of the test solution or near the point of application in the chromatogram obtained with 10 µL of the test solution.

*Detection B*: spray with *sodium nitrite solution R* until the coating is transparent; examine after 15 min.

*Results B*: the zones due to hyoscyamine in the chromatograms obtained with the test solution and the reference solution change from brown to reddish-brown but not to greyish-blue (atropine) and any secondary zones disappear.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 20.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

ASSAY

a) Determine the loss on drying (*2.2.32*) on 2.000 g by drying in an oven at 105 °C.

b) Moisten 10.0 g with a mixture of 5 mL of *ammonia R*, 10 mL of *ethanol (96 per cent) R* and 30 mL of *peroxide-free ether R* and mix thoroughly. Transfer the mixture to a suitable percolator, if necessary with the aid of the extracting mixture. Allow to macerate for 4 h and percolate with a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the liquid flowing from the percolator, dissolve the residue in *0.25 M sulfuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Concentrate the percolate to about 50 mL by distilling on a water-bath and transfer it to

a separating funnel, rinsing with *peroxide-free ether R*. Add a quantity of *peroxide-free ether R* equal to at least 2.1 times the volume of the percolate to produce a liquid of a density well below that of water. Shake the solution with no fewer than 3 quantities, each of 20 mL, of *0.25 M sulfuric acid*, separate the 2 layers by centrifugation if necessary and transfer the acid layers to a 2nd separating funnel. Make the acid layer alkaline with *ammonia R* and shake with 3 quantities, each of 30 mL, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the sodium sulfate with 3 quantities, each of 10 mL, of *chloroform R*. Add the washings to the chloroform extract, evaporate to dryness on a water-bath and heat in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 20.0 mL of *0.01 M sulfuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with *0.02 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content of total alkaloids, expressed as hyoscyamine, using the following expression:

$$\frac{57.88(20 - n)}{(100 - d)m}$$

$d$ = loss on drying, as a percentage;

$n$ = volume of *0.02 M sodium hydroxide*, in millilitres;

$m$ = mass of the herbal drug, in grams.

STORAGE

In an airtight container.

**07/2019:1811**
**corrected 10.0**

# SWEET ORANGE OIL

## Aurantii dulcis aetheroleum

DEFINITION

Essential oil obtained without heating, by suitable mechanical treatment from the fresh peel of the fruit of *Citrus* × *sinensis* (L.) Osbeck. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, pale yellow or orange, mobile liquid, which may become cloudy when chilled.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

Examine the chromatograms obtained in the test for bergapten.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Bergapten: a greenish-yellow fluorescent zone | |
| _____ | _____ |
| | Many blue fluorescent zones |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| | A brown fluorescent zone |
| Linalyl acetate: a brownish-orange fluorescent zone | A faint brownish-orange fluorescent zone (linalyl acetate) |
| _____ | _____ |
| | Many orange fluorescent zones |
| Linalol: a brownish-orange fluorescent zone | A brownish-orange fluorescent zone (linalol) |
| Bergapten: a faint greenish-yellow fluorescent zone | |
| | Many brownish-orange fluorescent zones |
| _____ | _____ |
| | Many blue fluorescent zones |
| **Reference solution** | **Test solution** |

B. It complies with the limits of the test for chromatographic profile.

TESTS

**Relative density** (*2.2.5*): 0.842 to 0.850.

**Refractive index** (*2.2.6*): 1.470 to 1.476.

**Optical rotation** (*2.2.7*): + 94° to + 99°.

**Peroxide value** (*2.5.5, Method B*): maximum 20.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test.

**Bergapten**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 0.2 mL of the substance to be examined in 1 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 2 mg of *bergapten R*, 10 µL of *linalol R* and 20 µL of *linalyl acetate R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (15:85 *V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution shows no greenish-yellow fluorescent zone corresponding to that of bergapten in the chromatogram obtained with the reference solution.

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min; examine the plate in ultraviolet light at 365 nm.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dilute 200 µL of the substance to be examined to 10.0 mL with *heptane R*.

*Reference solution (a)*. Dilute 5 µL of *α-pinene R*, 5 µL of *sabinene R*, 5 µL of *β-pinene R*, 5 µL of *β-myrcene R*, 5 µL of *octanal R*, 70 µL of *limonene R*, 5 µL of *linalol R*, 5 µL of *decanal R*, 10 µL of *citral R* (composed of neral and geranial) and 5 µL of *valencene R* to 5.0 mL with *heptane R*.

*Reference solution (b)*. Dilute 5 µL of *limonene R* to 50.0 mL with *heptane R*. Dilute 0.1 mL of the solution to 5.0 mL with *heptane R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 60 m, Ø = 0.25 mm;

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– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.4 mL/min.

*Split ratio*: 1:70.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 90 | 50 → 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*Identification of peaks*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to sabinene and β-pinene.

Determine the percentage content of each of these components. The percentages are within the following ranges:

– *α-pinene*: 0.4 per cent to 0.6 per cent;

– *sabinene*: 0.2 per cent to 1.1 per cent;

– *β-pinene*: 0.02 per cent to 0.3 per cent;

– *β-myrcene*: 1.5 per cent to 2.5 per cent;

– *octanal*: 0.1 per cent to 0.4 per cent;

– *limonene*: 92.0 per cent to 97.0 per cent;

– *linalol*: 0.2 per cent to 0.7 per cent;

– *decanal*: 0.1 per cent to 0.4 per cent;

– *neral*: 0.02 per cent to 0.10 per cent;

– *geranial*: 0.03 per cent to 0.20 per cent;

– *valencene*: 0.02 per cent to 0.5 per cent.

*Reporting threshold*: 0.01 per cent (reference solution (b)).

**Residue on evaporation**: 1.0 per cent to 5.0 per cent.

Evaporate 5.0 g to dryness on a water-bath and dry at 100-105 °C for 4 h.

STORAGE

At a temperature not exceeding 25 °C.

**04/2018:2634**

# SZECHWAN LOVAGE RHIZOME

## Ligustici chuanxiong rhizoma

DEFINITION

Whole or fragmented rhizome, gently baked to dryness, of *Ligusticum sinense* Oliv. 'Chuanxiong' (*Conioselinum anthriscoides* (H.Boissieu) Pimenov & Kljuykov, syn. *Ligusticum chuanxiong* Hort. ex S.H.Qiu, Y.Q.Zeng, K.Y.Pan, Y.C.Tang & J.M.Xu), with rootlets removed, collected in summer when the nodes of the stem become obviously swollen and purplish.

*Content*: minimum 3.5 mL/kg of essential oil.

IDENTIFICATION

A. *Whole drug.* The whole rhizome is irregularly knotty and fist-like, 2-7 cm in diameter. Externally, greyish-brown to yellowish-brown, rough and shrunken, with many nodular stem bases on the upper part of the rhizome, each with parallel and raised annulations and concave, subrounded stem scars. Numerous tuberculous rootlet scars are visible on the lower part and at the nodes.

*Fragmented drug.* The fragmented rhizome occurs as thick slices or irregular pieces. The slices have a conspicuously irregular outline. The cut surface is whitish-yellow to brownish-yellow. The texture is compact, difficult to break; the fracture shows a yellowish-white or greyish-yellow medulla, scattered with yellowish-brown oil dots. The cambium occurs as an undulate ring.

B. Microscopic examination (*2.8.23*). The powder is pale yellow to brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2634.-1): brownish-yellow cork fragments, showing polyhedral cells with slightly sinuous walls (surface view [A]) or rectangular cells (transverse section [E]); very numerous fragments of parenchyma [B, G] with slightly thickened cells; cluster crystals of calcium oxalate (10-25 µm in diameter), isolated [F] or included in the parenchyma cells [G], more or less numerous depending on the sample; reticulate or scalariform spiral vessels (14-50 µm in diameter), isolated or included in the parenchyma with thin-walled cells [D]; secretory canals, usually broken with thin-walled secretory cells containing droplets of oil, are sometimes visible (longitudinal section [C], transverse section [H]); very rarely fusiform fibres with thickened walls. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows very numerous starch granules, isolated [Ba] or included in parenchyma cells [B]; the granules are simple, rounded, ovoid, oblong or reniform, 5-16 µm in diameter and up to 22 µm long; the punctiform, slit- or V-shaped hilum may be visible. Occasional 2- to 4-compound starch granules may be present.

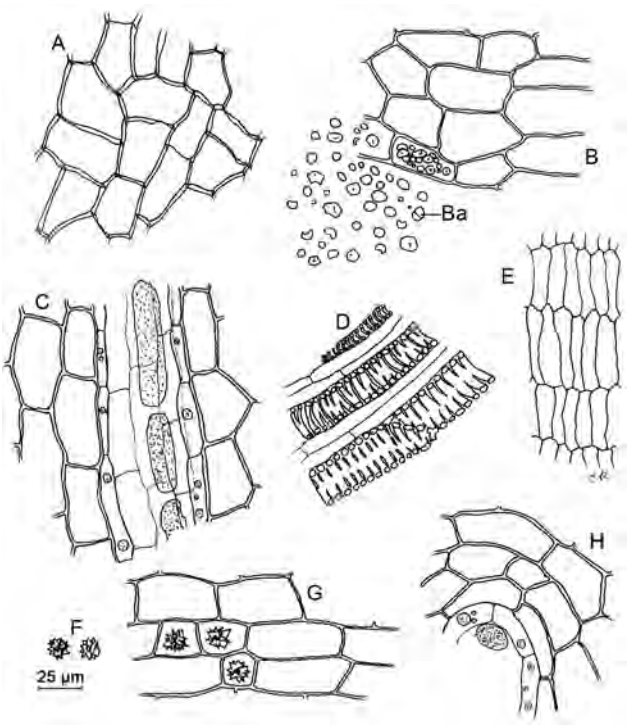


Figure 2634.-1. – *Illustration for identification test B of powdered herbal drug of szechwan lovage rhizome*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R* and mix for 10 min. Centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *imperatorin R* and 1 mg of *osthole R* in 1.0 mL of *methanol R.*

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (1:10:90 *V/V/V*).

*Application*: 4 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| | A bluish fluorescent zone |
| | A weak quenching zone |
| Osthole: a blue fluorescent zone | 2 quenching zones |
| Imperatorin: a quenching zone | |
| _____ | _____ |
| | 2 quenching zones |
| **Reference solution** | **Test solution** |

*Detection B*: treat with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 120 °C for 3 min and examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A reddish zone |
| _____ | _____ |
| | A weak yellowish zone (*Z*-ligustilide) |
| | 2 pink zones |
| Osthole: a violet zone | |
| Imperatorin: a yellow zone | A brownish zone |
| _____ | _____ |
| | A brownish zone |
| | A reddish-violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 25.0 g of the freshly crushed herbal drug, a 500 mL round-bottomed flask, 200 mL of *water R* as the distillation liquid and 0.50 mL of *trimethylpentane R* in the graduated tube. Distil at a rate of 3-3.5 mL/min for 3 h.

**01/2008:1837**
**corrected 7.0**

# TEA TREE OIL

## Melaleucae aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the foliage and terminal branchlets of *Melaleuca alternifolia* (Maiden and Betch) Cheel, *M. linariifolia* Smith, *M. dissitiflora* F. Mueller and/or other species of *Melaleuca*.

CHARACTERS

*Appearance*: clear, mobile, colourless or pale yellow liquid. Characteristic odour.

IDENTIFICATION

*First identification: B.*

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*Second identification*: *A*.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.1 mL of the substance to be examined in 5 mL of *heptane R*.

*Reference solution*. Dissolve 30 µL of *cineole R*, 60 µL of *terpinen-4-ol R* and 10 mg of *α-terpineol R* in 10 mL of *heptane R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *heptane R* (20:80 *V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*. Heat at 100-105 °C for 5-10 min while observing. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Cineole: a violet-brown zone | A violet-brown zone, less intense (cineole) |
| Terpinen-4-ol: a brownish-violet zone | A brownish-violet zone terpinen-4-ol) |
| α-terpineol: a violet or brownish-violet zone | A violet or brownish-violet zone (α-terpineol) |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.885 to 0.906.

**Refractive index** (*2.2.6*): 1.475 to 1.482.

**Optical rotation** (*2.2.7*): + 5° to + 15°.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.15 mL of the substance to be examined in 10 mL of *hexane R*.

*Reference solution*. Dissolve 5 µL of *α-pinene R*, 5 µL of *sabinene R*, 15 µL of *α-terpinene R*, 5 µL of *limonene R*, 5 µL of *cineole R*, 30 µL of *γ-terpinene R*, 5 µL of *p-cymene R*, 5 µL of *terpinolene R*, 60 µL of *terpinen-4-ol R*, 5 µL of *aromadendrene R* and 5 mg of *α-terpineol R* in 10 mL of *hexane R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m (a film thickness of 1 µm may be used) to 60 m (a film thickness of 0.2 µm may be used), Ø = 0.25-0.53 mm,
- *stationary phase*: *macrogol 20 000 R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 50 |
| | 1 - 37 | 50 → 230 |
| | 37 - 45 | 230 |
| Injection port | | 240 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of the reference solution. Record the retention times of these substances.

*System suitability*: reference solution:

- *resolution*: minimum 2.7 between the peaks due to terpinen-4-ol and aromadendrene.

Using the retention times determined from the chromatogram obtained with the reference solution, locate the components of the reference solution in the chromatogram obtained with the test solution. Disregard the peak due to hexane.

Determine the percentage content of these components. The percentages are within the following ranges:

- *α-pinene*: 1.0 per cent to 6.0 per cent,
- *sabinene*: maximum 3.5 per cent,
- *α-terpinene*: 5.0 per cent to 13.0 per cent,
- *limonene*: 0.5 per cent to 4.0 per cent,
- *cineole*: maximum 15.0 per cent,
- *γ-terpinene*: 10.0 per cent to 28.0 per cent,
- *p-cymene*: 0.5 per cent to 12.0 per cent,
- *terpinolene*: 1.5 per cent to 5.0 per cent,
- *terpinen-4-ol*: minimum 30.0 per cent,
- *aromadendrene*: maximum 7.0 per cent,
- *α-terpineol*: 1.5 per cent to 8.0 per cent.

STORAGE

At a temperature not exceeding 25 °C.

**04/2019:2483**

# THOMSON KUDZUVINE ROOT

## Puerariae thomsonii radix

DEFINITION

Whole or fragmented, dried root of *Pueraria montana* (Lour.) Merr. var. *thomsonii* (Benth.) M.R.Almeida (syn. *Pueraria thomsonii* (Benth.)), with the outer bark removed.

*Content*: minimum 0.4 per cent of total isoflavonoids, expressed as puerarin ($C_{21}H_{20}O_9$; $M_r$ 416.4) (dried drug), of which minimum 55 per cent consists of puerarin.

IDENTIFICATION

A. Cylindrical, subfusiform or semi-cylindrical, 12-15 cm long and 4-8 cm in diameter, sometimes in longitudinally or obliquely cut thick slices, varying in size. Externally yellowish-white or pale brown. The root is heavy, texture hard and starchy. A transverse section shows pale brown concentric rings formed by fibres. A longitudinal section shows several longitudinal striations formed by fibres.

B. Microscopic examination (*2.8.23*). The powder is yellowish-white. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2483.-1): thick-walled

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lignified fibres, which occur in bundles, surrounded by a calcium oxalate prism sheath [A]; rare sclereids, subrounded or elliptical, about 50 µm in diameter [E]; fragments of xylem [F] consisting of relatively large vessels with hexagonal or elliptical bordered-pits, arranged very densely [Fa] and xylem parenchyma cells with slightly thickened and pitted walls [Fb], fragments of parenchyma [C]; isolated prism crystals [B]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules [D], simple or 2-20 compound; the individual granules, 15-30 µm in diameter, are spheroidal, semi-rounded or polygonal with a punctiform, slit-shaped or stellate hilum.

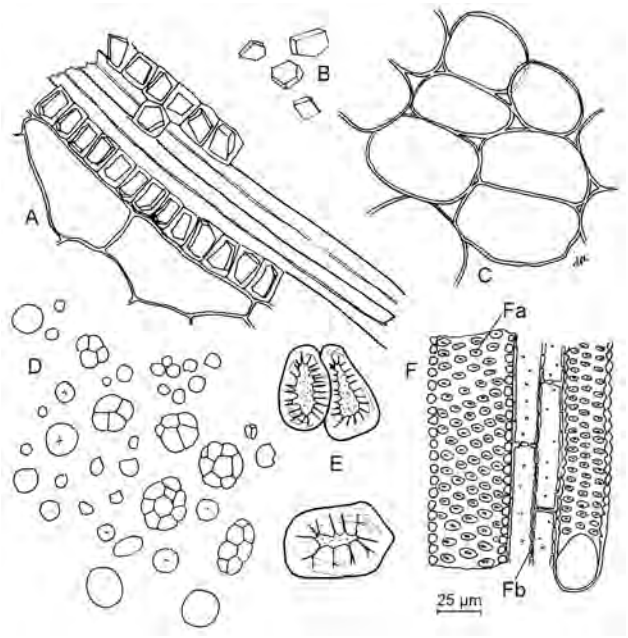


Figure 2483.-1 – *Illustration for identification test B of powdered herbal drug of Thomson kudzuvine root*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Sonicate 0.5 g of the powdered herbal drug (355) (*2.9.12*) with 5 mL of *methanol R*, then centrifuge; use the supernatant.

*Reference solution.* Dissolve 5 mg of *daidzin R* and 5 mg of *puerarin R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *methylene chloride R*, *methanol R*, *ethyl acetate R* (10:20:22:40 *V/V/V/V*); use the lower layer.

*Application*: 7 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A weak quenching zone |
| _____ | _____ |
| Daidzin: a quenching zone | A weak quenching zone |
| Puerarin: a quenching zone | A weak quenching zone |
| _____ | _____ |
| | Several quenching zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 1.00 g of the powdered herbal drug (355) (*2.9.12*) into a 250 mL conical flask, add 50.0 mL of *ethanol (30 per cent V/V) R* and weigh. Heat under a reflux condenser for 30 min. Allow to cool and weigh again. Adjust to the initial mass with *ethanol (30 per cent V/V) R*, mix well and filter.

*Reference solution.* Introduce an amount of *kudzuvine root dry extract HRS* corresponding to 3.0 mg of puerarin into a 250 mL conical flask, add 50.0 mL of *ethanol (30 per cent V/V) R* and weigh. Heat under a reflux condenser for 30 min. Allow to cool and weigh again. Adjust to the initial mass with *ethanol (30 per cent V/V) R*, mix well and filter.

*Column*: 2 columns coupled in series:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*.

*Mobile phase*:

- *mobile phase A*: *glacial acetic acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16.5 | 90 → 71 | 10 → 29 |

*Flow rate*: 3.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *kudzuvine root dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to the isoflavonoids (puerarin, 3-methoxypuerarin, 6-*O*″-D-xylosylpuerarin and daidzin).

*Relative retention* with reference to puerarin (retention time = about 3.4 min): 6-*O*″-D-xylosylpuerarin = about 1.15; daidzin = about 1.4.

*System suitability*: reference solution:

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to 3-methoxypuerarin and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to puerarin.

Herbal drugs

Calculate the percentage content of puerarin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = area of the peak due to puerarin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to puerarin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *kudzuvine root dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of puerarin in *kudzuvine root dry extract HRS*.

Calculate the percentage content of total isoflavonoids (puerarin, 6-*O*″-D-xylosylpuerarin and daidzin) using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1}$$

$A_1$ = sum of the areas of the peaks due to the isoflavonoids (puerarin, 6-*O*″-D-xylosylpuerarin and daidzin) in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to puerarin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *kudzuvine root dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of puerarin in *kudzuvine root dry extract HRS*.

**07/2014:0865**

# THYME

## Thymi herba

DEFINITION

Whole leaves and flowers separated from the previously dried stems of *Thymus vulgaris* L. or *Thymus zygis* L. or a mixture of both species.

*Content*:

– *essential oil*: minimum 12 mL/kg (anhydrous drug);

– *sum of the contents of thymol and carvacrol* (both $C_{10}H_{14}O$; $M_r$ 150.2): minimum 40 per cent in the essential oil.

CHARACTERS

Strong odour reminiscent of thymol.

IDENTIFICATION

A. The leaf of *Thymus vulgaris* is usually 4-12 mm long and up to 3 mm wide, sessile or with a very short petiole. The lamina is tough, entire, lanceolate or ovate, covered on both surfaces by a grey or greenish-grey indumentum; the edges are markedly rolled up towards the abaxial surface. The midrib is depressed on the adaxial surface and is very prominent on the abaxial surface. The calyx is green, often with violet spots and is tubular; at the end are 2 lips of which the upper one is bent back and at the end has 3 lobes, the lower is longer and has 2 hairy teeth. After flowering, the calyx tube is closed by a crown of long, stiff hairs. The corolla, about twice as long as the calyx, is usually brownish in the dry state and is slightly bilabiate.

The leaf of *Thymus zygis* is usually 1.7-6.5 mm long and 0.4-1.2 mm wide; it is acicular or linear-lanceolate and the edges are markedly rolled towards the abaxial surface. Both surfaces of the lamina are green or greenish-grey and the midrib is sometimes violet; the edges, in particular at the base, have long, white hairs. The dried flowers are very similar to those of *T. vulgaris*.

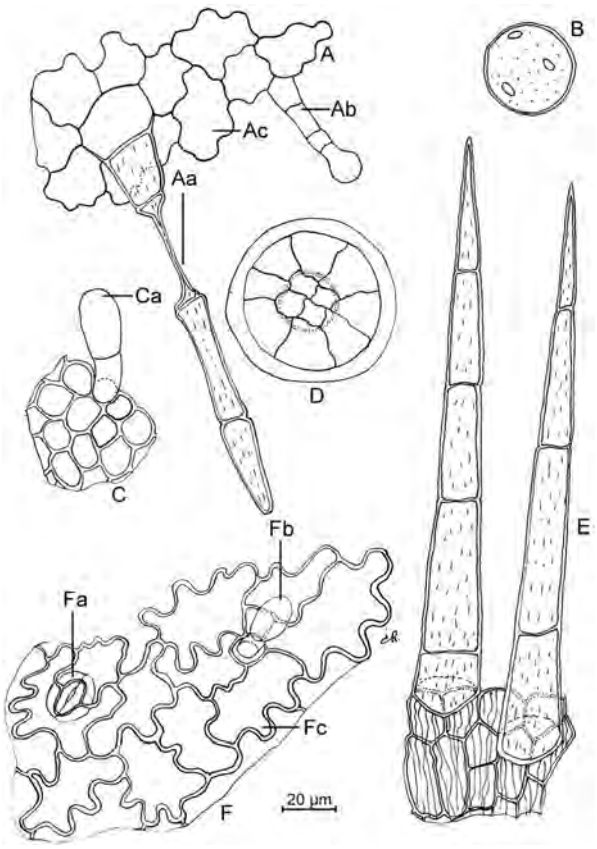


Figure 0865.-1. – *Illustration for identification test B of powdered herbal drug of thyme*

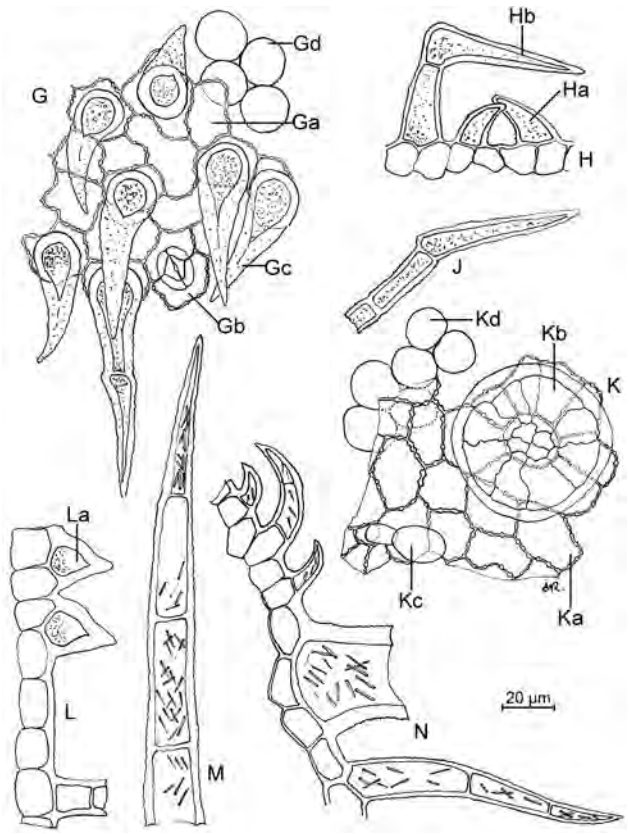


Figure 0865.-2. – *Illustration for identification test B of powdered herbal drug of thyme*

B. Microscopic examination (*2.8.23*). The powder of both species is greyish-green or greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0865.-1 and Figure 0865.-2): fragments of the outer epidermis of the corolla (surface view [A, C, F]), consisting of cells with wavy and slightly thickened [Fc] or unthickened [Ac] walls, numerous uniseriate, multicellular, covering trichomes, often with 1 cell collapsed [Aa], glandular trichomes with a unicellular head and a unicellular [Ca, Fb] or multicellular [Ab] stalk, diacytic stomata (*2.8.3*) [Fa] and glandular trichomes generally with 12 cells [D]; cells of the epidermis from the base of the corolla, isodiametric with slightly thickened walls [C]; pollen grains, relatively rare, spherical and smooth, with 6 germinal slit-like pores, measuring about 35 µm in diameter [B]; the powder of *T. zygis* also contains numerous thick bundles of fibres from the main veins and from fragments of stems; the epidermises of the leaves (surface view [G, K]) have cells with anticlinal walls that are sinuous and beaded [Ga, Ka], and diacytic stomata (*2.8.3*) [Gb]; numerous glandular trichomes made up of 12 secretory cells, the cuticle of which is generally raised by the secretion to form a globular or ovoid, bladder-like covering [Kb]; glandular trichomes with a unicellular stalk and a globular or ovoid head [Kc]; in both species, the adaxial epidermis bears covering trichomes with warty walls that are shaped as pointed teeth [Gc], and is usually associated with underlying palisade parenchyma [Gd, Kd]; the abaxial epidermis (transverse section [H, L]) bears covering trichomes of different types: unicellular, straight or slightly curved [Ha, La]; bicellular or tricellular, articulated and most often elbow-shaped [Hb, J] (*T. vulgaris*); bicellular or tricellular, more or less straight [N], or very large, multicellular [M], at the base of the lamina (*T. zygis*); fragments of calyx covered by numerous, uniseriate trichomes with 5-6 cells and a weakly striated cuticle (surface view [E]).

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge or filter; use the supernatant or the filtrate.

*Reference solution*. Dissolve 1 mg of *rutoside trihydrate R* and 1 mg of *rosmarinic acid R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (1:1:15 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 20 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min, treat the still-hot plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | 2 red fluorescent zones |
| Rosmarinic acid: a blue fluorescent zone | A blue fluorescent zone (rosmarinic acid) |
| | 1 or 2 blue fluorescent zones |
| | 2 yellow or orange fluorescent zones |
| | A green fluorescent zone may be present |
| Rutoside: an orange-yellow fluorescent zone | |
| **Reference solution** | **Test solution** |

D. Examine the chromatograms obtained in the assay for thymol and carvacrol.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Foreign matter** (*2.8.2*): maximum 10 per cent of stems and maximum 2 per cent of other foreign matter. Stems must not be more than 1 mm in diameter and 15 mm in length.

***Thymus serpyllum* L.** Adulteration with *T. serpyllum* L. is indicated by the presence of leaves with long trichomes at their base and with weakly pubescent other parts.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (355) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 30.0 g of the herbal drug, a 1000 mL round-bottomed flask and 400 mL of *water R* as the distillation liquid. Distil at a rate of 2-3 mL/min for 2 h without *xylene R* in the graduated tube.

**Thymol and carvacrol**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Filter the essential oil obtained in the determination of essential oil over a small amount of *anhydrous sodium sulfate R* and dilute to 5.0 mL with *heptane R* by rinsing the apparatus and the anhydrous sodium sulfate. Dilute a volume of the filtered solution corresponding to 100 µL of the essential oil to 5.0 mL with *heptane R*.

*Reference solution (a).* Dissolve 0.20 g of *thymol R* and 50 mg of *carvacrol R* in *heptane R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 10 µL of *carvacrol R* to 10.0 mL with *heptane R*. Dilute 100 µL of the solution to 10.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30-60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *nitrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 1-2 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 45 | 40 → 220 |
| Injection port | | 190 |
| Detector | | 210 |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to thymol and carvacrol.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of the reference solution in the chromatogram obtained with the test solution.

Determine the percentage content of thymol and carvacrol. Disregard any peak due to the solvent or with an area less than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**01/2012:1374**
**corrected 10.0**

# THYME OIL, THYMOL TYPE

## Thymi typo thymolo aetheroleum

DEFINITION

Essential oil obtained by steam distillation from the fresh flowering aerial parts of *Thymus vulgaris* L., *T. zygis* L. or a mixture of both species.

CHARACTERS

*Appearance*: clear, yellow or very dark reddish-brown, mobile liquid.

Odour reminiscent of thymol.

*Solubility*: miscible with anhydrous ethanol and with light petroleum.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.2 mL of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 5 mg of *thymol R* and 10 µL of *carvacrol R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methylene chloride R*.

*Application*: 10 µL [or 4 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A pink zone |
| _____ | _____ |
| Thymol: an orange-brown zone | An intense orange-brown zone (thymol) |
| Carvacrol: an orange-grey zone | A faint orange-grey zone (carvacrol) may be present |
| _____ | _____ |
| | A pink zone |
| | A violet zone |
| | A brownish-grey zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the characteristic peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.915 to 0.935.

**Refractive index** (*2.2.6*): 1.490 to 1.505.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 200 µL of the substance to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 µL of *β-myrcene R*, 5 µL of *α-terpinene R*, 20 µL of *p-cymene R*, 10 µL of *γ-terpinene R*, 5 µL of *linalol R*, 5 µL of *terpinen-4-ol R*, 40 mg of *thymol R* and 5 µL of *carvacrol R* in 5 mL of *heptane R*.

*Reference solution (b).* Dissolve 10 µL of *carvacrol R* in *heptane R* and dilute to 10.0 mL with the same solvent. Dilute 100 µL of the solution to 10.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

Herbal drugs

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 75 | 65 → 215 |
| Injection port | | 230 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to thymol and carvacrol.

*Identification of peaks*: using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution. The peak due to α-thujene elutes with a relative retention of about 0.8 with reference to β-myrcene. The peak due to carvacrol methyl ether elutes with a relative retention of about 0.9 with reference to thymol.

Determine the percentage content of these components. The limits are within the following ranges:

– *α-thujene*: 0.2 per cent to 1.5 per cent;
– *β-myrcene*: 1.0 per cent to 3.0 per cent;
– *α-terpinene*: 0.9 per cent to 2.6 per cent;
– *p-cymene*: 14.0 per cent to 28.0 per cent;
– *γ-terpinene*: 4.0 per cent to 12.0 per cent;
– *linalol*: 1.5 per cent to 6.5 per cent;
– *terpinen-4-ol*: 0.1 per cent to 2.5 per cent;
– *carvacrol methyl ether*: 0.05 per cent to 1.5 per cent;
– *thymol*: 37.0 per cent to 55.0 per cent;
– *carvacrol*: 0.5 per cent to 5.5 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

STORAGE

At a temperature not exceeding 25 °C.

**01/2008:1596**

# TOLU BALSAM

## Balsamum tolutanum

DEFINITION

Oleo-resin obtained from the trunk of *Myroxylon balsamum* (L.) Harms var. *balsamum*.

*Content*: 25.0 per cent to 50.0 per cent of free or combined acids, expressed as cinnamic acid ($C_9H_8O_2$; $M_r$ 148.2) (dried drug).

CHARACTERS

*Appearance*: hard, friable, brownish to reddish-brown mass; thin fragments are brownish-yellow when examined against the light.

Reminiscent odour of vanillin.

*Solubility*: practically insoluble in water, very soluble to freely soluble in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Stir 0.40 g of the fragmented drug with 10 mL of *methylene chloride R* for 5 min and filter.

*Reference solution.* Dissolve 50 mg of *benzyl cinnamate R* in *methylene chloride R*, add 50 µL of *benzyl benzoate R* and dilute to 10 mL with *methylene chloride R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *light petroleum R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R* and heat at 100-105 °C for 5 min. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the test and reference solutions. Furthermore, other coloured zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Benzyl benzoate: a greyish-blue zone | a greyish-blue zone |
| Benzyl cinnamate: a greyish-green zone | a greyish-green zone |
| **Reference solution** | **Test solution** |

TESTS

**Acid value**: 100 to 160.

Dissolve 0.5 g of the fragmented drug in 50 mL of *alcohol R*. Add 0.5 mL of *acid blue 93 solution R* and 5.0 mL of *0.5 M alcoholic potassium hydroxide*. Stir vigorously and titrate with *0.5 M hydrochloric acid* until the colour changes from brownish-red to blackish-green ($n_1$ mL of *0.5 M hydrochloric acid*). Carry out a blank test in the same manner ($n_2$ mL of *0.5 M hydrochloric acid*). Calculate the acid value in the same manner as the saponification value (*2.5.6*).

**Matter insoluble in alcohol**: maximum 5 per cent.

Boil 2.0 g of the fragmented drug with 25 mL of *alcohol (90 per cent V/V) R* and filter. Wash the residue with *alcohol (90 per cent V/V) R*, boiling until completely extracted, then dry the residue at 100-105 °C. Weigh the residue.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 2.000 g of the fragmented drug by spreading on a flat evaporating dish 9 cm in diameter and allowing to dry *in vacuo* for 4 h.

**Total ash** (*2.4.16*): maximum 0.3 per cent.

ASSAY

Boil 1.500 g under a reflux condenser with 25 mL of *0.5 M alcoholic potassium hydroxide* for 1 h. Evaporate the ethanol and heat the residue with 50 mL of *water R* until the substance is homogeneously distributed. After cooling, add 80 mL of *water R* and a solution of 1.5 g of *magnesium sulfate R* in 50 mL of *water R*. Mix, and allow to stand for 10 min. Filter through a pleated filter paper and wash the residue with 20 mL of *water R*. Combine the filtrate and the washings, acidify with *hydrochloric acid R* and extract with 4 quantities, each of 40 mL, of *ether R*. Discard the aqueous layer. Combine the organic extracts and wash with 2 quantities, each of 20 mL, and with 3 quantities, each of 10 mL, of a 50 g/L solution of *sodium hydrogen carbonate R*. Discard the ether layer. Combine the aqueous extracts, acidify with *hydrochloric acid R* and stir once with 30 mL, twice with 20 mL and once with 10 mL of *methylene chloride R*. Dry the combined methylene chloride extracts over *anhydrous sodium sulfate R*. Filter through a pleated filter and wash the residue with 10 mL of *methylene chloride R*. Reduce the combined methylene chloride extracts to 10 mL by distillation and eliminate the remaining methylene chloride in a current of air. Dissolve

the residue with heating in 10 mL of *alcohol R* previously neutralised to *phenol red solution R*. After cooling, titrate with *0.1 M sodium hydroxide*, using the same indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 14.82 mg of total acids, expressed as cinnamic acid.

STORAGE

Do not store in powdered form.

**07/2010:1478**

# TORMENTIL

## Tormentillae rhizoma

DEFINITION

Whole or cut, dried rhizome, freed from the roots, of *Potentilla erecta* (L.) Raeusch. (*P. tormentilla* Stokes).

*Content*: minimum 7 per cent of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1) (dried drug).

IDENTIFICATION

A. The rhizome is cylindrically spindle-shaped, with a very irregular appearance, often forming, twisted, knotty tubers, up to 10 cm long and 1-2 cm thick, very hard and scarcely branched. The surface is brown to reddish-brown, rugose and has remains of roots and transversely elongated depressed whitish scars from the stems. At the top of the rhizome the remains of numerous aerial stems may be present. The fracture is short and granular, dark red to brownish-yellow.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: coarsely serrate cluster crystals of calcium oxalate, up to 60 µm in diameter; fragments of thin-walled parenchyma containing reddish-brown tannin; groups of narrow, bordered-pitted vessels with lateral pores; thick-walled and pitted, polygonal parenchyma; groups and fragments of sclerenchymatous thick-walled fibres; occasional fragments of cork with thin-walled, brown, tabular cells. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows spherical or elliptical starch granules, up to about 20 µm in length.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *water R*, shake for 10 min and filter. Shake the filtrate with 2 quantities, each of 10 mL, of *ethyl acetate R* and filter the combined upper phases over 6 g of *anhydrous sodium sulfate R*. Evaporate the filtrate to dryness under reduced pressure and dissolve the residue in 1.0 mL of *ethyl acetate R*.

*Reference solution.* Dissolve 1.0 mg of *catechin R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *ether R*, *hexane R*, *ethyl acetate R* (20:20:20:40 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 10-15 min.

*Detection*: spray with a freshly prepared 5 g/L solution of *fast blue B salt R*. Reddish zones appear. Expose the plate to ammonia vapour, the zones become more intense turning reddish-brown. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fainter zones are present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Catechin: an intense reddish-brown zone | A more intense reddish-brown zone (catechin)<br>A fainter zone<br>An intense zone<br>Fainter zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of root and stems as well as rhizomes with black fracture and maximum 2 per cent of other foreign matter.

**Cadmium** (*2.4.27*): maximum 2.0 ppm.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

**Tannins** (*2.8.14*). Use 0.500 g of the powdered herbal drug (180) (*2.9.12*).

**01/2008:1895**

# TORMENTIL TINCTURE

## Tormentillae tinctura

DEFINITION

Tincture produced from *Tormentil (1478)*.

*Content*: minimum 1.5 per cent *m/m* of tannins, expressed as pyrogallol ($C_6H_6O_3$; $M_r$ 126.1).

PRODUCTION

The tincture is produced from 1 part of comminuted drug and 5 parts of ethanol (70 per cent *V/V*) by a suitable procedure.

CHARACTERS

Red or reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Mix 1.0 mL of the tincture to be examined with 1.0 mL of *alcohol (70 per cent V/V) R*.

*Reference solution.* Dissolve 1.0 mg of *catechin R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ether R*, *glacial acetic acid R*, *hexane R*, *ethyl acetate R* (20:20:20:40 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air for 10-15 min.

*Detection*: spray with a freshly prepared 5 g/L solution of *fast blue B salt R*. Reddish zones appear. Expose the plate to ammonia vapour, the zones become more intense, turning reddish-brown. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Catechin: an intense zone | An intense zone (catechin) |
| ——— | ——— |
| | A fainter zone |
| | An intense zone |
| | Fainter zones |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol content** (*2.9.10*): 64 per cent *V/V* to 69 per cent *V/V*.

**Methanol and 2-propanol** (*2.9.11*): maximum 0.05 per cent *V/V* of methanol and maximum 0.05 per cent *V/V* of 2-propanol.

ASSAY

**Tannins** (*2.8.14*). Use 2.50 g of the tincture to be examined.

**01/2009:0532**

# TRAGACANTH

## Tragacantha

[9000-65-1]

DEFINITION

Air-hardened, gummy exudate, flowing naturally or obtained by incision from the trunk and branches of *Astragalus gummifer* Labill. and certain other species of *Astragalus* from western Asia.

IDENTIFICATION

A. Tragacanth occurs in thin, flattened, ribbon-like, white or pale yellow, translucent strips, about 30 mm long and 10 mm wide and up to 1 mm thick, more or less curved, horny, with a short fracture; the surface is marked by fine longitudinal striae and concentric transverse ridges. It may also contain pieces similar in shape but somewhat thicker, more opaque and more difficult to fracture.

B. Microscopic examination (*2.8.23*). The powder is white or almost white and forms a mucilaginous gel with about 10 times its mass of *water R*. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows in the gummy mass numerous stratified cellular membranes that turn slowly violet when treated with *iodinated zinc chloride solution R*. The gummy mass includes starch grains, isolated or in small groups, usually rounded in shape and sometimes deformed, with diameters varying between 4 µm and 10 µm, occasionally up to 20 µm, and a central hilum visible between crossed nicol prisms.

C. Examine the chromatograms obtained in the test for acacia.
*Results*: the chromatogram obtained with the test solution shows 3 zones due to galactose, arabinose and xylose. A faint yellowish zone at the solvent front and a greyish-green zone between the zones due to galactose and arabinose may be present.

D. Moisten 0.5 g of the powdered herbal drug (355) (*2.9.12*) with 1 mL of *ethanol (96 per cent) R* and add gradually, while shaking, 50 mL of *water R* until a homogeneous mucilage is obtained. To 5 mL of the mucilage add 5 mL of *water R* and 2 mL of *barium hydroxide solution R*. A slight flocculent precipitate is formed. Heat on a water-bath for 10 min. An intense yellow colour develops.

TESTS

**Acacia**. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 100 mg of the powdered herbal drug (355) (*2.9.12*) in a thick-walled centrifuge test-tube, add 2 mL of a 100 g/L solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the test-tube and heat the mixture at 120 °C for 1 h. Centrifuge the resulting hydrolysate, transfer the clear supernatant carefully into a 50 mL flask, add 10 mL of *water R* and evaporate the solution to dryness under reduced pressure. To the resulting clear film add 0.1 mL of *water R* and 0.9 mL of *methanol R*. Centrifuge to separate the amorphous precipitate, collect the supernatant and, if necessary, dilute to 1 mL with *methanol R*.

*Reference solution*. Dissolve 10 mg of *arabinose R*, 10 mg of *galactose R*, 10 mg of *rhamnose R* and 10 mg of *xylose R* in 1 mL of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: 16 g/L solution of *sodium dihydrogen phosphate R*, *butanol R*, *acetone R* (10:40:50 *V/V/V*).

*Application*: 10 µL as bands.

*Development A*: over a path of 10 cm.

*Drying A*: in a current of warm air for a few minutes.

*Development B*: over a path of 15 cm using the same mobile phase.

*Drying B*: at 110 °C for 10 min.

*Detection*: spray with *anisaldehyde solution R* and dry at 110 °C for 10 min.

*Results*: the chromatogram obtained with the reference solution shows 4 clearly separated coloured zones due to galactose (greyish-green or green), arabinose (yellowish-green), xylose (greenish-grey or yellowish-grey) and rhamnose (yellowish-green), in order of increasing $R_F$ value; the chromatogram obtained with the test solution does not show a yellowish-green zone corresponding to the zone of rhamnose in the chromatogram obtained with the reference solution.

**Methylcellulose**. Examine the chromatograms obtained in the test for acacia.

*Results*: the chromatogram obtained with the test solution does not show a red zone near the solvent front.

**Sterculia gum**

A. Place 0.2 g of the powdered herbal drug (355) (*2.9.12*) in a 10 mL ground-glass-stoppered cylinder graduated in 0.1 mL. Add 10 mL of *ethanol (60 per cent V/V) R* and shake. Any gel formed occupies not more than 1.5 mL.

B. To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 100 mL of *water R* and shake. Add 0.1 mL of *methyl red solution R*. Not more than 5.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Foreign matter**: maximum 1.0 per cent.

Place 2.0 g of the powdered herbal drug (355) (*2.9.12*) in a 250 mL round-bottomed flask and add 95 mL of *methanol R*. Swirl to moisten the powder and add 60 mL of *hydrochloric acid R1*. Add a few glass beads about 4 mm in diameter and heat on a water-bath under a reflux condenser for 3 h, shaking occasionally. Remove the glass beads and filter the hot suspension *in vacuo* through a sintered-glass filter (160) (*2.1.2*). Rinse the flask with a small quantity of *water R* and pass the rinsings through the filter. Wash the residue on the filter with about 40 mL of *methanol R* and dry to constant

mass at 110 °C (about 1 h). Allow to cool in a desiccator and weigh. The residue weighs a maximum of 20 mg.

**Flow time**: minimum 10 s, or minimum 50 s if the substance to be examined is to be used for the preparation of emulsions.

Place 1.0 g of the powdered herbal drug (125-250) (*2.9.12*) in a 1000 mL round-bottomed flask with a ground-glass stopper, add 8.0 mL of *ethanol (96 per cent) R* and close the flask. Disperse the suspension over the inner surface of the flask by shaking, taking care not to wet the stopper. Open the flask and add as a single portion 72.0 mL of *water R*. Stopper the flask and shake vigorously for 3 min. Allow to stand for 24 h and shake vigorously again for 3 min. Eliminate air bubbles by applying vacuum above the mucilage for 5 min. Transfer the mucilage to a 50 mL cylinder. Dip in the mucilage a piece of glass tubing 200 mm long and 6.0 mm in internal diameter and graduated at 20 mm and 120 mm from the lower end; the tubing must not be rinsed with surface-active substances. When the mucilage has reached the upper mark, close the tube with a finger. Withdraw the closed tube, remove the finger and measure with a stop-watch the time needed for the meniscus to reach the lower graduation. Carry out this operation 4 times and determine the average value of the last 3 determinations.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states whether or not the contents are suitable for preparing emulsions.

**01/2015:1441**

# TURMERIC, JAVANESE

## Curcumae zanthorrhizae rhizoma

DEFINITION

Dried rhizome, cut in slices, of *Curcuma zanthorrhiza* Roxb. (syn. *C. zanthorrhiza* D. Dietrich).

*Content*:

– *essential oil*: minimum 50 mL/kg (anhydrous drug);

– *dicinnamoyl methane derivatives, expressed as curcumin* ($C_{21}H_{20}O_6$; $M_r$ 368.4): minimum 1.0 per cent (anhydrous drug).

CHARACTERS

Aromatic odour.

IDENTIFICATION

A. Orange-yellow or yellowish-brown or greyish-brown slices, mostly peeled 1.5-6 mm thick and 15-50 mm, more rarely up to 70 mm, in diameter. Fragments of the brownish-grey cork are sporadically present. The transverse surface is yellow with dark spots in the paler centre. The fracture is short and finely grained.

B. Microscopic examination (*2.8.23*). The powder is orange-yellow or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1441.-1): fragments of parenchyma [C, E] with large, rounded or ovoid cells containing orange-yellow or yellowish-brown secretory cells [Ea]; fragments of spiral [Fa, G] or reticulate [F] vessels; rare fragments of cork (surface view [B], side view [D]); fragments of epidermis [H] with fragments of thick-walled unicellular covering trichomes [Ha] with a pointed tip [Hb]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous ovoid or irregular starch granules [A], about 30-50 µm long and about 10-30 µm wide, with a more or less visible eccentric hilum.

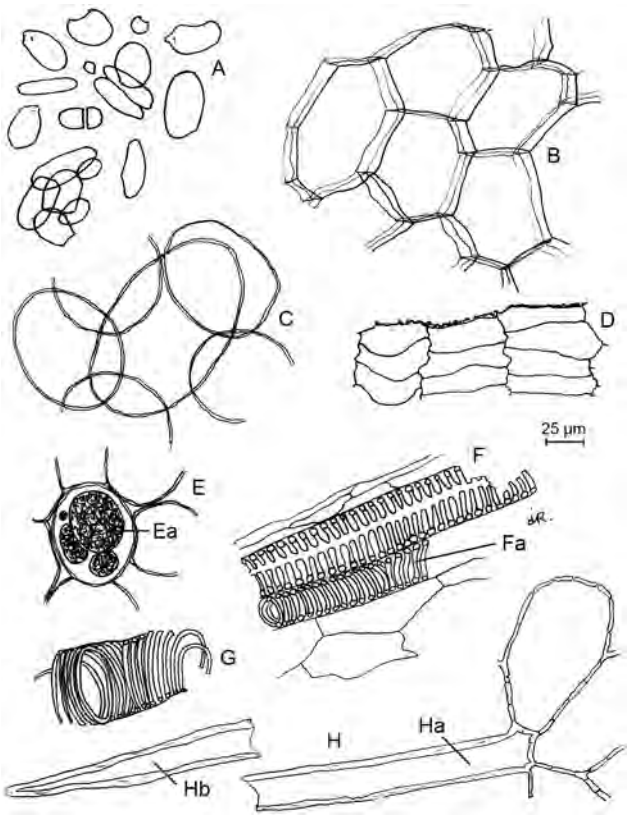


Figure 1441.-1. – *Illustration for identification test B of powdered herbal drug of Javanese turmeric*

C. Thin-layer chromatography (*2.2.27*). Examine the chromatograms obtained in the test for *Curcuma longa* L.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Curcuminoids: a greenish fluorescent zone | A greenish fluorescent zone (curcuminoids) |
| ——— | ——— |
| Curcuminoids: 2 greenish fluorescent zones | A greenish fluorescent zone (curcuminoids) |
| **Reference solution** | **Test solution** |

Herbal drugs

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint pink zone |
| | An intense reddish zone |
| | A dark zone |
| ——— | ——— |
| Thymol: a dark zone | |
| | A pinkish-red zone |
| Curcuminoids: a brown zone | A brown zone (curcuminoids) |
| ——— | ——— |
| Curcuminoids: 2 yellow zones | A yellow zone (curcuminoids) |
| **Reference solution** | **Test solution** |

TESTS

***Curcuma longa L.*** Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the freshly powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (96 per cent) R*, shake, allow to stand for 30 min with occasional shaking and filter; use the filtrate.

*Reference solution.* Dissolve 20 mg of *curcuminoids R* and 10 mg of *thymol R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Results A*: the chromatogram obtained with the test solution does not show a very intense greenish fluorescent zone in the lower third.

*Detection B*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 10 min; examine in ultraviolet light at 365 nm.

**Water** (*2.2.13*): maximum 120 mL/kg, determined on 20.0 g of the powdered herbal drug (500) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 8.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 2.5 g of the freshly powdered herbal drug (500) (*2.9.12*), a 2 L round-bottomed flask, 400 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2 mL/min for 3 h.

**Dicinnamoyl methane derivatives**. Disperse 0.500 g of the powdered herbal drug (500) (*2.9.12*) in 30 mL of *ethanol (96 per cent) R* in a 100 mL round-bottomed flask. Heat under a reflux condenser for 2.5 h. Cool and filter into a volumetric flask, rinse the round-bottomed flask and the filter with *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at 425 nm using *ethanol (96 per cent) R* as the compensation liquid.

Calculate the percentage content of dicinnamoyl methane derivatives, expressed as curcumin, using the following expression:

$$\frac{A \times 5000}{1607 \times m}$$

i.e. taking the specific absorbance of curcumin to be 1607.

$A$ = absorbance at 425 nm,

$m$ = mass of the herbal drug to be examined, in grams.

**01/2015:2543**
**corrected 10.0**

# TURMERIC RHIZOME

## Curcumae longae rhizoma

DEFINITION

Whole, cured (by boiling or steaming), dried rhizome of *Curcuma longa* L. (syn. *Curcuma domestica* Valeton) with roots and outer surface removed.

*Content*:

– *essential oil*: minimum 25 mL/kg (anhydrous drug);

– *dicinnamoyl methane derivatives, expressed as curcumin* ($C_{21}H_{20}O_6$; $M_r$ 368.4): minimum 2.0 per cent (anhydrous drug).

CHARACTERS

Spicy odour.

IDENTIFICATION

A. The rhizome is ovate, oblong-ovoid, pyriform or cylindrical, often shortly branched, up to 6 cm long and 15 mm thick. The primary rhizome shows scars from the lateral branches. The surface is slightly dusty, spotted and brownish-yellow, yellow or brownish-grey, finely striated. The fracture is granular, smooth, non-fibrous, slightly glossy, uniformly orange-yellow; it shows a narrow cortex that is darker on the outside.

B. Microscopic examination (*2.8.23*). The powder is orange-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2543.-1): fragments of parenchyma including some secretory cells that contain masses of brownish-yellow oil [G]; reticulate or pitted vessels [B, D]; rare fragments of epidermis (surface view [F]), with cells whose walls are slightly and irregularly thickened [Fa] and scars of covering trichomes [Fb]; occasional long and flexuous, thick-walled, unicellular covering trichomes, fragmented and free [E] or attached to fragments of epidermis [J]; rare fragments of cork (surface view [A], side view [H]), sometimes covered by epidermis [Ha]. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows starch granules, free or included in parenchymatous cells, usually gelatinised and agglomerated in a starchy paste; occasional ovoid starch granules, often deformed by curing [C], are also present.

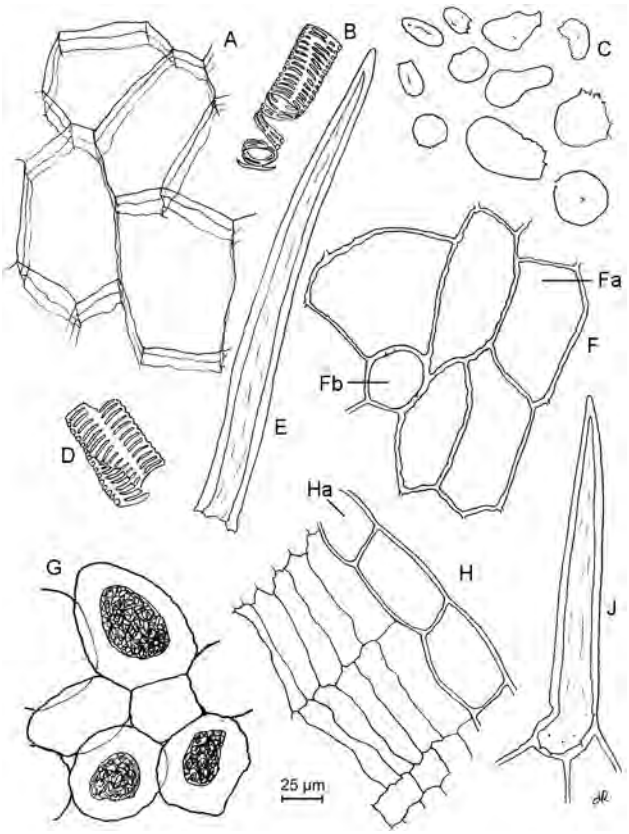


Figure 2543.-1. – *Illustration for identification test B of powdered herbal drug of turmeric rhizome*

C. Thin-layer chromatography (*2.2.27*). Examine the chromatograms obtained in the test for *Curcuma zanthorrhiza* Roxb.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Curcuminoids: a greenish fluorescent zone | A greenish fluorescent zone (curcuminoids) |
| ——— | ——— |
| Curcuminoids: 2 greenish fluorescent zones | 2 greenish fluorescent zones (curcuminoids) |
| **Reference solution** | **Test solution** |

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A faint pink zone |
| | An intense reddish zone |
| ——— | ——— |
| Thymol: a dark zone | |
| | A pinkish-red zone |
| Curcuminoids: a brown zone | A brown zone (curcuminoids) |
| ——— | ——— |
| Curcuminoids: 2 yellow zones | 2 yellow zones (curcuminoids) |
| **Reference solution** | **Test solution** |

TESTS

***Curcuma zanthorrhiza* Roxb.** (syn. *Curcuma zanthorrhiza* D. Dietr.). Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the freshly powdered herbal drug (355) (*2.9.12*) add 10 mL of *ethanol (96 per cent) R*, shake, allow to stand for 30 min with occasional shaking and filter; use the filtrate.

*Reference solution.* Dissolve 20 mg of *curcuminoids R* and 10 mg of *thymol R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL [or 3 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 365 nm.

*Detection B*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 10 min; examine in ultraviolet light at 365 nm.

*Results B*: the chromatogram obtained with the test solution shows no dark zone just above the zone due to thymol in the chromatogram obtained with the reference solution.

**Water** (*2.2.13*): maximum 120 mL/kg, determined on 15.0 g of the powdered herbal drug (500) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 2.5 g of the freshly powdered herbal drug (500) (*2.9.12*), a 2 L round-bottomed flask, 400 mL of *water R* as the distillation liquid and 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2 mL/min for 3 h.

**Dicinnamoyl methane derivatives**. Disperse 0.500 g of the powdered herbal drug (500) (*2.9.12*) in 30 mL of *ethanol (96 per cent) R* in a 100 mL round-bottomed flask. Heat under a reflux condenser for 2.5 h. Cool and filter into a volumetric flask, rinse the round-bottomed flask and the filter with *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at 425 nm using *ethanol (96 per cent) R* as the compensation liquid.

Calculate the percentage content of dicinnamoyl methane derivatives, expressed as curcumin, using the following expression:

$$\frac{A \times 5000}{1607 \times m}$$

i.e. taking the specific absorbance of curcumin to be 1607.

$A$ = absorbance at 425 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**07/2014:1627**
**corrected 9.4**

# TURPENTINE OIL

## Terebinthinae aetheroleum

DEFINITION

Essential oil obtained by steam distillation, followed by rectification at a temperature below 180 °C, from the oleoresin obtained by tapping *Pinus pinaster* Aiton and/or *Pinus massoniana* D.Don. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

Odour reminiscent of α-pinene and β-pinene.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1 mL of the oil to be examined in 10 mL of *toluene R*.

*Reference solution.* Dissolve 10 µL of *β-caryophyllene R* and 10 mg of *caryophyllene oxide R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 10 µL [or 2 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present below the zone due to caryophyllene oxide in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| β-Caryophyllene: a pink zone | A pink zone |
| Caryophyllene oxide: a pink zone | A pink zone (caryophyllene oxide) |
| | A brownish-violet zone |
| **Reference solution** | **Test solution** |

B. Examine the chromatograms obtained in the test for chromatographic profile.

*Results*: the peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Relative density** (*2.2.5*): 0.856 to 0.872.

**Refractive index** (*2.2.6*): 1.465 to 1.475.

**Optical rotation** (*2.2.7*): − 40° to − 28°.

**Acid value** (*2.5.1*): maximum 1.0.

**Peroxide value** (*2.5.5, Method B*): maximum 20.

**Fatty oils and resinified essential oils** (*2.8.7*). It complies with the test.

**Chromatographic profile**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dilute 1.0 mL of the oil to be examined in *heptane R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 30 µL of *α-pinene R*, 10 mg of *camphene R*, 20 µL of *β-pinene R*, 10 µL of *car-3-ene R*, 10 µL of *β-myrcene R*, 20 µL of *limonene R*, 10 µL of *longifolene R*, 10 µL of *β-caryophyllene R* and 10 mg of *caryophyllene oxide R* in 1.0 mL of *heptane R*.

*Reference solution (b).* Dissolve 5 µL of *β-caryophyllene R* in *heptane R* and dilute to 10.0 mL with the same solvent. Dilute 0.1 mL of the solution to 1.0 mL with *heptane R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 60 |
| | 10 - 80 | 60 → 200 |
| | 80 - 120 | 200 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Elution order*: order indicated in the composition of reference solution (a); record the retention times of these substances.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to car-3-ene and β-myrcene.

Using the retention times determined from the chromatogram obtained with reference solution (a), locate the components of reference solution (a) in the chromatogram obtained with the test solution.

Determine the percentage content of these components. The limits are within the following ranges:

- *α-pinene*: 70.0 per cent to 85.0 per cent;
- *camphene*: 0.5 per cent to 2.0 per cent;
- *β-pinene*: 5.0 per cent to 20.0 per cent;
- *car-3-ene*: maximum 1.0 per cent;
- *β-myrcene*: 0.4 per cent to 1.5 per cent;
- *limonene*: 1.0 per cent to 7.0 per cent;
- *longifolene*: 0.2 per cent to 4.0 per cent;
- *β-caryophyllene*: 0.1 per cent to 3.0 per cent;
- *caryophyllene oxide*: maximum 1.0 per cent;
- *disregard limit*: the area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Residue on evaporation** (*2.8.9*): maximum 2.5 per cent, determined after heating on a water-bath for 3 h.

STORAGE

At a temperature not exceeding 25 °C.

04/2019:2937

# TYPHAE POLLEN

## Typhae pollis

DEFINITION

Dried pollen of *Typha angustifolia* L., *Typha orientalis* C.Presl or other species of the genus *Typha* with single pollen grains (monads).

*Content*: minimum 0.8 per cent of total flavonoids, expressed as typhaneoside ($C_{34}H_{42}O_{20}$; $M_r$ 771) (dried drug).

IDENTIFICATION

A. Yellow or brownish-yellow, granular powder, mixed with small, irregular, mostly translucent, fragments of floral organs. The powder floats on water and disperses rapidly. The texture is fine and light, feels silky when rubbed between the fingers and easily adheres to the skin.

B. Microscopic examination (*2.8.23*). The powder is yellow to brownish-yellow. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2937.-1): single, light yellow pollen grains (monads), usually rounded, subtriangular or subrectangular with numerous intermediary forms, 17-32 µm in diameter, with a single, more or less distinct, germinal pore and a finely reticulate exine; a few collapsed grains [F]. The following fragments of floral organs may be present: fragments of stamens, either containing pollen grains [C] or showing the endothecium [G]; the endothecium is covered by a cuticle and, in side view [D], shows cells with walls thickened on 3 sides, whereas in surface view or from an angle [Ga, H], the cell walls appear as wave-shaped or punctiform thickenings; fragments of bracts consisting of either slightly lignified, fusiform cells [A] or slightly thickened, narrow, elongated, rectangular cells sometimes containing fine annular vessels [E]; raphides of calcium oxalate, either free [J] or included in rounded, thin-walled, parenchymatous cells; styloid crystals of calcium oxalate, usually broken [B].

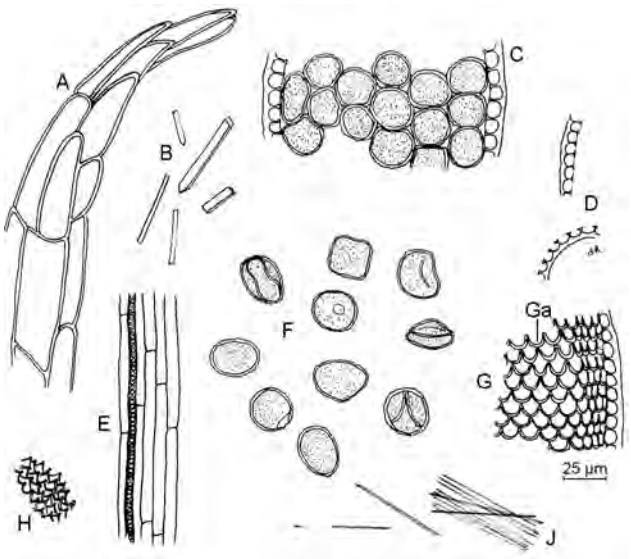


Figure 2937.-1. – *Illustration for identification test B of powdered herbal drug of typhae pollen*

C. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5.0 mL of *methanol R* and sonicate for 15 min. Centrifuge or filter and use the supernatant or the filtrate. If necessary, filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 3.0 mg of *isorhamnetin-3-O-neohesperidoside R* and 3.0 mg of *typhaneoside R* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 3 mg of *chlorogenic acid R* and 3 mg of *isorhamnetin-3-O-neohesperidoside R* in *methanol R* and dilute to 10 mL with the same solvent.

*Intensity marker*: typhaneoside.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: 7 cm from the lower edge of the plate.

*Drying*: in a current of cold air for 5 min.

*Detection*: heat at 100 °C for 3 min; treat the warm plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then with a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; examine in ultraviolet light at 366 nm.

*System suitability*: reference solution (c):

– the chromatogram shows in the middle third 2 distinct zones, which may be touching; the lower zone (isorhamnetin-3-*O*-neohesperidoside) shows a green fluorescence and the upper zone (chlorogenic acid) shows a light blue fluorescence.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution. Furthermore, in the chromatogram obtained with the test solution, other faint to very faint blue, green and/or orange fluorescent zones may be present.

| Top of the plate | |
|---|---|
| Isorhamnetin-3-*O*-neohesperidoside: a green fluorescent zone, intense | A green fluorescent zone, intense (isorhamnetin-3-*O*-neohesperidoside)<br>A yellow fluorescent zone, faint |
| Typhaneoside: a green fluorescent zone, intense | A green fluorescent zone, intense (typhaneoside)<br>A yellow fluorescent zone, faint |
| **Reference solution (a)** | **Test solution** |

TESTS

**Foreign matter**: maximum 10 per cent.

Weigh 10 g of the herbal drug onto a sieve (125) (*2.1.4*) and sieve gently with light tapping. The material remaining on the sieve weighs a maximum of 1.0 g.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 4.0 per cent.

Herbal drugs

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 1.0 g of the sieved herbal drug obtained in the test for foreign matter into a round-bottomed flask and add 50 mL of *methanol R*. Heat in a water-bath under a reflux condenser at 90 °C for 30 min. Allow to cool and filter. Repeat the extraction with a further 50 mL of *methanol R*. Allow to cool and filter. Combine the filtrates and evaporate to dryness under reduced pressure. Dissolve the residue in *methanol R* and dilute to 10.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.22 µm).

*Reference solution (a).* Dissolve 8.0 mg of *typhaneoside CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 0.230 g of *typhae pollen dry extract for system suitability HRS* in *methanol R* and dilute to 10 mL with the same solvent. Weigh and sonicate for 10 min. Allow to cool, weigh again and compensate for the loss of solvent with *methanol R*. Shake thoroughly and filter through a membrane filter (nominal pore size 0.22 µm).

*Column:*

– *size*: *l* = 0.15 m, Ø = 2.1 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm);

– *temperature*: 25 °C.

*Mobile phase*: *acetonitrile R*, 0.1 per cent *V/V* solution of *anhydrous formic acid R* (12.5:87.5 *V/V*).

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 2 µL.

*Run time*: 30 min.

*Identification of peaks*: use the chromatogram supplied with *typhae pollen dry extract for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to flavonoids 1, 2, 3, 5 and 6; use the chromatogram obtained with reference solution (a) to identify the peak due to typhaneoside (flavonoid 4).

*Relative retention* with reference to typhaneoside (retention time = about 16.2 min): flavonoid 1 = about 0.55; flavonoid 2 = about 0.76; flavonoid 3 = about 0.90; flavonoid 5 = about 1.23; flavonoid 6 = about 1.41.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.3 between the peaks due to flavonoid 3 and typhaneoside.

Calculate the percentage content of total flavonoids, expressed as typhaneoside, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1 \times 5}$$

$A_1$ = sum of the areas of the peaks due to typhaneoside and flavonoids 1, 2, 3, 5 and 6 in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to typhaneoside in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *typhaneoside CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of typhaneoside in *typhaneoside CRS*.

**01/2018:2729**

# UNCARIA STEM WITH HOOKS

## Uncariae rhynchophyllae ramulus cum uncis

DEFINITION

Fragments of dried branch or stem of *Uncaria rhynchophylla* (Miq.) Miq. ex Havil., each bearing paired hooks.

*Content*: minimum 0.2 per cent of total alkaloids, expressed as isorhynchophylline ($C_{22}H_{28}N_2O_4$; $M_r$ 384.5) (dried drug).

IDENTIFICATION

A. The glabrous, reddish-brown fragments of the stem are about 2-3 cm long and bear paired hooks. The stem is cylindrical or sub-square, 2-5 mm in diameter, with fine longitudinal striations. The hooks, rounded and curved downwards, are paired, opposite to each other on the stem; occasionally there is only 1 hook; the hooks are about 1-5 cm long, with an acute apex and a relatively broad base. Petiole scars and arc-shaped stipule scars are visible on the stem, below the hook. A transverse section of the stem shows either a central cavity or a spongy, whitish pith.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown or yellowish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2729.-1): brown fragments of the epidermis composed of irregular polygonal cells (surface view [D]); fragments of the dermal tissue (transverse section [A]) consisting of the epidermis [Ab] covered by a thick cuticle [Aa] and collenchymatous cells of the parenchyma [Ac]; fragments of phloem parenchyma with some cells containing sandy crystals of calcium oxalate (longitudinal section [J]); numerous pericyclic fibres, often in groups, with thick unchannelled walls [C]; sclereids of the cortical parenchyma with slightly thickened walls and oblique pits, varying greatly in shape and size, sometimes elongated [G], sometimes rectangular [H], isolated or in small groups; fragments of the xylem [E, K] consisting of large bordered-pitted vessels [Ka], bordered-pitted tracheids [Kb], annular vessels [Ea], these vessels are often associated with the outer layers of the pith with cells with channelled walls [Eb]; numerous rectangular cells of the medullary rays (longitudinal section [F]) with cells whose walls are regularly thickened and pitted; fragments of the pith with rounded to ovoid cells with slightly pitted walls [B].

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 1 mL of *concentrated ammonia R*. After 30 min, add 25 mL of *ethyl acetate R* and sonicate for 20 min. Filter and evaporate the filtrate to dryness under reduced pressure. Dissolve the residue in 1 mL of *methanol R*, centrifuge and use the supernatant.

*Reference solution.* Dissolve 1 mg of *isorhynchophylline R* and 1 mg of *rhynchophylline R* in 2 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (1:15:125 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

Herbal drugs

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other quenching zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A quenching zone |
| _____ | _____ |
| Isorhynchophylline: a quenching zone | A quenching zone (isorhynchophylline) |
| _____ | _____ |
| | A quenching zone |
| Rhynchophylline: a quenching zone | A quenching zone (rhynchophylline) |
| | A quenching zone |
| **Reference solution** | **Test solution** |

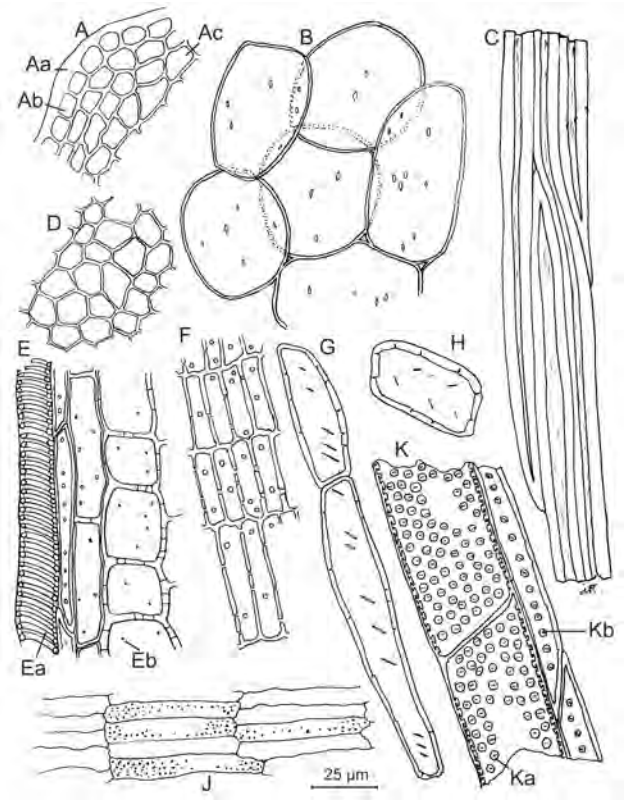


Figure 2729.-1. – *Illustration for identification test B of powdered herbal drug of uncaria stem with hooks*

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*). *Use freshly prepared solutions. Store and inject them at 5 °C, using a cooled autosampler.*

*Test solution.* To 0.500 g of the powdered herbal drug (710) (*2.9.12*) add 3.0 mL of *concentrated ammonia R*. After 30 min, add 50.0 mL of a mixture of equal volumes of *methanol R* and *methylene chloride R*. Sonicate for 30 min. Filter and evaporate the filtrate to dryness under reduced pressure. Dissolve the residue in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *isorhynchophylline CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 25.0 mL with the same mixture of solvents. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5.0 mg of *uncaria stem with hooks dry extract for system suitability HRS* in 1.0 mL of *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with extended pH range R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dilute 0.15 mL of *diethylamine R* to 1 L with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 15 | 75 → 70 | 25 → 30 |
| 15 - 25 | 70 → 55 | 30 → 45 |
| 25 - 35 | 55 | 45 |
| 35 - 40 | 55 → 10 | 45 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram supplied with *uncaria stem with hooks dry extract for system suitability HRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to alkaloids 1, 2, 3, 4, 6, 7, 8 and 9; use the chromatogram obtained with reference solution (a) to identify the peak due to isorhynchophylline.

*Relative retention* with reference to isorhynchophylline (retention time = about 28 min): alkaloid 1 = about 0.50; alkaloid 2 = about 0.58; alkaloid 3 = about 0.94; alkaloid 4 = about 0.97; alkaloid 6 = about 1.05; alkaloid 7 = about 1.13; alkaloid 8 = about 1.22; alkaloid 9 = about 1.27. Additional peaks may be present.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to alkaloid 4 and isorhynchophylline.

Calculate the percentage content of total alkaloids expressed as isorhynchophylline using the following expression:

$$\frac{(\Sigma A_1) \times m_2 \times p}{A_2 \times m_1 \times 25}$$

$\Sigma A_1$ = sum of the areas of the peaks due to alkaloids 1, 2, 3, 4, 6, 7, 8, 9 and the peak due to isorhynchophylline, in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to isorhynchophylline in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *isorhynchophylline CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of isorhynchophylline in *isorhynchophylline CRS*.

**07/2010:2400**

# VALERIAN DRY AQUEOUS EXTRACT

## Valerianae extractum aquosum siccum

DEFINITION

Extract produced from *Valerian root (0453)*.

*Content*: minimum 0.02 per cent of sesquiterpenic acids, expressed as valerenic acid ($C_{15}H_{22}O_2$; $M_r$ 234.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using water at not less than 60 °C.

CHARACTERS

*Appearance*: brown or brownish, hygroscopic powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 1.0 g of the extract to be examined in 10 mL of *methanol R* and sonicate for 10 min. Filter the supernatant through a membrane filter (nominal pore size 0.45 µm). Use the filtrate as the test solution.

*Reference solution.* Dissolve 5 mg of *acetoxyvalerenic acid R* and 5 mg of *valerenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. A faint violet zone due to valerenic acid may be present in the chromatogram obtained with the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____<br>Valerenic acid: a violet zone | _____ |
| Acetoxyvalerenic acid: a violet zone | A violet zone (acetoxyvalerenic acid) |
| _____ | _____<br>A violet zone (hydroxyvalerenic acid) |
| **Reference solution** | **Test solution** |

TESTS

**Loss on drying** (*2.8.17*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution.* In a 300 mL conical flask suspend 1.00 g of the extract to be examined in 40 mL of *water R* whilst swirling. Add 40 mL of *methanol R* and swirl for 1 h at 200 r/min. Filter the suspension into a volumetric flask and rinse the conical flask with 3 quantities, each of 5 mL, of the solvent mixture. Dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve a quantity of *valerian dry extract HRS* corresponding to 1.0 mg of valerenic acid in *methanol R* and dilute to 10.0 mL with the same solvent. Sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, 5 g/L solution of *phosphoric acid R* (20:80 *V/V*);
- *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 55 | 45 |
| 5 - 18 | 55 → 20 | 45 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *valerian dry extract HRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to acetoxyvalerenic acid and hydroxyvalerenic acid.

*Relative retention* with reference to valerenic acid (retention time = about 19 min): hydroxyvalerenic acid = about 0.2; acetoxyvalerenic acid = about 0.5.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression:

$$\frac{(A_1 + A_2) \times m_2 \times p \times 0.2}{A_3 \times m_1}$$

$A_1$ = area of the peak due to hydroxyvalerenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to valerenic acid in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *valerian dry extract HRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of valerenic acid in *valerian dry extract HRS*.

Herbal drugs

07/2014:1898

# VALERIAN DRY HYDROALCOHOLIC EXTRACT

## Valerianae extractum hydroalcoholicum siccum

DEFINITION

Extract produced from *Valerian root (0453)*.

*Content*: minimum 0.25 per cent *m/m* of sesquiterpenic acids, expressed as valerenic acid ($C_{15}H_{22}O_2$; $M_r$ 234.3) (anhydrous extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using ethanol (30-90 per cent *V/V*) or methanol (40-55 per cent *V/V*).

CHARACTERS

*Appearance*: brown, hygroscopic powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 1 g of the extract to be examined in 10 mL of *methanol R* and sonicate for 10 min. Filter the supernatant through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 5 mg of *acetoxyvalerenic acid R* and 5 mg of *valerenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other violet zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Valerenic acid: a violet zone | A violet zone (valerenic acid) |
| Acetoxyvalerenic acid: a violet zone | A violet zone (acetoxyvalerenic acid) |
| ——— | ——— |
| | 2 faint or very faint violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Suspend 1.00 g of the extract to be examined in 50.0 mL of *methanol R1*, sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve a quantity of *valerian dry extract HRS* corresponding to 0.5 mg of valerenic acid in *methanol R1* and dilute to 10.0 mL with the same solvent. Sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, 5 g/L solution of *phosphoric acid R* (20:80 *V/V*);
- *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 55 | 45 |
| 5 - 18 | 55 → 20 | 45 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *valerian dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to hydroxyvalerenic acid, acetoxyvalerenic acid and valerenic acid.

*System suitability*: reference solution:

- *relative retention* with reference to valerenic acid (retention time = about 19 min): hydroxyvalerenic acid = about 0.2; acetoxyvalerenic acid = about 0.5.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression:

$$\frac{(A_1 + A_2 + A_3) \times m_2 \times p \times 5}{A_4 \times m_1}$$

$A_1$ = area of the peak due to hydroxyvalerenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to valerenic acid in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *valerian dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of valerenic acid in *valerian dry extract HRS*.

04/2017:0453

# VALERIAN ROOT

## Valerianae radix

DEFINITION

Dried, whole or fragmented underground parts of *Valeriana officinalis* L. *s.l.*, including the rhizome surrounded by the roots and stolons.

Herbal drugs

*Content*:

– *essential oil*: minimum 4 mL/kg (dried drug);

– *sesquiterpenic acids*: minimum 0.17 per cent *m/m*, expressed as valerenic acid ($C_{15}H_{22}O_2$; $M_r$ 234.3) (dried drug).

IDENTIFICATION

A. The rhizome is yellowish-grey or pale brownish-grey, obconical or cylindrical, up to about 50 mm long and 30 mm in diameter; the base is elongated or compressed, usually entirely covered by numerous roots. The apex usually exhibits a cup-shaped scar from the aerial parts; stem bases are rarely present. When cut longitudinally, the pith exhibits a central cavity transversed by septa. The roots are numerous, almost cylindrical, of the same colour as the rhizome, 1-3 mm in diameter and sometimes more than 100 mm long. A few filiform fragile secondary roots are present. The fracture is short. The stolons show prominent nodes separated by longitudinally striated internodes, each 20-50 mm long, with a fibrous fracture.

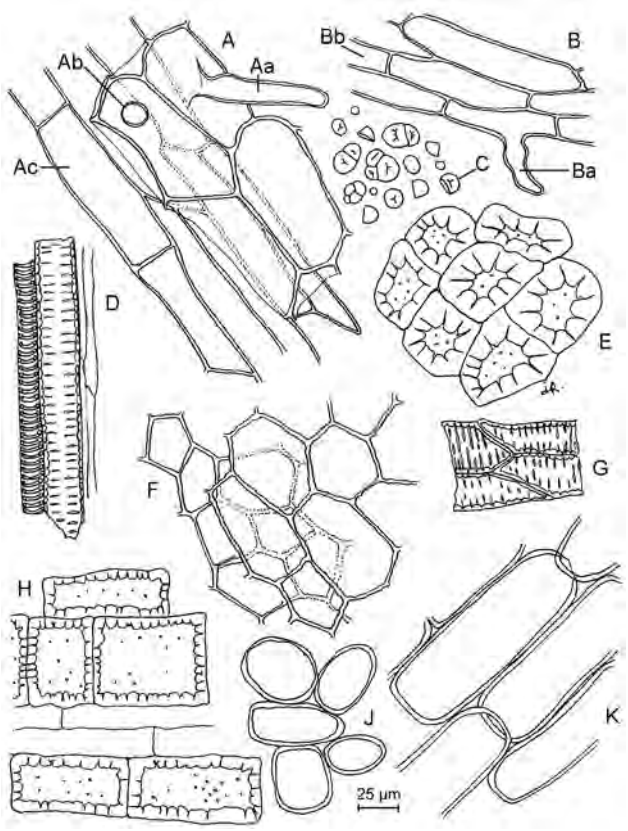


Figure 0453.-1. – *Illustration for identification test B of powdered herbal drug of valerian root*

B. Microscopic examination (*2.8.23*). The powder is pale yellowish-grey or pale greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 0453.-1): occasional groups of rectangular sclereids with moderately thickened walls and a large lumen, from the stem base [H]; very numerous fragments of parenchyma with large ovoid cells (longitudinal section [K], transverse section [J]); spiral, reticulate or pitted lignified vessels, isolated or in small groups [D, G]; thin-walled, elongated cells of the piliferous layer (surface view [A], transverse section [B]), some with root hairs [Aa, Ba] or their scars [Ab]; the piliferous layer is usually accompanied by an underlying layer of cells with slightly thickened and elongated walls [Ac, Bb]; fragments of dermal tissue from the rhizome composed of 1 or 2 layers of polygonal cells with irregularly thickened walls [F]; a few groups of sclereids with thick walls and a narrow lumen [E] from the pith of the rhizome. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*. The powder shows numerous starch granules, simple or 2- to 6-compound, but frequently separated, rounded or irregular and up to about 15 µm in diameter; most of the granules show a rather indistinct cleft or radiate hilum [C].

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Suspend 1 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *methanol R* and sonicate for 10 min. Filter the supernatant through a membrane filter (nominal pore size 0.45 µm). Use the filtrate.

*Reference solution*. Dissolve 5 mg of *acetoxyvalerenic acid R* and 5 mg of *valerenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other violet zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Valerenic acid: a violet zone | A violet zone (valerenic acid) |
| Acetoxyvalerenic acid: a violet zone | A violet zone (acetoxyvalerenic acid) |
| | 2 faint or very faint violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stem bases and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of well-homogenised powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 40.0 g of freshly powdered herbal drug (500) (*2.9.12*), a 2000 mL flask, 500 mL of *water R* as the distillation liquid and 0.50 mL of *1,2,4-trimethylbenzene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 4 h.

**Sesquiterpenic acids**. Liquid chromatography (*2.2.29*).

*Test solution*. Place 1.50 g of the powdered herbal drug (710) (*2.9.12*) in a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *methanol R1*. Mix and heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Place the filter with the residue in the 100 mL round-bottomed flask. Add 20 mL of *methanol R1* and heat on a water-bath under the reflux condenser for 15 min. Allow to cool and filter. Combine the filtrates and dilute to 50.0 mL with *methanol R1*, rinsing the round-bottomed flask and the filter.

*Reference solution*. Dissolve an amount of *valerian dry extract HRS* corresponding to 1.0 mg of valerenic acid in *methanol R1* and dilute to 10.0 mL with the same solvent. Sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, 5 g/L solution of *phosphoric acid R* (20:80 *V/V*);

– *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 55 | 45 |
| 5 - 18 | 55 → 20 | 45 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Peak identification*: use the chromatogram supplied with *valerian dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to acetoxyvalerenic acid and valerenic acid.

*System suitability*: reference solution:

– *relative retention* with reference to valerenic acid (retention time = about 19 min): acetoxyvalerenic acid = about 0.5.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression:

$$\frac{(A_1 + A_2) \times m_2 \times p \times 5}{A_3 \times m_1}$$

$A_1$ = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to valerenic acid in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *valerian dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of valerenic acid in *valerian dry extract HRS*.

**04/2017:2526**

# VALERIAN ROOT, CUT

## Valerianae radix minutata

DEFINITION

Dried, cut underground parts of *Valeriana officinalis* L. *s.l.*, including the rhizome, roots and stolons.

It is produced from *Valerian root (0453)* for the purpose of being used in herbal teas.

*Content*:

– *essential oil*: minimum 3 mL/kg (dried drug);

– *sesquiterpenic acids*: minimum 0.10 per cent *m/m* expressed as valerenic acid ($C_{15}H_{22}O_2$; $M_r$ 234.3) (dried drug).

IDENTIFICATION

A. Microscopic examination (*2.8.23*). The powder is pale yellowish-grey or pale greyish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2526.-1): occasional groups of rectangular sclereids with moderately thickened walls and a large lumen, from the stem base [H]; very numerous fragments of parenchyma with large ovoid cells (longitudinal section [K], transverse section [J]); spiral, reticulate or pitted lignified vessels, isolated or in small groups [D, G]; thin-walled, elongated cells of the piliferous layer (surface view [A], transverse section [B]), some with root hairs [Aa, Ba] or their scars [Ab]; the piliferous layer is usually accompanied by an underlying layer of cells with slightly thickened and elongated walls [Ac, Bb]; fragments of dermal tissue from the rhizome composed of 1 or 2 layers of polygonal cells with irregularly thickened walls [F]; a few groups of sclereids with thick walls and a narrow lumen [E] from the pith of the rhizome. Examine under a microscope using a 50 per cent *V/V* solution of *glycerol R*.

The powder shows numerous starch granules, simple or 2- to 6-compound, but frequently separated, rounded or irregular and up to about 15 µm in diameter; most of the granules show a rather indistinct cleft or radiate hilum [C].

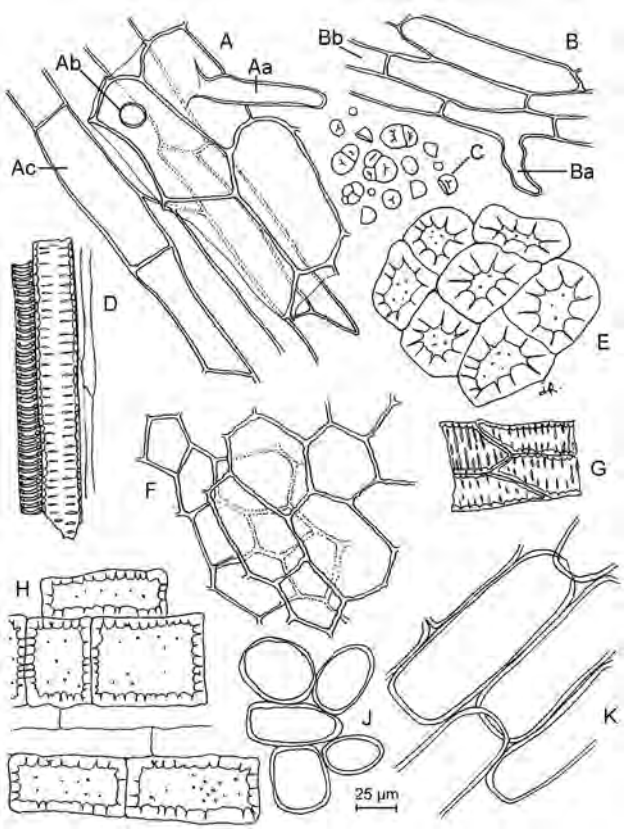


Figure 2526.-1. – *Illustration for identification test A of powdered herbal drug of cut valerian root*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 1 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *methanol R* and sonicate for 10 min. Filter the supernatant through a membrane filter (nominal pore size 0.45 µm). Use the filtrate.

*Reference solution.* Dissolve 5 mg of *acetoxyvalerenic acid R* and 5 mg of *valerenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other violet zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Valerenic acid: a violet zone | A violet zone (valerenic acid) |
| Acetoxyvalerenic acid: a violet zone | A violet zone (acetoxyvalerenic acid) |
| ——— | ——— |
| | 2 faint or very faint violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stem bases and maximum 2 per cent of other foreign matter, determined on the herbal drug prior to cutting.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of well-homogenised powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 5.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 40.0 g of freshly powdered herbal drug (500) (*2.9.12*), a 2000 mL flask, 500 mL of *water R* as the distillation liquid and 0.50 mL of *1,2,4-trimethylbenzene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 4 h.

**Sesquiterpenic acids**. Liquid chromatography (*2.2.29*).

*Test solution.* Place 1.50 g of the powdered herbal drug (710) (*2.9.12*) in a 100 mL round-bottomed flask with a ground-glass neck. Add 20 mL of *methanol R1*. Mix and heat on a water-bath under a reflux condenser for 30 min. Allow to cool and filter. Place the filter with the residue in the 100 mL round-bottomed flask. Add 20 mL of *methanol R1* and heat on a water-bath under the reflux condenser for 15 min. Allow to cool and filter. Combine the filtrates and dilute to 50.0 mL with *methanol R1*, rinsing the round-bottomed flask and the filter.

*Reference solution.* Dissolve an amount of *valerian dry extract HRS* corresponding to 1.0 mg of valerenic acid in *methanol R1* and dilute to 10.0 mL with the same solvent. Sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, 5 g/L solution of *phosphoric acid R* (20:80 *V/V*);

– *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 55 | 45 |
| 5 - 18 | 55 → 20 | 45 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Peak identification*: use the chromatogram supplied with *valerian dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to acetoxyvalerenic acid and valerenic acid.

*System suitability*: reference solution:

– *relative retention* with reference to valerenic acid (retention time = about 19 min): acetoxyvalerenic acid = about 0.5.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression:

$$\frac{(A_1 + A_2) \times m_2 \times p \times 5}{A_3 \times m_1}$$

$A_1$ = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to valerenic acid in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *valerian dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of valerenic acid in *valerian dry extract HRS*.

**07/2010:1899**

# VALERIAN TINCTURE

## Valerianae tinctura

DEFINITION

Tincture produced from *Valerian root (0453)*.

*Content*: minimum 0.015 per cent *m/m* of sesquiterpenic acids, expressed as valerenic acid ($C_{15}H_{22}O_2$; $M_r$ 234.3).

PRODUCTION

The tincture is produced from 1 part of the drug and 5 parts of ethanol (60 to 80 per cent *V/V*) by an appropriate procedure.

CHARACTERS

*Appearance*: brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 5 mL of the tincture to be examined with 5 mL of *ethanol (70 per cent V/V) R*.

*Reference solution.* Dissolve 5 mg of *acetoxyvalerenic acid R* and 5 mg of *valerenic acid R* in 20 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:38:60 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other violet zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| Valerenic acid: a violet zone | A violet zone (valerenic acid) |
| Acetoxyvalerenic acid: a violet zone | A violet zone (acetoxyvalerenic acid) |
| _____ | _____ |
| | 2 faint or very faint violet zones |
| **Reference solution** | **Test solution** |

TESTS

**Ethanol** (*2.9.10*): 95 per cent to 105 per cent of the quantity stated on the label.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dilute 10.0 g of the tincture to be examined to 50.0 mL with *methanol R1*.

*Reference solution.* Dissolve an amount of *valerian dry extract HRS* corresponding to 1.0 mg of valerenic acid in *methanol R1* and dilute to 10.0 mL with the same solvent. Sonicate for 10 min and filter through a membrane filter (nominal pore size 0.45 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, 5 g/L solution of *phosphoric acid R* (20:80 *V/V*);
- *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 55 | 45 |
| 5 - 18 | 55 → 20 | 45 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Peak identification*: use the chromatogram supplied with *valerian dry extract HRS* and the chromatogram obtained with the reference solution to identify the peaks due to acetoxyvalerenic acid and valerenic acid.

*System suitability*: reference solution:

- *relative retention* with reference to valerenic acid (retention time = about 19 min): acetoxyvalerenic acid = about 0.5.

Calculate the percentage content of sesquiterpenic acids, expressed as valerenic acid, using the following expression:

$$\frac{(A_1 + A_2) \times m_2 \times p \times 5}{A_3 \times m_1}$$

$A_1$ = area of the peak due to acetoxyvalerenic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to valerenic acid in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to valerenic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *valerian dry extract HRS* used to prepare the reference solution, in grams;

$p$ = percentage content of valerenic acid in *valerian dry extract HRS*.

**07/2012:1854**

# VERBENA HERB

## Verbenae herba

DEFINITION

Whole or fragmented, dried aerial parts of *Verbena officinalis* L. collected during flowering.

*Content*: minimum 1.5 per cent of verbenalin ($C_{17}H_{24}O_{10}$; $M_r$ 388.4) (dried drug).

IDENTIFICATION

A. The stem is greenish-brown, quadrangular, longitudinally grooved and roughly hairy, especially on the angles. The larger leaves are petiolate and deeply pinnately lobed, with bluntly dentate margins, the smaller leaves are sessile, not lobed, with crenate or dentate margins; the surfaces are rough and covered with bristly hairs, particularly over the veins, which are prominent on the lower surface. The flowers are numerous, arranged in a slender spike in the axils of leaf-like bracts; the tubular calyx has 5 acutely pointed lobes with the pale pink or lilac corolla forming a tube about twice as long as the calyx.

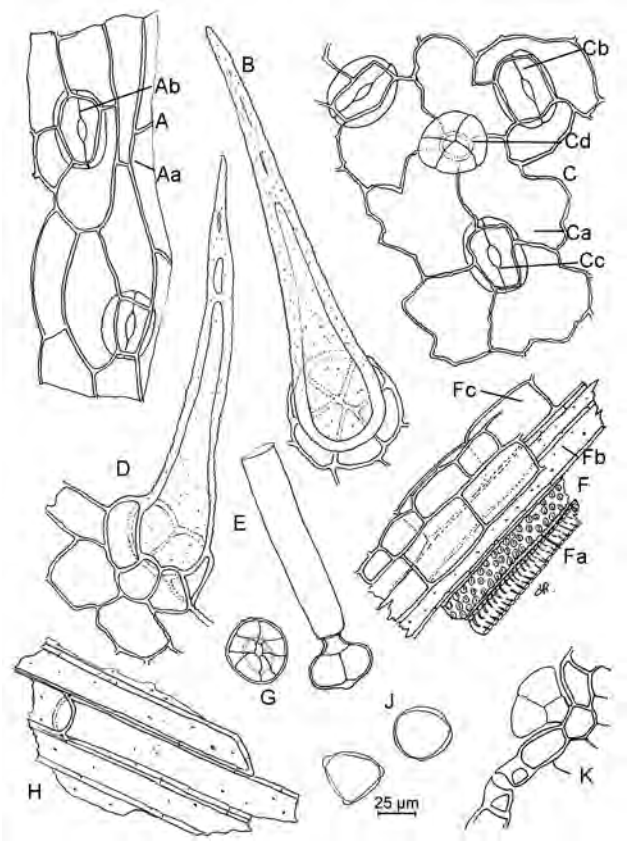


Figure 1854.-1. – *Illustration for identification test B of powdered herbal drug of verbena herb*

B. Microscopic examination (*2.8.23*). The powder is greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1854.-1): fragments of the leaves, which in surface view [C] show sinuous-walled epidermal cells [Ca] with anisocytic [Cb] or anomocytic [Cc] stomata (*2.8.3*), more numerous on the lower epidermis; fragments of stem epidermis [A] consisting of long, polygonal or rectangular epidermal cells [Aa] with thickened walls and stomata [Ab]; covering trichomes, unicellular, thick-walled, up to 500 µm long, wide at the base and arising from the centre of a single ring of domed, spherical epidermal cells (surface view [B], side view [D]); occasional glandular trichomes of 2 types: (a) long stalk with a flattened head about 35 µm in diameter and consisting of 4-8 radiating cells in side view [E] or in surface view of the head [G], and (b) short unicellular stalk and an enlarged ovate head composed of 4 radiating cells (surface view [Cd], transverse section [K]); triangular-ovoid or rounded pollen grains about 30 µm in diameter, with 3 pores and a smooth exine [J]; many fragments of stems [F] consisting of groups of fibres [Fb], vessels [Fa] and fragments of parenchyma [Fc]; isolated fragments of fibres [H].

C. Examine the chromatograms obtained in test B for *Aloysia citrodora*.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A brown or green zone |
| Arbutin: a blue or brown zone | |
| | An intense brownish-grey zone |
| Rutoside: a dark brownish-yellow zone | |
| **Reference solution** | **Test solution** |

TESTS

***Aloysia citrodora***

A. A lemon-like odour indicates the presence of *Aloysia citrodora*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (710) (*2.9.12*) add 5 mL of *methanol R*. Heat in a water-bath at 60 °C for 10 min. Cool and filter.

*Reference solution*. Dissolve 10 mg of *arbutin R* and 10 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *glacial acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 10 mm [or 8 mm].

*Development*: over a path of 12 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for about 10 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no intense blue or violet zone approximately at the position of rutoside in the chromatogram obtained with the reference solution.

Herbal drugs

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Internal standard solution.* Dissolve 10.0 mg of *ferulic acid R* in *ethanol (60 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

*Test solution.* To 1.00 g of the powdered herbal drug (710) (*2.9.12*) add 50.0 mL of the internal standard solution and stir with a magnetic stirrer for 2 h. Centrifuge for 15 min and filter the supernatant using a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve the contents of a vial of *verbenalin CRS* in the internal standard solution and dilute to 5.0 mL with the same solution.

*Precolumn*:

- *size*: *l* = 0.01 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*:

- *mobile phase A*: 0.3 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 93 → 83 | 7 → 17 |
| 20 - 30 | 83 | 17 |
| 30 - 35 | 83 → 75 | 17 → 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*System suitability*: test solution:

- the chromatogram obtained is similar to the chromatogram shown in Figure 1854.-2;
- *resolution*: minimum 3.5 between the peaks due to ferulic acid and acteoside.

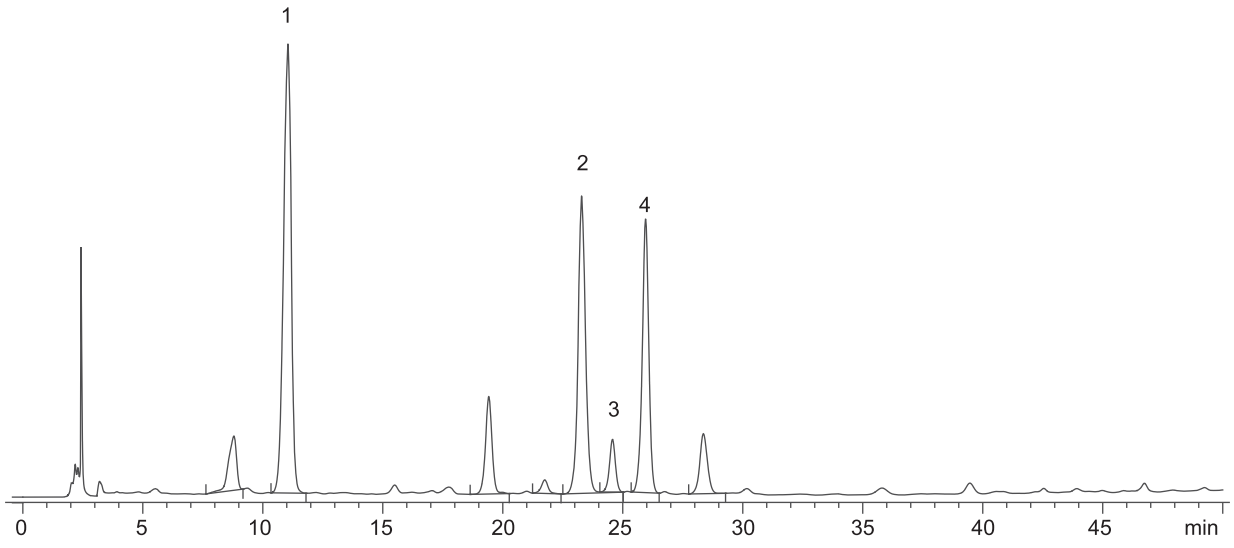


1. verbenalin 2. ferulic acid 3. unknown substance (may be absent) 4. acteoside

Figure 1854.-2. – *Chromatogram for the assay of verbena herb: test solution*

Calculate the percentage content of verbenalin using the following expression:

$$\frac{A_1 \times A_4 \times m_2 \times 1000}{A_2 \times A_3 \times m_1}$$

$A_1$ = area of the peak due to verbenalin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to verbenalin in the chromatogram obtained with the reference solution;

$A_3$ = area of the peak due to ferulic acid in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to ferulic acid in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug used to prepare the test solution, in grams;

$m_2$ = mass of verbenalin in the reference solution, in grams.

**01/2019:1835**

# WHITE HOREHOUND

## Marrubii herba

DEFINITION

Whole or fragmented dried flowering aerial parts of *Marrubium vulgare* L.

*Content*: minimum 0.7 per cent of marrubiin ($C_{20}H_{28}O_4$; $M_r$ 332.4) (dried drug).

IDENTIFICATION

A. The stems are up to 50 cm long, quadrangular, up to 7 mm wide, young stems are densely covered with whitish downy hairs, older stems are greenish-grey and less hairy. The lower leaves are broadly ovate to almost orbicular, upper leaves less broadly ovate, both petiolate; lamina 1.5-4 cm long, 1-3.5 cm wide, apex sub-acute, base tapering or somewhat cordate, margin dentate to crenate, petiole up to 3 cm long; venation pinnate, prominent on the lower surface, distinctly depressed on the upper surface. Both leaf surfaces are densely covered with fine, white, woolly hairs, older leaves having fewer hairs on the dark greyish-green upper surface. The flowers are small, sessile in dense axillary clusters. The calyx is 5 mm long, persistent, with 5 long and 5 short, alternating, hooked, recurved fringing spines; throat of calyx with an internal ring of long silky hairs; corolla 7 mm long, dull white, 4-lobed, upper lobe 2-lipped, lower-lobe 3-lipped; 4 short stamens; style with bifid stigma.

B. Microscopic examination (*2.8.23*). The powder is greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1835.-1): numerous covering trichomes of different types, isolated or associated with fragments of the epidermis of the leaves: a) covering trichomes in tufts [B, H] with 3-20 rigid branches of various sizes with smooth, regularly thickened walls, sometimes sessile [B], sometimes arising from a multicellular stalk [H], some branches are unicellular [Ba, Ha], others are multicellular (2-6 cells), 100-200 µm long, thick-walled and swollen at the junctions [Bb, Hb]; b) unicellular covering trichomes; c) multicellular covering trichomes similar to the branches of the trichomes in tufts; glandular trichomes of different types: a) glandular trichomes with a unicellular stalk and a unicellular [D], bicellular [Hc] or quadricellular head (surface view [Gb]); b) glandular trichomes with a multicellular stalk and a unicellular head [A]; c) glandular trichomes of lamiaceous type with a unicellular stalk and an 8-celled head covered with a cuticle (surface view [Gd]); fragments of the epidermis of the leaves [G] consisting of polygonal cells with slightly sinuous walls [Ga], stomata of the diacytic type (*2.8.3*) [Gc], covering trichomes and glandular trichomes; covering trichomes from the inner surface of the calyx, twisted or coiled, up to 1000 µm long, bi- or tricellular, thickened at the cell junctions, with a flexuous, very elongated distal cell [C]; fragments of palisade parenchyma (surface view [Bc]) containing small needle-shaped crystals of calcium oxalate; fragments of vascular tissue from the stems and veins [F]; fragments of petals with papillose cells [J]; spherical pollen grains, about 25 µm in diameter with a smooth exine [E].

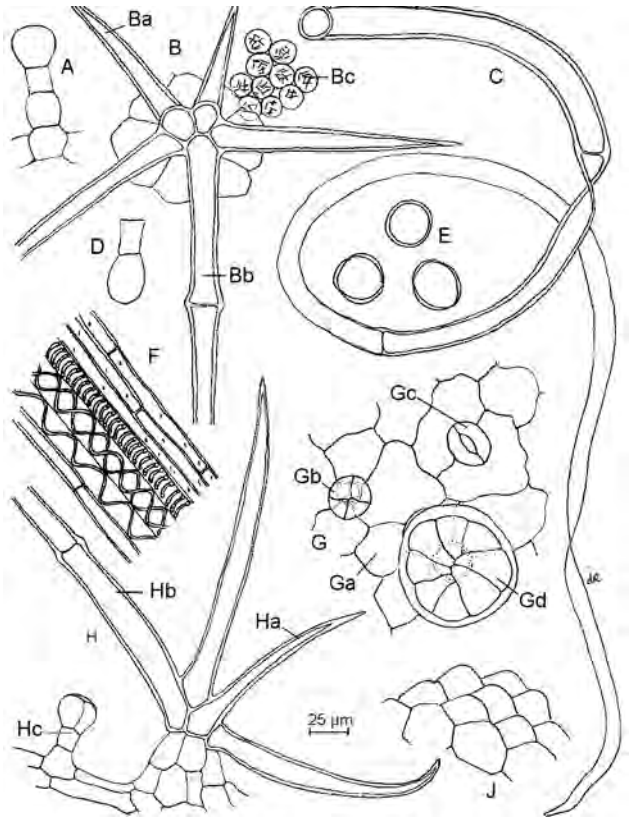


Figure 1835.-1. – *Illustration for identification test B of powdered herbal drug of white horehound*

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 2 mL of *dilute hydrochloric acid R* and 8 mL of *methanol R*. Heat under a reflux condenser for 30 min, cool and filter.

*Test solution (b).* To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *methanol R*. Heat under a reflux condenser for 30 min, cool and filter.

*Reference solution.* Dissolve 10 mg of *cholesterol R* and 10 mg of *guaiazulene R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methanol R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL [or 5 µL] of test solutions (a) and (b) and 10 µL [or 2 µL] of the reference solution, as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

Herbal drugs

*Detection*: treat with a 5 g/L solution of *vanillin R* in a mixture of 20 volumes of *ethanol (96 per cent) R* and 80 volumes of *sulfuric acid R* and examine in daylight immediately after heating at 130 °C for 5-10 min.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and test solutions (a) and (b). Further zones in the chromatograms obtained with test solutions (a) and (b) may be present. The zone due to marrubiin in the chromatogram obtained with test solution (a) is more intense than that in the chromatogram obtained with test solution (b). During extraction with hydrochloric acid and methanol, conversion of pre-marrubiin to marrubiin takes place which leads to an increase in intensity of the zone.

| Top of the plate | | |
|---|---|---|
| Guaiazulene: a reddish-violet zone | A bluish-violet zone | A bluish-violet zone |
| ——— | | ——— |
| | A bluish-violet zone | A bluish-violet zone |
| ——— | | ——— |
| Cholesterol: a bluish-violet zone | An intense bluish-violet zone (marrubiin) | A bluish-violet zone (marrubiin) |
| | A bluish-violet zone | A bluish-violet zone |
| | A bluish-violet zone | A bluish-violet zone |
| **Reference solution** | **Test solution (a)** | **Test solution (b)** |

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Reduce 50 g of the herbal drug to a powder (250) (*2.9.12*) and homogenise. To 1.00 g of the powdered herbal drug in a 50 mL round-bottomed flask add 15 mL of a mixture of 2 volumes of *dilute hydrochloric acid R* and 8 volumes of *methanol R*. Heat in a water bath at 80 °C under a reflux condenser for 30 min. Allow to cool to room temperature and filter through a plug of adsorbent cotton into a 25 mL volumetric flask. Dilute to 25.0 mL with *methanol R* by rinsing the round-bottomed flask and the filter.

*Reference solution.* Dissolve 2.0 mg of *marrubiin CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*;
- *mobile phase B*: dilute 0.5 mL of *phosphoric acid R* to 1000 mL with *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 40 → 90 | 60 → 10 |
| 15 - 20 | 90 → 40 | 10 → 60 |
| 20 - 25 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 217 nm.

*Injection*: 20 µL.

Locate the peak due to marrubiin by comparison with the chromatogram obtained with the reference solution.

Calculate the percentage content of marrubiin from the following expression:

$$\frac{A_1 \times m_2 \times p \times 2.5}{A_2 \times m_1}$$

$A_1$ = area of the peak due to marrubiin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to marrubiin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in milligrams;

$m_2$ = mass of *marrubiin CRS* used to prepare the reference solution, in milligrams;

$p$ = percentage content of marrubiin in *marrubiin CRS*.

**01/2008:1855**
**corrected 6.0**

# WILD PANSY (FLOWERING AERIAL PARTS)

## Violae herba cum flore

DEFINITION

Dried flowering aerial parts of *Viola arvensis* Murray and/or *Viola tricolor* L.

*Content*: minimum 1.5 per cent of flavonoids, expressed as violanthin ($C_{27}H_{30}O_{14}$; $M_r$ 578.5) (dried drug).

IDENTIFICATION

A. The stem is angular and hollow. The leaves are oval, petiolate, with a cordate base or elongated and obtuse, with lyrate stipules, divided in the middle. The flowers, with a long peduncle, are zygomorphic, with 5 oval, lanceolate sepals, an appendage pointed outwards and 5 petals of which the lower one bears a spur; in *Viola arvensis*, the petals are shorter than the calyx, the lower petal is cream coloured, with black lines, the 4 upper petals may be cream coloured or violet blue; in *Viola tricolor*, the petals are longer than the calyx and violet coloured, more or less tinged with yellow. The androecium consisting of 5 stamens bears at the apex a membranous connective appendage with 2 spurs. The trilocular ovary shows a short style and globular stigmata. The fruit are navicular capsules, three-lobed, yellowish brown, 5 mm to 10 mm long. The pale yellow, pyriform seeds are about 1 mm long, bearing a caruncle.

B. Microscopic examination (*2.8.23*). The powder is greenish. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epidermis of the leaves in surface view with wavy-walled cells and anomocytic stomata (*2.8.3*); conical unicellular covering trichomes, widened at the base and sharply pointed at the apex, with a striated cuticle; glandular trichomes with a multicellular head, and a short, multicellular stalk in the indentations of the leaf margins; cluster crystals of calcium oxalate, sometimes included in parenchyma; fragments of the corolla with wavy-walled epidermal cells, those from the mid-region papillose and with some extended to form flask or bottle-shaped projections, those from the base of the petals with covering trichomes up to about 300 µm long with characteristic hump-like swellings along their length;

spherical or polyhedral pollen grains, 60 µm to 80 µm in diameter, with finely pitted exines and 5 pores (*Viola arvensis*) or 4 pores (*Viola tricolor*); occasional fragments of spiral and reticulate vessels and groups of fibres from the stem.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Heat in a water-bath at 65 °C for 5 min, with frequent stirring, 2.0 g of the powdered herbal drug (355) (*2.9.12*) in 10 mL of *alcohol (70 per cent V/V) R*. Cool and filter.

*Reference solution.* Dissolve 2.5 mg of *rutoside trihydrate R*, 2.5 mg of *hyperoside R* and 1.0 mg of *caffeic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous formic acid R*, *acetic acid R*, *water R*, *ethyl acetate R* (11:11:27:100 *V/V/V/V*).

*Application*: 10 µL, as bands.

*Development*: over a path of 12 cm.

*Drying*: at 100-105 °C.

*Detection*: spray with a solution containing 10 g/L of *diphenylboric acid aminoethyl ester R* and 50 g/L of *macrogol 400 R* in *methanol R*. Allow the plate to dry in air for 30 min. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Caffeic acid: a greenish-blue to light blue fluorescent zone | A blue fluorescent zone |
| Hyperoside: a yellowish-brown fluorescent zone | A yellowish-green fluorescent zone |
| Rutoside: a yellowish-brown fluorescent zone | An intense yellowish-brown fluorescent zone (rutoside) |
| | A yellowish-green fluorescent zone |
| | A yellowish-green fluorescent zone |
| | A yellowish-green fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent.

**Swelling index** (*2.8.4*): minimum 9, determined on the powdered herbal drug (355) (*2.9.12*).

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 15.0 per cent.

ASSAY

*Stock solution.* In a 200 mL flask, introduce 0.300 g of the powdered herbal drug (250) (*2.9.12*) and 40 mL of *alcohol (60 per cent V/V) R*. Heat in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter through a plug of absorbent cotton into a 100 mL volumetric flask. Transfer the absorbent cotton with the drug residue back into the 200 mL flask, add 40 mL of *alcohol (60 per cent V/V) R* and heat again in a water-bath at 60 °C for 10 min, shaking frequently. Allow to cool and filter into the same 100 mL volumetric flask as used previously. Rinse the 200 mL flask with a further quantity of *alcohol (60 per cent V/V) R*, filter and transfer to the same 100 mL volumetric flask. Dilute to volume with *alcohol (60 per cent V/V) R* and filter.

*Test solution.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 mL volumetric flask as used previously. Add 10.0 mL of a solution containing 25.0 g/L of *boric acid R* and 20.0 g/L of *oxalic acid R* in *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

*Compensation liquid.* Introduce 5.0 mL of the stock solution into a round-bottomed flask and evaporate to dryness under reduced pressure. Take up the residue with 8 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into a 25 mL volumetric flask. Rinse the round-bottomed flask with 3 mL of a mixture of 10 volumes of *methanol R* and 100 volumes of *glacial acetic acid R* and transfer into the same 25 mL volumetric flask as used previously. Add 10.0 mL of *anhydrous formic acid R* and dilute to 25.0 mL with *anhydrous acetic acid R*.

Measure the absorbance (*2.2.25*) of the test solution at 405 nm after 30 min.

Calculate the percentage content of total flavonoids, expressed as violanthin from the expression:

$$\frac{A \times 1.25}{m}$$

taking the specific absorbance of violanthin to be 400.

$A$ = measured absorbance at 405 nm,

$m$ = mass of the herbal drug to be examined, in grams.

**07/2014:1891**

# WILD THYME

## Serpylli herba

DEFINITION

Whole or cut, dried, flowering aerial parts of *Thymus serpyllum* L.

*Content*: minimum 3.0 mL/kg of essential oil (dried drug).

IDENTIFICATION

A. The stem is much branched, up to about 1.5 mm in diameter, cylindrical or indistinctly quadrangular, green, reddish or purplish, the older stems brown and woody, the younger stems pubescent. The leaves are opposite, 3-12 mm long and up to 4 mm wide, elliptical to ovate-lanceolate with an obtuse apex, cuneate and shortly petiolate at the base; the margin is entire and markedly ciliate, especially near the base; both surfaces are more or less glabrous but distinctly punctate. The inflorescence is composed of about 6-12 flowers in rounded to ovoid, terminal heads. The calyx is tubular, 2-lipped with the upper lip dividing to form 3 teeth, the lower lip with 2 teeth, edged with long hairs; the inner surfaces are strongly pubescent, the hairs forming a closed tube after flowering. The corolla is purplish-violet

or red, 2-lipped, the lower lip with 3 lobes and the upper lip notched, the inner surface is strongly pubescent; 4 epipetalous stamens project from the corolla tube.

B. Microscopic examination (*2.8.23*). The powder is greyish-green or greenish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1891.-1): fragments of the leaf epidermises [A, B, F] covered by a finely striated cuticle and consisting of cells with sinuous anticlinal walls [Aa, Ba, Fa] and diacytic stomata (*2.8.3*) [Ab, Bb, Fb]; cells of the adaxial leaf epidermis [B] with wavy, irregularly thickened anticlinal walls [Ba]; numerous covering trichomes on both epidermises and the leaf margins, with some of the cells containing very small crystals of calcium oxalate [Af, Ca, Fd], the majority are short, conical, unicellular, with thickened and warty walls (surface view [Bc], side view [Fc]); fewer multicellular covering trichomes, long, tapering to a point, composed of up to 8 cells, slightly swollen at the joints, with finely pitted walls, on an epidermis [Ae] or fragmented [C]; abundant glandular trichomes, mostly multicellular of the lamiaceous type [Ac] with a unicellular stalk and a glandular head consisting of 12 inconspicuous cells, others with a unicellular stalk and a unicellular globular or ovoid head [Ad]; purplish-violet fragments of the corolla whose inner epidermis consists of cells with rounded papillae [D] and whose outer epidermis [E], with a striated cuticle, consists of cells with lobed walls [Ea], unicellular [Eb] or multicellular [Ec] uniseriate covering trichomes, glandular trichomes with a unicellular head and a unicellular stalk [Ed] and glandular trichomes of the lamiaceous type; relatively rare pollen grains, spherical, about 30 µm in diameter, with a finely pitted exine and 6 germinal pores [G].

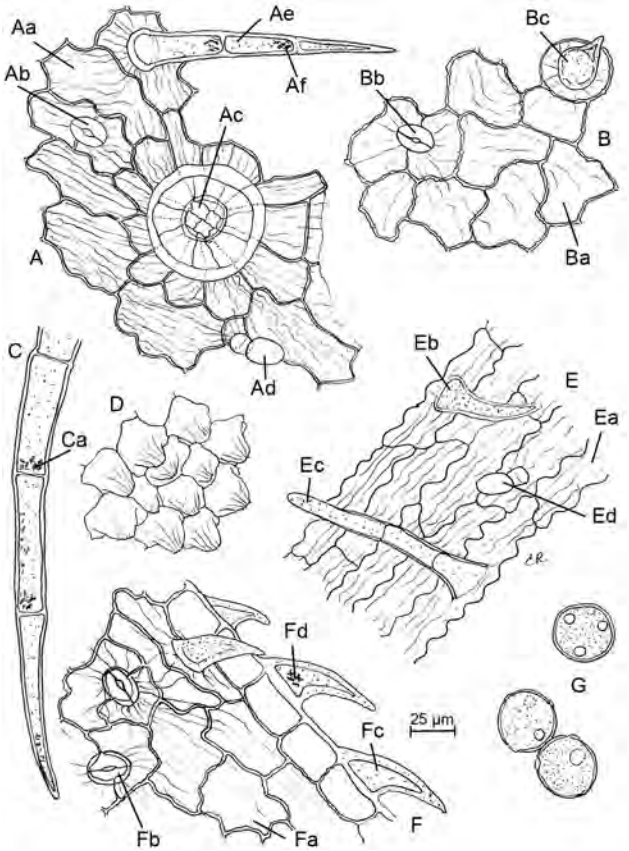


Figure 1891.-1.– *Illustration for identification test B of powdered herbal drug of wild thyme*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge or filter; use the supernatant or the filtrate.

*Reference solution*. Dissolve 1 mg of *rutoside trihydrate R* and 1 mg of *rosmarinic acid R* in 5 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (1:1:15 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands of 20 mm [or 8 mm].

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: heat at 100 °C for 3 min; treat the still-hot plate with a 5 g/L solution of *diphenylboric acid aminoethyl ester R* in *ethyl acetate R*, then treat with a 50 g/L solution of *macrogol 400 R* in *methylene chloride R*; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| | A red fluorescent zone |
| Rosmarinic acid: a blue fluorescent zone | A blue fluorescent zone (rosmarinic acid) |
| | 1 or 2 blue fluorescent zones |
| | A yellow or orange fluorescent zone |
| | A green or blue fluorescent zone may be present |
| Rutoside: an orange-yellow fluorescent zone | |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent, determined on 30 g.

***Thymus vulgaris* L. or *Thymus zygis* L.** Adulteration with *T. vulgaris* L. or *T. zygis* L. is indicated by the presence of acicular to linear-lanceolate leaves with a strongly bent margin, the adaxial surface showing covering trichomes shaped as pointed teeth with warty walls, the abaxial surface showing many types of warty covering trichomes: unicellular, straight or slightly curved, bicellular or tricellular, often elbow-shaped, and bicellular or tricellular, more or less straight.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 3.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 50.0 g of the cut herbal drug, a 1000 mL round-bottomed flask and 500 mL of *water R* as the distillation liquid. Distil at a rate of 2-3 mL/min for 2 h without *xylene R* in the graduated tube.

**01/2013:1583**

# WILLOW BARK

## Salicis cortex

DEFINITION

Whole or fragmented dried bark of young branches or whole dried pieces of current-year twigs of various species of genus *Salix* including *S. purpurea* L., *S. daphnoides* Vill. and *S. fragilis* L.

*Content*: minimum 1.5 per cent of total salicylic derivatives, expressed as salicin ($C_{13}H_{18}O_7$; $M_r$ 286.3) (dried drug).

IDENTIFICATION

A. The bark is 1-2 mm thick and occurs in flexible, elongated, quilled or curved pieces. The outer surface is smooth or slightly wrinkled longitudinally and greenish-yellow or brownish-grey. The inner surface is smooth or finely striated longitudinally and white, pale yellow or reddish-brown, depending on the species. The fracture is short in the outer part and coarsely fibrous in the inner region. The diameter of current-year twigs is not greater than 10 mm. The wood is white or pale yellow.

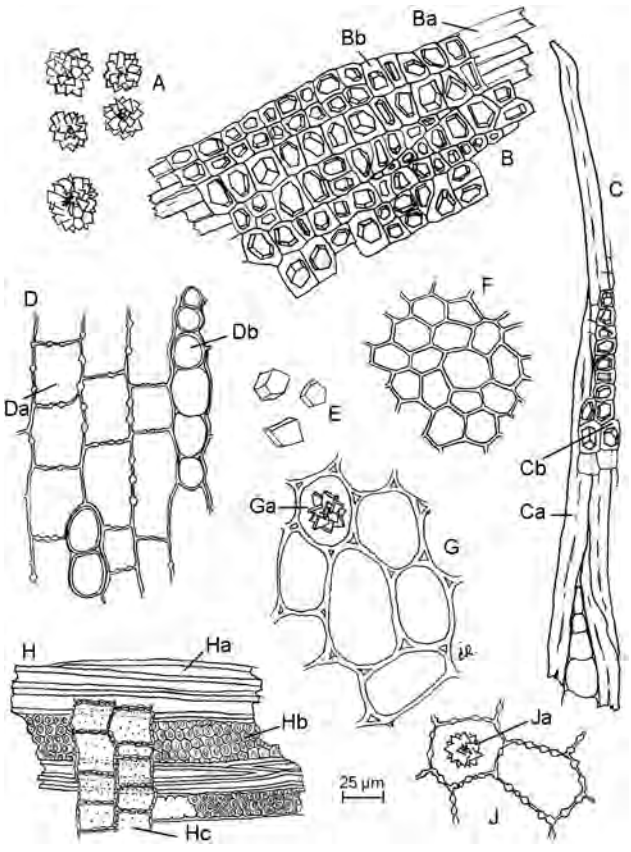


Figure 1583.-1. – *Illustration for identification test B of powdered herbal drug of willow bark*

B. Microscopic examination (*2.8.23*). The powder is pale yellow, greenish-yellow or light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1583.-1): bundles [B, C] of narrow fibres [Ba, Ca], up to about 600 µm long, with very thick walls and surrounded by a crystal sheath containing prisms of calcium oxalate [Bb, Cb]; parenchymatous cells of the cortex [D, J], with thick, pitted and deeply beaded walls [Da], and containing large cluster crystals of calcium oxalate [Ga, Ja]; some parenchyma cells are collenchymatous [G]; uniseriate medullary rays (tangential section [Db]); thickened cork cells (surface view [F]); numerous scattered prism crystals [E] and cluster crystals [A] of calcium oxalate; fragments of brownish collenchyma from the buds may also be present. Twigs show, additionally, wood fragments [H] composed of lignified fibres [Ha] and vessels [Hb], sometimes accompanied by medullary rays [Hc].

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* To 1.0 g of the powdered herbal drug (355) (*2.9.12*) add 10 mL of *methanol R*. Heat on a water-bath at about 50 °C, with frequent shaking, for 10 min. Cool and filter.

*Test solution (b).* To 5.0 mL of test solution (a) add 1.0 mL of a 50 g/L solution of *anhydrous sodium carbonate R* and heat in a water-bath at about 60 °C for 10 min. Cool and filter if necessary.

*Reference solution.* Dissolve 2 mg of *salicin R* and 2 mg of *chlorogenic acid R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in a current of warm air.

*Detection*: treat with a mixture of 5 volumes of *sulfuric acid R* and 95 volumes of *methanol R*. Heat at 100-105 °C for 5 min and examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and test solutions (a) and (b). Furthermore, other zones may be present in the chromatograms obtained with test solutions (a) and (b).

| Top of the plate | | |
|---|---|---|
| Salicin: a reddish-violet zone | Several reddish-violet zones may be present<br>A weak reddish-violet zone (salicin) | A reddish-violet zone (salicin) |
| Chlorogenic acid: a brown zone | | |
| **Reference solution** | **Test solution (a)** | **Test solution (b)** |

TESTS

**Foreign matter** (*2.8.2*): maximum 3 per cent of twigs with a diameter greater than 10 mm and maximum 2 per cent of other foreign matter.

**Cadmium** (*2.4.27*): maximum 2.0 ppm.

**Loss on drying** (*2.2.32*): maximum 11 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (355) (*2.9.12*) add 40 mL of *methanol R* and 40.0 mL of a 4.2 g/L solution of *sodium hydroxide R*. Heat in a water-bath at about 60 °C under a reflux condenser, with frequent shaking, for about 1 h. After cooling, add 4.0 mL of a 103.0 g/L solution of *hydrochloric acid R*. Filter the suspension into a 100 mL

volumetric flask, wash and dilute to 100.0 mL with a mixture of equal volumes of *methanol R* and *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 5.0 mg of *picein R* in 25.0 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R* (solution A). Dissolve 15.0 mg of *salicin CRS* in 25 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*; add 5.0 mL of solution A and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *tetrahydrofuran R*, 0.5 per cent *V/V* solution of *phosphoric acid R* (1.8:98.2 *V/V*);
- *mobile phase B*: *tetrahydrofuran R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 17 | 100 → 90 | 0 → 10 |
| 17 - 23 | 90 | 10 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*Retention time*: salicin = about 6.4 min; picein = about 7.7 min.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to salicin and picein.

Calculate the percentage content of total salicylic derivatives, expressed as salicin, using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to salicin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to salicin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *salicin CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of salicin in *salicin CRS*.

**04/2008:2312**

# WILLOW BARK DRY EXTRACT

## Salicis corticis extractum siccum

DEFINITION

Dry extract produced from *Willow bark (1583)*.

*Content*: minimum 5.0 per cent of total salicylic derivatives, expressed as salicin ($C_{13}H_{18}O_7$; $M_r$ 286.3) (dried extract).

PRODUCTION

The extract is produced from the herbal drug by a suitable procedure using either water or a hydroalcoholic solvent equivalent in strength to a maximum of 80 per cent *V/V* ethanol.

CHARACTERS

*Appearance*: yellowish-brown amorphous powder.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution (a).* To 0.200 g of the extract to be examined add 5 mL of *methanol R*. Sonicate for 5 min, filter and dilute to 10 mL with *methanol R*.

*Test solution (b).* To 5.0 mL of test solution (a) add 1.0 mL of a 50 g/L solution of *anhydrous sodium carbonate R* and heat in a water-bath at about 60 °C for 10 min. Cool and filter if necessary.

*Reference solution.* Dissolve 2.0 mg of *salicin R* and 2.0 mg of *chlorogenic acid R* in 1.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (8:15:77 *V/V/V*).

*Application*: 10 µL [or 2 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in a current of warm air.

*Detection*: spray with a mixture of 5 volumes of *sulfuric acid R* and 95 volumes of *methanol R*. Heat at 100-105 °C for 5 min and examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and test solutions (a) and (b). Furthermore, other zones may be present in the chromatogram obtained with test solutions (a) and (b).

| Top of the plate | | |
|---|---|---|
| | Several reddish-violet zones may be present | |
| Salicin: a reddish-violet zone | A weak reddish-violet zone (salicin) | A reddish-violet zone (salicin) |
| Chlorogenic acid: a brown zone | | |
| **Reference solution** | **Test solution (a)** | **Test solution (b)** |

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 0.300 g of the extract to be examined add 40 mL of *methanol R* and 40.0 mL of *0.1 M sodium hydroxide*. Heat in a water-bath at about 60 °C under a reflux condenser, with frequent shaking, for about 1 h. After cooling, add 4.0 mL of *1 M hydrochloric acid*. Filter the suspension into a 100 mL volumetric flask, then wash and dilute to 100.0 mL with a mixture of equal volumes of *water R* and *methanol R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 5.0 mg of *picein R* in 25.0 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R* (solution A). Dissolve 15.0 mg of *salicin CRS* in 25 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*. Add 5.0 mL of solution A and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

Herbal drugs

*Mobile phase*:
- *mobile phase A*: *tetrahydrofuran R*, 0.5 per cent *V/V* solution of *phosphoric acid R* (1.8:98.2 *V/V*);
- *mobile phase B*: *tetrahydrofuran R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 17 | 100 → 90 | 0 → 10 |
| 17 - 23 | 90 | 10 |
| 23 - 25 | 90 → 100 | 10 → 0 |
| 25 - 40 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*Retention time*: salicin = about 6.4 min; picein = about 7.7 min.

*System suitability*: reference solution:
- *resolution*: minimum 1.5 between the peaks due to salicin and picein.

Calculate the percentage content of total salicylic derivatives, expressed as salicin, from the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to salicin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to salicin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the extract to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *salicin CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of salicin in *salicin CRS*.

**04/2011:1380**

# WORMWOOD

## Absinthii herba

DEFINITION

Basal leaves or slightly leafy, flowering tops, or mixture of these dried, whole or cut organs of *Artemisia absinthium* L.

*Content*: minimum 2 mL/kg of essential oil (dried drug).

IDENTIFICATION

A. The leaves are greyish or greenish, densely tomentose on both surfaces. The basal leaves, with long petioles, have triangular or oval bipinnatisect or tripinnatisect lamina, with rounded or lanceolate segments. The cauline leaves are less segmented and the apical leaves are lanceolate. The stem of the flower-bearing region is greenish-grey, tomentose, up to 2.5 mm in diameter and usually with 5 flattened longitudinal grooves. The capitula are arranged as loose, axillary panicles, inserted at the level of the lanceolate or slightly pinnatisect leaves; they are spherical or flattened hemispherical, 2-4 mm in diameter and consist of a grey, tomentose involucre, the outer bracts linear, inner layer ovate, blunt at the apices with scarious margins, a receptacle with very long paleae up to 1 mm or more long, numerous yellow, tubular, hermaphroditic florets about 2 mm long and few yellow, ray florets.

B. Microscopic examination (*2.8.23*). The powder is greenish-grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1380.-1.): many T-shaped trichomes [A] with a short uniseriate stalk consisting of 1-5 small cells, perpendicularly capped by a very long, undulating terminal cell tapering at the ends; fragments of epidermises (surface view [D]) with sinuous or wavy walls, anomocytic stomata (*2.8.3*) [Da], covering trichomes [Db] and glandular trichomes containing oil [Dc] or not containing oil [Dd], each with a short, biseriate, 2-celled stalk and a biseriate head with 2-4 cells; free glandular trichomes (side view [C]); fragments of the corollas of the tubular and ray florets, some containing small cluster crystals of calcium oxalate [H]; numerous paleae each composed of a small cell forming a stalk and a very long, cylindrical and thin-walled terminal cell about 1-1.5 mm long, either whole [E] or limited to the distal part [B]; spheroidal pollen grains, about 30 µm in diameter, with 3 pores and a finely warty exine [G]; fragments of vascular tissue from the leaves [F] or the stems [J] consisting of vessels with spiral or annular thickenings [Fa], or with bordered pits [Ja], fibres [Fb, Jb] and parenchymatous cells with pitted, moderately thickened walls [Fc, Jc].

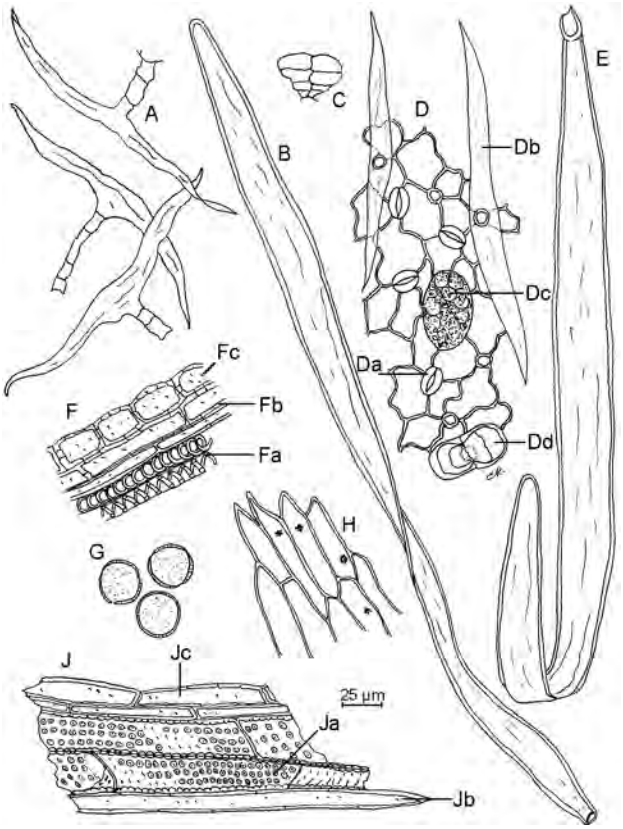


Figure 1380.-1. – *Illustration for identification test B of powdered herbal drug of wormwood*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Place 2 g of the powdered herbal drug (355) (*2.9.12*) in 50 mL of boiling *water R* and allow to stand for 5 min, shaking the flask several times. After cooling, add 5 mL of a 100 g/L solution of *lead acetate R*. Mix and filter. Rinse the flask and the residue on the filter with 20 mL of *water R*. Shake the filter with 50 mL of *methylene chloride R*. Separate the organic layer, dry over *anhydrous sodium sulfate R*, filter and evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 0.5 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 2 mg of *methyl red R* and 2 mg of *resorcinol R* in 10.0 mL of *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetone R*, *glacial acetic acid R*, *toluene R*, *methylene chloride R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: spray with *acetic anhydride - sulfuric acid solution R* and examine in daylight.

*Results A*: the chromatogram obtained with the test solution shows a blue zone due to artabsin shortly above a red zone due to methyl red in the chromatogram obtained with the reference solution.

*Detection B*: examine in daylight while heating at 100-105 °C for 5 min.

*Results B*: the chromatogram obtained with the reference solution shows in the middle third a red zone due to methyl red and below it a light pink zone due to resorcinol. The chromatogram obtained with the test solution shows an intense red or brownish-red zone due to absinthin with a similar $R_F$ value to that of the zone due to resorcinol in the chromatogram obtained with the reference solution. Other zones are visible, but less intense than that due to absinthin.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems with a diameter greater than 4 mm and maximum 2 per cent of other foreign matter.

**Bitterness value** (*2.8.15*): minimum 10 000.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 12.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.0 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 50.0 g of the cut drug, a 1000 mL round-bottomed flask and 500 mL of *water R* as the distillation liquid. Add 0.5 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for not less than 3 h.

**07/2014:1382**

# YARROW

## Millefolii herba

DEFINITION

Whole or cut, dried flowering tops of *Achillea millefolium* L.

*Content*:

– *essential oil*: minimum 2 mL/kg (dried drug);
– *proazulenes, expressed as chamazulene* ($C_{14}H_{16}$; $M_r$ 184.3): minimum 0.02 per cent (dried drug).

IDENTIFICATION

A. The leaves are green or greyish-green, faintly pubescent on the upper surface and more pubescent on the lower surface, 2-3 pinnately divided with linear lobes and a finely pointed whitish tip. The capitula are arranged in a corymb at the end of the stem. Each capitulum, 3-5 mm in diameter, consists of the receptacle, usually 4-5 ligulate ray-florets and 3-20 tubular disk-florets. The involucre consists of 3 rows of imbricate lanceolate, pubescent green bracts arranged with a brownish or whitish, membranous margin. The receptacle is slightly convex and, in the axillae of paleae, bears ligulate ray-florets with a three-lobed, whitish or reddish ligule and tubular disk-florets with a radial, five-lobed, yellowish or light brownish corolla. The pubescent green, partly brown or violet stems are longitudinally furrowed, up to 3 mm thick with a light-coloured medulla.

B. Microscopic examination (*2.8.23*). The powder is green or greyish-green. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 1382.-1): fragments of the stem epidermis (surface view [K]), with cells having a smooth cuticle and anomocytic stomata (*2.8.3*); fragments of leaf and bract epidermises (surface view [B]), with cells having wavy and irregularly thickened walls, a finely striated cuticle and anomocytic stomata (*2.8.3*); very rare glandular trichomes with a short stalk and a head formed of 2 rows of 3-5 cells enclosed in a bladder-like membrane [H]; uniseriate, whole or fragmented covering trichomes [A] consisting of 4-6 small, more or less isodiametric cells at the base and a thick-walled, often somewhat tortuous terminal cell, about 400 µm to greater than 1000 µm long; fragments of the ligulate corolla with papillary epidermal cells [D]; fragments of the corolla tubes, with sinuous epidermal cells, covered by a thin striated cuticle (surface view [F]); small-celled parenchyma from the corolla tubes containing cluster crystals of calcium oxalate [E]; groups of lignified and pitted cells from the bracts [G]; spherical pollen grains, about 30 µm in diameter, with 3 germinal pores and a spiny exine [C]; groups of sclerenchymatous fibres and small vessels with spiral or annular thickening, from the stem [J].

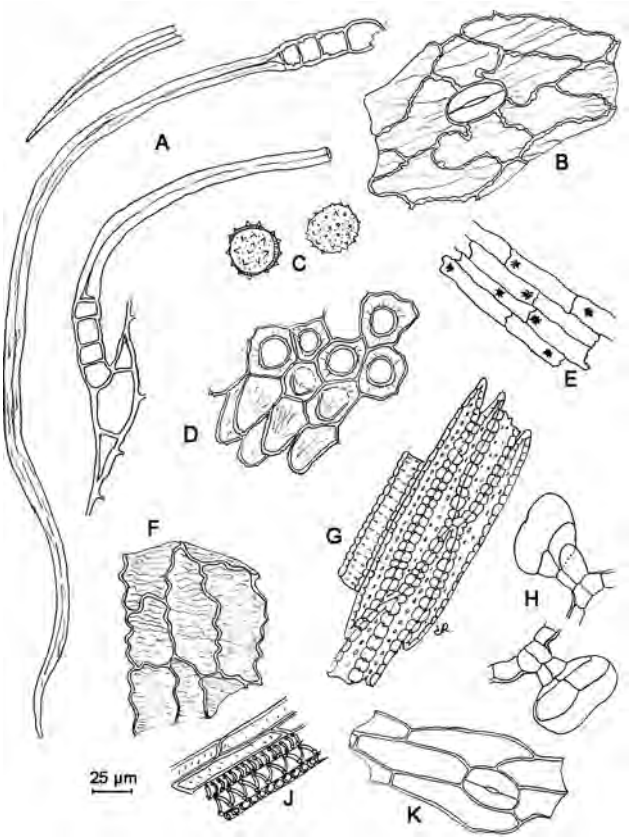


Figure 1382.-1. – *Illustration for identification test B of powdered herbal drug of yarrow*

C. To 2.0 g of the powdered herbal drug (710) (*2.9.12*) add 25 mL of *ethyl acetate R*, shake for 5 min and filter. Evaporate to dryness on a water-bath and dissolve the residue in 0.5 mL of *toluene R* (solution A). To 0.1 mL of this solution add 2.5 mL of *dimethylaminobenzaldehyde solution R8* and heat on a water-bath for 2 min. Allow to cool. Add 5 mL of *light petroleum R* and shake the mixture vigorously. The aqueous layer shows a blue or greenish-blue colour.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Use solution A prepared in identification test C.

*Reference solution.* Dissolve 10 mg of *cineole R* and 10 mg of *guaiazulene R* in 20 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 20 µL as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: treat with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows in the upper part a red zone (guaiazulene) and in the middle part a blue or greyish-blue zone (cineole). The chromatogram obtained with the test solution shows a violet zone a little above the zone due to guaiazulene in the chromatogram obtained with the reference solution; below this zone a reddish-violet zone; below which, 1-2 not clearly separated greyish-violet or greyish zones (which changes to greenish-grey after a few hours) and a reddish-violet zone a little above the zone due to cineole in the chromatogram obtained with the reference solution. Furthermore, other faint zones may be present.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of stems with a diameter greater than 3 mm and maximum 2 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 0.500 g of the powdered herbal drug (355) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 10.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.5 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 20.0 g of cut herbal drug, a 1000 mL round-bottomed flask and 500 mL of a mixture of 1 volume of *water R* and 9 volumes of *ethylene glycol R* as the distillation liquid. Add 0.50 mL of *1,2,4-trimethylbenzene R* in the graduated tube. Distil at a rate of 3-4 mL/min for 4 h.

Stop cooling at the end of distillation and continue distilling until the blue, steam-volatile components have reached the lower end of the cooler. Immediately start cooling again, to avoid warming the separation space. Stop the distillation after 10 min.

**Proazulenes**. To ensure that as little water as possible is transferred, transfer the blue mixture of essential oil and 1,2,4-trimethylbenzene obtained in the assay of essential oil into a 50 mL volumetric flask with the aid of small portions of *xylene R*, rinsing the graduated tube of the apparatus with *xylene R*, and dilute to 50.0 mL with the same solvent. Measure the absorbance (*2.2.25*) at 608 nm using *xylene R* as the compensation liquid.

Calculate the percentage content of proazulenes, expressed as chamazulene, using the following expression:

$$\frac{A \times 2.1}{m}$$

i.e. taking the specific absorbance of chamazulene to be 23.8.

$A$ = absorbance at 608 nm;

$m$ = mass of the herbal drug to be examined, in grams.

**01/2017:2656**

# ZANTHOXYLUM BUNGEANUM PERICARP

## Zanthoxyli bungeani pericarpium

DEFINITION

Dried pericarp of the ripe fruit, with seeds removed, of *Zanthoxylum bungeanum* Maxim.

*Content*: minimum 15 mL/kg of essential oil (anhydrous drug).

IDENTIFICATION

A. Single, dehiscent spherical capsules, split along the ventral suture, 4-5 mm in diameter; the outer surface is dark red or brownish-red, with numerous convex, translucent verrucose oil dots; the inner surface is light yellow and smooth; the endocarp is mostly separated from the mesocarp at the base and is rolled up.

B. Microscopic examination (*2.8.23*). The powder is reddish-brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters: fragments of the epicarp, covered by a striated cuticle, consisting of polygonal cells, with rigid walls and anomocytic stomata (*2.8.3*) with 5-7 subsidiary cells, some cells of the epicarp contain orange-yellow granular contents; fragments of the mesocarp consisting of parenchymatous cells some of which contain cluster crystals of calcium oxalate, elongated cells of the oil glands containing droplets of essential oil, vascular bundles with spiral vessels accompanied by fibres with regularly thickened, pitted walls and pointed ends, sometimes bifurcated, prisms of calcium oxalate, especially in the area of the vascular bundles; fragments of the endocarp consisting of several layers of narrow, elongated, thick-walled, pitted cells in a parquetry arrangement, with the arrangements in the different layers oriented crosswise to each other.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 0.5 g of the powdered herbal drug (355) (*2.9.12*) add 5 mL of *methanol R*. Sonicate for 10 min. Centrifuge or filter. Use the supernatant or filtrate.

*Reference solution.* Dissolve 1 mg of *chlorogenic acid R* and 1 mg of *emodin R* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (10:20:80 *V/V/V*).

*Application*: 5 µL as bands of 8 mm.

*Development*: over a path of 6 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 366 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Emodin: a yellow fluorescent zone | A red fluorescent zone<br>A green fluorescent zone |
| _____ | _____ |
| | A red fluorescent zone may be present<br>A faint green fluorescent zone |
| | 2 green fluorescent zones |
| | A green fluorescent zone |
| _____ | _____ |
| | A green fluorescent zone |
| Chlorogenic acid: a blue fluorescent zone | A blue fluorescent zone |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent of seeds and maximum 2 per cent of other foreign matter.

**Water** (*2.2.13*): maximum 100 mL/kg, determined on 20.0 g of the powdered herbal drug (710) (*2.9.12*).

**Total ash** (*2.4.16*): maximum 8.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 1.5 per cent.

ASSAY

**Essential oil** (*2.8.12*). Use 15.0 g of freshly powdered herbal drug (710) (*2.9.12*), a 500 mL round-bottomed flask, 250 mL of *water R* as the distillation liquid and 0.50 mL of *xylene R* in the graduated tube. Distil at a rate of 2-3 mL/min for 2 h.

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**01/2008:90006**
**corrected 10.0**

# HOMOEOPATHIC PREPARATIONS: INTRODUCTION

*All general texts and other monographs of the European Pharmacopoeia that are relevant to homoeopathy are applicable.*

*The 'Homoeopathy' chapter of the European Pharmacopoeia contains general monographs and individual monographs describing starting materials and preparations used virtually exclusively for homoeopathic medicines. Reference to these monographs for other purposes may be authorised by licensing authorities.*

**04/2017:1038**

# HOMOEOPATHIC PREPARATIONS

## Praeparationes homoeopathicae

DEFINITION

Homoeopathic preparations are prepared from substances, products or preparations called stocks, in accordance with a homoeopathic manufacturing procedure. A homoeopathic preparation is usually designated by the Latin name of the stock, followed by an indication of the degree of dilution and/or potentisation, if applicable.

**Raw materials**

Raw materials for the production of homoeopathic preparations may be of natural or synthetic origin.

For raw materials of zoological or human origin, adequate measures are taken to minimise the risk of agents of infection, including viruses (*5.1.7*), in the homoeopathic preparations. For this purpose, it is demonstrated that:

- the method of production includes a step or steps that have been shown to remove or inactivate agents of infection;
- where applicable, raw materials of zoological origin comply with the monograph *Products with risk of transmitting agents of animal spongiform encephalopathies (1483)*;
- where applicable, the animals and the tissues used to obtain the raw materials comply with the health requirements of the competent authorities for animals for human consumption;
- for materials of human origin, the donor follows the recommendations applicable to human blood donors and to donated blood (see *Human plasma for fractionation (0853)*), unless otherwise justified and authorised.

A raw material of botanical, zoological or human origin may be used either in the fresh state or in the dried state. Where appropriate, fresh material may be kept deep-frozen. Raw materials of botanical origin comply with the requirements of the monograph *Herbal drugs for homoeopathic preparations (2045)*.

Where justified and authorised for transportation or storage purposes, fresh plant material may be kept in ethanol (96 per cent) or in ethanol of a suitable concentration, provided the whole material including the storage medium is used for processing.

Raw materials comply with any requirements of the relevant monographs of the European Pharmacopoeia.

**Vehicles**

Vehicles are excipients used for the preparation of certain stocks or for the potentisation process. They may include, for example: purified water, ethanol of a suitable concentration, glycerol and lactose.

Vehicles comply with any requirements of the relevant monographs of the European Pharmacopoeia.

**Stocks**

Stocks are substances, products or preparations used as starting materials for the production of homoeopathic preparations. A stock is usually one of the following: a mother tincture or a glycerol macerate, for raw materials of botanical, zoological or human origin, or the substance itself, for raw materials of chemical or mineral origin.

Mother tinctures comply with the requirements of the monograph *Mother tinctures for homoeopathic preparations (2029)*.

Glycerol macerates are liquid preparations obtained from raw materials of botanical, zoological or human origin by using glycerol or a mixture of glycerol and either ethanol of a suitable concentration or a solution of sodium chloride of a suitable concentration.

**Potentisation**

Dilutions and triturations are obtained from stocks by a process of potentisation in accordance with a homoeopathic manufacturing procedure: this means successive dilutions and succussions, or successive appropriate triturations, or a combination of the 2 processes.

The potentisation steps are usually one of the following:

- 1 part of the stock plus 9 parts of the vehicle; they may be designated as 'D', 'DH' or 'X' (decimal);
- 1 part of the stock plus 99 parts of the vehicle; they may be designated as 'C' or 'CH' (centesimal).

The number of potentisation steps defines the degree of dilution; for example, 'D3', '3 DH' or '3X' means 3 decimal potentisation steps, and 'C3', '3 CH' or '3C' means 3 centesimal potentisation steps.

'LM' potencies are manufactured according to a specific procedure with a 50 000 dilution factor by alternate steps of liquid dilution and impregnation of pillules. The number of potentisation steps defines the degree of dilution, for example, 3rd LM means 3 successive LM dilutions.

**Dosage forms**

A dosage form of a homoeopathic preparation complies with any relevant dosage form monograph in the European Pharmacopoeia, and with the following:

- for the purpose of dosage forms for homoeopathic use, 'active substances' are considered to be 'dilutions or triturations of homoeopathic stocks' or 'homoeopathic stocks' (in case of a mother tincture or a glycerol macerate);
- these dosage forms can contain one or more 'active substances';
- they are prepared using appropriate excipients.

*Homoeopathic dosage form 'pillule'*

Pillules for homoeopathic use are solid preparations obtained from sucrose, lactose or other suitable excipients. *Pillules for homoeopathic preparations (2153)* are intended for impregnation or coating with one or more homoeopathic preparations. The impregnated pillules comply with the requirements of the monograph *Homoeopathic pillules, impregnated (2079)*. The coated pillules comply with the requirements of the monograph *Homoeopathic pillules, coated (2786)*. Both are intended for sublingual or oral use.

*Homoeopathic dosage form 'tablet'*

Tablets for homoeopathic use are solid preparations obtained from sucrose, lactose or other suitable excipients according to the monograph *Tablets (0478)*. They may be prepared either by compressing one or more 'active substances' with

the excipients or by impregnating preformed tablets with one or more liquid 'active substances'. The preformed tablets for impregnation are obtained from sucrose, lactose or other suitable excipients according to the monograph *Tablets (0478)*. Tablets for homoeopathic use are intended for sublingual or oral use.

*Homoeopathic dosage forms 'parenteral preparation', 'eye preparation', 'nasal preparation'*

For the last potentisation step(s), an ethanol-free vehicle is used to minimise the content of ethanol in the final preparation.

The residual ethanol content (*2.9.10*) is not greater than 1 per cent *V/V* unless otherwise justified and authorised.

*Manufacturing methods*

Homoeopathic preparations are produced using the methods described in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*. The preparations obtained using the manufacturing methods listed in Table 1038.-1 are restricted to the production of the corresponding dosage forms indicated in the right-hand column of table.

The competent authority has the right to accept or reject particular combinations of manufacturing method and substance.

Table 1038.-1.

| Manufacturing methods | Dosage forms |
|---|---|
| 1.4.1, 1.4.2, 1.4.3, 1.4.4 | Eye drops<br>Coated homoeopathic pillules<br>Solutions for injection |
| 2.1.2 | Eye drops<br>Solutions for injection<br>Nasal preparations |
| 2.2.1, 2.2.2, 2.2.3 | Eye drops<br>Coated homoeopathic pillules<br>Solutions for injection<br>Nasal preparations<br>Ointments, creams and gels<br>Oral powders (triturations)<br>Suppositories |
| 2.2.4 | Solutions for injection |
| 3.1.2, 3.2.2 | Eye drops<br>Coated homoeopathic pillules<br>Solutions for injection<br>Nasal preparations<br>Ointments, creams and gels<br>Suppositories |

**01/2012:2045**

# HERBAL DRUGS FOR HOMOEOPATHIC PREPARATIONS

## Plantae medicinales ad praeparationes homoeopathicas

DEFINITION

Herbal drugs for homoeopathic preparations are mainly whole plants or parts of plants, fragmented or broken, and include algae, fungi or lichens, in an unprocessed state, usually in fresh form. The state, fresh or dried, in which the drug is used, is defined in the individual monograph of the European Pharmacopoeia or, in its absence, in the individual monograph of an official national pharmacopoeia of a member state. In the absence of such a monograph, the state in which the herbal drug is used has to be defined. Certain exudates that have not been subjected to a specific treatment are also considered to be herbal drugs for homoeopathic preparations. Herbal drugs for homoeopathic preparations are precisely defined by the botanical scientific name of the source species according to the binomial system (genus, species, variety and author).

*Whole* describes a herbal drug for homoeopathic preparations that has not been reduced in size and is presented, dried or undried, as harvested.

*Fragmented* describes a herbal drug for homoeopathic preparations that has been reduced in size after harvesting to permit ease of handling, drying and/or packaging.

*Broken* describes a herbal drug for homoeopathic preparations in which the more fragile parts of the plant have broken during drying, packaging or transportation.

For dried herbal drugs for homoeopathic preparations, *cut* describes size reduction, other than powdering, that reduces the particle size below that which is described in the macroscopic identity of the herbal drug for homoeopathic preparations.

PRODUCTION

Herbal drugs for homoeopathic preparations are obtained from cultivated or wild plants. Suitable collection, cultivation, harvesting, sorting, drying, fragmentation and storage conditions are essential to guarantee the quality of herbal drugs for homoeopathic preparations.

Herbal drugs for homoeopathic preparations are, as far as possible, free from impurities such as soil, dust, dirt and other contaminants such as fungal, insect and other animal contaminants. They do not present signs of decay.

If a decontaminating treatment has been used, it is necessary to demonstrate that the constituents of the plant are not affected and that no harmful residues remain. The use of ethylene oxide is prohibited for the decontamination of herbal drugs for homoeopathic preparations.

Fresh herbal drugs are processed as rapidly as possible after harvesting. Where justified and authorised for transportation or storage purposes, fresh plant material may be deep-frozen; it may also be kept in ethanol (96 per cent) or in ethanol of a suitable concentration, provided the whole material including the storage medium is used for processing.

Adequate measures have to be taken in order to ensure that the microbiological quality of homoeopathic preparations containing 1 or more herbal drugs comply with the recommendations given in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

IDENTIFICATION

Herbal drugs for homoeopathic preparations are identified using their macroscopic and, where necessary, microscopic descriptions and any further tests that may be required (for example, thin-layer chromatography).

TESTS

The tests for foreign matter and loss on drying should be performed before any further processing of the fresh plant.

**Foreign matter** (*2.8.2*). Where a fresh plant is used as a starting material for the manufacture of homoeopathic preparations, the content of foreign matter is as low as possible; if necessary, the maximum content of foreign matter is indicated in the individual monograph.

Where a dried plant is used as a starting material for the manufacture of homoeopathic preparations, carry out a test for foreign matter, unless otherwise prescribed in the individual monograph. The content of foreign matter is not more than 2 per cent *m/m*, unless otherwise prescribed or justified and authorised.

**Adulteration**. A specific appropriate test may apply to herbal drugs for homoeopathic preparations liable to be falsified.

**Loss on drying** (*2.2.32*). Carry out a test for loss on drying on dried herbal drugs for homoeopathic preparations.

If a fresh plant is processed more than 24 h after harvesting, a test for loss on drying should be carried out. The minimum limit is indicated in the individual monograph.

**Water** (*2.2.13*). A determination of water is carried out on herbal drugs for homoeopathic preparations with a high essential oil content.

**Pesticides** (*2.8.13*). Herbal drugs for homoeopathic preparations comply with the requirements for pesticide residues. The requirements take into account the origin and the nature of the plant, where necessary the preparation in which the plant might be used and, where available, knowledge of the complete record of treatment of the batch of the plant. Where justified, the test for pesticides may be performed on the mother tincture according to the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

*If appropriate, herbal drugs for homoeopathic preparations comply with other tests, such as the following, for example.*

**Total ash** (*2.4.16*).

**Bitterness value** (*2.8.15*).

**Heavy metals** (*2.4.27*). Unless otherwise stated in an individual monograph or unless otherwise justified and authorised:

- *cadmium*: maximum 1.0 ppm;
- *lead*: maximum 5.0 ppm;
- *mercury*: maximum 0.1 ppm.

If justified by the nature or origin of the herbal drug or if required by the competent authority, suitable limits for the content of other heavy metals such as arsenic or nickel are defined.

Where justified, the test for heavy metals may be performed on the mother tincture according to the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

**Aflatoxin $B_1$** (*2.8.18*). Where appropriate, limits for aflatoxins may be required.

**Ochratoxin A** (*2.8.22*). Where appropriate, a limit for ochratoxin A may be required.

**Radioactive contamination**. In some specific circumstances, the risk of radioactive contamination is to be considered.

ASSAY

Where applicable, herbal drugs for homoeopathic preparations are assayed by an appropriate method.

STORAGE

Store dried herbal drugs protected from light.

**01/2017:2029**

# MOTHER TINCTURES FOR HOMOEOPATHIC PREPARATIONS

## Tincturae maternae ad praeparationes homoeopathicas

DEFINITION

Mother tinctures for homoeopathic preparations are liquid preparations obtained by the solvent action of a suitable vehicle upon raw materials. The raw materials are usually in the fresh form but may be dried. Mother tinctures for homoeopathic preparations may also be obtained from plant juices, with or without the addition of a vehicle. For some preparations, the matter to be extracted may undergo a preliminary treatment.

PRODUCTION

Mother tinctures for homoeopathic preparations are prepared by maceration, percolation, digestion, infusion, decoction, fermentation or as described in the individual monographs, usually using ethanol of suitable concentration.

Mother tinctures for homoeopathic preparations are obtained using a fixed proportion of raw material to solvent, taking the moisture content of the raw material into account, unless otherwise justified and authorised.

If fresh plants are used, suitable procedures are used to ensure freshness. The competent authorities may require that the freshness is demonstrated by means of a suitable test.

Where the mother tincture contains ethanol, it is tested for 2-propanol (*2.9.11*), with a maximum limit of 0.05 per cent *V/V*, unless assurance of compliance with this limit is provided by a detailed knowledge of the ethanol supply chain and the mother tincture manufacturing process.

Mother tinctures for homoeopathic preparations are usually clear. A slight sediment may form on standing and that is acceptable as long as the composition of the tincture is not changed significantly.

The manufacturing process is defined so that it is reproducible.

**Production by maceration**. Unless otherwise prescribed, reduce the matter to be extracted to pieces of suitable size, mix thoroughly and extract according to the prescribed extraction method with the prescribed extraction solvent. Allow to stand in a closed vessel for the prescribed time. The residue is separated from the extraction solvent and, if necessary, pressed out. In the latter case, the 2 liquids obtained are combined.

**Adjustment of the contents**. Adjustment of the content of constituents may be carried out if necessary, either by adding the extraction solvent of suitable concentration, or by adding another mother tincture for homoeopathic preparations of the vegetable or animal matter used for the preparation.

CHARACTERS

Where appropriate, the appearance and odour of the mother tincture are determined.

IDENTIFICATION

Where applicable, at least 1 chromatographic identification test is carried out.

TESTS

The limits in an individual monograph are set to include official methods of production. Specific limits will apply to each defined method of production.

*If the test for relative density is carried out, the test for ethanol need not be carried out, and vice versa.*

**Relative density** (*2.2.5*). The mother tincture for homoeopathic preparations complies with the limits prescribed in the monograph.

**Ethanol** (*2.9.10*). The ethanol content complies with that prescribed in the monograph.

**Methanol** (*2.9.11*): maximum 0.05 per cent *V/V*, unless otherwise prescribed or justified and authorised.

**Dry residue** (*2.8.16*). Where applicable, the mother tincture for homoeopathic preparations complies with the limits prescribed in the monograph.

**Pesticides** (*2.8.13*). Where applicable, the mother tincture for homoeopathic preparations complies with the test. This requirement is met if the herbal drug has been shown to comply with the test.

Justification is provided in cases where the test for pesticides is performed on the mother tincture, instead of on the herbal drug according to the requirements of the general monograph *Herbal drugs for homoeopathic preparations (2045)*. Limits are set, taking into consideration the nature and the origin of the herbal drug. The dilution factor of the mother tincture and the limit of quantification of the method are also taken into account when setting these limits.

**Heavy metals** (*2.4.27*). Justification is provided in cases where the test for heavy metals is performed on the mother tincture, instead of on the herbal drug according to the requirements of the general monograph *Herbal drugs for homoeopathic preparations (2045)*. Limits are set, taking into consideration the nature and the origin of the herbal drug. The dilution factor of the mother tincture and the limit of quantification of the method are also taken into account when setting these limits.

If required by the competent authority, suitable limits for the content of other heavy metals such as arsenic or nickel may be defined.

**Aflatoxin $B_1$** (*2.8.18*). Where appropriate, limits for aflatoxins may be required. Justification is provided in cases where the test for aflatoxin $B_1$ is performed on the mother tincture, instead of on the herbal drug. Limits are set, taking into consideration the nature and the origin of the herbal drug. The dilution factor of the mother tincture and the limit of quantification of the method are also taken into account when setting these limits.

ASSAY

Where applicable, an assay with quantitative limits is performed.

STORAGE

Protected from light. A maximum storage temperature may be specified.

LABELLING

The label states:

- that the product is a mother tincture for homoeopathic preparations (designated as 'TM' or 'Ø');
- the name of the raw material using the Latin title of the European Pharmacopoeia monograph where one exists;
- the method of preparation;
- the ethanol content or other solvent content, in per cent *V/V*, in the mother tincture;
- the ratio of raw material to mother tincture;
- where applicable, the storage conditions.

**01/2017:2371**
**corrected 9.2**

# METHODS OF PREPARATION OF HOMOEOPATHIC STOCKS AND POTENTISATION

## Via praeparandi stirpes homoeopathicas et potentificandi

Homoeopathic stocks are prepared, using suitable methods, from raw materials that comply with the requirements of the monograph *Homoeopathic preparations (1038)*. The methods described below, combined with established methods for potentisation, are examples of methods, but other methods described in an official national pharmacopoeia of a Member State may equally be used.

Where material of animal or human origin is to be used, particular reference is made to the requirements concerning the use of such raw material of zoological or human origin in the monograph *Homoeopathic preparations (1038)*.

In the preparation of liquid dilutions, the ethanol of the concentration prescribed in the method may, if necessary, be replaced by ethanol (36 per cent *V/V*) or ethanol (18 per cent *V/V*).

When the individual monograph allows that the mother tincture be prepared from more than one plant species, the mother tincture can be prepared from the specified parts of an individual plant species or from any mixture thereof. If for the preparation of a mother tincture the loss on drying has to be determined, the herbal drug or mixture of herbal drug with ethanol has to be processed immediately after the value of the loss on drying has been determined.

Unless otherwise stated, mother tinctures are prepared by maceration lasting 10-30 days.

Maceration may be replaced by long maceration (maximum 60 days) or very long maceration (maximum 180 days), provided it is demonstrated that the quality of the resulting mother tincture is the same as that of the mother tincture prepared by maceration.

Unless otherwise stated in the individual monograph, the term 'part(s)' denotes 'mass part(s)'. Unless otherwise stated in the method, the maximum temperature for the preparation is 25 °C.

### 1. MOTHER TINCTURES AND LIQUID POTENTISATIONS

#### *METHOD 1.1. HYDROALCOHOLIC MOTHER TINCTURES PREPARED WITHOUT HEATING*

**METHOD 1.1.1 (HOMÖOPATHISCHES ARZNEIBUCH (HAB) 1a: MOTHER TINCTURES AND LIQUID DILUTIONS)**

Method 1.1.1 is used for fresh herbal drugs containing generally more than 70 per cent of expressed juice and no essential oil or resin or mucilage. Mother tinctures prepared according to Method 1.1.1 are mixtures of equal parts of expressed juices and ethanol (90 per cent *V/V*).

Express the comminuted herbal drug. Immediately mix the expressed juice with an equal mass of ethanol (90 per cent *V/V*). Allow to stand in a closed container for not less than 5 days, then filter.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (50 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (50 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

2 parts of the mother tincture;

8 parts of ethanol (50 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (50 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2.

The 1st 'centesimal' dilution (C1) is made from:

2 parts of the mother tincture;

98 parts of ethanol (50 per cent *V/V*).

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st 'centesimal' dilution;

99 parts of ethanol (50 per cent *V/V*).

Subsequent centesimal dilutions are produced as stated for C2.

#### METHOD 1.1.2 (HAB 1b: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.2 is used where the latex of a herbal drug is to be processed.

Mother tinctures prepared according to Method 1.1.2 are mixtures of fresh plant latex with ethanol (36 per cent *V/V*). Mix the fresh latex with 2 parts by mass of ethanol (36 per cent *V/V*) and filter.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (36 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (36 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (36 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 1.1.3 (HAB 2a: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.3 is used for fresh herbal drugs containing generally less than 70 per cent of expressed juice and more than 60 per cent moisture (loss on drying) and no essential oil or resin.

Mother tinctures prepared according to Method 1.1.3 (ethanol content approximately 50 per cent *V/V*) are prepared by maceration as described below.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol (90 per cent *V/V*) and allow to stand in well-closed containers.

Use the following expression to calculate the amount ($A_2$), in kilograms, of ethanol (90 per cent *V/V*) required for the mass ($m$) of raw material, then subtract the amount of ethanol (90 per cent *V/V*) already added and add the difference to the mixture.

$$\frac{m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (50 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (50 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

2 parts of the mother tincture;

8 parts of ethanol (50 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (50 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2.

The 1st 'centesimal' dilution (C1) is made from:

2 parts of the mother tincture;

98 parts of ethanol (50 per cent *V/V*).

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st 'centesimal' dilution;

99 parts of ethanol (50 per cent *V/V*).

Subsequent centesimal dilutions are produced as stated for C2.

#### METHOD 1.1.4 (HAB 2b: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.4 is used for fresh herbal drugs containing generally less than 70 per cent of expressed juice and more than 60 per cent moisture (loss on drying) and no essential oil or resin.

Mother tinctures prepared according to Method 1.1.4 (ethanol content approximately 36 per cent *V/V*) are prepared by maceration as described below.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol (70 per cent *V/V*) and allow to stand in well-closed containers.

Use the following expression to calculate the amount ($A_2$), in kilograms, of ethanol (70 per cent *V/V*) required for the mass (*m*) of raw material, then subtract the amount of ethanol (70 per cent *V/V*) already added and add the difference to the mixture.

$$\frac{m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (36 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (36 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

2 parts of the mother tincture;

8 parts of ethanol (36 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2.

**METHOD 1.1.5 (HAB 3a: MOTHER TINCTURES AND LIQUID DILUTIONS)**

Method 1.1.5 is used for fresh herbal drugs containing essential oil or resin, or generally less than 60 per cent moisture.

Mother tinctures prepared according to Method 1.1.5 (ethanol content approximately 65 per cent *V/V*) are prepared by maceration as described below.

Comminute appropriately the herbal drug.

Determine the water content (*2.2.13*) or loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol (90 per cent *V/V*) and allow to stand in well-closed containers.

Use the following expression to calculate the amount ($A_3$), in kilograms, of ethanol (90 per cent *V/V*) required for the mass (*m*) of raw material, then subtract the amount of ethanol (90 per cent *V/V*) already added and add the difference to the mixture.

$$\frac{2 \times m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (70 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (70 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (70 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (70 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2. Use ethanol (50 per cent *V/V*) for dilutions from D4 onwards.

The 1st 'centesimal' dilution (C1) is made from:

3 parts of the mother tincture;

97 parts of ethanol (70 per cent *V/V*).

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st 'centesimal' dilution;

99 parts of ethanol (50 per cent *V/V*).

Subsequent centesimal dilutions are produced as stated for C2.

**METHOD 1.1.6 (HAB 3b: MOTHER TINCTURES AND LIQUID DILUTIONS)**

Method 1.1.6 is used for fresh herbal drugs containing essential oils or resins or generally less than 60 per cent moisture.

Mother tinctures prepared according to Method 1.1.6 (ethanol content approximately 57 per cent *V/V*) are prepared by maceration as described below.

Comminute appropriately the herbal drug.

Determine the water content (*2.2.13*) or loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol (80 per cent *V/V*) and allow to stand in well-closed containers.

Use the following expression to calculate the amount ($A_3$), in kilograms, of ethanol (80 per cent *V/V*) required for the mass (*m*) of raw material, then subtract the amount of ethanol (80 per cent *V/V*) already added and add the difference to the mixture.

$$\frac{2 \times m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the

above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (50 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (50 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (50 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (36 per cent *V/V*).

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D3.

#### METHOD 1.1.7 (HAB 3C: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.7 is used for fresh herbal drugs containing generally less than 60 per cent moisture (loss on drying).

Mother tinctures prepared according to Method 1.1.7 (ethanol content approximately 35 per cent *V/V*) are prepared by maceration as described below.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol (50 per cent *V/V*) and allow to stand in well-closed containers.

Use the following expression to calculate the amount ($A_3$), in kilograms, of ethanol (50 per cent *V/V*) required for the mass ($m$) of raw material, then subtract the amount of ethanol (50 per cent *V/V*) already added and add the difference to the mixture.

$$\frac{2 \times m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol (36 per cent *V/V*) required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol (36 per cent *V/V*). Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (36 per cent *V/V*).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 1.1.8 (HAB 4a: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.8 is generally used for dried herbal drugs.

Mother tinctures prepared according to Method 1.1.8 are prepared by maceration or percolation as described below, using 1 part of dried herbal drug and 10 parts of ethanol of the appropriate concentration (anhydrous, 96 per cent *V/V*, 90 per cent *V/V*, 80 per cent *V/V*, 70 per cent *V/V*, 50 per cent *V/V*, 36 per cent *V/V*, 18 per cent *V/V*), unless otherwise prescribed in the individual monograph.

*Production by maceration.* Unless otherwise prescribed, comminute the herbal drug, mix thoroughly with ethanol of the appropriate concentration and allow to stand in a closed container for an appropriate time. Separate the residue from the ethanol and, if necessary, press out. In the latter case, combine the 2 liquids obtained.

*Production by percolation.* If necessary, comminute the herbal drug. Mix thoroughly with a portion of ethanol of the appropriate concentration and allow to stand for an appropriate time. Transfer to a percolator and allow the percolate to flow slowly, at room temperature, making sure that the herbal drug to be extracted is always covered with the remaining ethanol. The residue may be pressed out and the expressed liquid combined with the percolate.

If adjustment to a given concentration is necessary, calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required to obtain the concentration specified or used for production, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of percolate or macerate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the percolate or macerate.

Mix the macerate or percolate with the calculated amount of ethanol of the appropriate concentration. Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of ethanol of the same concentration.

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of ethanol of the same concentration.

Unless a different ethanol concentration is specified, use ethanol (50 per cent *V/V*) for subsequent decimal dilutions from D4 onwards and proceed as stated for D3.

The 1st 'centesimal' dilution (C1) is made from:

10 parts of the mother tincture (D1);

90 parts of ethanol of the same concentration.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st 'centesimal' dilution;

99 parts of ethanol (50 per cent *V/V*), unless a different ethanol concentration is specified.

Subsequent centesimal dilutions are produced as stated for C2.

#### METHOD 1.1.9 (HAB 4b: MOTHER TINCTURES AND LIQUID DILUTIONS)

Method 1.1.9 is generally used for animal matter.

Mother tinctures prepared according to Method 1.1.9 are prepared by maceration or percolation as described below, using 1 part of animal matter and 10 parts of ethanol of the appropriate concentration (anhydrous, 96 per cent *V/V*, 90 per cent *V/V*, 80 per cent *V/V*, 70 per cent *V/V*, 50 per cent *V/V*, 36 per cent *V/V*, 18 per cent *V/V*), unless otherwise prescribed in the individual monograph.

*Production by maceration*. Unless otherwise prescribed, comminute the animal matter, mix thoroughly with ethanol of the appropriate concentration and allow to stand in a closed container for an appropriate time. Separate the residue from the ethanol and, if necessary, press out. In the latter case, combine the 2 liquids obtained.

*Production by percolation*. If necessary, comminute the animal matter. Mix thoroughly with a portion of ethanol of the appropriate concentration and allow to stand for an appropriate time. Transfer to a percolator and allow the percolate to flow slowly at room temperature, making sure that the animal matter to be extracted is always covered with the remaining ethanol. The residue may be pressed out and the expressed liquid combined with the percolate.

If adjustment to a given concentration is necessary, calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required to obtain the concentration specified or used for production, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of percolate or macerate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the percolate or macerate.

Mix the macerate or percolate with the calculated amount of ethanol of the appropriate concentration. Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of ethanol of the same concentration.

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of ethanol of the same concentration.

Unless a different ethanol concentration is specified, use ethanol (50 per cent *V/V*) for subsequent decimal dilutions from D4 onwards and proceed as stated for D3.

The 1st 'centesimal' dilution (C1) is made from:

10 parts of the mother tincture (D1);

90 parts of ethanol of the same concentration.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st 'centesimal' dilution;

99 parts of ethanol (50 per cent *V/V*), unless a different ethanol concentration is specified.

Subsequent centesimal dilutions are produced as stated for C2.

#### METHOD 1.1.10 (FRENCH PHARMACOPOEIA)

Method 1.1.10 is generally used for herbal drugs. The state of the herbal drug, fresh or dried, is specified in the individual monograph.

Mother tinctures prepared according to Method 1.1.10 are prepared by maceration.

Comminute appropriately the herbal drug. Take a sample and determine the loss on drying at 105 °C for 2 h (*2.2.32*) or the water content (*2.2.13*). Taking this value into account, calculate and add to the herbal drug the quantities of ethanol of the appropriate concentration required to produce, unless otherwise prescribed, a 1 in 10 mother tincture (1:10 mother tincture) with a suitable ethanol content. Allow to macerate for at least 10 days, with sufficient shaking.

Separate the residue from the ethanol and strain under pressure if necessary. Allow the combined liquids to stand for 48 h and filter. For mother tinctures with a required assay content, adjustment may be carried out, if necessary, by adding ethanol of the same concentration as used for the preparation of the tincture.

**Potentisation**

The 1st decimal dilution (D1) is made from:

1 part of the mother tincture;

9 parts of ethanol of the appropriate concentration.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st decimal dilution;

9 parts of ethanol of the appropriate concentration.

Subsequent decimal dilutions are produced as stated for D2, using ethanol of the appropriate concentration.

The 1st centesimal dilution (C1) is made from:

1 part of the mother tincture;

99 parts of ethanol of the appropriate concentration.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution;

99 parts of ethanol of the appropriate concentration.

Subsequent centesimal dilutions are produced as stated for C2, using ethanol of the appropriate concentration.

#### METHOD 1.1.11 (FRENCH PHARMACOPOEIA)

Method 1.1.11 is generally used for animal matter.

Mother tinctures prepared according to Method 1.1.11 are prepared by maceration.

The mass ratio of raw material to mother tincture is usually 1 to 20. To the raw material, appropriately comminuted, add the quantity of ethanol of the appropriate concentration required to produce a 1 in 20 mother tincture. Allow to macerate for at least 10 days, with sufficient shaking. Decant and filter. Allow to stand for 48 h and filter again. For mother tinctures with a required assay content, adjustment may be carried out, if necessary, by adding ethanol of the same concentration as used for the preparation of the tincture.

**Potentisation**

The 1st decimal dilution (D1) is made from:

1 part of the mother tincture;

9 parts of ethanol of the appropriate concentration.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st decimal dilution;

9 parts of ethanol of the appropriate concentration.

Subsequent decimal dilutions are produced as stated for D2, using ethanol of the appropriate concentration.

The 1st centesimal dilution (C1) is made from:

1 part of the mother tincture;

99 parts of ethanol of the appropriate concentration.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution;

99 parts of ethanol of the appropriate concentration.

Subsequent centesimal dilutions are produced as stated for C2, using ethanol of the appropriate concentration.

*METHOD 1.2. HYDROALCOHOLIC MOTHER TINCTURES PREPARED WITH HEATING*

**METHODS 1.2.1, 1.2.2. ETHANOLIC DIGESTIONS (HAB 18a, 18b: HEAT-TREATED MOTHER TINCTURES)**

Methods 1.2.1 and 1.2.2 are used for fresh herbal drugs containing generally less than 70 per cent of expressed juice and more than 60 per cent moisture (loss on drying) and no essential oil or resin.

Mother tinctures prepared according to Methods 1.2.1 (ethanol content approximately 50 per cent *V/V*) and 1.2.2 (ethanol content approximately 36 per cent *V/V*) are ethanolic digestions prepared by heat treatment and additional maceration as described below.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol of the concentration prescribed below and allow to stand in well-closed containers:

– Method 1.2.1: ethanol (90 per cent *V/V*);

– Method 1.2.2: ethanol (70 per cent *V/V*).

Use the following expression to calculate the amount ($A_2$), in kilograms, of ethanol of the appropriate concentration required for the mass ($m$) of raw material, then subtract the amount of ethanol of the appropriate concentration already added and add the difference to the mixture.

$$\frac{m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Warm the mixture containing the total amount of ethanol of the appropriate concentration to 37 °C in a covered container and maintain at this temperature for 1 h, swirling from time to time. Cool, allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the concentration prescribed below:

– Method 1.2.1: ethanol (50 per cent *V/V*);

– Method 1.2.2: ethanol (36 per cent *V/V*).

Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

2 parts of the mother tincture;

8 parts of ethanol (50 per cent *V/V*) (Method 1.2.1) or ethanol (36 per cent *V/V*) (Method 1.2.2).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (36 per cent *V/V*) (Method 1.2.1) or ethanol (18 per cent *V/V*) (Method 1.2.2).

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D3.

**METHODS 1.2.3, 1.2.4, 1.2.5. ETHANOLIC DIGESTIONS (HAB 18c, 18d, 18e: HEAT-TREATED MOTHER TINCTURES)**

Methods 1.2.3, 1.2.4 and 1.2.5 are used for fresh herbal drugs containing essential oil or resin or generally less than 60 per cent moisture.

Mother tinctures prepared according to Methods 1.2.3 (ethanol content approximately 65 per cent *V/V*), 1.2.4 (ethanol content approximately 57 per cent *V/V*) and 1.2.5 (ethanol content approximately 35 per cent *V/V*) are ethanolic digestions prepared by heat treatment and additional maceration as described below.

Comminute appropriately the herbal drug.

Determine the water content (*2.2.13*) or loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol of the concentration prescribed below and allow to stand in well-closed containers:

– Method 1.2.3: ethanol (90 per cent *V/V*);

– Method 1.2.4: ethanol (80 per cent *V/V*);

– Method 1.2.5: ethanol (50 per cent *V/V*).

Use the following expression to calculate the amount ($A_3$), in kilograms, of ethanol of the appropriate concentration required for the mass ($m$) of raw material, then subtract the amount of ethanol of the appropriate concentration already added and add the difference to the mixture.

$$\frac{2 \times m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Warm the mixture containing the total amount of ethanol of the appropriate concentration to 37 °C in a covered container and maintain at this temperature for 1 h, swirling from time to time. Cool, allow to stand for not less than 10 days, swirling from time to time, then express the mixture and filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the concentration prescribed below:

- Method 1.2.3: ethanol (70 per cent *V/V*);
- Method 1.2.4: ethanol (50 per cent *V/V*);
- Method 1.2.5: ethanol (36 per cent *V/V*).

Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (70 per cent *V/V*) (Method 1.2.3), ethanol (50 per cent *V/V*) (Method 1.2.4) or ethanol (36 per cent *V/V*) (Method 1.2.5).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (50 per cent *V/V*) (Method 1.2.3), ethanol (36 per cent *V/V*) (Method 1.2.4) or ethanol (18 per cent *V/V*) (Method 1.2.5).

Subsequent decimal dilutions are produced accordingly. In this process the ethanol concentration is reduced with each step, according to the sequence 70 per cent *V/V* - 50 per cent *V/V* - 36 per cent *V/V* - 18 per cent *V/V*.

#### METHOD 1.2.6. ETHANOLIC DIGESTIONS (HAB 18f: HEAT-TREATED MOTHER TINCTURES)

Method 1.2.6 is used for dried herbal drugs.

Mother tinctures prepared according to Method 1.2.6 are ethanolic digestions prepared by heat treatment and additional maceration as described below, using 1 part of dried herbal drug and 10 parts of ethanol of the appropriate concentration (96 per cent *V/V*, 90 per cent *V/V*, 80 per cent *V/V*, 70 per cent *V/V*, 50 per cent *V/V*, 36 per cent *V/V*, 18 per cent *V/V*), unless otherwise prescribed in the individual monograph.

Unless otherwise prescribed, comminute appropriately the herbal drug, mix thoroughly with the total amount of ethanol of the appropriate concentration, warm the mixture to 37 °C in a covered container and maintain at this temperature for 1 h, swirling from time to time. Cool, then allow to stand in a closed container for an appropriate time. After sedimentation, decant the supernatant and, if necessary, press out. In the latter case, combine the 2 liquids obtained. Filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required to obtain the concentration specified or used for production, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of the filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the calculated amount of ethanol of the appropriate concentration. Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of ethanol of the same concentration.

Subsequent decimal dilutions are produced accordingly. In this process the ethanol concentration is reduced with each step, according to the sequence 96 per cent *V/V* - 90 per cent *V/V* - 80 per cent *V/V* - 70 per cent *V/V* - 50 per cent *V/V* - 36 per cent *V/V* - 18 per cent *V/V*.

#### METHODS 1.2.7, 1.2.8. ETHANOLIC DECOCTIONS (HAB 19a, 19b: HEAT-TREATED MOTHER TINCTURES)

Methods 1.2.7 and 1.2.8 are used for fresh herbal drugs containing generally less than 70 per cent of expressed juice and more than 60 per cent moisture (loss on drying) and no essential oil or resin.

Mother tinctures prepared according to Methods 1.2.7 (ethanol content approximately 50 per cent *V/V*) and 1.2.8 (ethanol content approximately 36 per cent *V/V*) are ethanolic decoctions prepared by heat treatment and additional maceration as described below.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol of the concentration prescribed below and allow to stand in well-closed containers:

- Method 1.2.7: ethanol (90 per cent *V/V*);
- Method 1.2.8: ethanol (70 per cent *V/V*).

Use the following expression to calculate the amount ($A_2$), in kilograms, of ethanol of the appropriate concentration required for the mass of raw material, then subtract the amount of ethanol of the appropriate concentration already added and add the difference to the mixture.

$$\frac{m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Heat the mixture containing the total amount of ethanol of the appropriate concentration to boiling under reflux, and maintain for 30 min, unless otherwise specified in the individual monograph. Cool or allow to cool, allow to stand in a closed container for 12-36 h, then express the mixture and filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the concentration prescribed below:

– Method 1.2.7: ethanol (50 per cent *V/V*);

– Method 1.2.8: ethanol (36 per cent *V/V*).

Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

2 parts of the mother tincture;

8 parts of ethanol (50 per cent *V/V*) (Method 1.2.7) or ethanol (36 per cent *V/V*) (Method 1.2.8).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (36 per cent *V/V*) (Method 1.2.7) or ethanol (18 per cent *V/V*) (Method 1.2.8).

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D3.

**METHODS 1.2.9, 1.2.10, 1.2.11. ETHANOLIC DECOCTIONS (HAB 19c, 19d, 19e: HEAT-TREATED MOTHER TINCTURES)**

Methods 1.2.9, 1.2.10 and 1.2.11 are used for fresh herbal drugs containing essential oil or resin or generally less than 65 per cent moisture.

Mother tinctures prepared according to Methods 1.2.9 (ethanol content approximately 65 per cent *V/V*), 1.2.10 (ethanol content approximately 57 per cent *V/V*) and 1.2.11 (ethanol content approximately 35 per cent *V/V*) are ethanolic decoctions prepared by heat treatment and additional maceration as described below.

Comminute appropriately the herbal drug.

Determine the water content (*2.2.13*) or loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

To the comminuted herbal drug immediately add not less than half the mass of ethanol of the concentration prescribed below and allow to stand in well-closed containers:

– Method 1.2.9: ethanol (90 per cent *V/V*);

– Method 1.2.10: ethanol (80 per cent *V/V*);

– Method 1.2.11: ethanol (50 per cent *V/V*).

Use the following expression to calculate the amount ($A_3$), in kilograms, of ethanol of the appropriate concentration required for the mass of raw material, then subtract the amount of ethanol of the appropriate concentration already added and add the difference to the mixture.

$$\frac{2 \times m \times T}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Heat the mixture containing the total amount of ethanol of the appropriate concentration to boiling under reflux, and maintain for 30 min, unless otherwise specified in the individual monograph. Cool or allow to cool, then allow to stand in a closed container for 12-36 h, express the mixture and filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the concentration prescribed below:

– Method 1.2.9: ethanol (70 per cent *V/V*);

– Method 1.2.10: ethanol (50 per cent *V/V*);

– Method 1.2.11: ethanol (36 per cent *V/V*).

Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of ethanol (70 per cent *V/V*) (Method 1.2.9), ethanol (50 per cent *V/V*) (Method 1.2.10) or ethanol (36 per cent *V/V*) (Method 1.2.11).

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of ethanol (50 per cent *V/V*) (Method 1.2.9), ethanol (36 per cent *V/V*) (Method 1.2.10) or ethanol (18 per cent *V/V*) (Method 1.2.11).

Subsequent decimal dilutions are produced accordingly. In this process the ethanol concentration is reduced with each step, according to the sequence 70 per cent *V/V* - 50 per cent *V/V* - 36 per cent *V/V* - 18 per cent *V/V*.

**METHOD 1.2.12. ETHANOLIC DECOCTIONS (HAB 19f: HEAT-TREATED MOTHER TINCTURES)**

Method 1.2.12 is used for dried herbal drugs.

Mother tinctures prepared according to Method 1.2.12 are ethanolic decoctions prepared by heat treatment and additional maceration as described below, using 1 part of dried herbal drug and 10 parts of ethanol of the appropriate concentration (96 per cent *V/V*, 90 per cent *V/V*, 80 per cent *V/V*, 70 per cent *V/V*, 50 per cent *V/V*, 36 per cent *V/V*, 18 per cent *V/V*), unless otherwise prescribed in the individual monograph.

Unless otherwise prescribed, comminute appropriately the herbal drug, mix thoroughly with the total amount of ethanol of the appropriate concentration, heat to boiling under reflux

and maintain for 30 min. Cool or allow to cool, then allow to stand in a closed container for 12-36 h. After sedimentation, decant the supernatant and, if necessary, press out. In the latter case, combine the 2 liquids obtained. Filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required to obtain the concentration specified or used for production, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the appropriate concentration. Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of ethanol of the same concentration.

Subsequent decimal dilutions are produced accordingly. In this process the ethanol concentration is reduced with each step, according to the sequence 96 per cent *V/V* - 90 per cent *V/V* - 80 per cent *V/V* - 70 per cent *V/V* - 50 per cent *V/V* - 36 per cent *V/V* - 18 per cent *V/V*.

#### METHOD 1.2.13. ETHANOLIC INFUSIONS (HAB 20: HEAT-TREATED MOTHER TINCTURES)

Method 1.2.13 is used for dried herbal drugs.

Mother tinctures prepared according to Method 1.2.13 are ethanolic infusions prepared by heat treatment and additional maceration as described below using 1 part of dried herbal drug and 10 parts of ethanol of the appropriate concentration (90 per cent *V/V*, 80 per cent *V/V*, 70 per cent *V/V*, 50 per cent *V/V*, 36 per cent *V/V*, 18 per cent *V/V*), unless otherwise prescribed in the individual monograph. The quantities of ethanol (96 per cent *V/V*) and purified water required to achieve the specified ethanol concentration are added separately as described below.

Unless otherwise prescribed, comminute appropriately the herbal drug, mix thoroughly with the total amount of ethanol (96 per cent *V/V*), cover and allow to stand for 15 min. Add the purified water (heated to boiling) and heat the mixture to boiling under reflux for 5 min. Cool, or allow to cool, then allow to stand in a closed container for 12-36 h. After sedimentation, decant the supernatant and, if necessary, press out. In the latter case, combine the 2 liquids obtained. Filter the resulting liquid.

**Adjustment to any value as specified in the individual monograph**

Determine the percentage dry residue (*2.8.16*) or, where prescribed, the percentage assay content of the above-mentioned filtrate. Calculate the amount ($A_1$), in kilograms, of ethanol of the appropriate concentration required to obtain the concentration specified or used for production, using the following expression:

$$\frac{m \times (N_x - N_0)}{N_0}$$

$m$ = mass of filtrate, in kilograms;

$N_0$ = percentage dry residue or percentage assay content as required in the individual monograph;

$N_x$ = percentage dry residue or percentage assay content of the filtrate.

Mix the filtrate with the required amount of ethanol of the appropriate concentration. Allow to stand for not less than 5 days, then filter if necessary.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of ethanol of the same concentration.

Subsequent decimal dilutions are produced accordingly. In this process the ethanol concentration is reduced with each step, according to the sequence 90 per cent *V/V* - 80 per cent *V/V* - 70 per cent *V/V* - 50 per cent *V/V* - 36 per cent *V/V* - 18 per cent *V/V*.

### *METHOD 1.3. AQUEOUS MOTHER TINCTURES PREPARED WITHOUT HEATING*

#### METHOD 1.3.1. AQUEOUS MACERATES (HAB 49: AQUEOUS MOTHER TINCTURES)

Method 1.3.1 is used for fresh herbal drugs.

Mother tinctures prepared according to Method 1.3.1 are aqueous macerates prepared by short maceration with water as described below. This method is used solely in the manufacture of injections and eye preparations.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

Use the following expression to calculate the amount, in kilograms, of water required for the mass of raw material:

$$\frac{m \times (300 - T)}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Add the comminuted herbal drug to the calculated amount of water. Allow to stand for 2 h, then express the mixture and filter the resulting liquid. The mother tincture is used immediately, unless otherwise justified.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of water for injection.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of water for injection.

Subsequent decimal dilutions are produced as stated for D2.

### *METHOD 1.4. AQUEOUS MOTHER TINCTURES PREPARED WITH HEATING*

#### METHOD 1.4.1. AQUEOUS DIGESTIONS (HAB 24b: HEAT-TREATED AQUEOUS MOTHER TINCTURES)

Method 1.4.1 is used for fresh herbal drugs.

Mother tinctures prepared according to Method 1.4.1 are aqueous digestions prepared by heat treatment with water as described below. This method is solely used in the preparation of injections, eye preparations and coated homoeopathic pillules.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

Use the following expression to calculate the amount, in kilograms, of water required for the mass of raw material:

$$\frac{m \times (400 - T)}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Heat the mixture containing the total amount of water in a covered container to 37 °C and maintain at this temperature for 1 h, swirling from time to time. Express the mixture and filter the resulting liquid. The mother tincture is used immediately, unless otherwise justified.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

4 parts of the mother tincture;

6 parts of water for injection.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of water for injection.

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 1.4.2. AQUEOUS DECOCTIONS (HAB 23b: HEAT-TREATED AQUEOUS MOTHER TINCTURES)

Method 1.4.2 is used for fresh herbal drugs.

Mother tinctures prepared according to Method 1.4.2 are aqueous decoctions prepared by heat treatment with water as described below. This method is solely used in the preparation of injections, eye preparations and coated homoeopathic pillules.

Comminute appropriately the herbal drug.

Take a sample and determine the loss on drying (*2.2.32*). Unless otherwise prescribed, determine the loss on drying on 2.00-5.00 g of the comminuted herbal drug and dry at 105 °C for 2 h.

Use the following expression to calculate the amount, in kilograms, of water required for the mass of raw material:

$$\frac{m \times (300 - T)}{100}$$

$m$ = mass of raw material, in kilograms;

$T$ = percentage loss on drying of the sample.

Heat the calculated amount of water to above 90 °C and add the comminuted herbal drug. Maintain the mixture at this temperature under reflux for 30 min, then express the mixture and filter the resulting liquid. The mother tincture is used immediately, unless otherwise justified.

**Potentisation**

The 1st 'decimal' dilution (D1) is made from:

3 parts of the mother tincture;

7 parts of water for injection.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st 'decimal' dilution;

9 parts of water for injection.

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 1.4.3. AQUEOUS DECOCTIONS (HAB 23a: HEAT-TREATED AQUEOUS MOTHER TINCTURES)

Method 1.4.3 is used for dried herbal drugs.

Mother tinctures prepared according to Method 1.4.3 are aqueous decoctions prepared by heat treatment with water as described below. This method is solely used in the preparation of injections, eye preparations and coated homoeopathic pillules.

Comminute appropriately the herbal drug.

Mix 1 part of the comminuted dried herbal drug with 10 parts of boiling water and boil under reflux for 30 min, unless otherwise specified in the individual monograph. Filter while hot. If gentle pressure applied to the residue does not achieve a final mass of mother tincture equal to 10 parts, pour a sufficient amount of boiling water over the residue and express gently. Use the resulting extract to make the mother tincture up to 10 parts. Filter the combined liquid. The filtrate is the mother tincture. The mother tincture is used immediately, unless otherwise justified.

For starch-containing material process 1 part of herbal drug with 100 parts of water. In that case the mother tincture corresponds to the 2nd decimal dilution (Ø = D2).

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of water for injection.

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 1.4.4. AQUEOUS INFUSIONS (HAB 24a: HEAT-TREATED AQUEOUS MOTHER TINCTURES)

Method 1.4.4 is used for dried herbal drugs.

Mother tinctures prepared according to Method 1.4.4 are aqueous infusions prepared by heat treatment with water and additional maceration as described below. This method is solely used in the preparation of injections, eye preparations and coated homoeopathic pillules.

Comminute appropriately the herbal drug.

1 part of dried herbal drug is extracted with 10 parts of water.

Grind 1 part of the comminuted herbal drug in a mortar thoroughly with 3-5 parts of water, allow to stand for 15 min, then add the remainder of the water, which has been heated to boiling point. Place the mixture in a water-bath and maintain at a temperature above 90 °C for 5 min, swirling from time to time. Cover, allow to cool, then separate the residue from the liquid. If gentle pressure applied to the residue does not achieve a final mass of mother tincture equal to 10 parts, pour a sufficient amount of cold water over the residue and express gently. Use the resulting extract to make the mother tincture up to 10 parts. Filter the combined liquid. The filtrate is the mother tincture. The mother tincture is used immediately, unless otherwise justified.

**Potentisation**

The mother tincture corresponds to the 1st 'decimal' dilution (Ø = D1).

The 2nd decimal dilution (D2) is made from:

1 part of the mother tincture (D1);

9 parts of water for injection.

Subsequent decimal dilutions are produced as stated for D2.

### 2. GLYCEROL MACERATES

*METHOD 2.1*

Method 2.1 is used for maceration of raw materials of animal or herbal origin in glycerol (85 per cent) or glycerol/ethanol mixtures of appropriate concentration. Pathological material is excluded.

The raw materials are finely minced before use, where appropriate.

#### METHODS 2.1.1, 2.1.2 (HAB 42a AND 42b: MOTHER TINCTURES AND LIQUID DILUTIONS THEREOF)

Raw materials of animal origin - freshly killed animals or parts thereof - are used. Animals are processed immediately after being killed.

**Maceration**

Disperse 1 part of finely minced animal material in:

– 9 parts (decimal dilutions) or 99 parts (centesimal dilutions) of glycerol (85 per cent) for Method 2.1.1; or

– 2.1 parts of glycerol (85 per cent) for Method 2.1.2.

Allow to macerate for at least 2 h, then succuss. Filter when necessary.

Where justified, 1 part of glycerol (85 per cent) may be added to 1 part of animal material before mincing. Where very small amounts of animal material are used, the dilution may be prepared by dispersing 1 part of finely minced animal material in 99 parts of glycerol (85 per cent) (C1 or 'D2' if to be used for further decimal dilutions).

**Potentisation**

*Method 2.1.1*

The 2nd decimal dilution (D2) is made from:

1 part of the glycerol macerate D1;

9 parts of glycerol (85 per cent) or ethanol (18 per cent *V/V*).

Subsequent decimal dilutions are produced as stated for D2 but with ethanol (18 per cent *V/V*) as the vehicle.

The 2nd centesimal dilution (C2) is made from:

1 part of the glycerol macerate C1;

99 parts of ethanol (18 per cent *V/V*).

Subsequent centesimal dilutions are produced as stated for C2.

*Method 2.1.2*

The 1st 'decimal' dilution (D1) is made from:

3 parts of the glycerol macerate;

7 parts of water for injections.

The 2nd decimal dilution (D2) is made from:

1 part of D1;

9 parts of water for injections.

Subsequent decimal dilutions are produced as stated for D2.

#### METHOD 2.1.3 (FRENCH PHARMACOPOEIA)

Raw materials of herbal or animal origin are used.

**Maceration**

Comminute the raw material appropriately. Take a sample and determine the loss on drying at 105 °C for 2 h (*2.2.32*) or the water content (*2.2.13*). Taking this value into account, calculate and add to the raw material the quantity of the ethanol/glycerol mixture of the appropriate concentration to produce, unless otherwise prescribed, a 1 in 20 glycerol macerate. Allow to macerate for at least 21 days, with sufficient shaking. Decant and strain under pressure if necessary. Allow the combined liquids to stand for 48 h and filter.

**Potentisation**

The 1st decimal dilution (D1) is made from:

1 part of the glycerol macerate;

9 parts of a water/ethanol/glycerol mixture of appropriate concentration.

The 2nd decimal dilution (D2) is made from:

1 part of the 1st decimal dilution;

9 parts of a water/ethanol/glycerol mixture of appropriate concentration.

Subsequent decimal dilutions are produced as stated for D2 or using another appropriate vehicle.

The 1st centesimal dilution (C1) is made from:

1 part of the glycerol macerate;

99 parts of a water/ethanol/glycerol mixture of appropriate concentration.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution;

99 parts of a water/ethanol/glycerol mixture of appropriate concentration.

Subsequent centesimal dilutions are produced as stated for C2 or using another appropriate vehicle.

*METHOD 2.2*

#### METHODS 2.2.1, 2.2.2, 2.2.3, 2.2.4 (HAB 41a, 41b, 41c AND 41d: GLYCEROL MOTHER TINCTURES AND LIQUID DILUTIONS THEREOF)

Method 2.2 is used for maceration of raw materials of animal origin in a glycerol solution containing sodium chloride. Pathological material is excluded.

Raw materials from freshly killed animals, parts or secretions thereof are used in Methods 2.2.1, 2.2.2 and 2.2.3. Lower animals are killed with carbon dioxide in a covered vessel. All animals are processed immediately after being killed.

Blood components from live horses are used in method 2.2.4.

**Sample collection and/or pre-treatment**

The raw materials used in Methods 2.2.1, 2.2.2 and 2.2.3 are finely minced before use, where appropriate.

The blood used in Method 2.2.4 is collected by a veterinarian. Blood obtained from animals killed by bleeding must not be used. Take 200 mL of this blood and add 15 IU of heparin sodium and 0.625 mL of a 9 g/kg solution of sodium chloride per millilitre. Separate the blood components by fractional centrifugation and resuspend each individual cell sediment in 1.1 mL of a 9 g/kg solution of sodium chloride. These cell suspensions are processed into the glycerol macerate.

**Maceration**

Mix 1 part of finely minced animal material, secretions or blood cell suspensions, according to the method used, with 5 parts of a sodium chloride solution of the appropriate concentration (see Table 2371.-1) and 95 parts of glycerol. Allow to stand protected from light for at least 7 days, then decant. If necessary for Methods 2.2.1, 2.2.2 and 2.2.3, centrifuge before decanting, then filter the supernatant if necessary. The decanted liquid or the filtrate respectively is the glycerol macerate.

Any sediment present must be resuspended before processing the glycerol macerate.

Table 2371.-1

| Methods 2.2.1 and 2.2.4 | Method 2.2.2 | Method 2.2.3 |
|---|---|---|
| 15 g/kg solution of sodium chloride in purified water | 40 g/kg solution of sodium chloride in purified water | 80 g/kg solution of sodium chloride in purified water |

**Vehicle**

0.2 parts of sodium hydrogen carbonate and 8.8 parts of sodium chloride in 991 parts of water for injections or purified water as appropriate.

**Potentisation**

The glycerol macerate corresponds to the 2nd decimal dilution ('D2') or the 1st centesimal dilution (C1).

The 3rd decimal dilution (D3) is made from:

1 part of the 2nd decimal dilution;

9 parts of the appropriate vehicle.

Subsequent decimal dilutions are produced as stated for D3.

Where appropriate, the 4th decimal dilution (D4) is made from 1 part of the 3rd decimal dilution, 5.6 parts of the vehicle and 3.4 parts of water for injections.

The 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution;

99 parts of the appropriate vehicle.

Subsequent centesimal dilutions are produced as stated for C2.

## 3. LIQUID DILUTIONS

*METHOD 3.1*

Methods 3.1.1, 3.1.2 and 3.1.3 are used for dissolution of any suitable inorganic or organic starting material, for example minerals or venoms.

Unless otherwise specified, dissolve 1 part of the starting material in 9 parts (D1) or 99 parts (C1) of the liquid vehicle and succuss.

Where justified and authorised, in case of insufficient solubility of the starting material in the specified vehicle, directly produce the first possible dilution. For example, if the starting material is slightly soluble, dissolve 1 part of the starting material in 99 parts of the vehicle (C1 or 'D2' if to be used for further decimal dilutions).

**METHODS 3.1.1, 3.1.2 (HAB 5a, 5b: SOLUTIONS, AQUEOUS SOLUTIONS)**

**Vehicles**

The vehicles in Table 2371.-2 may be used.

Table 2371.-2

| Method 3.1.1 | Method 3.1.2 |
|---|---|
| Anhydrous ethanol | Water for injections |
| Ethanol (96 per cent *V/V*) | Purified water |
| Ethanol (90 per cent *V/V*) | |
| Ethanol (80 per cent *V/V*) | |
| Ethanol (70 per cent *V/V*) | |
| Ethanol (50 per cent *V/V*) | |
| Ethanol (36 per cent *V/V*) | |
| Ethanol (18 per cent *V/V*) | |
| Purified water | |
| Glycerol (85 per cent) | |

For Method 3.1.1, if ethanol (18 per cent *V/V*) is used, the starting material may be dissolved in 7.58 parts of purified water and the ethanol concentration adjusted by adding 1.42 parts of ethanol (96 per cent *V/V*) to the solution, for decimal dilutions. For centesimal dilutions, use 83.4 parts of purified water for 15.6 parts of ethanol (96 per cent *V/V*).

For Method 3.1.2, if the starting material is not stable and/or soluble in water, glycerol (85 per cent) may be added at a concentration of not more than 35 per cent of the vehicle, for potentisation up to D4.

**Potentisation**

Unless otherwise specified, the 2nd decimal dilution (D2) is made from:

1 part of the 1st decimal dilution (D1);

9 parts of ethanol (50 per cent *V/V*) for Method 3.1.1 or 9 parts of water for injections (or purified water, as appropriate) for Method 3.1.2.

Subsequent decimal dilutions are produced as stated for D2.

Unless otherwise specified, the 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution (C1);

99 parts of ethanol (50 per cent *V/V*) for Method 3.1.1 or 99 parts of water for injections (or purified water, as appropriate) for Method 3.1.2.

Subsequent centesimal dilutions are produced as stated for C2.

**Additives**

For Method 3.1.1, if a reaction such as precipitation is observed in the final dilution, the following additives may be used to enhance stability and/or solubility, unless otherwise specified:

- glacial acetic acid;
- concentrated hydrochloric acid;
- lactic acid;
- sodium hydroxide.

Where solutions or dilutions have been pH-adjusted, they must not be potentised further.

**METHOD 3.1.3**

**Vehicles**

Suitable vehicles, for example, ethanol of an appropriate concentration, glycerol or purified water may be used alone or combined.

**Potentisation**

Unless otherwise specified, the 2nd decimal dilution (D2) is made from:

1 part of the 1st decimal dilution (D1);

9 parts of the appropriate vehicle.

Subsequent decimal dilutions are produced as stated for D2.

Unless otherwise specified, the 2nd centesimal dilution (C2) is made from:

1 part of the 1st centesimal dilution (C1);

99 parts of the appropriate vehicle.

Subsequent centesimal dilutions are produced as stated for C2.

*METHOD 3.2*

Method 3.2 is generally used to produce liquid dilutions of triturations of substances that for the most part are sparingly soluble to practically insoluble.

**METHODS 3.2.1, 3.2.2 (HAB 8a, 8b: LIQUID PREPARATIONS MADE FROM TRITURATIONS, AQUEOUS PREPARATIONS MADE FROM TRITURATIONS)**

Preparations made according to Method 3.2.1 and Method 3.2.2 are produced from triturations D4, D5 and D6 or from triturations C4, C5 and C6, prepared according to method 4.1.1 by at least 2 potentisation steps.

**Vehicles**

The vehicles in Table 2371.-3 may be used.

Table 2371.-3

| Method 3.2.1 | Method 3.2.2 |
|---|---|
| 1st potentisation:<br>Purified water | All potentisations:<br>Water for injections<br>Purified water |
| 2nd potentisation:<br>Ethanol (36 per cent *V/V*) | |
| Further potentisations:<br>Ethanol (50 per cent *V/V*) | |

**Potentisation**

For the first liquid potentisation, dissolve 1 part of the trituration in 9 parts (decimal dilutions) or 99 parts (centesimal dilutions) of the specified vehicle (see Table 2371.-3) and succuss. For further potentisations, proceed in the same manner with 1 part of the previous dilution.

The D6, D7, C6 and C7 dilutions produced by the above method are not to be used for the preparation of further dilutions. For the preparation of higher dilutions use the D8 or C8 dilution.

**METHOD 3.2.3**

Preparations made according to Method 3.2.3 are produced from triturations D2 onwards and from triturations C1, C2, C3 and C4, prepared according to method 4.1.2.

**Vehicles**

Suitable vehicles such as ethanol of an appropriate concentration or purified water may be used.

**Potentisation**

Unless otherwise specified, the first liquid decimal dilution (Dn-1) is made from:

1 part of the decimal trituration Dn-2;

9 parts of purified water or of another suitable vehicle in appropriate proportions.

The following decimal dilution (Dn) is made from:

1 part of the first liquid decimal dilution Dn-1;

9 parts of a suitable vehicle.

Subsequent decimal dilutions are produced as stated for Dn.

Unless otherwise specified, the first liquid centesimal dilution (Cn-1) is made from:

1 part of the centesimal trituration Cn-2;

99 parts of purified water or of another suitable vehicle in appropriate proportions.

The following centesimal dilution (Cn) is made from:

1 part of the first liquid centesimal dilution Cn-1;

99 parts of a suitable vehicle.

Subsequent centesimal dilutions are produced as stated for Cn.

4. TRITURATIONS

*METHOD 4.1*

Method 4.1 is used for triturations, that is solid dilutions, of raw materials or of triturations prepared according to Methods 4.2.1 or 4.2.2. The duration and intensity of the trituration are such that homogeneity and potentisation are achieved.

**Vehicle**

Unless otherwise specified, lactose monohydrate is used.

**METHOD 4.1.1 (HAB 6: TRITURATIONS)**

Triturations are prepared manually or mechanically. Mechanical trituration must be used for quantities exceeding 1 kg. The resulting particle size of the raw material in the first decimal or centesimal dilution does not exceed 100 µm, unless otherwise prescribed in the individual monograph.

**Ratios of raw material to vehicle**

| Decimal triturations | Centesimal triturations |
|---|---|
| The 1st decimal trituration (D1) is made from: | The 1st centesimal trituration (C1) is made from: |
| 1 part of the raw material | 1 part of the raw material |
| 9 parts of the vehicle | 99 parts of the vehicle |
| Subsequent decimal triturations (Dn) are produced as stated for D1, using 1 part of the previous trituration (Dn-1). | Subsequent centesimal triturations (Cn) are produced as stated for C1, using 1 part of the previous trituration (Cn-1). |

Where fresh plant material is used, the quantity of vehicle added is such so as to obtain 10 parts of the trituration (decimal trituration) or 100 parts of the trituration (centesimal trituration) from 1 part of the raw material (replace the mass of water lost from the fresh plant by an equivalent amount of the vehicle). A suitable gentle drying process may need to be applied to the solid dilution.

Where justified and authorised, it may be necessary to directly produce a C1 or 'D2' if to be used for further decimal triturations as the first solid trituration, made from 1 part of raw material and 99 parts of vehicle.

**Trituration**

Unless otherwise justified and authorised, the method consists of dividing the vehicle into 3 equal parts and adding the raw material to the first part, then adding the second and third part of the vehicle, thoroughly triturating after each addition.

For mechanical trituration, use a machine allowing the requirements for particle size of the first decimal or centesimal solid trituration to be met. A machine fitted with a scraping device may be used to ensure even trituration. The time required to prepare one trituration is at least 1 h, unless otherwise justified and authorised.

For manual trituration, divide the vehicle into 3 equal parts and briefly triturate the first part in a porcelain mortar. Add the raw material, triturate the mixture for 6 min, scrape down for 4 min with an appropriate non-metallic device (for example, a porcelain spatula). Triturate for a further 6 min, scrape down again for a further 4 min, then add the second part of the vehicle and continue as above. Proceed in the same manner with the rest of the vehicle. The minimum time required for the whole process is thus 1 h. Carry out the whole process again for each subsequent solid dilution.

Triturations from D5 or C5 onwards may also be prepared by intense mechanical treatment by a suitable mixing machine as follows: add the solid trituration to one third of the vehicle and mix. Add the second third of the vehicle, mix and proceed in the same manner with the last third of the vehicle. The whole process lasts minimum 1 hour, unless otherwise justified and authorised.

It is possible to change to a liquid medium from the 4th, 5th and 6th decimal or centesimal triturations, as described in Methods 3.2.1 and 3.2.2.

**METHOD 4.1.2 (FRENCH PHARMACOPOEIA)**

**Trituration**

Triturations are prepared as follows:

*Decimal triturations*

Reduce 1 part of the homoeopathic stock to a powder. Triturate carefully with a small quantity of the vehicle. Add the vehicle in small quantities until 9 parts of this vehicle have been used. The resulting trituration is the 1st decimal trituration (D1).

Triturate as described above 1 part of this trituration with 9 parts of the vehicle. The resulting trituration is the 2nd decimal trituration (D2).

In all cases, it is possible to change to a liquid medium after the 7th decimal trituration (D7) as described in Method 3.2.3.

*Centesimal triturations*

Proceed in the same manner but following a centesimal series.

In all cases, it is possible to change to a liquid medium after the 3rd centesimal trituration (C3) as described in Method 3.2.3.

*METHOD 4.2*

Method 4.2 is used for triturations, that is solid dilutions, of liquid preparations such as mother tinctures and solutions, their dilutions, mixtures and co-potentised mixtures.

Gradually impregnate the total amount of vehicle, gently dry the moist mixture, mill and sieve if necessary, then mix and triturate until homogeneity and potentisation are achieved. Trituration is further carried out as described for Method 4.1.1 or Method 4.1.2.

**Vehicle**

Unless otherwise specified, lactose monohydrate is used.

**METHOD 4.2.1 (HAB 7: TRITURATIONS)**

**Ratios of starting material to vehicle**

The quantity of vehicle added must always be such so as to obtain 10 parts of the trituration (decimal trituration) or 100 parts of the trituration (centesimal trituration) from the required number of parts of the liquid preparation (see Table 2371.-4), taking the mass of the dry residue into consideration. Where the dry residue is considered negligible, the quantity of vehicle added is 10 parts (decimal trituration) or 100 parts (centesimal trituration), for 1 part of the liquid preparation.

Table 2371.-4

| Decimal triturations | Centesimal triturations |
|---|---|
| **Mother tinctures prepared according to Methods 1.1.1, 1.1.3 and 1.1.4** | |
| The 1st 'decimal' trituration (D1) is made from: | The 1st 'centesimal' trituration (C1) is made from: |
| 2 parts of the mother tincture | 2 parts of the mother tincture |
| maximum 10 parts of the vehicle, taking the mass of the dry residue into consideration | maximum 100 parts of the vehicle, taking the mass of the dry residue into consideration |
| **Mother tinctures prepared according to Methods 1.1.2, 1.1.5, 1.1.6 and 1.1.7** | |
| The 1st 'decimal' trituration (D1) is made from: | The 1st 'centesimal' trituration (C1) is made from: |
| 3 parts of the mother tincture | 3 parts of the mother tincture |
| maximum 10 parts of the vehicle, taking the mass of the dry residue into consideration | maximum 100 parts of the vehicle, taking the mass of the dry residue into consideration |
| **Mother tinctures prepared according to Methods 1.1.8 and 1.1.9** | |
| *The mother tincture corresponds to the 1st decimal dilution (D1)* | |
| The 2nd decimal trituration (D2) is made from: | The 1st 'centesimal' trituration (C1) is made from: |
| 1 part of the mother tincture | 10 parts of the mother tincture |
| maximum 10 parts of the vehicle, taking the mass of the dry residue into consideration | maximum 100 parts of the vehicle, taking the mass of the dry residue into consideration |
| **Solutions prepared according to Method 3.1.1 or liquid dilutions, mixtures and co-potentised mixtures** | |
| Decimal trituration n+1 (Dn+1) is made from: | Centesimal trituration n+1 (Cn+1) is made from: |
| 1 part of the dilution (Dn) | 1 part of the dilution (Cn) |
| maximum 10 parts of the vehicle, taking the mass of the dry residue into consideration | maximum 100 parts of the vehicle, taking the mass of the dry residue into consideration |

**METHOD 4.2.2**

**Ratios of starting material to vehicle**

| **Mother tinctures prepared according to Methods 1.1.10 and 1.1.11** | |
|---|---|
| The 1st decimal trituration (D1) is made from: | The 1st centesimal trituration (C1) is made from: |
| 1 part of the mother tincture | 1 part of the mother tincture |
| 10 parts of the vehicle | 100 parts of the vehicle |

## 5. OTHER PREPARATIONS

*METHOD 5.1*

Method 5.1 is used for preparing homoeopathic preparations by co-potentising 2 or more stocks and/or dilutions thereof, where co-potentisation consists of mixing several stocks or dilutions of stocks then potentising them together in one or more potentisation steps.

**METHODS 5.1.1, 5.1.2, 5.1.3 (HAB 40a, 40b, 40c: CO-POTENTISED MIXTURES)**

The stocks and/or dilutions in Table 2371.-5 may be used.

Table 2371.-5

| Method 5.1.1 | Method 5.1.2 | Method 5.1.3 |
|---|---|---|
| Stocks | Aqueous preparations | Triturations |
| Solutions | Glycerol macerates and aqueous dilutions thereof | |
| Triturations | Triturations | |
| Liquid dilutions | | |
| Mother tinctures whose method of production specifies a 1/10 (or 1/100) dilution | | |

**Vehicles**

The choice of the vehicle is determined by and must comply with any special requirement for the particular stock as well as the dosage form (see table Table 2371.-6).

Table 2371.-6

| Method 5.1.1 | Method 5.1.2 | Method 5.1.3 |
|---|---|---|
| Ethanol (96 per cent *V/V*) | Water for injections | Lactose monohydrate |
| Ethanol (90 per cent *V/V*) | Purified water | |
| Ethanol (80 per cent *V/V*) | Sugar syrup (sucrose, purified water (64:36)) | |
| Ethanol (70 per cent *V/V*) | | |
| Ethanol (50 per cent *V/V*) | | |
| Ethanol (36 per cent *V/V*) | | |
| Ethanol (18 per cent *V/V*) | | |

For Method 5.1.1, when starting from a trituration and where justified, purified water is used for the 1st potentisation step.

For Method 5.1.2, when starting from a glycerol macerate containing sodium chloride, unless otherwise justified and authorised, the following vehicle is used: 0.2 parts of sodium hydrogen carbonate and 8.8 parts of sodium chloride in 991 parts of water for injections.

**Potentisation**

For each potentisation step, combine and succuss or triturate 1 part of the given mixture with 9 parts (decimal dilutions) or 99 parts (centesimal dilutions) of the appropriate vehicle.

**METHOD 5.1.4**

**Vehicles**

Ethanol of an appropriate concentration, purified water or lactose monohydrate may, for example, be used.

**Potentisation**

Potentisation may be performed as prescribed for Methods 5.1.1, 5.1.2 and 5.1.3, either on the last step or on several successive steps.

**METHOD 5.1.5**

**Vehicle**

Ethanol of an appropriate concentration, purified water or lactose monohydrate may, for example, be used.

**Potentisation**

For a co-potentisation of centesimal dilutions, each dilution (Cn-1) represents 1 per cent of the final product and the proportion of vehicle to be added is reduced by the proportion of the active substances [i.e. 100 per cent – (1 per cent × the number of active substances)]. The same procedure applies, in the appropriate proportions, when co-potentising decimal dilutions.

*METHOD 5.2*

Method 5.2 is used to prepare potencies with a 50 000 dilution factor (LM) by alternate steps of liquid dilution and impregnation of sucrose pillules (category 1, unless otherwise authorised).

**Solid and liquid potencies**

Use a dropper (*2.1.1*).

To prepare pillules of 1st LM potency, a C3 trituration of the substance to be potentised is processed as follows: dissolve 60 mg of the C3 trituration in a volume of ethanol (18 per cent *V/V*) or another authorised concentration, corresponding to 500 drops. To 1 drop of this solution add a volume of ethanol (90 per cent *V/V*) or another authorised concentration, corresponding to 100 drops and succuss at least 100 times. Use all of this solution to impregnate a mass of pillules corresponding to 50 000 pillules, then allow to dry in air.

To prepare pillules of 2nd LM potency, process pillules of 1st LM potency as follows: dissolve 1 pillule of 1st LM potency in 1 drop of purified water, add a volume of ethanol (90 per cent *V/V*) or another authorised concentration, corresponding to 100 drops and succuss at least 100 times. Use all of this solution to impregnate a mass of pillules corresponding to 50 000 pillules, then allow to dry in air.

Subsequent solid potencies are prepared as for the 2nd LM potency.

Liquid potencies (HAB 17)

To prepare a liquid LM potency, dissolve 1 pillule of the required potency in 10.0 mL of ethanol (18 per cent *V/V*) or another authorised concentration. The potency of the solution corresponds to the potency of the pillule dissolved therein.

**01/2017:2153**

# PILLULES FOR HOMOEOPATHIC PREPARATIONS

## Granula ad praeparationes homoeopathicas

DEFINITION

Solid preparations obtained from sucrose, lactose or other suitable excipients. They possess a suitable mechanical strength to resist handling without crumbling or breaking. They are intended for impregnation or coating with one or more homoeopathic preparations. The impregnated pillules comply with the requirements of the monograph *Homoeopathic pillules, impregnated (2079)*. The coated pillules comply with the requirements of the monograph *Homoeopathic pillules, coated (2786)*.

PRODUCTION

In the manufacture, packaging, storage and distribution of pillules for homoeopathic preparations, suitable measures are taken to ensure their microbiological quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use*.

If a system of sizing is used, the indications in Table 2153.-1 are used.

Table 2153.-1. – *Classification of pillules according to their mass and size*

| Category | Number of pillules for homoeopathic preparations | Mass (g) | Fineness (µm) |
|---|---|---|---|
| 1 | 470 - 530 | 1.0 | 1000 - 1600 |
| 2 | 160 - 333 | 1.0 | 1400 - 2000 |
| 3 | 110 - 130 | 1.0 | 1800 - 2500 |
| 4 | 70 - 90 | 1.0 | 2000 - 2800 |
| 5 | 40 - 50 | 1.0 | 2500 - 3350 |
| 6 | 16 - 30 | 1.0 | 3150 - 4500 |
| 7 | 10 | 0.9 - 1.1 | 4000 - 5600 |
| 8 | 5 | 0.9 - 1.1 | 5600 - 6700 |
| 9 | 3 | 0.9 - 1.1 | 7100 - 8000 |
| 10 | 2 | 0.9 - 1.1 | 8000 - 9500 |

*NOTE*: for categories 7-10, the mass is obtained by weighing the specified number of pillules.

CHARACTERS

*Appearance*: white or almost white spheroids.

*Solubility*: usually freely soluble in water.

IDENTIFICATION

The excipients used for the manufacture of pillules for homoeopathic preparations are identified by one or more suitable test(s).

TESTS

*If the test for fineness is carried out, the test for uniformity of mass need not be carried out, and vice versa.*

**Uniformity of mass.** Carry out the test using 20 pillules to constitute 1 unit. Weigh individually 20 units taken at random and determine the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 per cent and none deviate by more than 20 per cent.

**Fineness** (*2.9.35*): not less than 90 per cent *m/m* of the pillules are between the lower and upper limits of the corresponding category as indicated in Table 2153.-1.

**Uniformity of impregnation**: none of the individual values deviate by more than 10 per cent from the average of 10 determinations.

*Methylene blue impregnation solution. Use a freshly prepared solution.* Dissolve 1.000 g of *methylene blue R* in 50 mL of *ethanol (70 per cent V/V) R* and dilute to 1000.0 mL with the same solvent.

*Test solution.* Impregnate a suitable quantity of pillules for homoeopathic preparations with a suitable quantity of the methylene blue impregnation solution, to achieve a content of 10 µL of impregnation solution per gram of pillules. Dissolve 5.000 g (*m* g) of these impregnated pillules in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution.* Dilute 1.0 mL of the methylene blue impregnation solution to 100.0 mL with *water R*. To 5.0 mL of this solution add 5.000 g of pillules for homoeopathic preparations, dissolve in *water R* and dilute to 25.0 mL with the same solvent.

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at 665 nm. Calculate the percentage of impregnation of the pillules for homoeopathic preparations using the following expression:

$$\frac{A_1 \times 500}{A_2 \times m}$$

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the reference solution;

$m$ = mass of the impregnated pillules used to prepare the test solution, in grams.

Carry out 10 individual determinations.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^1$ CFU/g (*2.6.12*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

LABELLING

The label states:

- the composition of the pillules;
- where applicable, the size of the pillules.

**04/2017:2079**

# HOMOEOPATHIC PILLULES, IMPREGNATED

## Granula homoeopathica imbuta

DEFINITION

Solid preparations obtained from sucrose, lactose or other suitable excipients. They possess a suitable mechanical strength to resist handling without crumbling or breaking. Impregnated homoeopathic pillules are prepared by impregnation of *Pillules for homoeopathic preparations (2153)* with one or more liquid homoeopathic preparations. They are intended for sublingual or oral use.

PRODUCTION

The impregnation process takes place using liquid preparations containing ethanol usually at a concentration of at least 68 per cent *V/V* according to either of the following approaches:

- in proportions of 1 mass part of liquid to 100 mass parts of pillules;
- in proportions of 1 volume part of liquid to 100 mass parts of pillules.

In the manufacture, packaging, storage and distribution of homoeopathic pillules, suitable measures are taken to ensure their microbiological quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use.*

CHARACTERS

*Appearance*: white, almost white or slightly coloured spheroids.

*Solubility*: usually freely soluble in water.

TESTS

**Microbial contamination**. Unless otherwise justified, authorised and labelled, the pillules are intended for sublingual administration and the following acceptance criteria apply.

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^1$ CFU/g (*2.6.12*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

**01/2015:2786**

# HOMOEOPATHIC PILLULES, COATED

## Granula homoeopathica velata

DEFINITION

Solid preparations prepared from sucrose *Pillules for homoeopathic preparations (2153)* (category 5), by coating with a syrup made from homoeopathic preparations either potentised or mixed with sucrose syrup. Triturations can be incorporated separately. Coated homoeopathic pillules possess a suitable mechanical strength to resist handling without crumbling or breaking. They are intended for sublingual or oral use. Coated homoeopathic pillules may also be called 'globuli velati'.

PRODUCTION

In the manufacture, packaging, storage and distribution of coated homoeopathic pillules, suitable measures are taken to ensure their microbiological quality; recommendations on this aspect are provided in general chapter *5.1.4. Microbiological quality of non-sterile pharmaceutical preparations and substances for pharmaceutical use.*

For coating, use homoeopathic stocks (in case of a mother tincture or a glycerol macerate) and/or decimal dilutions thereof or decimal triturations and a syrup made from 64 parts of sucrose and 36 parts of purified water. The homoeopathic preparations used are prepared according to the general monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*.

To prepare 100 parts of coated homoeopathic pillules, the pillules are coated with the syrup using one of the following procedures, depending upon the type of homoeopathic preparation used.

**Aqueous dilutions**

Mix 1 part of an aqueous preparation with 9 parts of the syrup and potentise by succussion; evenly apply the mixture to $(100 - x)$ parts of pillules (where $x$ is the mass of sucrose in the syrup). The potency of the coated homoeopathic pillules corresponds to the potency of the liquid preparation used for coating.

**Triturations**

Mix 10 parts of a trituration with 20 parts of the syrup; evenly apply the mixture to $(100 - x - y)$ parts of pillules (where $x$ is the mass of sucrose in the syrup and $y$ is the mass of lactose monohydrate in the incorporated trituration). The potency of the coated homoeopathic pillules corresponds to the potency of the trituration used.

**Mixtures**

Unless otherwise justified and authorised, prepare a mixture obtained from 10 parts of a liquid preparation (obtained from aqueous preparations, triturations or aqueous dilutions of glycerol macerates) and 90 parts of the syrup, adding sufficient purified water to evenly apply the mixture to $(100 - x - y)$ parts of pillules (where $x$ is the mass of sucrose in the syrup and $y$ is the mass of lactose monohydrate in the incorporated triturations). The potency/potencies of the coated homoeopathic pillules correspond to the potency/potencies of the individual preparations used in the mixture.

TESTS

**Uniformity of mass.** Carry out the test using 20 coated homoeopathic pillules to constitute 1 unit. Weigh individually 20 units taken at random and determine the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 per cent and none deviate by more than 20 per cent.

**Microbial contamination.** Unless otherwise justified, authorised and labelled, coated homoeopathic pillules are intended for sublingual administration and the following acceptance criteria apply.

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^1$ CFU/g (*2.6.12*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

**04/2016:2695**

# ACIDUM PICRINICUM FOR HOMOEOPATHIC PREPARATIONS(1)

## Acidum picricum ad praeparationes homoeopathicas

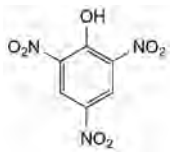

$C_6H_3N_3O_7$ $M_r$ 229.1
[88-89-1]

DEFINITION

2,4,6-Trinitrophenol (picric acid).

*Content*: 98.5 per cent to 101.5 per cent.

*The commercial substance is supplied immersed in water.*

*CAUTION: dry picric acid may explode if subjected to shock or excessive heat. Appropriate precautions must be taken and only very small quantities handled.*

CHARACTERS

*Appearance of picric acid*: light yellow.

IDENTIFICATION

A. Melting point (*2.2.14*): 121 °C to 123 °C, determined on the moistened substance to be examined dried in a desiccator to constant mass.

B. Suspend 0.1 g of the moistened substance to be examined in 1 mL of *water R*. Add 1 mL of *sodium sulfide solution R*. A red colour is produced.

TESTS

*Dry 5.0 g of the moistened substance to be examined in a desiccator to constant mass and use it to prepare solution S and to carry out the tests and the assay.*

**Solution S**. To 1.5 g of the dried substance to be examined add 30 mL of *distilled water R*, heat to boiling, allow to cool and filter. Dilute to 30 mL with *distilled water R*.

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*).

Dissolve 0.1 g of the dried substance to be examined in 10 mL of *distilled water R* and heat to 50 °C.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R* and filter if necessary.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 3 mL of solution S to 15 mL with *distilled water R* and filter if necessary.

ASSAY

Dissolve 0.200 g of the dried substance to be examined in 100 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, using 0.2 mL of *phenol red solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 22.91 mg of $C_6H_3N_3O_7$.

**07/2019:2824**

# ACIDUM SUCCINICUM FOR HOMOEOPATHIC PREPARATIONS(2)

## Acidum succinicum ad praeparationes homoeopathicas

$C_4H_6O_4$ $M_r$ 118.1
[110-15-6]

DEFINITION

Butanedioic acid (succinic acid).

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water and in ethanol (96 per cent), sparingly soluble in acetone.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Solution S (see Tests) is strongly acid (*2.2.4*).

B. Melting point (*2.2.14*): 184 °C to 189 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *succinic acid CRS*.

D. Neutralise 3 mL of solution S with *1 M sodium hydroxide*, then add 1 mL of *silver nitrate solution R2*. A white precipitate is formed.

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Oxalates**. Neutralise 5 mL of solution S with *dilute ammonia R1*, using 0.1 mL of *phenolphthalein solution R* as indicator. Add 0.1 mL of *acetic acid R* and 5 mL of *calcium sulfate solution R*. The solution remains clear for at least 20 min.

(1) FRENCH TITLE: Picricum acidum pour préparations homéopathiques
(2) FRENCH TITLE: Succinicum acidum pour préparations homéopathiques

ASSAY

Dissolve 0.500 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*) or using 0.2 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 59.05 mg of $C_4H_6O_4$.

**07/2018:2290**
**corrected 10.0**

# AGARICUS PHALLOIDES FOR HOMOEOPATHIC PREPARATIONS(3)

## Amanita phalloides ad praeparationes homoeopathicas

DEFINITION

Whole, fresh mushroom (fruiting body) *Amanita phalloides* (Vaill. ex Fr.) Link.

IDENTIFICATION

A. In young specimens, the cap, 50-150 mm in diameter, is hemispherical to campanulate with margins rolled inwards and is still covered by the white universal veil; in mature specimens, the cap is expanded, umbrella-like, convex to nearly plane and occasionally depressed; its colour is pale green along the margin and elsewhere greyish-green to yellowish-green, typically with fine grey streaks growing thicker towards the centre; the cuticle is peelable up to the middle of the cap as a fine membrane; the underlying flesh appears greenish-yellow, becoming more intense towards the centre of the cap, while in the other parts of the mushroom the flesh is uniformly whitish; lamellae are free, closely packed, white with a slight yellowish tinge; lamellulae are truncate; the stipe, about 10 cm high and 2 cm in diameter, is thin and solid, with a whitish, membranous, striate annulus and an enlarged and bulbous base that almost always shows a tough, white, membranous sac-like volva torn into irregular lobes at the top. In old specimens, the stipe is hollow, whitish and often covered with small greenish scales around the annulus. The spores are white.

B. Examined under a lens (x10), the upper surface is shiny, dry, appearing somewhat uneven, with no remains of the veil.

C. Examine under a microscope using a solution containing 1.5 g of *iodine R*, 5 g of *potassium iodide R* and 100 g of *chloral hydrate R* in 100 mL of *water R*. The spores are blackish-blue (starch reaction), short elliptical to subspherical, 8-11 µm long and 7-9 µm in diameter.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): minimum 85.0 per cent, determined on 5.0 g of the finely cut drug by drying in an oven at 105 °C for 2 h.

**Other *Amanita* species**. Veil remnants on caps are typical for most *Amanita* species, but not for *A. phalloides*. Therefore all mushrooms with veil remnants on the cap have to be discarded. The presence of veil remnants (patches) on the cap indicates adulteration with *A. citrina* (Schaeff.) Pers. (whitish-yellow cap with whitish to brownish patches) or with *A. muscaria* (L.: Fr.) Lamarck, *A. caesarea* (Scop.: Fr.) Pers., or *A. rubescens* Pers. (orange to bright red cap with white patches); a brownish cap indicates adulteration with *A. pantherina* (DC.) Krombh.; a greenish-white cap and a white stipe with a labile annulus indicates adulteration with *A. verna* (Bull.: Fr.) Lamarck.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

*Content*: 0.001 per cent *m/m* to 0.010 per cent *m/m* for the sum of α-amanitine and β-amanitine ($C_{39}H_{54}N_{10}O_{14}S$; $M_r$ 919).

PRODUCTION

The mother tincture is prepared according to the following methods as prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.5;
- method 1.1.10, using 5 parts of the cut drug for 100 parts of ethanol (45 per cent *V/V*) and maceration for 3 weeks.

CHARACTERS

*Appearance*: brownish-yellow, yellowish or green liquid.

IDENTIFICATION

Carry out test A when method 1.1.5 is used and carry out test B when method 1.1.10 is used.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Evaporate to dryness 20 mL of the mother tincture under vacuum at about 40 °C. Dissolve the residue in 1 mL of *water R* and add 1 mL of *methanol R*. Filter immediately.

*Reference solution*. Dissolve 10 mg of *rutoside trihydrate R* and 10 mg of *sennoside B R* in *methanol R* and dilute to 40 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (17:17:66 *V/V/V*).

*Application*: 40 µL [or 10 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in a current of warm air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: locate a quenching zone (sennoside B) in the lower third and a quenching zone (rutoside) in the middle third of the chromatogram obtained with the reference solution.

*Detection B*: treat immediately with a 1 per cent *V/V* solution of *cinnamic aldehyde R* in *methanol R* and allow to dry; treat with *hydrochloric acid R*; examine in daylight.

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

(3) FRENCH TITLE: Agaricus bulbosus pour préparations homéopathiques

| Top of the plate | | |
|---|---|---|
| Rutoside: a quenching zone | Rutoside: a yellow zone | A violet zone |
| Sennoside B: a quenching zone | | A violet zone<br>A violet zone<br>2 faint greyish-violet zones may be present |
| **Reference solution (detection A)** | **Reference solution (detection B)** | **Test solution (detection B)** |

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* The mother tincture to be examined.

*Reference solution.* Dissolve 2 mg of *gramine R* and 2 mg of *rutoside trihydrate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel $F_{254}$ plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R*, *methylene chloride R* (4:6:30:60 *V/V/V/V*).

*Application*: 40 µL [or 10 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in a current of warm air.

*Detection*: treat with a 5 per cent *V/V* solution of *cinnamic aldehyde R* in *methanol R* and expose to *hydrochloric acid R* vapour for 30 min; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Gramine: a yellow zone<br>Rutoside: a yellow zone | |
| | A pink zone<br>A pink zone |
| | An orange-yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.895 to 0.915 when method 1.1.5 is used.

**Ethanol** (*2.9.10*): 40 per cent *V/V* to 50 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 0.8 per cent.

**Mother tincture of Agaricus muscarius**. Thin-layer chromatography (*2.2.27*).

*Test solution.* The mother tincture to be examined.

*Reference solution.* Dissolve 10 mg of *leucine R* and 10 mg of *threonine R* in 5 mL of *water R* and dilute to 20 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *glacial acetic acid R*, *water R*, *acetone R*, *butanol R* (10:20:35:35 *V/V/V/V*).

*Application*: 20 µL [or 10 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in a current of warm air.

*Detection*: treat with a 1 g/L solution of *ninhydrin R* in *butanol R* and heat at 105 °C for 5-10 min; examine in daylight.

*Results*: the presence of noticeable zones in the chromatogram obtained with the test solution, in the same position as the zones due to leucine and threonine in the chromatogram obtained with the reference solution, indicates adulteration with mother tincture of Agaricus muscarius.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Evaporate 2.000 g of the mother tincture to be examined to dryness. Dissolve the residue in 2.0 mL of *water for chromatography R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 10.0 mg of *tryptophan CRS* in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.6 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.54 g of *ammonium acetate R* in 900 mL of *water for chromatography R*, adjust to pH 5.0 with *glacial acetic acid R* and dilute to 1 L with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 | 5 |
| 2 - 17 | 95 → 80 | 5 → 20 |
| 17 - 22 | 80 → 50 | 20 → 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 303 nm.

*Injection*: 10 µL.

*Relative retention* with reference to tryptophan (retention time = about 3 min): β-amanitine = about 2.9; α-amanitine = about 3.2.

*System suitability*: test solution:

- *resolution*: minimum 2.0 between the peaks due to β-amanitine and α-amanitine.

Calculate the percentage content *m/m* of α-amanitine and β-amanitine using the following expression:

$$\frac{(A_2 + A_3) \times m_1}{A_1 \times m_2} \times p \times K \times 0.1$$

$A_1$ = area of the peak due to tryptophan in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to α-amanitine in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to β-amanitine in the chromatogram obtained with the test solution;

$m_1$ = mass of *tryptophan CRS* used to prepare the reference solution, in grams;

$m_2$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$p$ = percentage content of tryptophan in *tryptophan CRS*;

$K$ = correction factor between α-amanitine, β-amanitine and tryptophan (0.1).

**07/2014:2023**

# ALLIUM SATIVUM FOR HOMOEOPATHIC PREPARATIONS[4]

## Allium sativum ad praeparationes homoeopathicas

DEFINITION

Fresh bulb of *Allium sativum* L.

CHARACTERS

It has a characteristic odour after cutting.

IDENTIFICATION

The bulb is generally 3 cm to 5 cm broad and almost spherical; the flat base bears the remnants of numerous short greyish-brown adventitious roots. The bulb consists of about 10 daughter bulbs (cloves) arranged roughly in a circle around a central axis. Individual daughter bulbs are 1 cm to 3 cm long, laterally compressed and convex on the dorsal side. Each daughter bulb has a tough, white or reddish skin around a fleshy tubular leaf, investing a more or less rounded elongated cone of leaf primordia and vegetative apex.

TESTS

**Water** (*2.2.13*): minimum 55.0 per cent, determined on 10.0 g of the finely cut drug, if performed to demonstrate the freshness of the drug.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of *Allium sativum* L. is prepared by maceration of the cut drug using alcohol of a suitable concentration.

CHARACTERS

*Appearance*: brownish-yellow liquid.

It has a peculiar and unpleasant aromatic odour.

IDENTIFICATION

A. To 2 mL of the mother tincture to be examined, add 0.2 mL of *dilute sodium hydroxide solution R*. A yellowish-white precipitate develops.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Extract 5 mL of the mother tincture to be examined with 2 quantities, each of 10 mL, of *ether R*. Combine the ether layers and dry over *anhydrous sodium sulfate R*. Filter and evaporate the filtrate in a water-bath at low temperature. Dissolve the residue in 0.4 mL of *methanol R*.

*Reference solution*. Dissolve 10 mg of *resorcinol R*, 10 mg of *thymol R* and 30 mg of *gallic acid R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *toluene R*, *di-isopropyl ether R* (10:40:50 *V/V/V*).

*Application*: 40 µL of the test solution and 10 µL of the reference solution.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm and identify gallic acid; spray with *anisaldehyde solution R*, heat to 105-110 °C for 5-10 min. Examine in daylight within 10 min.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Other zones may also be visible in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | An intense reddish-violet zone |
| Thymol: an orange-red zone | |
| | An intense reddish-violet zone |
| | A violet zone |
| | A yellowish or greenish zone |
| ——— | ——— |
| Resorcinol: an intense orange-red zone | |
| ——— | ——— |
| Gallic acid: a yellow zone | A violet zone |
| (UV at 254 nm: a fluorescent quenching zone) | A greenish-yellow zone |
| | A violet zone may be present |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.885 to 0.960.

**Ethanol** (*2.9.10*): 50 per cent *V/V* to 70 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 4.0 per cent.

STORAGE

In an airtight container.

**01/2018:2916**

# AMMONIUM CARBONICUM FOR HOMOEOPATHIC PREPARATIONS[5]

## Ammonii carbonas ad praeparationes homoeopathicas

DEFINITION

Mixture of varying proportions of diammonium carbonate ($(NH_4)_2CO_3$; $M_r$ 96.1), ammonium hydrogen carbonate ($NH_4HCO_3$; $M_r$ 79.1) and ammonium carbamate ($NH_2COONH_4$; $M_r$ 78.1).

*Content*: 30.0 per cent to 37.0 per cent of $NH_3$ ($M_r$ 17.03).

CHARACTERS

*Appearance*: white or almost white, translucent masses or white or almost white, crystalline powder, with a strong smell of ammonia.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in *water R* and dilute to 20 mL with the same solvent. The solution is slightly alkaline (*2.2.4*).

B. It gives the reaction of carbonates and bicarbonates (*2.3.1*).

(4) FRENCH TITLE: Allium sativum pour préparations homéopathiques
(5) FRENCH TITLE: Ammonium carbonicum pour préparations homéopathiques

C. It gives the reaction of ammonium salts and salts of volatile bases (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in 100 mL of *distilled water R*. Boil until the volume is reduced to about 10 mL. Verify that the emitted vapours no longer make *barium hydroxide solution R* cloudy. Cool and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Chlorides** (*2.4.4*): maximum 20 ppm.

Dissolve 2.5 g by gradually adding a mixture of 5 mL of *nitric acid R* and 10 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 60 ppm, determined on solution S.

ASSAY

Dissolve 2.5 g in *water R* and dilute to 100 mL with the same solvent. To 10.0 mL of the solution add 10.0 mL of *1 M hydrochloric acid*. Shake continuously until the effervescence ceases. Add 40 mL of *water R*. Titrate the excess of acid with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of $NH_3$.

STORAGE

In an airtight container.

**07/2014:2094**
**corrected 10.0**

# ANACARDIUM FOR HOMOEOPATHIC PREPARATIONS[6]

## Semecarpus anacardium ad praeparationes homoeopathicas

DEFINITION

Dried fruit of *Semecarpus anacardium* L. (*Anacardium orientale* L.).

*Content*: minimum 6.0 per cent *m/m* of total phenol derivatives expressed as eugenol ($C_{10}H_{12}O_2$; $M_r$ 164.2) (dried drug).

IDENTIFICATION

A. The dried fruit is oval and more or less heart-shaped; about 2 cm long, nearly 2 cm wide and 0.5 cm thick. Its surface is smooth, shiny and blackish. A transverse section shows a rather well developed, tough pericarp riddled with rather wide lacunae containing an abundant thick reddish-brown juice. The pericarp covers a white kernel under a reddish skin. The fruit may include the blackish, fleshy, wrinkled, cupuliferous receptacle.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of suitably cut herbal drug, add 10 mL of *ethanol (90 per cent V/V) R*. Heat under reflux on a water-bath at 60 °C for 15 min. Allow to cool and filter.

*Reference solution*. Dissolve 5 mg of *gallic acid R* and 5 mg of *caffeic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *toluene R* (15:85 *V/V*).

*Application*: 20 µL of the test solution and 10 µL of the reference solution, as bands.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a solution containing 10 g/L of *diphenylboric acid aminoethyl ester R* and 50 g/L of *macrogol 400 R* in *methanol R*. Examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other fainter zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| | A greenish-blue fluorescent zone |
| _____ | _____ |
| | Several violet-blue fluorescent zones |
| | A yellow fluorescent zone |
| Caffeic acid: a violet-blue fluorescent zone | |
| _____ | _____ |
| Gallic acid: a violet-blue fluorescent zone | A violet-blue fluorescent zone (gallic acid) |
| **Reference solution** | **Test solution** |

TESTS

***Anacardium occidentale* L.** Fruits of *Anacardium occidentale* L. are not present. These are up to 35 mm long, 30 mm large, 20 mm thick, light brown and distinctly kidney-shaped. The pericarp is smooth or slightly crinkled with dark marbling in places.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g of the finely divided herbal drug by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 5.0 per cent.

ASSAY

**Total phenol derivatives**. Absorption spectrophotometry (*2.2.25*).

*Stock solution*. Place 4.500 g of the crushed herbal drug in a flask. Add 200 mL of *ethanol (90 per cent V/V) R*. Boil in a water-bath under reflux for 4 h. Cool the flask. Quantitatively transfer into a volumetric flask. Dilute to 250.0 mL with *ethanol (90 per cent V/V) R*. Filter the liquid through a paper filter 125 mm in diameter. Discard the first 50 mL of the filtrate. Dilute 5.0 mL of filtrate to 50.0 mL with *ethanol (90 per cent V/V) R* and shake. Dilute 5.0 mL of this solution to 10.0 mL with *ethanol (90 per cent V/V) R* and shake.

*Test solution*. To 2.0 mL of stock solution add 1.0 mL of *phosphomolybdotungstic reagent R* and 10 mL of *water R*, mix and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. Wait exactly 3 min then filter the solution through a fibre-glass filter with a 1 µm mesh aperture, discarding the first 5 mL.

*Reference solution*. Dissolve 80.0 mg of *eugenol R* in *ethanol (90 per cent V/V) R* and dilute to 250.0 mL with the same solvent. Dilute 5.0 mL of the solution to 25.0 mL with *ethanol (90 per cent V/V) R*. To 2.0 mL of this solution add 1.0 mL of *phosphomolybdotungstic reagent R* and 10 mL of *water R*, mix and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. Wait exactly 3 min then filter the solution through a fibre-glass filter with a 1 µm mesh aperture, discarding the first 5 mL.

(6) FRENCH TITLE: Anacardium orientale pour préparations homéopathiques

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at 755 nm after 30 min using *water R* as compensation liquid.

Calculate the percentage content *m/m* of total phenol derivatives, expressed as eugenol, from the following expression:

$$\frac{A_1 \times m_2 \times 400}{A_2 \times m_1}$$

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the reference solution;

$m_1$ = mass of the drug to be examined, in milligrams;

$m_2$ = mass of eugenol in the reference solution, in milligrams.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

The mother tincture of Anacardium is prepared by maceration using ethanol of a suitable concentration from the dried fruit of *Semecarpus anacardium* L. (*Anacardium orientale* L.).

*Content*: 0.5 per cent *m/m* to 1.0 per cent *m/m* of total phenol derivatives expressed as eugenol.

CHARACTERS

*Appearance*: yellowish-brown or reddish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*) as described under Identification B of the drug with the following modification.

*Test solution.* The tincture to be examined.

*Results*: see identification B for the drug.

TESTS

**Relative density** (*2.2.5*): 0.815 to 0.845.

**Ethanol** (*2.9.10*): 85 per cent *V/V* to 95 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 1.50 per cent.

ASSAY

**Total phenol derivatives**. Absorption spectrophotometry (*2.2.25*) as described in the assay of the drug to be examined with the following modifications.

*Stock solution.* Place 8.000 g of the mother tincture to be examined in a volumetric flask and dilute to 250.0 mL with *ethanol (90 per cent V/V) R*. Dilute 5.0 mL of this solution to 20.0 mL with *ethanol (90 per cent V/V) R*.

*Test solution.* To 2.0 mL of stock solution add 1.0 mL of *phosphomolybdotungstic reagent R* and 10 mL of *water R*, mix and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. Wait exactly 3 min then filter the solution through a fibre-glass filter with a 1 µm mesh aperture, discarding the first 5 mL.

*Reference solution.* Dissolve 80.0 mg of *eugenol R* in *ethanol (90 per cent V/V) R* and dilute to 250.0 mL with the same solvent. Dilute 5.0 mL of the solution to 25.0 mL with *ethanol (90 per cent V/V) R*. To 2.0 mL of this solution add 1.0 mL of *phosphomolybdotungstic reagent R* and 10 mL of *water R*, mix and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. Wait exactly 3 min then filter the solution through a fibre-glass filter with a 1 µm mesh aperture, discarding the first 5 mL.

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at 755 nm after 30 min, using *water R* as compensation liquid.

Calculate the percentage content *m/m* of total phenol derivatives expressed as eugenol, using the following expression:

$$\frac{A_1 \times m_2 \times 80}{A_2 \times m_1}$$

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the reference solution;

$m_1$ = mass of the mother tincture to be examined, in milligrams;

$m_2$ = mass of eugenol in the reference solution, in milligrams.

**07/2014:2024**

# APIS FOR HOMOEOPATHIC PREPARATIONS[(7)]

## Apis mellifera ad praeparationes homoeopathicas

DEFINITION

Live worker honey bee (*Apis mellifera* L.).

CHARACTERS

Characters described under Identification.

PRODUCTION

If the bee has been exposed to treatment to prevent or cure diseases, appropriate measures are taken to ensure that the levels of residues are as low as possible.

IDENTIFICATION

The body is about 15 mm long, black, with a silky sheen, and covered with red hairs with a touch of grey. The broad tibiae are without spines. The posterior margins of the segments and legs are brown, with gradual transition to orange-red. The claws are two-membered, the maxillary palps single-membered. On the hind legs are baskets or scoops invested with bristles. The wings have 3 complete cubital cells, with the radial cell twice as long as it is wide; the 3 cells on the lower margin and the 3 middle cells are closed. A duct connects the barbed sting with the poison sac.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of *Apis mellifera* L. is prepared by maceration using alcohol of a suitable concentration.

CHARACTERS

Pale yellow liquid that may darken on storage.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* The mother tincture to be examined.

(7) FRENCH TITLE: Apis mellifica pour préparations homéopathiques

*Reference solution*. Dissolve 12 mg of *4-aminobutanoic acid R*, 12 mg of *leucine R* and 12 mg of *proline R* in 5 mL of *water R* and dilute to 50 mL with *alcohol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *ethanol R* (17:63 *V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 10 min; examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference and test solutions. Other zones may also be visible.

| Top of the plate | |
|---|---|
| ——— | ——— |
| | A pink zone |
| Leucine: a pink zone | A pink zone |
| | A pink zone |
| | A pink zone |
| ——— | ——— |
| Proline: an orange-yellow zone | An orange-yellow zone |
| 4-Aminobutanoic acid: a pink zone | A pink zone |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.890 to 0.910.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 0.30 per cent.

**07/2018:1599**

# ARSENICUM ALBUM FOR HOMOEOPATHIC PREPARATIONS[8]

## Arsenii trioxidum ad praeparationes homoeopathicas

$As_2O_3$ $M_r$ 197.8
[1327-53-3]

DEFINITION

*Content*: 99.5 per cent to 100.5 per cent of $As_2O_3$.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble to sparingly soluble in water. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Dissolve 20 mg in 1 mL of *dilute hydrochloric acid R*, add 4 mL of *water R* and 0.1 mL of *sodium sulfide solution R*. The resulting yellow precipitate is soluble in *dilute ammonia R1*.

B. Dissolve 20 mg in 1 mL of *hydrochloric acid R1*, add 5 mL of *hypophosphorous reagent R* and heat for 15 min on a water-bath. A black precipitate develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Prepare a 100 g/L solution in *dilute ammonia R1*, heating if necessary.

**Sulfides**: maximum 20 ppm.

Dissolve 1.0 g in 10.0 mL of *dilute sodium hydroxide solution R*. Add 0.05 mL of *lead acetate solution R*. Any colour in the test solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 10.0 mL of a 0.015 g/L solution of *sodium sulfide R* in *dilute sodium hydroxide solution R* and 0.05 mL of *lead acetate solution R*.

ASSAY

Dissolve 40.0 mg in a mixture of 10 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R*. Add 10 mL of *dilute hydrochloric acid R* and 3 g of *sodium hydrogen carbonate R* and mix. Add 1 mL of *starch solution R* and titrate with *0.05 M iodine*.

1 mL of *0.05 M iodine* is equivalent to 4.946 mg of $As_2O_3$.

**07/2018:2141**

# AURUM CHLORATUM NATRONATUM FOR HOMOEOPATHIC PREPARATIONS[9]

## Natrii tetrachloroauras dihydricus ad praeparationes homoeopathicas

$Na[AuCl_4],2H_2O$ $M_r$ 397.8
[13874-02-7]

DEFINITION

Sodium tetrachloroaurate(1-) dihydrate.

*Content*: 97.0 per cent to 101.0 per cent of $Na[AuCl_4],2H_2O$.

CHARACTERS

*Appearance*: orange-yellow, hygroscopic powder or crystals.

*Solubility*: very soluble or freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 20 mg in 2.0 mL of *0.1 M nitric acid*. Add 0.1 g of *oxalic acid R* and boil in a water-bath for 1 h. A deposit of metallic gold is formed.

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

C. Solution S gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Ignite 0.20 g in a porcelain crucible at 600 °C ± 50 °C for 30 min. Allow to cool and extract with 3 mL of *water R*, heating if necessary. Use the supernatant.

**Free hydrochloric acid**. When a glass rod impregnated with *concentrated ammonia R* is held close to the substance to be examined, no white fumes are produced.

(8) FRENCH TITLE: Arsenicum album pour préparations homéopathiques
(9) FRENCH TITLE: Aurum muriaticum natronatum pour préparations homéopathiques

**Nitrates**: maximum 200 ppm.

Dissolve 0.20 g in 4.0 mL of *nitrate-free water R*. Add 0.6 g of *zinc R* and 10 mL of *dilute sulfuric acid R*. Heat the solution on a water-bath for 30 min, allow to cool and filter. Rinse the filter with *nitrate-free water R* and dilute the filtrate to 20.0 mL with the same solvent. To 5.0 mL of this solution add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5.0 mL of *nitrogen-free sulfuric acid R*. Heat in a water-bath at 50 °C for 15 min, centrifuge if necessary and use the clear supernatant. Any blue colour in the solution is not more intense than that in a reference solution prepared at the same time and in the same manner using a mixture of 1.0 mL of *nitrate standard solution (10 ppm $NO_3$) R* and 3.0 mL of *nitrate-free water R*.

ASSAY

Dissolve 40.0 mg in 10 mL of *potassium iodide solution R*. Allow to stand for 5 min. Titrate with *0.01 M sodium thiosulfate* until decolourised. Shortly before reaching the endpoint, add 0.5 mL of *starch solution R*.

1 mL of *0.01 M sodium thiosulfate* is equivalent to 1.989 mg of $Na[AuCl_4],2H_2O$.

STORAGE

In an airtight container, protected from light.

**01/2017:2142**

# BARIUM CHLORATUM FOR HOMOEOPATHIC PREPARATIONS(10)

## Barii chloridum dihydricum ad praeparationes homoeopathicas

$BaCl_2,2H_2O$ $M_r$ 244.3
[10326-27-9]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of $BaCl_2,2H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble or practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 0.1 g in 1 mL of *water R*. Add 0.3 mL of *dilute sulfuric acid R*. A white precipitate is formed; it is insoluble in *dilute hydrochloric acid R* and in *dilute nitric acid R*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

ASSAY

Dissolve 0.200 g in 100 mL of *water R*. Add 100 mL of *methanol R*, 10 mL of *concentrated ammonia R* and 2 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to colourless.

1 mL of *0.1 M sodium edetate* is equivalent to 24.43 mg of $BaCl_2,2H_2O$.

**01/2016:2489**
**corrected 10.0**

# BELLADONNA FOR HOMOEOPATHIC PREPARATIONS(11)

## Atropa belladonna ad praeparationes homoeopathicas

DEFINITION

Whole, fresh, flowering plant of *Atropa belladonna* L., harvested at the end of flowering, with the ligneous base of the stems removed.

IDENTIFICATION

The root crown is short, thick, cylindrical, with one or more heads, and with many branching roots extending from it; these are grey or brown, fleshy, cylindrical or fusiform, very rarely twisted. The erect stem of the youngest part is cylindrical and pubescent. The petiolate leaves are alternate, simple, oval, attenuated at both ends, lacking stipules, with pinnate venation, up to 20 cm long and 10 cm wide; on the flowering stems they are paired and unequal in size. The drooping, pedunculate, solitary flowers appear between the petioles of the paired leaves; they are actinomorphous, pentameric, with superior ovaries; they have a gamosepalous, glandular, green calyx, which grows larger after flowering. The corolla, 2-3 cm long, is campanulate, gamopetalous, greenish-yellow with violet veins in the upper part; it is hairy and deciduous, divided into 5 narrow, rounded lobes. The 5 alternipetalous stamens, attached at the base of the corolla, have long filaments which are hairy in the lower part; they bear bilocular, introrse anthers which dehisce longitudinally. The superior ovary consists of 2 carpels with axile placentation. The style is simple, ending in a slightly cordiform stigma, extending beyond the stamens. The fruit is a spherical, fleshy berry, the size of a cherry, green then black and shiny when ripe. The seeds are numerous, reniform with a testa showing markings.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): minimum 75.0 per cent, determined on 5.0 g of the finely cut drug by drying in an oven at 105 °C for 2 h.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

*Content*: 0.020 per cent *m/m* to 0.050 per cent *m/m* of hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4).

(10) FRENCH TITLE: Baryta muriatica pour préparations homéopathiques
(11) FRENCH TITLE: Belladonna pour préparations homéopathiques

PRODUCTION

The mother tincture is prepared from the comminuted herbal drug according to the following methods prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.3;
- method 1.1.10, using ethanol (45 per cent *V/V*) and a maceration time of 3-5 weeks.

CHARACTERS

*Appearance*: brown liquid.

IDENTIFICATION

Examine the chromatograms obtained in the test for atropine.

*Detection A*: spray with *potassium iodobismuthate solution R2* until orange or brown zones become visible against a yellow background. Examine in daylight.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present, in particular in the middle of the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Hyoscine: an orange or brown zone | An orange or brown zone, sometimes very faint or absent (hyoscine) |
| _____ | _____ |
| _____ | _____ |
| Hyoscyamine: an orange or brown zone | An orange or brown zone (hyoscyamine) |
| **Reference solution** | **Test solution** |

The zone due to hyoscine in the chromatogram obtained with the reference solution and that with the same $R_F$ in the chromatogram obtained with the test solution are located at the limit between the middle third and the upper third and they may therefore appear in either part of the chromatogram.

TESTS

**Relative density** (*2.2.5*): 0.932 to 0.947 when method 1.1.3 is used.

**Ethanol** (*2.9.10*): 40 per cent *V/V* to 50 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 1.4 per cent.

**Mother tincture of Hyoscyamus niger – mother tincture of Datura stramonium.**

Concentrate 10 mL of the mother tincture to be examined on a water bath until a volume of 5 mL is obtained. Add 10 mL of *water R* and filter. Shake the filtrate with 5 mL of *methylene chloride R* and collect the organic layer. Filter and evaporate the filtrate to dryness under reduced pressure. Take up the residue with 10 mL of hot *water R*, and add 0.1 mL of *dilute ammonia R1*. A greenish-blue fluorescence appears when examined under ultraviolet light at 365 nm.

The absence of greenish-blue fluorescence indicates substitution by the mother tincture of Hyoscyamus niger and/or by the mother tincture of Datura stramonium.

**Atropine**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Evaporate 10 mL of the mother tincture to be examined on a water-bath. Take up the residue with 5 mL of *dilute sulfuric acid R1* and filter. Make alkaline with *concentrated ammonia R* and extract with 15 mL of *1,1-dimethylethyl methyl ether R*. Dry the ether layer over *anhydrous sodium sulfate R* and filter. Evaporate to dryness on a water-bath and dissolve the residue in 1 mL of *methanol R*.

*Reference solution*. Dissolve 50 mg of *hyoscyamine sulfate R* in 9 mL of *methanol R* (solution A). Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R* (solution B). To 8 mL of solution A add 1.8 mL of solution B and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: at 105 °C for 15 min; allow to cool.

*Detection B*: after detection A (see Identification), spray with *sodium nitrite solution R* until the yellow background disappears. Examine in daylight.

*Result B*: no greyish-blue zone (atropine) appears in place of the brown or reddish-brown zone due to hyoscyamine in the chromatogram obtained with the test solution.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Introduce 1.500 g of the mother tincture to be examined into a volumetric flask and dilute to 10.0 mL with *water for chromatography R*. Apply the mixture to a cartridge filled with a cation-exchange material (60 µm; 60 mg), pre-conditioned with 2 mL of *methanol R2* and then 2 mL of *water for chromatography R*. Rinse the volumetric flask with 1 mL of a mixture of 2 volumes of *anhydrous formic acid R* and 98 volumes of *water for chromatography R*, and apply the rinsings to the cartridge. Wash the cartridge with 2 mL of a mixture of 2 volumes of *anhydrous formic acid R* and 98 volumes of *water for chromatography R* and then with 2 mL of *methanol R2*. Dry the cartridge with the aid of a vacuum and elute with 3.5 mL of a mixture of 2 volumes of *concentrated ammonia R* and 98 volumes of *methanol R2*. Expel the solvent remaining in the cartridge into the eluate, add 40 µL of *glacial acetic acid R* and dilute to 5.0 mL with *water for chromatography R*.

*Reference solution (a)*. Dissolve 10.0 mg of *hyoscyamine sulfate CRS* in *methanol R2* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 10.0 mg of *hyoscine hydrobromide R* in *methanol R2* and dilute to 10.0 mL with the same solvent.

*Reference solution (c)*. Mix 3.0 mL of reference solution (a) and 2.5 mL of reference solution (b) and dilute to 25.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *triethylamine R*, *acetonitrile for chromatography R*, *phosphate buffer solution pH 7.0 R6* (0.01:5:95 *V/V/V*);
- *mobile phase B*: *triethylamine R*, *phosphate buffer solution pH 7.0 R6*, *acetonitrile for chromatography R* (0.01:40:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 10 | 10 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solution (c).

Homoeopathic preparations

*Retention time*: hyoscyamine = about 12 min; hyoscine = about 13.5 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 5 between the peaks due to hyoscyamine and hyoscine;
- *symmetry factor*: maximum 2.0 for the peak due to hyoscyamine.

Calculate the percentage content *m/m* of hyoscyamine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 16.667}$$

$A_1$ = area of the peak due to hyoscyamine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hyoscyamine in the chromatogram obtained with reference solution (c);

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *hyoscyamine sulfate CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of anhydrous hyoscyamine in *hyoscyamine sulfate CRS*.

**07/2014:2143**

# CADMIUM SULFURICUM FOR HOMOEOPATHIC PREPARATIONS(12)

## Cadmii sulfas hydricus ad praeparationes homoeopathicas

$CdSO_4,8/3H_2O$ $M_r$ 256.5

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of sulfates (*2.3.1*).

B. To 2 mL of solution S (see Tests) add 2 mL of *sodium sulfide solution R*. A yellow precipitate is formed.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.3 mL of *methyl orange solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Nitrates**: maximum 100 ppm.

Dissolve 1.0 g in *water R* and dilute to 20.0 mL with the same solvent. To 1.0 mL of this solution add 0.2 mL of a 10 g/L solution of *sulfanilic acid R* in *acetic acid R* and 0.2 mL of a recently prepared 3 g/L solution of *naphthylamine R* in *acetic acid R*. Add a turning of *zinc R*. A pink colour is produced within 5 min. It is not more intense than that of a mixture of 0.5 mL of *nitrate standard solution (10 ppm $NO_3$) R* and 0.5 mL of *water R*, prepared at the same time.

**Zinc sulfate, alkaline-earth sulfates, rare-earth sulfates**. Dissolve 1.0 g in 17 mL of *water R*. Add 0.5 mL of *hydrochloric acid R* and 1 g of *thioacetamide R*. Heat in a water-bath for 10 min. Dilute to 20.0 mL with *water R* and filter. Evaporate 10.0 mL of this solution to dryness in an oven. Ignite the residue at about 800 ± 50 °C to constant mass. The residue weighs a maximum of 2 mg.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 5 mL of solution S.

**Water** (*2.5.12*): 16.0 per cent to 20.0 per cent, determined on 80 mg. Shake for 10 min before carrying out the determination.

ASSAY

Dissolve 0.200 g in 50 mL of *water R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and 50 mg of *mordant black 11 triturate R1*. Titrate with *0.1 M sodium edetate* until the colour changes from red to green.

1 mL of *0.1 M sodium edetate* is equivalent to 20.85 mg of $CdSO_4$.

**07/2018:2996**

# CALCIUM FLUORATUM FOR HOMOEOPATHIC PREPARATIONS(13)

## Calcii fluoridum ad praeparationes homoeopathicas

$CaF_2$ $M_r$ 78.1
[7789-75-5]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent of $CaF_2$.

CHARACTERS

*Appearance*: fine, white or almost white powder.

*Solubility*: practically insoluble in water, slightly soluble in mineral acids.

IDENTIFICATION

A. To 0.80 g add 20 mL of *hydrochloric acid R* and heat to boiling under a reflux condenser until complete dissolution (about 30 min). After cooling, add 0.1 mL of *phenolphthalein solution R*, and then *concentrated ammonia R* until a pink colour is obtained. Add *glacial acetic acid R* until the solution is decolourised, then add 1 mL in excess. Filter and dilute to 40 mL with *water R*. Dilute 1 mL of the solution obtained to 5 mL with *distilled water R* and add 2 mL of *ammonium oxalate solution R*. A white precipitate is formed which dissolves in 2 mL of *dilute hydrochloric acid R*.

B. In a lead or platinum crucible, mix 10 mg with 20 mg of *anhydrous colloidal silica R* and a few drops of *sulfuric acid R*, with the aid of a copper wire, in order to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of *water R* is suspended, and warm gently. A white ring is rapidly formed around the drop of water.

(12) FRENCH TITLE: Cadmium sulfuricum pour préparations homéopathiques
(13) FRENCH TITLE: Calcarea fluorica pour préparations homéopathiques

TESTS

**Free acid**. Shake 5.0 g with 2 g of *calcium chloride R* and 100 mL of *water R* for 5 min. Heat to 70 °C and filter. To 40 mL of the filtrate, maintained at 70 °C, add 0.1 mL of *methyl red solution R*. Not more than 1.0 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to yellow.

ASSAY

Introduce 0.150 g into a 500 mL conical flask and add 8 mL of *hydrochloric acid R*. Boil for 3-4 min on a preheated hot plate and allow to cool. Add 300 mL of *water R*, followed by *strong sodium hydroxide solution R* until the first appearance of persistent opalescence (about pH 14). Add 0.13 g of *calconecarboxylic acid triturate R* and titrate with *0.1 M sodium edetate* until the colour changes from red-violet to pure blue. The opalescence caused by the strong sodium hydroxide solution disappears during the course of the titration. If still visible at the end of the titration, it can be dissolved by adding a few drops of *hydrochloric acid R*.

1 mL of *0.1 M sodium edetate* is equivalent to 7.81 mg of $CaF_2$.

**01/2017:2144**

# CALCIUM IODATUM FOR HOMOEOPATHIC PREPARATIONS(14)

## Calcii iodidum tetrahydricum ad praeparationes homoeopathicas

$CaI_2,4H_2O$ $M_r$ 366.0
[13640-62-5]

DEFINITION

*Content*: 97.0 per cent to 102.0 per cent of $CaI_2$ (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: very soluble or freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of calcium (*2.3.1*).

B. Solution S (see Tests) gives reaction (b) of iodides (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

**Free iodine, iodates**. To 5 mL of solution S add 2 mL of *methylene chloride R*. Shake and allow to stand. The organic layer is colourless (*2.2.2, Method I*) (free iodine). Add 0.2 mL of *dilute sulfuric acid R*. Shake and allow to stand. The organic layer remains colourless (*2.2.2, Method I*) (iodates).

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on 10 mL of solution S.

**Water** (*2.5.12*): 18.0 per cent to 22.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Add 5 mL of *dilute nitric acid R* and 25.0 mL of *0.1 M silver nitrate*. Shake. Add 2 mL of *ferric ammonium sulfate solution R2* and titrate with *0.1 M ammonium thiocyanate* until the colour changes to reddish-yellow.

1 mL of *0.1 M silver nitrate* is equivalent to 14.70 mg of $CaI_2$.

STORAGE

In an airtight container.

**07/2014:2486**
**corrected 10.0**

# COCCULUS FOR HOMOEOPATHIC PREPARATIONS(15)

## Anamirta cocculus ad praeparationes homoeopathicas

DEFINITION

Dried, ripe fruit of *Anamirta cocculus* (L.) Wight & Arn. (syn. *A. paniculata* Colebr.).

*Content*: minimum 0.80 per cent of picrotoxinin ($C_{15}H_{16}O_6$; $M_r$ 292.3) (dried drug).

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, B, C.*

A. The fruits are dark greyish-brown or black, reniform or sub-spherical, about 6-10 mm in diameter and 9-12 mm long; the outer surface is irregularly wrinkled with a ridge about 4-6 mm long running between the pale, circular scar left by the stalk and the small beak of the remains of the stigma. The pericarp is hard, about 1 mm thick and the inner surface is brownish-grey, hard and woody. Cut transversely, the fruit shows a single, cup-shaped seed into the hollow of which an ingrowth of the mesocarp and endocarp projects. Cut longitudinally, the endosperm shows the presence of 2 narrow cavities in each of which is enclosed 1 of the foliaceous cotyledons.

B. Microscopic examination (*2.8.23*). Reduce to a powder (710) (*2.9.12*). The powder is brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2486.-1): fragments of the epicarp (surface view [D]) consisting of thin-walled, polygonal cells, about 30-50 µm in diameter [Da], anomocytic stomata (*2.8.3*) [Db], and cells in a pattern consisting of a cell with slightly thickened walls, pitted at the centre, surrounded by 4-6 cells [Dc]; fragments of the epicarp and outer layers of the mesocarp (transverse section [C]) showing the epicarp covered by a fine cuticle [Ca] and cells of the mesocarp, ovoid or rounded, some containing prisms of calcium oxalate [Cb]; numerous fragments of the inner layers of the mesocarp and of the endocarp [A] consisting of sclereids [Aa] and short fibres with pitted walls [Ab]; fragments of the endocarp consisting of layers of variously oriented fibres (surface view [E]); sclereids and isolated fibres [B]; vascular bundles (longitudinal section [F]), accompanied by fibres [Fa]; fragments of the endosperm [G, H] containing very numerous small acicular crystals.

(14) FRENCH TITLE: Calcarea iodata pour préparations homéopathiques
(15) FRENCH TITLE: Cocculus indicus pour préparations homéopathiques

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.00 g of the powdered herbal drug (710) (*2.9.12*) add 20 mL of *ethanol (90 per cent V/V) R*, shake for 2 h and then centrifuge (1000 *g*). Use the supernatant.

*Reference solution.* Dissolve 10 mg of *picrotin R* and 10 mg of *picrotoxinin R* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *methanol R*, *ethyl acetate R*, *heptane R* (10:40:50 *V/V/V*).

*Application*: 40 µL [or 10 µL] as bands of 20 mm [or 10 mm].

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Above the zone due to picrotoxinin, several pink or violet zones may also be visible in the chromatogram obtained with the test solution.

| **Top of the plate** | |
|---|---|
| Picrotoxinin: a blue zone | A blue zone (picrotoxinin) |
| Picrotin: a blue zone | A blue zone (picrotin) |
| **Reference solution** | **Test solution** |

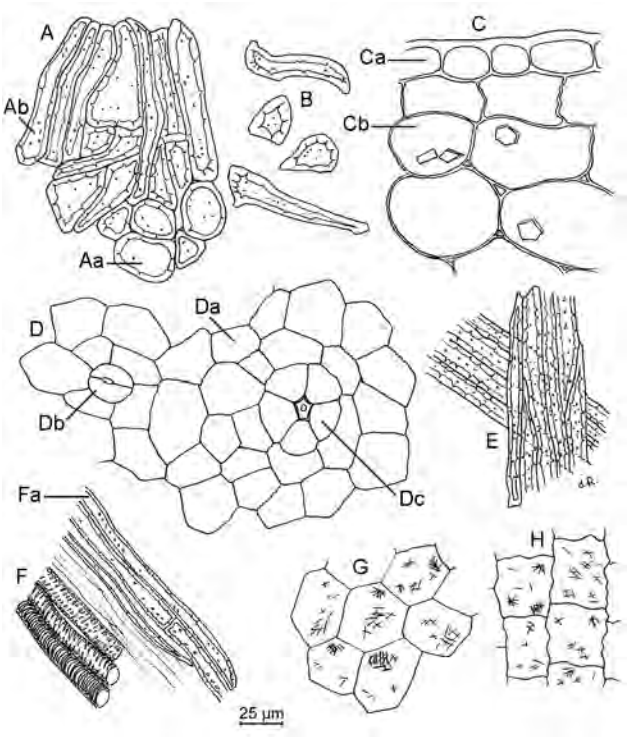


Figure 2486.-1. – *Illustration for identification test B of powdered herbal drug of Cocculus*

D. Examine the chromatograms obtained in the assay.

*Results*: the peaks due to picrotoxinin and picrotin in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with the reference solution.

TESTS

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 6.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 2.000 g of the powdered herbal drug (710) (*2.9.12*) add 20.0 mL of *ethanol (90 per cent V/V) R*, shake for 2 h and then centrifuge at 1000 *g* for 5 min. Dilute 2.0 mL of the supernatant to 20.0 mL with the mobile phase and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution.* Dissolve 5.0 mg of *picrotin CRS* and 5.0 mg of *picrotoxinin CRS* in 10.0 mL of *acetonitrile R*. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, *water R* (30:70 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of *picrotoxinin CRS*.

*Retention time*: picrotin = about 6 min; picrotoxinin = about 9.5 min.

*System suitability:* reference solution:

– *resolution*: minimum 2.0 between the peaks due to picrotin and picrotoxinin.

Calculate the percentage content of picrotoxinin using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to picrotoxinin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to picrotoxinin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *picrotoxinin CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of picrotoxinin in *picrotoxinin CRS*.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

*Content*: 0.07 per cent *m/m* to 0.15 per cent *m/m* of picrotoxinin ($C_{15}H_{16}O_6$).

PRODUCTION

The mother tincture is prepared from the dried, ripe fruit of *A. cocculus* (L.) Wight & Arn. according to the following methods prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.8 using the powdered herbal drug (710) (*2.9.12*) and ethanol (90 per cent *V/V*); use ethanol (70 per cent *V/V*) to prepare the 4th decimal dilution and ethanol (50 per cent *V/V*) for subsequent dilutions;
- method 1.1.10 using the crushed drug in fragments of about 2-3 mm, ethanol (90 per cent *V/V*) and a maceration time of about 3 weeks.

CHARACTERS

*Appearance*: yellow or dark yellow liquid.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*) as described in identification test C for the herbal drug with the following modification.

*Test solution.* The mother tincture to be examined.

B. Examine the chromatograms obtained in the assay.

*Results*: the peaks due to picrotoxinin and picrotin in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with the reference solution.

TESTS

**Relative density** (*2.2.5*): 0.830 to 0.845 when method 1.1.8 is used.

**Ethanol** (*2.9.10*): 85 per cent *V/V* to 95 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 0.7 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the assay of the herbal drug with the following modification.

*Test solution.* Dilute 0.500 g of the mother tincture to be examined to 10.0 mL with the mobile phase and filter using a membrane filtre (nominal pore size 0.45 µm).

Calculate the percentage content of picrotoxinin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to picrotoxinin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to picrotoxinin in the chromatogram obtained with the reference solution;

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *picrotoxinin CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of picrotoxinin in *picrotoxinin CRS*.

(16) FRENCH TITLE: Crocus sativus pour préparations homéopathiques

**07/2014:1624**

# CROCUS FOR HOMOEOPATHIC PREPARATIONS(16)

## Croci sativi stigma ad praeparationes homoeopathicas

DEFINITION

Dried stigmas of *Crocus sativus* L. usually joined by the base to a short style.

CHARACTERS

Characteristic, aromatic odour.

IDENTIFICATION

A. The dark brick-red stigmas, when dry, are 20 mm to 40 mm long and after soaking with water, about 35 mm to 50 mm long. The tubes, gradually widening at the top, are incised on one side, the upper margin is open and finely crenated. The style connecting the 3 stigmas is pale yellow and not more than 5 mm long.

B. Examine under a microscope using *chloral hydrate solution R*. It shows the following diagnostic characters: elongated epidermal cells, frequently with a short, central papilla; in water they release a yellow colouring matter; the upper border of the stigma has finger-shaped papillae, up to 150 µm long; between them are single, globular pollen grains, about 100 µm wide, with a finely pitted exine, vascular bundles with small spirally thickened vessels and no fibres.

C. Carefully crush pieces of the herbal drug to coarse particles and moisten with 0.2 mL of *phosphomolybdic acid solution R*. The particles turn blue within 1-2 min or they have a blue areole around them.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Carefully crush 0.1 g of the herbal drug with a glass rod and moisten with 0.2 mL of *water R*. After 3 min add 5 mL of *methanol R*, allow to stand for 20 min, protected from light, and filter through a plug of glass wool.

*Reference solution.* Dissolve 5 mg of *naphthol yellow R* in 5 mL of *methanol R* and add a solution of 5 mg of *Sudan red G R* in 5 mL of *methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *2-propanol R*, *ethyl acetate R* (10:25:65 *V/V/V*).

*Application*: 10 µL of the test solution and 5 µL of the reference solution as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection A*: examine in daylight.

*Results A*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| A red zone | |
| A yellow zone | |
| | 2 yellow zones |
| | An intense yellow zone (crocine) |
| **Reference solution** | **Test solution** |

*Detection B*: examine in ultraviolet light at 254 nm.

Homoeopathic preparations

*Results B*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| A red zone | 1 or 2 quenching zones |
| A yellow zone | A quenching zone |
| **Reference solution** | **Test solution** |

*Detection C*: treat with *anisaldehyde solution R* and examine in daylight while heating at 100-105 °C for 5-10 min.

*Results C*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| A red zone | 1 or 2 red to reddish-violet zones |
| A blue to bluish-green zone | A red to reddish-violet zone |
| | 2 blue to bluish-green zones |
| | An intense blue to bluish-green zone (crocine) |
| **Reference solution** | **Test solution** |

E. Dilute 0.1 mL of the test solution (see Identification D) with 1 mL of *methanol R*. Deposit 0.1 mL of this solution on a filter paper, allow to dry and spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R*. Examine in ultraviolet light at 365 nm. The spot shows an intense orange-yellow fluorescence.

TESTS

**Colouring intensity**. Introduce 0.10 g into a 5 mL volumetric flask and dilute to 5.0 mL with *distilled water R*. Close the flask and shake every 30 min for 8 h. Then allow to stand for 16 h. Dilute 1.0 mL to 500.0 mL with *distilled water R*. The absorbance (*2.2.25*) measured at 440 nm using *distilled water R* as the compensation liquid, is not less than 0.44.

**Foreign matter**. Examine the herbal drug microscopically. No parts with rough walls, no crystals and no pollen grains containing 3 germinal pores are present.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 7.0 per cent, determined on the residue obtained in the test for loss on drying.

**07/2014:2146**

# CUPRUM ACETICUM FOR HOMOEOPATHIC PREPARATIONS(17)

## Cupri acetas monohydricus ad praeparationes homoeopathicas

$Cu(C_2H_3O_2)_2,H_2O$ $M_r$ 199.7
[6046-93-1]

(17) FRENCH TITLE: Cuprum aceticum pour préparations homéopathiques

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of $Cu(C_2H_3O_2)_2,H_2O$.

CHARACTERS

*Appearance*: greenish-blue crystals or green powder.

*Solubility*: soluble in water, slightly soluble or very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of acetates (*2.3.1*).

B. Dissolve 0.1 g in 10 mL of *water R* and add *dilute ammonia R1* dropwise. A dark blue colour is produced.

TESTS

**Solution S**. Dissolve 3.0 g in a mixture of 40 mL of *distilled water R* and 0.6 mL of *glacial acetic acid R*, with heating at 70 °C. Cool and dilute to 45 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Impurities not precipitating with hydrogen sulfide**: maximum 0.1 per cent, calculated as sulfates.

To 2.000 g add 92 mL of *water R* and 8.0 mL of *dilute sulfuric acid R*. Heat to 70 °C. Pass a current of *hydrogen sulfide R* until there is no longer precipitation of copper sulfide. Allow to cool and stand, then filter. Evaporate to dryness 50.0 mL of the filtrate in a crucible. Ignite the residue at about 600 ± 50 °C to constant mass.

**Chlorides** (*2.4.4*): maximum 50 ppm, determined on solution S.

**Sulfates** (*2.4.13*): maximum 150 ppm, determined on solution S.

**Iron** (*2.4.9*): maximum 20 ppm.

Dissolve 0.500 g in 10 mL of *water R*. Transfer to a separating funnel. Add 20 mL of *hydrochloric acid R1* and 10 mL of *methyl isobutyl ketone R*. Shake vigorously for 3 min. Allow to stand. Transfer the organic layer to a second separating funnel and add 10 mL of *water R*. Shake vigorously for 3 min. Allow to stand. The aqueous layer complies with the limit test for iron.

**Nickel**: maximum 10 ppm.

To the residue obtained in the test for impurities not precipitating with hydrogen sulfide, add 2.0 mL of *hydrochloric acid R* and 1.0 mL of *sulfuric acid R*. Evaporate to dryness. Dissolve the residue in a mixture of 3.0 mL of *dilute sulfuric acid R* and 17.0 mL of *water R*. To 4.0 mL of this solution add 4.0 mL of *water R*, 5.0 mL of *bromine water R*, 7.0 mL of *dilute ammonia R1* and 3.0 mL of a 10 g/L solution of *dimethylglyoxime R* in *ethanol (90 per cent V/V) R*. This solution is not more intensely coloured within 1 min than a solution prepared as follows: mix 4.0 mL of a 1 ppm solution of nickel (Ni) prepared from *nickel standard solution (10 ppm Ni) R*, 4.0 mL of *water R* and 5.0 mL of *bromine water R*; carefully add 7.0 mL of *dilute ammonia R1* and 3.0 mL of a 10 g/L solution of *dimethylglyoxime R* in *ethanol (90 per cent V/V) R*.

ASSAY

Dissolve 0.400 g in *water R* and dilute to 50 mL with the same solvent. Add 6.0 mL of *glacial acetic acid R*, 10.0 g of *potassium iodide R* and 1 mL of *starch solution R*. Titrate with *0.1 M sodium thiosulfate*.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 19.97 mg of $Cu(C_2H_3O_2)_2,H_2O$.

**04/2016:1610**

# CUPRUM METALLICUM FOR HOMOEOPATHIC PREPARATIONS[18]

## Cuprum ad praeparationes homoeopathicas

Cu $A_r$ 63.5
[7440-50-8]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent of Cu.

CHARACTERS

*Appearance*: reddish-brown powder.

*Solubility*: practically insoluble in water, soluble in nitric acid, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. To 2 mL of solution S (see Tests) add 0.5 mL of *potassium ferrocyanide solution R*. A reddish-brown precipitate is formed.

B. To 5 mL of solution S add 0.6 mL of *ammonia R*. A blue precipitate is formed. Add 2 mL of *ammonia R*. The precipitate disappears; the solution has an intense blue colour.

TESTS

**Solution S**. Dissolve 2.0 g in 10 mL of *nitric acid R*. After nitrous fumes are no longer evolved, dilute to 60 mL with *distilled water R*.

**Acidity or alkalinity**. To 5.0 g add 20 mL of *carbon dioxide-free water R*. Boil for 1 min. Cool. Filter and dilute to 25.0 mL with *carbon dioxide-free water R*. To 10 mL of the solution add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 100 ppm, determined on solution S.

**Sulfates** (*2.4.13*): maximum 300 ppm, determined on solution S.

**Iron**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 1.00 g in 5 mL of *nitric acid R* and dilute to 50.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted as necessary with a 1 per cent *V/V* solution of *nitric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Flame*: air-acetylene.

**Lead**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use the test solution prepared for the test for iron.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R*, diluted as necessary with a 1 per cent *V/V* solution of *nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Flame*: air-acetylene.

**Zinc**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use the test solution prepared for the test for iron.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with a 1 per cent *V/V* solution of *nitric acid R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Flame*: air-acetylene.

ASSAY

Dissolve 0.100 g in 5 mL of *nitric acid R*. Heat to expel the nitrous fumes. Add 200 mL of *water R* and neutralise (*2.2.3*) with *dilute ammonia R1*. Add 1 g of *ammonium chloride R* and 3 mg of *murexide R*. Titrate with *0.1 M sodium edetate* until the colour changes from green to violet.

1 mL of *0.1 M sodium edetate* is equivalent to 6.354 mg of Cu.

**01/2019:2705**

# DIGITALIS FOR HOMOEOPATHIC PREPARATIONS[19]

## Digitalis purpurea ad praeparationes homoeopathicas

DEFINITION

Fresh leaf of *Digitalis purpurea* L, collected just before or during flowering.

IDENTIFICATION

A. The flower is light to dark violet. The leaf is variable in size, usually 10-50 cm long and 4-15 cm wide, simple, entire, lanceolate, oblong, and ending in a subacute apex. The margins of the lamina are crenate or dentate. It is thick with a velvety or rough texture. The lamina is decurrent, attenuated along the midrib, the whole forming a winged, triangular petiole, with purple-pink spots at the base. The venation is pinnate, with the lateral veins leaving the midrib at about 45°; they anastomose near the leaf margin forming arcs, and are connected to each other by an extensive network of tertiary veinlets. The upper surface is greyish-green and pubescent, but sometimes almost glabrous. The veins are sunken, forming depressed lines around bulging areas in the lamina. The lower surface is paler and very tomentose; the whitish veins are prominent giving the surface a honeycomb-like appearance.

B. From non-flowering plants, take a fragment of the lower epidermis of the leaf. Examine under a microscope using *chloral hydrate solution R*. The leaf has a smooth cuticle and shows the following diagnostic characters: epidermal cells 30-75 µm long, with distinctly sinuous anticlinal walls; numerous anomocytic stomata (*2.8.3*); covering trichomes often articulated and bent at right angles, uniseriate, usually with 3-5 cells, sometimes collapsed, and a terminal cell covered in a sometimes smooth but usually verrucose or slightly striated cuticle; glandular trichomes with unicellular stalks and globular bicellular heads; glandular trichomes with multicellular, uniseriate stalks and unicellular heads.

TESTS

**Foreign matter** (*2.8.2*): maximum 5 per cent.

(18) FRENCH TITLE: Cuprum metallicum pour préparations homéopathiques
(19) FRENCH TITLE: Digitalis purpurea pour préparations homéopathiques

***Digitalis lanata***. The presence of brownish-yellow flowers, of oval lanceolate, narrow leaves, with entire margins or with dentate margins only near the apex, the existence of a few glandular trichomes of epidermal cells with very characteristic beaded walls, and the absence of covering trichomes, indicates adulteration with *Digitalis lanata* Ehrh.

***Digitalis lutea***. The presence of brownish-yellow flowers, of sessile, lanceolate, denticulate, almost glabrous leaves and the scarcity of smooth covering trichomes, indicates adulteration with *Digitalis lutea* L.

***Digitalis grandiflora***. The presence of brownish-yellow flowers, of oblong or oval leaves with serrate margins, non-reticulate venation and veins bearing rare, very large covering trichomes with large pits, indicates adulteration with *Digitalis grandiflora* All.

**Loss on drying** (*2.2.32*): minimum 70.0 per cent, determined on 5.0 g of the comminuted herbal drug by drying in an oven at 105 °C for 2 h.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

The mother tincture is prepared from the fresh leaf of *Digitalis purpurea* L, collected just before or during flowering.

*Content*: 0.003 per cent *m/m* to 0.013 per cent *m/m* of digitoxin ($C_{41}H_{64}O_{13}$; $M_r$ 765).

PRODUCTION

The mother tincture is prepared from the comminuted herbal drug according to the following methods prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.3;
- method 1.1.10, using ethanol (65 per cent *V/V*) and a maceration time of 3-5 weeks.

CHARACTERS

*Appearance*: light greenish-brown or brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. Evaporate 10 mL of the mother tincture to be examined to dryness under reduced pressure. Take up the residue in 1 mL of a mixture of equal volumes of *ethyl acetate R* and *methanol R*.

*Reference solution*. Dissolve 5 mg of *digitoxin R* and 2 mg of *gitoxin R* in a mixture of equal volumes of *ethyl acetate R* and *methanol R*, and dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *water R*, *methanol R*, *ethyl acetate R* (7.5:10:75 *V/V/V*).

*Application*: 20 µL [or 10 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with a mixture of 2 volumes of a 10 g/L solution of *chloramine R* and 8 volumes of a 250 g/L solution of *trichloroacetic acid R* in *ethanol (96 per cent) R*, then heat at 100-105 °C for 10 min; examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Digitoxin: a bluish-green fluorescent zone | A bluish-green fluorescent zone (digitoxin) |
| Gitoxin: a light blue fluorescent zone | A light blue fluorescent zone (gitoxin) |
| ——— | ——— |
| | A faint brownish-yellow fluorescent zone may be present |
| | A faint light blue fluorescent zone may be present |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.935 to 0.955 when method 1.1.3 is used.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 2.5 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dilute 8.0 g of the mother tincture to be examined to 10.0 mL with *water for chromatography R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a)*. Dissolve 10.0 mg of *digitoxin for LC assay CRS* in *methanol R2* and dilute to 10.0 mL with the same solvent. Dilute 2.00 mL of the solution to 20.0 mL with a mixture of equal volumes of *methanol R2* and *water for chromatography R*.

*Reference solution (b)*. Dissolve 10.0 mg of *lanatoside C R* and 10.0 mg of *digoxin R* in *methanol R2* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with a mixture of equal volumes of *methanol R2* and *water for chromatography R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 32 | 80 → 40 | 20 → 60 |
| 32 - 33 | 40 → 80 | 60 → 20 |
| 33 - 38 | 80 | 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Retention time*: digitoxin = about 25 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to lanatoside C and digoxin.

Calculate the percentage content of digitoxin using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to digitoxin in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to digitoxin in the chromatogram obtained with reference solution (a);

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *digitoxin for LC assay CRS* used to prepare reference solution (a), in grams;

$p$ = percentage content of *digitoxin for LC assay CRS*.

**07/2014:2026**

# FERRUM METALLICUM FOR HOMOEOPATHIC PREPARATIONS(20)

## Ferrum ad praeparationes homoeopathicas

Fe $A_r$ 55.85
[7439-89-6]

DEFINITION

Iron obtained by reduction or sublimation as a fine blackish-grey powder.

*Content*: 97.5 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: fine, blackish-grey powder, without metallic lustre.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves with heating in dilute mineral acids.

IDENTIFICATION

Dissolve 50 mg in 2 mL of *dilute sulfuric acid R* and dilute to 10 mL with *water R*. The solution gives reaction (a) of iron (*2.3.1*).

TESTS

**Solution S**. To 10.0 g add 40 mL of *water R*. Boil for 1 min. Cool, filter and dilute to 50.0 mL with *water R*.

**Alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Substances insoluble in hydrochloric acid**. Dissolve 2.00 g in 40 mL of *hydrochloric acid R*. Heat on a water-bath. As soon as fumes are no longer evolved, filter through a sintered-glass filter (16) (*2.1.2*). Rinse with *water R*. Dry the residue in an oven at 100-105 °C for 1 h. The residue weighs a maximum of 20 mg (1.0 per cent).

**Substances soluble in water**. Evaporate 10.0 mL of solution S on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfides and phosphides**. In a 100 mL conical flask carefully mix 1.0 g with 10 mL of *dilute hydrochloric acid R*. Within 30 s *lead acetate paper R* moistened with *water R* and placed over the mouth of the flask is not coloured more intensely than light brown by the resulting fumes.

**Arsenic** (*2.4.2*): maximum 5 ppm.

Boil 0.2 g in 25 mL of *dilute hydrochloric acid R* until completely dissolved. The solution complies with limit test A.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dissolve 1.00 g in a mixture of 60 mL of *dilute hydrochloric acid R* and 10 mL of *dilute hydrogen peroxide solution R*. Reduce to a volume of 5 mL and dilute to 50.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Flame*: air-acetylene.

**Lead**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. In a separating funnel, place 20 mL of the test solution prepared for the test for copper. Add 25 mL of *lead-free hydrochloric acid R*. Stir with 3 quantities, each of 25 mL, of *di-isopropyl ether R*. Collect the aqueous layer. Add 0.10 g of *sodium sulfate decahydrate R*. Evaporate to dryness. Take up the residue with 1 mL of *lead-free nitric acid R* and dilute to 20 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R*, diluting with a 10 per cent *V/V* solution of *nitric acid R* containing 5 g/L of *sodium sulfate decahydrate R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217 nm.

*Flame*: air-acetylene.

ASSAY

Stir for 10 min 0.100 g in a hot solution of 1.25 g of *copper sulfate pentahydrate R* in 20 mL of *water R* in a 100 mL conical flask with a ground-glass stopper. Filter rapidly and wash the filter. Combine the filtrate and the washings, acidify with *dilute sulfuric acid R* and titrate with *0.02 M potassium permanganate* until a pink colour is obtained.

1 mL of *0.02 M potassium permanganate* is equivalent to 5.585 mg of Fe.

LABELLING

The label indicates whether the substance is obtained by reduction or sublimation.

(20) FRENCH TITLE: Ferrum metallicum pour préparations homéopathiques

**01/2008:2092**
**corrected 9.0**

# HEDERA HELIX FOR HOMOEOPATHIC PREPARATIONS[21]

## Hedera helix ad praeparationes homoeopathicas

DEFINITION

Fresh, young, fully developed but not yet lignified branch of *Hedera helix* L., harvested immediately before or at the beginning of flowering.

IDENTIFICATION

The fresh, young branches of *Hedera helix* L. are thin and flexible, climbing; they cling to their support by stem-roots. The leaves are alternate, simple and petiolate. The petiole shows a cylindrical section. The upper surface of the leaves is glabrous and shiny, darker than the lower surface. The lamina is usually divided into 3-5 more or less deeply cut lobes on sterile branches; it is oval, with a pointed apex on fertile branches. The inflorescences are arranged in a simple semi-globular corymb and grouped in terminal clusters. The pedicels of the umbel are covered in whitish hairs. Each flower shows 5 small teeth formed by the upper part of the sepals and 5 petals covered in very small inverted hairs.

TESTS

**Foreign matter** (*2.8.2*): if required by the competent authority, maximum 5 per cent.

**Loss on drying** (*2.2.32*): if required by the competent authority, minimum 50 per cent, determined on 5.0 g of the finely cut drug by drying in an oven at 105 °C for 2 h.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of *Hedera helix* L. is prepared by maceration using ethanol of a suitable concentration.

*Content*: minimum 0.15 per cent *m/m* of hederacoside C ($C_{59}H_{96}O_{26}$; $M_r$ 1221).

CHARACTERS

*Appearance*: dark greenish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* The mother tincture to be examined.

*Reference solution.* Dissolve 1 mg of *α-hederin R* and 1 mg of *hederacoside C R* in *methanol R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (1:1:4 *V/V/V*).

*Application*: 20 µL as bands.

*Development*: over half of the plate.

*Drying*: in air.

*Detection*: spray with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R* and heat at 100-105 °C for 10 min. Examine in daylight.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Other faint zones may also be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| α-Hederin: a violet zone | A violet zone (α-hederin) |
| Hederacoside C: a brown zone | A brown zone (hederacoside C) |
| ——— | A greyish-brown zone ——— |
| | A yellow zone |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.890 to 0.925.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 2.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* In a 20.0 mL volumetric flask, dilute 3.000 g of the mother tincture to be examined to 20.0 mL with the mobile phase.

*Reference solution.* In a 50.0 mL volumetric flask, dissolve 20.0 mg of *hederacoside C R* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *water R*, adjusted to pH 3 with *phosphoric acid R*, and 65 volumes of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Retention time*: hederacoside C = about 8 min.

Calculate the percentage content *m/m* of hederacoside C using the following expression:

$$\frac{A_1 \times m_2 \times C \times 0.4}{A_2 \times m_1}$$

$A_1$ = area of the peak due to hederacoside C in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hederacoside C in the chromatogram obtained with the reference solution;

$m_1$ = mass of the mother tincture in the test solution, in grams;

$m_2$ = mass of *hederacoside C R* in the reference solution, in grams;

$C$ = percentage content of *hederacoside C R*.

(21) FRENCH TITLE: Hedera helix pour préparations homéopathiques

**04/2016:2671 corrected 9.1**

# HISTAMINUM FOR HOMOEOPATHIC PREPARATIONS(22)

## Histaminum ad praeparationes homoeopathicas

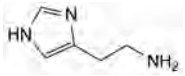

$C_5H_9N_3$ $M_r$ 111.1
[51-45-6]

DEFINITION

2-(1*H*-Imidazol-4-yl)ethan-1-amine.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: slightly yellow, deliquescent crystals.

*Solubility*: soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 82 °C to 85 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *histamine CRS*.

TESTS

**Solution S**. Dissolve 0.3 g in 2.75 mL of *2 M hydrochloric acid R* and dilute to 10 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method I*).

**Histidine**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Solution S.

*Reference solution (a)*. Dissolve 25 mg of *histidine hydrochloride monohydrate CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Mix 1 mL of the test solution and 1 mL of reference solution (a).

*Plate*: *TLC silica gel G plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetonitrile R* (5:20:75 *V/V/V*).

*Application*: 1 µL of the test solution and reference solution (a); 2 µL of reference solution (b).

*Development*: over a path of 10 cm [or 7 cm].

*Drying*: at 100-105 °C for 15 min.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *histidine*: any spot due to histidine in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (1.7 per cent).

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 0.5 g.

ASSAY

Dissolve 50.0 mg in 5 mL of *anhydrous formic acid R* and add 20 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 5.557 mg of $C_5H_9N_3$.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2012:2500 corrected 10.0**

# HYDRASTIS CANADENSIS FOR HOMOEOPATHIC PREPARATIONS(23)

## Hydrastis canadensis ad praeparationes homoeopathicas

The herbal drug complies with the requirements of the monograph *Goldenseal rhizome (1831)*.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

The mother tincture is prepared from the whole or cut, dried rhizome and roots of *Hydrastis canadensis* L.

*Content*:

– *hydrastine* ($C_{21}H_{21}NO_6$; $M_r$ 383.4): 0.10 per cent to 0.40 per cent;

– *berberine* ($C_{20}H_{18}NO_4$; $M_r$ 336.4): 0.20 per cent to 0.50 per cent.

PRODUCTION

The mother tincture is prepared by the following methods prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

– Method 1.1.8, using the powdered herbal drug (710) (*2.9.12*) and ethanol (70 per cent *V/V*) [or ethanol (62 per cent *m/m*)];

– Method 1.1.10, using the fragmented herbal drug (pieces about 1 cm in diameter), ethanol (65 per cent *V/V*) and maceration for 3-5 weeks.

CHARACTERS

*Appearance*: yellowish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. The mother tincture to be examined.

*Reference solution*. Immediately before use, dissolve 5 mg of *hydrastine hydrochloride R* and 5 mg of *berberine chloride R* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

(22) FRENCH TITLE: Histaminum pour préparations homéopathiques
(23) FRENCH TITLE: Hydrastis canadensis pour préparations homéopathiques

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 20 µL [or 5 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: see below the sequence of fluorescent zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint fluorescent zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Berberine: a bright yellow fluorescent zone<br>Hydrastine: a deep blue fluorescent zone | A bright yellow fluorescent zone (berberine)<br>A deep blue fluorescent zone (hydrastine) |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.890 to 0.905 when method 1.1.8 is used.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 1.2 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dilute about 1.000 g, accurately weighed, of the mother tincture to be examined to 20.0 mL with the mobile phase.

*Reference solution*. Immediately before use, dissolve 10.0 mg of *hydrastine hydrochloride CRS* and 10.0 mg of *berberine chloride CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 9.93 g of *potassium dihydrogen phosphate R* in 730 mL of *water R*, add 270 mL of *acetonitrile R* and mix.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*Elution order*: hydrastine, berberine.

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the peaks due to hydrastine and berberine.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to hydrastine and berberine.

Calculate the percentage content *m/m* of hydrastine using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.913}{A_2 \times m_1 \times 5}$$

Calculate the percentage content *m/m* of berberine using the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.905}{A_2 \times m_1 \times 5}$$

(24) FRENCH TITLE: Hyoscyamus niger pour préparations homéopathiques

$A_1$ = area of the peak due to hydrastine or to berberine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to hydrastine or to berberine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *hydrastine hydrochloride CRS* or mass of *berberine chloride CRS* used to prepare the reference solution, in grams;

$p$ = percentage content of *hydrastine hydrochloride CRS* or percentage content of *berberine chloride CRS*.

**01/2008:2091**
**corrected 9.0**

# HYOSCYAMUS FOR HOMOEOPATHIC PREPARATIONS(24)

## Hyoscyamus niger ad praeparationes homoeopathicas

DEFINITION

Whole, fresh flowering plant of *Hyoscyamus niger* L.

IDENTIFICATION

Hyoscyamus is an annual or biennial plant, with a well developed taproot. The robust, erect stem is hollow and subcylindrical and up to 80 cm long. The soft, viscid, dull dark-green leaves are densely pubescent on both surfaces, especially on the veins. The lower leaves are petiolate and are arranged in a rosette; the lower cauline leaves are semi-amplexicaul and the upper ones are completely amplexicaul. The lamina, up to 25 cm long, is oblong to ovate with 2 to 5 broadly dentate lobes on each side. The midrib is well developed. The secondary veins arise at a wide angle from the midrib and terminate in the apices of the lobes. The flowering tops are densely pubescent and form a short drooping cluster. Each flower arises in the axils of a large bract. The gamosepalous calyx is covered with dense cotton-like hairs and has 5 triangular-ovate lobes, each ending in a short point that becomes spiny. The gamopetalous corolla, with 5 nearly equal lobes, is yellowish and with a delicate, brown to blackish-violet venation. The fruit, sometimes present at the base of the inflorescences, is a pyxis distinctly swollen at the base.

TESTS

**Foreign matter** (*2.8.2*): if required by the competent authority, maximum 5 per cent.

**Loss on drying** (*2.2.32*): if required by the competent authority, minimum 50 per cent, determined on 5.0 g of the finely cut drug by drying in an oven at 105 °C for 2 h.

***Hyoscyamus albus* L.** The presence of middle and upper leaves with a petiole and of fruits barely swollen at the base indicates adulteration by *Hyoscyamus albus* L.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of *Hyoscyamus niger* L. is prepared by maceration of the drug, using ethanol of a suitable concentration.

*Content*: 0.002 per cent *m/m* to 0.01 per cent *m/m* of total alkaloids, expressed as hyoscyamine ($C_{17}H_{23}NO_3$; $M_r$ 289.4).

CHARACTERS

*Appearance*: dark greenish-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Evaporate 10 mL of the mother tincture to be examined in a water-bath at 40 °C, under reduced pressure. Take up the residue with 1 mL of *ammonia R*, and shake with 2 quantities, each of 10 mL, of *ether R*. Combine the ether layers, dry over *anhydrous sodium sulfate R* and filter. Evaporate on a water-bath and dissolve the residue in 0.50 mL of *methanol R*.

*Reference solution (a).* Dissolve 50 mg of *hyoscyamine sulfate R* in 10 mL of *methanol R* (solution A). Dissolve 15 mg of *hyoscine hydrobromide R* in 10 mL of *methanol R* (solution B). Mix 4 mL of solution A and 2 mL of solution B and dilute to 10 mL with *methanol R*.

*Reference solution (b).* Dissolve 20 mg of *atropine sulfate R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 15 min.

*Detection A*: spray with *dilute potassium iodobismuthate solution R* until orange zones become visible. Examine in daylight.

*Results A*: see below the sequence of the zones present in the chromatograms obtained with the reference solutions and the test solution. Other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | | |
|---|---|---|
| Hyoscine: an orange zone | | An orange zone (hyoscine) |
| Hyoscyamine: an orange zone | Atropine: an orange zone | A orange zone (hyoscyamine/atropine) |
| | | Faint orange zones (line of application) |
| **Reference solution (a)** | **Reference solution (b)** | **Test solution** |

*Detection B*: subsequently spray with *sodium nitrite solution R* until the yellow background disappears. Examine in daylight after 15 min.

*Results B*: see test for atropine.

TESTS

**Relative density** (*2.2.5*): 0.930 to 0.960.

**Atropine**. Examine the chromatograms obtained in the test for identification.

*Results*: the zone due to hyoscyamine in the chromatogram obtained with the test solution changes from orange to reddish-brown but not to greyish-blue (atropine).

**Ethanol** (*2.9.10*): 40 per cent *V/V* to 50 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 1.2 per cent.

ASSAY

Evaporate 100.0 g of the mother tincture to be examined, at a low temperature under reduced pressure, until a residue of about 10 g is obtained. Quantitatively transfer the residue to a separating funnel using a few millilitres of *ethanol (70 per cent V/V) R*. Add 5 mL of *concentrated ammonia R* and 25 mL of *water R*. Extract with successive fractions of a mixture of 1 volume of *chloroform R* and 3 volumes of *peroxide-free ether R* until the alkaloids are completely extracted. Evaporate to dryness a few millilitres of the last organic fraction. Take up the residue in *0.25 M sulfuric acid* and verify the absence of alkaloids using *potassium tetraiodomercurate solution R*. Combine the organic layers and extract several times with *0.25 M sulfuric acid*. Separate the layers by centrifugation if necessary and transfer the acid layers to a second separating funnel. Make the acid layer alkaline with *ammonia R* and shake with at least 3 quantities, each of 30 mL, of *chloroform R*. Combine the chloroform layers, add 4 g of *anhydrous sodium sulfate R* and allow to stand for 30 min with occasional shaking. Decant the chloroform and wash the anhydrous sodium sulfate with 3 quantities, each of 10 mL, of *chloroform R*. Combine the chloroform fractions, evaporate to dryness on a water-bath and dry in an oven at 100-105 °C for 15 min. Dissolve the residue in a few millilitres of *chloroform R*, add 10.0 mL of *0.005 M sulfuric acid* and remove the chloroform by evaporation on a water-bath. Titrate the excess of acid with *0.01 M sodium hydroxide* using *methyl red mixed solution R* as indicator.

Calculate the percentage content *m/m* of total alkaloids, expressed as hyoscyamine, from the expression:

$$\frac{0.2894(10 - n)}{m}$$

$n$ = volume of *0.01 M sodium hydroxide* used, in millilitres;

$m$ = mass of the mother tincture used, in grams.

**01/2008:2028**
**corrected 9.0**

# HYPERICUM FOR HOMOEOPATHIC PREPARATIONS[(25)]

## Hypericum perforatum ad praeparationes homoeopathicas

DEFINITION

Whole, fresh plant of *Hypericum perforatum* L., at the beginning of the flowering period.

IDENTIFICATION

The perennial plant consists of a spindle-shaped root and a branched rhizome, giving rise to long, decumbent runners. The cylindrical, erect stem is woody at the base, 0.2 m to 1 m long, branched in the upper part, with 2 raised longitudinal lines.

The leaves are opposite, sessile, exstipulate, oblong-oval and 15 mm to 30 mm long. The leaf margins show black glandular dots, and many small translucent oil glands are present on the entire surface and are visible by transmitted light.

The flowers are regular and form corymbose clusters at the apex of the stem. They have 5 green, lanceolate sepals with acuminate apices, and black oil glands near the entire margins; 5 orange-yellow petals, much longer than the sepals, with black oil glands near the terminal margins only;

(25) FRENCH TITLE: Hypericum perforatum pour préparations homéopathiques

3 staminal blades, each divided into many orange-yellow stamens and 3 carpels surmounted by red styles. Each petal is asymmetrically linear-ovate in shape, with one of the margin entire and the other dentate.

TESTS

**Foreign matter** (*2.8.2*): maximum 4 per cent of fruits and maximum 1 per cent of other foreign matter.

**Loss on drying** (*2.2.32*): if performed to demonstrate the freshness of the drug, minimum 55 per cent, determined on 5.0 g of finely cut drug by drying in an oven at 105 °C.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of *Hypericum perforatum* L. is prepared by maceration using alcohol of a suitable concentration.

CHARACTERS

Dark red to brownish red liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. The mother tincture to be examined.

*Reference solution*. Dissolve 5 mg of *rutoside trihydrate R*, 1 mg of *hypericin R* and 5 mg of *hyperoside R* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethyl acetate R* (6:9:90 *V/V/V*).

*Application*: 10 µL of the test solution and 5 µL of the reference solution, as 10 mm bands.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 10 min.

*Detection*: spray with a 10 g/L solution of *diphenylboric acid aminoethyl ester R* in *methanol R* and then a 50 g/L solution of *macrogol 400 R* in *methanol R*. Examine the plates after 30 min in ultraviolet light at 365 nm.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. In the chromatogram obtained with the test solution, the zone due to rutoside may be weak or even absent. The chromatogram obtained with the test solution shows a group of zones that may be blue or yellow, with a $R_F$ similar to that of the zone due to hyperoside in the chromatogram obtained with the reference solution. Other weak zones may also be visible.

| **Top of the plate** | |
|---|---|
| | A yellow to blue zone |
| Hypericin: a red zone | 2 red zones |
| ——— | ——— |
| | Several zones |
| ——— | ——— |
| Hyperoside: a yellow to orange zone | Blue or yellow zones |
| Rutoside: a yellow to orange zone | A yellow to orange zone |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.900 to 0.920.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 75 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 1.3 per cent.

**01/2017:2513**
**corrected 10.0**

# IGNATIA FOR HOMOEOPATHIC PREPARATIONS(26)

## Strychnos ignatii ad praeparationes homoeopathicas

DEFINITION

Dried, ripe seed of *Strychnos ignatii* P.J.Bergius.

*Content*: minimum 1.80 per cent for the sum of the contents of brucine ($C_{23}H_{26}N_2O_4$; $M_r$ 394.5) and strychnine ($C_{21}H_{22}N_2O_2$; $M_r$ 334.4), of which minimum 65 per cent consists of strychnine (dried drug).

IDENTIFICATION

A. The seed is grey, brown and dull, up to 3 cm long and 10-25 mm thick. It is irregular, with 3-5 distinct sides: one of these is usually wider, convex and glabrous; the others are angular and flattened and show the remains of testa hairs forming lighter zones in the depressions. The stony granular texture resembles that of pebbles from a river bed; the hilum is found on the most rounded end and forms a small, light brown depression. The fracture shows a compact, semi-translucent, horny endosperm; the embryo is located in the centre and is about 10-15 mm long, with a foliaceous cotyledon.

B. *CAUTION: take all necessary handling precautions when reducing this toxic herbal drug to a powder.*

Wash the herbal drug rapidly in cold water, then expose to steam; once sufficiently softened, cut into thin slices and crush in a suitable apparatus. Allow to dry, finish reducing to a powder (710) (*2.9.12*) and pass through a covered sieve.

Microscopic examination (*2.8.23*). The powder is light brown. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2513.-1): oil droplets [D]; fragments of endosperm [B, C, F] consisting of thick-walled cells of various sizes, the smallest located at the periphery of the endosperm [Cb] and the largest towards the centre of the seed [F]; a few fragments of the outer layer of the endosperm (surface view [J], transverse section [Ca]), with polygonal cells sometimes associated with the inner layer of the testa, composed of cells with indistinct walls (surface view [E], transverse section [Cd]); sclerified covering trichomes [A, K], sheared off, not enlarged at the base [Aa] and with walls composed of small, oblique, sclerified strips, tightly fused longitudinally [Ab, Ka]; numerous fragments of strips [G, H] and rare rounded tips of covering trichomes [K].

(26) FRENCH TITLE: Ignatia amara pour préparations homéopathiques

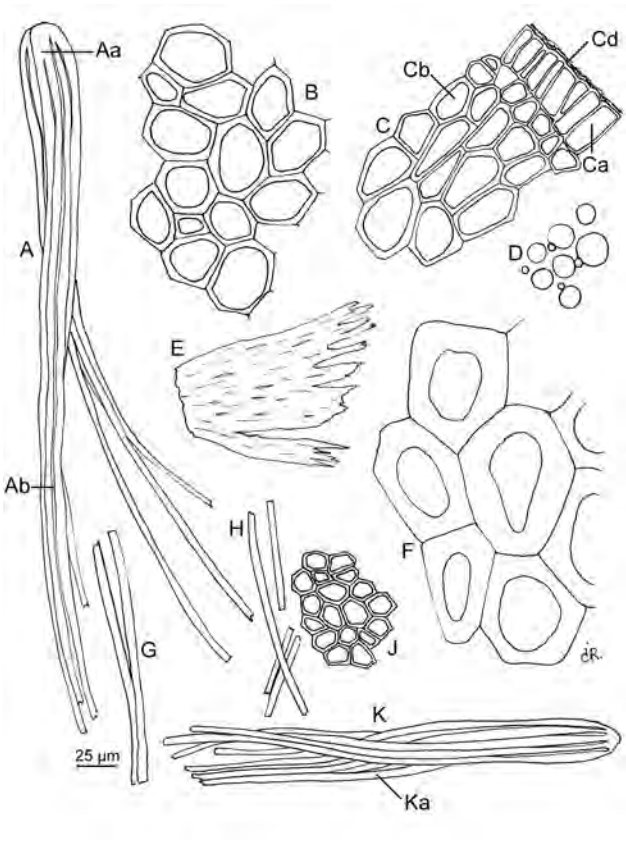


Figure 2513.-1. – *Illustration for identification test B of powdered herbal drug of Ignatia*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (710) (*2.9.12*) add 20 mL of *ethanol (70 per cent V/V) R*, allow to macerate for 15 min at room temperature, with stirring, and centrifuge. Use the supernatant.

*Reference solution.* Dissolve 10 mg of *brucine R* and 10 mg of *strychnine R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:5:95 *V/V/V*); use the lower layer.

*Application*: 10 µL [or 5 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air, then in an oven at 105-110 °C for 15 min; allow to cool.

*Detection*: spray with *iodoplatinate reagent R* and examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____<br>_____ | _____<br>_____ |
| Strychnine: a violet zone<br>Brucine: a blue zone | A violet zone (strychnine)<br>A blue zone (brucine) |
| **Reference solution** | **Test solution** |

TESTS

*CAUTION: when the powdered herbal drug (710) (2.9.12) is used, take the necessary precautions as indicated under Identification B.*

**Foreign matter** (*2.8.2*): maximum 1.0 per cent.

***Strychnos nux-vomica* L.** The presence of flattened discoid seeds and the presence in the powdered herbal drug, examined under a microscope, of testa cells transformed into hairs, with a sclerified base and a lignified tip, bent at a right angle and with 7-10 lignified ridges, and of numerous sclerified rods, indicate adulteration with *Strychnos nux-vomica* L.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.00 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.5 per cent.

**Aflatoxins** (*2.8.18*): maximum 2 µg/kg (aflatoxin $B_1$) and maximum 4 µg/kg (sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$).

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (710) (*2.9.12*) add 10.0 mL of *ethanol (60 per cent V/V) R*. Boil gently, with stirring, under a reflux condenser. After 30 min, cool and filter into a 20.0 mL volumetric flask. Wash the filter with *ethanol (60 per cent V/V) R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A.

*Reference solution.* Dissolve 10.0 mg of *brucine CRS* and 10.0 mg of *strychnine CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *triethylamine R*, *acetonitrile for chromatography R*, *methanol R2*, *tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R* (0.1:7.5:7.5:85 *V/V/V/V*);
- *mobile phase B*: *triethylamine R*, *tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R*, *acetonitrile for chromatography R*, *methanol R2* (0.1:15:42.5:42.5 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 25 | 100 → 70 | 0 → 30 |
| 25 - 30 | 70 → 65 | 30 → 35 |
| 30 - 31 | 65 → 0 | 35 → 100 |
| 31 - 32 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Elution order*: brucine, strychnine.

*System suitability*: reference solution:

- *resolution*: minimum 3.0 between the peaks due to brucine and strychnine.

Calculate the percentage contents of brucine and strychnine using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to brucine or strychnine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to brucine or strychnine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *brucine CRS* or *strychnine CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of brucine in *brucine CRS* or strychnine in *strychnine CRS*.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

The mother tincture is prepared from the dried, ripe seed of *Strychnos ignatii* P.J.Bergius.

*Content*: 0.18 per cent *m/m* to 0.36 per cent *m/m* for the sum of the contents of brucine ($C_{23}H_{26}N_2O_4$; $M_r$ 394.5) and strychnine ($C_{21}H_{22}N_2O_2$; $M_r$ 334.4), of which minimum 65 per cent is strychnine.

PRODUCTION

The mother tincture is prepared by the following methods prescribed in the general monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.8, using the powdered herbal drug (710) (*2.9.12*) and ethanol (70 per cent *V/V*);
- method 1.1.10, using the powdered herbal drug, ethanol (65 per cent *V/V*) and a maceration time of 3-5 weeks.

CHARACTERS

*Appearance*: brownish-yellow liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*) as described in identification test C for the herbal drug with the following modification.

*Test solution*. The mother tincture to be examined.

TESTS

**Relative density** (*2.2.5*): 0.890 to 0.904 when method 1.1.8 is used.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 1.2 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the assay of the herbal drug with the following modification.

*Test solution*. Dilute 2.000 g of the mother tincture to be examined to 20.0 mL with *ethanol (60 per cent V/V) R*.

Calculate the percentage contents of brucine and strychnine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to brucine or strychnine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to brucine or strychnine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *brucine CRS* or *strychnine CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of brucine in *brucine CRS* or strychnine in *strychnine CRS*.

**07/2014:2501**

# KALIUM BICHROMICUM FOR HOMOEOPATHIC PREPARATIONS(27)

## Kalii bichromas ad praeparationes homoeopathicas

$K_2Cr_2O_7$ $M_r$ 294.2
[7778-50-9]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of $K_2Cr_2O_7$.

CHARACTERS

*Appearance*: orange crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (b) of potassium (*2.3.1*).

B. Dissolve 10 mg in 5 mL of *water R*. Add 0.25 mL of *dilute sulfuric acid R*, 0.5 mL of *strong hydrogen peroxide solution R* and 1 mL of *ether R*. Shake. The upper layer is blue.

TESTS

**Solution S1**. Dissolve 5.0 g in *distilled water R* and dilute to 50.0 mL with the same solvent.

**Solution S2**. To 20.0 mL of solution S1 add 20 mL of *hydrochloric acid R* and 50 mL of *tributyl phosphate R*. Stir for 2 min. Remove the lower layer and shake it with 10 mL of *ether R*. Evaporate the lower layer to dryness under reduced pressure. Dissolve the residue in 10 mL of *distilled water R*. Add *dilute ammonia R1* until the solution is neutral to *blue litmus paper R* and dilute to 20.0 mL with *distilled water R*.

**Appearance of solution**. Solution S1 is clear (*2.2.1*).

**Calcium** (*2.4.3*): maximum 500 ppm.

Dilute 2.0 mL of solution S2 to 15 mL with *distilled water R*.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dissolve 1.0 g in 15 mL of *dilute nitric acid R*. Use 1 mL of *nitric acid R* instead of the prescribed *dilute nitric acid R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S2 to 15 mL with *distilled water R*.

ASSAY

Dissolve 0.100 g in 25 mL of *water R*. Add 2 g of *potassium iodide R* and 25 mL of *dilute sulfuric acid R*. Allow to stand in the dark for 10 min. Add 150 mL of *water R*. Titrate with *0.1 M sodium thiosulfate* until the colour changes from blue to green, adding 1 mL of *starch solution R* near the end of the titration.

(27) FRENCH TITLE: Kalium bichromicum pour préparations homéopathiques

1 mL of *0.1 M sodium thiosulfate* is equivalent to 4.903 mg of $K_2Cr_2O_7$.

**01/2017:2676**

# MAGNESIUM FLUORATUM FOR HOMOEOPATHIC PREPARATIONS(28)

## Magnesium fluoratum ad praeparationes homoeopathicas

$MgF_2$ $M_r$ 62.3
[7783-40-6]

DEFINITION

*Content*: 98.5 per cent to 100.5 per cent of $MgF_2$.

CHARACTERS

*Appearance*: white or almost white powder or crystals.

*Solubility*: practically insoluble in water, very slightly soluble in dilute nitric acid and in concentrated sulfuric acid, practically insoluble in anhydrous acetic acid.

IDENTIFICATION

A. Mix 0.2 g with 2 g of *potassium hydrogen sulfate R* in a platinum crucible and melt at 800 °C. Cool, carefully take up the melt in 20 mL of *water R* and boil briefly. Cool and filter. Dilute 0.5 mL of the filtrate to 2 mL with *water R*. The solution gives the reaction of magnesium (*2.3.1*).

B. Mix 0.2 g with 1 g of *anhydrous sodium carbonate R* in a platinum crucible and melt at 850 °C. Cool, take up the melt in 10 mL of *dilute acetic acid R* and boil briefly. Cool, filter and dilute the filtrate to 20 mL with *water R*. To 0.4 mL of this solution add dropwise a mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. The colour changes from red to yellow.

TESTS

**Solution S**. Boil 5.0 g with 100.0 mL of *distilled water R* under a reflux condenser for about 5 min. Allow to cool, then filter under vacuum.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Carbonates**. Suspend 0.5 g in 5 mL of *carbon dioxide-free water R* and add 5 mL of *dilute acetic acid R*. Quickly stopper the test tube with a stopper fitted with a glass tube bent twice through 90° and which dips at the other end in *barium hydroxide solution R*. Warm the mixture. The barium hydroxide solution remains clear.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Water-soluble matter**: maximum 0.5 per cent.

Boil 2.00 g with 100 mL of *water R* for 5 min. Filter the hot solution, then cool the filtrate and dilute to 100 mL with *water R*. Evaporate 50 mL of this solution to dryness in an evaporating dish and dry at 100-105 °C. The residue weighs a maximum of 5 mg.

ASSAY

Thoroughly mix 0.100 g with 2 g of *potassium hydrogen sulfate R* in a platinum crucible and melt at 800 °C. Allow to cool, carefully take up the melt in 10 mL of *dilute hydrochloric acid R* and heat to dissolve. Dilute the solution to 100.0 mL with *water R*. Transfer 50.0 mL of this solution to a 500 mL conical flask and dilute to 300 mL with *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 6.23 mg of $MgF_2$.

**01/2017:2505**
**corrected 10.0**

# MAGNESIUM PHOSPHORICUM FOR HOMOEOPATHIC PREPARATIONS(29)

## Magnesii hydrogenophosphas trihydricus ad praeparationes homoeopathicas

$MgHPO_4,3H_2O$ $M_r$ 174.3
[7782-75-4]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute acids.

IDENTIFICATION

A. Dissolve 0.1 g in a mixture of 2 mL of *dilute nitric acid R* and 8 mL of *water R*. The solution gives reaction (b) of phosphates (*2.3.1*).

B. Dissolve 0.1 g in a mixture of 2 mL of *dilute nitric acid R* and 8 mL of *water R*. Add 10 mL of *ammonium molybdate solution R* and filter. The filtrate gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 30 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with the same acid.

**Arsenic** (*2.4.2, Method B*): maximum 5 ppm, determined on 1.0 g.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Magnesium dihydrogen phosphate and magnesium phosphate**. Dissolve 2.00 g in 30.0 mL of *1 M hydrochloric acid*. Add 20 mL of *water R* and 0.05 mL of *methyl orange solution R*. Titrate the excess of hydrochloric acid with *1 M sodium hydroxide*. The volume of *1 M hydrochloric acid* used is between 11.0 mL and 12.5 mL.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*.

ASSAY

Dissolve 0.280 g in a mixture of 1 mL of *hydrochloric acid R1* and 5 mL of *water R*. Add 25.0 mL of *0.1 M sodium edetate* and dilute to 200 mL with *water R*. Neutralise with *concentrated ammonia R*, add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Titrate the excess of *0.1 M sodium edetate* with *0.1 M zinc sulfate* until the colour changes from pure blue to violet.

(28) FRENCH TITLE: Magnesia fluorata pour préparations homéopathiques
(29) FRENCH TITLE: Magnesia phosphorica pour préparations homéopathiques

1 mL of *0.1 M sodium edetate* is equivalent to 17.43 mg of $MgHPO_4,3H_2O$.

**01/2017:2514**
**corrected 10.0**

# NUX-VOMICA FOR HOMOEOPATHIC PREPARATIONS(30)

## Strychnos nux-vomica ad praeparationes homoeopathicas

DEFINITION

Dried, ripe seed of *Strychnos nux-vomica* L.

*Content*: minimum 1.50 per cent for the sum of the contents of brucine ($C_{23}H_{26}N_2O_4$; $M_r$ 394.5) and strychnine ($C_{21}H_{22}N_2O_2$; $M_r$ 334.4), of which 43 per cent to 67 per cent is strychnine (dried drug).

IDENTIFICATION

A. The seed is discoid, with a slightly raised margin, 20-25 mm in diameter and about 5 mm thick. It is not quite flat, with one surface slightly convex and the other slightly concave. Some seeds are irregularly curved. The colour of the seed ranges from light grey to greenish-grey and its satiny appearance is due to a silky down, consisting of dense hairs radiating from a central point on each of the faces. One of the surfaces has a raised point at the centre (the hilum), from which extends a radial ridge ending in a slight protuberance on the edge of the seed (the micropyle). A grey horny endosperm makes up most of the seed. The embryo, which is located in the central cavity, is small (about 6 mm long), with 2 cotyledons; the radicle is turned towards the micropyle.

B. *CAUTION: take all necessary handling precautions when reducing this toxic herbal drug to a powder.*

Wash the herbal drug rapidly in cold water, then expose to vapour from boiling water; once sufficiently softened, cut into thin slices and crush in a suitable apparatus. Allow to dry, finish reducing to a powder (710) (*2.9.12*) and pass through a covered sieve.

Microscopic examination (*2.8.23*). The powder is grey. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2514.-1): fragments of the outer testa (surface view [B], transverse section [C]), with cells with an enlarged, sclerified, strongly thickened and channelled base [Ba, Cb], transformed into curved or straight hairs, usually broken, with 7-10 lignified ridges [Bb, Ca] and a rounded lignified tip [D]; numerous rods or strips, highly variable in length and 5-15 µm wide, from the lignified ridges of the hairs [A]; numerous fragments of endosperm [F], consisting of very thick-walled polyhedral cells, some of which contain oil and aleurone grains; a few fragments of the outer layers of the endosperm (surface view [E], transverse section [C]); in surface view [E] the cells are polyhedral [Ea], sometimes associated with the brown pigmented layer of the testa formed of cells with indistinct walls [Eb]; in transverse section [C] the cells are elongated [Cc], sometimes associated with the pigmented layer [Cd] and the outer testa [Cb].

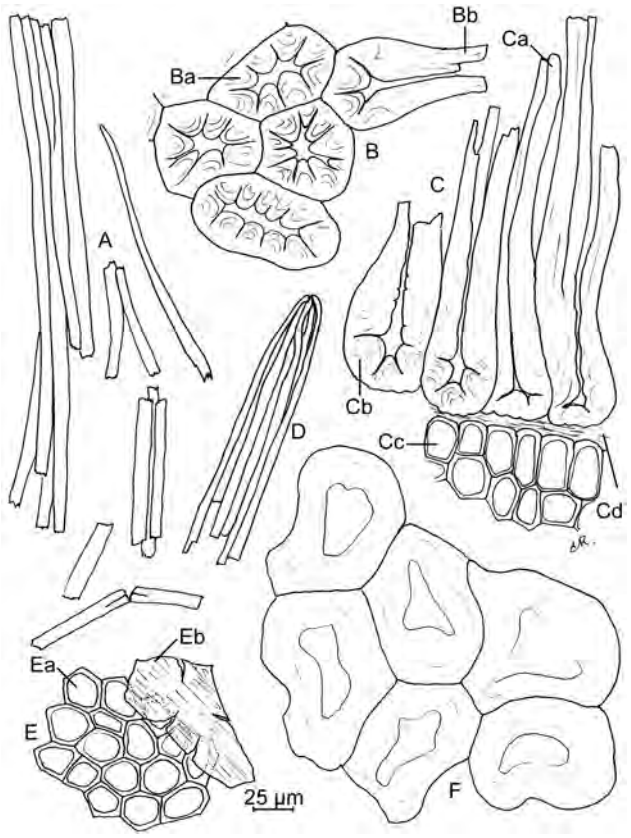


Figure 2514.-1. – *Illustration for identification test B of powdered herbal drug of Nux-vomica for homoeopathic preparations*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 2.0 g of the powdered herbal drug (710) (*2.9.12*) add 20 mL of *ethanol (70 per cent V/V) R*, allow to macerate for 15 min at room temperature, with stirring, and centrifuge. Use the supernatant.

*Reference solution.* Dissolve 10 mg of *brucine R* and 10 mg of *strychnine R* in 10 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:5:95 *V/V/V*); use the lower layer.

*Application*: 10 µL [or 5 µL] as bands.

*Development*: over a path of 15 cm [or 6 cm].

*Drying*: in air, then in an oven at 105-110 °C for 15 min; allow to cool.

*Detection*: spray with *iodoplatinate reagent R* and examine immediately in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| _____ | _____ |
| _____ | _____ |
| Strychnine: a violet zone | A violet zone (strychnine) |
| Brucine: a blue zone | A blue zone (brucine) |
| **Reference solution** | **Test solution** |

(30) FRENCH TITLE: Nux-vomica pour préparations homéopathiques

TESTS

When the powdered herbal drug (710) (*2.9.12*) is used, take the necessary precautions as indicated under Identification B.

**Foreign matter** (*2.8.2*): maximum 1.0 per cent.

***Strychnos ignatii* P.J. Bergius**. The presence of irregularly shaped seeds that are neither discoid nor flattened and the presence in the powdered herbal drug, examined under a microscope, of sclerified hairs, usually sheared off, not thickened at the base and with walls composed of small, oblique, sclerified strips, tightly fused longitudinally, indicate adulteration with *Strychnos ignatii* P.J. Bergius.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.00 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 3.0 per cent.

**Aflatoxins** (*2.8.18*): maximum 2 µg/kg (aflatoxin $B_1$) and maximum 4 µg/kg (sum of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$).

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* To 1.000 g of the powdered herbal drug (710) (*2.9.12*) add 10.0 mL of *ethanol (60 per cent V/V) R*. Boil gently, under a reflux condenser, with stirring. After 30 min, cool and filter into a 20.0 mL volumetric flask. Wash the filter with *ethanol (60 per cent V/V) R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A.

*Reference solution.* Dissolve 10.0 mg of *brucine CRS* and 10.0 mg of *strychnine CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *triethylamine R*, *acetonitrile for chromatography R*, *methanol R2*, *tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R* (0.1:7.5:7.5:85 *V/V/V/V*);
- *mobile phase B*: *triethylamine R*, *tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R*, *acetonitrile for chromatography R*, *methanol R2* (0.1:15:42.5:42.5 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 25 | 100 → 70 | 0 → 30 |
| 25 - 30 | 70 → 65 | 30 → 35 |
| 30 - 31 | 65 → 0 | 35 → 100 |
| 31 - 32 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Elution order*: brucine, strychnine.

*System suitability*: reference solution:

- *resolution*: minimum 3.0 between the peaks due to brucine and strychnine.

Calculate the percentage content of brucine and strychnine using the following expression:

$$\frac{A_1 \times m_2 \times p \times 2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to brucine or to strychnine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to brucine or to strychnine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the herbal drug to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *brucine CRS* or of *strychnine CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of brucine in *brucine CRS*, or strychnine in *strychnine CRS*.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

The mother tincture is prepared from the dried ripe seed of *Strychnos nux-vomica* L.

*Content*: 0.15 per cent *m/m* to 0.30 per cent *m/m* for the sum of the contents of brucine ($C_{23}H_{26}N_2O_4$; $M_r$ 394.5) and strychnine ($C_{21}H_{22}N_2O_2$; $M_r$ 334.4), of which 43 per cent to 67 per cent is strychnine.

PRODUCTION

The mother tincture is prepared by the following methods prescribed in the general monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.8, using the powdered herbal drug (710) (*2.9.12*) and ethanol (70 per cent *V/V*);
- method 1.1.10, using the powdered herbal drug, ethanol (65 per cent *V/V*) and a maceration time of 3-5 weeks.

CHARACTERS

*Appearance*: yellow or light brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*) as described in identification test C for the herbal drug with the following modification.

*Test solution.* The mother tincture to be examined.

TESTS

**Relative density** (*2.2.5*): 0.888 to 0.903 when method 1.1.8 is used.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 1.0 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the assay of the herbal drug with the following modifications.

*Test solution.* Dilute 2.000 g of the mother tincture to be examined to 20.0 mL with *ethanol (60 per cent V/V) R*.

Calculate the percentage content of brucine and strychnine using the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$ = area of the peak due to brucine or to strychnine in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to brucine or to strychnine in the chromatogram obtained with the reference solution;

$m_1$ = mass of the mother tincture to be examined used to prepare the test solution, in grams;

$m_2$ = mass of *brucine CRS* or of *strychnine CRS* used to prepare the reference solution, in grams;

$p$ = assigned percentage content of brucine in *brucine CRS*, or strychnine in *strychnine CRS*.

**01/2016:2683**

# PETROLEUM RECTIFICATUM FOR HOMOEOPATHIC PREPARATIONS(31)

## Petroleum ad praeparationes homoeopathicas

DEFINITION

Petroleum spirit distilling between 180 °C and 220 °C obtained by rectification of crude oil.

CHARACTERS

*Appearance*: clear, colourless, highly flammable liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Relative density (see Tests).

B. Refractive index (see Tests).

C. Distillation range (see Tests).

TESTS

**Solution S**. Dissolve 1 g in 99 g of *ethanol (90 per cent V/V) R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of the substance to be examined, add 5 mL of *carbon dioxide-free water R* and 0.25 mL of *phenol red solution R* and shake. The aqueous phase is yellow. Add 0.05 mL of *0.01 M sodium hydroxide* and shake. The aqueous phase is red.

**Relative density** (*2.2.5*): 0.752 to 0.762.

**Refractive index** (*2.2.6*): 1.422 to 1.426.

**Distillation range** (*2.2.11*): minimum 90 per cent *m/m* distils between 180 °C and 220 °C.

**Aromatic hydrocarbons**. The absorbance (*2.2.25*) of solution S between 250 nm and 400 nm is not greater than 0.100.

**Non-volatile matter**: maximum 0.04 per cent.

Evaporate 5.000 g to dryness on a water-bath and dry the residue in an oven at 100-105 °C for 2 h. The residue weighs a maximum of 2.0 mg.

STORAGE

In an airtight container.

LABELLING

The label states that the substance is obtained by rectification.

**07/2017:2844**

# SELENIUM FOR HOMOEOPATHIC PREPARATIONS(32)

## Selenium ad praeparationes homoeopathicas

Se $A_r$ 79.0
[7782-49-2]

DEFINITION

Amorphous selenium.

*Content*: 98.0 per cent to 101.0 per cent of Se (dried substance).

CHARACTERS

*Appearance*: black, reddish-brown or red powder, softens at 50 °C to 60 °C.

*Solubility*: practically insoluble in water, soluble in concentrated sulfuric acid giving a green solution.

IDENTIFICATION

A. Warm 5 mL of solution S1 (see Tests) on a water-bath and add 50 mg of *hydrazine sulfate R*. A red colour is produced.

B. To 5 mL of solution S1 add 0.5 mL of *dilute hydrochloric acid R* and 2 mL of a 100 g/L solution of *thiourea R*. A red precipitate is formed.

TESTS

**Solution S1**. Dissolve 20 mg in 1 mL of *nitric acid R*, warming on a water-bath. Continue warming until the evolution of brown fumes has ceased, then cool and add 10 mL of *water R*.

**Solution S2**. To the residue obtained in the test for sulfated ash add 2 mL of *nitric acid R* and 3 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue in a mixture of 1 mL of *dilute hydrochloric acid R* and 25 mL of hot *water R*. Cool, filter and dilute to 50.0 mL by washing the filter with *water R*.

**Appearance of solution**. Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Sulfur**: maximum 0.2 per cent.

To 0.100 g add 1 mL of *nitric acid R* dropwise, then add 2 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Add 2 mL of *hydrochloric acid R* and evaporate again to dryness on a water-bath. Dissolve the residue in 6 mL of a mixture of 1 mL of *dilute hydrochloric acid R* and 30 mL of *distilled water R*, then filter and dilute the filtrate to 20 mL by washing the filter with *distilled water R*. Dilute 5 mL of this solution to 15 mL with *distilled water R*. The solution complies with the limit test for sulfates (*2.4.13*) (0.6 per cent of sulfate corresponds to 0.2 per cent of sulfur).

**Water-soluble selenium compounds**: maximum 5 ppm.

*Test solution*. Shake 1.0 g with 20 mL of *water R* for 30 min and filter, washing the filter with 2 quantities, each of 5 mL, of *water R*. Adjust the filtrate to pH 2 with *dilute hydrochloric acid R*, and add 5.0 mL of a freshly prepared 1 g/L solution of *2,3-naphthalenediamine R* in *0.1 M hydrochloric acid*. Dilute to 50 mL with *water R* and allow to stand, protected from light, for 2 h. Shake the mixture with 10 mL of *toluene R* for

(31) FRENCH TITLE: Pétrole pour préparations homéopathiques
(32) FRENCH TITLE: Sélénium pour préparations homéopathiques.

3 min and allow the layers to separate for 3 min. Isolate the organic layer and dilute to 10.0 mL with *toluene R*.

*Reference solution*. Mix 5.0 mL of *selenium standard solution (1 ppm Se) R* and 25 mL of *water R*, and prepare in the same manner as for the filtrate in the test solution.

*Compensation liquid*. Treat 30 mL of *water R* in the same manner as for the filtrate in the test solution.

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at 380 nm by comparison with the compensation liquid.

The absorbance of the test solution is not greater than that of the reference solution.

**Iron** (*2.4.9*): maximum 100 ppm.

To 5 mL of solution S2 add 5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.000 g.

ASSAY

Dissolve 0.2000 g in 10 mL of *nitric acid R*. Warm until the evolution of brown fumes has ceased and the solution is only slightly yellow. Cool, add 2 g of *sulfamic acid R* and dilute to 100.0 mL with *water R*. To 10.0 mL of this solution add 50 mL of *water R* and 2.0 g of *potassium iodide R*. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium thiosulfate* is equivalent to 1.974 mg of Se.

**01/2016:2289**
**corrected 10.0**

# STAPHYSAGRIA FOR HOMOEOPATHIC PREPARATIONS(33)

## Delphinium staphisagria ad praeparationes homoeopathicas

DEFINITION

Dried, ripe seed of *Delphinium staphisagria* L.

*Content*: minimum 1.5 per cent of total alkaloids, expressed as delphinine ($C_{33}H_{45}NO_9$; $M_r$ 599.7) (dried drug).

IDENTIFICATION

A. The isolated seed has an irregular, flattened, tetrahedral shape, measuring 3-7 mm on each side; one of the sides is convex; the others are flat or concave. The surface is generally dark brown, rarely light brown, and shows a raised network defining alveoli; on the surface there are occasionally greyish spots. The seed coat is brittle and encloses an abundant endosperm, which is whitish to dark brown. The more or less wrinkled seeds are sometimes found as 5-6 seeds fitting together into an ovoid mass.

B. Microscopic examination (*2.8.23*). Reduce to a powder (710) (*2.9.12*). The powder consists of a mixture of dark brown and white particles with oily constituents. Examine under a microscope using *chloral hydrate solution R*. The powder shows the following diagnostic characters (Figure 2289.-1): fragments of the episperm consisting of a layer of large cells with regularly thickened walls and a wide lumen, elongated (surface view [A]), more or less isodiametric (transverse section [D]); the outside surface of the episperm cells generally exhibits numerous short, unequal, irregularly bent, cylindrical papillae, often with a globoid head, that look like cuticular papillae but are the conidiophores of a fungus present in almost every seed [Aa, Da]; underlying the episperm is an intermediate area, partly collapsed, composed of 5-6 layers of tangentially elongated, colourless, thin-walled cells [Cd, Db]; fragments of the inner testa, usually associated with the endosperm [B, C]; the inner testa comprises brown cells that, in transverse section, form a layer and are regular, 4-7 µm wide, with numerous U-shaped thickenings [Ca], whereas in surface view [Ba] they are elongated and about 70-100 µm long, with thin, finely striated walls; the endosperm consists of pentagonal or hexagonal cells, isodiametric or slightly elongated, with straight, thin walls [Bb, Cb], containing aleurone grains and oil droplets [Bc, Cc].

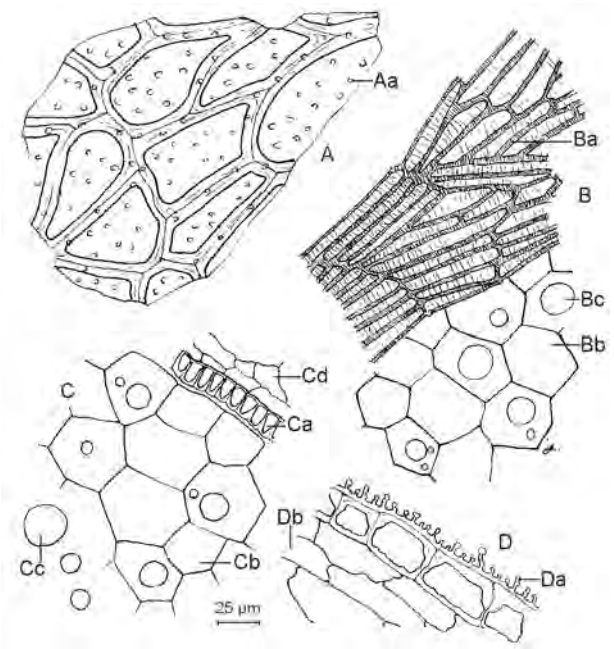


Figure 2289.-1. – *Illustration for identification test B of powdered herbal drug of Staphysagria for homoeopathic preparations*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g of the powdered herbal drug (710) (*2.9.12*) add 10 mL of *ethanol (90 per cent V/V) R* and boil under a reflux condenser for 15 min. Allow to cool and filter.

*Reference solution*. Dissolve 10 mg of *hyoscine hydrobromide R* and 10 mg of *papaverine hydrochloride R* in *ethanol (90 per cent V/V) R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *anhydrous ethanol R*, *acetone R*, *toluene R* (3:7:45:45 *V/V/V/V*).

*Application*: 30 µL [or 20 µL] as bands.

*Development*: over a path of 10 cm [or 6 cm].

*Drying*: in air.

*Detection*: treat with *dilute potassium iodobismuthate solution R*, then with *sodium nitrite solution R* until the yellow background disappears; examine in daylight.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

(33) FRENCH TITLE: Staphysagria pour préparations homéopathiques

| Top of the plate | |
|---|---|
| Papaverine: an orange zone<br>Hyoscine: an orange zone | 3-4 orange zones |
| **Reference solution** | **Test solution** |

TESTS

**Foreign matter** (*2.8.2*): maximum 1 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g of the powdered herbal drug (710) (*2.9.12*) by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 7.0 per cent.

ASSAY

To 2.000 g of the powdered herbal drug (710) (*2.9.12*) add 20 mL of *ethanol (90 per cent V/V) R*, and boil under a reflux condenser for 15 min. Allow to cool and filter through a plug of absorbent cotton, rinsing the filter with *ethanol (90 per cent V/V) R*. Add 2 mL of *dilute hydrochloric acid R* and evaporate to about 5 mL under reduced pressure. Add 20 mL of *water R*. Shake with 4 quantities, each of 25 mL, of *light petroleum R*. Discard the organic layers. Add to the aqueous layer 3 mL of *dilute ammonia R1* and shake with 4 quantities, each of 25 mL, of *1,1-dimethylethyl methyl ether R*. Dry the combined organic layers over *anhydrous sodium sulfate R*, filter and evaporate to dryness under reduced pressure at about 40 °C. Dissolve the residue in 10 mL of *anhydrous acetic acid R*. Titrate with *0.02 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.02 M perchloric acid* is equivalent to 11.99 mg of delphinine ($C_{33}H_{45}NO_9$).

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

DEFINITION

*Content*: 0.10 per cent *m/m* to 0.40 per cent *m/m* of total alkaloids, expressed as delphinine ($C_{33}H_{45}NO_9$; $M_r$ 599.7).

PRODUCTION

The mother tincture is prepared from the dried seed of *Delphinium staphisagria* L. according to the following methods prescribed in the monograph *Methods of preparation of homoeopathic stocks and potentisation (2371)*:

- method 1.1.8 using the powdered herbal drug (710) (*2.9.12*) and *ethanol (90 per cent V/V) R*; use *ethanol (70 per cent V/V) R* to prepare the 4th decimal dilution, and use *ethanol (50 per cent V/V) R* for subsequent dilutions;
- method 1.1.10 using the fragmented herbal drug and *ethanol (65 per cent V/V) R*.

CHARACTERS

*Appearance*: pale yellow liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*) as described in identification test C for the herbal drug with the following modification.

*Test solution.* The mother tincture to be examined.

*Results*: see below the sequence of zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, other faint zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| Papaverine: an orange zone<br>Hyoscine: an orange zone | 2-4 orange zones |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.830 to 0.840 when method 1.1.8 is used.

**Ethanol** (*2.9.10*): 60 per cent *V/V* to 70 per cent *V/V* when method 1.1.10 is used.

**Dry residue** (*2.8.16*): minimum 0.7 per cent.

ASSAY

Potentiometric titration (*2.2.20*) as described in the assay of the herbal drug with the following modifications.

To 20.00 g of the mother tincture to be examined add 2 mL of *dilute hydrochloric acid R* and evaporate to about 5 mL under reduced pressure. Continue as described in the assay of the herbal drug, beginning at the words "Add 20 mL of *water R*".

**04/2011:2515**
**corrected 9.0**

# SULFUR FOR HOMOEOPATHIC PREPARATIONS(34)

## Sulfur ad praeparationes homoeopathicas

S $A_r$ 32.07
[7704-34-9]

DEFINITION

Obtained by sublimation.

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: yellow powder.

*Solubility*: practically insoluble in water, soluble in carbon disulfide, slightly soluble in vegetable oils.

mp: about 120 °C.

IDENTIFICATION

A. Heated in the presence of air, it burns with a blue flame, emitting sulfur dioxide, which changes the colour of moistened *blue litmus paper R* to red.

B. Heat 0.1 g with 0.5 mL of *bromine water R* until decolourised. Add 5 mL of *water R* and filter. The solution gives reaction (a) of sulfates (*2.3.1*).

(34) FRENCH TITLE: Sulfur pour préparations homéopathiques

TESTS

**Solution S**. To 5.0 g add 50 mL of *carbon dioxide-free water R* prepared from *distilled water R*. Allow to stand for 30 min with frequent shaking and filter.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Odour** (*2.3.4*). It has no perceptible odour of hydrogen sulfide.

**Acidity or alkalinity**. To 5 mL of solution S add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.15 mL of *methyl red solution R*. The solution is orange-red.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Sulfides**. To 10 mL of solution S add 2 mL of *buffer solution pH 3.5 R* and 1 mL of a freshly prepared 1.6 g/L solution of *lead nitrate R* in *carbon dioxide-free water R*. Shake. After 1 min any colour in the solution is not more intense than that in a reference solution prepared at the same time using 1 mL of *lead standard solution (10 ppm Pb) R*, 9 mL of *carbon dioxide-free water R*, 2 mL of *buffer solution pH 3.5 R* and 1.2 mL of *thioacetamide reagent R*.

**Arsenic** (*2.4.2, Method B*): maximum 8 ppm.

Shake 2.5 g with 50 mL of *dilute ammonia R1* for 1 h and filter. Evaporate 25 mL of the filtrate to dryness. Add 2 mL of *water R* and 3 mL of *nitric acid R* to the residue and evaporate to dryness. The residue complies with the test.

Prepare the standard using 1 mL of *arsenic standard solution (10 ppm As) R*.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Carry out the oxygen-flask method (*2.5.10*), using 60.0 mg in a 1000 mL combustion flask with a polytetrafluoroethylene joint. Absorb the combustion products in a mixture of 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of *water R*. Heat to boiling, boil gently for 2 min and cool. Using 0.2 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide* until the colour changes from colourless to red. Carry out a blank titration under the same conditions.

1 mL of *0.1 M sodium hydroxide* is equivalent to 1.603 mg of S.

STORAGE

Protected from light.

**07/2014:2030**

# URTICA DIOICA FOR HOMOEOPATHIC PREPARATIONS(35)

## Urtica dioica ad praeparationes homoeopathicas

DEFINITION

Whole, fresh, flowering plant of *Urtica dioica* L.

CHARACTERS

The plant causes an itching, burning sensation on the skin.

(35) FRENCH TITLE: Urtica dioica pour préparations homéopathiques

IDENTIFICATION

A. The perennial plant has a taproot that sends out creeping subterranean rhizomes, more or less 4-angled in transverse section, from which extend adventious secondary roots and very numerous brownish hairy rootlets. The stipes are erect, generally unbranched, 3-5 mm in diameter and 0.3-1.5 m high, rarely up to 2.5 m high, 4-angled, greyish-green and covered in short hairs and stinging hairs.

   The decussate leaves are 30-150 mm long and 20-80 mm wide. The petiole is hispid and usually slightly less than one-third the length of the lamina. The leaf blade is ovate, acuminate, cordate or rounded at the base, and coarsely dentate; the apical tooth is distinctly larger than the lateral teeth. The upper side of the leaves is dark green and usually matt, both sides bear short serried hairs intermingled with long stinging hairs. The 2 stipules are linear-subulate and free. The inflorescences growing from the leaf axils are complex, the flowers unisexual, and, particularly in male plants, generally distinctly longer than the petiole. After shedding their pollen, male inflorescences are erect at an oblique angle or horizontal; female inflorescences are pendent when the fruit is ripe. All flowers have long stalks. The perianth of the male flowers is divided half-way down into equal green lobes, widest at their base, with short bristles and stinging hairs at the margins. The stamens are equal and opposite to the perianth segments, each with a long, whitish filament that curves inwards before pollen is shed and spreads out afterwards. The ovary is rudimentary, button or cup-shaped. The perianth of the female flowers is downy or bristly on the outside and consists of outer, and 2 inner segments; the inner segments are about twice the length of the outer ones. The hypogynous, ovate, unilocular ovary bears a large capitate stigma with a brush-like shock of hair. As the one-seeded fruit grows ripe, the 2 inner segments of the perianth fold around it like wings.

B. It complies with the test for *Urtica urens* (see Tests).

TESTS

***Urtica urens***. The margin of the lamina is not serrate with teeth twice as long as wide. The clusters of flowers in the axils are longer than the petiole of the leaf. Unisexual, apetalous flowers are not together on the same plant and in the same cluster.

**Foreign matter** (*2.8.2*): maximum 5 per cent.

**Loss on drying** (*2.2.32*): minimum 65.0 per cent, determined on 5.0 g of finely cut herbal drug by drying in an oven at 105 °C for 2 h, if performed to demonstrate the freshness of the herbal drug.

## Mother tincture

The mother tincture complies with the requirements of the general monograph *Mother tinctures for homoeopathic preparations (2029)*.

PRODUCTION

The mother tincture of Urtica dioica is prepared by maceration using ethanol of a suitable concentration.

CHARACTERS

*Appearance*: greenish-brown or orange-brown liquid.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. The mother tincture to be examined.

*Reference solution*. Dissolve 10 mg of *phenylalanine R* and 10 mg of *serine R* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel plate R*.

Homoeopathic preparations

*Mobile phase*: *glacial acetic acid R*, *water R*, *acetone R*, *butanol R* (10:20:35:35 *V/V/V/V*).

*Application*: 20 µL, as bands.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 1 g/L solution of *ninhydrin R* in *ethanol (96 per cent) R*, heat at 105-110 °C for 5-10 min and examine in daylight within 10 min.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Phenylalanine: a violet to reddish-brown zone | 4 red to violet zones |
| ——— | ——— |
| Serine: a reddish-violet zone | A violet zone |
| | A violet zone |
| **Reference solution** | **Test solution** |

TESTS

**Relative density** (*2.2.5*): 0.930 to 0.950.

**Ethanol** (*2.9.10*): 40 per cent *V/V* to 56 per cent *V/V*.

**Methanol** (*2.9.11*): maximum 0.10 per cent *V/V*.

**Dry residue** (*2.8.16*): minimum 1.1 per cent.

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Corrections are to be taken into account as soon as possible and not later than the end of the month following the month of publication (see the publication schedule opposite).

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See the information given in general chapter *5.8. Pharmacopoeial harmonisation*.

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# COMBISTATS

www.edqm.eu/combistats

CombiStats is a computer program for the statistical analysis of data from biological assays in agreement with general chapter 5.3. *Statistical analysis of results of biological assays and tests* of the 10th Edition of the European Pharmacopoeia.

# KEY TO MONOGRAPHS



*Version date of the text*

**01/2012:0884**
**corrected 10.0**

*Text reference number*

*Modification to be taken into account as soon as possible and not later than the end of the month following the month of publication of Ph. Eur. 10.0 (see section IV. Contents of the 10th Edition)*

*Link to further information on the text (e.g. Knowledge database) for smartphones/tablets with camera and barcode reader app*

## CARBIMAZOLE(1)

Carbimazolum

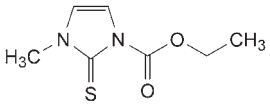

$C_7H_{10}N_2O_2S$

[22232-54-8] $M_r$ 186.2

*CAS number*

DEFINITION

Ethyl 3-methyl-2-thioxo-2,3-dihydro-1*H*-imidazole-1-carboxylate.

*Chemical name in accordance with IUPAC nomenclature rules*

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

*For the meaning of black and white diamonds see chapter 5.8. Pharmacopoeial harmonisation*

♦CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in acetone and in ethanol (96 per cent). ♦

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

*Application of the first and second identification is defined in the General Notices (chapter 1)*

◊A. Melting point (*2.2.14*): 122 °C to 125 °C.◊

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *carbimazole CRS*.

*Reference standard available from the EDQM (see http://crs.edqm.eu)*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reagent described in chapter 4*

*Reference solution*. Dissolve 10 mg of *carbimazole CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel GF254 plate R*.

*Further information on certain reagents available in the Knowledge database (http://go.edqm.eu/knowledge)*

*Mobile phase*: *acetone R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air for 30 min.

*Vertical line in the margin indicating where the text has been modified*

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Horizontal line in the margin indicating where part of the text has been deleted*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Reference to a general chapter*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in 10.0 mL of a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Use this solution within 5 min of preparation.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter 5.8. *Pharmacopoeial harmonisation*.

SPECIMEN

*IMPORTANT NOTICE*

# GENERAL MONOGRAPHS

The European Pharmacopoeia contains a number of general monographs covering classes of products. These general monographs give requirements that are applicable to all products in the given class or, in some cases, to any product in the given class for which there is a specific monograph in the Pharmacopoeia (see *1. General Notices*, General monographs). Where no restriction on scope of a general monograph is given in a preamble, it is applicable to all products in the class defined, irrespective of whether there is an individual monograph for the product in the Pharmacopoeia.

Whenever a monograph is used, it is essential to ascertain whether there is a general monograph applicable to the product in question. The general monographs listed below are published in the General Monographs section (unless otherwise stated). This list is updated where necessary and republished in each supplement.

Allergen products (1063)

Chemical precursors for radiopharmaceutical preparations (2902)

Dosage Forms monographs
*(published in the Dosage Forms section or the Homoeopathic Preparations section, as appropriate)*

Essential oils (2098)

Herbal drug extracts (0765)

Herbal drug preparations (1434)

Herbal drugs (1433)

Herbal drugs for homoeopathic preparations (2045)
*(published in the Homoeopathic Preparations section)*

Herbal teas (1435)

Herbal teas, instant (2620)

Homoeopathic preparations (1038)
*(published in the Homoeopathic Preparations section)*

Immunosera for human use, animal (0084)

Immunosera for veterinary use (0030)

Live biotherapeutic products for human use (3053)

Methods of preparation of homoeopathic stocks and potentisation (2371)
*(published in the Homoeopathic Preparations section)*

Monoclonal antibodies for human use (2031)

Mother tinctures for homoeopathic preparations (2029)
*(published in the Homoeopathic Preparations section)*

Pharmaceutical preparations (2619)

Products of fermentation (1468)

Products with risk of transmitting agents of animal spongiform encephalopathies (1483)

Radiopharmaceutical preparations (0125)

Recombinant DNA technology, products of (0784)

Substances for pharmaceutical use (2034)

Vaccines for human use (0153)

Vaccines for veterinary use (0062)

Vegetable fatty oils (1579)

# EUROPEAN PHARMACOPOEIA

## TENTH EDITION

## Volume II

*Published in accordance with the*
*Convention on the Elaboration of a European Pharmacopoeia*
*(European Treaty Series No. 50)*

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Strasbourg

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**01/2017:2589**

# ABACAVIR SULFATE

## Abacaviri sulfas

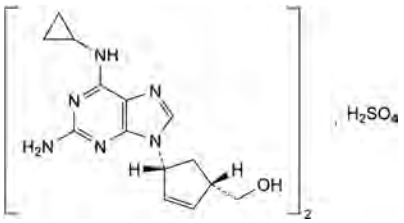

$C_{28}H_{38}N_{12}O_6S$ $M_r$ 671
[188062-50-2]

DEFINITION

Bis[[(1*S*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-enyl]methanol] sulfate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *abacavir sulfate CRS*.

B. Enantiomeric purity (see Tests).

C. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solution A*. Mix 0.5 mL of *trifluoroacetic acid R* and 100 mL of *methanol R*.

*Solution B*. Mix 30 volumes of *methanol R*, 30 volumes of *2-propanol R* and 40 volumes of *heptane R*.

*Test solution*. Dissolve 40 mg of the substance to be examined in 30 mL of solution A. Sonicate until dissolution is complete. Add 30 mL of *2-propanol R* and dilute to 100.0 mL with *heptane R*.

*Reference solution (a)*. Dissolve 2 mg of *abacavir for system suitability CRS* (containing impurities A and D) in 1.5 mL of solution A. Sonicate until dissolution is complete. Add 1.5 mL of *2-propanol R* and dilute to 5.0 mL with *heptane R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution B. Dilute 1.0 mL of this solution to 10.0 mL with solution B.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R* (10 µm);
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *diethylamine R*, *2-propanol R*, *heptane R* (0.1:15:85 *V/V/V*);
– *mobile phase B*: *heptane R*, *2-propanol R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 27 | 100 → 0 | 0 → 100 |
| 27 - 37 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 286 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *abacavir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and D.

*Relative retention* with reference to abacavir (retention time = about 17 min): impurity D = about 0.8; impurity A = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurities D and A; minimum 1.5 between the peaks due to impurity A and abacavir.

*Limit*:

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and transfer them to low-adsorption, inert glass vials.*

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent. Sonicate until dissolution is complete.

*Reference solution (a)*. Dissolve 2.5 mg of *abacavir for peak identification CRS* (containing impurities B and D) in 10.0 mL of *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dilute 0.5 mL of *trifluoroacetic acid R* in 1000 mL of *water R*;
– *mobile phase B*: *water R*, *methanol R* (15:85 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 25 | 95 → 70 | 5 → 30 |
| 25 - 40 | 70 → 10 | 30 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *abacavir for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and D.

*Relative retention* with reference to abacavir (retention time = about 22 min): impurity D = about 1.04; impurity B = about 1.3.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to abacavir.

*Limits*:

– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 60.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.54 mg of $C_{28}H_{38}N_{12}O_6S$.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F.*

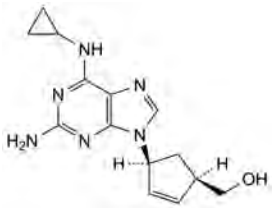

A. [(1*R*,4*S*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-enyl]methanol,

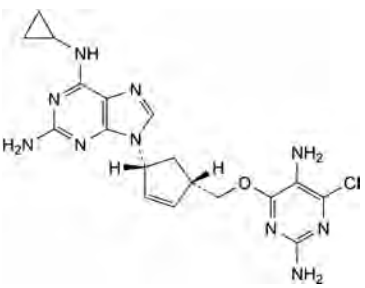

B. 6-(cyclopropylamino)-9-[(1*R*,4*S*)-4-[[(2,5-diamino-6-chloropyrimidin-4-yl)oxy]methyl]cyclopent-2-enyl]-9*H*-purin-2-amine,

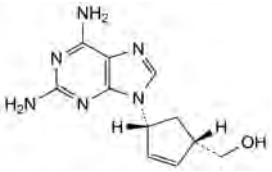

C. [(1*S*,4*R*)-4-(2,6-diamino-9*H*-purin-9-yl)cyclopent-2-enyl]methanol,

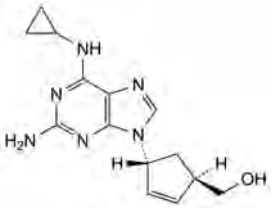

D. [(1*R*,4*R*)-4-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopent-2-enyl]methanol,

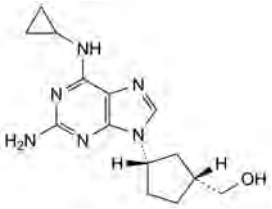

E. [(1*R*,3*S*)-3-[2-amino-6-(cyclopropylamino)-9*H*-purin-9-yl]cyclopentyl]methanol,

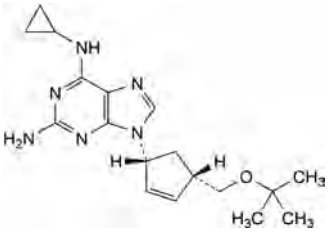

F. 6-(cyclopropylamino)-9-[(1*R*,4*S*)-4-[[(1,1-dimethylethyl)oxy]methyl]cyclopent-2-enyl]-9*H*-purine-2-amine.

**07/2019:0308**

# ACACIA, DRIED DISPERSION

## Acaciae gummi dispersione desiccatum

DEFINITION

Powder obtained from a dispersion of *Acacia (0307)* after a drying process.

CHARACTERS

It dissolves completely, after about 20 min, in twice its mass of water. The liquid obtained is colourless or yellowish, dense, viscous, adhesive, translucent and weakly acid to blue litmus paper. It is practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Examine under a microscope using *ethanol (96 per cent) R* as the mounting medium. The preparation to be examined consists of predominantly spheroidal or irregular and angular particles varying in size (4-500 µm), with 1 or more rounded cavities containing 1 or several air bubbles; a few flat fragments are also present. Only traces of starch granules are visible and no plant tissue is observed.

B. Examine the chromatograms obtained in the test for glucose and fructose.

*Results*: see below the sequence of zones present in the chromatograms obtained with reference solution (a) and the test solution.

| Top of the plate | |
|---|---|
| | 3 blue zones, very faint |
| Rhamnose: a greenish-brown zone | A greenish-brown zone, very faint to equivalent (rhamnose) |
| Xylose: a brownish-grey zone | |
| ——— | ——— |
| Arabinose: a brownish-grey zone | A brownish-grey zone, intense (arabinose) |
| Glucose: a greyish-blue zone | |
| Galactose: a greyish-blue zone | A greyish-blue zone, intense (galactose) |
| ——— | ——— |
| | 1 or 2 brownish-grey zones, very faint to equivalent |
| | 1 or 2 blue zones, faint to equivalent |
| **Reference solution (a)** | **Test solution** |

C. Dissolve 1 g of the preparation to be examined in 2 mL of *water R* by stirring frequently for 20 min. Add 2 mL of *ethanol (96 per cent) R*. After shaking, a white gelatinous mucilage is formed that becomes fluid upon addition of 10 mL of *water R*.

TESTS

**Solution S**. Dissolve 3.0 g of the preparation to be examined in 25 mL of *water R* by stirring for 10 min. Allow to stand for 20 min and dilute to 30 mL with *water R*.

**Glucose and fructose**. High-performance thin-layer chromatography (*2.8.25*).

*Test solution*. To 0.1 g in a thick-walled centrifuge tube add 2 mL of a 100 g/L solution of *trifluoroacetic acid R* and shake vigorously. Stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge, transfer 1 mL of the clear supernatant into a 10 mL flask and add 5 mL of *methanol R*.

*Reference solution (a)*. Dissolve 5 mg of *arabinose R*, 5 mg of *galactose R*, 5 mg of *glucose R*, 5 mg of *rhamnose R* and 5 mg of *xylose R* in 1 mL of *water R* and dilute to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 5 mg of *galactose R* and 5 mg of *glucose R* in 1 mL of *water R* and dilute to 10 mL with *methanol R*.

*Intensity marker*: galactose.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 4 µL of the test solution and reference solutions (a) and (b), and 2 µL of reference solution (c), as bands of 8 mm.

*Development A*: 70 mm from the lower edge of the plate, in an unsaturated tank.

*Drying A*: in air.

*Development B*: 70 mm from the lower edge of the plate, in an unsaturated tank, using freshly prepared mobile phase.

*Drying B*: in air.

*Detection*: treat with a solution prepared as follows: dissolve 4 g of *diphenylamine R* and 4 mL of *aniline R* in 160 mL of *acetone R* and add *phosphoric acid R* until the precipitate formed dissolves again (about 30 mL). Heat at 120 °C for 5-10 min and examine in daylight.

*System suitability*: reference solution (c):

– the chromatogram shows in the middle third 2 distinct zones, which may be touching; the lower zone (galactose) and the upper zone (glucose) are greyish-blue.

*Results*: the chromatogram obtained with the test solution shows no greyish-blue zone and no reddish zone between the zones due to galactose and arabinose in the chromatogram obtained with reference solution (a).

**Starch, dextrin and agar**. To 10 mL of solution S, previously boiled and cooled, add 0.1 mL of *0.05 M iodine*. No blue or reddish-brown colour develops.

**Sterculia gum**

A. Place 0.2 g in a 10 mL ground-glass-stoppered cylinder graduated in 0.1 mL. Add 10 mL of *ethanol (60 per cent V/V) R* and shake. Any gel formed occupies not more than 1.5 mL.

B. To 1.0 g add 100 mL of *water R* and shake. Add 0.1 mL of *methyl red solution R*. Not more than 5.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Tannins**. To 10 mL of solution S add 0.1 mL of *ferric chloride solution R1*. A gelatinous precipitate is formed, but neither the precipitate nor the liquid is dark blue.

**Tragacanth**. Examine the chromatograms obtained in the test for glucose and fructose.

*Results*: the chromatogram obtained with the test solution shows no faint to intense brownish-grey zone corresponding to the zone due to xylose in the chromatogram obtained with reference solution (a).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 4.0 per cent.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are*

*recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for acacia dried dispersion used as a viscosity-increasing agent and/or suspending agent in aqueous preparations.*

**Apparent viscosity**. Determine the dynamic viscosity using a capillary viscometer (*2.2.9*) or a rotating viscometer (*2.2.10*) on a 100 g/L solution of acacia, dried dispersion (dried substance).

**01/2017:1585**

# ACAMPROSATE CALCIUM

## Acamprosatum calcicum

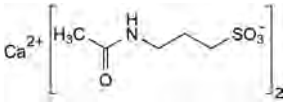

$C_{10}H_{20}CaN_2O_8S_2$ $M_r$ 400.5
[77337-73-6]

DEFINITION

Calcium bis[3-(acetylamino)propane-1-sulfonate].

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of acamprosate calcium*.

B. It gives reaction (a) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 7.0 for solution S.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.40 g of the substance to be examined in *distilled water R* and dilute to 20.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *borate buffer solution pH 10.4 R*. Place 3.0 mL of the solution obtained in a 25 mL ground-glass-stoppered tube. Add 0.15 mL of a freshly prepared 5 g/L solution of *fluorescamine R* in *acetonitrile R*. Shake immediately and vigorously for 30 s. Place in a water-bath at 50 °C for 30 min. Cool under a stream of cold water. Centrifuge and filter the supernatant through a suitable membrane filter (nominal pore size 0.45 µm), 25 mm in diameter.

*Reference solution*. Dissolve 50 mg of *acamprosate impurity A CRS* in *distilled water R* and dilute to 200.0 mL with the same solvent. Dilute 0.4 mL of the solution to 100.0 mL with *borate buffer solution pH 10.4 R*. Treat 3.0 mL of this solution in the same way as the test solution

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 170 $m^2$/g and a pore size of 12 nm.

*Mobile phase*: *acetonitrile R*, *methanol R*, *0.1 M phosphate buffer solution pH 6.5 R* (10:10:80 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 261 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of impurity A

*Retention times*: fluorescamine = about 4 min; impurity A = about 8 min; acamprosate is not detected by this system.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.4 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

To 4 g of *cation-exchange resin R* (75-150 µm) add 20 mL of *distilled water R* and stir magnetically for 10 min. Introduce this suspension into a glass column, 45 cm long and 2.2 cm in internal diameter, equipped with a polytetrafluoroethylene flow cap covered by a glass-wool plug. Allow a few millilitres of this solution to flow, then place a plug of glass wool over the resin. Pass 50 mL of *1 M hydrochloric acid* through the column. The pH of the eluate is close to 1. Wash with 3 quantities, each of 200 mL, of *distilled water R* to obtain an eluate at pH 6. Dissolve 0.100 g of the substance to be examined in 15 mL of *distilled water R*. Pass through the column and wash with 3 quantities, each of 25 mL, of *distilled water R*, collecting the eluate. Allow to elute until an eluate at pH 6 is obtained. Titrate the solution obtained with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* corresponds to 20.02 mg of $C_{10}H_{20}CaN_2O_8S_2$.

IMPURITIES

A. 3-aminopropane-1-sulfonic acid (homotaurine).

**01/2017:2089**

# ACARBOSE

## Acarbosum

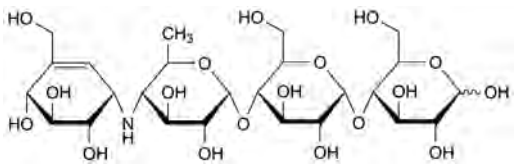

$C_{25}H_{43}NO_{18}$ $M_r$ 646
[56180-94-0]

DEFINITION

*O*-4,6-Dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose, which is produced by certain strains of *Actinoplanes utahensis*.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, hygroscopic, amorphous powder.

*Solubility*: very soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *acarbose for identification CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 7.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 168 to + 183 (anhydrous substance).

Dilute 2.0 mL of solution S to 10.0 mL with *water R*.

**Absorbance** (*2.2.25*): maximum 0.15 at 425 nm for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve the contents of a vial of *acarbose CRS* in 5.0 mL of *water R*.

*Reference solution (b)*. Dissolve the contents of a vial of *acarbose for peak identification CRS* (acarbose containing impurities A, B, C, D, E, F and G) in 1 mL of *water R*.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 750 volumes of *acetonitrile R1* and 250 volumes of a solution containing 0.60 g/L of *potassium dihydrogen phosphate R* and 0.35 g/L of *disodium hydrogen phosphate dihydrate R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of acarbose.

*Identification of impurities*: use the chromatogram supplied with *acarbose for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to acarbose (retention time = about 16 min): impurity D = about 0.5; impurity B = about 0.8; impurity A = about 0.9; impurity C = about 1.2; impurity E = about 1.7; impurity F = about 1.9; impurity G = about 2.2.

*System suitability*: reference solution (b):

- the chromatogram obtained is similar to the chromatogram supplied with *acarbose for peak identification CRS*;
- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to acarbose.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.63; impurity D = 0.75; impurity E = 1.25; impurity F = 1.25; impurity G = 1.25;
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent);
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *impurity A*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent);
- *impurity B*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurities F, G*: for each impurity, not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity E*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{25}H_{43}NO_{18}$ taking into account the assigned content of *acarbose CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H.*

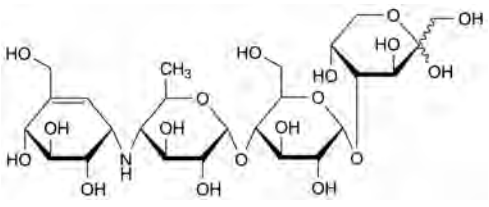

A. *O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-*arabino*-hex-2-ulopyranose,

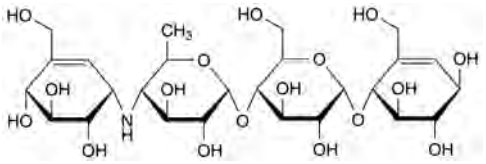

B. (1*R*,4*R*,5*S*,6*R*)-4,5,6-trihydroxy-2-(hydroxymethyl)cyclohex-2-enyl 4-*O*-[4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl]-α-D-glucopyranoside,

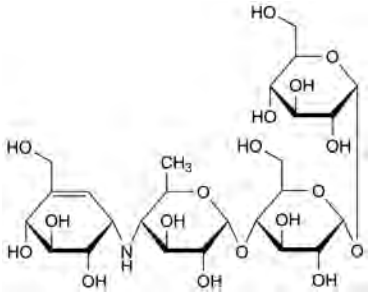

C. α-D-glucopyranosyl 4-*O*-[4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl]-α-D-glucopyranoside,

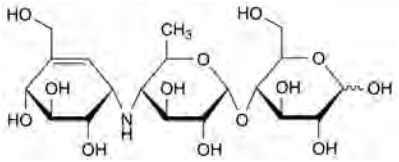

D. 4-*O*-[4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl]-D-glucopyranose,

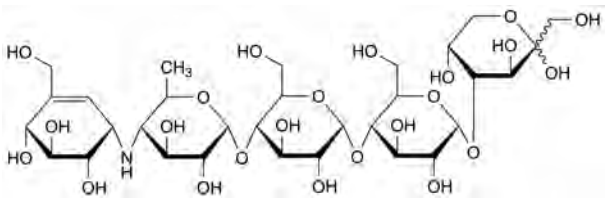

E. *O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-*arabino*-hex-2-ulopyranose (4-*O*-α-acarbosyl-D-fructopyranose),

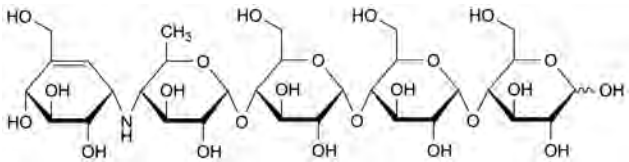

F. *O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-D-glucopyranose (4-*O*-α-acarbosyl-D-glucopyranose),

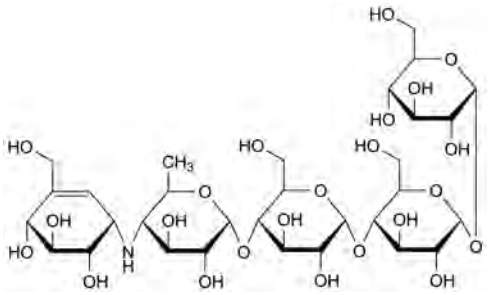

G. α-D-glucopyranosyl *O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranoside (α-D-glucopyranosyl α-acarboside),

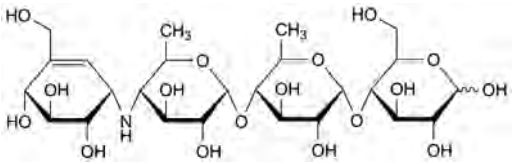

H. *O*-4,6-dideoxy-4-[[(1*S*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-enyl]amino]-α-D-glucopyranosyl-(1→4)-*O*-6-deoxy-α-D-glucopyranosyl-(1→4)-D-glucopyranose.

**01/2017:0871**

# ACEBUTOLOL HYDROCHLORIDE

## Acebutololi hydrochloridum

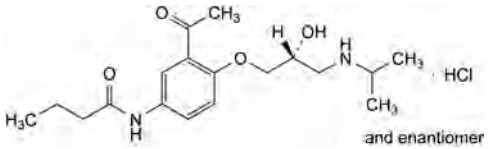


$C_{18}H_{29}ClN_2O_4$ $M_r$ 372.9
[34381-68-5]

DEFINITION

*N*-[3-Acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenyl]butanamide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), very slightly soluble in acetone and in methylene chloride.

mp: about 143 °C.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20.0 mg in a 0.1 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid solution. Dilute 5.0 mL of this solution to 100.0 mL with a 0.1 per cent *V/V* solution of *hydrochloric acid R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 233 nm and 322 nm.

*Specific absorbance at the absorption maximum*: 555 to 605 at 233 nm.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *acebutolol hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *acebutolol hydrochloride CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *pindolol CRS* in *methanol R* and dilute to 20 mL with the same solvent. To 1 mL of this solution add 1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *perchloric acid R*, *methanol R*, *water R* (5:395:600 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 5.0 to 7.0.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 0.5 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *acebutolol impurity I CRS* in 1.0 mL of mobile phase A.

*Reference solution (c)*. Mix 2.0 mL of reference solution (a) and 1.0 mL of reference solution (b) and dilute to 10.0 mL with mobile phase A.

*Reference solution (d)*. Dissolve 5.0 mg of *acebutolol impurity C CRS* in 10 mL of *acetonitrile R* and dilute to 25.0 mL with mobile phase A. Dilute 0.5 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (e)*. Dissolve 5.0 mg of *acebutolol impurity B CRS* in 10.0 mL of *acetonitrile R* and dilute to 25.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 50.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 2.0 mL of *phosphoric acid R*, and 3.0 mL of *triethylamine R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: mix equal volumes of *acetonitrile R* and mobile phase A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 98 | 2 |
| 2 - 30.5 | 98 → 10 | 2 → 90 |
| 30.5 - 41 | 10 | 90 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 25 µL.

*System suitability*: reference solution (c):

- *resolution*: minimum 7.0 between the peaks due to impurity I and acebutolol.

*Limits*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *impurity I*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *ethanol (96 per cent) R* and add 1 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.29 mg of $C_{18}H_{29}ClN_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K.*

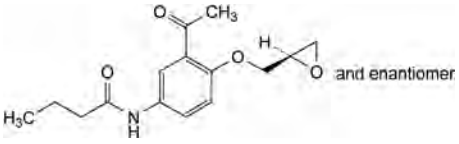


A. *N*-[3-acetyl-4-[(2*RS*)-oxiran-2-ylmethoxy]phenyl]-butanamide,

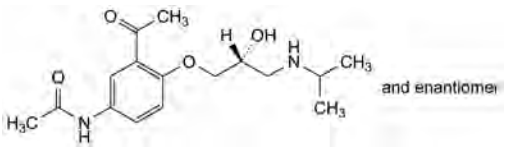


B. *N*-[3-acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenyl]acetamide (diacetolol),

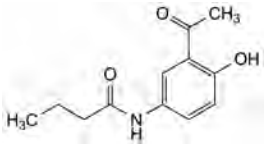

C. *N*-(3-acetyl-4-hydroxyphenyl)butanamide,

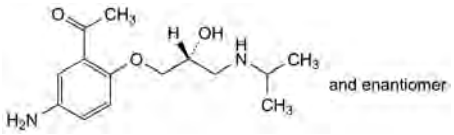


D. 1-[5-amino-2-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenyl]ethanone,

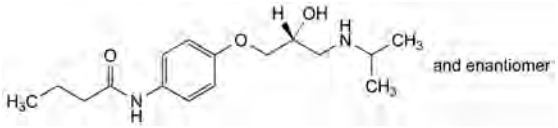


E. *N*-[4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]-propoxy]phenyl]butanamide,

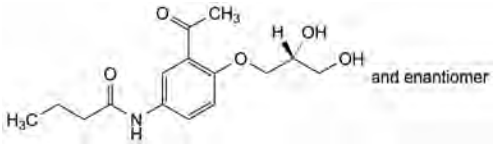


F. *N*-[3-acetyl-4-[(2*RS*)-2,3-dihydroxypropoxy]phenyl]-butanamide,

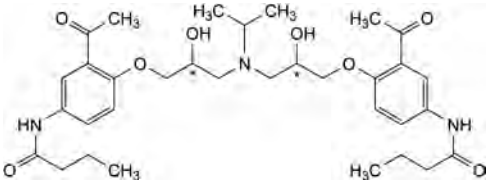

G. *N*,*N*′-[[(1-methylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy(3-acetyl-1,4-phenylene)]]dibutanamide (biamine),

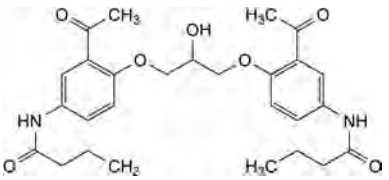

H. *N*,*N*′-[(2-hydroxypropane-1,3-diyl)bis[oxy(3-acetyl-1,4-phenylene)]]dibutanamide,

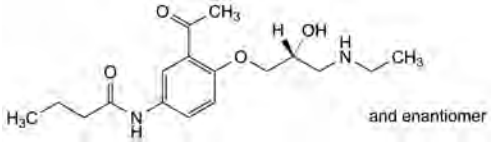


I. *N*-[3-acetyl-4-[(2*RS*)-3-(ethylamino)-2-hydroxy-propoxy]phenyl]butanamide,

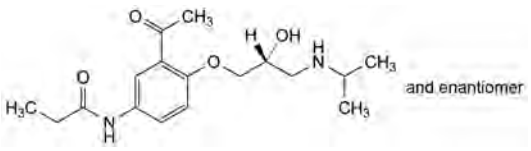


J. *N*-[3-acetyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenyl]propanamide,

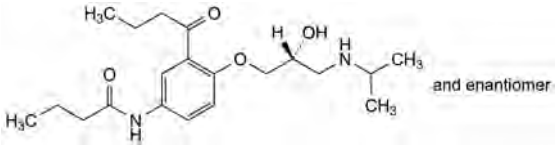


K. *N*-[3-butanoyl-4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)-amino]propoxy]phenyl]butanamide.

**01/2018:1281**

# ACECLOFENAC

## Aceclofenacum

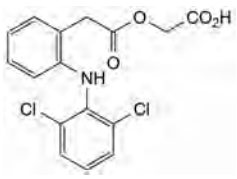

$C_{16}H_{13}Cl_2NO_4$ $M_r$ 354.2
[89796-99-6]

DEFINITION

[[[2-[(2,6-Dichlorophenyl)amino]phenyl]acetyl]oxy]acetic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 50.0 mL with *methanol R*.

*Spectral range*: 220-370 nm.

*Absorption maximum*: 275 nm.

*Specific absorbance at the absorption maximum*: 320 to 350.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison: Ph. Eur. reference spectrum of aceclofenac.*

C. Dissolve about 10 mg in 10 mL of *ethanol (96 per cent) R*. To 1 mL of the solution, add 0.2 mL of a mixture, prepared immediately before use, of equal volumes of a 6 g/L solution of *potassium ferricyanide R* and a 9 g/L solution of *ferric chloride R*. Allow to stand protected from light for 5 min. Add 3 mL of a 10.0 g/L solution of *hydrochloric acid R*. Allow to stand protected from light for 15 min. A blue colour develops and a precipitate is formed.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: mobile phase A, mobile phase B (30:70 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 21.6 mg of *diclofenac sodium CRS* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) and dilute to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 4.0 mg of *aceclofenac impurity F CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve 2.0 mg of *aceclofenac impurity H CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (f).* Mix 1.0 mL of reference solution (b), 1.0 mL of reference solution (d) and 1.0 mL of reference solution (e) and dilute to 100.0 mL with the solvent mixture.

*Reference solution (g).* Dissolve 5.0 mg of *aceclofenac impurity I CRS* in the solvent mixture and dilute to 50.0 mL with solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (h).* Dissolve 4 mg of *aceclofenac for peak identification CRS* (containing impurities B, C, D, E and G) in 2 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent;
- *temperature*: 40 °C.

*Mobile phase:*

- *mobile phase A*: 1.12 g/L solution of *phosphoric acid R* adjusted to pH 7.0 with a 42 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: *water R*, *acetonitrile R* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 70 → 50 | 30 → 50 |
| 25 - 30 | 50 → 20 | 50 → 80 |
| 30 - 50 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL of the test solution and reference solutions (c), (d), (e), (f), (g) and (h).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram supplied with *aceclofenac for peak identification CRS* and the chromatogram obtained with reference solution (h) to identify the peaks due to impurities B, C, D, E and G; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity F; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity H; use the chromatogram obtained with reference solution (g) to identify the peak due to impurity I.

*Relative retention* with reference to aceclofenac (retention time = about 11 min): impurity A = about 0.8; impurity G = about 1.3; impurity H = about 1.5; impurity I = about 2.3; impurity D = about 3.1; impurity B = about 3.2; impurity E = about 3.3; impurity C = about 3.5; impurity F = about 3.7.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to impurity A and aceclofenac.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurities B, C, D, E, G*: for each impurity, not more than the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (f) (0.2 per cent);
- *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.2 per cent);
- *impurity H*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.15 per cent);
- *impurity I*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (g) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (f) (0.10 per cent);
- *total*: maximum 0.7 per cent;
- *disregard limit*: 0.25 times the area of the peak due to aceclofenac in the chromatogram obtained with reference solution (f) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 40 mL of *methanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.42 mg of $C_{16}H_{13}Cl_2NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

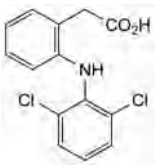


A. [2-[(2,6-dichlorophenyl)amino]phenyl]acetic acid (diclofenac),

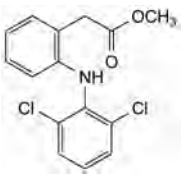


B. methyl [2-[(2,6-dichlorophenyl)amino]phenyl]acetate (methyl ester of diclofenac),

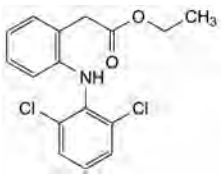

C. ethyl [2-[(2,6-dichlorophenyl)amino]phenyl]acetate (ethyl ester of diclofenac),

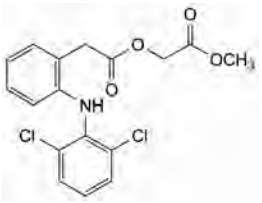

D. methyl [[[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxy]acetate (methyl ester of aceclofenac),

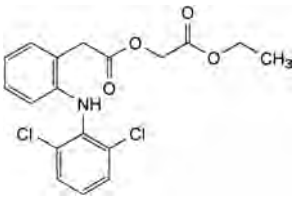

E. ethyl [[[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxy]acetate (ethyl ester of aceclofenac),

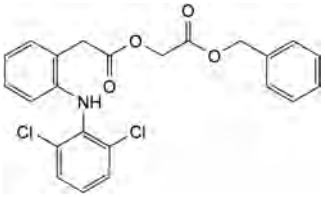

F. benzyl [[[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxy]acetate (benzyl ester of aceclofenac),

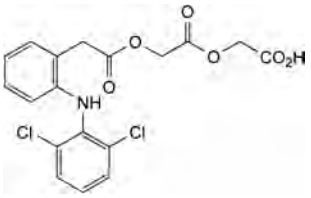

G. [[[[[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxy]acetyl]oxy]acetic acid (acetic aceclofenac),

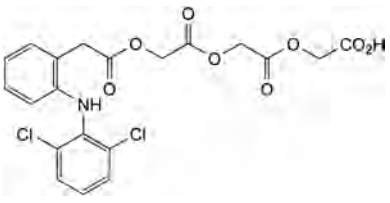

H. [[[[[[[2-[(2,6-dichlorophenyl)amino]phenyl]-acetyl]oxy]acetyl]oxy]acetyl]oxy]acetic acid (diacetic aceclofenac),

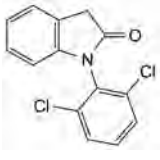

I. 1-(2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one.

**01/2017:1686**

# ACEMETACIN

## Acemetacinum

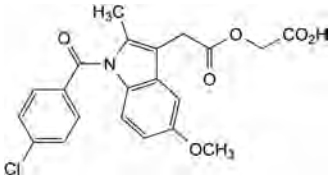

$C_{21}H_{18}ClNO_6$ $M_r$ 415.8
[53164-05-9]

DEFINITION

[[[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]oxy]acetic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or greenish-yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *acemetacin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *acetonitrile for chromatography R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 50.0 mL with *acetonitrile for chromatography R*. Dilute 1.0 mL of this solution to 100.0 mL with *acetonitrile for chromatography R*.

*Reference solution (b)*. Dissolve 5.0 mg of *acemetacin impurity A CRS* and 10.0 mg of *indometacin CRS* (impurity B) in *acetonitrile for chromatography R*, and dilute to 50.0 mL with the same solvent.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 20.0 mL with *acetonitrile for chromatography R*.

*Reference solution (d)*. To 1 mL of reference solution (b), add 10 mL of the test solution and dilute to 20 mL with *acetonitrile for chromatography R*.

*Reference solution (e)*. Dissolve the contents of a vial of *acemetacin impurity mixture CRS* (containing impurities C, D, E and F) in 1.0 mL of the test solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 6.5 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 9 | 95 → 65 | 5 → 35 |
| 9 - 16 | 65 | 35 |
| 16 - 28 | 65 → 20 | 35 → 80 |
| 28 - 34 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Identification of impurities*:

- use the chromatogram supplied with *acemetacin impurity mixture CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities C, D, E and F;
- use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to acemetacin (retention time = about 15 min): impurity A = about 0.7; impurity B = about 0.9; impurity F = about 1.2; impurity C = about 1.3; impurity D = about 1.5; impurity E = about 2.2.

*System suitability*: reference solution (d):

- *peak-to-valley ratio*: minimum 15, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to acemetacin.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.3; impurity D = 1.4; impurity F = 1.3;
- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *impurities C, D, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 20 mL of *acetone R* and add 10 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.58 mg of $C_{21}H_{18}ClNO_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

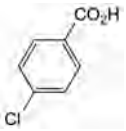

A. 4-chlorobenzoic acid,

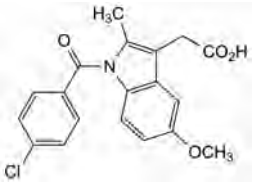

B. [1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid (indometacin),

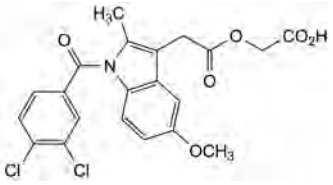

C. [[[1-(3,4-dichlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]oxy]acetic acid,

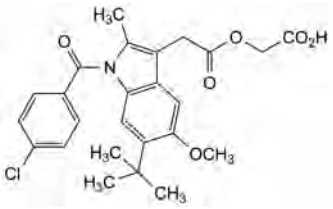

D. [[[1-(4-chlorobenzoyl)-6-(1,1-dimethylethyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]oxy]acetic acid,

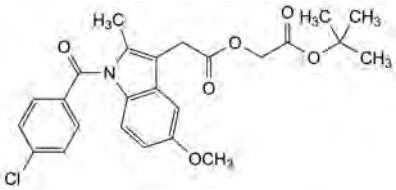

E. 1,1-dimethylethyl [[[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]oxy]acetate,

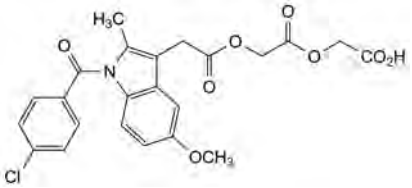

F. [[[[[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]oxy]acetyl]oxy]acetic acid.

01/2017:1282

# ACESULFAME POTASSIUM

## Acesulfamum kalicum

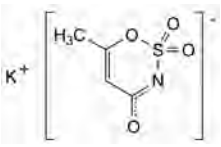

$C_4H_4KNO_4S$ $M_r$ 201.2
[55589-62-3]

DEFINITION

Potassium 6-methyl-1,2,3-oxathiazin-4-olate 2,2-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, very slightly soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *acesulfame potassium CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 5 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.
*Reference solution (a).* Dissolve 5 mg of *acesulfame potassium CRS* in *water R* and dilute to 5 mL with the same solvent.
*Reference solution (b).* Dissolve 5 mg of *acesulfame potassium CRS* and 5 mg of *saccharin sodium R* in *water R* and dilute to 5 mL with the same solvent.
*Plate*: *cellulose for chromatography R* as the coating substance.
*Mobile phase*: *concentrated ammonia R*, *acetone R*, *ethyl acetate R* (10:60:60 *V/V/V*).
*Application*: 5 µL as bands.
*Development*: twice over 2/3 of the plate.
*Drying*: in a current of warm air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated zones.
*Results*: the principal zone in the chromatogram obtained with the test solution is similar in position and size to the principal zone in the chromatogram obtained with reference solution (a).

C. 0.5 mL of solution S (see Tests) gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.80 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 50 mg of *acetylacetamide R* (impurity A) in *water R* and dilute to 25 mL with the same solvent. To 5 mL of the solution add 45 mL of *water R* and dilute to 100 mL with *methanol R*.

*Reference solution (b).* To 10 mL of reference solution (a) add 1 mL of the test solution and dilute to 20 mL with *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *water R*, *ethanol (96 per cent) R*, *ethyl acetate R* (2:15:74 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air until the solvents are completely removed.

*Detection*: spray with *phosphoric vanillin solution R* and heat at 120 °C for about 10 min; examine in daylight.

*System suitability*: the chromatogram obtained with reference solution (a) shows a clearly visible spot and the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Limit*:
– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.125 per cent).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 4.0 mg of *acesulfame potassium impurity B CRS* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 200.0 mL with *water R*.

*Reference solution (b).* Dissolve 0.100 g of the substance to be examined in reference solution (a) and dilute to 10.0 mL with the same solution.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: mix 40 volumes of *acetonitrile R* and 60 volumes of a 3.3 g/L solution of *tetrabutylammonium hydrogen sulfate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 234 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of acesulfame.

*Relative retention* with reference to acesulfame (retention time = about 5.3 min): impurity B = about 1.6.

*System suitability*:
– *signal-to-noise ratio*: minimum 10 for the peak due to impurity B in the chromatogram obtained with reference solution (a);
– *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to acesulfame, in the chromatogram obtained with reference solution (b).

*Limit*:
– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (20 ppm).

**Fluorides**: maximum 3 ppm.

Potentiometry (*2.2.36, Method I*).

*Test solution.* Dissolve 3.000 g of the substance to be examined in *distilled water R*, add 15.0 mL of *total-ionic-strength-adjustment buffer R1* and dilute to 50.0 mL with *distilled water R*.

*Reference solutions.* To 0.5 mL, 1.0 mL, 1.5 mL and 3.0 mL of *fluoride standard solution (10 ppm F) R* add 15.0 mL of *total-ionic-strength-adjustment buffer R1* and dilute to 50.0 mL with *distilled water R*.

*Indicator electrode*: fluoride-selective.

*Reference electrode*: silver-silver chloride.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.12 mg of $C_4H_4KNO_4S$.

IMPURITIES

*Specified impurities: A, B.*

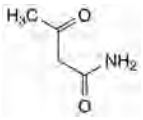

A. 3-oxobutanamide (acetylacetamide),

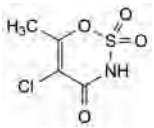

B. 5-chloro-6-methyl-1,2,3-oxathiazin-4(3*H*)-one 2,2-dioxide.

**01/2017:0454**

# ACETAZOLAMIDE

## Acetazolamidum

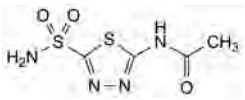

$C_4H_6N_4O_3S_2$ $M_r$ 222.2
[59-66-5]

DEFINITION

*N*-(5-Sulfamoyl-1,3,4-thiadiazol-2-yl)acetamide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Solution A*. Dissolve 30.0 mg in *0.01 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *0.01 M sodium hydroxide*.

*Solution B*. Dilute 25.0 mL of solution A to 100.0 mL with *0.01 M sodium hydroxide*.

*Spectral range*: 230-260 nm for solution A; 260-350 nm for solution B.

*Absorption maximum*: at 240 nm for solution A; at 292 nm for solution B.

*Specific absorbance at the absorption maximum*: 162 to 176 for solution A; 570 to 620 for solution B.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *acetazolamide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

C. Introduce about 20 mg into a test-tube and add 4 mL of *dilute hydrochloric acid R* and 0.2 g of *zinc powder R*. Immediately place a piece of *lead acetate paper R* over the mouth of the tube. The paper shows a brownish-black colour.

D. Dissolve about 25 mg in a mixture of 0.1 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*. Add 0.1 mL of *copper sulfate solution R*. A greenish-blue precipitate is formed.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ or $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in 10 mL of *1 M sodium hydroxide*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *acetazolamide for system suitability CRS* (containing impurities A, B, C, D, E and F) in 1.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped propoxybenzene silica gel for chromatography R* (4 µm).

*Mobile phase*: *acetonitrile for chromatography R*, 6.8 g/L solution of *potassium dihydrogen phosphate R* (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 25 µl.

*Run time*: 3.5 times the retention time of acetazolamide.

*Identification of impurities*: use the chromatogram supplied with *acetazolamide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to acetazolamide (retention time = about 8 min): impurity E = about 0.3; impurity D = about 0.4; impurity B = about 0.6; impurity C = about 1.4; impurity A = about 2.1; impurity F = about 2.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities E and D.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 2.3; impurity C = 2.6; impurity D = 1.6;
- *impurities A, B, C, D, E, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 0.4 g add 20 mL of *distilled water R* and dissolve by heating to boiling. Allow to cool with frequent shaking and filter.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 mL of *dimethylformamide R*. Titrate with *0.1 M ethanolic sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 22.22 mg of $C_4H_6N_4O_3S_2$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): G.

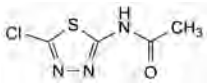

A. *N*-(5-chloro-1,3,4-thiadiazol-2-yl)acetamide,

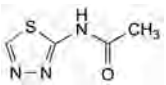

B. *N*-(1,3,4-thiadiazol-2-yl)acetamide,

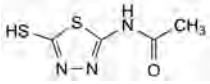

C. *N*-(5-sulfanyl-1,3,4-thiadiazol-2-yl)acetamide,

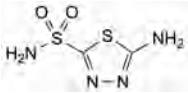

D. 5-amino-1,3,4-thiadiazole-2-sulfonamide,

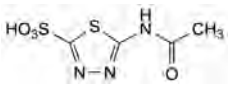

E. 5-acetamido-1,3,4-thiadiazole-2-sulfonic acid,

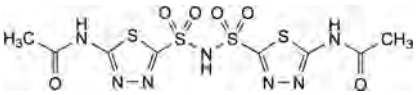

F. *N*-[5-[(5-acetamido-1,3,4-thiadiazol-2-yl)sulfonyl]-sulfamoyl-1,3,4-thiadiazol-2-yl]acetamide,

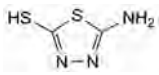

G. 5-amino-1,3,4-thiadiazole-2-thiol.

**01/2017:0590**

# ACETIC ACID, GLACIAL

## Acidum aceticum glaciale

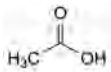

$C_2H_4O_2$ $M_r$ 60.1

[64-19-7]

DEFINITION

*Content*: 99.0 per cent *m/m* to 100.5 per cent *m/m*.

CHARACTERS

*Appearance*: crystalline mass or clear, colourless, volatile liquid.

*Solubility*: miscible with water, with ethanol (96 per cent) and with methylene chloride.

IDENTIFICATION

A. A 100 g/L solution is strongly acid (*2.2.4*).

B. To 0.03 mL add 3 mL of *water R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives reaction (b) of acetates (*2.3.1*).

TESTS

**Solution S**. Dilute 20 mL to 100 mL with *distilled water R*.

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Freezing point** (*2.2.18*): minimum 14.8 °C.

**Reducing substances**. Dilute 2.0 mL to 10.0 mL with *water R*. Add 0.1 mL of *0.02 M potassium permanganate*. Heat on a water-bath for 1 min, the colour remains pink.

**Chlorides** (*2.4.4*): maximum 25 mg/L.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 50 mg/L, determined on solution S.

**Iron** (*2.4.9*): maximum 5 ppm.

Dissolve the residue obtained in the test for residue on evaporation by heating with 2 quantities, each of 15 mL, of *water R* and dilute to 50.0 mL with *water R*. Dilute 5.0 mL of the solution to 10.0 mL with *water R*.

**Residue on evaporation**: maximum 0.01 per cent.

Evaporate 20 g to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 2.0 mg.

ASSAY

Weigh accurately a conical flask with a ground-glass stopper containing 25 mL of *water R*. Add 1.0 mL of the substance to be examined and weigh again accurately. Add 0.5 mL of *phenolphthalein solution R* and titrate with *1 M sodium hydroxide*.

1 mL of *1 M sodium hydroxide* is equivalent to 60.1 mg of $C_2H_4O_2$.

STORAGE

In an airtight container.

**04/2019:0872**

# ACETONE

## Acetonum

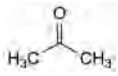

$C_3H_6O$ $M_r$ 58.08

[67-64-1]

DEFINITION

Propanone.

CHARACTERS

*Appearance*: volatile, clear, colourless liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

The vapour is flammable.

IDENTIFICATION

A. Relative density (see Tests).

B. To 1 mL, add 3 mL of *dilute sodium hydroxide solution R* and 0.3 mL of a 25 g/L solution of *sodium nitroprusside R*. An intense red colour is produced which becomes violet with the addition of 3.5 mL of *acetic acid R*.

C. To 10 mL of a 0.1 per cent *V/V* solution of the substance to be examined in *ethanol (50 per cent V/V) R*, add 1 mL of a 10 g/L solution of *nitrobenzaldehyde R* in *ethanol (50 per cent V/V) R* and 0.5 mL of *strong sodium hydroxide solution R*. Allow to stand for about 2 min and acidify with *acetic acid R*. A greenish-blue colour is produced.

TESTS

**Appearance of solution**. To 10 mL add 10 mL of *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL add 5 mL of *carbon dioxide-free water R*, 0.15 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.7 mL of *0.01 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The solution is red or orange.

**Relative density** (*2.2.5*): 0.790 to 0.793.

**Reducing substances**. To 30 mL add 0.1 mL of *0.02 M potassium permanganate* and allow to stand in the dark for 2 h. The mixture is not completely decolourised.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Reference solution (a)*. To 0.5 mL of *methanol R* add 0.5 mL of *2-propanol R* and dilute to 100.0 mL with the test solution. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Reference solution (b)*. Dilute 100 µL of *benzene R* to 100.0 mL with the test solution. Dilute 0.20 mL of this solution to 100.0 mL with the test solution.

*Column*:

– *material*: fused silica,

– *size*: *l* = 50 m, Ø = 0.3 mm,

– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 21 cm/s.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 11 | 45 → 100 |
| | 11 - 20 | 100 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Retention time*: impurity C = about 7.5 min.

*System suitability*:

– *resolution*: minimum 5.0 between the peak due to impurity A (2nd peak) and the peak due to impurity B (3rd peak) in the chromatogram obtained with reference solution (a);

– *signal-to-noise ratio*: minimum 5 for the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Limits*:

– *impurities A, B*: for each impurity, not more than the difference between the areas of the corresponding peaks in the chromatogram obtained with reference solution (a) and the areas of the corresponding peaks in the chromatogram obtained with the test solution (0.05 per cent *V/V*),

– *impurity C*: not more than the difference between the area of the peak due to impurity C in the chromatogram obtained with reference solution (b) and the area of the corresponding peak in the chromatogram obtained with the test solution (2 ppm *V/V*),

– *any other impurity*: for each impurity, not more than the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (0.05 per cent *V/V*).

**Matter insoluble in water**. Dilute 1.0 mL to 20 mL with *water R*. The solution is clear (*2.2.1*).

**Residue on evaporation**: maximum 50 ppm.

Evaporate 20.0 g to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 1 mg.

**Water** (*2.5.12*): maximum 3 g/L, determined on 10.0 mL.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

A. methanol,

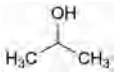

B. propan-2-ol (isopropanol),

C. benzene.

01/2017:1485

# ACETYLCHOLINE CHLORIDE

## Acetylcholini chloridum

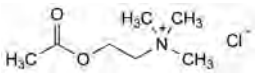

$C_7H_{16}ClNO_2$ $M_r$ 181.7
[60-31-1]

DEFINITION

2-(Acetyloxy)-*N*,*N*,*N*-trimethylethanaminium chloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder or colourless crystals, very hygroscopic.

*Solubility*: very soluble in water, freely soluble in alcohol, slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 149 °C to 152 °C.

Introduce the substance to be examined into a capillary tube. Dry in an oven at 100-105 °C for 3 h. Seal the tube and determine the melting point.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *acetylcholine chloride CRS*.

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal zone in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal zone in the chromatogram obtained with reference solution (b).

D. To 15 mg add 10 mL of *dilute sodium hydroxide solution R*, 2 mL of *0.02 M potassium permanganate* and heat. The vapours formed change the colour of *red litmus paper R* to blue.

E. 0.5 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

**Acidity**. Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*. Add 0.05 mL of *phenolphthalein solution R*. Not more than 0.4 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 0.30 g of the substance to be examined in *methanol R* and dilute to 3.0 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dilute 1 mL of test solution (a) to 100 mL with *methanol R*.

*Reference solution (b).* Dissolve 20.0 mg of *acetylcholine chloride CRS* in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution (c).* Dissolve 20 mg of *choline chloride R* in *methanol R*, add 0.4 mL of test solution (a) and dilute to 2.0 mL with *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: mix 20 volumes of a 40 g/L solution of *ammonium nitrate R*, 20 volumes of *methanol R* and 60 volumes of *acetonitrile R*.

*Application*: 5 µL as bands of 10 mm by 2 mm.

*Development*: over 2/3 of the plate.

*Detection*: spray with *potassium iodobismuthate solution R3*.

*System suitability*: the chromatogram obtained with reference solution (c) shows 2 clearly separated zones.

*Limits*:

– *any impurity*: any zones in the chromatogram obtained with test solution (a), apart from the principal zone, are not more intense than the principal zone in the chromatogram obtained with reference solution (a) (1 per cent).

**Trimethylamine**. Dissolve 0.1 g in 10 mL of *sodium carbonate solution R* and heat to boiling. No vapours appear which turn *red litmus paper R* blue.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.200 g in 20 mL of *carbon dioxide-free water R*. Neutralise with *0.01 M sodium hydroxide* using 0.15 mL of *phenolphthalein solution R* as indicator. Add 20.0 mL of *0.1 M sodium hydroxide* and allow to stand for 30 min. Titrate with *0.1 M hydrochloric acid*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.17 mg of $C_7H_{16}ClNO_2$.

STORAGE

In ampoules, protected from light.

IMPURITIES

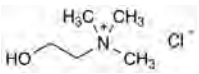

A. 2-hydroxy-*N*,*N*,*N*-trimethylethanaminium chloride (choline chloride),

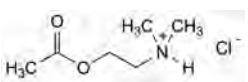

B. 2-(acetyloxy)-*N*,*N*-dimethylethanaminium chloride,

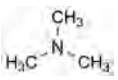

C. *N*,*N*-dimethylmethanamine.

**01/2017:0967**

# ACETYLCYSTEINE

## Acetylcysteinum

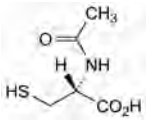

$C_5H_9NO_3S$ $M_r$ 163.2
[616-91-1]

DEFINITION

(2*R*)-2-(Acetylamino)-3-sulfanylpropanoic acid.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 104 °C to 110 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium bromide R*.

*Comparison*: *acetylcysteine CRS*.

D. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

E. To 0.5 mL of solution S (see Tests) add 0.05 mL of a 50 g/L solution of *sodium nitroprusside R* and 0.05 mL of *concentrated ammonia R*. A dark violet colour develops.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.0 to 2.8.

To 2 mL of solution S add 8 mL of *carbon dioxide-free water R* and mix.

**Specific optical rotation** (*2.2.7*): + 21.0 to + 27.0 (dried substance).

In a 25 mL volumetric flask, mix 1.25 g with 1 mL of a 10 g/L solution of *sodium edetate R*. Add 7.5 mL of a 40 g/L solution of *sodium hydroxide R*, mix and dissolve. Dilute to 25.0 mL with *phosphate buffer solution pH 7.0 R2*.

**Related substances**. Liquid chromatography (*2.2.29*). *Except where otherwise prescribed, prepare the solutions immediately before use.*

*Test solution (a).* Suspend 0.80 g of the substance to be examined in 1 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 50.0 mL with *water R*.

*Test solution (c).* Use test solution (a) after storage for at least 1 h.

*Reference solution (a).* Suspend 4.0 mg of *acetylcysteine CRS*, 4.0 mg of *L-cystine R* (impurity A), 4.0 mg of *L-cysteine R* (impurity B), 4.0 mg of *acetylcysteine impurity C CRS* and 4.0 mg of *acetylcysteine impurity D CRS* in 1 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

*Reference solution (b).* Suspend 4.0 mg of *acetylcysteine CRS* in 1 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: stir 3 volumes of *acetonitrile R* and 97 volumes of *water R* in a beaker; adjust to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL, 3 times; inject *0.01 M hydrochloric acid* as a blank.

*Run time*: 5 times the retention time of acetylcysteine (about 30 min).

*Retention time*: impurity A = about 2.2 min; impurity B = about 2.4 min; 2-methyl-2-thiazoline-4-carboxylic acid, originating in test solution (c) = about 3.3 min; acetylcysteine = about 6.4 min; impurity C = about 12 min; impurity D = about 14 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurities A and B and minimum 2.0 between the peaks due to impurities C and D.

From the chromatogram obtained with test solution (a), calculate the percentage content of the known impurities ($T_1$) and the unknown impurities ($T_2$) using the following equations:

$$T_1 = \frac{A_1 \times m_2 \times 100}{A_2 \times m_1}$$

$$T_2 = \frac{A_3 \times m_3 \times 100}{A_4 \times m_1}$$

$A_1$ = peak area of individual impurity (impurity A, impurity B, impurity C and impurity D) in the chromatogram obtained with test solution (a);

$A_2$ = peak area of the corresponding individual impurity (impurity A, impurity B, impurity C and impurity D) in the chromatogram obtained with reference solution (a);

$A_3$ = peak area of unknown impurity in the chromatogram obtained with test solution (a);

$A_4$ = peak area of acetylcysteine in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the substance to be examined in test solution (a);

$m_2$ = mass of the individual impurity in reference solution (a);

$m_3$ = mass of acetylcysteine in reference solution (b).

*Limits*:

– *impurities A, B, C, D*: for each impurity, maximum 0.5 per cent;
– *any other impurity*: for each impurity, maximum 0.5 per cent;
– *total*: maximum 0.5 per cent;

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak with a retention time of about 3.3 min due to 2-methyl-2-thiazoline-4-carboxylic acid.

**Zinc**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 1.00 g in *0.001 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (5 mg/mL Zn) R*, diluting with *0.001 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.8 nm.

*Atomisation device*: air-acetylene flame.

Use a correction procedure for non-specific absorption.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 70 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 60 mL of *water R* and add 10 mL of *dilute hydrochloric acid R*. After cooling in iced water, add 10 mL of *potassium iodide solution R* and titrate with *0.05 M iodine*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.05 M iodine* is equivalent to 16.32 mg of $C_5H_9NO_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

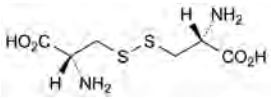

A. 3,3′-disulfanediylbis[(2*R*)-2-aminopropanoic acid] (L-cystine),

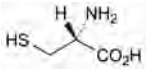

B. (2*R*)-2-amino-3-sulfanylpropanoic acid (L-cysteine),

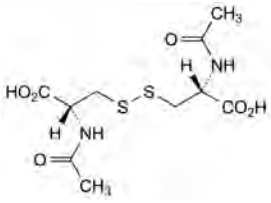

C. (2*R*,2′*R*)-3,3′-disulfanediylbis[2-(acetylamino)propanoic acid] (*N*,*N*′-diacetyl-L-cystine),

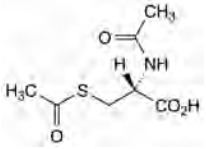

D. (2*R*)-2-(acetylamino)-3-(acetylsulfanyl)propanoic acid (*N*,*S*-diacetyl-L-cysteine).

**01/2008:2168**
**corrected 6.7**

# β-ACETYLDIGOXIN

## β-Acetyldigoxinum

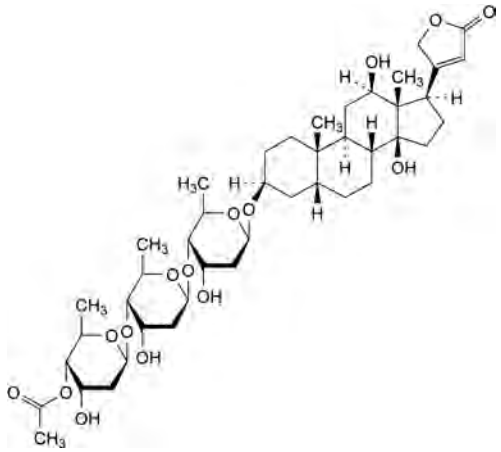

$C_{43}H_{66}O_{15}$ $M_r$ 823

[5355-48-6]

DEFINITION

3β-[(4-*O*-Acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *β-acetyldigoxin CRS.*

TESTS

**Specific optical rotation** (*2.2.7*): + 26.2 to + 28.2 (dried substance).

Dissolve 0.50 g in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 25.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix equal volumes of *methanol R2* and *acetonitrile for chromatography R.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10.0 mg of *β-acetyldigoxin CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *gitoxin CRS* (impurity D) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. To 5.0 mL of this solution, add 0.5 mL of reference solution (a) and dilute to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5.0 mg of *β-acetyldigoxin for peak identification CRS* (containing impurities A and B) in 10.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 70 | 30 |
| 10 - 20 | 70 → 35 | 30 → 65 |
| 20 - 20.1 | 35 → 70 | 65 → 30 |
| 20.1 - 25 | 70 | 30 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatograms obtained with reference solutions (c) and (d) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to β-acetyldigoxin (retention time = about 9 min): impurity B = about 0.3; impurity A = about 0.7; impurity D = about 1.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to β-acetyldigoxin and impurity D;

– *symmetry factor*: maximum 2.5 for the peak due to β-acetyldigoxin.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurity D*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *any other impurity*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *sum of impurities other than A, B and D*: not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{43}H_{66}O_{15}$ from the declared content of *β-acetyldigoxin CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, G, H.*

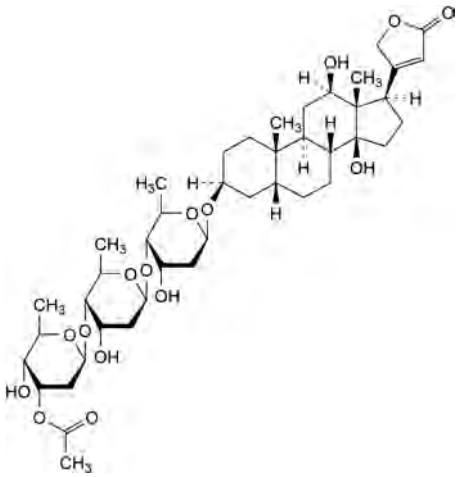

A. 3β-[(3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (α-acetyldigoxin),

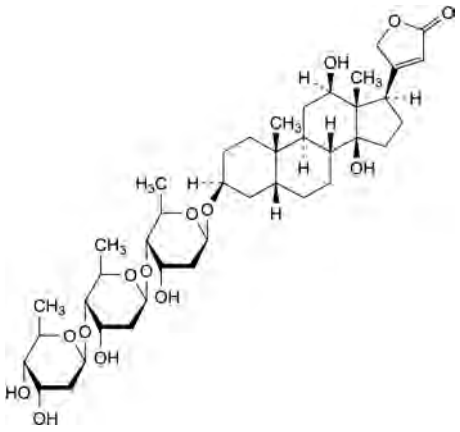

B. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (digoxin),

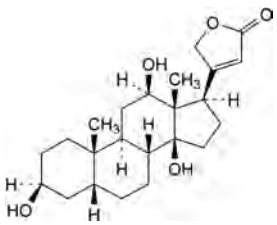

C. 3β,12β,14-trihydroxy-5β-card-20(22)-enolide (digoxigenin),

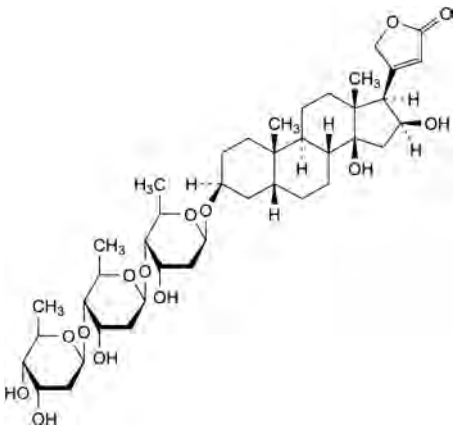

D. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14,16β-dihydroxy-5β-card-20(22)-enolide (gitoxin),

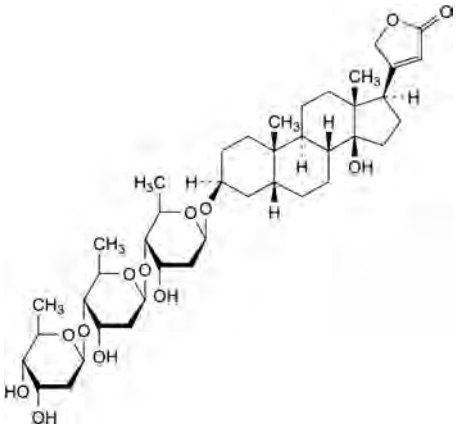

E. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-5β-card-20(22)-enolide (digitoxin),

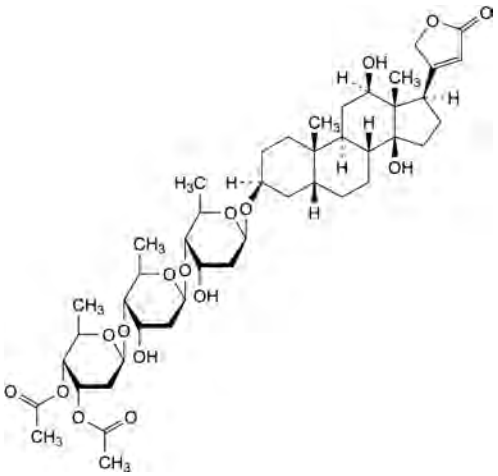

F. 3β-[(3,4-*O*-diacetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (diacetyldigoxin),

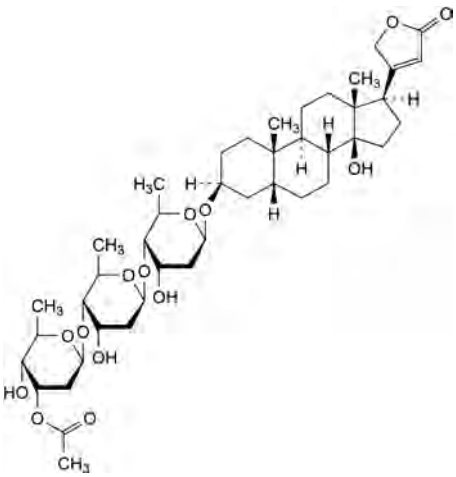

G. 3β-[(3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-5β-card-20(22)-enolide (α-acetyldigitoxin),

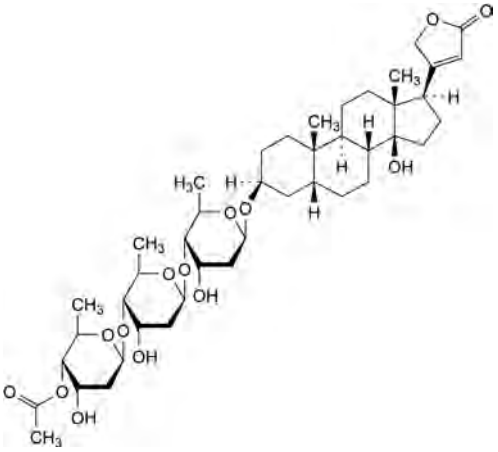

H. 3β-[(4-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-5β-card-20(22)-enolide (β-acetyldigitoxin).

**01/2018:2903**

# ACETYLENE INTERMIX (1 PER CENT) IN NITROGEN

## Acetylenum (1 per centum) in nitrogenio intermixtum

DEFINITION

A mixture containing 1 per cent *V/V* of acetylene in *Low-oxygen nitrogen (1685)*.

*Content*: 0.95 per cent *V/V* to 1.05 per cent *V/V* of acetylene ($C_2H_2$) in nitrogen ($N_2$).

This monograph applies to acetylene intermix (1 per cent) in nitrogen used in the preparation of lung function test gas mixtures for medicinal use.

PRODUCTION

The acetylene used in the manufacturing process is produced by hydrolysis of calcium carbide.

Prior to using the gas in the manufacturing process, the acetylene may be passed through an activated charcoal filter.

The acetylene is stored in cylinders, which may be filled with a porous mass with acetone the only solvent permitted.

CHARACTERS

*Appearance*: colourless gas.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the peak due to acetylene in the chromatogram obtained with the gas to be examined is similar in retention time and size to the peak due to acetylene in the chromatogram obtained with the reference gas.

B. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas. Nitrogen R1.*

*Column*:

– *material*: stainless steel;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 20 mL/min.

*Temperature*:

– *column*: 80 °C;
– *detector*: 130 °C.

*Detection*: thermal conductivity.

*Injection*: 10 µL.

*Retention time*: nitrogen = about 2 min.

*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with the reference gas.

TESTS

**Acetone**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Mixture containing 250 ppm *V/V* of *acetone R* in *nitrogen R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 10 m, Ø = 0.53 mm;
– *stationary phase*: *polyorganosiloxane for oxygen-containing compounds R* (film thickness 10 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 50 mL/min.

*Temperature*:

– *column*: 200 °C;
– *injection port*: 240 °C;
– *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 25 µL.

*Retention time*: acetone = about 1 min.

*Calculation of percentage content*:

– use the concentration of acetone in the reference gas.

*Limit*:

– *acetone*: maximum 250 ppm *V/V*.

**Arsine**: maximum 0.25 ppm *V/V*, determined using an arsine detector tube (*2.1.6*).

**Phosphine**: maximum 0.2 ppm *V/V*, determined using a phosphine detector tube (*2.1.6*).

**Hydrogen sulfide**: maximum 0.2 ppm *V/V*, determined using a hydrogen sulfide detector tube (*2.1.6*).

**Water** (*2.5.28*): maximum 10 ppm *V/V*.

ASSAY

Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Mixture containing 1.0 per cent *V/V* of *acetylene R* in *nitrogen R1*.

*Column*:

– *material*: stainless steel;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: 3 per cent *squalane R* on alumina.

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 20 mL/min.

*Temperature*:

– *column*: 100 °C;
– *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 100 µL.

*Retention time*: acetylene = about 6 min.

Calculate the percentage content of $C_2H_2$.

STORAGE

As a compressed gas, in appropriate high-pressure cylinders complying with the legal regulations.

LABELLING

The label states the nominal content, in per cent *V/V*, of acetylene in nitrogen.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

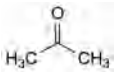

A. propan-2-one (acetone),

B. arsane (arsine),

C. phosphane (phosphine),

D. hydrogen sulfide,

E. water.

**01/2017:0309**

# ACETYLSALICYLIC ACID

## Acidum acetylsalicylicum

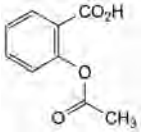

$C_9H_8O_4$ $M_r$ 180.2

[50-78-2]

DEFINITION

2-(Acetyloxy)benzoic acid.

*Content*: 99.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 143 °C (instantaneous method).

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *acetylsalicylic acid CRS*.

B. To 0.2 g add 4 mL of *dilute sodium hydroxide solution R* and boil for 3 min. Cool and add 5 mL of *dilute sulfuric acid R*. A crystalline precipitate is formed. Filter, wash the precipitate and dry at 100-105 °C. The melting point (*2.2.14*) is 156 °C to 161 °C.

C. In a test tube mix 0.1 g with 0.5 g of *calcium hydroxide R*. Heat the mixture and expose to the fumes produced a piece of filter paper impregnated with 0.05 mL of *nitrobenzaldehyde solution R*. A greenish-blue or greenish-yellow colour develops on the paper. Moisten the paper with *dilute hydrochloric acid R*. The colour becomes blue.

D. Dissolve with heating about 20 mg of the precipitate obtained in identification test B in 10 mL of *water R* and cool. The solution gives reaction (a) of salicylates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in 9 mL of *ethanol (96 per cent) R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *acetonitrile for chromatography R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *salicylic acid R* (impurity C) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *salicylic acid R* (impurity C) in the mobile phase and dilute to 10.0 mL with the mobile phase. To 1.0 mL of the solution add 0.2 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve with the aid of ultrasound the contents of a vial of *acetylsalicylic acid for peak identification CRS* (containing impurities A, B, D, E and F) in 1.0 mL of *acetonitrile R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *phosphoric acid R*, *acetonitrile for chromatography R*, *water R* (2:400:600 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 237 nm.

*Injection*: 10 µL.

*Run time*: 7 times the retention time of acetylsalicylic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C; use the chromatogram supplied with *acetylsalicylic acid for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, D, E and F.

*Relative retention* with reference to acetylsalicylic acid (retention time = about 5 min): impurity A = about 0.7; impurity B = about 0.8; impurity C = about 1.3; impurity D = about 2.3; impurity E = about 3.2; impurity F = about 6.0.

*System suitability*: reference solution (b):
- *resolution*: minimum 6.0 between the peaks due to acetylsalicylic acid and impurity C.

*Limits*:
- *impurities A, B, C, D, E, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a flask with a ground-glass stopper, dissolve 1.000 g in 10 mL of *ethanol (96 per cent) R*. Add 50.0 mL of *0.5 M sodium hydroxide*. Close the flask and allow to stand for 1 h. Using 0.2 mL of *phenolphthalein solution R* as indicator, titrate with *0.5 M hydrochloric acid*. Carry out a blank titration.

1 mL of *0.5 M sodium hydroxide* is equivalent to 45.04 mg of $C_9H_8O_4$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

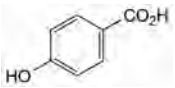


A. 4-hydroxybenzoic acid,

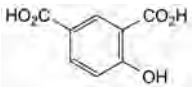


B. 4-hydroxybenzene-1,3-dicarboxylic acid (4-hydroxyisophthalic acid),

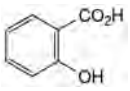


C. 2-hydroxybenzenecarboxylic acid (salicylic acid),

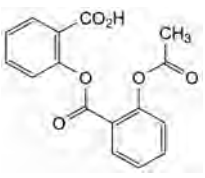


D. 2-[[2-(acetyloxy)benzoyl]oxy]benzoic acid (acetylsalicylsalicylic acid),

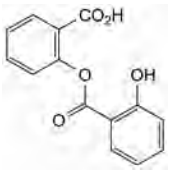

E. 2-[(2-hydroxybenzoyl)oxy]benzoic acid (salsalate, salicylsalicylic acid),

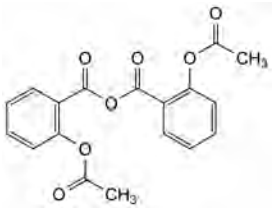

F. 2-(acetyloxy)benzoic anhydride (acetylsalicylic anhydride).

**01/2017:1383**

# *N*-ACETYLTRYPTOPHAN

## *N*-Acetyltryptophanum

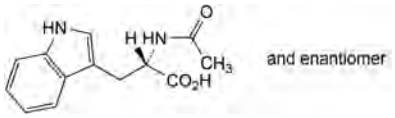


$C_{13}H_{14}N_2O_3$ $M_r$ 246.3
[87-32-1]

DEFINITION

(*RS*)-2-Acetylamino-3-(1*H*-indol-3-yl)propanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

Tryptophan used for the production of *N*-acetyltryptophan complies with the test for impurity A and other related substances in the monograph on *Tryptophan (1272)*.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, or colourless crystals.

*Solubility*: slightly soluble in water, very soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

mp: about 205 °C.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D, E.*

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *N-acetyltryptophan CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 50 mg of the substance to be examined in 0.2 mL of *concentrated ammonia R* and dilute to 10 mL with *water R*.
*Reference solution (a).* Dissolve 50 mg of *N-acetyltryptophan CRS* in 0.2 mL of *concentrated ammonia R* and dilute to 10 mL with *water R*.
*Reference solution (b).* Dissolve 10 mg of *tryptophan R* in the test solution and dilute to 2 mL with the test solution.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (25:25:40 *V/V/V*).
*Application*: 2 µL.
*Development*: over a path of 10 cm.
*Drying*: in an oven at 100-105 °C for 15 min.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 2 mg in 2 mL of *water R*. Add 2 mL of *dimethylaminobenzaldehyde solution R6*. Heat on a water-bath. A blue or greenish-blue colour develops.

E. It gives the reaction of acetyl (*2.3.1*). Proceed as described for substances hydrolysable only with difficulty.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ or $GY_7$ (*2.2.2, Method II*).

Dissolve 1.0 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 100 mL with the same alkaline solution.

**Optical rotation** (*2.2.7*): – 0.1° to + 0.1°.

Dissolve 2.50 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with the same alkaline solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test and reference solutions immediately before use.*

*Buffer solution pH 2.3.* Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 1000 mL of *water R*. Add about 700 mL of a 2.9 g/L solution of *phosphoric acid R* and adjust to pH 2.3 with the same acid solution.

*Solvent mixture*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 4.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *1,1´-ethylidenebistryptophan CRS* in 1 mL of reference solution (b).

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:
– *mobile phase A*: *acetonitrile R*, buffer solution pH 2.3 (115:885 *V/V*);
– *mobile phase B*: *acetonitrile R*, buffer solution pH 2.3 (350:650 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 45 | 100 → 0 | 0 → 100 |
| 45 - 65 | 0 | 100 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Retention time*: *N*-acetyltryptophan = about 29 min; 1,1′-ethylidenebis(tryptophan) = about 34 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 8.0 between the peaks due to *N*-acetyltryptophan and 1,1′-ethylidenebis(tryptophan); if necessary, adjust the time programme for the elution gradient (an increase in the duration of elution with mobile phase A produces longer retention times and a better resolution);
- *symmetry factor*: maximum 3.5 for the peak due to 1,1′-ethylidenebistryptophan in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurities A, B, C, D, E, F, G, H, I, J, K, L*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.01 times the area of the principal peak the chromatogram obtained with reference solution (a) (0.01 per cent).

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 0.10 g.

Prepare the standard using 0.2 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 10 ppm.

Dissolve 1.0 g in 50 mL of *hydrochloric acid R1*, with heating at 50 °C. Allow to cool. In a separating funnel, shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Examine the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 mL of *methanol R*. Add 50 mL of *anhydrous ethanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.63 mg of $C_{13}H_{14}N_2O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J, K, L.*

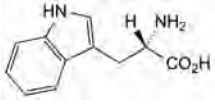

A. (*S*)-2-amino-3-(1*H*-indol-3-yl)propanoic acid (tryptophan),

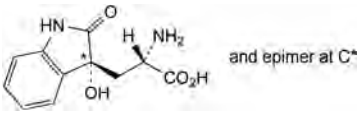


B. (*S*)-2-amino-3-[(3*RS*)-3-hydroxy-2-oxo-2,3-dihydro-1*H*-indol-3-yl]propanoic acid (dioxyindolylalanine),

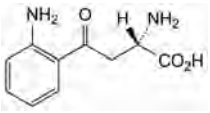

C. (*S*)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid (kynurenine),

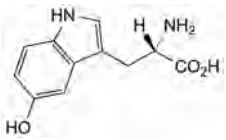

D. (*S*)-2-amino-3-(5-hydroxy-1*H*-indol-3-yl)propanoic acid (5-hydroxytryptophan),

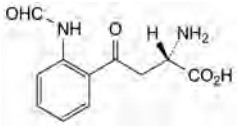

E. (*S*)-2-amino-4-[2-(formylamino)phenyl]-4-oxobutanoic acid (*N*-formylkynurenine),

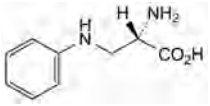

F. (*S*)-2-amino-3-(phenylamino)propanoic acid (3-phenylaminoalanine),

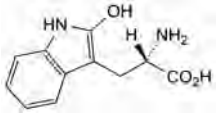

G. (*S*)-2-amino-3-(2-hydroxy-1*H*-indol-3-yl)propanoic acid (2-hydroxytryptophan),

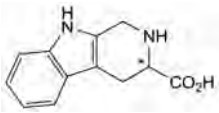

H. (3*RS*)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,

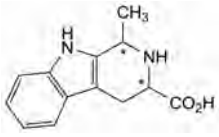

I. 1-methyl-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid,

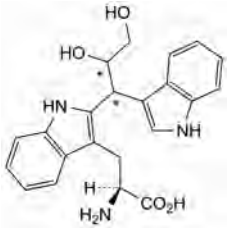

J. (*S*)-2-amino-3-[2-[2,3-dihydroxy-1-(1*H*-indol-3-yl)propyl]-1*H*-indol-3-yl]propanoic acid,

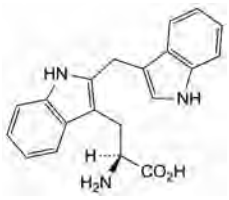

K. (*S*)-2-amino-3-[2-(1*H*-indol-3-ylmethyl)-1*H*-indol-3-yl]propanoic acid,

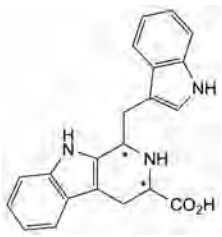

L. 1-(1*H*-indol-3-ylmethyl)-1,2,3,4-tetrahydro-9*H*-β-carboline-3-carboxylic acid.

**01/2017:1384**
**corrected 10.0**

# *N*-ACETYLTYROSINE

## *N*-Acetyltyrosinum

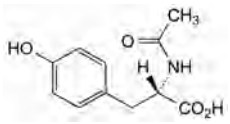

$C_{11}H_{13}NO_4$ $M_r$ 223.2
[537-55-3]

DEFINITION

(2*S*)-2-(Acetylamino)-3-(4-hydroxyphenyl)propanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in cyclohexane.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *N-acetyltyrosine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 80 mg of the substance to be examined in a mixture of 3 volumes of *glacial acetic acid R*, 3 volumes of *water R* and 94 volumes of *anhydrous ethanol R*, and dilute to 10 mL with the same mixture of solvents.
*Reference solution.* Dissolve 80 mg of *N-acetyltyrosine CRS* in a mixture of 3 volumes of *glacial acetic acid R*, 3 volumes of *water R* and 94 volumes of *anhydrous ethanol R*, and dilute to 10 mL with the same mixture of solvents.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *water R*, *glacial acetic acid R*, *ethyl acetate R* (10:15:75 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Solution S (see Tests) is strongly acid (*2.2.4*).

TESTS

**Solution S**. Dissolve 2.50 g in *water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 46 to + 49 (dried substance).
Dilute 10.0 mL of solution S to 25.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*
*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.
*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.
*Reference solution (b).* Dissolve 20.0 mg of *tyrosine CRS* (impurity A) in 2 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 20.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.
*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with mobile phase A.
*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 20.0 mL with the test solution.
*Column*:
– *size*: *l* = 0.15 m, Ø = 3 mm;
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 40 °C.
*Mobile phase*:
– *mobile phase A*: mix 1.0 mL of *phosphoric acid R* and 1000 mL of *water for chromatography R*;
– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 97 | 3 |
| 2 - 15 | 97 → 62 | 3 → 38 |

*Flow rate*: 0.7 mL/min.
*Detection*: spectrophotometer at 219 nm.
*Injection*: 2 µL of the test solution and reference solutions (a), (c) and (d).
*Relative retention* with reference to *N*-acetyltyrosine (retention time = about 6 min): impurity A = about 0.5.
*System suitability*: reference solution (d):
– *resolution*: minimum 5.0 between the principal peak and the peak due to impurity A.
*Limits*:
– *impurity A*: not more than 0.8 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.8 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: maximum 1.0 per cent;

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 20 mL with the same solvent.

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 0.100 g.

Prepare the standard using 0.2 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 20 ppm.

In a separating funnel, dissolve 0.5 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. The aqueous layer complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.180 g in 50 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 22.32 mg of $C_{11}H_{13}NO_4$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

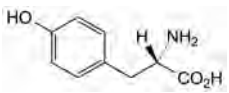

A. (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid (tyrosine),

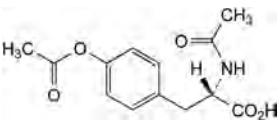

B. (2*S*)-2-(acetylamino)-3-[4-(acetoxy)phenyl]propanoic acid (diacetyltyrosine).

**01/2014:0968**

# ACICLOVIR

## Aciclovirum

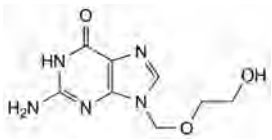

$C_8H_{11}N_5O_3$ $M_r$ 225.2
[59277-89-3]

DEFINITION

2-Amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in heptane. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *aciclovir CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 0.25 g in a 4 g/L solution of *sodium hydroxide R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *dimethyl sulfoxide R*, *water R* (20:80 *V/V*).

*Phosphate buffer solution pH 2.5*. Dissolve 3.48 g of *dipotassium hydrogen phosphate R* in 1000 mL of *water R* and adjust to pH 2.5 with *phosphoric acid R*.

*Phosphate buffer solution pH 3.1*. Dissolve 3.48 g of *dipotassium hydrogen phosphate R* in 1000 mL of *water R* and adjust to pH 3.1 with *phosphoric acid R*.

*Test solution*. Dissolve 25 mg of the substance to be examined in 5.0 mL of *dimethyl sulfoxide R* and dilute to 25.0 mL with *water R*.

*Reference solution (a)*. Dissolve 5 mg of *aciclovir for system suitability CRS* (containing impurities A, B, J, K, N, O and P) in 1 mL of *dimethyl sulfoxide R* and dilute to 5.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *aciclovir for peak identification 1 CRS* (containing impurities C and I) in 200 µL of *dimethyl sulfoxide R* and dilute to 1.0 mL with *water R*.

*Reference solution (d)*. Dissolve the contents of a vial of *aciclovir for peak identification 2 CRS* (containing impurities F and G) in 1.0 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, phosphate buffer solution pH 3.1 (1:99 *V/V*);

– *mobile phase B*: *acetonitrile R*, phosphate buffer solution pH 2.5 (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 27 | 100 → 80 | 0 → 20 |
| 27 - 40 | 80 | 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *aciclovir for peak identification 1 CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and I; use the chromatogram supplied with *aciclovir for peak identification 2 CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, F, G, J, K, N, O and P.

*Relative retention* with reference to aciclovir (retention time = about 13 min): impurity B = about 0.4; impurity P = about 0.7; impurity C = about 0.9; impurity N = about 1.37; impurities O and Q = about 1.42; impurity I = about 1.57; impurity J = about 1.62; impurity F = about 1.7; impurity A = about 1.8; impurities K and R = about 2.5; impurity G = about 2.6.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to impurity C and aciclovir in the chromatogram obtained with reference solution (c); minimum 1.5 between the peaks due to impurities F and A and minimum 1.5 between the peaks due to impurities K and G in the chromatogram obtained with reference solution (d).

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity I by 1.5;

– *impurity B*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);

– *sum of impurities O and Q*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *sum of impurities K and R*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurities A, G, J, N, P*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurities C, F, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);

– *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14, Method D*): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.150 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 22.52 mg of $C_8H_{11}N_5O_3$.

IMPURITIES

*Specified impurities: A, B, C, F, G, I, J, K, N, O, P, Q, R.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *L, M*.

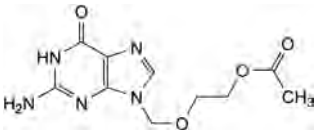

A. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl acetate,

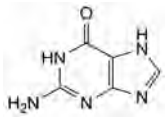

B. 2-amino-1,7-dihydro-6*H*-purin-6-one (guanine),

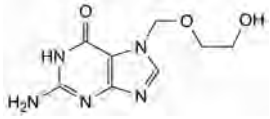

C. 2-amino-7-[(2-hydroxyethoxy)methyl]-1,7-dihydro-6*H*-purin-6-one,

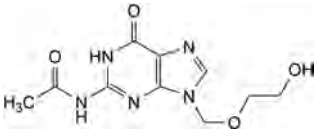

F. *N*-[9-[(2-hydroxyethoxy)methyl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl]acetamide,

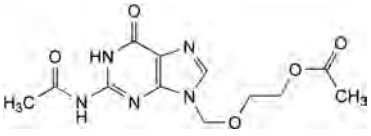

G. 2-[[2-(acetylamino)-6-oxo-1,6-dihydro-9*H*-purin-9-yl]methoxy]ethyl acetate,

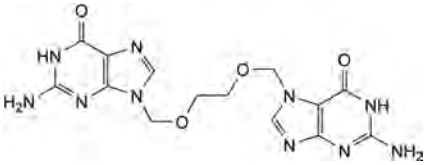

I. 2-amino-7-[[2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethoxy]methyl]-1,7-dihydro-6*H*-purin-6-one,

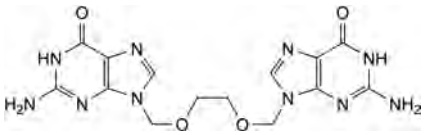

J. 9,9′-[ethylenebis(oxymethylene)]bis(2-amino-1,9-dihydro-6*H*-purin-6-one),

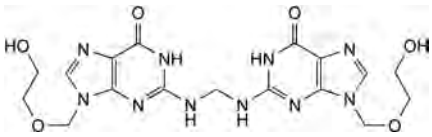

K. 2,2′-(methylenediimino)bis[9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one],

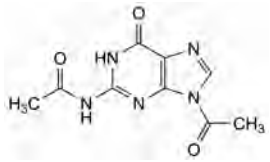

L. *N*-(9-acetyl-6-oxo-6,9-dihydro-1*H*-purin-2-yl)acetamide ($N^2$,9-diacetylguanine),

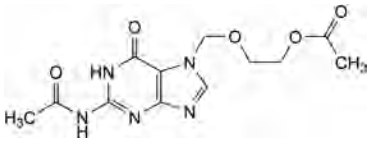

M. 2-[[2-(acetylamino)-6-oxo-1,6-dihydro-7*H*-purin-7-yl]methoxy]ethyl acetate,

N. unknown structure,

O. unknown structure,

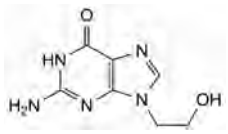

P. 2-amino-9-(2-hydroxyethyl)-1,9-dihydro-6*H*-purin-6-one,

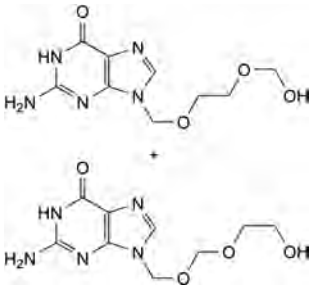

Q. mixture of 2-amino-9-[[2-(hydroxymethoxy)ethoxy]methyl]-1,9-dihydro-6*H*-purin-6-one and 2-amino-9-[[2-(hydroxyethoxy)methoxy]methyl]-1,9-dihydro-6*H*-purin-6-one,

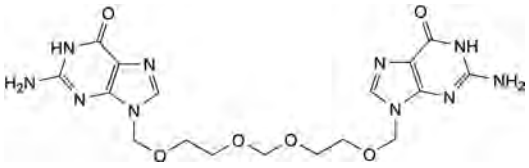

R. 9,9′-[methylenebis(oxyethyleneoxymethylene)]bis(2-amino-1,9-dihydro-6*H*-purin-6-one).

**07/2018:1385**

# ACITRETIN

## Acitretinum

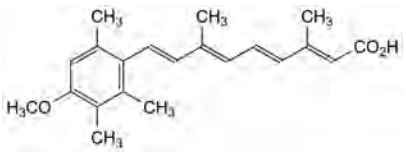

$C_{21}H_{26}O_3$ $M_r$ 326.4
[55079-83-9]

DEFINITION

(2*E*,4*E*,6*E*,8*E*)-9-(4-Methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or greenish-yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in tetrahydrofuran, slightly soluble in acetone and in ethanol (96 per cent), very slightly soluble in cyclohexane.

It is sensitive to air, heat and light, especially in solution.

It shows polymorphism (*5.9*).

*Carry out all operations as rapidly as possible and avoid exposure to actinic light; use freshly prepared solutions.*

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *acitretin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R* by heating under reflux; filter, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 5 mL of *tetrahydrofuran R* and dilute to 100.0 mL with *anhydrous ethanol R*.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 25.0 mL with *anhydrous ethanol R*.

*Reference solution (a).* Dissolve 25.0 mg of *acitretin CRS* in 5 mL of *tetrahydrofuran R* and dilute to 100.0 mL with *anhydrous ethanol R*. Dilute 10.0 mL of the solution to 25.0 mL with *anhydrous ethanol R*.

*Reference solution (b).* Dissolve 1 mg of *tretinoin CRS* in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent. Mix 5.0 mL of the solution with 2.5 mL of reference solution (a) and dilute to 100.0 mL with *anhydrous ethanol R*.

*Reference solution (c).* Dilute 1.0 mL of the test solution (a) to 100.0 mL with *anhydrous ethanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Reference solution (d).* Dissolve 2.5 mg of *acitretin for impurity A identification CRS* in 0.5 mL of *tetrahydrofuran R* and dilute to 10.0 mL with *anhydrous ethanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography for separation of polycyclic aromatic hydrocarbons R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: 0.3 per cent *V/V* solution of *glacial acetic acid R* in a mixture of 8 volumes of *water for chromatography R* and 92 volumes of *anhydrous ethanol R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 360 nm.

*Autosampler*: set at 4 °C.

*Injection*: 10 µL of test solution (a) and reference solutions (b), (c) and (d).

*Run time*: 2.5 times the retention time of acitretin.

*Identification of impurities*: use the chromatogram supplied with *acitretin for impurity A identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to acitretin (retention time = about 6 min): impurity A = about 0.8; tretinoin = about 0.85.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to tretinoin and acitretin.

*Calculation of percentage contents*:

– for each impurity, use the concentration of acitretin in reference solution (c).

*Limits*:

– *impurity A*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 100 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{21}H_{26}O_3$ taking into account the assigned content of *acitretin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of inert gas.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

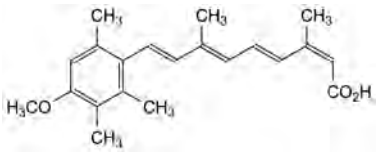

A. (2*Z*,4*E*,6*E*,8*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid,

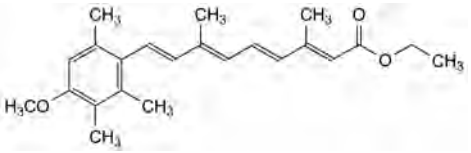

B. ethyl (2*E*,4*E*,6*E*,8*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoate.

**01/2017:2445**

# ADAPALENE

## Adapalenum

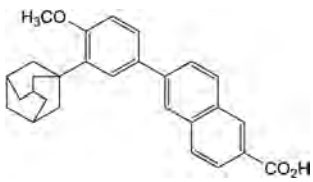

$C_{28}H_{28}O_3$ $M_r$ 412.5

[106685-40-9]

DEFINITION

6-(4-Methoxy-3-tricyclo[3.3.1.1$^{3,7}$]dec-1-ylphenyl)naphthalene-2-carboxylic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in tetrahydrofuran, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *adapalene CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.2 g in *tetrahydrofuran R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *tetrahydrofuran R*, *acetonitrile R*, *water R* (20:37:43 *V/V/V*).

*Test solution (a)*. Dissolve 40.0 mg of the substance to be examined in 10 mL of *tetrahydrofuran R*, add 7 mL of the solvent mixture and dilute to 20.0 mL with *tetrahydrofuran R*.

*Test solution (b)*. Dissolve 20.0 mg of the substance to be examined in 50 mL of *tetrahydrofuran R*, add 35 mL of the solvent mixture and dilute to 100.0 mL with *tetrahydrofuran R*. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *tetrahydrofuran R*. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2.4 mg of *adapalene impurity C CRS* in 2 mL of *tetrahydrofuran R* and dilute to 20.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with the solvent mixture. To 2.0 mL of this solution add 2.0 mL of reference solution (a) and dilute to 20.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *adapalene for peak identification CRS* (containing impurities A, C and D) in 0.5 mL of *tetrahydrofuran R* and dilute to 1.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 20.0 mg of *adapalene CRS* in 50 mL of *tetrahydrofuran R*, add 35 mL of the solvent mixture and dilute to 100.0 mL with *tetrahydrofuran R*. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped phenylsilyl silica gel for chromatography R* (5 µm) with a carbon loading of 7.5 per cent;
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *glacial acetic acid R*, *water R* (0.1:100 *V/V*);
- *mobile phase B*: *tetrahydrofuran R*, *acetonitrile R* (35:65 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.5 | 50 | 50 |
| 2.5 - 40 | 50 → 28 | 50 → 72 |
| 40 - 42 | 28 | 72 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 25 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *adapalene for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, C and D.

*Relative retention* with reference to adapalene (retention time = about 20 min): impurity A = about 0.3; impurity C = about 0.9; impurity D = about 1.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.5 between the peaks due to impurity C and adapalene;
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity C.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity C = 7; impurity D = 1.4;
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of adapalene from the declared content of *adapalene CRS*.

IMPURITIES

*Specified impurities: A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

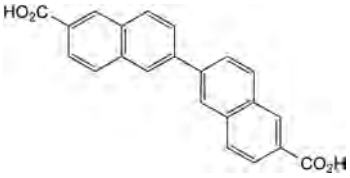


A. 2,2′-binaphthalene-6,6′-dicarboxylic acid,

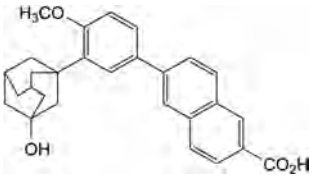


B. 6-[3-(3-hydroxytricyclo[3.3.1.1$^{3,7}$]dec-1-yl)-4-methoxyphenyl]naphthalene-2-carboxylic acid,

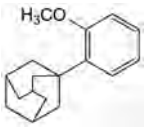


C. 1-(2-methoxyphenyl)tricyclo[3.3.1.1$^{3,7}$]decane,

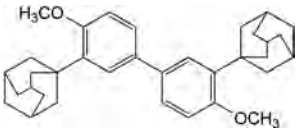


D. 1,1′-[4,4′-bis(methoxy)biphenyl-3,3′-diyl]bis(tricyclo[3.3.1.1$^{3,7}$]decane).

**01/2017:0800**

# ADENINE

## Adeninum

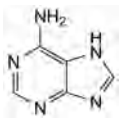


$C_5H_5N_5$ $M_r$ 135.1

[73-24-5]

DEFINITION

Adenine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7*H*-purin-6-amine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water and in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *adenine CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 1 g add 3.5 mL of *propionic anhydride R* and boil for 15 min with stirring. Cool. To the resulting crystalline mass add 15 mL of *light petroleum R* and heat to boiling with vigorous stirring. Cool and filter. Wash the precipitate with two quantities, each of 5 mL, of *light petroleum R*. Dissolve the precipitate in 10 mL of *water R* and boil for 1 min. Filter the mixture at 30 °C to 40 °C. Allow to cool. Filter, and dry the precipitate at 100 °C to 105 °C for 1 h. The melting point (*2.2.14*) of the precipitate is 237 °C to 241 °C.

TESTS

**Solution S**. Suspend 2.5 g in 50 mL of *distilled water R* and boil for 3 min. Cool and dilute to 50 mL with *distilled water R*. Filter. Use the filtrate as solution S.

**Appearance of solution**. Dissolve 0.5 g in *dilute hydrochloric acid R* and dilute to 50 mL with the same acid. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is blue. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is yellow.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *dilute acetic acid R*, with heating if necessary, and dilute to 10 mL with the same acid.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *dilute acetic acid R*.

*Reference solution (a).* Dissolve 10 mg of *adenine CRS* in *dilute acetic acid R*, with heating if necessary, and dilute to 10 mL with the same acid.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 20 mL with *dilute acetic acid R*.

*Reference solution (c).* Dissolve 10 mg of *adenine CRS* and 10 mg of *adenosine R* in *dilute acetic acid R*, with heating if necessary, and dilute to 10 mL with the same acid.

Apply to the plate 5 µL of each solution. Develop over a path of 12 cm using a mixture of 20 volumes of *concentrated ammonia R*, 40 volumes of *ethyl acetate R* and 40 volumes of *propanol R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

**Chlorides** (*2.4.4*). To 10 mL of solution S add 1 mL of *concentrated ammonia R* and 3 mL of *silver nitrate solution R2*. Filter. Wash the precipitate with a little *water R* and dilute the filtrate to 15 mL with *water R*. The solution complies with the limit test for chlorides (100 ppm). When carrying out the test, add 2 mL of *dilute nitric acid R* instead of 1 mL of *dilute nitric acid R*.

**Sulfates** (*2.4.13*). Dilute 10 mL of solution S to 15 mL with *distilled water R*. The solution complies with the limit test for sulfates (300 ppm).

**Ammonium**. Prepare a cell consisting of two watch-glasses 60 mm in diameter placed edge to edge. To the inner wall of the upper watch-glass stick a piece of *red litmus paper R* 5 mm square and wetted with a few drops of *water R*. Finely powder the substance to be examined, place 0.5 g in the lower watch-glass and suspend in 0.5 mL of *water R*. To the suspension add 0.30 g of *heavy magnesium oxide R*. Briefly triturate with a glass rod. Immediately close the cell by putting the two watch-glasses together. Heat at 40 °C for 15 min. The litmus paper is not more intensely blue coloured than a standard prepared at the same time and in the same manner using 0.05 mL of *ammonium standard solution (100 ppm* $NH_4$*) R*, 0.5 mL of *water R* and 0.30 g of *heavy magnesium oxide R* (10 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in a mixture of 20 mL of *acetic anhydride R* and 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.51 mg of $C_5H_5N_5$.

**01/2009:1486**

# ADENOSINE

## Adenosinum

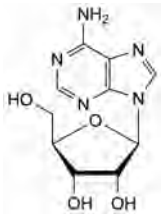


$C_{10}H_{13}N_5O_4$ $M_r$ 267.2

[58-61-7]

DEFINITION

9-β-D-Ribofuranosyl-9*H*-purin-6-amine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in hot water, practically insoluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute mineral acids.

mp: about 234 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *adenosine CRS*.

TESTS

**Solution S**. Suspend 5.0 g in 100 mL of *distilled water R* and heat to boiling. Allow to cool, filter with the aid of vacuum and dilute to 100 mL with *distilled water R*.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S, add 0.1 mL of *bromocresol purple solution R* and 0.1 mL of *0.01 M hydrochloric acid*. The solution is yellow. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is violet-blue.

**Specific optical rotation** (*2.2.7*): – 45 to – 49 (dried substance).

Dissolve 1.25 g in *1 M hydrochloric acid* and dilute to 50.0 mL with the same acid. Examine within 10 min of preparing the solution.

**Related substances**

Liquid chromatography (*2.2.29*).

*Solvent mixture*. Dissolve 6.8 g of *potassium hydrogen sulfate R* and 3.4 g of *tetrabutylammonium hydrogen sulfate R* in *water R*, adjust to pH 6.5 with a 60 g/L solution of *potassium hydroxide R* and dilute to 1000 mL with the same solvent. Use a freshly prepared solvent mixture.

*Test solution*. Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *adenine R* (impurity A) and 5 mg of *inosine R* (impurity G) in the mobile phase and dilute to 50 mL with the mobile phase. Dilute 4 mL of this solution to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, solvent mixture (40:60 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of adenosine.

*Relative retention* with reference to adenosine (retention time = about 13 min): impurity A = about 0.3; impurity G = about 0.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and G.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity G = 1.4;
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Ammonium** (*2.4.1, Method B*): maximum 10 ppm, determined on 0.5 g.

Prepare the standard using 5 mL of *ammonium standard solution (1 ppm $NH_4$) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g, warming slightly if necessary, in a mixture of 20 mL of *acetic anhydride R* and 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.72 mg of $C_{10}H_{13}N_5O_4$.

IMPURITIES

*Specified impurities*: *A, G*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, H*.

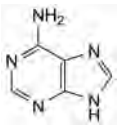


A. 7*H*-purin-6-amine (adenine),

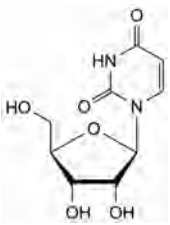


F. 1-β-D-ribofuranosylpyrimidine-2,4(1*H*,3*H*)-dione (uridine),

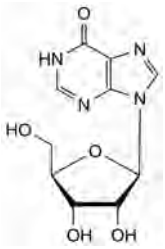


G. 9-β-D-ribofuranosyl-1,9-dihydro-6*H*-purin-6-one (inosine),

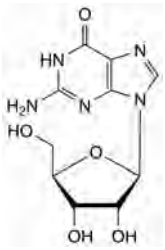

H. 2-amino-9-β-D-ribofuranosyl-1,9-dihydro-6*H*-purin-6-one (guanosine).

**01/2017:1586**

# ADIPIC ACID

## Acidum adipicum

$C_6H_{10}O_4$ $M_r$ 146.1
[124-04-9]

DEFINITION

Hexanedioic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in boiling water, freely soluble in ethanol (96 per cent) and in methanol, soluble in acetone.

IDENTIFICATION

A. Melting point (*2.2.14*): 151 °C to 154 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *adipic acid CRS*.

TESTS

**Solution S**. Dissolve 5.0 g with heating in *distilled water R* and dilute to 50 mL with the same solvent. Allow to cool and to crystallise. Filter through a sintered-glass filter (40) (*2.1.2*). Wash the filter with *distilled water R*. Collect the filtrate and the washings until a volume of 50 mL is obtained.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20 mg of *glutaric acid R* in 1.0 mL of the test solution and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase, dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 $m^2$/g and a pore size of 10 nm,
- *temperature*: 30 °C.

*Mobile phase*: mix 3 volumes of *acetonitrile R* and 97 volumes of a 24.5 g/L solution of *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 209 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of adipic acid.

*System suitability*: reference solution (a):
- *resolution*: minimum 9.0 between the peaks due to glutaric acid and adipic acid.

*Limits*:
- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Nitrates**: maximum 30 ppm.

To 1 mL of solution S add 2 mL of *concentrated ammonia R*, 0.5 mL of a 10 g/L solution of *manganese sulfate R*, 1 mL of a 10 g/L solution of *sulfanilamide R* and dilute to 20 mL with *water R*. Add 0.10 g of *zinc powder R* and cool in iced water for 30 min; shake from time to time. Filter and cool 10 mL of the filtrate in iced water. Add 2.5 mL of *hydrochloric acid R1* and 1 mL of a 10 g/L solution of *naphthylethylenediamine dihydrochloride R*. Allow to stand at room temperature. After 15 min the mixture is not more intensely coloured than a standard prepared at the same time and in the same manner, using 1.5 mL of *nitrate standard solution (2 ppm $NO_3$) R* instead of 1 mL of solution S. The test is invalid if a blank solution prepared at the same time and in the same manner, using 1 mL of *water R* instead of 1 mL of solution S, is more intensely coloured than a 2 mg/L solution of *potassium permanganate R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 3 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent.

Melt 1.0 g completely over a gas burner, then ignite the melted substance with the burner. After ignition, lower or remove the flame in order to prevent the substance from boiling and keep it burning until completely carbonised. Carry out the test for sulfated ash using the residue.

ASSAY

Dissolve 60.0 mg in 50 mL of *water R*. Add 0.2 mL of *phenolphthalein solution R* and titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 7.31 mg of $C_6H_{10}O_4$.

IMPURITIES

A. pentanedioic acid (glutaric acid),

B. butanedioic acid (succinic acid),

C. heptanedioic acid (pimelic acid).

07/2008:2303

# ADRENALINE

## Adrenalinum

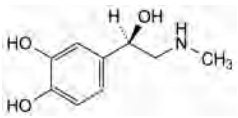

$C_9H_{13}NO_3$ $M_r$ 183.2
[51-43-4]

DEFINITION

4-[(1*R*)-1-Hydroxy-2-(methylamino)ethyl]benzene-1,2-diol.

Synthetic product.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder, becoming coloured on exposure to air and light.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It dissolves in hydrochloric acid.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *adrenaline CRS*.

B. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 1.000 g in a 25.75 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solvent. Examine the solution immediately.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 50.0 to − 54.0 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Solvent mixture A*. Dissolve 5.0 g of *potassium dihydrogen phosphate R* and 2.6 g of *sodium octanesulfonate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent (it is usually necessary to stir for at least 30 min to achieve complete dissolution). Adjust to pH 2.8 with *phosphoric acid R*.

*Solvent mixture B*: *acetonitrile R1*, solvent mixture A (13:87 *V/V*).

*Test solution*. Dissolve 40 mg of the substance to be examined in 5 mL of *0.1 M hydrochloric acid* and dilute to 50.0 mL with solvent mixture B.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solvent mixture B. Dilute 1.0 mL of this solution to 10.0 mL with solvent mixture B.

*Reference solution (b)*. Dissolve 1.5 mg of *noradrenaline tartrate CRS* (impurity B) and 1.5 mg of *adrenalone hydrochloride R* (impurity C) in solvent mixture B, add 1.0 mL of the test solution and dilute to 100 mL with solvent mixture B.

*Reference solution (c)*. Dissolve the contents of a vial of *adrenaline impurity mixture CRS* (containing impurities D and E) in 1.0 mL of the blank solution.

*Reference solution (d)*. Dissolve 4 mg of *adrenaline with impurity F CRS* in 0.5 mL of *0.1 M hydrochloric acid* and dilute to 5 mL with solvent mixture B.

*Blank solution*: *0.1 M hydrochloric acid*, solvent mixture B (1:9 *V/V*).

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, solvent mixture A (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R1*, solvent mixture A (45:55 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 92 → 50 | 8 → 50 |
| 15 - 20 | 50 → 92 | 50 → 8 |
| 20 - 25 | 92 | 8 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *adrenaline impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and E; use the chromatogram supplied with *adrenaline with impurity F CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity F.

*Relative retention* with reference to adrenaline (retention time = about 4 min): impurity F = about 0.2; impurity B = about 0.8; impurity C = about 1.3; impurity D = about 3.3; impurity E = about 3.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.0 between the peaks due to impurity B and adrenaline.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.7; impurity E = 0.6;
- *impurities B, C, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 18 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.32 mg of $C_9H_{13}NO_3$.

STORAGE

Under nitrogen, protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, F.*

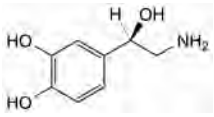

B. (1*R*)-2-amino-1-(3,4-dihydroxyphenyl)ethanol (noradrenaline),

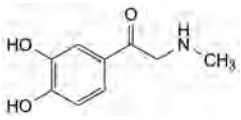

C. 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanone (adrenalone),

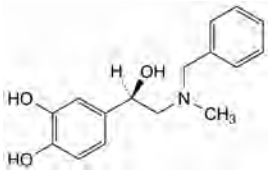

D. 4-[(1*R*)-2-(benzylmethylamino)-1-hydroxyethyl]benzene-1,2-diol,

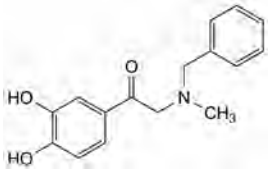

E. 2-(benzylmethylamino)-1-(3,4-dihydroxyphenyl)ethanone,

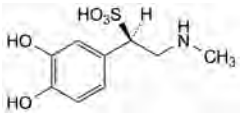

F. (1*R*)-1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanesulfonic acid.

**01/2008:0254**

# ADRENALINE TARTRATE

## Adrenalini tartras

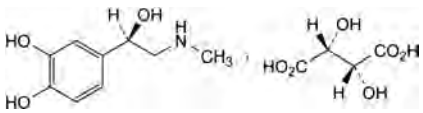

$C_{13}H_{19}NO_9$ $M_r$ 333.3

[51-42-3]

DEFINITION

(1*R*)-1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanol hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or greyish-white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 5 g in 50 mL of a 5 g/L solution of *sodium metabisulfite R* and make alkaline by addition of *ammonia R*. Keep the mixture at room temperature for at least 15 min and filter. Reserve the filtrate for identification test C. Wash the precipitate with 3 quantities, each of 10 mL, of *methanol R*. Dry at 80 °C. The specific optical rotation (*2.2.7*) of the residue (adrenaline base) is − 53.5 to − 50, determined using a 20.0 g/L solution in *0.5 M hydrochloric acid*.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of adrenaline base prepared as described under identification test A.

*Comparison*: use adrenaline base prepared as described under identification test A from 50 mg of *adrenaline tartrate CRS* dissolved in 5 mL of a 5 g/L solution of *sodium metabisulfite R*. Keep the mixture at room temperature for at least 30 min. Filter through a sintered-glass filter (*2.1.2*).

C. 0.2 mL of the filtrate obtained in identification test A gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent. Examine the solution immediately.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Solvent mixture A*. Dissolve 5.0 g of *potassium dihydrogen phosphate R* and then 2.6 g of *sodium octanesulfonate R* in *water for chromatography R*, and dilute to 1000 mL with the same solvent (it is usually necessary to stir for at least 30 min to achieve complete dissolution). Adjust to pH 2.8 with *phosphoric acid R*.

*Solvent mixture B*: *acetonitrile R1*, solvent mixture A (130:870 *V/V*).

*Test solution*. Dissolve 75 mg of the substance to be examined in 5 mL of *0.1 M hydrochloric acid* and dilute to 50 mL with solvent mixture B.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solvent mixture B. Dilute 1.0 mL of this solution to 10.0 mL with solvent mixture B.

*Reference solution (b)*. Dissolve 1.5 mg of *noradrenaline tartrate CRS* (impurity B) and 1.5 mg of *adrenalone hydrochloride R* (impurity C) in solvent mixture B, add 1.0 mL of the test solution and dilute to 100.0 mL with solvent mixture B.

*Reference solution (c)*. Dissolve the contents of a vial of *adrenaline impurity mixture CRS* (impurities D and E) in 0.1 mL of *0.1 M hydrochloric acid* and 0.9 mL of solvent mixture B.

*Reference solution (d)*. Dissolve 7.5 mg of *adrenaline tartrate with impurity A CRS* in 0.5 mL of *0.1 M hydrochloric acid* and dilute to 5.0 mL with solvent mixture B.

*Blank solution*: *0.1 M hydrochloric acid*, solvent mixture B (1:9 *V/V*).

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, solvent mixture A (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R1*, solvent mixture A (45:55 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 92 → 50 | 8 → 50 |
| 15 - 20 | 50 → 92 | 50 → 8 |
| 20 - 25 | 92 | 8 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *adrenaline impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and E; use the chromatogram supplied with *adrenaline tartrate with impurity A CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to adrenaline (retention time = about 4 min): impurity B = about 0.8; impurity C = about 1.3; impurity A = about 3.2; impurity D = about 3.3; impurity E = about 3.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity B and adrenaline.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.7; impurity E = 0.6;

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *impurities B, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurities D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 18 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*, heating gently if necessary. Titrate with *0.1 M perchloric acid* until a bluish-green colour is obtained, using 0.1 mL of *crystal violet solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 33.33 mg of $C_{13}H_{19}NO_9$.

STORAGE

In an airtight container, or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

A. unknown structure,

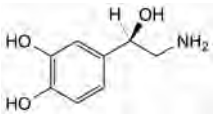

B. (1*R*)-2-amino-1-(3,4-dihydroxyphenyl)ethanol (noradrenaline),

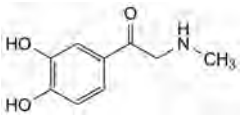

C. 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanone (adrenalone),

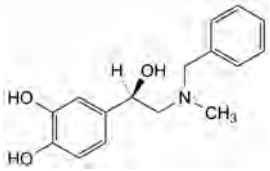

D. 4-[(1*R*)-2-(benzylmethylamino)-1-hydroxyethyl]benzene-1,2-diol,

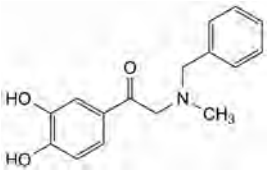

E. 2-(benzylmethylamino)-1-(3,4-dihydroxyphenyl)ethanone.

**01/2009:1238**

# AIR, MEDICINAL

## Aer medicinalis

DEFINITION

Compressed ambient air.

*Content*: 20.4 per cent *V/V* to 21.4 per cent *V/V* of oxygen ($O_2$).

CHARACTERS

*Appearance*: colourless gas.

*Solubility*: at 20 °C at a pressure of 101 kPa, 1 volume dissolves in about 50 volumes of water.

PRODUCTION

**Carbon dioxide**: maximum 500 ppm *V/V*, determined using an infrared analyser (*2.5.24*).

*Gas to be examined.* Filter the substance to be examined to avoid stray light phenomena.

*Reference gas (a).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing less than 1 ppm *V/V* of *carbon dioxide R1*.

*Reference gas (b).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing 500 ppm *V/V* of *carbon dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using an infrared analyser (*2.5.25*).

*Gas to be examined.* Filter the substance to be examined to avoid stray light phenomena.

*Reference gas (a).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing less than 1 ppm *V/V* of *carbon monoxide R*.

*Reference gas (b).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing 5 ppm *V/V* of *carbon monoxide R*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

**Sulfur dioxide**: maximum 1 ppm *V/V*, determined using an ultraviolet fluorescence analyser (Figure 1238.-1).

The apparatus consists of the following:

- a system generating ultraviolet radiation with a wavelength of 210 nm, made up of an ultraviolet lamp, a collimator, and a selective filter; the beam is blocked periodically by a chopper rotating at high speeds;
- a reaction chamber, through which flows the gas to be examined;
- a system that detects radiation emitted at a wavelength of 350 nm, made up of a selective filter, a photomultiplier tube and an amplifier.

*Gas to be examined.* Filter the substance to be examined.

*Reference gas (a).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*.

*Reference gas (b).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing 0.5 ppm *V/V* to 2 ppm *V/V* of *sulfur dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of sulfur dioxide in the gas to be examined.

**Oil**: maximum 0.1 mg/m$^3$, determined using an oil detector tube (*2.1.6*), when an oil-lubricated compressor is used for the production.

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total, determined using a chemiluminescence analyser (*2.5.26*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a).* Use a mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing less than 0.05 ppm *V/V* of nitrogen monoxide and nitrogen dioxide.

*Reference gas (b).* Use a mixture of 2 ppm *V/V* of *nitrogen monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide in the gas to be examined.

**Water**: maximum 67 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*), except where the competent authority decides that the following limit applies to medicinal air generated on-site and distributed in pipe-line systems operating at a pressure not greater than 10 bar and a temperature not less than 5 °C: maximum 870 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

**Assay**. Determine the concentration of oxygen in air using a paramagnetic analyser (*2.5.27*).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B.*

A. In a conical flask containing the substance to be examined, place a glowing wood splinter. The splinter remains glowing.

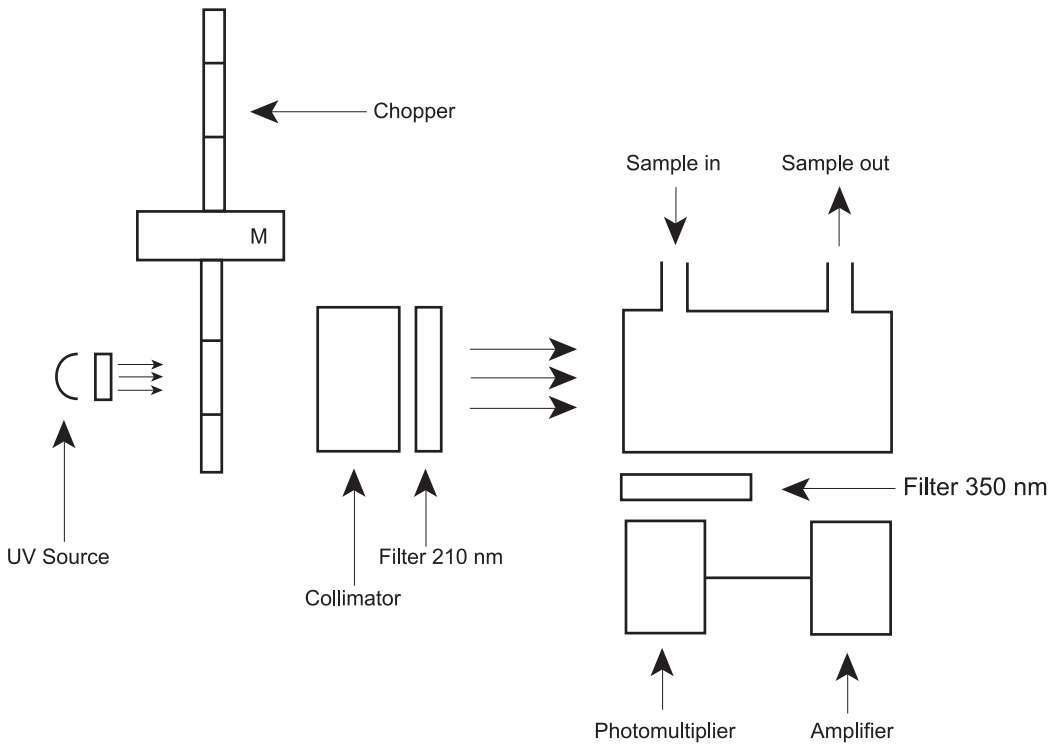


Figure 1238.-1. – *UV fluorescence analyser*

B. Use a gas burette (Figure 1238.-2) of 25 mL capacity in the form of a chamber in the middle of which is a tube graduated in 0.2 per cent between 19.0 per cent and 23.0 per cent, and isolated at each end by a tap with a conical barrel. The lower tap is joined to a tube with an olive-shaped nozzle and is used to introduce the gas into the apparatus. A cylindrical funnel above the upper tap is used to introduce the absorbent solution. Wash the burette with *water R* and dry. Open the 2 taps. Connect the nozzle to the source of the gas to be examined and set the flow rate to 1 L/min. Flush the burette by passing the gas to be examined through it for 1 min. Close the lower tap of the burette and immediately afterwards the upper tap. Rapidly disconnect the burette from the source of the gas to be examined. Rapidly give a half turn to the upper tap to eliminate any excess pressure in the burette. Keeping the burette vertical, fill the funnel with a freshly prepared mixture of 21 mL of a 560 g/L solution of *potassium hydroxide R* and 130 mL of a 200 g/L solution of *sodium dithionite R*. Open the upper tap slowly. The solution absorbs the oxygen and enters the burette. Allow to stand for 10 min without shaking. Read the level of the liquid meniscus on the graduated part of the burette. This figure represents the percentage *V/V* of oxygen. The value read is 20.4 to 21.4.

C. It complies with the limits of the assay.

TESTS

**Carbon dioxide**: maximum 500 ppm *V/V*, determined using a carbon dioxide detector tube (*2.1.6*).

**Sulfur dioxide**: maximum 1 ppm *V/V*, determined using a sulfur dioxide detector tube (*2.1.6*).

**Oil**: maximum 0.1 mg/m$^3$, determined using an oil detector tube (*2.1.6*), when an oil-lubricated compressor is used for the production.

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V*, determined using a nitrogen monoxide and nitrogen dioxide detector tube (*2.1.6*).

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube (*2.1.6*).

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*), except where the competent authority decides that the following limit applies to medicinal air generated on-site and distributed in pipe-line systems operating at a pressure not greater than 10 bar and a temperature not less than 5 °C: maximum 870 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

STORAGE

As a gas, in suitable containers complying with the legal regulations or as a gas supplied by a pipe network.

LABELLING

Where applicable, the label states the production method, as regards to the use of an oil - lubricated compression.

IMPURITIES

A. $CO_2$: carbon dioxide,

B. $SO_2$: sulfur dioxide,

C. NO: nitrogen monoxide,

D. $NO_2$: nitrogen dioxide,

E. oil,

F. CO: carbon monoxide,

G. $H_2O$: water.

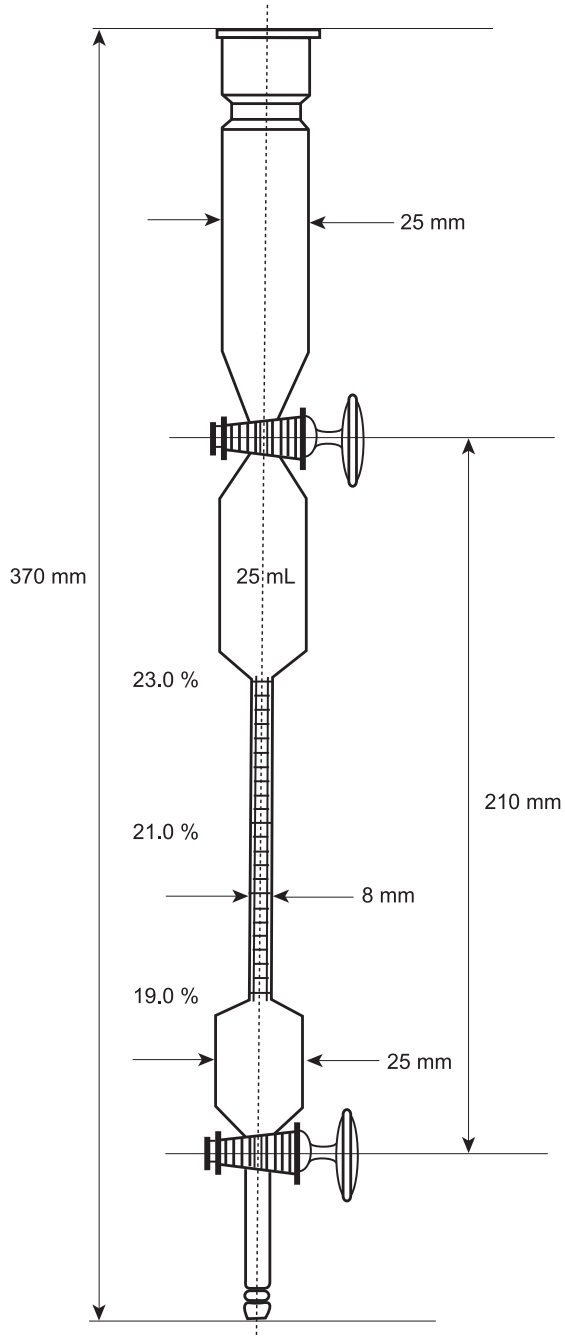


Figure 1238.-2. – *Gas burette*

**01/2008:1684**

# AIR, SYNTHETIC MEDICINAL

## Aer medicinalis artificiosus

DEFINITION

Mixture of *Nitrogen (1247)* and *Oxygen (0417)*.

*Content*: 95.0 per cent to 105.0 per cent of the nominal value which is between 21.0 per cent *V/V* to 22.5 per cent *V/V* of oxygen ($O_2$).

CHARACTERS

Colourless and odourless gas.

*Solubility*: at a temperature of 20 °C and a pressure of 101 kPa, 1 volume dissolves in about 50 volumes of water.

PRODUCTION

**Water** (*2.5.28*): maximum 67 ppm *V/V*.

**Assay** (*2.5.27*). Carry out the determination of oxygen in gases.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B.*

A. In a conical flask containing the substance to be examined, place a glowing splinter of wood. The splinter remains glowing.

B. Use a gas burette (Figure 1684.-1) of 25 mL capacity in the form of a chamber, in the middle of which is a tube graduated in 0.2 per cent between 19.0 per cent and 23.0 per cent, and isolated at each end by a tap with a conical barrel. The lower tap is joined to a tube with an olive-shaped nozzle and is used to introduce the gas into the apparatus. A cylindrical funnel above the upper tap is used to introduce the absorbent solution. Wash the burette with *water R* and dry. Open both taps. Connect the nozzle to the source of the substance to be examined and set the flow rate to 1 L/min. Flush the burette by passing the substance to be examined through it for 1 min. Close the lower tap of the burette and immediately afterwards the upper tap. Rapidly disconnect the burette from the source of the substance to be examined. Rapidly give a half turn of the upper tap to eliminate any excess pressure in the burette. Keeping the burette vertical, fill the funnel with a freshly prepared mixture of 21 mL of a 560 g/L solution of *potassium hydroxide R* and 130 mL of a 200 g/L solution of *sodium dithionite R*. Open the upper tap slowly. The solution absorbs the oxygen and enters the burette. Allow to stand for 10 min without shaking. Read the level of the liquid meniscus on the graduated part of the burette. This figure represents the percentage *V/V* of oxygen. The value read is 95.0 per cent to 105.0 per cent of the nominal value.

C. It complies with the limits of the assay.

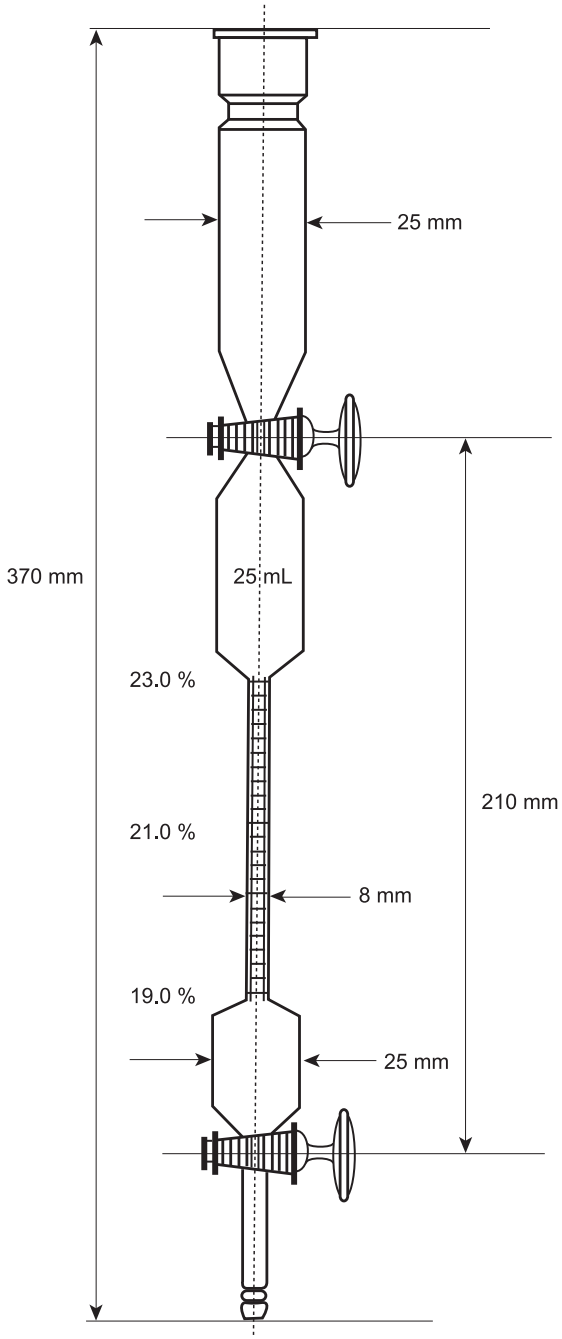


Figure 1684.-1.– *Gas burette*

TESTS

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

STORAGE

As a compressed gas in suitable containers complying with the legal regulations or as a compressed gas supplied by a pipe network, after mixing of the components.

LABELLING

The label states the nominal content of $O_2$ in per cent *V/V*.

IMPURITIES

A. $H_2O$: water.

**01/2017:0752**

# ALANINE

## Alaninum

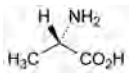


$C_3H_7NO_2$ $M_r$ 89.1

[56-41-7]

DEFINITION

(2*S*)-2-Aminopropanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *alanine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.
*Reference solution*. Dissolve 10 mg of *alanine CRS* in *water R* and dilute to 50 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.5 g in a mixture of 0.25 mL of *hydrochloric acid R1*, 0.5 mL of a 100 g/L solution of *sodium nitrite R* and 1 mL of *water R*. Shake; gas is given off. Add 2 mL of *dilute sodium hydroxide solution R*, followed by 0.25 mL of *iodinated potassium iodide solution R*. After about 30 min, a yellow precipitate is formed.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dilute 10 mL of solution S to 20 mL with *water R*.

**Specific optical rotation** (*2.2.7*): + 13.5 to + 15.5 (dried substance).

Dissolve 2.50 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:
- for any ninhydrin-positive substance detected at 570 nm, use the concentration of alanine in reference solution (a);
- for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:
- *any ninhydrin-positive substance*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:
- *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 8.91 mg of $C_3H_7NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B*.

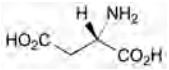

A. (2*S*)-2-aminobutanedioic acid (aspartic acid),

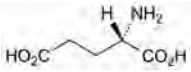

B. (2*S*)-2-aminopentanedioic acid (glutamic acid).

**04/2018:1386**
**corrected 9.6**

# ALBENDAZOLE

## Albendazolum

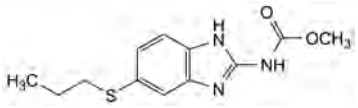

$C_{12}H_{15}N_3O_2S$ $M_r$ 265.3
[54965-21-8]

DEFINITION

Methyl *N*-[5-(propylsulfanyl)-1*H*-benzimidazol-2-yl]carbamate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous formic acid, very slightly soluble in methylene chloride, practically insoluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *albendazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.10 g in a mixture of 10 volumes of *anhydrous formic acid R* and 90 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *sulfuric acid R*, *methanol R* (1:99 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 5 mL of the solvent mixture and immediately dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *albendazole for system suitability CRS* (containing impurities B, C, E, F and H) in 1 mL of the solvent mixture and dilute to 10 mL with the mobile phase.

*Reference solution (c).* Dilute 1 mL of the solvent mixture to 10 mL with the mobile phase. Use 1 mL of this solution to dissolve the contents of a vial of *albendazole impurity mixture CRS* (containing impurities A and D).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 1.67 g/L solution of *ammonium dihydrogen phosphate R*, *methanol R* (30:70 *V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of albendazole.

*Identification of impurities*: use the chromatogram supplied with *albendazole impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and D; use the chromatogram supplied with *albendazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B + C, E, F and H.

*Relative retention* with reference to albendazole (retention time = about 11 min): impurity D = about 0.35; impurities B and C = about 0.40; impurity E = about 0.45; impurity A = about 0.48; impurity F = about 0.57; impurity H = about 0.66.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities B + C and E.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.7; impurities B and C = 1.4; impurity D = 1.9; impurity E = 1.4;
- for each impurity, use the concentration of albendazole in reference solution (a).

*Limits*:

- *impurity H*: maximum 0.6 per cent;
- *impurity F*: maximum 0.5 per cent;
- *impurity A*: maximum 0.4 per cent;
- *sum of impurities B and C*: maximum 0.4 per cent;
- *impurity E*: maximum 0.3 per cent;
- *impurity D*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.250 g in 3 mL of *anhydrous formic acid R* and add 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.53 mg of $C_{12}H_{15}N_3O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, I, J, K, L.*

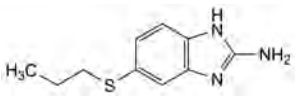

A. 5-(propylsulfanyl)-1*H*-benzimidazol-2-amine,

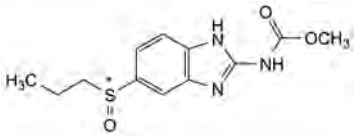

B. methyl *N*-[5-(propylsulfinyl)-1*H*-benzimidazol-2-yl]-carbamate,

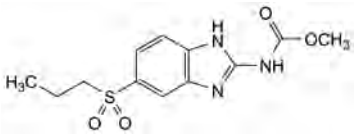

C. methyl *N*-[5-(propylsulfonyl)-1*H*-benzimidazol-2-yl]-carbamate,

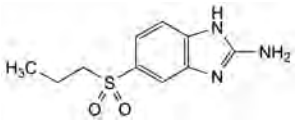

D. (2-amino-1*H*-benzimidazol-5-yl)propyl-λ⁶-sulfanedione,

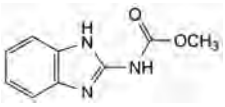

E. methyl *N*-(1*H*-benzimidazol-2-yl)carbamate,

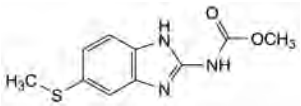

F. methyl *N*-[5-(methylsulfanyl)-1*H*-benzimidazol-2-yl]-carbamate,

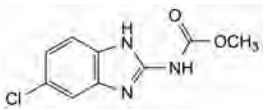

G. methyl *N*-(5-chloro-1*H*-benzimidazol-2-yl)carbamate,

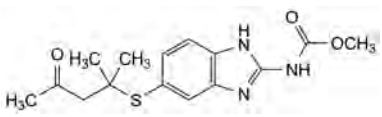

H. methyl *N*-[5-[(2-methyl-4-oxopentan-2-yl)sulfanyl]-1*H*-benzimidazol-2-yl]carbamate,

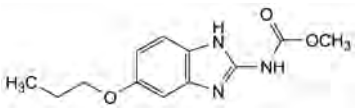

I. methyl *N*-(5-propoxy-1*H*-benzimidazol-2-yl)carbamate,

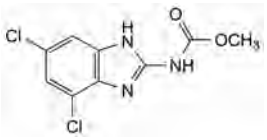

J. methyl *N*-(4,6-dichloro-1*H*-benzimidazol-2-yl)carbamate,

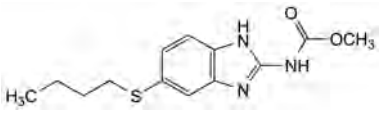

K. methyl *N*-[5-(butylsulfanyl)-1*H*-benzimidazol-2-yl]carbamate,

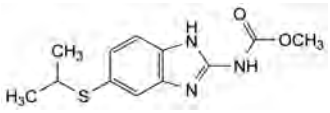

L. methyl *N*-[5-[(propan-2-yl)sulfanyl]-1*H*-benzimidazol-2-yl]carbamate.

**01/2008:1285**

# ALCURONIUM CHLORIDE

## Alcuronii chloridum

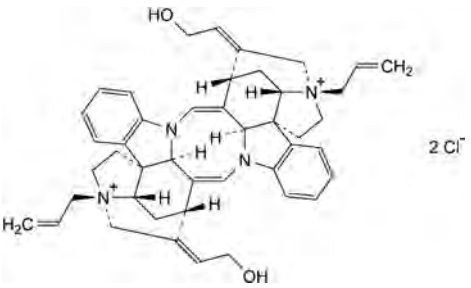

$C_{44}H_{50}Cl_2N_4O_2$ $M_r$ 738

[15180-03-7]

DEFINITION

(1*R*,3a*S*,10*S*,11a*S*,12*R*,14a*S*,19a*S*,20b*S*,21*S*,22a*S*,23*E*,26*E*)-23,26-bis(2-Hydroxyethylidene)-1,12-bis(prop-2-enyl)-2,3,11,11a,13,14,22,22a-octahydro-10*H*,21*H*-1,21:10,12-diethano-19a*H*,20b*H*-[1,5]diazocino[1,2,3-*lm*:5,6,7-*l′m′*]dipyrrolo[2′,3′-*d*:2″,3″:*d*′]dicarbazolediium dichloride (4,4′-didesmethyl-4,4′-bis(prop-2-enyl)toxiferin I dichloride).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly greyish-white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

*Carry out the identification, tests and assay as rapidly as possible avoiding exposure to actinic light.*

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *alcuronium chloride CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *alcuronium chloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: mix 15 volumes of a 58.4 g/L solution of *sodium chloride R*, 35 volumes of *dilute ammonia R2* and 50 volumes of *methanol R*.

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air for 10 min.

*Detection*: spray with *0.1 M ammonium and cerium nitrate*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$, $BY_6$ or $B_6$ (*2.2.2, Method I*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Specific optical rotation** (*2.2.7*): – 430 to – 451 (anhydrous substance), determined on solution S.

**Propan-2-ol** (*2.4.24, System A*): maximum 1.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 100 mL of *methanol R*, 200 mL of *acetonitrile R* and 200 mL of a 6.82 g/L solution of *potassium dihydrogen phosphate R*. Dissolve 1.09 g of *sodium laurylsulfonate for chromatography R* in the mixture and adjust the apparent pH to 8.0 with a 100 g/L solution of *sodium hydroxide R*.

*Test solution.* Dissolve 0.20 g of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 0.5 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 4.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (d).* To 5.0 mL of the test solution add 5.0 mg of *allylstrychnine bromide CRS*, dissolve in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 200 mL of *methanol R*, 400 mL of *acetonitrile R* and 400 mL of a 6.82 g/L solution of *potassium dihydrogen phosphate R*. Dissolve 2.18 g of *sodium laurylsulfonate for chromatography R* in the mixture and adjust the apparent pH to 5.4 with a 100 g/L solution of *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of alcuronium.

*System suitability*: reference solution (d):

– *resolution*: minimum 4.0 between the peaks due to *N*-allylstrychnine and alcuronium.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one of the peaks has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g by stirring in 70 mL of *acetic anhydride R* for 1 min. Titrate with *0.1 M perchloric acid* until the colour changes from violet-blue to greenish-blue, using 0.1 mL of *crystal violet solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 36.9 mg of $C_{44}H_{50}Cl_2N_4O_2$.

STORAGE

In an airtight container under nitrogen, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B.*

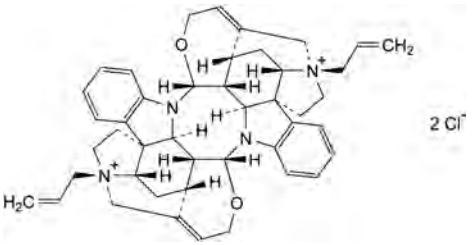


A. (1*R*,3a*S*,9*R*,9a*R*,10*R*,11a*S*,12*R*,14a*S*,19a*S*,20*R*,-20a*R*,20b*S*,21*R*,22a*S*)-1,12-bis(prop-2-enyl)-2,3,9a,11,11a,13,14,19a,20a,21,22,22a-dodecahydro-10*H*,20b*H*-1,23:12,27-dimethano-9,10:20,21-bis(epoxyprop[2]eno)-9*H*,20*H*-[1,5]diazocino[1,2,3-*lm*:5,6,7-*l′m′*]dipyrrolo[2′,3′-*d*:2″,3″:*d*′]dicarbazolediium dichloride (4,4′-diallylcaracurin V dichloride),

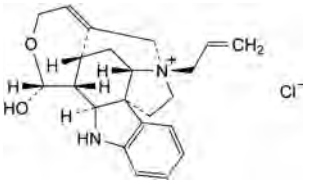


B. (4b*S*,7*R*,7a*S*,8a*R*,13*R*,13a*R*,13b*S*)-13-hydroxy-7-(prop-2-enyl)-5,6,7a,8,8a,11,13,13a,13b,14-decahydro-7,9-methano-7*H*-oxepino[3,4-*a*]pyrrolo[2,3-*d*]carbazolium chloride ((4*R*,17*R*)-4-allyl-17,18-epoxy-17-hydroxy-19,20-didehydrocuranium chloride).

01/2020:1286

# ALFACALCIDOL

## Alfacalcidolum

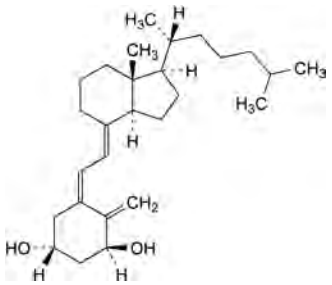

$C_{27}H_{44}O_2$ $M_r$ 400.6
[41294-56-8]

DEFINITION

(1*S*,3*R*,5*Z*,7*E*)-9,10-Secocholesta-5,7,10(19)-triene-1-3-diol.

*Content*: 97.0 per cent to 102.0 per cent.

A reversible isomerisation to pre-alfacalcidol takes place in solution, depending on temperature and time. The activity is due to both compounds (see Assay).

CHARACTERS

*Appearance*: white or almost white crystals.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in fatty oils.

It is sensitive to air, heat and light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of alfacalcidol.*

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Carry out the test as rapidly as possible, avoiding exposure to light and air, and prepare the solutions immediately before use.*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in 25.0 mL of *acetonitrile R* and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 5.0 mg of *alfacalcidol CRS* in 25.0 mL of *acetonitrile R* and dilute to 50.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 1.0 mL of this solution to 20.0 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 2 mg of *alfacalcidol for system suitability A CRS* (containing impurities A and D) in 10 mL of *acetonitrile R* and dilute to 20 mL with *water R*.

*Reference solution (d).* Dissolve 2 mg of *alfacalcidol for impurity B identification CRS* in 10 mL of *acetonitrile R* and dilute to 20 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 2.1 mm;
– *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
– *temperature*: 32 °C.

*Mobile phase*: *methanol R, water for chromatography R, acetonitrile R* (15:17:68 *V/V/V*).

*Flow rate*: 0.3 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 5 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of alfacalcidol.

*Identification of impurities*: use the chromatogram supplied with *alfacalcidol for system suitability A CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and D and pre-alfacalcidol; use the chromatogram supplied with *alfacalcidol for impurity B identification CRS* and the chromatogram obtained with reference solution (d) to identify impurity B.

*Relative retention* with reference to alfacalcidol (retention time = about 15 min): pre-alfacalcidol = about 0.91; impurity A = about 0.94; impurity D = about 0.96; impurity B = about 1.1.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity D and alfacalcidol;
– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to pre-alfacalcidol and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A;
– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity D.

*Limits*:

– *impurities A, B, D*: for each impurity, maximum 0.5 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to pre-alfacalcidol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{44}O_2$ taking into account the assigned content of *alfacalcidol CRS* and, if present, the peak due to pre-alfacalcidol.

STORAGE

Under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

IMPURITIES

*Specified impurities: A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

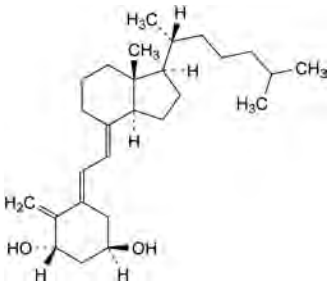

A. (1*S*,3*R*,5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1,3-diol (*trans*-alfacalcidol),

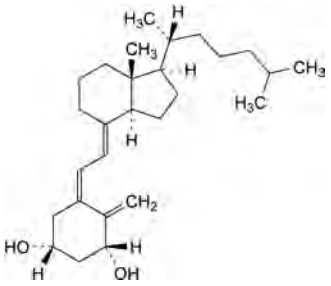

B. (1*R*,3*R*,5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1,3-diol (1β-calcidol),

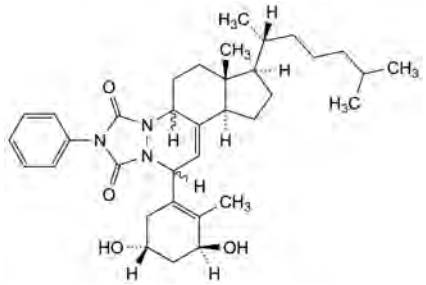

C. 6ξ-[(3*S*,5*R*)-3,5-dihydroxy-2-methylcyclohex-1-en-1-yl]-2-phenyl-2,5,10-triaza-17β-[(2*R*)-6-methylheptan-2-yl]-4-nor-9ξ-estr-7-ene-1,3-dione,

D. unknown structure.

**01/2017:1487 corrected 9.3**

# ALFADEX

## Alfadexum

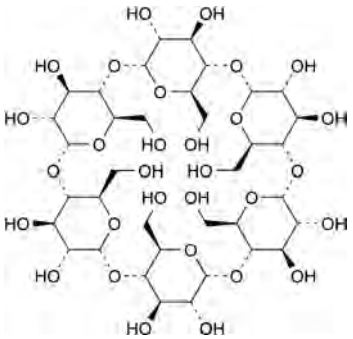

$[C_6H_{10}O_5]_6$ $M_r$ 973
[10016-20-3]

DEFINITION

Cyclohexakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltohexaose or α-cyclodextrin).

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in propylene glycol, practically insoluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Dissolve 0.2 g in 2 mL of *iodine solution R4* by warming on a water-bath, and allow to stand at room temperature; a yellowish-brown precipitate is formed.

TESTS

**Solution S**. Dissolve 1.000 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 5.0 to 8.0.

Mix 1 mL of a 223.6 g/L solution of *potassium chloride R* and 30 mL of solution S.

**Specific optical rotation** (*2.2.7*): + 147 to + 152 (dried substance), determined on solution S.

**Reducing sugars**: maximum 0.2 per cent.

*Test solution*. To 1 mL of solution S add 1 mL of *cupri-tartaric solution R4*. Heat on a water-bath for 10 min, cool to room temperature. Add 10 mL of *ammonium molybdate reagent R1* and allow to stand for 15 min.

*Reference solution*. Prepare a reference solution at the same time and in the same manner as the test solution, using 1 mL of a 0.02 g/L solution of *glucose R*.

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at the absorption maximum at 740 nm using *water R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Light-absorbing impurities**. Examine solution S between 230 nm and 750 nm. Between 230 nm and 350 nm, the absorbance (*2.2.25*) is not greater than 0.10. Between 350 nm and 750 nm, the absorbance (*2.2.25*) is not greater than 0.05.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.25 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a)*. Dissolve 25.0 mg of *betadex CRS* (impurity A), 25.0 mg of *gammacyclodextrin CRS* (impurity B) and 50.0 mg of *alfadex CRS* in *water R*, then dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 50.0 mL with *water R*.

*Reference solution (c)*. Dissolve 25.0 mg of *alfadex CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (10 μm).

*Mobile phase*: *methanol R*, *water R* (10:90 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: differential refractometer.

*Equilibration*: with the mobile phase for about 3 h.

*Injection*: 50 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of alfadex.

*Relative retention* with reference to alfadex (retention time = about 10 min): impurity B = about 0.7; impurity A = about 2.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity B and alfadex; if necessary, adjust the concentration of methanol in the mobile phase.

*Limits*:

– *impurities A, B*: for each impurity, not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *sum of impurities other than A and B*: not more than 0.5 times the area of the peak due to alfadex in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 11 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solutions (a) and (c).

*System suitability*:

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the peak due to alfadex after 5 injections of reference solution (a).

Calculate the percentage content of $[C_6H_{10}O_5]_6$ from the assigned content of *alfadex CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

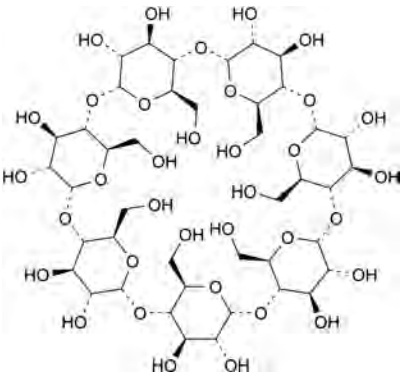

A. cycloheptakis-(1→4)-(α-D-glucopyranosyl) (betadex or cyclomaltoheptaose or β-cyclodextrin),

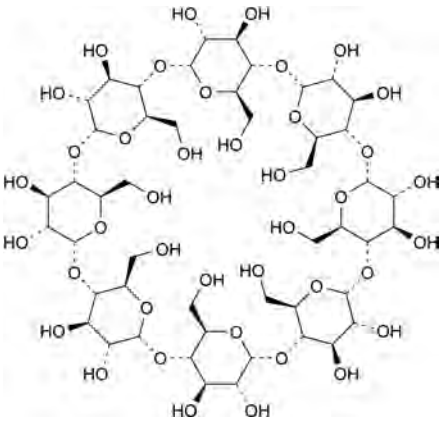

B. cyclooctakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltooctaose or γ-cyclodextrin).

**04/2019:1062**

# ALFENTANIL HYDROCHLORIDE HYDRATE

## Alfentanili hydrochloridum hydricum

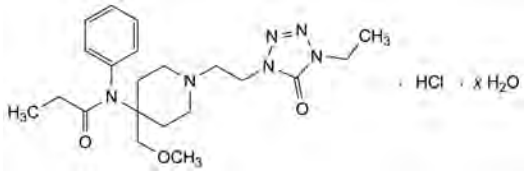

$C_{21}H_{33}ClN_6O_3$, $xH_2O$ $M_r$ 453.0 (anhydrous substance)
Anhydrous alfentanil hydrochloride: [69049-06-5]

DEFINITION

*N*-[1-[2-(4-Ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide hydrochloride hydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methanol.

mp: about 140 °C, with decomposition.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *alfentanil hydrochloride hydrate CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve 50 mg in a mixture of 0.4 mL of *ammonia R* and 2 mL of *water R*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g in *water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* In order to prepare impurity E *in situ*, dissolve 10 mg of the substance to be examined in 10.0 mL of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Neutralise with 10.0 mL of *dilute sodium hydroxide solution R* and evaporate to dryness on a water-bath. Cool and take up the residue in 10 mL of *methanol R*. Filter.

*Reference solution (b).* Dissolve the contents of a vial of *alfentanil impurity D CRS* in 1 mL of *methanol R*.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Blank solution*: *methanol R*.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: 5 g/L solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 40 | 10 → 60 |
| 15 - 20 | 40 | 60 |
| 20 - 25 | 40 → 90 | 60 → 10 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to alfentanil (retention time = about 8 min): impurity D = about 0.8; impurity E = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to impurity E and alfentanil.

*Calculation of percentage contents*:

- for each impurity, use the concentration of alfentanil hydrochloride hydrate in reference solution (c).

*Limits*:

- *impurity D*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.4 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.0 per cent to 4.0 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.350 g in 50 mL of a mixture of 1 volume of *ethanol (96 per cent) R* and 4 volumes of *water R* and add 5.0 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 45.30 mg of $C_{21}H_{33}ClN_6O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F, G, H.*

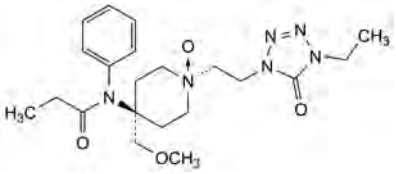

A. (1*s*,4*s*)-1-[2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)-4-(*N*-phenylpropanamido)piperidine 1-oxide,

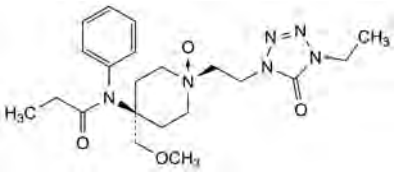

B. (1*r*,4*r*)-1-[2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)-4-(*N*-phenylpropanamido)piperidine 1-oxide,

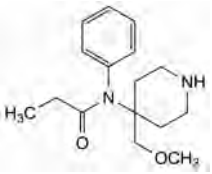

C. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,

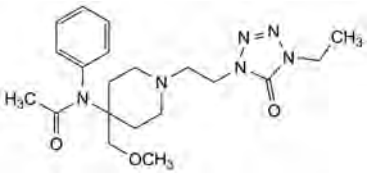

D. *N*-[1-[2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylacetamide,

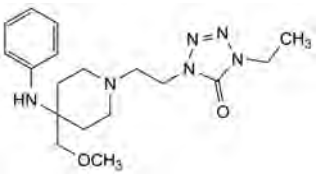

E. 1-ethyl-4-[2-[4-(methoxymethyl)-4-(phenylamino)piperidin-1-yl]ethyl]-1,4-dihydro-5*H*-tetrazol-5-one,

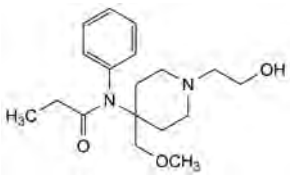

F. *N*-[1-(2-hydroxyethyl)-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,

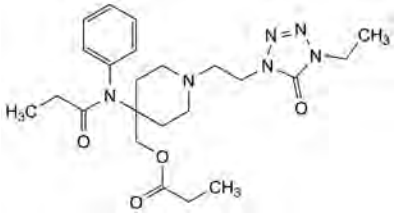

G. *N*-[1-[2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-[(propanoyloxy)methyl]piperidin-4-yl]-*N*-phenylpropanamide,

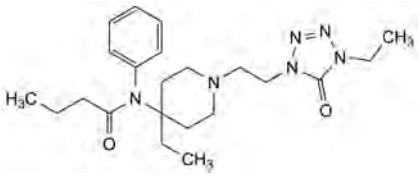

H. *N*-[1-[2-(4-ethyl-5-oxo-4,5-dihydro-1*H*-tetrazol-1-yl)ethyl]-4-(methoxymethyl)piperidin-4-yl]-*N*-phenylbutanamide.

**04/2017:1287**

# ALFUZOSIN HYDROCHLORIDE

## Alfuzosini hydrochloridum

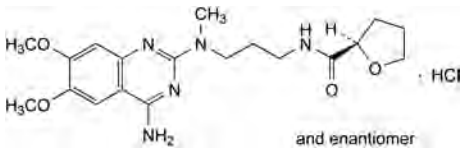

$C_{19}H_{28}ClN_5O_4$ $M_r$ 425.9

[81403-68-1]

DEFINITION

(2*RS*)-*N*-[3-[(4-Amino-6,7-dimethoxyquinazolin-2-yl)methylamino]propyl]oxolan-2-carboxamide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *alfuzosin hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent. Use a freshly prepared solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *alfuzosin for system suitability A CRS* (containing impurities B, F and G) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 4 mg of *alfuzosin for peak identification CRS* (containing impurity D) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C; if necessary, increase the temperature slightly to achieve the required resolution between the peaks due to impurity G and alfuzosin.

*Mobile phase*: mix 1 volume of *tetrahydrofuran R*, 20 volumes of *acetonitrile R* and 80 volumes of a solution prepared as follows: dilute 5.0 mL of *perchloric acid R* in 900 mL of *water for chromatography R*, adjust to pH 3.5 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of alfuzosin.

*Identification of impurities*: use the chromatogram supplied with *alfuzosin for system suitability A CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, F and G; use the chromatogram supplied with *alfuzosin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to alfuzosin (retention time = about 9 min): impurity D = about 0.4; impurity B = about 0.57; impurity F = about 0.63; impurity G = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities B and F; minimum 1.5 between the peaks due to impurity G and alfuzosin.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 0.6;
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity F*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*) : maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 40 mL of *anhydrous acetic acid R* and 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 42.59 mg of $C_{19}H_{28}ClN_5O_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, G.*

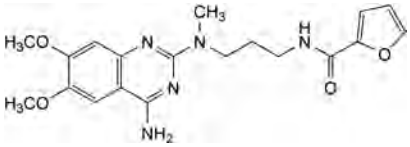

A. *N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)methylamino]propyl]furan-2-carboxamide,

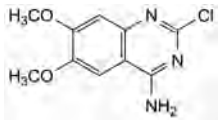

B. 2-chloro-6,7-dimethoxyquinazolin-4-amine,

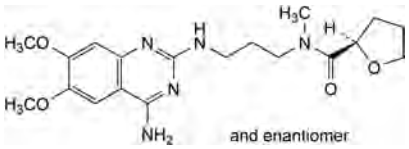


C. (2*RS*)-*N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)amino]propyl]-*N*-methyloxolan-2-carboxamide,

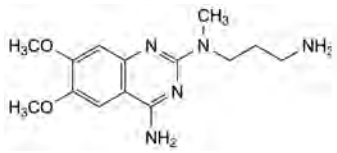

D. $N^2$-(3-aminopropyl)-6,7-dimethoxy-$N^2$-methylquinazolin-2,4-diamine,

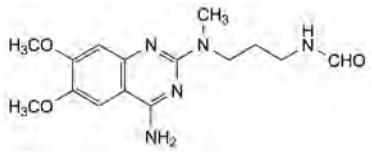

E. *N*-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)methylamino]propyl]formamide,

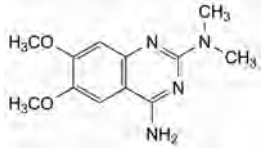

F. 6,7-dimethoxy-$N^2$,$N^2$-dimethylquinazoline-2,4-diamine,

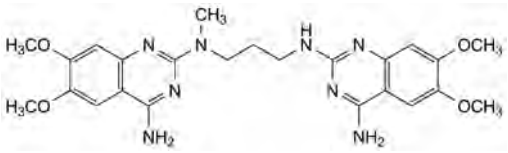

G. $N^2$-[3-[(4-amino-6,7-dimethoxyquinazolin-2-yl)amino]propyl]-6,7-dimethoxy-$N^2$-methylquinazoline-2,4-diamine.

**01/2017:0591**

# ALGINIC ACID

## Acidum alginicum

DEFINITION

Mixture of polyuronic acids $[(C_6H_8O_6)_n]$ composed of residues of D-mannuronic and L-guluronic acids, obtained mainly from algae belonging to the Phaeophyceae. A small proportion of the carboxyl groups may be neutralised.

*Content*: 19.0 per cent to 25.0 per cent of carboxyl groups ($-CO_2H$) (dried substance).

CHARACTERS

*Appearance*: white or pale yellowish-brown, crystalline or amorphous powder.

*Solubility*: very slightly soluble or practically insoluble in ethanol (96 per cent), practically insoluble in organic solvents. It swells in water but does not dissolve; it dissolves in solutions of alkali hydroxides.

IDENTIFICATION

A. To 0.2 g add 20 mL of *water R* and 0.5 mL of *sodium carbonate solution R*. Shake and filter. To 5 mL of the filtrate add 1 mL of *calcium chloride solution R*. A voluminous gelatinous mass is formed.

B. To 5 mL of the filtrate obtained in identification test A add 0.5 mL of a 123 g/L solution of *magnesium sulfate R*. No voluminous gelatinous mass is formed.

C. To 5 mg add 5 mL of *water R*, 1 mL of a freshly prepared 10 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and 5 mL of *hydrochloric acid R*. Boil gently for 3 min, cool, add 5 mL of *water R*, and shake with 15 mL of *di-isopropyl ether R*. Carry out a blank test. The upper layer obtained with the substance to be examined exhibits a deeper bluish-red colour than that obtained with the blank.

TESTS

**Chlorides**: maximum 1.0 per cent.

To 2.50 g add 50 mL of *dilute nitric acid R*, shake for 1 h and dilute to 100.0 mL with *dilute nitric acid R*. Filter. To 50.0 mL of the filtrate add 10.0 mL of *0.1 M silver nitrate* and 5 mL of *toluene R*. Titrate with *0.1 M ammonium thiocyanate*, using 2 mL of *ferric ammonium sulfate solution R2* as indicator and shaking vigorously towards the end-point.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 0.1000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 8.0 per cent (dried substance), determined on 0.100 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

To 0.2500 g add 25 mL of *water R*, 25.0 mL of *0.1 M sodium hydroxide* and 0.2 mL of *phenolphthalein solution R*. Titrate with *0.1 M hydrochloric acid*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 4.502 mg of carboxyl groups (-$CO_2H$).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for alginic acid used as disintegrant and/or binder.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Settling volume**. Place 75 mL of *water R* in a 100 mL graduated cylinder and add 1.5 g of the substance to be examined in 0.5 g portions, shaking vigorously after each addition. Dilute to 100.0 mL with *water R* and shake again until the substance is homogeneously distributed. Allow to stand for 4 h and determine the volume of the settled mass.

*The following characteristic may be relevant for alginic acid used as gelling agent or viscosity-increasing agent.*

**Apparent viscosity**. Determine the dynamic viscosity using a rotating viscometer (*2.2.10*).

Prepare a 20 g/L suspension of alginic acid (dried substance) and add *0.1 M sodium hydroxide* until a solution is obtained.

**01/2014:2650**
**corrected 10.0**

# ALIMEMAZINE HEMITARTRATE

## Alimemazini hemitartras

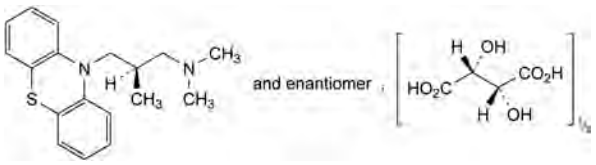


$C_{20}H_{25}N_2O_3S$ $M_r$ 373.5
[4330-99-8]

DEFINITION

(*2RS*)-*N*,*N*,2-Trimethyl-3-(10*H*-phenothiazin-10-yl)propan-1-amine hemi[(2*R*,3*R*)-2,3-dihydroxybutanedioate].

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or very slightly yellowish powder.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in toluene.

It deteriorates when exposed to air and light.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *alimemazine hemitartrate CRS*.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 5.0 to 6.5. *Carry out the test protected from light and use a freshly prepared solution.*

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and use freshly prepared solutions.*

*Solvent mixture*: *acetonitrile R*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 35 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 3.5 mg of *alimemazine for system suitability CRS* (containing impurities A, B and C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *methanol R*, 3.854 g/L solution of *ammonium acetate R* (10:40:50 *V/V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 253 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of alimemazine.

*Identification of impurities*: use the chromatogram supplied with *alimemazine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to alimemazine (retention time = about 27 min): impurity A = about 0.1; impurity B = about 0.5; impurity C = about 1.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to alimemazine and impurity C.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 4.4; impurity C = 0.4;
- for each impurity, use the concentration of alimemazine hemitartrate in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.3 per cent;
- *impurities A, C*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.35 mg of $C_{20}H_{25}N_2O_3S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

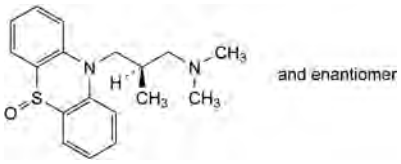


A. (2*RS*)-*N*,*N*,2-trimethyl-3-(5-oxido-10*H*-phenothiazin-10-yl)propan-1-amine,

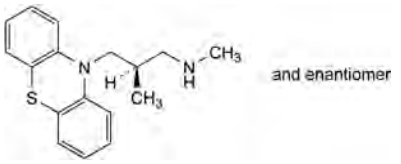


B. (2*RS*)-*N*,2-dimethyl-3-(10*H*-phenothiazin-10-yl)propan-1-amine,

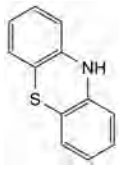

C. 10*H*-phenothiazine.

**01/2008:1288**
**corrected 6.0**

# ALLANTOIN

## Allantoinum

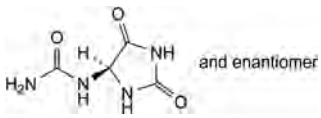


$C_4H_6N_4O_3$ $M_r$ 158.1
[97-59-6]

DEFINITION

Allantoin contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-(2,5-dioxoimidazolidin-4-yl)urea.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, very slightly soluble in alcohol.

It melts at about 225 °C, with decomposition.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *allantoin CRS*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Boil 20 mg with a mixture of 1 mL of *dilute sodium hydroxide solution R* and 1 mL of *water R*. Allow to cool. Add 1 mL of *dilute hydrochloric acid R*. To 0.1 mL of the solution add 0.1 mL of a 100 g/L solution of *potassium bromide R*, 0.1 mL of a 20 g/L solution of *resorcinol R* and 3 mL of *sulfuric acid R*. Heat for 5 min to 10 min on a water-bath. A dark blue colour develops, which becomes red after cooling and pouring into about 10 mL of *water R*.

D. Heat about 0.5 g. Ammonia vapour is evolved, which turns *red litmus paper R* blue.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R*, with heating if necessary, and dilute to 100 mL with the same solvent.

**Acidity or alkalinity**. To 5 mL of solution S add 5 mL of *carbon dioxide-free water R*, 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Optical rotation** (*2.2.7*). The angle of optical rotation, determined on solution S, is − 0.10° to + 0.10°.

**Reducing substances**. Shake 1.0 g with 10 mL of *water R* for 2 min. Filter. Add 1.5 mL of *0.02 M potassium permanganate*. The solution must remain violet for at least 10 min.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable *cellulose for chromatography R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 5.0 mL of *water R* with heating. Allow to cool. Dilute to 10 mL with *methanol R*. *Use the solution immediately after preparation.*

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 1 volume of *methanol R* and 1 volume of *water R*.

*Reference solution (a).* Dissolve 10 mg of *allantoin CRS* in a mixture of 1 volume of *methanol R* and 1 volume of *water R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 10 mg of *urea R* in 10 mL of *water R*. Dilute 1 mL of this solution to 10 mL with *methanol R*.

*Reference solution (c).* Mix 1 mL of reference solution (a) and 1 mL of reference solution (b).

Apply to the plate 10 µL of test solution (a) and 5 µL each of test solution (b), reference solution (a), reference solution (b) and reference solution (c). Develop over a path of 10 cm using a mixture of 15 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air. Spray the plate with a 5 g/L solution of *dimethylaminobenzaldehyde R* in a mixture of 1 volume of *hydrochloric acid R* and 3 volumes of *methanol R*. Dry the plate in a current of hot air. Examine in daylight after 30 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*). Not more than 0.1 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 120.0 mg in 40 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.81 mg of $C_4H_6N_4O_3$.

IMPURITIES

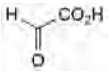

A. glyoxylic acid,

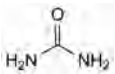

B. carbamide (urea).

**01/2017:0576**

# ALLOPURINOL

## Allopurinolum

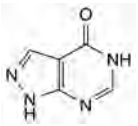

$C_5H_4N_4O$ $M_r$ 136.1
[315-30-0]

DEFINITION

1,5-Dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10 mg in 1 mL of a 4 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. Dilute 10.0 mL of this solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 250 nm.

*Absorption minimum*: at 231 nm.

*Absorbance ratio*: $A_{231}/A_{250}$ = 0.52 to 0.62.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *allopurinol CRS*.

C. Dissolve 0.3 g in 2.5 mL of *dilute sodium hydroxide solution R* and add 50 mL of *water R*. Add slowly and with shaking 5 mL of *silver nitrate solution R1*. A white precipitate is formed which does not dissolve on the addition of 5 mL of *ammonia R*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *concentrated ammonia R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *allopurinol CRS* in *concentrated ammonia R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous ethanol R*, *methylene chloride R* (40:60 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions. Store and inject them at 8 °C, using a cooled autosampler.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 2.5 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 50.0 mL with the mobile phase.

*Test solution (b).* Dissolve 20.0 mg of the substance to be examined in 5.0 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 250.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *allopurinol impurity A CRS*, 5 mg of *allopurinol impurity B CRS* and 5.0 mg of *allopurinol impurity C CRS* in 5.0 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 20.0 mg of *allopurinol CRS* in 5.0 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 250.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 1.25 g/L solution of *potassium dihydrogen phosphate R*.

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: twice the retention time of allopurinol.

*Elution order*: impurity A, impurity B, impurity C, allopurinol.

*Retention time*: allopurinol = about 10 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.1 between the peaks due to impurities B and C.

*Limits*:

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *sum of impurities other than A, B and C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurities D and E**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions. Store and inject them at 8 °C, using a cooled autosampler.*

*Solution A*: 1.25 g/L solution of *potassium dihydrogen phosphate R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 5.0 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 100.0 mL with solution A.

*Reference solution.* Dissolve 5.0 mg of *allopurinol impurity D CRS* and 5.0 mg of *allopurinol impurity E CRS* in 5.0 mL of a 4 g/L solution of *sodium hydroxide R* and dilute immediately to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Column*:
– *size*: *l* = 0.05 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *methanol R*, 1.25 g/L solution of *potassium dihydrogen phosphate R* (10:90 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of impurity E.

*Retention times*: impurity D = about 3.6 min; impurity E = about 4.5 min.

*System suitability*: reference solution:
– *resolution*: minimum 2.0 between the peaks due to impurities D and E.

*Limits*:
– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent);
– *impurity E*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Impurity F**. Liquid chromatography (*2.2.29*).

Under the following conditions, any hydrazine in the sample reacts with benzaldehyde to give benzaldehyde azine.

*Solvent mixture.* Mix equal volumes of *dilute sodium hydroxide solution R* and *methanol R*.

*Solution A*. Dissolve 2.0 g of *benzaldehyde R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Prepare immediately before use.

*Test solution.* Dissolve 250.0 mg of the substance to be examined in 5 mL of the solvent mixture. Add 4 mL of solution A, mix and allow to stand for 2.5 h at room temperature. Add 5.0 mL of *hexane R* and shake for 1 min. Allow the layers to separate and use the upper layer.

*Reference solution.* Dissolve 10.0 mg of *hydrazine sulfate R* in the solvent mixture by sonicating for about 2 min and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL to 20.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture. To 5.0 mL of the solution obtained, add 4 mL of solution A, mix and allow to stand for 2.5 h at room temperature. Add 5.0 mL of *hexane R* and shake for 1 min. Allow the layers to separate and use the upper layer.

*Blank solution.* To 5 mL of the solvent mixture add 4 mL of solution A, mix and allow to stand for 2.5 h at room temperature. Add 5.0 mL of *hexane R* and shake for 1 min. Allow the layers to separate and use the upper layer.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *cyanosilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm;
– *temperature*: 30 °C.

*Mobile phase*: *2-propanol R*, *hexane R* (5:95 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 310 nm.

*Injection*: 20 µL.

*Relative retention* with reference to benzaldehyde (retention time = about 2.8 min): benzaldehyde azine = about 0.8.

*System suitability*: reference solution:
– *resolution*: minimum 2 between the peaks due to benzaldehyde azine and benzaldehyde;
– *signal-to-noise ratio*: minimum 20 for the peak due to benzaldehyde azine.

*Limit*:
– *impurity F*: the area of the peak due to benzaldehyde azine in the chromatogram obtained with the test solution is not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm of hydrazine sulfate equivalent to 2.5 ppm of hydrazine).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_5H_4N_4O$ from the declared content of *allopurinol CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

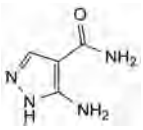

A. 5-amino-1*H*-pyrazole-4-carboxamide,

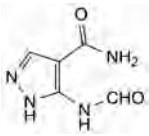

B. 5-(formylamino)-1*H*-pyrazole-4-carboxamide,

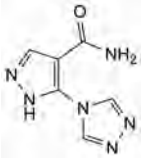

C. 5-(4*H*-1,2,4-triazol-4-yl)-1*H*-pyrazole-4-carboxamide,

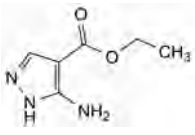

D. ethyl 5-amino-1*H*-pyrazole-4-carboxylate,

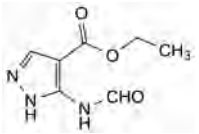

E. ethyl 5-(formylamino)-1*H*-pyrazole-4-carboxylate,

F. diazane (hydrazine).

**01/2017:2010**
**corrected 10.0**

# ALMAGATE

## Almagatum

$Al_2Mg_6C_2O_{20}H_{14},4H_2O$ $M_r$ 630
[66827-12-1]

DEFINITION

Hydrated aluminium magnesium hydroxycarbonate.

*Content*:

- aluminium: 15.0 per cent to 17.0 per cent (calculated as $Al_2O_3$),
- magnesium: 36.0 per cent to 40.0 per cent (calculated as MgO),
- carbonic acid: 12.5 per cent to 14.5 per cent (calculated as $CO_2$).

CHARACTERS

*Appearance*: white or almost white, fine, crystalline powder.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It dissolves with effervescence and heating in dilute mineral acids.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of almagate*.

B. Dissolve 0.15 g in *dilute hydrochloric acid R* and dilute to 20 mL with the same acid. 2 mL of the solution gives the reaction of aluminium (*2.3.1*).

C. 2 mL of the solution prepared under identification test B gives the reaction of magnesium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 9.1 to 9.7.

Disperse 4.0 g in 100 mL of *carbon dioxide-free water R*, stir for 2 min and filter.

**Neutralising capacity**. *Carry out the test at 37 °C*. Disperse 0.5 g in 100 mL of *water R*, heat, add 100.0 mL of *0.1 M hydrochloric acid*, previously heated and stir continuously; the pH (*2.2.3*) of the solution between 5 min and 20 min is not less than 3.0 and not greater than 4.5. Add 10.0 mL of *0.5 M hydrochloric acid*, previously heated, stir continuously for 1 h and titrate with *0.1 M sodium hydroxide* to pH 3.5; not more than 20.0 mL of *0.1 M sodium hydroxide* is required.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dissolve 0.33 g in 5 mL of *dilute nitric acid R* and dilute to 100 mL with *water R*. Prepare simultaneously the standard by diluting 0.7 mL of *dilute nitric acid R* to 5 mL with *water R* and adding 10 mL of *chloride standard solution (5 ppm Cl) R*.

**Sulfates** (*2.4.13*): maximum 0.4 per cent.

Dissolve 0.25 g in 5 mL of *dilute hydrochloric acid R* and dilute to 100 mL with *distilled water R*. Prepare simultaneously the standard by adding 0.8 mL of *dilute hydrochloric acid R* to 15 mL of *sulfate standard solution (10 ppm $SO_4$) R*.

**Sodium**: maximum 150 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dissolve 0.25 g in 50 mL of a 103 g/L solution of *hydrochloric acid R*.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with a 103 g/L solution of *hydrochloric acid R*.

**Loss on ignition**: 43.0 per cent to 49.0 per cent, determined on 1.000 g by ignition at 900 ± 50 °C.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

ASSAY

**Aluminium**. Dissolve 1.000 g in 5 mL of *hydrochloric acid R*, heating if necessary. Allow to cool to room temperature and dilute to 100.0 mL with *water R* (solution A). Introduce 10.0 mL of solution A into a 250 mL conical flask, add 25.0 mL of *0.05 M sodium edetate*, 20 mL of *buffer solution pH 3.5 R*, 40 mL of *anhydrous ethanol R* and 2 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *anhydrous ethanol R*. Titrate the excess of sodium edetate with *0.05 M zinc sulfate* until the colour changes from greenish-violet to pink.

1 mL of *0.05 M sodium edetate* is equivalent to 2.549 mg of $Al_2O_3$.

**Magnesium**. Introduce 10.0 mL of solution A prepared in the assay of aluminium into a 500 mL conical flask, add 200 mL of *water R*, 20 mL of *triethanolamine R* with shaking, 10 mL of *ammonium chloride buffer solution pH 10.0 R* and 50 mg of *mordant black 11 triturate R*. Titrate with *0.05 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.05 M sodium edetate* is equivalent to 2.015 mg of MgO.

**Carbonic acid**: 12.5 per cent to 14.5 per cent.

*Test sample*. Place 7.00 mg of the substance to be examined in a tin capsule. Seal the capsule.

*Reference sample*. Place 7.00 mg of *almagate CRS* in a tin capsule. Seal the capsule.

Introduce separately the test sample and the reference sample into a combustion chamber of a CHN analyser purged with *helium for chromatography R* and maintained at a temperature of 1020 °C. Simultaneously, introduce *oxygen R* at a pressure of 40 kPa and a flow rate of 20 mL/min and allow complete combustion of the sample. Sweep the combustion gases through a reduction reactor and separate the gases formed by gas chromatography (*2.2.28*).

*Column*:

- *size*: *l* = 2 m, Ø = 4 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 100 mL/min.

*Temperature*:

- *column*: 65 °C;
- *detector*: 190 °C.

*Detection*: thermal conductivity.

*Run time*: 16 min.

*System suitability*:

– average percentage of carbon in 5 reference samples must be within ± 0.2 per cent of the value assigned to the CRS; the difference between the upper and the lower values of the percentage of carbon in these samples must be below 0.2 per cent.

Calculate the percentage content of carbonic acid in the test sample according to the following formula:

$$C \times K \times \frac{A}{m}$$

$C$ = percentage content of carbonic acid in the reference sample;

$K$ = mean value for the 5 reference samples of the ratio of the mass in milligrams to the area of the peak due to carbonic acid;

$A$ = area of the peak due to carbonic acid in the chromatogram obtained with the test sample;

$m$ = sample mass, in milligrams.

STORAGE

In an airtight container.

**01/2010:1064**

# ALMOND OIL, REFINED

## Amygdalae oleum raffinatum

DEFINITION

Fatty oil obtained from the ripe seeds of *Prunus dulcis* (Mill.) D.A. Webb var. *dulcis* or *Prunus dulcis* (Mill.) D.A. Webb var. *amara* (DC.) Buchheim or a mixture of both varieties by cold expression. It is then refined. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: pale yellow, clear liquid.

*Solubility*: slightly soluble in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.916.

It solidifies at about − 18 °C.

IDENTIFICATION

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Specific absorbance** (*2.2.25*): 0.2 to 6.0, determined at the absorption maximum at 270 nm.

To 0.100 g add *cyclohexane R* and dilute to 10.0 mL with the same solvent. Adapt the concentration of the solution so that the absorbance lies between 0.5 and 1.5, measured in a 1 cm cell.

**Acid value** (*2.5.1*): maximum 0.5, determined on 5.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 0.9 per cent, determined on 5.0 g.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

– *saturated fatty acids of chain length less than $C_{16}$*: maximum 0.1 per cent;
– *palmitic acid*: 4.0 per cent to 9.0 per cent;
– *palmitoleic acid*: maximum 0.8 per cent;
– *margaric acid*: maximum 0.2 per cent;
– *stearic acid*: maximum 3.0 per cent;
– *oleic acid*: 62.0 per cent to 86.0 per cent;
– *linoleic acid*: 20.0 per cent to 30.0 per cent;
– *linolenic acid*: maximum 0.4 per cent;
– *arachidic acid*: maximum 0.2 per cent;
– *eicosenoic acid*: maximum 0.3 per cent;
– *behenic acid*: maximum 0.2 per cent;
– *erucic acid*: maximum 0.1 per cent.

**Sterols** (*2.4.23*).

*Composition of the sterol fraction of the oil*:

– *cholesterol*: maximum 0.7 per cent;
– *campesterol*: maximum 5.0 per cent;
– *stigmasterol*: maximum 4.0 per cent;
– *β-sitosterol*: 73.0 per cent to 87.0 per cent;
– *Δ5-avenasterol*: minimum 5.0 per cent;
– *Δ7-stigmastenol*: maximum 3.0 per cent;
– *Δ7-avenasterol*: maximum 3.0 per cent;
– *brassicasterol*: maximum 0.3 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light.

**01/2015:0261**

# ALMOND OIL, VIRGIN

## Amygdalae oleum virginale

DEFINITION

Fatty oil obtained by cold expression from the ripe seeds of *Prunus dulcis* (Mill.) D.A.Webb var. *dulcis* or *Prunus dulcis* (Mill.) D.A.Webb var. *amara* (DC.) Buchheim or a mixture of both varieties.

CHARACTERS

*Appearance*: yellow, clear liquid.

*Solubility*: slightly soluble in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.916.

It solidifies at about − 18 °C.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Absorbance (see Tests).

B. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

C. Composition of fatty acids (see Tests).

TESTS

**Specific absorbance** (*2.2.25*): maximum 0.2, determined at the absorption maximum at 270 nm. The ratio of the absorbance measured at 232 nm to that measured at 270 nm is greater than 7.

To 0.100 g add *cyclohexane R* and dilute to 10.0 mL with the same solvent. Adapt the concentration of the solution so that the absorbance lies between 0.5 and 1.5, measured in a 1 cm cell.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 15.0.

**Unsaponifiable matter** (*2.5.7*): maximum 0.9 per cent, determined on 5.0 g.

**Composition of fatty acids**. (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *saturated fatty acids of chain length less than $C_{16}$*: maximum 0.1 per cent,
- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *palmitoleic acid*: maximum 0.8 per cent,
- *margaric acid*: maximum 0.2 per cent,
- *stearic acid*: maximum 3.0 per cent,
- *oleic acid*: 62.0 per cent to 86.0 per cent,
- *linoleic acid*: 20.0 per cent to 30.0 per cent,
- *linolenic acid*: maximum 0.4 per cent,
- *arachidic acid*: maximum 0.2 per cent,
- *eicosenoic acid*: maximum 0.3 per cent,
- *behenic acid*: maximum 0.2 per cent,
- *erucic acid*: maximum 0.1 per cent.

**Sterols** (*2.4.23*).

*Composition of sterol fraction of the oil*:

- *cholesterol*: maximum 0.7 per cent,
- *campesterol*: maximum 4.0 per cent,
- *stigmasterol*: maximum 3.0 per cent,
- *β-sitosterol*: 73.0 per cent to 87.0 per cent,
- *Δ5-avenasterol*: minimum 10.0 per cent,
- *Δ7-stigmastenol*: maximum 3.0 per cent,
- *Δ7-avenasterol*: maximum 3.0 per cent,
- *brassicasterol*: maximum 0.3 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light.

**01/2008:1065**
**corrected 10.0**

# ALPRAZOLAM

## Alprazolamum

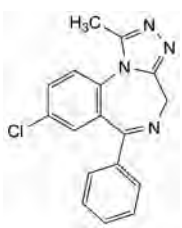

$C_{17}H_{13}ClN_4$ $M_r$ 308.8
[28981-97-7]

DEFINITION

8-Chloro-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]-benzodiazepine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone and in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Dissolve the substance to be examined in the smallest necessary quantity of *ethyl acetate R* and evaporate to dryness on a water-bath. Thoroughly mix 5.0 mg of the substance to be examined with 5.0 mg of *alprazolam CRS*. The melting point (*2.2.14*) of the mixture does not differ by more than 2 °C from the melting point of the substance to be examined.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *alprazolam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethyl acetate R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *alprazolam CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *alprazolam CRS* and 10 mg of *midazolam CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R*, *ethyl acetate R* (2:15:20:80 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

- the chromatogram shows 2 clearly separately spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 7.7 g of *ammonium acetate R* in 1000 mL of *water for chromatography R* and adjust to pH 4.2 with *glacial acetic acid R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2 mg of *alprazolam CRS* and 2 mg of *triazolam CRS* in *dimethylformamide R* and dilute to 100 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 0.5 mL of this solution to 10.0 mL with *dimethylformamide R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: buffer solution, *methanol R* (44:56 *V/V*);
- *mobile phase B*: buffer solution, *methanol R* (5:95 *V/V*);
- *temperature*: 40 °C;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 98 | 2 |
| 15 - 35 | 98 → 1 | 2 → 99 |
| 35 - 40 | 1 | 99 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL; inject *dimethylformamide R* as a blank.

*Retention time*: triazolam = about 9 min; alprazolam = about 10 min.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to triazolam and alprazolam.

*Limits*:
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 50 mL of a mixture of 2 volumes of *acetic anhydride R* and 3 volumes of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Titrate to the 2nd point of inflexion.

1 mL of *0.1 M perchloric acid* is equivalent to 15.44 mg of $C_{17}H_{13}ClN_4$.

STORAGE

Protected from light.

IMPURITIES

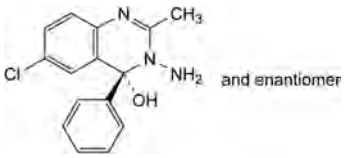


A. (4*RS*)-3-amino-6-chloro-2-methyl-4-phenyl-3,4-dihydroquinazolin-4-ol,

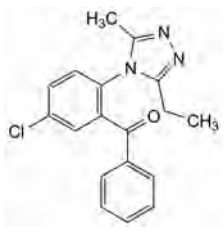


B. [5-chloro-2-[3-(hydroxymethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

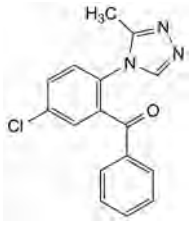


C. [5-chloro-2-[3-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

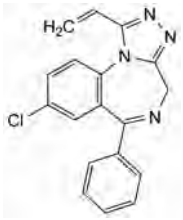


D. 8-chloro-1-ethenyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepine,

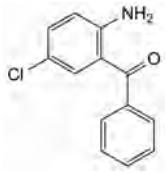


E. (2-amino-5-chlorophenyl)phenylmethanone,

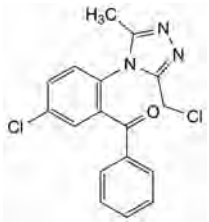


F. [5-chloro-2-[3-(chloromethyl)-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

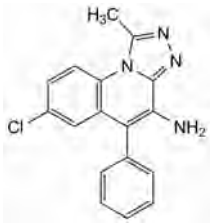


G. 7-chloro-1-methyl-5-phenyl[1,2,4]triazolo[4,3-*a*]quinolin-4-amine,

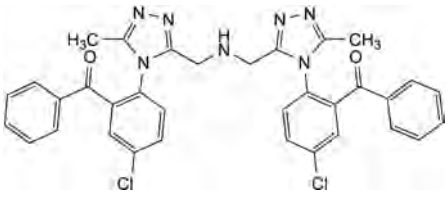


H. bis[[4-(2-benzoyl-4-chlorophenyl)-5-methyl-4*H*-1,2,4-triazol-3-yl]methyl]amine,

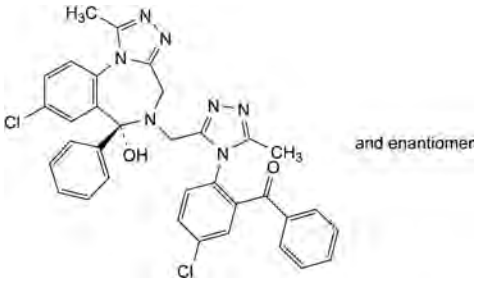


I. [5-chloro-2-[3-[[(6*RS*)-8-chloro-6-hydroxy-1-methyl-6-phenyl-4*H*-[1,2,4]triazolo[4,3-*a*][1,4]benzodiazepin-5(6*H*)-yl]methyl]-5-methyl-4*H*-1,2,4-triazol-4-yl]phenyl]phenylmethanone,

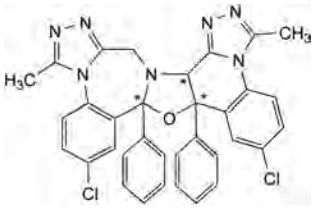

J. 2,17-dichloro-6,13-dimethyl-18b,19a-diphenyl-8b,19a-dihydro-10*H*,18b*H*-[1,2,4]triazolo[4‴,3‴:1″,2″]-quinolo[3″,4″:4′,5′]oxazolo[3′,2′-*d*]-1,2,4-triazolo[4,3-*a*]-[1,4]benzodiazepine.

**01/2017:0876**
**corrected 10.0**

# ALPRENOLOL HYDROCHLORIDE

## Alprenololi hydrochloridum

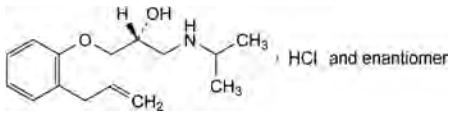


$C_{15}H_{24}ClNO_2$ $M_r$ 285.8
[13707-88-5]

DEFINITION

(2*RS*)-1-[(1-Methylethyl)amino]-3-[2-(prop-2-enyl)phenoxy]-propan-2-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 108 °C to 112 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *alprenolol hydrochloride CRS.*

C. Examine the chromatograms obtained in the test for impurity D.
*Detection*: examine in daylight, after exposure to iodine vapour for 30 min.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_9$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*; the solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*; the solution is yellow.

**Impurity C**: maximum 0.1 per cent.

Dissolve 0.25 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent. The absorbance (*2.2.25*) measured at 297 nm is not greater than 0.20.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 50 mL with *methanol R.*

*Reference solution (a).* Dissolve 10 mg of *alprenolol hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *alprenolol hydrochloride CRS* and 10 mg of *oxprenolol hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dilute 5 mL of test solution (b) to 50 mL with *methanol R.*

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: place 2 beakers each containing 30 mL of *ammonia R* at the bottom of the tank containing a mixture of 5 volumes of *methanol R* and 95 volumes of *ethyl acetate R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm in a tank saturated for at least 1 h.

*Drying*: at 100 °C for 15 min.

*Detection*: expose to iodine vapour for up to 6 h.

*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.

*Limits*: test solution (a):
– *impurity D*: any spot with an $R_F$ value greater than that of the principal spot is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 4.0 mg of *alprenolol hydrochloride CRS* and 0.8 mg of *4-isopropylphenol R* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 4.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
– *size*: *l* = 0.15 m, Ø = 4 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 0.656 g of *sodium octanesulfonate R* with 150 mL of *acetonitrile R* and dilute to 500 mL with phosphate buffer pH 2.8 prepared as follows: mix 1.78 g of *phosphoric acid R* and 15.6 g of *sodium dihydrogen phosphate R* and dilute to 2000 mL with *water R.*

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Equilibration*: with the mobile phase for about 1 h.

*Injection*: 20 µL.

*Run time*: twice the retention time of alprenolol.

*Retention time*: alprenolol = about 11 min; 4-isopropylphenol = about 18 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 5 between the peaks due to alprenolol and 4-isopropylphenol; if necessary, adjust the concentration of sodium octanesulfonate and/or acetonitrile in the mobile phase (increase the concentration of sodium octanesulfonate to increase the retention time of alprenolol and increase the concentration of acetonitrile to decrease the retention times of both compounds).

*Limits*:

– *unspecified impurities*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.04 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 25 mL of a mixture of equal volumes of *anhydrous ethanol R* and *water R*. Add 10 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.58 mg of $C_{15}H_{24}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

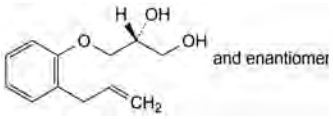


A. (2*RS*)-3-[2-(prop-2-enyl)phenoxy]propan-1,2-diol,

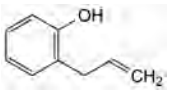


B. 2-(prop-2-enyl)phenol,

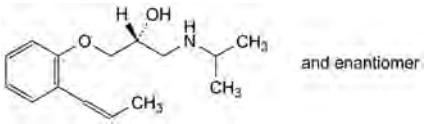


C. (2*RS*)-1-[(1-methylethyl)amino]-3-[2-(prop-1-enyl)phenoxy]propan-2-ol,

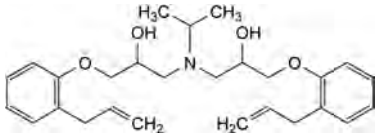


D. 1,1′-[(1-methylethyl)imino]bis[3-[2-(prop-2-enyl)phenoxy]propan-2-ol].

**04/2015:1488**

# ALPROSTADIL

## Alprostadilum

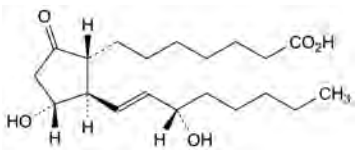


$C_{20}H_{34}O_5$ $M_r$ 354.5
[745-65-3]

DEFINITION

7-[(1*R*,2*R*,3*R*)-3-Hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid.

*Content*: 95.0 per cent to 102.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in acetone, slightly soluble in ethyl acetate.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): − 70 to − 60 (anhydrous substance).

Immediately before use, dissolve 50 mg in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *alprostadil CRS*.

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 100 µL of the test solution to 20.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (b).* Dissolve 1.0 mg of *dinoprostone impurity C CRS* (alprostadil impurity H) and 1.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solution (c).* In order to prepare impurities A and B *in situ*, dissolve 1 mg of the substance to be examined in 100 µL of *1 M sodium hydroxide* (the solution becomes brownish-red), wait for 3 min and add 100 µL of a 112 g/L solution of *phosphoric acid R* (yellowish-white opalescent solution); dilute to 5.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

**System A**

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (4 µm) with a pore size of 6 nm;
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent; adjust to pH 2.5 with a 2.9 g/L solution of *phosphoric acid R* (approximately 600 mL is required); to 740 mL of the buffer solution add 260 mL of *acetonitrile R1*;
- *mobile phase B*: dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent; adjust to pH 2.5 with a 2.9 g/L solution of *phosphoric acid R* (approximately 600 mL is required); to 200 mL of the buffer solution add 800 mL of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 75 | 100 | 0 |
| 75 - 76 | 100 → 0 | 0 → 100 |
| 76 - 86 | 0 | 100 |
| 86 - 87 | 0 → 100 | 100 → 0 |
| 87 - 102 | 100 | 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Retention time*: alprostadil = about 63 min.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity H and alprostadil in the chromatogram obtained with reference solution (b).

**System B**

Use the same conditions as for system A with the following mobile phase and elution programme:

- *mobile phase A*: dissolve 3.9 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent; adjust to pH 2.5 with a 2.9 g/L solution of *phosphoric acid R* (approximately 600 mL is required); to 600 mL of the buffer solution add 400 mL of *acetonitrile R1*;
- *mobile phase B*: use mobile phase B as described under system A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 | 0 |
| 50 - 51 | 100 → 0 | 0 → 100 |
| 51 - 61 | 0 | 100 |
| 61 - 62 | 0 → 100 | 100 → 0 |
| 62 - 72 | 100 | 0 |

*Relative retention* with reference to alprostadil (retention time = about 7 min): impurity A = about 2.4; impurity B = about 2.6.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B in the chromatogram obtained with reference solution (c).

Carry out the test according to system A and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the impurities listed in Table 1488.-1 by the corresponding correction factor;

Table 1488.-1.

| Impurity | Relative retention (system A) | Relative retention (system B) | Correction factor |
|---|---|---|---|
| impurity G | 0.80 | - | 0.7 |
| impurity F | 0.88 | - | 0.8 |
| impurity D | 0.90 | - | 1.0 |
| impurity H | 0.96 | - | 0.7 |
| impurity E | 1.10 | - | 0.7 |
| impurity C | - | 1.36 | 1.9 |
| impurity K | - | 1.85 | 0.06 |
| impurity A | - | 2.32 | 0.7 |
| impurity B | - | 2.45 | 1.5 |
| impurity I | - | 4.00 | 1.0 |
| impurity J | - | 5.89 | 1.0 |

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *any other impurity*: not more than 1.8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.9 per cent), and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent). Evaluate impurities appearing at relative retentions less than 1.2 by system A and impurities appearing at relative retentions greater than 1.2 by system B;
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 50 mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances, system A. *Prepare the solutions protected from light.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 25.0 mL with the same mixture of solvents. Dilute 3.0 mL of the solution to 20.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution.* Dissolve 5.0 mg of *alprostadil CRS* in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 25.0 mL with the same mixture of solvents. Dilute 6.0 mL of the solution to 20.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Injection*: 20 µL.

Calculate the percentage content of $C_{20}H_{34}O_5$ taking into account the assigned content of *alprostadil CRS*.

STORAGE

At a temperature of 2 °C to 8 °C.

IMPURITIES

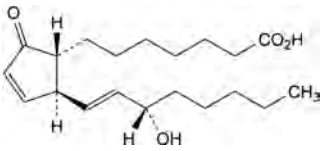

A. 7-[(1*R*,2*S*)-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-3-enyl]heptanoic acid (prostaglandin $A_1$),

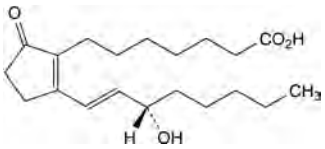

B. 7-[2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-1-enyl]heptanoic acid (prostaglandin $B_1$),

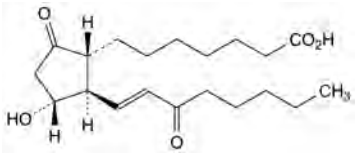

C. 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*)-3-oxooct-1-enyl]-5-oxocyclopentyl]heptanoic acid (15-oxoprostaglandin $E_1$),

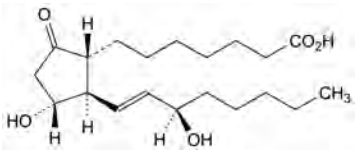

D. 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*R*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (15-epiprostaglandin $E_1$),

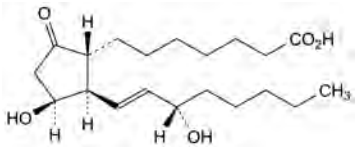

E. 7-[(1*R*,2*R*,3*S*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (11-epiprostaglandin $E_1$),

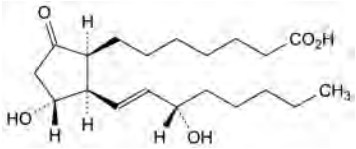

F. 7-[(1*S*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoic acid (8-epiprostaglandin $E_1$),

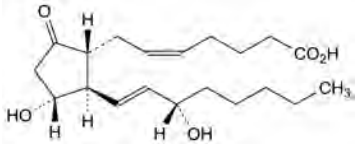

G. (5*Z*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (dinoprostone),

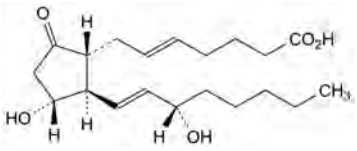

H. (5*E*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxy-oct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid ((5*E*)-prostaglandin $E_2$),

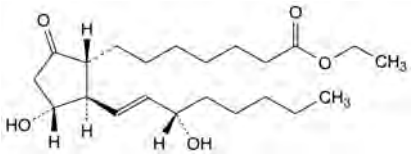

I. ethyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoate (prostaglandin $E_1$, ethyl ester),

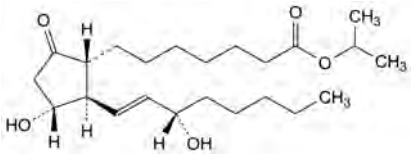

J. 1-methylethyl 7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]heptanoate (prostaglandin $E_1$, isopropyl ester),

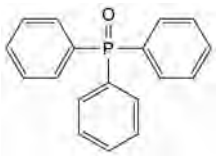

K. triphenylphosphine oxide.

**07/2013:1170**
**corrected 8.0**

# ALTEPLASE FOR INJECTION

## Alteplasum ad iniectabile

DEFINITION

Alteplase for injection is a sterile, freeze-dried preparation of alteplase, a tissue plasminogen activator produced by recombinant DNA technology. It has a potency of not less than 500 000 IU per milligram of protein.

Tissue plasminogen activator binds to fibrin clots and activates plasminogen, leading to the generation of plasmin and to the degradation of fibrin clots or blood coagulates.

Alteplase consists of 527 amino acids with a calculated relative molecular mass of 59 050 without consideration of the carbohydrate moieties attached at positions Asn 117, Asn 184 and Asn 448. The total relative molecular mass is approximately 65 000. Alteplase is cleaved by plasmin between amino-acids 275 and 276 into a two-chain form (A chain and B chain) that are connected by a disulfide bridge between Cys 264 and Cys 395. The single-chain form and the two-chain form show comparable fibrinolytic activity *in vitro*.

PRODUCTION

Alteplase is produced by recombinant DNA synthesis in cell culture; the fermentation takes place in serum-free medium.

The purification process is designed to remove efficiently potential impurities, such as antibiotics, DNA and protein contaminants derived both from the host cell and from the production medium, and potential viral contaminants.

If alteplase is stored in bulk form, stability (maintenance of potency) in the intended storage conditions must be demonstrated.

The production, purification and product consistency are checked by a number of analytical methods described below, carried out routinely as in-process controls.

**Protein content**. The protein concentration of alteplase solutions is determined by measuring the absorbance (*2.2.25*) of the protein solution at 280 nm and at 320 nm, using formulation buffer as the compensation liquid. If dilution of alteplase samples is necessary, the samples are diluted in formulation buffer. For the calculation of the alteplase concentration, the absorbance value ($A_{280} - A_{320}$) is divided by the specific absorption coefficient for alteplase of 1.9.

**Potency**. The potency of alteplase is determined in an *in vitro* clot-lysis assay as described under Assay. The specific activity of bulk alteplase is approximately 580 000 IU per milligram of alteplase.

***N*-terminal sequence**. *N*-terminal sequencing is applied to determine the correct *N*-terminal sequence and to determine semiquantitatively additional cleavage sites in the alteplase molecule, for example at position AA 275-276 or at position AA 27-28. The *N*-terminal sequence must conform with the sequence of human tissue plasminogen activator.

**Isoelectric focusing**. The consistency in the microheterogeneity of glycosylation of the alteplase molecule can be demonstrated by isoelectric focusing (IEF). A complex banding pattern with 10 major and several minor bands in the pH range 6.5-8.5 is observed. Denaturing conditions are applied to achieve a good separation of differently charged variants of alteplase. The broad charge distribution observed is due to a population of molecules, which differ in the fine structure of biantenary and triantenary complex-type carbohydrate residues, with different degrees of substitution with sialic acids. The banding pattern of alteplase test samples must be consistent with the pattern of alteplase reference standard.

**Single-chain alteplase content**. The alteplase produced by CHO (Chinese hamster ovary) cells in serum-free medium is predominantly single-chain alteplase. The single-chain form can be separated from the two-chain form by gel-permeation liquid chromatography under reducing conditions as described under Single-chain content (see Tests). The single-chain alteplase content in bulk samples must be higher than 60 per cent.

**Tryptic-peptide mapping**. The primary structure of the alteplase molecule is verified by tryptic-peptide mapping as described under Identification B. The reduced and carboxymethylated molecule is cleaved by trypsin into about 50 peptides, which are separated by reverse-phase liquid chromatography. A characteristic chromatogram (fingerprint) is obtained. The identity of the tryptic-peptide map of a given alteplase sample with the profile of a well-characterised reference standard is an indirect confirmation of the amino-acid sequence, because even single amino-acid exchanges in individual peptides can be detected by this sensitive technique. In addition, complex peaks of the glycopeptides can be isolated from the tryptic-peptide map and separated in a second dimension, either by reverse-phase liquid chromatography under modified conditions or by capillary electrophoresis. By this two-dimensional separation of glycopeptide variants, lot-to-lot consistency of the microheterogeneity of glycosylation can be demonstrated.

The tryptic-peptide map of alteplase samples must be consistent with the tryptic-peptide map of alteplase reference standard.

**Monomer content**. The monomer content of alteplase is measured by gel-permeation liquid chromatography under non-reduced conditions as described under Monomer content (see Tests). The monomer content of alteplase bulk samples must be higher than 95 per cent.

**Type I/Type II alteplase content**. CHO cells produce 2 glycosylation variants of alteplase. Type I alteplase contains 1 polymannose-type glycosylation at position Asn 117 and 2 complex-type glycosylation sites at positions Asn 184 and Asn 448. Type II alteplase is only glycosylated at positions Asn 117 and Asn 448.

The ratio of Type I/Type II alteplase is constant in the range of 45 to 65 per cent of Type I and 35 to 55 per cent of Type II. The content of alteplase Type I and Type II can be determined by a densitometric scan of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. Plasmin-treated samples of alteplase, which are reduced and carboxymethylated before loading on the gel, are separated into 3 bands: Type I alteplase A-chain (AA 1-275), Type II alteplase A-chain (AA 1-275) and alteplase B-chain (AA 276-527). The ratio of Type I/Type II alteplase is determined from a calibration curve, which is obtained by a densitometric scan of defined mixtures of purified Type I alteplase and Type II alteplase standards.

**SDS-PAGE**. SDS-PAGE (silver staining) is used to demonstrate purity of the alteplase bulk material and the integrity of the alteplase molecule. For alteplase bulk samples, no additional protein bands compared to reference standard or degradation products must occur in SDS-PAGE gels at a loading amount of 2.5 µg alteplase protein per lane and a limit of detection of 5 ng per protein (BSA) band.

**Bacterial endotoxins** (*2.6.14*): less than 1 IU per milligram of alteplase.

**Sialic acids**. Proceed using a suitable validated method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*. The sialic acids content for the test samples must be in the range of 70 to 130 per cent compared to alteplase reference standard, which contains about 3 moles of sialic acids per mole of alteplase.

**Neutral sugars**. Dilute alteplase samples and the reference standard in the assay buffer, containing 34.8 g/L of *arginine R*, 0.1 g/L of *polysorbate 80 R* and adjusted to pH 7.4 with *phosphoric acid R*, to a protein concentration of 50 µg/mL. Prepare the following concentrations of mannose in the same assay buffer for a calibration curve: 20, 30, 40, 50 and 60 µg/mL. Pipette 2 mL of alteplase samples and reference standard, as well as 2 mL of each mannose concentration in duplicate in reagent tubes. Add 50 µL of *phenol R*, followed by 5 mL of *sulfuric acid R*, in each reagent tube. Incubate the mixture for 30 min at room temperature. Measure the absorbance at 492 nm for each tube. Read the content of neutral sugars from the mannose calibration curve. The neutral sugar content is expressed in moles of neutral sugar per mole of alteplase, taking into account the dilution factor

for alteplase samples and reference standard and using a relative molecular mass of 180.2 for mannose and a relative molecular mass of 59 050 for the alteplase protein moiety. The neutral sugar content of the alteplase samples must be in the range of 70 to 130 per cent compared to alteplase reference standard, which contains about 12 moles of neutral sugar per mole of alteplase.

CHARACTERS

White or slightly yellow powder or solid friable mass.

*Reconstitute the preparation as stated on the label immediately before carrying out the Identification, Tests (except those for solubility and water) and Assay.*

IDENTIFICATION

A. The assay serves also to identify the preparation.

B. Tryptic-peptide mapping. Examine by liquid chromatography (*2.2.29*).

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a solution containing about 1 mg of alteplase per millilitre. Dialyse about 2.5 mL of the solution for at least 12 h into a solution containing 480 g/L of *urea R*, 44 g/L of *tris(hydroxymethyl)aminomethane R* and 1.5 g/L of *sodium edetate R* and adjusted to pH 8.6, using a membrane with a cut-off point corresponding to a relative molecular mass of 10 000 for globular proteins. Measure the volume of the solution, transfer it to a clean test-tube and add per millilitre 10 µL of a 156 g/L solution of *dithiothreitol R*. Allow to stand for 4 h, cool in iced water and add per millilitre of solution 25 µL of a freshly prepared 190 g/L solution of *iodoacetic acid R*. Allow to stand in the dark for 30 min. Add per millilitre 50 µL of dithiothreitol solution to stop the reaction. Dialyse for 24 h against an 8 g/L solution of *ammonium hydrogen carbonate R*. Add 1 part of *trypsin for peptide mapping R* to 100 parts of the protein and allow to stand for 6 h to 8 h. Repeat the addition of trypsin and allow to stand for a total of 24 h.

*Reference solution.* Prepare as for the test solution using a suitable reference standard instead of the preparation to be examined.

The chromatographic procedure may be carried out using:

– a column 0.1 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm to 10 µm);

*Mobile phase A.* 8 g/L solution of *sodium dihydrogen phosphate R*, adjusted to pH 2.85 with *phosphoric acid R*, filtered and degassed;

*Mobile phase B.* 75 per cent *V/V* solution of *acetonitrile R* in mobile phase A;

– as detector a spectrophotometer set at 210 nm.

Equilibrate the system with mobile phase A at a flow rate of 1 mL/min. After injection of the solution, increase the proportion of mobile phase B at a rate of 0.44 per cent per minute until the ratio of mobile phase A to mobile phase B is 60:40, then increase the proportion of mobile phase B at a rate of 1.33 per cent per minute until the ratio of mobile phase A to mobile phase B is 20:80 and then continue elution with this mixture for a further 10 min. Record the chromatogram for the reference solution: the test is not valid unless the resolution of peaks 6 (peptides 268-275) and 7 (peptides 1-7) is at least 1.5; $w_{h1}$ and $w_{h2}$ are not more than 0.4 min. Inject about 100 µL of the test solution and record the chromatogram. Verify the identity of the peaks by comparison with the chromatograms of the reference solution. There should not be any additional significant peaks or shoulders, a significant peak or shoulder being defined as one with an area response equal to or greater than 5 per cent of peak 19 (peptides 278-296); no significant peak is missing. A type chromatogram for identification of the peaks cited is shown in Figure 1170.-1.

TESTS

**Appearance of solution**. The reconstituted preparation is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.1 to 7.5.

**Solubility**. Add the volume of the liquid stated on the label. The preparation dissolves completely within 2 min at 20 °C to 25 °C.

**Protein content**. Prepare a solution of the substance to be examined with an accurately known concentration of about 1 g/L. Using a 34.8 g/L solution of *arginine R* adjusted to pH 7.3 with *phosphoric acid R*, dilute an accurately measured volume of the solution of the substance to be examined so that the absorbance measured at the maximum at about 280 nm is 0.5 to 1.0 (*test solution*). Measure the absorbance (*2.2.25*) of the solution at the maximum at about 280 nm and at 320 nm using the arginine solution as the compensation liquid. Calculate the protein content in the portion of alteplase taken from the following expression:

$$\frac{V(A_{280} - A_{320})}{1.9}$$

in which $V$ is the volume of the test solution, $A_{280}$ is the absorbance at the maximum at about 280 nm and $A_{320}$ is the absorbance at 320 nm.

**Single-chain content**. Examine by liquid chromatography (*2.2.29*).

*Test solution.* Dissolve the preparation to be examined in *water R* to obtain a solution containing about 1 mg of alteplase

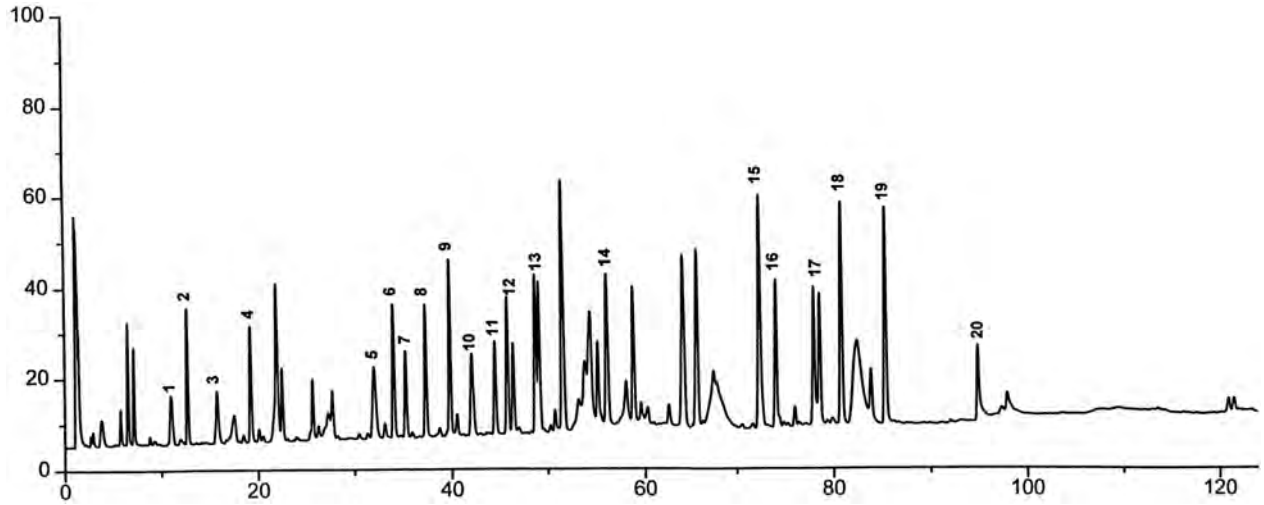


Figure 1170.-1. – *Chromatogram for tryptic-peptide mapping of alteplase*

per millilitre. Place about 1 mL of the solution in a tube, add 3 mL of a 3 g/L solution of *dithiothreitol R* in the mobile phase, place a cap on the tube and heat at about 80 °C for 3 min to 5 min.

The chromatographic procedure may be carried out using:

- a column 0.6 m long and 7.5 mm in internal diameter packed with silica-based, rigid, hydrophilic gel with spherical particles 10 µm to 13 µm in diameter, suitable for size-exclusion chromatography;
- as mobile phase at a flow rate of 0.5 mL/min a solution containing 30 g/L of *sodium dihydrogen phosphate R* and 1 g/L of *sodium dodecyl sulfate R*, adjusted to pH 6.8 with *dilute sodium hydroxide solution R*;
- as detector a spectrophotometer set at 214 nm.

Inject about 50 µL of the test solution and record the chromatogram. The chromatogram shows 2 major peaks corresponding to single-chain and two-chain alteplase. Calculate the relative amount of single-chain alteplase from the peak area values.

The test is not valid unless: the number of theoretical plates calculated on the basis of the single-chain alteplase peak is at least 1000. The content of single-chain alteplase is not less than 60 per cent of the total amount of alteplase-related substances found.

**Monomer content**. Examine by liquid chromatography (*2.2.29*).

*Test solution.* Reconstitute the preparation to be examined to obtain a solution containing about 1 mg per millilitre.

The chromatographic procedure may be carried out using:

- a column 0.6 m long and 7.5 mm in internal diameter packed with silica-based rigid, hydrophilic gel with spherical particles 10 µm to 13 µm in diameter, suitable for size-exclusion chromatography;
- as mobile phase at a flow rate of 0.5 mL/min a solution containing 30 g/L of *sodium dihydrogen phosphate R* and 1 g/L of *sodium dodecyl sulfate R*, adjusted to pH 6.8 with *dilute sodium hydroxide solution R*;
- as detector a spectrophotometer set at 214 nm.

Inject the test solution and record the chromatogram. The test is not valid unless the number of theoretical plates calculated for the alteplase monomer peak is at least 1000. Measure the response for all peaks, i.e. peaks corresponding to alteplase species of different molecular masses. Calculate the relative content of monomer from the area values of these peaks. The monomer content for alteplase must be at least 95 per cent.

**Water** (*2.5.12*). Not more than 4.0 per cent, determined by the semi-micro determination of water.

**Bacterial endotoxins** (*2.6.14*): less than 1 IU per milligram of protein.

**Sterility** (*2.6.1*). It complies with the test for sterility.

## ASSAY

The potency of alteplase is determined by comparing its ability to activate plasminogen to form plasmin with the same capacity of a reference preparation calibrated in International Units. The formation of plasmin is measured by the determination of the lysis time of a fibrin clot in given conditions.

The International Unit is the activity of a stated quantity of the International Standard of alteplase. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Solvent buffer.* A solution containing 1.38 g/L of *sodium dihydrogen phosphate monohydrate R*, 7.10 g/L of *anhydrous disodium hydrogen phosphate R*, 0.20 g/L of *sodium azide R* and 0.10 g/L of *polysorbate 80 R*.

*Human thrombin solution.* A solution of *human thrombin R* containing 33 IU/mL in solvent buffer.

*Human fibrinogen solution.* A 2 g/L solution of *fibrinogen R* in solvent buffer.

*Human plasminogen solution.* A 1 g/L solution of *human plasminogen R* in solvent buffer.

*Test solutions.* Using a solution of the substance to be examined containing 1 g/L, prepare serial dilutions using solvent buffer, for example 1:5000, 1:10 000, 1:20 000.

*Reference solutions.* Using a solution of a suitable reference standard having an accurately known concentration of about 1 g/L (580 000 IU of alteplase per millilitre), prepare 5 serial dilutions using *water R* to obtain reference solutions having known concentrations in the range 9.0 IU/mL to 145 IU/mL.

To each of a set of labelled glass test-tubes, add 0.5 mL of human thrombin solution. Allocate each test and reference solution to a separate tube and add to each tube 0.5 mL of the solution allocated to it. To each of a second set of labelled glass tubes, add 20 µL of human plasminogen solution, and 1 mL of human fibrinogen solution, mix and store on ice. Beginning with the reference/thrombin mixture containing the lowest number of International Units per millilitre, record the time and separately add 200 µL of each of the thrombin mixtures to the test tubes containing the plasminogen-fibrinogen mixture. Using a vortex mixer, intermittently mix the contents of each tube for a total of 15 s and carefully place in a rack in a circulating water-bath at 37 °C. A visibly turbid clot forms within 30 s and bubbles subsequently form within the clot. Record the clot-lysis time as the time between the first addition of alteplase solution and the moment when the last bubble rises to the surface. Using a least-squares fit, determine the equation of the line using the logarithms of the concentrations of the reference preparation in International Units per millilitre versus the logarithms of the values of their clot-lysis times in seconds, according to the following equation:

$$\log_{10} t = a + b\left(\log_{10} U_S\right)$$

in which $t$ is the clot-lysis time, $U_S$ the activity in International Units per millilitre of the reference preparation, $b$ is the slope and $a$ the $y$-intercept of the line. The test is not valid unless the correlation coefficient is − 0.9900 to − 1.0000. From the line equation and the clot-lysis time for the test solution, calculate the logarithm of the activity $U_A$ from the following equation:

$$\log_{10} U_A = \frac{\left[\left(\log_{10} t\right) - a\right]}{b}$$

Calculate the alteplase activity in International Units per millilitre from the following expression:

$$D \times U_A$$

in which $D$ is the dilution factor for the test solution. Calculate the specific activity in the portion of the substance to be examined from the following expression:

$$\frac{U_A}{P}$$

in which $P$ is the concentration of protein obtained in the test for protein content.

The estimated potency is not less than 90 per cent and not more than 110 per cent of the stated potency.

## STORAGE

Store in a colourless, glass container, under vacuum or under an inert gas, protected from light, at a temperature of 2 °C to 30 °C.

## LABELLING

The label states:

- the number of International Units per container;
- the amount of protein per container;
- the name and volume of the liquid to be used for reconstitution.

**07/2008:2185**

# ALTIZIDE

## Altizidum

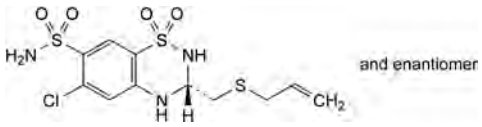


$C_{11}H_{14}ClN_3O_4S_3$ $M_r$ 383.9
[5588-16-9]

DEFINITION

(3*RS*)-6-Chloro-3-[(prop-2-enylsulfanyl)methyl]-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in methanol, practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *altizide CRS*.

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and 50 mg of the reference substance separately in 2 mL of *acetone R* and evaporate the solvent. Precipitate by adding 1 mL of *methylene chloride R*. Evaporate to dryness and record new spectra using the residues.

TESTS

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *acetone R* and dilute to 2.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10.0 mg of *altizide impurity B CRS* in *acetone R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b)*. To 1.0 mL of reference solution (a) add 1.0 mL of the test solution.

*Reference solution (c)*. Dilute 5.0 mL of reference solution (a) to 10.0 mL with *acetone R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetone R*, *methylene chloride R* (25:75 *V/V*).

*Application*: 10 µL of the test solution and reference solutions (b) and (c).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a mixture of equal volumes of a 10 g/L solution of *potassium permanganate R* and a 50 g/L solution of *sodium carbonate R*, prepared immediately before use. Allow to stand for 30 min and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*: any spot due to impurity B is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, except reference solution (b).*

*Test solution*. Dissolve 50 mg of the substance to be examined in 5 mL of *acetonitrile R* and dilute to 25 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. In order to produce impurity A *in situ*, dissolve 50 mg of the substance to be examined in 5 mL of *acetonitrile R* and dilute to 25 mL with *water R*. Allow to stand for 30 min.

*Reference solution (c)*. Dissolve 4 mg of *furosemide CRS* in 2 mL of *acetonitrile R*, add 2 mL of the test solution and dilute to 100 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, *water R* previously adjusted to pH 2.0 with *perchloric acid R* (25:75 *V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 5 µL.

*Run time*: twice the retention time of altizide.

*Relative retention* with reference to altizide (retention time = about 25 min): impurity A = about 0.15; furosemide = about 1.05.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.0 between the peaks due to altizide and furosemide.

*Limits*:

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 50.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances, with the following modifications.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 2 mL of *acetonitrile R* and dilute to 25.0 mL with the mobile phase.

*Reference solution*. Dissolve 25.0 mg of *altizide CRS* in 2 mL of *acetonitrile R* and dilute to 25.0 mL with the mobile phase.

Calculate the percentage content of $C_{11}H_{14}ClN_3O_4S_3$ from the declared content of *altizide CRS*.

IMPURITIES

*Specified impurities: A, B.*

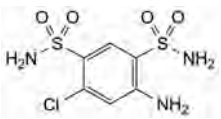

A. 4-amino-6-chlorobenzene-1,3-disulfonamide,

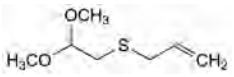

B. 3-[(2,2-dimethoxyethyl)sulfanyl]prop-1-ene.

**01/2017:0006**

# ALUM

## Alumen

$AlK(SO_4)_2,12H_2O$ $M_r$ 474.4
[7784-24-9]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent of $AlK(SO_4)_2,12H_2O$.

CHARACTERS

*Appearance*: granular powder or colourless, transparent, crystalline masses.

*Solubility*: freely soluble in water, very soluble in boiling water, soluble in glycerol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of sulfates (*2.3.1*).

B. Solution S gives the reaction of aluminium (*2.3.1*).

C. Shake 10 mL of solution S with 0.5 g of *sodium hydrogen carbonate R* and filter. The filtrate gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 3.5.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Ammonium** (*2.4.1*): maximum 0.2 per cent.

To 1 mL of solution S add 4 mL of *water R*. Dilute 0.5 mL of this solution to 14 mL with *water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*. Use in this test 0.3 mL of *thioglycollic acid R*.

ASSAY

Dissolve 0.900 g in 20 mL of *water R* and carry out the complexometric titration of aluminium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 47.44 mg of $AlK(SO_4)_2,12H_2O$.

**01/2017:0971**

# ALUMINIUM CHLORIDE HEXAHYDRATE

## Aluminii chloridum hexahydricum

$AlCl_3,6H_2O$ $M_r$ 241.4
[7784-13-6]

DEFINITION

*Content*: 95.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder or colourless crystals, deliquescent.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), soluble in glycerol.

IDENTIFICATION

A. Dilute 0.1 mL of solution S2 (see Tests) to 2 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

B. Dilute 0.3 mL of solution S2 to 2 mL with *water R*. The solution gives the reaction of aluminium (*2.3.1*).

TESTS

**Solution S1**. Dissolve 10.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Solution S2**. Dilute 50 mL of solution S1 to 100 mL with *water R*.

**Appearance of solution**. Solution S2 is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S1.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S1.

**Alkali and alkaline-earth metals**: maximum 0.5 per cent.

To 20 mL of solution S2 add 100 mL of *water R* and heat to boiling. To the hot solution add 0.2 mL of *methyl red solution R*. Add *dilute ammonia R1* until the colour of the indicator changes to yellow and dilute to 150 mL with *water R*. Heat to boiling and filter. Evaporate 75 mL of the filtrate to dryness on a water-bath and ignite to constant mass. The residue weighs a maximum of 2.5 mg.

**Water** (*2.5.12*): 42.0 per cent to 48.0 per cent, determined on 50.0 mg.

ASSAY

Dissolve 0.500 g in 25.0 mL of *water R*. Carry out the complexometric titration of aluminium (*2.5.11*). Titrate with *0.1 M zinc sulfate* until the colour of the indicator changes from greyish-green to pink. Carry out a blank titration.

1 mL of *0.1 M sodium edetate* is equivalent to 24.14 mg of $AlCl_3,6H_2O$.

STORAGE

In an airtight container.

**01/2017:1664**
**corrected 10.0**

# ALUMINIUM HYDROXIDE, HYDRATED, FOR ADSORPTION

## Aluminii hydroxidum hydricum ad adsorptionem

$[AlO(OH)],xH_2O$

DEFINITION

*Content*: 90.0 per cent to 110.0 per cent of the content of aluminium stated on the label.

*NOTE*: *shake the gel vigorously for at least 30 s immediately before examining.*

CHARACTERS

*Appearance*: white or almost white, translucent, viscous, colloidal gel. A supernatant may be formed upon standing.

*Solubility*: a clear or almost clear solution is obtained with alkali hydroxide solutions and mineral acids.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium.

To 10 mL of solution S add about 0.5 mL of *dilute hydrochloric acid R* and about 0.5 mL of *thioacetamide reagent R*. No precipitate is formed. Add dropwise 5 mL of *dilute sodium hydroxide solution R*. Allow to stand for 1 h. A gelatinous white precipitate is formed which dissolves upon addition of 5 mL of *dilute sodium hydroxide solution R*. Gradually add 5 mL of *ammonium chloride solution R* and allow to stand for 30 min. The gelatinous white precipitate is re-formed.

TESTS

**Solution S**. Add 1 g to 4 mL of *hydrochloric acid R*. Heat at 60 °C for 1 h, cool, dilute to 50 mL with *distilled water R* and filter if necessary.

**pH** (*2.2.3*): 5.5 to 8.5.

**Adsorption power**. Dilute the substance to be examined with *distilled water R* to obtain an aluminium concentration of 5 mg/mL. Prepare *bovine albumin R* solutions with the following concentrations of bovine albumin: 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 3 mg/mL, 5 mg/mL and 10 mg/mL. If necessary, adjust the gel and the *bovine albumin R* solutions to pH 6.0 with *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*.

For adsorption, mix 1 part of the diluted gel with 4 parts of each of the solutions of *bovine albumin R* and allow to stand at room temperature for 1 h. During this time shake the mixture vigorously at least 5 times. Centrifuge or filter through a non-protein-retaining filter. Immediately determine the protein content (*2.5.33, Method 2*) of either the supernatant or the filtrate.

It complies with the test if no bovine albumin is detectable in the supernatant or filtrate of the 2 mg/mL *bovine albumin R* solution (maximum level of adsorption) and in the supernatant or filtrate of *bovine albumin R* solutions of lower concentrations. Solutions containing 3 mg/mL, 5 mg/mL and 10 mg/mL *bovine albumin R* may show bovine albumin in the supernatant or filtrate, proportional to the amount of bovine albumin in the solutions.

**Sedimentation**. If necessary, adjust the substance to be examined to pH 6.0 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Dilute with *distilled water R* to obtain an aluminium concentration of approximately 5 mg/mL. If the aluminium content of the substance to be examined is lower than 5 mg/mL, adjust to pH 6.0 and dilute with a 9 g/L solution of *sodium chloride R* to obtain an aluminium concentration of about 1 mg/mL. After shaking for at least 30 s, place 25 mL of the preparation in a 25 mL graduated cylinder and allow to stand for 24 h.

It complies with the test if the volume of the clear supernatant is less than 5 mL for the gel with an aluminium content of about 5 mg/mL.

It complies with the test if the volume of the clear supernatant is less than 20 mL for the gel with an aluminium content of about 1 mg/mL.

**Chlorides** (*2.4.4*): maximum 0.33 per cent.

Dissolve 0.5 g in 10 mL of *dilute nitric acid R* and dilute to 500 mL with *water R*.

**Nitrates**: maximum 100 ppm.

Place 5 g in a test-tube immersed in ice-water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 5 mL of *nitrate standard solution (100 ppm $NO_3$) R*.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 2 mL of solution S to 20 mL with *water R*.

**Ammonium** (*2.4.1, Method B*): maximum 50 ppm, determined on 1.0 g.

Prepare the standard using 0.5 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Arsenic** (*2.4.2, Method A*): maximum 1 ppm, determined on 1 g.

**Iron** (*2.4.9*): maximum 15 ppm, determined on 0.67 g.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU of endotoxin per milligram of aluminium, if intended for use in the manufacture of an adsorbed product without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 2.50 g in 10 mL of *hydrochloric acid R*, heating for 30 min at 100 °C on a water-bath. Cool and dilute to 20 mL with *water R*. To 10 mL of the solution, add *concentrated ammonia R* until a precipitate is obtained. Add the smallest quantity of *hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 mL with *water R*. Carry out the complexometric titration of aluminium (*2.5.11*). Carry out a blank titration.

STORAGE

At a temperature not exceeding 30 °C. Do not allow to freeze. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the declared content of aluminium.

**01/2020:1388**

# ALUMINIUM MAGNESIUM SILICATE

## Aluminii magnesii silicas

DEFINITION

Mixture of colloidal-size particles of montmorillonite and saponite, practically free from grit and non-swellable ore.

The requirements for viscosity and ratio of aluminium content to magnesium content differ for the several types of aluminium magnesium silicate, as shown in the table below.

| Type | Viscosity (mPa·s) | | Al content / Mg content | |
|---|---|---|---|---|
| | min. | max. | min. | max. |
| IA | 225 | 600 | 0.5 | 1.2 |
| IB | 150 | 450 | 0.5 | 1.2 |
| IC | 800 | 2200 | 0.5 | 1.2 |
| IIA | 100 | 300 | 1.4 | 2.8 |

CHARACTERS

*Appearance*: almost white, coarse powder, granules or flakes (types IA, IC and IIA); almost white, fine powder (type IB).

*Solubility*: practically insoluble in water and in organic solvents.

It swells in water to produce a colloidal dispersion.

IDENTIFICATION

Carry out either tests A, B, C, F or tests D, E.

A. In a platinum crucible mix 1.0 g with 5.0 g of *anhydrous lithium metaborate R*. Heat slowly at first and ignite at 1000-1200 °C for 15 min. Allow to cool and crush the residue. 0.25 g of the residue gives the reaction of silicates (*2.3.1*).

B. Dissolve 1.0 g of the residue obtained in identification test A in a mixture of 5 mL of *dilute hydrochloric acid R* and 10 mL of *water R*. Filter to obtain a clear solution and add *ammonium chloride buffer solution pH 10.0 R*. A white, gelatinous precipitate is formed. Centrifuge and keep the supernatant for identification test C. Dissolve the precipitate in *dilute hydrochloric acid R*. Add dropwise *dilute sodium hydroxide solution R*. A white gelatinous precipitate is formed. Filter and add a few drops of *phenolphthalein solution R* to the residue. The residue turns pink. Wash the residue with *water R* until the pink colour is completely discharged and the residue remains white upon addition of a drop of *phenolphthalein solution R*. Sprinkle a few crystals of *sodium fluoride R* on the residue. The residue, in contact with the crystals, turns pink again in a short time.

C. To 2 mL of the supernatant obtained after centrifugation in identification test B, add 1 mL of *dilute ammonia R1* and 1 mL of *ammonium chloride solution R*. Upon the addition of *dilute ammonia R1* a white precipitate may form, which dissolves after addition of the *ammonium chloride solution R*. Add 1 mL of *disodium hydrogen phosphate solution R*. A white precipitate is formed.

D. X-ray diffraction (*2.9.33*), oriented sample.

Add 2 g in small portions to 100 mL of *water R*, with vigorous shaking. Allow to stand for at least 12 h to ensure complete hydration. Place 2 mL of the resulting mixture on a suitable glass slide and allow to dry in air at room temperature to produce an oriented film. Place the slide in a vacuum desiccator over *ethylene glycol R*. Evacuate the desiccator and close the stopcock so that the ethylene glycol saturates the chamber. Allow to stand for at least 12 h. Record the X-ray diffraction pattern and calculate the *d* values: the largest peak corresponds to a *d* value between 1.5 nm and 1.72 nm.

E. X-ray diffraction (*2.9.33*), random sample.

Prepare a random powder sample, record the X-ray diffraction pattern and determine the *d* values in the region between 0.148 nm and 0.154 nm. Peaks are found between 0.1492 nm and 0.1504 nm and between 0.1510 nm and 0.1540 nm.

F. It complies with the limits of the assay.

TESTS

**pH** (*2.2.3*): 9.0 to 10.0.

Disperse 5.0 g in 100 mL of *carbon dioxide-free water R*.

**Viscosity** (*2.2.10*). Weigh a quantity of the substance to be examined equivalent to 25.0 g of the dried substance and immediately transfer to a suitable 1 L blender jar containing a quantity of *water R*, at 25 ± 2 °C, that is sufficient to produce a mixture weighing 500 g. Blend for exactly 3 min, at 14 000-15 000 r/min. The heat generated during blending causes the temperature to rise to above 30 °C. Transfer the contents of the blender to a 600 mL beaker and allow to stand for 5 min. The sample temperature should be 33 ± 3 °C.

Using a suitable rotating viscometer equipped with a spindle as specified below, operate the viscometer at 60 r/min for exactly 6 min and record the scale reading.

For type IA, use a spindle with a cylinder 1.87 cm in diameter and 0.69 cm high attached to a shaft 0.32 cm in diameter, the distance from the top of the cylinder to the lower tip of the shaft being 2.54 cm, and an immersion depth of 5.00 cm (No. 2 spindle); if the scale reading is greater than 90 per cent of the full scale, repeat the measurement using a spindle similar to the No. 2 spindle but with a cylinder 1.27 cm in diameter and 0.16 cm high (No. 3 spindle).

For type IC, use a No. 3 spindle; if the scale reading is greater than 90 per cent of the full scale, repeat the measurement using a spindle with a cylindrical shaft 0.32 cm in diameter and an immersion depth of 4.05 cm (No. 4 spindle).

For types IB and IIA, use a No. 2 spindle.

*Limits*:

| Type | Viscosity (mPa·s) | |
|---|---|---|
| | min. | max. |
| IA | 225 | 600 |
| IB | 150 | 450 |
| IC | 800 | 2200 |
| IIA | 100 | 300 |

**Acid demand**. Weigh a quantity of the substance to be examined equivalent to 5.00 g of the dried substance and disperse in 500 mL of *water R* using a suitable blender fitted with a 1 L jar. With constant mixing, add 3.0 mL portions of *0.1 M hydrochloric acid* at 5 s, 65 s, 125 s, 185 s, 245 s, 305 s, 365 s, 425 s, 485 s, 545 s, 605 s, 665 s and 725 s and add a 1.0 mL portion at 785 s. The pH (*2.2.3*) determined at 840 s is not greater than 4.0.

**Arsenic** (*2.4.2, Method A*): maximum 3 ppm.

Transfer 16.6 g to a 250 mL beaker containing 100 mL of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil gently, with occasional stirring, for 15 min. Allow the insoluble matter to settle and decant the supernatant through a rapid-flow filter paper into a 250 mL volumetric flask, retaining as much sediment as possible in the beaker. To the residue in the beaker add 25 mL of hot *dilute hydrochloric acid R*, stir, heat to boiling, allow the insoluble matter to settle and decant the supernatant through the filter into the volumetric flask. Repeat the extraction with 4 additional quantities, each of 25 mL, of hot *dilute hydrochloric acid R*, decanting each supernatant through the filter into the volumetric flask. At the last extraction, transfer as much of the insoluble matter as possible onto the filter. Allow the combined filtrates to cool to room temperature and dilute to 250.0 mL with *dilute hydrochloric acid R*. Dilute 5.0 mL of this solution to 25.0 mL with *dilute hydrochloric acid R*.

**Lead**: maximum 15 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Transfer 10.0 g to a 250 mL beaker containing 100 mL of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil for 15 min. Allow to cool to room temperature and allow the insoluble matter to settle. Decant the supernatant through a rapid-flow filter paper into a 400 mL beaker. To the insoluble matter in the 250 mL beaker add 25 mL of hot *water R*. Stir, allow the insoluble matter to settle and decant the supernatant through the filter into the 400 mL beaker. Repeat the extraction with 2 additional quantities, each of 25 mL, of *water R*, decanting each time the supernatant through the filter into the 400 mL beaker. Wash the filter with 25 mL of hot *water R*, collecting this filtrate in the 400 mL beaker. Concentrate the combined filtrates to about 20 mL by gently boiling. If a precipitate appears, add about 0.1 mL of *nitric acid R*, heat to boiling and allow to cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 50 mL volumetric flask. Transfer the remaining contents of the 400 mL beaker through the filter paper and into the flask with *water R*. Dilute this solution to 50.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluted as necessary with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217 nm.

*Atomisation device*: oxidising air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 110 °C.

**Microbial contamination**.

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

**Aluminium**. Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* In a platinum crucible mix 0.200 g with 1.0 g of *anhydrous lithium metaborate R*. Heat slowly at first and ignite at 1000-1200 °C for 15 min. Allow to cool, place the crucible in a 100 mL beaker containing 25 mL of a 40 per cent *V/V* solution of *dilute nitric acid R* and add 50 mL of a 40 per cent *V/V* solution of *dilute nitric acid R*, filling and submerging the crucible. Place a polytetrafluoroethylene-coated magnetic stirring bar in the crucible and stir gently with a magnetic stirrer until dissolution is complete. Transfer the solution to a 200 mL volumetric flask, wash the beaker, crucible and magnetic stirrer bar with *water R*, collecting the washings in the volumetric flask, and dilute to 200.0 mL with *water R* (solution A). To 20.0 mL of solution A add 20 mL of a 10 g/L solution of *sodium chloride R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Dissolve, with gentle heating, 1.000 g of *aluminium R* in a mixture of 10 mL of *hydrochloric acid R* and 10 mL of *water R*. Allow to cool, then dilute to 1000.0 mL with *water R* (1 mg of aluminium per millilitre). Into 4 identical volumetric flasks, each containing 0.20 g of *sodium chloride R*, introduce 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL of this solution respectively, and dilute to 100.0 mL with *water R*.

*Blank solution.* Dissolve 0.20 g of *sodium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309 nm.

*Atomisation device*: acetylene-nitrous oxide flame.

**Magnesium**. Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 25.0 mL of solution A, prepared in the assay for aluminium, to 50.0 mL with *water R*. To 5.0 mL of this solution add 20.0 mL of *lanthanum chloride solution R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Place 1.000 g of *magnesium R* in a 250 mL beaker containing 20 mL of *water R* and carefully add 20 mL of *hydrochloric acid R*, warming if necessary to dissolve. Transfer the solution to a volumetric flask and dilute to 1000.0 mL with *water R* (1 mg of magnesium per millilitre). Dilute 5.0 mL of this solution to 500.0 mL with *water R*. Into 4 identical volumetric flasks, introduce 5.0 mL, 10.0 mL, 15.0 mL and 20.0 mL of the solution respectively. To each flask add 20.0 mL of *lanthanum chloride solution R* and dilute to 100.0 mL with *water R*.

*Blank solution.* Dilute 20 mL of *lanthanum chloride solution R* to 100.0 mL with *water R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285 nm.

*Atomisation device*: air-acetylene flame.

LABELLING

The label states the ratio of aluminium content to magnesium content, the viscosity and the corresponding type (see Definition).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for aluminium magnesium silicate used as viscosity-increasing agent and stabiliser.*

**Viscosity** (see Tests).

**01/2017:0311**

# ALUMINIUM OXIDE, HYDRATED

## Aluminii oxidum hydricum

DEFINITION

*Content*: 47.0 per cent to 60.0 per cent of $Al_2O_3$ ($M_r$ 102.0).

CHARACTERS

*Appearance*: white or almost white, amorphous powder.

*Solubility*: practically insoluble in water. It dissolves in dilute mineral acids and in solutions of alkali hydroxides.

IDENTIFICATION

Solution S (see Tests) gives the reaction of aluminium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in 15 mL of *hydrochloric acid R*, heating on a water-bath. Dilute to 100 mL with *distilled water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

**Alkaline impurities**. Shake 1.0 g with 20 mL of *carbon dioxide-free water R* for 1 min and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. Any pink colour disappears on the addition of 0.3 mL of *0.1 M hydrochloric acid*.

**Neutralising capacity**. *Carry out the test at 37 °C.* Disperse 0.5 g in 100 mL of *water R*, heat, add 100.0 mL of *0.1 M hydrochloric acid*, previously heated, and stir continuously; the pH (*2.2.3*) of the solution after 10 min, 15 min and 20 min is not less than 1.8, 2.3 and 3.0 respectively and is at no time greater than 4.5. Add 10.0 mL of *0.5 M hydrochloric acid*, previously heated, stir continuously for 1 h and titrate with *0.1 M sodium hydroxide* to pH 3.5; not more than 35.0 mL of *0.1 M sodium hydroxide* is required.

**Chlorides** (*2.4.4*): maximum 1 per cent.

Dissolve 0.1 g with heating in 10 mL of *dilute nitric acid R* and dilute to 100 mL with *water R*. Dilute 5 mL of the solution to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1 per cent.

Dilute 4 mL of solution S to 100 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 10 mL of solution S.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of bile-tolerant gram-negative bacteria (*2.6.13*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Dissolve 0.800 g in 10 mL of *hydrochloric acid R1*, heating on a water-bath. Cool and dilute to 50.0 mL with *water R*. To 10.0 mL of the solution add *dilute ammonia R1* until a precipitate begins to appear. Add the smallest quantity of *dilute hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 mL with *water R*. Carry out the complexometric titration of aluminium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 5.098 mg of $Al_2O_3$.

STORAGE

In an airtight container, at a temperature not exceeding 30 °C.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hydrated aluminium oxide used as adsorbent.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26*).

**01/2017:2166**

# ALUMINIUM PHOSPHATE GEL

## Aluminii phosphatis liquamen

DEFINITION

Hydrated $AlPO_4$ in gel form.

*Content*: 19.0 per cent to 21.0 per cent of $AlPO_4$.

CHARACTERS

*Appearance*: gel.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

A. Solution S (see Tests) gives reaction (b) of phosphates (*2.3.1*).

B. Solution S gives the reaction of aluminium (*2.3.1*).

C. It complies with the assay.

TESTS

**Solution S**. Dissolve 2.00 g in *dilute hydrochloric acid R* and dilute to 100 mL with the same acid.

**pH** (*2.2.3*): 6.0 to 8.0.

**Peroxides**: maximum 150 ppm, expressed as hydrogen peroxide.

*Test solution.* Dissolve with heating 1.0 g of the substance to be examined in 5 mL of *dilute hydrochloric acid R*, then add 5 mL of *water R* and 2 mL of *divanadium pentoxide solution in sulfuric acid R*.

*Reference solution.* Dilute 1.0 mL of *dilute hydrogen peroxide solution R* to 200.0 mL with *water R*. To 1 mL of this solution add 9 mL of *water R* and 2 mL of *divanadium pentoxide solution in sulfuric acid R*.

The test solution is not more intensely coloured than the reference solution.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dissolve 1.3 g in 5 mL of *dilute nitric acid R* and dilute to 200 mL with *water R*.

**Soluble phosphates**: maximum 0.5 per cent, expressed as $PO_4$.

*Test solution.* Centrifuge 10.0 g until a clear supernatant is obtained. To 2.00 mL of the supernatant add 20.0 mL of a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with *water R*. To 10.0 mL of this solution add 10.0 mL of *nitro-molybdovanadic reagent R* and dilute to 50.0 mL with *water R*. Allow to stand protected from light for 15 min.

*Reference solution.* Add 10.0 mL of *nitro-molybdovanadic reagent R* to 10.0 mL of a 143 mg/L solution of *potassium dihydrogen phosphate R* and dilute to 50.0 mL with *water R*. Allow to stand protected from light for 15 min.

Measure the absorbances (*2.2.25*) of the 2 solutions at 400 nm. The absorbance of the test solution is not greater than that of the reference solution.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Dilute 25 mL of solution S to 100 mL with *distilled water R*.

**Soluble aluminium**: maximum 50 ppm.

To 16.0 g add 50 mL of *water R*. Heat to boiling for 5 min. Cool and centrifuge. Separate the supernatant. Wash the residue with 20 mL of *water R* and centrifuge. Separate the supernatant and add to the first supernatant. To the combined supernatants add 5 mL of *hydrochloric acid R* and 20 mL of *water R*. Introduce all of this solution into a 500 mL conical flask and carry out the complexometric titration of aluminium (*2.5.11*) using *0.01 M sodium edetate*.

**Arsenic** (*2.4.2, Method A*): maximum 1 ppm, determined on 1.0 g.

**Acid neutralising capacity**. Add 2.0 g to 30 mL of *0.1 M hydrochloric acid* heated to 37 °C and maintain at 37 °C while shaking. Determine the pH after 15 min. The pH (*2.2.3*) of the mixture is 2.0 to 2.5.

**Residue on ignition**: 19.0 per cent to 23.0 per cent.

Heat 0.500 g at 50 °C for 5 hours, then ignite at 500 ± 50 °C until constant mass.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of bile-tolerant gram-negative bacteria (*2.6.13*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Dissolve with heating 0.300 g in 5 mL of *dilute hydrochloric acid R*. Add 45 mL of *water R*, 10.0 mL of *0.1 M sodium edetate* and 30 mL of a mixture of equal volumes of *ammonium acetate solution R* and *dilute acetic acid R*. Heat to boiling and

maintain boiling for 3 min. Cool, then add 25 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M zinc sulfate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M zinc sulfate* is equivalent to 12.2 mg of $AlPO_4$.

STORAGE

In an airtight container.

**01/2017:1598**

# ALUMINIUM PHOSPHATE, HYDRATED

## Aluminii phosphas hydricus

$AlPO_4,xH_2O$ $M_r$ 122.0 (anhydrous substance)

DEFINITION

*Content*: 94.0 per cent to 102.0 per cent of $AlPO_4$ ($M_r$ 122.0) (ignited substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

A. Solution S (see Tests) gives reaction (b) of phosphates (*2.3.1*).

B. Solution S gives the reaction of aluminium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.00 g in *dilute hydrochloric acid R* and dilute to 100 mL with the same acid.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 7.2

Shake 4.0 g with *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 1.3 per cent.

Dissolve 50.0 mg in 10 mL of *dilute nitric acid R* and dilute to 200 mL with *water R*.

**Soluble phosphates**: maximum 1.0 per cent, calculated as $PO_4^{3-}$.

*Test solution*. Stir 5.0 g with 150 mL of *water R* for 2 h. Filter and wash the filter with 50 mL of *water R*. Combine the filtrate and the washings and dilute to 250.0 mL with *water R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (a)*. Dissolve 2.86 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (b)*. Dilute 1 mL of reference solution (a) to 5 mL with *water R*.

*Reference solution (c)*. Dilute 3 mL of reference solution (a) to 5 mL with *water R*.

Treat each solution as follows. To 5.0 mL add 4 mL of *dilute sulfuric acid R*, 1 mL of *ammonium molybdate solution R*, 5 mL of *water R* and 2 mL of a solution containing 0.10 g of *4-methylaminophenol sulfate R*, 0.5 g of *anhydrous sodium sulfite R* and 20.0 g of *sodium metabisulfite R* in 100 mL of *water R*. Shake and allow to stand for 15 min. Dilute to 25.0 mL with *water R* and allow to stand for a further 15 min. Measure the absorbance (*2.2.25*) at 730 nm. Calculate the content of soluble phosphates from a calibration curve prepared using reference solutions (a), (b) and (c) after treatment.

**Sulfates** (*2.4.13*): maximum 0.6 per cent.

Dilute 8 mL of solution S to 100 mL with *distilled water R*.

**Arsenic** (*2.4.2*): maximum 1 ppm.

1.0 g complies with limit test A.

**Loss on ignition**. 10.0 per cent to 20.0 per cent, determined on 1.000 g at 800 ± 50 °C.

**Neutralising capacity**. Add 0.50 g to 30 mL of *0.1 M hydrochloric acid* previously heated to 37 °C and maintain at this temperature for 15 min while stirring. The pH (*2.2.3*) of the mixture after 15 min at 37 °C is 2.0 to 2.5.

ASSAY

Dissolve 0.400 g in 10 mL of *dilute hydrochloric acid R* and dilute to 100.0 mL with *water R*. To 10.0 mL of the solution, add 10.0 mL of *0.1 M sodium edetate* and 30 mL of a mixture of equal volumes of *ammonium acetate solution R* and *dilute acetic acid R*. Boil for 3 min, then cool. Add 25 mL of *ethanol (96 per cent) R* and 1 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *ethanol (96 per cent) R*. Titrate the excess of sodium edetate with *0.1 M zinc sulfate* until the colour changes to pink.

1 mL of *0.1 M sodium edetate* is equivalent to 12.20 mg of $AlPO_4$.

STORAGE

In an airtight container.

**01/2009:1676**
**corrected 7.0**

# ALUMINIUM SODIUM SILICATE

## Aluminii natrii silicas

DEFINITION

Silicic acid aluminium sodium salt of synthetic origin.

*Content*:

– *aluminium* (Al; $M_r$ 26.98): 2.7 per cent to 7.9 per cent (dried substance);

– *sodium* (Na; $M_r$ 22.99): 3.7 per cent to 6.3 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine, light, amorphous powder.

*Solubility*: practically insoluble in water and in organic solvents.

IDENTIFICATION

A. Transfer 1.0 g to a 100 mL beaker and add 10 mL of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil for 15 min. Allow to cool to room temperature, mix and centrifuge the solution. 2 mL of the supernatant gives the reaction of aluminium (*2.3.1*).

B. 2 mL of the supernatant obtained in identification test A gives reaction (a) of sodium (*2.3.1*).

C. 0.2 g gives the reaction of silicates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 9.5 to 11.5.

Disperse 5.0 g in 100 mL of *carbon dioxide-free water R*.

**Arsenic** (*2.4.2, Method A*): maximum 3 ppm.

Transfer 8.3 g to a 250 mL beaker containing 50 mL of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil gently, with occasional stirring, for 15 min. Centrifuge, and decant the supernatant through a rapid-flow filter paper into a 250 mL volumetric flask. To the residue in the beaker, add 25 mL of hot *dilute hydrochloric acid R*, stir, centrifuge, and decant the supernatant through the same filter into the volumetric flask. Repeat the extraction with 3 additional quantities, each of 25 mL, of hot *dilute hydrochloric acid R*, filtering each supernatant through this filter into the volumetric flask. Allow the combined filtrates to cool to room temperature and dilute to 250.0 mL with *dilute hydrochloric acid R*. Dilute 10.0 mL of the solution to 25.0 mL with *water R*.

**Lead**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Transfer 5.0 g to a 250 mL beaker containing 50 mL of *dilute hydrochloric acid R*. Mix, cover with a watch glass and boil for 15 min. Allow to cool to room temperature. Centrifuge, and decant the supernatant through a rapid-flow filter paper into a 250 mL beaker. To the insoluble matter add 25 mL of hot *water R*. Stir vigorously, centrifuge, and decant the supernatant through the same filter into the beaker. Repeat the extraction with 2 additional quantities, each of 25 mL, of hot *water R*, decanting each supernatant through the filter into the beaker. Wash the filter with 25 mL of hot *water R*, collecting the filtrate in the beaker. Concentrate the combined filtrates by gently boiling to about 15 mL. Add about 0.05 mL of *heavy metal-free nitric acid R*, heat to boiling and allow to cool to room temperature. Filter the concentrated extracts through a rapid-flow filter paper into a 25 mL volumetric flask. Transfer the remaining contents of the beaker through the filter paper and into the volumetric flask with *water R* and dilute to 25.0 mL with the same solvent.

*Reference solutions.* Into 4 separate 100 mL volumetric flasks, introduce respectively 3.0 mL, 5.0 mL, 10.0 mL and 15.0 mL of *lead standard solution (10 ppm Pb) R*, add 0.20 mL of *heavy metal-free nitric acid R* and dilute to 100.0 mL with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217.0 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Loss on ignition**: 5.0 per cent to 11.0 per cent (dried substance), determined on 1.000 g by ignition in a platinum crucible to constant mass at 1000 ± 25 °C.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

**Aluminium**. Atomic absorption spectrometry (*2.2.23, Method I*).

*Acid mixture.* Add 50 mL of *nitric acid R* to 500 mL of *water R*. Dissolve in this solution 17 g of *tartaric acid R* and dilute to 1000 mL with *water R*.

*Blank solution.* Dissolve 1.4 g of *anhydrous lithium metaborate R* in 60 mL of the acid mixture and dilute to 200 mL with *water R*.

*Test solution.* In a platinum crucible mix 0.200 g with 1.4 g of *anhydrous lithium metaborate R*. Heat slowly at first and ignite at 1100 ± 25 °C for 15 min. Cool, then place the crucible in a 100 mL beaker containing 60 mL of the acid mixture. Place a polytetrafluoroethylene-coated magnetic stirring bar in the crucible and stir gently with a magnetic stirrer for 16 h. Transfer the contents of the crucible into a 200 mL volumetric flask. Wash the crucible, the magnetic stirring bar and the beaker with *water R* and dilute to 200.0 mL with the same solvent (solution A). To 10.0 mL of this solution, add 1.0 mL of *lanthanum chloride solution R* and dilute to 50.0 mL with *water R*.

*Reference solutions.* Into 5 separate 50 mL volumetric flasks, introduce respectively 1.0 mL, 2.5 mL, 5.0 mL, 7.5 mL and 10.0 mL of *aluminium standard solution (100 ppm Al) R*, add 1 mL of *lanthanum chloride solution R* and 10 mL of the blank solution, and dilute to 50.0 mL with *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: acetylene-nitrous oxide flame.

**Sodium**. Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* To 2.0 mL of solution A, prepared in the assay of aluminium, add 1 mL of a 12.5 g/L solution of *caesium chloride R* and dilute to 20.0 mL with *water R*.

*Reference solutions.* Into 5 separate 200 mL volumetric flasks, each containing 10 mL of a 12.5 g/L solution of *caesium chloride R*, introduce respectively 1.0 mL, 2.0 mL, 4.0 mL, 6.0 mL and 10.0 mL of *sodium standard solution (200 ppm Na) R* and dilute to 200.0 mL with *water R*.

*Wavelength*: 589.0 nm.

**07/2012:1663**
**corrected 10.0**

# ALUMINIUM STEARATE

## Aluminii stearas

DEFINITION

Aluminium salts of a mixture of solid organic acids consisting mainly of variable proportions of aluminium stearate and aluminium palmitate. The organic acids are obtained from sources of vegetable or animal origin.

*Content*:

- *aluminium* (Al; $A_r$ 26.98): 3.0 per cent to 9.0 per cent (dried substance);
- *stearic acid in the fatty acid fraction*: minimum 40.0 per cent;
- *sum of stearic acid and palmitic acid in the fatty acid fraction*: minimum 90.0 per cent.

CHARACTERS

*Appearance*: white or almost white, very fine, light powder.

*Solubility*: practically insoluble in water and in anhydrous ethanol.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Freezing point (*2.2.18*): minimum 53 °C, determined on the residue obtained in the preparation of solution S (see Tests).

B. Acid value (*2.5.1*): 195 to 210.
Dissolve 0.200 g of the residue obtained in the preparation of solution S in 25 mL of the prescribed mixture of solvents.

C. Examine the chromatograms obtained in the assay of stearic acid and palmitic acid.
*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatogram obtained with the reference solution.

D. 1 mL of solution S gives the reaction of aluminium (*2.3.1*). The addition of 0.5 mL of *dilute hydrochloric acid R* described in the general method is omitted.

TESTS

**Solution S**. To 5.0 g add 50 mL of *peroxide-free ether R*, 20 mL of *dilute nitric acid R* and 20 mL of *distilled water R* and heat gently under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 4 mL, of *distilled water R*. Combine the aqueous layers, wash with 15 mL of *peroxide-free ether R* and dilute to 50.0 mL with *distilled water R* (solution S). Evaporate the ether layer to dryness and dry the residue at 100-105 °C. Keep the residue for identification tests A and B.

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R* and boil for 1 min with continuous shaking. Cool and filter. To 10 mL of the filtrate add 0.05 mL of *bromothymol blue solution R4*. Not more than 0.05 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dilute 0.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 0.3 mL of solution S to 15 mL with *distilled water R*.

**Cadmium**: maximum 3 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution*. Dilute 25 mL of *cadmium- and lead-free nitric acid R* to 100.0 mL with *water R*.

*Modifier solution*. Dissolve 20 g of *ammonium dihydrogen phosphate R* and 1 g of *magnesium nitrate R* in *water R* and dilute to 100 mL with the same solvent. Alternatively, use an appropriate matrix modifier as recommended by the graphite furnace atomic absorption (GFAA) spectrometer manufacturer.

*Test solution*. Place 0.100 g of the substance to be examined in a polytetrafluoroethylene digestion bomb and add 2.5 mL of *cadmium- and lead-free nitric acid R*. Close and seal the bomb according to the manufacturer's operating instructions. *When using a digestion bomb, be thoroughly familiar with the safety and operating instructions. Carefully follow the bomb manufacturer's instructions regarding care and maintenance of these digestion bombs. Do not use metal-jacketed bombs or liners that have been used with hydrochloric acid due to contamination from corrosion of the metal jacket by hydrochloric acid*. Heat the bomb in an oven at 170 °C for 3 h. Cool the bomb slowly in air to room temperature according to the bomb manufacturer's instructions. Place the bomb in a fume cupboard and open carefully as corrosive gases may be expelled. Dissolve the residue in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Prepare a solution containing 0.00165 µg/mL of *cadmium nitrate tetrahydrate R* in the blank solution (equivalent to 0.006 µg/mL of Cd).

Dilute 1.0 mL of the test solution to 10.0 mL with the blank solution. Prepare mixtures of this solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.25:0.75 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:0.75:0.25 *V/V/V*). To each mixture add 50 µL of the modifier solution and mix. These solutions contain respectively 0 µg, 0.0015 µg, 0.0030 µg and 0.0045 µg of cadmium per millilitre from the reference solution. Keep the remaining test solution for use in the test for lead and nickel.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

*Operating conditions*: use the temperature programme recommended for cadmium by the GFAA manufacturer. An example of temperature parameters for GFAA analysis of cadmium is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 600 | 10 | 30 |
| Atomisation | 1800 | 0 | 5 |

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution*. Use the solution described in the test for cadmium.

*Modifier solution*. Use the solution described in the test for cadmium.

*Test solution*. Use the solution described in the test for cadmium.

*Reference solution*. Prepare a solution of 0.100 µg/mL of Pb by suitable dilutions of *lead standard solution (100 ppm Pb) R* with the blank solution.

Prepare mixtures of the test solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:1.0:0 *V/V/V*). To each mixture add 50 µL of the modifier solution and mix. These solutions contain respectively 0 µg, 0.025 µg and 0.05 µg of lead per millilitre from the reference solution.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

*Operating conditions*: use the temperature programme recommended for lead by the GFAA manufacturer. An example of temperature parameters for GFAA analysis of lead is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 450 | 10 | 30 |
| Atomisation | 2000 | 0 | 5 |

**Nickel**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution*. Use the solution described in the test for cadmium.

*Modifier solution*. Dissolve 20 g of *ammonium dihydrogen phosphate R* in *water R* and dilute to 100 mL with the same solvent. Alternatively, use an appropriate matrix modifier as recommended by the GFAA spectrometer manufacturer.

*Test solution*. Use the solution described in the test for cadmium.

*Reference solution*. Prepare a solution of 0.050 µg/mL of Ni by suitable dilutions of a 0.2477 µg/mL solution of *nickel nitrate hexahydrate R* with the blank solution.

Prepare mixtures of the test solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:1.0:0 *V/V/V*). To each mixture add 50 µL of the modifier solution and mix. These solutions contain respectively 0 µg, 0.0125 µg and 0.025 µg of nickel per millilitre from the reference solution.

*Source*: nickel hollow-cathode lamp.

*Wavelength*: 232.0 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

*Operating conditions*: use the temperature programme recommended for nickel by the GFAA manufacturer. An example of temperature parameters for GFAA analysis of nickel is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 1000 | 20 | 30 |
| Atomisation | 2300 | 0 | 5 |

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

**Aluminium**. To 0.250 g in a 250 mL conical flask add 20 mL of *methanol R* and, slowly, 2 mL of *sulfuric acid R*. Heat the solution for 30 min under reflux on a water-bath, swirling frequently. Allow to cool. Add 100 mL of *water R* and adjust to about pH 1 by adding approximately 12 mL of *dilute sodium hydroxide solution R*. Add 20.0 mL of *0.1 M sodium edetate* and adjust to between pH 5 and pH 6 by the addition of *sodium acetate R*. Add 70 mg of *xylenol orange triturate R* and titrate immediately and quickly with *0.1 M zinc sulfate* until the colour changes from yellow to pinkish-violet.

1 mL of *0.1 M sodium edetate* is equivalent to 2.698 mg of Al.

**Stearic acid and palmitic acid**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. In a conical flask fitted with a reflux condenser, dissolve 0.100 g of the substance to be examined in 5 mL of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 mL of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 mL of *saturated sodium chloride solution R*. Shake and allow the layers to separate. Dry the organic layer over 0.1 g of *anhydrous sodium sulfate R* previously washed with *heptane R*. Dilute 1.0 mL of the solution to 10.0 mL with *heptane R*.

*Reference solution*. Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of the substance to be examined.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 70 |
| | 2 - 36 | 70 → 240 |
| | 36 - 41 | 240 |
| Injection port | | 220 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to methyl stearate: methyl palmitate = about 0.9.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to methyl palmitate and methyl stearate;
– *repeatability*: maximum relative standard deviation of 3.0 per cent for the areas of the peaks due to methyl palmitate and methyl stearate after 6 injections; maximum relative standard deviation of 1.0 per cent for the ratio of the areas of the peaks due to methyl palmitate to the areas of the peaks due to methyl stearate after 6 injections.

**01/2017:0165**

# ALUMINIUM SULFATE

## Aluminii sulfas

$Al_2(SO_4)_3,xH_2O$ $M_r$ 342.1 (anhydrous substance)

DEFINITION

*Content*: 51.0 per cent to 59.0 per cent of $Al_2(SO_4)_3$.

It contains a variable quantity of water of crystallisation.

CHARACTERS

*Appearance*: colourless, lustrous crystals or crystalline masses.

*Solubility*: soluble in cold water, freely soluble in hot water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

B. Solution S gives the reaction of aluminium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.5 to 4.0.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Alkali and alkaline-earth metals**: maximum 0.4 per cent.

To 20 mL of solution S add 100 mL of *water R*, heat and add 0.1 mL of *methyl red solution R*. Add *dilute ammonia R1* until the colour of the indicator changes to yellow. Dilute to 150 mL with *water R*, heat to boiling and filter. Evaporate 75 mL of the filtrate to dryness on a water-bath and ignite. The residue weighs a maximum of 2 mg.

**Ammonium** (*2.4.1*): maximum 500 ppm.

Dilute 0.4 mL of solution S to 14 mL with *water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*. Use 0.3 mL of *thioglycollic acid R* in this test.

ASSAY

Dissolve 0.500 g in 20 mL of *water R*. Carry out the complexometric titration of aluminium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 17.11 mg of $Al_2(SO_4)_3$.

STORAGE

In an airtight container.

**01/2018:2156**
**corrected 10.0**

# ALVERINE CITRATE

## Alverini citras

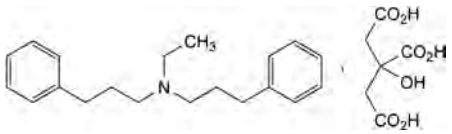

$C_{26}H_{35}NO_7$ $M_r$ 473.6

[5560-59-8]

DEFINITION

*N*-Ethyl-3-phenyl-*N*-(3-phenylpropyl)propan-1-amine dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *alverine citrate CRS*.

TESTS

**pH** (*2.2.3*): 3.5 to 4.5.

Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure. *Use freshly prepared solutions.*

*Test solution*. Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 20 mL with the same solvent. Add 2 mL of *concentrated ammonia R* and shake with 3 quantities, each of 15 mL, of *methylene chloride R*. To the combined lower layers add *anhydrous sodium sulfate R*, shake, filter, and evaporate the filtrate by suitable means at a temperature not exceeding 30 °C. Take up the residue with *methylene chloride R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *alverine impurity D CRS* (impurity D citrate) in 5 mL of *water R*, add 1 mL of *concentrated ammonia R* and shake with 3 quantities, each of 5 mL, of *methylene chloride R*. To the combined lower layers add *anhydrous sodium sulfate R*, shake, filter, and evaporate the filtrate by suitable means at a temperature not exceeding 30 °C. Take up the residue with *methylene chloride R*, add 0.2 mL of the test solution and dilute to 2.0 mL with *methylene chloride R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 20.0 mL with *methylene chloride R*.

*Reference solution (c)*. Dissolve 20 mg of *alverine impurity C CRS* in *methylene chloride R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.45 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.2 mL/min.

*Split ratio*: 1:11.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 7 | 120 |
| | 7 - 13 | 120 → 240 |
| | 13 - 21 | 240 |
| | 21 - 24 | 240 → 290 |
| | 24 - 39 | 290 |
| Injection port | | 290 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to alverine (retention time = about 18 min): impurity C = about 0.5; impurity D = about 0.97.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity D and alverine.

*Limits*:

- *impurities C, D*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent (reference solution (b)).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.375 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.36 mg of $C_{26}H_{35}NO_7$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E.*

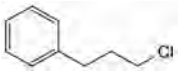

A. 1-chloro-3-phenylpropane,

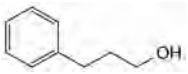

B. 3-phenylpropan-1-ol,

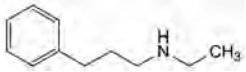

C. *N*-ethyl-3-phenylpropan-1-amine,

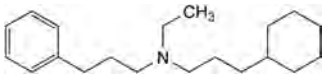

D. *N*-(3-cyclohexylpropyl)-*N*-ethyl-3-phenylpropan-1-amine,

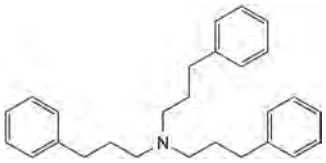

E. 3-phenyl-*N*,*N*-bis(3-phenylpropyl)propan-1-amine.

**01/2017:0463**
**corrected 10.0**

# AMANTADINE HYDROCHLORIDE

## Amantadini hydrochloridum

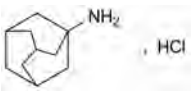

$C_{10}H_{18}ClN$ $M_r$ 187.7
[665-66-7]

DEFINITION

Tricyclo[3.3.1.1$^{3,7}$]decan-1-amine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent). It sublimes on heating.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amantadine hydrochloride CRS.*

B. To 0.1 g add 1 mL of *pyridine R*, mix and add 0.1 mL of *acetic anhydride R*. Heat to boiling for about 10 s. Pour the hot solution into 10 mL of *dilute hydrochloric acid R*, cool to 5 °C and filter. The precipitate, washed with *water R* and dried *in vacuo* at 60 °C for 1 h, melts (*2.2.14*) at 147 °C to 151 °C.

C. Dissolve 0.2 g in 1 mL of *0.1 M hydrochloric acid*. Add 1 mL of a 500 g/L solution of *sodium nitrite R*. A white precipitate is formed.

D. 1 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 2 mL of solution S to 10 mL with *carbon dioxide-free water R*. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 0.500 g of *adamantane R* in *methylene chloride R* and dilute to 10.0 mL with the same solvent.

*Test solution*. Weigh 0.5 g of the substance to be examined into a centrifuge tube. Add 9 mL of *methylene chloride R* and 10 mL of a 210 g/L solution of *sodium hydroxide R*. Shake for 10 min. Discard the upper layer. Dry the lower layer over *anhydrous sodium sulfate R*. Filter and collect the filtrate in a volumetric flask. Add 0.1 mL of the internal standard solution and dilute to 10.0 mL with *methylene chloride R*.

*Reference solution*. Weigh 5 mg of *amantadine hydrochloride CRS* into a centrifuge tube. Add 9 mL of *methylene chloride R* and 10 mL of a 210 g/L solution of *sodium hydroxide R*. Shake for 10 min. Discard the upper layer. Dry the lower layer over *anhydrous sodium sulfate R*. Filter and collect the filtrate in a volumetric flask. Add 1.0 mL of the internal standard solution and dilute to 100.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *base-deactivated phenyl(5)methyl(95)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 4 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 70 |
| | 5 - 23 | 70 → 250 |
| | 23 - 40 | 250 |
| Injection port | | 220 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to amantadine (retention time = about 14 min): internal standard = about 0.8.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to the internal standard and amantadine.

*Limits*:

- *unspecified impurities*: calculate the ratio ($R_1$) of the area of the peak due to amantadine to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the

area of any peak, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than $R_1$ (0.10 per cent);

– *total*: calculate the ratio ($R_2$) of 3 times the area of the peak due to amantadine to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than $R_2$ (0.3 per cent);

– *disregard limit*: calculate the ratio ($R_3$) of 0.5 times the area of the peak due to amantadine to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: disregard any peak with a ratio less than $R_3$ (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.77 mg of $C_{10}H_{18}ClN$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B*.

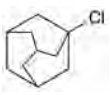


A. 1-chlorotricyclo[3.3.1.1$^{3,7}$]decane,

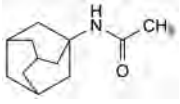


B. *N*-(tricyclo[3.3.1.1$^{3,7}$]dec-1-yl)acetamide.

**01/2017:1489**
**corrected 9.2**

# AMBROXOL HYDROCHLORIDE

## Ambroxoli hydrochloridum

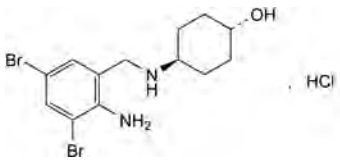


$C_{13}H_{19}Br_2ClN_2O$ $M_r$ 414.6
[23828-92-4]

DEFINITION

*trans*-4-[(2-Amino-3,5-dibromobenzyl)amino]cyclohexanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20.0 mg in *dilute sulfuric acid R1* and dilute to 100.0 mL with the same acid. Dilute 2.0 mL of the solution to 10.0 mL with *dilute sulfuric acid R1*.

*Spectral range*: 200-350 nm.

*Absorption maxima*: at 245 nm and 310 nm.

*Absorbance ratio*: $A_{245}/A_{310}$ = 3.2 to 3.4.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ambroxol hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *ambroxol hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R, propanol R, ethyl acetate R, hexane R* (1:10:20:70 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 25 mg in 2.5 mL of *water R*, mix with 1.0 mL of *dilute ammonia R1* and allow to stand for 5 min. Filter and acidify the filtrate with *dilute nitric acid R*. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.75 g in *methanol R* and dilute to 15 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* In order to prepare impurity B *in situ*, dissolve 5 mg of the substance to be examined in 0.2 mL of *methanol R*, add 0.04 mL of a mixture of 1 volume of *formaldehyde solution R* and 99 volumes of *water R*. Heat at 60 °C for 5 min. Evaporate to dryness under a current of nitrogen. Dissolve the residue in 5 mL of *water R* and dilute to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: a mixture of equal volumes of *acetonitrile R* and a solution prepared as follows: dissolve 1.32 g of *ammonium phosphate R* in 900 mL of *water R*, adjust to pH 7.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of ambroxol.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to ambroxol (retention time = about 9 min): impurity B = about 0.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity B and ambroxol.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent),
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 70 mL of *ethanol (96 per cent) R* and add 5 mL of *0.01 M hydrochloric acid.* Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide.* Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.46 mg of $C_{13}H_{19}Br_2ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

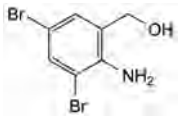


A. (2-amino-3,5-dibromophenyl)methanol,

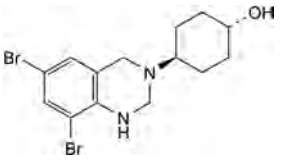


B. *trans*-4-(6,8-dibromo-1,4-dihydroquinazolin-3(2*H*)-yl)-cyclohexanol,

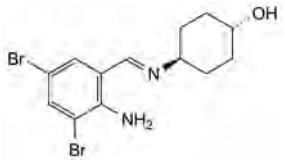


C. *trans*-4-[[(*E*)-2-amino-3,5-dibromobenzyliden]amino]-cyclohexanol,

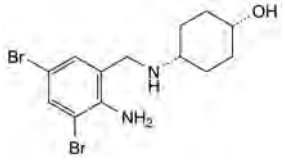


D. *cis*-4-[(2-amino-3,5-dibromobenzyl)amino]cyclohexanol,

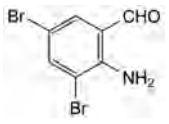


E. 2-amino-3,5-dibromobenzaldehyde.

**04/2019:0368**

# AMFETAMINE SULFATE

## Amfetamini sulfas

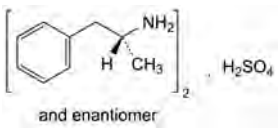


$C_{18}H_{28}N_2O_4S$ $M_r$ 368.5
[60-13-9]

DEFINITION

Bis[(2*RS*)-1-phenylpropan-2-amine] sulfate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: C, D.*

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amfetamine sulfate CRS.*

C. To 50 mL of solution S add 5 mL of *strong sodium hydroxide solution R* and 0.5 mL of *benzoyl chloride R* and shake. Continue to add *benzoyl chloride R* in portions of 0.5 mL, shaking after each addition, until no further precipitate is formed. Filter, wash the precipitate with *water R*, recrystallise twice from a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*, then dry at 100-105 °C. The crystals melt (*2.2.14*) at 131 °C to 135 °C.

D. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.04° to + 0.04° (measured in a 2 dm tube), determined on solution S.

**Acidity or alkalinity**. To 25 mL of solution S add 0.1 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 5 mL of *trifluoroacetic acid R* and 900 mL of *water for chromatography R*, adjust to pH 2.2 with *concentrated ammonia R* and dilute to 1000 mL with *acetonitrile R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *1-phenylpropan-2-ol R* (impurity A) and 5 mg of *benzaldehyde R* (impurity D) in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 1 mL of the solution to 100 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: solvent mixture;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 1 - 16 | 100 → 65 | 0 → 35 |
| 16 - 21 | 65 → 0 | 35 → 100 |
| 21 - 23 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 257 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and D.

*Relative retention* with reference to amfetamine (retention time = about 8 min): impurity D = about 1.6; impurity A = about 1.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to impurities D and A.

*Calculation of percentage contents:*

– for each impurity, use the concentration of amfetamine sulfate in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.85 mg of $C_{18}H_{28}N_2O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

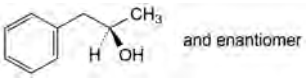

and enantiomer

A. (2*RS*)-1-phenylpropan-2-ol,

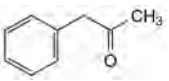


B. 1-phenylpropan-2-one,

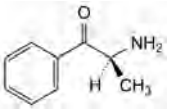


C. (2*S*)-2-amino-1-phenylpropan-1-one (cathinone),

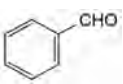


D. benzaldehyde.

01/2017:0873

# AMIDOTRIZOIC ACID DIHYDRATE

## Acidum amidotrizoicum dihydricum

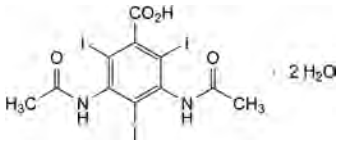

$C_{11}H_9I_3N_2O_4,2H_2O$ $M_r$ 650
[50978-11-5]

DEFINITION

3,5-Bis(acetylamino)-2,4,6-triiodobenzoic acid dihydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: A.

*Second identification*: B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amidotrizoic acid dihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in a 3 per cent *V/V* solution of *ammonia R* in *methanol R* and dilute to 5 mL with the same solution.
*Reference solution*. Dissolve 25 mg of *amidotrizoic acid dihydrate CRS* in a 3 per cent *V/V* solution of *ammonia R* in *methanol R* and dilute to 5 mL with the same solution.
*Plate*: *TLC silica gel $GF_{254}$ plate R*.
*Mobile phase*: *anhydrous formic acid R*, *methyl ethyl ketone R*, *toluene R* (20:25:60 *V/V/V*).
*Application*: 2 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air until the solvents have evaporated.
*Detection*: in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Heat 50 mg gently in a small porcelain dish over a naked flame. Violet vapour is evolved.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *dilute sodium hydroxide solution R* and dilute to 20 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Dissolve 0.250 g of *sodium hydroxide R* and 0.860 g of *sodium dihydrogen phosphate R* in 50 mL of *water R* and dilute to 1000 mL with the same solvent.

*Test solution*. Dissolve 40.0 mg of the substance to be examined in 10.0 mL of the solvent mixture with the aid of ultrasound.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *amidotrizoic acid for system suitability CRS* (impurities A, B, C and D) in 1.0 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*. Dissolve 3.4 g of *tetrabutylammonium hydrogen sulfate R* in a mixture of 230 mL of *acetonitrile R* and 770 mL of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 236 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of amidotrizoic acid.

*Identification of impurities*: use the chromatogram supplied with *amidotrizoic acid for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to amidotrizoic acid (retention time = about 5 min): impurity B = about 0.8; impurity C = about 0.9; impurity A = about 1.4; impurity D = about 1.8.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to impurities B and C in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 25 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurities A, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent), except for the peaks due to impurities A and D.

**Halides expressed as chlorides** (*2.4.4*): maximum 150 ppm.

Dissolve 0.55 g in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 15 mL of *water R*. Add 6 mL of *dilute nitric acid R* and filter.

**Free aromatic amines**. *Maintain the solutions and reagents in iced water, protected from bright light*. To 0.50 g in a 50 mL volumetric flask add 15 mL of *water R*. Shake and add 1 mL of *dilute sodium hydroxide solution R*. Cool in iced water, add 5 mL of a freshly prepared 5 g/L solution of *sodium nitrite R* and 12 mL of *dilute hydrochloric acid R*. Shake gently and allow to stand for exactly 2 min after adding the hydrochloric acid. Add 10 mL of a 20 g/L solution of *ammonium sulfamate R*. Allow to stand for 5 min, shaking frequently, and add 0.15 mL of a 100 g/L solution of *α-naphthol R* in *ethanol (96 per cent) R*. Shake and allow to stand for 5 min. Add 3.5 mL of *buffer solution pH 10.9 R*, mix and dilute to 50.0 mL with *water R*. The absorbance (*2.2.25*), measured within 20 min at 485 nm using as the compensation liquid a solution prepared at the same time and in the same manner but omitting the substance to be examined, is not greater than 0.30.

**Loss on drying** (*2.2.32*): 4.5 per cent to 7.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.150 g in a 250 mL round-bottomed flask add 5 mL of *strong sodium hydroxide solution R*, 20 mL of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 mL of *dilute sulfuric acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 20.47 mg of $C_{11}H_9I_3N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E.*

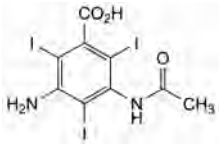

A. 3-(acetylamino)-5-amino-2,4,6-triiodobenzoic acid,

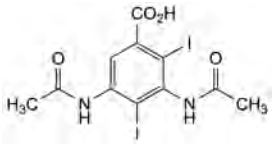

B. 3,5-bis(acetylamino)-2,4-diiodobenzoic acid,

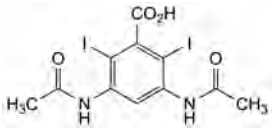

C. 3,5-bis(acetylamino)-2,6-diiodobenzoic acid,

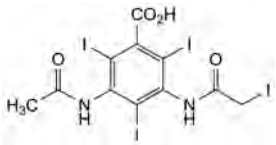

D. 3-(acetylamino)-5-[(iodoacetyl)amino]-2,4,6-triiodobenzoic acid,

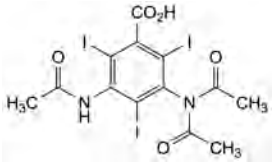

E. 3-(acetylamino)-5-(diacetylamino)-2,4,6-triiodobenzoic acid.

**07/2014:1289**

# AMIKACIN

## Amikacinum

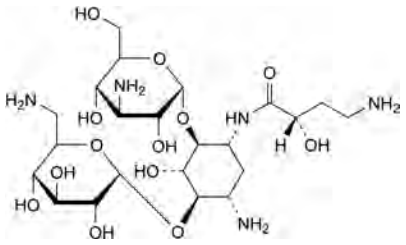

$C_{22}H_{43}N_5O_{13}$ $M_r$ 585.6
[37517-28-5]

DEFINITION

6-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine.

Antimicrobial substance obtained from kanamycin A.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, slightly soluble in methanol, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amikacin CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 25 mg of *amikacin CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *kanamycin monosulfate CRS* in 1 mL of the test solution and dilute to 10 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *ammonia R*, *methanol R* (25:30:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 9.5 to 11.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 97 to + 105 (anhydrous substance).

Dissolve 0.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of *amikacin for system suitability CRS* (containing impurities A, B, F and H) in mobile phase A and dilute to 10 mL with mobile phase A.

*Reference solution (d)*. Dissolve 5.0 mg of *amikacin impurity I CRS* in mobile phase A and dilute to 20.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: a mixture prepared with *carbon dioxide-free water R*, containing 1.8 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 1.4 per cent *V/V* of *tetrahydrofuran R*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas;
- *mobile phase B*: a mixture prepared with *carbon dioxide-free water R*, containing 1.8 g/L of *sodium octanesulfonate R*, 28 g/L of *anhydrous sodium sulfate R1*, 1.4 per cent *V/V* of *tetrahydrofuran R*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 38.0 | 100 → 30 | 0 → 70 |
| 38.0 - 38.1 | 30 → 0 | 70 → 100 |
| 38.1 - 68 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Post-column solution*: mixture of 1 volume of *carbonate-free sodium hydroxide solution R* and 24 volumes of previously degassed *carbon dioxide-free water R*, which is added in a pulseless manner to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate of post-column solution*: 0.3 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and – 0.15 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *amikacin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, F and H; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity I.

*Relative retention* with reference to amikacin (retention time = about 28 min): impurity I = about 0.13; impurity F = about 0.92; impurity B = about 0.95; impurity A = about 1.62; impurity H = about 1.95.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to amikacin; if necessary, adjust the volume of tetrahydrofuran in the mobile phase.

*Calculation of percentage contents*:

- for impurity I, use the concentration of impurity I in reference solution (d);
- for impurities other than I, use the concentration of amikacin in reference solution (a).

*Limits*:

- *impurities A, B, F, H, I*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.5 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.1 per cent.

**Water** (*2.5.12*): maximum 8.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution*. Dissolve 50.0 mg of *amikacin CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: a mixture prepared with *carbon dioxide-free water R*, containing 1.8 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 5.8 per cent *V/V* of *acetonitrile R1*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: 1.3 times the retention time of amikacin.

*Retention time*: amikacin = about 30 min.

*System suitability*: reference solution:

- *symmetry factor*: maximum 1.5 for the peak due to amikacin; if necessary, adjust the amount of *acetonitrile R1* in the mobile phase; peak splitting may be observed when the retention time becomes too short;
- *repeatability*: maximum relative standard deviation of 1.5 per cent after 6 injections.

Calculate the percentage content of $C_{22}H_{43}N_5O_{13}$ taking into account the assigned content of *amikacin CRS*.

IMPURITIES

*Specified impurities: A, B, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, G.*

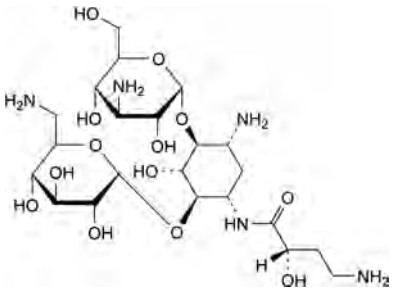

A. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,

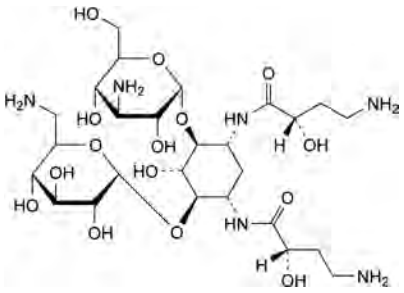

B. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1,3-*N*-bis[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,

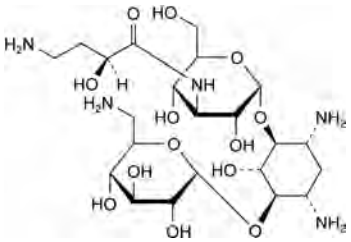

C. 4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-6-*O*-[3-[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-3-deoxy-α-D-glucopyranosyl]-2-deoxy-D-streptamine,

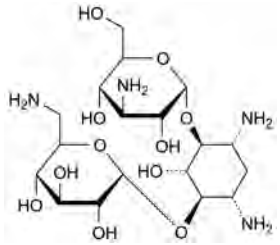

D. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine (kanamycin),

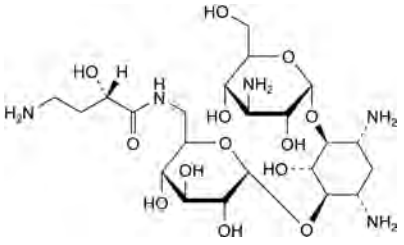

E. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-[6-[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-6-deoxy-α-D-glucopyranosyl]-2-deoxy-L-streptamine,

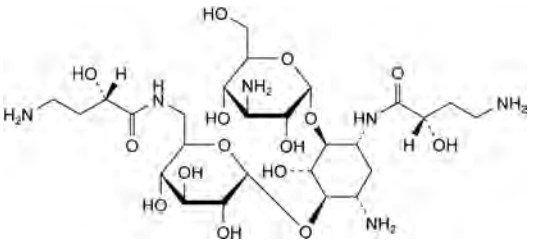

F. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-[6-[(2*S*)-4-amino-2-hydroxybutanoyl]amino-6-deoxy-α-D-glucopyranosyl]-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine,

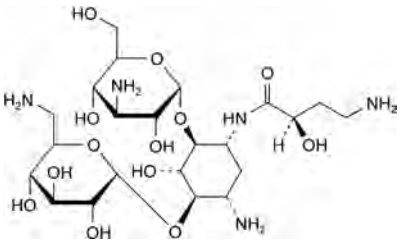

G. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*R*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine,

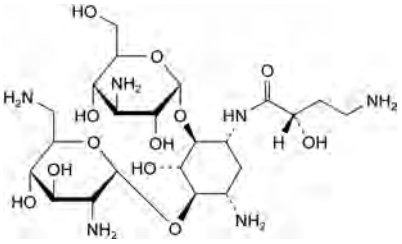

H. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine,

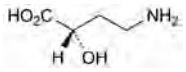

I. (2*S*)-4-amino-2-hydroxybutanoic acid.

**01/2019:1290**
**corrected 10.0**

# AMIKACIN SULFATE

## Amikacini sulfas

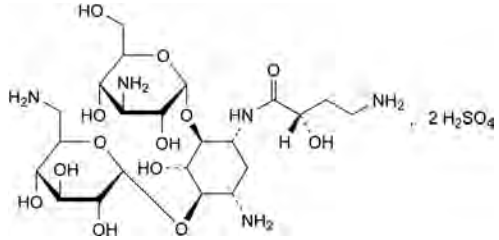

$C_{22}H_{47}N_5O_{21}S_2$ $M_r$ 782
[39831-55-5]

DEFINITION

6-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine sulfate.

Antimicrobial substance obtained from kanamycin A.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amikacin sulfate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 25 mg of *amikacin sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *kanamycin monosulfate CRS* in 1 mL of the test solution and dilute to 10 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *ammonia R*, *methanol R* (25:30:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 2.0 to 4.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 76 to + 84 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 33 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of *amikacin for system suitability CRS* (containing impurities A, B, F and H) in mobile phase A and dilute to 10 mL with mobile phase A.

*Reference solution (d)*. Dissolve 6.6 mg of *amikacin impurity I CRS* in mobile phase A and dilute to 20.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: a mixture prepared with *carbon dioxide-free water R*, containing 1.8 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 1.4 per cent *V/V* of *tetrahydrofuran R*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas;
– *mobile phase B*: a mixture prepared with *carbon dioxide-free water R*, containing 1.8 g/L of *sodium octanesulfonate R*, 28 g/L of *anhydrous sodium sulfate R1*, 1.4 per cent *V/V* of *tetrahydrofuran R*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 38.0 | 100 → 30 | 0 → 70 |
| 38.0 - 38.1 | 30 → 0 | 70 → 100 |
| 38.1 - 68 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Post-column solution*: mixture of 1 volume of *carbonate-free sodium hydroxide solution R* and 24 volumes of previously degassed *carbon dioxide-free water R*, which is added in a pulseless manner to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate of post-column solution*: 0.3 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and − 0.15 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *amikacin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, F and H; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity I.

*Relative retention* with reference to amikacin (retention time = about 28 min): impurity I = about 0.13; impurity F = about 0.92; impurity B = about 0.95; impurity A = about 1.62; impurity H = about 1.95.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to amikacin; if necessary, adjust the volume of tetrahydrofuran in the mobile phase.

*Calculation of percentage contents*:

– for impurity I, use the concentration of impurity I in reference solution (d);
– for impurities other than I, use the concentration of amikacin sulfate in reference solution (a).

*Limits*:

– *impurities A, B, F, H, I*: for each impurity, maximum 0.5 per cent;
– *any other impurity*: for each impurity, maximum 0.5 per cent;
– *total*: maximum 1.5 per cent;
– *reporting threshold*: 0.1 per cent.

**Sulfate**: 23.3 per cent to 25.8 per cent (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*.

Titrate with *0.1 M sodium edetate* adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of sulfate ($SO_4$).

**Loss on drying** (*2.2.32*): maximum 13.0 per cent, determined on 0.500 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 5 mL of a solution containing 25 mg of the substance to be examined in *water for injections R*.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution.* Dissolve 37.4 mg of *amikacin CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mixture containing 1.8 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 5.8 per cent *V/V* of *acetonitrile R1*, and 5 per cent *V/V* of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*; degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: 1.3 times the retention time of amikacin.

*Retention time*: amikacin = about 30 min.

*System suitability*: reference solution:

- *symmetry factor*: maximum 1.5 for the peak due to amikacin; if necessary, adjust the amount of *acetonitrile R1* in the mobile phase; peak splitting may be observed when the retention time becomes too short;
- *repeatability*: maximum relative standard deviation of 1.5 per cent after 6 injections.

Calculate the percentage content of $C_{22}H_{47}N_5O_{21}S_2$ taking into account the assigned content of *amikacin CRS* and a correction factor of 1.335.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, G.*

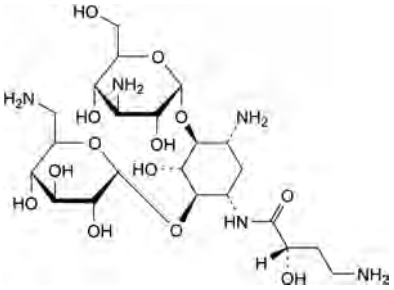

A. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,

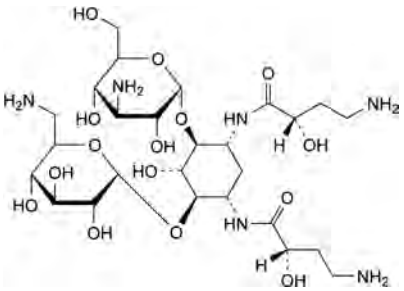

B. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1,3-*N*-bis[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-L-streptamine,

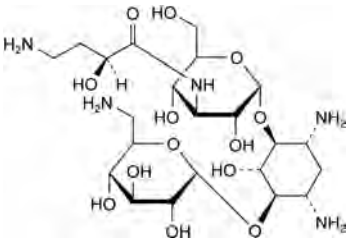

C. 4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-6-*O*-[3-[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-3-deoxy-α-D-glucopyranosyl]-2-deoxy-D-streptamine,

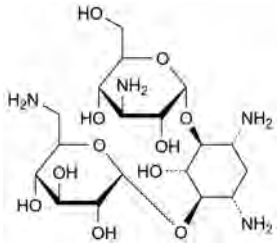

D. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine (kanamycin),

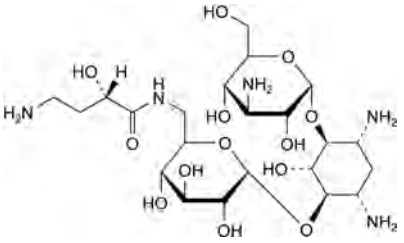

E. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-6-*O*-[6-[[(2*S*)-4-amino-2-hydroxybutanoyl]amino]-6-deoxy-α-D-glucopyranosyl]-2-deoxy-L-streptamine,

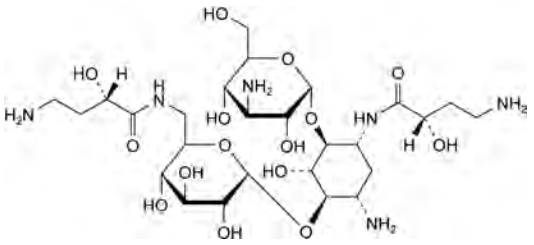

F. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-[6-[(2*S*)-4-amino-2-hydroxybutanoyl]amino-6-deoxy-α-D-glucopyranosyl]-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine,

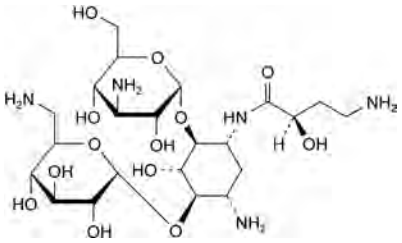

G. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*R*)-4-amino-2-hydroxybutanoyl]-2-deoxy-D-streptamine,

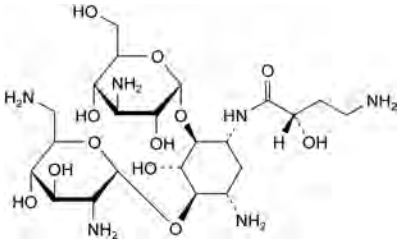

H. 6-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-1-*N*-[(2*S*)-4-amino-2-hydroxybutanoyl]-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine,

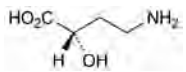

I. (2*S*)-4-amino-2-hydroxybutanoic acid.

**04/2019:0651**

# AMILORIDE HYDROCHLORIDE DIHYDRATE

## Amiloridi hydrochloridum dihydricum

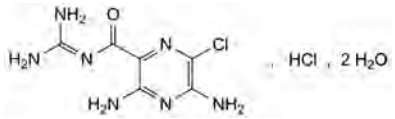

$C_6H_9Cl_2N_7O,2H_2O$ $M_r$ 302.1
[17440-83-4]

DEFINITION

3,5-Diamino-6-chloro-*N*-(diaminomethylidene)pyrazine-2-carboxamide hydrochloride dihydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: pale yellow or greenish-yellow powder.

*Solubility*: slightly soluble in water and in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amiloride hydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution*. Dissolve 5 mg of *amiloride hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *concentrated ammonia R*, *propanol R* (30:70 *V/V*).
*Application*: 5 µL; the volume may be adapted according to the type of plate used.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent. 2 mL of the solution gives reaction (a) of chlorides (*2.3.1*); acidify with 5 mL of *dilute acetic acid R*, instead of *dilute nitric acid R*.

D. Water (see Tests).

TESTS

**Free acid**. Dissolve 1.0 g in a mixture of 50 mL of *methanol R* and 50 mL of *water R* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to reach the end-point.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 2.76 g of *sodium dihydrogen phosphate monohydrate R* in 850 mL of *water for chromatography R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1.0 L with *water for chromatography R*.

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *methanol R1* and dilute to 10.0 mL with solution A.

*Reference solution (a)*. Dissolve 2 mg of *amiloride impurity A CRS* in 0.5 mL of *methanol R1*, add 0.5 mL of the test solution and dilute to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 4 mg of *amiloride for peak identification CRS* (containing impurity C) in 0.5 mL of *methanol R1* and dilute to 2.0 mL with solution A.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 0.8 g of *sodium hexanesulfonate monohydrate R* in a mixture of 80 mL of *acetonitrile R1* and 920 mL of solution A.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of amiloride.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *amiloride for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to amiloride (retention time = about 10 min): impurity C = about 0.5; impurity A = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity A and amiloride.

*Calculation of percentage contents*:

– for each impurity, use the concentration of amiloride hydrochloride dihydrate in reference solution (c).

*Limits*:

– *impurity C*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 11.0 per cent to 13.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 26.61 mg of $C_6H_9Cl_2N_7O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

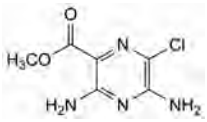

A. methyl 3,5-diamino-6-chloropyrazine-2-carboxylate,

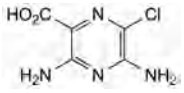

B. 3,5-diamino-6-chloropyrazine-2-carboxylic acid,

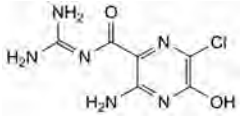

C. 3-amino-6-chloro-*N*-(diaminomethylidene)-5-hydroxypyrazine-2-carboxamide.

**01/2017:1687**
**corrected 10.0**

# 4-AMINOBENZOIC ACID

## Acidum 4-aminobenzoicum

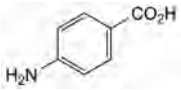

$C_7H_7NO_2$ $M_r$ 137.1
[150-13-0]

DEFINITION

4-Aminobenzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 186 °C to 189 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *4-aminobenzoic acid CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 20 mL with the same solvent.
*Reference solution (a)*. Dissolve 20 mg of *4-aminobenzoic acid CRS* in *methanol R* and dilute to 20 mL with the same solvent.
*Reference solution (b)*. Dissolve 10 mg of *4-nitrobenzoic acid R* in 10 mL of reference solution (a).
*Plate*: suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm as the coating substance.
*Mobile phase*: *glacial acetic acid R*, *hexane R*, *methylene chloride R* (5:20:75 *V/V/V*).
*Application*: 1 µL.
*Development*: over a path of 10 cm.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution.* Dissolve 25.0 mg of *4-nitrobenzoic acid R* and 25.0 mg of *benzocaine R* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4.0 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of a mixture of 70 volumes of *acetonitrile R* and 80 volumes of *methanol R*, and 80 volumes of a solution containing 1.5 g/L of *potassium dihydrogen phosphate R* and 2.5 g/L of *sodium octanesulfonate R* adjusted to pH 2.2 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*Run time*: 11 times the retention time of 4-aminobenzoic acid.

*Relative retention* with reference to 4-aminobenzoic acid (retention time = about 3 min): impurity A = about 4; impurity B = about 9.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.2 per cent),
- *any other impurity*: not more than 0.5 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.1 per cent),
- *total*: not more than 2.5 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.5 per cent),
- *disregard limit*: 0.1 times the area of the peak due to impurity A in the chromatogram obtained with the reference solution (0.02 per cent).

**Impurity C and impurity D**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 20.0 mg of *lauric acid R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 1.000 g of the substance to be examined in 10.0 mL of an 84 g/L solution of *sodium hydroxide R* and extract with 2 quantities, each of 10 mL, of *methylene chloride R*. Combine and wash with 5 mL of *water R*; filter through *anhydrous sodium sulfate R*. Wash the filter with *methylene chloride R*. Evaporate in a water-bath at 50-60 °C to obtain a volume of about 1-5 mL. Add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *methylene chloride R*.

*Reference solution (a).* Dissolve 20.0 mg of *aniline R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20.0 mg of *p-toluidine R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dilute 0.50 mL of reference solution (a), 0.50 mL of reference solution (b) and 10.0 mL of the internal standard solution to 100.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 130 |
| | 4 - 6.5 | 130 → 180 |
| | 6.5 - 11.5 | 180 |
| Injection port | | 280 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 2 µL; inject the test solution and reference solution (c).

*Retention time*: internal standard = about 9.5 min.

*Limits*:

- *impurity C*: calculate the ratio (*R*) of the area of the peak due to impurity C to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); calculate the ratio of the area of the peak due to impurity C to the area of the peak due to the internal standard from the chromatogram obtained with the test solution: this ratio is not greater than *R* (10 ppm),
- *impurity D*: calculate the ratio (*R*) of the area of the peak due to impurity D to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); calculate the ratio of the area of the peak due to impurity D to the area of the peak due to the internal standard from the chromatogram obtained with the test solution: this ratio is not greater than *R* (10 ppm).

**Iron** (*2.4.9*): maximum 40 ppm.

Dissolve 0.250 g in 3 mL of *ethanol (96 per cent) R* and dilute to 10.0 mL with *water R*.

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g with heating in 50 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 13.71 mg of $C_7H_7NO_2$.

STORAGE

Protected from light.

IMPURITIES

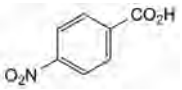


A. 4-nitrobenzoic acid,

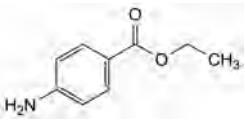


B. ethyl 4-aminobenzoate (benzocaine),

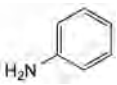


C. aniline,

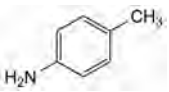


D. 4-methylaniline (*p*-toluidine).

**01/2017:0874**

# AMINOCAPROIC ACID

## Acidum aminocaproicum

$C_6H_{13}NO_2$ $M_r$ 131.2
[60-32-2]

DEFINITION

Aminocaproic acid contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 6-aminohexanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in alcohol.

It melts at about 205 °C with decomposition.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *aminocaproic acid CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with the test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.5 g in 4 mL of a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Evaporate to dryness by heating on a water-bath. Dry the residue in a desiccator. Dissolve the residue in about 2 mL of boiling *ethanol R*. Allow to cool and maintain at 4 °C to 8 °C for 3 h. Filter under reduced pressure. The residue washed with about 10 mL of *acetone R* and dried at 60 °C for 30 min, melts (*2.2.14*) at 131 °C to 133 °C.

D. Dissolve about 5 mg in 0.5 mL of *distilled water R*. Add 3 mL of *dimethylformamide R* and 2 mL of *ascorbic acid solution R*. Heat on a water-bath. An orange colour develops.

TESTS

**Solution S**. dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*) and remains clear (*2.2.1*) on standing for 24 h.

**pH** (*2.2.3*). The pH of solution S is 7.5 to 8.0.

**Absorbance** (*2.2.25*).

A. The absorbance of solution S at 287 nm is not more than 0.10 and at 450 nm is not more than 0.03.

B. Place 2.0 g in an even layer in a shallow dish 9 cm in diameter, cover and allow to stand at 98 °C to 102 °C for 72 h. Dissolve in *water R* and dilute to 10.0 mL with the same solvent. The absorbance of the solution at 287 nm is not more than 0.15 and at 450 nm is not more than 0.03.

**Ninhydrin-positive substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 50 mL with *water R*.

*Reference solution (a).* Dissolve 10 mg of *aminocaproic acid CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dilute 5 mL of test solution (b) to 20 mL with *water R*.

*Reference solution (c).* Dissolve 10 mg of *aminocaproic acid CRS* and 10 mg of *leucine CRS* in *water R* and dilute to 25 mL with the same solvent.

Apply separately to the plate 5 µL of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with the test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 20 mL of *anhydrous acetic acid R*. Using 0.1 mL of *crystal violet solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from bluish-violet to bluish-green.

1 mL of *0.1 M perchloric acid* is equivalent to 13.12 mg of $C_6H_{13}NO_2$.

**01/2017:1291**

# AMINOGLUTETHIMIDE

## Aminoglutethimidum

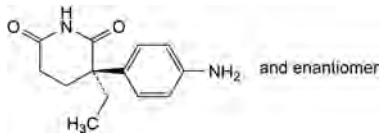


$C_{13}H_{16}N_2O_2$ $M_r$ 232.3
[125-84-8]

DEFINITION

(3*RS*)-3-(4-Aminophenyl)-3-ethylpiperidine-2,6-dione.

*Content*: 98.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in methanol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 150 °C to 154 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *aminoglutethimide CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *aminoglutethimide CRS* in *acetone R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 25 mg of *aminoglutethimide CRS* and 25 mg of *glutethimide CRS* in *acetone R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *glacial acetic acid R*, *methanol R*, *ethyl acetate R* (0.5:15:85 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separed spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *acetate buffer solution pH 5.0 R* (50:50 *V/V*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5.0 mg of *aminoglutethimide impurity A CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 10.0 mL with reference solution (a).

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 27 volumes of *methanol R* and 73 volumes of *acetate buffer solution pH 5.0 R*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 4 times the retention time of aminoglutethimide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to aminoglutethimide (retention time = about 9 min): impurity A = about 1.3.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to aminoglutethimide and impurity A.

*Limits*:

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *sum of impurities other than A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *total*: maximum 2.0 per cent for the sum of the contents of all impurities;

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Impurity D**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Use shaking, not sonication or heat, to dissolve the reference substance and the substance to be examined.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent.

*Reference solution.* Dissolve 3.0 mg of *aminoglutethimide impurity D CRS* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *dimethyl sulfoxide R*.

*Column*:

– *size*: *l* = 0.12 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.285 g of *sodium edetate R* in *water R*, add 7.5 mL of *dilute acetic acid R* and 50 mL of *0.1 M potassium hydroxide* and dilute to 1000 mL with *water R*; adjust to pH 5.0 with *glacial acetic acid R*; mix 350 mL of this solution with 650 mL of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 328 nm.

*Injection*: 10 µL.

*System suitability*: test solution:

– *number of theoretical plates*: minimum 3300, calculated for the principal peak;

– *mass distribution ratio*: 2.0 to 5.0 for the principal peak;

– *symmetry factor*: maximum 1.2 for the principal peak.

*Limit*:

– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (300 ppm).

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 6 mL of solution S to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.23 mg of $C_{13}H_{16}N_2O_2$.

IMPURITIES

*Specified impurities: A, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

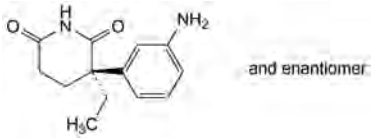


A. (3*RS*)-3-(3-aminophenyl)-3-ethylpiperidine-2,6-dione (3-aminoglutethimide),

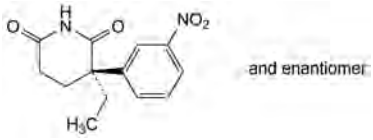


B. (3*RS*)-3-ethyl-3-(3-nitrophenyl)piperidine-2,6-dione,

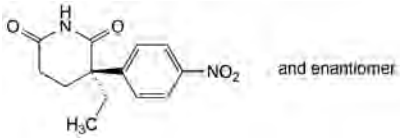


C. (3*RS*)-3-ethyl-3-(4-nitrophenyl)piperidine-2,6-dione,

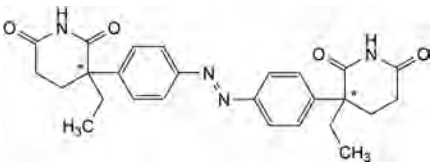

D. 3,3′-[diazenediylbis(4,1-phenylene)]bis(3-ethylpiperidine-2,6-dione) (azoglutethimide).

**01/2017:0803**

# AMIODARONE HYDROCHLORIDE

## Amiodaroni hydrochloridum

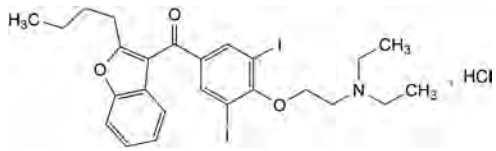


$C_{25}H_{30}ClI_2NO_3$ $M_r$ 682
[19774-82-4]

DEFINITION

(2-Butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl]methanone hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amiodarone hydrochloride CRS.*

B. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ or $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 3.2 to 3.8.

Dissolve 1.0 g in *carbon dioxide-free water R*, heating at 80 °C, cool and dilute to 20 mL with the same solvent.

**Impurity H**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use and keep protected from bright light.*

*Test solution.* Dissolve 0.500 g of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *(2-chloroethyl)diethylamine hydrochloride R* (impurity H) in *methylene chloride R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with *methylene chloride R.*

*Reference solution (b).* Mix 2.0 mL of the test solution and 2.0 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous formic acid R*, *methanol R*, *methylene chloride R* (5:10:85 *V/V/V*).

*Application*: 50 µL of the test solution and reference solution (a); 100 µL of reference solution (b).

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: spray with *potassium iodobismuthate solution R1* and then with *dilute hydrogen peroxide solution R*; examine immediately in daylight.

*System suitability*: reference solution (b):

– the spot due to impurity H is clearly visible.

*Limit*:

– *impurity H*: any spot due to impurity H is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 4.9.* To 800 mL of *water R* add 3.0 mL of *glacial acetic acid R*, adjust to pH 4.9 with *dilute ammonia R1* and dilute to 1000 mL with *water R.*

*Test solution.* Dissolve 0.125 g of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 25.0 mL with the same mixture of solvents.

*Reference solution.* Dissolve 5 mg of *amiodarone impurity D CRS*, 5 mg of *amiodarone impurity E CRS* and 5.0 mg of *amiodarone hydrochloride CRS* in *methanol R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R.*

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: buffer solution pH 4.9, *methanol R*, *acetonitrile R* (30:30:40 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of amiodarone.

*Relative retention* with reference to amiodarone (retention time = about 24 min): impurity A = about 0.26; impurity D = about 0.29; impurity E = about 0.37; impurity B = about 0.49; impurity C = about 0.55; impurity G = about 0.62; impurity F = about 0.69.

*System suitability*: reference solution:

– *resolution*: minimum 3.5 between the peaks due to impurities D and E.

*Limits*:

– *impurities A, B, C, D, E, F, G*: for each impurity, not more than the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.10 per cent);
– *total*: not more than 2.5 times the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.5 per cent);
– *disregard limit*: 0.25 times the area of the peak due to amiodarone in the chromatogram obtained with the reference solution (0.05 per cent).

**Iodides**: maximum 150 ppm.

*Prepare the test and reference solutions simultaneously.*

*Solution A*. Add 1.50 g of the substance to be examined to 40 mL of *water R* at 80 °C and shake until completely dissolved. Cool and dilute to 50.0 mL with *water R*.

*Test solution*. To 15.0 mL of solution A add 1.0 mL of *0.1 M hydrochloric acid* and 1.0 mL of *0.05 M potassium iodate*. Dilute to 20.0 mL with *water R*. Allow to stand protected from light for 4 h.

*Reference solution*. To 15.0 mL of solution A add 1.0 mL of *0.1 M hydrochloric acid*, 1.0 mL of an 88.2 mg/L solution of *potassium iodide R* and 1.0 mL of *0.05 M potassium iodate*. Dilute to 20.0 mL with *water R*. Allow to stand protected from light for 4 h.

Measure the absorbances (*2.2.25*) of the solutions at 420 nm, using a mixture of 15.0 mL of solution A and 1.0 mL of *0.1 M hydrochloric acid* diluted to 20.0 mL with *water R* as the compensation liquid. The absorbance of the test solution is not greater than half the absorbance of the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 50 °C at a pressure not exceeding 0.3 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.600 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 75 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 68.18 mg of $C_{25}H_{30}ClI_2NO_3$.

STORAGE

Protected from light, at a temperature not exceeding 30 °C.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H.*

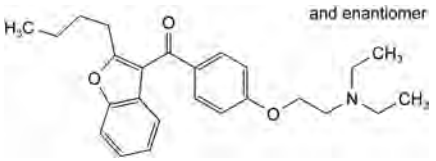


A. (2-butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]phenyl]methanone,

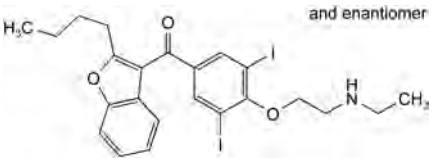


B. (2-butylbenzofuran-3-yl)[4-[2-(ethylamino)ethoxy]-3,5-diiodophenyl]methanone,

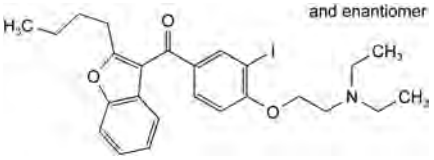


C. (2-butylbenzofuran-3-yl)[4-[2-(diethylamino)ethoxy]-3-iodophenyl]methanone,

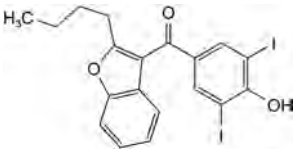

D. (2-butylbenzofuran-3-yl)(4-hydroxy-3,5-diiodophenyl)-methanone,

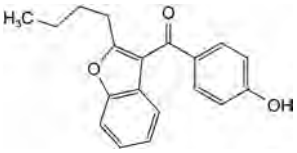

E. (2-butylbenzofuran-3-yl)(4-hydroxyphenyl)methanone,

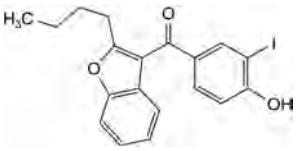

F. (2-butylbenzofuran-3-yl)(4-hydroxy-3-iodophenyl)methanone,

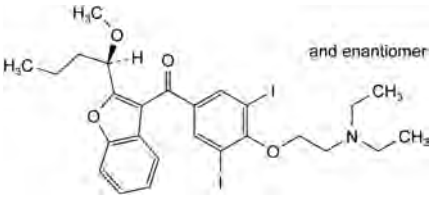


G. [4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl][2-[(1*RS*)-1-methoxybutyl]benzofuran-3-yl]methanone,

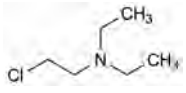

H. 2-chloro-*N*,*N*-diethylethanamine (2-chlorotriethylamine, (2-chloroethyl)diethylamine).

**01/2019:1490**

# AMISULPRIDE

## Amisulpridum

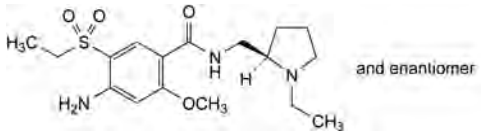


$C_{17}H_{27}N_3O_4S$ $M_r$ 369.5
[71675-85-9]

DEFINITION

4-Amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-(ethylsulfonyl)-2-methoxybenzamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in anhydrous ethanol.

mp: about 126 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amisulpride CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in 3 mL of a mixture of 1 volume of *acetic acid R* and 4 volumes of *water R*, and dilute to 20 mL with *water R*.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *sulpiride impurity A CRS* (amisulpride impurity A) in *methanol R* and dilute to 25 mL with the same solvent. Dilute 2 mL of the solution to 20 mL with *methanol R*.

*Reference solution (b)*. Dilute 1 mL of the test solution to 10 mL with reference solution (a).

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: 50 per cent *V/V* solution of *concentrated ammonia R*, *anhydrous ethanol R*, *di-isopropyl ether R* (10:25:65 *V/V/V*); use the upper layer obtained after shaking the mixture.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*Retardation factors*: impurity A = about 0.2; amisulpride = about 0.5.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *methanol R*, mobile phase A (12:16:72 *V/V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in 16 mL of *methanol R*, add 12 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *amisulpride for system suitability CRS* (containing impurity B) in 1 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 0.7 g of *sodium octanesulfonate R* in 930 mL of *water for chromatography R* and add 45.0 mL of a 5 per cent *V/V* solution of *dilute sulfuric acid R*; adjust to pH 2.3 with *dilute sulfuric acid R* and dilute to 1000 mL with *water for chromatography R*;
– *mobile phase B*: *methanol R1* ;
– *mobile phase C*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 18 | 72 | 16 | 12 |
| 18 - 35 | 72 → 50 | 16 → 38 | 12 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to amisulpride (retention time = about 17 min): impurity B = about 1.1.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to amisulpride.

*Calculation of percentage contents*:

– for each impurity, use the concentration of amisulpride in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.3 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g with shaking in a mixture of 5 mL of *acetic anhydride R* and 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.95 mg of $C_{17}H_{27}N_3O_4S$.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H*.

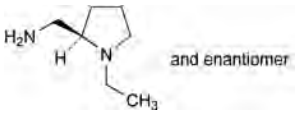


A. [(2*RS*)-1-ethylpyrrolidin-2-yl]methanamine,

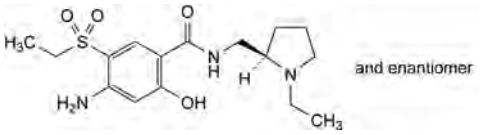


B. 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-(ethylsulfonyl)-2-hydroxybenzamide,

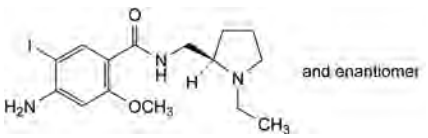


C. 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-iodo-2-methoxybenzamide,

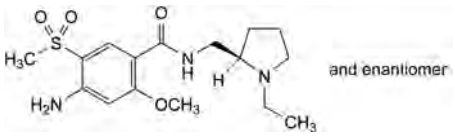


D. 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-2-methoxy-5-(methylsulfonyl)benzamide,

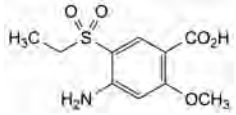


E. 4-amino-5-(ethylsulfonyl)-2-methoxybenzoic acid,

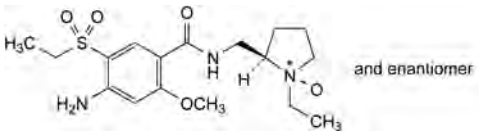


F. 4-amino-*N*-[[(2*RS*)-1-ethyl-1-oxidopyrrolidin-2-yl]methyl]-5-(ethylsulfonyl)-2-methoxybenzamide,

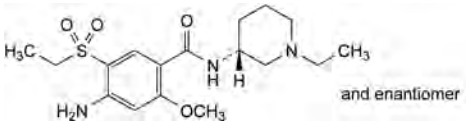


G. 4-amino-*N*-[(3*RS*)-1-ethylpiperidin-3-yl]-5-(ethylsulfonyl)-2-methoxybenzamide,

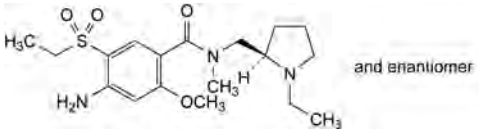


H. 4-amino-*N*-[[(2*RS*)-1-ethylpyrrolidin-2-yl]methyl]-5-(ethylsulfonyl)-2-methoxy-*N*-methylbenzamide.

**01/2017:0464**

# AMITRIPTYLINE HYDROCHLORIDE

## Amitriptylini hydrochloridum

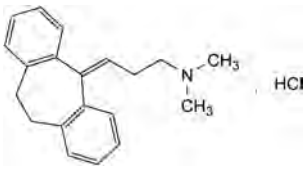


$C_{20}H_{24}ClN$ $M_r$ 313.9
[549-18-8]

DEFINITION

3-(10,11-Dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amitriptyline hydrochloride CRS*.

B. 20 mg gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 1.25 g in *water R* and dilute to 25 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *dibenzosuberone CRS* (impurity A) and 5.0 mg of *cyclobenzaprine hydrochloride CRS* (impurity B) in 5.0 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 5.23 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 7.0 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of amitriptyline.

*Relative retention* with reference to amitriptyline (retention time = about 14 min): impurity B = about 0.9; impurity A = about 2.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity B and amitriptyline.

*Limits*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *impurity A*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the peak due to amitriptyline in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the peak due to amitriptyline in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the peak due to amitriptyline in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.39 mg of $C_{20}H_{24}ClN$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G.*

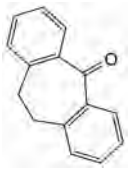

A. 10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-one (dibenzosuberone),

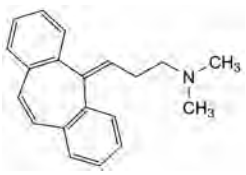

B. 3-(5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*,*N*-dimethylpropan-1-amine (cyclobenzaprine),

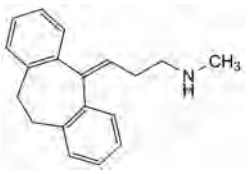

C. 3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*-methylpropan-1-amine (nortriptyline),

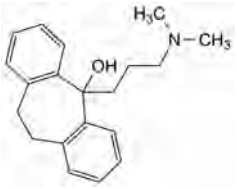

D. 5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ol,

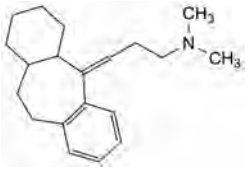

E. *N*,*N*-dimethyl-3-(1,2,3,4,4a,10,11,11a-octahydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)propan-1-amine,

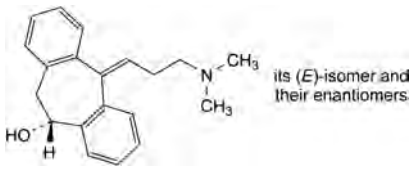


F. (5*EZ*,10*RS*)-5-[3-(dimethylamino)propylidene]-10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-10-ol,

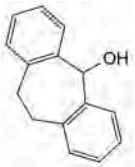

G. 10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ol (dibenzosuberol).

**04/2016:1491**

# AMLODIPINE BESILATE

## Amlodipini besilas

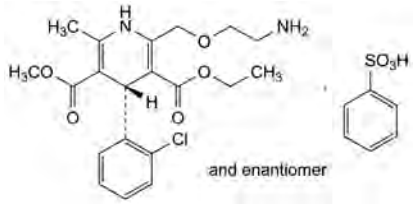


$C_{26}H_{31}ClN_2O_8S$ $M_r$ 567.1
[111470-99-6]

DEFINITION

3-Ethyl 5-methyl (4*RS*)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate benzenesulfonate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl benzenesulfonate esters are genotoxic and are potential impurities in amlodipine besilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general method *2.5.41. Methyl, ethyl and isopropyl benzenesulfonate in active substances* is available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, sparingly soluble in anhydrous ethanol, slightly soluble in 2-propanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amlodipine besilate CRS*.

TESTS

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.5 mg of *amlodipine impurity B CRS* and 2.5 mg of *amlodipine impurity G CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 2.5 mg of *amlodipine for peak identification CRS* (containing impurities D, E and F) in 5 mL of the mobile phase.

*Reference solution (d).* Dissolve 5.0 mg of *amlodipine impurity A CRS* in *acetonitrile R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 50.0 mg of *amlodipine besilate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: 2.3 g/L solution of *ammonium acetate R*, *methanol R* (30:70 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 237 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (c) and (d).

*Run time*: twice the retention time of amlodipine.

*Identification of impurities*: use the chromatogram supplied with *amlodipine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D, E and F; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to amlodipine (retention time = about 20 min): impurity G = about 0.21; impurity B = about 0.25; impurity D = about 0.5; impurity F = about 0.8; impurity E = about 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities G and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.7; impurity F = 0.7;
- *impurity D*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *impurities E, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 0.8 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to benzene sulfonate (relative retention = about 0.14).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (e).

Calculate the percentage content of $C_{26}H_{31}ClN_2O_8S$ taking into account the assigned content of *amlodipine besilate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, G, H.*

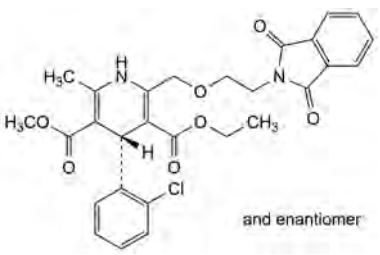


A. 3-ethyl 5-methyl (4*RS*)-4-(2-chlorophenyl)-2-[[2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)ethoxy]methyl]-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate,

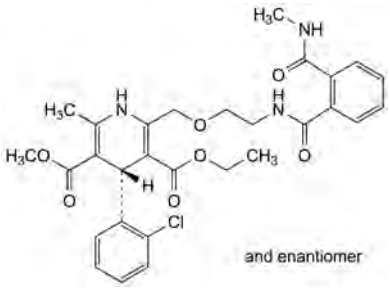


B. 3-ethyl 5-methyl (4*RS*)-4-(2-chlorophenyl)-6-methyl-2-[[2-[[2-(methylcarbamoyl)benzoyl]amino]-ethoxy]methyl]-1,4-dihydropyridine-3,5-dicarboxylate,

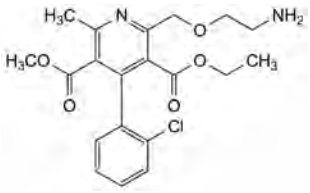

D. 3-ethyl 5-methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methylpyridine-3,5-dicarboxylate,

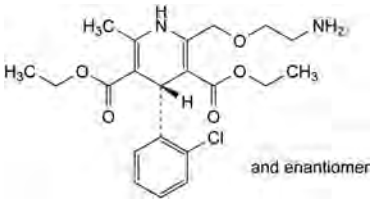


E. diethyl (4*RS*)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate,

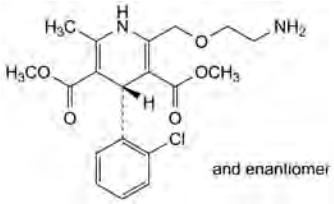


F. dimethyl (4*RS*)-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate,

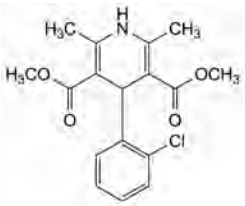

G. dimethyl 4-(2-chlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

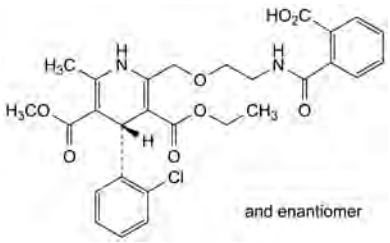


H. 2-[[2-[[(4*RS*)-4-(2-chlorophenyl)-3-(ethoxycarbonyl)-5-(methoxycarbonyl)-6-methyl-1,4-dihydropyridin-2-yl]methoxy]ethyl]carbamoyl]benzoic acid.

**01/2017:0877**

# AMMONIA SOLUTION, CONCENTRATED

## Ammoniae solutio concentrata

$NH_3$ $M_r$ 17.03

DEFINITION

*Content*: 25.0 per cent *m/m* to 30.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless liquid, very caustic.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. Relative density (*2.2.5*): 0.892 to 0.910.

B. It is strongly alkaline (*2.2.4*).

C. To 0.5 mL add 5 mL of *water R*. Bubble air through the solution and lead the gaseous mixture obtained over the surface of a solution containing 1 mL of *0.1 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The colour changes from red to yellow. Add 1 mL of *sodium cobaltinitrite solution R*. A yellow precipitate is formed.

TESTS

**Solution S**. Evaporate 220 mL almost to dryness on a water-bath. Cool, add 1 mL of *dilute acetic acid R* and dilute to 20 mL with *distilled water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

To 2 mL add 8 mL of *water R*.

**Oxidisable substances**. Cautiously add, whilst cooling, 8.8 mL to 100 mL of *dilute sulfuric acid R*. Add 0.75 mL of *0.002 M potassium permanganate*. Allow to stand for 5 min. The solution remains faintly pink.

**Pyridine and related substances**: maximum 2 ppm, calculated as pyridine.

Measure the absorbance (*2.2.25*) at 252 nm using *water R* as the compensation liquid. The absorbance is not greater than 0.06.

**Carbonates**: maximum 60 ppm.

To 10 mL in a test-tube with a ground-glass neck add 10 mL of *calcium hydroxide solution R*. Stopper immediately and mix. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of a 0.1 g/L solution of *anhydrous sodium carbonate R*.

**Chlorides** (*2.4.4*): maximum 1 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 5 ppm.

Dilute 3 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 0.25 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Residue on evaporation**: maximum 20 mg/L.

Evaporate 50 mL to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 1 mg.

ASSAY

Weigh accurately a flask with a ground-glass neck containing 50.0 mL of *1 M hydrochloric acid*. Add 2 mL of the substance to be examined and re-weigh. Add 0.1 mL of *methyl red solution R* as indicator. Titrate with *1 M sodium hydroxide* until the colour changes from red to yellow.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of $NH_3$.

STORAGE

Protected from air, at a temperature not exceeding 20 °C.

**01/2019:2081**

# AMMONIO METHACRYLATE COPOLYMER (TYPE A)

## Ammonio methacrylatis copolymerum A

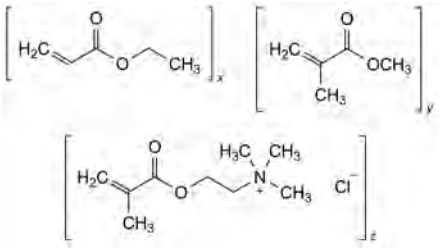

DEFINITION

Poly[ethyl propenoate-*co*-methyl 2-methylprop-2-enoate-*co*-*N*,*N*,*N*-trimethyl-2-[(2-methylprop-2-enoyl)oxy]ethan-1-aminium chloride] having a mean relative molecular mass of about 150 000.

The ratio of ethyl acrylate (ethyl propenoate) groups to methyl methacrylate (methyl 2-methylprop-2-enoate) groups to ammonio methacrylate (*N*,*N*,*N*-trimethyl-2-[(2-methylprop-2-enoyl)oxy]ethan-1-aminium chloride) groups is about 1:2:0.2.

*Content of ammonio methacrylate groups*: 8.9 per cent to 12.3 per cent (dried substance).

CHARACTERS

*Appearance*: colourless to white or almost white granules or powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in methylene chloride giving clear to cloudy solutions. Due to the polymeric nature of the substance, a stirring time of up to 5 h may be necessary.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ammonio methacrylate copolymer CRS*.

B. Viscosity (see Tests).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve a quantity of the substance to be examined corresponding to 12.5 g of the dried substance in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

**Viscosity** (*2.2.10*): maximum 15 mPa·s, determined on solution S.

*Apparatus*: rotating viscometer.

*Dimensions*:

- *spindle*: diameter = 25.15 mm; height = 90.74 mm; shaft diameter = 4.0 mm;
- *cylinder*: diameter = 27.62 mm; height = 0.135 m.

*Stirring speed*: 30 r/min.

*Volume of solution*: 16 mL of solution S.

*Temperature*: 20 °C.

**Appearance of a film**. Spread 2 mL of solution S evenly on a glass plate. Upon drying a clear film is formed.

**Monomers**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 3.5 g of *sodium perchlorate R* in *water R* and dilute to 100 mL with the same solvent.

*Test solution*. Dissolve 5.00 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent. To 10.0 mL of this solution add 5.0 mL of solution A, dropwise, while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant solution.

*Reference solution*. Dissolve 50.0 mg of *ethyl acrylate R* and 10.0 mg of *methyl methacrylate R* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Add 10 mL of this solution to 5 mL of solution A.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4.6 mm;
- *stationary phase*: irregular *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R2* and 80 volumes of *water for chromatography R* previously ajusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 50 µL.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (100 ppm);
- *impurity B*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (50 ppm).

**Methanol** (*2.4.24, System A*): maximum 1.5 per cent.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 5 h.

ASSAY

Dissolve 1.000 g in 75 mL of *glacial acetic acid R* at about 50 °C within about 30 min. Allow to cool to room temperature and add 25 mL of a 6 g/L solution of *copper acetate R* in *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.77 mg of $C_9H_{18}O_2NCl$ (ammonio methacrylate groups).

IMPURITIES

*Specified impurities: A, B.*

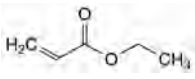

A. ethyl propenoate (ethyl acrylate),

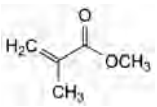

B. methyl 2-methylprop-2-enoate (methyl methacrylate).

**01/2019:2082**

# AMMONIO METHACRYLATE COPOLYMER (TYPE B)

## Ammonio methacrylatis copolymerum B

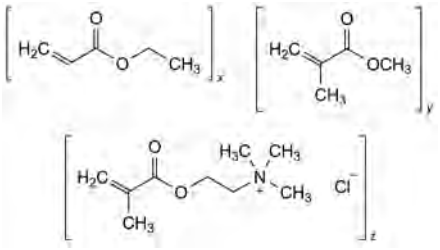

DEFINITION

Poly[ethyl propenoate-*co*-methyl 2-methylprop-2-enoate-*co*-*N*,*N*,*N*-trimethyl-2-[(2-methylprop-2-enoyl)oxy]ethan-1-aminium chloride] having a mean relative molecular mass of about 150 000.

The ratio of ethyl acrylate (ethyl propenoate) groups to methyl methacrylate (methyl 2-methylprop-2-enoate) groups to ammonio methacrylate (*N*,*N*,*N*-trimethyl-2-[(2-methylprop-2-enoyl)oxy]ethan-1-aminium chloride) groups is about 1:2:0.1.

*Content of ammonio methacrylate groups*: 4.5 per cent to 7.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless to white or almost white granules or powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in methylene chloride giving clear to cloudy solutions. Due to the polymeric nature of the substance, a stirring time of up to 5 h may be necessary.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ammonio methacrylate copolymer CRS*.

B. Viscosity (see Tests).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve a quantity of the substance to be examined corresponding to 12.5 g of the dried substance in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

**Viscosity** (*2.2.10*): maximum 15 mPa·s, determined on solution S.

*Apparatus*: rotating viscometer.

*Dimensions*:

- *spindle*: diameter = 25.15 mm; height = 90.74 mm; shaft diameter = 4.0 mm;
- *cylinder*: diameter = 27.62 mm; height = 0.135 m.

*Stirring speed*: 30 r/min.

*Volume of solution*: 16 mL of solution S.

*Temperature*: 20 °C.

**Appearance of a film**. Spread 2 mL of solution S evenly on a glass plate. Upon drying a clear film is formed.

**Monomers**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 3.5 g of *sodium perchlorate R* in *water R* and dilute to 100 mL with the same solvent.

*Test solution*. Dissolve 5.00 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent. To 10.0 mL of this solution add 5.0 mL of solution A, dropwise, while continuously stirring. Remove the precipitated polymer by centrifugation. Use the clear supernatant solution.

*Reference solution*. Dissolve 50.0 mg of *ethyl acrylate R* and 10.0 mg of *methyl methacrylate R* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Add 10 mL of this solution to 5 mL of solution A.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4.6 mm;
- *stationary phase*: irregular *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R2* and 80 volumes of *water for chromatography R* previously ajusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 50 µL.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (100 ppm);
- *impurity B*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (50 ppm).

**Methanol** (*2.4.24, System A*): maximum 1.5 per cent.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 5 h.

ASSAY

Dissolve 2.000 g in 75 mL of *glacial acetic acid R* at about 50 °C within about 30 min. Allow to cool to room temperature and add 25 mL of a 6 g/L solution of *copper acetate R* in *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.77 mg of $C_9H_{18}O_2NCl$ (ammonio methacrylate groups).

IMPURITIES

*Specified impurities: A, B.*

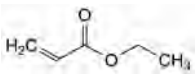

A. ethyl propenoate (ethyl acrylate),

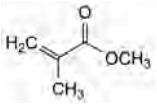

B. methyl 2-methylprop-2-enoate (methyl methacrylate).

01/2017:1389

# AMMONIUM BROMIDE

## Ammonii bromidum

$NH_4Br$ $M_r$ 97.9
[12124-97-9]

DEFINITION

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

It becomes yellow when exposed to light or air.

IDENTIFICATION

A. It gives reaction (a) of bromides (*2.3.1*).

B. 10 mL of solution S (see Tests) gives the reaction of ammonium salts (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromates**. To 10 mL of solution S add 1 mL of *starch solution R*, 0.1 mL of a 100 g/L solution of *potassium iodide R* and 0.25 mL of *0.5 M sulfuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

**Chlorides and sulfates**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.400 g of the substance to be examined in 50 mL of *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dilute 25.0 mL of test solution (a) to 50.0 mL with *water for chromatography R*.

*Reference solution (a)*. To 25.0 mL of test solution (a) add 1.0 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 12.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (b)*. Dilute 10.0 mL of test solution (a) to 100.0 mL with *water for chromatography R*. To 2.0 mL of this solution add 8.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 20.0 mL with *water for chromatography R*.

*Blank solution*: *water for chromatography R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 2 mm;

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (13 µm).

*Mobile phase*: dissolve 0.600 g of *potassium hydroxide R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent.

*Flow rate*: 0.4 mL/min.

*Detection*: conductivity detector equipped with a suitable ion suppressor.

*Injection*: 50 µL of test solution (b), reference solutions (a) and (b) and the blank solution.

*Run time*: 2.5 times the retention time of bromide.

*Retention time*: chloride = about 5 min; bromide = about 8 min; sulfate = about 16 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 8.0 between the peaks due to chloride and bromide.

*Calculation of percentage contents*:

– for chlorides, use the concentration of chloride in reference solution (a); correct the area of the peak due to chloride in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to chloride in the chromatogram obtained with test solution (b);

– for sulfates, use the concentration of sulfate in reference solution (a); correct the area of the peak due to sulfate in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to sulfate in the chromatogram obtained with test solution (b).

*Limits*:

– *chlorides*: maximum 0.6 per cent;

– *sulfates*: maximum 0.01 per cent.

**Iodides**. To 5 mL of solution S add 0.15 mL of *ferric chloride solution R1* and 2 mL of *methylene chloride R*. Shake and allow to separate. The lower layer is colourless (*2.2.2, Method I*).

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium and alkaline-earth metals** (*2.4.7*): maximum 200 ppm, calculated as Ca.

10.0 g complies with the test for magnesium and alkaline-earth metals. The volume of *0.01 M sodium edetate* used does not exceed 5.0 mL.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 9.794 mg of $NH_4Br$.

Calculate the percentage content of $NH_4Br$ using the following expression:

$$a - 2.763\,b$$

*a* = percentage content of $NH_4Br$ and $NH_4Cl$ obtained in the assay and calculated as $NH_4Br$;

*b* = percentage content of Cl obtained in the test for chlorides.

STORAGE

In an airtight container, protected from light.

01/2017:0007

# AMMONIUM CHLORIDE

## Ammonii chloridum

$NH_4Cl$ $M_r$ 53.49
[12125-02-9]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. It gives the reactions of chlorides (*2.3.1*).

B. 10 mL of solution S (see Tests) gives the reaction of ammonium salts (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromides and iodides**. To 10 mL of solution S add 0.1 mL of *dilute hydrochloric acid R* and 0.05 mL of *chloramine solution R*. After 1 min, add 2 mL of *chloroform R* and shake vigorously. The chloroform layer remains colourless (*2.2.2, Method I*).

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.00 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 1.000 g in 20 mL of *water R* and add a mixture of 5 mL of *formaldehyde solution R*, previously neutralised to *phenolphthalein solution R*, and 20 mL of *water R*. After 1-2 min, titrate slowly with *1 M sodium hydroxide*, using a further 0.2 mL of the same indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 53.49 mg of $NH_4Cl$.

**01/2017:1772**

# AMMONIUM GLYCYRRHIZATE

## Ammonii glycyrrhizas

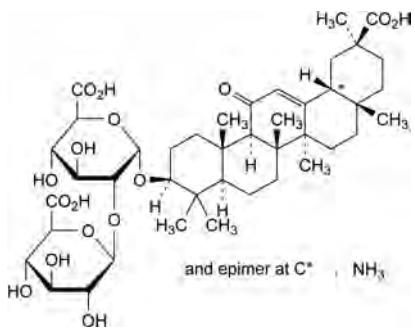


$C_{42}H_{65}NO_{16}$ $M_r$ 840

[53956-04-0]

DEFINITION

Mixture of ammonium 18α- and 18β-glycyrrhizate (ammonium salt of (20β)-3β-[[2-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid]oxy]-11-oxoolean-12-en-29-oic acid), the 18β-isomer being the main component.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, hygroscopic powder.

*Solubility*: slightly soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in acetone. It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ammonium glycyrrhizate CRS*.

B. Dissolve 0.1 g in 20 mL of *water R*, add 2 mL of *dilute sodium hydroxide solution R* and heat cautiously. On heating, the solution gives off vapours that may be identified by the alkaline reaction of wet litmus paper (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method I*).

Dissolve 1.0 g in *ethanol (20 per cent V/V) R* and dilute to 100.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 49.0 to + 54.0 (anhydrous substance).

Dissolve 0.5 g in *ethanol (50 per cent V/V) R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50 mg of *ammonium glycyrrhizate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 μm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (6:380:614 *V/V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 μL.

*Run time*: 3 times the retention time of 18β-glycyrrhizic acid.

*Relative retention* with reference to 18β-glycyrrhizic acid (retention time = about 8 min): impurity A = about 0.8; 18α-glycyrrhizic acid = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to 18β-glycyrrhizic acid and 18α-glycyrrhizic acid.

*Limits*:

– *18α-glycyrrhizic acid*: not more than twice the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (10.0 per cent),

– *impurity A*: not more than the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (5.0 per cent),

– *any other impurity*: for each impurity, not more than 0.4 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (2.0 per cent),
– *sum of other impurities*: not more than 1.4 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (7.0 per cent),
– *disregard limit*: 0.04 times the sum of the areas of the peaks in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.600 g in 60 mL of *anhydrous acetic acid R* heating at 80 °C if necessary. Cool. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 84.0 mg of $C_{42}H_{65}NO_{16}$.

STORAGE

In an airtight container.

IMPURITIES

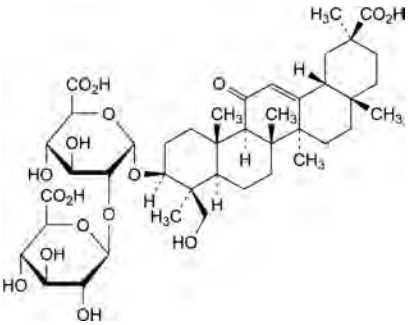

A. (4β,20β)-3β-[[2-*O*-(β-D-glucopyranosyluronic acid)-α-D-glucopyranosyluronic acid]oxy]-23-hydroxy-11-oxoolean-12-en-29-oic acid (24-hydroxyglycyrrhizinic acid).

**01/2017:1390**

# AMMONIUM HYDROGEN CARBONATE

## Ammonii hydrogenocarbonas

$NH_4HCO_3$ $M_r$ 79.1
[1066-33-7]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: fine, white or almost white, crystalline powder or white or almost white crystals, slightly hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

It volatilises rapidly at 60 °C. The volatilisation takes place slowly at ambient temperatures if the substance is slightly moist. It is in a state of equilibrium with ammonium carbamate.

IDENTIFICATION

A. It gives the reaction of carbonates and bicarbonates (*2.3.1*).

B. Dissolve 50 mg in 2 mL of *water R*. The solution gives the reaction of ammonium salts (*2.3.1*).

TESTS

**Solution S**. Dissolve 14.0 g in 100 mL of *distilled water R*. Boil to remove the ammonia, allow to cool and dilute to 100.0 mL with *distilled water R*.

**Chlorides** (*2.4.4*): maximum 70 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 70 ppm, determined on solution S.

**Iron** (*2.4.9*): maximum 40 ppm.

Dilute 1.8 mL of solution S to 10 mL with *water R*.

ASSAY

Dissolve cautiously 0.500 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection, or at the point of inflection if only 1 point is detected.

1 mL of *1 M hydrochloric acid* is equivalent to 79.1 mg of $NH_4HCO_3$.

STORAGE

In an airtight container.

**01/2008:0594**
**corrected 6.0**

# AMOBARBITAL

## Amobarbitalum

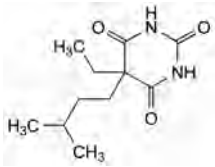

$C_{11}H_{18}N_2O_3$ $M_r$ 226.3
[57-43-2]

DEFINITION

Amobarbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5-ethyl-5-(3-methylbutyl)pyrimidin-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very slightly soluble in water, freely soluble in alcohol, soluble in methylene chloride. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *amobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 157 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *amobarbital CRS*.

C. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 mL with the same solvent.

*Reference solution.* Dissolve 0.1 g of *amobarbital CRS* in *alcohol R* and dilute to 100 mL with the same solvent.

Apply separately to the plate 10 µL of each solution. Develop over a path of 18 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 6 mL of *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 1.0 g add 50 mL of *water R* and boil for 2 min. Allow to cool and filter. To 10 mL of the filtrate add 0.15 mL of *methyl red solution R* and 0.1 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.2 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 mL with the same solvent.

*Reference solution.* Dilute 0.5 mL of the test solution to 100 mL with *alcohol R*.

Apply separately to the plate 20 µL of each solution. Develop over a path of 15 cm using the lower layer from a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine the plate immediately in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 5 mL of *pyridine R*. Add 0.5 mL of *thymolphthalein solution R* and 10 mL of *silver nitrate solution in pyridine R*. Titrate with *0.1 M ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 11.31 mg of $C_{11}H_{18}N_2O_3$.

**01/2008:0166**
**corrected 6.0**

# AMOBARBITAL SODIUM

## Amobarbitalum natricum

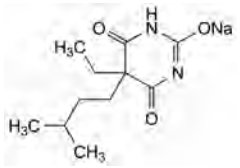


$C_{11}H_{17}N_2NaO_3$ $M_r$ 248.3
[64-43-7]

DEFINITION

Amobarbital sodium contains not less than 98.5 per cent and not more than the equivalent of 102.0 per cent of sodium derivative of 5-ethyl-5-(3-methylbutyl)pyrimidin-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, granular powder, hygroscopic, very soluble in carbon dioxide-free water (a small fraction may be insoluble), freely soluble in alcohol.

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Acidify 10 mL of solution S (see Tests) with *dilute hydrochloric acid R* and shake with 20 mL of *ether R*. Separate the ether layer, wash with 10 mL of *water R*, dry over *anhydrous sodium sulfate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100 °C to 105 °C (test residue). Repeat the operations using 0.1 g of *amobarbital sodium CRS* (reference residue). Determine the melting point (*2.2.14*) of the test residue. Mix equal parts of the test residue and the reference residue and determine the melting point of the mixture. The difference between the melting points (which are about 157 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing the spectrum obtained with the reference residue prepared from *amobarbital sodium CRS* with that obtained with the test residue (see identification test A).

C. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.1 g of the substance to be examined in *alcohol R* and dilute to 100 mL with the same solvent.

*Reference solution.* Dissolve 0.1 g of *amobarbital sodium CRS* in *alcohol R* and dilute to 100 mL with the same solvent.

Apply separately to the plate 10 µL of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

E. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *alcohol (50 per cent V/V) R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*). Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent. Disregard any slight residue. The pH of the solution is not more than 11.0.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 mL with the same solvent.

*Reference solution*. Dilute 0.5 mL of the test solution to 100 mL with *alcohol R*.

Apply separately to the plate 20 µL of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine the plate immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent). Disregard any spot at the point of application.

**Loss on drying** (*2.2.32*). Not more than 3.0 per cent, determined on 0.50 g by drying in an oven at 130 °C.

ASSAY

Dissolve 0.200 g in 5 mL of *ethanol R*. Add 0.5 mL of *thymolphthalein solution R* and 10 mL of *silver nitrate solution in pyridine R*. Titrate with *0.1 M ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 24.83 mg of $C_{11}H_{17}N_2NaO_3$.

STORAGE

Store in an airtight container.

**04/2019:2756**

# AMOROLFINE HYDROCHLORIDE

## Amorolfini hydrochloridum

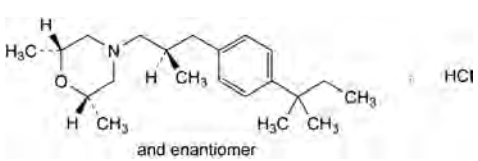


$C_{21}H_{36}ClNO$ $M_r$ 354.0
[78613-38-4]

DEFINITION

(2*RS*,6*SR*)-2,6-Dimethyl-4-[(2*RS*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]morpholine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amorolfine hydrochloride CRS*.

B. To 20 mg add 4.0 mL of *water R*, and acidify with *dilute nitric acid R*. A precipitate is formed. Centrifuge, and to 2 mL of the supernatant add 0.4 mL of *silver nitrate solution R1*. A curdled, white precipitate is formed. Centrifuge and wash the precipitate with 3 quantities, each of 1 mL, of *water R*. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves easily with the possible exception of a few large particles which dissolve slowly.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 3.5 g of *dipotassium hydrogen phosphate R* in 1000 mL of *water for chromatography R* and adjust to pH 7.0 with *phosphoric acid R*.

*Test solution*. Dissolve 20 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 4 mg of *amorolfine for system suitability CRS* (containing impurities D, E, I and J) in mobile phase A and dilute to 5 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 4 mg of *amorolfine for peak identification CRS* (containing impurity M) in mobile phase A and dilute to 5 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, buffer solution, *methanol R2* (5:35:60 *V/V/V*);
- *mobile phase B*: buffer solution, *acetonitrile R1*, *methanol R2* (10:30:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 25 | 90 → 0 | 10 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *amorolfine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities D, E, I and J; use the chromatogram supplied with *amorolfine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurity M (peaks 1 and 2).

*Relative retention* with reference to amorolfine (retention time = about 15 min): impurity M (peak 1) = about 0.56; impurity M (peak 2) = about 0.60; impurity D = about 0.85; impurity J = about 0.97; impurity I = about 1.05; impurity E (peak 1) = about 1.14; impurity E (peak 2) = about 1.17.

*System suitability*:
- *resolution*: minimum 2.0 between the peaks due to impurity J and amorolfine in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

– for each impurity, use the concentration of amorolfine hydrochloride in reference solution (b).

*Limits*:

– *impurity D*: maximum 0.2 per cent;
– *impurity E*: maximum 0.2 per cent for the sum of the areas of the 2 peaks;
– *impurity I*: maximum 0.15 per cent;
– *impurity M* : maximum 0.15 per cent for each peak;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 10.0 mL of *0.01 M hydrochloric acid* and 40 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.40 mg of $C_{21}H_{36}ClNO$.

IMPURITIES

*Specified impurities: D, E, I, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, F, G, H, J, K, L, O.*

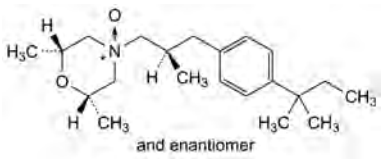


A. (2*RS*,4Ξ,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]morpholine 4-oxide,

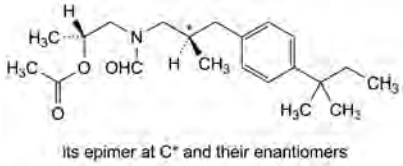


B. mixture of (2*RS*)-1-[*N*-[(2*R*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]formamido]propan-2-yl acetate and (2*RS*)-1-[*N*-[(2*S*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]formamido]propan-2-yl acetate,

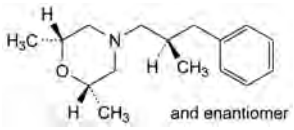


C. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-phenylpropyl]morpholine,

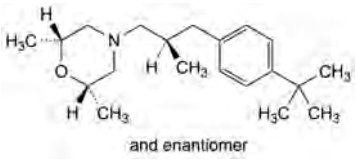


D. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-3-(4-*tert*-butylphenyl)-2-methylpropyl]morpholine,

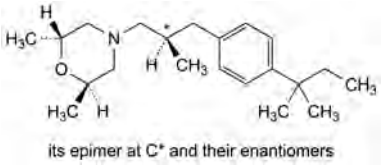


E. mixture of (2*RS*,6*RS*)-2,6-dimethyl-4-[(2*R*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]morpholine and (2*RS*,6*RS*)-2,6-dimethyl-4-[(2*S*)-2-methyl-3-[4-(2-methylbutan-2-yl)phenyl]propyl]morpholine,

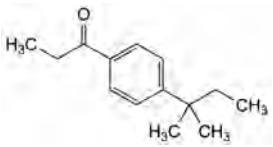

F. 1-[4-(2-methylbutan-2-yl)phenyl]propan-1-one,

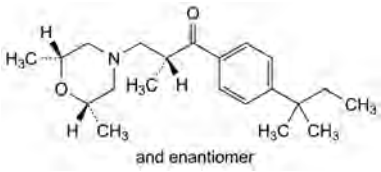


G. (2*RS*)-3-[(2*RS*,6*SR*)-2,6-dimethylmorpholin-4-yl]-1-[4-(2-methylbutan-2-yl)phenyl]-2-methylpropan-1-one,

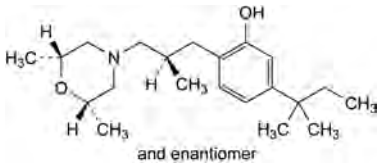


H. 2-[(2*RS*)-3-[(2*RS*,6*SR*)-2,6-dimethylmorpholin-4-yl]-2-methylpropyl]-5-(2-methylbutan-2-yl)phenol,

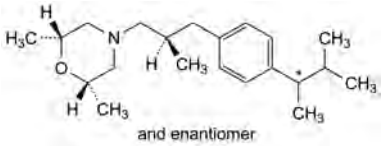


I. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-[4-[(2Ξ)-3-methylbutan-2-yl]phenyl]propyl]morpholine,

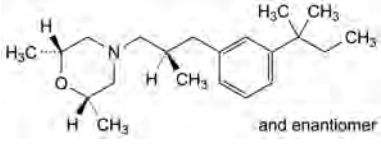


J. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-[3-(2-methylbutan-2-yl)phenyl]propyl]morpholine,

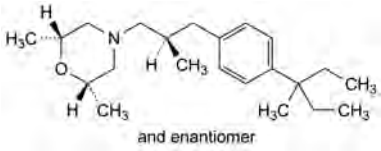


K. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-[4-(3-methylpentan-3-yl)phenyl]propyl]morpholine,

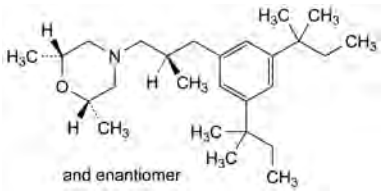


L. (2*RS*,6*SR*)-4-[(2*RS*)-3-[3,5-bis(2-methylbutan-2-yl)phenyl]-2-methylpropyl]-2,6-dimethylmorpholine,

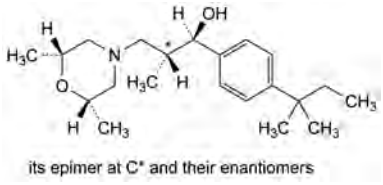


M. mixture of (1*RS*,2*RS*)-3-[(2*RS*,6*SR*)-2,6-dimethylmorpholin-4-yl]-2-methyl-1-[4-(2-methylbutan-2-yl)phenyl]propan-1-ol and (1*RS*,2*SR*)-3-[(2*RS*,6*SR*)-2,6-dimethylmorpholin-4-yl]-2-methyl-1-[4-(2-methylbutan-2-yl)phenyl]propan-1-ol,

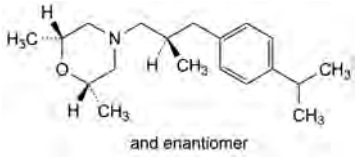


O. (2*RS*,6*SR*)-2,6-dimethyl-4-[(2*RS*)-2-methyl-3-[4-(propan-2-yl)phenyl]propyl]morpholine.

**01/2017:0577**
**corrected 10.0**

# AMOXICILLIN SODIUM

## Amoxicillinum natricum

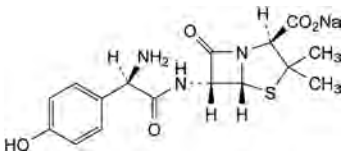

$C_{16}H_{18}N_3NaO_5S$ $M_r$ 387.4
[34642-77-8]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 89.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic, powder.

*Solubility*: very soluble in water, sparingly soluble in anhydrous ethanol, very slightly soluble in acetone.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.250 g in 5 mL of *water R*, add 0.5 mL of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the crystals and wash with 2-3 mL of a mixture of 1 volume of *water R* and 9 volumes of *acetone R*, then dry in an oven at 60 °C for 30 min.

*Comparison*: *amoxicillin trihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (a)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (b)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*), it may show an initial, but transient, pink colour, and after 5 min, its absorbance (*2.2.25*) at 430 nm is not greater than 0.20.

Dissolve 1.0 g in *water R* and dilute to 10.0 mL with the same solvent. Examine immediately after dissolution.

**pH** (*2.2.3*): 8.0 to 10.0.

Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 240 to + 290 (anhydrous substance).

Dissolve 62.5 mg in a 4 g/L solution of *potassium hydrogen phthalate R* and dilute to 25.0 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b)*. Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A. *Prepare immediately before use*.

*Reference solution (a)*. Dissolve 30.0 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 4.0 mg of *cefadroxil CRS* in mobile phase A and dilute to 50 mL with mobile phase A. To 5.0 mL of this solution add 5.0 mL of reference solution (a) and dilute to 100 mL with mobile phase A.

*Reference solution (c).* Dilute 2.0 mL of reference solution (a) to 20.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 20.0 mL with mobile phase A.

*Reference solution (d).* To 0.20 g of *amoxicillin trihydrate R* add 1.0 mL of *water R*. Shake and add dropwise *dilute sodium hydroxide solution R* to obtain a solution. The pH of the solution is about 8.5. Store the solution at room temperature for 4 h. Dilute 0.5 mL of this solution to 50.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 1 volume of *acetonitrile R* and 99 volumes of a 25 per cent *V/V* solution of *0.2 M potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*;
- *mobile phase B*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 25 per cent *V/V* solution of *0.2 M potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 92 | 8 |
| $t_R$ - ($t_R$ + 25) | 92 → 0 | 8 → 100 |
| ($t_R$ + 25) - ($t_R$ + 40) | 0 | 100 |
| ($t_R$ + 40) - ($t_R$ + 55) | 92 | 8 |

$t_R$ = retention time of amoxicillin determined with reference solution (c)

If the mobile phase has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of reference solutions (b) and (c) with isocratic elution at the initial mobile phase composition and 50 µL of test solution (b) and reference solution (d) according to the elution gradient described under Mobile phase; inject mobile phase A as a blank according to the elution gradient described under Mobile phase.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the 3 principal peaks eluted after the main peak corresponding to impurity C, amoxicillin dimer (impurity J; *n* = 1) and amoxicillin trimer (impurity J; *n* = 2).

*Relative retention* with reference to amoxicillin: impurity C = about 3.4; impurity J (*n* = 1) = about 4.1; impurity J (*n* = 2) = about 4.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to amoxicillin and cefadroxil; if necessary, adjust the ratio A:B of the mobile phase.

*Limits*:

- *impurity J* (*n* = 1): not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3 per cent);
- *any other impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent);
- *total*: not more than 9 times the area of the principal peak in the chromatogram obtained with reference solution (c) (9 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method A or B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.400 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of amoxicillin sodium by multiplying the percentage content of amoxicillin by 1.060.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

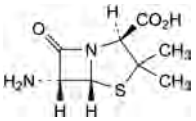

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

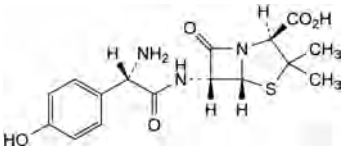

B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid (L-amoxicillin),

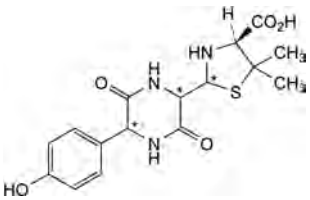

C. (4*S*)-2-[5-(4-hydroxyphenyl)-3,6-dioxopiperazin-2-yl]-5,5-dimethylthiazolidine-4-carboxylic acid (amoxicillin diketopiperazines),

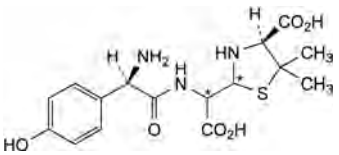

D. (4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of amoxicillin),

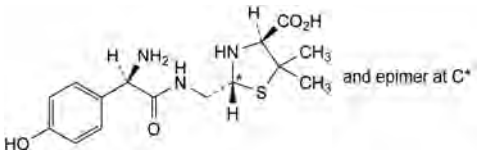


E. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]-amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of amoxicillin),

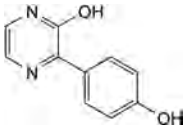

F. 3-(4-hydroxyphenyl)pyrazin-2-ol,

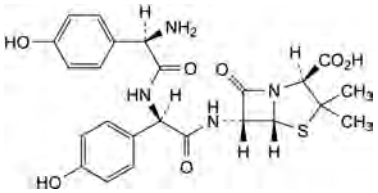

G. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-(4-hydroxyphenyl)glycylamoxicillin),

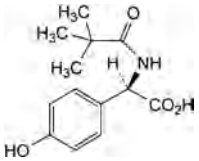

H. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-(4-hydroxyphenyl)acetic acid,

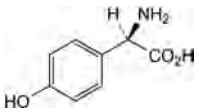

I. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,

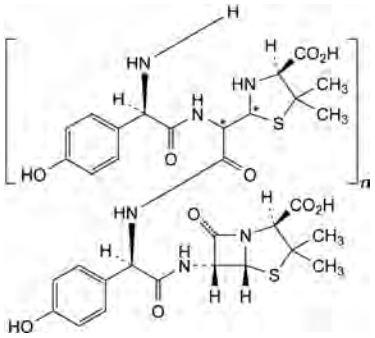

J. co-oligomers of amoxicillin and penicilloic acids of amoxicillin,

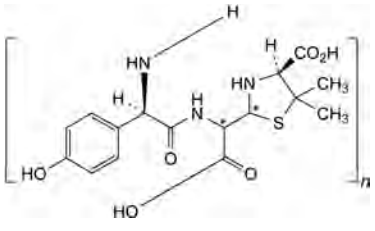

K. oligomers of penicilloic acids of amoxicillin.

**01/2013:0260**

# AMOXICILLIN TRIHYDRATE

## Amoxicillinum trihydricum

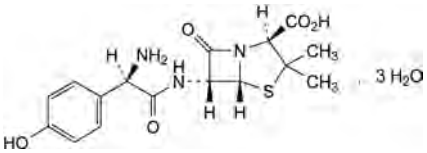

$C_{16}H_{19}N_3O_5S,3H_2O$ $M_r$ 419.4
[61336-70-7]

DEFINITION

(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-Amino-2-(4-hydroxyphenyl)acetyl]-amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in fatty oils. It dissolves in dilute acids and dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *amoxicillin trihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.
*Reference solution (a)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.
*Reference solution (b)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.
*Plate*: *TLC silanised silica gel plate R*.
*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.
*Application*: 1 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

TESTS

**Solution S**. With the aid of ultrasound or gentle heating, dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 290 to + 315 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 5.0.* To 250 mL of *0.2 M potassium dihydrogen phosphate R* add *dilute sodium hydroxide solution R* to pH 5.0 and dilute to 1000.0 mL with *water R*.

*Test solution (a).* Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b).* Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A. *Prepare immediately before use.*

*Reference solution (a).* Dissolve 30.0 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 4.0 mg of *cefadroxil CRS* in mobile phase A and dilute to 50 mL with mobile phase A. To 5.0 mL of this solution add 5.0 mL of reference solution (a) and dilute to 100 mL with mobile phase A.

*Reference solution (c).* Dilute 2.0 mL of reference solution (a) to 20.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, buffer solution pH 5.0 (1:99 *V/V*);
- *mobile phase B*: *acetonitrile R*, buffer solution pH 5.0 (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 92 | 8 |
| $t_R$ - ($t_R$ + 25) | 92 → 0 | 8 → 100 |
| ($t_R$ + 25) - ($t_R$ + 40) | 0 | 100 |
| ($t_R$ + 40) - ($t_R$ + 55) | 92 | 8 |

$t_R$ = retention time of amoxicillin determined with reference solution (c)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of reference solutions (b) and (c) with isocratic elution at the initial mobile phase composition and 50 µL of test solution (b) according to the elution gradient described under Mobile phase; inject mobile phase A as a blank according to the elution gradient described under Mobile phase.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to amoxicillin and cefadroxil; if necessary, adjust the ratio A:B of the mobile phase.

*Limit*:

- *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method A or B*): maximum 20 ppm.

**Water** (*2.5.12*): 11.5 per cent to 14.5 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{16}H_{19}N_3O_5S$ taking into account the assigned content of *amoxicillin trihydrate CRS*.

STORAGE

In an airtight container.

IMPURITIES

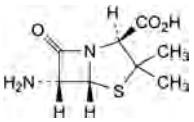

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

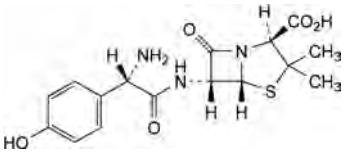

B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylic acid (L-amoxicillin),

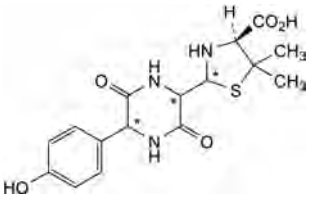

C. (4*S*)-2-[5-(4-hydroxyphenyl)-3,6-dioxopiperazin-2-yl]-5,5-dimethylthiazolidine-4-carboxylic acid (amoxicillin diketopiperazines),

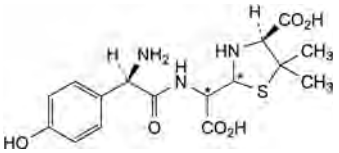

D. (4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]-amino]carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of amoxicillin),

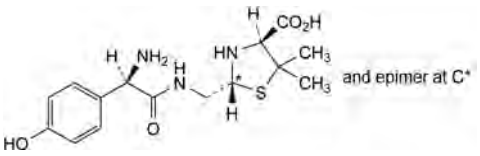


E. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]-amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of amoxicillin),

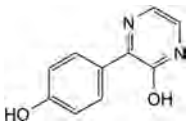

F. 3-(4-hydroxyphenyl)pyrazin-2-ol,

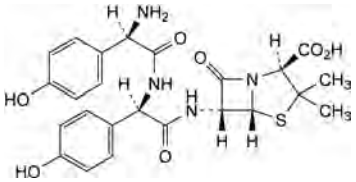

G. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-(4-hydroxyphenyl)glycylamoxicillin),

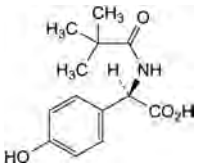

H. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-(4-hydroxyphenyl)acetic acid,

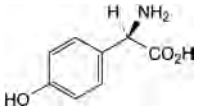

I. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,

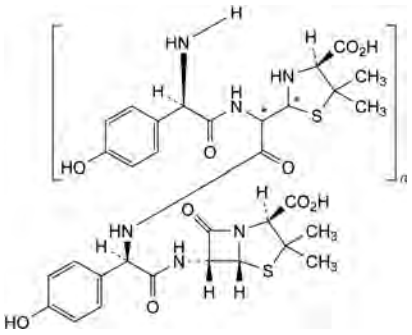

J. co-oligomers of amoxicillin and of penicilloic acids of amoxicillin,

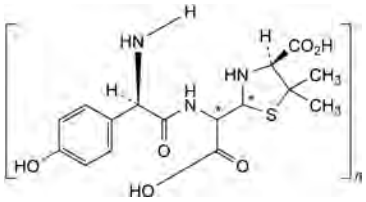

K. oligomers of penicilloic acids of amoxicillin,

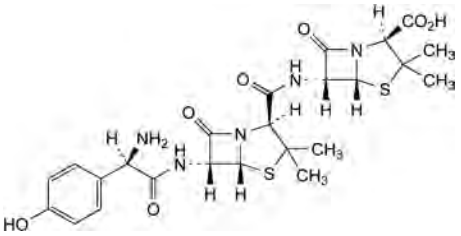

L. (2*S*,5*R*,6*R*)-6-[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA amoxicillin amide).

**01/2009:1292**
**corrected 10.0**

# AMPHOTERICIN B

## Amphotericinum B

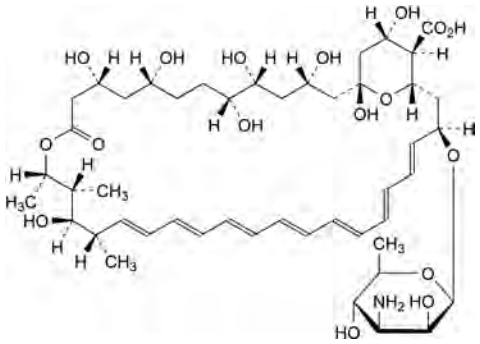

$C_{47}H_{73}NO_{17}$ $M_r$ 924
[1397-89-3]

DEFINITION

Mixture of antifungal polyenes produced by the growth of certain strains of *Streptomyces nodosus* or obtained by any other means. It consists mainly of amphotericin B which is (1*R*,3*S*,5*R*,6*R*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,19*E*,21*E*,23*E*,25*E*,27*E*,29*E*,31*E*,33*R*,35*S*,36*R*,37*S*)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylic acid.

*Content*: minimum 750 IU/mg (dried substance).

CHARACTERS

*Appearance*: yellow or orange, hygroscopic powder.

*Solubility*: practically insoluble in water, soluble in dimethyl sulfoxide and in propylene glycol, slightly soluble in dimethylformamide, very slightly soluble in methanol, practically insoluble in ethanol (96 per cent).

It is sensitive to light in dilute solutions.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25 mg in 5 mL of *dimethyl sulfoxide R* and dilute to 50 mL with *methanol R*. Dilute 2 mL of the solution to 200 mL with *methanol R*.

*Spectral range*: 300-450 nm.

*Absorption maxima*: at 362 nm, 381 nm and 405 nm.

*Absorbance ratios*:

– $A_{362}/A_{381}$ = 0.57 to 0.61;

– $A_{381}/A_{405}$ = 0.87 to 0.93.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *amphotericin B CRS*.

If the spectra obtained show differences, dry the substance to be examined and reference substance at 60 °C at a pressure not exceeding 0.7 kPa for 1 h and record new spectra.

C. To 1 mL of a 0.5 g/L solution in *dimethyl sulfoxide R*, add 5 mL of *phosphoric acid R* to form a lower layer, avoiding mixing the 2 liquids. A blue ring is immediately produced at the junction of the liquids. Mix, an intense blue colour is produced. Add 15 mL of *water R* and mix; the solution becomes pale yellow.

D. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution at 383 nm is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light and use within 24 h of preparation, except for reference solution (c) which should be injected immediately after its preparation.*

*Solvent mixture*: 10 g/L solution of *ammonium acetate R*, *N-methylpyrrolidone R*, *methanol R* (1:1:2 *V/V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 15 mL of *N-methylpyrrolidone R* and within 2 h dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *amphotericin B CRS* in 15 mL of *N-methylpyrrolidone R* and within 2 h dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20.0 mg of *nystatin CRS* in 15 mL of *N-methylpyrrolidone R* and within 2 h dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with reference solution (a). Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (d).* In order to prepare impurities B and C, dissolve 10 mg of the substance to be examined in 5 mL of *N-methylpyrrolidone R* and within 2 h add 35 mL of a mixture of 1 volume of *methanol R* and 4 volumes of *anhydrous ethanol R*. Add 0.10 mL of *dilute hydrochloric acid R*, mix and incubate at 25 °C for 2.5 h. Add 10 mL of 10 g/L solution of *ammonium acetate R* and mix.

*Reference solution (e).* Dissolve 4 mg of *amphotericin B for peak identification CRS* (containing impurities A and B) in 5 mL of *N-methylpyrrolidone R* and within 2 h dilute to 50 mL with the solvent mixture.

*Blank solution.* The solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 20 °C.

*Mobile phase*:

– *mobile phase A*: mix 1 volume of *methanol R*, 3 volumes of *acetonitrile R* and 6 volumes of a 4.2 g/L solution of *citric acid monohydrate R* previously adjusted to pH 4.7 using *concentrated ammonia R*;

– *mobile phase B*: mix 12 volumes of *methanol R*, 20 volumes of a 4.2 g/L solution of *citric acid monohydrate R* previously adjusted to pH 3.9 using *concentrated ammonia R* and 68 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 23 | 100 → 70 | 0 → 30 |
| 23 - 33 | 70 → 0 | 30 → 100 |
| 33 - 40 | 0 | 100 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer:

– at 303 nm: detection of tetraenes;

– at 383 nm: detection of heptaenes.

*Injection*: 20 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatograms supplied with *amphotericin B for peak identification CRS* and the chromatograms obtained with reference solution (e) to identify the peaks due to impurities A and B.

*Relative retention* with reference to amphotericin B (retention time = about 16 min): impurity B = about 0.75; impurity A = about 0.8; nystatin = about 0.85.

*System suitability at 383 nm*: reference solution (d):

– *resolution*: minimum 1.5 between the 2 peaks presenting a relative retention of about 0.7.

*Limits*:

– *impurity A at 303 nm*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent); if intended for use in the manufacture of parenteral preparations: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent);

– *any other impurity at 303 nm*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *impurity B at 383 nm*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent);

– *any other impurity at 383 nm*: for each impurity, not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *total at 303 and 383 nm*: maximum 15.0 per cent;

– *disregard limit at 303 nm*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);

– *disregard limit at 383 nm*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 3.0 per cent, determined on 1.0 g; if intended for use in the manufacture of parenteral preparations: maximum 0.5 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 1.0 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

*Protect all solutions from light throughout the assay.* Dissolve 25.0 mg in *dimethyl sulfoxide R* and dilute, with shaking, to 25.0 mL with the same solvent. Under constant stirring

of this stock solution, dilute with *dimethyl sulfoxide R* to obtain solutions of appropriate concentrations (the following concentrations have been found suitable: 44.4, 66.7 and 100 IU/mL). Prepare final solutions by diluting 1:20 with 0.2 M phosphate buffer solution pH 10.5 so that they all contain 5 per cent *V/V* of dimethyl sulfoxide. Prepare the reference and the test solutions simultaneously. Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C in an airtight container. If the substance is sterile, store in a sterile, tamper-evident container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

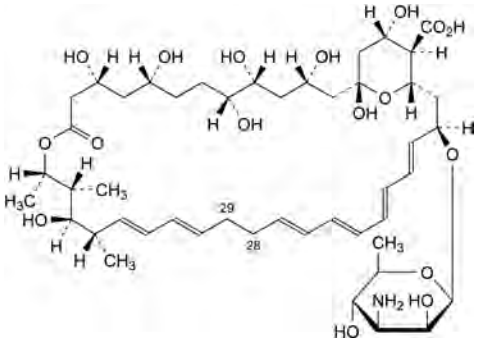

A. amphotericin A (28,29-dihydro-amphotericin B),

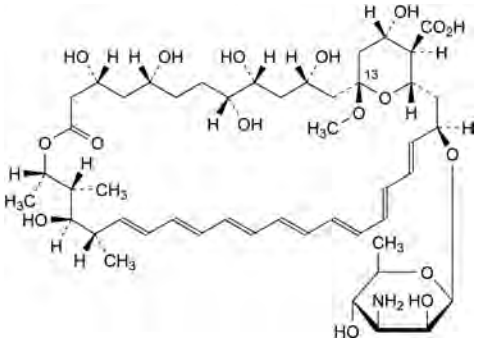

B. amphotericin X1 (13-*O*-methyl-amphotericin B),

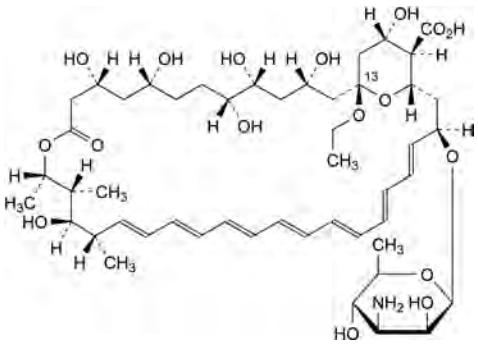

C. amphotericin X2 (13-*O*-ethyl-amphotericin B).

**01/2017:0167**

# AMPICILLIN

## Ampicillinum

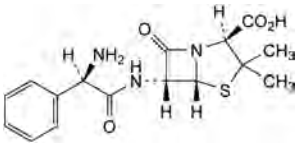

$C_{16}H_{19}N_3O_4S$ $M_r$ 349.4

[69-53-4]

DEFINITION

(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, practically insoluble in acetone, in ethanol (96 per cent) and in fatty oils. It dissolves in dilute solutions of acids and of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium bromide R*.

*Comparison*: *anhydrous ampicillin CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (a)*. Dissolve 25 mg of *anhydrous ampicillin CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (b)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *anhydrous ampicillin CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. Water (see Tests).

TESTS

**Appearance of solution**. The solutions are not more opalescent than reference suspension II (*2.2.1*).

Dissolve 1.0 g in 10 mL of *1 M hydrochloric acid*. Separately dissolve 1.0 g in 10 mL of *dilute ammonia R2*. Examine immediately after dissolution.

**pH** (*2.2.3*): 3.5 to 5.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 40 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 280 to + 305 (anhydrous substance).

Dissolve 62.5 mg in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 27.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b)*. *Prepare immediately before use.* Dissolve 27.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 27.0 mg of *anhydrous ampicillin CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2.0 mg of *cefradine CRS* in mobile phase A and dilute to 50 mL with mobile phase A. To 5.0 mL of this solution add 5.0 mL of reference solution (a).

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 50 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;
– *mobile phase B*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 400 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 85 | 15 |
| $t_R$ - ($t_R$ + 30) | 85 → 0 | 15 → 100 |
| ($t_R$ + 30) - ($t_R$ + 45) | 0 | 100 |
| ($t_R$ + 45) - ($t_R$ + 60) | 85 | 15 |

$t_R$ = retention time of ampicillin determined with reference solution (c)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of reference solutions (b) and (c) with isocratic elution at the initial mobile phase composition and 50 µL of test solution (b) according to the elution gradient described under Mobile phase; inject mobile phase A as a blank according to the elution gradient described under Mobile phase.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to ampicillin and cefradin; if necessary, adjust the ratio A:B of the mobile phase.

*Limit*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{16}H_{19}N_3O_4S$ from the declared content of *anhydrous ampicillin CRS*.

STORAGE

In an airtight container, at a temperature not exceeding 30 °C.

IMPURITIES

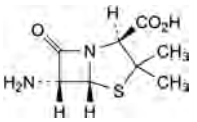

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

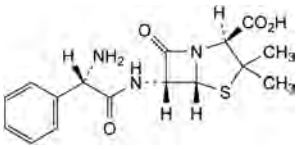

B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

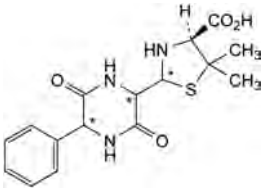

C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

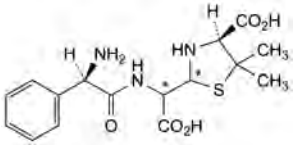

D. (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-carboxymethyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

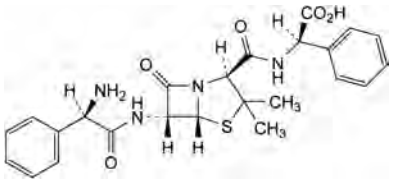

E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]-amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),

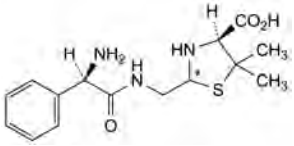

F. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penilloic acids of ampicillin),

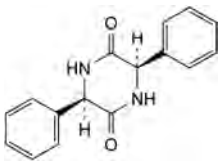

G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,

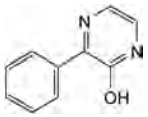

H. 3-phenylpyrazin-2-ol,

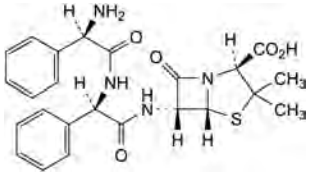

I. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(2*R*)-2-amino-2-phenylacetyl]-amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),

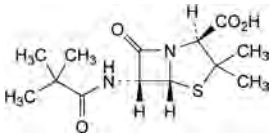

J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

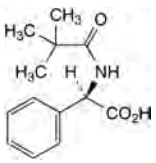

K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,

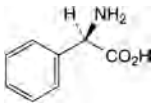

L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

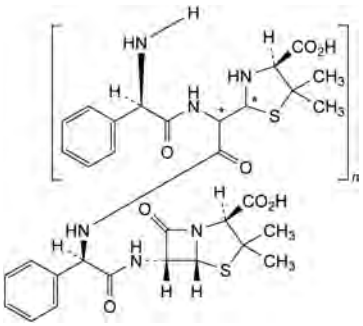

M. co-oligomers of ampicillin and of penicilloic acids of ampicillin.

**01/2017:0578**
**corrected 10.0**

# AMPICILLIN SODIUM

## Ampicillinum natricum

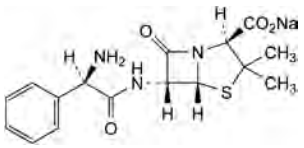

$C_{16}H_{18}N_3NaO_4S$ $M_r$ 371.4
[69-52-3]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 91.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in acetone, practically insoluble in fatty oils and in liquid paraffin.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.250 g in 5 mL of *water R*, add 0.5 mL of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the crystals through a small sintered-glass filter (40) (*2.1.2*), applying suction, wash with 2-3 mL of a mixture of 1 volume of *water R* and 9 volumes of *acetone R*, then dry in an oven at 60 °C for 30 min.

*Comparison*: *ampicillin trihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (a)*. Dissolve 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Reference solution (b)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. Solutions A and B are not more opalescent than reference suspension II (*2.2.1*) and the absorbance (*2.2.25*) of solution B at 430 nm is not greater than 0.15.

Place 1.0 g in a conical flask and add slowly and with continuous swirling 10 mL of *1 M hydrochloric acid* (solution A). Separately dissolve 1.0 g in *water R* and dilute to 10.0 mL with the same solvent (solution B). Examine immediately after dissolution.

**pH** (*2.2.3*): 8.0 to 10.0.

Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent. Measure 10 min after dissolution.

**Specific optical rotation** (*2.2.7*): + 258 to + 287 (anhydrous substance).

Dissolve 62.5 mg in a 4 g/L solution of *potassium hydrogen phthalate R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b).* Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A. *Prepare immediately before use.*

*Reference solution (a).* Dissolve 27.0 mg of *anhydrous ampicillin CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 2.0 mg of *cefradine CRS* in mobile phase A and dilute to 50 mL with mobile phase A. To 5.0 mL of this solution add 5.0 mL of reference solution (a).

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with mobile phase A.

*Reference solution (d).* To 0.20 g of the substance to be examined add 1.0 mL of *water R*. Heat the solution at 60 °C for 1 h. Dilute 0.5 mL of this solution to 50.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 50 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;

– *mobile phase B*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 400 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 85 | 15 |
| $t_R$ - ($t_R$ + 30) | 85 → 0 | 15 → 100 |
| ($t_R$ + 30) - ($t_R$ + 45) | 0 | 100 |
| ($t_R$ + 45) - ($t_R$ + 60) | 85 | 15 |

$t_R$ = retention time of ampicillin determined with reference solution (c)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of reference solutions (b) and (c) with isocratic elution at the initial mobile phase composition and 50 µL of test solution (b) and reference solution (d) according to the elution gradient described under Mobile phase; inject mobile phase A as a blank according to the elution gradient described under Mobile phase.

*Identification of peaks*: use the chromatogram obtained with reference solution (d) to identify the peaks due to ampicillin and ampicillin dimer.

*Relative retention* with reference to ampicillin: ampicillin dimer = about 2.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to ampicillin and cefradin; if necessary adjust the ratio A:B of the mobile phase.

*Limits*:

– *ampicillin dimer*: not more than 4.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (4.5 per cent);

– *any other impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Methylene chloride**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 1.0 mL of *ethylene chloride R* in *water R* and dilute to 500.0 mL with the same solvent.

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dissolve 1.0 g of the substance to be examined in *water R*, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Reference solution.* Dissolve 1.0 mL of *methylene chloride R* in *water R* and dilute to 500.0 mL with the same solvent. To 1.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Column*:

– *material*: glass;

– *size*: *l* = 1.5 m, Ø = 4 mm;

– *stationary phase*: *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *macrogol 1000 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 40 mL/min.

*Temperature*:

– *column*: 60 °C;

– *injection port*: 100 °C;

– *detector*: 150 °C.

*Detection*: flame ionisation.

Calculate the content of methylene chloride taking its density at 20 °C to be 1.325 g/mL.

*Limit*:

– *methylene chloride*: maximum 0.2 per cent *m/m*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of ampicillin sodium by multiplying the percentage content of ampicillin by 1.063.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

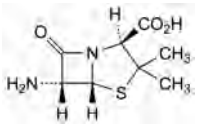

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

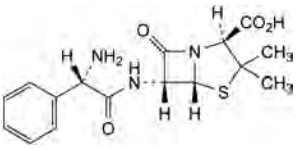

B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

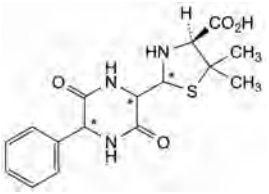

C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

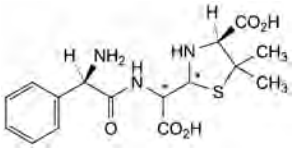

D. (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

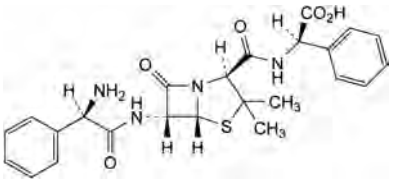

E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),

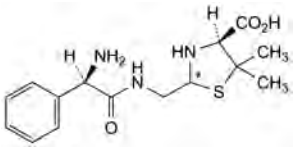

F. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ampicillin),

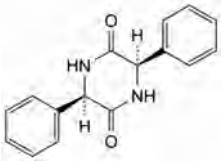

G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,

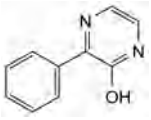

H. 3-phenylpyrazin-2-ol,

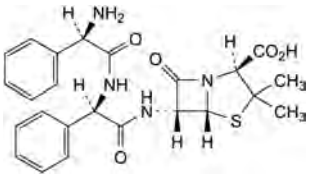

I. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(2*R*)-2-amino-2-phenylacetyl]-amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),

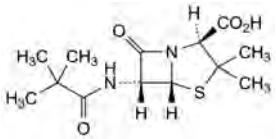

J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

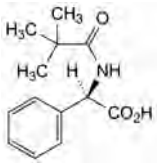

K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,

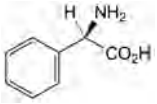

L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

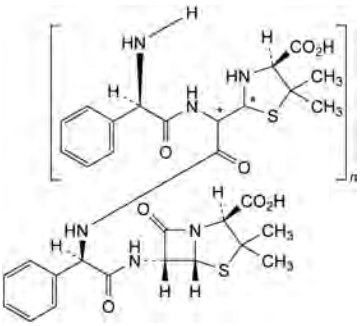

M. co-oligomers of ampicillin and of penicilloic acids of ampicillin,

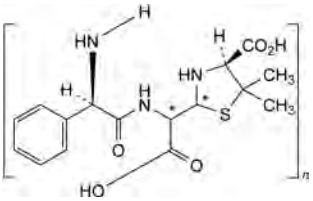

N. oligomers of penicilloic acids of ampicillin.

**01/2008:0168**
**corrected 6.0**

# AMPICILLIN TRIHYDRATE

## Ampicillinum trihydricum

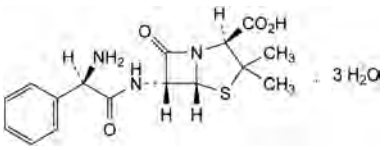

$C_{16}H_{19}N_3O_4S,3H_2O$ $M_r$ 403.5
[7177-48-2]

DEFINITION

(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid trihydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent) and in fatty oils. It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ampicillin trihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *sodium hydrogen carbonate solution R*.
*Reference solution (a)*. Dissolve 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.
*Reference solution (b)*. Dissolve 25 mg of *amoxicillin trihydrate CRS* and 25 mg of *ampicillin trihydrate CRS* in 10 mL of *sodium hydrogen carbonate solution R*.
*Plate*: *TLC silanised silica gel plate R*.
*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.
*Application*: 1 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

D. Water (see Tests).

TESTS

**Appearance of solution**. The solutions are not more opalescent than reference suspension II (*2.2.1*).

Dissolve 1.0 g in 10 mL of *1 M hydrochloric acid*. Separately dissolve 1.0 g in 10 mL of *dilute ammonia R2*. Examine immediately after dissolution.

**pH** (*2.2.3*): 3.5 to 5.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 40 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 280 to + 305 (anhydrous substance).

Dissolve 62.5 mg in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b)*. Dissolve 31.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A. *Prepare immediately before use.*

*Reference solution (a)*. Dissolve 27.0 mg of *anhydrous ampicillin CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2 mg of *cefradine CRS* in mobile phase A and dilute to 50 mL with mobile phase A. To 5 mL of this solution, add 5 mL of reference solution (a).

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with mobile phase A.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
– *mobile phase A*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 50 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;
– *mobile phase B*: mix 0.5 mL of *dilute acetic acid R*, 50 mL of *0.2 M potassium dihydrogen phosphate R* and 400 mL of *acetonitrile R*, then dilute to 1000 mL with *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 85 | 15 |
| $t_R$ - ($t_R$ + 30) | 85 → 0 | 15 → 100 |
| ($t_R$ + 30) - ($t_R$ + 45) | 0 | 100 |
| ($t_R$ + 45) - ($t_R$ + 60) | 85 | 15 |

$t_R$ = retention time of ampicillin determined with reference solution (c)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of reference solutions (b) and (c) with isocratic elution at the initial mobile phase composition and 50 µL of test solution (b) according to the elution gradient described under Mobile phase; inject mobile phase A as a blank according to the elution gradient described under Mobile phase.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to ampicillin and cefradin; if necessary, adjust the ratio A:B of the mobile phase.

*Limit*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): 12.0 per cent to 15.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of ampicillin from the declared content of *anhydrous ampicillin CRS*.

STORAGE

In an airtight container.

IMPURITIES

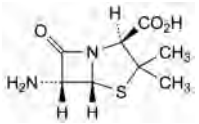

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

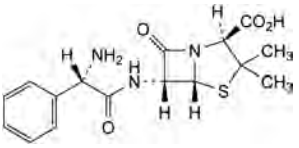

B. (2*S*,5*R*,6*R*)-6-[[(2*S*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (L-ampicillin),

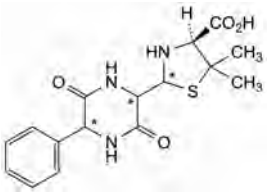

C. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

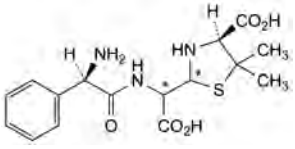

D. (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]carboxymethyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

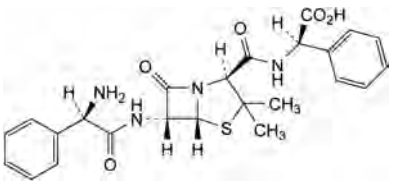

E. (2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetic acid (ampicillinyl-D-phenylglycine),

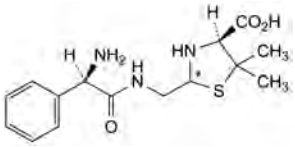

F. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penilloic acids of ampicillin),

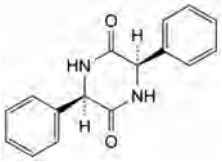

G. (3*R*,6*R*)-3,6-diphenylpiperazine-2,5-dione,

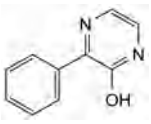

H. 3-phenylpyrazin-2-ol,

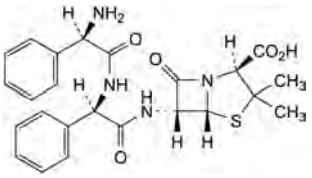

I. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (D-phenylglycylampicillin),

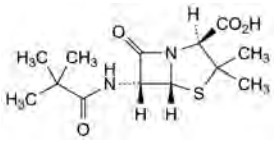

J. (2*S*,5*R*,6*R*)-6-[(2,2-dimethylpropanoyl)amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

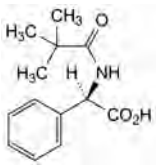

K. (2*R*)-2-[(2,2-dimethylpropanoyl)amino]-2-phenylacetic acid,

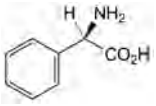

L. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

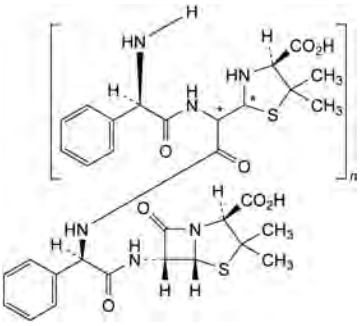

M. co-oligomers of ampicillin and of penicilloic acids of ampicillin,

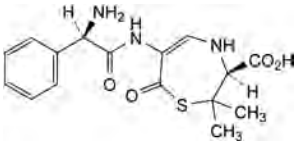

N. (3*S*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2,2-dimethyl-7-oxo-2,3,4,7-tetrahydro-1,4-thiazepine-3-carboxylic acid.

**01/2011:2405**

# AMYLMETACRESOL

## Amylmetacresolum

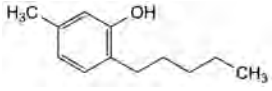

$C_{12}H_{18}O$ $M_r$ 178.3
[1300-94-3]

DEFINITION

5-Methyl-2-pentylphenol.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: clear or almost clear liquid, or solid crystalline mass, colourless or slightly yellow when freshly prepared. The substance changes colour during storage by darkening and/or discolouration to dark yellow, brownish-yellow or pink.

*Solubility*: practically insoluble in water, very soluble in acetone and in ethanol (96 per cent).

It solidifies at about 22 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: film between 2 plates of *potassium bromide R*.

*Comparison*: *amylmetacresol CRS*.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 0.100 g of *butylhydroxytoluene R* in *2-propanol R* and dilute to 10.0 mL with the same solvent.

*Test solution (a).* Dissolve 0.1000 g of the substance to be examined in *2-propanol R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* To 2.0 mL of test solution (a) add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *2-propanol R*.

*Reference solution (a).* Dissolve 10 mg of *m-cresol R* (impurity B) and 10 mg of *p-cresol R* (impurity D) in *2-propanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve the contents of a vial of *amylmetacresol for peak identification CRS* (containing impurities A, G and K) in 1.0 mL of *2-propanol R*.

*Reference solution (c).* Dissolve 0.1000 g of *amylmetacresol CRS* in *2-propanol R* and dilute to 10.0 mL with the same solvent. To 2.0 mL of this solution add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *2-propanol R*.

*Reference solution (d).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *2-propanol R*. Dilute 1.0 mL of this solution to 20.0 mL with *2-propanol R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 33 cm/s.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 17.5 | 100 → 240 |
| | 17.5 - 32.5 | 240 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL of test solution (a) and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *amylmetacresol for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, G and K.

*Relative retention* with reference to amylmetacresol (retention time = about 16 min): impurity G (diastereoisomer 1) = about 0.51; impurity G (diastereoisomer 2) = about 0.53; impurity D = about 0.77; impurity B = about 0.78; impurity K = about 0.95; impurity A = about 0.99.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurities D and B.

*Limits*:
- *impurity A*: maximum 0.6 per cent;
- *impurities G* (sum of the 2 diastereoisomers), *K*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *disregard limit*: the area of the peak due to amylmetacresol in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modification.

*Injection*: 1.0 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{12}H_{18}O$ from the declared content of *amylmetacresol CRS*.

STORAGE

In an airtight, non-metallic container, protected from light.

IMPURITIES

*Specified impurities: A, G, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, H, I, J.*

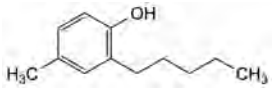

A. 4-methyl-2-pentylphenol,

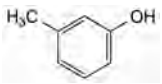

B. 3-methylphenol (*m*-cresol),

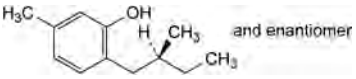


C. 5-methyl-2-[(2*RS*)-2-methylbutyl]phenol,

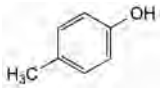

D. 4-methylphenol (*p*-cresol),

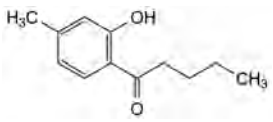

E. 1-(2-hydroxy-4-methylphenyl)pentan-1-one,

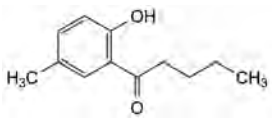

F. 1-(2-hydroxy-5-methylphenyl)pentan-1-one,

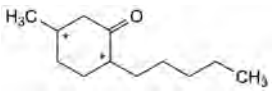

G. 5-methyl-2-pentylcyclohexanone,

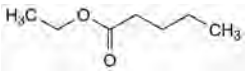

H. ethyl pentanoate,

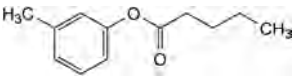

I. 3-methylphenyl pentanoate,

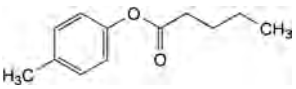

J. 4-methylphenyl pentanoate,

K. unknown structure.

**04/2013:2406**
**corrected 9.3**

# ANASTROZOLE

## Anastrozolum

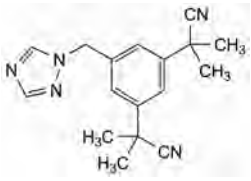

$C_{17}H_{19}N_5$ $M_r$ 293.4

[120511-73-1]

DEFINITION

2,2′-[5-(1*H*-1,2,4-Triazol-1-ylmethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, freely soluble in anhydrous ethanol, practically insoluble in cyclohexane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anastrozole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2.5 mg of *anastrozole impurity E CRS* in 20.0 mL of the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with test solution (a).

*Reference solution (c).* Dissolve 25.0 mg of *anastrozole CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R, water for chromatography R* (0.1:100 *V/V*);
- *mobile phase B*: *phosphoric acid R, acetonitrile R1* (0.1:100 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 | 5 |
| 2 - 54 | 95 → 35 | 5 → 65 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to anastrozole (retention time = about 29 min): impurity E = about 1.05.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.5 between the peaks due to anastrozole and impurity E.

*Calculation of percentage contents*:

- for each impurity, use the concentration of anastrozole in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 50.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{17}H_{19}N_5$ taking into account the assigned content of *anastrozole CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I.*

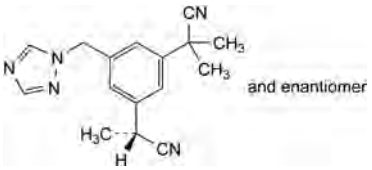


A. 2-[3-[(1*RS*)-1-cyanoethyl]-5-(1*H*-1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile,

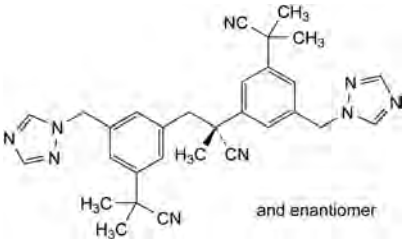


B. (2*RS*)-2,3-bis[3-(1-cyano-1-methylethyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)phenyl]-2-methylpropanenitrile,

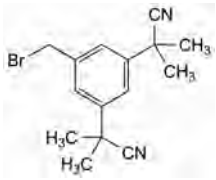


C. 2,2′-[5-(bromomethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile),

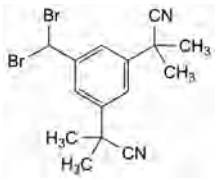


D. 2,2′-[5-(dibromomethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile),

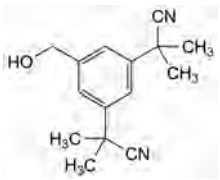


E. 2,2′-[5-(hydroxymethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile),

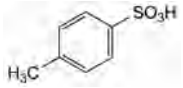


F. 4-methylbenzenesulfonic acid,

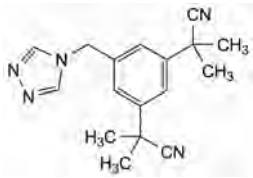


G. 2,2′-[5-(4*H*-1,2,4-triazol-4-ylmethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile),

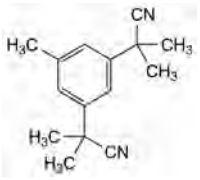

H. 2,2′-(5-methylbenzene-1,3-diyl)bis(2-methylpropanenitrile),

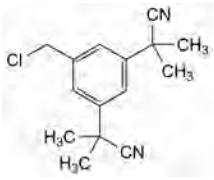

I. 2,2′-[5-(chloromethyl)benzene-1,3-diyl]bis(2-methylpropanenitrile).

01/2017:2621

# ANIMAL EPITHELIA AND OUTGROWTHS FOR ALLERGEN PRODUCTS

## Fragmenta epithelii phaneraeque bestiarum ad producta allergenica

DEFINITION

Animal epithelia and outgrowths for allergen products consist of hair, epithelium fragments, dander, feathers and other structures that grow from the epidermis of mammals or birds.

Animal epithelia and outgrowths may contain proteins deposited from the saliva and/or secretions from the sebaceous glands of the animal. They may be further processed (e.g. cut or washed) using qualified methods or are unprocessed.

PRODUCTION

Animal epithelia and outgrowths for allergen products are obtained from healthy animals selected to avoid possible transmissible agents of disease. The exact species and/or variety of animal is stated. Typical production steps, including animal management, source material collection and purification, are specified. The origin, quality, and traceability of the source material must be demonstrated.

It is expected that, where applicable, the animal care and husbandry follows the principles described for the protection of vertebrate animals used for experimental and other scientific purposes. A responsible veterinarian or another competent person confirms the identity of the species and that the animals are healthy. It is verified that the skin is visibly clean and intact before harvest and that the animals have not been recently treated with preparations for cutaneous application, such as antiparasitic drugs.

The collection of animal epithelia and outgrowths must be performed without injuring the skin of the animal. Confirmation that measures are in place to prevent cross-contamination by animal epithelia and outgrowths from other animals is provided, including during animal management, collection and processing. Methods involving the grinding of whole skin and/or pelts must not be used.

Where major changes to the production of the animal epithelia and outgrowths take place (e.g. when a new process or supplier is introduced), such changes are qualified.

Microbial contamination of the animal epithelia and outgrowths may be unavoidable and should be monitored on a representative number of batches of source material according to a justified sampling plan and each time a new supplier and/or a new process for the source material production is introduced; if a determination of microbial contamination is not applicable, this must be justified. Microbial contamination values and potential increases in microbial contamination are monitored during stability studies, in order to assess this aspect along with the source material characteristics upon storage.

Control methods and acceptance criteria relating to identity and purity of the animal epithelia and outgrowths are established. The acceptance criteria must ensure the consistency of the animal epithelia and outgrowths source material from a qualitative and quantitative point of view. The animal epithelia and outgrowths source material is stored under controlled conditions justified by stability data. The collection and production, as well as the handling of the source material, are such that consistent composition is ensured from batch to batch.

*ANIMAL EPITHELIA AND OUTGROWTHS FOR ALLERGEN PRODUCTS REFERENCE BATCH*

An appropriate reference batch is established for each animal epithelia and outgrowths source material. The nature of the reference batch depends on the testing approach to verify batch-to-batch consistency and to establish acceptable quality. The reference batch may be, for example, an internal reference preparation (if available), a source material extract or a sample of a production batch. Its characterisation must be described. The extent of characterisation of the reference batch depends on the nature of the animal epithelia and outgrowths source material, knowledge of the allergenic components and availability of suitable reagents. The reference batch is stored under controlled conditions ensuring its stability.

*BATCH-TO-BATCH CONSISTENCY*

To establish batch-to-batch consistency, one or more of the following tests are performed on each batch. The choice of tests must be justified.

**Total protein** (*2.5.33*).

**Protein profile**. Determined by using suitable electrophoresis methods (*2.2.31*, *2.2.54*).

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific antibodies.

**Major allergen content**. Determined by using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

**Total allergenic activity**. Determined by testing inhibition of the binding capacity of specific immunoglobulin E antibodies or by a suitable equivalent *in vitro* method.

CHARACTERS

Animal epithelia and outgrowths for allergen products are supplied as coloured powders or other materials such as feathers, dander or hairs.

IDENTIFICATION

The identity of animal epithelia and outgrowths is confirmed by their relevant macroscopic and microscopic characteristics in comparison to those of a reference batch or reference documents. Identity may also be confirmed using other methods such as ELISA or by genetic identification, if performed by generally accepted methods.

TESTS

**Foreign matter**. Foreign matter is defined as vermin (e.g. mites and fleas), dirt, and foreign animal epithelia and outgrowths. Foreign matter is determined by appropriate tests

(e.g. microscopic examination, ELISA), visual inspection and/or tactile inspection. Foreign matter is below a predefined and justified limit.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*). The water content of dried material is determined; specification limits must be supported by batch analysis and stability data.

STORAGE

The source materials are stored under controlled conditions justified by stability data.

LABELLING

The label states:

- the species of the source animal;
- the nature of the animal epithelia and outgrowths.

**01/2017:0972**

# ANTAZOLINE HYDROCHLORIDE

## Antazolini hydrochloridum

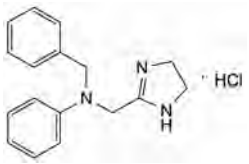

$C_{17}H_{20}ClN_3$ $M_r$ 301.8
[2508-72-7]

DEFINITION

Antazoline hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N*-benzyl-*N*-[(4,5-dihydro-1*H*-imidazol-2-yl)methyl]aniline hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, sparingly soluble in water, soluble in alcohol, slightly soluble in methylene chloride.

It melts at about 240 °C, with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *antazoline hydrochloride CRS*. Examine the substances as discs prepared using *potassium chloride R*.

B. Examine the chromatograms obtained in the test for related substances in daylight after spraying. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

C. To 5 mL of solution S (see Tests) add, drop by drop, *dilute sodium hydroxide solution R* until an alkaline reaction is produced. Filter. The precipitate, washed with two quantities, each of 10 mL, of *water R* and dried in a desiccator under reduced pressure, melts (*2.2.14*) at 119 °C to 123 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, heating at 60 °C if necessary. Allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance. Heat the plate at 110 °C for 15 min before using.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 5 mL with *methanol R*.

*Reference solution (a).* Dilute 0.5 mL of test solution (a) to 100 mL with *methanol R*.

*Reference solution (b).* Dissolve 20 mg of *antazoline hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (c).* Dissolve 20 mg of *xylometazoline hydrochloride CRS* in 1 mL of test solution (a) and dilute to 5 mL with *methanol R*.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *diethylamine R*, 10 volumes of *methanol R* and 85 volumes of *ethyl acetate R*. Dry the plate in a current of warm air for 15 min. Examine in ultraviolet light at 254 nm. The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots. Spray with a mixture of equal volumes of a 200 g/L solution of *ferric chloride R* and a 5 g/L solution of *potassium ferricyanide R*. Examine immediately in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.250 g in 100 mL of *alcohol R*. Add 0.1 mL of *phenolphthalein solution R1*. Titrate with *0.1 M alcoholic potassium hydroxide*.

1 mL of *0.1 M alcoholic potassium hydroxide* is equivalent to 30.18 mg of $C_{17}H_{20}ClN_3$.

IMPURITIES

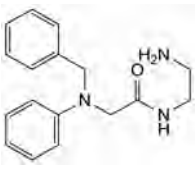

A. *N*-(2-aminoethyl)-2-(benzylphenylamino)acetamide.

01/2020:0209

# ANTICOAGULANT AND PRESERVATIVE SOLUTIONS FOR HUMAN BLOOD

## Solutiones anticoagulantes et sanguinem humanum conservantes

DEFINITION

Anticoagulant and preservative solutions for human blood are sterile and pyrogen-free solutions prepared with water for injections, filtered, distributed in the final containers and sterilised. The content of sodium citrate ($C_6H_5Na_3O_7,2H_2O$), glucose monohydrate ($C_6H_{12}O_6,H_2O$) or glucose ($C_6H_{12}O_6$) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4,2H_2O$) is not less than 95.0 per cent and not more than 105.0 per cent of that stated in the formulae below. The content of citric acid monohydrate ($C_6H_8O_7,H_2O$) or citric acid ($C_6H_8O_7$) is not less than 90.0 per cent and not more than 110.0 per cent of that stated in the formulae below. Subject to agreement by the competent authority, other substances, such as red-cell preservatives, may be included in the formula provided that their name and concentration are stated on the label.

Anticoagulant and preservative solutions for human blood are presented in airtight, tamper-evident containers of glass (*3.2.1*) or plastic (*3.3.4*).

## Anticoagulant acid-citrate-glucose solutions (ACD)

| | A | B |
|---|---|---|
| *Sodium citrate (0412)* | 22.0 g | 13.2 g |
| *Citric acid monohydrate (0456)* | 8.0 g | 4.8 g |
| or *Citric acid (0455)* | 7.3 g | 4.4 g |
| *Glucose monohydrate (0178)** | 24.5 g | 14.7 g |
| or *Glucose (0177)** | 22.3 g | 13.4 g |
| *Water for injections (0169)* to | 1000.0 mL | 1000.0 mL |
| Volume to be used per 100 mL of blood | 15.0 mL | 25.0 mL |

*The competent authority may require that the substances comply with the test for pyrogens given in the monographs on *Glucose monohydrate (0178)* and *Glucose (0177)*, respectively.

CHARACTERS

A colourless or faintly yellow, clear liquid, practically free from particles.

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. Dilute 2 mL of the solution to be examined (for formula A) or 3 mL (for formula B) to 100 mL with a mixture of 2 volumes of *water R* and 3 volumes of *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *glucose monohydrate CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

*Reference solution (b)*. Dissolve 10 mg of *glucose R*, 10 mg of *fructose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

Apply separately to the plate 2 µL of each solution and thoroughly dry the points of application. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The volumes of solvents have to be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

B. To 2 mL add 5 mL of *cupri-citric solution R*. Heat to boiling. An orange precipitate is formed and the solution becomes yellow.

C. To 2 mL (for formula A) add 3 mL of *water R* or to 4 mL (for formula B) add 1 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

D. 0.5 mL gives reaction (b) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*). The pH of the solution to be examined is 4.7 to 5.3.

**Hydroxymethylfurfural**. To 2.0 mL add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R* and 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using 2.0 mL of a solution containing 5 ppm of *hydroxymethylfurfural R* for formula A or 3 ppm of *hydroxymethylfurfural R* for formula B.

**Sterility** (*2.6.1*). They comply with the test for sterility.

**Pyrogens** (*2.6.8*). They comply with the test for pyrogens. Dilute with a pyrogen-free, 9 g/L solution of *sodium chloride R* to obtain a solution containing approximately 5 g/L of sodium citrate. Inject 10 mL of the diluted solution per kilogram of the rabbit's mass.

ASSAY

**Citric acid**. To 10.0 mL (for formula A) or to 20.0 mL (for formula B) add 0.1 mL of *phenolphthalein solution R1*. Titrate with *0.2 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.2 M sodium hydroxide* is equivalent to 14.01 mg of $C_6H_8O_7,H_2O$ or to 12.81 mg of $C_6H_8O_7$.

**Sodium citrate**. Prepare a chromatography column 0.10 m long and 10 mm in internal diameter and filled with *strongly acidic ion-exchange resin R* (300 µm to 840 µm). Maintain a 1 cm layer of liquid above the resin at all times. Wash the column with 50 mL of de-ionised *water R* at a flow rate of 12-14 mL/min.

Dilute 10.0 mL of the solution to be examined (for formula A) or 15.0 mL (for formula B) to about 40 mL with de-ionised *water R* in a beaker and transfer to the column reservoir, washing the beaker 3 times with a few millilitres of de-ionised *water R*. Allow the solution to run through the column at a flow rate of 12-14 mL/min and collect the eluate. Wash the column with 2 quantities, each of 30 mL, and with one quantity of 50 mL, of de-ionised *water R*. The column can be used for 3 successive determinations before regeneration with 3 times its volume of *dilute hydrochloric acid R*. Titrate the combined eluate and washings (about 150 mL) with *0.2 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

Calculate the content of sodium citrate in grams per litre from the following expressions:

For formula A: $1.961n - 1.40C$

or $1.961n - 1.53C'$

For formula B: $1.307n - 1.40C$

or $1.307n - 1.53C'$

$n$ = number of millilitres of *0.2 M sodium hydroxide* used in the titration;

$C$ = content of citric acid monohydrate in grams per litre determined as prescribed above;

$C'$ = content of citric acid in grams per litre determined as prescribed above.

**Reducing sugars**. Dilute 5.0 mL (for formula A) or 10.0 mL (for formula B) to 100.0 mL with *water R*. Introduce 25.0 mL of the solution into a 250 mL conical flask with ground-glass neck and add 25.0 mL of *cupri-citric solution R1*. Add a few pieces of porous material, attach a reflux condenser, heat so that boiling begins within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Add 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R* with caution and in small quantities. Titrate with *0.1 M sodium thiosulfate* using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator ($n_1$ mL). Carry out a blank titration using 25.0 mL of *water R* ($n_2$ mL).

Calculate the content of reducing sugars as glucose or as glucose monohydrate, as appropriate, from Table 0209.-1.

Table 0209.-1

| Volume of *0.1 M sodium thiosulfate* ($n_2 - n_1$ mL) | Glucose (mg) | Glucose monohydrate (mg) |
|---|---|---|
| 8 | 19.8 | 21.6 |
| 9 | 22.4 | 24.5 |
| 10 | 25.0 | 27.2 |
| 11 | 27.6 | 30.2 |
| 12 | 30.3 | 33.1 |
| 13 | 33.0 | 36.1 |
| 14 | 35.7 | 39.0 |
| 15 | 38.3 | 42.1 |
| 16 | 41.3 | 45.2 |

STORAGE

Store in an airtight, tamper-evident container, protected from light.

LABELLING

The label states:

- the composition and volume of the solution;
- the maximum amount of blood to be collected in the container.

## Anticoagulant citrate-phosphate-glucose solution (CPD)

| | |
|---|---|
| *Sodium citrate (0412)* | 26.3 g |
| *Citric acid monohydrate (0456)* | 3.27 g |
| or *Citric acid (0455)* | 2.99 g |
| *Glucose monohydrate (0178)** | 25.5 g |
| or *Glucose (0177)** | 23.2 g |
| *Sodium dihydrogen phosphate dihydrate (0194)* | 2.51 g |
| *Water for injections (0169)* to | 1000.0 mL |
| Volume to be used per 100 mL of blood | 14.0 mL |

*The competent authority may require that the substances comply with the test for pyrogens given in the monographs on *Glucose monohydrate (0178)* and *Glucose (0177)*, respectively.

CHARACTERS

A colourless or faintly yellow, clear liquid, practically free from particles.

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. Dilute 2 mL of the solution to be examined to 100 mL with a mixture of 2 volumes of *water R* and 3 volumes of *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *glucose monohydrate CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

*Reference solution (b)*. Dissolve 10 mg of *glucose R*, 10 mg of *fructose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

Apply separately to the plate 2 µL of each solution and thoroughly dry the starting points. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*. The volumes of solvents have to be measured accurately since a slight excess of water produces cloudiness. Dry the plate in a current of warm air. Repeat the development immediately, after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

B. To 2 mL add 5 mL of *cupri-citric solution R*. Heat to boiling. An orange precipitate is formed and the solution becomes yellow.

C. To 2 mL add 3 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

D. 1 mL gives reaction (b) of phosphates (*2.3.1*).

E. 0.5 mL gives reaction (b) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*). The pH of the solution is 5.3 to 5.9.

**Hydroxymethylfurfural**. To 2.0 mL add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R* and 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*), determined at 550 nm after allowing the mixture to stand for 2 min to

3 min, is not greater than that of a standard prepared at the same time in the same manner using 2.0 mL of a solution containing 5 ppm of *hydroxymethylfurfural R*.

**Sterility** (*2.6.1*). They comply with the test for sterility.

**Pyrogens** (*2.6.8*). They comply with the test for pyrogens. Dilute with a pyrogen-free, 9 g/L solution of *sodium chloride R* to obtain a solution containing approximately 5 g/L of sodium citrate. Inject 10 mL of the diluted solution per kilogram of the rabbit's mass.

ASSAY

**Sodium dihydrogen phosphate**. Dilute 10.0 mL to 100.0 mL with *water R*. To 10.0 mL of this solution add 10.0 mL of *nitro-molybdovanadic reagent R*. Mix and allow to stand at 20 °C to 25 °C for 30 min. At the same time and in the same manner, prepare a reference solution using 10.0 mL of a standard solution containing 0.219 g of *potassium dihydrogen phosphate R* per litre. Measure the absorbance (*2.2.25*) of the 2 solutions at 450 nm using as the compensation liquid a solution prepared in the same manner using 10 mL of *water R*. Calculate the content of sodium dihydrogen phosphate dihydrate (*P*) in grams per litre from the expression:

$$\frac{11.46 \times C \times A_1}{A_2}$$

$C$ = concentration of *potassium dihydrogen phosphate R* in the standard solution in grams per litre;

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the reference solution.

**Citric acid**. To 20.0 mL add 0.1 mL of *phenolphthalein solution R1* and titrate with *0.2 M sodium hydroxide*.

Calculate the content of citric acid monohydrate (*C*), or citric acid (*C′*), in grams per litre from the equations:

$$C = 0.7005n - 0.4490P$$

$$C' = 0.6404n - 0.4105P$$

$n$ = number of millilitres of *0.2 M sodium hydroxide* used in the titration;

$P$ = content of sodium dihydrogen phosphate dihydrate in grams per litre determined as prescribed above.

**Sodium citrate**. Prepare a chromatography column 0.10 m long and 10 mm in internal diameter and filled with *strongly acidic ion-exchange resin R* (300 µm to 840 µm). Maintain a 1 cm layer of liquid above the resin at all times. Wash the column with 50 mL of de-ionised *water R* at a flow rate of 12-14 mL/min.

Dilute 10.0 mL of the solution to be examined to about 40 mL with de-ionised *water R* in a beaker and transfer to the column reservoir, washing the beaker 3 times with a few millilitres of de-ionised *water R*. Allow the solution to run through the column at a flow rate of 12-14 mL/min and collect the eluate. Wash the column with 2 quantities, each of 30 mL, and with one quantity of 50 mL, of de-ionised *water R*. The column can be used for 3 successive determinations before regeneration with 3 times its volume of *dilute hydrochloric acid R*. Titrate the combined eluate and washings (about 150 mL) with *0.2 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

Calculate the content of sodium citrate in grams per litre from the following expressions:

$$1.961n - 1.257P - 1.40C$$

$$1.961n - 1.257P - 1.53C'$$

$n$ = number of millilitres of *0.2 M sodium hydroxide* used in the titration;

$P$ = content of sodium dihydrogen phosphate dihydrate in grams per litre determined as prescribed above;

$C$ = content of citric acid monohydrate in grams per litre determined as prescribed above;

$C'$ = content of citric acid in grams per litre determined as prescribed above.

**Reducing sugars**. Dilute 5.0 mL to 100.0 mL with *water R*. Introduce 25.0 mL of the solution into a 250 mL conical flask with ground-glass neck and add 25.0 mL of *cupri-citric solution R1*. Add a few pieces of porous material, attach a reflux condenser, heat so that boiling begins within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Add 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R* with caution and in small quantities. Titrate with *0.1 M sodium thiosulfate* using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator ($n_1$ mL). Carry out a blank titration using 25.0 mL of *water R* ($n_2$ mL).

Calculate the content of reducing sugars as glucose or as glucose monohydrate, as appropriate, from Table 0209.-1.

STORAGE

Store in an airtight, tamper-evident container, protected from light.

LABELLING

The label states:

- the composition and volume of the solution;
- the maximum amount of blood to be collected in the container.

**07/2013:1928**
**corrected 10.0**

# ANTI-T LYMPHOCYTE IMMUNOGLOBULIN FOR HUMAN USE, ANIMAL

## Immunoglobulinum anti-T lymphocytorum ex animali ad usum humanum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, obtained from serum or plasma of animals, mainly rabbits or horses, immunised with human lymphocytic antigens.

The immunoglobulin has the property of diminishing the number and function of immunocompetent cells, in particular T-lymphocytes. The preparation contains principally immunoglobulin G. It may contain antibodies against other lymphocyte subpopulations and against other cells. The preparation is intended for intravenous administration, after dilution with a suitable diluent where applicable. The preparation may contain excipients such as stabilisers.

Applicable provisions of the monograph on *Immunosera for human use, animal (0084)* are stated below.

PRODUCTION

*GENERAL PROVISIONS*

The production method has been shown to yield consistently immunoglobulins of acceptable safety, potency in man and stability.

Any reagent of biological origin used in production shall be free of contamination with bacteria, fungi and viruses. The method of preparation includes a step or steps that have been shown to remove or inactivate known agents of infection.

During development studies, it shall be demonstrated that the production method yields a product that:

- does not transmit infectious agents,
- is characterised by a defined pattern of immunological activity, notably: antigen binding, complement-dependent and independent cytotoxicity, cytokine release, induction of T-cell activation, cell death,
- does not contain antibodies that cross-react with human tissues to a degree that would impair clinical safety,
- has a defined maximum content of anti-thrombocyte antibody activity,
- has a defined maximum content of haemoglobin.

The product has been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated.

*Reference preparation*. A batch shown to be suitable for checking the validity of the assay and whose efficacy has been demonstrated in clinical trials, or a batch representative thereof.

*ANIMALS*

The animals used are of a species approved by the competent authority, are healthy and exclusively reserved for production of anti-T lymphocyte immunoglobulin. They are tested and shown to be free from a defined list of infectious agents. The introduction of animals into a closed herd follows specified procedures, including definition of quarantine measures. Where appropriate, tests for additional specific agents are considered depending on the geographical localisation of the establishment used for the breeding and production of the animals. The feed originates from a controlled source and no animal proteins are added. The suppliers of animals are certified by the competent authority.

If the animals are treated with antibiotics, a suitable withdrawal period is allowed before collection of blood or plasma. The animals are not treated with penicillin antibiotics. If a live vaccine is administered, a suitable waiting period is imposed between vaccination and collection of serum or plasma for immunoglobulin production.

The species, origin and identification number of the animals are specified.

*IMMUNISATION*

The antigens used are identified and characterised, where appropriate. They are identified by their names and a batch number; information on the source and preparation are recorded.

The selected animals are isolated for at least 1 week before being immunised according to a defined schedule with booster injections at suitable intervals. Adjuvants may be used.

Animals are kept under general health surveillance and specific antibody production is controlled at each cycle of immunisation.

Animals are thoroughly examined before collection of blood or plasma. If an animal shows any pathological lesion not related to the immunisation process, it is not used, nor are any other of the animals in the group concerned, unless it is evident that their use will not impair the safety of the product.

Human antigens such as continuously growing T-lymphocyte cell lines or thymocytes are used to immunise the animals. Cells may be subjected to a sorting procedure. The immunising antigens are shown to be free from infectious agents by validated methods for relevant blood-borne pathogens, notably hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) and other relevant adventitious agents originating from the preparation of the antigen. The cells used comply with defined requirements for purity of the cell population and freedom from adventitious agents.

*COLLECTION OF BLOOD OR PLASMA*

Collection of blood is made by venepuncture or plasmapheresis. The puncture area is shaved, cleaned and disinfected. The animals may be anaesthetised under conditions that do not influence the quality of the product.

No antimicrobial preservative is added to the plasma and serum samples. The blood or plasma is collected in such a manner as to maintain sterility of the product. The blood or plasma collection is conducted at a site separate from the area where the animals are kept or bred and the area where the immunoglobulin is purified. If the serum or plasma is stored before further processing, precautions are taken to avoid microbial contamination.

Several single plasma or serum samples may be pooled before purification. The single or pooled samples are tested before purification for the following tests.

**Tests for contaminating viruses**. Each pool is tested for contaminating viruses by suitable *in vitro* tests including inoculation to cell cultures capable of detecting a wide range of viruses relevant for the particular product. Where applicable, *in vitro* tests for contaminating viruses are carried out on the adsorbed pool, after the last production stage that may introduce viral contaminants.

*PURIFICATION AND VIRAL INACTIVATION*

The immunoglobulins are concentrated and purified by fractional precipitation, chromatography, immuno-adsorption or by other suitable chemical or physical methods. The methods are selected and validated to avoid contamination at all steps of processing and to avoid formation of protein aggregates that effect immunobiological characteristics of the product.

Unless otherwise justified and authorised, validated procedures are applied for removal and/or inactivation of viruses.

After purification and treatment for removal and/or inactivation of viruses, a stabiliser may be added to the intermediate product, which may be stored for a period defined in the light of stability data.

Only an intermediate product that complies with the following requirements may be used in the preparation of the final bulk.

If the method of preparation includes a step for adsorption of cross-reacting anti-human antibodies using material from human tissues and/or red blood cells, the human materials are submitted to a validated procedure for inactivation of infectious agents, unless otherwise justified and authorised. If erythrocytes are used for adsorption, the donors for such materials comply with the requirements for donors of blood and plasma of the monograph on *Human plasma for fractionation (0853)*. If other human material is used, it is shown by validated methods to be free from relevant blood-borne pathogens, notably HBV, HCV and HIV. If substances are used for inactivation or removal of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the anti-T lymphocyte immunoglobulin.

*FINAL BULK*

The final bulk is prepared from a single intermediate product or from a pool of intermediate products obtained from animals of the same species. No antimicrobial preservative is added either during the manufacturing procedure or for preparation of the final bulk solution. During manufacturing, the solution is passed through a bacteria-retentive filter.

*FINAL LOT*

The final bulk of anti-T-lymphocyte immunoglobulin is distributed aseptically into sterile, tamper-evident containers. The containers are closed as to prevent contamination.

Only a final lot that complies with the requirements prescribed below under Identification, Tests and Assay may be released for use.

CHARACTERS

*Appearance*:

– *liquid preparation*: clear or slightly opalescent, colourless or pale yellow liquid;
– *freeze-dried preparation*: white or slightly yellow powder or solid friable mass, which after reconstitution gives a liquid preparation corresponding to the description above.

IDENTIFICATION

A. Using a suitable range of species-specific antisera, carry out precipitation tests on the preparation to be examined. It is recommended that the test be carried out using antisera specific to the plasma proteins of each species of domestic animal commonly used in the preparation of materials of biological origin in the country concerned and antisera specific to human plasma proteins. The preparation is shown to contain proteins originating from the animal used for the anti-T lymphocyte immunoglobulin production.

B. Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal serum of the animal used for production, compare this serum and the preparation to be examined, both diluted to a concentration that will allow a clear gammaglobulin precipitation arc to be obtained on the gel. The main component of the preparation to be examined corresponds to the IgG component of normal serum of the animal used for production.

C. The preparation complies with the assay.

TESTS

**Solubility**. For the freeze-dried preparation, to a container add the volume of the liquid stated on the label. The preparation dissolves completely within the time stated on the label.

**Extractable volume** (*2.9.17*). It complies with the requirement for extractable volume.

**pH** (*2.2.3*). The pH is within the limits approved for the particular product.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg after dilution, where applicable.

**Total protein** (*2.5.33*): 90 per cent to 110 per cent of the amount stated on the label.

**Stabiliser**. Determine the amount of stabiliser by a suitable physico-chemical method. The preparation contains not less than 80 per cent and not more than 120 per cent of the quantity stated on the label.

**Distribution of molecular size**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range 2-20 g/L is usually suitable.

*Reference solution.* Dilute *human immunoglobulin (molecular size) BRP* with a 9 g/L solution of *sodium chloride R* to the same protein concentration as the test solution.

*Column*:

– *size*: *l* = 0.6 m, Ø = 7.5 mm,
– *stationary phase*: *silica gel for size-exclusion chromatography R*, a grade suitable for fractionation of globular proteins in the molecular mass range of 20 000 to 200 000.

*Mobile phase*: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R* and 11.688 g of *sodium chloride R* in 1 L of *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 50-600 µg of protein.

*Retention time*: identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter than that of dimer corresponds to polymers and aggregates.

*System suitability*:

– *reference solution*: the principal peak corresponds to IgG monomer and there is a peak corresponding to dimer with a retention time relative to monomer of 0.85 ± 0.05,
– *test solution*: the relative retentions of monomer and dimer are 1 ± 0.05 with reference to the corresponding peaks in the chromatogram obtained with the reference solution.

*Limits*:

– *total monomer and dimer*: at least 95 per cent of the total area of the peaks;
– *total polymers and aggregates*: maximum 5 per cent of the total area of the peaks.

**Purity**. Polyacrylamide gel electrophoresis (*2.2.31*), under non-reducing and reducing conditions.

*Resolving gel.* Non-reducing conditions: 8 per cent acrylamide; reducing conditions: 12 per cent acrylamide.

*Test solution.* Dilute the preparation to be examined to a protein concentration of 0.5-2 mg/mL.

*Reference solution.* Dilute the reference preparation to the same protein concentration as the test solution.

*Application*: 10 µL.

*Detection*: Coomassie staining.

*Results*: compared with the electropherogram of the reference solution, no additional bands are found in the electropherogram of the test solution.

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method A*). The 1 to 64 dilution does not show agglutination.

Where applicable, dilute the preparation to be examined as prescribed for use before preparing the dilutions for the test.

**Haemolysins**. Prepare a 1 to 64 dilution of the preparation to be examined, diluted if necessary as stated on the label. Take 6 aliquots of the 1 to 64 dilution. To 1 volume of 3 of the aliquots, add 1 volume of a 10 per cent *V/V* suspension of group A1, group B and group O erythrocytes in a 9 g/L solution of *sodium chloride R*, respectively. To 1 volume of the remaining 3 aliquots, add 1 volume of a 10 per cent *V/V* suspension of group A1, group B and group O erythrocytes in a 9 g/L solution of *sodium chloride R*, respectively, and to each aliquot 1 volume of fresh group AB serum (as a source of complement). Mix and incubate at 37 °C for 1 h. Examine the supernatant liquids for haemolysis. No signs of haemolysis are present.

**Thrombocyte antibodies**. Examined by a suitable method, the level of thrombocyte antibodies is shown to be below that approved for the specific product.

**Water** (*2.5.12*): maximum 3 per cent.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*). Unless otherwise justified and authorised, it complies with the test for pyrogens. Unless otherwise prescribed, inject 1 mL per kilogram of the rabbit's body mass.

ASSAY

The biological activity is determined by measuring the complement-dependent cytotoxicity on target cells. Flow cytometry is performed with read-out of dead cells stained using propidium iodide. The activity is expressed as the concentration of anti-T lymphocyte immunoglobulin in milligrams per millilitre which mediates 50 per cent cytotoxicity.

*Lymphocyte separation medium.* Commercial separation media with low viscosity and a density of 1.077 g/mL.

*Complement.* Commercial complement is suitable.

*Buffered salt solution pH 7.2.* Dissolve 8.0 g of *sodium chloride R*, 0.2 g of *potassium chloride R*, 3.18 g of *disodium hydrogen phosphate dodecahydrate R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Buffer solution for flow cytometry.* Add 40 mL of 0.1 per cent *V/V sodium azide R* and 10 mL of foetal calf serum to 440 mL of buffered salt solution pH 7.2. The foetal calf serum is inactivated at 56 °C for 30 min prior to use. Store at 4 °C.

*Propidium iodide solution.* Dissolve *propidium iodide R* in buffered salt solution pH 7.2, to a concentration of 1 mg/mL. Store this stock solution at 2-8 °C and use within 1 month. For the assay, dilute this solution with buffer solution for flow cytometry, to obtain a concentration of 5 µg/mL. Store at 2-8 °C and use within 3 h.

*Microtitre plates.* Plates used to prepare immunoglobulin dilutions are U- or V-bottomed polystyrene or poly(vinyl chloride) plates without surface treatment.

*Micronic tubes.* Suitable for flow cytometry measurement.

*Cell suspension.* Collect blood in anticoagulant from at least one healthy donor. Immediately isolate the peripheral blood mononuclear cells (PBMC) by gradient centrifugation in lymphocyte separation medium so that the PBMC form a visible clean interface between the plasma and the separation medium. Collect the layer containing the cells and dispense into centrifuge tubes containing buffered salt solution pH 7.2. Centrifuge at 400 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Repeat the centrifugation and resuspension procedure of the cells twice. After the third centrifugation, resuspend the cell pellet in 1 mL of buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent is required. Adjust the cell number to $7 \times 10^6$/mL by adding buffer solution for flow cytometry. Store the cell suspension at 4 °C and use within 12 h.

If necessary, the first PBMC pellet may be resuspended in buffered salt solution pH 7.2 containing 20 per cent foetal calf serum and stored overnight at 2 °C. Centrifuge at 400 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent is required. Adjust the cell number to $7 \times 10^6$/mL by adding buffer solution for flow cytometry.

It is also possible for cells to be immediately frozen and stored in nitrogen using the following method.

*Buffer solution for freezing.* To 20 mL of cell culture medium, add 25 mL of foetal calf serum and 5 mL of dimethyl sulfoxide (DMSO). Store this solution at 2-8 °C and use within 3 h.

$20 \times 10^6$ cells per ampoule are frozen. These ampoules are stored in liquid nitrogen.

*Buffer solution for thawing.* To 450 mL of cell culture medium, add 50 mL of foetal calf serum. Store this solution at 2-8 °C and use within 3 h.

Each ampoule is thawed in a water-bath at 37 °C with shaking. Cell suspension is repeated in a buffer solution for thawing. Centrifuge at 200 *g* at 2-8 °C for 10 min. Discard the supernatant. Suspend the cell pellet in buffer solution for flow cytometry. Repeat the procedure for centrifugation and resuspension of cells once. After the second centrifugation, resuspend the cells pellet in 1 mL of buffer solution for flow cytometry. Determine the number and vitality of the cells using a haemocytometer. Cell viability of at least 90 per cent is required. Adjust the cell number to $7 \times 10^6$/mL by adding buffer solution for flow cytometry. Store the cell suspension at 4 °C and use within 3 h.

*Test solutions.* For freeze-dried preparations, reconstitute as stated on the label. Prepare 3 independent series of not fewer than 7 dilutions using buffer solution for flow cytometry as diluent.

*Reference solutions.* For freeze-dried preparations, reconstitute according to the instructions for use. Prepare 3 independent dilution series of not fewer than 7 dilutions using buffer solution for flow cytometry as diluent.

Distribute 75 µL of each of the dilutions of the test solution or reference solution to each of a series of wells of a microtitre plate. Add 25 µL of the cell suspension of PBMC into each well. Add 25 µL of rabbit complement to each of the wells. Incubate at 37 °C for 30 min.

Centrifuge the plates at 200 *g* at 4 °C for 8 min, discard the supernatant and keep the plate on ice. Preparation for flow cytometry measurement is done step-wise by using a certain number of wells in order to allow labelling with *propidium iodide R* solution and measurement within a defined time period. Resuspend carefully the cell pellet of a certain number of wells with 200 µL of propidium iodide solution. Transfer the suspension into tubes. Incubate at 25 °C for 10 min then place immediately on ice.

Proceed with fluorescence measurement in a flow cytometer. Define a region including all propidium iodide-positive cells on the basis of Forward-Scattered, light (FSC) and flourescence (FL2 or FL3 for propidium iodide). Measure the percentage of propidium iodide-positive cells, without gating but excluding debris. Analyse at least 3000 cells for each of the test and reference solutions.

Use the percentages of dead cells to estimate the potency as the concentration in milligrams per millilitre of the preparation to be examined necessary to induce 50 per cent of cytotoxicity by fitting a sigmoidal dose response curve to the data obtained with the test and the reference preparations and by using a 4-parameter logistic model (see, for example, chapter *5.3*) and suitable software. The test is not valid unless the percentage of propidium iodide-positive cells at the lower asymptote of the curve is less then 15 per cent and the percentage of propidium iodide-positive cells at the upper asymptote of the curve is at least 80 per cent.

The estimated activity is 70 per cent to 130 per cent of the activity approved for the particular product.

The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

## STORAGE

Protected from light at the temperature stated on the label.

*Expiry date.* The expiry date is calculated from the beginning of the assay.

## LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content,
- for freeze-dried preparations:
  - the name and the volume of the reconstitution liquid to be added,
  - the quantity of protein in the container,
  - that the immunoserum is to be used immediately after reconstitution,
  - the time required for complete dissolution,
- the animal species of origin,
- the name and amount of stabiliser, where applicable,
- the dilution to be made before use of the product.

01/2020:0136

# APOMORPHINE HYDROCHLORIDE HEMIHYDRATE

## Apomorphini hydrochloridum hemihydricum

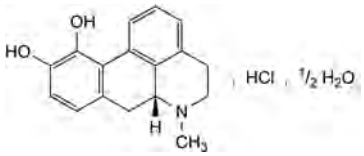


$C_{17}H_{18}ClNO_2,½H_2O$ $M_r$ 312.8
[41372-20-7]

DEFINITION

(6a*R*)-6-Methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-10,11-diol hydrochloride hemihydrate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish-brown or green-tinged greyish, crystalline powder or crystals; on exposure to air and light, the green tinge becomes more pronounced.

*Solubility*: sparingly soluble in water and in ethanol (96 per cent), practically insoluble in toluene.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid solution. Dilute 10.0 mL of the solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 230-350 nm

*Absorption maximum*: at 273 nm.

*Shoulder*: at 300-310 nm.

*Specific absorbance at the absorption maximum*: 530 to 570.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *apomorphine hydrochloride hemihydrate CRS*.

C. To 5 mL of solution S (see Tests) add a few millilitres of *sodium hydrogen carbonate solution R* until a permanent, white precipitate is formed. The precipitate slowly becomes greenish. Add 0.25 mL of *0.05 M iodine* and shake. The precipitate becomes greyish-green. Collect the precipitate. The precipitate dissolves in *methylene chloride R* giving a violet-blue solution and in *ethanol (96 per cent) R* giving a blue solution.

D. To 2 mL of solution S (see Tests) add 0.1 mL of *nitric acid R*. Mix and filter. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.25 g without heating in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ or $GY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): – 52 to – 48 (dried substance).

Dissolve 0.25 g in a 2.06 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 20.0 mL with the same solution.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with a 1 per cent *V/V* solution of *glacial acetic acid R*. Dilute 1.0 mL of this solution to 10.0 mL with a 1 per cent *V/V* solution of *glacial acetic acid R*.

*Reference solution (b).* Dissolve 12.5 mg of *apomorphine impurity B CRS* in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 10.0 mL with the same solution.

*Reference solution (c).* Dilute 2.0 mL of reference solution (b) to 10.0 mL with a 1 per cent *V/V* solution of *glacial acetic acid R*. Dilute 2.0 mL of this solution to 100.0 mL with a 1 per cent *V/V* solution of *glacial acetic acid R*.

*Reference solution (d).* Dissolve 25 mg of *boldine R* in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 10 mL with the same solution. To 1 mL of this solution add 1 mL of the test solution and dilute to 10 mL with a 1 per cent *V/V* solution of *glacial acetic acid R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: 1.1 g/L solution of *sodium octanesulfonate R*, adjusted to pH 2.2 with a 50 per cent *m/m* solution of *phosphoric acid R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 32 | 85 → 68 | 15 → 32 |
| 32 - 37 | 68 | 32 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to apomorphine (retention time = about 18 min): impurity B = about 0.4; boldine = about 0.9.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.5 between the peaks due to boldine and apomorphine.

*Calculation of percentage contents*:

– for impurity B, use the concentration of impurity B in reference solution (c);
– for impurities other than B, use the concentration of apomorphine hydrochloride hemihydrate in reference solution (a).

*Limits*:

– *impurity B*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): 2.5 per cent to 4.2 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the first 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.38 mg of $C_{17}H_{18}ClNO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

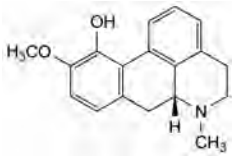

A. (6a*R*)-10-methoxy-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinolin-11-ol (apocodeine),

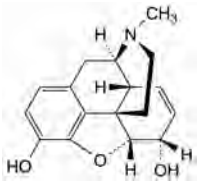

B. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol (morphine),

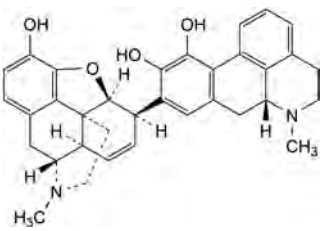

C. (6a*R*)-9-[7,8-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan-6α-yl]-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-10,11-diol (morphine-apomorphine dimer).

**01/2017:2757**

# APREPITANT

## Aprepitantum

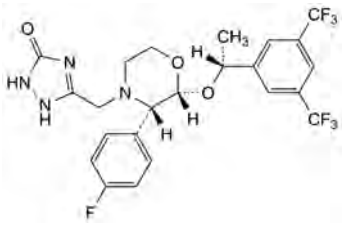

$C_{23}H_{21}F_7N_4O_3$ $M_r$ 534.4
[170729-80-3]

DEFINITION

5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-Bis(trifluoromethyl)phenyl]-ethoxy]-3-(4-fluorophenyl)morpholin-4-yl]methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *aprepitant CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): + 66.0 to + 70.0 (anhydrous substance), measured at 25 °C.

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (50:50 *V/V*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 40.0 mg of *aprepitant CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 4 mg of *aprepitant for system suitability CRS* (containing impurity A) in 5.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);

– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 65 | 35 |
| 2 - 22 | 65 → 20 | 35 → 80 |
| 22 - 32 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *aprepitant for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to aprepitant (retention time = about 15 min): impurity A = about 0.97.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity A and aprepitant.

*Limits*:

– *impurity A*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent (reference solution (b)).

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 0.200 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{23}H_{21}F_7N_4O_3$ taking into account the assigned content of *aprepitant CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

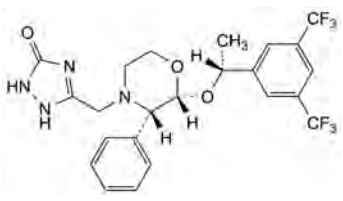

A. 5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]-ethoxy]-3-phenylmorpholin-4-yl]methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one,

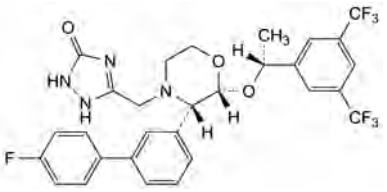

B. 5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]-ethoxy]-3-(4′-fluorobiphenyl-3-yl)morpholin-4-yl]-methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one,

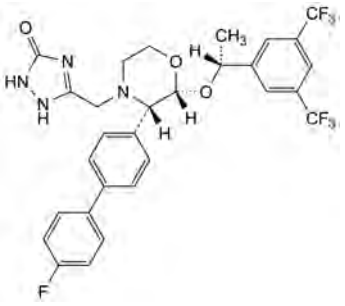

C. 5-[[(2*R*,3*S*)-2-[(1*R*)-1-[3,5-bis(trifluoromethyl)phenyl]-ethoxy]-3-(4′-fluorobiphenyl-4-yl)morpholin-4-yl]-methyl]-1,2-dihydro-3*H*-1,2,4-triazol-3-one.

**01/2019:0580**
**corrected 10.0**

# APROTININ

## Aprotininum

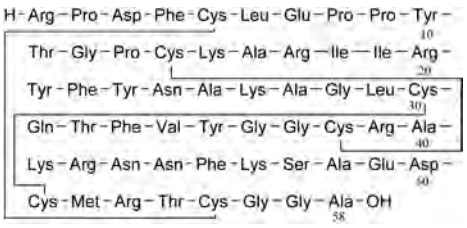


$C_{284}H_{432}N_{84}O_{79}S_7$ $M_r$ 6511
[9087-70-1]

DEFINITION

Aprotinin is a polypeptide consisting of a chain of 58 amino acids. It inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin and trypsin. It contains not less than 3.0 Ph. Eur. U. of aprotinin activity per milligram, calculated with reference to the dried substance.

PRODUCTION

The animals from which aprotinin is derived must fulfil the requirements for the health of animals suitable for human consumption.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

**Histamine** (*2.6.10*): maximum 0.2 µg of histamine base per 3 Ph. Eur. U.

CHARACTERS

*Appearance*: almost white hygroscopic powder.

*Solubility*: soluble in water and in isotonic solutions, practically insoluble in organic solvents.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Solution S (see Tests).

*Reference solution.* Dilute *aprotinin solution BRP* in *water R* to obtain a concentration of 15 Ph. Eur. U./mL.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *glacial acetic acid R* (80:100 *V/V*) containing 100 g/L of *sodium acetate R*.

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: spray with a solution of 0.1 g of *ninhydrin R* in a mixture of 6 mL of a 10 g/L solution of *cupric chloride R*, 21 mL of *glacial acetic acid R* and 70 mL of *anhydrous ethanol R*. Dry the plate at 60 °C.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Determine the ability of the substance to be examined to inhibit trypsin activity using the method described below.

*Test solution.* Dilute 1 mL of solution S to 50 mL with *buffer solution pH 7.2 R*.

*Trypsin solution.* Dissolve 10 mg of *trypsin BRP* in *0.002 M hydrochloric acid* and dilute to 100 mL with the same acid.

*Casein solution.* Dissolve 0.2 g of *casein R* in *buffer solution pH 7.2 R* and dilute to 100 mL with the same buffer solution.

*Precipitating solution*: *glacial acetic acid R*, *water R*, *anhydrous ethanol R* (1:49:50 *V/V/V*).

Mix 1 mL of the test solution with 1 mL of the trypsin solution. Allow to stand for 10 min and add 1 mL of the casein solution. Incubate at 35 °C for 30 min. Cool in iced water and add 0.5 mL of the precipitating solution. Shake and allow to stand at room temperature for 15 min. The solution is cloudy. Carry out a blank test under the same conditions using *buffer solution pH 7.2 R* instead of the test solution. The solution is not cloudy.

TESTS

**Solution S**. Prepare a solution of the substance to be examined containing 15 Ph. Eur. U./mL, calculated from the activity stated on the label.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Absorbance** (*2.2.25*): maximum 0.80 by measuring at the absorption maximum at 277 nm.

Prepare a solution of the substance to be examined containing 3.0 Ph. Eur. U./mL.

**Des-Ala-aprotinin and des-Ala-des-Gly-aprotinin**. Capillary zone electrophoresis (*2.2.47*): use the normalisation procedure.

*Test solution.* Prepare a solution of the substance to be examined in *water R* containing not less than 1 Ph. Eur. U./mL.

*Reference solution.* Dilute *aprotinin solution BRP* in *water R* to obtain the same concentration as the test solution.

*Capillary*:

- *material*: uncoated fused silica;
- *size*: effective length = 45-60 cm, Ø = 75 µm.

*Temperature*: 25 °C.

*CZE buffer.* Dissolve 8.21 g of *potassium dihydrogen phosphate R* in 400 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R*, dilute to 500.0 mL with *water R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Detection*: spectrophotometer at 214 nm.

*Between-run rinsing*: rinse the capillary for at least 1 min with *0.1 M sodium hydroxide* filtered through a membrane filter (nominal pore size 0.45 µm) and for 2 min with the CZE buffer.

*Injection*: under pressure or vacuum (for example, 3 s at a differential pressure of 3.5 kPa).

*Migration*: apply a field strength of 0.2 kV/cm, using the CZE buffer as the electrolyte in both buffer reservoirs.

*Run time*: 30 min.

*Identification of impurities*: use the electropherogram supplied with *aprotinin solution BRP* and the electropherogram obtained with the reference solution to identify the peaks due to impurities A and B.

*Relative migration* with reference to aprotinin (migration time = about 22 min): impurity A = about 0.98; impurity B = about 0.99.

*System suitability*: reference solution after at least 6 injections:

- *migration time*: aprotinin = 19.0 min to 25.0 min;
- *resolution*: minimum 0.8 between the peaks due to impurities A and B; minimum 0.5 between the peaks due to impurity B and aprotinin;
- *peak distribution*: the electropherogram obtained is qualitatively and quantitatively similar to the electropherogram supplied with *aprotinin solution BRP*;
- *height of the principal peak*: at least 1000 times the height of the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height.

*Limits*:

- *impurity A*: maximum 8.0 per cent;
- *impurity B*: maximum 7.5 per cent.

**Pyroglutamyl-aprotinin and related compounds**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Prepare a solution of the substance to be examined in mobile phase A, containing about 5 Ph. Eur. U./mL.

*Reference solution.* Dissolve the contents of a vial of *aprotinin for system suitability CRS* in 2.0 mL of mobile phase A.

*Column*:

- *size*: *l* = 0.075 m, Ø = 7.5 mm;
- *stationary phase*: *strong cation-exchange silica gel for chromatography R* (10 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.52 g of *potassium dihydrogen phosphate R* and 7.26 g of *disodium hydrogen phosphate dihydrate R* in 1000 mL of *water for chromatography R*; filter and degas;
- *mobile phase B*: dissolve 3.52 g of *potassium dihydrogen phosphate R*, 7.26 g of *disodium hydrogen phosphate dihydrate R* and 66.07 g of *ammonium sulfate R* in 1000 mL of *water for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 21 | 92 → 64 | 8 → 36 |
| 21 - 30 | 64 → 0 | 36 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 40 µL.

*Relative retention* with reference to aprotinin (retention time = 17.0 min to 20.0 min): impurity C = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to impurity C and aprotinin;
- *symmetry factor*: maximum 1.3 for the peak due to aprotinin.

*Limits*:

- *impurity C*: maximum 1.0 per cent;
- *any other impurity*: maximum 0.5 per cent;
- *sum of impurities other than C*: maximum 1.0 per cent.

**Aprotinin oligomers**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Prepare a solution of the substance to be examined in *water R* containing about 5 Ph. Eur. U./mL.

*Reference solution.* Treat the substance to be examined to obtain about 2 per cent aprotinin oligomers. For example, heat freeze-dried aprotinin at about 110 °C for about 4 h. Then dissolve in *water R* to obtain a concentration of about 5 Ph. Eur. U./mL.

*Column*: 3 columns coupled in series:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 20 000 to 10 000 000 (8 µm).

*Mobile phase*: *acetonitrile R*, *glacial acetic acid R*, *water for chromatography R* (2:2:6 *V/V/V*); filter and degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 277 nm.

*Injection*: 100 µL.

*Run time*: 40 min.

*Relative retention* with reference to aprotinin monomer (retention time = 24.5 min to 25.5 min): aprotinin dimer = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 1.3 between the peaks due to aprotinin dimer and monomer;
- *symmetry factor*: maximum 2.5 for the peak due to aprotinin monomer.

*Limit*:

- *total*: maximum 1.0 per cent.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 0.100 g by drying *in vacuo*.

**Bacterial endotoxins** (*2.6.14*): less than 0.14 IU per European Pharmacopoeia Unit of aprotinin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

## ASSAY

The activity of aprotinin is determined by measuring its inhibitory action on a solution of trypsin of known activity. The inhibiting activity of the aprotinin is calculated from the difference between the initial activity and the residual activity of the trypsin.

The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatals of trypsin.

Use a reaction vessel with a capacity of about 30 mL, provided with:

- a device that will maintain a temperature of 25 ± 0.1 °C;
- a stirring device, such as a magnetic stirrer;
- a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 mL and the pH-meter is provided with a wide reading scale and glass-silver-silver chloride or other suitable electrodes.

*Test solution.* Prepare a solution of the substance to be examined in *0.0015 M borate buffer solution pH 8.0 R* expected to contain 1.67 Ph. Eur. U./mL (about 0.6 mg (*m* mg) per millilitre).

*Trypsin solution.* Prepare a solution of *trypsin BRP* containing about 0.8 microkatals per millilitre (about 1 mg/mL), using *0.001 M hydrochloric acid* as the solvent. Use a freshly prepared solution and keep in iced water.

*Trypsin and aprotinin solution.* To 4.0 mL of the trypsin solution add 1.0 mL of the test solution. Dilute immediately to 40.0 mL with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

*Dilute trypsin solution.* Dilute 0.5 mL of the trypsin solution to 10.0 mL with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 mL of *0.0015 M borate buffer solution pH 8.0 R* and 1.0 mL of a freshly prepared 6.9 g/L solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with *0.1 M sodium hydroxide*. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 mL of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of *0.1 M sodium hydroxide* and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second ($n_1$ mL). Carry out, under the same conditions, a titration using 1.0 mL of the dilute trypsin solution. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second ($n_2$ mL).

Calculate the aprotinin activity in European Pharmacopoeia Units per milligram using the following expression:

$$\frac{4000(2n_2 - n_1)}{m}$$

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

## STORAGE

In an airtight, tamper-evident container, protected from light.

## LABELLING

The label states:

- the number of European Pharmacopoeia Units of aprotinin activity per milligram;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

## IMPURITIES

A. aprotinin-(1-56)-peptide,

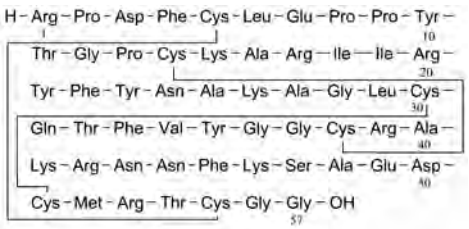


B. aprotinin-(1-57)-peptide,

C. (5-oxoprolyl)aprotinin (pyroglutamylaprotinin).

**01/2019:0579**
**corrected 10.0**

# APROTININ CONCENTRATED SOLUTION

## Aprotinini solutio concentrata

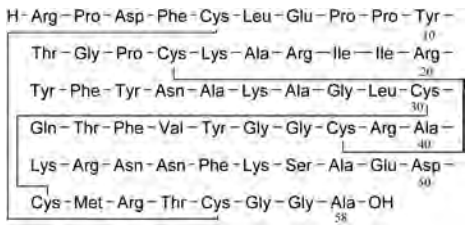


$C_{284}H_{432}N_{84}O_{79}S_7$ $M_r$ 6511

DEFINITION

Aprotinin concentrated solution is a solution of aprotinin, a polypeptide consisting of a chain of 58 amino acids, which inhibits stoichiometrically the activity of several proteolytic enzymes such as chymotrypsin, kallikrein, plasmin and trypsin. It contains not less than 15.0 Ph. Eur. U. of aprotinin activity per millilitre.

PRODUCTION

The animals from which aprotinin is derived must fulfil the requirements for the health of animals suitable for human consumption.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

**Histamine** (*2.6.10*): maximum 0.2 µg of histamine base per 3 Ph. Eur. U.

CHARACTERS

*Appearance*: clear, colourless liquid.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Solution S (see Tests).

*Reference solution.* Dilute *aprotinin solution BRP* in *water R* to obtain a concentration of 15 Ph. Eur. U./mL.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R*, *glacial acetic acid R* (80:100 *V/V*) containing 100 g/L of *sodium acetate R.*

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: spray with a solution of 0.1 g of *ninhydrin R* in a mixture of 6 mL of a 10 g/L solution of *cupric chloride R*, 21 mL of *glacial acetic acid R* and 70 mL of *anhydrous ethanol R*. Dry the plate at 60 °C.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Determine the ability of the preparation to be examined to inhibit trypsin activity using the method described below.

*Test solution.* Dilute 1 mL of solution S to 50 mL with *buffer solution pH 7.2 R.*

*Trypsin solution.* Dissolve 10 mg of *trypsin BRP* in *0.002 M hydrochloric acid* and dilute to 100 mL with the same acid.

*Casein solution.* Dissolve 0.2 g of *casein R* in *buffer solution pH 7.2 R* and dilute to 100 mL with the same buffer solution.

*Precipitating solution*: *glacial acetic acid R*, *water R*, *anhydrous ethanol R* (1:49:50 *V/V/V*).

Mix 1 mL of the test solution with 1 mL of the trypsin solution. Allow to stand for 10 min and add 1 mL of the casein solution. Incubate at 35 °C for 30 min. Cool in iced water and add 0.5 mL of the precipitating solution. Shake and allow to stand at room temperature for 15 min. The solution is cloudy. Carry out a blank test under the same conditions using *buffer solution pH 7.2 R* instead of the test solution. The solution is not cloudy.

TESTS

**Solution S**. Prepare a solution containing 15 Ph. Eur. U./mL, if necessary by dilution, on the basis of the activity stated on the label.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Absorbance** (*2.2.25*): maximum 0.80 by measuring at the absorption maximum at 277 nm.

Prepare a solution containing 3.0 Ph. Eur. U./mL.

**Des-Ala-aprotinin and des-Ala-des-Gly-aprotinin**. Capillary zone electrophoresis (*2.2.47*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of not less than 1 Ph Eur. U./mL.

*Reference solution.* Dilute *aprotinin solution BRP* in *water R* to obtain the same concentration as the test solution.

*Capillary*:

– *material*: uncoated fused silica;

– *size*: effective length = 45-60 cm, Ø = 75 µm.

*Temperature*: 25 °C.

*CZE buffer.* Dissolve 8.21 g of *potassium dihydrogen phosphate R* in 400 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R*, dilute to 500.0 mL with *water R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Detection*: spectrophotometer at 214 nm.

*Between-run rinsing*: rinse the capillary for at least 1 min with *0.1 M sodium hydroxide* filtered through a membrane filter (nominal pore size 0.45 µm) and for 2 min with the CZE buffer.

*Injection*: under pressure or vacuum (for example, 3 s at a differential pressure of 3.5 kPa).

*Migration*: apply a field strength of 0.2 kV/cm, using the CZE buffer as the electrolyte in both buffer reservoirs.

*Run time*: 30 min.

*Identification of impurities*: use the electropherogram supplied with *aprotinin solution BRP* and the electropherogram obtained with the reference solution to identify the peaks due to impurities A and B.

*Relative migration* with reference to aprotinin (migration time = about 22 min): impurity A = about 0.98; impurity B = about 0.99.

*System suitability*: reference solution after at least 6 injections:

– *migration time*: aprotinin = 19.0 min to 25.0 min;

– *resolution*: minimum 0.8 between the peaks due to impurities A and B; minimum 0.5 between the peaks due to impurity B and aprotinin;
– *peak distribution*: the electropherogram obtained is qualitatively and quantitatively similar to the electropherogram supplied with *aprotinin solution BRP*;
– *height of the principal peak*: at least 1000 times the height of the baseline noise. If necessary, adjust the sample load to give peaks of a sufficient height.

*Limits*:
– *impurity A*: maximum 8.0 per cent;
– *impurity B*: maximum 7.5 per cent.

**Pyroglutamyl-aprotinin and related compounds**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in mobile phase A to a concentration of about 5 Ph. Eur. U./mL.

*Reference solution.* Dissolve the contents of a vial of *aprotinin for system suitability CRS* in 2.0 mL of mobile phase A.

*Column*:
– *size*: *l* = 0.075 m, Ø = 7.5 mm;
– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (10 µm);
– *temperature*: 40 °C.

*Mobile phase*:
– *mobile phase A*: dissolve 3.52 g of *potassium dihydrogen phosphate R* and 7.26 g of *disodium hydrogen phosphate dihydrate R* in 1000 mL of *water for chromatography R*; filter and degas;
– *mobile phase B*: dissolve 3.52 g of *potassium dihydrogen phosphate R*, 7.26 g of *disodium hydrogen phosphate dihydrate R* and 66.07 g of *ammonium sulfate R* in 1000 mL of *water for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 21 | 92 → 64 | 8 → 36 |
| 21 - 30 | 64 → 0 | 36 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 40 µL.

*Relative retention* with reference to aprotinin (retention time = 17.0 min to 20.0 min): impurity C = about 0.9.

*System suitability*: reference solution:
– *resolution*: minimum 1.5 between the peaks due to impurity C and aprotinin;
– *symmetry factor*: maximum 1.3 for the peak due to aprotinin.

*Limits*:
– *impurity C*: maximum 1.0 per cent;
– *any other impurity*: maximum 0.5 per cent;
– *sum of impurities other than C*: maximum 1.0 per cent.

**Aprotinin oligomers**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of about 5 Ph. Eur. U./mL.

*Reference solution.* Treat the substance to be examined to obtain about 2 per cent aprotinin oligomers. For example, heat freeze-dried aprotinin at about 110 °C for about 4 h. Then dissolve in *water R* to obtain a concentration of about 5 Ph. Eur. U./mL.

*Column*: 3 columns coupled in series:
– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 20 000 to 10 000 000 (8 µm).

*Mobile phase*: *acetonitrile R*, *glacial acetic acid R*, *water for chromatography R* (2:2:6 *V/V/V*); filter and degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 277 nm.

*Injection*: 100 µL.

*Run time*: 40 min.

*Relative retention* with reference to aprotinin monomer (retention time = 24.5 min to 25.5 min): aprotinin dimer = about 0.9.

*System suitability*: reference solution:
– *resolution*: minimum 1.3 between the peaks due to aprotinin dimer and monomer;
– *symmetry factor*: maximum 2.5 for the peak due to aprotinin monomer.

*Limit*:
– *total*: maximum 1.0 per cent.

**Specific activity of the dry residue**: minimum 3.0 Ph. Eur. U. of aprotinin activity per milligram of dry residue.

Evaporate 25.0 mL to dryness in a water-bath, dry the residue at 110 °C for 15 h and weigh. From the mass of the residue and the activity determined as described below, calculate the number of European Pharmacopoeia Units per milligram of dry residue.

**Bacterial endotoxins** (*2.6.14*): less than 0.14 IU per European Pharmacopoeia Unit of aprotinin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The activity of aprotinin is determined by measuring its inhibitory action on a solution of trypsin of known activity. The inhibiting activity of the aprotinin is calculated from the difference between the initial activity and the residual activity of the trypsin.

The inhibiting activity of aprotinin is expressed in European Pharmacopoeia Units. 1 Ph. Eur. U. inhibits 50 per cent of the enzymatic activity of 2 microkatals of trypsin.

Use a reaction vessel with a capacity of about 30 mL, provided with:
– a device that will maintain a temperature of 25 ± 0.1 °C;
– a stirring device, such as a magnetic stirrer;
– a lid with 5 holes for accommodating the electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of the reagents.

An automatic or manual titration apparatus may be used. In the latter case the burette is graduated in 0.05 mL and the pH-meter is provided with a wide reading scale and glass-silver-silver chloride or other suitable electrodes.

*Test solution.* With *0.0015 M borate buffer solution pH 8.0 R* prepare an appropriate dilution (*D*) of the aprotinin concentrated solution expected, on the basis of the stated potency, to contain 1.67 Ph. Eur. U./mL.

*Trypsin solution.* Prepare a solution of *trypsin BRP* containing about 0.8 microkatals per millilitre (about 1 mg/mL), using *0.001 M hydrochloric acid* as the solvent. Use a freshly prepared solution and keep in iced water.

*Trypsin and aprotinin solution.* To 4.0 mL of the trypsin solution add 1.0 mL of the test solution. Dilute immediately to 40.0 mL with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water. Use within 6 h of preparation.

*Dilute trypsin solution.* Dilute 0.5 mL of the trypsin solution to 10.0 mL with *0.0015 M borate buffer solution pH 8.0 R*. Allow to stand at room temperature for 10 min and then keep in iced water.

Maintain an atmosphere of nitrogen in the reaction flask and stir continuously; introduce 9.0 mL of *0.0015 M borate buffer solution pH 8.0 R* and 1.0 mL of a freshly prepared

6.9 g/L solution of *benzoylarginine ethyl ester hydrochloride R*. Adjust to pH 8.0 with *0.1 M sodium hydroxide*. When the temperature has reached equilibrium at 25 ± 0.1 °C, add 1.0 mL of the trypsin and aprotinin solution and start a timer. Maintain at pH 8.0 by the addition of *0.1 M sodium hydroxide* and note the volume added every 30 s. Continue the reaction for 6 min. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second ($n_1$ mL). Carry out, under the same conditions, a titration using 1.0 mL of the dilute trypsin solution. Determine the number of millilitres of *0.1 M sodium hydroxide* used per second ($n_2$ mL).

Calculate the aprotinin activity in European Pharmacopoeia Units per millilitre using the following expression:

$$4000(2n_2 - n_1) \times D$$

*D* = dilution factor of the aprotinin concentrated solution to be examined in order to obtain a solution containing 1.67 Ph. Eur. U./mL.

The estimated activity is not less than 90 per cent and not more than 110 per cent of the activity stated on the label.

STORAGE

In an airtight, tamper-evident container, protected from light.

LABELLING

The label states:

- the number of European Pharmacopoeia Units of aprotinin activity per millilitre;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

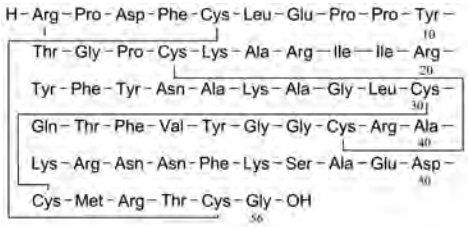


A. aprotinin-(1-56)-peptide,

B. aprotinin-(1-57)-peptide,

C. (5-oxoprolyl)aprotinin (pyroglutamylaprotinin).

**01/2020:1171**

# ARACHIS OIL, HYDROGENATED

## Arachidis oleum hydrogenatum

DEFINITION

Oil obtained by refining, bleaching, hydrogenating and deodorising oil obtained from the shelled seeds of *Arachis hypogaea* L. Each type of hydrogenated arachis oil is characterised by its nominal drop point.

CHARACTERS

*Appearance*: white or faintly yellowish, soft mass which melts to a clear, pale yellow liquid when heated.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride and in light petroleum (bp: 65-70 °C), very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Drop point (see Tests).

B. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

C. Composition of fatty acids (see Tests).

TESTS

**Drop point** (*2.2.17*): 32 °C to 43 °C, and within 3 °C of the nominal value.

**Acid value** (*2.5.1*): maximum 0.5.

Dissolve 10.0 g in 50 mL of the prescribed solvent by heating on a water-bath.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

Dissolve 5.0 g in 30 mL of the prescribed solvent by heating on a water-bath.

**Unsaponifiable matter** (*2.5.7*): maximum 1.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*) with the following modifications. Use the mixture of calibrating substances in Table 2.4.22.-3.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 0.7 mL/min.

*Split ratio*: 1:100.

*Temperature*:

- *column*: 180 °C for 20 min;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Composition of the fatty-acid fraction of the substance*:

- *saturated fatty acids of chain length less than $C_{14}$*: maximum 0.5 per cent;
- *myristic acid*: maximum 0.5 per cent;
- *palmitic acid*: 5.0 per cent to 16.0 per cent;
- *stearic acid*: 3.0 per cent to 19.0 per cent;
- *oleic acid and isomers*: 54.0 per cent to 78.0 per cent;

– *linoleic acid and isomers*: maximum 10.0 per cent;
– *arachidic acid*: 1.0 per cent to 3.0 per cent;
– *eicosenoic acids*: maximum 2.1 per cent;
– *behenic acid*: 1.0 per cent to 5.0 per cent;
– *erucic acid and isomers*: maximum 0.5 per cent;
– *lignoceric acid*: 0.5 per cent to 3.0 per cent.

STORAGE

Protected from light.

LABELLING

The label states the nominal drop point.

**01/2019:0263**

# ARACHIS OIL, REFINED

## Arachidis oleum raffinatum

DEFINITION

The refined fatty oil obtained from the shelled seeds of *Arachis hypogaea* L. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, yellowish, viscous liquid.

*Solubility*: very slightly soluble in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.915.

It solidifies at about 2 °C.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5, determined on 10.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids**. (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

– *saturated fatty acids of chain length less than* $C_{16}$: maximum 0.4 per cent;
– *palmitic acid*: 5.0 per cent to 14.0 per cent;
– *stearic acid*: 1.3 per cent to 6.5 per cent;
– *oleic acid*: 35.0 per cent to 76.0 per cent;
– *linoleic acid*: 8.0 per cent to 43.0 per cent;
– *linolenic acid*: maximum 0.6 per cent;
– *arachidic acid*: 0.5 per cent to 3.0 per cent;
– *eicosenoic acid*: 0.5 per cent to 3.0 per cent;
– *behenic acid*: 1.0 per cent to 5.0 per cent;
– *erucic acid*: maximum 0.5 per cent;
– *lignoceric acid*: 0.5 per cent to 3.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light.

**01/2017:0806**

# ARGININE

## Argininum

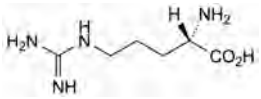

$C_6H_{14}N_4O_2$ $M_r$ 174.2
[74-79-3]

DEFINITION

(2*S*)-2-Amino-5-guanidinopentanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Solution S (see Tests) is strongly alkaline (*2.2.4*).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *arginine CRS*.

If the spectra obtained show differences, dry the substance to be examined and the reference substance in an oven at 105 °C and record new spectra.

D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.

*Reference solution*. Dissolve 10 mg of *arginine CRS* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 105 °C until the ammonia disappears completely.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve about 25 mg in 2 mL of *water R*. Add 1 mL of *α-naphthol solution R* and 2 mL of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 25.5 to + 28.5 (dried substance).

Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of arginine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 5 mL of solution S add 0.5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 10 mL of solution S, add 1.7 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 17.42 mg of $C_6H_{14}N_4O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

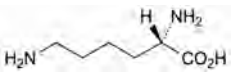

A. (2*S*)-2,6-diaminohexanoic acid (lysine),

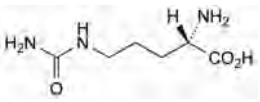

B. (2*S*)-2-amino-5-(carbamoylamino)pentanoic acid (citrulline),

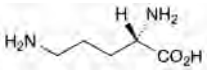

C. (2*S*)-2,5-diaminopentanoic acid (ornithine).

**01/2017:2096**

# ARGININE ASPARTATE

## Arginini aspartas

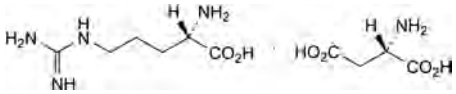

$C_{10}H_{21}N_5O_6$ $M_r$ 307.3
[7675-83-4]

DEFINITION

(2*S*)-2-Amino-5-guanidinopentanoic acid (2*S*)-2-aminobutanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white granules or powder.

*Solubility*: very soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *arginine aspartate CRS*.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances.
*Results*: the 2 principal spots in the chromatogram obtained with test solution (b) are similar in position, colour and size to the 2 principal spots in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 25 to + 27 (dried substance).

Dissolve 2.50 g in *dilute hydrochloric acid R* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a)*. Dissolve 0.20 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *water R*.

*Reference solution (a)*. Dissolve 25 mg of *arginine R* and 25 mg of *aspartic acid R* in *water R* and dilute to 25 mL with the same solvent.

*Reference solution (b)*. Dilute 2 mL of reference solution (a) to 50 mL with *water R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *ammonia R*, *propanol R* (36:64 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 10 min.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Limit*: test solution (a):

– *any impurity*: any spots, apart from the 2 principal spots, are not more intense than each of the 2 principal spots in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 0.5 g add 2.5 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*. Examine after 30 min.

**Ammonium** (*2.4.1*): maximum 100 ppm, determined on 100 mg.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C for 24 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 2 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 10.24 mg of $C_{10}H_{21}N_5O_6$.

**01/2017:0805**
**corrected 10.0**

# ARGININE HYDROCHLORIDE

## Arginini hydrochloridum

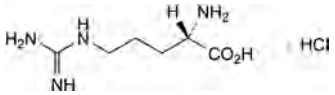


$C_6H_{15}ClN_4O_2$ $M_r$ 210.7
[1119-34-2]

DEFINITION

(2*S*)-2-Amino-5-guanidinopentanoic acid hydrochloride.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *arginine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.
*Reference solution*. Dissolve 10 mg of *arginine hydrochloride CRS* in *water R* and dilute to 50 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: at 105 °C until the ammonia disappears completely.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 25 mg in 2 mL of *water R*. Add 1 mL of *α-naphthol solution R* and 2 mL of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 21.0 to + 23.5 (dried substance).

Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50 mL with solution A. Dilute 1 mL of the solution to 200 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of arginine hydrochloride in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.07 mg of $C_6H_{15}ClN_4O_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

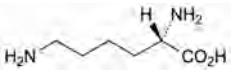

A. (2*S*)-2,6-diaminohexanoic acid (lysine),

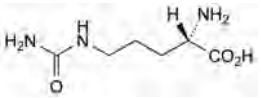

B. (2*S*)-2-amino-5-(carbamoylamino)pentanoic acid (citrulline),

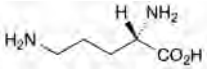

C. (2*S*)-2,5-diaminopentanoic acid (ornithine).

**07/2010:2407**

# ARGON

## Argon

Ar $A_r$ 39.95
[7440-37-1]

DEFINITION

Gas obtained by fractional distillation of ambient air.

*Content*: minimum 99.995 per cent *V/V* of Ar, calculated by deduction of the sum of impurities found when performing the test for impurities and the water content.

This monograph applies to argon for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 29 volumes of water.

IDENTIFICATION

A. Verify that the gas is not oxygen using a paramagnetic analyser (*2.5.27*).

B. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Use the following mixture of gases in *argon R1*: *methane R1* (5 ppm *V/V*), *nitrogen R1* (5 ppm *V/V*), *oxygen R* (5 ppm *V/V*).

*Column*:

– *material*: stainless steel;
– *size*: *l* = 2 m, Ø = 3 mm;
– *stationary phase*: *molecular sieve for chromatography R* (particle size 150-180 µm, pore size 0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 10 mL/min.

*Temperature*:

– *column*: 50 °C;
– *detector*: 150 °C.

*Detection*: thermal conductivity.

*Injection*: 25 µL.

*System suitability*: reference gas:

– *resolution*: minimum 3.0 between the peaks due to argon/oxygen and nitrogen and minimum 2.0 between the peaks due to nitrogen and methane.

*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with the reference gas.

TESTS

**Impurities**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Use the following mixture of gases in *argon R1*: *methane R1* (5 ppm *V/V*), *nitrogen R1* (5 ppm *V/V*), *oxygen R* (5 ppm *V/V*).

*Column*:

– *material*: stainless steel;
– *size*: *l* = 4 m, Ø = 4 mm;
– *stationary phase*: *molecular sieve for chromatography R* (particle size 150-180 µm, pore size 0.5 nm).

*Carrier gas*: *argon R1*.

*Flow rate*: 70 mL/min.

*Temperature*:

– *column*: 80 °C;
– *detector*: 40 °C.

*Detection*: discharge ionisation.

*Injection*: 1 mL.

*Sample rate*: 100 mL/min.

*Relative retention* with reference to impurity C (retention time = about 4.7 min): impurity A = about 0.4; impurity B = about 0.7.

*System suitability*: reference gas:

– *resolution*: minimum 3.0 between the peaks due to impurities A and B and minimum 2.0 between the peaks due to impurities B and C.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (5.0 ppm *V/V*);
– *total*: maximum 0.0040 per cent of the sum of the areas of all the peaks (40.0 ppm *V/V*).

**Water** (*2.5.28*): maximum 10.0 ppm *V/V*, determined using an electrolytic hygrometer.

STORAGE

In gaseous or liquid state, in suitable containers, complying with the legal regulations.

IMPURITIES

*Specified impurities*: *A, D.*

*Other detectable impurities*: *B, C.*

A. oxygen,

B. nitrogen,

C. methane,

D. water.

**04/2014:2617**
**corrected 10.0**

# ARIPIPRAZOLE

## Aripiprazolum

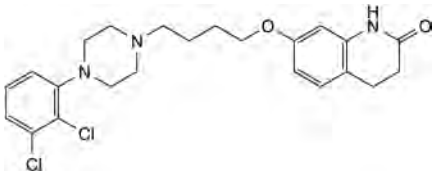

$C_{23}H_{27}Cl_2N_3O_2$ $M_r$ 448.4

[129722-12-9]

DEFINITION

7-[4-[4-(2,3-Dichlorophenyl)piperazin-1-yl]butoxy]-3,4-dihydroquinolin-2(1*H*)-one.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystals or crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, very slighty soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *aripiprazole CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. If intended for use in the manufacture of parenteral preparations, the solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in a mixture of 10 volumes of *acetic acid R* and 90 volumes of *anhydrous ethanol R* and dilute to 20 mL with the same mixture of solvents. Sonicate for about 15 min, shaking occasionally, until dissolution is complete.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Solvent mixture*: *acetic acid R*, *methanol R*, *acetonitrile R*, *water R* (1:10:30:60 *V/V/V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *aripiprazole impurity F CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 50 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *aripiprazole CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, 0.05 per cent *V/V* solution of *trifluoroacetic acid R* (10:90 *V/V*);
- *mobile phase B*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*, *acetonitrile R* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 10 | 80 → 65 | 20 → 35 |
| 10 - 20 | 65 → 10 | 35 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Relative retention* with reference to aripiprazole (retention time = about 11 min): impurity F = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to aripiprazole and impurity F.

*Calculation of percentage contents*:

- for each impurity, use the concentration of aripiprazole in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

Dissolve 1.0 mg of the substance to be examined in 20 mL of a 5.17 g/L solution of *hydrochloric acid R*.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

- *symmetry factor*: maximum 2.0.

Calculate the percentage content of $C_{23}H_{27}Cl_2N_3O_2$ taking into account the assigned content of *aripiprazole CRS*.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

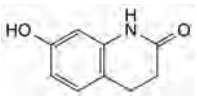


A. 7-hydroxy-3,4-dihydroquinolin-2(1*H*)-one,

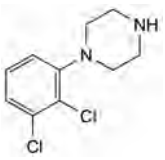


B. 1-(2,3-dichlorophenyl)piperazine,

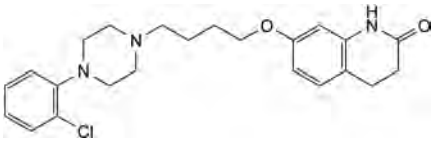


C. 7-[4-[4-(2-chlorophenyl)piperazin-1-yl]butoxy]-3,4-dihydroquinolin-2(1*H*)-one,

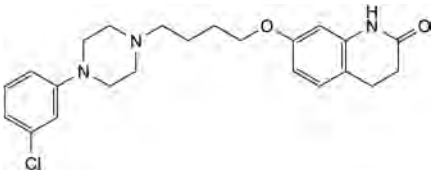


D. 7-[4-[4-(3-chlorophenyl)piperazin-1-yl]butoxy]-3,4-dihydroquinolin-2(1*H*)-one,

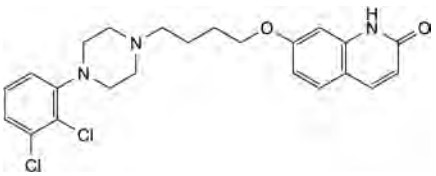


E. 7-[4-[4-(2,3-dichlorophenyl)piperazin-1-yl]butoxy]-quinolin-2(1*H*)-one,

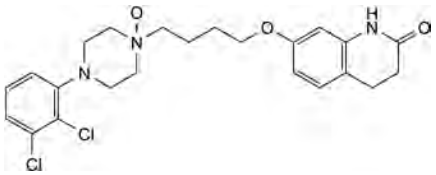

F. 7-[4-[4-(2,3-dichlorophenyl)-1-oxidopiperazin-1-yl]butoxy]-3,4-dihydroquinolin-2(1*H*)-one,

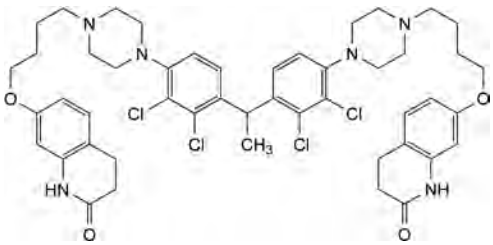

G. 7,7′-[ethane-1,1-diylbis[(2,3-dichlorobenzene-4,1-diyl)piperazine-4,1-diylbutane-4,1-diyloxy]]bis[3,4-dihydroquinolin-2(1*H*)-one].

**01/2017:1688**
**corrected 10.0**

# ARTICAINE HYDROCHLORIDE

## Articaini hydrochloridum

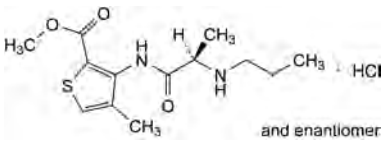


$C_{13}H_{21}ClN_2O_3S$ $M_r$ 320.8
[23964-57-0]

DEFINITION

Methyl 4-methyl-3-[[(2*RS*)-2-(propylamino)propanoyl]-amino]thiophene-2-carboxylate hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 50.0 mg in a 1 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of the solution to 100.0 mL with a 1 g/L solution of *hydrochloric acid R*. Examined between 200 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 272 nm. The specific absorbance at the maximum is 290 to 320.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: place dropwise 20 µL of the test solution on 300 mg discs.

*Test solution*. Dissolve 0.1 g in 5 mL of *water R*, add 3 mL of a saturated solution of *sodium hydrogen carbonate R* and shake twice with 2 mL of *methylene chloride R*. Combine the methylene chloride layers, dilute to 5.0 mL with *methylene chloride R* and dry over *anhydrous sodium sulfate R*.

*Comparison*: *articaine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 5 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 20 mg of *articaine hydrochloride CRS* in 5 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *triethylamine R*, *ethyl acetate R*, *heptane R* (10:35:65 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 4.2 to 5.2.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *articaine impurity A CRS* and 2.5 mg of *articaine impurity E CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: mix 25 volumes of *acetonitrile R* and 75 volumes of a solution prepared as follows: dissolve 2.02 g of *sodium heptanesulfonate R* and 4.08 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. Adjust to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of articaine.

*Relative retention* with reference to articaine (retention time = about 9 min): impurity A = about 0.8; impurity E = about 0.86.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.2 between the peaks due to impurities A and E.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *sum of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.08 mg of $C_{13}H_{21}ClN_2O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, J.*

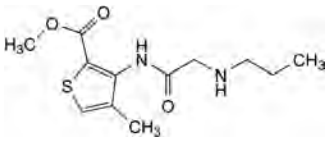

A. methyl 4-methyl-3-[[2-(propylamino)acetyl]amino]thiophene-2-carboxylate (acetamidoarticaine),

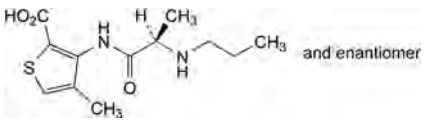


B. 4-methyl-3-[[(2*RS*)-2-(propylamino)propanoyl]amino]thiophene-2-carboxylic acid (articaine acid),

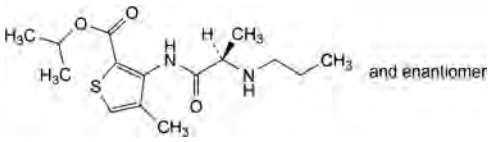


C. 1-methylethyl 4-methyl-3-[[(2*RS*)-2-(propylamino)propanoyl]amino]thiophene-2-carboxylate (articaine isopropyl ester),

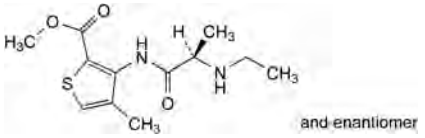


D. methyl 3-[[(2*RS*)-2-(ethylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (ethylarticaine),

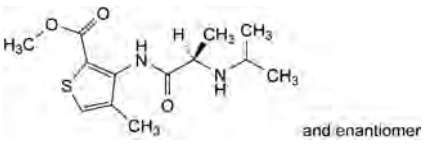


E. methyl 4-methyl-3-[[(2*RS*)-2-[(1-methylethyl)amino]propanoyl]amino]thiophene-2-carboxylate (isopropylarticaine),

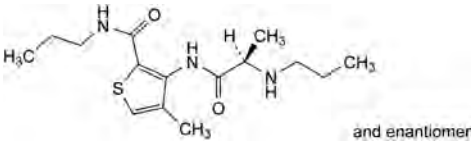


F. 4-methyl-*N*-propyl-3-[[(2*RS*)-2-(propylamino)propanoyl]amino]thiophene-2-carboxamide (articaine acid propionamide),

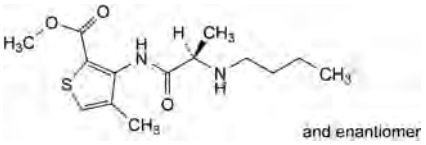


G. methyl 3-[[(2*RS*)-2-(butylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (butylarticaine),

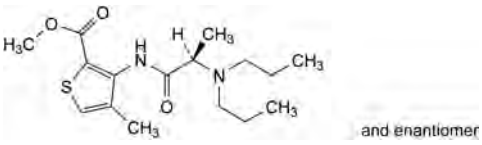


H. methyl 3-[[(2*RS*)-2-(dipropylamino)propanoyl]amino]-4-methylthiophene-2-carboxylate (dipropylarticaine),

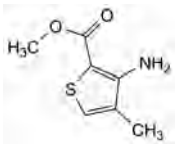

I. methyl 3-amino-4-methylthiophene-2-carboxylate (3-aminoarticaine),

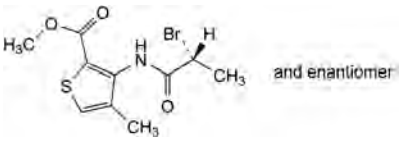


J. methyl 3-[[(2*RS*)-2-bromopropanoyl]amino]-4-methylthiophene-2-carboxylate (bromo compound).

**07/2019:0253**

# ASCORBIC ACID

## Acidum ascorbicum

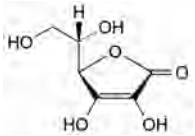

$C_6H_8O_6$ $M_r$ 176.1
[50-81-7]

DEFINITION

(5*R*)-5-[(1*S*)-1,2-Dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one.

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, becoming discoloured on exposure to air and moisture.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

mp: about 190 °C, with decomposition.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.10 g in *water R* and dilute immediately to 100.0 mL with the same solvent. Add 1.0 mL of the solution to 10 mL of a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

*Absorption maximum*: at 243 nm, determined immediately after dissolution.

*Specific absorbance at the absorption maximum*: 545 to 585.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ascorbic acid CRS*.

C. pH (*2.2.3*): 2.1 to 2.6 for solution S (see Tests).

D. To 1 mL of solution S add 0.2 mL of *dilute nitric acid R* and 0.2 mL of *silver nitrate solution R2*. A grey precipitate is formed.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 20.5 to + 21.5.

Dissolve 2.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Impurity E**: maximum 0.2 per cent.

*Test solution.* Dissolve 0.25 g in 5 mL of *water R*. Neutralise using *dilute sodium hydroxide solution R*, then add 1 mL of *dilute acetic acid R* and 0.5 mL of *calcium chloride solution R*.

*Reference solution.* Dissolve 70 mg of *oxalic acid R* (dihydrate of impurity E) in *water R* and dilute to 500 mL with the same solvent; to 5 mL of the solution add 1 mL of *dilute acetic acid R* and 0.5 mL of *calcium chloride solution R*.

Allow the solutions to stand for 1 h. Any opalescence in the test solution is not more intense than that in the reference solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Phosphate buffer solution.* Dissolve 6.8 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to about 175 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm) and dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 0.500 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *ascorbic acid impurity C CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *ascorbic acid impurity D CRS* and 5.0 mg of *ascorbic acid CRS* in the mobile phase, add 2.5 mL of reference solution (a) and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1 mL of the test solution to 200 mL with the mobile phase. Mix 1 mL of this solution and 1 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: phosphate buffer solution, *acetonitrile R1* (25:75 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of ascorbic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to ascorbic acid (retention time = about 11 min): impurity D = about 0.4; impurity C = about 1.7.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to ascorbic acid and impurity C in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 20 for the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Limits*:

- *impurities C, D*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than C and D*: not more than twice the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Copper**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.0 g in *0.1 M nitric acid* and dilute to 25.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) using *copper standard solution (10 ppm Cu) R*, diluting with *0.1 M nitric acid*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Atomisation device*: air-acetylene flame.

Adjust the zero of the apparatus using *0.1 M nitric acid*.

**Iron**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 5.0 g in *0.1 M nitric acid* and dilute to 25.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) using *iron standard solution (20 ppm Fe) R*, diluting with *0.1 M nitric acid*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

Adjust the zero of the apparatus using *0.1 M nitric acid*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 10 mL of *dilute sulfuric acid R* and 80 mL of *carbon dioxide-free water R*. Add 1 mL of *starch solution R*. Titrate with *0.05 M iodine* until a persistent violet-blue colour is obtained.

1 mL of *0.05 M iodine* is equivalent to 8.81 mg of $C_6H_8O_6$.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

*Specified impurities: C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, F, G, H.*

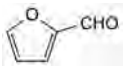

A. furan-2-carbaldehyde,

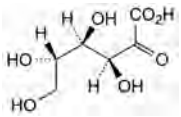

C. L-*xylo*-hex-2-ulosonic acid (L-sorbosonic acid),

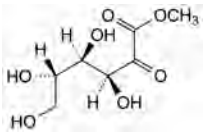

D. methyl L-*xylo*-hex-2-ulosonate (methyl L-sorbosonate),

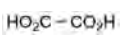

E. oxalic acid,

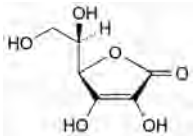

F. (5*R*)-5-[(1*R*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one,

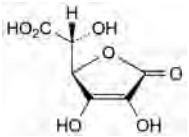

G. (*R*)-[(2*R*)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]hydroxyacetic acid,

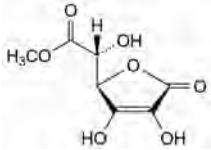

H. methyl (*R*)-[(2*R*)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]hydroxyacetate.

**01/2017:0807**

# ASCORBYL PALMITATE

## Ascorbylis palmitas

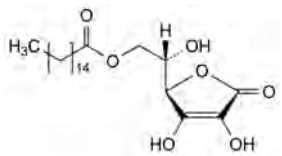

$C_{22}H_{38}O_7$ $M_r$ 414.5

[137-66-6]

DEFINITION

(2*S*)-2-[(2*R*)-3,4-Dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]-2-hydroxyethyl hexadecanoate.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in methanol, practically insoluble in methylene chloride and in fatty oils.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ascorbyl palmitate CRS*.

C. Dissolve about 10 mg in 5 mL of *methanol R*. The solution decolourises *dichlorophenolindophenol standard solution R*.

TESTS

**Solution S**. Dissolve 2.50 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method I*).

**Specific optical rotation** (*2.2.7*): + 21 to + 24 (dried substance), determined on solution S.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *ethanol (96 per cent) R*. Add 30 mL of *water R* and titrate with *0.05 M iodine* until a yellow colour is obtained.

1 mL of *0.05 M iodine* is equivalent to 20.73 mg of $C_{22}H_{38}O_7$.

STORAGE

In an airtight container, protected from light.

07/2019:2086

# ASPARAGINE MONOHYDRATE

## Asparaginum monohydricum

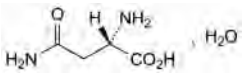

$C_4H_8N_2O_3,H_2O$ $M_r$ 150.1
[5794-13-8]

DEFINITION

(2*S*)-2,4-Diamino-4-oxobutanoic acid monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *A, B, D.*

*Second identification*: *A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *asparagine monohydrate CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *asparagine monohydrate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R, water R, butanol R* (25:25:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 110 °C for 15 min.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve with heating 2.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 33.7 to + 36.0 (dried substance).

Dissolve 2.50 g in a 309.0 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R.*

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R.*

*Reference solution (c).* Dissolve 5.0 mg of *aspartic acid R* (impurity A) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R.*

*Reference solution (d).* Dissolve 3.0 mg of *asparagine impurity C CRS* in 40 mL of *water R* using sonification and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R.*

*Reference solution (e).* Mix 5.0 mL of reference solution (c) with 2.5 mL of reference solution (a) and dilute to 10.0 mL with *water R.*

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: dissolve 13.6 g of *potassium dihydrogen phosphate R* and 2.16 g of *sodium octanesulfonate R* in about 900 mL of *water for chromatography R.* Adjust to pH 2.2 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R.* Add 5 mL of *acetonitrile R1.*

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of asparagine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to asparagine (retention time = about 6.6 min): impurity C = about 0.6; impurity A = about 1.2.

*System suitability*: reference solution (e):

- *resolution*: minimum 5.0 between the peaks due to asparagine and impurity A.

*Calculation of percentage contents*:

- for impurity A, use the concentration of impurity A in reference solution (c);
- for impurity C, use the concentration of impurity C in reference solution (d);
- for impurities other than A and C, use the concentration of asparagine monohydrate in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *impurity C*: maximum 0.1 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.8 per cent;
- *reporting threshold*: 0.03 per cent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 12.5 mL of solution S to 15 mL with *water R.*

**Sulfates** (*2.4.13*): maximum 200 ppm.

To 0.75 g add 2.5 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R.* Examine after 30 min.

**Ammonium** (*2.4.1, Method B*): maximum 0.1 per cent, determined on 10 mg.

Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R.*

**Iron** (*2.4.9*): maximum 10 ppm.

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 10 mL with the same acid. Shake 3 times with 10 mL of *methyl isobutyl ketone R1* for 3 min. Wash the combined organic

phases with 10 mL of *water R* for 3 min. The aqueous phase complies with the limit test for iron.

**Loss on drying** (*2.2.32*): 10.5 per cent to 12.5 per cent, determined on 1.000 g by drying in an oven at 130 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.110 g in 5 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.21 mg of $C_4H_8N_2O_3$.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, G, H.*

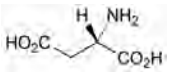

A. (2*S*)-2-aminobutanedioic acid (aspartic acid),

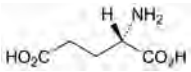

B. (2*S*)-2-aminopentanedioic acid (glutamic acid),

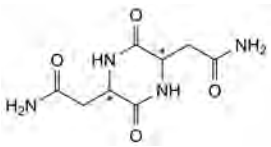

C. 2,2′-[(2Ξ,5Ξ)-3,6-dioxopiperazine-2,5-diyl]diacetamide,

D. (2*E*)-but-2-enedioic acid (fumaric acid),

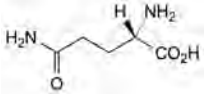

E. (2*S*)-2,5-diamino-5-oxopentanoic acid (glutamine),

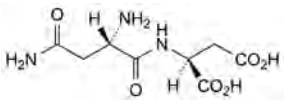

F. (2*S*)-2-[[(2*S*)-2,4-diamino-4-oxobutanoyl]amino]butanedioic acid (asparaginylaspartic acid),

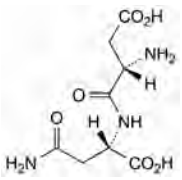

G. (2*S*)-4-amino-2-[[(2*S*)-2-amino-3-carboxypropanoyl]amino]-4-oxobutanoic acid (α-aspartylasparagine),

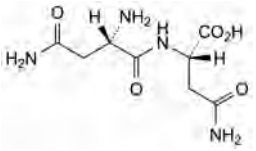

H. (2*S*)-4-amino-2-[[(2*S*)-2,4-diamino-4-oxobutanoyl]amino]-4-oxobutanoic acid (asparaginylasparagine).

**01/2017:0973**

# ASPARTAME

## Aspartamum

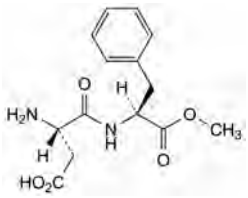

$C_{14}H_{18}N_2O_5$ $M_r$ 294.3
[22839-47-0]

DEFINITION

(3*S*)-3-Amino-4-[[(2*S*)-1-methoxy-1-oxo-3-phenylpropan-2-yl]amino]-4-oxobutanoic acid (methyl α-L-aspartyl-L-phenylalaninate).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: sparingly soluble or slightly soluble in water and in ethanol (96 per cent), practically insoluble in hexane and in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent.

*Spectral range*: 230-300 nm.

*Absorption maxima*: at 247 nm, 252 nm, 258 nm and 264 nm.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *aspartame CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 15 mg of the substance to be examined in 2.5 mL of *water R* and dilute to 10 mL with *acetic acid R.*

*Reference solution.* Dissolve 15 mg of *aspartame CRS* in 2.5 mL of *water R* and dilute to 10 mL with *acetic acid R.*

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R, anhydrous formic acid R, methanol R, methylene chloride R* (2:4:30:64 *V/V/V/V*).

*Application*: 20 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*Results*: the spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with the reference solution.

D. Dissolve about 20 mg in 5 mL of *methanol R* and add 1 mL of *alkaline hydroxylamine solution R1*. Heat on a water-bath for 15 min. Allow to cool and adjust to about pH 2 with *dilute hydrochloric acid R*. Add 0.1 mL of *ferric chloride solution R1*. A brownish-red colour is produced.

TESTS

**Solution S**. Dissolve 0.8 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

**Conductivity** (*2.2.38*): maximum 30 µS·cm$^{-1}$.

Dissolve 0.80 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution ($C_1$) and that of the water used for preparing the solution ($C_2$). The readings must be stable within 1 per cent over a period of 30 s.
Calculate the conductivity of the solution of the substance to be examined using the following expression:

$$C_1 - 0.992\ C_2$$

**Specific optical rotation** (*2.2.7*): + 14.5 to + 16.5 (dried substance).

Dissolve 2.00 g in a 690 g/L solution of *anhydrous formic acid R* and dilute to 50.0 mL with the same solution. Measure within 30 min of preparation.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.60 g of the substance to be examined in a mixture of 1.5 volumes of *glacial acetic acid R* and 98.5 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 4.5 mg of *aspartame impurity A CRS* in a mixture of 1.5 volumes of *glacial acetic acid R* and 98.5 volumes of *water R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (b)*. Dissolve 30.0 mg of *phenylalanine R* (impurity C) in a mixture of 15 volumes of *glacial acetic acid R* and 85 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (c)*. Dilute 5.0 mL of the test solution to 10.0 mL with *water R*. Dilute 3.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (d)*. Dissolve 30.0 mg of *L-aspartyl-L-phenylalanine R* (impurity B) in a mixture of 15 volumes of *glacial acetic acid R* and 85 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 1.0 mL of the solution to 10.0 mL with *water R*. Mix 1.0 mL of this solution with 1.0 mL of reference solution (b).

*Column*

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile R* and 90 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.7 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of aspartame.

*System suitability*: reference solution (d):

- *resolution*: minimum 3.5 between the peaks due to impurities B and C.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *sum of impurities other than A and C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent);
- *disregard limit*: disregard any peak due to the solvent.

**Loss on drying** (*2.2.32*): maximum 4.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 1.5 mL of *anhydrous formic acid R* and 60 mL of *anhydrous acetic acid R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.43 mg of $C_{14}H_{18}N_2O_5$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

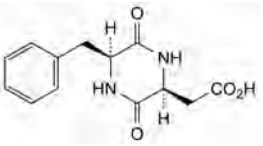


A. 2-[(2*S*,5*S*)-5-benzyl-3,6-dioxopiperazin-2-yl]acetic acid,

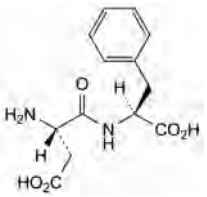


B. (3*S*)-3-amino-4-[[(1*S*)-1-carboxy-2-phenylethyl]amino]-4-oxobutanoic acid (α-L-aspartyl-L-phenylalanine),

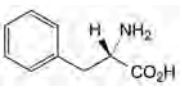


C. (2*S*)-2-amino-3-phenylpropanoic acid (L-phenylalanine).

**01/2018:0797**

# ASPARTIC ACID

## Acidum asparticum

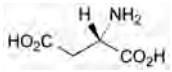

$C_4H_7NO_4$ $M_r$ 133.1
[56-84-8]

DEFINITION

(2*S*)-2-Aminobutanedioic acid (L-aspartic acid).

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: carry out either tests A, C or tests C, D.*

*Second identification: A, B, E.*

A. Specific optical rotation (*2.2.7*): + 24.0 to + 26.0 (dried substance).
Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

B. A suspension of 1 g in 10 mL of *water R* is strongly acid (*2.2.4*).

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *aspartic acid CRS*.

D. Enantiomeric purity (see Tests).

E. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in 2 mL of *dilute ammonia R1* and dilute to 50 mL with *water R*.
*Reference solution*. Dissolve 10 mg of *aspartic acid CRS* in 2 mL of *dilute ammonia R1* and dilute to 50 mL with *water R*.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same acid.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.100 g of D-*aspartic acid R* (impurity I) in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 0.100 g of the substance to be examined in 90 mL of *water R*, add 0.3 mL of reference solution (a) and dilute to 100.0 mL with *water R*.

*Reference solution (c)*. Dilute 0.3 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: L-*penicillamine coated silica gel for chiral separations R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *2-propanol R*, 0.5 g/L solution of *copper sulfate pentahydrate R* (5:95 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to aspartic acid (retention time = about 12 min): impurity I = about 0.85.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurity I and aspartic acid.

*Calculation of percentage content*:
- for impurity I, use the concentration of impurity I in reference solution (c).

*Limit*:
- *impurity I*: maximum 0.3 per cent.

**Other dicarboxylic acids**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in 2.0 mL of a 618 g/L solution of *hydrochloric acid R* and dilute to 10.0 mL with *water R*.

*Reference solution (a)*. Dissolve 20.0 mg of *malic acid R* (impurity A) in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 10.0 mg of *maleic acid R* (impurity H) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with reference solution (a).

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R*.

*Reference solution (e)*. Dissolve 10.0 mg of *fumaric acid R* (impurity B) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *cation-exchange resin R* (9 µm);
- *temperature*: 30 °C.

*Mobile phase*: 0.39 g/L solution of *sulfuric acid R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of impurity H.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and H; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity B.

*Relative retention* with reference to impurity H (retention time = about 7.5 min): impurity A = about 1.2; impurity B = about 2.0.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to impurities H and A.

*Calculation of percentage contents*:
- for impurity A, use the concentration of impurity A in reference solution (d);
- for impurities B and H, use the concentration of impurity H in reference solution (b);
- for impurities other than B and H, use the concentration of impurity A in reference solution (d).

*Limits*:
- *impurity A*: maximum 0.2 per cent;
- *impurity B*: maximum 0.10 per cent;
- *impurity H*: maximum 0.10 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test and reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Reference solution (e).* Dissolve 30.0 mg of *alanine R* (impurity D), 60.0 mg of *asparagine R* (impurity G) and 30.0 mg of *glutamic acid R* (impurity C) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test solution, blank solution and reference solutions (a), (b), (d) and (e) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:
- for impurities C, D and G, use the concentration of each impurity in reference solution (e);
- for any ninhydrin-positive substance detected at 570 nm, use the concentration of aspartic acid in reference solution (a);
- for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:
- *impurities C, D, G*: for each impurity, maximum 0.2 per cent;
- *any ninhydrin-positive substance*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 3 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. Add 1 mL of *water R* instead of 1 mL of *dilute nitric acid R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in 4 mL of *hydrochloric acid R* and dilute to 15 mL with *distilled water R*. Carry out the evaluation of the test after 30 min.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modification.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit:*
- *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *carbon dioxide-free water R*, with slight heating if necessary. Cool and titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 13.31 mg of $C_4H_7NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, H, G, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

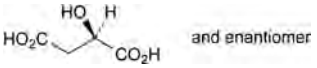

and enantiomer

A. (2*RS*)-2-hydroxybutanedioic acid (malic acid),

B. (2*E*)-but-2-enedioic acid (fumaric acid),

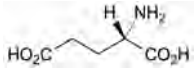


C. (2*S*)-2-aminopentanedioic acid (glutamic acid),

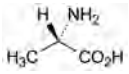

D. (2*S*)-2-aminopropanoic acid (alanine),

E. butanedioic acid (succinic acid),

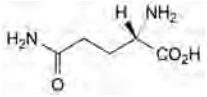

F. (2*S*)-2,5-diamino-5-oxopentanoic acid (L-glutamine),

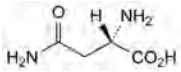

G. (2*S*)-2,4-diamino-4-oxobutanoic acid (asparagine),

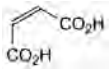

H. (2*Z*)-but-2-enedioic acid (maleic acid),

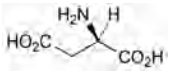

I. (2*R*)-2-aminobutanedioic acid (D-aspartic acid).

**04/2019:2898**
**corrected 10.0**

# ATAZANAVIR SULFATE

## Atazanaviri sulfas

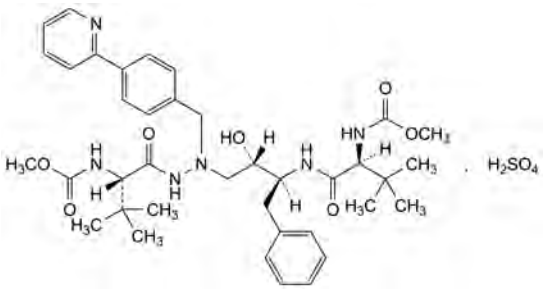

$C_{38}H_{54}N_6O_{11}S$ $M_r$ 803
[229975-97-7]

DEFINITION

Methyl [(5*S*,10*S*,11*S*,14*S*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-8-[[4-(pyridin-2-yl)phenyl]methyl]-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate sulfate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, slightly hygroscopic, crystalline powder that may contain agglomerates.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *atazanavir sulfate CRS*.

C. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): − 44 to − 40 (anhydrous substance), measured at 25 °C.

Dissolve 0.100 g in 8 mL of *methanol R*, using sonication if necessary, and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix equal volumes of *acetonitrile R1* and a freshly prepared 2.73 g/L solution of *potassium dihydrogen phosphate R* in *water for chromatography R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*.

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in 40 mL of the solvent mixture, sonicate for 3 min and dilute to 50.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in 40 mL of the solvent mixture, sonicate for 3 min and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *atazanavir sulfate CRS* in 40 mL of the solvent mixture, sonicate for 3 min and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 4 mg of *atazanavir for system suitability CRS* (containing impurity F) in 8 mL of the solvent mixture, sonicate for 3 min and dilute to 10 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2.0 mg of *atazanavir impurity K CRS* in 9 mL of the solvent mixture, sonicate for 3 min and dilute to 10.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 100.0 mL with the solvent mixture. Dilute 3.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.0 µm);
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: mix 25 volumes of *acetonitrile R1* and 75 volumes of a freshly prepared 2.73 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*;
- *mobile phase B*: mix 25 volumes of a freshly prepared 2.73 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*, and 75 volumes of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 45 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *atazanavir for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to atazanavir (retention time = about 30 min): impurity F = about 0.99.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to impurity F and atazanavir.

*Calculation of percentage contents*:
- for each impurity, use the concentration of atazanavir sulfate in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard any peak with a relative retention with reference to atazanavir of less than 0.2.

**Impurity K**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 8 | 95 → 0 | 5 → 100 |
| 8 - 14 | 0 | 100 |

*Injection*: 20 µL of test solution (b) and reference solution (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity K.

*Relative retention* with reference to atazanavir (retention time = about 10 min): impurity K = about 0.4.

*Calculation of percentage content*:

- for impurity K, use the concentration of impurity K in reference solution (d).

*Limit*:

- *impurity K*: maximum 0.15 per cent.

**Water** (*2.5.32*): maximum 2.5 per cent, determined on 0.100 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: solvent mixture.

*Injection*: test solution (a) and reference solutions (a) and (c).

*Run time*: 1.6 times the retention time of atazanavir.

*Relative retention* with reference to atazanavir (retention time = about 9.5 min): impurity F = about 0.94.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity F and atazanavir.

Calculate the percentage content of $C_{38}H_{54}N_6O_{11}S$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *atazanavir sulfate CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J.*

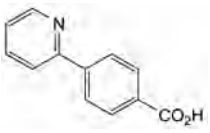

A. 4-(pyridin-2-yl)benzoic acid,

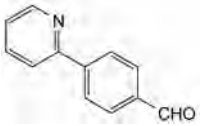

B. 4-(pyridin-2-yl)benzaldehyde,

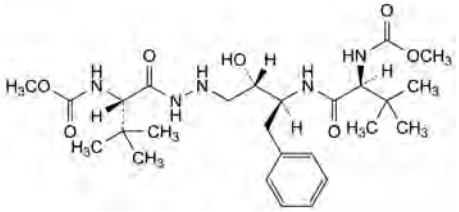

C. methyl [(5*S*,10*S*,11*S*,14*S*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate,

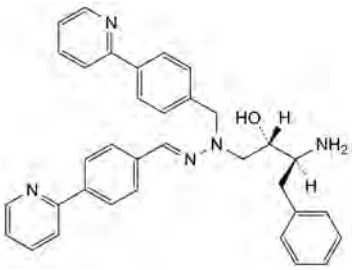

D. (2*S*,3*S*)-3-amino-4-phenyl-1-[(*E*)-1-[[4-(pyridin-2-yl)phenyl]methyl]-2-[[4-(pyridin-2-yl)phenyl]methylidene]hydrazin-1-yl]butan-2-ol,

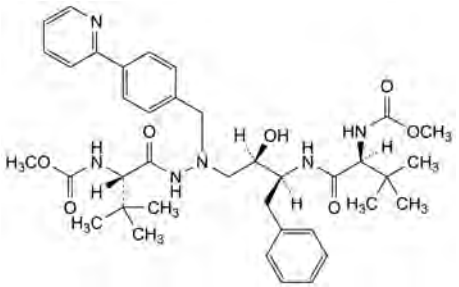

E. methyl [(5*S*,10*R*,11*S*,14*S*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-8-[[4-(pyridin-2-yl)phenyl]methyl]-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate,

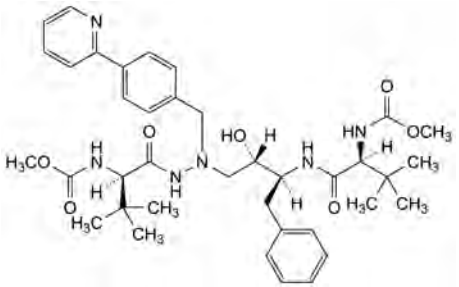

F. methyl [(5*R*,10*S*,11*S*,14*S*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-8-[[4-(pyridin-2-yl)phenyl]methyl]-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate,

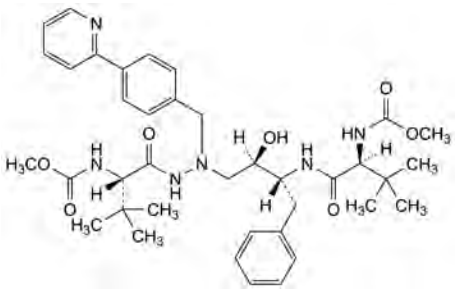

G. methyl [(5*S*,10*S*,11*S*,14*R*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-8-[[4-(pyridin-2-yl)phenyl]methyl]-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate,

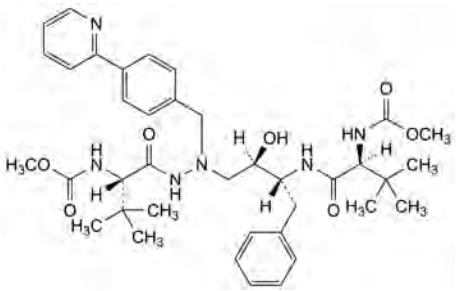

H. methyl [(5*S*,10*R*,11*R*,14*S*)-11-benzyl-5-*tert*-butyl-10-hydroxy-15,15-dimethyl-3,6,13-trioxo-8-[[4-(pyridin-2-yl)phenyl]methyl]-2-oxa-4,7,8,12-tetraazahexadecan-14-yl]carbamate,

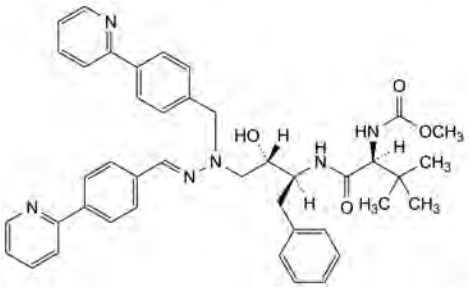

I. methyl [(2*S*)-1-[[(2*S*,3*S*)-3-hydroxy-1-phenyl-4-[(*E*)-1-[[4-(pyridin-2-yl)phenyl]methyl]-2-[[4-(pyridin-2-yl)phenyl]methylidene]hydrazin-1-yl]butan-2-yl]amino]-3,3-dimethyl-1-oxobutan-2-yl]carbamate,

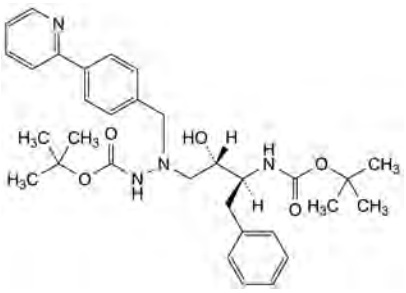

J. *tert*-butyl 2-[(2*S*,3*S*)-3-(*tert*-butoxyformamido)-2-hydroxy-4-phenylbutyl]-2-[[4-(pyridin-2-yl)phenyl]methyl]hydrazine-1-carboxylate,

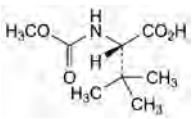

K. (2*S*)-2-(methoxyformamido)-3,3-dimethylbutanoic acid.

**04/2017:0703**

# ATENOLOL

## Atenololum

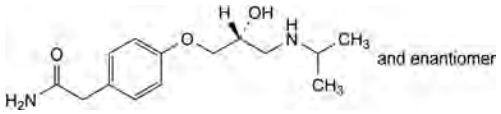


$C_{14}H_{22}N_2O_3$ $M_r$ 266.3
[29122-68-7]

DEFINITION

2-[4-[(2*RS*)-2-Hydroxy-3-[(propan-2-yl)amino]propoxy]-phenyl]acetamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, soluble in anhydrous ethanol, slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 152 °C to 155 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.100 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *methanol R.*

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 275 nm and 282 nm.

*Absorbance ratio*: $A_{275}/A_{282}$ = 1.15 to 1.20.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *atenolol CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 1.0 mL of *methanol R.*

*Reference solution.* Dissolve 10 mg of *atenolol CRS* in 1.0 mL of *methanol R.*

*Plate*: *TLC silanised silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R1*, *methanol R* (1:99 *V/V*).

*Application*: 10 µL.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 0.10 g in *water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than degree 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): + 0.10° to − 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 20 mL of the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2 mg of *atenolol for system suitability CRS* (containing impurities B, F, G, I and J) in 1.0 mL of the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium octanesulfonate R* and 0.4 g of *tetrabutylammonium hydrogen sulfate R* in 1 L of a mixture of 20 volumes of *tetrahydrofuran R*, 180 volumes of *methanol R2* and 800 volumes of a 3.4 g/L solution of *potassium dihydrogen phosphate R*; adjust the apparent pH to 3.0 with *phosphoric acid R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of atenolol.

*Identification of impurities*: use the chromatogram supplied with *atenolol for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, F, G, I and J.

*Relative retention* with reference to atenolol (retention time = about 8 min): impurity B = about 0.3; impurity J = about 0.7; impurity I = about 0.8; impurity F = about 2.0 (pair of peaks); impurity G = about 3.5.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.4 between the peaks due to impurities J and I.

*Limits*:

- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities F, G, I, J*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dissolve 50 mg in a mixture of 1 mL of *dilute nitric acid R* and 15 mL of *water R*. The solution, without further addition of *dilute nitric acid R*, complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.63 mg of $C_{14}H_{22}N_2O_3$.

IMPURITIES

*Specified impurities: B, F, G, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, H.*

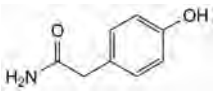

A. 2-(4-hydroxyphenyl)acetamide,

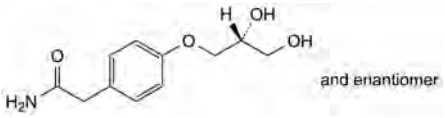


B. 2-[4-[(2*RS*)-2,3-dihydroxypropoxy]phenyl]acetamide,

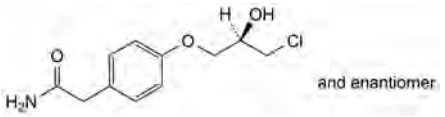


D. 2-[4-[(2*RS*)-3-chloro-2-hydroxypropoxy]phenyl]-acetamide,

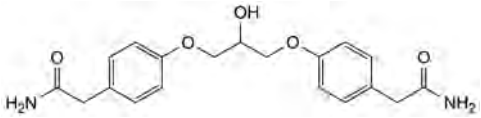

E. 2,2′-[(2-hydroxypropane-1,3-diyl)bis(oxy-4,1-phenylene)]diacetamide,

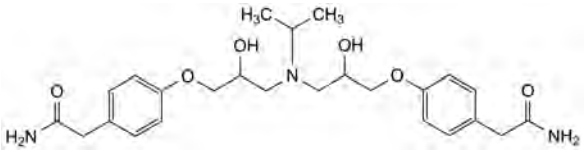

F. 2,2′-[[(propan-2-yl)azanediyl]bis[(2-hydroxypropane-3,1-diyl)oxy-4,1-phenylene]]diacetamide,

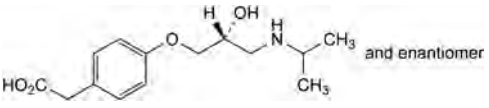


G. [4-[(2*RS*)-2-hydroxy-3-[(propan-2-yl)amino]-propoxy]phenyl]acetic acid,

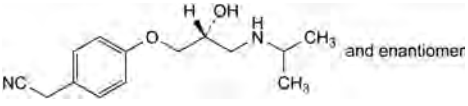


H. [4-[(2*RS*)-2-hydroxy-3-[(propan-2-yl)amino]-propoxy]phenyl]acetonitrile,

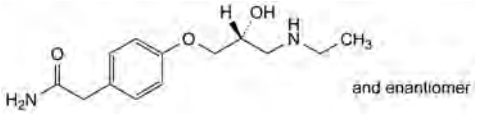


I. 2-[4-[(2*RS*)-3-(ethylamino)-2-hydroxypropoxy]-phenyl]acetamide,

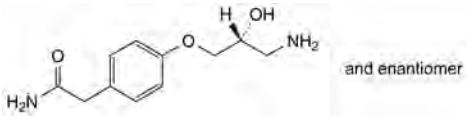


J. 2-[4-[(2*RS*)-3-amino-2-hydroxypropoxy]phenyl]-acetamide.

**01/2017:2640**

# ATOMOXETINE HYDROCHLORIDE

## Atomoxetini hydrochloridum

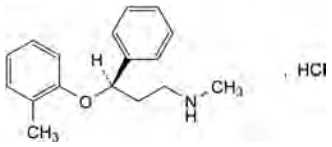


$C_{17}H_{22}ClNO$ $M_r$ 291.8
[82248-59-7]

DEFINITION

(3*R*)-*N*-Methyl-3-(2-methylphenoxy)-3-phenylpropan-1-amine hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *atomoxetine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. Isomeric purity (see Tests).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Isomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 35.0 mg of the substance to be examined in 2.5 mL of *anhydrous ethanol R*, sonicate until dissolution is complete and dilute to 10.0 mL with *heptane R*.

*Reference solution (a).* Dissolve 3.5 mg of *atomoxetine impurity B CRS* and 1 mg of *atomoxetine impurity D CRS* in 5 mL of *anhydrous ethanol R*, sonicate until dissolution is complete and dilute to 20.0 mL with *heptane R*.

*Reference solution (b).* Dissolve 35.0 mg of the substance to be examined in 2.5 mL of *anhydrous ethanol R*. Add 1.0 mL of reference solution (a) and dilute to 10.0 mL with *heptane R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *heptane R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (5 µm).

*Mobile phase*: mix 1.5 mL of *diethylamine R*, 2.0 mL of *trifluoroacetic acid R* and 150.0 mL of *2-propanol R* and dilute to 1000 mL with *heptane R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.3 times the retention time of atomoxetine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and D.

*Relative retention* with reference to atomoxetine (retention time = about 12 min): impurity B = about 0.5; impurity D = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.8 between the peaks due to impurities B and D.

*Limits*:

– *impurity B*: maximum 0.5 per cent;
– *impurity D*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *disregard limit*: the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak with a relative retention with reference to atomoxetine of about 0.7 (impurity A).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 5.9 g of *sodium octanesulfonate monohydrate R* in 1000 mL of a 2.9 g/L solution of *phosphoric acid R* previously adjusted to pH 2.5 with a 280 g/L solution of *potassium hydroxide R*.

*Test solution (a).* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 7.5 mg of *3-(methylamino)-1-phenylpropan-1-ol R* (impurity H) and 5 mg of *mandelic acid R* (impurity E) in test solution (b) and dilute to 50 mL with test solution (b).

*Reference solution (c).* Dissolve 5 mg of *atomoxetine for impurity A identification CRS* in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (d).* Dissolve 25.0 mg of *atomoxetine hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*: *propanol R*, solution A (27:73 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: 2.5 times the retention time of atomoxetine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities E and H; use the chromatogram supplied with *atomoxetine for impurity A identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to atomoxetine (retention time = about 10 min): impurity E = about 0.2; impurity H = about 0.3; impurity A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurities E and H.

*Calculation of percentage contents*:

– for each impurity, use the concentration of atomoxetine hydrochloride in reference solution (a).

*Limits*:

– *impurity A*: maximum 0.3 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{17}H_{22}ClNO$ taking into account the assigned content of *atomoxetine hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, G, H.*

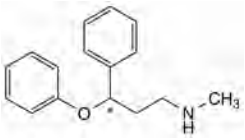

A. *N*-methyl-3-phenoxy-3-phenylpropan-1-amine,

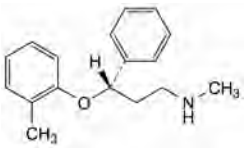

B. (3*S*)-*N*-methyl-3-(2-methylphenoxy)-3-phenylpropan-1-amine,

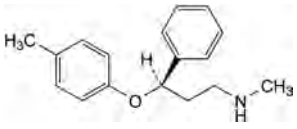

C. (3*R*)-*N*-methyl-3-(4-methylphenoxy)-3-phenylpropan-1-amine,

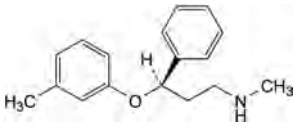

D. (3*R*)-*N*-methyl-3-(3-methylphenoxy)-3-phenylpropan-1-amine,

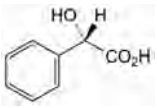

E. (2*S*)-2-hydroxy-2-phenylacetic acid (L-mandelic acid),

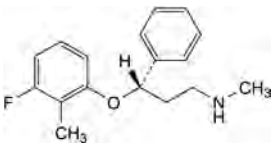

F. (3*S*)-3-(3-fluoro-2-methylphenoxy)-*N*-methyl-3-phenylpropan-1-amine,

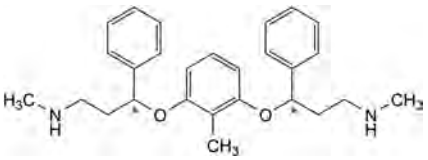

G. 3,3′-[(2-methylbenzene-1,3-diyl)bis(oxy)]bis(*N*-methyl-3-phenylpropan-1-amine),

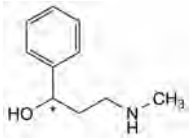

H. 3-(methylamino)-1-phenylpropan-1-ol.

**01/2017:2191**

# ATORVASTATIN CALCIUM TRIHYDRATE

## Atorvastatinum calcicum trihydricum

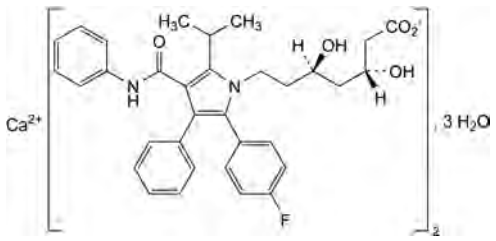

$C_{66}H_{68}CaF_2N_4O_{10},3H_2O$ $M_r$ 1209

[344423-98-9]

DEFINITION

Calcium (3*R*,5*R*)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoate trihydrate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *atorvastatin calcium trihydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Enantiomeric purity (see Tests).

C. Water (see Tests).

D. Ignite. The residue gives reaction (b) of calcium (*2.3.1*). Filtration may be necessary in case the residue does not completely dissolve.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *anhydrous ethanol R*, *methanol R* (50:50 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 4 mL of the solvent mixture and dilute to 10.0 mL with *hexane R*.

*Reference solution (a).* Dissolve 2 mg of *atorvastatin impurity E CRS* in *methanol R* and dilute to 20.0 mL with the same solvent (solution A). Dissolve 10 mg of the substance to be examined in 1.25 mL of *methanol R*, add 0.75 mL of solution A and 2 mL of *anhydrous ethanol R* and dilute to 10.0 mL with *hexane R*.

*Reference solution (b).* To 2.0 mL of the test solution add 40.0 mL of the solvent mixture and dilute to 100.0 mL with *hexane R*. To 3.0 mL of this solution add 5 mL of the solvent mixture and dilute to 20.0 mL with *hexane R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *amylose derivative of silica gel for chromatography R* (10 µm).

*Mobile phase*: *trifluoroacetic acid R*, *anhydrous ethanol R*, *hexane R* (0.1:6:94 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 244 nm.

*Injection*: 20 µL.

*Run time*: 1.2 times the retention time of atorvastatin.

*Relative retention* with reference to atorvastatin (retention time = about 44 min): impurity E = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity E and atorvastatin.

*Limit*:

– *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 40.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dissolve 50 mg of the substance to be examined in *dimethylformamide R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 40.0 mg of *atorvastatin calcium trihydrate CRS* in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (b) to 100.0 mL with *dimethylformamide R*. Dilute 1.0 mL of this solution to 10.0 mL with *dimethylformamide R*.

*Reference solution (c).* Dissolve 2.5 mg of *atorvastatin impurity A CRS*, 2.5 mg of *atorvastatin impurity B CRS*, 2.5 mg of *atorvastatin impurity C CRS*, 2.5 mg of *atorvastatin impurity D CRS* and 2.5 mg of the substance to be examined in *dimethylformamide R* and dilute to 50.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: *tetrahydrofuran R*, *acetonitrile R*, 3.9 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R* (12:21:67 *V/V/V*);

– *mobile phase B*: *tetrahydrofuran R*, 3.9 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R*, *acetonitrile R* (12:27:61 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 100 | 0 |
| 40 - 70 | 100 → 20 | 0 → 80 |
| 70 - 85 | 20 → 0 | 80 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 244 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to atorvastatin (retention time = about 33 min): impurity A = about 0.8; impurity B = about 0.9; impurity C = about 1.2; impurity D = about 2.1.

If necessary, adjust the mobile phase by increasing or decreasing the percentage of acetonitrile or the pH of the ammonium acetate solution to achieve a retention time of about 33 min for atorvastatin. For example, raising the pH would decrease the retention time of atorvastatin.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity B and atorvastatin.

*Limits*:

– *impurities A, B*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *impurities C, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to dimethylformamide.

**Sodium**: maximum 0.4 per cent (anhydrous substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Solvent mixture*: *hydrochloric acid R*, *water R*, *methanol R* (2:25:75 *V/V/V*).

*Test solution.* Dissolve 5.0 mg in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (50 ppm Na) R*, diluting with the solvent mixture.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 589.0 nm.

*Atomisation device*: air-acetylene flame.

**Water** (*2.5.12*): 3.5 per cent to 5.5 per cent, determined on 0.130 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage content of $C_{66}H_{68}CaF_2N_4O_{10}$ from the declared content of *atorvastatin calcium trihydrate CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G, H.*

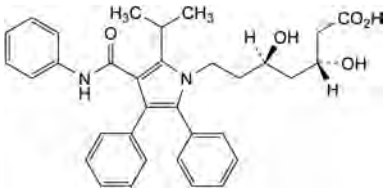

A. (3*R*,5*R*)-3,5-dihydroxy-7-[5-(1-methylethyl)-2,3-diphenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]heptanoic acid (desfluoroatorvastatin),

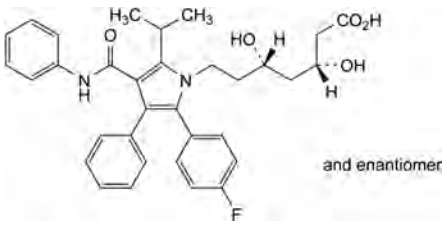


B. (3*RS*,5*SR*)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid,

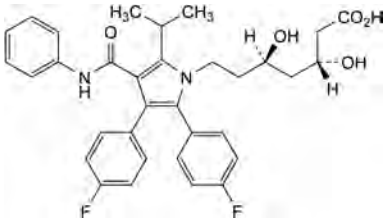

C. (3*R*,5*R*)-7-[2,3-bis(4-fluorophenyl)-5-(1-methylethyl)-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid (fluoroatorvastatin),

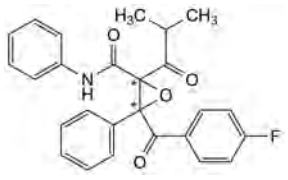

D. 3-[(4-fluorophenyl)carbonyl]-2-(2-methylpropanoyl)-*N*,3-diphenyloxirane-2-carboxamide,

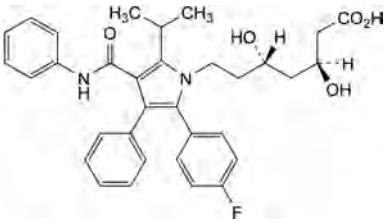

E. (3*S*,5*S*)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid (*ent*-atorvastatin),

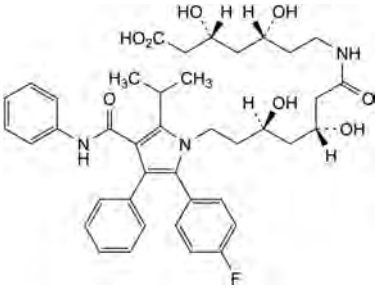

F. (3*R*,5*R*)-7-[[(3*R*,5*R*)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-3,5-dihydroxyheptanoyl]amino]-3,5-dihydroxyheptanoic acid,

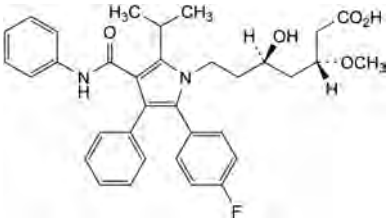

G. (3*R*,5*R*)-7-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]-5-hydroxy-3-methoxyheptanoic acid (3-*O*-methylatorvastatin),

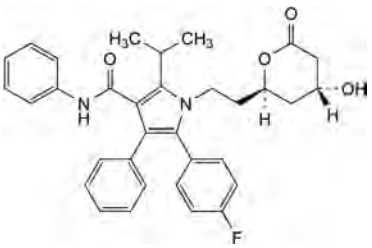

H. (4*R*,6*R*)-6-[2-[2-(4-fluorophenyl)-5-(1-methylethyl)-3-phenyl-4-(phenylcarbamoyl)-1*H*-pyrrol-1-yl]ethyl]-4-hydroxytetrahydro-2*H*-pyran-2-one.

**07/2013:2192**

# ATOVAQUONE

## Atovaquonum

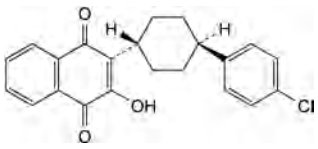

$C_{22}H_{19}ClO_3$ $M_r$ 366.8

[95233-18-4]

DEFINITION

2-[*trans*-4-(4-Chlorophenyl)cyclohexyl]-3-hydroxynaphthalene-1,4-dione.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *atovaquone CRS*.

If the spectra obtained show differences, dissolve 0.1 g of the substance to be examined and 0.1 g of the reference substance separately in 2.5 mL of a 50 g/L solution of *potassium hydroxide R* in *methanol R*. Filter the solutions and add each filtrate dropwise to a mixture of 0.8 mL of *acetic acid R* and 1.5 mL of *methanol R*, stirring continuously. Filter, wash the residues with *methanol R* and then with *water R*, and dry under vacuum at 55 °C. Record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *water R*, *acetonitrile R1* (20:80 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 25.0 mg of *atovaquone CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2.5 mg of *atovaquone for system suitability CRS* (containing impurities B and C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *phosphoric acid R*, *methanol R2*, *water for chromatography R*, *acetonitrile R1* (0.5:17.5:30:52.5 *V/V/V/V*).

*Flow rate*: 2.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of atovaquone.

*Identification of impurities*: use the chromatogram supplied with *atovaquone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to atovaquone (retention time = about 15 min): impurity B = about 0.85; impurity C = about 0.90.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity C and atovaquone;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of atovaquone in reference solution (c).

*Limits*:

- *impurity B*: maximum 0.5 per cent;
- *impurity C*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 0.100 g using the evaporation technique:

- *temperature*: 160 °C;
- *heating time*: 3 min;
- *flow rate*: 50 mL/min.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{19}ClO_3$ taking into account the assigned content of *atovaquone CRS*.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D*.

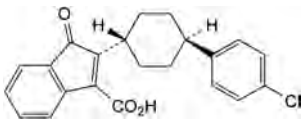


A. 2-[*trans*-4-(4-chlorophenyl)cyclohexyl]-1-oxo-1*H*-indene-3-carboxylic acid,

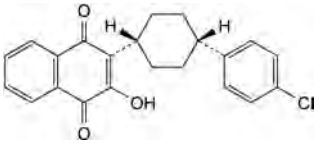


B. 2-[*cis*-4-(4-chlorophenyl)cyclohexyl]-3-hydroxynaphthalene-1,4-dione,

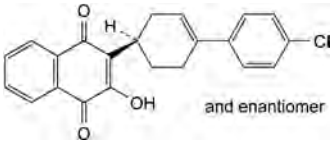


C. 2-[(1*RS*)-4-(4-chlorophenyl)cyclohex-3-en-1-yl]-3-hydroxynaphthalene-1,4-dione,

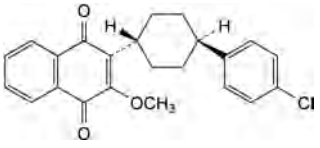


D. 2-[*trans*-4-(4-chlorophenyl)cyclohexyl]-3-methoxynaphthalene-1,4-dione.

04/2016:1970

# ATRACURIUM BESILATE

## Atracurii besilas

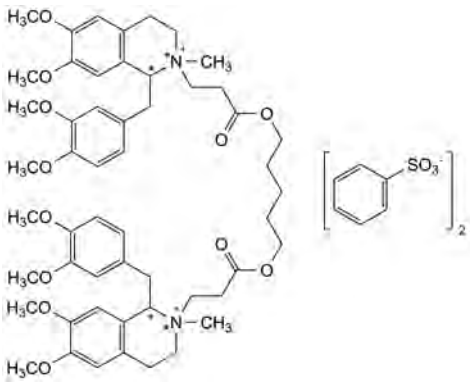

$C_{65}H_{82}N_2O_{18}S_2$ $M_r$ 1243
[64228-81-5]

DEFINITION

Mixture of the *cis-cis*, *cis-trans* and *trans-trans* isomers of 2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-1,3-diyl)]]bis[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzenesulfonate.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl benzenesulfonate esters are genotoxic and are potential impurities in atracurium besilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general method *2.5.41. Methyl, ethyl and isopropyl benzenesulfonate in active substances* is available to assist manufacturers.

CHARACTERS

*Appearance*: white or yellowish-white, slightly hygroscopic powder.

*Solubility*: soluble in water, very soluble in acetonitrile, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *atracurium besilate CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the 3 principal isomeric peaks in the chromatogram obtained with test solution (a) are similar in retention time to those in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.00 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Test solution (b).* Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 50.0 mg of *atracurium besilate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 20.0 mg of *methyl benzenesulfonate R* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 50 µL of the solution to 100.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 2.0 mg of *atracurium for peak identification CRS* (containing impurities A1, A2, B, C1, C2, D1, D2, E, G and K) in 2.0 mL of mobile phase A.

*Reference solution (e).* Dissolve 2.0 mg of *atracurium for impurity F identification CRS* in 2.0 mL of mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 5 volumes of *methanol R*, 20 volumes of *acetonitrile R* and 75 volumes of a 10.2 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.1 with *phosphoric acid R*;
– *mobile phase B*: mix 20 volumes of *acetonitrile R*, 30 volumes of *methanol R* and 50 volumes of a 10.2 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.1 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 15 | 80 → 40 | 20 → 60 |
| 15 - 25 | 40 | 60 |
| 25 - 30 | 40 → 0 | 60 → 100 |
| 30 - 45 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (d) and (e).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) and the chromatogram supplied with *atracurium for peak identification CRS* to identify the peaks due to impurities A1, A2, B, C1, C2, D1, D2, E, G and K; use the chromatogram obtained with reference solution (e) and the chromatogram supplied with *atracurium for impurity F identification CRS* to identify the peak due to impurity F.

*Relative retention* with reference to the atracurium *cis-cis* isomer (retention time = about 30 min): impurity E = about 0.2; impurity F = about 0.25; impurity G = about 0.3; impurity D1 = about 0.45; impurity D2 = about 0.5; atracurium *trans-trans* isomer = about 0.8; atracurium *cis-trans* isomer = about 0.9; impurity A1 = about 1.04; impurity I1 = about 1.07; impurity H1 = about 1.07 (shoulder on the front of peak A2); impurity A2 (major isomer) = about 1.08; impurity K1 = about 1.09 (shoulder on the tail of peak A2); impurity I2 (major isomer) = about 1.12; impurity H2 (major isomer) = about 1.12; impurity K2 (major isomer) = about 1.12; impurity B = about 1.15; impurity C1 = about 1.2; impurity C2 (major isomer) = about 1.3.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to the atracurium *trans-trans* isomer and the atracurium *cis-trans* isomer, and minimum 1.5 between the peaks due to the atracurium *cis-trans* isomer and the atracurium *cis-cis* isomer in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity A1 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the atracurium *cis-cis* isomer in the chromatogram obtained with reference solution (d).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity G by 0.5;
- *impurity E*: not more than 1.5 times the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *impurities A, D*: for each impurity, for the sum of the areas of the 2 isomer peaks, not more than 1.5 times the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *impurity C*: for the sum of the areas of the 2 isomer peaks, not more than the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurities F, G*: for each impurity, not more than the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurities H, I, K*: for the sum of the areas of the isomer peaks of these impurities, not more than the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3.5 times the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (3.5 per cent);
- *disregard limit*: 0.05 times the sum of the areas of the peaks due to the atracurium *cis-cis*, *trans-trans* and *cis-trans* isomers in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity J**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 15 | 80 → 75 | 20 → 25 |
| 15 - 25 | 75 | 25 |
| 25 - 30 | 75 → 55 | 25 → 45 |
| 30 - 38 | 55 → 0 | 45 → 100 |
| 38 - 45 | 0 | 100 |

*Detection*: spectrophotometer at 217 nm.

*Injection*: 100 µL of test solution (b) and reference solution (c).

*Retention time*: impurity J = about 25 min; atracurium *trans-trans* isomer = about 38 min.

*Limit*:

- *impurity J*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (10 ppm).

**Isomer composition**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification. Use the normalisation procedure.

*Injection*: test solution (a).

*Limits*:

- *atracurium cis-cis isomer*: 55.0 per cent to 60.0 per cent,
- *atracurium cis-trans isomer*: 34.5 per cent to 38.5 per cent,
- *atracurium trans-trans isomer*: 5.0 per cent to 6.5 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

## ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage content of $C_{65}H_{82}N_2O_{18}S_2$ from the sum of the areas of the peaks due to the 3 isomers.

## STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

## IMPURITIES

*Specified impurities: A, C, D, E, F, G, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

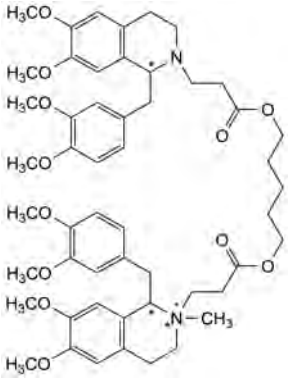

A. 1-(3,4-dimethoxybenzyl)-2-[13-[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]-3,11-dioxo-4,10-dioxatridecyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium (A1 = *trans* isomer, A2 = *cis* isomer),

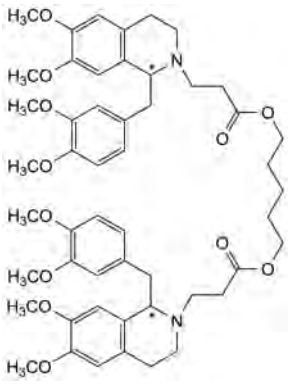

B. pentane-1,5-diyl bis[3-[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propanoate],

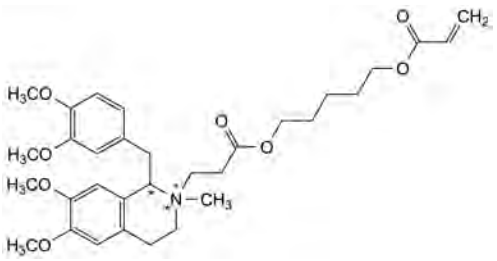

C. 1-(3,4-dimethoxybenzyl)-2-(3,11-dioxo-4,10-dioxatridec-12-enyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium (C1 = *trans* isomer, C2 = *cis* isomer),

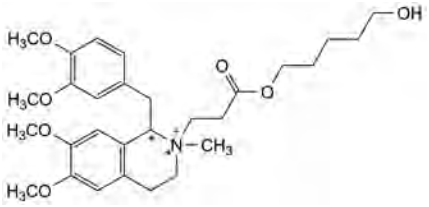

D. 1-(3,4-dimethoxybenzyl)-2-[3-[(5-hydroxypentyl)oxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium (D1 = *trans* isomer, D2 = *cis* isomer),

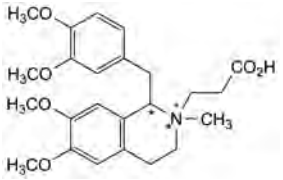

E. 2-(2-carboxyethyl)-1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

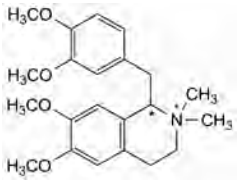

F. 1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium,

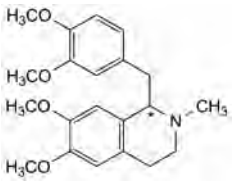

G. 1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline,

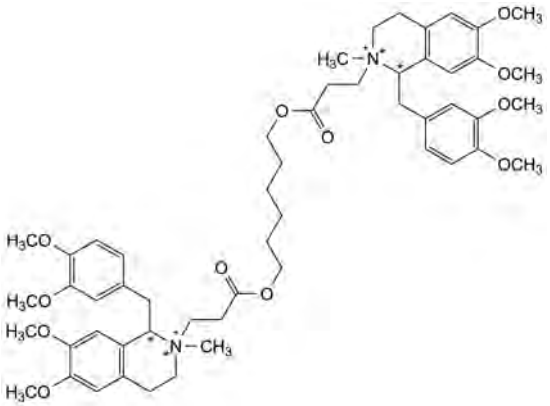

H. 2,2′-[hexane-1,6-diylbis[oxy(3-oxopropane-1,3-diyl)]]bis[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium] (H1 = *cis-trans* isomer, H2 = *cis-cis* isomer),

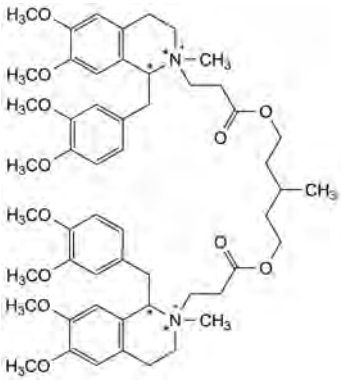

I. 2,2′-[(3-methylpentane-1,5-diyl)bis[oxy(3-oxopropane-1,3-diyl)]]bis[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium] (I1 = *cis-trans* isomer, I2 = *cis-cis* isomer),

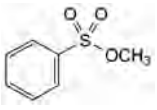

J. methyl benzenesulfonate,

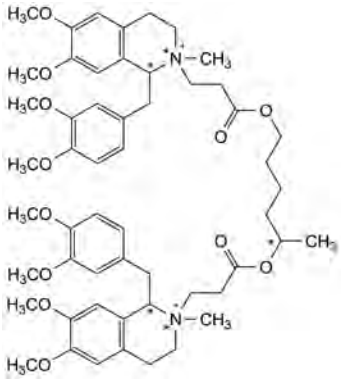

K. 2,2′-[hexane-1,5-diylbis[oxy(3-oxopropane-1,3-diyl)]]bis[1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium].

**07/2010:2056**
**corrected 10.0**

# ATROPINE

## Atropinum

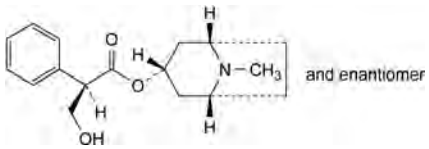


$C_{17}H_{23}NO_3$ $M_r$ 289.4
[51-55-8]

DEFINITION

(1*R*,3*R*,5*S*)-8-Methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *A, B, E.*

*Second identification*: *A, C, D, E.*

A. Melting point (*2.2.14*): 115 °C to 119 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *atropine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution*. Dissolve 10 mg of *atropine CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).
*Application*: 10 µL.
*Development*: over half of the plate.
*Drying*: at 100-105 °C for 15 min.
*Detection*: after cooling, spray with *dilute potassium iodobismuthate solution R*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Place about 3 mg in a porcelain crucible and add 0.2 mL of *fuming nitric acid R*. Evaporate to dryness on a water-bath. Dissolve the residue in 0.5 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*; a violet colour develops.

E. Optical rotation (see Tests).

TESTS

**Optical rotation** (*2.2.7*): − 0.70° to + 0.05° (measured in a 2 dm tube).

Dissolve 1.25 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 24 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 5 mg of *atropine impurity B CRS* in the test solution and dilute to 20 mL with the test solution. Dilute 5 mL of this solution to 25 mL with mobile phase A.

*Reference solution (c)*. Dissolve the contents of a vial of *atropine for peak identification CRS* (containing impurities A, D, E, F, G and H) in 1 mL of mobile phase A.

*Reference solution (d)*. Dissolve 5 mg of *tropic acid R* (impurity C) in mobile phase A and dilute to 10 mL with mobile phase A. Dilute 1 mL of the solution to 100 mL with mobile phase A. Dilute 1 mL of this solution to 10 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 3.5 g of *sodium dodecyl sulfate R* in 606 mL of a 7.0 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with a 5.8 g/L solution of *phosphoric acid R*, and mix with 320 mL of *acetonitrile R1*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 | 5 |
| 2 - 20 | 95 → 70 | 5 → 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *atropine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, D, E, F, G and H; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to atropine (retention time = about 11 min): impurity C = about 0.2; impurity E = about 0.67; impurity D = about 0.73; impurity F = about 0.8; impurity B = about 0.89; impurity H = about 0.93; impurity G = about 1.1; impurity A = about 1.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.5 between the peaks due to impurity B and atropine.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity C = 0.6;
- *impurities E, H*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, B, C, D, F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

ASSAY

Dissolve 0.250 g in 40 mL of *anhydrous acetic acid R*, heating if necessary, and allow to cool. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.94 mg of $C_{17}H_{23}NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

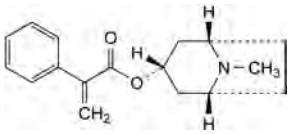

A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 2-phenylpropenoate (apoatropine),

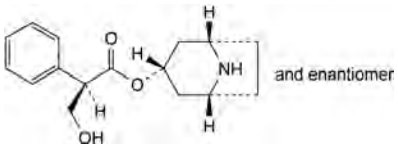


B. (1*R*,3*r*,5*S*)-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate (noratropine),

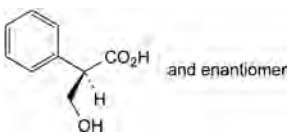


C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (tropic acid),

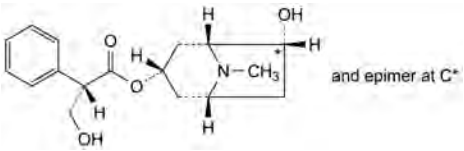


D. (1*R*,3*S*,5*R*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo-[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (6-hydroxyhyoscyamine),

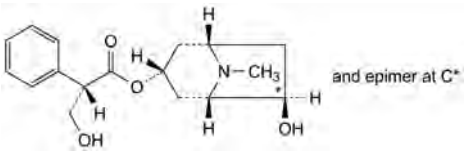


E. (1*S*,3*R*,5*S*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo-[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (7-hydroxyhyoscyamine),

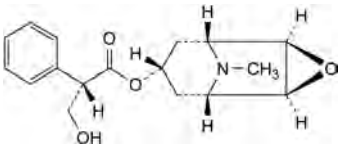

F. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo-[3.3.1.$0^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscine),

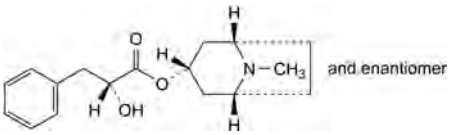


G. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-2-hydroxy-3-phenylpropanoate (littorine),

H. unknown structure.

**04/2008:0068**
**corrected 10.0**

# ATROPINE SULFATE

## Atropini sulfas

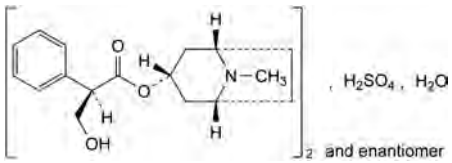


$C_{34}H_{48}N_2O_{10}S,H_2O$ $M_r$ 695

[5908-99-6]

DEFINITION

Bis[(1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate] sulfate monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: C, D, E, F.*

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *atropine sulfate CRS*.

C. Dissolve about 50 mg in 5 mL of *water R* and add 5 mL of *picric acid solution R*. The precipitate, washed with *water R* and dried at 100-105 °C for 2 h, melts (*2.2.14*) at 174 °C to 179 °C.

D. To about 1 mg add 0.2 mL of *fuming nitric acid R* and evaporate to dryness in a water-bath. Dissolve the residue in 2 mL of *acetone R* and add 0.1 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives the reactions of sulfates (*2.3.1*).

F. It gives the reaction of alkaloids (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.5 to 6.2.

Dissolve 0.6 g in *carbon dioxide-free water R* and dilute to 30 mL with the same solvent.

**Optical rotation** (*2.2.7*): – 0.50° to + 0.05° (measured in a 2 dm tube).

Dissolve 2.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 24 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *atropine impurity B CRS* in the test solution and dilute to 20 mL with the test solution. Dilute 5 mL of this solution to 25 mL with mobile phase A.

*Reference solution (c).* Dissolve the contents of a vial of *atropine for peak identification CRS* (containing impurities A, D, E, F, G and H) in 1 mL of mobile phase A.

*Reference solution (d).* Dissolve 5 mg of *tropic acid R* (impurity C) in mobile phase A and dilute to 10 mL with mobile phase A. Dilute 1 mL of the solution to 100 mL with mobile phase A. Dilute 1 mL of this solution to 10 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: dissolve 3.5 g of *sodium dodecyl sulfate R* in 606 mL of a 7.0 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with a 5.8 g/L solution of *phosphoric acid R*, and mix with 320 mL of *acetonitrile R1*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 | 5 |
| 2 - 20 | 95 → 70 | 5 → 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *atropine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, D, E, F, G and H; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to atropine (retention time = about 11 min): impurity C = about 0.2; impurity E = about 0.67; impurity D = about 0.73; impurity F = about 0.8; impurity B = about 0.89; impurity H = about 0.93; impurity G = about 1.1; impurity A = about 1.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurity B and atropine.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity C = 0.6;

– *impurities E, H*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *impurities A, B, C, D, F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 2.0 per cent to 4.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 30 mL of *anhydrous acetic acid R*, warming if necessary. Cool the solution. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 67.68 mg of $C_{34}H_{48}N_2O_{10}S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

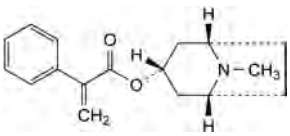


A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 2-phenylpropenoate (apoatropine),

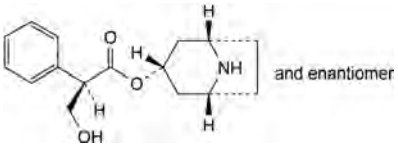


B. (1*R*,3*r*,5*S*)-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate (noratropine),

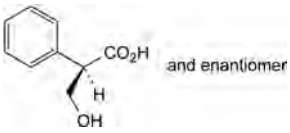


C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (tropic acid),

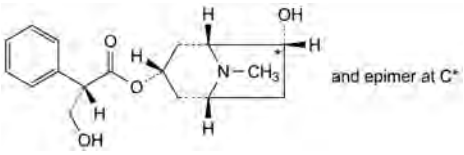


D. (1*R*,3*S*,5*R*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo-[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (6-hydroxyhyoscyamine),

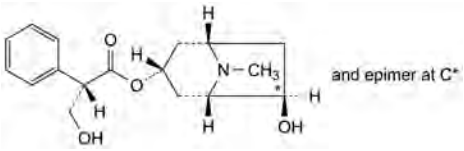


E. (1*S*,3*R*,5*S*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (7-hydroxyhyoscyamine),

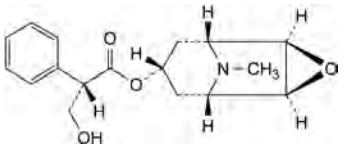

F. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscine),

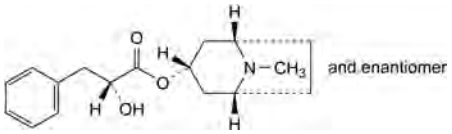


G. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-2-hydroxy-3-phenylpropanoate (littorine),

H. unknown structure.

**04/2010:1708**
**corrected 7.0**

# AZAPERONE FOR VETERINARY USE

## Azaperonum ad usum veterinarium

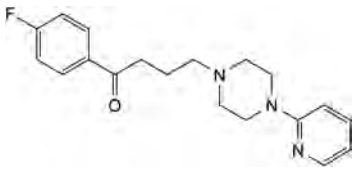

$C_{19}H_{22}FN_3O$ $M_r$ 327.4
[1649-18-9]

DEFINITION

1-(4-Fluorophenyl)-4-[4-(pyridin-2-yl)piperazin-1-yl]butan-1-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *azaperone CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in 25 mL of a 14 g/L solution of *tartaric acid R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *azaperone CRS* and 6.0 mg of *benperidol CRS* in *methanol R* and dilute to 200.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of the solution to 20.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.4 g of *anhydrous sodium sulfate R* in 900 mL of *water R*, add 16.0 mL of *0.01 M sulfuric acid* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 95 → 20 | 5 → 80 |
| 15 - 20 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to azaperone (retention time = about 9 min): impurity A = about 0.9; impurity B = about 1.1; impurity C = about 1.15.

*System suitability*: reference solution (a):

- *resolution*: minimum 8.0 between the peaks due to azaperone and to benperidol.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *sum of impurities B and C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 70 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 16.37 mg of $C_{19}H_{22}FN_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

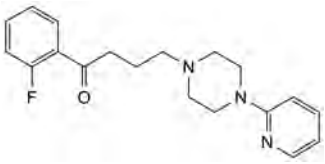

A. 1-(2-fluorophenyl)-4-[4-(pyridin-2-yl)piperazin-1-yl]butan-1-one,

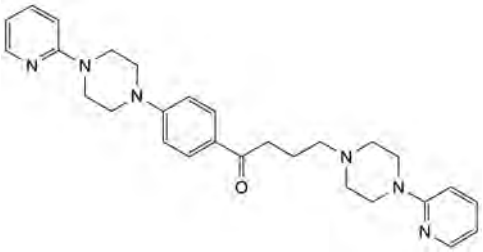

B. 4-[4-(pyridin-2-yl)piperazin-1-yl]-1-[4-[4-(pyridin-2-yl)piperazin-1-yl]phenyl]butan-1-one,

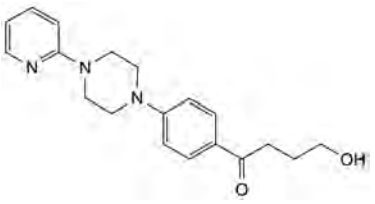

C. 4-hydroxy-1-[4-[4-(pyridin-2-yl)piperazin-1-yl]phenyl]butan-1-one.

**07/2010:0369**
**corrected 7.0**

# AZATHIOPRINE

## Azathioprinum

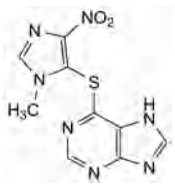

$C_9H_7N_7O_2S$ $M_r$ 277.3
[446-86-6]

DEFINITION

6-[(1-Methyl-4-nitro-1*H*-imidazol-5-yl)sulfanyl]-7*H*-purine.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale-yellow powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It is soluble in dilute solutions of alkali hydroxides and sparingly soluble in dilute mineral acids.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *azathioprine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. 2.76 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Test solution.* Dissolve 10 mg of the substance to be examined in 35 mL of a 0.8 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with solution A.

*Reference solution (a).* Dissolve 5 mg of *azathioprine impurity A CRS* and 5 mg of *mercaptopurine R* (impurity B) in 8.75 mL of a 0.8 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with solution A. To 1.0 mL of this solution, add 35 mL of a 0.8 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with solution A.

*Reference solution (b).* Dissolve 2.5 mg of *azathioprine impurity G CRS* and 2.5 mg of the substance to be examined in 8.8 mL of a 0.8 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with solution A. To 1.0 mL of this solution, add 17.5 mL of a 0.8 g/L solution of *sodium hydroxide R* and dilute to 50.0 mL with solution A.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, solution A (5:95 *V/V*);
- *mobile phase B*: solution A, *methanol R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 15 | 100 → 0 | 0 → 100 |
| 15 - 20 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B. Use the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to azathioprine (retention time = about 15 min): impurity A = about 0.3; impurity B = about 0.4; impurity G = about 0.97.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurities A and B in the chromatogram obtained with reference solution (a); minimum 2.0 between the peaks due to impurity G and azathioprine in the chromatogram obtained with reference solution (b).

*Limits*:

- *impurities A, B*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 27.73 mg of $C_9H_7N_7O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G.*

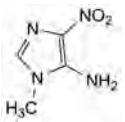

A. 1-methyl-4-nitro-1*H*-imidazol-5-amine,

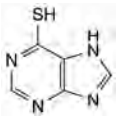

B. 7*H*-purine-6-thiol (mercaptopurine),

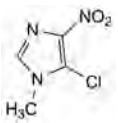

C. 5-chloro-1-methyl-4-nitro-1*H*-imidazole,

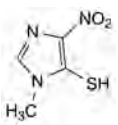

D. 1-methyl-4-nitro-1*H*-imidazole-5-thiol,

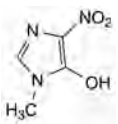

E. 1-methyl-4-nitro-1*H*-imidazol-5-ol,

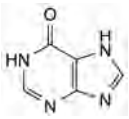

F. 1,7-dihydro-6*H*-purin-6-one (hypoxanthine),

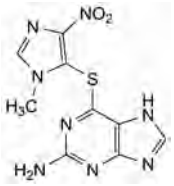

G. 6-[(1-methyl-4-nitro-1*H*-imidazol-5-yl)sulfanyl]-7*H*-purin-2-amine (thiamiprine).

**04/2016:1633**
**corrected 10.0**

# AZELASTINE HYDROCHLORIDE

## Azelastini hydrochloridum

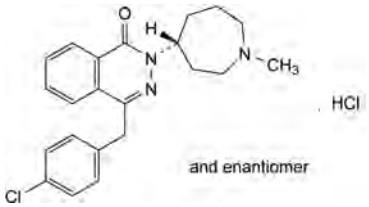


$C_{22}H_{25}Cl_2N_3O$ $M_r$ 418.4
[79307-93-0]

DEFINITION

4-[(4-Chlorophenyl)methyl]-2-[(4*RS*)-1-methylhexahydro-1*H*-azepin-4-yl]phthalazin-1(2*H*)-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *azelastine hydrochloride CRS*.

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *bromothymol blue solution R1*. Not more than 0.1 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (45:55 *V/V*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 1 mg of *benzohydrazide R* (impurity A), 1 mg of *azelastine impurity B CRS*, 1 mg of *2-[2-(4-chlorophenyl)acetyl]benzoic acid R* (impurity C), 1 mg of *azelastine impurity D CRS* and 1 mg of *azelastine impurity E CRS* in the test solution and dilute to 20.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cyanosilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 2.16 g of *sodium octanesulfonate R* and 0.68 g of *potassium dihydrogen phosphate R* in 740 mL of *water for chromatography R*; adjust to pH 3.0-3.1 with *dilute phosphoric acid R*, add 260 mL of *acetonitrile R1* and mix.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of azelastine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E. The elution order may vary, especially for impurity E, but the peak area of each impurity in reference solution (b) is different, so a clear identification of the impurities is possible.

*Relative retention* with reference to azelastine (retention time = about 10 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.4; impurity D = about 0.6; impurity E = about 0.8.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity E and azelastine and minimum 1.5 between the peaks due to impurities C and D in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 90 for the principal peak in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.6; impurity B = 4.5; impurity C = 2.0; impurity E = 2.8;
- for each impurity, use the concentration of azelastine hydrochloride in reference solution (a).

*Limits*:

- *impurities A, B, C, E*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.300 g in 5 mL of *anhydrous formic acid R*. Add 30 mL of *acetic anhydride R*. Titrate quickly with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1.0 mL of *0.1 M perchloric acid* is equivalent to 41.84 mg of $C_{22}H_{25}Cl_2N_3O$.

IMPURITIES

*Specified impurities: A, B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

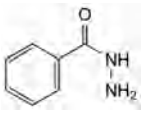

A. benzohydrazide (benzoyldiazane),

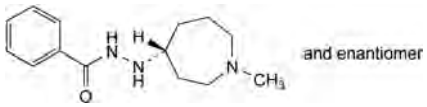


B. *N'*-[(4*RS*)-1-methylhexahydro-1*H*-azepin-4-yl]-benzohydrazide,

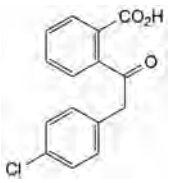

C. 2-[2-(4-chlorophenyl)acetyl]benzoic acid,

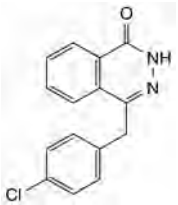

D. 4-[(4-chlorophenyl)methyl]phthalazin-1(2*H*)-one,

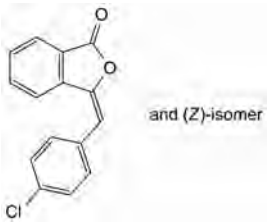


E. (3*E*)-3-[(4-chlorophenyl)methylidene]-2-benzofuran-1(3*H*)-one.

**01/2018:1649 corrected 9.6**

# AZITHROMYCIN

## Azithromycinum

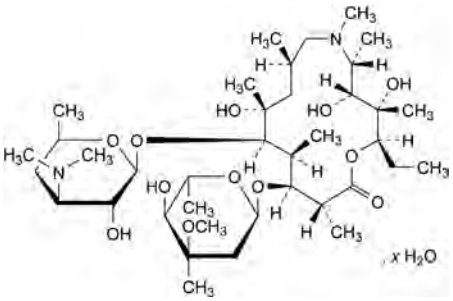

$C_{38}H_{72}N_2O_{12},xH_2O$ $M_r$ 749 (anhydrous substance)
with $x$ = 1 or 2

Azithromycin monohydrate: [121470-24-4]
Azithromycin dihydrate: [117772-70-0]

DEFINITION

(2*R*,3*S*,4*R*,5*R*,8*R*,10*R*,11*R*,12*S*,13*S*,14*R*)-13-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one. The degree of hydration is 1 or 2.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *azithromycin CRS*.

If the spectra obtained in the solid state show differences, prepare further spectra using 90 g/L solutions in *methylene chloride R*.

TESTS

**Solution S**. Dissolve 0.500 g in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.0 to 11.0.

Dissolve 0.100 g in 25.0 mL of *methanol R* and dilute to 50.0 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 49 to − 45 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Prepare a 1.73 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 10.0 with *ammonia R*. Transfer 350 mL of this solution to a suitable container. Add 300 mL of *acetonitrile R* and 350 mL of *methanol R*. Mix well.

*Test solution*. Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *azithromycin for system suitability CRS* (containing impurities F, H and J) in 1.0 mL of the solvent mixture and sonicate for 5 min.

*Reference solution (c)*. Dissolve 8.0 mg of *azithromycin for peak identification CRS* (containing impurities A, B, C, E, F, G, I, J, L, M, N, O and P) in 1.0 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (5 μm);
- *temperature*: 60 °C.

*Mobile phase*:
- *mobile phase A*: 1.80 g/L solution of *anhydrous disodium hydrogen phosphate R* adjusted to pH 8.9 with *dilute phosphoric acid R* or with *dilute sodium hydroxide solution R*;
- *mobile phase B*: *methanol R2, acetonitrile R1* (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 50 → 45 | 50 → 55 |
| 25 - 30 | 45 → 40 | 55 → 60 |
| 30 - 80 | 40 → 25 | 60 → 75 |
| 80 - 81 | 25 → 50 | 75 → 50 |
| 81 - 93 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 μL.

*Identification of impurities*: use the chromatogram supplied with *azithromycin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, E, F, G, I, J, L, M, N, O and P; use the chromatogram supplied with *azithromycin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity H.

*Relative retention* with reference to azithromycin (retention time = 45-50 min): impurity L = about 0.29; impurity M = about 0.37; impurity E = about 0.43; impurity F = about 0.51; impurity D = about 0.54; impurity J = about 0.54; impurity Q = about 0.54; impurity I = about 0.61; impurity C = about 0.73; impurity N = about 0.76; impurity H = about 0.79; impurity A = about 0.83; impurity P = about 0.92; impurity O = about 1.23; impurity G = about 1.26; impurity B = about 1.31.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 1.4, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity F.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity F = 0.3; impurity G = 0.2; impurity H = 0.1; impurity L = 2.3; impurity M = 0.6; impurity N = 0.7;
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *impurities A, C, E, F, H, I, L, M, N, O, P*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *sum of impurities D, J and Q*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

- *impurity G*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard the peaks eluting before impurity L and after impurity B.

**Water** (*2.5.12*): 1.8 per cent to 6.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A*. Mix 60 volumes of *acetonitrile R* and 40 volumes of a 6.7 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 8.0 with *phosphoric acid R*.

*Test solution.* Dissolve 53.0 mg of the substance to be examined in 2 mL of *acetonitrile R* and dilute to 100.0 mL with solution A.

*Reference solution (a).* Dissolve 53.0 mg of *azithromycin CRS* in 2 mL of *acetonitrile R* and dilute to 100.0 mL with solution A.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *azithromycin impurity A CRS* in 0.5 mL of *acetonitrile R* and dilute to 10 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl vinyl polymer for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 60 volumes of *acetonitrile R1* and 40 volumes of a 6.7 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 11.0 with a 560 g/L solution of *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of azithromycin.

*Retention time*: azithromycin = about 10 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity A and azithromycin.

Calculate the percentage content of $C_{38}H_{72}N_2O_{12}$ taking into account the assigned content of *azithromycin CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J, L, M, N, O, P, Q.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *K.*

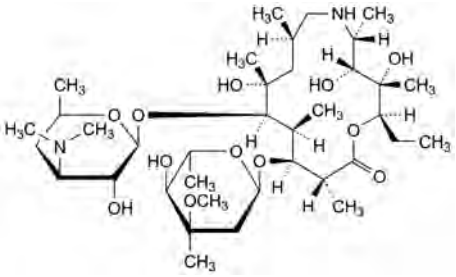

A. 6-demethylazithromycin,

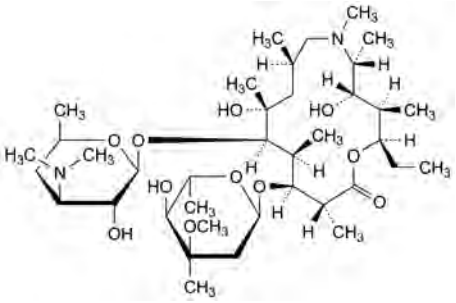

B. 3-deoxyazithromycin (azithromycin B),

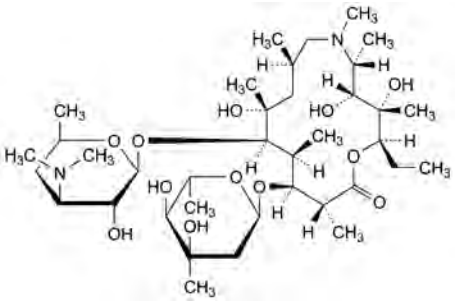

C. 3″-*O*-demethylazithromycin (azithromycin C),

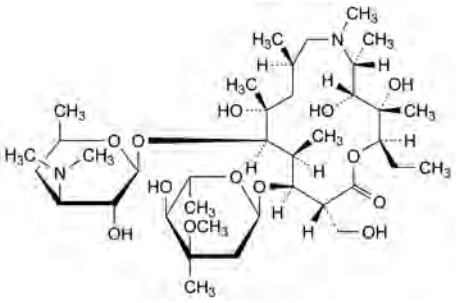

D. 14-demethyl-14-(hydroxymethyl)azithromycin (azithromycin F),

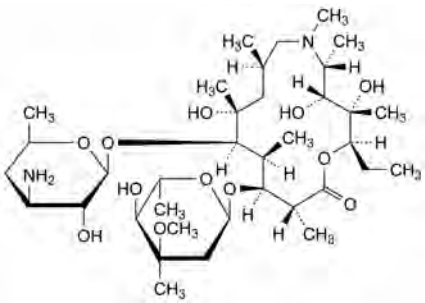

E. 3′-(*N*,*N*-didemethyl)azithromycin (aminoazithromycin),

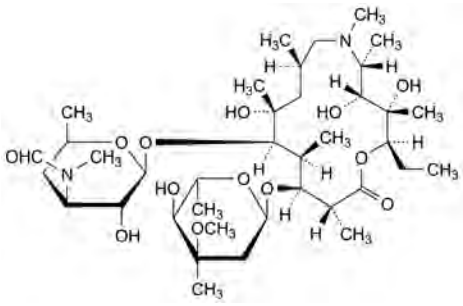

F. 3′-*N*-demethyl-3′-*N*-formylazithromycin,

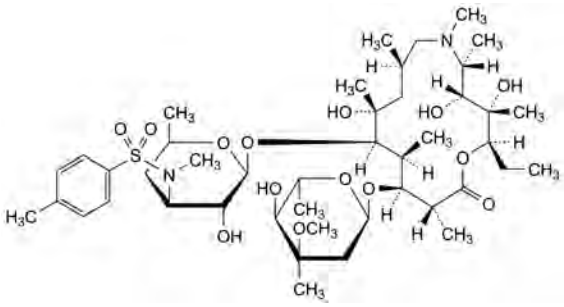

G. 3′-*N*-demethyl-3′-*N*-[(4-methylphenyl)sulfonyl]-azithromycin,

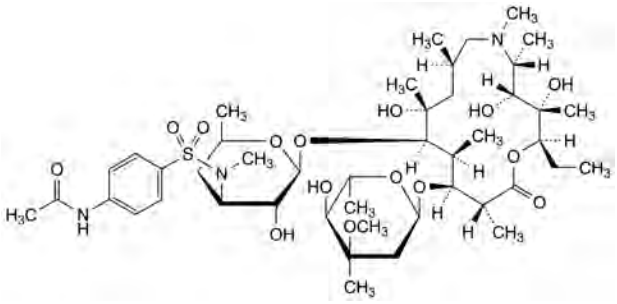

H. 3′-*N*-[[4-(acetylamino)phenyl]sulfonyl]-3′-*N*-demethylazithromycin,

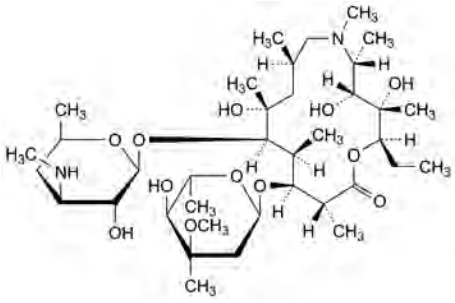

I. 3′-*N*-demethylazithromycin,

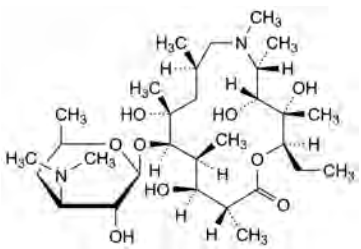

J. 13-*O*-decladinosylazithromycin,

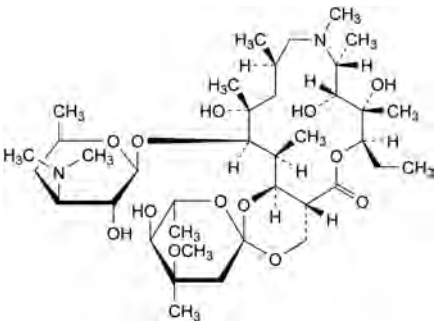

K. $C^{14}$,1″-epoxyazithromycin (azithromycin E),

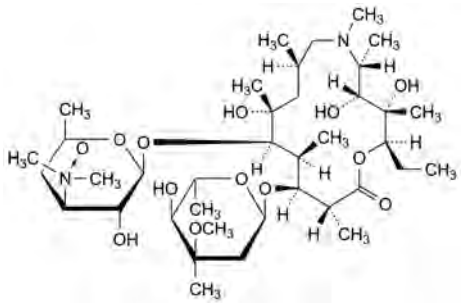

L. azithromycin 3′-*N*-oxide,

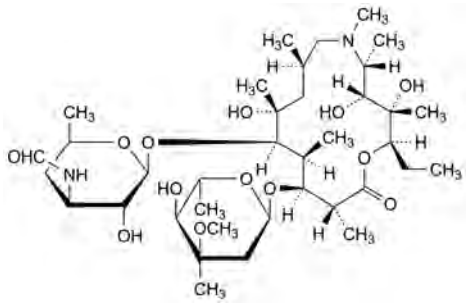

M. 3′-(*N*,*N*-didemethyl)-3′-*N*-formylazithromycin,

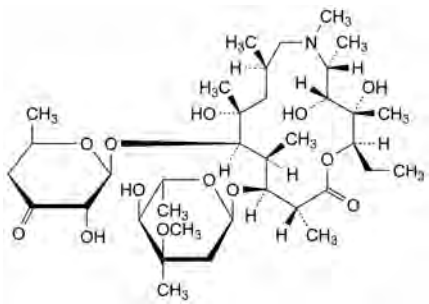

N. 3′-de(dimethylamino)-3′-oxoazithromycin,

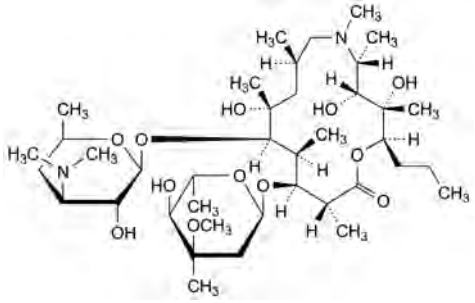

O. 2-desethyl-2-propylazithromycin,

P. unknown structure,

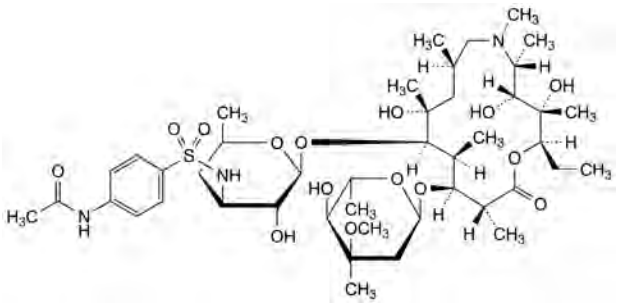

Q. 3′-*N*-[[4-(acetylamino)phenyl]sulfonyl]-3′-(*N*,*N*-didemethyl)azithromycin.

# B

Bacampicillin hydrochloride.. 1911
Bacitracin.. 1913
Bacitracin zinc.. 1917
Baclofen.. 1921
Bambuterol hydrochloride.. 1922
Barbital.. 1923
Barium sulfate.. 1924
Basic butylated methacrylate copolymer.. 1925
Beclometasone dipropionate.. 1926
Beclometasone dipropionate monohydrate.. 1929
Beeswax, white.. 1931
Beeswax, yellow.. 1932
Benazepril hydrochloride.. 1932
Bendroflumethiazide.. 1934
Benperidol.. 1935
Benserazide hydrochloride.. 1936
Bentonite.. 1938
Benzalkonium chloride.. 1938
Benzalkonium chloride solution.. 1940
Benzbromarone.. 1942
Benzethonium chloride.. 1943
Benzocaine.. 1944
Benzoic acid.. 1945
Benzoyl peroxide, hydrous.. 1946
Benzydamine hydrochloride.. 1947
Benzyl alcohol.. 1949
Benzyl benzoate.. 1950
Benzylpenicillin (benzathine) tetrahydrate.. 1951
Benzylpenicillin potassium.. 1953
Benzylpenicillin (procaine) monohydrate.. 1955
Benzylpenicillin sodium.. 1957
Betacarotene.. 1959
Betadex.. 1960
Betahistine dihydrochloride.. 1962
Betahistine mesilate.. 1963
Betamethasone.. 1964
Betamethasone acetate.. 1966
Betamethasone dipropionate.. 1968
Betamethasone sodium phosphate.. 1970
Betamethasone valerate.. 1971
Betaxolol hydrochloride.. 1973
Bezafibrate.. 1975
Bicalutamide.. 1976
Bifonazole.. 1977
Biotin.. 1979
Biperiden hydrochloride.. 1980
Bisacodyl.. 1982
Bismuth subcarbonate.. 1983
Bismuth subgallate.. 1984
Bismuth subnitrate, heavy.. 1985
Bismuth subsalicylate.. 1986
Bisoprolol fumarate.. 1986
Bleomycin sulfate.. 1989
Boldine.. 1990
Borage (starflower) oil, refined.. 1992
Borax.. 1992
Boric acid.. 1993
Botulinum toxin type A for injection.. 1993
Botulinum toxin type B for injection.. 1995
Bovine serum.. 1996
Brimonidine tartrate.. 1998
Bromazepam.. 1999
Bromhexine hydrochloride.. 2000
Bromocriptine mesilate.. 2001
Bromperidol.. 2003
Bromperidol decanoate.. 2005
Brompheniramine maleate.. 2007
Brotizolam.. 2008
Budesonide.. 2009
Bufexamac.. 2011
Buflomedil hydrochloride.. 2012
Bumetanide.. 2014
Bupivacaine hydrochloride.. 2015
Buprenorphine.. 2017
Buprenorphine hydrochloride.. 2019
Buserelin.. 2020
Buspirone hydrochloride.. 2022
Busulfan.. 2024
Butyl parahydroxybenzoate.. 2025
Butylhydroxyanisole.. 2026
Butylhydroxytoluene.. 2027

**01/2008:0808**
**corrected 6.1**

# BACAMPICILLIN HYDROCHLORIDE

## Bacampicillini hydrochloridum

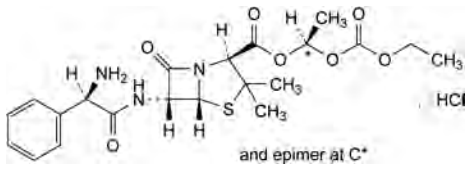


$C_{21}H_{28}ClN_3O_7S$ $M_r$ 502.0
[37661-08-8]

DEFINITION

(1*RS*)-1-[(Ethoxycarbonyl)oxy]ethyl (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate hydrochloride.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder or granules, hygroscopic.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent), soluble in methylene chloride.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bacampicillin hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of *methanol R.*

*Reference solution (a).* Dissolve 10 mg of *bacampicillin hydrochloride CRS* in 2 mL of *methanol R.*

*Reference solution (b).* Dissolve 10 mg of *bacampicillin hydrochloride CRS*, 10 mg of *talampicillin hydrochloride CRS* and 10 mg of *pivampicillin CRS* in 2 mL of *methanol R.*

*Plate*: *TLC silanised silica gel plate R.*

*Mobile phase*: mix 10 volumes of a 272 g/L solution of *sodium acetate R* adjusted to pH 5.0 with *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *ethanol (96 per cent) R.*

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: spray with *ninhydrin solution R1* and heat at 60 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R.* Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a dark yellow colour develops.

D. Dissolve about 25 mg in 2 mL of *water R.* Add 2 mL of *dilute sodium hydroxide solution R* and shake. Wait a few minutes and add 3 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R1.* A white precipitate is formed. Add 0.5 mL of *concentrated ammonia R.* The precipitate dissolves.

TESTS

**Appearance of solution**. Dissolve 0.200 g in 20 mL of *water R*; the solution is not more opalescent than reference suspension II (*2.2.1*). Dissolve 0.500 g in 10 mL of *water R*; the absorbance (*2.2.25*) of the solution at 430 nm is not greater than 0.10.

**pH** (*2.2.3*): 3.0 to 4.5.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 175 to + 195 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test solution and reference solutions (a), (b) and (d) immediately before use.*

*Phosphate buffer A.* Dissolve 1.4 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to about 800 mL with the same solvent. Adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000.0 mL with *water R.*

*Phosphate buffer B.* Dissolve 2.75 g of *sodium dihydrogen phosphate monohydrate R* and 2.3 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to about 1800 mL with the same solvent. Adjust to pH 6.8, if necessary, using *dilute phosphoric acid R* or *dilute sodium hydroxide solution R* and dilute to 2000.0 mL with *water R.*

*Test solution.* Dissolve 30.0 mg of the substance to be examined in phosphate buffer A and dilute to 100.0 mL with phosphate buffer A.

*Reference solution (a).* Dissolve 30.0 mg of *bacampicillin hydrochloride CRS* in phosphate buffer A and dilute to 100.0 mL with phosphate buffer A.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with phosphate buffer A.

*Reference solution (c).* Dissolve 30 mg of the substance to be examined in phosphate buffer B and dilute to 100 mL with phosphate buffer B. Heat at 80 °C for about 30 min.

*Reference solution (d).* Dissolve 20 mg of *ampicillin trihydrate CRS* (impurity I) in phosphate buffer A and dilute to 250 mL with phosphate buffer A. Dilute 5 mL of this solution to 100 mL with phosphate buffer A.

*Column*:

– *size*: *l* = 0.05 m, Ø = 3.9 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *acetonitrile R1* and 70 volumes of a 0.06 per cent *m/m* solution of *tetrahexylammonium hydrogen sulfate R* in phosphate buffer B.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3.5 times the retention time of bacampicillin.

*System suitability*:

– the peak due to impurity I is separated from the peaks due to the solvent in the chromatogram obtained with reference solution (d);

– *relative retention* with reference to bacampicillin: degradation product eluting just after bacampicillin = 1.12 to 1.38 in the chromatogram obtained with reference solution (c); if necessary, adjust the concentration of tetrahexylammonium hydrogen sulfate in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Butyl acetate and ethyl acetate** (*2.4.24, System A*): maximum 2.0 per cent of butyl acetate, maximum 4.0 per cent of ethyl acetate and maximum 5.0 per cent for the sum of the contents.

*Sample solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

Use the method of standard additions.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 60 °C;

– *equilibration time*: 20 min.

***N*,*N*-Dimethylaniline** (*2.4.26, Method A*): maximum 20 ppm.

**Water** (*2.5.12*): maximum 0.8 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{21}H_{28}ClN_3O_7S$ from the declared content of *bacampicillin hydrochloride CRS*.

STORAGE

In an airtight container.

IMPURITIES

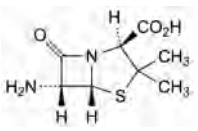

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

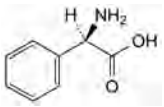

B. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

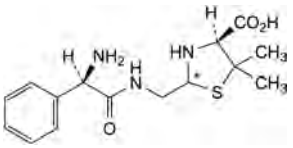

C. (2*RS*,4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ampicillin),

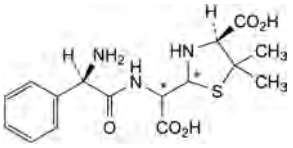

D. (4*S*)-2-[[[(2*R*)-2-amino-2-phenylacetyl]amino]-carboxymethyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ampicillin),

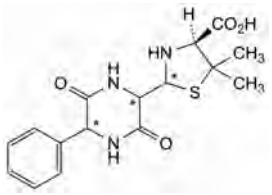

E. (4*S*)-2-(3,6-dioxo-5-phenylpiperazin-2-yl)-5,5-dimethylthiazolidine-4-carboxylic acid (diketopiperazines of ampicillin),

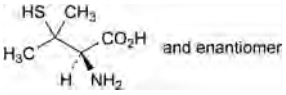


F. (2*RS*)-2-amino-3-methyl-3-sulfanylbutanoic acid (DL-penicillamine),

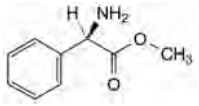

G. methyl (2*R*)-2-amino-2-phenylacetate (methyl D-phenylglycinate),

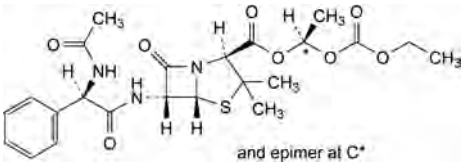


H. (1*RS*)-1-[(ethoxycarbonyl)oxy]ethyl (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-(acetylamino)-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (*N*-acetylbacampicillin),

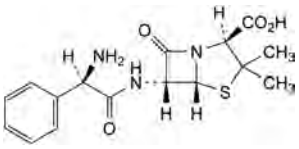

I. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ampicillin).

**04/2017:0465**
**corrected 10.0**

# BACITRACIN

## Bacitracinum

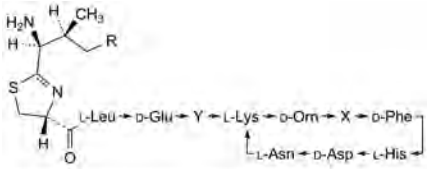


| Name | Mol. Formula | X | Y | R |
|---|---|---|---|---|
| Bacitracin A | $C_{66}H_{103}N_{17}O_{16}S$ | L-Ile | L-Ile | $CH_3$ |
| Bacitracin B1 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Ile | L-Ile | H |
| Bacitracin B2 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Val | L-Ile | $CH_3$ |
| Bacitracin B3 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Ile | L-Val | $CH_3$ |

[1405-87-4]

DEFINITION

Mixture of antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* or *Bacillus subtilis*, the main components being:

- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin A);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B1);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B2);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B3).

*Content*: minimum 60 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in a 3.4 g/L solution of *hydrochloric acid R* and dilute to 1.0 mL with the same solution.

*Reference solution.* Dissolve 10 mg of *bacitracin zinc CRS* in a 3.4 g/L solution of *hydrochloric acid R* and dilute to 1.0 mL with the same solution.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (14:29:57 *V/V/V*).

*Application*: 10 µL.

*Development*: over half of the plate.

*Drying*: at 100-105 °C.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position, size and colour to the spots in the chromatogram obtained with the reference solution.

B. Composition (see Tests).

C. Ignite 0.2 g. There is no significant yellow-coloured residue at high temperature. Allow to cool. Dissolve the residue in 0.1 mL of *dilute hydrochloric acid R*. Add 5 mL of *water R* and 0.2 mL of *strong sodium hydroxide solution R*. No white precipitate is formed.

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 6.0 to 7.0 for solution S.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Solution A*. 40 g/L solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide solution R*.

*Solution B*. In a volumetric flask, dissolve 54.4 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to 2000 mL with the same solvent. Adjust to pH 6.0 with a 34.8 g/L solution of *dipotassium hydrogen phosphate R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 20.0 mg of *bacitracin for system suitability CRS* in solution A and dilute to 10.0 mL with the same solution.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c).* In order to prepare impurities E, F, G and H *in situ*, heat about 4 mL of reference solution (a) in a water-bath for 30 min. Cool to room temperature.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped, charged surface, ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 28 ± 2 °C.

*Mobile phase*: *acetonitrile R*, solution B, *water for chromatography R*, *methanol R1* (43:100:300:557 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 100 µL of the test solution and reference solutions (a) and (b).

*Run time*: 3 times the retention time of bacitracin A.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurity M and bacitracins A, B1, B2 and B3 (see Figure 0465.-1).

*Relative retention* with reference to bacitracin A (retention time = about 20 min): impurity A = about 0.44; impurity B = about 0.52; impurity C = about 0.55; bacitracin B1 = about 0.65; bacitracin B2 = about 0.67; bacitracin B3 = about 0.81; impurity M = about 0.87; impurity N = about 0.90; impurity L = about 0.93; impurity O = about 1.2; impurities P and Q = about 1.3; impurity F = about 1.6; impurity G = about 1.8; impurity H = about 2.1; impurity E = about 2.8.

If necessary, adjust the composition of the mobile phase by changing the amount of organic modifier whilst keeping the ratio constant between methanol and acetonitrile.

*System suitability*:

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to bacitracin B2 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B1 in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 1.1, where $H_p$ = height above the baseline of the peak due to impurity M and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B3 in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 50 for the peak due to bacitracin A in the chromatogram obtained with reference solution (b).

*Limits*:

- *bacitracin A*: minimum 45.0 per cent;
- *sum of bacitracins A, B1, B2 and B3*: minimum 77.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition with the following modifications. Use the normalisation procedure.

*Injection*: test solution and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, L, M, N, O, P and Q (see Figure 0465.-1); use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E, F, G and H (see Figure 0465.-2).

*Limits*:

- *sum of impurities L and N*: maximum 8.0 per cent;
- *impurity E*: maximum 4.0 per cent;
- *impurity A*: maximum 3.5 per cent;
- *impurities B, M*: for each impurity, maximum 3.0 per cent;
- *impurity C*: maximum 2.5 per cent;
- *sum of impurities O, P and Q*: maximum 2.5 per cent;
- *sum of impurities F and G*: maximum 2.0 per cent;
- *impurity H*: maximum 1.0 per cent;
- *any other impurity*: for each impurity, maximum 2.0 per cent;
- *total*: maximum 23.0 per cent;
- *reporting threshold*: 0.25 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.1 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *bacitracin zinc CRS* as the reference substance.

STORAGE

In an airtight container at 2 °C to 8 °C. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, E, F, G, H, L, M, N, O, P, Q.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It

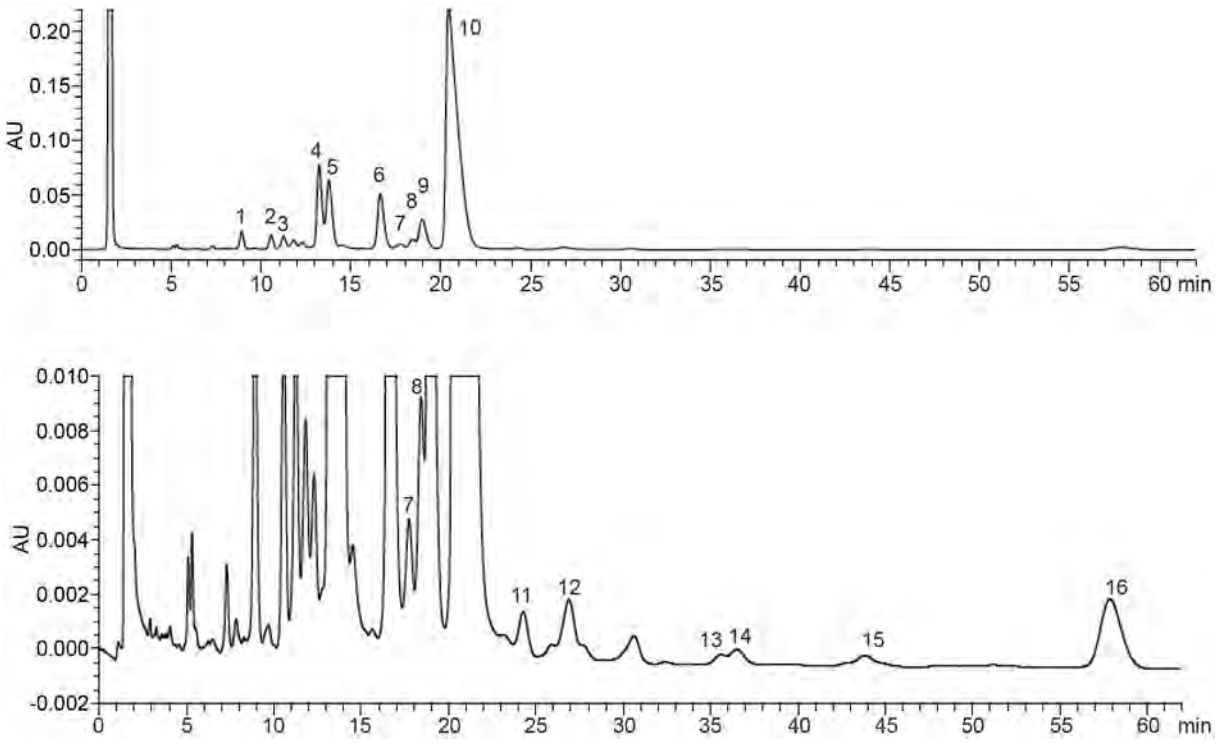


1. impurity A
2. impurity B
3. impurity C
4. bacitracin B1
5. bacitracin B2
6. bacitracin B3
7. impurity M
8. impurity N
9. impurity L
10. bacitracin A
11. impurity O
12. impurities P and Q
13. impurity F
14. impurity G
15. impurity H
16. impurity E

Figure 0465.-1. – *Chromatogram for the test for composition of bacitracin: test solution*

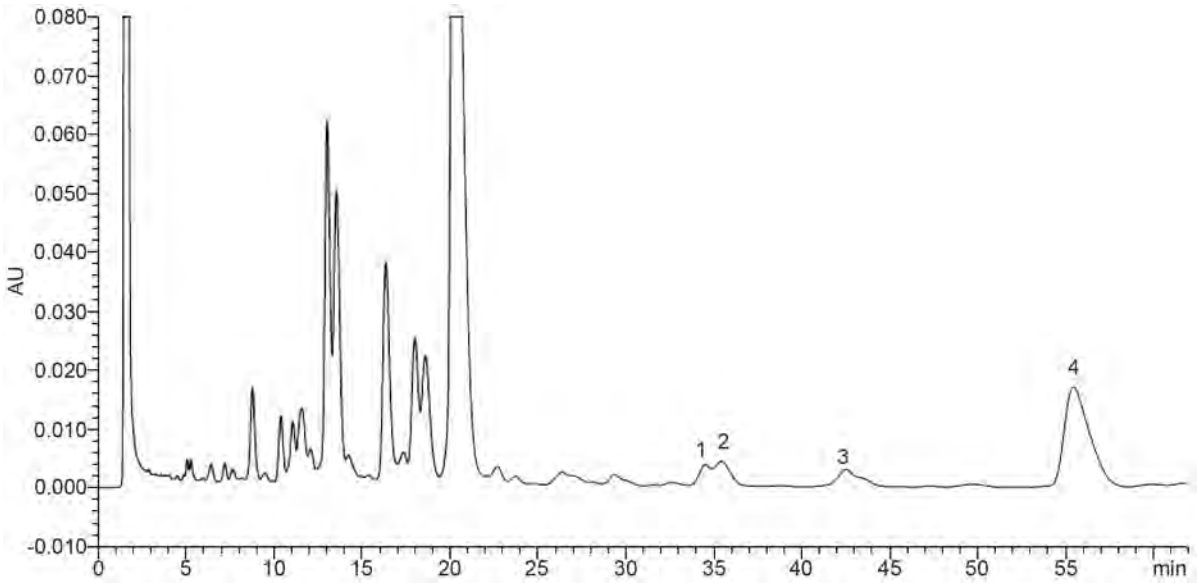


1. impurity F 2. impurity G 3. impurity H 4. impurity E

Figure 0465.-2. – *Chromatogram for the test for related substances of bacitracin: reference solution (c)*

is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, I, J, K.*

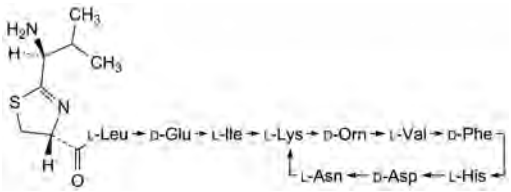


A. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D1, bacitracin C2),

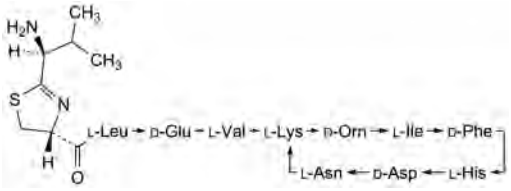


B. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D2, bacitracin C3),

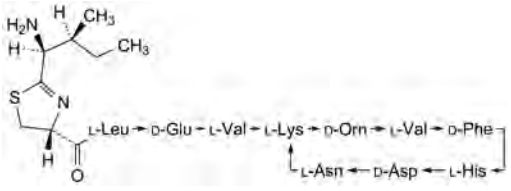


C. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D3, bacitracin C1a),

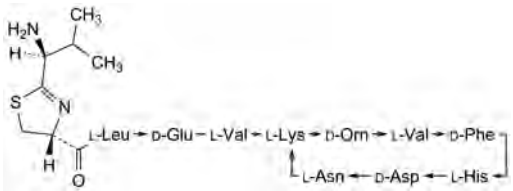


D. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin E),

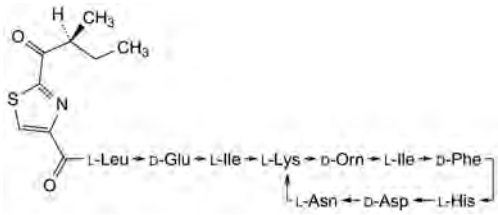


E. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin F),

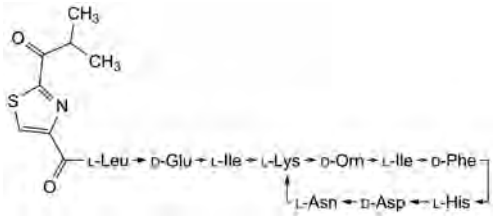


F. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H1),

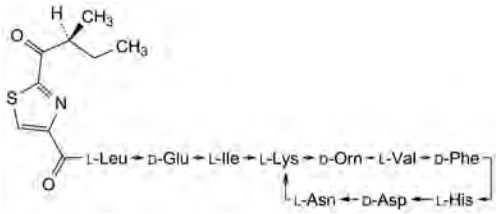


G. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H2),

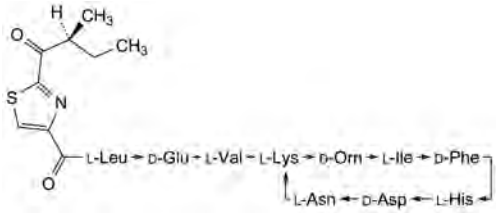


H. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H3),

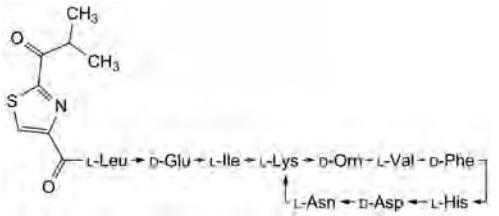


I. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I1),

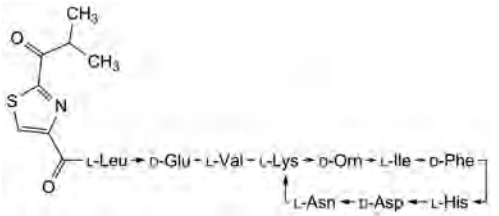


J. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I2),

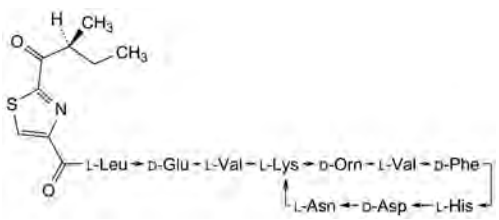


K. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I3),

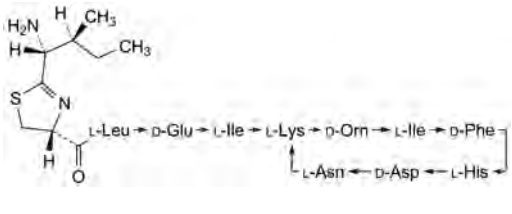


L. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*R*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin X),

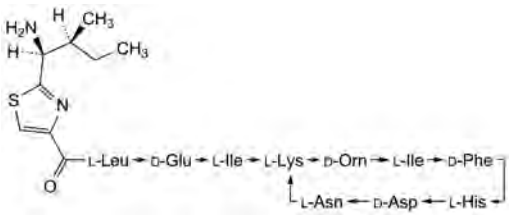


M. 4,10-anhydro[*N*-[[2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin Y),

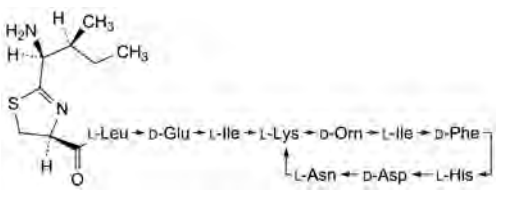


N. 4,10-anhydro[*N*-[[(4*S*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin Z),

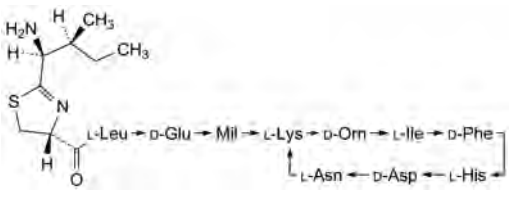


O. Mil = 5-methylene-L-isoleucine: 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-5-methylene-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J1),

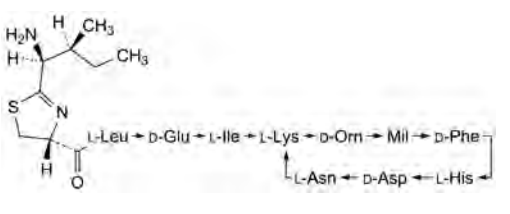


P. Mil = 5-methylene-L-isoleucine: 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-5-methylene-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J2),

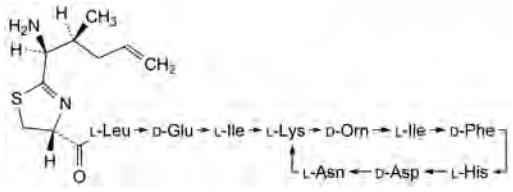


Q. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylpent-4-en-1-yl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J3).

**04/2017:0466**
**corrected 10.0**

# BACITRACIN ZINC

## Bacitracinum zincum

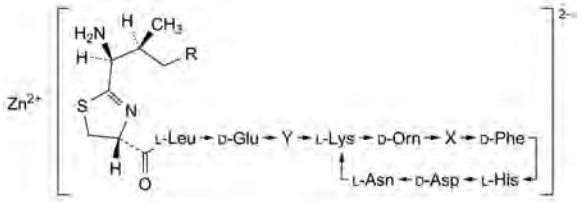


| Name | Mol. Formula | X | Y | R |
|---|---|---|---|---|
| Bacitracin A | $C_{66}H_{103}N_{17}O_{16}S$ | L-Ile | L-Ile | $CH_3$ |
| Bacitracin B1 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Ile | L-Ile | H |
| Bacitracin B2 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Val | L-Ile | $CH_3$ |
| Bacitracin B3 | $C_{65}H_{101}N_{17}O_{16}S$ | L-Ile | L-Val | $CH_3$ |

[1405-89-6]

DEFINITION

Zinc complex of bacitracin, which consists of a mixture of antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* or *Bacillus subtilis*, the main components being:

- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin A);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B1);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B2);
- 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin B3).

*Content*: minimum 60 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or light yellowish-grey, hygroscopic powder.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 0.5 mL of *dilute hydrochloric acid R* and dilute to 1.0 mL with *water R*.

*Reference solution.* Dissolve 10 mg of *bacitracin zinc CRS* in 0.5 mL of *dilute hydrochloric acid R* and dilute to 1.0 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (14:29:57 *V/V/V*).

*Application*: 10 µL.

*Development*: over half of the plate.

*Drying*: at 100-105 °C.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position, size and colour to the spots in the chromatogram obtained with the reference solution.

B. Composition (see Tests).

C. Ignite about 0.15 g, allow to cool and dissolve the residue in 1 mL of *dilute hydrochloric acid R*. Add 4 mL of *water R*. The solution gives the reaction of zinc (*2.3.1*).

TESTS

**pH** (*2.2.3*): 6.0 to 7.5.

Shake 1.0 g for about 1 min with 10 mL of *carbon dioxide-free water R* and filter.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Solution A*. 40 g/L solution of *sodium edetate R* adjusted to pH 7.0 with *dilute sodium hydroxide solution R*.

*Solution B*. In a volumetric flask, dissolve 54.4 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to 2000 mL with the same solvent. Adjust to pH 6.0 with a 34.8 g/L solution of *dipotassium hydrogen phosphate R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Test solution.* Dissolve 0.100 g of the substance to be examined in solution A and dilute to 50.0 mL with the same solution.

*Reference solution (a).* Dissolve 20.0 mg of *bacitracin for system suitability CRS* in solution A and dilute to 10.0 mL with the same solution.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c).* In order to prepare impurities E, F, G and H *in situ*, heat about 4 mL of reference solution (a) in a water-bath for 30 min. Cool to room temperature.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped, charged surface, ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 28 ± 2 °C.

*Mobile phase*: *acetonitrile R*, solution B, *water for chromatography R*, *methanol R1* (43:100:300:557 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 100 µL of the test solution and reference solutions (a) and (b).

*Run time*: 3 times the retention time of bacitracin A.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurity M and bacitracins A, B1, B2 and B3 (see Figure 0466.-1).

*Relative retention* with reference to bacitracin A (retention time = about 20 min): impurity A = about 0.44; impurity B = about 0.52; impurity C = about 0.55; bacitracin B1 = about 0.65; bacitracin B2 = about 0.67; bacitracin B3 = about 0.81; impurity M = about 0.87; impurity N = about 0.90; impurity L = about 0.93; impurity O = about 1.2; impurities P and Q = about 1.3; impurity F = about 1.6; impurity G = about 1.8; impurity H = about 2.1; impurity E = about 2.8.

If necessary, adjust the composition of the mobile phase by changing the amount of organic modifier whilst keeping the ratio constant between methanol and acetonitrile.

*System suitability*:

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to bacitracin B2 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B1 in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 1.1, where $H_p$ = height above the baseline of the peak due to impurity M and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bacitracin B3 in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 50 for the peak due to bacitracin A in the chromatogram obtained with reference solution (b).

*Limits*:

- *bacitracin A*: minimum 45.0 per cent;
- *sum of bacitracins A, B1, B2 and B3*: minimum 77.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition with the following modifications. Use the normalisation procedure.

*Injection*: test solution and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, L, M, N, O, P and Q (see Figure 0466.-1); use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E, F, G, and H (see Figure 0466.-2).

*Limits*:

- *sum of impurities L and N*: maximum 8.0 per cent;
- *impurity E*: maximum 4.0 per cent;
- *impurity A*: maximum 3.5 per cent;
- *impurities B, M*: for each impurity, maximum 3.0 per cent;
- *impurity C*: maximum 2.5 per cent;
- *sum of impurities O, P and Q*: maximum 2.5 per cent;
- *sum of impurities F and G*: maximum 2.0 per cent;
- *impurity H*: maximum 1.0 per cent;
- *any other impurity*: for each impurity, maximum 2.0 per cent;
- *total*: maximum 23.0 per cent;
- *reporting threshold*: 0.25 per cent.

  The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Zinc**: 3.5 per cent to 5.5 per cent (dried substance).

Dissolve 0.200 g in a mixture of 2.5 mL of *dilute acetic acid R* and 2.5 mL of water. Add 50 mL of *water R*, 50 mg of *xylenol orange triturate R* and sufficient *hexamethylenetetramine R* to produce a red colour. Add 2 g of *hexamethylenetetramine R* in excess. Titrate with *0.01 M sodium edetate* until a yellow colour is obtained.

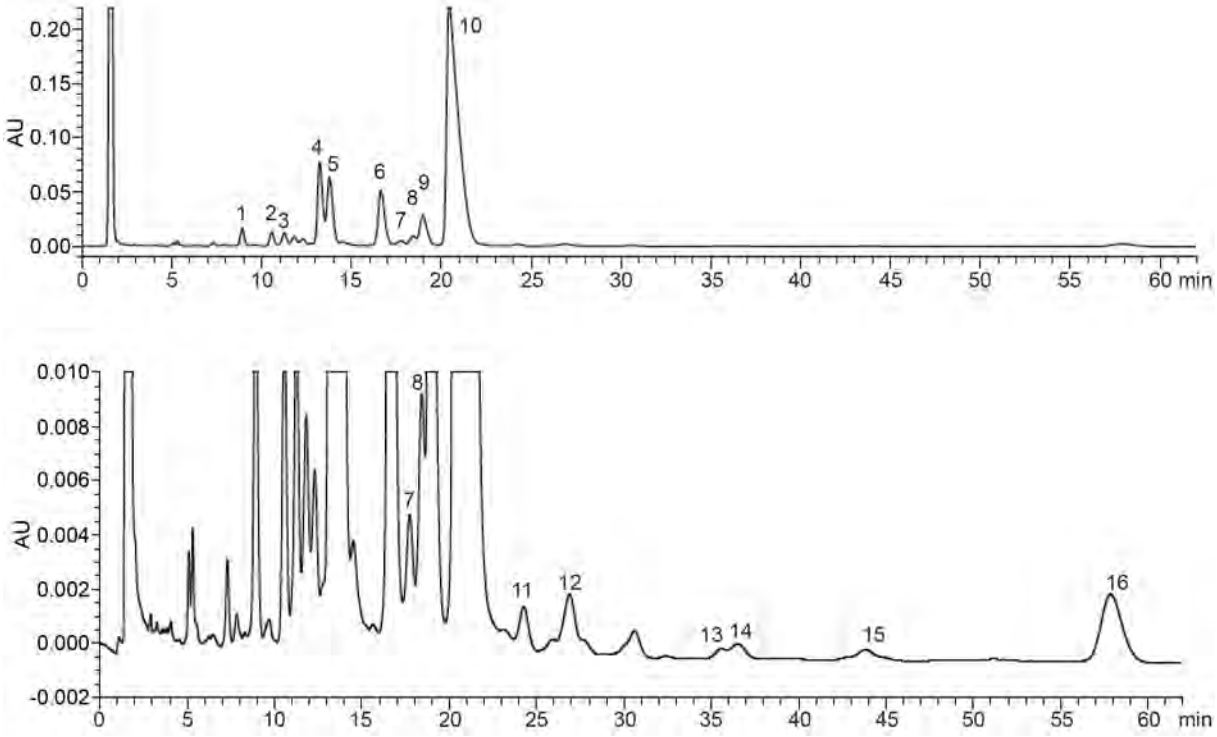


1. impurity A
2. impurity B
3. impurity C
4. bacitracin B1
5. bacitracin B2
6. bacitracin B3
7. impurity M
8. impurity N
9. impurity L
10. bacitracin A
11. impurity O
12. impurities P and Q
13. impurity F
14. impurity G
15. impurity H
16. impurity E

Figure 0466.-1. – *Chromatogram for the test for composition of bacitracin zinc: test solution*

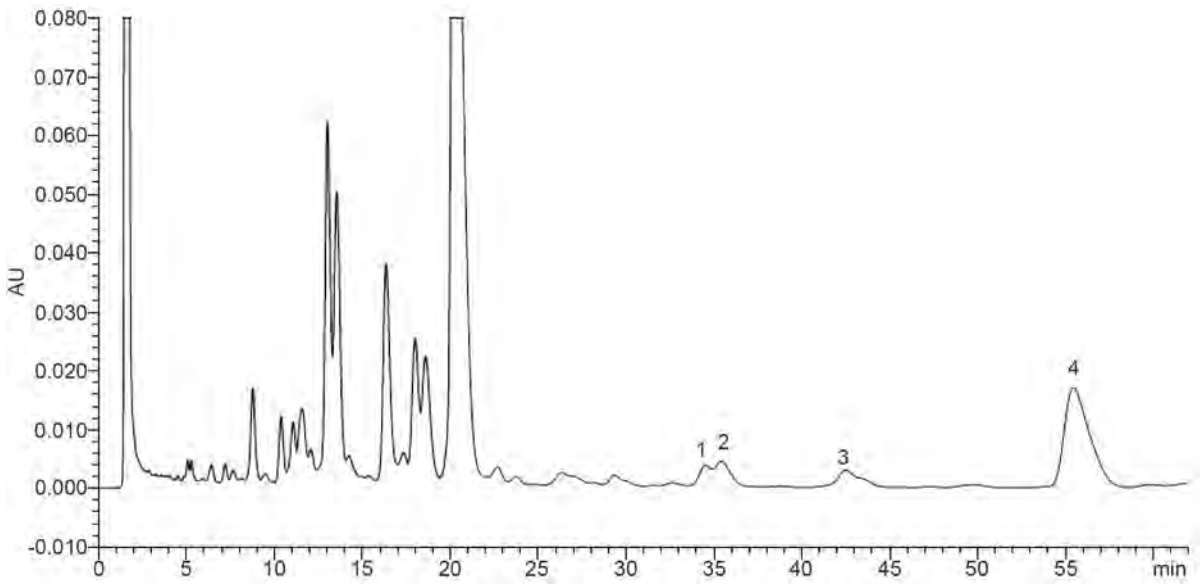


1. impurity F 2. impurity G 3. impurity H 4. impurity E

Figure 0466.-2. – *Chromatogram for the test for related substances of bacitracin zinc: reference solution (c)*

1 mL of *0.01 M sodium edetate* is equivalent to 0.654 mg of Zn.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.1 kPa for 3 h.

ASSAY

Suspend 50.0 mg in 5 mL of *water R*, add 0.5 mL of *dilute hydrochloric acid R* and dilute to 100.0 mL with *water R*. Allow the solution to stand for 30 min. Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, E, F, G, H, L, M, N, O, P, Q.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, I, J, K.*

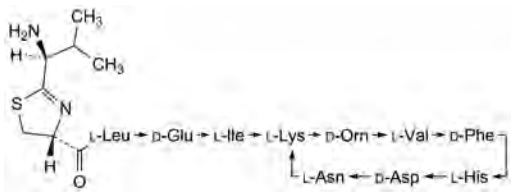


A. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D1, bacitracin C2),

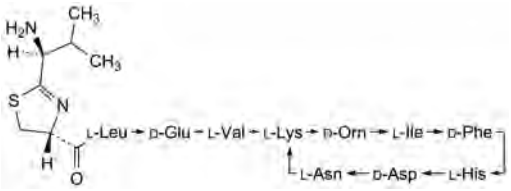


B. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D2, bacitracin C3),

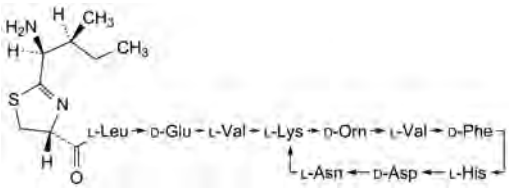


C. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin D3, bacitracin C1a),

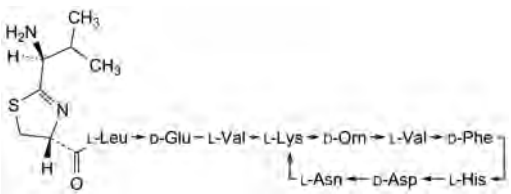


D. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*)-1-amino-2-methylpropyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin E),

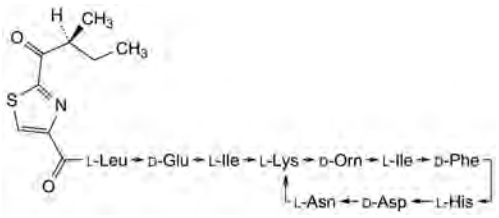


E. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine], (bacitracin F),

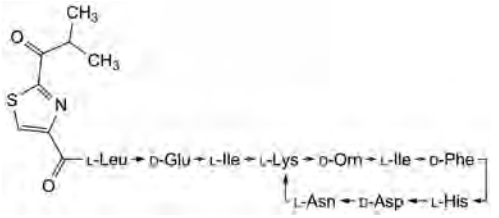


F. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H1),

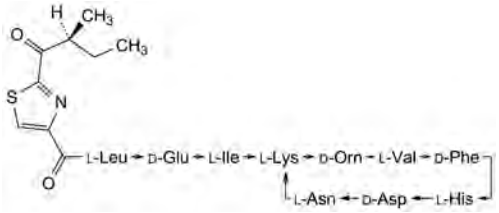


G. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H2),

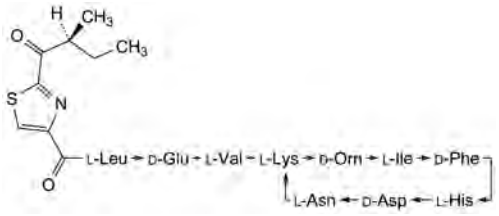


H. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin H3),

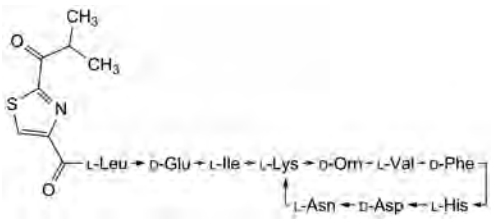


I. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I1),

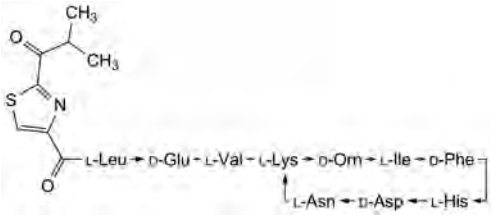


J. 4,10-anhydro[*N*-[[2-(2-methyl-1-oxopropyl)-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I2),

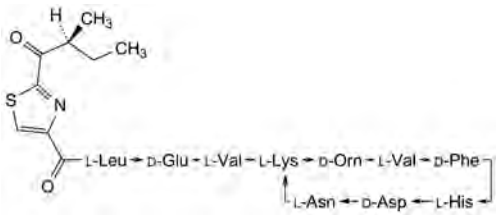


K. 4,10-anhydro[*N*-[[2-[(2*S*)-2-methyl-1-oxobutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-valyl-L-lysyl-D-ornithyl-L-valyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin I3),

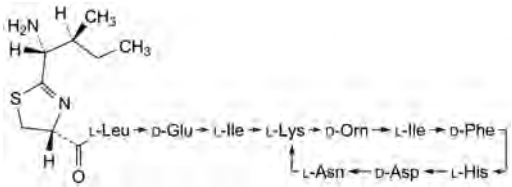


L. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*R*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin X),

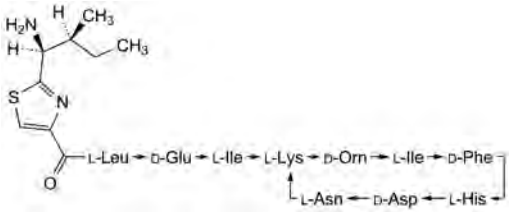


M. 4,10-anhydro[*N*-[[2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin Y),

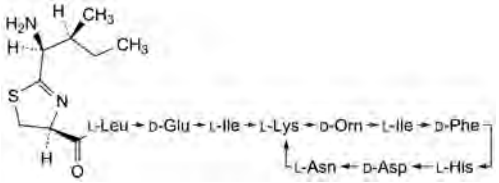


N. 4,10-anhydro[*N*-[[(4*S*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin Z),

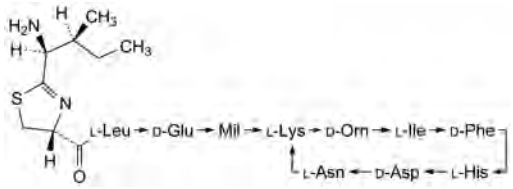


O. Mil = 5-methylene-L-isoleucine: 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-5-methylene-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J1),

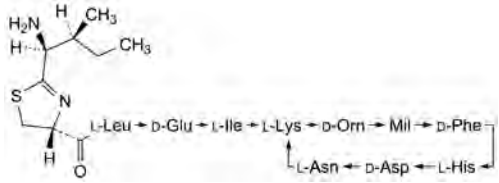


P. Mil = 5-methylene-L-isoleucine: 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylbutyl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-5-methylene-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J2),

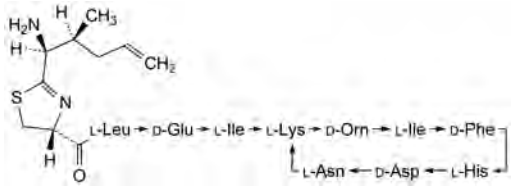


Q. 4,10-anhydro[*N*-[[(4*R*)-2-[(1*S*,2*S*)-1-amino-2-methylpent-4-en-1-yl]-4,5-dihydro-1,3-thiazol-4-yl]carbonyl]-L-leucyl-D-α-glutamyl-L-isoleucyl-L-lysyl-D-ornithyl-L-isoleucyl-D-phenylalanyl-L-histidyl-D-α-aspartyl-L-asparagine] (bacitracin J3).

**01/2008:0653**

# BACLOFEN

## Baclofenum

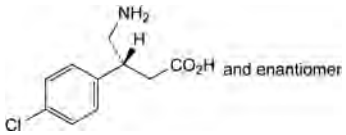


$C_{10}H_{12}ClNO_2$ $M_r$ 213.7
[1134-47-0]

DEFINITION

(3*RS*)-4-Amino-3-(4-chlorophenyl)butanoic acid.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in acetone. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 70 mg in *water R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 220-320 nm.

*Absorption maxima*: at 259 nm, 266 nm and 275 nm.

*Resolution* (*2.2.25*): minimum 1.5 for the absorbance ratio.

*Specific absorbance at the absorption maxima*:

- at 259 nm: 9.8 to 10.8;
- at 266 nm: 11.5 to 12.7;
- at 275 nm: 8.4 to 9.3.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs prepared using 3 mg of substance and 300 mg of *potassium bromide R*.

*Comparison*: *baclofen CRS*.

If the spectra obtained in the solid state show differences, dissolve 0.1 g of each of the substances separately in 1 mL of *dilute sodium hydroxide solution R* and add 10 mL of *ethanol (96 per cent) R* and 1 mL of *dilute acetic acid R*. Allow to stand for 1 h. Filter, wash the precipitate with *ethanol (96 per cent) R* and dry *in vacuo*. Prepare new discs and record the spectra.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution*. Dissolve 10 mg of *baclofen CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *chloroform R*, *ethyl acetate R* (5:5:20:30:40 *V/V/V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 12 cm.

*Drying*: allow the solvents to evaporate.

*Detection*: spray with *ninhydrin solution R3* until the plate is slightly wet. Place in an oven maintained at 100 °C for 10 min. Examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.50 g in *1 M sodium hydroxide* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *baclofen impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dilute 2.0 mL of the test solution and 2.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: dissolve 1.822 g of *sodium hexanesulfonate R* in 1 L of a mixture of 560 volumes of *water R*, 440 volumes of *methanol R* and 5 volumes of *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 5 times the retention time of baclofen.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to baclofen and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1500 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.37 mg of $C_{10}H_{12}ClNO_2$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

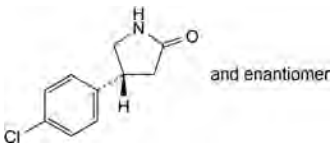


A. (4*RS*)-4-(4-chlorophenyl)pyrrolidin-2-one,

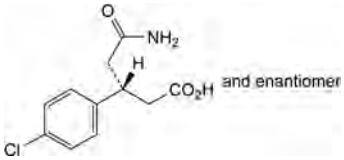


B. (3*RS*)-5-amino-3-(4-chlorophenyl)-5-oxopentanoic acid.

**01/2008:1293**

# BAMBUTEROL HYDROCHLORIDE

## Bambuteroli hydrochloridum

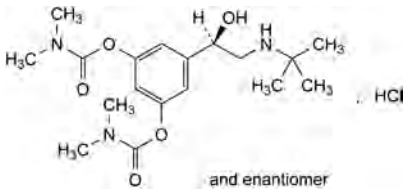


$C_{18}H_{30}ClN_3O_5$ $M_r$ 403.9
[81732-46-9]

DEFINITION

5-[(1*RS*)-2-[(1,1-Dimethylethyl)amino]-1-hydroxyethyl]-1,3-phenylene bis(dimethylcarbamate) hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *bambuterol hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in a mixture of 1 volume of *water R* and 6 volumes of *acetone R*, cool in ice to precipitate and dry both precipitates *in vacuo* at 50 °C to constant weight. Record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 4.0 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 5.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1.0 mg of *formoterol fumarate dihydrate CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Mix 0.8 mL of this solution with 0.4 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.3 g of *sodium octanesulfonate R* in 430 mL of a mixture of 25 volumes of *acetonitrile R1* and 75 volumes of *methanol R*; then mix this solution with 570 mL of 0.050 M phosphate buffer pH 3.0 prepared as follows: dissolve 6.90 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 1000 mL with *water R*, adjust to pH 3.0 with a 50 g/L solution of *dilute phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL; inject the mobile phase as a blank.

*Run time*: 1.5 times the retention time of bambuterol.

*Retention time*: formoterol = about 7 min; bambuterol = about 9 min. If necessary, adjust the composition of the mobile phase; increase the content of phosphate buffer to increase the retention time.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to bambuterol and formoterol.

*Limits*:

– *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to the mobile phase.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.320 g in 50 mL of *ethanol (96 per cent) R* and add 5 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 40.39 mg of $C_{18}H_{30}ClN_3O_5$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

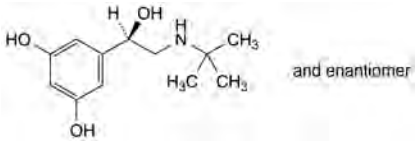


A. (1*RS*)-1-(3,5-dihydroxyphenyl)-2-[(1,1-dimethylethyl)-amino]ethanol (terbutaline),

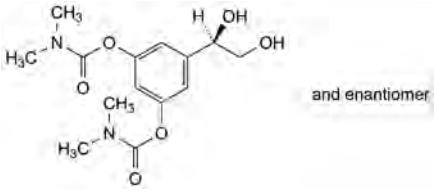


B. 5-[(1*RS*)-1,2-dihydroxyethyl]-1,3-phenylene bis(dimethylcarbamate),

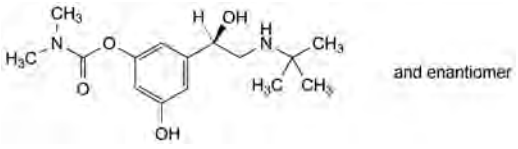


C. 3-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-5-hydroxyphenyl dimethylcarbamate,

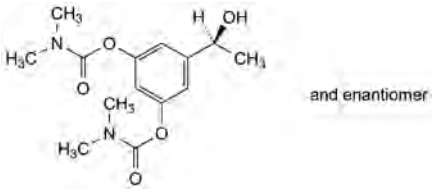


D. 5-[(1*RS*)-1-hydroxyethyl]-1,3-phenylene bis(dimethylcarbamate),

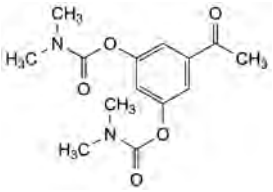


E. 5-acetyl-1,3-phenylene bis(dimethylcarbamate),

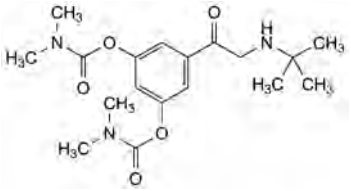


F. 5-[[(1,1-dimethylethyl)amino]acetyl]-1,3-phenylene bis(dimethylcarbamate).

**01/2008:0170**
**corrected 6.0**

# BARBITAL

## Barbitalum

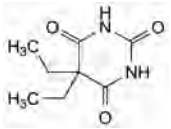


$C_8H_{12}N_2O_3$ $M_r$ 184.2
[57-44-3]

DEFINITION

Barbital contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 5,5-diethylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, slightly soluble in water, soluble in boiling water and in alcohol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *barbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 190 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *barbital CRS*.

C. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 75 mg of the substance to be examined in *alcohol R* and dilute to 25 mL with the same solvent.

*Reference solution*. Dissolve 75 mg of *barbital CRS* in *alcohol R* and dilute to 25 mL with the same solvent.

Apply separately to the plate 10 µL of each solution. Develop over a path of 18 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 6 mL of *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity**. Boil 1.0 g with 50 mL of *water R* for 2 min, allow to cool and filter. To 10 mL of the filtrate add 0.15 mL of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to produce a pure yellow colour.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 1.0 g of the substance to be examined in *alcohol R* and dilute to 100 mL with the same solvent.

*Reference solution*. Dilute 0.5 mL of the test solution to 100 mL with *alcohol R*.

Apply separately to the plate 20 µL of each solution. Develop over a path of 15 cm using the lower layer of a mixture of 5 volumes of *concentrated ammonia R*, 15 volumes of *alcohol R* and 80 volumes of *chloroform R*. Examine immediately in ultraviolet light at 254 nm. Spray with *diphenylcarbazone mercuric reagent R*. Allow the plate to dry in air and spray with freshly prepared *alcoholic potassium hydroxide solution R* diluted 1 in 5 with *aldehyde-free alcohol R*. Heat at 100 °C to 105 °C for 5 min and examine immediately. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 85.0 mg in 5 mL of *pyridine R*. Add 0.5 mL of *thymolphthalein solution R* and 10 mL of *silver nitrate solution in pyridine R*. Titrate with *0.1 M ethanolic sodium hydroxide* until a pure blue colour is obtained. Carry out a blank titration.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 9.21 mg of $C_8H_{12}N_2O_3$.

**01/2017:0010**

# BARIUM SULFATE

## Barii sulfas

$BaSO_4$ $M_r$ 233.4
[7727-43-7]

CHARACTERS

*Appearance*: fine, white or almost white powder, free from gritty particles.

*Solubility*: practically insoluble in water and in organic solvents. It is very slightly soluble in acids and in solutions of alkali hydroxides.

IDENTIFICATION

A. Boil a suspension of 0.2 g with 5 mL of a 500 g/L solution of *sodium carbonate R* for 5 min, add 10 mL of *water R*, filter and acidify a part of the filtrate with *dilute hydrochloric acid R*. The solution gives the reactions of sulfates (*2.3.1*).

B. Wash the residue collected in the preceding test with 3 successive small quantities of *water R*. To the residue add 5 mL of *dilute hydrochloric acid R*, filter and add to the filtrate 0.3 mL of *dilute sulfuric acid R*. A white precipitate is formed that is insoluble in *dilute sodium hydroxide solution R*.

TESTS

**Solution S**. To 20.0 g add 40 mL of *distilled water R* and 60 mL of *dilute acetic acid R*. Boil for 5 min, filter and dilute the cooled filtrate to 100 mL with *distilled water R*.

**Acidity or alkalinity**. Heat 5.0 g with 20 mL of *carbon dioxide-free water R* on a water-bath for 5 min and filter. To 10 mL of the filtrate add 0.05 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Acid-soluble substances**: maximum 0.3 per cent.

Evaporate 25 mL of solution S to dryness on a water-bath and dry to constant mass at 100-105 °C. The residue weighs a maximum of 15 mg.

**Oxidisable sulfur compounds**. Shake 1.0 g with 5 mL of *water R* for 30 s and filter. To the filtrate add 0.1 mL of *starch solution R*, dissolve 0.1 g of *potassium iodide R* in the mixture, add 1.0 mL of a freshly prepared 3.6 mg/L solution of *potassium iodate R* and 1 mL of *1 M hydrochloric acid* and shake well. The colour of the solution is more intense than that of a standard prepared at the same time and in the same manner, but omitting the potassium iodate.

**Soluble barium salts**: maximum 10 ppm.

To 2.5 mL of a 0.2 mg/L solution of *barium nitrate R* in a mixture of 30 volumes of *ethanol (96 per cent) R* and 70 volumes of *water R*, add 10 mL of *dilute sulfuric acid R*. Shake and allow to stand for 5 min. To 1 mL of this solution add 10 mL of solution S. Prepare a standard in the same manner using 10 mL of *barium standard solution (2 ppm Ba) R* instead of solution S.

After 10 min, any opalescence in the test solution is not more intense than that in the standard.

**Loss on ignition**: maximum 2.0 per cent, determined on 1.0 g at 600 ± 50 °C.

**04/2018:1975**

# BASIC BUTYLATED METHACRYLATE COPOLYMER

## Copolymerum methacrylatis butylati basicum

DEFINITION

Copolymer of 2-(dimethylamino)ethyl methacrylate, butyl methacrylate and methyl methacrylate having a mean relative molecular mass of about 47 000. The ratio of 2-(dimethylamino)ethyl methacrylate groups to butyl methacrylate and methyl methacrylate groups is about 2:1:1.

*Content of (dimethylamino)ethyl groups*: 20.8 per cent to 25.5 per cent (dried substance).

CHARACTERS

*Appearance*: colourless or yellowish granules or white or almost white powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride. It dissolves slowly in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *basic butylated methacrylate copolymer CRS*.

B. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 12.5 g in a mixture of 35.0 g of *acetone R* and 52.5 g of *2-propanol R*.

**Viscosity** (*2.2.10*): 3 mPa·s to 6 mPa·s, determined on solution S.

*Apparatus*: rotating viscometer.

*Dimensions*:

- *spindle*: diameter = 25.15 mm, height = 90.74 mm, shaft diameter = 4 mm;
- *cylinder*: diameter = 27.62 mm, height = 0.135 m.

*Rotating speed*: 30 r/min.

*Volume of solution*: 16 mL of solution S.

*Temperature*: 20 °C.

**Absorbance** (*2.2.25*): maximum 0.30 at 420 nm, determined on solution S.

**Appearance of a film**. Spread 1.0 mL of solution S evenly on a glass plate. Upon drying a clear film is formed.

**Monomers**: maximum 0.1 per cent for each monomer (butyl methacrylate, methyl methacrylate and 2-(dimethylamino)ethyl methacrylate), determined by procedures A and B.

A. Butyl methacrylate and methyl methacrylate. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *phosphate buffer solution pH 2.0 R* (40:60 *V/V*).

*Test solution*. Dissolve 1.00 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution*. Dissolve 20.0 mg of *butyl methacrylate CRS* (impurity A) and 10.0 mg of *methyl methacrylate CRS* (impurity B) in 3.0 mL of *butanol R* and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 250.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: *phosphate buffer solution pH 2.0 R*, *methanol R2* (45:55 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 50 µL.

*System suitability*: reference solution:

- *resolution*: minimum 5 between the peaks due to impurities A and B.

Calculate the percentage contents of impurities A and B using the following expression:

$$100 \times 10^{-6} \times 50 \times \frac{C}{M} \times \frac{A_T}{A_R}$$

$C$ = concentration of the monomer in the reference solution, in micrograms per millilitre;

$M$ = mass of substance to be examined in the test solution, in grams;

$A_T$ = area of the peak due to the monomer in the chromatogram obtained with the test solution;

$A_R$ = area of the peak due to the monomer in the chromatogram obtained with the reference solution.

B. 2-(Dimethylamino)ethyl methacrylate. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 1.00 g of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent.

*Reference solution*. Dissolve 10.0 mg of *2-(dimethylamino)ethyl methacrylate CRS* (impurity C) in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 50.0 mL with *tetrahydrofuran R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 25 volumes of a 3.404 g/L solution of *potassium dihydrogen phosphate R* and 75 volumes of *tetrahydrofuran R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 50 µL.

Calculate the percentage content of impurity C as described under procedure A.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 110 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 4 mL of *water R* and 96 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 7.21 mg of $C_4H_{10}N$.

STORAGE

In an airtight container.

IMPURITIES

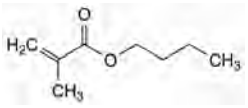

A. butyl 2-methylprop-2-enoate (butyl methacrylate),

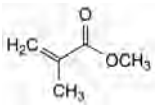

B. methyl 2-methylprop-2-enoate (methyl methacrylate),

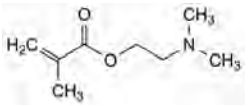

C. 2-(dimethylamino)ethyl 2-methylprop-2-enoate (2-(dimethylamino)ethyl methacrylate).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for basic butylated methacrylate copolymer used as film former in tablets.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take the film obtained in the test for appearance of a film (see Tests), place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* and stir. It dissolves within 1 h. Take another film, place it in a flask containing *phosphate buffer solution pH 6.8 R* and stir. It does not dissolve within 2 h.

**01/2017:0654**

# BECLOMETASONE DIPROPIONATE

## Beclometasoni dipropionas

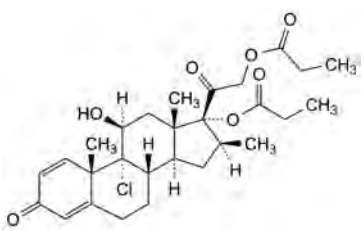

$C_{28}H_{37}ClO_7$ $M_r$ 521.0

[5534-09-8]

DEFINITION

9-Chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *anhydrous beclometasone dipropionate CRS*.

B. Treat 25 mg by the oxygen-flask method (*2.5.10*). Use a mixture of 1 mL of *1 M sodium hydroxide* and 20 mL of *water R* to absorb the combustion products. The solution gives reaction (a) of chlorides (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 108 to + 115 (dried substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (45:55 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in 28 mL of mobile phase B and dilute to 50.0 mL with mobile phase A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of test solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *beclometasone dipropionate for system suitability CRS* (containing impurity D) in 3 mL of mobile phase B and dilute to 5 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of *beclometasone dipropionate for peak identification CRS* (containing impurities A, B, C, L and M) in 3 mL of mobile phase B and dilute to 5 mL with mobile phase A. Use 1 mL of this solution to dissolve the contents of a vial of *beclometasone dipropionate impurities F and N CRS*.

*Reference solution (d)*. Dissolve 50.0 mg of *anhydrous beclometasone dipropionate CRS* in 28 mL of mobile phase B and dilute to 50.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical difunctional bonded *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.35 with *phosphoric acid R*;
- *mobile phase B*: *tetrahydrofuran R*, *acetonitrile R*, *methanol R* (5:23:25 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 40 | 60 |
| 4 - 12 | 40 → 45 | 60 → 55 |
| 12 - 59 | 45 | 55 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µl of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *beclometasone dipropionate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, F, L, M and N; use the chromatogram supplied with *beclometasone dipropionate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to beclometasone dipropionate (retention time = about 25 min): impurity A = about 0.3; impurity B = about 0.6; impurity D = about 1.1; impurity M = about 1.2; impurity L = about 1.3; impurity C = about 1.8; impurity N = about 2.0; impurity F = about 2.2.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to beclometasone dipropionate.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity F = 1.3; impurity M = 2.0;
- *impurity L*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *impurities B, F, M*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities A, D, N*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{28}H_{37}ClO_7$ from the declared content of *anhydrous beclometasone dipropionate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, F, L, M, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, H, I, J, O, Q, R, S, U, V.*

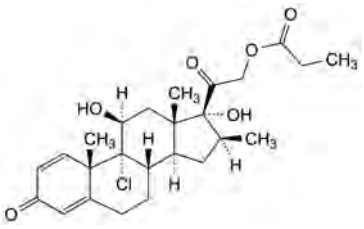

A. 9-chloro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl propanoate (beclometasone 21-propionate),

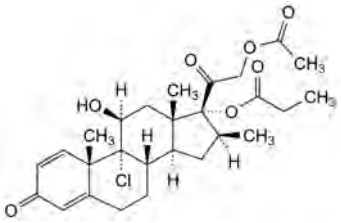

B. 21-(acetyloxy)-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (beclometasone 21-acetate 17-propionate),

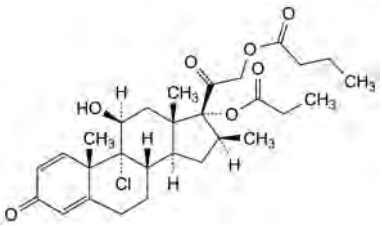

C. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxo-17-(propanoyloxy)-pregna-1,4-dien-21-yl butanoate (beclometasone 21-butyrate 17-propionate),

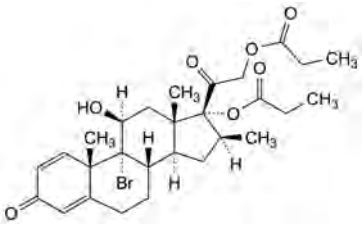

D. 9-bromo-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

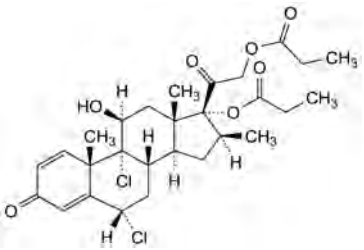

E. 6α,9-dichloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

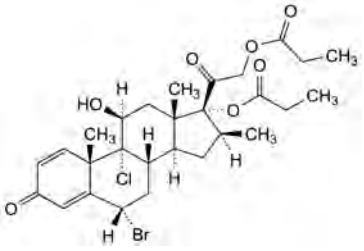

F. 6α-bromo-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

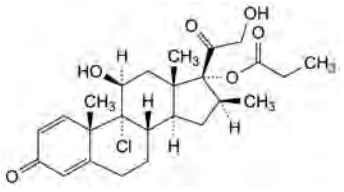

H. 9-chloro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (beclometasone 17-propionate),

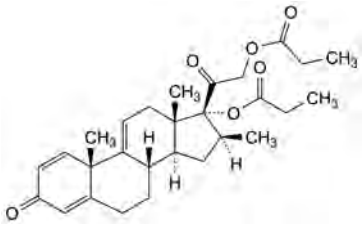

I. 16β-methyl-3,20-dioxopregna-1,4,9(11)-triene-17,21-diyl dipropanoate,

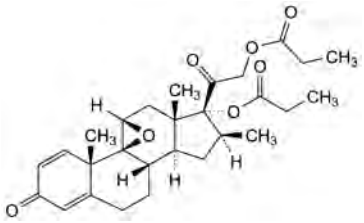

J. 9,11β-epoxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-diene-17,21-diyl dipropanoate,

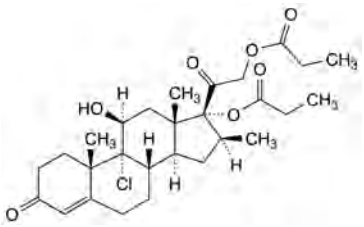

L. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregn-4-ene-17,21-diyl dipropanoate,

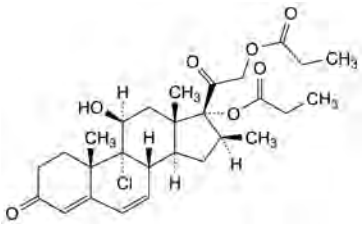

M. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-4,6-diene-17,21-diyl dipropanoate,

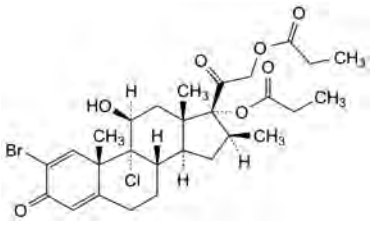

N. 2-bromo-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

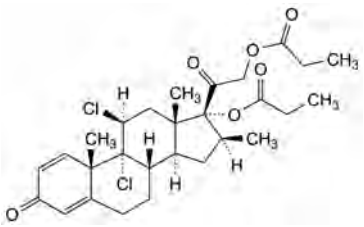

O. 9,11β-dichloro-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

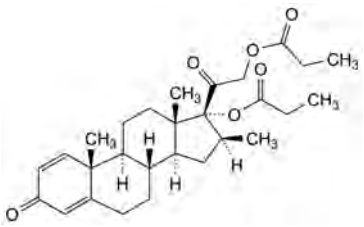

Q. 16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

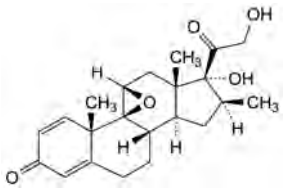

R. 9,11β-epoxy-17,21-dihydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione,

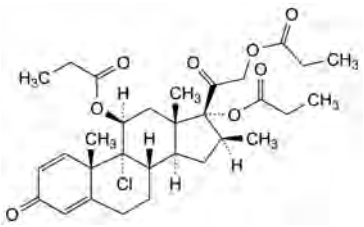

S. 9-chloro-16β-methyl-3,20-dioxopregna-1,4-diene-11β,17,21-triyl tripropanoate (beclometasone tripropionate),

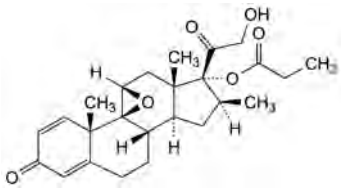

U. 9,11β-epoxy-21-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-dien-17-yl propanoate,

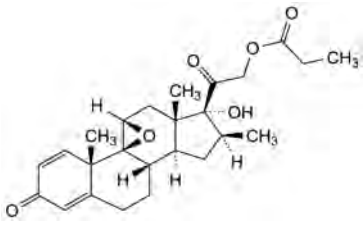

V. 9,11β-epoxy-17-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-dien-21-yl propanoate.

**01/2009:1709**
**corrected 7.0**

# BECLOMETASONE DIPROPIONATE MONOHYDRATE

## Beclometasoni dipropionas monohydricus

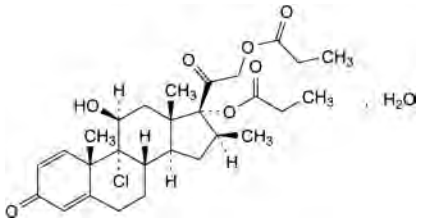

$C_{28}H_{37}ClO_7,H_2O$ $M_r$ 539.1

DEFINITION

9-Chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate monohydrate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *beclometasone dipropionate monohydrate CRS*.

B. Treat 25 mg by the oxygen-flask method (*2.5.10*). Use a mixture of 1 mL of *1 M sodium hydroxide* and 20 mL of *water R* to absorb the combustion products. The solution gives reaction (a) of chlorides (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 108 to + 115 (dried substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (45:55 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in 28 mL of mobile phase B and dilute to 50.0 mL with mobile phase A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of test solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *beclometasone dipropionate for system suitability CRS* (containing impurity D) in 3 mL of mobile phase B and dilute to 5 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of *beclometasone dipropionate for peak identification CRS* (containing impurities B, C and L) in 3 mL of mobile phase B and dilute to 5 mL with mobile phase A. Use 1 mL of this solution to dissolve the contents of a vial of *beclometasone dipropionate impurities F and N CRS*.

*Reference solution (d)*. Dissolve 50.0 mg of *anhydrous beclometasone dipropionate CRS* in 28 mL of mobile phase B and dilute to 50.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical difunctional bonded *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:
- *mobile phase A*: 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.35 with *phosphoric acid R*;
- *mobile phase B*: *tetrahydrofuran R*, *acetonitrile R*, *methanol R* (5:23:25 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 40 | 60 |
| 4 - 12 | 40 → 45 | 60 → 55 |
| 12 - 59 | 45 | 55 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µl of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *beclometasone dipropionate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C, F and L; use the chromatogram supplied with *beclometasone dipropionate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to beclometasone dipropionate (retention time = about 25 min): impurity B = about 0.6; impurity D = about 1.1; impurity L = about 1.3; impurity C = about 1.8; impurity F = about 2.2.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to beclometasone dipropionate.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 1.3;
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities C, F, L*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): 2.8 per cent to 3.8 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{28}H_{37}ClO_7$ from the declared content of *anhydrous beclometasone dipropionate CRS*.

IMPURITIES

*Specified impurities: B, C, F, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, H, I, J, M, N, O, Q, R, S, U, V.*

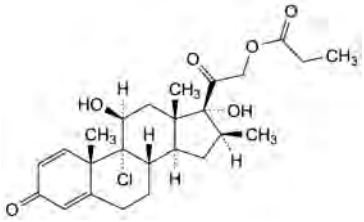

A. 9-chloro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl propanoate (beclometasone 21-propionate),

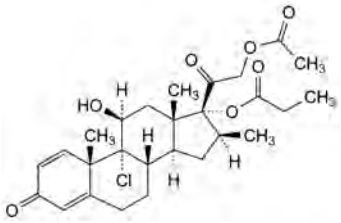

B. 21-(acetyloxy)-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (beclometasone 21-acetate 17-propionate),

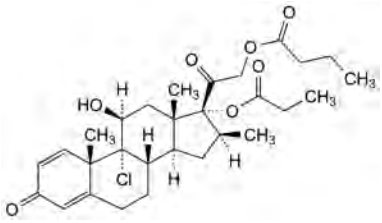

C. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxo-17-(propanoyloxy)-pregna-1,4-dien-21-yl butanoate (beclometasone 21-butyrate 17-propionate),

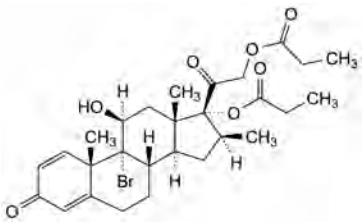

D. 9-bromo-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

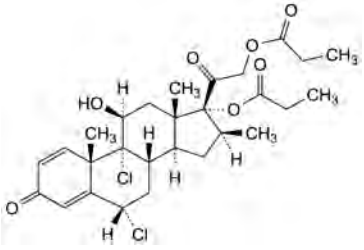

E. 6α,9-dichloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

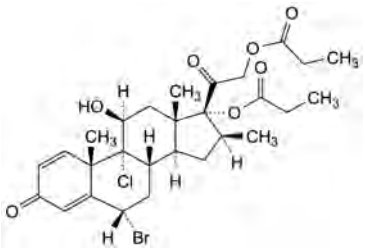

F. 6α-bromo-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

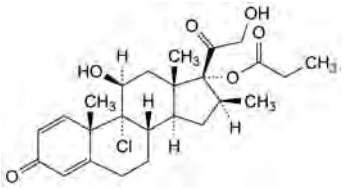

H. 9-chloro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (beclometasone 17-propionate),

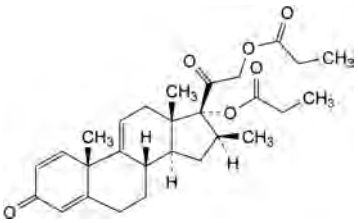

I. 16β-methyl-3,20-dioxopregna-1,4,9(11)-triene-17,21-diyl dipropanoate,

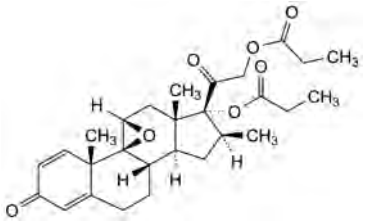

J. 9,11β-epoxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-diene-17,21-diyl dipropanoate,

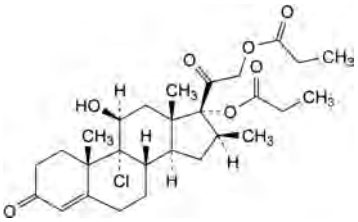

L. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregn-4-ene-17,21-diyl dipropanoate,

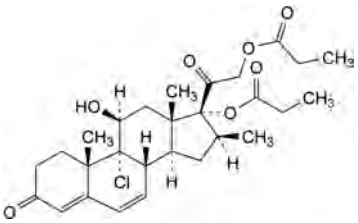

M. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-4,6-diene-17,21-diyl dipropanoate,

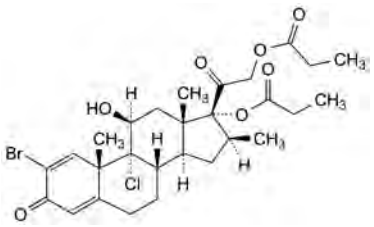

N. 2-bromo-9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

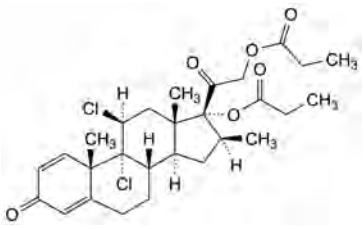

O. 9,11β-dichloro-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

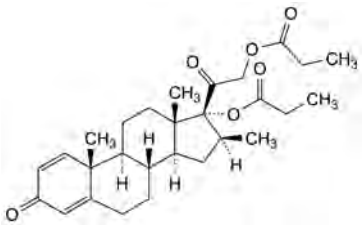

Q. 16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate,

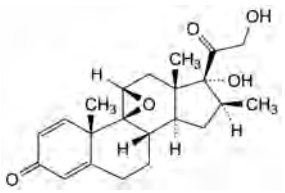

R. 9,11β-epoxy-17,21-dihydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione,

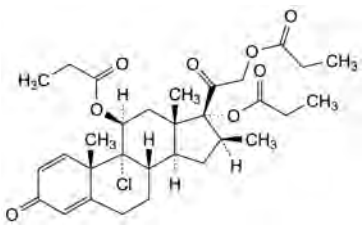

S. 9-chloro-16β-methyl-3,20-dioxopregna-1,4-diene-11β,17,21-triyl tripropanoate (beclometasone tripropionate),

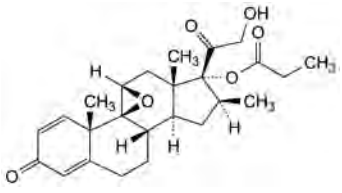

U. 9,11β-epoxy-21-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-dien-17-yl propanoate,

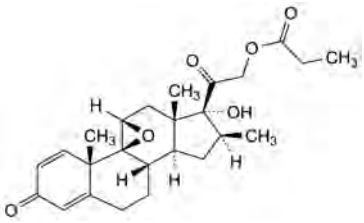

V. 9,11β-epoxy-17-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-dien-21-yl propanoate.

**01/2008:0069**

# BEESWAX, WHITE

## Cera alba

DEFINITION

Wax obtained by bleaching yellow beeswax.

CHARACTERS

*Appearance*: white or yellowish-white pieces or plates, translucent when thin, with a fine-grained, matt and non-crystalline fracture; when warmed in the hand they become soft and malleable.

It has an odour similar to that of yellow beeswax, though fainter and never rancid. It is tasteless and does not stick to the teeth.

*Solubility*: practically insoluble in water, partially soluble in hot ethanol (90 per cent *V/V*) and completely soluble in fatty and essential oils.

*Relative density*: about 0.960.

TESTS

**Drop point** (*2.2.17*): 61 °C to 66 °C.

Melt the beeswax by heating on a water-bath, pour onto a glass plate and allow to cool to a semi-solid mass. Fill the metal cup by inserting the wider end into the beeswax and repeating the procedure until beeswax extrudes from the narrow opening. Remove the excess with a spatula and insert the thermometer immediately. Remove the beeswax displaced. Allow to stand at room temperature for at least 12 h before determining the drop point.

**Acid value**: 17.0 to 24.0.

To 2.00 g (*m* g), in a 250 mL conical flask fitted with a reflux condenser, add 40 mL of *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 20 mL of *ethanol (96 per cent) R* and 0.5 mL of *phenolphthalein solution R1* and titrate the hot solution with *0.5 M alcoholic potassium hydroxide* until a red colour persists for at least 10 s ($n_1$ mL). Carry out a blank test ($n_2$ mL).

$$\text{Acid value} = \frac{28.05(n_1 - n_2)}{m}$$

**Ester value** (*2.5.2*): 70 to 80.

**Saponification value**: 87 to 104.

To 2.00 g (*m* g), in a 250 mL conical flask fitted with a reflux condenser, add 30 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and heat under a reflux condenser for 3 h. Titrate the hot solution immediately with *0.5 M hydrochloric acid*, using 1 mL of *phenolphthalein solution R1* as indicator ($n_1$ mL). Reheat the solution to boiling several times during the course of the titration. Carry out a blank test ($n_2$ mL).

$$\text{Saponification value} = \frac{28.05(n_2 - n_1)}{m}$$

**Ceresin, paraffins and certain other waxes**. To 3.0 g, in a 100 mL round-bottomed flask, add 30 mL of a 40 g/L solution of *potassium hydroxide R* in *aldehyde-free alcohol R* and boil gently under a reflux condenser for 2 h. Remove the condenser and immediately insert a thermometer. Place the flask in a water-bath at 80 °C and allow to cool, swirling the solution continuously. No precipitate is formed until 65 °C, although the solution may be slightly opalescent. Beginning at 65 °C, the solution may become cloudy and precipitates may be formed. At 59 °C, the solution is cloudy.

**Glycerol and other polyols**: maximum 0.5 per cent *m/m*, calculated as glycerol.

To 0.20 g add 10 mL of *alcoholic potassium hydroxide solution R* and heat on a water-bath under a reflux condenser for 30 min. Add 50 mL of *dilute sulfuric acid R*, cool and filter. Rinse the flask and the filter with *dilute sulfuric acid R*. Combine the filtrate and washings and dilute to 100.0 mL with *dilute sulfuric acid R*. Place 1.0 mL of the solution in a test-tube, add 0.5 mL of a 10.7 g/L solution of *sodium periodate R*, mix and allow to stand for 5 min. Add 1.0 mL of *decolorised fuchsin solution R* and mix. Any precipitate disappears. Place the tube in a beaker containing water at 40 °C. During cooling observe for 10-15 min. Any violet-blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 1.0 mL of a 10 mg/L solution of *glycerol R* in *dilute sulfuric acid R*.

**01/2008:0070**

# BEESWAX, YELLOW

## Cera flava

DEFINITION

Wax obtained by melting the walls of the honeycomb made by the honey-bee, *Apis mellifera* L., with hot water and removing foreign matter.

CHARACTERS

*Appearance*: yellow or light brown pieces or plates with a fine-grained, matt and non-crystalline fracture; when warmed in the hand they become soft and malleable.

It has a faint odour, characteristic of honey. It is tasteless and does not stick to the teeth.

*Solubility*: practically insoluble in water, partially soluble in hot ethanol (90 per cent *V/V*) and completely soluble in fatty and essential oils.

*Relative density*: about 0.960.

TESTS

**Drop point** (*2.2.17*): 61 °C to 66 °C.

Melt the beeswax by heating on a water-bath, pour onto a glass plate and allow to cool to a semi-solid mass. Fill the metal cup by inserting the wider end into the beeswax and repeating the procedure until beeswax extrudes from the narrow opening. Remove the excess with a spatula and insert the thermometer immediately. Remove the beeswax displaced. Allow to stand at room temperature for at least 12 h before determining the drop point.

**Acid value**: 17.0 to 22.0.

To 2.00 g (*m* g), in a 250 mL conical flask fitted with a reflux condenser, add 40 mL of *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 20 mL of *ethanol (96 per cent) R* and 0.5 mL of *phenolphthalein solution R1* and titrate the hot solution with *0.5 M alcoholic potassium hydroxide* until a red colour persists for at least 10 s ($n_1$ mL). Carry out a blank test ($n_2$ mL).

$$\text{Acid value} = \frac{28.05(n_1 - n_2)}{m}$$

**Ester value** (*2.5.2*): 70 to 80.

**Saponification value**: 87 to 102.

To 2.00 g (*m* g), in a 250 mL conical flask fitted with a reflux condenser, add 30 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *xylene R* and a few glass beads. Heat until the substance is dissolved. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and heat under a reflux condenser for 3 h. Titrate the hot solution immediately with *0.5 M hydrochloric acid*, using 1 mL of *phenolphthalein solution R1* as indicator ($n_1$ mL). Reheat the solution to boiling several times during the course of the titration. Carry out a blank test ($n_2$ mL).

$$\text{Saponification value} = \frac{28.05(n_2 - n_1)}{m}$$

**Ceresin, paraffins and certain other waxes**. To 3.0 g, in a 100 mL round-bottomed flask, add 30 mL of a 40 g/L solution of *potassium hydroxide R* in *aldehyde-free alcohol R* and boil gently under a reflux condenser for 2 h. Remove the condenser and immediately insert a thermometer. Place the flask in a water-bath at 80 °C and allow to cool, swirling the solution continuously. No precipitate is formed until 65 °C, although the solution may be slightly opalescent. Beginning at 65 °C, the solution may become cloudy and precipitates may be formed. At 59 °C, the solution is cloudy.

**Glycerol and other polyols**: maximum 0.5 per cent *m/m*, calculated as glycerol.

To 0.20 g add 10 mL of *alcoholic potassium hydroxide solution R* and heat on a water-bath under a reflux condenser for 30 min. Add 50 mL of *dilute sulfuric acid R*, cool and filter. Rinse the flask and the filter with *dilute sulfuric acid R*. Combine the filtrate and washings and dilute to 100.0 mL with *dilute sulfuric acid R*. Place 1.0 mL of the solution in a test-tube, add 0.5 mL of a 10.7 g/L solution of *sodium periodate R*, mix and allow to stand for 5 min. Add 1.0 mL of *decolorised fuchsin solution R* and mix. Any precipitate disappears. Place the tube in a beaker containing water at 40 °C. During cooling observe for 10-15 min. Any violet-blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 1.0 mL of a 10 mg/L solution of *glycerol R* in *dilute sulfuric acid R*.

**01/2017:2388**

# BENAZEPRIL HYDROCHLORIDE

## Benazeprili hydrochloridum

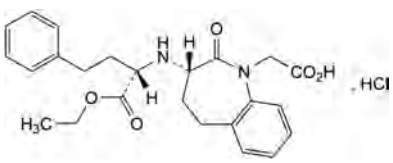


$C_{24}H_{29}ClN_2O_5$ $M_r$ 461.0
[86541-74-4]

DEFINITION

[(3*S*)-3-[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetic acid hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: slightly soluble in water, freely soluble in anhydrous ethanol, very slightly soluble in ethyl acetate, practically insoluble in cyclohexane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): − 141 to − 136 (dried substance).

Dissolve 1.000 g in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benazepril hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Enantiomeric purity (see Tests).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *benazepril hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *benazepril for system suitability CRS* (containing impurities B, C, D, E, F and G) in 1.0 mL of test solution (a).

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.30 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: add 0.2 mL of *glacial acetic acid R* to 1000 mL of a mixture of 360 volumes of *water R* and 640 volumes of *methanol R2*; add 0.81 g of *tetrabutylammonium bromide R* and stir to dissolve.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 25 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 3 times the retention time of benazepril.

*Relative retention* with reference to benazepril (retention time = about 6 min): impurity E = about 0.3; impurity F = about 0.4; impurity C = about 0.5; impurity B = about 1.8; impurity D = about 2.0; impurity G = about 2.5.

*Identification of impurities*: use the chromatogram supplied with *benazepril for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D, E, F and G.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to benazepril and impurity B and minimum 1.5 between the peaks due to impurities E and F.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.5; impurity F = 0.7;
- *impurity B*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurities D, E, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 6.0.* Dissolve 3.58 g of *disodium hydrogen phosphate dodecahydrate R* and 9.66 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *benazepril impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: spherical *silica gel AGP for chiral chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *methanol R2*, buffer solution pH 6.0 (20:80 *V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3.5 times the retention time of benazepril.

*Relative retention* with reference to benazepril (retention time = about 6 min): impurity A = about 1.9.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to benazepril.

*Limit*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{24}H_{29}ClN_2O_5$ from the declared content of *benazepril hydrochloride CRS*.

STORAGE

Protected from light, in an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

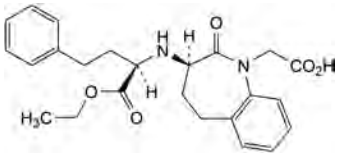

A. [(3*R*)-3-[[(1*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]-amino]-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetic acid,

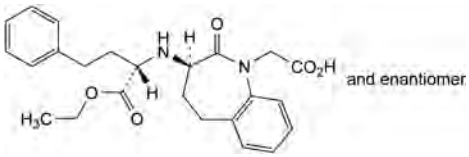


B. [(3*RS*)-3-[[(1*SR*)-1-(ethoxycarbonyl)-3-phenylpropyl]-amino]-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetic acid,

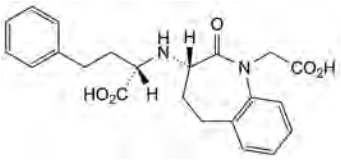

C. (2*S*)-2-[[(3*S*)-1-(carboxymethyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-3-yl]amino]-4-phenylbutanoic acid,

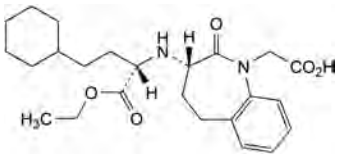

D. [(3*S*)-3-[[(1*S*)-3-cyclohexyl-1-(ethoxycarbonyl)propyl]-amino]-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetic acid,

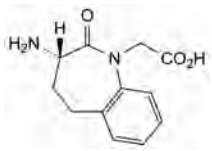

E. [(3*S*)-3-amino-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetic acid,

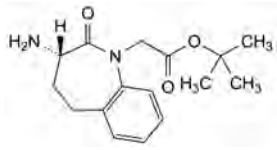

F. 1,1-dimethylethyl [(3*S*)-3-amino-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-1-yl]acetate,

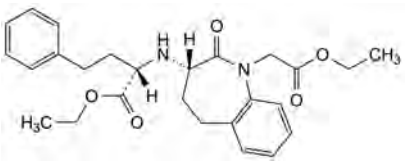

G. ethyl (2*S*)-2-[[(3*S*)-1-(2-ethoxy-2-oxoethyl)-2-oxo-2,3,4,5-tetrahydro-1*H*-1-benzazepin-3-yl]amino]-4-phenylbutanoate.

**01/2008:0370**
**corrected 6.0**

# BENDROFLUMETHIAZIDE

## Bendroflumethiazidum

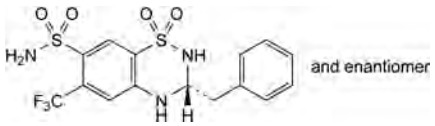


$C_{15}H_{14}F_3N_3O_4S_2$ $M_r$ 421.4
[73-48-3]

DEFINITION

(3*RS*)-3-Benzyl-6-(trifluoromethyl)-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bendroflumethiazide CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 40 volumes of *methanol R* and 60 volumes of a 2.0 g/L solution of *citric acid monohydrate R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2 mg of *bendroflumethiazide impurity A CRS* and 2.5 mg of *altizide CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture. Mix 1 mL of this solution with 1 mL of the test solution and dilute to 100 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 15 volumes of *tetrahydrofuran R*, 25 volumes of *methanol R* and 60 volumes of a 2.0 g/L solution of *citric acid monohydrate R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of bendroflumethiazide.

*Relative retention* with reference to bendroflumethiazide (retention time = about 8 min): impurity A = about 0.2; altizide = about 0.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 10 between the peaks due to altizide and bendroflumethiazide.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *dimethyl sulfoxide R*. Titrate to the 2nd point of inflexion with *0.1 M tetrabutylammonium hydroxide in 2-propanol*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 21.07 mg of $C_{15}H_{14}F_3N_3O_4S_2$.

IMPURITIES

*Specified impurities: A.*

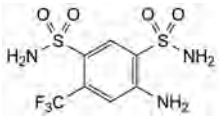

A. 4-amino-6-(trifluoromethyl)benzene-1,3-disulfonamide.

**07/2011:1172**

# BENPERIDOL

## Benperidolum

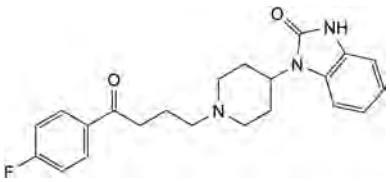

$C_{22}H_{24}FN_3O_2$ $M_r$ 381.4

[2062-84-2]

DEFINITION

1-[1-[4-(4-Fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benperidol CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methyl isobutyl ketone R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (a).* Dissolve 30 mg of *benperidol CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dissolve 30 mg of *benperidol CRS* and 30 mg of *droperidol CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetone R*, *methanol R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 5 mL of *anhydrous ethanol R*. Add 0.5 mL of *dinitrobenzene solution R* and 0.5 mL of *2 M alcoholic potassium hydroxide R*. A violet colour is produced which becomes brownish-red after 20 min.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.5 mg of *benperidol CRS* and 2.5 mg of *droperidol CRS* in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 10 g/L solution of *tetrabutylammonium hydrogen sulfate R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 → 60 | 0 → 40 |
| 15 - 20 | 60 | 40 |
| 20 - 25 | 100 | 0 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL.

*Relative retention* with reference to benperidol (retention time = about 6.5 min): impurity A = about 0.2; impurity B = about 0.9; droperidol = about 1.1; impurity D = about 1.2; impurity E = about 1.3; impurity C = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to benperidol and droperidol.

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 38.14 mg of $C_{22}H_{24}FN_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

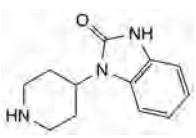

A. 1-(piperidin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,

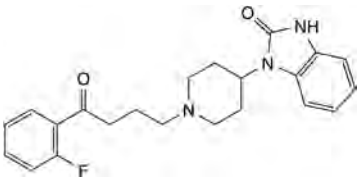

B. 1-[1-[4-(2-fluorophenyl)-4-oxobutyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

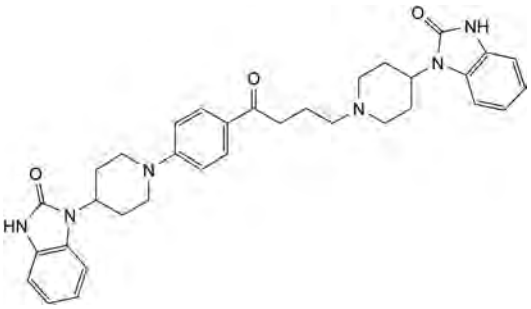

C. 1-[1-[4-oxo-4-[4-[4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]phenyl]butyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

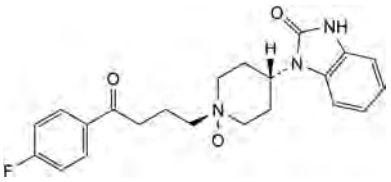

D. *cis*-1-[1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl 1-oxide]-1,3-dihydro-2*H*-benzimidazol-2-one,

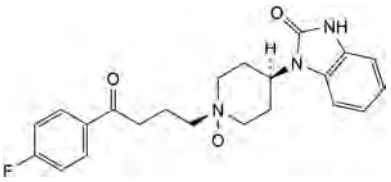

E. *trans*-1-[1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl 1-oxide]-1,3-dihydro-2*H*-benzimidazol-2-one.

**01/2020:1173**

# BENSERAZIDE HYDROCHLORIDE

## Benserazidi hydrochloridum

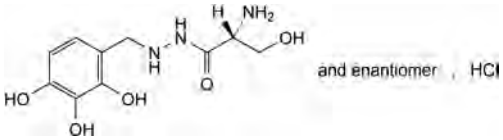


$C_{10}H_{16}ClN_3O_5$ $M_r$ 293.7
[14919-77-8]

DEFINITION

(2*RS*)-2-Amino-3-hydroxy-*N*′-[(2,3,4-trihydroxyphenyl)methyl]propanehydrazide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white or orange-white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benserazide hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in hot *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Solution S (see Tests) gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *All solutions must be injected immediately or stored at 4 °C.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5.0 mg of *benserazide impurity A CRS* and 5.0 mg of *benserazide impurity C CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 5 mg of *benserazide for peak identification A CRS* (containing impurity B) in 5 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.2 g of *sodium heptanesulfonate monohydrate R* and 6.8 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, add 50 mL of *methanol R2* and adjust to pH 3.5 with *phosphoric acid R*;
- *mobile phase B*: dissolve 2.2 g of *sodium heptanesulfonate monohydrate R* and 6.8 g of *potassium dihydrogen phosphate R* in 500 mL of *water for chromatography R*, adjust to pH 3.5 with *phosphoric acid R* and add 500 mL of *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 → 0 | 0 → 100 |
| 15 - 25 | 0 | 100 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C; use the chromatogram supplied with *benserazide for peak identification A CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; doubling of the peak due to impurity C, related to separation of the (*EZ*)-isomers, may be observed.

*Relative retention* with reference to benserazide (retention time = about 9 min): impurity A = about 0.6; impurity C = about 1.2; impurity B = about 1.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to benserazide and impurity C; use the 1st peak of impurity C if 2 peaks occur.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.7;
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity C*: not more than the area of the corresponding peak or pair of peaks in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g. Use as the solvent a solution containing 30 mL of *formamide R*, 30 mL of *methanol R* and 7.0 g of *salicylic acid R* (the *salicylic acid R* must be added to the solvent mixture in the titration vessel).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.250 g in 5 mL of *anhydrous formic acid R*. Add 70 mL of *anhydrous acetic acid R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.37 mg of $C_{10}H_{16}ClN_3O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

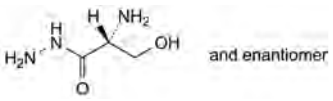


A. (2*RS*)-2-amino-3-hydroxypropanehydrazide,

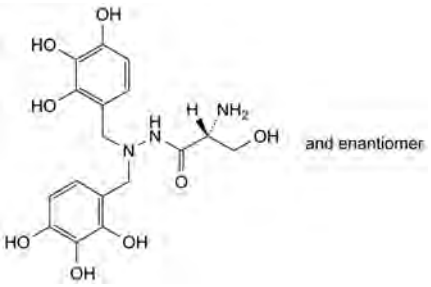


B. (2*RS*)-2-amino-3-hydroxy-*N*′,*N*′-bis[(2,3,4-trihydroxyphenyl)methyl]propanehydrazide,

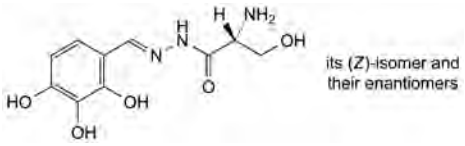


C. (2*RS*)-2-amino-3-hydroxy-*N*′-[(*EZ*)-(2,3,4-trihydroxyphenyl)methylidene]propanehydrazide.

**01/2017:0467**

# BENTONITE

## Bentonitum

DEFINITION

Natural clay containing a high proportion of montmorillonite, a native hydrated aluminium silicate in which some aluminium and silicon atoms may be replaced by other atoms such as magnesium and iron.

CHARACTERS

*Appearance*: very fine, homogeneous, greyish-white powder with a more or less yellowish or pinkish tint.

*Solubility*: practically insoluble in water and in aqueous solutions.

It swells with a little water forming a malleable mass.

IDENTIFICATION

A. To 0.5 g in a metal crucible add 1 g of *potassium nitrate R* and 3 g of *sodium carbonate R* and heat until the mixture melts. Allow to cool. To this residue add 20 mL of boiling *water R*, mix and filter. Wash the insoluble residue with 50 mL of *water R*. To this residue add 1 mL of *hydrochloric acid R* and 5 mL of *water R*. Filter. To the filtrate add 1 mL of *strong sodium hydroxide solution R* and filter. To this filtrate add 3 mL of *ammonium chloride solution R*. A gelatinous white precipitate is formed.

B. Add 2.0 g in 20 portions to 100 mL of a 10 g/L solution of *sodium laurilsulfate R* in a 100 mL graduated cylinder about 30 mm in diameter. Allow 2 min between additions for each portion to settle. Allow to stand for 2 h. The apparent volume of the sediment is not less than 22 mL.

C. 0.25 g gives the reaction of silicates (*2.3.1*).

TESTS

**Alkalinity**. To 2 g add 100 mL of *carbon dioxide-free water R* and shake for 5 min. To 5 mL of this suspension add 0.1 mL of *thymolphthalein solution R*. The liquid becomes bluish. Add 0.1 mL of *0.1 M hydrochloric acid*. The liquid is decolourised within 5 min.

**Coarse particles**: maximum 0.5 per cent.

To 20 g add 1000 mL of *water R* and mix for 15 min using a high-speed mixer capable of operating at not less than 5000 r/min. Transfer the suspension to a wet sieve (75), tared after drying at 100-105 °C. Wash with 3 quantities, each of 500 mL, of *water R*, ensuring that any agglomerates have been dispersed. Dry the sieve at 100-105 °C and weigh. The particles on the sieve weigh a maximum of 0.1 g.

**Loss on drying** (*2.2.32*): maximum 15 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for bentonite used as viscosity-increasing agent or suspending agent.*

**Sedimentation volume**. To 6.0 g add 200 mL of *water R* and mix for 20 min using a high-speed mixer capable of operating at 10 000 r/min. Transfer 100 mL of this suspension to a graduated cylinder. Allow to stand for 24 h. The volume of the clear supernatant is not greater than 2 mL.

**Swelling power with water**: see Identification B.

**04/2009:0372**
**corrected 10.0**

# BENZALKONIUM CHLORIDE

## Benzalkonii chloridum

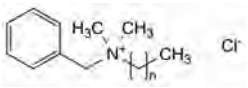

[8001-54-5]

DEFINITION

Mixture of alkylbenzyldimethylammonium chlorides, the alkyl groups mainly having chain lengths of $C_{12}$, $C_{14}$ and $C_{16}$.

*Content*: 95.0 per cent to 104.0 per cent of alkylbenzyldimethylammonium chlorides (anhydrous substance) calculated using the average relative molecular mass (see Tests).

CHARACTERS

*Appearance*: white or yellowish-white powder or gelatinous, yellowish-white fragments, hygroscopic. On heating it forms a clear molten mass.

*Solubility*: very soluble in water and in ethanol (96 per cent). An aqueous solution froths copiously when shaken.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 80 mg in *water R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 257 nm, 263 nm and 269 nm.

*Shoulder*: at about 250 nm.

B. Examine the chromatograms obtained in the test for average relative molecular mass and ratio of alkyl components.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to the principal peaks in the chromatogram obtained with the reference solution.

C. To 2 mL of solution S (see Tests) add 0.1 mL of *glacial acetic acid R* and, dropwise, 1 mL of *sodium tetraphenylborate solution R*. A white precipitate is formed. Filter. Dissolve the precipitate in a mixture of 1 mL of *acetone R* and 5 mL of *ethanol (96 per cent) R*, heating to not more than 70 °C. Add *water R* dropwise to the warm solution until a slight opalescence forms. Heat gently until the solution is clear and allow to cool. White crystals separate. Filter, wash with 3 quantities, each of 10 mL, of *water R* and dry *in vacuo* (*2.2.32*) at a temperature not exceeding 50 °C. The crystals melt (*2.2.14*) at 127 °C to 133 °C.

D. To 5 mL of *dilute sodium hydroxide solution R* add 0.1 mL of *bromophenol blue solution R1* and 5 mL of *methylene chloride R* and shake. The methylene chloride layer is colourless. Add 0.1 mL of solution S and shake. The methylene chloride layer becomes blue.

E. To 2 mL of solution S add 1 mL of *dilute nitric acid R*. A white precipitate is formed which dissolves on the addition of 5 mL of *ethanol (96 per cent) R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.1 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Average relative molecular mass and ratio of alkyl components**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.400 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution*. Dissolve the contents of a vial of *benzalkonium chloride for system suitability CRS* in 5.0 mL of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped nitrile silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 45 volumes of *acetonitrile R* and 55 volumes of a 13.6 g/L solution of *sodium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of homologues*: use the chromatogram supplied with *benzalkonium chloride for system suitability CRS* and the chromatogram obtained with the reference solution to identify the peaks due to $C_{12}$, $C_{14}$ and $C_{16}$.

*Relative retention* with reference to $C_{12}$ homologue (retention time = about 6 min): $C_{14}$ homologue = about 1.3; $C_{16}$ homologue = about 1.7.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to the $C_{12}$ and $C_{14}$ homologues.

Calculate the average relative molecular mass of the sample by summing the products for each homologue, using the following expression:

$$W\left(\frac{A}{B}\right)$$

*A* = area of the peak due to the given homologue in the chromatogram obtained with the test solution;

*B* = sum of the areas of the peaks due to all homologues in the chromatogram obtained with the test solution;

*W* = relative molecular mass for the given homologue: 340, 368 and 396 for the $C_{12}$, $C_{14}$ and $C_{16}$ homologues, respectively.

Calculate the percentage of each homologue, using the following expression:

$$100\left(\frac{C}{D}\right)$$

*C* = product of the relative molecular mass of the given homologue and the area of the corresponding peak in the chromatogram obtained with the test solution;

*D* = sum of the *C* values for all homologues quantified.

*Limits*:

– *$C_{12}$ homologue*: minimum 40 per cent;

– *$C_{14}$ homologue*: minimum 20 per cent;

– *sum of $C_{12}$ and $C_{14}$ homologues*: minimum 70 per cent.

**Impurities A, B and C**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.50 g of the substance to be examined in *methanol R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 25.0 mg of *benzyl alcohol CRS* (impurity A) in *methanol R1* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 75.0 mg of *benzaldehyde CRS* (impurity B) in *methanol R1* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *methanol R1*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 1.09 g of *sodium hexanesulfonate R* and 6.9 g of *sodium dihydrogen phosphate monohydrate R* in *water R*; adjust to pH 3.5 with *phosphoric acid R* and dilute to 1000.0 mL with the same solvent;

– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 | 20 |
| 10 - 14 | 80 → 50 | 20 → 50 |
| 14 - 35 | 50 | 50 |
| 35 - 36 | 50 → 20 | 50 → 80 |
| 36 - 55 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm for impurities A and C, and at 257 nm for impurity B.

*Injection*: 20 µL.

*Relative retention* with reference to impurity A (retention time = about 10 min): impurity B = about 1.3; impurity C = about 2.4.

*System suitability*: at 210 nm:

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c);

– *symmetry factor*: minimum 0.6 for the peak due to impurity A in the chromatogram obtained with reference solution (a).

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.3;
– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *impurity C*: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Amines and amine salts**. Dissolve 5.0 g with heating in 20 mL of a mixture of 3 volumes of *1 M hydrochloric acid* and 97 volumes of *methanol R* and add 100 mL of *2-propanol R*. Pass a stream of *nitrogen R* slowly through the solution. Titrate with up to 12.0 mL of *0.1 M tetrabutylammonium hydroxide* and record the potentiometric titration curve (*2.2.20*). If the curve shows 2 points of inflexion, the volume of titrant added between the 2 points is not greater than 5.0 mL. If the curve shows no point of inflexion, the substance to be examined does not comply with the test. If the curve shows 1 point of inflexion, repeat the test but add 3.0 mL of a 25.0 g/L solution of *dimethyldecylamine R* in *2-propanol R* before the titration. If the titration curve after addition of 12.0 mL of the titrant shows only 1 point of inflexion, the substance to be examined does not comply with the test.

**Water** (*2.5.12*): maximum 10 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.00 g in *water R* and dilute to 100.0 mL with the same solvent. Transfer 25.0 mL of the solution to a separating funnel, add 25 mL of *methylene chloride R*, 10 mL of *0.1 M sodium hydroxide* and 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R*. Shake well, allow to separate and discard the methylene chloride layer. Shake the aqueous layer with 3 quantities, each of 10 mL, of *methylene chloride R* and discard the methylene chloride layers. To the aqueous layer add 40 mL of *hydrochloric acid R*, allow to cool and titrate with *0.05 M potassium iodate* until the deep-brown colour is almost discharged. Add 5 mL of *methylene chloride R* and continue the titration, shaking vigorously, until the methylene chloride layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 mL of the freshly prepared 50 g/L solution of *potassium iodide R*, 20 mL of *water R* and 40 mL of *hydrochloric acid R*.

1 mL of *0.05 M potassium iodate* is equivalent to $\frac{x}{10}$ mg of benzalkonium chloride where $x$ is the average relative molecular mass of the sample.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C.*

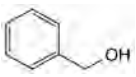


A. benzyl alcohol,

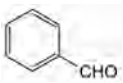


B. benzaldehyde,

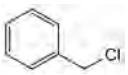


C. (chloromethyl)benzene.

**04/2009:0371**
**corrected 10.0**

# BENZALKONIUM CHLORIDE SOLUTION

## Benzalkonii chloridi solutio

DEFINITION

Aqueous solution of a mixture of alkylbenzyldimethylammonium chlorides, the alkyl groups mainly having chain lengths of $C_{12}$, $C_{14}$ and $C_{16}$.

*Content*: 475 g/L to 525 g/L of alkylbenzyldimethylammonium chlorides, calculated using the average relative molecular mass (see Tests). The solution may contain ethanol (96 per cent).

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish liquid.

*Solubility*: miscible with water and with ethanol (96 per cent). It froths copiously when shaken.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dilute 0.3 mL to 100.0 mL with *water R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 257 nm, 263 nm and 269 nm.

*Shoulder*: at about 250 nm.

B. Examine the chromatograms obtained in the test for average relative molecular mass and ratio of alkyl components.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to the principal peaks in the chromatogram obtained with the reference solution.

C. To 0.05 mL add 2 mL of *water R*, 0.1 mL of *glacial acetic acid R* and, dropwise, 1 mL of *sodium tetraphenylborate solution R*. A white precipitate is formed. Filter. Dissolve the precipitate in a mixture of 1 mL of *acetone R* and 5 mL of *ethanol (96 per cent) R*, heating to not more than 70 °C. Add *water R* dropwise to the warm solution until a slight opalescence forms. Heat gently until the solution is clear and allow to cool. White crystals separate. Filter, wash with 3 quantities, each of 10 mL, of *water R* and dry *in vacuo* (*2.2.32*) at a temperature not exceeding 50 °C. The crystals melt (*2.2.14*) at 127 °C to 133 °C.

D. To 5 mL of *dilute sodium hydroxide solution R* add 0.1 mL of *bromophenol blue solution R1* and 5 mL of *methylene chloride R* and shake. The methylene chloride layer is colourless. Add 0.05 mL of the solution to be examined and shake. The methylene chloride layer becomes blue.

E. To 0.05 mL add 1 mL of *dilute nitric acid R*. A white precipitate is formed which dissolves on the addition of 5 mL of *ethanol (96 per cent) R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dilute 2.0 g to 100 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.1 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Average relative molecular mass and ratio of alkyl components**. Liquid chromatography (*2.2.29*).

*Test solution*. Determine the density (*2.2.5*) of the solution to be examined. Dilute a quantity of the solution to be examined equivalent to about 0.400 g of benzalkonium chloride to 100.0 mL with *water R*.

*Reference solution*. Dissolve the contents of a vial of *benzalkonium chloride for system suitability CRS* in 5.0 mL of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped nitrile silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 45 volumes of *acetonitrile R* and 55 volumes of a 13.6 g/L solution of *sodium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of homologues*: use the chromatogram supplied with *benzalkonium chloride for system suitability CRS* and the chromatogram obtained with the reference solution to identify the peaks due to homologues $C_{12}$, $C_{14}$ and $C_{16}$.

*Relative retention* with reference to $C_{12}$ homologue (retention time = about 6 min): $C_{14}$ homologue = about 1.3; $C_{16}$ homologue = about 1.7.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to the $C_{12}$ and $C_{14}$ homologues.

Calculate the average relative molecular mass of the sample by summing the products for each homologue, using the following expression:

$$W\left(\frac{A}{B}\right)$$

*A* = area of the peak due to the given homologue in the chromatogram obtained with the test solution;

*B* = sum of the areas of the peaks due to all homologues in the chromatogram obtained with the test solution;

*W* = relative molecular mass for the given homologue: 340, 368 and 396 for the $C_{12}$, $C_{14}$ and $C_{16}$ homologues, respectively.

Calculate the percentage of each homologue, using the following expression:

$$100\left(\frac{C}{D}\right)$$

*C* = product of the relative molecular mass of the given homologue and the area of the corresponding peak in the chromatogram obtained with the test solution;

*D* = sum of the *C* values for all homologues quantified.

*Limits*:

- *$C_{12}$ homologue*: minimum 40 per cent;
- *$C_{14}$ homologue*: minimum 20 per cent;
- *sum of $C_{12}$ and $C_{14}$ homologues*: minimum 70 per cent.

**Impurities A, B and C**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Determine the density (*2.2.5*) of the solution to be examined. Dilute a quantity of the solution to be examined equivalent to 2.5 g of benzalkonium chloride to 50.0 mL with *methanol R1*.

*Reference solution (a)*. Dissolve 25.0 mg of *benzyl alcohol CRS* (impurity A) in *methanol R1* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 75.0 mg of *benzaldehyde CRS* (impurity B) in *methanol R1* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with *methanol R1*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.09 g of *sodium hexanesulfonate R* and 6.9 g of *sodium dihydrogen phosphate monohydrate R* in *water R*; adjust to pH 3.5 with *phosphoric acid R* and dilute to 1000.0 mL with the same solvent;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 | 20 |
| 10 - 14 | 80 → 50 | 20 → 50 |
| 14 - 35 | 50 | 50 |
| 35 - 36 | 50 → 20 | 50 → 80 |
| 36 - 55 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm for impurities A and C, and at 257 nm for impurity B.

*Injection*: 20 µL.

*Relative retention* with reference to impurity A (retention time = about 10 min): impurity B = about 1.3; impurity C = about 2.4.

*System suitability*: at 210 nm:

- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c);
- *symmetry factor*: minimum 0.6 for the peak due to impurity A in the chromatogram obtained with reference solution (a).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.3;
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *impurity C*: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Amines and amine salts**. Mix 10.0 g, while heating, with 20 mL of a mixture of 3 volumes of *1 M hydrochloric acid* and 97 volumes of *methanol R* and add 100 mL of *2-propanol R*. Pass a stream of *nitrogen R* slowly through the solution. Titrate with up to 12.0 mL of *0.1 M tetrabutylammonium hydroxide* and record the potentiometric titration curve (*2.2.20*). If the curve shows 2 points of inflexion, the volume of titrant added

between the 2 points is not greater than 5.0 mL. If the curve shows no point of inflexion, the solution to be examined does not comply with the test. If the curve shows 1 point of inflexion, repeat the test but add 3.0 mL of a 25.0 g/L solution of *dimethyldecylamine R* in *2-propanol R* before the titration. If the titration curve after the addition of 12.0 mL of the titrant shows only 1 point of inflexion, the solution to be examined does not comply with the test.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Determine the density (*2.2.5*) of the solution to be examined. Dilute 4.00 g to 100.0 mL with *water R*. Transfer 25.0 mL of the solution to a separating funnel, add 25 mL of *methylene chloride R*, 10 mL of *0.1 M sodium hydroxide* and 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R*. Shake well, allow to separate and discard the methylene chloride layer. Shake the aqueous layer with 3 quantities, each of 10 mL, of *methylene chloride R* and discard the methylene chloride layers. To the aqueous layer add 40 mL of *hydrochloric acid R*, allow to cool and titrate with *0.05 M potassium iodate* until the deep-brown colour is almost discharged. Add 5 mL of *methylene chloride R* and continue the titration, shaking vigorously, until the methylene chloride layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 mL of the freshly prepared 50 g/L solution of *potassium iodide R*, 20 mL of *water R* and 40 mL of *hydrochloric acid R*.

1 mL of *0.05 M potassium iodate* is equivalent to $\frac{x}{10}$ mg of benzalkonium chloride where $x$ is the average relative molecular mass of the sample.

LABELLING

The label states the content of ethanol (96 per cent), if any.

IMPURITIES

*Specified impurities: A, B, C.*

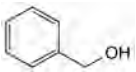


A. benzyl alcohol,

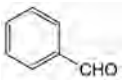


B. benzaldehyde,

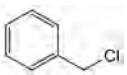


C. (chloromethyl)benzene.

**01/2017:1393**

# BENZBROMARONE

## Benzbromaronum

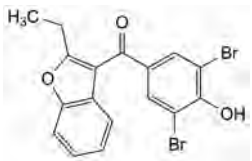


$C_{17}H_{12}Br_2O_3$ $M_r$ 424.1
[3562-84-3]

DEFINITION

(3,5-Dibromo-4-hydroxyphenyl)(2-ethylbenzofuran-3-yl)-methanone.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, sparingly soluble in ethanol (96 per cent).

mp: about 152 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *benzbromarone CRS*.

B. By means of a copper wire, previously ignited, introduce a small amount of the substance to be examined into the non-luminous part of a flame. The colour of the flame becomes green.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.25 g in *dimethylformamide R* and dilute to 25 mL with the same solvent.

**Acidity or alkalinity**. Shake 0.5 g with 10 mL of *carbon dioxide-free water R* for 1 min and filter. To 2.0 mL of the filtrate add 0.1 mL of *methyl red solution R* and 0.1 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.3 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in 30 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *benzarone CRS* (impurity C) in the mobile phase and dilute to 20 mL with the mobile phase. To 5 mL of this solution add 1 mL of the test solution and dilute to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R*, *methanol R* (5:25:300:990 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 231 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of benzbromarone.

*Relative retention* with reference to benzbromarone: impurity A = about 0.6; impurity B = about 2.

*System suitability*: reference solution (b):

- *resolution*: minimum 10.0 between the peaks due to impurity C (1st peak) and benzbromarone (2nd peak).

*Limits*:

- *impurity A*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity B*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

- *sum of impurities other than A and B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Halides expressed as chlorides** (*2.4.4*): maximum 400 ppm.

Shake 1.25 g with a mixture of 5 mL of *dilute nitric acid R* and 15 mL of *water R*. Filter. Rinse the filter with *water R* and dilute the filtrate to 25 mL with the same solvent. Dilute 2.5 mL of this solution to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 125 ppm.

Moisten the residue obtained in the test for sulfated ash with 2 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Add 0.05 mL of *hydrochloric acid R* and 10 mL of *water R*, heat to boiling and maintain boiling for 1 min. Allow to cool. Rinse the crucible with *water R*, collect the rinsings and dilute to 25 mL with *water R*. Dilute 2 mL of this solution to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 mL of *methanol R*. Stir until completely dissolved and add 10 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 42.41 mg of $C_{17}H_{12}Br_2O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

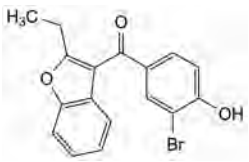

A. (3-bromo-4-hydroxyphenyl)(2-ethylbenzofuran-3-yl)methanone,

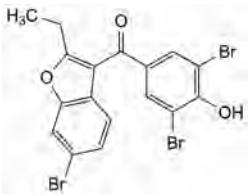

B. (6-bromo-2-ethylbenzofuran-3-yl)(3,5-dibromo-4-hydroxyphenyl)methanone,

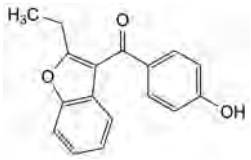

C. (2-ethylbenzofuran-3-yl)(4-hydroxyphenyl)methanone (benzarone).

**01/2008:0974**
**corrected 6.0**

# BENZETHONIUM CHLORIDE

## Benzethonii chloridum

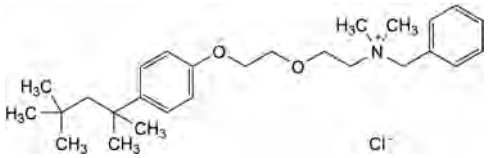

$C_{27}H_{42}ClNO_2$ $M_r$ 448.1
[121-54-0]

DEFINITION

*N*-Benzyl-*N*,*N*-dimethyl-2-[2-[4-(1,1,3,3-tetramethylbutyl)-phenoxy]ethoxy]ethanaminium chloride.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: very soluble in water and in ethanol (96 per cent), freely soluble in methylene chloride.

An aqueous solution froths copiously when shaken.

IDENTIFICATION

A. Melting point (*2.2.14*): 158 °C to 164 °C, after drying at 105 °C for 4 h.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution*. Dissolve 25 mg of *benzethonium chloride CRS* in *water R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (5:5:100 *V/V/V*).

*Application*: 20 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of warm air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 5 mL of *dilute sodium hydroxide solution R* add 0.1 mL of *bromophenol blue solution R1* and 5 mL of *methylene chloride R* and shake. The lower layer is colourless. Add 0.1 mL of solution S (see Tests) and shake. A blue colour develops in the lower layer.

D. To 2 mL of solution S add 1 mL of *dilute nitric acid R*. A white precipitate is formed which dissolves upon addition of 5 mL of *ethanol (96 per cent) R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 25 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.3 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.1 mL of *methyl red solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is orange-red.

**Volatile bases and salts of volatile bases** (*2.4.1, Method B*): maximum 50 ppm, determined on 0.20 g.

Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*. Replace heavy magnesium oxide by 2.0 mL of *strong sodium hydroxide solution R*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 100.0 mL with the same solvent. Transfer 25.0 mL of the solution to a separating funnel, add 10 mL of a 4 g/L solution of *sodium hydroxide R*, 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R* and 25 mL of *methylene chloride R*. Shake vigorously, allow to separate and discard the lower layer. Shake the upper layer with 3 quantities, each of 10 mL, of *methylene chloride R* and discard the lower layers. To the upper layer add 40 mL of *hydrochloric acid R*, allow to cool and titrate with *0.05 M potassium iodate* until the deep brown colour is almost discharged. Add 4 mL of *methylene chloride R* and continue the titration, shaking vigorously, until the lower layer is no longer brown. Carry out a blank titration using a mixture of 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R*, 20 mL of *water R* and 40 mL of *hydrochloric acid R*.

1 mL of *0.05 M potassium iodate* is equivalent to 44.81 mg of $C_{27}H_{42}ClNO_2$.

STORAGE

Protected from light.

**07/2015:0011**
**corrected 9.4**

# BENZOCAINE

## Benzocainum

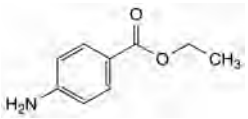

$C_9H_{11}NO_2$ $M_r$ 165.2
[94-09-7]

DEFINITION

Ethyl 4-aminobenzoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benzocaine CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (50:50 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 5 mL of *acetonitrile R1* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *4-nitrobenzoic acid R* (impurity E) in 10.0 mL of the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *perchloric acid R* to 100 mL with *water for chromatography R*; dilute 1 mL of the solution to 100 mL with *water for chromatography R*; mix 9 volumes of this solution and 1 volume of *acetonitrile R1*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 15 | 100 → 38.5 | 0 → 61.5 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to benzocaine (retention time = about 10 min): impurity E = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity E and benzocaine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of benzocaine in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using 0.400 g dissolved in a mixture of 25 mL of *hydrochloric acid R* and 50 mL of *water R*.

1 mL of *0.1 M sodium nitrite* is equivalent to 16.52 mg of $C_9H_{11}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H.*

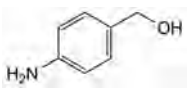

A. (4-aminophenyl)methanol,

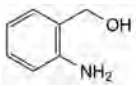

B. (2-aminophenyl)methanol,

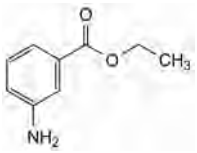

C. ethyl 3-aminobenzoate,

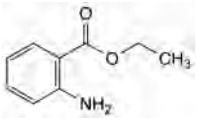

D. ethyl 2-aminobenzoate,

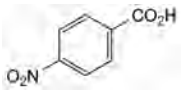

E. 4-nitrobenzoic acid,

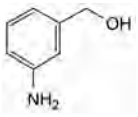

F. (3-aminophenyl)methanol,

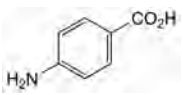

G. 4-aminobenzoic acid,

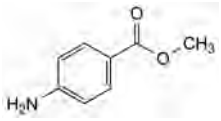

H. methyl 4-aminobenzoate.

**01/2017:0066**

# BENZOIC ACID

## Acidum benzoicum

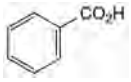

$C_7H_6O_2$ $M_r$ 122.1
[65-85-0]

DEFINITION

Benzenecarboxylic acid.

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, soluble in boiling water, freely soluble in ethanol (96 per cent) and in fatty oils.

IDENTIFICATION

A. Melting point (*2.2.14*): 121 °C to 124 °C.

B. Solution S (see Tests) gives reaction (a) of benzoates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Carbonisable substances**. Dissolve 0.5 g with shaking in 5 mL of *sulfuric acid R*. After 5 min, the solution is not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method I*).

**Oxidisable substances**. Dissolve 0.2 g in 10 mL of boiling *water R*. Cool, shake and filter. To the filtrate add 1 mL of *dilute sulfuric acid R* and 0.2 mL of *0.02 M potassium permanganate*. After 5 min, the solution is still coloured pink.

**Halogenated compounds and halides**: maximum 300 ppm.

*All glassware used must be chloride-free and may be prepared by soaking overnight in a 500 g/L solution of nitric acid R, rinsed with water R and stored full of water R. It is recommended that glassware be reserved for this test.*

*Solution (a)*. Dissolve 6.7 g in a mixture of 40 mL of *1 M sodium hydroxide* and 50 mL of *ethanol (96 per cent) R* and dilute to 100.0 mL with *water R*. To 10.0 mL of this solution add 7.5 mL of *dilute sodium hydroxide solution R* and 0.125 g of *nickel-aluminium alloy R* and heat on a water-bath for 10 min. Allow to cool to room temperature, filter into a 25 mL volumetric flask and wash with 3 quantities, each of 2 mL, of *ethanol (96 per cent) R*. Dilute the filtrate and washings to 25.0 mL with *water R*. This solution is used to prepare solution A.

*Solution (b)*. In the same manner, prepare a similar solution without the substance to be examined. This solution is used to prepare solution B.

In four 25 mL volumetric flasks, place separately 10 mL of solution (a), 10 mL of solution (b), 10 mL of *chloride standard solution (8 ppm Cl) R* (used to prepare solution C) and 10 mL of *water R*. To each flask add 5 mL of *ferric ammonium sulfate solution R5*, mix and add dropwise and with swirling 2 mL of *nitric acid R* and 5 mL of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 mL with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm the absorbance (*2.2.25*) of solution A using solution B as the compensation liquid, and

the absorbance of solution C using the solution obtained with 10 mL of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 mL of *ethanol (96 per cent) R* and titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenol red solution R* as indicator, until the colour changes from yellow to violet-red.

1 mL of *0.1 M sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

**01/2008:0704**
**corrected 7.0**

# BENZOYL PEROXIDE, HYDROUS

## Benzoylis peroxidum cum aqua

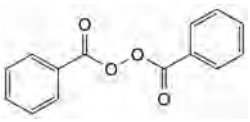

$C_{14}H_{10}O_4$ $M_r$ 242.2 (anhydrous substance)
Anhydrous benzoyl peroxide: [94-36-0]

DEFINITION

*Content*:

– *dibenzoyl peroxide*: 70.0 per cent to 77.0 per cent;
– *water*: minimum 20.0 per cent.

CHARACTERS

*Appearance*: white or almost white, amorphous or granular powder.

*Solubility*: practically insoluble in water, soluble in acetone, soluble in methylene chloride with the separation of water, slightly soluble in ethanol (96 per cent).

It loses water rapidly on exposure to air with a risk of explosion.

*Mix the entire sample thoroughly before carrying out the following tests.*

IDENTIFICATION

*First identification: B*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Solution A*. Dissolve 80.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*.

*Solution B*. Dilute 10.0 mL of solution A to 100.0 mL with *ethanol (96 per cent) R*.

*Spectral ranges*: 250-300 nm for solution A; 220-250 nm for solution B.

*Absorption maxima*: at 274 nm for solution A; at 235 nm for solution B.

*Shoulder*: at about 282 nm for solution A.

*Absorbance ratio*: $A_{235}/A_{274}$ = 1.17 to 1.21.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of hydrous benzoyl peroxide*.

C. Dissolve about 25 mg in 2 mL of *acetone R*. Add 1 mL of a 10 g/L solution of *diethylphenylenediamine sulfate R* and mix. A red colour develops which quickly darkens and becomes dark violet within 5 min.

D. To 1 g add 5 mL of *ethanol (96 per cent) R*, 5 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R*. Boil the mixture under reflux for 20 min. Cool. The solution gives reaction (c) of benzoates (*2.3.1*).

TESTS

**Acidity**. Dissolve a quantity of the substance to be examined containing the equivalent of 1.0 g of dibenzoyl peroxide in 25 mL of *acetone R*, add 75 mL of *water R* and filter. Wash the residue with two quantities, each of 10 mL, of *water R*. Combine the filtrate and the washings and add 0.25 mL of *phenolphthalein solution R1*. Not more than 1.25 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator. Carry out a blank test.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve a quantity of the substance to be examined containing the equivalent of 0.10 g of dibenzoyl peroxide in *acetonitrile R* and dilute to 50 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b)*. Dissolve 30.0 mg of *benzoic acid R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 50.0 mg of *ethyl benzoate R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 50.0 mg of *benzaldehyde R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (e)*. Dissolve 30.0 mg of *benzoic acid R* and 30.0 mg of *benzaldehyde R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (1:500:500 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL loop injector.

*Run time*: 2 times the retention time of dibenzoyl peroxide.

*Relative retention* with reference to dibenzoyl peroxide (retention time = about 28.4 min): impurity B = about 0.15; impurity A = about 0.2; impurity C = about 0.4.

*System suitability*: reference solution (e):

– *resolution*: minimum 6 between the peaks due to benzoic acid and benzaldehyde.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.25 per cent);
– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Chlorides** (*2.4.4*): maximum 0.4 per cent.

Dissolve a quantity of the substance to be examined containing the equivalent of 0.5 g of dibenzoyl peroxide in 15 mL of *acetone R*. Add, while stirring, 50 mL of *0.05 M nitric acid*. Allow to stand for 10 min and filter. Wash the residue with 2 quantities, each of 10 mL, of *0.05 M nitric acid*. Combine the filtrate and the washings and dilute to 100 mL with *0.05 M nitric acid*. Dilute 2.5 mL of the solution to 15.0 mL with *water R*.

ASSAY

*Solution (a)*. Dissolve 2.500 g immediately before use in 75 mL of *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

**Dibenzoyl peroxide**. To 5.0 mL of solution (a) add 20 mL of *acetone R* and 3 mL of a 500 g/L solution of *potassium iodide R* and mix. Allow to stand for 1 min. Titrate with *0.1 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 12.11 mg of $C_{14}H_{10}O_4$.

**Water** (*2.5.12*). Carry out the semi-micro determination of water, using 5.0 mL of solution (a). Use as the solvent a mixture of 20.0 mL of *anhydrous methanol R* and 3.0 mL of a 100 g/L solution of *potassium iodide R* in *dimethylformamide R*. After adding solution (a), stir for 5 min before starting the titration. Carry out a blank determination.

Calculate the percentage content of water using the following expression:

$$\frac{(n_1 - n_2) \times w \times 2}{m} + (p \times 0.0744)$$

$n_1$ = number of millilitres of *iodosulfurous reagent R* used in the sample determination,

$n_2$ = number of millilitres of *iodosulfurous reagent R* used in the blank determination,

$w$ = water equivalent of *iodosulfurous reagent R* in milligrams of water per millilitre of reagent,

$m$ = mass of the substance to be examined used for the preparation of solution (a) in grams,

$p$ = percentage content of dibenzoyl peroxide.

STORAGE

In a container that has been treated to reduce static discharge and that has a device for release of excess pressure, at a temperature of 2 °C to 8 °C, protected from light.

IMPURITIES

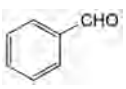


A. benzaldehyde,

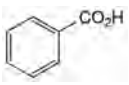


B. benzoic acid,

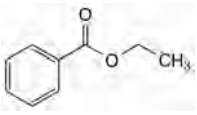


C. ethyl benzoate.

**01/2020:2759**

# BENZYDAMINE HYDROCHLORIDE

## Benzydamini hydrochloridum

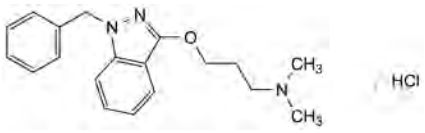


$C_{19}H_{24}ClN_3O$ $M_r$ 345.9
[132-69-4]

DEFINITION

3-[(1-Benzyl-1*H*-indazol-3-yl)oxy]-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

**Impurity G**: maximum 5 ppm, determined by a suitable, validated method.

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride, slightly soluble in acetone, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *benzydamine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *benzydamine for system suitability CRS* (containing impurities A, B and D) in 5 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amidoalkylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 1.36 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, adjust to pH 3.0 with *phosphoric acid R*, add 1.16 g of *sodium octanesulfonate R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 50 | 50 |
| 2 - 22 | 50 → 35 | 50 → 65 |
| 22 - 29 | 35 | 65 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 320 nm.

*Equilibration*: at least 10 min with the mobile phase at the initial composition.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *benzydamine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to benzydamine (retention time = about 12 min): impurity A = about 1.36; impurity D = about 1.43; impurity B = about 2.0.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurities A and D in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 38 for the principal peak in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity D = 1.4;
- for each impurity, use the concentration of benzydamine hydrochloride in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.5 per cent;
- *impurity A*: maximum 0.2 per cent;
- *impurity D*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.59 mg of $C_{19}H_{24}ClN_3O$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F.*

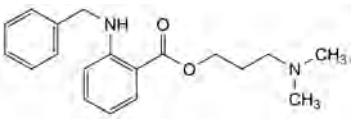

A. 3-(dimethylamino)propyl 2-(benzylamino)benzoate,

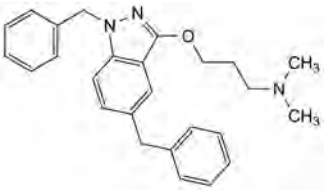

B. 3-[(1,5-dibenzyl-1*H*-indazol-3-yl)oxy]-*N*,*N*-dimethylpropan-1-amine,

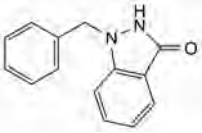

C. 1-benzyl-1,2-dihydro-3*H*-indazol-3-one,

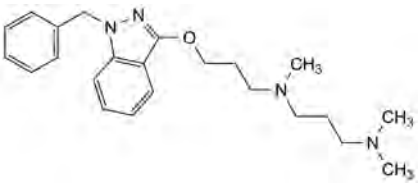

D. $N^1$-[3-[(1-benzyl-1*H*-indazol-3-yl)oxy]propyl]-$N^1$,$N^3$,$N^3$-trimethylpropane-1,3-diamine,

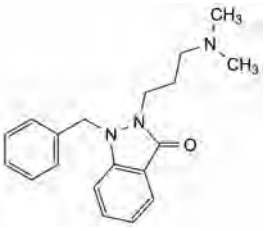

E. 1-benzyl-2-[3-(dimethylamino)propyl]-1,2-dihydro-3*H*-indazol-3-one,

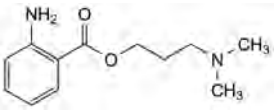

F. 3-(dimethylamino)propyl 2-aminobenzoate,

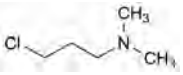

G. 3-chloro-*N*,*N*-dimethylpropan-1-amine.

**04/2018:0256**
**corrected 10.0**

# BENZYL ALCOHOL[(1)]

## Alcohol benzylicus

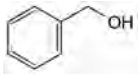

$C_7H_8O$ $M_r$ 108.1
[100-51-6]

DEFINITION

Phenylmethanol.

*Content*: 98.0 per cent to 100.5 per cent.

♦CHARACTERS

*Appearance*: clear, colourless, oily liquid.

*Solubility*: soluble in water, miscible with ethanol (96 per cent) and with fatty and essential oils.

*Relative density*: 1.043 to 1.049.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benzyl alcohol CRS*.♦

TESTS

♦**Appearance of solution**. Shake 2.0 mL with 60 mL of *water R*. It dissolves completely. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).♦

**Acidity**. To 10 mL add 10 mL of *ethanol (96 per cent) R* and 1 mL of *phenolphthalein solution R*. Not more than 1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Refractive index** (*2.2.6*): 1.538 to 1.541.

**Peroxide value** (*2.5.5, Method A*): maximum 5.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Standard solution (a)*. Dissolve 0.100 g of *ethylbenzene R* in the test solution and dilute to 10.0 mL with the same solution. Dilute 2.0 mL of this solution to 20.0 mL with the test solution.

*Standard solution (b)*. Dissolve 2.000 g of *dicyclohexyl R* in the test solution and dilute to 10.0 mL with the same solution. Dilute 2.0 mL of this solution to 20.0 mL with the test solution.

*Reference solution (a)*. Dissolve 0.750 g of *benzaldehyde R* and 0.500 g of *cyclohexylmethanol R* in the test solution and dilute to 25.0 mL with the test solution. Add 1.0 mL of this solution to a mixture of 2.0 mL of standard solution (a) and 3.0 mL of standard solution (b) and dilute to 20.0 mL with the test solution.

*Reference solution (b)*. Dissolve 0.250 g of *benzaldehyde R* and 0.500 g of *cyclohexylmethanol R* in the test solution and dilute to 25.0 mL with the test solution. Add 1.0 mL of this solution to a mixture of 2.0 mL of standard solution (a) and 2.0 mL of standard solution (b) and dilute to 20.0 mL with the test solution.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 25 cm/s at 50 °C.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 34 | 50 → 220 |
| | 34 - 69 | 220 |
| Injection port | | 200 |
| Detector | | 310 |

*Detection*: flame ionisation.

*Benzyl alcohol not intended for parenteral administration*

*Injection*: without air-plug, 0.1 µL of the test solution and reference solution (a).

*Relative retention* with reference to benzyl alcohol (retention time = about 26 min): ethylbenzene = about 0.28; dicyclohexyl = about 0.59; impurity A = about 0.68; impurity B = about 0.71.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurities A and B.

If any peaks in the chromatogram obtained with the test solution have the same retention time as the peaks due to ethyl benzene or dicyclohexyl, subtract the areas of any such peaks from the peak areas at these retention times in the chromatograms obtained with reference solutions (a) or (b) (corrected peak areas of ethyl benzene and dicyclohexyl). Any such peaks in the chromatogram obtained with the test solution are to be included in the assessments for the sum of other peaks.

*Limits*:

– *impurity A*: not more than the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.15 per cent);
– *impurity B*: not more than the difference between the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) and the area of the peak due to impurity B in the chromatogram obtained with the test solution (0.10 per cent);
– *sum of other peaks with a relative retention less than that of benzyl alcohol*: not more than 4 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (a) corrected if necessary as described above (0.04 per cent);
– *sum of peaks with a relative retention greater than that of benzyl alcohol*: not more than the area of the peak due to dicyclohexyl in the chromatogram obtained with reference solution (a) corrected if necessary as described above (0.3 per cent);
– *disregard limit*: 0.01 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (a) corrected if necessary as described above (0.0001 per cent).

*Benzyl alcohol intended for parenteral administration*

*Injection*: without air-plug, 0.1 µL of the test solution and reference solution (b).

*Relative retention* with reference to benzyl alcohol (retention time = about 26 min): ethylbenzene = about 0.28; dicyclohexyl = about 0.59; impurity A = about 0.68; impurity B = about 0.71.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurities A and B.

If any peaks in the chromatogram obtained with the test solution have the same retention times as the peaks due to ethyl benzene or dicyclohexyl, subtract the areas of

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

any such peaks from the peak areas at these retention times in the chromatograms obtained with reference solutions (a) or (b) (corrected peak areas of ethyl benzene and dicyclohexyl). Any such peaks in the chromatogram obtained with the test solution are to be included in the assessments for the sum of other peaks.

*Limits*:

- *impurity A*: not more than the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (b) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.05 per cent);
- *impurity B*: not more than the difference between the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) and the area of the peak due to impurity B in the chromatogram obtained with the test solution (0.10 per cent);
- *sum of other peaks with a relative retention less than that of benzyl alcohol*: not more than twice the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (b) corrected if necessary as described above (0.02 per cent);
- *sum of peaks with a relative retention greater than that of benzyl alcohol*: not more than the area of the peak due to dicyclohexyl in the chromatogram obtained with reference solution (b) corrected if necessary as described above (0.2 per cent);
- *disregard limit*: 0.01 times the area of the peak due to ethylbenzene in the chromatogram obtained with reference solution (b) corrected if necessary as described above (0.0001 per cent).

**Residue on evaporation**: maximum 0.05 per cent.

After ensuring that the substance to be examined complies with the test for peroxide value, evaporate 10.0 g to dryness in a tared quartz or porcelain crucible or platinum dish on a hot plate at a temperature not exceeding 200 °C. Ensure that the substance to be examined does not boil during evaporation. Dry the residue on the hot plate for 1 h and allow to cool in a desiccator. The residue weighs a maximum of 5 mg.

ASSAY

To 0.900 g (*m* g) add 15.0 mL of a freshly prepared mixture of 1 volume of *acetic anhydride R* and 7 volumes of *anhydrous pyridine R* and heat under a reflux condenser on a water-bath for 30 min. Cool and add 25 mL of *water R*. Using 0.25 mL of *phenolphthalein solution R* as indicator, titrate with *1 M sodium hydroxide* ($n_1$ mL). Carry out a blank titration ($n_2$ mL).

Calculate the percentage content of $C_7H_8O$ using the following expression:

$$\frac{10.81(n_2 - n_1)}{m}$$

♦STORAGE

In an airtight container, under nitrogen, protected from light and at a temperature between 2 °C and 8 °C.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.♦

IMPURITIES

*Specified impurities: A, B.*

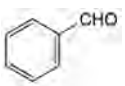


A. benzaldehyde,

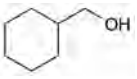


B. cyclohexylmethanol.

**01/2008:0705**
**corrected 10.0**

# BENZYL BENZOATE

## Benzylis benzoas

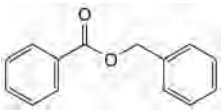


$C_{14}H_{12}O_2$ $M_r$ 212.2
[120-51-4]

DEFINITION

Phenylmethyl benzoate.

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: colourless or almost colourless crystals or colourless or almost colourless, oily liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent), with methylene chloride and with fatty and essential oils.

bp: about 320 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of benzyl benzoate.*

B. To 2 g add 25 mL of *alcoholic potassium hydroxide solution R* and boil under a reflux condenser for 2 h. Remove the ethanol on a water-bath, add 50 mL of *water R* and distill. Collect about 25 mL of distillate and use it for identification test C. Acidify the liquid remaining in the distillation flask with *dilute hydrochloric acid R*. A white precipitate is formed that, when washed with *water R* and dried *in vacuo* melts (*2.2.14*) at 121 °C to 124 °C.

C. To the distillate obtained in identification test B add 2.5 g of *potassium permanganate R* and 5 mL of *dilute sodium hydroxide solution R*. Boil under a reflux condenser for 15 min, cool and filter. Acidify the filtrate with *dilute hydrochloric acid R*. A white precipitate is formed that, when washed with *water R* and dried *in vacuo*, melts (*2.2.14*) at 121 °C to 124 °C.

TESTS

**Acidity**. Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent. Titrate with *0.1 M sodium hydroxide* using *phenolphthalein solution R* as indicator. Not more than 0.2 mL is required to change the colour of the indicator to pink.

**Relative density** (*2.2.5*): 1.118 to 1.122.

**Refractive index** (*2.2.6*): 1.568 to 1.570.

**Freezing point** (*2.2.18*): minimum 17.0 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 2.000 g add 50.0 mL of *0.5 M alcoholic potassium hydroxide* and boil gently under a reflux condenser for 1 h. Titrate the hot solution with *0.5 M hydrochloric acid* using 1 mL of *phenolphthalein solution R* as indicator. Carry out a blank determination.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 106.1 mg of $C_{14}H_{12}O_2$.

STORAGE

In an airtight, well-filled container, protected from light.

**01/2019:0373**
**corrected 10.0**

# BENZYLPENICILLIN (BENZATHINE) TETRAHYDRATE

## Benzathini benzylpenicillinum tetrahydricum

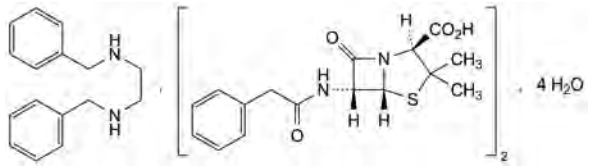

$C_{48}H_{56}N_6O_8S_2,4H_2O$ $M_r$ 981
[41372-02-5]

DEFINITION

$N^1$,$N^2$-Dibenzylethane-1,2-diamine bis[(2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate] tetrahydrate.

Salt obtained from *Benzylpenicillin sodium (0114)* or *Benzylpenicillin potassium (0113)* produced by the growth of certain strains of *Penicillium notatum* or related micro-organisms.

*Content*:

– *benzathine benzylpenicillin*: 94.5 per cent to 102.0 per cent (anhydrous substance) without correction for dispersing or suspending agents;
– *benzathine*: 24.0 per cent to 27.0 per cent (anhydrous substance).

Dispersing or suspending agents (e.g. lecithin and polysorbate 80) may be added.

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: very slightly soluble in water, freely soluble in dimethylformamide and in formamide, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *benzathine benzylpenicillin CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *methanol R.*
*Reference solution.* Dissolve 25 mg of *benzathine benzylpenicillin CRS* in 5 mL of *methanol R.*
*Plate*: *TLC silanised silica gel plate R.*
*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 7.0 with *ammonia R.*
*Application*: 1 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution:
– the chromatogram shows 2 clearly separated spots.
*Results*: the 2 principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the 2 principal spots in the chromatogram obtained with the reference solution.

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R.* Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.

D. To 0.1 g add 2 mL of *1 M sodium hydroxide* and shake for 2 min. Shake the mixture with 2 quantities, each of 3 mL, of *ether R.* Evaporate the combined ether layers to dryness and dissolve the residue in 1 mL of *ethanol (50 per cent V/V) R.* Add 5 mL of *picric acid solution R*, heat at 90 °C for 5 min and allow to cool slowly. Separate the crystals and recrystallise from *ethanol (25 per cent V/V) R* containing 10 g/L of *picric acid R.* The crystals melt (*2.2.14*) at about 214 °C.

TESTS

**Acidity or alkalinity**. To 0.50 g add 100 mL of *carbon dioxide-free water R* and shake for 5 min. Filter through a sintered-glass filter (*2.1.2*). To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1.* The solution is green or yellow. Not more than 0.2 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and by diluting to volume immediately after dissolution.*

*Solution A*. Prepare a solution containing 1.3 g/L of *disodium hydrogen phosphate dodecahydrate R* and 6.8 g/L of *potassium dihydrogen phosphate R.*

*Test solution (a).* Dissolve 40.0 mg of the substance to be examined in 50 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Test solution (b).* Dissolve 70.0 mg of the substance to be examined in 25 mL of *methanol R* and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dissolve 40.0 mg of *benzathine benzylpenicillin CRS* in 50 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (b).* Dissolve 3 mg of *benzathine benzylpenicillin for peak identification CRS* (containing impurities A, B, C, D, E, F, G, H, I, J and K) in 1 mL of *methanol R* and dilute to 2 mL with solution A.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with a mixture of equal volumes of *methanol R* and solution A.

*Reference solution (d).* Dilute 3.0 mL of reference solution (c) to 100.0 mL with a mixture of equal volumes of *methanol R* and solution A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 10 volumes of a 34 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with *phosphoric acid R*, 30 volumes of *methanol R1* and 60 volumes of *water for chromatography R*;

– *mobile phase B*: mix 5 volumes of a 34 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with *phosphoric acid R*, 25 volumes of *water for chromatography R* and 70 volumes of *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 16 | 85 → 0 | 15 → 100 |
| 16 - 26 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *benzathine benzylpenicillin for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G, H, I, J and K.

*Relative retention* with reference to benzylpenicillin (retention time = about 7 min): impurity A = about 0.18; benzathine = about 0.30; impurity D = about 0.36; impurity G = about 0.38; impurity J = about 0.44; impurity E = about 0.51 and 0.60; impurity B = about 0.69; impurity F = about 0.84 and 0.88; impurity H = about 1.22; impurity I = about 1.42; impurity C = about 1.75; impurity K = about 2.90.

*System suitability*:

– *resolution*: minimum 1.0 between the peaks due to the epimers of impurity F and minimum 1.5 between the peaks due to impurities D and G in the chromatogram obtained with reference solution (b);

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 1.9; impurity F = 1.5;

– for each impurity, use the concentration of benzylpenicillin in reference solution (c).

*Limits*:

– *impurity C*: maximum 2.0 per cent;

– *impurity K*: maximum 1.0 per cent;

– *impurity J*: maximum 0.5 per cent;

– *impurities E (sum of isomers), F (sum of epimers)*: for each impurity, maximum 0.3 per cent;

– *impurities A, B, D, G, H, I*: for each impurity, maximum 0.2 per cent;

– *any other impurity*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 3.5 per cent;

– *reporting threshold*: 0.05 per cent; disregard the peak due to benzathine.

**Water** (*2.5.12*): 5.0 per cent to 8.0 per cent, determined on 0.200 g.

**Bacterial endotoxins** (*2.6.14, Method E*): less than 0.13 IU/mL, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

Suspend 20 mg in 20 mL of a solution of *0.1 M sodium hydroxide* diluted 1 to 100, shake thoroughly and centrifuge. Examine the supernatant.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (15:85 *V/V*).

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 1.8 for the peak due to benzylpenicillin.

Calculate the percentage contents of benzathine ($C_{16}H_{20}N_2$) and benzathine benzylpenicillin ($C_{48}H_{56}N_6O_8S_2$) taking into account the assigned content of *benzathine benzylpenicillin CRS*.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J, K.*

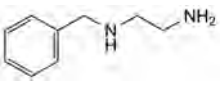

A. $N^1$-benzylethane-1,2-diamine,

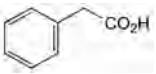

B. phenylacetic acid,

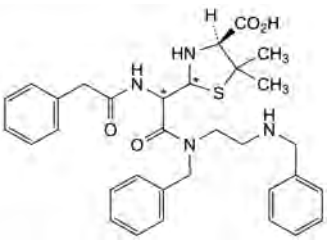

C. (2*Ξ*,4*S*)-2-[(1*Ξ*)-2-[benzyl[2-(benzylamino)ethyl]amino]-2-oxo-1-(2-phenylacetamido)ethyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (benzylpenicilloic acids benzathide),

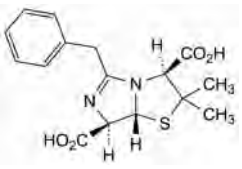

D. (3*S*,7*R*,7a*R*)-5-benzyl-2,2-dimethyl-2,3,7,7a-tetrahydroimidazo[5,1-*b*][1,3]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),

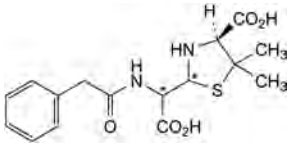

E. (2Ξ,4*S*)-2-[(Ξ)-carboxy(2-phenylacetamido)methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

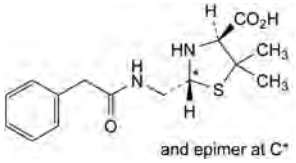


F. (2*RS*,4*S*)-5,5-dimethyl-2-[(2-phenylacetamido)methyl]-1,3-thiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin),

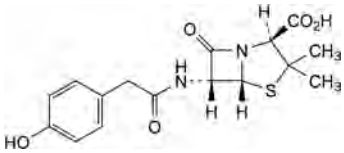

G. (2*S*,5*R*,6*R*)-6-[2-(4-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

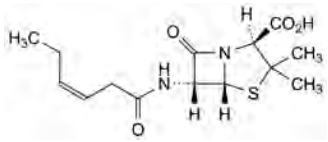

H. (2*S*,5*R*,6*R*)-6-[(3*Z*)-hex-3-enamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (isopenicillin F),

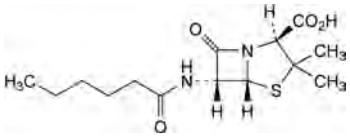

I. (2*S*,5*R*,6*R*)-6-hexanamido-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (dihydropenicillin F),

J. unknown structure,

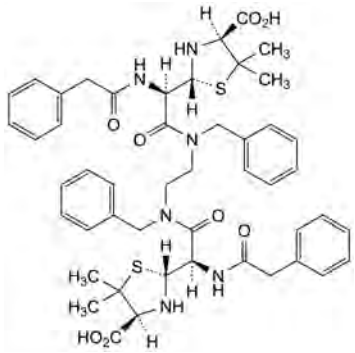

K. (2*R*,2′*R*,4*S*,4′*S*)-2,2′-[(4*R*,11*R*)-6,9-dibenzyl-2,5,10,13-tetraoxo-1,14-diphenyl-3,6,9,12-tetraazatetradecane-4,11-diyl]bis(5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid).

**07/2017:0113**
**corrected 10.0**

# BENZYLPENICILLIN POTASSIUM

## Benzylpenicillinum kalicum

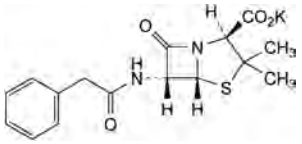

$C_{16}H_{17}KN_2O_4S$ $M_r$ 372.5
[113-98-4]

DEFINITION

Potassium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)-amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Substance produced by the growth of certain strains of *Penicillium notatum* or related organisms.

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benzylpenicillin potassium CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *water R.*

*Reference solution (a).* Dissolve 25 mg of *benzylpenicillin potassium CRS* in 5 mL of *water R.*

*Reference solution (b).* Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 mL of *water R.*

*Plate*: *TLC silanised silica gel plate R.*

*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R.*

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.

D. It gives reaction (a) of potassium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.5 to 7.5.

Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 3.0 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *benzylpenicillin sodium CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *benzylpenicillin for system suitability CRS* (containing impurities A, B, C, D, E, F, G and H) in 0.35 mL of *methanol R1* and add 0.65 mL of *water R*.

*Reference solution (c).* Dilute 1.0 mL of test solution (b) to 100.0 mL with *water R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 30 volumes of *methanol R1* and 60 volumes of *water for chromatography R*;
- *mobile phase B*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 35 volumes of *water for chromatography R* and 55 volumes of *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 70 | 30 |
| 7 - 17 | 70 → 0 | 30 → 100 |
| 17 - 22 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *benzylpenicillin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G and H.

*Relative retention* with reference to benzylpenicillin (retention time = about 7 min): impurity A = about 0.22; impurity D = about 0.33; impurity C = about 0.35; impurity E = about 0.48 and 0.55; impurity B = about 0.62; impurity F = about 0.81 and 0.83; impurity G = about 1.47; impurity H = about 1.90.

*System suitability*:

- *resolution*: minimum 1.2 between the peaks due to the epimers of impurity F and minimum 1.5 between the peaks due to impurities D and C in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity D = 0.6; impurity E = 2.0; impurity F = 1.7;
- for each impurity, use the concentration of benzylpenicillin potassium in reference solution (c).

*Limits*:

- *impurity F*: maximum 2.0 per cent for the sum of the 2 epimers;
- *impurity E*: maximum 1.0 per cent for the sum of the isomers;
- *impurity B*: maximum 0.5 per cent;
- *impurities A, C, D, G, H*: for each impurity, maximum 0.2 per cent;
- *any other impurity*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A, mobile phase B (70:30 *V/V*).

*Flow rate*: 1.2 mL/min.

*Injection*: 10 µL of test solution (a) and reference solution (a).

Calculate the percentage content of $C_{16}H_{17}KN_2O_4S$ taking into account the assigned content of *benzylpenicillin sodium CRS* and a conversion factor of 1.045.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

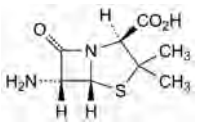

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

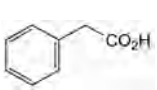

B. phenylacetic acid,

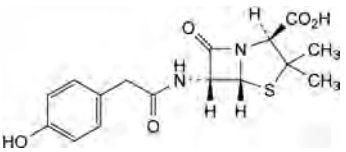

C. (2*S*,5*R*,6*R*)-6-[[(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

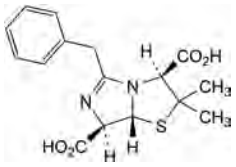

D. (3*S*,7*R*,7a*R*)-5-benzyl-2,2-dimethyl-2,3,7,7a-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),

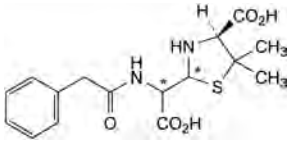

E. (4*S*)-2-[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

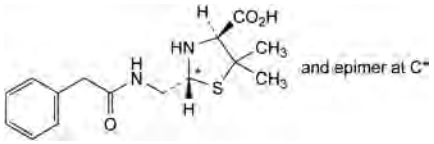


F. (2*RS*,4*S*)-5,5-dimethyl-2-[[(phenylacetyl)amino]methyl]-thiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin),

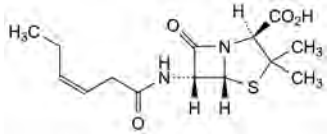

G. (2*S*,5*R*,6*R*)-6-[(3*Z*)-hex-3-enoylamino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

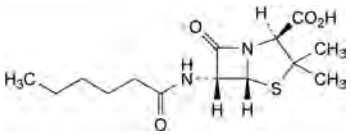

H. (2*S*,5*R*,6*R*)-6-(hexanoylamino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (dihydropenicillin F).

**07/2019:0115**
**corrected 10.0**

# BENZYLPENICILLIN (PROCAINE) MONOHYDRATE

## Benzylpenicillinum procainum monohydricum

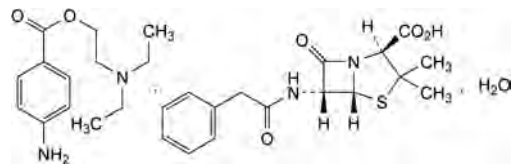

$C_{29}H_{38}N_4O_6S,H_2O$ $M_r$ 588.7
[6130-64-9]

DEFINITION

2-(Diethylamino)ethyl 4-aminobenzoate (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid monohydrate.

Salt obtained from *Benzylpenicillin sodium (0114)* or *Benzylpenicillin potassium (0113)* produced by the growth of certain strains of *Penicillium notatum* or related micro-organisms.

*Content*:

– *procaine benzylpenicillin*: 96.0 per cent to 102.0 per cent (anhydrous substance) without correction for dispersing or suspending agents;
– *procaine* ($C_{13}H_{20}N_2O_2$; $M_r$ 236.3): 39.0 per cent to 42.0 per cent (anhydrous substance).

Dispersing or suspending agents (for example, lecithin and polysorbate 80) may be added.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: slightly soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *procaine benzylpenicillin CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *acetone R.*
*Reference solution.* Dissolve 25 mg of *procaine benzylpenicillin CRS* in 5 mL of *acetone R.*
*Plate*: *TLC silanised silica gel plate R.*
*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 7.0 with *ammonia R.*
*Application*: 1 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution:
– the chromatogram shows 2 clearly separated spots.
*Results*: the 2 principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the 2 principal spots in the chromatogram obtained with the reference solution.

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R.* Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.

D. Dissolve 0.1 g in 2 mL of *dilute hydrochloric acid R.* The solution, which may be turbid, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 7.5.
Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 15 mL with the same solvent. Shake until dissolution is complete.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test solutions immediately before use.*
*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).
*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in 25 mL of *methanol R* and dilute to 50.0 mL with *water R.*
*Test solution (b).* Dissolve 0.100 g of the substance to be examined in 10 mL of *methanol R* and dilute to 20.0 mL with *water R.*

*Reference solution (a).* Dissolve 50.0 mg of *procaine benzylpenicillin CRS* in 25 mL of *methanol R* and dilute to 50.0 mL with *water R*.

*Reference solution (b).* Dissolve 12.0 mg of *4-aminobenzoic acid R* (impurity A) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 10 mg of *procaine benzylpenicillin for peak identification CRS* (containing impurities B, C, D, E, F, G, H, I and J) in 1 mL of *methanol R* and add 1 mL of *water R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (e).* Dilute 1.0 mL of reference solution (d) to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 30 volumes of *methanol R1* and 60 volumes of *water for chromatography R*;
- *mobile phase B*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 35 volumes of *water for chromatography R* and 55 volumes of *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 70 | 30 |
| 7 - 17 | 70 → 0 | 30 → 100 |
| 17 - 22 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *procaine benzylpenicillin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C, D, E, F, G, H, I and J; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to benzylpenicillin (retention time = about 7 min): procaine = about 0.19; impurity A = about 0.22; impurity D = about 0.33; impurity F = about 0.35; impurity B = about 0.48 and 0.55; impurity E = about 0.62; impurity C = about 0.81 and 0.83; impurity I = about 0.93; impurity G = about 1.47; impurity H = about 1.90; impurity J = about 2.37.

*System suitability*:

- *resolution*: minimum 1.0 between the peaks due to the epimers of impurity C and minimum 1.2 between the peaks due to impurities D and F in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to benzylpenicillin in the chromatogram obtained with reference solution (e).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity D by 0.4;
- for impurity A, use the concentration of impurity A in reference solution (b);
- for impurities other than A, use the concentration of procaine benzylpenicillin monohydrate in reference solution (d) taking into account the area of the peak due to benzylpenicillin in the chromatogram obtained with reference solution (d).

*Limits*:

- *impurities F, G, H, I, J*: for each impurity, maximum 0.5 per cent;
- *impurities B (sum of isomers), C (sum of epimers)*: for each impurity, maximum 0.4 per cent;
- *impurities E, D*: for each impurity, maximum 0.2 per cent;
- *impurity A*: maximum 0.024 per cent;
- *any other impurity*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to procaine.

**Water** (*2.5.12*): 2.8 per cent to 4.2 per cent, determined on 0.300 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (30:70 *V/V*).

*Injection*: 10 µL of test solution (a) and reference solution (a).

*Run time*: 3 times the retention time of benzylpenicillin.

Calculate the percentage content of procaine ($C_{13}H_{20}N_2O_2$) taking into account the area of the peak due to procaine and the assigned content of procaine ($C_{13}H_{20}N_2O_2$) in *procaine benzylpenicillin CRS*.

Calculate the percentage content of procaine benzylpenicillin ($C_{29}H_{38}N_4O_6S$) taking into account the area of the peak due to benzylpenicillin and the assigned content of procaine benzylpenicillin ($C_{29}H_{38}N_4O_6S$) in *procaine benzylpenicillin CRS*.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

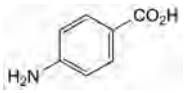


A. 4-aminobenzoic acid,

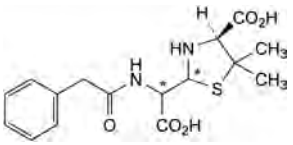


B. (2*Ξ*,4*S*)-2-[(*Ξ*)-carboxy(2-phenylacetamido)methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

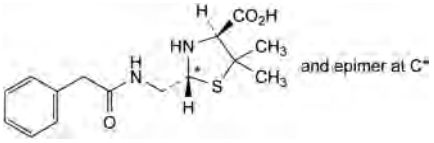


C. (2*RS*,4*S*)-5,5-dimethyl-2-[(2-phenylacetamido)methyl]-1,3-thiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin),

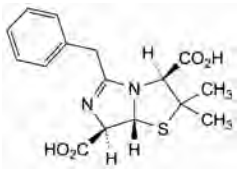

D. (3*S*,7*R*,7a*R*)-5-benzyl-2,2-dimethyl-2,3,7,7a-tetrahydroimidazo[5,1-*b*][1,3]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),

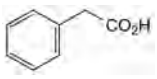

E. phenylacetic acid,

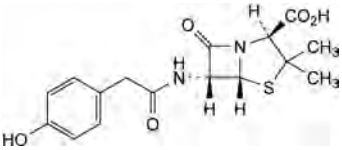

F. (2*S*,5*R*,6*R*)-6-[2-(4-hydroxyphenyl)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

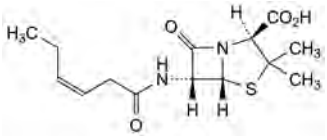

G. (2*S*,5*R*,6*R*)-6-[(3*Z*)-hex-3-enamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (iso-penicillin F),

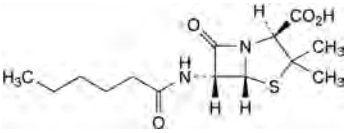

H. (2*S*,5*R*,6*R*)-6-hexanamido-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (dihydropenicillin F),

I. unknown structure,

J. unknown structure.

**07/2017:0114**
**corrected 10.0**

# BENZYLPENICILLIN SODIUM

## Benzylpenicillinum natricum

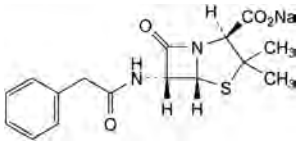

$C_{16}H_{17}N_2NaO_4S$ $M_r$ 356.4
[69-57-8]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(phenylacetyl)-amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Substance produced by the growth of certain strains of *Penicillium notatum* or related organisms.

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *benzylpenicillin sodium CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *water R*.
*Reference solution (a).* Dissolve 25 mg of *benzylpenicillin sodium CRS* in 5 mL of *water R*.
*Reference solution (b).* Dissolve 25 mg of *benzylpenicillin sodium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 mL of *water R*.
*Plate*: *TLC silanised silica gel plate R.*
*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.
*Application*: 1 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is practically colourless. Place the test-tube on a water-bath for 1 min; a reddish-brown colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.5 to 7.5.
Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*).
Dissolve 3.0 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*
*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.
*Test solution (b).* Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.
*Reference solution (a).* Dissolve 50.0 mg of *benzylpenicillin sodium CRS* in *water R* and dilute to 50.0 mL with the same solvent.
*Reference solution (b).* Dissolve 5 mg of *benzylpenicillin for system suitability CRS* (containing impurities A, B, C, D, E, F, G and H) in 0.35 mL of *methanol R1* and add 0.65 mL of *water R*.
*Reference solution (c).* Dilute 1.0 mL of test solution (b) to 100.0 mL with *water R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 30 volumes of *methanol R1* and 60 volumes of *water for chromatography R*;
- *mobile phase B*: mix 10 volumes of a 68 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.4 with a 500 g/L solution of *phosphoric acid R*, 35 volumes of *water for chromatography R* and 55 volumes of *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 70 | 30 |
| 7 - 17 | 70 → 0 | 30 → 100 |
| 17 - 22 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *benzylpenicillin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G and H.

*Relative retention* with reference to benzylpenicillin (retention time = about 7 min): impurity A = about 0.22; impurity D = about 0.33; impurity C = about 0.35; impurity E = about 0.48 and 0.55; impurity B = about 0.62; impurity F = about 0.81 and 0.83; impurity G = about 1.47; impurity H = about 1.90.

*System suitability*:

- *resolution*: minimum 1.2 between the peaks due to the epimers of impurity F and minimum 1.5 between the peaks due to impurities D and C in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.3; impurity D = 0.6; impurity E = 2.0; impurity F = 1.7;
- for each impurity, use the concentration of benzylpenicillin sodium in reference solution (c).

*Limits*:

- *impurity E*: maximum 2.0 per cent for the sum of the isomers;
- *impurity F*: maximum 1.0 per cent for the sum of the 2 epimers;
- *impurity B*: maximum 0.5 per cent;
- *impurities A, C, D, G, H*: for each impurity, maximum 0.2 per cent;
- *any other impurity*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent *m/m*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A, mobile phase B (70:30 *V/V*).

*Flow rate*: 1.2 mL/min.

*Injection*: 10 µL of test solution (a) and reference solution (a).

Calculate the percentage content of $C_{16}H_{17}N_2NaO_4S$ taking into account the assigned content of *benzylpenicillin sodium CRS*.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

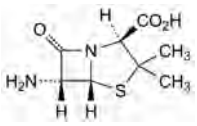

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

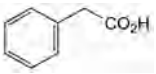

B. phenylacetic acid,

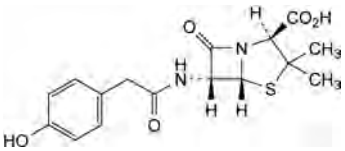

C. (2*S*,5*R*,6*R*)-6-[[(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid,

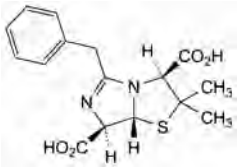

D. (3*S*,7*R*,7a*R*)-5-benzyl-2,2-dimethyl-2,3,7,7a-tetrahydroimidazo[5,1-*b*]thiazole-3,7-dicarboxylic acid (penillic acid of benzylpenicillin),

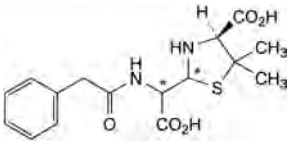

E. (4*S*)-2-[carboxy[(phenylacetyl)amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of benzylpenicillin),

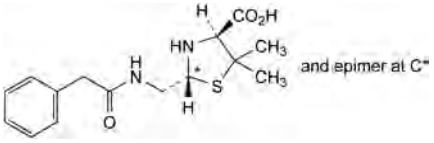


F. (2*RS*,4*S*)-5,5-dimethyl-2-[[(phenylacetyl)amino]methyl]-thiazolidine-4-carboxylic acid (penilloic acids of benzylpenicillin),

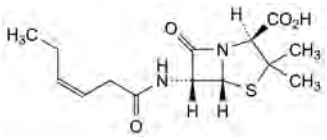

G. (2*S*,5*R*,6*R*)-6-[(3*Z*)-hex-3-enoylamino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (iso-penicillin F),

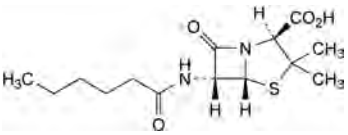

H. (2*S*,5*R*,6*R*)-6-(hexanoylamino)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (dihydropenicillin F).

**04/2019:1069**

# BETACAROTENE

## Betacarotenum

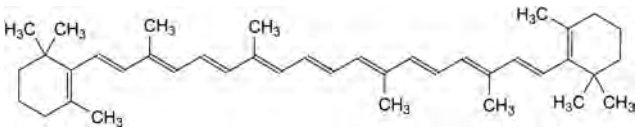

$C_{40}H_{56}$ $M_r$ 536.9

[7235-40-7]

DEFINITION

*Main component*: 1,1′-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaene-1,18-diyl]bis(2,6,6-trimethylcyclohexene) (β,β-carotene, betacarotene, *all-trans*-betacarotene, *all-E*-betacarotene).

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: brown-red or brownish-red, crystalline powder.

*Solubility*: practically insoluble in water, soluble in tetrahydrofuran, practically insoluble in anhydrous ethanol.

It is sensitive to air, heat and light, especially in solution.

IDENTIFICATION

*Carry out all operations as rapidly as possible. Prepare the solutions immediately before use.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with *tetrahydrofuran R*. Dilute 3.0 mL of this solution to 100.0 mL with *cyclohexane R*.

*Absorption maximum*: 455 nm.

*Shoulder*: about 427 nm.

*Absorbance ratio*: $A_{455}/A_{483}$ = 1.14 to 1.18.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

*Carry out all operations as rapidly as possible. Prepare the solutions immediately before use.*

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Stabilised tetrahydrofuran*: *tetrahydrofuran R* containing 0.25 g/L of *butylhydroxytoluene R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in stabilised tetrahydrofuran and dilute to 200.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *anhydrous ethanol R*.

*Reference solution (a).* Dissolve 50.0 mg of *betacarotene CRS* in stabilised tetrahydrofuran and dilute to 200.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *anhydrous ethanol R*.

*Reference solution (b).* Dissolve 5 mg of *betacarotene for system suitability CRS* (containing impurities C, D, E, G and H) in stabilised tetrahydrofuran and dilute to 20 mL with the same solvent. Dilute 1 mL of the solution to 20 mL with *anhydrous ethanol R*.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with stabilised tetrahydrofuran. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amidoalkylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 50 mg of *butylhydroxytoluene R* in 20 mL of *2-propanol R*, then add 0.2 mL of *N,N-diisopropylethylamine R*, 25 mL of a 2.0 g/L solution of *ammonium acetate R*, 455 mL of *acetonitrile R* and 450 mL of *methanol R*; warm to room temperature and dilute to 1000 mL with *methanol R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 448 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.7 times the retention time of betacarotene.

*Identification of impurities*: use the chromatogram supplied with *betacarotene for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C, D, E, G and H.

*Relative retention* with reference to betacarotene (retention time = about 28 min): impurity G = about 0.6; impurity C = about 0.95; impurity D = about 1.05; impurity E = about 1.15; impurity H = about 2.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity C and betacarotene;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to betacarotene.

*Limits*:

- *impurity E*: maximum 4.0 per cent;
- *impurity D*: maximum 2.0 per cent;
- *impurity C*: maximum 0.6 per cent;
- *impurity G*: maximum 0.4 per cent;
- *impurity H*: maximum 0.4 per cent;
- *unspecified impurities*: for each impurity, maximum 0.15 per cent;
- *sum of impurities other than D and E*: maximum 5.0 per cent;
- *reporting threshold*: 0.05 per cent (0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c)).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying *in vacuo* at 40 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification:

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{40}H_{56}$ taking into account the assigned content of *betacarotene CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: C, D, E, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, F.*

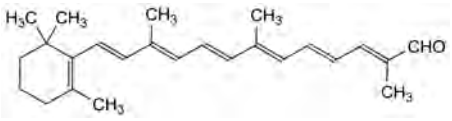

A. (2*E*,4*E*,6*E*,8*E*,10*E*,12*E*)-2,7,11-trimethyl-13-(2,6,6-trimethylcyclohex-1-en-1-yl)trideca-2,4,6,8,10,12-hexaenal (12′-apo-β-caroten-12′-al),

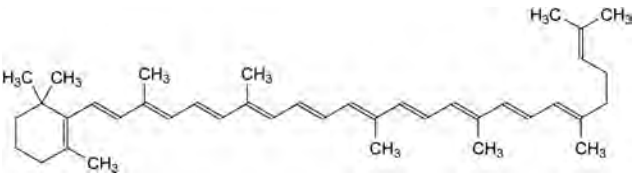

B. 1-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*,19*E*)-3,7,12,16,20,24-hexamethylpentacosa-1,3,5,7,9,11,13,15,17,19,23-undecaen-1-yl]-2,6,6-trimethylcyclohexene (β,Ψ-carotene, γ-carotene),

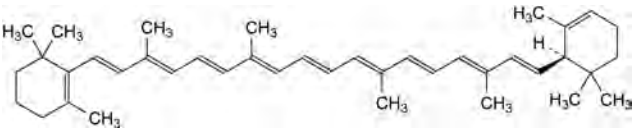

C. 1,3,3-trimethyl-2-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyl-18-[(1*R*)-2,6,6-trimethylcyclohex-2-en-1-yl]octadeca-1,3,5,7,9,11,13,15,17-nonaen-1-yl]cyclohexene ((6′*R*)-β,ε-carotene, α-carotene),

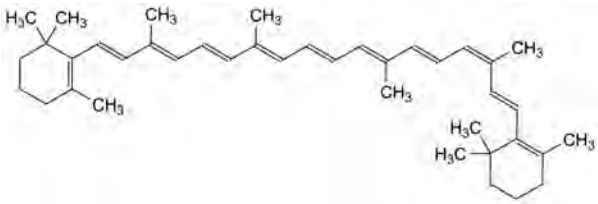

D. 1,1′-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*Z*,17*E*)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaene-1,18-diyl]bis(2,6,6-trimethylcyclohexene) (9-*cis*-β,β-carotene, 9-*cis*-betacarotene),

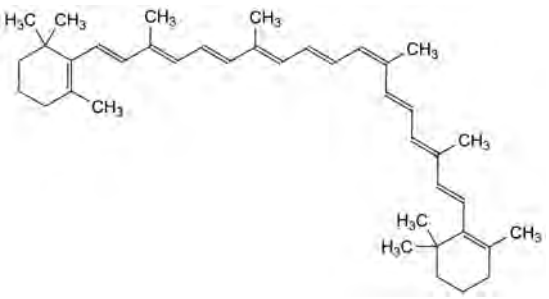

E. 1,1′-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*Z*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaene-1,18-diyl]bis(2,6,6-trimethylcyclohexene) (13-*cis*-β,β-carotene, 13-*cis*-betacarotene),

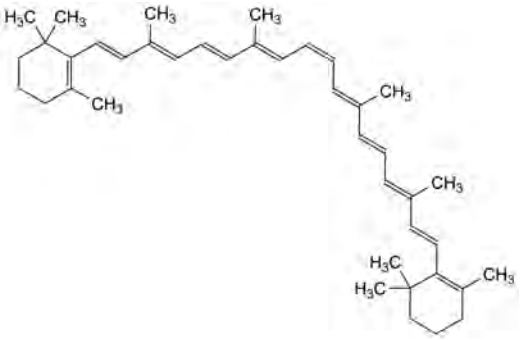

F. 1,1’-[(1*E*,3*E*,5*E*,7*E*,9*Z*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaene-1,18-diyl]bis(2,6,6-trimethylcyclohexene) (15-cis-β,β-carotene, 15-*cis*-betacarotene),

G. unknown structure (oxidation products),

H. unknown structure.

**07/2019:1070**

# BETADEX

## Betadexum

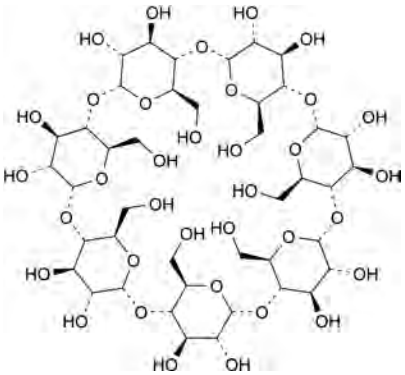

$[C_6H_{10}O_5]_7$ $M_r$ 1135

[7585-39-9]

DEFINITION

Cycloheptakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltoheptaose or β-cyclodextrin).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline, hygroscopic powder.

*Solubility*: sparingly soluble in water and in propylene glycol, practically insoluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Dissolve 0.2 g in 2 mL of *iodine solution R4* by warming on a water-bath, and allow to stand at room temperature. A yellowish-brown precipitate is formed.

TESTS

**Solution S**. Dissolve 1.000 g in *carbon dioxide-free water R* with heating, allow to cool and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 5.0 to 8.0.

To 10 mL of solution S add 0.1 mL of a saturated solution of *potassium chloride R*.

**Specific optical rotation** (*2.2.7*): + 160 to + 164 (dried substance), determined on solution S.

**Reducing sugars**: maximum 0.2 per cent.

*Test solution*. To 1 mL of solution S add 1 mL of *cupri-tartaric solution R4*. Heat on a water-bath for 10 min, cool to room temperature. Add 10 mL of *ammonium molybdate reagent R1* and allow to stand for 15 min.

*Reference solution*. Prepare a reference solution at the same time and in the same manner as the test solution, using 1 mL of a 0.02 g/L solution of *glucose R*.

Measure the absorbance (*2.2.25*) of the test solution and the reference solution at the absorption maximum at 740 nm using *water R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Light-absorbing impurities**. Examine solution S between 230 nm and 750 nm. Between 230 nm and 350 nm, the absorbance (*2.2.25*) is not greater than 0.10. Between 350 nm and 750 nm, the absorbance (*2.2.25*) is not greater than 0.05.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.250 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a)*. Dissolve 25.0 mg of *alfadex CRS* (impurity A), 25.0 mg of *gammacyclodextrin CRS* (impurity B) and 50.0 mg of *betadex CRS* in *water R*, then dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Reference solution (c)*. Dissolve 25.0 mg of *betadex CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: *methanol R*, *water for chromatography R* (10:90 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: differential refractometer.

*Equilibration*: with the mobile phase for about 3 h.

*Injection*: 50 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 1.5 times the retention time of betadex.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to betadex (retention time = about 10 min): impurity B = about 0.3; impurity A = about 0.45.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurities B and A; if necessary, adjust the concentration of methanol in the mobile phase.

*Calculation of percentage contents*:
- for impurities A and B, use the concentration of the corresponding impurity in reference solution (b);
- for impurities other than A and B, use the concentration of betadex in reference solution (b).

*Limits*:
- *impurities A, B*: for each impurity, maximum 0.25 per cent;
- *sum of impurities other than A and B*: maximum 0.5 per cent;
- *reporting threshold*: 0.15 per cent.

**Residual solvents**. Head-space gas chromatography (*2.2.28*): use the standard additions method.

*Internal standard*: *ethylene chloride R*.

*Stock solution A*. To 20 µL of *ethylene chloride R* add 0.5 mL of *dimethyl sulfoxide R* and dilute to 25.0 mL with *water R*.

*Stock solution B*. To 25 µL of *trichloroethylene R* add 25 µL of *toluene R* and 0.5 mL of *dimethyl sulfoxide R*, then dilute to 50.0 mL with *water R*.

*Test solutions (a), (b), (c) and (d)*. In each of 4 identical vials, introduce 0.5 g of the substance to be examined, 0.10 g of *calcium chloride R*, 30 µL of *α-amylase solution R* and 1 mL of reference solutions (a), (b), (c) and (d), respectively, then add 9.0 mL of *water R*. Prepare test solutions (b), (c) and (d) in triplicate.

*Reference solution (a)*. Dilute 250 µL of stock solution A to 10.0 mL with *water R*.

*Reference solution (b)*. To 100 µL of stock solution B add 250 µL of stock solution A and dilute to 10.0 mL with *water R*.

*Reference solution (c)*. To 200 µL of stock solution B add 250 µL of stock solution A and dilute to 10.0 mL with *water R*.

*Reference solution (d)*. To 300 µL of stock solution B add 250 µL of stock solution A and dilute to 10.0 mL with *water R*.

*Blank solution*. In a vial identical to those used for the test solutions, introduce 0.10 g of *calcium chloride R*, 30 µL of *α-amylase solution R*, 0.5 mL of *dimethyl sulfoxide R* and 10.0 mL of *water R*.

*Column*:
- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.7 mL/min.

*Static head-space conditions that may be used: if the equipment has different setting parameters, adjust the equipment settings so as to comply with the system suitability criterion*:
- *equilibration temperature*: 45 °C;
- *equilibration time*: 2 h;
- *syringe temperature*: 50 °C;
- *injection speed*: 500 µL/s.

*Temperature*:
- *column*: 50 °C;
- *injection port*: 140 °C;
- *detector*: 280 °C.

*Detection*: flame ionisation.

*Injection*: 200 µL.

*Relative retention* with reference to the internal standard (retention time = about 13 min): trichloroethylene = about 0.6; toluene = about 0.8.

*System suitability*: test solutions (b), (c) and (d):

– *repeatability*: maximum relative standard deviations of the ratios of the areas of the peaks due to trichloroethylene and toluene to that of the peak due to ethylene chloride of 10.0 per cent, for each set of triplicate test solutions and each residual solvent.

Calculate the content of trichloroethylene and of toluene taking their relative densities to be 1.46 and 0.87, respectively.

*Limits*:

– *trichloroethylene*: maximum 10 ppm;

– *toluene*: maximum 10 ppm.

**Loss on drying** (*2.2.32*): maximum 16.0 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solutions (a) and (c).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the area of the peak due to betadex, determined on 5 injections.

Calculate the percentage content of $[C_6H_{10}O_5]_7$ using the chromatogram obtained with reference solution (c) and taking into account the assigned content of *betadex CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

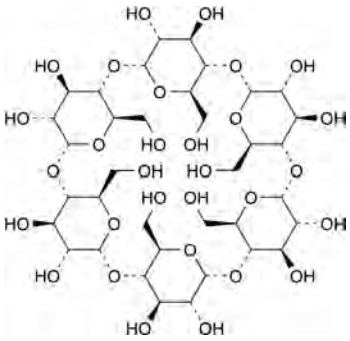

A. cyclohexakis-(1→4)-(α-D-glucopyranosyl) (alfadex or cyclomaltohexaose or α-cyclodextrin),

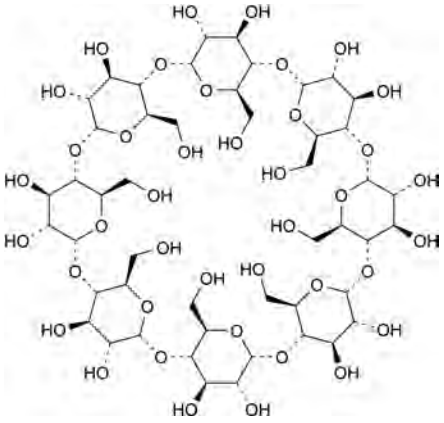

B. cyclooctakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltooctaose or γ-cyclodextrin).

**01/2008:1665**
**corrected 6.0**

# BETAHISTINE DIHYDROCHLORIDE

## Betahistini dihydrochloridum

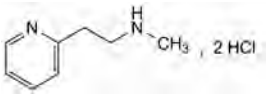

$C_8H_{14}Cl_2N_2$ $M_r$ 209.1
[5579-84-0]

DEFINITION

*N*-Methyl-2-(pyridin-2-yl)ethanamine dihydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow powder, very hygroscopic.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), practically insoluble in 2-propanol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 150 °C to 154 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *betahistine dihydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of *ethanol (96 per cent) R*.
*Reference solution.* Dissolve 10 mg of *betahistine dihydrochloride CRS* in 2 mL of *ethanol (96 per cent) R*.
*Plate*: *TLC silica gel $GF_{254}$ plate R*.
*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *methanol R* (0.75:15:30 *V/V/V*).
*Application*: 2 µL.
*Development*: over 2/3 of the plate.
*Drying*: at 110 °C for 10 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R*, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.0 to 3.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10 mg of *betahistine dihydrochloride CRS* and 10 mg of *2-vinylpyridine R* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 2.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.0 g of *sodium dodecyl sulfate R* in a mixture of 15 mL of a 10 per cent *V/V* solution of *sulfuric acid R*, 35 mL of a 17 g/L solution of *tetrabutylammonium hydrogen sulfate R* and 650 mL of *water R*; adjust to pH 3.3 using *dilute sodium hydroxide solution R* and mix with 300 mL of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of betahistine.

*Relative retention* with reference to betahistine (retention time = about 7 min): impurity B = about 0.2; impurity A = about 0.3; impurity C = about 3.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.5 between the peaks due to 2-vinylpyridine and betahistine.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.4;

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times of the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 50 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added to reach the second point of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 10.46 mg of $C_8H_{14}Cl_2N_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C.*

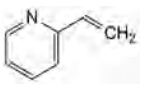

A. 2-ethenylpyridine (2-vinylpyridine),

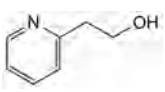

B. 2-(pyridin-2-yl)ethanol,

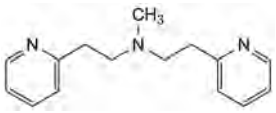

C. *N*-methyl-2-(pyridin-2-yl)-*N*-[2-(pyridin-2-yl)ethyl]ethanamine.

**01/2017:1071**

# BETAHISTINE MESILATE

## Betahistini mesilas

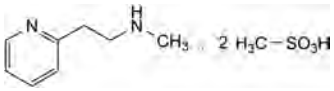

$C_{10}H_{20}N_2O_6S_2$ $M_r$ 328.4

[54856-23-4]

DEFINITION

*N*-Methyl-2-(pyridin-2-yl)ethanamine bis(methanesulfonate).

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in betahistine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, very hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), very slightly soluble in 2-propanol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 108 °C to 112 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *betahistine mesilate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 2 mL with the same solvent.
*Reference solution*. Dissolve 10 mg of *betahistine mesilate CRS* in *ethanol (96 per cent) R* and dilute to 2 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *methanol R* (0.75:15:30 *V/V/V*).
*Application*: 2 µL.
*Development*: over 3/4 of the plate.
*Drying*: at 110 °C for 10 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g add 5 mL of *dilute hydrochloric acid R* and shake for about 5 min. Add 1 mL of *barium chloride solution R1*. The solution remains clear. To a further 0.1 g add 0.5 g of *anhydrous sodium carbonate R*, mix and ignite until a white residue is obtained. Allow to cool and dissolve the residue in 7 mL of *water R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.0 to 3.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10 mg of *betahistine mesilate CRS* and 10 mg of *2-vinylpyridine R* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.0 g of *sodium dodecyl sulfate R* in a mixture of 15 volumes of a 10 per cent *V/V* solution of *sulfuric acid R*, 35 volumes of a 17 g/L solution of *tetrabutylammonium hydrogen sulfate R* and 650 volumes of *water R*; adjust to pH 3.3 using *dilute sodium hydroxide solution R* and mix with 300 volumes of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of betahistine mesilate.

*Retention time*: betahistine mesilate = about 8 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.5 between the peaks due to impurity A and betahistine mesilate.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**2-Propanol** (*2.4.24*): maximum 0.5 per cent.

**Chlorides** (*2.4.4*): maximum 35 ppm.

To 14 mL of solution S add 1 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 250 ppm.

Dilute 6 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.50 g.

ASSAY

Dissolve 0.140 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.42 mg of $C_{10}H_{20}N_2O_6S_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A*.

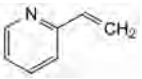

A. 2-ethenylpyridine (2-vinylpyridine).

**01/2008:0312**
**corrected 6.0**

# BETAMETHASONE

## Betamethasonum

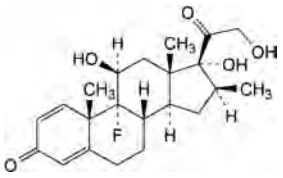

$C_{22}H_{29}FO_5$ $M_r$ 392.5
[378-44-9]

DEFINITION

9-Fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in anhydrous ethanol, very slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E.*

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of this solution in a stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at 419 nm is not greater than 0.10.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *betamethasone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20 mg of *betamethasone CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *dexamethasone CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *butanol R* saturated with *water R*, *toluene R*, *ether R* (5:10:85 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which may, however, not be completely separated.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

E. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add this solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

TESTS

**Specific optical rotation** (*2.2.7*): + 118 to + 126 (dried substance).

Dissolve 0.125 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *methanol R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 2 mg of *betamethasone CRS* and 2 mg of *methylprednisolone CRS* in mobile phase A, then dilute to 100.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: in a 1000 mL volumetric flask mix 250 mL of *acetonitrile R* with 700 mL of *water R* and allow to equilibrate; dilute to 1000 mL with *water R* and mix again;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 40 | 100 → 0 | 0 → 100 |
| 40 - 41 | 0 → 100 | 100 → 0 |
| 41 - 46 | 100 | 0 |

*Flow rate*: 2.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with mobile phase B for at least 30 min and then with mobile phase A for 5 min. For subsequent chromatograms, use the conditions described from 40 min to 46 min.

*Injection*: 20 µL; inject the mixture of equal volumes of *acetonitrile R* and *methanol R* as a blank.

*Retention time*: methylprednisolone = about 11.5 min; betamethasone = about 12.5 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to methylprednisolone and betamethasone; if necessary, adjust the concentration of acetonitrile in mobile phase A.

*Limits*:

– *impurities A, B, C, D, E, F, G, H, I, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and not more than 1 such peak has an area greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 238.5 nm.

Calculate the content of $C_{22}H_{29}FO_5$ taking the specific absorbance to be 395.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J.*

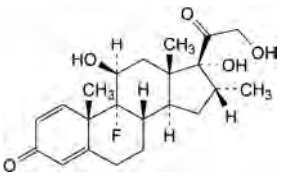


A. 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone),

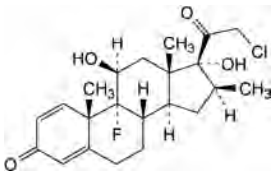

B. 21-chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione,

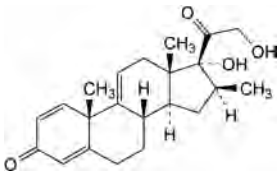

C. 17,21-dihydroxy-16β-methylpregna-1,4,9(11)-triene-3,20-dione,

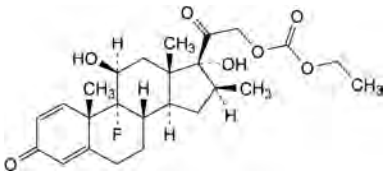

D. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl ethoxycarboxylate,

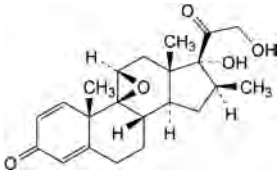

E. 9,11β-epoxy-17,21-dihydroxy-16β-methyl-9β-pregna-1,4-diene-3,20-dione,

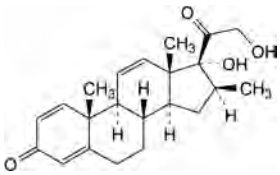

F. 17,21-dihydroxy-16β-methylpregna-1,4,11-triene-3,20-dione,

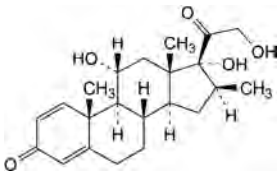

G. 11α,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione,

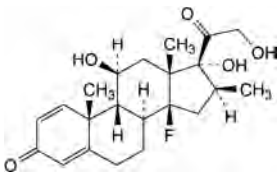

H. 14-fluoro-11β,17,21-trihydroxy-16β-methyl-8α,9β,14β-pregna-1,4-diene-3,20-dione,

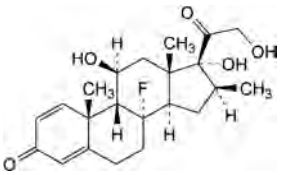

I. 8-fluoro-11β,17,21-trihydroxy-16β-methyl-8α,9β-pregna-1,4-diene-3,20-dione,

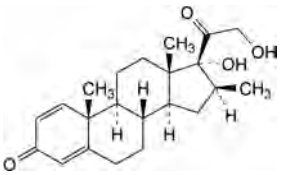

J. 17,21-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione.

**01/2008:0975**

# BETAMETHASONE ACETATE

## Betamethasoni acetas

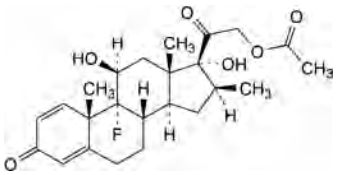

$C_{24}H_{31}FO_6$ $M_r$ 434.5
[987-24-6]

DEFINITION

9-Fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-21-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E, F.*

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at 419 nm is not greater than 0.10.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *betamethasone acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20 mg of *betamethasone acetate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *prednisolone acetate CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R.* Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add this solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. About 10 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 120 to + 128 (anhydrous substance).

Dissolve 0.250 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 4 mL of *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2 mg of *betamethasone acetate CRS* and 2 mg of *dexamethasone acetate CRS* (impurity B) in the mobile phase, then dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: in a 1000 mL volumetric flask mix 380 mL of *acetonitrile R* with 550 mL of *water R* and allow to equilibrate; dilute to 1000 mL with *water R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of betamethasone acetate.

*Retention time*: betamethasone acetate = about 19 min; impurity B = about 22 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.3 between the peaks due to betamethasone acetate and impurity B; if necessary, adjust slightly the concentration of acetonitrile in the mobile phase.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *total*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.25 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 240 nm.

Calculate the content of $C_{24}H_{31}FO_6$ taking the specific absorbance to be 350.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

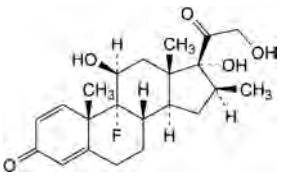

A. 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone),

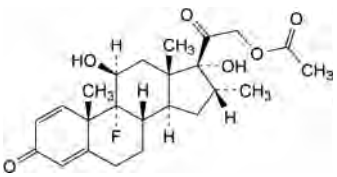

B. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (dexamethasone acetate),

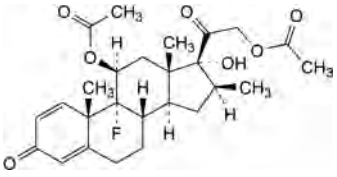

C. 9-fluoro-17-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-11β,21-diyl diacetate (betamethasone 11,21-diacetate),

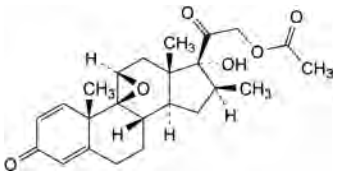

D. 9,11β-epoxy-17-hydroxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-diene-21-yl acetate.

**01/2018:0809**

# BETAMETHASONE DIPROPIONATE

## Betamethasoni dipropionas

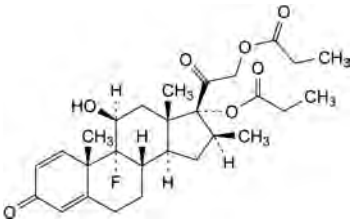

$C_{28}H_{37}FO_7$ $M_r$ 504.6
[5593-20-4]

DEFINITION

9-Fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of the solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at 419 nm is not more than 0.10.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *betamethasone dipropionate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution A). Dilute 2 mL of solution A to 10 mL with *methylene chloride R*.
*Test solution (b).* Transfer 2 mL of solution A to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h. Allow to cool.
*Reference solution (a).* Dissolve 25 mg of *betamethasone dipropionate CRS* in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution B). Dilute 2 mL of solution B to 10 mL with *methylene chloride R*.
*Reference solution (b).* Transfer 2 mL of solution B to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a current of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 2 h. Allow to cool.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.
*Application*: 5 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.
*Detection B*: spray with *alcoholic solution of sulfuric acid R*, heat at 120 °C for 10 min or until the spots appear, and allow to cool; examine in daylight and in ultraviolet light at 365 nm.
*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution; the principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an $R_F$ value distinctly lower than that of the principal spot in each of the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a deep reddish-brown colour develops. Add this solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Specific optical rotation** (*2.2.7*): + 84 to + 88 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 60.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *betamethasone dipropionate for system suitability A CRS* (containing impurities B, C, D, E, G and I) in the mobile phase and dilute to 2.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 60.0 mg of *betamethasone dipropionate CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *betamethasone dipropionate for peak identification CRS* (containing impurity H) in the mobile phase and dilute to 2.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (2.5 µm);
- *temperature*: 20 ± 2 °C.

*Mobile phase*: mix 35 mL of *water for chromatography R* and 56 mL of *acetonitrile R* and allow to equilibrate; dilute to 100 mL with *water for chromatography R* and mix.

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL of test solution (a) and reference solutions (a), (b) and (d).

*Run time*: 3 times the retention time of betamethasone dipropionate.

*Identification of impurities*: use the chromatogram supplied with *betamethasone dipropionate for system suitability A CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C, D, E, G and I; use the chromatogram supplied with *betamethasone dipropionate for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity H.

*Relative retention* with reference to betamethasone dipropionate (retention time = about 10 min): impurity B = about 0.4; impurity C = about 0.5; impurity D = about 0.7; impurity I = about 1.16; impurity E = about 1.22; impurity H = about 1.7; impurity G = about 2.1.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to betamethasone dipropionate; minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity E.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 1.3; impurity H = 1.4;
- *impurity C*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, H*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities D, E, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity I*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{28}H_{37}FO_7$ taking into account the assigned content of *betamethasone dipropionate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, C, D, E, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, F.*

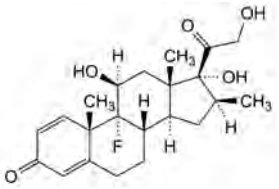

A. 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone),

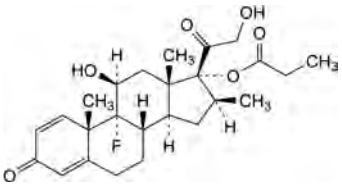

B. 9-fluoro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (betamethasone 17-propionate),

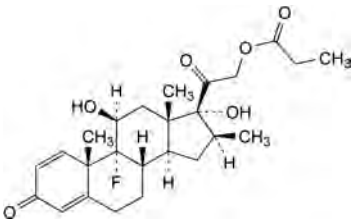

C. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl propanoate (betamethasone 21-propionate),

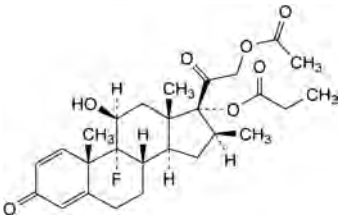

D. 21-(acetyloxy)-9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (betamethasone 21-acetate 17-propionate),

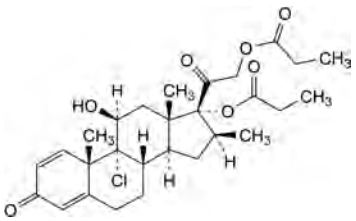

E. 9-chloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate (beclometasone dipropionate),

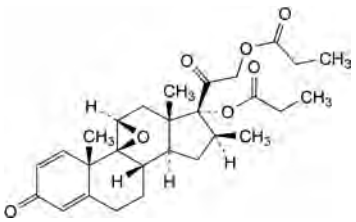

F. 9,11β-epoxy-16β-methyl-3,20-dioxo-9β-pregna-1,4-diene-17,21-diyl dipropanoate (9β,11β-epoxybetamethasone dipropionate),

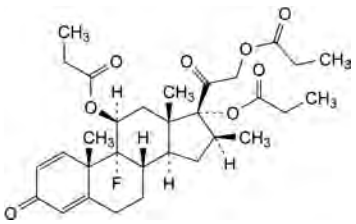

G. 9-fluoro-16β-methyl-3,20-dioxopregna-1,4-diene-11β,17,21-triyl tripropanoate (betamethasone tripropionate),

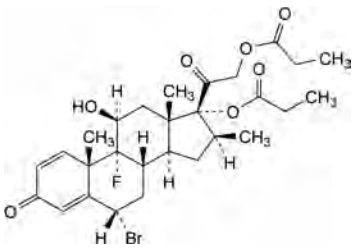

H. 6α-bromo-9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-diene-17,21-diyl dipropanoate (6α-bromobetamethasone dipropionate),

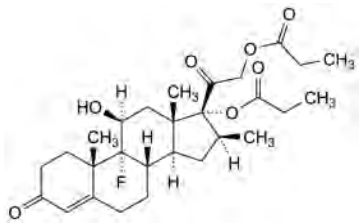

I. 9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregn-4-ene-17,21-diyl dipropanoate (1,2-dihydrobetamethasone dipropionate).

**01/2008:0810**

# BETAMETHASONE SODIUM PHOSPHATE

## Betamethasoni natrii phosphas

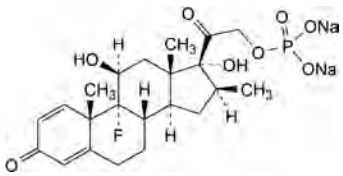

$C_{22}H_{28}FNa_2O_8P$ $M_r$ 516.4
[151-73-5]

DEFINITION

9-Fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate.

*Content*: 96.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, very hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E, F.*

A. Dissolve 10.0 mg in 5 mL of *water R* and dilute to 100.0 mL with *anhydrous ethanol R*. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at the absorption maximum at 450 nm is not more than 0.10.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *betamethasone sodium phosphate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *betamethasone sodium phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *prednisolone sodium phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent. Dilute 5 mL of this solution to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which may, however, not be completely separated.

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, an intense reddish-brown colour develops. Add the solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. To about 40 mg add 2 mL of *sulfuric acid R* and heat gently until white fumes are evolved. Add *nitric acid R* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 mL of *water R*, heat until white fumes are again evolved, cool, add 10 mL of *water R* and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (*2.3.1*) and reaction (b) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 9.0.

Dilute 1 mL of solution S to 5 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 98 to + 104 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25 mg of *betamethasone sodium phosphate CRS* and 25 mg of *dexamethasone sodium phosphate CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: in a 250 mL conical flask, weigh 1.360 g of *potassium dihydrogen phosphate R* and 0.600 g of *hexylamine R*, mix and allow to stand for 10 min and then dissolve in 185 mL of *water R*; add 65 mL of *acetonitrile R*, mix and filter (0.45 µm).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 45 min.

*Injection*: 20 µL.

*Run time*: twice the retention time of betamethasone sodium phosphate.

*Retention time*: betamethasone sodium phosphate = about 14 min; dexamethasone sodium phosphate = about 15.5 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to betamethasone sodium phosphate and dexamethasone sodium phosphate; if necessary, increase the concentration of acetonitrile or increase the concentration of water in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent), and not more than 1 such peak has an area greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);

– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent);

– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Inorganic phosphate**: maximum 1 per cent.

Dissolve 50 mg in *water R* and dilute to 100 mL with the same solvent. To 10 mL of this solution add 5 mL of *molybdovanadic reagent R*, mix and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of *phosphate standard solution (5 ppm $PO_4$) R*.

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 250.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241 nm.

Calculate the content of $C_{22}H_{28}FNa_2O_8P$ taking the specific absorbance to be 297.

STORAGE

In an airtight container, protected from light.

**01/2009:0811**

# BETAMETHASONE VALERATE

## Betamethasoni valeras

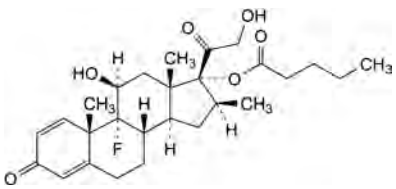

$C_{27}H_{37}FO_6$ $M_r$ 476.6

[2152-44-5]

DEFINITION

9-Fluoro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in ethanol (96 per cent).

mp: about 192 °C, with decomposition.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *betamethasone 17-valerate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**Specific optical rotation** (*2.2.7*): + 77 to + 83 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Prepare the solutions immediately before use.*

*Solvent mixture*: *glacial acetic acid R*, mobile phase (1:1000 *V/V*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 12.5 mg of *betamethasone valerate for system suitability CRS* (containing impurities D and G) in 5.0 mL of the solvent mixture. Use 1.0 mL of this solution to dissolve the contents of a vial of *betamethasone valerate impurity mixture CRS* (containing impurities C, H and I).

*Reference solution (c).* Dissolve 6 mg of *betamethasone CRS* (impurity A) and 3 mg of *betamethasone 21-valerate CRS* (impurity E) in 30.0 mL of the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 239 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of betamethasone valerate.

*Identification of impurities*: use the chromatogram supplied with *betamethasone valerate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C, D, G, H and I; use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and E.

*Relative retention* with reference to betamethasone valerate (retention time = about 20 min): impurity A = about 0.3; impurity I = about 0.6; impurity C = about 0.8; impurity H = about 1.3; impurity D = about 1.4; impurity E = about 1.6; impurity G = about 2.0.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.7 between the peaks due to impurities H and D.

*Limits*:

- *impurity A*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *impurities E, G*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities C, H, I*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 50.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 240 nm.

Calculate the content of $C_{27}H_{37}FO_6$ taking the specific absorbance to be 325.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, C, E, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, F.*

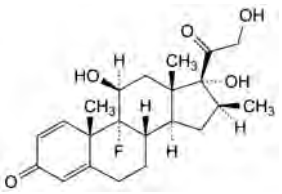


A. 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone),

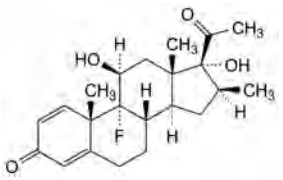

B. 9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione (21-deoxy-betamethasone),

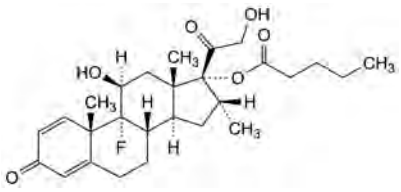

C. 9-fluoro-11β,21-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate (dexamethasone 17-valerate),

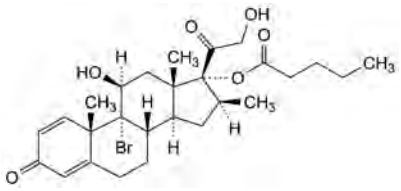

D. 9-bromo-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate (9-bromo-betamethasone valerate),

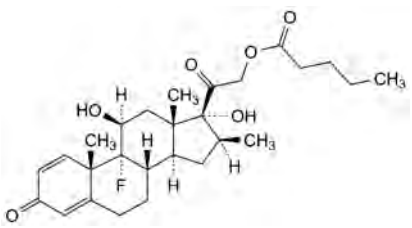

E. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl pentanoate (betamethasone 21-valerate),

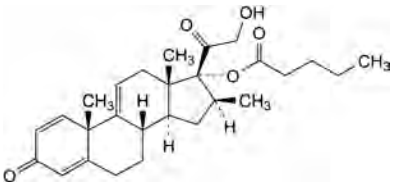

F. 21-hydroxy-16β-methyl-3,20-dioxopregna-1,4,9(11)-trien-17-yl pentanoate (betamethasone valerate δ-9(11)),

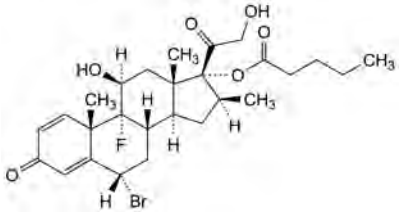

G. 6α-bromo-9-fluoro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate (6α-bromo-betamethasone valerate),

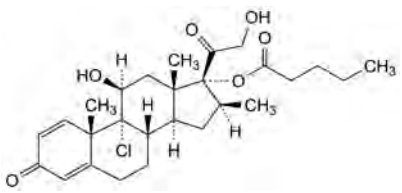

H. 9-chloro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl pentanoate (beclomethasone 17-valerate),

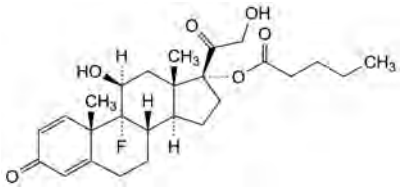

I. 9-fluoro-11β,21-dihydroxy-3,20-dioxopregna-1,4-dien-17-yl pentanoate (9-fluoro-prednisolone 17-valerate).

**01/2017:1072**

# BETAXOLOL HYDROCHLORIDE

## Betaxololi hydrochloridum

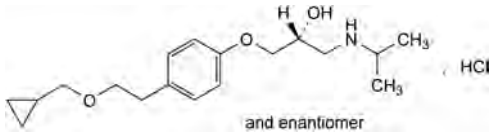


$C_{18}H_{30}ClNO_3$ $M_r$ 343.9
[63659-19-8]

DEFINITION

(2*RS*)-1-[4-[2-(Cyclopropylmethoxy)ethyl]phenoxy]-3-[(1-methylethyl)amino]propan-2-ol hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), soluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 113 °C to 117 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *betaxolol hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 1 mL of *methanol R.*
*Reference solution (a).* Dissolve 20 mg of *betaxolol hydrochloride CRS* in 2 mL of *methanol R.*
*Reference solution (b).* Dissolve 10 mg of *oxprenolol hydrochloride CRS* in 1 mL of reference solution (a).
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*
*Mobile phase*: *perchloric acid R*, *methanol R*, *water R* (0.5:50:50 *V/V/V*).
*Application*: 2 µL.
*Development*: over a path of 10 cm.
*Drying*: in air.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with a 50 g/L solution of *vanillin R* in a mixture of 5 volumes of *sulfuric acid R*, 10 volumes of *glacial acetic acid R* and 85 volumes of *methanol R*, heat at 100-105 °C until the colour of the spots reaches maximum intensity (10-15 min), and examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 25 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent. Add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare reference solutions (c) and (d) immediately before use.*

*Test solution*. Dissolve 10 mg of the substance to be examined in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 8 mg of the substance to be examined and 4 mg of *betaxolol impurity A CRS* in 20.0 mL of the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 2 mg of *betaxolol impurity C CRS* in 50 mL of the mobile phase. Dilute 5 mL of the solution to 20 mL with the mobile phase.

*Reference solution (d)*. Dissolve 10 mg of *betaxolol for peak identification CRS* (containing impurities B, D and E) in 5 mL of reference solution (c).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 175 mL of *acetonitrile R* and 175 mL of *methanol R* and dilute to 1 L with a 3.4 g/L solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Run time*: 4.5 the retention time of betaxolol.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *betaxolol for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B, C, D and E.

*Relative retention* with reference to betaxolol (retention time = about 8 min): impurity B = about 0.3; impurity A = about 0.8; impurity D = about 1.5; impurity E = about 2.2; impurity C = about 4.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity A and betaxolol.

*Limits*:

- *impurities A, B, C, D, E*: for each impurity, not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.39 mg of $C_{18}H_{30}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

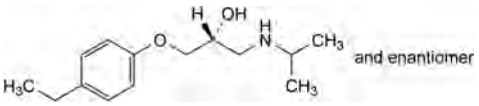


A. (2*RS*)-1-(4-ethylphenoxy)-3-[(1-methylethyl)amino]-propan-2-ol,

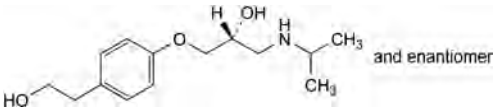


B. (2*RS*)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol,

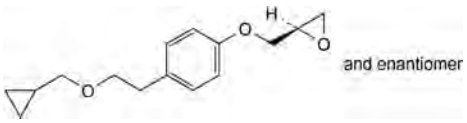


C. (2*RS*)-2-[[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]-methyl]oxirane,

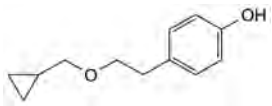


D. 4-[2-(cyclopropylmethoxy)ethyl]phenol,

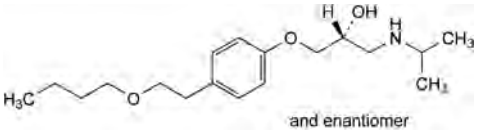


E. (2*RS*)-1-[4-(2-butoxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol.

**01/2017:1394**

# BEZAFIBRATE

## Bezafibratum

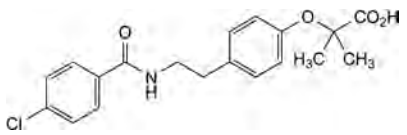

$C_{19}H_{20}ClNO_4$ $M_r$ 361.8
[41859-67-0]

DEFINITION

2-[4-[2-[(4-Chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, sparingly soluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 181 °C to 185 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bezafibrate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R* and evaporate to dryness. Dry the residues *in vacuo* at 80 °C for 1 h and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *bezafibrate CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *methyl ethyl ketone R*, *xylene R* (2.7:30:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over half of the plate.

*Drying*: at 120 °C for at least 15 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 1.0 g in *dimethylformamide R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 10.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (c).* To 1 mL of the test solution, add 1 mL of *0.1 M hydrochloric acid* and evaporate to dryness on a hot plate. Dissolve the residue in 20 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 40 volumes of a 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.3 with *phosphoric acid R*, and 60 volumes of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 228 nm.

*Injection*: 20 µL.

*Run time*: the time necessary to detect the ester, which, depending on the route of synthesis, may be impurity C, D or E.

*Relative retention* with reference to bezafibrate (retention time = about 6.0 min): impurity A = about 0.5; impurity B = about 0.6; impurity C = about 1.5; impurity D = about 2.3; impurity E = about 6.2.

*System suitability*:

– *resolution*: minimum 5.0 between the 2 principal peaks in the chromatogram obtained with reference solution (c);
– *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.75 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dilute 10 mL of solution S to 50 mL with *water R*. Filter the resultant suspension through a wet filter previously washed with *water R* until free from chlorides. Prepare the standard using 9 mL of *chloride standard solution (5 ppm Cl) R* and 6 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 25 volumes of *water R* and 75 volumes of *ethanol (96 per cent) R*. Using 0.1 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide* until a pink colour is obtained. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.18 mg of $C_{19}H_{20}ClNO_4$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

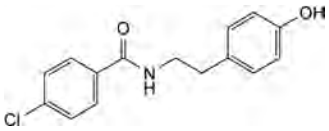

A. 4-chloro-*N*-[2-(4-hydroxyphenyl)ethyl]benzamide (chlorobenzoyltyramine),

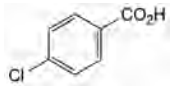

B. 4-chlorobenzoic acid,

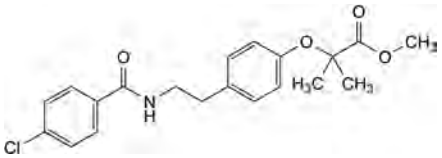

C. methyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]phenoxy]-2-methylpropanoate,

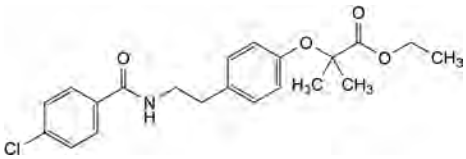

D. ethyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]-phenoxy]-2-methylpropanoate,

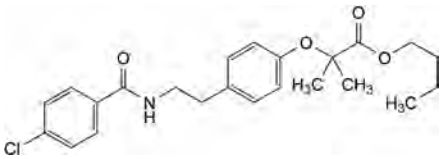

E. butyl 2-[4-[2-[(4-chlorobenzoyl)amino]ethyl]-phenoxy]-2-methylpropanoate.

**01/2017:2196**

# BICALUTAMIDE

## Bicalutamidum

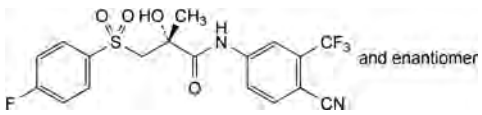


$C_{18}H_{14}F_4N_2O_4S$ $M_r$ 430.4
[90357-06-5]

DEFINITION

(2*RS*)-*N*-[4-Cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanamide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, slightly soluble in anhydrous ethanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bicalutamide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *phosphoric acid R*, *acetonitrile R1*, *water R* (0.05:50:50 *V/V/V*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *bicalutamide for system suitability CRS* (containing impurities B and C) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25.0 mg of *bicalutamide CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *acetonitrile R1*, *water R* (1.9:100:1900 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *water R*, *acetonitrile R1* (1.9:100:1900 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 92 | 8 |
| 3 - 23 | 92 → 67 | 8 → 33 |
| 23 - 43 | 67 → 50 | 33 → 50 |
| 43 - 50 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *bicalutamide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to bicalutamide (retention time = about 38 min): impurity B = about 0.98; impurity C = about 1.1.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bicalutamide.

*Limits*:

- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{14}F_4N_2O_4S$ taking into account the assigned content of *bicalutamide CRS*.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F, H, J, K, L, M.*

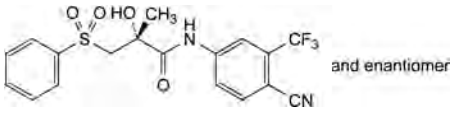


A. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methyl-3-(phenylsulfonyl)propanamide,

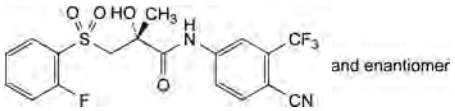


B. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(2-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanamide,

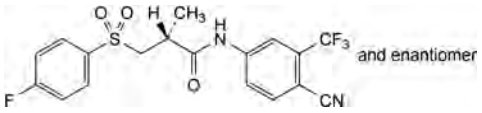


C. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfonyl]-2-methylpropanamide,

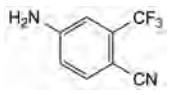

D. 4-amino-2-(trifluoromethyl)benzonitrile,

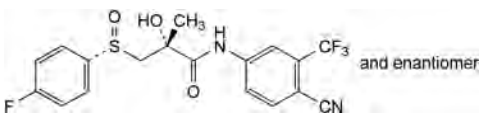


E. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(*RS*)-(4-fluorophenyl)sulfinyl]-2-hydroxy-2-methylpropanamide,

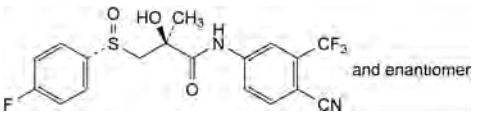


F. (2*SR*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(*RS*)-(4-fluorophenyl)sulfinyl]-2-hydroxy-2-methylpropanamide,

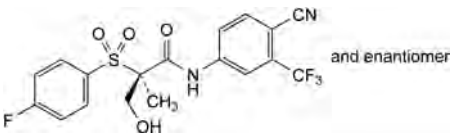


H. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-[(4-fluorophenyl)sulfonyl]-3-hydroxy-2-methylpropanamide,

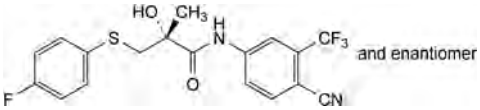


J. (2*RS*)-*N*-[4-cyano-3-(trifluoromethyl)phenyl]-3-[(4-fluorophenyl)sulfanyl]-2-hydroxy-2-methylpropanamide,

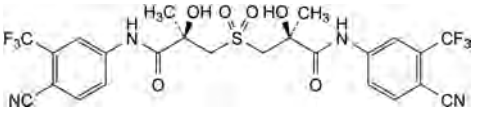

K. (2*R*,2′*S*)-3,3′-sulfonylbis[*N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methylpropanamide],

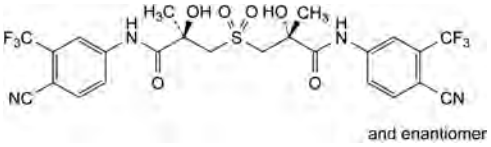


L. (2*RS*,2′*RS*)-3,3′-sulfonylbis[*N*-[4-cyano-3-(trifluoromethyl)phenyl]-2-hydroxy-2-methylpropanamide],

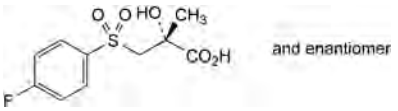


M. (2*RS*)-3-[(4-fluorophenyl)sulfonyl]-2-hydroxy-2-methylpropanoic acid.

**04/2012:1395**

# BIFONAZOLE

## Bifonazolum

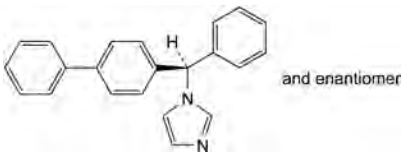


$C_{22}H_{18}N_2$ $M_r$ 310.4
[60628-96-8]

DEFINITION

1-[(*RS*)-(Biphenyl-4-yl)phenylmethyl]-1*H*-imidazole.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bifonazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *2-propanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 3.2.* Mix 2.0 mL of *phosphoric acid R* with 980 mL of *water R*, adjust to pH 3.2 (*2.2.3*) with *triethylamine R* and dilute to 1000.0 mL with *water R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 25 mL of *acetonitrile R* and dilute to 50.0 mL with buffer solution pH 3.2.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with buffer solution pH 3.2. Dilute 1.0 mL of this solution to 10.0 mL with buffer solution pH 3.2.

*Reference solution (b).* Dissolve 2 mg of *bifonazole for system suitability CRS* (containing impurities A, B, C, D and E) in 2 mL of *acetonitrile R* and dilute to 10.0 mL with buffer solution pH 3.2.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution pH 3.2 (20:80 *V/V*);
- *mobile phase B*: buffer solution pH 3.2, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 60 | 40 |
| 8 - 12 | 60 → 10 | 40 → 90 |
| 12 - 30 | 10 | 90 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram supplied with *bifonazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to bifonazole (retention time = about 4 min): impurity C = about 0.2; impurity B = about 0.7; impurity A = about 3.2; impurity D = about 3.6; impurity E = about 5.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity B and bifonazole.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2;
- *impurities B, D*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities A, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.04 mg of $C_{22}H_{18}N_2$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

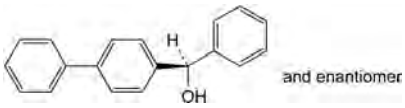


A. (*RS*)-(biphenyl-4-yl)phenylmethanol,

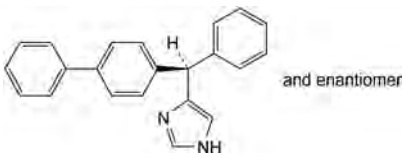


B. 4-[(*RS*)-(biphenyl-4-yl)phenylmethyl]-1*H*-imidazole,

C. 1*H*-imidazole,

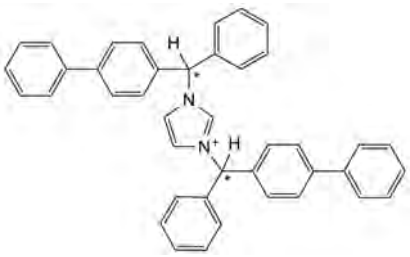


D. 1,3-bis[(biphenyl-4-yl)phenylmethyl]-1*H*-imidazolium ion,

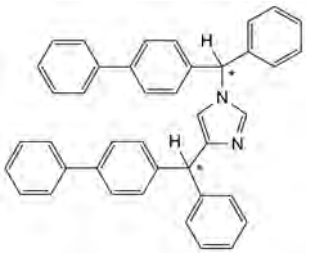


E. 1,4-bis[(biphenyl-4-yl)phenylmethyl]-1*H*-imidazole.

**01/2020:1073**

# BIOTIN

## Biotinum

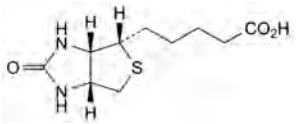

$C_{10}H_{16}N_2O_3S$ $M_r$ 244.3
[58-85-5]

DEFINITION

5-[(3a*S*,4*S*,6a*R*)-2-Oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent), practically insoluble in acetone. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: A.

*Second identification*: B.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *biotin CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Prepare the solutions immediately before use and keep protected from bright light.*

*Test solution*. Dissolve 5 mg of the substance to be examined in *glacial acetic acid R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 5 mg of *biotin CRS* in *glacial acetic acid R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (5 µm).

*Mobile phase*: *methanol R*, *glacial acetic acid R*, *toluene R* (5:25:75 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of warm air.

*Detection*: allow to cool and spray with *4-dimethylaminocinnamaldehyde solution R*; examine immediately in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 0.250 g in a 4 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with the same alkaline solution.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 89 to + 93 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and keep protected from bright light.*

*Solvent mixture*: *water R*, *acetonitrile R* (50:50 *V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture using sonication and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *biotin for system suitability CRS* (containing impurities A, C and E) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanesulfonic acid R*, *acetonitrile R1*, *water for chromatography R* (1:25:1000 *V/V/V*);
- *mobile phase B*: *methanesulfonic acid R*, *water for chromatography R*, *acetonitrile R1* (1:25:1000 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 20 | 95 → 0 | 5 → 100 |
| 20 - 28 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm from 0 to 5 min and at 210 nm from 5 to 28 min.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *biotin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C and E.

*Relative retention* with reference to biotin (retention time = about 12 min): bromide = about 0.2; impurity C = about 0.25; impurity A = about 1.1; impurity E = about 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to biotin and impurity A.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity E by 0.2;
- for each impurity, use the concentration of biotin in reference solution (a).

*Limits*:

- *impurities A, E*: for each impurity, maximum 0.5 per cent;
- *impurity C*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.05 per cent, disregard the peak due to the bromide ion.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Suspend 0.200 g in 5 mL of *dimethylformamide R*. Heat until the substance has dissolved completely. Add 50 mL of *ethanol R* and titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 24.43 mg of $C_{10}H_{16}N_2O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, F, G, H.*

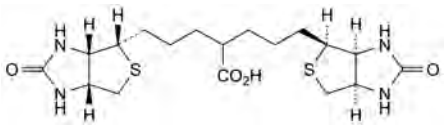

A. 5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]-2-[[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]propyl]pentanoic acid,

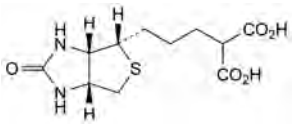

B. 4-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]butane-1,1-dicarboxylic acid,

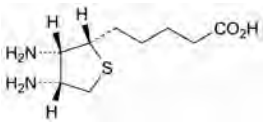

C. 5-[(2*S*,3*S*,4*R*)-3,4-diaminothiolan-2-yl]pentanoic acid,

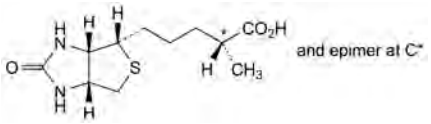


D. (2*RS*)-2-methyl-5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid,

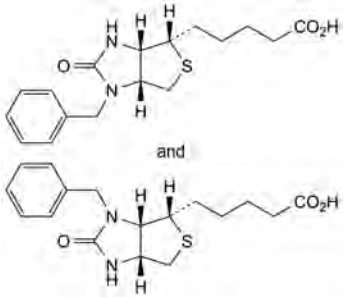


E. 5-[(3a*S*,4*S*,6a*R*)-1-benzyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid and 5-[(3a*S*,4*S*,6a*R*)-3-benzyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid,

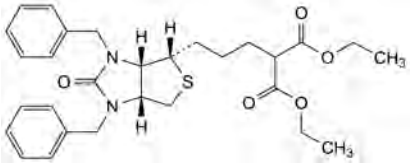

F. diethyl 4-[(3a*S*,4*S*,6a*R*)-1,3-dibenzyl-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]butane-1,1-dicarboxylate,

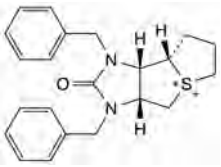

G. (3a*R*,8a*S*,8b*S*)-1,3-dibenzyl-2-oxodecahydrothieno[1′,2′:1,2]thieno[3,4-*d*]imidazol-5-ium,

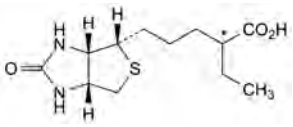

H. 2-ethyl-5-[(3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl]pentanoic acid.

**01/2017:1074**
**corrected 10.0**

# BIPERIDEN HYDROCHLORIDE

## Biperideni hydrochloridum

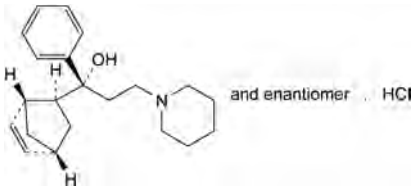


$C_{21}H_{30}ClNO$ $M_r$ 347.9

[1235-82-1]

DEFINITION

(1*RS*)-1-[(1*RS*,2*SR*,4*RS*)-Bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in ethanol (96 per cent), very slightly soluble in methylene chloride.

mp: about 280 °C, with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *biperiden hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution (a).* Dissolve 25 mg of *biperiden hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution (b).* Dissolve 5 mg of *biperiden impurity A CRS* in reference solution (a) and dilute to 2 mL with the same solution.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *diethylamine R, methanol R, toluene R* (1:1:20 *V/V/V*).
*Application*: 5 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *dilute potassium iodobismuthate solution R* and then with *sodium nitrite solution R* and examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

- the chromatogram shows 2 clearly separated spots.

C. To about 20 mg add 5 mL of *phosphoric acid R*. A green colour develops.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.10 g in *carbon dioxide-free water R*, heating gently if necessary, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.5 for solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dilute 0.5 mL of the test solution to 100 mL with *methanol R*. Dilute 10 mL of this solution to 50 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *biperiden impurity A CRS* in *methanol R* and dilute to 5 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *methanol R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 0.4 mL/min.

*Split ratio*: 1:250.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 200 |
| | 5 - 40 | 200 → 270 |
| Injection port | | 250 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Run time*: twice the retention time of biperiden.

*Relative retention* with reference to biperiden: impurities A, B and C = between 0.95 and 1.05.

*System suitability*:

- *resolution*: minimum 2.5 between the peak due to biperiden (1st peak) and the peak due to impurity A (2nd peak) in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 6 for the principal peak in the chromatogram obtained with reference solution (a).

*Limits*:

- *impurities A, B, C*: for each impurity, maximum 0.50 per cent of the area of the principal peak;
- *any other impurity*: for each impurity, maximum 0.10 per cent of the area of the principal peak;
- *total of impurities A, B and C*: maximum 1.0 per cent of the area of the principal peak;
- *total of impurities other than A, B and C*: maximum 0.50 per cent of the area of the principal peak;
- *disregard limit*: 0.05 per cent of the area of the principal peak.

**Impurity F** (*2.4.24*): maximum 2 ppm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 mL of *ethanol (96 per cent) R*. In a closed vessel, titrate with *0.1 M alcoholic potassium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M alcoholic potassium hydroxide* is equivalent to 34.79 mg of $C_{21}H_{30}ClNO$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

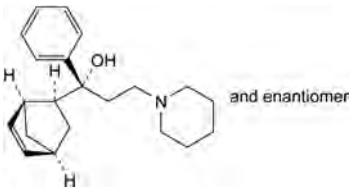


A. (1*RS*)-1-[(1*SR*,2*SR*,4*SR*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol (*endo* form),

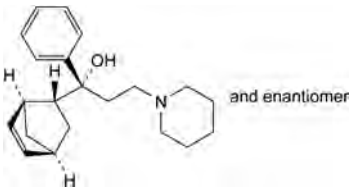


B. (1*RS*)-1-[(1*SR*,2*RS*,4*SR*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol,

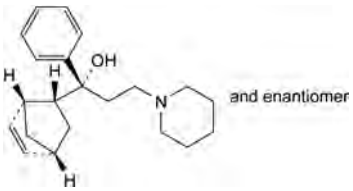


C. (1*RS*)-1-[(1*RS*,2*RS*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-1-phenyl-3-(piperidin-1-yl)propan-1-ol,

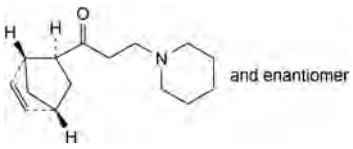


D. 1-[(1*RS*,2*SR*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-3-(piperidin-1-yl)propan-1-one,

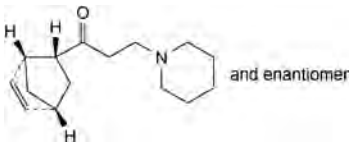


E. 1-[(1*RS*,2*RS*,4*RS*)-bicyclo[2.2.1]hept-5-en-2-yl]-3-(piperidin-1-yl)propan-1-one,

F. benzene.

**01/2008:0595**
**corrected 6.0**

# BISACODYL

## Bisacodylum

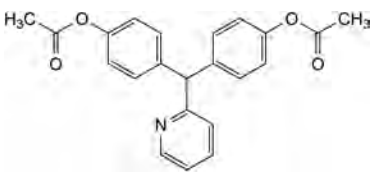


$C_{22}H_{19}NO_4$ $M_r$ 361.4
[603-50-9]

DEFINITION

4,4′-(Pyridin-2-ylmethylene)diphenyl diacetate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent). It dissolves in dilute mineral acids.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 131 °C to 135 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in a 6 g/L solution of *potassium hydroxide R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 10.0 mL of this solution to 100.0 mL with a 6 g/L solution of *potassium hydroxide R* in *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 248 nm.

*Shoulder*: at 290 nm.

*Specific absorbance at the absorption maximum*: 632 to 672.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bisacodyl CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *chloroform R*, evaporate to dryness and record new spectra using the residues.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *bisacodyl CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *methyl ethyl ketone R*, *xylene R* (50:50 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm.

*Drying*: in air, if necessary heating at 100-105 °C.

*Detection*: spray with a mixture of equal volumes of *0.05 M iodine* and *dilute sulfuric acid R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R*, shake, heat to boiling, cool and filter. Add 0.2 mL of *0.01 M sodium hydroxide* and 0.1 mL of *methyl red solution R*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *glacial acetic acid R*, *acetonitrile R*, *water R* (4:30:66 *V/V/V*).

*Test solution.* Dissolve 50 mg of the substance to be examined in 25 mL of *acetonitrile R* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2.0 mg of *bisacodyl for system suitability CRS* (containing impurities A, B, C, D and E) in 1.0 mL of *acetonitrile R* and dilute to 2.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5.0 mg of *bisacodyl for peak identification CRS* (containing impurity F) in 2.5 mL of *acetonitrile R* and dilute to 5.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 45 volumes of *acetonitrile R* and 55 volumes of a 1.58 g/L solution of *ammonium formate R* previously adjusted to pH 5.0 with *anhydrous formic acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of bisacodyl.

*Identification of impurities*: use the chromatogram supplied with *bisacodyl for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to bisacodyl (retention time = about 13 min): impurity A = about 0.2; impurity B = about 0.4; impurity C = about 0.45; impurity D = about 0.8; impurity E = about 0.9; impurity F = about 2.6.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bisacodyl.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.7;
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurities C, E*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.14 mg of $C_{22}H_{19}NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

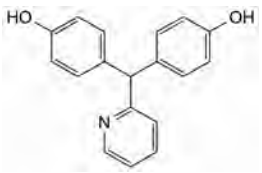


A. 4,4′-(pyridin-2-ylmethylene)diphenol,

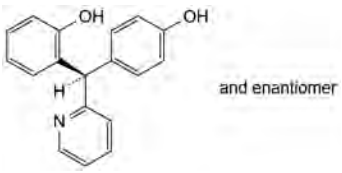


B. 2-[(*RS*)-(4-hydroxyphenyl)(pyridin-2-yl)methyl]phenol,

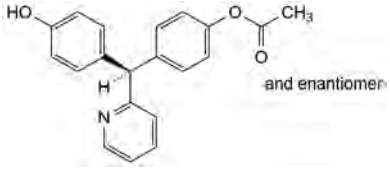


C. 4-[(*RS*)-(4-hydroxyphenyl)(pyridin-2-yl)methyl]phenyl acetate,

D. unknown structure,

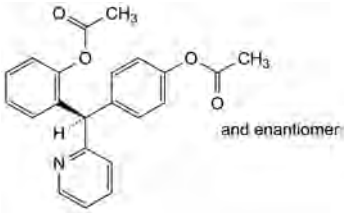


E. 2-[(*RS*)-[4-(acetyloxy)phenyl](pyridin-2-yl)methyl]phenyl acetate,

F. unknown structure.

**01/2008:0012**
**corrected 7.0**

# BISMUTH SUBCARBONATE

## Bismuthi subcarbonas

DEFINITION

*Content*: 80.0 per cent to 82.5 per cent of Bi ($A_r$ 209.0) (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves with effervescence in mineral acids.

IDENTIFICATION

A. It gives the reaction of carbonates (*2.3.1*).

B. It gives the reactions of bismuth (*2.3.1*).

TESTS

**Solution S**. Shake 5.0 g with 10 mL of *water R* and add 20 mL of *nitric acid R*. Heat to dissolve, cool and dilute to 100 mL with *water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 500 ppm.

To 6.6 mL of solution S add 4 mL of *nitric acid R* and dilute to 50 mL with *water R*.

**Nitrates**: maximum 0.4 per cent.

To 0.25 g in a 125 mL conical flask, add 20 mL of *water R*, 0.05 mL of *indigo carmine solution R1* and then, as a single addition but with caution, 30 mL of *sulfuric acid R*. Titrate immediately with *indigo carmine solution R1* until a stable blue colour is obtained. Not more than *n* mL of the titrant is required, *n* being the volume corresponding to 1 mg of $NO_3$.

**Alkali and alkaline-earth metals**: maximum 1.0 per cent.

To 1.0 g add 10 mL of *water R* and 10 mL of *acetic acid R*. Boil for 2 min, cool and filter. Wash the residue with 20 mL of *water R*. To the combined filtrate and washings add 2 mL of *dilute hydrochloric acid R* and 20 mL of *water R*. Boil and pass *hydrogen sulfide R* through the boiling solution until no further precipitate is formed. Filter, wash the residue with *water R*, evaporate the combined filtrate and washings to dryness on a water-bath and add 0.5 mL of *sulfuric acid R*. Ignite gently and allow to cool. The residue weighs a maximum of 10 mg.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm.

To 0.5 g in a distillation flask add 5 mL of *water R* and 7 mL of *sulfuric acid R*, allow to cool and add 5 g of *reducing mixture R* and 10 mL of *hydrochloric acid R*. Heat the contents of the flask to boiling gradually over 15-30 min and continue heating at such a rate that the distillation proceeds steadily until

the volume in the flask is reduced by half or until 5 min after the air-condenser has become full of steam. It is important that distillation be discontinued before fumes of sulfur trioxide appear. Collect the distillate in a tube containing 15 mL of *water R* cooled in ice-water. Wash down the condenser with *water R* and dilute the distillate to 25 mL with the same solvent. Prepare the standard using a mixture of 2.5 mL of *arsenic standard solution (1 ppm As) R* and 22.5 mL of *water R*.

**Copper**: maximum 50 ppm.

To 5 mL of solution S, add 2 mL of *ammonia R* and dilute to 50 mL with *water R*. Filter. To 10 mL of the filtrate add 1 mL of a 1 g/L solution of *sodium diethyldithiocarbamate R*. The solution is not more intensely coloured than a standard prepared at the same time in the same manner using a mixture of 0.25 mL of *copper standard solution (10 ppm Cu) R* and 9.75 mL of *water R* instead of 10 mL of the filtrate.

**Lead**: maximum 20 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 12.5 g in 75 mL of a mixture of equal volumes of *lead-free nitric acid R* and *water R*. Boil for 1 min, cool and dilute to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using appropriate quantities of lead standard solution and a 37 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

*Atomisation device*: air-acetylene flame.

**Silver**: maximum 25 ppm.

To 2.0 g add 1 mL of *water R* and 4 mL of *nitric acid R*. Heat gently until dissolved and dilute to 11 mL with *water R*. Cool and add 2 mL of *1 M hydrochloric acid*. Allow to stand protected from light for 5 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 10 mL of *silver standard solution (5 ppm Ag) R*, 1 mL of *nitric acid R* and 2 mL of *1 M hydrochloric acid*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.500 g in 3 mL of *nitric acid R* and dilute to 250 mL with *water R*. Carry out the complexometric titration of bismuth (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

STORAGE

Protected from light.

**01/2008:1493**
**corrected 7.0**

# BISMUTH SUBGALLATE

## Bismuthi subgallas

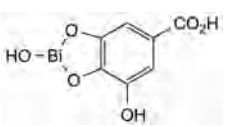


$C_7H_5BiO_6$ $M_r$ 394.1
[99-26-3]

DEFINITION

Complex of bismuth and gallic acid.

*Content*: 48.0 per cent to 51.0 per cent of Bi ($A_r$ 209.0) (dried substance).

CHARACTERS

*Appearance*: yellow powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in mineral acids with decomposition and in solutions of alkali hydroxides, producing a reddish-brown liquid.

IDENTIFICATION

A. Mix 0.1 g with 5 mL of *water R* and 0.1 mL of *phosphoric acid R*. Heat to boiling and maintain boiling for 2 min. Cool and filter. To the filtrate, add 1.5 mL of *ferric chloride solution R1*; a blackish-blue colour develops.

B. It gives reaction (b) of bismuth (*2.3.1*).

TESTS

**Solution S**. In a porcelain or quartz dish, ignite 1.0 g, increasing the temperature very gradually. Heat in a muffle furnace at 600 ± 50 °C for 2 h. Cool and dissolve the residue with warming in 4 mL of a mixture of equal volumes of *lead-free nitric acid R* and *water R* and dilute to 20 mL with *water R*.

**Acidity**. Shake 1.0 g with 20 mL of *water R* for 1 min and filter. To the filtrate add 0.1 mL of *methyl red solution R*. Not more than 0.15 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 0.5 g add 10 mL of *dilute nitric acid R*. Heat on a water-bath for 5 min and filter. Dilute 5 mL of the filtrate to 15 mL with *water R*.

**Nitrates**: maximum 0.2 per cent.

To 1.0 g add 25 mL of *water R* then 25 mL of a mixture of 2 volumes of *sulfuric acid R* and 9 volumes of *water R*. Heat at about 50 °C for 1 min with stirring and filter. To 10 mL of the filtrate, carefully add 30 mL of *sulfuric acid R*. The solution is not more intensely brownish-yellow than a reference solution prepared at the same time as follows: to 0.4 g of *gallic acid R*, add 20 mL of *nitrate standard solution (100 ppm $NO_3$) R* and 30 mL of a mixture of 2 volumes of *sulfuric acid R* and 9 volumes of *water R*, then filter; to 10 mL of the filtrate, carefully add 30 mL of *sulfuric acid R*.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (10 ppm Cu) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 20 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

*Atomisation device*: air-acetylene flame.

**Silver**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *silver standard solution (5 ppm Ag) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: silver hollow-cathode lamp.

*Wavelength*: 328.1 nm.

*Atomisation device*: air-acetylene flame.

**Substances not precipitated by ammonia**: maximum 1.0 per cent.

In a porcelain or quartz dish, ignite 2.0 g, increasing the temperature very gradually to 600 ± 50 °C; allow to cool. Moisten the residue with 2 mL of *nitric acid R*, evaporate to dryness on a water-bath and carefully heat and ignite once more at 600 ± 50 °C. After cooling, dissolve the residue in 5 mL of *nitric acid R* and dilute to 20 mL with *water R*. To 10 mL of this solution, add *concentrated ammonia R* until alkaline and filter. Wash the residue with *water R* and evaporate the combined filtrate and washings to dryness on a water-bath. Add 0.3 mL of *dilute sulfuric acid R* and ignite. The residue weighs a maximum of 10 mg.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

To 0.300 g add 10 mL of a mixture of equal volumes of *nitric acid R* and *water R*, heat to boiling and maintain boiling for 2 min. Add 0.1 g of *potassium chlorate R*, heat to boiling and maintain boiling for 1 min. Add 10 mL of *water R* and heat until the solution becomes colourless. To the hot solution, add 200 mL of *water R* and 50 mg of *xylenol orange triturate R*. Titrate with *0.1 M sodium edetate* until a yellow colour is obtained.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

STORAGE

Protected from light.

**01/2008:1494**
**corrected 7.0**

# BISMUTH SUBNITRATE, HEAVY

## Bismuthi subnitras ponderosus

$4[BiNO_3(OH)_2],BiO(OH)$ $M_r$ 1462
[1304-85-4]

DEFINITION

*Content*: 71.0 per cent to 74.0 per cent of Bi ($A_r$ 209.0) (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in mineral acids with decomposition.

IDENTIFICATION

A. Dilute 1 mL of solution S1 (see Tests) to 5 mL with *water R* and add 0.3 mL of *potassium iodide solution R*. A black precipitate is formed which dissolves into an orange solution with the addition of 2 mL of *potassium iodide solution R*.

B. It gives reaction (b) of bismuth (*2.3.1*).

C. It gives the reaction of nitrates (*2.3.1*).

D. pH (*2.2.3*): maximum 2.0 for solution S2 (see Tests).

TESTS

**Solution S1**. Shake 5.0 g by gently heating in 10 mL of *water R* and add 20 mL of *nitric acid R*. Heat until dissolution, cool and dilute to 100 mL with *water R*.

**Solution S2**. Place 1.00 g in a 20 mL volumetric flask and add 2.0 mL of *lead-free nitric acid R*. Allow acid attack to take place without heating and if necessary warm slightly at the end to completely dissolve the test sample. Add 10 mL of *water R*, shake and add, in small fractions, 4.5 mL of *lead-free ammonia R*; shake and allow to cool. Dilute to 20.0 mL with *water R*, shake again and allow the solids to settle. The clear supernatant solution is solution S2.

**Acidity**. Suspend 1.0 g in 15 mL of *water R* and shake several times. Allow to stand for 5 min and filter. To 10 mL of the filtrate, add 0.5 mL of *phenolphthalein solution R1*. Not more than 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 5.0 mL of solution S1, add 3 mL of *nitric acid R* and dilute to 15 mL with *water R*.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S2.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (10 ppm Cu) R* and diluting with a 37 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 20 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S2.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* and diluting with a 37 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

*Atomisation device*: air-acetylene flame.

**Silver**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S2.

*Reference solutions.* Prepare the reference solutions using *silver standard solution (5 ppm Ag) R* and diluting with a 37 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: silver hollow-cathode lamp.

*Wavelength*: 328.1 nm.

*Atomisation device*: air-acetylene flame.

**Substances not precipitated by ammonia**: maximum 1.0 per cent.

To 20 mL of solution S1, add *concentrated ammonia R* until an alkaline reaction is produced and filter. Wash the residue with *water R*, and evaporate the combined filtrate and washings to dryness on a water-bath. To the residue, add 0.3 mL of *dilute sulfuric acid R* and ignite. The residue weighs a maximum of 10 mg.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve with heating 0.250 g in 10 mL of a mixture of 2 volumes of *perchloric acid R* and 5 volumes of *water R*. To the hot solution, add 200 mL of *water R* and 50 mg of *xylenol orange triturate R*. Titrate with *0.1 M sodium edetate* until a yellow colour is obtained.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

**01/2008:1495 corrected 10.0**

# BISMUTH SUBSALICYLATE

## Bismuthi subsalicylas

$C_7H_3BiO_4$ $M_r$ 362.1
[14882-18-9]

DEFINITION

Complex of bismuth and salicylic acid.

*Content*: 56.0 per cent to 59.4 per cent of Bi ($A_r$ 209.0) (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in alcohol. It dissolves in mineral acids with decomposition.

IDENTIFICATION

A. To 0.5 g add 10 mL of *hydrochloric acid R1*. Heat on a water-bath for 5 min. Cool and filter. Retain the filtrate for identification test B. Wash the residue with *dilute hydrochloric acid R* and then with *water R*. Dissolve the residue in 0.5-1 mL of *dilute sodium hydroxide solution R*. Add 15 mL of *water R*. Neutralise with *dilute hydrochloric acid R*. The solution gives reaction (a) of salicylates (*2.3.1*).

B. The filtrate obtained in identification test A gives reaction (b) of bismuth (*2.3.1*).

TESTS

**Solution S**. In a porcelain or quartz dish, ignite 1.0 g, increasing the temperature very gradually. Heat in a muffle furnace at 600 ± 25 °C for 2 h. Cool and dissolve the residue with warming in 4 mL of a mixture of equal volumes of *lead-free nitric acid R* and *water R* and dilute to 20 mL with *water R*.

**Acidity**. Shake 2.0 g with 30 mL of *ether R* for 1 min and filter. To the filtrate add 30 mL of *alcohol R* and 0.1 mL of *thymol blue solution R*. Not more than 0.35 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.250 g in a mixture of 2 mL of *nitric acid R*, 5 mL of *water R* and 8 mL of *methanol R*.

**Nitrates**: maximum 0.4 per cent.

To 0.1 g add 10 mL of *water R* and, with caution, 20 mL of *sulfuric acid R* and stir. The solution is not more intensely yellow coloured than a reference solution prepared at the same time using 0.1 g of *salicylic acid R*, 6 mL of *water R*, 4 mL of *nitrate standard solution (100 ppm $NO_3$) R* and 20 mL of *sulfuric acid R*.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (10 ppm Cu) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 20 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm (depending on the apparatus, the line at 217.0 nm may be used).

*Atomisation device*: air-acetylene flame.

**Silver**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *silver standard solution (5 ppm Ag) R* and diluting with a 6.5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: silver hollow-cathode lamp.

*Wavelength*: 328.1 nm.

*Atomisation device*: air-acetylene flame.

**Soluble bismuth**: maximum 40 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Suspend 5.0 g in 100 mL of *water R*. Stir constantly for 2 h at 20-23 °C. Filter through filter paper (slow filtration) then through a cellulose micropore membrane filter (0.1 µm). To 10.0 mL of clear filtrate, add 0.1 mL of *nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *bismuth standard solution (100 ppm Bi) R* and diluting with a mixture of equal volumes of *dilute nitric acid R* and *water R*.

*Source*: bismuth hollow-cathode lamp.

*Wavelength*: 223.06 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve with heating 0.300 g in 10 mL of a mixture of 2 volumes of *perchloric acid R* and 5 volumes of *water R*. To the hot solution, add 200 mL of *water R* and 50 mg of *xylenol orange triturate R*. Titrate with *0.1 M sodium edetate* until a yellow colour is obtained.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

STORAGE

Protected from light.

**01/2012:1710**

# BISOPROLOL FUMARATE

## Bisoprololi fumaras

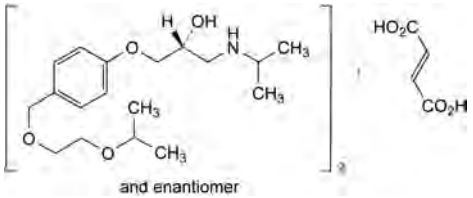

and enantiomer

$C_{40}H_{66}N_2O_{12}$ $M_r$ 767
[104344-23-2]

DEFINITION

(2*RS*)-1-[4-[[2-(1-Methylethoxy)ethoxy]methyl]phenoxy]-3-[(1-methylethyl)amino]propan-2-ol fumarate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: very soluble in water, freely soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bisoprolol fumarate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate and dry the residues at 60 °C at a pressure not exceeding 0.7 kPa and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (20:80 *V/V*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *bisoprolol for peak identification CRS* (containing impurities A and E) in 1.0 mL of the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *bisoprolol for system suitability CRS* (containing impurity G) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 ± 2 °C.

*Mobile phase*:

- *mobile phase A*: 10 g/L solution of *phosphoric acid R*;
- *mobile phase B*: 10 g/L solution of *phosphoric acid R* in *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 95 | 5 |
| 4 - 8 | 95 → 80 | 5 → 20 |
| 8 - 15 | 80 | 20 |
| 15 - 34 | 80 → 20 | 20 → 80 |
| 34 - 36 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *bisoprolol for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to fumaric acid and impurities A and E; use the chromatogram supplied with *bisoprolol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity G.

*Relative retention* with reference to bisoprolol (retention time = about 18 min): impurity A = about 0.5; impurity G = about 1.1; impurity E = about 1.2.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to bisoprolol.

*Limits*:

- *impurity G*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to fumaric acid.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 38.35 mg of $C_{40}H_{66}N_2O_{12}$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, K, L, N, Q, R, S, T, U.*

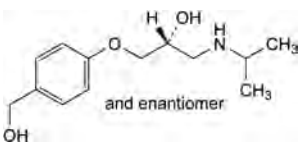


A. (2*RS*)-1-(4-hydroxymethyl-phenoxy)-3-isopropylamino-propan-2-ol,

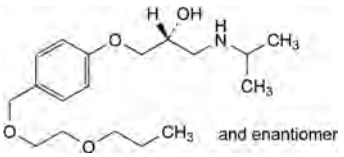


B. (2*RS*)-1-isopropylamino-3-[4-(2-propoxy-ethoxymethyl)-phenoxy]propan-2-ol,

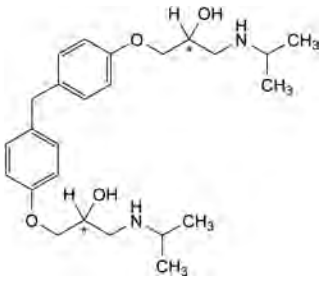

C. 1-[4-[4-(2-hydroxy-3-isopropylamino-propoxy)-benzyl]phenoxy]-3-isopropylaminopropan-2-ol,

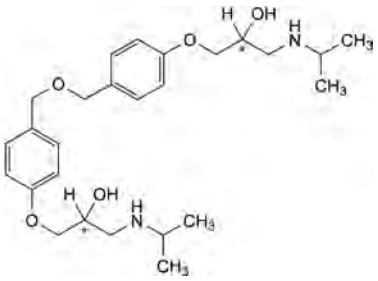

D. 1-[4-[4-(2-hydroxy-3-isopropylaminopropoxy)benzyloxyl-methyl]phenoxy]-3-isopropylaminopropan-2-ol,

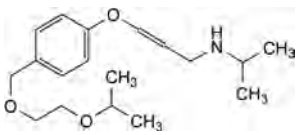

E. (*EZ*)-[3-[4-(2-isopropoxy-ethoxymethyl)phenoxy]allyl]-isopropylamine,

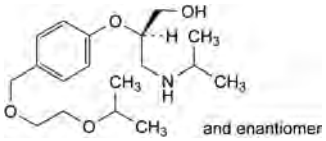

F. (2*RS*)-2-[4-(2-isopropoxy-ethoxymethyl)phenoxy]-3-isopropylaminopropan-2-ol,

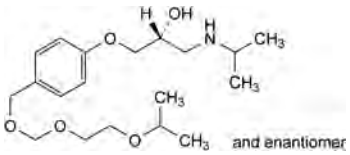

G. (2*RS*)-1-[4-[[(2-isopropoxyethoxy)methoxy]methyl]phenoxy]-3-isopropylaminopropan-2-ol,

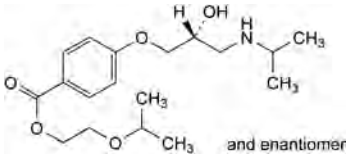

K. 2-isopropoxyethyl 4-[[(2*RS*)-2-hydroxy-3-(isopropylamino)propyl]oxy]benzoate,

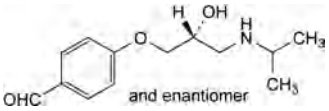

L. 4-[[(2*RS*)-2-hydroxy-3-(isopropylamino)propyl]oxy]-benzaldehyde,

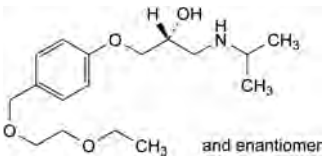

N. (2*RS*)-1-[4-[(2-ethoxyethoxy)methyl]phenoxy]-3-isopropylaminopropan-2-ol,

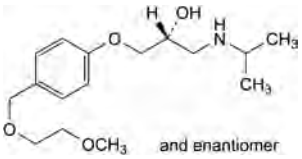

Q. (2*RS*)-1-(isopropylamino)-3-[4-(2-methoxyethoxy)methyl]phenoxypropan-2-ol,

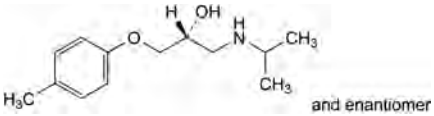

R. (2*RS*)-1-(isopropylamino)-3-(4-methylphenoxy)propan-2-ol,

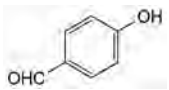

S. 4-hydroxybenzaldehyde,

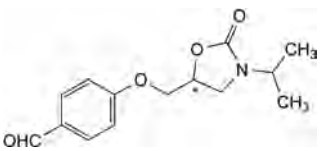

T. 4-[(3-isopropyl-2-oxo-1,3-oxazolidin-5-yl)methoxy]-benzaldehyde,

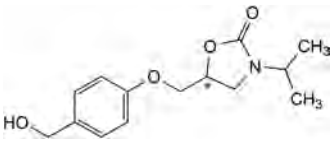

U. 5-[[4-(hydroxymethyl)phenoxy]methyl]-3-isopropyl-1,3-oxazolidin-2-one.

**01/2008:0976**
**corrected 10.0**

# BLEOMYCIN SULFATE

## Bleomycini sulfas

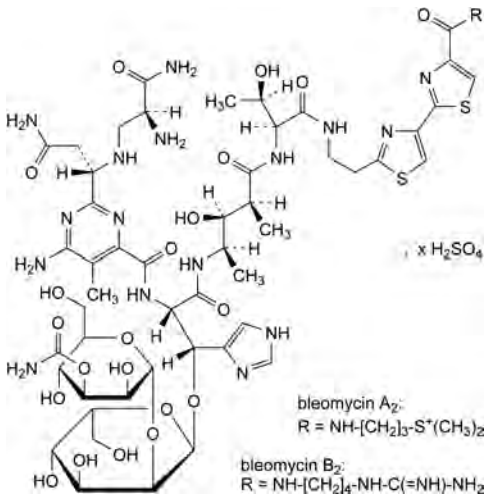


[9041-93-4]

DEFINITION

Sulfate of a mixture of glycopeptides produced by *Streptomyces verticillus* or by any other means; the 2 principal components of the mixture are *N*-[3-(dimethylsulfonio)propyl]bleomycinamide (bleomycin $A_2$) and *N*-[4-(carbamimidoylamino)butyl]bleomycinamide (bleomycin $B_2$).

*Potency*: minimum 1500 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, very hygroscopic powder.

*Solubility*: very soluble in water, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for composition.
*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with reference solution (a).

B. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.10.

Dissolve 0.200 g in *water R* and dilute to 10.0 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 6.0.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *bleomycin sulfate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.5 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*:
- *mobile phase A*: *methanol R*;
- *mobile phase B*: dissolve 0.960 g of *sodium pentanesulfonate R* in 900 mL of acetic acid (4.8 g/L $C_2H_4O_2$), add 1.86 g of *sodium edetate R*, dilute to 1000 mL with the same solvent and adjust to pH 4.3 with *ammonia R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 10 → 40 | 90 → 60 |
| 60 - end | 40 | 60 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: until impurity D is eluted (about 80 min).

*Relative retention* with reference to bleomycin $A_2$: impurity D = 1.5 to 2.5.

*System suitability*:
- *resolution*: minimum 5 between the peaks due to bleomycin $A_2$ (1st principal peak) and bleomycin $B_2$ (2nd principal peak) in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 2 per cent for the principal peak after 6 injections of reference solution (a).

*Limits*:
- *bleomycin $A_2$*: 55 per cent to 70 per cent;
- *bleomycin $B_2$*: 25 per cent to 32 per cent;
- *sum of bleomycin $A_2$ and $B_2$*: minimum 85 per cent;
- *impurity D*: maximum 5.5 per cent;
- *sum of impurities other than D*: maximum 9.5 per cent;
- *disregard limit*: 0.1 per cent of the total.

**Copper**: maximum 200 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 50 mg in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dilute 1.0 mL of *copper standard solution (10 ppm Cu) R* to 10.0 mL with *water R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 50 mg by drying at 60 °C at a pressure not exceeding 0.67 kPa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*), using the diffusion method. Use *bleomycin sulfate CRS* as the chemical reference substance.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

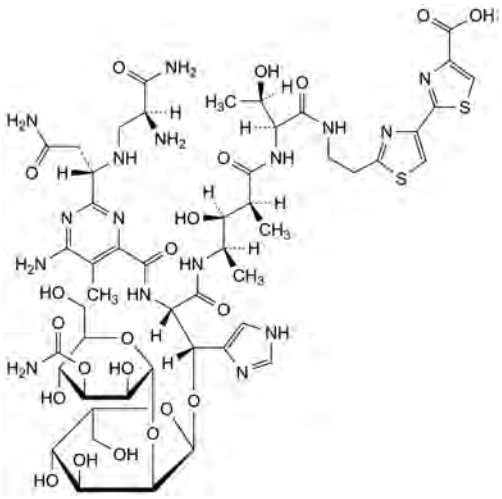

A. bleomycinic acid,

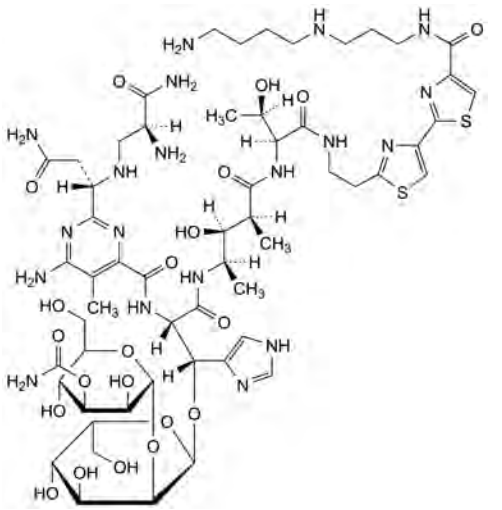

B. bleomycin $A_5$,

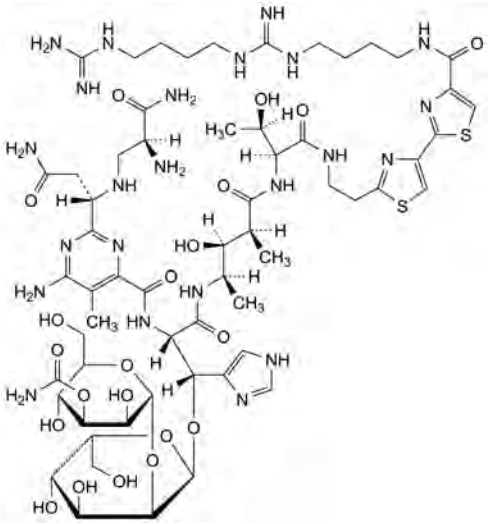

C. bleomycin $B_4$,

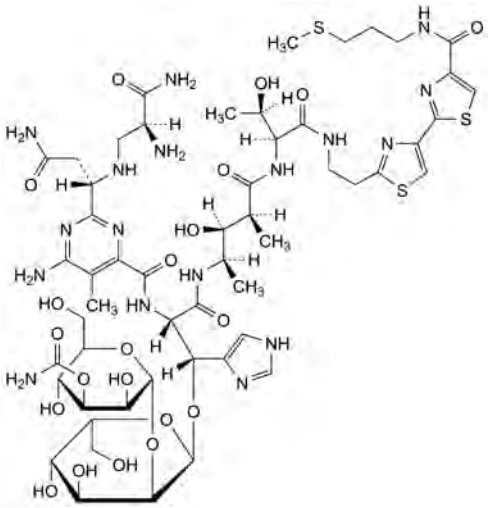

D. demethylbleomycin $A_2$.

**01/2020:2971**

# BOLDINE

## Boldinum

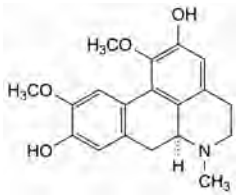

$C_{19}H_{21}NO_4$ $M_r$ 327.4
[476-70-0]

DEFINITION

(6a*S*)-1,10-Dimethoxy-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-2,9-diol, of vegetable origin.

*Content*: 98.5 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or yellowish, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute acid solutions.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *boldine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 121.0 to + 127.0 (anhydrous substance).

Dissolve 0.500 g in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 12.0 mg of the substance to be examined in 8 mL of *methanol R* using sonication and dilute to 10.0 mL with *methanol R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 12 mg of *boldine for system suitability CRS* (containing impurities C and E) in 8 mL of *methanol R* using sonication and dilute to 10 mL with *methanol R*.

*Reference solution (c).* In order to prepare impurity A *in situ*, add 0.5 mL of *strong hydrogen peroxide solution R* to 5 mL of reference solution (b) and stir for 1 h.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.54 g of *ammonium acetate R* in 950 mL of *water for chromatography R*, adjust to pH 8.50 ± 0.05 with *diethylamine R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 38 | 85 → 64 | 15 → 36 |
| 38 - 50 | 64 → 20 | 36 → 80 |
| 50 - 53 | 20 | 80 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *boldine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, C and E.

*Relative retention* with reference to boldine (retention time = about 23 min): impurity A = about 0.25; impurity C = about 0.6; impurity E = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 10.0 between the peaks due to impurity C and boldine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of boldine in reference solution (a).

*Limits*:

- *impurity C*: maximum 1.5 per cent;
- *impurity A*: maximum 0.3 per cent;
- *impurity E*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

**2-Propanol** (*2.4.24*): maximum 1.0 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g using the evaporation technique at 120 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.74 mg of $C_{19}H_{21}NO_4$.

IMPURITIES

*Specified impurities: A, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D.*

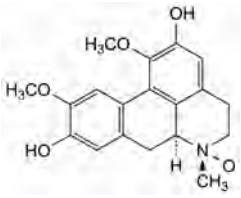


A. (6*S*,6a*S*)-2,9-dihydroxy-1,10-dimethoxy-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline *N*-oxide,

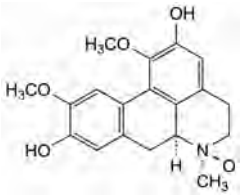


B. (6*R*,6a*S*)-2,9-dihydroxy-1,10-dimethoxy-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline *N*-oxide,

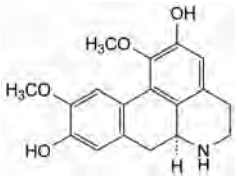


C. (6a*S*)-1,10-dimethoxy-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinoline-2,9-diol,

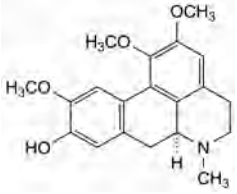


D. (6a*S*)-1,2,10-trimethoxy-6-methyl-5,6,6a,7-tetrahydro-4*H*-dibenzo[*de*,*g*]quinolin-9-ol,

E. unknown structure.

01/2020:2105

# BORAGE (STARFLOWER) OIL, REFINED

## Boraginis officinalis oleum raffinatum

DEFINITION

Fatty oil obtained from seeds of *Borago officinalis* L. by extraction and/or expression. It is then refined. A suitable antioxidant may be added.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, light yellow or yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.921.

*Refractive index*: about 1.476.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5, or maximum 0.3 if intended for use in the manufacture of parenteral preparations.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 2.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *saturated fatty acids of chain length less than* $C_{16}$: maximum 0.3 per cent;
- *palmitic acid*: 9.0 per cent to 12.0 per cent;
- *palmitoleic acid*: maximum 0.6 per cent;
- *stearic acid*: 2.0 per cent to 6.0 per cent;
- *oleic acid*: 12.0 per cent to 22.0 per cent;
- *linoleic acid*: 30.0 per cent to 41.0 per cent;
- *gamma-linolenic acid*: 17.0 per cent to 27.0 per cent;
- *alpha-linolenic acid*: maximum 0.5 per cent;
- *arachidic acid*: maximum 0.5 per cent;
- *eicosenoic acid*: 2.8 per cent to 4.4 per cent;
- *erucic acid*: maximum 3.0 per cent;
- *nervonic acid*: maximum 4.5 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

Under an inert gas, in a well-filled, airtight container, protected from light.

LABELLING

The label states:

- where applicable, that the oil is suitable for use in the manufacture of parenteral preparations;
- the name of the inert gas.

01/2017:0013

# BORAX

## Borax

$Na_2B_4O_7,10H_2O$ $M_r$ 381.4

[1303-96-4]

DEFINITION

Disodium tetraborate decahydrate.

*Content*: 99.0 per cent to 103.0 per cent of $Na_2B_4O_7,10H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, colourless crystals or crystalline masses, efflorescent.

*Solubility*: soluble in water, very soluble in boiling water, freely soluble in glycerol.

IDENTIFICATION

A. To 1 mL of solution S (see Tests) add 0.1 mL of *sulfuric acid R* and 5 mL of *methanol R* and ignite. The flame has a green border.

B. To 5 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution is red. On the addition of 5 mL of *glycerol (85 per cent) R* the colour disappears.

C. Solution S gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 4.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.0 to 9.6 for solution S.

**Sulfates** (*2.4.13*): maximum 50 ppm, determined on solution S.

Use in this test 1.0 mL of *acetic acid R*. Prepare the standard using a mixture of 3 mL of *sulfate standard solution (10 ppm* $SO_4$*) R* and 12 mL of *distilled water R*.

**Ammonium** (*2.4.1*): maximum 10 ppm.

Dilute 6 mL of solution S to 14 mL with *water R*. Prepare the standard using a mixture of 2.5 mL of *ammonium standard solution (1 ppm* $NH_4$*) R* and 7.5 mL of *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm, determined on 5 mL of solution S.

**Calcium** (*2.4.3*): maximum 100 ppm, determined on solution S.

Prepare the standard using a mixture of 6 mL of *calcium standard solution (10 ppm Ca) R* and 9 mL of *distilled water R*.

ASSAY

Dissolve 20 g of *mannitol R* in 100 mL of *water R*, heating if necessary, cool and add 0.5 mL of *phenolphthalein solution R* and neutralise with *0.1 M sodium hydroxide* until a pink colour is obtained. Add 3.00 g of the substance to be examined, heat until dissolution is complete, cool, and titrate with *1 M sodium hydroxide* until the pink colour reappears.

1 mL of *1 M sodium hydroxide* is equivalent to 0.1907 g of $Na_2B_4O_7,10H_2O$.

**01/2017:0001**

# BORIC ACID

## Acidum boricum

$H_3BO_3$ $M_r$ 61.8
[10043-35-3]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, colourless, shiny plates greasy to the touch, or white or almost white crystals.

*Solubility*: soluble in water and in ethanol (96 per cent), freely soluble in boiling water and in glycerol (85 per cent).

IDENTIFICATION

A. Dissolve 0.1 g by gently heating in 5 mL of *methanol R*, add 0.1 mL of *sulfuric acid R* and ignite the solution. The flame has a green border.

B. Solution S (see Tests) is acid (*2.2.4*).

TESTS

**Solution S**. Dissolve 3.3 g in 80 mL of boiling *distilled water R*, cool and dilute to 100 mL with *carbon dioxide-free water R* prepared from *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.8 to 4.8 for solution S.

**Solubility in ethanol (96 per cent)**. The solution is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

Dissolve 1.0 g in 10 mL of boiling *ethanol (96 per cent) R*.

**Organic matter**. It does not darken on progressive heating to dull redness.

**Sulfates** (*2.4.13*): maximum 450 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

ASSAY

Dissolve 1.000 g with heating in 100 mL of *water R* containing 15 g of *mannitol R*. Titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 mL of *1 M sodium hydroxide* is equivalent to 61.8 mg of $H_3BO_3$.

**01/2019:2113**
**corrected 10.0**

# BOTULINUM TOXIN TYPE A FOR INJECTION

## Toxinum botulinicum A ad iniectabile

DEFINITION

Botulinum toxin type A for injection is a dried preparation containing purified botulinum neurotoxin type A, which may be present in the form of a complex with haemagglutinins and non-toxic proteins. Botulinum neurotoxin type A or its haemagglutinin complex is prepared by a suitable purification process of the liquid supernatant from a broth-culture of a suitable strain of *Clostridium botulinum* type A.

The purified complexes consist of several proteins and can be of various sizes. The largest complex (relative molecular mass of about 900 000) consists of a 150 000 relative molecular mass neurotoxin, a 130 000 relative molecular mass non-toxic protein and various haemagglutinins ranging between relative molecular mass 14 000 and 43 000. The purified toxin moiety is composed of only the same 150 000 relative molecular mass neurotoxin as is found in the 900 000 relative molecular mass neurotoxin complex, which is initially produced as a single chain and further cleaved (nicked) by endogenous proteases into a fully active, disulfide-linked, 54 000 relative molecular mass light chain and a 97 000 relative molecular mass heavy chain.

The preparation is reconstituted before use, as stated on the label.

PRODUCTION

*GENERAL PROVISIONS*

Production of the toxin is based on seed cultures, managed in a defined seed-lot system in which the ability to produce toxin is conserved. The production method must be shown to yield consistently product of activity and profile comparable to that of lots shown in clinical studies to be of adequate safety and efficacy.

The production method and stability of the finished product and relevant intermediates are evaluated using the tests below. Such tests include the specific toxin activity per milligram of protein of purified toxin in an appropriate functional model of toxin activity and may be supported by tests confirming the presence of botulinum toxin type A, and, if appropriate, associated non-toxic proteins.

*BACTERIAL SEED LOTS*

A highly toxigenic strain of *C. botulinum* of known toxin type A and confirmed absence of genes encoding other botulinum toxins (particularly botulinum toxin types B and F), with known origin and history, is grown using suitable media. The bacterial strain, used for the master seed lot, shall be identified by historical records that include information on its origin and the tests used to characterise the strain. These will include morphological, cultural, biochemical, genetic and serological properties of the strain. The master seed lot and the working seed lot, where applicable, must be demonstrated to have identical profiles. Only a seed lot that complies with the following requirements may be used.

**Identification**. Each seed lot is identified as containing pure cultures of *C. botulinum* type A bacteria with no extraneous bacterial or fungal contamination.

**Microbial purity**. Each seed lot complies with the requirements for absence of contaminating micro-organisms. The purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media and examination of colony morphology.

**Phenotypic parameters**. Each seed lot must have a known fatty acid profile, sugar fermentation profile (glucose, lactose, mannose, etc.) and proteolytic activity and must demonstrate relevant lipase, lecithinase and gelatinase activity.

**Genetic purity**. Each seed lot must have information on the toxin gene sequence and comply with requirements for the absence of other genes encoding other toxin serotypes.

**Production of active toxin**. A bacterial strain producing a high yield of active toxin, as determined by an acute toxicity assay, is suitable. Seed lots demonstrate a capability of producing at least a minimum toxicity level appropriate for the manufacturing process and scale.

*MANUFACTURER'S REFERENCE PREPARATIONS*

During development, reference preparations are established for subsequent verification of batch consistency during production and for control of the bulk purified toxin and finished product. They are derived from representative batches of botulinum toxin type A that are characterised as described under Bulk Purified Toxin.

The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their suitability.

*BULK PURIFIED TOXIN*

*C. botulinum* type A strain is grown anaerobically, in suitable media, from which cultures are selected for step-up incubations under a suitably controlled anaerobic atmosphere through the seed culture and bulk fermentation stages to allow maximum production of toxin. The toxin is purified by suitable methods to remove nucleic acids and components likely to cause adverse reactions.

Only a purified toxin that complies with the following requirements may be used in the preparation of the final bulk. For each test and for each product, limits of acceptance are established and each new purified toxin must comply with these limits.

**Residual reagents**. Removal of residual reagents used in purification steps is confirmed by suitable limit tests or by validation of the process.

**Nucleic acids**. Removal of nucleic acids is confirmed by suitable limit tests or by validation of the process.

**Immunological identity**. The presence of specific type A toxin is confirmed by a suitable immunochemical method (*2.7.1*).

**Specific activity**. The specific activity is confirmed in a mouse model of toxicity or by *in vivo/ex vivo* methods validated with respect to the $LD_{50}$ assay and expressed in mouse $LD_{50}$ units per milligram of protein. Specific activity must not be less than $1 \times 10^8$ mouse $LD_{50}$ units per milligram of protein for the 150 000 relative molecular mass neurotoxin and must not be less than $1 \times 10^7$ mouse $LD_{50}$ units per milligram of protein for the 900 000 relative molecular mass neurotoxin complex.

**Protein**. The total protein concentration is determined by a suitable method. An acceptable value is established for the product and each batch must be shown to comply with the limits.

**Protein profile**. Identity and protein composition are determined by polyacrylamide gel electrophoresis (*2.2.31*) under reducing or non-reducing conditions or by other suitable physicochemical methods such as size-exclusion chromatography (*2.2.30*), comparing with suitable reference standards.

**Total viable count**. It complies with the limits approved for the particular product.

*FINAL BULK*

The final bulk is prepared by adding approved excipients to the bulk purified toxin. The solution is filtered through a bacteria-retentive filter. If human albumin is added, it complies with the monograph *Human albumin solution (0255)*.

*FINAL LOT*

The final bulk is distributed aseptically into sterile, tamper-evident containers. Uniformity of fill is verified during filling and the test for uniformity of content (*2.9.6*) is not required. The containers are closed so as to prevent contamination.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

**pH** (*2.2.3*). The pH of the reconstituted product is within ± 0.5 pH units of the limit approved for the particular product.

**Water**: not more than the limit approved for the particular product.

## IDENTIFICATION

The presence of botulinum toxin type A is confirmed by a suitable immunochemical method (*2.7.1*).

## TESTS

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU per vial.

## ASSAY

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The $LD_{50}$ assay is associated with severe suffering of animals and manufacturers are strongly encouraged to develop and validate assays that will reduce the number of animals used, or refine or replace the test procedure with the goal of promoting animal welfare.

The potency of the reconstituted product is determined by an $LD_{50}$ assay in mice or by a method validated with respect to the $LD_{50}$ assay. The potency is expressed in terms of the $LD_{50}$ for mice or relative to the reference preparation.

For determination of the $LD_{50}$, graded doses of the product are injected intraperitoneally into groups of mice and the $LD_{50}$ is calculated by the usual statistical methods (*5.3*) from the mouse lethality in each group. A suitable reference preparation is assayed in parallel; the potency of the toxin is expressed relative to the reference or the value found for the reference is within suitable limits defined in terms of the assigned potency.

After validation with respect to the $LD_{50}$ assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare, for example mouse bioassays using paralysis as the end-point, *ex vivo* assays using mouse phrenic nerve diaphragm, endopeptidase assays *in vitro* and cell-based assays.

For alternative replacement methods the potency is calculated with respect to a suitable reference preparation calibrated in mouse $LD_{50}$ units.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

The test may be repeated but when more than 1 test is performed, the results of all valid tests must be combined in the estimate of potency.

## LABELLING

The label states:

- the number of units of toxin per vial with a statement that units are product specific and not applicable to other preparations containing botulinum toxin type A;
- the name and the volume of the diluent to be added for reconstitution of the dried product.

**01/2019:2581**
**corrected 10.0**

# BOTULINUM TOXIN TYPE B FOR INJECTION

## Toxinum botulinicum B ad iniectabile

DEFINITION

Botulinum toxin type B for injection is a liquid preparation containing purified botulinum neurotoxin type B, which may be present in the form of a complex with haemagglutinins and non-toxic proteins. Botulinum neurotoxin type B or its haemagglutinin complex is prepared by a suitable purification process of the liquid supernatant from a broth-culture of a suitable strain of *Clostridium botulinum* type B. Suitable stabilisers may be added.

The toxin is present in its native form as a complex of neurotoxin and non-toxin proteins and haemagglutinins with a total relative molecular mass of approximately 700 000. The neurotoxin is synthesised by the bacterium as a single-chain polypeptide of approximately 150 000 relative molecular mass that is activated during the fermentation process via a proteolytic cleavage (nicking) by endogenous proteases. The nicked protein is a fully active double-chain polypeptide consisting of a heavy chain (100 000 relative molecular mass) and a light chain (50 000 relative molecular mass), connected by a disulfide bond.

PRODUCTION

*GENERAL PROVISIONS*

Production of the toxin is based on seed cultures, managed in a defined seed-lot system in which the ability to produce toxin is conserved. The production method must be shown to yield consistently product of activity and profile comparable to that of lots shown in clinical studies to be of adequate safety and efficacy.

The production method and stability of the finished product and relevant intermediates are evaluated using the tests below. Such tests include the specific toxin activity per milligram of protein of purified toxin in an appropriate functional model of toxin activity and may be supported by tests confirming the presence of botulinum toxin type B, and, if appropriate, associated non-toxic proteins.

*BACTERIAL SEED LOTS*

A highly toxigenic strain of *C. botulinum* of known toxin type B and confirmed absence of genes encoding other botulinum toxins (particularly botulinum toxin types A and F), with known origin and history, is grown using suitable media. The bacterial strain, used for the master seed lot, shall be identified by historical records that include information on its origin and the tests used to characterise the strain. These will include morphological, cultural, biochemical, genetic and serological properties of the strain. The master seed lot and the working seed lot, where applicable, must be demonstrated to have identical profiles. Only a seed lot that complies with the following requirements may be used.

**Identification**. Each seed lot is identified as containing pure cultures of *C. botulinum* type B bacteria with no extraneous bacterial or fungal contamination.

**Microbial purity**. Each seed lot complies with the requirements for absence of contaminating micro-organisms. The purity of bacterial cultures is verified by methods of suitable sensitivity. These may include inoculation into suitable media and examination of colony morphology.

**Phenotypic parameters**. Each seed lot must have a known fatty acid profile, sugar fermentation profile (glucose, lactose, mannose, etc.) and proteolytic activity and must demonstrate relevant lipase, lecithinase and gelatinase activity.

**Genetic purity**. Each seed lot must have information on the toxin gene genomic location and on the toxin gene sequence, and comply with requirements for the absence of other genes encoding other toxin serotypes.

**Production of active toxin**. A bacterial strain producing a high yield of active toxin, as determined by an acute toxicity assay, is suitable. Seed lots demonstrate a capability of producing at least a minimum toxicity level appropriate for the manufacturing process and scale.

*MANUFACTURER'S REFERENCE PREPARATIONS*

During development, reference preparations are established for subsequent verification of batch consistency during production and for control of the bulk purified toxin and finished product. They are derived from representative batches of botulinum toxin type B that are characterised as described under Bulk Purified Toxin.

The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their suitability.

*BULK PURIFIED TOXIN*

*C. botulinum* type B strain is grown anaerobically, in suitable media, from which cultures are selected for step-up incubations under a suitably controlled anaerobic atmosphere through the seed culture and bulk fermentation stages to allow maximum production of toxin. The toxin is purified by suitable methods to remove nucleic acids and components likely to cause adverse reactions.

Only a purified toxin that complies with the following requirements may be used in the preparation of the final bulk. For each test and for each product, limits of acceptance are established and each new purified toxin must comply with these limits.

**Residual reagents**. Removal of residual reagents used in purification steps is confirmed by suitable limit tests or by validation of the process.

**Nucleic acids**. Removal of nucleic acids is confirmed by suitable limit tests or by validation of the process.

**Immunological identity**. The presence of specific type B toxin is confirmed by a suitable immunochemical method (*2.7.1*).

**Specific activity**. The specific activity is confirmed in a mouse model of toxicity or by *in vivo/ex vivo* methods validated with respect to the $LD_{50}$ assay and expressed in mouse $LD_{50}$ units per milligram of protein. Specific activity must not be less than $1 \times 10^8$ mouse $LD_{50}$ units per milligram of protein.

**Protein**. The total protein concentration is determined by a suitable method. An acceptable value is established for the product and each batch must be shown to comply with the limits.

**Protein profile**. Identity and protein composition are determined by polyacrylamide gel electrophoresis (*2.2.31*) under reducing or non-reducing conditions or by other suitable physicochemical methods such as size-exclusion chromatography (*2.2.30*), comparing with suitable reference standards.

**Total viable count**. It complies with the limits approved for the particular product.

*FINAL BULK*

The final bulk is prepared by adding approved excipients to the bulk purified toxin. The solution is filtered through a bacteria-retentive filter. If human albumin is added, it complies with the monograph *Human albumin solution (0255)*.

*FINAL LOT*

The final bulk is distributed aseptically into sterile, tamper-evident containers. Uniformity of fill is verified during filling and the test for uniformity of content (*2.9.6*) is not required. The containers are closed so as to prevent contamination.

Only a final lot that is within the limits approved for the particular product and is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

**pH** (*2.2.3*). The pH of the product is within ± 0.5 pH units of the limit approved for the particular product.

IDENTIFICATION

The presence of botulinum toxin type B is confirmed by a suitable immunochemical method (*2.7.1*).

TESTS

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU per vial.

ASSAY

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. The $LD_{50}$ assay is associated with severe suffering of animals and manufacturers are strongly encouraged to develop and validate assays that will reduce the number of animals used, or refine or replace the test procedure with the goal of promoting animal welfare.

The potency of the product is determined by an $LD_{50}$ assay in mice or by a method validated with respect to the $LD_{50}$ assay. The potency is expressed in terms of the $LD_{50}$ for mice or relative to the reference preparation.

For determination of the $LD_{50}$, graded doses of the product are injected intraperitoneally into groups of mice and the $LD_{50}$ is calculated by the usual statistical methods (*5.3*) from the mouse lethality in each group. A suitable reference preparation is assayed in parallel; the potency of the toxin is expressed relative to the reference or the value found for the reference is within suitable limits defined in terms of the assigned potency.

After validation with respect to the $LD_{50}$ assay (reference method), the product may also be assayed by other methods that are preferable in terms of animal welfare, for example mouse bioassays using paralysis as the end-point, *ex vivo* assays using mouse phrenic nerve diaphragm, endopeptidase assays *in vitro* and cell-based assays.

For alternative replacement methods the potency is calculated with respect to a suitable reference preparation calibrated in mouse $LD_{50}$ units.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

The test may be repeated but when more than 1 test is performed, the results of all valid tests must be combined in the estimate of potency.

LABELLING

The label states the number of units of toxin per vial with a statement that units are product specific and not applicable to other preparations containing botulinum toxin type B.

**01/2008:2262**

# BOVINE SERUM

## Serum bovinum

DEFINITION

Liquid fraction of blood obtained from the ox (*Bos taurus* L.) and from which cells, fibrin and clotting factors have been removed.

Different types of bovine serum are used:

- *adult bovine serum* obtained at slaughter from cattle that are declared fit for human consumption;
- *calf serum* obtained at slaughter from animals, fit for human consumption, before the age of 12 months;
- *new-born calf serum* obtained at slaughter from animals before the age of 20 days;
- *foetal bovine serum* obtained from normal foetuses from dams fit for human consumption;
- *donor bovine serum* obtained by repeated bleeding of donor animals from controlled donor herds.

*This monograph provides a general quality specification for bovine serum. Various measures are applied during the production of bovine serum aimed at obtaining a product that is acceptable as regards viral safety. No single measure, nor the combination of measures outlined below can guarantee complete viral safety but they rather reduce the risk involved in the use of serum in the manufacture of medicinal products. It is therefore necessary for the manufacturer of a medicinal product to take account of this when choosing the serum for a particular use by making a risk assessment.*

PRODUCTION

All stages of serum production are submitted to a suitable quality management system.

Traceability of serum is maintained from the final container to the abattoir of origin (for blood collected from slaughtered animals) or to the herd of origin (for blood collected from donor animals).

Further guarantee of the safety and quality of serum may be ensured by the use of a controlled donor herd. Where serum is obtained from such a herd, the animals are subjected to regular veterinary examination to ascertain their health status. Animals introduced into the herd are traceable as regards source, breeding and rearing history. The introduction of animals into the herd follows specified procedures, including defined quarantine measures. During the quarantine period the animals are observed and tested to establish that they are free from all agents and antibodies from which the donor herd is claimed to be free. It may be necessary to test the animals in quarantine for freedom from additional agents, depending on factors such as information available on their breeding and rearing history. It is recommended that animals in the herd should not be vaccinated against bovine viral diarrhoea virus. Tests are carried out for any agent and/or antibody from which the herd is claimed to be free.

Serum is obtained by separation of the serum from blood cells and clot under conditions designed to minimise microbial contamination. Serum from a number of animals is pooled and a batch number is allocated to the pool. Appropriate steps are taken to ensure homogeneity of the harvested material, intermediate pools and the final batch. Suitable measures (for example filtration) are taken to ensure sterility or a low bioburden. Before further processing, the serum is tested for sterility or bioburden. General and specific tests for viral contaminants are carried out as described below.

A step or steps for virus inactivation/removal are applied to serum intended for production of immunological veterinary medicinal products. Unless otherwise justified and authorised for a particular medicinal product, a step or steps for virus inactivation/removal are applied to serum intended for production of human and non-immunological veterinary medicinal products.

*INACTIVATION*

The inactivation procedure applied is validated with respect to a suitable representative range of viruses covering different types (enveloped, non-enveloped, DNA, RNA viruses). The optimal choice of relevant and model viruses depends strongly on the specific inactivation/removal procedure; representative viruses with different degrees of resistance to the type of treatment must be included. Bovine viral diarrhoea virus must be included in the viruses used for validation. Serum free from antibodies against bovine viral diarrhoea virus is used in part or all of the validation studies.

For bovine serum intended for use in immunological veterinary medicinal products, for inactivation by gamma irradiation a minimum dose of 30 kGy is applied, unless otherwise justified and authorised.

Critical parameters for the method of virus inactivation/removal are established and the parameters used in the validation study are strictly adhered to during subsequent application of the procedures to each batch of serum.

For inactivation by gamma irradiation, critical parameters include:

- the temperature;
- packaging configuration;
- distribution of dosimeters to assess the effective dose received by the product whatever its position;
- the minimum and maximum dose received.

*QUALITY CONTROL TESTS APPLIED TO EACH BATCH*

A suitable sample size for each batch is established. Specific tests for viral contaminants are validated with respect to sensitivity and specificity. The cell cultures used for general tests for viral contaminants are shown to be sensitive to a suitable range of potential contaminants. Control cells used in the tests are cultivated, where relevant, with a bovine serum controlled and inactivated as described in this monograph. Serum free from antibodies to bovine viral diarrhoea virus is required for validation of the effect of antibodies on the detection limits for bovine viral diarrhoea virus.

**Tests carried out on the batch prior to treatment**

The following tests are carried out on the serum (before any virus inactivation/removal steps, where applicable).

*Tests for viral contaminants.* General tests supplemented by specific tests are carried out.

*General tests.* Validated tests are carried out by inoculation of the serum on at least 2 distinct cell lines, one of which is of bovine origin. The cell lines used are suitable for detecting haemadsorbing viruses such as bovine parainfluenza virus 3 and cytopathic agents such as bovine herpesvirus 1.

*Specific tests for viral contaminants (if not detected by general tests), where relevant in view of the country of origin of the serum*: bluetongue virus, bovine adenovirus, bovine parvovirus, bovine respiratory syncytial virus, bovine viral diarrhoea virus, rabies virus and reovirus. Depending on the country of origin, specific tests for other viruses may be needed. The animal health status of countries is defined by the 'Office International des Epizooties' (OIE).

For serum to be subjected to a virus inactivation/removal procedure, if evidence of viral contamination is found in any of the tests described above, the serum is acceptable only if the virus is identified and shown to be present in an amount that has been shown in a validation study to be effectively inactivated.

For serum that is not to be subjected to a virus inactivation/removal procedure, if evidence of viral contamination is found in any of the tests described above, the serum is not acceptable.

A test for bovine viral diarrhoea virus antibodies is carried out; an acceptance criterion for the titre is established taking account of the risk assessment.

*Composition.* The content of a suitable selection of the following components is determined and shown to be within the expected range for the type of serum: cholesterol, α-, β- and γ-globulin, albumin, creatinine, bilirubin, glucose, serum aspartate transaminase (SAST, formerly SGOT - serum glutamic-oxaloacetic transaminase), serum alanine transaminase (SALT, formerly SGPT - glutamic-pyruvic transaminase), phosphorus, potassium, calcium, sodium and pH.

**Tests carried out on the batch post-treatment**

If bovine viral diarrhoea virus was detected before virus inactivation/removal, the following test for bovine viral diarrhoea virus is carried out after virus inactivation/removal.

*Test for bovine viral diarrhoea virus.* A validated test for bovine viral diarrhoea virus is carried out, for example by inoculation into susceptible cell cultures, followed by not fewer than 3 subcultures and detection by immunostaining. No evidence of the presence of bovine viral diarrhoea virus is found.

IDENTIFICATION

A. The electrophoretic pattern corresponds to that for serum and is consistent with the type (foetal or other) of bovine serum.

B. Bovine origin is confirmed by a suitable immunochemical method (*2.7.1*).

TESTS

**Osmolality** (*2.2.35*): 280 mosmol/kg to 365 mosmol/kg for foetal bovine serum and 240 mosmol/kg to 340 mosmol/kg for other types.

**Total protein** (*2.5.33*): 30 mg/mL to 45 mg/mL for foetal bovine serum and minimum 35 mg/mL for other types.

**Haemoglobin**: maximum 4 mg/mL, determined by a validated method, such as spectrophotometry.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mL for donor bovine serum, less than 25 IU/mL for foetal bovine serum, less than 100 IU/mL for other types.

**Sterility** (*2.6.1*). It complies with the test. Use 10 mL for each medium.

**Mycoplasmas** (*2.6.7*). It complies with the test.

STORAGE

Frozen at − 10 °C or below.

LABELLING

The label states:

- the type of serum;
- where applicable, that the serum has been inactivated and the inactivation method;
- where the serum has been inactivated by gamma irradiation, the target minimum dose of the irradiation procedure.

**07/2014:2760**
**corrected 10.0**

# BRIMONIDINE TARTRATE

## Brimonidini tartras

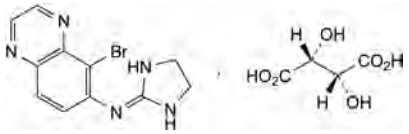

$C_{15}H_{16}BrN_5O_6$ $M_r$ 442.2
[70359-46-5]

DEFINITION

5-Bromo-*N*-(imidazolidin-2-ylidene)quinoxalin-6-amine (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish or slightly brownish powder.

*Solubility*: soluble in water, practically insoluble in anhydrous ethanol and in toluene.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *brimonidine tartrate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 9.0 to + 10.5 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 65.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve the contents of a vial of *brimonidine for system suitability CRS* (containing impurity E) in 1.0 mL of *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 2.6 g of *sodium heptanesulfonate R* in 310 mL of *methanol R*, add 2.5 mL of *triethylamine R* and 7.5 mL of *glacial acetic acid R*, and dilute to 1000 mL with *water R*. Use a freshly prepared mixture.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of brimonidine.

*Identification of impurities*: use the chromatogram supplied with *brimonidine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to brimonidine (retention time = about 19 min): impurity E = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurity E and brimonidine.

*Calculation of percentage contents*:
- for each impurity, use the concentration of brimonidine tartrate in reference solution (a).

*Limits*:
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 70 mL of *anhydrous acetic acid R* using sonication until complete dissolution. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 44.22 mg of $C_{15}H_{16}BrN_5O_6$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

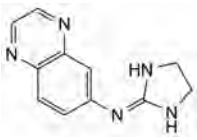

A. *N*-(imidazolidin-2-ylidene)quinoxalin-6-amine,

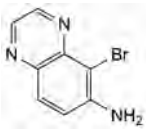

B. 5-bromoquinoxalin-6-amine,

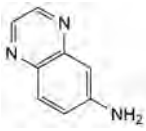

C. quinoxalin-6-amine,

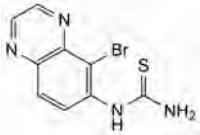

D. 1-(5-bromoquinoxalin-6-yl)thiourea,

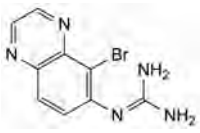

E. 2-(5-bromoquinoxalin-6-yl)guanidine,

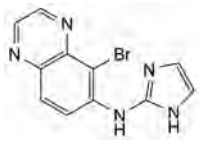

F. 5-bromo-*N*-(1*H*-imidazol-2-yl)quinoxalin-6-amine,

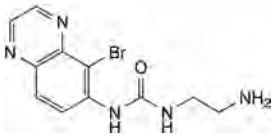

G. 1-(2-aminoethyl)-3-(5-bromoquinoxalin-6-yl)urea.

**01/2008:0879**

# BROMAZEPAM

## Bromazepamum

$C_{14}H_{10}BrN_3O$ $M_r$ 316.2
[1812-30-2]

DEFINITION

7-Bromo-5-(pyridin-2-yl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble or sparingly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bromazepam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 9 mL of a mixture of 1 volume of *acetonitrile R* and 8 volumes of *methanol R*. Dilute to 20.0 mL with an 11.33 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with a 100 g/L solution of *potassium hydroxide R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *bromazepam for system suitability CRS* (containing impurities A, B, C, D and E) in 5 mL of a mixture of 1 volume of *acetonitrile R* and 8 volumes of *methanol R*. Dilute to 10.0 mL with an 11.33 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with a 100 g/L solution of *potassium hydroxide R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 50 °C.

*Mobile phase*: mix 5 volumes of *acetonitrile R*, 45 volumes of *methanol R* and 50 volumes of an 11.33 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with a 100 g/L solution of *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of bromazepam.

*Identification of impurities*: use the chromatogram supplied with *bromazepam for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to bromazepam (retention time = about 5 min): impurity D = about 1.4; impurity A = about 1.5; impurity C = about 1.6; impurity E = about 2.1; impurity B = about 2.2.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to bromazepam and impurity D and minimum 1.2 between the peaks due to impurities A and C.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.3; impurity B = 1.8; impurity E = 2.1;
- *impurities A, B, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 2.7 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 20 mL of *anhydrous acetic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.62 mg of $C_{14}H_{10}BrN_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

A. (2-amino-5-bromophenyl)(pyridin-2-yl)methanone,

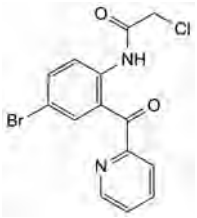

B. *N*-[4-bromo-2-(pyridin-2-ylcarbonyl)phenyl]-2-chloroacetamide,

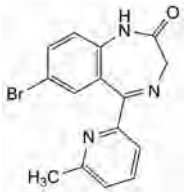

C. 7-bromo-5-(6-methylpyridin-2-yl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

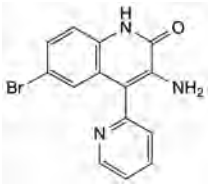

D. 3-amino-6-bromo-4-(pyridin-2-yl)quinolin-2(1*H*)-one,

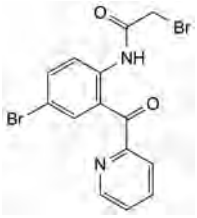

E. 2-bromo-*N*-[4-bromo-2-(pyridin-2-ylcarbonyl)-phenyl]acetamide.

**04/2017:0706**
**corrected 10.0**

# BROMHEXINE HYDROCHLORIDE

## Bromhexini hydrochloridum

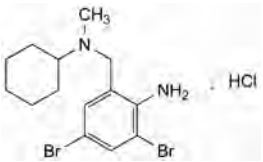

$C_{14}H_{21}Br_2ClN_2$ $M_r$ 412.6
[611-75-6]

DEFINITION

2,4-Dibromo-6-[[cyclohexyl(methyl)amino]methyl]aniline hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bromhexine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *bromhexine hydrochloride CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R*, *acetone R* (1:29:70 *V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 20 mg in 1 mL of *methanol R* and add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.6 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 1.26 g of *ammonium formate R* in 950 mL of *water for chromatography R* and adjust to pH 4.4 with *anhydrous formic acid R*. Dilute to 1000.0 mL with *water for chromatography R*.

*Solvent mixture*: *acetonitrile for chromatography R*, *water for chromatography R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *bromhexine for system suitability CRS* (containing impurities C and D) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *bromhexine hydrochloride CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.6 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile for chromatography R*, buffer solution (40:60 *V/V*).

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 3.0 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: twice the retention time of bromhexine.

*Identification of impurities*: use the chromatogram supplied with *bromhexine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and D.

*Relative retention* with reference to bromhexine (retention time = about 10 min): impurity C = about 0.2; impurity D = about 0.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurities C and D.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity C by 1.6;
- for each impurity, use the concentration of bromhexine hydrochloride in reference solution (b).

*Limits*:

- *impurity C*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{14}H_{21}Br_2ClN_2$ taking into account the assigned content of *bromhexine hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E.*

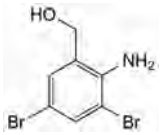

A. (2-amino-3,5-dibromophenyl)methanol,

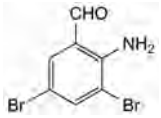

B. 2-amino-3,5-dibromobenzaldehyde,

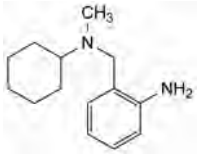

C. 2-[[cyclohexyl(methyl)amino]methyl]aniline,

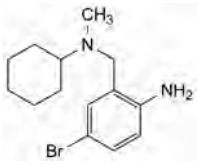

D. 4-bromo-2-[[cyclohexyl(methyl)amino]methyl]aniline,

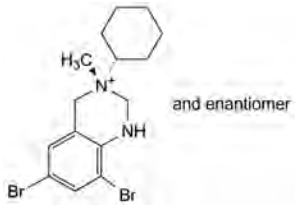


E. (3*RS*)-6,8-dibromo-3-cyclohexyl-3-methyl-1,2,3,4-tetrahydroquinazolin-3-ium.

**04/2016:0596**

# BROMOCRIPTINE MESILATE

## Bromocriptini mesilas

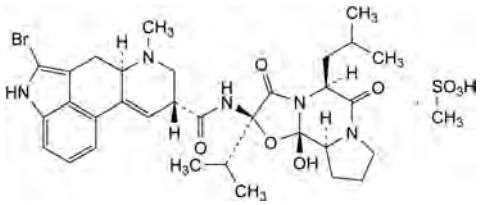

$C_{33}H_{44}BrN_5O_8S$ $M_r$ 751

[22260-51-1]

DEFINITION

(6a*R*,9*R*)-5-Bromo-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide monomethanesulfonate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in bromocriptine mesilate. The manufacturing process should be developed

taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or slightly coloured, fine crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), sparingly soluble in methylene chloride.

It is very sensitive to light.

*The identification, tests and assay are to be carried out as rapidly as possible, protected from light.*

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in 10 mL of *methanol R* and dilute to 200.0 mL with *0.01 M hydrochloric acid.*

*Spectral range*: 250-380 nm.

*Absorption maximum*: at 305 nm.

*Absorption minimum*: at 270 nm.

*Specific absorbance at the absorption maximum*: 120 to 135 (dried substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bromocriptine mesilate CRS.*

C. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *ethanol (96 per cent) R*, *methanol R*, *methylene chloride R* (30:30:40 *V/V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution.* Dissolve 10 mg of *bromocriptine mesilate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R*, *water R*, *2-propanol R*, *methylene chloride R*, *ether R* (0.1:1.5:3:88:100 *V/V/V/V/V*).

*Application*: 10 µL.

*Development*: immediately in an unsaturated tank, over a path of 15 cm.

*Drying*: in a current of cold air for 2 min.

*Detection*: spray with *ammonium molybdate solution R3* and dry at 100 °C until the spots appear (about 10 min).

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g add 5 mL of *dilute hydrochloric acid R* and shake for about 5 min. Filter and add 1 mL of *barium chloride solution R1.* The filtrate remains clear. To a further 0.1 g add 0.5 g of *anhydrous sodium carbonate R*, mix and ignite until a white residue is obtained. Allow to cool and dissolve the residue in 7 mL of *water R* (solution A). Solution A gives reaction (a) of sulfates (*2.3.1*).

E. Solution A obtained in identification test D gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$, $BY_5$ or $Y_5$ (*2.2.2, Method II*).

Dissolve 0.25 g in *methanol R* and dilute to 25 mL with the same solvent.

**pH** (*2.2.3*): 3.1 to 3.8.

Dissolve 0.2 g in a mixture of 2 volumes of *methanol R* and 8 volumes of *carbon dioxide-free water R* and dilute to 20 mL with the same mixture of solvents.

**Specific optical rotation** (*2.2.7*): + 95 to + 105 (dried substance).

Dissolve 0.100 g in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *buffer solution pH 2.0 R*, *methanol R* (50:50 *V/V*).

*Test solution.* Dissolve 0.500 g of the substance to be examined in 5.0 mL of *methanol R* and dilute to 10.0 mL with *buffer solution pH 2.0 R.*

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *bromocriptine mesilate for system suitability CRS* (containing impurities A and B) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.791 g/L solution of *ammonium carbonate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 90 → 40 | 10 → 60 |
| 30 - 45 | 40 | 60 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *bromocriptine mesilate for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to bromocriptine: impurity C = about 1.2.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.1 between the peaks due to impurities A and B.

*Limits*:

- *impurity A*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent);
- *impurity C*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurities B, D, E, F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent) and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent), apart from the peak due to impurity A.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 0.500 g by drying *in vacuo* at 80 °C for 5 h.

ASSAY

Dissolve 0.500 g in 80 mL of a mixture of 10 volumes of *anhydrous acetic acid R* and 70 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 75.1 mg of $C_{33}H_{44}BrN_5O_8S$.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding – 15 °C.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

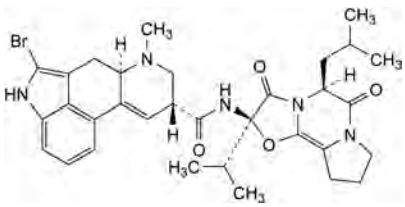

A. (6a*R*,9*R*)-5-bromo-7-methyl-*N*-[(2*R*,5*S*)-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxo-2,3,5,6,9,10-hexahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2-bromoanhydro-α-ergocryptine),

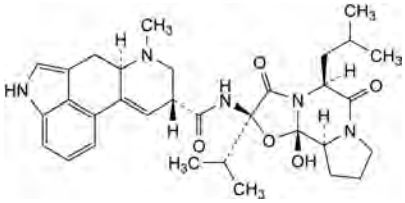

B. (6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (α-ergocryptine),

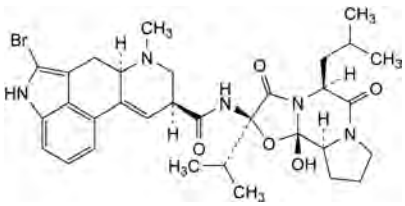

C. (6a*R*,9*S*)-5-bromo-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide ((8*S*)-2-bromo-α-ergocryptine),

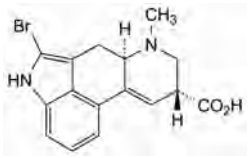

D. (6a*R*,9*R*)-5-bromo-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxylic acid,

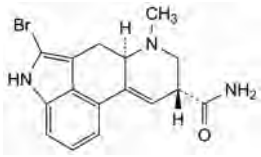

E. (6a*R*,9*R*)-5-bromo-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide,

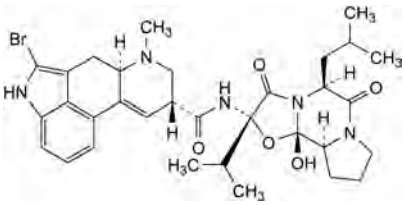

F. (6a*R*,9*R*)-5-bromo-*N*-[(2*S*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide ((2′*S*)-2-bromo-α-ergocryptine),

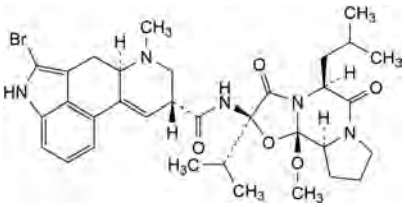

G. (6a*R*,9*R*)-5-bromo-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-methoxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2-bromo-10′b-*O*-methyl-α-ergocryptine).

**07/2011:1178**

# BROMPERIDOL

## Bromperidolum

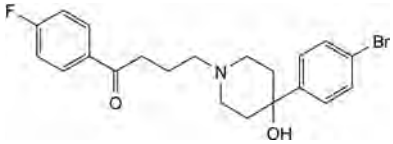

$C_{21}H_{23}BrFNO_2$ $M_r$ 420.3
[10457-90-6]

DEFINITION

4-[4-(4-Bromophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol and in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Melting point (*2.2.14*): 156 °C to 159 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bromperidol CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *bromperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *bromperidol CRS* and 10 mg of *haloperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R.*

*Mobile phase*: *tetrahydrofuran R*, *methanol R*, 58 g/L solution of *sodium chloride R* (10:45:45 *V/V/V*).

*Application*: 1 µL.

*Development*: in an unsaturated tank, over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which may, however, not be completely separated.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 5 mL of *anhydrous ethanol R*. Add 0.5 mL of *dinitrobenzene solution R* and 0.5 mL of *2 M alcoholic potassium hydroxide R*. A violet colour is produced that becomes brownish-red after 20 min.

E. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 0.2 g in 20 mL of a 1 per cent *V/V* solution of *lactic acid R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.5 mg of *bromperidol CRS* and 5.0 mg of *haloperidol CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 17 g/L solution of *tetrabutylammonium hydrogen sulfate R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 50 | 10 → 50 |
| 15 - 20 | 50 | 50 |
| 20 - 25 | 90 | 10 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to bromperidol (retention time = about 6 min): impurity A = about 0.5; impurity B = about 0.8; haloperidol = about 0.9; impurity C = about 1.4; impurity D = about 1.5; impurity E = about 1.8; impurity F = about 1.85.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to haloperidol and bromperidol.

*Limits*:

– *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 42.03 mg of $C_{21}H_{23}BrFNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

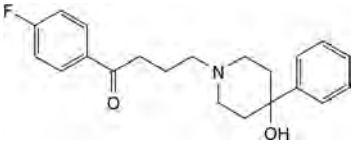


A. 1-(4-fluorophenyl)-4-(4-hydroxy-4-phenylpiperidin-1-yl)butan-1-one,

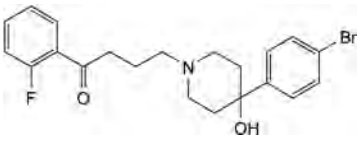


B. 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(2-fluorophenyl)butan-1-one,

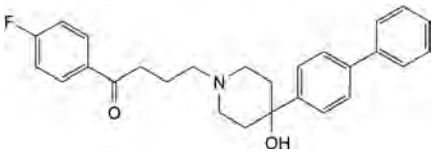

C. 4-[4-(biphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one,

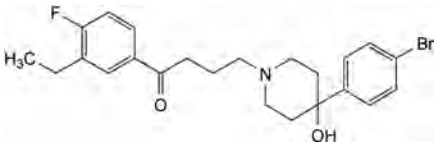

D. 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(3-ethyl-4-fluorophenyl)butan-1-one,

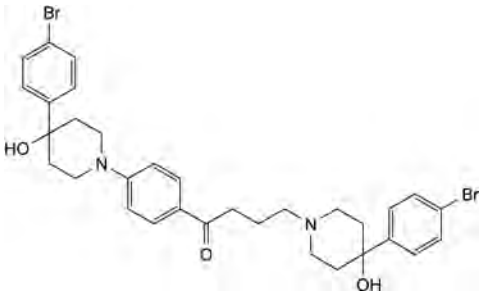

E. 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-[4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]phenyl]butan-1-one,

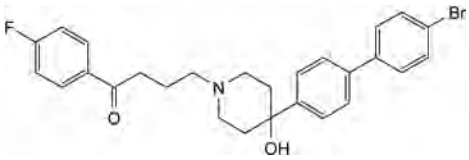

F. 4-[4-(4′-bromobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one.

**07/2011:1397**

# BROMPERIDOL DECANOATE

## Bromperidoli decanoas

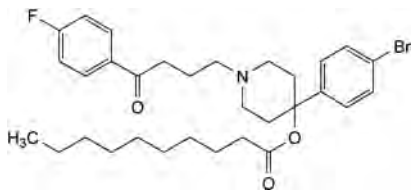

$C_{31}H_{41}BrFNO_3$ $M_r$ 574.6
[75067-66-2]

DEFINITION

4-(4-Bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, soluble in ethanol (96 per cent).

mp: about 60 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *bromperidol decanoate CRS*.

B. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methylene chloride R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.5 mg of *bromperidol decanoate CRS* and 2.5 mg of *haloperidol decanoate CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.1 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: 27 g/L solution of *tetrabutylammonium hydrogen sulfate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 80 → 40 | 20 → 60 |
| 30 - 35 | 40 | 60 |
| 35 - 40 | 40 → 80 | 60 → 20 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to bromperidol decanoate (retention time = about 24 min): impurity G = about 0.10; impurity L = about 0.15; impurity H = about 0.8; impurity A = about 0.89; impurity I = about 0.91; impurity B = about 0.96; haloperidol decanoate = about 0.98; impurity F = about 1.10; impurity C = about 1.15; impurity K = about 1.2; impurity E = about 1.23; impurity D = about 1.25.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to haloperidol decanoate and bromperidol decanoate.

*Limits*:
- *impurities A, B, C, D, E, F, G, H, I, J, K*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 30 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.450 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 57.46 mg of $C_{31}H_{41}BrFNO_3$.

STORAGE

Protected from light, at a temperature below 25 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *L.*

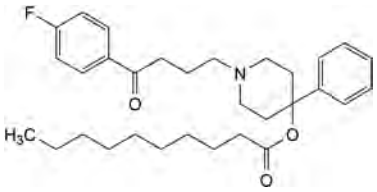

A. 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-phenylpiperidin-4-yl decanoate,

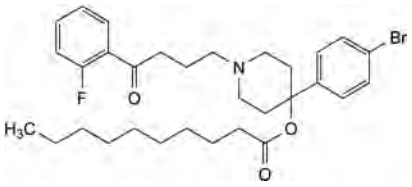

B. 4-(4-bromophenyl)-1-[4-(2-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate,

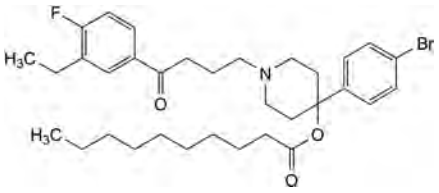

C. 4-(4-bromophenyl)-1-[4-(3-ethyl-4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate,

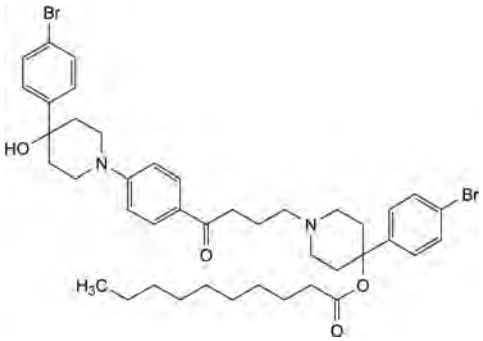

D. 4-(4-bromophenyl)-1-[4-[4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]phenyl]-4-oxobutyl]piperidin-4-yl decanoate,

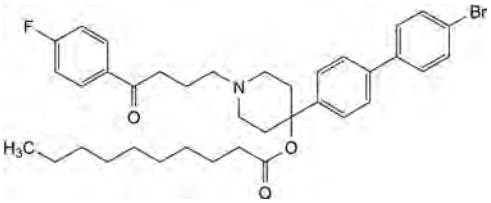

E. 4-(4′-bromobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

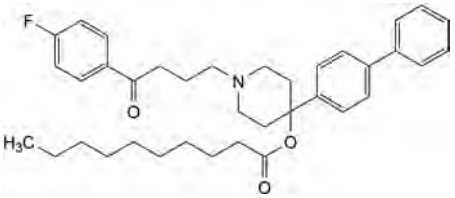

F. 4-(biphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

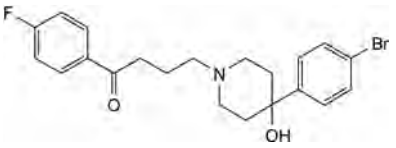

G. 4-[4-(4-bromophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one (bromperidol),

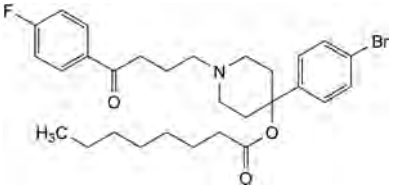

H. 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl octanoate,

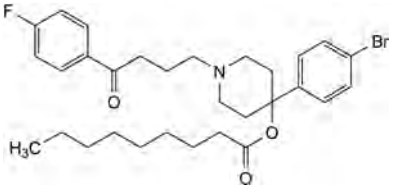

I. 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl nonanoate,

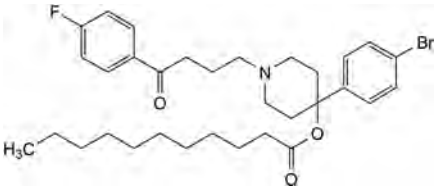

J. 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl undecanoate,

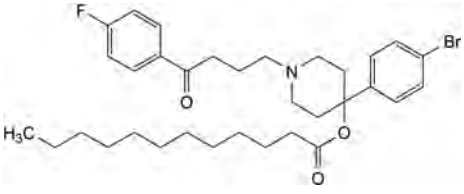

K. 4-(4-bromophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl dodecanoate,

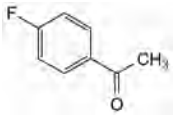

L. 1-(4-fluorophenyl)ethanone.

**01/2017:0977**
**corrected 10.0**

# BROMPHENIRAMINE MALEATE

## Brompheniramini maleas

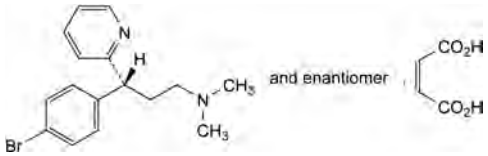


$C_{20}H_{23}BrN_2O_4$ $M_r$ 435.3
[980-71-2]

DEFINITION

(3*RS*)-3-(4-Bromophenyl)-*N*,*N*-dimethyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butenedioate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

*First identification*: *C, F.*

*Second identification*: *A, B, D, E, F.*

A. Melting point (*2.2.14*): 130 °C to 135 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 65 mg in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution. Dilute 5.0 mL of this solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 220-320 nm.

*Absorption maximum*: at 265 nm.

*Specific absorbance at the absorption maximum*: 190 to 210.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *brompheniramine maleate CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Dissolve 56 mg of *maleic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *water R*, *anhydrous formic acid R*, *methanol R*, *di-isopropyl ether R* (3:7:20:70 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of air for 5 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution shows 2 clearly separated spots; the upper spot is similar in position and size to the spot in the chromatogram obtained with the reference solution.

E. To 0.15 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue in 10 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of bromides (*2.3.1*).

F. Optical rotation (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0.

Dissolve 0.20 g in 20 mL of *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): − 0.20° to + 0.20° (measured in a 2 dm tube).

Dissolve 2.5 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in 10.0 mL of *methylene chloride R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 10.0 mL with *methylene chloride R*.

*Reference solution (b).* Dissolve 10 mg of *chlorphenamine maleate CRS* (impurity A) and 10 mg of *pheniramine maleate CRS* (impurity C) in *methylene chloride R* and dilute to 5 mL with the same solvent. To 2.5 mL of the solution add 2.5 mL of the test solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *nitrogen for chromatography R.*

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:5.

*Temperature*:

- *column*: 205 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 1.2 times the retention time of brompheniramine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C.

*Relative retention* with reference to brompheniramine (retention time = about 34 min): impurity C = about 0.4; impurity A = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and brompheniramine.

*Limits*:

- *impurities A, C*: for each impurity, not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.260 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.77 mg of $C_{20}H_{23}BrN_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C.*

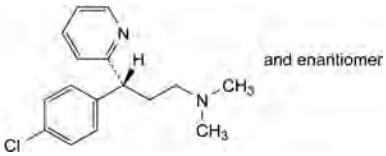


A. (3*RS*)-3-(4-chlorophenyl)-*N*,*N*-dimethyl-3-(pyridin-2-yl)propan-1-amine (chlorphenamine),

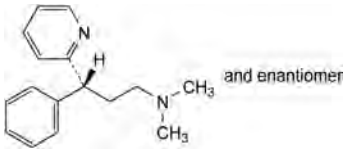


C. (3*RS*)-*N*,*N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine (pheniramine).

**01/2008:2197**
**corrected 7.0**

# BROTIZOLAM

## Brotizolamum

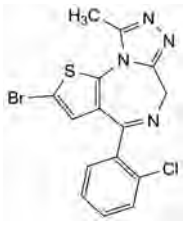


$C_{15}H_{10}BrClN_4S$ $M_r$ 393.7
[57801-81-7]

DEFINITION

2-Bromo-4-(2-chlorophenyl)-9-methyl-6*H*-thieno-[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]diazepine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish powder.

*Solubility*: practically insoluble in water, sparingly soluble or slightly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *brotizolam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL of *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *brotizolam impurity B CRS* in 50 mL of *acetonitrile R*. Dilute 2 mL of this solution to 20 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 2 g/L solution of *sodium heptanesulfonate monohydrate R*;
- *mobile phase B*: mix 25 volumes of a 2 g/L solution of *sodium heptanesulfonate R* and 75 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 63 | 37 |
| 4 - 15 | 63 → 12 | 37 → 88 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 5 µL.

*Relative retention* with reference to brotizolam (retention time = about 7.4 min): impurity A = about 0.5; impurity B = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and brotizolam.

*Limits*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 0.67 g in 20.0 mL of *methanol R*, mix and filter.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 25 mL of *glacial acetic acid R* and 50 mL of *acetic anhydride R*. Titrate to the second point of inflexion with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.68 mg of $C_{15}H_{10}BrClN_4S$.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

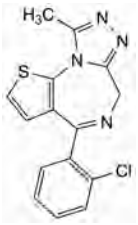

A. 4-(2-chlorophenyl)-9-methyl-6*H*-thieno[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]diazepine (desbromobrotizolam),

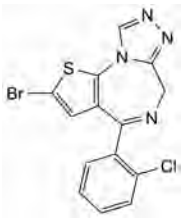

B. 2-bromo-4-(2-chlorophenyl)-6*H*-thieno[3,2-*f*][1,2,4]-triazolo[4,3-*a*][1,4]diazepine (desmethylbrotizolam).

**01/2010:1075**

# BUDESONIDE

## Budesonidum

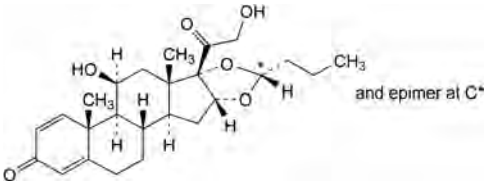


$C_{25}H_{34}O_6$ $M_r$ 430.5

[51333-22-3]

DEFINITION

Mixture of the C-22*S* (epimer A) and the C-22*R* (epimer B) epimers of 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *budesonide CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 25 mg of *budesonide CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 12.5 mg of *triamcinolone acetonide CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*; heat at 120 °C for 10 min or until the spots appear and allow to cool; examine the chromatograms in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

C. Dissolve about 2 mg in 2 mL of *sulfuric acid R*. Within 5 min a yellow colour develops. Within 30 min the colour changes to brown or reddish-brown. Cautiously add the solution to 10 mL of *water R* and mix. The colour fades and a clear solution remains.

D. Dissolve about 1 mg in 2 mL of a solution containing 2 g of *phosphomolybdic acid R* dissolved in a mixture of 10 mL of *dilute sodium hydroxide solution R*, 15 mL of *water R* and 25 mL of *glacial acetic acid R*. Heat for 5 min on a water-bath. Cool in iced water for 10 min and add 3 mL of *dilute sodium hydroxide solution R*. The solution is blue.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R*, *phosphate buffer solution pH 3.2 R* (32:68 *V/V*).

*Test solution (a).* Dissolve 50 mg of the substance to be examined in 15 mL of *acetonitrile R* and dilute to 50 mL with *phosphate buffer solution pH 3.2 R.*

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in 15 mL of *acetonitrile R* and dilute to 50.0 mL with *phosphate buffer solution pH 3.2 R.*

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *budesonide for system suitability CRS* (containing impurities A, D, G, K and L) in 1.5 mL of *acetonitrile R* and dilute to 5 mL with *phosphate buffer solution pH 3.2 R.*

*Reference solution (c).* Dissolve 25.0 mg of *budesonide CRS* in 15 mL of *acetonitrile R* and dilute to 50.0 mL with *phosphate buffer solution pH 3.2 R.*

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *anhydrous ethanol R, acetonitrile R, phosphate buffer solution pH 3.2 R* (2:32:68 *V/V/V*);

– *mobile phase B*: *acetonitrile R, phosphate buffer solution pH 3.2 R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 38 | 100 | 0 |
| 38 - 50 | 100 → 0 | 0 → 100 |
| 50 - 60 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *budesonide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, D, G, K and L.

*Relative retention* with reference to budesonide epimer B (retention time = about 17 min): impurity A = about 0.1; epimers of impurity D = about 0.63 and 0.67; impurity L = about 0.95; epimers of impurity G = about 1.2 and 1.3; epimers of impurity K = about 2.9 and 3.0.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the 1st of the 2 peaks due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to budesonide epimer A (the 2nd of the 2 principal peaks); and minimum 3, where $H_p$ = height above the baseline of the peak due to impurity L and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to budesonide epimer B (the 1st of the 2 principal peaks).

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.8; impurity K = 1.3;

– *impurities A, L*: for each impurity, not more than twice the sum of the areas of the 2 peaks due to the budesonide epimers in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurities D, K*: for each impurity, for the sum of the areas of the 2 epimer peaks, not more than twice the sum of the areas of the 2 peaks due to the budesonide epimers in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each individual peak, not more than the sum of the areas of the 2 peaks due to the budesonide epimers in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the sum of the areas of the 2 peaks due to the budesonide epimers in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the sum of the areas of the 2 peaks due to the budesonide epimers in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Epimer A**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 21 | 100 | 0 |
| 21 - 22 | 100 → 0 | 0 → 100 |
| 22 - 31 | 0 | 100 |

*Injection*: 20 µL of test solution (b) and reference solutions (b) and (c).

*Retention time*: budesonide epimer B = about 17 min; budesonide epimer A = about 19 min.

*System suitability*:

– *resolution*: minimum 1.5 between the 2 principal peaks (budesonide epimers A and B) in the chromatogram obtained with reference solution (c);

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity L and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to budesonide epimer B (the 1st of the 2 principal peaks) in the chromatogram obtained with reference solution (b).

*Limit*:

– *epimer A*: 40.0 per cent to 51.0 per cent of the sum of the areas of the 2 peaks due to the budesonide epimers.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*). Examine the chromatograms obtained in the test for epimer A.

Calculate the percentage content of $C_{25}H_{34}O_6$ from the sum of the areas of the 2 peaks due to the budesonide epimers and the declared content of *budesonide CRS*.

IMPURITIES

*Specified impurities: A, D, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, F, G, H, I, J.*

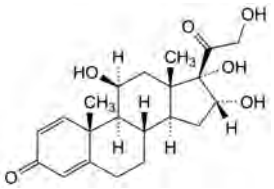

A. 11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione,

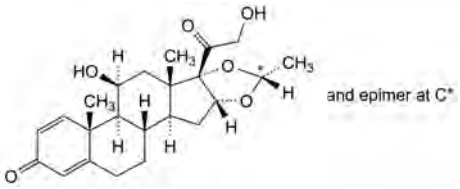


B. 16α,17-[(1*RS*)-ethylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione,

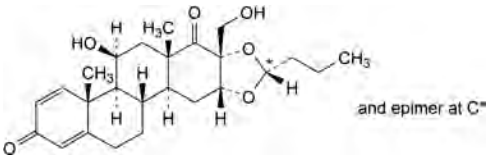


C. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β-hydroxy-17-(hydroxymethyl)-*D*-homoandrosta-1,4-diene-3,17a-dione,

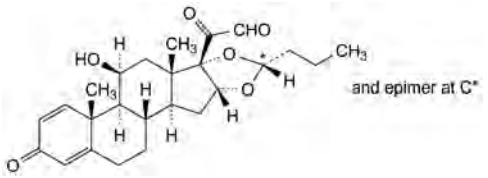


D. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β-hydroxy-3,20-dioxopregna-1,4-dien-21-al,

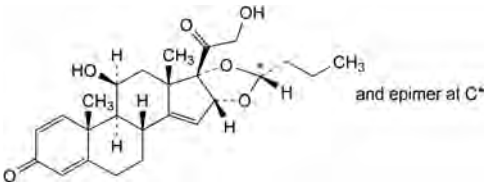


E. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-1,4,14-triene-3,20-dione,

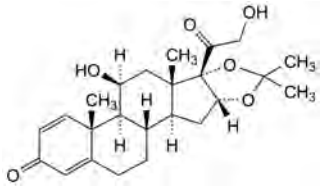

F. 16α,17-[1-methylethylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione,

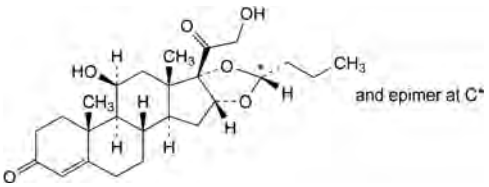


G. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregn-4-ene-3,20-dione.

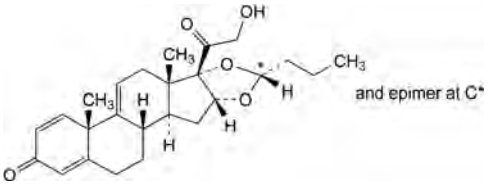


H. 16α,17-[(1*RS*)-butylidenebis(oxy)]-21-hydroxypregna-1,4,9(11)-triene-3,20-dione,

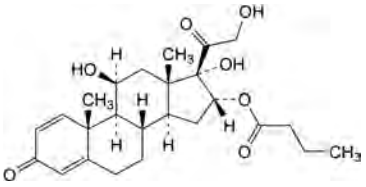

I. 11β,17,21-trihydroxy-3,20-dioxopregna-1,4-dien-16α-yl butanoate,

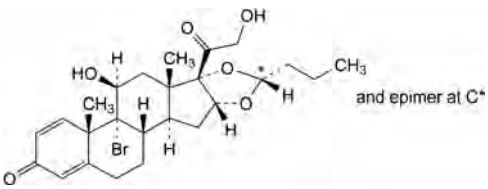


J. 16α,17-[(1*RS*)-butylidenebis(oxy)]-9α-bromo-11β,21-dihydroxypregna-1,4-diene-3,20-dione,

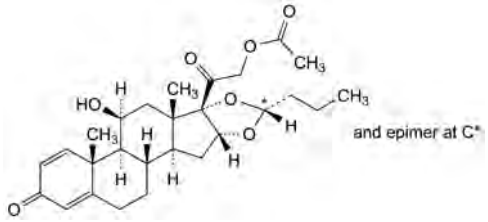


K. 16α,17-[(1*RS*)-butylidenebis(oxy)]-11β,21-dihydroxypregna-1,4-diene-3,20-dione-21-acetate,

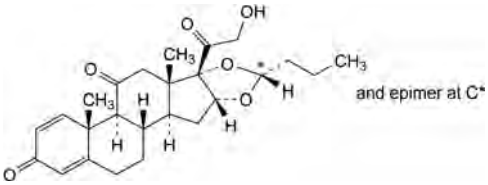


L. 16α,17-[(1*RS*)-butylidenebis(oxy)]-21-hydroxypregna-1,4-diene-3,11,20-trione.

**07/2015:1179**

# BUFEXAMAC

## Bufexamacum

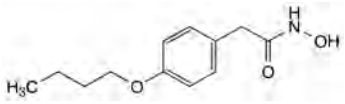

$C_{12}H_{17}NO_3$ $M_r$ 223.3

[2438-72-4]

DEFINITION

2-(4-Butoxyphenyl)-*N*-hydroxyacetamide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, slightly soluble in ethyl acetate and in methanol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 20 mg in *methanol R* and dilute to 20 mL with the same solvent. Dilute 1 mL of the solution to 50 mL with *methanol R*.

*Spectral range*: 210-360 nm.

*Absorption maxima*: at 228 nm, 277 nm and 284 nm.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bufexamac CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *bufexamac CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *salicylic acid R* in reference solution (a) and dilute to 5 mL with the same solution.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *dioxan R*, *toluene R* (4:20:90 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 25.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *bufexamac CRS*, 5 mg of *bufexamac impurity A CRS*, 5 mg of *bufexamac impurity B CRS*, 5 mg of *bufexamac impurity C CRS* and 5 mg of *bufexamac impurity D CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 30 volumes of a 1.4 g/L solution of *dipotassium hydrogen phosphate R* and 70 volumes of *methanol R*, then adjust to pH 3.6 with *dilute phosphoric acid R*;
– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 13 | 100 → 70 | 0 → 30 |
| 13 - 40 | 70 | 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to bufexamac (retention time = about 5.7 min): impurity D = about 1.3; impurity A = about 1.8; impurity B = about 3.0; impurity C = about 5.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to bufexamac and impurity D.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *dimethylformamide R*. Titrate with *0.1 M lithium methoxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M lithium methoxide* is equivalent to 22.33 mg of $C_{12}H_{17}NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

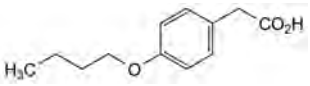

A. 2-(4-butoxyphenyl)acetic acid,

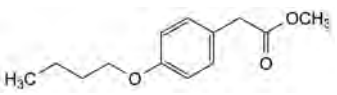

B. methyl 2-(4-butoxyphenyl)acetate,

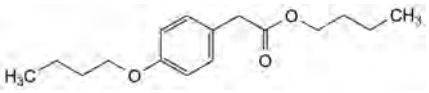

C. butyl 2-(4-butoxyphenyl)acetate,

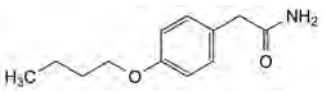

D. 2-(4-butoxyphenyl)acetamide.

**01/2017:1398**

# BUFLOMEDIL HYDROCHLORIDE

## Buflomedili hydrochloridum

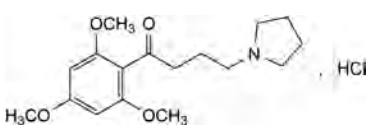

$C_{17}H_{26}ClNO_4$ $M_r$ 343.9

[35543-24-9]

DEFINITION

4-(Pyrrolidin-1-yl)-1-(2,4,6-trimethoxyphenyl)butan-1-one hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, microcrystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), very slightly soluble in acetone.

mp: about 195 °C, with decomposition.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 275 nm.

*Specific absorbance at the absorption maximum*: 143 to 149.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *buflomedil hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 2 mL with the same solvent.

*Reference solution.* Dissolve 40 mg of *buflomedil hydrochloride CRS* in *methanol R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *triethylamine R*, *2-propanol R*, *toluene R* (5:50:50 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 0.5 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *buflomedil impurity B CRS* in the mobile phase, add 0.5 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *buflomedil for peak identification CRS* (containing impurities A and C) in 1.0 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 45 volumes of *acetonitrile R1* and 55 volumes of a 9.25 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of buflomedil.

*Identification of impurities*: use the chromatogram supplied with *buflomedil for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to buflomedil (retention time = about 5 min): impurity B = about 0.6; impurity C = about 0.7; impurity A = about 1.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity B and impurity C.

*Limits*:

- *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 15 mL of *anhydrous acetic acid R* and add 35 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.39 mg of $C_{17}H_{26}ClNO_4$.

IMPURITIES

*Specified impurities: A, B, C.*

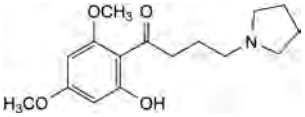


A. 1-(2-hydroxy-4,6-dimethoxyphenyl)-4-(pyrrolidin-1-yl)butan-1-one,

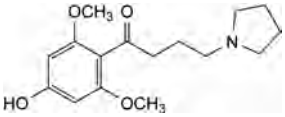


B. 1-(4-hydroxy-2,6-dimethoxyphenyl)-4-(pyrrolidin-1-yl)butan-1-one,

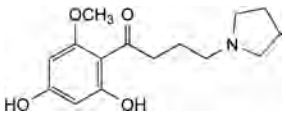

C. 1-(2,4-dihydroxy-6-methoxyphenyl)-4-(pyrrolidin-1-yl)butan-1-one.

**01/2008:1076**
**corrected 8.4**

# BUMETANIDE

## Bumetanidum

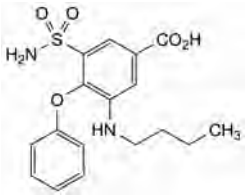

$C_{17}H_{20}N_2O_5S$ $M_r$ 364.4
[28395-03-1]

DEFINITION

3-(Butylamino)-4-phenoxy-5-sulfamoylbenzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in ethanol (96 per cent), slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

mp: about 233 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *bumetanide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.1 g in a 6 g/L solution of *potassium hydroxide R* and dilute to 20 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *bumetanide impurity A CRS* and 2 mg of *bumetanide impurity B CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*: mix 70 volumes of *methanol R*, 25 volumes of *water for chromatography R* and 5 volumes of a 27.2 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with a 280 g/L solution of *potassium hydroxide R*; add *tetrahexylammonium bromide R* to this mixture to obtain a concentration of 2.17 g/L.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of bumetanide.

*Relative retention* with reference to bumetanide (retention time = about 6 min): impurity B = about 0.4; impurity A = about 0.6; impurity C = about 2.5; impurity D = about 4.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and impurity B.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *alcohol R*. Add 0.1 mL of *phenol red solution R*. Titrate with *0.1 M sodium hydroxide* until a violet-red colour is obtained. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.44 mg of $C_{17}H_{20}N_2O_5S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

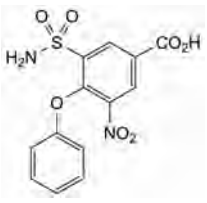

A. 3-nitro-4-phenoxy-5-sulfamoylbenzoic acid,

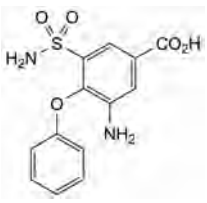

B. 3-amino-4-phenoxy-5-sulfamoylbenzoic acid,

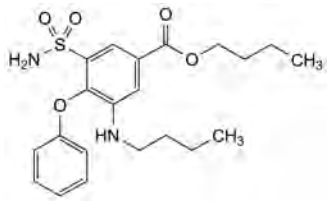

C. butyl 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoate,

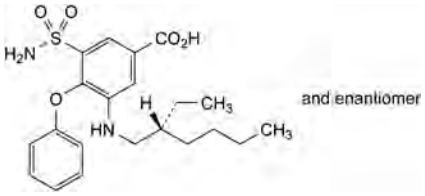


D. 3-[[(2*RS*)-2-ethylhexyl]amino]-4-phenoxy-5-sulfamoylbenzoic acid.

**01/2017:0541**
**corrected 10.0**

# BUPIVACAINE HYDROCHLORIDE

## Bupivacaini hydrochloridum

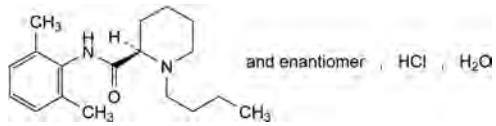


$C_{18}H_{29}ClN_2O,H_2O$ $M_r$ 342.9
[73360-54-0]

DEFINITION

(2*RS*)-1-Butyl-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide hydrochloride monohydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, D, E.*

*Second identification*: *B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *bupivacaine hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution.* Dissolve 25 mg of *bupivacaine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel G plate R.*
*Mobile phase*: *concentrated ammonia R, methanol R* (0.1:100 *V/V*).
*Application*: 5 µL.
*Development*: over a path of 10 cm.
*Drying*: in air.
*Detection*: spray with *dilute potassium iodobismuthate solution R.*
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 0.1 g in 10 mL of *water R*, add 2 mL of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 15 mL, of *1,1-dimethylethyl methyl ether R*. Dry the combined upper layers over *anhydrous sodium sulfate R* and filter. Evaporate the filtrate, recrystallise the residue from *ethanol (90 per cent V/V) R* and dry under reduced pressure. The crystals melt (*2.2.14*) at 105 °C to 108 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

E. Optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *0.01 M sodium hydroxide*; the pH (*2.2.3*) is not less than 4.7. Add 0.4 mL of *0.01 M hydrochloric acid*; the pH is not greater than 4.7.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 1.0 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 25 mg of *methyl behenate R* in *methylene chloride R* and dilute to 500 mL with the same solvent.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *water R*, add 2.5 mL of *dilute sodium hydroxide solution R* and extract with 2 quantities, each of 5 mL, of the internal standard solution. Filter the lower layer.

*Reference solution (a).* Dissolve 10 mg of the substance to be examined, 10 mg of *bupivacaine impurity B CRS* and 10 mg of *bupivacaine impurity E CRS* in 2.5 mL of *water R*, add 2.5 mL of *dilute sodium hydroxide solution R* and extract with 2 quantities, each of 5 mL, of the internal standard solution. Filter the lower layer and dilute to 20 mL with the internal standard solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the internal standard solution.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 10.0 mL with the internal standard solution.

*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with the internal standard solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 2.5 mL/min.

*Split ratio*: 1:12.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 180 |
| | 0 - 10 | 180 → 230 |
| | 10 - 15 | 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and E.

*Relative retention* with reference to bupivacaine (retention time = about 10 min): impurity B = about 0.7; impurity E = about 1.1; internal standard = about 1.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to bupivacaine and impurity E.

*Limits*:

– *impurity B*: calculate the ratio ($R_1$) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (c); from the chromatogram obtained with the test solution, calculate the ratio of the area of the peak due to impurity B to the area of the peak due to the internal standard: this ratio is not greater than $R_1$ (0.5 per cent);
– *unspecified impurities*: calculate the ratio ($R_2$) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (d); from the chromatogram obtained with the test solution, calculate for each impurity the ratio of the area of any peak, apart from the principal peak, the peak due to impurity B and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than $R_2$ (0.10 per cent);
– *total*: calculate the ratio ($R_3$) of the area of the principal peak to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than $R_3$ (1.0 per cent);
– *disregard limit*: ratio less than 0.05 times $R_3$ (0.05 per cent).

**Impurity F**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5.0 mg of *bupivacaine impurity F CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 20 mg of *methyl benzoate R* and 25 mg of *bupivacaine impurity F CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 3.0 mL of the solution to 50.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: dissolve 0.23 g of *sodium dihydrogen phosphate monohydrate R* and 3.626 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1000 mL with the same solvent; mix equal volumes of this solution (pH 8.0) and *acetonitrile R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 15 | 100 → 80 | 0 → 20 |
| 15 - 25 | 80 | 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity F.

*Relative retention* with reference to bupivacaine (retention time = about 20 min): impurity F = about 0.3; methyl benzoate = about 0.4.

*System suitability*:

– *resolution*: minimum 4.0 between the peaks due to impurity F and methyl benzoate in the chromatogram obtained with reference solution (b);
– *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (a).

*Limit*:

– *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (10 ppm).

**Loss on drying** (*2.2.32*): 4.5 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 mL of *water R* and 25 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M ethanolic sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 32.49 mg of $C_{18}H_{29}ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E.*

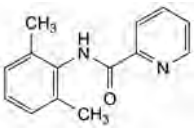

A. *N*-(2,6-dimethylphenyl)pyridine-2-carboxamide,

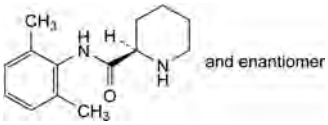


B. (2*RS*)-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide,

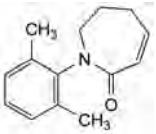

C. 1-(2,6-dimethylphenyl)-1,5,6,7-tetrahydro-2*H*-azepin-2-one,

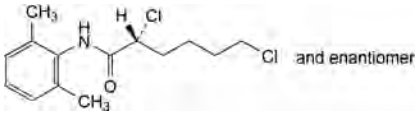


D. (2*RS*)-2,6-dichloro-*N*-(2,6-dimethylphenyl)hexanamide,

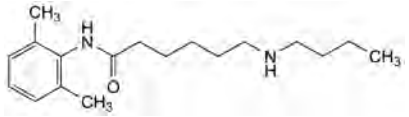


E. 6-(butylamino)-*N*-(2,6-dimethylphenyl)hexanamide,

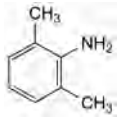


F. 2,6-dimethylaniline.

**07/2009:1180**
**corrected 7.0**

# BUPRENORPHINE

## Buprenorphinum

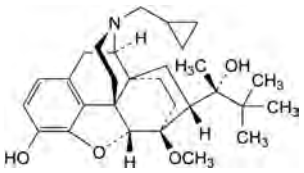


$C_{29}H_{41}NO_4$ $M_r$ 467.6
[52485-79-7]

DEFINITION

(2*S*)-2-[17-(Cyclopropylmethyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone, soluble in methanol, slightly soluble in cyclohexane. It dissolves in dilute solutions of acids.

mp: about 217 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *buprenorphine CRS*.

TESTS

**Solution S**. Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): – 103 to – 107 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of *buprenorphine for system suitability CRS* (containing impurities A, B, F, G, H and J) in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of *acetonitrile R* and 90 volumes of the following solution: dissolve 5.44 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 4.5 with a 5 per cent *V/V* solution of *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 89 | 11 |
| 2 - 12 | 89 → 64 | 11 → 36 |
| 12 - 15 | 64 → 41 | 36 → 59 |
| 15 - 20 | 41 → 39 | 59 → 61 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *buprenorphine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, F, G, H and J.

*Relative retention* with reference to buprenorphine (retention time = about 8.5 min): impurity B = about 0.4; impurity J = about 1.1; impurity F = about 1.27; impurity H = about 1.33; impurity A = about 1.40; impurity G = about 1.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to buprenorphine and impurity J.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity G by 0.3;
- *impurity H*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *impurities A, B, F, J*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.400 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 46.76 mg of $C_{29}H_{41}NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, F, G, H, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, I.*

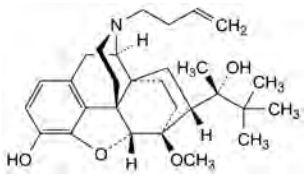

A. (2*S*)-2-[17-(but-3-enyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol,

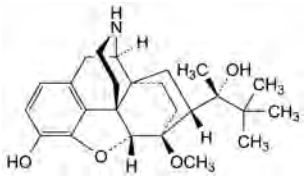

B. (2*S*)-2-(4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl)-3,3-dimethylbutan-2-ol (norbuprenorphine),

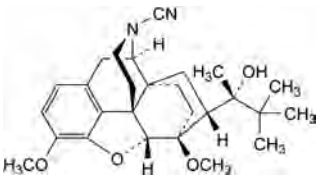

C. 4,5α-epoxy-7α-[(1*S*)-1-hydroxy-1,2,2-trimethylpropyl]-3,6-dimethoxy-6α,14-ethano-14α-morphinan-17-carbonitrile,

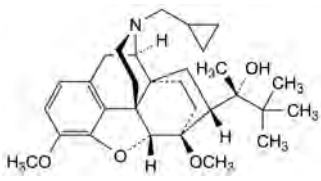

D. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3,6-dimethoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol (3-*O*-methylbuprenorphine),

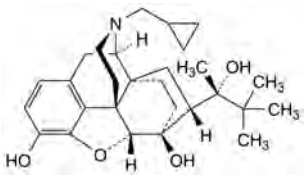

E. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3,6-dihydroxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol (6-*O*-desmethylbuprenorphine),

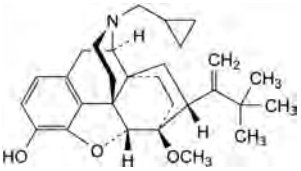

F. 17-(cyclopropylmethyl)-4,5α-epoxy-6-methoxy-7α-[1-(1,1-dimethylethyl)ethenyl]-6α,14-ethano-14α-morphinan-3-ol,

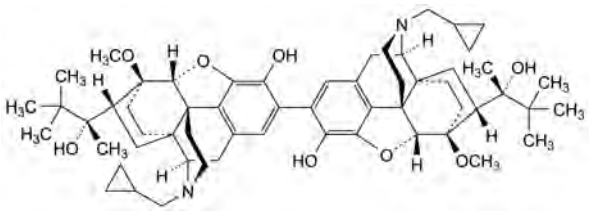

G. 17,17′-di(cyclopropylmethyl)-4,5α;4′,5α′-diepoxy-7α,7α′-di[(1*S*)-1-hydroxy-1,2,2-trimethylpropyl]-6,6′-dimethoxy-2,2′-bi(6α,14-ethano-14α-morphinan)-3,3′-diol (2,2′-bibuprenorphine),

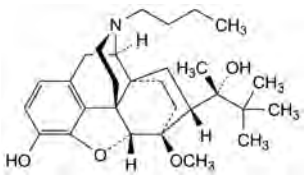

H. (2*S*)-2-[17-butyl-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]3,3-dimethylbutan-2-ol,

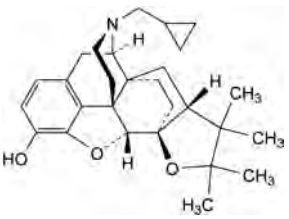

I. 17-(cyclopropylmethyl)-4″,4″,5″,5″-tetramethyl-4″,5″-dihydro-(7β*H*)-6α,14-ethano-(5β*H*)-difurano-[2′,3′,4′,5′:4,12,13,5;2″,3″:6,7]-14α-morphinan-3-ol,

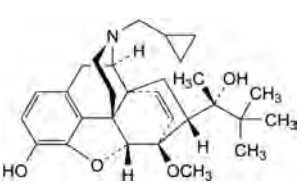

J. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-etheno-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol.

**07/2009:1181**
**corrected 6.6**

# BUPRENORPHINE HYDROCHLORIDE

## Buprenorphini hydrochloridum

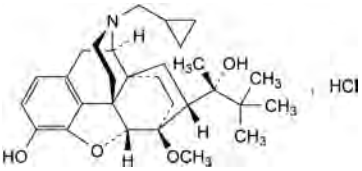

$C_{29}H_{42}ClNO_4$ $M_r$ 504.1
[53152-21-9]

DEFINITION

(2*S*)-2-[17-(Cyclopropylmethyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *buprenorphine hydrochloride CRS*.

B. 3 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in 5.0 mL of *methanol R* and, while stirring, dilute to 25.0 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10.0 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): – 92 to – 98 (dried substance).

Dissolve 0.200 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of *buprenorphine for system suitability CRS* (containing impurities A, B, F, G, H and J) in 1.0 mL of *methanol R*.

*Column*:
- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 10 volumes of *acetonitrile R* and 90 volumes of the following solution: dissolve 5.44 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 4.5 with a 5 per cent *V/V* solution of *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 89 | 11 |
| 2 - 12 | 89 → 64 | 11 → 36 |
| 12 - 15 | 64 → 41 | 36 → 59 |
| 15 - 20 | 41 → 39 | 59 → 61 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *buprenorphine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, F, G, H and J.

*Relative retention* with reference to buprenorphine (retention time = about 8.5 min): impurity B = about 0.4; impurity J = about 1.1; impurity F = about 1.27; impurity H = about 1.33; impurity A = about 1.40; impurity G = about 1.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to buprenorphine and impurity J.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity G by 0.3;
- *impurity H*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *impurities A, B, F, J*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by heating in an oven at 115-120 °C.

ASSAY

Dissolve 0.400 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 50.41 mg of $C_{29}H_{42}ClNO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, F, G, H, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, I.*

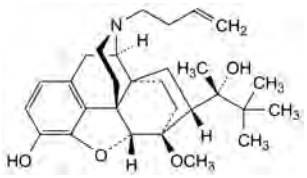

A. (2*S*)-2-[17-(but-3-enyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol,

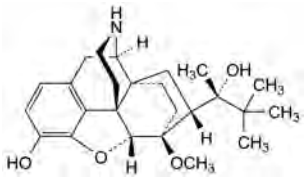

B. (2*S*)-2-(4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl)-3,3-dimethylbutan-2-ol (norbuprenorphine),

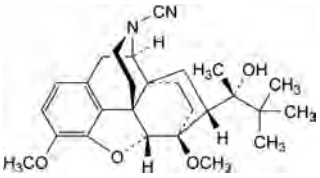

C. 4,5α-epoxy-7α-[(1*S*)-1-hydroxy-1,2,2-trimethylpropyl]-3,6-dimethoxy-6α,14-ethano-14α-morphinan-17-carbonitrile,

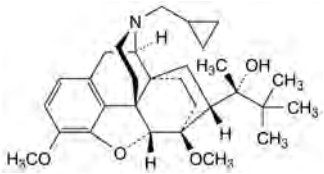

D. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3,6-dimethoxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol (3-*O*-methylbuprenorphine),

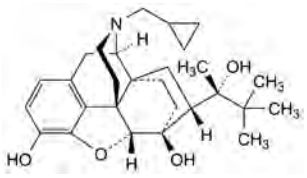

E. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3,6-dihydroxy-6α,14-ethano-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol (6-*O*-desmethylbuprenorphine),

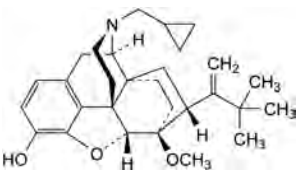

F. 17-(cyclopropylmethyl)-4,5α-epoxy-6-methoxy-7α-[1-(1,1-dimethylethyl)ethenyl]-6α,14-ethano-14α-morphinan-3-ol,

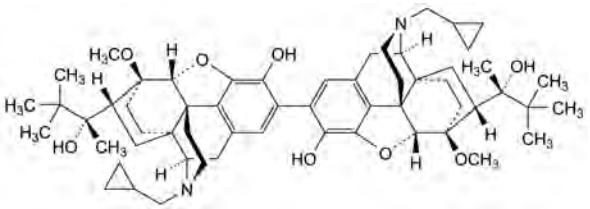

G. 17,17′-di(cyclopropylmethyl)-4,5α;4′,5α′-diepoxy-7α,7α′-di[(1*S*)-1-hydroxy-1,2,2-trimethylpropyl]-6,6′-dimethoxy-2,2′-bi(6α,14-ethano-14α-morphinan)-3,3′-diol (2,2′-bibuprenorphine),

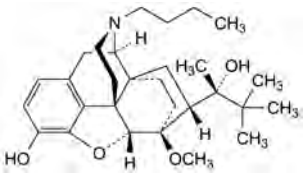

H. (2*S*)-2-[17-butyl-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-ethano-14α-morphinan-7α-yl]3,3-dimethylbutan-2-ol,

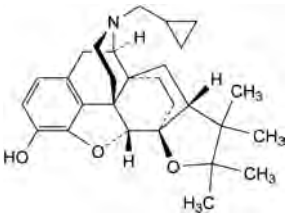

I. 17-(cyclopropylmethyl)-4″,4″,5″,5″-tetramethyl-4″,5″-dihydro-(7β*H*)-6α,14-ethano-(5β*H*)-difurano-[2′,3′,4′,5′:4,12,13,5;2″,3″:6,7]-14α-morphinan-3-ol,

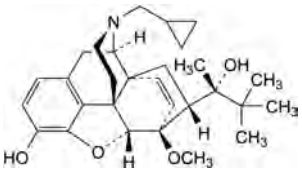

J. (2*S*)-2-[17-(cyclopropylmethyl)-4,5α-epoxy-3-hydroxy-6-methoxy-6α,14-etheno-14α-morphinan-7α-yl]-3,3-dimethylbutan-2-ol.

**01/2016:1077**
**corrected 10.0**

# BUSERELIN

## Buserelinum

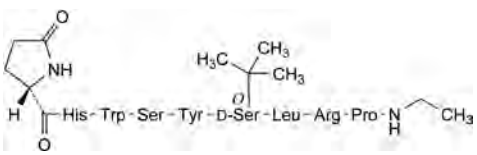

$C_{60}H_{86}N_{16}O_{13}$ $M_r$ 1239
[57982-77-1]

DEFINITION

5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-*O*-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl-*N*-ethyl-L-prolinamide.

Synthetic nonapeptide analogue of human gonadotrophin-releasing hormone GnRH with agonistic activity to gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous, acetic acid-free substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder, hygroscopic.

*Solubility*: sparingly soluble in water and in dilute acids.

IDENTIFICATION

Carry out either tests A and B or tests A and C.

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

B. Nuclear magnetic resonance spectrometry (*2.2.64*).

*Preparation*: 4 mg/mL solution in a mixture of 20 volumes of *deuterated acetic acid R* and 80 volumes of *deuterium oxide R*.

*Comparison*: dissolve the contents of a vial of *buserelin for NMR identification CRS* in a mixture of 20 volumes of *deuterated acetic acid R* and 80 volumes of *deuterium oxide R* to obtain a concentration of 4 mg/mL.

*Operating conditions*:

– *field strength*: minimum 300 MHz;

– *temperature*: 27 °C.

*Results*: examine the $^1$H NMR spectrum from 0 to 9 ppm. The $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *buserelin for NMR identification CRS*.

C. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/6 of the sum of the number of moles of glutamic acid, histidine, tyrosine, leucine, arginine and proline as equal to 1. The values fall within the following limits: serine 1.4 to 2.0; proline 0.8 to 1.2; glutamic acid 0.9 to 1.1; leucine 0.9 to 1.1; tyrosine 0.9 to 1.1; histidine 0.9 to 1.1; arginine 0.9 to 1.1. Not more than traces of other amino acids are present.

TESTS

**Appearance of solution**. A 10 g/L solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): – 58 to – 49 (anhydrous, acetic acid-free substance), determined on a 10 g/L solution.

**Specific absorbance** (*2.2.25*): 49 to 56, measured at the absorption maximum at 278 nm (anhydrous, acetic acid-free substance).

Dissolve 10.0 mg in 100.0 mL of *0.01 M hydrochloric acid*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 5.0 mg of the substance to be examined in 5.0 mL of the mobile phase.

*Reference solution (a)*. Dissolve the contents of a vial of *D-His-buserelin CRS* (impurity A) in the mobile phase. Dilute an appropriate volume of this solution in the mobile phase to obtain a final concentration of 1 mg/mL. Add 1.0 mL of the test solution to 1.0 mL of this solution.

*Reference solution (b)*. Dissolve the contents of a vial of *buserelin CRS* in the mobile phase. Dilute an appropriate volume of this solution in the mobile phase to obtain a final concentration of 1.0 mg/mL.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve the contents of a vial of *buserelin for peak identification CRS* (containing impurities F and G) in the mobile phase. Dilute an appropriate volume of this solution in the mobile phase to obtain a final concentration of 1 mg/mL.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 42 °C.

*Mobile phase*: mix 200 mL of *acetonitrile R* and 700 mL of an 11.2 g/L solution of *phosphoric acid R* previously adjusted to pH 2.5 with *triethylamine R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (c) and (d).

*Run time*: 60 min.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities F and G.

*Relative retention* with reference to buserelin (retention time = about 23 min): impurity F = about 0.83; impurity A = about 0.91; impurity G = about 1.26.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity A and buserelin.

*Calculation of percentage contents*:

– for each impurity, use the concentration of buserelin in reference solution (c).

*Limits*:

– *impurity A*: maximum 2.5 per cent;

– *impurity F*: maximum 1.0 per cent;

– *impurity G*: maximum 1.0 per cent;

– *unspecified impurities*: for each impurity, maximum 0.5 per cent;

– *total*: maximum 4.0 per cent;

– *reporting threshold*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 3.0 per cent to 7.0 per cent.

*Test solution*. Dissolve 20.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of solvents.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 80.0 mg.

**Bacterial endotoxins** (*2.6.14*): less than 55.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of buserelin ($C_{60}H_{86}N_{16}O_{13}$) taking into account the assigned content of $C_{60}H_{86}N_{16}O_{13}$ in *buserelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states:

– the mass of peptide in the container;

– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities*: *A, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

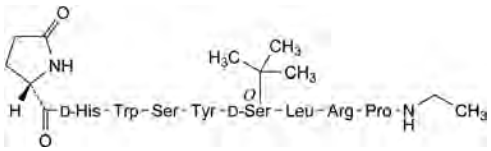


A. [2-D-histidine]buserelin,

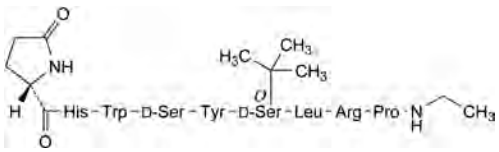


B. [4-D-serine]buserelin,

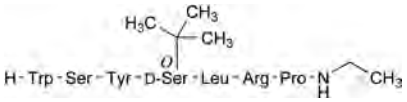


C. buserelin-(3-9)-peptide,

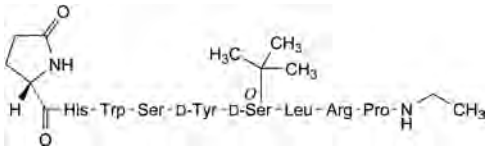


D. [5-D-tyrosine]buserelin,

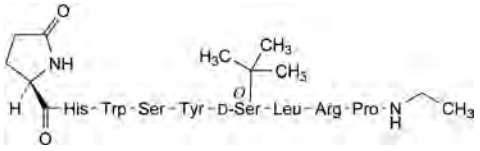


E. [1-(5-oxo-D-proline)]buserelin,

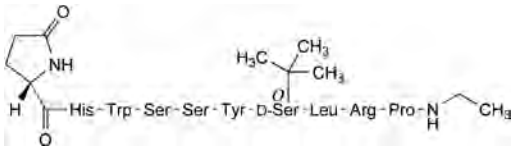


F. endo-3a-serine-buserelin,

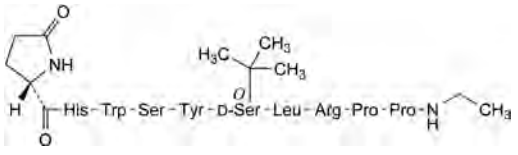


G. endo-8a-proline-buserelin.

**01/2008:1711**
**corrected 6.0**

# BUSPIRONE HYDROCHLORIDE

## Buspironi hydrochloridum

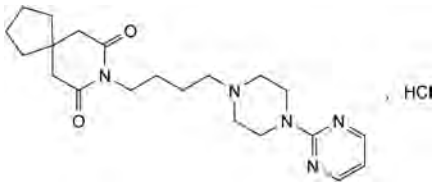


$C_{21}H_{32}ClN_5O_2$ $M_r$ 422.0
[33386-08-2]

DEFINITION

8-[4-[4-(Pyrimidin-2-yl)piperazin-1-yl]butyl]-8-azaspiro[4.5]decane-7,9-dione hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *buspirone hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *buspirone for system suitability CRS* (containing impurities E, G, J, L and N) in 2.0 mL of mobile phase A and sonicate for 10 min.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 950 volumes of a solution containing 6.8 g/L of *potassium dihydrogen phosphate R* and 0.93 g/L of *sodium hexanesulfonate monohydrate R*, previously adjusted to pH 3.4 with *phosphoric acid R* and 50 volumes of *acetonitrile R1*;
– *mobile phase B*: mix 250 volumes of a solution containing 3.4 g/L of *potassium dihydrogen phosphate R* and 3.52 g/L of *sodium hexanesulfonate monohydrate R*, previously adjusted to pH 2.2 with *phosphoric acid R* and 750 volumes of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 90 | 10 |
| 6 - 34 | 90 → 42 | 10 → 58 |
| 34 - 45 | 42 | 58 |
| 45 - 55 | 42 → 0 | 58 → 100 |
| 55 - 56 | 0 → 100 | 100 → 0 |
| 56 - 60 | 100 | 0 |
| 60 - 61 | 100 → 90 | 0 → 10 |

*Flow rate*: 1 mL/min.

*Detection*: variable wavelength spectrophotometer capable of operating at 240 nm and at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *buspirone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities E, G, J, L and N.

*Relative retention at 240 nm* with reference to buspirone (retention time = about 25 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.6; impurity D = about 0.7; impurity E = about 0.8; impurity F = about 0.9; impurity G = about 1.05; impurity H = about 1.1; impurity I = about 1.2; impurity J = about 1.5.

*Relative retention at 210 nm* with reference to buspirone (retention time = about 25 min): impurity K = about 0.6; impurity L = about 1.7; impurity M = about 1.8; impurity N = about 1.9.

*System suitability*: reference solution (b):

- *peak-to-valley ratio at 240 nm*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to buspirone;
- *resolution at 210 nm*: minimum 4.0 between the peaks due to impurity L and impurity N;
- the chromatograms obtained are similar to the chromatograms supplied with *buspirone for system suitability CRS*.

*Limits*: spectrophotometer at 240 nm:

- *correction factor*: for the calculation of content, multiply the peak area of impurity J by 2,
- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *impurity J*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

*Limits*: spectrophotometer at 210 nm:

- *impurity K*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity eluting with a relative retention greater than 1.6*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 10 mL of *glacial acetic acid R* and add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.10 mg of $C_{21}H_{32}ClN_5O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: E, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, G, H, I, L, M, N.*

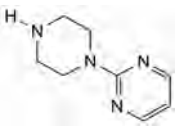

A. 2-(piperazin-1-yl)pyrimidine,

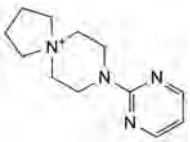

B. 8-(pyrimidin-2-yl)-8-aza-5-azoniaspiro[4.5]decane,

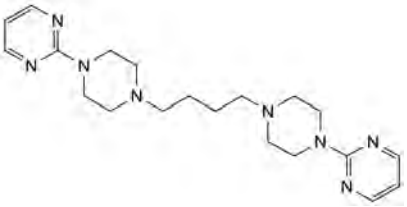

C. 2,2′-[butane-1,4-diylbis(piperazine-1,4-diyl)]dipyrimidine,

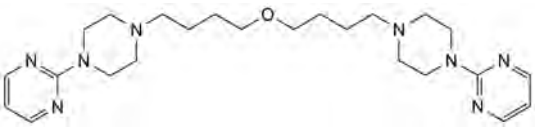

D. 2,2′-[oxybis[butane-1,4-diyl(piperazine-1,4-diyl)]]dipyrimidine,

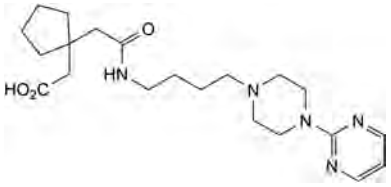

E. [1-[2-oxo-2-[[4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl]amino]ethyl]cyclopentyl]acetic acid,

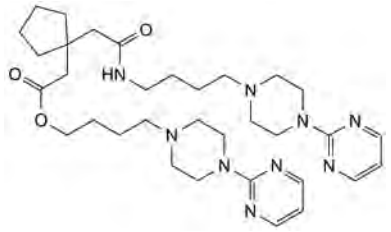

F. 4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl [1-[2-oxo-2-[[4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl]amino]ethyl]cyclopentyl]acetate,

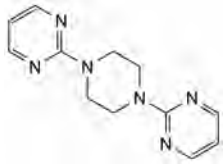

G. 2,2′-(piperazine-1,4-diyl)dipyrimidine,

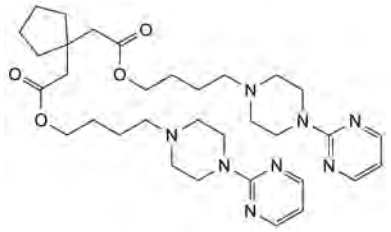

H. bis[4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl] (cyclopentane-1,1-diyl)diacetate,

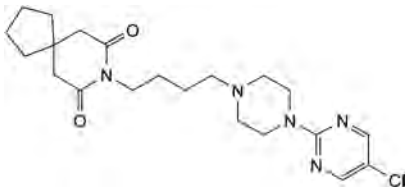

I. 8-[4-[4-(5-chloropyrimidin-2-yl)piperazin-1-yl]butyl]-8-azaspiro[4.5]decane-7,9-dione,

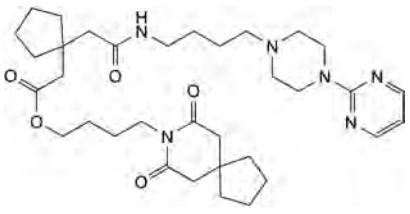

J. 4-(7,9-dioxo-8-azaspiro[4.5]dec-8-yl)butyl [1-[2-oxo-2-[[4-[4-(pyrimidin-2-yl)piperazin-1-yl]butyl]amino]ethyl]-cyclopentyl]acetate,

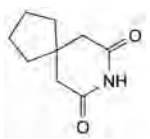

K. 8-azaspiro[4.5]decane-7,9-dione,

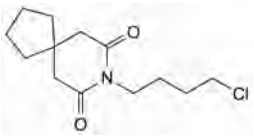

L. 8-(4-chlorobutyl)-8-azaspiro[4.5]decane-7,9-dione,

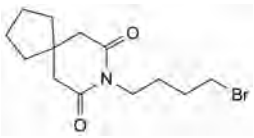

M. 8-(4-bromobutyl)-8-azaspiro[4.5]decane-7,9-dione,

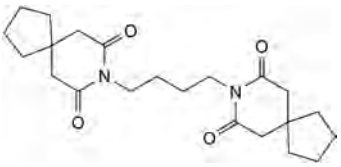

N. 8,8′-(butane-1,4-diyl)bis(8-azaspiro[4.5]decane-7,9-dione).

**01/2008:0542**

# BUSULFAN

## Busulfanum

$C_6H_{14}O_6S_2$ $M_r$ 246.3
[55-98-1]

DEFINITION

Butane-1,4-diyl di(methanesulfonate).

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone and in acetonitrile, very slightly soluble in ethanol (96 per cent).

mp: about 116 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *busulfan CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 2 mL of *acetone R.*

*Reference solution.* Dissolve 20 mg of *busulfan CRS* in 2 mL of *acetone R.*

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *acetone R, toluene R* (50:50 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: spray with *anisaldehyde solution R* and heat at 120 °C.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 0.1 g add 5 mL of *1 M sodium hydroxide*. Heat until a clear solution is obtained. Allow to cool. To 2 mL of the solution add 0.1 mL of *potassium permanganate solution R*. The colour changes from purple through violet to blue and finally to green. Filter and add 1 mL of *ammoniacal silver nitrate solution R*. A precipitate is formed.

D. To 0.1 g add 0.1 g of *potassium nitrate R* and 0.25 g of *sodium hydroxide R*, mix and heat to fusion. Allow to cool and dissolve the residue in 5 mL of *water R*. Adjust to pH 1-2 using *dilute hydrochloric acid R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 0.25 g in 20 mL of *acetonitrile R*, dilute to 25 mL with *water R* and examine immediately.

**Acidity**. Dissolve 0.20 g with heating in 50 mL of *anhydrous ethanol R*. Add 0.1 mL of *methyl red solution R*. Not more than 0.05 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.250 g add 50 mL of *water R*. Shake. Boil under a reflux condenser for 30 min and, if necessary, make up to the initial volume with *water R*. Allow to cool. Using 0.3 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 12.32 mg of $C_6H_{14}O_6S_2$.

STORAGE

In an airtight container, protected from light.

**07/2011:0881**

# BUTYL PARAHYDROXYBENZOATE

## Butylis parahydroxybenzoas

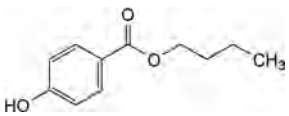


$C_{11}H_{14}O_3$ $M_r$ 194.2
[94-26-8]

DEFINITION

Butyl 4-hydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 68 °C to 71 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *butyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.

*Reference solution (a).* Dissolve 10 mg of *butyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *propyl parahydroxybenzoate R* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).

*Application*: 2 µL of test solution (b) and reference solutions (a) and (b).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. To 2 mL of solution S add 3 mL of *ethanol (96 per cent) R*, 5 mL of *carbon dioxide-free water R* and 0.1 mL of *bromocresol green solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A), 5 mg of *propyl parahydroxybenzoate R* (impurity D) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 50.0 mg of *butyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *butyl parahydroxybenzoate impurity E CRS* (iso-butyl parahydroxybenzoate) in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (e).* Dilute 0.5 mL of reference solution (d) to 50.0 mL with reference solution (b).

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (50:50 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (c) and (e).

*Run time*: 1.5 times the retention time of butyl parahydroxybenzoate.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and D; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity E.

*Relative retention* with reference to butyl parahydroxybenzoate (retention time = about 22 min): impurity A = about 0.1; impurity D = about 0.5; impurity E = about 0.9.

*System suitability*:
- *resolution*:
  - minimum 5.0 between the peaks due to impurity D and butyl parahydroxybenzoate in the chromatogram obtained with reference solution (a);
  - minimum 1.5 between the peaks due to impurity E and butyl parahydroxybenzoate in the chromatogram obtained with reference solution (e).

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{11}H_{14}O_3$ from the declared content of *butyl parahydroxybenzoate CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

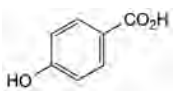

A. 4-hydroxybenzoic acid,

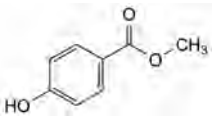

B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

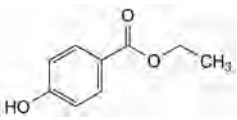

C. ethyl 4-hydroxybenzoate (ethyl parahydroxybenzoate),

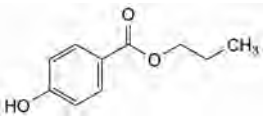

D. propyl 4-hydroxybenzoate (propyl parahydroxybenzoate),

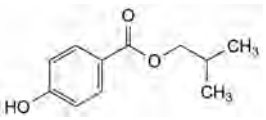

E. 2-methylpropyl 4-hydroxybenzoate (iso-butyl parahydroxybenzoate).

**01/2017:0880**

# BUTYLHYDROXYANISOLE

## Butylhydroxyanisolum

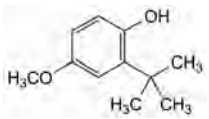

$C_{11}H_{16}O_2$ $M_r$ 180.3

[25013-16-5]

DEFINITION

Butylhydroxyanisole is 2-(1,1-dimethylethyl)-4-methoxyphenol containing not more than 10 per cent of 3-(1,1-dimethylethyl)-4-methoxyphenol.

CHARACTERS

A white, yellowish or slightly pinkish, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in alcohol and in fatty oils. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To 0.5 mL of solution S (see Tests) add 10 mL of *aminopyrazolone solution R* and 1 mL of *potassium ferricyanide solution R*. Mix and add 10 mL of *methylene chloride R*. Shake vigorously. After separation, the organic layer is red.

C. Dissolve about 10 mg in 2 mL of *alcohol R*. Add 1 mL of a 1 g/L solution of *testosterone propionate R* in *alcohol R* and 2 mL of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 10 min and allow to cool. A red colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *alcohol R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.25 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methylene chloride R*.

*Reference solution (a).* Dissolve 25 mg of *butylhydroxyanisole CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of reference solution (a) to 20 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve 50 mg of *hydroquinone R* in 5 mL of *alcohol R* and dilute to 100 mL with *methylene chloride R*. Dilute 1 mL of this solution to 10 mL with *methylene chloride R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 10 cm using *methylene chloride R*. Allow the plate to dry in air and spray with a freshly prepared mixture of 10 volumes of *potassium ferricyanide solution R*, 20 volumes of *ferric chloride solution R1* and 70 volumes of *water R*. In the chromatogram obtained with test solution (a): any violet-blue spot with an $R_F$ value of about 0.35 (corresponding to 3-(1,1-dimethylethyl)-4-methoxyphenol) is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (10 per cent); any spot corresponding to hydroquinone is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent); any spot, apart from the principal spot and any spots corresponding to 3-(1,1-dimethylethyl)-4-methoxyphenol and hydroquinone, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

STORAGE

Store protected from light.

IMPURITIES

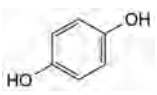


A. benzene-1,4-diol (hydroquinone).

**01/2008:0581**

# BUTYLHYDROXYTOLUENE

## Butylhydroxytoluenum

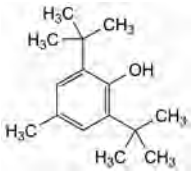


$C_{15}H_{24}O$ $M_r$ 220.4
[128-37-0]

DEFINITION

Butylhydroxytoluene is 2,6-bis(1,1-dimethylethyl)-4-methylphenol.

CHARACTERS

A white or yellowish-white, crystalline powder, practically insoluble in water, very soluble in acetone, freely soluble in alcohol and in vegetable oils.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Freezing-point (see Tests).

B. Dissolve 0.500 g in *ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *ethanol R*. Examined between 230 nm and 300 nm (*2.2.25*), the solution shows an absorption maximum at 278 nm. The specific absorbance at the maximum is 80 to 90.

C. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *butylhydroxytoluene CRS*.

D. Dissolve about 10 mg in 2 mL of *alcohol R*. Add 1 mL of a 1 g/L solution of *testosterone propionate R* in *alcohol R* and 2 mL of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 10 min and allow to cool. A blue colour develops.

TESTS

**Appearance of solution**. Dissolve 1.0 g in *methanol R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ or $BY_5$ (*2.2.2, Method II*).

**Freezing-point** (*2.2.18*): 69 °C to 70 °C.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution.* Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dilute 1 mL of the test solution to 200 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using *methylene chloride R*. Dry the plate in air and spray with a freshly prepared mixture of 10 volumes of *potassium ferricyanide solution R*, 20 volumes of *ferric chloride solution R1* and 70 volumes of *water R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

# C


Cabergoline.. 2031
Caffeine.. 2032
Caffeine monohydrate.. 2033
Calcifediol monohydrate.. 2035
Calcipotriol.. 2036
Calcipotriol monohydrate.. 2038
Calcitonin (salmon).. 2040
Calcitriol.. 2043
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Calcium gluconate, anhydrous.. 2052
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Calcium hydrogen phosphate.. 2055
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Calcium hydroxide.. 2057
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Calcium lactate monohydrate.. 2058
Calcium lactate pentahydrate.. 2059
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Carbomers.. 2085
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Carbon monoxide intermix (5 per cent) in nitrogen.. 2089
Carboplatin.. 2089
Carboprost trometamol.. 2090
Carisoprodol.. 2091
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Carrageenan.. 2098
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Castor oil, hydrogenated.. 2102
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Cefalexin monohydrate.. 2108
Cefalotin sodium.. 2109
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Cefatrizine propylene glycol.. 2113
Cefazolin sodium.. 2114
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Cefixime.. 2119
Cefoperazone sodium.. 2120
Cefotaxime sodium.. 2122
Cefoxitin sodium.. 2124
Cefpodoxime proxetil.. 2126
Cefprozil monohydrate.. 2128
Cefradine.. 2130
Ceftazidime pentahydrate.. 2132
Ceftazidime pentahydrate with sodium carbonate for injection.. 2134
Ceftriaxone sodium.. 2136
Cefuroxime axetil.. 2138
Cefuroxime sodium.. 2139
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Celiprolol hydrochloride.. 2142
Cellulose acetate.. 2143
Cellulose acetate butyrate.. 2144
Cellulose acetate phthalate.. 2145
Cellulose, microcrystalline.. 2146
Cellulose, powdered.. 2150
Cetirizine dihydrochloride.. 2153
Cetostearyl alcohol.. 2155
Cetostearyl alcohol (type A), emulsifying.. 2156
Cetostearyl alcohol (type B), emulsifying.. 2157
Cetostearyl isononanoate.. 2159
Cetrimide.. 2159
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Cetyl palmitate.. 2160
Cetylpyridinium chloride.. 2161
Charcoal, activated.. 2162
Chenodeoxycholic acid .. 2163
Chitosan hydrochloride.. 2164
Chloral hydrate.. 2165
Chlorambucil.. 2166
Chloramphenicol.. 2167
Chloramphenicol palmitate.. 2168
Chloramphenicol sodium succinate.. 2170
Chlorcyclizine hydrochloride.. 2171
Chlordiazepoxide.. 2172
Chlordiazepoxide hydrochloride.. 2173
Chlorhexidine diacetate.. 2174
Chlorhexidine digluconate solution.. 2176
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Chloroquine sulfate.. 2186
Chlorphenamine maleate.. 2187
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Chlorprothixene hydrochloride.. 2189
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Chlortetracycline hydrochloride.. 2193
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Cholecalciferol concentrate (powder form).. 2198
Cholecalciferol concentrate (water-dispersible form).. 2199
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Ciclopirox olamine.. 2209
Ciclosporin.. 2210
Cilastatin sodium.. 2212
Cilazapril.. 2214
Cimetidine.. 2215
Cimetidine hydrochloride.. 2217
Cinchocaine hydrochloride.. 2218
Cineole.. 2220
Cinnarizine.. 2221
Ciprofibrate.. 2222
Ciprofloxacin.. 2223
Ciprofloxacin hydrochloride.. 2225
Cisatracurium besilate.. 2226
Cisplatin.. 2231
Citalopram hydrobromide.. 2232
Citalopram hydrochloride.. 2234
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Clazuril for veterinary use.. 2241
Clebopride malate.. 2243
Clemastine fumarate.. 2244
Clenbuterol hydrochloride.. 2246
Clindamycin hydrochloride.. 2247
Clindamycin phosphate.. 2248
Clioquinol.. 2251
Clobazam.. 2252
Clobetasol propionate.. 2253
Clobetasone butyrate.. 2255
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Clonazepam.. 2263
Clonidine hydrochloride.. 2264
Clopamide.. 2265
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Clopidogrel hydrochloride.. 2268
Clopidogrel hydrogen sulfate.. 2270
Closantel sodium dihydrate for veterinary use.. 2272
Clotrimazole.. 2273
Cloxacillin sodium.. 2275
Clozapine.. 2276
Cocaine hydrochloride.. 2278
Cocoa butter.. 2279
Coconut oil, refined.. 2279
Cocoyl caprylocaprate.. 2280
Codeine hydrochloride dihydrate.. 2281
Codeine monohydrate.. 2283
Codeine phosphate hemihydrate.. 2285
Codeine phosphate sesquihydrate.. 2288
Codergocrine mesilate.. 2290
Cod-liver oil, farmed.. 2291
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Cod-liver oil (type B).. 2299
Colchicine.. 2303
Colestyramine.. 2305
Colistimethate sodium.. 2306
Colistin sulfate.. 2309
Copovidone.. 2311
Copper sulfate.. 2313
Copper sulfate pentahydrate.. 2314
Cortisone acetate.. 2315
Cotton, absorbent.. 2316
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Cresol, crude.. 2318
Croscarmellose sodium.. 2318
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Crotamiton.. 2321
Cyanocobalamin.. 2322
Cyclizine hydrochloride.. 2323
Cyclopentolate hydrochloride.. 2324
Cyclophosphamide.. 2326
Cyproheptadine hydrochloride.. 2326
Cyproterone acetate.. 2327
Cysteine hydrochloride monohydrate.. 2329
Cystine.. 2331
Cytarabine.. 2332

**01/2008:1773**

# CABERGOLINE

## Cabergolinum

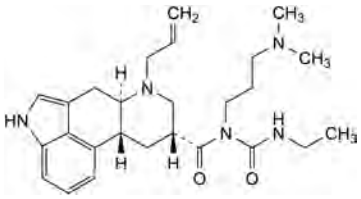

$C_{26}H_{37}N_5O_2$ $M_r$ 451.6
[81409-90-7]

DEFINITION

1-Ethyl-3-[3-(dimethylamino)propyl]-3-[[(6a*R*,9*R*,10a*R*)-7-(prop-2-enyl)-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]carbonyl]urea.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), very slightly soluble in hexane. It is slightly soluble in 0.1 M hydrochloric acid.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cabergoline CRS*.

If the spectra obtained in the solid state show differences, dissolve 50 mg of the substance to be examined and 50 mg of the reference substance separately in 1 mL of *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): − 77 to − 83 (anhydrous substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protected from light.*

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 30.0 mg of *cabergoline CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Suspend 50 mg of the substance to be examined in 10 mL of *0.1 M sodium hydroxide*. Stir for about 15 min. To 1 mL of the suspension add 1 mL of *0.1 M hydrochloric acid* and dilute to 10 mL with the mobile phase. Sonicate until dissolution is complete. The main degradation product obtained is impurity A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 16 volumes of *acetonitrile R* and 84 volumes of a freshly prepared 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*. Add 0.2 volumes of *triethylamine R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 4 times the retention time of cabergoline.

*Relative retention* with reference to cabergoline (retention time = about 12 min): impurity D = about 0.3; impurity B = about 0.6; impurity A = about 0.8; impurity C = about 2.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to cabergoline and impurity A.

*Limits*:

– *impurities A, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *impurities B, D*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{26}H_{37}N_5O_2$ from the areas of the peaks and the declared content of *cabergoline CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

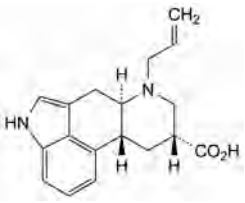

A. (6a*R*,9*R*,10a*R*)-7-(prop-2-enyl)-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxylic acid,

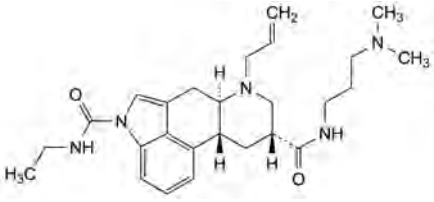

B. (6a*R*,9*R*,10a*R*)-$N^9$-[3-(dimethylamino)propyl]-$N^4$-ethyl-7-(prop-2-enyl)-6a,7,8,9,10,10a-hexahydroindolo[4,3-*fg*]quinoline-4,9(6*H*)-dicarboxamide,

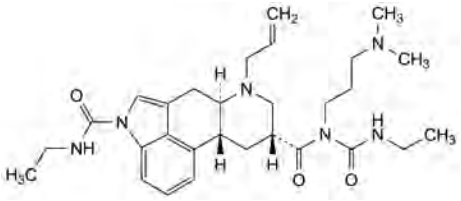

C. (6a*R*,9*R*,10a*R*)-$N^9$-[3-(dimethylamino)propyl]-$N^4$-ethyl-$N^9$-(ethylcarbamoyl)-7-(prop-2-enyl)-6a,7,8,9,10,10a-hexahydroindolo[4,3-*fg*]quinoline-4,9(6*H*)-dicarboxamide,

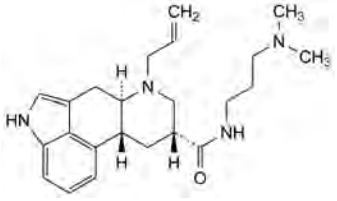

D. (6a*R*,9*R*,10a*R*)-*N*-[3-(dimethylamino)propyl]-7-(prop-2-enyl)-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide.

**01/2020:0267**

# CAFFEINE

## Coffeinum

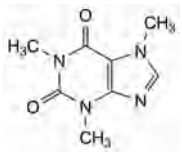

$C_8H_{10}N_4O_2$ $M_r$ 194.2
[58-08-2]

DEFINITION

1,3,7-Trimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or silky crystals.

*Solubility*: sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol (96 per cent). It dissolves in concentrated solutions of alkali benzoates or salicylates.

It sublimes readily.

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E, F.*

A. Melting point (*2.2.14*): 234 °C to 239 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *caffeine CRS*.

C. To 2 mL of a saturated solution add 0.05 mL of *iodinated potassium iodide solution R*. The solution remains clear. Add 0.1 mL of *dilute hydrochloric acid R*; a brown precipitate is formed. Neutralise with *dilute sodium hydroxide solution R*; the precipitate dissolves.

D. In a ground-glass-stoppered tube, dissolve about 10 mg in 0.25 mL of a mixture of 0.5 mL of *acetylacetone R* and 5 mL of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 7 min. Cool and add 0.5 mL of *dimethylaminobenzaldehyde solution R2*. Heat again in a water-bath at 80 °C for 7 min. Allow to cool and add 10 mL of *water R*; an intense blue colour develops.

E. Loss on drying (see Tests).

F. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g with heating in 30 mL of *carbon dioxide-free water R* prepared from *distilled water R*, cool and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S add 0.05 mL of *bromothymol blue solution R1*; the solution is green or yellow. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *caffeine for system suitability CRS* (containing impurities A, C, D and F) in the mobile phase and dilute to 5 mL with the mobile phase. Dilute 2 mL of this solution to 10 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *tetrahydrofuran R*, 25 volumes of *acetonitrile R* and 955 volumes of a solution containing 0.82 g/L of *anhydrous sodium acetate R* previously adjusted to pH 4.5 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of caffeine.

*Identification of impurities*: use the chromatogram supplied with *caffeine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C, D and F.

*Relative retention* with reference to caffeine (retention time = about 8 min): impurity C = about 0.38; impurity D = about 0.42; impurity F = about 0.6; impurity A = about 0.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurities C and D and minimum 2.5 between the peaks due to impurities F and A.

*Limits*:
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 500 ppm, determined on 15 mL of solution S.

Prepare the standard using a mixture of 7.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 7.5 mL of *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g with heating in 5 mL of *anhydrous acetic acid R*. Allow to cool, add 10 mL of *acetic anhydride R* and 20 mL of *toluene R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.42 mg of $C_8H_{10}N_4O_2$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

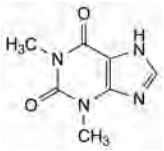

A. 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theophylline),

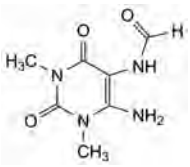

B. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

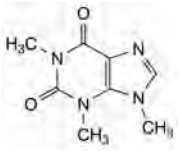

C. 1,3,9-trimethyl-3,9-dihydro-1*H*-purine-2,6-dione (isocaffeine),

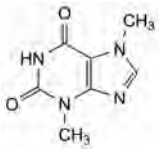

D. 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theobromine),

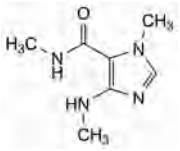

E. *N*,1-dimethyl-4-(methylamino)-1*H*-imidazole-5-carboxamide (caffeidine),

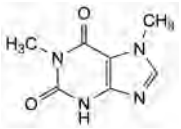

F. 1,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

**01/2020:0268**

# CAFFEINE MONOHYDRATE

## Coffeinum monohydricum

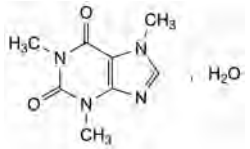

$C_8H_{10}N_4O_2,H_2O$ $M_r$ 212.2
[5743-12-4]

DEFINITION

1,3,7-Trimethyl-3,7-dihydro-1*H*-purine-2,6-dione monohydrate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or silky crystals.

*Solubility*: sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol (96 per cent). It dissolves in concentrated solutions of alkali benzoates or salicylates.

It sublimes readily.

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E, F.*

A. Melting point (*2.2.14*): 234 °C to 239 °C, determined after drying at 100-105 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined at 100-105 °C before use.

*Comparison*: *caffeine CRS*.

C. To 2 mL of a saturated solution add 0.05 mL of *iodinated potassium iodide solution R*; the solution remains clear. Add 0.1 mL of *dilute hydrochloric acid R*; a brown precipitate is formed. Neutralise with *dilute sodium hydroxide solution R*; the precipitate dissolves.

D. In a glass-stoppered tube, dissolve about 10 mg in 0.25 mL of a mixture of 0.5 mL of *acetylacetone R* and 5 mL of *dilute sodium hydroxide solution R*. Heat in a water-bath at 80 °C for 7 min. Cool and add 0.5 mL of *dimethylaminobenzaldehyde solution R2*. Heat again in a water-bath at 80 °C for 7 min. Allow to cool and add 10 mL of *water R*; an intense blue colour develops.

E. Loss on drying (see Tests).

F. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g with heating in 30 mL of *carbon dioxide-free water R* prepared from *distilled water R*, cool, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S add 0.05 mL of *bromothymol blue solution R1*; the solution is green or yellow. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.110 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *caffeine for system suitability CRS* (containing impurities A, C, D and F) in the mobile phase and dilute to 5 mL with the mobile phase. Dilute 2 mL of this solution to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*. Mix 20 volumes of *tetrahydrofuran R*, 25 volumes of *acetonitrile R* and 955 volumes of a solution containing 0.82 g/L of *anhydrous sodium acetate R* previously adjusted to pH 4.5 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of caffeine.

*Identification of impurities*: use the chromatogram supplied with *caffeine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C, D and F.

*Relative retention* with reference to caffeine (retention time = about 8 min): impurity C = about 0.38; impurity D = about 0.42; impurity F = about 0.6; impurity A = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities C and D; minimum 2.5 between the peaks due to impurities F and A.

*Limits*:

- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 500 ppm, determined on 15 mL of solution S.

Prepare the standard using a mixture of 7.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 7.5 mL of *distilled water R*.

**Loss on drying** (*2.2.32*): 5.0 per cent to 9.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g, previously dried at 100-105 °C, with heating in 5 mL of *anhydrous acetic acid R*. Allow to cool, and add 10 mL of *acetic anhydride R* and 20 mL of *toluene R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.42 mg of $C_8H_{10}N_4O_2$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

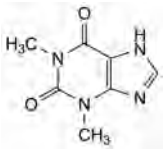

A. 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theophylline),

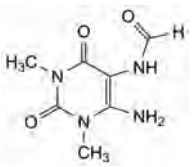

B. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

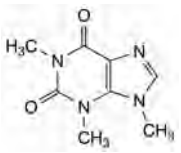

C. 1,3,9-trimethyl-3,9-dihydro-1*H*-purine-2,6-dione (isocaffeine),

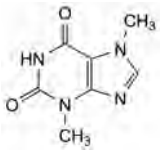

D. 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theobromine),

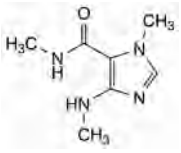

E. *N*,1-dimethyl-4-(methylamino)-1*H*-imidazole-5-carboxamide (caffeidine),

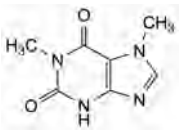

F. 1,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

**01/2019:1295**

# CALCIFEDIOL MONOHYDRATE

## Calcifediolum monohydricum

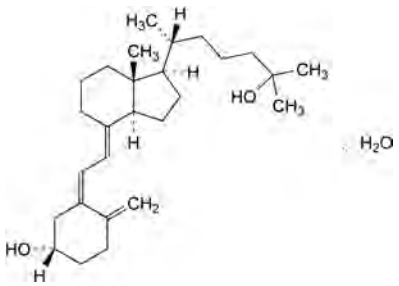

$C_{27}H_{44}O_2,H_2O$ $M_r$ 418.7
[63283-36-3]

DEFINITION

(3*S*,5*Z*,7*E*)-9,10-Secocholesta-5,7,10(19)-triene-3,25-diol monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

A reversible isomerisation to pre-calcifediol takes place in solution, depending on temperature and time. The activity is due to both compounds (see Assay).

CHARACTERS

*Appearance*: white or almost white crystals.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in fatty oils.

It is sensitive to air, heat and light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of calcifediol.*

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Carry out the test as rapidly as possible, avoiding exposure to actinic light and air.*

*Test solution.* Dissolve 5.0 mg of the substance to be examined without heating in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *calcifediol CRS* without heating in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Heat 2 mL of reference solution (a) in a water-bath at 80 °C under a reflux condenser for 2 h and cool.

*Reference solution (d).* Dissolve 1 mg of *calcifediol for impurity D identification CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R, methanol R* (20:80 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 50 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of calcifediol.

*Identification of impurities*: use the chromatogram supplied with *calcifediol for impurity D identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention* with reference to calcifediol (retention time = about 11 min): pre-calcifediol = about 1.3; impurity D = about 1.7.

*System suitability*: reference solution (c):
- *resolution*: minimum 5.0 between the peaks due to calcifediol and pre-calcifediol.

*Limits*:
- *impurity D*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent (0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b)); disregard the peak due to pre-calcifediol.

**Water** (*2.5.32*): 3.8 per cent to 5.0 per cent, determined on 10.0 mg by direct introduction of the sample.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{44}O_2$ taking into account the assigned content of *calcifediol CRS* and, if necessary, the peak due to pre-calcifediol.

STORAGE

Under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

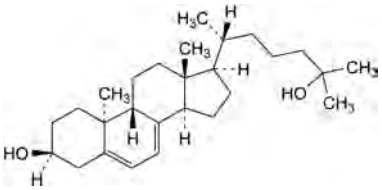

A. 9β,10α-cholesta-5,7-diene-3β,25-diol,

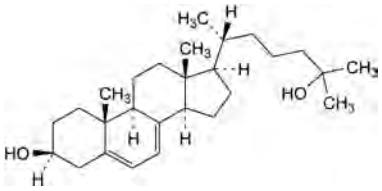

B. cholesta-5,7-diene-3β,25-diol,

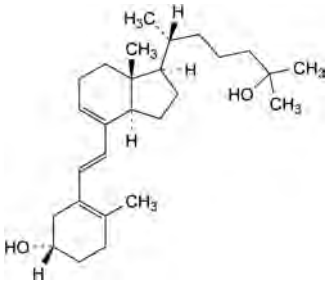

C. (3*S*,6*E*)-9,10-secocholesta-5(10),6,8-triene-3,25-diol,

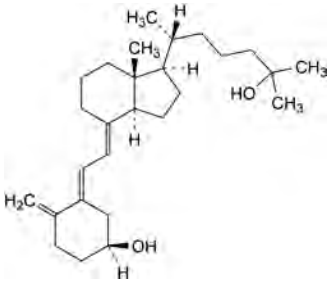

D. (3*S*,5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-3,25-diol.

**01/2019:2011**

# CALCIPOTRIOL

## Calcipotriolum

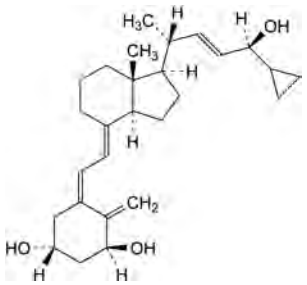

$C_{27}H_{40}O_3$ $M_r$ 412.6
[112965-21-6]

DEFINITION

(5*Z*,7*E*,22*E*,24*S*)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol.

*Content*: 95.5 per cent to 102.0 per cent (dried substance).

A reversible isomerisation to pre-calcipotriol takes place in solution, depending on temperature and time. The activity is due to both compounds.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It is sensitive to heat and light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of anhydrous calcipotriol*.

B. Loss on drying (see Tests).

TESTS

*Carry out the tests for related substances and the assay as rapidly as possible and protected from actinic light and air.*

**Related substances**

A. Thin-layer chromatography (*2.2.27*).

*Solution A*. To 1 mL of *triethylamine R* add 9 mL of *chloroform R*.

*Test solution*. Dissolve 1 mg of the substance to be examined in 100 µL of solution A.

*Reference solution (a)*. To 10 µL of the test solution add 990 µL of solution A.

*Reference solution (b)*. To 250 µL of reference solution (a) add 750 µL of solution A.

*Reference solution (c)*. To 100 µL of reference solution (a) add 900 µL of solution A.

*Reference solution (d)*. Place 2 mg of the substance to be examined in a vial and dissolve in 200 µL of solution A. Close the vial and keep it in a water-bath at 60 °C for 2 h.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *2-methylpropanol R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Development*: over 2/3 of the plate.

*Drying*: in air, then at 140 °C for 10 min.

*Detection*: spray the hot plate with an *alcoholic solution of sulfuric acid R*, dry at 140 °C for not more than 1 min and examine in ultraviolet light at 366 nm.

*Relative retention* with reference to calcipotriol ($R_F$ = about 0.4): impurity G = about 0.4; impurity H = about 0.4; pre-calcipotriol = about 0.9; impurity A = about 1.2.

*System suitability*: reference solution (d):

– the chromatogram shows a secondary spot due to pre-calcipotriol.

*Limits*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent);
– *impurities G, H*: any spot due to impurity G or H is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent for the sum);
– *unspecified impurities*: any other spot is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

B. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 1.32 g of *ammonium phosphate R* in *water R* and dilute to 10.0 mL with the same solvent.

*Solvent mixture*: solution A, *water R*, *methanol R* (0.3:29.7:70 *V/V/V*).

*Test solution (a)*. Dissolve 2 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 2.00 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 1 mg of *calcipotriol for system suitability CRS* (containing impurities B, C and D) in the solvent mixture and dilute to 2.5 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2.00 mg of *calcipotriol monohydrate CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *water for chromatography R, methanol R* (30:70 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: twice the retention time of calcipotriol.

*Identification of impurities*: use the chromatogram supplied with *calcipotriol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C and D.

*Relative retention* with reference to calcipotriol (retention time = about 13.5 min): impurity B = about 0.86; impurity C = about 0.92; impurity D = about 1.3.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to calcipotriol.

*Limits*:

- *impurity B*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying**: maximum 1.0 per cent, determined on 5 mg by thermogravimetry (*2.2.34*). Heat to 105 °C at a rate of 10 °C/min and maintain at 105 °C for 60 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{27}H_{40}O_3$ taking into account the assigned content of *calcipotriol monohydrate CRS* and, if necessary, the peak due to pre-calcipotriol.

STORAGE

In an airtight container, protected from light, at − 20 °C or below.

IMPURITIES

**Test A for related substances**: A, G, H, I.

**Test B for related substances**: B, C, D, E, F.

*Specified impurities: A, B, C, D, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, I.*

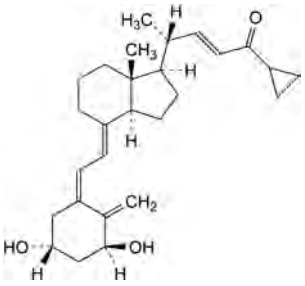

A. (5*Z*,7*E*,22*E*)-24-cyclopropyl-1α,3β-dihydroxy-9,10-secochola-5,7,10(19),22-tetraen-24-one,

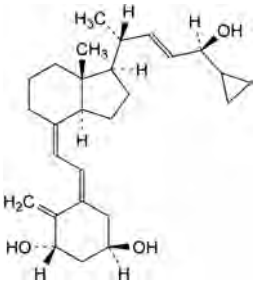

B. (5*Z*,7*Z*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol ((7*Z*)-calcipotriol),

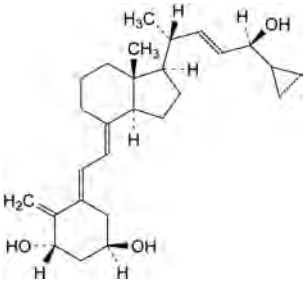

C. (5*E*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol ((5*E*)-calcipotriol),

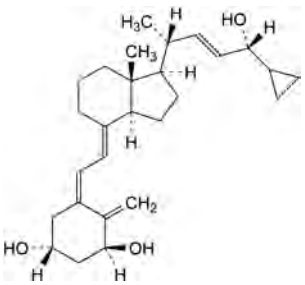

D. (5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol (24-*epi*-calcipotriol),

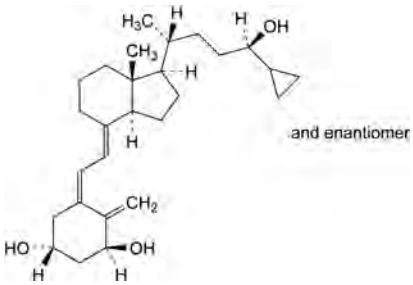


E. *rac*-(5*Z*,7*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19)-triene-1α,3β,24-triol,

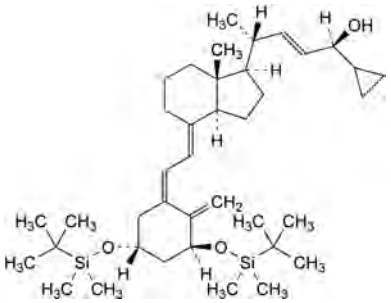

F. (5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-1α,3β-bis[[(1,1-dimethylethyl)dimethylsilyl]oxy]-9,10-secochola-5,7,10(19),22-tetraen-24-ol,

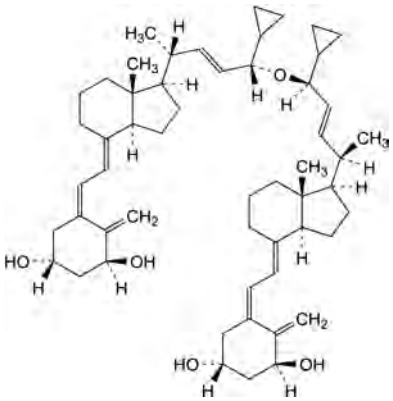

G. 24,24′-oxybis[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β-diol],

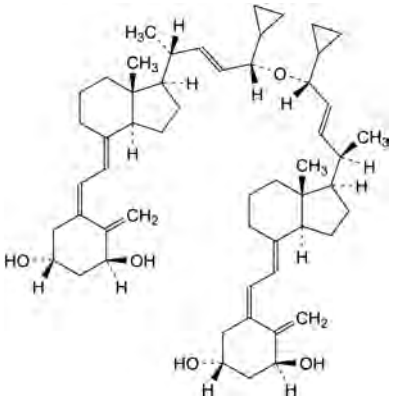

H. (5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-24-[[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-1α,3β-dihydroxy-9,10-secochola-5,7,10(19),22-tetraen-24-yl]oxy]-9,10-secochola-5,7,10(19),22-tetraene-1α,3β-diol,

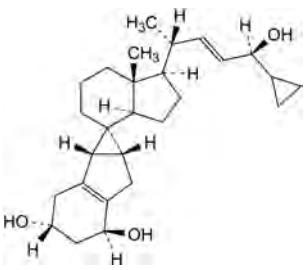

I. (6*S*,7*R*,8*R*,22*E*,24*S*)-24-cyclopropyl-6,8:7,19-dicyclo-9,10-secochola-5(10),22-diene-1α,3β,24-triol (suprasterol of calcipotriol).

**01/2019:2284**

# CALCIPOTRIOL MONOHYDRATE

## Calcipotriolum monohydricum

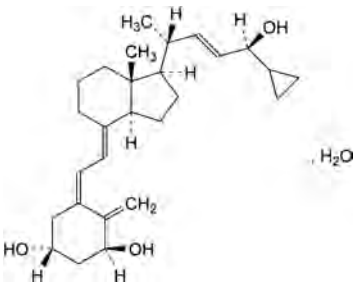

$C_{27}H_{40}O_3,H_2O$ $M_r$ 430.6
[147657-22-5]

DEFINITION

(5*Z*,7*E*,22*E*,24*S*)-24-Cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol monohydrate.

*Content*: 95.5 per cent to 102.0 per cent (anhydrous substance).

A reversible isomerisation to pre-calcipotriol takes place in solution, depending on temperature and time. The activity is due to both compounds.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It is sensitive to light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of calcipotriol monohydrate.*

B. Water (see Tests).

TESTS

*Carry out the tests for related substances and the assay as rapidly as possible and protected from actinic light and air.*

**Related substances**

A. Thin-layer chromatography (*2.2.27*).

*Solution A*. To 1 mL of *triethylamine R* add 9 mL of *chloroform R*.

*Test solution.* Dissolve 1 mg of the substance to be examined in 100 µL of solution A.

*Reference solution (a).* To 10 µL of the test solution add 990 µL of solution A.

*Reference solution (b).* To 250 µL of reference solution (a) add 750 µL of solution A.

*Reference solution (c).* To 100 µL of reference solution (a) add 900 µL of solution A.

*Reference solution (d).* Place 2 mg of the substance to be examined in a vial and dissolve in 200 µL of solution A. Close the vial and keep it in a water bath at 60 °C for 2 h.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *2-methylpropanol R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Development*: over 2/3 of the plate.

*Drying*: in air, then at 140 °C for 10 min.

*Detection*: spray the hot plate with an *alcoholic solution of sulfuric acid R*, dry at 140 °C for not more than 1 min and examine in ultraviolet light at 366 nm.

*Relative retention* with reference to calcipotriol ($R_F$ = about 0.4): impurity G = about 0.4; impurity H = about 0.4; pre-calcipotriol = about 0.9; impurity A = about 1.2.

*System suitability*: reference solution (d):

– the chromatogram shows a secondary spot due to pre-calcipotriol.

*Limits*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent);
– *impurities G, H*: any spot due to impurity G or H is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent for the sum);
– *unspecified impurities*: any other spot is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

B. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 1.32 g of *ammonium phosphate R* in *water R* and dilute to 10.0 mL with the same solvent.

*Solvent mixture*: solution A, *water R*, *methanol R* (0.3:29.7:70 *V/V/V*).

*Test solution (a).* Dissolve 2 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 2.00 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 1 mg of *calcipotriol for system suitability CRS* (containing impurities B, C and D) in the solvent mixture and dilute to 2.5 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2.00 mg of *calcipotriol monohydrate CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *water for chromatography R*, *methanol R* (30:70 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: twice the retention time of calcipotriol.

*Identification of impurities*: use the chromatogram supplied with *calcipotriol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C and D.

*Relative retention* with reference to calcipotriol (retention time = about 13.5 min): impurity B = about 0.86; impurity C = about 0.92; impurity D = about 1.3.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to calcipotriol.

*Limits*:

– *impurity B*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 3.3 per cent to 5.0 per cent, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{27}H_{40}O_3$ taking into account the assigned content of *calcipotriol monohydrate CRS* and, if necessary, the peak due to pre-calcipotriol.

STORAGE

In an airtight container, protected from light.

IMPURITIES

**Test A for related substances**: A, G, H, I.

**Test B for related substances**: B, C, D, E, F.

*Specified impurities: A, B, C, D, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, I.*

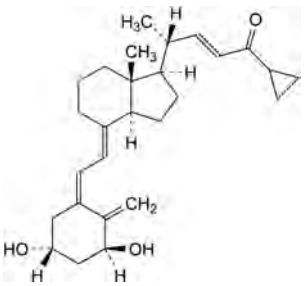


A. (5*Z*,7*E*,22*E*)-24-cyclopropyl-1α,3β-dihydroxy-9,10-secochola-5,7,10(19),22-tetraen-24-one,

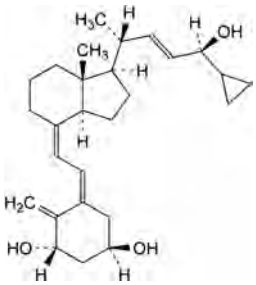

B. (5*Z*,7*Z*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol ((7*Z*)-calcipotriol),

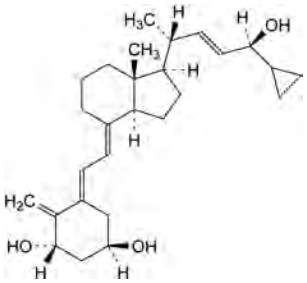

C. (5*E*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol ((5*E*)-calcipotriol),

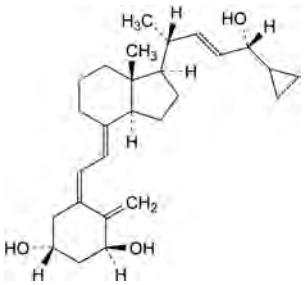

D. (5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β,24-triol (24-*epi*-calcipotriol),

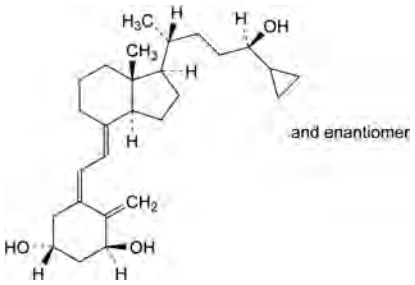


E. *rac*-(5*Z*,7*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19)-triene-1α,3β,24-triol,

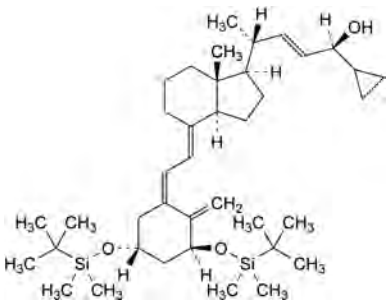

F. (5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-1α,3β-bis[[(1,1-dimethylethyl)dimethylsilyl]oxy]-9,10-secochola-5,7,10(19),22-tetraen-24-ol,

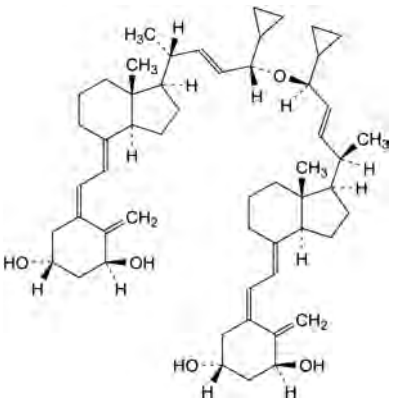

G. 24,24′-oxybis[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-9,10-secochola-5,7,10(19),22-tetraene-1α,3β-diol],

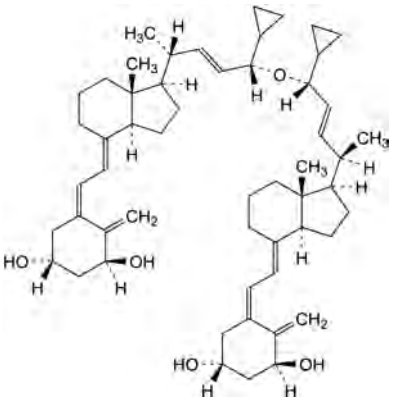

H. (5*Z*,7*E*,22*E*,24*R*)-24-cyclopropyl-24-[[(5*Z*,7*E*,22*E*,24*S*)-24-cyclopropyl-1α,3β-dihydroxy-9,10-secochola-5,7,10(19),22-tetraen-24-yl]oxy]-9,10-secochola-5,7,10(19),22-tetraene-1α,3β-diol,

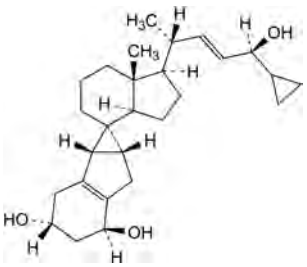

I. (6*S*,7*R*,8*R*,22*E*,24*S*)-24-cyclopropyl-6,8:7,19-dicyclo-9,10-secochola-5(10),22-diene-1α,3β,24-triol (suprasterol of calcipotriol).

**01/2008:0471**
**corrected 10.0**

# CALCITONIN (SALMON)

## Calcitoninum salmonis

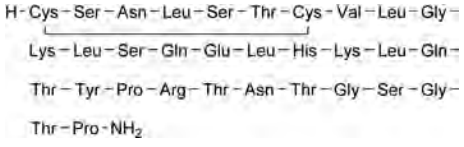


$C_{145}H_{240}N_{44}O_{48}S_2$ $M_r$ 3432

DEFINITION

Polypeptide having the structure determined for salmon calcitonin I. It lowers the calcium concentration in plasma of mammals by diminishing the rate of bone resorption. It is obtained by chemical synthesis or by a method based on recombinant DNA (rDNA) technology. It is available as an acetate.

*Content*: 90.0 per cent to 105.0 per cent of the peptide $C_{145}H_{240}N_{44}O_{48}S_2$ (anhydrous and acetic acid-free substance).

By convention, for the purpose of labelling calcitonin (salmon) preparations, 1 mg of calcitonin (salmon) ($C_{145}H_{240}N_{44}O_{48}S_2$) is equivalent to 6000 IU of biological activity.

PRODUCTION

The following requirements apply only to calcitonin (salmon) produced by a method based on rDNA technology.

Prior to release, the following tests are carried out on each batch of calcitonin (salmon), unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell or vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

*The following requirement applies only to calcitonin (salmon) obtained by chemical synthesis.*

B. Amino acid analysis (*2.2.56*).

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking as equivalent to 1 the sum, divided by 20, of the number of moles of aspartic acid, glutamic acid, proline, glycine, valine, leucine, histidine, arginine and lysine. The values fall within the following limits: aspartic acid: 1.8 to 2.2; glutamic acid: 2.7 to 3.3; proline: 1.7 to 2.3; glycine: 2.7 to 3.3; valine: 0.9 to 1.1; leucine: 4.5 to 5.3; histidine: 0.9 to 1.1; arginine: 0.9 to 1.1; lysine: 1.8 to 2.2; serine: 3.2 to 4.2; threonine: 4.2 to 5.2; tyrosine: 0.7 to 1.1; half-cystine: 1.4 to 2.1.

*The following requirement applies only to calcitonin (salmon) produced by a method based on rDNA technology.*

C. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Prepare a 1 mg/mL solution of the substance to be examined. Transfer 1.0 mL to a clean tube. Add 100 µL of *1 M tris-hydrochloride buffer solution pH 8.0 R* and 20 µL of a freshly prepared 1.0 mg/mL solution of *trypsin for peptide mapping R*. Allow to stand at 2-8 °C for 16-20 h. Stop the reaction by adding 10 µL of a 50 per cent *V/V* solution of *trifluoroacetic acid R*. Cap the vial and mix. Centrifuge the vials to remove air bubbles.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *calcitonin (salmon) CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

– *mobile phase A*: mix 1 mL of *trifluoroacetic acid R* and 1000 mL of *water R*; filter and degas;

– *mobile phase B*: mix 0.850 mL of *trifluoroacetic acid R*, 200 mL of *water R* and 800 mL of *acetonitrile for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 → 65 | 0 → 35 |
| 50 - 60 | 65 → 40 | 35 → 60 |
| 60 - 60.1 | 40 → 0 | 60 → 100 |
| 60.1 - 65.1 | 0 | 100 |
| 65.1 - 65.2 | 0 → 100 | 100 → 0 |
| 65.2 - 80.2 | 100 | 0 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 20 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of calcitonin (salmon) digest supplied with *calcitonin (salmon) CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution: the retention times of the fragment peaks in the chromatogram obtained with the test solution are within 5 per cent of the retention times of the fragments obtained with the reference solution; the peak area ratios of the fragment peaks in the chromatogram obtained with the test solution, normalised to the area of peak $T_2$, are within 5 per cent of the corresponding peak ratios in the chromatogram obtained with the reference solution.

TESTS

**Acetic acid** (*2.5.34*): 4.0 per cent to 15.0 per cent.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*The following requirement applies to calcitonin (salmon), whether obtained by chemical synthesis or by a method based on rDNA technology.*

A. *Test solution.* Prepare a 1.0 mg/mL solution of the substance to be examined in mobile phase A.

*Reference solution.* Dissolve the contents of a vial of *calcitonin (salmon) CRS* in mobile phase A to obtain a concentration of 1.0 mg/mL.

*Resolution solution.* Dissolve the contents of a vial of *N-acetyl-Cys[1]-calcitonin CRS* in 400 µL of mobile phase A and add 100 µL of the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 65 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 3.26 g of *tetramethylammonium hydroxide R* in 900 mL of *water R*, adjust to pH 2.5 with *phosphoric acid R* and mix with 100 mL of *acetonitrile for chromatography R*; filter and degas;

– *mobile phase B*: dissolve 1.45 g of *tetramethylammonium hydroxide R* in 400 mL of *water R*, adjust to pH 2.5 with *phosphoric acid R* and mix with 600 mL of *acetonitrile for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 72 → 48 | 28 → 52 |
| 30 - 32 | 48 → 72 | 52 → 28 |
| 32 - 55 | 72 | 28 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Relative retention* with reference to calcitonin (salmon) (retention time = about 20 min): impurity B = about 0.8; impurity C = about 0.9; impurity D = about 1.05; impurity A = about 1.15.

*System suitability*: resolution solution:

– *resolution*: minimum 5.0 between the peaks due to calcitonin (salmon) and impurity A,

– *symmetry factor*: maximum 2.5 for the peak due to impurity A.

*Limits*:

– *impurities A, B, C, D*: for each impurity, maximum 3.0 per cent; other unidentified, specified impurities may occur that co-elute with impurities A, B, C and D; the acceptance criterion applies irrespective of whether these impurities co-elute;

– *total*: maximum 5.0 per cent;

– *disregard limit*: 0.1 per cent.

*The following requirement applies only to calcitonin (salmon) produced by a method based on rDNA technology.*

B. *Test solution.* Prepare a 0.5 mg/mL solution of the substance to be examined. To 1.0 mL of this solution add 100 µL of *0.25 M citrate buffer solution pH 3.0 R*.

*Resolution solution.* Prepare a 1 mg/mL solution of the substance to be examined. Mix 1 volume of the solution and 1 volume of *calcitonin-Gly CRS*. To 1.0 mL of this mixture add 100 µL of *0.25 M citrate buffer solution pH 3.0 R*.

*Column*:

– *size*: *l* = 0.20 m, Ø = 4.6 mm;

– *stationary phase*: a suitable polysulfoethylaspartamide ion-exchange gel (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 15 volumes of *acetonitrile for chromatography R* and 85 volumes of a 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with a 600 g/L solution of *potassium hydroxide R*;

– *mobile phase B*: mix 15 volumes of *acetonitrile for chromatography R* and 85 volumes of a solution containing 2.72 g/L of *potassium dihydrogen phosphate R* and 29.22 g/L of *sodium chloride R* adjusted to pH 4.6 with a 600 g/L solution of *potassium hydroxide R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 → 0 | 0 → 100 |
| 10 - 15 | 0 | 100 |
| 15 - 15.1 | 0 → 100 | 100 → 0 |
| 15.1 - 22.1 | 100 | 0 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL; rinse the injector with a 40 per cent *V/V* solution of *acetonitrile for chromatography R*.

*Relative retention* with reference to calcitonin (salmon) (retention time = about 9 min): impurity G = about 0.4; impurity F = about 0.6; impurity E = about 0.9.

*System suitability*: resolution solution:

– *resolution*: minimum 3.0 between the peaks due to impurity E and calcitonin (salmon).

*Limits*:

– *impurity E*: maximum 0.6 per cent;

– *impurities F, G*: for each impurity, maximum 0.2 per cent.

**Water** (*2.5.32*): maximum 10.0 per cent.

**Acetic acid and water**: maximum 20 per cent, calculated by adding together the percentage contents of acetic acid and water determined by the methods described above.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances. Use method A for calcitonin (salmon) obtained by chemical synthesis and method B for calcitonin (salmon) obtained by a method based on rDNA technology.

Calculate the content of calcitonin (salmon) ($C_{145}H_{240}N_{44}O_{48}S_2$) from the area of the principal peak in each of the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{145}H_{240}N_{44}O_{48}S_2$ in *calcitonin (salmon) CRS*. Proceed with tangential integration of the peak areas.

STORAGE

Protected from light at a temperature between 2 °C and 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

– the calcitonin peptide content ($C_{145}H_{240}N_{44}O_{48}S_2$);

– the origin: synthetic or rDNA technology.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

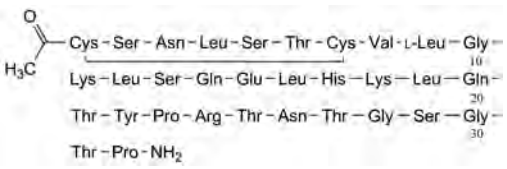


A. acetylcalcitonin (salmon),

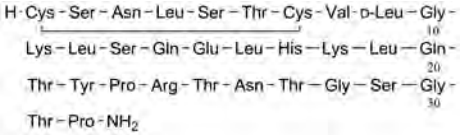


B. [9-D-leucine]calcitonin (salmon),

C. des-22-tyrosine-calcitonin (salmon),

D. *O*-acetylated calcitonin (salmon),

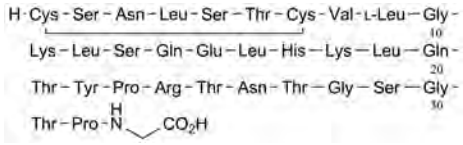


E. salmon calcitoninylglycine,

F. [1,7-bis(3-sulfo-L-alanine)]calcitonin (salmon),

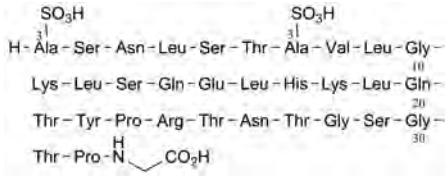


G. [1,7-bis(3-sulfo-L-alanine)]calcitoninylglycine (salmon).

**01/2013:0883**

# CALCITRIOL

## Calcitriolum

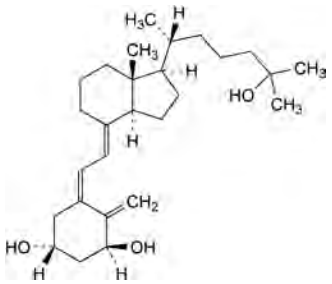


$C_{27}H_{44}O_3$ $M_r$ 416.6
[32222-06-3]

DEFINITION

(5*Z*,7*E*)-9,10-Secocholesta-5,7,10(19)-triene-1α,3β,25-triol.

*Content*: 97.0 per cent to 103.0 per cent.

A reversible isomerisation to pre-calcitriol takes place in solution, depending on temperature and time. The activity is due to both compounds (see Assay).

CHARACTERS

*Appearance*: white or almost white crystals.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in fatty oils.

It is sensitive to air, heat and light.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of calcitriol.*

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Carry out the test as rapidly as possible, avoiding exposure to actinic light and air.*

*Test solution.* Dissolve 1.00 mg of the substance to be examined without heating in 10.0 mL of the mobile phase.

*Reference solution (a).* Dissolve 1.00 mg of *calcitriol CRS* without heating in 10.0 mL of the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Heat 2 mL of reference solution (a) at 80 °C for 30 min.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 450 volumes of a 1.0 g/L solution of *tris(hydroxymethyl)aminomethane R* adjusted to pH 7.0-7.5 with *phosphoric acid R*, and 550 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 50 µL.

*Run time*: twice the retention time of calcitriol.

*Relative retention* with reference to calcitriol (retention time = about 14 min): impurity C = about 0.4; pre-calcitriol = about 0.88; impurity A = about 0.95; impurity B = about 1.1.

*System suitability*:
- *resolution*: minimum 3.5 between the peaks due to pre-calcitriol and calcitriol in the chromatogram obtained with reference solution (c);
- *number of theoretical plates*: minimum 10 000, calculated for the peak due to calcitriol in the chromatogram obtained with reference solution (a).

*Limits*:
- *impurities A, B, C*: for each impurity, maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to pre-calcitriol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):
- *repeatability*: maximum relative standard deviation of 1 per cent for the peak due to calcitriol after 6 injections.

Calculate the percentage content of $C_{27}H_{44}O_3$ taking into account the assigned content of *calcitriol CRS* and, if necessary, the peak due to pre-calcitriol.

STORAGE

Under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

IMPURITIES

*Specified impurities: A, B, C.*

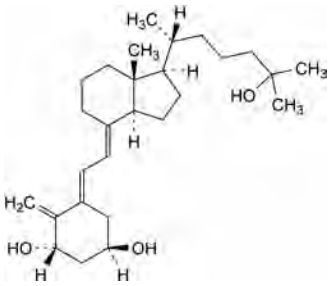

A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1α,3β,25-triol (*trans*-calcitriol),

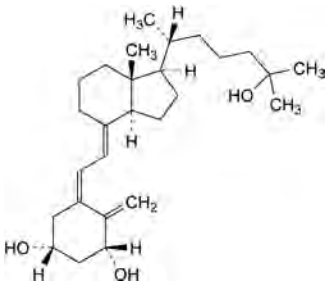

B. (5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1β,3β,25-triol (1β-calcitriol),

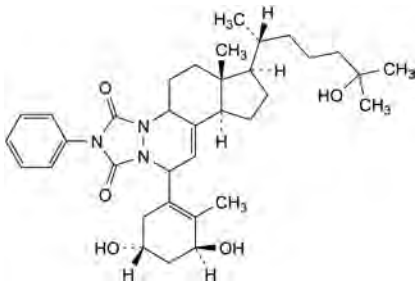

C. (6a*R*,7*R*,9a*R*)-11-[(3*S*,5*R*)-3,5-dihydroxy-2-methylcyclohex-1-enyl]-7-[(1*R*)-5-hydroxy-1,5-dimethylhexyl]-6a-methyl-2-phenyl-5,6,6a,7,8,9,9a,11-octahydro-1*H*,4a*H*-cyclopenta[*f*][1,2,4]triazolo[1,2-*a*]cinnoline-1,3(2*H*)-dione (triazoline adduct of pre-calcitriol).

**01/2017:2128**

# CALCIUM ACETATE

## Calcii acetas

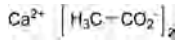

$C_4H_6CaO_4$ $M_r$ 158.2

[62-54-4]

DEFINITION

Calcium diacetate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (b) of calcium (*2.3.1*).

B. It gives reaction (b) of acetates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.2 to 8.2.

Dilute 5.0 mL of solution S to 10.0 mL with *carbon dioxide-free water R*.

**Readily oxidisable substances**. Dissolve 2.0 g in boiling *water R* and dilute to 100 mL with boiling *water R*, add a few glass beads, 6 mL of *5 M sulfuric acid R* and 0.3 mL of *0.02 M potassium permanganate*, mix, boil gently for 5 min and allow the precipitate to settle. The pink colour in the supernatant is not completely discharged.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dissolve 0.15 g in *water R* and dilute to 15 mL with the same solvent.

**Fluorides**: maximum 50 ppm.

Potentiometry (*2.2.36, Method I*).

*Test solution.* In a 50 mL volumetric flask, dissolve 0.200 g in a 10.3 g/L solution of *hydrochloric acid R*, add 5.0 mL of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. To 20.0 mL of the solution add 20.0 mL of *total-ionic-strength-adjustment buffer R* and 3 mL of an 82 g/L solution of *anhydrous sodium acetate R*. Adjust to pH 5.2 with *ammonia R* and dilute to 50.0 mL with *distilled water R*.

*Reference solutions.* To 0.25 mL, 0.5 mL, 0.75 mL and 1.0 mL of *fluoride standard solution (10 ppm F) R* add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

*Indicator electrode*: fluoride selective.

*Reference electrode*: silver-silver chloride.

Take into account the addition of fluoride to the test solution for the calculation.

**Nitrates**. To 10.0 mL of solution S add 5 mg of *sodium chloride R*, 0.05 mL of *indigo carmine solution R* and add with stirring, 10 mL of *nitrogen-free sulfuric acid R*. The blue colour remains for at least 10 min.

**Sulfates** (*2.4.13*): maximum 600 ppm.

Dissolve 0.25 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Aluminium** (*2.4.17*): maximum 1 ppm, if intended for use in the manufacture of peritoneal dialysis solutions, haemofiltration solutions or haemodialysis solutions.

*Test solution.* Dissolve 4.0 g of the substance to be examined in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution.* Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution.* Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Arsenic** (*2.4.2*): maximum 3 ppm.

3.3 mL of solution S complies with test A.

**Barium**: maximum 50 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Dissolve 5.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *barium standard solution (0.1 per cent Ba) R*, diluted as necessary with *water R*.

*Wavelength*: 455.4 nm.

**Iron** (*2.4.9*): maximum 20 ppm, if intended for use in the manufacture of parenteral preparations or haemodialysis solutions.

Dilute 5 mL of solution S to 10 mL of *water R*.

**Magnesium**: maximum 500 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *magnesium standard solution (0.1 per cent Mg) R*, diluted as necessary with *water R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-acetylene flame.

**Potassium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations or haemodialysis solutions.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *potassium standard solution (0.2 per cent K) R*, diluted as necessary with *water R*.

*Wavelength*: 766.5 nm.

**Sodium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations or haemodialysis solutions.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

**Strontium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations or haemodialysis solutions.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* Dissolve 2.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *strontium standard solution (1.0 per cent Sr) R*, diluted as necessary with *water R*.

*Wavelength*: 460.7 nm.

**Water** (*2.5.12*): maximum 7.0 per cent, determined on 0.100 g. Add 2 mL of *anhydrous acetic acid R* to the titration vessel in addition to the methanol. Clean the titration vessel after each determination.

ASSAY

Dissolve 0.150 g in 100 mL of *water R* and carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 15.82 mg of $C_4H_6CaO_4$.

STORAGE

In an airtight container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations, peritoneal dialysis solutions, haemofiltration solutions or haemodialysis solutions.

**01/2017:1182**

# CALCIUM ASCORBATE

## Calcii ascorbas

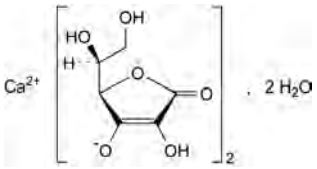


$C_{12}H_{14}CaO_{12},2H_2O$ $M_r$ 426.3
[5743-28-2]

DEFINITION

Calcium di[(*R*)-2-[(*S*)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2*H*-furan-3-olate] dihydrate.

*Content*: 99.0 per cent to 100.5 per cent of $C_{12}H_{14}CaO_{12},2H_2O$.

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of calcium ascorbate.*

C. Dilute 1 mL of solution S (see Tests) to 10 mL with *water R*. To 2 mL of the solution add 0.2 mL of a 100 g/L solution of *ferrous sulfate R*. A deep violet colour develops.

D. To 1 mL of solution S add 0.2 mL of *dilute nitric acid R* and 0.2 mL of *silver nitrate solution R2*. A grey precipitate is formed.

E. The substance gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.00 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*). Examine the colour of the solution immediately after preparation of the solution.

**pH** (*2.2.3*): 6.8 to 7.4 for solution S.

**Specific optical rotation** (*2.2.7*): + 95 to + 97 (dried substance), determined using freshly prepared solution S.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Fluorides**: maximum 10 ppm.

Potentiometry (*2.2.36, Method I*).

*Test solution.* In a 50 mL volumetric flask, dissolve 1.000 g in a 10.3 g/L solution of *hydrochloric acid R*, add 5.0 mL of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. To 20.0 mL of the solution add 20.0 mL of *total-ionic-strength-adjustment buffer R* and 3 mL of an 82 g/L solution of *anhydrous sodium acetate R*. Adjust to pH 5.2 with *ammonia R* and dilute to 50.0 mL with *distilled water R*.

*Reference solutions.* To 0.25 mL, 0.5 mL, 1.0 mL, 2.0 mL and 5.0 mL of *fluoride standard solution (10 ppm F) R* add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

*Indicator electrode*: fluoride selective.

*Reference electrode*: silver-silver chloride.

Take into account the addition of fluoride to the test solution for the calculation.

**Copper**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.0 g in a 9.7 g/L solution of *nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (10 ppm Cu) R*, diluting with a 9.7 g/L solution of *nitric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Atomisation device*: air-acetylene flame.

**Iron**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 5.0 g in a 9.7 g/L solution of *nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (10 ppm Fe) R*, diluting with a 9.7 g/L solution of *nitric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 0.1 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

ASSAY

Dissolve 80.0 mg in a mixture of 10 mL of *dilute sulfuric acid R* and 80 mL of *carbon dioxide-free water R*. Add 1 mL of *starch solution R*. Titrate with *0.05 M iodine* until a persistent violet-blue colour is obtained.

1 mL of *0.05 M iodine* is equivalent to 10.66 mg of $C_{12}H_{14}CaO_{12},2H_2O$.

STORAGE

In a non-metallic container, protected from light.

**01/2017:0014**

# CALCIUM CARBONATE

## Calcii carbonas

$CaCO_3$ $M_r$ 100.1
[471-34-1]

DEFINITION

*Content*: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water.

IDENTIFICATION

A. It gives the reaction of carbonates (*2.3.1*).

B. 0.2 mL of solution S (see Tests) gives the reactions of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 80 mL of *dilute acetic acid R*. When the effervescence ceases, boil for 2 min. Allow to cool, dilute to 100 mL with *dilute acetic acid R* and filter, if necessary, through a sintered-glass filter (*2.1.2*).

**Substances insoluble in acetic acid**: maximum 0.2 per cent.

Wash any residue obtained during the preparation of solution S with 4 quantities, each of 5 mL, of hot *water R* and dry at 100-105 °C for 1 h. The residue weighs a maximum of 10 mg.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dilute 3 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.25 per cent.

Dilute 1.2 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Barium**. To 10 mL of solution S add 10 mL of *calcium sulfate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 10 mL of solution S and 10 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 200 ppm.

Dissolve 50 mg in 5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*.

**Magnesium and alkali metals**: maximum 1.5 per cent.

Dissolve 1.0 g in 12 mL of *dilute hydrochloric acid R*. Boil the solution for about 2 min and add 20 mL of *water R*, 1 g of *ammonium chloride R* and 0.1 mL of *methyl red solution R*. Add *dilute ammonia R1* until the colour of the indicator changes and then add 2 mL in excess. Heat to boiling and add 50 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100 mL with *water R* and filter through a suitable filter. To 50 mL of the filtrate add 0.25 mL of *sulfuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 ± 50 °C. The residue weighs a maximum of 7.5 mg.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 200 ± 10 °C.

ASSAY

Dissolve 0.150 g in a mixture of 3 mL of *dilute hydrochloric acid R* and 20 mL of *water R*. Boil for 2 min, allow to cool and dilute to 50 mL with *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 10.01 mg of $CaCO_3$.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium carbonate used as filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2017:0015**

# CALCIUM CHLORIDE DIHYDRATE

## Calcii chloridum dihydricum

$CaCl_2,2H_2O$ $M_r$ 147.0
[10035-04-8]

DEFINITION

*Content*: 97.0 per cent to 103.0 per cent of $CaCl_2,2H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

B. It gives the reactions of calcium (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of freshly prepared solution S add 0.1 mL of *phenolphthalein solution R*. If the solution is red, not more than 0.2 mL of *0.01 M hydrochloric acid* is required to discharge the colour and if the solution is colourless, not more than 0.2 mL of *0.01 M sodium hydroxide* is required to turn it red.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Aluminium**. To 10 mL of solution S add 2 mL of *ammonium chloride solution R* and 1 mL of *dilute ammonia R1* and boil the solution. No turbidity or precipitate is formed.

If intended for use in the manufacture of dialysis solutions, the above test is replaced by the following test for aluminium (*2.4.17*): maximum 1 ppm.

*Prescribed solution*. Dissolve 4 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Magnesium and alkali metals**: maximum 0.5 per cent.

To a mixture of 20 mL of solution S and 80 mL of *water R* add 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*, heat to boiling and pour into the boiling solution a hot solution of 5 g of *ammonium oxalate R* in 75 mL of *water R*. Allow to stand for 4 h, dilute to 200 mL with *water R* and filter through a suitable filter. To 100 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

ASSAY

Dissolve 0.280 g in 100 mL of *water R* and carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 14.70 mg of $CaCl_2,2H_2O$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

STORAGE

In an airtight container.

**01/2017:0707**

# CALCIUM CHLORIDE HEXAHYDRATE

## Calcii chloridum hexahydricum

$CaCl_2,6H_2O$ $M_r$ 219.1
[7774-34-7]

DEFINITION

*Content*: 97.0 per cent to 103.0 per cent of $CaCl_2,6H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline mass or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It solidifies at about 29 °C.

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

B. It gives the reactions of calcium (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 15.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of freshly prepared solution S add 0.1 mL of *phenolphthalein solution R*. If the solution is red, not more than 0.2 mL of *0.01 M hydrochloric acid* is required to discharge the colour and if the solution is colourless, not more than 0.2 mL of *0.01 M sodium hydroxide* is required to turn it red.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Aluminium**. To 10 mL of solution S add 2 mL of *ammonium chloride solution R* and 1 mL of *dilute ammonia R1*. Heat to boiling. No turbidity or precipitate is formed.

If intended for use in the manufacture of dialysis solutions, the above test is replaced by the following test for aluminium (*2.4.17*): maximum 1 ppm.

*Prescribed solution*. Dissolve 6 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. After at least 15 min, any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 7 ppm, determined on solution S.

**Magnesium and alkali metals**: maximum 0.3 per cent.

To a mixture of 20 mL of solution S and 80 mL of *water R* add 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*, heat to boiling and pour into the boiling solution a hot solution of 5 g of *ammonium oxalate R* in 75 mL of *water R*. Allow to stand for 4 h, dilute to 200 mL with *water R* and filter through a suitable filter. To 100 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

ASSAY

Dissolve 0.200 g in 100 mL of *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.91 mg of $CaCl_2,6H_2O$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

**01/2017:1183**

# CALCIUM DOBESILATE MONOHYDRATE

## Calcii dobesilas monohydricus

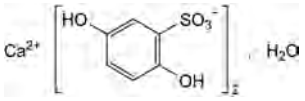


$C_{12}H_{10}CaO_{10}S_2,H_2O$ $M_r$ 436.4
[20123-80-2]

DEFINITION

Calcium di(2,5-dihydroxybenzenesulfonate) monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very soluble in water, freely soluble in anhydrous ethanol, very slightly soluble in 2-propanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 0.100 g in *water R* and dilute to 200.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Spectral range*: 210-350 nm.

*Absorption maxima*: at 221 nm and 301 nm.

*Specific absorbance at the absorption maximum at 301 nm*: 174 to 181.

B. Mix 1 mL of *ferric chloride solution R2*, 1 mL of a freshly prepared 10 g/L solution of *potassium ferricyanide R* and 0.1 mL of *nitric acid R*. To this mixture add 5 mL of freshly prepared solution S (see Tests): a blue colour and a precipitate are immediately produced.

C. 2 mL of freshly prepared solution S gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S, when freshly prepared, is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Keep all solutions at 2-8 °C.*

*Buffer solution*. Dissolve 1.2 g of *anhydrous sodium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, adjust to pH 6.5 with *disodium hydrogen phosphate solution R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 10 mg of the substance to be examined and 10 mg of *hydroquinone R* (impurity A) in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 100 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, buffer solution (10:90 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of dobesilate.

*Relative retention* with reference to dobesilate (retention time = about 6 min): impurity A = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 8.0 between the peaks due to dobesilate and impurity A.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.6;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Iron** (*2.4.9*): maximum 10 ppm, determined on 10 mL of solution S.

**Water** (*2.5.12*): 4.0 per cent to 6.0 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.200 g in a mixture of 10 mL of *water R* and 40 mL of *dilute sulfuric acid R*. Titrate with *0.1 M cerium sulfate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M cerium sulfate* is equivalent to 10.45 mg of $C_{12}H_{10}CaO_{10}S_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

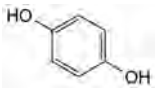

A. benzene-1,4-diol (hydroquinone).

**04/2019:0978**
**corrected 10.0**

# CALCIUM FOLINATE HYDRATE

## Calcii folinas hydricus

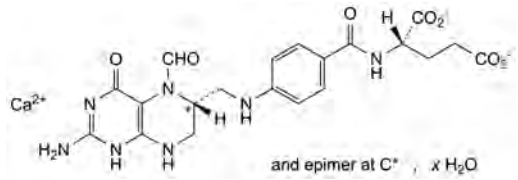

$C_{20}H_{21}CaN_7O_7,xH_2O$ $M_r$ 511.5 (anhydrous substance)
[2060570-47-8]

DEFINITION

Calcium (2*S*)-2-[4-[[[(6*RS*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]-benzamido]pentanedioate hydrate.

*Content*:

– *calcium folinate* ($C_{20}H_{21}CaN_7O_7$): 97.0 per cent to 102.0 per cent (anhydrous substance);
– *calcium* (Ca; $A_r$ 40.08): 7.54 per cent to 8.14 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or light yellow, amorphous or crystalline, hygroscopic powder.

*Solubility*: sparingly soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

The amorphous form may produce supersaturated solutions in water.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *calcium folinate CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R* and add dropwise sufficient *acetone R* to produce a precipitate. Allow to stand for 15 min, collect the precipitate by centrifugation, wash the precipitate with 2 small quantities of *acetone R* and dry. Record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 15 mg of the substance to be examined in a 3 per cent *V/V* solution of *ammonia R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 15 mg of *calcium folinate CRS* in a 3 per cent *V/V* solution of *ammonia R* and dilute to 5 mL with the same solvent.

*Plate*: *cellulose for chromatography $F_{254}$ R* as the coating substance.

*Mobile phase*: the lower layer of a mixture of 1 volume of *isoamyl alcohol R* and 10 volumes of a 50 g/L solution of *citric acid monohydrate R* previously adjusted to pH 8 with *ammonia R*.

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (b) of calcium (*2.3.1*).

TESTS

*Carry out the tests as rapidly as possible, protected from actinic light.*

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R*, heating at 40 °C if necessary, and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 6.8 to 8.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 14.4 to + 18.0 (anhydrous substance), determined on solution S.

**Absorbance** (*2.2.25*): maximum 0.60, determined at 420 nm on solution S.

**Ethanol**. Head-space gas chromatography (*2.2.28*): use the standard additions method.

*Test solution.* Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dilute 0.750 g of *anhydrous ethanol R* to 1000.0 mL with *water R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 10 m, Ø = 0.32 mm;
– *stationary phase*: *styrene-divinylbenzene copolymer R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 4 mL/min.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 80 °C;
– *equilibration time*: 20 min;
– *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 125 → 185 |
| | 6 - 15 | 185 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: at least 3 times.

*Limit*:

– *ethanol*: maximum 3.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (c).* Dissolve 5 mg of *calcium folinate for system suitability CRS* (containing impurities A, E and F) in 5 mL of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 165 mL of *methanol R* and 835 mL of a solution containing 4.0 mL of *tetrabutylammonium dihydrogen phosphate solution R* and 1.42 g of *disodium hydrogen phosphate dihydrate R* in *water for chromatography R*, previously adjusted to pH 7.7 with *phosphoric acid R* or *dilute sodium hydroxide solution R*.

*Flow rate*: 1.25 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3.5 times the retention time of folinic acid.

*Identification of impurities*: use the chromatogram supplied with *calcium folinate for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, E and F.

*Relative retention* with reference to folinic acid (retention time = about 13.9 min): impurity E = about 0.4; impurity A = about 0.6; impurity F = about 0.7.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to impurities A and F.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity E = 0.6; impurity F = 0.6;
- for each impurity, use the concentration of calcium folinate hydrate in reference solution (b).

*Limits*:

- *impurities A, E, F*: for each impurity, maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides**: maximum 0.5 per cent.

Dissolve 0.300 g in 50 mL of *water R* heating at 40 °C if necessary. Add 10 mL of *dilute nitric acid R* and titrate with *0.005 M silver nitrate* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.005 M silver nitrate* is equivalent to 0.177 mg of Cl.

**Water** (*2.5.12*): 10.0 per cent to 17.0 per cent.

Dissolve 0.100 g in a mixture of 15 mL of *formamide R* and 50 mL of the titration solvent. Stir for about 6 min before titrating and use a suitable titrant that does not contain pyridine.

ASSAY

*Carry out the assays as rapidly as possible, protected from actinic light.*

**Calcium**. Dissolve 0.400 g in 150 mL of *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

**Calcium folinate**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{20}H_{21}CaN_7O_7$ taking into account the assigned content of *calcium folinate CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, G, I.*

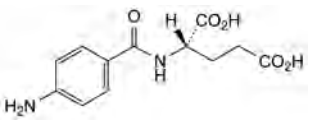

A. (2*S*)-2-(4-aminobenzamido)pentanedioic acid,

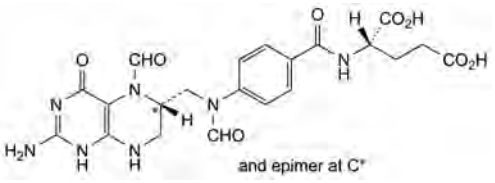


B. (2*S*)-2-[4-[[[(6*RS*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl](formyl)amino]-benzamido]pentanedioic acid (5,10-diformyltetrahydrofolic acid),

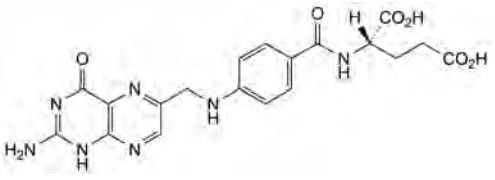

C. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzamido]pentanedioic acid (folic acid),

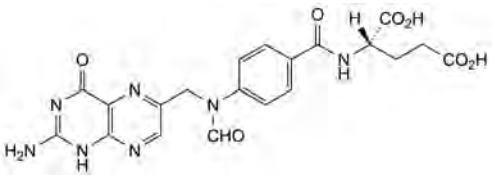

D. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl](formyl)amino]benzamido]pentanedioic acid (10-formylfolic acid),

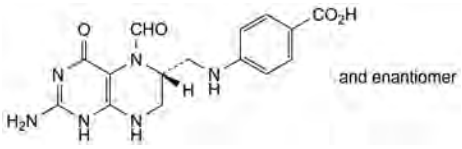


E. 4-[[[(6*RS*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoic acid (5-formyltetrahydropteroic acid),

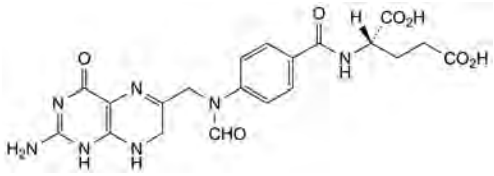

F. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl](formyl)amino]benzamido]pentanedioic acid (10-formyldihydrofolic acid),

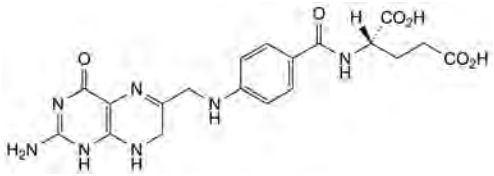

G. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino]benzamido]pentanedioic acid (dihydrofolic acid),

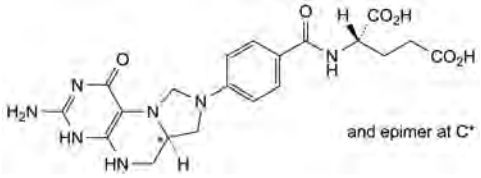


I. (2*S*)-2-[4-[(6a*RS*)-3-amino-1-oxo-1,2,5,6,6a,7-hexahydroimidazo[1,5-*f*]pteridin-8(9*H*)-yl]benzamido]pentanedioic acid ((6a*RS*)-5,10-methylenetetrahydrofolic acid).

**01/2017:1399**
**corrected 10.0**

# CALCIUM GLUCOHEPTONATE

## Calcii glucoheptonas

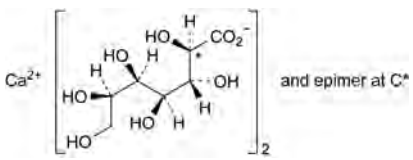


$C_{14}H_{26}CaO_{16}$ $M_r$ 490.4

DEFINITION

Mixture in variable proportions, of calcium di(D-*glycero*-D-*gulo*-heptonate) and calcium di(D-*glycero*-D-*ido*-heptonate).

*Content*: 98.0 per cent to 102.0 per cent of calcium 2,3,4,5,6,7-hexahydroxyheptanoate (dried substance).

CHARACTERS

*Appearance*: white or very slightly yellow, amorphous powder, hygroscopic.

*Solubility*: very soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 1 mL of *water R*.

*Reference solution (a).* Dissolve 20 mg of *calcium glucoheptonate CRS* in 1 mL of *water R*.

*Reference solution (b).* Dissolve 10 mg of *calcium gluconate CRS* in 0.5 mL of the test solution and dilute to 1 mL with *water R*.

*Plate*: *cellulose for chromatography R1* as the coating substance.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *acetone R*, *butanol R* (20:20:30:30 *V/V/V/V*); use a freshly prepared mixture.

*Application*: 10 µL as bands of 20 mm by 2 mm.

*Development*: in a tank previously allowed to saturate for 10 min, over a path of 12 cm.

*Drying*: in air.

*Detection*: spray with *0.02 M potassium permanganate*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. 0.2 mL of solution S (see Tests) gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.0 for solution S.

**Reducing sugars**: maximum 1 per cent, expressed as glucose.

Dissolve 1.0 g in 5 mL of *water R* with the aid of gentle heat. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* until the precipitate dissolves, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R* added towards the end of the titration, as indicator. Not less than 12.6 mL of *0.05 M sodium thiosulfate* is required.

**Cyanide**. Dissolve 5.0 g in 50 mL of *water R* and add 2.0 g of *tartaric acid R*. Place this solution in a distillation apparatus (*2.2.11*). The plain bend adapter attached to the end of the condenser has a vertical part that is long enough to extend to 1 cm from the bottom of a 50 mL test-tube used as a receiver. Place 10 mL of *water R* and 2 mL of *0.1 M sodium hydroxide* into the receiver. Distil, collect 25 mL of distillate and dilute to 50 mL with *water R*. To 25 mL of this solution add 25 mg of *ferrous sulfate R* and boil for a short time. After cooling to about 70 °C add 10 mL of *hydrochloric acid R1*. After 30 min, filter the solution and wash the filter. A yellow spot appears on the filter; there is no blue or green spot.

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 5 mL of solution S, add 10 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Iron** (*2.4.9*): maximum 40 ppm.

Dilute 2.5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 167 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.800 g in a mixture of 2 mL of *3 M hydrochloric acid* and 150 mL of *water R*. While stirring, add 12.5 mL of *0.1 M sodium edetate*, 15 mL of *1 M sodium hydroxide* and 0.3 g of *hydroxynaphthol blue, sodium salt R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 49.04 mg of $C_{14}H_{26}CaO_{16}$.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

**01/2017:0172**

# CALCIUM GLUCONATE

## Calcii gluconas

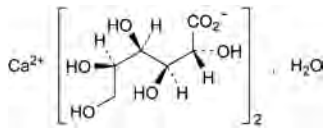

$C_{12}H_{22}CaO_{14},H_2O$ $M_r$ 448.4
[18016-24-5]

DEFINITION

Calcium bis[(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanoate] monohydrate (calcium di(D-gluconate) monohydrate).

*Content*: 98.5 per cent to 102.0 per cent of $C_{12}H_{22}CaO_{14},H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: sparingly soluble in water, freely soluble in boiling water.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Reference solution*. Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for 20 min; allow to cool.

*Detection*: spray with a solution containing 10 g/L of *cerium sulfate R* and 25 g/L of *ammonium molybdate R* in *dilute sulfuric acid R* and heat at 105 °C for about 10 min.

*Results*: after 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Solution S (see Tests) gives the reactions of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *water R* heated to 60 °C and dilute to 50 mL with the same solvent.

**Appearance of solution**. At 60 °C, solution S is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*). After cooling, it is not more opalescent than reference suspension II (*2.2.1*).

**Organic impurities and boric acid**. Introduce 0.5 g into a porcelain dish previously rinsed with *sulfuric acid R* and placed in a bath of iced water. Add 2 mL of cooled *sulfuric acid R* and mix. No yellow or brown colour develops. Add 1 mL of *chromotrope II B solution R*. A violet colour develops and does not become dark blue. The solution is not more intensely coloured than that of a mixture of 1 mL of *chromotrope II B solution R* and 2 mL of cooled *sulfuric acid R*.

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 12.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dissolve 10.0 g with heating in a mixture of 10 mL of *acetic acid R* and 90 mL of *distilled water R*.

**Magnesium and alkali metals**: maximum 0.4 per cent.

Dissolve 1.00 g in 100 mL of boiling *water R*, add 10 mL of *ammonium chloride solution R*, 1 mL of *ammonia R* and, dropwise, 50 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 200 mL with *water R* and filter. Evaporate 100 mL of the filtrate to dryness and ignite. The residue weighs a maximum of 2 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.8000 g in 20 mL of hot *water R*, allow to cool and dilute to 300 mL with *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 44.84 mg of $C_{12}H_{22}CaO_{14},H_2O$.

**01/2017:2364**

# CALCIUM GLUCONATE, ANHYDROUS

## Calcii gluconas anhydricus

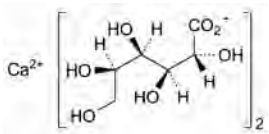

$C_{12}H_{22}CaO_{14}$ $M_r$ 430.4

DEFINITION

Anhydrous calcium D-gluconate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: sparingly soluble in water, freely soluble in boiling water.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Reference solution*. Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for 20 min, then allow to cool.

*Detection*: spray with a solution containing 25 g/L of *ammonium molybdate R* and 10 g/L of *cerium sulfate R* in *dilute sulfuric acid R*, and heat at 100-105 °C for about 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Solution S (see Tests) gives the reactions of calcium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 1.0 g in *water R* heated to 60 °C and dilute to 50 mL with the same solvent.

**Appearance of solution**. At 60 °C, solution S is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*). After cooling, it is not more opalescent than reference suspension II (*2.2.1*).

**Organic impurities and boric acid**. Place 0.5 g in a porcelain dish previously rinsed with *sulfuric acid R* and placed in a bath of iced water. Add 2 mL of cooled *sulfuric acid R* and mix. No yellow or brown colour develops. Add 1 mL of *chromotrope II B solution R*. A violet colour develops and does not become dark blue. Compare the colour obtained with that of a mixture of 1 mL of *chromotrope II B solution R* and 2 mL of cooled *sulfuric acid R*.

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 12.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dissolve 10.0 g with heating in a mixture of 10 mL of *acetic acid R* and 90 mL of *distilled water R*.

**Magnesium and alkali metals**: maximum 0.4 per cent (expressed as MgO).

Dissolve 1.00 g in 100 mL of boiling *water R*, add 10 mL of *ammonium chloride solution R*, 1 mL of *ammonia R* and, dropwise, 50 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 200 mL with *water R* and filter. Evaporate 100 mL of the filtrate to dryness and ignite. The residue weighs a maximum of 2 mg.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 16 h.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.350 g in 20 mL of hot *water R*, allow to cool and dilute to 300 mL with *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 43.04 mg of $C_{12}H_{22}CaO_{14}$.

**01/2017:0979**

# CALCIUM GLUCONATE FOR INJECTION

## Calcii gluconas ad iniectabile

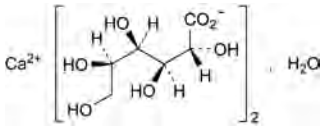

$C_{12}H_{22}CaO_{14},H_2O$ $M_r$ 448.4

[18016-24-5]

DEFINITION

Calcium bis[(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanoate] monohydrate (calcium di(D-gluconate) monohydrate).

*Content*: 99.0 per cent to 101.0 per cent of $C_{12}H_{22}CaO_{14},H_2O$.

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: sparingly soluble in water, freely soluble in boiling water.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Reference solution*. Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for 20 min; allow to cool.

*Detection*: spray with a solution containing 10 g/L of *cerium sulfate R* and 25 g/L of *ammonium molybdate R* in *dilute sulfuric acid R* and heat at 105 °C for about 10 min.

*Results*: after 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. About 20 mg gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. To 10.0 g add 90 mL of boiling *distilled water R* and boil with stirring, for not more than 10 s, until completely dissolved, then dilute to 100.0 mL with the same solvent.

**Appearance of solution**. At 60 °C, solution S is not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*). After cooling to 20 °C, it is not more opalescent than reference suspension II (*2.2.1*).

**pH** (*2.2.3*): 6.4 to 8.3.

Dissolve 1.0 g in 20 mL of *carbon dioxide-free water R*, heating on a water-bath.

**Organic impurities and boric acid**. Introduce 0.5 g into a porcelain dish previously rinsed with *sulfuric acid R* and placed in a bath of iced water. Add 2 mL of cooled *sulfuric acid R* and mix. No yellow or brown colour develops. Add 1 mL of *chromotrope II B solution R*. A violet colour develops and does not become dark blue. The solution is not more intensely coloured than that of a mixture of 1 mL of *chromotrope II B solution R* and 2 mL of cooled *sulfuric acid R*.

**Oxalates**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 1.00 g of the substance to be examined in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Reference solution.* Dissolve 1.00 g of the substance to be examined in *water for chromatography R*, add 0.5 mL of a 0.152 g/L solution of *sodium oxalate R* in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Precolumn*:

- *size*: *l* = 30 mm, Ø = 4 mm;
- *stationary phase*: suitable strong anion-exchange resin (30-50 µm).

*Columns 1 and 2*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: suitable strong anion-exchange resin (30-50 µm).

*Anion-suppresser column*: connected in series with the precolumn and analytical columns and equipped with a micromembrane that separates the mobile phase from the suppressor regeneration solution, flowing countercurrent to the mobile phase.

*Mobile phase*: dissolve 0.212 g of *anhydrous sodium carbonate R* and 63 mg of *sodium hydrogen carbonate R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent.

*Flow rate of the mobile phase*: 2 mL/min.

*Suppressor regeneration solution*: 1.23 g/L solution of *sulfuric acid R* in *water for chromatography R*.

*Flow rate of the suppressor regeneration solution*: 4 mL/min.

*Detection*: conductance.

*Injection*: 50 µL.

*System suitability*: reference solution:

- *repeatability*: maximum relative standard deviation of 2.0 per cent for the area of the peak due to oxalate after 5 injections.

Inject 50 µL of each solution 3 times. Calculate the content of oxalates in parts per million using the following expression:

$$\frac{S_T \times 50}{S_R - S_T}$$

$S_T$ = area of the peak due to oxalate in the chromatogram obtained with the test solution;

$S_R$ = area of the peak due to oxalate in the chromatogram obtained with the reference solution.

*Limit*:

- *oxalates*: maximum 100 ppm.

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 50 ppm.

To 10 mL of previously filtered solution S add 5 mL of *water R*.

**Phosphates** (*2.4.11*): maximum 100 ppm.

Dilute 1 mL of solution S to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 50 ppm, determined on previously filtered solution S.

Prepare the standard using a mixture of 7.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 7.5 mL of *distilled water R*.

**Iron**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Introduce 2.0 g into a 100 mL polytetrafluoroethylene beaker and add 5 mL of *nitric acid R*. Boil, evaporating almost to dryness. Add 1 mL of *strong hydrogen peroxide solution R* and evaporate again almost to dryness. Repeat the hydrogen peroxide treatment until a clear solution is obtained. Using 2 mL of *nitric acid R*, transfer the solution into a 25 mL volumetric flask. Dilute to 25.0 mL with *dilute hydrochloric acid R*. In the same manner, prepare a compensation solution using 0.65 g of *calcium chloride R1* instead of the substance to be examined.

*Reference solutions.* Prepare the reference solutions from *iron standard solution (20 ppm Fe) R*, diluting with *dilute hydrochloric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

Carry out a basic correction using a deuterium lamp.

**Magnesium and alkali metals**: maximum 0.4 per cent.

To 0.50 g add a mixture of 1.0 mL of *dilute acetic acid R* and 10.0 mL of *water R* and rapidly boil, whilst shaking, until completely dissolved. To the boiling solution add 5.0 mL of *ammonium oxalate solution R* and allow to stand for at least 6 h. Filter through a sintered-glass filter (1.6) (*2.1.2*) into a porcelain crucible. Carefully evaporate the filtrate to dryness and ignite. The residue weighs not more than 2 mg.

**Bacterial endotoxins** (*2.6.14*): less than 167 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.350 g in 20 mL of hot *water R*, allow to cool and dilute to 300 mL with *water R*. Carry out the complexometric titration of calcium (*2.5.11*). Use 50 mg of *calconecarboxylic acid triturate R*.

1 mL of *0.1 M sodium edetate* is equivalent to 44.84 mg of $C_{12}H_{22}CaO_{14},H_2O$.

**01/2017:0980**

# CALCIUM GLYCEROPHOSPHATE

## Calcii glycerophosphas

$C_3H_7CaO_6P$ $M_r$ 210.1

DEFINITION

Mixture in variable proportions of the calcium salt of (*RS*)-2,3-dihydroxypropyl phosphate and of 2-hydroxy-1-(hydroxymethyl)ethyl phosphate which may be hydrated.

*Content*: 18.6 per cent to 19.4 per cent of Ca (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Mix 1 g with 1 g of *potassium hydrogen sulfate R* in a test tube fitted with a glass tube. Heat strongly and direct the white vapour towards a piece of filter paper impregnated with a freshly prepared 10 g/L solution of *sodium nitroprusside R*. The filter paper develops a blue colour in contact with *piperidine R*.

B. Ignite 0.1 g in a crucible. Take up the residue with 5 mL of *nitric acid R* and heat on a water-bath for 1 min. Filter. The filtrate gives reaction (b) of phosphates (*2.3.1*).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.5 g at room temperature in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 150 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*).

**Acidity or alkalinity**. To 100 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 1.5 mL of *0.1 M hydrochloric acid* or 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Citric acid**. Shake 5.0 g with 20 mL of *carbon dioxide-free water R* and filter. To the filtrate add 0.15 mL of *sulfuric acid R* and filter again. To the filtrate add 5 mL of *mercuric sulfate solution R* and heat to boiling. Add 0.5 mL of a 3.2 g/L solution of *potassium permanganate R* and again heat to boiling. No precipitate is formed.

**Glycerol and ethanol (96 per cent)-soluble substances**: maximum 0.5 per cent.

Shake 1.000 g with 25 mL of *ethanol (96 per cent) R* for 1 min. Filter. Evaporate the filtrate on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs a maximum of 5 mg.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dissolve 0.1 g in a mixture of 2 mL of *acetic acid R* and 8 mL of *water R* and dilute to 15 mL with *water R*.

**Phosphates** (*2.4.11*): maximum 400 ppm.

Dilute 2.5 mL of solution S to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent, determined on solution S.

**Arsenic** (*2.4.2, Method A*): maximum 3 ppm.

Dissolve 0.33 g in *water R* and dilute to 25 mL with the same solvent.

**Iron** (*2.4.9*): maximum 50 ppm, detemined on 0.20 g.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g by drying in an oven at 150 °C for 4 h.

ASSAY

Dissolve 0.200 g in *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

**01/2019:0981**

# CALCIUM HYDROGEN PHOSPHATE(1)

## Calcii hydrogenophosphas

$CaHPO_4$ $M_r$ 136.1
[7757-93-9]

DEFINITION

*Content*: 97.5 per cent to 102.5 per cent.

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder, or colourless crystals.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute hydrochloric acid and in dilute nitric acid.♦

IDENTIFICATION

A. Dissolve with heating 0.1 g in 10 mL of *dilute hydrochloric acid R*. Add 2.5 mL of *dilute ammonia R1*, shake, and add 5 mL of a 35 g/L solution of *ammonium oxalate R*. A white precipitate is produced.

B. Dissolve 0.1 g in 5 mL of *dilute nitric acid R*, add 2 mL of *ammonium molybdate solution R* and heat at 70 °C for 1-2 min. A yellow precipitate is produced.

◊C. It complies with the limits of the assay.◊

TESTS

♦**Solution S**. Dissolve 2.5 g in 20 mL of *dilute hydrochloric acid R*, filter if necessary and add *dilute ammonia R1* until a precipitate is formed. Add just sufficient *dilute hydrochloric acid R* to dissolve the precipitate and dilute to 50 mL with *distilled water R*.♦

**Acid-insoluble substances**: maximum 0.2 per cent.

Dissolve 5.0 g in 40 mL of *water R*, add 10 mL of *hydrochloric acid R* and heat to boiling for 5 min. Cool, then collect the insoluble substances using ashless filter paper. Wash with *water R* until turbidity is no longer produced when *silver nitrate solution R2* is added. Ignite the residue and the filter paper at 600 ± 50 °C. The residue weighs not more than 10 mg.

**Carbonates**. Shake 1.0 g with 5 mL of *carbon dioxide-free water R* and add 2 mL of *hydrochloric acid R*. No effervescence is produced.

**Chlorides**: maximum 0.25 per cent.

*Test solution*. Dissolve 0.20 g in a mixture of 20 mL of *water R* and 13 mL of *dilute nitric acid R* by warming if necessary, dilute to 100 mL with *water R* and filter if necessary. Use 50 mL of this solution.

*Reference solution*. To 0.70 mL of *0.01 M hydrochloric acid*, add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*.

Add 1 mL of *silver nitrate solution R2* to the test solution and to the reference solution and mix. After standing for 5 min protected from light, any opalescence in the test solution, by viewing vertically or horizontally against a black background, is not more intense than that in the reference solution.

♦**Fluorides**: maximum 100 ppm.

Potentiometry (*2.2.36, Method II*).

*Chelating solution*. Dissolve 45 g of *cyclohexylenedinitrilotetraacetic acid R* in 75 mL of *sodium hydroxide solution R* and dilute to 250 mL with *water R*.

(1) This monograph has undergone the process of Pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*Test solution.* Dissolve 1.000 g in 4 mL of *hydrochloric acid R1*, add 20 mL of chelating solution, 2.7 mL of *glacial acetic acid R* and 2.8 g of *sodium chloride R*, adjust to pH 5-6 with *sodium hydroxide solution R* and dilute to 50.0 mL with *water R*.

*Reference solution.* Dissolve 4.42 g of *sodium fluoride R*, previously dried at 300 °C for 12 h, in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 50.0 mL of this solution to 500.0 mL with *total-ionic-strength-adjustment buffer R* (200 ppm F).

*Indicator electrode*: fluoride-selective.

*Reference electrode*: silver-silver chloride.

Carry out the measurement on 20.0 mL of the test solution. Add at least 3 times 0.10 mL of the reference solution and carry out the measurement after each addition. Calculate the concentration of fluorides using the calibration curve. ♦

**Sulfates**: maximum 0.5 per cent.

*Test solution.* Dissolve 0.5 g in a mixture of 5 mL of *water R* and 5 mL of *dilute hydrochloric acid R* and dilute to 100 mL with *water R*. Filter if necessary. To 20 mL of this solution, add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*.

*Reference solution.* To 1.0 mL of *0.005 M sulfuric acid*, add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Filter if necessary.

To the test solution and to the reference solution, add 2 mL of a 120 g/L solution of *barium chloride R* and allow to stand for 10 min. Any opalescence in the test solution, by viewing vertically or horizontally against a black background, is not more intense than that in the reference solution.

♦ **Arsenic** (*2.4.2, Method A*): maximum 10 ppm, determined on 2 mL of solution S. ♦

**Barium**. To 0.5 g, add 10 mL of *water R* and heat to boiling. While stirring, add 1 mL of *hydrochloric acid R* dropwise. Allow to cool and filter if necessary. Add 2 mL of a 10 g/L solution of *dipotassium sulfate R* and allow to stand for 10 min. No turbidity is produced.

♦ **Iron** (*2.4.9*): maximum 400 ppm.

Dilute 0.5 mL of solution S to 10 mL with *water R*. ♦

**Loss on ignition**: 6.6 per cent to 8.7 per cent, determined on 1.000 g to constant mass at 800-825 °C.

ASSAY

Dissolve 0.4 g in 12 mL of *dilute hydrochloric acid R* by heating on a water bath if necessary and dilute to 200.0 mL with *water R*. To 20.0 mL of this solution add 25.0 mL of *0.02 M sodium edetate*, 50 mL of *water R*, 5 mL of *ammonium chloride buffer solution pH 10.7 R* and about 25 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with *0.02 M zinc sulfate*. Carry out a blank titration.

1 mL of *0.02 M sodium edetate* is equivalent to 2.721 mg of $CaHPO_4$.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium hydrogen phosphate used as filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**Powder flow** (*2.9.36*).

**01/2017:0116**

# CALCIUM HYDROGEN PHOSPHATE DIHYDRATE

## Calcii hydrogenophosphas dihydricus

$CaHPO_4,2H_2O$ $M_r$ 172.1
[7789-77-7]

DEFINITION

*Content*: 98.0 per cent to 105.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute hydrochloric acid and in dilute nitric acid.

IDENTIFICATION

A. Dissolve with heating 0.1 g in 10 mL of *dilute hydrochloric acid R*. Add 2.5 mL of *dilute ammonia R1*, shake and add 5 mL of a 35 g/L solution of *ammonium oxalate R*. A white precipitate is produced.

B. Dissolve 0.1 g in 5 mL of *dilute nitric acid R*, add 2 mL of *ammonium molybdate solution R* and heat at 70 °C for 2 min. A yellow precipitate is produced.

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.5 g in 20 mL of *dilute hydrochloric acid R*, filter if necessary and add *dilute ammonia R1* until a precipitate is formed. Add just sufficient *dilute hydrochloric acid R* to dissolve the precipitate and dilute to 50 mL with *distilled water R*.

**Acid-insoluble substances**: maximum 0.2 per cent.

Dissolve 5.0 g in 40 mL of *water R*, add 10 mL of *hydrochloric acid R* and heat to boiling for 5 min. Cool, then collect the insoluble substances using ashless filter paper. Wash with *water R* until turbidity is no longer produced when *silver nitrate solution R2* is added to the filtrate. Ignite at 600 ± 50 °C. The residue weighs not more than 10 mg.

**Carbonates**. Shake 0.5 g with 5 mL of *carbon dioxide-free water R* and add 1 mL of *hydrochloric acid R*. No effervescence is produced.

**Chlorides**: maximum 0.25 per cent.

*Test solution.* Dissolve 0.20 g in a mixture of 20 mL of *water R* and 13 mL of *dilute nitric acid R* by warming if necessary, dilute to 100 mL with *water R* and filter if necessary. Use 50 mL of this solution.

*Reference solution.* To 0.70 mL of *0.01 M hydrochloric acid*, add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*.

Add 1 mL of *silver nitrate solution R2* to the test solution and to the reference solution and mix. After standing for 5 min protected from light, any opalescence in the test solution is not more intense than that in the reference solution.

**Fluorides**: maximum 100 ppm.

Potentiometry (*2.2.36, Method II*).

*Chelating solution*. Dissolve 45 g of *cyclohexylenedinitrilotetraacetic acid R* in 75 mL of *sodium hydroxide solution R* and dilute to 250 mL with *water R*.

*Test solution*. Dissolve 1.000 g in 4 mL of *hydrochloric acid R1*, add 20 mL of chelating solution, 2.7 mL of *glacial acetic acid R* and 2.8 g of *sodium chloride R*, adjust to pH 5-6 with *sodium hydroxide solution R* and dilute to 50.0 mL with *water R*.

*Reference solution*. Dissolve 4.42 g of *sodium fluoride R*, previously dried at 300 °C for 12 h, in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 50.0 mL of this solution to 500.0 mL with *total-ionic-strength-adjustment buffer R* (200 ppm F).

*Indicator electrode*: fluoride-selective.

*Reference electrode*: silver-silver chloride.

Carry out the measurement on 20.0 mL of the test solution. Add at least 3 times 0.10 mL of the reference solution and carry out the measurement after each addition. Calculate the concentration of fluorides using the calibration curve.

**Sulfates**: maximum 0.5 per cent.

*Test solution*. Dissolve 0.5 g in a mixture of 5 mL of *water R* and 5 mL of *dilute hydrochloric acid R* and dilute to 100 mL with *water R*. Filter if necessary. To 20 mL of this solution, add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*.

*Reference solution*. To 1.0 mL of *0.005 M sulfuric acid*, add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Filter if necessary.

To the test solution and to the reference solution, add 2 mL of a 120 g/L solution of *barium chloride R* and allow to stand for 10 min. Any opalescence in the test solution is not more intense than that in the reference solution.

**Arsenic** (*2.4.2, Method A*): maximum 10 ppm, determined on 2 mL of solution S.

**Barium**. To 0.5 g, add 10 mL of *water R* and heat to boiling. While stirring, add 1 mL of *hydrochloric acid R* dropwise. Allow to cool and filter if necessary. Add 2 mL of a 10 g/L solution of *dipotassium sulfate R* and allow to stand for 10 min. No turbidity is produced.

**Iron** (*2.4.9*): maximum 400 ppm.

Dilute 0.5 mL of solution S to 10 mL with *water R*.

**Loss on ignition**: 24.5 per cent to 26.5 per cent, determined on 1.000 g by ignition to constant mass at 800-825 °C.

ASSAY

Dissolve 0.4 g in 12 mL of *dilute hydrochloric acid R* by heating on a water bath if necessary and dilute to 200 mL with *water R*. To 20.0 mL of this solution add 25.0 mL of *0.02 M sodium edetate*, 50 mL of *water R*, 5 mL of *ammonium chloride buffer solution pH 10.7 R* and about 25 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with *0.02 M zinc sulfate*. Carry out a blank titration.

1 mL of *0.02 M sodium edetate* is equivalent to 3.44 mg of $CaHPO_4,2H_2O$.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium hydrogen phosphate dihydrate used as filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**Powder flow** (*2.9.36*).

**01/2017:1078**

# CALCIUM HYDROXIDE

## Calcii hydroxidum

$Ca(OH)_2$ $M_r$ 74.1
[1305-62-0]

DEFINITION

*Content*: 95.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: practically insoluble in water.

IDENTIFICATION

A. To 0.80 g in a mortar, add 10 mL of *water R* and 0.5 mL of *phenolphthalein solution R* and mix. The suspension turns red. On addition of 17.5 mL of *1 M hydrochloric acid*, the suspension becomes colourless without effervescing. The red colour occurs again when the mixture is triturated for 1 min. On addition of a further 6 mL of *1 M hydrochloric acid* and triturating, the solution becomes colourless.

B. Dissolve about 0.1 g in *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. 5 mL of the solution give reaction (b) of calcium (*2.3.1*).

TESTS

**Matter insoluble in hydrochloric acid**: maximum 0.5 per cent.

Dissolve 2.0 g in 30 mL of *hydrochloric acid R*. Boil the solution and filter. Wash the residue with hot *water R*. The residue weighs a maximum of 10 mg.

**Carbonates**: maximum 5.0 per cent of $CaCO_3$.

Add 5.0 mL of *1 M hydrochloric acid* to the titrated solution obtained under Assay and titrate with *1 M sodium hydroxide* using 0.5 mL of *methyl orange solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 50.05 mg of $CaCO_3$.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dissolve 0.30 g in a mixture of 2 mL of *nitric acid R* and 10 mL of *water R* and dilute to 30 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.4 per cent.

Dissolve 0.15 g in a mixture of 5 mL of *dilute hydrochloric acid R* and 10 mL of *distilled water R* and dilute to 60 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm.

Dissolve 0.50 g in 5 mL of *brominated hydrochloric acid R* and dilute to 50 mL with *water R*. Use 25 mL of this solution.

**Magnesium and alkali metals**: maximum 4.0 per cent calculated as sulfates.

Dissolve 1.0 g in a mixture of 10 mL of *hydrochloric acid R* and 40 mL of *water R*. Boil and add 50 mL of a 63 g/L solution of *oxalic acid R*. Neutralise with *ammonia R* and dilute to

200 mL with *water R*. Allow to stand for 1 h and filter through a suitable filter. To 100 mL of the filtrate, add 0.5 mL of *sulfuric acid R*. Cautiously evaporate to dryness and ignite. The residue weighs a maximum of 20 mg.

ASSAY

To 1.500 g in a mortar, add 20-30 mL of *water R* and 0.5 mL of *phenolphthalein solution R*. Titrate with *1 M hydrochloric acid* by triturating the substance until the red colour disappears. The final solution is used in the tests for carbonates.

1 mL of *1 M hydrochloric acid* is equivalent to 37.05 mg of $Ca(OH)_2$.

**01/2017:2118**

# CALCIUM LACTATE

## Calcii lactas

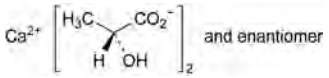


$C_6H_{10}CaO_6$ $M_r$ 218.2

DEFINITION

Calcium bis(2-hydroxypropanoate) or mixture of calcium (2*R*)-, (2*S*)- and (2*RS*)-2-hydroxypropanoates.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: soluble in water, freely soluble in boiling water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Loss on drying (see Tests).

B. It gives the reaction of lactates (*2.3.1*).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is colourless. Not more than 2.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Magnesium and alkali salts**: maximum 1 per cent.

To 20 mL of solution S add 20 mL of *water R*, 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*. Heat to boiling and rapidly add 40 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100.0 mL with *water R* and filter. To 50.0 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness and ignite the residue to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve 0.200 g in *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.82 mg of $C_6H_{10}CaO_6$.

**01/2017:2117**

# CALCIUM LACTATE MONOHYDRATE

## Calcii lactas monohydricus

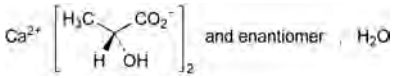


$C_6H_{10}CaO_6,H_2O$ $M_r$ 236.0

DEFINITION

Calcium bis(2-hydroxypropanoate) or mixture of calcium (2*R*)-, (2*S*)- and (2*RS*)-2-hydroxypropanoates monohydrates.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: soluble in water, freely soluble in boiling water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Loss on drying (see Tests).

B. It gives the reaction of lactates (*2.3.1*).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.4 g (equivalent to 5.0 g of the dried substance) with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is colourless. Not more than 2.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Magnesium and alkali salts**: maximum 1 per cent.

To 20 mL of solution S add 20 mL of *water R*, 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*. Heat to boiling and rapidly add 40 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100.0 mL with *water R* and filter. To 50.0 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness and ignite the residue to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

**Loss on drying** (*2.2.32*): 5.0 per cent to 8.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve a quantity equivalent to 0.200 g of the dried substance in *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.82 mg of $C_6H_{10}CaO_6$.

**01/2017:0468**

# CALCIUM LACTATE PENTAHYDRATE

## Calcii lactas pentahydricus

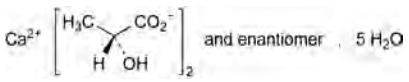


$C_6H_{10}CaO_6,5H_2O$ $M_r$ 308.3

DEFINITION

Calcium bis(2-hydroxypropanoate) or mixture of calcium (2*R*)-, (2*S*)- and (2*RS*)-2-hydroxypropanoates pentahydrates.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder, slightly efflorescent.

*Solubility*: soluble in water, freely soluble in boiling water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Loss on drying (see Tests).

B. It gives the reaction of lactates (*2.3.1*).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 7.1 g (equivalent to 5.0 g of the dried substance) with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is colourless. Not more than 2.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Magnesium and alkali salts**: maximum 1 per cent.

To 20 mL of solution S add 20 mL of *water R*, 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*. Heat to boiling and rapidly add 40 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100.0 mL with *water R* and filter. To 50.0 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness and ignite the residue to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

**Loss on drying** (*2.2.32*): 22.0 per cent to 27.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve a quantity equivalent to 0.200 g of the dried substance in *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.82 mg of $C_6H_{10}CaO_6$.

**01/2017:0469**

# CALCIUM LACTATE TRIHYDRATE

## Calcii lactas trihydricus

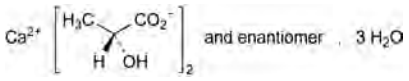


$C_6H_{10}CaO_6,3H_2O$ $M_r$ 272.3

DEFINITION

Calcium bis(2-hydroxypropanoate) or mixture of calcium (2*R*)-, (2*S*)- and (2*RS*)-2-hydroxypropanoates trihydrates.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: soluble in water, freely soluble in boiling water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Loss on drying (see Tests).

B. It gives the reaction of lactates (*2.3.1*).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 6.2 g (equivalent to 5.0 g of the dried substance) with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is colourless. Not more than 2.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Barium**. To 10 mL of solution S add 1 mL of *calcium sulfate solution R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of *distilled water R* and 10 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Magnesium and alkali salts**: maximum 1 per cent.

To 20 mL of solution S add 20 mL of *water R*, 2 g of *ammonium chloride R* and 2 mL of *dilute ammonia R1*. Heat to boiling and rapidly add 40 mL of hot *ammonium oxalate solution R*. Allow to stand for 4 h, dilute to 100.0 mL with *water R* and filter. To 50.0 mL of the filtrate add 0.5 mL of *sulfuric acid R*. Evaporate to dryness and ignite the residue to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5 mg.

**Loss on drying** (*2.2.32*): 15.0 per cent to 20.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve a quantity equivalent to 0.200 g of the dried substance in *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.82 mg of $C_6H_{10}CaO_6$.

**04/2019:1606**
**corrected 10.0**

# CALCIUM LEVOFOLINATE HYDRATE

## Calcii levofolinas hydricus

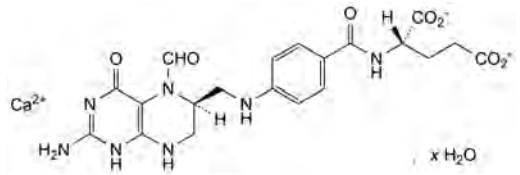


$C_{20}H_{21}CaN_7O_7,xH_2O$ $M_r$ 511.5 (anhydrous substance)

DEFINITION

Calcium (2*S*)-2-[4-[[[(6*S*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]-benzamido]pentanedioate hydrate.

*Content*:

- *calcium levofolinate* ($C_{20}H_{21}CaN_7O_7$; $M_r$ 511.5): 97.0 per cent to 102.0 per cent (anhydrous substance);
- *calcium* (Ca; $A_r$ 40.08): 7.54 per cent to 8.14 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or light yellow, amorphous or crystalline, hygroscopic powder.

*Solubility*: slightly soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison: calcium folinate CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R* and add dropwise sufficient *acetone R* to produce a precipitate. Allow to stand for 15 min, collect the precipitate by centrifugation, wash the precipitate twice with a minimum quantity of *acetone R* and dry. Record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 15 mg of the substance to be examined in a 3 per cent *V/V* solution of *ammonia R* and dilute to 5 mL with the same solvent.

*Reference solution*. Dissolve 15 mg of *calcium folinate CRS* in a 3 per cent *V/V* solution of *ammonia R* and dilute to 5 mL with the same solvent.

*Plate*: *cellulose for chromatography $F_{254}$ R* as the coating substance.

*Mobile phase*: the lower layer of a mixture of 1 volume of *isoamyl alcohol R* and 10 volumes of a 50 g/L solution of *citric acid monohydrate R* previously adjusted to pH 8 with *ammonia R*.

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (b) of calcium (*2.3.1*).

TESTS

*Carry out the tests and the assay as rapidly as possible, protected from actinic light.*

**Solution S**. Dissolve 0.40 g in *carbon dioxide-free water R*, heating at 40 °C if necessary, and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 7.5 to 8.5 for solution S.

**Specific optical rotation** (*2.2.7*): – 15.0 to – 10.0 (anhydrous substance), measured at 25 °C.

Dissolve 0.200 g in *tris(hydroxymethyl)aminomethane solution R* previously adjusted to pH 8.1 with *sodium hydroxide solution R* or *hydrochloric acid R1* and dilute to 20.0 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.25, determined at 420 nm on solution S.

**Ethanol**. Head-space gas chromatography (*2.2.28*): use the standard additions method.

*Test solution*. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dilute 0.750 g of *anhydrous ethanol R* to 1000.0 mL with *water R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 10 m, Ø = 0.32 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 4 mL/min.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 80 °C;
- *equilibration time*: 20 min;
- *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 80 → 220 |
| Injection port | | 110 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: at least 3 times.

*Limit*:

– *ethanol*: maximum 3.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (c).* Dissolve 5 mg of *calcium folinate for system suitability CRS* (containing impurities A, E, F and I) in 5 mL of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 165 mL of *methanol R* and 835 mL of a solution containing 4.0 mL of *tetrabutylammonium dihydrogen phosphate solution R* and 1.42 g of *disodium hydrogen phosphate dihydrate R* in *water for chromatography R*, previously adjusted to pH 7.7 with *phosphoric acid R* or *dilute sodium hydroxide solution R*.

*Flow rate*: 1.25 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 4 times the retention time of folinic acid.

*Identification of impurities*: use the chromatogram supplied with *calcium folinate for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, E, F and I.

*Relative retention* with reference to folinic acid (retention time = about 13.9 min): impurity E = about 0.4; impurity A = about 0.6; impurity F = about 0.7; impurity I = about 1.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the peaks due to impurities A and F.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity E = 0.6; impurity F = 0.6; impurity I = 0.6;
– for each impurity, use the concentration of calcium levofolinate hydrate in reference solution (b).

*Limits*:

– *impurities A, E*: for each impurity, maximum 0.3 per cent;
– *impurities F, I*: for each impurity, maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.20 per cent;
– *total*: maximum 1.5 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Impurity H**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *calcium folinate CRS* in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4 mm;
– *stationary phase*: *human albumin coated silica gel for chiral separation R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: dissolve 9.72 g of *sodium dihydrogen phosphate R* in 890 mL of *water for chromatography R* and adjust to pH 5.0 with *sodium hydroxide solution R*; add 100 mL of *2-propanol R* and 10 mL of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 286 nm.

*Injection*: 10 µL.

*Retention times*: levofolinic acid = about 9 min; impurity H = about 19 min.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to levofolinic acid and impurity H in the chromatogram obtained with reference solution (a). The sum of the areas of the 2 peaks is 100 per cent. The peak area of impurity H is 48 per cent to 52 per cent. In the chromatogram obtained with reference solution (b) 2 clearly visible peaks are obtained.

*Limit*:

– *impurity H*: maximum 0.5 per cent.

**Chlorides**: maximum 0.5 per cent.

Dissolve 0.300 g in 50 mL of *water R* heating at 40 °C if necessary. Add 10 mL of *dilute nitric acid R* and titrate with *0.005 M silver nitrate* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.005 M silver nitrate* is equivalent to 0.177 mg of Cl.

**Water** (*2.5.12*): 10.0 per cent to 17.0 per cent.

Dissolve 0.100 g in a mixture of 15 mL of *formamide R* and 50 mL of the titration solvent. Stir for about 6 min before titrating and use a suitable titrant that does not contain pyridine.

## ASSAY

*Carry out the assays as rapidly as possible, protected from actinic light.*

**Calcium**. Dissolve 0.400 g in 150 mL of *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

**Calcium folinate**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{20}H_{21}CaN_7O_7$ taking into account the assigned content of *calcium folinate CRS*.

## STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, E, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, G.*

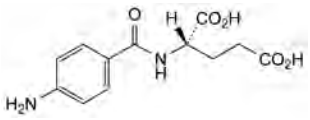

A. (2*S*)-2-(4-aminobenzamido)pentanedioic acid,

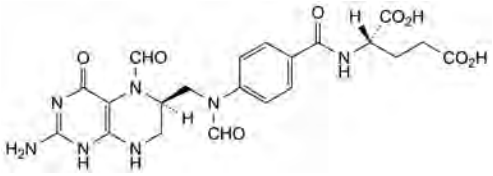

B. (2*S*)-2-[4-[[[(6*R*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl](formyl)amino]-benzamido]pentanedioic acid (5,10-diformyl-tetrahydrolevofolic acid),

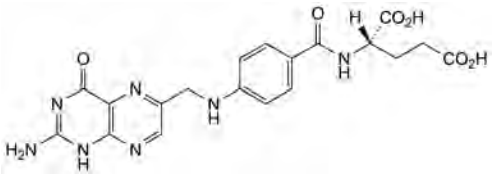

C. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzamido]pentanedioic acid (folic acid),

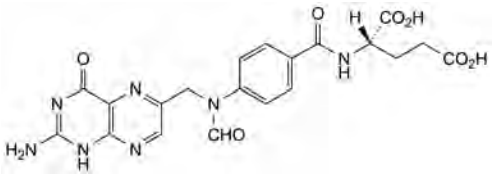

D. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl](formyl)amino]benzamido]pentanedioic acid (10-formylfolic acid),

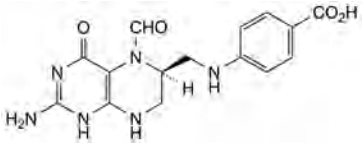

E. 4-[[[(6*S*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzoic acid ((6*S*)-5-formyltetrahydropteroic acid),

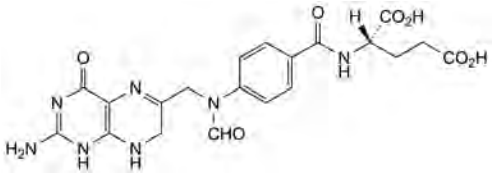

F. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl](formyl)amino]benzamido]pentanedioic acid (10-formyldihydrofolic acid),

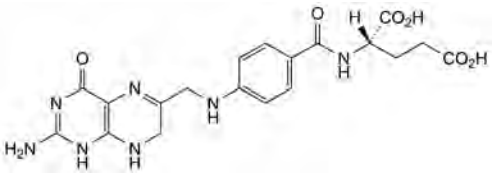

G. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl)methyl]amino]benzamido]pentanedioic acid (dihydrofolic acid),

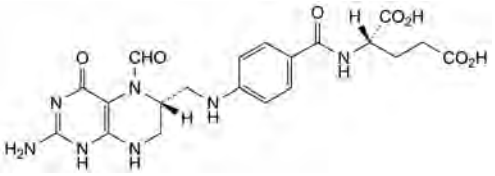

H. (2*S*)-2-[4-[[[(6*R*)-2-amino-5-formyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl]methyl]amino]benzamido]-pentanedioic acid (dextrofolinic acid),

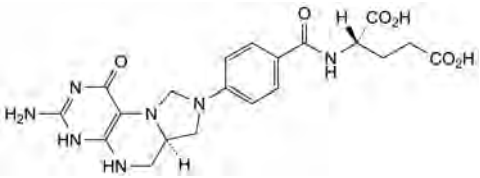

I. (2*S*)-2-[4-[(6a*R*)-3-amino-1-oxo-1,2,5,6,6a,7-hexahydro-imidazo[1,5-*f*]pteridin-8(9*H*)-yl]benzamido]pentanedioic acid ((6a*R*)-5,10-methylenetetrahydrofolic acid).

**01/2017:1296**

# CALCIUM LEVULINATE DIHYDRATE

## Calcii laevulinas dihydricus

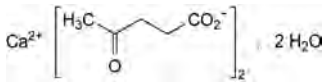

$C_{10}H_{14}CaO_6,2H_2O$ $M_r$ 306.3

[5743-49-7]

DEFINITION

Calcium di(4-oxopentanoate) dihydrate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *calcium levulinate dihydrate CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 60 mg of the substance to be examined in *water R* and dilute to 1 mL with the same solvent.

*Reference solution.* Dissolve 60 mg of *calcium levulinate dihydrate CRS* in *water R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 20 min and allow to cool.

*Detection*: spray with a 30 g/L solution of *potassium permanganate R*. Dry in a current of warm air for about 5 min or until the spots become yellow. Examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 1 mL of solution S (see Tests), add 20 mL of a 2.5 g/L solution of *dinitrophenylhydrazine R* in *dilute hydrochloric acid R*. Allow to stand for 15 min. Filter, wash the precipitate with *water R*. Dry the precipitate in an oven at 100-105 °C. The melting point (*2.2.14*) is 203 °C to 210 °C.

D. It gives reaction (b) of calcium (*2.3.1*).

E. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.8 to 7.8 for solution S.

**Oxidisable substances**. To 1 mL of solution S, add 10 mL of *water R*, 1 mL of *dilute sulfuric acid R* and 0.25 mL of a 3.0 g/L solution of *potassium permanganate R*. Mix. After 5 min, the violet colour of the mixture is still visible.

**Sucrose and reducing sugars**. To 5 mL of solution S add 2 mL of *hydrochloric acid R1* and dilute to 10 mL with *water R*. Heat to boiling for 5 min and allow to cool. Add 10 mL of *sodium carbonate solution R*. Allow to stand for 5 min, dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and heat to boiling for 1 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Magnesium and alkali metals**: maximum 1.0 per cent.

To 10 mL of solution S, add 80 mL of *water R*, 10 mL of *ammonium chloride solution R* and 1 mL of *ammonia R*. Heat to boiling. To the boiling solution, add dropwise 50 mL of warm *ammonium oxalate solution R*. Allow to stand for 4 h, then dilute to 200 mL with *water R* and filter. To 100 mL of the filtrate, add 0.5 mL of *sulfuric acid R*. Evaporate to dryness on a water-bath and ignite to constant mass at 600 ± 50 °C. The residue weighs a maximum of 5.0 mg.

**Loss on drying** (*2.2.32*): 11.0 per cent to 12.5 per cent, determined on 0.200 g by drying at 105 °C.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 4 mL of a solution containing per millilitre 50 mg of the substance to be examined.

ASSAY

Dissolve 0.240 g in 50 mL of *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 27.03 mg of $C_{10}H_{14}CaO_6$.

STORAGE

Protected from light.

**07/2019:0470**

# CALCIUM PANTOTHENATE

## Calcii pantothenas

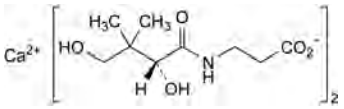


$C_{18}H_{32}CaN_2O_{10}$ $M_r$ 476.5

[137-08-6]

DEFINITION

Calcium bis[3-[(2*R*)-2,4-dihydroxy-3,3-dimethyl-butanamido]propanoate].

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, slightly hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. To 1 mL of solution S (see Tests) add 1 mL of *dilute sodium hydroxide solution R* and 0.1 mL of *copper sulfate solution R*. A blue colour develops.

D. It gives reaction (a) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.8 to 8.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 25.5 to + 27.5 (dried substance), determined on solution S.

**Impurity A and other aminocarboxylic acid impurities**: maximum 0.50 per cent.

Dissolve 8.000 g in 40 mL of *water R* and dilute to 100.0 mL with the same solvent. Add 25 mL of *formaldehyde solution R* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 8.91 mg of $C_3H_7NO_2$.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.600 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 20.0 mg of *calcium pantothenate CRS* in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (c).* Dissolve 3.0 mg of *pantolactone CRS* (impurity C) in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (d).* Mix 1 mL of reference solution (a) and 1.0 mL of reference solution (c) and dilute to 10.0 mL with *water R*.

*Reference solution (e).* Dissolve 3 mg of *pantothenate for peak identification CRS* (containing impurities B, E and H) in 0.5 mL of *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *acetonitrile R1*, 1.56 g/L solution of *sodium dihydrogen phosphate R* in *water for chromatography R*, adjusted to pH 2.5 with *phosphoric acid R* (1:99 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 5 µL of test solution (a) and reference solutions (b), (c), (d) and (e).

*Run time*: 7 times the retention time of calcium pantothenate.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C; use the chromatogram supplied with *pantothenate for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities B, E and H.

*Relative retention* with reference to calcium pantothenate (retention time = about 4 min): impurity B = about 0.5; impurity C = about 0.8; impurity E = about 1.7; impurity H = about 3.5.

*System suitability*: reference solution (d):

- *resolution*: minimum 3.0 between the peaks due to impurity C and calcium pantothenate;
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity C.

*Calculation of percentage contents*:

- for impurities B and C, use the concentration of impurity C in reference solution (c);
- for impurities other than B and C, use the concentration of calcium pantothenate in reference solution (b).

*Limits*:

- *impurity B*: maximum 0.8 per cent;
- *impurity C*: maximum 0.5 per cent;
- *impurity E*: maximum 0.25 per cent;
- *impurity H*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard any peak due to impurity A eluting before 1 min.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.83 mg of $C_{18}H_{32}CaN_2O_{10}$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, E, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, G.*

A. 3-aminopropanoic acid (β-alanine),

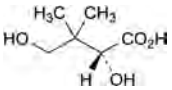

B. (2*R*)-2,4-dihydroxy-3,3-dimethylbutanoic acid (pantoic acid),

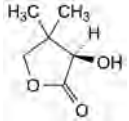

C. (3*R*)-3-hydroxy-4,4-dimethyldihydrofuran-2(3*H*)-one (pantolactone),

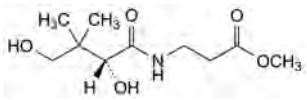

D. methyl 3-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanamido]propanoate (methyl pantothenate),

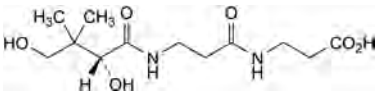

E. 3-[3-[(2*R*)-2,4-dihydroxy-3,3-dimethylbutanamido]propanamido]propanoic acid (β-alanyl pantothenamide),

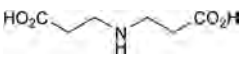

F. 3,3′-azanediyldipropanoic acid,

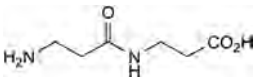

G. 3-(3-aminopropanamido)propanoic acid (β-alanyl-β-alanine),

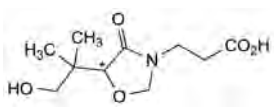

H. 3-[(5Ξ)-5-(1-hydroxy-2-methylpropan-2-yl)-4-oxo-1,3-oxazolidin-3-yl]propanoic acid.

**01/2017:1052**

# CALCIUM PHOSPHATE

## Tricalcii phosphas

DEFINITION

Mixture of calcium phosphates.

*Content*: 35.0 per cent to 40.0 per cent of Ca ($A_r$ 40.08).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water. It dissolves in dilute hydrochloric acid and in dilute nitric acid.

IDENTIFICATION

A. Dissolve 0.1 g in 5 mL of a 25 per cent *V/V* solution of *nitric acid R*. The solution gives reaction (b) of phosphates (*2.3.1*).

B. It gives reaction (b) of calcium (*2.3.1*). Filter before adding *potassium ferrocyanide solution R*.

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.50 g in 20 mL of *dilute hydrochloric acid R*. If the solution is not clear, filter it. Add *dilute ammonia R1* dropwise until a precipitate is formed. Dissolve the precipitate by adding *dilute hydrochloric acid R* and dilute to 50 mL with *distilled water R*.

**Chlorides** (*2.4.4*): maximum 0.15 per cent.

Dissolve 0.22 g in a mixture of 1 mL of *nitric acid R* and 10 mL of *water R* and dilute to 100 mL with *water R*.

**Fluorides**: maximum 75 ppm.

Potentiometry (*2.2.36, Method II*).

*Test solution.* Dissolve 0.250 g in *0.1 M hydrochloric acid*, add 5.0 mL of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 mL with *0.1 M hydrochloric acid*. To 20.0 mL of this solution add 20.0 mL of *total-ionic-strength-adjustment buffer R* and 3 mL of an 82 g/L solution of *anhydrous sodium acetate R*. Adjust to pH 5.2 with *ammonia R* and dilute to 50.0 mL with *distilled water R*.

*Reference solution. Fluoride standard solution (10 ppm F) R.*

*Indicator electrode*: fluoride-selective.

*Reference electrode*: silver-silver chloride.

Carry out the measurements on the test solution, then add at least 3 quantities, each of 0.5 mL, of the reference solution, carrying out a measurement after each addition. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 1 mL of solution S to 25 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 400 ppm.

Dilute 0.5 mL of solution S to 10 mL with *water R*.

**Acid-insoluble matter**: maximum 0.2 per cent.

Dissolve 5.0 g in a mixture of 10 mL of *hydrochloric acid R* and 30 mL of *water R*. Filter, wash the residue with *water R* and dry to constant mass at 100-105 °C. The residue weighs a maximum of 10 mg.

**Loss on ignition**: maximum 8.0 per cent, determined on 1.000 g by ignition at 800 ± 50 °C for 30 min.

ASSAY

Dissolve 0.200 g in a mixture of 1 mL of *hydrochloric acid R1* and 5 mL of *water R*. Add 25.0 mL of *0.1 M sodium edetate* and dilute to 200 mL with *water R*. Adjust to about pH 10 with *concentrated ammonia R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and a few milligrams of *mordant black 11 triturate R*. Titrate the excess sodium edetate with *0.1 M zinc sulfate* until the colour changes from blue to violet.

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium phosphate is used as a filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**Powder flow** (*2.9.36*).

**07/2010:0882**
**corrected 9.0**

# CALCIUM STEARATE

## Calcii stearas

[1592-23-0]

DEFINITION

Mixture of calcium salts of different fatty acids consisting mainly of stearic (octadecanoic) acid [$(C_{17}H_{35}COO)_2Ca$; $M_r$ 607] and palmitic (hexadecanoic) acid [$(C_{15}H_{31}COO)_2Ca$; $M_r$ 550.9] with minor proportions of other fatty acids.

*Content*:

– *calcium*: 6.4 per cent to 7.4 per cent ($A_r$ 40.08) (dried substance);

– *stearic acid in the fatty acid fraction*: minimum 40.0 per cent;

– *sum of stearic acid and palmitic acid in the fatty acid fraction*: minimum 90.0 per cent.

CHARACTERS

*Appearance*: fine, white or almost white, crystalline powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Freezing point (*2.2.18*): minimum 53 °C, for the residue obtained in the preparation of solution S (see Tests).

B. Acid value (*2.5.1*): 195 to 210.

Dissolve 0.200 g of the residue obtained in the preparation of solution S in 25 mL of the prescribed mixture of solvents.

C. Examine the chromatograms obtained in the test for fatty acid composition.

*Results*: the retention times of the principal peaks in the chromatogram obtained with the test solution are approximately the same as those of the principal peaks in the chromatogram obtained with the reference solution.

D. Neutralise 5 mL of solution S to *red litmus paper R* using *strong sodium hydroxide solution R*. The solution gives reaction (b) of calcium (*2.3.1*).

TESTS

**Solution S**. To 5.0 g add 50 mL of *peroxide-free ether R*, 20 mL of *dilute nitric acid R* and 20 mL of *distilled water R*. Boil under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 5 mL, of *distilled water R*. Combine the aqueous layers, wash with 15 mL of *peroxide-free ether R* and dilute the aqueous layer to 50 mL with *distilled water R* (solution S). Evaporate the ether layer to dryness and dry the residue at 100-105 °C. Keep the residue for identification tests A and B.

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R* and boil for 1 min with continuous shaking. Cool and filter. To 10 mL of the filtrate add 0.05 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dilute 0.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.3 per cent.

Dilute 0.5 mL of solution S to 15 mL with *distilled water R*.

**Cadmium**: maximum 3 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Place 50.0 mg in a polytetrafluoroethylene digestion bomb and add 0.5 mL of a mixture of 1 volume of *hydrochloric acid R* and 5 volumes of *cadmium- and lead-free nitric acid R*. Allow to digest at 170 °C for 5 h. Allow to cool. Dissolve the residue in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solutions*. Prepare the reference solutions using *cadmium standard solution (10 ppm Cd) R*, diluted if necessary with a 1 per cent *V/V* solution of *hydrochloric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: graphite furnace.

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Use the solution described in the test for cadmium.

*Reference solutions*. Prepare the reference solutions using *lead standard solution (10 ppm Pb) R*, diluted if necessary with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm; 217.0 nm may be used depending on the apparatus.

*Atomisation device*: graphite furnace.

**Nickel**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Use the solution described in the test for cadmium.

*Reference solutions*. Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted if necessary with *water R*.

*Source*: nickel hollow-cathode lamp.

*Wavelength*: 232.0 nm.

*Atomisation device*: graphite furnace.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

**Calcium**. To 0.500 g in a 250 mL conical flask add 50 mL of a mixture of equal volumes of *anhydrous ethanol R* and *butanol R*, 5 mL of *concentrated ammonia R*, 3 mL of *ammonium chloride buffer solution pH 10.0 R*, 30.0 mL of *0.1 M sodium edetate* and 15 mg of *mordant black 11 triturate R*. Heat to 45-50 °C until the solution is clear. Cool and titrate with *0.1 M zinc sulfate* until the colour changes from blue to violet. Carry out a blank titration.

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

**Composition of fatty acids**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 mL of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 mL of *heptane R* through the condenser. Boil under a reflux condenser for 10 min. Allow to cool. Add 20 mL of *saturated sodium chloride solution R*. Shake and allow the layers to separate. Remove about 2 mL of the organic layer and dry over 0.2 g of *anhydrous sodium sulfate R*. Dilute 1.0 mL of the solution to 10.0 mL with *heptane R*.

*Reference solution*. Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of calcium stearate.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 70 |
| | 2 - 36 | 70 → 240 |
| | 36 - 41 | 240 |
| Injection port | | 220 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to methyl stearate: methyl palmitate = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to methyl palmitate and methyl stearate.

Calculate the content of palmitic acid and stearic acid. Disregard the peak due to the solvent.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in*

*the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium stearate used as a lubricant in tablets and capsules.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26, Method I*). Determine the specific surface area in the $P/P_o$ range of 0.05 to 0.15.

*Sample outgassing*: 2 h at 40 °C.

**01/2017:0982**

# CALCIUM SULFATE DIHYDRATE

## Calcii sulfas dihydricus

$CaSO_4,2H_2O$ $M_r$ 172.2
[10101-41-4]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent of $CaSO_4,2H_2O$.

CHARACTERS

*Appearance*: white or almost white fine powder.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Loss on ignition (see Tests).

B. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

C. Solution S gives reaction (a) of calcium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in 50 mL of a 10 per cent *V/V* solution of *hydrochloric acid R* by heating at 50 °C for 5 min. Allow to cool.

**Acidity or alkalinity**. Shake 1.5 g with 15 mL of *carbon dioxide-free water R* for 5 min. Allow to stand for 5 min and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R* and 0.25 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.30 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.2 mL of *methyl red solution R*. The solution is reddish-orange.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Shake 0.5 g with 15 mL of *water R* for 5 min. Allow to stand for 15 min and filter. Dilute 5 mL of the filtrate to 15 mL with *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 10 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 100 ppm.

To 0.25 g add a mixture of 5 mL of *hydrochloric acid R* and 20 mL of *water R*. Heat to boiling, cool and filter.

**Loss on ignition**: 18.0 per cent to 22.0 per cent, determined on 1.000 g by ignition to constant mass at 800 ± 50 °C.

ASSAY

Dissolve 0.150 g in 120 mL of *water R*. Carry out the complexometric titration of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 17.22 mg of $CaSO_4,2H_2O$.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for calcium sulfate dihydrate used as filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**Powder flow** (*2.9.36*).

**01/2015:1400**

# D-CAMPHOR

## D-Camphora

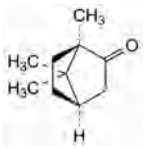


$C_{10}H_{16}O$ $M_r$ 152.2
[464-49-3]

DEFINITION

(1*R*,4*R*)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or friable, crystalline masses.

Highly volatile even at room temperature.

*Solubility*: slightly soluble in water, very soluble in alcohol and in light petroleum, freely soluble in fatty oils, very slightly soluble in glycerol.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 175 °C to 179 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *racemic camphor CRS*.

D. Dissolve 1.0 g in 30 mL of *methanol R*. Add 1.0 g of *hydroxylamine hydrochloride R* and 1.0 g of *anhydrous sodium acetate R*. Boil under a reflux condenser for 2 h. Allow to cool and add 100 mL of *water R*. Filter, wash the

precipitate obtained with 10 mL of *water R* and recrystallise from 10 mL of a mixture of 4 volumes of *alcohol R* and 6 volumes of *water R*. The crystals, dried *in vacuo*, melt (*2.2.14*) at 118 °C to 121 °C.

TESTS

*Carry out the weighings and dissolution rapidly.*

**Solution S**. Dissolve 2.50 g in 10 mL of *alcohol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): + 41.0 to + 44.0, determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 2.50 g of the substance to be examined in *heptane R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *heptane R*.

*Reference solution (b)*. Dilute 10.0 mL of reference solution (a) to 20.0 mL with *heptane R*.

*Reference solution (c)*. Dissolve 0.50 g of *borneol R* in *heptane R* and dilute to 25.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *heptane R*.

*Reference solution (d)*. Dissolve 50 mg of *linalol R* and 50 mg of *bornyl acetate R* in *heptane R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *size*: *l* = 30 m, Ø = 0.25 mm,

– *stationary phase*: *macrogol 20 000 R* (0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:70.

*Flow rate*: 45 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 50 |
| | 10 - 35 | 50 → 100 |
| | 35 - 45 | 100 → 200 |
| | 45 - 55 | 200 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (d).

– *resolution*: minimum 3.0 between the peaks due to bornyl acetate and to linalol.

*Limits*:

– *borneol*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent),

– *any other impurity*: not more than half of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

– *total of other impurities*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent),

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Halogens**: maximum 100 ppm.

Dissolve 1.0 g in 10 mL of *2-propanol R* in a distillation flask. Add 1.5 mL of *dilute sodium hydroxide solution R* and 50 mg of *nickel-aluminium alloy R*. Heat on a water-bath until the *2-propanol R* has evaporated. Allow to cool and add 5 mL of *water R*. Mix and filter through a wet filter previously washed with *water R* until free from chlorides. Dilute the filtrate to 10.0 mL with *water R*. To 5.0 mL of the solution, add *nitric acid R* dropwise until the precipitate which forms is redissolved and dilute to 15 mL with *water R*. The solution complies with the limit test for chlorides (*2.4.4*).

**Residue on evaporation** (*2.8.9*): maximum 0.05 per cent.

Evaporate 2.0 g on a water-bath and dry in an oven at 100-105 °C for 1 h. The residue weighs a maximum of 1 mg.

**Water**. Dissolve 1 g in 10 mL of *light petroleum R*. The solution is clear (*2.2.1*).

IMPURITIES

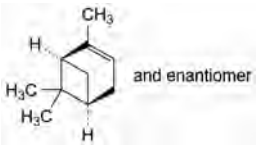


A. 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (α-pinene),

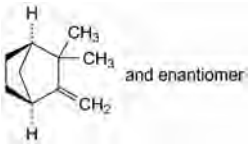


B. 2,2-dimethyl-3-methylenebicyclo[2.2.1]heptane (camphene),

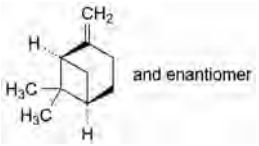


C. 6,6-dimethyl-2-methylenebicyclo[3.1.1]heptane (β-pinene),

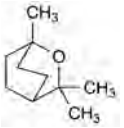


D. 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane (cineole),

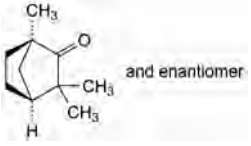


E. 1,3,3-trimethylbicyclo[2.2.1]heptan-2-one (fenchone),

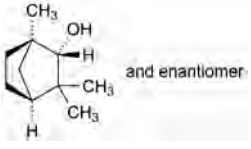


F. *exo*-1,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (fenchol),

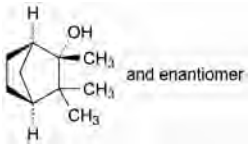


G. *exo*-2,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (camphene hydrate),

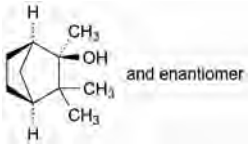


H. *endo*-2,3,3-trimethylbicyclo[2.2.1]heptan-2-ol (methylcamphenilol),

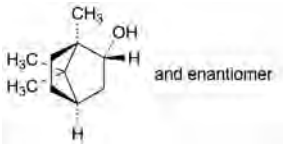


I. *exo*-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (*exo*-borneol),

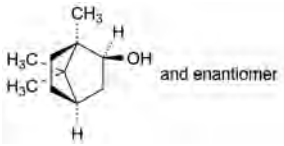


J. *endo*-1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol (*endo*-borneol).

**01/2008:0655**
**corrected 6.0**

# CAMPHOR, RACEMIC

## Camphora racemica

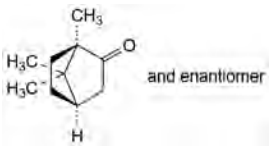


$C_{10}H_{16}O$ $M_r$ 152.2
[76-22-2]

DEFINITION

(1*RS*,4*RS*)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or friable, crystalline masses, highly volatile even at room temperature.

*Solubility*: slightly soluble in water, very soluble in ethanol (96 per cent) and in light petroleum, freely soluble in fatty oils, very slightly soluble in glycerol.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Optical rotation (see Tests).

B. Melting point (*2.2.14*): 172 °C to 180 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: mulls in *liquid paraffin R*.
*Comparison*: *racemic camphor CRS*.

D. Dissolve 1.0 g in 30 mL of *methanol R*. Add 1.0 g of *hydroxylamine hydrochloride R* and 1.0 g of *anhydrous sodium acetate R*. Boil under a reflux condenser for 2 h. Allow to cool and add 100 mL of *water R*. A precipitate is formed. Filter, wash with 10 mL of *water R* and recrystallise from 10 mL of a mixture of 4 volumes of *ethanol (96 per cent) R* and 6 volumes of *water R*. The crystals, dried *in vacuo*, melt (*2.2.14*) at 118 °C to 121 °C.

TESTS

*Carry out the weighings rapidly.*

**Solution S**. Dissolve 2.50 g in 10 mL of *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 1.0 g in 10 mL of *ethanol (96 per cent) R* and add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Optical rotation** (*2.2.7*): – 0.15° to + 0.15°, determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *hexane R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50 mg of the substance to be examined and 50 mg of *bornyl acetate R* in *hexane R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 200.0 mL with *hexane R*.

*Column*:
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *macrogol 20 000 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:
- *column*: 130 °C;
- *injection port and detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 3 times the retention time of camphor.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to camphor and bornyl acetate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:
- *any impurity*: for each impurity, not more than 2 per cent of the area of the principal peak;
- *total*: not more than 4 per cent of the area of the principal peak;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b).

**Halogens**: maximum 100 ppm.

Dissolve 1.0 g in 10 mL of *2-propanol R* in a distillation flask. Add 1.5 mL of *dilute sodium hydroxide solution R* and 50 mg of *nickel-aluminium alloy R*. Heat on a water-bath until the *2-propanol R* has evaporated. Allow to cool and add 5 mL of *water R*. Mix and filter through a wet filter previously washed with *water R* until free from chlorides. Dilute the filtrate to 10.0 mL with *water R*. To 5.0 mL of this solution, add *nitric acid R* dropwise until the precipitate which forms is redissolved and dilute to 15 mL with *water R*. The solution complies with the limit test for chlorides (*2.4.4*).

**Water**. Dissolve 1 g in 10 mL of *light petroleum R*. The solution is clear (*2.2.1*).

**Residue on evaporation**: maximum 0.05 per cent.

Evaporate 2.0 g on a water-bath and dry at 100-105 °C for 1 h. The residue weighs not more than 1 mg.

**01/2020:2573**



# CANDESARTAN CILEXETIL

## Candesartanum cilexetili

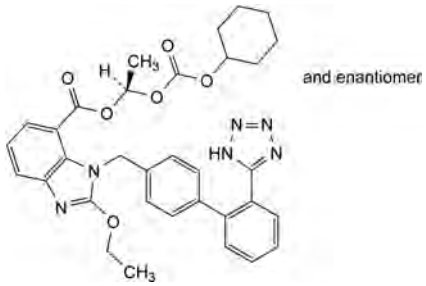


$C_{33}H_{34}N_6O_6$ $M_r$ 611

[145040-37-5]

DEFINITION

(1*RS*)-1-[[(Cyclohexyloxy)carbonyl]oxy]ethyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

PRODUCTION

As *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are classified as probable human carcinogens, manufacturers must ensure that their manufacturing process does not generate such impurities and develop appropriate control strategies. To allow manufacturers to make the necessary changes to their process, a transition period has been agreed by competent authorities and strict temporary limits on levels of these impurities introduced in the Test section.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride and slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *candesartan cilexetil CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 50.0 mL of the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *candesartan cilexetil for system suitability CRS* (containing impurities A, B and F) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2.5 mg of *candesartan cilexetil for peak identification CRS* (containing impurities G and H) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: *glacial acetic acid R*, *water for chromatography R*, *acetonitrile R* (1:43:57 *V/V/V*);
- *mobile phase B*: *glacial acetic acid R*, *water for chromatography R*, *acetonitrile R* (1:10:90 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 33 | 100 → 0 | 0 → 100 |
| 33 - 40 | 0 | 100 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *candesartan cilexetil for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and F; use the chromatogram supplied with *candesartan cilexetil for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities G and H.

*Relative retention* with reference to candesartan cilexetil (retention time = about 11 min): impurity G = about 0.2; impurity A = about 0.4; impurity B = about 0.5; impurity F = about 2.0; impurity H = about 3.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurities A and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurities A and G = 0.7; impurity H = 1.6;
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities A, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 60.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Nitrosamines**. Carry out the test by a suitable method.

The substance to be examined does not contain either NDMA or NDEA above the limits provided below or both impurities at whatever level:

– *N-nitrosodimethylamine (NDMA)*: maximum 3.000 ppm;

– *N-nitrosodiethylamine (NDEA)*: maximum 0.820 ppm.

ASSAY

Dissolve 0.500 g in 60 mL of *glacial acetic acid R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*) at the 1st inflexion point.

1 mL of *0.1 M perchloric acid* is equivalent to 61.1 mg of $C_{33}H_{34}N_6O_6$.

IMPURITIES

*Specified impurities: A, B, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, I.*

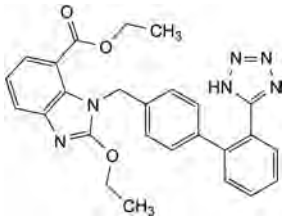

A. ethyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate,

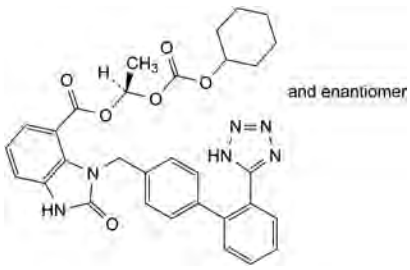


B. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 2-oxo-3-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-2,3-dihydro-1*H*-benzimidazole-4-carboxylate,

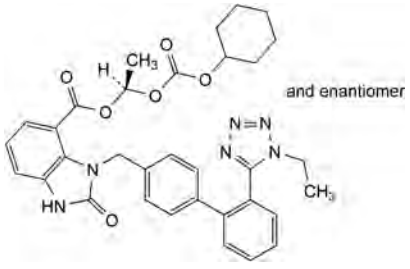


C. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 3-[[2′-(1-ethyl-1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-2-oxo-2,3-dihydro-1*H*-benzimidazole-4-carboxylate,

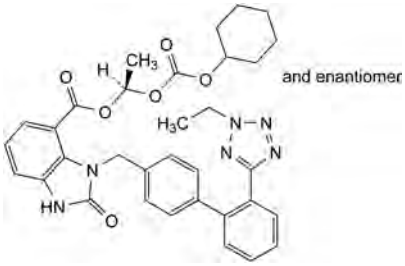


D. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 3-[[2′-(2-ethyl-2*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-2-oxo-2,3-dihydro-1*H*-benzimidazole-4-carboxylate,

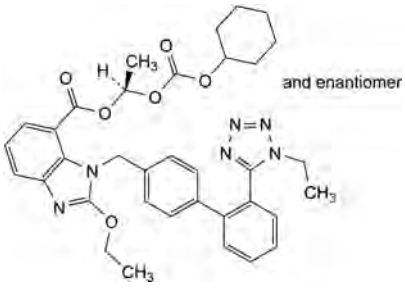


E. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 2-ethoxy-1-[[2′-(1-ethyl-1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate,

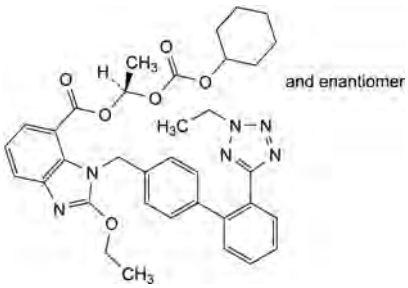


F. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 2-ethoxy-1-[[2′-(2-ethyl-2*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate,

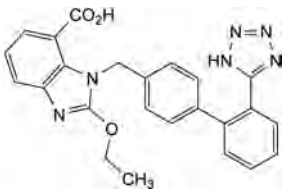

G. 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylic acid (candesartan),

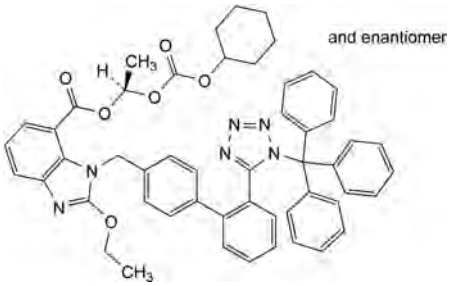


H. (1*RS*)-1-[[(cyclohexyloxy)carbonyl]oxy]ethyl 2-ethoxy-1-[[2′-[1-(triphenylmethyl)-1*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate (*N*-tritylcandesartan),

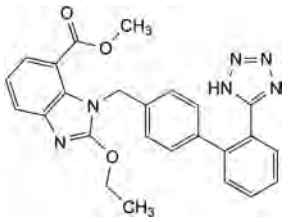

I. methyl 2-ethoxy-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-benzimidazole-7-carboxylate.



**04/2014:2762**

# CAPECITABINE

## Capecitabinum

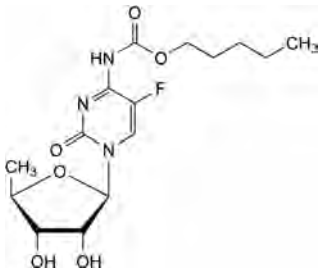

$C_{15}H_{22}FN_3O_6$ $M_r$ 359.3
[154361-50-9]

DEFINITION

Pentyl [1-(5-deoxy-β-D-ribofuranosyl)-5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl]carbamate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *capecitabine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 96.0 to + 100.0 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C.*

*Solvent mixture*: *acetonitrile R*, *methanol R*, *water R* (5:35:60 *V/V/V*).

*Test solution.* Dissolve 60.0 mg of the substance to be examined in 80 mL of the solvent mixture, sonicate until dissolution is complete and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 60.0 mg of *capecitabine CRS* in 80 mL of the solvent mixture, sonicate until dissolution is complete and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 3 mg of *capecitabine impurity A CRS*, 3 mg of *capecitabine impurity B CRS* and 5 mg of *capecitabine impurity D CRS* in 80 mL of the solvent mixture, sonicate until dissolution is complete and dilute to 100.0 mL with the solvent mixture. Dilute 1 mL of the solution to 50 mL with the test solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R*, *methanol R*, 0.1 per cent *V/V* solution of *glacial acetic acid R* (5:35:60 *V/V/V*);
- *mobile phase B*: *acetonitrile R*, 0.1 per cent *V/V* solution of *glacial acetic acid R*, *methanol R* (5:15:80 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 20 | 100 → 49 | 0 → 51 |
| 20 - 30 | 49 | 51 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to capecitabine (retention time = about 17 min): impurity A = about 0.18; impurity B = about 0.19; impurities D and E = about 0.95.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to impurities A and B; minimum 2.0 between the peaks due to impurity D and capecitabine.

*Calculation of percentage contents*:
- for each impurity, use the concentration of capecitabine in reference solution (b);
- *correction factor*: multiply the peak area of impurity B by 1.3.

*Limits*:
- *impurities A, B*: for each impurity, maximum 0.3 per cent;
- *sum of impurities D and E*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.03 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent.

Inject 1.0 mL of a 0.200 g/mL solution of the substance to be examined in *methanol R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{15}H_{22}FN_3O_6$ taking into account the assigned content of *capecitabine CRS*.

IMPURITIES

*Specified impurities: A, B, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, F, G.*

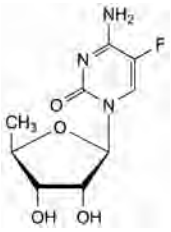

A. 4-amino-1-(5-deoxy-β-D-ribofuranosyl)-5-fluoropyrimidin-2(1*H*)-one (5′-deoxy-5-fluorocytidine),

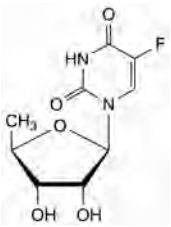

B. 1-(5-deoxy-β-D-ribofuranosyl)-5-fluoropyrimidine-2,4(1*H*,3*H*)-dione (5′-deoxy-5-fluorouridine),

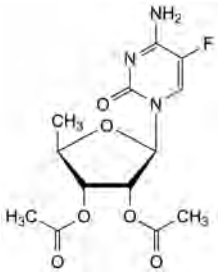

C. 1-(2,3-di-*O*-acetyl-5-deoxy-β-D-ribofuranosyl)-4-amino-5-fluoropyrimidin-2(1*H*)-one,

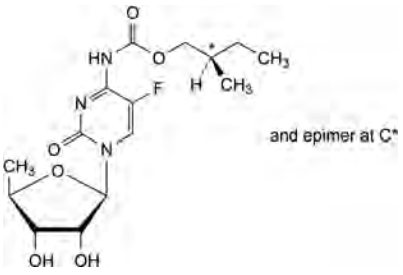


D. (2*RS*)-2-methylbutyl [1-(5-deoxy-β-D-ribofuranosyl)-5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl]carbamate,

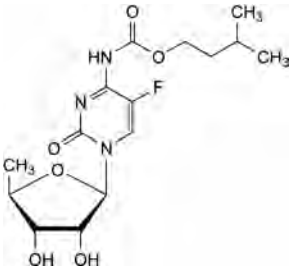

E. 3-methylbutyl [1-(5-deoxy-β-D-ribofuranosyl)-5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl]carbamate,

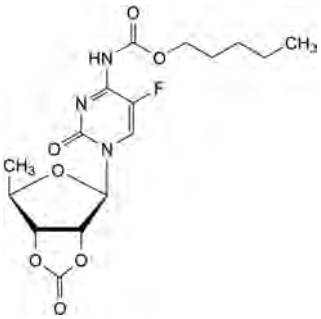

F. pentyl [5-fluoro-1-[(3a*R*,4*R*,6*R*,6a*R*)-6-methyl-2-oxotetrahydrofuro[3,4-*d*][1,3]dioxol-4-yl]-2-oxo-1,2-dihydropyrimidin-4-yl]carbamate,

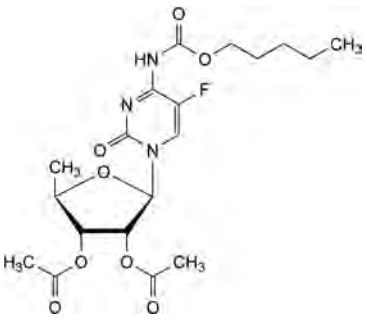

G. pentyl [1-(2,3-di-*O*-acetyl-5-deoxy-β-D-ribofuranosyl)-5-fluoro-2-oxo-1,2-dihydropyrimidin-4-yl]carbamate.

**01/2017:1401**

# CAPRYLIC ACID

## Acidum caprylicum

$C_8H_{16}O_2$ $M_r$ 144.2
[124-07-2]

DEFINITION

Octanoic acid.

*Content*: 99.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish, oily liquid.

*Solubility*: very slightly soluble in water, very soluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Relative density (see Tests).

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 0.909 to 0.912.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 0.10 g of the substance to be examined in *ethyl acetate R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.10 g of *caprylic acid CRS* in *ethyl acetate R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *ethyl acetate R*. Dilute 5.0 mL of this solution to 50.0 mL with *ethyl acetate R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 100 |
| | 1 - 25 | 100 → 220 |
| | 25 - 35 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 5 for the principal peak.

*Limits*:

- *any impurity*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.7 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.125 g in 25 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 14.42 mg of $C_8H_{16}O_2$.

IMPURITIES

A. hexanoic acid,

B. heptanoic acid,

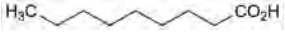

C. nonanoic acid,

D. decanoic acid,

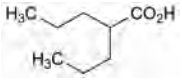

E. 2-propylpentanoic acid (valproic acid),

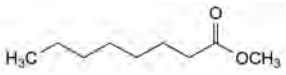

F. methyl octanoate,

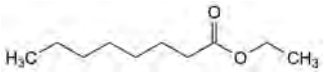

G. ethyl octanoate,

H. methyl decanoate,

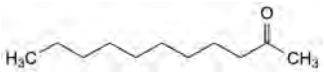

I. undecan-2-one,

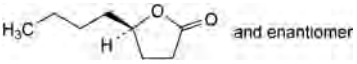


J. 5-butyltetrahydrofuran-2-one (γ-hydroxyoctanoic acid lactone).

**04/2017:1184**

# CAPRYLOCAPROYL MACROGOLGLYCERIDES

## Macrogolglyceridorum caprylocaprates

DEFINITION

Mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 200 and 400.

They are obtained by partial alcoholysis of medium-chain triglycerides using macrogol, or by esterification of glycerol and macrogol with caprylic (octanoic) acid and capric (decanoic) acid, or by mixing glycerol esters and condensates of ethylene oxide with caprylic acid and capric acid. They may contain free macrogols.

CHARACTERS

*Appearance*: pale-yellow, oily liquid.

*Solubility*: dispersible in hot water, freely soluble in methylene chloride.

*Density*: about 1.0 at 20 °C.

*Refractive index*: about 1.4 at 20 °C.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 50 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.9 ($R_{st}$ 1) and spots due to 1,3-diglycerides ($R_{st}$ 0.7), to 1,2-diglycerides ($R_{st}$ 0.6), to monoglycerides ($R_{st}$ 0.1) and to esters of macrogol ($R_{st}$ 0).

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Viscosity** (*2.2.9*). Carry out the determination at 20 ± 0.5 °C.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Viscosity (mPa·s) |
|---|---|---|
| 4 | 200 | 30 to 50 |
| 6 | 300 | 60 to 80 |
| 8 | 400 | 80 to 110 |

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*). Use 1.0 g.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Hydroxyl value |
|---|---|---|
| 4 | 200 | 80 to 120 |
| 6 | 300 | 140 to 180 |
| 8 | 400 | 170 to 205 |

**Peroxide value** (*2.5.5, Method A*): maximum 6.0, determined on 2.0 g.

**Saponification value** (*2.5.6*). Use 2.0 g.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Saponification value |
|---|---|---|
| 4 | 200 | 265 to 285 |
| 6 | 300 | 170 to 190 |
| 8 | 400 | 85 to 105 |

**Alkaline impurities**. Introduce 5.0 g into a test-tube and carefully add a mixture, neutralised if necessary with *0.01 M hydrochloric acid* or with *0.01 M sodium hydroxide*, of 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*, 0.3 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Shake and allow to stand. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

**Free glycerol**: maximum 5.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat if necessary. After cooling, add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate the iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

– *caproic acid*: maximum 2.0 per cent;

– *caprylic acid*: 50.0 per cent to 80.0 per cent;

– *capric acid*: 20.0 per cent to 50.0 per cent;

– *lauric acid*: maximum 3.0 per cent;

– *myristic acid*: maximum 1.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of ethylene oxide units per molecule (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for caprylocaproyl macrogolglycerides used as self-emulsifying agents and solubilisers.*

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**Composition of fatty acids** (see Tests).

**01/2017:1079**
**corrected 10.0**

# CAPTOPRIL

## Captoprilum

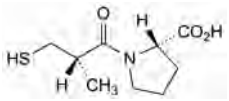


$C_9H_{15}NO_3S$ $M_r$ 217.3
[62571-86-2]

DEFINITION

(2*S*)-1-[(2*S*)-2-Methyl-3-sulfanylpropanoyl]pyrrolidine-2-carboxylic acid.

*Content*: 98.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in methanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *captopril CRS*.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.0 to 2.6 for solution S.

**Specific optical rotation** (*2.2.7*): – 132 to – 127 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Impurity F**. Gas chromatography (*2.2.28*).

*Reagent solution.* Add 2.8 mL of *acetyl chloride R* dropwise to 17.2 mL of *anhydrous methanol R* at 0 °C and mix. Allow to stand for 20 min at room temperature before use.

*Test solution.* Introduce 20.0 mg of the substance to be examined into a vial and add 1.0 mL of the reagent solution. Mix and heat at 60 °C for 30 min. Evaporate to dryness under a stream of *nitrogen R*. Dissolve the residue in 0.5 mL of *ethyl acetate R*, add 0.5 mL of *pentafluoropropionic anhydride R*, mix and heat at 60 °C for 30 min. Evaporate to dryness under a stream of *nitrogen R*. Dissolve the residue in 1.0 mL of *butyl acetate R*.

*Reference solution (a).* Dissolve the contents of a vial of *captopril for system suitability CRS* (containing impurity F) in 1.0 mL of the reagent solution. Prepare as described for the test solution.

*Reference solution (b).* Mix 0.25 mL of reference solution (a) and 0.75 mL of *butyl acetate R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.2 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 200 |
| | 10 - 14 | 200 → 240 |
| | 14 - 34 | 240 |
| Injection port | | 270 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to captopril (retention time = about 6 min): impurity F = about 0.96.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity F and captopril in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity F in the chromatogram obtained with reference solution (b).

Calculate the percentage content of impurity F using the following expression:

$$\frac{A}{A+B} \times 100$$

*A* = area of the peak due to impurity F in the chromatogram obtained with the test solution;

*B* = area of the peak due to captopril in the chromatogram obtained with the test solution.

*Limit*:

- *impurity F*: maximum 0.2 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *phosphoric acid R*, *acetonitrile R1*, *water R* (0.08:10:90 *V/V/V*).

*Test solution.* Dissolve 0.125 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 4.0 mg of *captopril impurity J CRS*, 5.0 mg of *captopril impurity B CRS*, 5.0 mg of *captopril impurity C CRS* and 5.0 mg of *captopril impurity D CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture. Prepare immediately before use.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *captopril impurity E CRS* in *acetonitrile R* and dilute to 25.0 mL with the same solvent. Dilute 4 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (c).* In order to prepare impurity A *in situ*, introduce 1.0 mL of the test solution into a volumetric flask and add 230 µL of *0.05 M iodine*. If the solution is not colourless, add *0.1 M sodium thiosulfate* dropwise until it becomes colourless, and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.3 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (10 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *water R* (0.08:100 *V/V*);
- *mobile phase B*: *phosphoric acid R*, *acetonitrile R1*, *water R* (0.08:50:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 50 | 10 → 50 |
| 20 - 45 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C, D and J; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to captopril (retention time = about 15 min): impurity C = about 0.6; impurity D = about 0.8; impurity E = about 0.9; impurity B = about 1.17; impurity J = about 1.22; impurity A = about 1.7.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurities B and J in the chromatogram obtained with reference solution (a);
- *resolution*: minimum 2.0 between the peaks due to impurity E and captopril in the chromatogram obtained with reference solution (b).

*Limits*:

- *impurity A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent);

- *impurity J*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities B, C, D*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: maximum 1.2 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 30 mL of *water R*. Titrate with *0.05 M iodine*, determining the end-point potentiometrically (*2.2.20*). Use a combined platinum electrode.

1 mL of *0.05 M iodine* is equivalent to 21.73 mg of $C_9H_{15}NO_3S$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, L, M, N, O.*

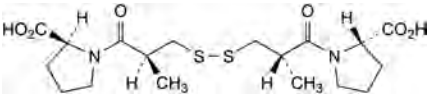

A. 1,1′-[disulfanediylbis[(2*S*)-2-methyl-1-oxopropane-3,1-diyl]]bis[(2*S*)-pyrrolidine-2-carboxylic] acid (captopril disulfide),

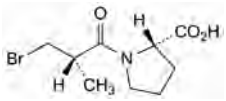

B. (2*S*)-1-[(2*S*)-3-bromo-2-methylpropanoyl]-pyrrolidine-2-carboxylic acid,

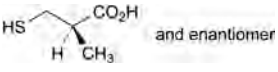


C. (2*RS*)-2-methyl-3-sulfanylpropanoic acid,

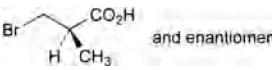


D. (2*RS*)-3-bromo-2-methylpropanoic acid,

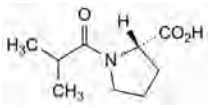

E. (2*S*)-1-(2-methylpropanoyl)pyrrolidine-2-carboxylic acid,

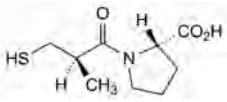

F. (2*S*)-1-[(2*R*)-2-methyl-3-sulfanylpropanoyl]pyrrolidine-2-carboxylic acid (*epi*-captopril),

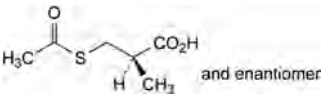


G. (2*RS*)-3-(acetylsulfanyl)-2-methylpropanoic acid,

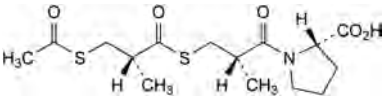

H. (2*S*)-1-[(2*S*)-3-[[(2*R*)-3-(acetylsulfanyl)-2-methylpropanoyl]sulfanyl]-2-methylpropanoyl]pyrrolidine-2-carboxylic acid,

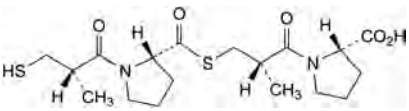

I. (2*S*)-1-[(2*S*)-3-[[[(2*S*)-1-[(2*S*)-2-methyl-3-sulfanylpropanoyl]pyrrolidin-2-yl]carbonyl]sulfanyl]-2-methylpropanoyl]pyrrolidine-2-carboxylic acid,

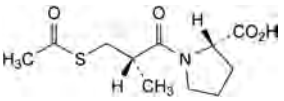

J. (2*S*)-1-[(2*S*)-3-(acetylsulfanyl)-2-methylpropanoyl]pyrrolidine-2-carboxylic acid (acetylcaptopril),

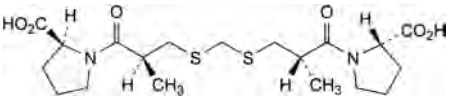

L. 1,1′-[methylenebis[sulfanediyl[(2*S*)-2-methyl-1-oxopropane-3,1-diyl]]]bis[(2*S*)-pyrrolidine-2-carboxylic] acid,

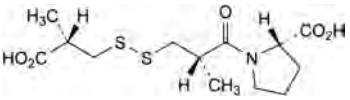

M. (2*S*)-1-[(2*S*)-3-[[(2*S*)-2-carboxypropyl]disulfanyl]-2-methylpropanoyl]pyrrolidine-2-carboxylic acid,

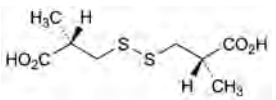

N. 3,3′-disulfanediylbis[(2*S*)-2-methylpropanoic] acid,

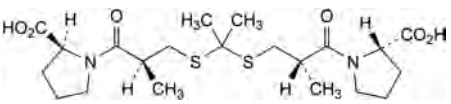

O. 1,1′-[propane-2,2-diylbis[sulfanediyl[(2*S*)-2-methyl-1-oxopropane-3,1-diyl]]]bis[(2*S*)-pyrrolidine-2-carboxylic] acid.

01/2017:1971

# CARBACHOL

## Carbacholum

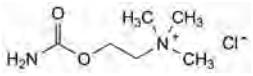

$C_6H_{15}ClN_2O_2$ $M_r$ 182.7
[51-83-2]

DEFINITION

2-(Carbamoyloxy)-*N,N,N*-trimethylethanaminium chloride.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, hygroscopic powder.

*Solubility*: very soluble in water, sparingly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *carbachol CRS*.

B. Examine the chromatograms obtained in the test for related substances.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. 0.5 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 2.0 mL of solution S, add 0.05 mL of *methyl red mixed solution R*. Not more than 0.2 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Test solution (b).* Dilute 2.0 mL of test solution (a) to 20.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *carbachol CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dissolve 8 mg of *choline chloride R* and 8 mg of *acetylcholine chloride CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL to 10.0 mL with *methanol R*.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: *water R, methanol R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: spray with *potassium iodobismuthate solution R3*.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Limits*: in the chromatogram obtained with test solution (a):

– *any impurity*: any spot, apart from the principal spot, is not more intense than one or other of the 2 principal spots in the chromatogram obtained with reference solution (b) (1 per cent). Compare the spots with the spot of the most appropriate colour in the chromatogram obtained with reference solution (b).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g of the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.150 g in a mixture of 10 mL of *anhydrous acetic acid R* and 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.27 mg of $C_6H_{15}ClN_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

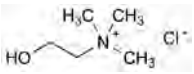

A. 2-hydroxy-*N,N,N*-trimethylethanaminium chloride (choline chloride).

01/2017:0543

# CARBAMAZEPINE

## Carbamazepinum

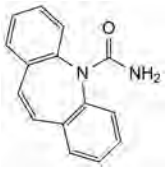

$C_{15}H_{12}N_2O$ $M_r$ 236.3
[298-46-4]

DEFINITION

5*H*-Dibenzo[*b,f*]azepine-5-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in acetone and in ethanol (96 per cent).

It shows polymorphism (*5.9*). The acceptable crystalline form corresponds to *carbamazepine CRS*.

IDENTIFICATION

A. Melting point (*2.2.14*): 189 °C to 193 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *carbamazepine CRS*.
*Preparation*: examine the substances as discs without prior treatment.

TESTS

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R*, shake for 15 min and filter. To 10 mL of the filtrate add 0.05 mL of *phenolphthalein solution R1* and 0.5 mL of *0.01 M sodium hydroxide*; the solution is red. Add 1.0 mL of *0.01 M hydrochloric acid*; the solution is colourless. Add 0.15 mL of *methyl red solution R*; the solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 60.0 mg of the substance to be examined in *methanol R2* and dilute to 20.0 mL with the same solvent. Sonicate. Dilute 10.0 mL of this solution to 20.0 mL with *water R*.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 50.0 mL with a mixture of equal volumes of *methanol R2* and *water R*.

*Reference solution (a).* Dissolve 7.5 mg of *carbamazepine CRS*, 7.5 mg of *carbamazepine impurity A CRS* and 7.5 mg of *iminodibenzyl R* (impurity E) in *methanol R2* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with a mixture of equal volumes of *methanol R2* and *water R*.

*Reference solution (b).* Dissolve 60.0 mg of *carbamazepine CRS* in *methanol R2* and dilute to 20.0 mL with the same solvent. Sonicate. Dilute 5.0 mL of this solution to 50.0 mL with a mixture of equal volumes of *methanol R2* and *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *nitrile silica gel for chromatography R1* (10 µm).

*Mobile phase*: *tetrahydrofuran R*, *methanol R2*, *water R* (3:12:85 *V/V/V*); to 1000 mL of this solution add 0.2 mL of *anhydrous formic acid R* and 0.5 mL of *triethylamine R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solution (a).

*Run time*: 8 times the retention time of carbamazepine.

*Relative retention* with reference to carbamazepine (retention time = about 10 min): impurity A = about 0.9; impurity E = about 3.5.

*System suitability*:

– *resolution*: minimum 1.7 between the peaks due to impurity A and carbamazepine in the chromatogram obtained with reference solution (a).

*Limits*:

– *impurities A, E*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: not more than the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the peak due to carbamazepine in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 140 ppm.

Suspend 0.715 g in 20 mL of *water R* and boil for 10 min. Cool and dilute to 20 mL with *water R*. Filter through a membrane filter (nominal pore size 0.8 µm). Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (b).

*System suitability*:

– *repeatability*: reference solution (b).

Calculate the percentage content of $C_{15}H_{12}N_2O$ from the declared content of *carbamazepine CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G.*

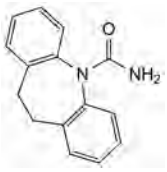


A. 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide (10,11-dihydrocarbamazepine),

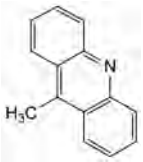


B. 9-methylacridine,

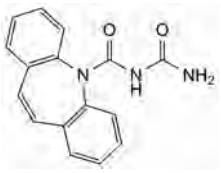


C. (5*H*-dibenzo[*b,f*]azepin-5-ylcarbonyl)urea (*N*-carbamoylcarbamazepine),

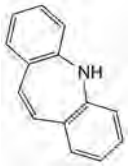


D. 5*H*-dibenzo[*b,f*]azepine (iminostilbene),

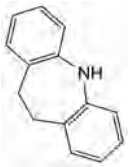


E. 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine (iminodibenzyl),

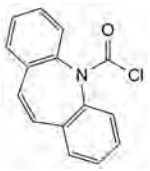

F. 5*H*-dibenzo[*b,f*]azepine-5-carbonyl chloride (5-chlorocarbonyliminostilbene),

G. 10-bromo-5*H*-dibenzo[*b,f*]azepine-5-carboxamide (10-bromocarbamazepine).

**01/2017:1185**

# CARBASALATE CALCIUM

## Carbasalatum calcicum

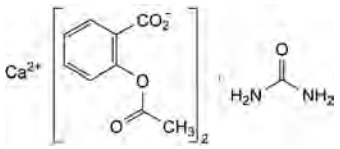

$C_{19}H_{18}CaN_2O_9$ $M_r$ 458.4
[5749-67-7]

DEFINITION

Equimolecular compound of calcium di[2-(acetyloxy)benzoate] and urea.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in dimethylformamide, practically insoluble in acetone and in anhydrous methanol.

*Protect the substance from moisture during handling. Examination in aqueous solutions has to be performed immediately after preparation.*

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.250 g in *water R* and dilute to 100.0 mL with the same solvent. To 1.0 mL of the solution add 75 mL of *water R* and 5 mL of *dilute hydrochloric acid R*, mix and dilute to 100.0 mL with *water R*. Examine immediately.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 228 nm and 276 nm.

*Specific absorbance at the absorption maxima*:

– at 228 nm: 363 to 379,
– at 276 nm: 49 to 53.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of carbasalate calcium.*

C. Dissolve 0.1 g in 10 mL of *water R*, boil for 2 min and cool. The solution gives reaction (a) of salicylates (*2.3.1*).

D. Heat 0.2 g with 0.2 g of *sodium hydroxide R*; a yellow or yellowish-brown colour is produced and the vapour turns *red litmus paper R* blue.

E. It gives reaction (a) of calcium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

Dissolve 2.5 g in 50 mL of *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *phosphoric acid R*, *methanol R*, *acetonitrile for chromatography R* (0.5:8:92 *V/V/V*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in 5 mL of the solvent mixture, sonicate for 15 min and dilute to 10.0 mL with the solvent mixture. Filter the solution through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (a).* Dissolve 10.0 mg of *salicylic acid CRS* (impurity C) in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *carbasalate impurity B CRS* in 20.0 mL of the solvent mixture.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Mix 1.0 mL of this solution with 5.0 mL of reference solution (a), add 1.0 mL of reference solution (c) and dilute to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: *phosphoric acid R*, *acetonitrile for chromatography R*, *water R* (0.5:40:60 *V/V/V*).

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (d).

*Run time*: 8 times the retention time of acetylsalicylic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and C.

*Relative retention* with reference to acetylsalicylic acid (retention time = about 2 min): impurity C = about 1.3; impurity B = about 2.5.

*System suitability*: reference solution (d):

– *resolution*: minimum 5.0 between the peaks due to acetylsalicylic acid and impurity C.

*Limits*:

– *impurity C*: not more than 5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *impurity B*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
– *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Sodium**: maximum 0.1 per cent.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.0 g in 500.0 mL of *water R*.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 1.000 g. Use a mixture of 15 mL of *anhydrous methanol R* and 15 mL of *dimethylformamide R* as the solvent.

ASSAY

In a flask with a ground-glass stopper, dissolve 0.400 g in 25 mL of *water R*. Add 25.0 mL of *0.1 M sodium hydroxide*. Close the flask and allow to stand for 2 h. Titrate with *0.1 M hydrochloric acid*, using 0.2 mL of *phenolphthalein solution R*. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 22.92 mg of $C_{19}H_{18}CaN_2O_9$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D.*

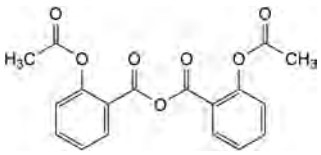

A. 2-(acetyloxy)benzoic anhydride,

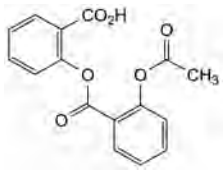

B. 2-[[2-(acetyloxy)benzoyl]oxy]benzoic acid (acetylsalicyl-salicylic acid),

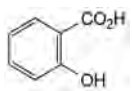

C. 2-hydroxybenzenecarboxylic acid (salicylic acid),

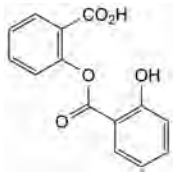

D. 2-[(2-hydroxybenzoyl)oxy]benzoic acid (salicylsalicylic acid).

**01/2017:0755**
**corrected 9.4**

# CARBIDOPA

## Carbidopum

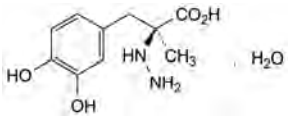

$C_{10}H_{14}N_2O_4,H_2O$ $M_r$ 244.2
[38821-49-7]

DEFINITION

(2*S*)-3-(3,4-Dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid monohydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in an 8.5 g/L solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 10.0 mL of this solution to 100.0 mL with an 8.5 g/L solution of *hydrochloric acid R* in *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 283 nm.

*Specific absorbance at the absorption maximum*: 135 to 150 (dried substance).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *carbidopa CRS*.

D. Shake vigorously about 5 mg with 10 mL of *water R* for 1 min and add 0.3 mL of *ferric chloride solution R2*. An intense green colour is produced, which quickly changes to reddish-brown.

E. Suspend about 20 mg in 5 mL of *water R* and add 5 mL of *cupri-tartaric solution R*. On heating, the colour of the solution changes to dark brown and a red precipitate is formed.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ or $B_6$ (*2.2.2, Method II*).

Dissolve 0.25 g in 25 mL of *1 M hydrochloric acid*.

**Specific optical rotation** (*2.2.7*): − 26.5 to − 22.5 (dried substance).

Using an ultrasonic bath, dissolve completely 0.250 g in *aluminium chloride solution R* and dilute to 25.0 mL with the same solution.

**Hydrazine**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.50 g in *dilute hydrochloric acid R* and dilute to 2.0 mL with the same acid.

*Test solution (b).* Place 25 g of *strongly basic anion-exchange resin R* into each of 2 conical flasks with ground-glass stoppers. To each, add 150 mL of *carbon dioxide-free water R* and shake from time to time during 30 min. Decant the liquid from both flasks and repeat the process with further quantities, each of 150 mL, of *carbon dioxide-free water R*.

Take two 100 mL measuring cylinders 3.5-4.5 cm in internal diameter and label these A and B. Into cylinder A, transfer as completely as possible the resin from 1 conical flask using 60 mL of *carbon dioxide-free water R*; into cylinder B, transfer the 2nd quantity of resin, this time using 20 mL of *carbon dioxide-free water R*.

Into each cylinder, insert a gas-inlet tube, the end of which has an internal diameter of 2-3 mm and which reaches almost to the bottom of the cylinder. Pass a rapid stream of *nitrogen for chromatography R* through each mixture so that homogeneous suspensions are formed. After 30 min, without interrupting the gas flow, add 1.0 mL of test solution (a) to cylinder A; after 1 min stop the gas flow into cylinder A and transfer the contents, through a moistened filter paper, into cylinder B. After 1 min, stop the gas flow to cylinder B and pour the solution immediately through a moistened filter paper into a freshly prepared mixture of 1 mL of a 200 g/L solution of *salicylaldehyde R* in *methanol R* and 20 mL of *phosphate buffer solution pH 5.5 R* in a conical flask; shake thoroughly for 1 min and heat in a water-bath at 60 °C for 15 min. The liquid becomes clear. Allow to cool, add 2.0 mL of *toluene R* and shake vigorously for 2 min. Transfer the mixture into a centrifuge tube and centrifuge.

Separate the toluene layer in a 100 mL separating funnel and shake vigorously with 2 quantities, each of 20 mL, of a 200 g/L solution of *sodium metabisulfite R* and finally with 2 quantities, each of 50 mL, of *water R*. Separate the toluene layer.

*Reference solution (a).* Dissolve 10 mg of *hydrazine sulfate R* in *dilute hydrochloric acid R* and dilute to 50 mL with the same acid. Dilute 1.0 mL of this solution to 10.0 mL with *dilute hydrochloric acid R*.

*Reference solution (b).* Prepare the solution at the same time and in the same manner as described for test solution (b) using 1.0 mL of reference solution (a) instead of 1.0 mL of test solution (a).

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: *water R*, *methanol R* (10:20 *V/V*).

*Application*: 10 µL of test solution (b) and reference solution (b).

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Limit*:

– *hydrazine*: any spot showing a yellow fluorescence is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (20 ppm).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Buffer solution.* Dissolve 6.9 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of *water for chromatography R*, adjust to pH 2.2 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase, add 20 µL of *hydrochloric acid R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *carbidopa for system suitability CRS* (containing impurities A, D, E, I and J) in the mobile phase, add 4 µL of *hydrochloric acid R* and dilute to 2.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *carbidopa impurity mixture CRS* (impurities F and H) in 1.0 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
– *temperature*: 25 °C.

*Mobile phase*: *ethanol (96 per cent) R*, buffer solution (7:93 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of carbidopa.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, D + E, F, H, I and J.

*Relative retention* with reference to carbidopa (retention time = about 3 min): impurity A = about 0.9; impurities D and E = about 1.9; impurity H = about 2.5; impurity I = about 3.7; impurity J = about 4.0; impurity F = about 4.4.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to impurity A and carbidopa in the chromatogram obtained with reference solution (b); minimum 1.5 between the peaks due to impurities I and J in the chromatogram obtained with reference solution (b);
– *signal-to-noise ratio*: minimum 30 for the principal peak in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurities D and E = 1.5; impurity I = 1.5; impurity J = 1.5;
– for each impurity, use the concentration of carbidopa in reference solution (a).

*Limits*:

– *impurity A*: maximum 0.5 per cent;
– *impurity J*: maximum 0.25 per cent;
– *sum of impurities D and E*: maximum 0.2 per cent;
– *impurities F, H, I*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): 6.9 per cent to 7.9 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g with gentle heating in 75 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.62 mg of $C_{10}H_{14}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E, F, H, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, G.*

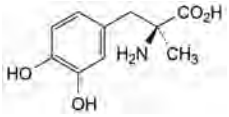

A. (2*S*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid (L-methyldopa),

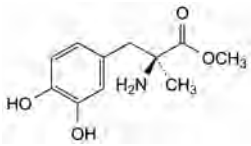

B. methyl (2*S*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoate,

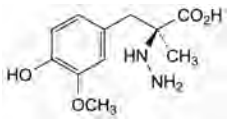

C. (2*S*)-2-hydrazino-3-(4-hydroxy-3-methoxyphenyl)-2-methylpropanoic acid (3-*O*-methylcarbidopa),

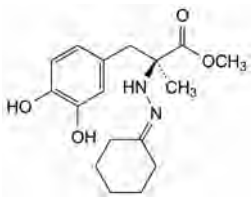

D. methyl (2*S*)-2-(2-cyclohexylidenehydrazino)-3-(3,4-dihydroxyphenyl)-2-methylpropanoate,

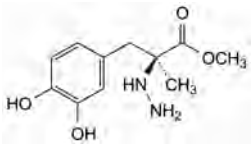

E. methyl (2*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoate,

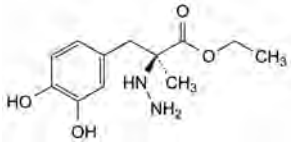

F. ethyl (2*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropanoate,

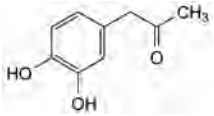

G. 1-(3,4-dihydroxyphenyl)propan-2-one,

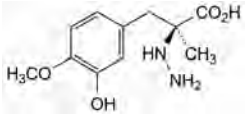

H. (2*S*)-2-hydrazino-3-(3-hydroxy-4-methoxyphenyl)-2-methylpropanoic acid,

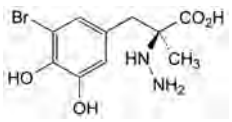

I. (2*S*)-3-(3-bromo-4,5-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid,

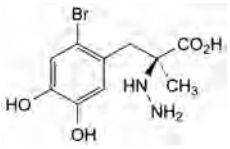

J. (2*S*)-3-(2-bromo-4,5-dihydroxyphenyl)-2-hydrazino-2-methylpropanoic acid.

**04/2014:0884**
**corrected 10.0**

# CARBIMAZOLE

## Carbimazolum

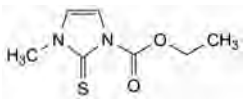

$C_7H_{10}N_2O_2S$ $M_r$ 186.2
[22232-54-8]

DEFINITION

Ethyl 3-methyl-2-thioxo-2,3-dihydro-1*H*-imidazole-1-carboxylate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 122 °C to 125 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *carbimazole CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10.0 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *carbimazole CRS* in *methylene chloride R* and dilute to 10.0 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *acetone R, methylene chloride R* (20:80 *V/V*).
*Application*: 10 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air for 30 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in a mixture of 0.05 mL of *dilute hydrochloric acid R* and 50 mL of *water R*. Add 1 mL of *potassium iodobismuthate solution R*. A red precipitate is formed.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (20:80 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *thiamazole CRS* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Mix 1 mL of the solution with 2 mL of the test solution and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *thiamazole CRS* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *carbimazole CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b) and (c).

*Run time*: 1.5 times the retention time of carbimazole.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to carbimazole (retention time = about 6 min): impurity A = about 0.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity A and carbimazole.

*Limits*:

- *impurity A*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: maximum 0.6 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (d).

Calculate the percentage content of $C_7H_{10}N_2O_2S$ taking into account the assigned content of *carbimazole CRS*.

IMPURITIES

*Specified impurities: A.*

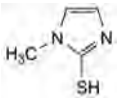

A. 1-methyl-1*H*-imidazole-2-thiol (thiamazole).

**01/2017:0885**

# CARBOCISTEINE

## Carbocisteinum

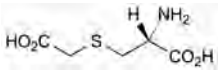

$C_5H_9NO_4S$ $M_r$ 179.2

[638-23-3]

DEFINITION

Carbocisteine contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2-amino-3-[(carboxymethyl)sulfanyl]propanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water and in alcohol. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *carbocisteine CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.1 g in 4.5 mL of *dilute sodium hydroxide solution R*. Heat on a water-bath for 10 min. Cool and add 1 mL of a 25 g/L solution of *sodium nitroprusside R*. A dark red colour is produced, which changes to brown and then to yellow within a few minutes.

TESTS

**Solution S**. Disperse 5.00 g in 20 mL of *water R* and add dropwise with shaking 2.5 mL of *strong sodium hydroxide solution R*. Adjust to pH 6.3 with *1 M sodium hydroxide* and dilute to 50.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). Shake 0.2 g with 20 mL of *carbon dioxide-free water R*. The pH of the suspension is 2.8 to 3.0.

**Specific optical rotation** (*2.2.7*): – 32.5 to – 35.5, determined on solution S and calculated with reference to the dried substance.

**Ninhydrin-positive substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *dilute ammonia R2* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 50 mL with *water R*.

*Reference solution (a)*. Dissolve 10 mg of *carbocisteine CRS* in *dilute ammonia R2* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dilute 5 mL of test solution (b) to 20 mL with *water R*.

*Reference solution (c)*. Dissolve 10 mg of *carbocisteine CRS* and 10 mg of *arginine hydrochloride CRS* in 5 mL of *dilute ammonia R2* and dilute to 25 mL with *water R*.

Apply separately to the plate 5 µL of each solution. Allow the plate to dry in air. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Dry the plate in a current of warm air. Spray with *ninhydrin solution R* and heat at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

**Chlorides** (*2.4.4*). Dissolve 33 mg in 5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution, without further addition of nitric acid, complies with the limit test for chlorides (0.15 per cent).

**Sulfates** (*2.4.13*). Dissolve 0.5 g in 5 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*. The solution complies with the limit test for sulfates (300 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*). Not more than 0.3 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 10 mL of *anhydrous formic acid R* with slight heating and shake until dissolution is complete. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.92 mg of $C_5H_9NO_4S$.

STORAGE

Store protected from light.

**01/2017:1299**

# CARBOMERS

## Carbomera

DEFINITION

High-molecular-mass polymers of acrylic acid cross-linked with alkenyl ethers of sugars or polyalcohols.

*Content*: 56.0 per cent to 68.0 per cent of carboxylic acid (-$CO_2H$) groups (dried substance).

CHARACTERS

*Appearance*: white or almost white, fluffy, hygroscopic powder.

*Solubility*: swells in water and in other polar solvents after dispersion and neutralisation with sodium hydroxide solution.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Main bands*: at 1710 ± 5 $cm^{-1}$, 1454 ± 5 $cm^{-1}$, 1414 ± 5 $cm^{-1}$, 1245 ± 5 $cm^{-1}$, 1172 ± 5 $cm^{-1}$, 1115 ± 5 $cm^{-1}$ and 801 ± 5 $cm^{-1}$, with the strongest band at 1710 ± 5 $cm^{-1}$.

B. Adjust a 10 g/L dispersion to about pH 7.5 with *1 M sodium hydroxide*. A highly viscous gel is formed.

C. Add 2 mL of a 100 g/L solution of *calcium chloride R*, with continuous stirring, to 10 mL of the gel from identification test B. A white precipitate is immediately produced.

D. Add 0.5 mL of *thymol blue solution R* to 10 mL of a 10 g/L dispersion. An orange colour is produced. Add 0.5 mL of *cresol red solution R* to 10 mL of a 10 g/L dispersion. A yellow colour is produced.

TESTS

**Free acrylic acid**. Liquid chromatography (*2.2.29*).

*Test solution*. Mix 0.125 g of the substance to be examined with a 25 g/L solution of *aluminium potassium sulfate R* and dilute to 25.0 mL with the same solution. Heat the suspension at 50 °C for 20 min with shaking, then shake the suspension at room temperature for 60 min. Centrifuge and use the clear supernatant solution as the test solution.

*Reference solution*. Dissolve 62.5 mg of *acrylic acid R* in a 25 g/L solution of *aluminium potassium sulfate R* and dilute to 100.0 mL with the same solution. Dilute 1.0 mL of this solution to 50.0 mL with a 25 g/L solution of *aluminium potassium sulfate R*.

*Column*:
- *size*: *l* = 0.12 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1.361 g/L solution of *potassium dihydrogen phosphate R*, adjusted to pH 2.5 using *dilute phosphoric acid R*;
- *mobile phase B*: mixture of equal volumes of a 1.361 g/L solution of *potassium dihydrogen phosphate R* and *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 100 | 0 |
| 8 - 9 | 100 → 0 | 0 → 100 |
| 9 - 20 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Retention time*: acrylic acid = about 6.0 min.

*Limit*:
- *acrylic acid*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.25 per cent).

**Benzene**. Gas chromatography (*2.4.24, System A*).

*Solution A*. Dissolve 0.100 g of *benzene R* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Test solution*. Weigh 50.0 mg of the substance to be examined into an injection vial and add 5.0 mL of *water R* and 1.0 mL of *dimethyl sulfoxide R*.

*Reference solution*. Weigh 50.0 mg of the substance to be examined into an injection vial and add 4.0 mL of *water R*, 1.0 mL of *dimethyl sulfoxide R* and 1.0 mL of solution A.

*Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous dispersion.*

*Static head-space conditions that may be used*:

– *equilibration temperature*: 80 °C;
– *equilibration time*: 60 min;
– *transfer-line temperature*: 90 °C.

*Injection*: 1 mL of the gaseous phase of the test solution and 1 mL of the gaseous phase of the reference solution; repeat these injections twice more.

*System suitability:*

– *repeatability*: maximum relative standard deviation of the differences in area between the analyte peaks obtained from the 3 replicate pair injections of the reference solution and the test solution is 15 per cent.

*Limit*:

– *benzene*: the mean area of the peak due to benzene in the chromatograms obtained with the test solution is not greater than 0.5 times the mean area of the peak due to benzene in the chromatograms obtained with the reference solution (2 ppm).

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 60 min.

**Sulfated ash** (*2.4.14*): maximum 4.0 per cent, determined on 1.0 g.

ASSAY

Slowly add 50 mL of *water R* to 0.120 g whilst stirring and heating at 60 °C for 15 min. Stop heating, add 150 mL of *water R* and continue stirring for 30 min. Add 2 g of *potassium chloride R* and titrate with *0.2 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.2 M sodium hydroxide* is equivalent to 9.0 mg of carboxylic acid (-$CO_2H$) groups.

STORAGE

In an airtight container.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for carbomers used as viscosity-increasing agents and gelling agents.*

**Apparent viscosity** (*2.2.10*): the nominal apparent viscosity is typically between 300 mPa·s and 115 000 mPa·s. For a product with a nominal apparent viscosity of 20 000 mPa·s or greater, the apparent viscosity is typically 70.0 per cent to 130.0 per cent of the nominal value; for a product with a nominal apparent viscosity of less than 20 000 mPa·s, the apparent viscosity is typically 50.0 per cent to 150.0 per cent of the nominal value.

Dry the substance to be examined *in vacuo* at 80 °C for 1 h. Carefully add 2.50 g of the previously dried substance to be examined to 500 mL of *water R* in a 1000 mL beaker while stirring continuously at 1000 ± 50 r/min, with the stirrer shaft set at an angle of 60° to one side of the beaker. Add the previously dried substance over a period of 45-90 s, at a uniform rate, ensuring that loose agglomerates of powder are broken up, and continue stirring at 1000 ± 50 r/min for 15 min. Remove the stirrer and place the beaker containing the dispersion in a water-bath at 25 ± 1 °C for 30 min. Insert the stirrer to a depth necessary to ensure that air is not drawn into the dispersion and, while stirring at 300 ± 25 r/min, titrate to pH 7.3-7.8 by adding a 180 g/L solution of *sodium hydroxide R* below the surface, determining the end-point potentiometrically (*2.2.20*). The total volume of the 180 g/L solution of *sodium hydroxide R* used is about 6.2 mL. Allow 2-3 min before the final pH determination. If the final pH exceeds 7.8, discard the preparation and prepare another using a smaller amount of sodium hydroxide for titration. Return the neutralised preparation to the water-bath at 25 °C for 1 h, then perform the viscosity determination without delay to avoid slight viscosity changes that occur 75 min after neutralisation. Determine the viscosity using a rotating viscometer with a spindle rotating at 20 r/min, using a spindle suitable for the expected apparent viscosity.

**Carboxylic acid groups**: see Assay.

**01/2008:0375**

# CARBON DIOXIDE

## Carbonei dioxidum

$CO_2$ $M_r$ 44.01
[124-38-9]

DEFINITION

*Content*: minimum 99.5 per cent *V/V* of $CO_2$ in the gaseous phase.

This monograph applies to carbon dioxide for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 1 volume of water.

PRODUCTION

*Examine the gaseous phase.*

*If the test is performed on a cylinder of gas, keep the cylinder of the substance to be examined at room temperature for not less than 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.*

**Carbon monoxide**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 5 ppm *V/V* of *carbon monoxide R* in *nitrogen R1*.

*Column*:

– *material*: stainless steel,
– *size*: *l* = 2 m, Ø = 4 mm,
– *stationary phase*: an appropriate molecular sieve for chromatography (0.5 nm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 60 mL/min.

*Temperature*:

– *column*: 50 °C,
– *injection port and detector*: 130 °C.

*Detection*: flame ionisation with methaniser.

*Injection*: loop injector.

Adjust the injected volumes and the operating conditions so that the height of the peak due to carbon monoxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

*Limit*:

– *carbon monoxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (5 ppm *V/V*).

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total, determined using a chemiluminescence analyser (*2.5.26*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a). Carbon dioxide R1.*

*Reference gas (b).* A mixture containing 2 ppm *V/V* of *nitrogen monoxide R* in *carbon dioxide R1* or in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide in the gas to be examined.

If nitrogen is used instead of carbon dioxide in reference gas (b), multiply the result obtained by the quenching correction factor in order to correct the quenching effect on the analyser response caused by the carbon dioxide matrix effect.

The quenching correction factor is determined by applying a known reference mixture of nitrogen monoxide in carbon dioxide and comparing the actual content with the content indicated by the analyser which has been calibrated with a $NO/N_2$ reference mixture.

$$\text{Quenching correction factor} = \frac{\text{actual nitrogen monoxide content}}{\text{indicated nitrogen monoxide content}}$$

**Total sulfur**: maximum 1 ppm *V/V*, determined using an ultraviolet fluorescence analyser after oxidation of the sulfur compounds by heating at 1000 °C (Figure 0375.-1).

The apparatus consists of the following:

– a system generating ultraviolet radiation with a wavelength of 210 nm, made up of an ultraviolet lamp, a collimator, and a selective filter; the beam is blocked periodically by a chopper rotating at high speed,

– a reaction chamber through which flows the previously filtered gas to be examined,

– a system that detects radiation emitted at a wavelength of 350 nm, made up of a selective filter, a photomultiplier tube and an amplifier.

*Gas to be examined.* The substance to be examined.

*Reference gas (a). Carbon dioxide R1.*

*Reference gas (b).* A mixture containing between 0.5 ppm *V/V* and 2 ppm *V/V* of *hydrogen sulfide R1* in *carbon dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Pass the gas to be examined through a quartz oven heated to 1000 °C. *Oxygen R* is circulated in the oven at a tenth of the flow rate of the gas to be examined. Measure the sulfur dioxide content in the gaseous mixture leaving the oven.

**Water**: maximum 67 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

**Assay**. Infrared analyser (*2.5.24*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Carbon dioxide R1.*

*Reference gas (b).* A mixture containing 95.0 per cent *V/V* of *carbon dioxide R1* and 5.0 per cent *V/V* of *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of carbon dioxide.*

B. Place a glowing splinter of wood in an atmosphere of the substance to be examined. It is extinguished.

C. Pass a stream of the substance to be examined through *barium hydroxide solution R*. A white precipitate is formed which dissolves with effervescence in *dilute acetic acid R*.

TESTS

*Examine the gaseous phase.*

*If the test is performed on a cylinder of gas, keep the cylinder of the substance to be examined at room temperature for not less than 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.*

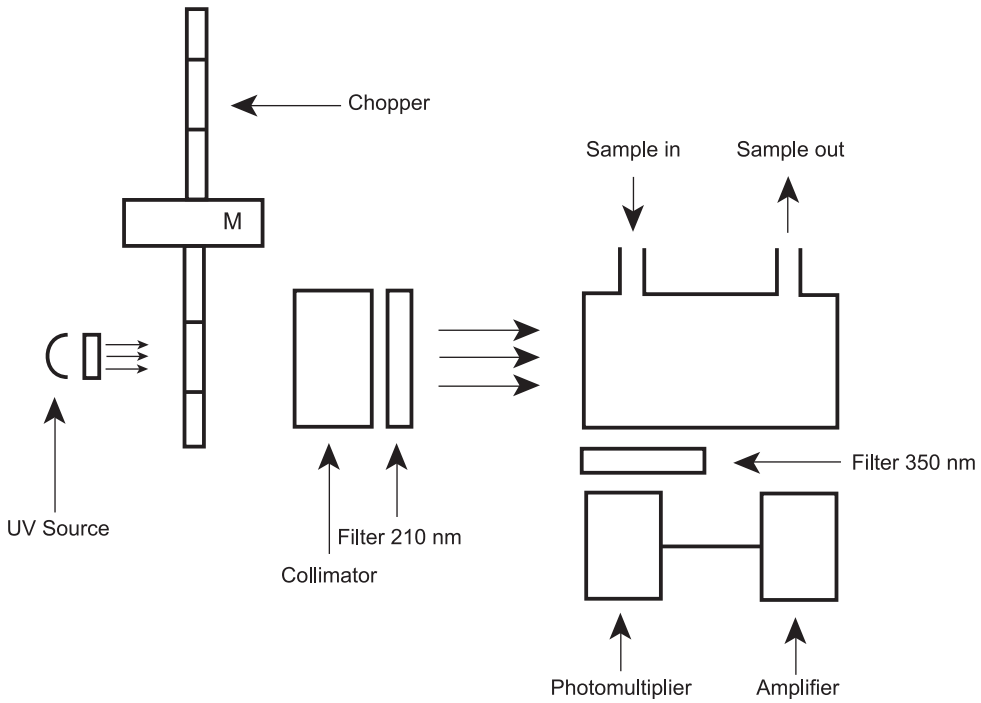


Figure 0375.-1.– *UV Fluorescence Analyser*

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube (*2.1.6*).

**Hydrogen sulfide**: maximum 1 ppm *V/V*, determined using a hydrogen sulfide detector tube (*2.1.6*).

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total, determined using a nitrogen monoxide and nitrogen dioxide detector tube (*2.1.6*).

**Sulfur dioxide**: maximum 2 ppm *V/V*, determined using a sulfur dioxide detector tube (*2.1.6*).

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

STORAGE

Store liquefied under pressure in suitable containers complying with the legal regulations.

IMPURITIES

A. NO: nitrogen monoxide,

B. $NO_2$: nitrogen dioxide,

C. CO: carbon monoxide,

D. total sulfur,

E. $H_2O$: water.

**01/2011:2408**
**corrected 7.2**

# CARBON MONOXIDE

## Carbonei monoxidum

CO $M_r$ 28.00
[630-08-0]

DEFINITION

Gas obtained by steam reforming (catalytic oxidation) of hydrocarbons.

*Content*: minimum 99.5 per cent *V/V* of CO.

This monograph applies to carbon monoxide for medicinal use.

CHARACTERS

*Appearance*: colourless, flammable gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 2.266 volumes of carbon monoxide dissolve in 100 volumes of water.

IDENTIFICATION

*Carry out either test A or B.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of carbon monoxide.*

B. It complies with the limits of the assay.

TESTS

**Carbon dioxide**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 300 ppm *V/V* of *carbon dioxide R1* in *carbon monoxide R.*

*Column*:
- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: an appropriate divinylbenzene porous polymer (149-177 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 30 mL/min.

*Temperature*:
- *column*: 50 °C;
- *detector*: 220 °C.

*Detection*: thermal conductivity.

*Injection*: 1 mL.

*Run time*: 3 min.

*Relative retention* with reference to carbon monoxide (retention time = about 0.4 min): carbon dioxide = about 3.5.

*Limit*:
- *carbon dioxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (300 ppm *V/V*).

**Methane**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 100 ppm *V/V* of *methane R* in *carbon monoxide R.*

*Column*:
- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 4 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (177-250 µm).

*Carrier gas*: *nitrogen for chromatography R.*

*Flow rate*: 10 mL/min.

*Temperature*:
- *column*: 95 °C;
- *detector*: 240 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL.

*Run time*: 3 min.

*Retention time*: methane = about 1.8 min.

*Limit*:
- *methane*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (100 ppm *V/V*).

**Hydrogen**. Gas chromatography.

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 300 ppm *V/V* of *hydrogen for chromatography R* in *carbon monoxide R.*

*Column*:
- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: molecular sieve for chromatography (149-177 µm) with a nominal pore size of 0.5 nm.

*Carrier gas*: *argon for chromatography R.*

*Flow rate*: 30 mL/min.

*Temperature*:
- *column*: 100 °C;
- *detector*: 160 °C.

*Detection*: thermal conductivity.

*Injection*: 1 mL.

*Run time*: 4 min.

*Relative retention* with reference to carbon monoxide (retention time = about 2.3 min): hydrogen = about 0.4.

*Limit*:
- *hydrogen*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (300 ppm *V/V*).

**Nickel tetracarbonyl and iron pentacarbonyl**: not detectable, using a detector tube having a limit of detection of 0.1 ppm *V/V* (*2.1.6*).

**Water**: maximum 10 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

ASSAY

Infrared analyser (*2.5.25*).

*Gas to be examined.* The substance to be examined, previously filtered to avoid stray light phenomena.

*Reference gas (a). Carbon monoxide R.*

*Reference gas (b).* A mixture containing 95.0 per cent *V/V* of *carbon monoxide R* and 5.0 per cent *V/V* of *nitrogen R1.*

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

STORAGE

Under pressure in suitable containers complying with the legal regulations.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

A. $CO_2$: carbon dioxide,

B. $CH_4$: methane,

C. $H_2$: hydrogen,

D. $Ni(CO)_4$: nickel tetracarbonyl,

E. $Fe(CO)_5$: iron pentacarbonyl,

F. $H_2O$: water.

**01/2018:2904**

# CARBON MONOXIDE INTERMIX (5 PER CENT) IN NITROGEN

## Carbonei monoxidum (5 per centum) in nitrogenio intermixtum

DEFINITION

A mixture containing 5 per cent *V/V* of *Carbon monoxide (2408)* in *Low-oxygen nitrogen (1685).*

*Content*: 4.75 per cent *V/V* to 5.25 per cent *V/V* of carbon monoxide (CO) in nitrogen ($N_2$).

This monograph applies to carbon monoxide intermix (5 per cent) in nitrogen used in the preparation of lung function test gas mixtures for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

IDENTIFICATION

Carry out either tests A, C or tests B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of carbon monoxide.*

B. It complies with the limits of the assay.

C. Gas chromatography (*2.2.28*).
*Gas to be examined.* The substance to be examined.
*Reference gas. Nitrogen R1.*
*Column*:
– *material*: stainless steel;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).
*Carrier gas*: *helium for chromatography R.*
*Flow rate*: 20 mL/min.
*Temperature*:
– *column*: 80 °C;
– *detector*: 130 °C.
*Detection*: thermal conductivity.
*Injection*: 10 µL.
*Retention time*: nitrogen = about 2 min.
*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with the reference gas.

TESTS

**Water** (*2.5.28*): maximum 10 ppm *V/V*.

ASSAY

Infrared analyser (*2.5.25*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray-light phenomena.

*Reference gas (a).* Mixture containing 5.0 per cent *V/V* of *carbon monoxide R* in *nitrogen R1.*

*Reference gas (b). Nitrogen R1.*

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

STORAGE

As a compressed gas, in appropriate high-pressure cylinders complying with the legal regulations.

LABELLING

The label states the nominal content, in per cent *V/V*, of carbon monoxide in nitrogen.

IMPURITIES

*Specified impurities: A.*

A. water.

**07/2019:1081**

# CARBOPLATIN

## Carboplatinum

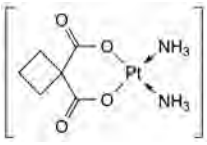

$C_6H_{12}N_2O_4Pt$ $M_r$ 371.3
[41575-94-4]

DEFINITION

(*SP*-4-2)-Diammine[cyclobutan-1,1-di(carboxylato-κ*O*)(2-)]platinum.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless, crystalline powder.

*Solubility*: sparingly soluble in water, very slightly soluble in acetone and in ethanol (96 per cent).

mp: about 200 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of carboplatin.*

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Impurity B and acidity**: maximum 0.5 per cent, calculated as impurity B.

To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.7 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solution*. Dilute 0.5 mL of the test solution to 200.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile for chromatography R* (13:87 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of carboplatin.

*Relative retention* with reference to carboplatin (retention time = about 7 min): impurity A = about 0.3.

*System suitability*: test solution:

- *number of theoretical plates*: minimum 5000; if necessary, adjust the concentration of acetonitrile in the mobile phase;
- *symmetry factor*: maximum 2.0; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 0.5 g in *water R*, heating slightly if necessary, and dilute to 20 mL with the same solvent. Filter if necessary. Dilute 10 mL of this solution to 15 mL with *water R*. Prepare the standard using 5 mL of *chloride standard solution (5 ppm Cl) R*.

**Ammonium** (*2.4.1, Method B*): maximum 100 ppm, determined on 0.20 g.

Prepare the standard using 0.2 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Use the residue obtained in the test for loss on drying. Ignite 0.200 g of the residue to constant mass at 800 ± 50 °C.

1 mg of the residue is equivalent to 1.903 mg of $C_6H_{12}N_2O_4Pt$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

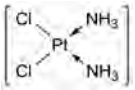


A. (*SP*-4-2)-diamminedichloridoplatinum(II) (cisplatin),

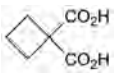


B. cyclobutane-1,1-dicarboxylic acid.

**01/2008:1712**

# CARBOPROST TROMETAMOL

## Carboprostum trometamolum

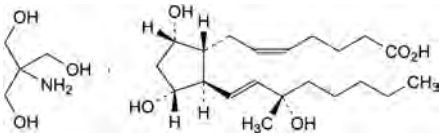


$C_{25}H_{47}NO_8$ $M_r$ 489.7

[58551-69-2]

DEFINITION

2-Amino-2-(hydroxymethyl)propane-1,3-diol (5*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(1*E*,3*S*)-3-hydroxy-3-methyloct-1-enyl]cyclopentyl]hept-5-enoate ((15*S*)-15-methyl-$PGF_2$).

*Content*: 94.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of carboprost trometamol.*

TESTS

**Specific optical rotation** (*2.2.7*): + 18 to + 24 (anhydrous substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 15.0 mg of the substance to be examined in a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water for chromatography R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 15.0 mg of *carboprost trometamol CRS* (containing impurity A) in a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water for chromatography R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) and 0.15 mL of *(15R)-15-methylprostaglandin $F_{2\alpha}$ R* (impurity B) to 100.0 mL with a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water for chromatography R*.

*Reference solution (c).* Dilute 2.0 mL of the test solution to 20.0 mL with a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water for chromatography R*. Dilute 2.0 mL of this solution to 20.0 mL with a mixture of 23 volumes of *acetonitrile R* and 77 volumes of *water for chromatography R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 8-10 nm and a carbon loading of 12-19 per cent.

*Mobile phase*: mix 23 volumes of *acetonitrile R1* and 77 volumes of a 2.44 g/L solution of *sodium dihydrogen phosphate R* in *water for chromatography R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: 1.3 times the retention time of carboprost.

*Relative retention* with reference to carboprost (retention time = about 80 min): impurity B = about 0.85; impurity A = about 0.9.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *carboprost trometamol CRS* to identify the peak due to impurity A.

*System suitability*:

- *resolution*: minimum 3.4 between the peaks due to impurity B and carboprost in the chromatogram obtained with reference solution (b);
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B in the chromatogram obtained with reference solution (a).

*Limits*:

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent),
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent),
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (4.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 50 mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mix 27 volumes of *acetonitrile R1* and 73 volumes of a 2.44 g/L solution of *sodium dihydrogen phosphate R* in *water for chromatography R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Injection*: test solution and reference solution (a).

*Run time*: 1.2 times the retention time of carboprost.

*Retention time*: carboprost = about 29 min.

Calculate the percentage content of $C_{25}H_{47}NO_8$ using the declared content of *carboprost trometamol CRS*.

STORAGE

At a temperature below – 15 ° C.

IMPURITIES

*Specified impurities: A, B.*

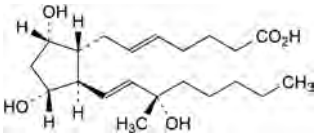

A. (5*E*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(1*E*,3*S*)-3-hydroxy-3-methyloct-1-enyl]cyclopentyl]hept-5-enoic acid,

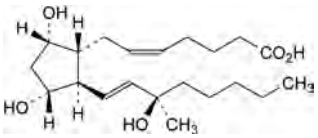

B. (5*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(1*E*,3*R*)-3-hydroxy-3-methyloct-1-enyl]cyclopentyl]hept-5-enoic acid.

**01/2017:1689**

# CARISOPRODOL

## Carisoprodolum

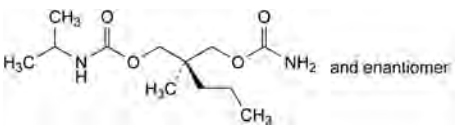


$C_{12}H_{24}N_2O_4$ $M_r$ 260.3

[78-44-4]

DEFINITION

(2*RS*)-2-[(Carbamoyloxy)methyl]-2-methylpentyl (1-methylethyl)carbamate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 92 °C to 95 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *carisoprodol CRS*.

C. Examine the chromatograms obtained in the test for related substances.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (d).

D. Dissolve 0.2 g in 15 mL of a 28 g/L solution of *potassium hydroxide R* in *alcohol R* and boil under a reflux condenser for 15 min. Add 0.5 mL of *glacial acetic acid R* and 1 mL of a 50 g/L solution of *cobalt nitrate R* in *ethanol R*. An intense blue colour develops.

TESTS

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 2.5 g in *alcohol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methylene chloride R*.

*Reference solution (a).* Dissolve 5.0 mg of *meprobamate CRS* in *methylene chloride R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 50 mL with *methylene chloride R*.

*Reference solution (c).* Dilute 5 mL of reference solution (b) to 10 mL with *methylene chloride R*.

*Reference solution (d).* Dissolve 20 mg of *carisoprodol CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (e).* Dissolve the contents of a vial of *carisoprodol impurity A CRS* in 5 mL of reference solution (d) and dilute to 50 mL with *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetone R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air for 15 min.

*Detection*: spray with a solution prepared as follows: dissolve 5 g of *phosphomolybdic acid R* in a mixture of 50 mL of *glacial acetic acid R* and 10 mL of *sulfuric acid R*, and dilute to 100 mL with *glacial acetic acid R*. Heat the plate at 100-105 °C for 30 min.

*System suitability*:

- the chromatogram obtained with reference solution (c) shows 1 clearly visible spot;
- the chromatogram obtained with reference solution (e) shows 2 clearly separated spots.

*Limits*: in the chromatogram obtained with test solution (a):

- *impurity D*: any spot due to impurity D is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *any other impurity*: any spot, apart from the principal spot and any spot due to impurity D, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 15 mL of a 25 per cent *V/V* solution of *sulfuric acid R* and boil under a reflux condenser for 3 h. Cool, dissolve by cautiously adding 30 mL of *water R*, cool again and place in a steam-distillation apparatus. Add 40 mL of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate into 40 mL of a 40 g/L solution of *boric acid R* until the total volume in the receiver reaches about 200 mL. Add 0.25 mL of *methyl red mixed solution R*. Titrate with *0.1 M hydrochloric acid*, until the colour changes from green to violet. Carry out a blank titration.

1 mL of *0.1 M hydrochloric acid* is equivalent to 13.02 mg of $C_{12}H_{24}N_2O_4$.

IMPURITIES

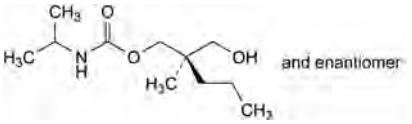


A. (2*RS*)-2-(hydroxymethyl)-2-methylpentyl (1-methylethyl)-carbamate,

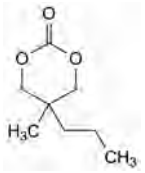


B. 5-methyl-5-propyl-1,3-dioxan-2-one,

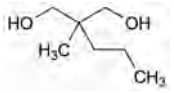


C. 2-methyl-2-propylpropane-1,3-diol,

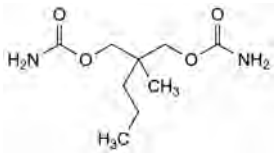


D. 2-methyl-2-propylpropane-1,3-diyl dicarbamate (meprobamate).

**01/2017:2360**

# CARMELLOSE(2)

## Carmellosum

[9000-11-7]

DEFINITION

Carboxymethylether of cellulose.

Partly *O*-carboxymethylated cellulose.

♦CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: practically insoluble in anhydrous ethanol. It swells with water to form a suspension and becomes viscid in 1 M sodium hydroxide.♦

IDENTIFICATION

A. pH (*2.2.3*): 3.5 to 5.0.
Suspend 1.0 g in 100 mL of *carbon dioxide-free water R*.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *carmellose CRS*.

TESTS

**Chlorides**: maximum 0.36 per cent.

Shake 0.8 g with 50 mL of *water R*, dissolve in 10 mL of *1 M sodium hydroxide* and dilute to 100 mL with *water R*. Heat on a water-bath a mixture of 10 mL of *dilute nitric acid R* and 20 mL of this solution until a flocculent precipitate is produced. Cool, centrifuge and take out the supernatant. Wash the precipitate with 3 quantities, each of 10 mL, of *water R*, centrifuging each time. Combine the supernatant and the washings and dilute to 100 mL with *water R*. To 25 mL of this solution add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R* (test

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

solution). Prepare the reference solution in the same manner, using 0.40 mL of *0.01 M hydrochloric acid*. Add 1 mL of *silver nitrate solution R2* to the test solution and the reference solution. Allow to stand protected from light for 5 min. Any opalescence in the test solution is not more intense than that in the reference solution.

**Sulfates**: maximum 0.72 per cent.

Shake 0.40 g with 25 mL of *water R*, dissolve in 5 mL of *1 M sodium hydroxide* and add 20 mL of *water R*. Heat this solution with 2.5 mL of *hydrochloric acid R* in a water-bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant. Wash the precipitate with 3 quantities, each of 10 mL, of *water R*, centrifuging each time. Combine the supernatant and the washings, and dilute to 100 mL with *water R*. Filter, and discard the first 5 mL of the filtrate. To 25 mL of the filtrate add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R* (test solution). Prepare the reference solution in the same manner, using 1.5 mL of *0.005 M sulfuric acid*. Add 2 mL of a 120 g/L solution of *barium chloride R* to the test solution and the reference solution. Mix and allow to stand for 10 min. The white turbidity produced in the test solution is not thicker than that in the reference solution.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent (dried substance), determined on 1.0 g.

♦STORAGE

In an airtight container.♦

**04/2018:0886**

## CARMELLOSE CALCIUM(3)

### Carmellosum calcicum

[9050-04-8]

DEFINITION

Calcium salt of partly *O*-carboxymethylated cellulose. Calcium salt of a polycarboxymethyl ether of cellulose.

♦CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic after drying.

*Solubility*: practically insoluble in acetone, in ethanol (96 per cent) and in toluene. It swells with water to form a suspension.♦

IDENTIFICATION

A. Shake 0.1 g thoroughly with 10 mL of *water R*. Add 2 mL of a 42 g/L solution of *sodium hydroxide R* and allow to stand for 10 min (solution A). Dilute 1 mL of solution A to 5 mL with *water R*. To 0.05 mL of this solution add 0.5 mL of a 0.5 g/L solution of *chromotropic acid, sodium salt R* in a 75 per cent *m/m* solution of *sulfuric acid R* and heat on a water-bath for 10 min. A reddish-violet colour develops.

B. Shake 5 mL of solution A obtained in identification test A with 10 mL of *acetone R*. A white, flocculent precipitate is produced.

C. Shake 5 mL of solution A obtained in identification test A with 1 mL of *ferric chloride solution R1*. A brown, flocculent precipitate is formed.

D. Ignite 1 g to ash and dissolve the residue in a mixture of 5 mL of *acetic acid R* and 10 mL of *water R*. Filter if necessary and heat the filtrate to boiling. Cool and neutralise with *dilute ammonia R1*. The solution gives reaction (a) of calcium (*2.3.1*).

TESTS

**Solution S**. Shake 1.0 g with 50 mL of *distilled water R*, add 5 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *distilled water R*.

**Alkalinity**. Shake 1.0 g thoroughly with 50 mL of *carbon dioxide-free water R* and add 0.1 mL of *phenolphthalein solution R*. No red colour develops.

**Chlorides** (*2.4.4*): maximum 0.36 per cent.

Heat 28 mL of solution S with 10 mL of *dilute nitric acid R* on a water-bath until a flocculent precipitate is produced. Cool, centrifuge and separate the supernatant. Wash the precipitate with 3 quantities, each of 10 mL, of *water R*, centrifuging each time. Combine the supernatant and the washings and dilute to 100 mL with *water R*. To 25 mL add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Dilute 10 mL of the solution to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1 per cent.

Heat 20 mL of solution S with 1 mL of *hydrochloric acid R* on a water-bath until a flocculent precipitate is produced. Cool, centrifuge and separate the supernatant. Wash the precipitate with 3 quantities, each of 10 mL, of *distilled water R*, centrifuging each time. Combine the supernatant and the washings and dilute to 100 mL with *distilled water R*. To 25 mL add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): 10.0 per cent to 20.0 per cent, determined on 1.0 g of the dried substance.

♦STORAGE

In an airtight container.♦

**01/2020:0472**

## CARMELLOSE SODIUM

### Carmellosum natricum

[9004-32-4]

DEFINITION

Carboxymethylcellulose sodium. Sodium salt of a partly *O*-carboxymethylated cellulose.

*Content*: 6.5 per cent to 10.8 per cent of sodium (Na) (dried substance).

CHARACTERS

*Appearance*: white or almost white, granular powder, hygroscopic after drying.

*Solubility*: practically insoluble in acetone, in anhydrous ethanol and in toluene. It is easily dispersed in water giving colloidal solutions.

IDENTIFICATION

A. To 10 mL of solution S (see Tests) add 1 mL of *copper sulfate solution R*. A blue, cotton-like precipitate is formed.

B. Boil 5 mL of solution S for a few minutes. No precipitate is formed.

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

C. To the residue obtained in the determination of the sulfated ash, add 1 mL of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 mL of *water R*. The solution gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Sprinkle a quantity of the substance to be examined equivalent to 1.0 g of the dried substance onto 90 mL of *carbon dioxide-free water R* at 40 °C to 50 °C stirring vigorously. Continue stirring until a colloidal solution is obtained, cool and dilute to 100 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 6.0 to 8.0.

**Viscosity**: 75 per cent to 140 per cent of the value stated on the label.

While stirring, introduce a quantity of the substance to be examined equivalent to 2.00 g of the dried substance into 50 mL of *water R* heated to 90 °C. For a product of low viscosity, use, if necessary, the quantity required to give the concentration indicated on the label. Allow to cool, dilute to 100.0 mL with *water R* and stir until dissolution is complete. Determine the viscosity (*2.2.10*) using a rotating viscometer at 20 °C and a shear rate of 10 $s^{-1}$. If it is impossible to obtain a shear rate of exactly 10 $s^{-1}$, use a shear rate slightly higher and a rate slightly lower and interpolate.

**Sodium glycolate**: maximum 0.4 per cent (dried substance).

*Test solution.* Place a quantity of the substance to be examined equivalent to 0.500 g of dried substance in a beaker. Add 5 mL of *acetic acid R* and 5 mL of *water R*. Stir until dissolution is complete (about 30 min). Add 80 mL of *acetone R* and 2 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and filter with *acetone R* and dilute the filtrate to 100.0 mL with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant.

*Reference solution.* In a volumetric flask, dissolve 0.310 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*), in *water R* and dilute to 1000.0 mL with the same solvent. Place 5.0 mL of this solution in a volumetric flask, add 5 mL of *acetic acid R* and allow to stand for about 30 min. Add 80 mL of *acetone R* and 2 g of *sodium chloride R* and dilute to 100.0 mL with *acetone R*.

Place 2.0 mL of each solution in a separate 25 mL volumetric flask. Heat on a water-bath to eliminate acetone. Cool to room temperature and add 5.0 mL of *2,7-dihydroxynaphthalene solution R* to each flask. Shake and add 15.0 mL of *2,7-dihydroxynaphthalene solution R*. Close the flasks with aluminium foil and heat on a water-bath for 20 min. Cool under running water and dilute to 25.0 mL with *sulfuric acid R*. Within 10 min, transfer 10.0 mL of each solution to a flat-bottomed tube. Examine the solutions viewing vertically. The test solution is not more intensely coloured than the reference solution.

**Chlorides** (*2.4.4*): maximum 0.25 per cent.

Dilute 2 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): 20.0 per cent to 33.3 per cent, determined on 1.0 g using a mixture of equal volumes of *sulfuric acid R* and *water R* and calculated with reference to the dried substance. These limits correspond to a content of 6.5 per cent to 10.8 per cent of sodium (Na).

LABELLING

The label states:

– the viscosity in millipascal seconds for a 20 g/L solution;

– for a product of low viscosity, the concentration of the solution to be used and the viscosity in millipascal seconds.

**01/2020:1186**

# CARMELLOSE SODIUM, LOW-SUBSTITUTED

## Carmellosum natricum substitutum humile

[9050-32-4]

DEFINITION

Low-substituted sodium carboxymethylcellulose. Sodium salt of a partly *O*-(carboxymethylated) cellulose.

*Content*: 2.0 per cent to 4.5 per cent of sodium (Na) (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or short fibres.

*Solubility*: practically insoluble in acetone, in anhydrous ethanol and in toluene. It swells in water to form a gel.

IDENTIFICATION

A. Shake 1 g with 100 mL of a 100 g/L solution of *sodium hydroxide R*. A suspension is produced.

B. Shake 1 g with 50 mL of *water R*. Transfer 1 mL of the mixture to a test tube, add 1 mL of *water R* and 0.05 mL of a freshly prepared 40 g/L solution of *α-naphthol R* in *methanol R*. Incline the test tube and add carefully 2 mL of *sulfuric acid R* down the side so that it forms a lower layer. A reddish-purple colour develops at the interface.

C. Sulfated ash (*2.4.14*) (see Tests).

D. To the residue obtained in the determination of the sulfated ash, add 1 mL of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 6.0 to 8.5.

Shake 1 g with 100 mL of *carbon dioxide-free water R* for 5 min. Centrifuge.

**Sodium chloride and sodium glycolate**: maximum 0.5 per cent (dried substance) for the sum of the percentage contents.

*Sodium chloride.* Place 5.00 g in a 250 mL conical flask, add 50 mL of *water R* and 5 mL of *strong hydrogen peroxide solution R* and heat on a water bath for 20 min, stirring occasionally to ensure total hydration. Cool, add 100 mL of *water R* and 10 mL of *nitric acid R*. Titrate with *0.05 M silver nitrate* determining the end-point potentiometrically (*2.2.20*) using a silver-based indicator electrode and a double-junction reference electrode containing a 100 g/L solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket.

1 mL of *0.05 M silver nitrate* is equivalent to 2.922 mg of NaCl.

*Sodium glycolate.* Place a quantity of the substance to be examined equivalent to 0.500 g of the dried substance in a beaker. Add 5 mL of *glacial acetic acid R* and 5 mL of *water R* and stir to ensure total hydration (about 30 min). Add 80 mL of *acetone R* and 2 g of *sodium chloride R*. Stir for several minutes to ensure complete precipitation of the carboxymethylcellulose. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and filter with *acetone R* and dilute the filtrate to 100.0 mL with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant as the test solution.

Prepare the reference solutions as follows: in a 100 mL volumetric flask, dissolve 0.100 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*), in *water R* and dilute to 100.0 mL with the same solvent. Transfer 0.5 mL, 1.0 mL, 1.5 mL and 2.0 mL of the solution to separate volumetric flasks; dilute the contents of each flask to 5.0 mL with *water R*, add 5 mL of *glacial acetic acid R*, dilute to 100.0 mL with *acetone R* and mix.

Transfer 2.0 mL of the test solution and 2.0 mL of each of the reference solutions to separate 25 mL volumetric flasks. Heat the uncovered flasks in a water-bath to eliminate the acetone. Allow to cool and add 5.0 mL of *2,7-dihydroxynaphthalene solution R* to each flask. Mix, add a further 15.0 mL of *2,7-dihydroxynaphthalene solution R* and mix again. Close the flasks with aluminium foil and heat in a water-bath for 20 min. Cool and dilute to 25.0 mL with *sulfuric acid R*.

Measure the absorbance (*2.2.25*) of each solution at 540 nm. Prepare a blank using 2.0 mL of a solution containing 5 per cent *V/V* each of *glacial acetic acid R* and *water R* in *acetone R*. Prepare a standard curve using the absorbances obtained with the reference solutions. From the standard curve and the absorbance of the test solution, determine the mass *a*, in milligrams, of glycolic acid in the substance to be examined and calculate the content of sodium glycolate from the following expression:

$$\frac{10 \times 1.29 \times a}{(100 - b)m}$$

1.29 = factor converting glycolic acid to sodium glycolate,

*b* = loss on drying as a percentage,

*m* = mass of the substance to be examined, in grams.

**Water-soluble substances**: maximum 70.0 per cent.

Disperse 5.00 g in 400.0 mL of *water R* and stir for 1 min every 10 min during the first 30 min. Allow to stand for 1 h and centrifuge, if necessary. Decant 100.0 mL of the supernatant onto a fast filter paper in a vacuum filtration funnel, apply vacuum and collect 75.0 mL of the filtrate. Evaporate to dryness and dry the residue at 100-105 °C for 4 h.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): 6.5 per cent to 13.5 per cent (dried substance), corresponding to a content of 2.0 per cent to 4.5 per cent of Na.

Use 1.0 g with a mixture of equal volumes of *sulfuric acid R* and *water R*.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for low-substituted carmellose sodium used as disintegrant.*

**Settling volume**: 15.0 mL to 35.0 mL.

In a 100 mL graduated cylinder, place 20 mL of *2-propanol R*, add 5.0 g of the substance to be examined and shake vigorously. Dilute to 30 mL with *2-propanol R* then to 50 mL with *water R* and shake vigorously. Within 15 min, repeat the shaking 3 times. Allow to stand for 4 h and determine the volume of the settled mass.

**01/2020:1187**

# CARMUSTINE

## Carmustinum

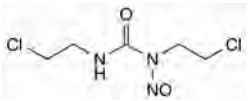

$C_5H_9Cl_2N_3O_2$ $M_r$ 214.1
[154-93-8]

DEFINITION

*N*,*N*′-Bis(2-chloroethyl)-*N*-nitrosourea.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellowish, granular powder.

*Solubility*: very slightly soluble in water, very soluble in methylene chloride, freely soluble in anhydrous ethanol.

mp: about 31 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the melted substance prepared as a film.

*Comparison*: *carmustine CRS*.

TESTS

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in 0.5 mL of *methanol R*.

*Reference solution (a).* Dissolve 30.0 mg of *chloroethylamine hydrochloride R* (impurity B) in 25 mL of *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 0.20 g of the substance to be examined in 0.5 mL of reference solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*:

– *mobile phase A*: *ethyl acetate R*;

– *mobile phase B*: *methanol R*, *ethyl acetate R* (30:70 *V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate with mobile phase A, allow to dry in air for 5 min; over 1/3 of the plate with mobile phase B, allow to dry in air for 10 min.

*Detection*: spray with *diethylamine R* and heat at 105 °C for 20 min; allow to cool and spray with *silver nitrate solution R2*. Expose to ultraviolet light at 365 nm until brown to black spots appear and examine in daylight.

*Retardation factors*: impurity B = about 0.2; carmustine = about 0.9.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity B*: any spot due to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).
*Carry out the test protected from light. Prepare the solutions immediately before use.*

*Solvent mixture*: *ethanol (96 per cent) R*, *water R* (10:90 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 1 mL of *ethanol (96 per cent) R* and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10.0 mg of *carmustine impurity A CRS* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 25.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* To 1 mL of the test solution add 1 mL of reference solution (b) and dilute to 100 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 2.7 g of *potassium dihydrogen phosphate R* in 1000 mL of *water for chromatography R*, add 1 mL of *triethylamine R* and adjust to pH 3.5 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 85 | 15 |
| 10 - 60 | 85 → 30 | 15 → 70 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm and, for impurity A, at 205 nm.

*Autosampler*: set at 5 °C.

*Injection*: 20 µL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to carmustine (retention time = about 36 min): impurity A = about 0.3.

*System suitability*: reference solution (d):

- *resolution*: minimum 5.0 between the peaks due to impurity A and carmustine.

*Calculation of percentage contents*:

- for impurity A, use the concentration of impurity A in reference solution (c);
- for impurities other than A, use the concentration of carmustine in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.100 g in 30 mL of *anhydrous ethanol R* and dilute to 100.0 mL with *water R*. Dilute 3.0 mL of the solution to 100.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 230 nm.

Calculate the content of $C_5H_9Cl_2N_3O_2$ taking the specific absorbance to be 270.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B.*

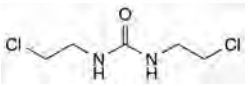

A. *N*,*N*′-bis(2-chloroethyl)urea,

B. 2-chloroethan-1-amine.

**01/2016:0597**

# CARNAUBA WAX

## Cera carnauba

[8015-86-9]

DEFINITION

Purified wax obtained from the leaves of *Copernicia cerifera* Mart.

CHARACTERS

*Appearance*: pale yellow or yellow powder, flakes or hard masses.

*Solubility*: practically insoluble in water, soluble on heating in ethyl acetate and in xylene, practically insoluble in ethanol (96 per cent).

*Relative density*: about 0.97.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined with heating in 5 mL of *chloroform R*. Use the warm solution.

*Reference solution.* Dissolve 5 mg of *menthol R*, 5 µL of *menthyl acetate R* and 5 mg of *thymol R* in 10 mL of *toluene R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *chloroform R* (2:98 *V/V*).

*Application*: 30 µL of the test solution and 10 µL of the reference solution as bands 20 mm by 3 mm.

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: spray with a freshly prepared 200 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* (about 10 mL for a 20 cm plate). Heat at 100-105 °C for 10-15 min.

*Results*: the chromatogram obtained with the reference solution shows in the lower part a dark blue zone (menthol), above this zone a reddish zone (thymol) and in the upper part a dark blue zone (menthyl acetate). The chromatogram obtained with the test solution shows a large blue zone (triacontanol = melissyl alcohol) at a level between the thymol and menthol zones in the chromatogram obtained with the reference solution. Further blue zones are visible in the upper part of the chromatogram obtained with the test solution, at levels between those of the menthyl acetate and thymol zones in the chromatogram obtained with the reference solution; above these zones, further zones are visible in the chromatogram obtained with the test solution; the zone with the highest $R_F$ value is very pronounced. A number of faint zones are visible below the triacontanol zone and the point of application is coloured blue.

TESTS

**Melting point** (*2.2.15*): 80 °C to 88 °C.

Melt the substance to be examined carefully on a water-bath before introduction into the capillary tubes. Allow the tubes to stand in the refrigerator for 24 h or at 0 °C for 2 h.

**Acid value**: 2 to 7.

To 2.000 g (*m* g) in a 250 mL conical flask fitted with a reflux condenser add 40 mL of *xylene R* and a few glass beads. Heat with stirring until the substance is completely dissolved. Add 20 mL of *ethanol (96 per cent) R* and 1 mL of *bromothymol blue solution R3* and titrate the hot solution with *0.5 M alcoholic potassium hydroxide* until a green colour persisting for at least 10 s is obtained ($n_1$ mL). Carry out a blank test ($n_2$ mL). Calculate the acid value using the following expression:

$$\frac{28.05(n_1 - n_2)}{m}$$

**Saponification value**: 78 to 95.

To 2.000 g (*m* g) in a 250 mL conical flask fitted with a reflux condenser add 40 mL of *xylene R* and a few glass beads. Heat with stirring until the substance is completely dissolved. Add 20 mL of *ethanol (96 per cent) R* and 20.0 mL of *0.5 M alcoholic potassium hydroxide*. Boil under a reflux condenser for 3 h. Add 1 mL of *phenolphthalein solution R1* and titrate the hot solution immediately with *0.5 M hydrochloric acid* until the red colour disappears. Repeat the heating and titration until the colour no longer reappears on heating ($n_3$ mL). Carry out a blank test ($n_4$ mL). Calculate the saponification value using the following expression:

$$\frac{28.05(n_4 - n_3)}{m}$$

**Total ash** (*2.4.16*): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

Protected from light.

**07/2008:2201**
**corrected 7.8**

# CARPROFEN FOR VETERINARY USE

## Carprofenum ad usum veterinarium

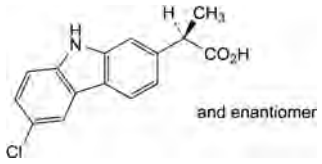


$C_{15}H_{12}ClNO_2$ $M_r$ 273.7
[53716-49-7]

DEFINITION

(2*RS*)-2-(6-Chloro-9*H*-carbazol-2-yl)propanoic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in methanol, slightly soluble in 2-propanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *carprofen CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_3$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.5 mg of *carprofen for system suitability CRS* (containing impurity C) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*: mix 30 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R* and 70 volumes of *methanol R2*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of carprofen.

*Retention time*: carprofen = about 10 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity C and carprofen.

*Limits*:

- *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent. 12 mL of the solution complies with test B. Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *ethanol (96 per cent) R*. Add 1.0 mL of *0.1 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.37 mg of $C_{15}H_{12}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H.*

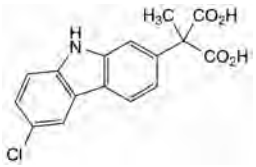

A. 2-(6-chloro-9*H*-carbazol-2-yl)-2-methylpropanedioic acid,

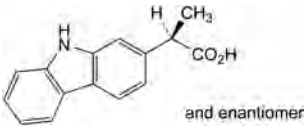


B. (2*RS*)-2-(9*H*-carbazol-2-yl)propanoic acid,

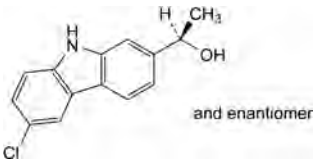


C. (1*RS*)-1-(6-chloro-9*H*-carbazol-2-yl)ethanol,

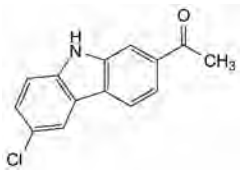

D. 1-(6-chloro-9*H*-carbazol-2-yl)ethanone,

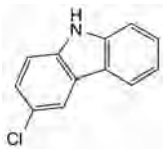

E. 3-chloro-9*H*-carbazole,

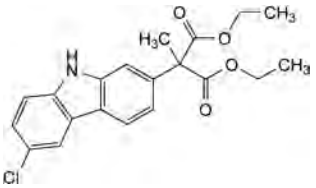

F. diethyl 2-(6-chloro-9*H*-carbazol-2-yl)-2-methylpropanedioate,

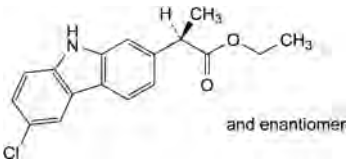


G. ethyl (2*RS*)-2-(6-chloro-9*H*-carbazol-2-yl)propanoate,

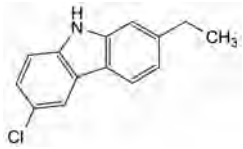

H. 6-chloro-2-ethyl-9*H*-carbazole.

**04/2015:2138**

# CARRAGEENAN

## Carrageenanum

DEFINITION

Polysaccharides extracted from different Rhodophyceae with boiling water or aqueous alkali solutions. Carrageenan is separated by alcohol precipitation, potassium chloride precipitation, gel pressing, drum drying or freezing. The alcohol used during separation and purification is generally 2-propanol. The main components are potassium, sodium, calcium or magnesium salts of the sulfate esters of D-galactose and 3,6-anhydro-D-galactose copolymers. They exist in different proportions depending on the biological origin of the polymer.

The prevalent copolymers are designated as κ-, ι- and λ-carrageenan.

CHARACTERS

*Appearance*: yellowish, brownish, or white or almost white powder.

*Solubility*: soluble in water giving a viscous or colloidal solution, insoluble in organic solvents.

IDENTIFICATION

A. Prepare a 20 g/L dispersion and heat in a water-bath at 80 °C. Mix 1 volume of this solution and about 4 volumes of *water R* and add 2-3 drops of a 0.5 g/L solution of *methylene blue R* in *ethanol (96 per cent) R*. A blue precipitate is formed.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: prepare a 2 g/L solution of the substance to be examined; cast 4.5 mL of the solution into a plastic flat-bottomed weighing boat about 35 mm in diameter and allow to dry completely in an air-flow oven at 60 °C until a film, about 10 µm thick, is obtained (about 4 h).

Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000-1100 $cm^{-1}$ region. Absorption maxima are 1065 $cm^{-1}$ and 1020 $cm^{-1}$ for gelling and non-gelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 $cm^{-1}$ are shown in Table 2138.-1.

Table 2138.-1. – *Characteristic absorption bands for carrageenan identification by infrared absorption spectrophotometry*

| Wavenumber ($cm^{-1}$) | Molecular structure | Absorbance relative to the absorbance at 1050 $cm^{-1}$ | | |
|---|---|---|---|---|
| | | κ | ι | λ |
| 1220 - 1260 | Ester sulfate | 0.7 - 1.2 | 1.2 -2.0 | 1.4 - 2.0 |
| 928 - 933 | 3,6-Anhydro-D-galactose | 0.3 - 0.6 | 0.2 - 0.5 | ≤ 0.2 |
| 840 - 850 | Galactose-4-sulfate | 0.3 - 0.5 | 0.2 - 0.4 | - |
| 825 - 830 | Galactose-2-sulfate | - | - | 0.2 - 0.4 |
| 810 - 820 | Galactose-6-sulfate | - | - | 0.1 - 0.3 |
| 800 - 805 | 3,6-Anhydro-D-galactose-2-sulfate | ≤ 0.2 | 0.2 - 0.4 | - |

Table 2138.-1 shows absorbance ratios corresponding to copolymer types isolated from carrageenans. Native extracts of carrageenan from most cold-water seaweed species comprise κ-, ι- and λ-structures from the natural mix of haploid and diploid plants. Such extracts exhibit, in practice, the characteristic absorbance peaks of κ-, ι- and λ-structures and yield absorbance ratios somewhere between the above-mentioned individual ranges.

TESTS

**Viscosity** (*2.2.10*): minimum 5 mPa·s. Heat a 15 g/L dispersion (dried substance) at 80 °C for at least 15 min to dissolve. Compensate for any loss of water by evaporation, allow to cool to 75 °C and carry out the test at this temperature.

**Arsenic** (*2.4.27*): maximum 3.0 ppm.

**Cadmium** (*2.4.27*): maximum 2.0 ppm.

**Lead** (*2.4.27*): maximum 5.0 ppm.

**Mercury** (*2.4.27*): maximum 1.0 ppm.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 40.0 per cent.

**Ash insoluble in hydrochloric acid** (*2.8.1*): maximum 2.0 per cent.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for carrageenan used as viscosity-increasing agent.*

**Gel formation**. Prepare a 20 g/L dispersion and heat in a water-bath at 80 °C (solution A). Allow to cool; it becomes more viscous upon cooling and may form a gel.

To 10 mL of solution A, while still hot, add 4 drops of a 100 g/L solution of *potassium chloride R*, mix and allow to cool. A 'brittle' gel indicates a carrageenan of a predominantly κ-type; an 'elastic' gel indicates a predominantly ι-type; if the solution does not form a gel, the carrageenan is of a predominantly λ-type; a weak or pourable gel obtained on cooling indicates a carrageenan comprising a mixture of κ- and λ-types, which can be confirmed by infrared absorption spectrophotometry.

**Viscosity** (see Tests).

**01/2008:1972**
**corrected 6.0**

# CARTEOLOL HYDROCHLORIDE

## Carteololi hydrochloridum

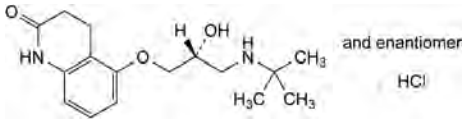


$C_{16}H_{25}N_2O_3Cl$ $M_r$ 328.8
[51781-21-21-6]

DEFINITION

5-[(2*RS*)-3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystals or crystalline powder.

*Solubility*: soluble in water, sparingly soluble in methanol, slightly soluble in ethanol 96 per cent, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of carteolol hydrochloride.*

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.300 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 5.0 to 6.0.

Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 10 mg of *carteolol for system suitability CRS* in the mobile phase and dilute to 5 mL with the mobile phase.

*Reference solution (d)*. Dilute 5.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 1 volume of *methanol R2*, 20 volumes of *acetonitrile R* and 79 volumes of a 2.82 g/L solution of *sodium hexanesulfonate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 252 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *carteolol for system suitability CRS* to identify the peak due to impurity H.

*System suitability*:

- the chromatogram obtained with reference solution (c) is similar to the chromatogram provided with *carteolol for system suitability CRS*; the peaks due to impurity H and carteolol show base-line separation;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (d);
- *number of theoretical plates*: minimum 6000, calculated for the principal peak in the chromatogram obtained with reference solution (a).

*Limits*:

- *impurity H*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.88 mg of $C_{16}H_{25}N_2O_3Cl$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, I.*

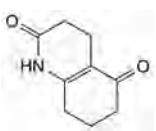

A. 4,6,7,8-tetrahydroquinoline-2,5(1*H*,3*H*)-dione,

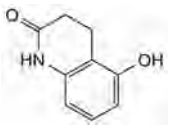

B. 5-hydroxy-3,4-dihydroquinolin-2(1*H*)-one,

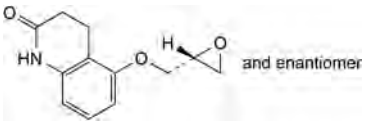

C. 5-[[(2*RS*)-oxiran-2-yl]methoxy]-3,4-dihydroquinolin-2(1*H*)-one,

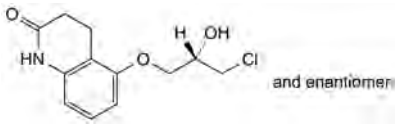

D. 5-[(2*RS*)-3-chloro-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

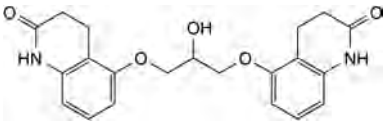

E. 5,5′-[(2-hydroxypropan-1,3-diyl)bis(oxy)]bis(3,4-dihydroquinolin-2(1*H*)-one),

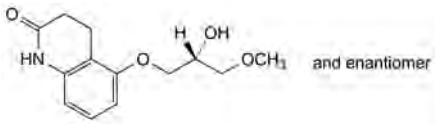

F. 5-[(2*RS*)-2-hydroxy-3-methoxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

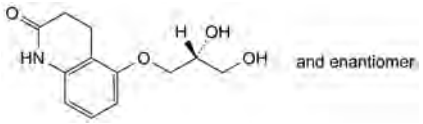

G. 5-[(2*RS*)-2,3-dihydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one,

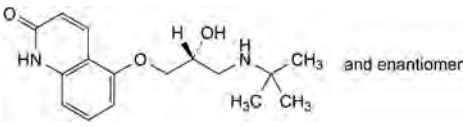

H. 5-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]quinolin-2(1*H*)-one,

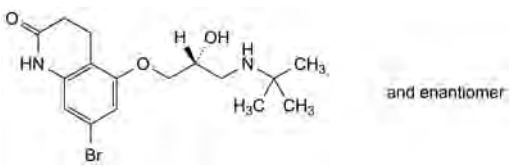

I. 7-bromo-5-[(2*RS*)-3-[(1,1-(dimethylethyl)amino]-2-hydroxypropoxy]-3,4-dihydroquinolin-2(1*H*)-one.

**01/2017:1745**

# CARVEDILOL

## Carvedilolum

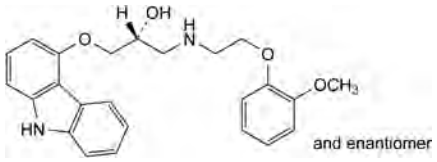


$C_{24}H_{26}N_2O_4$ $M_r$ 406.5
[72956-09-3]

DEFINITION

(2*RS*)-1-(9*H*-Carbazol-4-yloxy)-3-[[2-(2-methoxyphenoxy)-ethyl]amino]propan-2-ol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent). It is practically insoluble in dilute acids.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *carvedilol CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *carvedilol impurity C CRS* in 5.0 mL of the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 4.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *carvedilol for system suitability CRS* (containing impurities A and D) in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.150 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 55 °C.

*Mobile phase*: dissolve 1.77 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 650 mL with the same solvent; adjust to pH 2.0 with *phosphoric acid R* and add 350 mL of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of carvedilol.

*Identification of impurities*: use the chromatogram supplied with *carvedilol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and D; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to carvedilol (retention time = about 4 min): impurity A = about 0.5; impurity C = about 2.9; impurity D = about 3.8.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to impurity A and carvedilol in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2.0;
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.02 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than C*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 40.65 mg of $C_{24}H_{26}N_2O_4$.

IMPURITIES

*Specified impurities*: *A, C, D*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

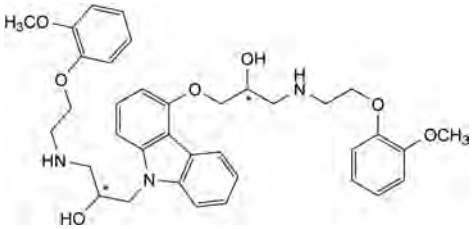

A. 1-[[9-[2-hydroxy-3-[[2-(2-methoxyphenoxy)ethyl]amino]-propyl]-9*H*-carbazol-4-yl]oxy]-3-[[2-(2-methoxyphenoxy)ethyl]amino]propan-2-ol,

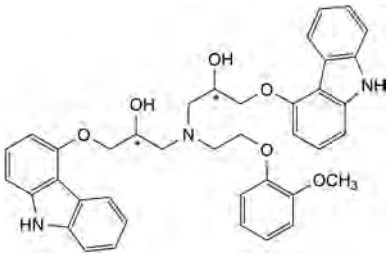

B. 1,1′-[[2-(2-methoxyphenoxy)ethyl]nitrilo]bis[3-(9*H*-carbazol-4-yloxy)propan-2-ol],

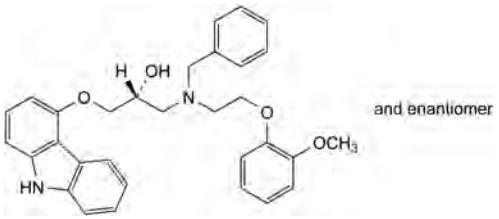


C. (2*RS*)-1-[benzyl[2-(2-methoxyphenoxy)ethyl]amino]-3-(9*H*-carbazol-4-yloxy)propan-2-ol,

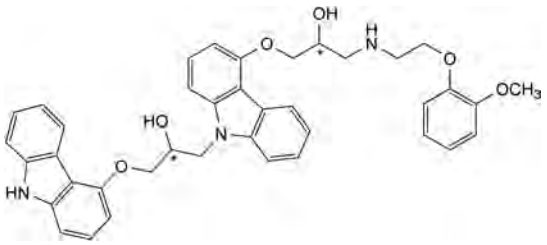

D. 1-(9*H*-carbazol-4-yloxy)-3-[4-[2-hydroxy-3-[[2-(2-methoxyphenoxy)ethyl]amino]propoxy]-9*H*-carbazol-9-yl]propan-2-ol.

**01/2020:1497**

# CASTOR OIL, HYDROGENATED

## Ricini oleum hydrogenatum

DEFINITION

Fatty oil obtained by hydrogenation of *Virgin castor oil (0051)*. It consists mainly of the triglyceride of 12-hydroxystearic ((12Ξ)-12-hydroxyoctadecanoic) acid.

CHARACTERS

*Appearance*: fine, almost white or pale yellow powder or almost white or pale yellow masses or flakes.

*Solubility*: practically insoluble in water, slightly soluble in methylene chloride, very slightly soluble in anhydrous ethanol, practically insoluble in light petroleum.

IDENTIFICATION

A. Melting point (*2.2.14*): 83 °C to 88 °C.

B. Hydroxyl value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 4.0, determined on 10.0 g dissolved in 75 mL of hot *ethanol (96 per cent) R*.

**Hydroxyl value** (*2.5.3, Method A*): 145 to 165, determined on a warm solution.

**Iodine value** (*2.5.4, Method A*): maximum 5.0.

**Alkaline impurities**. Dissolve 1.0 g with gentle heating in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3 mL of *toluene R*. Add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*. Not more than 0.2 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*) with the following modifications. Use the mixture of calibrating substances in Table 2.4.22.-3.

*Test solution*. Introduce 75 mg of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve in 2 mL of *1,1-dimethylethyl methyl ether R1* by shaking and heat gently (50-60 °C). Add, when still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for at least 5 min. Add 5 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Use the upper layer.

*Reference solution*. Dissolve 50 mg of *methyl 12-hydroxystearate CRS* and 50 mg of *methyl stearate CRS* in 10.0 mL of *1,1-dimethylethyl methyl ether R1*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m; Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 55 | 215 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*:

- *symmetry factor*: 0.7 to 1.5 for the peak due to methyl stearate in the chromatogram obtained with the test solution.

Calculate the fraction of each fatty acid using the following expression:

$$A_{x,s,c} / \sum A_{x,s,c} \times 100 \text{ per cent } m / m$$

$A_{x,s,c}$ = corrected peak area of the fatty acid in the test solution:

$$A_{x,s,c} = A_{x,s} \times R_c$$

$R_c$ = relative correction factor for the peak due to methyl 12-hydroxystearate:

$$R_c = \frac{m_{1,r} \times A_{2,r}}{A_{1,r} \times m_{2,r}}$$

$R_c$ = 1 for peaks corresponding to each of the other specified fatty acids or any unspecified fatty acid;

$m_{1,r}$ = mass of methyl 12-hydroxystearate in the reference solution;

$m_{2,r}$ = mass of methyl stearate in the reference solution;

$A_{1,r}$ = area of the peak due to methyl 12-hydroxystearate in the chromatogram obtained with the reference solution;

$A_{2,r}$ = area of the peak due to methyl stearate in the chromatogram obtained with the reference solution;

$A_{x,s}$ = area of the peaks due to any specified or unspecified fatty acid methyl esters.

*Composition of the fatty-acid fraction of the oil*:

- *palmitic acid*: maximum 2.0 per cent;
- *stearic acid*: 7.0 per cent to 14.0 per cent;
- *arachidic acid*: maximum 1.0 per cent;
- *12-oxostearic acid*: maximum 5.0 per cent;
- *12-hydroxystearic acid*: 78.0 per cent to 91.0 per cent;
- *any other fatty acid*: maximum 3.0 per cent.

STORAGE

In a well-filled container.

IMPURITIES

A. 12-oxooctadecanoic acid (12-oxostearic acid).

**01/2020:2367**

# CASTOR OIL, REFINED

## Ricini oleum raffinatum

DEFINITION

Fatty oil obtained from the seeds of *Ricinus communis* L. by cold expression. It is then refined. A suitable antioxidant may be added.

PRODUCTION

During the expression step, the temperature of the oil must not exceed 50 °C.

CHARACTERS

*Appearance*: clear, almost colourless or slightly yellow, viscous, hygroscopic liquid.

*Solubility*: slightly soluble in light petroleum, miscible with ethanol (96 per cent) and with glacial acetic acid.

*Relative density*: about 0.958.

*Refractive index*: about 1.479.

*Viscosity*: about 1000 mPa·s.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B.*

A. A mixture of 2 mL of the substance to be examined and 8 mL of *ethanol (96 per cent) R* is clear (*2.2.1*).

B. Specific absorbance (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_3$ or $Y_3$ (*2.2.2, Method I*).

**Optical rotation** (*2.2.7*): + 3.5° to + 6.0°.

**Specific absorbance** (*2.2.25*): 0.7 to 1.5, determined at the absorption maximum at 270 nm.

To 1.00 g add *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 0.8.

Dissolve 5.00 g in 25 mL of the prescribed mixture of solvents.

**Hydroxyl value** (*2.5.3, Method A*): minimum 160.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 0.8 per cent, determined on 5.0 g.

**Oil obtained by extraction and adulteration**. In a ground-glass-stoppered tube about 125 mm long and 18 mm in internal diameter, thoroughly mix 3 mL of the substance to be examined with 3 mL of *carbon disulfide R*. Shake for 3 min with 1 mL of *sulfuric acid R*. The mixture is less intensely coloured than a freshly prepared mixture of 3.2 mL of *ferric chloride solution R1*, 2.3 mL of *water R* and 0.5 mL of *dilute ammonia R1*.

**Composition of fatty acids**. Gas chromatography (*2.4.22*) with the following modifications.

Use the mixture of calibrating substances in Table 2.4.22.-3.

*Test solution*. Introduce 75 mg of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve in 2 mL of *1,1-dimethylethyl methyl ether R1* with shaking and heat gently (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and shake vigorously for at least 5 min. Add 5 mL of *distilled water R* and shake vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Use the upper layer.

*Reference solution*. Dissolve 50 mg of *methyl ricinoleate CRS* and 50 mg of *methyl stearate CRS* in 10.0 mL of *1,1-dimethylethyl methyl ether R1*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 55 | 215 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*:

- *symmetry factor*: 0.7 to 1.5 for the peak due to methyl stearate in the chromatogram obtained with the test solution.

Calculate the percentage content of each fatty acid by the normalisation procedure.

Correct the area of the peak due to methyl ricinoleate, by multiplying by a factor *R* calculated using the following expression:

$$\frac{m_1 \times A_2}{A_1 \times m_2}$$

$m_1$ = mass of methyl ricinoleate in the reference solution;

$m_2$ = mass of methyl stearate in the reference solution;

$A_1$ = area of the peak due to methyl ricinoleate in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to methyl stearate in the chromatogram obtained with the reference solution.

*Composition of the fatty-acid fraction of the oil*:

- *palmitic acid*: maximum 2.0 per cent;
- *stearic acid*: maximum 2.5 per cent;
- *oleic acid*: 2.5 per cent to 6.0 per cent;
- *linoleic acid*: 2.5 per cent to 7.0 per cent;
- *linolenic acid*: maximum 1.0 per cent;
- *eicosenoic acid*: maximum 1.0 per cent;
- *ricinoleic acid*: 85.0 per cent to 92.0 per cent;
- *any other fatty acid*: maximum 1.0 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent, or maximum 0.2 per cent if intended for use in the manufacture of parenteral preparations, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light. If intended for use in the manufacture of parenteral preparations, store under an inert gas.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations and the name of the inert gas.

**01/2020:0051**

# CASTOR OIL, VIRGIN

## Ricini oleum virginale

DEFINITION

Fatty oil obtained by cold expression from the seeds of *Ricinus communis* L. A suitable antioxidant may be added.

PRODUCTION

During the expression step, the temperature of the oil must not exceed 50 °C.

CHARACTERS

*Appearance*: clear at 40 °C, slightly yellow, viscous, hygroscopic liquid.

*Solubility*: slightly soluble in light petroleum, miscible with ethanol (96 per cent) and with glacial acetic acid.

*Relative density*: about 0.958.

*Refractive index*: about 1.479.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B.*

A. A mixture of 2 mL of the substance to be examined and 8 mL of *ethanol (96 per cent) R* is clear (*2.2.1*).

B. Specific absorbance (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Optical rotation** (*2.2.7*): + 3.5° to + 6.0°.

**Specific absorbance** (*2.2.25*): maximum 0.7, determined at the absorption maximum at 270 nm.

To 1.00 g add *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 1.5.

Dissolve 5.00 g in 25 mL of the prescribed mixture of solvents.

**Hydroxyl value** (*2.5.3, Method A*): minimum 160.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Unsaponifiable matter** (*2.5.7*): maximum 0.8 per cent, determined on 5.0 g.

**Composition of fatty acids**. Gas chromatography (*2.4.22*) with the following modifications.

Use the mixture of calibrating substances in Table 2.4.22.-3.

*Test solution.* Introduce 75 mg of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve in 2 mL of *1,1-dimethylethyl methyl ether R1* with shaking and heat gently (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for at least 5 min. Add 5 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Use the upper layer.

*Reference solution.* Dissolve 50 mg of *methyl ricinoleate CRS* and 50 mg of *methyl stearate CRS* in 10.0 mL of *1,1-dimethylethyl methyl ether R1*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 55 | 215 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*:

- *symmetry factor*: 0.7 to 1.5 for the peak due to methyl stearate in the chromatogram obtained with the test solution.

Calculate the percentage content of each fatty acid by the normalisation procedure.

Correct the area of the peak due to methyl ricinoleate, by multiplying by a factor *R* calculated using the following expression:

$$\frac{m_1 \times A_2}{A_1 \times m_2}$$

$m_1$ = mass of methyl ricinoleate in the reference solution;

$m_2$ = mass of methyl stearate in the reference solution;

$A_1$ = area of the peak due to methyl ricinoleate in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to methyl stearate in the chromatogram obtained with the reference solution.

*Composition of the fatty-acid fraction of the oil:*
- *palmitic acid*: maximum 2.0 per cent;
- *stearic acid*: maximum 2.5 per cent;
- *oleic acid* : 2.5 per cent to 6.0 per cent;
- *linoleic acid* : 2.5 per cent to 7.0 per cent;
- *linolenic acid* : maximum 1.0 per cent;
- *eicosenoic acid* : maximum 1.0 per cent;
- *ricinoleic acid* : 85.0 per cent to 92.0 per cent;
- *any other fatty acid*: maximum 1.0 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

**01/2017:0986**

# CEFACLOR

## Cefaclorum

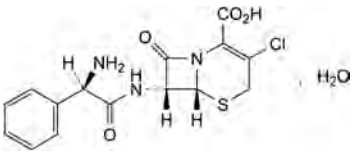

$C_{15}H_{14}ClN_3O_4S,H_2O$ $M_r$ 385.8

[70356-03-5]

DEFINITION

(6*R*,7*R*)-7-[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent of $C_{15}H_{14}ClN_3O_4S$ (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow powder.

*Solubility*: slightly soluble in water, practically insoluble in methanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefaclor CRS*.

TESTS

**pH** (*2.2.3*): 3.0 to 4.5.

Suspend 0.250 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 101 to + 111 (anhydrous substance).

Dissolve 0.250 g in a 10 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 10.0 mL of a 2.7 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Reference solution (a).* Dissolve 2.5 mg of *cefaclor CRS* and 5.0 mg of *delta-3-cefaclor CRS* (impurity D) in 100.0 mL of a 2.7 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a 2.7 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 4.0 with *phosphoric acid R*;
- *mobile phase B*: mix 450 mL of *acetonitrile R* with 550 mL of mobile phase A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 95 → 75 | 5 → 25 |
| 30 - 45 | 75 → 0 | 25 → 100 |
| 45 - 55 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (a):
- *resolution*: minimum 2 between the peaks due to cefaclor and impurity D; if necessary, adjust the acetonitrile content in the mobile phase;
- *symmetry factor*: maximum 1.2 for the peak due to cefaclor; if necessary, adjust the acetonitrile content in the mobile phase.

*Limits*:
- *any impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Water** (*2.5.12*): 3.0 per cent to 6.5 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 15.0 mg of *cefaclor CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 3.0 mg of *cefaclor CRS* and 3.0 mg of *delta-3-cefaclor CRS* (impurity D) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: add 220 mL of *methanol R* to a mixture of 780 mL of *water R*, 10 mL of *triethylamine R* and 1 g of *sodium pentanesulfonate R*, then adjust to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL.

*System suitability*:

- *resolution*: minimum 2.5 between the peaks due to cefaclor and impurity D in the chromatogram obtained with reference solution (b); if necessary, adjust the concentration of methanol in the mobile phase;
- *symmetry factor*: maximum 1.5 for the peak due to cefaclor in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

IMPURITIES

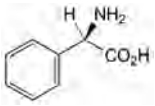

A. (2*R*)-2-amino-2-phenylacetic acid (phenylglycine),

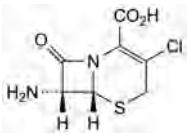

B. (6*R*,7*R*)-7-amino-3-chloro-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid,

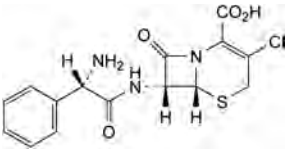

C. (6*R*,7*R*)-7-[[(2*S*)-2-amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

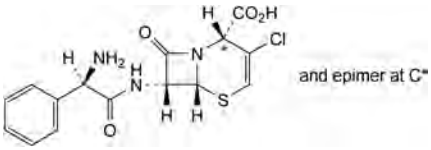


D. (2*R*,6*R*,7*R*)- and (2*S*,6*R*,7*R*)-7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-3-cefaclor),

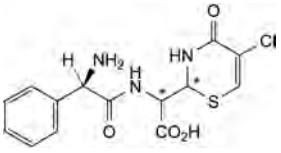

E. 2-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-(5-chloro-4-oxo-3,4-dihydro-2*H*-1,3-thiazin-2-yl)acetic acid,

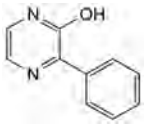

F. 3-phenylpyrazin-2-ol,

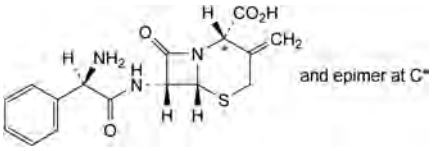


G. (2*R*,6*R*,7*R*)- and (2*S*,6*R*,7*R*)-7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methylene-8-oxo-5-thia-1-azabicyclo[4.2.0]octane-2-carboxylic acid (isocefalexine),

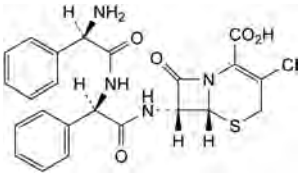

H. (6*R*,7*R*)-7-[[(2*R*)-2-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3-chloro-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*N*-phenylglycyl cefaclor).

**04/2008:0813**
**corrected 7.0**

# CEFADROXIL MONOHYDRATE

## Cefadroxilum monohydricum

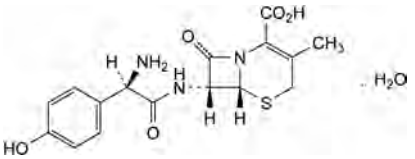

$C_{16}H_{17}N_3O_5S,H_2O$ $M_r$ 381.4
[66592-87-8]

DEFINITION

(6*R*,7*R*)-7-[[(2*R*)-2-Amino-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefadroxil CRS*.

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

Suspend 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 165 to + 178 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 10.0 mg of *D-α-(4-hydroxyphenyl)glycine CRS* (impurity A) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10.0 mg of *7-aminodesacetoxycephalosporanic acid CRS* (impurity B) in *phosphate buffer solution pH 7.0 R5* and dilute to 10.0 mL with the same buffer solution.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) to 100.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 10 mg of *dimethylformamide R* and 10 mg of *dimethylacetamide R* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (e).* Dilute 1.0 mL of reference solution (c) to 25.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm,
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *phosphate buffer solution pH 5.0 R*,
– *mobile phase B*: *methanol R2*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 98 | 2 |
| 1 - 20 | 98 → 70 | 2 → 30 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (c), (d) and (e).

*Relative retention* with reference to cefadroxil (retention time = about 6 min): dimethylformamide = about 0.4; dimethylacetamide = about 0.75.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to impurities A and B in the chromatogram obtained with reference solution (c),
– *signal-to-noise ratio*: minimum 10 for the 2nd peak in the chromatogram obtained with reference solution (e).

*Limits*:

– *impurity A*: not more than the area of the 1st peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
– *any other impurity*: for each impurity, not more than the area of the 2nd peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
– *total*: not more than 3 times the area of the 2nd peak in the chromatogram obtained with reference solution (c) (3.0 per cent),
– *disregard limit*: 0.05 times the area of the 2nd peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peaks due to dimethylformamide and dimethylacetamide.

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): 4.0 per cent to 6.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *cefadroxil CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *cefadroxil CRS* and 50 mg of *amoxicillin trihydrate CRS* in the mobile phase and dilute to 100 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, a 2.72 g/L solution of *potassium dihydrogen phosphate R* (4:96 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to cefadroxil and to amoxicillin.

Calculate the percentage content of cefadroxil.

STORAGE

Protected from light.

IMPURITIES

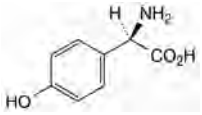

A. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid,

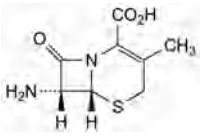

B. (6*R*,7*R*)-7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA),

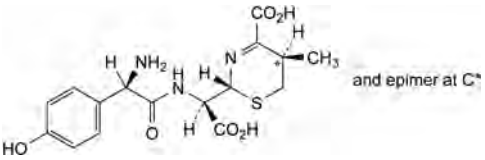


C. (2*R*,5*RS*)-2-[(*R*)-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]carboxymethyl]-5-methyl-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid,

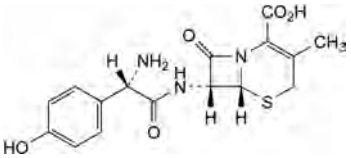

D. (6*R*,7*R*)-7-[[(2*S*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid (L-cefadroxil),

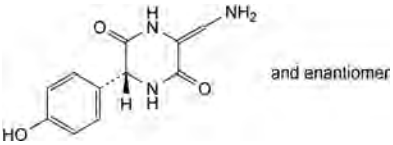


E. (6*RS*)-3-(aminomethylene)-6-(4-hydroxyphenyl)piperazine-2,5-dione,

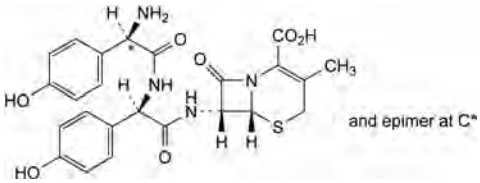


F. (6*R*,7*R*)-7-[[(2*R*)-2-[[(2*RS*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

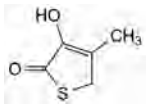

G. 3-hydroxy-4-methylthiophen-2(5*H*)-one,

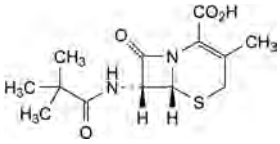

H. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA pivalamide).

**04/2008:0708**
**corrected 7.0**

# CEFALEXIN MONOHYDRATE

## Cefalexinum monohydricum

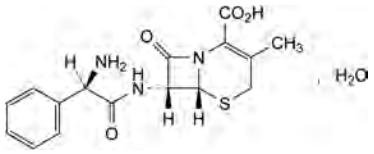

$C_{16}H_{17}N_3O_4S,H_2O$ $M_r$ 365.4
[23325-78-2]

DEFINITION

(6*R*,7*R*)-7-[[(2*R*)-2-Amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefalexin monohydrate CRS*.

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 149 to + 158 (anhydrous substance).

Dissolve 0.125 g in *phthalate buffer solution pH 4.4 R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 10.0 mg of *D-phenylglycine R* in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 10.0 mg of *7-aminodesacetoxycephalosporanic acid CRS* in *phosphate buffer solution pH 7.0 R5* and dilute to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) to 100.0 mL with mobile phase A.

*Reference solution (d)*. Dissolve 10 mg of *dimethylformamide R* and 10 mg of *dimethylacetamide R* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (e)*. Dilute 1.0 mL of reference solution (c) to 20.0 mL with mobile phase A.

*Reference solution (f)*. Dissolve 10 mg of *cefotaxime sodium CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A. To 1.0 mL of this solution add 1.0 mL of the test solution and dilute to 100 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 5.0 R*;
- *mobile phase B*: *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 98 | 2 |
| 1 - 20 | 98 → 70 | 2 → 30 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (c), (d), (e) and (f).

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurities A and B in the chromatogram obtained with reference solution (c) and minimum 1.5 between the peaks due to cefalexin and cefotaxime in the chromatogram obtained with reference solution (f).

*Limits*:

- *impurity B*: not more than the area of the 2nd peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *any other impurity*: not more than the area of the 1st peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *total*: not more than 3 times the area of the 1st peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *disregard limit*: the area of the 2nd peak in the chromatogram obtained with reference solution (e) (0.05 per cent); disregard any peaks due to dimethylformamide or dimethylacetamide.

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): 4.0 per cent to 8.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *cefalexin monohydrate CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *cefradine CRS* in 20 mL of reference solution (a) and dilute to 100 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *acetonitrile R*, 13.6 g/L solution of *potassium dihydrogen phosphate R*, *water R* (2:5:10:83 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to cefalexin and cefradine.

Calculate the percentage content of cefalexin monohydrate.

STORAGE

Protected from light.

IMPURITIES

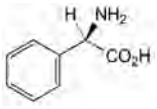

A. (2*R*)-2-amino-2-phenylacetic acid (D-phenylglycine),

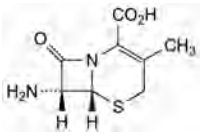

B. (6*R*,7*R*)-7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-aminodesacetoxycephalosporanic acid, 7-ADCA),

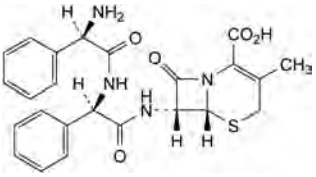

C. (6*R*,7*R*)-7-[[(2*R*)-2-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

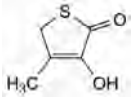

D. 3-hydroxy-4-methylthiophen-2(5*H*)-one,

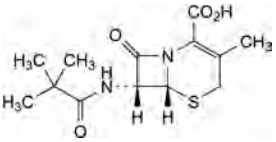

E. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA pivalamide),

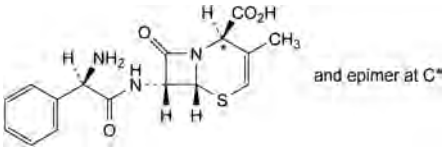


F. (2*RS*,6*R*,7*R*)-7-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-2-cefalexin).

**04/2013:0987**
**corrected 10.0**

# CEFALOTIN SODIUM

## Cefalotinum natricum

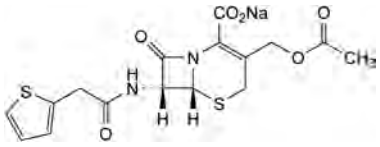

$C_{16}H_{15}N_2NaO_6S_2$ $M_r$ 418.4

[58-71-9]

DEFINITION

Sodium (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[(thiophen-2-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefalotin sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 450 nm is not greater than 0.20.

**pH** (*2.2.3*): 4.5 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 124 to + 134 (anhydrous substance).

Dissolve 1.25 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 75.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 75.0 mg of *cefalotin sodium CRS* in *water R* and dilute to 25.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *water R*.

*Reference solution (c).* Mix 1 mL of test solution (a), 1 mL of *hydrochloric acid R1* and 8 mL of *water R*. Heat at 60 °C for 12 min and cool to room temperature in iced water. Inject immediately.

*Reference solution (d).* Dissolve 5 mg of *cefalotin for impurity B identification CRS* in *water R* and dilute to 5 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 3 volumes of *acetonitrile R1* and 97 volumes of a 1.742 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: mix 40 volumes of *acetonitrile R1* and 60 volumes of a 1.742 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 2.5 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 0 | 0 → 100 |
| 30 - 35 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Relative retention* with reference to cefalotin (retention time = about 26 min): impurity C = about 0.2; impurity B = about 0.7; impurity D = about 0.88; impurity A = about 0.96.

*System suitability*: reference solution (c):

- *resolution*: minimum 7.0 between the peaks due to impurity D and cefalotin.

*Limits*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity D*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *any other impurity*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**N,N-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.500 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.13 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mix 14 volumes of *acetonitrile R* and 86 volumes of a 6.967 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 6.0 with *phosphoric acid R*.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 5 µL of test solution (b) and reference solution (a).

*Run time*: 1.5 times the retention time of cefalotin (retention time = about 10 min).

Calculate the percentage content of $C_{16}H_{15}N_2NaO_6S_2$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *cefalotin sodium CRS*.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

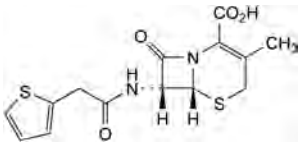

A. (6*R*,7*R*)-3-methyl-8-oxo-7-[(thiophen-2-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetoxycefalotin),

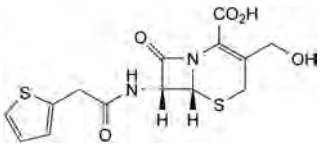

B. (6*R*,7*R*)-3-(hydroxymethyl)-8-oxo-7-[(thiophen-2-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetylcefalotin),

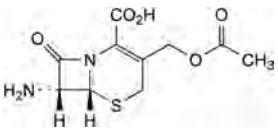

C. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA),

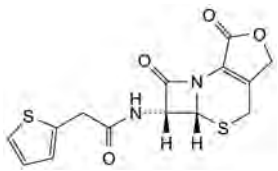

D. (5a*R*,6*R*)-6-[(thiophen-2-ylacetyl)amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (cefalotin lactone).

**01/2017:1402**
**corrected 10.0**

# CEFAMANDOLE NAFATE

## Cefamandoli nafas

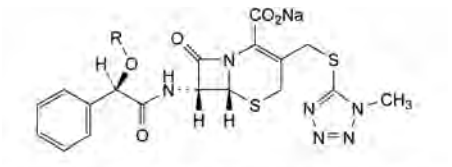

| Compound | R | Molecular Formula | $M_r$ |
|---|---|---|---|
| Cefamandole nafate | CHO | $C_{19}H_{17}N_6NaO_6S_2$ | 512.5 |
| Cefamandole sodium | H | $C_{18}H_{17}N_6NaO_5S_2$ | 484.5 |

Cefamandole nafate: [42540-40-9]
Cefamandole sodium: [30034-03-8]

DEFINITION

Mixture of sodium (6*R*,7*R*)-7-[[(2*R*)-2-(formyloxy)-2-phenylacetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate and sodium (6*R*,7*R*)-7-[[(2*R*)-2-hydroxy-2-phenylacetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (cefamandole sodium), with sodium carbonate.

Semi-synthetic product derived from a fermentation product.

*Content*:

– *cefamandole nafate* ($C_{19}H_{17}N_6NaO_6S_2$): 93.0 per cent to 102.0 per cent (anhydrous and sodium carbonate-free substance), for the sum of the content of cefamandole nafate and cefamandole sodium expressed as cefamandole nafate;
– *cefamandole sodium* ($C_{18}H_{17}N_6NaO_5S_2$): maximum 10.0 per cent (anhydrous and sodium carbonate-free substance);
– *sodium carbonate* ($Na_2CO_3$): 4.8 per cent to 6.4 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefamandole nafate CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 475 nm is not greater than 0.03.

**pH**: 6.0 to 8.0 for solution S, measured after 30 min.

**Specific optical rotation** (*2.2.7*): – 35.0 to – 45.0 (anhydrous and sodium carbonate-free substance).

Dissolve 1.00 g in *acetate buffer solution pH 4.7 R1* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 18 volumes of *acetonitrile R* and 75 volumes of a 10 per cent *V/V* solution of *triethylamine R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1 mL of the test solution to 10 mL with the solvent mixture, then heat at 60 °C for 30 min.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase: octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *triethylamine phosphate solution*: dissolve 2.0 g of *sodium pentanesulfonate R* in 350 mL of *water R*, add 40 mL of *triethylamine R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 700 mL with *water R*;
– *mobile phase A:* mix 1 volume of the triethylamine phosphate solution and 2 volumes of *water R*;
– *mobile phase B*: mix equal volumes of the triethylamine phosphate solution, *methanol R* and *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 1 - 35 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL loop injector.

*Relative retention* with reference to cefamandole nafate (retention time = about 24 min): cefamandole = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to cefamandole and cefamandole nafate.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.3 per cent *m/m*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

**Cefamandole nafate**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *cefamandole nafate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1 mL of the test solution to 10 mL with the mobile phase, then heat at 60 °C for 30 min.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R* and 75 volumes of a 10 per cent *V/V* solution of *triethylamine R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL loop injector.

*System suitability*:

- *resolution*: minimum 7.0 between the 2 principal peaks in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 0.8 per cent after a series of single injections of not less than 3 freshly prepared reference solutions (a).

Calculate the percentage content of cefamandole nafate ($C_{19}H_{17}N_6NaO_6S_2$) from the sum of the contents of cefamandole nafate and cefamandole sodium expressed as cefamandole nafate, using the declared content of *cefamandole nafate CRS*.

1 mg of cefamandole sodium is equivalent to 1.0578 mg of cefamandole nafate.

**Sodium carbonate**. Dissolve 0.500 g in 50 mL of *water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 5.3 mg of $Na_2CO_3$.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states that the substance contains sodium carbonate.

IMPURITIES

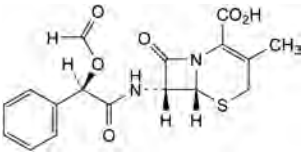

A. (6*R*,7*R*)-7-[[(2*R*)-2-(formyloxy)-2-phenylacetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (formylmandeloyl-7-amino-desacetoxy-cephalosporanic acid),

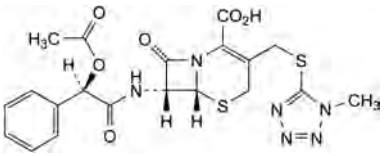

C. (6*R*,7*R*)-7-[[(2*R*)-2-(acetyloxy)-2-phenylacetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*O*-acetylcefamandole),

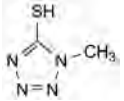

D. 1-methyl-1*H*-tetrazole-5-thiol,

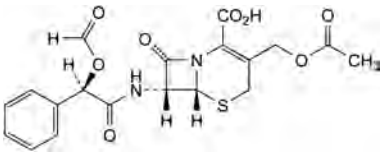

E. (6*R*,7*R*)-7-[[(2*R*)-2-(formyloxy)-2-phenylacetyl]amino]-3-[(acetyloxy)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (formylmandeloyl-7-ACA).

**01/2008:1650**
**corrected 10.0**

# CEFAPIRIN SODIUM

## Cefapirinum natricum

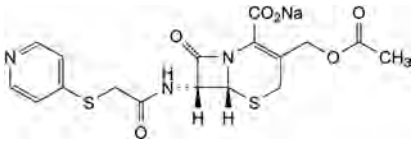

$C_{17}H_{16}N_3NaO_6S_2$ $M_r$ 445.5
[24356-60-3]

DEFINITION

Sodium (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[[[(pyridin-4-yl)sulfanyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow powder.

*Solubility*: soluble in water, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefapirin sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 2.0 g in *water R* and dilute to 10.0 mL with the same solvent. The solution is clear (*2.2.1*). Dilute 5.0 mL to 10.0 mL with *water R*. The absorbance (*2.2.25*) of this solution at 450 nm is not greater than 0.25.

**pH** (*2.2.3*): 6.5 to 8.5.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 150 to + 165 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 42 mg of the substance to be examined in the mobile phase and dilute to 200.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 42 mg of *cefapirin sodium CRS* in the mobile phase and dilute to 200.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 20.0 mL with the mobile phase.

*Reference solution (d).* Mix 1 mL of the test solution, 8 mL of the mobile phase and 1 mL of *hydrochloric acid R1*. Heat at 60 °C for 10 min.

*Column*:

- *size*: *l* = 0.30 m, Ø = 4 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 80 mL of *dimethylformamide R*, 4.0 mL of *glacial acetic acid R* and 20 mL of a 4.5 per cent *m/m* solution of *potassium hydroxide R*. Dilute to 2 L with *water R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of cefapirin.

*Relative retention* with reference to cefapirin (retention time = about 13 min): impurity B = about 0.3; impurity C = about 0.5; impurity A = about 0.75.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to cefapirin and impurity A.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and not more than 1 such peak has an area greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{16}N_3NaO_6S_2$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C.*

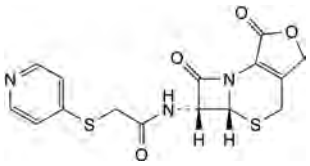

A. (5a*R*,6*R*)-6-[[[(pyridin-4-yl)sulfanyl]acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (deacetylcefapirin lactone),

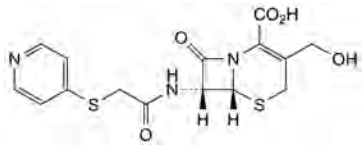

B. (6*R*,7*R*)-3-(hydroxymethyl)-8-oxo-7-[[[(pyridin-4-yl)sulfanyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetylcefapirin),

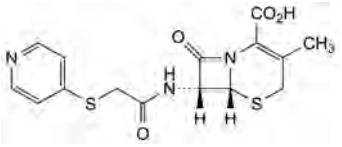

C. (6*R*,7*R*)-3-methyl-8-oxo-7-[[[(pyridin-4-yl)sulfanyl]acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetoxycefapirin).

**01/2008:1403**

# CEFATRIZINE PROPYLENE GLYCOL

## Cefatrizinum propylen glycolum

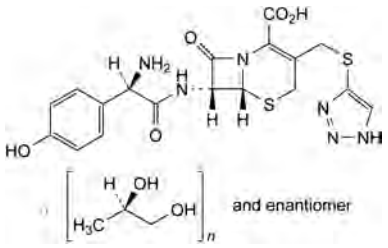


$C_{18}H_{18}N_6O_5S_2,(C_3H_8O_2)_n$ $M_r$ 462.5 (base)

DEFINITION

Mixture of (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-8-oxo-3-[[(1*H*-1,2,3-triazol-4-yl)sulfanyl]-methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid and propane-1,2-diol in molecular proportions of about 1:1.

*Content*: 95.0 per cent to 102.0 per cent of $C_{18}H_{18}N_6O_5S_2$ (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefatrizine propylene glycol CRS.*

B. Examine the chromatograms obtained in the test for propylene glycol.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**Specific optical rotation** (*2.2.7*): + 63 to + 69 (anhydrous substance).

Dissolve 0.400 g in *1 M hydrochloric acid* and dilute to 20.0 mL with the same acid.

**Propylene glycol**. Gas chromatography (*2.2.28*).

*Solvent mixture*: *acetone R*, *water R* (20:80 *V/V*).

*Internal standard solution.* Dissolve 1.0 g of *dimethylacetamide R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution.* Introduce 0.40 g of the substance to be examined into a ground-glass-stoppered test-tube. Add 3.0 mL of the internal standard solution, 1.0 mL of the solvent mixture and 2.0 mL of *hydrochloric acid R*. Seal the test-tube and shake.

*Reference solution (a).* Dissolve 2.0 g of *propylene glycol R* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Introduce into a ground-glass-stoppered test-tube 1.0 mL of reference solution (a) and 1.0 mL of the internal standard solution.

*Column*:

– *material*: stainless steel;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (150-180 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 200 °C;
– *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solution (b).

*Limit*:

– *propylene glycol*: 13.0 per cent to 18.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 60.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 60.0 mg of *cefatrizine propylene glycol CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 30.0 mg of *cefatrizine impurity A CRS* in *buffer solution pH 7.0 R* and dilute to 100.0 mL with the same buffer solution.

*Reference solution (c).* Dilute 0.6 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 100.0 mL with *buffer solution pH 7.0 R*.

*Reference solution (e).* To 1.0 mL of reference solution (a) add 1.0 mL of reference solution (b) and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 5 volumes of *acetonitrile R* and 95 volumes of a 2.72 g/L solution of *potassium dihydrogen phosphate R* in *water R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL of the test solution and reference solutions (c), (d) and (e).

*Run time*: at least twice the retention time of cefatrizine.

*System suitability*: reference solution (e):

– *resolution*: minimum 5.0 between the peaks due to cefatrizine and impurity A.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent);
– *sum of impurities other than A*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.1 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.03 per cent).

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{18}H_{18}N_6O_5S_2$ from the declared content of $C_{18}H_{18}N_6O_5S_2$ in *cefatrizine propylene glycol CRS*.

IMPURITIES

*Specified impurities: A.*

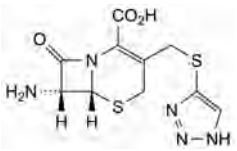

A. (6*R*,7*R*)-7-amino-8-oxo-3-[[(1*H*-1,2,3-triazol-4-yl)sulfanyl]methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA triazole).

**04/2019:0988**
**corrected 10.0**

# CEFAZOLIN SODIUM

## Cefazolinum natricum

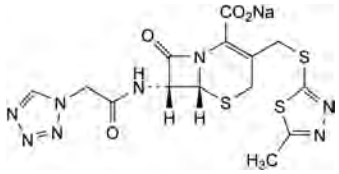

$C_{14}H_{13}N_8NaO_4S_3$ $M_r$ 476.5
[27164-46-1]

DEFINITION

Sodium (6*R*,7*R*)-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, very hygroscopic.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.150 g in 5 mL of *water R*, add 0.5 mL of *dilute acetic acid R*, swirl and allow to stand for 10 min in iced water. Filter the precipitate and rinse with 1-2 mL of *water R*. Dissolve in a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Evaporate the solvent almost to dryness, then dry in an oven at 60 °C for 30 min.

*Comparison*: *cefazolin CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.15.

**pH** (*2.2.3*): 4.0 to 6.0 for solution S.

**Specific optical rotation** (*2.2.7*): – 24 to – 15 (anhydrous substance).

Dissolve 1.25 g in *water R* and dilute to 25.0 mL with the same solvent.

**Absorbance** (*2.2.25*). Dissolve 0.100 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *sodium hydrogen carbonate solution R*. Examined between 220 nm and 350 nm, the solution shows an absorption maximum at 272 nm. The specific absorbance at the maximum is 260 to 300 (anhydrous substance).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 20 mg of the substance to be examined in 10 mL of a 2 g/L solution of *sodium hydroxide R*. Allow to stand for 15-30 min. Dilute 1.0 mL of the solution to 20 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: solution containing 14.54 g/L of *disodium hydrogen phosphate dodecahydrate R* and 3.53 g/L of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 98 | 2 |
| 2 - 4 | 98 → 85 | 2 → 15 |
| 4 - 10 | 85 → 60 | 15 → 40 |
| 10 - 11.5 | 60 → 35 | 40 → 65 |
| 11.5 - 12 | 35 | 65 |
| 12 - 15 | 35 → 98 | 65 → 2 |
| 15 - 21 | 98 | 2 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to cefazolin and impurity L (see Figure 0988.-1).

*Limits*:

- *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *total*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.15 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

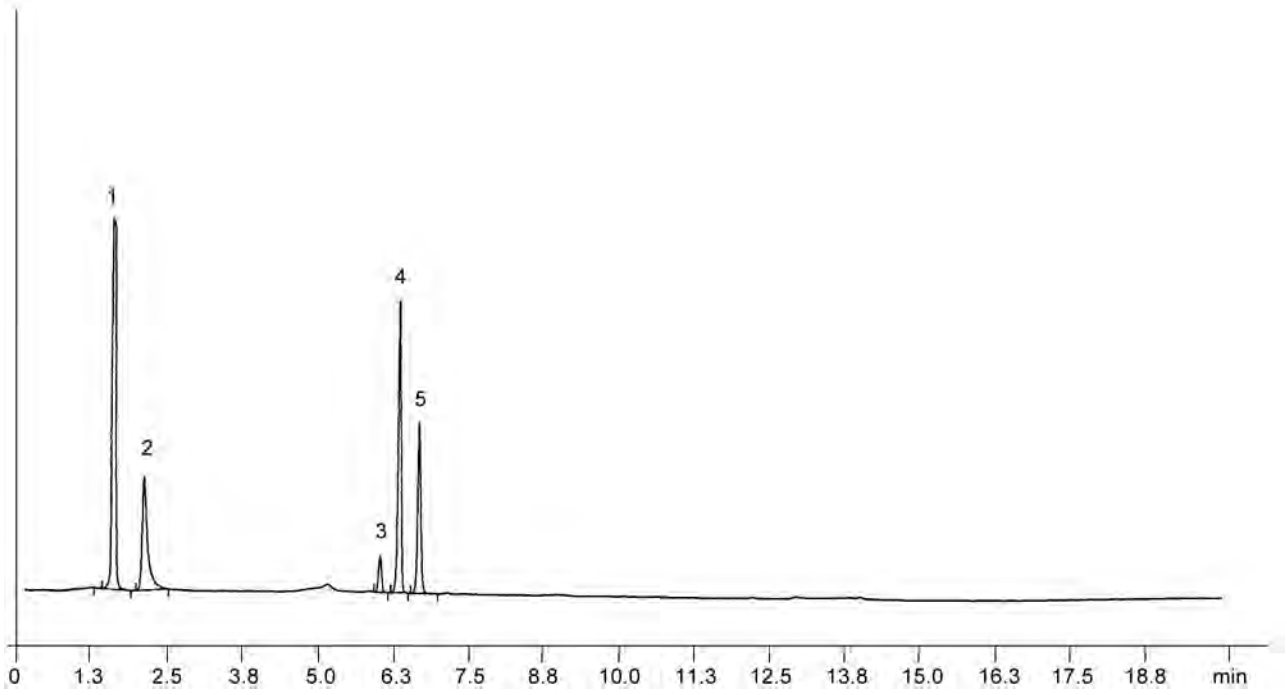


1. impurity J 2. impurity E 3. unknown structure 4. cefazolin 5. impurity L

Figure 0988.-1. – *Chromatogram for the test for related substances of cefazolin sodium: reference solution (b) (in situ degradation)*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *cefazolin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *cefuroxime sodium CRS* in 10.0 mL of reference solution (a) and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile R* and 90 volumes of a solution containing 2.77 g/L of *disodium hydrogen phosphate dodecahydrate R* and 1.86 g/L of *citric acid monohydrate R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to cefazolin and cefuroxime in the chromatogram obtained with reference solution (b);
- *symmetry factor*: 0.8 to 3.0 for the peak due to cefazolin in the chromatogram obtained with reference solution (a).

Calculate the percentage content of $C_{14}H_{13}N_8NaO_4S_3$ taking into account the assigned content of *cefazolin CRS* and a conversion factor of 1.048.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, G, H, I, J, K, L.*

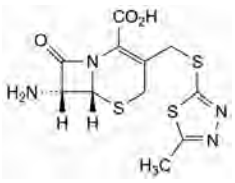

A. (6*R*,7*R*)-7-amino-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

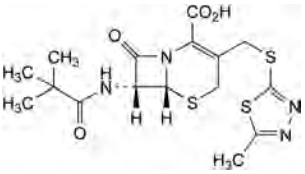

B. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

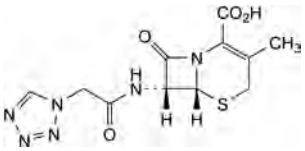

C. (6*R*,7*R*)-3-methyl-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

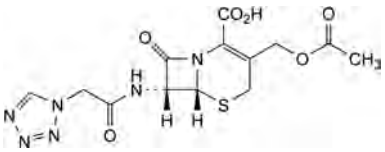

D. (6*R*,7*R*)-3-[(acetyloxy)methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

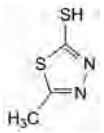

E. 5-methyl-1,3,4-thiadiazol-2-thiol (MMTD),

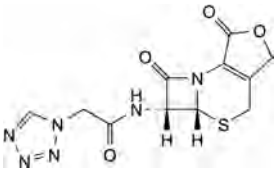

G. (5a*R*,6*R*)-6-[(1*H*-tetrazol-1-ylacetyl)amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,

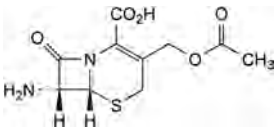

H. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA),

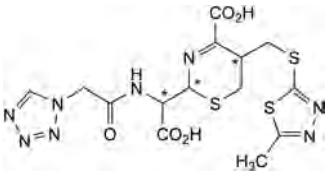

I. 2-[carboxy[(1*H*-tetrazol-1-ylacetyl)amino]methyl]-5-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid (cefazoloic acid),

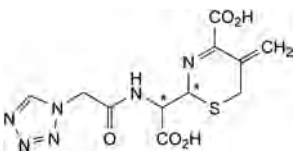

J. 2-[carboxy[(1*H*-tetrazol-1-ylacetyl)amino]methyl]-5-methylidene-5,6-dihydro-2*H*-1,3-thiazine-4-carboxylic acid,

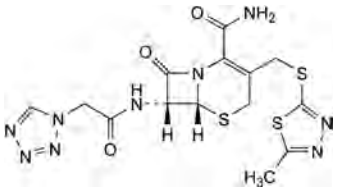

K. (6*R*,7*R*)-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxamide (cefazolinamide),

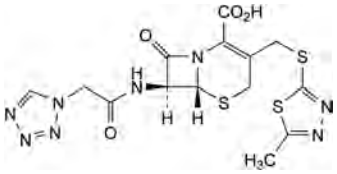

L. (6*R*,7*S*)-3-[[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanyl]methyl]-8-oxo-7-[(1*H*-tetrazol-1-ylacetyl)amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

**07/2011:2126**
**corrected 10.0**

# CEFEPIME DIHYDROCHLORIDE MONOHYDRATE

## Cefepimi dihydrochloridum monohydricum

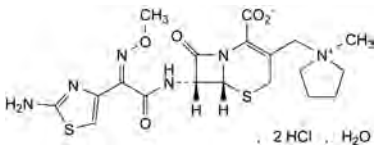

$C_{19}H_{26}Cl_2N_6O_5S_2,H_2O$ $M_r$ 571.5
[123171-59-5]

DEFINITION

(6*R*,7*R*)-7-[[(2*Z*)-(2-Aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[(1-methylpyrrolidinio)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate dihydrochloride monohydrate. Semi-synthetic product derived from a fermentation product.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefepime dihydrochloride monohydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 40 to + 45 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Impurity G**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *0.01 M nitric acid* and dilute to 10.0 mL with the same acid.

*Reference solution (a)*. Dilute 0.250 g of *N-methylpyrrolidine R* (impurity G) to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 100.0 mL with *0.01 M nitric acid*.

*Reference solution (b)*. Dilute 0.250 g of *pyrrolidine R* to 100 mL with *0.01 M nitric acid*. Dilute 2 mL of the solution to 100 mL with *0.01 M nitric acid*. Mix 5 mL of this solution with 5 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.05 m, Ø = 4.6 mm;
– *stationary phase*: *strong cation-exchange resin R* (5 µm).

*Mobile phase*: mix 1 volume of *acetonitrile R* and 100 volumes of *0.01 M nitric acid*; filter through a 0.2 µm filter.

*Flow rate*: 1 mL/min.

*Detection*: conductivity detector.

*Injection*: 100 µL.

*Run time*: 1.1 times the retention time of cefepime.

*Retention time*: cefepime = about 50 min, eluting as a broadened peak.

*System suitability*:

– *symmetry factor*: maximum 2.5 for the peak due to impurity G in the chromatogram obtained with reference solution (a);
– *repeatability*: maximum relative standard deviation of 5.0 per cent after 6 injections of reference solution (a);
– *peak-to-valley ratio*: minimum 3 between the peaks due to pyrrolidine and impurity G in the chromatogram obtained with reference solution (b).

Calculate the percentage content of impurity G in the test solution using reference solution (a).

*Limit*:

– *impurity G*: maximum 0.5 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or keep refrigerated at 4-8 °C for not more than 12 h.*

*Test solution*. Dissolve 70.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A. Sonicate for 30 s and stir for about 5 min.

*Reference solution (a)*. Dissolve 70.0 mg of *cefepime dihydrochloride monohydrate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A. Sonicate for 30 s and stir for about 5 min.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 10.0 mL with mobile phase A. Dilute 2.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 7 mg of *cefepime dihydrochloride monohydrate for system suitability CRS* (containing impurities A, B and F) in mobile phase A and dilute to 5 mL with mobile phase A.

*Reference solution (d)*. Dissolve 2 mg of *cefepime impurity E CRS* in mobile phase A and dilute to 25.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of *acetonitrile R* and 90 volumes of a 0.68 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.0 with a 0.5 M potassium hydroxide solution prepared from *potassium hydroxide R*;
- *mobile phase B*: mix equal volumes of *acetonitrile R* and a 0.68 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.0 with a 0.5 M potassium hydroxide solution prepared from *potassium hydroxide R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 30 | 100 → 50 | 0 → 50 |
| 30 - 35 | 50 | 50 |
| 35 - 36 | 50 → 100 | 50 → 0 |
| 36 - 45 | 100 | 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *cefepime dihydrochloride monohydrate for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and F; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity E.

*Relative retention* with reference to cefepime (retention time = about 7 min): impurity E = about 0.4; impurity F = about 0.8; impurity A = about 2.5; impurity B = about 4.1.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity F and cefepime.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity B = 1.4; impurity E = 1.8;
- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities B, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity E*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 3.0 per cent to 4.5 per cent, determined on 0.400 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.04 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A.

*Injection*: test solution and reference solution (a).

*Run time*: 1.4 times the retention time of cefepime.

Calculate the percentage content of $C_{19}H_{26}Cl_2N_6O_5S_2$ from the declared content of *cefepime dihydrochloride monohydrate CRS*.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

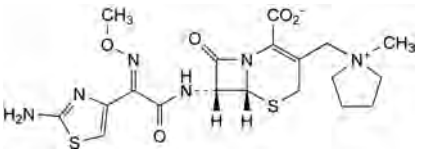

A. (6*R*,7*R*)-7-[[(2*E*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[(1-methylpyrrolidinio)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (*anti*-cefepime),

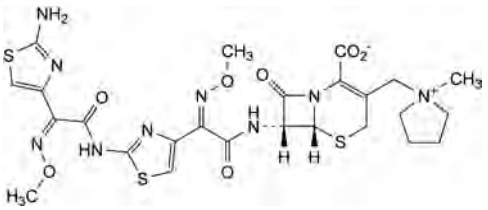

B. (6*R*,7*R*)-7-[[(2*Z*)-[2-[[(2*Z*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]thiazol-4-yl](methoxyimino)acetyl]amino]-3-[(1-methylpyrrolidinio)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

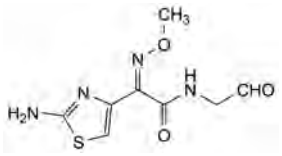

C. (2*Z*)-2-(2-aminothiazol-4-yl)-*N*-(formylmethyl)-2-(methoxyimino)acetamide,

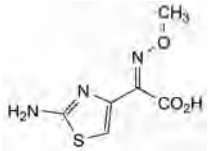

D. (2*Z*)-(2-aminothiazol-4-yl)(methoxyimino)acetic acid,

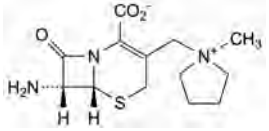

E. (6*R*,7*R*)-7-amino-3-[(1-methylpyrrolidinio)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

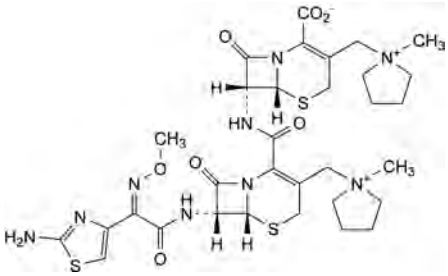

F. (6*R*,7*R*)-7-[[[(6*R*,7*R*)-7-[[(2*Z*)-(2-aminothiazol-4-yl)-(methoxyimino)acetyl]amino]-3-[(1-methylpyrrolidinio)-methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-2-yl]-carbonyl]amino]-3-[(1-methylpyrrolidinio)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

G. 1-methylpyrrolidine (*N*-methylpyrrolidine).

**04/2018:1188**

# CEFIXIME

## Cefiximum

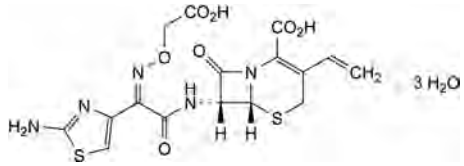

$C_{16}H_{15}N_5O_7S_2,3H_2O$ $M_r$ 507.5

DEFINITION

(6*R*,7*R*)-7-[[(*Z*)-2-(2-Aminothiazol-4-yl)-2-[(carboxy-methoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid trihydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: slightly soluble in water, soluble in methanol, sparingly soluble in anhydrous ethanol, practically insoluble in ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefixime CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**pH** (*2.2.3*): 2.6 to 4.1.

Suspend 0.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *cefixime CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* In order to prepare impurity D *in situ*, dissolve 10 mg of *cefixime CRS* in 10 mL of *water R*, heat on a water-bath for 45 min and cool. Inject immediately.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.
- *Mobile phase*: mix 250 volumes of *acetonitrile R* and 750 volumes of a tetrabutylammonium hydroxide solution prepared as follows: dissolve 8.2 g of *tetrabutylammonium hydroxide R* in *water for chromatography R* and dilute to 800 mL with the same solvent; adjust to pH 6.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Autosampler*: set at 4 °C.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of cefixime.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to cefixime and impurity D; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *any impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Ethanol** (*2.4.24*). Head-space gas chromatography (*2.2.28*): use the standard additions method.

*Sample solution.* Dissolve 0.250 g of the substance to be examined in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

*Limit*:

- *ethanol*: maximum 1.0 per cent.

**Water** (*2.5.12*): 9.0 per cent to 12.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 3.0 for the peak due to cefixime;
- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of $C_{16}H_{15}N_5O_7S_2$ taking into account the assigned content of *cefixime CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

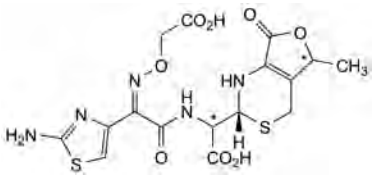

A. 2-[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)-imino]acetyl]amino]-2-[(2*R*)-5-methyl-7-oxo-1,2,5,7-tetrahydro-4*H*-furo[3,4-*d*][1,3]thiazin-2-yl]acetic acid,

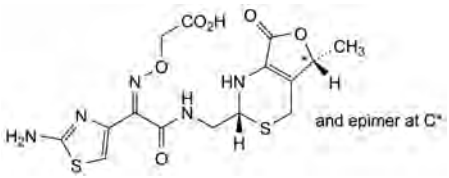


B. 2-[[[(*Z*)-1-(2-aminothiazol-4-yl)-2-[[[(2*R*,5*RS*)-5-methyl-7-oxo-1,2,5,7-tetrahydro-4*H*-furo[3,4-*d*][1,3]thiazin-2-yl]methyl]amino]-2-oxoethylidene]amino]oxy]acetic acid,

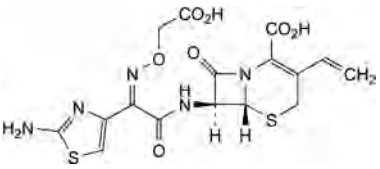

C. (6*R*,7*S*)-7-[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefixime 7-epimer),

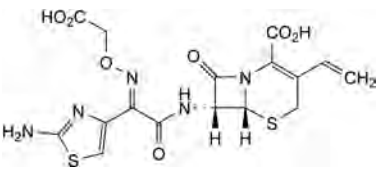

D. (6*R*,7*R*)-7-[[(*E*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefixime (*E*)-isomer),

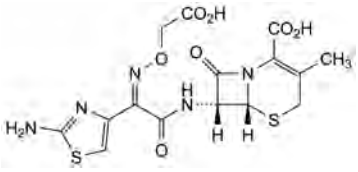

E. (6*R*,7*R*)-7-[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(carboxymethoxy)imino]acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

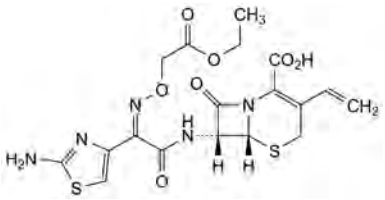

F. (6*R*,7*R*)-7-[[(*Z*)-2-(2-aminothiazol-4-yl)-2-[(2-ethoxy-2-oxoethoxy)imino]acetyl]amino]-3-ethenyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

**01/2017:1404**
**corrected 10.0**

# CEFOPERAZONE SODIUM

## Cefoperazonum natricum

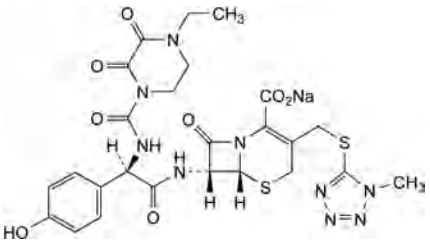

$C_{25}H_{26}N_9NaO_8S_2$ $M_r$ 668

[62893-20-3]

DEFINITION

Sodium (6*R*,7*R*)-7-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, hygroscopic powder.

*Solubility*: freely soluble in water, soluble in methanol, slightly soluble in ethanol (96 per cent).

If crystalline, it shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve the substance to be examined in *methanol R* and evaporate to dryness; examine the residue.

*Comparison*: *Ph. Eur. reference spectrum of cefoperazone sodium*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (a) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.15.

Dissolve 2.5 g in *water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *cefoperazone dihydrate CRS* in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 884 volumes of *water R*, 110 volumes of *acetonitrile R*, 3.5 volumes of a 60 g/L solution of *acetic acid R* and 2.5 volumes of a triethylammonium acetate solution prepared as follows: dilute 14 mL of *triethylamine R* and 5.7 mL of *glacial acetic acid R* to 100 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of cefoperazone.

*Retention time*: cefoperazone = about 15 min.

*System suitability*: reference solution (a):

– *number of theoretical plates*: minimum 5000, calculated for the principal peak; if necessary, adjust the content of *acetonitrile R* in the mobile phase;

– *symmetry factor*: maximum 1.6 for the principal peak; if necessary, adjust the content of *acetonitrile R* in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *total*: not more than 4.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.5 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Acetone** (*2.4.24, System B*): maximum 2.0 per cent.

*Sample solution.* Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Solvent solution.* Dissolve 0.350 g of *acetone R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *water R*.

Prepare each of 4 injection vials as shown in the table below:

| Vial No. | Sample solution (mL) | Solvent solution (mL) | *Water R* (mL) |
|---|---|---|---|
| 1 | 1.0 | 0 | 4.0 |
| 2 | 1.0 | 1.0 | 3.0 |
| 3 | 1.0 | 2.0 | 2.0 |
| 4 | 1.0 | 3.0 | 1.0 |

*Static head-space conditions that may be used*:

– *equilibration time*: 15 min;

– *transfer-line temperature*: 110 °C.

*Temperature*:

– *Column*: 40 °C for 10 min.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.20 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of cefoperazone sodium by multiplying the percentage content of cefoperazone by 1.034.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

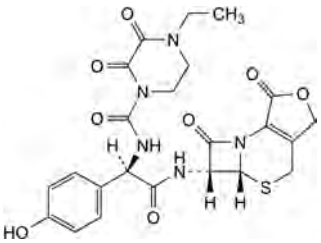

A. (5a*R*,6*R*)-6-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)-carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,

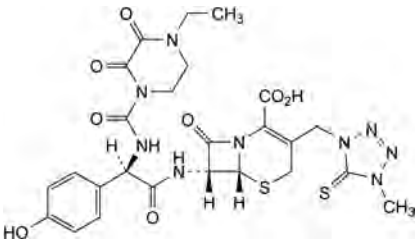

B. (6*R*,7*R*)-7-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)-carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[(4-methyl-5-thioxo-4,5-dihydro-1*H*-tetrazol-1-yl)methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

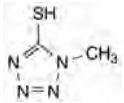

C. 1-methyl-1*H*-tetrazole-5-thiol,

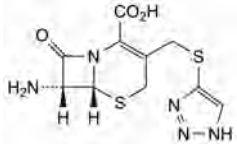

D. (6*R*,7*R*)-7-amino-8-oxo-3-[(1*H*-1,2,3-triazol-4-yl-sulfanyl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-TACA),

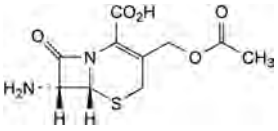

E. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-amino-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ACA),

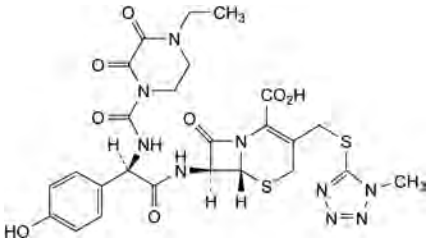

F. (6*R*,7*S*)-7-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazine-1-yl)-carbonyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-3-[[(1-methyl-1*H*-tetrazol-5-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

**01/2008:0989**
**corrected 10.0**

# CEFOTAXIME SODIUM

## Cefotaximum natricum

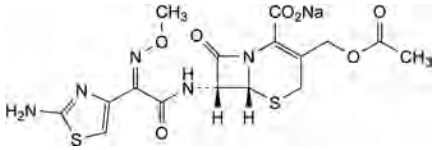

$C_{16}H_{16}N_5NaO_7S_2$ $M_r$ 477.4
[64485-93-4]

DEFINITION

Sodium (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefotaxime sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*). Add 1 mL of *glacial acetic acid R* to 10 mL of solution S. The solution, examined immediately, is clear.

**pH** (*2.2.3*): 4.5 to 6.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 58.0 to + 64.0 (anhydrous substance).

Dissolve 0.100 g in *water R* and dilute to 10.0 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.40 at 430 nm for solution S.

**Specific absorbance** (*2.2.25*): 360 to 390, determined at the absorption maximum at 235 nm (anhydrous substance).

Dissolve 20.0 mg in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*: mobile phase B, mobile phase A (14:86 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with the same solution.

*Reference solution (a)*. Dissolve 8.0 mg of *cefotaxime acid CRS* in solution A and dilute to 10.0 mL with the same solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A.

*Reference solution (c)*. Add 1.0 mL of *dilute hydrochloric acid R* to 4.0 mL of the test solution. Heat the solution at 40 °C for 2 h. Add 5.0 mL of *buffer solution pH 6.6 R* and 1.0 mL of *dilute sodium hydroxide solution R*.

*Reference solution (d)*. Dissolve 4 mg of *cefotaxime for peak identification CRS* (containing impurities A, B, C, E and F) in 5 mL of solution A.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: 7.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R* adjusted to pH 6.25 using *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 86 | 14 |
| 7 - 9 | 86 → 82 | 14 → 18 |
| 9 - 16 | 82 | 18 |
| 16 - 45 | 82 → 60 | 18 → 40 |
| 45 - 50 | 60 | 40 |
| 50 - 55 | 60 → 86 | 40 → 14 |
| 55 - 60 | 86 | 14 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *cefotaxime for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, E and F.

*Relative retention* with reference to cefotaxime (retention time = about 13 min): impurity B = about 0.3; impurity A = about 0.5; impurity E = about 0.6; impurity C = about 1.9; impurity D = about 2.3; impurity F = about 2.4; impurity G = about 3.1.

*System suitability*: reference solution (c):
- *resolution*: minimum 3.5 between the peaks due to impurity E and cefotaxime;
- *symmetry factor*: maximum 2.0 for the peak due to cefotaxime.

*Limits*:
- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethanol** (*2.4.24, System A*): maximum 1.0 per cent.

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent *m/m*.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.05 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

## ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{16}H_{16}N_5NaO_7S_2$ by multiplying the percentage content of cefotaxime by 1.048.

## STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

## IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

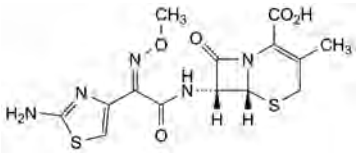

A. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetoxycefotaxime),

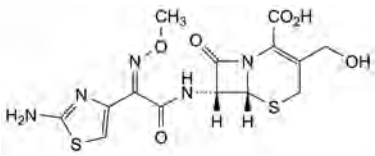

B. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (deacetylcefotaxime),

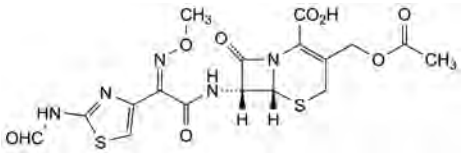

C. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*Z*)-2-[2-(formylamino)thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*N*-formylcefotaxime),

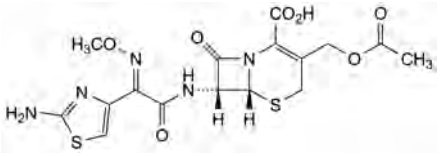

D. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*E*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid ((*E*)-cefotaxime),

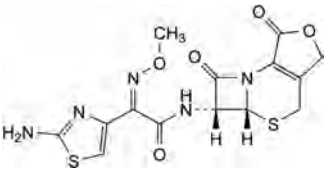

E. (5a*R*,6*R*)-6-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (deacetylcefotaxime lactone),

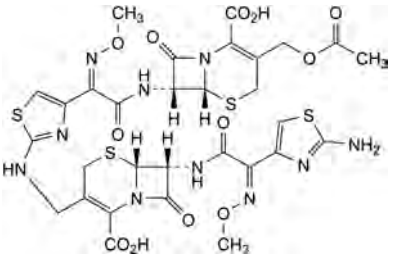

F. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*Z*)-2-[2-[[[(6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-2-yl]methyl]amino]thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefotaxime dimer),

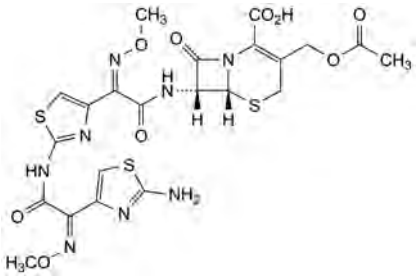

G. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(2*Z*)-2-[2-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]-amino]thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (ATA cefotaxime).

**01/2013:0990**
**corrected 10.0**

# CEFOXITIN SODIUM

## Cefoxitinum natricum

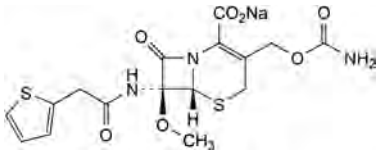

$C_{16}H_{16}N_3NaO_7S_2$ $M_r$ 449.4
[33564-30-6]

DEFINITION

Sodium (6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-8-oxo-7-[[(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: very soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefoxitin sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.2 to 7.0.

Dilute 2 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 206 to + 214 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 1.0 g of *potassium dihydrogen phosphate R* and 1.8 g of *anhydrous disodium hydrogen phosphate R* in 1000 mL of *water R*. To 100 mL of the solution add 800 mL of *water R*, adjust to pH 7.0 with *phosphoric acid R* or a 40 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 50 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with solution A.

*Reference solution (c).* Dissolve 5 mg of *cefoxitin for peak identification CRS* (containing impurities A, B, E, H, I and J) in solution A and dilute to 5 mL with solution A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (3.0 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: 1.0 g/L solution of *ammonium formate R* adjusted to pH 2.7 with *anhydrous formic acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 92 | 8 |
| 5 - 50 | 92 → 74 | 8 → 26 |
| 50 - 85 | 74 | 26 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *cefoxitin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, E, H, I and J.

*Relative retention* with reference to cefoxitin (retention time = about 30 min): impurity A = about 0.83; impurity I = about 0.98; impurity H = about 1.06; impurity E = about 1.11; impurity B = about 1.18; impurity J = about 1.66.

*System suitability*: reference solution (c):
- *resolution*: minimum 2.0 between the peaks due to impurities H and E;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cefoxitin.

*Limits*:
- *impurity I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurities E, H*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity J*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, B*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.13 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 25.0 mg of *cefoxitin sodium CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20.0 mg of *2-(2-thienyl)acetic acid R* in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (c).* Mix 1.0 mL of reference solution (a) and 5.0 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetic acid R, acetonitrile R, water R* (1:19:81 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: 12 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.5 between the 2 principal peaks.

Calculate the percentage content of $C_{16}H_{16}N_3NaO_7S_2$ taking into account the assigned content of *cefoxitin sodium CRS*.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A, B, E, H, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, F, G.*

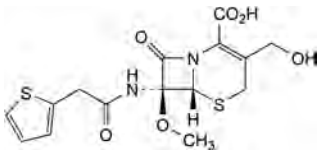

A. (6*R*,7*S*)-3-(hydroxymethyl)-7-methoxy-8-oxo-7-[[(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (decarbamoylcefoxitin),

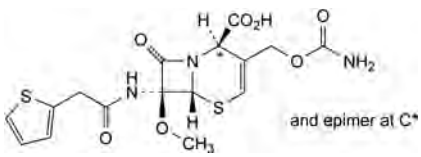


B. (2*RS*,6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-8-oxo-7-[[(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylic acid (delta-3-cefoxitin),

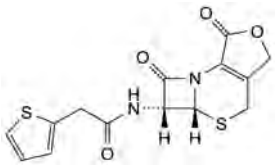

C. (5a*R*,6*R*)-6-[[(thiophen-2-yl)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (cefalotin lactone),

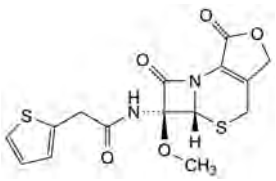

D. (5a*R*,6*S*)-6-methoxy-6-[[(thiophen-2-yl)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (cefoxitin lactone),

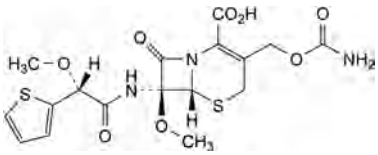

E. (6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-7-[[(2*R*)-2-methoxy-2-(thiophen-2-yl)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid ((*R*)-methoxy cefoxitin),

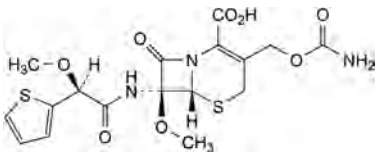

F. (6*R*,7*S*)-3-[(carbamoyloxy)methyl]-7-methoxy-7-[[(2*S*)-2-methoxy-2-(thiophen-2-yl)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid ((*S*)-methoxy cefoxitin),

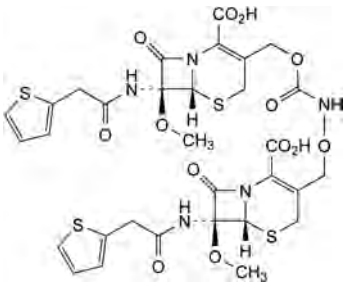

G. (6*R*,7*S*)-3-[[[[[[(6*R*,7*S*)-2-carboxy-7-methoxy-8-oxo-7-[[2-(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]oxy]carbamoyl]-oxy]methyl]-7-methoxy-8-oxo-7-[[2-(thiophen-2-yl)acetyl]amino]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefoxitin dimer),

H. unknown structure,

I. unknown structure,

J. unknown structure.

01/2011:2341

# CEFPODOXIME PROXETIL

## Cefpodoximum proxetili

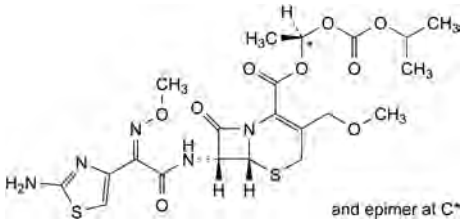


$C_{21}H_{27}N_5O_9S_2$ $M_r$ 557.6
[87239-81-4]

DEFINITION

(1*RS*)-1-[[(1-Methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]-amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 94.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow or light brown, amorphous powder.

*Solubility*: very slightly soluble or practically insoluble in water, very soluble in acetonitrile and in methanol, freely soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefpodoxime proxetil CRS*.

TESTS

**Diastereoisomer ratio**. Liquid chromatography (*2.2.29*) as described under Assay. Use the normalisation procedure.

*Limit*: test solution:

– the ratio of the area of the peak due to cefpodoxime proxetil diastereoisomer II to the sum of the areas of the peaks due to cefpodoxime proxetil diastereoisomers I and II is between 0.5 and 0.6.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C.*

*Solvent mixture*: *glacial acetic acid R*, *acetonitrile R*, *water R* (2:99:99 *V/V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *cefpodoxime proxetil for peak identification CRS* (containing impurities B, C and D) in 5.0 mL of the solvent mixture.

*Reference solution (c)*. Dissolve 5 mg of *cefpodoxime proxetil for impurity H identification CRS* in 5.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: maintain at a constant temperature of 20 °C.

*Mobile phase*:

– *mobile phase A*: *anhydrous formic acid R*, *methanol R*, *water R* (1:400:600 *V/V/V*);
– *mobile phase B*: *anhydrous formic acid R*, *water R*, *methanol R* (1:50:950 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 65 | 95 | 5 |
| 65 - 145 | 95 → 15 | 5 → 85 |
| 145 - 155 | 15 | 85 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *cefpodoxime proxetil for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C and D; use the chromatogram supplied with *cefpodoxime proxetil for impurity H identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurity H.

*Relative retention* with reference to cefpodoxime proxetil diastereoisomer II (retention time = about 58 min): diastereoisomer I of impurity B = about 0.68; diastereoisomer I of cefpodoxime proxetil = about 0.74; impurity C = about 0.82; diastereoisomer II of impurity B = about 0.85; impurity D (2 peaks) = about 0.88 and 1.13; peaks due to diastereoisomers of impurity H: between about 1.9 and 2.3.

*System suitability*:

– the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *cefpodoxime proxetil for peak identification CRS*;
– *resolution*: minimum 6.0 between the peaks due to cefpodoxime proxetil diastereoisomers I and II in the chromatogram obtained with reference solution (a);
– *peak-to-valley ratio*: minimum 1.1, where $H_p$ = height above the baseline of the peak due to diastereoisomer II of impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Limits*:

– *impurity C*: not more than twice the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (2.0 per cent);
– *impurity D (sum of the 2 diastereoisomers)*: not more than the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *impurity H (sum of the diastereoisomers)*: not more than the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *impurity B (sum of the 2 diastereoisomers)*: not more than 0.5 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *any other impurity*: for each impurity, not more than 0.2 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *total*: not more than 4 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (4.0 per cent);
– *disregard limit*: 0.05 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A*: 20 mg/L solution of *anhydrous citric acid R* in *acetonitrile R*.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution.* Dissolve 30.0 mg of *cefpodoxime proxetil CRS* in solution A and dilute to 50.0 mL with solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *methanol R*, *water R* (9:11 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of cefpodoxime proxetil diastereoisomer II.

*Retention time*: cefpodoxime proxetil diastereoisomer II = about 30 min.

*System suitability*: reference solution:

- *resolution*: minimum 4.0 between the peaks due to cefpodoxime proxetil diastereoisomers I and II.

Calculate the percentage content of $C_{21}H_{27}N_5O_9S_2$ from the sum of the areas of the 2 peaks due to the diastereoisomers and using the declared content of *cefpodoxime proxetil CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, D, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, E, F, G.*

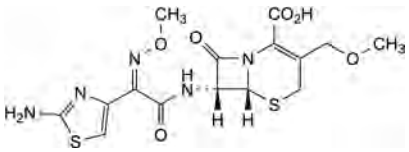

A. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefpodoxime),

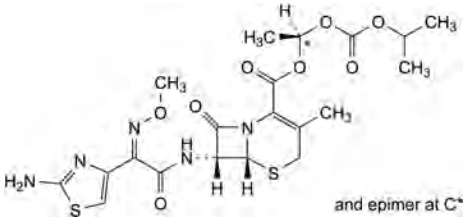


B. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (ADCA-analogue of cefpodoxime proxetil),

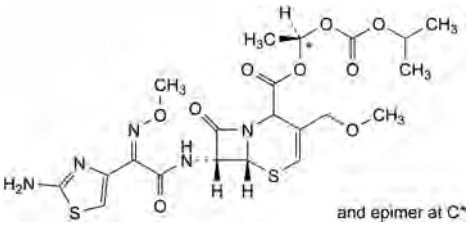


C. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate (delta-2-cefpodoxime proxetil),

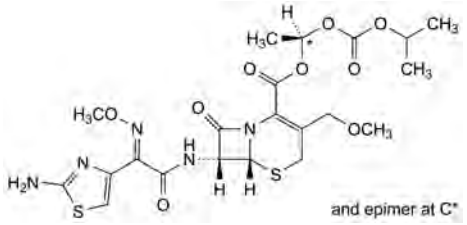


D. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*E*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (anti-cefpodoxime proxetil),

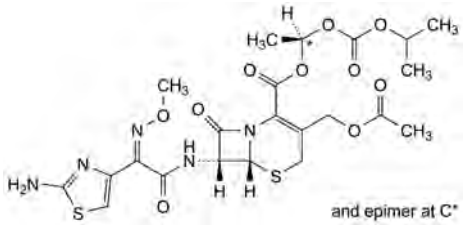


E. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-3-(acetoxymethyl)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (ACA-analogue of cefpodoxime proxetil),

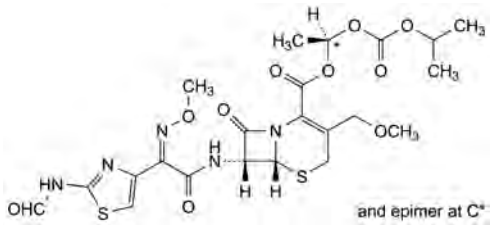


F. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-2-[(2-formylamino)thiazol-4-yl)-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (*N*-formyl cefpodoxime proxetil),

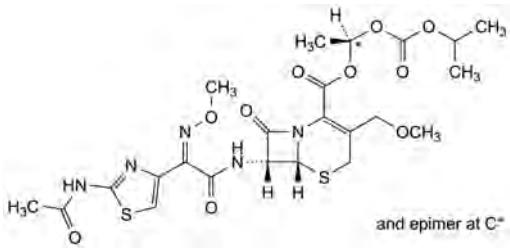


G. (1*RS*)-1-[[(1-methylethoxy)carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-[2-(2-acetylamino)thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (*N*-acetyl-cefpodoxime proxetil),

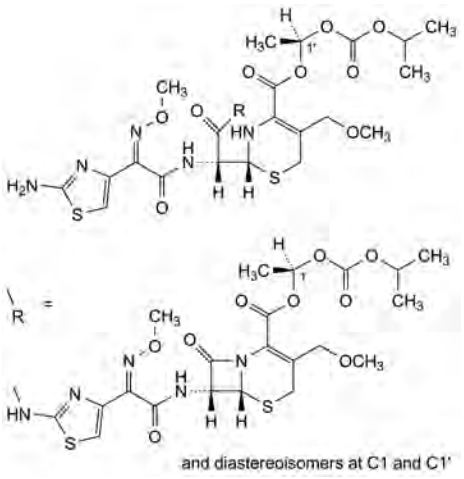


H. mixture of the diastereoisomers of 1-[[(1-methylethoxy)-carbonyl]oxy]ethyl (6*R*,7*R*)-7-[[(2*Z*)-2-[2-[[(2*R*)-2-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetyl]amino]-2-[(2*R*)-5-(methoxymethyl)-4-[[1-[[(1-methylethoxy)-carbonyl]oxy]ethoxy]carbonyl]-3,6-dihydro-2*H*-1,3-thiazin-2-yl]acetyl]amino]thiazol-4-yl]-2-(methoxyimino)acetyl]amino]-3-(methoxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (cefpodoxime proxetil dimer).

**01/2017:2342**
**corrected 9.4**

# CEFPROZIL MONOHYDRATE

## Cefprozilum monohydricum

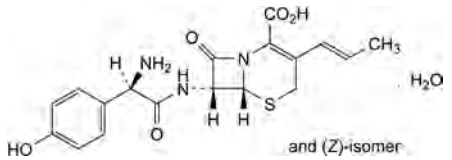


$C_{18}H_{19}N_3O_5S, H_2O$ $M_r$ 407.4
[121123-17-9]

DEFINITION

Mixture of the 2 diastereoisomers of (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-8-oxo-3-[(1*EZ*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellow, crystalline powder, slightly hygroscopic.

*Solubility*: slightly soluble in water and in methanol, practically insoluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefprozil CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 0.125 g of the substance to be examined in 1 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with mobile phase A.

*Test solution (b).* Dissolve 30.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *cefprozil for peak identification CRS* (containing impurities B, H and M) in 0.05 mL of a 103 g/L solution of *hydrochloric acid R* and add 1 mL of mobile phase A.

*Reference solution (c).* Dissolve 3 mg of *cefprozil CRS* and 6 mg of *cefprozil impurity mixture CRS* (containing impurities D and F) in 2 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with mobile phase A.

*Reference solution (d).* Dissolve 30.0 mg of *cefprozil CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (e).* Dissolve 10.0 mg of *cefadroxil CRS* (impurity B) in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (f).* Dissolve 10.0 mg of *cefprozil impurity A CRS* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 11.5 g of *ammonium dihydrogen phosphate R* in *water for chromatography R*, adjust to pH 4.4 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*, mobile phase A (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 81 | 19 |
| 8 - 20 | 81 → 36 | 19 → 64 |
| 20 - 25 | 36 | 64 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b), (c), (e) and (f).

*Identification of impurities*: use the chromatogram supplied with *cefprozil for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, H and M; use the chromatogram supplied with *cefprozil impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and F; impurities G and I are identified by their relative retention.

*Relative retention* with reference to cefprozil (*Z*)-isomer (retention time = about 7 min): impurity A = about 0.4; impurity B = about 0.5; impurity D = about 0.7; impurity F = about 0.9; cefprozil (*E*)-isomer = about 1.4; impurity G = about 1.7; impurity H = about 2.0; impurity I = about 2.1; impurity M = about 2.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.4 between the peaks due to impurity F and cefprozil (*Z*)-isomer.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 2.3;

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.5 per cent);
- *impurities D, G, H, I, M*: for each impurity, not more than 0.3 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.2 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: maximum 2.0 per cent;
- *disregard limit*: 0.05 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.05 per cent).

**(*E*)-isomer ratio**. Liquid chromatography (*2.2.29*) as described under Assay.

Determine the area of the peak due to the (*E*)-isomer in the chromatogram obtained with test solution (b) and reference solution (d). Calculate the ratio of the (*E*)-isomer to the sum of both cefprozil isomers, as determined under Assay.

*Limit*:

- *(E)-isomer ratio*: 0.06 to 0.11.

**Water** (*2.5.12*): 3.5 per cent to 6.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

## ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (18:82 *V/V*).

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL of test solution (b) and reference solution (d).

*Run time*: twice the retention time of cefprozil (*Z*)-isomer.

*Elution order*: (*Z*)-isomer, (*E*)-isomer.

*Retention time*: cefprozil (*Z*)-isomer = about 8 min.

*System suitability*: reference solution (d):

- *resolution*: minimum 2.5 between the peaks due to cefprozil (*Z*)-isomer and the (*E*)-isomer.

Calculate the percentage content of the sum of both isomers of cefprozil ($C_{18}H_{19}N_3O_5S$) taking into account the assigned contents of both (*E*)-isomer and (*Z*)-isomer of *cefprozil CRS*.

## STORAGE

In an airtight container.

## IMPURITIES

*Specified impurities*: *A, B, D, G, H, I, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, J, K, L, N.*

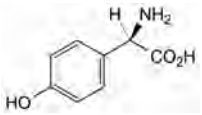

A. (2*R*)-2-amino-2-(4-hydroxyphenyl)acetic acid (*p*-hydroxyphenylglycine),

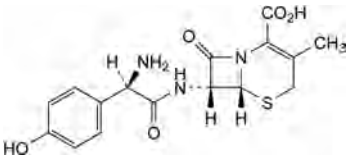

B. (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]-amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefadroxil),

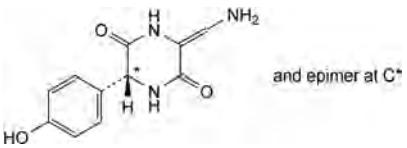


C. (6*RS*)-3-(aminomethylene)-6-(4-hydroxyphenyl)-piperazine-2,5-dione,

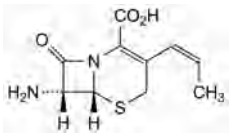

D. (6*R*,7*R*)-7-amino-8-oxo-3-[(1*Z*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

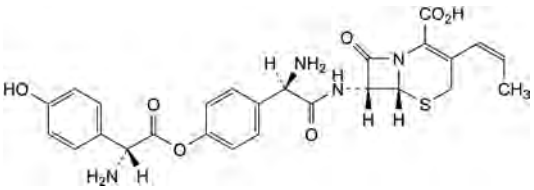

E. (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-[4-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]oxy]phenyl]acetyl]amino]-8-oxo-3-[(1*Z*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

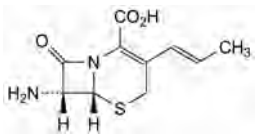

F. (6*R*,7*R*)-7-amino-8-oxo-3-[(1*E*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

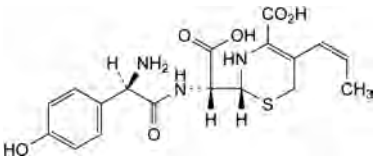

G. (2*R*)-2-[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino-2-[(2*R*)-4-carboxy-5-[(1*Z*)-prop-1-enyl]-3,6-dihydro-2*H*-1,3-thiazin-2-yl]-acetic acid,

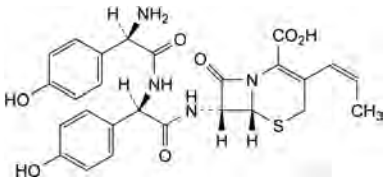

H. (6*R*,7*R*)-7-[[(2*R*)-2-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino]-2-(4-hydroxyphenyl)acetyl]amino]-8-oxo-3-[(1*Z*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

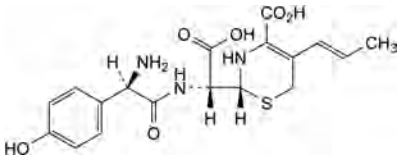

I. (2*R*)-2-[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino-2-[(2*R*)-4-carboxy-5-[(1*E*)-prop-1-enyl]-3,6-dihydro-2*H*-1,3-thiazin-2-yl]-acetic acid,

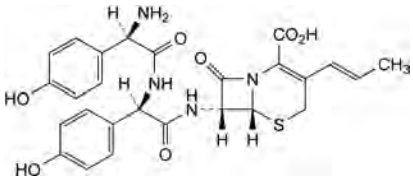

J. (6*R*,7*R*)-7-[[(2*R*)-2-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-2-(4-hydroxyphenyl)acetyl]-amino]-8-oxo-3-[(1*E*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

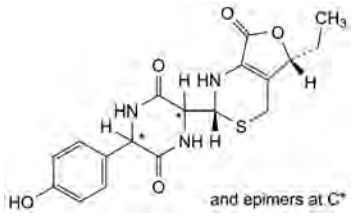


K. mixture of 4 diastereoisomers of (3*RS*,6*RS*)-3-[(2*R*,5*R*)-5-ethyl-7-oxo-1,2,5,7-tetrahydro-4*H*-furo[3,4-*d*][1,3]thiazin-2-yl]-6-(4-hydroxyphenyl)piperazine-2,5-dione,

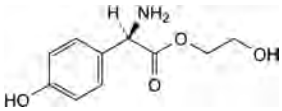

L. 2-hydroxyethyl (2*R*)-2-amino-2-(4-hydroxyphenyl)acetate,

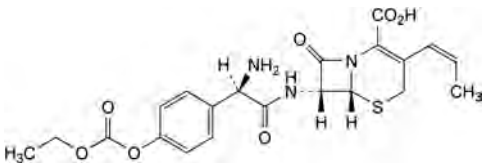

M. (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-[4-[(ethoxycarbonyl)-oxy]phenyl]acetyl]amino]-8-oxo-3-[(1*Z*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

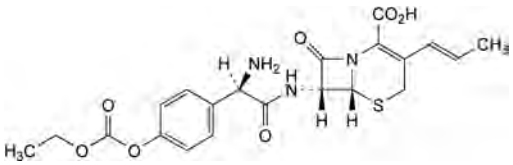

N. (6*R*,7*R*)-7-[[(2*R*)-2-amino-2-[4-(ethoxycarbonyl)-oxy]phenyl]acetyl]amino]-8-oxo-3-[(1*E*)-prop-1-enyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

**01/2014:0814**

# CEFRADINE

## Cefradinum

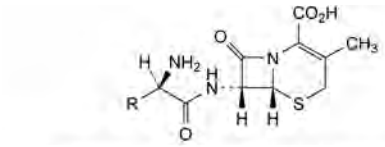

| Compound | R | Mol. Formula | $M_r$ |
|---|---|---|---|
| cefradine | | $C_{16}H_{19}N_3O_4S$ | 349.4 |
| cefalexin | | $C_{16}H_{17}N_3O_4S$ | 347.4 |
| 4',5'-dihydrocefradine | | $C_{16}H_{21}N_3O_4S$ | 351.4 |

Cefradine: [38821-53-3]

DEFINITION

*Main component*: (6*R*,7*R*)-7-[[(2*R*)-amino(cyclohexa-1,4-dienyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (cefradine).

Semi-synthetic product derived from a fermentation product.

*Content*:

- *cefradine*: minimum 90.0 per cent (anhydrous substance);
- *cefalexin*: maximum 5.0 per cent (anhydrous substance);
- *4′,5′-dihydrocefradine*: maximum 2.0 per cent (anhydrous substance);
- *sum of the percentage contents of cefradine, cefalexin and 4′,5′-dihydrocefradine*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, hygroscopic powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent) and in hexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefradine CRS*.

If the spectra obtained in the solid state show differences, dissolve 30 mg of the substance to be examined and 30 mg of the reference substance separately in 10 mL of *methanol R*, evaporate to dryness at 40 °C at a pressure less than 2 kPa and record new spectra using the residues.

TESTS

**Solution S**. Dissolve 2.50 g in *sodium carbonate solution R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*). Allow solution S to stand for 5 min. The absorbance (*2.2.25*) of solution S measured at 450 nm is not greater than 0.60.

**pH** (*2.2.3*): 3.5 to 6.0.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 80.0 to + 90.0 (anhydrous substance).

Dissolve 0.250 g in *acetate buffer solution pH 4.6 R* and dilute to 25.0 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.300 g of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 3.0 mg of *cyclohexa-1,4-dienylglycine CRS* (impurity B) in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 3 mg of the substance to be examined and 3 mg of *cefalexin monohydrate CRS* in mobile phase A and dilute to 25 mL with mobile phase A.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 6 mg of *cefradine for peak identification CRS* (containing impurities C, D and E) in 1.0 mL of mobile phase A.

*Reference solution (e).* Dissolve the contents of a vial of *cefradine impurity mixture CRS* (impurities A and G) in 1.0 mL of mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *dilute phosphoric acid R*;
- *mobile phase B*: *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.5 | 99.5 → 97 | 0.5 → 3 |
| 2.5 - 11 | 97 → 75 | 3 → 25 |
| 11 - 13 | 75 → 60 | 25 → 40 |
| 13 - 16 | 60 | 40 |
| 16 - 19 | 60 → 20 | 40 → 80 |
| 19 - 19.1 | 20 → 99.5 | 80 → 0.5 |
| 19.1 - 25 | 99.5 | 0.5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *cefradine for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C, D and E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B; use the chromatogram supplied with *cefradine impurity mixture CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities A and G.

*Relative retention* with reference to cefradine (retention time = about 15 min): impurity A = about 0.27; impurity B = about 0.32; impurity C = about 0.53; impurity D = about 0.63; impurity E = about 0.80; impurity F = about 0.92; cefalexin = about 0.95; 4′,5′-dihydrocefradine = about 1.06; impurity G = about 1.32.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to cefalexin and cefradine.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 3.4;
- *impurities A, B, C, D, E, F, G*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.25 per cent);
- *any other impurity*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.25 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peaks due to cefalexin and 4′,5′-dihydrocefradine.

***N*,*N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *phosphate buffer solution pH 5.0 R* and dilute to 100.0 mL with the same solution.

*Reference solution (a).* Dissolve 50.0 mg of *cefradine CRS* (containing 4′,5′-dihydrocefradine) in *phosphate buffer solution pH 5.0 R* and dilute to 100.0 mL with the same solution.

*Reference solution (b).* Dissolve 5.0 mg of *cefalexin monohydrate CRS* in *phosphate buffer solution pH 5.0 R* and dilute to 100.0 mL with the same solution.

*Reference solution (c).* Dilute 1 mL of reference solution (a) to 10 mL with *phosphate buffer solution pH 5.0 R*. Mix 5 mL of this solution and 5 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *phosphate buffer solution pH 5.0 R* (25:75 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL.

*Run time*: twice the retention time of cefradine.

*Relative retention* with reference to cefradine (retention time = about 3 min): cefalexin = about 0.7; 4′,5′-dihydrocefradine = about 1.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 4.0 between the peaks due to cefalexin and cefradine.

Calculate the percentage content of cefradine using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *cefradine CRS*. Calculate the percentage content of cefalexin using the chromatogram obtained with reference solution (b) and taking into account the assigned content of *cefalexin monohydrate CRS*. Calculate the percentage content of 4′,5′-dihydrocefradine using the chromatogram obtained with reference solution (b), taking into account the assigned content of *cefalexin monohydrate CRS* and multiplying the area of the peak due to 4′,5′-dihydrocefradine by a correction factor of 1.6.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

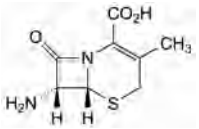

A. (6*R*,7*R*)-7-amino-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-aminodeacetoxycephalosporanic acid, 7-ADCA),

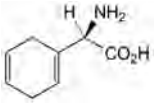

B. (2*R*)-amino(cyclohexa-1,4-dienyl)acetic acid (D-dihydrophenylglycine, cyclohexa-1,4-dienylglycine),

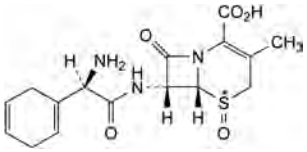

C. (6*R*,7*R*)-7-[[(2*R*)-amino(cyclohexa-1,4-dienyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 5-oxide (isomer 1),

D. (6*R*,7*R*)-7-[[(2*R*)-amino(cyclohexa-1,4-dienyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid 5-oxide (isomer 2),

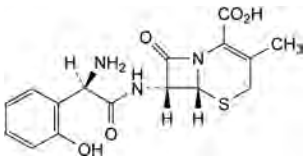

E. (6*R*,7*R*)-7-[[(2*R*)-amino(2-hydroxyphenyl)acetyl]amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

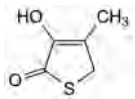

F. 3-hydroxy-4-methylthiophen-2(5*H*)-one,

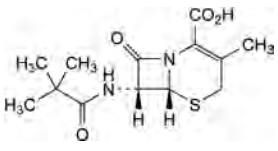

G. (6*R*,7*R*)-7-[(2,2-dimethylpropanoyl)amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (7-ADCA pivalamide).

**01/2018:1405**
**corrected 10.0**

# CEFTAZIDIME PENTAHYDRATE

## Ceftazidimum pentahydricum

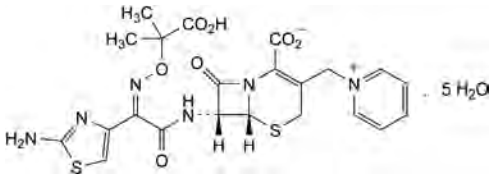

$C_{22}H_{22}N_6O_7S_2,5H_2O$ $M_r$ 637
[78439-06-2]

DEFINITION

(6*R*,7*R*)-7-[[(2*Z*)-2-(2-Aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate pentahydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in methanol, practically insoluble in acetone and in ethanol (96 per cent). It dissolves in acid and alkali solutions.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ceftazidime CRS*.

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 4.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Suspend 0.150 g of the substance to be examined in 5 mL of *acetonitrile R*, dissolve by adding *water R* and dilute to 100 mL with *water R*.

*Reference solution (a)*. To 1.0 mL of the test solution add 5.0 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 5.0 mL with *water R*.

*Reference solution (b)*. In order to prepare impurity B *in situ*, expose 5 mL of the test solution to ultraviolet light at 254 nm for about 24 h.

*Reference solution (c)*. Dissolve the contents of a vial of *ceftazidime for peak identification CRS* (containing impurities A and G) in 2.0 mL of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: solution containing 3.6 g/L of *disodium hydrogen phosphate dodecahydrate R* and 1.4 g/L of *potassium dihydrogen phosphate R*, adjusted to pH 3.4 with a 10 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 96 → 89 | 4 → 11 |
| 4 - 5 | 89 | 11 |
| 5 - 8 | 89 → 84 | 11 → 16 |
| 8 - 11 | 84 → 80 | 16 → 20 |
| 11 - 15 | 80 → 50 | 20 → 50 |
| 15 - 18 | 50 → 20 | 50 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Relative retention* with reference to ceftazidime (retention time = about 8 min): impurity F = about 0.4; impurity G = about 0.8; impurity A = about 0.9; impurity B = about 1.4.

*Identification of impurities*: use the chromatogram supplied with *ceftazidime for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and G; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*System suitability*: reference solution (c):

– *resolution*: minimum 4.0 between the peaks due to impurity A and ceftazidime.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity G by 3.0;
– *impurities A, B, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to impurity F.

**Impurity F**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Phosphate buffer solution.* Prepare a 10 per cent *V/V* solution of *phosphate buffer solution pH 7.0 R4*.

*Test solution.* Dissolve 0.500 g of the substance to be examined in phosphate buffer solution and dilute to 100.0 mL with the same solution.

*Reference solution (a).* Dissolve 1.00 g of *pyridine R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 200.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with phosphate buffer solution.

*Reference solution (b).* Dilute 1 mL of the test solution to 200 mL with phosphate buffer solution. To 1 mL of this solution add 20 mL of reference solution (a) and dilute to 200 mL with phosphate buffer solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 8 volumes of a 28.8 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 7.0 with *ammonia R*, 24 volumes of *acetonitrile R* and 68 volumes of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 255 nm.

*Injection*: 20 µL.

*Run time*: 10 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 7.0 between the peaks due to ceftazidime and impurity F.

*Limit*:

– *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (500 ppm).

**Water** (*2.5.12*): 13.0 per cent to 15.0 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

## ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *ceftazidime CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *ceftazidime for peak identification CRS* (containing impurities A and G) in 3.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *hexylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 4.3 g of *disodium hydrogen phosphate dodecahydrate R* and 2.7 g of *potassium dihydrogen phosphate R* in 980 mL of *water R*, then add 20 mL of *acetonitrile R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 20 µL.

*Run time*: 6 min.

*Relative retention* with reference to ceftazidime (retention time = about 4.5 min): impurity A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity A and ceftazidime.

Calculate the content of ceftazidime ($C_{22}H_{22}N_6O_7S_2$) taking into account the assigned content of $C_{22}H_{22}N_6O_7S_2$ in *ceftazidime CRS*.

## STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

## IMPURITIES

*Specified impurities*: *A, B, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, H.*

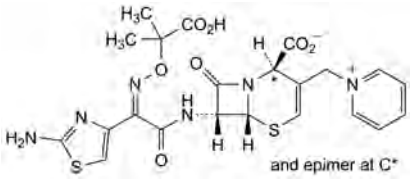


A. (2*RS*,6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate (Δ-2-ceftazidime),

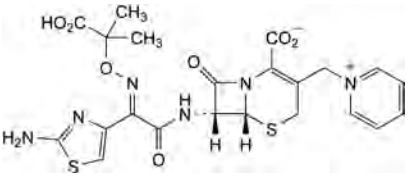

B. (6*R*,7*R*)-7-[[(2*E*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

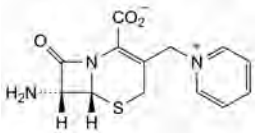

C. (6*R*,7*R*)-7-amino-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

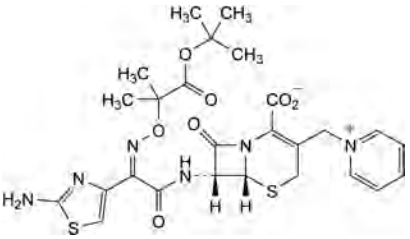

E. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[[2-(1,1-dimethylethoxy)-1,1-dimethyl-2-oxoethoxy]imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

F. pyridine,

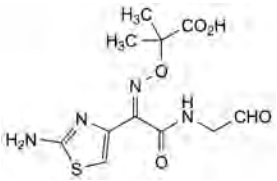

G. 2-[[[(1*Z*)-1-(2-aminothiazol-4-yl)-2-[(oxoethyl)amino]-2-oxoethylidene]amino]oxy]-2-methylpropanoic acid,

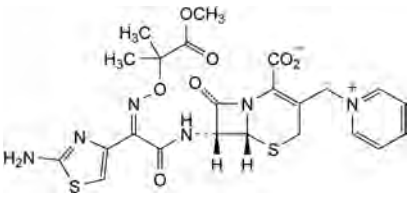

H. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[(2-methoxy-1,1-dimethyl-2-oxoethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

**01/2018:2344**
**corrected 10.0**

# CEFTAZIDIME PENTAHYDRATE WITH SODIUM CARBONATE FOR INJECTION

## Ceftazidimum pentahydricum et natrii carbonas ad iniectabile

DEFINITION

Sterile mixture of *Ceftazidime pentahydrate (1405)* and *Anhydrous sodium carbonate (0773)*.

Semi-synthetic product derived from a fermentation product.

*Content*:

– *ceftazidime*: 93.0 per cent to 105.0 per cent (dried and carbonate-free substance);
– *sodium carbonate*: 8.0 per cent to 10.0 per cent.

CHARACTERS

*Appearance*: white or pale yellow powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. It gives the reaction of carbonates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.60 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 425 nm is not greater than 0.50.

**pH** (*2.2.3*): 5.0 to 7.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Suspend 0.150 g of the substance to be examined in 5 mL of *acetonitrile R*, dissolve by adding *water R* and dilute to 100 mL with *water R*.

*Reference solution (a)*. To 1.0 mL of the test solution add 5.0 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 5.0 mL with *water R*.

*Reference solution (b)*. In order to prepare impurity B *in situ*, expose 5 mL of the test solution to ultraviolet light at 254 nm for about 24 h.

*Reference solution (c)*. Dissolve the contents of a vial of *ceftazidime for peak identification CRS* (containing impurities A and G) in 2.0 mL of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: solution containing 3.6 g/L of *disodium hydrogen phosphate dodecahydrate R* and 1.4 g/L of *potassium dihydrogen phosphate R*, adjusted to pH 3.4 with a 10 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 96 → 89 | 4 → 11 |
| 4 - 5 | 89 | 11 |
| 5 - 8 | 89 → 84 | 11 → 16 |
| 8 - 11 | 84 → 80 | 16 → 20 |
| 11 - 15 | 80 → 50 | 20 → 50 |
| 15 - 18 | 50 → 20 | 50 → 80 |
| 18 - 22 | 20 | 80 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Relative retention* with reference to ceftazidime (retention time = about 8 min): impurity F = about 0.4; impurity G = about 0.8; impurity A = about 0.9; impurity B = about 1.4.

*Identification of impurities*: use the chromatogram supplied with *ceftazidime for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and G; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*System suitability*: reference solution (c):

– *resolution*: minimum 4.0 between the peaks due to impurity A and ceftazidime.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity G by 3.0;

– *impurities A, B, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to impurity F.

**Impurity F**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Phosphate buffer solution.* Prepare a 10 per cent *V/V* solution of *phosphate buffer solution pH 7.0 R4*.

*Test solution.* Dissolve 0.500 g of the substance to be examined in phosphate buffer solution and dilute to 100.0 mL with the same solution.

*Reference solution (a).* Dissolve 1.00 g of *pyridine R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 200.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with phosphate buffer solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with phosphate buffer solution. To 1.0 mL of this solution add 20.0 mL of reference solution (a) and dilute to 200.0 mL with phosphate buffer solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 8 volumes of a 28.8 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 7.0 with *ammonia R*, 24 volumes of *acetonitrile R* and 68 volumes of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 255 nm.

*Injection*: 20 µL.

*Run time*: 10 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 7.0 between the peaks due to ceftazidime and impurity F.

*Limit*:

– *impurity F*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

**Loss on drying** (*2.2.32*): maximum 13.5 per cent, determined on 0.300 g. Dry at 25 °C at a pressure not exceeding 0.67 kPa for 4 h then heat the residue at 100 °C at a pressure not exceeding 0.67 kPa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

**Ceftazidime**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *ceftazidime CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *ceftazidime for peak identification CRS* (containing impurities A and G) in 3.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *hexylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 4.3 g of *disodium hydrogen phosphate dodecahydrate R* and 2.7 g of *potassium dihydrogen phosphate R* in 980 mL of *water R*, then add 20 mL of *acetonitrile R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 20 µL.

*Run time*: 6 min.

*Relative retention* with reference to ceftazidime (retention time = about 4.5 min): impurity A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity A and ceftazidime.

Calculate the content of ceftazidime ($C_{22}H_{22}N_6O_7S_2$) taking into account the assigned content of $C_{22}H_{22}N_6O_7S_2$ in *ceftazidime CRS*.

**Sodium carbonate**. Atomic absorption spectrometry (*2.2.23, Method I*).

*Caesium chloride buffer solution.* To 12.7 g of *caesium chloride R* add 500 mL of *water R* and 86 mL of *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

*Sodium standard solution (1000 mg/L).* Dissolve 3.70 g of *sodium nitrate R* in *water R* and dilute to 500 mL with the same solvent, add 48.5 g of *nitric acid R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 650.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent. To 10.0 mL of this solution add 5.0 mL of caesium chloride buffer solution and dilute to 50.0 mL with *water R*.

*Reference solution.* Into 4 identical flasks, each containing 20.0 mL of caesium chloride buffer solution, introduce respectively 0 mL, 5.00 mL, 10.00 mL and 15.00 mL of sodium standard solution (1000 mg/L) and dilute to 200.0 mL with *water R*.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 330.2 nm to 330.3 nm.

*Atomisation device*: air-acetylene flame.

Calculate the percentage content of sodium carbonate.

STORAGE

In a sterile, airtight, tamper-evident container, protected from light and humidity.

LABELLING

The label states the percentage content *m/m* of ceftazidime.

IMPURITIES

*Specified impurities*: *A, B, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, H.*

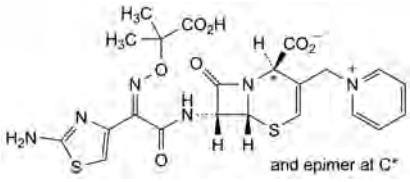


A. (2*RS*,6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate (Δ-2-ceftazidime),

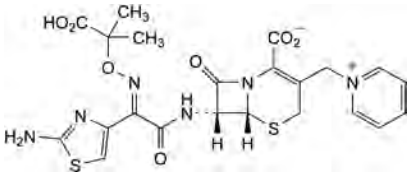

B. (6*R*,7*R*)-7-[[(2*E*)-2-(2-aminothiazol-4-yl)-2-[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

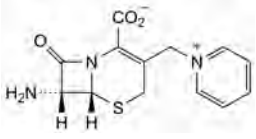

C. (6*R*,7*R*)-7-amino-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

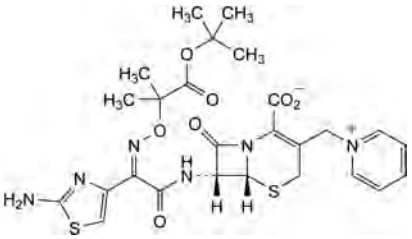

E. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[[2-(1,1-dimethylethoxy)-1,1-dimethyl-2-oxoethoxy]imino]acetyl]-amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate,

F. pyridine,

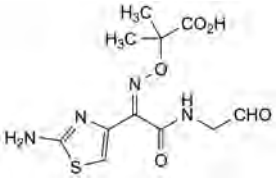

G. 2-[[[(1*Z*)-1-(2-aminothiazol-4-yl)-2-[(oxoethyl)amino]-2-oxoethylidene]amino]oxy]-2-methylpropanoic acid,

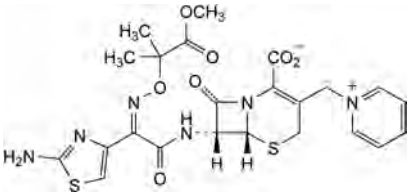

H. (6*R*,7*R*)-7-[[(2*Z*)-2-(2-aminothiazol-4-yl)-2-[(2-methoxy-1,1-dimethyl-2-oxoethoxy)imino]acetyl]amino]-8-oxo-3-[(pyridin-1-ium-1-yl)methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

**01/2008:0991**
**corrected 10.0**

# CEFTRIAXONE SODIUM

## Ceftriaxonum natricum

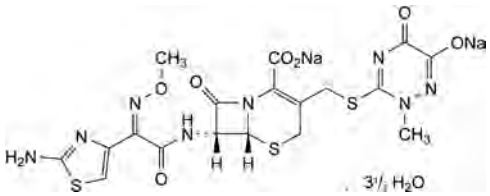

$C_{18}H_{16}N_8Na_2O_7S_3,3½H_2O$ $M_r$ 662
[104376-79-6]

DEFINITION

Disodium (6*R*,7*R*)-7-[[(2*Z*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[[(2-methyl-6-oxido-5-oxo-2,5-dihydro-1,2,4-triazin-3-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate 3.5 hydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: almost white or yellowish, slightly hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol, very slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ceftriaxone sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.40 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ or $BY_5$ (*2.2.2*).

Dilute 2 mL of solution S to 20 mL with *water R*.

**pH** (*2.2.3*): 6.0 to 8.0 for solution S.

**Specific optical rotation** (*2.2.7*): − 155 to − 170 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 30.0 mg of *ceftriaxone sodium CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *ceftriaxone sodium CRS* and 5.0 mg of *ceftriaxone impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.0 g of *tetradecylammonium bromide R* and 2.0 g of *tetraheptylammonium bromide R* in a mixture of 440 mL of *water R*, 55 mL of *0.067 M phosphate buffer solution pH 7.0 R*, 5.0 mL of citrate buffer solution pH 5.0 prepared by dissolving 20.17 g of *citric acid monohydrate R* in 800 mL of *water R*, adjusting to pH 5.0 with *strong sodium hydroxide solution R* and diluting to 1000.0 mL with *water R*, and 500 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of ceftriaxone.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to ceftriaxone and impurity A.

*Limits*:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (4.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): 8.0 per cent to 11.0 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.08 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{18}H_{16}N_8Na_2O_7S_3$ from the declared content of *ceftriaxone sodium CRS*.

STORAGE

In an airtight container protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

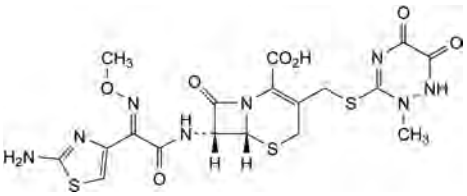

A. (6*R*,7*R*)-7-[[(2*E*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-3-[[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid ((*E*)-isomer),

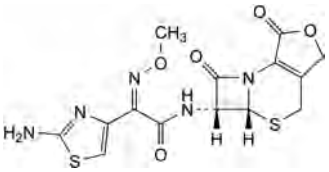

B. (5a*R*,6*R*)-6-[[(2*Z*)-(2-aminothiazol-4-yl)(methoxyimino)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto-[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,

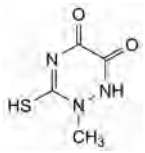

C. 2-methyl-3-sulfanyl-1,2-dihydro-1,2,4-triazine-5,6-dione,

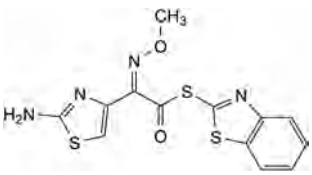

D. *S*-benzothiazol-2-yl (2*Z*)-(2-aminothiazol-4-yl)(methoxyimino)thioacetate,

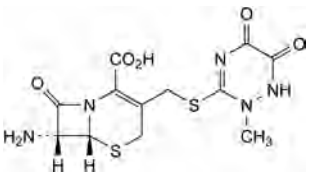

E. (6*R*,7*R*)-7-amino-3-[[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl)sulfanyl]methyl]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid.

01/2008:1300
corrected 10.0

# CEFUROXIME AXETIL

## Cefuroximum axetili

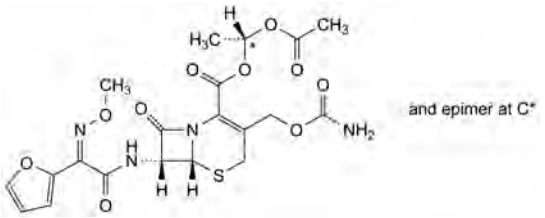


$C_{20}H_{22}N_4O_{10}S$ $M_r$ 510.5
[64544-07-6]

DEFINITION

Mixture of the 2 diastereoisomers of (1*RS*)-1-(acetyloxy)ethyl (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, soluble in acetone, in ethyl acetate and in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cefuroxime axetil CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the peaks due to cefuroxime axetil diastereoisomers A and B in the chromatogram obtained with reference solution (d).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the test solution and reference solution (d) immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* In order to prepare *in situ* impurity A, heat 5 mL of the test solution at 60 °C for 1 h.

*Reference solution (c).* In order to prepare *in situ* impurity B, expose 5 mL of the test solution to ultraviolet light at 254 nm for 24 h.

*Reference solution (d).* Dissolve 10.0 mg of *cefuroxime axetil CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *trimethylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 23 g/L solution of *ammonium dihydrogen phosphate R* (38:62 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the pair of peaks due to impurity A and use the chromatogram obtained with reference solution (c) to identify the pair of peaks due to impurity B.

*Relative retention* with reference to cefuroxime axetil diastereoisomer A: cefuroxime axetil diastereoisomer B = about 0.9, impurity A = about 1.2; impurity B = 1.7 and 2.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to cefuroxime axetil diastereoisomer A and impurity A.

*Limits*:

– *impurity A*: maximum 1.5 per cent for the sum of the pair of peaks;
– *impurity B*: maximum 1.0 per cent for the sum of the pair of peaks;
– *impurity E*: maximum 0.5 per cent;
– *any other impurity*: for each impurity, maximum 0.5 per cent;
– *total*: maximum 3.0 per cent;
– *disregard limit*: 0.05 times the area of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Diastereoisomer ratio**. Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Limit*: test solution:

– the ratio of the area of the peak due to cefuroxime axetil diastereoisomer A to the sum of the areas of the peaks due to cefuroxime axetil diastereoisomers A and B is between 0.48 and 0.55.

**Acetone** (*2.4.24*): maximum 1.1 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.400 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (d).

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to cefuroxime axetil diastereoisomers A and B;
– *repeatability*: maximum relative standard deviation of 2.0 per cent for the sum of the peaks due to cefuroxime axetil diastereoisomers A and B after 6 injections.

Calculate the percentage content of $C_{20}H_{22}N_4O_{10}S$ from the sum of the areas of the 2 diastereoisomer peaks and the declared content of $C_{20}H_{22}N_4O_{10}S$ in *cefuroxime axetil CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

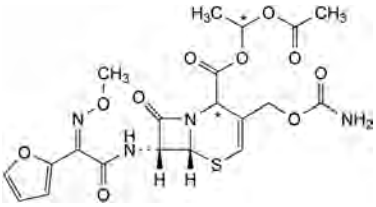

A. 1-(acetyloxy)ethyl (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-3-ene-2-carboxylate ($\Delta^3$-isomers),

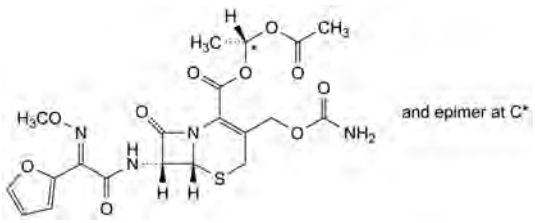


B. (1*RS*)-1-(acetyloxy)ethyl (6*R*,7*R*)-3-[(carbamoyloxy)-methyl]-7-[[(*E*)-2-(furan-2-yl)-2-(methoxyimino)acetyl]-amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate ((*E*)-isomers),

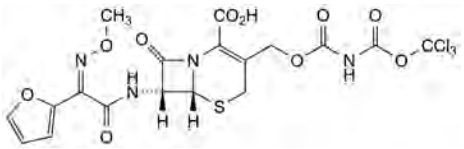

C. (6*R*,7*R*)-7-[[(*Z*)-2-(furan-2-yl)-2-(methoxyimino)acetyl]-amino]-8-oxo-3-[[[(trichloroacetyl)carbamoyl]oxy]-methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

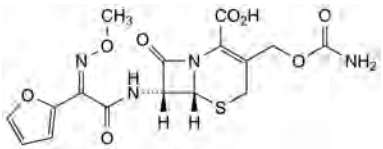

D. cefuroxime.

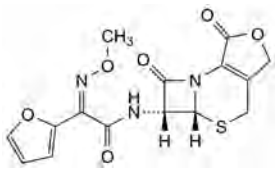

E. (5a*R*,6*R*)-6-[[(2*Z*)-2-(furan-2-yl)-2-(methoxy-imino)acetyl]amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione (descarbamoylcefuroxime lactone).

**01/2008:0992**
**corrected 10.0**

# CEFUROXIME SODIUM

## Cefuroximum natricum

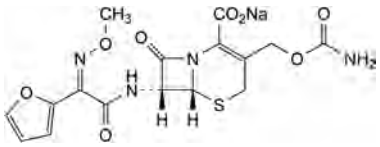

$C_{16}H_{15}N_4NaO_8S$ $M_r$ 446.4
[56238-63-2]

DEFINITION

Sodium (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cefuroxime sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*). The absorbance (*2.2.25*) of solution S measured at 450 nm is not greater than 0.25.

**pH** (*2.2.3*): 5.5 to 8.5.

Dilute 2 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 59 to + 66 (anhydrous substance).

Dissolve 0.500 g in *acetate buffer solution pH 4.6 R* and dilute to 25.0 mL with the same buffer solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or keep at 2-8 °C.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 25.0 mg of *cefuroxime sodium CRS* in *water R* and dilute to 25.0 mL with the same solvent. Dilute 5.0 mL to 50.0 mL with *water R*.

*Reference solution (b).* Place 20 mL of reference solution (a) in a water-bath at 80 °C for 15 min. Cool and inject immediately.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *hexylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 1 volume of *acetonitrile R* and 99 volumes of an acetate buffer solution pH 3.4, prepared by dissolving 6.01 g of *glacial acetic acid R* and 0.68 g of *sodium acetate R* in *water R* and diluting to 1000 mL with the same solvent.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL loop injector; inject test solution (a) and reference solutions (b) and (c).

*Run time*: 4 times the retention time of cefuroxime.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to cefuroxime and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent *m/m*.

**Water** (*2.5.12*): maximum 3.5 per cent, determined on 0.400 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of cefuroxime sodium.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

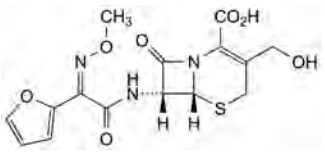

A. (6*R*,7*R*)-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]-amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid (descarbamoyl-cefuroxime),

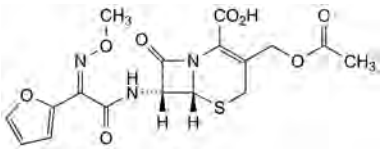

B. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

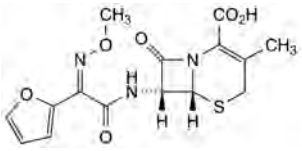

C. (6*R*,7*R*)-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]-amino]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct-2-ene-2-carboxylic acid,

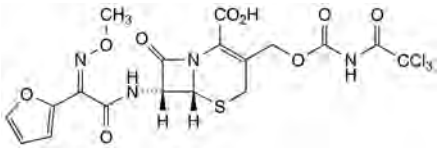

D. (6*R*,7*R*)-7-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]-amino]-8-oxo-3-[[[(trichloroacetyl)carbamoyl]oxy]-methyl]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

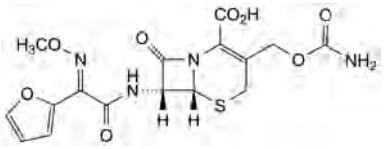

E. (6*R*,7*R*)-3-[(carbamoyloxy)methyl]-7-[[(*E*)-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (*trans*-cefuroxime),

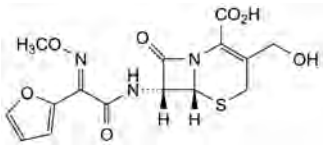

F. (6*R*,7*R*)-7-[[(*E*)-(furan-2-yl)(methoxyimino)acetyl]-amino]-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid,

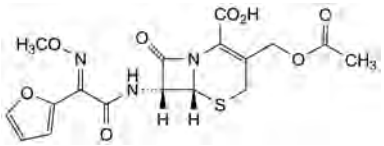

G. (6*R*,7*R*)-3-[(acetyloxy)methyl]-7-[[(*E*)-(furan-2-yl)(methoxyimino)acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid,

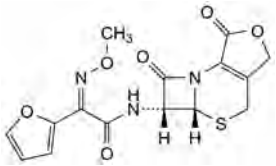

H. (5a*R*,6*R*)-6-[[(*Z*)-(furan-2-yl)(methoxyimino)acetyl]-amino]-5a,6-dihydro-3*H*,7*H*-azeto[2,1-*b*]furo[3,4-*d*][1,3]thiazine-1,7(4*H*)-dione,

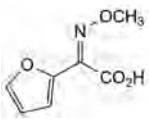

I. (*Z*)-(furan-2-yl)(methoxyimino)acetic acid.

**01/2017:2591**

# CELECOXIB

## Celecoxibum

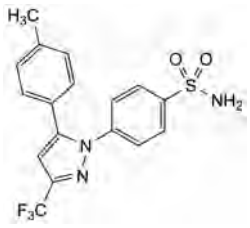

$C_{17}H_{14}F_3N_3O_2S$ $M_r$ 381.4
[169590-42-5]

DEFINITION

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzenesulfonamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or amorphous powder.

*Solubility*: practically insoluble in water, freely soluble to soluble in anhydrous ethanol, soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *celecoxib CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R2* (25:75 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *celecoxib CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 3 mg of *celecoxib impurity A CRS* and 3 mg of *celecoxib impurity B CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 25.0 mL with reference solution (a).

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*: mix 10 volumes of *acetonitrile R1*, 30 volumes of *methanol R2* and 60 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 25 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.5 times the retention time of celecoxib.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to celecoxib (retention time = about 27 min): impurity A = about 0.9; impurity B = about 1.1.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity A and celecoxib and minimum 1.8 between the peaks due to celecoxib and impurity B in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- for all impurities, use the concentration of celecoxib in reference solution (c).

*Limits*:

- *impurity A*: maximum 0.4 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.400 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{14}F_3N_3O_2S$ taking into account the assigned content of *celecoxib CRS*.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

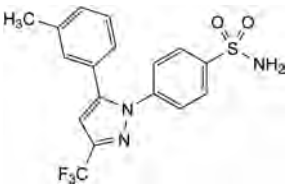

A. 4-[5-(3-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide,

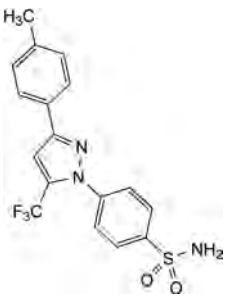

B. 4-[3-(4-methylphenyl)-5-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide.

01/2008:1632
corrected 6.0

# CELIPROLOL HYDROCHLORIDE

## Celiprololi hydrochloridum

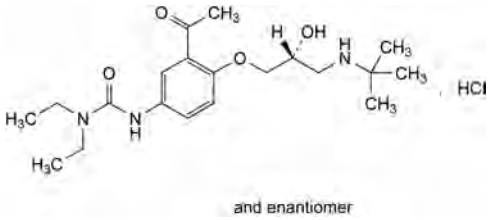


$C_{20}H_{34}ClN_3O_4$ $M_r$ 416.0
[57470-78-7]

DEFINITION

3-[3-Acetyl-4-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]-1,1-diethylurea hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or very slightly yellow, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *celiprolol hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 1.0 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 2 mg of the substance to be examined and 2 mg of *acebutolol hydrochloride R* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined in 2 mL of mobile phase A and allow to stand for 24 h (for identification of impurity A).

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 10 mg of *celiprolol for peak identification CRS* in mobile phase A and dilute to 2 mL with mobile phase A.

*Reference solution (e). This solution is only prepared if required (see below) and is used to determine the identity of impurity I which co-elutes with impurity H (the 2 impurities originate from different routes of synthesis).* Dissolve the contents of a vial of *celiprolol impurity I CRS* in mobile phase A and dilute to 2.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 91 mL of *tetrahydrofuran R*, 63 mL of *acetonitrile R1*, 0.6 mL of *pentafluoropropanoic acid R* and 0.2 mL of *trifluoroacetic acid R*; dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 → 80 | 0 → 20 |
| 50 - 51 | 80 → 100 | 20 → 0 |
| 51 - 65 | 100 | 0 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *celiprolol for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B, E and F.

*Relative retention* with reference to celiprolol (retention time = about 10 min): impurity A = about 0.3; impurity D = about 0.7; impurity G = about 1.2; impurity B = about 1.4; impurity F = about 1.6; impurity C = about 2.2; impurity H or I = about 2.5; impurity E = about 3.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to celiprolol and acebutolol.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 4.0; impurity B = 1.5; impurity E = 2.3; impurity F = 0.5; impurity I = 1.7;
- *any impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent), and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- if any of the above limits are exceeded and if a peak occurs with a relative retention of about 2.5 (impurity H or I), the identity of this peak has to be clarified by use of a UV spectrum recorded with a diode array detector; if this spectrum is different from the one obtained with reference solution (e), no correction factor is applied;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.350 g under an atmosphere of nitrogen in 50 mL of *ethanol (96 per cent) R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.60 mg of $C_{20}H_{34}ClN_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

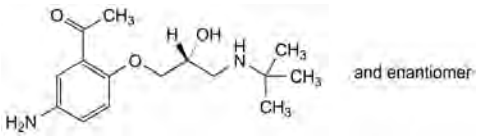


A. 1-[5-amino-2-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]ethanone,

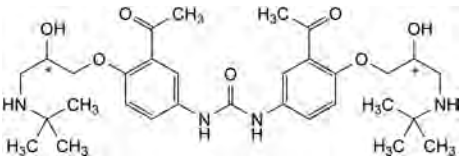

B. 1,3-bis[3-acetyl-4-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]urea,

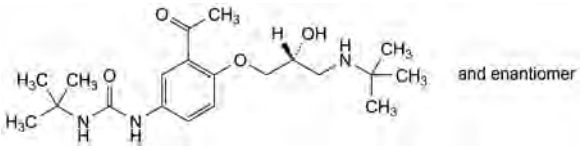


C. 1-[3-acetyl-4-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]phenyl]-3-(1,1-dimethylethyl)urea,

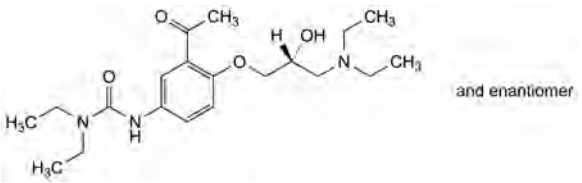


D. 3-[3-acetyl-4-[(2*RS*)-3-(diethylamino)-2-hydroxypropoxy]phenyl]-1,1-diethylurea,

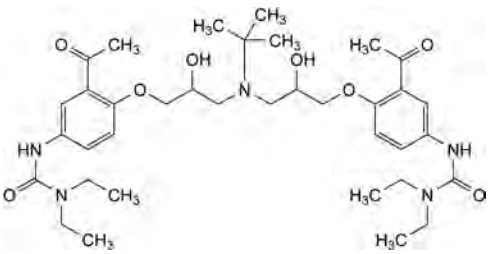

E. 1,1′-[[(1,1-dimethylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy(3-acetyl-1,4-phenylene)]]bis(3,3-diethylurea),

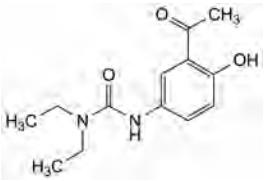

F. 3-(3-acetyl-4-hydroxyphenyl)-1,1-diethylurea,

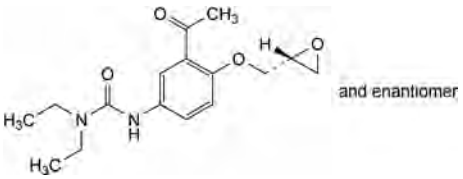


G. 3-[3-acetyl-4-[[(*RS*)-oxiranyl]methoxy]phenyl]-1,1-diethylurea,

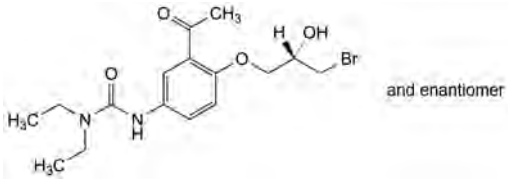


H. 3-[3-acetyl-4-[(2*RS*)-3-bromo-2-hydroxypropoxy]phenyl]-1,1-diethylurea (bromhydrin compound),

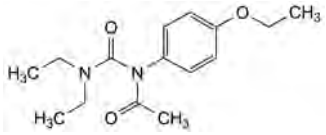

I. 1-acetyl-1-(4-ethoxyphenyl)-3,3-diethylurea.

**04/2019:0887**

# CELLULOSE ACETATE

## Cellulosi acetas

DEFINITION

Partly or completely *O*-acetylated cellulose.

*Content*:

– *acetyl groups* ($C_2H_3O$; $M_r$ 43.04): 29.0 per cent to 44.8 per cent (dried substance); 90.0 per cent to 110.0 per cent of the nominal content (dried substance).

CHARACTERS

*Appearance*: white, yellowish-white or greyish-white, hygroscopic powder or granules.

*Solubility*: practically insoluble in water, soluble in acetone, in formic acid and in a mixture of equal volumes of methanol and methylene chloride, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cellulose acetate CRS*.

*Preparation*: prepare a 20 g/L solution of cellulose acetate, previously dried, in *acetone R* (mono- or diester) or in *methylene chloride R* (di- or triester), and spread 1 drop of the solution between 2 sodium chloride plates; separate the plates, heat them both at 105 °C for 1 h, and reassemble the dried plates.

TESTS

**Free acid**: maximum 0.1 per cent, calculated as acetic acid (dried substance).

To 5.00 g in a 250 mL conical flask, add 150 mL of *carbon dioxide-free water R*, insert the stopper, swirl the suspension gently and allow to stand for 3 h. Filter, then wash the flask and the filter with *carbon dioxide-free water R*, adding the washings to the filtrate. Add 0.1 mL of *phenolphthalein solution R1* and titrate the combined filtrate and washings with *0.01 M sodium hydroxide* until a pale pink colour is obtained.

1 mL of *0.01 M sodium hydroxide* is equivalent to 0.6005 mg of free acid, calculated as acetic acid.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

A. Cellulose acetate containing not more than 42.0 per cent of acetyl groups.

To 2.000 g in a 500 mL conical flask, add 100 mL of *acetone R* and 5-10 mL of *water R*. Close the flask and stir with a magnetic stirrer until dissolution is complete. Add 30.0 mL of *1 M sodium hydroxide* with constant stirring. A finely divided precipitate of regenerated cellulose, free from lumps, is obtained. Close the flask and stir with a magnetic stirrer for 30 min. Add 100 mL of *water R* at 80 °C, washing down the sides of the flask, stir for 2 min and cool to room temperature. Titrate with *0.5 M sulfuric acid*, using 0.1 mL of *phenolphthalein solution R1* as indicator. Carry out a blank titration.

Calculate the percentage content of acetyl groups using the following expression:

$$\frac{4.305(n_2 - n_1)}{(100 - d) \times m} \times 100$$

$d$ = loss on drying as a percentage;

$m$ = mass of the substance to be examined, in grams;

$n_1$ = volume of *0.5 M sulfuric acid* used in the titration, in millilitres;

$n_2$ = volume of *0.5 M sulfuric acid* used in the blank titration, in millilitres.

B. Cellulose acetate containing more than 42.0 per cent of acetyl groups.

To 2.000 g in a 500 mL conical flask, add 30 mL of *dimethyl sulfoxide R* and 100 mL of *acetone R*. Close the flask and stir with a magnetic stirrer for 16 h. Add 30.0 mL of *1 M sodium hydroxide* with constant stirring. Close the flask and stir with a magnetic stirrer for 6 min. Allow to stand without stirring for 60 min. Resume stirring and add 100 mL of *water R* at 80 °C, washing down the sides of the flask, stir for 2 min and cool to room temperature. Titrate with *0.5 M hydrochloric acid*, using 0.1 mL of *phenolphthalein solution R1* as indicator. Add 0.5 mL of *0.5 M hydrochloric acid* in excess, stir for 5 min and allow to stand for 30 min. Titrate with *0.5 M sodium hydroxide* until a persistent pink colour is obtained, stirring with a magnetic stirrer. Calculate the net amount of *0.5 M sodium hydroxide* consumed, in millimoles, taking the mean of 2 blank titrations into consideration.

Calculate the percentage content of acetyl groups using the following expression:

$$\frac{4.305 \times n}{(100 - d) \times m} \times 100$$

$d$ = loss on drying as a percentage;

$m$ = mass of the substance to be examined, in grams;

$n$ = net amount of *0.5 M sodium hydroxide* consumed, in millimoles.

STORAGE

In an airtight container.

LABELLING

The label states the nominal percentage content of acetyl groups.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for cellulose acetate used as film former.*

**Viscosity**. Dissolve 10 g in a mixture of 50 mL of *methanol R* and 50 mL of *methylene chloride R* by shaking. Determine the viscosity of this solution at 20 ± 0.1 °C using a rotating viscometer (*2.2.10*).

**Acetyl groups**: see Assay.

*The following characteristics may be relevant for cellulose acetate used as matrix former in prolonged-release tablets.*

**Viscosity**: see test above.

**Acetyl groups**: see Assay.

**Molecular mass distribution** (*2.2.30*).

**Particle-size distribution** (*2.9.31*).

**Powder flow** (*2.9.36*).

**01/2017:1406**

# CELLULOSE ACETATE BUTYRATE

## Cellulosi acetas butyras

DEFINITION

Partly or completely *O*-acetylated and *O*-butyrated cellulose.

*Content*:

- *acetyl groups* ($C_2H_3O$): 2.0 per cent to 30.0 per cent (dried substance); 90.0 per cent to 110.0 per cent of that stated on the label (dried substance);
- *butyryl groups* ($C_4H_7O$): 16.0 per cent to 53.0 per cent (dried substance); 90.0 per cent to 110.0 per cent of that stated on the label (dried substance).

CHARACTERS

*Appearance*: white, yellowish-white or greyish-white powder or granules, slightly hygroscopic.

*Solubility*: practically insoluble in water, soluble in acetone, in formic acid and in a mixture of equal volumes of methanol and methylene chloride, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cellulose acetate butyrate CRS*.

The intensity of the bands may vary according to the degree of substitution.

B. It complies with the limits of the assay.

TESTS

**Acidity**. To 5.00 g in a 250 mL conical flask, add 150 mL of *carbon dioxide-free water R*, insert the stopper, swirl the suspension gently and allow to stand for 3 h. Filter, wash the flask and the filter with *carbon dioxide-free water R*. Combine the filtrate and washings. Add 0.1 mL of *phenolphthalein solution R1*. Not more than 3.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. To 1.000 g of the substance to be examined in a 500 mL conical flask, add 100 mL of *acetone R* and 10 mL of *water R*. Close the flask and stir with a magnetic stirrer until dissolution is complete. Add 30.0 mL of *1 M sodium hydroxide* with constant stirring. Close the flask and stir with a magnetic stirrer for 30 min. Add 100 mL of hot *water R* at 80 °C, washing down the sides of the flask and stir for 2 min. Cool, centrifuge or filter the suspension and wash the residue with *water R*. Combine the filtrate and washings, adjust to pH 3 with *dilute phosphoric acid R* and dilute to 500.0 mL with *water R*.

*Reference solution*. Dissolve 0.200 g of *glacial acetic acid R* and 0.400 g of *butyric acid R* in *water R*, adjust to pH 3 with *dilute phosphoric acid R* and dilute to 500.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *methanol R*;
- *mobile phase B*: *phosphate buffer solution pH 3.0 R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 5 | 95 |
| 30 - 35 | 5 → 20 | 95 → 80 |
| 35 - 60 | 20 | 80 |
| 60 - 61 | 5 | 95 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

Calculate the percentage content of acetic acid and butyric acid using the chromatograms obtained with the 2 solutions. To calculate the percentage content of acetyl ($C_2H_3O$) and of butyryl ($C_4H_7O$) groups, multiply the percentage content of acetic acid and butyric acid by 0.717 and 0.807, respectively.

STORAGE

In an airtight container.

LABELLING

The label states the nominal percentage content of acetyl and butyryl groups.

**01/2017:0314**

# CELLULOSE ACETATE PHTHALATE(4)

## Cellulosi acetas phthalas

[9004-38-0]

DEFINITION

Partly *O*-acetylated and *O*-phthalylated cellulose.

*Content*:

- *phthaloyl groups* ($C_8H_5O_3$; $M_r$ 149.1): 30.0 per cent to 36.0 per cent (anhydrous and acid-free substance);
- *acetyl groups* ($C_2H_3O$; $M_r$ 43.04): 21.5 per cent to 26.0 per cent (anhydrous and acid-free substance).

♦CHARACTERS

*Appearance*: white or almost white, free-flowing powder or colourless flakes, hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in diethylene glycol, practically insoluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.♦

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cellulose acetate phthalate CRS*.

TESTS

**Viscosity** (*2.2.9*): 45 mPa·s to 90 mPa·s, determined at 25 ± 0.2 °C.

Dissolve 15 g, calculated with reference to the anhydrous substance, in 85 g of a mixture of 1 part by mass of *water R* and 249 parts by mass of *acetone R*.

**Free acid**: maximum 3.0 per cent, calculated as phthalic acid (anhydrous substance).

Shake 3.0 g for 2 h with 100 mL of a 50 per cent *V/V* solution of *methanol R* and filter. Wash the flask and the filter with 2 quantities, each of 10 mL, of a 50 per cent *V/V* solution of *methanol R*. Combine the filtrate and washings, add 0.1 mL of *phenolphthalein solution R1* and titrate with *0.1 M sodium hydroxide* until a faint pink colour is obtained. Carry out a blank titration using 120 mL of a 50 per cent *V/V* solution of *methanol R*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 8.3 mg of free acid, calculated as phthalic acid.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

Carry out the test using a mixture of 2 volumes of *methylene chloride R* and 3 volumes of *anhydrous ethanol R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

(4) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

ASSAY

**Phthaloyl groups**. Dissolve 1.000 g in 50 mL of a mixture of 2 volumes of *acetone R* and 3 volumes of *ethanol (96 per cent) R*. Add about 0.1 mL of *phenolphthalein solution R1* and titrate with *0.1 M sodium hydroxide*. Carry out a blank titration.

Calculate the percentage content of phthaloyl groups ($P$) using the following expression:

$$\frac{14910(n_1 - n_2)}{(100 - a)(100 - S)m} - \frac{179.5S}{(100 - S)}$$

$a$ = percentage content of water (see Tests);

$m$ = mass of the substance to be examined, in grams;

$n_1$ = volume of *0.1 M sodium hydroxide* used in the titration, in millilitres;

$n_2$ = volume of *0.1 M sodium hydroxide* used in the blank titration, in millilitres;

$S$ = percentage content of free acid (see Tests).

**Acetyl groups**. To 0.100 g add 25.0 mL of *0.1 M sodium hydroxide* and heat on a water-bath under a reflux condenser for 30 min. Cool, add about 0.1 mL of *phenolphthalein solution R1* and titrate with *0.1 M hydrochloric acid*. Carry out a blank titration.

Calculate the percentage content of acetyl groups using the following expression:

$$\left[\frac{4305(n_2 - n_1)}{(100 - a)(100 - S)m} - \frac{51.82S}{(100 - S)}\right] - 0.5772P$$

$a$ = percentage content of water (see Tests);

$m$ = mass of the substance to be examined, in grams;

$n_1$ = volume of *0.1 M hydrochloric acid* used in the titration, in millilitres;

$n_2$ = volume of *0.1 M hydrochloric acid* used in the blank titration, in millilitres;

$P$ = percentage content of phthaloyl groups;

$S$ = percentage content of free acid (see Tests).

STORAGE

In an airtight container.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for cellulose acetate phthalate used as film former in gastro-resistant tablets and capsules.*

**Viscosity**: see Tests.

**Solubility of a film**. Dissolve about 0.15 g in 1 mL of *acetone R* and pour onto a clear glass plate. A film is formed. Take a piece of the film and place it in a flask containing *0.1 M hydrochloric acid*. It does not dissolve. Then place the piece of film in a flask containing *phosphate buffer solution pH 6.8 R*. It dissolves.

**Phthaloyl groups**: see Assay.

**Acetyl groups**: see Assay.

**01/2020:0316**

# CELLULOSE, MICROCRYSTALLINE[5]

## Cellulosum microcristallinum

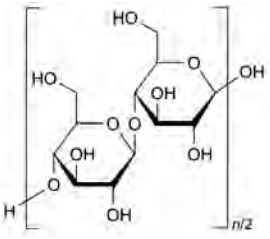


$C_{6n}H_{10n+2}O_{5n+1}$
[9004-34-6]

DEFINITION

Purified, partly depolymerised cellulose prepared by treating alpha-cellulose, obtained as a pulp from fibrous plant material, with mineral acids.

♦CHARACTERS

*Appearance*: white or almost white, fine or granular, slightly hygroscopic powder.

*Solubility*: practically insoluble in water, in acetone, in anhydrous ethanol, in toluene, in dilute acids and in a 50 g/L solution of sodium hydroxide.♦

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *microcrystalline cellulose CRS*.

Disregard any band between 800 $cm^{-1}$ and 825 $cm^{-1}$ or between 950 $cm^{-1}$ and 1000 $cm^{-1}$.

B. Place about 10 mg on a watch-glass and disperse in 2 mL of *iodinated zinc chloride solution R*. The substance becomes violet-blue.

C. The degree of polymerisation is not more than 350.

Transfer 1.300 g to a 125 mL conical flask. Add 25.0 mL of *water R* and 25.0 mL of *cupriethylenediamine hydroxide solution R*. Immediately purge the solution with *nitrogen R*, insert the stopper and shake until completely dissolved. Transfer an appropriate volume of the solution to a suitable capillary viscometer (*2.2.9*). Equilibrate the solution at 25 ± 0.1 °C for at least 5 min. Record the flow time ($t_1$) in seconds between the 2 marks on the viscometer. Calculate the kinematic viscosity ($v_1$) of the solution using the following expression:

$$t_1(k_1)$$

$k_1$ = viscometer constant.

(5) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Dilute a suitable volume of *cupriethylenediamine hydroxide solution R* with an equal volume of *water R* and measure the flow time ($t_2$) using a suitable capillary viscometer. Calculate the kinematic viscosity ($v_2$) of the solvent using the following expression:

$$t_2(k_2)$$

$k_2$ = viscometer constant.

Determine the relative viscosity ($\eta_{rel}$) of the substance to be examined using the following expression:

$$v_1 / v_2$$

Determine the intrinsic viscosity ($[\eta]_c$) by interpolation, using the intrinsic viscosity table (Table 0316.-1).

Calculate the degree of polymerisation ($P$) using the following expression:

$$\frac{95[\eta]_c}{m[(100 - b) / 100]}$$

$m$ = mass of the substance to be examined, in grams;

$b$ = loss on drying, in per cent.

TESTS

◊**Solubility**. Dissolve 50 mg in 10 mL of *ammoniacal solution of copper tetrammine R*. It dissolves completely, leaving no residue.◊

**pH** (*2.2.3*): 5.0 to 7.5 for the supernatant.

Shake 5 g with 40 mL of *carbon dioxide-free water R* for 20 min and centrifuge.

**Conductivity** (*2.2.38*). The conductivity of the test solution does not exceed the conductivity of the water by more than 75 µS·cm$^{-1}$.

Use as test solution the supernatant obtained in the test for pH. Measure the conductivity of the supernatant after a stable reading has been obtained and measure the conductivity of the water used to prepare the test solution.

**Ether-soluble substances**: maximum 0.05 per cent (5.0 mg) for the difference between the mass of the residue and the mass obtained from a blank determination.

Place 10.0 g in a chromatography column about 20 mm in internal diameter and pass 50 mL of *peroxide-free ether R* through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish, with the aid of a current of air in a fume cupboard. After all ether has evaporated, dry the residue at 105 °C for 30 min, allow to cool in a desiccator and weigh. Carry out a blank determination.

**Water-soluble substances**: maximum 0.25 per cent (12.5 mg) for the difference between the mass of the residue and the mass obtained from a blank determination.

Shake 5.0 g with 80 mL of *water R* for 10 min. Filter through a filter paper with the aid of vacuum into a tared flask. Evaporate to dryness on a water-bath avoiding charring. Dry at 105 °C for 1 h, allow to cool in a desiccator and weigh. Carry out a blank determination.

Table 0316.-1. – *Intrinsic viscosity table*

| Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$ | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $[\eta]_c$ | | | | | | | | | |
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 1.1 | 0.098 | 0.106 | 0.115 | 0.125 | 0.134 | 0.143 | 0.152 | 0.161 | 0.170 | 0.180 |
| 1.2 | 0.189 | 0.198 | 0.207 | 0.216 | 0.225 | 0.233 | 0.242 | 0.250 | 0.259 | 0.268 |
| 1.3 | 0.276 | 0.285 | 0.293 | 0.302 | 0.310 | 0.318 | 0.326 | 0.334 | 0.342 | 0.350 |
| 1.4 | 0.358 | 0.367 | 0.375 | 0.383 | 0.391 | 0.399 | 0.407 | 0.414 | 0.422 | 0.430 |
| 1.5 | 0.437 | 0.445 | 0.453 | 0.460 | 0.468 | 0.476 | 0.484 | 0.491 | 0.499 | 0.507 |
| 1.6 | 0.515 | 0.522 | 0.529 | 0.536 | 0.544 | 0.551 | 0.558 | 0.566 | 0.573 | 0.580 |
| 1.7 | 0.587 | 0.595 | 0.602 | 0.608 | 0.615 | 0.622 | 0.629 | 0.636 | 0.642 | 0.649 |
| 1.8 | 0.656 | 0.663 | 0.670 | 0.677 | 0.683 | 0.690 | 0.697 | 0.704 | 0.710 | 0.717 |
| 1.9 | 0.723 | 0.730 | 0.736 | 0.743 | 0.749 | 0.756 | 0.762 | 0.769 | 0.775 | 0.782 |
| 2.0 | 0.788 | 0.795 | 0.802 | 0.809 | 0.815 | 0.821 | 0.827 | 0.833 | 0.840 | 0.846 |
| 2.1 | 0.852 | 0.858 | 0.864 | 0.870 | 0.876 | 0.882 | 0.888 | 0.894 | 0.900 | 0.906 |
| 2.2 | 0.912 | 0.918 | 0.924 | 0.929 | 0.935 | 0.941 | 0.948 | 0.953 | 0.959 | 0.965 |
| 2.3 | 0.971 | 0.976 | 0.983 | 0.988 | 0.994 | 1.000 | 1.006 | 1.011 | 1.017 | 1.022 |
| 2.4 | 1.028 | 1.033 | 1.039 | 1.044 | 1.050 | 1.056 | 1.061 | 1.067 | 1.072 | 1.078 |
| 2.5 | 1.083 | 1.089 | 1.094 | 1.100 | 1.105 | 1.111 | 1.116 | 1.121 | 1.126 | 1.131 |
| 2.6 | 1.137 | 1.142 | 1.147 | 1.153 | 1.158 | 1.163 | 1.169 | 1.174 | 1.179 | 1.184 |
| 2.7 | 1.190 | 1.195 | 1.200 | 1.205 | 1.210 | 1.215 | 1.220 | 1.225 | 1.230 | 1.235 |
| 2.8 | 1.240 | 1.245 | 1.250 | 1.255 | 1.260 | 1.265 | 1.270 | 1.275 | 1.280 | 1.285 |
| 2.9 | 1.290 | 1.295 | 1.300 | 1.305 | 1.310 | 1.314 | 1.319 | 1.324 | 1.329 | 1.333 |
| 3.0 | 1.338 | 1.343 | 1.348 | 1.352 | 1.357 | 1.362 | 1.367 | 1.371 | 1.376 | 1.381 |
| 3.1 | 1.386 | 1.390 | 1.395 | 1.400 | 1.405 | 1.409 | 1.414 | 1.418 | 1.423 | 1.427 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 3.2 | 1.432 | 1.436 | 1.441 | 1.446 | 1.450 | 1.455 | 1.459 | 1.464 | 1.468 | 1.473 |
| 3.3 | 1.477 | 1.482 | 1.486 | 1.491 | 1.496 | 1.500 | 1.504 | 1.508 | 1.513 | 1.517 |
| 3.4 | 1.521 | 1.525 | 1.529 | 1.533 | 1.537 | 1.542 | 1.546 | 1.550 | 1.554 | 1.558 |
| 3.5 | 1.562 | 1.566 | 1.570 | 1.575 | 1.579 | 1.583 | 1.587 | 1.591 | 1.595 | 1.600 |
| 3.6 | 1.604 | 1.608 | 1.612 | 1.617 | 1.621 | 1.625 | 1.629 | 1.633 | 1.637 | 1.642 |
| 3.7 | 1.646 | 1.650 | 1.654 | 1.658 | 1.662 | 1.666 | 1.671 | 1.675 | 1.679 | 1.683 |
| 3.8 | 1.687 | 1.691 | 1.695 | 1.700 | 1.704 | 1.708 | 1.712 | 1.715 | 1.719 | 1.723 |
| 3.9 | 1.727 | 1.731 | 1.735 | 1.739 | 1.742 | 1.746 | 1.750 | 1.754 | 1.758 | 1.762 |
| 4.0 | 1.765 | 1.769 | 1.773 | 1.777 | 1.781 | 1.785 | 1.789 | 1.792 | 1.796 | 1.800 |
| 4.1 | 1.804 | 1.808 | 1.811 | 1.815 | 1.819 | 1.822 | 1.826 | 1.830 | 1.833 | 1.837 |
| 4.2 | 1.841 | 1.845 | 1.848 | 1.852 | 1.856 | 1.859 | 1.863 | 1.867 | 1.870 | 1.874 |
| 4.3 | 1.878 | 1.882 | 1.885 | 1.889 | 1.893 | 1.896 | 1.900 | 1.904 | 1.907 | 1.911 |
| 4.4 | 1.914 | 1.918 | 1.921 | 1.925 | 1.929 | 1.932 | 1.936 | 1.939 | 1.943 | 1.946 |
| 4.5 | 1.950 | 1.954 | 1.957 | 1.961 | 1.964 | 1.968 | 1.971 | 1.975 | 1.979 | 1.982 |
| 4.6 | 1.986 | 1.989 | 1.993 | 1.996 | 2.000 | 2.003 | 2.007 | 2.010 | 2.013 | 2.017 |
| 4.7 | 2.020 | 2.023 | 2.027 | 2.030 | 2.033 | 2.037 | 2.040 | 2.043 | 2.047 | 2.050 |
| 4.8 | 2.053 | 2.057 | 2.060 | 2.063 | 2.067 | 2.070 | 2.073 | 2.077 | 2.080 | 2.083 |
| 4.9 | 2.087 | 2.090 | 2.093 | 2.097 | 2.100 | 2.103 | 2.107 | 2.110 | 2.113 | 2.116 |
| 5.0 | 2.119 | 2.122 | 2.125 | 2.129 | 2.132 | 2.135 | 2.139 | 2.142 | 2.145 | 2.148 |
| 5.1 | 2.151 | 2.154 | 2.158 | 2.160 | 2.164 | 2.167 | 2.170 | 2.173 | 2.176 | 2.180 |
| 5.2 | 2.183 | 2.186 | 2.190 | 2.192 | 2.195 | 2.197 | 2.200 | 2.203 | 2.206 | 2.209 |
| 5.3 | 2.212 | 2.215 | 2.218 | 2.221 | 2.224 | 2.227 | 2.230 | 2.233 | 2.236 | 2.240 |
| 5.4 | 2.243 | 2.246 | 2.249 | 2.252 | 2.255 | 2.258 | 2.261 | 2.264 | 2.267 | 2.270 |
| 5.5 | 2.273 | 2.276 | 2.279 | 2.282 | 2.285 | 2.288 | 2.291 | 2.294 | 2.297 | 2.300 |
| 5.6 | 2.303 | 2.306 | 2.309 | 2.312 | 2.315 | 2.318 | 2.320 | 2.324 | 2.326 | 2.329 |
| 5.7 | 2.332 | 2.335 | 2.338 | 2.341 | 2.344 | 2.347 | 2.350 | 2.353 | 2.355 | 2.358 |
| 5.8 | 2.361 | 2.364 | 2.367 | 2.370 | 2.373 | 2.376 | 2.379 | 2.382 | 2.384 | 2.387 |
| 5.9 | 2.390 | 2.393 | 2.396 | 2.400 | 2.403 | 2.405 | 2.408 | 2.411 | 2.414 | 2.417 |
| 6.0 | 2.419 | 2.422 | 2.425 | 2.428 | 2.431 | 2.433 | 2.436 | 2.439 | 2.442 | 2.444 |
| 6.1 | 2.447 | 2.450 | 2.453 | 2.456 | 2.458 | 2.461 | 2.464 | 2.467 | 2.470 | 2.472 |
| 6.2 | 2.475 | 2.478 | 2.481 | 2.483 | 2.486 | 2.489 | 2.492 | 2.494 | 2.497 | 2.500 |
| 6.3 | 2.503 | 2.505 | 2.508 | 2.511 | 2.513 | 2.516 | 2.518 | 2.521 | 2.524 | 2.526 |
| 6.4 | 2.529 | 2.532 | 2.534 | 2.537 | 2.540 | 2.542 | 2.545 | 2.547 | 2.550 | 2.553 |
| 6.5 | 2.555 | 2.558 | 2.561 | 2.563 | 2.566 | 2.568 | 2.571 | 2.574 | 2.576 | 2.579 |
| 6.6 | 2.581 | 2.584 | 2.587 | 2.590 | 2.592 | 2.595 | 2.597 | 2.600 | 2.603 | 2.605 |
| 6.7 | 2.608 | 2.610 | 2.613 | 2.615 | 2.618 | 2.620 | 2.623 | 2.625 | 2.627 | 2.630 |
| 6.8 | 2.633 | 2.635 | 2.637 | 2.640 | 2.643 | 2.645 | 2.648 | 2.650 | 2.653 | 2.655 |
| 6.9 | 2.658 | 2.660 | 2.663 | 2.665 | 2.668 | 2.670 | 2.673 | 2.675 | 2.678 | 2.680 |
| 7.0 | 2.683 | 2.685 | 2.687 | 2.690 | 2.693 | 2.695 | 2.698 | 2.700 | 2.702 | 2.705 |
| 7.1 | 2.707 | 2.710 | 2.712 | 2.714 | 2.717 | 2.719 | 2.721 | 2.724 | 2.726 | 2.729 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 7.2 | 2.731 | 2.733 | 2.736 | 2.738 | 2.740 | 2.743 | 2.745 | 2.748 | 2.750 | 2.752 |
| 7.3 | 2.755 | 2.757 | 2.760 | 2.762 | 2.764 | 2.767 | 2.769 | 2.771 | 2.774 | 2.776 |
| 7.4 | 2.779 | 2.781 | 2.783 | 2.786 | 2.788 | 2.790 | 2.793 | 2.795 | 2.798 | 2.800 |
| 7.5 | 2.802 | 2.805 | 2.807 | 2.809 | 2.812 | 2.814 | 2.816 | 2.819 | 2.821 | 2.823 |
| 7.6 | 2.826 | 2.828 | 2.830 | 2.833 | 2.835 | 2.837 | 2.840 | 2.842 | 2.844 | 2.847 |
| 7.7 | 2.849 | 2.851 | 2.854 | 2.856 | 2.858 | 2.860 | 2.863 | 2.865 | 2.868 | 2.870 |
| 7.8 | 2.873 | 2.875 | 2.877 | 2.879 | 2.881 | 2.884 | 2.887 | 2.889 | 2.891 | 2.893 |
| 7.9 | 2.895 | 2.898 | 2.900 | 2.902 | 2.905 | 2.907 | 2.909 | 2.911 | 2.913 | 2.915 |
| 8.0 | 2.918 | 2.920 | 2.922 | 2.924 | 2.926 | 2.928 | 2.931 | 2.933 | 2.935 | 2.937 |
| 8.1 | 2.939 | 2.942 | 2.944 | 2.946 | 2.948 | 2.950 | 2.952 | 2.955 | 2.957 | 2.959 |
| 8.2 | 2.961 | 2.963 | 2.966 | 2.968 | 2.970 | 2.972 | 2.974 | 2.976 | 2.979 | 2.981 |
| 8.3 | 2.983 | 2.985 | 2.987 | 2.990 | 2.992 | 2.994 | 2.996 | 2.998 | 3.000 | 3.002 |
| 8.4 | 3.004 | 3.006 | 3.008 | 3.010 | 3.012 | 3.015 | 3.017 | 3.019 | 3.021 | 3.023 |
| 8.5 | 3.025 | 3.027 | 3.029 | 3.031 | 3.033 | 3.035 | 3.037 | 3.040 | 3.042 | 3.044 |
| 8.6 | 3.046 | 3.048 | 3.050 | 3.052 | 3.054 | 3.056 | 3.058 | 3.060 | 3.062 | 3.064 |
| 8.7 | 3.067 | 3.069 | 3.071 | 3.073 | 3.075 | 3.077 | 3.079 | 3.081 | 3.083 | 3.085 |
| 8.8 | 3.087 | 3.089 | 3.092 | 3.094 | 3.096 | 3.098 | 3.100 | 3.102 | 3.104 | 3.106 |
| 8.9 | 3.108 | 3.110 | 3.112 | 3.114 | 3.116 | 3.118 | 3.120 | 3.122 | 3.124 | 3.126 |
| 9.0 | 3.128 | 3.130 | 3.132 | 3.134 | 3.136 | 3.138 | 3.140 | 3.142 | 3.144 | 3.146 |
| 9.1 | 3.148 | 3.150 | 3.152 | 3.154 | 3.156 | 3.158 | 3.160 | 3.162 | 3.164 | 3.166 |
| 9.2 | 3.168 | 3.170 | 3.172 | 3.174 | 3.176 | 3.178 | 3.180 | 3.182 | 3.184 | 3.186 |
| 9.3 | 3.188 | 3.190 | 3.192 | 3.194 | 3.196 | 3.198 | 3.200 | 3.202 | 3.204 | 3.206 |
| 9.4 | 3.208 | 3.210 | 3.212 | 3.214 | 3.215 | 3.217 | 3.219 | 3.221 | 3.223 | 3.225 |
| 9.5 | 3.227 | 3.229 | 3.231 | 3.233 | 3.235 | 3.237 | 3.239 | 3.241 | 3.242 | 3.244 |
| 9.6 | 3.246 | 3.248 | 3.250 | 3.252 | 3.254 | 3.256 | 3.258 | 3.260 | 3.262 | 3.264 |
| 9.7 | 3.266 | 3.268 | 3.269 | 3.271 | 3.273 | 3.275 | 3.277 | 3.279 | 3.281 | 3.283 |
| 9.8 | 3.285 | 3.287 | 3.289 | 3.291 | 3.293 | 3.295 | 3.297 | 3.298 | 3.300 | 3.302 |
| 9.9 | 3.304 | 3.305 | 3.307 | 3.309 | 3.311 | 3.313 | 3.316 | 3.318 | 3.320 | 3.321 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| 10 | 3.32 | 3.34 | 3.36 | 3.37 | 3.39 | 3.41 | 3.43 | 3.45 | 3.46 | 3.48 |
| 11 | 3.50 | 3.52 | 3.53 | 3.55 | 3.56 | 3.58 | 3.60 | 3.61 | 3.63 | 3.64 |
| 12 | 3.66 | 3.68 | 3.69 | 3.71 | 3.72 | 3.74 | 3.76 | 3.77 | 3.79 | 3.80 |
| 13 | 3.80 | 3.83 | 3.85 | 3.86 | 3.88 | 3.89 | 3.90 | 3.92 | 3.93 | 3.95 |
| 14 | 3.96 | 3.97 | 3.99 | 4.00 | 4.02 | 4.03 | 4.04 | 4.06 | 4.07 | 4.09 |
| 15 | 4.10 | 4.11 | 4.13 | 4.14 | 4.15 | 4.17 | 4.18 | 4.19 | 4.20 | 4.22 |
| 16 | 4.23 | 4.24 | 4.25 | 4.27 | 4.28 | 4.29 | 4.30 | 4.31 | 4.33 | 4.34 |
| 17 | 4.35 | 4.36 | 4.37 | 4.38 | 4.39 | 4.41 | 4.42 | 4.43 | 4.44 | 4.45 |
| 18 | 4.46 | 4.47 | 4.48 | 4.49 | 4.50 | 4.52 | 4.53 | 4.54 | 4.55 | 4.56 |
| 19 | 4.57 | 4.58 | 4.59 | 4.60 | 4.61 | 4.62 | 4.63 | 4.64 | 4.65 | 4.66 |

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for microcrystalline cellulose used as binder, diluent or disintegrant.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2017:0315**

# CELLULOSE, POWDERED(6)

## Cellulosi pulvis

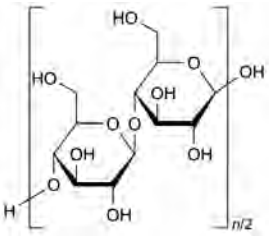

$C_{6n}H_{10n+2}O_{5n+1}$

DEFINITION

Purified, mechanically disintegrated cellulose prepared by processing alpha-cellulose obtained as a pulp from fibrous plant material.

♦CHARACTERS

*Appearance*: white or almost white, fine or granular powder.

*Solubility*: practically insoluble in water, slightly soluble in a 50 g/L solution of sodium hydroxide, practically insoluble in acetone, in anhydrous ethanol, in toluene, in dilute acids and in most organic solvents.♦

IDENTIFICATION

A. Place about 10 mg on a watch-glass and disperse in 2 mL of *iodinated zinc chloride solution R*. The substance becomes violet-blue.

B. The degree of polymerisation is greater than 440.

Transfer 0.250 g to a 125 mL conical flask. Add 25.0 mL of *water R* and 25.0 mL of *cupriethylenediamine hydroxide solution R*. Immediately purge the solution with *nitrogen R*, insert the stopper and shake until completely dissolved. Transfer an appropriate volume of the solution to a suitable capillary viscometer (*2.2.9*). Equilibrate the solution at 25 ± 0.1 °C for at least 5 min. Record the flow time ($t_1$) in seconds between the 2 marks on the viscometer. Calculate the kinematic viscosity ($v_1$) of the solution using the following expression:

$$t_1(k_1)$$

$k_1$ = viscometer constant.

Dilute a suitable volume of *cupriethylenediamine hydroxide solution R* with an equal volume of *water R* and measure the flow time ($t_2$) using a suitable capillary viscometer. Calculate the kinematic viscosity ($v_2$) of the solvent using the following expression:

$$t_2(k_2)$$

$k_2$ = viscometer constant.

Determine the relative viscosity ($\eta_{rel}$) of the substance to be examined using the following expression:

$$v_1 / v_2$$

Determine the intrinsic viscosity ($[\eta]_c$) by interpolation, using the intrinsic viscosity table (Table 0315.-1).

Calculate the degree of polymerisation ($P$) using the following expression:

$$\frac{95[\eta]_c}{m[(100 - b) / 100]}$$

$m$ = mass of the substance to be examined, in grams;

$b$ = loss on drying, in per cent.

TESTS

◊**Solubility**. Dissolve 50 mg in 10 mL of *ammoniacal solution of copper tetrammine R*. It dissolves completely, leaving no residue.◊

**pH** (*2.2.3*): 5.0 to 7.5 for the supernatant.

Mix 10 g with 90 mL of *carbon dioxide-free water R* and allow to stand with occasional stirring for 1 h.

**Ether-soluble substances**: maximum 0.15 per cent (15.0 mg) for the difference between the mass of the residue and the mass obtained from a blank determination.

Place 10.0 g in a chromatography column about 20 mm in internal diameter and pass 50 mL of *peroxide-free ether R* through the column. Evaporate the eluate to dryness in a previously dried and tared evaporating dish, with the aid of a current of air in a fume cupboard. After all the ether has evaporated, dry the residue at 105 °C for 30 min, allow to cool in a desiccator and weigh. Carry out a blank determination.

**Water-soluble substances**: maximum 1.5 per cent (15.0 mg) for the difference between the mass of the residue and the mass obtained from a blank determination.

Shake 6.0 g with 90 mL of *carbon dioxide-free water R* for 10 min. Filter with the aid of vacuum into a tared flask. Discard the first 10 mL of the filtrate and pass the filtrate through the same filter a second time, if necessary, to obtain a clear filtrate. Evaporate a 15.0 mL portion of the filtrate to dryness in a tared evaporating dish without charring. Dry at 105 °C for 1 h, allow to cool in a desiccator and weigh. Carry out a blank determination.

**Loss on drying** (*2.2.32*): maximum 6.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

(6) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent (dried substance), determined on 1.0 g.

♦**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for powdered cellulose used as diluent or disintegrant.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

Table 0315.-1. – *Intrinsic viscosity table*

| Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$ | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | $[\eta]_c$ | | | | | |
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 1.1 | 0.098 | 0.106 | 0.115 | 0.125 | 0.134 | 0.143 | 0.152 | 0.161 | 0.170 | 0.180 |
| 1.2 | 0.189 | 0.198 | 0.207 | 0.216 | 0.225 | 0.233 | 0.242 | 0.250 | 0.259 | 0.268 |
| 1.3 | 0.276 | 0.285 | 0.293 | 0.302 | 0.310 | 0.318 | 0.326 | 0.334 | 0.342 | 0.350 |
| 1.4 | 0.358 | 0.367 | 0.375 | 0.383 | 0.391 | 0.399 | 0.407 | 0.414 | 0.422 | 0.430 |
| 1.5 | 0.437 | 0.445 | 0.453 | 0.460 | 0.468 | 0.476 | 0.484 | 0.491 | 0.499 | 0.507 |
| 1.6 | 0.515 | 0.522 | 0.529 | 0.536 | 0.544 | 0.551 | 0.558 | 0.566 | 0.573 | 0.580 |
| 1.7 | 0.587 | 0.595 | 0.602 | 0.608 | 0.615 | 0.622 | 0.629 | 0.636 | 0.642 | 0.649 |
| 1.8 | 0.656 | 0.663 | 0.670 | 0.677 | 0.683 | 0.690 | 0.697 | 0.704 | 0.710 | 0.717 |
| 1.9 | 0.723 | 0.730 | 0.736 | 0.743 | 0.749 | 0.756 | 0.762 | 0.769 | 0.775 | 0.782 |
| 2.0 | 0.788 | 0.795 | 0.802 | 0.809 | 0.815 | 0.821 | 0.827 | 0.833 | 0.840 | 0.846 |
| 2.1 | 0.852 | 0.858 | 0.864 | 0.870 | 0.876 | 0.882 | 0.888 | 0.894 | 0.900 | 0.906 |
| 2.2 | 0.912 | 0.918 | 0.924 | 0.929 | 0.935 | 0.941 | 0.948 | 0.953 | 0.959 | 0.965 |
| 2.3 | 0.971 | 0.976 | 0.983 | 0.988 | 0.994 | 1.000 | 1.006 | 1.011 | 1.017 | 1.022 |
| 2.4 | 1.028 | 1.033 | 1.039 | 1.044 | 1.050 | 1.056 | 1.061 | 1.067 | 1.072 | 1.078 |
| 2.5 | 1.083 | 1.089 | 1.094 | 1.100 | 1.105 | 1.111 | 1.116 | 1.121 | 1.126 | 1.131 |
| 2.6 | 1.137 | 1.142 | 1.147 | 1.153 | 1.158 | 1.163 | 1.169 | 1.174 | 1.179 | 1.184 |
| 2.7 | 1.190 | 1.195 | 1.200 | 1.205 | 1.210 | 1.215 | 1.220 | 1.225 | 1.230 | 1.235 |
| 2.8 | 1.240 | 1.245 | 1.250 | 1.255 | 1.260 | 1.265 | 1.270 | 1.275 | 1.280 | 1.285 |
| 2.9 | 1.290 | 1.295 | 1.300 | 1.305 | 1.310 | 1.314 | 1.319 | 1.324 | 1.329 | 1.333 |
| 3.0 | 1.338 | 1.343 | 1.348 | 1.352 | 1.357 | 1.362 | 1.367 | 1.371 | 1.376 | 1.381 |
| 3.1 | 1.386 | 1.390 | 1.395 | 1.400 | 1.405 | 1.409 | 1.414 | 1.418 | 1.423 | 1.427 |
| 3.2 | 1.432 | 1.436 | 1.441 | 1.446 | 1.450 | 1.455 | 1.459 | 1.464 | 1.468 | 1.473 |
| 3.3 | 1.477 | 1.482 | 1.486 | 1.491 | 1.496 | 1.500 | 1.504 | 1.508 | 1.513 | 1.517 |
| 3.4 | 1.521 | 1.525 | 1.529 | 1.533 | 1.537 | 1.542 | 1.546 | 1.550 | 1.554 | 1.558 |
| 3.5 | 1.562 | 1.566 | 1.570 | 1.575 | 1.579 | 1.583 | 1.587 | 1.591 | 1.595 | 1.600 |
| 3.6 | 1.604 | 1.608 | 1.612 | 1.617 | 1.621 | 1.625 | 1.629 | 1.633 | 1.637 | 1.642 |
| 3.7 | 1.646 | 1.650 | 1.654 | 1.658 | 1.662 | 1.666 | 1.671 | 1.675 | 1.679 | 1.683 |
| 3.8 | 1.687 | 1.691 | 1.695 | 1.700 | 1.704 | 1.708 | 1.712 | 1.715 | 1.719 | 1.723 |
| 3.9 | 1.727 | 1.731 | 1.735 | 1.739 | 1.742 | 1.746 | 1.750 | 1.754 | 1.758 | 1.762 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 4.0 | 1.765 | 1.769 | 1.773 | 1.777 | 1.781 | 1.785 | 1.789 | 1.792 | 1.796 | 1.800 |
| 4.1 | 1.804 | 1.808 | 1.811 | 1.815 | 1.819 | 1.822 | 1.826 | 1.830 | 1.833 | 1.837 |
| 4.2 | 1.841 | 1.845 | 1.848 | 1.852 | 1.856 | 1.859 | 1.863 | 1.867 | 1.870 | 1.874 |
| 4.3 | 1.878 | 1.882 | 1.885 | 1.889 | 1.893 | 1.896 | 1.900 | 1.904 | 1.907 | 1.911 |
| 4.4 | 1.914 | 1.918 | 1.921 | 1.925 | 1.929 | 1.932 | 1.936 | 1.939 | 1.943 | 1.946 |
| 4.5 | 1.950 | 1.954 | 1.957 | 1.961 | 1.964 | 1.968 | 1.971 | 1.975 | 1.979 | 1.982 |
| 4.6 | 1.986 | 1.989 | 1.993 | 1.996 | 2.000 | 2.003 | 2.007 | 2.010 | 2.013 | 2.017 |
| 4.7 | 2.020 | 2.023 | 2.027 | 2.030 | 2.033 | 2.037 | 2.040 | 2.043 | 2.047 | 2.050 |
| 4.8 | 2.053 | 2.057 | 2.060 | 2.063 | 2.067 | 2.070 | 2.073 | 2.077 | 2.080 | 2.083 |
| 4.9 | 2.087 | 2.090 | 2.093 | 2.097 | 2.100 | 2.103 | 2.107 | 2.110 | 2.113 | 2.116 |
| 5.0 | 2.119 | 2.122 | 2.125 | 2.129 | 2.132 | 2.135 | 2.139 | 2.142 | 2.145 | 2.148 |
| 5.1 | 2.151 | 2.154 | 2.158 | 2.160 | 2.164 | 2.167 | 2.170 | 2.173 | 2.176 | 2.180 |
| 5.2 | 2.183 | 2.186 | 2.190 | 2.192 | 2.195 | 2.197 | 2.200 | 2.203 | 2.206 | 2.209 |
| 5.3 | 2.212 | 2.215 | 2.218 | 2.221 | 2.224 | 2.227 | 2.230 | 2.233 | 2.236 | 2.240 |
| 5.4 | 2.243 | 2.246 | 2.249 | 2.252 | 2.255 | 2.258 | 2.261 | 2.264 | 2.267 | 2.270 |
| 5.5 | 2.273 | 2.276 | 2.279 | 2.282 | 2.285 | 2.288 | 2.291 | 2.294 | 2.297 | 2.300 |
| 5.6 | 2.303 | 2.306 | 2.309 | 2.312 | 2.315 | 2.318 | 2.320 | 2.324 | 2.326 | 2.329 |
| 5.7 | 2.332 | 2.335 | 2.338 | 2.341 | 2.344 | 2.347 | 2.350 | 2.353 | 2.355 | 2.358 |
| 5.8 | 2.361 | 2.364 | 2.367 | 2.370 | 2.373 | 2.376 | 2.379 | 2.382 | 2.384 | 2.387 |
| 5.9 | 2.390 | 2.393 | 2.396 | 2.400 | 2.403 | 2.405 | 2.408 | 2.411 | 2.414 | 2.417 |
| 6.0 | 2.419 | 2.422 | 2.425 | 2.428 | 2.431 | 2.433 | 2.436 | 2.439 | 2.442 | 2.444 |
| 6.1 | 2.447 | 2.450 | 2.453 | 2.456 | 2.458 | 2.461 | 2.464 | 2.467 | 2.470 | 2.472 |
| 6.2 | 2.475 | 2.478 | 2.481 | 2.483 | 2.486 | 2.489 | 2.492 | 2.494 | 2.497 | 2.500 |
| 6.3 | 2.503 | 2.505 | 2.508 | 2.511 | 2.513 | 2.516 | 2.518 | 2.521 | 2.524 | 2.526 |
| 6.4 | 2.529 | 2.532 | 2.534 | 2.537 | 2.540 | 2.542 | 2.545 | 2.547 | 2.550 | 2.553 |
| 6.5 | 2.555 | 2.558 | 2.561 | 2.563 | 2.566 | 2.568 | 2.571 | 2.574 | 2.576 | 2.579 |
| 6.6 | 2.581 | 2.584 | 2.587 | 2.590 | 2.592 | 2.595 | 2.597 | 2.600 | 2.603 | 2.605 |
| 6.7 | 2.608 | 2.610 | 2.613 | 2.615 | 2.618 | 2.620 | 2.623 | 2.625 | 2.627 | 2.630 |
| 6.8 | 2.633 | 2.635 | 2.637 | 2.640 | 2.643 | 2.645 | 2.648 | 2.650 | 2.653 | 2.655 |
| 6.9 | 2.658 | 2.660 | 2.663 | 2.665 | 2.668 | 2.670 | 2.673 | 2.675 | 2.678 | 2.680 |
| 7.0 | 2.683 | 2.685 | 2.687 | 2.690 | 2.693 | 2.695 | 2.698 | 2.700 | 2.702 | 2.705 |
| 7.1 | 2.707 | 2.710 | 2.712 | 2.714 | 2.717 | 2.719 | 2.721 | 2.724 | 2.726 | 2.729 |
| 7.2 | 2.731 | 2.733 | 2.736 | 2.738 | 2.740 | 2.743 | 2.745 | 2.748 | 2.750 | 2.752 |
| 7.3 | 2.755 | 2.757 | 2.760 | 2.762 | 2.764 | 2.767 | 2.769 | 2.771 | 2.774 | 2.776 |
| 7.4 | 2.779 | 2.781 | 2.783 | 2.786 | 2.788 | 2.790 | 2.793 | 2.795 | 2.798 | 2.800 |
| 7.5 | 2.802 | 2.805 | 2.807 | 2.809 | 2.812 | 2.814 | 2.816 | 2.819 | 2.821 | 2.823 |
| 7.6 | 2.826 | 2.828 | 2.830 | 2.833 | 2.835 | 2.837 | 2.840 | 2.842 | 2.844 | 2.847 |
| 7.7 | 2.849 | 2.851 | 2.854 | 2.856 | 2.858 | 2.860 | 2.863 | 2.865 | 2.868 | 2.870 |
| 7.8 | 2.873 | 2.875 | 2.877 | 2.879 | 2.881 | 2.884 | 2.887 | 2.889 | 2.891 | 2.893 |
| 7.9 | 2.895 | 2.898 | 2.900 | 2.902 | 2.905 | 2.907 | 2.909 | 2.911 | 2.913 | 2.915 |
| 8.0 | 2.918 | 2.920 | 2.922 | 2.924 | 2.926 | 2.928 | 2.931 | 2.933 | 2.935 | 2.937 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.00 | 0.01 | 0.02 | 0.03 | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 |
| 8.1 | 2.939 | 2.942 | 2.944 | 2.946 | 2.948 | 2.950 | 2.952 | 2.955 | 2.957 | 2.959 |
| 8.2 | 2.961 | 2.963 | 2.966 | 2.968 | 2.970 | 2.972 | 2.974 | 2.976 | 2.979 | 2.981 |
| 8.3 | 2.983 | 2.985 | 2.987 | 2.990 | 2.992 | 2.994 | 2.996 | 2.998 | 3.000 | 3.002 |
| 8.4 | 3.004 | 3.006 | 3.008 | 3.010 | 3.012 | 3.015 | 3.017 | 3.019 | 3.021 | 3.023 |
| 8.5 | 3.025 | 3.027 | 3.029 | 3.031 | 3.033 | 3.035 | 3.037 | 3.040 | 3.042 | 3.044 |
| 8.6 | 3.046 | 3.048 | 3.050 | 3.052 | 3.054 | 3.056 | 3.058 | 3.060 | 3.062 | 3.064 |
| 8.7 | 3.067 | 3.069 | 3.071 | 3.073 | 3.075 | 3.077 | 3.079 | 3.081 | 3.083 | 3.085 |
| 8.8 | 3.087 | 3.089 | 3.092 | 3.094 | 3.096 | 3.098 | 3.100 | 3.102 | 3.104 | 3.106 |
| 8.9 | 3.108 | 3.110 | 3.112 | 3.114 | 3.116 | 3.118 | 3.120 | 3.122 | 3.124 | 3.126 |
| 9.0 | 3.128 | 3.130 | 3.132 | 3.134 | 3.136 | 3.138 | 3.140 | 3.142 | 3.144 | 3.146 |
| 9.1 | 3.148 | 3.150 | 3.152 | 3.154 | 3.156 | 3.158 | 3.160 | 3.162 | 3.164 | 3.166 |
| 9.2 | 3.168 | 3.170 | 3.172 | 3.174 | 3.176 | 3.178 | 3.180 | 3.182 | 3.184 | 3.186 |
| 9.3 | 3.188 | 3.190 | 3.192 | 3.194 | 3.196 | 3.198 | 3.200 | 3.202 | 3.204 | 3.206 |
| 9.4 | 3.208 | 3.210 | 3.212 | 3.214 | 3.215 | 3.217 | 3.219 | 3.221 | 3.223 | 3.225 |
| 9.5 | 3.227 | 3.229 | 3.231 | 3.233 | 3.235 | 3.237 | 3.239 | 3.241 | 3.242 | 3.244 |
| 9.6 | 3.246 | 3.248 | 3.250 | 3.252 | 3.254 | 3.256 | 3.258 | 3.260 | 3.262 | 3.264 |
| 9.7 | 3.266 | 3.268 | 3.269 | 3.271 | 3.273 | 3.275 | 3.277 | 3.279 | 3.281 | 3.283 |
| 9.8 | 3.285 | 3.287 | 3.289 | 3.291 | 3.293 | 3.295 | 3.297 | 3.298 | 3.300 | 3.302 |
| 9.9 | 3.304 | 3.305 | 3.307 | 3.309 | 3.311 | 3.313 | 3.316 | 3.318 | 3.320 | 3.321 |

Intrinsic viscosity $[\eta]_c$ at different values of relative viscosity $\eta_{rel}$

| | $[\eta]_c$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $\eta_{rel}$ | 0.0 | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 |
| 10 | 3.32 | 3.34 | 3.36 | 3.37 | 3.39 | 3.41 | 3.43 | 3.45 | 3.46 | 3.48 |
| 11 | 3.50 | 3.52 | 3.53 | 3.55 | 3.56 | 3.58 | 3.60 | 3.61 | 3.63 | 3.64 |
| 12 | 3.66 | 3.68 | 3.69 | 3.71 | 3.72 | 3.74 | 3.76 | 3.77 | 3.79 | 3.80 |
| 13 | 3.80 | 3.83 | 3.85 | 3.86 | 3.88 | 3.89 | 3.90 | 3.92 | 3.93 | 3.95 |
| 14 | 3.96 | 3.97 | 3.99 | 4.00 | 4.02 | 4.03 | 4.04 | 4.06 | 4.07 | 4.09 |
| 15 | 4.10 | 4.11 | 4.13 | 4.14 | 4.15 | 4.17 | 4.18 | 4.19 | 4.20 | 4.22 |
| 16 | 4.23 | 4.24 | 4.25 | 4.27 | 4.28 | 4.29 | 4.30 | 4.31 | 4.33 | 4.34 |
| 17 | 4.35 | 4.36 | 4.37 | 4.38 | 4.39 | 4.41 | 4.42 | 4.43 | 4.44 | 4.45 |
| 18 | 4.46 | 4.47 | 4.48 | 4.49 | 4.50 | 4.52 | 4.53 | 4.54 | 4.55 | 4.56 |
| 19 | 4.57 | 4.58 | 4.59 | 4.60 | 4.61 | 4.62 | 4.63 | 4.64 | 4.65 | 4.66 |

**04/2013:1084**

# CETIRIZINE DIHYDROCHLORIDE

## Cetirizini dihydrochloridum

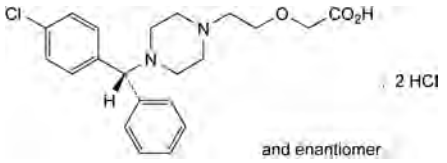


$C_{21}H_{27}Cl_3N_2O_3$ $M_r$ 461.8
[83881-52-1]

DEFINITION

(*RS*)-2-[2-[4-[(4-Chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid dihydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20.0 mg in 50 mL of a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of this solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 210-350 nm.

*Absorption maximum*: at 231 nm.

*Specific absorbance at the absorption maximum*: 359 to 381.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cetirizine dihydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *cetirizine dihydrochloride CRS* in *water R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *chlorphenamine maleate CRS* in *water R* and dilute to 5 mL with the same solvent. Mix 1 mL of the solution and 1 mL of reference solution (a).

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *ammonia R*, *methanol R*, *methylene chloride R* (1:10:90 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 1.2 to 1.8 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *cetirizine dihydrochloride CRS* and 2 mg of *cetirizine impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *cetirizine for peak identification CRS* (containing impurities B, C, D, E and F) in 5.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *dilute sulfuric acid R*, *water R*, *acetonitrile R* (0.4:6.6:93 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of cetirizine.

*Identification of impurities*: use the chromatogram supplied with *cetirizine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C, D, E and F; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to cetirizine (retention time = about 9 min): impurity D = about 0.6; impurity B = about 0.8; impurity C = about 0.9; impurity E = about 1.2; impurity F = about 1.37; impurity A = about 1.42.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cetirizine.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity C = 1.9; impurity D = 0.6; impurity E = 1.3; impurity F = 1.9;

– *impurities A, B, C, D, E, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 70 mL of a mixture of 30 volumes of *water R* and 70 volumes of *acetone R*. Titrate with *0.1 M sodium hydroxide* to the 2nd point of inflexion. Determine the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.39 mg of $C_{21}H_{27}Cl_3N_2O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

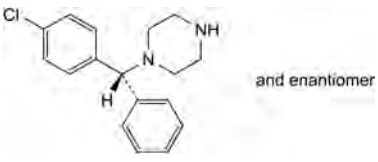


A. (*RS*)-1-[(4-chlorophenyl)phenylmethyl]piperazine,

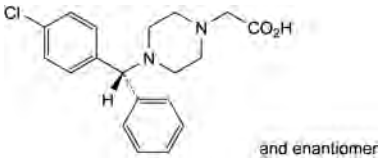


B. (*RS*)-2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]acetic acid,

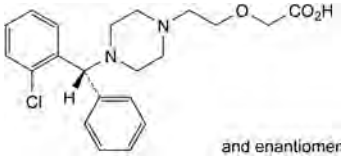


C. (*RS*)-2-[2-[4-[(2-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]acetic acid,

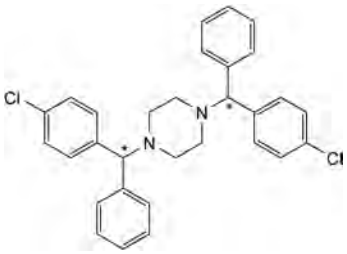


D. 1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine,

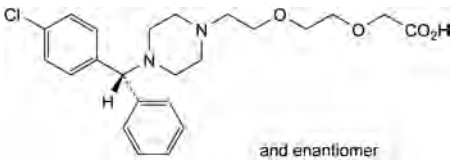


E. (*RS*)-2-[2-[2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]ethoxy]acetic acid (ethoxycetirizine),

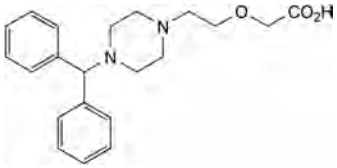


F. 2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]acetic acid,

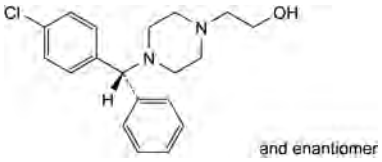


G. (*RS*)-2-[4-[(4-chlorophenyl)phenylmethyl]piperazin-1-yl]ethan-1-ol.

**01/2008:0702**
**corrected 10.0**

# CETOSTEARYL ALCOHOL

## Alcohol cetylicus et stearylicus

DEFINITION

Mixture of solid aliphatic alcohols, mainly octadecan-1-ol (stearyl alcohol; $C_{18}H_{38}O$; $M_r$ 270.5) and hexadecan-1-ol (cetyl alcohol; $C_{16}H_{34}O$; $M_r$ 242.4), of animal or vegetable origin.

*Content*:

– *stearyl alcohol*: minimum 40.0 per cent,

– *sum of the contents of stearyl alcohol and cetyl alcohol*: minimum 90.0 per cent.

CHARACTERS

*Appearance*: white or pale yellow, wax-like mass, plates, flakes or granules.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in light petroleum. When melted, it is miscible with fatty oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the principal peaks in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

Dissolve 0.50 g in 20 mL of boiling *ethanol (96 per cent) R*. Allow to cool.

**Melting point** (*2.2.14*): 49 °C to 56 °C.

**Acid value** (*2.5.1*): maximum 1.0.

**Hydroxyl value** (*2.5.3, Method A*): 208 to 228.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

Dissolve 2.00 g in *methylene chloride R* and dilute to 25 mL with the same solvent.

**Saponification value** (*2.5.6*): maximum 2.0.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.100 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 60 mg of *cetyl alcohol CRS* and 40 mg of *stearyl alcohol CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *ethanol (96 per cent) R*.

*Column*:

– *size*: *l* = 30 m, Ø = 0.32 mm,

– *stationary phase*: *methylpolysiloxane R* (1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| | 20 - 40 | 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to cetyl alcohol and stearyl alcohol.

Calculate the percentage contents of $C_{16}H_{34}O$ and $C_{18}H_{38}O$.

04/2014:0801
corrected 10.0

# CETOSTEARYL ALCOHOL (TYPE A), EMULSIFYING

## Alcohol cetylicus et stearylicus emulsificans A

DEFINITION

Mixture of cetostearyl alcohol and sodium cetostearyl sulfate. A suitable buffer may be added.

*Content*:

– *cetostearyl alcohol*: minimum 80.0 per cent (anhydrous substance);
– *sodium cetostearyl sulfate*: minimum 7.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, waxy mass, plates, flakes or granules.

*Solubility*: soluble in hot water giving an opalescent solution, practically insoluble in cold water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, C, D.*

*Second identification*: *A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.1 g of the substance to be examined in 10 mL of *trimethylpentane R*, heating on a water-bath. Shake with 2 mL of *ethanol (70 per cent V/V) R* and allow to separate. Use the lower layer as test solution (b). Dilute 1 mL of the upper layer to 8 mL with *trimethylpentane R*.

*Test solution (b).* Use the lower layer obtained in the preparation of test solution (a).

*Reference solution (a).* Dissolve 24 mg of *cetyl alcohol CRS* and 16 mg of *stearyl alcohol CRS* in 10 mL of *trimethylpentane R*.

*Reference solution (b).* Dissolve 20 mg of *sodium cetostearyl sulfate R* in 10 mL of *ethanol (70 per cent V/V) R*, heating on a water-bath.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: *water R*, *acetone R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 50 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*; heat at 120 °C until spots appear (about 5 min).

*Results*:

– the 2 principal spots in the chromatogram obtained with test solution (a) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (a);
– 2 of the spots in the chromatogram obtained with test solution (b) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (b).

B. Examine the chromatograms obtained in the assay of cetostearyl alcohol.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatograms obtained with reference solutions (a) and (b).

C. It gives a yellow colour to a non-luminous flame.

D. To 0.3 g add 20 mL of *anhydrous ethanol R* and heat to boiling on a water-bath with shaking. Filter the mixture immediately, evaporate to dryness and take up the residue in 7 mL of *water R*. To 1 mL of the solution add 0.1 mL of a 1 g/L solution of *methylene blue R*, 2 mL of *dilute sulfuric acid R* and 2 mL of *methylene chloride R* and shake. A blue colour develops in the lower layer.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

Dissolve 2.00 g in 25 mL of *methylene chloride R*.

**Saponification value** (*2.5.6*): maximum 2.0.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.50 g.

ASSAY

**Cetostearyl alcohol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.200 g of *1-nonadecanol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 0.200 g of the substance to be examined in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (a).* Dissolve 0.100 g of *cetyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (b).* Dissolve 0.100 g of *stearyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Column*:

– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.25 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: cetyl alcohol, stearyl alcohol, 1-nonadecanol.

Calculate the percentage content of cetyl alcohol in the substance to be examined using the following expression and taking into account the assigned content of *cetyl alcohol CRS*:

$$A_x \times \frac{A_2}{A_1} \times \frac{m_{x,y}}{A_{x,y}} \times \frac{1}{m} \times 100$$

$A_x$ = area of the peak due to cetyl alcohol in the chromatogram obtained with the test solution;

$A_{x,y}$ = area of the peak due to *cetyl alcohol CRS* in the chromatogram obtained with reference solution (a);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a);

$m$ = mass of the substance to be examined in the test solution, in milligrams;

$m_{x,y}$ = mass of *cetyl alcohol CRS* in reference solution (a), in milligrams.

Calculate the percentage content of stearyl alcohol in the substance to be examined using the following expression and taking into account the assigned content of *stearyl alcohol CRS*:

$$A_z \times \frac{A_3}{A_1} \times \frac{m_{z,y}}{A_{z,y}} \times \frac{1}{m} \times 100$$

$A_z$ = area of the peak due to stearyl alcohol in the chromatogram obtained with the test solution;

$A_{z,y}$ = area of the peak due to *stearyl alcohol CRS* in the chromatogram obtained with reference solution (b);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (b);

$m$ = mass of the substance to be examined in the test solution, in milligrams;

$m_{z,y}$ = mass of *stearyl alcohol CRS* in reference solution (b), in milligrams.

The percentage content of cetostearyl alcohol corresponds to the sum of the percentage contents of cetyl alcohol and stearyl alcohol.

**Sodium cetostearyl sulfate**. Disperse 0.300 g in 25 mL of *methylene chloride R*. Add 50 mL of *water R* and 10 mL of *dimidium bromide-sulfan blue mixed solution R*. Titrate with *0.004 M benzethonium chloride*, using sonication, heating, and allowing the layers to separate before each addition, until the colour of the lower layer changes from pink to grey.

1 mL of *0.004 M benzethonium chloride* is equivalent to 1.434 mg of sodium cetostearyl sulfate.

LABELLING

The label states, where applicable, the name and concentration of any added buffer.

**04/2014:0802**
**corrected 10.0**

# CETOSTEARYL ALCOHOL (TYPE B), EMULSIFYING

## Alcohol cetylicus et stearylicus emulsificans B

DEFINITION

Mixture of cetostearyl alcohol and sodium laurilsulfate. A suitable buffer may be added.

*Content*:

– *cetostearyl alcohol*: minimum 80.0 per cent (anhydrous substance);

– *sodium laurilsulfate*: minimum 7.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, waxy mass, plates, flakes or granules.

*Solubility*: soluble in hot water giving an opalescent solution, practically insoluble in cold water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C, D.*

*Second identification: A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.1 g of the substance to be examined in 10 mL of *trimethylpentane R*, heating on a water-bath. Shake with 2 mL of *ethanol (70 per cent V/V) R* and allow to separate. Use the lower layer as test solution (b). Dilute 1 mL of the upper layer to 8 mL with *trimethylpentane R*.

*Test solution (b).* Use the lower layer obtained in the preparation of test solution (a).

*Reference solution (a).* Dissolve 24 mg of *cetyl alcohol CRS* and 16 mg of *stearyl alcohol CRS* in 10 mL of *trimethylpentane R*.

*Reference solution (b).* Dissolve 20 mg of *sodium laurilsulfate CRS* in 10 mL of *ethanol (70 per cent V/V) R*, heating on a water-bath.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: *water R*, *acetone R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 50 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*; heat at 120 °C until spots appear (about 5 min).

*Results*:

– the 2 principal spots in the chromatogram obtained with test solution (a) are similar in position and colour to the principal spots in the chromatogram obtained with reference solution (a);

– 1 of the spots in the chromatogram obtained with test solution (b) is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (b).

B. Examine the chromatograms obtained in the assay of cetostearyl alcohol.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatograms obtained with reference solutions (a) and (b).

C. It gives a yellow colour to a non-luminous flame.

D. To 0.3 g add 20 mL of *anhydrous ethanol R* and heat to boiling on a water-bath with shaking. Filter the mixture immediately, evaporate to dryness and take up the residue in 7 mL of *water R*. To 1 mL of the solution add 0.1 mL of a 1 g/L solution of *methylene blue R*, 2 mL of *dilute sulfuric acid R* and 2 mL of *methylene chloride R* and shake. A blue colour develops in the lower layer.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

Dissolve 2.00 g in 25 mL of *methylene chloride R*.

**Saponification value** (*2.5.6*): maximum 2.0.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.50 g.

ASSAY

**Cetostearyl alcohol**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 0.200 g of *1-nonadecanol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Test solution*. Dissolve 0.200 g of the substance to be examined in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (a)*. Dissolve 0.100 g of *cetyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (b)*. Dissolve 0.100 g of *stearyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: cetyl alcohol, stearyl alcohol, 1-nonadecanol.

Calculate the percentage content of cetyl alcohol in the substance to be examined using the following expression and taking into account the assigned content of *cetyl alcohol CRS*:

$$A_x \times \frac{A_2}{A_1} \times \frac{m_{x,y}}{A_{x,y}} \times \frac{1}{m} \times 100$$

$A_x$ = area of the peak due to cetyl alcohol in the chromatogram obtained with the test solution;

$A_{x,y}$ = area of the peak due to *cetyl alcohol CRS* in the chromatogram obtained with reference solution (a);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a);

$m$ = mass of the substance to be examined in the test solution, in milligrams;

$m_{x,y}$ = mass of *cetyl alcohol CRS* in reference solution (a), in milligrams.

Calculate the percentage content of stearyl alcohol in the substance to be examined using the following expression and taking into account the assigned content of *stearyl alcohol CRS*:

$$A_z \times \frac{A_3}{A_1} \times \frac{m_{z,y}}{A_{z,y}} \times \frac{1}{m} \times 100$$

$A_z$ = area of the peak due to stearyl alcohol in the chromatogram obtained with the test solution;

$A_{z,y}$ = area of the peak due to *stearyl alcohol CRS* in the chromatogram obtained with reference solution (b);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (b);

$m$ = mass of the substance to be examined in the test solution, in milligrams;

$m_{z,y}$ = mass of *stearyl alcohol CRS* in reference solution (b), in milligrams.

The percentage content of cetostearyl alcohol corresponds to the sum of the percentage contents of cetyl alcohol and stearyl alcohol.

**Sodium laurilsulfate**. Disperse 0.300 g in 25 mL of *methylene chloride R*. Add 50 mL of *water R* and 10 mL of *dimidium bromide-sulfan blue mixed solution R*. Titrate with *0.004 M benzethonium chloride*, using sonication, heating, and allowing the layers to separate before each addition, until the colour of the lower layer changes from pink to grey.

1 mL of *0.004 M benzethonium chloride* is equivalent to 1.154 mg of sodium laurilsulfate.

LABELLING

The label states, where applicable, the name and concentration of any added buffer.

**01/2017:1085**

# CETOSTEARYL ISONONANOATE

## Cetostearylis isononanoas

DEFINITION

Mixture of esters of cetostearyl alcohol with isononanoic acid, mainly 3,5,5-trimethylhexanoic acid.

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish liquid.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in light petroleum, miscible with fatty oils and with liquid paraffins.

*Viscosity*: 15 mPa·s to 30 mPa·s.

*Relative density*: 0.85 to 0.86.

*Refractive index*: 1.44 to 1.45.

IDENTIFICATION

A. On cooling, turbidity occurs below 15 °C.

B. Saponification value (see Tests).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of cetostearyl isononanoate.*

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): maximum 5.0, determined on 4.0 g. Add 5.0 mL of acetylating reagent.

**Iodine value** (*2.5.4, Method A*): maximum 1.0.

**Saponification value** (*2.5.6*): 135 to 148, determined on 1.0 g.

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 10.0 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent, determined on 2.0 g.

**01/2008:0378**
**corrected 6.0**

# CETRIMIDE

## Cetrimidum

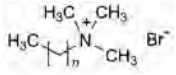

DEFINITION

Cetrimide consists of trimethyltetradecylammonium bromide and may contain smaller amounts of dodecyl- and hexadecyl-trimethylammonium bromides.

*Content*: 96.0 per cent to 101.0 per cent of alkyltrimethylammonium bromides, calculated as $C_{17}H_{38}BrN$ ($M_r$ 336.4) (dried substance).

CHARACTERS

*Appearance*: white or almost white, voluminous, free-flowing powder.

*Solubility*: freely soluble in water and in alcohol.

IDENTIFICATION

A. Dissolve 0.25 g in *alcohol R* and dilute to 25.0 mL with the same solvent. At wavelengths from 260 nm to 280 nm, the absorbance (*2.2.25*) of the solution has a maximum of 0.05.

B. Dissolve about 5 mg in 5 mL of *buffer solution pH 8.0 R*. Add about 10 mg of *potassium ferricyanide R*. A yellow precipitate is formed. Prepare a blank in the same manner but omitting the substance to be examined: a yellow solution is observed but no precipitate is formed.

C. Solution S (see Tests) froths copiously when shaken.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 0.10 g of *trimethyltetradecylammonium bromide CRS* in *water R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silanised silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetone R*, 270 g/L solution of *sodium acetate R*, *methanol R* (20:35:45 *V/V/V*).

*Application*: 1 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of hot air.

*Detection*: allow to cool; expose the plate to iodine vapour and examine in daylight.

*Result*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.1 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Amines and amine salts**. Dissolve 5.0 g in 30 mL of a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol R* and add 100 mL of *2-propanol R*. Pass a stream of *nitrogen R* slowly through the solution. Gradually add 15.0 mL of *0.1 M tetrabutylammonium hydroxide* and record the potentiometric titration curve (*2.2.20*). If the curve shows 2 points of inflexion, the volume of titrant added between the 2 points is not greater than 2.0 mL.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 100.0 mL with the same solvent. Transfer 25.0 mL of the solution to a separating funnel, add 25 mL of *chloroform R*, 10 mL of *0.1 M sodium hydroxide* and 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R*. Shake, allow to separate and discard the chloroform layer. Shake the aqueous layer with 3 quantities, each of 10 mL, of *chloroform R* and discard the chloroform layers. Add 40 mL of *hydrochloric acid R*, allow to cool and titrate with *0.05 M potassium iodate* until the deep brown colour is almost discharged. Add 2 mL of *chloroform R* and continue the titration, shaking vigorously, until the colour of the chloroform layer no longer changes. Carry out a blank

titration on a mixture of 10.0 mL of the freshly prepared 50 g/L solution of *potassium iodide R*, 20 mL of *water R* and 40 mL of *hydrochloric acid R*.

1 mL of *0.05 M potassium iodate* is equivalent to 33.64 mg of $C_{17}H_{38}BrN$.

**01/2008:0540**
**corrected 10.0**

# CETYL ALCOHOL

## Alcohol cetylicus

DEFINITION

Mixture of solid alcohols, mainly hexadecan-1-ol ($C_{16}H_{34}O$; $M_r$ 242.4), of animal or vegetable origin.

*Content*: minimum 95.0 per cent of $C_{16}H_{34}O$.

CHARACTERS

*Appearance*: white or almost white, unctuous mass, powder, flakes or granules.

*Solubility*: practically insoluble in water, freely soluble or sparingly soluble in ethanol (96 per cent). When melted, it is miscible with vegetable and animal oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

Dissolve 0.50 g in 20 mL of boiling *ethanol (96 per cent) R*. Allow to cool.

**Melting point** (*2.2.14*): 46 °C to 52 °C.

**Acid value** (*2.5.1*): maximum 1.0.

**Hydroxyl value** (*2.5.3, Method A*): 218 to 238.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

Dissolve 2.00 g in *methylene chloride R* and dilute to 25 mL with the same solvent.

**Saponification value** (*2.5.6*): maximum 2.0.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.100 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50 mg of *cetyl alcohol CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dissolve 50 mg of *stearyl alcohol R* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (c)*. Mix 1 mL of reference solution (a) and 1 mL of reference solution (b) and dilute to 10 mL with *ethanol (96 per cent) R*.

*Column*:

- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *methylpolysiloxane R* (1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| | 20 - 40 | 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solutions (a) and (c).

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to cetyl alcohol and stearyl alcohol.

Calculate the percentage content of $C_{16}H_{34}O$.

**01/2020:1906**

# CETYL PALMITATE

## Cetylis palmitas

DEFINITION

Mixture of esters of $C_{14}$-$C_{18}$ alcohols with lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic) and stearic (octadecanoic) acids ('Cetyl esters wax').

*Content* (expressed as hexadecyl hexadecanoate): 10.0 per cent to 20.0 per cent for Cetyl palmitate 15, 60.0 per cent to 70.0 per cent for Cetyl palmitate 65 and minimum 90.0 per cent for Cetyl palmitate 95.

CHARACTERS

*Appearance*: white or almost white, waxy plates, flakes or powder.

*Solubility*: practically insoluble in water, soluble in boiling anhydrous ethanol and in methylene chloride, slightly soluble in light petroleum, practically insoluble in anhydrous ethanol.

mp: about 45 °C for Cetyl palmitate 15 and Cetyl palmitate 65 and about 52 °C for Cetyl palmitate 95.

IDENTIFICATION

A. It complies with the limits of the assay and the chromatogram obtained with the test solution shows the typical main peak(s).

B. Saponification value (see Tests).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 4.0 g in *methylene chloride R* and dilute to 20 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 4.0.

Dissolve 10.0 g in 50 mL of the solvent mixture described by heating under reflux on a water-bath for 5 min.

**Hydroxyl value** (*2.5.3, Method A*): maximum 20.0.

Carry out the titration at a temperature between 55 °C and 70 °C, shaking the flask towards the end of the titration.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Saponification value** (*2.5.6*): 105 to 120.

Heat under reflux for 2 h.

**Alkaline impurities**. Dissolve 2.0 g with gentle heating in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3 mL of *toluene R*. Add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the solution to yellow.

**Water** (*2.5.12*): maximum 0.3 per cent, determined on 1.0 g using a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *hexane R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 20.0 mg of *cetyl palmitate 95 CRS* in *hexane R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20.0 mg of *cetyl palmitate 15 CRS* in *hexane R* and dilute to 20.0 mL with the same solvent.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 10 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 2.65 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 6.5 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 100 → 300 |
| | 10 - 15 | 300 |
| Injection port | | 350 |
| Detector | | 350 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to cetyl palmitate (retention time = about 9 min): cetyl alcohol = about 0.3; palmitic acid = about 0.4; lauric ester = about 0.8; myristic ester = about 0.9; stearic ester = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to cetyl palmitate and cetyl stearate.

STORAGE

At a temperature not exceeding 25 °C.

LABELLING

The label states the type of cetyl palmitate.

**01/2008:0379**
**corrected 6.0**

# CETYLPYRIDINIUM CHLORIDE

## Cetylpyridinii chloridum

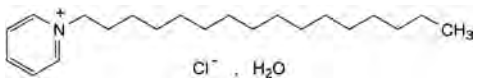


$C_{21}H_{38}ClN,H_2O$ $M_r$ 358.0
[6004-24-6]

DEFINITION

Cetylpyridinium chloride contains not less than 96.0 per cent and not more than the equivalent of 101.0 per cent of 1-hexadecylpyridinium chloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, slightly soapy to the touch, soluble in water and in alcohol. An aqueous solution froths copiously when shaken.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.10 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*. Examined between 240 nm and 300 nm (*2.2.25*), the solution shows an absorption maximum at 259 nm and 2 shoulders at about 254 nm and at about 265 nm. The specific absorbance at the maximum is 126 to 134, calculated with reference to the anhydrous substance.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *cetylpyridinium chloride CRS*. Examine the substances in the solid state.

C. To 5 mL of *dilute sodium hydroxide solution R* add 0.1 mL of *bromophenol blue solution R1* and 5 mL of *chloroform R* and shake. The chloroform layer is colourless. Add 0.1 mL of solution S (see Tests) and shake. The chloroform layer becomes blue.

D. Solution S gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Acidity**. To 50 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 2.5 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Amines and amine salts**. Dissolve 5.0 g with heating in 20 mL of a mixture of 3 volumes of *1 M hydrochloric acid* and 97 volumes of *methanol R* and add 100 mL of *2-propanol R*. Pass a stream of *nitrogen R* slowly through the solution. Gradually add 12.0 mL of *0.1 M tetrabutylammonium hydroxide* and record the potentiometric titration curve (*2.2.20*). If the curve shows 2 points of inflexion, the volume of titrant added between the two points is not greater than 5.0 mL. If the curve shows no point of inflexion, the substance to be examined does not comply with the test. If the curve shows one point of inflexion, repeat the test but add 3.0 mL of a 25.0 g/L solution of *dimethyldecylamine R* in *2-propanol R*

before the titration. If the titration curve after the addition of 12.0 mL of the titrant shows only one point of inflexion, the substance to be examined does not comply with the test.

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.300 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.00 g in *water R* and dilute to 100.0 mL with the same solvent. Transfer 25.0 mL of the solution to a separating funnel, add 25 mL of *chloroform R*, 10 mL of *0.1 M sodium hydroxide* and 10.0 mL of a freshly prepared 50 g/L solution of *potassium iodide R*. Shake well, allow to separate and discard the chloroform layer. Shake the aqueous layer with three quantities, each of 10 mL, of *chloroform R* and discard the chloroform layers. To the aqueous layer add 40 mL of *hydrochloric acid R*, allow to cool and titrate with *0.05 M potassium iodate* until the deep-brown colour is almost discharged. Add 2 mL of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer no longer changes colour. Carry out a blank titration on a mixture of 10.0 mL of the freshly prepared 50 g/L solution of *potassium iodide R*, 20 mL of *water R* and 40 mL of *hydrochloric acid R*.

1 mL of *0.05 M potassium iodate* is equivalent to 34.0 mg of $C_{21}H_{38}ClN$.

**01/2009:0313**
**corrected 7.0**

# CHARCOAL, ACTIVATED

## Carbo activatus

DEFINITION

Obtained from vegetable matter by suitable carbonisation processes intended to confer a high adsorption power.

CHARACTERS

*Appearance*: black, light powder free from grittiness.

*Solubility*: practically insoluble in all usual solvents.

IDENTIFICATION

A. When heated to redness it burns slowly without a flame.

B. Adsorption power (see Tests).

TESTS

**Solution S**. To 2.0 g in a conical flask with a ground-glass neck add 50 mL of *dilute hydrochloric acid R*. Boil gently under a reflux condenser for 1 h, filter and wash the filter with *dilute hydrochloric acid R*. Evaporate the combined filtrate and washings to dryness on a water-bath, dissolve the residue in *0.1 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

**Acidity or alkalinity**. To 2.0 g add 40 mL of *water R* and boil for 5 min. Cool, restore to the original mass with *carbon dioxide-free water R* and filter. Reject the first 20 mL of the filtrate. To 10 mL of the filtrate add 0.25 mL of *bromothymol blue solution R1* and 0.25 mL of *0.02 M sodium hydroxide*. The solution is blue. Not more than 0.75 mL of *0.02 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid-soluble substances**: maximum 3 per cent.

To 1.0 g add 25 mL of *dilute nitric acid R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (10) (*2.1.2*) and wash with 10 mL of hot *water R*. Evaporate the combined filtrate and washings to dryness on a water-bath, add to the residue 1 mL of *hydrochloric acid R*, evaporate to dryness again and dry the residue to constant mass at 100-105 °C. The residue weighs a maximum of 30 mg.

**Alkali-soluble coloured substances**. To 0.25 g add 10 mL of *dilute sodium hydroxide solution R* and boil for 1 min. Cool, filter and dilute the filtrate to 10 mL with *water R*. The solution is not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

**Ethanol (96 per cent) soluble substances**: maximum 0.5 per cent.

To 2.0 g add 50 mL of *ethanol (96 per cent) R* and boil under a reflux condenser for 10 min. Filter immediately, cool, and dilute to 50 mL with *ethanol (96 per cent) R*. The filtrate is not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*). Evaporate 40 mL of the filtrate to dryness and dry to constant mass at 100-105 °C. The residue weighs a maximum of 8 mg.

**Fluorescent substances**. In an intermittent-extraction apparatus, treat 10.0 g with 100 mL of *cyclohexane R1* for 2 h. Collect the liquid and dilute to 100 mL with *cyclohexane R1*. Examine in ultraviolet light at 365 nm. The fluorescence of the solution is not more intense than that of a solution of 83 µg of *quinine R* in 1000 mL of *0.005 M sulfuric acid* examined under the same conditions.

**Sulfides**. To 1.0 g in a conical flask add 5 mL of *hydrochloric acid R1* and 20 mL of *water R*. Heat to boiling. The fumes released do not turn *lead acetate paper R* brown.

**Copper**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R* and diluting with *0.1 M hydrochloric acid*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 325.0 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (100 ppm Pb) R* and diluting with *0.1 M hydrochloric acid*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm; 217.0 nm may be used depending on the apparatus.

*Atomisation device*: air-acetylene flame.

**Zinc**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R* and diluting with *0.1 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 214.0 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 15 per cent, determined on 1.00 g by drying in an oven at 120 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 5.0 per cent, determined on 1.0 g.

**Adsorption power**. To 0.300 g in a 100 mL ground-glass-stoppered conical flask add 25.0 mL of a freshly prepared solution of 0.5 g of *phenazone R* in 50 mL of *water R*. Shake thoroughly for 15 min. Filter and reject the first 5 mL of filtrate. To 10.0 mL of the filtrate add 1.0 g of *potassium bromide R* and 20 mL of *dilute hydrochloric acid R*. Using 0.1 mL of *methyl red solution R* as indicator, titrate with *0.0167 M potassium bromate* until the red colour

is discharged. Titrate slowly (1 drop every 15 s) towards the end of the titration. Carry out a blank titration using 10.0 mL of the phenazone solution.

Calculate the quantity of phenazone adsorbed per 100 g of activated charcoal from the following expression:

$$\frac{2.353(a-b)}{m}$$

$a$ = number of millilitres of *0.0167 M potassium bromate* used for the blank;

$b$ = number of millilitres of *0.0167 M potassium bromate* used for the test;

$m$ = mass in grams of the substance to be examined.

Minimum 40 g of phenazone is adsorbed per 100 g of activated charcoal, calculated with reference to the dried substance.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

STORAGE

In an airtight container.

**01/2017:1189**

# CHENODEOXYCHOLIC ACID

## Acidum chenodeoxycholicum

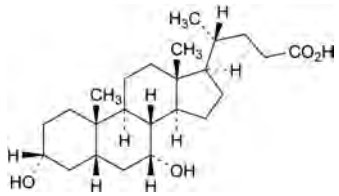

$C_{24}H_{40}O_4$ $M_r$ 392.6

[474-25-9]

DEFINITION

Chenodeoxycholic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3α,7α-dihydroxy-5β-cholan-24-oic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water, freely soluble in alcohol, soluble in acetone, slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *chenodeoxycholic acid CRS*. Examine the substances prepared as discs using *potassium bromide R*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 1 mL of *sulfuric acid R*. Add 0.1 mL of *formaldehyde solution R* and allow to stand for 5 min. Add 5 mL of *water R*. The suspension obtained is greenish-blue.

TESTS

**Specific optical rotation** (*2.2.7*). Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent. The specific optical rotation is + 11.0 to + 13.0, calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution (a).* Dissolve 0.40 g of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

*Reference solution (a).* Dissolve 40 mg of *chenodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 20 mg of *lithocholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents. Dilute 2 mL of the solution to 100 mL with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

*Reference solution (c).* Dissolve 20 mg of *ursodeoxycholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 50 mL with the same mixture of solvents.

*Reference solution (d).* Dissolve 20 mg of *cholic acid CRS* in a mixture of 1 volume of *water R* and 9 volumes of *acetone R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (e).* Dilute 0.5 mL of test solution (a) to 20 mL with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Dilute 1 mL of the solution to 10 mL with a mixture of 1 volume of *water R* and 9 volumes of *acetone R*.

*Reference solution (f).* Dissolve 10 mg of *chenodeoxycholic acid CRS* in reference solution (c) and dilute to 25 mL with the same solution.

Apply separately to the plate 5 µL of each solution. Develop in an unsaturated tank over a path of 15 cm using a mixture of 1 volume of *glacial acetic acid R*, 30 volumes of *acetone R* and 60 volumes of *methylene chloride R*. Dry the plate at 120 °C for 10 min. Spray the plate immediately with a 47.6 g/L solution of *phosphomolybdic acid R* in a mixture of 1 volume of *sulfuric acid R* and 20 volumes of *glacial acetic acid R* and heat again at 120 °C until blue spots appear on a lighter background. In the chromatogram obtained with test solution (a): any spot corresponding to lithocholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent); any spot corresponding to ursodeoxycholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (1 per cent); any spot corresponding to cholic acid is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.5 per cent); any spot apart from the principal spot and any spots corresponding to lithocholic acid, ursodeoxycholic acid and cholic acid, is not more intense than the principal spot in the chromatogram obtained with reference solution (e) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (f) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *alcohol R*, previously neutralised to 0.2 mL of *phenolphthalein solution R*. Add 50 mL of *water R* and titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 39.26 mg of $C_{24}H_{40}O_4$.

IMPURITIES

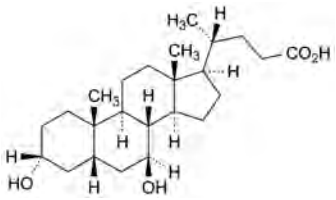

A. 3α-7β-dihydroxy-5β-cholan-24-oic acid (ursodeoxycholic acid),

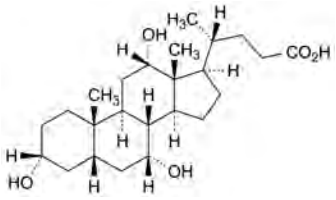

B. 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid),

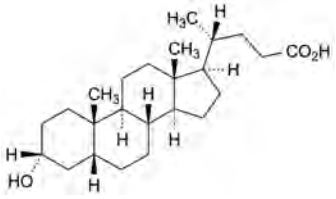

C. 3α-hydroxy-5β-cholan-24-oic acid (lithocholic acid),

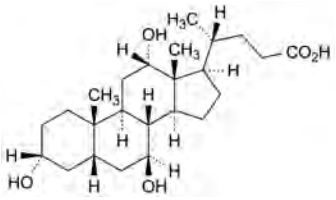

D. 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid (ursocholic acid),

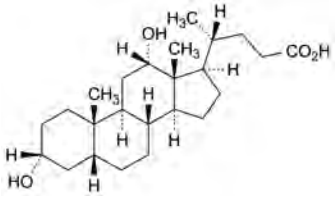

E. 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid),

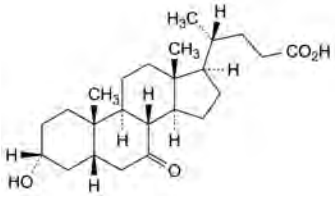

F. 3α-hydroxy-7-oxo-5β-cholan-24-oic acid,

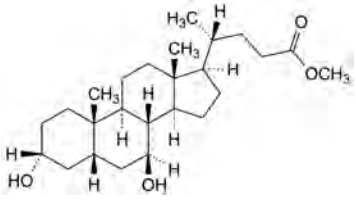

G. methyl 3α,7β-dihydroxy-5β-cholan-24-oate.

**01/2017:1774**

# CHITOSAN HYDROCHLORIDE

## Chitosani hydrochloridum

DEFINITION

Chitosan hydrochloride is the chloride salt of an unbranched binary heteropolysaccharide consisting of the two units *N*-acetyl-D-glucosamine and D-glucosamine, obtained by partial deacetylation of chitin normally leading to a degree of deacetylation of 70.0 per cent to 95.0 per cent. Chitin is extracted from the shells of shrimp and crab.

PRODUCTION

The animals from which chitosan hydrochloride is derived must fulfil the requirements for the health of animals suitable for human consumption to the satisfaction of the competent authority. It must have been shown to what extent the method of production allows inactivation or removal of any contamination by viruses or other infectious agents.

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: sparingly soluble in water, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *chitosan hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

C. Dilute 50 mL of solution S (see Tests) to 250 mL with a 25 per cent *V/V* solution of *ammonia R*. A voluminous gelatinous mass is formed.

D. To 10 mL of solution S add 90 mL of *acetone R*. A voluminous gelatinous mass is formed.

TESTS

**Solution S**. Dissolve 1.0 g in 100 mL of *water R* and stir vigorously for 20 min with a mechanical stirrer.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Matter insoluble in water**: maximum 0.5 per cent.

Add 2.00 g to 400.0 mL of *water R* while stirring until no further dissolution takes place. Transfer the solution to a 2 L beaker, and add 200 mL of *water R*. Boil the solution gently for 2 h, covering the beaker during the operation. Filter through a sintered-glass filter (40) (*2.1.2*), wash the residue with water and dry to constant weight in an oven at 100-105 °C. The residue weighs a maximum of 10 mg.

**pH** (*2.2.3*): 4.0 to 6.0 for solution S.

**Viscosity** (*2.2.10*): 80 per cent to 120 per cent of the value stated on the label, determined on solution S.

Determine the viscosity using a rotating viscometer at 20 °C with a spindle rotating at 20 r/min, using a suitable spindle for the range of the expected viscosity.

**Degree of deacetylation**

*Test solution*. Dissolve 0.250 g in *water R* and dilute to 50.0 mL with the same solvent, stirring vigorously. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Measure the absorbance (*2.2.25*) from 200 nm to 205 nm as the first derivative of the absorbance curve. Determine the pH of the solution.

*Reference solutions*. Prepare solutions of 1.0 µg/mL, 5.0 µg/mL, 15.0 µg/mL and 35.0 µg/mL of *N-acetylglucosamine R* in *water R*. Measure the absorbance (*2.2.25*) from 200 nm to 205 nm of each solution as the first derivative of the absorption curve. Make a standard curve by plotting the first derivative at 202 nm as a function of the concentration of *N*-acetylglucosamine, and calculate the slope of the curve by least squares linear regression. Use the standard curve to determine the equivalent amount of *N*-acetylglucosamine for the substance to be examined.

Calculate the degree of deacetylation (molar) using the following expression:

$$\frac{100 \times M_1 \times (C_1 - C_2)}{(M_1 \times C_1) - [(M_1 - M_3) \times C_2]}$$

$C_1$ = concentration of chitosan hydrochloride in the test solution in micrograms per millilitre;

$C_2$ = concentration of *N*-acetylglucosamine in the test solution, as determined from the standard curve prepared using the reference solution in micrograms per millilitre;

$M_1$ = 203 (relative molecular mass of *N*-acetylglucosamine unit ($C_8H_{13}NO_5$) in polymer);

$M_3$ = relative molecular mass of chitosan hydrochloride.

$M_3$ is calculated from the pH in solution, assuming a pKa value of 6.8, using the following equations:

$$M_3 = f \times M_2 + (1 - f) \times (M_2 + 36.5)$$

$$f = \frac{p}{1 + p}$$

$$p = 10^{(\mathrm{pH} - \mathrm{pKa})}$$

$M_2$ = 161 (relative molecular mass of deacetylated unit (glucosamine) ($C_6H_{11}NO_4$) in polymer).

**Chlorides**: 10.0 per cent to 20.0 per cent.

Introduce 0.200 g into a 250 mL borosilicate flask fitted with a reflux condenser. Add 40 mL of a mixture of 1 volume of *nitric acid R* and 2 volumes of *water R*. Boil gently under a reflux condenser for 5 min. Cool and add 25 mL of *water R* through the condenser. Add 16.0 mL of *0.1 M silver nitrate*, shake vigorously and titrate with *0.1 M ammonium thiocyanate*, using 1 mL of *ferric ammonium sulfate solution R2* as indicator, and shaking vigorously towards the end-point. Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 3.55 mg of Cl.

**Loss on drying** (*2.2.32*): maximum 10 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

STORAGE

At a temperature of 2 °C to 8 °C, protected from moisture and light.

LABELLING

The label states the nominal viscosity in millipascal seconds for a 10 g/L solution in *water R*.

**01/2017:0265**

# CHLORAL HYDRATE

## Chlorali hydras

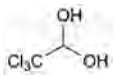


$C_2H_3Cl_3O_2$ $M_r$ 165.4
[302-17-0]

DEFINITION

2,2,2-Trichloroethane-1,1-diol.

*Content*: 98.5 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: colourless, transparent crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. To 10 mL of solution S (see Tests) add 2 mL of *dilute sodium hydroxide solution R*. The mixture becomes cloudy and, when heated, gives off an odour of chloroform.

B. To 1 mL of solution S add 2 mL of *sodium sulfide solution R*. A yellow colour develops which quickly becomes reddish-brown. On standing for a short time, a red precipitate may be formed.

TESTS

**Solution S**. Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 30 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Chloral alcoholate**. Warm 1.0 g with 10 mL of *dilute sodium hydroxide solution R*, filter the supernatant solution and add *0.05 M iodine* dropwise until a yellow colour is obtained. Allow to stand for 1 h. No precipitate is formed.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Non-volatile residue**: maximum 0.1 per cent.

Evaporate 2.000 g on a water-bath. The residue weighs a maximum of 2 mg.

ASSAY

Dissolve 4.000 g in 10 mL of *water R* and add 40.0 mL of *1 M sodium hydroxide*. Allow to stand for exactly 2 min and titrate with *0.5 M sulfuric acid*, using 0.1 mL of *phenolphthalein solution R* as indicator. Titrate the neutralised solution with *0.1 M silver nitrate*, using 0.2 mL of *potassium chromate solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* used by deducting from the volume of *1 M sodium hydroxide*, added at the beginning of the titration, the volume of *0.5 M sulfuric acid* used in the 1st titration and two-fifteenths of the volume of *0.1 M silver nitrate* used in the 2nd titration.

1 mL of *1 M sodium hydroxide* is equivalent to 0.1654 g of $C_2H_3Cl_3O_2$.

STORAGE

In an airtight container.

04/2011:0137

# CHLORAMBUCIL

## Chlorambucilum

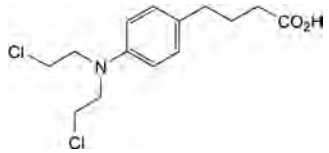


$C_{14}H_{19}Cl_2NO_2$ $M_r$ 304.2
[305-03-3]

DEFINITION

4-[4-[Bis(2-chloroethyl)amino]phenyl]butanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chlorambucil CRS.*

TESTS

**Impurity G**. Liquid chromatography (*2.2.29*). *The solutions are stable for 8 h at room temperature or for 24 h at 4-8 °C; protect them from light.*

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *chlorambucil with impurity G CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 1 per cent *V/V* solution of *trifluoroacetic acid R* (50:50 *V/V*).

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of chlorambucil.

*Relative retention* with reference to chlorambucil (retention time = about 11 min): impurity G = about 1.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to chlorambucil and impurity G.

*Limit*:

- *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Solvent mixture*: 10.3 g/L solution of *hydrochloric acid R*, *acetonitrile for chromatography R* (10:90 *V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *chlorambucil for system suitability CRS* (containing impurities B and E) in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 1.9 g/L solution of *ammonium acetate R* adjusted to pH 3.9 with *acetic acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 60 | 40 |
| 5 - 15 | 60 → 10 | 40 → 90 |
| 15 - 25 | 10 | 90 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *chlorambucil for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and E.

*Relative retention* with reference to chlorambucil (retention time = about 12 min): impurity B = about 0.5; impurity E = about 1.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and chlorambucil.

*Limits*:

- *impurity E*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *impurity B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 10 mL of *acetone R* and add 10 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.42 mg of $C_{14}H_{19}Cl_2NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, F.*

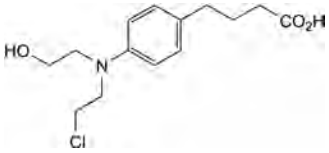

A. 4-[4-[(2-chloroethyl)(2-hydroxyethyl)amino]phenyl]butanoic acid,

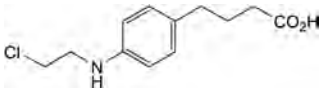

B. 4-[4-[(2-chloroethyl)amino]phenyl]butanoic acid,

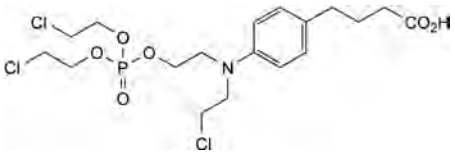

C. 4-[4-[[2-[[bis(2-chloroethoxy)phosphoryl]oxy]ethyl](2-chloroethyl)amino]phenyl]butanoic acid,

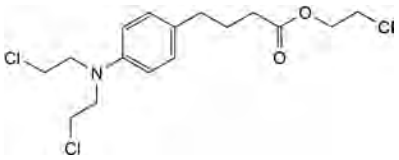

D. 2-chloroethyl 4-[4-[bis(2-chloroethyl)amino]phenyl]-butanoate,

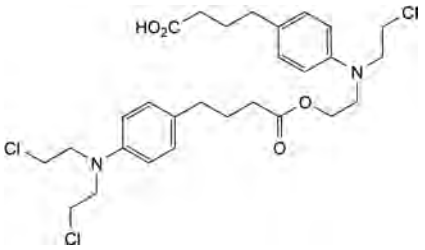

E. 4-[4-[[2-[[4-[4-[bis(2-chloroethyl)amino]phenyl]-butanoyl]oxy]ethyl](2-chloroethyl)amino]phenyl]butanoic acid,

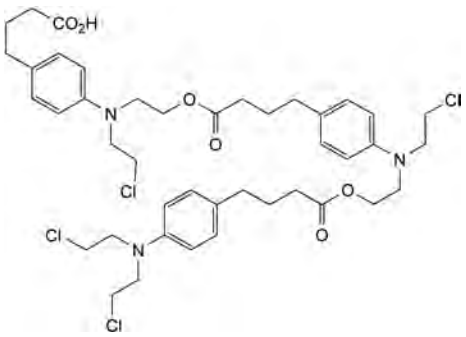

F. 4-[4-[[2-[[4-[4-[[2-[[4-[4-[bis(2-chloroethyl)amino]-phenyl]butanoyl]oxy]ethyl](2-chloroethyl)amino]-phenyl]butanoyl]oxy]ethyl](2-chloroethyl)amino]-phenyl]butanoic acid,

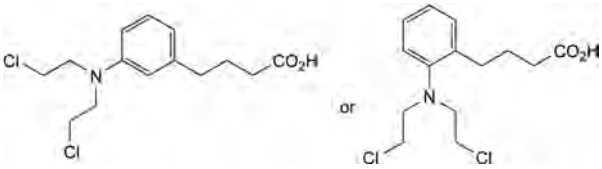

G. 4-[2-[bis(2-chloroethyl)amino]phenyl]butanoic acid or 4-[3-[bis(2-chloroethyl)amino]phenyl]butanoic acid (*meta* or *ortho* chlorambucil).

**04/2019:0071**
**corrected 10.0**

# CHLORAMPHENICOL

## Chloramphenicolum

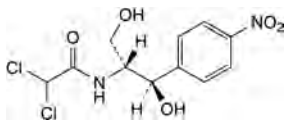

$C_{11}H_{12}Cl_2N_2O_5$ $M_r$ 323.1

[56-75-7]

DEFINITION

2,2-Dichloro-*N*-[(1*R*,2*R*)-1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white, greyish-white or yellowish-white, fine, crystalline powder or fine crystals, needles or elongated plates.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent) and in propylene glycol.

A solution in anhydrous ethanol is dextrorotatory and a solution in ethyl acetate is laevorotatory.

IDENTIFICATION

*First identification: A.*

*Second identification: B.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chloramphenicol CRS.*

B. Melting point (*2.2.14*).

*Determination A*: determine the melting point of the substance to be examined.

*Results A*: 149 °C to 153 °C.

*Determination B*: mix equal parts of the substance to be examined and *chloramphenicol CRS* and determine the melting point of the mixture.

*Results B*: the absolute difference between the melting point of the mixture and the value obtained in determination A is not greater than 2 °C.

TESTS

**Acidity or alkalinity**. To 0.1 g add 20 mL of *carbon dioxide-free water R*, shake and add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.1 mL of *0.02 M hydrochloric acid* or *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): + 18.5 to + 20.5.

Dissolve 1.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 2.0 g of *sodium heptanesulfonate R* in 900 mL of *water for chromatography R*. Add 6.8 g of *potassium dihydrogen phosphate R* and 5 mL of *triethylamine R*. Adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in 10 mL of *methanol R* and dilute to 200.0 mL with mobile phase A.

*Test solution (b)*. Dissolve 25.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 20.0 mg of *chloramphenicol CRS* in 10 mL of *methanol R* and dilute to 200.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 12.5 mg of *4-nitrobenzaldehyde R* (impurity B) in 2 mL of *methanol R* and dilute to 50 mL with mobile phase A.

*Reference solution (d)*. Dissolve 5 mg of *chloramphenicol for peak identification CRS* (containing impurity A) in 1 mL of *methanol R*, add 1 mL of reference solution (c) and dilute to 10 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, solution A (32:68 *V/V*);
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 13 | 100 | 0 |
| 13 - 25 | 100 → 60 | 0 → 40 |
| 25 - 33 | 60 | 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 277 nm.

*Injection*: 10 µL of test solution (b) and reference solutions (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *chloramphenicol for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A and B.

*Relative retention* with reference to chloramphenicol (retention time = about 14 min): impurity A = about 0.7; impurity B = about 0.9.

*System suitability*: reference solution (d):

- *resolution*: minimum 2.0 between the peaks due to impurity B and chloramphenicol.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity A by 0.7;
- for each impurity, use the concentration of chloramphenicol in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 1.00 g add 10 mL of *nitric acid R* and 20 mL of *water R* and shake for 5 min. Filter through a filter paper previously washed by filtering 5 mL portions of *water R* until 5 mL of filtrate no longer becomes opalescent on addition of 0.1 mL of *nitric acid R* and 0.1 mL of *silver nitrate solution R1*. 15 mL of the filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A.

*Injection*: test solution (a) and reference solution (a).

*Run time*: 1.5 times the retention time of chloramphenicol.

Calculate the percentage content of $C_{11}H_{12}Cl_2N_2O_5$ taking into account the assigned content of *chloramphenicol CRS*.

STORAGE

Protected from light. If the substance is sterile, the container is also sterile, airtight and tamper-evident.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

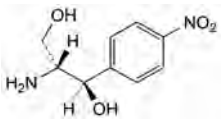


A. (1*R*,2*R*)-2-amino-1-(4-nitrophenyl)propane-1,3-diol,

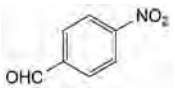


B. 4-nitrobenzaldehyde.

**01/2008:0473**
**corrected 10.0**

# CHLORAMPHENICOL PALMITATE

## Chloramphenicoli palmitas

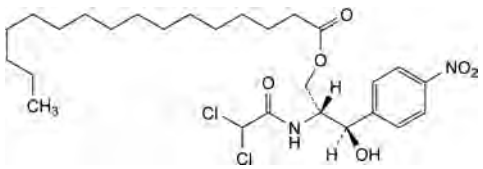


$C_{27}H_{42}Cl_2N_2O_6$ $M_r$ 561.6
[530-43-8]

DEFINITION

Chloramphenicol palmitate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*R*,3*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl hexadecanoate, calculated with reference to the dried substance.

Semi-synthetic product derived from a fermentation product.

CHARACTERS

A white or almost white, fine, unctuous powder, practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent), very slightly soluble in hexane.

It melts at 87 °C to 95 °C.

It shows polymorphism (*5.9*). The thermodynamically stable form has low bioavailability following oral administration.

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using *TLC silanised silica gel plate R*.

*Test solution*. Dissolve 50 mg of the substance to be examined in a mixture of 1 mL of *1 M sodium hydroxide* and 5 mL of *acetone R* and allow to stand for 30 min. Add 1.1 mL of *1 M hydrochloric acid* and 3 mL of *acetone R*.

*Reference solution (a)*. Dissolve 10 mg of *chloramphenicol CRS* in *acetone R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *palmitic acid R* in *acetone R* and dilute to 5 mL with the same solvent.

*Reference solution (c)*. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 5 mL with the same solvent.

Apply to the plate 4 µL of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of a 100 g/L solution of *ammonium acetate R* and 70 volumes of *ethanol (96 per cent) R*. Allow the plate to dry in air and spray with a solution containing 0.2 g/L of *dichlorofluorescein R* and 0.1 g/L of *rhodamine B R* in *ethanol (96 per cent) R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows 3 spots corresponding in position to the principal spots in the chromatograms obtained with reference solutions (a), (b) and (c).

B. Dissolve 0.2 g in 2 mL of *pyridine R*, add 2 mL of a 100 g/L solution of *potassium hydroxide R* and heat on a water-bath. A red colour is produced.

C. Dissolve about 10 mg in 5 mL of *ethanol (96 per cent) R* and add 4.5 mL of *dilute sulfuric acid R* and 50 mg of *zinc powder R*. Allow to stand for 10 min and if necessary decant the supernatant or filter. Cool the solution in iced water and add 0.5 mL of *sodium nitrite solution R*. Allow to stand for 2 min and add 1 g of *urea R*, 2 mL of *strong sodium hydroxide solution R* and 1 mL of *β-naphthol solution R*. A red colour develops.

TESTS

**Acidity**. Dissolve 1.0 g in 5 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *ether R*, warming to 35 °C. Add 0.2 mL of *phenolphthalein solution R*. Not more than 0.4 mL of *0.1 M sodium hydroxide* is required to produce a pink colour persisting for 30 s.

**Specific optical rotation** (*2.2.7*). Dissolve 1.25 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent. The specific optical rotation is + 22.5 to + 25.5.

**Free chloramphenicol**: maximum 450 ppm. Dissolve 1.0 g, with gentle heating, in 80 mL of *xylene R*. Cool and shake with 3 quantities, each of 15 mL, of *water R*. Dilute the combined aqueous extracts to 50 mL with *water R* and shake with 10 mL of *toluene R*. Allow to separate and discard the toluene layer. Centrifuge a portion of the aqueous layer and measure the absorbance (*A*) (*2.2.25*) at the maximum at 278 nm using as the compensation liquid a blank solution having an absorbance not greater than 0.05.

Calculate the content of free chloramphenicol in parts per million from the expression:

$$\frac{A \times 10^4}{5.96}$$

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.1 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *chloramphenicol palmitate isomer CRS* in *acetone R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *acetone R*.

*Reference solution (b)*. Dissolve 20 mg of *chloramphenicol dipalmitate CRS* in *acetone R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *acetone R*.

*Reference solution (c)*. Dissolve 5 mg of *chloramphenicol CRS* in *acetone R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *acetone R*.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R*, 40 volumes of *chloroform R* and 50 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution, any spots due to chloramphenicol palmitate isomer and chloramphenicol dipalmitate are not more intense than the corresponding spots in the chromatograms obtained with reference solutions (a) and (b) respectively (2.0 per cent) and any spot, apart from the principal spot and the spots due to chloramphenicol palmitate isomer and chloramphenicol dipalmitate, is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C at a pressure not exceeding 0.1 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 90.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 250.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) of the solution at the maximum at 271 nm.

Calculate the content of $C_{27}H_{42}Cl_2N_2O_6$ taking the specific absorbance to be 178.

STORAGE

Protected from light.

IMPURITIES

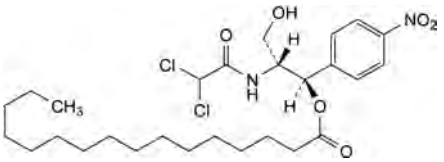


A. (1*R*,2*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-1-(4-nitrophenyl)propyl hexadecanoate (chloramphenicol palmitate isomer),

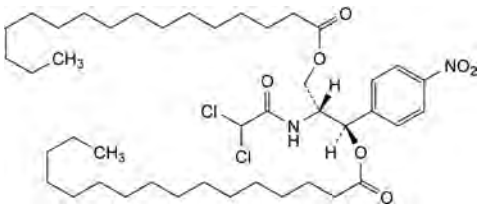

B. (1*R*,2*R*)-2-[(dichloroacetyl)amino]-1-(4-nitrophenyl)propane-1,3-diyl bishexadecanoate (chloramphenicol dipalmitate).



**01/2008:0709**
**corrected 10.0**

# CHLORAMPHENICOL SODIUM SUCCINATE

## Chloramphenicoli natrii succinas

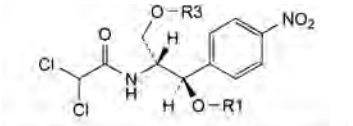


$C_{15}H_{15}Cl_2N_2NaO_8$ $M_r$ 445.2

DEFINITION

Mixture in variable proportions of sodium (2*R*,3*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-3-(4-nitrophenyl)propyl butanedioate (3 isomer) and of sodium (1*R*,2*R*)-2-[(dichloroacetyl)amino]-3-hydroxy-1-(4-nitrophenyl)propyl butanedioate (1 isomer).

Semi-synthetic product derived from a fermentation product.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 2 mL of *acetone R*.

*Reference solution (a).* Dissolve 20 mg of *chloramphenicol sodium succinate CRS* in 2 mL of *acetone R*.

*Reference solution (b).* Dissolve 20 mg of *chloramphenicol CRS* in 2 mL of *acetone R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *dilute acetic acid R*, *methanol R*, *chloroform R* (1:14:85 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the 2 principal spots in the chromatogram obtained with the test solution are similar in position and size to the 2 principal spots in the chromatogram obtained with reference solution (a); their positions are different from that of the principal spot in the chromatogram obtained with reference solution (b).

B. Dissolve about 10 mg in 1 mL of *ethanol (50 per cent V/V) R*, add 3 mL of a 10 g/L solution of *calcium chloride R* and 50 mg of *zinc powder R* and heat on a water-bath for 10 min. Filter the hot solution and allow to cool. Add 0.1 mL of *benzoyl chloride R* and shake for 1 min. Add 0.5 mL of *ferric chloride solution R1* and 2 mL of *chloroform R* and shake. The upper layer is light violet-red or purple.

C. Dissolve 50 mg in 1 mL of *pyridine R*. Add 0.5 mL of *dilute sodium hydroxide solution R* and 1.5 mL of *water R*. Heat in a water-bath for 3 min. A red colour develops. Add 2 mL of *nitric acid R* and cool under running water. Add 1 mL of *0.1 M silver nitrate*. A white precipitate is formed slowly.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 6.4 to 7.0.

Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 5.0 to + 8.0 (anhydrous substance).

Dissolve 0.50 g in *water R* and dilute to 10.0 mL with the same solvent.

**Chloramphenicol and chloramphenicol disodium disuccinate**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *chloramphenicol CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase (solution A). Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *chloramphenicol disodium disuccinate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase (solution B). Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25 mg of the substance to be examined in the mobile phase, add 5 mL of solution A and 5 mL of solution B and dilute to 100 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 20 g/L solution of *phosphoric acid R*, *methanol R*, *water R* (5:40:55 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (c):

– the 2 peaks corresponding to those in the chromatograms obtained with reference solutions (a) and (b) are clearly separated from the peaks corresponding to the 2 principal peaks in the chromatogram obtained with the test solution; if necessary, adjust the methanol content of the mobile phase.

*Limits*:

– *chloramphenicol*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
– *chloramphenicol disodium disuccinate*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of pyrogens, it complies with the test

for pyrogens. Inject per kilogram of the rabbit's mass 2.5 mL of a solution in *water for injections R* containing 2 mg of the substance to be examined per millilitre.

ASSAY

Dissolve 0.200 g in *water R* and dilute to 500.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 276 nm.

Calculate the content of $C_{15}H_{15}Cl_2N_2NaO_8$, taking the specific absorbance to be 220.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container, protected from light.

**01/2008:1086**
**corrected 7.0**

# CHLORCYCLIZINE HYDROCHLORIDE

## Chlorcyclizini hydrochloridum

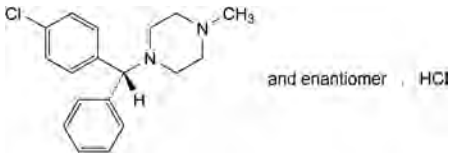


$C_{18}H_{22}Cl_2N_2$ $M_r$ 337.3
[14362-31-3]

DEFINITION

Chlorcyclizine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-1-[(4-chlorophenyl)phenylmethyl]-4-methylpiperazine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in methylene chloride, soluble in alcohol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 10.0 mg in a 5 g/L solution of *sulfuric acid R* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with a 5 g/L solution of *sulfuric acid R*. Examined between 215 nm and 300 nm (*2.2.25*), the solution shows an absorption maximum at 231 nm. The specific absorbance at the maximum is 475 to 525, calculated with reference to the dried substance.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *chlorcyclizine hydrochloride CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances (see Tests). The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. The pH of the solution is 5.0 to 6.0.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a plate coated with a suitable silica gel.

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 5 mL of test solution (a) to 100 mL with *methanol R*.

*Reference solution (a).* Dissolve 10 mg of *chlorcyclizine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *methylpiperazine R* in *methanol R* and dilute to 50 mL with the same solvent.

*Reference solution (c).* Dilute 1 mL of test solution (b) to 25 mL with *methanol R*.

*Reference solution (d).* Dissolve 10 mg of *hydroxyzine hydrochloride CRS* and 10 mg of *chlorcyclizine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

Apply separately to the plate 10 µL of each solution and develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 13 volumes of *methanol R* and 85 volumes of *methylene chloride R*. Allow the plate to dry in air and expose it to iodine vapour for 10 min. In the chromatogram obtained with test solution (a): any spot corresponding to methylpiperazine is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); any spot, apart from the principal spot and any spot corresponding to methylpiperazine, is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 1 mL of *0.1 M hydrochloric acid* and 50 mL of *methanol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the two points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.73 mg of $C_{18}H_{22}Cl_2N_2$.

STORAGE

Store protected from light.

IMPURITIES

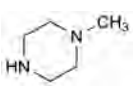


A. *N*-methylpiperazine.

**01/2008:0656**
**corrected 6.0**

# CHLORDIAZEPOXIDE

## Chlordiazepoxidum

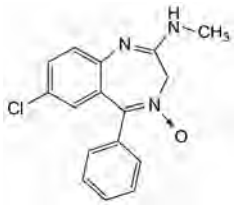

$C_{16}H_{14}ClN_3O$ $M_r$ 299.8
[58-25-3]

DEFINITION

7-Chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amine 4-oxide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or light yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chlordiazepoxide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from bright light and prepare the solutions immediately before use.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *chlordiazepoxide impurity A CRS* in the mobile phase, add 25.0 mL of the test solution and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 4.0 mg of *aminochlorobenzophenone R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 6 times the retention time of chlordiazepoxide.

*Relative retention* with reference to chlordiazepoxide (retention time = about 3.6 min): impurity A = about 0.7; impurity B = about 2.3; impurity C = about 3.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and chlordiazepoxide.

*Limits*:

- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent),
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g, with heating if necessary, in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.98 mg of $C_{16}H_{14}ClN_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

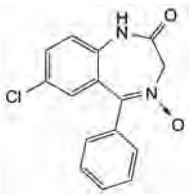

A. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide,

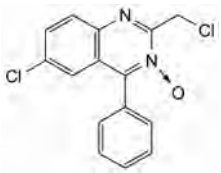

B. 6-chloro-2-(chloromethyl)-4-phenylquinazoline 3-oxide,

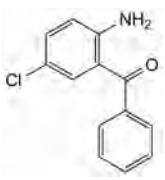

C. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone).

**01/2008:0474**

# CHLORDIAZEPOXIDE HYDROCHLORIDE

## Chlordiazepoxidi hydrochloridum

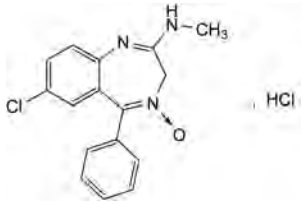

$C_{16}H_{15}Cl_2N_3O$ $M_r$ 336.2
[438-41-5]

DEFINITION

7-Chloro-*N*-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-amine 4-oxide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: soluble in water, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *chlordiazepoxide hydrochloride CRS*.
If the spectra obtained in the solid state show differences, dissolve 100 mg in 9 mL of *water R* and add 1 mL of *dilute sodium hydroxide solution R*. Extract with 10 mL of *methylene chloride R* in a separating funnel. Evaporate the organic layer and dry the residue obtained at 100-105 °C. Proceed in the same way with the reference substance. Record new spectra using the residues.

B. Dissolve 50 mg in 5 mL of *water R*, add 1 mL of *dilute ammonia R1*, mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the following operations protected from bright light and prepare the solutions immediately before use.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *chlordiazepoxide impurity A CRS* in the mobile phase, add 25.0 mL of the test solution and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 4.0 mg of *aminochlorobenzophenone R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 6 times the retention time of chlordiazepoxide.

*Relative retention* with reference to chlordiazepoxide (retention time = about 3.6 min): impurity A = about 0.7; impurity B = about 2.3; impurity C = about 3.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to impurity A and chlordiazepoxide.

*Limits*:
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent),
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 33.62 mg of $C_{16}H_{15}Cl_2N_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

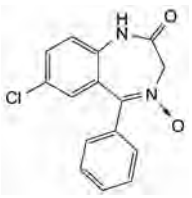

A. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide,

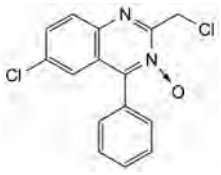

B. 6-chloro-2-(chloromethyl)-4-phenylquinazoline 3-oxide,

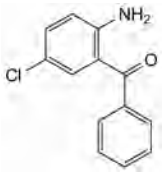

C. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone).

**04/2018:0657**

# CHLORHEXIDINE DIACETATE

## Chlorhexidini diacetas

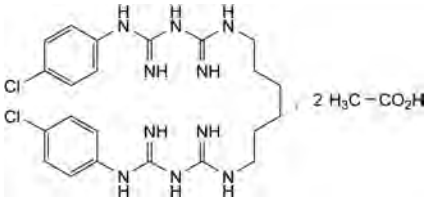

$C_{26}H_{38}Cl_2N_{10}O_4$ $M_r$ 626
[56-95-1]

DEFINITION

$N^1$,$N^{1\prime}$-(Hexane-1,6-diyl)bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide] diacetate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, microcrystalline powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent), slightly soluble in glycerol and in propylene glycol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chlorhexidine diacetate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 5 mg of *chlorhexidine diacetate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethanol (96 per cent) R*, *methylene chloride R* (7:10:40:50 *V/V/V/V*).

*Application*: 5 µL; the volume may be adapted based on the type of plate used.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: treat with *copper sulfate solution R* and examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of acetates (*2.3.1*).

TESTS

**Impurity P (chloroaniline)**: maximum 500 ppm.

*Test solution.* Dissolve 0.20 g in 25 mL of *water R* with shaking if necessary. Add 1 mL of *hydrochloric acid R* and dilute to 30 mL with *water R*. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

*Reference solutions.* Prepare reference solutions representing respectively 50 ppm, 100 ppm, 200 ppm, 500 ppm and 600 ppm of *chloroaniline R* (impurity P) in the test sample as follows: dilute 1.0 mL, 2.0 mL, 4.0 mL, 10.0 mL and 12.0 mL of a solution containing 0.010 g/L of *chloroaniline R* (impurity P) in *dilute hydrochloric acid R* to 20 mL with *water R*. Then, add 10 mL of *water R*. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer each solution quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

Measure the absorbance (*2.2.25*) of each reference solution at 556 nm and plot a calibration curve.

Measure the absorbance (*2.2.25*) of the test solution at 556 nm. Determine the concentration of chloroaniline from the calibration curve.

**Related substances**. Liquid chromatography (*2.2.29*). *Store the solutions at a temperature not exceeding 12 °C.*

*Test solution.* Dissolve 0.140 g of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *chlorhexidine for system suitability CRS* (containing impurities A, B, H, I, K, N and O) in 1.0 mL of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase:*

- *mobile phase A*: mix 20 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R* and 80 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water for chromatography R*;
- *mobile phase B*: mix 10 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water for chromatography R* and 90 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 32 | 100 → 80 | 0 → 20 |
| 32 - 37 | 80 | 20 |
| 37 - 47 | 80 → 70 | 20 → 30 |
| 47 - 54 | 70 | 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *chlorhexidine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, H, I, K, N and O.

*Relative retention* with reference to chlorhexidine (retention time = about 35 min): impurity N = about 0.35; impurity B = about 0.36; impurity A = about 0.6; impurity H = 0.85; impurity O = about 0.90; impurity I = about 0.91; impurity K = about 1.4.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity N.

*Limits*:

- *sum of impurities I and O*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity K*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities A, H, N*: for each impurity, not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 3.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.15 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 100 mL of *anhydrous acetic acid R* and titrate with *0.1 M perchloric acid*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 15.64 mg of $C_{26}H_{38}Cl_2N_{10}O_4$.

IMPURITIES

*Specified impurities*: *A, H, I, K, N, O, P.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E, F, G, M.*

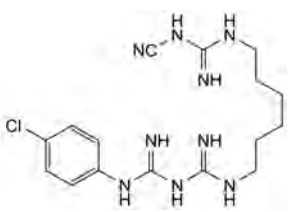

A. $N^1$-(4-chlorophenyl)-$N^3$-[6-[(*N*-cyanocarbamimidoyl)-amino]hexyl]imidodicarbonimidic diamide,

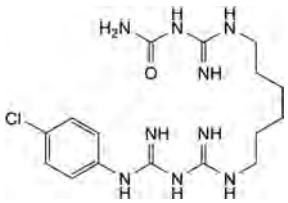

B. *N*-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]carbamimidoyl]urea,

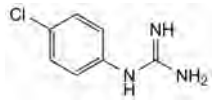

E. *N*-(4-chlorophenyl)guanidine,

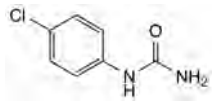

F. *N*-(4-chlorophenyl)urea,

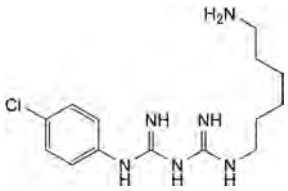

G. $N^1$-(6-aminohexyl)-$N^3$-(4-chlorophenyl)imido-dicarbonimidic diamide,

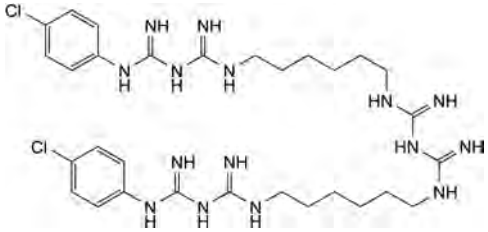

H. $N^1$,$N^{1\prime}$-[azanediylbis(carbonimidoylazanediylhexane-6,1-diyl)]bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide],

I. unknown structure,

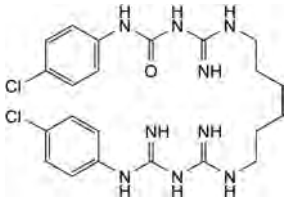

K. *N*-(4-chlorophenyl)-*N′*-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)-carbamimidoyl]carbamimidoyl]amino]hexyl]-carbamimidoyl]urea,

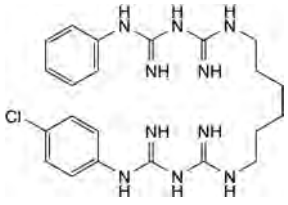

M. $N^1$-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]-$N^3$-phenylimido-dicarbonimidic diamide,

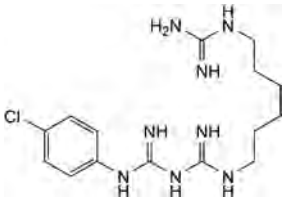

N. $N^1$-[6-(carbamimidoylamino)hexyl]-$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide,

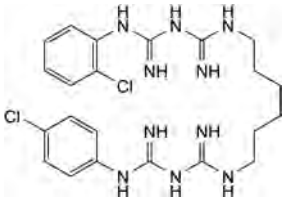

O. $N^1$-[6-[[*N*-[*N*-(2-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]-$N^3$-(4-chlorophenyl)-imidodicarbonimidic diamide,

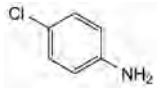

P. 4-chloroaniline.

**01/2018:0658**

# CHLORHEXIDINE DIGLUCONATE SOLUTION

## Chlorhexidini digluconatis solutio

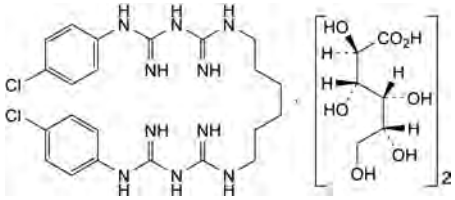

$C_{34}H_{54}Cl_2N_{10}O_{14}$ $M_r$ 898
[18472-51-0]

DEFINITION

$N^1$,$N^{1\prime}$-(Hexane-1,6-diyl)bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide] di-D-gluconate.

*Content*: 190 g/L to 210 g/L.

CHARACTERS

*Appearance*: almost colourless or pale-yellowish liquid.

*Solubility*: miscible with water, with not more than 3 parts of acetone and with not more than 5 parts of ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 1 mL add 40 mL of *water R*, cool in iced water, make alkaline to *titan yellow paper R* by adding dropwise, and with stirring, *strong sodium hydroxide solution R* and add 1 mL in excess. Filter, wash the precipitate with *water R* until the washings are free from alkali and recrystallise from *ethanol (70 per cent V/V) R*. Dry at 100-105 °C. Examine the residue.

*Comparison*: *chlorhexidine CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 10.0 mL of the preparation to be examined to 50 mL with *water R*.

*Reference solution.* Dissolve 25 mg of *calcium gluconate CRS* in 1 mL of *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 1/2 of the plate.

*Drying*: at 100 °C for 20 min and allow to cool.

*Detection*: spray with a solution containing 25 g/L of *ammonium molybdate R* and 10 g/L of *cerium sulfate R* in *dilute sulfuric acid R*, and heat at 110 °C for about 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 1 mL add 40 mL of *water R*, cool in iced water, make alkaline to *titan yellow paper R* by adding dropwise, and with stirring, *strong sodium hydroxide solution R* and add 1 mL in excess. Filter, wash the precipitate with *water R* until the washings are free from alkali and recrystallise from *ethanol (70 per cent V/V) R*. Dry at 100-105 °C. The residue melts (*2.2.14*) at 132 °C to 136 °C.

D. To 0.05 mL add 5 mL of a 10 g/L solution of *cetrimide R*, 1 mL of *strong sodium hydroxide solution R* and 1 mL of *bromine water R*; a deep red colour is produced.

TESTS

**Relative density** (*2.2.5*): 1.06 to 1.07.

**pH** (*2.2.3*): 5.5 to 7.0.

Dilute 5.0 mL to 100 mL with *carbon dioxide-free water R*.

**Impurity P (chloroaniline)**: maximum 500 ppm, calculated with reference to chlorhexidine digluconate solution.

*Test solution.* Dilute 0.20 g of the preparation to be examined to 30 mL with *water R*. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

*Reference solutions.* Prepare reference solutions representing respectively 50 ppm, 100 ppm, 200 ppm, 500 ppm and 600 ppm of *chloroaniline R* (impurity P) in the test sample as follows: dilute 1.0 mL, 2.0 mL, 4.0 mL, 10.0 mL and 12.0 mL of a solution containing 0.010 g/L of *chloroaniline R* (impurity P) in *dilute hydrochloric acid R* to 20 mL with *water R*. Then, add 10 mL of *water R*. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer each solution quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

Measure the absorbance (*2.2.25*) of each reference solution at 556 nm and plot a calibration curve.

Measure the absorbance (*2.2.25*) of the test solution at 556 nm. Determine the concentration of chloroaniline from the calibration curve.

**Related substances**. Liquid chromatography (*2.2.29*). *Store the solutions at a temperature not exceeding 12 °C.*

*Test solution.* Dilute 1.0 mL of the preparation to be examined to 100.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *chlorhexidine for system suitability CRS* (containing impurities A, B, F, G, H, I, J, K, L, N and O) in 1.0 mL of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R* and 80 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water R*;
- *mobile phase B*: mix 10 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water R* and 90 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 32 | 100 → 80 | 0 → 20 |
| 32 - 37 | 80 | 20 |
| 37 - 47 | 80 → 70 | 20 → 30 |
| 47 - 54 | 70 | 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *chlorhexidine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, F, G, H, I, J, K, L, N and O.

*Relative retention* with reference to chlorhexidine (retention time = about 35 min): impurity L = about 0.23; impurity Q = about 0.24; impurity G = about 0.25; impurity N = about 0.35; impurity B = about 0.36; impurity F = about 0.5; impurity A = about 0.6; impurity H = about 0.85; impurity O = about 0.90; impurity I = about 0.91; impurity J = about 0.96; impurity K = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurities L and G;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity N.

*Limits*:

- *impurity N*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity H*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities A, J , K*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *sum of impurities I and O*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity G*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities B, F, L, Q*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

ASSAY

Determine the density (*2.2.5*) of the preparation to be examined. Transfer 1.00 g to a 250 mL beaker and add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.44 mg of $C_{34}H_{54}Cl_2N_{10}O_{14}$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, F, G, H, I, J, K, L, N, O, P, Q.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, M*.

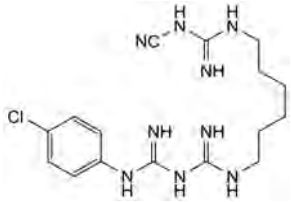

A. $N^1$-(4-chlorophenyl)-$N^3$-[6-[(*N*-cyanocarbamimidoyl)amino]hexyl]imidodicarbonimidic diamide,

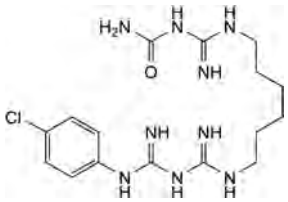

B. *N*-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]carbamimidoyl]amino]hexyl]carbamimidoyl]urea,

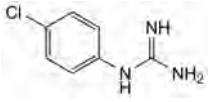

E. *N*-(4-chlorophenyl)guanidine,

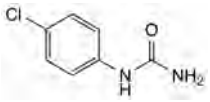

F. *N*-(4-chlorophenyl)urea,

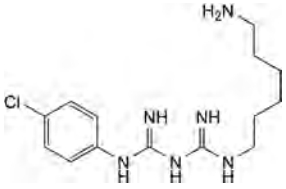

G. $N^1$-(6-aminohexyl)-$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide,

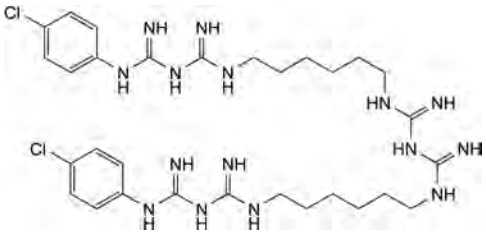

H. $N^1$,$N^{1\prime}$-[azanediylbis(carbonimidoylazanediylhexane-6,1-diyl)]bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide],

I. unknown structure,

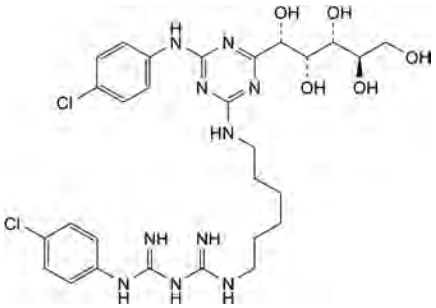

J. 1-(4-chlorophenyl)-5-[6-[[4-[(4-chlorophenyl)amino]-6-[(1*S*,2*R*,3*R*,4*R*)-1,2,3,4,5-pentahydroxypentyl]-1,3,5-triazin-2-yl]amino]hexyl]biguanide,

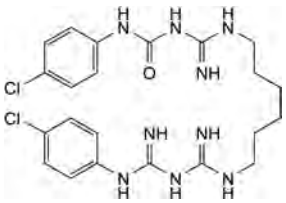

K. *N*-(4-chlorophenyl)-*N′*-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]carbamimidoyl]amino]hexyl]carbamimidoyl]urea,

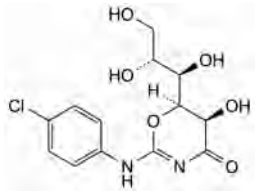

L. (5*R*,6*S*)-2-[(4-chlorophenyl)amino]-5-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-5,6-dihydro-4*H*-1,3-oxazin-4-one,

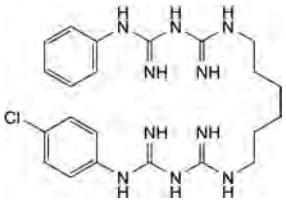

M. $N^1$-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]carbamimidoyl]amino]hexyl]-$N^3$-phenylimidodicarbonimidic diamide,

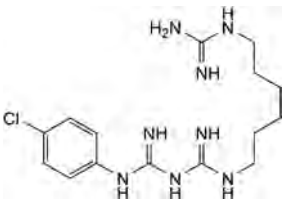

N. $N^1$-[6-(carbamimidoylamino)hexyl]-$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide,

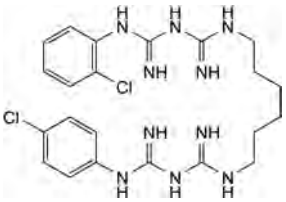

O. $N^1$-[6-[[*N*-[*N*-(2-chlorophenyl)carbamimidoyl]carbamimidoyl]amino]hexyl]-$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide,

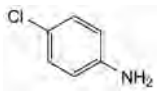

P. 4-chloroaniline,

Q. unknown structure.

**04/2018:0659**

# CHLORHEXIDINE DIHYDROCHLORIDE

## Chlorhexidini dihydrochloridum

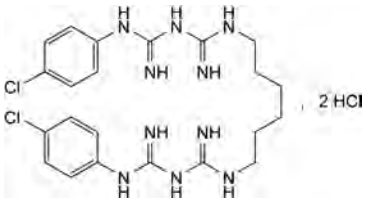

$C_{22}H_{32}Cl_4N_{10}$ $M_r$ 578.4
[3697-42-5]

DEFINITION

$N^1$,$N^{1\prime}$-(Hexane-1,6-diyl)bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide] dihydrochloride.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in propylene glycol, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *chlorhexidine dihydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 5 mg of *chlorhexidine dihydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethanol (96 per cent) R*, *methylene chloride R* (7:10:40:50 *V/V/V/V*).

*Application*: 5 µL; the volume may be adapted based on the type of plate used.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: treat with *copper sulfate solution R* and examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurity P (chloroaniline)**: maximum 300 ppm.

*Test solution*. To 0.20 g add 1 mL of *hydrochloric acid R*, shake for about 30 s, dilute to 30 mL with *water R* and shake until a clear solution is obtained. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

*Reference solutions*. Prepare reference solutions representing respectively 50 ppm, 100 ppm, 200 ppm, 300 ppm and 500 ppm of *chloroaniline R* (impurity P) in the test sample as follows: dilute 1.0 mL, 2.0 mL, 4.0 mL, 6.0 mL and 10.0 mL of a solution containing 0.010 g/L of *chloroaniline R* (impurity P) in *dilute hydrochloric acid R* to 20 mL with *water R*. Then, add 10 mL of *water R*. Add rapidly and with thorough mixing after each addition: 5 mL of a 103 g/L solution of *hydrochloric acid R*, 0.35 mL of *sodium nitrite solution R*, 2 mL of a 50 g/L solution of *ammonium sulfamate R*, 5 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and 1 mL of *ethanol (96 per cent) R*; transfer each solution quantitatively to a volumetric flask, dilute to 50.0 mL with *water R* and allow to stand for 30 min.

Measure the absorbance (*2.2.25*) of each reference solution at 556 nm and plot a calibration curve.

Measure the absorbance (*2.2.25*) of the test solution at 556 nm. Determine the concentration of chloroaniline from the calibration curve.

**Related substances**. Liquid chromatography (*2.2.29*). *Store the solutions at a temperature not exceeding 12 °C.*

*Test solution*. Dissolve 0.130 g of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 5 mg of *chlorhexidine for system suitability CRS* (containing impurities A, B, H, I, K, N and O) in 1.0 mL of mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R* and 80 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water for chromatography R*;
- *mobile phase B*: mix 10 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *water for chromatography R* and 90 volumes of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 32 | 100 → 80 | 0 → 20 |
| 32 - 37 | 80 | 20 |
| 37 - 47 | 80 → 70 | 20 → 30 |
| 47 - 54 | 70 | 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *chlorhexidine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, H, I, K, N and O.

*Relative retention* with reference to chlorhexidine (retention time = about 35 min): impurity N = about 0.35; impurity B = about 0.36; impurity A = about 0.6; impurity H = about 0.85; impurity O = about 0.90; impurity I = about 0.91; impurity K = about 1.4.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity N.

*Limits*:

- *impurity H*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity K*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *sum of impurities I and O*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurities A, N*: for each impurity, not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 100.0 mg in 5 mL of *anhydrous formic acid R* and add 70 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.46 mg of $C_{22}H_{32}Cl_4N_{10}$.

IMPURITIES

*Specified impurities: A, H, I, K, N, O, P.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E, F, G, M.*

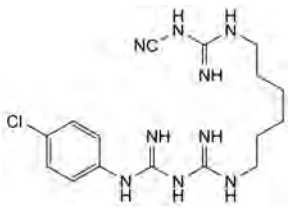

A. $N^1$-(4-chlorophenyl)-$N^3$-[6-[(*N*-cyanocarbamimidoyl)-amino]hexyl]imidodicarbonimidic diamide,

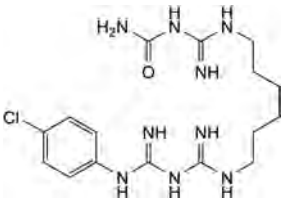

B. *N*-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]carbamimidoyl]urea,

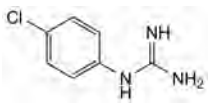

E. *N*-(4-chlorophenyl)guanidine,

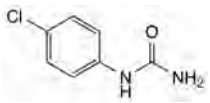

F. *N*-(4-chlorophenyl)urea,

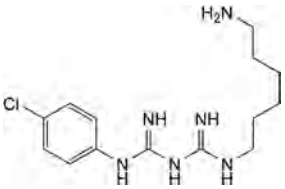

G. $N^1$-(6-aminohexyl)-$N^3$-(4-chlorophenyl)imido-dicarbonimidic diamide,

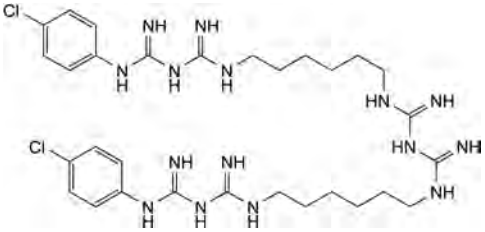

H. $N^1$,$N^{1\prime}$-[azanediylbis(carbonimidoylazanediylhexane-6,1-diyl)]bis[$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide],

I. unknown structure,

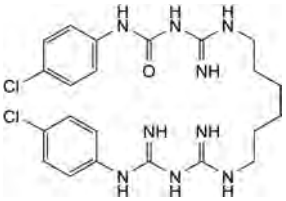

K. *N*-(4-chlorophenyl)-*N*′-[*N*-[6-[[*N*-[*N*-(4-chlorophenyl)-carbamimidoyl]carbamimidoyl]amino]hexyl]-carbamimidoyl]urea,

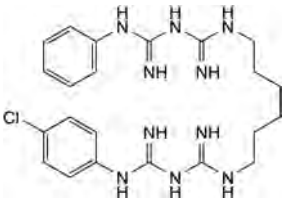

M. $N^1$-[6-[[*N*-[*N*-(4-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]-$N^3$-phenylimido-dicarbonimidic diamide,

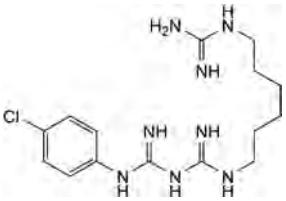

N. $N^1$-[6-(carbamimidoylamino)hexyl]-$N^3$-(4-chlorophenyl)imidodicarbonimidic diamide,

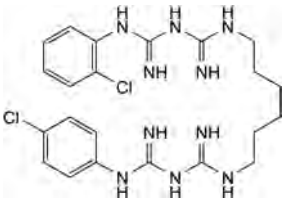

O. $N^1$-[6-[[*N*-[*N*-(2-chlorophenyl)carbamimidoyl]-carbamimidoyl]amino]hexyl]-$N^3$-(4-chlorophenyl)-imidodicarbonimidic diamide,

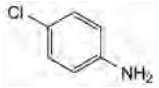

P. 4-chloroaniline.

**07/2014:2702**

# CHLORMADINONE ACETATE

## Chlormadinoni acetas

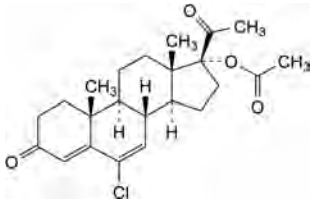

$C_{23}H_{29}ClO_4$ $M_r$ 404.9

[302-22-7]

DEFINITION

6-Chloro-3,20-dioxopregna-4,6-dien-17-yl acetate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetonitrile, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chlormadinone acetate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 14.0 to − 10.0 (dried substance).

Dissolve 0.200 g in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 20 mg of the substance to be examined in mobile phase B and dilute to 10.0 mL with mobile phase B.

*Test solution (b).* Dissolve 10.0 mg of the substance to be examined in mobile phase B and dilute to 20.0 mL with mobile phase B.

*Reference solution (a).* Dissolve 4 mg of *chlormadinone acetate for system suitability CRS* (containing impurities A, B, E and K) in mobile phase B and dilute to 2.0 mL with mobile phase B.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (c).* Dissolve 5.0 mg of *chlormadinone acetate impurity G CRS* in mobile phase B and dilute to 50.0 mL with mobile phase B. Dilute 1.0 mL of the solution to 50.0 mL with mobile phase B.

*Reference solution (d).* Dissolve 10.0 mg of *chlormadinone acetate CRS* in mobile phase B and dilute to 20.0 mL with mobile phase B.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 60 | 40 |
| 8 - 30 | 60 → 5 | 40 → 95 |
| 30 - 33 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 236 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *chlormadinone acetate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, E and K; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity G.

*Relative retention* with reference to chlormadinone acetate (retention time = about 15 min): impurity K = about 0.75; impurity G = about 0.80; impurity A = about 0.95; impurity E = about 1.04; impurity B = about 1.2.

*System suitability*:

- *signal-to-noise ratio*: minimum 35 for the principal peak in the chromatogram obtained with reference solution (b);
- *peak-to-valley ratio*: minimum 1.6, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to chlormadinone acetate in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity K by 1.7;
- for impurities E, B and K, use the concentration of chlormadinone acetate in reference solution (b);
- for impurities other than E, B and K, use the concentration of impurity G in reference solution (c).

*Limits*:

- *impurity B*: maximum 0.2 per cent;
- *impurities A, E, G, K*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (45:55 *V/V*).

*Injection*: test solution (b) and reference solution (d).

*Run time*: twice the retention time of chlormadinone acetate.

*Retention time*: chlormadinone acetate = about 12 min.

Calculate the percentage content of $C_{23}H_{29}ClO_4$ taking into account the assigned content of *chlormadinone acetate CRS*.

IMPURITIES

*Specified impurities: A, B, E, G, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, F, H, I, J, L.*

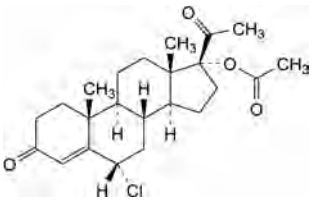

A. 6α-chloro-3,20-dioxopregn-4-en-17-yl acetate,

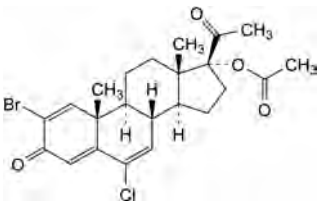

B. 2-bromo-6-chloro-3,20-dioxopregna-1,4,6-trien-17-yl acetate,

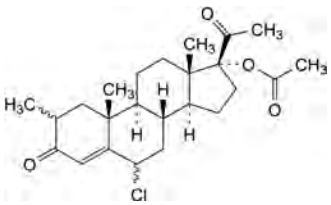

C. 6ξ-chloro-2ξ-methyl-3,20-dioxopregn-4-en-17-yl acetate,

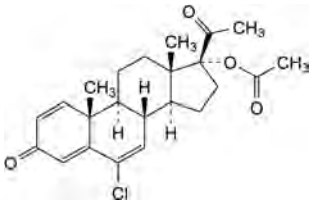

D. 6-chloro-3,20-dioxopregna-1,4,6-trien-17-yl acetate,

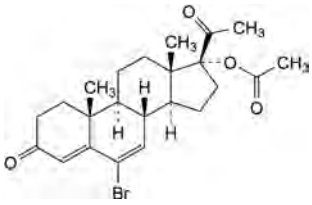

E. 6-bromo-3,20-dioxopregna-4,6-dien-17-yl acetate,

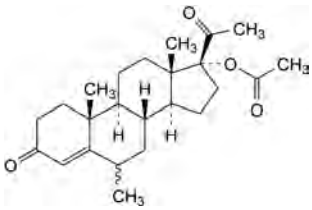

F. 6ξ-methyl-3,20-dioxopregn-4-en-17-yl acetate,

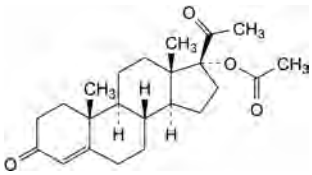

G. 3,20-dioxopregn-4-en-17-yl acetate,

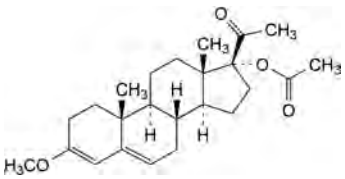

H. 3-methoxy-20-oxopregna-3,5-dien-17-yl acetate,

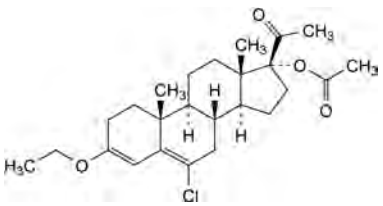

I. 6-chloro-3-ethoxy-20-oxopregna-3,5-dien-17-yl acetate,

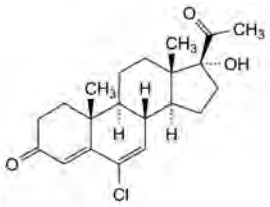

J. 6-chloro-17-hydroxypregna-4,6-diene-3,20-dione,

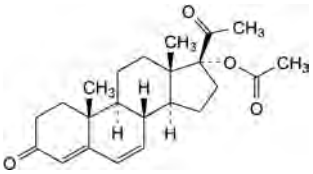

K. 3,20-dioxopregna-4,6-dien-17-yl acetate,

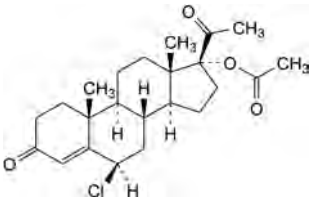

L. 6β-chloro-3,20-dioxopregn-4-en-17-yl acetate.

**01/2019:0382**
**corrected 10.0**

# CHLOROBUTANOL

## Chlorobutanolum

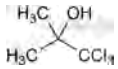

$C_4H_7Cl_3O$ $M_r$ 177.5
[57-15-8]

DEFINITION

1,1,1-Trichloro-2-methylpropan-2-ol.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, sublimes readily.

*Solubility*: slightly soluble in water, very soluble in ethanol (96 per cent), soluble in glycerol (85 per cent).

mp: about 95 °C (without previous drying).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *chlorobutanol CRS*.

B. Water (see Tests).

TESTS

**Solution S**. Dissolve 5 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Acidity**. To 4 mL of solution S add 15 mL of *ethanol (96 per cent) R* and 0.1 mL of *bromothymol blue solution R1*. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Impurities A and B**. Head-space gas chromatography (*2.2.28*).

*Solvent mixture*: *water R*, *dimethylformamide R* (40:60 *V/V*).

*Test solution.* Introduce 2.000 g of the substance to be examined into a vial, add 5.0 mL of the solvent mixture and close the vial immediately.

*Reference solution (a).* Dissolve 60.0 mg of *chloroform R* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well. Dilute 4.0 mL of the solution to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Reference solution (b).* Dissolve 0.500 g of *acetone R* (impurity B) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well.

*Reference solution (c).* Dilute 8.0 mL of reference solution (b) to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Reference solution (d).* Dissolve 50.0 mg of *2-propanol R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well. Mix 8.0 mL of the solution and 8.0 mL of reference solution (b), dilute to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *nitrogen R*.

*Flow rate*: 1.7 mL/min.

*Split ratio*: 1:3.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 90 °C;
- *equilibration time*: 20 min;
- *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 25 | 40 |
| | 25 - 37 | 40 → 220 |
| | 37 - 42 | 220 |
| Injection port | | 220 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to 2-propanol.

*Relative retention* with reference to impurity B (retention time = about 14 min): 2-propanol = about 1.1; impurity A = about 2.0.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurity B and 2-propanol.

*Calculation of contents*:

- for impurity A, use the concentration of impurity A in reference solution (a);
- for impurity B, use the concentration of impurity B in reference solution (c).

*Limits*:

- *impurity B*: maximum 0.10 per cent;
- *impurity A*: maximum 60 ppm.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dissolve 0.17 g in 5 mL of *ethanol (96 per cent) R* and dilute to 15 mL with *water R*. When preparing the standard, replace the 5 mL of *water R* by 5 mL of *ethanol (96 per cent) R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in 20 mL of *ethanol (96 per cent) R* in a 50 mL centrifuge tube. Add 10 mL of *dilute sodium hydroxide solution R*, cap tightly and heat in a water-bath for 10 min. Cool and transfer quantitatively to a titration vessel using 100 mL of *water R*. Add 20 mL of *dilute nitric acid R* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.92 mg of $C_4H_7Cl_3O$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

A. trichloromethane (chloroform),

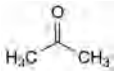

B. propan-2-one (acetone).

**01/2019:0383**
**corrected 10.0**

# CHLOROBUTANOL HEMIHYDRATE

## Chlorobutanolum hemihydricum

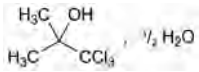

$C_4H_7Cl_3O,½H_2O$ $M_r$ 186.5
[6001-64-5]

DEFINITION

1,1,1-Trichloro-2-methylpropan-2-ol hemihydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, sublimes readily.

*Solubility*: slightly soluble in water, very soluble in ethanol (96 per cent), soluble in glycerol (85 per cent).

mp: about 78 °C (without previous drying).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *chlorobutanol hemihydrate CRS*.

B. Water (see Tests).

TESTS

**Solution S**. Dissolve 5 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Acidity**. To 4 mL of solution S add 15 mL of *ethanol (96 per cent) R* and 0.1 mL of *bromothymol blue solution R1*. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Impurities A and B**. Head-space gas chromatography (*2.2.28*).

*Solvent mixture*: *water R, dimethylformamide R* (40:60 *V/V*).

*Test solution*. Introduce 2.000 g of the substance to be examined into a vial, add 5.0 mL of the solvent mixture and close the vial immediately.

*Reference solution (a)*. Dissolve 60.0 mg of *chloroform R* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well. Dilute 4.0 mL of the solution to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Reference solution (b)*. Dissolve 0.500 g of *acetone R* (impurity B) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well.

*Reference solution (c)*. Dilute 8.0 mL of reference solution (b) to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Reference solution (d)*. Dissolve 50.0 mg of *2-propanol R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Shake well. Mix 8.0 mL of the solution and 8.0 mL of reference solution (b), dilute to 200.0 mL with the solvent mixture and transfer 5.0 mL to an injection vial.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *nitrogen R*.

*Flow rate*: 1.7 mL/min.

*Split ratio*: 1:3.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 90 °C;
- *equilibration time*: 20 min;
- *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 25 | 40 |
| | 25 - 37 | 40 → 220 |
| | 37 - 42 | 220 |
| Injection port | | 220 |
| Detector | | 230 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to 2-propanol.

*Relative retention* with reference to impurity B (retention time = about 14 min): 2-propanol = about 1.1; impurity A = about 2.0.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurity B and 2-propanol.

*Calculation of contents*:

- for impurity A, use the concentration of impurity A in reference solution (a);
- for impurity B, use the concentration of impurity B in reference solution (c).

*Limits*:

- *impurity B*: maximum 0.10 per cent;
- *impurity A*: maximum 60 ppm.

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 1 mL of solution S add 4 mL of *ethanol (96 per cent) R* and dilute to 15 mL with *water R*. When preparing the standard, replace the 5 mL of *water R* by 5 mL of *ethanol (96 per cent) R*.

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in 20 mL of *ethanol (96 per cent) R* in a 50 mL centrifuge tube. Add 10 mL of *dilute sodium hydroxide solution R*, cap tightly and heat in a water-bath for 10 min. Cool and transfer quantitatively to a titration vessel using 100 mL of *water R*. Add 20 mL of *dilute nitric acid R* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.92 mg of $C_4H_7Cl_3O$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

A. trichloromethane (chloroform),

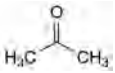

B. propan-2-one (acetone).

**01/2011:0384**
**corrected 10.0**

# CHLOROCRESOL

## Chlorocresolum

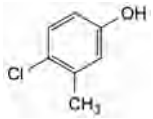

$C_7H_7ClO$ $M_r$ 142.6
[59-50-7]

DEFINITION

4-Chloro-3-methylphenol.

*Content*: 98.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or compacted crystalline masses supplied as pellets or colourless or white crystals.

*Solubility*: slightly soluble in water, very soluble in ethanol (96 per cent), freely soluble in fatty oils. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

A. Melting point (*2.2.14*): 64 °C to 67 °C.

B. To 0.1 g add 0.2 mL of *benzoyl chloride R* and 0.5 mL of *dilute sodium hydroxide solution R*. Shake vigorously until a white, crystalline precipitate is formed. Add 5 mL of *water R* and filter. The precipitate, recrystallised from 5 mL of *methanol R* and dried at 70 °C, melts (*2.2.14*) at 85 °C to 88 °C.

C. To 5 mL of solution S (see Tests) add 0.1 mL of *ferric chloride solution R1*. A bluish colour is produced.

TESTS

**Solution S**. To 3.0 g, finely powdered, add 60 mL of *carbon dioxide-free water R*, shake for 2 min and filter.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 1.25 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

**Acidity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R*. The solution is orange or red. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to produce a pure yellow colour.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 1.0 g of the substance to be examined in *acetone R* and dilute to 100 mL with the same solvent.

*Reference solution*. Dilute 1.0 mL of the test solution to 100.0 mL with *acetone R*. Dilute 5.0 mL of this solution to 100.0 mL with *acetone R*.

*Column*:

- *material*: glass;
- *size*: *l* = 1.80 m, Ø = 3-4 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 3-5 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 125 °C;
- *injection port*: 210 °C;
- *detector*: 230 °C.

*Detection*: flame ionisation.

*Run time*: 3 times the retention time of chlorocresol.

*Retention time*: chlorocresol = about 8 min.

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

**Non-volatile matter**: maximum 0.1 per cent.

Evaporate 2.0 g to dryness on a water-bath and dry the residue at 100-105 °C. The residue weighs not more than 2 mg.

ASSAY

In a ground-glass-stoppered flask, dissolve 70.0 mg in 30 mL of *glacial acetic acid R*. Add 25.0 mL of *0.0167 M potassium bromate*, 20 mL of a 150 g/L solution of *potassium bromide R* and 10 mL of *hydrochloric acid R*. Allow to stand protected from light for 15 min. Add 1 g of *potassium iodide R* and 100 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, shaking vigorously and using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.0167 M potassium bromate* is equivalent to 3.565 mg of $C_7H_7ClO$.

STORAGE

Protected from light.

**01/2017:0544**

# CHLOROQUINE PHOSPHATE

## Chloroquini phosphas

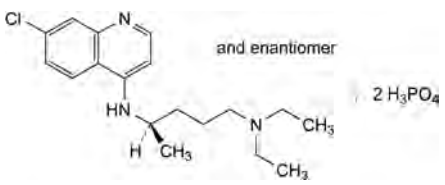


$C_{18}H_{32}ClN_3O_8P_2$ $M_r$ 515.9
[50-63-5]

DEFINITION

Chloroquine phosphate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of $N^4$-(7-chloroquinolin-4-yl)-$N^1$,$N^1$-diethylpentane-1,4-diamine bis(dihydrogen phosphate), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, very slightly soluble in alcohol and in methanol.

It exists in 2 forms, one of which melts at about 195 °C and the other at about 218 °C.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.100 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Examined between 210 nm and 370 nm (*2.2.25*), the solution shows absorption maxima at 220 nm, 235 nm, 256 nm, 329 nm and 342 nm. The specific absorbances at the maxima are respectively 600 to 660, 350 to 390, 300 to 330, 325 to 355 and 360 to 390.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with the base isolated from *chloroquine sulfate CRS*. Record the spectra using solutions prepared as follows: dissolve separately 0.1 g of the substance to be examined and 80 mg of the reference substance in 10 mL of *water R*, add 2 mL of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 20 mL, of *methylene chloride R*; combine the organic layers, wash with *water R*, dry over *anhydrous sodium sulfate R*, evaporate to dryness and dissolve the residues separately, each in 2 mL of *methylene chloride R*.

C. Dissolve 25 mg in 20 mL of *water R* and add 8 mL of *picric acid solution R1*. The precipitate, washed with *water R*, with *alcohol R* and finally with *methylene chloride R*, melts (*2.2.14*) at 206-209 °C.

D. Dissolve 0.1 g in 10 mL of *water R*, add 2 mL of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 20 mL, of *methylene chloride R*. The aqueous layer, acidified by the addition of *nitric acid R*, gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ or $GY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 3.8 to 4.3.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dilute 1 mL of the test solution to 100 mL with *water R*.

*Reference solution (b).* Dilute 5 mL of reference solution (a) to 10 mL with *water R*.

Apply to the plate 2 µL of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 40 volumes of *cyclohexane R* and 50 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.79 mg of $C_{18}H_{32}ClN_3O_8P_2$.

STORAGE

In an airtight container, protected from light.

**01/2017:0545**

# CHLOROQUINE SULFATE

## Chloroquini sulfas

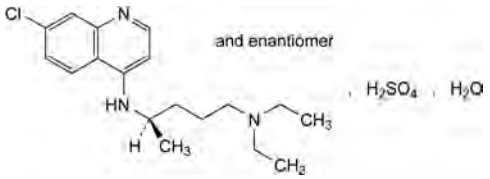


$C_{18}H_{28}ClN_3O_4S,H_2O$ $M_r$ 436.0

DEFINITION

Chloroquine sulfate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of $N^4$-(7-chloroquinolin-4-yl)-$N^1$,$N^1$-diethylpentane-1,4-diamine sulfate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in methanol, very slightly soluble in ethanol (96 per cent).

It melts at about 208 °C (instantaneous method).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.100 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Examined between 210 nm and 370 nm (*2.2.25*), the solution shows absorption maxima at 220 nm, 235 nm, 256 nm, 329 nm and 342 nm. The specific absorbances at the maxima are respectively 730 to 810, 430 to 470, 370 to 410, 400 to 440 and 430 to 470.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with the base isolated from *chloroquine sulfate CRS*. Record the spectra using solutions prepared as follows: dissolve separately 0.1 g of the substance to be examined and of the reference substance in 10 mL of *water R*, add 2 mL of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 20 mL, of *methylene chloride R*; combine the organic layers, wash with *water R*, dry over *anhydrous sodium sulfate R*, evaporate to dryness and dissolve the residues separately each in 2 mL of *methylene chloride R*.

C. Dissolve 25 mg in 20 mL of *water R* and add 8 mL of *picric acid solution R1*. The precipitate, washed with *water R*, with *ethanol (96 per cent) R* and finally with *ether R*, melts (*2.2.14*) at 206 °C to 209 °C.

D. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ or $GY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 4.0 to 5.0.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dilute 1 mL of the test solution to 100 mL with *water R*.

*Reference solution (b).* Dilute 5 mL of reference solution (a) to 10 mL with *water R*.

Apply separately to the plate 2 µL of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *diethylamine R*, 40 volumes of *cyclohexane R* and 50 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Water** (*2.5.12*): 3.0 per cent to 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.8 mg of $C_{18}H_{28}ClN_3O_4S$.

STORAGE

Store in an airtight container, protected from light.

**01/2017:0386**

# CHLORPHENAMINE MALEATE

## Chlorphenamini maleas

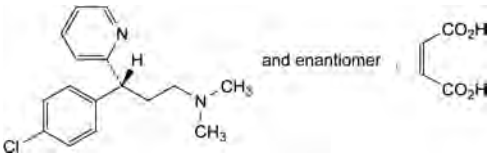


$C_{20}H_{23}ClN_2O_4$ $M_r$ 390.9

[113-92-8]

DEFINITION

(3*RS*)-3-(4-Chlorophenyl)-*N*,*N*-dimethyl-3-(pyridin-2-yl)propan-1-amine hydrogen (*Z*)-butenedioate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 130 °C to 135 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *chlorphenamine maleate CRS*.

C. Optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 0.5 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5 mg of *chlorphenamine impurity C CRS* in 5 mL of the test solution and dilute to 50.0 mL with the mobile phase. Dilute 2 mL of this solution to 20 mL with the mobile phase.

*Reference solution (d)*. Dissolve 5 mg of *2,2′-dipyridylamine R* (impurity B) in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (e)*. Dissolve the contents of a vial of *chlorphenamine impurity A CRS* in 2 mL of the test solution. Sonicate for 5 min.

*Column*:

– *size*: *l* = 0.30 m, Ø = 3.9 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 8.57 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of chlorphenamine.

*Relative retention* with reference to chlorphenamine (retention time = about 11 min): maleic acid = about 0.2; impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.9; impurity D = about 3.0.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity C and chlorphenamine.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity B = 1.4;

– *impurity A*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurities B, C, D*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.54 mg of $C_{20}H_{23}ClN_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

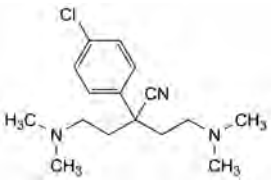


A. 2-(4-chlorophenyl)-4-(dimethylamino)-2-[2-(dimethylamino)ethyl]butanenitrile,

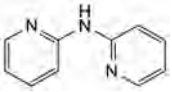


B. *N*-(pyridin-2-yl)pyridin-2-amine (2,2′-dipyridylamine),

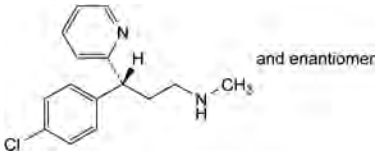


C. (3*RS*)-3-(4-chlorophenyl)-*N*-methyl-3-(pyridin-2-yl)propan-1-amine,

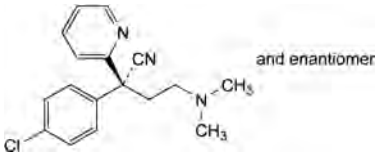


D. (2*RS*)-2-(4-chlorophenyl)-4-(dimethylamino)-2-(pyridin-2-yl)butanenitrile.



**01/2020:0475**

# CHLORPROMAZINE HYDROCHLORIDE

## Chlorpromazini hydrochloridum

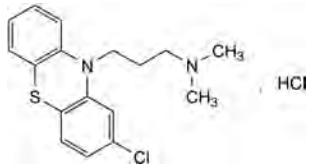


$C_{17}H_{20}Cl_2N_2S$ $M_r$ 355.3
[69-09-0]

DEFINITION

3-(2-Chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It decomposes on exposure to air and light.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*). *Prepare the solutions protected from bright light and measure the absorbances immediately.*

*Test solution.* Dissolve 50.0 mg in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 500.0 mL with the same solution. Dilute 5.0 mL of the solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 230-340 nm.

*Absorption maxima*: 254 nm and 306 nm.

*Specific absorbance at the absorption maximum at 254 nm*: 890 to 960.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: 60 g/L solutions in *methylene chloride R*.

*Comparison*: *chlorpromazine hydrochloride CRS*.

C. Identification of phenothiazines by thin-layer chromatography (*2.3.3*): use *chlorpromazine hydrochloride CRS* to prepare the reference solution.

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 3.5 to 4.5. *Carry out the test protected from light and use freshly prepared solutions.*

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Impurity F**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use and protect from light.*

*Solvent mixture*: *diethylamine R*, *methanol R* (5:95 *V/V*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *chlorpromazine impurity F CRS* in 2.0 mL of the solvent mixture.

*Reference solution (b).* Dilute 300 µL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 0.10 g of the substance to be examined in the solvent mixture, add 1.0 mL of reference solution (a) and dilute to 5.0 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetone R*, *diethylamine R*, *cyclohexane R* (10:10:80 *V/V/V*).

*Application*: 10 µL of the test solution and reference solutions (b) and (c).

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Retardation factors*: impurity F = about 0.5; chlorpromazine = about 0.6.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots due to impurity F and chlorpromazine.

*Limit*:

– *impurity F*: any spot due to impurity F is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.15 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 4 mg of *chlorpromazine impurity D CRS* in the mobile phase and dilute to 10 mL with the mobile phase. To 1 mL of the solution add 1 mL of the test solution and dilute to 100 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 4.0 mg of *chlorpromazine impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 4 mg of *promazine hydrochloride CRS* (impurity C) and 4.0 mg of *chlorpromazine impurity E CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 0.2 volumes of *thiodiethylene glycol R*, 50 volumes of *acetonitrile R* and 50 volumes of a 0.5 per cent *V/V* solution of *trifluoroacetic acid R* previously adjusted to pH 5.3 with *tetramethylethylenediamine R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of chlorpromazine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C and E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to chlorpromazine (retention time = about 8 min): impurity A = about 0.4; impurity B = about 0.5; impurity C = about 0.7; impurity D = about 0.9; impurity E = about 3.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity D and chlorpromazine.

*Limits*:

– *impurities B, C, D*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
– *impurity E*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: maximum 1.0 per cent;
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.1 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.53 mg of $C_{17}H_{20}Cl_2N_2S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

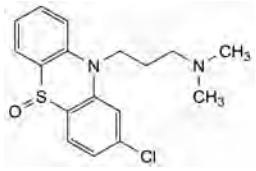

A. 3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine *S*-oxide (chlorpromazine sulfoxide),

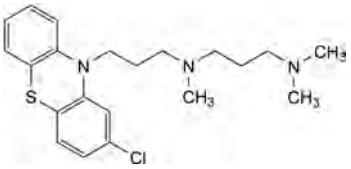

B. *N*-[3-(2-chloro-10*H*-phenothiazin-10-yl)propyl]-*N*,*N*′,*N*′-trimethylpropane-1,3-diamine,

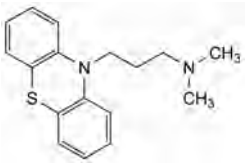

C. 3-(10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine (promazine),

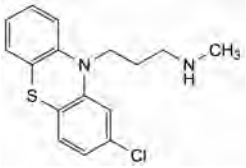

D. 3-(2-chloro-10*H*-phenothiazin-10-yl)-*N*-methylpropan-1-amine (desmethylchlorpromazine),

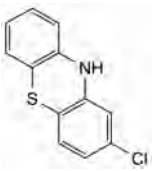

E. 2-chloro-10*H*-phenothiazine,

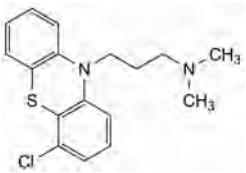

F. 3-(4-chloro-10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine.

**07/2019:0815**

# CHLORPROTHIXENE HYDROCHLORIDE

## Chlorprothixeni hydrochloridum

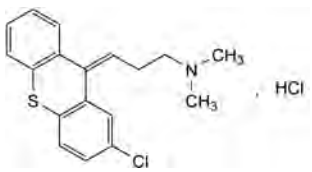

$C_{18}H_{19}Cl_2NS$ $M_r$ 352.3
[6469-93-8]

DEFINITION

(3*Z*)-3-(2-Chloro-9*H*-thioxanthen-9-ylidene)-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent), slightly soluble in methylene chloride.

mp: about 220 °C.

IDENTIFICATION

*First identification*: *A, E.*

*Second identification*: *B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 25 mg in 1 mL of *water R*, add 0.1 mL of *dilute sodium hydroxide solution R* and shake with 2 mL of *methylene chloride R*; separate the organic layer and wash with 0.5 mL of *water R*; evaporate the organic layer to dryness and dry the residue at 40-50 °C; examine the residue as a disc.

*Comparison*: repeat the operations using 25 mg of *chlorprothixene hydrochloride CRS*.

B. Dissolve 0.2 g in a mixture of 5 mL of *dioxan R* and 5 mL of a 1.5 g/L solution of *sodium nitrite R*. Add 0.8 mL of *nitric acid R*. After 10 min, add the solution to 20 mL of *water R*. Filter the precipate formed after 1 h. The filtrate is used immediately for identification test C. Dissolve the precipitate by warming in about 15 mL of *ethanol (96 per cent) R* and add the solution to 10 mL of *water R*. Filter and dry the precipitate at 100-105 °C for 2 h. The melting point (*2.2.14*) is 152 °C to 154 °C.

C. To 1 mL of the filtrate obtained in identification test B, add 0.2 mL of a suspension of 50 mg of *fast red B salt R* in 1 mL of *ethanol (96 per cent) R*. Add 1 mL of *0.5 M alcoholic potassium hydroxide*. A dark red colour is produced. Carry out a blank test.

D. Dissolve about 20 mg in 2 mL of *nitric acid R*. A red colour is produced. Add 5 mL of *water R* and examine in ultraviolet light at 365 nm. The solution shows green fluorescence.

E. Dissolve 20 mg in 2 mL of *water R*, acidify with *dilute nitric acid R* and allow to stand for 5 min. Centrifuge. The supernatant gives reaction (a) of chlorides (*2.3.1*) starting from 'add 0.4 mL of *silver nitrate solution R1*'.

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.4 to 5.2 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from bright light.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *chlorprothixene for system suitability CRS* (containing impurities C and F) in 1 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: solution containing 6.0 g/L of *potassium dihydrogen phosphate R*, 2.9 g/L of *sodium laurilsulfate R* and 9 g/L of *tetrabutylammonium bromide R* in a mixture of 50 volumes of *methanol R*, 400 volumes of *acetonitrile R* and 550 volumes of *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: for about 30 min with the mobile phase.

*Injection*: 20 µL.

*Run time*: twice the retention time of chlorprothixene.

*Identification of impurities*: use the chromatogram supplied with *chlorprothixene for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and F.

*Relative retention* with reference to chlorprothixene (retention time = about 10 min): impurity C = about 1.25; impurity F = about 1.33.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to chlorprothixene and impurity C.

*Limits*:

- *impurity F*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.23 mg of $C_{18}H_{19}Cl_2NS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

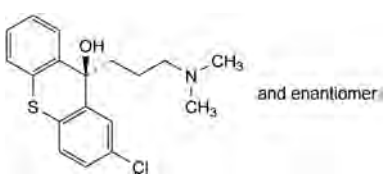


A. (3*RS*)-2-chloro-9-[3-(dimethylamino)propyl]-9*H*-thioxanthen-9-ol,

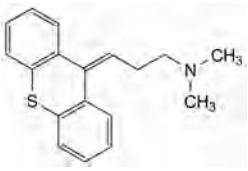

B. *N,N*-dimethyl-3-(9*H*-thioxanthen-9-ylidene)propan-1-amine,

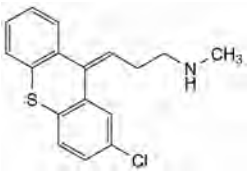

C. (3*Z*)-3-(2-chloro-9*H*-thioxanthen-9-ylidene)-*N*-methylpropan-1-amine,

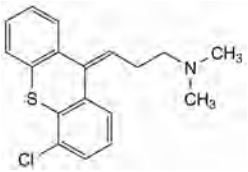

D. (3*Z*)-3-(4-chloro-9*H*-thioxanthen-9-ylidene)-*N,N*-dimethylpropan-1-amine,

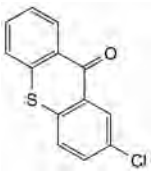

E. 2-chloro-9*H*-thioxanthen-9-one,

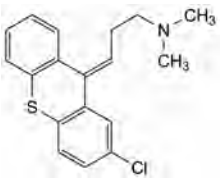

F. (3*E*)-3-(2-chloro-9*H*-thioxanthen-9-ylidene)-*N,N*-dimethylpropan-1-amine ((*E*)-chlorprothixene).

**01/2020:0546**

# CHLORTALIDONE

## Chlortalidonum

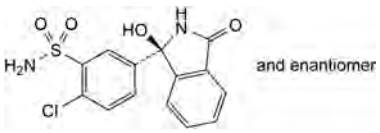


$C_{14}H_{11}ClN_2O_4S$ $M_r$ 338.8
[77-36-1]

DEFINITION

2-Chloro-5-[(1*RS*)-1-hydroxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulfonamide.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: very slightly soluble in water, soluble in acetone and in methanol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *chlortalidone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Acidity**. Dissolve 1.0 g with heating in a mixture of 25 mL of *acetone R* and 25 mL of *carbon dioxide-free water R*. Cool. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Not more than 0.75 mL of *0.1 M sodium hydroxide* is required.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Mix 2 volumes of a 2 g/L solution of *sodium hydroxide R*, 48 volumes of mobile phase B and 50 volumes of mobile phase A.

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 10.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *chlortalidone for system suitability CRS* (containing impurities B and G) in 1 mL of the solvent mixture. Dilute 400 µL of the solution to 2 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 50.0 mg of *chlortalidone CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.32 g of *ammonium phosphate R* in about 900 mL of *water for chromatography R* and adjust to pH 5.5 with *dilute phosphoric acid R*; dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16 | 65 | 35 |
| 16 - 21 | 65 → 50 | 35 → 50 |
| 21 - 35 | 50 | 50 |
| 35 - 45 | 50 → 65 | 50 → 35 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *chlortalidone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and G.

*Relative retention* with reference to chlortalidone (retention time = about 7 min): impurity B = about 0.7; impurity G = about 6.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and chlortalidone.

*Limits*:

- *impurity B*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *impurity G*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 12 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 350 ppm.

Triturate 0.3 g finely, add 30 mL of *water R*, shake for 5 min and filter. 15 mL of the filtrate complies with the test. Prepare the standard using 10 mL of *chloride standard solution (5 ppm Cl) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{14}H_{11}ClN_2O_4S$ taking into account the assigned content of *chlortalidone CRS*.

IMPURITIES

*Specified impurities: B, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F, H, I.*

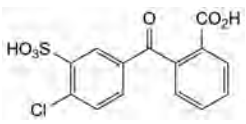

A. 2-(4-chloro-3-sulfobenzoyl)benzoic acid,

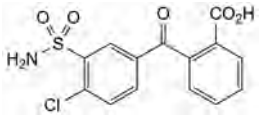

B. 2-(4-chloro-3-sulfamoylbenzoyl)benzoic acid,

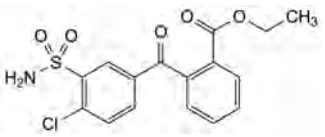

C. ethyl 2-(4-chloro-3-sulfamoylbenzoyl)benzoate,

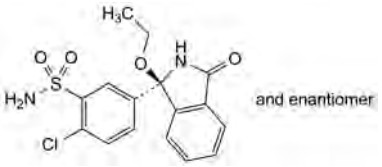


D. 2-chloro-5-[(1*RS*)-1-ethoxy-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulfonamide,

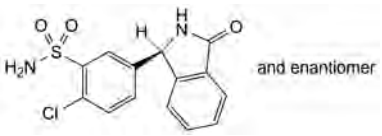


E. 2-chloro-5-[(1*RS*)-3-oxo-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulfonamide,

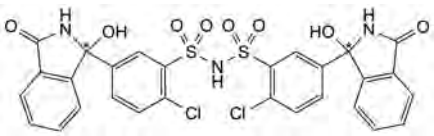

F. (1$^1\Xi$,7$^1\Xi$)-2$^4$,6$^6$-dichloro-1$^1$,7$^1$-dihydroxy-1$^2$,1$^3$,7$^2$,7$^3$-tetrahydro-1$^1$*H*,7$^1$*H*-3λ$^6$,5λ$^6$-dithia-4-aza-1,7(1)-diisoindola-2,6(1,3)-dibenzaheptaphan-1$^3$,3,3,5,5,7$^3$-hexone,

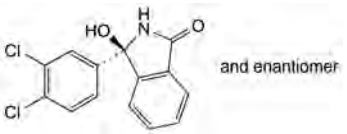


G. (3*RS*)-3-(3,4-dichlorophenyl)-3-hydroxy-2,3-dihydro-1*H*-isoindol-1-one,

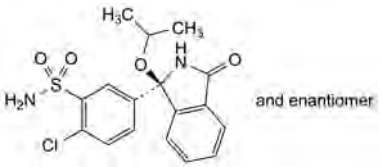


H. 2-chloro-5-[(1*RS*)-3-oxo-1-[(propan-2-yl)oxy]-2,3-dihydro-1*H*-isoindol-1-yl]benzenesulfonamide,

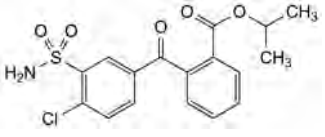

I. propan-2-yl 2-(4-chloro-3-sulfamoylbenzoyl)benzoate.

**01/2017:0173**
**corrected 10.0**

# CHLORTETRACYCLINE HYDROCHLORIDE

## Chlortetracyclini hydrochloridum

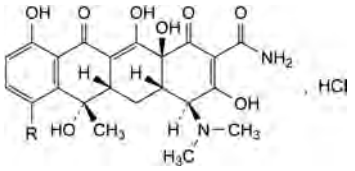

| Compound | R | Molecular formula | $M_r$ |
|---|---|---|---|
| Chlortetracycline hydrochloride | Cl | $C_{22}H_{24}Cl_2N_2O_8$ | 515.3 |
| Tetracycline hydrochloride | H | $C_{22}H_{25}ClN_2O_8$ | 480.9 |

Chlortetracycline hydrochloride: [64-72-2]
Tetracycline hydrochloride: [64-75-5]

DEFINITION

Mixture of antibiotics, the main component being the hydrochloride of (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (chlortetracycline hydrochloride), a substance produced by the growth of certain strains of *Streptomyces aureofaciens* or obtained by any other means.

*Content*:

– *chlortetracycline hydrochloride* ($C_{22}H_{24}Cl_2N_2O_8$): minimum 89.5 per cent (anhydrous substance);
– *tetracycline hydrochloride* ($C_{22}H_{25}ClN_2O_8$): maximum 6.0 per cent (anhydrous substance);
– *sum of the contents of chlortetracycline hydrochloride and tetracycline hydrochloride*: 94.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow powder.

*Solubility*: slightly soluble in water and in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, C.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *chlortetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *chlortetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *doxycycline R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A deep blue colour develops and becomes bluish-green. Add the solution to 2.5 mL of *water R*. The colour becomes brownish.

C. It gives reaction (a) of chlorides (*2.3.1*).

D. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 2.3 to 3.3.

Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*, heating slightly.

**Specific optical rotation** (*2.2.7*): – 250 to – 235 (anhydrous substance).

Dissolve 0.125 g in *water R* and dilute to 50.0 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.40 at 460 nm.

Dissolve 0.125 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Reference solution (a).* Dissolve 25.0 mg of *chlortetracycline hydrochloride CRS* in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with mobile phase B.

*Reference solution (d).* Dissolve 5 mg of *chlortetracycline for system suitability CRS* (containing impurities A, B, D, E, G, H, J, K and L) in mobile phase B and dilute to 5 mL with mobile phase B.

*Reference solution (e).* Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in mobile phase B and dilute to 25.0 mL with mobile phase B. Dilute 5.0 mL of this solution to 100.0 mL with mobile phase B.

*Column*:

– *size*: *l* = 0.075 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (3.5 µm);
– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: to 725 mL of *water R* add 50 mL of *perchloric acid solution R*, shake and add 225 mL of *dimethyl sulfoxide R*;
– *mobile phase B*: to 250 mL of *water R* add 50 mL of *perchloric acid solution R*, shake and add 700 mL of *dimethyl sulfoxide R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 46 | 100 → 0 | 0 → 100 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *chlortetracycline for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, D, E, G, H, J, K and L.

*Relative retention* with reference to chlortetracycline (retention time = about 26 min): impurity D = about 0.5; tetracycline = about 0.6; impurity E = about 0.7; impurity B = about 0.8, impurity A = about 0.86; impurity G = about 0.9; impurity H = about 1.1; impurity J = about 1.4, impurity K = about 1.67; impurity L = about 1.71.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to tetracycline and impurity E; minimum 1.5 between the peaks due to impurities A and G; minimum 1.5 between the peaks due to impurities K and L; if necessary, adjust the concentration of dimethyl sulfoxide in mobile phase A.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 1.4; impurity J = 0.3; impurity K = 0.4; impurity L = 0.4;
- *impurity A*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent);
- *impurities B, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity J*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurities D, G, H, L*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity K*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solutions (a) and (e).

Calculate the percentage content of $C_{22}H_{24}Cl_2N_2O_8$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *chlortetracycline hydrochloride CRS*. Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$ using the chromatogram obtained with reference solution (e) and taking into account the assigned content of *tetracycline hydrochloride CRS*.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, D, E, G, H, J, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, F, I.*

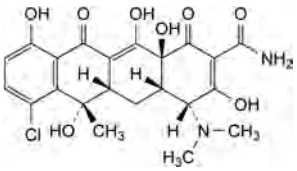

A. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epichlortetracycline),

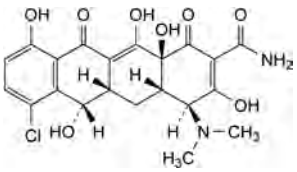

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (demeclocycline),

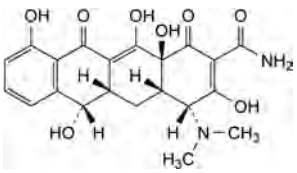

C. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidemethyltetracycline),

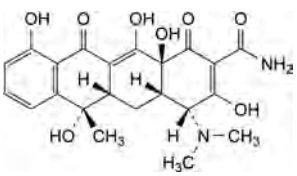

D. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epitetracycline),

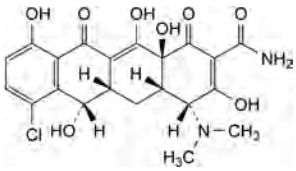

E. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidemethylchlortetracycline),

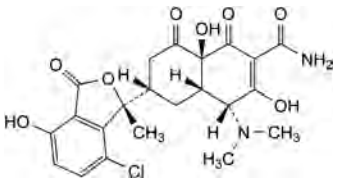

F. (4*R*,4a*S*,6*S*,8a*S*)-6-[(1*R*)-7-chloro-4-hydroxy-1-methyl-3-oxo-1,3-dihydro-2-benzofuran-1-yl]-4-(dimethylamino)-3,8a-dihydroxy-1,8-dioxo-1,4,4a,5,6,7,8,8a-octahydronaphthalene-2-carboxamide (4-epiisochlortetracycline),

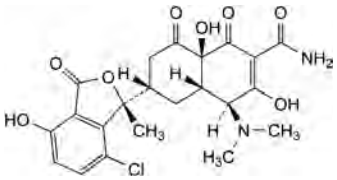

G. (4*S*,4a*S*,6*S*,8a*S*)-6-[(1*R*)-7-chloro-4-hydroxy-1-methyl-3-oxo-1,3-dihydro-2-benzofuran-1-yl]-4-(dimethylamino)-3,8a-dihydroxy-1,8-dioxo-1,4,4a,5,6,7,8,8a-octahydronaphthalene-2-carboxamide (isochlortetracycline),

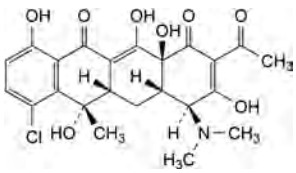

H. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-acetyl-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarboxamidochlortetracycline),

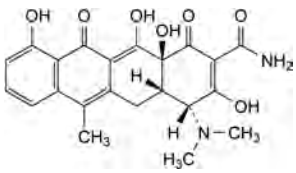

I. (4*R*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,11,12a-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline),

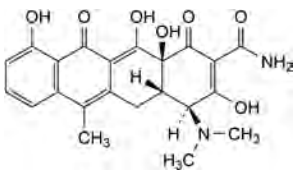

J. (4*S*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,11,12a-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

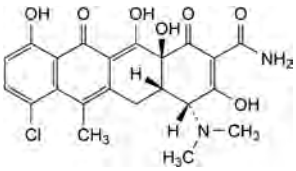

K. (4*R*,4a*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,11,12a-hexahydrotetracene-2-carboxamide (4-epianhydrochlortetracycline),

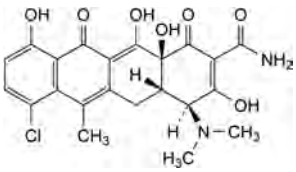

L. (4*S*,4a*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,11,12a-hexahydrotetracene-2-carboxamide (anhydrochlortetracycline).

**01/2013:0072**

# CHOLECALCIFEROL

## Cholecalciferolum

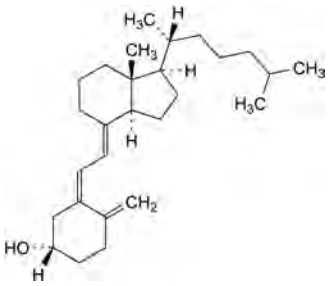

$C_{27}H_{44}O$ $M_r$ 384.6
[67-97-0]

DEFINITION

(5*Z*,7*E*)-9,10-Secocholesta-5,7,10(19)-trien-3β-ol.

*Content*: 97.0 per cent to 102.0 per cent.

A reversible isomerisation to pre-cholecalciferol takes place in solution, depending on temperature and time. The activity is due to both compounds (see Assay).

1 mg of cholecalciferol is equivalent to 40 000 IU of antirachitic activity (vitamin D) in rats.

CHARACTERS

*Appearance*: white or almost white crystals.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in trimethylpentane and in fatty oils.

It is sensitive to air, heat and light. Solutions in solvents without an antioxidant are unstable and are to be used immediately.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cholecalciferol CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 105 to + 112, determined within 30 min of preparing the solution.

Dissolve 0.200 g rapidly in *aldehyde-free alcohol R* without heating and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, avoiding exposure to actinic light and air.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *trimethylpentane R* without heating and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *cholecalciferol CRS* in *trimethylpentane R* without heating and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of *cholecalciferol for system suitability CRS* (containing impurity A) to 5.0 mL with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool (formation of pre-cholecalciferol).

*Reference solution (c).* Dilute 10.0 mL of reference solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *pentanol R*, *hexane R* (0.3:99.7 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of cholecalciferol.

*Relative retention* with reference to cholecalciferol (retention time = about 19 min): pre-cholecalciferol = about 0.5; impurity A = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to pre-cholecalciferol and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peak due to pre-cholecalciferol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{44}O$ taking into account the assigned content of *cholecalciferol CRS* and, if necessary, the peak due to pre-cholecalciferol.

STORAGE

Under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E*.

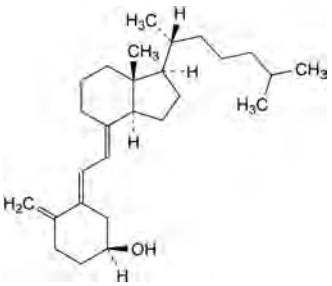

A. (5*E*,7*E*)-9,10-secocholesta-5,7,10(19)-trien-3β-ol (*trans*-cholecalciferol, *trans*-vitamin $D_3$),

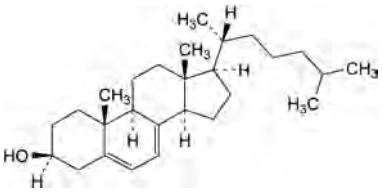

B. cholesta-5,7-dien-3β-ol (7,8-didehydrocholesterol, provitamin $D_3$),

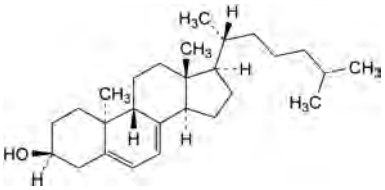

C. 9β,10α-cholesta-5,7-dien-3β-ol (lumisterol$_3$),

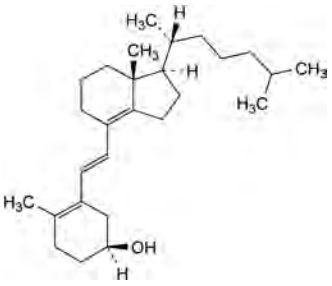

D. (6*E*)-9,10-secocholesta-5(10),6,8(14)-trien-3β-ol (iso-tachysterol$_3$),

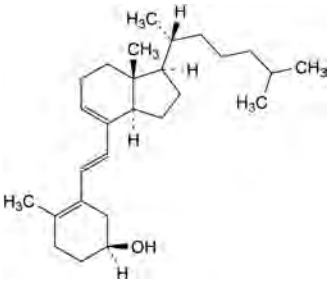

E. (6*E*)-9,10-secocholesta-5(10),6,8-trien-3β-ol (tachysterol$_3$).

**01/2019:0575**

# CHOLECALCIFEROL CONCENTRATE (OILY FORM)

## Cholecalciferolum densatum oleosum

DEFINITION

Solution of *Cholecalciferol (0072)* in a suitable vegetable fatty oil, authorised by the competent authority.

*Content*: 90.0 per cent to 110.0 per cent of the cholecalciferol content stated on the label, which is not less than 500 000 IU/g.

It may contain suitable stabilisers such as antioxidants.

CHARACTERS

*Appearance*: clear, yellow liquid.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol, miscible with solvents of fats.

Partial solidification may occur, depending on the temperature.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Prepare a solution in *cyclohexane R* containing the equivalent of about 400 IU/mL.

*Spectral range*: 250-300 nm.

*Absorption maximum*: at 267 nm.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

Dissolve 5.0 g in 25 mL of the prescribed mixture of solvents.

**Peroxide value** (*2.5.5, Method A*): maximum 20.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 400 000 IU, in 10.0 mL of *toluene R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *cholecalciferol CRS* without heating in 10.0 mL of *toluene R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of *cholecalciferol for system suitability CRS* to 5.0 mL with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

*Reference solution (c).* Dissolve 0.10 g of *cholecalciferol CRS* without heating in *toluene R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Dilute 5.0 mL of reference solution (c) to 50.0 mL with the mobile phase. Keep the solution in iced water.

*Reference solution (e).* Place 5.0 mL of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser protected from light and under *nitrogen R* for 45 min. Cool and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *pentanol R*, *hexane R* (3:997 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: the chosen volume of each solution (the same volume for reference solution (a) and for the test solution); automatic injection device or sample loop recommended.

*Relative retention* with reference to cholecalciferol (retention time = about 19 min): pre-cholecalciferol = about 0.4; *trans*-cholecalciferol = about 0.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.0 between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol; if necessary adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution;
– *repeatability*: maximum relative standard deviation of 1.0 per cent for the peak due to cholecalciferol after 6 injections.

Calculate the conversion factor (*f*) using the following expression:

$$\frac{K - L}{M}$$

$K$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d);

$L$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e);

$M$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Calculate the content of cholecalciferol in International Units per gram using the following expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + \left(f \times S_p\right)}{S'_D} \times 40\,000 \times 1000$$

$m$ = mass of the preparation to be examined in the test solution, in milligrams;

$m'$ = mass of *cholecalciferol CRS* in reference solution (a), in milligrams;

$V$ = volume of the test solution (100 mL);

$V'$ = volume of reference solution (a) (100 mL);

$S_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution;

$S'_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a);

$S_p$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution;

$f$ = conversion factor.

STORAGE

In an airtight, well-filled container, protected from light. The contents of an opened container are to be used as soon as possible; any unused part is to be protected by an atmosphere of nitrogen.

LABELLING

The label states:

- the number of International Units per gram;
- the method of restoring the solution if partial solidification occurs.

**01/2017:0574**

# CHOLECALCIFEROL CONCENTRATE (POWDER FORM)

## Cholecalciferoli pulvis

DEFINITION

Powder concentrate obtained by dispersing an oily solution of *Cholecalciferol (0072)* in an appropriate matrix, which is usually based on a combination of gelatin and carbohydrates of suitable quality, authorised by the competent authority.

*Content*: 90.0 per cent to 110.0 per cent of the cholecalciferol content stated on the label, which is not less than 100 000 IU/g.

It may contain suitable stabilisers such as antioxidants.

CHARACTERS

*Appearance*: white or yellowish-white, small particles.

*Solubility*: practically insoluble, swells, or forms a dispersion in water, depending on the formulation.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Place 10.0 mL of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 0.4 mL of *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R*.

*Reference solution (a).* Dissolve 10 mg of *cholecalciferol CRS* in *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R* and dilute to 4 mL with the same solution.

*Reference solution (b).* Dissolve 10 mg of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R* and dilute to 4 mL with the same solution.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: a 0.1 g/L solution of *butylhydroxytoluene R* in a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R.*

*Application*: 20 µL.

*Development*: immediately, protected from light, over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *sulfuric acid R.*

*Results*: the chromatogram obtained with the test solution shows immediately a bright yellow principal spot, which rapidly becomes orange-brown, then gradually greenish-grey, remaining so for 10 min. This spot is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows immediately at the same level an orange principal spot, which gradually becomes reddish-brown and remains so for 10 min.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Place 5.0 mL of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 50.0 mL of *cyclohexane R*.

*Spectral range*: 250-300 nm.

*Absorption maximum*: at 265 nm.

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce into a saponification flask a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 100 000 IU. Add 5 mL of *water R*, 20 mL of *anhydrous ethanol R*, 1 mL of *sodium ascorbate solution R* and 3 mL of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R*. Heat in a water-bath under a reflux condenser for 30 min. Cool rapidly under running water. Transfer the liquid to a separating funnel with the aid of 2 quantities, each of 15 mL, of *water R*, 1 quantity of 10 mL of *ethanol (96 per cent) R* and 2 quantities, each of 50 mL, of *pentane R*. Shake vigorously for 30 s. Allow to stand until the 2 layers are clear. Transfer the lower aqueous-alcoholic layer to a 2nd separating funnel and shake with a mixture of 10 mL of *ethanol (96 per cent) R* and 50 mL of *pentane R*. After separation, transfer the aqueous-alcoholic layer to a 3rd separating funnel and the pentane layer to the 1st separating funnel, washing the 2nd separating funnel with 2 quantities, each of 10 mL, of *pentane R* and adding the washings to the 1st separating funnel. Shake the aqueous-alcoholic layer with 50 mL of *pentane R* and add the pentane layer to the 1st funnel. Wash the pentane layer with 2 quantities, each of 50 mL, of a freshly prepared 30 g/L solution of *potassium hydroxide R* in *ethanol (10 per cent V/V) R*, shaking vigorously, then wash with successive quantities, each of 50 mL, of *water R* until the washings have an approximate pH of 7 to 8, using a *pH indicator strip R*. Transfer the washed pentane extract to a ground-glass-stoppered flask. Evaporate the contents of the flask to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 5.0 mL of *toluene R* and add 20.0 mL of the mobile phase to obtain a solution containing about 4000 IU/mL.

*Reference solution (a).* Dissolve 10.0 mg of *cholecalciferol CRS*, without heating, in 10.0 mL of *toluene R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of *cholecalciferol for system suitability CRS* to 5.0 mL with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

*Reference solution (c).* Dissolve 0.10 g of *cholecalciferol CRS*, without heating, in *toluene R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Dilute 5.0 mL of reference solution (c) to 50.0 mL with the mobile phase. Keep the solution in iced water.

*Reference solution (e).* Place 5.0 mL of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace the air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser, protected from light and under *nitrogen R*, for 45 min. Cool and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *pentanol R*, *hexane R* (3:997 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: the chosen volume of each solution (the same volume for reference solution (a) and for the test solution); automatic injection device or sample loop recommended.

*Relative retention* with reference to cholecalciferol: pre-cholecalciferol = about 0.4; *trans*-cholecalciferol = about 0.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.0 between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol; if necessary, adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution;

– *repeatability*: maximum relative standard deviation of 1.0 per cent for the peak due to cholecalciferol after 6 injections.

Calculate the conversion factor (*f*) using the following expression:

$$\frac{K - L}{M}$$

$K$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d);

$L$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e);

$M$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Calculate the content of cholecalciferol in International Units per gram using the following expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + (f \times S_p)}{S'_D} \times 40\,000 \times 1000$$

$m$ = mass of the preparation to be examined in the test solution, in milligrams;

$m'$ = mass of *cholecalciferol CRS* in reference solution (a), in milligrams;

$V$ = volume of the test solution (25 mL);

$V'$ = volume of reference solution (a) (100 mL);

$S_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution;

$S'_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a);

$S_p$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution;

$f$ = conversion factor.

STORAGE

In an airtight, well-filled container, protected from light. The contents of an opened container are to be used as soon as possible; any unused part is to be protected by an atmosphere of nitrogen.

LABELLING

The label states the number of International Units per gram.

**01/2017:0598**

# CHOLECALCIFEROL CONCENTRATE (WATER-DISPERSIBLE FORM)

## Cholecalciferolum in aqua dispergibile

DEFINITION

Solution of *Cholecalciferol (0072)* in a suitable vegetable fatty oil, authorised by the competent authority, to which suitable solubilisers have been added.

*Content*: 90.0 per cent to 115.0 per cent of the cholecalciferol content stated on the label, which is not less than 100 000 IU/g.

It may contain suitable stabilisers such as antioxidants.

CHARACTERS

*Appearance*: slightly yellowish liquid of variable opalescence and viscosity.

Highly concentrated solutions may become cloudy at low temperatures or form a gel at room temperature.

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: A, B, D.*

A. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Place 10.0 mL of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 0.4 mL of *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R*.

*Reference solution (a).* Dissolve 10 mg of *cholecalciferol CRS* in *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R* and dilute to 4 mL with the same solution.

*Reference solution (b).* Dissolve 10 mg of *ergocalciferol CRS* in *ethylene chloride R* containing 10 g/L of *squalane R* and 0.1 g/L of *butylhydroxytoluene R* and dilute to 4 mL with the same solution.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: a 0.1 g/L solution of *butylhydroxytoluene R* in a mixture of equal volumes of *cyclohexane R* and *peroxide-free ether R.*

*Application*: 20 µL.

*Development*: immediately, protected from light, over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *sulfuric acid R.*

*Results*: the chromatogram obtained with the test solution shows immediately a bright yellow principal spot, which rapidly becomes orange-brown, then gradually greenish-grey, remaining so for 10 min. This spot is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The chromatogram obtained with reference solution (b) shows

immediately at the same level an orange principal spot, which gradually becomes reddish-brown and remains so for 10 min.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Place 5.0 mL of the test solution prepared for the assay in a suitable flask and evaporate to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 50.0 mL of *cyclohexane R*.

*Spectral range*: 250-300 nm.

*Absorption maximum*: at 265 nm.

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

D. Mix about 1 g with 10 mL of *water R* previously warmed to 50 °C, and cool to 20 °C. Immediately after cooling, a uniform, slightly opalescent and slightly yellow dispersion is obtained.

TESTS

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

Liquid chromatography (*2.2.29*).

*Test solution.* Introduce into a saponification flask a quantity of the preparation to be examined, weighed with an accuracy of 0.1 per cent, equivalent to about 100 000 IU. Add 5 mL of *water R*, 20 mL of *anhydrous ethanol R*, 1 mL of *sodium ascorbate solution R* and 3 mL of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R*. Heat in a water-bath under a reflux condenser for 30 min. Cool rapidly under running water. Transfer the liquid to a separating funnel with the aid of 2 quantities, each of 15 mL, of *water R*, 1 quantity of 10 mL of *ethanol (96 per cent) R* and 2 quantities, each of 50 mL, of *pentane R*. Shake vigorously for 30 s. Allow to stand until the 2 layers are clear. Transfer the aqueous-alcoholic layer to a 2nd separating funnel and shake with a mixture of 10 mL of *ethanol (96 per cent) R* and 50 mL of *pentane R*. After separation, transfer the aqueous-alcoholic layer to a 3rd separating funnel and the pentane layer to the 1st separating funnel, washing the 2nd separating funnel with 2 quantities, each of 10 mL, of *pentane R* and adding the washings to the 1st separating funnel. Shake the aqueous-alcoholic layer with 50 mL of *pentane R* and add the pentane layer to the 1st funnel. Wash the pentane layer with 2 quantities, each of 50 mL, of a freshly prepared 30 g/L solution of *potassium hydroxide R* in *ethanol (10 per cent V/V) R*, shaking vigorously, and then wash with successive quantities, each of 50 mL, of *water R* until the washings have an approximate pH of 7 to 8, using a *pH indicator strip R*. Transfer the washed pentane extract to a ground-glass-stoppered flask. Evaporate the contents of the flask to dryness under reduced pressure by swirling in a water-bath at 40 °C. Cool under running water and restore atmospheric pressure with *nitrogen R*. Dissolve the residue immediately in 5.0 mL of *toluene R* and add 20.0 mL of the mobile phase to obtain a solution containing about 4000 IU/mL.

*Reference solution (a).* Dissolve 10.0 mg of *cholecalciferol CRS*, without heating, in 10.0 mL of *toluene R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of *cholecalciferol for system suitability CRS* to 5.0 mL with the mobile phase. Heat in a water-bath at 90 °C under a reflux condenser for 45 min and cool.

*Reference solution (c).* Dissolve 0.10 g of *cholecalciferol CRS*, without heating, in *toluene R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Dilute 5.0 mL of reference solution (c) to 50.0 mL with the mobile phase. Keep the solution in iced water.

*Reference solution (e).* Place 5.0 mL of reference solution (c) in a volumetric flask, add about 10 mg of *butylhydroxytoluene R* and displace the air from the flask with *nitrogen R*. Heat in a water-bath at 90 °C under a reflux condenser, protected from light and under *nitrogen R*, for 45 min. Cool and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *pentanol R*, *hexane R* (3:997 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: the chosen volume of each solution (the same volume for reference solution (a) and for the test solution); automatic injection device or sample loop recommended.

*Relative retention* with reference to cholecalciferol: pre-cholecalciferol = about 0.4; *trans*-cholecalciferol = about 0.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.0 between the peaks due to pre-cholecalciferol and *trans*-cholecalciferol; if necessary, adjust the proportions of the constituents and the flow rate of the mobile phase to obtain this resolution;
– *repeatability*: maximum relative standard deviation of 1.0 per cent for the peak due to cholecalciferol after 6 injections.

Calculate the conversion factor (*f*) using the following expression:

$$\frac{K - L}{M}$$

$K$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (d);

$L$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (e);

$M$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with reference solution (e).

The value of *f* determined in duplicate on different days may be used during the entire procedure.

Calculate the content of cholecalciferol in International Units per gram using the following expression:

$$\frac{m'}{V'} \times \frac{V}{m} \times \frac{S_D + (f \times S_p)}{S'_D} \times 40\,000 \times 1000$$

$m$ = mass of the preparation to be examined in the test solution, in milligrams;

$m'$ = mass of *cholecalciferol CRS* in reference solution (a), in milligrams;

$V$ = volume of the test solution (25 mL);

$V'$ = volume of reference solution (a) (100 mL);

$S_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with the test solution;

$S'_D$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (a);

$S_p$ = area (or height) of the peak due to pre-cholecalciferol in the chromatogram obtained with the test solution;

$f$ = conversion factor.

STORAGE

In an airtight, well-filled container, protected from light, at the temperature stated on the label.

The contents of an opened container are to be used as soon as possible; any unused part is to be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram;
- the storage temperature.

**07/2014:0993**
**corrected 10.0**

# CHOLESTEROL

## Cholesterolum

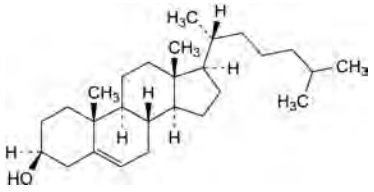

$C_{27}H_{46}O$ $M_r$ 386.7
[57-88-5]

DEFINITION

Cholest-5-en-3β-ol.

*Content*:

- *cholesterol*: minimum 95.0 per cent (dried substance);
- *total sterols*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone and in ethanol (96 per cent).

It is sensitive to light.

IDENTIFICATION

A. Melting point (*2.2.14*): 147 °C to 150 °C.

B. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10 mg of the substance to be examined in *ethylene chloride R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *cholesterol CRS* in *ethylene chloride R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ethyl acetate R, toluene R* (33:66 *V/V*).

*Application*: 20 µL.

*Development*: immediately, protected from light, over a path of 15 cm.

*Drying*: in air.

*Detection*: spray 3 times with *antimony trichloride solution R*; examine within 3-4 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 5 mg in 2 mL of *methylene chloride R*. Add 1 mL of *acetic anhydride R*, 0.01 mL of *sulfuric acid R* and shake. A pink colour is produced which rapidly changes to red, then to blue and finally to brilliant green.

TESTS

**Solubility in ethanol (96 per cent)**. In a stoppered flask, dissolve 0.5 g in 50 mL of *ethanol (96 per cent) R* at 50 °C. Allow to stand for 2 h. No deposit or turbidity is formed.

**Acidity**. Dissolve 1.0 g in 10 mL of *ether R*, add 10.0 mL of *0.1 M sodium hydroxide* and shake for about 1 min. Heat gently to eliminate ether and then boil for 5 min. Cool, add 10 mL of *water R* and 0.1 mL of *phenolphthalein solution R* as indicator and titrate with *0.1 M hydrochloric acid* until the pink colour just disappears, stirring the solution vigorously throughout the titration. Carry out a blank titration. The difference between the volumes of *0.1 M hydrochloric acid* required to change the colour of the indicator in the blank and in the test is not more than 0.3 mL.

**Loss on drying** (*2.2.32*): maximum 0.3 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.100 g of *pregnenolone isobutyrate CRS* in *heptane R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the internal standard solution and dilute to 25.0 mL with the same solution.

*Reference solution.* Dissolve 25.0 mg of *cholesterol CRS* in the internal standard solution and dilute to 25.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 2 mL/min.

*Split ratio*: 1:25.

*Temperature*:

- *column*: 275 °C;
- *injection port*: 285 °C;
- *detector*: 300 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*System suitability*: reference solution:

- *resolution*: minimum 10.0 between the peaks due to pregnenolone isobutyrate and cholesterol;
- *symmetry factor*: minimum 0.6 for the peak due to cholesterol.

Calculate the percentage content of cholesterol taking into account the assigned content of *cholesterol CRS*. Calculate the percentage content of total sterols by adding together the contents of cholesterol and other substances with a retention time less than or equal to 1.5 times the retention time of cholesterol. Disregard the peaks due to the internal standard and the solvent.

STORAGE

Protected from light.

LABELLING

The label states the source material for the production of cholesterol (for example bovine brain and spinal cord, wool fat or chicken eggs).

IMPURITIES

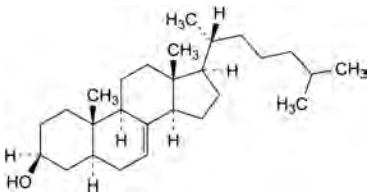

A. 5α-cholest-7-en-3β-ol (lathosterol),

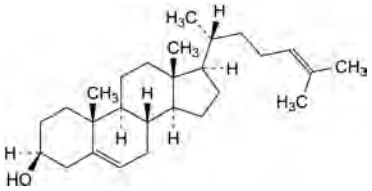

B. cholesta-5,24-dien-3β-ol (desmosterol),

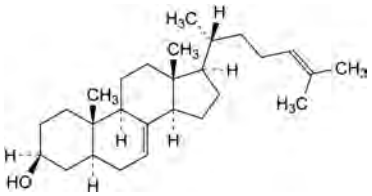

C. 5α-cholesta-7,24-dien-3β-ol.

**01/2020:2397**

# CHOLESTEROL FOR PARENTERAL USE

## Cholesterolum ad usum parenteralem

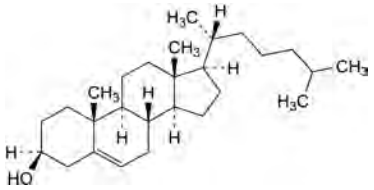

$C_{27}H_{46}O$ $M_r$ 386.7

[57-88-5]

DEFINITION

Cholest-5-en-3β-ol obtained from *Wool fat (0134)*.

*Content*:

– *cholesterol*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone and in ethanol (96 per cent).

It is sensitive to light.

IDENTIFICATION

A. Melting point (*2.2.14*): 147 °C to 150 °C.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

C. Dissolve about 5 mg in 2 mL of *methylene chloride R*. Add 1 mL of *acetic anhydride R* and 0.01 mL of *sulfuric acid R* and shake. A pink colour is produced which rapidly changes to red, then to blue and finally to bright green.

TESTS

**Solubility in ethanol (96 per cent)**. In a stoppered flask, dissolve 0.5 g in 50 mL of *ethanol (96 per cent) R* at 50 °C. Allow to stand for 2 h. The solution is clear.

**Acidity**. Dissolve 1.0 g in 10 mL of *ether R*, add 10.0 mL of *0.1 M sodium hydroxide* and shake for about 1 min. Heat gently to eliminate the ether and then boil for 5 min. Cool, add 10 mL of *water R* and 0.1 mL of *phenolphthalein solution R* as indicator and titrate with *0.1 M hydrochloric acid* until the pink colour just disappears, stirring the solution vigorously throughout the titration. Carry out a blank titration. The difference between the volumes of *0.1 M hydrochloric acid* required to change the colour of the indicator in the blank titration and in the test is not more than 0.1 mL.

**Peroxide value** (*2.5.5, Method A*): maximum 10.

**Other sterols**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 0.100 g of *pregnenolone isobutyrate CRS* in *heptane R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the internal standard solution and dilute to 25.0 mL with the same solution.

*Reference solution.* Dissolve 25.0 mg of *cholesterol CRS* in the internal standard solution and dilute to 25.0 mL with the same solution.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 μm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2 mL/min.

*Split ratio*: 1:25.

*Temperature*:

– *column*: 275 °C;
– *injection port*: 285 °C;
– *detector*: 300 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 μL.

*Relative retention* with reference to cholesterol (retention time = about 8.5 min): pregnenolone isobutyrate = about 0.8.

*System suitability*: reference solution:

– *resolution*: minimum 10.0 between the peaks due to pregnenolone isobutyrate and cholesterol.

*Limits*:

– *total of other substances with a retention time less than or equal to 1.5 times the retention time of cholesterol*: maximum 0.5 per cent;
– *disregard limit*: 0.05 per cent; disregard the peak due to the internal standard.

**Benzoyl ureas**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in 200 mL of *heptane R* using a magnetic stirrer and add 10 mL of *acetonitrile R*. Shake and allow the layers to separate. Isolate the lower layer (acetonitrile) and add 10 mL of *acetonitrile R* to

the heptane layer and extract again. Combine the lower layers and evaporate to dryness by suitable means. Add 0.5 mL of *acetonitrile R* then 0.5 mL of *water R* to the residue. Suspend with the aid of ultrasound for about 5 min. Centrifuge the suspension for 5 min and use the supernatant.

*Reference solution (a).* Dissolve 10.0 mg of *diflubenzuron R* (impurity A) and 10.0 mg of *triflumuron R* (impurity B) in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 0.1 mL of the solution to 100.0 mL with *acetonitrile R*.

*Reference solution (b).* Mix 0.5 mL of reference solution (a) and 0.5 mL of *water R*.

*Reference solution (c).* Dissolve 1.0 g of the substance to be examined in 200 mL of *heptane R* using a magnetic stirrer. Add 0.5 mL of reference solution (a) and 9.5 mL of *acetonitrile R*. Shake and allow the layers to separate. Isolate the lower layer (acetonitrile) and add 10 mL of *acetonitrile R* to the heptane layer and extract again. Combine the lower layers and evaporate to dryness by suitable means. Add 0.5 mL of *acetonitrile R* then 0.5 mL of *water R* to the residue. Suspend with the aid of ultrasound for about 5 min. Centrifuge the suspension for 5 min and use the supernatant.

*Column*:
- *size*: *l* = 0.25 m, Ø = 3 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R*, *water for chromatography R* (50:50 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 20.5 | 100 → 0 | 0 → 100 |
| 20.5 - 30 | 0 | 100 |

After elution of the components, a gradient is applied to prevent a strong drifting baseline due to cholesterol during the following run.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 100 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Retention time*: impurity A = about 10 min; impurity B = about 18 min.

*Limits*:
- *impurity A*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.05 ppm);
- *impurity B*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.05 ppm).

**Loss on drying** (*2.2.32*): maximum 0.1 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*): less than 0.1 IU/mg.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for other sterols with the following modification:

*System suitability*: reference solution:
- *symmetry factor*: minimum 0.6 for the peak due to cholesterol.

Calculate the percentage content of $C_{27}H_{46}O$ taking into account the assigned content of *cholesterol CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

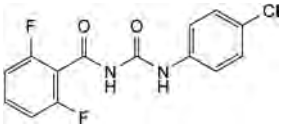

A. 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea (diflubenzuron),

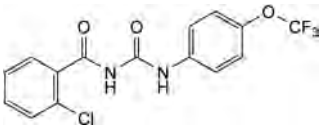

B. 1-(2-chlorobenzoyl)-3-[(4-trifluoromethoxy)phenyl]urea (triflumuron).

**01/2017:2064**

# CHONDROITIN SULFATE SODIUM

## Chondroitini natrii sulfas

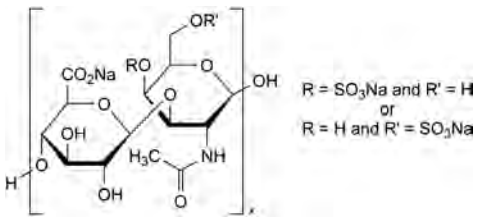


$H_2O(C_{14}H_{19}NNa_2O_{14}S)_x$

DEFINITION

Natural copolymer based mainly on the 2 disaccharides: [4)-(β-D-glucopyranosyluronic acid)-(1→3)-[2-(acetylamino)-2-deoxy-β-D-galactopyranosyl 4-sulfate]-(1→] and [4)-(β-D-glucopyranosyluronic acid)-(1→3)-[2-(acetylamino)-2-deoxy-β-D-galactopyranosyl 6-sulfate]-(1→], sodium salt. On complete hydrolysis it liberates D-galactosamine, D-glucuronic acid, acetic acid and sulfuric acid. It is obtained from cartilage of both terrestrial and marine origins. Depending on the animal species of origin, it shows different proportions of 4-sulfate and 6-sulfate groups.

*Content*: 95 per cent to 105 per cent (dried substance).

PRODUCTION

The animals from which chondroitin sulfate sodium is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.

*Comparison*: for chondroitin sulfate sodium of terrestrial origin use *chondroitin sulfate sodium CRS* and for chondroitin sulfate sodium of marine origine use *chondroitin sulfate sodium (marine) CRS*.

B. Solution S1 (see Tests) gives reaction (b) of sodium (*2.3.1*).

C. Examine the electropherograms obtained in the test for related substances.

*Results*: the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with reference solution (a).

TESTS

**Solution S1**. Dissolve 2.500 g in 50.0 mL of *carbon dioxide-free water R*.

**Solution S2**. Dilute 1.0 mL of solution S1 to 10.0 mL with *water R*.

**pH** (*2.2.3*): 5.5 to 7.5 for solution S1.

**Specific optical rotation** (*2.2.7*): – 20 to – 30 (terrestrial origin) or – 12 to – 19 (marine origin) (dried substance), determined on solution S1.

**Intrinsic viscosity**: 0.01 $m^3$/kg to 0.15 $m^3$/kg.

*Test solution (a).* Weigh 5.000 g ($m_{0p}$) of the substance to be examined and add about 80 mL of an 11.7 g/L solution of *sodium chloride R* at room temperature. Dissolve by shaking at room temperature for 30 min. Dilute to 100.0 mL with an 11.7 g/L solution of *sodium chloride R*. Filter through a membrane filter (nominal pore size 0.45 µm) and discard the first 10 mL. The concentration of test solution (a) is only indicative and must be adjusted after an initial measurement of the viscosity of test solution (a).

*Test solution (b).* To 15.0 mL of test solution (a) add 5.0 mL of an 11.7 g/L solution of *sodium chloride R*.

*Test solution (c).* To 10.0 mL of test solution (a) add 10.0 mL of an 11.7 g/L solution of *sodium chloride R*.

*Test solution (d).* To 5.0 mL of test solution (a) add 15.0 mL of an 11.7 g/L solution of *sodium chloride R*.

Determine the flow-time (*2.2.9*) for an 11.7 g/L solution of *sodium chloride R* ($t_0$) and the flow times for the 4 test solutions ($t_1$, $t_2$, $t_3$ and $t_4$), at 25.00 ± 0.03 °C. Use an appropriate suspended level viscometer (specifications: viscometer constant = about 0.005 $mm^2/s^2$, kinematic viscosity range = 1-5 $mm^2/s$, internal diameter of tube $R$ = 0.53 mm, volume of bulb $C$ = 5.6 mL, internal diameter of tube $N$ = 2.8-3.2 mm) with a funnel-shaped lower capillary end. Use the same viscometer for all measurements; measure all outflow times in triplicate. The test is not valid unless the results do not differ by more than 0.35 per cent from the mean and if the flow time $t_1$ is not less than $1.6 \times t_0$ and not more than $1.8 \times t_0$. If this is not the case, adjust the concentration of test solution (a) and repeat the procedure.

*Calculation of the relative viscosities*

Since the densities of the chondroitin sulfate solutions and of the solvent are almost equal, the relative viscosities $\eta_{ri}$ (being $\eta_{r1}$, $\eta_{r2}$, $\eta_{r3}$ and $\eta_{r4}$) can be calculated from the ratio of the flow times for the respective solutions $t_i$ (being $t_1$, $t_2$, $t_3$ and $t_4$) to the flow time of the solvent $t_0$, but taking into account the kinetic energy correction factor for the capillary ($B$ = 30 800 $s^3$), as shown below:

$$\frac{t_i - \frac{B}{t_i^2}}{t_0 - \frac{B}{t_0^2}}$$

*Calculation of the concentrations*

Calculate the concentration $c_1$ (expressed in kg/$m^3$) of chondroitin sulfate sodium in test solution (a) using the following expression:

$$m_{0p} \times \frac{x}{100} \times \frac{100 - h}{100} \times 10$$

$x$ = percentage content of chondroitin sulfate sodium as determined in the assay;

$h$ = loss on drying as a percentage.

Calculate the concentration $c_2$ (expressed in kg/$m^3$) of chondroitin sulfate sodium in test solution (b) using the following expression:

$$c_1 \times 0.75$$

Calculate the concentration $c_3$ (expressed in kg/$m^3$) of chondroitin sulfate sodium in test solution (c) using the following expression:

$$c_1 \times 0.50$$

Calculate the concentration $c_4$ (expressed in kg/$m^3$) of chondroitin sulfate sodium in test solution (d) using the following expression:

$$c_1 \times 0.25$$

*Calculation of the intrinsic viscosity*

The specific viscosity $\eta_{si}$ of the test solution (being $\eta_{s1}$, $\eta_{s2}$, $\eta_{s3}$ and $\eta_{s4}$) is calculated from the relative viscosities $\eta_{ri}$ (being $\eta_{r1}$, $\eta_{r2}$, $\eta_{r3}$ and $\eta_{r4}$) according to the following expression:

$$\eta_{ri} - 1$$

The intrinsic viscosity [η], defined as

$$[\eta] = \lim_{c \to 0} \left( \frac{\eta_s}{c} \right)$$

is calculated by linear least-squares regression analysis using the following equation:

$$\frac{\eta_{si}}{c_i} = c_i \times k_H + [\eta]$$

$c_i$ = concentration of the substance to be examined expressed in kg/$m^3$;

$k_H$ = Huggins' constant.

**Related substances**. Electrophoresis (*2.2.31*).

*Buffer solution A* (0.1 M barium acetate pH 5.0). Dissolve 25.54 g of *barium acetate R* in 900 mL of *water R*. Adjust to pH 5.0 with *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

*Buffer solution B* (1 M barium acetate pH 5.0). Dissolve 255.43 g of *barium acetate R* in 900 mL of *water R*. Adjust to pH 5.0 with *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

*Staining solution.* Dissolve 1.0 g of *toluidine blue R* and 2.0 g of *sodium chloride R* in 1000 mL of *0.01 M hydrochloric acid*. Filter.

*Test solution.* Prepare a 30 mg/mL solution of the substance to be examined in *water R*.

*Reference solution (a).* Prepare a 30 mg/mL solution of *chondroitin sulfate sodium CRS* in *water R*.

*Reference solution (b).* Dilute 2.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Reference solution (c).* Mix equal volumes of reference solution (b) and *water R*.

*Procedure.* Allow the electrophoresis support to cool the plate to 10 °C. Pre-equilibrate the agarose gel for 1 min in buffer solution A. Remove excess liquid by careful decanting. Dry the gel for approximately 5 min. Place 400 mL of buffer solution B into each of the containers of the electrophoresis equipment. Transfer 1 µL of each solution to the slots of the agarose gel. Pipette a few millilitres of a 50 per cent *V/V* solution of *glycerol R* onto the cooled plate of the electrophoresis equipment and place the gel in the middle of the ceramic plate. Place a wick, saturated with buffer solution B, at the

positive and negative sides of the agarose gel. Ensure that there is good contact between the electrophoresis buffer and the agarose gel. Perform the electrophoresis under the following conditions: 75 mA/gel, resulting in a voltage of 100-150 V (maximum 300-400 V) for a gel of about 12 cm × 10 cm. Carry out the electrophoresis for 12 min. Place the gel in a mixture consisting of 10 volumes of *anhydrous ethanol R* and 90 volumes of buffer solution A for 2 min. Carry out the electrophoresis for 20 min. Place the gel in a mixture consisting of 30 volumes of *anhydrous ethanol R* and 70 volumes of buffer solution A for 2 min. Carry out the electrophoresis for 20 min. Stain the gel in the staining solution for 10 min. Destain the gel for 15 min under running tap water followed by 10-15 min with *water R* until the band in the electropherogram obtained with reference solution (c) is visible. Allow the gel to dry.

*System suitability*:

- the electropherogram obtained with reference solution (c) shows a visible band;
- the band in the electropherogram obtained with reference solution (b) is clearly visible and similar in position to the band in the electropherogram obtained with reference solution (a).

*Results*: any secondary band in the electropherogram obtained with the test solution is not more intense than the band in the electropherogram obtained with reference solution (b) (2 per cent).

**Protein** (*2.5.33, Method 2*): maximum 3.0 per cent (dried substance).

*Test solution.* Dilute 1.0 mL of solution S1 to 50.0 mL with *0.1 M sodium hydroxide*.

*Reference solutions.* Dissolve about 0.100 g of *bovine albumin R*, accurately weighed, in *0.1 M sodium hydroxide* and dilute to 50.0 mL with the same solvent. Carry out all additional dilutions using *0.1 M sodium hydroxide*.

**Chlorides** (*2.4.4*): maximum 0.5 per cent.

Dilute 1 mL of solution S2 to 15 mL with *water R*. Do not add diluted nitric acid. Prepare the standard using 5 mL of *chloride standard solution (5 ppm Cl) R* and 10 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Staphylococcus aureus* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

Absence of bile-tolerant gram-negative bacteria (*2.6.13*).

ASSAY

*Test solution (a).* Weigh 0.100 g ($m_1$) of the substance to be examined, dissolve in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a).* Weigh 0.100 g ($m_0$) of *chondroitin sulfate sodium CRS*, previously dried as described in the test for loss on drying, dissolve in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with *water R*.

*Titrant solution (a).* Weigh 4.000 g of *cetylpyridinium chloride monohydrate R* and dilute to 1000 mL with *water R*.

*Titrant solution (b).* Weigh 1.000 g of *cetylpyridinium chloride monohydrate R* and dilute to 1000 mL with *water R*.

Perform either visual or photometric titration as follows:

*Visual titration.* Titrate 40.0 mL of reference solution (a) and 40.0 mL of test solution (a) with titrant solution (a). The solution becomes turbid. At the end point, the liquid appears clear, with an almost-white precipitate in suspension. The precipitate is more apparent if 0.1 mL of a 1 per cent solution of *methylene blue R* is added before starting the titration. The precipitated particles are more apparent against the blue background.

*Photometric titration.* Titrate 50.0 mL of reference solution (b) and 50.0 mL of test solution (b) with titrant solution (b). To determine the end point, use a suitable autotitrator equipped with a phototrode at a suitable wavelength (none is critical) in the visible range.

Calculate the percentage content of chondroitin sulfate sodium using the following expression:

$$\frac{v_1 \times m_0}{v_0 \times m_1} \times \frac{100}{100 - h} \times Z$$

$v_0$ = volume of appropriate titrant solution when titrating the appropriate reference solution, in millilitres;

$v_1$ = volume of appropriate titrant solution when titrating the appropriate test solution, in millilitres;

$h$ = loss on drying of the substance to be examined, as a percentage;

$Z$ = percentage content of $H_2O(C_{14}H_{19}NNa_2O_{14}S)_x$ in *chondroitin sulfate sodium CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the origin of the substance (marine or terrestrial).

**01/2016:0476**

# CHYMOTRYPSIN

## Chymotrypsinum

[9004-07-3]

DEFINITION

Chymotrypsin is a proteolytic enzyme obtained by the activation of chrymotrypsinogen extracted from the pancreas of beef (*Bos taurus* L.). It has an activity of not less than 5.0 microkatals per milligram. In solution it has maximal enzymic activity at about pH 8; the activity is reversibly inhibited at pH 3, the pH at which it is most stable.

PRODUCTION

The animals from which chymotrypsin is derived must fulfil the requirements for the health of animals suitable for human consumption. Furthermore, the tissues used shall not include any specified risk material as defined by any relevant international or, where appropriate, national legislation.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

**Histamine** (*2.6.10*): not more than 1 µg (calculated as histamine base) per 5 microkatals of chymotrypsin activity. Before carrying out the test, heat the solution of the substance to be examined on a water-bath for 30 min.

CHARACTERS

*Appearance*: white or almost white, crystalline or amorphous powder, hygroscopic if amorphous.

*Solubility*: sparingly soluble in water.

IDENTIFICATION

A. Dilute 1 mL of solution S (see Tests) to 10 mL with *water R*. In a depression in a white spot-plate, mix 0.05 mL of this solution with 0.2 mL of the substrate solution. A purple colour develops.

*Substrate solution*. To 24.0 mg of *acetyltyrosine ethyl ester R* add 0.2 mL of *ethanol (96 per cent) R* and swirl to dissolve. Add 2.0 mL of *0.067 M phosphate buffer solution pH 7.0 R* and 1 mL of *methyl red mixed solution R* and dilute to 10.0 mL with *water R*.

B. Dilute 0.5 mL of solution S to 5 mL with *water R*. Add 0.10 mL of a 20 g/L solution of *tosylphenylalanylchloromethane R* in *ethanol (96 per cent) R*. Adjust to pH 7.0 and shake for 2 h. In a depression in a white spot-plate, mix 0.05 mL of this solution with 0.2 mL of the substrate solution (see Identification test A). No colour develops within 3 min of mixing.

TESTS

**Solution S**. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*).

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Specific absorbance** (*2.2.25*): 18.5 to 22.5, determined at the absorption maximum at 281 nm; maximum 8, determined at the absorption minimum at 250 nm.

Dissolve 30.0 mg in *0.001 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

**Trypsin**.

*Substrate solution*. To 98.5 mg of *tosylarginine methyl ester hydrochloride R*, suitable for assaying trypsin, add 5 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and swirl to dissolve. Add 2.5 mL of *methyl red mixed solution R* and dilute to 25.0 mL with *water R*.

*Test solution*. Transfer to a depression in a white spot-plate 0.01 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and 0.1 mL of solution S. Add 0.2 mL of the substrate solution.

*Reference solution*. At the same time and in the same manner as for the test solution, prepare a solution using the substance to be examined to which not more than 1 per cent *m/m* of *trypsin BRP* has been added.

Start a timer. No colour appears in the test solution within 3-5 min after the addition of the substrate solution. A purple colour is produced in the control solution.

**Loss on drying** (*2.2.32*): not more than 5.0 per cent, determined on 0.100 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 2 h.

ASSAY

The activity of chymotrypsin is determined by comparing the rate at which it hydrolyses *acetyltyrosine ethyl ester R* with the rate at which *chymotrypsin BRP* hydrolyses the same substrate under the same conditions.

*Apparatus*. Use a reaction vessel of about 30 mL capacity provided with:

- a device that will maintain a temperature of 25.0 ± 0.1 °C;
- a stirring device, for example a magnetic stirrer;
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. For the latter, the burette is graduated in 0.005 mL and the pH meter is provided with a wide-range scale and glass-silver-silver chloride or other suitable electrodes.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in *0.001 M hydrochloric acid* and dilute to 250.0 mL with the same acid.

*Reference solution*. Dissolve 25.0 mg of *chymotrypsin BRP* in *0.001 M hydrochloric acid* and dilute to 250.0 mL with the same acid.

Store the solutions at 0-5 °C. Warm 1 mL of each solution to about 25 °C over 15 min and use 50 µL of each solution (corresponding to about 25 nanokatals) for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 mL of *0.01 M calcium chloride solution R* to the reaction vessel and, while stirring, add 0.35 mL of *0.2 M acetyltyrosine ethyl ester R*. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min), adjust to pH 8.0 exactly with *0.02 M sodium hydroxide*. Add 50 µL of the test solution (equivalent to about 5 µg of the substance to be examined) and start a timer. Maintain at pH 8.0 by the addition of *0.02 M sodium hydroxide*, noting the volume added every 30 s. Calculate the volume of *0.02 M sodium hydroxide* used per second between 30 s and 210 s. Carry out a titration in the same manner using the reference solution and calculate the volume of *0.02 M sodium hydroxide* used per second.

Calculate the activity in microkatals per milligram using the following expression:

$$\frac{m' \times V}{m \times V'} \times A$$

$m$ = mass of the substance to be examined, in milligrams;

$m'$ = mass of *chymotrypsin BRP*, in milligrams;

$V$ = volume of *0.02 M sodium hydroxide* used per second by the test solution;

$V'$ = volume of *0.02 M sodium hydroxide* used per second by the reference solution;

$A$ = activity of *chymotrypsin BRP*, in microkatals per milligram.

STORAGE

In an airtight container at 2 °C to 8 °C, protected from light.

LABELLING

The label states:

- the quantity of chymotrypsin and the total activity in microkatals per container;
- for the amorphous substance, that it is hygroscopic.

**01/2017:2703**

# CICLESONIDE

## Ciclesonidum

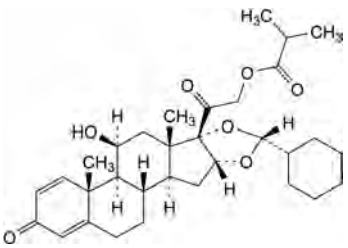

$C_{32}H_{44}O_7$ $M_r$ 540.7

[126544-47-6]

DEFINITION

(2′*R*)-2′-Cyclohexyl-11β-hydroxy-3,20-dioxo-16β*H*-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 2-methylpropanoate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble to soluble in acetone and in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciclesonide CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *ciclesonide CRS* in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 3 mg of *ciclesonide impurity B CRS*, 3 mg of *ciclesonide impurity C CRS* and 5 mg of *ciclesonide containing impurity A CRS* in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (c).* Dissolve 50 mg of the substance to be examined in *anhydrous ethanol R*, add 1.0 mL of reference solution (b) and dilute to 50.0 mL with *anhydrous ethanol R*.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with *anhydrous ethanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*: *water R*, *anhydrous ethanol R* (38:62 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 243 nm.

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*Run time*: 2.2 times the retention time of ciclesonide.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to ciclesonide (retention time = about 16 min): impurity B = about 0.4; impurity C = about 0.9; impurity A = about 1.4.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity C and ciclesonide.

*Calculation of percentage contents*:

- for each impurity, use the concentration of ciclesonide in reference solution (d).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurities B, C*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total of unspecified impurities*: maximum 0.2 per cent;
- *total*: maximum 1.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*Run time*: 1.6 times the retention time of ciclesonide.

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.2 for the peak due to ciclesonide.

Calculate the percentage content of $C_{32}H_{44}O_7$ taking into account the assigned content of *ciclesonide CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

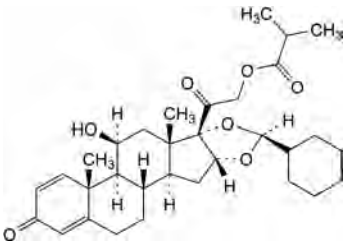

A. (2′*S*)-2′-cyclohexyl-11β-hydroxy-3,20-dioxo-16β*H*-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 2-methylpropanoate (*S*-epimer of ciclesonide),

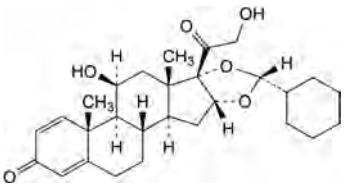

B. (2′*R*)-2′-cyclohexyl-11β,21-dihydroxy-16β*H*-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-diene-3,20-dione,

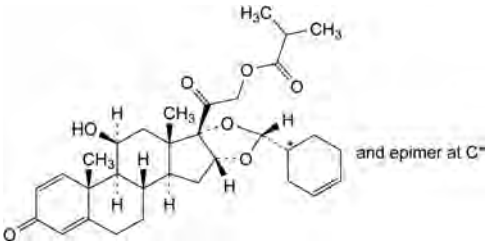


C. (2′*R*)-2′-[(1*RS*)-cyclohex-3-enyl]-11β-hydroxy-3,20-dioxo-16β*H*-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 2-methylpropanoate.

01/2017:1407
corrected 10.0

# CICLOPIROX

## Ciclopiroxum

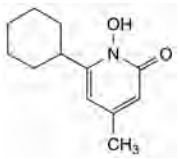

$C_{12}H_{17}NO_2$ $M_r$ 207.3
[29342-05-0]

DEFINITION

6-Cyclohexyl-1-hydroxy-4-methylpyridin-2(1*H*)-one.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Melting point (*2.2.14*): 140 °C to 145 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciclopirox CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 20 mg of *ciclopirox CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Pretreatment*: before use, predevelop with the mobile phase until the solvent front has migrated to the top of the plate. Allow to dry in air for 5 min.

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethanol (96 per cent) R* (10:15:75 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 10 min.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: spray with a 20 g/L solution of *ferric chloride R* in *anhydrous ethanol R*.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test avoiding exposure to actinic light. All materials in direct contact with the substance to be examined like column materials, reagents, solvents, etc. should contain only very low amounts of extractable metal cations.*

*Solvent mixture*: *acetonitrile R*, mobile phase (10:90 *V/V*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in 15 mL of the solvent mixture, using an ultrasonic bath if necessary, and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 15.0 mg of *ciclopirox impurity A CRS* and 15.0 mg of *ciclopirox impurity B CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 200.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Mix 5.0 mL of reference solution (a) and 5.0 mL of the test solution.

*Column*:

- *size*: *l* = 0.08 m, Ø = 4 mm;
- *stationary phase*: *nitrile silica gel for chromatography R2* (5 µm).

In order to ensure desorption of interfering metal ions, every new column is to be rinsed with the rinsing solution over a period of not less than 15 h and then with the mobile phase for not less than 5 h at a flow rate of 0.2 mL/min.

*Rinsing solution*: *glacial acetic acid R*, *acetylacetone R*, *acetonitrile R*, *water R* (0.1:0.1:50:50 *V/V/V/V*).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, 0.96 g/L solution of *sodium edetate R* (0.01:23:77 *V/V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 220 nm and at 298 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d); inject the solvent mixture as a blank.

*Run time*: 2.5 times the retention time of ciclopirox.

*Retention time*: ciclopirox = 8 min to 11 min; if necessary adjust the ratio of the 0.96 g/L solution of sodium edetate to acetonitrile in the mobile phase.

*Relative retention* with reference to ciclopirox: impurity A = about 0.5; impurity C = about 0.9; impurity B = about 1.3.

*System suitability*: at 298 nm:

- *resolution*: minimum 2.0 between the peaks due to ciclopirox and impurity B in the chromatogram obtained with reference solution (d);
- *symmetry factor*: 0.8 to 2.0 for the principal peak in the chromatogram obtained with the test solution.

*Limits*:

- *impurity A at 220 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, C at 298 nm*: for each impurity, not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities at 298 nm*: for each impurity, not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *sum of impurities other than B at 298 nm*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit at 298 nm*: 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 20 mL of *methanol R*. Add 20 mL of *water R* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.73 mg of $C_{12}H_{17}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

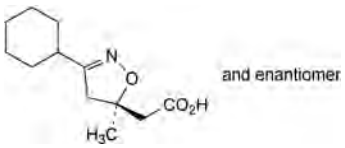


A. [(5*RS*)-3-cyclohexyl-5-methyl-4,5-dihydro-1,2-oxazol-5-yl]acetic acid,

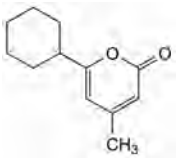

B. 6-cyclohexyl-4-methyl-2*H*-pyran-2-one,

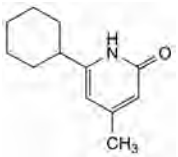

C. 6-cyclohexyl-4-methylpyridin-2(1*H*)-one.

**01/2017:1302**

# CICLOPIROX OLAMINE

## Ciclopirox olaminum

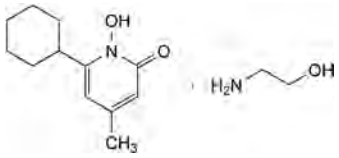

$C_{14}H_{24}N_2O_3$ $M_r$ 268.4

[41621-49-2]

DEFINITION

6-Cyclohexyl-1-hydroxy-4-methylpyridin-2(1*H*)-one and 2-aminoethanol.

*Content*:

– *ciclopirox* ($C_{12}H_{17}NO_2$; $M_r$ 207.3): 76.0 per cent to 78.5 per cent (dried substance);

– *2-aminoethanol* ($C_2H_7NO$; $M_r$ 61.1): 22.2 per cent to 23.3 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder.

*Solubility*: sparingly soluble in water, very soluble in ethanol (96 per cent) and in methylene chloride, slightly soluble in ethyl acetate, practically insoluble in cyclohexane.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciclopirox olamine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethyl acetate R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 25 mg of *ciclopirox olamine CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Pretreatment*: before use, predevelop 2 plates with the mobile phase until the solvent front has migrated to the top of the plates. Allow to dry in air for 5 min.

*Mobile phase*: *concentrated ammonia R*, *water R*, *anhydrous ethanol R* (10:15:75 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 10 min.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: spray 1 plate with *ferric chloride solution R3*.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection C*: spray the 2nd second plate with *ninhydrin solution R*. Heat at 110 °C until the spots appear.

*Results C*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 8.0 to 9.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test avoiding exposure to actinic light. All materials in direct contact with the substance to be examined, such as column materials, reagents, solvents, etc. should contain only small amounts of extractable metal cations.*

*Solvent mixture*: *acetonitrile R*, mobile phase (10:90 *V/V*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined (corresponding to about 30 mg of ciclopirox) in a mixture of 20 µL of *anhydrous acetic acid R*, 2 mL of *acetonitrile R*, and 15 mL of the mobile phase, using an ultrasonic bath if necessary. Dilute the solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 15.0 mg of *ciclopirox impurity A CRS* and 15.0 mg of *ciclopirox impurity B CRS* in a mixture of 1 mL of *acetonitrile R* and 7 mL of the mobile phase, and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 200.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 2.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d).* Mix 5.0 mL of reference solution (a) and 5.0 mL of the test solution.

*Column*:

- *size*: *l* = 80 mm, Ø = 4 mm;
- *stationary phase*: *nitrile silica gel for chromatography R* (5 µm).

In order to ensure desorption of interfering metal ions, every new column is to be rinsed with the rinsing solution over a period of not less than 15 h and then with the mobile phase for not less than 5 h at a flow rate of 0.2 mL/min.

*Rinsing solution*: *acetylacetone R*, *anhydrous acetic acid R*, *acetonitrile R*, *water R* (0.1:0.1:50:50 *V/V/V/V*).

*Mobile phase*: *anhydrous acetic acid R*, *acetonitrile R*, 0.96 g/L solution of *sodium edetate R* (0.01:23:77 *V/V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 220 nm and at 298 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 2.5 times the retention time of ciclopirox.

*Retention time*: ciclopirox = 8 min to 11 min; if necessary adjust the ratio of the 0.96 g/L solution of sodium edetate to acetonitrile in the mobile phase.

*Relative retention* with reference to ciclopirox: impurity A = about 0.5; impurity C = about 0.9; impurity B = about 1.3.

*System suitability*: at 298 nm:

- *resolution*: minimum of 2.0 between the peaks due to impurity B and ciclopirox in the chromatogram obtained with reference solution (d);
- *symmetry factor*: 0.8 to 2.0 for the principal peak in the chromatogram obtained with the test solution.

*Limits*:

- *impurity A at 220 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, C at 298 nm*: for each impurity, not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities at 298 nm*: for each impurity, not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *sum of impurities other than B at 298 nm*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit at 298 nm*: 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying under high vacuum.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**2-Aminoethanol**. Dissolve 0.250 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 6.108 mg of $C_2H_7NO$.

**Ciclopirox**. Dissolve 0.200 g in 2 mL of *methanol R*. Add 38 mL of *water R*, swirl and titrate immediately with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

Use *0.1 M sodium hydroxide*, the titre of which has been determined under the conditions prescribed above using 0.100 g of *benzoic acid RV*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.73 mg of $C_{12}H_{17}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

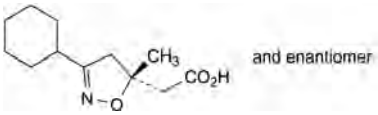


A. [(5*RS*)-3-cyclohexyl-5-methyl-4,5-dihydro-1,2-oxazol-5-yl]acetic acid,

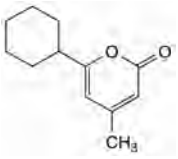


B. 6-cyclohexyl-4-methyl-2*H*-pyran-2-one,

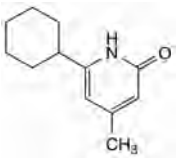


C. 6-cyclohexyl-4-methylpyridin-2(1*H*)-one.

**01/2017:0994**
**corrected 10.0**

# CICLOSPORIN

## Ciclosporinum

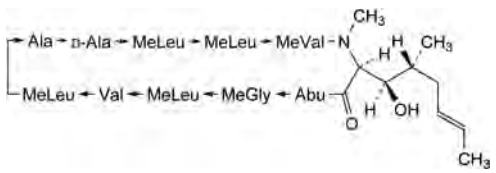


$C_{62}H_{111}N_{11}O_{12}$ $M_r$ 1203
[59865-13-3]

DEFINITION

Cyclo[[(2*S*,3*R*,4*R*,6*E*)-3-hydroxy-4-methyl-2-(methylamino)-oct-6-enoyl]-L-2-aminobutanoyl-*N*-methylglycyl-*N*-methyl-L-leucyl-L-valyl-*N*-methyl-L-leucyl-L-alanyl-D-alanyl-*N*-methyl-L-leucyl-*N*-methyl-L-leucyl-*N*-methyl-L-valyl] (ciclosporin A).

Substance produced by *Beauveria nivea (Tolypocladium inflatum* Gams) or obtained by any other means.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciclosporin CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$, $BY_5$ or $R_7$ (*2.2.2, Method II*).

Dissolve 1.5 g in *anhydrous ethanol R* and dilute to 15 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 193 to – 185 (dried substance).

Dissolve 0.125 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 30.0 mg of *ciclosporin CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 2.0 mL of reference solution (a) to 200.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *ciclosporin for system suitability CRS* in 5.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3-5 µm);
– *temperature*: 80 °C.

The column is connected to the injection port by a steel capillary tube about 1 m long, having an internal diameter of 0.25 mm and maintained at 80 °C.

*Mobile phase*: *phosphoric acid R*, *1,1-dimethylethyl methyl ether R*, *acetonitrile R*, *water R* (0.1:5:43:52 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.7 times the retention time of ciclosporin.

*System suitability*: reference solution (c):

– *retention time*: ciclosporin = 25 min to 30 min; if necessary, adjust the ratio of acetonitrile to water in the mobile phase;
– *peak-to-valley ratio*: minimum 1.4, where $H_p$ = height above the baseline of the peak due to ciclosporin U and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ciclosporin; if necessary, adjust the ratio of 1,1-dimethylethyl methyl ether to acetonitrile in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g at 60 °C at a pressure not exceeding 15 Pa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.84 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Dissolve 50 mg of the substance to be examined in a mixture of 280 mg of *ethanol (96 per cent) R* and 650 mg of *polyoxyethylated castor oil R* and dilute to the required concentration using water for BET.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{62}H_{111}N_{11}O_{12}$ taking into account the assigned content of *ciclosporin CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

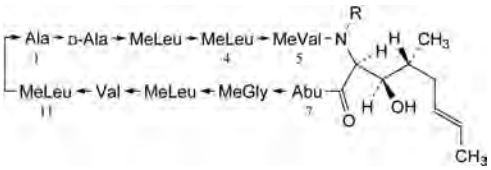


A. different ciclosporins [difference from ciclosporin (R = $CH_3$: ciclosporin A)]: ciclosporin B [7-L-Ala]; ciclosporin C [7-L-Thr]; ciclosporin D [7-L-Val]; ciclosporin E [5-L-Val]; ciclosporin G [7-(L-2-aminopentanoyl)]; ciclosporin H [5-D-MeVal]; ciclosporin L [R = H]; ciclosporin T [4-L-Leu]; ciclosporin U [11-L-Leu]; ciclosporin V [1-L-Abu],

B. [6-[(2*S*,3*R*,4*R*)-3-hydroxy-4-methyl-2-(methylamino)-octanoic acid]]ciclosporin A,

C. isociclosporin A.

**01/2017:1408 corrected 10.0**

# CILASTATIN SODIUM

## Cilastatinum natricum

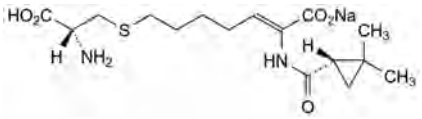

$C_{16}H_{25}N_2NaO_5S$ $M_r$ 380.4
[81129-83-1]

DEFINITION

Sodium (*Z*)-7-[[(2*R*)-2-amino-2-carboxyethyl]sulfanyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoate.

*Content*: 98.0 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or light yellow, amorphous, hygroscopic powder.

*Solubility*: very soluble in water and in methanol, slightly soluble in anhydrous ethanol, very slightly soluble in dimethyl sulfoxide, practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cilastatin sodium CRS*.

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 7.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 41.5 to + 44.5 (anhydrous substance).

Dissolve 0.250 g in a mixture of 1 volume of *hydrochloric acid R* and 120 volumes of *methanol R*, then dilute to 25.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*). Prepare the solutions immediately before use.

*Test solution*. Dissolve 32 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 3 mg of *cilastatin for system suitability 1 CRS* (containing impurities A, B, E, F, G (epimer 2) and H) in *water R* and dilute to 2.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 3 mg of *cilastatin for system suitability 2 CRS* (containing impurities C and G (epimer 1)) in *water R* and dilute to 2.0 mL with the same solvent.

*Reference solution (d)*. Dissolve 32 mg of *mesityl oxide R* (impurity D) in 100.0 mL of *water R*. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *phosphate buffer solution pH 3.25 R*;
– *mobile phase B*: *acetonitrile R1*, *phosphate buffer solution pH 3.25 R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 28 | 100 → 90 | 0 → 10 |
| 28 - 38 | 90 | 10 |
| 38 - 63 | 90 → 50 | 10 → 50 |
| 63 - 78 | 50 → 30 | 50 → 70 |
| 78 - 88 | 30 | 70 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *cilastatin for system suitability 1 CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, E, F, G (epimer 2) and H; use the chromatogram supplied with *cilastatin for system suitability 2 CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and G (epimer 1); use the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention* with reference to cilastatin (retention time = about 50 min): impurity E = about 0.2; impurity A (epimer 1) = about 0.60; impurity A (epimer 2) = about 0.62; impurity D = about 0.9; impurity F = about 0.98; impurity G (epimer 1) = about 1.02; impurity G (epimer 2) = about 1.05; impurity H = about 1.06; impurity B = about 1.17; impurity C = about 1.23.

*System suitability*:

– *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cilastatin in the chromatogram obtained with reference solution (b);
– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity G (epimer 1) and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cilastatin in the chromatogram obtained with reference solution (c).

*Calculation of percentage contents*:

– for all impurities, use the concentration of cilastatin sodium in reference solution (a);
– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.3; impurity E = 3.3; impurity G (epimer 1) and impurity G (epimer 2) = 1.6.

*Limits*:

– *impurities A (sum of the epimers)*: maximum 0.5 per cent;
– *impurity C*: maximum 0.4 per cent;
– *impurities E*: maximum 0.3 per cent;
– *impurities B, F, H*: for each impurity, maximum 0.1 per cent;
– *impurity G*: for each epimer, maximum 0.1 per cent;
– *unspecified impurities*: for each impurity, maximum 0.05 per cent;
– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.03 per cent; disregard any peak due to impurity D in the chromatogram obtained with reference solution (d).

**Impurity D, acetone and methanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.5 mL of *propanol R* in *water R* and dilute to 1000 mL with the same solvent.

*Test solution.* Dissolve 0.200 g of the substance to be examined in *water R*, add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Reference solution.* Dissolve 2.0 mL of *acetone R*, 0.5 mL of *methanol R* and 0.5 mL of *mesityl oxide R* (impurity D) in *water R* and dilute to 1000 mL with the same solvent. To 2.0 mL of this solution add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*. This solution contains 316 µg of acetone, 79 µg of methanol and 86 µg of impurity D per millilitre.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.53 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 9 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2.5 | 50 |
| | 2.5 - 5 | 50 → 70 |
| | 5 - 5.5 | 70 |
| Injection port | | 160 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

Calculate the percentage contents of acetone, methanol and impurity D using the following expression:

$$\left(\frac{C}{W}\right) \times \left(\frac{R_u}{R_s}\right)$$

$C$ = concentration of the solvent in the reference solution, in µg/mL;

$W$ = quantity of cilastatin sodium in the test solution, in milligrams;

$R_u$ = ratio of the area of the solvent peak to the area of the propanol peak in the chromatogram obtained with the test solution;

$R_s$ = ratio of the area of the solvent peak to the area of the propanol peak in the chromatogram obtained with the reference solution.

*Limits*:

– *acetone*: maximum 1.0 per cent *m/m*;

– *methanol*: maximum 0.5 per cent *m/m*;

– *impurity D*: maximum 0.4 per cent *m/m*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.100 g in 30 mL of *methanol R* and add 5 mL of *water R*. Add *0.1 M hydrochloric acid* to a pH of about 3.0. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Three jumps of potential are observed. Read the volume added between the 1st and the 3rd point of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 19.02 mg of $C_{16}H_{25}N_2NaO_5S$.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

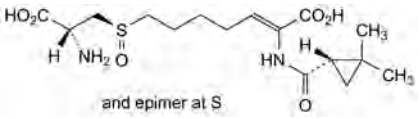


A. (*Z*)-7-[(*RS*)-[(2*R*)-2-amino-2-carboxyethyl]sulfinyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,

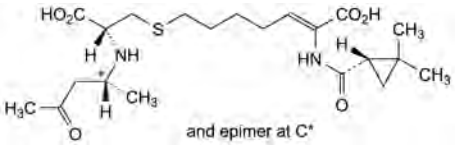


B. (*Z*)-7-[[(2*R*)-2-carboxy-2-[[(1*RS*)-1-methyl-3-oxobutyl]amino]ethyl]sulfanyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,

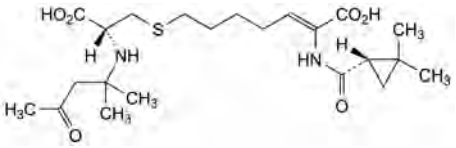

C. (*Z*)-7-[[(2*R*)-2-carboxy-2-[(1,1-dimethyl-3-oxobutyl)amino]ethyl]sulfanyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid,

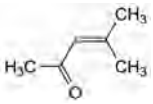

D. 4-methylpent-3-en-2-one (mesityl oxide),

E. 7-[[(2*R*)-2-amino-2-carboxyethyl]sulfanyl]-2-oxoheptanoic acid,

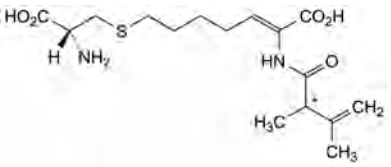

F. (*Z*)-7-[[(2*R*)-2-amino-2-carboxyethyl]sulfanyl]-2-[(2,3-dimethylbut-3-enoyl)amino]hept-2-enoic acid,

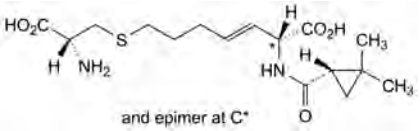


G. (*E*)-(2*RS*)-7-[[(2*R*)-2-amino-2-carboxyethyl]sulfanyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-3-enoic acid,

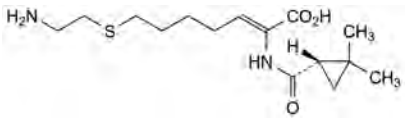

H. (*Z*)-7-[(2-aminoethyl)sulfanyl]-2-[[[(1*S*)-2,2-dimethylcyclopropyl]carbonyl]amino]hept-2-enoic acid.

**01/2008:1499**



# CILAZAPRIL

## Cilazaprilum

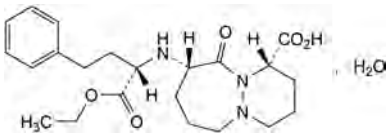

$C_{22}H_{31}N_3O_5,H_2O$ $M_r$ 435.5
[92077-78-6]

DEFINITION

(1*S*,9*S*)-9-[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylic acid monohydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cilazapril CRS*.

B. Specific optical rotation (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): – 383 to – 399 (anhydrous substance).

Dissolve 0.200 g in *0.067 M phosphate buffer solution pH 7.0 R*, with the aid of ultrasound if necessary, and dilute to 50.0 mL with the same buffer solution. Carry out the determination at 365 nm.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 2 mg of *cilazapril impurity A CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *cilazapril impurity A CRS* and 5 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *hexane R*, *methanol R*, *ethyl acetate R* (5:5:15:15:60 *V/V/V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in a current of cold air for 10 min.

*Detection*: spray with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with *dilute hydrogen peroxide solution R*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *cilazapril impurity D CRS* in the test solution and dilute to 10.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of *triethylamine R* and 750 volumes of *water R*, adjust to pH 2.30 with *phosphoric acid R*, and add 200 volumes of *tetrahydrofuran R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of cilazapril; when impurity A is present, it may be necessary to continue the chromatography until it is eluted.

*Relative retention* with reference to cilazapril: impurity B = about 0.6; impurity D = about 0.9; impurity C = about 1.6; impurity A = 4 to 5.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurity D and cilazapril;
– *symmetry factor*: maximum 3.0 for the peak due to cilazapril.

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity D*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurity C*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to impurity A.

**Water** (*2.5.12*): 3.5 per cent to 5.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 10 mL of *anhydrous ethanol R* and add 50 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.75 mg of $C_{22}H_{31}N_3O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

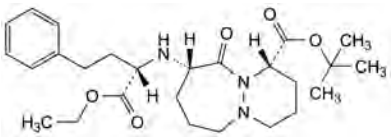

A. 1,1-dimethylethyl (1*S*,9*S*)-9-[[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylate,

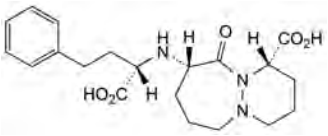

B. (1*S*,9*S*)-9-[[(*S*)-1-carboxy-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylic acid,

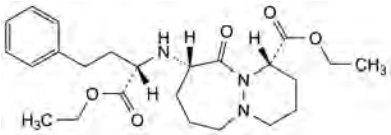

C. ethyl (1*S*,9*S*)-9-[[(*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]diazepine-1-carboxylate,

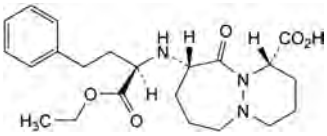

D. (1*S*,9*S*)-9-[[(*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]-amino]-10-oxooctahydro-6*H*-pyridazino[1,2-*a*][1,2]-diazepine-1-carboxylic acid.

**01/2017:0756**
**corrected 9.4**

# CIMETIDINE

## Cimetidinum

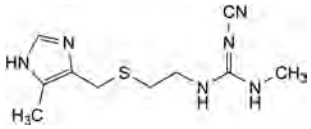

$C_{10}H_{16}N_6S$ $M_r$ 252.3

[51481-61-9]

DEFINITION

2-Cyano-1-methyl-3-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]guanidine.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute mineral acids.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 139 °C to 144 °C.

If necessary, dissolve the substance to be examined in *2-propanol R*, evaporate to dryness and determine the melting point again.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cimetidine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *cimetidine CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R* (15:20:65 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of cold air.

*Detection*: expose to iodine vapour until maximum contrast has been obtained and examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 3.0 g in 12 mL of *1 M hydrochloric acid* and dilute to 20 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 2.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *cimetidine for system suitability CRS* (containing impurities B, C, D, E, G and H) in 1.0 mL of mobile phase A.

*Reference solution (c)*. Dissolve 4 mg of *cimetidine for peak identification CRS* (containing impurity F) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Mobile phase A*: mix 0.4 volumes of *diethylamine R* and 780 volumes of a 1.1 g/L solution of *sodium hexanesulfonate R*; adjust to pH 2.8 with *phosphoric acid R*; add 250 volumes of *methanol R2*;

*Mobile phase B*: *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 100 | 0 |
| 60 - 65 | 100 → 90 | 0 → 10 |
| 65 - 120 | 90 | 10 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µl.

*Identification of impurities*: use the chromatogram supplied with *cimetidine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D, E, G and H; use the chromatogram supplied with *cimetidine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to cimetidine (retention time = about 18 min): impurity G = about 0.2; impurity E = about 0.4; impurity D = about 1.5; impurity C = about 1.6; impurity B = about 2.0; impurity H = about 2.3; impurity F = about 4.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities D and C.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 2.5; impurity D = 3.3; impurity E = 0.7; impurity G = 0.6.
– *impurities B, C, D, E, F, G, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.23 mg of $C_{10}H_{16}N_6S$

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, C, D, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, I, J.*

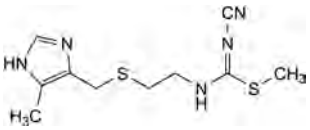

A. methyl 3-cyano-1-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]carbamimidothioate,

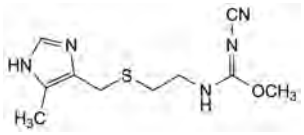

B. methyl 3-cyano-1-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]carbamimidate,

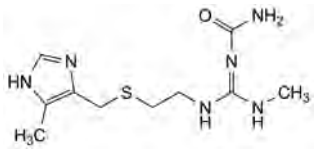

C. 1-[(methylamino)[[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]amino]methylidene]urea,

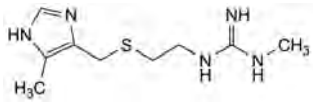

D. 1-methyl-3-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]guanidine,

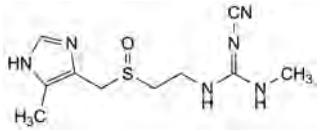

E. 2-cyano-1-methyl-3[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfinyl]ethyl]guanidine,

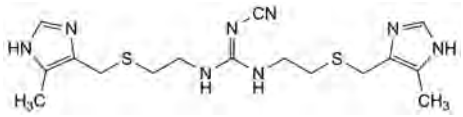

F. 2-cyano-1,3-bis[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]guanidine,

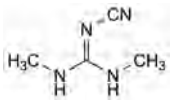

G. 2-cyano-1,3-dimethylguanidine,

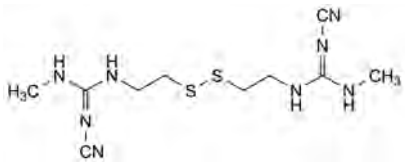

H. 1,1′-(disulfanediyldiethylene)bis(2-cyano-3-methylguanidine),

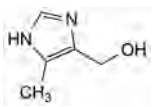

I. (5-methyl-1*H*-imidazol-4-yl)methanol,

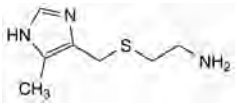

J. 2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]sulfanyl]-ethanamine.

**01/2017:1500**
**corrected 9.4**

# CIMETIDINE HYDROCHLORIDE

## Cimetidini hydrochloridum

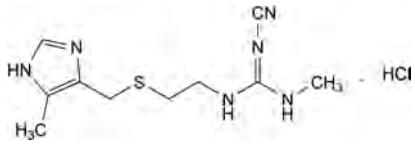

$C_{10}H_{17}ClN_6S$ $M_r$ 288.8
[70059-30-2]

DEFINITION

2-Cyano-1-methyl-3-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]guanidine hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 70 mg in *0.2 M sulfuric acid* and dilute to 100.0 mL with the same acid. Dilute 2.0 mL of this solution to 100.0 mL with *0.2 M sulfuric acid.*

*Specific absorbance at the absorption maximum at 218 nm*: 650 to 705.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cimetidine hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *cimetidine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R* (15:20:65 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of cold air

*Detection*: expose to iodine vapour until maximum contrast has been obtained and examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 3.0 g in 12 mL of *1 M hydrochloric acid* and dilute to 20 mL with *water R.*

**pH** (*2.2.3*): 4.0 to 5.0.

Dissolve 100 mg in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 2.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve the contents of a vial of *cimetidine for system suitability CRS* (containing impurities B, C, D, E, G and H) in 1.0 mL of mobile phase A.

*Reference solution (c).* Dissolve 4 mg of *cimetidine for peak identification CRS* (containing impurity F) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Mobile phase A*: mix 0.4 volumes of *diethylamine R* and 780 volumes of a 1.1 g/L solution of *sodium hexanesulfonate R.* Adjust to pH 2.8 with *phosphoric acid R* and add 250 volumes of *methanol R2*;

*Mobile phase B*: *methanol R2*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 100 | 0 |
| 60 - 65 | 100 → 90 | 0 → 10 |
| 65 - 120 | 90 | 10 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram supplied with *cimetidine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to the impurities B, C, D, E, G and H; use the chromatogram supplied with *cimetidine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to cimetidine (retention time = about 18 min): impurity G = about 0.2; impurity E = about 0.4; impurity D = about 1.5; impurity C = about 1.6; impurity B = about 2.0; impurity H = about 2.3; impurity F = about 4.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities D and C.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 2.5; impurity D = 3.3; impurity E = 0.7; impurity G = 0.6;
- *impurities B, C, D, E, F, G, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.88 mg of $C_{10}H_{17}ClN_6S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, I, J.*

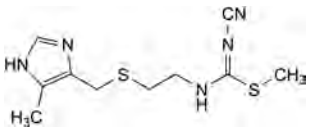

A. methyl 3-cyano-1-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]carbamimidothioate,

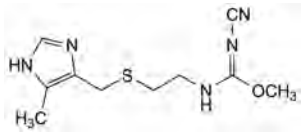

B. methyl 3-cyano-1-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]carbamimidate,

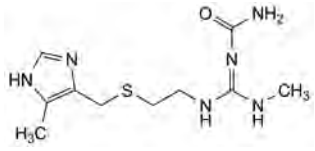

C. 1-[(methylamino)[[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]amino]methylidene]urea,

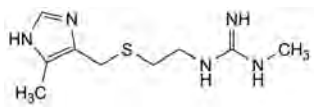

D. 1-methyl-3-[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfanyl]ethyl]guanidine,

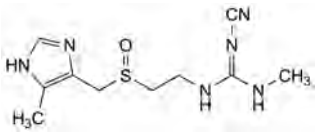

E. 2-cyano-1-methyl-3[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]sulfinyl]ethyl]guanidine,

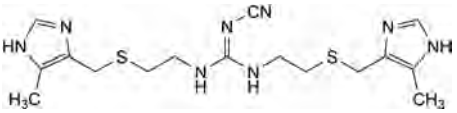

F. 2-cyano-1,3-bis[2-[[(5-methyl-1*H*-imidazol-4-yl)-methyl]-sulfanyl]ethyl]guanidine.

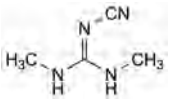

G. 2-cyano-1,3-dimethylguanidine,

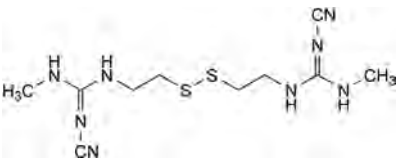

H. 1,1′-(disulfanediyldiethylene)bis(2-cyano-3-methylguanidine),

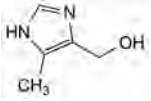

I. (5-methyl-1*H*-imidazol-4-yl)methanol,

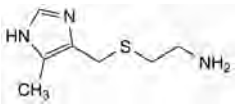

J. 2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]sulfanyl]-ethanamine.

**01/2019:1088**

# CINCHOCAINE HYDROCHLORIDE

## Cinchocaini hydrochloridum

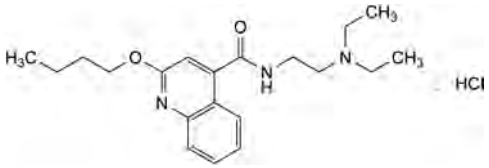

$C_{20}H_{30}ClN_3O_2$ $M_r$ 379.9
[61-12-1]

DEFINITION

2-Butoxy-*N*-[2-(diethylamino)ethyl]quinoline-4-carboxamide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic, very easily agglomerates.

*Solubility*: very soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 60.0 mg in a 103 g/L solution of *hydrochloric acid R* and dilute to 100 mL with the same acid solution. Dilute 2 mL of this solution to 100 mL with a 103 g/L solution of *hydrochloric acid R*.

*Special range*: 220-350 nm.

*Absorption maxima*: 246 nm and 319 nm.

*Absorbance ratio*: $A_{246}/A_{319}$ = 2.7 to 3.0.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cinchocaine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *cinchocaine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ammonia R*, *methanol R*, *acetone R*, *toluene R* (1:5:30:50 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air for 15 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.5 g in 5 mL of *water R*. Add 1 mL of *dilute ammonia R2.* A white precipitate is formed. Filter, wash the precipitate with 5 quantities, each of 10 mL, of *water R* and dry in a desiccator. It melts at 64 °C to 66 °C (*2.2.14*).

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.0.

Dilute 10 mL of solution S to 50 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, *water R* (10:90 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2 mg of *cinchocaine impurity A CRS* and 2 mg of *cinchocaine impurity B CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 10 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 3 mm;

– *stationary phase*: end-capped cross-linked *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: to 950 mL of *water for chromatography R* add 0.6 mL of *phosphoric acid R*, adjust to pH 2.0 with the same acid and dilute to 1000 mL with *water for chromatography R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 5.5 | 90 → 82 | 10 → 18 |
| 5.5 - 10.5 | 82 → 50 | 18 → 50 |
| 10.5 - 16.5 | 50 → 49 | 50 → 51 |
| 16.5 - 19.5 | 49 → 46 | 51 → 54 |
| 19.5 - 24.5 | 46 → 30 | 54 → 70 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 10 µL.

*Relative retention* with reference to cinchocaine (retention time = about 10.5 min): impurity B = about 0.5; impurity A = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurities B and A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of cinchocaine hydrochloride in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– reporting threshold: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 15.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.99 mg of $C_{20}H_{30}ClN_3O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

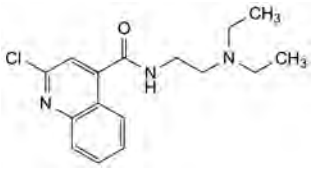

A. 2-chloro-*N*-[2-(diethylamino)ethyl]quinoline-4-carboxamide,

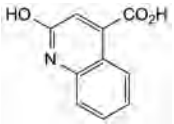

B. 2-hydroxyquinoline-4-carboxylic acid,

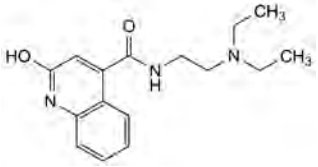

C. *N*-[2-(diethylamino)ethyl]-2-hydroxyquinoline-4-carboxamide,

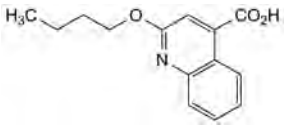

D. 2-butoxyquinoline-4-carboxylic acid.

**01/2008:1973**

# CINEOLE

## Cineolum

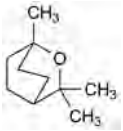

$C_{10}H_{18}O$ $M_r$ 154.3
[470-82-6]

DEFINITION

1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octane.

CHARACTERS

*Appearance*: clear colourless liquid.

*Solubility*: practically insoluble in water, miscible with alcohol and with methylene chloride.

It solidifies at about 0.5 °C.

IDENTIFICATION

A. Refractive index (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 1 mL of solution S (see Tests) to 25 mL with *alcohol R*.

*Reference solution.* Mix 80 mg of *cineole CRS* with *alcohol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: spray with *anisaldehyde solution R*, heat at 100-105 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 0.1 mL add 4 mL of *sulfuric acid R*. An orange-red colour develops. Add 0.2 mL of *formaldehyde solution R*. The colour changes to deep brown.

TESTS

**Solution S**. Dilute 2.00 g to 10.0 mL with *alcohol R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Chiral impurities**. The optical rotation (*2.2.7*) of solution S is − 0.10° to + 0.10°.

**Refractive index** (*2.2.6*): 1.456 to 1.460.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 1.0 g of *camphor R* in *heptane R* and dilute to 200 mL with the same solvent.

*Test solution (a).* Dissolve 2.5 g of the substance to be examined in *heptane R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* Dissolve 2.5 g of the substance to be examined in *heptane R*, add 5.0 mL of the internal standard solution and dilute to 25.0 mL with *heptane R*.

*Reference solution (a).* To 2.0 mL of test solution (a) add 20.0 mL of the internal standard solution and dilute to 100.0 mL with *heptane R*.

*Reference solution (b).* Dissolve 50 mg of *1,4-cineole R* and 50 mg of the substance to be examined in *heptane R* and dilute to 50.0 mL with the same solvent.

*Column*:

- *size*: *l* = 30 m, Ø = 0.25 mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 45 cm/s.

*Split-ratio*: 1:70.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 50 |
| | 10 - 35 | 50 → 100 |
| | 35 - 45 | 100 → 200 |
| | 45 - 55 | 200 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

- *resolution*: minimum 10 between the peaks due to impurity A and to cineole.

*Limits*:

- *total*: calculate the ratio (*R*) of the area of the peak due to cineole to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a); from the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to internal standard: this ratio is not greater than *R* (2 per cent),
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Residue on evaporation**: maximum 0.1 per cent.

To 2.0 g add 5 mL of *water R*, evaporate to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg.

STORAGE

In an airtight container, protected from light.

IMPURITIES

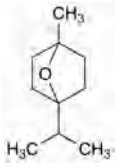

A. 1-methyl-4-(1-methylethyl)-7-oxabicyclo[2.2.1]heptane (1,4-cineole).

**01/2017:0816**

# CINNARIZINE

## Cinnarizinum

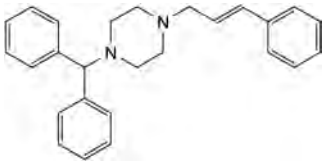

$C_{26}H_{28}N_2$ $M_r$ 368.5

[298-57-7]

DEFINITION

(*E*)-1-(Diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, slightly soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 118 °C to 122 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cinnarizine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 20 mL with the same solvent.
*Reference solution (a).* Dissolve 10 mg of *cinnarizine CRS* in *methanol R* and dilute to 20 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *cinnarizine CRS* and 10 mg of *flunarizine dihydrochloride CRS* in *methanol R* and dilute to 20 mL with the same solvent.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*
*Mobile phase*: 58.4 g/L solution of *sodium chloride R*, *methanol R*, *acetone R* (20:30:50 *V/V/V*).
*Application*: 5 µL.
*Development*: in an unsaturated tank, over 3/4 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.2 g of *anhydrous citric acid R* in 10 mL of *acetic anhydride R* in a water-bath at 80 °C and maintain the temperature of the water-bath at 80 °C for 10 min. Add about 20 mg of the substance to be examined. A purple colour develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 0.5 g in *methylene chloride R* and dilute to 20 mL with the same solvent.

**Acidity or alkalinity**. Suspend 0.5 g in 15 mL of *water R*. Boil for 2 min. Cool and filter. Dilute the filtrate to 20 mL with *carbon dioxide-free water R*. To 10 mL of this solution add 0.1 mL of *phenolphthalein solution R* and 0.25 mL of *0.01 M sodium hydroxide*. The solution is pink. To 10 mL of the solution add 0.1 mL of *methyl red solution R* and 0.25 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 12.5 mg of *cinnarizine CRS* and 15.0 mg of *flunarizine dihydrochloride CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 20.0 mL with *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 20.0 mL with *methanol R*.

*Column*:
– *size*: *l* = 0.1 m, Ø = 4.0 mm;
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
– *mobile phase A*: 10 g/L solution of *ammonium acetate R*;
– *mobile phase B*: 0.2 per cent *V/V* solution of *glacial acetic acid R* in *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 75 → 10 | 25 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to cinnarizine (retention time = about 11 min): impurity A = about 0.4; flunarizine = about 1.05; impurity B = about 1.1; impurity C = about 1.2; impurity D = about 1.6; impurity E = about 1.8.

*System suitability*: reference solution (a):
– *resolution*: minimum 5.0 between the peaks due to cinnarizine and flunarizine.

*Limits*:
– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 18.43 mg of $C_{26}H_{28}N_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

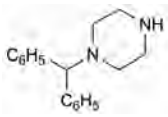

A. 1-(diphenylmethyl)piperazine,

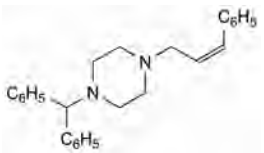

B. (*Z*)-1-(diphenylmethyl)-4-(3-phenylprop-2-enyl)piperazine,

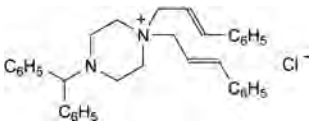

C. 4-(diphenylmethyl)-1,1-bis[(*E*)-3-phenylprop-2-enyl]piperazinium chloride,

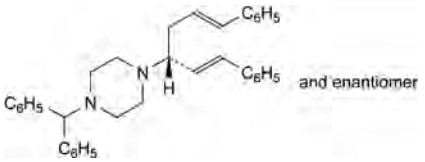

D. 1-(diphenylmethyl)-4-[(1*RS*,3*E*)-4-phenyl-1-[(*E*)-2-phenylethenyl]but-3-enyl]piperazine,

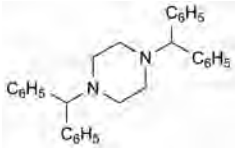

E. 1,4-bis(diphenylmethyl)piperazine.

**01/2008:2013**

# CIPROFIBRATE

## Ciprofibratum

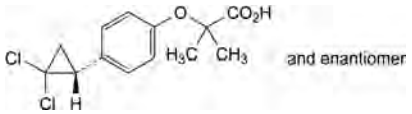

$C_{13}H_{14}Cl_2O_3$ $M_r$ 289.2
[52214-84-3]

DEFINITION

2-[4-[(1*RS*)-2,2-Dichlorocyclopropyl]phenoxy]-2-methylpropanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, soluble in toluene.

mp: about 115 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciprofibrate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method II*).

Dissolve 1.0 g in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 50 mL with the same mixture of solvents.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Reference solution (b)*. Dissolve the contents of a vial of *ciprofibrate for system suitability CRS* in 2.0 mL of a mixture of equal volumes of *acetonitrile R* and *water R*.

*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
– *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.2 with *phosphoric acid R*,
– *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 75 → 30 | 25 → 70 |
| 30 - 40 | 30 | 70 |
| 40 - 42 | 30 → 75 | 70 → 25 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *ciprofibrate for system suitability CRS* to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to ciprofibrate (retention time = about 18 min): impurity A = about 0.7; impurity B = about 0.8; impurity C = about 0.95; impurity D = about 1.3; impurity E = about 1.5.

*System suitability*: reference solution (b):

- *resolution*: baseline separation between the peaks due to impurity C and ciprofibrate.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2.3,
- *impurities A, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity E*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total of other impurities*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 350 ppm.

To 0.190 g add 20 mL of *water R* and treat in an ultrasonic bath for 8 min. Filter. 15 mL of the filtrate complies with the test.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 mL of *water R* and 40 mL of *anhydrous ethanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.92 mg of $C_{13}H_{14}Cl_2O_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

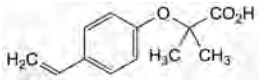

A. 2-(4-ethenylphenoxy)-2-methylpropanoic acid,

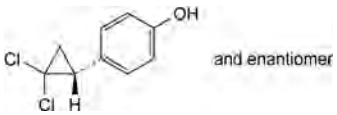


B. 4-[(1*RS*)-2,2-dichlorocyclopropyl]phenol,

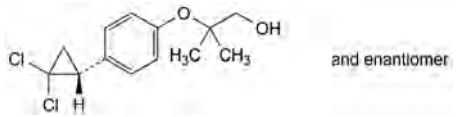


C. 2-[4-[(1*RS*)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropan-1-ol,

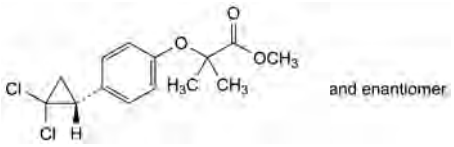


D. methyl 2-[4-[(1*RS*)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropanoate,

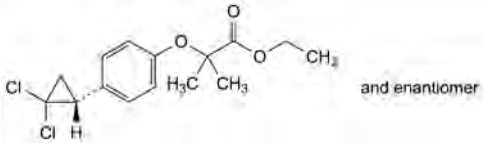


E. ethyl 2-[4-[(1*RS*)-2,2-dichlorocyclopropyl]phenoxy]-2-methylpropanoate.

**01/2017:1089**

# CIPROFLOXACIN

## Ciprofloxacinum

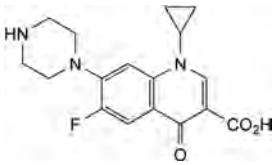

$C_{17}H_{18}FN_3O_3$ $M_r$ 331.4
[85721-33-1]

DEFINITION

1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or pale yellow, crystalline powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, very slightly soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ciprofloxacin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

Dissolve 0.25 g in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 20 mL with the same solution.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *dilute ammonia R1* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *ciprofloxacin impurity A CRS* in a mixture of 0.1 mL of *dilute ammonia R1* and 90 mL of *water R* and dilute to 100 mL with *water R*. Dilute 2 mL of the solution to 10 mL with *water R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetonitrile R*, *concentrated ammonia R*, *methanol R*, *methylene chloride R* (10:20:40:40 *V/V/V/V*).

*Application*: 5 µL.

*Development*: at the bottom of a chromatographic tank, place an evaporating dish containing 50 mL of *concentrated ammonia R*. Expose the plate to the ammonia vapour for 15 min in the closed tank. Withdraw the plate, transfer to a 2nd chromatographic tank and develop over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 25.0 mg of the substance to be examined add 0.2 mL of *dilute phosphoric acid R* and dilute to 50.0 mL with the mobile phase. Treat in an ultrasonic bath until a clear solution is obtained.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 5.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2.5 mg of *ciprofloxacin hydrochloride for peak identification CRS* (containing impurities B, C, D and E) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 13 volumes of *acetonitrile R* and 87 volumes of a 2.45 g/L solution of *phosphoric acid R*, previously adjusted to pH 3.0 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 50 µL.

*Run time*: twice the retention time of ciprofloxacin.

*Identification of impurities*: use the chromatogram supplied with *ciprofloxacin hydrochloride for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D and E.

*Relative retention* with reference to ciprofloxacin (retention time = about 9 min): impurity E = about 0.4; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.3 between the peaks due to impurities B and C.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity C = 0.6; impurity D = 1.4; impurity E = 6.7;
– *impurities B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying under vacuum at 120 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 80 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.14 mg of $C_{17}H_{18}FN_3O_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

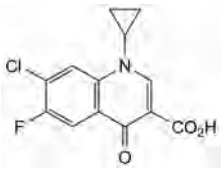


A. 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (fluoroquinolonic acid),

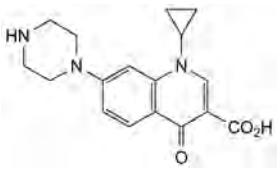


B. 1-cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (desfluoro compound),

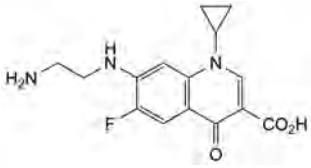


C. 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (ethylenediamine compound),

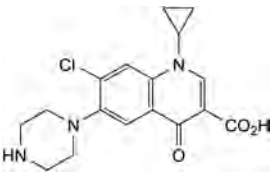


D. 7-chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

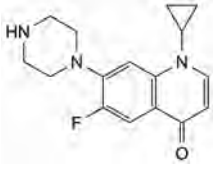


E. 1-cyclopropyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1*H*)-one (decarboxylated compound),

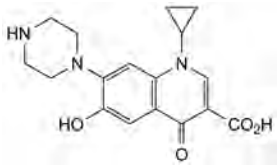

F. 1-cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

**01/2017:0888**

# CIPROFLOXACIN HYDROCHLORIDE

## Ciprofloxacini hydrochloridum

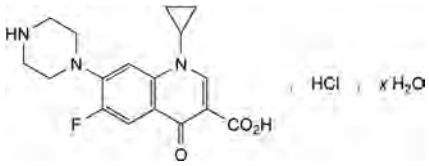

$C_{17}H_{19}ClFN_3O_3,xH_2O$ $M_r$ 367.8 (anhydrous)

DEFINITION

1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid hydrochloride. It contains a variable quantity of water.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: pale yellow, crystalline, slightly hygroscopic powder.

*Solubility*: soluble in water, slightly soluble in methanol, very slightly soluble in anhydrous ethanol, practically insoluble in acetone, in ethyl acetate and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ciprofloxacin hydrochloride CRS*.

B. 0.1 g gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**pH** (*2.2.3*): 3.5 to 4.5 for solution S.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *ciprofloxacin impurity A CRS* in a mixture of 0.1 mL of *dilute ammonia R1* and 90 mL of *water R* and dilute to 100 mL with *water R*. Dilute 2 mL of the solution to 10 mL with *water R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetonitrile R*, *concentrated ammonia R*, *methanol R*, *methylene chloride R* (10:20:40:40 *V/V/V/V*).

*Application*: 5 µL.

*Development*: at the bottom of a chromatographic tank, place an evaporating dish containing 50 mL of *concentrated ammonia R*. Expose the plate to the ammonia vapour for 15 min in the closed tank. Withdraw the plate, transfer to a 2nd chromatographic tank and develop over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *ciprofloxacin hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2.5 mg of *ciprofloxacin hydrochloride for peak identification CRS* (containing impurities B, C, D and E) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 13 volumes of *acetonitrile R* and 87 volumes of a 2.45 g/L solution of *phosphoric acid R* previously adjusted to pH 3.0 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.3 times the retention time of ciprofloxacin.

*Identification of impurities*: use the chromatogram supplied with *ciprofloxacin hydrochloride for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D and E.

*Relative retention* with reference to ciprofloxacin (retention time = about 9 min): impurity E = about 0.4; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.3 between the peaks due to impurities B and C.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity C = 0.6; impurity D = 1.4; impurity E = 6.7;
– *impurity E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
– *impurities B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 6.7 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{19}ClFN_3O_3$ taking into account the assigned content of *ciprofloxacin hydrochloride CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

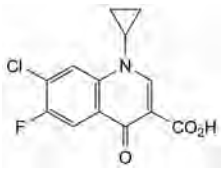

A. 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (fluoroquinolonic acid),

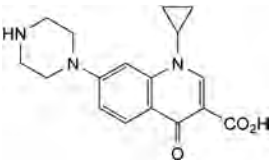

B. 1-cyclopropyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (desfluoro compound),

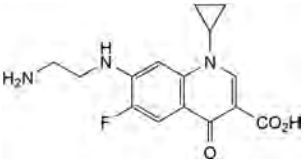

C. 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (ethylenediamine compound),

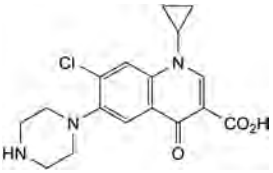

D. 7-chloro-1-cyclopropyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

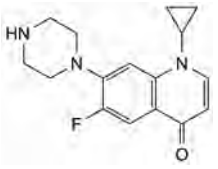

E. 1-cyclopropyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1*H*)-one (decarboxylated compound),

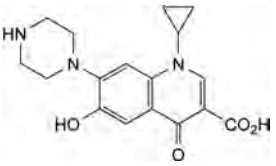

F. 1-cyclopropyl-6-hydroxy-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

**04/2016:2763**
**corrected 9.4**

# CISATRACURIUM BESILATE

## Cisatracurii besilas

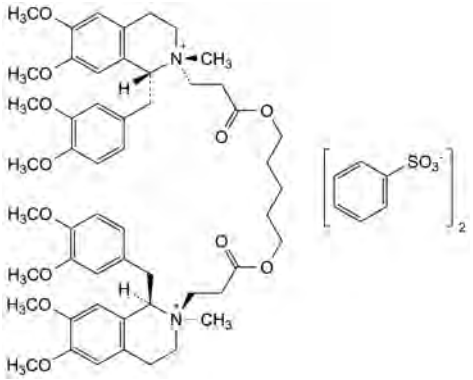

$C_{65}H_{82}N_2O_{18}S_2$ $M_r$ 1243
[96946-42-8]

DEFINITION

2,2′-[Pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[(1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium] dibenzenesulfonate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl benzenesulfonate esters are genotoxic and are potential impurities in cisatracurium besilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general method *2.5.41. Methyl, ethyl and isopropyl benzenesulfonate in active substances* is available to assist manufacturers.

CHARACTERS

*Appearance*: white or yellowish, hygroscopic powder.

*Solubility*: soluble in water, very soluble in anhydrous ethanol, freely soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cisatracurium besilate CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 1.00 g in *water R* and dilute to 100 mL with the same solvent.

**Diastereoisomers**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 0.100 g of the substance to be examined in a 0.1 per cent *V/V* solution of *diethylamine R* in *methanol R* and dilute to 25.0 mL with the same solution. Heat in a water-bath at 60 °C for 15 min then cool to room temperature.

*Reference solution*. Dissolve 8 mg of *laudanosine CRS* in a 0.1 per cent *V/V* solution of *diethylamine R* in *methanol R* and dilute to 2.0 mL with the same solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm).

*Mobile phase*: *propanol R1*, 0.1 per cent *V/V* solution of *diethylamine R* in *heptane R* (15:85 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of *R*-laudanosine.

*Elution order*: *S*-laudanosine; *R*-laudanosine.

*Retention time*: *R*-laudanosine = about 18 min.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to *S*-laudanosine and *R*-laudanosine.

Calculate the percentage content of the sum of impurities S, T, U and V by multiplying the percentage of *S*-laudanosine by 2.

*Limit*:

- *sum of impurities S, T, U, V*: maximum 0.5 per cent; disregard any peak other than *S*-laudanosine and *R*-laudanosine.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*. Mix 20 volumes of *acetonitrile R* and 80 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.1 with *phosphoric acid R*.

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *cisatracurium besilate CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2.0 mg of *cisatracurium for system suitability CRS* (containing impurities D, F, N, O, P, Q and H and/or T and/or V) in 1.0 mL of the solvent mixture.

*Reference solution (d)*. Dissolve 2.0 mg of *cisatracurium for peak identification CRS* (containing impurities A, C, I (epimer 1) and I (epimer 2) and/or K and/or L and/or M) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (2 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *methanol R*, 15 volumes of *acetonitrile R* and 80 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *methanol R*, *acetonitrile R* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 92 → 82 | 8 → 18 |
| 1 - 8 | 82 → 80 | 18 → 20 |
| 8 - 10 | 80 → 70 | 20 → 30 |
| 10 - 12 | 70 → 45 | 30 → 55 |
| 12 - 20 | 45 | 55 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Autosampler*: set at 4 °C.

*Injection*: 5 µL of test solution (a) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *cisatracurium for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D, F, H + T + V, N, O, P, Q; use the chromatogram supplied with *cisatracurium for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, C, I (epimer 1) and I (epimer 2) + K + L + M.

*Relative retention* with reference to cisatracurium (retention time = about 9 min): impurity A = about 0.3; impurity C = about 0.4; impurity D = about 0.45; impurity F = about 0.55; impurities H, T and V = about 0.97; impurity I (epimer 1) = about 1.25; impurities I (epimer 2), K, L and M = about 1.3; impurity N = about 1.4; impurity O = about 1.5; impurity P = about 1.6; impurity Q = about 1.65.

Impurity M can coelute or be separated from impurities I (epimer 2), K and L.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity H and/or T and/or V and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cisatracurium.

*Limits*:

- *correction factor*: multiply the peak area of impurity C by 0.5;
- *sum of impurities H, T, V*: maximum 0.8 per cent;
- *impurity Q*: maximum 0.7 per cent;
- *impurities A, C, F, O, P*: for each impurity, maximum 0.5 per cent;
- *impurity D*: maximum 0.4 per cent;
- *impurity N*: maximum 0.3 per cent;
- *sum of impurities I, K, L, M*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 2.5 per cent;
- *reporting threshold*: 0.05 per cent (reference solution (b)).

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications:

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 2.5 for the principal peak.

Calculate the percentage content of $C_{65}H_{82}N_2O_{18}S_2$ taking into account the assigned content of *cisatracurium besilate CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

**Test for diastereoisomers**: S.

**Test for related substances**: *A, B, C, D, E, F, G, H, I, K, L, M, N, O, P, Q, R, T, U, V, W*.

*Specified impurities: A, C, D, F, H, I, K, L, M, N, O, P, Q, S, T, V*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E, G, R, U, W*.

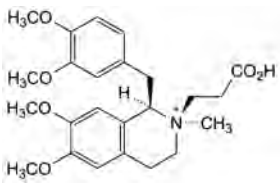

A. (1*R*,2*R*)-2-(2-carboxyethyl)-1-[(3,4-dimethoxyphenyl)-methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

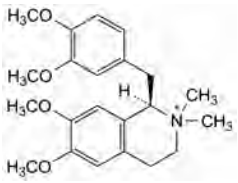

B. (1*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium,

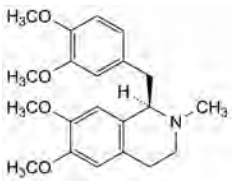

C. (1*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (1*R*-laudanosine),

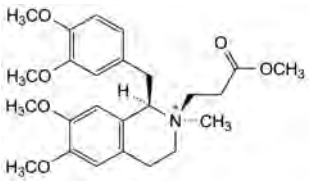

D. (1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-(3-methoxy-3-oxopropyl)-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

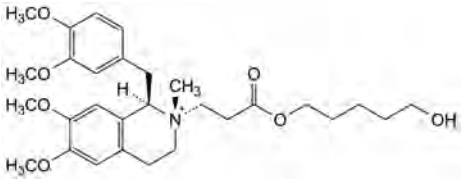

E. (1*R*,2*S*)-1-[(3,4-dimethoxyphenyl)methyl]-2-[3-[(5-hydroxypentyl)oxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

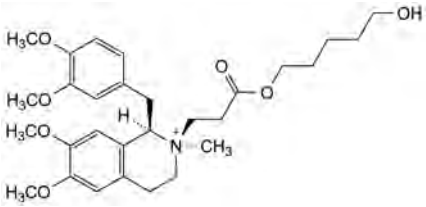

F. (1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-2-[3-[(5-hydroxypentyl)oxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

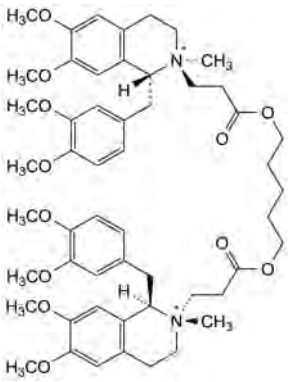

G. 2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[(1*R*,2*S*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

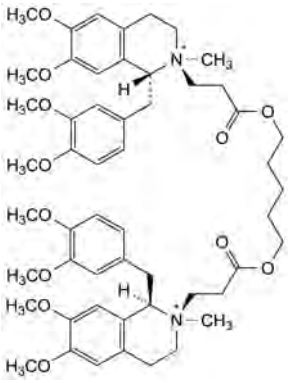

H. (1*R*,1′*R*,2*R*,2′*S*)-2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[1-[(3,4-dimethoxyphenyl)-methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

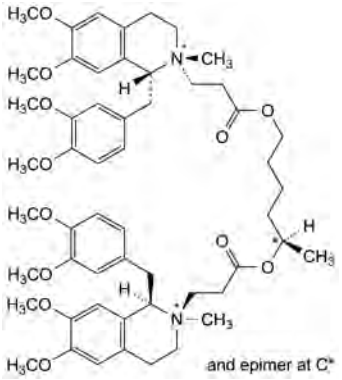


I. 2,2′-[[(1*RS*)-1-methylpentane-1,5-diyl]bis[oxy(3-oxopropane-3,1-diyl)]]bis[(1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

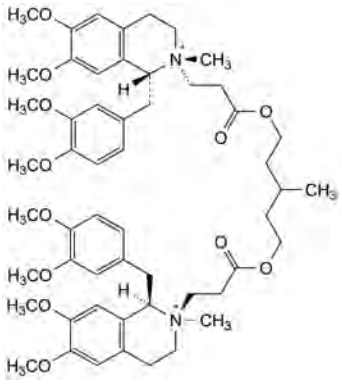

K. 2,2′-[(3-methylpentane-1,5-diyl)bis[oxy(3-oxopropane-3,1-diyl)]]bis[(1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

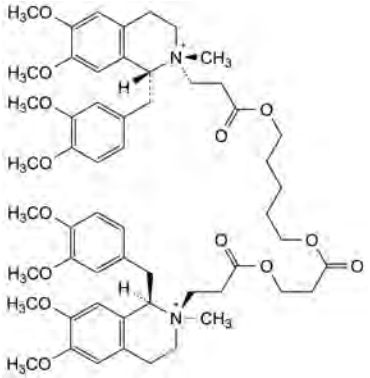

L. (1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-2-[3-[3-[[5-[[3-[(1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-3,4-dihydroisoquinolinium-2(1*H*)-yl]propanoyl]oxy]pentyl]oxy]-3-oxopropoxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

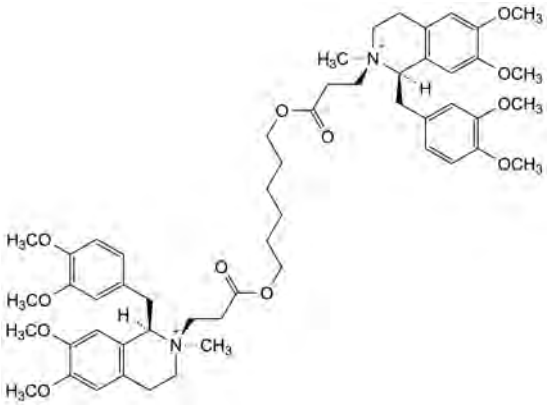

M. 2,2′-[hexane-1,6-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[(1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

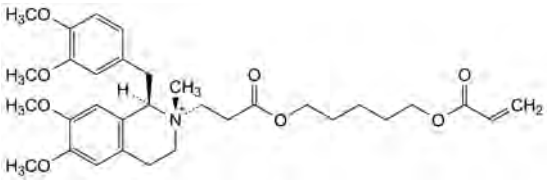

N. (1*R*,2*S*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-2-[3-[[5-(prop-2-enoyloxy)pentyl]oxy]-3-oxopropyl]-1,2,3,4-tetrahydroisoquinolinium,

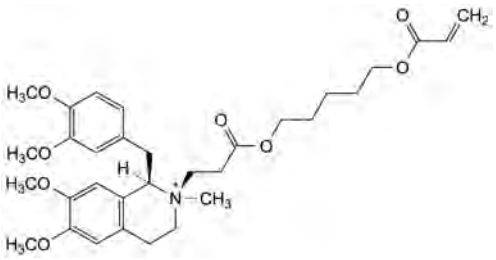

O. (1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-2-[3-[[5-(prop-2-enoyloxy)pentyl]oxy]-3-oxopropyl]-1,2,3,4-tetrahydroisoquinolinium,

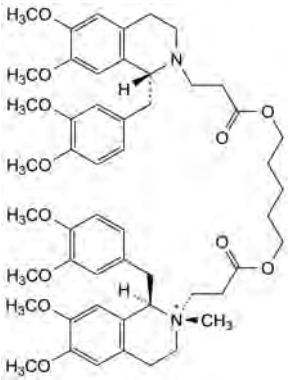

P. (1*R*,2*S*)-1-[(3,4-dimethoxyphenyl)methyl]-2-[3-[[5-[[3-[(1*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propanoyl]oxy]pentyl]oxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

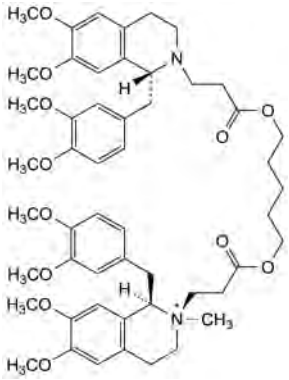

Q. (1*R*,2*R*)-1-[(3,4-dimethoxyphenyl)methyl]-2-[3-[[5-[[3-[(1*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propanoyl]oxy]pentyl]oxy]-3-oxopropyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium,

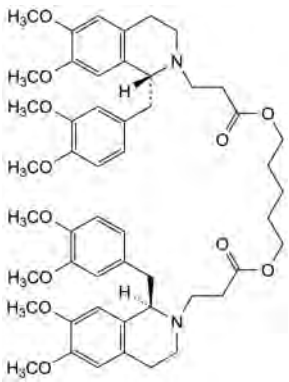

R. pentane-1,5-diyl bis[3-[(1*R*)-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-3,4-dihydroisoquinolin-2(1*H*)-yl]propanoate],

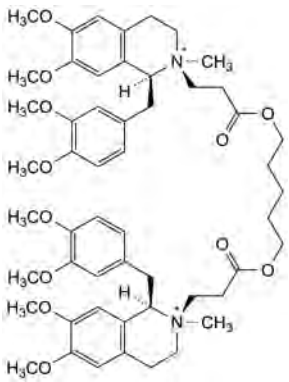

S. (1*R*,1′*S*,2*R*,2′*S*)-2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

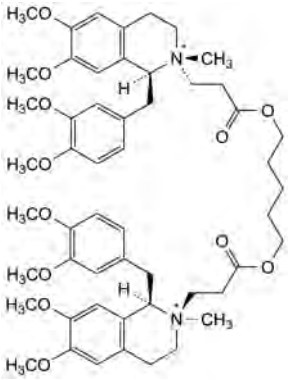

T. (1*R*,1′*S*,2*R*,2′*R*)-2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

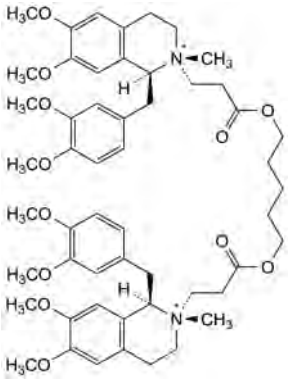

U. (1*R*,1′*S*,2*S*,2′*R*)-2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

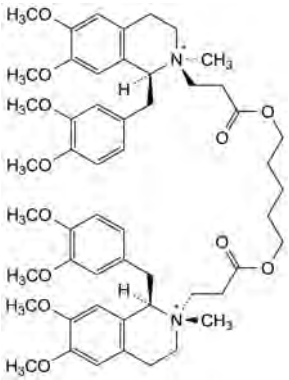

V. (1*S*,1′*R*,2*S*,2′*S*)-2,2′-[pentane-1,5-diylbis[oxy(3-oxopropane-3,1-diyl)]]bis[1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinolinium],

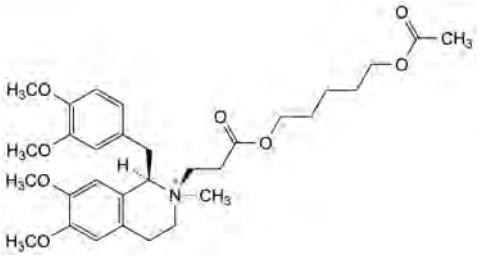

W. (1*R*,2*R*)-2-[3-[[5-(acetyloxy)pentyl]oxy]]-1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxy-2-methyl-3-oxopropyl-1,2,3,4-tetrahydroisoquinolinium.

**01/2009:0599**
**corrected 7.0**

# CISPLATIN

## Cisplatinum

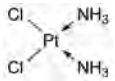


$PtCl_2(NH_3)_2$ $M_r$ 300.0
[15663-27-1]

DEFINITION

*cis*-Diamminedichloroplatinum(II).

*Content*: 97.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: yellow powder, or yellow or orange-yellow crystals.

*Solubility*: slightly soluble in water, sparingly soluble in dimethylformamide, practically insoluble in ethanol (96 per cent).

*Carry out identification test B, the tests (except that for silver) and the assay protected from light.*

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cisplatin CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dilute 1 mL of solution S2 (see Tests) to 10 mL with *dimethylformamide R.*
*Reference solution.* Dissolve 10 mg of *cisplatin CRS* in 5 mL of *dimethylformamide R.*
*Plate*: *cellulose for chromatography R1* as the coating substance.
*Pretreatment*: activate the plate by heating at 150 °C for 1 h.
*Mobile phase*: *acetone R*, *dimethylformamide R* (10:90 *V/V*).
*Application*: 2 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with a 50 g/L solution of *stannous chloride R* in a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Examine after 1 h.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Add 50 mg to 2 mL of *dilute sodium hydroxide solution R* in a glass dish. Evaporate to dryness. Dissolve the residue in a mixture of 0.5 mL of *nitric acid R* and 1.5 mL of *hydrochloric acid R*. Evaporate to dryness. The residue is orange. Dissolve the residue in 0.5 mL of *water R* and add 0.5 mL of *ammonium chloride solution R*. A yellow, crystalline precipitate is formed.

TESTS

**Solution S1**. Dissolve 25 mg in a 9 g/L solution of *sodium chloride R* in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Solution S2**. Dissolve 0.20 g in *dimethylformamide R* and dilute to 10 mL with the same solvent.

**Appearance of solution S1**. Solution S1 is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

**Appearance of solution S2**. Solution S2 is clear (*2.2.1*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S1, measured immediately after preparation.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Do not heat or sonicate any platinum-containing solution. All solutions are to be used within 4 h.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in a 9.0 g/L solution of *sodium chloride R* and dilute to 25.0 mL with the same solution.

*Reference solution (a).* Dissolve 25.0 mg of *cisplatin CRS* in a 9.0 g/L solution of *sodium chloride R* and dilute to 25.0 mL with the same solution.

*Reference solution (b).* Dissolve 5.0 mg of *cisplatin impurity A CRS* in a 9.0 g/L solution of *sodium chloride R* and dilute to 50.0 mL with the same solution.

*Reference solution (c).* Dissolve 5.6 mg of *cisplatin impurity B CRS* in a 9.0 g/L solution of *sodium chloride R* and dilute to 100.0 mL with the same solution.

*Reference solution (d).* Mix 0.05 mL of the test solution with 5.0 mL of reference solution (b) and 5.0 mL of reference solution (c) and dilute to 25.0 mL with a 9.0 g/L solution of *sodium chloride R.*

*Reference solution (e).* Dilute 5.0 mL of reference solution (d) to 20.0 mL with a 9.0 g/L solution of *sodium chloride R.*

*Blank solution*: 9.0 g/L solution of *sodium chloride R.*

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 1.08 g of *sodium octanesulfonate R*, 1.70 g of *tetrabutylammonium hydrogen sulfate R* and 2.72 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to 950 mL with the same solvent. Adjust to pH 5.9 with *1 M sodium hydroxide* and dilute to 1000 mL with *water for chromatography R.*

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution, reference solutions (d) and (e), and the blank solution.

*Run time*: 7 times the retention time of cisplatin.

The displacement peak is the latest eluting peak of the group of injection peaks in the chromatogram obtained with the blank solution.

*Identification of cisplatin aquo complex*: use the chromatogram supplied with *cisplatin CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to cisplatin aquo complex.

*Relative retention* with reference to cisplatin (retention time = about 3.8 min): displacement peak = about 0.5; impurity A = about 0.6; impurity B = about 0.7; cisplatin aquo complex = about 1.2.

*System suitability*: reference solution (d):
- *resolution*: minimum 2.5 between the peaks due to impurities A and B, the displacement peak and the peak due to impurity A are well separated.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (2.0 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (1.0 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to cisplatin in the chromatogram obtained with reference solution (d) (0.10 per cent);
– *sum of impurities other than A and B*: not more than 2.5 times the area of the peak due to cisplatin in the chromatogram obtained with reference solution (d) (0.5 per cent);
– *disregard limit*: the area of the peak due to cisplatin in the chromatogram obtained with reference solution (e) (0.05 per cent). Disregard any peak due to the cisplatin aquo complex.

**Silver**: maximum 250 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 0.100 g in 15 mL of *nitric acid R*, heating to 80 °C. Cool and dilute to 25.0 mL with *water R*.

*Reference solutions.* To suitable volumes (10 mL to 30 mL) of *silver standard solution (5 ppm Ag) R* add 50 mL of *nitric acid R* and dilute to 100.0 mL with *water R*.

*Source*: silver hollow-cathode lamp, preferably using a transmission band of 0.5 nm.

*Wavelenth*: 328 nm.

*Atomisation device*: fuel-lean air-acetylene flame.

Carry out a blank determination.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $PtCl_2(NH_3)_2$ from the sum of the areas of the peaks due to cisplatin and cisplatin aquo complex and from the declared content of *cisplatin CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

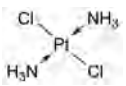


A. *trans*-diamminedichloroplatinum(II) (transplatin),

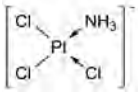


B. amminetrichloroplatinate(–),

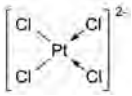


C. tetrachloroplatinate(2–).

**01/2017:2288**
**corrected 9.3**

# CITALOPRAM HYDROBROMIDE

## Citaloprami hydrobromidum

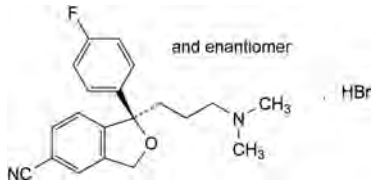


$C_{20}H_{22}BrFN_2O$ $M_r$ 405.3
[59729-32-7]

DEFINITION

(1*RS*)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile hydrobromide.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water and in anhydrous ethanol.

IDENTIFICATION

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *citalopram hydrobromide CRS*.

C. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A (solution A). Dilute 1.0 mL of solution A to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve the contents of a vial of *citalopram for system suitability CRS* (containing impurities B, D and G) in 1.0 mL of solution A.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (4 µm);
– *temperature*: 40 °C.

*Mobile phase*:
– *mobile phase A*: dissolve 1.58 g of *ammonium formate R* in 500 mL of a mixture of 4 volumes of *acetonitrile for chromatography R*, 32 volumes of *methanol R1* and 64 volumes of *water for chromatography R*;
– *mobile phase B*: dissolve 1.58 g of *ammonium formate R* in 500 mL of a mixture of 32 volumes of *water for chromatography R* and 68 volumes of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 25 | 100 → 40 | 0 → 60 |
| 25 - 30 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm and, for impurity G, at 254 nm.

*Injection*: 40 µL.

*Identification of impurities*: use the chromatogram supplied with *citalopram for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, D and G.

*Relative retention* with reference to citalopram (retention time = about 19 min): impurity G = about 0.5; impurity B = about 0.7; impurity D = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity D and citalopram at 230 nm.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity G by 0.6;

– *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurity B*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *impurity G at 254 nm*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *sum of impurities other than G*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of *ethanol (96 per cent) R* and add 0.5 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 40.53 mg of $C_{20}H_{22}BrFN_2O$.

IMPURITIES

*Specified impurities: B, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F.*

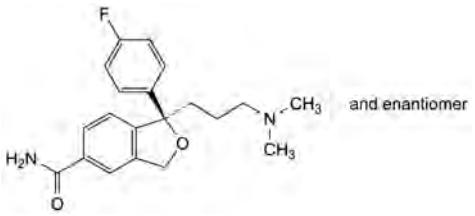


A. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide,

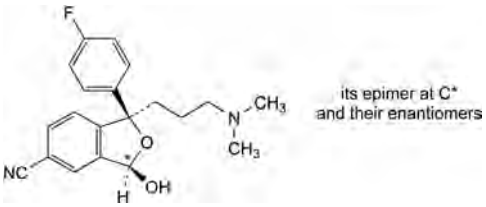


B. 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3dihydroisobenzofuran-5-carbonitrile,

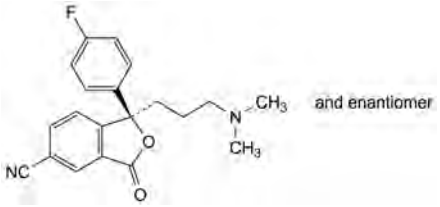


C. (3*RS*)-6-cyano-3-[3-(dimethylamino)propyl]-3-(4-fluorophenyl)isobenzofuran-1(3*H*)-one,

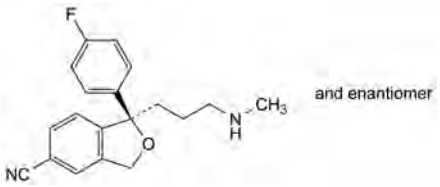


D. (1*RS*)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile,

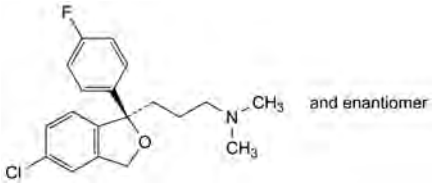


E. 3-[(1*RS*)-5-chloro-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine,

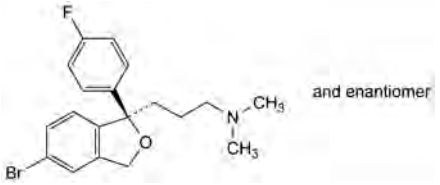


F. 3-[(1*RS*)-5-bromo-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine,

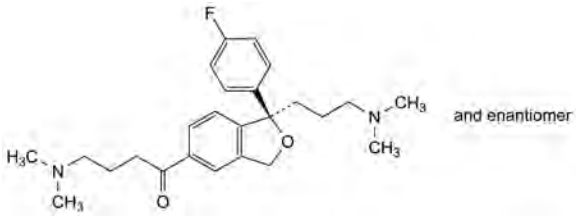


G. 4-(dimethylamino)-1-[(1*RS*)-1-[3-(dimethylamino)-propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-yl]butan-1-one.



**01/2017:2203**
**corrected 9.3**

# CITALOPRAM HYDROCHLORIDE

## Citaloprami hydrochloridum

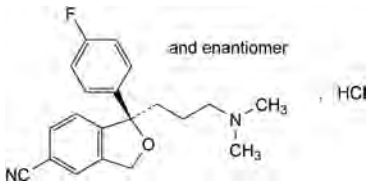


$C_{20}H_{22}ClFN_2O$ $M_r$ 360.9
[85118-27-0]

DEFINITION

(1*RS*)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carbonitrile hydrochloride.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in anhydrous ethanol.

IDENTIFICATION

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *citalopram hydrochloride CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S, examined immediately after preparation, is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A (solution A). Dilute 1.0 mL of solution A to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *citalopram for system suitability CRS* (impurities B and D) in 1.0 mL of solution A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (4 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 1.58 g of *ammonium formate R* in 500 mL of a mixture of 4 volumes of *acetonitrile for chromatography R*, 32 volumes of *methanol R1* and 64 volumes of *water for chromatography R*;
- *mobile phase B*: dissolve 1.58 g of *ammonium formate R* in 500 mL of a mixture of 32 volumes of *water for chromatography R* and 68 volumes of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 25 | 100 → 40 | 0 → 60 |
| 25 - 30 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 40 µL.

*Identification of impurities*: use the chromatogram supplied with *citalopram for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and D.

*Relative retention* with reference to citalopram (retention time = about 19 min): impurity B = about 0.7; impurity D = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurity D and citalopram.

*Limits*:
- *impurity B*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 0.5 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.09 mg of $C_{20}H_{22}ClFN_2O$.

IMPURITIES

*Specified impurities*: *B*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F*.

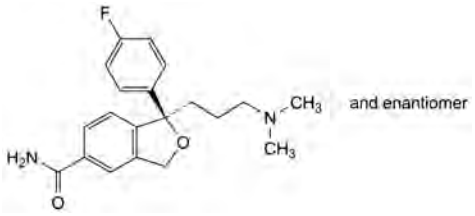


A. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-5-carboxamide,

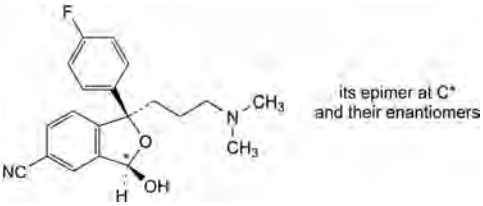


B. 1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydroisobenzofuran-5-carbonitrile,

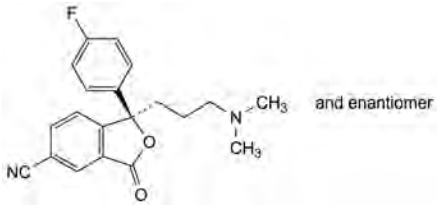


C. (3*RS*)-6-cyano-3-[3-(dimethylamino)propyl]-3-(4-fluorophenyl)isobenzofuran-1(3*H*)-one,

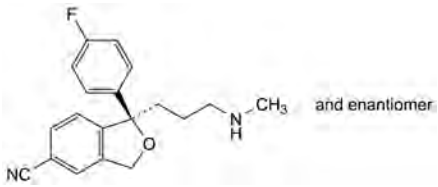


D. (1*RS*)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile,

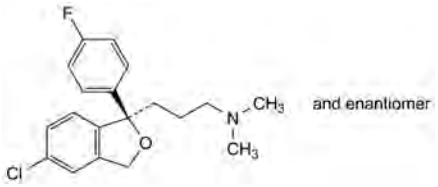


E. 3-[(1*RS*)-5-chloro-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine,

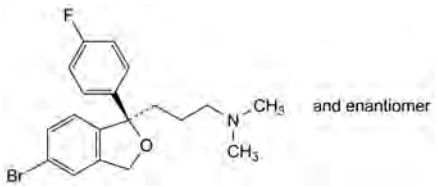


F. 3-[(1*RS*)-5-bromo-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine.

**01/2017:0455**

# CITRIC ACID[(7)]

## Acidum citricum

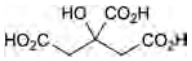


$C_6H_8O_7$ $M_r$ 192.1
[77-92-9]

DEFINITION

2-Hydroxypropane-1,2,3-tricarboxylic acid.

*Content*: 99.5 per cent to 100.5 per cent (anhydrous substance).

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder, colourless crystals or granules.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

mp: about 153 °C, with decomposition.♦

IDENTIFICATION

*First identification: B,◊E.*

*Second identification: A, C, D, E.*

A. Dissolve 1 g in 10 mL of *water R*. The solution is strongly acidic (*2.2.4*).◊

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined and the reference substance at 105 ± 2 °C for 2 h.

*Comparison*: *anhydrous citric acid CRS*.

◊C. Add about 5 mg to a mixture of 1 mL of *acetic anhydride R* and 3 mL of *pyridine R*. A red colour develops.

D. Dissolve 0.5 g in 5 mL of *water R*, neutralise using *1 M sodium hydroxide* (about 7 mL), add 10 mL of *calcium chloride solution R* and heat to boiling. A white precipitate is formed.

E. Water (see Tests).◊

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless or not more intensely coloured than reference solution $Y_7$, $BY_7$ or $GY_7$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10 mL with the same solvent.

**Readily carbonisable substances**. To 1.0 g in a cleaned test tube add 10 mL of *sulfuric acid R* and immediately heat the mixture in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly immediately afterwards. The solution is not more intensely coloured than a mixture of 1 mL of red primary solution and 9 mL of yellow primary solution (*2.2.2, Method I*).

**Oxalic acid**: maximum 360 ppm, calculated as anhydrous oxalic acid.

Dissolve 0.80 g in 4 mL of *water R*. Add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules. Boil for 1 min. Allow to stand for 2 min. Transfer the supernatant to a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of a 50 g/L solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 mL of a 0.1 g/L solution of *oxalic acid R*.

(7) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dissolve 2.0 g in *distilled water R* and dilute to 30 mL with the same solvent.

**Aluminium** (*2.4.17*): maximum 0.2 ppm, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution.* Dissolve 20 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution.* Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution.* Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 2.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

♦**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.♦

ASSAY

Dissolve 0.550 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 64.03 mg of $C_6H_8O_7$.

♦LABELLING

The label states, where applicable, that the substance is intended for use in the manufacture of dialysis solutions.♦

**01/2017:0456**

# CITRIC ACID MONOHYDRATE(8)

## Acidum citricum monohydricum

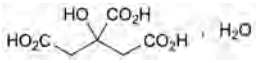

$C_6H_8O_7,H_2O$ $M_r$ 210.1

[5949-29-1]

DEFINITION

2-Hydroxypropane-1,2,3-tricarboxylic acid monohydrate.

*Content*: 99.5 per cent to 100.5 per cent (anhydrous substance).

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder, colourless crystals or granules, efflorescent.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).♦

IDENTIFICATION

*First identification: B,◊E.*

*Second identification: A, C, D, E.*

A. Dissolve 1 g in 10 mL of *water R*. The solution is strongly acidic (*2.2.4*).◊

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined and the reference substance at 105 ± 2 °C for 2 h.

*Comparison*: *citric acid monohydrate CRS*.

◊C. Add about 5 mg to a mixture of 1 mL of *acetic anhydride R* and 3 mL of *pyridine R*. A red colour develops.

D. Dissolve 0.5 g in 5 mL of *water R*, neutralise using *1 M sodium hydroxide* (about 7 mL), add 10 mL of *calcium chloride solution R* and heat to boiling. A white precipitate is formed.

E. Water (see Tests).◊

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless or not more intensely coloured than reference solution $Y_7$, $BY_7$ or $GY_7$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10 mL with the same solvent.

**Readily carbonisable substances**. To 1.0 g in a cleaned test tube add 10 mL of *sulfuric acid R* and immediately heat the mixture in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly immediately afterwards. The solution is not more intensely coloured than a mixture of 1 mL of red primary solution and 9 mL of yellow primary solution (*2.2.2, Method I*).

**Oxalic acid**: maximum 360 ppm, calculated as anhydrous oxalic acid.

Dissolve 0.80 g in 4 mL of *water R*. Add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules. Boil for 1 min. Allow to stand for 2 min. Transfer the supernatant to a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of a 50 g/L solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 mL of a 0.1 g/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dissolve 2.0 g in *distilled water R* and dilute to 30 mL with the same solvent.

**Aluminium** (*2.4.17*): maximum 0.2 ppm, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution.* Dissolve 20 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution.* Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution.* Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Water** (*2.5.12*): 7.5 per cent to 9.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

♦**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.♦

ASSAY

Dissolve 0.550 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 64.03 mg of $C_6H_8O_7$.

♦STORAGE

In an airtight container.

LABELLING

The label states, where applicable, that the substance is intended for use in the manufacture of dialysis solutions.♦

(8) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**01/2011:2174**
**corrected 10.0**

# CLADRIBINE

## Cladribinum

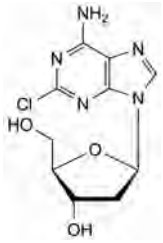

$C_{10}H_{12}ClN_5O_3$ $M_r$ 285.7
[4291-63-8]

DEFINITION

2-Chloro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-9*H*-purin-6-amine.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in dimethyl sulfoxide, slightly soluble in methanol, practically insoluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cladribine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined in the minimum volume of *methanol R* and evaporate to dryness. Dry the precipitate at 100 °C for 2 h and record a new spectrum using the residue.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Disperse 0.15 g in *water R*, dilute to 50 mL with the same solvent and sonicate until dissolution is complete.

**Specific optical rotation** (*2.2.7*): – 21.0 to – 27.0 (anhydrous substance).

Dissolve 0.25 g in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 2.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *2-deoxy-D-ribose R* (impurity E) in *dimethylformamide R* and dilute to 25.0 mL with the same solvent. Dilute 3.0 mL of this solution to 10.0 mL with *dimethylformamide R*.

*Reference solution (b)*. Dissolve 10.0 mg of *2-deoxy-D-ribose R* (impurity E) in *dimethylformamide R* and dilute to 5.0 mL with the same solvent. Mix 9 volumes of this solution with 1 volume of the test solution.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *ethyl acetate R* (20:40:40 *V/V/V*).

*Application*: 5 µL as bands of 10 mm; thoroughly dry the points of application in a current of warm air.

*Development*: over 2/3 of the plate.

*Drying*: in air, then heat at 45 °C for 10 min.

*Detection*: spray with a solution containing 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*; heat at 110 °C for 20 min or until the spots appear.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity E*: any spot due to impurity E is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 20.0 mg of *cladribine CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 1.0 mg of *cladribine impurity C CRS* in reference solution (b) and dilute to 25.0 mL with the same solution.

*Reference solution (e)*. Dilute 5.0 mL of reference solution (c) to 10.0 mL with the solvent mixture.

*Reference solution (f)*. Dissolve 3 mg of *cladribine for peak identification CRS* (containing impurities A, B, C and D) in 2 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

– *mobile phase C*: 50 g/L solution of *phosphoric acid R* in *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 10 | 80 → 70 | 10 → 20 | 10 |
| 10 - 25 | 70 → 20 | 20 → 70 | 10 |
| 25 - 30 | 20 | 70 | 10 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 252 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (c), (d), (e) and (f).

*Identification of impurities*: use the chromatogram supplied with *cladribine for peak identification CRS* and the chromatogram obtained with reference solution (f) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to cladribine (retention time = about 10 min): impurity A = about 0.33; impurity B = about 0.44; impurity C = about 0.73; impurity D = about 0.92.

*System suitability*: reference solution (d):

– *resolution*: minimum 4.5 between the peaks due to impurity C and cladribine.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.7; impurity C = 0.8;

– *impurities A, C*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);

– *impurities B, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 3 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{10}H_{12}ClN_5O_3$ from the declared content of *cladribine CRS*.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G.*

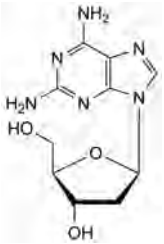

A. 9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-9*H*-purin-2,6-diamine,

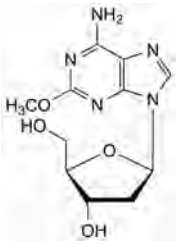

B. 9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-2-methoxy-9*H*-purin-6-amine,

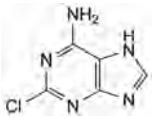

C. 2-chloro-7*H*-purin-6-amine (2-chloroadenine),

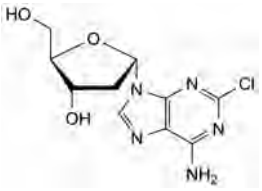

D. 2-chloro-9-(2-deoxy-α-D-*erythro*-pentofuranosyl)-9*H*-purin-6-amine,

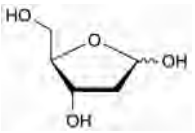

E. 2-deoxy-D-*erythro*-pentofuranose (2-deoxy-D-ribose),

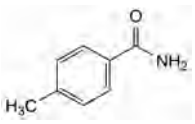

F. 4-methylbenzamide,

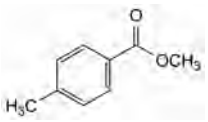

G. methyl 4-methylbenzoate.

**04/2018:1651**

# CLARITHROMYCIN

## Clarithromycinum

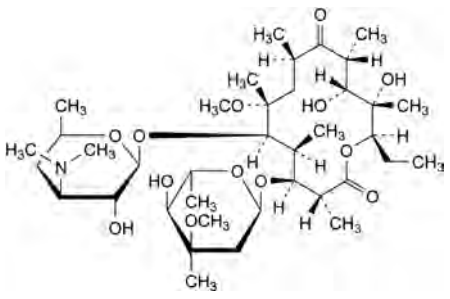

$C_{38}H_{69}NO_{13}$ $M_r$ 748

[81103-11-9]

DEFINITION

(3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-12,13-dihydroxy-7-methoxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (6-*O*-methylerythromycin A).

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clarithromycin CRS*.

TESTS

**Solution S**. Dissolve 0.500 g in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear or not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): – 102 to – 94 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 75.0 mg of the substance to be examined in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. Dissolve 75.0 mg of *clarithromycin CRS* in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 100.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (d)*. Dissolve 3.0 mg of *clarithromycin for peak identification CRS* in 1.0 mL of *acetonitrile R1* and dilute to 2.0 mL with *water R*.

*Blank solution*. Dilute 25.0 mL of *acetonitrile R1* to 50.0 mL with *water R* and mix.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: a 4.76 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 4.4 with *dilute phosphoric acid R* or a 45 g/L solution of *potassium hydroxide R*, filtered through a C18 filtration kit;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 32 | 75 → 40 | 25 → 60 |
| 32 - 34 | 40 | 60 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL of the blank solution, the test solution and reference solutions (b), (c) and (d).

*Relative retention r* (not $r_G$) with reference to clarithromycin (retention time = about 11 min): impurity I = about 0.38; impurity A = about 0.42; impurity J = about 0.63; impurity L = about 0.74; impurity B = about 0.79; impurity M = about 0.81; impurity C = about 0.89; impurity D = about 0.96; impurity N = about 1.15; impurity E = about 1.27; impurity F = about 1.33; impurity P = about 1.35; impurity O = about 1.41; impurity K = about 1.59; impurity G = about 1.72; impurity H = about 1.82.

*System suitability*:

- *symmetry factor*: maximum 1.7 for the peak due to clarithromycin in the chromatogram obtained with reference solution (b);
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clarithromycin in the chromatogram obtained with reference solution (d).

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 0.27; impurity H = 0.15; use the chromatogram supplied with *clarithromycin for peak identification CRS* to identify the peaks;
- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent), and not more than 4 such peaks have an area greater than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.4 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent); disregard the peaks eluting before impurity I and after impurity H.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{38}H_{69}NO_{13}$ taking into account the assigned content of *clarithromycin CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P.*

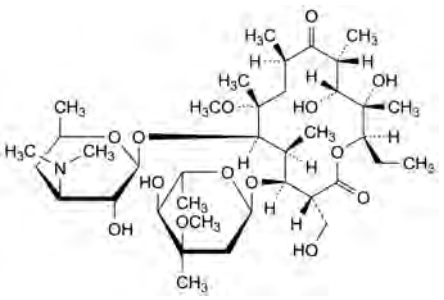

A. 2-demethyl-2-(hydroxymethyl)-6-*O*-methylerythromycin A (clarithromycin F),

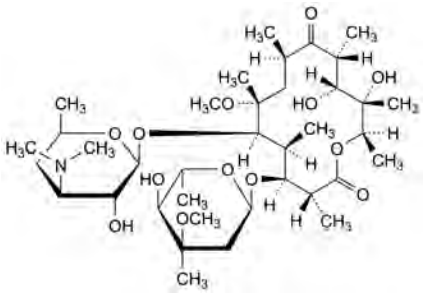

B. 6-*O*-methyl-15-norerythromycin A,

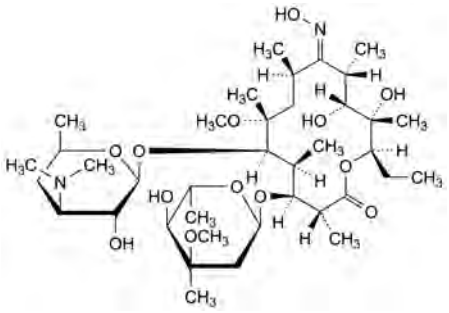

C. 6-*O*-methylerythromycin A (*E*)-9-oxime,

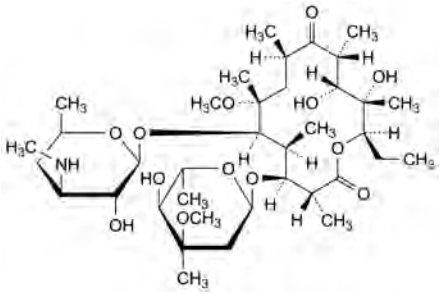

D. 3″-*N*-demethyl-6-*O*-methylerythromycin A,

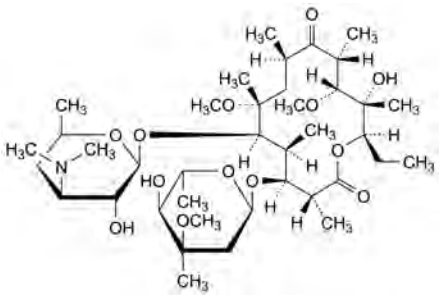

E. 6,11-di-*O*-methylerythromycin A,

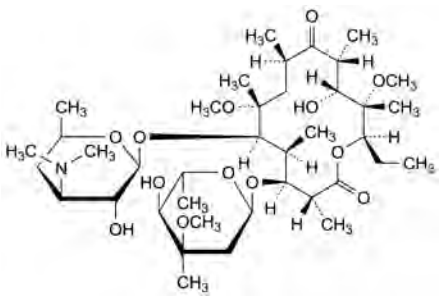

F. 6,12-di-*O*-methylerythromycin A,

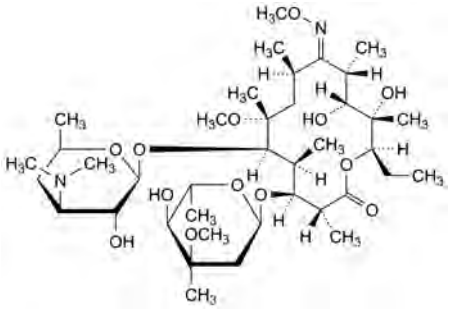

G. 6-*O*-methylerythromycin A (*E*)-9-(*O*-methyloxime),

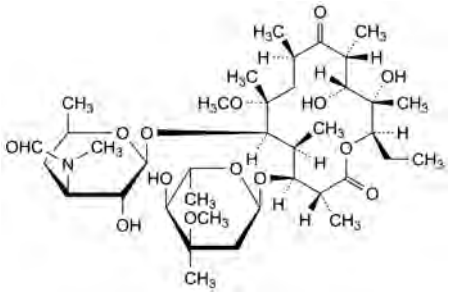

H. 3″-*N*-demethyl-3′-*N*-formyl-6-*O*-methylerythromycin A,

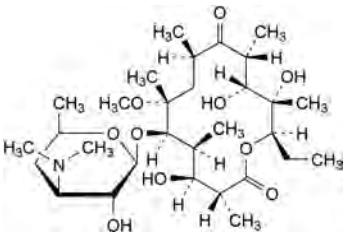

I. 3-*O*-decladinosyl-6-*O*-methylerythromycin A,

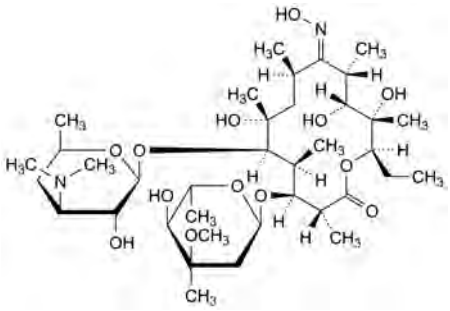

J. erythromycin A (*E*)-9-oxime,

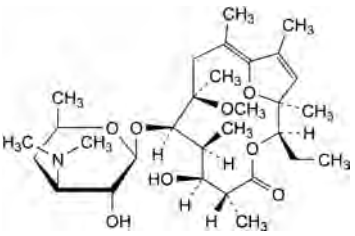

K. (1*S*,2*R*,5*R*,6*S*,7*S*,8*R*,9*R*,11*Z*)-2-ethyl-6-hydroxy-9-methoxy-1,5,7,9,11,13-hexamethyl-8-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-3,15-dioxabicyclo[10.2.1]pentadeca-11,13-dien-4-one (3-*O*-decladinosyl-8,9:10,11-dianhydro-6-*O*-methylerythromycin A-9,12-hemiketal),

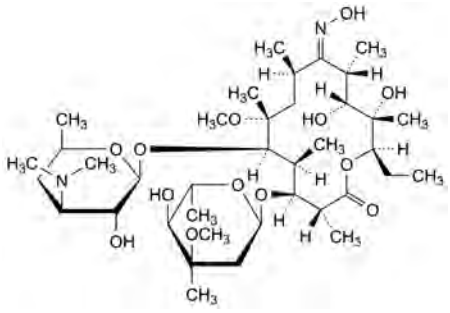

L. 6-*O*-methylerythromycin A (*Z*)-9-oxime,

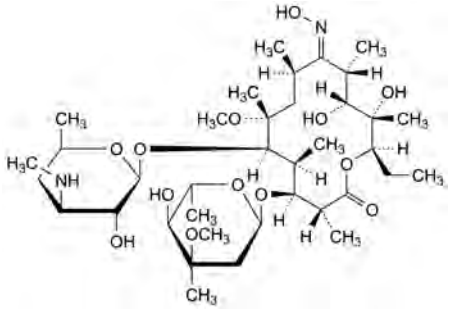

M. 3″-*N*-demethyl-6-*O*-methylerythromycin A (*E*)-9-oxime,

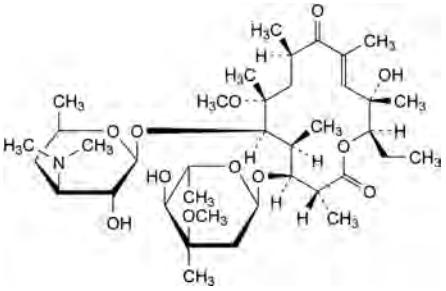

N. (10*E*)-10,11-didehydro-11-deoxy-6-*O*-methylerythromycin A,

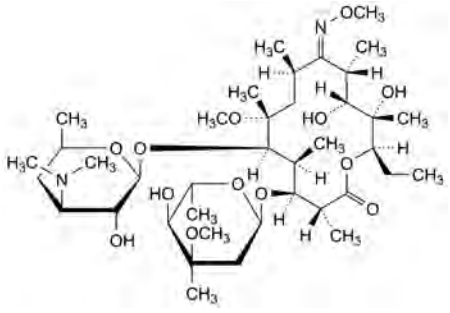

O. 6-*O*-methylerythromycin A (*Z*)-9-(*O*-methyloxime),

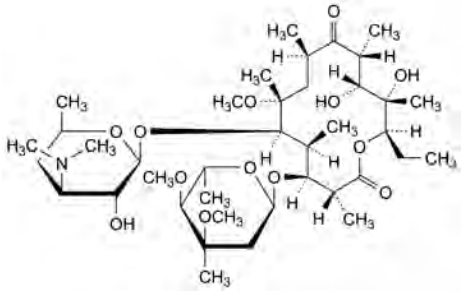

P. 4′,6-di-*O*-methylerythromycin A.

**07/2010:1714**

# CLAZURIL FOR VETERINARY USE

## Clazurilum ad usum veterinarium

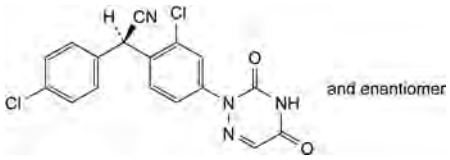


$C_{17}H_{10}Cl_2N_4O_2$ $M_r$ 373.2
[101831-36-1]

DEFINITION

(2*RS*)-[2-Chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl](4-chlorophenyl)acetonitrile.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or light yellow powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Melting point (*2.2.14*): 199 °C to 203 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clazuril CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *tetrahydrofuran R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *clazuril for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H and I) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 100 volumes of a 7.7 g/L solution of *ammonium acetate R* adjusted to pH 6.2 with a 10 per cent *V/V* solution of *anhydrous formic acid R*, 150 volumes of *acetonitrile R* and 750 volumes of *water R*;
- *mobile phase B*: mix 50 volumes of *water R*, 100 volumes of a 7.7 g/L solution of *ammonium acetate R* adjusted to pH 6.2 with a 10 per cent *V/V* solution of *anhydrous formic acid R* and 850 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *clazuril for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to clazuril (retention time = about 16 min): impurity A = about 0.6; impurity B = about 0.78; impurity C = about 0.80; impurity D = about 0.86; impurity E = about 0.9; impurity F = about 0.95; impurity G = about 0.98; impurity H = about 1.1; impurity I = about 1.2.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clazuril,
- the chromatogram obtained is similar to the chromatogram supplied with *clazuril for system suitability CRS*.

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 1.4; impurity H = 0.8;
- *impurities A, B, C, D, E, F, G, H, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peaks due to the solvents.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve about 0.260 g in 35 mL of *tetrahydrofuran R* and add 35 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.32 mg of $C_{17}H_{10}Cl_2N_4O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

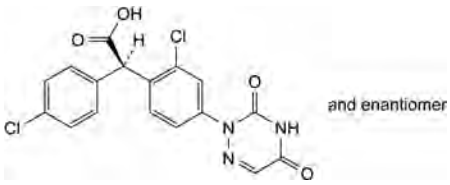


A. (2*RS*)-[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl](4-chlorophenyl)acetic acid,

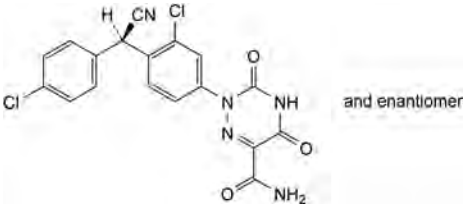


B. 2-[3-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,

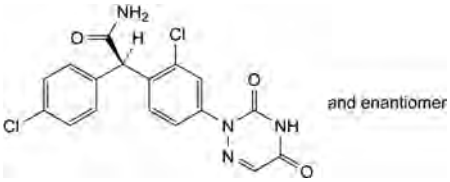


C. (2*RS*)-2-[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl]-2-(4-chlorophenyl)acetamide,

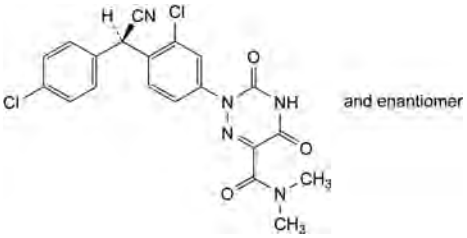


D. 2-[3-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-*N*,*N*-dimethyl-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,

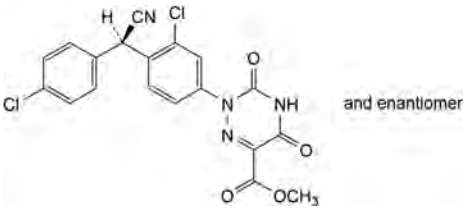


E. methyl 2-[3-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,

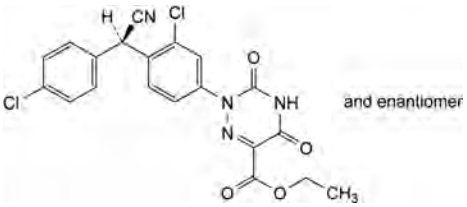


F. ethyl 2-[3-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,

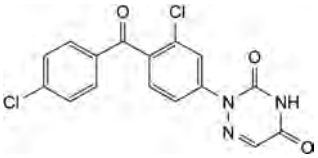


G. 2-[3-chloro-4-(4-chlorobenzoyl)phenyl]-1,2,4-triazine-3,5(2*H*,4*H*)-dione,

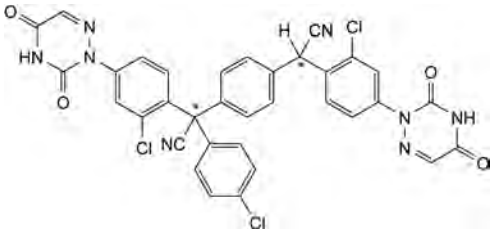

H. [2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl][4-[[2-chloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl]cyanomethyl]phenyl](4-chlorophenyl)acetonitrile,

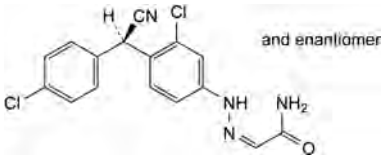

I. (*Z*)-2-[[3-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]phenyl]diazanylidene]acetamide.

**01/2017:1303**

# CLEBOPRIDE MALATE

## Clebopridi malas

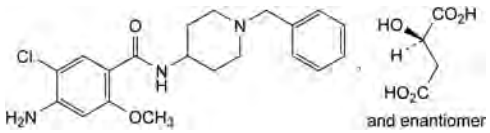

$C_{24}H_{30}ClN_3O_7$ $M_r$ 508.0
[57645-91-7]

DEFINITION

4-Amino-*N*-(1-benzylpiperidin-4-yl)-5-chloro-2-methoxybenzamide acid (*RS*)-2-hydroxybutanedioate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water and in methanol, slightly soluble in anhydrous ethanol, practically insoluble in methylene chloride.

mp: about 164 °C, with decomposition.

IDENTIFICATION

*First identification*: B, C.

*Second identification*: A, C, D.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 20.0 mg in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 270 nm and 307 nm.

*Specific absorbance at the absorption maxima*:

– at 270 nm: 252 to 278;

– at 307 nm: 204 to 226.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clebopride malate CRS*.

C. Dissolve 20 mg in 1 mL of *sulfuric acid R*, add 1 mL of *β-naphthol solution R1* and mix. The solution examined in daylight is yellow with blue fluorescence.

D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in *anhydrous ethanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *clebopride malate CRS* in *anhydrous ethanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *clebopride malate CRS* and 5 mg of *metoclopramide hydrochloride CRS* in *anhydrous ethanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *acetone R*, *methanol R*, *toluene R* (2:14:14:70 *V/V/V/V*).

*Application*: 5 µL as bands of 10 mm by 3 mm.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated zones.

*Results*: the principal zone in the chromatogram obtained with the test solution is similar in position and size to the principal zone in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S, examined immediately after preparation, is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**pH** (*2.2.3*): 3.8 to 4.2 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of the substance to be examined and 10 mg of *metoclopramide hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.12 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R1* and 80 volumes of a 1 g/L solution of *sodium heptanesulfonate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of clebopride.

*Relative retention* with reference to clebopride (retention time = about 15 min): metoclopramide = about 0.45.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to metoclopramide and clebopride.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the 2 peaks eluting within the first 2 min.

**Chlorides**: maximum 100 ppm.

*Prepare the solutions at the same time.*

*Test solution.* Dissolve 0.530 g in 20.0 mL of *anhydrous acetic acid R*, add 6 mL of *dilute nitric acid R* and dilute to 50.0 mL with *water R*.

*Reference solution.* To 1.5 mL of *0.001 M hydrochloric acid* add 20.0 mL of *anhydrous acetic acid R* and 6 mL of *dilute nitric acid R* and dilute to 50.0 mL with *water R*.

Transfer both recently prepared solutions to separate test-tubes. Add to each tube 1 mL of *silver nitrate solution R2*. Allow to stand for 5 min protected from light. Examine the tubes laterally against a black background. Any opalescence in the test solution is not more intense than that in the reference solution.

**Sulfates**: maximum 100 ppm.

*Prepare the solutions at the same time.*

*Test solution.* Dissolve 3.00 g in 20.0 mL of *glacial acetic acid R*, heating gently if necessary. Allow to cool and dilute to 50.0 mL with *water R*.

*Reference solution.* To 9 mL of *sulfate standard solution (10 ppm $SO_4$) R1* add 6 mL of *glacial acetic acid R*.

Into 2 test-tubes introduce 1.5 mL of *sulfate standard solution (10 ppm $SO_4$) R1* and add 1 mL of a 250 g/L solution of *barium chloride R*. Shake and allow to stand for 1 min. To one of the tubes add 15 mL of the test solution and to the other add 15 mL of the reference solution. After 5 min, any opalescence in the tube containing the test solution is not more intense than that in the tube containing the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 50.80 mg of $C_{24}H_{30}ClN_3O_7$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

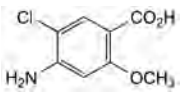

A. 4-amino-5-chloro-2-methoxybenzoic acid,

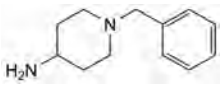

B. 1-benzylpiperidin-4-amine,

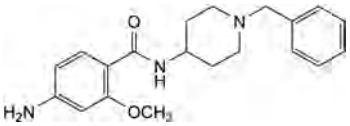

C. 4-amino-*N*-(1-benzylpiperidin-4-yl)-2-methoxybenzamide.

**04/2014:1190**
**corrected 10.0**

# CLEMASTINE FUMARATE

## Clemastini fumaras

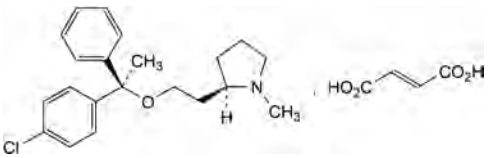

$C_{25}H_{30}ClNO_5$ $M_r$ 460.0
[14976-57-9]

DEFINITION

(2*R*)-2-[2-[(1*R*)-1-(4-Chlorophenyl)-1-phenylethoxy]ethyl]-1-methylpyrrolidine hydrogen (*E*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, sparingly soluble in ethanol (70 per cent *V/V*), practically insoluble in heptane.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clemastine fumarate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.
*Reference solution.* Dissolve 20.0 mg of *clemastine fumarate CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *methanol R*, *tetrahydrofuran R* (1:20:80 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of cold air for 5 min.
*Detection*: spray with a freshly prepared mixture of 1 volume of *potassium iodobismuthate solution R* and 10 volumes of *dilute acetic acid R* and then with *dilute hydrogen peroxide solution R*; cover the plate immediately with a glass plate of the same size and examine the chromatograms after 2 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *fumaric acid CRS* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *water R, anhydrous formic acid R, di-isopropyl ether R* (5:25:70 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 30 min and allow to cool.

*Detection*: spray with a 16 g/L solution of *potassium permanganate R* and examine in daylight.

*Results*: the principal spot with the highest $R_F$ value in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 0.500 g in *methanol R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.2 to 4.2.

Suspend 1.0 g in 10 mL of *carbon dioxide-free water R.*

**Specific optical rotation** (*2.2.7*): + 15.0 to + 18.0 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Phosphate buffer solution pH 7.1.* Mix 1.9 volumes of a 138 g/L solution of *sodium dihydrogen phosphate monohydrate R*, 6.8 volumes of an 89 g/L solution of *disodium hydrogen phosphate dihydrate R* and 91.3 volumes of *water for chromatography R.*

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (20:80 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 30 mL of the solvent mixture with the aid of ultrasound and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *clemastine for system suitability CRS* (containing impurity B) in 1.0 mL of the solvent mixture with the aid of ultrasound for about 5 min.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: phosphate buffer solution pH 7.1;
- *mobile phase B*: phosphate buffer solution pH 7.1, *acetonitrile R1* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 45 | 55 |
| 3 - 23 | 45 → 5 | 55 → 95 |
| 23 - 26 | 5 | 95 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 90 µL.

*Identification of impurities*: use the chromatogram supplied with *clemastine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to clemastine (retention time = about 17 min): fumaric acid = about 0.1; impurity B = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity B and clemastine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of clemastine fumarate in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to fumaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 46.00 mg of $C_{25}H_{30}ClNO_5$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

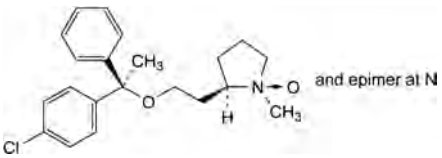


A. (1*RS*,2*R*)-2-[2-[(1*R*)-1-(4-chlorophenyl)-1-phenylethoxy]-ethyl]-1-methylpyrrolidine 1-oxide,

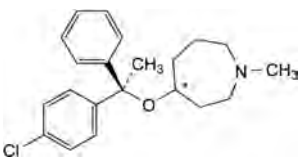


B. 4-[(1*R*)-1-(4-chlorophenyl)-1-phenylethoxy]-1-methylazepane,

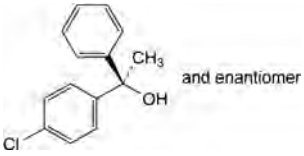


C. (1*RS*)-1-(4-chlorophenyl)-1-phenylethanol.

01/2008:1409

# CLENBUTEROL HYDROCHLORIDE

## Clenbuteroli hydrochloridum

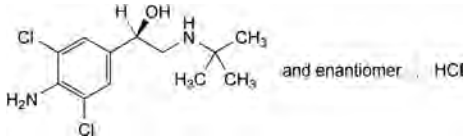


$C_{12}H_{19}Cl_3N_2O$ $M_r$ 313.7
[21898-19-1]

DEFINITION

(1*RS*)-1-(4-Amino-3,5-dichlorophenyl)-2-[(1,1-dimethylethyl)amino]ethanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent), slightly soluble in acetone.

mp: about 173 °C, with decomposition.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clenbuterol hydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 10 mL of *methanol R*.

*Reference solution.* Dissolve 10 mg of *clenbuterol hydrochloride CRS* in 10 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ammonia R*, *anhydrous ethanol R*, *toluene R* (0.15:10:15 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a 10 g/L solution of *sodium nitrite R* in *1 M hydrochloric acid* and dip after 10 min in a 4 g/L solution of *naphthylethylenediamine dihydrochloride R* in *methanol R*. Allow to dry in air.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in 10 mL of *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 0.30 g in *water R* and dilute to 10.0 mL with the same solvent. Filter if necessary.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Disperse 100.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 0.1 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 5 mg of *clenbuterol impurity B CRS* in 10 mL of the mobile phase, add 2.5 mL of the test solution and dilute to 25.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 40 °C.

*Mobile phase*: mix 200 volumes of *acetonitrile R*, 200 volumes of *methanol R* and 600 volumes of a solution prepared as follows: dissolve 3.0 g of *sodium decanesulfonate R* and 5.0 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL.

*Run time*: 1.5 times the retention time of clenbuterol.

*Retention time*: clenbuterol = about 29 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity B and clenbuterol.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.37 mg of $C_{12}H_{19}Cl_3N_2O$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

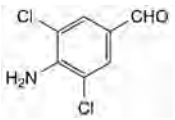


A. 4-amino-3,5-dichlorobenzaldehyde,

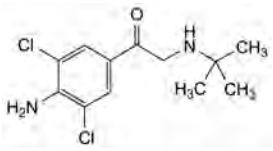

B. 1-(4-amino-3,5-dichlorophenyl)-2-[(1,1-dimethylethyl)-amino]ethanone,

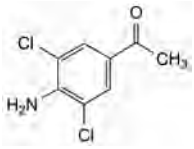

C. 1-(4-amino-3,5-dichlorophenyl)ethanone,

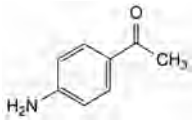

D. 1-(4-aminophenyl)ethanone,

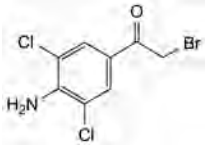

E. 1-(4-amino-3,5-dichlorophenyl)-2-bromoethanone,

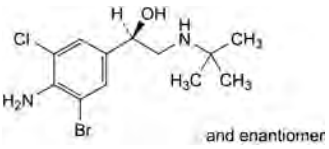


F. (1*RS*)-1-(4-amino-3-bromo-5-chlorophenyl)-2-[(1,1-dimethylethyl)amino]ethanol.

**01/2018:0582**

# CLINDAMYCIN HYDROCHLORIDE

## Clindamycini hydrochloridum

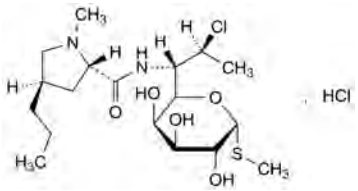

$C_{18}H_{34}Cl_2N_2O_5S$ $M_r$ 461.5
[21462-39-5]

DEFINITION

Methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside hydrochloride. It contains a variable quantity of water.

Semi-synthetic product derived from a fermentation product.

*Content*: 92.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clindamycin hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *clindamycin hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *clindamycin hydrochloride CRS* and 10 mg of *lincomycin hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: mix 19 volumes of *2-propanol R*, 38 volumes of a 150 g/L solution of *ammonium acetate R* adjusted to pH 9.6 with *ammonia R*, and 43 volumes of *ethyl acetate R*. Use the upper layer.

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 1 g/L solution of *potassium permanganate R*.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 2 mL of *dilute hydrochloric acid R* and heat on a water-bath for 3 min. Add 3 mL of *sodium carbonate solution R* and 1 mL of a 20 g/L solution of *sodium nitroprusside R*. A violet-red colour develops.

D. Dissolve 0.1 g in *water R* and dilute to 10 mL with the same solvent. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 3.0 to 5.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 135 to + 150 (anhydrous substance).

Dissolve 1.000 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *clindamycin hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 45 volumes of *acetonitrile R1* and 55 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.5 with a 250 g/L solution of *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of clindamycin.

*Relative retention* with reference to clindamycin (retention time = about 10 min): impurity B = about 0.7; impurity C = about 0.8.

*System suitability:* reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurities B and C; minimum 2.0 between the peaks due to impurity C and clindamycin.

*Calculation of percentage contents*:

– for each impurity, use the concentration of clindamycin hydrochloride in reference solution (b).

*Limits*:

– *impurity C*: maximum 4.0 per cent;
– *impurity B*: maximum 2.0 per cent;
– *any other impurity*: for each impurity, maximum 0.5 per cent;
– *total*: maximum 6.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.0 per cent to 6.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{18}H_{34}Cl_2N_2O_5S$ taking into account the assigned content of *clindamycin hydrochloride CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F.*

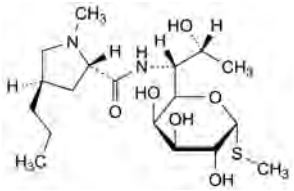

A. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (lincomycin),

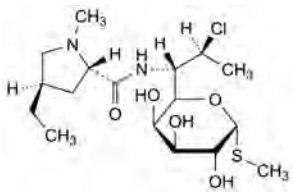

B. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-4-ethyl-1-methylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin B),

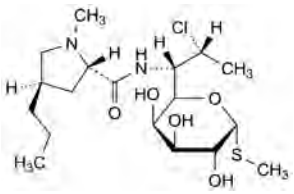

C. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (7-epiclindamycin),

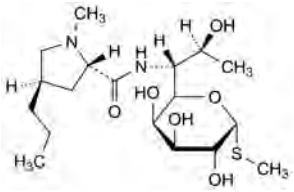

D. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (7-epilincomycin),

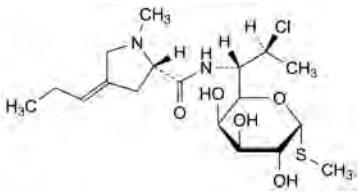

E. methyl (5*R*)-5-[(1*S*,2*S*)-2-chloro-1-[[(4*Z*)-1-methyl-4-propylidene-L-prolyl]amino]propyl]-1-thio-β-L-arabinopyranoside,

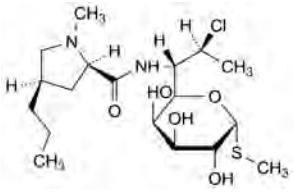

F. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*R*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside.

**01/2015:0996**
**corrected 10.0**

# CLINDAMYCIN PHOSPHATE

## Clindamycini phosphas

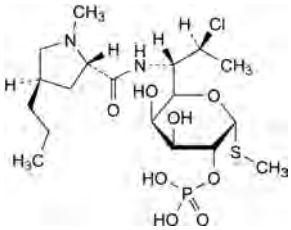

$C_{18}H_{34}ClN_2O_8PS$ $M_r$ 505.0
[24729-96-2]

DEFINITION

Methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-2-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clindamycin phosphate CRS*.

If the spectra obtained in the solid state show differences, dissolve 50 mg of the substance to be examined and the reference substance separately in 0.2 mL of *water R* and heat until completely dissolved. Evaporate to dryness under reduced pressure, dry the residues at 100-105 °C for 2 h and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *clindamycin phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *lincomycin hydrochloride CRS* in 5 mL of reference solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 30 min.

*Detection*: spray with a 1 g/L solution of *potassium permanganate R*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 2 mL of *dilute hydrochloric acid R* and heat in a water-bath for 3 min. Add 4 mL of *sodium carbonate solution R* and 1 mL of a 20 g/L solution of *sodium nitroprusside R*. Prepare a standard in the same manner using *clindamycin phosphate CRS*. The colour of the test solution corresponds to that of the standard.

D. Boil 0.1 g under a reflux condenser with a mixture of 5 mL of *strong sodium hydroxide solution R* and 5 mL of *water R* for 90 min. Cool and add 5 mL of *nitric acid R*. Extract with 3 quantities, each of 15 mL, of *methylene chloride R* and discard the extracts. Filter the upper layer through a paper filter. The filtrate gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R*. Heat gently if necessary. Cool and dilute to 25.0 mL with *carbon dioxide-free water R*.

**Appearance of the solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 30.0 mg of *clindamycin phosphate CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 3.0 mg of *clindamycin phosphate for system suitability CRS* (containing impurities B, E, F, G, I, J, K and L) in mobile phase A and dilute to 1.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: mix 21 volumes of *acetonitrile R1* and 79 volumes of a 13.6 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 6.0 with a 450 g/L solution of *potassium hydroxide R*;
– *mobile phase B*: mix 40 volumes of a 13.6 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 6.0 with a 450 g/L solution of *potassium hydroxide R*, and 60 volumes of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 13 | 100 | 0 |
| 13 - 18 | 100 → 50 | 0 → 50 |
| 18 - 39 | 50 | 50 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *clindamycin phosphate for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, E, F, G, I, J, K and L.

*Relative retention* with reference to clindamycin phosphate (retention time = about 12 min): impurity F = about 0.15; impurity G = about 0.19; impurity I = about 0.34; impurity B = about 0.45; impurity L = about 0.64; impurity J = about 1.20; impurity E = about 1.73; impurity K = about 1.90.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the peaks due to impurities F and G.

*Calculation of percentage contents*:

– for each impurity, use the concentration of clindamycin phosphate in reference solution (b).

*Limits*:

– *impurities B, L*: for each impurity, maximum 1.0 per cent;
– *impurities E, F*: for each impurity, maximum 0.5 per cent;
– *impurities G, I, J, K*: for each impurity, maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 2.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.6 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 3.0 for the peak due to clindamycin phosphate.

Calculate the percentage content of $C_{18}H_{34}ClN_2O_8PS$ taking into account the assigned content of *clindamycin phosphate CRS*.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: B, E, F, G, I, J, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, H.*

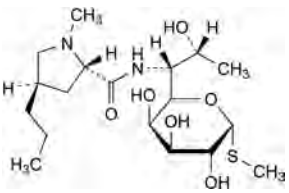

A. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (lincomycin),

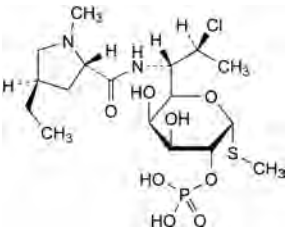

B. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-4-ethyl-1-methylpyrrolidin-2-yl]carbonyl]amino]-2-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin B 2-phosphate),

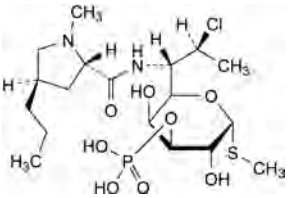

C. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-3-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin 3-phosphate),

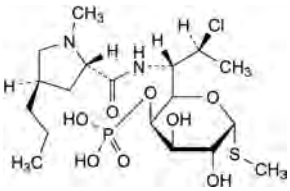

D. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-4-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin 4-phosphate),

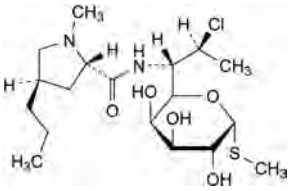

E. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin),

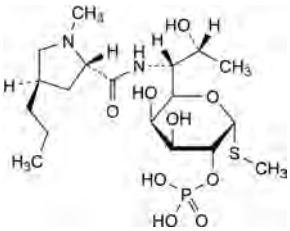

F. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-2-*O*-phosphono-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (lincomycin 2-phosphate),

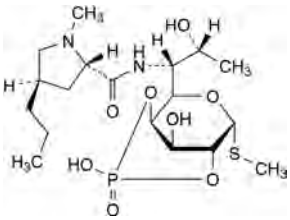

G. methyl 6,8-dideoxy-2,4-*O*-(hydroxyphosphoryl)-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (2,4-phosphatidyl lincomycin),

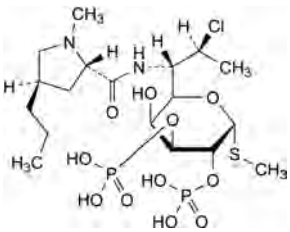

H. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-2,3-di-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin 2,3-bisphosphate),

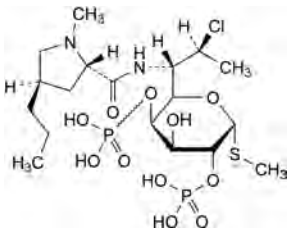

I. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-2,4-di-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (clindamycin 2,4-bisphosphate),

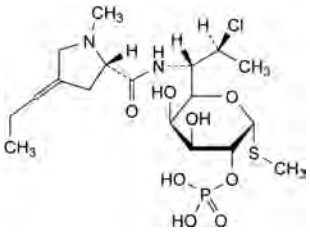

J. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*)-1-methyl-4-propylidenepyrrolidin-2-yl]carbonyl]amino]-2-*O*-phosphono-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (propylidene analog of clindamycin 2-phosphate),

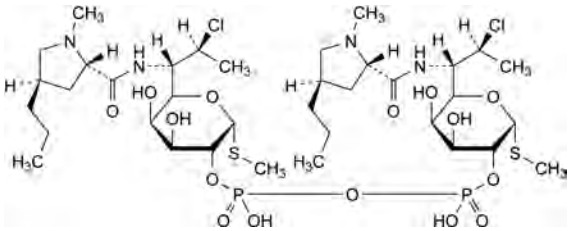

K. 2,2′-oxybis(hydroxyphosphoryl)bis[methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside] (diclindamycin pyrophosphate),

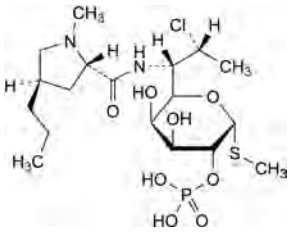

L. methyl 7-chloro-6,7,8-trideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-2-*O*-phosphono-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (7-epiclindamycin 2-phosphate).

**01/2008:2111**
**corrected 10.0**

# CLIOQUINOL

## Clioquinolum

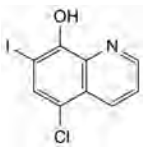

$C_9H_5ClINO$ $M_r$ 305.5
[130-26-7]

DEFINITION

5-Chloro-7-iodoquinolin-8-ol.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white, light yellow, brownish-yellow or yellowish-grey powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble or slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Dissolve 40.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL to 100.0 mL with *methanol R* (solution A). Examined between 280 nm and 350 nm (*2.2.25*), solution A shows an absorption maximum at 321 nm. Dilute 10.0 mL of solution A to 100.0 mL with *methanol R* (solution B). Examined between 230 nm and 280 nm, solution B shows an absorption maximum at 255 nm. The specific absorbance at this absorption maximum is 1530 to 1660.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *clioquinol CRS*.

C. When heated, violet fumes are produced.

D. Dissolve about 1 mg in 5 mL of *ethanol (96 per cent) R*. Add 0.05 mL of *ferric chloride solution R1*. A dark green colour develops.

TESTS

**Acidity or alkalinity**. Shake 0.5 g with 10 mL of *carbon dioxide-free water R* and filter. To the filtrate add 0.2 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent, heating gently if necessary. Dilute 10.0 mL of the solution to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20.0 mg of *5-chloroquinolin-8-ol R*, 10.0 mg of *5,7-dichloroquinolin-8-ol R*, 5 mg of the substance to be examined and 10.0 mg of *5,7-diiodoquinolin-8-ol R* in *methanol R*, heating gently if necessary and dilute to 20.0 mL with the same solvent. Dilute 4.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.50 g of *sodium edetate R* in 350 mL of *water R*, add 4.0 mL of *hexylamine R* and mix. Adjust to pH 3.0 with *phosphoric acid R*. Add 600 mL of *methanol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of clioquinol.

*Relative retention* with reference to clioquinol (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 1.3.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.0 between the peaks due to clioquinol and impurity C.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2.0 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent),
- *total of the nominal contents of impurities A, B, C and unspecified impurities*: maximum 3.0 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Halides**: maximum 140 ppm, expressed as chlorides.

Shake 0.5 g with 25 mL of *water R* for 1 min and filter. To the filtrate add 0.5 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R2*. Allow to stand for 5 min. Any opalescence is not more intense than that in a standard prepared at the same time by adding 0.5 mL of *silver nitrate solution R2* to 25 mL of *water R* containing 0.2 mL of *0.01 M hydrochloric acid* and 0.5 mL of *dilute nitric acid R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 mL of *acetic anhydride R* and add 30 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.55 mg of total quinolines, calculated as clioquinol.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

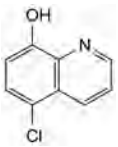

A. 5-chloroquinolin-8-ol,

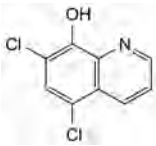

B. 5,7-dichloroquinolin-8-ol,

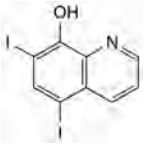

C. 5,7-diiodoquinolin-8-ol.

**01/2008:1974**
**corrected 6.0**

# CLOBAZAM

## Clobazamum

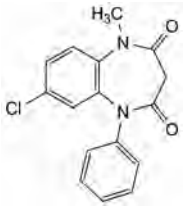

$C_{16}H_{13}ClN_2O_2$ $M_r$ 300.7
[22316-47-8]

DEFINITION

7-Chloro-1-methyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of clobazam.*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *clobazam impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *chlordiazepoxide CRS* and 5 mg of *clonazepam CRS* in the mobile phase and dilute to 50 mL with the mobile phase. Dilute 1 mL of the solution to 100 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of clobazam.

*Retention time*: clobazam = about 15 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.3 between the peaks due to chlordiazepoxide and clonazepam.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

- *any other impurity*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *total of other impurities*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 250.0 mL with *alcohol R*. Measure the absorbance (*2.2.25*) at the maximum at 232 nm.

Calculate the content of $C_{16}H_{13}ClN_2O_2$ taking the specific absorbance to be 1380.

IMPURITIES

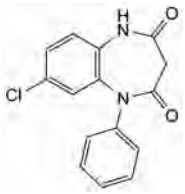

A. 7-chloro-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione,

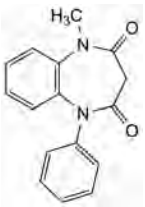

B. 1-methyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione,

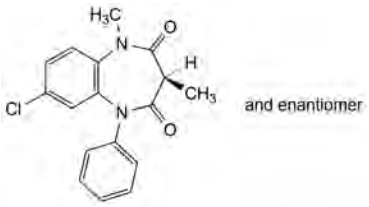


C. (3*RS*)-7-chloro-1,3-dimethyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione,

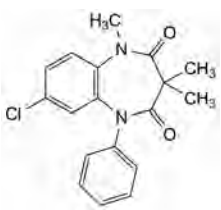

D. 7-chloro-1,3,3-trimethyl-5-phenyl-1,5-dihydro-3*H*-1,5-benzodiazepine-2,4-dione,

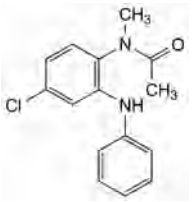

E. *N*-[4-chloro-2-(phenylamino)phenyl]-*N*-methylacetamide,

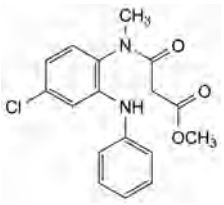

F. methyl 3-[[4-chloro-2-(phenylamino)phenyl]methyl-amino]-3-oxopropanoate.

**01/2008:2127**
**corrected 6.0**

# CLOBETASOL PROPIONATE

## Clobetasoli propionas

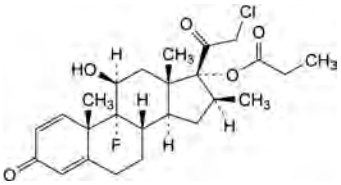

$C_{25}H_{32}ClFO_5$ $M_r$ 467.0
[25122-46-7]

DEFINITION

21-Chloro-9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clobetasol propionate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 112 to + 118 (dried substance).

Dissolve 0.500 g in *acetone R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Test solution (b)*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20.0 mg of *clobetasol propionate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *clobetasol impurity J CRS* in 2.0 mL of the mobile phase. To 0.5 mL of this solution add 0.5 mL of test solution (b) and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *clobetasol for peak identification CRS* (containing impurities A, B, C, D, E, L and M) in 2 mL of the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of test solution (a) to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 10 volumes of *methanol R*, 42.5 volumes of a 7.85 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 5.5 with a 100 g/L solution of *sodium hydroxide R* and 47.5 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of clobetasol propionate.

*Identification of impurities*: use the chromatogram supplied with *clobetasol for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E, L and M.

*Relative retention* with reference to clobetasol propionate (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.6; impurity C = about 0.9; impurity J = about 1.1; impurity D = about 1.2; impurity L = about 1.3; impurity M = about 1.6; impurity E = about 2.1.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to clobetasol propionate and impurity J in the chromatogram obtained with reference solution (b);
- the chromatogram obtained with reference solution (c) is similar to the chromatogram supplied with *clobetasol for peak identification CRS*.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.6; impurity C = 1.5;
- *impurity E*: not more than 1.4 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.7 per cent);
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *impurities B, C*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurities A, L, M*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{25}H_{32}ClFO_5$ using the chromatogram obtained with reference solution (a) and the declared content of *clobetasol propionate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G, H, I, J, K.*

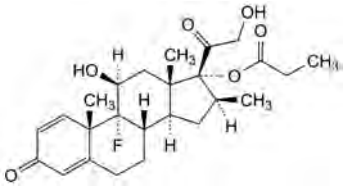

A. 9-fluoro-11β,21-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate (betamethasone 17-propionate),

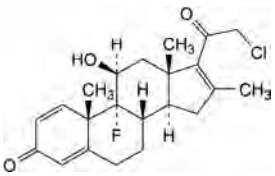

B. 21-chloro-9-fluoro-11β-hydroxy-16-methylpregna-1,4,16-triene-3,20-dione,

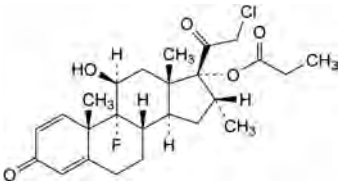

C. 21-chloro-9-fluoro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate,

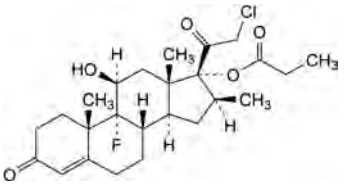

D. 21-chloro-9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregn-4-en-17-yl propanoate (1,2-dihydroclobetasol 17-propionate),

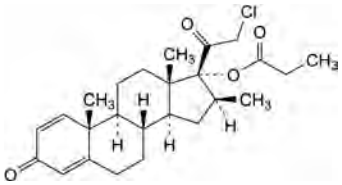

E. 21-chloro-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate,

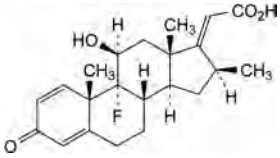

F. 9-fluoro-11β-hydroxy-16β-methyl-3-oxopregna-1,4,17(20)-trien-21-oic acid,

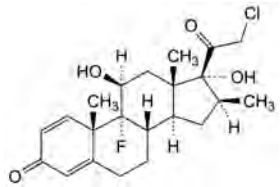

G. 21-chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione (clobetasol),

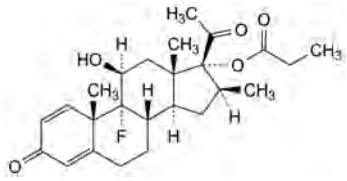

H. 9-fluoro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl propanoate,

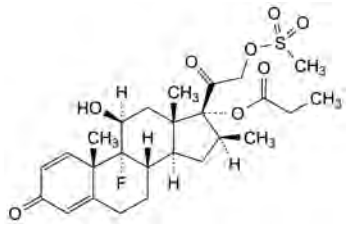

I. 9-fluoro-11β-hydroxy-16β-methyl-21-[(methylsulfonyl)-oxy]-3,20-dioxopregna-1,4-dien-17-yl propanoate,

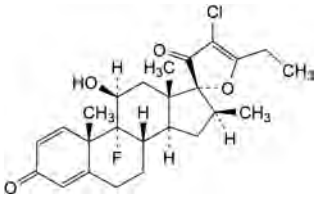

J. (17*R*)-4′-chloro-5′-ethyl-9-fluoro-11β-hydroxy-16β-methylspiro[androsta-1,4-diene-17,2′(3′*H*)-furan]-3,3′-dione (17α-spiro compound),

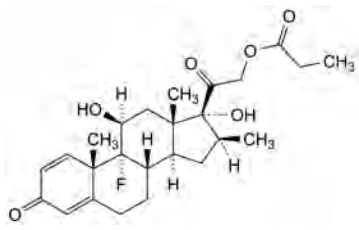

K. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl propanoate (betamethasone 21-propionate),

L. unknown structure,

M. unknown structure.

**01/2010:1090**
**corrected 8.4**

# CLOBETASONE BUTYRATE

## Clobetasoni butyras

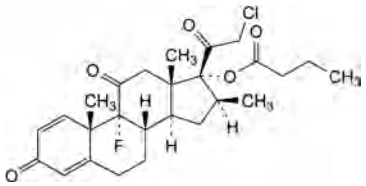

$C_{26}H_{32}ClFO_5$ $M_r$ 479.0
[25122-57-0]

DEFINITION

21-Chloro-9-fluoro-16β-methyl-3,11,20-trioxopregna-1,4-dien-17-yl butanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, slightly soluble in ethanol (96 per cent).

mp: about 178 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clobetasone butyrate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 131 to + 138 (dried substance).

Dissolve 0.250 g in *ethanol R1* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *anhydrous formic acid R*, *acetonitrile R*, *water R* (0.1:43:57 *V/V/V*).

*Test solution.* Dissolve 65 mg of the substance to be examined in 5.0 mL of *acetonitrile R* and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 13 mg of *clobetasone butyrate for system suitability CRS* (containing impurity F) in 1 mL of *acetonitrile R* and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *anhydrous formic acid R*, *water R* (0.1:99.9 *V/V*);
- *mobile phase B*: *anhydrous formic acid R*, *acetonitrile R* (0.1:99.9 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 57 | 43 |
| 3 - 26 | 57 → 43 | 43 → 57 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 241 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *clobetasone butyrate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity F.

*Relative retention* with reference to clobetasone butyrate (retention time = about 14 min): impurity F = about 0.9.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to impurity F and clobetasone butyrate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 20.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 235 nm.

Calculate the content of $C_{26}H_{32}ClFO_5$, taking the specific absorbance to be 327.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F, G, H, I.*

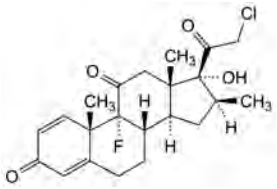

A. 21-chloro-9-fluoro-17-hydroxy-16β-methylpregna-1,4-diene-3,11,20-trione (clobetasone),

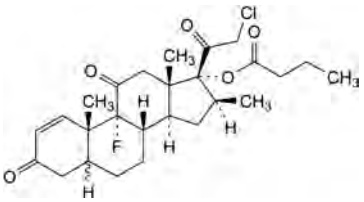

C. 21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregn-1-en-17-yl butanoate (4,5-dihydroclobetasone butyrate),

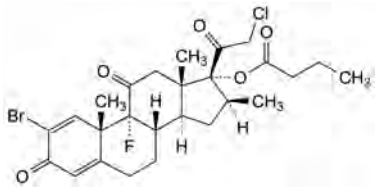

D. 2-bromo-21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregna-1,4-dien-17-yl butanoate (2-bromoclobetasone butyrate),

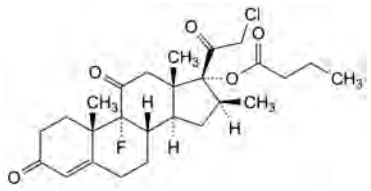

E. 21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregn-4-en-17-yl butanoate (1,2-dihydroclobetasone butyrate),

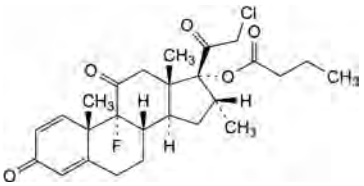

F. 21-chloro-9-fluoro-16α-methyl-3,11,20-trioxopregna-1,4-dien-17-yl butanoate (16α-methyl clobetasone butyrate),

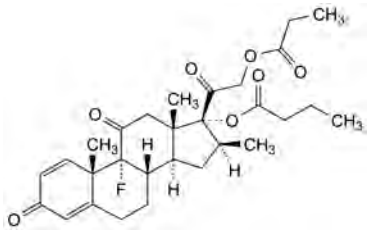

G. 9-fluoro-16β-methyl-3,11,20-trioxo-21-(propanoyloxy)-pregna-1,4-dien-17-yl butanoate,

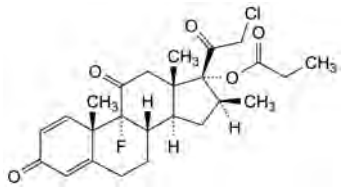

H. 21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregna-1,4-dien-17-yl propanoate (17-*O*-propionyl clobetasone),

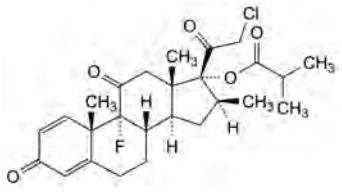

I. 21-chloro-9-fluoro-16β-methyl-3,11,20-trioxopregna-1,4-dien-17-yl 2-methylpropanoate (17-*O*-isobutyryl clobetasone).

**01/2017:1777**
**corrected 10.0**

# CLODRONATE DISODIUM TETRAHYDRATE

## Dinatrii clodronas tetrahydricus

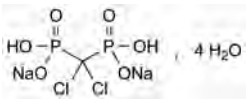

$CH_2Cl_2Na_2O_6P_2,4H_2O$ $M_r$ 360.9

DEFINITION

Disodium (dichloromethylene)bis(hydrogen phosphonate) tetrahydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent), slightly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clodronate disodium tetrahydrate CRS*.

B. Dissolve 0.5 g in 10 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 4.5, for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in 30 mL of *water R*, sonicate for 10 min and dilute to 50.0 mL with *water R* (*test stock solution*). Dilute 10.0 mL of the test stock solution to 20.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 50.0 mL with *water R*.

*Reference solution (b)*. Dissolve 1 mg of *clodronate impurity D CRS* in 10 mL of *water R*, sonicate for 10 min and dilute to 20.0 mL with *water R*. Mix 2.0 mL of this solution with 10.0 mL of the test stock solution and dilute to 20.0 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of a 0.3 g/L solution of *phosphoric acid R* (impurity B) to 100.0 mL with *water R*.

*Precolumn*:
- *size*: *l* = 0.05 m, Ø = 4 mm;
- *stationary phase*: *anion-exchange resin R*;
- *particle size*: 9 µm.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *anion-exchange resin R*;
- *particle size*: 9 µm.

*Mobile phase*:
- *mobile phase A*: 0.21 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*; close immediately, mix and use under helium pressure;
- *mobile phase B*: 4.2 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*; close immediately, mix and use under helium pressure;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 90 → 60 | 10 → 40 |
| 10 - 22 | 60 → 50 | 40 → 50 |
| 22 - 23 | 50 → 20 | 50 → 80 |
| 23 - 25 | 20 | 80 |

*Flow rate*: 1 mL/min.

*Detection*: conductivity detector. Use a self-regenerating anion suppressor.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*Relative retention* with reference to clodronate (retention time = about 13 min): impurities A and B = about 0.7; impurity D = about 1.1.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clodronate.

*Limits*:
- *sum of impurities A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 18.5 per cent to 21.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.140 g in 10 mL of *water R*. Add 10 mL of *strong sodium hydroxide solution R* and some glass beads. Boil until the solution is completely decolourised (about 10 min). Cool in an ice-bath and add 30 mL of *water R* and 10 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 14.44 mg of $CH_2Cl_2Na_2O_6P_2$.

IMPURITIES

*Specified impurities*: *A, B*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical*

*use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

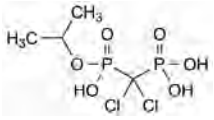

A. [dichloro[hydroxy(1-methylethoxy)phosphino-yl]methyl]phosphonic acid,

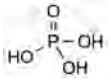

B. phosphoric acid,

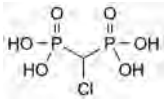

D. (chloromethylene)bis(phosphonic acid).

**01/2017:2054**

# CLOFAZIMINE

## Clofaziminum

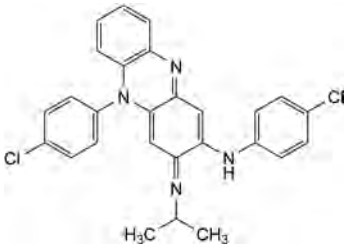

$C_{27}H_{22}Cl_2N_4$ $M_r$ 473.4
[2030-63-9]

DEFINITION

*N*,5-Bis(4-chlorophenyl)-3-[(1-methylethyl)imino]-3,5-dihydrophenazin-2-amine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: reddish-brown, fine powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, very slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clofazimine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *clofazimine CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *propanol R*, *methylene chloride R* (6:85 *V/V*).

*Application*: 5 µL.

*First development*: over 2/3 of the plate.

*Drying*: horizontally in air for 5 min.

*Second development*: over 2/3 of the plate.

*Drying*: in air for 5 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 2 mg in 3 mL of *acetone R* and add 0.1 mL of *hydrochloric acid R*. An intense violet colour is produced. Add 0.5 mL of a 200 g/L solution of *sodium hydroxide R*; the colour changes to orange-red.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *clofazimine for system suitability CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.25 g of *sodium laurilsulfate R*, 0.85 g of *tetrabutylammonium hydrogen sulfate R* and 0.885 g of *disodium hydrogen phosphate dodecahydrate R* in *water R*. Adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 500 mL with *water R*. Mix 35 volumes of this solution and 65 volumes of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of clofazimine.

*Identification of impurities*: use the chromatogram supplied with *clofazimine for system suitability CRS* to identify the peak due to impurity B.

*Relative retention* with reference to clofazimine (retention time = about 15 min): impurity A = about 0.7; impurity B = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: baseline separation between the peaks due to impurity B and clofazimine.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 5 mL of *methylene chloride R* and add 20 mL of *acetone R* and 5 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.34 mg of $C_{27}H_{22}Cl_2N_4$.

IMPURITIES

*Specified impurities: A, B.*

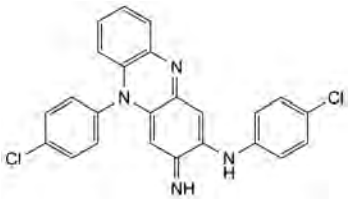

A. *N*,5-bis(4-chlorophenyl)-3-imino-3,5-dihydrophenazin-2-amine,

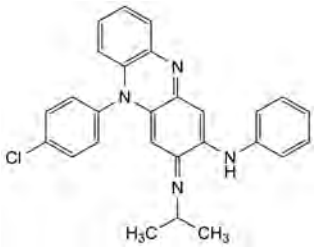

B. 5-(4-chlorophenyl)-3-[(1-methylethyl)imino]-*N*-phenyl-3,5-dihydrophenazin-2-amine.

**01/2008:0318**
**corrected 10.0**

# CLOFIBRATE

## Clofibratum

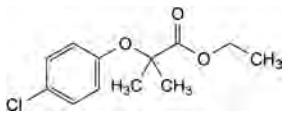

$C_{12}H_{15}ClO_3$ $M_r$ 242.7
[637-07-0]

DEFINITION

Ethyl 2-(4-chlorophenoxy)-2-methylpropionate.

CHARACTERS

*Appearance*: clear, almost colourless liquid.

*Solubility*: very slightly soluble in water, miscible with ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clofibrate CRS.*

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).
*Test solution (a).* Dissolve 0.10 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *methanol R*.
*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with *methanol R*.
*Spectral range*: 250-350 nm for test solution (a); 220-250 nm for test solution (b).
*Absorption maxima*: at 280 nm and 288 nm for test solution (a); at 226 nm for test solution (b).
*Specific absorbances at the absorption maxima*:
– at 226 nm: about 460 for test solution (b);
– at 280 nm: about 44 for test solution (a);
– at 288 nm: about 31 for test solution (a).

TESTS

**Relative density** (*2.2.5*): 1.138 to 1.147.

**Refractive index** (*2.2.6*): 1.500 to 1.505.

**Acidity**. To 1.0 g add 10 mL of *anhydrous ethanol R* and 0.1 mL of *phenol red solution R*. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Volatile related substances**. Gas chromatography (*2.2.28*).
*Test solution.* To 10.0 g of the substance to be examined add a mixture of 10 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R*. Shake, separate the lower (organic) layer, wash with 5 mL of *water R* and add the washings to the aqueous layer. Dry the organic layer with *anhydrous sodium sulfate R* and use as the test solution. Reserve the aqueous layer for the test for 4-chlorophenol.
*Reference solution (a).* Dissolve 0.12 g of the substance to be examined in *chloroform R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *chloroform R*.
*Reference solution (b).* Dissolve 0.12 g of *methyl 2-(4-chlorophenoxy)-2-methylpropionate CRS* in the substance to be examined and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with the substance to be examined. Dilute 1.0 mL of this solution to 10.0 mL with the substance to be examined.
*Column*:
– *size*: *l* = 1.5 m, Ø = 4 mm;
– *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (250-420 µm) impregnated with 30 per cent *m/m* of *methylpolysiloxane R*; or *silanised diatomaceous earth for gas chromatography R* (150-180 µm) impregnated with 10 per cent *m/m* of *methylpolysiloxane R*;
– *temperature*: 185 °C.
*Carrier gas*: *nitrogen for chromatography R.*
*Detection*: flame ionisation.
*Injection*: 2 µL.
*System suitability*: reference solution (b):
– *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to methyl 2-(4-chlorophenoxy)-2-methylpropionate and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clofibrate.
*Limit*:
– *total*: not more than 10 times the area of the peak due to clofibrate in the chromatogram obtained with reference solution (a) (0.1 per cent).

**4-Chlorophenol**. Gas chromatography (*2.2.28*) as described in the test for volatile related substances with the following modifications.
*Test solution.* Shake the aqueous layer reserved in the test for volatile related substances with 2 quantities, each of

5 mL, of *chloroform R* and discard the organic layers. Acidify the aqueous layer by the dropwise addition of *hydrochloric acid R*. Shake with 3 quantities, each of 3 mL, of *chloroform R*. Combine the organic layers and dilute to 10.0 mL with *chloroform R*.

*Reference solution*. Dissolve 0.25 g of *chlorophenol R* in *chloroform R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *chloroform R*.

*Limit*:

– *4-chlorophenol*: not more than the area of the peak due to 4-chlorophenol in the chromatogram obtained with the reference solution (25 ppm).



**01/2008:0997**
**corrected 9.5**

# CLOMIFENE CITRATE

## Clomifeni citras

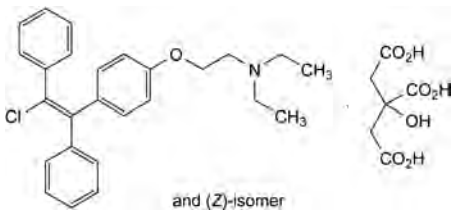


$C_{32}H_{36}ClNO_8$ $M_r$ 598.1
[50-41-9]

DEFINITION

Mixture of the (*E*)- and (*Z*)-isomers of 2-[4-(2-chloro-1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine dihydrogen citrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clomifene citrate CRS*.

B. Dissolve about 5 mg in 5 mL of a mixture of 1 volume of *acetic anhydride R* and 5 volumes of *pyridine R*, then heat in a water-bath. A deep red colour is produced.

TESTS

*Prepare the solutions protected from light in brown-glass vessels. Ensure minimum exposure of the solutions to daylight until they are required for chromatography.*

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 12.5 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 12.5 mg of *clomifene citrate for performance test CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 400 mL of *acetonitrile for chromatography R* with 600 mL of *water for chromatography R* and add 8.0 mL of *diethylamine R*; adjust to pH 6.2 with about 1-2 mL of *phosphoric acid R*, taking care to reduce progressively the volume of each addition as the required pH is approached.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 233 nm.

*Equilibration*: with the mobile phase for about 1 h.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of clomifene.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 15, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clomifene; if necessary, adjust the concentration of acetonitrile in the mobile phase;
– the chromatogram obtained is similar to the chromatogram supplied with *clomifene citrate for performance test CRS*.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
– *impurities B, C, D, E, F, G, H*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *total*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent);
– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak with a retention time relative to the clomifene peak of 0.2 or less.

**(*Z*)-isomer**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 25 mL of *0.1 M hydrochloric acid*, add 5 mL of *1 M sodium hydroxide* and shake with 3 quantities, each of 25 mL, of *ethanol-free chloroform R*. Wash the combined extracts with 10 mL of *water R*, dry over *anhydrous sodium sulfate R* and dilute to 100 mL with *ethanol-free chloroform R*. To 20 mL of this solution add 0.1 mL of *triethylamine R* and dilute to 100 mL with *hexane R*.

*Reference solution*. Dissolve 25 mg of *clomifene citrate CRS* in 25 mL of *0.1 M hydrochloric acid*, add 5 mL of *1 M sodium hydroxide* and shake with 3 quantities, each of 25 mL, of *ethanol-free chloroform R*. Wash the combined extracts with 10 mL of *water R*, dry over *anhydrous sodium sulfate R* and dilute to 100 mL with *ethanol-free chloroform R*. To 20 mL of this solution add 0.1 mL of *triethylamine R* and dilute to 100 mL with *hexane R*.

*Column*:

– *size*: *l* = 0.3 m, Ø = 4 mm;
– *stationary phase*: *silica gel for chromatography R* (10 µm).

*Mobile phase*: *triethylamine R*, *ethanol-free chloroform R*, *hexane R* (1:200:800 *V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Equilibration*: with the mobile phase for about 2 h.

*Injection*: 50 µL.

*Identification of peaks*: the chromatogram obtained with the reference solution shows a peak due to the (*E*)-isomer just before a peak due to the (*Z*)-isomer.

*System suitability*: reference solution:

– *resolution*: minimum 1.0 between the peaks due to the (*E*)- and (*Z*)-isomers; if necessary, adjust the relative proportions of ethanol-free chloroform and hexane in the mobile phase.

Measure the area of the peak due to the (*Z*)-isomer in the chromatograms obtained with the test solution and the reference solution. Calculate the content of the (*Z*)-isomer, as a percentage of the total clomifene citrate present, from the declared content of *clomifene citrate CRS*.

*Limit*:

– *(Z)-isomer*: 30.0 per cent to 50.0 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.000 g.

ASSAY

Dissolve 0.500 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 59.81 mg of $C_{32}H_{36}ClNO_8$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H.*

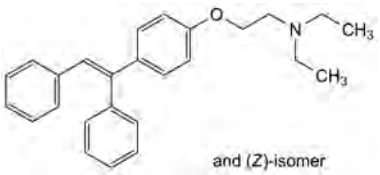


A. 2-[4-(1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine,

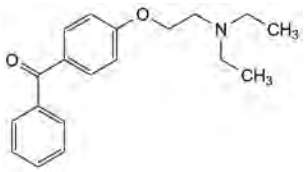

B. [4-[2-(diethylamino)ethoxy]phenyl]phenylmethanone,

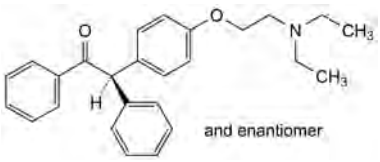


C. (2*RS*)-2-[4-[2-(diethylamino)ethoxy]phenyl]-1,2-diphenylethanone,

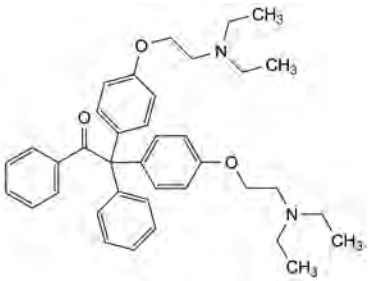

D. 2,2-bis[4-[2-(diethylamino)ethoxy]phenyl]-1,2-diphenylethanone,

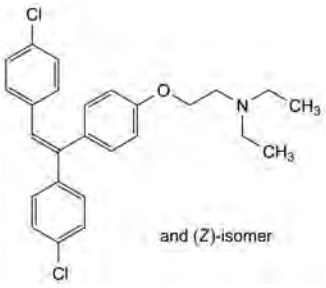


E. 2-[4-[1,2-bis(4-chlorophenyl)ethenyl]phenoxy]-*N,N*-diethylethanamine,

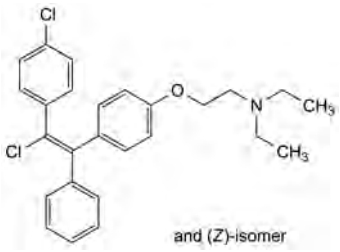


F. 2-[4-[2-chloro-2-(4-chlorophenyl)-1-phenylethenyl]-phenoxy]-*N,N*-diethylethanamine,

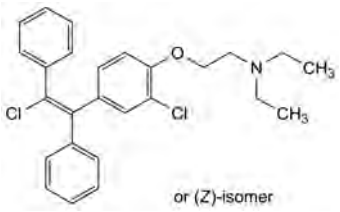


GH. 2-[2-chloro-4-(2-chloro-1,2-diphenylethenyl)phenoxy]-*N,N*-diethylethanamine (G. higher-melting-point isomer; H. lower-melting-point isomer).

**01/2017:0889**

# CLOMIPRAMINE HYDROCHLORIDE

## Clomipramini hydrochloridum

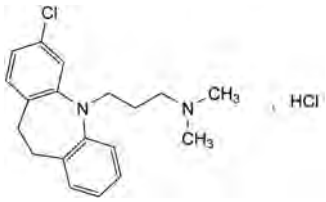


$C_{19}H_{24}Cl_2N_2$ $M_r$ 351.3
[17321-77-6]

DEFINITION

3-(3-Chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N,N*-dimethylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water and in methylene chloride, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clomipramine hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve about 50 mg in 5 mL of *water R* and add 1 mL of *dilute ammonia R1*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 3.5 to 5.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protected from light.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

*Reference solution (a).* Dissolve 22.6 mg of *imipramine hydrochloride CRS* (impurity B), 4.0 mg of *clomipramine impurity C CRS*, 4.0 mg of *clomipramine impurity D CRS* and 2.0 mg of *clomipramine impurity F CRS* in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 100.0 mL with the same mixture of mobile phases. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

*Reference solution (c).* Dissolve 10.0 mg of *clomipramine hydrochloride CRS* and 3.0 mg of *clomipramine impurity C CRS* in a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A and dilute to 20.0 mL with the same mixture of mobile phases. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 25 volumes of mobile phase B and 75 volumes of mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped cyanopropylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.2 g of *sodium dihydrogen phosphate R* in *water R*, add 1.1 mL of *nonylamine R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 75 | 25 |
| 10 - 20 | 75 → 65 | 25 → 35 |
| 20 - 32 | 65 | 35 |
| 32 - 34 | 65 → 75 | 35 → 25 |
| 34 - 44 | 75 | 25 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Relative retention* with reference to clomipramine (retention time = about 8 min): impurity B = about 0.7; impurity C = about 0.9; impurity D = about 1.7; impurity F = about 3.4.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to clomipramine and impurity C.

*Limits*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurities C, D*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of unspecified impurities*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.13 mg of $C_{19}H_{24}Cl_2N_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C, D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, E, G.*

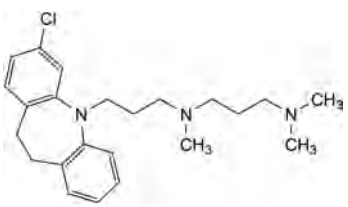


A. *N*-[3-(3-chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)propyl]-*N,N′,N′*-trimethylpropane-1,3-diamine,

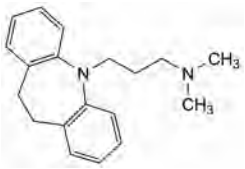

B. 3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine (imipramine),

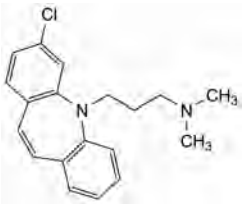

C. 3-(3-chloro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine,

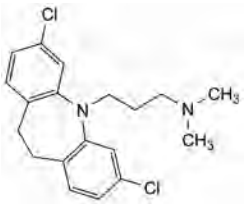

D. 3-(3,7-dichloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine,

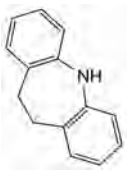

E. 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine (iminodibenzyl),

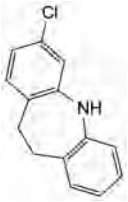

F. 3-chloro-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine,

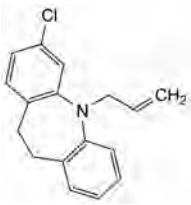

G. 3-chloro-5-(prop-2-en-1-yl)-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine.

**01/2008:0890**
**corrected 6.0**

# CLONAZEPAM

## Clonazepamum

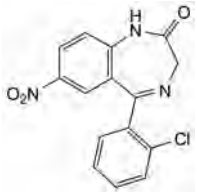

$C_{15}H_{10}ClN_3O_3$ $M_r$ 315.7
[1622-61-3]

DEFINITION

5-(2-Chlorophenyl)-7-nitro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: slightly yellowish, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in alcohol and in methanol.

mp: about 239 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of clonazepam*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solvent mixture*: *tetrahydrofuran R*, *methanol R*, *water R* (10:42:48 *V/V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *flunitrazepam R* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 1.0 mg of *clonazepam impurity B CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of *tetrahydrofuran R*, 42 volumes of *methanol R* and 48 volumes of a 6.6 g/L solution of *ammonium phosphate R* previously adjusted to pH 8.0 with a 40 g/L solution of *sodium hydroxide R* or *dilute phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of clonazepam.

*Relative retention* with reference to clonazepam (retention time = about 7 min): impurity B = about 2.1; impurity A = about 2.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.8 between the peaks due to flunitrazepam and to clonazepam.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent)

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.275 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.57 mg of $C_{15}H_{10}ClN_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

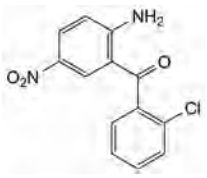

A. (2-amino-5-nitrophenyl)(2-chlorophenyl)methanone,

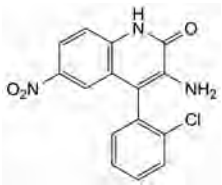

B. 3-amino-4-(2-chlorophenyl)-6-nitroquinolin-2(1*H*)-one.

**01/2008:0477**
**corrected 10.0**

# CLONIDINE HYDROCHLORIDE

## Clonidini hydrochloridum

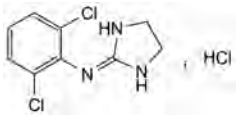

$C_9H_{10}Cl_3N_3$ $M_r$ 266.6
[4205-91-8]

DEFINITION

2,6-Dichloro-*N*-(imidazolidin-2-ylidene)aniline hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in anhydrous ethanol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 30.0 mg in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

*Spectral range*: 245-350 nm.

*Absorption maxima*: at 272 nm and 279 nm.

*Point of inflexion*: at 265 nm.

*Specific absorbance at the absorption maxima*:

– at 272 nm: about 18;

– at 279 nm: about 16.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clonidine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 5 mg of *clonidine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *glacial acetic acid R*, *butanol R*, *water R* (10:40:50 *V/V/V*); allow to separate, filter the upper layer and use the filtrate.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R2*. Allow to dry in air for 1 h. Spray again with *potassium iodobismuthate solution R2* and then immediately spray with a 50 g/L solution of *sodium nitrite R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 50 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 5 mg of *clonidine impurity B CRS* in 2 mL of *acetonitrile R* and dilute to 5 mL with mobile phase A. To 1 mL of this solution, add 1 mL of the test solution and dilute to 10 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *propylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 4 g of *potassium dihydrogen phosphate R* in 1000 mL of *water for chromatography R*, and adjust to pH 4.0 with *phosphoric acid R*;
- *mobile phase B*: mobile phase A, *acetonitrile R1* (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 90 | 10 |
| 0 - 15 | 90 → 30 | 10 → 70 |
| 15 - 15.1 | 30 → 90 | 70 → 10 |
| 15.1 - 20 | 90 | 10 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to clonidine and impurity B.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M ethanolic sodium hydroxide* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 26.66 mg of $C_9H_{10}Cl_3N_3$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

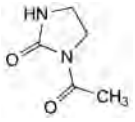

A. 1-acetylimidazolidin-2-one,

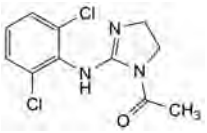

B. 1-acetyl-2-[(2,6-dichlorophenyl)amino]-4,5-dihydro-1*H*-imidazole,

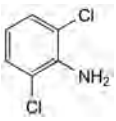

C. 2,6-dichloroaniline.

**01/2017:1747**

# CLOPAMIDE

## Clopamidum

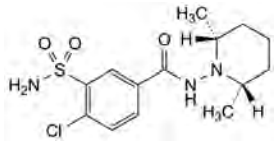

$C_{14}H_{20}ClN_3O_3S$ $M_r$ 345.8

[636-54-4]

DEFINITION

4-Chloro-*N*-[(2*RS*,6*SR*)-2,6-dimethylpiperidin-1-yl]-3-sulfamoylbenzamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

The production method is evaluated to determine the potential for formation of an *N*-nitroso compound (*cis*-2,6-dimethyl-1-nitrosopiperidine). Where necessary, the production method is validated to demonstrate that the *N*-nitroso compound is absent in the final product.

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water and in anhydrous ethanol, sparingly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clopamide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 100 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *clopamide for system suitability CRS* (containing impurities B, C and H) in 1.0 mL of *methanol R*.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 40.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *ammonium acetate R* in 950 mL of *water R*, adjust to pH 2.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;
- *mobile phase C*: *water R*, *tetrahydrofuran for chromatography R* (20:80 *V/V*); this mobile phase allows adequate rinsing of the system;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 35 | 95 → 75 | 5 → 25 | 0 |
| 35 - 45 | 75 → 35 | 25 → 65 | 0 |
| 45 - 50 | 35 → 30 | 65 → 0 | 0 → 70 |
| 50 - 60 | 30 | 0 | 70 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *clopamide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C and H.

*Relative retention* with reference to clopamide (retention time = about 33 min): impurity C = about 0.8; impurity H = about 1.2; impurity B = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 3 between the peaks due to impurity C and clopamide.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.5; impurity H = 0.4;
- *impurities B, C, H*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.280 g in 70 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.58 mg of $C_{14}H_{20}ClN_3O_3S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, G.*

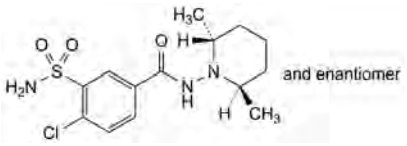


A. 4-chloro-*N*-[(2*RS*,6*RS*)-2,6-dimethylpiperidin-1-yl]-3-sulfamoylbenzamide (*trans*-clopamide),

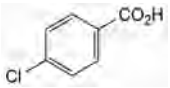

B. 4-chlorobenzoic acid,

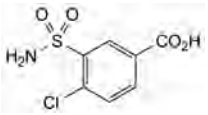

C. 4-chloro-3-sulfamoylbenzoic acid,

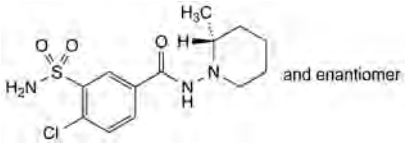


G. 4-chloro-*N*-[(2*RS*)-2-methylpiperidin-1-yl]-3-sulfamoylbenzamide,

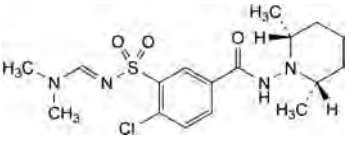

H. 4-chloro-3-[(*E*)-[(dimethylamino)methylene]sulfamoyl]-*N*-[(2*RS*,6*SR*)-2,6-dimethylpiperidin-1-yl]benzamide.

**01/2017:2790**
**corrected 10.0**

# CLOPIDOGREL BESILATE

## Clopidogreli besilas

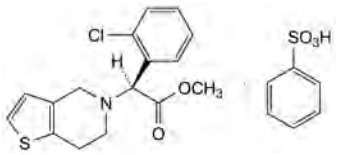


$C_{22}H_{22}ClNO_5S_2$ $M_r$ 480.0
[744256-69-7]

DEFINITION

Methyl (2*S*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetate benzenesulfonate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl benzenesulfonate esters are genotoxic and are potential impurities in clopidogrel besilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general method *2.5.41. Methyl, ethyl and isopropyl benzenesulfonate in active substances* is not suitable for clopidogrel besilate since it was observed that methyl benzenesulfonate was obtained during the gas chromatography analysis as an artefact originating from degradation. Another suitable and validated method should be used. The content of each alkyl benzenesulfonate is not more than 3 ppm.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): + 47.0 to + 51.0 (anhydrous substance).
Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clopidogrel besilate CRS*.

C. Enantiomeric purity (see Tests).

D. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.
*Injection*: test solution and reference solution (d).
*Results*:
- the peak due to besilate in the chromatogram obtained with the test solution is similar in retention time to the corresponding peak in the chromatogram obtained with reference solution (d);
- the ratio of the area of the peak due to the besilate to the area of the peak due to clopidogrel in the chromatogram obtained with the test solution is minimum 0.1.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 46.0 mg of the substance to be examined in 10.0 mL of *anhydrous ethanol R* and dilute to 20.0 mL with *heptane R*.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *heptane R*.

*Reference solution (a).* Dissolve 10 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in 2.5 mL of *anhydrous ethanol R* and dilute to 5.0 mL with *heptane R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *heptane R*. Dilute 1.5 mL of this solution to 10.0 mL with *heptane R*.

*Reference solution (c).* Dissolve 34.0 mg of *clopidogrel hydrochloride CRS* in 10.0 mL of *anhydrous ethanol R* and dilute to 20.0 mL with *heptane R*. Dilute 1.0 mL of the solution to 10.0 mL with *heptane R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *anhydrous ethanol R*, *heptane R* (15:85 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 1.25 times the retention time of clopidogrel.

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to clopidogrel (retention time = about 18 min): impurity C = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurities C and B.

*Calculation of percentage content*:
- for impurity C, use the concentration of clopidogrel besilate in reference solution (b).

*Limit*:
- *impurity C*: maximum 0.15 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, *acetonitrile R1* (40:60 *V/V*).

*Test solution.* Dissolve 74 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *clopidogrel impurity A CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 32 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in the solvent mixture, add 0.5 mL of reference solution (a) and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25 mg of *sodium benzenesulfonate R* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 5 volumes of *methanol R2* and 95 volumes of a 0.96 g/L solution of *sodium pentanesulfonate monohydrate R* adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: *methanol R2*, *acetonitrile R1* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 89.5 | 10.5 |
| 3 - 48 | 89.5 → 31.5 | 10.5 → 68.5 |
| 48 - 68 | 31.5 | 68.5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to clopidogrel (retention time = about 27 min): besilate = about 0.05; impurity A = about 0.4; impurity B = about 1.1.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clopidogrel.

*Calculation of percentage contents*:

– for each impurity, use the concentration of clopidogrel besilate in reference solution (c).

*Limits*:

– *impurities A, B*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent; disregard the peak due to the besilate.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for enantiomeric purity with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{22}H_{22}ClNO_5S_2$ taking into account the assigned content of *clopidogrel hydrochloride CRS*, and a conversion factor of 1.34.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G.*

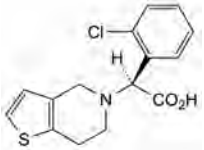

A. (2*S*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetic acid,

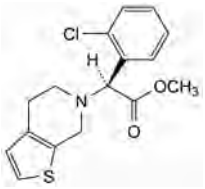

B. methyl (2*S*)-2-(2-chlorophenyl)-2-[4,7-dihydrothieno-[2,3-*c*]pyridin-6(5*H*)-yl]acetate,

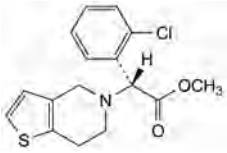

C. methyl (2*R*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno-[3,2-*c*]pyridin-5(4*H*)-yl]acetate,

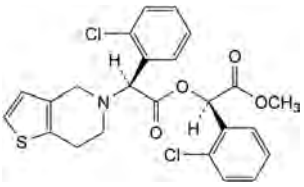

D. (1*R*)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl (2*S*)-2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl)acetate,

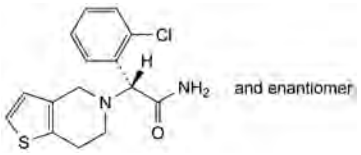


E. (2*RS*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetamide,

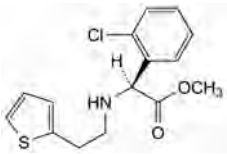

F. methyl (2*S*)-2-(2-chlorophenyl)-2-[[2-(thiophen-2-yl)ethyl]amino]acetate,

G. unknown structure.

**01/2017:2791**
**corrected 10.0**

# CLOPIDOGREL HYDROCHLORIDE

## Clopidogreli hydrochloridum

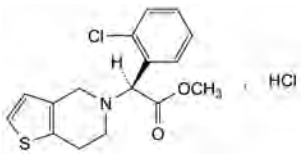

$C_{16}H_{17}Cl_2NO_2S$ $M_r$ 358.3
[120202-65-5]

DEFINITION

Methyl (2*S*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetate hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish powder.

*Solubility*: practically insoluble in water, very soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): + 65.0 to + 69.0 (anhydrous substance).
Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *clopidogrel hydrochloride CRS*.

C. Enantiomeric purity (see Tests).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 34.0 mg of the substance to be examined in 10.0 mL of *anhydrous ethanol R* and dilute to 20.0 mL with *heptane R*.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *heptane R*.

*Reference solution (a).* Dissolve 10 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in 2.5 mL of *anhydrous ethanol R* and dilute to 5.0 mL with *heptane R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *heptane R*. Dilute 1.5 mL of this solution to 10.0 mL with *heptane R*.

*Reference solution (c).* Dissolve 34.0 mg of *clopidogrel hydrochloride CRS* in 10.0 mL of *anhydrous ethanol R* and dilute to 20.0 mL with *heptane R*. Dilute 1.0 mL of the solution to 10.0 mL with *heptane R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *anhydrous ethanol R*, *heptane R* (15:85 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 1.25 times the retention time of clopidogrel.

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to clopidogrel (retention time = about 18 min): impurity C = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurities C and B.

*Calculation of percentage content*:
- for impurity C, use the concentration of clopidogrel hydrochloride in reference solution (b).

*Limit*:
- *impurity C*: maximum 0.15 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, *acetonitrile R1* (40:60 *V/V*).

*Test solution.* Dissolve 55 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *clopidogrel impurity A CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 32 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in the solvent mixture, add 0.5 mL of reference solution (a) and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 5 volumes of *methanol R2* and 95 volumes of a 0.96 g/L solution of *sodium pentanesulfonate monohydrate R* adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: *methanol R2*, *acetonitrile R1* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 89.5 | 10.5 |
| 3 - 48 | 89.5 → 31.5 | 10.5 → 68.5 |
| 48 - 68 | 31.5 | 68.5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to clopidogrel (retention time = about 27 min): impurity A = about 0.4; impurity B = about 1.1.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clopidogrel.

*Calculation of percentage contents*:
- for each impurity, use the concentration of clopidogrel hydrochloride in reference solution (c).

*Limits*:
- *impurities A, B*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.4 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g. Replace the solvent after each titration.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for enantiomeric purity with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{16}H_{17}Cl_2NO_2S$ taking into account the assigned content of *clopidogrel hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G.*

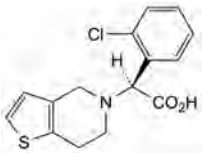

A. (2*S*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetic acid,

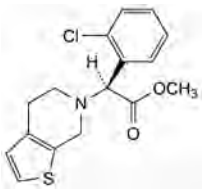

B. methyl (2*S*)-2-(2-chlorophenyl)-2-[4,7-dihydrothieno-[2,3-*c*]pyridin-6(5*H*)-yl]acetate,

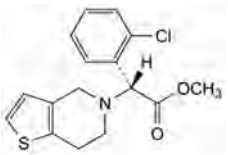

C. methyl (2*R*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno-[3,2-*c*]pyridin-5(4*H*)-yl]acetate,

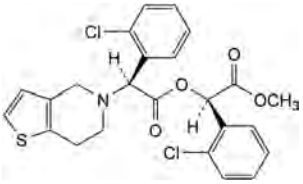

D. (1*R*)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl (2*S*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetate,

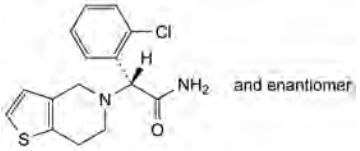


E. (2*RS*)-2-(2-chlorophenyl)-2-[6,7-dihydrothieno[3,2-*c*]-pyridin-5(4*H*)-yl]acetamide,

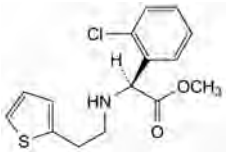

F. methyl (2*S*)-2-(2-chlorophenyl)-2-[[2-(thiophen-2-yl)ethyl]amino]acetate,

G. unknown structure.

**01/2017:2531**
**corrected 10.0**

# CLOPIDOGREL HYDROGEN SULFATE

## Clopidogreli hydrogenosulfas

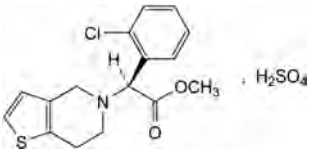

$C_{16}H_{18}ClNO_6S_2$ $M_r$ 419.9
[120202-66-6]

DEFINITION

Methyl (2*S*)-(2-chlorophenyl)[6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]acetate sulfate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in cyclohexane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): + 54.0 to + 58.0 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clopidogrel hydrogen sulfate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues (the substance may stick to the surface of the recipient used).

C. Enantiomeric purity (see Tests).

D. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

Dissolve 1.0 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 0.1 g of the substance to be examined in 25.0 mL of *anhydrous ethanol R* and dilute to 50.0 mL with *heptane R*.

*Reference solution*. Dissolve 10 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in 2.5 mL of *anhydrous ethanol R* and dilute to 5 mL with *heptane R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *anhydrous ethanol R*, *heptane R* (15:85 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 1.25 times the retention time of clopidogrel.

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with the reference solution to identify the peaks due to impurities B and C.

*Relative retention* with reference to clopidogrel (retention time = about 18 min): impurity C = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to impurities C and B;
- *signal-to-noise ratio*: minimum 20 for the peak due to impurity C.

*Limit*:

- *impurity C*: maximum 0.5 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, *acetonitrile R* (40:60 *V/V*).

*Test solution*. Dissolve 65 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *clopidogrel impurity A CRS* in the solvent mixture and dilute to 25 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 32 mg of *clopidogrel for system suitability CRS* (containing impurities B and C) in the solvent mixture, add 0.5 mL of reference solution (a) and dilute to 5 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *methanol R1* and 95 volumes of a 0.96 g/L solution of *sodium pentanesulfonate monohydrate R* adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: *methanol R1*, *acetonitrile for chromatography R* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 89.5 | 10.5 |
| 3 - 48 | 89.5 → 31.5 | 10.5 → 68.5 |
| 48 - 68 | 31.5 | 68.5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *clopidogrel for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to clopidogrel (retention time = about 25 min): impurity A = about 0.4; impurity B = about 1.1.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to clopidogrel.

*Limits*:

- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

Replace the solvent after each titration.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in a mixture of 10 mL of *acetone R*, 10 mL of *methanol R* and 30 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). A precipitate may be formed during the titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.99 mg of $C_{16}H_{18}ClNO_6S_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

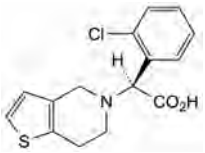


A. (2*S*)-(2-chlorophenyl)[6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]acetic acid,

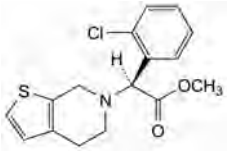


B. methyl (2*S*)-(2-chlorophenyl)[4,7-dihydrothieno[2,3-*c*]pyridin-6(5*H*)-yl]acetate,

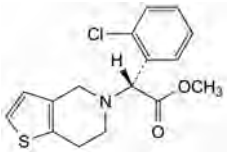


C. methyl (2*R*)-(2-chlorophenyl)[6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]acetate,

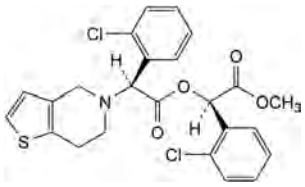

D. methyl (2*R*)-(2-chlorophenyl)[(2*S*)-(2-chlorophenyl)[6,7-dihydrothieno[3,2-*c*]pyridin-5(4*H*)-yl]acetyloxy]acetate.

**04/2019:1716**

# CLOSANTEL SODIUM DIHYDRATE FOR VETERINARY USE

## Closantelum natricum dihydricum ad usum veterinarium

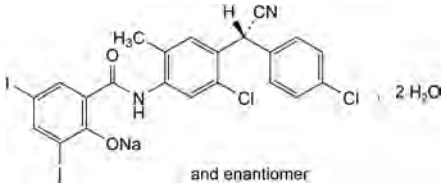


$C_{22}H_{13}Cl_2I_2N_2NaO_2,2H_2O$ $M_r$ 685 (anhydrous substance)
Anhydrous closantel sodium: [61438-64-0]

DEFINITION

*N*-[5-Chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide sodium salt dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow powder, slightly hygroscopic.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent), soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*), without recrystallisation.

*Comparison*: *closantel sodium dihydrate CRS*.

B. Dissolve 0.1 g in 2 mL of *ethanol (96 per cent) R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

Dissolve 0.50 g in *ethanol (96 per cent) R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *closantel for system suitability CRS* (containing impurities A to J) in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 25.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: to 100 mL of a 7.7 g/L solution of *ammonium acetate R* previously adjusted to pH 4.3 with *acetic acid R*, add 50 mL of *acetonitrile for chromatography R* and 850 mL of *water for chromatography R*;
- *mobile phase B*: to 100 mL of a 7.7 g/L solution of *ammonium acetate R* previously adjusted to pH 4.3 with *acetic acid R*, add 50 mL of *water for chromatography R* and 850 mL of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 50 | 50 |
| 2 - 22 | 50 → 20 | 50 → 80 |
| 22 - 27 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *closantel for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F, G, H, I and J.

*Relative retention* with reference to closantel (retention time = about 16 min): impurity A = about 0.07; impurity B = about 0.48; impurity C = about 0.62; impurity D = about 0.65; impurity E = about 0.82; impurity F = about 0.89; impurity G = about 0.93; impurity H = about 1.13; impurity I = about 1.16; impurity J = about 1.55.

*System suitability*: reference solution (a):
- *resolution*: baseline separation between the peaks due to impurity G and closantel;
- the chromatogram obtained is similar to the chromatogram supplied with *closantel for system suitability CRS*.

*Limits*:
- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity B = 1.3;
- *impurity G*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities F, H, I*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities A, B, C, D, E, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *total*: not more than 7.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 4.8 per cent to 5.8 per cent, determined on 0.250 g.

Use a mixture of 1 volume of *dimethylformamide R* and 4 volumes of *methanol R* as the solvent.

ASSAY

Dissolve 0.500 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 68.5 mg of $C_{22}H_{13}Cl_2I_2N_2NaO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

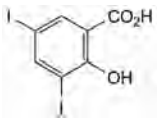

A. 2-hydroxy-3,5-diiodobenzoic acid,

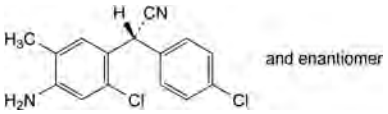

B. (2*RS*)-(4-amino-2-chloro-5-methylphenyl)(4-chlorophenyl)ethanenitrile,

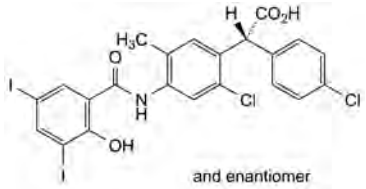

C. (2*RS*)-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl](4-chlorophenyl)acetic acid,

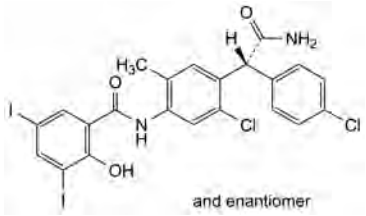

D. *N*-[4-[(1*RS*)-2-amino-1-(4-chlorophenyl)-2-oxoethyl]-5-chloro-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide,

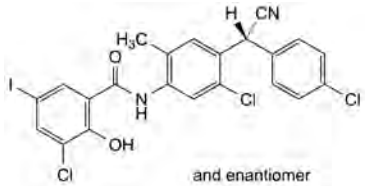

E. 3-chloro-*N*-[5-chloro-4-[(*RS*)-(4-chlorophenyl)cyano-methyl]-2-methylphenyl]-2-hydroxy-5-iodobenzamide,

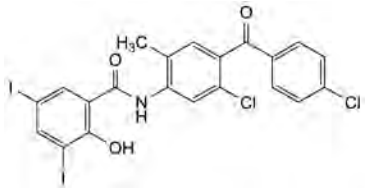

F. *N*-[5-chloro-4-(4-chlorobenzoyl)-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide,

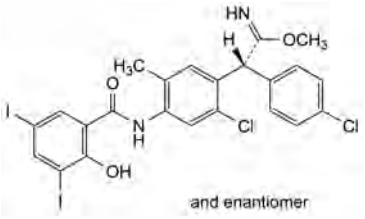

G. methyl (2*RS*)-2-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl]-2-(4-chlorophenyl)acetimidate,

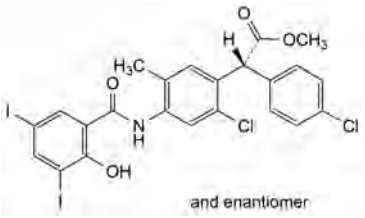

H. methyl (2*RS*)-[2-chloro-4-[(2-hydroxy-3,5-diiodobenzoyl)amino]-5-methylphenyl](4-chlorophenyl)acetate,

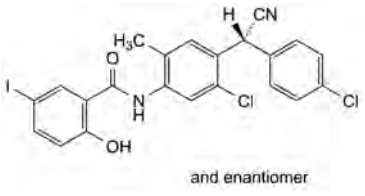

I. *N*-[5-chloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-5-iodobenzamide,

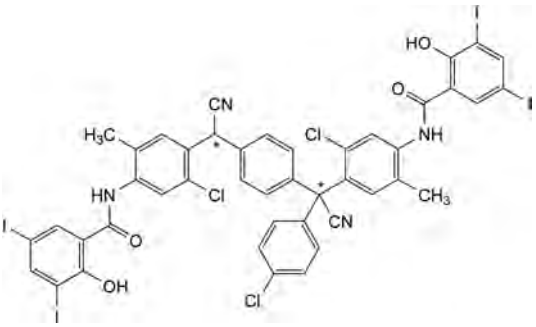

J. *N*-[5-chloro-4-[[4-[[2-chloro-4-[(2-hydroxy-3,5-diiodo-benzoyl)amino]-5-methylphenyl]cyanomethyl]phenyl]-(4-chlorophenyl)cyanomethyl]-2-methylphenyl]-2-hydroxy-3,5-diiodobenzamide.

**04/2008:0757**

# CLOTRIMAZOLE

## Clotrimazolum

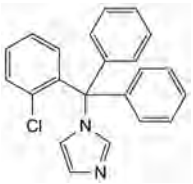

$C_{22}H_{17}ClN_2$ $M_r$ 344.8

[23593-75-1]

DEFINITION

1-[(2-Chlorophenyl)diphenylmethyl]-1*H*-imidazole.

*Content*: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 141 °C to 145 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clotrimazole CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *clotrimazole CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R1*, *propanol R*, *toluene R* (0.5:10:90 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R1*.

*Reference solution (b).* Dissolve the contents of a vial of *clotrimazole for peak identification CRS* (containing impurities A, B and F) in 1.0 mL of *acetonitrile R1*.

*Reference solution (c).* Dissolve 5.0 mg of *imidazole CRS* (impurity D) and 5.0 mg of *clotrimazole impurity E CRS* in *acetonitrile R1* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 25.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *potassium dihydrogen phosphate R* and 0.5 g of *tetrabutylammonium hydrogen sulfate R1* in *water R* and dilute to 1000 mL with the same solvent;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 25 | 75 → 20 | 25 → 80 |
| 25 - 30 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Relative retention* with reference to clotrimazole (retention time = about 12 min): impurity D = about 0.1; impurity F = about 0.9; impurity B = about 1.1; impurity E = about 1.5; impurity A = about 1.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity F and clotrimazole;
- the chromatogram obtained is similar to the chromatogram supplied with *clotrimazole for peak identification CRS*.

*Limits*:

- *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities D, E*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 80 mL of *anhydrous acetic acid R*. Using 0.3 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from brownish-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 34.48 mg of $C_{22}H_{17}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

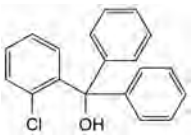

A. (2-chlorophenyl)diphenylmethanol,

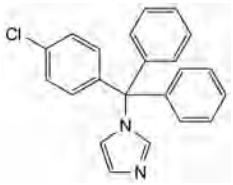

B. 1-[(4-chlorophenyl)diphenylmethyl]-1*H*-imidazole,

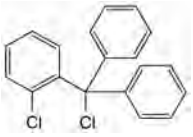

C. 1-chloro-2-(chlorodiphenylmethyl)benzene,

D. imidazole,

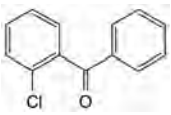

E. (2-chlorophenyl)phenylmethanone (2-chlorobenzophenone),

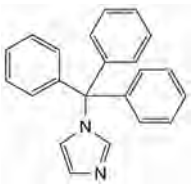

F. 1-(triphenylmethyl)-1*H*-imidazole (deschloroclotrimazole).

**01/2008:0661**
**corrected 10.0**

# CLOXACILLIN SODIUM

## Cloxacillinum natricum

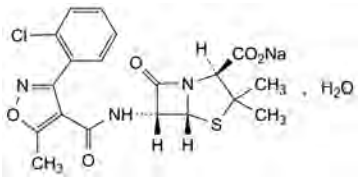

$C_{19}H_{17}ClN_3NaO_5S,H_2O$ $M_r$ 475.9
[7081-44-9]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[[3-(2-chlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *cloxacillin sodium CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in 5 mL of *water R*.
*Reference solution (a)*. Dissolve 25 mg of *cloxacillin sodium CRS* in 5 mL of *water R*.
*Reference solution (b)*. Dissolve 25 mg of *cloxacillin sodium CRS*, 25 mg of *dicloxacillin sodium CRS* and 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R*.
*Plate*: *TLC silanised silica gel plate R*.
*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R*, then adjust to pH 5.0 with *glacial acetic acid R*.
*Application*: 1 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear; examine in daylight.
*System suitability*: reference solution (b):
– the chromatogram shows 3 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; the solution becomes yellow.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.04.

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 160 to + 169 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 50.0 mg of *cloxacillin sodium CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 5.0 mL of test solution (b) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *cloxacillin sodium CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R* and 75 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 5 times the retention time of cloxacillin.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to cloxacillin (1st peak) and flucloxacillin (2nd peak).

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): 3.0 per cent to 4.5 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.20 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*:

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

Calculate the percentage content of $C_{19}H_{17}ClN_3NaO_5S$ from the declared content of *cloxacillin sodium CRS*.

STORAGE

In an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

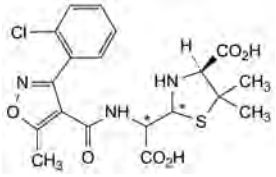

A. (4*S*)-2-[carboxy[[[3-(2-chlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acid of cloxacillin),

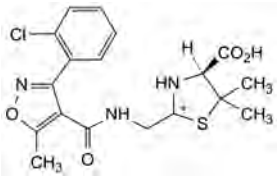

B. (2*RS*,4*S*)-2-[[[[3-(2-chlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penilloic acid of cloxacillin),

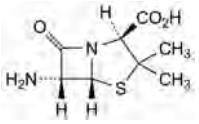

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

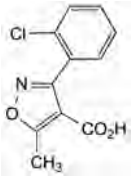

D. 3-(2-chlorophenyl)-5-methyl-1,2-oxazole-4-carboxylic acid,

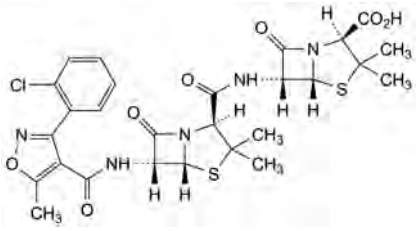

E. (2*S*,5*R*,6*R*)-6-[[[(2*S*,5*R*,6*R*)-6-[[[3-(2-chlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA cloxacillin amide).

**01/2017:1191**

# CLOZAPINE

## Clozapinum

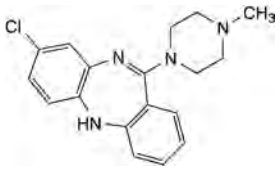

$C_{18}H_{19}ClN_4$ $M_r$ 326.8
[5786-21-0]

DEFINITION

8-Chloro-11-(4-methylpiperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]-diazepine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent). It dissolves in dilute acetic acid.

IDENTIFICATION

A. Melting point (*2.2.14*): 182 °C to 186 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *clozapine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R2* (20:80 *V/V*).

*Solution A*. Dissolve 2.04 g of *potassium dihydrogen phosphate R* in 1000 mL of *water R* and adjust to pH 2.4 ± 0.05 with *dilute phosphoric acid R*.

*Test solution*. Dissolve 75 mg of the substance to be examined in 80 mL of *methanol R2* and dilute to 100 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *clozapine for peak identification CRS* (containing impurities A, B, C and D) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile for chromatography R*, *methanol R2*, solution A (1:1:8 *V/V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*, *methanol R2*, solution A (4:4:2 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 24 | 100 → 0 | 0 → 100 |
| 24 - 29 | 0 | 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 257 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *clozapine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to clozapine (retention time = about 11 min): impurity C = about 0.9; impurity D = about 1.1; impurity A = about 1.6; impurity B = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity C and clozapine;
- the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *clozapine for peak identification CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 2.7;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurities B, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.34 mg of $C_{18}H_{19}ClN_4$.

IMPURITIES

*Specified impurities: A, B, C, D.*

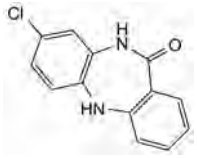

A. 8-chloro-5,10-dihydro-11*H*-dibenzo[*b*,*e*][1,4]diazepin-11-one,

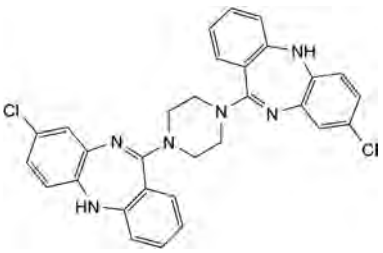

B. 11,11′-(piperazine-1,4-diyl)bis(8-chloro-5*H*-dibenzo[*b*,*e*][1,4]diazepine),

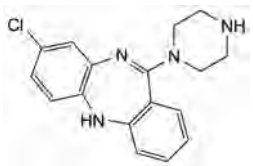

C. 8-chloro-11-(piperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine,

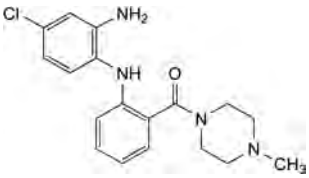

D. 1-[2-[(2-amino-4-chlorophenyl)amino]benzoyl]-4-methylpiperazine.

**01/2008:0073 corrected 6.0**

# COCAINE HYDROCHLORIDE

## Cocaini hydrochloridum

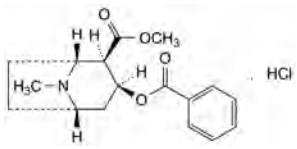

$C_{17}H_{22}ClNO_4$ $M_r$ 339.8
[53-21-4]

DEFINITION

Methyl (1*R*,2*R*,3*S*,5*S*)-3-(benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in alcohol, slightly soluble in methylene chloride.

mp: about 197 °C, with decomposition.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D, E.*

A. Dissolve 20.0 mg in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of the solution to 50.0 mL with *0.01 M hydrochloric acid*. Examined between 220 nm and 350 nm (*2.2.25*), the solution shows 2 absorption maxima, at 233 nm and 273 nm. The specific absorbance at 233 nm is 378 to 402.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of cocaine hydrochloride*.

C. Dissolve 0.1 g in 5 mL of *water R* and add 1 mL of *dilute ammonia R2*. A white precipitate is formed. Initiate crystallisation by scratching the wall of the tube with a glass rod. The crystals, washed with *water R* and dried *in vacuo*, melt (*2.2.14*) at 96 °C to 99 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

E. It gives the reaction of alkaloids (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): – 70 to – 73 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 20.0 mL with the same solvent.

**Readily carbonisable substances**. To 0.2 g add 2 mL of *sulfuric acid R*. After 15 min, the solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method I*).

**Related substances**. Examine by liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 25 mg of the substance to be examined in *0.01 M sodium hydroxide* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *0.01 M sodium hydroxide*. Allow the solution to stand for 15 min.

*Column:*

– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 335 $m^2/g$, a pore size of 10 nm and a carbon loading of 19.1 per cent,
– *temperature*: 35 °C.

*Mobile phase*: *triethylamine R*, *tetrahydrofuran R*, *acetonitrile R*, *water R* (0.5:100:430:479.5 *V/V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 216 nm.

*Injection*: 20 µL.

*Relative retention* with reference to cocaine (retention time = about 7.4 min): degradation product = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum of 5 between the peaks due to cocaine and to the degradation product.

*Limits*:

– *any impurity eluting after the principal peak*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.98 mg of $C_{17}H_{22}ClNO_4$.

STORAGE

Protected from light.

IMPURITIES

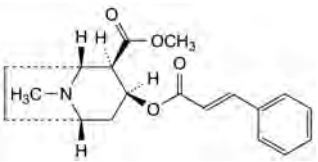

A. methyl (1*R*,2*R*,3*S*,5*S*)-8-methyl-3-[[(*E*)-3-phenylpropenoyl]oxy]-8-azabicyclo[3.2.1]octane-2-carboxylate (cinnamoylcocaine),

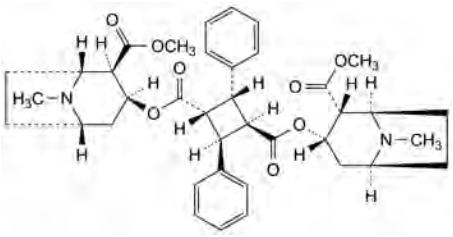

B. bis[(1*R*,2*R*,3*S*,5*S*)-2-(methoxycarbonyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl] (1*r*,2*c*,3*t*,4*t*)-2,4-diphenylcyclobutane-1,3-dicarboxylate (α-truxilline),

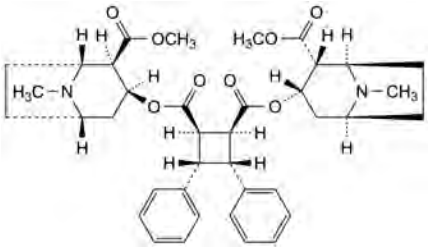

C. bis[(1*R*,2*R*,3*S*,5*S*)-2-(methoxycarbonyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl] (1*r*,2*c*,3*t*,4*t*)-3,4-diphenylcyclobutane-1,2-dicarboxylate (β-truxilline).

**01/2020:2607**

# COCOA BUTTER

## Theobromatis oleum

DEFINITION

Solid fat obtained from the roasted seeds of *Theobroma cacao* L.

CHARACTERS

*Appearance*: yellowish-white, solid mass.

*Solubility*: freely soluble in boiling anhydrous ethanol and in light petroleum, slightly soluble in ethanol (96 per cent).

*Relative density*: about 0.895 at 40 °C.

*Refractive index*: about 1.457 at 40 °C.

IDENTIFICATION

A. Melting point (*2.2.15*): 31 °C to 35 °C.

Introduce 10 g of the substance to be examined into a beaker and melt at 55 °C. Cool in a water-bath to 25 °C and stir continuously until it assumes a paste-like consistency, taking care to avoid the introduction of air bubbles. Place the beaker in a water-bath maintained at 32-33 °C. Continue stirring for about 30 min until the substance reaches the temperature of the water-bath and changes to a liquid cream. Pour into another beaker, and allow to solidify at room temperature for at least 2 h.

Introduce the substance into the capillary tubes and allow to stand at 2-8 °C for at least 48 h.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 4.0.

**Peroxide value** (*2.5.5, Method A*): maximum 3.0.

The *starch solution R* must be introduced before starting the titration.

**Saponification value** (*2.5.6*): 188 to 198, determined on 2.5 g.

**Alkaline impurities**

*Solvent mixture*. Dilute 15 mL of *water R* to 500 mL with *acetone R* and mix. Add 2.5 mL of a 1 g/L solution of *bromophenol blue R* in *ethanol (50 per cent V/V) R* and mix again. If the solution is blue or yellow instead of green, neutralise with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*, respectively, to obtain a green solution.

Melt 50 g of the substance to be examined at about 50 °C and mix thoroughly. In a 150 mL conical flask, introduce 10.0 g of the melted substance and add 50 mL of the solvent mixture. Stir vigorously and allow the 2 layers to separate. Not more than 2 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow; this coloration must persist after vigorous stirring.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*) with the following modifications.

Use the mixture of calibrating substances in Table 2.4.22.-1.

*Column*:

- *material*: fused silica, glass or quartz;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 70 → 205 |
| | 15 - 25 | 205 |
| | 25 - 27.5 | 205 → 230 |
| | 27.5 - 50 | 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Composition of the fatty-acid fraction of the substance*:

- *lauric acid*: maximum 0.5 per cent;
- *myristic acid*: maximum 0.5 per cent;
- *palmitic acid*: 24.0 per cent to 31.0 per cent;
- *stearic acid*: 30.0 per cent to 38.0 per cent;
- *oleic acid*: 31.0 per cent to 38.0 per cent;
- *linoleic acid*: maximum 4.5 per cent;
- *arachidic acid*: maximum 1.5 per cent.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 25 °C.

**01/2015:1410**

# COCONUT OIL, REFINED

## Cocois oleum raffinatum

[8001-31-8]

DEFINITION

Fatty oil obtained from the dried, solid part of the endosperm of *Cocos nucifera* L., then refined.

CHARACTERS

*Appearance*: white or almost white, unctuous mass.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride and in light petroleum (bp: 65-70 °C), very slightly soluble in ethanol (96 per cent).

*Refractive index*: about 1.449, determined at 40 °C.

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

TESTS

**Melting point** (*2.2.14*): 23 °C to 26 °C.

**Acid value** (*2.5.1*): maximum 0.5, determined on 20.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method B*). Refined coconut oil is melted under gentle heating to a homogeneous liquid prior to sampling.

*Reference solution.* Dissolve 15.0 mg of *tricaproin CRS*, 80.0 mg of *tristearin CRS*, 0.150 g of *tricaprin CRS*, 0.200 g of *tricaprylin CRS*, 0.450 g of *trimyristin CRS* and 1.25 g of *trilaurin CRS* in a mixture of 2 volumes of *methylene chloride R* and 8 volumes of *heptane R*, then dilute to 50 mL with the same mixture of solvents heating at 45-50 °C. Transfer 2 mL of this mixture to a 10 mL centrifuge tube with a screw cap and evaporate the solvent in a current of *nitrogen R*. Dissolve with 1 mL of *heptane R* and 1 mL of *dimethyl carbonate R* and mix vigorously under gentle heating (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for about 5 min. Add 3 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µL of the organic phase.

Calculate the percentage content of each fatty acid using the following expression:

$$\frac{A_{x,s,c}}{\sum A_{x,s,c}} \times 100 \text{ per cent } m / m$$

$A_{x,s,c}$ is the corrected peak area of each fatty acid in the test solution:

$$A_{x,s,c} = A_{x,s} \times R_c$$

$R_c$ is the relative correction factor:

$$R_c = \frac{m_{x,r} \times A_{1,r}}{A_{x,r} \times m_{1,r}}$$

for the peaks due to caproic, caprylic, capric, lauric and myristic acid methyl esters.

$m_{x,r}$ = mass of tricaproin, tricaprylin, tricaprin, trilaurin or trimyristin in the reference solution, in milligrams;

$m_{1,r}$ = mass of tristearin in the reference solution, in milligrams:

$A_{x,r}$ = area of the peaks due to caproic, caprylic, capric, lauric and myristic acid methyl esters in the reference solution;

$A_{1,r}$ = area of the peak due to stearic acid methyl ester in the reference solution;

$A_{x,s}$ = area of the peaks due to any specified or unspecified fatty acid methyl esters;

$R_c$ = 1 for the peaks due to each of the remaining specified fatty acid methyl esters or any unspecified fatty acid methyl ester.

*Composition of the fatty-acid fraction of the oil*:

- *caproic acid ($R_{Rt}$ 0.11)*: maximum 1.5 per cent,
- *caprylic acid ($R_{Rt}$ 0.23)*: 5.0 per cent to 11.0 per cent,
- *capric acid ($R_{Rt}$ 0.56)*: 4.0 per cent to 9.0 per cent,
- *lauric acid ($R_{Rt}$ 0.75)*: 40.0 per cent to 50.0 per cent,
- *myristic acid ($R_{Rt}$ 0.85)*: 15.0 per cent to 20.0 per cent,
- *palmitic acid ($R_{Rt}$ 0.93)*: 7.0 per cent to 12.0 per cent,
- *stearic acid ($R_{Rt}$ 1.00)*: 1.5 per cent to 5.0 per cent,
- *oleic acid ($R_{Rt}$ 1.01)*: 4.0 per cent to 10.0 per cent,
- *linoleic acid ($R_{Rt}$ 1.03)*: 1.0 per cent to 3.0 per cent,
- *linolenic acid ($R_{Rt}$ 1.06)*: maximum 0.2 per cent,
- *arachidic acid ($R_{Rt}$ 1.10)*: maximum 0.2 per cent,
- *eicosenoic acid ($R_{Rt}$ 1.11)*: maximum 0.2 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light.

**04/2015:1411**

# COCOYL CAPRYLOCAPRATE

## Cocoylis caprylocapras

DEFINITION

Mixture of esters of saturated $C_{12}$ - $C_{18}$ alcohols with caprylic (octanoic) and capric (decanoic) acids obtained by the reaction of these acids with vegetable saturated fatty alcohols.

CHARACTERS

*Appearance*: slightly yellowish liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent) and with liquid paraffin.

*Relative density*: about 0.86.

*Refractive index*: about 1.445.

*Viscosity*: about 11 mPa·s.

IDENTIFICATION

A. Freezing point (*2.2.18*): maximum 15 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cocoyl caprylocaprate CRS*.

C. Composition of fatty acids and fatty alcohols (see Tests).

TESTS

**Appearance**. The substance to be examined is not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method I*).

**Acid value** (*2.5.1*): maximum 0.5, determined on 5.00 g.

**Hydroxyl value** (*2.5.3, Method A*): maximum 5.0, determined on 4.0 g. Add 5.0 mL of acetylating reagent.

**Iodine value** (*2.5.4, Method A*): maximum 1.0.

**Saponification value** (*2.5.6*): 160 to 173.

**Composition of fatty acids and fatty alcohols** (*2.4.22, Method C*). Use the chromatogram obtained with the following reference solution for identification of the peaks due to the fatty alcohols.

*Reference solution.* Dissolve the amounts of the substances listed in the following table in 10 mL of *heptane R*.

| Substance | Amount (mg) |
|---|---|
| *Methyl caproate R* | 10 |
| *Methyl caprylate R* | 90 |
| *Methyl decanoate R* | 50 |
| *Methyl laurate R* | 20 |
| *Methyl myristate R* | 10 |
| *Methyl palmitate R* | 10 |
| *Methyl stearate R* | 10 |
| *Decanol R* | 10 |
| *Lauryl alcohol R* | 100 |
| *Myristyl alcohol R* | 40 |
| *Cetyl alcohol CRS* | 30 |
| *Stearyl alcohol CRS* | 20 |

Consider the sum of the areas of the peaks due to the fatty acids listed below to be equal to 100 and the sum of the areas of the peaks due to the fatty alcohols listed below to be equal to 100.

*Composition of the fatty acid fraction of the substance*:
- *caproic acid*: maximum 2.0 per cent,
- *caprylic acid*: 50.0 per cent to 80.0 per cent,
- *capric acid*: 20.0 per cent to 50.0 per cent,
- *lauric acid*: maximum 3.0 per cent,
- *myristic acid*: maximum 2.0 per cent.

*Composition of the fatty alcohol fraction of the substance*:
- *capric alcohol*: maximum 3.0 per cent,
- *lauryl alcohol*: 48.0 per cent to 63.0 per cent,
- *myristyl alcohol*: 18.0 per cent to 27.0 per cent,
- *cetyl alcohol*: 6.0 per cent to 13.0 per cent,
- *stearyl alcohol*: 9.0 per cent to 16.0 per cent.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 5.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.0 g.

**07/2018:1412**
**corrected 10.0**

# CODEINE HYDROCHLORIDE DIHYDRATE

## Codeini hydrochloridum dihydricum

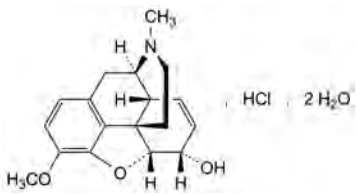


$C_{18}H_{22}ClNO_3,2H_2O$ $M_r$ 371.9

DEFINITION

4,5α-Epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol hydrochloride dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small, colourless crystals.

*Solubility*: soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

*First identification: A, D, F.*

*Second identification: B, C, D, E, F.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *codeine hydrochloride dihydrate CRS.*

B. To 5 mL of solution S (see Tests) add 1 mL of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C. It melts (*2.2.15*) at 155 °C to 159 °C.

C. To about 10 mg add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 mL of *nitric acid R*. The colour changes to red.

D. Solution S gives reaction (a) of chlorides (*2.3.1*).

E. It gives the reaction of alkaloids (*2.3.1*).

F. Water (see Tests).

TESTS

**Solution S**. Dissolve 2.00 g in *carbon dioxide-free water R*, prepared from *distilled water R*, and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S add 5 mL of *carbon dioxide-free water R*. Add 0.05 mL of *methyl red solution R* and 0.2 mL of *0.02 M hydrochloric acid*; the solution is red. Add 0.4 mL of *0.02 M sodium hydroxide*; the solution becomes yellow.

**Specific optical rotation** (*2.2.7*): − 117 to − 121 (anhydrous substance).

Dilute 5.0 mL of solution S to 10.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 0.5 per cent *V/V* solution of *phosphoric acid R*.

*Test solution*. Dissolve 0.190 g of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 3 mg of *codeine for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H and I) in 1 mL of solution A.

*Column*:
- *size*: *l* = 0.075 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl multi-layered organosilica polymer R* (1.9 µm).
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: mix 4 volumes of *acetonitrile R* and 96 volumes of a 20 g/L solution of *glacial acetic acid R* previously adjusted to pH 4.5 with a 500 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 7.33 | 100 → 93 | 0 → 7 |
| 7.33 - 10.33 | 93 → 67 | 7 → 33 |
| 10.33 - 12 | 67 | 33 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 3 µL.

*Identification of impurities*: use the chromatogram supplied with *codeine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to codeine (retention time = about 6.0 min): impurity B = about 0.3; impurity E = about 0.4; impurity F = about 0.8; impurity H = about 0.9; impurity C = about 1.2; impurity I = about 1.4; impurity D = about 1.45; impurity A = about 1.5; impurity G = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurities F and H; minimum 1.5 between the peaks due to impurities D and A.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity G = 0.2; impurity I = 1.3;
- for each impurity, use the concentration of codeine hydrochloride dihydrate in reference solution (a).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurity H*: maximum 0.25 per cent;
- *impurities C, D, E*: for each impurity, maximum 0.2 per cent;
- *impurities B, F, G, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 5 mL of solution S to 20 mL with *distilled water R*.

**Water** (*2.5.12*): 8.0 per cent to 10.5 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.59 mg of $C_{18}H_{22}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L, M.*

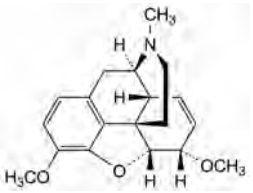

A. 4,5α-epoxy-3,6α-dimethoxy-17-methyl-7,8-didehydromorphinan (methylcodeine),

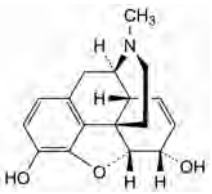

B. 4,5α-epoxy-17-methyl-7,8-didehydromorphinan-3,6α-diol (morphine),

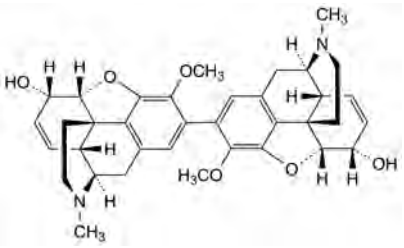

C. 4,5α:4′,5′α-diepoxy-3,3′-dimethoxy-17,17′-dimethyl-7,7′,8,8′-tetradehydro-2,2′-bimorphinan-6α,6′α-diol (codeine dimer),

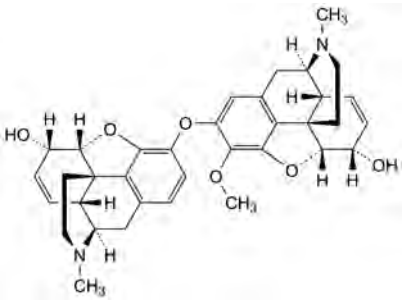

D. 2-[(4,5α-epoxy-6α-hydroxy-17-methyl-7,8-didehydromorphinan-3-yl)oxy]-4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol (3-*O*-(codein-2-yl)morphine),

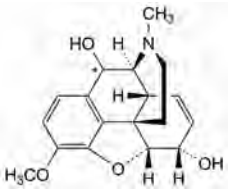

E. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydro-morphinan-6α,10ξ-diol,

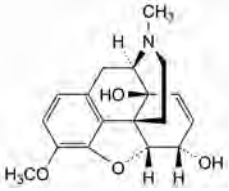

F. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydro-morphinan-6α,14-diol,

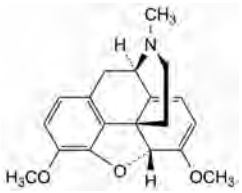

G. 4,5α-epoxy-3,6-dimethoxy-17-methyl-6,7,8,14-tetradehydromorphinan (thebaine),

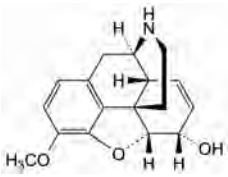

H. 4,5α-epoxy-3-methoxy-7,8-didehydromorphinan-6α-ol (norcodeine),

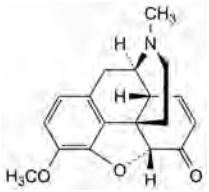

I. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydro-morphinan-6-one (codeinone),

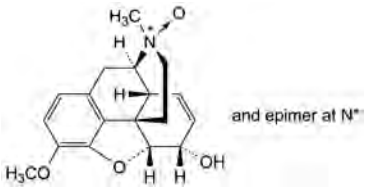


J. (17*RS*)-4,5α-epoxy-6α-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan 17-oxide (codeine *N*-oxide),

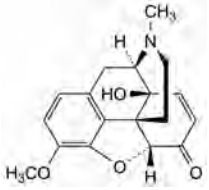

K. 4,5α-epoxy-14-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6-one (14-hydroxycodeinone),

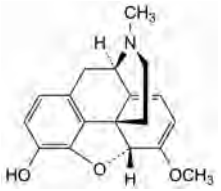

L. 4,5α-epoxy-6-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-3-ol (oripavine),

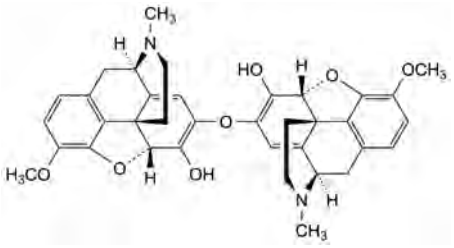

M. 7,7′-oxybis(4,5α-epoxy-3-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-6-ol) (7,7′-oxybis(6-*O*-demethylthebaine)).

**07/2018:0076**
**corrected 10.0**

# CODEINE MONOHYDRATE

## Codeinum monohydricum

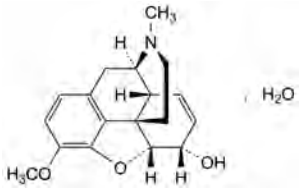

$C_{18}H_{21}NO_3,H_2O$ $M_r$ 317.4
[6059-47-8]

DEFINITION

4,5α-Epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C, F.*

*Second identification: A, B, D, E, F.*

A. Melting point (*2.2.14*): 155 °C to 159 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* To 2.0 mL of solution S (see Tests) add 50 mL of *water R*, then 10 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with *water R*.

*Spectral range*: 250-350 nm.

*Absorption maximum*: at 284 nm.

*Specific absorbance at the absorption maximum*: about 50 (dried substance).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *codeine CRS*.

D. To about 10 mg add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 mL of *nitric acid R*. The colour changes to red.

E. It gives the reaction of alkaloids (*2.3.1*).

F. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 50 mg in *water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 142 to − 146 (dried substance).

Dissolve 0.50 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 0.5 per cent *V/V* solution of *phosphoric acid R*.

*Test solution*. Dissolve 0.160 g of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 3 mg of *codeine for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H and I) in 1 mL of solution A.

*Column*:

- *size*: *l* = 0.075 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl multi-layered organosilica polymer R* (1.9 µm).
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 4 volumes of *acetonitrile R* and 96 volumes of a 20 g/L solution of *glacial acetic acid R* previously adjusted to pH 4.5 with a 500 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 7.33 | 100 → 93 | 0 → 7 |
| 7.33 - 10.33 | 93 → 67 | 7 → 33 |
| 10.33 - 12 | 67 | 33 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 3 µL.

*Identification of impurities*: use the chromatogram supplied with *codeine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to codeine (retention time = about 6.0 min): impurity B = about 0.3; impurity E = about 0.4; impurity F = about 0.8; impurity H = about 0.9; impurity C = about 1.2; impurity I = about 1.4; impurity D = about 1.45; impurity A = about 1.5; impurity G = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurities F and H; minimum 1.5 between the peaks due to impurities D and A.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity G = 0.2; impurity I = 1.3;
- for each impurity, use the concentration of codeine monohydrate in reference solution (a).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurity H*: maximum 0.25 per cent;
- *impurities C, D, E*: for each impurity, maximum 0.2 per cent;
- *impurities B, F, G, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): 4.0 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.94 mg of $C_{18}H_{21}NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L, M.*

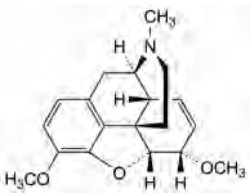

A. 4,5α-epoxy-3,6α-dimethoxy-17-methyl-7,8-didehydromorphinan (methylcodeine),

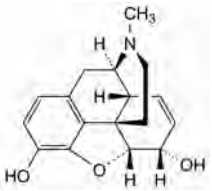

B. 4,5α-epoxy-17-methyl-7,8-didehydromorphinan-3,6α-diol (morphine),

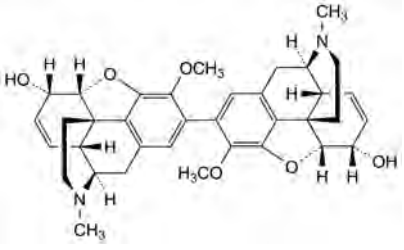

C. 4,5α:4′,5′α-diepoxy-3,3′-dimethoxy-17,17′-dimethyl-7,7′,8,8′-tetradehydro-2,2′-bimorphinan-6α,6′α-diol (codeine dimer),

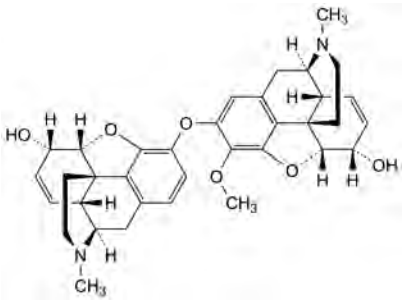

D. 2-[(4,5α-epoxy-6α-hydroxy-17-methyl-7,8-didehydromorphinan-3-yl)oxy]-4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol (3-*O*-(codein-2-yl)morphine),

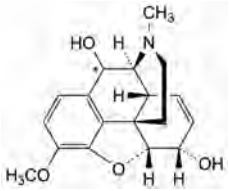

E. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α,10ξ-diol,

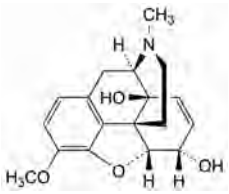

F. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α,14-diol,

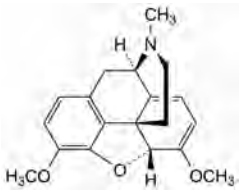

G. 4,5α-epoxy-3,6-dimethoxy-17-methyl-6,7,8,14-tetradehydromorphinan (thebaine),

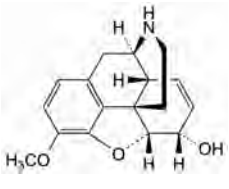

H. 4,5α-epoxy-3-methoxy-7,8-didehydromorphinan-6α-ol (norcodeine),

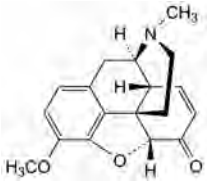

I. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6-one (codeinone),

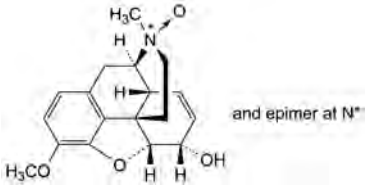


J. (17*RS*)-4,5α-epoxy-6α-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan 17-oxide (codeine *N*-oxide),

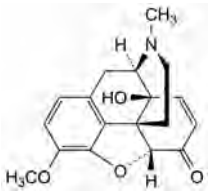

K. 4,5α-epoxy-14-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6-one (14-hydroxycodeinone),

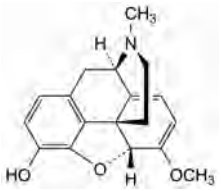

L. 4,5α-epoxy-6-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-3-ol (oripavine),

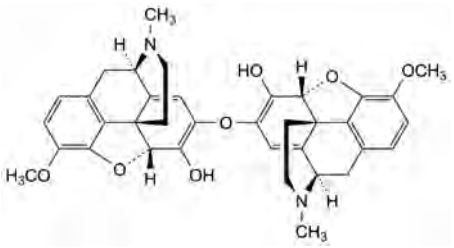

M. 7,7′-oxybis(4,5α-epoxy-3-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-6-ol) (7,7′-oxybis(6-*O*-demethylthebaine)).

**07/2018:0074**
**corrected 10.0**

# CODEINE PHOSPHATE HEMIHYDRATE

## Codeini phosphas hemihydricus

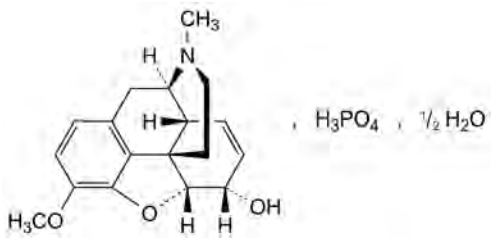


$C_{18}H_{24}NO_7P,½H_2O$ $M_r$ 406.4
[41444-62-6]

DEFINITION

4,5α-Epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol phosphate hemihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small, colourless crystals.

*Solubility*: freely soluble in water, slightly soluble or very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E, F.*

*Second identification: A, C, D, E, F, G.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dilute 1.0 mL of solution S (see Tests) to 100.0 mL with *water R*. To 25.0 mL of this solution add 25 mL of *water R*, then 10 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with *water R*.

*Spectral range*: 250-350 nm.

*Absorption maximum*: at 284 nm.

*Specific absorbance at the absorption maximum*: about 38 (dried substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.20 g in 4 mL of *water R*. Add 1 mL of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C.

*Comparison*: *codeine CRS*.

C. Dissolve 0.20 g in 4 mL of *water R*. Add 1 mL of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 155 °C to 159 °C.

D. To about 10 mg add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 mL of *nitric acid R*. The colour changes to red.

E. Loss on drying (see Tests).

F. Solution S gives reaction (a) of phosphates (*2.3.1*).

G. It gives the reaction of alkaloids (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): − 98 to − 102 (dried substance).

Dilute 5.0 mL of solution S to 10.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 0.5 per cent *V/V* solution of *phosphoric acid R*.

*Test solution.* Dissolve 0.190 g of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 3 mg of *codeine for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H and I) in 1 mL of solution A.

*Column*:

- *size*: *l* = 0.075 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl multi-layered organosilica polymer R* (1.9 µm).
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 4 volumes of *acetonitrile R* and 96 volumes of a 20 g/L solution of *glacial acetic acid R* previously adjusted to pH 4.5 with a 500 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 7.33 | 100 → 93 | 0 → 7 |
| 7.33 - 10.33 | 93 → 67 | 7 → 33 |
| 10.33 - 12 | 67 | 33 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 3 µL.

*Identification of impurities*: use the chromatogram supplied with *codeine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to codeine (retention time = about 6.0 min): impurity B = about 0.3; impurity E = about 0.4; impurity F = about 0.8; impurity H = about 0.9; impurity C = about 1.2; impurity I = about 1.4; impurity D = about 1.45; impurity A = about 1.5; impurity G = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurities F and H; minimum 1.5 between the peaks due to impurities D and A.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity G = 0.2; impurity I = 1.3;
- for each impurity, use the concentration of codeine phosphate hemihydrate in reference solution (a).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurity H*: maximum 0.25 per cent;
- *impurities C, D, E*: for each impurity, maximum 0.2 per cent;
- *impurities B, F, G, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 5 mL of solution S to 20 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): 1.5 per cent to 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 39.74 mg of $C_{18}H_{24}NO_7P$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L, M.*

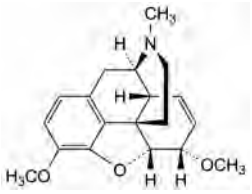

A. 4,5α-epoxy-3,6α-dimethoxy-17-methyl-7,8-didehydromorphinan (methylcodeine),

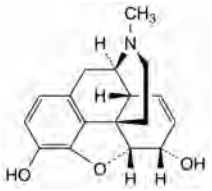

B. 4,5α-epoxy-17-methyl-7,8-didehydromorphinan-3,6α-diol (morphine),

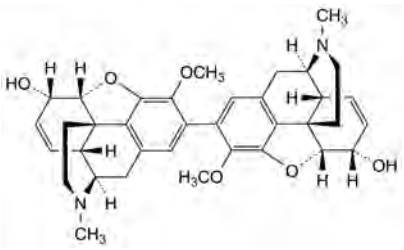

C. 4,5α:4′,5′α-diepoxy-3,3′-dimethoxy-17,17′-dimethyl-7,7′,8,8′-tetradehydro-2,2′-bimorphinan-6α,6′α-diol (codeine dimer),

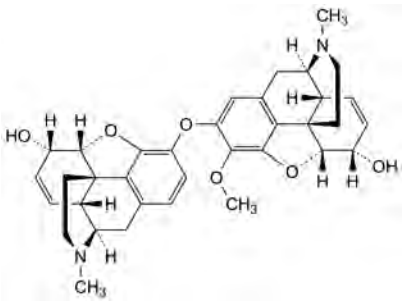

D. 2-[(4,5α-epoxy-6α-hydroxy-17-methyl-7,8-didehydromorphinan-3-yl)oxy]-4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α-ol (3-*O*-(codein-2-yl)morphine),

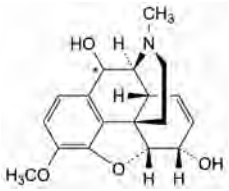

E. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α,10ξ-diol,

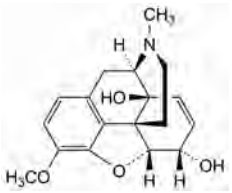

F. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6α,14-diol,

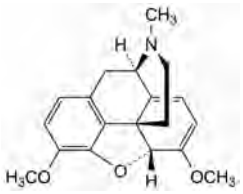

G. 4,5α-epoxy-3,6-dimethoxy-17-methyl-6,7,8,14-tetradehydromorphinan (thebaine),

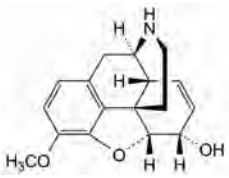

H. 4,5α-epoxy-3-methoxy-7,8-didehydromorphinan-6α-ol (norcodeine),

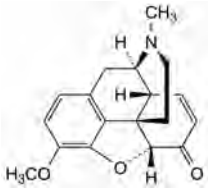

I. 4,5α-epoxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6-one (codeinone),

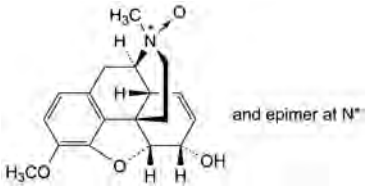


J. (17*RS*)-4,5α-epoxy-6α-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan 17-oxide (codeine *N*-oxide),

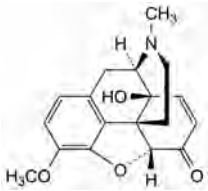

K. 4,5α-epoxy-14-hydroxy-3-methoxy-17-methyl-7,8-didehydromorphinan-6-one (14-hydroxycodeinone),

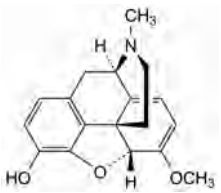

L. 4,5α-epoxy-6-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-3-ol (oripavine),

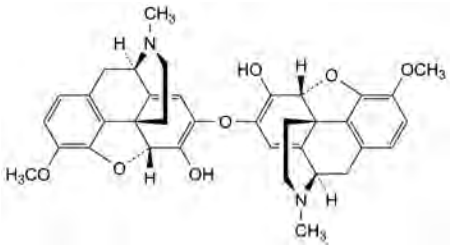

M. 7,7′-oxybis(4,5α-epoxy-3-methoxy-17-methyl-6,7,8,14-tetradehydromorphinan-6-ol) (7,7′-oxybis(6-*O*-demethylthebaine)).

**01/2008:0075**
**corrected 6.0**

# CODEINE PHOSPHATE SESQUIHYDRATE

## Codeini phosphas sesquihydricus

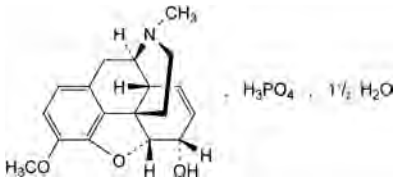

$C_{18}H_{24}NO_7P,1½H_2O$ $M_r$ 424.4
[5913-76-8]

DEFINITION

7,8-Didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol phosphate sesquihydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small, colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, E, F.*

*Second identification*: *A, C, D, E, F, G.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dilute 1.0 mL of solution S (see Tests) to 100.0 mL with *water R*. To 25.0 mL of this solution add 25 mL of *water R* then 10 mL of *1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

*Spectral range*: 250-350 nm.

*Absorption maximum*: at 284 nm.

*Specific absorbance at the absorption maximum*: about 38 (dried substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.20 g in 4 mL of *water R*. Add 1 mL of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. Wash the precipitate with *water R* and dry at 100-105 °C. Examine the dried precipitate prepared as discs using *potassium bromide R*.

*Comparison*: *Ph. Eur. reference spectrum of codeine.*

C. Dissolve 0.20 g in 4 mL of *water R*. Add 1 mL of a mixture of equal volumes of *strong sodium hydroxide solution R* and *water R* and initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod and cooling in iced water. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 155 °C to 159 °C.

D. To about 10 mg add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R2* and heat on a water-bath. A blue colour develops. Add 0.05 mL of *nitric acid R*. The colour changes to red.

E. Loss on drying (see Tests).

F. Solution S gives reaction (a) of phosphates (*2.3.1*).

G. It gives the reaction of alkaloids (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): – 98 to – 102 (dried substance).

Dilute 5.0 mL of solution S to 10.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined and 0.100 g of *sodium octanesulfonate R* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *codeine impurity A CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. To 0.25 mL of the test solution add 2.5 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.08 g of *sodium octanesulfonate R* in a mixture of 20 mL of *glacial acetic acid R* and 250 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 10 µL.

*Run time*: 10 times the retention time of codeine.

*Relative retention* with reference to codeine (retention time = about 6 min): impurity B = about 0.6; impurity E = about 0.7; impurity A = about 2.0; impurity C = about 2.3; impurity D = about 3.6.

*System suitability*: reference solution (d):

– *resolution*: minimum 3 between the peaks due to codeine and impurity A.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.25;
– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *impurities B, C, D, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *sum of impurities other than A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 5 mL of solution S to 20 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): 5.0 per cent to 7.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.350 g in a mixture of 10 mL of *anhydrous acetic acid R* and 20 mL of *dioxan R*. Titrate with *0.1 M perchloric acid* using 0.05 mL of *crystal violet solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 39.74 mg of $C_{18}H_{24}NO_7P$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G.*

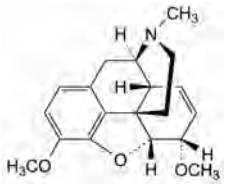

A. 7,8-didehydro-4,5α-epoxy-3,6α-dimethoxy-17-methylmorphinan (methylcodeine),

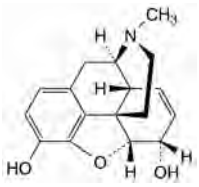

B. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol (morphine),

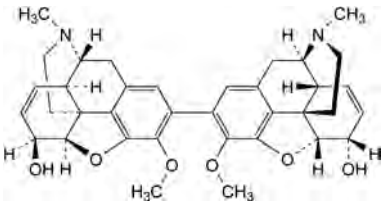

C. 7,7′,8,8′-tetradehydro-4,5α:4′,5′α-diepoxy-3,3′-dimethoxy-17,17′-dimethyl-2,2′-bimorphinanyl-6α,6′α-diol (codeine dimer),

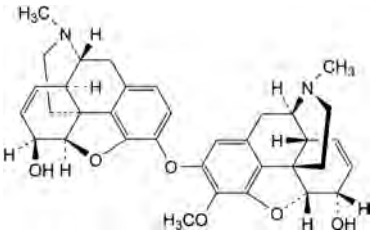

D. 7,8-didehydro-2-[(7,8-didehydro-4,5α-epoxy-6α-hydroxy-17-methylmorphinan-3-yl)oxy]-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (3-*O*-(codein-2-yl)morphine),

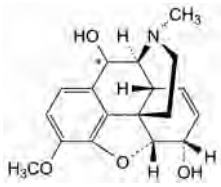

E. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α,10-diol,

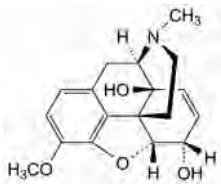

F. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α,14-diol,

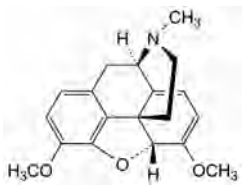

G. 6,7,8,14-tetradehydro-4,5α-epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine).

**07/2013:2060**

# CODERGOCRINE MESILATE

## Codergocrini mesilas

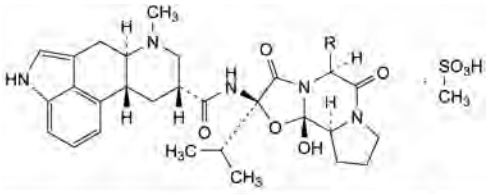

| Name | Mol. Formula | $M_r$ | R |
|---|---|---|---|
| dihydroergocornine mesilate | $C_{32}H_{45}N_5O_8S$ | 660 | $H_3C$, $CH_3$ |
| dihydroergocristine mesilate | $C_{36}H_{45}N_5O_8S$ | 708 | |
| α-dihydroergocryptine mesilate | $C_{33}H_{47}N_5O_8S$ | 674 | $CH_3$, $CH_3$ |
| β-dihydroergocryptine mesilate | $C_{33}H_{47}N_5O_8S$ | 674 | $H_3C$, H, $CH_3$ |

[8067-24-1]

DEFINITION

A mixture of:

- (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2,5-bis(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate (dihydroergocornine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate (dihydroergocristine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate (α-dihydroergocryptine mesilate);
- (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-[(1*RS*)-1-methylpropyl]-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate (β-dihydroergocryptine mesilate or epicriptine mesilate).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

It is considered that alkylsulfonate esters are genotoxic and are potential impurities in codergocrine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or yellowish powder.

*Solubility*: sparingly soluble in water, sparingly soluble to soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution.* Dissolve 0.20 g of *methanesulfonic acid R* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *concentrated ammonia R*, *butanol R*, *acetone R* (5:10:20:65 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air for not more than 1 min.

*Detection*: spray with a 1 g/L solution of *bromocresol purple R* in *methanol R*, adjusted to a violet-red colour with 0.05 mL of *dilute ammonia R1*.

*Drying*: in a current of hot air at 100 °C.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the test for composition.

*Results*: the 4 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 4 principal peaks in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*): 4.2 to 5.2.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20 mg of the substance to be examined in a mixture of 1 volume of *anhydrous ethanol R* and 2 volumes of a 10 g/L solution of *tartaric acid R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution.* Dissolve 20 mg of *codergocrine mesilate CRS* in a mixture of 1 volume of *anhydrous ethanol R* and 2 volumes of a 10 g/L solution of *tartaric acid R* and dilute to 10 mL with the same mixture of solvents.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *triethylamine R*, *acetonitrile R*, *water R* (2.5:25:75 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 20 min.

*Elution order*: dihydroergocornine, α-dihydroergocryptine, dihydroergocristine, β-dihydroergocryptine.

*System suitability*: test solution:
- *resolution*: minimum 3 between any 2 consecutive principal peaks.

*Composition*:
- *dihydroergocornine*: 30.0 per cent to 35.0 per cent;
- *α-dihydroergocryptine*: 20.0 per cent to 25.0 per cent;
- *dihydroergocristine*: 30.0 per cent to 35.0 per cent;
- *β-dihydroergocryptine*: 10.0 per cent to 13.0 per cent;
- *disregard limit*: 1.0 per cent.

**Related substances**. Thin-layer chromatography (*2.2.27*). *Perform the test as rapidly as possible and protected from direct light. Prepare the test solution last and immediately before application on the plate.*

*Test solution.* Dissolve 0.40 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5.0 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 40 mg of *dihydroergocristine mesilate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10.0 mL with the same mixture of solvents. Dilute 3.0 mL of the solution to 50.0 mL with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

*Reference solution (b).* To 2.0 mL of reference solution (a), add 1.0 mL of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

*Reference solution (c).* To 1.0 mL of reference solution (a), add 2.0 mL of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

*Reference solution (d).* To 1.0 mL of reference solution (a), add 5.0 mL of a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *methylene chloride R* (1:3:50:50 *V/V/V/V*).

*Application*: 10 µL.

*Drying*: in the dark for 2 min after the application of the last solution.

*First development*: in an unsaturated tank, over 2/3 of the plate.

*Drying*: in a current of cold air for not more than 1 min.

*Second development*: in an unsaturated tank, over 2/3 of the plate; use freshly prepared mobile phase.

*Drying*: in a current of cold air for not more than 1 min.

*Detection*: spray thoroughly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air until the spot in the chromatogram obtained with reference solution (d) is clearly visible.

*System suitability*: test solution:
- the chromatogram shows at least 3 separated secondary spots.

*Limits*:
- *any impurity*: any spots, apart from the principal spot, are not more intense than the spot in the chromatogram obtained with reference solution (a) (0.3 per cent); not more than 4 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent) and 2 of these may be more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying at 120 °C under high vacuum.

ASSAY

Dissolve 0.500 g in 60 mL of *pyridine R*. Pass a stream of *nitrogen R* over the surface of the solution and titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 68.04 mg of codergocrine mesilate (average $M_r$ = 680).

STORAGE

Protected from light.

**07/2012:2398**

# COD-LIVER OIL, FARMED

## Iecoris aselli domestici oleum

DEFINITION

Purified fatty oil obtained from the fresh livers of farmed cod, *Gadus morhua* L., solid substances being removed by cooling and filtering.

*Content*:
- *sum of the contents of EPA and DHA (expressed as triglycerides)*: 10.0 per cent to 28.0 per cent;
- *vitamin A*: 50 IU (15 µg) to 500 IU (150 µg) per gram;
- *vitamin $D_3$*: maximum 50 IU (1.3 µg) per gram.

A suitable antioxidant may be added.

PRODUCTION

The fish shall only be given feed with a composition that is in accordance with the relevant European Union or other applicable regulations.

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: clear, pale yellowish liquid.

*Solubility*: practically insoluble in water, miscible with light petroleum, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Examine the $^{13}C$ NMR spectra obtained in the test for positional distribution (β(2)-acyl) of fatty acids (see Tests). The spectra contain peaks between 172 ppm and 173 ppm with shifts similar to those in the spectrum shown in Figure 2398.-1.

   The positional distribution (β(2)-acyl) for cervonic (docosahexaenoic) acid (C22:6 n-3; DHA), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA) and moroctic acid (C18:4 n-3) complies with the limits.

B. Linoleic acid (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

**Anisidine value** (*2.5.36*): maximum 10.0.

**Peroxide value** (*2.5.5, Method B*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 2.0 g, and extracting with 3 quantities, each of 50 mL, of *peroxide-free ether R*.

**Stearin**. Heat at least 10 mL to 60-90 °C then allow to cool for 3 h in a bath of iced water or a thermostatically controlled bath at 0 ± 0.5 °C. If necessary, to eliminate insoluble matter, filter the sample after heating. The sample remains clear.

**Positional distribution (β(2)-acyl) of fatty acids**. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Test solution.* Dissolve 190-210 mg of the substance to be examined in 500 µL of *deuterated chloroform R*. Prepare at least 3 samples and examine within 3 days.

*Apparatus*: high-resolution FT-NMR spectrometer operating at minimum 300 MHz.

*Acquisition of $^{13}C$ NMR spectra.* The following parameters may be used:

- *sweep width*: 200 ppm (– 5 ppm to 195 ppm);
- *irradiation frequency offset*: 95 ppm;
- *time domain*: 64 K;
- *pulse delay*: 2 s;
- *pulse program*: zgig 30 (inverse gated, 30° excitation pulse);
- *dummy scans*: 4;
- *number of scans*: 4096.

*Processing and plotting.* The following parameters may be used:

- *size*: 64 K (zero-filling);
- *window multiplication*: exponential;
- *Lorentzian broadening factor*: 0.2 Hz.

Use the $CDCl_3$ signal for shift referencing. The shift of the central peak of the 1:1:1 triplet is set to 77.16 ppm.

Plot the spectral region δ 171.5-173.5 ppm. Compare the spectrum with the spectrum shown in Figure 2398.-1. The shift values lie within the ranges given in Table 2398.-1.

Table 2398.-1. – *Shift values*

| Signal | Shift range (ppm) |
|---|---|
| β DHA | 172.05 - 172.09 |
| α DHA | 172.43 - 172.47 |
| β EPA | 172.52 - 172.56 |
| α EPA | 172.90 - 172.94 |
| β C18:4 | 172.56 - 172.60 |
| α C18:4 | 172.95 - 172.99 |

*System suitability*:

- *signal-to-noise ratio*: minimum 5 for the smallest relevant peak corresponding to α C18:4 signal (in the range δ 172.95-172.99 ppm);
- *peak width at half-height*: maximum 0.02 ppm for the central $CDCl_3$ signal (at δ 77.16 ppm).

*Calculation of positional distribution (β(2)-acyl)*: use the following expression:

$$\frac{100 \times \beta}{\alpha + \beta}$$

$\alpha$ = peak area of the corresponding α-carbonyl peak;

$\beta$ = peak area of β-carbonyl peak from C22:6 n-3, C20:5 n-3 or C18:4 n-3, respectively.

*Limits*:

- *positional distribution (β(2)-acyl)*:
  - *cervonic (docosahexaenoic) acid (C22:6 n-3; DHA)*: 71 per cent to 81 per cent;
  - *timnodonic (eicosapentaenoic) acid (C20:5 n-3 EPA)*: 32 per cent to 40 per cent;
  - *moroctic acid (C18:4 n-3)*: 28 per cent to 38 per cent.

**Composition of fatty acids** (*2.4.29*). For identification of the peaks, see the chromatogram shown in Figure 2398.-2.

The 24 largest peaks of the methyl esters account for more than 90 per cent of the total area (these correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

**Linoleic acid** (*2.4.29*): 3.0 per cent to 11.0 per cent.

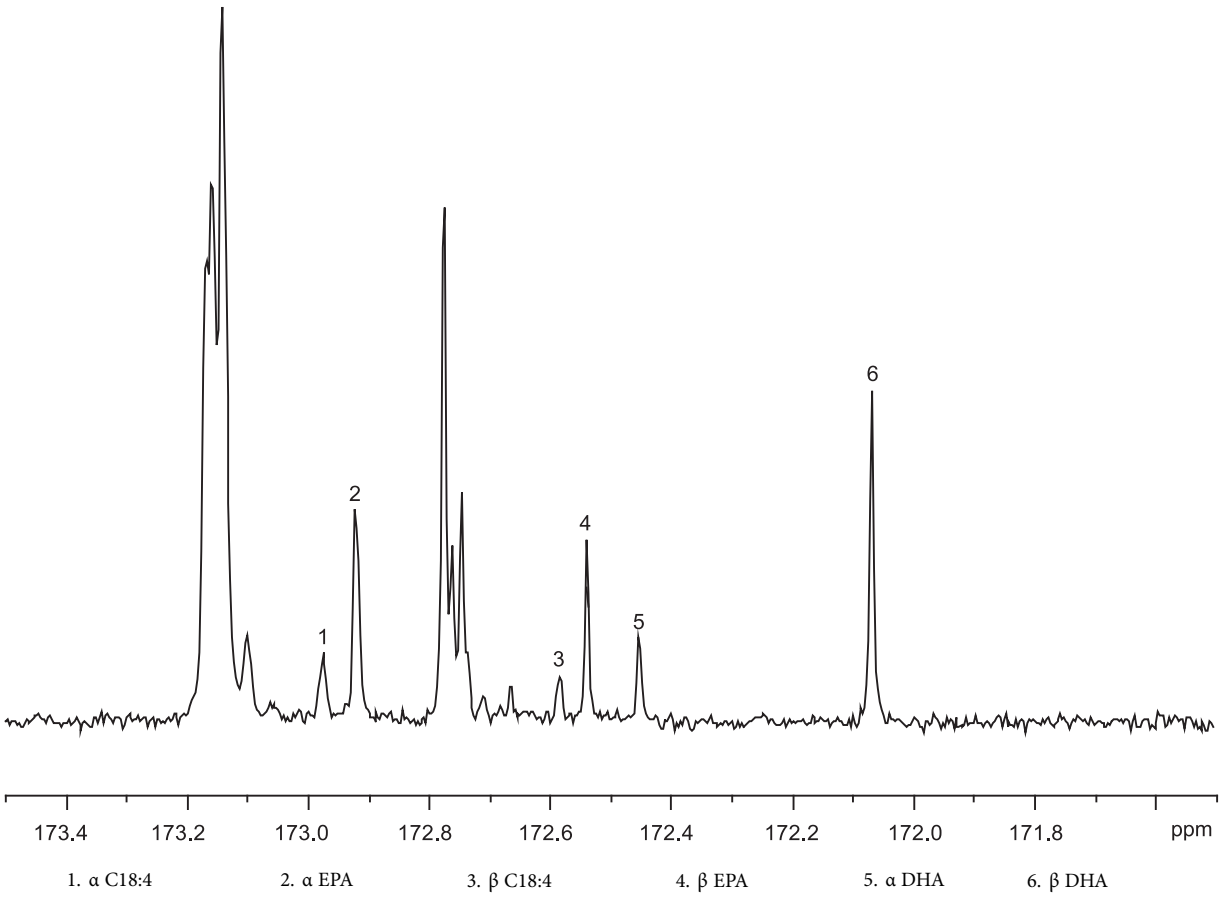


Figure 2398.-1. – *$^{13}C$ NMR spectrum: carbonyl region of farmed cod-liver oil*

ASSAY

**EPA and DHA** (*2.4.29*). See the chromatogram shown in Figure 2398.-2.

**Vitamin A**. *Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.*

Use method A. If method A is found not to be valid, use method B.

*METHOD A*

Ultraviolet absorption spectrophotometry (*2.2.25*).

*Test solution*. To 1.00 g in a round-bottomed flask, add 3 mL of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R* and 30 mL of *anhydrous ethanol R*. Boil under reflux in a current of *nitrogen R* for 30 min. Cool rapidly and add 30 mL of *water R*. Extract with 50 mL of *ether R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 mL, of *water R*, and evaporate to dryness under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C or in a rotary evaporator at a temperature not exceeding 30 °C under reduced pressure (water ejector). Dissolve the residue in sufficient *2-propanol R1* to give an expected concentration of vitamin A equivalent to 10-15 IU/mL.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in specially matched 1 cm cells, using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram, using the following expression:

$$A_{325} \times \frac{1821}{100m} \times V$$

$A_{325}$ = absorbance at 325 nm;

$m$ = mass of the substance to be examined, in grams;

$V$ = total volume of solution containing 10-15 IU of vitamin A per millilitre;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if $A_{325}$ has a value not greater than $A_{325,corr}$ /0.970, where $A_{325,corr}$ is the corrected absorbance at 325 nm and is given by the following equation:

$$A_{325,\,corr} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

*A* designates the absorbance at the wavelength indicated by the subscript.

If $A_{325}$ has a value greater than $A_{325,corr}$ /0.970, calculate the content of vitamin A using the following expression:

$$A_{325,\,corr} \times \frac{1821}{100m} \times V$$

The assay is not valid unless:

- the wavelength of maximum absorption lies between 323 nm and 327 nm;
- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

*METHOD B*

Liquid chromatography (*2.2.29*).

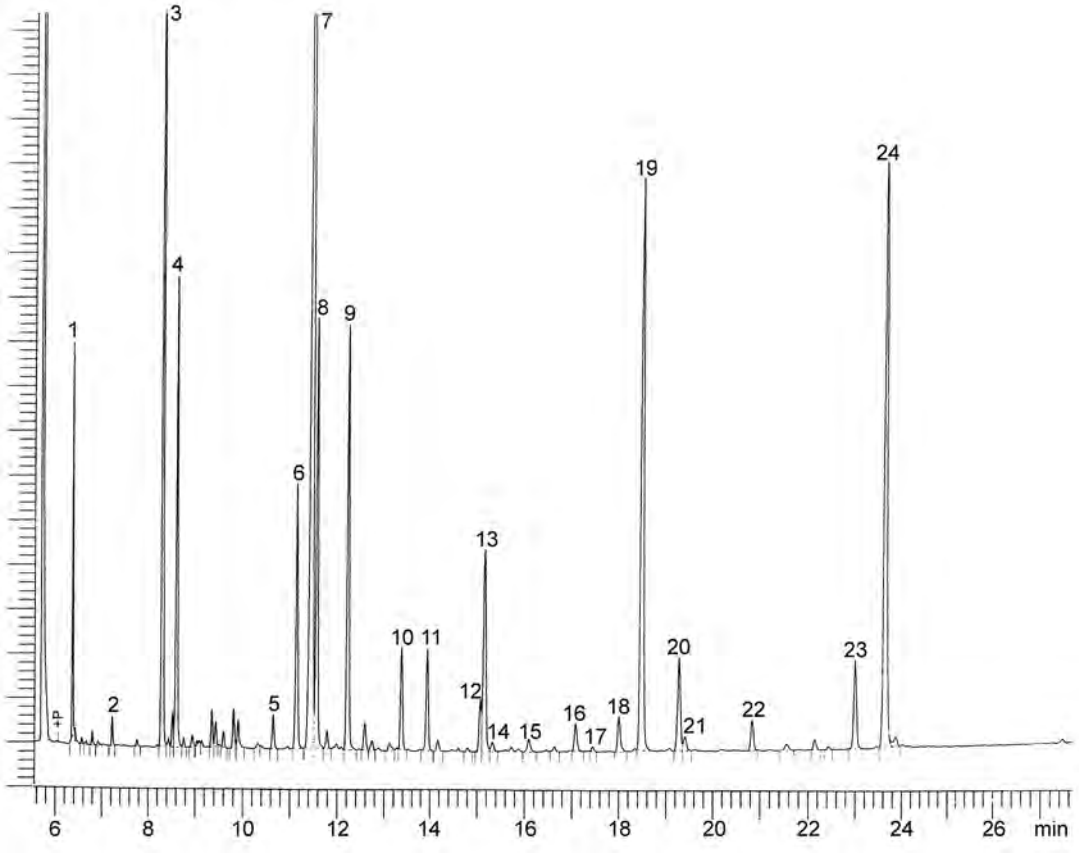


| | | | | | |
|---|---|---|---|---|---|
| 1. C14:0 | 5. C16:4 n-1 | 9. C18:2 n-6 | 13. C20:1 n-9 | 17. C20:3 n-3 | 21. C22:1 n-9 |
| 2. C15:0 | 6. C18:0 | 10 C18:3 n-3 | 14. C20:1 n-7 | 18. C20:4 n-3 | 22. C21:5 n-3 |
| 3. C16:0 | 7. C18:1 n-9 | 11. C18:4 n-3 | 15. C20:2 n-6 | 19. C20:5 n-3 | 23. C22:5 n-3 |
| 4. C16:1 n-7 | 8. C18:1 n-7 | 12. C20:1 n-11 | 16. C20:4 n-6 | 20. C22:1 n-11 | 24. C22:6 n-3 |

Figure 2398.-2. – *Chromatogram for the test for composition of fatty acids of farmed cod-liver oil*

*Test solution*. Prepare duplicates. To 2.00 g in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R* and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask suitable for a rotary evaporator. Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R*, transfer to a 25 mL volumetric flask and dilute to 25 mL with *2-propanol R*. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Reference solution (a).* Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 mL contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/mL and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) using the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

$A_{326}$ = absorbance at 326 nm;

$V_1$ = volume of reference solution (a) used;

$V_2$ = volume of the diluted solution;

1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

*Reference solution (b).* Proceed as described for the test solution but using 2.00 mL of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (b) with *2-propanol R1* to a presumed all-*trans*-retinol concentration of 10-15 IU/mL and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b), using the following expression:

$$A_{325} \times \frac{1821 \times V_3}{100 \times V_4}$$

$A_{325}$ = absorbance at 325 nm;

$V_3$ = volume of the diluted solution;

$V_4$ = volume of reference solution (b) used;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

*Column*:

– *size*: $l$ = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: *water R*, *methanol R* (3:97 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL; inject in triplicate the test solution and reference solution (b).

*Retention time*: all-*trans*-retinol = 5 ± 1 min.

*System suitability*:

– the chromatogram obtained with the test solution shows a peak corresponding to the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

– the results obtained with the duplicate test solutions do not differ by more than 5 per cent;

– the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

$A_1$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

$C$ = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/mL);

$V$ = volume of reference solution (a) treated (2.00 mL);

$m$ = mass of the substance to be examined in the test solution (2.00 g).

**Vitamin $D_3$.** Liquid chromatography (*2.2.29*). *Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

*Internal standard solution.* Dissolve 0.50 mg of *ergocalciferol CRS* in 100 mL of *anhydrous ethanol R*.

*Test solution (a).* To 4.00 g in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R*, and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask suitable for a rotary evaporator. Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure (water ejector) and fill with *nitrogen R*

when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 mL of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Test solution (b).* Prepare duplicates. To 4.00 g add 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*Reference solution (a).* Dissolve 0.50 mg of *cholecalciferol CRS* in 100.0 mL of *anhydrous ethanol R*.

*Reference solution (b).* In a round-bottomed flask, add 2.0 mL of reference solution (a) and 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*PURIFICATION*

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *nitrile silica gel for chromatography R* (10 µm).

*Mobile phase*: *isoamyl alcohol R*, *hexane R* (1.6:98.4 *V/V*).

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 350 µL of reference solution (b) and test solutions (a) and (b). Collect each eluate from 2 min before until 2 min after the retention time of cholecalciferol, in a ground-glass-stoppered tube containing 1 mL of a 1 g/L solution of *butylhydroxytoluene R* in *hexane R*. Evaporate separately to dryness at a temperature not exceeding 30 °C under a gentle current of *nitrogen R*. Dissolve each residue in 1.5 mL of *acetonitrile R*.

*DETERMINATION*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *phosphoric acid R*, 96 per cent *V/V* solution of *acetonitrile R* (0.2:99.8 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 2 quantities not exceeding 200 µL of each of the 3 solutions obtained under Purification.

*System suitability*:

- *resolution*: minimum 1.4 between the peaks due to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b);
- the results obtained with the test solution (b) duplicates do not differ by more than 5 per cent.

Calculate the content of vitamin $D_3$ in International Units per gram using the following expression, taking into account the assigned content of *cholecalciferol CRS*:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1}\right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

$m_1$ = mass of the sample in test solution (b), in grams;

$m_2$ = total mass of *cholecalciferol CRS* used for the preparation of reference solution (a), in micrograms (500 µg);

$A_1$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a);

$A_2$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b);

$A_3$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b);

$A_4$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b);

$A_5$ = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b);

$A_6$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b);

$V_1$ = total volume of reference solution (a) (100 mL);

$V_2$ = volume of reference solution (a) used for preparing reference solution (b) (2.0 mL).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the concentration of EPA and DHA as a sum;
- the number of International Units of vitamin A per gram;
- the number of International Units of vitamin $D_3$ per gram.

**01/2020:1192**

# COD-LIVER OIL (TYPE A)

## Iecoris aselli oleum A

DEFINITION

Purified fatty oil obtained from the fresh livers of wild cod, *Gadus morhua* L. and other species of *Gadidae*, solid substances being removed by cooling and filtering. A suitable antioxidant may be added.

*Content*:

- *vitamin A*: 600 IU (180 µg) to 2500 IU (750 µg) per gram;
- *vitamin $D_3$*: 60 IU (1.5 µg) to 250 IU (6.25 µg) per gram.

PRODUCTION

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: clear, yellowish liquid.

*Solubility*: practically insoluble in water, miscible with light petroleum, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, C.*

*Second identification: C, D.*

A. In the assay for vitamin A using method A, the test solution shows an absorption maximum (*2.2.25*) at 325 ± 2 nm. In the assay for vitamin A using method B, the chromatogram obtained with the test solution shows a peak corresponding to the peak due to all-*trans*-retinol in the chromatogram obtained with the reference solution.

B. In the assay for vitamin $D_3$, the chromatogram obtained with test solution (a) shows a peak corresponding to the peak due to cholecalciferol in the chromatogram obtained with reference solution (b).

C. Composition of fatty acids (see Tests).

D. To 0.1 g add 0.5 mL of *methylene chloride R* and 1 mL of *antimony trichloride solution R*. Mix. A deep blue colour develops in about 10 s.

TESTS

**Appearance**. The substance to be examined is not more intensely coloured than a reference solution prepared as follows: to 3.0 mL of red primary solution add 25.0 mL of yellow primary solution and dilute to 50.0 mL with a 10 g/L solution of *hydrochloric acid R* (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 0.917 to 0.930.

**Refractive index** (*2.2.6*): 1.477 to 1.484.

**Acid value** (*2.5.1*): maximum 2.0, determined on 20.00 g.

**Anisidine value** (*2.5.36*): maximum 30.0.

**Iodine value** (*2.5.4, Method B*): 150 to 180, determined on 0.14 g to 0.16 g.

Use *starch solution R2*.

**Peroxide value** (*2.5.5, Method B*): maximum 10.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Stearin**. Heat at least 10 mL to 60-90 °C and cool to room temperature. Immerse for 3 h in a bath of iced water or a thermostatically controlled bath at 0 ± 0.5 °C. If necessary, to eliminate insoluble matter, filter the sample after heating. The sample remains clear.

**Composition of fatty acids**. Gas chromatography (*2.2.28*).

| Trivial name of fatty acid | Nomenclature | Lower limit area (per cent) | Upper limit area (per cent) |
|---|---|---|---|
| *Saturated fatty acids:* | | | |
| Myristic acid | 14:0 | 2.0 | 6.0 |
| Palmitic acid | 16:0 | 7.0 | 14.0 |
| Stearic acid | 18:0 | 1.0 | 4.0 |
| *Mono-unsaturated fatty acids:* | | | |
| Palmitoleic acid | 16:1 n-7 | 4.5 | 11.5 |
| *cis*-Vaccenic acid | 18:1 n-7 | 2.0 | 7.0 |
| Oleic acid | 18:1 n-9 | 12.0 | 21.0 |
| Gadoleic acid | 20:1 n-11 | 1.0 | 5.5 |
| Gondoic acid | 20:1 n-9 | 5.0 | 17.0 |
| Erucic acid | 22:1 n-9 | 0 | 1.5 |
| Cetoleic acid (22:1 n-11) | 22:1 n-11+13 | 5.0 | 12.0 |
| *Poly-unsaturated fatty acids:* | | | |
| Linoleic acid | 18:2 n-6 | 0.5 | 3.0 |
| α-Linolenic acid | 18:3 n-3 | 0 | 2.0 |
| Moroctic acid | 18:4 n-3 | 0.5 | 4.5 |
| Timnodonic (eicosapentaenoic) acid (EPA) | 20:5 n-3 | 7.0 | 16.0 |
| Cervonic (docosahexaenoic) acid (DHA) | 22:6 n-3 | 6.0 | 18.0 |

*Test solution*. Dilute about 0.45 g of the substance to be examined to 10.0 mL with a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R*. Transfer 2.0 mL of this solution to a quartz tube and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Cool, add 2 mL of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 mL of *trimethylpentane R*, cap and vortex or shake vigorously for at least 30 s. Immediately add 5 mL of *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and vortex or shake vigorously for at least 15 s. Allow the upper layer to become clear and transfer it to a separate tube. Shake the methanol layer once more with 1 mL of *trimethylpentane R* and combine the trimethylpentane extracts. Wash the combined extracts with 2 quantities, each of 1 mL, of *water R* and dry over *anhydrous sodium sulfate R*. Prepare 2 solutions for each sample.

*Reference solution*. Dissolve 0.300 g of *methyl arachidate R*, 0.300 g of *methyl behenate R*, 0.300 g of *methyl palmitate R* and 0.300 g of *methyl stearate R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R* or *helium for chromatography R*, with an oxygen scrubber.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 55 | 170 → 225 |
| | 55 - 75 | 225 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL, twice.

*System suitability*:

- the 15 fatty acids to be tested are satisfactorily identified from the chromatogram shown in Figure 1192.-1;
- in the chromatogram obtained with the reference solution, multiply the areas of the peaks due to methyl palmitate, methyl stearate, methyl arachidate and methyl behenate by the corresponding response factors in Table 1192.-1; normalise the corrected areas of the peaks due to the fatty acid methyl esters to a sum of 100 per cent; the normalised area percentage of each fatty acid methyl ester is to be within ± 1.0 percentage unit of the corresponding weight percentage;

Table 1192.-1.

| Fatty acid methyl ester | Theoretical response factor |
|---|---|
| Methyl palmitate | 1.049 |
| Methyl stearate | 1.029 |
| Methyl arachidate | 1.013 |
| Methyl behenate | 1.000 |

- *resolution*: in the chromatogram obtained with the test solution:
  - minimum 1.3 between the peaks due to methyl oleate and methyl *cis*-vaccenate; the resolution between the peaks due to methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement.

Calculate the area per cent for each fatty acid methyl ester using the following expression:

$$\frac{A_x}{A_t} \times 100$$

$A_x$ = peak area of fatty acid $x$;

$A_t$ = sum of the peak areas (up to C22:6 n-3).

The calculation is not valid unless:

- the total area is based only on peaks due solely to fatty acid methyl esters;

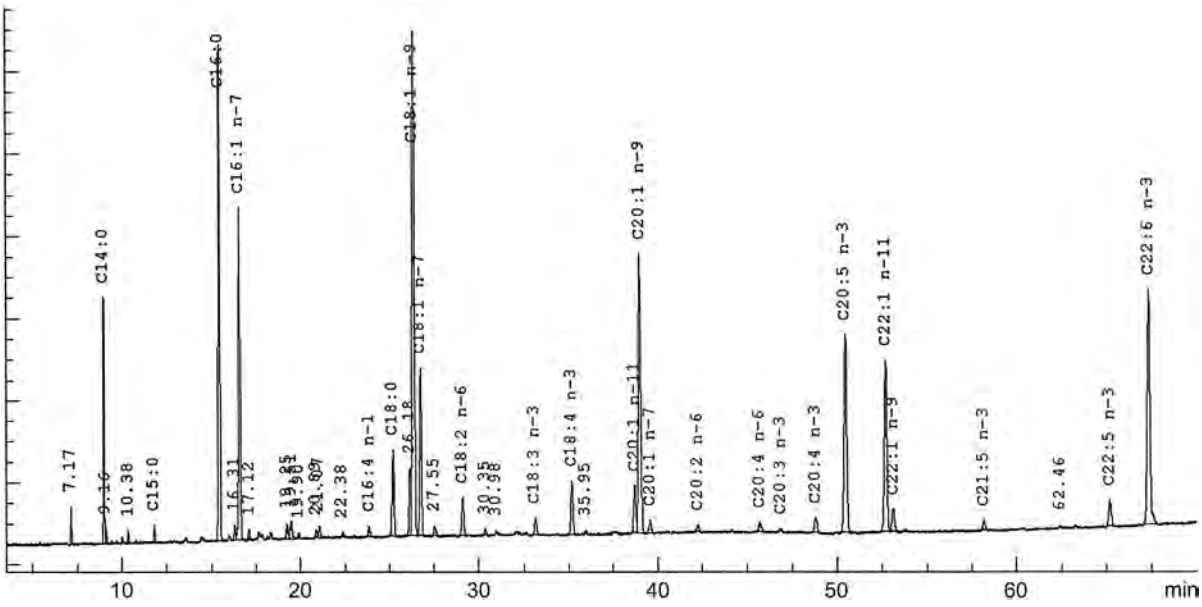


Figure 1192.-1. – *Chromatogram for the test for composition of fatty acids of cod-liver oil (type A)*

- the number of fatty acid methyl ester peaks exceeding 0.05 per cent of the total area is at least 24;
- the 24 largest peaks of the methyl esters account for more than 90 per cent of the total area (these correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

ASSAY

**Vitamin A**. *Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.*

Use method A. If method A is found not to be valid, use method B.

*METHOD A*

Ultraviolet absorption spectrophotometry (*2.2.25*).

*Test solution.* To 1.00 g in a round-bottomed flask, add 3 mL of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R* and 30 mL of *anhydrous ethanol R*. Boil under reflux in a current of *nitrogen R* for 30 min. Cool rapidly and add 30 mL of *water R*. Extract with 50 mL of *ether R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 mL, of *water R*, and evaporate to dryness under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C or by other suitable means at a temperature not exceeding 30 °C under reduced pressure. Dissolve the residue in sufficient *2-propanol R1* to give an expected concentration of vitamin A equivalent to 10-15 IU/mL.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in specially matched 1 cm cells, using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram, using the following expression:

$$A_{325} \times \frac{1821}{100m} \times V$$

$A_{325}$ = absorbance at 325 nm;

$m$ = mass of the substance to be examined, in grams;

$V$ = total volume of solution containing 10-15 IU of vitamin A per millilitre;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if $A_{325}$ has a value not greater than $A_{325,corr}/0.970$, where $A_{325,corr}$ is the corrected absorbance at 325 nm and is given by the following equation:

$$A_{325,corr} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

*A* designates the absorbance at the wavelength indicated by the subscript.

If $A_{325}$ has a value greater than $A_{325,corr}/0.970$, calculate the content of vitamin A using the following expression:

$$A_{325,corr} \times \frac{1821}{100m} \times V$$

The assay is not valid unless:

- the wavelength of the maximum absorption lies between 323 nm and 327 nm;
- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

*METHOD B*

Liquid chromatography (*2.2.29*).

*Test solution.* Prepare duplicates. To 2.00 g of the substance to be examined in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R* and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask. Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure and fill with *nitrogen R* when evaporation is completed.

Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R1*, transfer to a 25 mL volumetric flask and dilute to 25 mL with *2-propanol R1*. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Reference solution (a).* Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 mL contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/mL and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) using the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

$A_{326}$ = absorbance at 326 nm;

$V_1$ = volume of reference solution (a) used;

$V_2$ = volume of the diluted solution;

1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

*Reference solution (b).* Proceed as described for the test solution but using 2.00 mL of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (b) with *2-propanol R1* to a presumed all-*trans*-retinol concentration of 10-15 IU/mL and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b), using the following expression:

$$A_{325} \times \frac{1821 \times V_3}{100 \times V_4}$$

$A_{325}$ = absorbance at 325 nm;

$V_3$ = volume of the diluted solution;

$V_4$ = volume of reference solution (b) used;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: *water for chromatography R*, *methanol R* (3:97 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL; inject in triplicate the test solution and reference solution (b).

*Retention time*: all-*trans*-retinol = 5 ± 1 min.

*System suitability*:

- the chromatogram obtained with the test solution shows a peak corresponding to the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);
- the results obtained with the duplicate test solutions do not differ by more than 5 per cent;
- the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

$A_1$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

$C$ = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/mL);

$V$ = volume of reference solution (a) treated (2.00 mL);

$m$ = mass of the substance to be examined in the test solution (2.00 g).

**Vitamin $D_3$.** Liquid chromatography (*2.2.29*). *Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

*Internal standard solution.* Dissolve 0.50 mg of *ergocalciferol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* To 4.00 g of the substance to be examined in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R*, and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask. Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 mL of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Test solution (b).* Prepare duplicates. To 4.00 g of the substance to be examined add 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*Reference solution (a).* Dissolve 0.50 mg of *cholecalciferol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Into a round-bottomed flask, add 2.0 mL of reference solution (a) and 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*PURIFICATION*

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *nitrile silica gel for chromatography R* (10 µm).

*Mobile phase*: *isoamyl alcohol R*, *hexane R* (1.6:98.4 *V/V*).

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 350 µL of reference solution (b) and test solutions (a) and (b). Collect each eluate from 2 min before until 2 min after the retention time of cholecalciferol, in a ground-glass-stoppered tube containing 1 mL of a 1 g/L solution of *butylhydroxytoluene R* in *hexane R*. Evaporate separately to dryness at a temperature not exceeding 30 °C under a gentle current of *nitrogen R*. Dissolve each residue in 1.5 mL of *acetonitrile R*.

*DETERMINATION*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *phosphoric acid R*, 96 per cent *V/V* solution of *acetonitrile R* (0.2:99.8 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 2 quantities not exceeding 200 µL of each of the 3 solutions obtained under Purification.

*System suitability*:

- *resolution*: minimum 1.4 between the peaks due to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b);
- the results obtained with the test solution (b) duplicates do not differ by more than 5 per cent.

Calculate the content of vitamin $D_3$ in International Units per gram using the following expression, taking into account the assigned content of *cholecalciferol CRS*:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1}\right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

$m_1$ = mass of the substance to be examined in test solution (b), in grams;

$m_2$ = total mass of *cholecalciferol CRS* used for the preparation of reference solution (a), in micrograms (500 µg);

$A_1$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a);

$A_2$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b);

$A_3$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b);

$A_4$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b);

$A_5$ = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b);

$A_6$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b);

$V_1$ = total volume of reference solution (a) (100 mL);

$V_2$ = volume of reference solution (a) used to prepare reference solution (b) (2.0 mL).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units of vitamin A per gram;
- the number of International Units of vitamin $D_3$ per gram.

**01/2020:1193**

# COD-LIVER OIL (TYPE B)

## Iecoris aselli oleum B

DEFINITION

Purified fatty oil obtained from the fresh livers of wild cod, *Gadus morhua* L. and other species of *Gadidae*, solid substances being removed by cooling and filtering. A suitable antioxidant may be added.

*Content*:

- *vitamin A*: 600 IU (180 µg) to 2500 IU (750 µg) per gram;
- *vitamin $D_3$*: 60 IU (1.5 µg) to 250 IU (6.25 µg) per gram.

PRODUCTION

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: clear, yellowish liquid.

*Solubility*: practically insoluble in water, miscible with light petroleum, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, C.*

*Second identification: C, D.*

A. In the assay for vitamin A using method A, the test solution shows an absorption maximum (*2.2.25*) at 325 ± 2 nm. In the assay for vitamin A using method B, the chromatogram obtained with the test solution shows a peak corresponding to the peak due to all-*trans*-retinol in the chromatogram obtained with the reference solution.

B. In the assay for vitamin $D_3$, the chromatogram obtained with test solution (a) shows a peak corresponding to the peak due to cholecalciferol in the chromatogram obtained with reference solution (b).

C. Composition of fatty acids (see Tests).

D. To 0.1 g add 0.5 mL of *methylene chloride R* and 1 mL of *antimony trichloride solution R*. Mix. A deep blue colour develops in about 10 s.

TESTS

**Appearance**. The substance to be examined is not more intensely coloured than a reference solution prepared as follows: to 3.0 mL of red primary solution add 25.0 mL of yellow primary solution and dilute to 50.0 mL with a 10 g/L solution of *hydrochloric acid R* (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 0.917 to 0.930.

**Refractive index** (*2.2.6*): 1.477 to 1.484.

**Acid value** (*2.5.1*): maximum 2.0, determined on 20.00 g.

**Iodine value** (*2.5.4, Method B*): 150 to 180, determined on 0.14 g to 0.16 g.

Use *starch solution R2*.

**Peroxide value** (*2.5.5, Method B*): maximum 10.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Stearin**. Heat at least 10 mL to 60-90 °C and cool to room temperature. Immerse for 3 h in a bath of iced water or a thermostatically controlled bath at 0 ± 0.5 °C. If necessary, to eliminate insoluble matter, filter the sample after heating. The sample remains clear.

**Composition of fatty acids**. Gas chromatography (*2.2.28*).

| Trivial name of fatty acid | Nomenclature | Lower limit area (per cent) | Upper limit area (per cent) |
|---|---|---|---|
| *Saturated fatty acids:* | | | |
| Myristic acid | 14:0 | 2.0 | 6.0 |
| Palmitic acid | 16:0 | 7.0 | 14.0 |
| Stearic acid | 18:0 | 1.0 | 4.0 |
| *Mono-unsaturated fatty acids:* | | | |
| Palmitoleic acid | 16:1 n-7 | 4.5 | 11.5 |
| *cis*-Vaccenic acid | 18:1 n-7 | 2.0 | 7.0 |
| Oleic acid | 18:1 n-9 | 12.0 | 21.0 |
| Gadoleic acid | 20:1 n-11 | 1.0 | 5.5 |
| Gondoic acid | 20:1 n-9 | 5.0 | 17.0 |
| Erucic acid | 22:1 n-9 | 0 | 1.5 |
| Cetoleic acid (22:1 n-11) | 22:1 n-11+13 | 5.0 | 12.0 |
| *Poly-unsaturated fatty acids:* | | | |
| Linoleic acid | 18:2 n-6 | 0.5 | 3.0 |
| α-Linolenic acid | 18:3 n-3 | 0 | 2.0 |
| Moroctic acid | 18:4 n-3 | 0.5 | 4.5 |
| Timnodonic (eicosapentaenoic) acid (EPA) | 20:5 n-3 | 7.0 | 16.0 |
| Cervonic (docosahexaenoic) acid (DHA) | 22:6 n-3 | 6.0 | 18.0 |

*Test solution*. Dilute about 0.45 g of the substance to be examined to 10.0 mL with a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R*. Transfer 2.0 mL of this solution to a quartz tube and evaporate the solvent with a gentle current of *nitrogen R*. Add 1.5 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Cool, add 2 mL of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 mL of *trimethylpentane R*, cap and vortex or shake vigorously for at least 30 s. Immediately add 5 mL of *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and vortex or shake thoroughly for at least 15 s. Allow the upper layer to become clear and transfer to a separate tube. Shake the methanol layer once more with 1 mL of *trimethylpentane R* and combine the trimethylpentane extracts. Wash the combined extracts with 2 quantities, each of 1 mL, of *water R* and dry over *anhydrous sodium sulfate R*. Prepare 2 solutions for each sample.

*Reference solution*. Dissolve 0.300 g of *methyl arachidate R*, 0.300 g of *methyl behenate R*, 0.300 g of *methyl palmitate R* and 0.300 g of *methyl stearate R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R* or *helium for chromatography R*, with an oxygen scrubber.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 55 | 170 → 225 |
| | 55 - 75 | 225 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL, twice.

*System suitability*:

- the 15 fatty acids to be tested are satisfactorily identified from the chromatogram shown in Figure 1193.-1;
- in the chromatogram obtained with the reference solution, multiply the areas of the peaks due to methyl palmitate, methyl stearate, methyl arachidate and methyl behenate by the corresponding response factors in Table 1193.-1; normalise the corrected areas of the peaks due to the fatty acid methyl esters to a sum of 100 per cent; the normalised area percentage of each fatty acid methyl ester is to be within ± 1.0 percentage unit of the corresponding weight percentage;

Table 1193.-1.

| Fatty acid methyl ester | Theoretical response factor |
|---|---|
| Methyl palmitate | 1.049 |
| Methyl stearate | 1.029 |
| Methyl arachidate | 1.013 |
| Methyl behenate | 1.000 |

- *resolution*: in the chromatogram obtained with the test solution:
  - minimum 1.3 between the peaks due to methyl oleate and methyl *cis*-vaccenate; the resolution between the peaks due to methyl gadoleate and methyl gondoate is sufficient for purposes of identification and area measurement.

Calculate the area per cent for each fatty acid methyl ester using the following expression:

$$\frac{A_x}{A_t} \times 100$$

$A_x$ = peak area of fatty acid $x$;

$A_t$ = sum of the peak areas (up to C22:6 n-3).

The calculation is not valid unless:

- the total area is based only on peaks due solely to fatty acid methyl esters;
- the number of fatty acid methyl ester peaks exceeding 0.05 per cent of the total area is at least 24;
- the 24 largest peaks of the methyl esters account for more than 90 per cent of the total area (these correspond to, in common elution order: 14:0, 15:0, 16:0, 16:1 n-7, 16:4 n-1, 18:0, 18:1 n-9, 18:1 n-7, 18:2 n-6, 18:3 n-3, 18:4 n-3, 20:1 n-11, 20:1 n-9, 20:1 n-7, 20:2 n-6, 20:4 n-6, 20:3 n-3, 20:4 n-3, 20:5 n-3, 22:1 n-11, 22:1 n-9, 21:5 n-3, 22:5 n-3, 22:6 n-3).

ASSAY

**Vitamin A**. *Carry out the test as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (for example, copper and iron) and acids.*

Use method A. If method A is found not to be valid, use method B.

*METHOD A*

Ultraviolet absorption spectrophotometry (*2.2.25*).

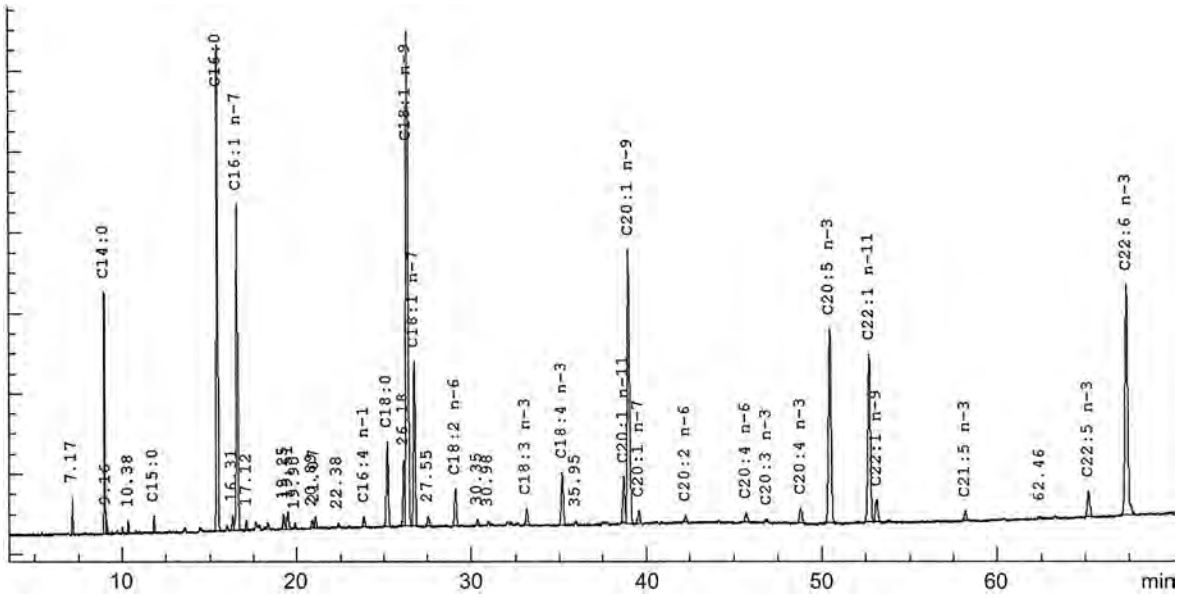


Figure 1193.-1. – *Chromatogram for the test for composition of fatty acids of cod-liver oil (type B)*

*Test solution.* To 1.00 g in a round-bottomed flask, add 3 mL of a freshly prepared 50 per cent *m/m* solution of *potassium hydroxide R* and 30 mL of *anhydrous ethanol R*. Boil under reflux in a current of *nitrogen R* for 30 min. Cool rapidly and add 30 mL of *water R*. Extract with 50 mL of *ether R*. Repeat the extraction 3 times and discard the lower layer after complete separation. Wash the combined upper layers with 4 quantities, each of 50 mL, of *water R* and evaporate to dryness under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C or by other suitable means at a temperature not exceeding 30 °C under reduced pressure. Dissolve the residue in sufficient *2-propanol R1* to give an expected concentration of vitamin A equivalent to 10-15 IU/mL.

Measure the absorbances of the solution at 300 nm, 310 nm, 325 nm and 334 nm and at the wavelength of maximum absorption with a suitable spectrophotometer in specially matched 1 cm cells, using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A, as all-*trans*-retinol, in International Units per gram using the following expression:

$$A_{325} \times \frac{1821}{100m} \times V$$

$A_{325}$ = absorbance at 325 nm;

$m$ = mass of the substance to be examined, in grams;

$V$ = total volume of solution containing 10-15 IU of vitamin A per millilitre;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

The above expression can be used only if $A_{325}$ has a value not greater than $A_{325,corr}/0.970$ where $A_{325,corr}$ is the corrected absorbance at 325 nm and is given by the following equation:

$$A_{325,corr} = 6.815A_{325} - 2.555A_{310} - 4.260A_{334}$$

*A* designates the absorbance at the wavelength indicated by the subscript.

If $A_{325}$ has a value greater than $A_{325,corr}/0.970$, calculate the content of vitamin A using the following expression:

$$A_{325,corr} \times \frac{1821}{100m} \times V$$

The assay is not valid unless:

- the wavelength of maximum absorption lies between 323 nm and 327 nm;
- the absorbance at 300 nm relative to that at 325 nm is at most 0.73.

*METHOD B*

Liquid chromatography (*2.2.29*).

*Test solution.* Prepare duplicates. To 2.00 g of the substance to be examined in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 15 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R* and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask.Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in *2-propanol R1*, transfer to a 25 mL volumetric flask and dilute to 25 mL with *2-propanol R1*. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Reference solution (a).* Prepare a solution of *retinol acetate CRS* in *2-propanol R1* so that 1 mL contains about 1000 IU of all-*trans*-retinol.

The exact concentration of reference solution (a) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (a) with *2-propanol R1* to a presumed concentration of 10-15 IU/mL and measure the absorbance at 326 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of vitamin A in International Units per millilitre of reference solution (a) using the following expression, taking into account the assigned content of *retinol acetate CRS*:

$$A_{326} \times \frac{1900 \times V_2}{100 \times V_1}$$

$A_{326}$ = absorbance at 326 nm;

$V_1$ = volume of reference solution (a) used;

$V_2$ = volume of the diluted solution;

1900 = conversion factor for the specific absorbance of *retinol acetate CRS*, in International Units.

*Reference solution (b).* Proceed as described for the test solution but using 2.00 mL of reference solution (a) in place of the substance to be examined.

The exact concentration of reference solution (b) is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dilute reference solution (b) with *2-propanol R1* to a presumed concentration of 10-15 IU/mL of all-*trans*-retinol and measure the absorbance at 325 nm in matched 1 cm cells using *2-propanol R1* as the compensation liquid.

Calculate the content of all-*trans*-retinol in International Units per millilitre of reference solution (b) using the following expression:

$$A_{325} \times \frac{1821 \times V_3}{100 \times V_4}$$

$A_{325}$ = absorbance at 325 nm;

$V_3$ = volume of the diluted solution;

$V_4$ = volume of reference solution (b) used;

1821 = conversion factor for the specific absorbance of all-*trans*-retinol, in International Units.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: *water for chromatography R*, *methanol R* (3:97 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL; inject in triplicate the test solution and reference solution (b).

*Retention time*: all-*trans*-retinol = 5 ± 1 min.

*System suitability*:

– the chromatogram obtained with the test solution shows a peak corresponding to the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

– the results obtained with the duplicate test solutions do not differ by more than 5 per cent;

– the recovery of all-*trans*-retinol in reference solution (b) as assessed by direct absorption spectrophotometry is greater than 95 per cent.

Calculate the content of vitamin A using the following expression:

$$A_1 \times \frac{C \times V}{A_2} \times \frac{1}{m}$$

$A_1$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to all-*trans*-retinol in the chromatogram obtained with reference solution (b);

$C$ = concentration of *retinol acetate CRS* in reference solution (a) as assessed prior to the saponification, in International Units per millilitre (= 1000 IU/mL);

$V$ = volume of reference solution (a) treated (2.00 mL);

$m$ = mass of the substance to be examined in the test solution (2.00 g).

**Vitamin $D_3$.** Liquid chromatography (*2.2.29*). *Carry out the assay as rapidly as possible, avoiding exposure to actinic light and air.*

*Internal standard solution.* Dissolve 0.50 mg of *ergocalciferol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* To 4.00 g of the substance to be examined in a round-bottomed flask, add 5 mL of a freshly prepared 100 g/L solution of *ascorbic acid R*, 10 mL of a freshly prepared 800 g/L solution of *potassium hydroxide R* and 100 mL of *anhydrous ethanol R*. Boil under a reflux condenser on a water-bath for 30 min. Add 100 mL of a 10 g/L solution of *sodium chloride R* and cool the solution to room temperature. Transfer the solution to a 500 mL separating funnel, rinsing the round-bottomed flask with about 75 mL of a 10 g/L solution of *sodium chloride R* and then with 150 mL of a mixture of equal volumes of *ether R* and *light petroleum R1*. Shake for 1 min. When the layers have separated completely, discard the lower layer and wash the upper layer, first with 50 mL of a 30 g/L solution of *potassium hydroxide R* in a 10 per cent *V/V* solution of *anhydrous ethanol R*, and then with 3 quantities, each of 50 mL, of a 10 g/L solution of *sodium chloride R*. Filter the upper layer through 5 g of *anhydrous sodium sulfate R* on a fast filter paper into a 250 mL flask.Wash the funnel with 10 mL of fresh extraction mixture, filter and combine the upper layers. Distil them at a temperature not exceeding 30 °C under reduced pressure and fill with *nitrogen R* when evaporation is completed. Alternatively, evaporate the solvent under a gentle current of *nitrogen R* at a temperature not exceeding 30 °C. Dissolve the residue in 1.5 mL of the mobile phase described under Purification. Gentle heating in an ultrasonic bath may be required. *A large fraction of the white residue is cholesterol, constituting approximately 50 per cent m/m of the unsaponifiable matter of cod-liver oil.*

*Test solution (b).* Prepare duplicates. To 4.00 g of the substance to be examined add 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*Reference solution (a).* Dissolve 0.50 mg of *cholecalciferol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* In a round-bottomed flask, add 2.0 mL of reference solution (a) and 2.0 mL of the internal standard solution and proceed as described for test solution (a).

*PURIFICATION*

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *nitrile silica gel for chromatography R* (10 µm).

*Mobile phase*: *isoamyl alcohol R*, *hexane R* (1.6:98.4 *V/V*).

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 350 µL of reference solution (b) and test solutions (a) and (b). Collect each eluate from 2 min before until 2 min after the retention time of cholecalciferol, in a ground-glass-stoppered tube containing 1 mL of a 1 g/L solution of *butylhydroxytoluene R* in *hexane R*. Evaporate

separately to dryness at a temperature not exceeding 30 °C under a gentle current of *nitrogen R*. Dissolve each residue in 1.5 mL of *acetonitrile R*.

*DETERMINATION*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *phosphoric acid R*, 96 per cent *V/V* solution of *acetonitrile R* (0.2:99.8 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 2 quantities not exceeding 200 µL of each of the 3 solutions obtained under Purification.

*System suitability*:

- *resolution*: minimum 1.4 between the peaks due to ergocalciferol and cholecalciferol in the chromatogram obtained with reference solution (b);
- the results obtained with the test solution (b) duplicates do not differ by more than 5 per cent.

Calculate the content of vitamin $D_3$ in International Units per gram using the following expression, taking into account the assigned content of *cholecalciferol CRS*:

$$\frac{A_2}{A_6} \times \frac{A_3}{A_4 - \left[\frac{A_5}{A_1}\right] \times A_2} \times \frac{m_2}{m_1} \times \frac{V_2}{V_1} \times 40$$

$m_1$ = mass of the substance to be examined in test solution (b), in grams;

$m_2$ = total mass of *cholecalciferol CRS* used for the preparation of reference solution (a), in micrograms (500 µg);

$A_1$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (a);

$A_2$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with test solution (b);

$A_3$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with reference solution (b);

$A_4$ = area (or height) of the peak due to ergocalciferol in the chromatogram obtained with test solution (b);

$A_5$ = area (or height) of a possible peak in the chromatogram obtained with test solution (a) with the same retention time as the peak co-eluting with ergocalciferol in test solution (b);

$A_6$ = area (or height) of the peak due to cholecalciferol in the chromatogram obtained with reference solution (b);

$V_1$ = total volume of reference solution (a) (100 mL);

$V_2$ = volume of reference solution (a) used to prepare reference solution (b) (2.0 mL).

STORAGE

In an airtight and well-filled container, protected from light. If no antioxidant is added, store under an inert gas.

Once the container has been opened, its contents are used as soon as possible and any part of the contents not used at once is protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units of vitamin A per gram;
- the number of International Units of vitamin $D_3$ per gram.

**04/2018:0758**

# COLCHICINE

## Colchicinum

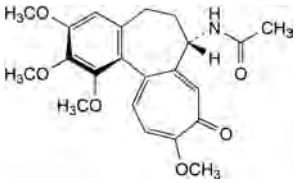

$C_{22}H_{25}NO_6$ $M_r$ 399.4

[64-86-8]

DEFINITION

*N*-[(7*S*,12a*M*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellowish-white, amorphous or crystalline powder.

*Solubility*: very soluble in water, rapidly recrystallising from concentrated solutions as the sesquihydrate, freely soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 5 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 25.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 230-400 nm.

*Absorption maxima*: 243 nm and 350 nm.

*Absorbance ratio*: $A_{243}/A_{350}$ = 1.7 to 1.9.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *colchicine CRS*.

C. To 0.5 mL of solution S (see Tests) add 0.5 mL of *dilute hydrochloric acid R* and 0.15 mL of *ferric chloride solution R1*. The solution is yellow and becomes dark green on boiling for 30 s. Cool, add 2 mL of *methylene chloride R* and shake. The organic layer is greenish-yellow.

D. Dissolve about 30 mg in 1 mL of *ethanol (96 per cent) R* and add 0.15 mL of *ferric chloride solution R1*. A brownish-red colour develops.

TESTS

**Solution S**. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_3$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Either the solution does not change colour or it becomes green. Not more than 0.1 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Specific optical rotation** (*2.2.7*): − 250 to − 235 (anhydrous substance).

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *colchicine for system suitability A CRS* (containing impurities A, E and G) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: mix 450 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* and 530 volumes of *methanol R*. After cooling to room temperature, adjust the volume to 1000 mL with *methanol R*. Adjust the apparent pH to 5.5 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of colchicine.

*Identification of impurities*: use the chromatogram supplied with *colchicine for system suitability A CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, E and G. (Impurity B is the conformational isomer of colchicine, which is formed *in situ* in solution).

*Relative retention* with reference to colchicine (retention time = about 7 min): impurity E = about 0.6; impurity B = about 0.9; impurity A = about 0.94; impurity G = about 1.4.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to colchicine; minimum 2, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity G by 1.6;
- for each impurity, use the concentration of colchicine in reference solution (b).

*Limits*:

- *impurity A*: maximum 3.0 per cent;
- *impurity G*: maximum 0.25 per cent;
- *impurity E*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 4.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to impurity B.

**Impurity F**: maximum 0.2 per cent.

Dissolve 50 mg in *water R* and dilute to 5 mL with the same solvent. Add 0.1 mL of *ferric chloride solution R1*. The solution is not more intensely coloured than a mixture of 1 mL of red primary solution, 2 mL of yellow primary solution and 2 mL of blue primary solution (*2.2.2, Method II*).

**Ethyl acetate** (*2.4.24*): maximum 6.0 per cent *m/m*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.250 g with gentle heating in a mixture of 20 mL of *acetic anhydride R* and 40 mL of *toluene R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 39.94 mg of $C_{22}H_{25}NO_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

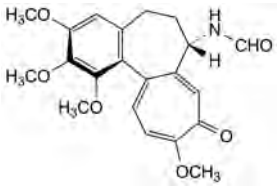

A. *N*-[(7*S*,12a*M*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]formamide (*N*-deacetyl-*N*-formylcolchicine),

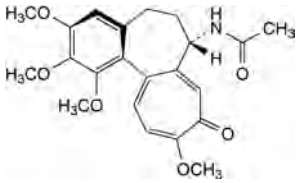

B. *N*-[(7*S*,12a*P*)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (conformational isomer),

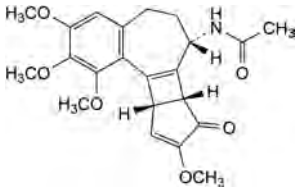

C. *N*-[(7*S*,7b*R*,10a*S*)-1,2,3,9-tetramethoxy-8-oxo-5,6,7,7b,8,10a-hexahydrobenzo[*a*]cyclopenta-[3,4]cyclobuta[1,2-*c*]cyclohepten-7-yl]acetamide (β-lumicolchicine),

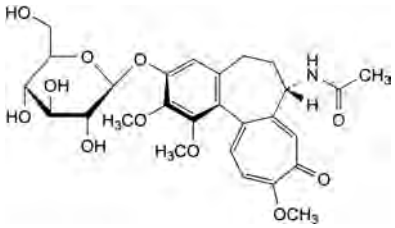

D. *N*-[(7*S*,12a*M*)-3-(β-D-glucopyranosyloxy)-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicoside),

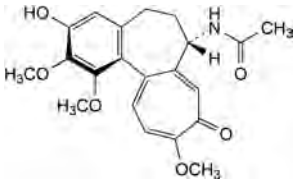

E. *N*-[(7*S*,12a*M*)-3-hydroxy-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (3-*O*-demethylcolchicine),

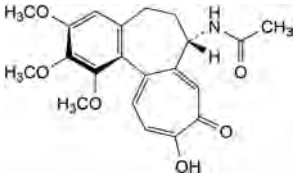

F. *N*-[(7*S*,12a*M*)-10-hydroxy-1,2,3-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchiceine),

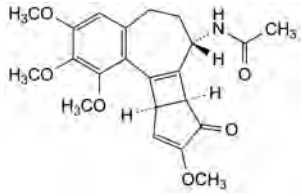

G. *N*-[(7*S*,7b*S*,10a*R*)-1,2,3,9-tetramethoxy-8-oxo-5,6,7,7b,8,10a-hexahydrobenzo[*a*]cyclopenta-[3,4]cyclobuta[1,2-*c*]cyclohepten-7-yl]acetamide (γ-lumicolchicine).

**01/2017:1775**
**corrected 10.0**

# COLESTYRAMINE

## Colestyraminum

[11041-12-6]

DEFINITION

Strongly basic anion-exchange resin in chloride form, consisting of styrene-divinylbenzene copolymer with quaternary ammonium groups.

*Nominal exchange capacity*: 1.8 g to 2.2 g of sodium glycocholate per gram (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder, hygroscopic.

*Solubility*: insoluble in water, in methylene chloride and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison: colestyramine CRS.*

B. Chloride (see Tests).

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

Suspend 0.100 g in 10 mL of *water R* and allow to stand for 10 min.

**Dialysable quaternary amines**: maximum 500 ppm, expressed as benzyltrimethylammonium chloride.

*Test solution.* Place a 25 cm piece of cellulose dialysis tubing having a molecular weight cut-off of 12 000-14 000 and an inflated diameter of 3-6 cm (flat width of 5-9 cm) in *water R* to hydrate until pliable, appropriately sealing one end. Introduce 2.0 g of the substance to be examined into the tube and add 10 mL of *water R*. Seal the tube and completely immerse it in 100 mL of *water R* in a suitable vessel and stir the liquid for 16 h to effect dialysis. Use the dialysate as test solution.

*Reference solution.* Prepare the reference solution in a similar manner but using 10 mL of a freshly prepared 0.1 g/L solution of *benzyltrimethylammonium chloride R* instead of the substance to be examined.

Transfer 5.0 mL of the test solution to a separating funnel and add 5 mL of a 3.8 g/L solution of *disodium tetraborate R*, 1 mL of a solution containing 1.5 g/L of *bromothymol blue R* and 4.05 g/L of *sodium carbonate R* and 10 mL of *chloroform R*. Shake the mixture vigourously for 1 min, allow the phases to separate and transfer the clear organic layer to a 25 mL volumetric flask. Repeat the extraction with a further 10 mL of *chloroform R*, combine the organic layers and dilute to 25 mL with *chloroform R*. Measure the absorbance (*2.2.25*) of the solution at the absorption maximum at 420 nm, using as compensation liquid a solution prepared in the same manner but using 5.0 mL of *water R* instead of the test solution.

Repeat the operation using 5.0 mL of the reference solution.

The absorbance obtained with the test solution is not greater than that obtained with the reference solution.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* Shake 5.0 g with 10 mL of *acetone R* for 30 min. Centrifuge and use the supernatant.

*Reference solution (a).* Dissolve 5 mg of *styrene R* in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *acetone R*.

*Reference solution (b).* Dissolve 0.35 mL of *styrene R* in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *acetone R*.

*Reference solution (c).* Dissolve 0.35 mL of *toluene R* in *acetone R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Mix 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c) with *acetone R* and dilute to 100.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.30 m, Ø = 3.9 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm) with a specific surface area of 330 $m^2$/g and a pore size of 12.5 nm.

*Mobile phase*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution and reference solutions (a) and (d).

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to impurity A and toluene.

*Limit*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 ppm).

**Chloride**: 13.0 per cent to 17.0 per cent (dried substance).

To 0.2 g add 100 mL of *water R* and 50 mg of *potassium nitrate R*. Add, with stirring, 2 mL of *nitric acid R* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 3.55 mg of Cl.

**Loss on drying** (*2.2.32*): maximum 12 per cent, determined on 1.000 g by drying *in vacuo* at 70 °C at a pressure not exceeding 7 kPa for 16 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**Exchange capacity**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 1.500 g of *sodium glycocholate R* in a solution containing 4 g/L of *potassium dihydrogen phosphate R* and 12 g/L of *dipotassium hydrogen phosphate R* and dilute to 100.0 mL with the same solution.

*Test solution*. Add 20.0 mL of solution A to a quantity of the substance to be examined equivalent to about 0.100 g of the dried substance. Shake mechanically for 2 h and centrifuge for 15 min. Dilute 5.0 mL of the supernatant to 50.0 mL with *water R*.

*Reference solution (a)*. Dilute 4.0 mL of solution A to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 60 mg of *sodium glycocholate R* and 30 mg of *sodium taurodeoxycholate R* in *water R* and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 10.9 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 50 µL.

*Run time*: twice the retention time of glycocholate.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to glycocholate and taurodeoxycholate.

Calculate the nominal exchange capacity using the following expression:

$$\frac{(2.5\,A_1 - A_2) \times m_1 \times 1.2}{12.5 \times A_1 \times m_2}$$

$A_1$ = area of the peak due to glycocholate in the chromatogram obtained with reference solution (a),

$A_2$ = area of the peak due to glycocholate in the chromatogram obtained with the test solution,

$m_1$ = mass, in milligrams, of *sodium glycocholate R* used in the preparation of solution A,

$m_2$ = mass, in milligrams, of the dried substance to be examined used in the preparation of the test solution,

1.2 = correction factor to convert the true exchange capacity to the conventionally used nominal exchange capacity.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

A. styrene.

**07/2017:0319**
**corrected 10.0**

# COLISTIMETHATE SODIUM

## Colistimethatum natricum

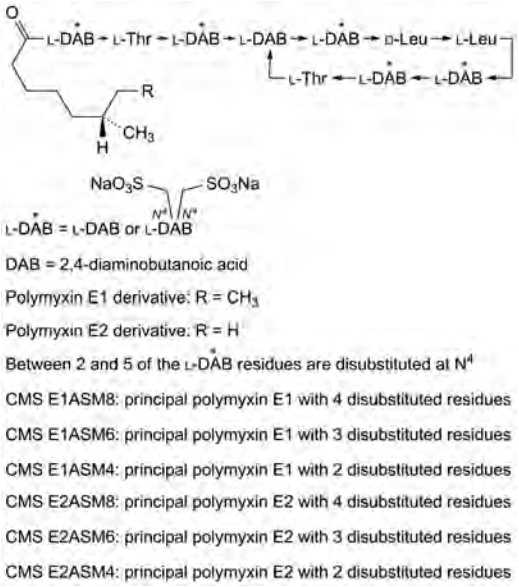


[8068-28-8]

DEFINITION

Colistimethate sodium is prepared from colistin by the action of formaldehyde and sodium hydrogen sulfite to form a mixture of di to penta bis-sulfomethylated primary amine derivatives, mainly polymyxins E1 and E2.

Semi-synthetic product derived from a fermentation product.

*Content*: minimum 11 500 IU/mg (dried substance).

PRODUCTION

The composition and purity of the colistin starting material used in the synthesis of colistimethate sodium are equivalent to that described in the monograph *Colistin sulfate (0320)*. In addition, the colistin starting material must have a polymyxin E1 content between 50 per cent and 75 per cent and a polymyxin E2 content between 5 per cent and 20 per cent, in order to comply with the composition limits for colistimethate sodium.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for composition.

*Results*: the peaks due to CMS E1ASM8, CMS E1ASM6, CMS E1ASM4, CMS E2ASM8, CMS E2ASM6 and CMS E2ASM4 in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (a).

B. It gives reaction (b) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.16 g in 10 mL of *water R*.

**pH** (*2.2.3*): 6.5 to 8.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Measure after 30 min.

**Free colistin**. Dissolve 80 mg in 3 mL of *water R*. Add 0.1 mL of a 100 g/L solution of *silicotungstic acid R*; 10-20 s after addition of the reagent, the solution is not more opalescent than reference suspension II (*2.2.1*).

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Buffer solution*: 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 6.5 with *1 M sodium hydroxide*.

*Test solution*. Dissolve 20 mg of the substance to be examined in 0.5 mL of *water R* and dilute to 10.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *colistimethate sodium for peak identification CRS* in 0.25 mL of *water R* and dilute to 5.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 2 mg of *E1 colistimethate sodium for peak identification CRS* in 0.25 mL of *water R* and dilute to 2.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 1.5 mg of *E2 colistimethate sodium for peak identification CRS* in 0.25 mL of *water R* and dilute to 5.0 mL with *methanol R*.

*Reference solution (d)*. Dilute 1.5 mL of reference solution (a) to 25.0 mL with *methanol R*.

*Precolumn*:

- *size*: *l* = 5 mm, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.7 µm).

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.7 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution (25:475 *V/V*);
- *mobile phase B*: *acetonitrile R1*, buffer solution (250:250 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 → 68 | 20 → 32 |
| 10 - 35 | 68 → 53 | 32 → 47 |

*Flow rate*: 0.30 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 5 °C.

*Injection*: 2.0 µL.

*Identification of peaks*: use the chromatogram supplied with *colistimethate sodium for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to CMS E1ASM8, CMS E1ASM6, CMS E1ASM4, CMS E2ASM8, CMS E2ASM6 and CMS E2ASM4 (see Figure 0319.-1).

The peak corresponding to the most abundant compound in the range of 11.0-14.5 min (CMS E1ASM6) in the chromatogram obtained with reference solution (a) is set as the identification reference peak (relative retention 1.00).

*Identification of peaks related to CMS E1 and CMS E2*: use the chromatogram supplied with *E1 colistimethate sodium for peak identification CRS* and *E2 colistimethate sodium for peak identification CRS*, and the chromatograms obtained with reference solutions (b) and (c) to identify all peaks related to CMS E1 and CMS E2 in the chromatogram obtained with the test solution (see Figures 0319.-2 and 0319.-3).

*Relative retention* with reference to CMS E1ASM6 (retention time = about 13 min): CMS E2ASM8 = about 0.22; CMS E1ASM8 = about 0.39; CMS E2ASM6 = about 0.71; CMS E2ASM4 = about 1.77; CMS E1ASM4 = about 2.35.

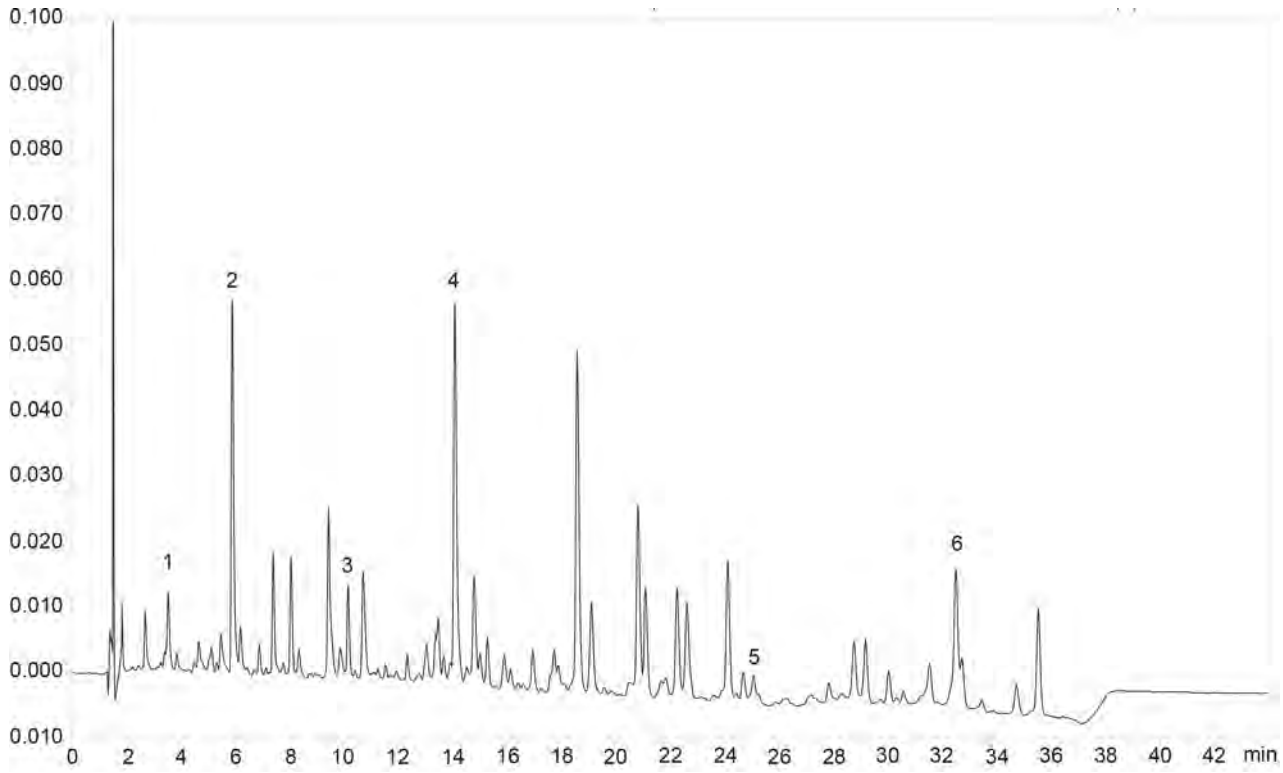


1. CMS E2ASM8
2. CMS E1ASM8
3. CMS E2ASM6
4. CMS E1ASM6
5. CMS E2ASM4
6. CMS E1ASM4

Figure 0319.-1. – *Chromatogram for the test for composition of colistimethate sodium: reference solution (a)*

Integrate all peaks above 0.05 per cent to establish the total area.

*System suitability*:

- the difference in the retention times of CMS E1ASM6 in 2 consecutive injections of reference solution (a) is less than 0.1 min; the drift in the retention time of CMS E1ASM6 from the beginning to the end of the sequence is less than 0.5 min;
- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak with a relative retention of about 2.37 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to CMS E1ASM4 in the chromatogram obtained with reference solution (a);
- *number of theoretical plates*: minimum 50 000, calculated for the peak due to CMS E1ASM6 in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 50 for the peak due to CMS E1ASM6 in the chromatogram obtained with reference solution (d).

*Limits*:

- *CMS E1ASM8*: 5.0 per cent to 9.5 per cent;
- *CMS E1ASM6*: 6.5 per cent to 9.5 per cent;
- *CMS E1ASM4*: 2.0 per cent to 5.0 per cent;
- *CMS E2ASM8*: 0.5 per cent to 2.0 per cent;
- *CMS E2ASM6*: 0.5 per cent to 2.5 per cent;
- *CMS E2ASM4*: maximum 1.5 per cent;
- *sum of the peaks related to CMS E1 and CMS E2*: minimum 77.0 per cent;
- *disregard limit*: 0.50 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition.

*Limits*:

- *any other impurity* (any peak not related to CMS E1 or CMS E2): for each impurity, maximum 1.5 per cent;
- *sum of impurities* (sum of all peaks not related to CMS E1 or CMS E2): maximum 5.5 per cent;
- *disregard limit*: 0.50 per cent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): 16 per cent to 21 per cent, determined on 0.50 g.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of pyrogens, it complies with the test. Inject, per kilogram of the rabbit's mass, 1 mL of a solution in *water for injections R* containing 2.5 mg of the substance to be examined per millilitre.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light. If the substance is sterile the container is also sterile and tamper-evident.

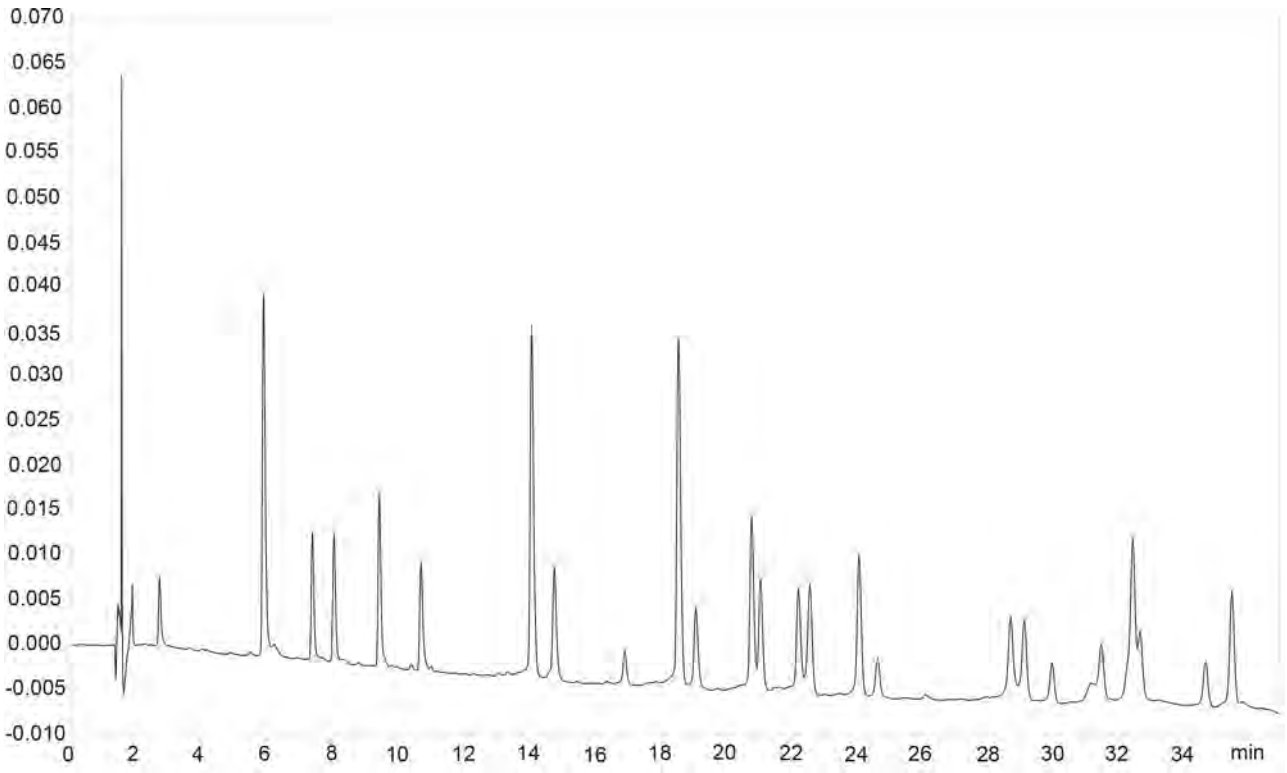


Figure 0319.-2. – *Chromatogram for the test for composition of colistimethate sodium: reference solution (b)*

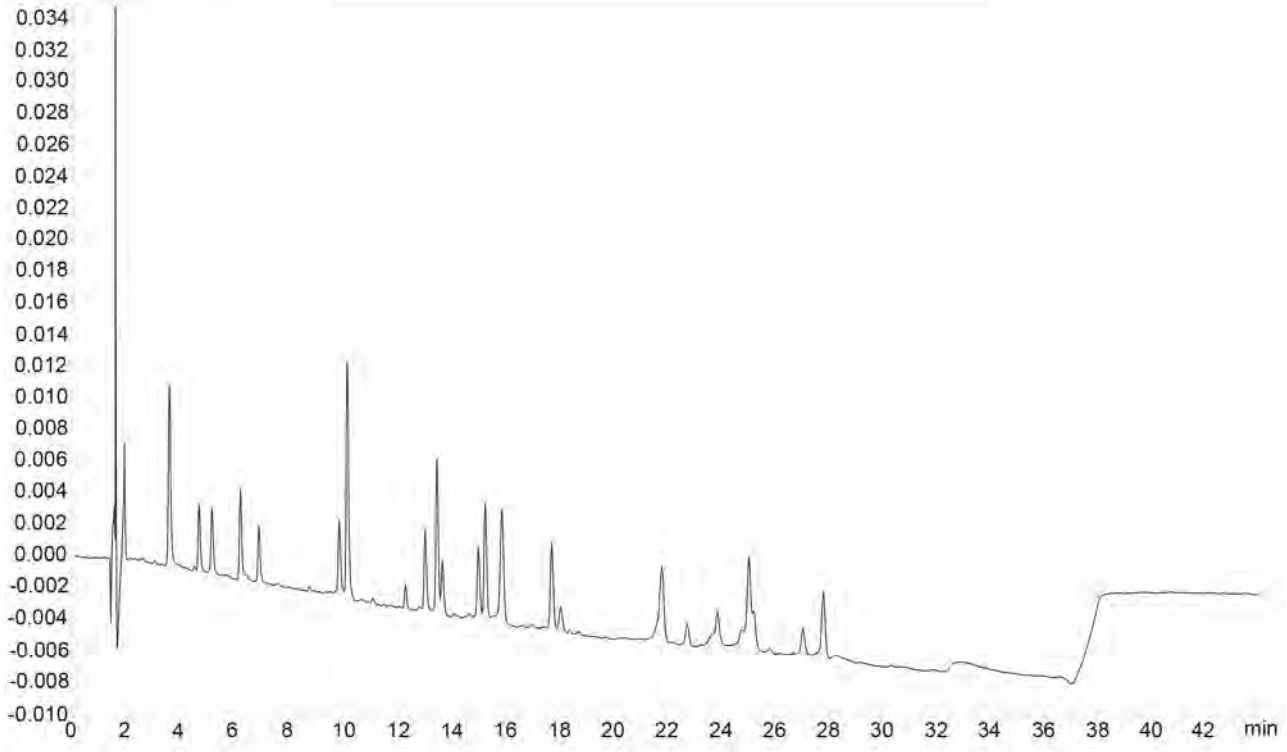


Figure 0319.-3. – *Chromatogram for the test for composition of colistimethate sodium: reference solution (c)*

**01/2019:0320**
**corrected 10.0**

# COLISTIN SULFATE

## Colistini sulfas

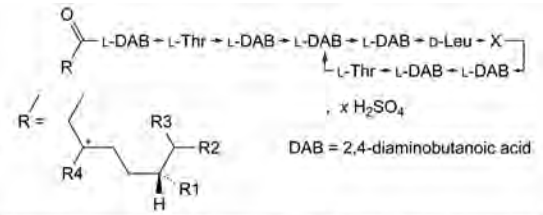


| polymyxin | X | R1 | R2 | R3 | R4 | Mol. Formula | $M_r$ |
|---|---|---|---|---|---|---|---|
| E1 | L-Leu | $CH_3$ | $CH_3$ | H | H | $C_{53}H_{100}N_{16}O_{13}$ | 1169 |
| E2 | L-Leu | $CH_3$ | H | H | H | $C_{52}H_{98}N_{16}O_{13}$ | 1155 |
| E3 | L-Leu | H | $CH_3$ | H | H | $C_{52}H_{98}N_{16}O_{13}$ | 1155 |
| E4 | L-Leu | H | H | H | H | $C_{51}H_{96}N_{16}O_{13}$ | 1141 |
| E6 | L-Leu | $CH_3$ | $CH_3$ | H | OH | $C_{53}H_{100}N_{16}O_{14}$ | 1185 |
| E1-7MOA | L-Leu | H | $CH_3$ | $CH_3$ | H | $C_{53}H_{100}N_{16}O_{13}$ | 1169 |
| E1-I | L-Ile | $CH_3$ | $CH_3$ | H | H | $C_{53}H_{100}N_{16}O_{13}$ | 1169 |
| E1-Nva | L-Nva | $CH_3$ | $CH_3$ | H | H | $C_{52}H_{98}N_{16}O_{13}$ | 1155 |
| E2-I | L-Ile | $CH_3$ | H | H | H | $C_{52}H_{98}N_{16}O_{13}$ | 1155 |
| E2-Val | L-Val | $CH_3$ | H | H | H | $C_{51}H_{96}N_{16}O_{13}$ | 1141 |

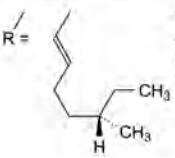


| polymyxin | X | Mol. Formula | $M_r$ |
|---|---|---|---|
| 2,3-dehydro E1 | L-Leu | $C_{53}H_{98}N_{16}O_{13}$ | 1167 |

[1264-72-8]

DEFINITION

A mixture of the sulfates of polypeptides produced by certain strains of *Bacillus polymyxa* var. *colistinus*.

*Content*: minimum 19 000 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Heat at 135 °C in a sealed tube for 5 h. Evaporate to dryness on a water-bath and continue the heating until moistened *blue litmus paper R* does not turn red. Dissolve the residue in 0.5 mL of *water R*.

*Reference solution (a).* Dissolve 20 mg of *leucine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *threonine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dissolve 20 mg of *phenylalanine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (d).* Dissolve 20 mg of *serine R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Carry out the following procedures protected from light.*

*Mobile phase*: *water R*, *phenol R* (25:75 *V/V*).

*Application*: 5 µL as bands of 10 mm, then place the plate in the chromatographic tank so that it is not in contact with the mobile phase, and allow it to become impregnated with the vapour of the mobile phase for at least 12 h.

*Development*: over half of the plate.

*Drying*: at 105 °C.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*Results*: the chromatogram obtained with the test solution shows zones corresponding to those in the chromatograms obtained with reference solutions (a) and (b), but shows no zones corresponding to those in the chromatograms

obtained with reference solutions (c) and (d); the chromatogram obtained with the test solution also shows a zone with a very low $R_F$ value (2,4-diaminobutyric acid).

B. Examine the chromatograms obtained in the test for composition.

*Results*: the peaks due to polymyxin E1 and polymyxin E2 in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (a).

C. Dissolve about 5 mg in 3 mL of *water R*. Add 3 mL of *dilute sodium hydroxide solution R*. Shake and add 0.5 mL of a 10 g/L solution of *copper sulfate pentahydrate R*. A violet colour is produced.

D. Dissolve about 50 mg in 1 mL of *1 M hydrochloric acid* and add 0.5 mL of *0.01 M iodine*. The solution remains coloured.

E. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.0 to 6.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 5.0 mg of the substance to be examined in 8 mL of *water R* and dilute to 10.0 mL with *acetonitrile R*.

*Reference solution (a)*. Dissolve 5.0 mg of *colistin for system suitability CRS* in 8 mL of *water R* and dilute to 10.0 mL with *acetonitrile R*.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.0 µm);
- *temperature*: 50 °C.

*Mobile phase*: mix 22 volumes of *acetonitrile R1* and 78 volumes of a solution prepared as follows: dissolve 4.46 g of *anhydrous sodium sulfate R* in 900 mL of *water for chromatography R*, adjust to pH 2.4 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of polymyxin E1.

*Identification of peaks*: use the chromatogram supplied with *colistin for system suitability CRS* to identify the peaks due to polymyxins E1, E2, E3, E4, E6, E1-7MOA, E1-I, E1-Nva, E2-I, E2-Val, 2,3-dehydro E1 and due to impurities A and B.

*Relative retention* with reference to polymyxin E1 (retention time = about 21 min): polymyxins E4 and E2-Val = about 0.28; polymyxin E6 = about 0.39; polymyxin E2-I = about 0.42; polymyxin E2 = about 0.50; impurity A = about 0.53; polymyxin E3 = about 0.56; polymyxin E1-Nva = about 0.59; polymyxin E1-I = about 0.82; polymyxin 2,3-dehydro E1 = about 0.90; polymyxin E1-7MOA = about 1.1; impurity B = about 1.3.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to polymyxin E6 and polymyxin E2-I, and minimum 3.0 between the peaks due to polymyxin 2,3-dehydro E1 and polymyxin E1 in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peak due to polymyxin E1 in the chromatogram obtained with reference solution (b);
- *peak-to-valley ratio*: minimum 1.1, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to polymyxin E2 in the chromatogram obtained with reference solution (a).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of polymyxin 2,3-dehydro E1 by 0.3;
- *polymyxin E1-I*: maximum 8.5 per cent;
- *polymyxin E3*: maximum 5.5 per cent;
- *polymyxin E1-7MOA*: maximum 5.0 per cent;
- *polymyxin E6*: maximum 4.5 per cent;
- *polymyxin E1-Nva*: maximum 4.5 per cent;
- *sum of polymyxins E4 and E2-Val*: maximum 3.0 per cent;
- *polymyxin E2-I*: maximum 2.5 per cent;
- *polymyxin 2,3-dehydro E1*: maximum 1.5 per cent;
- *sum of polymyxins E1, E2, E3, E4, E6, E1-7MOA, E1-I, E1-Nva, E2-I, E2-Val and 2,3-dehydro E1*: minimum 86.0 per cent;
- *disregard limit*: 0.35 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition.

*Limits*:

- *impurity B*: maximum 4.0 per cent;
- *any other impurity*: for each impurity, maximum 2.5 per cent, and not more than 4 such impurities exceed 1.0 per cent;
- *total*: maximum 11.0 per cent;
- *disregard limit*: 0.35 per cent.

**Sulfate**: 16.0 per cent to 18.0 per cent (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust to pH 11 with *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*, adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Loss on drying** (*2.2.32*): maximum 3.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

A. unknown structure,

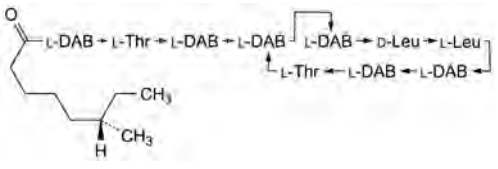


B. [$N^4$-Dab$^5$]polymyxin E1.

**01/2020:0891**

# COPOVIDONE

## Copovidonum

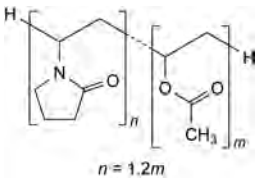


$(C_6H_9NO)_n(C_4H_6O_2)_m$ $M_r$ $(111.1)_n + (86.1)_m$

[25086-89-9]

DEFINITION

Copolymer of 1-ethenylpyrrolidin-2-one and ethenyl acetate in the mass proportion 3:2.

*Content*:

- *nitrogen* (N; $A_r$ 14.01): 7.0 per cent to 8.0 per cent (dried substance);
- *ethenyl acetate* ($C_4H_6O_2$; $M_r$ 86.10): 35.3 per cent to 42.0 per cent (dried substance).

♦CHARACTERS

*Appearance*: white or yellowish-white hygroscopic powder or flakes.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methylene chloride.♦

IDENTIFICATION

*First identification: A.*

◊*Second identification: B, C.*◊

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined and the reference substance at 105 °C for 3 h.

*Comparison*: *copovidone CRS*.

◊B. To 1 mL of solution S (see Tests) add 5 mL of *water R* and 0.2 mL of *0.05 M iodine*. A red colour appears.

C. Dissolve 0.7 g of *hydroxylamine hydrochloride R* in 10 mL of *methanol R*, add 20 mL of a 40 g/L solution of *sodium hydroxide R* and filter if necessary. To 5 mL of the solution add 0.1 g of the substance to be examined and boil for 2 min. Transfer 50 µL to a filter paper and add 0.1 mL of a mixture of equal volumes of *ferric chloride solution R1* and *hydrochloric acid R*. A violet colour appears.◊

TESTS

**Solution S**. Dissolve 10.0 g in *water R* and dilute to 100.0 mL with the same solvent. Add the substance to be examined to the *water R* in small portions with constant stirring.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $B_5$, $R_5$ or $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 7.0 for solution S.

**Viscosity, expressed as *K*-value**. Dilute 5.0 mL of solution S to 50.0 mL with *water R*. Allow to stand for 1 h and determine the viscosity (*2.2.9*) of the solution at 25 ± 0.1 °C. Calculate the *K*-value using the following expression:

$$\frac{1.5 \log v_{rel} - 1}{0.15 + 0.003c} + \frac{\sqrt{300c \log v_{rel} + (c + 1.5c \log v_{rel})^2}}{0.15c + 0.003c^2}$$

$c$ = concentration of the substance to be examined (dried substance), in grams per 100 mL;

$v_{rel}$ = kinematic viscosity of the solution relative to that of *water R*.

The *K*-value is 90.0 per cent to 110.0 per cent of the nominal *K*-value.

**Aldehydes**: maximum 500 ppm, expressed as acetaldehyde.

*Test solution*. Dissolve 1.0 g of the substance to be examined in *phosphate buffer solution pH 9.0 R* and dilute to 100.0 mL with the same solvent. Stopper the flask tightly and heat at 60 °C for 1 h. Allow to cool to room temperature.

*Reference solution*. Dissolve 0.140 g of *acetaldehyde ammonia trimer trihydrate R* in *water R* and dilute to 200.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *phosphate buffer solution pH 9.0 R*.

Into 3 identical spectrophotometric cells with a path length of 1 cm, introduce separately 0.5 mL of the test solution, 0.5 mL of the reference solution and 0.5 mL of *water R* (blank). To each cell add 2.5 mL of *phosphate buffer solution pH 9.0 R* and 0.2 mL of *nicotinamide-adenine dinucleotide solution R*. Mix and stopper tightly. Allow to stand at 22 ± 2 °C for 2-3 min and measure the absorbance (*2.2.25*) of each solution at 340 nm, using *water R* as the compensation liquid. To each cell, add 0.05 mL of *aldehyde dehydrogenase solution R*, mix and stopper tightly. Allow to stand at 22 ± 2 °C for 5 min. Measure the absorbance of each solution at 340 nm using *water R* as the compensation liquid.

Calculate the content of aldehydes, expressed as acetaldehyde, in parts per million, using the following expression:

$$\frac{(A_{t2} - A_{t1}) - (A_{b2} - A_{b1})}{(A_{s2} - A_{s1}) - (A_{b2} - A_{b1})} \times \frac{100\,000 \times C}{m}$$

$A_{t1}$ = absorbance of the test solution before the addition of aldehyde dehydrogenase;

$A_{t2}$ = absorbance of the test solution after the addition of aldehyde dehydrogenase;

$A_{s1}$ = absorbance of the reference solution before the addition of aldehyde dehydrogenase;

$A_{s2}$ = absorbance of the reference solution after the addition of aldehyde dehydrogenase;

$A_{b1}$ = absorbance of the blank before the addition of aldehyde dehydrogenase;

$A_{b2}$ = absorbance of the blank after the addition of aldehyde dehydrogenase;

$m$ = mass of the substance to be examined (dried substance) in the test solution, in grams;

$C$ = concentration of acetaldehyde in the reference solution, calculated from the mass of acetaldehyde ammonia trimer trihydrate and by multiplying by a factor of 0.72, in milligrams per millilitre.

**Peroxides**: maximum 400 ppm, expressed as $H_2O_2$.

Dissolve a quantity of the substance to be examined equivalent to 4.0 g of the dried substance in *water R* and dilute to 100.0 mL with the same solvent (stock solution). To 25.0 mL of the stock solution add 2 mL of *titanium trichloride-sulfuric acid reagent R*, mix and allow to stand for 30 min. The absorbance (*2.2.25*) of the solution, measured at 405 nm using a mixture of 25.0 mL of the stock solution and 2.0 mL of a 13 per cent *V/V* solution of *sulfuric acid R* as the compensation liquid, is not greater than 0.35.

**Hydrazine**. Thin-layer chromatography (*2.2.27*). *Use freshly prepared solutions.*

*Test solution*. Dissolve a quantity of the substance to be examined equivalent to 2.5 g of the dried substance in 25 mL of *water R* and mix. Add 0.5 mL of a 50 g/L solution of *salicylaldehyde R* in *methanol R*, mix and heat in a water-bath

at 60 °C for 15 min. Allow to cool, add 2.0 mL of *toluene R*, stopper tightly, shake vigorously for 2 min and centrifuge. Use the upper layer.

*Reference solution.* Dissolve 90 mg of *salicylaldehyde azine R* in *toluene R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *toluene R*.

*Plate*: *TLC silanised silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *methanol R* (1:2 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Retardation factor*: salicylaldehyde azine = about 0.3.

*Limit*:

– *hydrazine*: any spot due to salicylaldehyde azine is not more intense than the spot in the chromatogram obtained with the reference solution (1 ppm).

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in 5 mL of *methanol R*. Sonicate until dissolution is complete and dilute to 100.0 mL with *water R*.

*Reference solution.* Dissolve 0.150 g of *2-pyrrolidone R* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 3.0 mL of the solution to 100.0 mL with the mobile phase.

*Precolumn*:

– *size*: *l* = 0.010 m, Ø = 4 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: *methanol R2*, *water for chromatography R* (5:95 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL. After each injection of the test solution, elute and wash away the remaining sample by passing the mobile phase through the column backwards for about 30 min at the same flow rate as applied in the test. This process may be replaced by washing the precolumn only.

*Run time*: 4 times the retention time of impurity A.

*Identification of impurities*: use the chromatogram obtained with the reference solution to identify the peak due to impurity A.

*Retention time*: impurity A = about 7 min.

*System suitability*: reference solution:

– *symmetry factor*: maximum 1.5 for the peak due to impurity A;

– *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections.

Calculate the percentage content of impurity A using the following expression:

$$\frac{A_1}{A_2} \times \frac{m_2}{m_1} \times 3$$

$A_1$ = area of the peak due to impurity A in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to impurity A in the chromatogram obtained with the reference solution;

$m_1$ = mass of the substance to be examined (dried substance) in the test solution, in grams;

$m_2$ = mass of impurity A in the reference solution, in grams.

*Limit*:

– *impurity A*: maximum 0.5 per cent.

**Impurities B and C**. Liquid chromatography (*2.2.29*). *Store the test solution and the reference solution at a temperature not exceeding 10 °C and use them within 8 h.*

*Test solution.* Dissolve 0.250 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution.* Dissolve 50.0 mg of *1-vinylpyrrolidin-2-one R* (impurity B) and 50.0 mg of *vinyl acetate R* (impurity C) in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Precolumn*:

– *size*: *l* = 0.033 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R1*, *water for chromatography R* (8:92 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm for impurity C and at 235 nm for impurity B.

*Injection*: 20 µL. After each injection of the test solution, elute and wash away the remaining sample by passing the mobile phase through the column backwards for about 30 min at the same flow rate as applied in the test. This process may be replaced by washing the precolumn only. Store and inject the solutions at a temperature not exceeding 5 °C and use them within 8 h. Use a cooled autosampler.

*Run time*: twice the retention time of impurity C.

*Identification of impurities*: use the chromatogram obtained with the reference solution to identify the peaks due to impurities B and C.

*Retention time*: impurity B = about 17 min;
impurity C = about 22 min.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to impurities B and C when the chromatogram is recorded at 205 nm;

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the areas of the peaks due to impurities B and C determined on 6 injections. Use separate vials for each replicate injection.

Calculate the content of impurity B in parts per million using the following expression:

$$\frac{A_1}{A_2} \times \frac{m_2}{m_1} \times 0.05$$

$A_1$ = area of the peak due to impurity B in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to impurity B in the chromatogram obtained with the reference solution;

$m_1$ = mass of the substance to be examined (dried substance) in the test solution, in grams;

$m_2$ = mass of impurity B in the reference solution, in grams.

Calculate the content of impurity C in parts per million using the following expression:

$$\frac{A_1}{A_2} \times \frac{m_2}{m_1} \times 0.05$$

$A_1$ = area of the peak due to impurity C in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to impurity C in the chromatogram obtained with the reference solution;

$m_1$ = mass of the substance to be examined (dried substance) in the test solution, in grams;

$m_2$ = mass of impurity C in the reference solution, in grams.

*Limits*:

– *impurities B, C*: for each impurity, maximum 10 ppm.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**Ethenyl acetate**. Determine the saponification value (*2.5.6*) on 2.00 g of the substance to be examined. Multiply the result obtained by 0.1534 to obtain the percentage content of the ethenyl acetate component.

**Nitrogen**. Place 0.100 g of the substance to be examined (*m* mg) in a combustion flask and add 5 g of a mixture of 1 g of *copper sulfate pentahydrate R*, 1 g of *titanium dioxide R* and 33 g of *dipotassium sulfate R*. Wash any adhering particles from the neck into the flask with a small quantity of *water R*. Add 7 mL of *sulfuric acid R*, allowing it to run down the insides of the flask. Heat the flask gradually until the solution has a clear, yellowish-green colour, and the inside wall of the flask is free from any carbonised material, and then heat for a further 45 min. After cooling, add cautiously 20 mL of *water R*, and connect the flask to the distillation apparatus, which has been previously washed by passing steam through it. To the absorption flask add 30 mL of a 40 g/L solution of *boric acid R*, 3 drops of *bromocresol green-methyl red solution R* and sufficient *water R* to immerse the lower end of the condenser tube. Add 30 mL of *strong sodium hydroxide solution R* through the funnel, rinse the funnel cautiously with 10 mL of *water R*, immediately close the clamp on the rubber tube, then start distillation with steam to obtain 80-100 mL of distillate. Remove the absorption flask from the lower end of the condenser tube, rinsing the end part with a small quantity of *water R*, and titrate the distillate with *0.025 M sulfuric acid* until the colour of the solution changes from green through pale greyish-blue to pale greyish reddish-purple. Carry out a blank determination.

1 mL of *0.025 M sulfuric acid* is equivalent to 0.700 mg of N.

♦STORAGE

In an airtight container.♦

LABELLING

The label states the nominal *K*-value.

IMPURITIES

*Specified impurities: A, B, C.*

A. pyrrolidin-2-one (2-pyrrolidone),

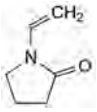

B. 1-ethenylpyrrolidin-2-one (1-vinylpyrrolidin-2-one),

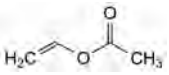

C. ethenyl acetate (vinyl acetate).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for copovidone used as binder in tablets and granules.*

**Viscosity** (*2.2.9*): determine the dynamic viscosity using a capillary viscometer on a 10 per cent solution (dried substance) or on a 20 per cent solution (dried substance) at 25 °C. It is typically about 8 mPa·s or about 23 mPa·s, respectively.

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

*The following characteristic may be relevant for copovidone used as film former in coated dosage forms and in aerosols.*

**Viscosity** (*2.2.9*): see above.

**01/2017:0893**

# COPPER SULFATE

## Cupri sulfas

$CuSO_4$ $M_r$ 159.6
[7758-98-7]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: greenish-grey powder, very hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in methanol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Add several drops of *dilute ammonia R2* to 1 mL of solution S (see Tests). A blue precipitate is formed. On further addition of *dilute ammonia R2* the precipitate dissolves and a dark blue colour is produced.

B. Loss on drying (see Tests).

C. Dilute 1 mL of solution S to 5 mL with *water R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.6 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Iron**: maximum 150 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 0.32 g in 10 mL of *water R*, add 2.5 mL of *lead-free nitric acid R* and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, adding 2.5 mL of *lead-free nitric acid R* and diluting to 25.0 mL with *water R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

*Copper may form explosive acetylides with acetylene. Therefore, clean the burner thoroughly before any residues become dry.*

**Lead**: maximum 80 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 1.6 g in 10 mL of *water R*, add 2.5 mL of *lead-free nitric acid R* and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (100 ppm Pb) R*, adding 2.5 mL of *lead-free nitric acid R* and diluting to 25.0 mL with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217.0 nm.

*Atomisation device*: air-acetylene flame.

*Copper may form explosive acetylides with acetylene. Therefore, clean the burner thoroughly before any residues become dry.*

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 250 ± 10 °C.

ASSAY

Dissolve 0.125 g in 50 mL of *water R*. Add 2 mL of *sulfuric acid R* and 3 g of *potassium iodide R*. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R*, added towards the end of the titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 15.96 mg of $CuSO_4$.

STORAGE

In an airtight container.

**01/2008:0894**
**corrected 7.0**

# COPPER SULFATE PENTAHYDRATE

## Cupri sulfas pentahydricus

$CuSO_4,5H_2O$ $M_r$ 249.7
[7758-99-8]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: blue, crystalline powder or transparent, blue crystals.

*Solubility*: freely soluble in water, soluble in methanol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Add several drops of *dilute ammonia R2* to 1 mL of solution S (see Tests). A blue precipitate is formed. On further addition of *dilute ammonia R2* the precipitate dissolves and a dark blue colour is produced.

B. Loss on drying (see Tests).

C. Dilute 1 mL of solution S to 5 mL with *water R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Iron**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 0.5 g in 10 mL of *water R*, add 2.5 mL of *lead-free nitric acid R* and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, adding 2.5 mL of *lead-free nitric acid R* and diluting to 25.0 mL with *water R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

*Copper may form explosive acetylides with acetylene. Therefore, clean the burner thoroughly before any residues become dry.*

**Lead**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.5 g in 10 mL of *water R*, add 2.5 mL of *lead-free nitric acid R* and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (100 ppm Pb) R*, adding 2.5 mL of *lead-free nitric acid R* and diluting to 25.0 mL with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217.0 nm.

*Atomisation device*: air-acetylene flame.

*Copper may form explosive acetylides with acetylene. Therefore, clean the burner thoroughly before any residues become dry.*

**Loss on drying** (*2.2.32*): 35.0 per cent to 36.5 per cent, determined on 0.500 g by drying in an oven at 250 ± 10 °C.

ASSAY

Dissolve 0.200 g in 50 mL of *water R*. Add 2 mL of *sulfuric acid R* and 3 g of *potassium iodide R*. Titrate with *0.1 M sodium thiosulfate*, adding 1 mL of *starch solution R* towards the end of the titration.

1 mL *0.1 M sodium thiosulfate* is equivalent to 24.97 mg of $CuSO_4,5H_2O$.

**01/2008:0321**
**corrected 6.0**

# CORTISONE ACETATE

## Cortisoni acetas

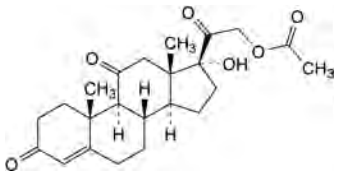

$C_{23}H_{30}O_6$ $M_r$ 402.5
[50-04-4]

DEFINITION

17-Hydroxy-3,11,20-trioxopregn-4-en-21-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in dioxan, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent) and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cortisone acetate CRS.*

If the spectra obtained in the solid state show differences, record new spectra using 50 g/L solutions in *methylene chloride R* in a 0.2 mm cell.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20 mg of *cortisone acetate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *hydrocortisone acetate R* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R.* Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution A). Dilute 2 mL of this solution to 10 mL with *methylene chloride R.*

*Test solution (b).* Transfer 2 mL of solution A to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2.5 h. Allow to cool.

*Reference solution (a).* Dissolve 25 mg of *cortisone acetate CRS* in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution B). Dilute 2 mL of this solution to 10 mL with *methylene chloride R.*

*Reference solution (b).* Transfer 2 mL of solution B to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2.5 h. Allow to cool.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an $R_F$ value distinctly lower than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a faint yellow colour develops. Add this solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

E. About 10 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 211 to + 220 (dried substance).

Dissolve 0.250 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 2 mg of *cortisone acetate CRS* and 2 mg of *hydrocortisone acetate CRS* (impurity A) in *acetonitrile R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: in a 1000 mL volumetric flask mix 400 mL of *acetonitrile R* with 550 mL of *water R* and allow to equilibrate; dilute to 1000 mL with *water R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL; inject *acetonitrile R* as a blank.

*Run time*: twice the retention time of cortisone acetate.

*Retention time*: impurity A = about 10 min; cortisone acetate = about 12 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.2 between the peaks due to impurity A and cortisone acetate; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 237 nm.

Calculate the content of $C_{23}H_{30}O_6$ taking the specific absorbance to be 395.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

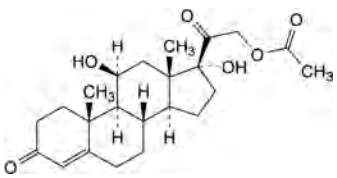


A. 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (hydrocortisone acetate).

01/2008:0036
corrected 7.0

# COTTON, ABSORBENT

## Lanugo gossypii absorbens

DEFINITION

Absorbent cotton consists of new fibres or good quality combers obtained from the seed-coat of various species of the genus *Gossypium* L., cleaned, purified, bleached and carefully carded. It may not contain any compensatory colouring matter.

CHARACTERS

It is white or almost white and is composed of fibres of average length not less than 10 mm, determined by a suitable method, and contains not more than traces of leaf residue, pericarp, seed-coat or other impurities. It offers appreciable resistance when pulled. It does not shed any appreciable quantity of dust when gently shaken.

IDENTIFICATION

A. Examined under a microscope, each fibre is seen to consist of a single cell, up to about 4 cm long and up to 40 µm wide, in the form of a flattened tube with thick and rounded walls and often twisted.

B. When treated with *iodinated zinc chloride solution R*, the fibres become violet.

C. To 0.1 g add 10 mL of *zinc chloride-formic acid solution R*. Heat to 40 °C and allow to stand for 2 h 30 min, shaking occasionally. It does not dissolve.

TESTS

**Solution S**. Place 15.0 g in a suitable vessel, add 150 mL of *water R*, close the vessel and allow to macerate for 2 h. Decant the solution, squeeze the residual liquid carefully from the sample with a glass rod and mix. Reserve 10 mL of the solution for the test for surface-active substances and filter the remainder.

**Acidity or alkalinity**. To 25 mL of solution S add 0.1 mL of *phenolphthalein solution R* and to another 25 mL add 0.05 mL of *methyl orange solution R*. Neither solution is pink.

**Foreign fibres**. Examined under a microscope, it is seen to consist exclusively of typical cotton fibres, except that occasionally a few isolated foreign fibres may be present.

**Fluorescence**. Examine a layer about 5 mm in thickness under ultraviolet light at 365 nm. It displays only a slight brownish-violet fluorescence and a few yellow particles. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibres.

**Neps**. Spread about 1 g evenly between 2 colourless transparent plates each 10 cm square. Examine for neps by transmitted light and compare with *Cotton wool standard for neps CRS*. The product to be examined is not more neppy than the standard.

**Absorbency**

*Apparatus.* A dry cylindrical copper wire basket 8.0 cm high and 5.0 cm in diameter. The wire of which the basket is constructed is about 0.4 mm in diameter, the mesh is 1.5 cm to 2.0 cm wide and the mass of the basket is 2.7 ± 0.3 g.

*Sinking time.* Not more than 10 s. Weigh the basket to the nearest centigram ($m_1$). Take a total of 5.00 g in approximately equal quantities from 5 different places in the product to be examined, place loosely in the basket and weigh the filled basket to the nearest centigram ($m_2$). Fill a beaker 11 cm to 12 cm in diameter to a depth of 10 cm with water at about 20 °C. Hold the basket horizontally and drop it from a height of about 10 mm into the water. Measure with a stopwatch the time taken for the basket to sink below the surface of the water. Calculate the result as the average of 3 tests.

*Water-holding capacity.* Not less than 23.0 g of water per gram. After the sinking time has been measured, remove the basket from the water, allow it to drain for exactly 30 s suspended in a horizontal position over the beaker, transfer it to a tared beaker ($m_3$) and weigh to the nearest centigram ($m_4$). Calculate the water-holding capacity per gram of absorbent cotton using the following expression:

$$\frac{m_4 - (m_2 + m_3)}{m_2 - m_1}$$

Calculate the result as the average of 3 tests.

**Ether-soluble substances**. Not more than 0.50 per cent. In an extraction apparatus, extract 5.00 g with *ether R* for 4 h at a rate of at least 4 extractions per hour. Evaporate the ether extract and dry the residue to constant mass at 100 °C to 105 °C.

**Extractable colouring matter**. In a narrow percolator, slowly extract 10.0 g with *alcohol R* until 50 mL of extract is obtained. The liquid obtained is not more intensely coloured (*2.2.2, Method II*) than reference solution $Y_5$, $GY_6$ or a reference solution prepared as follows: to 3.0 mL of blue primary solution add 7.0 mL of hydrochloric acid (10 g/L HCl). Dilute 0.5 mL of this solution to 10.0 mL with hydrochloric acid (10 g/L HCl).

**Surface-active substances**. Introduce the 10 mL portion of solution S reserved before filtration into a 25 mL graduated ground-glass-stoppered cylinder with an external diameter of 20 mm and a wall thickness of not greater than 1.5 mm, previously rinsed 3 times with *sulfuric acid R* and then with *water R*. Shake vigorously 30 times in 10 s, allow to stand for 1 min and repeat the shaking. After 5 min, any foam present must not cover the entire surface of the liquid.

**Water-soluble substances**. Not more than 0.50 per cent. Boil 5.000 g in 500 mL of *water R* for 30 min, stirring frequently. Replace the water lost by evaporation. Decant the liquid, squeeze the residual liquid carefully from the sample with a glass rod and mix. Filter the liquid whilst hot. Evaporate 400 mL of the filtrate (corresponding to 4/5 of the mass of the sample taken) and dry the residue to constant mass at 100 °C to 105 °C.

**Loss on drying** (*2.2.32*). Not more than 8.0 per cent, determined on 5.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.40 per cent. Introduce 5.00 g into a previously heated and cooled, tared crucible. Heat cautiously over a naked flame and then carefully to dull redness at 600 °C. Allow to cool, add a few drops of *dilute sulfuric acid R*, then heat and incinerate until all the black particles have disappeared. Allow to cool. Add a few drops of *ammonium carbonate solution R*. Evaporate and incinerate carefully, allow to cool and weigh again. Repeat the incineration for periods of 5 min to constant mass.

STORAGE

Store in a dust-proof package in a dry place.

**01/2020:1305**

# COTTONSEED OIL, HYDROGENATED

## Gossypii oleum hydrogenatum

DEFINITION

Product obtained by refining and hydrogenation of oil obtained from seeds of cultivated plants of various varieties of *Gossypium hirsutum* L. or of other species of *Gossypium*. The product consists mainly of triglycerides of palmitic and stearic acids.

CHARACTERS

*Appearance*: white or almost white mass or powder which melts to a clear, pale yellow liquid when heated.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride and in toluene, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

TESTS

**Melting point** (*2.2.14*): 57 °C to 70 °C.

**Acid value** (*2.5.1*): maximum 0.5.

Dissolve 10.0 g in 50 mL of a hot mixture of equal volumes of *ethanol (96 per cent) R* and *toluene R*, previously neutralised with *0.1 M potassium hydroxide* using 0.5 mL of *phenolphthalein solution R1* as indicator. Titrate the solution immediately while still hot.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.0 per cent, determined on 5.0 g.

**Alkaline impurities**. Dissolve with gentle heating 2.0 g in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3 mL of *toluene R*. Add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour to yellow.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*) with the following modifications. Use the mixture of calibrating substances in Table 2.4.22.-3.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 0.65 mL/min.

*Split ratio*: 1:100.

*Temperature*:

- *column*: 180 °C for 35 min;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Composition of the fatty-acid fraction of the substance*:

- *saturated fatty acids of chain length less than $C_{14}$*: maximum 0.2 per cent;
- *myristic acid*: maximum 1.0 per cent;
- *palmitic acid*: 19.0 per cent to 26.0 per cent;
- *stearic acid*: 68.0 per cent to 80.0 per cent;
- *oleic acid and isomers*: maximum 4.0 per cent;
- *linoleic acid and isomers*: maximum 1.0 per cent;

– *arachidic acid*: maximum 1.0 per cent;
– *behenic acid*: maximum 1.0 per cent;
– *lignoceric acid*: maximum 0.5 per cent.

STORAGE

Protected from light.

01/2008:1628

# CRESOL, CRUDE

## Cresolum crudum

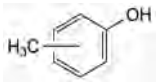


$C_7H_8O$ $M_r$ 108.1

DEFINITION

Mixture of 2-, 3- and 4-methylphenol.

CHARACTERS

*Appearance*: colourless or pale brown liquid.

*Solubility*: sparingly soluble in water, miscible with alcohol and with methylene chloride.

IDENTIFICATION

A. To 0.5 mL add 300 mL of *water R*, mix and filter. To 10 mL of the filtrate add 1 mL of *ferric chloride solution R1*. A blue colour is produced.

B. To 10 mL of the filtrate obtained in identification test A, add 1 mL of *bromine water R*. A pale yellow flocculent precipitate is produced.

C. Relative density (see Tests).

TESTS

**Solution S**. To 2.5 g of the substance to be examined add 50 mL of *water R*, shake for 1 min and filter through a moistened filter.

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is red.

**Relative density** (*2.2.5*): 1.029 to 1.044.

**Distillation range** (*2.2.11*): a maximum of 2.0 per cent *V/V* distils below 188 °C and a minimum of 80 per cent *V/V* distils between 195 °C and 205 °C.

**Sulfur compounds**. Place 20 mL in a small conical flask. Over the mouth of the flask fix a piece of filter paper moistened with *lead acetate solution R*. Heat on a water-bath for 5 min. Not more than a light yellow colour is produced on the filter paper.

**Residue on evaporation**: maximum 0.1 per cent.

Evaporate 2.0 g to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs not more than 2 mg.

STORAGE

Protected from light.

01/2020:0985

# CROSCARMELLOSE SODIUM(9)

## Carmellosum natricum conexum

DEFINITION

Cross-linked sodium carboxymethylcellulose.

Sodium salt of a cross-linked, partly *O*-carboxymethylated cellulose.

♦CHARACTERS

*Appearance*: white or greyish-white, hygroscopic powder.

*Solubility*: practically insoluble in acetone, in anhydrous ethanol and in toluene.♦

IDENTIFICATION

A. Mix 1 g with 100 mL of a solution containing 4 ppm of *methylene blue R*, stir the mixture and allow it to settle. The substance to be examined absorbs the methylene blue and settles as a blue, fibrous mass.

B. Mix 1 g with 50 mL of *water R*. Transfer 1 mL of the mixture to a small test-tube and add 1 mL of *water R* and 0.05 mL of a freshly prepared 40 g/L solution of *α-naphthol R* in *methanol R*. Incline the test-tube and carefully add 2 mL of *sulfuric acid R* down the side so that it forms a lower layer. A reddish-violet colour develops at the interface.

♦C. To the residue obtained in the test for sulfated ash add 1 mL of *hydrochloric acid R* and evaporate on a water-bath. Take up the residue in 20 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).♦

TESTS

**pH** (*2.2.3*): 5.0 to 7.0 for the suspension.

Shake 1 g with 100 mL of *carbon dioxide-free water R* for 5 min.

♦**Sodium chloride and sodium glycolate**: maximum 0.5 per cent (dried substance) for the sum of the percentage contents.

*Sodium chloride*. Place 5.00 g in a 250 mL conical flask, add 50 mL of *water R* and 5 mL of *strong hydrogen peroxide solution R* and heat on a water-bath for 20 min, stirring occasionally to ensure total hydration. Cool and add 100 mL of *water R* and 10 mL of *nitric acid R*. Titrate with *0.05 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*) using a silver indicator electrode and a double-junction reference electrode containing a 100 g/L solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket, and stirring constantly.

1 mL of *0.05 M silver nitrate* is equivalent to 2.922 mg of NaCl.

*Sodium glycolate*. Place a quantity of the substance to be examined equivalent to 0.500 g of the dried substance in a 100 mL beaker. Add 5 mL of *glacial acetic acid R* and 5 mL of *water R* and stir to ensure total hydration (about 15 min). Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Stir for several minutes to ensure complete precipitation of the carboxymethylcellulose. Filter through a fast filter paper impregnated with *acetone R* into a volumetric flask, rinse the beaker and the filter with 30 mL of *acetone R* and dilute the filtrate to 100.0 mL with the same solvent. Allow to stand for 24 h without shaking. Use the clear supernatant to prepare the test solution.

Prepare the reference solutions as follows: in a 100 mL volumetric flask, dissolve 0.100 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*) at room temperature overnight, in *water R* and dilute to 100.0 mL with the same solvent; use the solution within 30 days; transfer 1.0 mL, 2.0 mL, 3.0 mL

(9) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

and 4.0 mL of the solution to separate volumetric flasks, dilute the contents of each flask to 5.0 mL with *water R*, add 5 mL of *glacial acetic acid R*, dilute to 100.0 mL with *acetone R* and mix.

Transfer 2.0 mL of the test solution and 2.0 mL of each of the reference solutions to separate 25 mL volumetric flasks. Heat the uncovered flasks for 20 min on a water-bath to eliminate acetone. Allow to cool and add 5.0 mL of *2,7-dihydroxynaphthalene solution R* to each flask. Mix, add a further 15.0 mL of *2,7-dihydroxynaphthalene solution R* and mix again. Close the flasks with aluminium foil and heat on a water-bath for 20 min. Cool and dilute to 25.0 mL with *sulfuric acid R*.

Measure the absorbance (*2.2.25*) of each solution at 540 nm. Prepare a blank using 2.0 mL of a solution containing 5 per cent *V/V* each of *glacial acetic acid R* and *water R* in *acetone R*. Prepare a standard curve using the absorbances obtained with the reference solutions. From the standard curve and the absorbance of the test solution, determine the mass (*a*) of glycolic acid in the substance to be examined, in milligrams, and calculate the content of sodium glycolate using the following expression:

$$\frac{10 \times 1.29 \times a}{(100 - b)m}$$

1.29 = factor converting glycolic acid to sodium glycolate;

*b* = loss on drying as a percentage;

*m* = mass of the substance to be examined, in grams.

**Water-soluble substances**: maximum 10.0 per cent.

Disperse 10.00 g in 800.0 mL of *water R* and stir for 1 min every 10 min during the first 30 min. Allow to stand for 1 h and centrifuge if necessary. Decant 200.0 mL of the supernatant liquid onto a fast filter paper in a vacuum filtration funnel, apply vacuum and collect 150.0 mL of the filtrate. Evaporate to dryness and dry the residue at 100-105 °C for 4 h. ♦

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): 14.0 per cent to 28.0 per cent (dried substance), determined on 1.0 g.

♦**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*). ♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for croscarmellose sodium used as disintegrant.*

**Settling volume**. Place 75 mL of *water R* in a 100 mL graduated cylinder and add 1.5 g of the substance to be examined in 0.5 g portions, shaking vigorously after each addition. Dilute to 100.0 mL with *water R* and shake again until the substance is homogeneously distributed. Allow to stand for 4 h. The settling volume is between 10.0 mL and 30.0 mL.

**Degree of substitution**: 0.60 to 0.85 (dried substance).

Place 1.000 g in a 500 mL conical flask, add 300 mL of a 100 g/L solution of *sodium chloride R* and 25.0 mL of *0.1 M sodium hydroxide*, stopper the flask and allow to stand for 5 min, shaking occasionally. Add 0.25 mL of *m-cresol purple solution R* and about 15 mL of *0.1 M hydrochloric acid* from a burette. Insert the stopper and shake. If the solution is violet, add *0.1 M hydrochloric acid* in 1 mL portions until the solution becomes yellow, shaking after each addition. Titrate with *0.1 M sodium hydroxide* until the colour turns to violet.

Calculate the number of milliequivalents (*M*) of base required to neutralise the equivalent of 1 g of dried substance.

Calculate the degree of acid carboxymethyl substitution (*A*) using the following expression:

$$\frac{1150M}{(7102 - 412M - 80C)}$$

*C* = sulfated ash as a percentage.

Calculate the degree of sodium carboxymethyl substitution (*S*) using the following expression:

$$\frac{(162 + 58A)C}{(7102 - 80C)}$$

The degree of substitution is the sum of *A* and *S*.

**Particle size distribution** (*2.9.31* or *2.9.38*).

**Hausner ratio** (*2.9.36*).

**01/2017:0892**
**corrected 10.0**

# CROSPOVIDONE(10)

## Crospovidonum

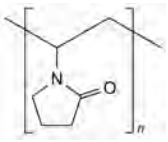

$(C_6H_9NO)_n$ $M_r$ $(111.1)_n$

[9003-39-8]

DEFINITION

Cross-linked homopolymer of 1-ethenylpyrrolidin-2-one.

*Content*: 11.0 per cent to 12.8 per cent of N ($A_r$ 14.01) (dried substance).

2 types of crospovidone are available, depending on the particle size: type A and type B.

♦CHARACTERS

*Appearance*: hygroscopic, white or yellowish-white powder or flakes.

*Solubility*: practically insoluble in water, in ethanol 96 per cent and in methylene chloride. ♦

IDENTIFICATION

♦A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *crospovidone CRS*. ♦

(10) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

B. Suspend 1 g in 10 mL of *water R*, add 0.1 mL of *0.05 M iodine* and shake for 30 s. Add 1 mL of *starch solution R* and shake. No blue colour develops within 30 s.

C. To 10 mL of *water R*, add 0.1 g and shake. A suspension is formed and no clear solution is obtained within 15 min.

D. *The analytical sieves must be clean and dry. For this purpose the sieves are washed in hot water and allowed to dry overnight in a drying cabinet at 105 °C.*

Place 20 g (dried substance) in a 1000 mL conical flask, add 500 mL of *water R* and shake the suspension for 30 min. Pour the suspension through a 63 µm analytical sieve, previously tared, and rinse the sieve with *water R* until the filtrate is clear. Dry the sieve and sample residue at 105 °C for 5 h in a drying cabinet without circulating air. Cool in a desiccator for 30 min and weigh.

Calculate the percentage sieving residue (fraction of sample particles having a diameter of more than 63 µm), using the following expression:

$$\frac{m_1 - m_3}{m_2} \times 100$$

$m_1$ = mass of the sieve and sample residue, after drying for 5 h, in grams;

$m_2$ = initial mass of the sample, in grams;

$m_3$ = mass of the sieve, in grams.

If the sieving residue fraction is more than 15 per cent, the substance is classified as type A; if the sieving residue fraction is less than or equal to 15 per cent, the substance is classified as type B.

TESTS

**Peroxides**. Type A: maximum 400 ppm expressed as $H_2O_2$; type B: maximum 1000 ppm expressed as $H_2O_2$.

Suspend 2.0 g in 50 mL of *water R*. To 25 mL of this suspension add 2 mL of *titanium trichloride-sulfuric acid reagent R*. Allow to stand for 30 min and filter. The absorbance (*2.2.25*) of the filtrate, measured at 405 nm using a mixture of 25 mL of a filtered 40 g/L suspension of the substance to be examined and 2 mL of a 13 per cent *V/V* solution of *sulfuric acid R* as the compensation liquid, has a maximum of 0.35.

For type B use 10 mL of the suspension and dilute to 25 mL with *water R* for the test.

**Water-soluble substances**: maximum 1.5 per cent.

Place 25.0 g in a 400 mL beaker, add 200 mL of *water R* and stir for 1 h using a magnetic stirrer. Transfer the suspension to a 250.0 mL volumetric flask, rinsing with *water R*, and dilute to volume with the same solvent. Allow the bulk of the solids to settle. Filter about 100 mL of the almost clear supernatant through a membrane filter (nominal pore size 0.45 µm), protected by superimposing a membrane filter (nominal pore size 3 µm). While filtering, stir the liquid above the membrane filter manually or by means of a mechanical stirrer, taking care not to damage the membrane filter. Transfer 50.0 mL of the clear filtrate to a tared 100 mL beaker, evaporate to dryness and dry at 105-110 °C for 3 h. The residue weighs a maximum of 75 mg.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution*. Suspend 1.250 g in 50.0 mL of *methanol R* and shake for 60 min. Leave the bulk to settle and filter through a membrane filter (nominal pore size 0.2 µm).

*Reference solution (a)*. Dissolve 50 mg of *1-vinylpyrrolidin-2-one R* (impurity A) in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *1-vinylpyrrolidin-2-one R* (impurity A) and 0.50 g of *vinyl acetate R* in *methanol R* and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 100 mL with the mobile phase.

*Precolumn*:

- *size*: $l$ = 0.025 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: $l$ = 0.25 m, Ø = 4 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile for chromatography R*, *water for chromatography R* (10:90 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 50 µL. After each injection of the test solution, wash the precolumn by passing the mobile phase backwards, at the same flow rate as applied in the test, for 30 min.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity A and vinyl acetate in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections of reference solution (a).

*Calculation of percentage content*:

- for impurity A, use the concentration of impurity A in reference solution (a).

*Limit*:

- *impurity A*: maximum 10 ppm.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Place 0.100 g of the substance to be examined ($m$ mg) in a combustion flask and add 5 g of a mixture of 1 g of *copper sulfate pentahydrate R*, 1 g of *titanium dioxide R* and 33 g of *dipotassium sulfate R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with a small quantity of *water R*. Add 7 mL of *sulfuric acid R*, allowing it to run down the inside wall of the flask. Gradually heat the flask until the solution has a clear, yellowish-green colour, and the inside wall of the flask is free from carbonised material, and then heat for a further 45 min. After cooling, cautiously add 20 mL of *water R*, and connect the flask to the distillation apparatus, which has been previously washed by passing steam through it. To the absorption flask add 30 mL of a 40 g/L solution of *boric acid R*, 0.15 mL of *bromocresol green-methyl red solution R* and sufficient *water R* to immerse the lower end of the condenser tube. Add 30 mL of *strong sodium hydroxide solution R* through a funnel, cautiously rinse the funnel with 10 mL of *water R*, immediately close the clamp attached to the rubber tube, then start the distillation with steam to obtain 80-100 mL of distillate. Remove the absorption flask from the lower end of the condenser tube, rinsing the end part with a small quantity of *water R*, and titrate the distillate with *0.025 M sulfuric acid* until the colour of the solution changes from green through pale greyish-blue to pale greyish red-purple. Carry out a blank determination and make any necessary correction.

1 mL of *0.025 M sulfuric acid* is equivalent to 0.700 mg of N.

STORAGE

In an airtight container.

♦LABELLING

The label states the type of crospovidone (type A or type B).♦

IMPURITIES

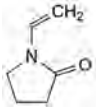

A. 1-ethenylpyrrolidin-2-one (1-vinylpyrrolidin-2-one).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for crospovidone used as disintegrant.*

**Hydration capacity**. Introduce 2.0 g into a 100 mL centrifuge tube and add 40 mL of *water R*. Shake vigorously until a suspension is obtained. Shake again 5 min and 10 min later, then centrifuge for 15 min at 750 *g*. Decant the supernatant and weigh the residue. The hydration capacity is the ratio of the mass of the residue to the initial mass of the sample. It is typically 3 to 9.

**Particle-size distribution** (*2.9.31*).

**Powder flow** (*2.9.36*).

*The following characteristic may be relevant for crospovidone used as suspension stabiliser.*

**Settling volume**. Introduce 10 g into a 100 mL graduated cylinder and add 90 mL of *water R*. Shake vigorously. Dilute to 100 mL with *water R*, washing the powder residues from the walls of the cylinder. Allow to stand for 24 h, then read the volume of the sediment. It is typically greater than 60 mL.

**07/2010:1194**

# CROTAMITON

## Crotamitonum

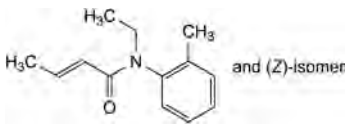


$C_{13}H_{17}NO$ $M_r$ 203.3

[483-63-6]

DEFINITION

*N*-Ethyl-*N*-(2-methylphenyl)but-2-enamide.

*Content*:

– *sum of the (E)- and (Z)-isomers*: 96.0 per cent to 102.0 per cent;

– *(Z)-isomer*: maximum 15.0 per cent.

CHARACTERS

*Appearance*: colourless or pale yellow, oily liquid.

*Solubility*: slightly soluble in water, miscible with ethanol (96 per cent).

At low temperatures it may partly or completely solidify.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in *cyclohexane R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *cyclohexane R*.

*Spectral range*: 220-300 nm.

*Absorption maximum*: at 242 nm.

*Specific absorbance at the absorption maximum*: 300 to 330.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *crotamiton CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *anhydrous ethanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 25 mg of *crotamiton CRS* in *anhydrous ethanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: shake 98 volumes of *methylene chloride R* with 2 volumes of *concentrated ammonia R*, dry over *anhydrous sodium sulfate R*, filter and mix 97 volumes of the filtrate with 3 volumes of *2-propanol R*.

*Application*: 5 µL.

*Development*: over a 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 10 mL of a saturated solution add a few drops of a 3 g/L solution of *potassium permanganate R*. A brown colour is produced and a brown precipitate is formed on standing.

TESTS

**Relative density** (*2.2.5*): 1.006 to 1.011.

**Refractive index** (*2.2.6*): 1.540 to 1.542.

**Free amines**: maximum 500 ppm, expressed as ethylaminotoluene.

Dissolve 5.00 g in 16 mL of *methylene chloride R* and add 4.0 mL of *glacial acetic acid R*. Add 0.1 mL of *metanil yellow solution R* and 1.0 mL of *0.02 M perchloric acid*. The solution is red-violet.

**Chlorides**: maximum 100 ppm.

Boil 5.0 g under a reflux condenser for 1 h with 25 mL of *ethanol (96 per cent) R* and 5 mL of a 200 g/L solution of *sodium hydroxide R*. Cool, add 5 mL of *water R* and shake with 25 mL of *ether R*. Dilute the lower layer to 20 mL with *water R*; add 5 mL of *nitric acid R*, dilute to 50 mL with *water R* and add 1 mL of a freshly prepared 50 g/L solution of *silver nitrate R*. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of a freshly prepared 50 g/L solution of *silver nitrate R* and a solution prepared by diluting 5 mL of a 200 g/L solution of *sodium hydroxide R* to 20 mL with *water R* and adding 1.5 mL of *0.01 M hydrochloric acid*, 5 mL of *nitric acid R* and diluting to 50 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *crotamiton CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 15.0 mg of *crotamiton impurity A CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 15 mg of *crotamiton impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with test solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *tetrahydrofuran R*, *cyclohexane R* (8:92 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Run time*: 2.5 times the retention time of the (*E*)-isomer.

*Relative retention* with reference to the (*E*)-isomer: (*Z*)-isomer = about 0.5; impurity A = about 0.8.

*System suitability*: reference solution (d):

– *resolution*: minimum 4.5 between the peaks due to impurity A and the (*E*)-isomer.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
– *unspecified impurities*: for each impurity, not more than 0.1 times the sum of the areas of the peaks due to the (*Z*)- and (*E*)- isomers in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *sum of impurities other than A*: not more than the sum of the areas of the peaks due to the (*Z*)- and (*E*)-isomers in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *disregard limit*: 0.02 times the sum of the areas of the peaks due to the (*Z*)- and (*E*)-isomers in the chromatogram obtained with reference solution (c) (0.02 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{13}H_{17}NO$ from the sum of the areas of the peaks due to the (*Z*)- and (*E*)-isomers in the chromatograms obtained. Calculate the content of the (*Z*)-isomer, as a percentage of the total content of the (*E*)- and (*Z*)-isomers, from the chromatogram obtained with test solution (b).

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

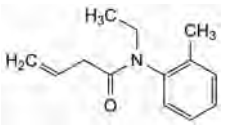

A. *N*-ethyl-*N*-(2-methylphenyl)but-3-enamide.

**07/2014:0547**

# CYANOCOBALAMIN

## Cyanocobalaminum

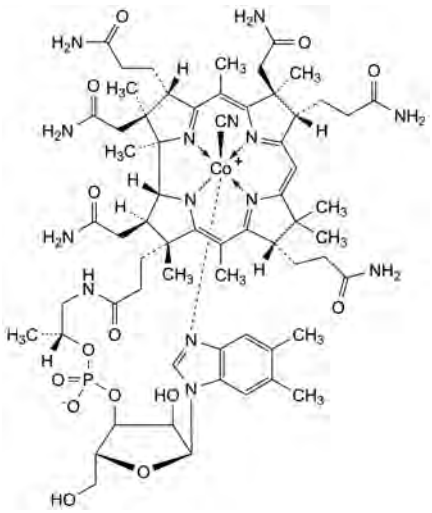

$C_{63}H_{88}CoN_{14}O_{14}P$ $M_r$ 1355

[68-19-9]

DEFINITION

α-(5,6-Dimethylbenzimidazol-1-yl)cobamide cyanide.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

This monograph applies to cyanocobalamin produced by fermentation.

CHARACTERS

*Appearance*: dark red, crystalline powder or dark red crystals.

*Solubility*: sparingly soluble in water and in ethanol (96 per cent), practically insoluble in acetone.

The anhydrous substance is very hygroscopic.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 2.5 mg in *water R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 260-610 nm.

*Absorption maxima*: at 278 nm, 361 nm and from 547 nm to 559 nm.

*Absorbance ratio*:

– $A_{361}$ / $A_{547\text{-}559}$ = 3.15 to 3.45;
– $A_{361}$ / $A_{278}$ = 1.70 to 1.90.

B. Thin-layer chromatography (*2.2.27*). *Carry out the test protected from light.*

*Test solution.* Dissolve 2 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Reference solution.* Dissolve 2 mg of *cyanocobalamin CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *dilute ammonia R1*, *methanol R*, *methylene chloride R* (9:30:45 *V/V/V*).

*Application*: 10 µL.

*Development*: in an unsaturated tank, over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase. *Use within 1 h.*

*Reference solution (a).* Dilute 3.0 mL of the test solution to 100.0 mL with the mobile phase. *Use within 1 h.*

*Reference solution (b).* Dilute 5.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase. *Use within 1 h.*

*Reference solution (c).* Dissolve 25 mg of the substance to be examined in 10 mL of *water R*, warming if necessary. Allow to cool and add 5 mL of a 1.0 g/L solution of *chloramine R* and 0.5 mL of *0.05 M hydrochloric acid*, then dilute to 25 mL with *water R*. Shake and allow to stand for 5 min. Dilute 1 mL of this solution to 10 mL with the mobile phase and inject immediately.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 26.5 volumes of *methanol R* and 73.5 volumes of a 10 g/L solution of *disodium hydrogen phosphate dodecahydrate R* adjusted to pH 3.5 with *phosphoric acid R* and use within 2 days.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 361 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of cyanocobalamin.

*System suitability*:

- the chromatogram obtained with reference solution (c) shows 2 principal peaks;
- *resolution*: minimum 2.5 between the 2 principal peaks in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (3 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 40.00 mg by drying *in vacuo* at 105 °C for 2 h.

ASSAY

Dissolve 100.0 mg in *water R* and dilute to 500.0 mL with the same solvent. Dilute 25.0 mL of the solution to 200.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 361 nm.

Calculate the content of $C_{63}H_{88}CoN_{14}O_{14}P$ taking the specific absorbance to be 207.

STORAGE

In an airtight container, protected from light.

**07/2008:1092**
**corrected 10.0**

# CYCLIZINE HYDROCHLORIDE

## Cyclizini hydrochloridum

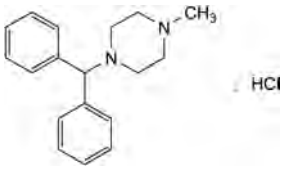


$C_{18}H_{23}ClN_2$ $M_r$ 302.8
[305-25-3]

DEFINITION

1-(Diphenylmethyl)-4-methylpiperazine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 20.0 mg in a 5 g/L solution of *sulfuric acid R* and dilute to 100.0 mL with the same acid solution.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with a 5 g/L solution of *sulfuric acid R*.

*Spectral range*: 240-350 nm for test solution (a); 210-240 nm for test solution (b).

*Resolution* (*2.2.25*): minimum 1.7.

*Absorption maxima*: at 258 nm and 262 nm for test solution (a); at 225 nm for test solution (b).

*Absorbance ratio*: $A_{262}/A_{258}$ = 1.0 to 1.1.

*Specific absorbance at the absorption maximum at 225 nm*: 370 to 410 for test solution (b).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cyclizine hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *cyclizine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (2:13:85 *V/V/V*).

*Application*: 20 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 30 min.

*Detection*: expose to iodine vapour for 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.5 g in 10 mL of *ethanol (60 per cent V/V) R*, heating if necessary. Cool in iced water. Add 1 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R*. Filter, wash the precipitate with *water R* and dry at 60 °C at a pressure not exceeding 0.7 kPa for 2 h. The melting point (*2.2.14*) is 105 °C to 108 °C.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.5 to 5.5.

Dissolve 0.5 g in a mixture of 40 volumes of *ethanol (96 per cent) R* and 60 volumes of *carbon dioxide-free water R* and dilute to 25 mL with the same mixture of solvents.

**Related substances**. Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.250 g of the substance to be examined in 4.0 mL of *methanol R* and dilute to 5.0 mL with *1 M sodium hydroxide*.

*Reference solution (a)*. Dissolve 25 mg of the substance to be examined in 10.0 mL of *methanol R*. Dilute 1.0 mL of this solution to 50.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined, 5.0 mg of *cyclizine impurity A CRS* and 5.0 mg of *cyclizine impurity B CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.33 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.50 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:25.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 100 → 240 |
| | 14 - 16 | 240 → 270 |
| | 16 - 30 | 270 |
| Injection port | | 250 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to cyclizine (retention time = about 15 min): impurity A = about 0.2; impurity B = about 0.7.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 50, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to methanol.

*Limits*:

- *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.120 g in 15 mL of *anhydrous formic acid R* and add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 15.14 mg of $C_{18}H_{23}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

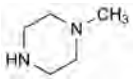

A. 1-methylpiperazine,

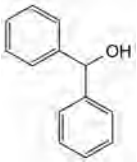

B. diphenylmethanol (benzhydrol).

**04/2009:1093**

# CYCLOPENTOLATE HYDROCHLORIDE

## Cyclopentolati hydrochloridum

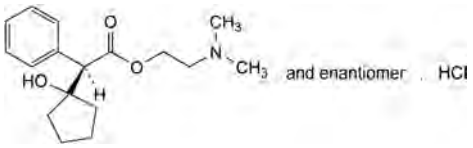


$C_{17}H_{26}ClNO_3$ $M_r$ 327.8

[5870-29-1]

DEFINITION

2-(Dimethylamino)ethyl (2*RS*)-(1-hydroxycyclopentyl)-(phenyl)acetate hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 135 °C to 141 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium chloride R*.

*Comparison*: *cyclopentolate hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in 5 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 10 mg of *cyclopentolate hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *butyl acetate R*, *2-propanol R* (5:15:30:50 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 30 min; examine in ultraviolet light at 365 nm.

*Result*: the principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.5 to 5.5.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 10 mg of *cyclopentolate for system suitability CRS* (containing impurity C) in *water R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped hexylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.66 g of *ammonium phosphate R* in *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*; mix and filter; mix 55 volumes of this solution and 45 volumes of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µl.

*Run time*: 2.5 times the retention time of cyclopentolate.

*Identification of impurities*: use the chromatogram supplied with *cyclopentolate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to cyclopentolate (retention time = about 4 min): impurity C = about 0.9.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 6, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to cyclopentolate.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2.0;
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 1.0 mL of *0.1 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.79 mg of $C_{17}H_{26}ClNO_3$.

IMPURITIES

*Specified impurities*: *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*, *B*.

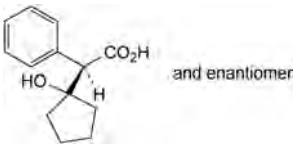


A. (2*RS*)-(1-hydroxycyclopentyl)(phenyl)acetic acid,

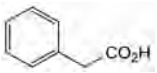


B. phenylacetic acid,

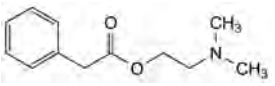


C. 2-(dimethylamino)ethyl phenylacetate.

01/2017:0711

# CYCLOPHOSPHAMIDE

## Cyclophosphamidum

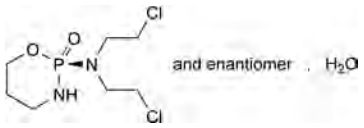


$C_7H_{15}Cl_2N_2O_2P,H_2O$ $M_r$ 279.1
[6055-19-2]

DEFINITION

Cyclophosphamide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*RS*)-*N*,*N*-bis(2-chloroethyl)tetrahydro-2*H*-1,3,2-oxazaphosphorin-2-amine 2-oxide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, soluble in water, freely soluble in alcohol.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *cyclophosphamide CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 51 °C) is not greater than 2 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *cyclophosphamide CRS*.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.1 g in 10 mL of *water R* and add 5 mL of *silver nitrate solution R1*; the solution remains clear. Boil, a white precipitate is formed which dissolves in *concentrated ammonia R* and is reprecipitated on the addition of *dilute nitric acid R*.

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 4.0 to 6.0, determined immediately after preparation of the solution.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *alcohol R* and dilute to 5 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *alcohol R*.

*Reference solution (a)*. Dissolve 10 mg of *cyclophosphamide CRS* in *alcohol R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dilute 0.1 mL of test solution (a) to 10 mL with *alcohol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *anhydrous formic acid R*, 4 volumes of *acetone R*, 12 volumes of *water R* and 80 volumes of *methyl ethyl ketone R*. Dry the plate in a current of warm air and heat at 110 °C for 10 min. At the bottom of a chromatographic tank, place an evaporating dish containing a 50 g/L solution of *potassium permanganate R* and add an equal volume of *hydrochloric acid R*. Place the plate whilst still hot in the tank and close the tank. Leave the plate in contact with the chlorine gas for 2 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of coating below the points of application gives at most a very faint blue colour with a drop of *potassium iodide and starch solution R*. Avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* and allow to stand for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent). Disregard any spot remaining at the point of application.

**Chlorides** (*2.4.4*). Dissolve 0.15 g in *water R* and dilute to 15 mL with the same solvent. The freshly prepared solution complies with the limit test for chlorides (330 ppm).

**Phosphates** (*2.4.11*). Dissolve 0.10 g in *water R* and dilute to 100 mL with the same solvent. The solution complies with the limit test for phosphates (100 ppm).

**Water** (*2.5.12*): 6.0 per cent to 7.0 per cent, determined on 0.300 g by the semi-micro determination of water.

ASSAY

Dissolve 0.100 g in 50 mL of a 1 g/L solution of *sodium hydroxide R* in *ethylene glycol R* and boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 25 mL of *water R*. Add 75 mL of *2-propanol R*, 15 mL of *dilute nitric acid R*, 10.0 mL of *0.1 M silver nitrate* and 2.0 mL of *ferric ammonium sulfate solution R2* and titrate with *0.1 M ammonium thiocyanate*.

1 mL of *0.1 M silver nitrate* is equivalent to 13.05 mg of $C_7H_{15}Cl_2N_2O_2P$.

07/2009:0817

# CYPROHEPTADINE HYDROCHLORIDE

## Cyproheptadini hydrochloridum

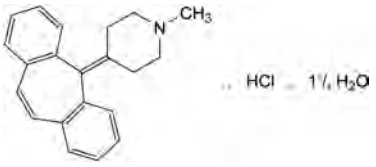


$C_{21}H_{22}ClN,1\frac{1}{2}H_2O$ $M_r$ 350.9
[41354-29-4]

DEFINITION

4-(5*H*-Dibenzo[*a*,*d*][7]annulen-5-ylidene)-1-methylpiperidine hydrochloride sesquihydrate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cyproheptadine hydrochloride CRS*.

B. A saturated solution gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Acidity**. Dissolve 0.10 g in *water R* and dilute to 25 mL with the same solvent. Add 0.1 mL of *methyl red solution R*. Not more than 0.15 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2.0 mg of *dibenzocycloheptene CRS* (impurity A), 2.0 mg of *dibenzosuberone CRS* (impurity B) and 2.0 mg of *cyproheptadine impurity C CRS* in mobile phase A, add 1.0 mL of the test solution and dilute to 100.0 mL with mobile phase A.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 6.12 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 4.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*; mix 60 volumes of this solution and 40 volumes of *acetonitrile for chromatography R*;
- *mobile phase B*: dissolve 6.12 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 4.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*; mix 40 volumes of this solution and 60 volumes of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10.0 | 100 | 0 |
| 10.0 - 10.1 | 100 → 0 | 0 → 100 |
| 10.1 - 35 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to cyproheptadine (retention time = about 8 min): impurity C = about 0.7; impurity B = about 2.6; impurity A = about 3.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 7.0 between the peaks due to impurity C and cyproheptadine.

*Limits*:
- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 7.0 per cent to 9.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.39 mg of $C_{21}H_{22}ClN$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

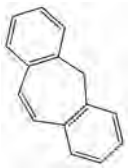

A. 5*H*-dibenzo[*a,d*][7]annulene (dibenzocycloheptene),

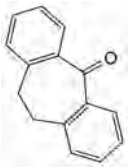


B. 10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-one (dibenzosuberone),

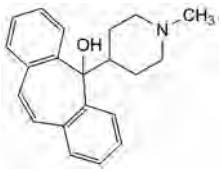


C. 5-(1-methylpiperidin-4-yl)-5*H*-dibenzo[*a,d*][7]annulen-5-ol.

**04/2012:1094**

# CYPROTERONE ACETATE

## Cyproteroni acetas

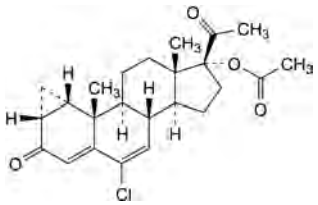


$C_{24}H_{29}ClO_4$ $M_r$ 416.9
[427-51-0]

DEFINITION

6-Chloro-3,20-dioxo-1β,2β-dihydro-3′*H*-cyclopropa-[1,2]pregna-1,4,6-trien-17-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in methanol, sparingly soluble in anhydrous ethanol.

mp: about 210 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
   *Comparison*: *cyproterone acetate CRS*.

B. Thin-layer chromatography (*2.2.27*).
   *Test solution.* Dissolve 20 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.
   *Reference solution.* Dissolve 10 mg of *cyproterone acetate CRS* in *methylene chloride R* and dilute to 5 mL with the same solvent.
   *Plate*: *TLC silica gel $F_{254}$ plate R*.
   *Mobile phase*: *cyclohexane R*, *ethyl acetate R* (50:50 *V/V*).
   *Application*: 5 μL.
   *Development*: twice over 3/4 of the plate; dry in air between the 2 developments.
   *Drying*: in air.
   *Detection*: examine in ultraviolet light at 254 nm.
   *Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 1 mg add 2 mL of *sulfuric acid R* and heat on a water-bath for 2 min. A red colour develops. Cool. Add this solution cautiously to 4 mL of *water R* and shake. The solution becomes violet.

D. Incinerate about 30 mg with 0.3 g of *anhydrous sodium carbonate R* over a naked flame for about 10 min. Cool and dissolve the residue in 5 mL of *dilute nitric acid R*. Filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

E. It gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 152 to + 157 (dried substance).

Dissolve 0.25 g in *acetone R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve the contents of a vial of *cyproterone impurity mixture CRS* (impurities F and I) in 1.0 mL of the test solution.

*Reference solution (c).* Dissolve 2 mg of *cyproterone acetate for peak identification CRS* (containing impurities B, C, E and G) in 2.0 mL of *acetonitrile R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 μm).

*Mobile phase*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 μL.

*Run time*: twice the retention time of cyproterone acetate.

*Identification of impurities*: use the chromatogram supplied with *cyproterone impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities F and I; use the chromatogram supplied with *cyproterone acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C, E and G.

*Relative retention* with reference to cyproterone acetate (retention time = about 22 min): impurity E = about 0.27; impurity G = about 0.3; impurity F = about 0.5; impurity B = about 0.7; impurity I = about 0.9; impurity C = about 1.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity I and cyproterone acetate.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.8; impurity E = 0.7;
- *impurity F*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity E*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities B, C, G*: for each impurity, not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 282 nm.

Calculate the content of $C_{24}H_{29}ClO_4$ taking the specific absorbance to be 414.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use*

*(2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, H, I, J.*

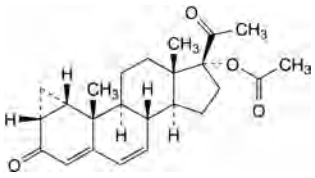

A. 3,20-dioxo-1β,2β-dihydro-3′*H*-cyclopropa-[1,2]pregna-1,4,6-trien-17-yl acetate,

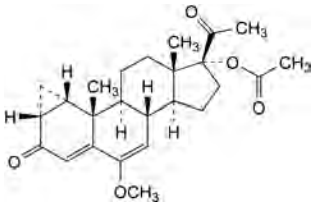

B. 6-methoxy-3,20-dioxo-1β,2β-dihydro-3′*H*-cyclopropa[1,2]pregna-1,4,6-trien-17-yl acetate,

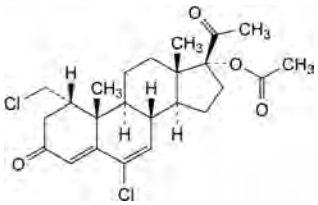

C. 6-chloro-1α-(chloromethyl)-3,20-dioxopregna-4,6-dien-17-yl acetate,

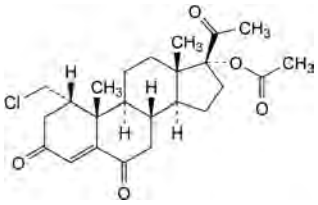

D. 1α-(chloromethyl)-3,6,20-trioxopregn-4-en-17-yl acetate,

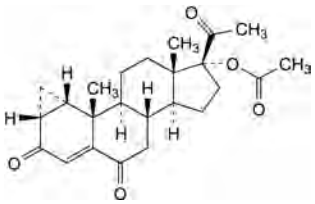

E. 3,6,20-trioxo-1β,2β-dihydro-3′*H*-cyclopropa[1,2]pregna-1,4-dien-17-yl acetate,

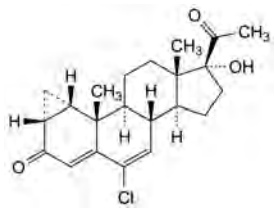

F. 6-chloro-17-hydroxy-1β,2β-dihydro-3′*H*-cyclopropa[1,2]-pregna-1,4,6-triene-3,20-dione,

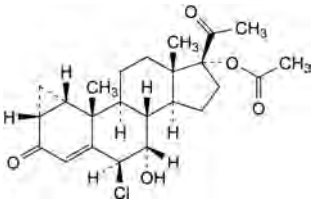

G. 6β-chloro-7α-hydroxy-3,20-dioxo-1β,2β-dihydro-3′*H*-cyclopropa[1,2]pregna-1,4-dien-17-yl acetate,

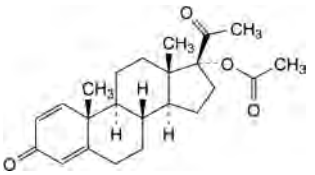

H. 3,20-dioxopregna-1,4-dien-17-yl acetate,

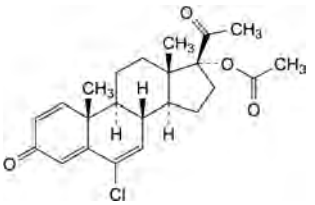

I. 6-chloro-3,20-dioxopregna-1,4,6-trien-17-yl acetate (delmadinone acetate),

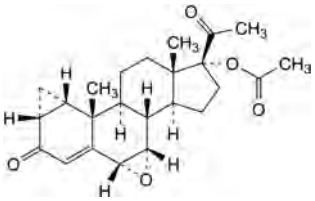

J. 6α,7α-epoxy-3,20-dioxo-1β,2β-dihydro-3′*H*-cyclopropa[1,2]pregna-1,4-dien-17-yl acetate.

**01/2017:0895**
**corrected 10.0**

# CYSTEINE HYDROCHLORIDE MONOHYDRATE

## Cysteini hydrochloridum monohydricum

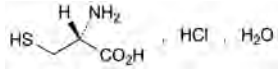

$C_3H_8ClNO_2S,H_2O$ $M_r$ 175.6
[7048-04-6]

DEFINITION

(2*R*)-2-Amino-3-sulfanylpropanoic acid hydrochloride monohydrate.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification*: *A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cysteine hydrochloride monohydrate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent. Add 10 mL of a 40 g/L solution of *N-ethylmaleimide R* in *ethanol (96 per cent) R*. Allow to stand for 5 min. Dilute 2 mL of the solution to 10 mL with *water R*.

*Reference solution*. Dissolve 20 mg of *cysteine hydrochloride monohydrate CRS* in *water R* and dilute to 10 mL with the same solvent. Add 10 mL of a 40 g/L solution of *N-ethylmaleimide R* in *ethanol (96 per cent) R*. Allow to stand for 5 min. Dilute 2 mL of the solution to 10 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 80 °C for 30 min.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in 1 mL of *dilute sodium hydroxide solution R*. Add 1 mL of a 30 g/L solution of *sodium nitroprusside R*. An intense violet colour develops which becomes brownish-red and then orange. Add 1 mL of *hydrochloric acid R*. The solution becomes green.

E. Dissolve about 50 mg in 5 mL of *water R*. Heat to about 60 °C on a water-bath and carefully add, dropwise, 5 mL of *strong hydrogen peroxide solution R*. Heat the water-bath to boiling and maintain the sample on the water-bath for 1 h. After cooling to room temperature reconstitute the sample to 10 mL with *water R*. 2 mL of the solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dilute 10 mL of solution S to 20 mL with *water R*.

**Specific optical rotation** (*2.2.7*): + 5.5 to + 7.0 (dried substance).

Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1. *Prepare the solutions immediately before use.*

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *L-cystine R* (impurity A) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (d)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (e)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50 mL with solution A. Dilute 1 mL of the solution to 200 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (e):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for impurity A, use the concentration of impurity A in reference solution (b);

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of cysteine hydrochloride monohydrate in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (c); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *impurity A at 570 nm*: maximum 0.5 per cent;

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (d) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 20 ppm.

In a separating funnel, dissolve 0.50 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): 8.0 per cent to 12.0 per cent, determined on 1.000 g by drying at a pressure not exceeding 0.7 kPa for 24 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a ground-glass stoppered flask dissolve 0.300 g of the substance to be examined and 4 g of *potassium iodide R* in 20 mL of *water R*. Cool the solution in iced water and add 3 mL of *hydrochloric acid R1* and 25.0 mL of *0.05 M iodine*. Stopper the flask and allow to stand in the dark for 20 min. Titrate with *0.1 M sodium thiosulfate* using 3 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 15.76 mg of $C_3H_8ClNO_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

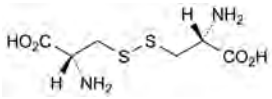

A. (2*R*,2′*R*)-3,3′-disulfanediylbis(2-aminopropanoic acid) (cystine),

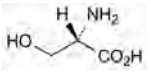

B. (2*S*)-2-amino-3-hydroxypropanoic acid (serine).

**01/2019:0998**

# CYSTINE

## Cystinum

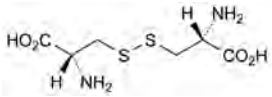

$C_6H_{12}N_2O_4S_2$ $M_r$ 240.3

[56-89-3]

DEFINITION

L-Cystine (3,3′-disulfanediylbis[(2*R*)-2-aminopropanoic acid]).

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *cystine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 1 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with *water R.*
*Reference solution.* Dissolve 10 mg of *cystine CRS* in 1 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with *water R.*
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g carefully add 1 mL of *strong hydrogen peroxide solution R* and 0.1 mL of *ferric chloride solution R1*. Allow to cool. Add 1 mL of *dilute hydrochloric acid R* and 5 mL of *water R*. Add 1 mL of *barium chloride solution R1*. Turbidity or a white precipitate develops within 3 min.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 10 mL with the same acid.

**Specific optical rotation** (*2.2.7*): − 224 to − 218 (dried substance).

Dissolve 0.50 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: a 10.3 g/L solution of *hydrochloric acid R* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions (a), (b) and (d) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of cystine in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides**: maximum 200 ppm.

Dissolve 0.5 g in 5 mL of *dilute nitric acid R* and dilute to 10 mL with the same solvent. Add 10 mL of *strong hydrogen peroxide solution R* and heat on a water-bath for 30 min. Cool and dilute to 50 mL with *water R*. Add 1 mL of *silver nitrate solution R2* and mix. Allow to stand for 5 min protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 mL of *chloride standard solution (50 ppm Cl) R*. Examine the tubes laterally against a black background.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in 5 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a flask with a ground-glass stopper, dissolve 0.100 g in a mixture of 2 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R*. Add 10 mL of a 200 g/L solution of *potassium bromide R*, 50.0 mL of *0.0167 M potassium bromate* and 15 mL of *dilute hydrochloric acid R*. Stopper the flask and cool in iced water. Allow to stand in the dark for 10 min. Add 1.5 g of *potassium iodide R*. After 1 min, titrate with *0.1 M sodium thiosulfate*, using 2 mL of *starch solution R*, added towards the end-point, as indicator. Carry out a blank titration.

1 mL of *0.0167 M potassium bromate* is equivalent to 2.403 mg of $C_6H_{12}N_2O_4S_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

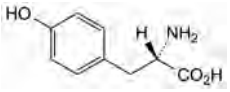


A. L-tyrosine ((2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid).

**01/2019:0760**
**corrected 10.0**

# CYTARABINE

## Cytarabinum

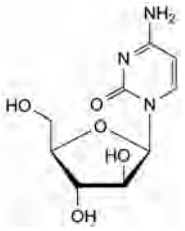


$C_9H_{13}N_3O_5$ $M_r$ 243.2
[147-94-4]

DEFINITION

4-Amino-1-β-D-arabinofuranosylpyrimidin-2(1*H*)-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

mp: about 215 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *cytarabine CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 154 to + 160 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 1.38 g of *sodium dihydrogen phosphate monohydrate R* and 1.42 g of *anhydrous disodium hydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 7 with a 4 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *uracil arabinoside CRS* (impurity A) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 0.5 mL of the solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 2 mg of *uridine R* (impurity B) and 2.5 mg of the substance to be examined in *water R* and dilute to 100 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, buffer solution (2:98 *V/V*);
- *mobile phase B*: *methanol R*, buffer solution (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to cytarabine (retention time = about 9 min): impurity B = about 1.2; impurity A = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to cytarabine and impurity B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of impurity A in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.250 g by drying *in vacuo* at 60 °C at a pressure of 0.2-0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 mL of *anhydrous acetic acid R*, warming if necessary. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.32 mg of $C_9H_{13}N_3O_5$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I.*

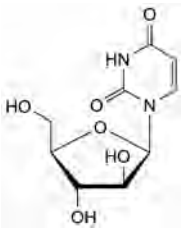

A. 1-β-D-arabinofuranosylpyrimidine-2,4(1*H*,3*H*)-dione (uracil arabinoside),

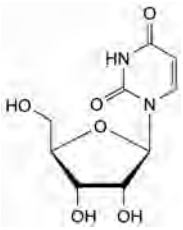

B. 1-β-D-ribofuranosylpyrimidine-2,4(1*H*,3*H*)-dione (uridine),

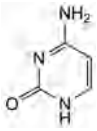

C. 4-aminopyrimidin-2(1*H*)-one (cytosine),

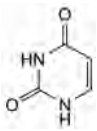

D. pyrimidin-2,4(1*H*,3*H*)-dione (uracil),

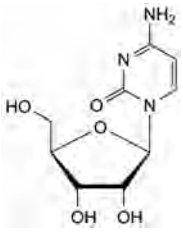

E. 4-amino-1-β-D-ribofuranosylpyrimidin-2(1*H*)-one (cytidine),

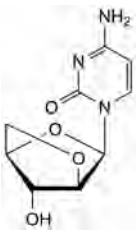

F. 4-amino-1-(2,5-anhydro-β-D-arabinofuranosyl)pyrimidin-2(1*H*)-one,

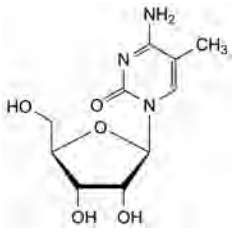

G. 4-amino-5-methyl-1-β-D-ribofuranosylpyrimidin-2(1*H*)-one (5-methylcytidine),

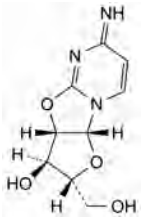

H. (2*R*,3*R*,3a*S*,9a*R*)-2-(hydroxymethyl)-6-imino-2,3,3a,9a-tetrahydro-6*H*-furo[2′,3′:4,5][1,3]oxazolo[3,2-*a*]pyrimidin-3-ol,

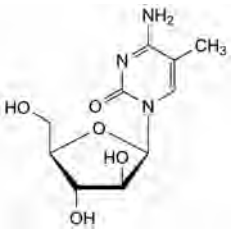

I. 4-amino-1-β-D-arabinofuranosyl-5-methylpyrimidin-2(1*H*)-one.

# D

Dacarbazine.. 2337
Dalteparin sodium.. 2338
Danaparoid sodium.. 2340
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Dihydroergotamine mesilate.. 2418
Dihydrostreptomycin sulfate for veterinary use.. 2420
Dihydrotachysterol.. 2422
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Dimercaprol.. 2426
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Dimethylacetamide.. 2428
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Diphenoxylate hydrochloride.. 2436
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Diprophylline.. 2441
Dipyridamole.. 2442
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Disodium edetate.. 2445
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Disodium phosphate dodecahydrate.. 2447
Disopyramide.. 2448
Disopyramide phosphate.. 2449
Disulfiram.. 2450
Dithranol.. 2451
Dobutamine hydrochloride.. 2452
Docetaxel.. 2453
Docetaxel trihydrate.. 2455
Docusate sodium.. 2457
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Domperidone maleate.. 2460
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Dopexamine dihydrochloride.. 2463
Dorzolamide hydrochloride.. 2464
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Doxapram hydrochloride.. 2467
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Doxorubicin hydrochloride.. 2471
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Droperidol.. 2478
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Dutasteride.. 2483
Dydrogesterone.. 2485

**01/2008:1691**
**corrected 10.0**

# DACARBAZINE

## Dacarbazinum

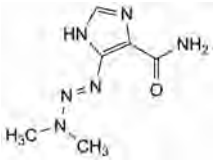

$C_6H_{10}N_6O$ $M_r$ 182.2
[4342-03-4]

DEFINITION

5-[(1*E*)-3,3-Dimethyltriaz-1-enyl]-1*H*-imidazole-4-carboxamide.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: slightly soluble in water and in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 15.0 mg in 100.0 mL of *0.1 M hydrochloric acid*. Dilute 5.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 200-400 nm.

*Absorption maximum*: at 323 nm.

*Shoulder*: at 275 nm.

*Specific absorbance at the absorption maximum*: 1024 to 1131.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dacarbazine CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 2.0 mg of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Dissolve 2.0 mg of *dacarbazine CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (1:2:5 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.25 g in a 210 g/L solution of *citric acid monohydrate R* and dilute to 25.0 mL with the same solution.

**Related substances**

A. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and protect them from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined and 75 mg of *citric acid monohydrate R* in *distilled water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *dacarbazine impurity A CRS* in *distilled water R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of this solution to 25.0 mL with *distilled water R*.

*Reference solution (b).* Dissolve 5.0 mg of *dacarbazine impurity B CRS* in *distilled water R*, add 0.5 mL of the test solution and dilute to 10.0 mL with *distilled water R*. Dilute 1.0 mL of this solution to 50.0 mL with *distilled water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 15.63 g/L solution of *glacial acetic acid R* containing 2.33 g/L of *sodium dioctyl sulfosuccinate R*. As the mobile phase contains sodium dioctyl sulfosuccinate, it must be freshly prepared every day, and the column must be flushed with a mixture of equal volumes of *methanol R* and *water for chromatography R*, after all tests have been completed or at the end of the day, for at least 2 h.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 25 µL of the test solution and reference solution (a).

*Run time*: 3 times the retention time of impurity A.

*Retention time*: impurity A = about 3 min.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities eluting after impurity A*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent).

B. Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Mobile phase*: mix 45 volumes of a 15.63 g/L solution of *glacial acetic acid R* containing 2.33 g/L of *sodium dioctyl sulfosuccinate R* with 55 volumes of *methanol R*.

*Injection*: 10 µL of the test solution and reference solution (b).

*Run time*: twice the retention time of dacarbazine.

*Relative retention* with reference to dacarbazine (retention time = about 12 min): impurity B = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity B and dacarbazine.

*Limits*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to dacarbazine in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the peak due to dacarbazine in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the peak due to dacarbazine in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity D**. Head-space gas chromatography (*2.2.28*).

*Test solution.* Introduce 0.200 g of the substance to be examined into a 20 mL vial and firmly attach the septum and cap. Using a 10 µL syringe, inject 5 µL of *water R* into the vial.

*Reference solution (a).* Dilute 2.5 mL of *dimethylamine solution R* (impurity D) to 100.0 mL with *water R* (solution A). Firmly attach the septum and cap to a 20 mL vial. Using a 10 µL syringe, inject 10 µL of solution A into the vial.

*Reference solution (b).* Firmly attach the septum and cap to a 20 mL vial. Using a 10 µL syringe, inject 10 µL of solution A and 10 µL of a 10 g/L solution of *triethylamine R* into the vial.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30.0 m, Ø = 0.53 mm;
- *stationary phase*: *base-deactivated macrogol R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 13 mL/min.

*Split ratio*: 1:1.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 10 min;
- *transfer-line temperature*: 90 °C;
- *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 35 |
| | 3 - 11 | 35 → 165 |
| Injection port | | 180 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 mL.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity D and triethylamine.

*Limit*:

- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.22 mg of $C_6H_{10}N_6O$.

STORAGE

At a temperature of 2 °C to 8 °C, protected from light.

IMPURITIES

*Specified impurities*: *A, B, D*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C*.

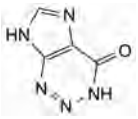

A. 3,7-dihydro-4*H*-imidazo[4,5-*d*]-1,2,3-triazin-4-one (2-azahypoxanthine),

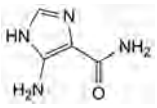

B. 5-amino-1*H*-imidazole-4-carboxamide,

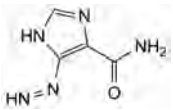

C. 5-diazenyl-1*H*-imidazole-4-carboxamide,

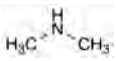

D. *N*-methylmethanamine.

**01/2008:1195**

# DALTEPARIN SODIUM

## Dalteparinum natricum

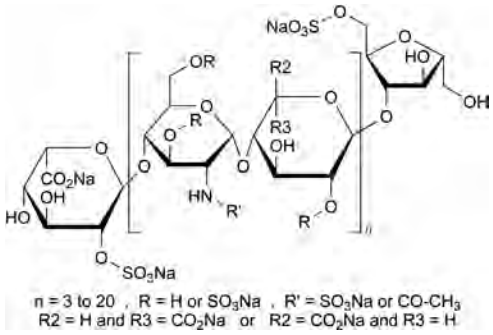


DEFINITION

Dalteparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by nitrous acid depolymerisation of heparin from porcine intestinal mucosa. The majority of the components have a 2-*O*-sulfo-α-L-idopyranosuronic acid structure at the non-reducing end and a 6-*O*-sulfo-2,5-anhydro-D-mannitol structure at the reducing end of their chain.

*Dalteparin sodium complies with the monograph Low-molecular-mass heparins (0828) with the modifications and additional requirements below.*

The mass-average relative molecular mass ranges between 5600 and 6400, with a characteristic value of about 6000.

The degree of sulfatation is 2.0 to 2.5 per disaccharide unit.

The potency is not less than 110 IU and not more than 210 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The anti-factor IIa activity is not less than 35 IU/mg and not more than 100 IU/mg, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 1.9 and 3.2.

## PRODUCTION

Dalteparin sodium is produced by a validated manufacturing and purification procedure under conditions designed to minimise the presence of N-NO groups.

The manufacturing procedure must have been shown to reduce any contamination by N-NO groups to approved limits using an appropriate, validated quantification method.

## IDENTIFICATION

Carry out identification test A as described in the monograph *Low-molecular-mass heparins (0828)* using *dalteparin sodium CRS*.

Carry out identification test C as described in the monograph *Low-molecular-mass heparins (0828)*. The following requirements apply.

The mass-average relative molecular mass ranges between 5600 and 6400. The mass percentage of chains lower than 3000 is not more than 13.0 per cent. The mass percentage of chains higher than 8000 ranges between 15.0 per cent and 25.0 per cent.

## TESTS

**Appearance of solution**. Dissolve 1 g in 10 mL of *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Nitrite**. Not more than 5 ppm. Examine by liquid chromatography (*2.2.29*). *Rinse all volumetric flasks at least three times with water R before the preparation of the solutions.*

*Test solution.* Dissolve 80.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent. Allow to stand for at least 30 min.

*Reference solution (a).* Dissolve 60.0 mg of *sodium nitrite R* in *water R* and dilute to 1000.0 mL with the same solvent.

*For the preparation of reference solution (b), use a pipette previously rinsed with reference solution (a).*

*Reference solution (b).* Dilute 1.00 mL of reference solution (a) to 50.0 mL with *water R*.

*Before preparing reference solutions (c), (d) and (e), rinse all pipettes with reference solution (b).*

*Reference solution (c).* Dilute 1.00 mL of reference solution (b) to 100.0 mL with *water R* (corresponding to 1 ppm of nitrite in the test sample).

*Reference solution (d).* Dilute 3.00 mL of reference solution (b) to 100.0 mL with *water R* (corresponding to 3 ppm of nitrite in the test sample).

*Reference solution (e).* Dilute 5.00 mL of reference solution (b) to 100.0 mL with *water R* (corresponding to 5 ppm of nitrite in the test sample).

The chromatographic procedure may be carried out using:

- a column 0.125 m long and 4.3 mm in internal diameter packed with a strong anion-exchange resin;
- as mobile phase at a flow rate of 1.0 mL/min a solution consisting of 13.61 g of *sodium acetate R* dissolved in *water R*, adjusted to pH 4.3 with *phosphoric acid R* and diluted to 1000 mL with *water R*;
- as detector an appropriate electrochemical device with the following characteristics and settings: a suitable working electrode, a detector potential of + 1.00 V versus Ag/AgCl reference electrode and a detector sensitivity of 0.1 μA full scale.

Inject 100 μL of reference solution (d). When the chromatograms are recorded in the prescribed conditions, the retention time for nitrite is 3.3 to 4.0 min. The test is not valid unless:

- the number of theoretical plates calculated for the nitrite peak is at least 7000 per metre per column (dalteparin sodium will block the binding sites of the stationary phase, which will cause shorter retention times and lower separation efficiency for the analyte; the initial performance of the column may be partially restored using a 58 g/L solution of *sodium chloride R* at a flow rate of 1.0 mL/min for 1 h; after regeneration the column is rinsed with 200 mL to 400 mL of *water R*);
- the symmetry factor for the nitrite peak is less than 3;
- the relative standard deviation of the peak area for nitrite obtained from 6 injections is less than 3.0 per cent.

Inject 100 μL each of reference solutions (c) and (e). The test is not valid unless:

- the correlation factor for a linear relationship between concentration and response for reference solutions (c), (d) and (e) is at least 0.995;
- the signal-to-noise ratio for reference solution (c) is not less than 5 (if the noise level is too high, electrode recalibration is recommended);
- a blank injection of *water R* does not give rise to spurious peaks.

Inject 100 μL of the test solution. Calculate the content of nitrite from the peak areas in the chromatogram obtained with reference solutions (c), (d) and (e).

**Boron**. Not more than 1 ppm, determined by inductively coupled plasma atomic emission spectroscopy.

Boron is determined by measurement of the emission from an inductively coupled plasma (ICP) at a wavelength specific to boron. The emission line at 249.733 nm is used. Use an appropriate apparatus, whose settings have been optimised as directed by the manufacturer.

*Test solution.* Dissolve 0.2500 g of the substance to be examined in about 2 mL of *water for chromatography R*, add 100 μL of *nitric acid R* and dilute to 10.00 mL with the same solvent.

*Reference solution (a).* Prepare a 1 per cent *V/V* solution of *nitric acid R* in *water for chromatography R* (blank).

*Reference solution (b).* Prepare a 11.4 μg/mL solution of *boric acid R* in a 1 per cent *V/V* solution of *nitric acid R* in *water for chromatography R* ($STD_{cal}$).

*Reference solution (c).* Dissolve 0.2500 g of a reference dalteparin sodium with no detectable boron in about 2 mL of *water for chromatography R*, add 100 μL of *nitric acid R* and dilute to 10.00 mL with the same solvent ($STD_0$).

*Reference solution (d).* Dissolve 0.2500 g of a reference dalteparin sodium with no boron detected in about 2 mL of a 1 per cent *V/V* solution of *nitric acid R* in *water for chromatography R*, add 10 μL of a 5.7 mg/mL solution of *boric acid R* and dilute to 10.00 mL with the same solvent ($STD_1$). This solution contains 1 μg/mL of boron.

Calculate the content of boron in the substance to be examined, using the following correction factor:

$$f = \frac{(STD_1 - STD_0) \times 2}{(STD_{cal} - \text{blank})}$$

**Loss on drying** (*2.2.32*). Not more than 5.0 per cent, determined on 1.000 g by drying in an oven at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

**01/2011:2090**
**corrected 10.0**

# DANAPAROID SODIUM

## Danaparoidum natricum

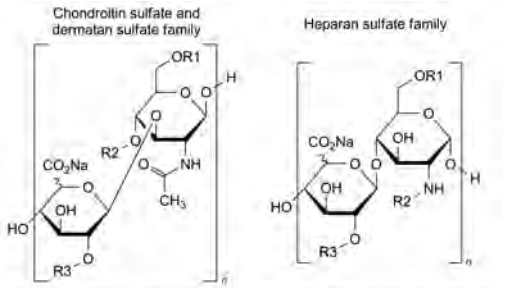


| ΔDi | R1 | R2 | R3 |
|---|---|---|---|
| -OS | H | H | H |
| -6S | $SO_3Na$ | H | H |
| -4S | H | $SO_3Na$ | H |
| -US | H | H | $SO_3Na$ |
| -(U,6)S | $SO_3Na$ | H | $SO_3Na$ |
| -(U,4)S | H | $SO_3Na$ | $SO_3Na$ |
| -(4,6)S | $SO_3Na$ | $SO_3Na$ | H |
| -(U,4,6)S | $SO_3Na$ | $SO_3Na$ | $SO_3Na$ |

| ΔDiHS | R1 | R2 | R3 |
|---|---|---|---|
| -OS | H | Ac | H |
| -6S | $SO_3Na$ | Ac | H |
| -NS | H | $SO_3Na$ | H |
| -US | H | Ac | $SO_3Na$ |
| -(U,N)S | H | $SO_3Na$ | $SO_3Na$ |
| -(6,N)S | $SO_3Na$ | $SO_3Na$ | H |
| -(U,N,6)S | $SO_3Na$ | $SO_3Na$ | $SO_3Na$ |

### DEFINITION

Preparation containing the sodium salts of a mixture of sulfated glycosaminoglycans present in porcine tissues. Danaparoid sodium is prepared from the intestinal mucosa of pigs. Its major constituents are heparan sulfate and dermatan sulfate. On complete hydrolysis it liberates D-glucosamine, D-galactosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulfuric acid. It has the characteristic property of enhancing the inactivation of activated factor X (factor Xa) by antithrombin. It has a negligible effect on the inactivation rate of thrombin by antithrombin.

*Potency*: 11.0 to 17.0 anti-factor Xa units per milligram (dried substance).

### PRODUCTION

The animals from which danaparoid sodium is derived must fulfil the requirements for the health of animals suitable for human consumption. It is prepared using a process that ensures that the relative proportion of active sulfated glycosaminoglycans is consistent. It is produced by methods of manufacturing designed to minimise or eliminate endotoxins and hypotensive substances.

### CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water.

### IDENTIFICATION

A. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay and Tests respectively, is not less than 22.

B. Molecular mass distribution (see Tests): the mass-average relative molecular mass ranges between 4000 and 7000.

### TESTS

**pH** (*2.2.3*): 5.5 to 7.0.

Dissolve 0.5 g of the dried substance to be examined in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Anti-factor IIa activity**: maximum 0.5 units per milligram (dried substance).

*Test solutions.* Prepare 2 independent series of dilutions in geometric progression of the substance to be examined in *phosphate buffer solution pH 6.5 R* and in the concentration range of 0.0005 to 0.005 units of anti-factor IIa activity per millilitre.

*Reference solutions.* Prepare 2 independent series of dilutions in geometric progression of *danaparoid sodium CRS* in *phosphate buffer solution pH 6.5 R* and in the concentration range of 0.0005 to 0.005 units of anti-factor IIa activity per millilitre.

Transfer 50 µL of each solution into the wells of a 96-well microtitre plate. To each well add 50 µL of *antithrombin III solution R3* and 50 µL of *human thrombin solution R1*. Shake the microtitre plate but do not allow bubbles to form. Incubate for 75 min. To each well add 50 µL of *chromogenic substrate R4*. Shake the microtitre plate. Measure the absorbances at 405 nm (*2.2.25*) using a suitable reading device, exactly 4 min after the addition of the chromogenic substrate. The reaction may be stopped using 75 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Determine the blank amidolytic activity in a similar manner, using *phosphate buffer solution pH 6.5 R* as the blank solution (minimum 10 blanks per microtitre plate). Calculate the activity of the substance to be examined in units of anti-factor IIa activity per milligram using a suitable statistical method, for example the parallel-line assay.

**Chondroitin sulfate and dermatan sulfate**. Chondroitin sulfate: maximum 8.5 per cent (dried substance); dermatan sulfate: 8.0 per cent to 16.0 per cent (dried substance).

Determine by selective enzymatic degradation.

*Test solutions.* Dry the substance to be examined at 60 °C over *diphosphorus pentoxide R* at a pressure of about 670 Pa for 3 h. Dissolve 0.200 g of the dried substance in 10.0 mL of *water R*. Dilute this solution as necessary to obtain 3 test solutions containing 20 mg/mL, 10 mg/mL and 5 mg/mL of the dried substance to be examined in *water R*.

*Chondroitin sulfate reference solutions.* Dry *chondroitin sulfate sodium CRS* over *diphosphorus pentoxide R* at room temperature at a pressure of about 670 Pa for 16 h. Prepare solutions containing 1 mg/mL, 2 mg/mL and 3 mg/mL of dried *chondroitin sulfate sodium CRS* in *water R*.

*Dermatan sulfate reference solutions.* Dry *dermatan sulfate CRS* over *diphosphorus pentoxide R* at room temperature at a pressure of about 670 Pa for 16 h. Prepare solutions containing 1 mg/mL, 2 mg/mL and 3 mg/mL of dried *dermatan sulfate CRS* in *water R*.

*Chondroitinase ABC solution.* Dissolve *chondroitinase ABC R* in *tris-sodium acetate-sodium chloride buffer solution pH 8.0 R* to obtain an activity of 0.5-1.0 units per millilitre.

*Chondroitinase AC solution.* Dissolve *chondroitinase AC R* in *tris-sodium acetate-sodium chloride buffer solution pH 7.4 R* to obtain an activity of 1.0-2.0 units per millilitre.

*Procedure*:

– *Degradation with chondroitinase ABC*: label 2 sets of 10 tubes in triplicate: T1, T2 and T3 for the test solutions; SD1, SD2 and SD3 for the dermatan sulfate reference solutions; SC1, SC2 and SC3 for the chondroitin sulfate reference solutions; and B for the blank (*water R*). To each tube add 1.25 mL of *tris-sodium acetate buffer solution pH 8.0 R* and 150 µL of the test solutions, dermatan sulfate reference solutions, chondroitin sulfate reference solutions or *water R*. To each tube in 1 set of tubes add 75 µL of chondroitinase ABC solution. To determine the blank response level, add 75 µL of *tris-sodium acetate-sodium chloride buffer solution pH 8.0 R* to each tube in the other set of tubes. Mix the contents of the tubes using a vortex mixer, cover with appropriate stoppers and incubate at 37 °C for at least 24 h.

– *Degradation with chondroitinase AC*: label 7 tubes in triplicate: T1, T2 and T3 for the test solutions; SC1, SC2 and SC3 for the chondroitin sulfate reference solutions; and B for the blank (*water R*). To each tube add 1.25 mL of *tris-sodium acetate buffer solution pH 7.4 R* and 150 µL of the test solutions, chondroitin sulfate reference solutions or *water R*. Add 75 µL of chondroitinase AC solution to each tube. Mix the contents of the tubes using a vortex mixer, cover with appropriate stoppers and incubate at 37 °C for at least 24 h. After the incubation period mix the contents of the tubes using a vortex mixer and dilute to 12 times with *water R*. Measure the absorbances (*2.2.25*) of the diluted solutions at 234 nm against *water R* using a suitable spectrophotometer.

*Calculation*: calculate the mean blank absorbance of each reference solution, i.e. the mean of the absorbances of the reference solutions to which no chondroitinase ABC has been added. Subtract the mean blank absorbance value from the individual absorbance of each reference solution. Calculate linear regression curves for the 2 chondroitin sulfate reference and the dermatan sulfate reference by plotting the blank-corrected absorbances against the concentrations.

Calculate the average percentage content of dermatan sulfate in the test solutions of all tested concentrations using the following expression:

$$\frac{A_2 - A_1 - \frac{(A_3 - A_1 - I_1) \times B_2}{B_1} - I_2 - I_3}{B_3 \times C} \times 100$$

$A_1$ = blank absorbance of the test solution;

$A_2$ = absorbance of the test solution with chondroitinase ABC;

$A_3$ = absorbance of the test solution with chondroitinase AC;

$B_1$ = gradient of the curve obtained with the chondroitin sulfate reference solutions with chondroitinase AC;

$B_2$ = gradient of the curve obtained with the chondroitin sulfate reference solutions with chondroitinase ABC;

$B_3$ = gradient of the curve obtained with the dermatan sulfate reference solutions with chondroitinase ABC;

$C$ = concentration of the test solution, in milligrams per millilitre;

$I_1$ = y-intercept of the curve obtained with the chondroitin sulfate reference solutions with chondroitinase AC;

$I_2$ = y-intercept of the curve obtained with the chondroitin sulfate reference solutions with chondroitinase ABC;

$I_3$ = y-intercept of the curve obtained with the dermatan sulfate reference solutions with chondroitinase ABC.

Calculate the average percentage content of chondroitin sulfate in the test solutions for all tested concentrations using the following expression:

$$\frac{(A_3 - A_1 - I_1) \times 100}{B_1 \times C}$$

**Molecular mass distribution**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of the mobile phase.

*Reference solution.* Dissolve 10 mg of *danaparoid sodium CRS* in 2 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.60 m, Ø = 7.5 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R* (10 µm) with a fractionation range for proteins with a relative molecular mass of approximately 5000-100 000;

– *temperature*: 30 °C.

*Mobile phase*: 28.4 g/L solution of *anhydrous sodium sulfate R* adjusted to pH 5.0 with *dilute sulfuric acid R*.

*Flow rate*: 0.9 mL/min ± 2 per cent.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 100 µL.

*Run time*: for a period of time ensuring complete elution of sample and solvent peaks (about 40 min).

*System suitability*: inject the reference solution twice. The difference between the retention times corresponding to the maxima of the peaks is not more than 5 s.

*Calibration*: calibration is achieved by taking the relevant part of the chromatogram obtained with the reference solution, i.e. excluding the sharp peak at the end of the chromatogram, and matching the chromatogram obtained with the test solution with the calibration table obtained with the reference solution. From the calibration curve obtained, determine the molecular mass distribution of the sample. A calibration table is supplied with *danaparoid sodium CRS*.

*Limits*:

– *chains with a relative molecular mass less than 2000*: maximum 13 per cent;

– *chains with a relative molecular mass less than 4000*: maximum 39 per cent;

– *chains with a relative molecular mass between 4000 and 8000*: minimum 50 per cent;

– *chains with a relative molecular mass higher than 8000*: maximum 19 per cent;

– *chains with a relative molecular mass higher than 10 000*: maximum 11 per cent.

**Nitrogen** (*2.5.9*): 2.4 per cent to 3.0 per cent (dried substance).

**Nucleic acids**: maximum 0.5 per cent (dried substance).

*Test solution.* Weigh about 50 mg of the dried substance to be examined into a centrifuge tube and dissolve in 200 µL of *water R*.

*Reference solution.* Dissolve about 50 mg of *ribonucleic acid CRS* in 5 mL of *0.1 M sodium hydroxide* and dilute to 20.0 mL with *water R*. Transfer 200 µL of the solution into a centrifuge tube.

Add 4.0 mL of a 50 g/L solution of *trichloroacetic acid R* to each tube and mix. Place all tubes in boiling water for 30 min. Allow to cool to room temperature. Add again 4.0 mL of a 50 g/L solution of *trichloroacetic acid R* to each tube and mix. If any of the test solutions is not clear, sonicate all the tubes in an ultrasonic bath for 10 min and centrifuge at 1500 *g* for 15 min. Dilute 1.0 mL of the clear supernatant to 4.0 mL with *water R*. Measure the absorbances of the diluted reference and test solutions at 265 nm (*2.2.25*) against a blank solution prepared in the same manner, and calculate the percentage nucleic acid content of the sample.

**Total protein** (*2.5.33, Method 2*): maximum 0.5 per cent.

Dissolve the substance to be examined in *water R*. Use *bovine albumin R* as the reference substance.

**Sodium**: 9.0 per cent to 11.0 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 0.125 g of the substance to be examined in 100.0 mL of a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid*.

*Reference solutions.* Prepare reference solutions containing 50 ppm, 100 ppm and 150 ppm of Na by diluting *sodium standard solution (1000 ppm Na) R* with a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid*.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 330.3 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 60 °C over *diphosphorus pentoxide R* at a pressure of 670 Pa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.02 IU per unit of anti-factor Xa activity, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The anticoagulant activity of danaparoid sodium is determined *in vitro* by an assay which determines its ability to accelerate the inhibition of factor Xa by antithrombin III (anti-factor Xa assay).

*Test solutions.* Prepare 2 independent series of dilutions in geometric progression of the substance to be examined in *tris(hydroxymethyl)aminomethane EDTA buffer solution pH 8.4 R* and in the concentration range of 0.1 to 0.32 units of anti-factor Xa activity per millilitre.

*Reference solutions.* Prepare 2 independent series of dilutions in geometric progression of *danaparoid sodium CRS* in *tris(hydroxymethyl)aminomethane EDTA buffer solution pH 8.4 R* and in the concentration range of 0.08 to 0.35 units of anti-factor Xa activity per millilitre.

Transfer 40 µL of each solution into the wells of a 96-well microtitre plate. Add 40 µL of *antithrombin III solution R4* to each well and shake the microtitre plate but do not allow bubbles to form. Add 40 µL of *bovine factor Xa solution R1* to each well. Exactly 2 min after the addition of the factor Xa solution, add 80 µL of *chromogenic substrate R5*. Measure the absorbance at 405 nm (*2.2.25*) using a suitable reading device, exactly 4 min after the addition of the factor Xa solution. The reaction may be stopped using 75 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Determine the blank amidolytic activity in the same manner, using *tris(hydroxymethyl)aminomethane EDTA buffer solution pH 8.4 R* as the blank (minimum 8 blanks per microtitre plate). Calculate the potency of the substance to be examined in units of anti-factor Xa activity per milligram using a suitable statistical method, for example the parallel-line assay.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the number of units of anti-factor Xa activity per milligram.

**01/2008:0077**
**corrected 6.0**

# DAPSONE

## Dapsonum

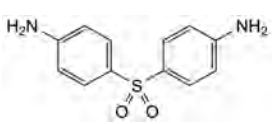


$C_{12}H_{12}N_2O_2S$ $M_r$ 248.3
[80-08-0]

DEFINITION

Dapsone contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4,4′-sulfonyldianiline, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish-white, crystalline powder, very slightly soluble in water, freely soluble in acetone, sparingly soluble in alcohol. It dissolves freely in dilute mineral acids.

IDENTIFICATION

A. Melting point (*2.2.14*): 175 °C to 181 °C.

B. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows 2 absorption maxima, at 260 nm and 295 nm. The specific absorbances at these maxima are 700 to 760 and 1150 to 1250, respectively.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 10 mg of *dapsone CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 10 mL with *methanol R*.

*Reference solution (c).* Dilute 2 mL of reference solution (b) to 10 mL with *methanol R*.

Apply separately to the plate 1 µL of test solution (b), 1 µL of reference solution (a), 10 µL of test solution (a), 10 µL of reference solution (b) and 10 µL of reference solution (c). Develop in an unsaturated tank over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 6 volumes of *methanol R*, 20 volumes of *ethyl acetate R* and 20 volumes of *heptane R*. Allow the plate to dry in air. Spray the plate with a 1 g/L solution of *4-dimethylaminocinnamaldehyde R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *alcohol R*. Examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and not more than 2 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *dilute hydrochloric acid R*. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*).

1 mL of *0.1 M sodium nitrite* is equivalent to 12.42 mg of $C_{12}H_{12}N_2O_2S$.

STORAGE

Store protected from light.

**01/2008:0662**
**corrected 10.0**

# DAUNORUBICIN HYDROCHLORIDE

## Daunorubicini hydrochloridum

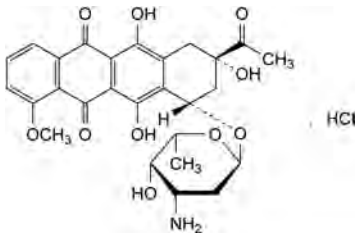

$C_{27}H_{30}ClNO_{10}$ $M_r$ 564.0
[23541-50-6]

DEFINITION

(8*S*,10*S*)-8-Acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance produced by certain strains of *Streptomyces coeruleorubidus* or of *Streptomyces peucetius* or obtained by any other means.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise the presence of histamine.

CHARACTERS

*Appearance*: crystalline, orange-red powder, hygroscopic.

*Solubility*: freely soluble in water and in methanol, slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *daunorubicin hydrochloride CRS*.

B. Dissolve about 10 mg in 0.5 mL of *nitric acid R*, add 0.5 mL of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 mL of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *daunorubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *doxorubicin hydrochloride CRS* and 10 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *daunorubicinone CRS* and 5.0 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 200.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mixture of equal volumes of *acetonitrile R* and a solution containing 2.88 g/L of *sodium laurilsulfate R* and 2.25 g/L of *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL; inject the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of daunorubicin.

*Relative retention* with reference to daunorubicin (retention time = about 15 min): impurity A = about 0.4; impurity D = about 0.5; epirubicin = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (b):
- *resolution*: minimum of 2.0 between the peaks due to impurity D and epirubicin.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (1.5 per cent),
- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent),
- *total of other impurities*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (2.5 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Butanol** (*2.4.24, System B*): maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 4.3 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{30}ClNO_{10}$.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

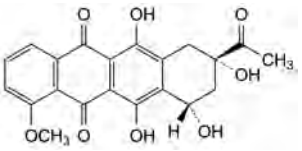

A. (8*S*,10*S*)-8-acetyl-6,8,10,11-tetrahydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicin aglycone, daunorubicinone),

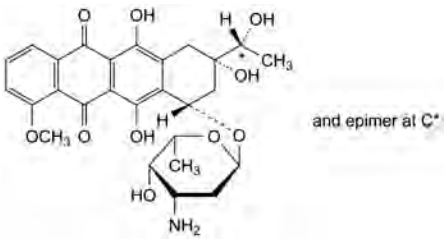


B. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicinol),

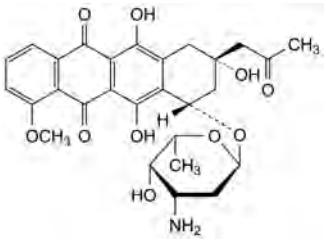

C. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-8-(2-oxopropyl)-7,8,9,10-tetrahydrotetracene-5,12-dione (feudomycin B),

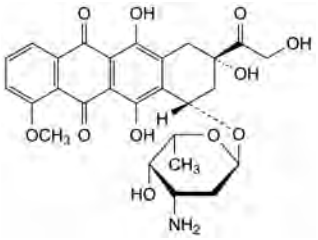

D. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicin),

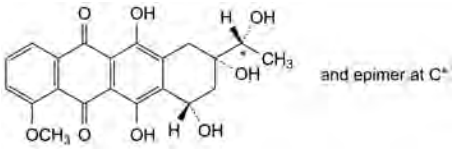


E. (8*S*,10*S*)-6,8,10,11-tetrahydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (13-dihydrodaunorubicinone),

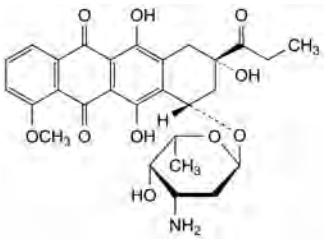

F. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-8-propanoyl-7,8,9,10-tetrahydrotetracene-5,12-dione (8-ethyldaunorubicin).

**01/2008:1307**

# DECYL OLEATE

## Decylis oleas

DEFINITION

Mixture consisting of decyl esters of fatty acids, mainly oleic (*cis*-9-octadecenoic) acid.

A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, pale yellow or colourless liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent), with methylene chloride and with light petroleum (bp: 40-60 °C).

IDENTIFICATION

A. Relative density (see Tests).

B. Saponification value (see Tests).

C. Oleic acid (see Tests).

TESTS

**Relative density** (*2.2.5*): 0.860 to 0.870.

**Acid value** (*2.5.1*): maximum 1.0, determined on 10.0 g.

**Iodine value** (*2.5.4, Method A*): 55 to 70.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Saponification value** (*2.5.6*): 130 to 140, determined on 2.0 g.

**Oleic acid** (*2.4.22, Method A*): minimum 60.0 per cent in the fatty acid fraction of the substance.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

Protected from light.

**07/2018:2236**

# DEFERIPRONE

## Deferipronum

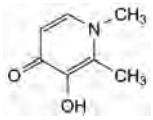

$C_7H_9NO_2$ $M_r$ 139.2

[30652-11-0]

DEFINITION

3-Hydroxy-1,2-dimethylpyridin-4-(1*H*)-one.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pinkish-white powder.

*Solubility*: sparingly soluble in water, slightly soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *deferiprone CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use only colourless glassware. Protect the solutions from light.*

*Solution A*. Dissolve 2.91 g of *sodium edetate R*, 4.01 g of *sodium octanesulfonate monohydrate R* and 6.20 g of *dipotassium hydrogen phosphate R* in *water for chromatography R* and dilute to 2000 mL with the same solvent; adjust to pH 3.0 with *phosphoric acid R*.

*Solution B*. Dissolve 0.73 g of *sodium edetate R*, 1.0 g of *sodium octanesulfonate monohydrate R* and 1.55 g of *dipotassium hydrogen phosphate R* in *water for chromatography R* and dilute to 2000 mL with the same solvent; adjust to pH 3.0 with *phosphoric acid R*.

*Solvent mixture*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Test solution (a)*. Dissolve 0.100 g of the substance to be examined in a volume of the mobile phase corresponding to about 2/3 of the final volume and dilute to 100.0 mL with the mobile phase.

*Test solution (b)*. Dissolve 50.0 mg of the substance to be examined in a volume of the solvent mixture corresponding to about 2/3 of the final volume and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 200.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2 mg of *maltol R* (impurity B) in the mobile phase and dilute to 100.0 mL with the mobile phase. To 2.5 mL of the solution add 10 mL of test solution (a) and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 50.0 mg of *deferiprone CRS* in a volume of the solvent mixture corresponding to about 2/3 of the final volume and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 200.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, solution A (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Preconditioning of the column*: rinse for 20 min with the mobile phase before each series of injections.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of deferiprone.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to deferiprone (retention time = about 12 min): impurity B = about 0.5.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity B and deferiprone.

*Calculation of percentage contents*:

- for each impurity, use the concentration of deferiprone in reference solution (b).

*Limits*:

- *impurity B*: maximum 0.10 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.03 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *acetonitrile R*, solution B (10:90 *V/V*).

*Injection*: 10 µL of test solution (b) and reference solution (c).

*Run time*: twice the retention time of deferiprone.

*Retention time*: deferiprone = about 7.7 min.

Calculate the percentage content of $C_7H_9NO_2$ taking into account the assigned content of *deferiprone CRS*.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

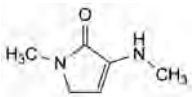

A. 1-methyl-3-(methylamino)-1,5-dihydro-2*H*-pyrrol-2-one,

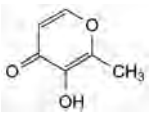

B. 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol),

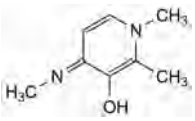

C. 1,2-dimethyl-4-[(Ξ)-methylimino]-1,4-dihydropyridin-3-ol.

**04/2019:2987**
**corrected 10.0**

# DEFERIPRONE ORAL SOLUTION

## Deferiproni solutio peroralis

DEFINITION

Oral solution of *Deferiprone (2236)*.

*It complies with the monograph Liquid preparations for oral use (0672) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of deferiprone ($C_7H_9NO_2$) stated on the label.

IDENTIFICATION

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay, with a diode array detector in the range of 210-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with test solution (b) is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (c).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use only colourless glassware. Protect the solutions from light.*

*Buffer solution.* Dissolve 0.25 g of *sodium edetate R*, 4.3 g of *sodium octanesulfonate monohydrate R* and 13.8 g of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R*, and dilute to 1000 mL with the same solvent; adjust to pH 2.1 with *phosphoric acid R*.

*Solvent mixture*: *methanol R*, buffer solution (20:80 *V/V*)

*Test solution (a).* To a volume of the preparation to be examined containing the equivalent of 100 mg of deferiprone, add the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (b) to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *maltol R* (impurity B) in the mobile phase and dilute to 100 mL with the mobile phase. Mix 2.5 mL of the solution and 10 mL of test solution (a) and dilute to 100 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *deferiprone CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *methanol R*, buffer solution (13:87 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of deferiprone.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to deferiprone (retention time = about 11 min): impurity B = about 0.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and deferiprone.

*Calculation of percentage contents*:

- for each impurity, use the concentration of deferiprone in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to impurity B.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: solvent mixture.

*Flow rate*: 1.1 mL/min.

*Injection*: test solution (b) and reference solution (c).

*Run time*: 1.5 times the retention time of deferiprone.

*Retention time*: deferiprone = about 7 min.

*System suitability*: reference solution (c):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of deferiprone ($C_7H_9NO_2$) taking into account the assigned content of *deferiprone CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph): *B*.

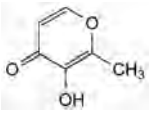


B. 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol).

**07/2019:2986**

# DEFERIPRONE TABLETS

## Deferiproni compressi

DEFINITION

Tablets containing *Deferiprone (2236)*.

*They comply with the monograph Tablets (0478) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of deferiprone ($C_7H_9NO_2$) stated on the label.

IDENTIFICATION

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay, with a diode array detector in the range of 210-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with test solution (b) is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (c).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use only colourless glassware. Protect the solutions from light.*

*Buffer solution.* Dissolve 2.91 g of *sodium edetate R*, 4.01 g of *sodium octanesulfonate monohydrate R* and 6.20 g of *dipotassium hydrogen phosphate R* in *water for chromatography R* and dilute to 2000 mL with the same solvent; adjust to pH 3.0 with *phosphoric acid R*.

*Test solution (a).* Crush 20 tablets to obtain a homogeneous powder. Dissolve an amount of the powder containing the equivalent of 100 mg of deferiprone in the mobile phase by sonicating for approximately 15 min and dilute to 100.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 200.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of test solution (b) to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *maltol R* (impurity B) in the mobile phase and dilute to 100 mL with the mobile phase. Mix 5 mL of the solution and 10 mL of test solution (a) and dilute to 100 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *deferiprone CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 200.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, buffer solution (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Preconditioning of the column*: rinse for 20 min with the mobile phase before each series of injections.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of deferiprone.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to deferiprone (retention time = about 12 min): impurity B = about 0.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and deferiprone.

*Calculation of percentage contents*:

- for each impurity, use the concentration of deferiprone in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to impurity B.

**Dissolution** (*2.9.3, Apparatus 2*).

*The tablets comply with the test and acceptance criterion as described below, unless otherwise justified and authorised.*

*Dissolution medium*: 10.3 g/L solution of *hydrochloric acid R*. Use 1000 mL of the medium.

*Rotation speed*: 50 r/min.

*Time*: 45 min.

*Analysis.* Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solutions.* The samples withdrawn from the dissolution vessel and filtered.

*Reference solution.* Dissolve a suitable quantity of *deferiprone CRS* in a suitable volume of the dissolution medium to obtain a concentration of deferiprone corresponding to the theoretical concentration of deferiprone in the test solution, based on the labelled content of the tablets.

When the test is carried out off-line, the solutions may be diluted according to the path length of the cell (e.g. 50-fold dilution for 500 mg tablets and 100-fold for 1000 mg tablets).

Measure the absorbances of the solutions at the absorption maximum at 275 nm, with a background correction at 490 nm.

Calculate the amount of dissolved deferiprone ($C_7H_9NO_2$), expressed as a percentage of the content stated on the label, taking into account the assigned content of *deferiprone CRS*.

*Acceptance criterion*:

- *Q* = 80 per cent after 45 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: 10 µL of test solution (b) and reference solution (c).

*Run time*: twice the retention time of deferiprone.

*System suitability*: reference solution (c):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of deferiprone ($C_7H_9NO_2$) taking into account the assigned content of *deferiprone CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph): *B*.

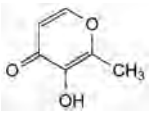

B. 3-hydroxy-2-methyl-4*H*-pyran-4-one (maltol).

**01/2018:0896**
**corrected 10.0**

# DEFEROXAMINE MESILATE

## Deferoxamini mesilas

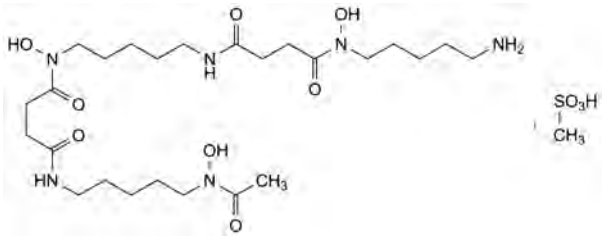

$C_{26}H_{52}N_6O_{11}S$ $M_r$ 657
[138-14-7]

DEFINITION

$N^1$-[5-(4-[(5-Aminopentyl)(hydroxy)amino]-4-oxobutanamido)pentyl]-$N^1$-hydroxy-$N^4$-[5-(*N*-hydroxyacetamido)pentyl]butanediamide methanesulfonate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in deferoxamine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in methanol, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *deferoxamine mesilate CRS*.

B. Dissolve 0.1 g in 5 mL of *dilute hydrochloric acid R*. Add 1 mL of *barium chloride solution R2*. The solution is clear. In a porcelain crucible, mix 0.1 g with 1 g of *anhydrous sodium carbonate R*, heat and ignite over a naked flame. Allow to cool. Dissolve the residue in 10 mL of *water R*, heating if necessary, and filter. The filtrate gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.0.

Dilute 1 mL of freshly prepared solution S to 10 mL with *carbon dioxide-free water R* prepared from *distilled water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

A. Deferoxamine mesilate produced by fermentation.

*Solvent mixture*: *acetonitrile R*, *water R* (6:94 *V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 10 mL of the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *deferoxamine for system suitability CRS* (containing impurity A) in mobile phase A and dilute to 5 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.075 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 32 °C.

*Mobile phase*:
- *mobile phase A*: 1.32 g/L solution of *ammonium phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: mixture of equal volumes of *acetonitrile for chromatography R* and mobile phase A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 88 | 12 |
| 4 - 20 | 88 → 80 | 12 → 20 |
| 20 - 35 | 80 → 57.5 | 20 → 42.5 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to deferoxamine (retention time = about 18 min): impurity A = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurity A and deferoxamine.

*Calculation of percentage contents*:
- for each impurity, use the concentration of deferoxamine mesilate in reference solution (a).

*Limits*:
- *impurity A*: maximum 2.0 per cent;
- *any other impurity*: for each impurity, maximum 0.8 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

B. Deferoxamine mesilate produced by a synthetic process.

*Solvent mixture*: mobile phase B, mobile phase A (20:80 *V/V*).

*Test solution.* Dissolve 0.125 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 12.5 mg of *deferoxamine for peak identification CRS* (containing impurities F, G, H, I and J) in the solvent mixture and dilute to 1 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 0.605 g of *sodium edetate R* in 900 mL of *water for chromatography R*, add 1.0 mL of *phosphoric acid R* and adjust to pH 6.0 with *concentrated ammonia R*; dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: dissolve 0.780 g of *sodium edetate R* in 750 mL of *water for chromatography R* and add 1.0 mL of *phosphoric acid R*; add 250 mL of *acetonitrile for chromatography R*, mix thoroughly and adjust the apparent pH to 6.0 with *concentrated ammonia R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 | 80 | 20 |
| 0 - 21.6 | 80 → 20 | 20 → 80 |
| 21.6 - 31.6 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *deferoxamine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities F, G, H, I and J.

*Relative retention* with reference to deferoxamine (retention time = about 12.9 min): impurity I = about 0.5; impurity F = about 0.96; impurity G = about 0.98; impurity H = about 1.2; impurities J and K = about 1.37.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to deferoxamine.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity I by 1.4;
- for each impurity, use the concentration of deferoxamine mesilate in reference solution (a).

*Limits*:

- *impurities F, H*: for each impurity, maximum 0.5 per cent;
- *impurities G, I*: for each impurity, maximum 0.3 per cent;
- *sum of impurities J and K*: maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.03 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dilute 2 mL of solution S to 20 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 5 mL of solution S to 20 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.025 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Since deferoxamine mesilate has an inhibitory effect on the bacterial endotoxins test, a suitable procedure is put in place to remove this inhibitory effect. The monocyte-activation test (*2.6.30*) has been found suitable to overcome this issue.

## ASSAY

Dissolve 0.500 g in 50 mL of *water R*. Add 4 mL of *dilute sulfuric acid R1*. Titrate with *0.1 M ferric ammonium sulfate*. To accelerate the titration, add 4.5 mL quickly, stop for 1.5 min and continue to titrate uniformly at a rate of about 0.2 mL/min. Determine the end-point potentiometrically (*2.2.20*) using a platinum indicator electrode and a suitable reference electrode.

1 mL of *0.1 M ferric ammonium sulfate* is equivalent to 65.68 mg of $C_{26}H_{52}N_6O_{11}S$.

## STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

## LABELLING

The label states the origin of the substance:

- produced by fermentation;
- produced by a synthetic process.

## IMPURITIES

**Test A for related substances**: A, B, C, J.

**Test B for related substances**: E, F, G, H, I, J, K.

*Specified impurities: A, F, G, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E.*

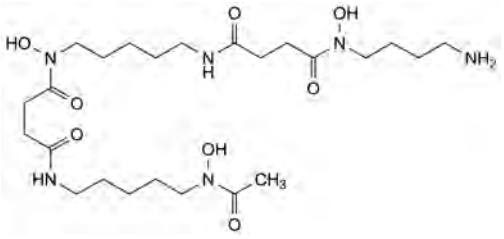

A. $N^1$-[5-(4-[(4-aminobutyl)(hydroxy)amino]-4-oxobutanamido)pentyl]-$N^1$-hydroxy-$N^4$-[5-(*N*-hydroxyacetamido)pentyl]butanediamide,

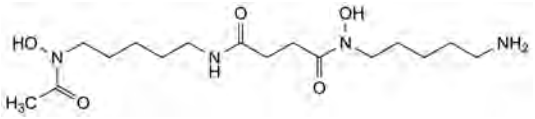

B. $N^1$-(5-aminopentyl)-$N^1$-hydroxy-$N^4$-[5-(*N*-hydroxyacetamido)pentyl]butanediamide,

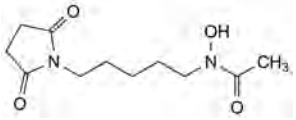

C. *N*-[5-(2,5-dioxopyrrolidin-1-yl)pentyl]-*N*-hydroxyacetamide,

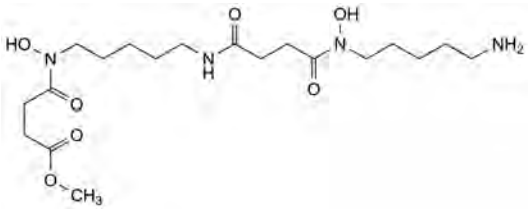

E. methyl 4-[[5-[[4-[(5-aminopentyl)(hydroxy)amino]-4-oxobutanamido]pentyl](hydroxy)amino]-4-oxobutanoate,

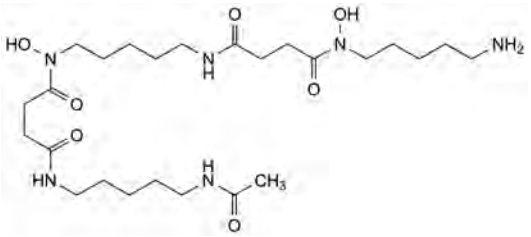

F. $N^1$-(5-acetamidopentyl)-$N^4$-[5-[4-[(5-aminopentyl)-(hydroxyl)amino]-4-oxobutanamido]pentyl]-$N^4$-hydroxybutanediamide,

G. unknown structure,

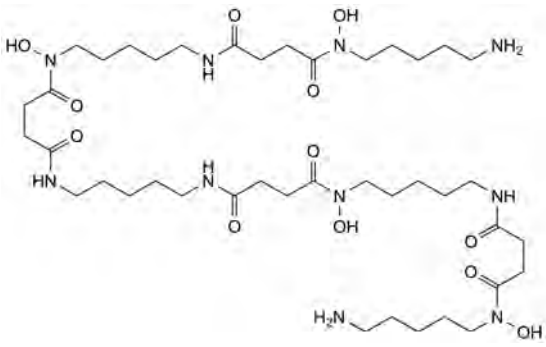

H. $N^1$,$N^{37}$-bis(5-aminopentyl)-$N^1$,$N^{37}$,11,27-tetrahydroxy-4,12,15,23,26,34-hexaoxo-5,11,16,22,27,33-hexaazaheptatriacontane-1,37-diamide,

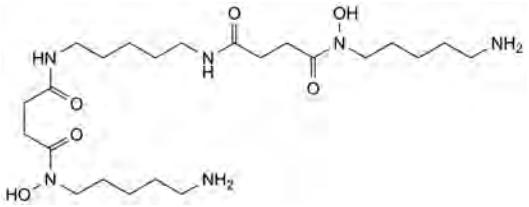

I. $N^1$-(5-aminopentyl)-$N^4$-[5-(4-[(5-aminopentyl)-(hydroxy)amino]-4-oxobutanamido)pentyl]-$N^1$-hydroxybutanediamide,

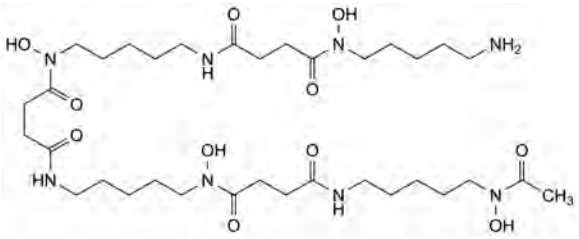

J. $N^1$-(5-aminopentyl)-$N^1$,11,22-trihydroxy-$N^{26}$-[5-(*N*-hydroxyacetamido)pentyl]-4,12,15,23-tetraoxo-5,11,16,22-tetraazahexacosane-1,26-diamide,

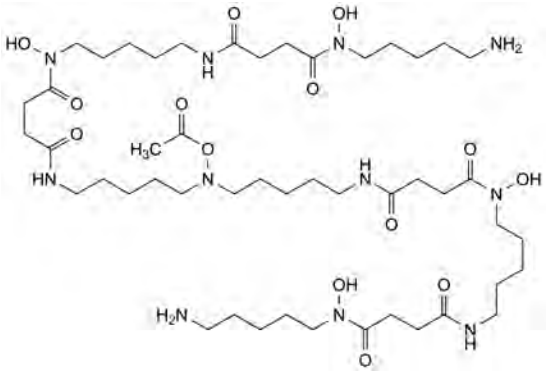

K. 22-(acetyloxy)-$N^1$,$N^{43}$-bis(5-aminopentyl)-$N^1$,$N^{43}$,11,33-tetrahydroxy-4,12,15,29,32,40-hexaoxo-5,11,16,22,28,33,39-heptaazatritetracontane-1,43-diamide.

**01/2008:2169**
**corrected 9.2**

# DEMBREXINE HYDROCHLORIDE MONOHYDRATE FOR VETERINARY USE

## Dembrexini hydrochloridum monohydricum ad usum veterinarium

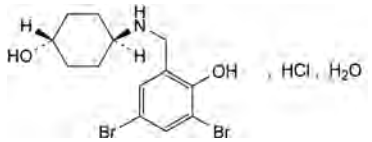

$C_{13}H_{18}Br_2ClNO_2,H_2O$ $M_r$ 433.6
[52702-51-9]

DEFINITION

*trans*-4-[(3,5-Dibromo-2-hydroxybenzyl)amino]cyclohexanol hydrochloride monohydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dembrexine hydrochloride monohydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 2.5 mg of *tribromophenol R* (impurity E) in *methanol R* and dilute to 50.0 mL with the same solvent. To 1.0 mL of this solution add 1.0 mL of the test solution and dilute to 10.0 mL with *methanol R*.

*Blank solution*. *Methanol R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 1.0 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.4 with a 0.5 M potassium hydroxide solution prepared from *potassium hydroxide R* and dilute to 1000 mL with *water R*; mix 80 volumes of this solution with 20 volumes of *methanol R*;
- *mobile phase B*: *methanol R*, *acetonitrile R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 75 | 25 |
| 7 - 15 | 75 → 50 | 25 → 50 |
| 15 - 20 | 50 | 50 |
| 20 - 25 | 50 → 75 | 50 → 25 |
| 25 - 30 | 75 | 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Relative retention* with reference to dembrexine (retention time = about 6 min): impurity A = about 2.3; impurity B = about 1.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 2 between the peaks due to dembrexine and impurity E.

*Limits*:
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Water** (*2.5.12*): 3.5 per cent to 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 40 mL of *methanol R*. Add 40 mL of *acetone R* and 1 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.56 mg of $C_{13}H_{18}Br_2ClNO_2$.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E.*

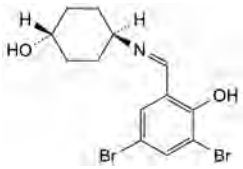

A. *trans*-4-[(3,5-dibromo-2-hydroxybenzylidene)amino]-cyclohexanol,

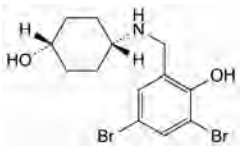

B. *cis*-4-[(3,5-dibromo-2-hydroxybenzyl)amino]cyclohexanol,

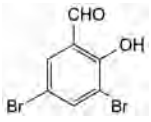

C. 3,5-dibromo-2-hydroxybenzaldehyde,

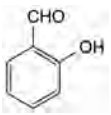

D. 2-hydroxybenzaldehyde (salicylaldehyde),

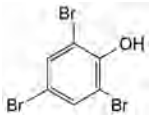

E. 2,4,6-tribromophenol.

**01/2017:0176**
**corrected 10.0**

# DEMECLOCYCLINE HYDROCHLORIDE

## Demeclocyclini hydrochloridum

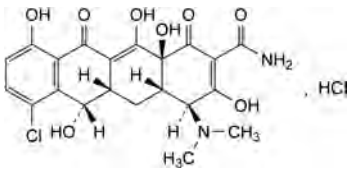

$C_{21}H_{22}Cl_2N_2O_8$ $M_r$ 501.3
[64-73-3]

DEFINITION

(4*S*,4a*S*,5a*S*,6*S*,12a*S*)-7-Chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by certain strains of *Streptomyces aureofaciens*.

*Content*: 89.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow powder.

*Solubility*: soluble or sparingly soluble in water, slightly soluble in alcohol, very slightly soluble in acetone. It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *demeclocycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *demeclocycline hydrochloride CRS*, 5 mg of *chlortetracycline hydrochloride R* and 5 mg of *tetracycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A violet colour develops. Add the solution to 2.5 mL of *water R*. The colour becomes yellow.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 2.0 to 3.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Buffer 1*: 22.2 g/L solution of *sodium edetate R* adjusted to pH 7.5 with a 40 g/L solution of *sodium hydroxide R*.

*Buffer 2*: 17.0 g/L solution of *tetrapropylammonium hydrogen sulfate R* adjusted to pH 7.5 with a 40 g/L solution of *sodium hydroxide R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in a 1.0 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solution.

*Reference solution (a)*. Dissolve 25.0 mg of *demeclocycline hydrochloride CRS* in a 1.0 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with a 1.0 g/L solution of *hydrochloric acid R*.

*Reference solution (c)*. Dissolve 5 mg of *demeclocycline for system suitability CRS* (containing impurities A, B, C, E and G) in a 1.0 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

*Column*:

- *size*: *l* = 0.075 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, *water R*, buffer 1, buffer 2 (2:28:35:35 *V/V/V/V*);
- *mobile phase B*: *acetonitrile R*, buffer 1, buffer 2 (30:35:35 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 83 | 17 |
| 5 - 15 | 83 → 30 | 17 → 70 |
| 15 - 25 | 30 | 70 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *demeclocycline for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, E and G.

*Relative retention* with reference to demeclocycline (retention time = about 14 min): impurity C = about 0.3; impurity B = about 0.7; impurity A = about 0.8; impurity E = about 1.2; impurity G = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of demeclocycline hydrochloride in reference solution (b).

*Limits*:

- *impurities A, B*: for each impurity, maximum 5.0 per cent;
- *impurities C, G*: for each impurity, maximum 0.3 per cent;
- *impurity E*: maximum 0.2 per cent;
- *any other impurity*: for each impurity, maximum 0.15 per cent;
- *total*: maximum 10.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{21}H_{22}Cl_2N_2O_8$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *demeclocycline hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F.*

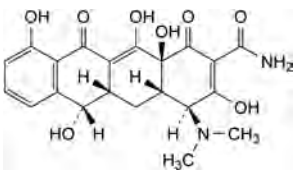

A. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (demethyltetracycline),

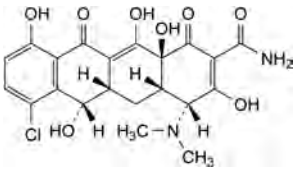

B. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidemeclocycline),

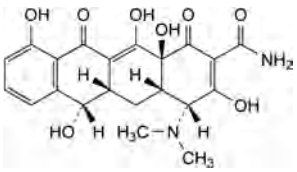

C. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidemethyltetracycline),

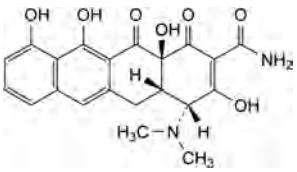

D. (4*R*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrodemethyltetracycline),

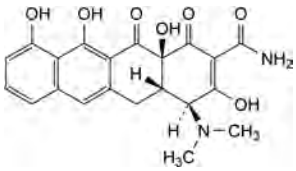

E. (4*S*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrodemethyltetracycline),

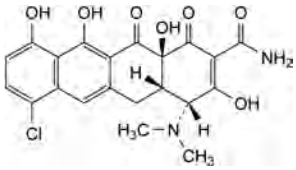

F. (4*R*,4a*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrodemeclocycline),

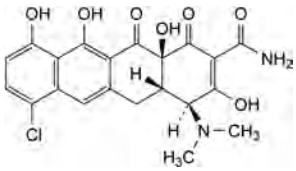

G. (4*S*,4a*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrodemeclocycline).

**01/2017:1308**

# DEPTROPINE CITRATE

## Deptropini citras

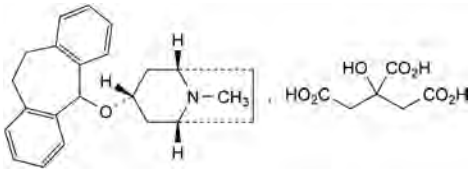

$C_{29}H_{35}NO_8$ $M_r$ 525.6
[2169-75-7]

DEFINITION

Deptropine citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,3*r*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-yloxy)-8-methyl-8-azabicyclo[3.2.1]octane dihydrogen citrate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, microcrystalline powder, very slightly soluble in water and in ethanol, practically insoluble in methylene chloride.

It melts at about 170 °C, with decomposition.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D, E.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *deptropine citrate CRS*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

C. To about 1 mg add 0.5 mL of *sulfuric acid R*. A stable red-orange colour develops.

D. Dissolve about 1 mg in 0.25 mL of *perchloric acid R* and warm gently until the solution becomes turbid. Add 5 mL of *glacial acetic acid R*; a pink colour with an intense green fluorescence appears.

E. To about 5 mg add 1 mL of *acetic anhydride R* and 5 mL of *pyridine R*. A purple colour develops.

TESTS

**pH** (*2.2.3*). Suspend 0.25 g in *carbon dioxide-free water R*, dilute to 25 mL with the same solvent and filter. The pH of the solution is 3.7 to 4.5.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 20 mg of *deptropine citrate CRS* in *methanol R* and dilute to 2 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *methanol R*.

*Reference solution (c).* Dissolve 5 mg of *tropine CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Dissolve 10 mg of *deptropine citrate CRS* and 10 mg of *tropine CRS* in *methanol R* and dilute to 25 mL with the same solvent.

Apply to the plate 40 µL of each solution. Develop over a path of 10 cm using a mixture of 8 volumes of *concentrated ammonia R* and 92 volumes of *butanol R*. Dry the plate at 100 °C to 105 °C until the ammonia has completely evaporated. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). Spray with *dilute potassium iodobismuthate solution R* and then with a 10 g/L solution of *sodium nitrite R*. Expose the plate to iodine vapours. Examine in daylight and in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spot corresponding to tropine is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent); any spot, apart from the principal spot and any spot corresponding to tropine, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

**Loss on drying** (*2.2.32*). Not more than 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 52.56 mg of $C_{29}H_{35}NO_8$.

STORAGE

Store protected from light.

IMPURITIES

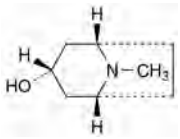

A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]octan-3-ol (tropine),

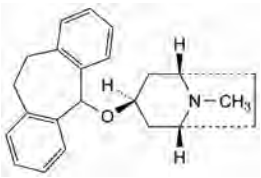

B. (1*R*,3*s*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-yloxy)-8-methyl-8-azabicyclo[3.2.1]octane (pseudodeptropine),

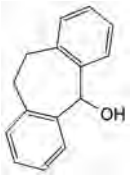

C. 10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ol (dibenzocycloheptadienol),

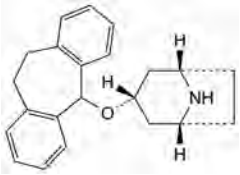

D. (1*R*,3*r*,5*S*)-3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-yloxy)-8-azabicyclo[3.2.1]octane (demethyldeptropine).

**01/2008:1413**
**corrected 6.0**

# DEQUALINIUM CHLORIDE

## Dequalinii chloridum

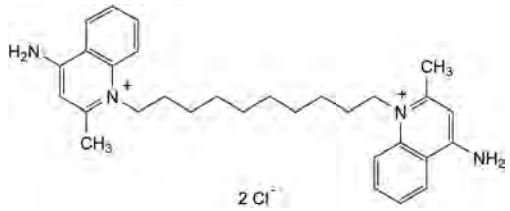

$C_{30}H_{40}Cl_2N_4$ $M_r$ 527.6
[522-51-0]

DEFINITION

1,1′-(Decane-1,10-diyl)bis(4-amino-2-methylquinolinium) dichloride (dried substance).

*Content*: 95.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve about 10 mg in *water R* and dilute to 100 mL with the same solvent. Dilute 10 mL of the solution to 100 mL with *water R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 240 nm and 326 nm.

*Shoulder*: at 336 nm.

*Absorbance ratios*:

- $A_{240}/A_{326}$ = 1.56 to 1.80;
- $A_{326}/A_{336}$ = 1.12 to 1.30.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Spectral range*: 600-2000 $cm^{-1}$.

*Comparison*: *dequalinium chloride CRS.*

C. To 5 mL of solution S (see Tests) add 5 mL of *potassium ferricyanide solution R*. A yellow precipitate is formed.

D. To 10 mL of solution S add 1 mL of *dilute nitric acid R*. A white precipitate is formed. Filter and reserve the filtrate for identification test E.

E. The filtrate from identification test D gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.2 g in 90 mL of *carbon dioxide-free water R*, heating if necessary, and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *dequalinium chloride for performance test CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *dequalinium chloride CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R.*

*Mobile phase*: dissolve 2 g of *sodium hexanesulfonate R* in 300 mL of *water R*; adjust to pH 4.0 with *acetic acid R* and add 700 mL of *methanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of dequalinium chloride.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dequalinium chloride. If necessary, adjust the concentration of methanol in the mobile phase.

*Limits*:

- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *total of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (10 per cent);
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Readily carbonisable substances**. Dissolve 20 mg in 2 mL of *sulfuric acid R*. After 5 min the solution is not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method I*).

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying at 105 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.200 g in 5 mL of *anhydrous formic acid R* and add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.38 mg of $C_{30}H_{40}Cl_2N_4$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

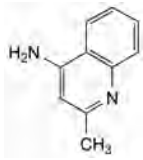

A. 2-methylquinolin-4-amine,

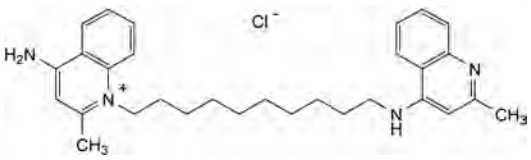

B. 4-amino-1-[10-[(2-methylquinolin-4-yl)amino]decyl]-2-methylquinolinium chloride,

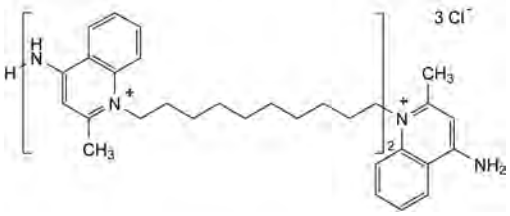

C. 1-[10-(4-amino-2-methylquinolinio)decyl]-4-[[10-(4-amino-2-methylquinolinio)decyl]amino]-2-methylquinolinium trichloride.

**07/2011:2537**

# 3-*O*-DESACYL-4′-MONOPHOSPHORYL LIPID A

## Adeps A 3-*O*-desacyl-4′-monophosphorylatus

DEFINITION

3-*O*-Desacyl-4′-monophosphoryl lipid A is a detoxified derivative of the lipopolysaccharide (LPS) of *Salmonella minnesota*, strain R595, which retains the immunostimulatory activities of the parent LPS. It consists of a mixture of congeners, all containing a backbone of β1′→6-linked disaccharide of 2-deoxy-2-aminoglucose phosphorylated at the 4′-position, but differing in the fatty acid substitutions at the 2, 2′ and 3′ positions. The immunostimulatory activities of 3-*O*-desacyl-4′-monophosphoryl lipid A combined with the vaccine include up-regulation of co-stimulatory molecules on antigen-presenting cells and secretion of pro-inflammatory cytokines, resulting in an enhanced immune response of the Th1-type against the antigens. 3-*O*-desacyl-4′-monophosphoryl lipid A is a lyophilised powder or a sterile liquid.

Requirements given in the sections up to and including the section Triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A also apply to formulations that do not proceed to the 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk.

PRODUCTION

*GENERAL PROVISIONS*

The production method shall have been shown to yield consistently a 3-*O*-desacyl-4′-monophosphoryl lipid A comparable in structure and function with a preparation of 3-*O*-desacyl-4′-monophosphoryl lipid A used as adjuvant in the particular vaccine of proven clinical efficacy and safety in man.

During development studies, and wherever revalidation is necessary, a test for residual endotoxin activity is carried out by injecting intravenously 12-day-old embryonated hens' eggs with 0.1 mL of dilutions of the test sample (8 eggs per dilution) of 3-*O*-desacyl-4′-monophosphoryl lipid A. Eggs are candled and read for mortality at 18-24 hours post-inoculation and the chick embryo 50 per cent lethal dose ($CELD_{50}$) is calculated. The residual endotoxin activity of the 3-*O*-desacyl-4′-monophosphoryl lipid A is acceptable if the $CELD_{50}$ is more than 100 µg.

An endotoxin standard of *Salmonella typhimurium* is prepared and selected dilutions are injected into each group of 8 eggs.

For a test to be valid, the $CELD_{50}$ of the endotoxin standard must not be more than 0.05 µg.

*Reference preparation*: a batch of 3-*O*-desacyl-4′-monophosphoryl lipid A shown to be comparable in structure and function with a preparation of 3-*O*-desacyl-4′-monophosphoryl lipid A used as adjuvant in the particular vaccine of proven clinical efficacy and safety in man or a batch representative thereof.

*BACTERIAL SEED LOTS*

The bacterial strain used for master seed lots shall be identified by historical records that include information on its origin and the tests used to characterise the strain, in particular genotypic and phenotypic information. Only a working seed lot that complies with the following requirements may be used.

**Identification**. The working seed lot is identified by suitable methods such as Gram staining and fatty acid profiling (*5.1.6*).

**Microbial Purity**. Each seed lot complies with the requirements for absence of contaminating organisms. Purity of bacterial cultures is verified by methods of suitable sensitivity and specificity.

*PROPAGATION AND HARVEST*

The bacteria is grown using a suitable liquid medium. At the end of cultivation, the culture is tested for purity and yield. The culture medium is separated from the bacterial mass by a suitable method, for example filtration. Only a harvest that is consistent with respect to the profiles for growth rate, pH, and $O_2$-consumption may be used for the extraction of LPS.

*TRIETHYLAMINE SALT OF 3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A*

LPS is extracted from the bacterial cells by successive alcohol and chloroform-methanol extractions and is then converted to 3-*O*-desacyl-4′-monophosphoryl lipid A by hydrolysis, then purified and salified by triethanolamine before freeze-drying. The freeze-dried triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A must comply with the following requirements.

**Appearance**. A visual description of the particular preparation after freeze-drying is established and approved by the competent authority; each batch of freeze-dried triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A must comply with this description.

**Protein**: less than 0.5 per cent *m/m*, determined using a suitable method, for example a reversed-phase HPLC method for amino acid analysis (*2.2.56*). The total amino acid content in micrograms is calculated by comparison to amino acid standards and is equal to the protein concentration.

**Nucleic acid**: maximum 0.3 per cent *m/m*, determined using a suitable method. For example, a fluorimetric method may be used where nucleic acids are extracted from the freeze-dried triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A, using a solution containing $NH_4OH$ and a suitable non-ionic detergent, and stained by a suitable fluorescent dye. The nucleic acid content in the test sample is interpolated from a calibration curve.

**Hexosamine** (*2.5.20*): 1000 nmol/mg to 1450 nmol/mg.

**Phosphorus** (*2.5.18*): 0.5 µmol/mg to 0.8 µmol/mg.

**Congener distribution**. The relative amount of tetraacyl, pentaacyl, hexaacyl and heptaacyl congener groups are determined by a suitable method, for example reversed-phase HPLC analysis (*2.2.29*).

The relative amount of each congener group in the triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A is:

- tetraacyl: 15 per cent to 35 per cent;
- pentaacyl: 35 per cent to 60 per cent;
- hexaacyl: 20 per cent to 40 per cent;
- heptaacyl: less than 0.5 per cent.

**Triethylamine**: 4.2 to 5.8 per cent *m/m*, determined by a suitable method, for example gas chromatography (*2.2.28*).

**Water** (*2.5.12*): maximum 6.7 per cent *m/m*.

**Free fatty acids**: maximum 2.6 per cent *m/m*, determined by a suitable method, for example reversed-phase HPLC analysis (*2.2.29*).

**2-Keto-3-deoxyoctonate**: less than 0.5 per cent *m/m*, determined by a suitable method. For example, a colorimetric method may be used where 2-keto-3-deoxyoctonate is released by hydrolysis (0.2 N $H_2SO_4$ at 100 °C for 30 min), oxidised by periodic acid, and reacted with sodium arsenite to yield β-formylpyruvic acid, which subsequently is coupled to thiobarbituric acid to give a red coloured chromophore with absorption maximum at 550 nm. The amount of 2-keto-3-deoxyoctonate is interpolated from a calibration curve.

**Identity**. The test for congener distribution also serves to identify the product.

**Microbial contamination**

TAMC: acceptance criterion $10^1$ CFU/10 mg (*2.6.12*).

**Pyrogens** (*2.6.8*). The triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A complies with the test for pyrogens. Inject into each rabbit per kilogram of body mass 3 mL of a solution containing 2.5 µg of 3-*O*-desacyl-4′-monophosphoryl lipid A.

*3-O-DESACYL-4′-MONOPHOSPHORYL LIPID A LIQUID BULK*

The triethylamine salt of 3-*O*-desacyl-4′-monophosphoryl lipid A is dispersed in a liquid suitable for the subsequent processing steps at a defined target concentration. If the salt is not soluble in water a microfluidisation step is necessary to prepare a stable aqueous suspension.
The liquid bulk is sterilised by filtration through a bacteria-retentive filter.

Only a 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk that complies with the requirements given below under Identification, Tests and Assay and that is within the limits approved for the particular product may be used for the preparation of 3-*O*-desacyl-4′-monophosphoryl lipid A in the final lots.

CHARACTERS

When dispersed in an aqueous solution: slightly turbid suspension.

When dissolved in an organic solvent: a description of its appearance is established and approved by the competent authority; the 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk complies with this description.

IDENTIFICATION

Congener distribution (see Tests).

TESTS

**Particle size**. Where applicable, the particle size in the microfluidised liquid bulk is determined by a suitable method, for example dynamic light scattering. The particle size for each batch of liquid bulk is within the limits approved for the particular product.

**Sterility** (*2.6.1*). It complies with the test, carried out using 10 mL for each medium.

**Congener distribution**. The relative amount of tetraacyl, pentaacyl, hexaacyl and heptaacyl congener groups are determined by a suitable method, for example reversed-phase HPLC analysis (*2.2.29*).

The relative amount of each congener group in the 3-*O*-desacyl-4′-monophosphoryl lipid A liquid bulk is:

- tetraacyl: 15 per cent to 35 per cent;
- pentaacyl: 35 per cent to 60 per cent;
- hexaacyl: 20 per cent to 40 per cent;
- heptaacyl: less than 0.5 per cent.

ASSAY

The 3-*O*-desacyl-4′-monophosphoryl lipid A content is determined by a suitable method, for example gas chromatographic quantification (*2.2.28*) of trifluoroacetic anhydride derivatised fatty acid methyl esters of the 3-*O*-desacyl-4′-monophosphoryl lipid A fatty acids dodecanoic acid (C12:0), tetradecanoic acid (C14:0), 3-hydroxy tetradecanoic acid (3-OH-C14:0) and hexadecanoic acid (C16:0) obtained by hydrolysis of 3-*O*-desacyl-4′-monophosphoryl lipid A in an aqueous/methanol (50:50 *V/V*) solution, containing 5 per cent of sodium hydroxide. To the test sample, a reference sample and the dilutions of the calibration curve, pentadecanoic acid

(C15:0) is added as an internal standard. The temperature gradient applied must allow the separation of the fatty acid methyl esters in about 40 min.

The sum of the ratios between the area for each individual fatty acid methyl ester (C12:0, C14:0, 3-OH-C14:0 and C16:0) and the area of the internal standard (ratio = area $C_x$ / area C15:0) is calculated. The 3-*O*-desacyl-4′-monophosphoryl lipid A quantity corresponding to the sum ratio value on the calibration curve, established with the dilutions of the 3-*O*-desacyl-4′-monophosphoryl lipid A standard, is reported.

The content of 3-*O*-desacyl-4′-monophosphoryl lipid A is not less than 80 per cent and not greater than 120 per cent of the estimated content.

**07/2019:1666**
**corrected 10.0**

# DESFLURANE

## Desfluranum

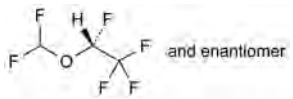


$C_3H_2F_6O$ $M_r$ 168.0
[57041-67-5]

DEFINITION

(2*RS*)-2-(Difluoromethoxy)-1,1,1,2-tetrafluoroethane.

CHARACTERS

*Appearance*: clear, colourless, mobile, heavy liquid.

*Solubility*: practically insoluble in water, miscible with anhydrous ethanol.

*Relative density*: 1.47, determined at 15 °C.

bp: about 22 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the substance in the gaseous state.

*Comparison*: *Ph. Eur. reference spectrum of desflurane.*

TESTS

*The substance to be examined must be cooled to a temperature below 10 °C and the tests must be carried out at a temperature below 20 °C.*

**Acidity or alkalinity**. To 20 mL add 20 mL of *carbon dioxide-free water R*, shake for 3 min and allow to stand. Collect the upper layer and add 0.2 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.01 M sodium hydroxide* or 0.6 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Reference solution (a)*. Introduce 25 mL of the substance to be examined into a 50 mL flask fitted with a septum, and add 0.50 mL of *desflurane impurity A CRS* and 1.0 mL of *isoflurane CRS* (impurity B). Add 50 µL of *acetone R* (impurity H), 10 µL of *chloroform R* (impurity F) and 50 µL of *methylene chloride R* (impurity E) to the solution, using an airtight syringe, and dilute to 50.0 mL with the substance to be examined. Dilute 5.0 mL of this solution to 50.0 mL with the substance to be examined. Store at a temperature below 10 °C.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 50.0 mL with the substance to be examined. Store at a temperature below 10 °C.

*Reference solution (c)*. Dilute 5.0 mL of reference solution (b) to 25.0 mL with the substance to be examined. Store at a temperature below 10 °C.

*Column*:
- *material*: fused silica;
- *size*: *l* = 105 m, Ø = 0.32 mm;
- *stationary phase*: *trifluoropropylmethylpolysiloxane R* (film thickness 1.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.0 mL/min.

*Split ratio*: 1:25.

*Temperature*:
- *column*: 30 °C;
- *injection port*: 150 °C;
- *detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 2.0 µL.

*Run time*: 35 min.

*Relative retention* with reference to desflurane (retention time = about 11.5 min): impurity C = about 1.06; impurity D = about 1.09; impurity A = about 1.14; impurity G = about 1.39; impurity E = about 1.5; impurity B = about 1.7; impurity F = about 2.2; impurity H = about 2.6.

*System suitability*: reference solution (a):
- *number of theoretical plates*: minimum 20 000, calculated for the peak due to impurity A;
- *symmetry factor*: maximum 2.0 for the peak due to impurity B.

*Limits*:
- *impurity B*: not more than the difference between the area of the corresponding peak in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (0.2 per cent *V/V*);
- *impurity A*: not more than the difference between the area of the corresponding peak in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (0.1 per cent *V/V*);
- *impurities C, D, G*: for each impurity, not more than the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (b) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.01 per cent *V/V*);
- *impurities E, H*: for each impurity, not more than the difference between the area of the corresponding peak in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (0.01 per cent *V/V*);
- *impurity F*: not more than the difference between the area of the corresponding peak in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (0.002 per cent *V/V*);
- *unspecified impurities*: for each impurity, not more than 0.5 times the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (b) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.005 per cent *V/V*);
- *sum of impurities other than A, B, C, D, E, F, G and H*: not more than the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (b) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.01 per cent *V/V*);

– *disregard limit*: the difference between the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) and the area of the peak due to impurity A in the chromatogram obtained with the test solution (0.002 per cent *V/V*).

**Fluorides**: maximum 10 ppm.

Potentiometry (*2.2.36, Method I*).

*Test solution.* To 10.0 mL in a separating funnel, add 10 mL of a mixture of 30.0 mL of *dilute ammonia R2* and 70.0 mL of *distilled water R*. Shake for 1 min and collect the upper layer. Repeat this extraction procedure twice, collecting the upper layer each time. Adjust the combined upper layers to pH 5.2 with *dilute hydrochloric acid R*. Add 5.0 mL of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 mL with *distilled water R*. To 20.0 mL of this solution add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

*Reference solutions.* To each of 1.0 mL, 2.0 mL, 3.0 mL, 4.0 mL and 5.0 mL of *fluoride standard solution (10 ppm F) R* add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

*Indicator electrode*: fluoride selective.

*Reference electrode*: silver-silver chloride.

Carry out the measurements on 20 mL of each solution. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

**Non-volatile matter**: maximum 100 mg/L.

Evaporate 20.0 mL to dryness with the aid of a stream of *nitrogen R*. The residue weighs not more than 2.0 mg.

STORAGE

In a glass bottle fitted with a polyethylene-lined cap. Before opening the bottle, cool the contents to below 10 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

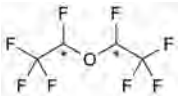

A. 1,1′-oxybis[(1Ξ)-1,2,2,2-tetrafluoroethane],

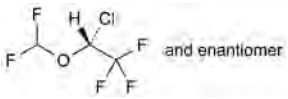


B. (2*RS*)-2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane (isofluorane),

C. dichlorofluoromethane,

D. trichlorofluoromethane,

E. dichloromethane (methylene chloride),

F. trichloromethane (chloroform),

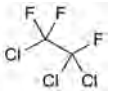

G. 1,1,2-trichloro-1,2,2-trifluoroethane,

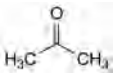

H. propan-2-one (acetone).

**01/2020:0481**

# DESIPRAMINE HYDROCHLORIDE

## Desipramini hydrochloridum

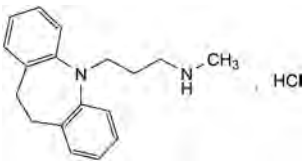

$C_{18}H_{23}ClN_2$ $M_r$ 302.8

[58-28-6]

DEFINITION

3-(10,11-Dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*-methylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water and in anhydrous ethanol, practically insoluble in heptane.

mp: about 214 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *desipramine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R*, warming to not more than 30 °C if necessary, and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S, examined immediately after preparation, is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.3 mL of *0.01 M sodium hydroxide*. The solution is yellow. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 5.2 g of *dipotassium hydrogen phosphate R* in 1000 mL of *water for chromatography R*, add 1 mL of *triethylamine R* and adjust to pH 6.4 with *phosphoric acid R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *imipramine hydrochloride R* (impurity A) in mobile phase A and dilute to 100 mL with mobile phase A. Mix 1 mL of the solution and 9 mL of the test solution, and dilute to 100 ml with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, *acetonitrile R*, buffer solution (11:14:75 *V/V/V*);
- *mobile phase B*: *methanol R*, *acetonitrile R*, buffer solution (28:34:38 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 37 | 85 → 0 | 15 → 100 |
| 37 - 52 | 0 | 100 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 40 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to desipramine (retention time = about 19 min): impurity A = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 25 between the peaks due to desipramine and impurity A.

*Calculation of percentage contents*:

- for each impurity, use the concentration of desipramine hydrochloride in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.28 mg of $C_{18}H_{23}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): A.

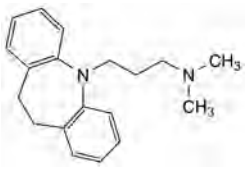

A. 3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine (imipramine).

**01/2008:0482**
**corrected 6.0**

# DESLANOSIDE

## Deslanosidum

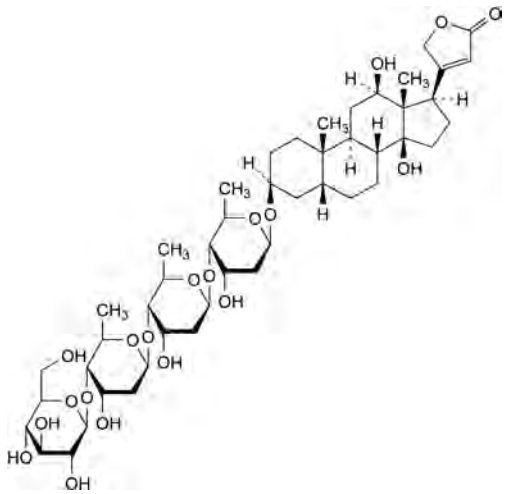

$C_{47}H_{74}O_{19}$ $M_r$ 943
[17598-65-1]

DEFINITION

Deslanoside contains not less than 95.0 per cent and not more than the equivalent of 105.0 per cent of 3β-[(*O*-β-D-glucopyranosyl-(1→4)-*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β,14β-card-20(22)-enolide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline or finely crystalline powder, hygroscopic, practically insoluble in water, very slightly soluble in alcohol. In an atmosphere of low relative humidity, it loses water.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *deslanoside CRS*. When comparing the spectra, special attention is given to the absence of a distinct absorption maximum at about 1260 $cm^{-1}$ and to the intensity of the absorption maximum at about 1740 $cm^{-1}$. Examine the substances in discs prepared by dissolving 1 mg of the substance to be examined or 1 mg of the reference substance in 0.3 mL of *methanol R* and triturating with about 0.4 g of dry, finely powdered *potassium bromide R* until the mixture is uniform and completely dry.

B. Examine the chromatograms obtained in the test for related substances. The principal zone in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal zone in the chromatogram obtained with reference solution (a).

C. Suspend about 0.5 mg in 0.2 mL of *alcohol (60 per cent V/V) R*. Add 0.1 mL of *dinitrobenzoic acid solution R* and 0.1 mL of *dilute sodium hydroxide solution R*. A violet colour develops.

D. Dissolve about 5 mg in 5 mL of *glacial acetic acid R* and add 0.05 mL of *ferric chloride solution R1*. Cautiously add 2 mL of *sulfuric acid R*, avoiding mixing the two liquids. Allow to stand; a brown but not reddish ring develops at the interface and a greenish-yellow, then bluish-green colour diffuses from it to the upper layer.

TESTS

**Solution S**. Dissolve 0.20 g in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 10 mL with the same mixture of solvents.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*). Dissolve 0.200 g in *anhydrous pyridine R* and dilute to 10.0 mL with the same solvent. The specific optical rotation is + 6.5 to + 8.5, calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a)*. Use solution S.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with a mixture of equal volumes of *chloroform R* and *methanol R*.

*Reference solution (a)*. Dissolve 20 mg of *deslanoside CRS* in a mixture of equal volumes of *chloroform R* and *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b)*. Dilute 2.5 mL of reference solution (a) to 10 mL with a mixture of equal volumes of *chloroform R* and *methanol R*.

*Reference solution (c)*. Dilute 1 mL of reference solution (a) to 10 mL with a mixture of equal volumes of *chloroform R* and *methanol R*.

Apply separately to the plate as 10 mm bands 5 µL of each solution. Develop immediately over a path of 15 cm using a mixture of 3 volumes of *water R*, 36 volumes of *methanol R* and 130 volumes of *methylene chloride R*. Dry the plate in a current of warm air, spray with a mixture of 5 volumes of *sulfuric acid R* and 95 volumes of *alcohol R* and heat at 140 °C for 15 min. Examine in daylight. In the chromatogram obtained with test solution (a), any zone, apart from the principal zone, is not more intense than the zone in the chromatogram obtained with reference solution (b) (2.5 per cent) and at most two such zones are more intense than the zone in the chromatogram obtained with reference solution (c) (1.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 5.0 per cent, determined on 0.500 g by drying *in vacuo* at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *alcohol R*. Prepare a reference solution in the same manner, using 50.0 mg of *deslanoside CRS* (undried). To 5.0 mL of each solution add 3.0 mL of *alkaline sodium picrate solution R* and allow to stand protected from bright light in a water-bath at 20 ± 1 °C for 40 min. Measure the absorbance (*2.2.25*) of each solution at the maximum at 484 nm, using as the compensation liquid a mixture of 3.0 mL of *alkaline sodium picrate solution R* and 5.0 mL of *alcohol R* prepared at the same time.

Calculate the content of $C_{47}H_{74}O_{19}$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Store in an airtight, glass container, protected from light, at a temperature below 10 °C.

**01/2017:2570**
**corrected 10.0**

# DESLORATADINE

## Desloratadinum

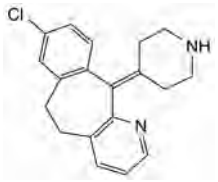


$C_{19}H_{19}ClN_2$ $M_r$ 310.8
[100643-71-8]

DEFINITION

8-Chloro-11-(piperidin-4-ylidene)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble or practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble or very slightly soluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *desloratadine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methyl isobutyl ketone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20.0 mg of *desloratadine CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 4 mg of *desloratadine for system suitability CRS* (containing impurities A and B) in the mobile phase and dilute to 5.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
– *temperature*: 35 °C.

*Mobile phase*: dissolve 0.865 g of *sodium dodecyl sulfate R* in *water R*, add 0.5 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *water R*; mix 57 volumes of this solution and 43 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 100 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of desloratadine.

*Identification of impurities*: use the chromatogram supplied with *desloratadine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to desloratadine (retention time = about 21 min): impurity A = about 0.8; impurity B = about 0.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the peaks due to impurity B and desloratadine.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.6; impurity B = 1.6;

– for each impurity, use the concentration of desloratadine in reference solution (b).

*Limits*:

– *impurity B*: maximum 0.3 per cent;

– *impurity A*: maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.4 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: 0.5 to 1.5 for the peak due to desloratadine.

Calculate the percentage content of $C_{19}H_{19}ClN_2$ taking into account the assigned content of *desloratadine CRS*.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

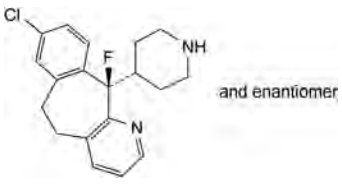


A. (11*RS*)-8-chloro-11-fluoro-11-(piperidin-4-yl)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine,

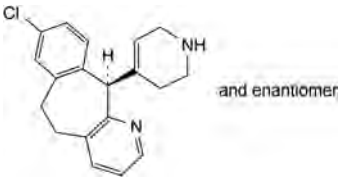


B. (11*RS*)-8-chloro-11-(1,2,3,6-tetrahydropyridin-4-yl)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine,

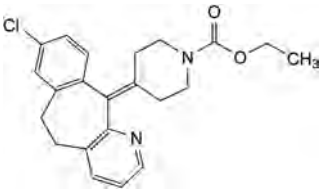


C. ethyl 4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta-[1,2-*b*]pyridin-11-ylidene)piperidine-1-carboxylate (loratadine).

**07/2009:0712**
**corrected 10.0**

# DESMOPRESSIN

## Desmopressinum

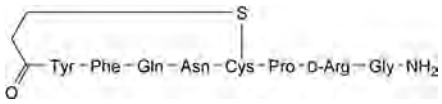


$C_{46}H_{64}N_{14}O_{12}S_2$ $M_r$ 1069
[16679-58-6]

DEFINITION

(3-Sulfanylpropanoyl)-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-D-arginylglycinamide cyclic (1→6)-disulfide.

Synthetic cyclic nonapeptide, available as an acetate.

*Content*: 95.0 per cent to 105.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white, fluffy powder.

*Solubility*: soluble in water, in ethanol (96 per cent) and in glacial acetic acid.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the retention time and size of the principal peak in the chromatogram obtained with the test solution are approximately the same as those of the principal peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/6 of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, arginine and phenylalanine as equal to 1. The values fall within the following limits: aspartic acid: 0.90 to 1.10; glutamic acid: 0.90 to 1.10; proline: 0.90 to 1.10; glycine: 0.90 to 1.10; arginine: 0.90 to 1.10; phenylalanine: 0.90 to 1.10; tyrosine: 0.70 to 1.05; half-cystine: 0.30 to 1.05. Lysine, isoleucine and leucine are absent; not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): – 72 to – 82 (anhydrous and acetic acid-free substance).

Dissolve 10.0 mg in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 5.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 1.0 mg of the substance to be examined in 2.0 mL of *water R*.

*Resolution solution.* Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* in 500 µL of *water R*.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *0.067 M phosphate buffer solution pH 7.0 R*; filter and degas;
- *mobile phase B*: *acetonitrile for chromatography R*, mobile phase A (50:50 *V/V*); filter and degas.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 76 | 24 |
| 4 - 18 | 76 → 58 | 24 → 42 |
| 18 - 35 | 58 → 48 | 42 → 52 |
| 35 - 40 | 48 → 76 | 52 → 24 |
| 40 - 50 | 76 | 24 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL.

*Retention time*: desmopressin = about 16 min; oxytocin = about 17 min.

*System suitability*: resolution solution:

- *resolution*: minimum 1.5 between the peaks due to desmopressin and oxytoxin.

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.5 per cent;
- *total*: maximum 1.5 per cent;
- *disregard limit*: 0.05 per cent.

**Acetic acid** (*2.5.34*): 3.0 per cent to 8.0 per cent.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 6.0 per cent, determined on 20.0 mg.

**Bacterial endotoxins** (*2.6.14*): less than 500 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Reference solution.* Dissolve the contents of a vial of *desmopressin CRS* in *water R* to obtain a concentration of 0.5 mg/mL.

*Mobile phase*: mobile phase B, mobile phase A (40:60 *V/V*).

*Flow rate*: 2.0 mL/min.

*Retention time*: desmopressin = about 5 min.

Calculate the content of desmopressin ($C_{46}H_{64}N_{14}O_{12}S_2$) from the declared content of $C_{46}H_{64}N_{14}O_{12}S_2$ in *desmopressin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the mass of peptide per container;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G*.

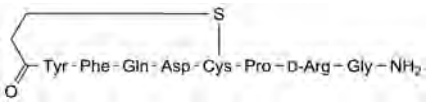


A. [5-L-aspartic acid]desmopressin,

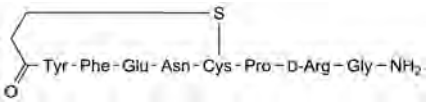


B. [4-L-glutamic acid]desmopressin,

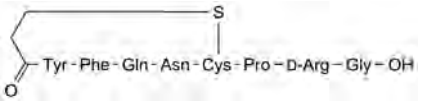


C. [9-glycine]desmopressin,

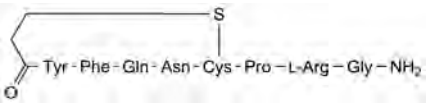


D. [8-L-arginine]desmopressin,

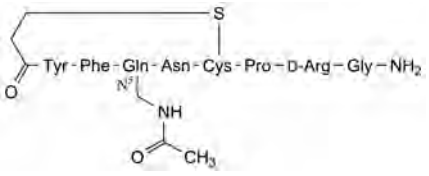


E. $N^{5.4}$-[(acetylamino)methyl]desmopressin,

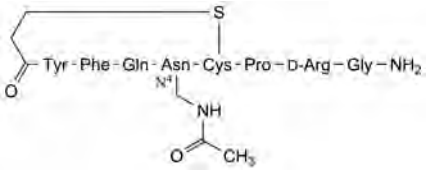


F. $N^{4.5}$-[(acetylamino)methyl]desmopressin,

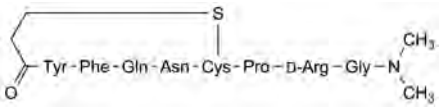


G. $N^{1.9}$,$N^{1.9}$-dimethyldesmopressin.

**01/2008:1717**

# DESOGESTREL

## Desogestrelum

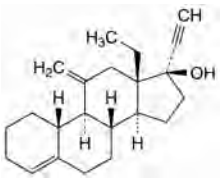

$C_{22}H_{30}O$ $M_r$ 310.5
[54024-22-5]

DEFINITION

13-Ethyl-11-methylidene-18,19-dinor-17α-pregn-4-en-20-yn-17-ol.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methanol, freely soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *desogestrel CRS*.

B. Specific optical rotation (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 53 to + 57 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 4 mg of *desogestrel for system suitability CRS* (containing impurities A, B, C and D) in 5 mL of *acetonitrile R1* and dilute to 10.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (d).* Dissolve 20.0 mg of *desogestrel CRS* in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: sterically protected *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 50 °C.

*Mobile phase*: *water R*, *acetonitrile R1* (27:73 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 15 µL of the test solution and reference solutions (a), (b) and (c).

*Run time*: 2.5 times the retention time of desogestrel.

*Identification of impurities*: use the chromatogram supplied with *desogestrel for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to desogestrel (retention time = about 22 min): impurity E = about 0.2; impurity D = about 0.25; impurity B = about 0.7; impurity A = about 0.95; impurity C = about 1.05.

*System suitability*: reference solution (a):
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to desogestrel.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak area of the following impurities by the corresponding correction factor: impurity A = 1.8, impurity D = 1.5;
- *impurities A, B, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at a pressure not exceeding 2 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (d).

Calculate the percentage content of $C_{22}H_{30}O$ from the areas of the peaks and the declared content of *desogestrel CRS*.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E.*

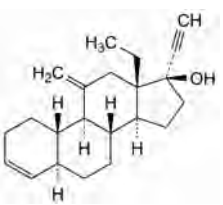

A. 13-ethyl-11-methylidene-18,19-dinor-5α,17α-pregn-3-en-20-yn-17-ol (desogestrel $\Delta^3$-isomer),

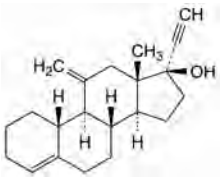

B. 11-methylidene-19-nor-17α-pregn-4-en-20-yn-17-ol,

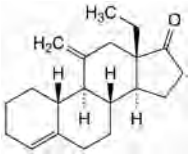

C. 13-ethyl-11-methylidenegon-4-en-17-one,

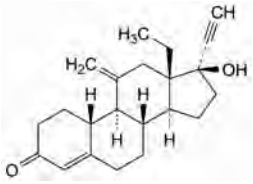

D. 13-ethyl-17-hydroxy-11-methylidene-18,19-dinor-17α-pregn-4-en-20-yn-3-one,

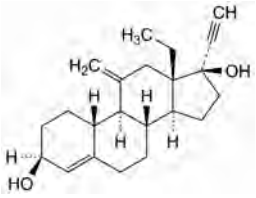

E. 13-ethyl-11-methylidene-18,19-dinor-17α-pregn-4-en-20-yne-3β,17-diol.

**04/2015:1414**
**corrected 10.0**

# DETOMIDINE HYDROCHLORIDE FOR VETERINARY USE

## Detomidini hydrochloridum ad usum veterinarium

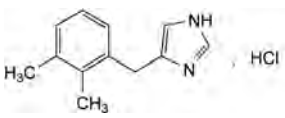

$C_{12}H_{15}ClN_2$ $M_r$ 222.7
[90038-01-0]

DEFINITION

4-[(2,3-Dimethylphenyl)methyl]-1*H*-imidazole hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *detomidine hydrochloride CRS*.
If the spectra obtained show differences, dry the substance to be examined and the reference substance separately in an oven at 105 °C and record new spectra.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.25 g in *water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 20 mL of the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 0.20 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 1 mg of *detomidine impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with reference solution (a).

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, 2.64 g/L solution of *ammonium phosphate R* (35:65 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of detomidine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to detomidine (retention time = about 6 min): impurity B = about 2.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to detomidine and impurity B.

*Calculation of percentage contents*:
- for each impurity, use the concentration of detomidine hydrochloride in reference solution (a).

*Limits*:
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.10 per cent.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g in 50 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 22.27 mg of $C_{12}H_{15}ClN_2$.

STORAGE

In an airtight container.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use*

*(2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

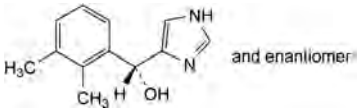


A. (*RS*)-(2,3-dimethylphenyl)(1*H*-imidazol-4-yl)methanol,

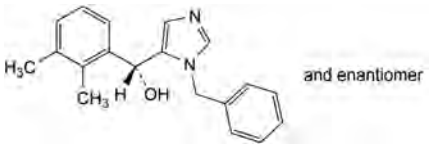


B. (*RS*)-(1-benzyl-1*H*-imidazol-5-yl)(2,3-dimethylphenyl)-methanol,

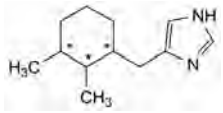


C. 4-[(2,3-dimethylcyclohexyl)methyl]-1*H*-imidazole.

**01/2014:0388**

# DEXAMETHASONE

## Dexamethasonum

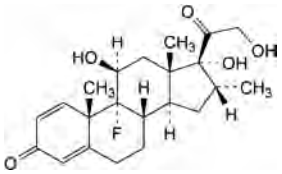


$C_{22}H_{29}FO_5$ $M_r$ 392.5
[50-02-2]

DEFINITION

9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in anhydrous ethanol, slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E.*

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of this solution in a stoppered test tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at the absorption maximum at 419 nm is not less than 0.4.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dexamethasone CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).
*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Reference solution (a).* Dissolve 20 mg of *dexamethasone CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.
*Reference solution (b).* Dissolve 10 mg of *betamethasone CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *butanol R* saturated with *water R*, *toluene R*, *ether R* (5:10:85 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.
*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).
*System suitability*: reference solution (b):
– the chromatogram shows 2 spots which may, however, not be completely separated.

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a faint reddish-brown colour develops. Add this solution to 10 mL of *water R* and mix; the colour is discharged.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank solution is red.

TESTS

**Specific optical rotation** (*2.2.7*): + 86 to + 92 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25 mg of the substance to be examined in 1.5 mL of *acetonitrile R* and add 5 mL of mobile phase A. Sonicate until dissolution is complete and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *dexamethasone for system suitability CRS* (containing impurities B, F and G) in 0.5 mL of *acetonitrile R* and add 1 mL of mobile phase A. Sonicate until dissolution is complete and dilute to 2.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 5 mg of *dexamethasone for peak identification CRS* (containing impurities J and K) in 0.5 mL of *acetonitrile R* and add 1 mL of mobile phase A. Sonicate until dissolution is complete and dilute to 2.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: mix 250 mL of *acetonitrile R* with 700 mL of *water R* and allow to equilibrate; dilute to 1000.0 mL with *water R* and mix again;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 40 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL; inject mobile phase A as a blank.

*Identification of impurities*: use the chromatogram supplied with *dexamethasone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, F and G; use the chromatogram supplied with *dexamethasone for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities J and K.

*Relative retention* with reference to dexamethasone (retention time = about 15 min): impurity J = about 0.90; impurity B = about 0.94; impurity K = about 1.3; impurity F = about 1.5; impurity G = about 1.7.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dexamethasone.

*Limits*:

– *impurity G*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *impurities B, F, J, K*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 238.5 nm.

Calculate the content of $C_{22}H_{29}FO_5$ taking the specific absorbance to be 394.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, F, G, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, H, I.*

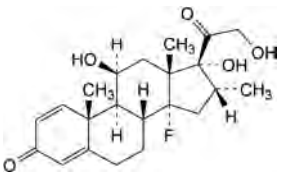

A. 14-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

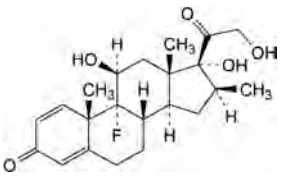

B. 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione (betamethasone),

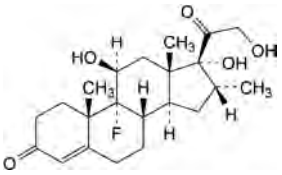

C. 9-fluoro-11β,17,21-trihydroxy-16α-methylpregn-4-ene-3,20-dione,

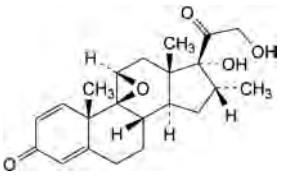

D. 9β,11β-epoxy-17,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

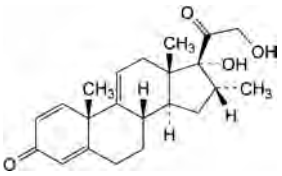

E. 17,21-dihydroxy-16α-methylpregna-1,4,9(11)-triene-3,20-dione,

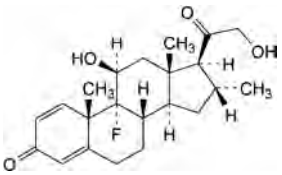

F. 9-fluoro-11β,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

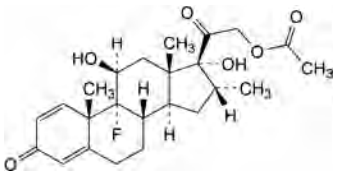

G. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (dexamethasone acetate),

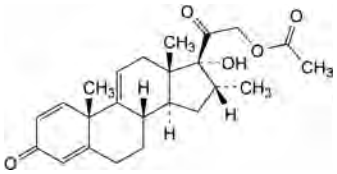

H. 17-hydroxy-16α-methyl-3,20-dioxopregna-1,4,9(11)-trien-21-yl acetate,

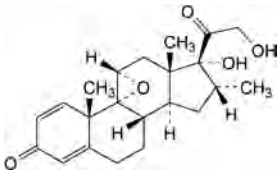

I. 9α,11α-epoxy-17,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

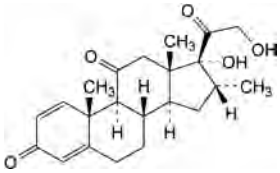

J. 17,21-dihydroxy-16α-methylpregna-1,4-diene-3,11,20-trione,

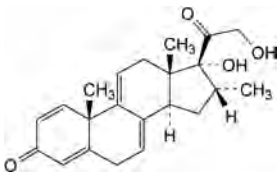

K. 17,21-dihydroxy-16α-methylpregna-1,4,7,9(11)-tetraene-3,20-dione.

**01/2017:0548**

# DEXAMETHASONE ACETATE

## Dexamethasoni acetas

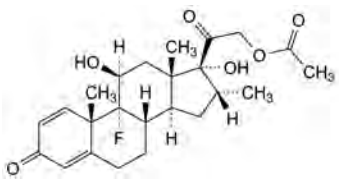

$C_{24}H_{31}FO_6$ $M_r$ 434.5
[1177-87-3]

DEFINITION

9-Fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, G.*

*Second identification: A, C, D, E, F.*

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at the absorption maximum at 419 nm is not less than 0.35.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dexamethasone acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 20 mg of *dexamethasone acetate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *cortisone acetate R* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*, heat at 120 °C for 10 min or until the spots appear, and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a faint reddish-brown colour develops. Add this solution to 10 mL of *water R* and mix. The colour is discharged and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl*

*nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. About 10 mg gives the reaction of acetyl (*2.3.1*).

G. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (e).

TESTS

**Specific optical rotation** (*2.2.7*): + 94 to + 99 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in about 4 mL of *acetonitrile R* and dilute to 10.0 mL with *water for chromatography R*.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 5.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *dexamethasone CRS* (impurity A) and 2 mg of *betamethasone acetate CRS* (impurity D) in the mobile phase, using sonication for about 10 min, and dilute to 100.0 mL with the mobile phase. Mix 1.0 mL of this solution and 6.0 mL of test solution (a) and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *dexamethasone acetate impurity E CRS* in 1.0 mL of the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *dexamethasone acetate for peak identification CRS* (containing impurity I) in about 0.8 mL of *acetonitrile R* and dilute to 2.0 mL with *water for chromatography R*.

*Reference solution (e).* Dissolve 25.0 mg of *dexamethasone acetate CRS* in about 4 mL of *acetonitrile R* and dilute to 10.0 mL with *water for chromatography R*. Dilute 1.0 mL of the solution to 5.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 380 mL of *acetonitrile R* and 550 mL of *water for chromatography R* and allow to equilibrate; dilute to 1000 mL with *water for chromatography R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (c) and (d).

*Run time*: 2.5 times the retention time of dexamethasone acetate.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and D; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E; use the chromatogram supplied with *dexamethasone acetate for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity I.

*Relative retention* with reference to dexamethasone acetate (retention time = about 22 min): impurity A = about 0.4; impurity D = about 0.9; impurity E = about 1.2; impurity I = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.3 between the peaks due to impurity D and dexamethasone acetate.

*Limits*:

- *impurity I*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity D*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities A, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* in an oven at 105 °C.

ASSAY

*Carry out the test protected from light.*

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *acetonitrile R*, *water for chromatography R* (45:55 *V/V*).

*Injection*: test solution (b) and reference solution (e).

*Run time*: 1.5 times the retention time of dexamethasone acetate.

*Retention time*: dexamethasone acetate = about 13 min.

Calculate the percentage content of $C_{24}H_{31}FO_6$ taking into account the assigned content of *dexamethasone acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F, G, H.*

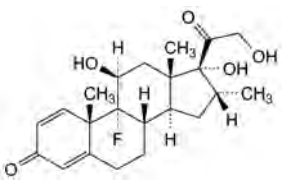


A. 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone),

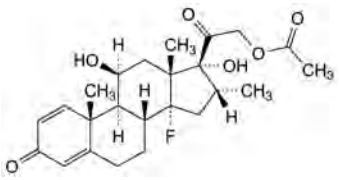

B. 14-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

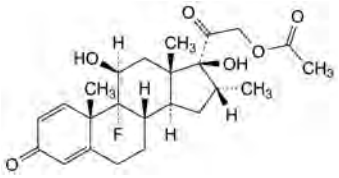

C. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxo-17α-pregna-1,4-dien-21-yl acetate,

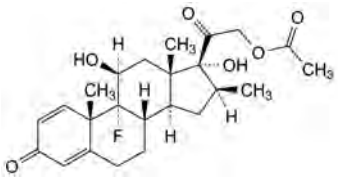

D. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (betamethasone acetate),

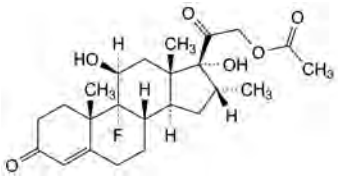

E. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregn-4-en-21-yl acetate,

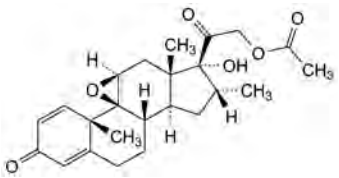

F. 9β,11β-epoxy-17-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

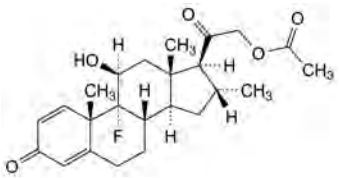

G. 9-fluoro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

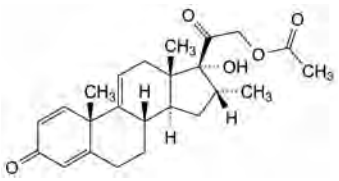

H. 17-hydroxy-16α-methyl-3,20-dioxopregna-1,4,9(11)-trien-21-yl acetate,

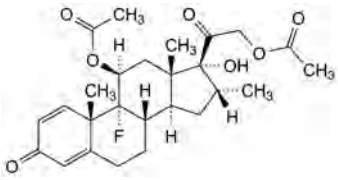

I. 9-fluoro-17-hydroxy-16α-methyl-3,20-dioxopregna-1,4-diene-11β,21-diyl diacetate (dexamethasone 11,21-diacetate).

**01/2008:2237**

# DEXAMETHASONE ISONICOTINATE

## Dexamethasoni isonicotinas

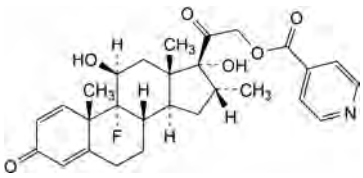

$C_{28}H_{32}FNO_6$ $M_r$ 497.6

[2265-64-7]

DEFINITION

9-Fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl pyridine-4-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol and in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dexamethasone isonicotinate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 142 to + 146 (dried substance).

Suspend 0.200 g in 4.0 mL of *ethyl acetate R* and dilute to 20.0 mL with *ethanol (96 per cent) R*. Treat in an ultrasonic bath until a clear solution is obtained.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare solutions immediately before use.*

*Test solution.* Suspend 50.0 mg in 7 mL of *acetonitrile R* and dilute to 10.0 mL with *water R*. Treat in an ultrasonic bath until a clear solution is obtained.

*Reference solution (a).* Suspend 5.0 mg of *dexamethasone CRS* and 5.0 mg of *dexamethasone acetate CRS* in 70 mL of *acetonitrile R*, add 1.0 mL of the test solution and dilute to 100.0 mL with *water R*. Treat in an ultrasonic bath until a clear solution is obtained.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R*.

*Reference solution (c).* Suspend 5 mg of *dexamethasone isonicotinate for impurity C identification CRS* in 0.7 mL of *acetonitrile R* and dilute to 1 mL with *water R*. Treat in an ultrasonic bath until a clear solution is obtained.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: *water R*,
- *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 68 | 32 |
| 2 - 20 | 68 → 50 | 32 → 50 |
| 20 - 25 | 50 → 68 | 50 → 32 |
| 25 - 35 | 68 | 32 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *dexamethasone isonicotinate for impurity C identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Relative retention* with reference to dexamethasone isonicotinate (retention time = about 12 min): impurity A = about 0.4; impurity C = about 0.6; impurity B = about 0.8.

*System suitability*: reference solution (a):
- *resolution*: minimum 5.0 between the peaks due to impurity B and dexamethasone isonicotinate.

*Limits*:
- *impurity A*: not more than 5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *impurity B*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *impurity C*: not more than 3 times the area of the peak due to dexamethasone isonicotinate in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *unspecified impurities*: for each impurity, not more than the area of the peak due to dexamethasone isonicotinate in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than 8 times the area of the peak due to dexamethasone isonicotinate in the chromatogram obtained with reference solution (b) (0.8 per cent),
- *disregard limit*: 0.5 times the area of the peak due to dexamethasone isonicotinate in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 102 °C under high vacuum for 4 h.

ASSAY

Dissolve 0.400 g in a mixture of 5 mL of *anhydrous formic acid R* and 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 49.76 mg of $C_{28}H_{32}FNO_6$.

IMPURITIES

*Specified impurities: A, B, C.*

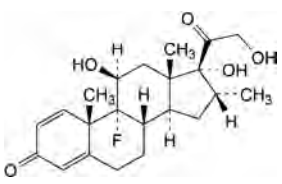

A. 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone),

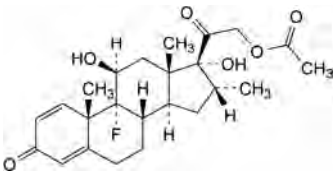

B. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (dexamethasone acetate),

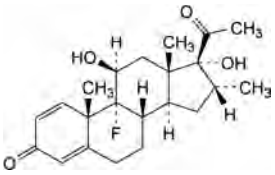

C. 9-fluoro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (21-deoxydexamethasone).

**04/2014:0549**

# DEXAMETHASONE SODIUM PHOSPHATE

## Dexamethasoni natrii phosphas

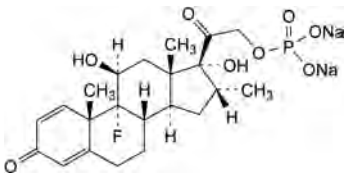

$C_{22}H_{28}FNa_2O_8P$ $M_r$ 516.4
[2392-39-4]

DEFINITION

9-Fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, G.*

*Second identification: A, C, D, E, F.*

A. Dissolve 10.0 mg in 5 mL of *water R* and dilute to 100.0 mL with *anhydrous ethanol R*. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) measured at the absorption maximum at 419 nm is at least 0.20.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dexamethasone sodium phosphate CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *dexamethasone sodium phosphate CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *prednisolone sodium phosphate CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*, heat at 120 °C for 10 min or until the spots appear, and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which may, however, not be completely separated.

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a faint yellowish-brown colour develops. Add this solution to 10 mL of *water R* and mix. The colour fades and a clear solution remains.

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

F. To 40 mg add 2 mL of *sulfuric acid R* and heat gently until white fumes are evolved, add *nitric acid R* dropwise, continue the heating until the solution is almost colourless and cool. Add 2 mL of *water R*, heat until white fumes are again evolved, cool, add 10 mL of *water R* and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (*2.3.1*) and reaction (b) of phosphates (*2.3.1*).

G. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 9.5.

Dilute 1 mL of solution S to 5 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 75 to + 83 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 7.0 g of *ammonium acetate R* in 1000 mL of *water R*.

*Test solution*. Dissolve 10 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 2 mg of *betamethasone sodium phosphate CRS* (impurity B) and 2 mg of *dexamethasone sodium phosphate CRS* in mobile phase A, then dilute to 100.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2 mg of *dexamethasone sodium phosphate for peak identification CRS* (containing impurities A, C, D, E, F and G) in mobile phase A and dilute to 2.0 mL with mobile phase A.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: mix 300 mL of solution A and 350 mL of *water R*, adjust to pH 3.8 with *acetic acid R*, then add 350 mL of *methanol R*;
– *mobile phase B*: adjust 300 mL of solution A to pH 4.0 with *acetic acid R*, then add 700 mL of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3.5 | 90 | 10 |
| 3.5 - 23.5 | 90 → 60 | 10 → 40 |
| 23.5 - 34.5 | 60 → 5 | 40 → 95 |
| 34.5 - 50 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *dexamethasone sodium phosphate for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C, D, E, F and G; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to dexamethasone sodium phosphate (retention time = about 22 min): impurity C = about 0.5; impurity D = about 0.6; impurity E = about 0.8; impurity F = about 0.92; impurity B = about 0.95; impurity A = about 1.37; impurity G = about 1.41.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity B and dexamethasone sodium phosphate.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.75;
– *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

- *impurity G*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurities B, C, D, E, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Inorganic phosphates**: maximum 1 per cent.

Dissolve 50 mg in *water R* and dilute to 100 mL with the same solvent. To 10 mL of this solution add 5 mL of *molybdovanadic reagent R*, mix and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 mL of *phosphate standard solution (5 ppm $PO_4$) R*.

**Ethanol** (*2.4.24, System A*): maximum 1.5 per cent.

**Water** (*2.5.12*): maximum 10.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *dexamethasone CRS* (impurity A) and 2 mg of *dexamethasone sodium phosphate CRS* in 2 mL of *tetrahydrofuran R*, then dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 30.0 mg of *dexamethasone sodium phosphate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 520 mL of *water R* with 2 mL of *phosphoric acid R*. Adjust the temperature to 20 °C, then adjust to pH 2.6 with *sodium hydroxide R*. Mix this solution with 36 mL of *tetrahydrofuran R* and 364 mL of *methanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of dexamethasone sodium phosphate.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to dexamethasone sodium phosphate (retention time = about 8 min): impurity A = about 2.0.

*System suitability*: reference solution (a):

- *resolution*: minimum 6.0 between the peaks due to dexamethasone sodium phosphate and impurity A.

Calculate the percentage content of $C_{22}H_{28}FNa_2O_8P$ using the chromatogram obtained with reference solution (b) and taking into account the assigned content of *dexamethasone sodium phosphate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H.*

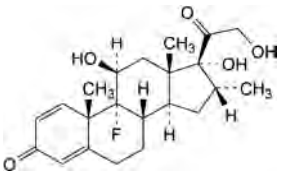

A. 9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (dexamethasone),

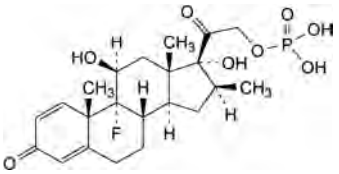

B. 9-fluoro-11β,17-dihydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-21-yl dihydrogen phosphate (betamethasone phosphate),

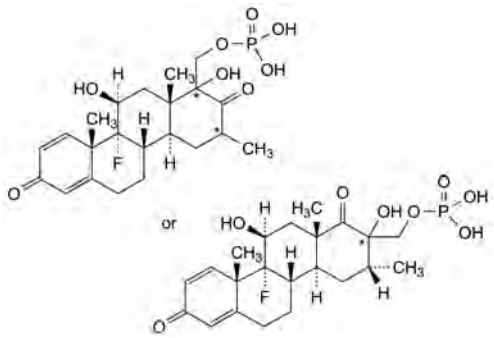

C, D, E, F. for each impurity, one or more diastereoisomer(s) of (9-fluoro-11β,17a-dihydroxy-16-methyl-3,17-dioxo-*D*-homo-androsta-1,4-dien-17a-yl)methyl dihydrogen phosphate (undefined stereochemistry at C-16 and C-17a), or (9-fluoro-11β,17-dihydroxy-16α-methyl-3,17a-dioxo-*D*-homo-androsta-1,4-dien-17-yl)methyl dihydrogen phosphate (undefined stereochemistry at C-17),

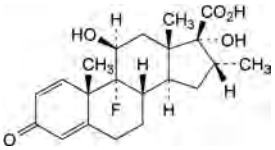

G. 9-fluoro-11β,17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid,

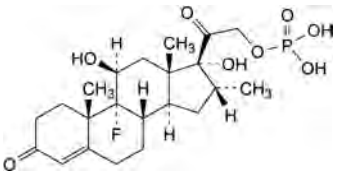

H. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregn-4-en-21-yl dihydrogen phosphate.

**04/2019:2752**

# DEXAMFETAMINE SULFATE

## Dexamfetamini sulfas

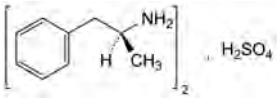

$C_{18}H_{28}N_2O_4S$ $M_r$ 368.5
[51-63-8]

DEFINITION

Bis[(2*S*)-1-phenylpropan-2-amine] sulfate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: C, D.*

A. Enantiomeric purity (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dexamfetamine sulfate CRS*.

C. To 50 mL of solution S add 5 mL of *strong sodium hydroxide solution R* and 0.5 mL of *benzoyl chloride R* and shake. Continue to add *benzoyl chloride R* in portions of 0.5 mL, shaking after each addition, until no further precipitate is formed. Filter, wash the precipitate with *water R*, recrystallise twice from a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*, then dry at 100-105 °C. The crystals melt (*2.2.14*) at 155 °C to 160 °C.

D. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 25 mL of solution S add 0.1 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *amfetamine sulfate CRS* (containing impurity E and dexamfetamine) in 5 mL of *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *vancomycin-bonded silica gel for chiral separation R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *concentrated ammonia R1*, *glacial acetic acid R*, *methanol R2* (0.1:1:998.9 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 1.5 times the retention time of dexamfetamine.

*Identification of peaks*: use the chromatogram supplied with *amfetamine sulfate CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to dexamfetamine and impurity E.

*Relative retention* with reference to dexamfetamine (retention time = about 10 min): impurity E = about 1.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.5 between the peaks due to dexamfetamine and impurity E.

*Calculation of percentage content*:
- for impurity E, use the concentration of dexamfetamine sulfate in reference solution (a).

*Limit*:
- *impurity E*: maximum 5.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 5 mL of *trifluoroacetic acid R* and 900 mL of *water for chromatography R*, adjust to pH 2.2 with *concentrated ammonia R* and dilute to 1000 mL with *acetonitrile R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *1-phenylpropan-2-ol R* (impurity A) and 5 mg of *benzaldehyde R* (impurity D) in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 1 mL of the solution to 100 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: solvent mixture;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 1 - 16 | 100 → 65 | 0 → 35 |
| 16 - 21 | 65 → 0 | 35 → 100 |
| 21 - 23 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 257 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and D.

*Relative retention* with reference to dexamfetamine (retention time = about 8 min): impurity D = about 1.6; impurity A = about 1.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to impurities D and A.

*Calculation of percentage contents*:
- for each impurity, use the concentration of dexamfetamine sulfate in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.85 mg of $C_{18}H_{28}N_2O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

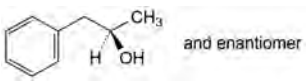


A. (2*RS*)-1-phenylpropan-2-ol,

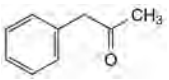


B. 1-phenylpropan-2-one,

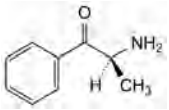


C. (2*S*)-2-amino-1-phenylpropan-1-one (cathinone),

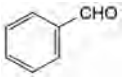


D. benzaldehyde,

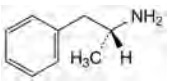


E. (2*R*)-1-phenylpropan-2-amine (levamfetamine).

**01/2017:1196**
**corrected 10.0**

# DEXCHLORPHENIRAMINE MALEATE

## Dexchlorpheniramini maleas

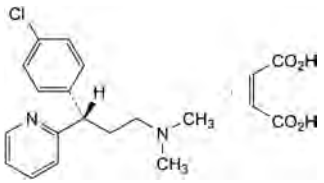


$C_{20}H_{23}ClN_2O_4$ $M_r$ 390.9
[2438-32-6]

DEFINITION

(3*S*)-3-(4-Chlorophenyl)-*N*,*N*-dimethyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butenedioate.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

*First identification: A, C, E.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 110 °C to 115 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *dexchlorpheniramine maleate CRS*.

D. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.
*Reference solution.* Dissolve 56 mg of *maleic acid R* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *water R*, *anhydrous formic acid R*, *methanol R*, *di-isopropyl ether R* (3:7:20:70 *V/V/V/V*).
*Application*: 5 µL.
*Development*: over a path of 12 cm.
*Drying*: in a current of air for a few minutes.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the chromatogram obtained with the test solution shows 2 clearly separated spots. The upper spot is similar in position and size to the spot in the chromatogram obtained with the reference solution.

E. To 0.15 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 10 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.5.

Dissolve 0.20 g in 20 mL of *water R*.

**Specific optical rotation** (*2.2.7*): + 22 to + 23 (dried substance), determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 1.0 mL of *methylene chloride R*.

*Reference solution.* Dissolve 5.0 mg of *brompheniramine maleate CRS* in 0.5 mL of *methylene chloride R* and add 0.5 mL of the test solution. Dilute 0.5 mL of this solution to 50.0 mL with *methylene chloride R*.

*Column*:

- *material*: glass;
- *size*: *l* = 2.3 m, Ø = 2 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (135-175 µm) impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 20 mL/min.

*Temperature*:

- *column*: 205 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 2.5 times the retention time of dexchlorpheniramine.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to dexchlorpheniramine and brompheniramine.

*Limits*:

- *impurity A*: not more than 0.8 times the area of the peak due to dexchlorpheniramine in the chromatogram obtained with the reference solution (0.4 per cent);
- *total*: not more than twice the area of the peak due to dexchlorpheniramine in the chromatogram obtained with the reference solution (1 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 3 mL of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 mL of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *dexchlorpheniramine maleate CRS* in 3 mL of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 mL of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *chlorphenamine maleate CRS* in 3 mL of *water R*. Add a few drops of *concentrated ammonia R* until an alkaline reaction is produced. Shake with 5 mL of *methylene chloride R*. Separate the layers. Evaporate the lower, methylene chloride layer to an oily residue on a water-bath. Dissolve the oily residue in *2-propanol R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 50 mL with *2-propanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chromatography R*.

*Mobile phase*: *diethylamine R*, *2-propanol R*, *hexane R* (3:20:980 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

Under these conditions the peak due to the (*S*)-isomer appears first.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to the (*R*)-enantiomer (impurity B) and the (*S*)-enantiomer in the chromatogram obtained with reference solution (b);
- the retention times of the principal peaks in the chromatograms obtained with the test solution and reference solution (a) are identical ((*S*)-enantiomer).

*Limits*:

- *(R)-enantiomer* (impurity B): not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 65 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.54 mg of $C_{20}H_{23}ClN_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

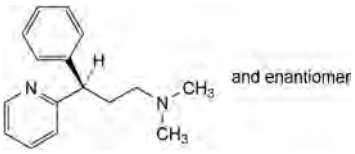

and enantiomer

A. (3*RS*)-*N*,*N*-dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine,

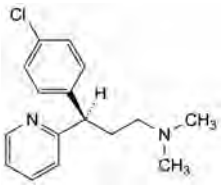


B. (3*R*)-3-(4-chlorophenyl)-*N*,*N*-dimethyl-3-(pyridin-2-yl)propan-1-amine ((*R*)-enantiomer).

**01/2017:0761**

# DEXPANTHENOL

## Dexpanthenolum

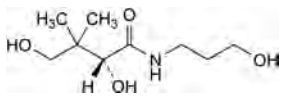


$C_9H_{19}NO_4$ $M_r$ 205.3

[81-13-0]

DEFINITION

Dexpanthenol contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-2,4-dihydroxy-*N*-(3-hydroxypropyl)-3,3-dimethylbutanamide, calculated with reference to the anhydrous substance.

CHARACTERS

A colourless or slightly yellowish, viscous hygroscopic liquid, or a white or almost white, crystalline powder, very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *dexpanthenol CRS*. Examine the substances using discs prepared as follows: dissolve the substance to be examined and the reference substance separately in 1.0 mL of *anhydrous ethanol R* to obtain a concentration of 5 mg/mL. Place dropwise 0.5 mL of this solution on a disc of *potassium bromide R*. Dry the disc at 100-105 °C for 15 min.

C. Examine the chromatograms obtained in the test for 3-aminopropanol. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 mL of solution S (see Tests) add 1 mL of *dilute sodium hydroxide solution R* and 0.1 mL of *copper sulfate solution R*. A blue colour develops.

TESTS

**Solution S**. Dissolve 2.500 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is not greater than 10.5.

**Specific optical rotation** (*2.2.7*). The specific optical rotation is + 29.0 to + 32.0, determined on solution S and calculated with reference to the anhydrous substance.

**3-Aminopropanol**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.25 g of the substance to be examined in *anhydrous ethanol R* and dilute to 5 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *anhydrous ethanol R*.

*Reference solution (a).* Dissolve the contents of a vial of *dexpanthenol CRS* in 1.0 mL of *anhydrous ethanol R* to obtain a concentration of 5 mg/mL.

*Reference solution (b).* Dissolve 25 mg of *3-aminopropanol R* in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *concentrated ammonia R*, 25 volumes of *methanol R* and 55 volumes of *butanol R*. Allow the plate to dry in air, spray with a 100 g/L solution of *trichloroacetic acid R* in *methanol R* and heat at 150 °C for 10 min. Spray with a 1 g/L solution of *ninhydrin R* in *methanol R* and heat at 120 °C until a colour appears. Any spot due to 3-aminopropanol in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Water** (*2.5.12*). Not more than 1.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.400 g add 50.0 mL of *0.1 M perchloric acid*. Boil under a reflux condenser for 5 h protected from humidity. Allow to cool. Add 50 mL of *dioxan R* by rinsing the condenser, protected from humidity. Add 0.2 mL of *naphtholbenzein solution R* and titrate with *0.1 M potassium hydrogen phthalate* until the colour changes from green to yellow. Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 20.53 mg of $C_9H_{19}NO_4$.

STORAGE

In an airtight container.

**04/2018:1506**

# DEXTRAN 1 FOR INJECTION

## Dextranum 1 ad iniectabile

DEFINITION

Low-molecular-weight fraction of dextran, consisting of a mixture of isomaltooligosaccharides.

*Average relative molecular mass*: about 1000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512 F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

*Appearance*: white or almost white hygroscopic powder.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 3.000 g in *water R*, heat on a water-bath and dilute to 100.0 mL with the same solvent. The specific optical rotation (*2.2.7*) is + 148 to + 164, calculated with reference to the dried substance. Dry an aliquot of the solution first on a water-bath and then to constant weight *in vacuo* at 70 °C. Calculate the dextran content after correction for the content of sodium chloride.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: to 1-2 mg add 1 or a few drops of *water R*. Grind in an agate mortar for 1-2 min. Add about 300 mg of *potassium bromide R* and mix to a slurry but do not grind. Dry *in vacuo* at 40 °C for 15 min. Crush the residue. If it is not dry, dry for another 15 min. Prepare a disc with the residue.

*Comparison*: repeat the operations using *dextran 1 CRS*.

C. Molecular-mass distribution (see Tests).

TESTS

**Solution S**. Dissolve 7.5 g in *carbon dioxide-free water R*, heat on a water-bath and dilute to 50 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.12, determined at 375 nm on solution S.

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is pink. Add

0.4 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.1 mL of *methyl red solution R*. The solution is red or orange.

**Nitrogen-containing substances**: maximum 110 ppm of N.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 mL of *bromocresol green solution R*, 0.5 mL of *methyl red solution R* and 20 mL of *water R*. Titrate with *0.01 M hydrochloric acid*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Sodium chloride**: maximum 1.5 per cent.

Accurately weigh 3-5 g and dissolve in 100 mL of *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

**Molecular-mass distribution**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dissolve 6.0-6.5 mg of the substance to be examined in 1.0 mL of the mobile phase.

*Reference solution (a)*. Dissolve 6.0-6.5 mg of *dextran 1 CRS* in 1.0 mL of the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *isomaltooligosaccharide CRS* in 1 mL of the mobile phase, and mix. This corresponds to approximately 45 µg of isomaltotriose (3 glucose units), approximately 45 µg of isomaltononaose (9 glucose units), and approximately 60 µg of sodium chloride per 100 µL.

*Column*: 2 columns coupled in series:
- *size*: *l* = 0.30 m, Ø = 10 mm;
- *stationary phase*: dextran covalently bound to highly cross-linked porous agarose beads, allowing resolution of oligosaccharides in the molecular mass range of 180 to 3000;
- *temperature*: 20-25 °C.

*Mobile phase*: 2.92 g/L solution of *sodium chloride R*.

*Flow rate*: 0.07-0.08 mL/min maintained constant to ± 1 per cent.

*Detection*: differential refractometer.

*Injection*: 100 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (b) to identify the peaks due to isomaltotriose, isomaltononaose and sodium chloride.

Determine the peak areas. Disregard any peak due to sodium chloride. Calculate the average relative molecular mass $M_w$ and the amount of the fraction with less than 3 and more than 9 glucose units, of *dextran 1 CRS* and of the substance to be examined, using the following expression:

$$M_w = \sum w_i \times m_i$$

$M_w$ = average molecular mass of the dextran;

$m_i$ = molecular mass of oligosaccharide *i*;

$w_i$ = weight proportion of oligosaccharide *i*.

Use the following $m_i$ values for the calculation:

| Oligosaccharide *i* | $m_i$ |
|---|---|
| glucose | 180 |
| isomaltose | 342 |
| isomaltotriose | 504 |
| isomaltotetraose | 666 |
| isomaltopentaose | 828 |
| isomaltohexaose | 990 |
| isomaltoheptaose | 1152 |
| isomaltooctaose | 1314 |
| isomaltononaose | 1476 |
| isomaltodecaose | 1638 |
| isomaltoundecaose | 1800 |
| isomaltododecaose | 1962 |
| isomaltotridecaose | 2124 |
| isomaltotetradecaose | 2286 |
| isomaltopentadecaose | 2448 |
| isomaltohexadecaose | 2610 |
| isomaltoheptadecaose | 2772 |
| isomaltooctadecaose | 2934 |
| isomaltononadecaose | 3096 |

*System suitability*: the values obtained for *dextran 1 CRS* are within the values stated on the label.

*Limits*:
- *average molecular mass ($M_w$)*: 850 to 1150;
- *fraction with less than 3 glucose units*: less than 15 per cent;
- *fraction with more than 9 glucose units*: less than 20 per cent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 5.000 g by drying in an oven at 105 °C for 5 h.

**Bacterial endotoxins** (*2.6.14*): less than 25 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**01/2017:0999**

# DEXTRAN 40 FOR INJECTION

## Dextranum 40 ad iniectabile

DEFINITION

Mixture of polysaccharides, principally of the α-1,6-glucan type.

*Average relative molecular mass*: about 40 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 195 to + 201 (dried substance).

Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dextran CRS*.

C. Molecular-mass distribution (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.1 mL of *methyl red solution R*. The solution is red or orange.

**Nitrogen-containing substances**: maximum 110 ppm N.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 mL of *bromocresol green solution R*, 0.5 mL of *methyl red solution R* and 20 mL of *water R*. Titrate with *0.01 M hydrochloric acid*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Residual solvents**. Gas chromatography (*2.2.28*).

*Internal standard*: *propanol R*.

*Test solution*. Dissolve 5 g of the substance to be examined in 100 mL of *water R* and distil. Collect the first 45 mL of the distillate, add 1 mL of a 25 g/L solution of *propanol R* and dilute to 50 mL with *water R*.

*Reference solution*. Mix 0.5 mL of a 25 g/L solution of *anhydrous ethanol R*, 0.5 mL of a 25 g/L solution of *propanol R* and 0.5 mL of a 2.5 g/L solution of *methanol R* and dilute to 25.0 mL with *water R*.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 1.8 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (125-150 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 25 mL/min.

*Temperature*:

- *column*: 190 °C;
- *injection port*: 240 °C;
- *detector*: 210 °C.

*Detection*: flame ionisation.

*Injection*: the chosen volume of each solution.

*Limits*:

- *ethanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent);
- *methanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent);
- *sum of solvents other than ethanol, methanol and propanol*: not more than the area of the peak due to the internal standard (0.5 per cent, calculated as propanol).

**Molecular-mass distribution** (*2.2.39*). The average molecular mass ($M_w$) is 35 000 to 45 000. The average molecular mass of the 10 per cent high fraction is not greater than 110 000. The average molecular mass of the 10 per cent low fraction is not less than 7000.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 0.50 g.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**01/2017:1000**

# DEXTRAN 60 FOR INJECTION

## Dextranum 60 ad iniectabile

DEFINITION

Mixture of polysaccharides, principally of the α-1,6-glucan type.

*Average relative molecular mass*: about 60 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 195 to + 201 (dried substance).

Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dextran CRS*.

C. Molecular-mass distribution (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.1 mL of *methyl red solution R*. The solution is red or orange.

**Nitrogen-containing substances**: maximum 110 ppm of N.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 mL of *bromocresol green solution R*, 0.5 mL of *methyl red solution R* and 20 mL of *water R*. Titrate with *0.01 M hydrochloric acid*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Residual solvents**. Gas chromatography (*2.2.28*).

*Internal standard*: *propanol R*.

*Test solution*. Dissolve 5 g of the substance to be examined in 100 mL of *water R* and distil. Collect the first 45 mL of the distillate, add 1 mL of a 25 g/L solution of *propanol R* and dilute to 50 mL with *water R*.

*Reference solution*. Mix 0.5 mL of a 25 g/L solution of *anhydrous ethanol R*, 0.5 mL of a 25 g/L solution of *propanol R* and 0.5 mL of a 2.5 g/L solution of *methanol R* and dilute to 25.0 mL with *water R*.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 1.8 m, Ø = 2 mm;

– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (125-150 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 25 mL/min.

*Temperature*:
– *column*: 190 °C;
– *injection port*: 240 °C;
– *detector*: 210 °C.

*Detection*: flame ionisation.

*Injection*: the chosen volume of each solution.

*Limits*:
– *ethanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent);
– *methanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent);
– *sum of solvents other than ethanol, methanol and propanol*: not more than the area of the peak due to the internal standard (0.5 per cent, calculated as propanol).

**Molecular-mass distribution** (*2.2.39*). The average molecular mass ($M_w$) is 54 000 to 66 000. The average molecular mass of the 10 per cent high fraction is not greater than 180 000. The average molecular mass of the 10 per cent low fraction is not less than 14 000.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 0.50 g.

**Bacterial endotoxins** (*2.6.14*): less than 16 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**01/2017:1001**

# DEXTRAN 70 FOR INJECTION

## Dextranum 70 ad iniectabile

DEFINITION

Mixture of polysaccharides, principally of the α-1,6-glucan type.

*Average relative molecular mass*: about 70 000.

PRODUCTION

It is obtained by hydrolysis and fractionation of dextrans produced by fermentation of sucrose using *Leuconostoc mesenteroides* strain NRRL B-512 = CIP 78.59 or substrains thereof (for example *L. mesenteroides* B-512F = NCTC 10817).

It is prepared in conditions designed to minimise the risk of microbial contamination.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 195 to + 201 (dried substance).
Dissolve 1.0 g in *water R*, heating on a water-bath, and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dextran CRS*.

C. Molecular-mass distribution (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R*, heating on a water-bath, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution remains colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.1 mL of *methyl red solution R*. The solution is red or orange.

**Nitrogen-containing substances**: maximum 110 ppm of N.
Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.200 g and heating for 2 h. Collect the distillate in a mixture of 0.5 mL of *bromocresol green solution R*, 0.5 mL of *methyl red solution R* and 20 mL of *water R*. Titrate with *0.01 M hydrochloric acid*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Residual solvents**. Gas chromatography (*2.2.28*).

*Internal standard*: *propanol R*.

*Test solution*. Dissolve 5 g of the substance to be examined in 100 mL of *water R* and distil. Collect the first 45 mL of the distillate, add 1 mL of a 25 g/L solution of *propanol R* and dilute to 50 mL with *water R*.

*Reference solution*. Mix 0.5 mL of a 25 g/L solution of *anhydrous ethanol R*, 0.5 mL of a 25 g/L solution of *propanol R* and 0.5 mL of a 2.5 g/L solution of *methanol R* and dilute to 25.0 mL with *water R*.

*Column*:
– *material*: stainless steel;
– *size*: *l* = 1.8 m, Ø = 2 mm;
– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (125-150 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 25 mL/min.

*Temperature*:
– *column*: 190 °C;
– *injection port*: 240 °C;
– *detector*: 210 °C.

*Detection*: flame ionisation.

*Injection*: the chosen volume of each solution.

*Limits*:
– *ethanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent);
– *methanol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent);
– *sum of solvents other than ethanol, methanol and propanol*: not more than the area of the peak due to the internal standard (0.5 per cent, calculated as propanol).

**Molecular-mass distribution** (*2.2.39*). The average molecular mass ($M_w$) is 64 000 to 76 000. The average molecular mass of the 10 per cent high fraction is not greater than 185 000. The average molecular mass of the 10 per cent low fraction is not less than 15 000.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 0.200 g by heating in an oven at 105 ± 2 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 0.50 g.

**Bacterial endotoxins** (*2.6.14*): less than 16 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

01/2017:2238

# DEXTRANOMER

## Dextranomerum

[56087-11-7]

DEFINITION

Three-dimensional network made of dextran chains *O*,*O*′-cross-linked with 2-hydroxypropane-1,3-diyl bridges and *O*-substituted with 2,3-dihydroxypropyl and 2-hydroxy-1-(hydroxymethyl)ethyl groups.

CHARACTERS

*Appearance*: white or almost white, spherical beads.

*Solubility*: practically insoluble in water. It swells in water and in electrolyte solutions.

PRODUCTION

The absorption capacity is determined using a 9.0 g/L solution of *sodium chloride R* containing 20 µL/L of *polysorbate 20 R* or another suitable solution, with a suitable, validated method.

The particle size is controlled to a minimum of 80 per cent of the number of dry beads within 100-300 µm and a maximum of 7 per cent of their number below 100 µm using a suitable, validated method.

IDENTIFICATION

A. The substance to be examined is practically insoluble in *water R*. It swells in *water R*.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: grind the substance to be examined in *acetone R*. Evaporate the solvent at room temperature and use the residue.

*Comparison*: *dextranomer CRS*.

TESTS

**pH** (*2.2.3*): 5.3 to 7.5.

Introduce 0.50 g to 30 mL of a freshly prepared 74.6 g/L solution of *potassium chloride R*. Allow to stand for 2 min. Determine the pH on the mucilage obtained.

**Boron**: maximum 30 ppm.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) (*2.2.57*).

*Test solution*. Introduce 3.0 g into a platinum dish and moisten with 5 mL of a 32.1 g/L solution of *magnesium nitrate R* in a mixture of equal volumes of *ethanol (96 per cent) R* and *distilled water R*. Evaporate to dryness on a water-bath. Ignite at 550 °C for 5 h. Take up the residue with 5 mL of *6 M hydrochloric acid R* and transfer to a 50 mL volumetric flask. Add about 20 mL of *distilled water R* and allow to digest for 1 h on a water-bath. Allow to cool and dilute to 50.0 mL with *distilled water R*.

*Reference solutions*. Prepare the reference solutions using a solution of *boric acid R* containing 10 ppm of boron. Proceed as described for the test solution.

*Wavelength*: 249.773 nm.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 15 h.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*), determined using the pour-plate method.

01/2017:1507
corrected 9.6

# DEXTRIN

## Dextrinum

[9004-53-9]

DEFINITION

Maize, potato or cassava starch partly hydrolysed and modified by heating with or without the presence of acids, alkalis or pH-control agents.

CHARACTERS

*Appearance*: white or almost white, free-flowing powder.

*Solubility*: very soluble in boiling water forming a mucilaginous solution, slowly soluble in cold water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. To 1 mL of the solution add 0.05 mL of *iodine solution R1*. A dark blue or reddish-brown colour is produced, which disappears on heating.

B. Centrifuge 5 mL of the mucilage obtained in identification test A. To the upper layer add 2 mL of *dilute sodium hydroxide solution R* and, dropwise with shaking, 0.5 mL of *copper sulfate solution R* and boil. A red precipitate is produced.

C. It is very soluble in boiling *water R*, forming a mucilaginous solution.

TESTS

**pH** (*2.2.3*): 2.0 to 8.0.

Disperse 5.0 g in 100 mL of *carbon dioxide-free water R*.

**Chlorides**: maximum 0.2 per cent.

Dissolve 2.5 g in 50 mL of boiling *water R*, dilute to 100 mL with *water R* and filter. Dilute 1 mL of the filtrate to 15 mL with *water R* and add 1 mL of *dilute nitric acid R*. Pour the mixture as a single addition into 1 mL of *silver nitrate solution R2* and allow to stand for 5 min protected from light. When viewed transversely against a black background any opalescence produced is not more intense than that obtained by treating a mixture of 10 mL of *chloride standard solution (5 ppm Cl) R* and 5 mL of *water R*, prepared in the same manner.

**Reducing sugars**: maximum 10 per cent, calculated as glucose $C_6H_{12}O_6$.

To a quantity of dextrin equivalent to 2.0 g (dried substance) add 100 mL of *water R*, shake for 30 min, dilute to 200.0 mL with *water R* and filter. To 10.0 mL of alkaline *cupri-tartaric solution R* add 20.0 mL of the filtrate, mix, and heat on a hot plate adjusted to bring the solution to boil within 3 min. Boil for 2 min, and cool immediately. Add 5 mL of a 300 g/L solution of *potassium iodide R* and 10 mL of *dilute sulfuric acid R*, mix, and titrate immediately with *0.1 M sodium thiosulfate*, using *starch solution R*, added towards the end of the titration, as indicator. Repeat the procedure beginning with "To 10.0 mL of...", using, in place of the filtrate, 20.0 mL of a 1 g/L solution of *glucose R*, accurately prepared. Perform a blank titration. $(V_B - V_U)$ is not greater than $(V_B - V_S)$, in which $V_B$, $V_U$ and $V_S$ are the number of millilitres of *0.1 M sodium thiosulfate* consumed in the titrations of the blank, the dextrin and the glucose, respectively.

**Loss on drying** (*2.2.32*): maximum 13.0 per cent, determined on 1.000 g by drying at 130 ± 5 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for dextrin used as filler and binder, in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

*The following characteristic may be relevant for dextrin used as viscosity-increasing agent.*

**Apparent viscosity** (*2.2.10*): typically 100 mPa·s to 350 mPa·s (dried substance), depending on the grade of dextrin.

In a beaker, prepare a 10-50 per cent slurry so that the viscosity value ranges from 100 mPa·s to 350 mPa·s. The total mass of the sample plus water must be 600 g. Mix with a plastic rod to obtain a homogeneous slurry. Place the beaker in a water-bath at 100 ± 1 °C. Introduce the paddle of a stirrer into the beaker and close the beaker with a lid. Start agitation at 250 r/min as rapidly as possible and carry on for exactly 30 min. Transfer the paste immediately to the beaker to be used for viscosity measurement, placed in a water-bath at 40 ± 1 °C. Stir until the temperature in the beaker is 40 ± 1 °C then measure the apparent viscosity using spindle no. 2 and a rotation speed of 100 r/min.

**07/2016:0020**

# DEXTROMETHORPHAN HYDROBROMIDE

## Dextromethorphani hydrobromidum

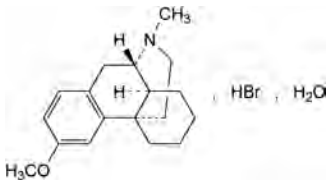


$C_{18}H_{26}BrNO,H_2O$ $M_r$ 370.3
[6700-34-1]

DEFINITION

*ent*-3-Methoxy-17-methylmorphinan hydrobromide monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 125 °C, with decomposition.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dextromethorphan hydrobromide CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 25 mg of *dextromethorphan hydrobromide CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel G plate R.*
*Mobile phase*: *concentrated ammonia R*, *methylene chloride R*, *methanol R*, *ethyl acetate R*, *toluene R* (2:10:13:20:55 *V/V/V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *potassium iodobismuthate solution R2.*
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 0.4 g in *carbon dioxide-free water R* with gentle heating, cool and dilute to 20 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Specific optical rotation** (*2.2.7*): + 28 to + 30 (anhydrous substance).

Dissolve 0.200 g in *0.1 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *dextromethorphan impurity A CRS* in 2 mL of the test solution and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.11 g of *docusate sodium R* in a mixture of 400 mL of *water R* and 600 mL of *acetonitrile R*, add 0.56 g of *sodium nitrate R* and adjust to apparent pH 2.0 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of dextromethorphan.

*Relative retention* with reference to dextromethorphan (retention time = about 22 min): impurity B = about 0.4; impurity C = about 0.8; impurity D = about 0.9; impurity A = about 1.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to dextromethorphan and impurity A.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.2;
- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent), and not more than 1 such peak has an area greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

***N,N*-Dimethylaniline**: maximum 10 ppm.

Dissolve 0.5 g with heating in 20 mL of *water R*. Allow to cool, add 2 mL of *dilute acetic acid R* and 1 mL of a 10 g/L solution of *sodium nitrite R* and dilute to 25 mL with *water R*. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 20 mL of a 0.25 mg/L solution of *N,N-dimethylaniline R*.

**Water** (*2.5.12*): 4.0 per cent to 5.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 20 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.23 mg of $C_{18}H_{26}BrNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

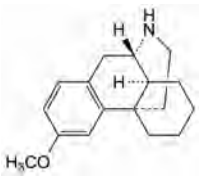

A. *ent*-3-methoxymorphinan,

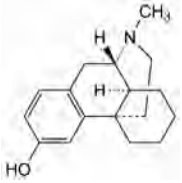

B. *ent*-17-methylmorphinan-3-ol,

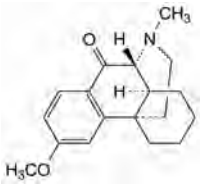

C. *ent*-3-methoxy-17-methylmorphinan-10-one,

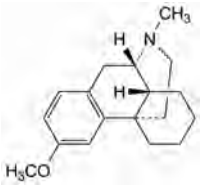

D. *ent*-(14*S*)-3-methoxy-17-methylmorphinan.

**01/2008:0021**
**corrected 6.0**

# DEXTROMORAMIDE TARTRATE

## Dextromoramidi tartras

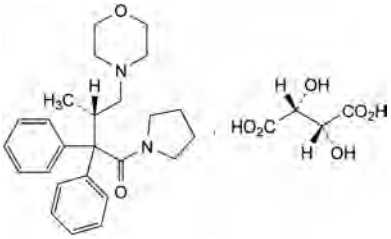

$C_{29}H_{38}N_2O_8$ $M_r$ 542.6
[2922-44-3]

DEFINITION

Dextromoramide tartrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 1-[(3*S*)-3-methyl-4-(morpholin-4-yl)-2,2-diphenylbutanoyl]pyrrolidine hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous or crystalline powder, soluble in water, sparingly soluble in alcohol.

It melts at about 190 °C, with slight decomposition.

IDENTIFICATION

A. Dissolve 75 mg in *1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows 3 absorption maxima, at 254 nm, 259 nm and 264 nm. The specific absorbances at the maxima are about 6.9, 7.7 and 6.5, respectively.

B. Dissolve about 50 mg in *water R* and dilute to 10 mL with the same solvent. To 2 mL of the solution add 3 mL of *ammoniacal silver nitrate solution R* and heat on a water-bath. A grey or black precipitate is formed.

C. It gives reaction (b) of tartrates (*2.3.1*).

TESTS

**pH** (*2.2.3*). Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent. The pH of the solution is 3.0 to 4.0.

**Specific optical rotation** (*2.2.7*). Dissolve 0.50 g in *0.1 M hydrochloric acid* and dilute to 10.0 mL with the same acid. The specific optical rotation is + 21 to + 23.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dilute 1 mL of the test solution to 100 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using *methanol R*. Allow the plate to dry in air and spray with *dilute potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.05 M perchloric acid* using 0.15 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.05 M perchloric acid* is equivalent to 27.13 mg of $C_{29}H_{38}N_2O_8$.

**01/2010:0713**

# DEXTROPROPOXYPHENE HYDROCHLORIDE

## Dextropropoxypheni hydrochloridum

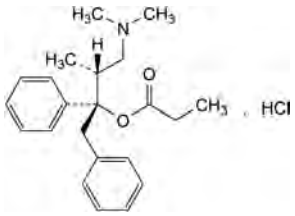

$C_{22}H_{30}ClNO_2$ $M_r$ 375.9
[1639-60-7]

DEFINITION

(1*S*,2*R*)-1-Benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl propanoate hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

mp: about 165 °C.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dextropropoxyphene hydrochloride CRS*.

C. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 30 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 10 mL of solution S to 25 mL with *carbon dioxide-free water R*. To 10 mL of this solution add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Specific optical rotation** (*2.2.7*): + 52 to + 57.

Dissolve 0.100 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *methanol R* (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2 mg of *dextropropoxyphene for system suitability CRS* (containing impurities A, B, C and D) in 1.0 mL of the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of *toluene R* to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 2.5 g of *ammonium phosphate R* in *water R*, adjust to pH 5.6 with *dilute phosphoric acid R* and dilute to 1000 mL with the same solvent;
- *mobile phase B*: *acetonitrile R1*.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 7 | 85 → 75 | 15 → 25 |
| 7 - 24 | 75 → 50 | 25 → 50 |
| 24 - 32 | 50 → 40 | 50 → 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *dextropropoxyphene for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D. Use the chromatogram obtained with reference solution (c) to identify the peak due to toluene.

*Relative retention* with reference to dextropropoxyphene (retention time = about 18 min): impurity A = about 0.8; impurity B = about 0.9; impurity D = about 1.1; impurity C = about 1.2.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dextropropoxyphene.

*Limits*:
- *impurities A, B*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to toluene (relative retention = about 1.24).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.270 g in 60 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.59 mg of $C_{22}H_{30}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

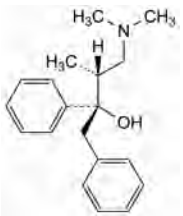

A. (2*S*,3*R*)-4-(dimethylamino)-1,2-diphenyl-3-methyl-butan-2-ol (oxyphene),

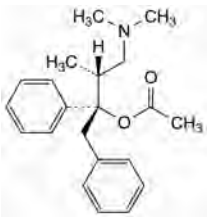

B. (1*S*,2*R*)-1-benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl acetate (acetoxyphene),

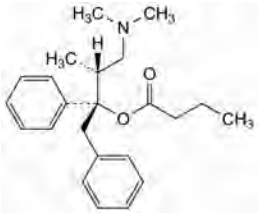

C. (1*S*,2*R*)-1-benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl butanoate (butyroxyphene),

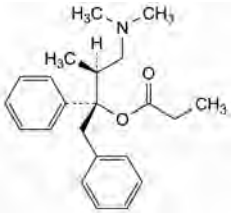

D. (1*S*,2*S*)-1-benzyl-3-(dimethylamino)-2-methyl-1-phenylpropyl propanoate (isopropoxyphene),

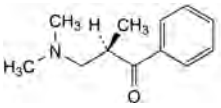


F. (2*RS*)-3-(dimethylamino)-2-methyl-1-phenylpropan-1-one.

**01/2020:2409**

# DIACEREIN

## Diacereinum

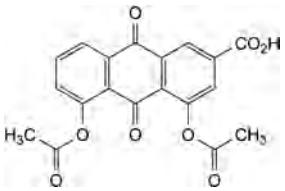

$C_{19}H_{12}O_8$ $M_r$ 368.3

[13739-02-1]

DEFINITION

4,5-Bis(acetyloxy)-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in dimethylacetamide, slightly soluble in tetrahydrofuran, practically insoluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *diacerein CRS*.

TESTS

**Impurities B and H**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solution A*. Dissolve 10 g of *sodium hydroxide R* in 500 mL of *water R*.

*Solution B*. Dissolve 14.7 g of *sodium chloride R* and 18.8 g of *glycine R* in 500 mL of *water R*.

*Solution C*. Mix 25.3 volumes of solution A and 74.6 volumes of solution B. If necessary, adjust to pH 9.5 using *dilute sodium hydroxide solution R* or *dilute sulfuric acid R*.

*Solution D*. Dilute 5 mL of *dilute sulfuric acid R* to 500 mL with *water R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in 30 mL of solution A and mix for 10 min. Add 70 mL of solution B and adjust to pH 9.5 with *dilute sodium hydroxide solution R* or *dilute sulfuric acid R*, if necessary. Extract with 3 quantities, each of 25 mL, of *methylene chloride R*. Combine the methylene chloride extracts and wash with 2 quantities,

each of 8 mL, of solution C and then once with 10 mL of solution D. Evaporate the organic layer to dryness at 33 °C, completing the drying procedure using compressed air. Dissolve the residue in 2.0 mL of the mobile phase.

*Reference solution (a).* Dissolve 7.5 mg of *diacerein impurity B CRS* in *tetrahydrofuran R* and dilute to 25.0 mL with the same solvent. Sonicate for not more than 30 s. Dilute 1.0 mL of the solution to 100.0 mL with solution A. Dilute 5.0 mL of this solution to 50.0 mL with solution A. Mix 5.0 mL of this solution with 25 mL of solution A for 10 min. Add 70 mL of solution B and adjust to pH 9.5 with *dilute sodium hydroxide solution R* or *dilute sulfuric acid R*, if necessary. Perform the extraction as described for the test solution. *Care should be taken that the time between dissolution of diacerein impurity B in tetrahydrofuran and extraction does not exceed 30 min.*

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 5.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: irregular *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 16 ± 1 °C.

*Mobile phase*: *tetrahydrofuran R*, *acetonitrile R*, 4 g/L solution of *citric acid monohydrate R* (8:27.5:64.5 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 100 µL.

*Run time*: 2.5 times the retention time of impurity B.

*Retention time*: impurity B = about 11 min.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limit*:

- *sum of impurities B and H*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (15 ppm).

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in 50 mL of *tetrahydrofuran R* and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *tetrahydrofuran R*. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* In order to prepare impurities D and E *in situ*, add 10.0 mL of a 0.4 g/L solution of *sodium hydroxide R* to 0.100 g of the substance to be examined. Add 40 mL of *tetrahydrofuran R* and dilute to 100 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *diacerein impurity mixture CRS* (impurities C and F) in a mixture of 0.5 mL of *tetrahydrofuran R* and 0.5 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: to 353 mL of *water for chromatography R* add 147 mL of *phosphoric acid R* and mix; dilute 2 mL of the solution to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 80 | 20 |
| 3 - 13 | 80 → 60 | 20 → 40 |
| 13 - 20 | 60 | 40 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *diacerein impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and F; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities D and E.

*Relative retention* with reference to diacerein (retention time = about 13.5 min): impurity D = about 1.1; impurity E = about 1.15; impurity C = about 1.2; impurity F = about 1.3.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurities D and E in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 100 for the principal peak in the chromatogram obtained with reference solution (a).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.4; impurity D = 1.3; impurity E = 1.3; impurity F = 9.5;
- *impurities D, E*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity F*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 20 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 60.0 mg of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 25.0 mL with the solvent mixture.

*Reference solution.* Dissolve 60.0 mg of *diacerein CRS* in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 25.0 mL with the solvent mixture.

Calculate the percentage content of $C_{19}H_{12}O_8$ taking into account the assigned content of *diacerein CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, F, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

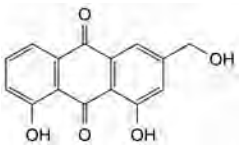

B. 1,8-dihydroxy-3-(hydroxymethyl)anthracene-9,10-dione (aloe-emodin),

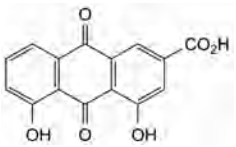

C. 4,5-dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid (rhein),

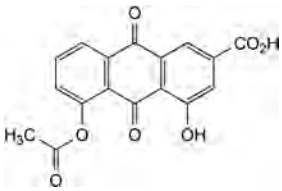

D. 5-(acetyloxy)-4-hydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid (monoacetyl rhein isomer A),

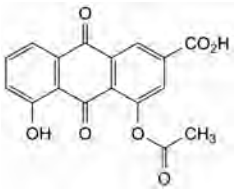

E. 4-(acetyloxy)-5-hydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid (monoacetyl rhein isomer B),

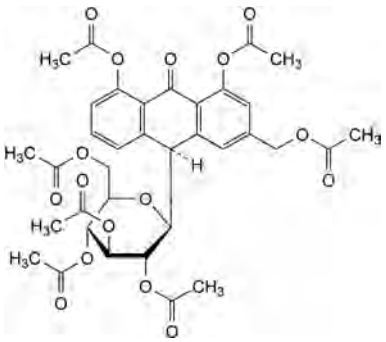

F. (10*S*)-3-[(acetyloxy)methyl]-10-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-9-oxo-9,10-dihydroanthracene-1,8-diyl diacetate (heptaacetyl aloin, heptaacetyl barbaloin),

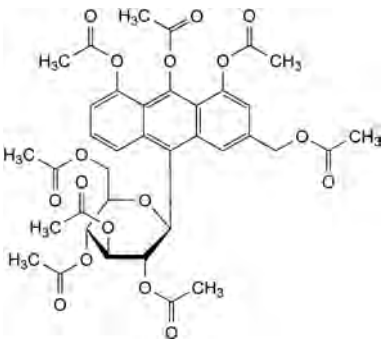

G. 3-[(acetyloxy)methyl]-10-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)anthracene-1,8,9-triyl triacetate,

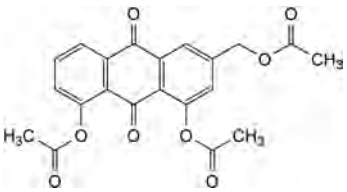

H. 3-[(acetyloxy)methyl]-9,10-dioxo-9,10-dihydroanthracene-1,8-diyl diacetate (triacetyl aloe-emodin).

**01/2017:0022**

# DIAZEPAM

## Diazepamum

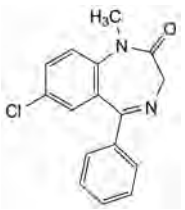

$C_{16}H_{13}ClN_2O$ $M_r$ 284.7

[439-14-5]

DEFINITION

7-Chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison: diazepam CRS.*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from bright light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 0.5 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *diazepam for system suitability CRS* (containing impurities A, B and E) in 1.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 22 volumes of *acetonitrile R*, 34 volumes of *methanol R* and 44 volumes of a 3.4 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.0 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: about 4 times the retention time of diazepam.

*Identification of impurities*: use the chromatogram supplied with *diazepam for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and E.

*Relative retention* with reference to diazepam (retention time = about 9 min): impurity E = about 0.7; impurity A = about 0.8; impurity B = about 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurities E and A and minimum 6.0 between the peaks due to impurity A and diazepam.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.3; impurity E = 1.3;
- *impurities A, B, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.47 mg of $C_{16}H_{13}ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, F.*

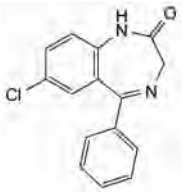

A. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (nordazepam),

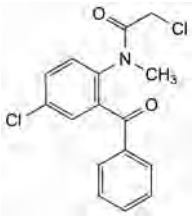

B. *N*-(2-benzoyl-4-chlorophenyl)-2-chloro-*N*-methylacetamide,

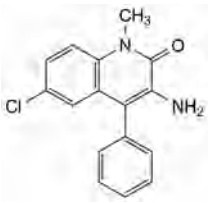

C. 3-amino-6-chloro-1-methyl-4-phenylquinolin-2(1*H*)-one,

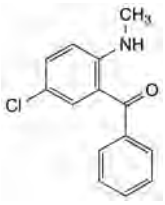

D. [5-chloro-2-(methylamino)phenyl]phenylmethanone,

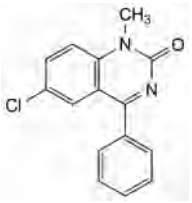

E. 6-chloro-1-methyl-4-phenylquinazolin-2(1*H*)-one,

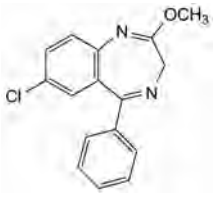

F. 7-chloro-2-methoxy-5-phenyl-3*H*-1,4-benzodiazepine.

01/2008:0550
corrected 6.0

# DIAZOXIDE

## Diazoxidum

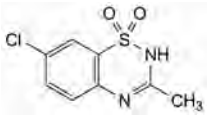

$C_8H_7ClN_2O_2S$ $M_r$ 230.7
[364-98-7]



DEFINITION

Diazoxide contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 7-chloro-3-methyl-2*H*-1,2,4-benzothiadiazine 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine or crystalline powder, practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in alcohol. It is very soluble in dilute solutions of the alkali hydroxides.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Dissolve 50.0 mg in 5 mL of *1 M sodium hydroxide* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *0.1 M sodium hydroxide*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 280 nm and a shoulder at 304 nm. The specific absorbance at the maximum is 570 to 610.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *diazoxide CRS*. Examine the substances prepared as discs using *potassium bromide R*.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. Dissolve about 20 mg in a mixture of 5 mL of *hydrochloric acid R* and 10 mL of *water R*. Add 0.1 g of *zinc powder R*. Boil for 5 min, cool and filter. To the filtrate add 2 mL of a 1 g/L solution of *sodium nitrite R* and mix. Allow to stand for 1 min and add 1 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. A red or violet-red colour develops.

TESTS

**Appearance of solution**. Dissolve 0.4 g in 2 mL of *1 M sodium hydroxide* and dilute to 20 mL with *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 0.5 g of the powdered substance to be examined add 30 mL of *carbon dioxide-free water R*, shake for 2 min and filter. To 10 mL of the filtrate add 0.2 mL of *0.01 M sodium hydroxide* and 0.15 mL of *methyl red solution R*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a)*. Dissolve 0.1 g of the substance to be examined in a mixture of 0.5 mL of *1 M sodium hydroxide* and 1 mL of *methanol R* and dilute to 5 mL with *methanol R*.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 5 mL with a mixture of 1 volume of *1 M sodium hydroxide* and 9 volumes of *methanol R*.

*Reference solution (a)*. Dilute 0.5 mL of test solution (a) to 100 mL with a mixture of 1 volume of *1 M sodium hydroxide* and 9 volumes of *methanol R*.

*Reference solution (b)*. Dissolve 20 mg of *diazoxide CRS* in a mixture of 0.5 mL of *1 M sodium hydroxide* and 1 mL of *methanol R* and dilute to 5 mL with *methanol R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 7 volumes of *concentrated ammonia R*, 25 volumes of *methanol R* and 68 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g with gentle heating in 50 mL of a mixture of 1 volume of *water R* and 2 volumes of *dimethylformamide R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 23.07 mg of $C_8H_7ClN_2O_2S$.

04/2016:2300

# DIBROMPROPAMIDINE DIISETHIONATE

## Dibrompropamidini diisetionas

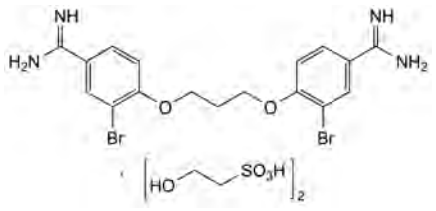

$C_{21}H_{30}Br_2N_4O_{10}S_2$ $M_r$ 722
[614-87-9]

DEFINITION

3,3′-Dibromo-4,4′-[propane-1,3-diylbis(oxy)]dibenzimidamide bis(2-hydroxyethanesulfonate).

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl 2-hydroxyethanesulfonate esters are potential impurities in dibrompropamidine diisetionate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting

materials, process capability and validation including, where necessary, demonstration that alkyl 2-hydroxyethanesulfonate esters are not detectable in the final product.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble or soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dibrompropamidine diisetionate CRS*.

B. Mix 0.1 g with 0.5 g of *anhydrous sodium carbonate R*, ignite and take up the residue with 20 mL of *water R*. Filter and neutralise the filtrate to *blue litmus paper R* with *nitric acid R*. The filtrate gives reaction (a) of bromides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 6.0.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *anhydrous formic acid R*, *methanol R*, *ethyl acetate R* (0.01:8:12 *V/V/V*).

*Test solution*. To 8 mL of *methanol R* add 20.0 mg of the substance to be examined and dissolve with the aid of an ultrasonic bath. Add 11 mL of *ethyl acetate R* then 10 µL of *anhydrous formic acid R* and mix. Dilute to 20.0 mL with *ethyl acetate R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *dibrompropamidine for system suitability CRS* (containing impurities A and B) in 4 mL of *methanol R* using an ultrasonic bath. Add 5 mL of *ethyl acetate R*, then 5 µL of *anhydrous formic acid R* and mix. Dilute to 10.0 mL with *ethyl acetate R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *strong cation-exchange silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 4 volumes of a 25 g/L solution of *ammonium formate R* in *methanol R* and 6 volumes of *ethyl acetate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 40 µL.

*Run time*: 1.5 times the retention time of dibrompropamidine.

*Identification of impurities*: use the chromatogram supplied with *dibrompropamidine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to dibrompropamidine (retention time = about 20 min): impurity A = about 0.4; impurity B = about 1.1.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dibrompropamidine.

*Limits*:
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide* under a current of *nitrogen R*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 36.12 mg of $C_{21}H_{30}Br_2N_4O_{10}S_2$.

IMPURITIES

*Specified impurities: A, B.*

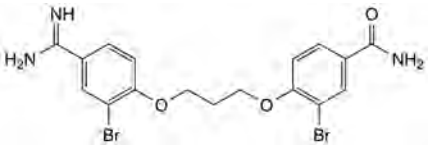

A. 3-bromo-4-[3-(2-bromo-4-carbamimidoylphenoxy)-propoxy]benzamide,

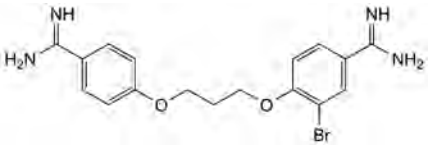

B. 3-bromo-4-[3-(4-carbamimidoylphenoxy)propoxy]-benzimidamide.

**01/2008:0762**
**corrected 10.0**

# DIBUTYL PHTHALATE

## Dibutylis phthalas

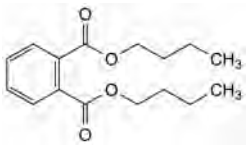

$C_{16}H_{22}O_4$ $M_r$ 278.3
[84-74-2]

DEFINITION

Dibutyl benzene-1,2-dicarboxylate.

*Content*: 99.0 per cent *m/m* to 101.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, oily liquid, colourless or very slightly yellow.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, C.*

*Second identification*: *A, D, E.*

A. Relative density (*2.2.5*): 1.043 to 1.048.

B. Refractive index (*2.2.6*): 1.490 to 1.495.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dibutyl phthalate CRS.*

D. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 50 mg of the substance to be examined in *ether R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 50 mg of *dibutyl phthalate CRS* in *ether R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R.*
*Mobile phase*: *heptane R*, *ether R* (30:70 *V/V*).
*Application*: 10 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. To about 0.1 mL add 0.25 mL of *sulfuric acid R* and 50 mg of *resorcinol R.* Heat in a water-bath for 5 min. Allow to cool. Add 10 mL of *water R* and 1 mL of *strong sodium hydroxide solution R.* The solution becomes yellow or brownish-yellow and shows a green fluorescence.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity**. Dissolve 20.0 g in 50 mL of *ethanol (96 per cent) R* previously neutralised to *phenolphthalein solution R1.* Add 0.2 mL of *phenolphthalein solution R1.* Not more than 0.50 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Gas chromatography (*2.2.28*).
*Internal standard solution.* Dissolve 60 mg of *bibenzyl R* in *methylene chloride R* and dilute to 20 mL with the same solvent.
*Test solution (a).* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20.0 mL with the same solvent.
*Test solution (b).* Dissolve 1.0 g of the substance to be examined in *methylene chloride R*, add 2.0 mL of the internal standard solution and dilute to 20.0 mL with *methylene chloride R.*
*Reference solution.* To 1.0 mL of test solution (a) add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *methylene chloride R.*
*Column*:
– *material*: glass;
– *size*: *l* = 1.5 m, Ø = 4 mm;
– *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (150-180 µm) impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R.*
*Carrier gas*: *nitrogen for chromatography R.*
*Flow rate*: 30 mL/min.
*Temperature*:
– *column*: 190 °C;
– *injection port and detector*: 225 °C.
*Detection*: flame ionisation.
*Injection*: 1 µL.
*Run time*: 3 times the retention time of dibutyl phthalate.
*Elution order*: bibenzyl, dibutyl phthalate.
*Retention time*: dibutyl phthalate = about 12 min.
*System suitability*:
– *resolution*: minimum 12 between the peaks due to bibenzyl and dibutyl phthalate in the chromatogram obtained with the reference solution;
– in the chromatogram obtained with test solution (a), there is no peak with the same retention time as the internal standard.
*Limit*:
– *total*: calculate the ratio (*R*) of the area of the peak due to dibutyl phthalate to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than *R* (1.0 per cent).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 10.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 0.750 g into a 250 mL borosilicate glass flask. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Heat in a water-bath under a reflux condenser for 1 h. Add 1 mL of *phenolphthalein solution R1* and titrate immediately with *0.5 M hydrochloric acid* until the colour changes from red to colourless. Carry out a blank titration. Calculate the volume of potassium hydroxide used in the saponification.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 69.59 mg of $C_{16}H_{22}O_4$.

STORAGE

In an airtight container.

**04/2014:2410**

# 2,4-DICHLOROBENZYL ALCOHOL

## Alcohol 2,4-dichlorobenzylicus

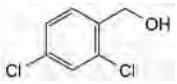


$C_7H_6Cl_2O$ $M_r$ 177.0
[1777-82-8]

DEFINITION

(2,4-Dichlorophenyl)methanol.
*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.
*Solubility*: very slightly soluble in water, very soluble in ethanol (96 per cent).
mp: about 59 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *2,4-dichlorobenzyl alcohol CRS.*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (50:50 *V/V*).

*Buffer solution*. Dissolve 0.68 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000.0 mL with *water R*.

*Test solution (a)*. Dissolve 0.100 g of the substance to be examined in 10.0 mL of *acetonitrile R1* and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 20.0 mg of *2,4-dichlorobenzyl alcohol impurity A CRS* and 20.0 mg of *2,4-dichlorobenzyl alcohol impurity C CRS* in 100.0 mL of *acetonitrile R1*. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 0.100 g of *2,4-dichlorobenzyl alcohol CRS* in 10.0 mL of *acetonitrile R1* and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R2*, *acetonitrile R1*, buffer solution (20:30:50 *V/V/V*);
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 100 | 0 |
| 7 - 17 | 100 → 20 | 0 → 80 |
| 17 - 30 | 20 | 80 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Relative retention* with reference to 2,4-dichlorobenzyl alcohol (retention time = about 7 min): impurity C = about 0.87; impurity A = about 0.91.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

- for each impurity, use the concentration of 2,4-dichlorobenzyl alcohol in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 0.500 g using the evaporation technique:

- *temperature*: 120 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_7H_6Cl_2O$ taking into account the assigned content of *2,4-dichlorobenzyl alcohol CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

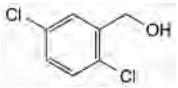

A. (2,5-dichlorophenyl)methanol,

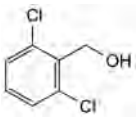

B. (2,6-dichlorophenyl)methanol,

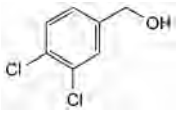

C. (3,4-dichlorophenyl)methanol,

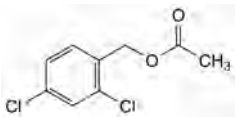

D. 2,4-dichlorobenzyl acetate,

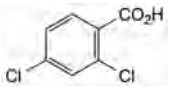

E. 2,4-dichlorobenzoic acid,

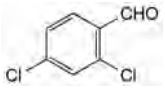

F. 2,4-dichlorobenzaldehyde,

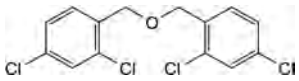

G. 1,1′-(oxydimethylene)bis(2,4-dichlorobenzene).

**01/2008:1718**
**corrected 7.0**

# DICLAZURIL FOR VETERINARY USE

## Diclazurilum ad usum veterinarium

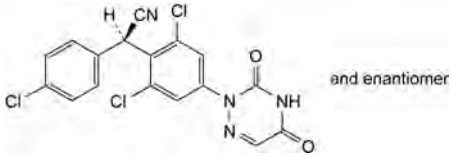


$C_{17}H_9Cl_3N_4O_2$ $M_r$ 407.6
[101831-37-2]

DEFINITION

(*RS*)-(4-Chlorophenyl)[2,6-dichloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl]acetonitrile.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or light yellow powder.

*Solubility*: practically insoluble in water, sparingly soluble in dimethylformamide, practically insoluble in alcohol and methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of diclazuril.*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *diclazuril for system suitability CRS* in *dimethylformamide R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of the solution to 20.0 mL with *dimethylformamide R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 10 volumes of a 6.3 g/L solution of *ammonium formate R* adjusted to pH 4.0 with *anhydrous formic acid R*, 15 volumes of *acetonitrile R* and 75 volumes of *water R*,
- *mobile phase B*: mix 10 volumes of a 6.3 g/L solution of *ammonium formate R* adjusted to pH 4.0 with *anhydrous formic acid R*, 85 volumes of *acetonitrile R* and 5 volumes of *water R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 5 µL.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum of 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to diclazuril.

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.9; impurity H = 1.4,
- *impurity D*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 75 mL of *dimethylformamide R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M tetrabutylammonium hydroxide*. Read the volume added at the second inflexion point. Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 20.38 mg of $C_{17}H_9Cl_3N_4O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

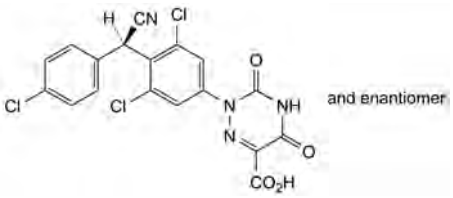


A. 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylic acid,

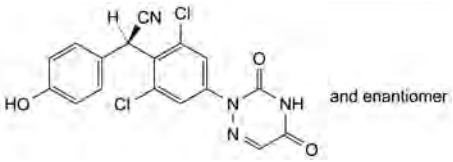


B. (*RS*)-[2,6-dichloro-4-(3,5-dioxo-4,5-dihydro-1,2,4-triazin-2(3*H*)-yl)phenyl](4-hydroxyphenyl)acetonitrile,

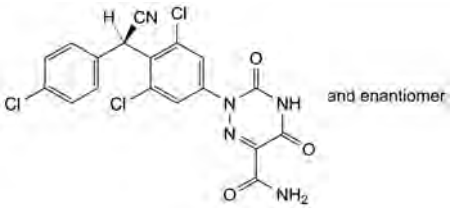


C. 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)cyanomethyl]-phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide,

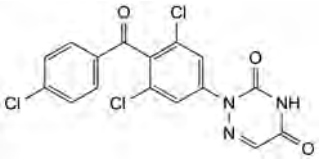


D. 2-[3,5-dichloro-4-(4-chlorobenzoyl)phenyl]-1,2,4-triazine-3,5(2*H*,4*H*)-dione,

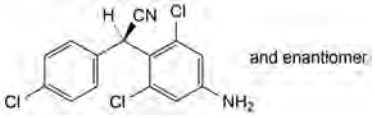


E. (*RS*)-(4-amino-2,6-dichlorophenyl)(4-chlorophenyl)-acetonitrile,

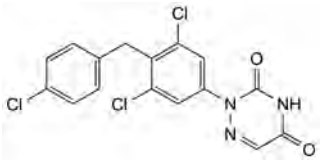

F. 2-[3,5-dichloro-4-(4-chlorobenzyl)phenyl]-1,2,4-triazine-3,5(2*H*,4*H*)-dione,

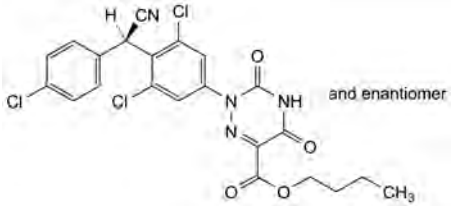


G. butyl 2-[3,5-dichloro-4-[(*RS*)-(4-chlorophenyl)-cyanomethyl]phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxylate,

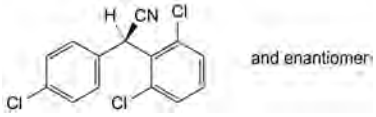


H. (*RS*)-(4-chlorophenyl)(2,6-dichlorophenyl)acetonitrile,

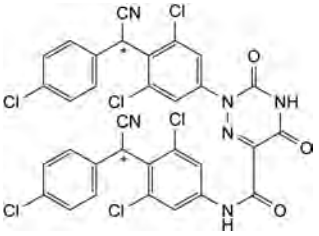

I. *N*,2-bis[3,5-dichloro-4-[(4-chlorophenyl)cyanomethyl]-phenyl]-3,5-dioxo-2,3,4,5-tetrahydro-1,2,4-triazine-6-carboxamide.

**01/2017:1508**
**corrected 10.0**

# DICLOFENAC POTASSIUM

## Diclofenacum kalicum

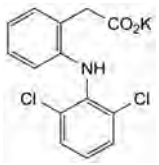

$C_{14}H_{10}Cl_2KNO_2$ $M_r$ 334.2
[15307-81-0]

DEFINITION

Potassium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, slightly hygroscopic, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), slightly soluble in acetone.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *diclofenac potassium CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution (a).* Dissolve 25 mg of *diclofenac potassium CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *indometacin R* in reference solution (a) and dilute to 2 mL with the same solution.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R.*
*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R* (10:10:80 *V/V/V*).
*Application*: 5 µL.
*Development*: over 1/2 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 10 mL of *ethanol (96 per cent) R.* To 1 mL of this solution add 0.2 mL of a mixture, prepared immediately before use, of equal volumes of a 6 g/L solution of *potassium ferricyanide R* and a 9 g/L solution of *ferric chloride R.* Allow to stand protected from light for 5 min. Add 3 mL of a 10 g/L solution of *hydrochloric acid R.* Allow to stand protected from light for 15 min. A blue colour develops and a precipitate is formed.

D. Suspend 0.5 g in 10 mL of *water R.* Stir and add *water R* until the substance is dissolved. Add 2 mL of *hydrochloric acid R1*, stir for 1 h and filter with the aid of vacuum. Neutralise with *sodium hydroxide solution R.* The solution gives reaction (b) of potassium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 440 nm is not greater than 0.05.
Dissolve 1.25 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.
*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.
*Reference solution (b).* Dissolve the contents of a vial of *diclofenac for system suitability CRS* (containing impurities A and F) in 1 mL of the mobile phase.
*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).
*Mobile phase*: mix 34 volumes of a solution containing 0.5 g/L of *phosphoric acid R* and 0.8 g/L of *sodium dihydrogen phosphate R*, previously adjusted to pH 2.5 with *phosphoric acid R*, and 66 volumes of *methanol R.*
*Flow rate*: 1.0 mL/min.
*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 1.6 times the retention time of diclofenac.

*Identification of impurities*: use the chromatogram supplied with *diclofenac for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and F.

*Relative retention* with reference to diclofenac (retention time = about 25 min): impurity A = about 0.4; impurity F = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to impurity F and diclofenac.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity F = 0.3;

– for each impurity, use the concentration of diclofenac potassium in reference solution (a).

*Limits*:

– *impurities A, F*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.4 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.250 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.42 mg of $C_{14}H_{10}Cl_2KNO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

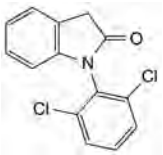

A. 1-(2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one,

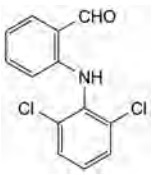

B. 2-[(2,6-dichlorophenyl)amino]benzaldehyde,

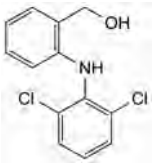

C. [2-[(2,6-dichlorophenyl)amino]phenyl]methanol,

D. [2-[(2-bromo-6-chlorophenyl)amino]phenyl]acetic acid,

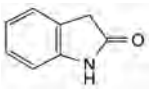

E. 1,3-dihydro-2*H*-indol-2-one,

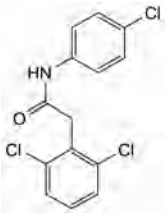

F. *N*-(4-chlorophenyl)-2-(2,6-dichlorophenyl)acetamide.

**01/2017:1002**
**corrected 10.0**

# DICLOFENAC SODIUM

## Diclofenacum natricum

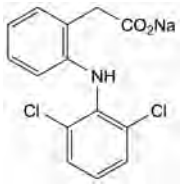

$C_{14}H_{10}Cl_2NNaO_2$ $M_r$ 318.1
[15307-79-6]

DEFINITION

Sodium [2-[(2,6-dichlorophenyl)amino]phenyl]acetate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, slightly hygroscopic, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), slightly soluble in acetone.

mp: about 280 °C, with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *diclofenac sodium CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *diclofenac sodium CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *indometacin R* in reference solution (a) and dilute to 2 mL with reference solution (a).

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R* (10:10:80 *V/V/V*).

*Application*: 5 µL.

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 10 mL of *ethanol (96 per cent) R*. To 1 mL of this solution add 0.2 mL of a mixture, prepared immediately before use, of equal volumes of a 6 g/L solution of *potassium ferricyanide R* and a 9 g/L solution of *ferric chloride R*. Allow to stand protected from light for 5 min. Add 3 mL of a 10 g/L solution of *hydrochloric acid R*. Allow to stand, protected from light, for 15 min. A blue colour develops and a precipitate is formed.

D. Dissolve 60 mg in 0.5 mL of *methanol R* and add 0.5 mL of *water R*. The solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 440 nm is not greater than 0.05.

Dissolve 1.25 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *diclofenac for system suitability CRS* (containing impurities A and F) in 1 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 34 volumes of a solution containing 0.5 g/L of *phosphoric acid R* and 0.8 g/L of *sodium dihydrogen phosphate R*, previously adjusted to pH 2.5 with *phosphoric acid R*, and 66 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 1.6 times the retention time of diclofenac.

*Identification of impurities*: use the chromatogram supplied with *diclofenac for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and F.

*Relative retention* with reference to diclofenac (retention time = about 25 min): impurity A = about 0.4; impurity F = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to impurity F and diclofenac.

*Calculation of percentage contents:*

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity F = 0.3;

– for each impurity, use the concentration of diclofenac sodium in reference solution (a).

*Limits*:

– *impurity A*: maximum 0.2 per cent;

– *impurity F*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.4 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.250 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.81 mg of $C_{14}H_{10}Cl_2NNaO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

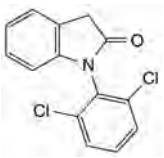


A. 1-(2,6-dichlorophenyl)-1,3-dihydro-2*H*-indol-2-one,

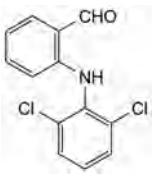


B. 2-[(2,6-dichlorophenyl)amino]benzaldehyde,

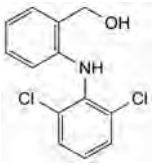


C. [2-[(2,6-dichlorophenyl)amino]phenyl]methanol,

D. [2-[(2-bromo-6-chlorophenyl)amino]phenyl]acetic acid,

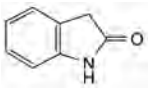

E. 1,3-dihydro-2*H*-indol-2-one,

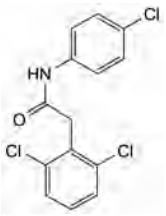

F. *N*-(4-chlorophenyl)-2-(2,6-dichlorophenyl)acetamide.



**01/2008:0663**
**corrected 10.0**

# DICLOXACILLIN SODIUM

## Dicloxacillinum natricum

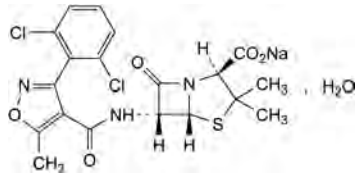

$C_{19}H_{16}Cl_2N_3NaO_5S,H_2O$ $M_r$ 510.3
[13412-64-1]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[[3-(2,6-dichlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *dicloxacillin sodium CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *water R.*

*Reference solution (a).* Dissolve 25 mg of *dicloxacillin sodium CRS* in 5 mL of *water R.*

*Reference solution (b).* Dissolve 25 mg of *cloxacillin sodium CRS*, 25 mg of *dicloxacillin sodium CRS* and 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R.*

*Plate*: *TLC silanised silica gel plate R.*

*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R.*

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and about 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; a yellow colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.04.

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 128 to + 143 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *dicloxacillin sodium CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of test solution (b) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *dicloxacillin sodium CRS* in the mobile phase, then dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R* and 75 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R.*

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 5 times the retention time of dicloxacillin.

*Retention time*: dicloxacillin = about 10 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to flucloxacillin (1st peak) and dicloxacillin (2nd peak).

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): 3.0 per cent to 4.5 per cent, determined on 0.300 g.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 mL of a solution in *water for injections R* containing 20 mg of the substance to be examined per millilitre.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

STORAGE

In an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

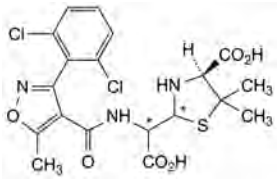

A. (4*S*)-2-[carboxy[[[3-(2,6-dichlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of dicloxacillin),

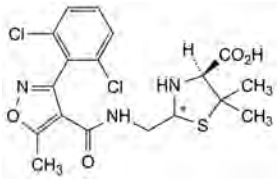

B. (2*RS*,4*S*)-2-[[[[3-(2,6-dichlorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penilloic acids of dicloxacillin),

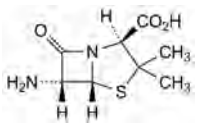

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

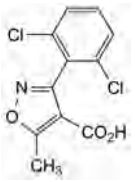

D. 3-(2,6-dichlorophenyl)-5-methyl-1,2-oxazole-4-carboxylic acid.

**04/2017:1197**
**corrected 10.0**

# DICYCLOVERINE HYDROCHLORIDE

## Dicycloverini hydrochloridum

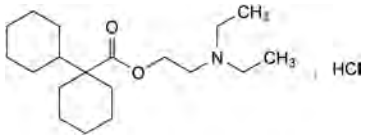

$C_{19}H_{36}ClNO_2$ $M_r$ 346.0

DEFINITION

2-(Diethylamino)ethyl 1,1′-(bicyclohexane)-1-carboxylate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dicycloverine hydrochloride CRS.*
If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 50 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *dicycloverine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel plate R*
*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *propanol R* (5:10:10:75 *V/V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of warm air.
*Detection*: spray with *dilute potassium iodobismuthate solution R*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 3 mL of a 1.0 g/L solution of *sodium laurilsulfate R* add 5 mL of *methylene chloride R* and 0.05 mL of a 2.5 g/L solution of *methylene blue R*, mix gently and allow to

separate; the lower layer is blue. Add 2 mL of a 20 g/L solution of the substance to be examined, mix gently and allow to separate; the upper layer is blue and the lower layer is colourless.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 5.5.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *dicycloverine for system suitability CRS* (containing impurity B) in 1 mL of the mobile phase.

*Reference solution (c).* Dissolve 7.5 mg of *dicycloverine impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: mix 15 volumes of a 4.62 g/L solution of *ammonium acetate R* previously adjusted to pH 8.0 with *2 M sodium hydroxide R* and 85 volumes of *methanol R2*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of dicycloverine.

*Identification of impurities*: use the chromatogram supplied with *dicycloverine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to dicycloverine (retention time = about 22 min): impurity A = about 0.5; impurity B = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity B and dicycloverine.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity B by 0.4;
- for impurity A, use the concentration of impurity A in reference solution (c);
- for impurities other than A, use the concentration of dicycloverine hydrochloride in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.5 per cent;
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.7 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 1.0 per cent, determined on 1.00 g by direct introduction of the sample.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.60 mg of $C_{19}H_{36}ClNO_2$.

IMPURITIES

*Specified impurities: A, B.*

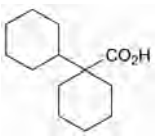


A. 1,1′-bi(cyclohexane)-1-carboxylic acid,

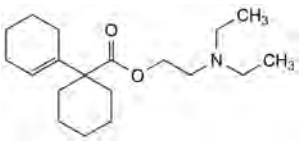


B. 2-(diethylamino)ethyl 1,1′-bi(cyclohexan)-1′-ene-1-carboxylate.

**01/2017:2200**

# DIDANOSINE

## Didanosinum

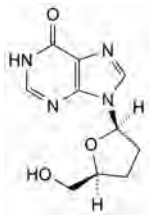


$C_{10}H_{12}N_4O_3$ $M_r$ 236.2
[69655-05-6]

DEFINITION

9-(2,3-Dideoxy-β-D-*glycero*-pentofuranosyl)-1,9-dihydro-6*H*-purin-6-one (2′,3′-dideoxyinosine).

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in dimethyl sulfoxide, slightly soluble in methanol and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *didanosine CRS*.

B. Specific optical rotation (*2.2.7*): – 28.2 to – 24.2 (anhydrous substance).
Dissolve 0.100 g in *water R* and dilute to 10.0 mL with the same solvent.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 8 volumes of mobile phase B and 92 volumes of mobile phase A.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 50.0 mL of the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *didanosine impurity A CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL to 20.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *didanosine for system suitability CRS* (containing impurities A to F) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5 mg of *didanosine impurity G CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL to 20 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 8 volumes of *methanol R* and 92 volumes of a 3.86 g/L solution of *ammonium acetate R* adjusted to pH 8.0 with *concentrated ammonia R*;
- *mobile phase B*: mix 30 volumes of *methanol R* and 70 volumes of a 3.86 g/L solution of *ammonium acetate R* adjusted to pH 8.0 with *concentrated ammonia R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 18 | 100 | 0 |
| 18 - 25 | 100 → 0 | 0 → 100 |
| 25 - 45 | 0 | 100 |
| 45 - 50 | 0 → 100 | 100 → 0 |
| 50 - 60 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *didanosine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A to F and use the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to didanosine (retention time = about 13-15 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.44; impurity D = about 0.48; impurity E = about 0.5; impurity F = about 0.8; impurity G = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to impurity C and impurity D.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, C, D, E, F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.62 mg of $C_{10}H_{12}N_4O_3$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I.*

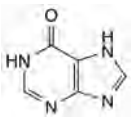

A. 1,7-dihydro-6*H*-purin-6-one (hypoxanthine),

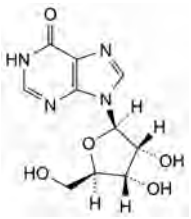

B. 9-β-D-ribofuranosyl-1,9-dihydro-6*H*-purin-6-one (inosine),

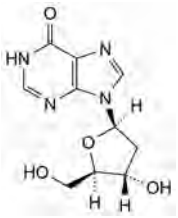

C. 9-(2-deoxy-β-D-*erythro*-pentofuranosyl)-1,9-dihydro-6*H*-purin-6-one (2′-deoxyinosine),

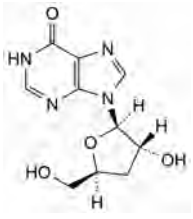

D. 9-(3-deoxy-β-D-*erythro*-pentofuranosyl)-1,9-dihydro-6*H*-purin-6-one (3′-deoxyinosine),

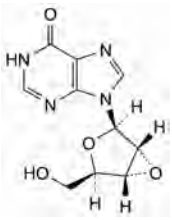

E. 9-(2,3-anhydro-β-D-ribofuranosyl)-1,9-dihydro-6*H*-purin-6-one (2′,3′-anhydroinosine),

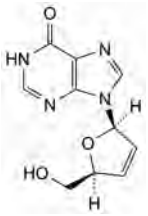

F. 9-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-1,9-dihydro-6*H*-purin-6-one (2′,3′-dideoxy-2′,3′-didehydroinosine),

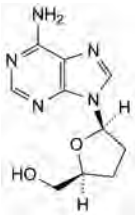

G. 9-(2,3-dideoxy-β-D-*glycero*-pentofuranosyl)-9*H*-purin-6-amine (2′,3′-dideoxyadenosine),

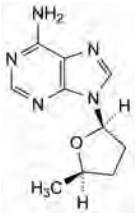

H. 9-(2,3,5-trideoxy-β-D-*glycero*-pentofuranosyl)-9*H*-purin-6-amine (2′,3′,5′-trideoxyadenosine),

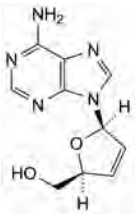

I. 9-(2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-9*H*-purin-6-amine (2′,3′-dideoxy-2′,3′-didehydroadenosine).



**04/2016:2732**

# DIENOGEST

## Dienogestum

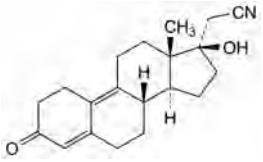

$C_{20}H_{25}NO_2$ $M_r$ 311.4
[65928-58-7]

DEFINITION

(17-Hydroxy-3-oxoestra-4,9-dien-17α-yl)acetonitrile.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white, almost white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dienogest CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 352 to − 344 (dried substance).

Dissolve 0.125 g in *methanol R2* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (40:60 *V/V*).

*Test solution (a).* Dissolve 20 mg of the substance to be examined in 8 mL of *acetonitrile R1* and dilute to 20.0 mL with *water R*.

*Test solution (b).* Dissolve 30.0 mg of the substance to be examined in 40 mL of *acetonitrile R1* and dilute to 100.0 mL with *water R*. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2.5 mg of *dienogest for system suitability CRS* (containing impurity F) in 2 mL of *acetonitrile R1* and dilute to 5.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 30.0 mg of *dienogest CRS* in 40 mL of *acetonitrile R1* and dilute to 100.0 mL with *water R*. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, *water for chromatography R* (10:90 *V/V*);

– *mobile phase B*: *water for chromatography R, acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 90 | 10 |
| 3 - 53 | 90 → 10 | 10 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 15 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *dienogest for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity F.

*Relative retention* with reference to dienogest (retention time = about 19 min): impurity F = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to dienogest and impurity F.

*Calculation of percentage contents*:

– for each impurity, use the concentration of dienogest in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 14 | 75 → 25 | 25 → 75 |

*Detection*: spectrophotometer at 215 nm.

*Injection*: test solution (b) and reference solution (c).

*Retention time*: dienogest = about 9 min.

Calculate the percentage content of $C_{20}H_{25}NO_2$ taking into account the assigned content of *dienogest CRS*.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, K, L, M*.

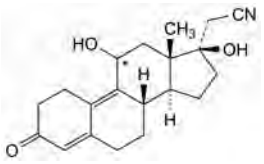

A. (11,17-dihydroxy-3-oxoestra-4,9-dien-17α-yl)acetonitrile,

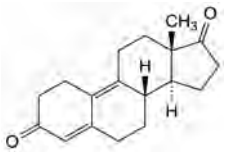

B. estra-4,9-diene-3,17-dione,

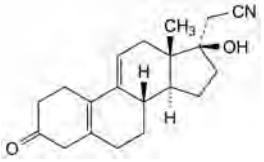

C. [17-hydroxy-3-oxoestra-5(10),9(11)-dien-17α-yl]-acetonitrile,

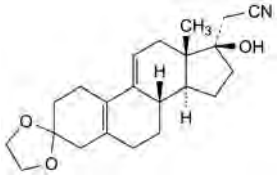

D. [17′-hydroxyspiro[1,3-dioxolan-2,3′-estra-5′(10′),9′(11′)-dien]-17′α-yl]acetonitrile,

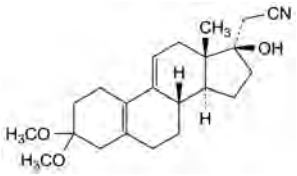

E. [17-hydroxy-3,3-dimethoxyestra-5(10),9(11)-dien-17α-yl]-acetonitrile,

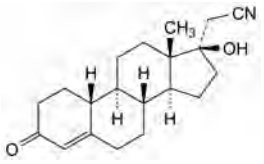

F. (17-hydroxy-3-oxoestr-4-en-17α-yl)acetonitrile,

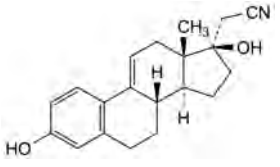

G. [3,17-dihydroxyestra-1,3,5(10),9(11)-tetraen-17α-yl]-acetonitrile,

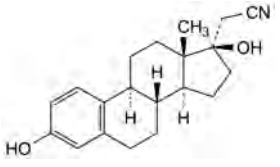

H. [3,17-dihydroxyestra-1,3,5(10)-trien-17α-yl]acetonitrile,

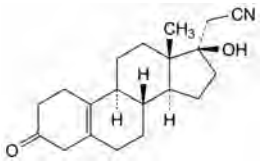

I. [17-hydroxy-3-oxoestr-5(10)-en-17α-yl]acetonitrile,

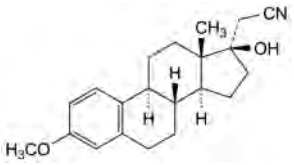

J. [17-hydroxy-3-methoxyestra-1,3,5(10)-trien-17α-yl]-acetonitrile,

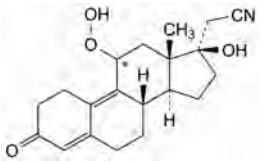

K. (11-hydroperoxy-17-hydroxy-3-oxoestra-4,9-dien-17α-yl)-acetonitrile,

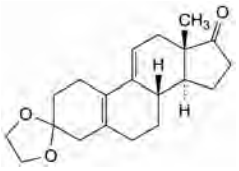

L. spiro[1,3-dioxolan-2,3′-estra-5′(10′),9′(11′)-dien]-17′-one,

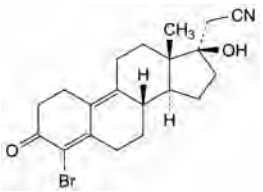

M. (4-bromo-17-hydroxy-3-oxoestra-4,9-dien-17α-yl)-acetonitrile.

**04/2008:0897**
**corrected 10.0**

# DIETHYL PHTHALATE

## Diethylis phthalas

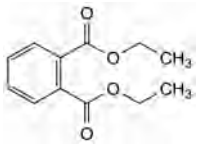

$C_{12}H_{14}O_4$ $M_r$ 222.2
[84-66-2]

DEFINITION

Diethyl benzene-1,2-dicarboxylate.

*Content*: 99.0 per cent *m/m* to 101.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless or very slightly yellow, oily liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, D, E.*

A. Relative density (*2.2.5*): 1.117 to 1.121.

B. Refractive index (*2.2.6*): 1.500 to 1.505.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: thin films.

*Comparison*: *diethyl phthalate CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *ether R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 50 mg of *diethyl phthalate CRS* in *ether R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *heptane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. To about 0.1 mL add 0.25 mL of *sulfuric acid R* and 50 mg of *resorcinol R*. Heat on a water-bath for 5 min. Allow to cool. Add 10 mL of *water R* and 1 mL of *strong sodium hydroxide solution R*. The solution becomes yellow or brownish-yellow and shows green fluorescence.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity**. Dissolve 20.0 g in 50 mL of *ethanol (96 per cent) R* previously neutralised to *phenolphthalein solution R1*. Add 0.2 mL of *phenolphthalein solution R1*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 60 mg of *naphthalene R* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Test solution (a)*. Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

*Test solution (b)*. Dissolve 1.0 g of the substance to be examined in *methylene chloride R*, add 2.0 mL of the internal standard solution and dilute to 20.0 mL with *methylene chloride R*.

*Reference solution*. To 1.0 mL of test solution (a) add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *methylene chloride R*.

*Column*:

- *material*: glass;
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (150-180 µm) impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 150 °C;

– *injection port and detector*: 225 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 3 times the retention time of diethyl phthalate.

*Elution order*: naphthalene, diethyl phtalate.

*System suitability*:

– *resolution*: minimum 10 between the peaks due to naphthalene and diethyl phthalate in the chromatogram obtained with the reference solution;
– in the chromatogram obtained with test solution (a), there is no peak with the same retention time as the internal standard.

*Limit*:

– *total*: calculate the ratio (*R*) of the area of the peak due to diethyl phthalate to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than *R* (1.0 per cent).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 10.0 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 0.750 g into a 250 mL borosilicate glass flask. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Boil in a water-bath under a reflux condenser for 1 h. Add 1 mL of *phenolphthalein solution R1* and titrate immediately with *0.5 M hydrochloric acid*. Carry out a blank titration. Calculate the volume of *0.5 M alcoholic potassium hydroxide* used in the saponification.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 55.56 mg of $C_{12}H_{14}O_4$.

STORAGE

In an airtight container.

**01/2017:0271**

# DIETHYLCARBAMAZINE CITRATE

## Diethylcarbamazini citras

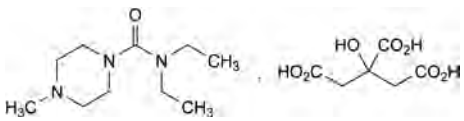

$C_{16}H_{29}N_3O_8$ $M_r$ 391.4
[1642-54-2]

DEFINITION

*N*,*N*-Diethyl-4-methylpiperazine-1-carboxamide dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: about 138 °C, with decomposition.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *diethylcarbamazine citrate CRS.*

B. Examine the chromatograms obtained in the test for impurities A and B.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 5 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

TESTS

**Solution S**. Shake 2.5 g with *water R* until dissolved and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Impurities A and B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 0.1 g of *diethylcarbamazine citrate CRS* in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *methylpiperazine R* (impurity A) in *methanol R* and dilute to 100 mL with the same solvent.

*Reference solution (c).* Dissolve 10 mg of *dimethylpiperazine R* (impurity B) in *methanol R* and dilute to 100 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *concentrated ammonia R*, *methyl ethyl ketone R*, *methanol R* (5:30:65 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C.

*Detection*: expose to iodine vapour for 30 min.

*Retardation factors*: impurity A = about 0.2; impurity B = about 0.5.

*Limits*:

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 31.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

*Test solution (a).* Suspend 0.30 g of the substance to be examined in solution A and dilute to 100 mL with solution A. Filter or centrifuge and use the clear filtrate or supernatant.

*Test solution (b).* Dissolve 10.0 mg of the substance to be examined in solution A and dilute to 100.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 10 mg of *citric acid monohydrate R* in solution A and dilute to 10 mL with solution A.

*Reference solution (c).* To 3 mL of test solution (a) add 0.5 mL of *strong hydrogen peroxide solution R* and maintain at 80 °C for 3 h. Dilute to 100 mL with solution A.

*Reference solution (d).* Dissolve 5.0 mg of *diethylcarbamazine citrate CRS* in solution A and dilute to 50.0 mL with solution A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 100 volumes of *methanol R2* and 900 volumes of a 10 g/L solution of *potassium dihydrogen phosphate R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: twice the retention time of diethylcarbamazine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to the citrate.

*Relative retention* with reference to diethylcarbamazine (retention time = about 7 min): citrate = about 0.2; degradation product = about 1.6.

*System suitability*: reference solution (c):

– *resolution*: minimum 5 between the peaks due to diethylcarbamazine and the degradation product.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to the citrate.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of test solution (b) and reference solution (d).

Calculate the percentage content of $C_{16}H_{29}N_3O_8$ from the declared content of *diethylcarbamazine citrate CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

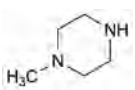

A. 1-methylpiperazine,

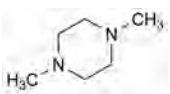

B. 1,4-dimethylpiperazine.

**01/2008:1198**
**corrected 10.0**

# DIETHYLENE GLYCOL MONOETHYL ETHER

## Diethylenglycoli aether monoethylicus

$C_6H_{14}O_3$ $M_r$ 134.2
[111-90-0]

DEFINITION

2-(2-Ethoxyethoxy)ethanol, produced by condensation of ethylene oxide and alcohol, followed by distillation.

CHARACTERS

*Appearance*: clear, colourless, hygroscopic liquid.

*Solubility*: miscible with water, with acetone and with alcohol, miscible in certain proportions with vegetable oils, not miscible with mineral oils.

*Relative density*: about 0.991.

IDENTIFICATION

A. Refractive index (*2.2.6*): 1.426 to 1.428.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of diethylene glycol monoethyl ether.*

TESTS

**Acid value** (*2.5.1*): maximum 0.1.

Mix 30.0 mL with 30 mL of *alcohol R* previously neutralised with *0.1 M potassium hydroxide* using *phenolphthalein solution R* as indicator. Titrate with *0.01 M alcoholic potassium hydroxide.*

**Peroxide value** (*2.5.5*): maximum 8.0, determined on 2.00 g.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.00 g of *decane R* to 100.0 mL with *methanol R*.

*Test solution.* To 5.00 g of the substance to be examined, add 0.1 mL of the internal standard solution and dilute to 10.0 mL with *methanol R*.

*Reference solution (a).* Dilute 25.0 mg of *ethylene glycol monomethyl ether R*, 80.0 mg of *ethylene glycol monoethyl ether R*, 0.310 g of *ethylene glycol R* and 0.125 g of *diethylene glycol R* to 100.0 mL with *methanol R*. To 1.0 mL of this solution add 0.1 mL of the internal standard solution and dilute to 10.0 mL with *methanol R*.

*Reference solution (b).* Dilute 25.0 mg of *ethylene glycol monoethyl ether R* and 25.0 mg of *ethylene glycol R* to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 5.0 mL with *methanol R*.

*Reference solution (c).* Dilute 1.00 g of the substance to be examined to 100.0 mL with *methanol R*. To 1.0 mL of this solution add 0.1 mL of the internal standard solution and dilute to 10.0 mL with *methanol R*.

*Column*:

– *material*: fused silica,

– *size*: *l* = 30 m, Ø = 0.32 mm,

– *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *nitrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 2.0 mL/min.

*Split ratio*: 1:80.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 120 |
| | 1 - 10 | 120 → 225 |
| | 10 - 12 | 225 |
| Injection port | | 275 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Relative retentions* with reference to diethylene glycol monoethyl ether (retention time = about 4 min): ethylene glycol monomethyl ether = about 0.4; ethylene glycol monoethyl ether = about 0.5; ethylene glycol = about 0.55; diethylene glycol = about 1.1.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to ethylene glycol monoethyl ether and to ethylene glycol in the chromatogram obtained with reference solution (b),
- *signal-to-noise ratio*: minimum 3.0 for the peak due to ethylene glycol monomethyl ether in the chromatogram obtained with reference solution (a),

*Limits* (take into account the impurity/internal standard peak area ratio):

- *ethylene glycol monomethyl ether*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (50 ppm),
- *ethylene glycol monoethyl ether*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (160 ppm),
- *ethylene glycol*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (620 ppm),
- *diethylene glycol*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (250 ppm),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Ethylene oxide**. Head-space gas chromatography (*2.2.28*).

*Test solution.* To 1.00 g of the substance to be examined in a vial, add 50 µL of *water R*.

*Reference solution.* To 1.00 g of the substance to be examined in a vial, add 50 µL of *ethylene oxide solution R4* and close tightly.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.1 mL/min.

*Static head-space conditions which may be used*:

- *equilibration temperature*: 80 °C,
- *equilibration time*: 45 min,
- *transfer line temperature*: 110 °C,
- *pressurisation time*: 2 min,
- *injection time*: 12 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 40 |
| | 5 - 18 | 40 → 200 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL.

The peak due to ethylene oxide is identified by injecting solutions of ethylene oxide of increasing concentration.

Determine the content of ethylene oxide (ppm) in the substance to be examined using the following expression:

$$\frac{S_T \times C}{(S_S \times M_T) - (S_T \times M_S)}$$

$S_T$ = area of the peak due to ethylene oxide in the chromatogram obtained with the test solution,

$S_S$ = area of the peak due to ethylene oxide in the chromatogram obtained with the reference solution,

$M_T$ = mass of the substance to be examined in the test solution, in grams,

$M_S$ = mass of the substance to be examined in the reference solution, in grams,

$C$ = mass of added ethylene oxide in the reference solution, in micrograms.

*Limit*:

- *ethylene oxide*: maximum 1 ppm.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 10.0 g.

STORAGE

Under an inert gas, in an airtight container.

LABELLING

The label states that the substance is stored under an inert gas.

**01/2020:1415**

# DIETHYLENE GLYCOL PALMITOSTEARATE

## Diethylenglycoli palmitostearas

DEFINITION

Mixture of diethylene glycol (2,2′-oxydi(ethan-1-ol)) mono- and diesters of palmitic (hexadecanoic) and stearic (octadecanoic) acids, produced by the condensation of diethylene glycol and stearic acid 50 of vegetable or animal origin (see *Stearic acid (1474)*).

*Content*:

- *monoesters*: 45.0 per cent to 60.0 per cent;
- *diesters*: 35.0 per cent to 55.0 per cent.

CHARACTERS

*Appearance*: white or almost white, waxy solid.

*Solubility*: practically insoluble in water, soluble in acetone and in hot ethanol (96 per cent).

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of monoesters).

TESTS

**Melting point** (*2.2.15*): 43 °C to 50 °C.

**Acid value** (*2.5.1*): maximum 4.0.

**Iodine value** (*2.5.4*): maximum 3.0.

**Saponification value** (*2.5.6*): 155 to 180, determined on 2.0 g.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance:*

- *stearic acid*: 40.0 per cent to 60.0 per cent;
- *sum of contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

**Free diethylene glycol**: maximum 2.5 per cent, determined as prescribed under Assay.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of equal volumes of *methanol R* and *methylene chloride R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* In a 15 mL flask, weigh 0.200 g (*m*). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* In four 15 mL flasks, weigh respectively 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *diethylene glycol R*. Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of diethylene glycol in milligrams per gram for each reference solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7.5 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to diethylene glycol: diesters = about 0.78; monoesters = about 0.84.

*Calculations*:

- *free diethylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration of diethylene glycol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free diethylene glycol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 270}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

270 = average rounded molar mass of palmitic acid and stearic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y}(100 - A - B)\right] - D$$

$A$ = percentage content of free diethylene glycol;

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters.

- *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{Y}{X+Y}(100 - A - B)$$

STORAGE

Protected from light.

**01/2008:0484**
**corrected 6.0**

# DIETHYLSTILBESTROL

## Diethylstilbestrolum

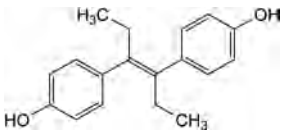


$C_{18}H_{20}O_2$ $M_r$ 268.4
[56-53-1]

DEFINITION

Diethylstilbestrol contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of (*E*)-4,4′-(1,2-diethylethene-1,2-diyl)diphenol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in alcohol. It dissolves in solutions of the alkali hydroxides.

It melts at about 172 °C.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Examined between 230 nm and 450 nm (*2.2.25*), the irradiated solution of the substance to be examined prepared as prescribed in the assay shows two absorption maxima, at 292 nm and 418 nm.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *diethylstilbestrol CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for mono-and dimethyl ethers. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 0.5 mg in 0.2 mL of *glacial acetic acid R*, add 1 mL of *phosphoric acid R* and heat on a water-bath for 3 min. A deep-yellow colour develops.

TESTS

**4,4′-Dihydroxystilbene and related ethers**. Dissolve 0.100 g in *ethanol R* and dilute to 10.0 mL with the same solvent. The absorbance (*2.2.25*) of the solution measured at 325 nm is not greater than 0.50.

**Mono- and dimethyl ethers**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a)*. Dissolve 0.2 g of the substance to be examined in 2 mL of *alcohol R*.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 20 mL with *alcohol R*.

*Reference solution (a)*. Dissolve 10 mg of *diethylstilbestrol CRS* in 2 mL of *alcohol R*.

*Reference solution (b)*. Dissolve 5 mg of *diethylstilbestrol monomethyl ether CRS* in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution (c)*. Dissolve 5 mg of *diethylstilbestrol dimethyl ether CRS* in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution (d)*. Dissolve 10 mg of *dienestrol CRS* in 2 mL of *alcohol R*. To 1 mL of this solution add 1 mL of reference solution (a).

Apply to the plate 1 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *diethylamine R* and 90 volumes of *toluene R*. Allow the plate to dry in air, spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 10 min. In the chromatogram obtained with test solution (a), any spots corresponding to diethylstilbestrol monomethyl ether and diethylstilbestrol dimethyl ether are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively (0.5 per cent). Diethylstilbestrol gives one or sometimes two spots. The test is not valid unless the chromatogram obtained with reference solution (d) shows at least two clearly separated spots having approximately the same intensity.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 20.0 mg in *ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *ethanol R*. To 25.0 mL of the resulting solution add 25.0 mL of a solution of 1 g of *dipotassium hydrogen phosphate R* in 55 mL of *water R*. Prepare in the same manner a reference solution using 20.0 mg of *diethylstilbestrol CRS*. Transfer an equal volume of each solution to separate 1 cm quartz cells and close the cells; place the two cells about 5 cm from a low-pressure, short-wave 2 W to 20 W mercury lamp and irradiate for about 5 min. Measure the absorbance (*2.2.25*) of the irradiated solutions at the maximum at 418 nm, using *water R* as the compensation liquid. Continue the irradiation for successive periods of 3 min to 15 min, depending on the power of the lamp, and repeat the measurement of the absorbances at 418 nm until the maximum absorbance (about 0.7) is obtained. If necessary, adjust the geometry of the irradiation apparatus to obtain a maximum, reproducible absorbance at 418 nm.

Calculate the content of $C_{18}H_{20}O_2$ from the measured absorbances and the concentrations of the solutions.

STORAGE

Store protected from light.

**04/2013:2239**

# DIFLOXACIN HYDROCHLORIDE TRIHYDRATE FOR VETERINARY USE

## Difloxacini hydrochloridum trihydricum ad usum veterinarium

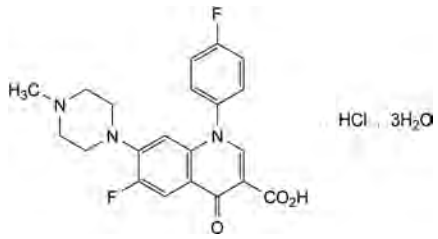


$C_{21}H_{20}ClF_2N_3O_3,3H_2O$ $M_r$ 490.0

Anhydrous difloxacin hydrochloride: [91296-86-5]

DEFINITION

6-Fluoro-1-(4-fluorophenyl)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid hydrochloride trihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or light yellow, crystalline powder.

*Solubility*: slightly soluble in water and in methanol, very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *difloxacin hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Suspend 30 mg in 2 mL of *water R*, acidify with *dilute nitric acid R* and filter. The clear filtrate gives reaction (a) of chlorides (*2.3.1*).

C. Water (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Solution A*. Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 900 mL of *water R* and adjust to pH 2.5 with *phosphoric acid R*; dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in 50.0 mL of the solvent mixture and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 6.0 mg of *difloxacin impurity G CRS* in *acetonitrile R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Mix 0.5 mL of reference solution (a), 1.0 mL of the test solution and 50 mL of the solvent mixture and dilute to 100.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 3 mg of *sarafloxacin hydrochloride R* (impurity B) in 100.0 mL of solution A. Dilute 1.0 mL of the solution to 50.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

Monographs D

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, *tetrahydrofuran R*, solution A (5:5:90 *V/V/V*);

– *mobile phase B*: *acetonitrile R*, solution A, *tetrahydrofuran R* (5:35:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 50 | 100 → 0 | 0 → 100 |
| 50 - 60 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 30 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to difloxacin (retention time = about 10 min): impurity B = about 1.2; impurity G = about 4.0.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to difloxacin.

*Limits*:

– *impurity G*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the peak due to difloxacin in the chromatogram obtained with reference solution (b) (0.20 per cent);

– *total*: maximum 1.0 per cent;

– *disregard limit*: 0.1 times the area of the peak due to difloxacin in the chromatogram obtained with reference solution (b) (0.10 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

*Solvent mixture*. Dissolve 30 g of *propylene glycol R* in 30 mL of *methanol R*, add 4 g of *arginine R* and dilute to 100 mL with *water R*.

0.25 g complies with test H. Prepare the reference solution using 0.5 mL of *lead standard solution (10 ppm Pb) R*. The substance precipitates after addition of *buffer solution pH 3.5 R*. Dilute to 20 mL with *methanol R*; the substance re-dissolves completely.

**Water** (*2.5.12*): 8.0 per cent to 12.0 per cent, determined on 0.100 g, using a mixture of 20 volumes of *formamide R* and 25 volumes of *methanol R* as solvent.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.150 g in 5 mL of *anhydrous formic acid R* and add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflexion.

1 mL of *0.1 M perchloric acid* is equivalent to 21.79 mg of $C_{21}H_{20}ClF_2N_3O_3$.

IMPURITIES

*Specified impurities: G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

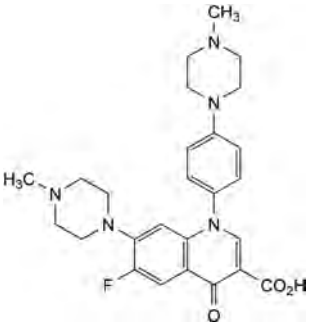

A. 6-fluoro-7-(4-methylpiperazin-1-yl)-1-[4-(4-methylpiperazin-1-yl)phenyl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

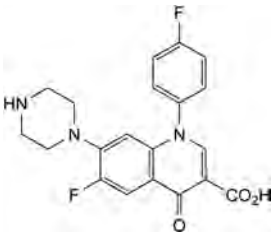

B. 6-fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid (sarafloxacin),

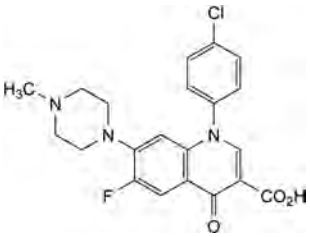

C. 1-(4-chlorophenyl)-6-fluoro-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

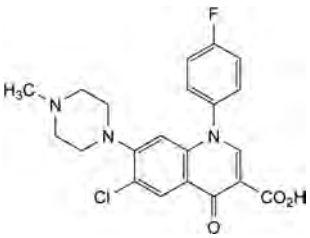

D. 6-chloro-1-(4-fluorophenyl)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

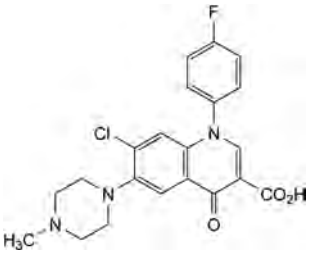

E. 7-chloro-1-(4-fluorophenyl)-6-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

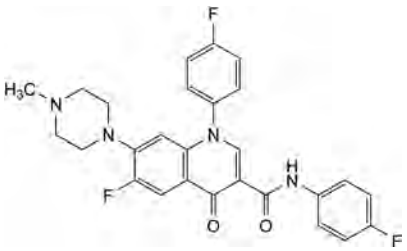

F. 6-fluoro-*N*,1-bis(4-fluorophenyl)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxamide,

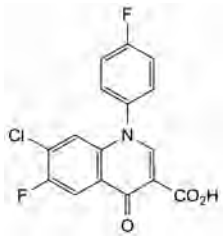

G. 7-chloro-6-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.

**01/2008:0078**
**corrected 6.0**

# DIGITOXIN

## Digitoxinum

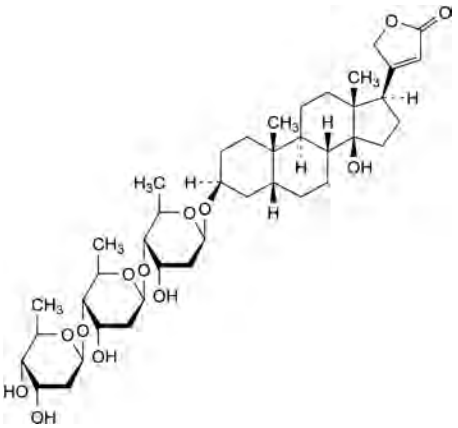

$C_{41}H_{64}O_{13}$ $M_r$ 765
[71-63-6]

DEFINITION

Digitoxin contains not less than 95.0 per cent and not more than the equivalent of 103.0 per cent of 3β-[(*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-5β,14β-card-20(22)-enolide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in a mixture of equal volumes of methanol and methylene chloride, slightly soluble in alcohol and in methanol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *digitoxin CRS*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Suspend about 0.5 mg in 0.2 mL of *alcohol (60 per cent V/V) R*. Add 0.1 mL of *dinitrobenzoic acid solution R* and 0.1 mL of *dilute sodium hydroxide solution R*. A violet colour develops.

D. Dissolve about 0.5 mg in 1 mL of *glacial acetic acid R*, heating gently, allow to cool and add 0.05 mL of *ferric chloride solution R1*. Cautiously add 1 mL of *sulfuric acid R*, avoiding mixing the two liquids. A brown ring develops at the interface and on standing a green, then blue colour passes to the upper layer.

TESTS

**Appearance of solution**. Dissolve 50 mg in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Specific optical rotation** (*2.2.7*). Dissolve 0.25 g in *chloroform R* and dilute to 10.0 mL with the same solvent. The specific optical rotation is + 16.0 to + 18.5.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel G plate R*.

*Test solution.* Dissolve 20 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 2 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 20 mg of *digitoxin CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 2 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 0.5 mL of reference solution (a) to 50 mL with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

*Reference solution (c).* Dissolve 10 mg of *gitoxin CRS* with stirring in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 50 mL with the same mixture of solvents.

*Reference solution (d).* Dilute 1 mL of reference solution (b) to 2 mL with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

*Reference solution (e).* Mix 1 mL of reference solution (a) and 1 mL of reference solution (c).

Apply to the plate 5 µL of each solution. Develop immediately over a path of 15 cm using a mixture of 15 volumes of *methanol R*, 40 volumes of *cyclohexane R* and 90 volumes of *methylene chloride R*. Dry the plate in a stream of cold air for 5 min. Repeat the development and dry the plate in a stream of cold air for 5 min. Spray with a mixture of 1 volume of *sulfuric acid R* and 9 volumes of *alcohol R* and heat at 130 °C for 15 min. Examine in daylight.

*Gitoxin.* Any spot corresponding to gitoxin in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c) (2.0 per cent).

*Other glycosides.* Any spot in the chromatogram obtained with the test solution, apart from the principal spot and the spot corresponding to gitoxin, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

The test is not valid unless the chromatogram obtained with reference solution (e) shows clearly separated spots corresponding to digitoxin, gitoxin and other glycosides and the spot in the chromatogram obtained with reference solution (d) is clearly visible.

**Loss on drying** (*2.2.32*). Not more than 1.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 40.0 mg in *alcohol R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *alcohol R*. Prepare a reference solution in the same manner, using 40.0 mg of *digitoxin CRS*. To 5.0 mL of each solution add 3.0 mL of *alkaline sodium picrate solution R*, allow to stand protected from bright light for 30 min and measure the absorbance (*2.2.25*) of each solution at the maximum at 495 nm, using as the compensation liquid a mixture of 5.0 mL of *alcohol R* and 3.0 mL of *alkaline sodium picrate solution R* prepared at the same time.

Calculate the content of $C_{41}H_{64}O_{13}$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Store protected from light.

**01/2008:0079**
**corrected 7.0**

# DIGOXIN

## Digoxinum

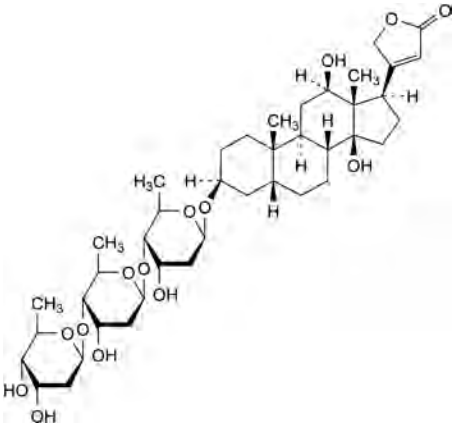

$C_{41}H_{64}O_{14}$ $M_r$ 781
[20830-75-5]

DEFINITION

3β-[(2,6-Dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, or colourless crystals.

*Solubility*: practically insoluble in water, soluble in a mixture of equal volumes of methanol and methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *digoxin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

Dissolve 50 mg in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

**Specific optical rotation** (*2.2.7*): + 13.9 to + 15.9 (dried substance).

Dissolve 0.50 g in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 25.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 100.0 mL of *methanol R*.

*Reference solution (a)*. Dissolve 10.0 mg of *digoxin CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 2.5 mg of *digoxigenin CRS* (impurity C) in *methanol R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (d)*. Dissolve 50.0 mg of *lanatoside C R* (impurity H) in *methanol R* and dilute to 100.0 mL with the same solvent. To 1.0 mL of this solution, add 1.0 mL of the test solution and dilute to 20.0 mL with *methanol R*.

*Reference solution (e)*. Dissolve 5.0 mg of *digoxin for peak identification CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, *water R* (10:90 *V/V*);
- *mobile phase B*: *water R*, *acetonitrile R* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 78 | 22 |
| 5 - 15 | 78 → 30 | 22 → 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *digoxin for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities A, B, C, E, F, G and K.

*Relative retention* with reference to digoxin (retention time = about 4.3 min): impurity C = about 0.3; impurity E = about 0.5; impurity F = about 0.6; impurity G = about 0.8; impurity L = about 1.4; impurity K = about 1.6; impurity B = about 2.2; impurity A = about 2.6.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurity H and digoxin.

*Limits*:

- *impurity F*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent);

- *impurity C*: not more than 5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *impurities E, K*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity G*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *impurities A, B*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity L*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *sum of impurities other than A, B, C, E, F, G, K, L*: not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
- *total*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

The thresholds indicated under Related Substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying *in vacuo* in an oven.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

## ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{41}H_{64}O_{14}$ from the declared content of *digoxin CRS*.

## STORAGE

Protected from light.

## IMPURITIES

*Specified impurities: A, B, C, E, F, G, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, H, I, J.*

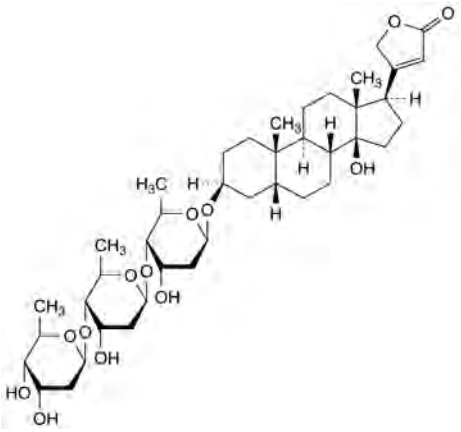

A. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxy-5β-card-20(22)-enolide (digitoxin),

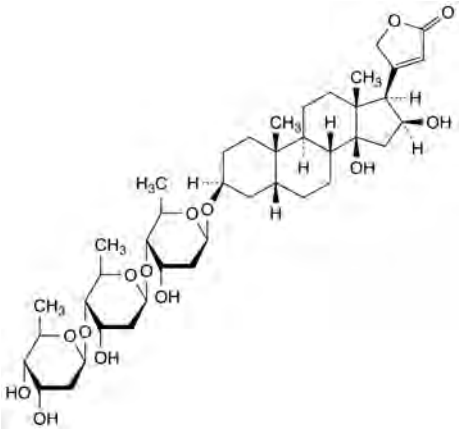

B. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14,16β-dihydroxy-5β-card-20(22)-enolide (gitoxin),

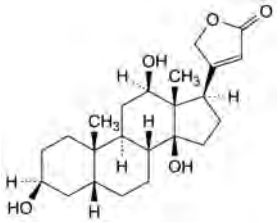

C. 3β,12β,14-trihydroxy-5β-card-20(22)-enolide (digoxigenin),

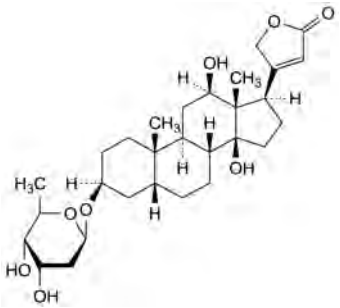

D. 3β-(2,6-dideoxy-β-D-*ribo*-hexopyranosyloxy)-12β,14-dihydroxy-5β-card-20(22)-enolide (digoxigenin monodigitoxoside),

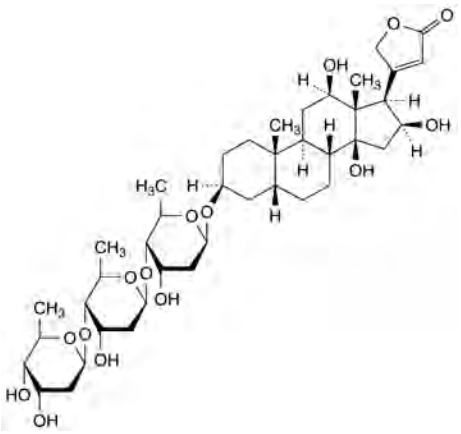

E. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14,16β-trihydroxy-5β-card-20(22)-enolide (diginatin),

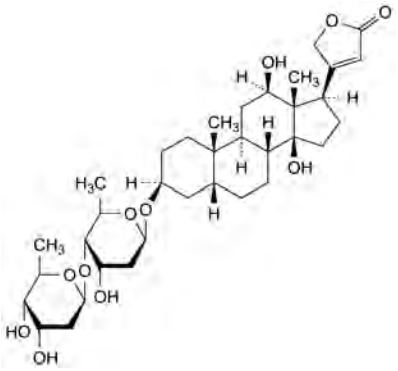

F. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (digoxigenin bisdigitoxoside),

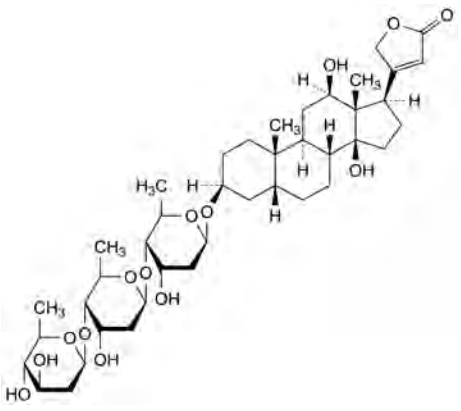

G. 3β-[(2,6-dideoxy-β-D-*arabino*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (neodigoxin),

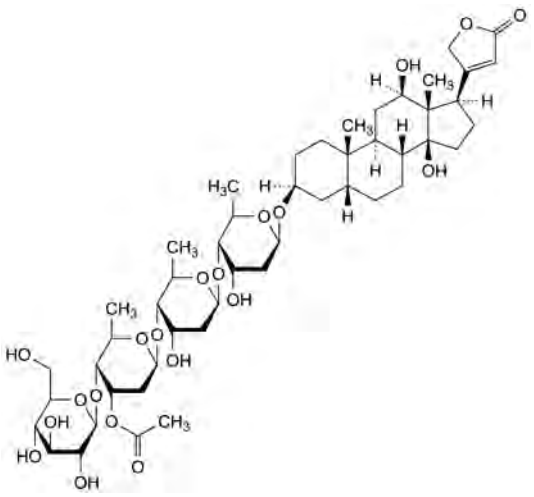

H. 3β-[(β-D-glucopyranosyl-(1→4)-3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (lanatoside C),

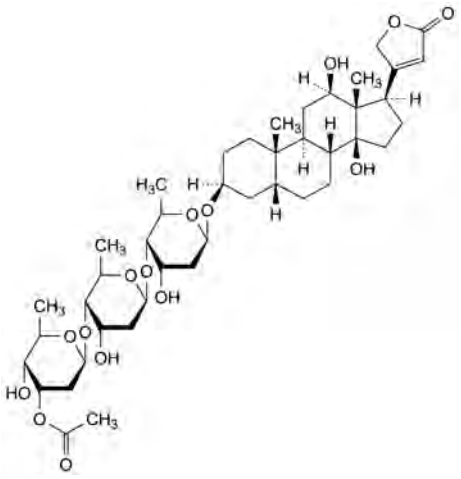

I. 3β-[(3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (α-acetyldigoxin),

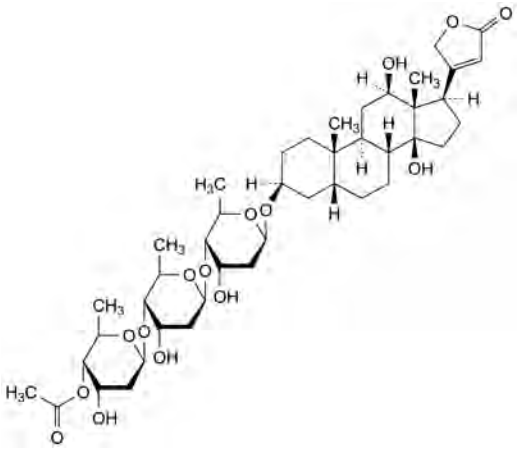

J. 3β-[(4-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (β-acetyldigoxin),

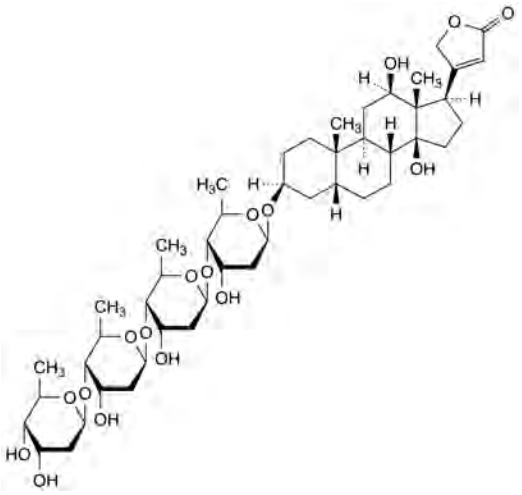

K. 3β-[(2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide (digoxigenin tetrakisdigitoxoside),

L. unknown structure.

**01/2016:1310**
**corrected 9.2**

# DIHYDRALAZINE SULFATE, HYDRATED

## Dihydralazini sulfas hydricus

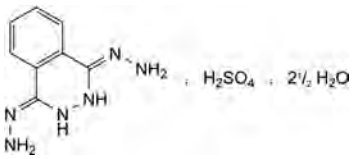

$C_8H_{12}N_6O_4S,2½H_2O$ $M_r$ 333.3

Dihydralazine sulfate, anhydrous: [7327-87-9]

DEFINITION

(Phthalazine-1,4(2*H*,3*H*)-diylidene)dihydrazine sulfate 2.5-hydrate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: slightly soluble in water, practically insoluble in anhydrous ethanol. It dissolves in dilute mineral acids.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of dihydralazine sulfate hydrated*.

B. Dissolve about 50 mg in 5 mL of *dilute hydrochloric acid R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in *dilute nitric acid R* and dilute to 10 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in a 6 g/L solution of *glacial acetic acid R* and dilute to 50.0 mL with the same solution.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase containing 0.5 g/L of *sodium edetate R*. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase containing 0.5 g/L of *sodium edetate R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase containing 0.5 g/L of *sodium edetate R*.

*Reference solution (c).* Dissolve 5 mg of *dihydralazine for system suitability CRS* in a 6 g/L solution of *glacial acetic acid R* and dilute to 5.0 mL with the same solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *nitrile silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 22 volumes of *acetonitrile R1* and 78 volumes of a solution containing 1.44 g/L of *sodium laurilsulfate R* and 0.75 g/L of *tetrabutylammonium bromide R*, then adjust to pH 3.0 with *dilute sulfuric acid R1*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of dihydralazine.

*Relative retention* with reference to dihydralazine: impurity A = about 0.8.

*System suitability*: reference solution (c):
- the peaks due to impurity A and dihydralazine are baseline separated as in the chromatogram supplied with *dihydralazine for system suitability CRS*.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Impurity B**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 40.0 mg of *hydrazine sulfate R* (impurity B) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*. To 0.50 mL of this solution, add 0.200 g of the substance to be examined and dissolve in 6 mL of *dilute hydrochloric acid R*, then dilute to 10.0 mL with *water R*. In a centrifuge tube with a ground-glass stopper, place immediately 0.50 mL of this solution and 2.0 mL of a 60 g/L solution of *benzaldehyde R* in a mixture of equal volumes of *methanol R* and *water R*. Shake for 90 s. Add 1.0 mL of *water R* and 5.0 mL of *heptane R*. Shake for 1 min and centrifuge. Use the upper layer.

*Reference solution.* Dissolve 40.0 mg of *hydrazine sulfate R* (impurity B) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 25.0 mL with *water R*. To 0.50 mL of this solution, add 6 mL of *dilute hydrochloric acid R* and dilute to 10.0 mL with *water R*. In a centrifuge tube with a ground-glass stopper, place 0.50 mL of this solution

and 2.0 mL of a 60 g/L solution of *benzaldehyde R* in a mixture of equal volumes of *methanol R* and *water R*. Shake for 90 s. Add 1.0 mL of *water R* and 5.0 mL of *heptane R*. Shake for 1 min and centrifuge. Use the upper layer.

*Blank solution.* Prepare in the same manner as for the reference solution but replacing the 0.50 mL of hydrazine sulfate solution by 0.50 mL of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 0.3 g/L solution of *sodium edetate R*, *acetonitrile R* (30:70 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 305 nm.

*Injection*: 20 µL.

*Relative retention* with reference to benzaldehyde: benzaldehyde azine (benzalazine) corresponding to impurity B = about 1.8.

*Limit*:

– *impurity B*: the area of the peak due to benzaldehyde azine is not greater than twice the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

**Iron** (*2.4.9*): maximum 20 ppm.

To the residue obtained in the test for sulfated ash add 0.2 mL of *sulfuric acid R* and heat carefully until the acid is almost completely eliminated. Allow to cool and dissolve the residue with heating in 5.5 mL of *hydrochloric acid R1*. Filter the hot solution through a filter previously washed 3 times with *dilute hydrochloric acid R*. Wash the crucible and the filter with 5 mL of *water R*. Combine the filtrate and the washings and neutralise with about 3.5 mL of *strong sodium hydroxide solution R*. Adjust to pH 3-4 with *acetic acid R* and dilute to 20 mL with *water R*. Prepare the standard with 5 mL of *iron standard solution (2 ppm Fe) R* and 5 mL of *water R*.

**Loss on drying** (*2.2.32*): 13.0 per cent to 15.0 per cent, determined on 1.000 g by drying in an oven at 50 °C at a pressure not exceeding 0.7 kPa for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 60.0 mg in 25 mL of *water R*. Add 35 mL of *hydrochloric acid R* and titrate slowly with *0.05 M potassium iodate*, determining the end-point potentiometrically (*2.2.20*), using a suitable reference electrode and a platinum indicator electrode.

1 mL of *0.05 M potassium iodate* is equivalent to 7.208 mg of $C_8H_{12}N_6O_4S$.

IMPURITIES

*Specified impurities: A, B, C.*

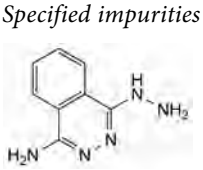

A. 4-hydrazinophthalazin-1-amine,

B. $H_2N$-$NH_2$: hydrazine,

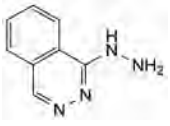

C. (phthalazin-1-yl)hydrazine (hydralazine).

**01/2008:1776**

# DIHYDROCODEINE HYDROGEN TARTRATE

## Dihydrocodeini hydrogenotartras

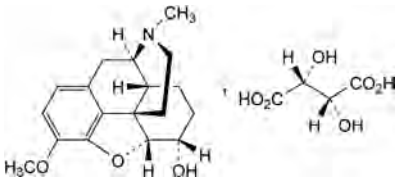

$C_{22}H_{29}NO_9$ $M_r$ 451.5

[5965-13-9]

DEFINITION

4,5α-Epoxy-3-methoxy-17-methylmorphinan-6α-ol hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in alcohol, practically insoluble in cyclohexane.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of dihydrocodeine hydrogen tartrate*.

B. To about 0.1 g add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R1* and heat on a water-bath. A brownish-yellow colour develops. Add 0.05 mL of *dilute nitric acid R*. The colour does not become red.

C. To 1 mL of solution S (see Tests) add 5 mL of *picric acid solution R*. Heat on a water-bath until a clear solution is obtained. Allow to cool. A precipitate is formed. Filter, wash with 5 mL of *water R* and dry at 100-105 °C. The crystals melt (*2.2.14*) at 220 °C to 223 °C.

D. It gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.2 to 4.2 for solution S.

**Specific optical rotation** (*2.2.7*): – 70.5 to – 73.5 (anhydrous substance).

Dilute 10.0 mL of solution S to 20.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *codeine phosphate R* in 2.0 mL of the test solution and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: to 1.0 g of *sodium heptanesulfonate R*, add 10.0 mL of *glacial acetic acid R* and 4.0 mL of a solution of 5.0 mL of *triethylamine R* diluted to 25.0 mL with a mixture of equal volumes of *water R* and *acetonitrile R*. Add 170 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 284 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of dihydrocodeine.

*Retention time*: dihydrocodeine = about 14 min.

*System suitability*: reference solution (a):

– *resolution*: minimum of 2 between the peaks due to dihydrocodeine and to impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *any other peak*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent); disregard any peak due to tartaric acid (relative retention with reference to dihydrocodeine = about 0.25),
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.7 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 45.15 mg of $C_{22}H_{29}NO_9$.

STORAGE

Protected from light.

IMPURITIES

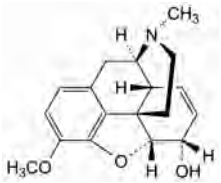

A. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (codeine),

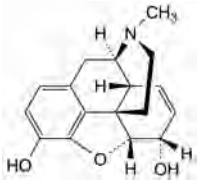

B. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol (morphine),

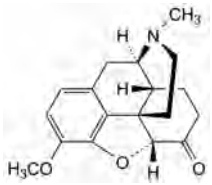

C. 4,5α-epoxy-3-methoxy-17-methylmorphinan-6-one (hydrocodone),

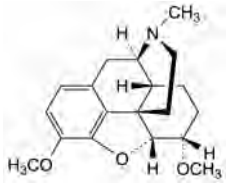

D. 4,5α-epoxy-3,6α-dimethoxy-17-methylmorphinan (tetrahydrothebaine).

**04/2016:1416**

# DIHYDROERGOCRISTINE MESILATE

## Dihydroergocristini mesilas

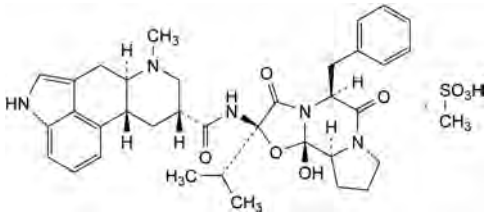

$C_{36}H_{45}N_5O_8S$ $M_r$ 708

[24730-10-7]

DEFINITION

(6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-Benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxo-octahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in dihydroergocristine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, fine crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *dihydroergocristine mesilate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution.* Dissolve 0.10 g of *dihydroergocristine mesilate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *dimethylformamide R*, *ether R* (2:15:85 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate protected from light.

*Drying*: in a current of cold air for 5 min.

*Detection*: spray with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air for 2 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution.* Dissolve 0.20 g of *methanesulfonic acid R* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5 mL with the same mixture of solvents. Dilute 1 mL of the solution to 10 mL with a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *water R*, *concentrated ammonia R*, *butanol R*, *acetone R* (5:10:20:65 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm protected from light.

*Drying*: in a current of cold air for not more than 1 min.

*Detection*: spray with a 1 g/L solution of *bromocresol purple R* in *methanol R*, adjusting the colour to violet-red with one drop of *dilute ammonia R1* and dry the plate in a current of hot air at 100 °C.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 0.50 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 43 to − 37 (dried substance).

Dissolve 0.250 g in *anhydrous pyridine R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test and preparation of the solutions protected from bright light.*

*Test solution.* Dissolve 75.0 mg of the substance to be examined in 10 mL of *acetonitrile R*. Add 10 mL of a 1.0 g/L solution of *phosphoric acid R* and dilute to 50.0 mL with *water R*.

*Reference solution.* Dissolve 20.0 mg of *codergocrine mesilate CRS* in 10 mL of *acetonitrile R*. Add 10 mL of a 1.0 g/L solution of *phosphoric acid R* and dilute to 50.0 mL with *water R*. Dilute 6.0 mL of the solution to 50.0 mL with a mixture of 20 volumes of *acetonitrile R*, 20 volumes of a 1.0 g/L solution of *phosphoric acid R* and 60 volumes of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

*Mobile phase*:

- *mobile phase A*: mix 100 volumes of *acetonitrile R* with 900 volumes of *water R* and add 10 volumes of *triethylamine R*;
- *mobile phase B*: mix 100 volumes of *water R* with 900 volumes of *acetonitrile R* and add 10 volumes of *triethylamine R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 75 | 25 |
| 5 - 20 | 75 → 25 | 25 → 75 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Relative retention* with reference to dihydroergocristine (retention time = about 13.7 min): impurity F = about 0.8; impurity H = about 0.9; impurity I = about 1.02.

*System suitability*: reference solution:

- the chromatogram shows 4 peaks;
- *resolution*: minimum 1 between the peaks due to dihydroergocristine and impurity I.

*Limits*:

- *any impurity*: not more than the area of the peak due to dihydroergocristine in the chromatogram obtained with the reference solution (1 per cent);
- *total*: not more than twice the area of the peak due to dihydroergocristine in the chromatogram obtained with the reference solution (2 per cent);
- *disregard limit*: 0.1 times the area of the peak due to dihydroergocristine in the chromatogram obtained with the reference solution (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 0.500 g by drying under high vacuum at 80 °C.

ASSAY

Dissolve 0.300 g in 60 mL of *pyridine R*. Pass a stream of *nitrogen R* over the surface of the solution and titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Note the volume used at the second point of inflexion.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 35.39 mg of $C_{36}H_{45}N_5O_8S$.

STORAGE

Store protected from light.

IMPURITIES

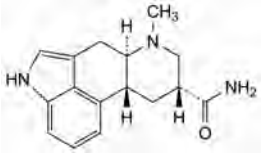


A. (6a*R*,9*R*,10a*R*)-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (6-methylergoline-8β-carboxamide),

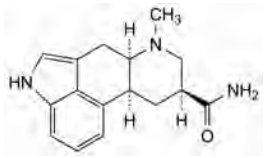

B. (6a*R*,9*S*,10a*S*)-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (6-methylisoergoline-8α-carboxamide),

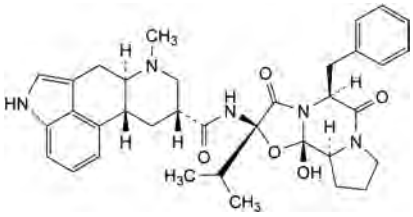

C. (6a*R*,9*R*,10a*R*)-*N*-[(2*S*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2′-epidihydroergocristine),

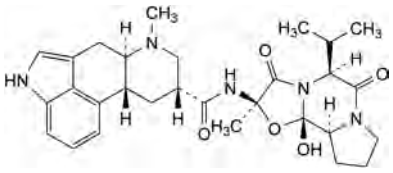

D. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-methyl-5-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergosine),

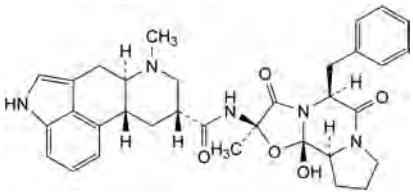

E. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline- 9-carboxamide (dihydroergotamine),

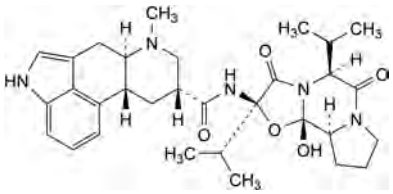

F. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)10b-hydroxy-2,5-bis(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergocornine),

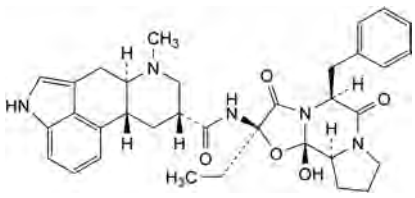

G. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-2-ethyl-10b-hydroxy-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergostine),

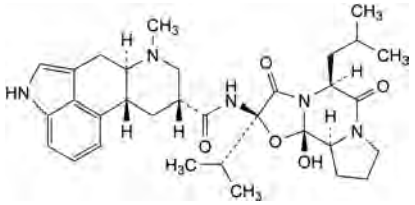

H. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-(2-methylpropyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (α-dihydroergocryptine),

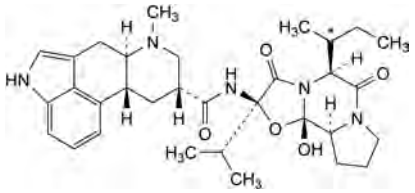

I. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-10b-hydroxy-2-(1-methylethyl)-5-[(1*RS*-1-methylpropyl]-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (β-dihydroergocryptine or epicriptine),

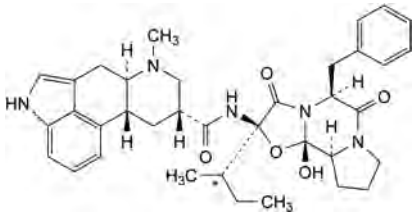

J. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-[(1*RS*)-1-methylpropyl]-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergosedmine),

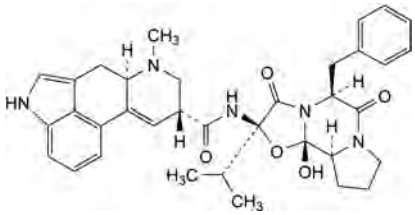

K. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergocristine),

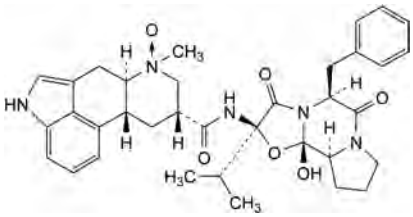

L. (6a*R*,7*RS*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide 7-oxide (dihydroergocristine 6-oxide).

**04/2016:0551**

Monographs D

# DIHYDROERGOTAMINE MESILATE

## Dihydroergotamini mesilas

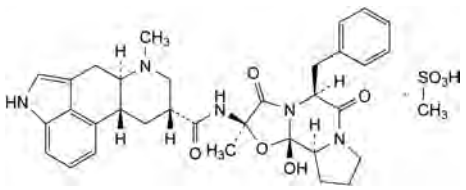

$C_{34}H_{41}N_5O_8S$ $M_r$ 680
[6190-39-2]

DEFINITION

(6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-Benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide methanesulfonate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in dihydroergotamine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, C.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 5.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 250-350 nm.

*Absorption maxima*: at 281 nm and 291 nm.

*Shoulder*: at 275 nm.

*Absorbance*: negligible above 320 nm.

*Specific absorbance at the absorption maximum at 281 nm*: 95 to 105 (dried substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dihydroergotamine mesilate CRS.*

C. Thin-layer chromatography (*2.2.27*). *Prepare the reference solution and the test solution immediately before use.*

*Solvent mixture*: *methanol R, methylene chloride R* (10:90 *V/V*).

*Test solution.* Dissolve 5 mg of the substance to be examined in the solvent mixture and dilute to 2.5 mL with the solvent mixture.

*Reference solution.* Dissolve 5 mg of *dihydroergotamine mesilate CRS* in the solvent mixture and dilute to 2.5 mL with the solvent mixture.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R, methanol R, ethyl acetate R, methylene chloride R* (1:6:50:50 *V/V/V/V*).

*Application*: 5 µL.

*Development*: protected from light, over a path of 15 cm; dry in a current of cold air for not longer than 1 min and repeat the development protected from light over a path of 15 cm using a freshly prepared amount of the mobile phase.

*Drying*: in a current of cold air.

*Detection*: spray abundantly with *dimethylaminobenzaldehyde solution R7* and dry in a current of hot air for about 2 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 g of the substance to be examined, add 5 mL of *dilute hydrochloric acid R* and shake for about 5 min. Filter, then add 1 mL of *barium chloride solution R1*. The filtrate remains clear. Mix 0.1 g of the substance to be examined with 0.4 g of powdered *sodium hydroxide R*, heat to fusion and continue to heat for 1 min. Cool, add 5 mL of *water R*, boil and filter. Acidify the filtrate with *hydrochloric acid R1* and filter again. The filtrate gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ or $BY_7$ (*2.2.2, Method II*).

Dissolve 0.10 g in a mixture of 0.1 mL of a 70 g/L solution of *methanesulfonic acid R* and 50 mL of *water R*.

**pH** (*2.2.3*): 4.4 to 5.4.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 47 to − 42 (dried substance).

Dissolve 0.250 g in *anhydrous pyridine R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R, water R* (50:50 *V/V*).

*Test solution.* Dissolve 70 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 7 mg of the substance to be examined and 6.8 mg of *ergotamine tartrate CRS* (impurity A) (equivalent to 7 mg of ergotamine mesilate) in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 5 mL of this solution to 10 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *dihydroergotamine for peak identification CRS* (containing impurities A, B, C, D and E) in the solvent mixture, add 100 µL of *dilute sulfuric acid R* and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 3 g/L solution of *sodium heptanesulfonate monohydrate R* adjusted to pH 2.0 with *phosphoric acid R*;
- *mobile phase B*: mobile phase A, *acetonitrile for chromatography R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 58 → 40 | 42 → 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *dihydroergotamine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to dihydroergotamine (retention time = about 6.5 min): impurity D = about 0.7; impurity C = about 0.86; impurity A = about 0.95; impurity B = about 1.2; impurity E = about 1.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity A and dihydroergotamine.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.3; impurity C = 1.3;
- *impurities B, E*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 0.500 g by drying at 105 °C at a pressure not exceeding 0.1 kPa for 5 h.

ASSAY

Dissolve 0.500 g in a mixture of 10 mL of *anhydrous acetic acid R* and 70 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 68.00 mg of $C_{34}H_{41}N_5O_8S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

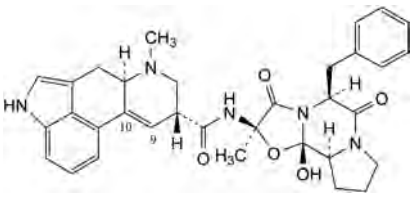

A. (6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergotamine),

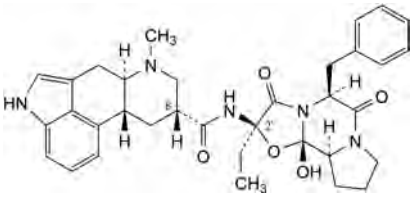

B. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-2-ethyl-10b-hydroxy-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (9,10-dihydroergostine),

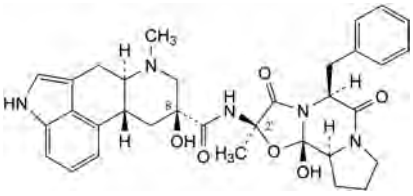

C. (6a*R*,9*S*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-9-hydroxy-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (8-hydroxy-9,10-dihydroergotamine),

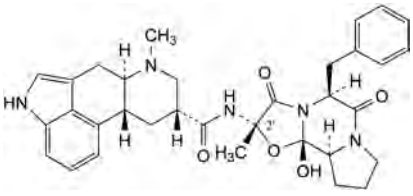

D. (6a*R*,9*R*,10a*R*)-*N*-[(2*S*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (2′-*epi*-9,10-dihydroergotamine),

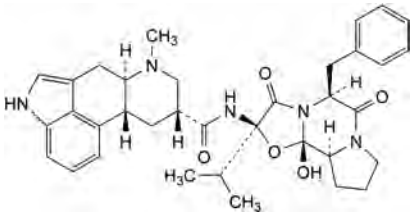

E. (6a*R*,9*R*,10a*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-(1-methylethyl)-3,6-dioxo-octahydro-8*H*-oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline-9-carboxamide (dihydroergocristine).

**01/2019:0485**
**corrected 10.0**

# DIHYDROSTREPTOMYCIN SULFATE FOR VETERINARY USE

## Dihydrostreptomycini sulfas ad usum veterinarium

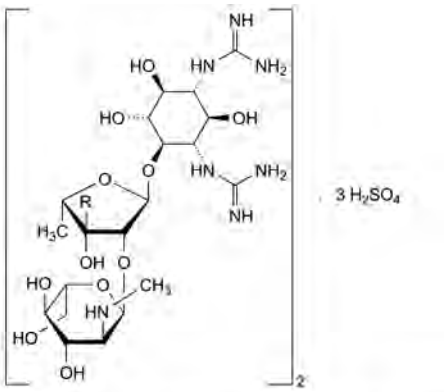

| Compound | R | Molec. Formula | $M_r$ |
|---|---|---|---|
| dihydrostreptomycin sulfate | $CH_2OH$ | $C_{42}H_{86}N_{14}O_{36}S_3$ | 1461 |
| streptomycin sulfate | CHO | $C_{42}H_{84}N_{14}O_{36}S_3$ | 1457 |

[5490-27-7]

DEFINITION

*Main compound*: bis[$N^1$,$N^3$-dicarbamimidoyl-4-*O*-[5-deoxy-2-*O*-[2-deoxy-2-(methylamino)-α-L-glucopyranosyl]-3-*C*-(hydroxymethyl)-α-L-lyxofuranosyl]-D-streptamine] trisulfate.

Sulfate of a substance obtained by catalytic hydrogenation of streptomycin or by any other means.

Semi-synthetic product derived from a fermentation product.

Stabilisers may be added.

*Content*:

– *sum of the percentage contents of dihydrostreptomycin sulfate and streptomycin sulfate*: 95.0 per cent to 102.0 per cent (dried substance);
– *streptomycin sulfate*: maximum 2.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in acetone, in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, E.*

*Second identification: B, C, D, E.*

A. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.
*Reference solution (a).* Dissolve the contents of a vial of *dihydrostreptomycin sulfate CRS* in 5.0 mL of *water R*.
*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 5.0 mL with *water R*.
*Reference solution (c).* Dissolve 10 mg of *kanamycin monosulfate CRS* and 10 mg of *neomycin sulfate CRS* in *water R*, add 2.0 mL of reference solution (a), mix thoroughly and dilute to 10 mL with *water R*.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: 70 g/L solution of *potassium dihydrogen phosphate R*.
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of warm air.
*Detection*: spray with a mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 460 g/L solution of *sulfuric acid R*; heat at 150 °C for 5-10 min.
*System suitability*: reference solution (c):
– the chromatogram shows 3 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

C. Dissolve 0.1 g in 2 mL of *water R* and add 1 mL of *α-naphthol solution R* and 2 mL of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.

D. Dissolve 10 mg in 5 mL of *water R* and add 1 mL of *1 M hydrochloric acid*. Heat in a water-bath for 2 min. Add 2 mL of a 5 g/L solution of *α-naphthol R* in *1 M sodium hydroxide* and heat in a water-bath for 1 min. A violet-pink colour is produced.

E. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*). Allow to stand protected from light at about 20 °C for 24 h; solution S is not more opalescent than reference suspension II (*2.2.1*).

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): – 91.0 to – 83.0 (dried substance).

Dissolve 0.200 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *dihydrostreptomycin sulfate CRS* (containing impurities A, B and C) in 5.0 mL of *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 50.0 mL with *water R*.

*Reference solution (d).* Dissolve 10 mg of *streptomycin sulfate CRS* in *water R* and dilute to 20 mL with the same solvent. Mix 0.1 mL of this solution with 1.0 mL of reference solution (a).

*Reference solution (e).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: solution containing 4.6 g/L of *anhydrous sodium sulfate R*, 1.5 g/L of *sodium octanesulfonate R*, 120 mL/L of *acetonitrile R1* and 50 mL/L of a 27.2 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with a 22.5 g/L solution of *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of dihydrostreptomycin.

*Identification of impurities*: use the chromatogram supplied with *dihydrostreptomycin sulfate CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to streptomycin and impurities A, B and C.

*Relative retention* with reference to dihydrostreptomycin (retention time = about 57 min): impurity A = about 0.2; impurity B = about 0.8; streptomycin = about 0.9; impurity C = about 0.95.

*System suitability*:

- peak-to-valley ratio (a): minimum 1.1, where $H_p$ = height above the baseline of the peak due to streptomycin and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity C in the chromatogram obtained with reference solution (d);
- peak-to-valley ratio (b): minimum 5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dihydrostreptomycin in the chromatogram obtained with reference solution (d);
- the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *dihydrostreptomycin sulfate CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.5;
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent); disregard the peak due to streptomycin.

**Heavy metals** (*2.4.8*): 20 ppm.

1.0 g complies with test C. Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solutions (a) and (e).

Calculate the percentage content of streptomycin sulfate using the chromatogram obtained with reference solution (e) and the assigned content of *dihydrostreptomycin sulfate CRS*.

Calculate the percentage content of dihydrostreptomycin sulfate using the chromatogram obtained with reference solution (a) and the assigned content of *dihydrostreptomycin sulfate CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

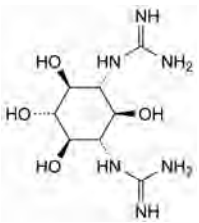

A. $N^1$,$N^3$-dicarbamimidoyl-D-streptamine (streptidine),

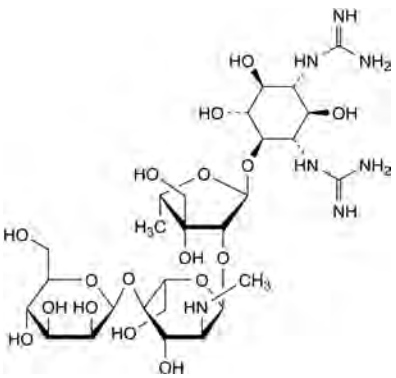

B. $N^1$,$N^3$-dicarbamimidoyl-4-*O*-[5-deoxy-2-*O*-[2-deoxy-4-*O*-β-D-mannopyranosyl-2-(methylamino)-α-L-glucopyranosyl]-3-*C*-(hydroxymethyl)-α-L-lyxofuranosyl]-D-streptamine (dihydrostreptomycin B),

C. unknown structure,

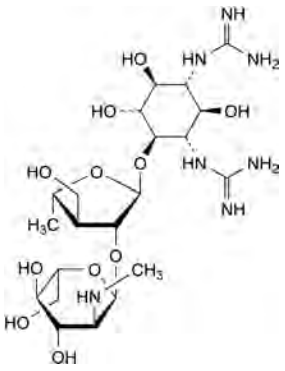

D. $N^1$,$N^3$-dicarbamimidoyl-4-*O*-[3,5-dideoxy-2-*O*-[2-deoxy-2-(methylamino)-α-L-glucopyranosyl]-3-*C*-(hydroxymethyl)-α-L-arabinofuranosyl]-D-streptamine (deoxydihydrostreptomycin).



**01/2019:2014**

# DIHYDROTACHYSTEROL

## Dihydrotachysterolum

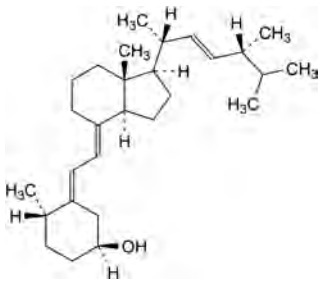

$C_{28}H_{46}O$ $M_r$ 398.7
[67-96-9]

DEFINITION

(5*E*,7*E*,22*E*)-9,10-Seco-10α-ergosta-5,7,22-trien-3β-ol.

*Content*: 97.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: colourless crystals or white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in hexane, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dihydrotachysterol CRS*.

If the spectra obtained in the solid state show differences, record new spectra using the residues after recrystallisation from *methanol R*.

TESTS

**Specific optical rotation** (*2.2.7*): + 99 to + 103.

Dissolve 0.500 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 10.00 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 1.0 mg of *dihydrotachysterol for system suitability CRS* (containing impurities A, B and C) in *acetonitrile R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 10.00 mg of *dihydrotachysterol CRS* in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (c)*. Dilute 5.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 5.0 mL of this solution to 50.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography for separation of polycyclic aromatic hydrocarbons R* (3 μm);
- *temperature*: 40 °C.

*Mobile phase*: *decanol R, water for chromatography R, acetonitrile R1* (1:25:1000 *V/V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: variable-wavelength spectrophotometer capable of operating at 251 nm and at 203 nm.

*Injection*: 5 μL of the test solution and reference solutions (a) and (c).

*Run time*: twice the retention time of dihydrotachysterol.

*Identification of impurities*: reference solution (a):

- use the chromatogram obtained at 203 nm and the chromatogram obtained at 203 nm supplied with *dihydrotachysterol for system suitability CRS* to identify the peak due to impurity A;
- use the chromatogram obtained at 251 nm and the chromatogram obtained at 251 nm supplied with *dihydrotachysterol for system suitability CRS* to identify the peaks due to impurities B and C.

*Relative retention* with reference to dihydrotachysterol (retention time = about 15 min): impurity B = about 0.9; impurity C = about 1.2; impurity A (not visible at 251 nm, detected at 203 nm) = about 1.2.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dihydrotachysterol in the chromatogram obtained at 251 nm.

Examine the chromatogram obtained at 203 nm for impurity A and the chromatogram obtained at 251 nm for impurities other than A.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurities B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total (including A)*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) at 251 nm (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.10 per cent, determined on 40.0 mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 251 nm.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{28}H_{46}O$ taking into account the assigned content of *dihydrotachysterol CRS*.

STORAGE

Under an inert gas, in an airtight container, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

IMPURITIES

*Specified impurities: A, B, C.*

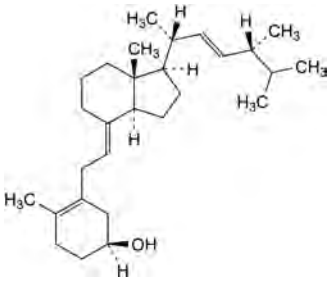

A. (7*E*,22*E*)-9,10-secoergosta-5(10),7,22-trien-3β-ol (dihydrovitamin $D_2$-I),

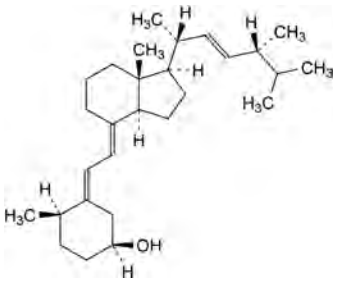

B. (5*E*,7*E*,22*E*)-9,10-secoergosta-5,7,22-trien-3β-ol (dihydrovitamin $D_2$-IV),

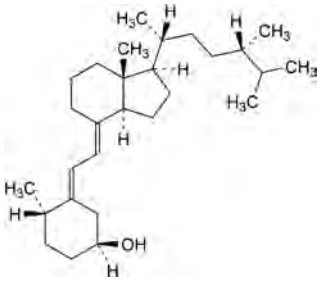

C. (5*E*,7*E*)-9,10-seco-10α-ergosta-5,7-dien-3β-ol (dihydrotachysterol$_4$).

**01/2017:1004**

# DILTIAZEM HYDROCHLORIDE

## Diltiazemi hydrochloridum

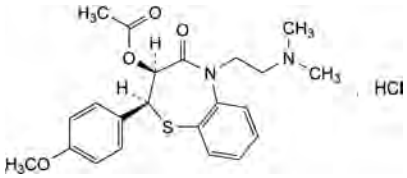

$C_{22}H_{27}ClN_2O_4S$ $M_r$ 451.0

[33286-22-5]

DEFINITION

Hydrochloride of (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, in methanol and in methylene chloride, slightly soluble in anhydrous ethanol.

mp: about 213 °C, with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *diltiazem hydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 5 mL with the same solvent.
*Reference solution*. Dissolve 50 mg of *diltiazem hydrochloride CRS* in *methylene chloride R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *acetic acid R*, *water R*, *methylene chloride R*, *anhydrous ethanol R* (1:3:10:12 *V/V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in 5 mL of *water R*. Add 1 mL of *ammonium reineckate solution R*. A pink precipitate is produced.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.3 to 5.3.

Dilute 2.0 mL of solution S to 10.0 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 115 to + 120 (dried substance).

Dilute 5.0 mL of solution S to 25.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 200.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *diltiazem for system suitability CRS* (containing impurity A) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5 mg of *diltiazem impurity F CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: mix 5 volumes of *anhydrous ethanol R*, 25 volumes of *acetonitrile R* and 70 volumes of a solution containing 6.8 g/L of *potassium dihydrogen phosphate R* and 0.1 mL/L of *N,N-dimethyloctylamine R*, adjusted to pH 4.5 with *dilute phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of diltiazem.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to diltiazem (retention time = about 5 min): impurity F = about 0.5; impurity A = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity A and diltiazem;

– *symmetry factor*: maximum 2.0 for the peak due to impurity A; if necessary, adjust the concentration of *N,N*-dimethyloctylamine in the mobile phase.

*Limits*:

– *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in a mixture of 2 mL of *anhydrous formic acid R* and 60 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 45.1 mg of $C_{22}H_{27}ClN_2O_4S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

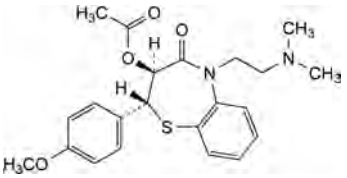

A. (2*R*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,

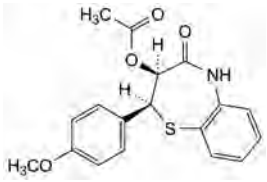

B. (2*S*,3*S*)-2-(4-methoxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,

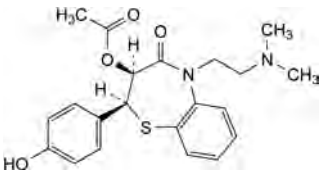

C. (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-2-(4-hydroxyphenyl)-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,

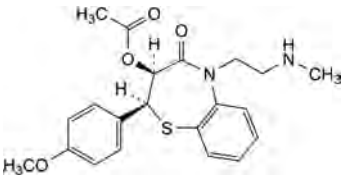

D. (2*S*,3*S*)-2-(4-methoxyphenyl)-5-[2-(methylamino)ethyl]-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepin-3-yl acetate,

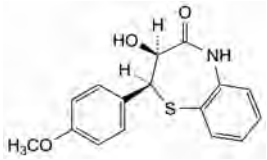

E. (2*S*,3*S*)-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one,

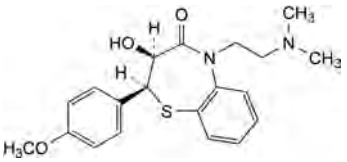

F. (2*S*,3*S*)-5-[2-(dimethylamino)ethyl]-3-hydroxy-2-(4-methoxyphenyl)-2,3-dihydro-1,5-benzothiazepin-4(5*H*)-one.

**07/2009:0601**

# DIMENHYDRINATE

## Dimenhydrinatum

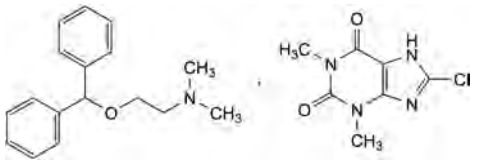

$C_{24}H_{28}ClN_5O_3$ $M_r$ 470.0
[523-87-5]

DEFINITION

Diphenhydramine [2-(diphenylmethoxy)-*N*,*N*-dimethylethanamine] 8-chlorotheophylline (8-chloro-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione).

*Content*:

– *diphenhydramine* ($C_{17}H_{21}NO$; $M_r$ 255.4): 53.0 per cent to 55.5 per cent (dried substance);

– *8-chlorotheophylline* ($C_7H_7ClN_4O_2$; $M_r$ 214.6): 44.0 per cent to 46.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *C*.

*Second identification*: *A, B, D*.

A. Melting point (*2.2.14*): 102 °C to 106 °C.

B. Dissolve 0.1 g in a mixture of 3 mL of *water R* and 3 mL of *ethanol (96 per cent) R*, add 6 mL of *water R* and 1 mL of *dilute hydrochloric acid R* and cool in iced water for 30 min, scratching the wall of the tube with a glass rod if necessary to initiate crystallisation. Dissolve about 10 mg of the precipitate obtained in 1 mL of *hydrochloric acid R*, add 0.1 g of *potassium chlorate R* and evaporate to dryness in a porcelain dish. A reddish residue is obtained that becomes violet-red when exposed to ammonia vapour.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dimenhydrinate CRS*.

D. Dissolve 0.2 g in 10 mL of *ethanol (96 per cent) R*. Add 10 mL of *picric acid solution R* and initiate crystallisation by scratching the wall of the tube with a glass rod. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 130 °C to 134 °C.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 7.1 to 7.6 for the filtrate.

To 0.4 g add 20 mL of *carbon dioxide-free water R*, shake for 2 min and filter.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (18:82 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 57 mg of *diphenhydramine hydrochloride CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5.0 mg of *diphenhydramine impurity A CRS* (impurity F) in 5.0 mL of reference solution (a) and dilute to 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve the contents of a vial of *dimenhydrinate for peak identification CRS* (containing impurities A and E) in 1.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 10.0 g of *triethylamine R2* in 950 mL of *water R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 2 | 82 | 18 | 1.2 |
| 2 - 15 | 82 → 50 | 18 → 50 | 1.2 |
| 15 - 20 | 50 → 20 | 50 → 80 | 1.2 → 2.0 |
| 20 - 30 | 20 | 80 | 2.0 |

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *dimenhydrinate for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A and E; use the chromatogram obtained with reference solution (c) to identify impurity F.

*Relative retention* with reference to diphenhydramine (retention time = about 13 min): impurity A = about 0.3; impurity E = about 0.7; impurity F = about 0.95.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity F and diphenhydramine.

*Limits*:

– *impurities A, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurity E*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

**Diphenhydramine**. Dissolve 0.200 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.54 mg of $C_{17}H_{21}NO$.

**8-Chlorotheophylline**. To 0.800 g add 50 mL of *water R*, 3 mL of *dilute ammonia R1* and 0.6 g of *ammonium nitrate R* and heat on a water-bath for 5 min. Add 25.0 mL of *0.1 M silver nitrate* and continue heating on a water-bath for 15 min with frequent swirling. Cool, add 25 mL of *dilute nitric acid R* and dilute to 250.0 mL with *water R*. Filter and discard the first 25 mL of the filtrate. Using 5 mL of *ferric ammonium sulfate solution R2* as indicator, titrate 100.0 mL of the filtrate with *0.1 M ammonium thiocyanate* until a yellowish-brown colour is obtained.

1 mL of *0.1 M silver nitrate* is equivalent to 21.46 mg of $C_7H_7ClN_4O_2$.

IMPURITIES

*Specified impurities: A, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, G, H, I, J, K.*

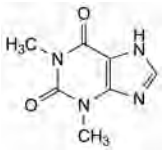

A. 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theophylline),

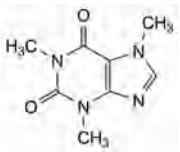

C. 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (caffeine),

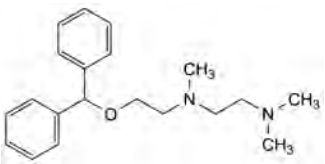

D. *N*-[2-(diphenylmethoxy)ethyl]-*N*,*N*′,*N*′-trimethylethane-1,2-diamine,

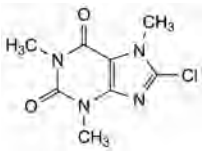

E. 8-chloro-1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (8-chlorocaffeine),

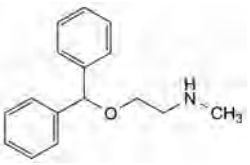

F. 2-(diphenylmethoxy)-*N*-methylethanamine (diphenhydramine impurity A),

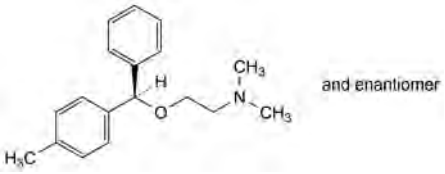


G. *N*,*N*-dimethyl-2-[(*RS*)-(4-methylphenyl)(phenyl)-methoxy]ethanamine (4-methyldiphenhydramine),

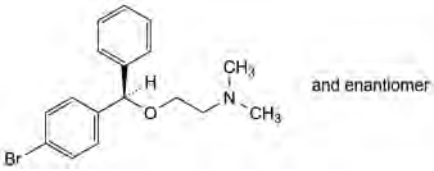


H. 2-[(*RS*)-(4-bromophenyl)-(phenyl)methoxy]-*N*,*N*-dimethylethanamine (4-bromodiphenhydramine),

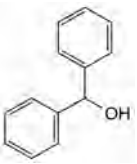

I. diphenylmethanol (benzhydrol),

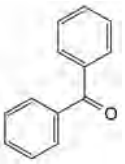

J. diphenylmethanone (benzophenone),

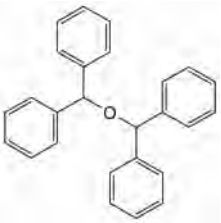

K. [oxybis(methanetriyl)]tetrabenzene.

**01/2008:0389**

# DIMERCAPROL

## Dimercaprolum

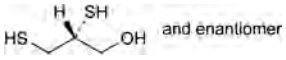


$C_3H_8OS_2$ $M_r$ 124.2
[59-52-9]

DEFINITION

(2*RS*)-2,3-Disulfanylpropan-1-ol.

*Content*: 98.5 per cent to 101.5 per cent.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow liquid.

*Solubility*: soluble in water and in arachis oil, miscible with ethanol (96 per cent) and with benzyl benzoate.

IDENTIFICATION

A. Dissolve 0.05 mL in 2 mL of *water R*. Add 1 mL of *0.05 M iodine*. The colour of the iodine is discharged immediately.

B. Dissolve 0.1 mL in 5 mL of *water R* and add 2 mL of *copper sulfate solution R*. A bluish-black precipitate is formed which quickly becomes dark grey.

C. In a ground-glass-stoppered tube, suspend 0.6 g of *sodium bismuthate R*, previously heated to 200 °C for 2 h, in a mixture of 2.8 mL of *dilute phosphoric acid R* and 6 mL of *water R*. Add 0.2 mL of the substance to be examined, mix and allow to stand for 10 min with frequent shaking. To 1 mL of the supernatant add 5 mL of a 4 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* and mix. Heat in a water-bath for 15 min. A violet-red colour develops.

TESTS

**Appearance**. It is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ or $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.25 mL of *bromocresol green solution R* and 0.3 mL of *0.01 M hydrochloric acid*. The solution is yellow. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Refractive index** (*2.2.6*): 1.568 to 1.574.

**Halides**. To 2.0 g add 25 mL of *alcoholic potassium hydroxide solution R* and boil under a reflux condenser for 2 h. Eliminate the ethanol by evaporation in a stream of hot air. Add 20 mL of *water R* and cool. Add 40 mL of *water R* and 10 mL of *strong hydrogen peroxide solution R*, boil gently for 10 min, cool and filter rapidly. Add 10 mL of *dilute nitric acid R* and 5.0 mL of *0.1 M silver nitrate*. Using 2 mL of *ferric ammonium sulfate solution R2* as indicator, titrate with *0.1 M ammonium thiocyanate* until a reddish-yellow colour is obtained. Carry out a blank titration. The difference between the titration volumes is not greater than 1.0 mL.

ASSAY

Dissolve 0.100 g in 40 mL of *methanol R*. Add 20 mL of *0.1 M hydrochloric acid* and 50.0 mL of *0.05 M iodine*. Allow to stand for 10 min and titrate with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 6.21 mg of $C_3H_8OS_2$.

STORAGE

In a well-filled, airtight container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2017:0763**

# DIMETHYL SULFOXIDE

## Dimethylis sulfoxidum

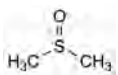

$C_2H_6OS$ $M_r$ 78.1
[67-68-5]

DEFINITION

Sulfinylbismethane.

CHARACTERS

*Appearance*: colourless liquid or colourless crystals, hygroscopic.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Relative density (see Tests).

B. Refractive index (see Tests).

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dimethyl sulfoxide CRS*.

D. Dissolve 50 mg of *nickel chloride R* in 5 mL of the substance to be examined. The solution is greenish-yellow. Heat in a water-bath at 50 °C. The colour changes to green or bluish-green. Cool. The colour changes to greenish-yellow.

TESTS

**Acidity**. Dissolve 50.0 g in 100 mL of *carbon dioxide-free water R*. Add 0.1 mL of *phenolphthalein solution R1*. Not more than 5.0 mL of *0.01 M sodium hydroxide* is required to produce a pink colour.

**Relative density** (*2.2.5*): 1.100 to 1.104.

**Refractive index** (*2.2.6*): 1.478 to 1.480.

**Freezing point** (*2.2.18*): minimum 18.3 °C.

**Absorbance** (*2.2.25*). Purge with *nitrogen R* for 15 min. The absorbance, measured using *water R* as the compensation liquid, is not more than 0.30 at 275 nm and not more than 0.20 at both 285 nm and 295 nm. Examined between 270 nm and 350 nm, the substance to be examined shows no absorption maximum.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.125 g of *bibenzyl R* in *acetone R* and dilute to 50 mL with the same solvent.

*Test solution (a).* Dissolve 5.0 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dissolve 5.0 g of the substance to be examined in *acetone R*, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *acetone R*.

*Reference solution.* Dissolve 50.0 mg of the substance to be examined and 50 mg of *dimethyl sulfone R* in *acetone R*, add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *acetone R*.

*Column*:

- *material*: glass;
- *size*: *l* = 1.5 m, Ø = 4 mm;
- *stationary phase*: *diatomaceous earth for gas chromatography R* (125-180 µm) impregnated with 10 per cent *m/m* of *polyethyleneglycol adipate R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 165 °C;
- *injection port and detector*: 190 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 4 times the retention time of dimethyl sulfoxide.

*Elution order*: dimethyl sulfoxide, dimethyl sulfone, bibenzyl.

*Retention time*: dimethyl sulfoxide = about 5 min.

*System suitability*:

– *resolution*: minimum 3 between the peaks due to dimethyl sulfoxide and dimethyl sulfone in the chromatogram obtained with the reference solution;

– in the chromatogram obtained with test solution (a) there is no peak with the same retention time as the internal standard.

*Limit*:

– *total*: calculate the ratio *R* of the area of the peak due to dimethyl sulfoxide to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with test solution (b), calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard to the area of the peak due to the internal standard: this ratio is not greater than *R* (0.1 per cent).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 10.0 g.

STORAGE

In an airtight, glass container, protected from light.

**01/2017:1667**

# DIMETHYLACETAMIDE

## Dimethylacetamidum

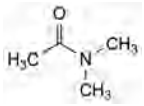

$C_4H_9NO$ $M_r$ 87.1

[127-19-5]

DEFINITION

*N*,*N*-Dimethylacetamide.

CHARACTERS

*Appearance*: clear, colourless, slightly hygroscopic liquid.

*Solubility*: miscible with water, with ethanol (96 per cent), and with most common organic solvents.

bp: about 165 °C.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Relative density (*2.2.5*): 0.941 to 0.944.

B. Refractive index (*2.2.6*): 1.435 to 1.439.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: films.
*Comparison*: *Ph. Eur. reference spectrum of dimethylacetamide.*

D. Dilute 50 mg with 1 mL of *methanol R*. Add 1 mL of a 15 g/L solution of *hydroxylamine hydrochloride R* and mix. Add 1 mL of *dilute sodium hydroxide solution R*, mix and allow to stand for 30 min. Add 1 mL of *dilute hydrochloric acid R* and add 1 mL of a 100 g/L solution of *ferric chloride R* in *0.1 M hydrochloric acid*. A reddish-brown colour develops, reaching a maximum intensity after about 5 min.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity**. Dilute 50 mL with 50 mL of *water R* previously adjusted with *0.02 M potassium hydroxide* or *0.02 M hydrochloric acid* to a bluish-green colour, using 0.5 mL of *bromothymol blue solution R1* as indicator. Not more than 5.0 mL of *0.02 M potassium hydroxide* is required to restore the initial (bluish-green) colour.

**Alkalinity**. To 50 mL add 50 mL of *water R* previously adjusted with *0.02 M potassium hydroxide* or *0.02 M hydrochloric acid* to a yellow colour, using 0.5 mL of *bromothymol blue solution R1* as indicator. Not more than 0.5 mL of *0.02 M hydrochloric acid* is required to restore the initial (yellow) colour.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* The substance to be examined.

*Reference solution (a).* Dilute a mixture of 1 mL of the substance to be examined and 1 mL of *dimethylformamide R* (impurity B) to 20 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 1 mL of the substance to be examined to 20.0 mL with *methylene chloride R*. Dilute 0.1 mL of the solution to 10.0 mL with *methylene chloride R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Linear velocity*: 30 cm/s.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 80 → 200 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to dimethylacetamide and impurity B in the chromatogram obtained with reference solution (a);

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

– *any impurity*: maximum 0.1 per cent;

– *total*: maximum 0.3 per cent;

– *disregard limit*: the area of the peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Non-volatile matter**: maximum 20 ppm.

Evaporate 50 g to dryness by suitable means at a pressure not exceeding 1 kPa and on a water-bath. Dry the residue in an oven at 170-175 °C. The residue weighs not more than 1 mg.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 0.100 g.

STORAGE

In an airtight container, protected from light.

IMPURITIES

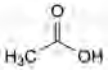

A. acetic acid,

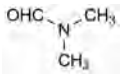

B. *N*,*N*-dimethylformamide,

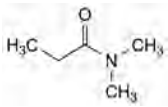

C. *N*,*N*-dimethylpropanamide,

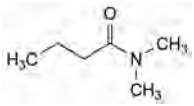

D. *N*,*N*-dimethylbutanamide.

**01/2017:0138**

# DIMETICONE

## Dimeticonum

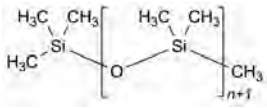

[9006-65-9]

DEFINITION

α-Trimethylsilyl-ω-methylpoly[oxy(dimethylsilanediyl)].

This poly(dimethylsiloxane) is obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. Different grades of dimeticone exist which are distinguished by a number indicating the nominal kinematic viscosity placed after the name.

Their degree of polymerisation ($n$ = 20 to 400) is such that their kinematic viscosities are nominally between 20 $mm^2 \cdot s^{-1}$ and 1300 $mm^2 \cdot s^{-1}$.

*Dimeticones with a nominal viscosity of 50 $mm^2 \cdot s^{-1}$ or lower are intended for external use only.*

CHARACTERS

*Appearance*: clear, colourless liquid of various viscosities.

*Solubility*: practically insoluble in water, very slightly soluble or practically insoluble in anhydrous ethanol, miscible with ethyl acetate, with methyl ethyl ketone and with toluene.

IDENTIFICATION

A. It is identified by its kinematic viscosity at 25 °C (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dimeticone CRS*.

The region of the spectrum from 850 $cm^{-1}$ to 750 $cm^{-1}$ is not taken into account.

C. Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

D. In a platinum crucible, prepare the sulfated ash (*2.4.14*) using 50 mg. The residue is a white powder that gives the reaction of silicates (*2.3.1*).

TESTS

**Acidity**. To 2.0 g add 25 mL of a mixture of equal volumes of *anhydrous ethanol R* and *ether R*, previously neutralised to 0.2 mL of *bromothymol blue solution R1*, and shake. Not more than 0.15 mL of *0.01 M sodium hydroxide* is required to change the colour of the solution to blue.

**Viscosity** (*2.2.9*): 90 per cent to 110 per cent of the nominal kinematic viscosity stated on the label, determined at 25 °C.

**Mineral oils**. Place 2 g in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

**Phenylated compounds**. Dissolve 5.0 g with shaking in 10 mL of *cyclohexane R*. At wavelengths from 250 nm to 270 nm, the absorbance (*2.2.25*) of the solution is not greater than 0.2.

**Volatile matter**: maximum 0.3 per cent, for dimeticones with a nominal viscosity greater than 50 $mm^2 \cdot s^{-1}$, determined on 1.00 g by heating in an oven at 150 °C for 2 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

LABELLING

The label states:

- the nominal kinematic viscosity by a number placed after the name of the product;
- where applicable, that the product is intended for external use.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for dimeticone used as emollient.*

**Viscosity** (see Tests).

**01/2008:1417**
**corrected 10.0**

# DIMETINDENE MALEATE

## Dimetindeni maleas

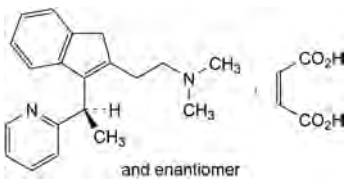


$C_{24}H_{28}N_2O_4$ $M_r$ 408.5
[3614-69-5]

DEFINITION

*N*,*N*-Dimethyl-2-[3-[(*RS*)-1-(pyridin-2-yl)ethyl]-1*H*-inden-2-yl]ethanamine (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *dimetindene maleate CRS*.

TESTS

**Solution S**. Dissolve 0.20 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than $Y_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Solvent mixture*: *acetone R*, *methylene chloride R* (50:50 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *2-ethylpyridine R* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 10.0 mL of this solution to 100.0 mL with the solvent mixture.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: about 30 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 60 |
| | 1 - 34.3 | 60 → 260 |
| | 34.3 - 46.3 | 260 |
| Injection port | | 240 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 2 µL; inject via a split injector with a split flow of 30 mL/min.

*Run time*: 1.3 times the retention time of dimetindene.

*Elution order*: impurity A and maleic acid appear during the first 8 min.

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 1.3 for the principal peak.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *impurities B, C, D, E, F, G, H, I*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities other than A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.1 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.43 mg of $C_{24}H_{28}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

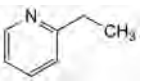

A. 2-ethylpyridine,

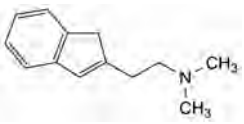

B. 2-(1*H*-inden-2-yl)-*N*,*N*-dimethylethanamine,

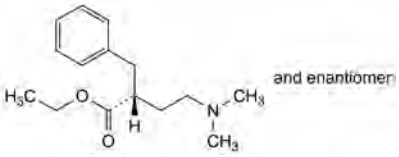


C. ethyl (2*RS*)-2-benzyl-4-(dimethylamino)butanoate,

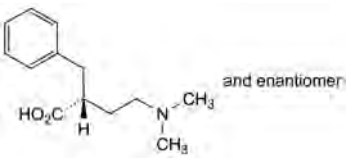


D. (2*RS*)-2-benzyl-4-(dimethylamino)butanoic acid,

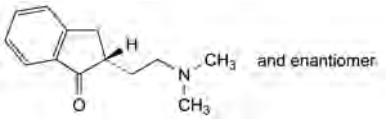


E. (2*RS*)-2-[2-(dimethylamino)ethyl]indan-1-one,

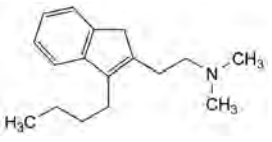

F. 2-(3-butyl-1*H*-inden-2-yl)-*N*,*N*-dimethylethanamine,

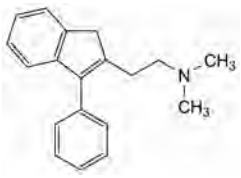

G. *N*,*N*-dimethyl-2-(3-phenyl-1*H*-inden-2-yl)ethanamine,

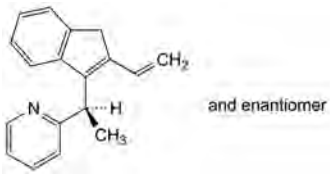


H. 2-[(1*RS*)-1-(2-ethenyl-1*H*-inden-3-yl)ethyl]pyridine,

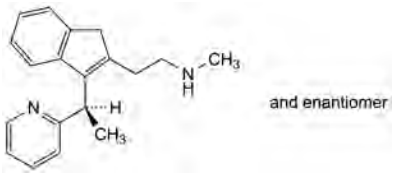


I. *N*-methyl-2-[3-[(1*RS*)-1-(pyridin-2-yl)ethyl]-1*H*-inden-2-yl]ethanamine.

**01/2008:1312**

# DINOPROST TROMETAMOL

## Dinoprostum trometamolum

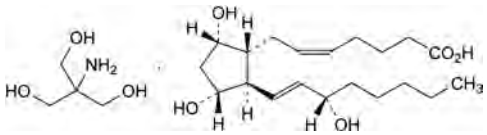

$C_{24}H_{45}NO_8$ $M_r$ 475.6
[38562-01-5]

DEFINITION

Trometamol (*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoate ($PGF_{2\alpha}$).

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in acetonitrile.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 19 to + 26 (anhydrous substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dinoprost trometamol CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (23:77 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a). Degradation of dinoprost trometamol to impurity B.* Dissolve 1 mg of the substance to be examined in 1 mL of the mobile phase and heat the solution on a water-bath at 85 °C for 5 min and cool.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 20.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

*Mobile phase*: dissolve 2.44 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with *water R*; adjust to pH 2.5 with *phosphoric acid R* (about 0.6 mL); mix 770 mL of this solution with 230 mL of *acetonitrile R1*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of the principal peak (to elute degradation products formed during heating) for reference solution (a) and 10 min after the elution of dinoprost for the test solution and reference solution (b).

*Retention time*: impurity B = about 55 min; impurity A = about 60 min; dinoprost = about 66 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurities B and A and minimum 2.0 between the peaks due to impurity A and dinoprost; if necessary, adjust the composition of the mobile phase by increasing the concentration of acetonitrile to decrease the retention times;
– *symmetry factor*: maximum 1.2 for the peaks due to impurities A and B.

*Limits*:

– *impurity A*: not more than twice the area of the principal peak obtained with reference solution (b) (2 per cent);
– *impurities B, C, D*: for each impurity, not more than 1.5 times the area of the principal peak obtained with reference solution (b) (1.5 per cent) and not more than one such peak has an area greater than 0.5 times the area of the principal peak obtained with reference solution (b) (0.5 per cent);
– *sum of impurities other than A*: not more than twice the area of the principal peak obtained with reference solution (b) (2 per cent);
– *disregard limit*: 0.05 times the area of the principal peak obtained with reference solution (b) (0.05 per cent); disregard any peak due to trometamol (retention time = about 1.5 min).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (23:77 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution.* Dissolve 10.0 mg of *dinoprost trometamol CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

Monographs D

*Mobile phase*: dissolve 2.44 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with *water R*; adjust to pH 2.5 with *phosphoric acid R* (about 0.6 mL); mix 730 mL of this solution with 270 mL of *acetonitrile R1*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Retention time*: dinoprost = about 23 min.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the peak due to dinoprost after 6 injections.

Calculate the percentage of dinoprost trometamol from the declared content of *dinoprost trometamol CRS*.

IMPURITIES

*Specified impurities: A, B, C, D.*

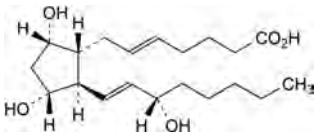

A. (*E*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((5*E*)-$PGF_{2\alpha}$; 5,6-*trans*-$PGF_{2\alpha}$),

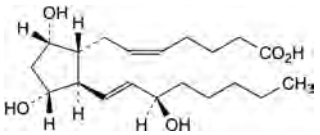

B. (*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*R*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((15*R*)-$PGF_{2\alpha}$;15-epi$PGF_{2\alpha}$),

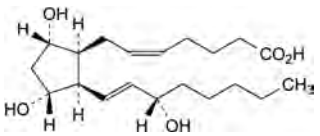

C. (*Z*)-7-[(1*S*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid ((8*S*)-$PGF_{2\alpha}$; 8-epi$PGF_{2\alpha}$),

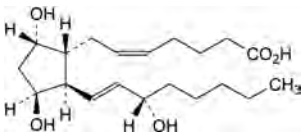

D. (*Z*)-7-[(1*R*,2*R*,3*S*,5*S*)-3,5-dihydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoic acid (11β-$PGF_{2\alpha}$; 11-epi$PGF_{2\alpha}$).

**01/2008:1311**
**corrected 9.2**

# DINOPROSTONE

## Dinoprostonum

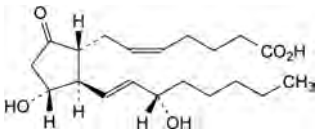

$C_{20}H_{32}O_5$ $M_r$ 352.5
[363-24-6]

DEFINITION

(*Z*)-7-[(1*R*,2*R*,3*R*)-3-Hydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid ($PGE_2$).

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, very soluble in methanol, freely soluble in alcohol.

The substance degrades at room temperature.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 90 to – 82 (anhydrous substance).

Immediately before use, dissolve 50.0 mg in *alcohol R* and dilute to 10.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dinoprostone CRS*.

TESTS

*Prepare the solutions immediately before use.*

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 10.0 mg of the substance to be examined in a 58 per cent *V/V* solution of *methanol R2* and dilute to 2.0 mL with the same solvent.

*Test solution (b).* Dissolve 20.0 mg of the substance to be examined in a 58 per cent *V/V* solution of *methanol R2* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 1 mg of *dinoprostone CRS* and 1 mg of *dinoprostone impurity C CRS* in a 58 per cent *V/V* solution of *methanol R2* and dilute to 10.0 mL with the same solvent. Dilute 4.0 mL of the solution to 10.0 mL with a 58 per cent *V/V* solution of *methanol R2*.

*Reference solution (b).* Dilute 0.5 mL of test solution (a) to 10.0 mL with a 58 per cent *V/V* solution of *methanol R2*. Dilute 1.0 mL of the solution to 10.0 mL with a 58 per cent *V/V* solution of *methanol R2*.

*Reference solution (c).* In order to prepare *in situ* the degradation compounds (impurity D and impurity E), dissolve 1 mg of the substance to be examined in 100 µL of *1 M sodium hydroxide* (the solution becomes brownish-red), wait 4 min, add 150 µL of *dilute acetic acid R1* (yellowish-white opalescent solution) and dilute to 5.0 mL with a 58 per cent *V/V* solution of *methanol R2*.

*Reference solution (d).* Dissolve 20 mg of *dinoprostone CRS* in a 58 per cent *V/V* solution of *methanol R2* and dilute to 20.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R*,

– *temperature*: 30 °C.

*Mobile phase*: mix 42 volumes of a 0.2 per cent *V/V* solution of *acetic acid R* and 58 volumes of *methanol R2*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL; inject test solution (a) and reference solutions (a), (b) and (c).

*Relative retention* with reference to dinoprostone (retention time = about 18 min): impurity C = about 1.2; impurity D = about 1.8; impurity E = about 2.0.

*System suitability*: reference solution (a):

- *resolution*: minimum of 3.8 between the peaks due to dinoprostone and to impurity C. If necessary adjust the concentration of the acetic acid solution and/or methanol (increase the concentration of the acetic acid solution to increase the retention time for dinoprostone and impurity C and increase the concentration of methanol to decrease the retention time for both compounds).

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.2; impurity E = 0.7,
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *total of other impurities*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

If any peak with a relative retention to dinoprostone of about 0.8 is greater than 0.5 per cent or if the total of other impurities is greater than 1.0 per cent, record the chromatogram of test solution (a) with a detector set at 230 nm. If the area of the peak at 230 nm is twice the area of the peak at 210 nm, multiply the area at 210 nm by 0.2 (correction factor for impurity F).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.50 g.

ASSAY

*Prepare the solutions immediately before use.*

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{20}H_{32}O_5$.

STORAGE

At a temperature not exceeding - 15 °C.

IMPURITIES

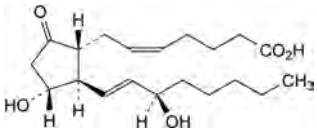

A. (*Z*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*R*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (15-epiPGE$_2$; (15*R*)-PGE$_2$),

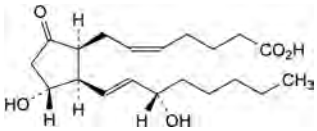

B. (*Z*)-7-[(1*S*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (8-epiPGE$_2$; (8S)-PGE$_2$),

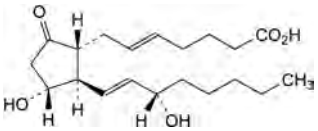

C. (*E*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (5-*trans*-PGE$_2$; (5*E*)-PGE$_2$),

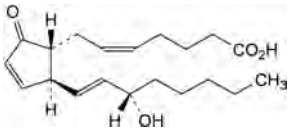

D. (*Z*)-7-[(1*R*,2*S*)-2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-3-enyl]hept-5-enoic acid (PGA$_2$),

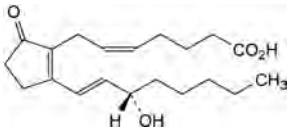

E. (*Z*)-7-[2-[(*E*)-(3*S*)-3-hydroxyoct-1-enyl]-5-oxocyclopent-1-enyl]hept-5-enoic acid (PGB$_2$),

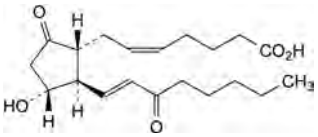

F. (*Z*)-7-[(1*R*,2*R*,3*R*)-3-hydroxy-2-[(*E*)-3-oxo-oct-1-enyl]-5-oxocyclopentyl]hept-5-enoic acid (15-oxo-PGE$_2$; 15-keto-PGE$_2$).

**01/2017:1611**

# DIOSMIN

## Diosminum

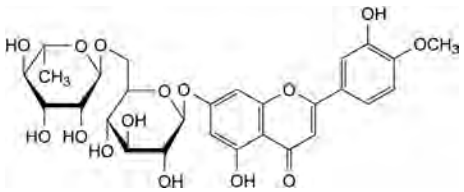

$C_{28}H_{32}O_{15}$ $M_r$ 609

[520-27-4]

DEFINITION

7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one.

Substance obtained through iodine-assisted oxidation of (2*S*)-7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (hesperidin) of natural origin.

*Content:* 90.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: greyish-yellow or light yellow, hygroscopic powder.

*Solubility*: practically insoluble in water, soluble in dimethyl sulfoxide, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *diosmin CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Iodine**: maximum 0.1 per cent.

Determine the total content of iodine by potentiometry, using an iodide-selective electrode (*2.2.36*), after oxygen combustion (*2.5.10*).

*Test solution.* Wrap 0.100 g of the substance to be examined in a piece of filter paper and place it in a sample carrier. Introduce into the flask 50 mL of a 0.2 g/L solution of *hydrazine R*. Flush the flask with oxygen for 10 min. Ignite the filter paper. Stir the contents of the flask immediately after the end of the combustion to dissolve completely the combustion products. Continue stirring for 1 h.

*Reference solution.* Dilute 2.0 mL of a 16.6 g/L solution of *potassium iodide R* to 100.0 mL with *water R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R*.

Introduce into a beaker 30 mL of a 200 g/L solution of *potassium nitrate R* in *0.1 M nitric acid*. Immerse the electrodes and stir for 10 min. The potential of the solution ($nT_1$) must remain stable. Add 1 mL of the test solution and measure the potential ($nT_2$).

Introduce into a beaker 30 mL of a 200 g/L solution of *potassium nitrate R* in *0.1 M nitric acid*. Immerse the electrodes and stir for 10 min. The potential of the solution must remain stable ($nR_1$). Add 80 µL of the reference solution and measure the potential ($nR_2$).

The absolute value $|nT_2 - nT_1|$ is not higher than the absolute value $|nR_2 - nR_1|$.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 25.0 mg of *diosmin CRS* in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *dimethyl sulfoxide R*.

*Reference solution (c).* Dissolve 5.0 mg of *diosmin for system suitability CRS* (containing impurities A, B, C, D, E and F) in *dimethyl sulfoxide R* and dilute to 5.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *glacial acetic acid R*, *methanol R*, *water R* (2:6:28:66 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 6 times the retention time of diosmin.

*Identification of impurities*: use the chromatogram supplied with *diosmin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to diosmin (retention time = about 4 min): impurity A = about 0.5; impurity B = about 0.6; impurity C = about 0.8; impurity D = about 2.2; impurity E = about 2.6; impurity F = about 4.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to impurities B and C.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.4; impurity F = 0.6;
- for each impurity, use the concentration of diosmin in reference solution (b).

*Limits*:

- *impurity B*: maximum 4.0 per cent;
- *impurities C, E*: for each impurity, maximum 3.0 per cent;
- *impurity F*: maximum 2.0 per cent;
- *impurity D*: maximum 0.6 per cent;
- *impurity A*: maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.4 per cent;
- *total*: maximum 8.5 per cent;
- *reporting threshold*: 0.10 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{28}H_{32}O_{15}$ taking into account the assigned content of *diosmin CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

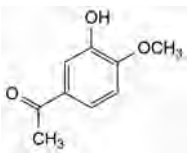


A. 1-(3-hydroxy-4-methoxyphenyl)ethan-1-one (acetoisovanillone),

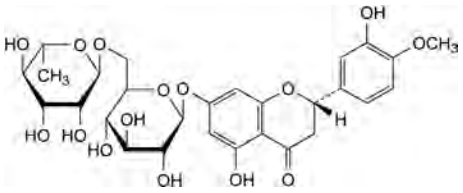

B. (2*S*)-7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (hesperidin),

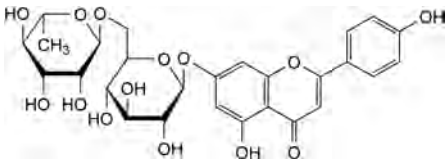

C. 7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (isorhoifolin),

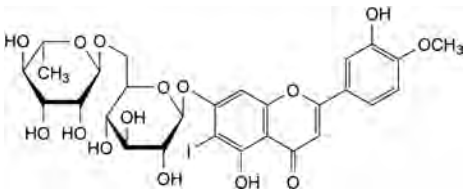

D. 7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-6-iodo-4*H*-1-benzopyran-4-one (6-iododiosmin),

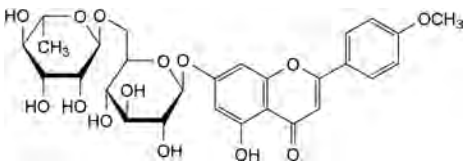

E. 7-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-methoxyphenyl)-4*H*-1-benzopyran-4-one (linarin),

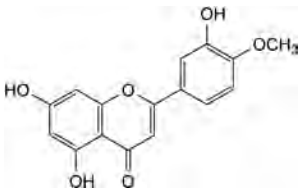

F. 5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one (diosmetin),

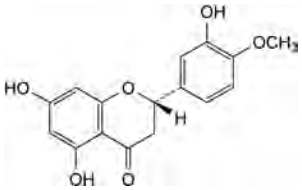

G. (2*S*)-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (hesperetin).

**01/2016:0023**

# DIPHENHYDRAMINE HYDROCHLORIDE

## Diphenhydramini hydrochloridum

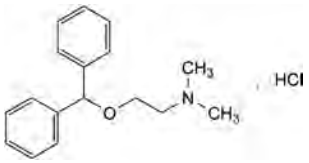

$C_{17}H_{22}ClNO$ $M_r$ 291.8
[147-24-0]

DEFINITION

2-(Diphenylmethoxy)-*N*,*N*-dimethylethanamine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 168 °C to 172 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 253 nm, 258 nm and 264 nm.

*Absorbance ratios*:

- $A_{258}/A_{253}$ = 1.1 to 1.3;
- $A_{258}/A_{264}$ = 1.2 to 1.4.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *diphenhydramine hydrochloride CRS.*

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S and a fivefold dilution of solution S are clear (*2.2.1*). Solution S is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.15 mL of *methyl red solution R* and 0.25 mL of *0.01 M hydrochloric acid*. The solution is pink. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 70 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *diphenhydramine impurity A CRS* and 5 mg of *diphenylmethanol R* in the mobile phase and dilute to 10.0 mL with the mobile phase. To 2.0 mL of this solution add 1.5 mL of the test solution and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 5.4 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 using *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 7 times the retention time of diphenhydramine.

*Relative retention* with reference to diphenhydramine (retention time = about 6 min): impurity A = about 0.9; impurity B = about 1.5; impurity C = about 1.8; impurity D = about 2.6; impurity E = about 5.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to diphenhydramine and to impurity A.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.7,

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),

– *any other impurity*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 29.18 mg of $C_{17}H_{22}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

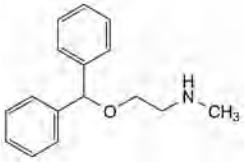

A. 2-(diphenylmethoxy)-*N*-methylethanamine,

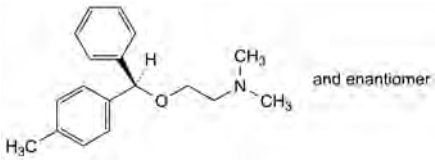


B. 2-[(*RS*)-(4-methylphenyl)phenylmethoxy]-*N,N*-dimethylethanamine,

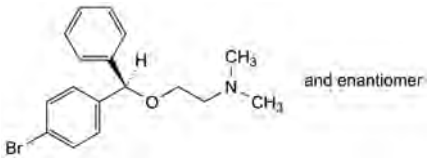


C. 2-[(*RS*)-(4-bromophenyl)phenylmethoxy]-*N,N*-dimethylethanamine,

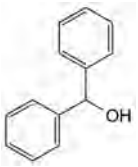


D. diphenylmethanol (benzhydrol),

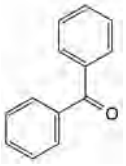


E. diphenylmethanone (benzophenone).

**04/2012:0819**

# DIPHENOXYLATE HYDROCHLORIDE

## Diphenoxylati hydrochloridum

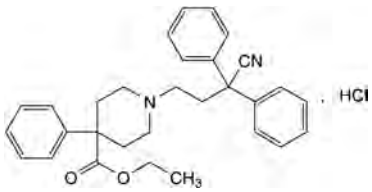


$C_{30}H_{33}ClN_2O_2$ $M_r$ 489.1
[3810-80-8]

DEFINITION

Ethyl 1-(3-cyano-3,3-diphenylpropyl)-4-phenylpiperidine-4-carboxylate hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *diphenoxylate hydrochloride CRS*.

B. Dissolve about 30 mg in 5 mL of *methanol R*. Add 0.25 mL of *nitric acid R* and 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled precipitate is formed. Centrifuge and rinse the precipitate with 3 quantities, each

of 2 mL, of *methanol R*. Carry out this operation rapidly and protected from bright light. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves easily.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methylene chloride R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Adjust 900 mL of *water R* to pH 2.3 with *phosphoric acid R* and dilute to 1000.0 mL with *water R*.

*Solvent mixture*: *acetonitrile R1*, solution A (50:50 *V/V*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 20 mL of the solvent mixture, sonicate for 2 min, cool and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2 mg of *diphenoxylate for system suitability CRS* (containing impurity A) in 2.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: solution A;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 75 | 25 |
| 5 - 40 | 75 → 15 | 25 → 85 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Relative retention* with reference to diphenoxylate (retention time = about 16 min): impurity A = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and diphenoxylate.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 40 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M ethanolic sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 48.91 mg of $C_{30}H_{33}ClN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C*.

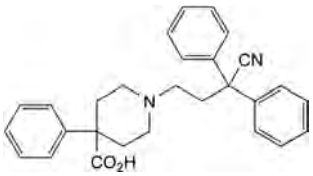


A. 1-(3-cyano-3,3-diphenylpropyl)-4-phenylpiperidine-4-carboxylic acid (diphenoxylic acid),

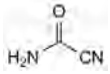


B. 1-cyanomethanamide,

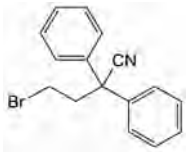


C. 4-bromo-2,2-diphenylbutanenitrile.

**01/2008:1719**
**corrected 7.0**

# DIPIVEFRINE HYDROCHLORIDE

## Dipivefrini hydrochloridum

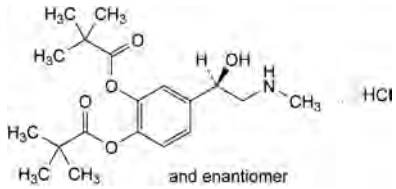


$C_{19}H_{30}ClNO_5$ $M_r$ 387.9
[64019-93-8]

DEFINITION

Hydrochloride of 4-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]-1,2-phenylene bis(2,2-dimethylpropanoate).

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very soluble in methanol, freely soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 160 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *dipivefrine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurities A and B**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

*Reference solution.* Dissolve 10.0 mg of *adrenaline R* and 10.0 mg of *adrenalone hydrochloride R* in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 1.0 mL of this solution to 10.0 mL with *0.01 M hydrochloric acid*. *Protect this solution from light.*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *anhydrous formic acid R*;
- *mobile phase B*: *methanol R2*, *acetonitrile R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 5 | 100 → 40 | 0 → 60 |
| 5 - 10 | 40 | 60 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Retention times*: impurity A = about 2.2 min; impurity B = about 3.2 min.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

- *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 40 volumes of *methanol R2* and 60 volumes of *acetonitrile R*. Mix 55 volumes of this mixture and 45 volumes of *0.01 M hydrochloric acid*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *dipivefrine for system suitability CRS* (containing impurities C, D and E) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5.0 mg of *dipivefrine hydrochloride CRS* in the solvent mixture and dilute to 2.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 25.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*: mix 45 volumes of a 2.7 g/L solution of *concentrated ammonia R* adjusted to pH 10.0 with *dilute acetic acid R* and 55 volumes of a mixture of 40 volumes of *methanol R2* and 60 volumes of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of dipivefrine.

*Relative retention* with reference to dipivefrine (retention time = about 7 min): impurities C and D = about 0.4; impurity E = about 1.3; impurity F = about 2.0.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to dipivefrine and impurity E.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurities C and D = 0.5; impurity E = 0.06;
- *sum of impurities C and D*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities E, F*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak with a mass distribution ratio less than 0.5.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of reference solutions (a) and (c).

*System suitability*: reference solution (c):

- *symmetry factor*: maximum 2.0 for the peak due to dipivefrine.

Calculate the percentage content of $C_{19}H_{30}ClNO_5$ using the chromatograms obtained with reference solutions (a) and (c) and the declared content of *dipivefrine hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

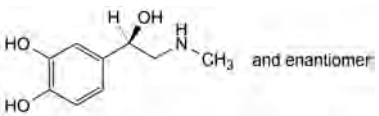


A. 4-[(1*RS*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol ((±)-adrenaline),

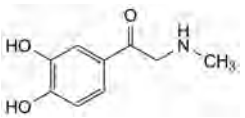


B. 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanone (adrenalone),

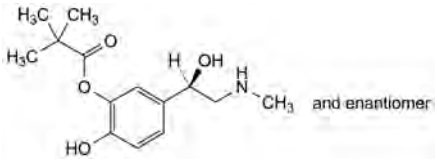


C. 2-hydroxy-5-[(1*RS*)-1-hydroxy-2-(methylamino)-ethyl]phenyl 2,2-dimethylpropanoate,

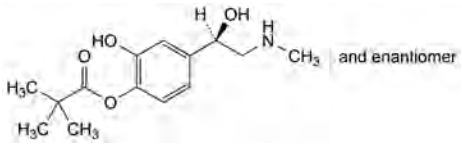


D. 2-hydroxy-4-[(1*RS*)-1-hydroxy-2-(methylamino)-ethyl]phenyl 2,2-dimethylpropanoate,

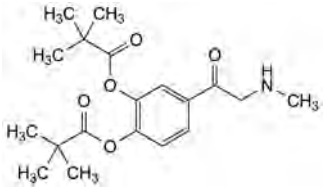

E. 4-[(methylamino)acetyl]-1,2-phenylene bis(2,2-dimethylpropanoate) (adrenalone dipivalate ester),

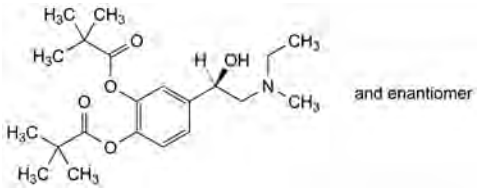


F. 4-[(1*RS*)-2-(ethylmethylamino)-1-hydroxyethyl]-1,2-phenylene bis(2,2-dimethylpropanoate).

**04/2019:0898**

# DIPOTASSIUM CLORAZEPATE MONOHYDRATE

## Dikalii clorazepas monohydricus

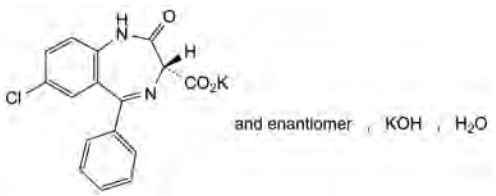


$C_{16}H_{11}ClK_2N_2O_4,H_2O$ $M_r$ 426.9

DEFINITION

Potassium (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine-3-carboxylate compound with potassium hydroxide (1:1) monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or light yellow, crystalline powder, hygroscopic.

*Solubility*: freely soluble to very soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

Solutions in water and in ethanol (96 per cent) are unstable and are to be used immediately.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 10.0 mg in a 0.3 g/L solution of *potassium carbonate R* and dilute to 100.0 mL with the same solution.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with a 0.3 g/L solution of *potassium carbonate R*.

*Spectral range*: 280-350 nm for test solution (a); 220-280 nm for test solution (b).

*Absorption maxima*: about 315 nm (broad) for test solution (a); 230 nm for test solution (b).

*Specific absorbance at the absorption maxima*:

- 230 nm: 800 to 870;
- 315 nm: 49 to 56.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of dipotassium clorazepate*.

C. Dissolve about 20 mg in 2 mL of *sulfuric acid R*. Observed in ultraviolet light at 365 nm, the solution shows yellow fluorescence.

D. Dissolve 0.5 g in 5 mL of *water R*. Add 0.1 mL of *thymol blue solution R*. The solution is violet-blue.

E. Place 1.0 g in a crucible and add 2 mL of *dilute sulfuric acid R*. Heat at first on a water-bath, then ignite until all black particles have disappeared. Allow to cool. Take up the residue with *water R* and dilute to 20 mL with the same solvent. The solution gives reaction (b) of potassium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

Rapidly dissolve 2.0 g with shaking in *water R* and dilute to 20.0 mL with the same solvent. Observe immediately.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 11.7 g of *dipotassium hydrogen phosphate R* in about 900 mL of *water R*, adjust to pH 7.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*. Mix equal volumes of this solution and *methanol R*.

*Solution B*: *methanol R*, 20 g/L solution of *potassium carbonate R* (30:70 *V/V*).

*Test solution (a).* Dissolve 10.0 mg of the substance to be examined in solution A and dilute to 20.0 mL with solution A.

*Test solution (b).* Dissolve 10.0 mg of the substance to be examined in solution B cooled to 0 °C and dilute to 20.0 mL with solution B.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 20.0 mL with solution A.

*Reference solution (b).* Dissolve 15 mg of *aminochlorobenzophenone R* (impurity A) and 15 mg of *ethyl clorazepate R* (impurity C) in *methanol R* and dilute to 20 mL with the same solvent. Dilute 1 mL of the solution to 20 mL with *methanol R*.

*Reference solution (c).* Dissolve 15 mg of *nordazepam R* (impurity B) in *methanol R* and dilute to 20 mL with the same solvent. Dilute 1 mL of the solution to 20 mL with solution B.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: dissolve 1.17 g of *dipotassium hydrogen phosphate R* in about 580 mL of *water for chromatography R*, adjust to pH 8.0 with *phosphoric acid R*, dilute to 650 mL with *water for chromatography R*, and add 450 mL of *methanol R1*;

– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 20 | 80 → 50 | 20 → 50 |
| 20 - 25 | 50 | 50 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Autosampler*: set at 4 °C.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*Relative retention* with reference to clorazepate (retention time = about 3 min): impurity B = about 4.6; impurity C = about 5.9; impurity A = about 6.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurities C and A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of dipotassium clorazepate monohydrate in reference solution (a).

*Limits*:

– *impurity C*: maximum 0.3 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.4 per cent;

– *reporting threshold*: 0.05 per cent; disregard the peak due to impurity B.

**Impurity B**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solutions (a) and (c).

*Calculation of percentage content*:

– *correction factor*: multiply the peak area of impurity B by 0.6;

– use the concentration of dipotassium clorazepate monohydrate in reference solution (a).

*Limit*:

– *impurity B*: maximum 0.2 per cent.

**Water** (*2.5.12*): 3.5 per cent to 5.5 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.130 g in 10 mL of *anhydrous acetic acid R*. Add 30 mL of *methylene chloride R*. Titrate with *0.1 M perchloric acid*, determining the 2 points of inflexion by potentiometry (*2.2.20*).

At the 2nd point of inflexion, 1 mL of *0.1 M perchloric acid* is equivalent to 13.03 mg of $C_{16}H_{11}ClK_2N_2O_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): A.

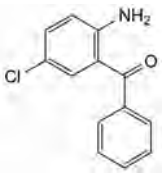

A. (2-amino-5-chlorophenyl)(phenyl)methanone (aminochlorobenzophenone),

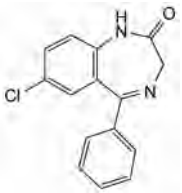

B. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (nordazepam),

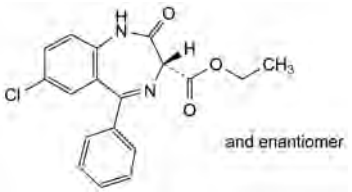


C. ethyl (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine-3-carboxylate (ethyl clorazepate).

**01/2017:1003**

# DIPOTASSIUM PHOSPHATE

## Dikalii phosphas

$K_2HPO_4$ $M_r$ 174.2
[7758-11-4]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals, very hygroscopic.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. Solution S gives reaction (b) of phosphates (*2.3.1*).

C. Solution S gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Reducing substances**. To 5 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate* and heat on a water-bath for 5 min. The solution remains faintly pink.

**Monopotassium phosphate**: maximum 2.5 per cent.

From the volume of *1 M hydrochloric acid* (10.0 mL) and of *1 M sodium hydroxide* ($n_1$ mL and $n_2$ mL) used in the assay, calculate the following ratio:

$$\frac{n_2 - 10}{10 - n_1}$$

This ratio is not greater than 0.025.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 2.5 mL of solution S add 10 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

To 1.5 mL of solution S add 2 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Sodium**: maximum 0.1 per cent, if intended for use in the manufacture of parenteral preparations.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 125-130 °C.

**Bacterial endotoxins** (*2.6.14*): less than 1.1 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.800 g (*m*) in 40 mL of *carbon dioxide-free water R* and add 10.0 mL of *1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *1 M sodium hydroxide*. Read the volume added at the 1st inflexion point ($n_1$ mL). Continue the titration to the 2nd inflexion point (total volume of *1 M sodium hydroxide* required, $n_2$ mL).

Calculate the percentage content of $K_2HPO_4$ from the following expression:

$$\frac{1742(10 - n_1)}{m(100 - d)}$$

*d* = percentage loss on drying.

STORAGE

In an airtight container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:0486**

# DIPROPHYLLINE

## Diprophyllinum

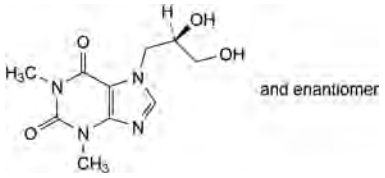


$C_{10}H_{14}N_4O_4$ $M_r$ 254.2
[479-18-5]

DEFINITION

7-[(2*RS*)-2,3-Dihydroxypropyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *diprophylline CRS*.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.25 mL of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.4 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 5 mg of *etofylline CRS* (impurity C) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 0.5 mL of the solution to 20.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with polar incorporated groups R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*: *methanol R*, *water R* (10:90 *V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of diprophylline.

*Relative retention* with reference to diprophylline (retention time = about 18 min): impurity C = about 1.1.

Monographs D

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to diprophylline.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 400 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.200 g in 3.0 mL of *anhydrous formic acid R* and add 50.0 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.42 mg of $C_{10}H_{14}N_4O_4$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

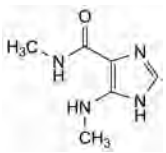

A. *N*-methyl-5-(methylamino)-1*H*-imidazole-4-carboxamide (theophyllidine),

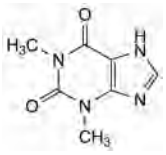

B. 1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theophylline),

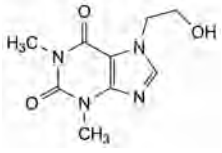

C. 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (etofylline),

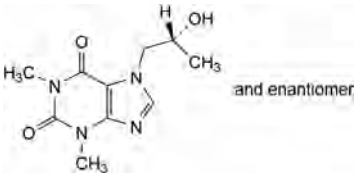


D. 7-[(2*RS*)-2-hydroxypropyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (proxyphylline).

**01/2014:1199**

# DIPYRIDAMOLE

## Dipyridamolum

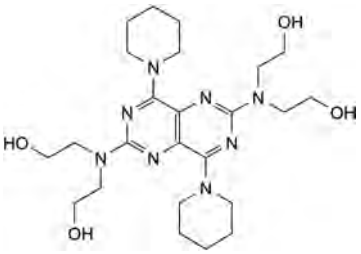

$C_{24}H_{40}N_8O_4$ $M_r$ 504.6

[58-32-2]

DEFINITION

2,2′,2″,2‴-[[4,8-Di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,6-diyl]dinitrilo]tetraethanol.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: bright yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in anhydrous ethanol. It dissolves in dilute mineral acids.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium bromide R*.

*Comparison*: *dipyridamole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *dipyridamole for peak identification CRS* (containing impurities A, B, C, D, E and F) in 1 mL of *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 1.0 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.0 with *0.5 M sodium hydroxide* and dilute to 1000 mL with *water R*;

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 40 | 60 |
| 5 - 19 | 40 → 5 | 60 → 95 |
| 19 - 24 | 5 → 40 | 95 → 60 |
| 24 - 29 | 40 | 60 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *dipyridamole for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to dipyridamole (retention time = about 8 min): impurity B = about 0.2; impurity F = about 0.3; impurity D = about 0.9; impurity E = about 1.3; impurity C = about 1.6; impurity A = about 2.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity D and dipyridamole;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.7;
- *impurities A, B, C*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities D, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 0.250 g add 10 mL of *water R* and shake vigorously. Filter, rinse the filter with 5 mL of *water R* and dilute to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 70 mL of *methanol R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 50.46 mg of $C_{24}H_{40}N_8O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G.*

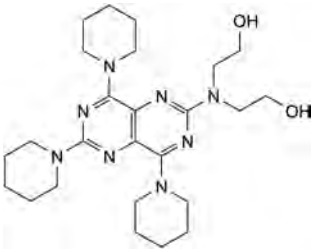

A. 2,2′-[[4,6,8-tri(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidin-2-yl]nitrilo]diethanol,

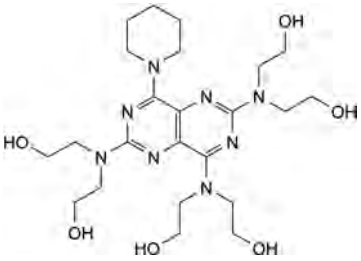

B. 2,2′,2″,2‴,2⁗,2⁗′[[8-(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,4,6-triyl]trinitrilo]hexaethanol,

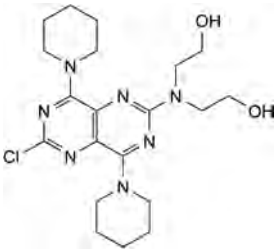

C. 2,2′-[[6-chloro-4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidin-2-yl]nitrilo]diethanol,

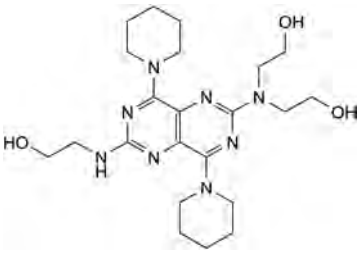

D. 2,2′-[[6-[(2-hydroxyethyl)amino]-4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidin-2-yl]nitrilo]diethanol,

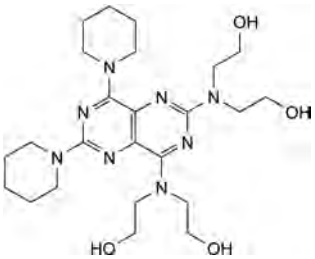

E. 2,2′,2″,2‴-[[6,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,4-diyl]dinitrilo]tetraethanol,

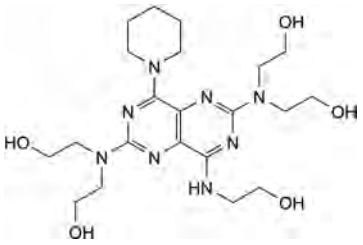

F. 2,2′,2″,2‴-[[4-[(2-hydroxyethyl)amino]-8-(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,6-diyl]dinitrilo]tetraethanol,

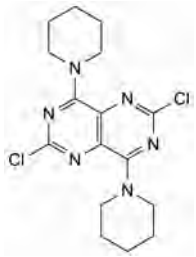

G. 2,6-dichloro-4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine.



**01/2017:1313**

# DIRITHROMYCIN

## Dirithromycinum

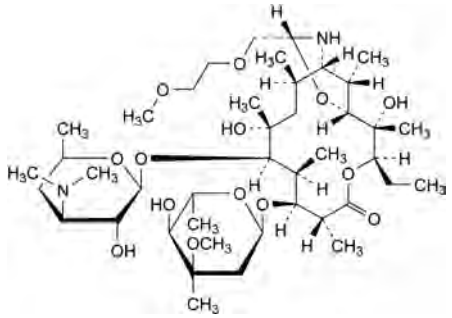

$C_{42}H_{78}N_2O_{14}$ $M_r$ 835

[62013-04-1]

DEFINITION

(1*R*,2*S*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*,13*S*,15*R*,17*S*)-9-[[3-(Dimethylamino)-3,4,6-trideoxy-β-D-*xylo*-hexopyranosyl]oxy]-3-ethyl-2,10-dihydroxy-15-[(2-methoxyethoxy)methyl]-2,6,8,10,12,17-hexamethyl-7-[(3-*C*-methyl-3-*O*-methyl-2,6dideoxy-α-L-*ribo*-hexopyranosyl)oxy]-4,16-dioxa-14azabicyclo[11.3.1]heptadecan-5-one (or (9*S*)-9,11-[imino[(1*R*)-2-(2-methoxyethoxy)ethylidene]oxy]-9-deoxo-11-deoxyerythromycin).

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent for the sum of the percentage contents of $C_{42}H_{78}N_2O_{14}$ and dirithromycin 15*S*-epimer (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, very soluble in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dirithromycin CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (a) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *acetonitrile R1* (30:70 *V/V*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *dirithromycin CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20 mg of *dirithromycin CRS* in the mobile phase and dilute to 10 mL with the mobile phase. Allow to stand for 24 h before use.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 9 volumes of *water R*, 19 volumes of *methanol R*, 28 volumes of a solution containing 1.9 g/L of *potassium dihydrogen phosphate R* and 9.1 g/L of *dipotassium hydrogen phosphate R* adjusted to pH 7.5 if necessary with a 100 g/L solution of *potassium hydroxide R*, and 44 volumes of *acetonitrile R1*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL of test solution (b) and reference solutions (b) and (c).

*Run time*: 3 times the retention time of dirithromycin.

*Relative retention* with reference to dirithromycin: impurity A = about 0.7; 15*S*-epimer = about 1.1.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to dirithromycin and its 15*S*-epimer; if necessary, adjust the concentration of the organic modifiers in the mobile phase.

*Limits*:

- *impurity A*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *disregard limit*: disregard the peak due to the 15*S*-epimer.

**Dirithromycin 15*S*-epimer**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (b).

*System suitability*: reference solution (b):

- *repeatability*: maximum relative standard deviation of 5.0 per cent after 6 injections.

*Limit*:

- *15S-epimer*: maximum 1.5 per cent.

**Acetonitrile** (*2.4.24, System A*): maximum 0.1 per cent.

Prepare the solutions using *dimethylformamide R* instead of *water R*.

*Sample solution.* Dissolve 0.200 g of the substance to be examined in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

*Static head-space injection conditions that may be used*:

– *equilibration temperature*: 120 °C;

– *equilibration time*: 60 min;

– *transfer-line temperature*: 125 °C.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

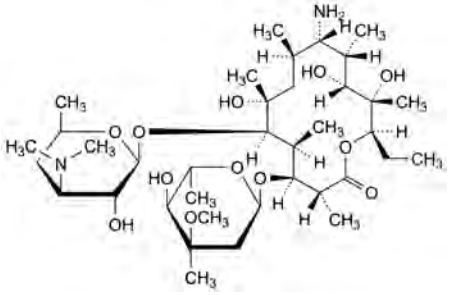

A. (9*S*)-9-amino-9-deoxoerythromycin,

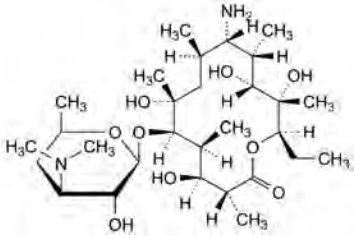

B. (9*S*)-9-amino-3-de(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)-9-deoxoerythromycin,

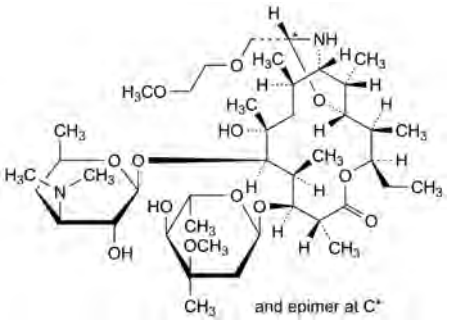


C. (9*S*)-9,11-[imino[(1*RS*)-2-(2-methoxyethoxy)ethylidene]-oxy]-9-deoxo-11,12-dideoxyerythromycin (dirithromycin B),

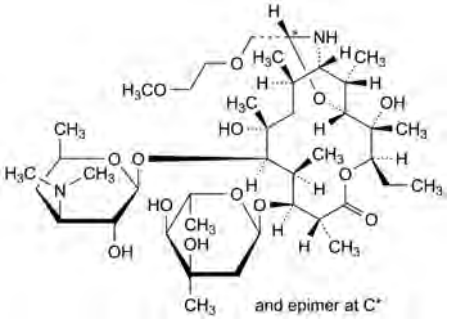


D. (9*S*)-9,11-[imino[(1*RS*)-2-(2-methoxyethoxy)ethylidene]-oxy]-3′-*O*-demethyl-9-deoxo-11-deoxyerythromycin (dirithromycin C),

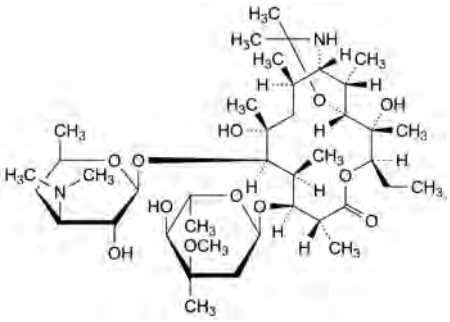

E. 9,11-[imino(1-methylethylidene)oxy]-9-deoxo-11-deoxyerythromycin.

**01/2017:0232**

# DISODIUM EDETATE

## Dinatrii edetas

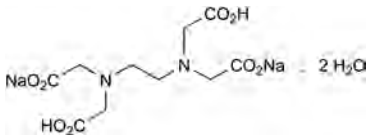

$C_{10}H_{14}N_2Na_2O_8,2H_2O$ $M_r$ 372.2

DEFINITION

Disodium dihydrogen (ethylenedinitrilo)tetraacetate dihydrate.

*Content*: 98.5 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *disodium edetate CRS*.

B. Dissolve 2 g in 25 mL of *water R*, add 6 mL of *lead nitrate solution R*, shake and add 3 mL of *potassium iodide solution R*. No yellow precipitate is formed. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2*. Add 3 mL of *ammonium oxalate solution R*. No precipitate is formed.

C. Dissolve 0.5 g in 10 mL of *water R* and add 0.5 mL of *calcium chloride solution R*. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 mL of *ammonium oxalate solution R*. No precipitate is formed.

D. It gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture.* Dissolve 10.0 g of *ferric sulfate pentahydrate R* in 20 mL of *0.5 M sulfuric acid* and add 780 mL of *water R*. Adjust to pH 2.0 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution.* Dissolve 40.0 mg of *nitrilotriacetic acid R* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. To 1.0 mL of the solution add 0.1 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: spherical *graphitised carbon for chromatography R1* (5 µm) with a specific surface area of 120 $m^2$/g and a pore size of 25 nm.

*Mobile phase*: dissolve 50.0 mg of *ferric sulfate pentahydrate R* in 50 mL of *0.5 M sulfuric acid* and add 750 mL of *water R*. Adjust to pH 1.5 with *0.5 M sulfuric acid* or *1 M sodium hydroxide*, add 20 mL of *ethylene glycol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL; filter the solutions and inject immediately.

*Run time*: 4 times the retention time of the iron complex of impurity A.

*Retention times*: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.

*System suitability*: reference solution:
- *resolution*: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid,
- *signal-to-noise ratio*: minimum 50 for the peak due to impurity A.

*Limit*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Iron** (*2.4.9*): maximum 80 ppm.

Dilute 2.5 mL of solution S to 10 mL with *water R*. Add 0.25 g of *calcium chloride R* to the test solution and the standard before the addition of the *thioglycollic acid R*.

ASSAY

Dissolve 0.300 g in *water R* and dilute to 300 mL with the same solvent. Add 2 g of *hexamethylenetetramine R* and 2 mL of *dilute hydrochloric acid R*. Titrate with *0.1 M lead nitrate*, using about 50 mg of *xylenol orange triturate R* as indicator.

1 mL of *0.1 M lead nitrate* is equivalent to 37.22 mg of $C_{10}H_{14}N_2Na_2O_8,2H_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

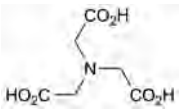


A. nitrilotriacetic acid.

**01/2017:1509**

# DISODIUM PHOSPHATE

## Dinatrii phosphas

$Na_2HPO_4$ $M_r$ 142.0
[7558-79-4]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. Loss on drying (see Tests).

C. Solution S gives reaction (b) of phosphates (*2.3.1*).

D. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Reducing substances**. To 10 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate* and heat on a water-bath for 5 min. The colour of the permanganate is not completely discharged.

**Monosodium phosphate**: maximum 2.5 per cent.

From the volume of *1 M hydrochloric acid* (25 mL) and of *1 M sodium hydroxide* ($n_1$ mL and $n_2$ mL) used in the assay, calculate the following ratio:

$$\frac{n_2 - 25}{25 - n_1}$$

This ratio is not greater than 0.025.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *dilute nitric acid R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 6 mL of solution S add 2 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 10 mL of solution S.

**Iron** (*2.4.9*): maximum 20 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

ASSAY

Dissolve 1.600 g (*m*) in 25.0 mL of *carbon dioxide-free water R* and add 25.0 mL of *1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *1 M sodium hydroxide*. Read the volume added at the 1st inflexion point ($n_1$ mL). Continue the titration to the 2nd inflexion point (total volume of *1 M sodium hydroxide* required, $n_2$ mL).

Calculate the percentage content of $Na_2HPO_4$ from the following expression:

$$\frac{1420(25 - n_1)}{m(100 - d)}$$

*d* = percentage loss on drying.

STORAGE

In an airtight container.

**01/2017:0602**

# DISODIUM PHOSPHATE DIHYDRATE

## Dinatrii phosphas dihydricus

$Na_2HPO_4,2H_2O$ $M_r$ 178.0
[10028-24-7]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. Loss on drying (see Tests).

C. Solution S gives reaction (b) of phosphates (*2.3.1*).

D. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Reducing substances**. To 5 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate* and heat on a water-bath for 5 min. The colour of the permanganate is not completely discharged.

**Monosodium phosphate**: maximum 2.5 per cent.

From the volume of *1 M hydrochloric acid* (25 mL) and of *1 M sodium hydroxide* ($n_1$ mL and $n_2$ mL) used in the assay, calculate the following ratio:

$$\frac{n_2 - 25}{25 - n_1}$$

This ratio is not greater than 0.025.

**Chlorides** (*2.4.4*): maximum 400 ppm.

To 2.5 mL of solution S add 10 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

To 3 mL of solution S add 2 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 40 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): 19.5 per cent to 21.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

ASSAY

Dissolve 2.000 g (*m*) in 50 mL of *water R* and add 25.0 mL of *1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *1 M sodium hydroxide*. Read the volume added at the 1st inflexion point ($n_1$ mL). Continue the titration to the 2nd inflexion point (total volume of *1 M sodium hydroxide* required, $n_2$ mL).

Calculate the percentage content of $Na_2HPO_4$ from the following expression:

$$\frac{1420(25 - n_1)}{m(100 - d)}$$

*d* = percentage loss on drying.

**01/2019:0118**

# DISODIUM PHOSPHATE DODECAHYDRATE

## Dinatrii phosphas dodecahydricus

$Na_2HPO_4,12H_2O$ $M_r$ 358.1
[10039-32-4]

DEFINITION

*Content*: 98.5 per cent to 102.5 per cent.

CHARACTERS

*Appearance*: colourless, transparent crystals, very efflorescent.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. Loss on drying (see Tests).

C. Solution S gives reaction (b) of phosphates (*2.3.1*).

D. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Reducing substances**. To 5 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate* and heat on a water-bath for 5 min. The colour of the permanganate is not completely discharged.

**Monosodium phosphate**: maximum 2.5 per cent.

From the volume of *1 M hydrochloric acid* ($n_3$ mL) and of *1 M sodium hydroxide* ($n_1$ mL and $n_2$ mL) used in the assay, calculate the following ratio:

$$\frac{n_2 - n_3}{n_3 - n_1}$$

This ratio is not greater than 0.025.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 2.5 mL of solution S add 10 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 3 mL of solution S add 2 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): 57.0 per cent to 61.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 4 h.

ASSAY

Dissolve 4.00 g ($m$) in 25 mL of *water R* and add 25.0 mL of *1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *1 M sodium hydroxide*. Read the volume added at the 1st inflexion point ($n_1$ mL). Continue the titration to the 2nd inflexion point (total volume of *1 M sodium hydroxide* required, $n_2$ mL). Carry out a blank titration ($n_3$ mL).

Calculate the percentage content of $Na_2HPO_4,12H_2O$ using the following expression:

$$\frac{3581(n_3 - n_1)}{m \times 100}$$

**01/2017:1006**

# DISOPYRAMIDE

## Disopyramidum

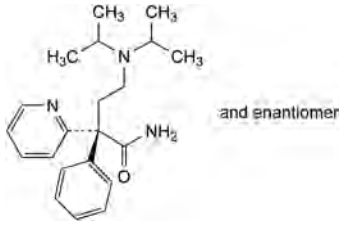


$C_{21}H_{29}N_3O$ $M_r$ 339.5
[3737-09-5]

DEFINITION

Disopyramide contains not less than 98.5 per cent and not more than the equivalent of 101.5 per cent of (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, slightly soluble in water, freely soluble in methylene chloride, soluble in alcohol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Dissolve 40.0 mg in a 5 g/L solution of *sulfuric acid R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 5.0 mL of this solution to 50.0 mL with a 5 g/L solution of *sulfuric acid R* in *methanol R*. Examined between 240 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 269 nm and a shoulder at 263 nm. The specific absorbance at the maximum is 190 to 210.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *disopyramide CRS*. Examine the substances as discs prepared by placing 50 µL of a 50 g/L solution in *methylene chloride R* on a disc of *potassium bromide R*. Dry the discs at 60 °C for 1 h before use.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *dilute potassium iodobismuthate solution R*. Examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *disopyramide CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 0.5 mL of test solution (b) to 20 mL with *methanol R*.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 30 volumes of *acetone R* and 30 volumes of *cyclohexane R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying at 80 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.7 kPa for 2 h.

**Sulfated ash** (*2.4.14*). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 30 mL of *anhydrous acetic acid R*. Add 0.2 mL of *naphtholbenzein solution R*. Titrate with *0.1 M perchloric acid* until the colour changes from yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 16.97 mg of $C_{21}H_{29}N_3O$.

STORAGE

Store protected from light.

IMPURITIES

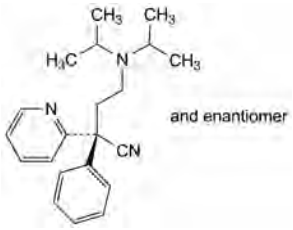


A. (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanenitrile (di-isopyronitrile),

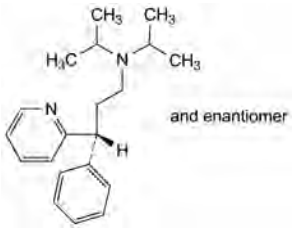


B. (3*RS*)-*N*,*N*-bis(1-methylethyl)-3-phenyl-3-(pyridin-2-yl)propan-1-amine,

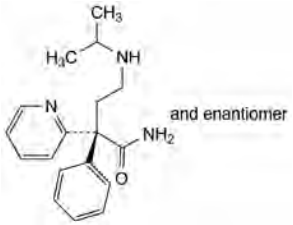


C. (2*RS*)-4-[(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide,

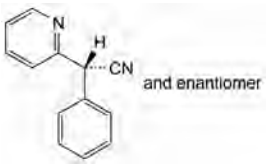


D. (*RS*)-phenyl(pyridin-2-yl)acetonitrile (pyronitrile).

**01/2017:1005**

# DISOPYRAMIDE PHOSPHATE

## Disopyramidi phosphas

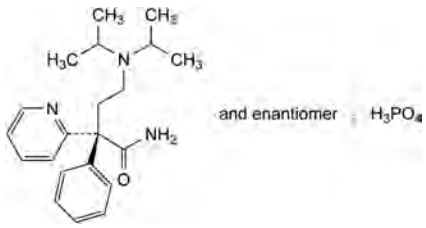


$C_{21}H_{32}N_3O_5P$ $M_r$ 437.5
[22059-60-5]

DEFINITION

Disopyramide phosphate contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide dihydrogen phosphate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, soluble in water, sparingly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Dissolve 50.0 mg in a 5 g/L solution of *sulfuric acid R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 5.0 mL of this solution to 50.0 mL with a 5 g/L solution of *sulfuric acid R* in *methanol R*. Examined between 240 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 269 nm and a shoulder at 263 nm. The specific absorbance at the maximum is 147 to 163.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *disopyramide phosphate CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). Spray with *dilute potassium iodobismuthate solution R*. Examine in daylight. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Solution S (see Tests) gives reaction (a) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 4.0 to 5.0.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 25 mg of *disopyramide phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 20 mL with *methanol R*.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 30 volumes of *acetone R* and 30 volumes of *cyclohexane R*. Dry the plate in a current of warm air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.180 g in 30 mL of *anhydrous acetic acid R*. Add 0.2 mL of *naphtholbenzein solution R*. Titrate with *0.1 M perchloric acid* until the colour changes from yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 21.88 mg of $C_{21}H_{32}N_3O_5P$.

STORAGE

Store protected from light.

IMPURITIES

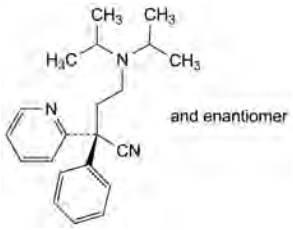


A. (2*RS*)-4-[bis(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanenitrile (di-isopyronitrile),

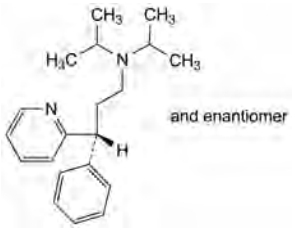


B. (3*RS*)-*N*,*N*-bis(1-methylethyl)-3-phenyl-3-(pyridin-2-yl)propan-1-amine,

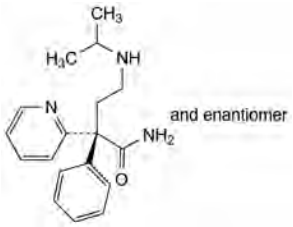


C. (2*RS*)-4-[(1-methylethyl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide,

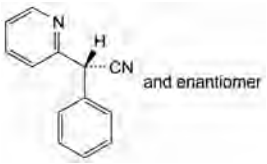


D. (*RS*)-phenyl(pyridin-2-yl)acetonitrile (pyronitrile).

**01/2017:0603**

# DISULFIRAM

## Disulfiramum

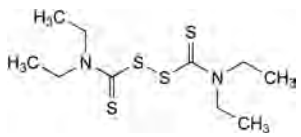


$C_{10}H_{20}N_2S_4$ $M_r$ 296.5

[97-77-8]

DEFINITION

Disulfiram contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of tetraethyldisulfanedicarbothioamide, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in alcohol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 70 °C to 73 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *disulfiram CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 10 mL of *methanol R*. Add 2 mL of a 0.5 g/L solution of *cupric chloride R* in *methanol R*. A yellow colour develops which becomes greenish-yellow.

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *ethyl acetate R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *ethyl acetate R*.

*Reference solution (a).* Dissolve 10 mg of *disulfiram CRS* in *ethyl acetate R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 20 mL with *ethyl acetate R*.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 30 volumes of *butyl acetate R* and 70 volumes of *hexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Diethyldithiocarbamate**. Dissolve 0.20 g in 10 mL of *peroxide-free ether R*, add 5 mL of *buffer solution pH 8.0 R* and shake vigorously. Discard the upper layer and wash the lower layer with 10 mL of *peroxide-free ether R*. Add to the lower layer 0.2 mL of a 4 g/L solution of *copper sulfate pentahydrate R* and 5 mL of *cyclohexane R*. Shake. Any yellow colour in the upper layer is not more intense than that of a standard prepared at the same time using 0.2 mL of a freshly prepared 0.15 g/L solution of *sodium diethyldithiocarbamate R* (150 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.450 g in 80 mL of *acetone R* and add 20 mL of a 20 g/L solution of *potassium nitrate R*. Titrate with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*), using a silver electrode and a silver-silver chloride double-junction electrode saturated with potassium nitrate.

1 mL of *0.1 M silver nitrate* is equivalent to 59.30 mg of $C_{10}H_{20}N_2S_4$.

STORAGE

Store protected from light.

IMPURITIES

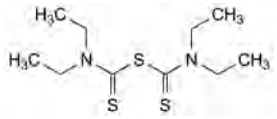

A. diethylthiocarbamic thioanhydride (sulfiram),

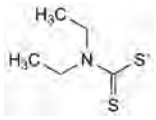

B. diethyldithiocarbamate.

**01/2016:1007**

# DITHRANOL

## Dithranolum

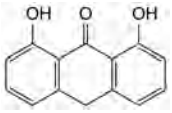

$C_{14}H_{10}O_3$ $M_r$ 226.2
[1143-38-0]

DEFINITION

1,8-Dihydroxyanthracen-9(10*H*)-one.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or brownish-yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

*Carry out all tests protected from bright light and use freshly prepared solutions.*

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 178 °C to 182 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dithranol CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *dithranol CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve about 5 mg of *dantron R* (impurity B) in 5 mL of reference solution (a).

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *hexane R*, *methylene chloride R* (50:50 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: place the plate in a tank saturated with ammonia vapour until the spots appear. Examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 5 mg add 0.1 g of *anhydrous sodium acetate R* and 1 mL of *acetic anhydride R*. Boil for 30 s. Add 20 mL of *ethanol (96 per cent) R*. Examined in ultraviolet light at 365 nm, the solution shows a blue fluorescence.

TESTS

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in 20 mL of *methylene chloride R*, add 1.0 mL of *glacial acetic acid R* and dilute to 100.0 mL with *hexane R*.

*Reference solution.* Dissolve 5.0 mg of *anthrone R* (impurity A), 5.0 mg of *dantron R* (impurity B), 5.0 mg of *dithranol impurity C CRS* and 5.0 mg of *dithranol CRS* in *methylene chloride R* and dilute to 5.0 mL with the same solvent. To 1.0 mL of this solution, add 19.0 mL of *methylene chloride R* and 1.0 mL of *glacial acetic acid R*, and dilute to 50.0 mL with *hexane R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *methylene chloride R*, *hexane R* (1:5:82 *V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of impurity C.

*Elution order*: dithranol, impurity B, impurity A, impurity C.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to dithranol and impurity B.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (1 per cent).

B. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 5 mL of *tetrahydrofuran R* and dilute to 25.0 mL with the mobile phase.

*Reference solution.* Dissolve 5.0 mg of *dithranol impurity D CRS* and 5.0 mg of *dithranol CRS* in 5 mL of *tetrahydrofuran R* and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.20 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *tetrahydrofuran R*, *water R* (2.5:40:60 *V/V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of dithranol.

*System suitability*: reference solution:

– *resolution*: minimum 2.5 between the peaks due to impurity D and dithranol.

*Limit*:

– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (2.5 per cent).

*Total (tests A + B)*: maximum 3.0 per cent for the sum of the contents of all impurities.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Shake 1.0 g with 20 mL of *water R* for 1 min and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous pyridine R*. Titrate with *0.1 M tetrabutylammonium hydroxide* under *nitrogen R*. Determine the end-point potentiometrically (*2.2.20*), using a glass indicator electrode and a suitable reference electrode.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 22.62 mg of $C_{14}H_{10}O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

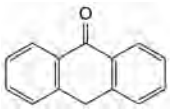

A. anthracen-9(10*H*)-one (anthrone),

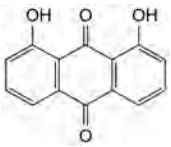

B. 1,8-dihydroxyanthracene-9,10-dione (dantron),

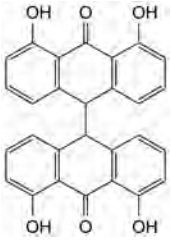

C. 4,4′,5,5′-tetrahydroxy-9,9′-bianthracenyl-10,10′(9*H*,9′*H*)-dione,

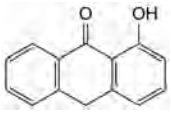

D. 1-hydroxyanthracen-9(10*H*)-one.

**01/2017:1200**

# DOBUTAMINE HYDROCHLORIDE

## Dobutamini hydrochloridum

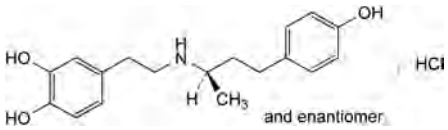


$C_{18}H_{24}ClNO_3$ $M_r$ 337.8
[49745-95-1]

DEFINITION

4-[2-[[(2*RS*)-4-(4-Hydroxyphenyl)-butan-2-yl]amino]ethyl]-benzene-1,2-diol hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dobutamine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*) using a mixture of equal volumes of *methanol R* and *water R*.

TESTS

**Acidity or alkalinity**. Dissolve 0.1 g in *water R* with gentle heating and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Absorbance** (*2.2.25*): maximum 0.04 at 480 nm.

Dissolve 0.5 g in a mixture of equal volumes of *methanol R* and *water R* with heating, if necessary, at 30-35 °C and dilute to 25 mL with the same mixture of solvents. Cool quickly. Examine immediately.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (35:65 *V/V*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 4.0 mL of the test solution to 100.0 mL with a 0.05 g/L solution of *anisaldehyde R* in the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *dobutamine impurity mixture CRS* (impurities A, B and C) in 1.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: dissolve 2.60 g of *sodium octanesulfonate R* in 1000 mL of *water R*, add 3 mL of *triethylamine R* and adjust to pH 2.5 with *phosphoric acid R*;
– *mobile phase B*: *acetonitrile R*, *methanol R* (18:82 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 65 | 35 |
| 5 - 20 | 65 → 20 | 35 → 80 |
| 20 - 25 | 20 | 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *dobutamine impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to dobutamine (retention time = about 12 min): impurity A = about 0.3; impurity B = about 0.5; anisaldehyde = about 0.7; impurity C = about 1.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to dobutamine and anisaldehyde.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.4;
– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.250 g in 10 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.79 mg of $C_{18}H_{24}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

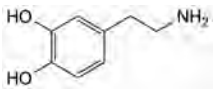


A. 4-(2-aminoethyl)benzene-1,2-diol (dopamine),

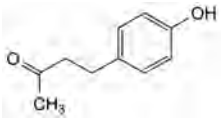


B. 4-(4-hydroxyphenyl)butan-2-one,

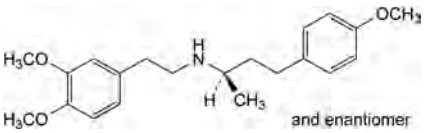


C. (2*RS*)-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-4-(4-methoxyphenyl)butan-2-amine.

**01/2017:2593**

# DOCETAXEL

## Docetaxelum

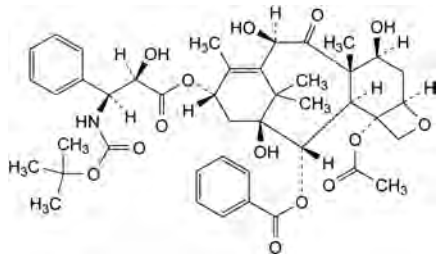

$C_{43}H_{53}NO_{14}$ $M_r$ 808

[114977-28-5]

DEFINITION

5β,20-Epoxy-1,7β,10β-trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate].

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, soluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *anhydrous docetaxel CRS*.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method I*).

Dissolve 1.0 g in *anhydrous ethanol R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 41.5 to – 38.5 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetic acid R*, *acetonitrile R1*, *water R* (0.05:50:50 *V/V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *docetaxel trihydrate CRS* in 2.5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *docetaxel for system suitability CRS* (containing impurities A, B and C) in 0.25 mL of *anhydrous ethanol R* and dilute to 5.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5 mg of *docetaxel impurity E CRS* in 2.5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 72 | 28 |
| 9 - 39 | 72 → 28 | 28 → 72 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *docetaxel for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity E.

*Relative retention* with reference to docetaxel (retention time = about 27 min): impurity E = about 0.2; impurity A = about 0.97; impurity B = about 1.08; impurity C = about 1.13.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to impurity A and docetaxel.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.6;
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *impurity E*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: maximum 0.8 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): maximum 1.5 per cent.

Inject 800 µL of a 25 mg/mL solution of the substance to be examined in *methanol R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.3 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{43}H_{53}NO_{14}$ taking into account the assigned content of *docetaxel trihydrate CRS*.

STORAGE

Protected from light, in an airtight container.

IMPURITIES

*Specified impurities*: *A, B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, G.*

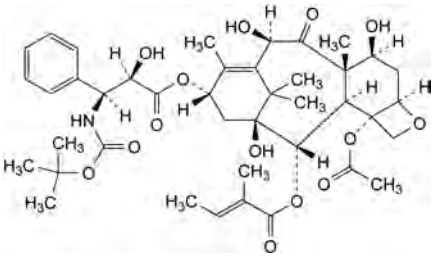

A. 5β,20-epoxy-1,7β,10β-trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] 2-[(2*E*)-2-methylbut-2-enoate] (2-*O*-desbenzoyl-2-*O*-tiglyldocetaxel),

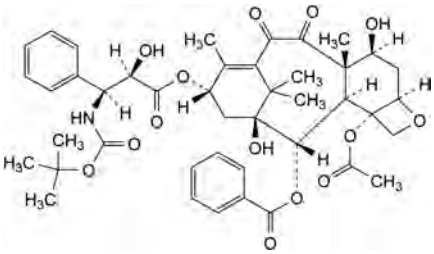

B. 5β,20-epoxy-1,7β-dihydroxy-9,10-dioxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (10-deoxy-10-oxodocetaxel),

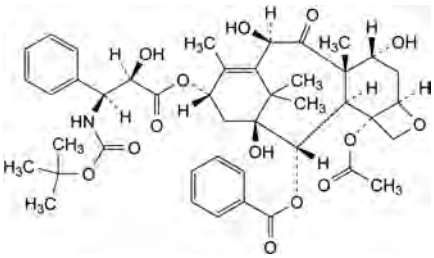

C. 5β,20-epoxy-1,7α,10β-trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (7-*epi*-docetaxel),

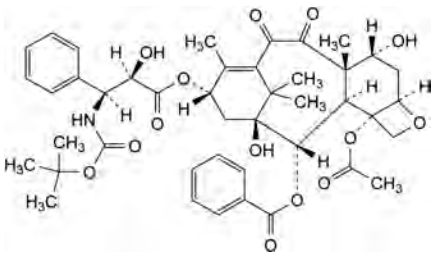

D. 5β,20-epoxy-1,7α-dihydroxy-9,10-dioxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (10-deoxy-10-oxo-7-*epi*-docetaxel),

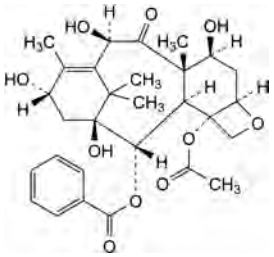

E. 5β,20-epoxy-4-(acetyloxy)-1,7β,10β,13α-tetrahydroxy-9-oxotax-11-en-2α-yl benzoate (10-desacetyl-baccatin III),

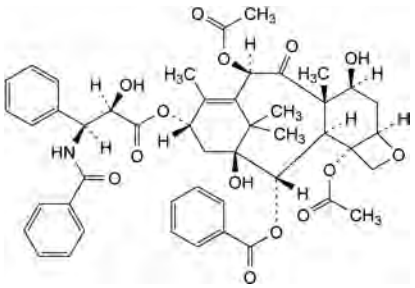

F. 5β,20-epoxy-1,7β-dihydroxy-9-oxotax-11-ene-2α,4,10β,13α-tetrayl 4,10-diacetate 2-benzoate 13-[(2*R*,3*S*)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoate] (paclitaxel),

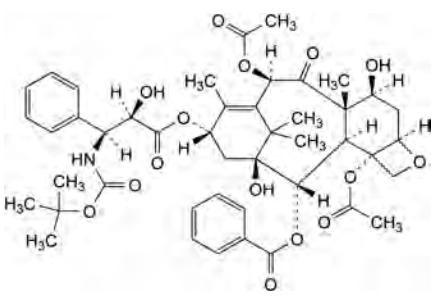

G. 5β,20-epoxy-1,7β-dihydroxy-9-oxotax-11-ene-2α,4,10β,13α-tetrayl 4,10-diacetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (10-acetyldocetaxel).

**01/2017:2449**

# DOCETAXEL TRIHYDRATE

## Docetaxelum trihydricum

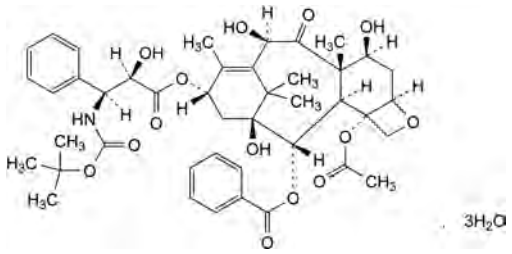

$C_{43}H_{53}NO_{14},3H_2O$ $M_r$ 862

[148408-66-6]

DEFINITION

5β,20-epoxy-1,7β,10β-Trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] trihydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, soluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *docetaxel trihydrate CRS*.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method I*).

Dissolve 1.0 g in *anhydrous ethanol R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 41.5 to − 38.5 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetic acid R*, *acetonitrile R1*, *water R* (0.05:50:50 *V/V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *docetaxel trihydrate CRS* in 2.5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5 mg of *docetaxel for system suitability CRS* (containing impurities A, B and C) in 0.25 mL of *anhydrous ethanol R* and dilute to 5.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);

– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: *water R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 72 | 28 |
| 9 - 39 | 72 → 28 | 28 → 72 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *docetaxel for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to docetaxel (retention time = about 27 min): impurity A = about 0.97; impurity B = about 1.08; impurity C = about 1.13.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to impurity A and docetaxel.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.6;

– *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurities B, C*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): 5.0 per cent to 7.0 per cent.

Inject 200 µL of a 100 mg/mL solution of the substance to be examined in *dimethylformamide R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.3 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{43}H_{53}NO_{14}$ taking into account the assigned content of *docetaxel trihydrate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

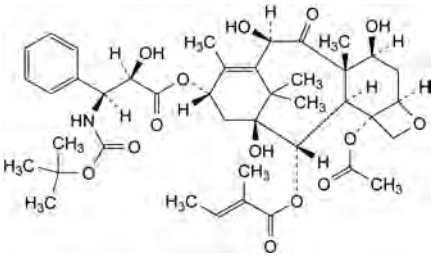

A. 5β,20-epoxy-1,7β,10β-trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] 2-[(2*E*)-2-methylbut-2-enoate] (2-*O*-desbenzoyl-2-*O*-tiglyldocetaxel),

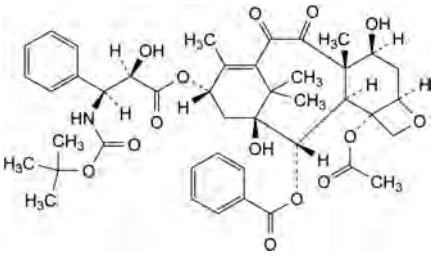

B. 5β,20-epoxy-1,7β-dihydroxy-9,10-dioxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (10-deoxy-10-oxodocetaxel),

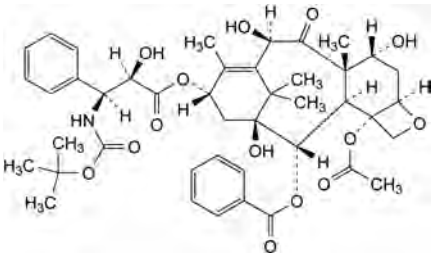

C. 5β,20-epoxy-1,7α,10β-trihydroxy-9-oxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (7-*epi*-docetaxel),

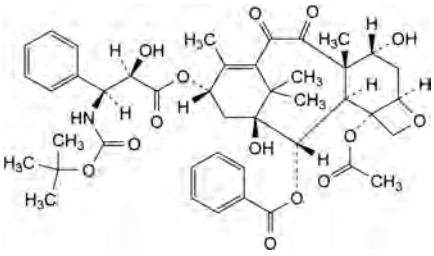

D. 5β,20-epoxy-1,7α-dihydroxy-9,10-dioxotax-11-ene-2α,4,13α-triyl 4-acetate 2-benzoate 13-[(2*R*,3*S*)-3-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-3-phenylpropanoate] (10-deoxy-10-oxo-7-*epi*-docetaxel).

**01/2017:1418**
**corrected 10.0**

# DOCUSATE SODIUM

## Natrii docusas

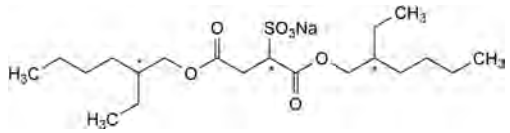

$C_{20}H_{37}NaO_7S$ $M_r$ 444.6
[577-11-7]

DEFINITION

Sodium 1,4-bis[(2-ethylhexyl)oxy]-1,4-dioxobutane-2-sulfonate.

*Content*: 98.0 to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, waxy masses or flakes, hygroscopic.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: place about 3 mg of the substance to be examined on a sodium chloride plate, add 0.05 mL of *acetone R* and immediately cover with another sodium chloride plate. Rub the plates together to dissolve the substance to be examined, slide the plates apart and allow the acetone to evaporate.

*Comparison*: *docusate sodium CRS*.

B. In a crucible, ignite 0.75 g in the presence of *dilute sulfuric acid R*, until an almost white residue is obtained. Allow to cool and take up the residue with 5 mL of *water R*. Filter. 2 mL of the filtrate gives reaction (a) of sodium (*2.3.1*).

TESTS

**Alkalinity**. Dissolve 1.0 g in 100 mL of a mixture of equal volumes of *methanol R* and *water R*, previously neutralised to *methyl red solution R*. Add 0.1 mL of *methyl red solution R*. Not more than 0.2 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related non-ionic substances**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 10 mg of *methyl behenate R* in *hexane R* and dilute to 50 mL with the same solvent.

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in 2.0 mL of the internal standard solution and dilute to 5.0 mL with *hexane R*. Pass the solution, at a rate of about 1.5 mL/min, through a column 10 mm in internal diameter, packed with 5 g of *basic aluminium oxide R* and previously washed with 25 mL of *hexane R*. Elute with 5 mL of *hexane R* and discard the eluate. Elute with 20 mL of a mixture of equal volumes of *ether R* and *hexane R*. Evaporate the eluate to dryness and dissolve the residue in 2.0 mL of *hexane R*.

*Test solution (b)*. Prepare as described for test solution (a) but dissolving 0.10 g of the substance to be examined in *hexane R*, diluting to 5.0 mL with the same solvent, and using a new column.

*Reference solution*. Dilute 2.0 mL of the internal standard solution to 5.0 mL with *hexane R*.

*Column*:

– *material*: glass;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 230 °C;
– *injection port and detector*: 280 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: 2.5 times the retention time of the internal standard.

*System suitability*: there is no peak with the same retention time as the internal standard in the chromatogram obtained with test solution (b).

*Limits*: test solution (a):

– *any impurity*: for each impurity, not more than the area of the peak due to the internal standard (0.4 per cent).

**Chlorides**: maximum 350 ppm.

Dissolve 5.0 g in 50 mL of *ethanol (50 per cent V/V) R*. Titrate with *0.01 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.01 M silver nitrate* is equivalent to 0.3545 mg of Cl.

**Sodium sulfate**: maximum 2 per cent.

Dissolve 0.25 g in 40 mL of a mixture of 20 volumes of *water R* and 80 volumes of *2-propanol R*. Adjust the pH to between 2.5 and 4.0 using *perchloric acid solution R*. Add 0.4 mL of *naphtharson solution R* and 0.1 mL of a 0.125 g/L solution of *methylene blue R*. Not more than 1.5 mL of *0.025 M barium perchlorate* is required to change the colour of the indicator from yellowish-green to yellowish-pink.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.250 g.

ASSAY

To 1.000 g in a 250 mL conical flask fitted with a reflux condenser add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and heat on a water-bath under reflux for 45 min. Allow to cool. Add 0.25 mL of *phenolphthalein solution R1* and titrate with *0.5 M hydrochloric acid* until the red colour disappears. Carry out a blank titration.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 0.1112 g of $C_{20}H_{37}NaO_7S$.

STORAGE

In an airtight container.

**01/2017:2078**

# DODECYL GALLATE

## Dodecylis gallas

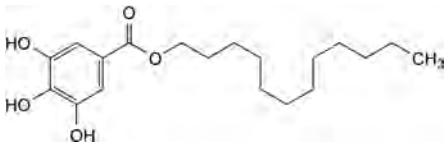

$C_{19}H_{30}O_5$ $M_r$ 338.4
[1166-52-5]

DEFINITION

Dodecyl 3,4,5-trihydroxybenzoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble or practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *dodecyl gallate CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 96 °C) is not greater than 2 °C.

B. Examine the chromatograms obtained in the test for impurity A.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20 mL with *acetone R*.

*Reference solution (a).* Dissolve 10 mg of *dodecyl gallate CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *gallic acid R* in *acetone R* and dilute to 20 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10 mL with *acetone R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 5 mL with test solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *ethyl formate R*, *toluene R* (10:40:50 *V/V/V*).

*Application*: 5 µL of test solutions (a) and (b) and reference solutions (a), (c) and (d).

*Development*: over 2/3 of the plate.

*Drying*: in air for 10 min.

*Detection*: spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol (96 per cent) R*.

*System suitability*: reference solution (d):

– the chromatogram shows 2 clearly separated principal spots.

*Limit*: test solution (a):

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 1.65 g add 50 mL of *water R*. Shake for 5 min. Filter. 15 mL of the filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 70 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 250.0 mL with the same solvent. Dilute 5.0 mL of the solution to 200.0 mL with *methanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 275 nm.

Calculate the content of $C_{19}H_{30}O_5$ taking the specific absorbance to be 321.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

*Specified impurities: A.*

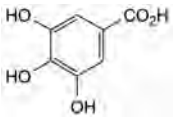

A. 3,4,5-trihydroxybenzoic acid (gallic acid).

**01/2017:1009**

# DOMPERIDONE

## Domperidonum

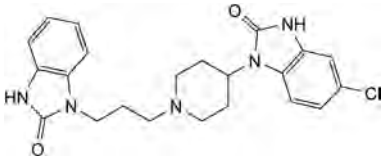

$C_{22}H_{24}ClN_5O_2$ $M_r$ 425.9

[57808-66-9]

DEFINITION

5-Chloro-1-[1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, slightly soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 244 °C to 248 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *domperidone CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *domperidone CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *domperidone CRS* and 20 mg of *droperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear; examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *domperidone CRS* and 15.0 mg of *droperidol CRS* in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium acetate R*;

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 70 → 0 | 30 → 100 |
| 10 - 12 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Relative retention* with reference to domperidone (retention time = about 6.5 min): impurity A = about 0.4; impurity B = about 0.65; impurity C = about 0.7; droperidol = about 1.1; impurity D = about 1.15; impurity E = about 1.2; impurity F = about 1.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to domperidone and droperidol.

*Limits*:

– *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid* until the colour changes from orange-yellow to green using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 42.59 mg of $C_{22}H_{24}ClN_5O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

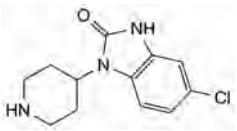

A. 5-chloro-1-(piperidin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,

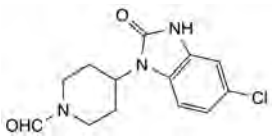

B. 4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1-formylpiperidine,

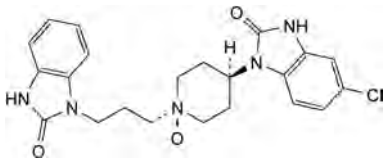

C. *cis*-4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidine 1-oxide,

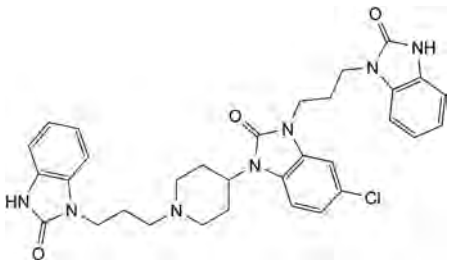

D. 5-chloro-3-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]-1-[1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

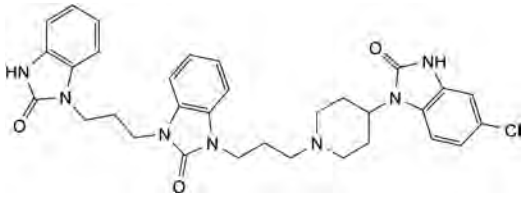

E. 1-[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-3-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]-1,3-dihydro-2*H*-benzimidazol-2-one,

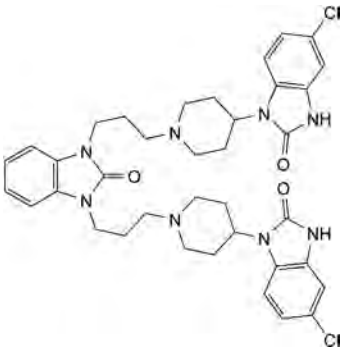

F. 1,3-bis[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-1,3-dihydro-2*H*-benzimidazol-2-one.

**01/2017:1008**



# DOMPERIDONE MALEATE

## Domperidoni maleas

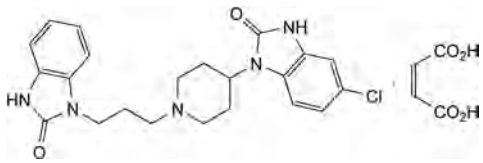

$C_{26}H_{28}ClN_5O_6$ $M_r$ 542.0
[83898-65-1]

DEFINITION

5-Chloro-1-[1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one hydrogen (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in dimethylformamide, slightly soluble in methanol, very slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B, C*.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *domperidone maleate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *2-propanol R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *domperidone maleate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *domperidone maleate CRS* and 20 mg of *droperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear. Examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Triturate 0.1 g with a mixture of 1 mL of *strong sodium hydroxide solution R* and 3 mL of *water R*. Shake with 3 quantities, each of 5 mL, of *ether R*. To 0.1 mL of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min. No colour develops. To the remainder of the aqueous layer add 2 mL of *bromine solution R*. Heat on a water-bath for 15 min and then heat to boiling. Cool. To 0.1 mL of this solution add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min. A violet colour develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use*.

*Test solution*. Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10.0 mg of *domperidone maleate CRS* and 15.0 mg of *droperidol CRS* in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Blank solution*: *dimethylformamide R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium acetate R*;
– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 70 → 0 | 30 → 100 |
| 10 - 12 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Equilibration*: with *methanol R* for at least 30 min and then with the mobile phase at the initial composition for at least 5 min.

*Injection*: 10 µL.

*Retention time*: domperidone = about 6.5 min; droperidol = about 7 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to domperidone and droperidol; if necessary, adjust the concentration of methanol in the mobile phase or adjust the time programme for the linear gradient.

*Limits*:

– *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to maleic acid at the beginning of the chromatogram.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Using 0.2 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from orange-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 54.20 mg of $C_{26}H_{28}ClN_5O_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

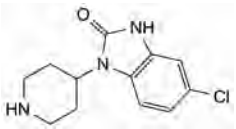

A. 5-chloro-1-(piperidin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,

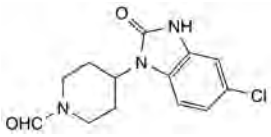

B. 4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1-formylpiperidine,

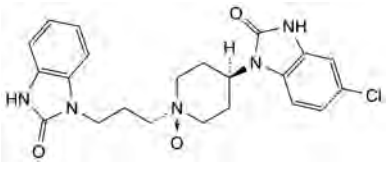

C. *cis*-4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidine 1-oxide,

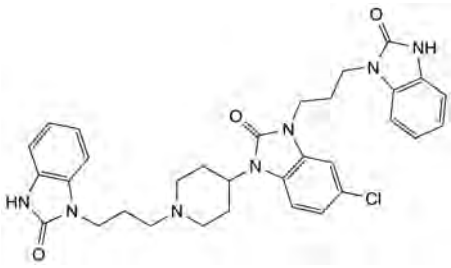

D. 5-chloro-3-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]-1-[1-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

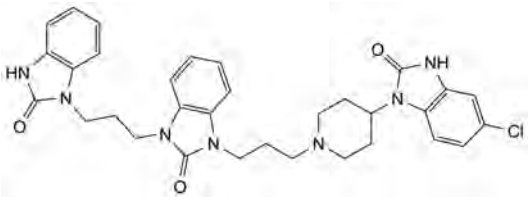

E. 1-[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-3-[3-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)propyl]-1,3-dihydro-2*H*-benzimidazol-2-one,

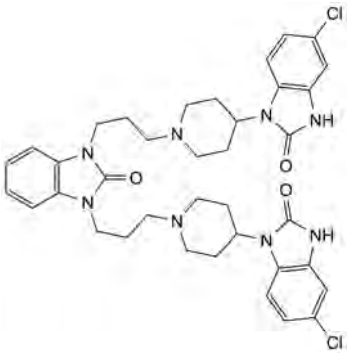

F. 1,3-bis[3-[4-(5-chloro-2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)piperidin-1-yl]propyl]-1,3-dihydro-2*H*-benzimidazol-2-one.

**01/2017:0664**

# DOPAMINE HYDROCHLORIDE

## Dopamini hydrochloridum

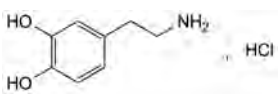

$C_8H_{12}ClNO_2$ $M_r$ 189.6
[62-31-7]

DEFINITION

4-(2-Aminoethyl)benzene-1,2-diol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), sparingly soluble in acetone and in methylene chloride.

IDENTIFICATION

*First identification: B, E.*

*Second identification*: *A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 40.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 280 nm.

*Specific absorbance at the absorption maximum*: 136 to 150.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dopamine hydrochloride CRS*.

C. Dissolve about 5 mg in a mixture of 5 mL of *1 M hydrochloric acid* and 5 mL of *water R*. Add 0.1 mL of *sodium nitrite solution R* containing 100 g/L of *ammonium molybdate R*. A yellow colour develops which becomes red on the addition of *strong sodium hydroxide solution R*.

D. Dissolve about 2 mg in 2 mL of *water R* and add 0.2 mL of *ferric chloride solution R2*. A green colour develops which changes to bluish-violet on the addition of 0.1 g of *hexamethylenetetramine R*.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ or $Y_6$ (*2.2.2, Method II*).

Dissolve 0.4 g in *water R* and dilute to 10 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.75 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 1.5 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Buffer solution.* Dissolve 21 g of *citric acid monohydrate R* in 200 mL of *1 M sodium hydroxide* and dilute to 1000 mL with *water R*. To 600 mL of this solution add 400 mL of *0.1 M hydrochloric acid*.

*Test solution.* Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 25 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10 mg of *3-O-methyldopamine hydrochloride R* (impurity B) and 10 mg of *4-O-methyldopamine hydrochloride R* (impurity A) in mobile phase A and dilute to 100 mL with mobile phase A. Dilute 6 mL of this solution to 25 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 1.08 g of *sodium octanesulfonate R* in 880 mL of the buffer solution and add 50 mL of *methanol R* and 70 mL of *acetonitrile R*;
- *mobile phase B*: dissolve 1.08 g of *sodium octanesulfonate R* in 700 mL of the buffer solution and add 100 mL of *methanol R* and 200 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 40 | 10 → 60 |
| 20 - 25 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Retention time*: dopamine = about 5 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurities B and A.

*Limits*:

- *unspecificied impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout the titration and stop the titration immediately after the end-point has been reached.*

Dissolve 0.150 g in 10 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.96 mg of $C_8H_{12}ClNO_2$.

STORAGE

In an airtight container, under nitrogen, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

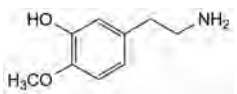


A. 5-(2-aminoethyl)-2-methoxyphenol (4-*O*-methyldopamine),

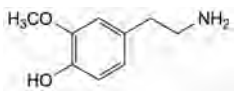


B. 4-(2-aminoethyl)-2-methoxyphenol (3-*O*-methyldopamine),

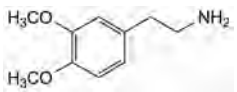


C. 2-(3,4-dimethoxyphenyl)ethanamine.

**01/2017:1748**

# DOPEXAMINE DIHYDROCHLORIDE

## Dopexamini dihydrochloridum

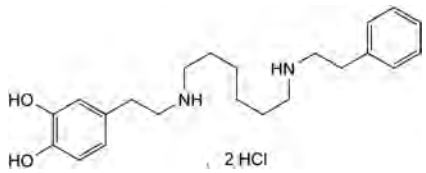


$C_{22}H_{34}Cl_2N_2O_2$ $M_r$ 429.4
[86484-91-5]

DEFINITION

4-[2-[[6-[(2-Phenylethyl)amino]hexyl]amino]ethyl]benzene-1,2-diol dihydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, sparingly soluble in ethanol (96 per cent) and in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dopexamine dihydrochloride CRS.*

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 0.10 g in *0.1 M hydrochloric acid* and dilute to 10 mL with the same acid.

**pH** (*2.2.3*): 3.7 to 5.7.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *dopexamine impurity B CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 5 mg of *dopexamine impurity F CRS* in mobile phase A and dilute to 100 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: mix 5 volumes of *buffer solution pH 2.5 R* and 95 volumes of *water R*;
- *mobile phase B*: mix 5 volumes of *buffer solution pH 2.5 R* and 95 volumes of a 60 per cent *V/V* solution of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 81 → 77 | 19 → 23 |
| 10 - 25 | 77 → 50 | 23 → 50 |
| 25 - 30 | 50 | 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Preconditioning of the column*: rinse for 5 min with a mixture of 19 volumes of mobile phase B and 81 volumes of mobile phase A.

*Injection*: 20 µL.

*Relative retention* with reference to dopexamine (retention time = about 5 min): impurity A = about 0.5; impurity B = about 2.0; impurity C = about 2.3; impurity D = about 2.8; impurity E = about 2.9; impurity F = about 3.0; impurity I = about 3.6; impurity J = about 5.0; impurity K = about 5.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 2 between the peaks due to dopexamine and impurity B.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity F = 0.7;
- *impurities A, B, C, D, E, F, I, K*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity J**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Detection*: spectrophotometer at 210 nm.

*Limit*:
- *impurity J*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg.

ASSAY

*Carry out the titration immediately after preparation of the test solution. In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.150 g in 10 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.47 mg of $C_{22}H_{34}Cl_2N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H.*

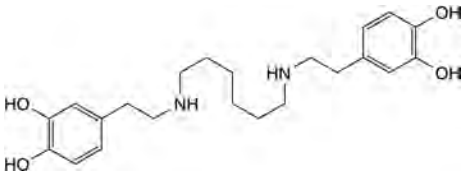

A. 4,4′-[hexane-1,6-diylbis(iminoethylene)]dibenzene-1,2-diol,

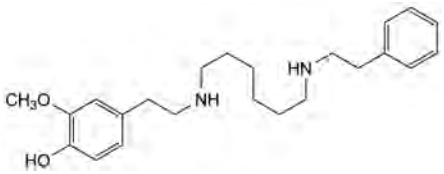

B. 2-methoxy-4-[2-[[6-[(2-phenylethyl)amino]hexyl]amino]-ethyl]phenol,

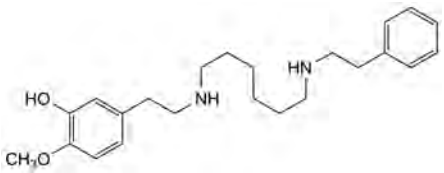

C. 2-methoxy-5-[2-[[6-[(2-phenylethyl)amino]hexyl]amino]-ethyl]phenol,

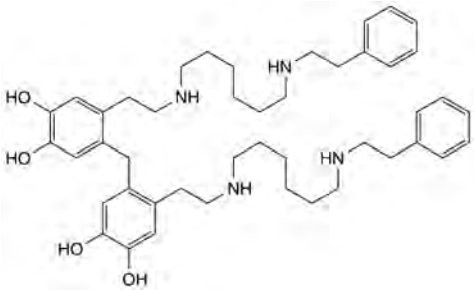

D. 4,4′-methylenebis[5-[2-[[6-[(2-phenylethyl)amino]hexyl]-amino]ethyl]benzene-1,2-diol],

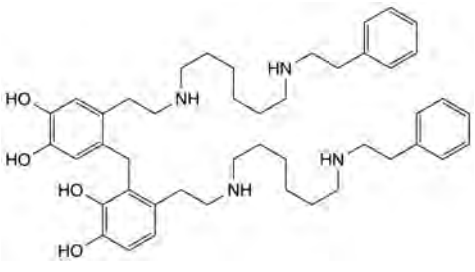

E. 3-[4,5-dihydroxy-2-[2-[[6-[(2-phenylethyl)amino]hexyl]-amino]ethyl]benzyl]-4-[2-[[6-[(2-phenylethyl)amino]-hexyl]amino]ethyl]benzene-1,2-diol,

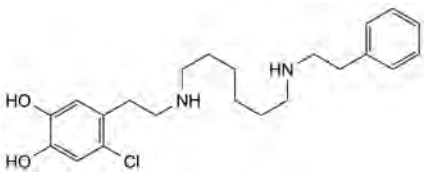

F. 4-chloro-5-[2-[[6-[(2-phenylethyl)amino]hexyl]amino]-ethyl]benzene-1,2-diol,

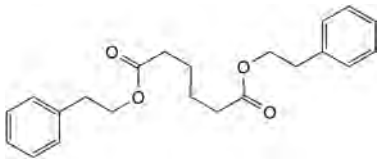

G. bis(2-phenylethyl) hexanedioate,

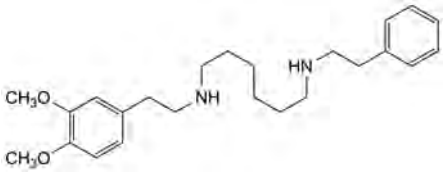

H. *N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*′-(2-phenylethyl)hexane-1,6-diamine,

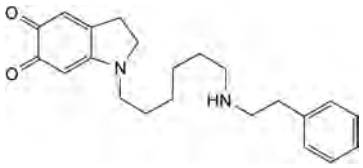

I. 1-[6-[(2-phenylethyl)amino]hexyl]-2,3-dihydro-1*H*-indole5,6-dione (dopexamine aminochrome),

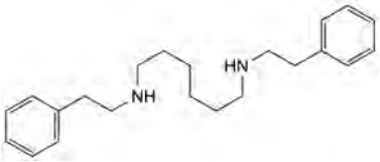

J. *N*,*N*′-bis(2-phenylethyl)hexane-1,6-diamine,

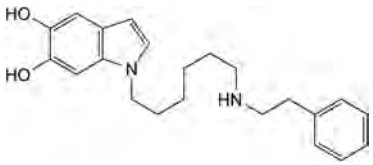

K. 1-[6-[(2-phenylethyl)amino]hexyl]-1*H*-indole-5,6-diol.

**01/2008:2359**

# DORZOLAMIDE HYDROCHLORIDE

## Dorzolamidi hydrochloridum

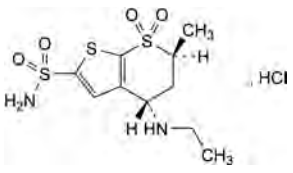

$C_{10}H_{17}ClN_2O_4S_3$ $M_r$ 360.9

[130693-82-2]

DEFINITION

(4*S*,6*S*)-4-(Ethylamino)-6-methyl-5,6-dihydro-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, slightly soluble in methanol, very slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dorzolamide hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. It complies with the test for impurity A (see Tests).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurity A**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *glacial acetic acid R*, *1,1-dimethylethyl methyl ether R* (3:10:87 *V/V/V*).

*Test solution.* In a centrifuge tube, dissolve 20.0 mg of the substance to be examined in 4 mL of *dilute ammonia R4*, add 4 mL of *ethyl acetate R*, and mix. Separate the organic layer and transfer it to a separate centrifuge tube. Add 4 mL of *ethyl acetate R* to the aqueous layer, mix, separate the organic layer, and combine it with the 1st extract. Evaporate the combined organic layers to dryness in a water-bath at 50 °C under a stream of *nitrogen R*. Dissolve the residue in 3 mL of *acetonitrile R*, add 0.06 mL of *(S)-(−)-α-methylbenzyl isocyanate R*, and heat in a water-bath at 50 °C for 5 min. Evaporate to dryness in a water-bath at 50 °C under a stream of *nitrogen R*. Dissolve the residue in 10 mL of the solvent mixture.

*Reference solution.* In a centrifuge tube, dissolve 18.0 mg of *dorzolamide hydrochloride CRS* and 2.0 mg of *dorzolamide impurity A CRS* in 4 mL of *dilute ammonia R4*, and proceed as indicated for the test solution beginning with "add 4 mL of *ethyl acetate R*, and mix".

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *acetonitrile R*, *heptane R*, *1,1-dimethylethyl methyl ether R* (0.2:2:35:63 *V/V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of dorzolamide.

*Relative retention* with reference to dorzolamide (retention time = about 10 min): impurity A = about 1.4.

*System suitability*: reference solution:

– *resolution*: minimum 4.0 between the peaks due to dorzolamide and impurity A.

Calculate the percentage content of impurity A using the following expression:

$$\frac{A}{A+B} \times 100$$

*A* = area of the peak due to impurity A in the chromatogram obtained with the test solution;

*B* = area of the peak due to dorzolamide in the chromatogram obtained with the test solution.

*Limit*:

– *impurity A*: maximum 0.5 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 2 mg of *dorzolamide for system suitability CRS* (containing impurity C) in 2 mL of mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: mix 65 mL of *acetonitrile R* and 935 mL of a 3.7 g/L solution of *potassium dihydrogen phosphate R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 30 | 100 → 50 | 0 → 50 |
| 30 - 37 | 50 → 100 | 50 → 0 |
| 37 - 44 | 100 | 0 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *dorzolamide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to dorzolamide (retention time = about 11 min): impurity C = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity C and dorzolamide.

*Limits*:

– *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*, using sonication if necessary. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 1st and the 3rd points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.05 mg of $C_{10}H_{17}N_2O_4S_3Cl$.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D.*

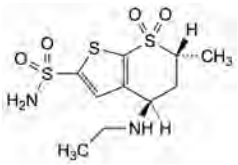

A. (4*R*,6*R*)-4-(ethylamino)-6-methyl-5,6-dihydro-4*H*-thieno-[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide,

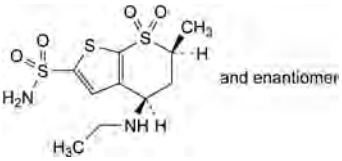


B. (4*RS*,6*SR*)-4-(ethylamino)-6-methyl-5,6-dihydro-4*H*-thieno[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide,

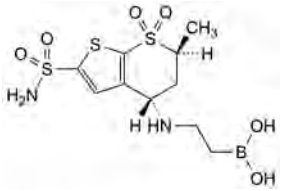

C. [2-[[(4*S*,6*S*)-6-methyl-7,7-dioxo-2-sulfamoyl-4,5,6,7-tetrahydro-7λ$^6$-thieno[2,3-*b*]thiopyran-4-yl]amino]ethyl]boronic acid,

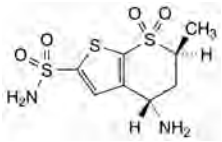

D. (4*S*,6*S*)-4-amino-6-methyl-5,6-dihydro-4*H*-thieno-[2,3-*b*]thiopyran-2-sulfonamide 7,7-dioxide.

**04/2019:1314**

# DOSULEPIN HYDROCHLORIDE

## Dosulepini hydrochloridum

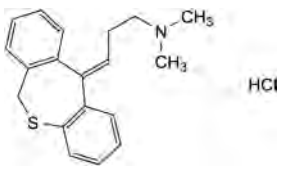

$C_{19}H_{22}ClNS$ $M_r$ 331.9

[897-15-4]

DEFINITION

(*E*)-3-(Dibenzo[*b*,*e*]thiepin-11(6*H*)-ylidene)-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or faintly yellow, crystalline powder.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in a 1 g/L solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 2.0 mL to 50.0 mL with a 1 g/L solution of *hydrochloric acid R* in *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: 231 nm and 306 nm.

*Shoulder*: about 260 nm.

*Specific absorbance at the absorption maximum at 231 nm*: 660 to 730.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dosulepin hydrochloride CRS*.

C. Dissolve about 1 mg in 5 mL of *sulfuric acid R*. A dark red colour is produced.

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1 g in *water R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 4.2 to 5.2.

Dissolve 1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 12.5 mg of *dosulepin impurity A CRS* in 5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 0.5 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *dosulepin for system suitability CRS* (containing impurity E) in 5 mL of *methanol R* and dilute to 20 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped cyanosilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: 0.83 per cent *V/V* solution of *perchloric acid R*, *propanol R*, *methanol R1*, *water for chromatography R* (1:10:30:60 *V/V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 5 µL.

*Run time*: 2.5 times the retention time of dosulepin.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *dosulepin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to dosulepin (retention time = about 14 min): impurity A = about 0.3; impurity E = about 0.92.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to dosulepin.

*Limits*:

- *impurity E*: not more than 5 per cent of the sum of the areas of the peak due to impurity E and the principal peak in the chromatogram obtained with the test solution (5 per cent);
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5 mL of *anhydrous acetic acid R* and 35 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.19 mg of $C_{19}H_{22}ClNS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

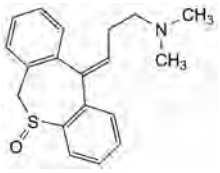

A. (*E*)-3-(5-oxo-5λ⁴-dibenzo[*b*,*e*]thiepin-11(6*H*)-ylidene)-*N*,*N*-dimethylpropan-1-amine,

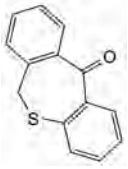

B. dibenzo[*b*,*e*]thiepin-11(6*H*)-one,

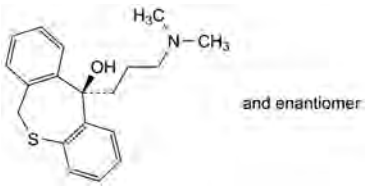


C. (11*RS*)-11-[3-(dimethylamino)propyl]-6,11-dihydrodibenzo[*b*,*e*]thiepin-11-ol,

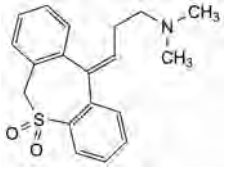

D. (*E*)-3-(5,5-dioxo-5λ⁶-dibenzo[*b*,*e*]thiepin-11(6*H*)-ylidene)-*N*,*N*-dimethylpropan-1-amine,

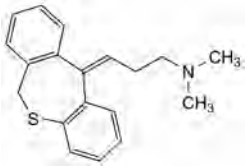

E. (*Z*)-3-(dibenzo[*b*,*e*]thiepin-11(6*H*)-ylidene)-*N*,*N*-dimethylpropan-1-amine.

**01/2017:1201**

# DOXAPRAM HYDROCHLORIDE

## Doxaprami hydrochloridum

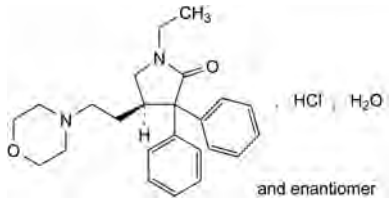


$C_{24}H_{31}ClN_2O_2,H_2O$ $M_r$ 433.0
[7081-53-0]

DEFINITION

(4*RS*)-1-Ethyl-4-[2-(morpholin-4-yl)ethyl]-3,3-diphenylpyrrolidin-2-one hydrochloride monohydrate.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *doxapram hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *doxapram hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: solution of *ammonia R* containing 17 g/L of $NH_3$, *2-propanol R*, *2-methylpropanol R* (10:10:80 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *dilute potassium iodobismuthate solution R* and examine immediately.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.000 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 10 mL of solution S to 25 mL with *water R*.

**pH** (*2.2.3*): 3.5 to 5.0.

Dilute 5 mL of solution S to 25 mL with *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 5.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *doxapram impurity B CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase. To 1.0 mL of the solution, add 1.0 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 50 volumes of *acetonitrile R* and 50 volumes of a 0.82 g/L solution of *sodium acetate R* adjusted to pH 4.5 with *glacial acetic acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of doxapram.

*Retention time*: doxapram = about 6 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to doxapram and impurity B.

*Limits*:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): 3.0 per cent to 4.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.50 mg of $C_{24}H_{31}ClN_2O_2$.

IMPURITIES

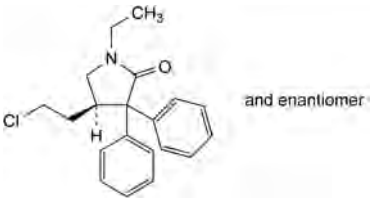


A. (4*RS*)-4-(2-chloroethyl)-1-ethyl-3,3-diphenylpyrrolidin-2-one,

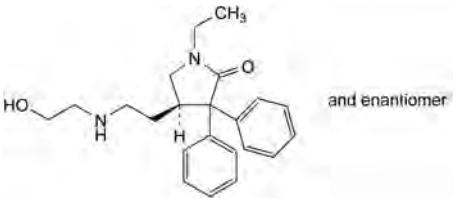


B. (4*RS*)-1-ethyl-4-[2-[(2-hydroxyethyl)amino]ethyl]-3,3-diphenylpyrrolidin-2-one.

**07/2019:2125**

# DOXAZOSIN MESILATE

## Doxazosini mesilas

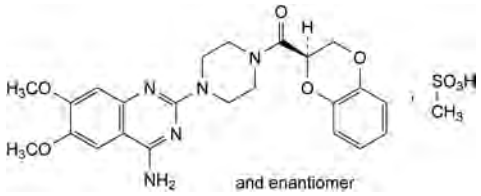


$C_{24}H_{29}N_5O_8S$ $M_r$ 547.6
[77883-43-3]

DEFINITION

[4-(4-Amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl][(2*RS*)-2,3-dihydro-1,4-benzodioxin-2-yl]methanone methanesulfonate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in doxazosin mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in a mixture of 15 volumes of water and 35 volumes of tetrahydrofuran, slightly soluble in methanol, practically insoluble in acetone.

It shows polymorphism (*5.9*); some forms may be hygroscopic.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *doxazosin mesilate CRS*.

If the spectra obtained in the solid state show differences, mix 1 part of the substance to be examined and 1 part of the reference substance separately with 10 parts of *anhydrous ethanol R* and heat to boiling. Continue heating the suspension under a reflux condenser for about 3 h. Cool and filter. Record new spectra using the previously dried residues on the filters.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in a mixture of 15 mL of *water R* and 35 mL of *tetrahydrofuran R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 5 mL of mobile phase B, adding *water R*, and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 25.0 mg of *doxazosin mesilate CRS* in 5 mL of mobile phase B, adding *water R*, and dilute to 50.0 mL with *water R*.

*Reference solution (c)*. Dissolve 5 mg of *doxazosin impurity D CRS* and 5 mg of *doxazosin impurity F CRS* in 5 mL of mobile phase B, adding *water R*, and dilute to 50 mL with *water R*. Dilute 1 mL of this solution to 5 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 1.5 g/L solution of *phosphoric acid R*;
- *mobile phase B*: 1.5 g/L solution of *phosphoric acid R* in *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 40 | 90 → 50 | 10 → 50 |
| 40 - 45 | 50 | 50 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and F.

*Relative retention* with reference to doxazosin (retention time = about 30 min): impurity D = about 0.5; impurity F = about 0.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 4.5 between the peaks due to impurities D and F.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{24}H_{29}N_5O_8S$ taking into account the assigned content of *doxazosin mesilate CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H*.

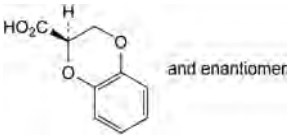


A. (2*RS*)-2,3-dihydro-1,4-benzodioxine-2-carboxylic acid,

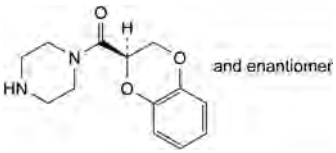


B. [(2*RS*)-2,3-dihydro-1,4-benzodioxin-2-yl](piperazin-1-yl)methanone,

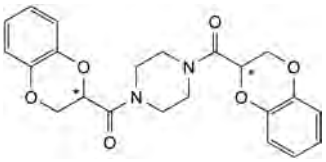


C. (piperazine-1,4-diyl)bis[[(2Ξ)-2,3-dihydro-1,4-benzodioxin-2-yl]methanone],

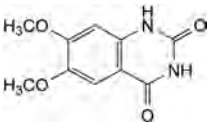


D. 6,7-dimethoxyquinazoline-2,4(1*H*,3*H*)-dione,

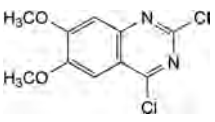


E. 2,4-dichloro-6,7-dimethoxyquinazoline,

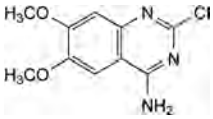

F. 2-chloro-6,7-dimethoxyquinazolin-4-amine,

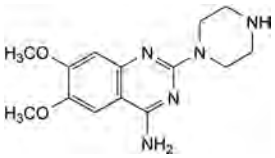

G. 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine,

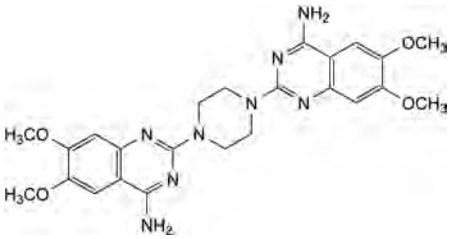

H. 2,2′-(piperazine-1,4-diyl)bis(6,7-dimethoxyquinazolin-4-amine).



**01/2017:1096**

# DOXEPIN HYDROCHLORIDE

## Doxepini hydrochloridum

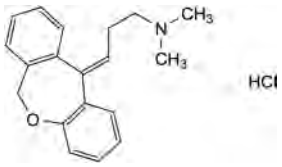

$C_{19}H_{22}ClNO$ $M_r$ 315.8
[1229-29-4]

DEFINITION

(*E*)-3-(Dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine hydrochloride.

*Content*: 98.0 per cent to 101.0 per cent of $C_{19}H_{22}ClNO$ (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: C, E.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 185 °C to 191 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in a 1 g/L solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 mL with the same acid solution. Dilute 5.0 mL to 50.0 mL with a 1 g/L solution of *hydrochloric acid R* in *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 297 nm.

*Specific absorbance at the absorption maximum*: 128 to 142.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *doxepin hydrochloride CRS*.

D. Dissolve about 5 mg in 2 mL of *sulfuric acid R*. A dark red colour is produced.

E. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 30 mL with the same solvent.

**Appearance of solution**. Dilute 10 mL of solution S to 25 mL with *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect them from light.*

*Phosphate buffer solution.* Dissolve 1.42 g of *anhydrous disodium hydrogen phosphate R* in *water R*, adjust to pH 7.7 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture.* Mix 1 volume of *1 M sodium hydroxide* and 250 volumes of the mobile phase.

*Test solution.* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *doxepin for system suitability CRS* (containing impurities A, B and C) in 1.0 mL of mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, phosphate buffer solution, *methanol R1* (20:30:50 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of doxepin.

*Identification of impurities*: use the chromatogram supplied with *doxepin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to doxepin (retention time = about 18 min): impurity A = about 0.5; impurity C = about 0.6; impurity B = about 0.7; the peak due to doxepin might show a shoulder caused by the (*Z*)-isomer (impurity D).

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and C, and minimum 1.5 between the peaks due to impurities C and B;
- the chromatogram obtained is similar to the chromatogram supplied with *doxepin for system suitability CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.7;
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**(*Z*)-Isomer**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4 mm;
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 220 $m^2$/g and a pore size of 80 nm;
- *temperature*: 50 °C.

*Mobile phase*: mix 30 volumes of *methanol R* and 70 volumes of a 30 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to the (*E*)-isomer (1st peak) and to the (*Z*)-isomer (2nd peak).

*Results*:

- calculate the ratio of the area of the peak due to the (*E*)-isomer to the area of the peak due to the (*Z*)-isomer: this ratio is 4.4 to 6.7 (13.0 per cent to 18.5 per cent of the (*Z*)-isomer).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5 mL of *anhydrous acetic acid R* and 35 mL of *acetic anhydride R*. Using 0.2 mL of *crystal violet solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from blue to green.

1 mL of *0.1 M perchloric acid* is equivalent to 31.58 mg of $C_{19}H_{22}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

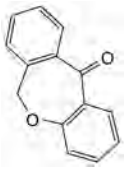

A. dibenzo[*b,e*]oxepin-11(6*H*)-one (doxepinone),

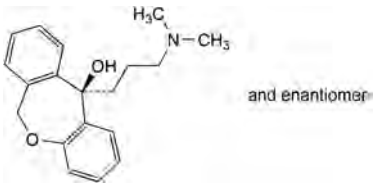

B. (11*RS*)-11-[3-(dimethylamino)propyl]-6,11-dihydrodibenzo[*b,e*]oxepin-11-ol (doxepinol),

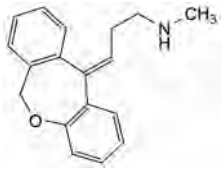

C. (*E*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N*-methylpropan-1-amine (desmethyldoxepin),

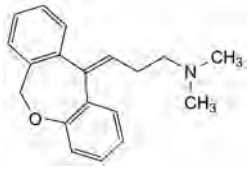

D. (*Z*)-3-(dibenzo[*b,e*]oxepin-11(6*H*)-ylidene)-*N,N*-dimethylpropan-1-amine.

**01/2008:0714**
**corrected 10.0**

# DOXORUBICIN HYDROCHLORIDE

## Doxorubicini hydrochloridum

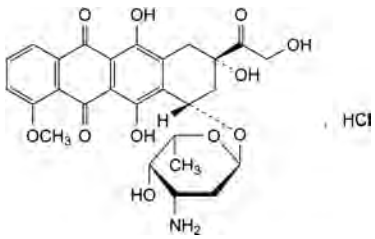

$C_{27}H_{30}ClNO_{11}$ $M_r$ 580.0
[25316-40-9]

DEFINITION

(8*S*,10*S*)-10-[(3-Amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance produced by certain strains of *Streptomyces coeruleorubidus* or *Streptomyces peucetius* or obtained by any other means.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: orange-red, crystalline powder, hygroscopic.

*Solubility*: soluble in water, slightly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *doxorubicin hydrochloride CRS*.

B. Dissolve about 10 mg in 0.5 mL of *nitric acid R*, add 0.5 mL of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 mL of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *doxorubicin hydrochloride CRS* and 10 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix equal volumes of *acetonitrile R* and a solution containing 2.88 g/L of *sodium laurilsulfate R* and 2.25 g/L of *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL; inject test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of doxorubicin.

*Retention time*: doxorubicin = about 8 min.

*System suitability*: reference solution (a):

- *resolution*: minimum of 2.0 between the peaks due to doxorubicin and to epirubicin.

*Limits*:

- *any impurity*: not more than the area of the peak due to doxorubicin in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.1 times the area of the peak due to doxorubicin in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethanol** (*2.4.24, System B*): maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 2.2 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{30}ClNO_{11}$.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

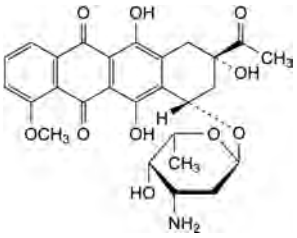

A. (8*S*,10*S*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicin),

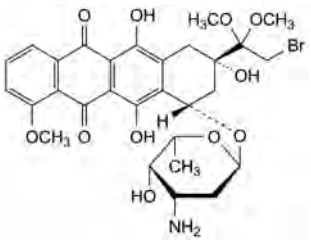

B. (8*S*,10*S*)-10[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-8-(2-bromo-1,1-dimethoxyethyl)-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione,

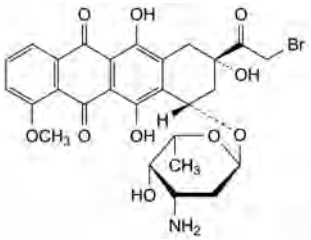

C. (8*S*,10*S*)-10[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-8-(bromoacetyl)-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione,

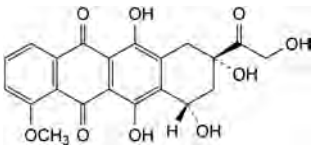

D. (8*S*,10*S*)-6,8,10,11-tetrahydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicin aglycone, doxorubicinone).

**01/2017:0272**
**corrected 10.0**

# DOXYCYCLINE HYCLATE

## Doxycyclini hyclas

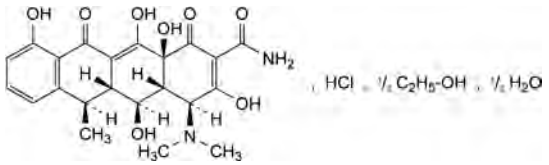

$(C_{22}H_{25}ClN_2O_8),½C_2H_6O,½H_2O$ $M_r$ 512.9

[24390-14-5]

DEFINITION

(4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-(Dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride hemiethanol hemihydrate.

Substance obtained from oxytetracycline or metacycline or by any other means.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent of $C_{22}H_{25}ClN_2O_8$ (anhydrous and ethanol-free substance).

CHARACTERS

*Appearance*: yellow, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in methanol, sparingly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.
   *Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A yellow colour develops.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 2.0 to 3.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.07 at 490 nm (anhydrous and ethanol-free substance).

Dissolve 0.10 g in a mixture of 1 volume of a 103 g/L solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. Carry out the measurement within 1 h of preparing the solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Suspend 111.6 g of *sodium edetate R* in 900 mL of *water for chromatography R*. Adjust to pH 7.0 with *concentrated ammonia R* to achieve complete dissolution, then dilute to 1 L with *water for chromatography R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Reference solution (a).* Dissolve 20.0 mg of *doxycycline hyclate CRS* in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Reference solution (b).* Dissolve 5 mg of *doxycycline for system suitability CRS* (containing impurities A, B, C and F) in a 1 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 13 volumes of *acetonitrile R*, 17 volumes of *water for chromatography R*, 35 volumes of a 67.9 g/L solution of *tetrabutylammonium hydrogen sulfate R* previously adjusted to pH 7.0 with *concentrated ammonia R* and 35 volumes of solution A.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of doxycycline.

*Identification of impurities*: use the chromatogram supplied with *doxycycline for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and F.

*Relative retention* with reference to doxycycline (retention time = about 21 min): impurity C = about 0.4; impurity A = about 0.7; impurity B = about 0.8; impurity F = about 1.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurities A and B; minimum 2.0 between the peaks due to impurity B and doxycycline.

*Calculation of percentage contents*:
- for each impurity, use the concentration of doxycycline hyclate in reference solution (c).

*Limits*:
- *impurity A*: maximum 2.0 per cent;
- *impurity F*: maximum 1.2 per cent;
- *impurity B*: maximum 0.5 per cent;
- *impurity C*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Ethanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 0.50 mL of *propanol R* to 1.0 L with *water R*.

*Test solution.* Dissolve 0.10 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the same solution.

*Reference solution.* Dilute 0.5 mL of *anhydrous ethanol R* to 100.0 mL with the internal standard solution. Dilute 1.0 mL of this solution to 10.0 mL with the internal standard solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 37 cm/s.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 7 | 70 |
| | 7 - 13 | 70 → 160 |
| | 13 - 15 | 160 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to propanol (retention time = about 7 min): ethanol = about 0.6.

Calculate the content of ethanol, taking the density (*2.2.5*) at 20 °C to be 0.790 g/mL.

*Limit*:
- *ethanol*: 4.3 per cent to 6.0 per cent.

**Water** (*2.5.12*): 1.4 per cent to 2.8 per cent, determined on 1.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.14 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$ taking into account the assigned content of *doxycycline hyclate CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

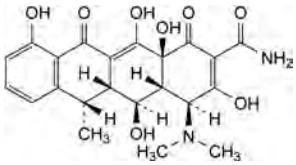

A. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (6-epidoxycycline),

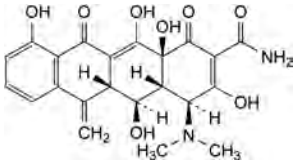

B. (4*S*,4a*R*,5*S*,5a*R*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methylene-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (metacycline),

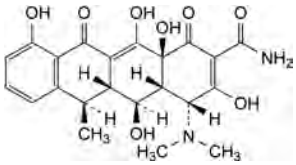

C. (4*R*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidoxycycline),

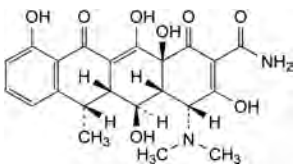

D. (4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epi-6-epidoxycycline),

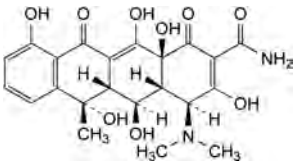

E. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (oxytetracycline),

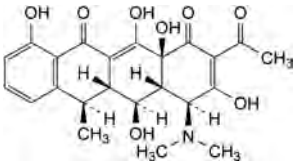

F. (4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyldoxycycline).

**01/2017:0820**
**corrected 10.0**

# DOXYCYCLINE MONOHYDRATE

## Doxycyclinum monohydricum

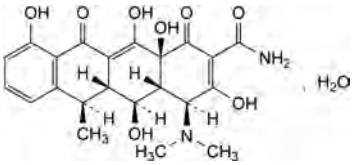

$C_{22}H_{24}N_2O_8,H_2O$ $M_r$ 462.5
[17086-28-1]

DEFINITION

(4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-(Dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide monohydrate.

Substance obtained from oxytetracycline or metacycline or by any other means.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent of $C_{22}H_{24}N_2O_8$ (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of mineral acids and in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A yellow colour develops.

C. Water (see Tests).

TESTS

**pH** (*2.2.3*): 5.0 to 6.5.

Suspend 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.07 at 490 nm (anhydrous substance).

Dissolve 0.10 g in a mixture of 0.5 volumes of *hydrochloric acid R* and 99.5 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. Carry out the measurement within 1 h of preparing the solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Suspend 111.6 g of *sodium edetate R* in 900 mL of *water R*. Adjust to pH 7.0 with *concentrated ammonia R* to achieve complete dissolution, then dilute to 1 L with *water R*.

*Test solution*. Dissolve 20.0 mg of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Reference solution (a)*. Dissolve 20.0 mg of *doxycycline hyclate CRS* in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Reference solution (b)*. Dissolve 5 mg of *doxycycline for system suitability CRS* (containing impurities A, B, C and F) in a 1 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm) ;
– *temperature*: 35 °C.

*Mobile phase*: mix 13 volumes of *acetonitrile R*, 17 volumes of *water R*, 35 volumes of a 67.9 g/L solution of *tetrabutylammonium hydrogen sulfate R* previously adjusted to pH 7.0 with *concentrated ammonia R* and 35 volumes of solution A.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of doxycycline.

*Identification of impurities*: use the chromatogram supplied with *doxycycline for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and F.

*Relative retention* with reference to doxycycline (retention time = about 21 min): impurity C = about 0.4; impurity A = about 0.7; impurity B = about 0.8; impurity F = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities A and B; minimum 2.0 between the peaks due to impurity B and doxycycline.

*Calculation of percentage contents*:

– for each impurity, use the concentration of doxycycline monohydrate in reference solution (c).

*Limits*:

– *impurity A*: maximum 2.0 per cent;
– *impurity F*: maximum 1.2 per cent;
– *impurity B*: maximum 0.5 per cent;
– *impurity C*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 3.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.6 per cent to 4.6 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}N_2O_8$ taking into account the assigned content of *doxycycline hyclate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

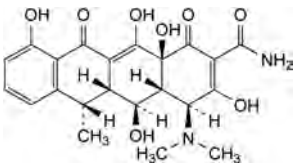

A. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (6-epidoxycycline),

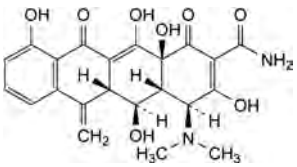

B. (4*S*,4a*R*,5*S*,5a*R*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methylene-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (metacycline),

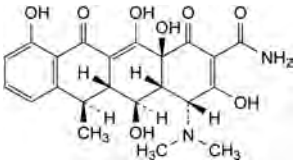

C. (4*R*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epidoxycycline),

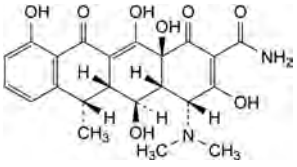

D. (4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epi-6-epidoxycycline),

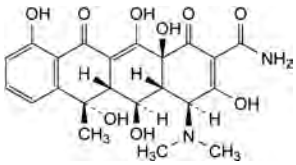

E. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (oxytetracycline),

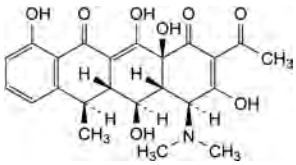

F. (4*S*,4a*R*,5*S*,5a*R*,6*R*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyldoxycycline).

**01/2013:1589**
**corrected 10.0**

# DOXYLAMINE HYDROGEN SUCCINATE

## Doxylamini hydrogenosuccinas

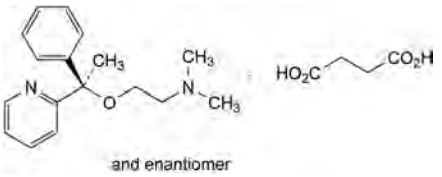


$C_{21}H_{28}N_2O_5$ $M_r$ 388.5
[562-10-7]

DEFINITION

*N*,*N*-Dimethyl-2-[(1*RS*)-1-phenyl-1-(pyridin-2-yl)ethoxy]ethanamine hydrogen butanedioate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *doxylamine hydrogen succinate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.4 g in *water R* and dilute to 20 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.650 g of the substance to be examined in 20 mL of a 10.3 g/L solution of *hydrochloric acid R*. Add 3 mL of a 100 g/L solution of *sodium hydroxide R* and extract with 3 quantities, each of 25 mL, of *methylene chloride R*. Combine the methylene chloride extracts and filter using hydrophobic phase-separation filter paper. Rinse the filter with 10 mL of *methylene chloride R* and combine the rinsings with the methylene chloride extracts. Evaporate the solvent under reduced pressure at a temperature not exceeding 40 °C. Dissolve the residue in 20.0 mL of *anhydrous ethanol R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *anhydrous ethanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Reference solution (b)*. Dissolve 50 mg of *doxylamine for system suitability CRS* (containing impurity C) in 10 mL of a 10.3 g/L solution of *hydrochloric acid R*. Add 1.5 mL of a 100 g/L solution of *sodium hydroxide R* and extract with 3 quantities, each of 25 mL, of *methylene chloride R*. Combine the methylene chloride extracts and filter using hydrophobic phase-separation filter paper. Rinse the filter with 10 mL of *methylene chloride R* and combine the rinsings with the methylene chloride extracts. Evaporate the solvent under reduced pressure at a temperature not exceeding 40 °C. Dissolve the residue in 5.0 mL of *anhydrous ethanol R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 7 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 160 → 220 |
| | 12 - 27 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram supplied with *doxylamine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to doxylamine (retention time = about 12 min): impurity C = about 0.96.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity C and doxylamine.

*Limits*:

- *impurity C*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.43 mg of $C_{21}H_{28}N_2O_5$.

IMPURITIES

*Specified impurities*: *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D*.

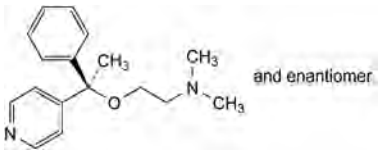


A. *N,N*-dimethyl-2-[(1*RS*)-1-phenyl-1-(pyridin-4-yl)ethoxy]ethanamine,

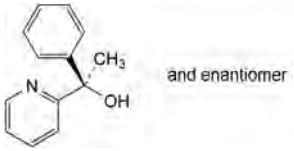


B. (1*RS*)-1-phenyl-1-(pyridin-2-yl)ethanol,

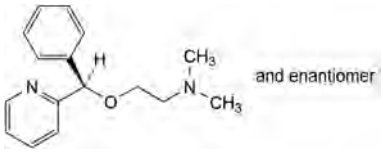


C. *N,N*-dimethyl-2-[(1*RS*)-1-phenyl-1-(pyridin-2-yl)methoxy]ethanamine,

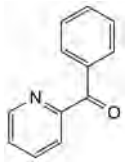


D. phenyl(pyridin-2-yl)methanone (2-benzoylpyridine).

**01/2020:3039**

# DRONEDARONE HYDROCHLORIDE

## Dronedaroni hydrochloridum

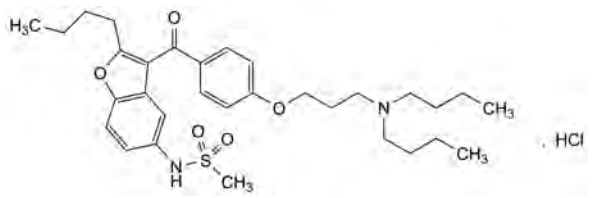


$C_{31}H_{45}ClN_2O_5S$ $M_r$ 593.2
[141625-93-6]

DEFINITION

*N*-[2-Butyl-3-[4-[3-(dibutylamino)propoxy]benzoyl]-1-benzofuran-5-yl]methanesulfonamide hydrochloride.

*Content*: 98.0 to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: practically insoluble in water, freely soluble in methanol, soluble in ethanol (96 per cent) and practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dronedarone hydrochloride CRS*.

B. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile for chromatography R*, mobile phase A (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture, using sonication, and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *dronedarone impurity B CRS* in *methanol R* and dilute to 25 mL with the same solvent. Dilute 1 mL of the solution to 20 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *diisopropylcyanosilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: to 2.0 mL of *triethylamine R* add 950 mL of *water for chromatography R*, adjust to pH 4.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 70 | 30 |
| 2 - 17 | 70 → 60 | 30 → 40 |
| 17 - 27 | 60 | 40 |
| 27 - 42 | 60 → 50 | 40 → 50 |
| 42 - 47 | 50 → 40 | 50 → 60 |
| 47 - 60 | 40 | 60 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 246 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to dronedarone (retention time = about 40 min): impurity B = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity B and dronedarone.

*Calculation of percentage contents*:

- for each impurity, use the concentration of dronedarone hydrochloride in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A*. To 2.0 mL of *triethylamine R* add 950 mL of *water for chromatography R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution*. Dissolve 50.0 mg of *dronedarone hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *diisopropylcyanosilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, solution A (50:50 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of dronedarone (retention time = about 9 min).

Calculate the percentage content of $C_{31}H_{45}ClN_2O_5S$ taking into account the assigned content of *dronedarone hydrochloride CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C*.

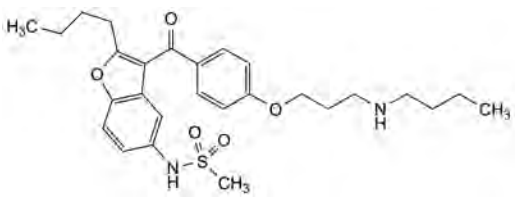

A. *N*-[2-butyl-3-[4-[3-(butylamino)propoxy]benzoyl]-1-benzofuran-5-yl]methanesulfonamide,

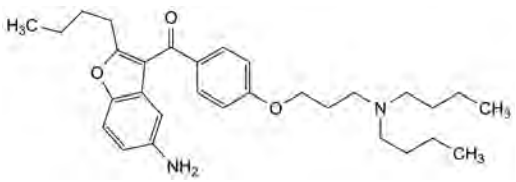

B. (5-amino-2-butyl-1-benzofuran-3-yl)[4-[3-(dibutylamino)propoxy]phenyl]methanone,

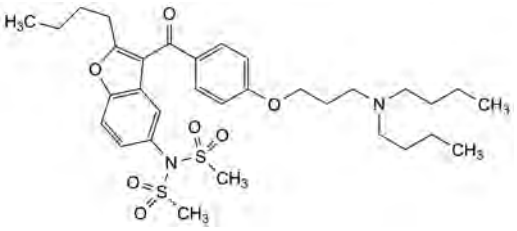

C. *N*-[2-butyl-3-[4-[3-(dibutylamino)propoxy]benzoyl]-1-benzofuran-5-yl]-*N*-(methanesulfonyl)methanesulfonamide.

**01/2017:1010**

# DROPERIDOL

## Droperidolum

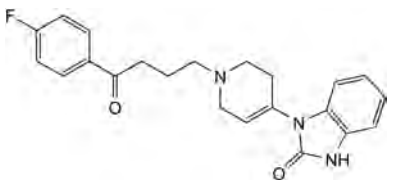

$C_{22}H_{22}FN_3O_2$ $M_r$ 379.4

[548-73-2]

DEFINITION

1-[1-[4-(4-Fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide and in methylene chloride, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *droperidol CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (a)*. Dissolve 30 mg of *droperidol CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b)*. Dissolve 30 mg of *droperidol CRS* and 30 mg of *benperidol CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *acetone R*, *methanol R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 5 mL of *anhydrous ethanol R*. Add 0.5 mL of *dinitrobenzene solution R* and 0.5 mL of *2 M alcoholic potassium hydroxide R*. A violet colour is produced and becomes brownish-red after 20 min.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.20 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.10 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 2.5 mg of *droperidol CRS* and 2.5 mg of *benperidol CRS* in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*;

– *mobile phase B*: 10 g/L solution of *tetrabutylammonium hydrogen sulfate R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 0 → 40 | 100 → 60 |
| 15 - 20 | 40 | 60 |
| 20 - 25 | 40 → 0 | 60 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL.

*Relative retention* with reference to droperidol (retention time = about 7 min): impurity A = about 0.2; impurity B = about 0.85; benperidol = about 0.9; impurity C = about 0.95; impurity D = about 1.2; impurity E = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to benperidol and droperidol.

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from orange-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 37.94 mg of $C_{22}H_{22}FN_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

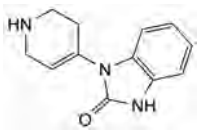


A. 1-(1,2,3,6-tetrahydropyridin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,

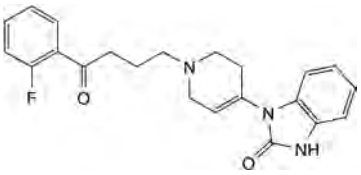


B. 1-[1-[4-(2-fluorophenyl)-4-oxobutyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

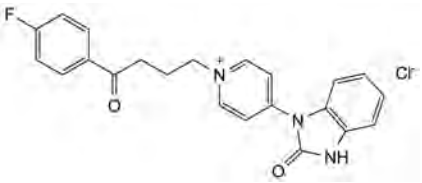


C. 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)pyridinium chloride,

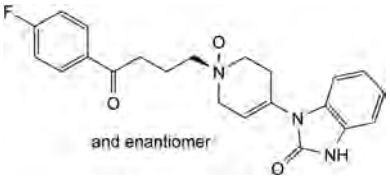


D. (1*RS*)-1-[4-(4-fluorophenyl)-4-oxobutyl]-4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-1,2,3,6-tetrahydropyridine 1-oxide,

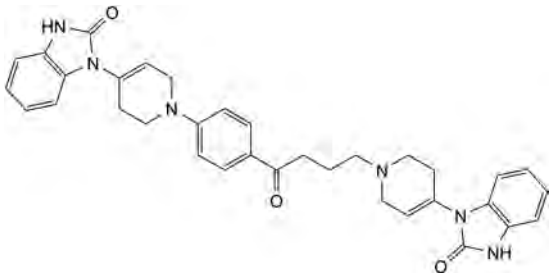

E. 1-[1-[4-[4-[4-(2-oxo-2,3-dihydro-1*H*-benzimidazol-1-yl)-3,6-dihydropyridin-1(2*H*)-yl]-1-oxobutyl]phenyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

**04/2015:2404**



# DROSPIRENONE

## Drospirenonum

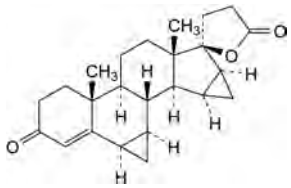

$C_{24}H_{30}O_3$ $M_r$ 366.5
[67392-87-4]

DEFINITION

3-Oxo-6α,7α,15α,16α-tetrahydro-3′*H*,3″*H*-dicyclopropa-[6,7:15,16]-17α-pregn-4-en-21,17-carbolactone.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *drospirenone CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 193 to − 187 (dried substance).

Dissolve 0.100 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Use 1.0 mL of this solution to dissolve the contents of a vial of *drospirenone impurity E CRS*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 30.0 mg of *drospirenone CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 3 mg of *drospirenone for peak identification CRS* (containing impurity A) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 63 | 37 |
| 2 - 16 | 63 → 52 | 37 → 48 |
| 16 - 23 | 52 | 48 |
| 23 - 31 | 52 → 20 | 48 → 80 |
| 31 - 39 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *drospirenone for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E.

*Relative retention* with reference to drospirenone (retention time = about 22 min): impurity E = about 1.1; impurity A = about 1.2.

*System suitability*: reference solution (a):
- *resolution*: minimum 5.0 between the peaks due to drospirenone and impurity E.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.5;
- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{24}H_{30}O_3$ taking into account the assigned content of *drospirenone CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, K.*

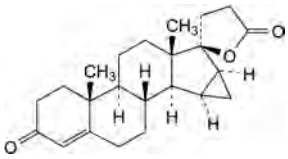

A. 3-oxo-15α,16α-dihydro-3′*H*-cyclopropa[15,16]-17α-pregn-4-ene-21,17-carbolactone (6,7-desmethylenedrospirenone),

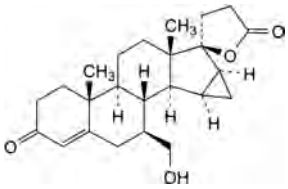

B. 7β-(hydroxymethyl)-3-oxo-15α,16α-dihydro-3′*H*-cyclopropa[15,16]-17α-pregn-4-ene-21,17-carbolactone (7β-hydroxymethyl derivative),

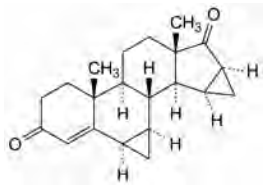

C. 6α,7α,15α,16α-tetrahydro-3′*H*,3″*H* -dicyclopropa-[6,7:15,16]androst-4-ene-3,17-dione (17-keto derivative),

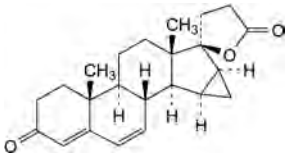

D. 3-oxo-15α,16α-dihydro-3′*H*-cyclopropa[15,16]-17α-pregna-4,6-diene-21,17-carbolactone (Δ6-drospirenone),

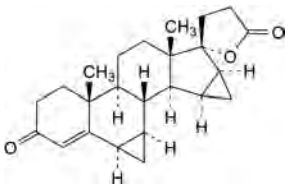

E. 3-oxo-6α,7α,15α,16α-tetrahydro-3′*H*,3″*H* -dicyclopropa[6,7:15,16]pregn-4-ene-21,17-carbolactone (17-epidrospirenone),

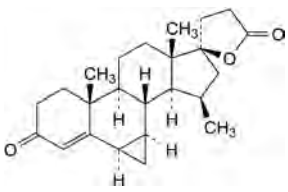

F. 15β-methyl-3-oxo-6α,7α-dihydro-3′*H*-cyclopropa[6,7]-17α-pregn-4-ene-21,17-carbolactone (3″-16-secodrospirenone),

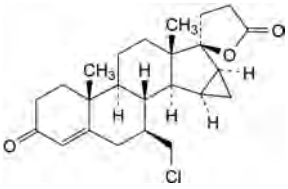

G. 7β-(chloromethyl)-3-oxo-15α,16α-dihydro-3′*H*-cyclopropa[15,16]-17α-pregn-4-ene-21,17-carbolactone (3′-chloro-3′,6-secodrospirenone),

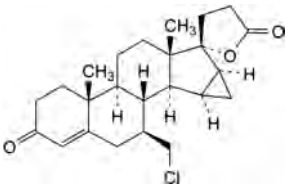

H. 7β-(chloromethyl)-3-oxo-15α,16α-dihydro-3′*H*-cyclopropa[15,16]pregn-4-ene-21,17-carbolactone (3′-chloro-3′,6-seco-17-epidrospirenone),

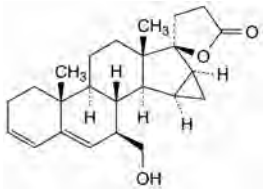

I. 7β-(hydroxymethyl)-15α,16α-dihydro-3′*H*-cyclopropa[15,16]-17α-pregna-3,5-diene-21,17-carbolactone (7β-hydroxymethyldiene derivative),

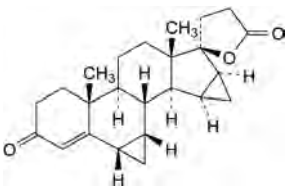

K. 3-oxo-6β,7β,15α,16α-tetrahydro-3′*H*,3″*H*-dicyclopropa-[6,7:15,16]-17α-pregn-4-ene-21,17-carbolactone (6α,7α-drospirenone).

**01/2017:2594**
**corrected 10.0**

# DULOXETINE HYDROCHLORIDE

## Duloxetini hydrochloridum

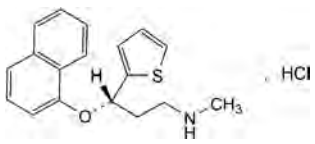

$C_{18}H_{20}ClNOS$ $M_r$ 333.9
[136434-34-9]

DEFINITION

(3*S*)-*N*-Methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, practically insoluble in hexane.

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): + 119 to + 127 (dried substance).
   Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent. Examine within 30 min of preparing the solution.

B. Infrared absorption spectrophotometry (*2.2.24*).
   *Comparison*: *duloxetine hydrochloride CRS*.

C. Enantiomeric purity (see Tests).

D. Dissolve 25 mg in 5 mL of *methanol R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 5.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *duloxetine impurity A CRS* and 5 mg of the substance to be examined in 100 mL of the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: add 2.0 mL of *diethylamine R* to 1000 mL of a mixture of 17 volumes of *2-propanol R* and 83 volumes of *hexane R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to duloxetine (retention time = about 7 min): impurity A = about 1.3.

*System suitability*:
- *resolution*: minimum 3.5 between the peaks due to duloxetine and impurity A in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

*Limit*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).
*Carry out the test protected from light. Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (25:75 *V/V*).

*Test solution (a)*. Dissolve 20 mg of the substance to be examined in 200.0 mL of the solvent mixture.

*Test solution (b)*. Dissolve 50.0 mg of the substance to be examined in 100.0 mL of the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 20 mg of *duloxetine for system suitability CRS* (containing impurity F) in the mobile phase and dilute to 200 mL with the mobile phase. In order to prepare impurities C and D *in situ*, heat the solution at 60 °C for 1 h (solution containing impurities C, D and F).

*Reference solution (c)*. Dissolve 50.0 mg of *duloxetine hydrochloride CRS* in 100.0 mL of the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Hexanesulfonate solution*: dissolve 10.3 g of *sodium hexanesulfonate monohydrate for ion-pair chromatography R* in a solution prepared as follows and dilute to 1000.0 mL with the same solution: dissolve 2.9 g (1.7 mL) of *phosphoric acid R* in 900 mL of *water for chromatography R*, adjust to pH 2.5 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Mobile phase*: *acetonitrile for chromatography R*, *propanol R*, hexanesulfonate solution (13:17:70 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of duloxetine.

*Identification of impurities*: use the chromatogram supplied with *duloxetine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C, D and F.

*Relative retention* with reference to duloxetine (retention time = about 16 min): impurity C = about 0.4; impurity D = about 0.5; impurity F = about 1.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities C and D;
- *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to duloxetine.

*Limits*:
- *impurity F*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{20}ClNOS$ taking into account the assigned content of *duloxetine hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, F*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use*

*(2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, G.*

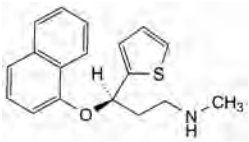

A. (3*R*)-*N*-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine,

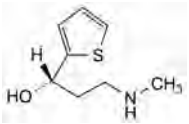

B. (1*S*)-3-(methylamino)-1-(thiophen-2-yl)propan-1-ol,

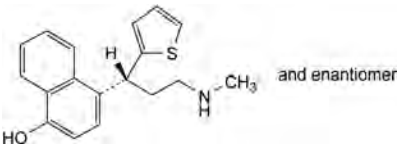


C. 4-[(1*RS*)-3-(methylamino)-1-(thiophen-2-yl)propyl]naphthalen-1-ol,

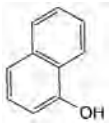

D. naphthalen-1-ol,

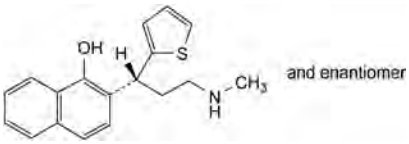


E. 2-[(1*RS*)-3-(methylamino)-1-(thiophen-2-yl)propyl]naphthalen-1-ol,

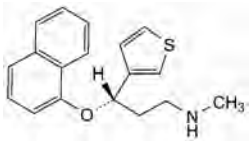

F. (3*S*)-*N*-methyl-3-(naphthalen-1-yloxy)-3-(thiophen-3-yl)propan-1-amine,

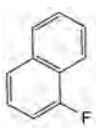

G. 1-fluoronaphthalene.

**01/2017:2641**

# DUTASTERIDE

## Dutasteridum

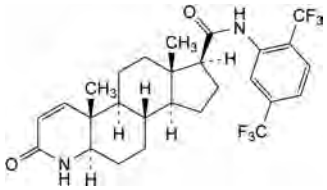

$C_{27}H_{30}F_6N_2O_2$ $M_r$ 528.5
[164656-23-9]

DEFINITION

*N*-[2,5-Bis(trifluoromethyl)phenyl]-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble or sparingly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *dutasteride CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 33.0 to + 39.0 (anhydrous substance).

Dissolve 0.100 g in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *acetonitrile R1* (40:60 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *dutasteride for system suitability CRS* (containing impurities A, B, C, E, F, G, H and I) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 50.0 mg of *dutasteride CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 0.25 volumes of *trifluoroacetic acid R*, 480 volumes of *water for chromatography R* and 520 volumes of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: 1.6 times the retention time of dutasteride.

*Identification of impurities*: use the chromatogram supplied with *dutasteride for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, E, F and G.

*Relative retention* with reference to dutasteride (retention time = about 36 min): impurity A = about 0.10; impurity B = about 0.11; impurity C = about 0.4; impurity E = about 0.9; impurity F = about 1.1; impurity G = about 1.2.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity E and dutasteride and minimum 1.5 between the peaks due to impurities A and B in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 30 for the peak due to dutasteride in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity F = 3.0;
- for each impurity, use the concentration of dutasteride in reference solution (a).

*Limits*:

- *impurity F*: maximum 0.4 per cent;
- *impurities E, G*: for each impurity, maximum 0.3 per cent;
- *impurities A, C*: for each impurity, maximum 0.2 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *reporting threshold*: 0.05 per cent.

B. Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R, acetonitrile R1* (20:80 *V/V*).

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Run time*: 5 times the retention time of dutasteride.

*Identification of impurities*: use the chromatogram supplied with *dutasteride for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities H and I.

*Relative retention* with reference to dutasteride (retention time = about 4 min): impurity H = about 3.4; impurity I = about 3.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities H and I.

*Calculation of percentage contents*:

- for each impurity, use the concentration of dutasteride in reference solution (a).

*Limits*:

- *impurity I*: maximum 0.5 per cent;
- *impurity H*: maximum 0.3 per cent;
- *unspecified impurities eluting after dutasteride*: for each impurity, maximum 0.10 per cent;
- *reporting threshold*: 0.05 per cent.

*Limit*:

- *total for tests A and B*: maximum 1.5 per cent.

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 0.100 g using the evaporation technique at 180 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (c).

Calculate the percentage content of $C_{27}H_{30}F_6N_2O_2$ taking into account the assigned content of *dutasteride CRS*.

IMPURITIES

*Specified impurities: A, B, C, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

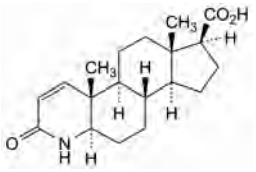

A. 3-oxo-4-aza-5α-androst-1-ene-17β-carboxylic acid,

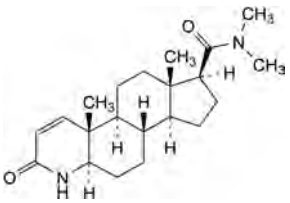

B. *N*,*N*-dimethyl-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide,

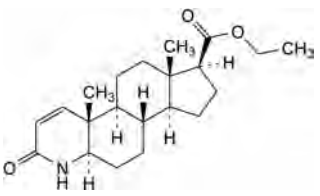

C. ethyl 3-oxo-4-aza-5α-androst-1-ene-17β-carboxylate,

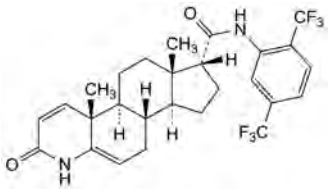

D. *N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-azaandrost-1,5-diene-17α-carboxamide,

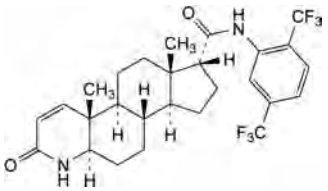

E. *N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-aza-5α-androst-1-ene-17α-carboxamide,

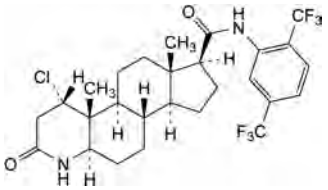

F. *N*-[2,5-bis(trifluoromethyl)phenyl]-1α-chloro-3-oxo-4-aza-5α-androstane-17β-carboxamide,

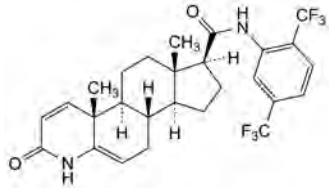

G. *N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-azaandrost-1,5-diene-17β-carboxamide,

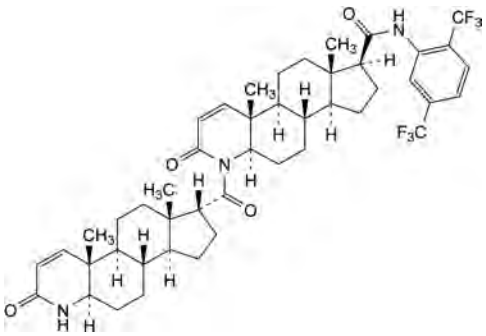

H. *N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-[3-oxo-4-aza-5α-androst-1-ene-17α-carbonyl]-4-aza-5α-androst-1-ene-17β-carboxamide (dutasteride dimer 1),

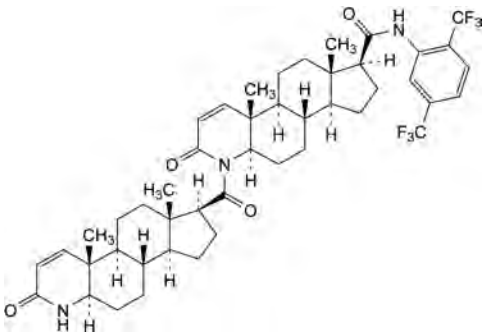

I. *N*-[2,5-bis(trifluoromethyl)phenyl]-3-oxo-4-[3-oxo-4-aza-5α-androst-1-ene-17β-carbonyl]-4-aza-5α-androst-1-ene-17β-carboxamide (dutasteride dimer 2).

**01/2009:2357**

# DYDROGESTERONE

## Dydrogesteronum

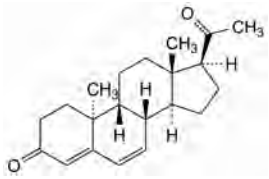

$C_{21}H_{28}O_2$ $M_r$ 312.5
[152-62-5]

DEFINITION

9β,10α-Pregna-4,6-diene-3,20-dione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *dydrogesterone CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): – 469 to – 485 (dried substance), measured at 25 °C.

Dissolve 0.100 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution (b).* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3.0 mg of *dydrogesterone impurity A CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10 mg of the substance to be examined in 10 mL of reference solution (a).

*Reference solution (d).* Dissolve 10 mg of the substance to be examined in 30 mL of *ethanol (96 per cent) R*. Add 1 mL of a 8.4 g/L solution of *sodium hydroxide R* and heat at 85 °C for 10 min. Cool to room temperature, add 1 mL of a 20.6 g/L solution of *hydrochloric acid R*, add 20 mL of *acetonitrile R*, 2 mg of *dydrogesterone impurity B CRS*, dilute to 100 mL with *water R* and mix. This solution contains dydrogesterone and impurities B and C.

*Reference solution (e).* Dissolve 20.0 mg of *dydrogesterone CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *ethanol (96 per cent) R*, *water R* (21:25:54 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm and at 385 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b), (c) and (d).

*Run time*: twice the retention time of dydrogesterone.

*Relative retention at 385 nm* with reference to dydrogesterone (retention time = about 13 min): impurity A = about 0.9.

*Relative retention at 280 nm* with reference to dydrogesterone (retention time = about 13 min): impurity B = about 1.1; impurity C = about 1.2.

*System suitability*:

- *resolution at 385 nm*: minimum 1.1 between the peaks due to impurity A and dydrogesterone in the chromatogram obtained with reference solution (c);
- *resolution at 280 nm*: minimum 4.5 between the peaks due to dydrogesterone and impurity B and minimum 1.5 between the peaks due to impurity B and impurity C in the chromatogram obtained with reference solution (d).

*Limits*:

- *impurity A at 385 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity B at 280 nm*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *impurity C at 280 nm*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities at 280 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total at 280 nm*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit at 280 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 280 nm.

*Injection*: test solution (b) and reference solution (e).

Calculate the percentage content of $C_{21}H_{28}O_2$ from the declared content of *dydrogesterone CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

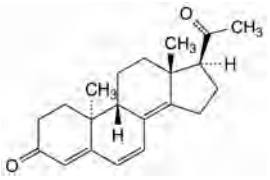

A. 9β,10α-pregna-4,6,8(14)-triene-3,20-dione,

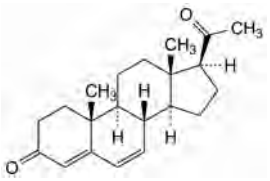

B. pregna-4,6-diene-3,20-dione,

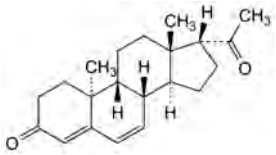

C. 9β,10α,17α-pregna-4,6-diene-3,20-dione.

# E

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Edetic acid.... 2492
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Ethylenediamine.... 2587
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Etidronate disodium.... 2589
Etilefrine hydrochloride.... 2589
Etodolac.... 2591
Etofenamate.... 2593
Etomidate.... 2595
Etoposide.... 2596
Eugenol.... 2599
Evening primrose oil, refined.... 2601
Everolimus.... 2601
Exemestane.... 2604

**01/2019:2015**

# EBASTINE

## Ebastinum

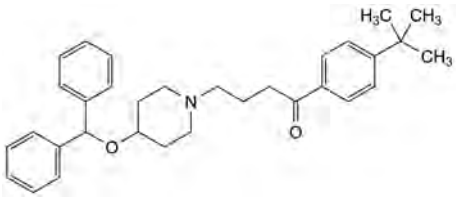

$C_{32}H_{39}NO_2$ $M_r$ 469.7
[90729-43-4]

DEFINITION

1-(4-*tert*-Butylphenyl)-4-[4-(diphenylmethoxy)piperidin-1-yl]butan-1-one.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, sparingly soluble in methanol.

mp: about 86 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ebastine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dilute 12 mL of *phosphoric acid R* and 12 mL of *diethylamine R* in 800 mL of *water for chromatography R*, adjust to pH 6.0 with *diethylamine R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *bis(diphenylmethyl) ether R* (impurity H) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 0.5 mL of the solution to 5.0 mL with the test solution. Dilute 3.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: solution A, *methanol R2*, *acetonitrile R1* (20:38:42 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 15 °C.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of ebastine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity H.

*Relative retention* with reference to ebastine (retention time = about 27 min): impurity H = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 2 between the peaks due to impurity H and ebastine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of ebastine in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Suspend 2.5 g in 25 mL of *dilute nitric acid R*. Boil under a reflux condenser for 10 min. Cool and filter.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 46.97 mg of $C_{32}H_{39}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H*.

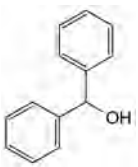

A. diphenylmethanol (benzhydrol),

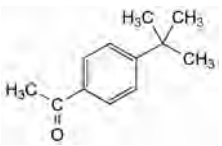

B. 1-(4-*tert*-butylphenyl)ethan-1-one,

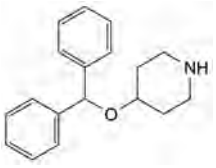

C. 4-(diphenylmethoxy)piperidine,

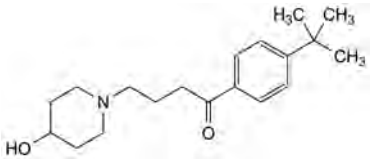

D. 1-(4-*tert*-butylphenyl)-4-(4-hydroxypiperidin-1-yl)butan-1-one,

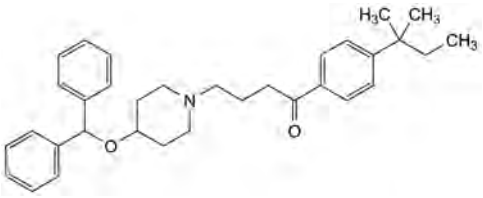

E. 4-[4-(diphenylmethoxy)piperidin-1-yl]-1-[4-(2-methylbutan-2-yl)phenyl]butan-1-one,

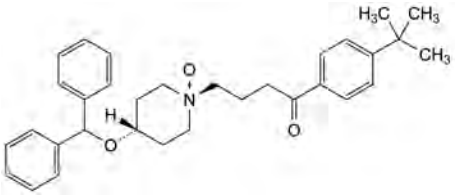

F. (1*s*,4*s*)-1-[4-(4-*tert*-butylphenyl)-4-oxobutyl]-4-(diphenylmethoxy)piperidine *N*-oxide,

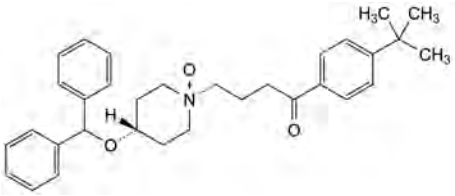

G. (1*r*,4*r*)-1-[4-(4-*tert*-butylphenyl)-4-oxobutyl]-4-(diphenylmethoxy)piperidine *N*-oxide,

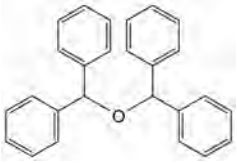

H. [oxybis(methanetriyl)]tetrakisbenzene (bis(diphenylmethyl) ether).

**07/2010:2049**
**corrected 7.0**

# ECONAZOLE

## Econazolum

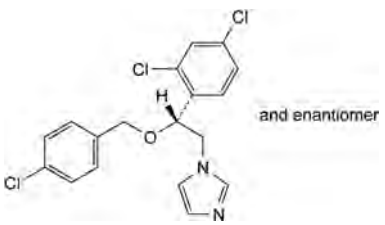


$C_{18}H_{15}Cl_3N_2O$ $M_r$ 381.7
[27220-47-9]

DEFINITION

1-[(2*RS*)-2-[(4-Chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)-ethyl]-1*H*-imidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Melting point (*2.2.14*): 88 °C to 92 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *econazole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *econazole for system suitability CRS* (containing impurities A, B and C) in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 25.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: *methanol R*, 0.77 g/L solution of *ammonium acetate R* (20:80 *V/V*);
- *mobile phase B*: *methanol R*, *acetonitrile R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 60 → 10 | 40 → 90 |
| 25 - 27 | 10 | 90 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *econazole for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to econazole (retention time = about 15 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 1.1.

*System suitability*: reference solution (a):
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to econazole.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
- *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 75 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 38.17 mg of $C_{18}H_{15}Cl_3N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

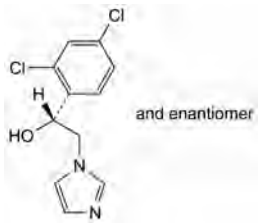


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

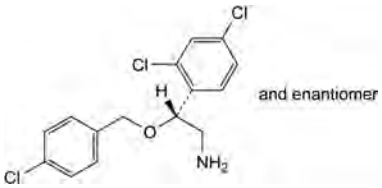


B. (2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)-ethanamine,

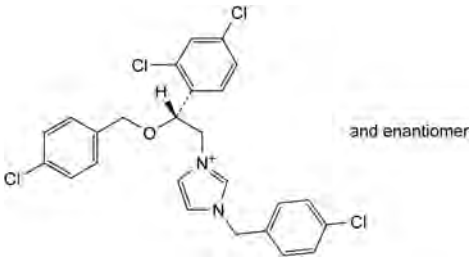


C. 1-(4-chlorobenzyl)-3-[(2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]imidazolium.

**07/2010:0665**
**corrected 7.0**

# ECONAZOLE NITRATE

## Econazoli nitras

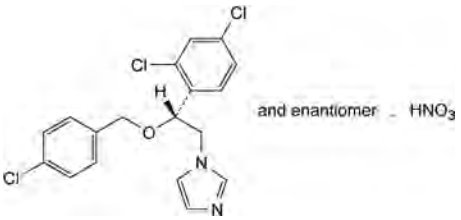


$C_{18}H_{16}Cl_3N_3O_4$ $M_r$ 444.7
[24169-02-6]

DEFINITION

1-[(2*RS*)-2-[(4-Chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)-ethyl]-1*H*-imidazole nitrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in methanol, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

mp: about 165 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *econazole nitrate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *econazole for system suitability CRS* (containing impurities A, B and C) in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 25.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 35 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R*, 0.77 g/L solution of *ammonium acetate R* (20:80 *V/V*);
– *mobile phase B*: *methanol R*, *acetonitrile R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 60 → 10 | 40 → 90 |
| 25 - 27 | 10 | 90 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *econazole for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to econazole (retention time = about 15 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 1.1.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to econazole.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
- *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to the nitrate ion at the beginning of the chromatogram.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 44.47 mg of $C_{18}H_{16}Cl_3N_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

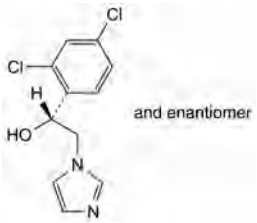


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

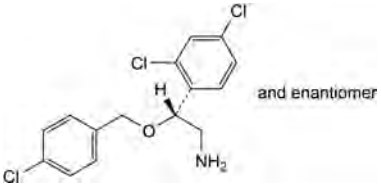


B. (2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)-ethanamine,

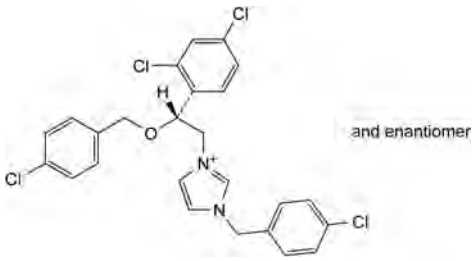


C. 1-(4-chlorobenzyl)-3-[(2*RS*)-2-[(4-chlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]imidazolium.

**01/2017:1612**

# EDETIC ACID

## Acidum edeticum

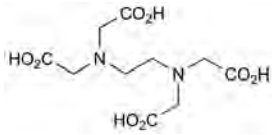


$C_{10}H_{16}N_2O_8$ $M_r$ 292.2
[60-00-4]

DEFINITION

(Ethylenedinitrilo)tetraacetic acid.

*Content*: 98.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs, after drying the substance to be examined in an oven at 100-105 °C for 2 h.

*Comparison*: *sodium edetate R*, treated as follows: dissolve 0.25 g of *sodium edetate R* in 5 mL of *water R*, add 1.0 mL of *dilute hydrochloric acid R*. Filter, wash the residue with 2 quantities, each of 5 mL, of *water R* and dry the residue in an oven at 100-105 °C for 2 h.

B. To 5 mL of *water R* add 0.1 mL of *ammonium thiocyanate solution R* and 0.1 mL of *ferric chloride solution R1* and mix. The solution is red. Add 0.5 mL of solution S (see Tests). The solution becomes yellowish.

C. To 10 mL of solution S add 0.5 mL of *calcium chloride solution R*. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 mL of *ammonium oxalate solution R*. No precipitate is formed.

TESTS

**Solution S**. Dissolve 5.0 g in 20 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture.* Dissolve 10.0 g of *ferric sulfate pentahydrate R* in 20 mL of *0.5 M sulfuric acid* and add 780 mL of *water R*.

Adjust to pH 2.0 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in 1.0 mL of *1 M sodium hydroxide* and dilute to 25.0 mL with the solvent mixture.

*Reference solution*. Dissolve 40.0 mg of *nitrilotriacetic acid R* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. To 1.0 mL of the solution add 0.1 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm,

– *stationary phase*: spherical *graphitised carbon for chromatography R1* (5 µm) with a specific surface area of 120 $m^2$/g and a pore size of 25 nm.

*Mobile phase*: dissolve 50.0 mg of *ferric sulfate pentahydrate R* in 50 mL of *0.5 M sulfuric acid* and add 750 mL of *water R*. Adjust to pH 1.5 with *0.5 M sulfuric acid* or *1 M sodium hydroxide*, add 20 mL of *ethylene glycol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL; filter the solutions and inject immediately.

*Run time*: 4 times the retention time of the iron complex of impurity A.

*Retention time*: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.

*System suitability*: reference solution:

– *resolution*: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid,

– *signal-to-noise ratio*: minimum 50 for the peak due to impurity A.

*Limit*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 10 mL of solution S add 8 mL of *nitric acid R* and stir for 10 min. A precipitate is formed. Filter and wash the filter with *water R*. Collect the filtrate and the washings and dilute to 20 mL with *water R*. Dilute 10 mL of this solution to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 80 ppm.

Dilute 2.5 mL of solution S to 10 mL with *water R* and add 0.25 g of *calcium chloride R* before adding the *thioglycollic acid R*. Allow to stand for 5 min. Also add 0.25 g of *calcium chloride R* to the standard.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 2.0 mL of *dilute sodium hydroxide solution R* and dilute to 300 mL with *water R*. Add 2 g of *hexamethylenetetramine R* and 2 mL of *dilute hydrochloric acid R*. Titrate with *0.1 M zinc sulfate* using about 50 mg of *xylenol orange triturate R* as indicator.

1 mL of *0.1 M zinc sulfate* corresponds to 29.22 mg of $C_{10}H_{16}N_2O_8$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

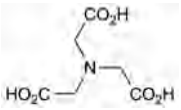

A. nitrilotriacetic acid.

**01/2008:2106**
**corrected 10.0**

# EDROPHONIUM CHLORIDE

## Edrophonii chloridum

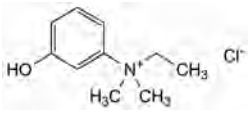

$C_{10}H_{16}ClNO$ $M_r$ 201.7
[116-38-1]

DEFINITION

*N*-Ethyl-3-hydroxy-*N*,*N*-dimethylanilinium chloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *edrophonium chloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 25 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10.0 mg of *3-dimethylaminophenol R* in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Mix 1.0 mL of the test solution and 1.0 mL of reference solution (a) and dilute to 100.0 mL with *water R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *styrene-divinylbenzene copolymer R* (8-10 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile R* and 90 volumes of a 7.7 g/L solution of *tetramethylammonium bromide R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 281 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of edrophonium.

*Relative retention* with reference to edrophonium (retention time = about 3.8 min): impurity A = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to edrophonium and impurity A.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *any other impurity*: for each impurity, not more than the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *total*: not more than 5 times the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.5 per cent),

– *disregard limit*: 0.5 times the area of the peak due to edrophonium in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 8.3 IU/mg.

ASSAY

Dissolve 0.150 g in 60 mL of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.17 mg of $C_{10}H_{16}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

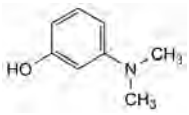


A. 3-(dimethylamino)phenol.

**07/2019:2315**

# EGG PHOSPHOLIPIDS FOR INJECTION

## Phospholipida ex ovo ad iniectabile

Egg lecithins: [93685-90-6]
Egg phosphatidylcholines: [97281-44-2]

DEFINITION

Mixture of natural phospholipids of egg yolk, and fractions thereof, for injection. A suitable stabiliser such as α-tocopherol may be added.

*Content*:

– *phosphatidylcholine*: minimum 60.0 per cent (anhydrous substance);

– *phosphatidylethanolamine*: maximum 20.0 per cent (anhydrous substance);

– *lysophosphatidylcholine*: maximum 3.0 per cent (anhydrous substance).

PRODUCTION

**Viral safety**. The requirements of general chapter *5.1.7* apply.

CHARACTERS

*Appearance*: almost white or yellow mass or powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and freely soluble in heptane.

IDENTIFICATION

*Carry out either test A or test B. If sphingomyelin is not detected with test A or B, carry out test C.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *phosphatidylethanolamine from egg yolk CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 15 mg of *lysophosphatidylcholine from egg yolk CRS* and 15 mg of *sphingomyelin from egg yolk R* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 15 mg of *phosphatidylcholine from egg yolk CRS* in *methanol R*, add 5 mL of reference solution (a) and 1 mL of reference solution (b) and dilute to 50.0 mL with *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *chloroform R* (4:25:65 *V/V/V*).

*Application*: 4 µL of the test solution and reference solution (c), as slim bands.

*Development*: over 2/3 of the plate.

*Detection*: spray with *copper sulfate solution R1* and heat at 170 °C for 5-15 min; the phospholipids appear as brownish-grey bands.

*Retardation factors*: lysophosphatidylcholine = about 0.15; sphingomyelin = about 0.2; phosphatidylcholine = about 0.3; phosphatidylethanolamine = about 0.6.

*System suitability*: reference solution (c):

– the chromatogram shows 4 clearly separated bands.

*Results*:

– the principal band in the chromatogram obtained with the test solution is similar in position and colour to the band due to phosphatidylcholine in the chromatogram obtained with reference solution (c);

– the band due to sphingomyelin is visible in the chromatogram obtained with the test solution.

B. Liquid chromatography (*2.2.29*) as described in the assay with the following modifications.

*Injection*: test solution and reference solution (h).

*Identification of peaks*: use the chromatogram obtained with reference solution (h) to identify the peak due to sphingomyelin.

*Relative retention* with reference to phosphatidylcholine (retention time = about 8 min): sphingomyelin = about 1.1 (may be eluted as 1 or 2 peaks).

*Results*:

– the principal peak in the chromatogram obtained with the test solution is due to phosphatidylcholine;

– the peak due to sphingomyelin in the chromatogram obtained with the test solution has a signal-to-noise ratio not less than 3.

C. Composition of fatty acids (*2.4.22, Method B*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the substance*:
- the oleic acid (C18:1) content is greater than that of linoleic acid (C18:2);
- arachidonic acid (C20:4) and docosahexaenoic acid (C22:6) are present.

TESTS

**Acid value** (*2.5.1*): maximum 20.

Dissolve 2.00 g in 50 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *ether R*, previously neutralised with *0.1 M potassium hydroxide* using 0.5 mL of *phenolphthalein solution R1* as indicator.

**Peroxide value** (*2.5.5, Method A*): maximum 3.

Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*).

**Nonphosphatidyl lipids**: maximum 7.0 per cent.

*Test solution.* Dissolve 0.500 g of the substance to be examined in *ether R* and dilute to 15.0 mL with the same solvent.

*Chromatography column*: *l* = 0.35-0.4 m, Ø = 10-20 mm.

*Preparation of the column.* Transfer 1 kg of silica gel with a particle size of 0.05-0.2 mm into a container fitted with a screw cap. Add 150 g of *water R*, shake thoroughly, and allow to stand for 24 h. Suspend 15 g of the prepared adsorbent in 50 mL of *ether R* and introduce into the column. Allow the *ether R* to pass through the column to a level of about 1 cm above the silica gel bed.

Transfer the test solution to the column. Rinse with 2 quantities, each of 15 mL, of *ether R*, allowing each to pass through the column before adding the next. After rinsing, elute with 105 mL of *ether R*. Combine the rinsings and eluate and evaporate to dryness. Remove any volatile components with a stream of nitrogen and dry the residue at 105 °C for 20 min.

Calculate the percentage content of nonphosphatidyl lipids using the following expression:

$$\frac{m_1}{m_2} \times 100$$

$m_1$ = mass of the residue, in milligrams;

$m_2$ = mass of the substance to be examined in the test solution, in milligrams.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500-1.00 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14, Method A*): less than 6 IU/g. The suitability of the method must be verified and a risk assessment must be carried out according to general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins* since the phospholipids can interfere with the test.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 8 volumes of *water R* and 46 volumes of *2-propanol R*, then add 46 volumes of *hexane R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*NOTE: if saturation of the peak due to phosphatidylcholine is observed, the test solution may be diluted with the solvent mixture to quantify this compound.*

*Reference solution ($a_1$).* Dissolve 60 mg of *phosphatidylcholine from egg yolk CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_1$), ($c_1$), ($d_1$), ($e_1$), ($f_1$).* Dilute reference solution ($a_1$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of phosphatidylcholine in the test solution.

*Reference solution ($a_2$).* Dissolve 40 mg of *phosphatidylethanolamine from egg yolk CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_2$), ($c_2$), ($d_2$), ($e_2$), ($f_2$).* Dilute reference solution ($a_2$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of phosphatidylethanolamine in the test solution.

*Reference solution ($a_3$).* Dissolve 40 mg of *lysophosphatidylcholine from egg yolk CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_3$), ($c_3$), ($d_3$), ($e_3$), ($f_3$).* Dilute reference solution ($a_3$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of lysophosphatidylcholine in the test solution.

*Reference solution (g).* Mix 5 mL of reference solution ($a_1$) and 5 mL of reference solution ($a_2$).

*Reference solution (h).* Dissolve 5 mg of *sphingomyelin from egg yolk R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *diol silica gel for chromatography R* (5 µm);
- *temperature*: 55 °C.

*Mobile phase*:
- *mobile phase A*: mix 0.08 volumes of *triethylamine R*, 1.5 volumes of *glacial acetic acid R*, 17 volumes of *2-propanol R* and 81.42 volumes of *hexane R*;
- *mobile phase B*: mix 0.08 volumes of *triethylamine R*, 1.5 volumes of *glacial acetic acid R*, 14 volumes of *water for chromatography R* and 84.42 volumes of *2-propanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.0 | 95 | 5 |
| 2.0 - 7.0 | 95 → 80 | 5 → 20 |
| 7.0 - 10.5 | 80 → 60 | 20 → 40 |
| 10.5 - 17.0 | 60 → 0 | 40 → 100 |
| 17.0 - 19.5 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria:
- *carrier gas*: *nitrogen R*;
- *flow rate*: 2.5 mL/min;
- *evaporator temperature*: 50 °C.

*Injection*: 20 µL of the test solution and reference solutions ($b_{1,2,3}$), ($c_{1,2,3}$), ($d_{1,2,3}$), ($e_{1,2,3}$), ($f_{1,2,3}$) and (g).

*Identification of peaks*: use the chromatograms obtained with reference solutions ($f_1$), ($f_2$) and ($f_3$) to identify the peaks due to phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine.

*Relative retention* with reference to phosphatidylcholine (retention time = about 8 min): phosphatidylethanolamine = about 0.8; lysophosphatidylcholine = about 1.2 (may be eluted as 1 or 2 peaks).

Establish calibration curves with the logarithm of the concentration of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine in

reference solutions ($b_{1,2,3}$), ($c_{1,2,3}$), ($d_{1,2,3}$), ($e_{1,2,3}$) and ($f_{1,2,3}$) as the abscissa and the logarithm of the corresponding peak areas as the ordinate.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to phosphatidylethanolamine and phosphatidylcholine in the chromatogram obtained with reference solution (g);
- *phosphatidylcholine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_1$);
  - *correlation coefficient*: minimum 0.995;
- *phosphatidylethanolamine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_2$);
  - *correlation coefficient*: minimum 0.995;
- *lysophosphatidylcholine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_3$);
  - *correlation coefficient*: minimum 0.99.

Calculate the percentage contents of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine using the calibration curves.

STORAGE

Under nitrogen, protected from light, at – 15 °C or below.

LABELLING

The label states:

- the specified content range of phosphatidylcholine;
- the specified content range of phosphatidylethanolamine.



**01/2008:2242**

# EMEDASTINE DIFUMARATE

## Emedastini difumaras

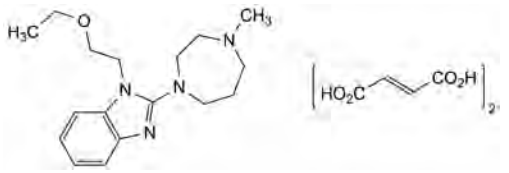

$C_{25}H_{34}N_4O_9$ $M_r$ 534.6

[87233-62-3]

DEFINITION

1-(2-Ethoxyethyl)-2-(4-methylhexahydro-1*H*-1,4-diazepin-1-yl)-1*H*-benzimidazole bis[hydrogen (2*E*)-butenedioate].

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish powder.

*Solubility*: soluble in water, sparingly soluble in anhydrous ethanol, very slightly soluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *emedastine difumarate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 2.50 g in *water R* and dilute to 50 mL with the same solvent.

**pH** (*2.2.3*): 3.0 to 4.5.

Dissolve 0.20 g in 100 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *emedastine impurity E CRS* in the mobile phase and dilute to 25 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined in the mobile phase. Add 0.5 mL of reference solution (a) and dilute to 10 mL with the mobile phase.

*Reference solution (c).* Dilute 5.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.9 g of *disodium hydrogen phosphate dodecahydrate R* and 2.5 g of *sodium dodecyl sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust to pH 2.4 with *phosphoric acid R*. Mix 550 volumes of this solution with 450 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of emedastine.

*Relative retention* with reference to emedastine (retention time = about 18 min): fumaric acid = about 0.1; impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.5; impurity D = about 0.7; impurity E = about 0.9; impurity F = about 1.4.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to emedastine.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peak due to fumaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.73 mg of $C_{25}H_{34}N_4O_9$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

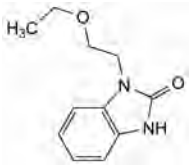

A. 1-(2-ethoxyethyl)-1,3-dihydro-2*H*-benzimidazol-2-one,

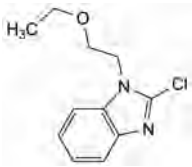

B. 2-chloro-1-(2-ethoxyethyl)-1*H*-benzimidazole,

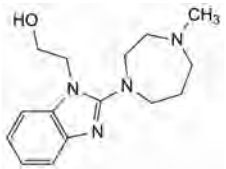

C. 2-[2-(4-methylhexahydro-1*H*-1,4-diazepin-1-yl)-1*H*-benzimidazol-1-yl]ethanol,

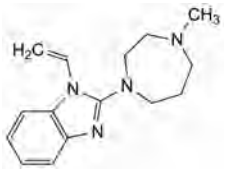

D. 1-ethenyl-2-(4-methylhexahydro-1*H*-1,4-diazepin-1-yl)-1*H*-benzimidazole,

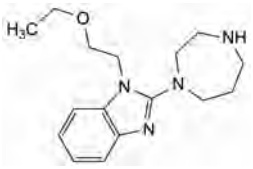

E. 1-(2-ethoxyethyl)-2-(hexahydro-1*H*-1,4-diazepin-1-yl)-1*H*-benzimidazole,

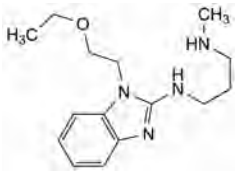

F. *N*-[1-(2-ethoxyethyl)-1*H*-benzimidazol-2-yl]-*N'*-methylpropane-1,3-diamine.

**01/2017:1420**
**corrected 10.0**

# ENALAPRIL MALEATE

## Enalaprili maleas

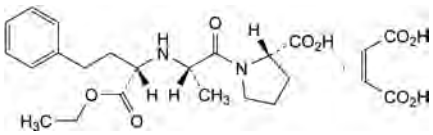

$C_{24}H_{32}N_2O_9$ $M_r$ 492.5
[76095-16-4]

DEFINITION

(2*S*)-1-[(2*S*)-2-[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]-amino]propanoyl]pyrrolidine-2-carboxylic acid hydrogen (*Z*)-butenedioate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *enalapril maleate CRS*.

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.4 to 2.9 for solution S.

**Specific optical rotation** (*2.2.7*): − 51 to − 48 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution A*. Dissolve 2.8 g of *sodium dihydrogen phosphate monohydrate R* in 950 mL of *water for chromatography R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Buffer solution B*. Dissolve 2.8 g of *sodium dihydrogen phosphate monohydrate R* in 950 mL of *water for chromatography R*, adjust to pH 6.8 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Solvent mixture*: *acetonitrile R1*, buffer solution A (5:95 *V/V*).

*Test solution (a)*. Dissolve 30 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 3 mg of *enalapril for system suitability CRS* (containing impurity A) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *enalapril impurity mixture A CRS* (impurities C and H) in 1.0 mL of the solvent mixture.

*Reference solution (d).* Dissolve 2.0 mg of *enalapril impurity G CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.1 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm);
- *temperature*: 70 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution B (5:95 *V/V*);
- *mobile phase B*: buffer solution B, *acetonitrile R1* (34:66 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 95 | 5 |
| 3 - 23 | 95 → 40 | 5 → 60 |
| 23 - 30 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 50 µL of test solution (a) and reference solutions (a), (b) and (c); for impurity G, test solution (b) and reference solution (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram supplied with *enalapril impurity mixture A CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and H; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to enalapril (retention time = about 13 min): maleic acid = about 0.1; impurity C = about 0.2; impurity A = about 1.1; impurity G = about 1.2; impurity H = about 1.3.

*System suitability*:

- *signal-to-noise ratio*: minimum 40 for the peak due to enalapril in the chromatogram obtained with reference solution (a); minimum 40 for the peak due to impurity G in the chromatogram obtained with reference solution (d);
- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to enalapril in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity H by 2.0;
- for impurity G, use the concentration of impurity G in reference solution (d);
- for impurities other than G, use the concentration of enalapril maleate in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *impurity H*: maximum 0.3 per cent;
- *impurities C, G*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Titrate to the 2nd point of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 16.42 mg of $C_{24}H_{32}N_2O_9$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, I.*

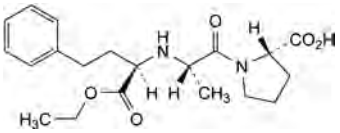

A. (2*S*)-1-[(2*S*)-2-[[(1*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

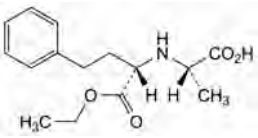

B. (2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoic acid,

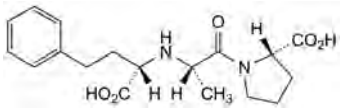

C. (2*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

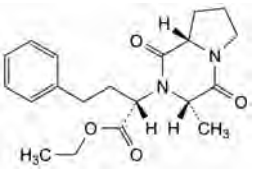

D. ethyl (2*S*)-2-[(3*S*,8a*S*)-3-methyl-1,4-dioxohexahydropyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoate,

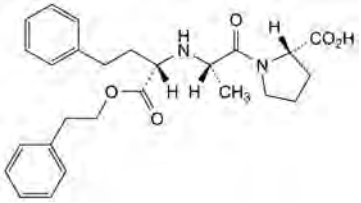

E. (2*S*)-1-[(2*S*)-2-[[(1*S*)-3-phenyl-1-[(2-phenylethoxy)carbonyl]propyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

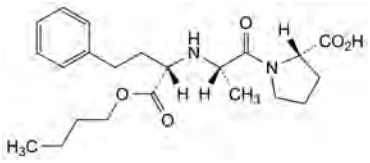

F. (2*S*)-1-[(2*S*)-2-[[(1*S*)-1-(butoxycarbonyl)-3-phenylpropyl]-amino]propanoyl]pyrrolidine-2-carboxylic acid,

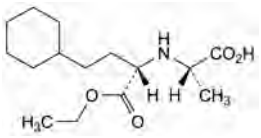

G. (2*S*)-2-[[(1*S*)-3-cyclohexyl-1-(ethoxycarbonyl)propyl]-amino]propanoic acid,

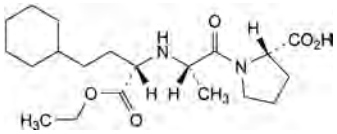

H. (2*S*)-1-[(2*S*)-2-[[(1*S*)-3-cyclohexyl-1-(ethoxycarbonyl)-propyl]amino]propanoyl]pyrrolidine-2-carboxylic acid,

I. 1*H*-imidazole.

**01/2017:1749**

# ENALAPRILAT DIHYDRATE

## Enalaprilatum dihydricum

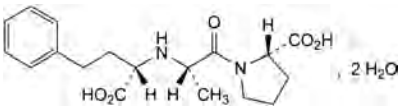

$C_{18}H_{24}N_2O_5,2H_2O$ $M_r$ 384.4
[84680-54-6]

DEFINITION

(2*S*)-1-[(2*S*)-2-[[(1*S*)-1-Carboxy-3-phenylpropyl]amino]-propanoyl)pyrrolidine-2-carboxylic acid dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: very slightly soluble or slightly soluble in water, sparingly soluble in methanol, practically insoluble in acetonitrile.

It shows pseudopolymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: mulls in *liquid paraffin R*.

*Comparison*: *enalaprilat dihydrate CRS*.

If the spectra obtained show differences, expose the substance to be examined and the reference substance to a 98 per cent relative humidity for 3 days using a chamber conditioned with a saturated solution of *calcium sulfate R*. Record new spectra.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.10 g in *water R* and dilute to 100.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 53.0 to – 56.0 (anhydrous substance).

Dissolve 0.200 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Buffer solution.* Dissolve 1.36 g of *potassium dihydrogen phosphate R* in 950 mL of *water R*. Adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture.* Buffer solution, *acetonitrile R1*, *methanol R1* (1:2:2 *V/V/V*).

*Dissolution mixture.* Solvent mixture, buffer solution (8:92 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 2.5 mL of *methanol R1* and dilute to 25.0 mL with the dissolution mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the dissolution mixture. Dilute 5.0 mL of this solution to 10.0 mL with the dissolution mixture.

*Reference solution (b).* Dissolve 5 mg of *enalaprilat for system suitability CRS* (containing impurity C) in 0.5 mL of *methanol R1* and dilute to 5 mL with the dissolution mixture.

*Reference solution (c).* Dissolve the contents of a vial of *enalaprilat impurity G CRS* in 1 mL of the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 70 °C.

*Mobile phase*:

– *mobile phase A*: solvent mixture, buffer solution (10:90 *V/V*);
– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 50 | 100 → 90 | 0 → 10 |
| 50 - 80 | 90 | 10 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *enalaprilat for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity G.

*Relative retention* with reference to enalaprilat (retention time = about 21 min): impurity C = about 1.2; impurity G = about 2.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to enalaprilat.

*Limits*:

– *impurities C, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 7.0 per cent to 11.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.1 IU/mg.

ASSAY

Dissolve 0.300 g in *glacial acetic acid R* and dilute to 50 mL with the same solvent. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.84 mg of $C_{18}H_{24}N_2O_5$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: C, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F.*

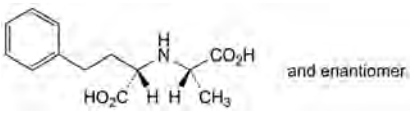


A. (2*SR*)-2-[[(1*SR*)-1-carboxyethyl]amino]-4-phenylbutanoic acid,

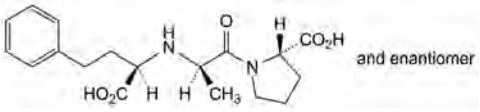


B. (2*SR*)-1-[(2*RS*)-2-[[(1*RS*)-1-carboxy-3-phenylpropyl]-amino]propanoyl]pyrrolidine-2-carboxylic acid,

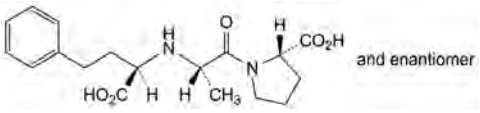


C. (2*SR*)-1-[(2*SR*)-2-[[(1*RS*)-1-carboxy-3-phenylpropyl]-amino]propanoyl]pyrrolidine-2-carboxylic acid,

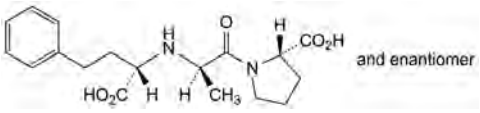


D. (2*SR*)-1-[(2*RS*)-2-[[(1*SR*)-1-carboxy-3-phenylpropyl]-amino]propanoyl]pyrrolidine-2-carboxylic acid,

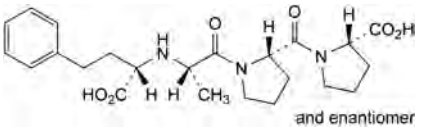


E. (2*SR*)-1-[[(2*SR*)-1-[(2*SR*)-2-[[(1*SR*)-1-carboxy-3-phenylpropyl]amino]propanoyl]pyrrolidin-2-yl]carbonyl]pyrrolidine-2-carboxylic acid,

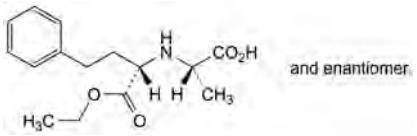


F. (2*SR*)-2-[[(1*SR*)-1-(ethoxycarbonyl)-3-phenylpropyl]-amino]propanoic acid,

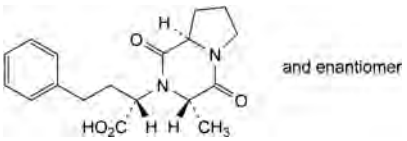


G. (2*SR*)-2-[(3*SR*,8a*RS*)-3-methyl-1,4-dioxohexahydro-pyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoic acid.

**07/2010:1720**
**corrected 10.0**

# ENILCONAZOLE FOR VETERINARY USE

## Enilconazolum ad usum veterinarium

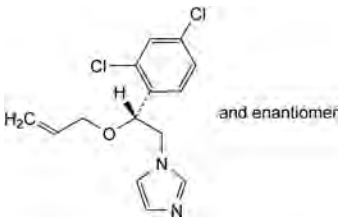


$C_{14}H_{14}Cl_2N_2O$ $M_r$ 297.2
[35554-44-0]

DEFINITION

1-[(2*RS*)-2-(2,4-Dichlorophenyl)-2-(prop-2-enyloxy)ethyl]-1*H*-imidazole.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: clear, yellowish, oily liquid or solid mass.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent), in methanol and in toluene.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *enilconazole CRS*.

TESTS

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 0.1 g in *methanol R* and dilute to 10 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use and protect from light.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *toluene R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *enilconazole CRS* and 10 mg of *enilconazole impurity E CRS* in *toluene R* and dilute to 100 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *toluene R*. Dilute 1.0 mL of this solution to 10.0 mL with *toluene R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.52 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:38.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6.4 | 100 → 260 |
| | 6.4 - 14 | 260 |
| Injection port | | 250 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E.

*Relative retention* with reference to enilconazole (retention time = about 10 min): impurity A = about 0.6; impurity B = about 0.7; impurity C = about 0.8; impurity D = about 0.9; impurity E = about 1.03; impurity F = about 1.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to enilconazole and impurity E.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent), and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 40 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.230 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid* using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 29.72 mg of $C_{14}H_{14}Cl_2N_2O$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

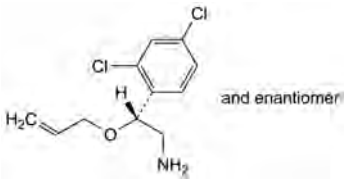


A. (2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-enyloxy)-ethanamine,

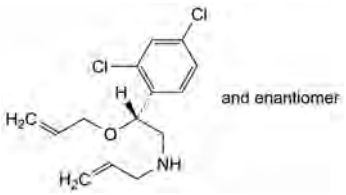


B. *N*-[(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-enyloxy)-ethyl]prop-2-en-1-amine,

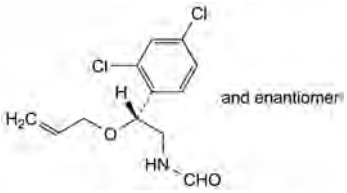


C. *N*-[(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-enyloxy)-ethyl]formamide,

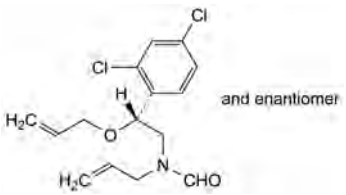


D. *N*-[(2*RS*)-2-(2,4-dichlorophenyl)-2-(prop-2-enyloxy)-ethyl]-*N*-(prop-2-enyl)formamide,

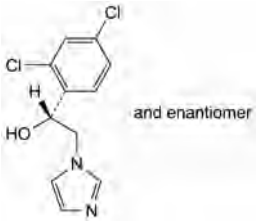


E. (1*RS*)-1-(2,4-dichlorophenyl)-2-(-1*H*-imidazol-1-yl)ethanol,

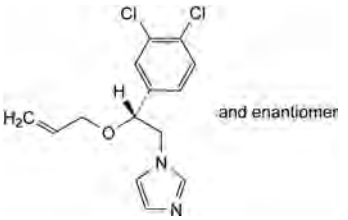


F. 1-[(2*RS*)-2-(3,4-dichlorophenyl)-2-(prop-2-enyloxy)ethyl]-1*H*-imidazole.

**04/2014:1097**

# ENOXAPARIN SODIUM

## Enoxaparinum natricum

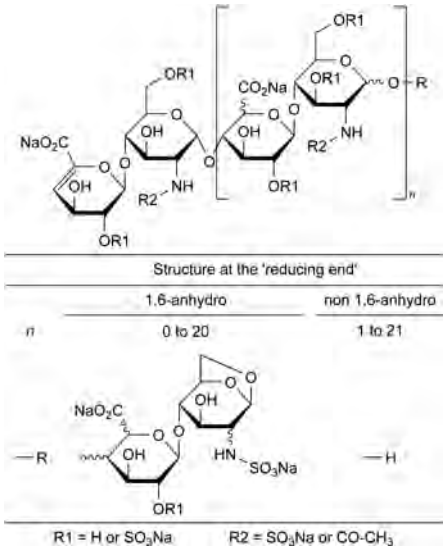


DEFINITION

Enoxaparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by alkaline depolymerisation of the benzyl ester derivative of heparin from porcine intestinal mucosa. Enoxaparin consists of a complex set of oligosaccharides that have not yet been completely characterised. Based on current knowledge, the majority of the components have a 4-enopyranose uronate structure at the non-reducing end of their chain. 15 per cent to 25 per cent of the components have a 1,6-anhydro structure at the reducing end of their chain.

*Enoxaparin sodium complies with the monograph Low-molecular-mass heparins (0828) with the modifications and additional requirements below.*

The mass-average relative molecular mass ranges between 3800 and 5000, with a characteristic value of about 4500.

The degree of sulfatation is about 2 per disaccharide unit.

The potency is not less than 90 IU and not more than 125 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The anti-factor IIa activity is not less than 20.0 IU and not more than 35.0 IU per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 3.3 and 5.3.

PRODUCTION

Enoxaparin is produced by alkaline depolymerisation of benzyl ester derivatives of heparin from porcine intestinal mucosa under conditions that yield a product complying with the structural requirements stated under Definition.

IDENTIFICATION

A. Carry out identification test A as described in the monograph *Low-molecular-mass heparins (0828)* using *enoxaparin sodium CRS*.

B. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solution A*. Dissolve 12 mg of *sodium tetrahydroborate R* in 400 µL of *water R* and mix using a vortex mixer.

*Heparinase solution (a).* Dissolve *heparinase I R* in *potassium phosphate buffer solution pH 7.0 R* to obtain an activity of 0.4 IU/mL. Store the solution at – 20 °C until use.

*Heparinase solution (b).* Dissolve *heparinase II R* in *potassium phosphate buffer solution pH 7.0 R* to obtain an activity of 0.4 IU/mL. Store the solution at – 20 °C until use.

*Heparinase solution (c).* Dissolve *heparinase III R* in *potassium phosphate buffer solution pH 7.0 R* to obtain an activity of 0.4 IU/mL. Store the solution at – 20 °C until use.

*Heparinase solution (d).* Mix equal volumes of heparinase solution (a), heparinase solution (b) and heparinase solution (c).

*Blank solution.* Gently mix by inversion 20 µL of *water R*, 70 µL of *sodium/calcium acetate buffer solution pH 7.0 R* and 100 µL of heparinase solution (d). Place in a water-bath at 25 °C for 48 h. Mix 60 µL of this solution and 10 µL of freshly prepared solution A. Mix and allow to stand at room temperature for 4 h.

*Test solution (a).* Dissolve 20 mg of the substance to be examined in 1 mL of *water R*.

*Test solution (b).* To 20 µL of test solution (a), add 70 µL of *sodium/calcium acetate buffer solution pH 7.0 R* and 100 µL of heparinase solution (d). Gently mix by inversion and place in a water-bath at 25 °C for 48 h.

*Test solution (c).* To 60 µL of test solution (b), add 10 µL of freshly prepared solution A. Mix and allow to stand at room temperature for 4 h.

*Reference solution (a).* Dissolve 20 mg of *enoxaparin sodium CRS* in 1 mL of *water R*.

*Reference solution (b).* To 20 µL of reference solution (a), add 70 µL of *sodium/calcium acetate buffer solution pH 7.0 R* and 100 µL of heparinase solution (d). Gently mix by inversion and place in a water-bath at 25 °C for 48 h.

*Reference solution (c).* To 60 µL of reference solution (b), add 10 µL of freshly prepared solution A. Mix and allow to stand at room temperature for 4 h.

*NOTE: heparinase solutions (a), (b) and (c) can be stored for 3 months at – 20 °C. Test solutions (a) and (b) and reference solutions (a) and (b) must be prepared at the same time; depolymerised test solutions are stable for 1 month at – 20 °C. Test solution (c) and reference solution (c) must also be prepared at the same time.*

*Precolumn*:

- *size*: *l* = 0.01 m, Ø = 4.6 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.280 g of *sodium dihydrogen phosphate R* in 950 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: dissolve 140 g of *sodium perchlorate R* in 950 mL of mobile phase A, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with mobile phase A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 97 → 65 | 3 → 35 |
| 20 - 50 | 65 → 0 | 35 → 100 |
| 50 - 60 | 0 | 100 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 234 nm.

*Injection*: 18 µL of the blank solution, test solution (c) and reference solutions (b) and (c).

*Identification of disaccharides*: use the chromatogram supplied with *enoxaparin sodium CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to the disaccharides listed in Table 1097.-1; use the chromatogram obtained with reference solution (b) to confirm the identity of the peaks due to the 1,6-anhydro derivatives.

Table 1097.-1. – *Correlation between the relative retention of peaks observed in chromatograms obtained with solutions of depolymerised and reduced enoxaparin, with reference to reduced ΔIS (retention time = about 30 min), and molecular masses of the enoxaparin derivatives*

| Derivatives | Relative retention | Molecular mass (Da) |
|---|---|---|
| Unidentified | < 0.20 | 741 |
| Reduced ΔIVA | 0.20 | 401 |
| Unidentified | 0.20 - 0.46 | 741 |
| Reduced ΔIVS | 0.46 | 461 |
| Unidentified | 0.46 - 0.48 | 483 |
| Reduced ΔIIA | 0.48 | 503 |
| Unidentified | 0.48 - 0.52 | 503 |
| 1,6-anhydro ΔIIS | 0.52 | 443 |
| Unidentified | 0.52 - 0.57 | 503 |
| Reduced ΔIIIA | 0.57 | 503 |
| Unidentified | 0.57 - 0.66 | 533 |
| Reduced ΔIIS | 0.66 | 563 |
| Unidentified | 0.66 - 0.76 | 563 |
| Reduced ΔIIIS | 0.76 | 563 |
| Unidentified | 0.76 - 0.85 | 583 |
| Reduced ΔIA | 0.85 | 605 |
| 1,6-anhydro ΔIS | 0.88 | 545 |
| Unidentified | 0.88 - 0.97 | 635 |
| Reduced ΔIIA-IVSglu | 0.97 | 1066 |
| Reduced ΔIS | 1.00 | 665 |
| ΔIS | 1.04 | 665 |
| Unidentified | 1.04 - 1.10 | 1228 |
| Reduced ΔIIA-IISglu | 1.10 | 1168 |
| Unidentified | 1.10 - 1.28 | 1228 |
| 1,6-anhydro ΔIS-IS | 1.28 | 1210 |
| Unidentified | > 1.28 | 1228 |

*NOTE: depending on the resolution of the column, 1,6-anhydro ΔIIS may be eluted in the form of 2 peaks (mannosamine and glucosamine forms), which are both taken into account as 1,6-anhydro ΔIIS.*

*Relative retention* with reference to reduced ΔIS (retention time = about 30 min): see Table 1097.-1.

*System suitability*:

- *peak area ratio*: maximum 1.15 for the peaks due to 1,6-anhydro ΔIS-IS and 1,6-anhydro ΔIS in the chromatogram obtained with reference solution (b); maximum 0.02 for the peaks due to ΔIS and reduced ΔIS in the chromatogram obtained with reference solution (c);
- *resolution*: minimum 1.5 between the peaks due to reduced ΔIA and 1,6-anhydro ΔIS in the chromatogram obtained with reference solution (c);
- the content of 1,6-anhydro derivatives in *enoxaparin sodium CRS* is within 1.5 per cent of the assigned content.

*Calculation*:

Calculate the molar percentage of the 3 main 1,6-anhydro derivatives using the relative molecular masses given in Table 1097.-1 and the following expression:

$$100 \times \frac{Mw}{\sum Mw_x \cdot A_x} \times (A_1 + A_2 + A_3)$$

$Mw$ = mass-average relative molecular mass of enoxaparin (as determined by identification test C);

$Mw_x$ = relative molecular mass attributed to derivative $x$ according to Table 1097.-1;

$A_x$ = area of the peak due to derivative $x$;

$A_1$ = area of the peak due to 1,6-anhydro ΔIS;

$A_2$ = area of the peak due to 1,6-anhydro ΔIIS;

$A_3$ = area of the peak due to 1,6-anhydro ΔIS-IS.

Disregard any peak observed with the blank solution.

Correct the value to the nearest unit.

*Limit*: 15 per cent to 25 per cent of components bearing the 1,6-anhydro structure at the reducing end of their chain.

C. Carry out identification test C as described in the monograph *Low-molecular-mass heparins (0828)*. The following requirements apply.

The mass-average relative molecular mass ranges between 3800 and 5000. The mass percentage of chains lower than 2000 ranges between 12.0 per cent and 20.0 per cent. The mass percentage of chains between 2000 and 8000 ranges between 68.0 per cent and 82.0 per cent.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 1.0 g in 10 mL of *water R*.

**pH** (*2.2.3*): 6.2 to 7.7.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Specific absorbance** (*2.2.25*): 14.0 to 20.0 (dried substance), determined at 231 nm.

Dissolve 50.0 mg in 100 mL of *0.01 M hydrochloric acid*.

**Benzyl alcohol**. Liquid chromatography (*2.2.29*).

*Internal standard solution*: 1 g/L solution of *3,4-dimethylphenol R* in *methanol R*.

*Test solution*. Dissolve about 0.500 g of the substance to be examined in 5.0 mL of *1 M sodium hydroxide*. Allow to stand for 1 h. Add 1.0 mL of *glacial acetic acid R* and 1.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Reference solution*. Prepare a 0.25 g/L solution of *benzyl alcohol R* in *water R*. Mix 0.50 mL of this solution with 1.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

Monographs E

*Precolumn*:

– *size*: *l* = 0.02 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *acetonitrile R*, *water R* (5:15:80 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 256 nm.

*Injection*: 20 µL.

From the chromatogram obtained with the reference solution, calculate the ratio ($R_1$) of the height of the peak due to benzyl alcohol to the height of the peak due to the internal standard. From the chromatogram obtained with the test solution, calculate the ratio ($R_2$) of the height of the peak due to benzyl alcohol to the height of the peak due to the internal standard.

Calculate the percentage content *m/m* of benzyl alcohol using the following expression:

$$\frac{0.0125 \times R_2}{m \times R_1}$$

*m* = mass of the substance to be examined, in grams.

*Limit*:

– *benzyl alcohol*: maximum 0.1 per cent *m/m*.

**Sodium** (*2.2.23, Method I*): 11.3 per cent to 13.5 per cent (dried substance).

**01/2017:1511**

# ENOXOLONE

## Enoxolonum

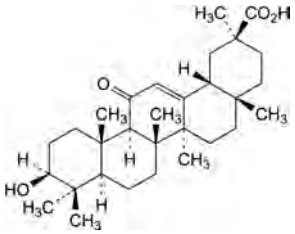

$C_{30}H_{46}O_4$ $M_r$ 470.7

[471-53-4]

DEFINITION

(20β)-3β-Hydroxy-11-oxo-olean-12-en-29-oic acid.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder.

*Solubility*: practically insoluble in water, soluble in ethanol, sparingly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B, C*.

A. Examine by infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *enoxolone CRS*.

If the spectra obtained in the solid state show differences, dissolve 0.2 g of the substance to be examined and 0.2 g of the reference substance separately in 6 mL of *ethanol R*. Boil under a reflux condenser for 1 h and add 6 mL of *water R*. A precipitate is formed. Cool to about 10 °C and filter with the aid of vacuum. Wash the precipitate with 10 mL of *alcohol R*, dry in an oven at 80 °C and record new spectra.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *enoxolone CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *methylene chloride R* (5:10:90 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 5 min.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in 10 mL of *methylene chloride R*. To 2 mL of this solution, add 1 mL of *acetic anhydride R* and 0.3 mL of *sulfuric acid R*. A pink colour is produced.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.1 g in *ethanol R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 145 to + 154 (dried substance).

Dissolve 0.50 g in *dioxan R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in the mobile phaseand dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 0.1 g of *18α-glycyrrhetinic acid R* in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent. To 2.0 mL of the solution, add 2.0 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),

– *temperature*: 30 °C.

*Mobile phase*: mix 430 volumes of *tetrahydrofuran R* and 570 volumes of a 1.36 g/L solution of *sodium acetate R* adjusted to pH 4.8 with *glacial acetic acid R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 20 µL loop injector; inject the test solution and the reference solutions.

*Run time*: 4 times the retention time of enoxolone.

*System suitability*:

– *resolution*: minimum of 2.0 between the peaks due to enoxolone and to 18α-glycyrrhetinic acid in the chromatogram obtained with reference solution (c).

*Limits*:

– *any impurity*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent),

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.330 g in 40 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 47.07 mg of $C_{30}H_{46}O_4$.

STORAGE

Protected from light.

IMPURITIES

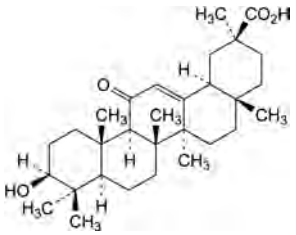

A. (20β)-3β-hydroxy-11-oxo-18α-olean-12-en-29-oic acid,

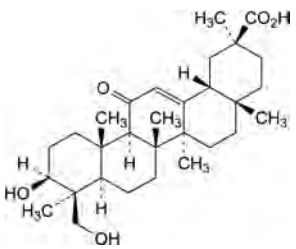

B. (4β,20β)-3β,23-dihydroxy-11-oxo-olean-12-en-29-oic acid.

**04/2010:2229**
**corrected 10.0**

# ENROFLOXACIN FOR VETERINARY USE

## Enrofloxacinum ad usum veterinarium

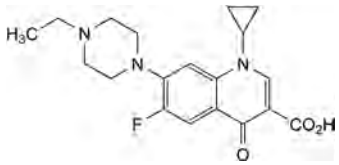

$C_{19}H_{22}FN_3O_3$ $M_r$ 359.4
[93106-60-6]

DEFINITION

1-Cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellowish or light yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *enrofloxacin CRS*.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

To 1.0 g of the substance to be examined add about 0.25 g of *potassium hydroxide R* and 7 mL of *water R*. Sonicate to dissolve and dilute to 10.0 mL with *water R*.

**Impurity A**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *methanol R*, *methylene chloride R* (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution*. Dissolve 5.0 mg of *ciprofloxacin impurity A CRS* (enrofloxacin impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 4.0 mL of the solution to 10.0 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 μm).

*Mobile phase*: *butanol R*, *water R*, *anhydrous acetic acid R*, *ethyl acetate R* (15:15:20:50 *V/V/V/V*).

*Application*: 10 μL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10 mg of *enrofloxacin for system suitability CRS* (containing impurities B and C) and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 15 volumes of *methanol R* and 85 volumes of a 2.9 g/L solution of *phosphoric acid R*, previously adjusted to pH 2.3 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of enrofloxacin.

*Identification of impurities*: use the chromatogram supplied with *enrofloxacin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to enrofloxacin (retention time = about 16 min): impurity C = about 0.6; impurity B = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity B and enrofloxacin.

*Limits*:

- *impurity B*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.20 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

Dissolve 1.5 g in a mixture of 5 mL of *dilute acetic acid R* and 10 mL of *water R*. Filter. 12 mL of the filtrate after adding 2 mL of *water R* (instead of buffer solution) complies with test E. Prepare the reference solution using 12 mL of *lead standard solution (2 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 2.000 g by drying *in vacuo* at 120 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 100 mL of *anhydrous acetic acid R* and titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.94 mg of $C_{19}H_{22}FN_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G.*

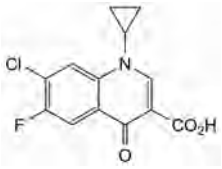


A. 7-chloro-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

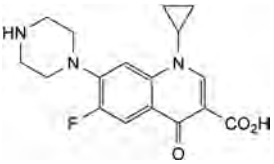


B. ciprofloxacin,

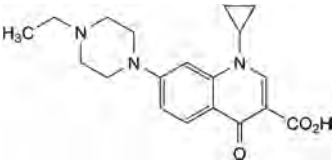


C. 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

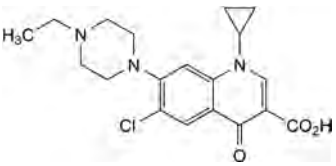


E. 6-chloro-1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

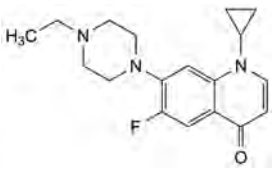


F. 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoroquinolin-4(1*H*)-one,

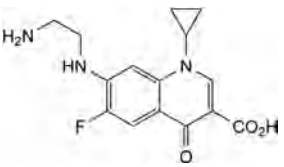


G. 7-[(2-aminoethyl)amino]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.

**01/2017:2574**

# ENTACAPONE

## Entacaponum

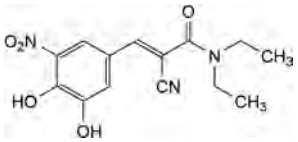

$C_{14}H_{15}N_3O_5$ $M_r$ 305.3
[130929-57-6]

DEFINITION

(2*E*)-2-Cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: greenish-yellow or yellow powder.

*Solubility*: practically insoluble in water, soluble or sparingly soluble in acetone, slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *entacapone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Solvent mixture*: *tetrahydrofuran R*, *methanol R* (30:70 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *entacapone impurity A CRS* in the solvent mixture, add 5.0 mL of test solution (a) and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 50.0 mg of *entacapone CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped propyl-2-phenylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*: mix 2 volumes of *tetrahydrofuran R*, 44 volumes of *methanol R* and 54 volumes of a 2.34 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 2.1 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of entacapone.

*Relative retention* with reference to entacapone (retention time = about 17 min): impurity A = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity A and entacapone.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{14}H_{15}N_3O_5$ from the declared content of *entacapone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I.*

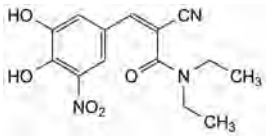

A. (2*Z*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide,

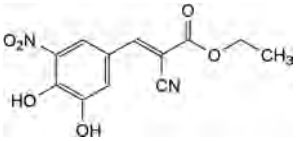

B. ethyl (2*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)prop-2-enoate,

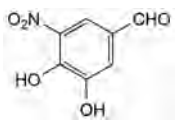

C. 3,4-dihydroxy-5-nitrobenzaldehyde,

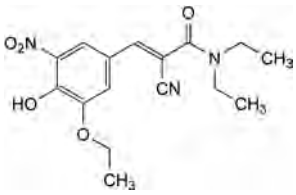

D. (2*E*)-2-cyano-3-(3-ethoxy-4-hydroxy-5-nitrophenyl)-*N*,*N*-diethylprop-2-enamide,

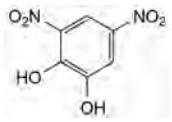

E. 3,5-dinitrobenzene-1,2-diol,

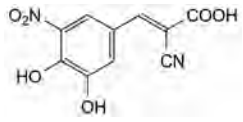

F. (2*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)prop-2-enoic acid,

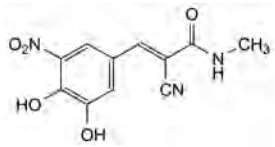

G. (2*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-*N*-methylprop-2-enamide,

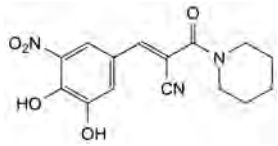

H. (2*E*)-3-(3,4-dihydroxy-5-nitrophenyl)-2-(piperidin-1-ylcarbonyl)prop-2-ennitrile,

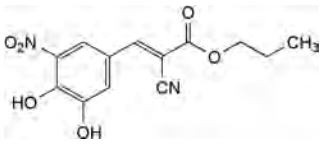

I. propyl (2*E*)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)prop-2-enoate.

**04/2019:2815**

# ENTECAVIR MONOHYDRATE

## Entecavirum monohydricum

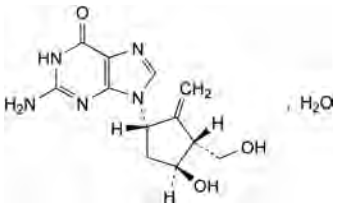

$C_{12}H_{15}N_5O_3,H_2O$ $M_r$ 295.3
[209216-23-9]

DEFINITION

2-Amino-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-1,9-dihydro-6*H*-purin-6-one monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, in anhydrous ethanol and in heptane, slightly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *entecavir monohydrate CRS*.

If the spectra obtained show differences, suspend 50 mg of the substance to be examined and 50 mg of the reference substance separately in 5 mL of hot *water R*. Swirl protected from light and heat until a clear solution is obtained. Allow to cool and filter the precipitates *in vacuo*. Dry the precipitates for 12 h in a desiccator and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): + 24 to + 30 (anhydrous substance), measured at 25 °C.

Dissolve 0.25 g in 10 mL of a mixture of equal volumes of *dimethylformamide R* and *methanol R* and dilute to 25.0 mL with the same mixture of solvents.

**Impurity F**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 10 mL of *methanol R* using sonication, and dilute to 25.0 mL with the same solvent.

*Reference solution.* Dissolve 2.5 mg of *entecavir impurity F CRS* in 20 mL of *methanol R* using sonication, and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 65 → 53 | 35 → 47 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Retention time*: impurity F = about 6 min.

*Calculation of percentage content*:

- for impurity F, use the concentration of impurity F in the reference solution.

*Limit*:

- *impurity F*: maximum 0.10 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 10 mL of *methanol R* using sonication, and dilute to 25.0 mL with the same solvent. Dilute 2.0 mL of the solution to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 25.0 mg of *entecavir monohydrate CRS* in 10 mL of *methanol R* using sonication, and dilute to 25.0 mL with the same solvent. Dilute 2.0 mL of the solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve the contents of a vial of *entecavir for system suitability CRS* (containing impurities A and C) in 1 mL of *methanol R* using sonication, and dilute to 5.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R, water for chromatography R* (3:97 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 100 | 0 |
| 8 - 50 | 100 → 77 | 0 → 23 |
| 50 - 75 | 77 → 17 | 23 → 83 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *entecavir for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and C.

*Relative retention* with reference to entecavir (retention time = about 21 min): impurity A = about 0.9; impurity C = about 1.03.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.5 between the peaks due to impurity A and entecavir; minimum 2.0 between the peaks due to entecavir and impurity C.

*Calculation of percentage contents*:

- for each impurity, use the concentration of entecavir monohydrate in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent; disregard any peak with a relative retention with reference to entecavir of about 3.4 (impurity F).

**Water** (*2.5.32*): 5.5 per cent to 7.0 per cent, determined on 30.0 mg by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{15}N_5O_3$ taking into account the assigned content of *entecavir monohydrate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

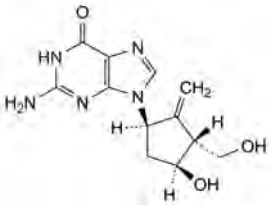

A. 2-amino-9-[(1*R*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-1,9-dihydro-6*H*-purin-6-one,

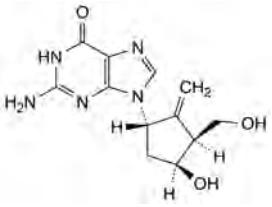

B. 2-amino-9-[(1*S*,3*S*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-1,9-dihydro-6*H*-purin-6-one,

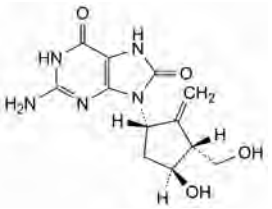

C. 2-amino-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-7,9-dihydro-1*H*-purine-6,8-dione,

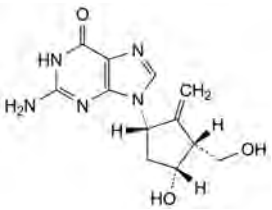

D. 2-amino-9-[(1*S*,3*R*,4*R*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-1,9-dihydro-6*H*-purin-6-one,

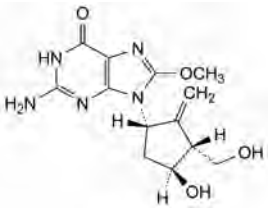

E. 2-amino-9-[(1*S*,3*R*,4*S*)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-8-methoxy-1,9-dihydro-6*H*-purin-6-one,

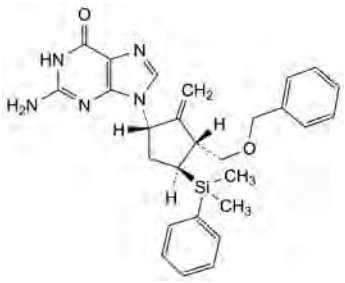

F. 2-amino-9-[(1*S*,3*R*,4*S*)-3-[(benzyloxy)methyl]-4-[dimethyl(phenyl)silyl]-2-methylidenecyclopentyl]-1,9-dihydro-6*H*-purin-6-one.

**01/2017:0488**

# EPHEDRINE

## Ephedrinum

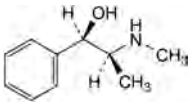

$C_{10}H_{15}NO$ $M_r$ 165.2
[299-42-3]

DEFINITION

Ephedrine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,2*S*)-2-methylamino-1-phenylpropan-1-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, soluble in water, very soluble in alcohol.

It melts at about 36 °C.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with the base isolated from *ephedrine hydrochloride CRS*. Examine the substances in discs prepared as follows: dissolve 40 mg of the substance to be examined in 1 mL of *water R*, add 1 mL of *dilute sodium hydroxide solution R* and 4 mL of *chloroform R* and shake; dry the organic layer over 0.2 g of *anhydrous sodium sulfate R*; prepare a blank disc using about 0.3 g of *potassium bromide R*; apply dropwise to the disc 0.1 mL of the organic layer, allowing the solvent to evaporate between applications; dry the disc at 50 °C for 2 min. Repeat the operations using 50 mg of *ephedrine hydrochloride CRS*.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 1 mL of *water R*. Add 0.2 mL of *strong sodium hydroxide solution R* and 0.2 mL of *copper sulfate solution R*. A violet colour is produced. Add 2 mL of *ether R* and shake. The ether layer is purple and the aqueous layer blue.

E. Water (see Tests).

TESTS

**Appearance of solution**. Dissolve 0.25 g in *water R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*). Dissolve 2.25 g in 15 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*. The specific optical rotation is − 41 to − 43, calculated with reference to the anhydrous substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 25 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 200 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

**Chlorides**. Dissolve 0.17 g in 10 mL of *water R*. Add 5 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R1*. Allow to stand for 2 min, protected from bright light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of *chloride standard solution (5 ppm Cl) R*, 5 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R1* (290 ppm).

**Water** (*2.5.12*). Not more than 0.5 per cent, determined on 2.000 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 mL of *alcohol R* and add 20.0 mL of *0.1 M hydrochloric acid*. Using 0.05 mL of *methyl red solution R* as indicator, titrate with *0.1 M sodium hydroxide* until a yellow colour is obtained.

1 mL of *0.1 M hydrochloric acid* is equivalent to 16.52 mg of $C_{10}H_{15}NO$.

STORAGE

Store protected from light.

**01/2008:0489**
**corrected 6.0**

# EPHEDRINE HEMIHYDRATE

## Ephedrinum hemihydricum

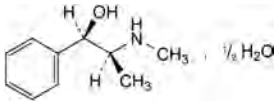

$C_{10}H_{15}NO,½H_2O$ $M_r$ 174.2
[50906-05-3]

DEFINITION

Ephedrine hemihydrate contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*R*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, soluble in water, very soluble in alcohol.

It melts at about 42 °C, determined without previous drying.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with the base isolated from *ephedrine hydrochloride CRS*. Examine the substances in discs prepared as follows: dissolve 40 mg of the substance to be examined in 1 mL of *water R*, add 1 mL of *dilute sodium hydroxide solution R* and 4 mL of *chloroform R* and shake; dry the organic layer over 0.2 g of *anhydrous sodium sulfate R*; prepare a blank disc using about 0.3 g of *potassium bromide R*; apply dropwise to the disc 0.1 mL of the organic layer, allowing the solvent to evaporate between applications; dry the disc at 50 °C for 2 min. Repeat the operations using 50 mg of *ephedrine hydrochloride CRS*.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 1 mL of *water R*. Add 0.2 mL of *strong sodium hydroxide solution R* and 0.2 mL of *copper sulfate solution R*. A violet colour is produced. Add 2 mL of *ether R* and shake. The ether layer is purple and the aqueous layer blue.

E. Water (see Tests).

TESTS

**Appearance of solution**. Dissolve 0.25 g in *water R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*). Dissolve 2.25 g in 15 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*. The specific optical rotation is − 41 to − 43, calculated with reference to the anhydrous substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.2 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 25 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 200 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

**Chlorides**. Dissolve 0.18 g in 10 mL of *water R*. Add 5 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R1*. Allow to stand for 2 min, protected from bright light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of *chloride standard solution (5 ppm Cl) R*, 5 mL of *dilute nitric acid R* and 0.5 mL of *silver nitrate solution R1* (280 ppm).

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.300 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 mL of *alcohol R* and add 20.0 mL of *0.1 M hydrochloric acid*. Using 0.05 mL of *methyl red solution R* as indicator, titrate with *0.1 M sodium hydroxide* until a yellow colour is obtained.

1 mL of *0.1 M hydrochloric acid* is equivalent to 16.52 mg of $C_{10}H_{15}NO$.

STORAGE

Store protected from light.

**01/2008:0487**
**corrected 6.0**

# EPHEDRINE HYDROCHLORIDE

## Ephedrini hydrochloridum

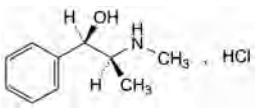

$C_{10}H_{16}ClNO$ $M_r$ 201.7
[50-98-6]

DEFINITION

(1*R*,2*S*)-2-(Methylamino)-1-phenylpropan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

mp: about 219 °C.

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ephedrine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *ephedrine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *concentrated ammonia R*, *2-propanol R* (5:15:80 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R*; heat at 110 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 mL of solution S (see Tests) add 1 mL of *water R*, 0.2 mL of *copper sulfate solution R* and 1 mL of *strong sodium hydroxide solution R*. A violet colour is produced. Add 2 mL of *methylene chloride R* and shake. The lower (organic) layer is dark grey and the upper (aqueous) layer is blue.

E. To 5 mL of solution S (see Tests) add 5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.00 g in *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Specific optical rotation** (*2.2.7*): − 33.5 to − 35.5 (dried substance).

Dilute 12.5 mL of solution S to 25.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 75 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *pseudoephedrine hydrochloride CRS* in the mobile phase and dilute to 50 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *phenylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: mix 6 volumes of *methanol R* and 94 volumes of a 11.6 g/L solution of *ammonium acetate R* adjusted to pH 4.0 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 257 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of ephedrine.

*Relative retention* with reference to ephedrine (retention time = about 8 min): impurity B = about 1.1; impurity A = about 1.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to ephedrine and impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.4;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *sum of impurities other than A*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.17 mg of $C_{10}H_{16}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

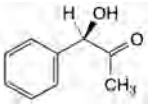


A. (–)-(1*R*)-1-hydroxy-1-phenylpropan-2-one,

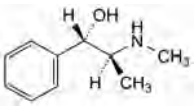


B. (1*S*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol (pseudoephedrine).

**01/2008:0715**
**corrected 6.0**

# EPHEDRINE HYDROCHLORIDE, RACEMIC

## Ephedrini racemici hydrochloridum

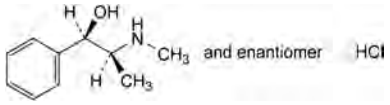


$C_{10}H_{16}ClNO$ $M_r$ 201.7
[134-71-4]

DEFINITION

Racemic ephedrine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (1*RS*,2*SR*)-2-(methylamino)-1-phenylpropan-1-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, soluble in ethanol (96 per cent).

It melts at about 188 °C.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *racemic ephedrine hydrochloride CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.1 mL of solution S (see Tests) add 1 mL of *water R*, 0.2 mL of *copper sulfate solution R* and 1 mL of *strong sodium hydroxide solution R*. A violet colour is produced. Add 2 mL of *ether R* and shake. The ether layer is purple and the aqueous layer is blue.

E. To 5 mL of solution S add 5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.00 g in *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.1 mL of *0.01 M sodium hydroxide*; the solution is yellow. Add 0.2 mL of *0.01 M hydrochloric acid*; the solution is red.

**Optical rotation** (*2.2.7*): + 0.2° to − 0.2°, determined on solution S.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *racemic ephedrine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (a) to 200 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 5 volumes of *chloroform R*, 15 volumes of *concentrated ammonia R* and 80 volumes of *2-propanol R*. Allow the plate to dry in air. Spray with *ninhydrin solution R* and heat at 110 °C for 5 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Disregard any spot of lighter colour than the background.

**Sulfates** (*2.4.13*). 15 mL of solution S complies with the limit test for sulfates (100 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g in 30 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the two points of inflexion.

1 mL of *0.1 M sodium hydroxide* corresponds to 20.17 mg of $C_{10}H_{16}ClNO$.

STORAGE

Store protected from light.

**01/2017:2411**

# EPINASTINE HYDROCHLORIDE

## Epinastini hydrochloridum

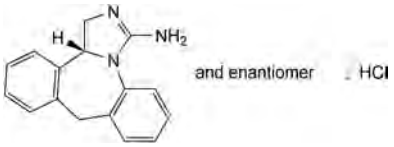


$C_{16}H_{16}ClN_3$ $M_r$ 285.8
[108929-04-0]

DEFINITION

(13b*RS*)-9,13b-Dihydro-1*H*-dibenzo[*c,f*]imidazo[1,5-*a*]azepin-3-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in methanol, sparingly soluble in methylene chloride, slightly soluble in acetonitrile.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
Comparison: *epinastine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

Monographs E

TESTS

**Acidity or alkalinity**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red mixed solution R* and 0.25 mL of *0.01 M sodium hydroxide*. The solution is green. Add 0.5 mL of *0.01 M hydrochloric acid*. The solution is reddish-violet.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 4.4*. Dissolve 3.8 g of *sodium pentanesulfonate monohydrate R* and 4.0 g of *potassium dihydrogen phosphate R* in *water R*, adjust to pH 4.4 with *phosphoric acid R* and dilute to 1000.0 mL with *water R*.

*Solvent mixture*: mobile phase B, mobile phase A (25:75 *V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 10.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *epinastine for system suitability CRS* (containing impurities A and B) in 10.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase* :

- *mobile phase A*: *methanol R2*, buffer solution pH 4.4 (15:85 *V/V*);
- *mobile phase B*: *methanol R2*, *acetonitrile R1* (15:85 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 80 | 20 |
| 4 - 13 | 80 → 30 | 20 → 70 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *epinastine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to epinastine (retention time = about 4 min): impurity A = about 1.2; impurity B = about 2.0.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to epinastine.

*Limits*:

- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 100 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 2 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.58 mg of $C_{16}H_{16}ClN_3$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

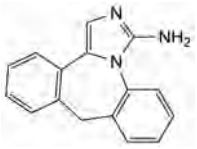

A. 9*H*-dibenzo[*c,f*]imidazo[1,5-*a*]azepin-3-amine,

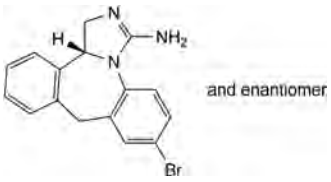


B. (13b*RS*)-7-bromo-9,13b-dihydro-1*H*-dibenzo[*c,f*]imidazo-[1,5-*a*]azepin-3-amine.

**01/2008:1590**
**corrected 10.0**

# EPIRUBICIN HYDROCHLORIDE

## Epirubicini hydrochloridum

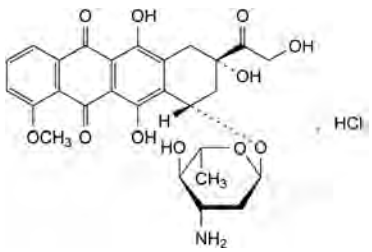


$C_{27}H_{30}ClNO_{11}$ $M_r$ 580.0
[56390-09-1]

DEFINITION

(8*S*,10*S*)-10-[(3-Amino-2,3,6-trideoxy-α-L-*arabino*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione hydrochloride.

Substance obtained by chemical transformation of a substance produced by certain strains of *Streptomyces peucetius*.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: orange-red powder.

*Solubility*: soluble in water and in methanol, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *epirubicin hydrochloride CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 0.5 mL of *nitric acid R*, add 0.5 mL of *water R* and heat over a flame for 2 min. Allow to cool and add 0.5 mL of *silver nitrate solution R1*. A white precipitate is formed.

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Allow the solutions to stand for 3 h before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *epirubicin hydrochloride CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *epirubicin hydrochloride CRS* and 10 mg of *doxorubicin hydrochloride CRS* in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (c).* Dissolve 10 mg of *doxorubicin hydrochloride CRS* in a mixture of 5 mL of *water R* and 5 mL of *phosphoric acid R*. Allow to stand for 30 min. Adjust to pH 2.6 with an 80 g/L solution of *sodium hydroxide R*. Add 15 mL of *acetonitrile R* and 10 mL of *methanol R*. Mix.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *trimethylsilyl silica gel for chromatography R* (6 µm);
– *temperature*: 35 °C.

*Mobile phase*: mix 17 volumes of *methanol R*, 29 volumes of *acetonitrile R* and 54 volumes of a solution containing 3.7 g/L of *sodium laurilsulfate R* and 2.8 per cent *V/V* of *dilute phosphoric acid R*.

*Flow rate*: 2.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3.5 times the retention time of epirubicin.

*Identification of impurities*: use the 2nd most abundant peak present in the chromatogram obtained with reference solution (c) to identify impurity A.

*Relative retention* with reference to epirubicin (retention time = about 9.5 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.8; impurity E = about 1.1; impurity D = about 1.5; impurity F = about 1.7; impurity G = about 2.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity C and epirubicin.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.7;
– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent);
– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent);
– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Acetone** (*2.4.24*): maximum 1.5 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.100 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.1 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{27}H_{30}ClNO_{11}$.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

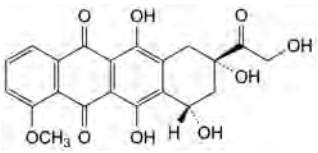

A. (8*S*,10*S*)-6,8,10,11-tetrahydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicinone),

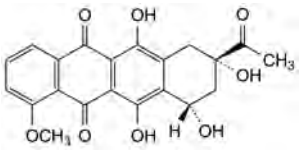

B. (8*S*,10*S*)-8-acetyl-6,8,10,11-tetrahydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicinone),

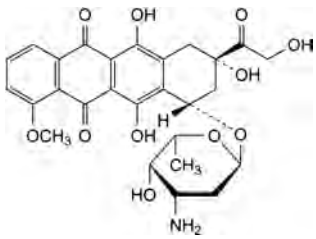

C. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (doxorubicin),

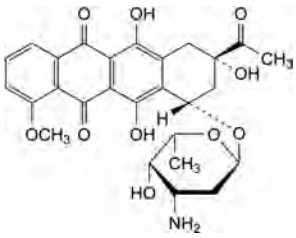

D. (8*S*,10*S*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (daunorubicin),

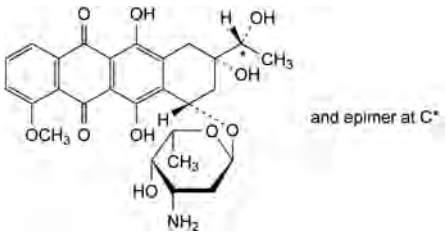


E. (8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-6,8,11-trihydroxy-8-[(1*RS*)-1-hydroxyethyl]-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (dihydrodaunorubicin),

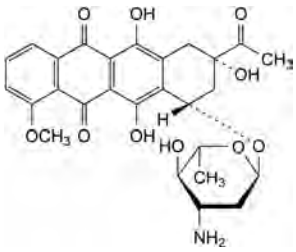

F. (8*S*,10*S*)-8-acetyl-10-[(3-amino-2,3,6-trideoxy-α-L-*arabino*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione (*epi*-daunorubicin),

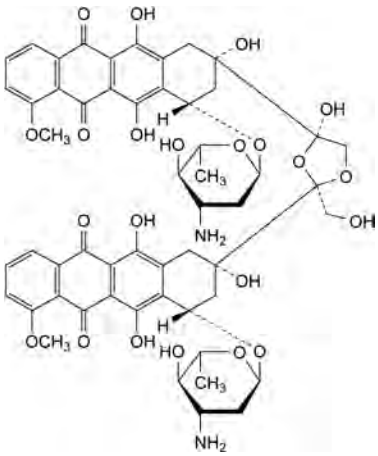

G. 8,8′-[(2*R*,4*R*)-4-hydroxy-2-(hydroxymethyl)-1,3-dioxolan-2,4-diyl]bis[(8*S*,10*S*)-10-[(3-amino-2,3,6-trideoxy-α-L-*arabino*-hexopyranosyl)oxy]-6,8,11-trihydroxy-1-methoxy-7,8,9,10-tetrahydrotetracene-5,12-dione] (epirubicin dimer).

**04/2015:2765**

# EPLERENONE

## Eplerenonum

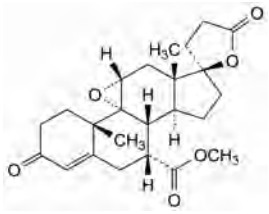

$C_{24}H_{30}O_6$ $M_r$ 414.5

[107724-20-9]

DEFINITION

9,11α-Epoxy-7α-(methoxycarbonyl)-3-oxo-17α-pregn-4-ene-21,17-carbolactone.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white, almost white or slightly yellow, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *eplerenone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): − 16.0 to − 14.0.

Dissolve 0.250 g in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *methanol R*, *water R* (25:25:50 *V/V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *eplerenone for system suitability CRS* (containing impurities A and D) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *eplerenone for peak identification CRS* (containing impurity B) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 25.0 mg of *eplerenone CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: *phosphoric acid R*, *acetonitrile R*, *methanol R* (0.1:40:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 54 | 46 |
| 25 - 32 | 54 → 40 | 46 → 60 |
| 32 - 45 | 40 | 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *eplerenone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and D; use the chromatogram supplied with *eplerenone for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to eplerenone (retention time = about 9 min): impurity D = about 0.71; impurity A = about 0.74; impurity B = about 1.2.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of eplerenone in reference solution (c).

*Limits*:

– *impurities A, B*: for each impurity, maximum 0.3 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.6 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{24}H_{30}O_6$ taking into account the assigned content of *eplerenone CRS*.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G.*

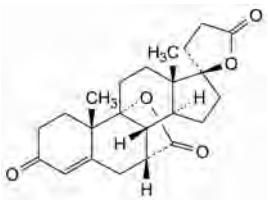

A. 3-oxo-17α-pregn-4-ene-7α,9:21,17-dicarbolactone,

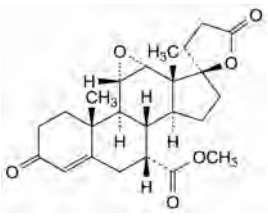

B. 11α,12α-epoxy-7α-(methoxycarbonyl)-3-oxo-17α-pregn-4-ene-21,17-carbolactone,

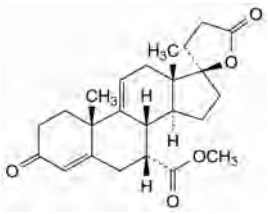

C. 7α-(methoxycarbonyl)-3-oxo-17α-pregna-4,9(11)-diene-21,17-carbolactone,

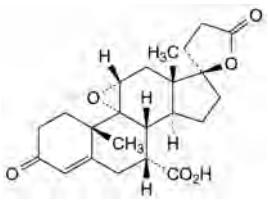

D. (2′*R*)-9,11α-epoxy-3,5′-dioxo-4′,5′-dihydro-3′*H*-spiro[androst-4-ene-17,2′-furan]-7α-carboxylic acid,

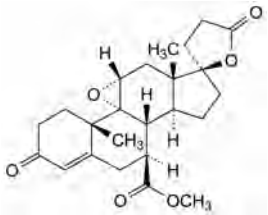

E. 9,11α-epoxy-7β-(methoxycarbonyl)-3-oxo-17α-pregn-4-ene-21,17-carbolactone,

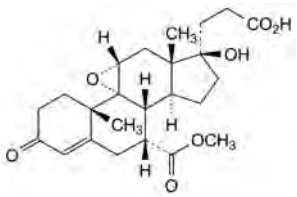

F. 9,11α-epoxy-17-hydroxy-7α-(methoxycarbonyl)-3-oxo-17α-pregn-4-ene-21-carboxylic acid,

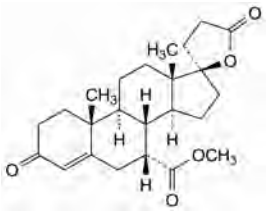

G. 7α-(methoxycarbonyl)-3-oxo-17α-pregn-4-ene-21,17-carbolactone.

**04/2019:0082**

# ERGOCALCIFEROL

## Ergocalciferolum

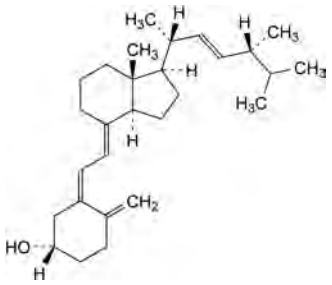

$C_{28}H_{44}O$ $M_r$ 396.7
[50-14-6]

DEFINITION

(3*S*,5*Z*,7*E*,22*E*)-9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol.

*Content*: 97.0 per cent to 102.0 per cent.

A suitable antioxidant may be added.

A reversible isomerisation to pre-ergocalciferol takes place in solution, depending on temperature and time. The activity is due to both compounds (see Assay).

1 mg of ergocalciferol is equivalent to 40 000 IU of antirachitic activity (vitamin D) in rats.

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder or white or almost white crystals.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in methanol, soluble in fatty oils.

It is sensitive to air, heat and light.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ergocalciferol CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 103 to + 107.

Dissolve 0.200 g rapidly and without heating in *aldehyde-free alcohol R* and dilute to 25.0 mL with the same solvent. Examine within 30 min of preparing the solution.

**Reducing substances**. Dissolve 0.1 g in *aldehyde-free alcohol R* and dilute to 10.0 mL with the same solvent. Add 0.5 mL of a 5 g/L solution of *tetrazolium blue R* in *aldehyde-free alcohol R* and 0.5 mL of *dilute tetramethylammonium hydroxide solution R*. Allow to stand for exactly 5 min and add 1.0 mL of *glacial acetic acid R*. Prepare a reference solution at the same time and in the same manner using 10.0 mL of a solution containing 0.2 µg/mL of *hydroquinone R* in *aldehyde-free alcohol R*. Measure the absorbance (*2.2.25*) of the 2 solutions at 525 nm using as the compensation liquid 10.0 mL of *aldehyde-free alcohol R* treated in the same manner. The absorbance of the test solution is not greater than that of the reference solution (20 ppm).

**Impurity B**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, avoiding exposure to actinic light and air.*

*Test solution*. Dissolve 25.0 mg of the substance to be examined without heating in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution*. Dissolve 5.0 mg of *ergosterol CRS* (impurity B) without heating in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *methanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of ergocalciferol.

*Relative retention* with reference to ergocalciferol (retention time = about 7 min): impurity B = about 1.6.

*Limit*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, avoiding exposure to actinic light and air.*

*Test solution*. Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *ergocalciferol for system suitability CRS* (containing impurities A, F and G) in 10 mL of the mobile phase. Heat in a water-bath at 90 °C under a reflux-condenser for 45 min and allow to cool (*in-situ* degradation to obtain pre-ergocalciferol). Dilute 3 mL of the solution to 25 mL with the mobile phase.

*Reference solution (b)*. Dissolve 15.0 mg of *ergocalciferol CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (c) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *methanol R*, *acetonitrile R* (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (c) and (d).

*Run time*: twice the retention time of ergocalciferol.

*Identification of impurities*: use the chromatogram supplied with *ergocalciferol for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, F and G, and pre-ergocalciferol.

*Relative retention* with reference to ergocalciferol (retention time = about 13 min): impurity F = about 0.6; impurity A = about 0.8; pre-ergocalciferol = about 0.9; impurity G = about 1.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity A and pre-ergocalciferol; minimum 2.5 between the peaks due to pre-ergocalciferol and ergocalciferol.

*Calculation of percentage contents*:

- for impurities A, F and G, use the concentration of ergocalciferol in reference solution (c);
- for impurities other than A, F and G, use the concentration of ergocalciferol in reference solution (d).

*Limits*:

- *impurity G*: maximum 1.5 per cent;
- *impurities A, F*: for each impurity, maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard any peak due to pre-ergocalciferol or the antioxidant.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{28}H_{44}O$ taking into account the assigned content of *ergocalciferol CRS*, and if present, the peak due to pre-ergocalciferol in the test solution.

STORAGE

Under an inert gas, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

The contents of an opened container are to be used immediately.

LABELLING

The label states the name and concentration of any added antioxidant.

IMPURITIES

*Specified impurities: A, B, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E.*

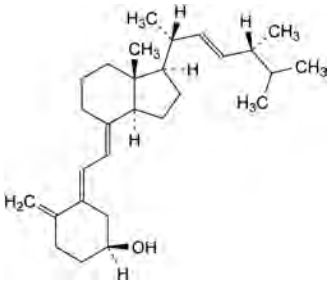

A. (3*S*,5*E*,7*E*,22*E*)-9,10-secoergosta-5,7,10(19),22-tetraen-3-ol (*trans*-vitamin $D_2$),

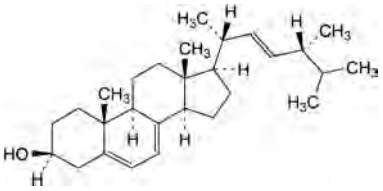

B. (22*E*)-ergosta-5,7,22-trien-3β-ol (ergosterol),

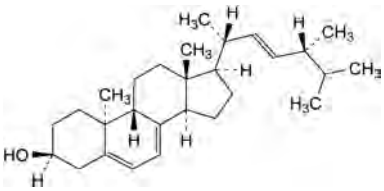

C. (22*E*)-9β,10α-ergosta-5,7,22-trien-3β-ol (lumisterol$_2$),

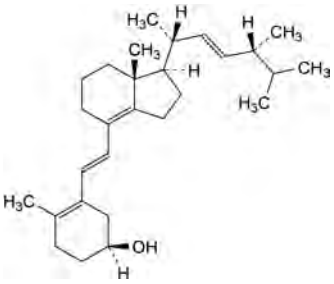

D. (3*S*,6*E*,22*E*)-9,10-secoergosta-5(10),6,8(14),22-tetraen-3-ol (iso-tachysterol$_2$),

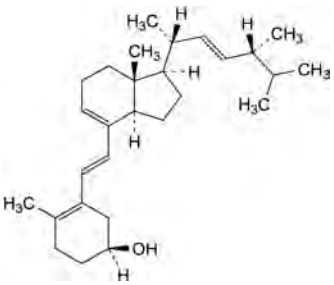

E. (3*S*,6*E*,22*E*)-9,10-secoergosta-5(10),6,8,22-tetraen-3-ol (tachysterol$_2$),

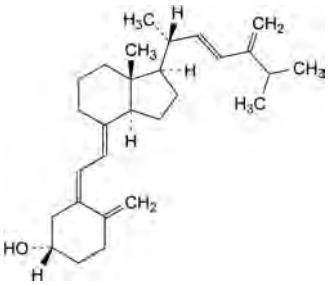

F. (3*S*,5*Z*,7*E*,22*E*)-9,10-secoergosta-5,7,10(19),22,24(24$^1$)-pentaen-3-ol,

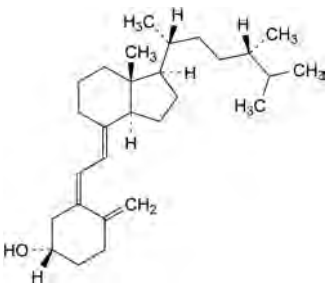

G. (3*S*,5*Z*,7*E*)-9,10-secoergosta-5,7,10(19)-trien-3-ol (vitamin $D_4$).

**01/2008:0223**
**corrected 6.0**

# ERGOMETRINE MALEATE

## Ergometrini maleas

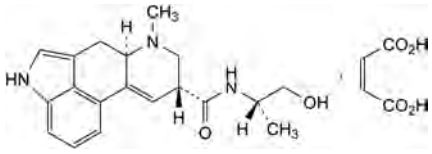

$C_{23}H_{27}N_3O_6$ $M_r$ 441.5
[129-51-1]

DEFINITION

Ergometrine maleate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of (6a*R*,9*R*)-*N*-[(*S*)-2-hydroxy-1-methylethyl]-7-methyl-4,6,6a,7,8,9-hexahydro-indolo[4,3-*fg*]quinoline-9-carboxamide (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white or slightly coloured, crystalline powder, sparingly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E.*

A. Dissolve 30 mg in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with *0.01 M hydrochloric acid*. Examined between 250 nm and 360 nm (*2.2.25*), the solution shows an absorption maximum at 311 nm and a minimum at 265 nm to 272 nm. The specific absorbance at the maximum is 175 to 195.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *ergometrine maleate CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.1 mL of solution S (see Tests) add 1 mL of *glacial acetic acid R*, 0.05 mL of *ferric chloride solution R1* and 1 mL of *phosphoric acid R* and heat in a water-bath at 80 °C. After about 10 min, a blue or violet colour develops which becomes more intense on standing.

E. Dissolve 0.1 g in a mixture of 0.5 mL of *dilute sulfuric acid R* and 2.5 mL of *water R*. Add 5 mL of *ether R* and 1 mL of *strong sodium hydroxide solution R* and shake. Separate the aqueous layer and shake with two quantities, each of 5 mL, of *ether R*. To 0.1 mL of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min. No colour develops. To the rest of the aqueous layer add 1 mL of *bromine water R*. Heat on a water-bath for 10 min, then heat to boiling and cool. To 0.2 mL of this solution add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min. A pinkish-violet colour develops.

TESTS

**Solution S**. Dissolve 0.100 g, without heating and protected from light, in 9 mL of *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ or $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 3.6 to 4.4.

**Specific optical rotation** (*2.2.7*): + 50 to + 56, determined on solution S and calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. *Carry out all operations as rapidly as possible, protected from light. Prepare the test and reference solutions immediately before use.*

*Test solution (a).* Dissolve 50 mg of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R* and dilute to 5.0 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

*Reference solution (a).* Dissolve 10 mg of *ergometrine maleate CRS* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

*Reference solution (c).* To 2.0 mL of reference solution (b) add 2.0 mL of a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol (80 per cent V/V) R*.

Apply separately to the plate 5 µL of each solution. Develop immediately over a path of 14 cm using a mixture of 3 volumes of *water R*, 25 volumes of *methanol R* and 75 volumes of *chloroform R*. Dry the plate in a current of cold air and spray with *dimethylaminobenzaldehyde solution R7*. Dry the plate in a current of warm air for about 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 2.0 per cent, determined on 0.20 g by drying over *diphosphorus pentoxide R* at 80 °C at a pressure not exceeding 2.7 kPa for 2 h.

ASSAY

Dissolve 0.150 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.05 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.05 M perchloric acid* is equivalent to 22.07 mg of $C_{23}H_{27}N_3O_6$.

STORAGE

Store in an airtight, glass container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2017:0224**

# ERGOTAMINE TARTRATE

## Ergotamini tartras

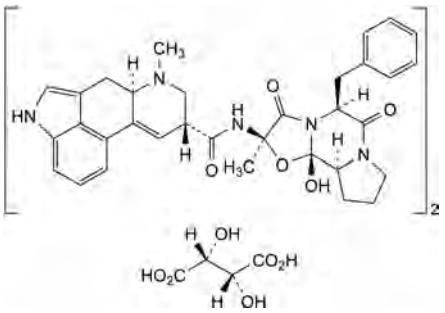

$C_{70}H_{76}N_{10}O_{16}$ $M_r$ 1313
[379-79-3]

DEFINITION

Bis[(6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-[1,3]oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide] (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

It may contain two molecules of methanol of crystallisation.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, slightly hygroscopic.

*Solubility*: slightly soluble in ethanol 96 per cent. Aqueous solutions slowly become cloudy owing to hydrolysis; this may be prevented by the addition of tartaric acid.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with *0.01 M hydrochloric acid.*

*Spectral range*: 250 nm and 360 nm.

*Absorption maximum*: at 311 to 321 nm.

*Absorption minimum*: at 265 to 275 nm.

*Specific absorbance at the absorption maximum*: 118 to 128 (dried substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ergotamine tartrate CRS.*

*Preparation*: as discs, triturate the substance to be examined and the reference substance separately with 0.2 mL of *methanol R* and then with *potassium bromide R* as prescribed in the general method.

C. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 5 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5.0 mL with the same mixture of solvents.

*Reference solution.* Dissolve 5 mg of *ergotamine tartrate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 5.0 mL with the same mixture of solvents.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous ethanol R*, *methylene chloride R*, *dimethylformamide R*, *ether R* (5:10:15:70 *V/V/V/V*).

*Application*: 5 µL; immediately expose the points of application to ammonia vapour for exactly 20 s by moving the line of application from side to side above a beaker 55 mm high and 45 mm in diameter containing about 20 mL of *concentrated ammonia R*; dry the line of application in a current of cold air for exactly 20 s.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air for about 2 min.

*Detection A*: examine for not more than 1 min in ultraviolet light at 365 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and fluorescence to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: spray the plate abundantly with *dimethylaminobenzaldehyde solution R7* and dry in a current of warm air for about 2 min; examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 mL of solution S (see Tests) add 1 mL of *glacial acetic acid R*, 0.05 mL of *ferric chloride solution R1* and 1 mL of *phosphoric acid R* and heat in a water-bath at 80 °C. After about 10 min, a blue or violet colour develops which becomes more intense on standing.

E. Dissolve about 10 mg in 1.0 mL of *0.1 M sodium hydroxide*. Transfer to a separating funnel and shake with 5 mL of *methylene chloride R*. Discard the organic layer. Neutralise the aqueous layer with a few drops of *dilute hydrochloric acid R*. 0.1 mL of this solution gives reaction (b) of tartrates (*2.3.1*). Pour the reaction mixture into 1 mL of *water R* to observe the colour change to red or brownish-red.

TESTS

*Carry out all operations as rapidly as possible, protected from light.*

**Solution S**. Triturate 50 mg finely with about 25 mg of *tartaric acid R* and dissolve with shaking in 10 mL of *water R.*

**pH** (*2.2.3*): 4.0 to 5.5 for the suspension.

Shake 25 mg, finely powdered, with 10 mL of *carbon dioxide-free water R.*

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (10:90 *V/V*).

*Solution A*. Dissolve 1.0 g of *potassium dihydrogen phosphate R* and 2.0 g of *sodium 1-propanesulfonate R* in 900 mL of *water R*, adjust to pH 4.3 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R.*

*Test solution.* Dissolve 30 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution.* Dissolve 3 mg of *ergotamine for system suitability CRS* (containing impurities A and C) in the solvent mixture and dilute to 5.0 mL with the solvent mixture. In order to prepare impurity B *in situ*, maintain the solution at room temperature for 1 h.

*Column*:

– *size*: *l* = 0.05 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (1.8 µm);
– *temperature*: 15 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, solution A (10:90 *V/V*);

– *mobile phase B*: solution A, *acetonitrile R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 80 → 50 | 20 → 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Autosampler*: set at 4 °C.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram supplied with *ergotamine for system suitability CRS* and the chromatogram obtained with the reference solution to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to ergotamine (retention time = about 3 min): impurity A = about 0.9; impurity B = about 1.1; impurity C = about 1.2.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to impurity A and ergotamine.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.3;
– *impurity C*: maximum 0.4 per cent;
– *impurities A, B*: for each impurity, maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.6 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 0.100 g by drying *in vacuo* at 95 °C for 6 h.

ASSAY

Dissolve 0.200 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.05 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.05 M perchloric acid* is equivalent to 32.84 mg of $C_{70}H_{76}N_{10}O_{16}$.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

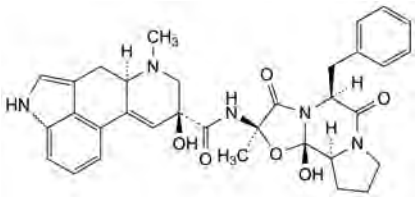

A. (6a*R*,9*S*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-[1,3]oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-9-hydroxy-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (8-hydroxyergotamine),

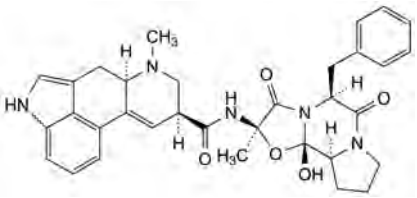

B. (6a*R*,9*S*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-2-methyl-3,6-dioxooctahydro-8*H*-[1,3]oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergotaminine),

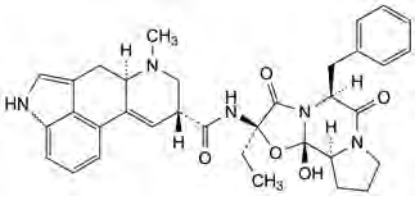

C. (6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-2-ethyl-10b-hydroxy-3,6-dioxooctahydro-8*H*-[1,3]oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergostine),

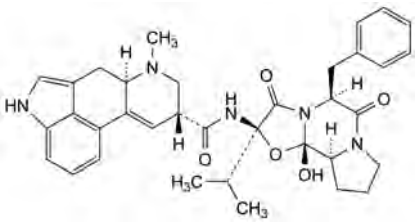

D. (6a*R*,9*R*)-*N*-[(2*R*,5*S*,10a*S*,10b*S*)-5-benzyl-10b-hydroxy-3,6-dioxo-2-(propan-2-yl)octahydro-8*H*-[1,3]oxazolo[3,2-*a*]pyrrolo[2,1-*c*]pyrazin-2-yl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergocristine).

**07/2019:1803**

# ERYTHRITOL

## Erythritolum

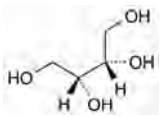

$C_4H_{10}O_4$ $M_r$ 122.1
[149-32-6]

DEFINITION

(2*R*,3*S*)-Butane-1,2,3,4-tetrol (*meso*-erythritol).

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or free-flowing granules.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 119 °C to 122 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *erythritol CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution, while gently stirring with a magnetic stirrer.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.50 g of *erythritol CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dilute 5.0 mL of reference solution (b) to 100.0 mL with *water R*.

*Reference solution (d)*. Dissolve 1.0 g of *erythritol R* and 1.0 g of *glycerol R* in *water R* and dilute to 20 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 8.0 mm;

– *stationary phase*: *cation-exchange resin R* (9 µm);

– *temperature*: 70 °C.

*Mobile phase*: 0.01 per cent *V/V* solution of *sulfuric acid R*.

*Flow rate*: 0.8 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of erythritol.

*Relative retention* with reference to erythritol (retention time = about 11 min): impurity A = about 0.77; impurity B = about 0.90; impurity C = about 0.94; impurity D = about 1.10.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to erythritol and impurity D.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);

– TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);

– absence of *Escherichia coli* (*2.6.13*);

– absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins:

– less than 4 IU/g for parenteral preparations having a concentration of 100 g/L or less of erythritol;

– less than 2.5 IU/g for parenteral preparations having a concentration of more than 100 g/L of erythritol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_4H_{10}O_4$ taking into account the assigned content of *erythritol CRS*.

LABELLING

The label states where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

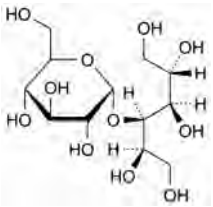

A. 4-*O*-α-D-glucopyranosyl-D-glucitol (D-maltitol),

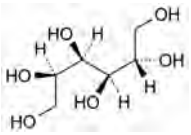

B. D-glucitol (D-sorbitol),

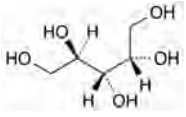

C. (2*R*,3*s*,4*S*)-pentane-1,2,3,4,5-pentol (*meso*-ribitol),

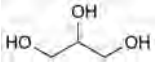

D. propane-1,2,3-triol (glycerol).

01/2017:0179

# ERYTHROMYCIN

## Erythromycinum

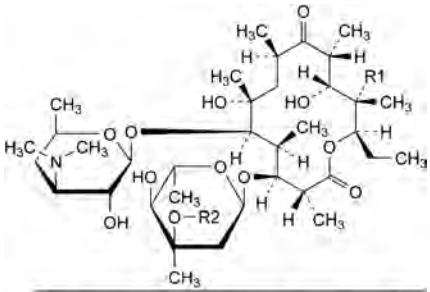

| Erythromycin | Mol. Formula | $M_r$ | R1 | R2 |
|---|---|---|---|---|
| A | $C_{37}H_{67}NO_{13}$ | 734 | OH | $CH_3$ |
| B | $C_{37}H_{67}NO_{12}$ | 718 | H | $CH_3$ |
| C | $C_{36}H_{65}NO_{13}$ | 720 | OH | H |



### DEFINITION

Mixture of macrolide antibiotics produced by a strain of *Streptomyces erythreus*.

*Main component*: (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A).

*Content*:

- *sum of erythromycins A, B and C*: 93.0 per cent to 102.0 per cent (anhydrous substance);
- *erythromycin B*: maximum 5.0 per cent (anhydrous substance);
- *erythromycin C*: maximum 5.0 per cent (anhydrous substance).

### CHARACTERS

*Appearance*: white or slightly yellow powder or colourless or slightly yellow crystals, slightly hygroscopic.

*Solubility*: slightly soluble in water (the solubility decreases as the temperature rises), freely soluble in ethanol (96 per cent), soluble in methanol.

It shows polymorphism (*5.9*).

### IDENTIFICATION

*First identification: A.*

*Second identification: B.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *erythromycin A CRS*.

Disregard any band in the region from 1980 $cm^{-1}$ to 2050 $cm^{-1}$.

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and of the reference substance separately in 1.0 mL of *methylene chloride R*, dry at 60 °C for 3 h at a pressure not exceeding 0.67 kPa and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *erythromycin A CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *spiramycin CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: mix 4 volumes of *2-propanol R*, 8 volumes of a 150 g/L solution of *ammonium acetate R* previously adjusted to pH 9.6 with *ammonia R*, and 9 volumes of *ethyl acetate R*; allow to settle and use the upper layer.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R1* and heat at 110 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a), and its position and colour are different from those of the spots in the chromatogram obtained with reference solution (b).

### TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 11.5 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture*: *methanol R*, solution A (40:60 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 40.0 mg of *erythromycin A CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 4 mg of *erythromycin for system suitability CRS* (containing impurities A, B, C, D, E, F, H and L) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (e)*. Dissolve 4 mg of *erythromycin for impurity M identification CRS* in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 65 °C; preheating the mobile phase may be required, for instance by extending the inlet tubing in the oven to 30 cm.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 7.0 R7*, *acetonitrile R1*, *water R* (5:35:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution pH 7.0 R7*, *water R*, *acetonitrile R1* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 100 | 0 |
| $t_R$ - ($t_R$ + 2) | 100 → 0 | 0 → 100 |
| ($t_R$ + 2) - ($t_R$ + 15) | 0 | 100 |

$t_R$ = retention time of erythromycin B, determined by injecting 10 µL of reference solution (b) and eluting with mobile phase A

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 100 µL.

*Identification of impurities*: use the chromatogram supplied with *erythromycin for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, E, F, H and L; use the chromatogram supplied with *erythromycin for impurity M identification CRS* and the chromatogram obtained with reference solution (e) to identify the peak due to impurity M; use the chromatogram obtained with reference solution (b) to identify the peaks due to erythromycins B and C.

*Relative retention* with reference to erythromycin A (retention time = about 23 min): impurity H = about 0.3; impurity A = about 0.4; impurity B = about 0.5; erythromycin C = about 0.55; impurity M = about 0.58; impurity L = about 0.63; impurity C = about 0.9; impurity D = about 1.61; erythromycin B = about 1.75; impurity F = about 1.81; impurity E = about 2.3.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.2 between the peaks due to impurity B and erythromycin C;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin B; minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin A. If necessary, adjust the concentration of *acetonitrile R1* in the mobile phases and/or the gradient to obtain the required separation.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 2; impurity E = 0.08; impurity F = 0.08; impurity L = 0.11;
- for each impurity, use the concentration of erythromycin A in reference solution (c).

*Limits*:

- *impurity C*: maximum 3.0 per cent;
- *impurities A, B*: for each impurity, maximum 2.0 per cent;
- *impurities D, E, F, H, M*: for each impurity, maximum 1.0 per cent;
- *impurity L*: maximum 0.4 per cent;
- *any other impurity*: for each impurity, maximum 0.4 per cent;
- *total*: maximum 7.0 per cent;
- *reporting threshold*: 0.2 per cent; disregard the peaks due to erythromycins B and C.

**Thiocyanate**: maximum 0.3 per cent.

*Prepare the solutions immediately before use and protect from actinic light.*

*Compensation liquid.* Dilute 1.0 mL of a 90 g/L solution of *ferric chloride R* to 50.0 mL with *methanol R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in 20 mL of *methanol R*, add 1.0 mL of a 90 g/L solution of *ferric chloride R* and dilute to 50.0 mL with *methanol R*.

*Prepare 2 independent reference solutions.*

*Reference solution.* Dissolve 0.100 g of *potassium thiocyanate R*, previously dried at 105 °C for 1 h, in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *methanol R*. To 5.0 mL of this solution, add 1.0 mL of a 90 g/L solution of *ferric chloride R* and dilute to 50.0 mL with *methanol R*.

Measure the absorbance (*2.2.25*) of each reference solution ($A_1$, $A_2$) and of the test solution ($A$) at the absorption maximum (about 492 nm).

*Suitability value*:

$$S = \frac{m_2 \times A_1}{m_1 \times A_2}$$

$m_1$, $m_2$ = mass of potassium thiocyanate used to prepare reference solutions $A_1$ and $A_2$ respectively, in grams.

The test is not valid unless $S$ is not less than 0.985 and not greater than 1.015.

Calculate the percentage content of thiocyanate using the following expression:

$$\frac{A \times 58.08 \times 0.5}{m \times 97.18} \times \left( \frac{m_1}{A_1} + \frac{m_2}{A_2} \right)$$

$m$ = mass of the substance to be examined used to prepare the test solution, in grams;

58.08 = relative molecular mass of the thiocyanate moiety;

97.18 = relative molecular mass of potassium thiocyanate.

**Water** (*2.5.12*): maximum 6.5 per cent, determined on 0.200 g.

Use a 100 g/L solution of *imidazole R* in *anhydrous methanol R* as the solvent.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solutions (a) and (b).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.0 for the peak due to erythromycin A;
- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of erythromycin A ($C_{37}H_{67}NO_{13}$) using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B ($C_{37}H_{67}NO_{12}$) and erythromycin C ($C_{36}H_{65}NO_{13}$) using the chromatogram obtained with reference solution (b).

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, H, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *I, J, K, N.*

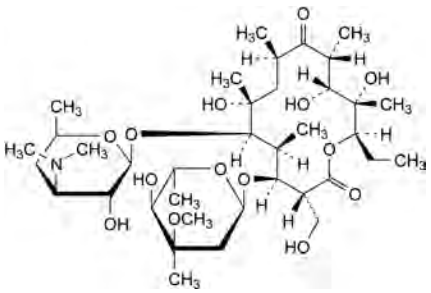

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin F),

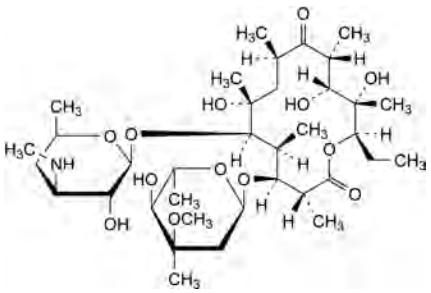

B. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethylerythromycin A),

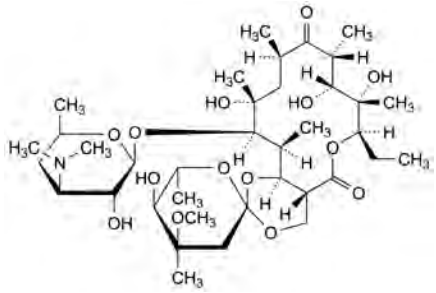

C. (2*S*,4a*R*,4′*R*,5′*S*,6′*S*,7*R*,8*S*,9*R*,10*R*,12*R*,14*R*,15*R*,16*S*,16a*S*)-7-ethyl-5′,8,9,14-tetrahydroxy-4′-methoxy-4′,6′,8,10,12,14,16-heptamethyl-15-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-hexadecahydrospiro[5*H*,11*H*-1,3-dioxino[5,4-*c*]oxacyclotetradecin-2,2′-pyrane]-5,11-dione (erythromycin E),

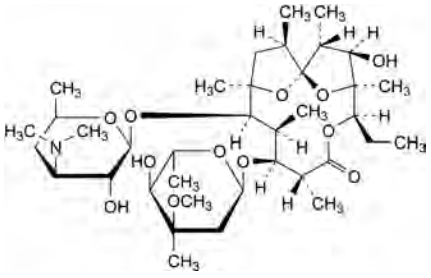

D. (1*S*,2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadecan-7-one (anhydroerythromycin A),

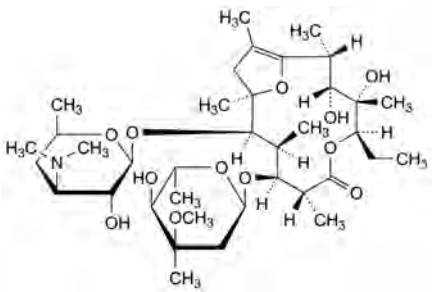

E. (2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3,4-dihydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15-dioxabicyclo[10.2.1]pentadec-1(14)-en-7-one (erythromycin A enol ether),

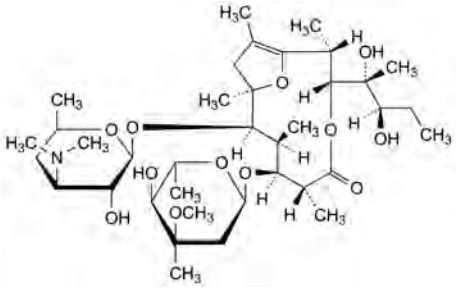

F. (2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridec-1(12)-en-5-one (pseudoerythromycin A enol ether),

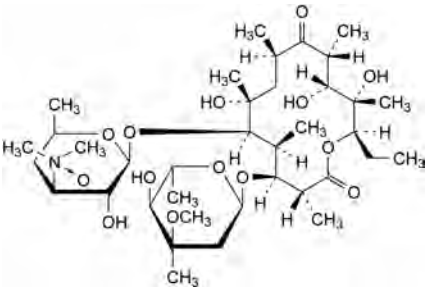

H. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione *N*-oxide (erythromycin A 3″-*N*-oxide),

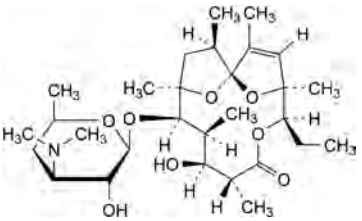

I. (1*S*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-5-ethyl-9-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadec-2-en-7-one (erythralosamine),

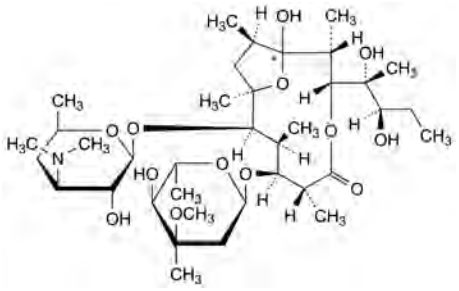

J. (1*RS*,2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-1-hydroxy-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridecan-5-one (pseudoerythromycin A hemiketal),

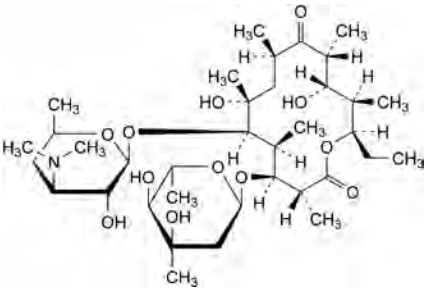

K. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin D),

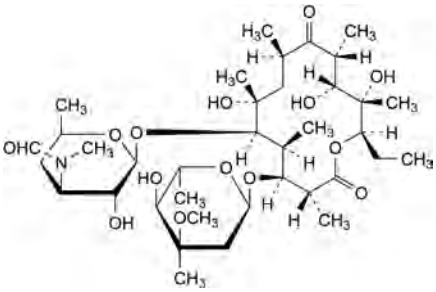

L. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(formylmethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-formyl erythromycin A),

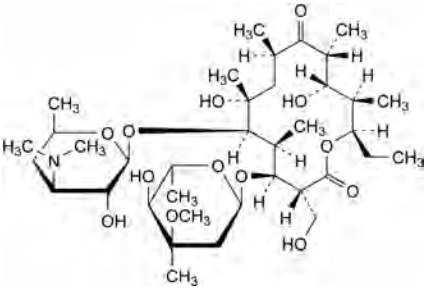

M. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin G),

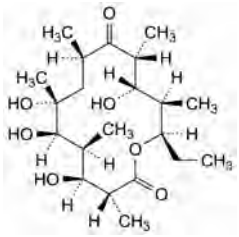

N. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-14-ethyl-4,6,7,12-tetrahydroxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione (erythronolide B).

**01/2017:0552**

# ERYTHROMYCIN ESTOLATE

## Erythromycini estolas

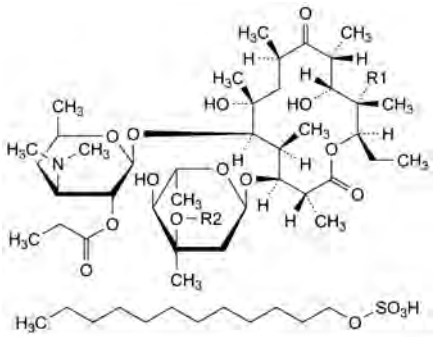

| Erythromycin (estolate) | Mol. Formula | $M_r$ | R1 | R2 |
|---|---|---|---|---|
| A | $C_{52}H_{97}NO_{18}S$ | 1056 | OH | $CH_3$ |
| B | $C_{52}H_{97}NO_{17}S$ | 1040 | H | $CH_3$ |
| C | $C_{51}H_{95}NO_{18}S$ | 1042 | OH | H |

DEFINITION

Mixture of the estolate esters of erythromycin.

*Main component*: (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-2-*O*-propanyl-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione dodecyl sulfate (erythromycin A 2″-propionate dodecyl sulfate).

Semi-synthetic product derived from a fermentation product obtained using a strain of *Streptomycines erythreus*.

*Content*:

– *sum of erythromycins A, B and C expressed as estolates*: 93.0 per cent to 102.0 per cent (anhydrous substance);
– *erythromycin B estolate*: maximum 5.0 per cent (anhydrous substance);
– *erythromycin C estolate*: maximum 5.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in acetone. It is practically insoluble in dilute hydrochloric acid.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *erythromycin estolate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use (apart from the test solution).*

*Solution A* (hydrolysis solution). Dissolve 11.5 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 0.150 g of the substance to be examined in 25 mL of *methanol R*. Add 20 mL of solution A, mix and allow to stand at room temperature for at least 12 h. Dilute to 50.0 mL with solution A.

*Reference solution (a).* Dissolve 40.0 mg of *erythromycin A CRS* in 12.0 mL of *methanol R* and dilute to 20.0 mL with solution A.

*Reference solution (b).* Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in 40.0 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with a mixture of 4 volumes of solution A and 6 volumes of *methanol R*.

*Reference solution (d).* Dissolve 4 mg of *erythromycin for system suitability CRS* (containing impurities A, B, C, D, E, F, H and L) in 0.4 mL of *methanol R* and add 0.6 mL of solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 65 °C; preheating the mobile phase may be required, for instance by extending the inlet tubing in the oven to 30 cm.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 7.0 R7*, *acetonitrile R1*, *water R* (5:35:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution ph 7.0 R7*, *water R*, *acetonitrile R1* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 100 | 0 |
| $t_R$ - ($t_R$ + 2) | 100 → 0 | 0 → 100 |
| ($t_R$ + 2) - ($t_R$ + 15) | 0 | 100 |

$t_R$ = retention time of erythromycin B, determined by injecting 20 µL of reference solution (b) and eluting with mobile phase A

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 200 µL.

*Identification of impurities*: use the chromatogram supplied with *erythromycin for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, E, F and L; use the chromatogram obtained with reference solution (b) to identify the peaks due to erythromycins B and C.

*Relative retention* with reference to erythromycin A (retention time = about 23 min): impurity A = about 0.4; impurity B = about 0.5; erythromycin C = about 0.55; impurity L = about 0.63; impurity C = about 0.9; impurity D = about 1.61; erythromycin B = about 1.75; impurity F = about 1.81; impurity E = about 2.3.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.2 between the peaks due to impurity B and erythromycin C;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin B; minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin A. If necessary, adjust the concentration of *acetonitrile R1* in the mobile phases and/or the gradient to obtain the required separation.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 2; impurity E = 0.08; impurity F = 0.08; impurity L = 0.11;
- for each impurity, use the concentration of erythromycin A in reference solution (c).

*Limits*:

- *impurity C*: maximum 3.0 per cent;
- *impurities A, B*: for each impurity, maximum 2.0 per cent;
- *impurities D, F*: for each impurity, maximum 1.0 per cent;
- *impurities eluting with relative retention between 2.1 and 2.4*: not more than 4 impurities; for 1 impurity, maximum 3.5 per cent; for each other impurity, maximum 1.0 per cent;
- *impurity L*: maximum 0.4 per cent;
- *any other impurity*: for each impurity, maximum 0.4 per cent;
- *total*: maximum 7.0 per cent;
- *reporting threshold*: 0.2 per cent; disregard the peaks due to erythromycins B and C and any peak with a relative retention to erythromycin A of less than 0.3 (hydrolysis peaks).

**Free erythromycin**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.250 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution.* Dissolve 75.0 mg of *erythromycin A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 25.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a solution containing 3.4 g/L of *potassium dihydrogen phosphate R* and 2.0 g/L of *triethylamine R*, previously adjusted to pH 3.0 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 195 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of erythromycin A for the reference solution and 4.5 times the retention time of the 1st peak of erythromycin propionate for the test solution.

*Retention time*: erythromycin A = about 5 min; 1st peak of erythromycin propionate = about 10 min.

*Limit*:

- *free erythromycin*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (6.0 per cent).

**Dodecyl sulfate**: 23.0 per cent to 25.5 per cent of $C_{12}H_{26}O_4S$ (anhydrous substance).

Dissolve 0.500 g in 25 mL of *dimethylformamide R*. Titrate with *0.1 M sodium methoxide* using 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* as indicator.

1 mL of *0.1 M sodium methoxide* is equivalent to 26.64 mg of $C_{12}H_{26}O_4S$.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

Use a 100 g/L solution of *imidazole R* in *anhydrous methanol R* as the solvent.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solutions (a) and (b).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 2.0 for the peak due to erythromycin A;

– *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of erythromycin A ($C_{37}H_{67}NO_{13}$) using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B ($C_{37}H_{67}NO_{12}$) and erythromycin C ($C_{36}H_{65}NO_{13}$) using the chromatogram obtained with reference solution (b). Express the result as erythromycin A estolate, erythromycin B estolate and erythromycin C estolate by multiplying the percentage content of erythromycin A by 1.4387, the percentage content of erythromycin B by 1.4485 and the percentage content of erythromycin C by 1.4472.

For the calculation of content of erythromycin estolate, use the sum of erythromycins A, B and C expressed as estolates as described above.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, J, K, M, N.*

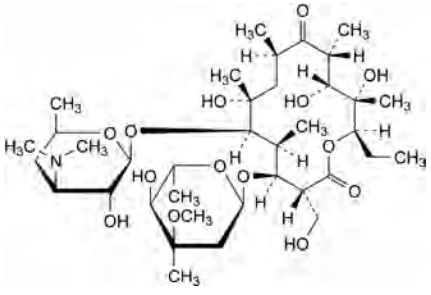

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin F),

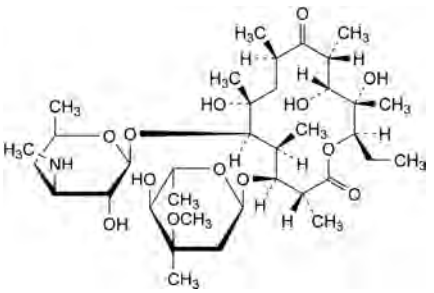

B. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethylerythromycin A),

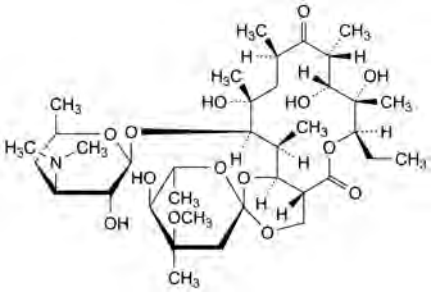

C. (2*S*,4a*R*,4′*R*,5′*S*,6′*S*,7*R*,8*S*,9*R*,10*R*,12*R*,14*R*,15*R*,16*S*,16a*S*)-7-ethyl-5′,8,9,14-tetrahydroxy-4′-methoxy-4′,6′,8,10,12,14,16-heptamethyl-15-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-hexadecahydrospiro[5*H*,11*H*-1,3-dioxino[5,4-*c*]oxacyclotetradecin-2,2′-pyrane]-5,11-dione (erythromycin E),

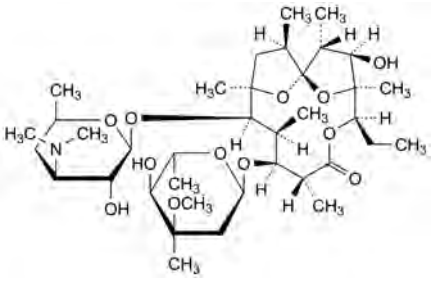

D. (1*S*,2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadecan-7-one (anhydroerythromycin A),

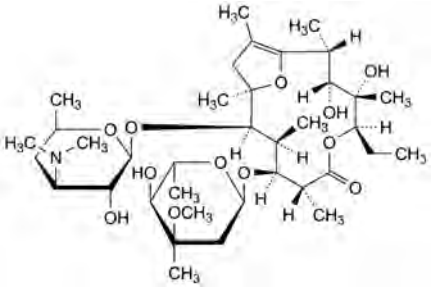

E. (2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3,4-dihydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15-dioxabicyclo[10.2.1]pentadec-1(14)-en-7-one (erythromycin A enol ether),

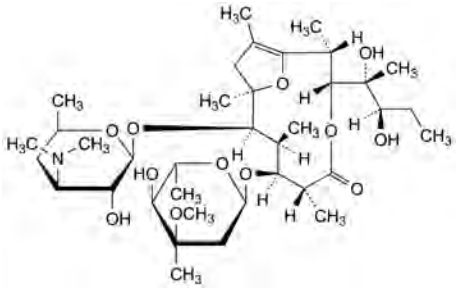

F. (2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridec-1(12)-en-5-one (pseudoerythromycin A enol ether),

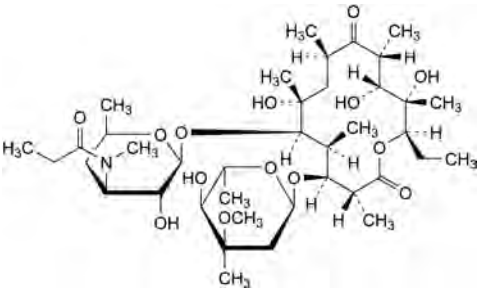

G. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylpropanoylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-propanoylerythromycin A),

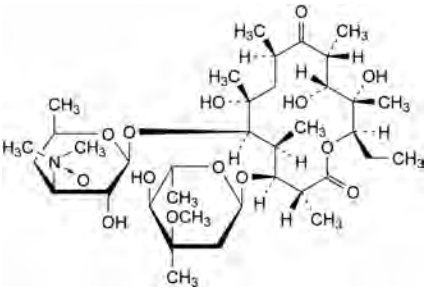

H. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione *N*-oxide (erythromycin A 3″-*N*-oxide),

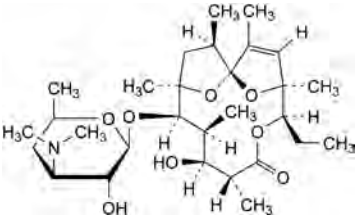

I. (1*S*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-5-ethyl-9-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadec-2-en-7-one (erythralosamine),

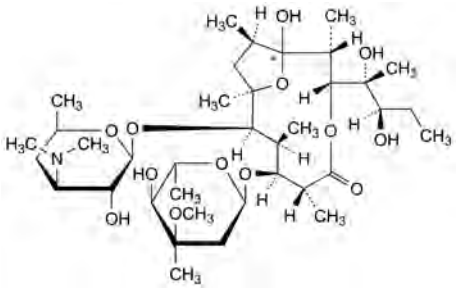

J. (1*RS*,2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-1-hydroxy-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridecan-5-one (pseudoerythromycin A hemiketal),

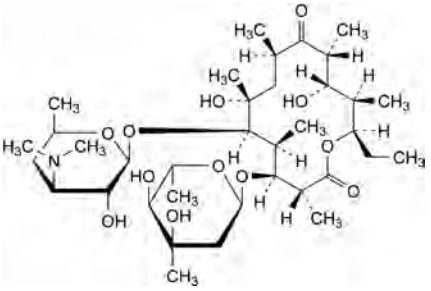

K. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin D),

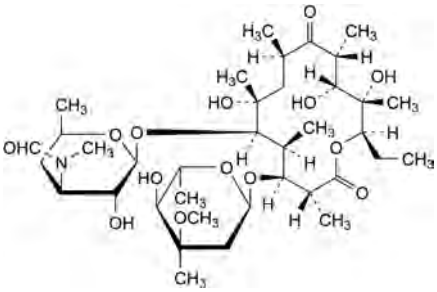

L. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(formylmethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-formylerythromycin A),

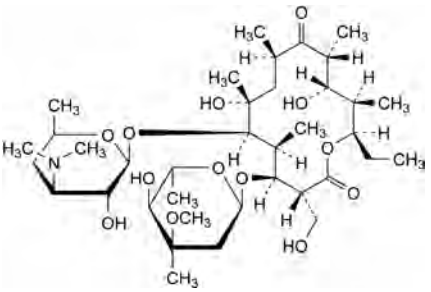

M. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin G),

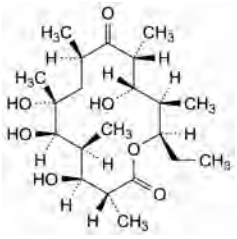

N. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-14-ethyl-4,6,7,12-tetrahydroxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione (erythronolide B).

**05/2017:0274**

# ERYTHROMYCIN ETHYLSUCCINATE

## Erythromycini ethylsuccinas

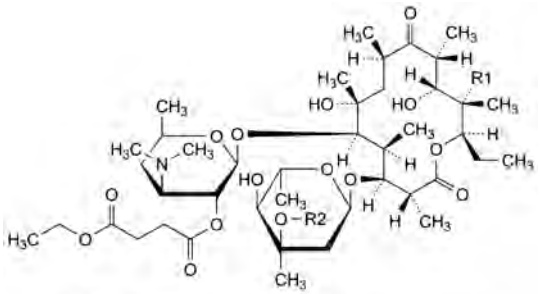

| Erythromycin (ethylsuccinate) | Mol. Formula | *M*r | R1 | R2 |
|---|---|---|---|---|
| A | $C_{43}H_{75}NO_{16}$ | 862 | OH | $CH_3$ |
| B | $C_{43}H_{75}NO_{15}$ | 846 | H | $CH_3$ |
| C | $C_{42}H_{73}NO_{16}$ | 848 | OH | H |

### DEFINITION

Mixture of the ethylsuccinate esters of erythromycin.

*Main component*: (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-2-*O*-(4-ethoxy-4-oxobutanoyl)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A 2″-(ethyl succinate)).

Semi-synthetic product derived from a fermentation product obtained using a strain of *Streptomyces erythreus*.

*Content*:

- *sum of erythromycins A, B and C expressed as ethylsuccinates*: 93.0 per cent to 102.0 per cent (anhydrous substance);
- *erythromycin B ethylsuccinate*: maximum 5.0 per cent (anhydrous substance);
- *erythromycin C ethylsuccinate*: maximum 5.0 per cent (anhydrous substance).

### CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol and in methanol.

### IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *erythromycin ethylsuccinate CRS*.

### TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Hydrolysis solution*. A 20 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 8.0 with *phosphoric acid R*.

*Test solution*. Dissolve 0.115 g of the substance to be examined in 25 mL of *methanol R*. Add 20 mL of the hydrolysis solution, mix and allow to stand at room temperature for at least 12 h. Dilute to 50.0 mL with the hydrolysis solution.

*Reference solution (a)*. Dissolve 40.0 mg of *erythromycin A CRS* in 10 mL of *methanol R* and dilute to 20.0 mL with the hydrolysis solution.

*Reference solution (b)*. Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in 50 mL of *methanol R*. Add 5.0 mL of reference solution (a) and dilute to 100.0 mL with the hydrolysis solution.

*Reference solution (c)*. Dissolve 2 mg of *N-demethylerythromycin A CRS* in 20 mL of reference solution (b).

*Reference solution (d)*. Dilute 3.0 mL of reference solution (a) to 100.0 mL with a mixture of equal volumes of *methanol R* and the hydrolysis solution.

*Reference solution (e)*. Dissolve 40 mg of *erythromycin A CRS*, previously heated at 130 °C for 3 h, in 10 mL of *methanol R* and dilute to 20 mL with the hydrolysis solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm) with a pore size of 100 nm;
- *temperature*: 70 °C using a water-bath for the column and at least one-third of the tubing preceding the column.

*Mobile phase*: to 50 mL of a 35 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 8.0 with *dilute phosphoric acid R*, add 400 mL of *water for chromatography R*, 165 mL of *2-methyl-2-propanol R* and 30 mL of *acetonitrile R1*, and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 200 µL of the test solution and reference solutions (a), (c), (d) and (e).

*Run time*: 5 times the retention time of erythromycin A; begin integration after the hydrolysis peak.

*Relative retention* with reference to erythromycin A (retention time = about 15 min): hydrolysis peak = less than 0.3; impurity B = about 0.45; erythromycin C = about 0.5; impurity C = about 0.9; impurity G = about 1.3; impurity D = about 1.4; impurity F = about 1.5; erythromycin B = about 1.8; impurity E = about 4.3.

*System suitability*: reference solution (c):

- *resolution*: minimum 0.8 between the peaks due to impurity B and erythromycin C and minimum 5.5 between the peaks due to impurity B and erythromycin A.

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.09; impurity F = 0.15; impurity G = 0.14; use the chromatogram obtained with reference solution (e) to identify the peaks due to impurities E and F;
- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (3.0 per cent);
- *total*: not more than 1.67 times the area of the principal peak in the chromatogram obtained with reference solution (d) (5.0 per cent);
- *disregard limit*: 0.02 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.06 per cent).

**Free erythromycin**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in *acetonitrile R1* and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dissolve 75.0 mg of *erythromycin A CRS* in *acetonitrile R1* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 25.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a solution containing 3.4 g/L of *potassium dihydrogen phosphate R* and 2.0 g/L of *triethylamine R*, previously adjusted to pH 3.0 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 195 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of erythromycin A (retention time = about 8 min) for the reference solution; twice the retention time of erythromycin ethylsuccinate (retention time = about 24 min) for the test solution.

*Limit*:

- *free erythromycin*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (6.0 per cent).

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.300 g.

Use a 100 g/L solution of *imidazole R* in *anhydrous methanol R* as the solvent.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use (apart from the test solution).*

*Solution A* (hydrolysis solution). Dissolve 11.5 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture*: *methanol R*, solution A (40:60 *V/V*).

*Test solution.* Dissolve 11.5 mg of the substance to be examined in 2.5 mL of *methanol R*. Add 2 mL of solution A, mix and allow to stand at room temperature for at least 12 h. Dilute to 5.0 mL with solution A.

*Reference solution (a).* Dissolve 40.0 mg of *erythromycin A CRS* in 10.0 mL of *methanol R* and dilute to 20.0 mL with solution A.

*Reference solution (b).* Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in 50.0 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 65 °C; preheating the mobile phase may be required, for instance by extending the inlet tubing in the oven to 30 cm.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 7.0 R7*, *acetonitrile R1*, *water for chromatography R* (5:35:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution pH 7.0 R7*, *water for chromatography R*, *acetonitrile R1* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 100 | 0 |
| $t_R$ - ($t_R$ + 2) | 100 → 0 | 0 → 100 |
| ($t_R$ + 2) - ($t_R$ + 15) | 0 | 100 |

$t_R$ = retention time of erythromycin B, determined by injecting 20 µL of reference solution (b) and eluting with mobile phase A

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 200 µL.

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.0 for the peak due to erythromycin A;
- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of erythromycin A ($C_{37}H_{67}NO_{13}$) using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B ($C_{37}H_{67}NO_{12}$) and erythromycin C ($C_{36}H_{65}NO_{13}$) using the chromatogram obtained with reference solution (b).

Express the results as erythromycin A ethylsuccinate, erythromycin B ethylsuccinate and erythromycin C ethylsuccinate by multiplying the percentage content of erythromycin A by 1.1744, the percentage content of erythromycin B by 1.1783 and the percentage content of erythromycin C by 1.1777.

For the calculation of content of erythromycin ethylsuccinate, use the sum of erythromycins A, B and C expressed as ethylsuccinates as described above.

STORAGE

In an airtight container, protected from light.

IMPURITIES

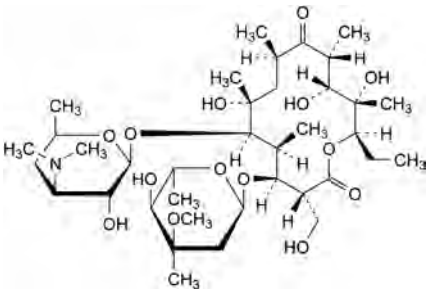

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin F),

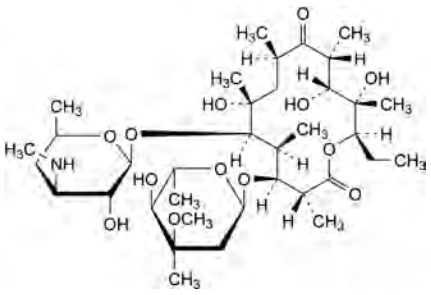

B. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethylerythromycin A),

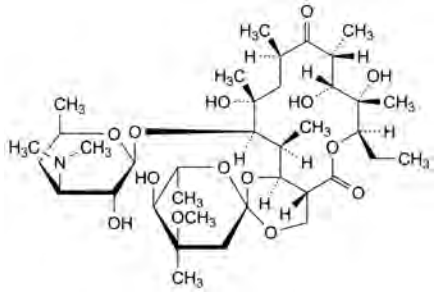

C. (2*S*,4a*R*,4′*R*,5′*S*,6′*S*,7*R*,8*S*,9*R*,10*R*,12*R*,14*R*,15*R*,16*S*,16a*S*)-7-ethyl-5′,8,9,14-tetrahydroxy-4′-methoxy-4′,6′,8,10,12,14,16-heptamethyl-15-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-hexadecahydrospiro[5*H*,11*H*-1,3-dioxino[5,4-*c*]oxacyclotetradecin-2,2′-pyrane]-5,11-dione (erythromycin E),

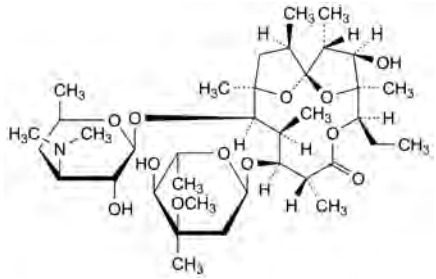

D. (1*S*,2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadecan-7-one (anhydroerythromycin A),

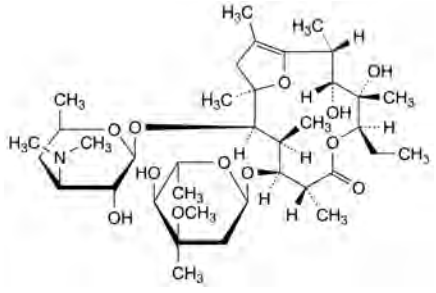

E. (2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3,4-dihydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15-dioxabicyclo[10.2.1]pentadec-1(14)-en-7-one (erythromycin A enol ether),

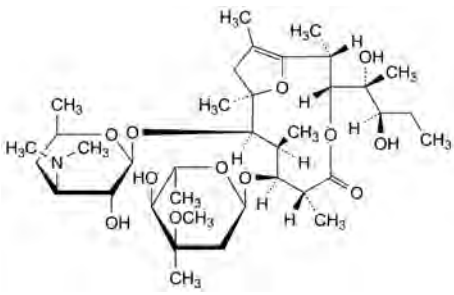

F. (2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridec-1(12)-en-5-one (pseudoerythromycin A enol ether),

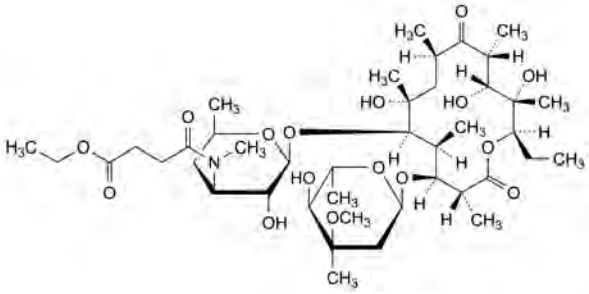

G. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-[(4-ethoxy-4-oxobutanoyl)methylamino]-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-(ethoxysuccinyl)erythromycin A).

**01/2017:1098**
**corrected 10.0**

# ERYTHROMYCIN LACTOBIONATE

## Erythromycini lactobionas

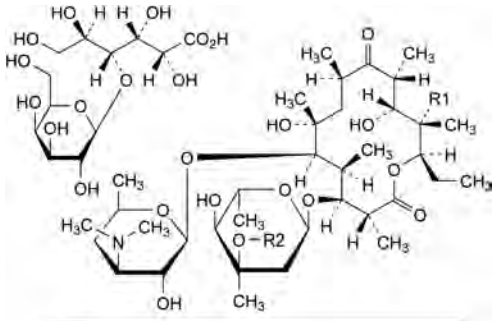

| Erythromycin (lactobionate) | Mol. Formula | $M_r$ | R1 | R2 |
|---|---|---|---|---|
| A | $C_{49}H_{89}NO_{25}$ | 1092 | OH | $CH_3$ |
| B | $C_{49}H_{89}NO_{24}$ | 1076 | H | $CH_3$ |
| C | $C_{48}H_{87}NO_{25}$ | 1078 | OH | H |

DEFINITION

Mixture of the lactobionate salts of erythromycin.

*Main component*: (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-

hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione 4-*O*-β-D-galactopyranosyl-D-gluconate (erythromycin A lactobionate).

Salt of a product obtained by fermentation using a strain of *Streptomyces erythreus*.

*Content*:

– *sum of erythromycins A, B and C expressed as lactobionates*: 93.0 per cent to 102.0 per cent (anhydrous substance);
– *erythromycin B lactobionate*: maximum 5.0 per cent (anhydrous substance);
– *erythromycin C lactobionate*: maximum 5.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, hygroscopic powder.

*Solubility*: soluble in water, freely soluble in anhydrous ethanol and in methanol, very slightly soluble in acetone and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *erythromycin lactobionate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in 20 mL of *water R*.

**pH** (*2.2.3*): 6.5 to 7.5.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 11.5 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture*: *methanol R*, solution A (40:60 *V/V*).

*Test solution*. Dissolve 60.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 40.0 mg of *erythromycin A CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 4 mg of *erythromycin for system suitability CRS* (containing impurities A, B, C, D, E, F, H and L) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
– *temperature*: 65 °C; preheating the mobile phase may be required, for instance by extending the inlet tubing in the oven to 30 cm.

*Mobile phase*:

– *mobile phase A*: *phosphate buffer solution pH 7.0 R7*, *acetonitrile R1*, *water R* (5:35:60 *V/V/V*);
– *mobile phase B*: *phosphate buffer solution pH 7.0 R7*, *water R*, *acetonitrile R1* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 100 | 0 |
| $t_R$ - ($t_R$ + 2) | 100 → 0 | 0 → 100 |
| ($t_R$ + 2) - ($t_R$ + 15) | 0 | 100 |

$t_R$ = retention time of erythromycin B, determined by injecting 10 µL of reference solution (b) and eluting with mobile phase A

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 100 µL.

*Identification of impurities*: use the chromatogram supplied with *erythromycin for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, E, F and L; use the chromatogram obtained with reference solution (b) to identify the peaks due to erythromycins B and C.

*Relative retention* with reference to erythromycin A (retention time = about 23 min): impurity A = about 0.4; impurity B = about 0.5; erythromycin C = about 0.55; impurity L = about 0.63; impurity C = about 0.9; impurity D = about 1.61; erythromycin B = about 1.75; impurity F = about 1.81; impurity E = about 2.3.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.2 between the peaks due to impurity B and erythromycin C;
– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin B; minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin A. If necessary, adjust the concentration of *acetonitrile R1* in the mobile phases and/or the gradient to obtain the required separation.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 2; impurity E = 0.08; impurity F = 0.08; impurity L = 0.11;
– for each impurity, use the concentration of erythromycin A in reference solution (c).

*Limits*:

– *impurity C*: maximum 3.0 per cent;
– *impurities A, B*: for each impurity, maximum 2.0 per cent;
– *impurities D, E, F*: for each impurity, maximum 1.0 per cent;
– *impurity L*: maximum 0.4 per cent,
– *any other impurity*: for each impurity, maximum 0.4 per cent;
– *total*: maximum 7.0 per cent;
– *reporting threshold*: 0.2 per cent; disregard the peaks due to erythromycins B and C.

**Free lactobionic acid**: maximum 1.0 per cent of $C_{12}H_{22}O_{12}$ (anhydrous substance).

Dissolve 0.400 g in 50 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Calculate the volume of *0.1 M sodium hydroxide* required per gram of the substance to be examined ($n_1$ mL). Dissolve 0.500 g in 40 mL of *anhydrous acetic acid R* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Calculate the volume of *0.1 M perchloric acid* required per gram of the substance to be examined ($n_2$ mL).

Calculate the percentage content of $C_{12}H_{22}O_{12}$ using the following expression:

$$3.580(n_1 - n_2)$$

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

Use a 100 g/L solution of *imidazole R* in *anhydrous methanol R* as the solvent.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solutions (a) and (b).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.0 for the peak due to erythromycin A;
- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of erythromycin A ($C_{37}H_{67}NO_{13}$) using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B ($C_{37}H_{67}NO_{12}$) and erythromycin C ($C_{36}H_{65}NO_{13}$) using the chromatogram obtained with reference solution (b).

Express the result as erythromycin A lactobionate, erythromycin B lactobionate and erythromycin C lactobionate by multiplying the percentage content of erythromycin A by 1.4877, the percentage content of erythromycin B by 1.4986 and the percentage content of erythromycin C by 1.4972.

For the calculation of content of erythromycin lactobionate, use the sum of erythromycins A, B and C expressed as lactobionates as described above.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I, J, K, M, N.*

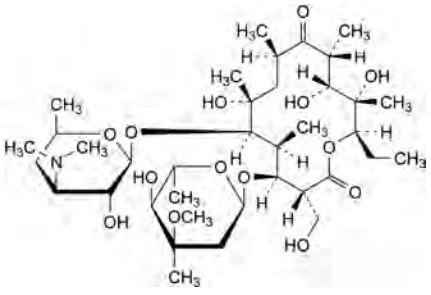

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin F),

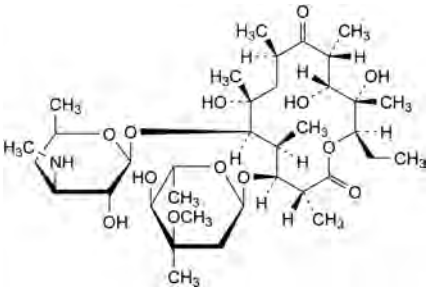

B. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethylerythromycin A),

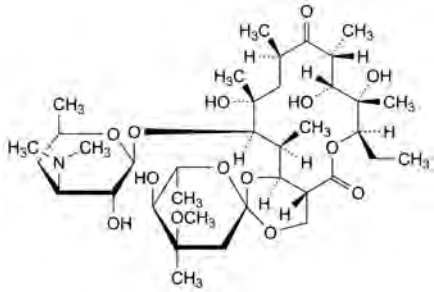

C. (2*S*,4a*R*,4′*R*,5′*S*,6′*S*,7*R*,8*S*,9*R*,10*R*,12*R*,14*R*,15*R*,16*S*)-16a*S*)-7-ethyl-5′,8,9,14-tetrahydroxy-4′-methoxy-4′,6′,8,10,12,14,16-heptamethyl-15-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-hexadecahydrospiro[5*H*,11*H*-1,3-dioxino[5,4-*c*]oxacyclotetradecin-2,2′-pyrane]-5,11-dione (erythromycin E),

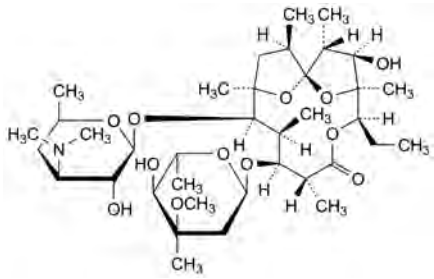

D. (1*S*,2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadecan-7-one (anhydroerythromycin A),

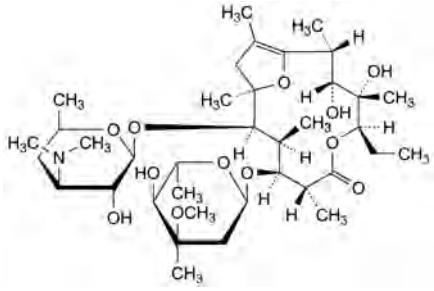

E. (2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3,4-dihydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15-dioxabicyclo[10.2.1]pentadec-1(14)-en-7-one (erythromycin A enol ether),

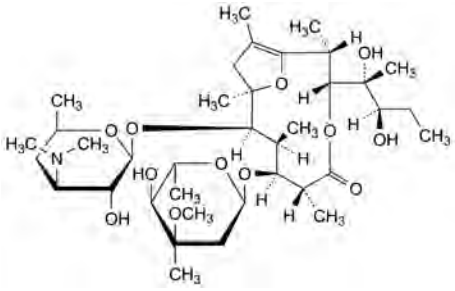

F. (2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridec-1(12)-en-5-one (pseudoerythromycin A enol ether),

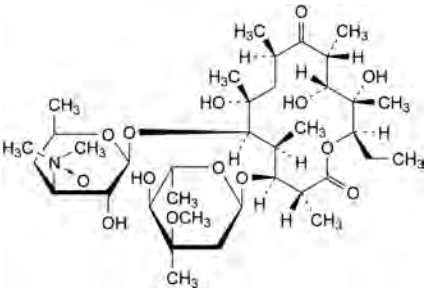

H. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione *N*-oxide (erythromycin A 3″-*N*-oxide),

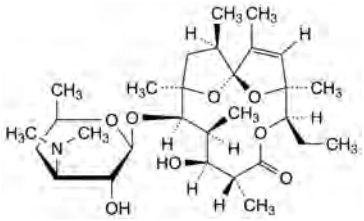

I. (1*S*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-5-ethyl-9-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadec-2-en-7-one (erythralosamine),

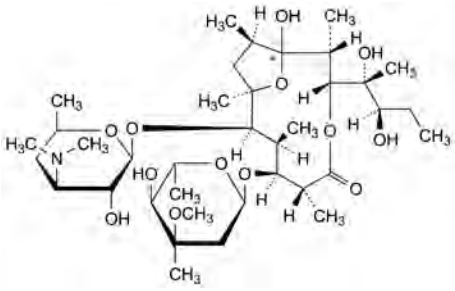

J. (1*RS*,2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-1-hydroxy-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridecan-5-one (pseudoerythromycin A hemiketal),

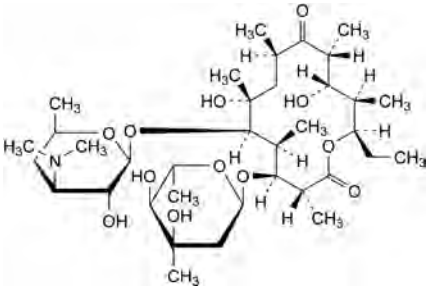

K. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin D),

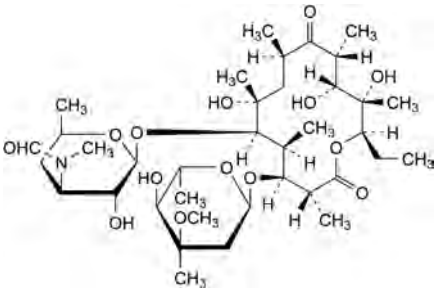

L. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(formylmethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-formylerythromycin A),

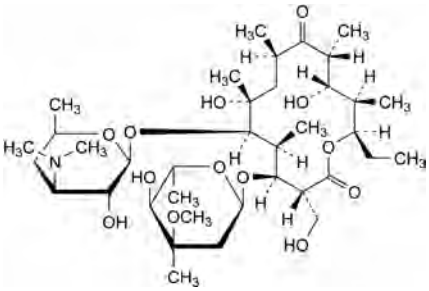

M. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin G),

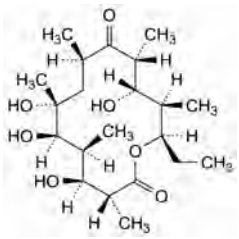

N. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-14-ethyl-4,6,7,12-tetrahydroxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione (erythronolide B).

**01/2017:0490**

# ERYTHROMYCIN STEARATE

## Erythromycini stearas

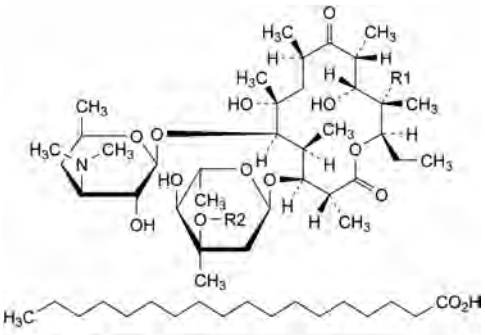

| Erythromycin (stearate) | Mol. Formula | $M_r$ | R1 | R2 |
|---|---|---|---|---|
| A | $C_{55}H_{103}NO_{15}$ | 1018 | OH | $CH_3$ |
| B | $C_{55}H_{103}NO_{14}$ | 1002 | H | $CH_3$ |
| C | $C_{54}H_{101}NO_{15}$ | 1004 | OH | H |

### DEFINITION

Mixture of the stearate salts of erythromycin and stearic acid.

*Main component*: (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione octadecanoate (erythromycin A stearate).

Salt of a product obtained by fermentation using a strain of *Streptomyces erythreus*.

*Content*:

- *sum of erythromycins A, B and C expressed as stearates*: 79.0 per cent to 102.0 per cent (anhydrous substance);
- *erythromycin B stearate*: maximum 5.0 per cent (anhydrous substance);
- *erythromycin C stearate*: maximum 5.0 per cent (anhydrous substance).

### CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methanol.

Solutions may be opalescent.

### IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *erythromycin stearate CRS*.

### TESTS

**Free stearic acid**: maximum 14.0 per cent (anhydrous substance) of $C_{18}H_{36}O_2$.

Dissolve 0.400 g in 50 mL of *methanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Calculate the volume of *0.1 M sodium hydroxide* required per gram of the substance to be examined ($n_1$ mL). Dissolve 0.500 g in 30 mL of *methylene chloride R*. If the solution is opalescent, filter and shake the residue with 3 quantities, each of 25 mL, of *methylene chloride R*. Filter, if necessary, and rinse the filter with *methylene chloride R*. Reduce the volume of the combined filtrate and rinsings to 30 mL by evaporation on a water-bath. Add 50 mL of *glacial acetic acid R* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Calculate the volume of *0.1 M perchloric acid* required per gram of the substance to be examined ($n_2$ mL).

Calculate the percentage content of $C_{18}H_{36}O_2$ using the following expression:

$$2.845(n_1 - n_2) \times \frac{100}{100 - h}$$

$h$ = percentage water content.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 11.5 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture*: *methanol R*, solution A (40:60 *V/V*).

*Test solution*. Dissolve 55.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Centrifuge and use the clear solution.

*Reference solution (a)*. Dissolve 40.0 mg of *erythromycin A CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10.0 mg of *erythromycin B CRS* and 10.0 mg of *erythromycin C CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 4 mg of *erythromycin for system suitability CRS* (containing impurities A, B, C, D, E, F, H and L) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (e)*. Dissolve 4 mg of *erythromycin stearate for impurity S identification CRS* in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 65 °C; preheating the mobile phase may be required, for instance by extending the inlet tubing in the oven to 30 cm.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 7.0 R7*, *acetonitrile R1*, *water R* (5:35:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution pH 7.0 R7*, *water R*, *acetonitrile R1* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 100 | 0 |
| $t_R$ - ($t_R$ + 2) | 100 → 0 | 0 → 100 |
| ($t_R$ + 2) - ($t_R$ + 15) | 0 | 100 |

$t_R$ = retention time of erythromycin B, determined by injecting 10 µL of reference solution (b) and eluting with mobile phase A

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 100 µL.

*Identification of impurities*: use the chromatogram supplied with *erythromycin for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, E, F and L; use the chromatogram supplied with *erythromycin stearate for impurity S identification CRS* and the chromatogram obtained with reference solution (e) to identify the peak due to impurity S; use the chromatogram obtained with reference solution (b) to identify the peaks due to erythromycins B and C.

*Relative retention* with reference to erythromycin A (retention time = about 23 min): impurity A = about 0.4; impurity B = about 0.5; erythromycin C = about 0.55; impurity L = about 0.63; impurity C = about 0.9; impurity D = about 1.61; erythromycin B = about 1.75; impurity F = about 1.81; impurity S = about 2.1; impurity E = about 2.3.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.2 between the peaks due to impurity B and erythromycin C;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin B; minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to erythromycin A. If necessary, adjust the concentration of *acetonitrile R1* in the mobile phases and/or the gradient to obtain the required separation.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 2; impurity E = 0.08; impurity F = 0.08; impurity L = 0.11;
- for each impurity, use the concentration of erythromycin A in reference solution (c).

*Limits*:

- *impurity C*: maximum 3.0 per cent;
- *impurities A, B*: for each impurity, maximum 2.0 per cent;
- *impurities D, E, F, S*: for each impurity, maximum 1.0 per cent;
- *impurity L*: maximum 0.4 per cent;
- *any other impurity*: for each impurity, maximum 0.4 per cent;
- *total*: maximum 7.0 per cent;
- *reporting threshold*: 0.2 per cent; disregard the peaks due to erythromycins B and C.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

Use a 100 g/L solution of *imidazole R* in *anhydrous methanol R* as the solvent.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solutions (a) and (b).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.0 for the peak due to erythromycin A;
- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

Calculate the percentage content of erythromycin A ($C_{37}H_{67}NO_{13}$) using the chromatogram obtained with reference solution (a). Calculate the percentage contents of erythromycin B ($C_{37}H_{67}NO_{12}$) and erythromycin C ($C_{36}H_{65}NO_{13}$) using the chromatogram obtained with reference solution (b).

Express the results as erythromycin A stearate, erythromycin B stearate and erythromycin C stearate by multiplying the percentage content of erythromycin A by 1.3869, the percentage content of erythromycin B by 1.3955 and the percentage content of erythromycin C by 1.3944.

For the calculation of content of erythromycin stearate, use the sum of erythromycins A, B and C expressed as stearates as described above.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, L, S.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I, J, K, M, N.*

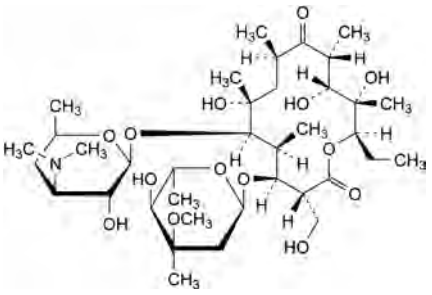

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin F),

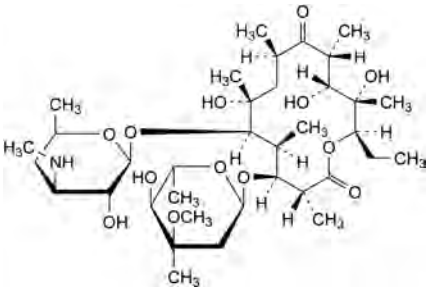

B. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(methylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethylerythromycin A),

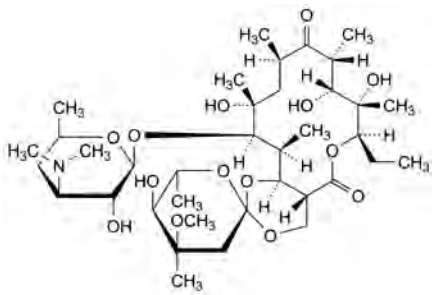

C. (2*S*,4a*R*,4′*R*,5′*S*,6′*S*,7*R*,8*S*,9*R*,10*R*,12*R*,14*R*,15*R*,16*S*,16a*S*)-7-ethyl-5′,8,9,14-tetrahydroxy-4′-methoxy-4′,6′,8,10,12,14,16-heptamethyl-15-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-hexadecahydrospiro[5*H*,11*H*-1,3-dioxino[5,4-*c*]oxacyclotetradecin-2,2′-pyrane]-5,11-dione (erythromycin E),

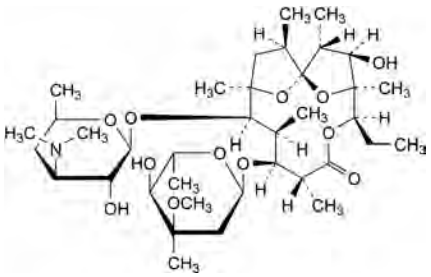

D. (1*S*,2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadecan-7-one (anhydroerythromycin A),

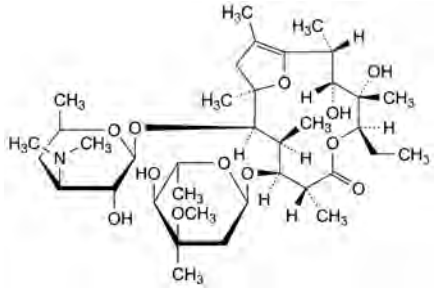

E. (2*R*,3*R*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*)-9-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-5-ethyl-3,4-dihydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15-dioxabicyclo[10.2.1]pentadec-1(14)-en-7-one (erythromycin A enol ether),

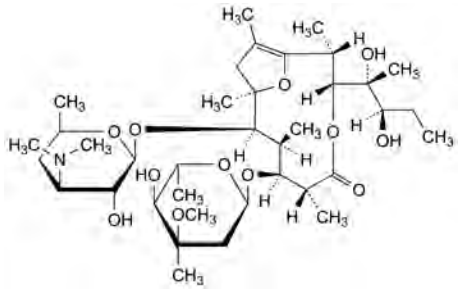

F. (2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridec-1(12)-en-5-one (pseudoerythromycin A enol ether),

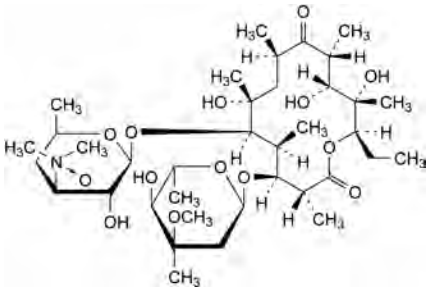

H. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione *N*-oxide (erythromycin A 3″-*N*-oxide),

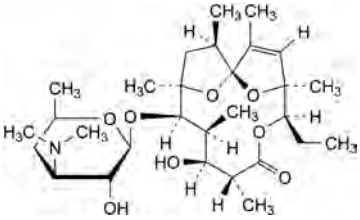

I. (1*S*,4*S*,5*R*,8*R*,9*S*,10*S*,11*R*,12*R*,14*R*)-5-ethyl-9-hydroxy-2,4,8,10,12,14-hexamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-6,15,16-trioxatricyclo[10.2.1.1$^{1,4}$]hexadec-2-en-7-one (erythralosamine),

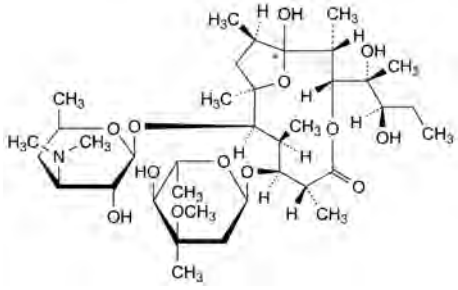

J. (1*RS*,2*R*,3*R*,6*R*,7*S*,8*S*,9*R*,10*R*,12*R*)-7-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-3-[(1*R*,2*R*)-1,2-dihydroxy-1-methylbutyl]-1-hydroxy-2,6,8,10,12-pentamethyl-9-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]-4,13-dioxabicyclo[8.2.1]tridecan-5-one (pseudoerythromycin A hemiketal),

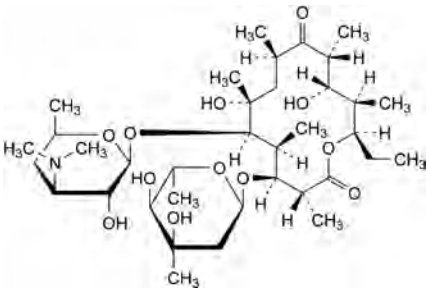

K. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin D),

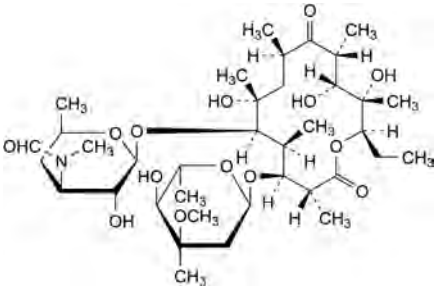

L. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(formylmethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (3″-*N*-demethyl-3″-*N*-formylerythromycin A),

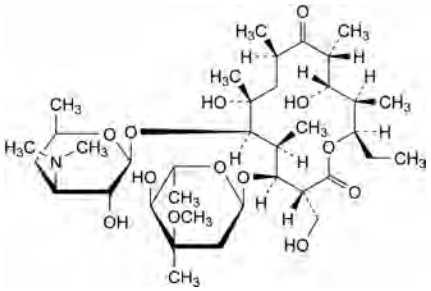

M. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3-(hydroxymethyl)-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin G),

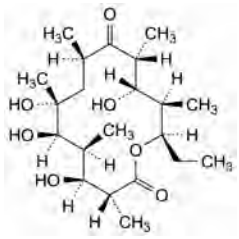

N. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*S*,13*R*,14*R*)-14-ethyl-4,6,7,12-tetrahydroxy-3,5,7,9,11,13-hexamethyloxacyclotetradecane-2,10-dione (erythronolide B),

S. unknown structure.

**01/2019:1316**
**corrected 10.0**

# ERYTHROPOIETIN CONCENTRATED SOLUTION

## Erythropoietini solutio concentrata

| | | | | |
|---|---|---|---|---|
| APPRLICDSR | VLERYLLEAK | EAENITTGCA | EHCSLNENIT | 40 |
| VPDTKVNFYA | WKRMEVGQQA | VEVWQGLALL | SEAVLRGQAL | 80 |
| LVNSSQPWEP | LQLHVDKAVS | GLRSLTTLLR | ALGAQKEAIS | 120 |
| PPDAASAAPL | RTITADTFRK | LFRVYSNFLR | GKLKLYTGEA | 160 |
| CRTGD | | | | 165 |

disulfide bridges: 7-161, 29-33

$M_r$ approx. 30 600

DEFINITION

Solution containing a family of closely-related glycoproteins which are indistinguishable from the naturally occurring human erythropoietin (urinary erythropoietin) in terms of amino acid sequence (165 amino acids) and average glycosylation pattern, at a concentration of 0.5-10 mg/mL. It may also contain buffer salts and other excipients. It has a potency of not less than 100 000 IU per milligram of active substance determined using the conditions described under Assay and in the test for protein.

PRODUCTION

Erythropoietin is produced in rodent cells *in vitro* by a method based on recombinant DNA technology.

Prior to release, the following tests are carried out on each batch of erythropoietin concentrated solution, unless exemption has been granted by the competent authority.

**Host cell-derived proteins**: the limit is approved by the competent authority.

**Host cell- and vector-derived DNA**: the limit is approved by the competent authority.

CHARACTERS

*Appearance*: clear or slightly turbid, colourless solution.

IDENTIFICATION

A. It gives the appropriate response when examined using the conditions described under Assay.

B. Capillary zone electrophoresis (*2.2.47*).

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of 1 mg/mL. Desalt 0.25 mL of the solution by passage through a micro-concentrator cartridge provided with a membrane with a molecular mass cut-off of not more than 10 000 Da. Add 0.2 mL of *water R* to the sample and desalt again. Repeat the desalting procedure once more. Dilute the sample with *water R*, determine its protein concentration as described under Tests and adjust to a concentration of approximately 1 mg/mL with *water R*.

*Reference solution.* Dissolve the contents of a vial of *erythropoietin for physicochemical tests CRS* in 0.10 mL of *water R*. Proceed with desalting as described for the test solution.

*Capillary*:

– *material*: uncoated fused silica;

– *size*: effective length = about 100 cm, Ø = 50 µm.

*Temperature*: 35 °C.

*CZE buffer concentrate (0.1 M sodium chloride, 0.1 M tricine, 0.1 M sodium acetate).* Dissolve 0.584 g of *sodium chloride R*, 1.792 g of *tricine R* and 0.820 g of *anhydrous sodium acetate R* in *water R* and dilute to 100.0 mL with the same solvent.

*1 M putrescine solution.* Dissolve 0.882 g of *putrescine R* in 10 mL of *water R*. Distribute in 0.5 mL aliquots.

*CZE buffer (0.01 M tricine, 0.01 M sodium chloride, 0.01 M sodium acetate, 7 M urea, 2.5 mM putrescine).* Dissolve 21.0 g of *urea R* in 25 mL of *water R* by warming in a water-bath at 30 °C. Add 5.0 mL of CZE buffer concentrate and 125 µL of 1 M putrescine solution. Dilute to 50.0 mL with *water R*. Using *dilute acetic acid R*, adjust to pH 5.55 at 30 °C and filter through a membrane filter (nominal pore size 0.45 µm).

*Detection*: spectrophotometer at 214 nm.

*Set the autosampler to store the samples at 4 °C during analysis.*

*Preconditioning of the capillary*: rinse the capillary for 60 min with *0.1 M sodium hydroxide* filtered through a membrane filter (nominal pore size 0.45 µm) and for 60 min with CZE buffer. Apply voltage for 12 h (20 kV).

*Between-run rinsing*: rinse the capillary for 10 min with *water R*, for 5 min with *0.1 M sodium hydroxide* filtered through a membrane filter (nominal pore size 0.45 µm) and for 10 min with CZE buffer.

*Injection*: under pressure or vacuum.

*Migration*: apply a field strength of 143 V/cm (15.4 kV for capillaries of 107 cm total length) for 80 min, using CZE buffer as the electrolyte in both buffer reservoirs.

*System suitability*: in the electropherogram obtained with the reference solution, a pattern of well separated peaks corresponding to the peaks in the electropherogram of erythropoietin supplied with *erythropoietin for physicochemical tests CRS* is seen, and the largest peak is at least 50 times greater than the baseline noise. If necessary, adjust the sample load to give peaks of sufficient height. Identify the peaks due to isoforms 1 to 8. Isoform 1 may not be visible. The peak due to isoform 8 is detected

and the resolution between the peaks due to isoforms 5 and 6 is not less than 1. Repeat the separation at least 3 times. The baseline is stable, showing little drift, and the distribution of peaks is qualitatively and quantitatively similar to the distribution of peaks in the electropherogram of erythropoietin supplied with *erythropoietin for physicochemical tests CRS*. The relative standard deviation of the migration time of the peak due to isoform 2 is less than 2 per cent.

*Limits*: identify the peaks due to isoforms 1 to 8 in the electropherogram obtained with the test solution by comparison with the electropherogram obtained with the reference solution. Calculate the percentage content of each isoform from the corresponding peak area. The percentages are within the following ranges:

| Isoform | Content (per cent) |
|---|---|
| 1 | 0 - 15 |
| 2 | 0 - 15 |
| 3 | 1 - 20 |
| 4 | 10 - 35 |
| 5 | 15 - 40 |
| 6 | 10 - 35 |
| 7 | 5 - 25 |
| 8 | 0 - 15 |

C. Polyacrylamide gel electrophoresis and immunoblotting.

(a) Polyacrylamide gel electrophoresis (*2.2.31*).

*Gel dimensions*: 0.75 mm thick, about 16 cm square.

*Resolving gel*: 12 per cent acrylamide.

*Sample buffer*: *concentrated SDS-PAGE sample buffer R*.

*Test solution (a).* Dilute the preparation to be examined in *water R* to obtain a concentration of 1.0 mg/mL. To 1 volume of this solution add 1 volume of sample buffer.

*Test solution (b).* Dilute the preparation to be examined in *water R* to obtain a concentration of 0.1 mg/mL. To 1 volume of this solution add 1 volume of sample buffer.

*Reference solution (a).* Dissolve the contents of a vial of *erythropoietin for physicochemical tests CRS* in 0.10 mL of *water R*. To 1 volume of this solution add 1 volume of sample buffer.

*Reference solution (b).* Dissolve the contents of a vial of *erythropoietin for physicochemical tests CRS* in *water R* and dilute with the same solvent to obtain a concentration of 0.1 mg/mL. To 1 volume of this solution add 1 volume of sample buffer.

*Reference solution (c).* A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa.

*Reference solution (d).* A solution of pre-stained molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa and suitable for the electrotransfer to an appropriate membrane.

*Sample treatment:* boil for 2 min.

*Application*: 20 µL, in the following order: reference solution (c), reference solution (a), test solution (a), empty well, reference solution (b), test solution (b), reference solution (d).

At the end of the separation, remove the gel-cassette from the apparatus and cut the gel into 2 parts: the first part containing reference solution (c), reference solution (a) and test solution (a); the second part containing reference solution (b), test solution (b) and reference solution (d).

*Detection*: by Coomassie staining on the first part of the gel.

*System suitability*: reference solution (c):

– the validation criteria are met.

*Results*: the electropherogram obtained with test solution (a) shows a single diffuse band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (a).

(b) Immunoblotting.

Transfer the second part of the gel onto a membrane suitable for the immobilisation of proteins, using commercially available electrotransfer equipment and following the manufacturer's instructions. After electrotransfer, incubate the membrane in a neutral isotonic buffer containing a suitable blocking agent (for example, 50 g/L of dried milk or 10 per cent *V/V* foetal calf serum), for 1-2 h, followed by incubation for 1-14 h in the same blocking solution with a suitable dilution of either a polyclonal or monoclonal anti-erythropoietin antibody. Detect erythropoietin-bound antibody using a suitable enzyme- or radiolabelled antibody (for example, an alkaline phosphatase-conjugated second antibody). The precise details of blocking agents, concentrations and incubation times should be optimised using the principles set out in *Immunochemical methods* (*2.7.1*).

*System suitability*: in the electropherogram obtained with reference solution (d), the molecular mass markers are resolved on the membrane into discrete bands, with a linear relationship between distance migrated and $\text{logarithm}_{10}$ of the molecular mass.

*Results*: the electropherogram obtained with test solution (b) shows a single broad band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution (b).

D. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Dilute the preparation to be examined in *tris acetate buffer solution pH 8.5 R* to a concentration of 1.0 mg/mL. Equilibrate the solution in *tris acetate buffer solution pH 8.5 R* using a suitable procedure (dialysis against *tris acetate buffer solution pH 8.5 R*, or membrane filtration using the procedure described under Identification B, but reconstituting the desalted sample with *tris acetate buffer solution pH 8.5 R*, are suitable). Transfer the dialysed solution to a polypropylene centrifuge tube. Freshly prepare a solution of *trypsin for peptide mapping R* at a concentration of 1 mg/mL in *water R*, and add 2 µL to 0.10 mL of the dialysed solution. Cap the tube and place in a water-bath at 37 °C for 18 h. Remove the sample from the water-bath and stop the reaction immediately by freezing.

*Reference solution.* Dissolve the contents of a vial of *erythropoietin for physicochemical tests CRS* in 0.10 mL of *water R*. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously, and under identical conditions.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*:

– *mobile phase A*: 0.06 per cent *V/V* solution of *trifluoroacetic acid R*;

– *mobile phase B*: to 100 mL of *water for chromatography R* add 0.6 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile R1*;

Monographs E

| Time (min) | Flow rate (mL/min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|---|
| 0 - 10 | 0.75 | 100 | 0 |
| 10 - 125 | 0.75 | 100 → 39 | 0 → 61 |
| 125 - 135 | 1.25 | 39 → 17 | 61 → 83 |
| 135 - 145 | 1.25 | 17 → 0 | 83 → 100 |
| 145 - 150 | 1.25 | 100 | 0 |

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 50 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of erythropoietin digest supplied with *erythropoietin for physicochemical tests CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

E. *N*-terminal sequence analysis.

The first 15 amino acids are: Ala - Pro - Pro - Arg - Leu - Ile - (no recovered peak) - Asp - Ser - Arg - Val - Leu - Glu - Arg - Tyr.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Desalt the equivalent of 50 µg of erythropoietin. For example, dilute a volume of the preparation to be examined equivalent to 50 µg of the active substance in 1 mL of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R*. Pre-wash a C18 reverse-phase sample preparation cartridge according to the instructions supplied and equilibrate the cartridge in a 0.1 per cent *V/V* solution of *trifluoroacetic acid R*. Apply the sample to the cartridge, and wash successively with a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* containing 0 per cent, 10 per cent and 50 per cent *V/V* of *acetonitrile R* according to the manufacturer's instructions. Lyophilise the 50 per cent *V/V acetonitrile R* eluate.

Redissolve the desalted sample in 50 µL of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* and couple to a sequencing cartridge using the protocol provided by the manufacturer. Run 15 sequencing cycles, using the reaction conditions for proline when running the 2nd and 3rd cycles.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino-acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids;
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

TESTS

**Protein** (*2.5.33, Method I*): 80 per cent to 120 per cent of the stated concentration.

*Test solution*. Dilute the preparation to be examined in a 4 g/L solution of *ammonium hydrogen carbonate R*.

Record the absorbance spectrum between 250 nm and 400 nm. Measure the value at the absorbance maximum (276-280 nm), after correction for any light scattering, measured up to 400 nm. Calculate the concentration of erythropoietin taking the specific absorbance to be 7.43.

**Dimers and related substances with molecular masses greater than that of erythropoietin**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined in the mobile phase to obtain a concentration of 0.2 mg/mL.

*Resolution solution*. Dissolve the contents of a vial of *erythropoietin for SEC system suitability CRS* in the mobile phase to obtain a concentration of 0.2 mg/mL.

*Reference solution*. Dilute 20 µL of the resolution solution to 1.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.5 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm), of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 20 000 to 200 000.

*Mobile phase*: dissolve 1.15 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium dihydrogen phosphate R* and 23.4 g of *sodium chloride R* in 900 mL of *water for chromatography R*, adjust to pH 7.4 if necessary, and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*Run time*: twice the retention time of erythropoietin monomer.

*Relative retention* with reference to erythropoietin monomer: erythropoietin dimer = about 0.9.

*System suitability*:

- the area of the principal peak in the chromatogram obtained with the reference solution is 1.5 per cent to 2.5 per cent of the area of the principal peak in the chromatogram obtained with the resolution solution;
- *resolution*: minimum 1.5 between the peaks due to erythropoietin dimer and erythropoietin monomer in the chromatogram obtained with the resolution solution.

*Limit*:

- *sum of the peaks eluted before the principal peak*: maximum 2.0 per cent; disregard any peak with a retention time greater than that of the principal peak.

**Sialic acids**: minimum 10 mol of sialic acids (calculated as *N*-acetylneuraminic acid) per mole of erythropoietin.

*Test solution (a)*. Dilute the preparation to be examined in the mobile phase used in the test for dimers and related substances with molecular masses greater than that of erythropoietin to obtain a concentration of 0.3 mg/mL.

*Test solution (b)*. To 0.5 mL of test solution (a) add 0.5 mL of the mobile phase used in the test for dimers and related substances with molecular masses greater than that of erythropoietin.

*Reference solution (a)*. Dissolve a suitable amount of *N-acetylneuraminic acid R* in *water R* to obtain a concentration of 0.1 mg/mL.

*Reference solution (b)*. To 0.8 mL of reference solution (a) add 0.2 mL of *water R*.

*Reference solution (c)*. To 0.6 mL of reference solution (a) add 0.4 mL of *water R*.

*Reference solution (d)*. To 0.4 mL of reference solution (a) add 0.6 mL of *water R*.

*Reference solution (e)*. To 0.2 mL of reference solution (a) add 0.8 mL of *water R*.

*Reference solution (f)*. Use *water R*.

Carry out the test in triplicate. Transfer 100 µL of each of the test and reference solutions to 10 mL glass test tubes. To each tube add 1.0 mL of *resorcinol reagent R*. Stopper the tubes and incubate at 100 °C for 30 min. Cool on ice. To each tube, add 2.0 mL of a mixture of 12 volumes of *butanol R* and 48 volumes of *butyl acetate R*. Mix vigorously, and allow the 2 phases to

separate. Ensuring that the upper phase is completely clear, remove the upper phase, taking care to exclude completely any of the lower phase. Measure the absorbance (*2.2.25*) of all samples at 580 nm. Using the calibration curve generated by the reference solutions, determine the content of sialic acids in test solutions (a) and (b) and calculate the mean. Calculate the number of moles of sialic acids per mole of erythropoietin assuming that the relative molecular mass of erythropoietin is 30 600 and that the relative molecular mass of *N*-acetylneuraminic acid is 309.

*System suitability*:

- the individual replicates agree to within ± 10 per cent of each other;
- the value obtained from reference solution (a) is between 1.5 and 3.3 times that obtained with test solution (a).

**Bacterial endotoxins** (*2.6.14*): less than 20 IU in the volume that contains 100 000 IU of erythropoietin.

ASSAY

The activity of the preparation is compared with that of *erythropoietin BRP* and expressed in International Units (IU).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency ($P$ = 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

Carry out the determination of potency by Method A or B.

**A. In polycythaemic mice**

The activity of the preparation is estimated by examining, under given conditions, its effect in stimulating the incorporation of $^{59}$Fe into circulating red blood cells of mice made polycythaemic by exposure to reduced atmospheric pressure.

The following schedule, using treatment in a hypobaric chamber, has been found to be suitable.

Induce polycythaemia in female mice of the same strain, weighing 16-18 g. Place the mice in a hypoxic chamber and reduce the pressure to 0.6 atmospheres. After 3 days at 0.6 atmospheres, further reduce the pressure to 0.4-0.5 atmospheres and maintain the animals at this pressure for a further 11 days (the partial vacuum is interrupted daily for a maximum of 1 h at about 11:00 a.m., in order to clean the cages and feed the animals). At the end of the specified period, return the mice to normal atmospheric conditions. Randomly distribute the mice into cages, each containing 6 animals, and mark them.

*Test solution (a).* Dilute the substance to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/mL.

*Test solution (b).* Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Test solution (c).* Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Reference solution (a).* Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 0.2 IU/mL.

*Reference solution (b).* Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Reference solution (c).* Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Radiolabelled ferric [$^{59}$Fe] chloride solution, concentrated.* Use a commercially available solution of [$^{59}$Fe]ferric chloride (approximate specific activity: 100-1000 MBq/mg of Fe).

*Radiolabelled [$^{59}$Fe]ferric chloride solution.* Dilute the concentrated radiolabelled [$^{59}$Fe]ferric chloride solution in *Sodium citrate buffer solution pH 7.8 (0.034 M sodium citrate, 0.101 M sodium chloride) R* to obtain a solution with an activity of $3.7 \times 10^4$ Bq/mL.

The concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

3 days after returning the animals to atmospheric pressure, inject each animal subcutaneously with 0.2 mL of one of the solutions. The 6 animals in each cage must each receive one of the 6 different treatments (3 test solutions and 3 reference solutions), and the order of injection must be separately randomised for each cage. A minimum of 8 cages is recommended. 2 days after injection of the test or reference solution, inject each animal intraperitoneally with 0.2 mL of radiolabelled [$^{59}$Fe]ferric chloride solution. The order of the injections must be the same as that of the erythropoietin injections, and the time interval between administration of the erythropoietin and the radiolabelled ferric chloride solution must be the same for each animal. After a further 48 h, anaesthetise each animal by injection of a suitable anaesthetic, record body weights and withdraw blood samples (0.65 mL) into haematocrit capillaries from the bifurcation of the aorta. After determining the packed cell volume for each sample, measure the radioactivity.

Calculate the response (percentage of iron-59 in total circulating blood) for each mouse using the expression:

$$\frac{A_s \times M \times 7.5}{A_t \times V_s}$$

$A_s$ = radioactivity in the sample;

$A_t$ = total radioactivity injected;

7.5 = total blood volume as per cent body weight;

$M$ = body weight, in grams;

$V_s$ = sample volume.

Calculate the potency by the usual statistical methods for a parallel line assay. Eliminate from the calculation any animal where the packed cell volume is less than 54 per cent, or where the body weight is more than 24 g.

**B. In normocythaemic mice**

The assay is based on the measurement of stimulation of reticulocyte production in normocythaemic mice.

The assay may be carried out using the following procedure:

*Test solution (a).* Dilute the preparation to be examined in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/mL.

*Test solution (b).* Mix equal volumes of test solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Test solution (c).* Mix equal volumes of test solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Reference solution (a).* Dissolve *erythropoietin BRP* in *phosphate-albumin buffered saline pH 7.2 R1* to obtain a concentration of 80 IU/mL.

*Reference solution (b).* Mix equal volumes of reference solution (a) and *phosphate-albumin buffered saline pH 7.2 R1*.

*Reference solution (c).* Mix equal volumes of reference solution (b) and *phosphate-albumin buffered saline pH 7.2 R1*.

The exact concentrations of the test solutions and reference solutions may need to be modified, based on the response range of the animals used.

At the beginning of the assay procedure, randomly distribute mice of a suitable age and strain (8-week old B6D2F1 mice are suitable) into 6 cages. A minimum of 8 mice per cage is recommended. Inject each animal subcutaneously with 0.5 mL of the appropriate treatment (one solution per cage) and put the animal in a new cage. Combine the mice in such a way that each cage housing the treated mice contains one mouse out of the 6 different treatments (3 test solutions and 3 reference solutions, 6 mice per cage). 4 days after the injections, collect blood samples from the animals and determine the number of reticulocytes using a suitable procedure.

The following method may be employed:

*The volume of blood, dilution procedure and fluorescent reagent may need to be modified to ensure maximum development and stability of fluorescence.*

*Colorant solution, concentrated.* Use a solution of thiazole orange suitable for the determination of reticulocytes. Prepare at a concentration twice that necessary for the analysis.

Proceed with the following dilution steps. Dilute whole blood 500-fold in the buffer used to prepare the colorant solution. Dilute this solution 2-fold in the concentrated colorant solution. After staining for 3-10 min, determine the reticulocyte count microfluorometrically in a flow cytometer. The percentage of reticulocytes is determined using a biparametric histogram: number of cells/red fluorescence (620 nm).

Calculate the potency by the usual statistical methods for a parallel line assay.

STORAGE

In an airtight container at a temperature below – 20 °C. Avoid repeated freezing and thawing.

LABELLING

The label states:

- the erythropoietin content in milligrams per millilitre;
- the activity in International Units per millilitre;
- the name and the concentration of any other excipients.

**01/2017:2758**

# ESCITALOPRAM

## Escitalopramum

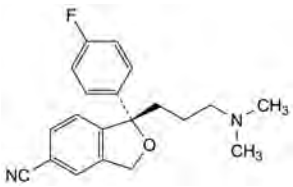


$C_{20}H_{21}FN_2O$ $M_r$ 324.4
[128196-01-0]

DEFINITION

(1*S*)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in toluene.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *escitalopram CRS*.

B. Enantiomeric purity (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 3.4 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*; adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 25 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *escitalopram for system suitability CRS* (containing impurity D) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, buffer solution (10:90 *V/V*);
- *mobile phase B*: buffer solution, *acetonitrile R* (35:65 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 2 | 95 | 5 | 1.0 |
| 2 - 37 | 95 → 65 | 5 → 35 | 1.0 |
| 37 - 47 | 65 → 0 | 35 → 100 | 1.0 |
| 47 - 62 | 0 | 100 | 2.0 |

*Detection*: spectrophotometer at 237 nm and at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *escitalopram for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to escitalopram (retention time = about 38 min): impurity D = about 0.98.

*System suitability*: reference solution (a) at 237 nm:

- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to escitalopram.

*Calculation of percentage contents*:

- for each impurity, use the concentration of escitalopram in reference solution (b) at 237 nm.

*Limits*:

- *unspecified impurities at 237 nm and at 254 nm*: for each impurity, maximum 0.10 per cent;
- *total at 237 nm*: maximum 0.5 per cent;
- *reporting threshold at 237 nm*: 0.05 per cent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *citalopram hydrobromide CRS* (containing equal amounts of impurity K and escitalopram) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *protein derivative of silica gel for chiral separation R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, *0.05 M phosphate buffer solution pH 7.0 R* (15:85 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 15 µL.

*Run time*: twice the retention time of escitalopram.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity K.

*Relative retention* with reference to escitalopram (retention time = about 11 min): impurity K = about 1.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.3 between the peaks due to escitalopram and impurity K;
- *symmetry factor*: maximum 4.0 for the peak due to escitalopram; maximum 4.0 for the peak due to impurity K.

*Limits*:

- *impurity K*: maximum 2.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *acetic anhydride R* and 75 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.44 mg of $C_{20}H_{21}FN_2O$.

IMPURITIES

*Specified impurities: K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, L, M.*

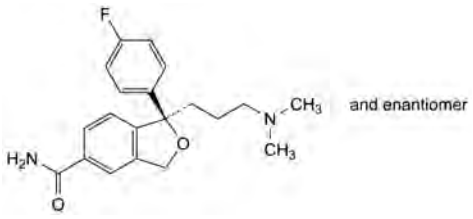


A. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carboxamide,

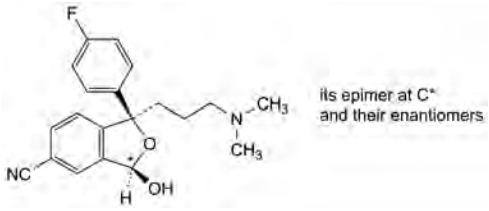


B. mixture of (1*RS*,3*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile and (1*RS*,3*SR*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile,

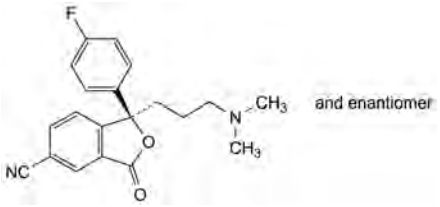


C. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-oxo-1,3-dihydro-2-benzofuran-5-carbonitrile,

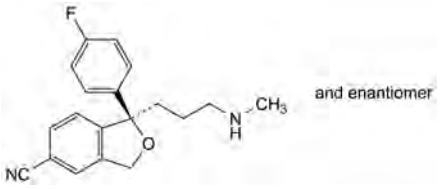


D. (1*RS*)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydro-2-benzofuran-5-carbonitrile,

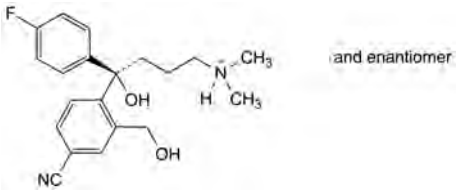


E. (4*RS*)-4-[4-cyano-2-(hydroxymethyl)phenyl]-4-(4-fluorophenyl)-4-hydroxy-*N*,*N*-dimethylbutan-1-aminium,

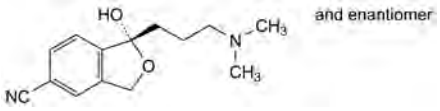


F. (1*RS*)-1-[3-(dimethylamino)propyl]-1-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile,

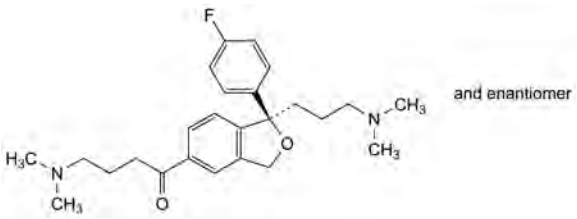


G. 4-(dimethylamino)-1-[(1*RS*)-1-[3-(dimethylamino)-propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-yl]butan-1-one,

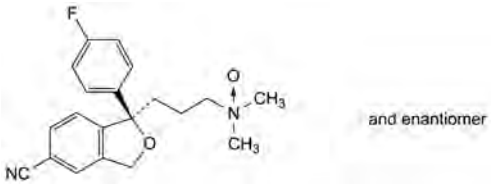


H. 3-[(1*RS*)-5-cyano-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine *N*-oxide,

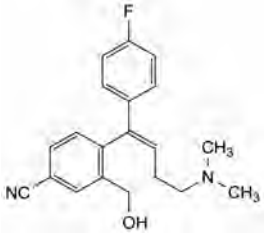


I. 4-[(1*Z*)-4-(dimethylamino)-1-(4-fluorophenyl)but-1-en-1-yl]-3-(hydroxymethyl)benzonitrile,

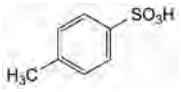

J. 4-methylbenzenesulfonic acid (*p*-toluenesulfonic acid),

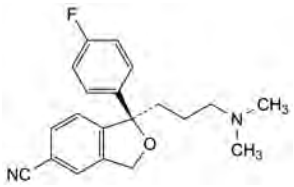

K. (1*R*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile,

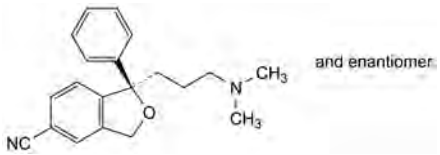


L. (1*RS*)-1-[3-(dimethylamino)propyl]-1-phenyl-1,3-dihydro-2-benzofuran-5-carbonitrile,

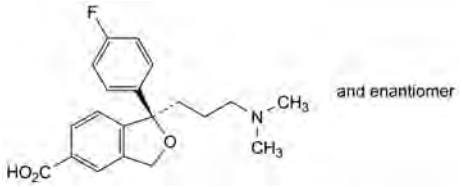


M. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carboxylic acid.

**07/2016:2733**

# ESCITALOPRAM OXALATE

## Escitaloprami oxalas

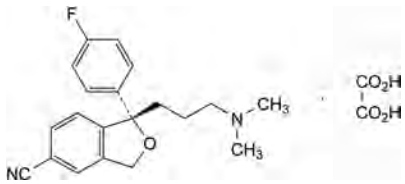

$C_{22}H_{23}FN_2O_5$ $M_r$ 414.4
[219861-08-2]

DEFINITION

(1*S*)-1-[3-(Dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile hydrogen oxalate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *escitalopram oxalate CRS*.

B. Enantiomeric purity (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 3.4 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1.0 L with *water R*.

*Test solution*. Dissolve 25 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 5 mg of *escitalopram for system suitability CRS* (containing impurity D) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R*, buffer solution (10:90 *V/V*);
- *mobile phase B*: buffer solution, *acetonitrile R* (35:65 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 2 | 95 | 5 | 1.0 |
| 2 - 37 | 95 → 65 | 5 → 35 | 1.0 |
| 37 - 47 | 65 → 0 | 35 → 100 | 1.0 |
| 47 - 62 | 0 | 100 | 2.0 |

*Detection*: spectrophotometer at 237 nm and at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *escitalopram for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to escitalopram (retention time = about 38 min): impurity D = about 0.98.

*System suitability*: reference solution (a) at 237 nm:
- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to escitalopram.

*Calculation of percentage contents*:
- for each impurity, use the concentration of escitalopram in reference solution (b) at 237 nm.

*Limits*:
- *unspecified impurities at 237 nm and at 254 nm*: for each impurity, maximum 0.10 per cent;
- *total at 237 nm*: maximum 0.5 per cent;
- *reporting threshold at 237 nm*: 0.05 per cent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *citalopram hydrobromide CRS* (containing equal amounts of impurity K and escitalopram) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *protein derivative of silica gel for chiral separation R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, *0.05 M phosphate buffer solution pH 7.0 R* (15:85 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 15 µL.

*Run time*: twice the retention time of escitalopram.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity K.

*Relative retention* with reference to escitalopram (retention time = about 11 min): impurity K = about 1.2.

*System suitability*: reference solution (a):
– *resolution*: minimum 1.3 between the peaks due to escitalopram and impurity K;
– *symmetry factor*: maximum 4.0 for the peak due to escitalopram; maximum 4.0 for the peak due to impurity K.

*Limits*:
– *impurity K*: maximum 2.0 per cent;
– *reporting threshold*: 0.10 per cent (reference solution (b)).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *acetic anhydride R* and 75 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.44 mg of $C_{22}H_{23}FN_2O_5$.

IMPURITIES

*Specified impurities: K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, L, M.*

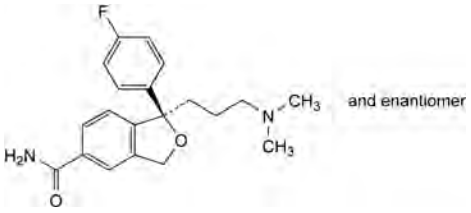


A. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carboxamide,

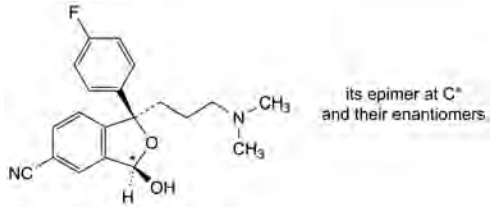


B. mixture of (1*RS*,3*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile and (1*RS*,3*SR*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile,

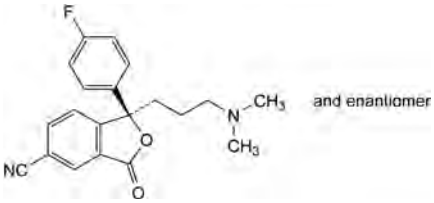


C. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-3-oxo-1,3-dihydro-2-benzofuran-5-carbonitrile,

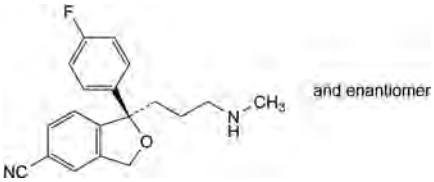


D. (1*RS*)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydro-2-benzofuran-5-carbonitrile,

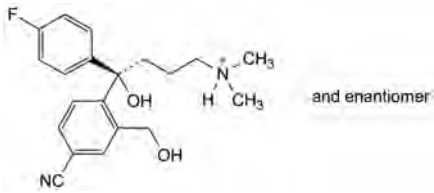


E. (4*RS*)-4-[4-cyano-2-(hydroxymethyl)phenyl]-4-(4-fluorophenyl)-4-hydroxy-*N*,*N*-dimethylbutan-1-aminium,

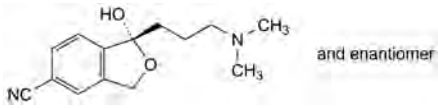


F. (1*RS*)-1-[3-(dimethylamino)propyl]-1-hydroxy-1,3-dihydro-2-benzofuran-5-carbonitrile,

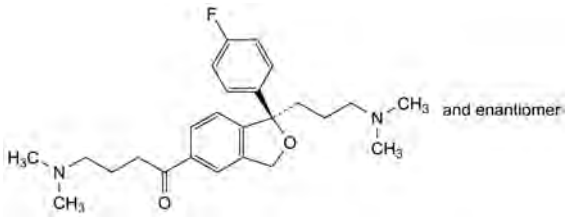


G. 4-(dimethylamino)-1-[(1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-yl]-butan-1-one,

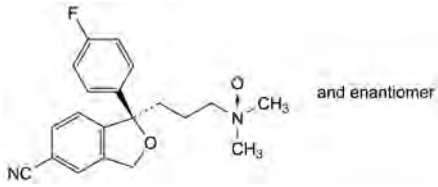


H. 3-[(1*RS*)-5-cyano-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-1-yl]-*N*,*N*-dimethylpropan-1-amine *N*-oxide,

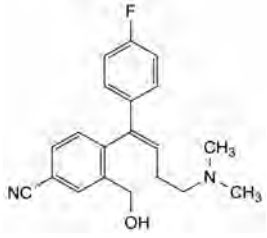


I. 4-[(1*Z*)-4-(dimethylamino)-1-(4-fluorophenyl)but-1-en-1-yl]-3-(hydroxymethyl)benzonitrile,

Monographs E

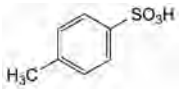

J. 4-methylbenzenesulfonic acid (*p*-toluenesulfonic acid),

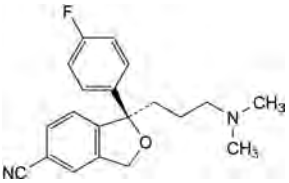

K. (1*R*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carbonitrile,

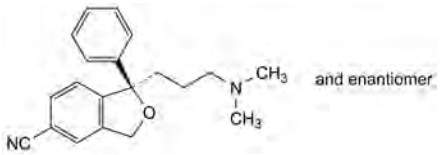

L. (1*RS*)-1-[3-(dimethylamino)propyl]-1-phenyl-1,3-dihydro-2-benzofuran-5-carbonitrile,

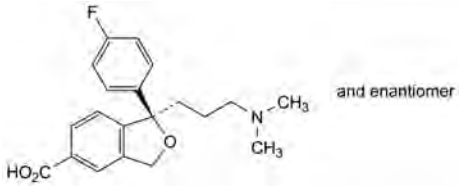

M. (1*RS*)-1-[3-(dimethylamino)propyl]-1-(4-fluorophenyl)-1,3-dihydro-2-benzofuran-5-carboxylic acid.

**01/2017:1742**
**corrected 9.3**

# ESKETAMINE HYDROCHLORIDE

## Esketamini hydrochloridum

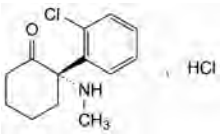

$C_{13}H_{17}Cl_2NO$ $M_r$ 274.2
[33643-47-9]

DEFINITION

(2*S*)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 85.0 to + 95.0.
Dilute 12.5 mL of solution S (see Tests) to 40.0 mL with *water R*.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of esketamine hydrochloride*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 8.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 4.5.
Dilute 12.5 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Impurity D**. Liquid chromatography (*2.2.29*).
*Test solution*. Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.
*Reference solution (a)*. Dissolve 5 mg of *esketamine impurity D CRS* in *water R*, add 20 mL of the test solution and dilute to 50 mL with *water R*. Dilute 10 mL of this solution to 100 mL with *water R*.
*Reference solution (b)*. Dilute 5.0 mL of the test solution to 25.0 mL with *water R*. Dilute 5.0 mL of this solution to 50.0 mL with *water R*.
*Reference solution (c)*. Dilute 2.5 mL of reference solution (b) to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.
*Precolumn*:
– *size*: *l* = 0.01 m, Ø = 3.0 mm;
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm);
– *temperature*: 30 °C.
*Column*:
– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm);
– *temperature*: 30 °C.
*Mobile phase*: mix 16 volumes of *methanol R* and 84 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with *potassium hydroxide R*.
*Flow rate*: 0.8 mL/min.
*Detection*: spectrophotometer at 215 nm.
*Injection*: 20 µL.
*Run time*: 20 min.
*Relative retention* with reference to esketamine (retention time = about 10 min): impurity D = about 1.3.
*System suitability*:
– *resolution*: minimum 2.0 between the peaks due to esketamine and impurity D in the chromatogram obtained with reference solution (a);
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c).
*Limit*:
– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.
*Reference solution (a)*. Dissolve 5 mg of *ketamine impurity A CRS* in the mobile phase (using ultrasound, if necessary) and dilute to 10 mL with the mobile phase. To 1 mL of the solution add 0.5 mL of the test solution and dilute to 100 mL with the mobile phase. Prepare immediately before use.
*Reference solution (b)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.95 g of *sodium hexanesulfonate R* in 1000 mL of a mixture of 25 volumes of *acetonitrile R* and 75 volumes of *water R* and add 4 mL of *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 10 times the retention time of esketamine.

*Relative retention* with reference to esketamine: impurity A = about 1.6; impurity B = about 3.3; impurity C = about 4.6.

*System suitability*: reference solution (a):

– *retention time*: esketamine = 3.0 min to 4.5 min;

– *resolution*: minimum 1.5 between the peaks due to impurity A and esketamine.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *methanol R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.42 mg of $C_{13}H_{17}Cl_2NO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

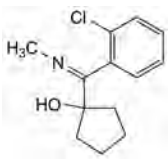

A. 1-[(2-chlorophenyl)(methylimino)methyl]cyclopentanol,

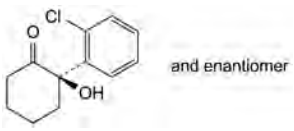


B. (2*RS*)-2-(2-chlorophenyl)-2-hydroxycyclohexanone,

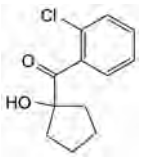

C. (2-chlorophenyl)(1-hydroxycyclopentyl)methanone,

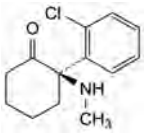

D. (2*R*)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone ((*R*)-ketamine).

**07/2014:2787**

# ESOMEPRAZOLE MAGNESIUM DIHYDRATE

## Esomeprazolum magnesicum dihydricum

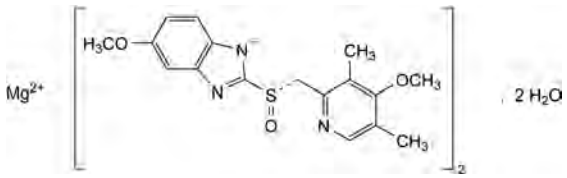

$C_{34}H_{36}MgN_6O_6S_2,2H_2O$ $M_r$ 749.2
[217087-10-0]

DEFINITION

Magnesium bis[5-methoxy-2-[(*S*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazol-1-ide] dihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly coloured powder, slightly hygroscopic.

*Solubility*: slightly soluble in water, soluble in methanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *esomeprazole magnesium dihydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Enantiomeric purity (see Tests).

C. Ignite about 0.5 g of the substance to be examined according to the procedure for the sulfated ash test (*2.4.14*). Dissolve the residue in 10 mL of *water R*. 2 mL of this solution gives the reaction of magnesium (*2.3.1*).

D. Water (see Tests).

TESTS

**Absorbance** (*2.2.25*): maximum 0.20 at 440 nm.

Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Buffer solution pH 6.0.* Mix 20 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and 70 mL of a 156.0 g/L solution of *sodium dihydrogen phosphate R*, then dilute to 1000 mL with *water R*. Dilute 250 mL of this solution to 1000 mL with *water R*.

*Buffer solution pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, then dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 40 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 50.0 mL with buffer solution pH 11.0. Dilute 1.0 mL of this solution to 25.0 mL with buffer solution pH 11.0.

*Reference solution.* Dissolve 2 mg of *omeprazole CRS* in buffer solution pH 11.0 and dilute to 50.0 mL with the same buffer solution. Dilute 1.0 mL of the solution to 10.0 mL with buffer solution pH 11.0.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.0 mm;
- *stationary phase*: $\alpha_1$*-acid-glycoprotein silica gel for chiral separation R* (5 µm).

*Mobile phase*: *acetonitrile R*, buffer solution pH 6.0 (13:87 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 20 µL.

*Relative retention* with reference to esomeprazole (retention time = about 5 min): impurity F = about 0.7.

*System suitability*: reference solution:

- *resolution*: minimum 3.0 between the peaks due to impurity F and esomeprazole.

*Limit*:

- *impurity F*: maximum 0.6 per cent; disregard any peak other than impurity F and esomeprazole.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 14 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 3 mg of *omeprazole for peak identification CRS* (containing impurity E) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 40 µL.

*Run time*: 4 times the retention time of esomeprazole.

*Identification of impurities*: use the chromatogram supplied with *omeprazole for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to esomeprazole (retention time = about 9 min): impurity E = about 0.4; impurity D = about 0.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity D and omeprazole.

*Limits*:

- *impurities D, E*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Magnesium**: 3.30 per cent to 3.55 per cent (anhydrous substance).

Dissolve 0.400 g in 25 mL of *methanol R*, sonicate until dissolution is complete. Add 25 mL of *water R*, 10 mL of *concentrated ammonia R*, 20.000 mL of *0.05 M sodium edetate* and about 50 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with *0.05 M zinc sulfate* until the colour changes from full blue to violet. Carry out a blank titration.

1 mL of *0.05 M sodium edetate* corresponds to 1.21525 mg of Mg.

**Water** (*2.5.12*): 4.5 per cent to 6.1 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer solution pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, then dilute to 100.0 mL with *water R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in about 10 mL of *methanol R*, add 10 mL of buffer solution pH 11.0 and dilute to 200.0 mL with *water R*.

*Reference solution.* Dissolve 10.0 mg of *omeprazole CRS* in about 10 mL of *methanol R*, add 10 mL of buffer solution pH 11.0 and dilute to 200.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of esomeprazole.

*Retention time*: esomeprazole = about 4 min.

Calculate the percentage content of $C_{34}H_{36}MgN_6O_6S_2$ taking into account the assigned content of *omeprazole CRS*.

1 g of omeprazole is equivalent to 1.032 g of esomeprazole magnesium.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

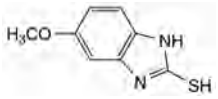

A. 5-methoxy-1*H*-benzimidazole-2-thiol,

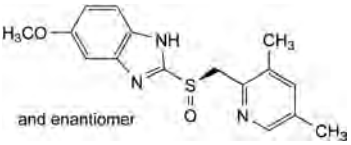


B. 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

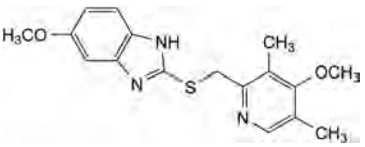

C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole (ufiprazole),

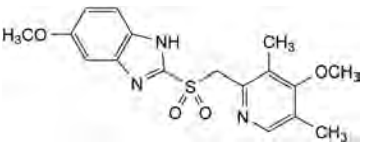

D. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole (omeprazole sulfone),

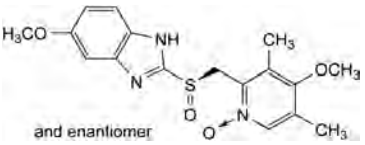


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide,

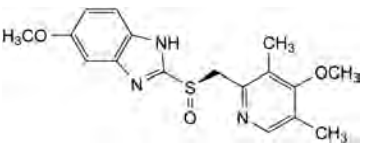

F. 5-methoxy-2-[(*R*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole ((*R*)-omeprazole).

**07/2014:2372**

# ESOMEPRAZOLE MAGNESIUM TRIHYDRATE

## Esomeprazolum magnesicum trihydricum

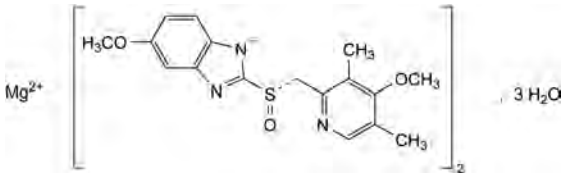

$C_{34}H_{36}MgN_6O_6S_2,3H_2O$ $M_r$ 767.2

[217087-09-7]

DEFINITION

Magnesium bis[5-methoxy-2-[(*S*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazol-1-ide] trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly coloured powder, slightly hygroscopic.

*Solubility*: slightly soluble in water, soluble in methanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *esomeprazole magnesium trihydrate CRS*.

B. Enantiomeric purity (see Tests).

C. Ignite about 0.5 g of the substance to be examined according to the procedure for the sulfated ash test (*2.4.14*). Dissolve the residue in 10 mL of *water R*. 2 mL of this solution gives the reaction of magnesium (*2.3.1*).

D. Water (see Tests).

TESTS

**Absorbance** (*2.2.25*): maximum 0.20 at 440 nm.

Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Buffer solution pH 6.0.* Mix 20 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and 70 mL of a 156.0 g/L solution of *sodium dihydrogen phosphate R*, then dilute to 1000 mL with *water R*. Dilute 250 mL of this solution to 1000 mL with *water R*.

*Buffer solution pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, then dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 40 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 50.0 mL with buffer solution pH 11.0. Dilute 1.0 mL of this solution to 25.0 mL with buffer solution pH 11.0.

*Reference solution.* Dissolve 2 mg of *omeprazole CRS* in buffer solution pH 11.0 and dilute to 50.0 mL with the same buffer solution. Dilute 1.0 mL of the solution to 10.0 mL with buffer solution pH 11.0.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.0 mm;

– *stationary phase*: $\alpha_1$*-acid-glycoprotein silica gel for chiral separation R* (5 µm).

*Mobile phase*: *acetonitrile R*, buffer solution pH 6.0 (13:87 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 20 µL.

*Relative retention* with reference to esomeprazole (retention time = about 5 min): impurity F = about 0.7.

*System suitability*: reference solution:

– *resolution*: minimum 3.0 between the peaks due to impurity F and esomeprazole.

*Limit*:

– *impurity F*: maximum 0.2 per cent; disregard any peak other than impurity F and esomeprazole.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 14 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 3 mg of *omeprazole for peak identification CRS* (containing impurity E) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 40 µL.

*Run time*: 4 times the retention time of esomeprazole.

*Identification of impurities*: use the chromatogram supplied with *omeprazole for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to esomeprazole (retention time = about 9 min): impurity E = about 0.4; impurity D = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity D and omeprazole.

*Limits*:

– *impurities D, E*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Magnesium**: 3.30 per cent to 3.55 per cent (anhydrous substance).

Dissolve 0.400 g in 25 mL of *methanol R*, sonicate until dissolution is complete. Add 25 mL of *water R*, 10 mL of *concentrated ammonia R*, 20.000 mL of *0.05 M sodium edetate* and about 50 mg of *mordant black 11 triturate R*. Titrate the excess of sodium edetate with *0.05 M zinc sulfate* until the colour changes from full blue to violet. Carry out a blank titration.

1 mL of *0.05 M sodium edetate* corresponds to 1.21525 mg of Mg.

**Water** (*2.5.12*): 6.2 per cent to 8.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer solution pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, then dilute to 100.0 mL with *water R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in about 10 mL of *methanol R*, add 10 mL of buffer solution pH 11.0 and dilute to 200.0 mL with *water R*.

*Reference solution.* Dissolve 10.0 mg of *omeprazole CRS* in about 10 mL of *methanol R*, add 10 mL of buffer solution pH 11.0 and dilute to 200.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of esomeprazole.

*Retention time*: esomeprazole = about 4 min.

Calculate the percentage content of $C_{34}H_{36}MgN_6O_6S_2$ taking into account the assigned content of *omeprazole CRS*.

1 g of omeprazole is equivalent to 1.032 g of esomeprazole magnesium.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

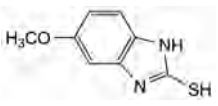


A. 5-methoxy-1*H*-benzimidazole-2-thiol,

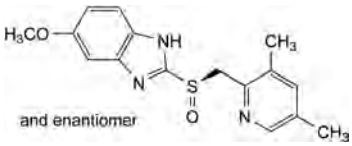


B. 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

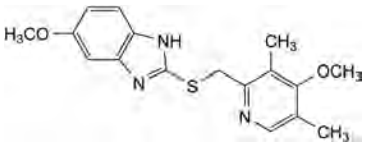


C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole (ufiprazole),

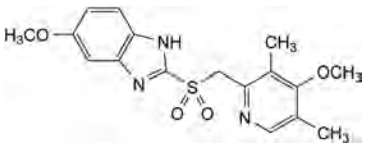


D. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole (omeprazole sulfone),

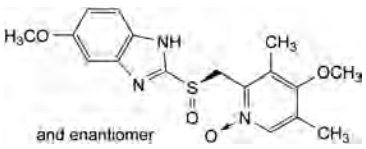


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide,

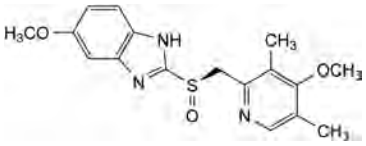


F. 5-methoxy-2-[(*R*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole ((*R*)-omeprazole).

**04/2019:2923**

# ESOMEPRAZOLE SODIUM

## Esomeprazolum natricum

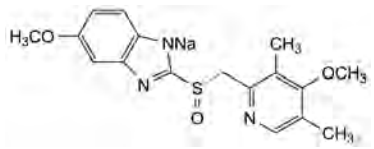


$C_{17}H_{18}N_3NaO_3S$ $M_r$ 367.4
[161796-78-7]

DEFINITION

Sodium 5-methoxy-2-[(*S*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methanesulfinyl]-1*H*-benzimidazol-1-ide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water and in ethanol (96 per cent), soluble in propylene glycol, very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *esomeprazole sodium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

B. Enantiomeric purity (see Tests).

C. Ignite 1 g and cool. Add 1 mL of *water R* to the residue and neutralise with *hydrochloric acid R*. Filter and dilute the filtrate to 4 mL with *water R*. 0.1 mL of the solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 10.3 to 11.3 for solution S.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Buffer solution pH 6.0.* Mix 20 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and 70 mL of a 156.0 g/L solution of *sodium dihydrogen phosphate R*, then dilute to 1000.0 mL with *water for chromatography R*. Dilute 250 mL of this solution to 1000.0 mL with *water for chromatography R*.

*Buffer solution pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, then dilute to 1000.0 mL with *water R*.

*Test solution.* Dissolve 40 mg of the substance to be examined in buffer solution pH 11.0 and dilute to 50.0 mL with the same buffer solution. Dilute 1.0 mL of this solution to 25.0 mL with buffer solution pH 11.0.

*Reference solution (a).* Dissolve 2 mg of *omeprazole CRS* in buffer solution pH 11.0 and dilute to 50 mL with the same buffer solution. Dilute 1 mL of the solution to 10 mL with buffer solution pH 11.0.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with buffer solution pH 11.0. Dilute 1.0 mL of this solution to 20.0 mL with buffer solution pH 11.0.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: $\alpha_1$*-acid-glycoprotein silica gel for chiral separation R* (5 µm).

*Mobile phase*: *acetonitrile R*, buffer solution pH 6.0 (15:85 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of esomeprazole.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to esomeprazole (retention time = about 4 min): impurity B = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.8 between the peaks due to impurity B and esomeprazole.

*Limit*:

– *impurity B*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent (reference solution (b)); disregard any peak other than impurity B and esomeprazole.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Buffer solution pH 7.6.* Dissolve 5.7 g of *disodium hydrogen phosphate dihydrate R* and 0.7 g of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent.

*Test solution.* Dissolve 10 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in mobile phase A and dilute to 50 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (3.5 µm).

*Mobile phase*:

– *mobile phase A*: mix 100 mL of *acetonitrile R* and 100 mL of buffer solution pH 7.6 and dilute to 1000.0 mL with *water for chromatography R*;

– *mobile phase B*: mix 10 mL of buffer solution pH 7.6 and 800 mL of *acetonitrile R* and dilute to 1000.0 mL with *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 12 | 100 → 80 | 0 → 20 |
| 12 - 32 | 80 → 0 | 20 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to esomeprazole (retention time = about 16 min): impurity D = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity D and omeprazole.

*Calculation of percentage contents*:

– for each impurity, use the concentration of esomeprazole sodium in reference solution (b).

*Limits*:

– *impurity D*: maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

ASSAY

Dissolve 0.300 g in 50 mL of *carbon dioxide-free water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 36.74 mg of $C_{17}H_{18}N_3NaO_3S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F, G.*

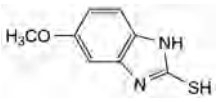

A. 5-methoxy-1*H*-benzimidazole-2-thiol,

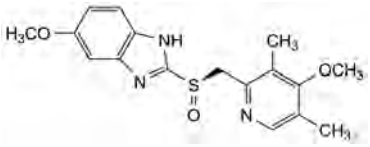

B. 5-methoxy-2-[(*R*)-(4-methoxy-3,5-dimethylpyridin-2-yl)methanesulfinyl]-1*H*-benzimidazole ((*R*)-omeprazole),

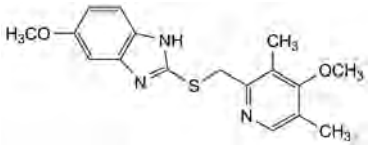

C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole (ufiprazole),

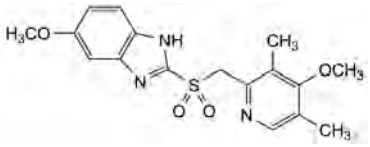

D. 5-methoxy-2-[(4-methoxy-3,5-dimethylpyridin-2-yl)methanesulfonyl]-1*H*-benzimidazole (omeprazole sulfone),

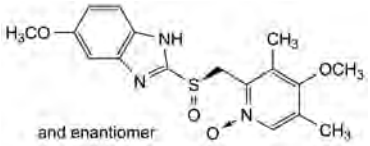


E. 4-methoxy-2-[[(*RS*)-5-methoxy-1*H*-benzimidazole-2-sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide,

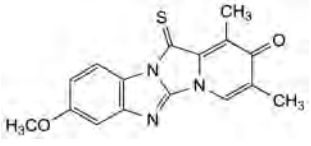

F. 8-methoxy-1,3-dimethyl-12-sulfanylidenepyrido[1′,2′:3,4]imidazo[1,2-a]benzimidazol-2(12*H*)-one,

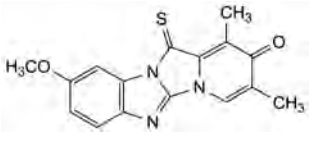

G. 9-methoxy-1,3-dimethyl-12-sulfanylidenepyrido[1′,2′:3,4]imidazo[1,2-*a*]benzimidazol-2(12*H*)-one.

**04/2008:0139**

# ESTRADIOL BENZOATE

## Estradioli benzoas

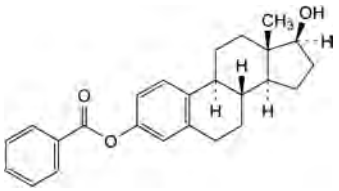

$C_{25}H_{28}O_3$ $M_r$ 376.5
[50-50-0]

DEFINITION

17β-Hydroxyestra-1,3,5(10)-trien-3-yl benzoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *estradiol benzoate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): + 55.0 to + 59.0 (dried substance).

Dissolve 0.250 g in *acetone R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *estradiol benzoate for system suitability CRS* (containing impurities A, B, C, E and G) in *acetonitrile R1* and dilute to 2.5 mL with the same solvent.

*Reference solution (b)*. Dilute 0.5 mL of the test solution to 100.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*, *acetonitrile R1* (40:60 *V/V*);
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 21 | 100 → 10 | 0 → 90 |
| 21 - 31 | 10 | 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *estradiol benzoate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, E and G.

*Relative retention* with reference to estradiol benzoate (retention time = about 19 min): impurity A = about 0.3; impurity E = about 1.1; impurity B = about 1.2; impurity G = about 1.3; impurity C = about 1.5.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to estradiol benzoate.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 3.3; impurity C = 0.7;
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, E, G*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity A*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 25.0 mg in *anhydrous ethanol R* and dilute to 250.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *anhydrous ethanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 231 nm.

Calculate the content of $C_{25}H_{28}O_3$ taking the specific absorbance to be 500.

IMPURITIES

*Specified impurities: A, B, C, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, H.*

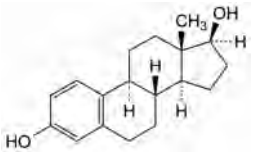

A. estra-1,3,5(10)-triene-3,17β-diol (estradiol),

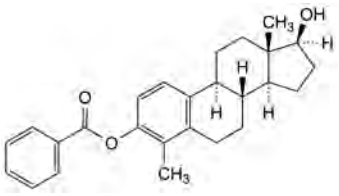

B. 17β-hydroxy-4-methylestra-1,3,5(10)-trien-3-yl benzoate,

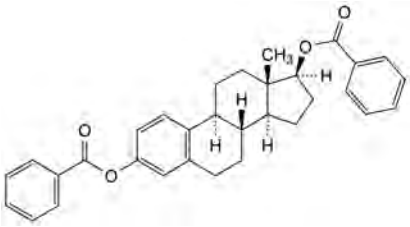

C. estra-1,3,5(10)-triene-3,17β-diyl dibenzoate,

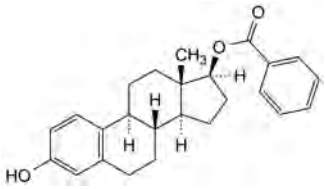

D. 3-hydroxyestra-1,3,5(10)-trien-17β-yl benzoate,

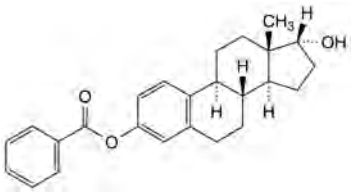

E. 17α-hydroxyestra-1,3,5(10)-trien-3-yl benzoate,

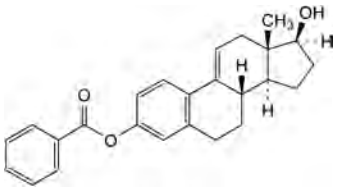

F. 17β-hydroxyestra-1,3,5(10),9(11)-tetraen-3-yl benzoate.

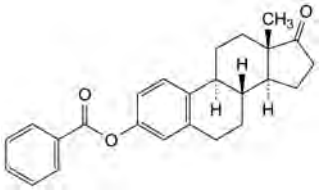

G. 17-oxoestra-1,3,5(10)-trien-3-yl benzoate (estrone benzoate),

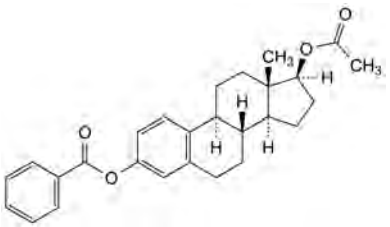

H. estra-1,3,5(10)-triene-3,17β-diyl 17-acetate 3-benzoate,

**04/2017:0821**

# ESTRADIOL HEMIHYDRATE

## Estradiolum hemihydricum

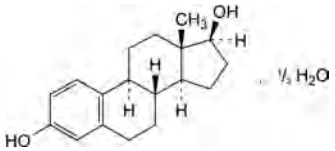

$C_{18}H_{24}O_2,½H_2O$ $M_r$ 281.4

DEFINITION

Estra-1,3,5(10)-triene-3,17β-diol hemihydrate.

*Content*: 97.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 175 °C to 180 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *estradiol hemihydrate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 50 mL with the same solvent.
*Reference solution (a)*. Dissolve 50 mg of *estradiol hemihydrate CRS* in *methanol R* and dilute to 50 mL with the same solvent.
*Reference solution (b)*. Dissolve 25 mg of *ethinylestradiol CRS* in reference solution (a) and dilute to 25 mL with reference solution (a).
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *ethanol (96 per cent) R*, *toluene R* (20:80 *V/V*).
*Application*: 5 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air until the solvent has evaporated.
*Detection*: heat at 110 °C for 10 min. Spray the hot plate with *alcoholic solution of sulfuric acid R*. Heat again at 110 °C for 10 min. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.
*System suitability*: the chromatogram obtained with reference solution (b) shows 2 spots which may however not be completely separated.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 1 mg add 0.5 mL of freshly prepared *sulfomolybdic reagent R2*. A blue colour develops which in ultraviolet light at 365 nm has an intense green fluorescence. Add 1 mL of *sulfuric acid R* and 9 mL of *water R*. The colour becomes pink with a yellowish fluorescence.

E. Water (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 76.0 to + 83.0 (anhydrous substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *acetonitrile R* and dilute to 25.0 mL with *methanol R2*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Mix equal volumes of a 1 mg/mL solution of the substance to be examined in *methanol R2* and of a 1 mg/mL solution of *2,3-dichloro-5,6-dicyanobenzoquinone R* in *methanol R2*. Allow to stand for 30 min before injection.

*Reference solution (c)*. Dissolve 5 mg of *estradiol for peak identification CRS* (containing impurities A, B and C) in 2 mL of *acetonitrile R* and dilute to 5.0 mL with *methanol R2*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: to 400 mL of *acetonitrile R* add 50 mL of *methanol R2* and 400 mL of *water for chromatography R*; allow to stand for 10 min, dilute to 1000 mL with *water for chromatography R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of estradiol.

*Identification of impurities*: use the chromatogram supplied with *estradiol for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to estradiol (retention time = about 13 min): impurity D = about 0.9; impurity B = about 1.1; impurity A = about 1.4; impurity C = about 1.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to estradiol and impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.4;
- *impurities A, B, C, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 2.9 per cent to 3.5 per cent, determined on 0.500 g.

ASSAY

Dissolve 20.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *0.1 M sodium hydroxide*. Allow to cool to room temperature. Measure the absorbance (*2.2.25*) of the solution at the maximum at 238 nm.

Calculate the content of $C_{18}H_{24}O_2$ taking the specific absorbance to be 335.

IMPURITIES

*Specified impurities: A, B, C, D.*

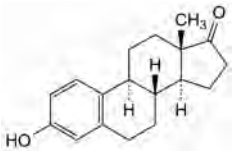

A. 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),

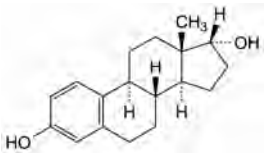

B. estra-1,3,5(10)-triene-3,17α-diol (17α-estradiol),

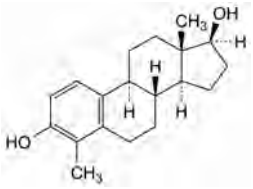

C. 4-methylestra-1,3,5(10)-triene-3,17β-diol,

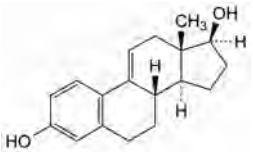

D. estra-1,3,5(10),9(11)-tetraene-3,17β-diol.

**04/2016:1614**

# ESTRADIOL VALERATE

## Estradioli valeras

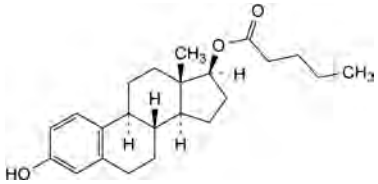

$C_{23}H_{32}O_3$ $M_r$ 356.5

[979-32-8]

DEFINITION

3-Hydroxyestra-1,3,5(10)-trien-17β-yl pentanoate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *estradiol valerate CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Solution S**. Dissolve 0.500 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 41 to + 47 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 20.0 mL with *acetonitrile R1*.

*Reference solution (a)*. Dissolve 3 mg of *estradiol valerate for system suitability CRS* (containing impurities A, C, D and E) in *acetonitrile R1* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R1*.

*Reference solution (c)*. Dissolve 50.0 mg of *estradiol valerate CRS* in *acetonitrile R1* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL of the solution to 20.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core alkylsilyl silica gel for chromatography R* (2.6 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 48 | 52 |
| 1 - 10 | 48 → 35 | 52 → 65 |
| 10 - 17.5 | 35 → 0 | 65 → 100 |
| 17.5 - 26 | 0 | 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 5 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *estradiol valerate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, C, D and E.

*Relative retention* with reference to estradiol valerate (retention time = about 9 min): impurity A = about 0.1; impurity C = about 0.9; impurity D = about 1.3; impurity E = about 1.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity C and estradiol valerate.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity C by 0.5;
- for each impurity, use the concentration of estradiol valerate in reference solution (b).

*Limits*:

- *impurity D*: maximum 0.4 per cent;
- *impurities A, C*: for each impurity, maximum 0.2 per cent;
- *impurity E*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{23}H_{32}O_3$ taking into account the assigned content of *estradiol valerate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, F, G, H, I, J.*

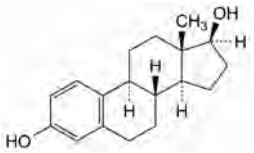


A. estra-1,3,5(10)-triene-3,17β-diol (estradiol),

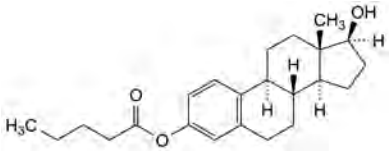


B. 17β-hydroxyestra-1,3,5(10)-trien-3-yl pentanoate,

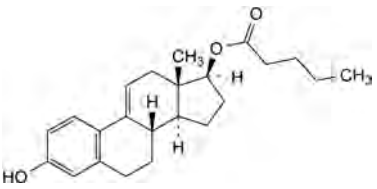


C. 3-hydroxyestra-1,3,5(10),9(11)-tetraen-17β-yl pentanoate,

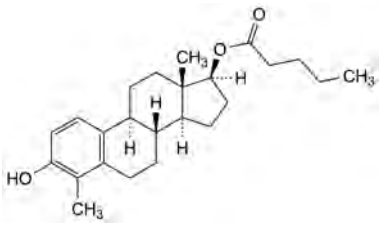

D. 3-hydroxy-4-methylestra-1,3,5(10)-trien-17β-yl pentanoate,

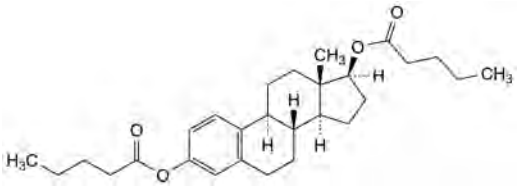

E. estra-1,3,5(10)-trien-3,17β-diyl dipentanoate,

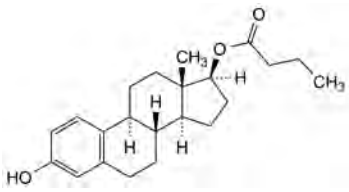

F. 3-hydroxyestra-1,3,5(10)-trien-17β-yl butanoate,

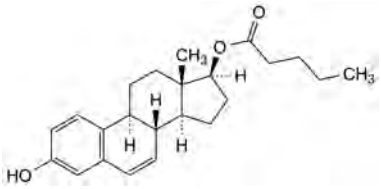

G. 3-hydroxyestra-1,3,5(10),6-tetraen-17β-yl pentanoate,

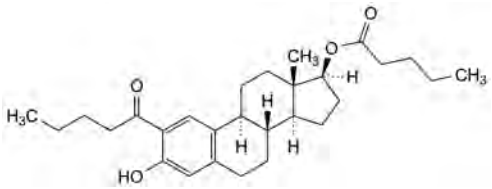

H. 3-hydroxy-2-pentanoylestra-1,3,5(10)-trien-17β-yl pentanoate,

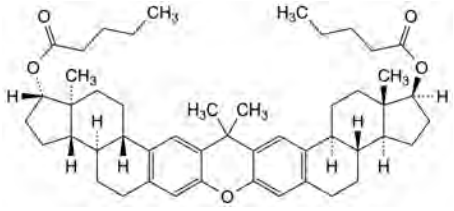

I. (1*S*,3a*S*,3b*R*,10a*R*,10b*S*,13*S*,13a*S*,15a*S*,18b*S*,20a*S*)-13a,17,17,20a-tetramethyl-2,3,3a,3b,4,5,9,10,10a,-10b,11,12,13,13a,14,15,15a,17,18b,19,20,20a-docosahydro-1*H*-bis(cyclopenta[5,6]naphtho)-[1,2-*b*:2′,1′-*i*]xanthene-1,13-diyl dipentanoate,

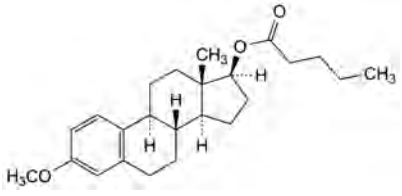

J. 3-methoxyestra-1,3,5(10)-trien-17β-yl pentanoate.

**07/2018:1203**

# ESTRIOL

## Estriolum

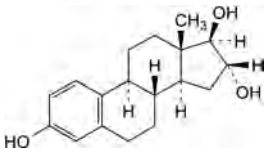

$C_{18}H_{24}O_3$ $M_r$ 288.4
[50-27-1]

DEFINITION

Estra-1,3,5(10)-triene-3,16α,17β-triol.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *estriol CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Specific optical rotation** (*2.2.7*): + 60 to + 65 (dried substance).

Dissolve 80 mg in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 25 mL of *methanol R* and dilute to 50.0 mL with *water R*.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *estriol for system suitability CRS* (containing impurities A, D, E and F) in 5 mL of *methanol R* and dilute to 10.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25.0 mg of *estriol CRS* in 25 mL of *methanol R* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2.1 mm;
– *stationary phase*: *end-capped, charged surface, ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R1*, *water for chromatography R* (28:72 *V/V*);
– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 100 | 0 |
| 9 - 23 | 100 → 57 | 0 → 43 |
| 23 - 28 | 57 | 43 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *estriol for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, D, E and F.

*Relative retention* with reference to estriol (retention time = about 11 min): impurity A = about 0.95; impurity F = about 1.45; impurity E = about 1.5; impurity D = about 1.8.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to estriol.

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity A by 0.5;
– for each impurity, use the concentration of estriol in reference solution (b).

*Limits*:

– *impurity F*: maximum 0.5 per cent;
– *impurity E*: maximum 0.3 per cent;
– *impurities A, D*: for each impurity, maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 5.5 | 90 → 30 | 10 → 70 |
| 5.5 - 7.5 | 30 | 70 |

*Injection*: 5 µL of test solution (b) and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to estriol (retention time = about 4 min): impurity A = about 0.9.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to estriol.

Calculate the percentage content of $C_{18}H_{24}O_3$ taking into account the assigned content of *estriol CRS*.

IMPURITIES

*Specified impurities: A, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, G, H, I, J, K.*

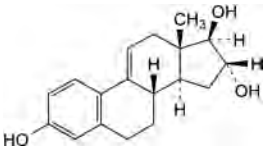

A. estra-1,3,5(10),9(11)-tetraene-3,16α,17β-triol (9,11-didehydroestriol),

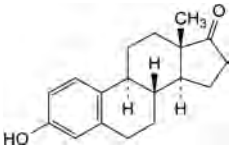

B. 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),

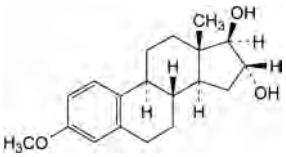

C. 3-methoxyestra-1,3,5(10)-triene-16α,17β-diol (estriol 3-methyl ether),

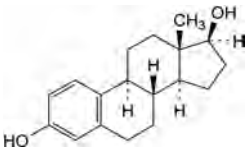

D. estra-1,3,5(10)-triene-3,17β-diol (estradiol),

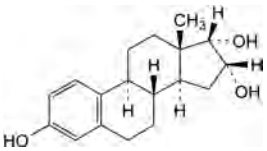

E. estra-1,3,5(10)-triene-3,16α,17α-triol (17-epi-estriol),

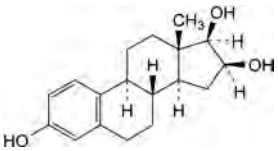

F. estra-1,3,5(10)-triene-3,16β,17β-triol (16-epi-estriol),

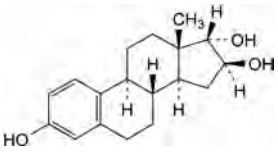

G. estra-1,3,5(10)-triene-3,16β,17α-triol (16,17-epi-estriol),

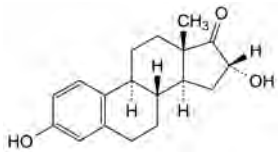

H. 3,16α-dihydroxyestra-1,3,5(10)-trien-17-one,

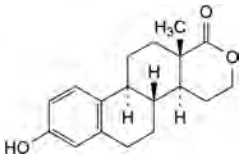

I. 3-hydroxy-17-oxa-17a-homoestra-1,3,5(10)-trien-17a-one,

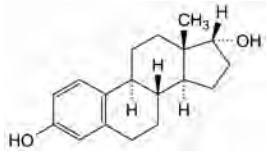

J. estra-1,3,5(10)-triene-3,17α-diol (17-epi-estradiol),

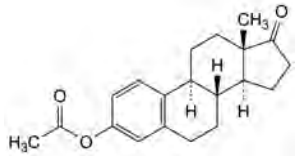

K. 17-oxoestra-1,3,5(10)-trien-3-yl acetate (estrone acetate).

**01/2008:1512**
**corrected 10.0**

# ESTROGENS, CONJUGATED

## Estrogeni coniuncti

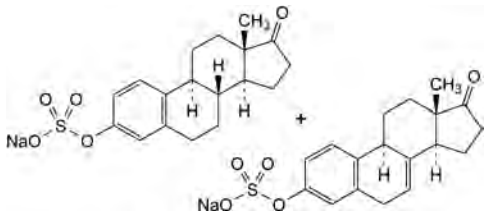

$C_{18}H_{21}NaO_5S + C_{18}H_{19}NaO_5S$ $M_r$ 372.4 + 370.4

DEFINITION

Mixture of various conjugated forms of estrogens obtained from the urine of pregnant mares or by synthesis, dispersed in a suitable powdered diluent.

The 2 principal components are 17-oxoestra-1,3,5(10)-trien-3-yl sodium sulfate (sodium estrone sulfate) and 17-oxoestra-1,3,5(10),7-tetraen-3-yl sodium sulfate (sodium equilin sulfate). Concomitants are sodium 17α-estradiol sulfate, sodium 17α-dihydroequilin sulfate and sodium 17β-dihydroequilin sulfate.

*Content* (percentages related to the labelled content):

- *sodium estrone sulfate*: 52.5 per cent to 61.5 per cent;
- *sodium equilin sulfate*: 22.5 per cent to 30.5 per cent;
- *sodium 17α-estradiol sulfate*: 2.5 per cent to 9.5 per cent;
- *sodium 17α-dihydroequilin sulfate*: 13.5 per cent to 19.5 per cent;
- *sodium 17β-dihydroequilin sulfate*: 0.5 per cent to 4.0 per cent;
- *sum of sodium estrone sulfate and sodium equilin sulfate*: 79.5 per cent to 88.0 per cent.

CHARACTERS

*Appearance*: almost white or brownish, amorphous powder.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks due to estrone and equilin in the chromatogram obtained with test solution (a) are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with reference solution (a).

B. Examine the chromatogram obtained in the test for chromatographic profile.

*Results*: the chromatogram obtained with test solution (b) exhibits additional peaks due to 17α-estradiol, 17α-dihydroequilin and 17β-dihydroequilin, at relative retentions with reference to 3-*O*-methylestrone (internal standard) of about 0.24, 0.30 and 0.35 respectively.

TESTS

**Chromatographic profile**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 8 mg of *3-O-methylestrone R* in 10.0 mL of *anhydrous ethanol R*. Dilute 2.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Acetate buffer solution pH 5.2*. Dissolve 10 g of *sodium acetate R* in 100 mL of *water R* and add 10 mL of *dilute acetic acid R*. Dilute to 500 mL with *water R* and adjust to pH 5.2 ± 0.1.

*Test solution (a)*. Considering the labelled content, transfer an accurately weighed quantity corresponding to about 2 mg of conjugated estrogens to a 50 mL centrifuge tube containing 15 mL of the acetate buffer solution pH 5.2 and 1 g of *barium chloride R*. Cap the tube tightly and shake for 30 min. If necessary, adjust to pH 5.0 ± 0.5 with *acetic acid R* or a 120 g/L solution of *sodium acetate R*. Sonicate for 30 s, then shake for 30 min. Add a suitable sulfatase preparation equivalent to 2500 units and shake mechanically for 10 min in a water-bath at 50 ± 1 °C. Swirl the tube by hand, then shake mechanically for 10 min in the water-bath. Allow to cool. Add 15.0 mL of *ethylene chloride R* to the mixture, immediately cap the tube tightly and shake for 15 min. Centrifuge for 10 min or until the lower layer is clear. Draw out the organic layer to a screw-cap tube, add 5 g of *anhydrous sodium sulfate R* and shake. Allow the solution to stand until clear. Protect the solution from any loss due to evaporation. Transfer 3.0 mL of the clear solution to a suitable centrifuge tube fitted with a screw cap. Add 1.0 mL of the internal standard solution. Evaporate the mixture to dryness with the aid of a stream of *nitrogen R*, maintaining the temperature below 50 °C. To the dry residue add 15 µL of *anhydrous pyridine R* and 65 µL of *N,O-bis(trimethylsilyl)trifluoroacetamide R* containing 1 per cent of *chlorotrimethylsilane R*. Immediately cap the tube tightly, mix thoroughly and allow to stand for 15 min. Add 0.5 mL of *toluene R* and mix mechanically.

*Test solution (b)*. Prepare as described in test solution (a), but do not add the sulfatase and use 6.0 mL of the upper layer instead of 3.0 mL. Prepare a blank in the same manner.

*Reference solution (a)*. Dissolve separately 8 mg of *estrone CRS*, 7 mg of *equilin CRS* and 5 mg of *17α-dihydroequilin CRS* in 10.0 mL of *anhydrous ethanol R*. Dilute together 2.0 mL, 1.0 mL and 1.0 mL respectively of these solutions to 10.0 mL with *anhydrous ethanol R*. Transfer 1.0 mL of this solution and 1.0 mL of the internal standard solution to a centrifuge tube fitted with a screw cap. Evaporate the mixture to dryness with the aid of a stream of *nitrogen R*, maintaining the temperature below 50 °C. To the dry residue add 15 µL of *anhydrous pyridine R* and 65 µL of *N,O-bis(trimethylsilyl)trifluoroacetamide R* containing 1 per cent of *chlorotrimethylsilane R*. Immediately cap the tube tightly, mix and allow to stand for 15 min. Add 0.5 mL of *toluene R*.

*Reference solution (b)*. Prepare as described in reference solution (a), but dilute tenfold with *anhydrous ethanol R* before adding the internal standard.

*Column*:
- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropyl(25)phenyl(25)methyl(50)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R*.

*Flow rate*: 2 mL/min.

*Split ratio*: 1:20 to 1:30.

*Temperature*:
- *column*: 220 °C;
- *injection port and detector*: 260 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to 3-*O*-methylestrone: 17α-dihydroequilin = about 0.30; estrone = about 0.80; equilin = about 0.87.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.2 between the peaks due to estrone and equilin; if necessary, adjust the temperature and the flow rate of the carrier gas.

In the chromatogram obtained with reference solution (a), measure the areas of the peaks due to 17α-dihydroequilin, estrone and 3-*O*-methylestrone.

In the chromatogram obtained with test solution (a), locate the peaks with relative retentions with reference to 3-*O*-methylestrone of 1 and about 0.24, 0.29, 0.30, 0.35, 0.56, 0.64, 0.90 and 1.3 and measure their areas.

Calculate the percentage content of the components occurring as sodium sulfate salts using expression (1) below.

In the chromatogram obtained with reference solution (b), measure the areas of the peaks due to estrone and 3-*O*-methylestrone.

In the chromatogram obtained with test solution (b), locate the peaks with relative retentions with reference to 3-*O*-methylestrone of about 0.30, 0.80 and 0.87 and measure the sum of the areas.

Calculate the percentage content of 17α-dihydroequilin, estrone and equilin occurring as free steroids using expression (2) below.

$$\frac{S_A' \times S_I \times m_R \times 137.8 \times 1000}{S_R \times S_I' \times m \times LC} \quad (1)$$

$$\frac{S_{FS}' \times S_I \times m_E \times 100 \times 1000}{S_E \times S_I' \times m \times LC} \quad (2)$$

$S_I$ = area of the peak due to the internal standard in the chromatogram obtained with the corresponding reference solution;

$S'_I$ = area of the peak due to the internal standard in the chromatogram obtained with the corresponding test solution;

$S_R$ = area of the peak due to the reference substance (Table 1512.-1) in the chromatogram obtained with the corresponding reference solution;

$S'_A$ = area of the peak due to the analyte in the chromatogram obtained with the corresponding test solution;

$m_R$ = mass of the reference substance (Table 1512.-1) in the corresponding reference solution, in milligrams;

$m$ = mass of the substance to be examined in the corresponding test solution, in milligrams;

$S'_{FS}$ = sum of the areas of the peaks due to 17α-dihydroequilin, estrone and equilin in the chromatogram obtained with the corresponding test solution;

$S_E$ = area of the peak due to *estrone CRS* in the chromatogram obtained with the corresponding reference solution;

$m_E$ = mass of *estrone CRS* in the corresponding reference solution, in milligrams;

$LC$ = labelled content, in milligrams per gram.

The percentages are within the following ranges:
- *sodium 17α-estradiol sulfate*: 2.5 per cent to 9.5 per cent;
- *sodium 17α-dihydroequilin sulfate*: 13.5 per cent to 19.5 per cent;
- *sodium 17β-dihydroequilin sulfate*: 0.5 per cent to 4.0 per cent;
- *sodium 17β-estradiol sulfate*: maximum 2.25 per cent;
- *sodium 17α-dihydroequilenin sulfate*: maximum 3.25 per cent;
- *sodium 17β-dihydroequilenin sulfate*: maximum 2.75 per cent;
- *sodium 8,9-didehydroestrone sulfate*: maximum 6.25 per cent;
- *sodium equilenin sulfate*: maximum 5.5 per cent;
- *sum of estrone, equilin and 17α-dihydroequilin*: maximum 1.3 per cent.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for chromatographic profile with the following modifications.

Table 1512.-1

| Relative retention (to 3-*O*-methylestrone) | Analyte | Quantified with reference to CRS | Present as |
|---|---|---|---|
| 0.24 | 17α-estradiol | *17α-dihydroequilin CRS* | sodium sulfate |
| 0.29 | 17β-estradiol | *estrone CRS* | sodium sulfate |
| 0.30 | 17α-dihydroequilin | *17α-dihydroequilin CRS* | free steroid, sodium sulfate (assay) |
| 0.35 | 17β-dihydroequilin | *17α-dihydroequilin CRS* | sodium sulfate |
| 0.56 | 17α-dihydroequilenin | *estrone CRS* | sodium sulfate |
| 0.64 | 17β-dihydroequilenin | *estrone CRS* | sodium sulfate |
| 0.80 | estrone | *estrone CRS* | free steroid, sodium sulfate (assay) |
| 0.87 | equilin | *equilin CRS* | free steroid, sodium sulfate (assay) |
| 0.90 | 8,9-didehydroestrone | *estrone CRS* | sodium sulfate |
| 1 | 3-*O*-methylestrone | (internal standard) | |
| 1.3 | equilenin | *estrone CRS* | sodium sulfate |

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the ratio of the area of the peak due to estrone to that due to the internal standard after at least 6 injections.

In the chromatogram obtained with reference solution (a), measure the areas of the peaks due to estrone or equilin and 3-*O*-methylestrone. In the chromatogram obtained with test solution (a), measure the areas of the peaks due to estrone, equilin and 3-*O*-methylestrone.

Calculate the percentage content of sodium estrone sulfate and sodium equilin sulfate using expression (1).

LABELLING

The label states:

– the name of the substance;
– the content of the substance;
– the nature of the diluent.

IMPURITIES AND CONCOMITANTS

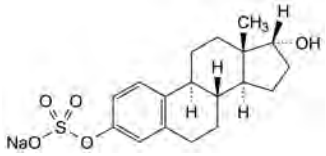

A. 17α-hydroxyestra-1,3,5(10)-trien-3-yl sodium sulfate (sodium 17α-estradiol sulfate),

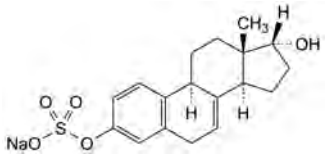

B. 17α-hydroxyestra-1,3,5(10),7-tetraen-3-yl sodium sulfate (sodium 17α-dihydroequilin sulfate),

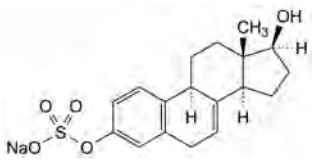

C. 17β-hydroxyestra-1,3,5(10),7-tetraen-3-yl sodium sulfate (sodium 17β-dihydroequilin sulfate),

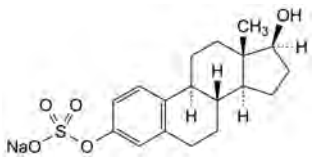

D. 17β-hydroxyestra-1,3,5(10)-trien-3-yl sodium sulfate (sodium 17β-estradiol sulfate),

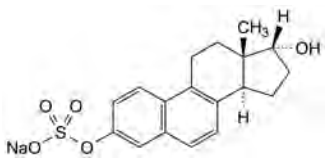

E. 17α-hydroxyestra-1,3,5(10),6,8-pentaen-3-yl sodium sulfate (sodium 17α-dihydroequilenin sulfate),

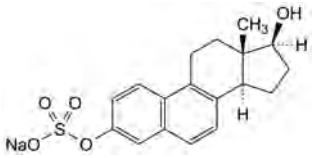

F. 17β-hydroxyestra-1,3,5(10),6,8-pentaen-3-yl sodium sulfate (sodium 17β-dihydroequilenin sulfate),

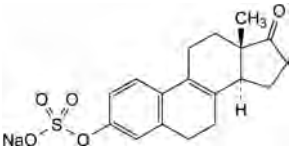

G. 17-oxoestra-1,3,5(10),8-tetraen-3-yl sodium sulfate (sodium 8,9-didehydroestrone sulfate),

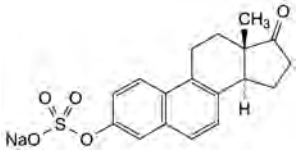

H. 17-oxoestra-1,3,5(10),6,8-pentaen-3-yl sodium sulfate (sodium equilenin sulfate),

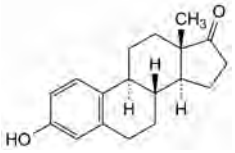

I. 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),

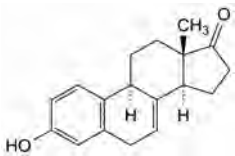

J. 3-hydroxyestra-1,3,5(10),7-tetraen-17-one (equilin),

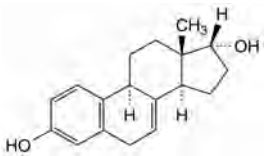

K. estra-1,3,5(10),7-tetraene-3,17α-diol (17α-dihydroequilin).

**01/2017:0457**

# ETACRYNIC ACID

## Acidum etacrynicum

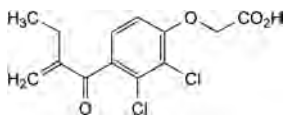

$C_{13}H_{12}Cl_2O_4$ $M_r$ 303.1

[58-54-8]

DEFINITION

[2,3-Dichloro-4-(2-methylenebutanoyl)phenoxy]acetic acid

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent). It dissolves in ammonia and in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 121 °C to 124 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Solvent mixture*: 103 g/L solution of *hydrochloric acid R*, *methanol R* (1:99 *V/V*).

*Test solution*: Dissolve 50.0 mg in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 10.0 mL of this solution to 100.0 mL with the solvent mixture.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 270 nm.

*Shoulder*: at about 285 nm.

*Specific absorbance at the absorption maximum*: 110 to 120.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *etacrynic acid CRS*.

D. Dissolve about 30 mg in 2 mL of *aldehyde-free alcohol R*. Dissolve 70 mg of *hydroxylamine hydrochloride R* in 0.1 mL of *water R*, add 7 mL of *alcoholic potassium hydroxide solution R* and dilute to 10 mL with *aldehyde-free alcohol R*. Allow to stand and add 1 mL of the supernatant to the solution of the substance to be examined. Heat the mixture on a water-bath for 3 min. After cooling, add 3 mL of *water R* and 0.15 mL of *hydrochloric acid R*. Examined in ultraviolet light at 254 nm, the mixture shows an intense blue fluorescence.

E. Dissolve about 25 mg in 2 mL of a 42 g/L solution of *sodium hydroxide R* and heat in a water-bath for 5 min. Cool and add 0.25 mL of a mixture of equal volumes of *sulfuric acid R* and *water R*. Add 0.5 mL of a 100 g/L solution of *chromotropic acid, sodium salt R* and, carefully, 2 mL of *sulfuric acid R*. An intense violet colour is produced.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *etacrynic acid for system suitability CRS* (containing impurities A, B and C) in 5.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 1 per cent *V/V* solution of *triethylamine R* adjusted to pH 6.8 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0-2.5 | 70 | 30 |
| 2.5-3 | 70→65 | 30→35 |
| 3-6 | 65 | 35 |
| 6-7 | 65→45 | 35→55 |
| 7-22 | 45 | 55 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *etacrynic acid for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to etacrynic acid (retention time = about 9 min): impurity A = about 0.8; impurity B = about 1.3; impurity C = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity A and etacrynic acid.

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity B = 0.6; impurity C = 1.3;
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, B*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 2.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure of 0.1-0.5 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 100 mL of *methanol R* and add 5 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.31 mg of $C_{13}H_{12}Cl_2O_4$.

IMPURITIES

*Specified impurities: A, B, C.*

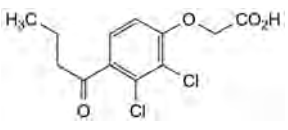


A. (4-butanoyl-2,3-dichlorophenoxy)acetic acid,

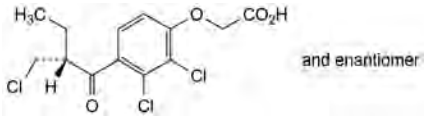


B. [2,3-dichloro-4-[2-(chloromethyl)butanoyl]phenoxy]acetic acid,

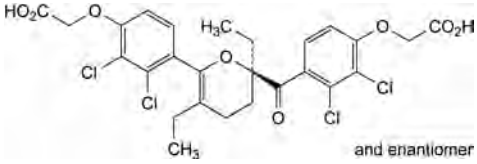


C. [4-[2-[4-(carboxymethoxy)-2,3-dichlorobenzoyl]-2,5-diethyl-3,4-dihydro-2*H*-pyran-6-yl]-2,3-dichlorophenoxy]acetic acid.

**01/2017:1204**

# ETAMSYLATE

## Etamsylatum

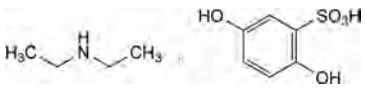

$C_{10}H_{17}NO_5S$ $M_r$ 263.3
[2624-44-4]

DEFINITION

*N*-Ethylethanamine 2,5-dihydroxybenzenesulfonate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in methanol, soluble in anhydrous ethanol, practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 127 °C to 134 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *etamsylate CRS.*

C. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).
*Test solution.* Dissolve 0.100 g in *water R* and dilute to 200.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Examine immediately.
*Spectral range*: 210-350 nm.
*Absorption maxima*: at 221 nm and 301 nm.
*Specific absorbance at the absorption maximum at 301 nm*: 145 to 151.

D. Into a test-tube, introduce 2 mL of freshly prepared solution S (see Tests) and 0.5 g of *sodium hydroxide R*. Warm the mixture and place a wet strip of *red litmus paper R* near the open end of the tube. The colour of the paper becomes blue.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S, when freshly prepared, is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.6 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Keep all solutions at 2-8 °C.*

*Buffer solution.* Dissolve 1.2 g of *anhydrous sodium dihydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust to pH 6.5 with *disodium hydrogen phosphate solution R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined and 10 mg of *hydroquinone R* (impurity A) in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to 100 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase:* spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, buffer solution (10:90 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 11 times the retention time of etamsylate.

*Relative retention* with reference to etamsylate (retention time = about 6 min): impurity A = about 1.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 8.0 between the peaks due to etamsylate and impurity A.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.5;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* in an oven at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 10 mL of *water R* and 40 mL of *dilute sulfuric acid R*. Titrate with *0.1 M cerium sulfate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M cerium sulfate* is equivalent to 13.16 mg of $C_{10}H_{17}NO_5S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

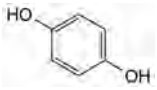

A. benzene-1,4-diol (hydroquinone).

**07/2019:2895**

# ETANERCEPT

## Etanerceptum

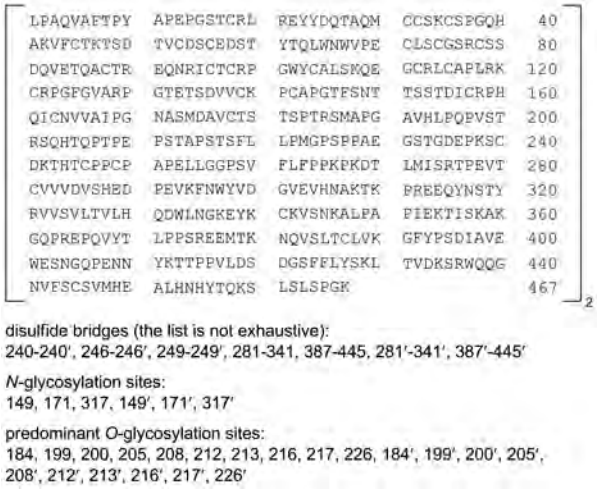


disulfide bridges (the list is not exhaustive):
240-240′, 246-246′, 249-249′, 281-341, 387-445, 281′-341′, 387′-445′

*N*-glycosylation sites:
149, 171, 317, 149′, 171′, 317′

predominant *O*-glycosylation sites:
184, 199, 200, 205, 208, 212, 213, 216, 217, 226, 184′, 199′, 200′, 205′, 208′, 212′, 213′, 216′, 217′, 226′

$C_{2224}H_{3472}N_{618}O_{701}S_{36}$ (monomer)

$M_r$ approx. 51 200 (monomer without glycosylation)

[185243-69-0]

DEFINITION

Dimeric fusion protein consisting of the extracellular ligand-binding portion of the human tumour necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept contains the $CH_2$ domain, the $CH_3$ domain and the hinge region, but not the $CH_1$ domain of IgG1. Etanercept consists of 934 amino acids and has an apparent molecular mass of approximately 150 kDa.

Etanercept represents a glycosylated protein with multiple *N*- and *O*-linked glycosylation sites. It shows full occupancy of *N*-linked glycans at N149, N171 and N317.

It contains one or more suitable buffering and/or stabilising agents.

*Content (milligrams of protein per millilitre)*: as approved by the competent authority.

*Potency*: $1.0 \times 10^6$ to $2.9 \times 10^6$ IU per milligram of protein.

PRODUCTION

Etanercept is produced in a suitable mammalian cell expression system by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process consistently produces a product with the expected *O*-glycan occupancy using a suitably qualified assay.

*Prior to release, the following tests are carried out on each batch of etanercept, unless exemption has been granted by the competent authority.*

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell and vector-derived DNA**. The limit is approved by the competent authority.

***N*-Glycan analysis**. Use a suitable method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*, section 2-3:

- release the glycans using one of the agents described in Table *2.2.59.*-1, for example peptide *N*-glycosidase F (PNGase F);
- label the released glycans with one of the fluorescent labelling agents described in Table *2.2.59.*-2, for example 2-aminobenzamide;
- analyse the labelled glycans by liquid chromatography (*2.2.29*) using fluorescence detection.

*The following procedure is given as an example.*

*Test solution*. To 4 µL of the preparation to be examined (about 25 mg/mL) add 21 µL of *water R*, 3 µL of *0.25 M sodium phosphate buffer solution pH 7.5 R* and 2 µL of a 500 000 U/mL solution of *peptide N-glycosidase F R*. Mix and incubate at 37 °C for 20-24 h. Label the released glycans with 2-aminobenzamide using a suitable procedure. The procedure employs a combination of reagents optimised and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labelled glycans from the reaction. Resuspend or dilute the labelled glycans in 100 µL of *water R*.

*Reference solution (a)*. Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of about 25 mg/mL. Carry out the release and labelling of glycans in the same manner as for the test solution. Resuspend or dilute the labelled glycans in 100 µL of *water R*.

*Reference solution (b)*. Use a suitable etanercept in-house reference preparation shown to be representative of batches tested clinically and batches used to demonstrate consistency of production. Dilute, if necessary, with *water R* to obtain a concentration of about 25 mg/mL. Carry out the release and labelling of glycans in the same manner as for the test solution. Resuspend or dilute the labelled glycans in 100 µL of *water R*.

*Blank solution*. Prepare at the same time and in the same manner as for the test solution but using *water R* instead of the preparation to be examined.

Analyse the labelled glycans by liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: an amide derivative of *silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 9.8 mL of *anhydrous formic acid R* and 500 mL of *water for chromatography R*, adjust to pH 4.0 with *ammonia R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 20 → 30 | 80 → 70 |
| 2 - 67.0 | 30 → 52 | 70 → 48 |
| 67.0 - 67.1 | 52 → 80 | 48 → 20 |
| 67.1 - 73.0 | 80 | 20 |

*Flow rate*: 0.4 mL/min.

*Detection*: fluorimeter at 330 nm for excitation and 420 nm for emission.

*Autosampler*: set at 2-8 °C.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram shown in Figure 2895.-1 to identify the 2 groups of oligosaccharides corresponding to neutral (peaks 1 to 5) and sialylated (peaks 6 to 9) *N*-glycans; record the retention time of each peak in both groups.

*System suitability*:

- the chromatogram obtained with reference solution (a) is qualitatively similar to the chromatogram supplied with *etanercept CRS* and peaks 1 to 9 are clearly visible;
- no significant peaks are observed in the chromatogram obtained with the blank solution.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution (b);
- the retention times of the peaks in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with reference solution (b);
- no additional peaks are observed in the chromatogram obtained with the test solution in comparison with the chromatogram obtained with reference solution (b).

Calculate the relative peak areas of the individual peaks corresponding to neutral and sialylated *N*-glycans with reference to the sum of the areas of all retained glycan peaks.

Calculate the percentage contents of the neutral and sialylated groups, using the following expressions:

$$\frac{A}{A+B} \times 100$$

$$\frac{B}{A+B} \times 100$$

*A* = sum of the areas of the peaks due to neutral *N*-glycans;

*B* = sum of the areas of the peaks due to sialylated *N*-glycans.

*NOTE: peaks 2 and 3 are separate peaks, but they are integrated together.*

*Limits*:

- *percentage of neutral N-glycans*: as approved by the competent authority;
- *percentage of sialylated N-glycans*: as approved by the competent authority.

CHARACTERS

*Appearance*: clear, almost colourless, slightly yellow or slightly brown liquid.

IDENTIFICATION

A. It complies with the limits of the assay (potency).

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of about 15 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of about 15 mg/mL.

*Reduction and alkylation.* To 200 µL of the test solution add 500 µL of *guanidine-tris(hydroxymethyl)aminomethane buffer solution pH 8.3 R* and 7 µL of a 154 g/L solution of *dithiothreitol R*. Mix and incubate at 65 °C for 15 min. Cool in an ice-bath for 5-10 min, then add 15.4 µL of a freshly prepared 185 g/L solution of *iodoacetamide R*. Mix and

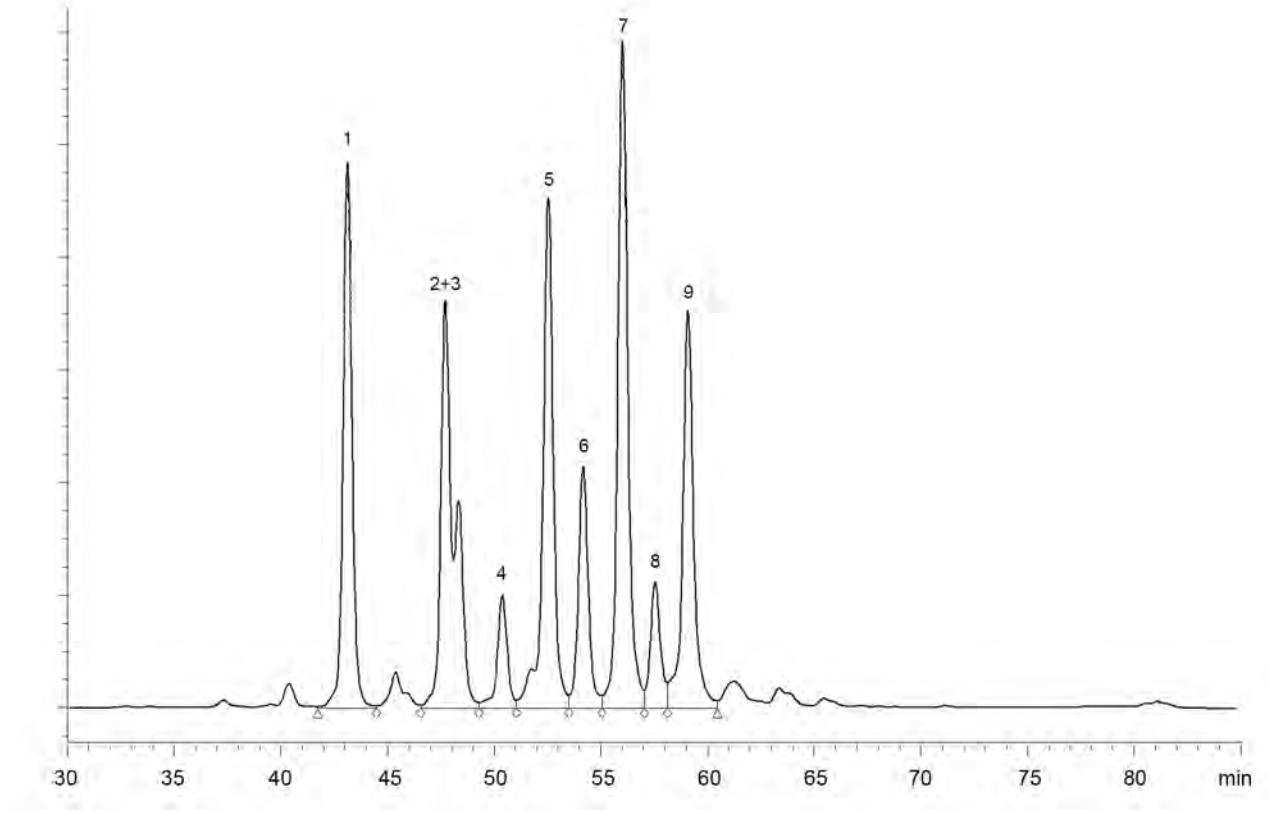


| Peak | Charged | Structure | Peak | Charged | Structure | Peak | Charged | Structure | Peak | Charged | Structure |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | No | Asialo-, agalacto-, biantennary, core-fucosylated | 4. | No | Asialo-, galactosylated biantennary | 6. | Yes | Monosialylated-, galactosylated biantennary | 8. | Yes | Disialylated, galactosylated biantennary |
| 2+3. | No | Asialo-, mono-galactosylated biantennary, core-fucosylated | 5. | No | Asialo-, galactosylated biantennary, core-fucosylated | 7. | Yes | Monosialylated-, galactosylated biantennary, core-fucosylated | 9. | Yes | Disialylated-, galactosylated biantennary, core-fucosylated |

Figure 2895.-1. – *Chromatogram for N-glycan analysis of etanercept*

allow to stand protected from light for 10 min. Add 1.4 µL of a 154 g/L solution of *dithiothreitol R*. Mix and allow to stand protected from light for 10 min.

*Digestion*. To 97 µL of the reduced test solution prepared previously, add 903 µL of *0.1 M tris-hydrochloride buffer solution pH 7.5 R*. Add 9.6 µL of a 500 000 U/mL solution of *peptide N-glycosidase F R* and incubate at 37 °C for 1 h. Add 40 µL of a 1 mg/mL solution of *trypsin for peptide mapping R* and incubate at 37 °C for 5 h. Heat at 95 °C for 5 min and cool in ice for 5 min. Adjust to pH 2 with about 30 µL of a 150 g/L solution of *trifluoroacetic acid R*.

Carry out the reduction/alkylation and digestion steps for the reference solution in the same manner as for the test solution.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.2 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm;
- *temperature*: 30-35 °C.

*Mobile phase*:

- *mobile phase A*: mix 3 g of *trifluoroacetic acid R* and 2000 mL of *water for chromatography R*;
- *mobile phase B*: mix 2 g of *trifluoroacetic acid R*, 330 mL of *water for chromatography R* and 1320 mL of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 98 | 2 |
| 5 - 125 | 98 → 50 | 2 → 50 |
| 125 - 140 | 50 → 5 | 50 → 95 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 200 µL.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *etanercept CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*). As approved by the competent authority.

**Sialic acid**. Use a suitable method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*, section 2-4:

- release the sialic acid by acid hydrolysis pre-treatment of the preparation to be examined;
- label the released sialic acid with a fluorescent labelling reagent, for example 1,2-diamino-4,5-methylenedioxybenzene, using a suitable procedure;
- analyse the labelled sialic acid by liquid chromatography (*2.2.29*) using fluorescence detection.

*The following procedure is given as an example.*

*Solution A*. Dissolve 8.71 g of *arginine R* in 40 mL of *water R*. Add 0.5 mL of a 10 g/L solution of *polysorbate 80 R*, mix and adjust to pH 7.3 with *phosphoric acid R*. Dilute to 250 mL with *water R*.

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of about 5 mg/mL. Further dilute with solution A to obtain a concentration of about 1 mg/mL. To 50 µL of this solution add 50 µL of a 480 g/L solution of *glacial acetic acid R* and incubate at 90 °C for 65 min. Cool, briefly centrifuge and evaporate to dryness. Label the released sialic acid using a suitable procedure; for example, add 15 µL of a 1.6 g/L solution of *1,2-diamino-4,5-methylenedioxybenzene dihydrochloride R* containing 78.1 g/L of *2-mercaptoethanol R* and 3.1 g/L of *sodium dithionite R* and incubate at 50 °C for 3 h. Dilute to 1 mL with *water R*.

*Reference solution (a)*. Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of about 5 mg/mL. Carry out the release and labelling of sialic acid in the same manner as for the test solution. Dilute to 1 mL with *water R*.

*Reference solution (b)*. Dissolve *N-acetylneuraminic acid R* in *water R* to obtain a concentration of about 1 mg/mL. Mix 40 µL of the solution, 40 µL of a 1 mg/mL solution of *bovine albumin R1* and 120 µL of solution A. Use 50 µL of this solution to carry out the release and labelling of sialic acid in the same manner as for the test solution. Dilute with *water R* to obtain a concentration of 0.01 µg/µL.

*Standard solutions*. Dilute reference solution (b) with *water R* to obtain a concentration of 2 ng/µL. Further dilute this solution to prepare a standard curve with concentrations in the range of 0.10-0.40 ng/µL (6 concentrations, typically 0.10 ng/µL, 0.15 ng/µL, 0.20 ng/µL, 0.25 ng/µL, 0.30 ng/µL, 0.40 ng/µL). Analyse the labelled sialic acid by liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 8 nm;
- *temperature*: 35 °C.

*Mobile phase*: mix 8 mL of *acetonitrile R*, 500 mL of *methanol R* and 1820 mL of *water for chromatography R*; mix thoroughly.

*Flow rate*: 1 mL/min.

*Detection*: fluorimeter at 374 nm for excitation and 448 nm for emission.

*Autosampler*: set at 2-8 °C.

*Injection*: 20 µL.

*Retention time*: sialic acid = about 10.5 min.

Calculate the sialic acid content in the preparation to be examined using the standard curve and the content of sialic acid (*N*-acetylneuraminic acid) in the standard solutions. Report the molar ratio (number of moles of sialic acid per mole of etanercept), using the molar mass of the monomer.

*System suitability*:

- the peak due to sialic acid in the chromatogram obtained with reference solution (a) is visible and is similar to the corresponding peak in the chromatogram supplied with *etanercept CRS*;
- *repeatability*: maximum relative standard deviation of 15 per cent for the sialic acid content expressed as the molar ratio, determined on 3 consecutive injections of reference solution (a);
- the coefficient of determination ($R^2$) calculated for the standard curve is not less than 0.9995.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution (a).

*Limit*:

- 8 to 19 moles of sialic acid per mole of etanercept.

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of about 2 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of about 2 mg/mL.

*Column*:
- *size*: *l* = 0.035 m, Ø = 4.6 mm;
- *stationary phase*: *resin for hydrophobic interaction chromatography R* (2.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 475.9 g of *ammonium sulfate R* in *water for chromatography R* and dilute to 1950 mL with the same solvent; adjust to pH 7.0 with *phosphoric acid R* and dilute to 2000 mL with *water for chromatography R*;
- *mobile phase B*: dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* in *water for chromatography R* and dilute to 1950 mL with the same solvent; adjust to pH 7.0 with *phosphoric acid R* and dilute to 2000 mL with *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: fluorimeter at 278 nm for excitation and 350 nm for emission.

*Autosampler*: set at 10 °C.

*Injection*: 5 µL; perform 3 injections.

*Relative retention* with reference to etanercept (retention time = about 28.5 min): peak 1 = 0.96; peak 3 = 1.12.

*System suitability*: reference solution:
- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *etanercept CRS*;
- peaks 1 and 3 are clearly separated from the peak due to etanercept.

*Results*:
- no additional peaks are observed in the chromatogram obtained with the test solution in comparison with the chromatogram obtained with the reference solution.

*Limits*:
- *peak 1*: maximum 5 per cent;
- *peak 3*: maximum 28 per cent;
- *sum of all peaks other than the principal peak*: maximum 30 per cent.

**Impurities with molecular masses greater than that of etanercept**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Solution A*. Dissolve 8.8 g of *sodium chloride R* and 15.6 g of *sodium dihydrogen phosphate R* in 900 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent.

*Solution B*. Dissolve 8.75 g of *sodium chloride R* and 14.2 g of *anhydrous disodium hydrogen phosphate R* in 900 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent.

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of 2.5 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of 2.5 mg/mL.

*Column*:
- *size*: *l* = 0.30 m, Ø = 8.0 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) with a pore size of 30 nm and of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 1000 000.

*Mobile phase*: mix 220 mL of solution A and 780 mL of solution B, and adjust to pH 7.2 with solution A or solution B.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 14 µL; perform at least 3 injections.

*Relative retention* with reference to etanercept monomer (retention time = about 7.8 min): aggregates = 0.84; high molecular mass species = 0.89.

Any shoulder appearing on the descending part of the peak due to etanercept monomer is included in its area.

*System suitability*: reference solution:
- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *etanercept CRS*;
- *resolution*: minimum 1.7 between the peaks due to the high molecular mass species and etanercept monomer;
- *number of theoretical plates*: minimum 3000 calculated for the peak due to etanercept monomer.

*Limit*:
- *sum of the peaks eluted before the principal peak*: maximum 8.0 per cent.

**Impurities with molecular masses differing from that of etanercept**. Polyacrylamide gel electrophoresis (*2.2.31*) under both reducing and non-reducing conditions.

*Gel dimensions*: 1.0 mm thick.

*Resolving gel*: 8-16 per cent acrylamide.

*Sample buffer (non-reducing conditions)*: *concentrated SDS-PAGE sample buffer R.*

*Sample buffer (reducing conditions)*: *concentrated SDS-PAGE sample buffer for reducing conditions R.*

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of 0.2 mg/mL. Mix 1 volume of this solution and 1 volume of sample buffer.

*Reference solution (a).* Dissolve the contents of a vial of *etanercept CRS* in *water R* to obtain a concentration of 0.2 mg/mL. Mix 1 volume of the solution and 1 volume of sample buffer.

*Reference solution (b).* 0.01 mg/mL solution of *bovine albumin R.*

*Reference solution (c).* Mix 1 volume of reference solution (b) and 4 volumes of *concentrated SDS-PAGE sample buffer R.*

*Reference solution (d).* Mix 1 volume of reference solution (b) and 19 volumes of *concentrated SDS-PAGE sample buffer R.*

*Reference solution (e).* A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 5-200 kDa.

*Sample treatment*: heat at 90-105 °C for 5 min and load onto the gel within 15 min.

*Application*: 10 µL.

*Detection*: by silver staining.

*Identification of bands*:
- *non-reducing conditions*: the band corresponding to etanercept of an apparent molecular mass of approximately 150 kDa is present; related protein bands with apparent molecular masses of approximately 60 kDa, 100 kDa, 120 kDa, 225 kDa, 250 kDa may also be present;
- *reducing conditions*: the band corresponding to etanercept of an apparent molecular mass of approximately 76 kDa is present; related protein bands with apparent molecular masses of approximately 25 kDa, 35 kDa, 55 kDa, 200 kDa may also be present.

*System suitability*:
- the bands in the electropherogram obtained with reference solution (a) are clearly visible;
- the band in the electropherogram obtained with reference solutions (b), (c) and (d) is clearly visible;
- all expected bands in the electropherogram obtained with reference solution (e) are visible and clearly separated.

*Results*:

- the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);
- the electropherogram obtained with the test solution shows no additional band that is more intense than that of the band in the electropherogram obtained with reference solution (d).

**Microbiological contamination** (*2.6.12*): maximum 1 CFU/mL.

ASSAY

**Protein** (*2.5.33, Method 1*).

*Test solution*. Dilute the preparation to be examined gravimetrically with a suitable buffer to obtain a concentration of 1.0 mg/mL. Prepare in triplicate.

*Reference solution*. Dissolve the contents of a vial of *etanercept CRS* in a suitable buffer to obtain a concentration of 1.0 mg/mL.

Record the absorbance spectrum between 250 nm and 400 nm. Measure the value at the absorbance maximum of 280 nm after correction for any light scattering measured up to 320 nm. Calculate the protein concentration of etanercept taking into account the assigned content of *etanercept CRS*.

**Potency**. The potency of etanercept is determined by comparison of dilutions of the test preparation with the dilutions of *etanercept BRP* using a suitable cell-based assay based on the inhibitory action of etanercept on the biological activity of TNF-α and a suitable readout for assessing this inhibitory effect.

*The following procedure is given as an example.*

Carry out an apoptosis-based assay based on the ability of etanercept to inhibit TNF-α induced apoptosis in histiocytic lymphoma cell line U937 (ATCC No. CRL-1593.2) via caspase activation. The U937 cells are incubated with varying dilutions of test and reference preparations of etanercept in the presence of TNF-α. They are then incubated with Caspase-Glo 3/7 reagent, which results in caspase cleavage of a luminogenic substrate, subsequent release of a luciferase substrate and generation of a luminescent signal. The luminescence produced is proportional to the amount of caspase activity present.

*Assay medium*. RPMI 1640 containing L-alanyl-L-glutamine, 6.0 g/L *HEPES R* (25 mM) and foetal bovine serum (7.5 per cent *V/V*).

*Test solutions*. Dilute the preparation to be examined with assay medium to obtain a concentration of about 72 ng/mL. Use this solution to prepare 11 additional sample dilutions (dilution steps of 1.2 or 1.4 have been found suitable).

*Reference solutions*. Reconstitute the contents of 1 ampoule of *etanercept BRP* with sterilised *water for injections R* to obtain a concentration of 10 000 IU/mL. Further dilute with assay medium to obtain a concentration of 144 IU/mL. Use this solution to prepare 11 additional dilutions to generate the standard curve (dilution steps of 1.2 or 1.4 have been found suitable).

*TNF-α working solution*. Dissolve the contents of a vial of TNF-α according to the supplier's instructions. Further dilute with assay medium to obtain a suitable working concentration. As the biological activity of TNF-α is likely to vary between different suppliers and also between different batches from the same supplier, this should be controlled by use of an appropriate standard (e.g. WHO International Standard for TNF-α).

*Method*.

*Plate preparation*. Add 600 µL of assay medium to the wells designated for cells only (column 1, rows A-D) on a cluster tube rack. Add 300 µL of assay medium and 300 µL of TNF-α working solution to the wells designated for the TNF-α controls (column 1, rows E-H). Add 300 µL of the test or reference solutions and 300 µL of TNF-α working solution to the sample wells (columns 2-12, rows A-H). Mix on a shaker for 5 min. Incubate at 36.0-38.0 °C for 30-60 min in a humidified incubator using 5 ± 2 per cent $CO_2$.

*NOTE: when using deep-well or 96-well plates instead of cluster tubes, adapt the volumes of sample, TNF-α working solution and assay medium accordingly.*

*Cell preparation*. A cell density between $3.0 \times 10^5$ and $1.0 \times 10^6$ cells per millilitre is suitable, and cell viability is not less than 95 per cent.

*Plating test solution, reference solution, controls and cells*. Transfer 60 µL from each cluster tube and add to the corresponding wells. Mix the cell suspension thoroughly and add 60 µL to each well. Mix the contents of the plates on a shaker for 5 min. Incubate the plates without lids at 36.0-38.0 °C for 2-2.5 h in a humidified incubator using 5 ± 2 per cent $CO_2$.

*Addition of Caspase-Glo 3/7 assay system*. Reconstitute the Caspase-Glo 3/7 assay system according to the manufacturer's instructions and add 100 µL to each well of the assay plates. Shake the plates, covered with black lids, on a plate shaker for 10-15 min. Incubate at room temperature for 30-60 min. Place the uncovered plates in a luminometer and read the luminescence for a minimum of 1 second per well.

Calculate the potency of the preparation to be examined using the four-parameter logistic curve model (*5.3*).

*System suitability*:

- *maximum value (TNF-α control) to minimum value (cell only) ratio*: minimum 3.0.

*Result*: the estimated potency is not less than 80 per cent and not more than 140 per cent relative to the reference solution. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

In an airtight container at − 20 °C or below.

LABELLING

The label states the content, in milligrams of protein per millilitre.

**07/2019:1591**
**corrected 10.0**

# ETHACRIDINE LACTATE MONOHYDRATE

## Ethacridini lactas monohydricus

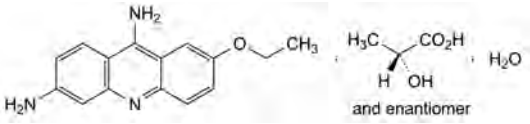


$C_{18}H_{21}N_3O_4,H_2O$ $M_r$ 361.4
[6402-23-9]

DEFINITION

7-Ethoxyacridine-3,9-diamine (2*RS*)-2-hydroxypropanoate monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow crystalline powder.

*Solubility*: sparingly soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: A.

*Second identification*: B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ethacridine lactate monohydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in 2 mL of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution*. Dissolve 5 mg of *ethacridine lactate monohydrate CRS* in 2 mL of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (17:17:66 *V/V/V*).

*Application*: 2 µL; the volume can be adapted based on the type of plate used.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C.

*Detection A*: examine in daylight.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: examine in ultraviolet light at 254 nm and at 366 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 50 mL of solution S (see Tests) add 10 mL of *dilute sodium hydroxide solution R*. Filter. To 5 mL of the filtrate, add 1 mL of *dilute sulfuric acid R*. 5 mL of the solution obtained gives the reaction of lactates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 7.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium octanesulfonate R* in a mixture of 300 mL of *acetonitrile R* and 700 mL of *phosphate buffer solution pH 2.8 R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 268 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of ethacridine.

*Retention time*: ethacridine = about 15 min.

*Limits*:

- *any impurity*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): 4.5 per cent to 5.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.270 g in 5.0 mL of *anhydrous formic acid R*. Add 60.0 mL of *acetic anhydride R* and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.34 mg of $C_{18}H_{21}N_3O_4$.

STORAGE

Protected from light.

IMPURITIES

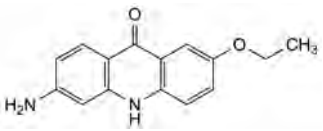

A. 6-amino-2-ethoxyacridin-9(10*H*)-one,

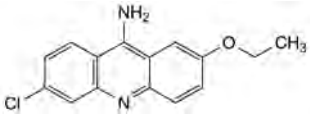

B. 6-chloro-2-ethoxyacridin-9-amine,

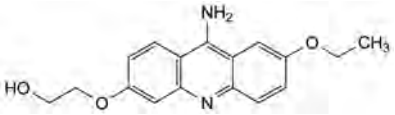

C. 2-[(9-amino-7-ethoxyacridin-3-yl)oxy]ethan-1-ol.

**01/2017:0553**

# ETHAMBUTOL HYDROCHLORIDE

## Ethambutoli hydrochloridum

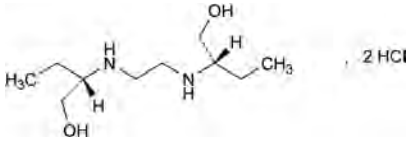

$C_{10}H_{26}Cl_2N_2O_2$ $M_r$ 277.2

[1070-11-7]

DEFINITION

(2*S*,2′*S*)-2,2′-(Ethylenediimino)dibutan-1-ol dihydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D, E.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ethambutol hydrochloride CRS*.

B. Examine the chromatograms obtained in the test for impurity A.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

C. Dissolve 0.1 g in 10 mL of *water R*. Add 0.2 mL of *copper sulfate solution R* and 0.5 mL of *dilute sodium hydroxide solution R*; a blue colour is produced.

D. It gives reaction (a) of chlorides (*2.3.1*).

E. Related substances (see Tests).

TESTS

**pH** (*2.2.3*): 3.7 to 4.0.

Dissolve 0.2 g in 10 mL of *carbon dioxide-free water R*.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 50.0 mg of *aminobutanol R* (impurity A) in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 50 mg of *ethambutol hydrochloride CRS* and 5 mg of *aminobutanol R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *methanol R* (10:15:75 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air; heat at 110 °C for 10 min.

*Detection*: cool then spray with *ninhydrin solution R1*; heat at 110 °C for 5 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Suspend 4.0 mg of the substance to be examined in 4.0 mL of *acetonitrile R1* and add 100 µL of *triethylamine R*. Sonicate the mixture for 5 min. Add 15 µL of *(R)-(+)-α-methylbenzyl isocyanate R* and heat at 70 °C for 20 min.

*Reference solution (a).* Dilute 0.50 mL of the test solution to 100.0 mL with *acetonitrile R1*.

*Reference solution (b).* Treat 4.0 mg of *ethambutol for system suitability CRS* (containing impurity B) as described for the test solution.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R*, *water R* (50:50 *V/V*);

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 71 | 29 |
| 30 - 35 | 71 → 0 | 29 → 100 |
| 35 - 37 | 0 | 100 |
| 37 - 38 | 0 → 71 | 100 → 29 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Relative retention* with reference to ethambutol (retention time = about 14 min): impurity B = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to ethambutol and impurity B.

*Limits*:

– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *unspecified impurities with a relative retention of 0.75 to 1.5 with reference to ethambutol*: for each impurity, not more than 0.2 times the area of the peak due to ethambutol in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total (impurity B and unspecified impurities with a relative retention of 0.75 to 1.5 with reference to ethambutol)*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *disregard limit*: 0.1 times the area of the peak due to ethambutol in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity D (1,2-dichloroethane)** (*2.4.24*): maximum 5 ppm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *water R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.72 mg of $C_{10}H_{26}Cl_2N_2O_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): C.

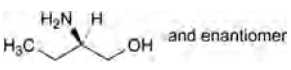


A. 2-aminobutan-1-ol,

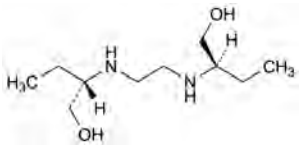

B. (2*R*,2′*S*)-2,2′-(ethylenediimino)dibutan-1-ol (meso-ethambutol),

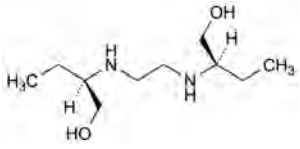

C. (2*R*,2′*R*)-2,2′-(ethylenediimino)dibutan-1-ol ((*R*,*R*)-ethambutol),

D. 1,2-dichloroethane (ethylene chloride).

**01/2015:1317**
**corrected 10.0**

# ETHANOL (96 PER CENT)[(1)]

## Ethanolum (96 per centum)

DEFINITION

*Content*:

– *ethanol* ($C_2H_6O$; $M_r$ 46.07): 95.1 per cent *V/V* (92.6 per cent *m/m*) to 96.9 per cent *V/V* (95.2 per cent *m/m*) at 20 °C, calculated from the relative density using the alcoholimetric tables (*5.5*);

– *water*.

♦CHARACTERS

*Appearance*: colourless, clear, volatile, flammable liquid, hygroscopic.

*Solubility*: miscible with water and with methylene chloride.

It burns with a blue, smokeless flame.

bp: about 78 °C.♦

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Relative density (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of ethanol (96 per cent).*

◊C. Mix 0.1 mL with 1 mL of a 10 g/L solution of *potassium permanganate R* and 0.2 mL of *dilute sulfuric acid R* in a test-tube. Cover immediately with a filter paper moistened with a freshly prepared solution containing 0.1 g of *sodium nitroprusside R* and 0.5 g of *piperazine hydrate R* in 5 mL of *water R*. After a few minutes, an intense blue colour appears on the paper and becomes paler after 10-15 min.

D. To 0.5 mL add 5 mL of *water R*, 2 mL of *dilute sodium hydroxide solution R*, then slowly add 2 mL of *0.05 M iodine*. A yellow precipitate is formed within 30 min.◊

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*) when compared with *water R*. Dilute 1.0 mL to 20 mL with *water R*. After standing for 5 min, the dilution remains clear (*2.2.1*) when compared with *water R*.

**Acidity or alkalinity**. To 20 mL add 20 mL of *carbon dioxide-free water R* and 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 1.0 mL of *0.01 M sodium hydroxide*. The solution is pink (30 ppm, expressed as acetic acid).

**Relative density** (*2.2.5*): 0.805 to 0.812.

**Absorbance** (*2.2.25*): maximum 0.40 at 240 nm, 0.30 between 250 nm and 260 nm, and 0.10 between 270 nm and 340 nm. The spectrum shows a steadily descending curve with no observable peaks or shoulders.

Examine between 235 nm and 340 nm, in a 5 cm cell using *water R* as the compensation liquid.

**Volatile impurities**. Gas chromatography (*2.2.28*).

*Test solution (a)*. The substance to be examined.

*Test solution (b)*. Add 150 µL of *4-methylpentan-2-ol R* to 500.0 mL of the substance to be examined.

*Reference solution (a)*. Dilute 100 µL of *anhydrous methanol R* to 50.0 mL with the substance to be examined. Dilute 5.0 mL of the solution to 50.0 mL with the substance to be examined.

*Reference solution (b)*. Dilute 50 µL of *anhydrous methanol R* and 50 µL of *acetaldehyde R* to 50.0 mL with the substance to be examined. Dilute 100 µL of the solution to 10.0 mL with the substance to be examined.

*Reference solution (c)*. Dilute 150 µL of *acetal R* to 50.0 mL with the substance to be examined. Dilute 100 µL of the solution to 10.0 mL with the substance to be examined.

*Reference solution (d)*. Dilute 100 µL of *benzene R* to 100.0 mL with the substance to be examined. Dilute 100 µL of the solution to 50.0 mL with the substance to be examined.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 40 |
| | 12 - 32 | 40 → 240 |
| | 32 - 42 | 240 |
| Injection port | | 200 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the 1st peak (acetaldehyde) and the 2nd peak (methanol).

*Limits*:

– *methanol* in the chromatogram obtained with test solution (a): not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (200 ppm *V/V*);

– *acetaldehyde + acetal*: maximum 10 ppm *V/V*, expressed as acetaldehyde.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Calculate the sum of the contents of acetaldehyde and acetal in parts per million *V/V* using the following expression:

$$\frac{10 \times A_E}{A_T - A_E} + \frac{30 \times C_E}{C_T - C_E} \times \frac{44.05}{118.2}$$

$A_E$ = area of the peak due to acetaldehyde in the chromatogram obtained with test solution (a),

$A_T$ = area of the peak due to acetaldehyde in the chromatogram obtained with reference solution (b),

$C_E$ = area of the peak due to acetal in the chromatogram obtained with test solution (a),

$C_T$ = area of the peak due to acetal in the chromatogram obtained with reference solution (c),

44.05 = molecular mass of acetaldehyde,

118.2 = molecular mass of acetal.

– *benzene*: maximum 2 ppm *V/V*.

Calculate the content of benzene in parts per million *V/V* using the following expression:

$$\frac{2B_E}{B_T - B_E}$$

$B_E$ = area of the peak due to benzene in the chromatogram obtained with test solution (a),

$B_T$ = area of the peak due to benzene in the chromatogram obtained with reference solution (d).

If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

– *total of other impurities* in the chromatogram obtained with test solution (b): not more than the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (300 ppm),

– *disregard limit*: 0.03 times the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (9 ppm).

**Residue on evaporation**: maximum 25 ppm *m/V*.

Evaporate 100 mL to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2.5 mg.

STORAGE

Protected from light.

◊IMPURITIES

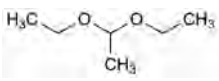

A. 1,1-diethoxyethane (acetal),

B. acetaldehyde,

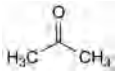

C. propan-2-one (acetone),

D. benzene,

E. cyclohexane,

F. methanol,

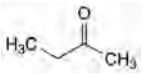

G. butan-2-one (methyl ethyl ketone),

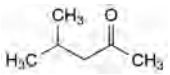

H. 4-methylpentan-2-one (methyl isobutyl ketone),

I. propan-1-ol (propanol),

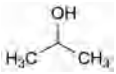

J. propan-2-ol (isopropyl alcohol),

K. butan-1-ol (butanol),

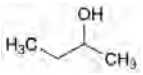

L. butan-2-ol,

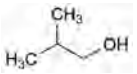

M. 2-methylpropan-1-ol (isobutanol),

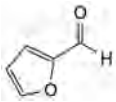

N. furane-2-carbaldehyde (furfural),

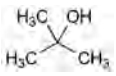

O. 2-methylpropan-2-ol (1,1-dimethylethyl alcohol),

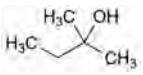

P. 2-methylbutan-2-ol,

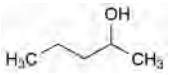

Q. pentan-2-ol,

R. pentan-1-ol (pentanol),

S. hexan-1-ol (hexanol),

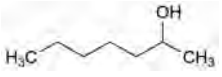

T. heptan-2-ol,

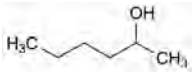

U. hexan-2-ol,

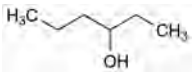

V. hexan-3-ol.◊

**01/2015:1318**
**corrected 10.0**

# ETHANOL, ANHYDROUS[(2)]

## Ethanolum anhydricum

$C_2H_6O$ $M_r$ 46.07
[64-17-5]

DEFINITION

*Content*: not less than 99.5 per cent *V/V* of $C_2H_6O$ (99.2 per cent *m/m*), at 20 °C, calculated from the relative density using the alcoholimetric tables (*5.5*).

♦CHARACTERS

*Appearance*: colourless, clear, volatile, flammable liquid, hygroscopic.

*Solubility*: miscible with water and with methylene chloride.

It burns with a blue, smokeless flame.

bp: about 78 °C.♦

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Relative density (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of anhydrous ethanol.*

◊C. Mix 0.1 mL with 1 mL of a 10 g/L solution of *potassium permanganate R* and 0.2 mL of *dilute sulfuric acid R* in a test-tube. Cover immediately with a filter paper moistened with a freshly prepared solution containing 0.1 g of *sodium nitroprusside R* and 0.5 g of *piperazine hydrate R* in 5 mL of *water R*. After a few minutes, an intense blue colour appears on the paper and becomes paler after 10-15 min.

D. To 0.5 mL add 5 mL of *water R*, 2 mL of *dilute sodium hydroxide solution R*, then slowly add 2 mL of *0.05 M iodine*. A yellow precipitate is formed within 30 min.◊

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*) when compared with *water R*. Dilute 1.0 mL to 20 mL with *water R*. After standing for 5 min, the dilution remains clear (*2.2.1*) when compared with *water R*.

**Acidity or alkalinity**. To 20 mL add 20 mL of *carbon dioxide-free water R* and 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 1.0 mL of *0.01 M sodium hydroxide*. The solution is pink (30 ppm, expressed as acetic acid).

**Relative density** (*2.2.5*): 0.790 to 0.793.

**Absorbance** (*2.2.25*): maximum 0.40 at 240 nm, 0.30 between 250 nm and 260 nm, and 0.10 between 270 nm and 340 nm. The spectrum shows a steadily descending curve with no observable peaks or shoulders.

Examine between 235 nm and 340 nm in a 5 cm cell using *water R* as the compensation liquid.

**Volatile impurities**. Gas chromatography (*2.2.28*).

*Test solution (a)*. The substance to be examined.

*Test solution (b)*. Add 150 µL of *4-methylpentan-2-ol R* to 500.0 mL of the substance to be examined.

*Reference solution (a)*. Dilute 100 µL of *anhydrous methanol R* to 50.0 mL with the substance to be examined. Dilute 5.0 mL of the solution to 50.0 mL with the substance to be examined.

*Reference solution (b)*. Dilute 50 µL of *anhydrous methanol R* and 50 µL of *acetaldehyde R* to 50.0 mL with the substance to be examined. Dilute 100 µL of the solution to 10.0 mL with the substance to be examined.

*Reference solution (c)*. Dilute 150 µL of *acetal R* to 50.0 mL with the substance to be examined. Dilute 100 µL of the solution to 10.0 mL with the substance to be examined.

*Reference solution (d)*. Dilute 100 µL of *benzene R* to 100.0 mL with the substance to be examined. Dilute 100 µL of the solution to 50.0 mL with the substance to be examined.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 40 |
| | 12 - 32 | 40 → 240 |
| | 32 - 42 | 240 |
| Injection port | | 200 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the 1st peak (acetaldehyde) and the 2nd peak (methanol).

*Limits*:

– *methanol* in the chromatogram obtained with test solution (a): not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (200 ppm *V/V*);
– *acetaldehyde + acetal*: maximum 10 ppm *V/V*, expressed as acetaldehyde.

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

Calculate the sum of the contents of acetaldehyde and acetal in parts per million *V/V* using the following expression:

$$\frac{10 \times A_E}{A_T - A_E} + \frac{30 \times C_E}{C_T - C_E} \times \frac{44.05}{118.2}$$

$A_E$ = area of the peak due to acetaldehyde in the chromatogram obtained with test solution (a),

$A_T$ = area of the peak due to acetaldehyde in the chromatogram obtained with reference solution (b),

$C_E$ = area of the peak due to acetal in the chromatogram obtained with test solution (a),

$C_T$ = area of the peak due to acetal in the chromatogram obtained with reference solution (c),

44.05 = molecular mass of acetaldehyde,

118.2 = molecular mass of acetal.

– *benzene*: maximum 2 ppm *V/V*.

Calculate the content of benzene in parts per million *V/V* using the following expression:

$$\frac{2B_E}{B_T - B_E}$$

$B_E$ = area of the peak due to benzene in the chromatogram obtained with test solution (a),

$B_T$ = area of the peak due to benzene in the chromatogram obtained with reference solution (d).

If necessary, the identity of benzene can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

– *total of other impurities* in the chromatogram obtained with test solution (b): not more than the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (300 ppm);

– *disregard limit*: 0.03 times the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (9 ppm).

**Residue on evaporation**: maximum 25 ppm *m/V*.

Evaporate 100 mL to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2.5 mg.

STORAGE

Protected from light.

◊IMPURITIES

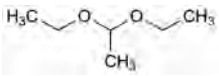

A. 1,1-diethoxyethane (acetal),

B. acetaldehyde,

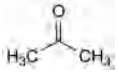

C. propan-2-one (acetone),

D. benzene,

E. cyclohexane,

F. methanol,

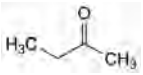

G. butan-2-one (methyl ethyl ketone),

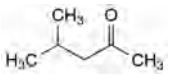

H. 4-methylpentan-2-one (methyl isobutyl ketone),

I. propan-1-ol (propanol),

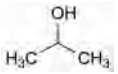

J. propan-2-ol (isopropyl alcohol),

K. butan-1-ol (butanol),

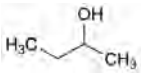

L. butan-2-ol,

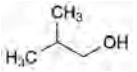

M. 2-methylpropan-1-ol (isobutanol),

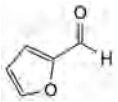

N. furane-2-carbaldehyde (furfural),

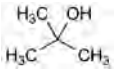

O. 2-methylpropan-2-ol (1,1-dimethylethyl alcohol),

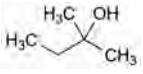

P. 2-methylbutan-2-ol,

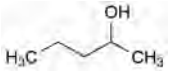

Q. pentan-2-ol,

R. pentan-1-ol (pentanol),

S. hexan-1-ol (hexanol),

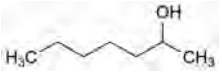

T. heptan-2-ol,

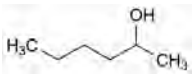

U. hexan-2-ol,

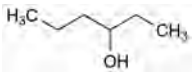

V. hexan-3-ol.◊

**07/2016:0650**

# ETHER

## Aether

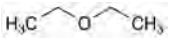

$C_4H_{10}O$ $M_r$ 74.1
[60-29-7]

DEFINITION

Diethyl ether.

It may contain a suitable non-volatile antioxidant at a suitable concentration.

CHARACTERS

*Appearance*: clear, colourless, volatile liquid.

*Solubility*: soluble in water, miscible with ethanol (96 per cent), with methylene chloride and with fatty oils.

It is highly flammable.

IDENTIFICATION

A. Relative density (see Tests).

B. Distillation range (see Tests).

TESTS

**Acidity**. To 20 mL of *ethanol (96 per cent) R* add 0.25 mL of *bromothymol blue solution R1* and, dropwise, *0.02 M sodium hydroxide* until a blue colour persists for 30 s. Add 25 mL of the substance to be examined, shake and add, dropwise, *0.02 M sodium hydroxide* until the blue colour reappears and persists for 30 s. Not more than 0.4 mL of *0.02 M sodium hydroxide* is required.

**Relative density** (*2.2.5*): 0.714 to 0.716.

**Distillation range** (*2.2.11*). *Do not distil if the substance to be examined does not comply with the test for peroxides.* It distils completely between 34.0 °C and 35.0 °C. Carry out the test using a suitable heating device and taking care to avoid directly heating the flask above the level of the liquid.

**Aldehydes**. To 10.0 mL in a ground-glass-stoppered cylinder add 1 mL of *alkaline potassium tetraiodomercurate solution R* and shake for 10 s. Allow to stand for 5 min, protected from light. The lower layer may show a yellow or reddish-brown opalescence but not a grey or black opalescence.

**Peroxides**. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance to be examined, mix and allow to stand protected from light for 5 min. No colour develops.

**Non-volatile matter**: maximum 20 mg/L.

*After ensuring that the substance to be examined complies with the test for peroxides*, evaporate 50 mL to dryness on a water-bath and dry the residue in an oven at 100-105 °C. The residue weighs a maximum of 1 mg.

**Substances with a foreign odour**. Moisten a disc of filter paper 80 mm in diameter with 5 mL of the substance to be examined and allow to evaporate. No foreign odour is perceptible immediately after the evaporation.

**Water** (*2.5.12*): maximum 2 g/L, determined on 20 mL.

STORAGE

In an airtight container, protected from light.

**07/2016:0367**

# ETHER, ANAESTHETIC

## Aether anaestheticus

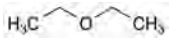

$C_4H_{10}O$ $M_r$ 74.1
[60-29-7]

DEFINITION

Diethyl ether.

It may contain a suitable non-volatile antioxidant at an appropriate concentration.

CHARACTERS

*Appearance*: clear, colourless liquid, volatile, very mobile.

*Solubility*: soluble in 15 parts of water, miscible with ethanol (96 per cent) and with fatty oils.

It is highly flammable.

IDENTIFICATION

A. Relative density (see Tests).

B. Distillation range (see Tests).

TESTS

**Acidity**. To 20 mL of *ethanol (96 per cent) R* add 0.25 mL of *bromothymol blue solution R1* and, dropwise, *0.02 M sodium hydroxide* until a blue colour persists for 30 s. Add 25 mL of the substance to be examined, shake and add, dropwise, *0.02 M sodium hydroxide* until the blue colour reappears and persists for 30 s. Not more than 0.4 mL of *0.02 M sodium hydroxide* is required.

**Relative density** (*2.2.5*): 0.714 to 0.716.

**Distillation range** (*2.2.11*). *Do not distil if the substance to be examined does not comply with the test for peroxides.* It distils completely between 34.0 °C and 35.0 °C. Carry out the test using a suitable heating device and taking care to avoid directly heating the flask above the level of the liquid.

**Acetone and aldehydes**. To 10.0 mL in a ground-glass-stoppered cylinder add 1 mL of *alkaline potassium tetraiodomercurate solution R* and shake for 10 s. Allow to stand for 5 min, protected from light. The lower layer shows only a slight opalescence.

If the substance to be examined does not comply with the test, distil 40 mL, *after ensuring that the substance to be examined complies with the test for peroxides*, until only 5 mL remains. Collect the distillate in a receiver cooled in a bath of iced water and repeat the test described above using 10.0 mL of the distillate.

**Peroxides**. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 15 mm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour develops.

**Non-volatile matter**: maximum 20 mg/L.

*After ensuring that the substance to be examined complies with the test for peroxides*, evaporate 50 mL to dryness on a water-bath and dry the residue in an oven at 100-105 °C. The residue weighs a maximum of 1 mg.

**Substances with a foreign odour**. Moisten a disc of filter paper 80 mm in diameter with 5 mL of the substance to be examined and allow to evaporate. No foreign odour is perceptible immediately after the evaporation.

**Water** (*2.5.12*): maximum 2 g/L, determined on 20 mL.

STORAGE

In an airtight container, protected from light. The contents of a partly filled container may deteriorate rapidly.

**04/2012:0140**

# ETHINYLESTRADIOL

## Ethinylestradiolum

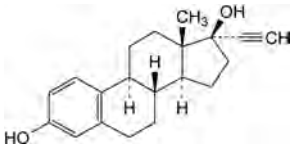


$C_{20}H_{24}O_2$ $M_r$ 296.4
[57-63-6]

DEFINITION

19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent). It dissolves in dilute alkaline solutions.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ethinylestradiol CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 25 mL with the solvent mixture.

*Reference solution*. Dissolve 25 mg of *ethinylestradiol CRS* in the solvent mixture and dilute to 25 mL with the solvent mixture.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *ethanol (96 per cent) R*, *toluene R* (10:90 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air until the solvent has evaporated.

*Detection*: heat at 110 °C for 10 min, spray the hot plate with *alcoholic solution of sulfuric acid R* and heat again at 110 °C for 10 min. Examine in daylight and in ultraviolet light at 365 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R1* (40:60 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 30 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2 mg of *estrone CRS* (impurity C) in 10.0 mL of the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture. Use 1.0 mL of this solution to dissolve the contents of a vial of *ethinylestradiol for system suitability CRS* (containing impurities B, F, H, I and K).

*Reference solution (c)*. Dissolve 50.0 mg of *ethinylestradiol CRS* in 30 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, *water R* (30:70 *V/V*);
- *mobile phase B*: *water R*, *acetonitrile R1* (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 100 | 0 |
| 35 - 65 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 30 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *ethinylestradiol for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, F, H, I and K.

*Relative retention* with reference to ethinylestradiol (retention time = about 35 min): impurity F = about 0.2; impurity H = about 0.5; impurity I = about 0.8; impurity B = about 0.88; impurity C = about 0.92; impurity K = about 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.2 between the peaks due to impurities I and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity I = 0.4;
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities H, I, K*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities C, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{20}H_{24}O_2$ from the declared content of *ethinylestradiol CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, F, H, I, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, G, J, L, M.*

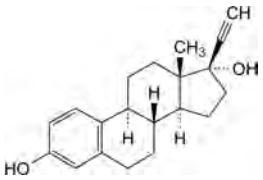

A. 19-norpregna-1,3,5(10)-trien-20-yne-3,17-diol (17β-ethinylestradiol),

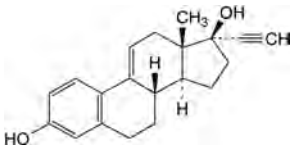

B. 19-nor-17α-pregna-1,3,5(10),9(11)-tetraen-20-yne-3,17-diol,

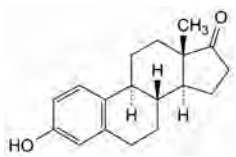

C. 3-hydroxyestra-1,3,5(10)-trien-17-one (estrone),

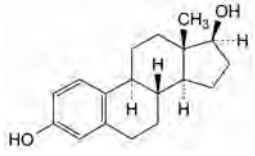

D. estra-1,3,5(10)-triene-3,17β-diol (estradiol),

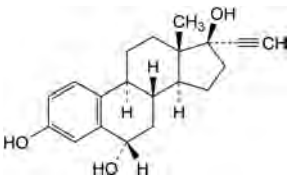

E. 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,6α,17-triol (6α-hydroxy-ethinylestradiol),

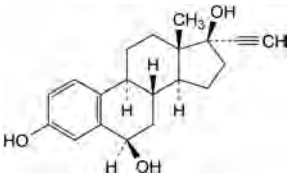

F. 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,6β,17-triol (6β-hydroxy-ethinylestradiol),

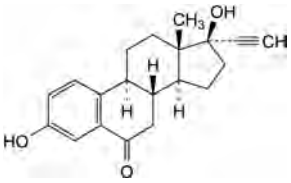

G. 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-6-one (6-oxo-ethinylestradiol),

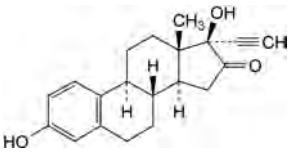

H. 3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-16-one (16-oxo-ethinylestradiol),

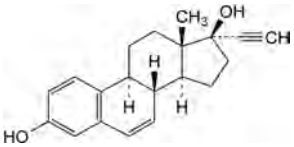

I. 19-nor-17α-pregna-1,3,5(10),6-tetraen-20-yne-3,17-diol,

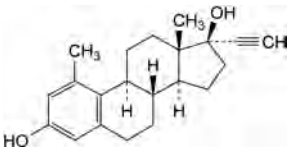

J. 1-methyl-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol (1-methyl-ethinylestradiol),

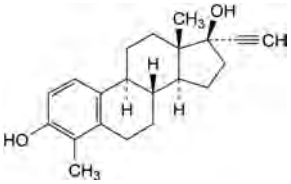

K. 4-methyl-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol (4-methyl-ethinylestradiol),

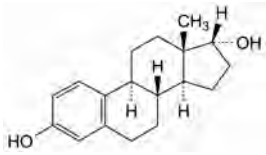

L. estra-1,3,5(10)-triene-3,17α-diol (17α-estradiol),

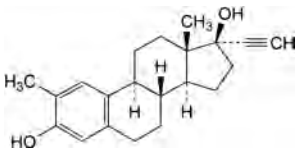

M. 2-methyl-19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol (2-methyl-ethinylestradiol).

**01/2017:0141**

# ETHIONAMIDE

## Ethionamidum

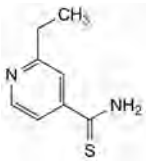

$C_8H_{10}N_2S$ $M_r$ 166.2
[536-33-4]

DEFINITION

2-Ethylpyridine-4-carbothioamide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance:* yellow, crystalline powder or small, yellow crystals.

*Solubility:* practically insoluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 158 °C to 164 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 290 nm.

*Specific absorbance at the absorption maximum*: 380 to 440.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison: ethionamide CRS.*

D. Dissolve about 10 mg in 5 mL of *methanol R*. Add 5 mL of *silver nitrate solution R2*. A dark brown precipitate is formed.

TESTS

**Acidity**. Dissolve 2.0 g in 20 mL of *methanol R*, heating to about 50 °C, and add 20 mL of *water R*. Cool slightly while shaking until crystallisation begins and then allow to cool to room temperature. Add 60 mL of *water R* and 0.2 mL of *cresol red solution R*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Dissolve 0.240 g of *ammonium dihydrogen phosphate R* in 700 mL of *water R* and add 300 mL of *acetonitrile R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *pyridine-4-carbonitrile R* (impurity B) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *ethionamide for system suitability CRS* (containing impurity A) in reference solution (b) and dilute to 5.0 mL with reference solution (b).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.287 g of *ammonium dihydrogen phosphate R* in 850 mL of *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 85 | 15 |
| 30 - 32 | 85 → 70 | 15 → 30 |
| 32 - 55 | 70 | 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 287 nm.

*Injection*: 5 µL of the test solution and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram supplied with *ethionamide for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to ethionamide (retention time = about 9 min): impurity A = about 0.4; impurity B = about 0.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of ethionamide in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.62 mg of $C_8H_{10}N_2S$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F.*

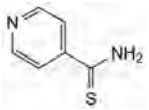

A. pyridine-4-carbothioamide,

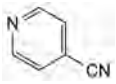

B. pyridine-4-carbonitrile,

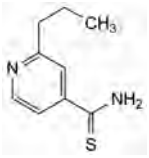

C. 2-propylpyridine-4-carbothioamide (prothionamide),

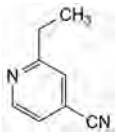

D. 2-ethylpyridine-4-carbonitrile,

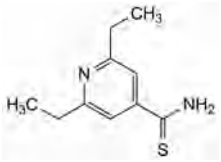

E. 2,6-diethylpyridine-4-carbothioamide,

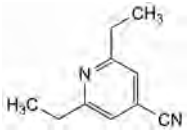

F. 2,6-diethylpyridine-4-carbonitrile.

**01/2019:0764**

# ETHOSUXIMIDE

## Ethosuximidum

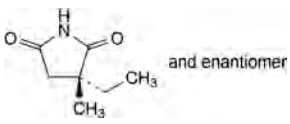


$C_7H_{11}NO_2$ $M_r$ 141.2
[77-67-8]

DEFINITION

(3*RS*)-3-Ethyl-3-methylpyrrolidine-2,5-dione.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, powder or waxy solid.

*Solubility*: freely soluble in water, very soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 45 °C to 50 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ethosuximide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *methanol R*.

*Reference solution*. Dissolve 20 mg of *ethosuximide CRS* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (17:17:66 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C for 10 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 25 mL with the same solvent.

**Cyanide**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.125 g of *potassium cyanide R* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. Dilute 0.5 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 0.50 g of the substance to be examined in *water R*, add 0.5 mL of reference solution (a) and dilute to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.075 m, Ø = 7.5 mm,

– *stationary phase*: spherical *weak anion-exchange resin R* (10 µm).

*Mobile phase*: dissolve 2.1 g of *lithium hydroxide R* and 85 mg of *sodium edetate R* in *water for chromatography R*, and dilute to 1000 mL with the same solvent.

*Flow rate*: 2.0 mL/min.

*Detection*: electrochemical detector (direct amperometry) with a silver working electrode, a silver-silver chloride reference electrode, held at + 0.05 V oxidation potential, and a detector sensitivity of 20 nA full scale.

*Injection*: 20 µL of the test solution and reference solution (b).

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to cyanide and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ethosuximide.

*Limit*:

– *cyanide*: not more than 0.5 times the height of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A. Store the solution at room temperature for at least 30 min before injection (*in situ* transformation of impurity B to impurity A).

*Reference solution (a).* Dissolve 5.0 mg of *ethosuximide impurity A CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with mobile phase A.

*Reference solution (c).* Mix 1 mL of reference solution (a) and 4 mL of the test solution.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2.1 mm;
– *stationary phase*: *end-capped solid core octadecylsilyl organosilica polymer compatible with 100 per cent aqueous mobile phases R* (2.6 µm);
– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: 15.6 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*;
– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 90 | 10 |
| 10 - 11 | 90 → 30 | 10 → 70 |
| 11 - 15 | 30 | 70 |

*Flow rate*: 0.25 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 3 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to ethosuximide (retention time = about 4 min): impurity A = about 1.7.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to ethosuximide and impurity A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of impurity A in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.2 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 20 mL of *dimethylformamide R* and carry out a potentiometric titration (*2.2.20*) using *0.1 M tetrabutylammonium hydroxide.* Protect the solution from atmospheric carbon dioxide throughout the titration. Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 14.12 mg of $C_7H_{11}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph Substances for pharmaceutical use (2034). It is therefore not necessary to identify these impurities for demonstration of compliance. See also 5.10. Control of impurities in substances for pharmaceutical use): A, B.*

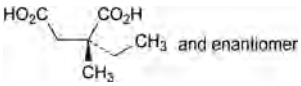


A. (2*RS*)-2-ethyl-2-methylbutanedioic acid,

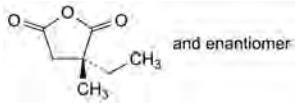


B. (3*RS*)-3-ethyl-3-methyldihydrofuran-2,5-dione.

**01/2008:0899**

# ETHYL ACETATE

## Ethylis acetas

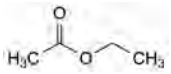


$C_4H_8O_2$ $M_r$ 88.1
[141-78-6]

DEFINITION

Ethyl ethanoate.

CHARACTERS

*Appearance*: clear, colourless, volatile liquid.

*Solubility*: soluble in water, miscible with acetone, with ethanol (96 per cent) and with methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Boiling point (*2.2.12*): 76 °C to 78 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of ethyl acetate.*

C. It gives the reaction of acetyl (*2.3.1*).

D. It gives the reaction of esters (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Mix 1 mL of the substance to be examined and 15 mL of *water R*.

**Acidity**. To 10 mL of *ethanol (96 per cent) R* add 0.1 mL of *phenolphthalein solution R* and *0.01 M sodium hydroxide* until the colour changes to pink. Add 5.5 mL of the substance to be examined and 0.25 mL of *0.02 M sodium hydroxide*. The solution remains pink for not less than 15 s.

**Relative density** (*2.2.5*): 0.898 to 0.902.

**Refractive index** (*2.2.6*): 1.370 to 1.373.

**Reaction with sulfuric acid**. Carefully add 2 mL to 10 mL of *sulfuric acid R*. After 15 min, the interface between the 2 liquids is not coloured.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Column*:

– *material*: glass;
– *size*: *l* = 2 m, Ø = 2 mm;
– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (136-173 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 18.8 | 90 → 240 |
| | 18.8 - 26.8 | 240 |
| Injection port | | 240 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Limit*:

– *total*: not more than 0.2 per cent of the area of the principal peak.

**Residue on evaporation**: maximum 30 ppm.

Evaporate 100.0 g to dryness on a water-bath and dry in an oven at 100-105 °C. The residue weighs not more than 3 mg.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 10.0 mL.

STORAGE

Protected from light, at a temperature not exceeding 30 °C.

IMPURITIES

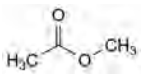

A. methyl ethanoate (methyl acetate),

B. ethanol,

C. methanol.

**01/2008:1319**

# ETHYL OLEATE

## Ethylis oleas

DEFINITION

Mixture consisting of the ethyl esters of fatty acids, mainly oleic (*cis*-9-octadecenoic) acid.

A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, pale yellow or colourless liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent), with methylene chloride and with light petroleum (bp: 40-60 °C).

IDENTIFICATION

A. Relative density (see Tests).
B. Saponification value (see Tests).
C. Oleic acid (see Tests).

TESTS

**Relative density** (*2.2.5*): 0.866 to 0.874.

**Acid value** (*2.5.1*): maximum 0.5, determined on 10.0 g.

**Iodine value** (*2.5.4, Method A*): 75 to 90.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Saponification value** (*2.5.6*): 177 to 188, determined on 2.0 g.

**Oleic acid** (*2.4.22, Method A*): minimum 60.0 per cent in the fatty acid fraction of the substance to be examined.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

Protected from light.

**07/2010:0900**

# ETHYL PARAHYDROXYBENZOATE

## Ethylis parahydroxybenzoas

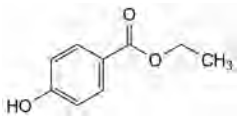

$C_9H_{10}O_3$ $M_r$ 166.2
[120-47-8]

DEFINITION

Ethyl 4-hydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 115 °C to 118 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ethyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.
*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.
*Reference solution (a)*. Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (b)*. Dissolve 10 mg of *methyl parahydroxybenzoate R* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).

*Application*: 2 µL of test solution (b) and reference solutions (a) and (b).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. To 2 mL of solution S add 3 mL of *ethanol (96 per cent) R*, 5 mL of *carbon dioxide-free water R* and 0.1 mL of *bromocresol green solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A), 5 mg of *methyl parahydroxybenzoate R* (impurity B) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50.0 mg of *ethyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 4 times the retention time of ethyl parahydroxybenzoate.

*Relative retention* with reference to ethyl parahydroxybenzoate (retention time = about 3.0 min): impurity A = about 0.5; impurity B = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity B and ethyl parahydroxybenzoate.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_9H_{10}O_3$ from the declared content of *ethyl parahydroxybenzoate CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

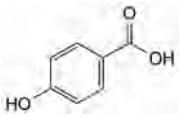


A. 4-hydroxybenzoic acid,

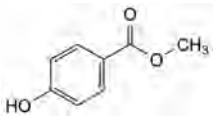


B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

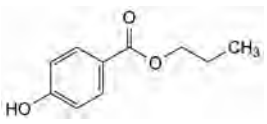


C. propyl 4-hydroxybenzoate (propyl parahydroxybenzoate),

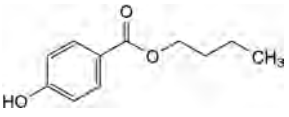


D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

**04/2019:0822**
**corrected 10.0**

# ETHYLCELLULOSE[3]

## Ethylcellulosum

DEFINITION

Partly *O*-ethylated cellulose.

It may contain a suitable antioxidant.

*Content*: 44.0 per cent to 51.0 per cent of ethoxy ($-OC_2H_5$) groups (dried substance).

♦CHARACTERS

*Appearance*: white or yellowish-white powder or granular powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride and in a mixture of 20 g of ethanol (96 per cent) and 80 g of toluene, slightly soluble in ethyl acetate and in methanol, practically insoluble in glycerol (85 per cent) and in propylene glycol. The solutions may show a slight opalescence.♦

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 40 mg in 1 mL of *methylene chloride R*; spread 2 drops of this solution between 2 sodium chloride plates then remove one of the plates to evaporate the solvent.

*Comparison*: *ethylcellulose CRS*.

◊B. It complies with the limits of the assay.◊

TESTS

**Acidity or alkalinity**. To 0.5 g add 25 mL of *carbon dioxide-free water R* and shake for 15 min. Filter through a sintered-glass filter (40) (*2.1.2*). To 10 mL of the solution add 0.1 mL of *phenolphthalein solution R* and 0.5 mL of *0.01 M sodium hydroxide*. The solution is pink. To 10 mL of the solution add 0.1 mL of *methyl red solution R* and 0.5 mL of *0.01 M hydrochloric acid*. The solution is red.

**Viscosity** (*2.2.9*): 80.0 per cent to 120.0 per cent of that stated on the label for a nominal viscosity greater than 6 mPa·s; 75.0 per cent to 140.0 per cent of that stated on the label for a nominal viscosity not greater than 6 mPa·s.

Shake a quantity of the substance to be examined equivalent to 5.00 g of the dried substance with 95 g of a mixture of 20 g of *ethanol (96 per cent) R* and 80 g of *toluene R* until the substance is dissolved. Determine the viscosity in mPa·s at 25 °C using a capillary viscometer.

**Acetaldehyde**: maximum 100 ppm.

Introduce 3.0 g into a 250 mL conical flask with a ground-glass stopper, add 10 mL of *water R* and stir mechanically for 1 h. Allow to stand for 24 h, filter and dilute the filtrate to 100.0 mL with *water R*. Transfer 5.0 mL of the filtrate to a 25 mL volumetric flask, add 5 mL of a 0.5 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* and heat in a water-bath at 60 °C for 5 min. Add 2 mL of *ferric chloride-sulfamic acid reagent R* and heat again in a water-bath at 60 °C for 5 min. Cool and dilute to 25.0 mL with *water R*. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using instead of the 5.0 mL of filtrate, 5.0 mL of a reference solution prepared by diluting 3.0 mL of *acetaldehyde standard solution (100 ppm $C_2H_4O$) R1* to 100.0 mL with *water R*.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Disperse 0.250 g in 50 mL of *water R*, heat to boiling and allow to cool, shaking occasionally. Filter and discard the first 10 mL of the filtrate. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution*. To 10 mL of *o-xylene R* add 0.5 mL of *octane R* and dilute to 100.0 mL with *o-xylene R*.

*Test solution*. To 30.0 mg (dried substance), add 60 mg of *adipic acid R* in a 5 mL pressure-tight reaction vial equipped with a pressure-tight membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap or another sealing system providing a sufficient air-tightness. Add 2.00 mL of the internal standard solution and 1.0 mL of *hydriodic acid R* and close immediately. Accurately weigh the vial (total mass before heating). Do not mix the contents of the vial by hand before heating. Place the vial in an oven or heat in a suitable heater, with continuous mechanical agitation, maintaining the internal temperature of the vial at 115 ± 2 °C for 70 min. Allow to cool and accurately weigh the vial (total mass after heating). If the difference between the total mass before heating and the total mass after heating is more than 10 mg, prepare a new test solution. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper layer as the test solution.

*Reference solution*. Place 60 mg of *adipic acid R* and 2.00 mL of the internal standard solution in another 5 mL reaction vial, add 1.0 mL of *hydriodic acid R* and close immediately. Accurately weigh the vial then inject 25 µL of *iodoethane R* through the septum into the vial, weigh again accurately and mix. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper layer as the reference solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.2 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 50 |
| | 3 - 8 | 50 → 100 |
| | 8 - 12 | 100 → 250 |
| | 12 - 20 | 250 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to octane (retention time = about 10 min): iodoethane = about 0.6.

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*System suitability*: reference solution:
- *resolution*: minimum 5.0 between the peaks due to iodoethane and octane;
- *repeatability*: maximum relative standard deviation of 2.0 per cent for the response factor of the principal peak determined on 6 injections.

Calculate the response factor ($R$) of *iodoethane R* using the following expression:

$$\frac{A_1 \times W_1 \times C}{A_2 \times 100}$$

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to iodoethane in the chromatogram obtained with the reference solution;

$W_1$ = mass of *iodoethane R* in the reference solution, in milligrams;

$C$ = percentage content of *iodoethane R*.

Calculate the percentage content *m/m* of ethoxy groups using the following expression:

$$\frac{A_4 \times R \times M_1 \times 100}{A_3 \times W_2 \times M_2}$$

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to iodoethane in the chromatogram obtained with the test solution;

$R$ = response factor;

$M_1$ = molar mass of the ethoxy group (45.1);

$M_2$ = molar mass of iodoethane (156.0);

$W_2$ = mass of the sample (dried substance) in the test solution, in milligrams.

LABELLING

The label states:
- the nominal viscosity in millipascal seconds for a 5 per cent *m/m* solution;
- the name and concentration of any added antioxidant.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for ethylcellulose used as binder and film former.*

**Viscosity** (see Tests).

**Degree of substitution** (see Assay).

*The following characteristics may be relevant for ethylcellulose used as matrix former in modified-release oral dosage forms.*

**Viscosity** (see Tests).

**Degree of substitution** (see Assay).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2020:1421**

# ETHYLENE GLYCOL MONOPALMITOSTEARATE

## Ethylenglycoli monopalmitostearas

DEFINITION

Mixture of ethylene glycol (ethane-1,2-diol) mono- and diesters of palmitic (hexadecanoic) and stearic (octadecanoic) acids, produced by the condensation of ethylene glycol and stearic acid 50 of vegetable or animal origin (see *Stearic acid (1474)*).

*Content*: minimum 50.0 per cent of monoesters.

CHARACTERS

*Appearance*: white or almost white, waxy solid.

*Solubility*: practically insoluble in water, soluble in acetone and in hot ethanol (96 per cent).

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of monoesters).

TESTS

**Melting point** (*2.2.15*): 54 °C to 60 °C.

**Acid value** (*2.5.1*): maximum 3.0.

**Iodine value** (*2.5.4*): maximum 3.0.

**Saponification value** (*2.5.6*): 170 to 195, determined on 2.0 g.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:
- *stearic acid*: 40.0 per cent to 60.0 per cent;
- *sum of contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

**Free ethylene glycol**: maximum 5.0 per cent, determined as prescribed under Assay.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Carry out the titration at 60 °C using a mixture of equal volumes of *methanol R* and *methylene chloride R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* In a 15 mL flask, weigh 0.200 g ($m$). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance ($M$).

*Reference solutions.* In four 15 mL flasks, weigh respectively 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *ethylene glycol R*. Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of ethylene glycol in milligrams per gram for each reference solution.

*Column*:
- *size*: $l$ = 0.6 m, Ø = 7.5 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to ethylene glycol: diesters = about 0.76; monoesters = about 0.83.

*Calculations*:

– *free ethylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration of ethylene glycol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free ethylene glycol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

– *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 270}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

270 = average rounded molar mass of palmitic acid and stearic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

– *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y}(100 - A - B)\right] - D$$

*A* = percentage content of free ethylene glycol;

*B* = percentage content of water (see Tests);

*D* = percentage content of free fatty acids;

*X* = area of the peak due to monoesters and free fatty acids;

*Y* = area of the peak due to diesters.

STORAGE

Protected from light.

**01/2017:0716**

# ETHYLENEDIAMINE

## Ethylendiaminum

$C_2H_8N_2$ $M_r$ 60.1

[107-15-3]

DEFINITION

Ethane-1,2-diamine.

*Content*: 98.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow liquid, hygroscopic.

*Solubility*: miscible with water and with anhydrous ethanol.

On exposure to air, white fumes are evolved. On heating, it evaporates completely.

IDENTIFICATION

A. Relative density (*2.2.5*): 0.895 to 0.905.

B. Boiling point (*2.2.12*): 116 °C to 118 °C.

C. To 0.2 mL add 0.5 mL of *acetic anhydride R*. Boil. A crystalline mass forms after cooling, which dissolves in 5 mL of *2-propanol R* with heating. Cool the solution and add 5 mL of *ether R*. If necessary, initiate crystallisation by scratching the walls of the test-tube with a glass rod. Filter through a sintered-glass filter (*2.1.2*), wash with several portions of *ether R* and dry at 100-105 °C. The residue melts (*2.2.14*) at 173 °C to 177 °C.

TESTS

**Solution S**. Mix 10 g with *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than the reference solution $BY_6$ (*2.2.2, Method II*).

**Carbonate**. A mixture of 4 mL of solution S and 6 mL of *calcium hydroxide solution R* is not more opalescent than reference suspension II (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 5 mL of solution S add 5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Ammonia and other bases**. Dissolve 1.2 g in 20 mL of *ethanol (96 per cent) R* and add, dropwise with stirring, 4.5 mL of *hydrochloric acid R*. Evaporate to dryness on a water-bath, breaking up any resulting cake with a glass rod, and dry at 100-105 °C for 1 h. 1 g of the residue is equivalent to 0.4518 g of $C_2H_8N_2$. Calculate the percentage content of $C_2H_8N_2$: it does not vary by more than 0.5 from the percentage content determined in the assay.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Residue on evaporation**: maximum 0.3 per cent.

Evaporate 5.00 g to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 15 mg.

ASSAY

Place 25.0 mL of *1 M hydrochloric acid* and 0.2 mL of *methyl red mixed solution R* in a flask. Add 0.600 g of the substance to be examined. Titrate with *1 M sodium hydroxide* until the colour changes from violet-red to green.

1 mL of *1 M hydrochloric acid* is equivalent to 30.05 mg of $C_2H_8N_2$.

STORAGE

In an airtight container, protected from light.

**01/2008:0491**

# ETHYLMORPHINE HYDROCHLORIDE

## Ethylmorphini hydrochloridum

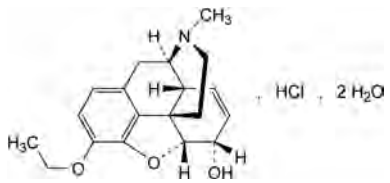

$C_{19}H_{24}ClNO_3,2H_2O$ $M_r$ 385.9

DEFINITION

7,8-Didehydro-4,5α-epoxy-3-ethoxy-17-methylmorphinan-6α-ol hydrochloride dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in alcohol, insoluble in cyclohexane.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of ethylmorphine hydrochloride*.

B. In a test-tube, dissolve 0.5 g in 6 mL of *water R* and add 15 mL of *0.1 M sodium hydroxide*. Scratch the wall of the tube with a glass rod. A white, crystalline precipitate is formed. Collect the precipitate, wash and dissolve in 20 mL of *water R* heated to 80 °C. Filter and cool in iced water. The crystals, after drying *in vacuo* for 12 h, melt (*2.2.14*) at 85 °C to 89 °C.

C. To about 10 mg add 1 mL of *sulfuric acid R* and 0.05 mL of *ferric chloride solution R2*. Heat on a water-bath. A blue colour develops. Add 0.05 mL of *nitric acid R*. The colour becomes red.

D. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R* and 0.2 mL of *0.02 M hydrochloric acid*, the solution is red. Add 0.4 mL of *0.02 M sodium hydroxide*, the solution becomes yellow.

**Specific optical rotation** (*2.2.7*): – 102 to – 105 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 25.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 12.5 mg of *codeine R* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 0.5 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Reference solution (d)*. To 1.0 mL of the test solution, add 1.0 mL of reference solution (b) and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*: add 1.25 g of *sodium heptanesulfonate R* to a mixture of 12.5 mL of *glacial acetic acid R* and 5 mL of a 20 per cent *V/V* solution of *triethylamine R* in a mixture of equal volumes of *methanol R* and *water R*. Dilute to 1000 mL with *water R*. To 550 mL of this solution add 450 mL of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of ethylmorphine.

*Relative retention* with reference to ethylmorphine (retention time = about 6.2 min): impurity B = about 0.7; impurity C = about 0.8; impurity D = about 1.3; impurity A = about 2.5.

*System suitability*: reference solution (d):

- *resolution*: minimum 5 between the peaks due to ethylmorphine and impurity C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.4,
- *impurities A, B, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total of impurities other than C*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 8.0 per cent to 10.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 30 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.99 mg of $C_{19}H_{24}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

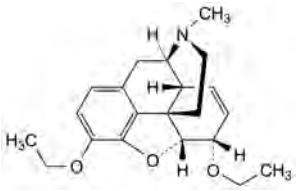

A. 7,8-didehydro-4,5α-epoxy-3,6α-diethoxy-17-methylmorphinan,

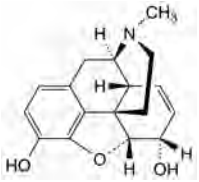

B. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol (morphine),

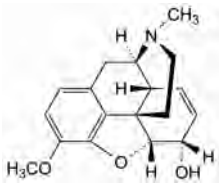

C. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (codeine),

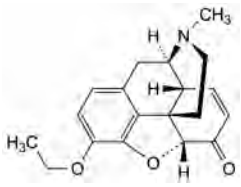

D. 7,8-didehydro-4,5α-epoxy-3-ethoxy-17-methylmorphinan-6-one (ethylmorphinone).

**01/2017:1778**

# ETIDRONATE DISODIUM

## Dinatrii etidronas

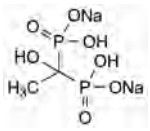

$C_2H_6Na_2O_7P_2$ $M_r$ 250.0
[7414-83-7]

DEFINITION

Disodium dihydrogen (1-hydroxyethylidene)bisphosphonate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *etidronate disodium CRS*.
The transmittance at about 2000 $cm^{-1}$ (5 µm) is not less than 40 per cent without compensation.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.2 to 5.2.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Impurities A and B**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* To 2.0 mL of a 0.3 g/L solution of *phosphoric acid R* add 2.0 mL of a 0.25 g/L solution of *phosphorous acid R* and dilute to 50.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *anion-exchange resin R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 0.2 mL of *anhydrous formic acid R* and 1000 mL of *water R*; adjust to pH 3.5 with an 80 g/L solution of *sodium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: differential refractometer.

*Injection*: 100 µL.

*System suitability*: reference solution:
- *resolution*: minimum 2.5 between the peaks due to impurity A and impurity B.

*Limits*:
- *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent).

**Water** (*2.5.32*): maximum 5.0 per cent.

Dissolve 50.0 mg in a mixture of equal volumes of *anhydrous acetic acid R* and *formamide R* and dilute to 5.0 mL with the same mixture of solvents. Use 1.0 mL of the solution.

ASSAY

Dissolve 0.100 g in 2 mL of *anhydrous formic acid R* and dilute to 50 mL with *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 12.50 mg of $C_2H_6Na_2O_7P_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

A. $H_3PO_4$: phosphoric acid,

B. $H_3PO_3$: phosphorous acid.

**01/2017:1205**

# ETILEFRINE HYDROCHLORIDE

## Etilefrini hydrochloridum

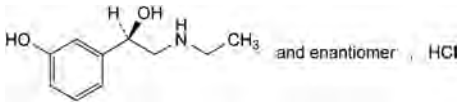


$C_{10}H_{16}ClNO_2$ $M_r$ 217.7
[943-17-9]

DEFINITION

(1*RS*)-2-(Ethylamino)-1-(3-hydroxyphenyl)ethanol hydrochloride.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 118 °C to 122 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium chloride R*.
*Comparison*: *etilefrine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Prepare the solutions protected from bright light and develop the chromatograms protected from light.*

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *etilefrine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *phenylephrine hydrochloride CRS* in 2 mL of reference solution (a) and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (5:25:70 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a 10 g/L solution of *potassium permanganate R*; examine in daylight after 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.2 mL of solution S (see Tests), add 1 mL of *water R*, 0.1 mL of *copper sulfate solution R* and 1 mL of *strong sodium hydroxide solution R*. A blue colour is produced. Add 2 mL of *ether R* and shake. The upper layer is colourless.

E. Dilute 1 mL of solution S to 10 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 4 mL of solution S to 10 mL with *carbon dioxide-free water R*. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 50.0 mL with *water R*.

*Reference solution (b).* Dissolve 10.0 mg of *etilefrine impurity A CRS* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (c).* To 10.0 mL of reference solution (a) add 5.0 mL of reference solution (b) and dilute to 20.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.1 g/L solution of *sodium laurilsulfate R* adjusted to pH 2.3 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of etilefrine.

*Relative retention* with reference to etilefrine (retention time = about 9 min): impurity E = about 0.5; impurity C = about 0.8; impurity B = about 0.9; impurity A = about 1.2; impurity F = about 1.7; impurity D = about 4.5.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to etilefrine and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),

– *impurities B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *sum of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent),

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak due to the solvent.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on 15 mL of solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 20 mL of *anhydrous acetic acid R* and 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.77 mg of $C_{10}H_{16}ClNO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

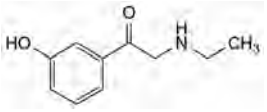


A. 2-(ethylamino)-1-(3-hydroxyphenyl)ethanone (etilefrone),

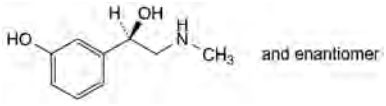


B. (1*RS*)-1-(3-hydroxyphenyl)-2-(methylamino)ethanol (phenylephrine),

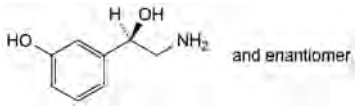


C. (1*RS*)-2-amino-1-(3-hydroxyphenyl)ethanol (norfenefrine),

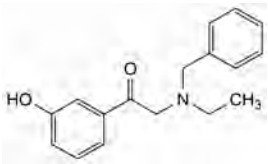


D. 2-(benzylethylamino)-1-(3-hydroxyphenyl)ethanone (benzyletilefrone),

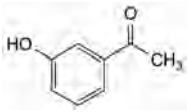


E. 1-(3-hydroxyphenyl)ethanone (3-hydroxyacetophenone),

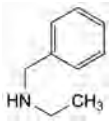


F. *N*-benzylethanamine (benzylethylamine).

**01/2017:1422**

# ETODOLAC

## Etodolacum

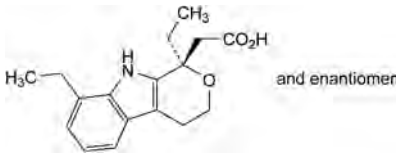


$C_{17}H_{21}NO_3$ $M_r$ 287.4
[41340-25-4]

DEFINITION

[(1*RS*)-1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 144 °C to 150 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *etodolac CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *etodolac CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R* previously activated by heating at 105 °C for 1 h.
Place the plate in an unsaturated tank containing a mixture of 20 volumes of a 25 g/L solution of *ascorbic acid R* and 80 volumes of *methanol R*. Allow the solution to ascend 1 cm above the line of application on the plate, remove the plate and allow it to dry for at least 30 min.
*Mobile phase*: *glacial acetic acid R*, *anhydrous ethanol R*, *toluene R* (0.5:30:70 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution.* Dissolve 20.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 50.0 mL with the same solvent.
*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 20.0 mL with *acetonitrile R1*.
*Reference solution (b).* Dissolve 4 mg of *etodolac impurity H CRS* in the test solution and dilute to 10 mL with the same solution. Dilute 0.5 mL of this solution to 50 mL with *acetonitrile R1*.
*Reference solution (c).* Dissolve the contents of a vial of *etodolac for peak identification CRS* (containing impurities A, B, C, D, E, F, G, H, I and K) in 1.0 mL of *acetonitrile R1*.
*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 35 °C.
*Mobile phase*:
– *mobile phase A*: 0.77 g/L solution of *ammonium acetate R*;
– *mobile phase B*: mobile phase A, *acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 80 → 50 | 20 → 50 |
| 25 - 42 | 50 | 50 |
| 42 - 48 | 50 → 80 | 50 → 20 |

*Flow rate*: 1 mL/min.
*Detection*: spectrophotometer at 225 nm.
*Injection*: 5 µL.
*Identification of impurities*: use the chromatogram supplied with *etodolac for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E, F, G, H, I and K.
*Relative retention* with reference to etodolac (retention time = about 16.7 min): impurity A = about 0.68; impurity B = about 0.83; impurity C = about 0.85; impurity H = about 1.09; impurity D = about 1.17;

impurity G = about 1.19; impurity E = about 1.20; impurity F = about 1.22; impurity I = about 1.50; impurity K = about 2.37.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to etodolac and impurity H.

*Limits*:

– *impurity C*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurities A, B, D, E, F, G, H, I, K*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides**: maximum 300 ppm.

Dissolve 1.0 g of the substance to be examined in 60 mL of *methanol R*, add 10 mL of *water R* and 20 mL of *dilute nitric acid R*. Titrate with *0.01 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.01 M silver nitrate* is equivalent to 0.3545 mg of Cl.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 mL of *methanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 28.74 mg of $C_{17}H_{21}NO_3$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, L.*

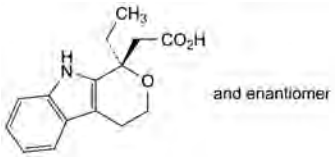


A. [(1*RS*)-1-ethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-desethyl etodolac),

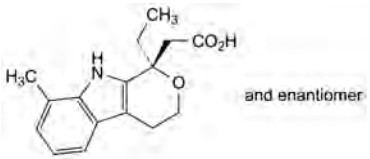


B. [(1*RS*)-1-ethyl-8-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-methyl etodolac),

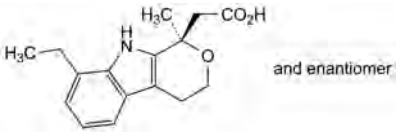


C. [(1*RS*)-8-ethyl-1-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-methyl etodolac),

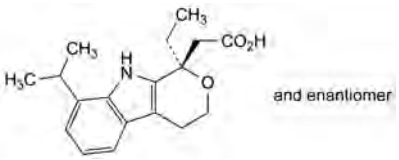


D. [(1*RS*)-1-ethyl-8-(1-methylethyl)-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-isopropyl etodolac),

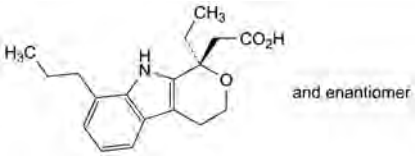


E. [(1*RS*)-1-ethyl-8-propyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (8-propyl etodolac),

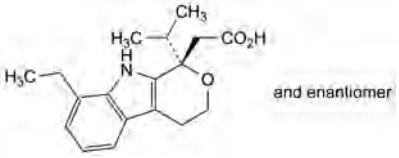


F. [(1*RS*)-8-ethyl-1-(1-methylethyl)-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-isopropyl etodolac),

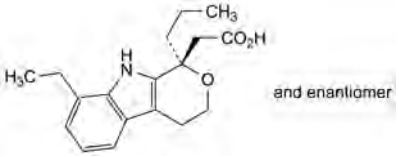


G. [(1*RS*)-8-ethyl-1-propyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetic acid (1-propyl etodolac),

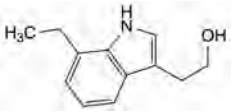


H. 2-(7-ethyl-1*H*-indol-3-yl)ethanol,

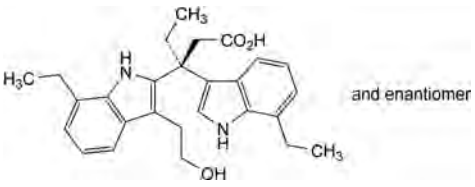


I. (3*RS*)-3-[7-ethyl-3-(2-hydroxyethyl)-1*H*-indol-2-yl]-3-(7-ethyl-1*H*-indol-3-yl)pentanoic acid (etodolac dimer),

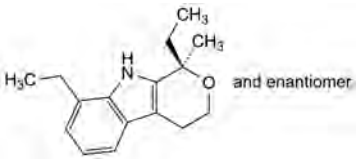


J. (1*RS*)-1,8-diethyl-1-methyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indole (decarboxy etodolac),

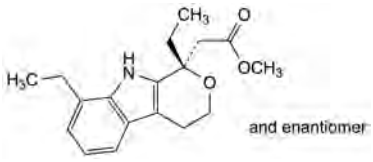


K. methyl [(1*RS*)-1,8-diethyl-1,3,4,9-tetrahydropyrano[3,4-*b*]indol-1-yl]acetate (etodolac methyl ester),

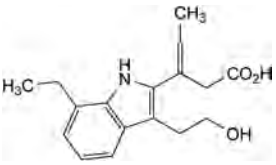


L. (*EZ*)-3-[7-ethyl-3-(2-hydroxyethyl)-1*H*-indol-2-yl]pent-3-enoic acid.

**01/2017:1513**
**corrected 10.0**

# ETOFENAMATE

## Etofenamatum

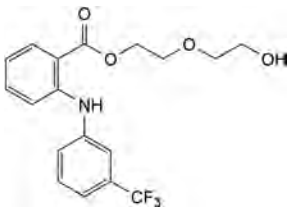


$C_{18}H_{18}F_3NO_4$ $M_r$ 369.4
[30544-47-9]

DEFINITION

2-(2-Hydroxyethoxy)ethyl 2-[[3-(trifluoromethyl)phenyl]-amino]benzoate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellowish, viscous liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent) and with ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *etofenamate CRS*.

*Preparation*: films.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_1$ (*2.2.2, Method II*).

**Impurity F**. Gas chromatography (*2.2.28*).

*Internal standard*: *tetradecane R*.

*Solution A*. Dissolve 6.0 mg of *tetradecane R* in *hexane R* and dilute to 10.0 mL with the same solvent.

*Solution B*. To 6.0 mg of *diethylene glycol R* in a 10 mL volumetric flask add 3 mL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat for 30 min at 50 °C. After cooling dilute to 10.0 mL with *N-methyltrimethylsilyl-trifluoroacetamide R*.

*Test solution*. To 0.200 g of the substance to be examined add 10 µL of solution A. Add 2 mL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat for 30 min at 50 °C.

*Reference solution*. To 2.0 mL of *N-methyltrimethylsilyl-trifluoroacetamide R* add 10 µL of solution A and 10 µL of solution B.

*Column*:

– *size*: *l* = 25 m, Ø = 0.20 mm;
– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.33 µm).

*Carrier gas*: *hydrogen for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) | Rate (°C/min) |
|---|---|---|---|
| Column | 0 - 13 | 60 → 150 | 7 |
| | 13 - 19 | 150 → 300 | 25 |
| | 19 - 34 | 300 | |
| Injection port | | 150 | |
| Detector | | 300 | |

*Detection*: flame ionisation.

*Injection*: 0.2 µL.

*Limit*:

– *impurity F*: maximum 0.1 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R* (40:60 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 30 mL of *methanol R* and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. Dissolve 10.0 mg of *etofenamate impurity G CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 0.2 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 0.2 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. To 5.0 mL of reference solution (a), add 5.0 mL of reference solution (b).

*Reference solution (d)*. Dissolve 10.0 mg of *etofenamate for peak identification CRS* (containing impurities A, B, C, D and E) in 6.0 mL of *methanol R* and dilute to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 1.3 g of *ammonium phosphate R* and 4.0 g of *tetrabutylammonium hydroxide R* in 900 mL of *water R*, adjust to pH 8.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*;
– *mobile phase B*: *methanol R*;

| Time | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 13 | 40 | 60 |
| 13 - 20 | 40 → 10 | 60 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 286 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *etofenamate for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D and E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity G.

*Relative retention* with reference to etofenamate (retention time = about 13 min): impurity A = about 0.2; impurity C = about 0.7; impurity G = about 0.85; impurity E = about 1.5; impurity B = about 1.6; impurity D = about 1.7.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.3 between the peaks due to impurity G and etofenamate.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.62; impurity C = 0.45; impurity D = 0.77;

– *impurity D*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurity A*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *impurities B, C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.2 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 3.000 g add 20 mL of *2-propanol R* and 20.0 mL of *1 M sodium hydroxide* and heat under reflux for 2 h. Add 0.1 mL of *bromothymol blue solution R1*. Titrate after cooling with *1 M hydrochloric acid* until the colour disappears. Carry out a blank titration.

1 mL of *1 M sodium hydroxide* is equivalent to 0.3694 g of $C_{18}H_{18}F_3NO_4$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

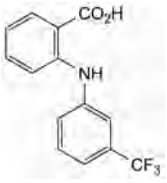

A. 2-[[3-(trifluoromethyl)phenyl]amino]benzoic acid (flufenamic acid),

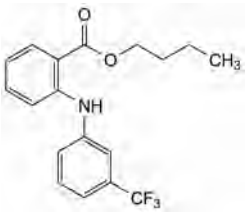

B. butyl 2-[[3-(trifluoromethyl)phenyl]amino]benzoate (butyl flufenamate),

C. *N*-phenyl-3-(trifluoromethyl)aniline,

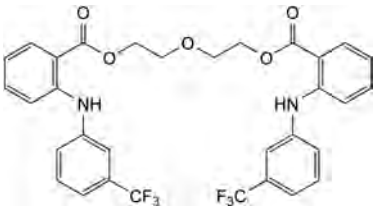

D. 2,2′-oxybis(ethylene) bis[2-[[3-(trifluoromethyl)phenyl]-amino]benzoate],

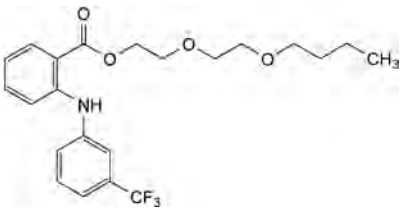

E. 2-(2-butoxyethoxy)ethyl 2-[[3-(trifluoromethyl)phenyl]-amino]benzoate,

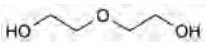

F. 2,2′-oxydiethanol,

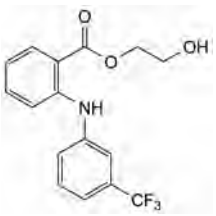

G. 2-hydroxyethyl 2-[[3-(trifluoromethyl)phenyl]amino]-benzoate.

**01/2008:1514**
**corrected 7.0**

# ETOMIDATE

## Etomidatum

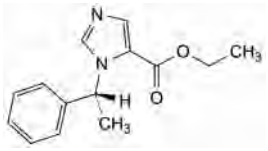

$C_{14}H_{16}N_2O_2$ $M_r$ 244.3
[33125-97-2]

DEFINITION

Ethyl 1-[(1*R*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 68 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *etomidate CRS*.

B. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 0.25 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 67 to + 70 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *anhydrous ethanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5.0 mg of *etomidate CRS* and 5.0 mg of *etomidate impurity B CRS* in the solvent mixture, then dilute to 250.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 25.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium carbonate R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 → 30 | 10 → 70 |
| 5 - 6 | 30 → 10 | 70 → 90 |
| 6 - 10 | 10 | 90 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*Retention time*: impurity B = about 4.5 min; etomidate = about 5.0 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to impurity B and etomidate; if necessary, adjust the concentration of ammonium carbonate in the mobile phase or the time programme of the linear gradient.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 40 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid* using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 24.43 mg of $C_{14}H_{16}N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

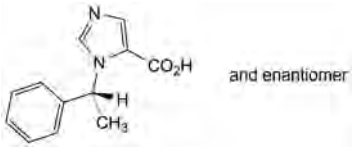


A. 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylic acid,

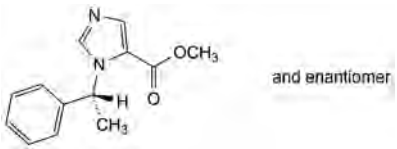


B. methyl 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate (metomidate),

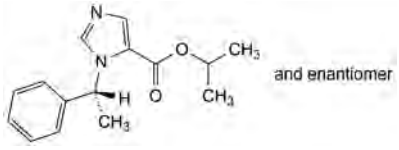


C. 1-methylethyl 1-[(1*RS*)-1-phenylethyl]-1*H*-imidazole-5-carboxylate.

**04/2017:0823**

# ETOPOSIDE

## Etoposidum

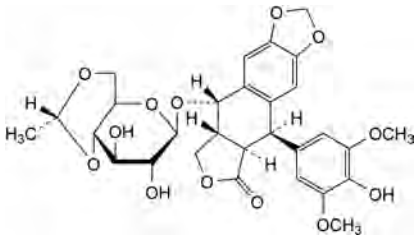

$C_{29}H_{32}O_{13}$ $M_r$ 588.6
[33419-42-0]

DEFINITION

(5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *etoposide CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 2 mL with the same mixture of solvents.
*Reference solution.* Dissolve 10 mg of *etoposide CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 2 mL with the same mixture of solvents.
*Plate*: *silica gel H R* as the coating substance.
*Mobile phase*: *water R*, *glacial acetic acid R*, *acetone R*, *methylene chloride R* (1.5:8:20:100 *V/V/V/V*).
*Application*: 5 µL as bands of 10 mm.
*Development*: immediately, over 6/7 of the plate.
*Drying*: in a current of warm air for 5 min.
*Detection*: spray with a mixture of 1 volume of *sulfuric acid R* and 9 volumes of *ethanol (96 per cent) R* and heat at 140 °C for 15 min. Cover the plate immediately with a glass plate of the same size. Examine in daylight.
*Results*: the principal zone in the chromatogram obtained with the test solution is similar in position, colour and size to the principal zone in the chromatogram obtained with the reference solution.

D. In a test-tube dissolve about 5 mg in 5 mL of *glacial acetic acid R* and add 0.05 mL of *ferric chloride solution R1*. Mix and cautiously add 2 mL of *sulfuric acid R*. Avoid mixing the 2 layers. Allow to stand for about 30 min; a pink to reddish-brown ring develops at the interface and the upper layer is yellow.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

Dissolve 0.6 g in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 mL with the same mixture of solvents.

**Specific optical rotation** (*2.2.7*): – 114 to – 106 (anhydrous substance).

Dissolve 50.0 mg in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution (a).* Dissolve 40 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 4 mg of *etoposide for system suitability CRS* (containing impurities B, C, D, E, N and O) in 1.0 mL of the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *etoposide CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *anhydrous formic acid R*, *triethylamine R*, *water for chromatography R* (1:1:998 *V/V/V*);
- *mobile phase B*: *anhydrous formic acid R*, *triethylamine R*, *acetonitrile R* (1:1:998 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 75 | 25 |
| 7 - 23 | 75 → 27 | 25 → 73 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *etoposide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D, E, N and O.

*Relative retention* with reference to etoposide (retention time = about 5 min): impurity D = about 0.4; impurity E = about 0.8; impurity C = about 1.1; impurity B = about 1.2; impurity N = about 3.1; impurity O = about 4.2.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to etoposide; and minimum 3.0, where $H_p$ = height above the baseline

of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity O by 1.7;
- *impurities B, C, D, E, N*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity O*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to the solvent.

**Water** (*2.5.32*): maximum 6.0 per cent, determined on 0.150 g using the evaporation technique at 130 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (c).

*System suitability*:

- *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections of reference solution (c).

Calculate the percentage content of $C_{29}H_{32}O_{13}$ taking into account the assigned content of *etoposide CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: B, C, D, E, N, O.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, F, G, H, I, J, K, L, M, P, Q, R.*

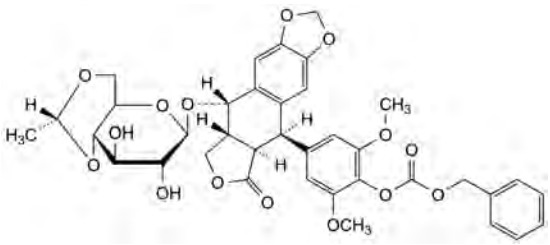

A. (5*R*,5a*R*,8a*R*,9*S*)-5-[4-[[(benzyloxy)carbonyl]oxy]-3,5-dimethoxyphenyl]-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4'-carbobenzoyloxyethylidene-lignan P),

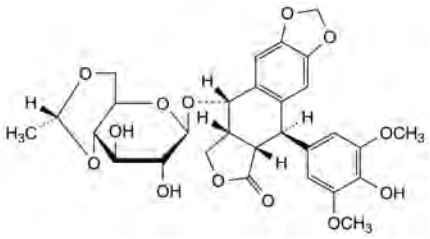

B. (5*R*,5a*S*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (picroethylidene-lignan P; *cis*-etoposide),

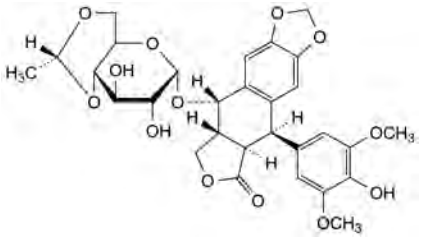

C. (5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-α-D-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (α-etoposide),

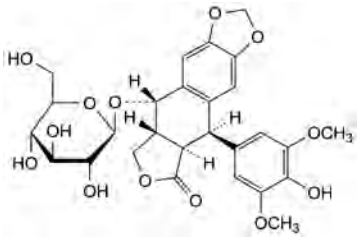

D. (5*R*,5a*R*,8a*R*,9*S*)-9-(β-D-glucopyranosyloxy)-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro-[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (lignan P),

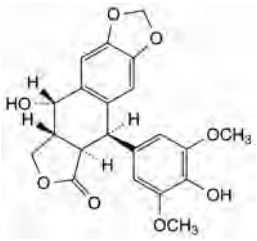

E. (5*R*,5a*R*,8a*R*,9*S*)-9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4′-desmethylepipodophyllotoxin),

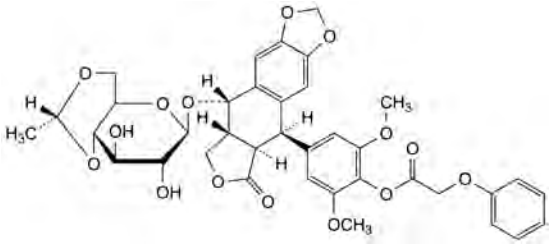

F. (5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-[4-[(phenoxyacetyl)oxy]-3,5-dimethoxyphenyl]-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4'-phenoxyacetyletoposide),

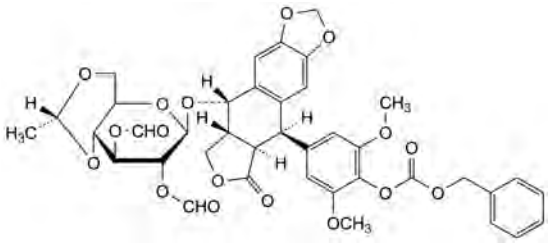

G. (5*R*,5a*R*,8a*R*,9*S*)-5-[4-[[(benzyloxy)carbonyl]oxy]-3,5-dimethoxyphenyl]-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-2,3-di-*O*-formyl-β-D-glucopyranosyl]oxy]-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4'-carbobenzoyloxydiformyl-ethylidene-lignan P),

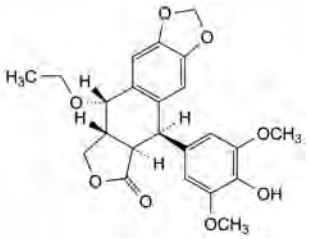

H. (5*R*,5a*R*,8a*R*,9*S*)-9-ethoxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4′-*O*-desmethyl-1-*O*-ethylepipodophyllotoxin),

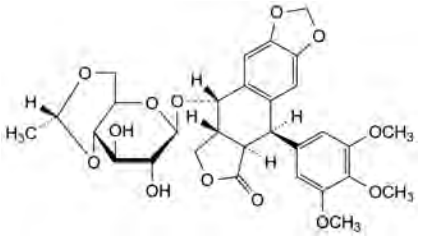

I. (5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-(3,4,5-trimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4-*O*-methylethylidene-lignan P),

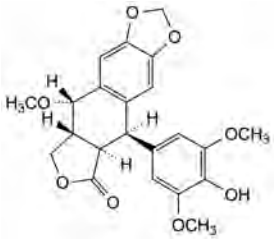

J. (5*R*,5a*R*,8a*R*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-9-methoxy-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]-benzodioxol-6(5a*H*)-one (4′-*O*-desmethyl-1-*O*-methylepipodophyllotoxin),

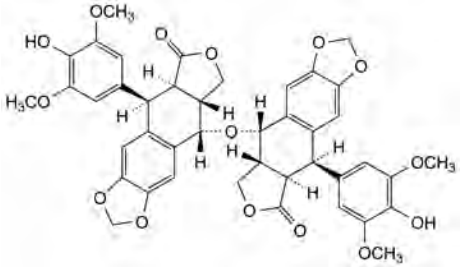

K. 9,9′-oxybis[(5*R*,5a*R*,8a*R*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro-[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one] (di-4′-*O*-desmethylepipodophyllotoxin),

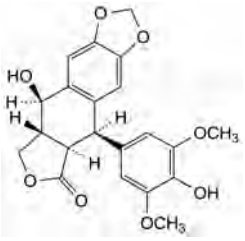

L. (5*R*,5a*R*,8a*R*,9*R*)-9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (4′-*O*-desmethylpodophyllotoxin),

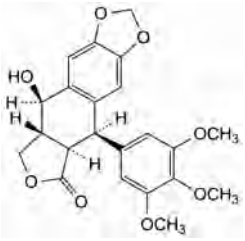

M. (5*R*,5a*R*,8a*R*,9*R*)-9-hydroxy-5-(3,4,5-trimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one (podophyllotoxin),

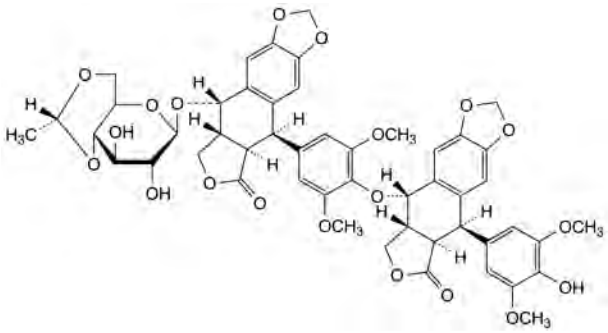

N. (5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-[4-[[(5*R*,5a*R*,8a*R*,9*S*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-6-oxo-5,5a,6,8,8a,9-hexahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-9-yl]oxy]-3,5-dimethoxyphenyl]-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one,

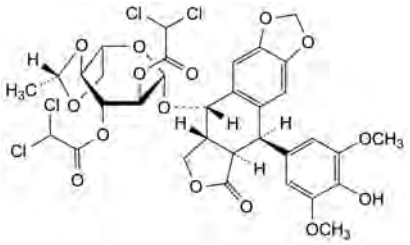

O. (5*R*,5a*R*,8a*R*,9*S*)-9-[[2,3-bis-*O*-(dichloroacetyl)-4,6-*O*-[(1*S*)-ethane-1,1-diyl]-β-L-glucopyranosyl]oxy]-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one,

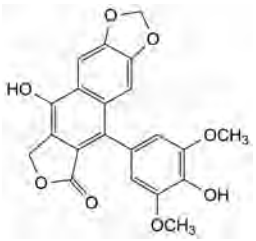

P. 9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(8*H*)-one,

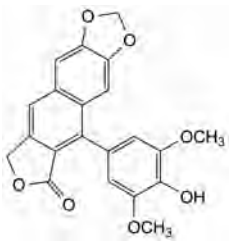

Q. 5-(4-hydroxy-3,5-dimethoxyphenyl)[2]benzofuro-[5,6-*f*][1,3]benzodioxol-6(8*H*)-one,

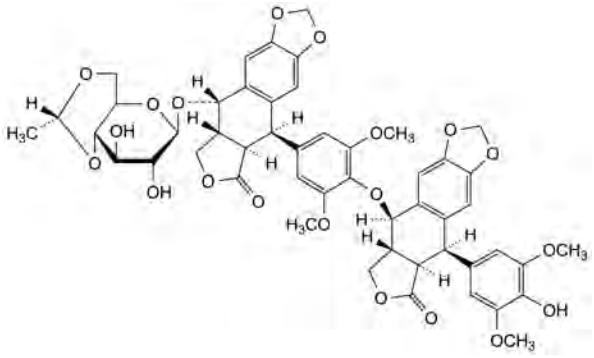

R. (5*R*,5a*R*,8a*R*,9*S*)-9-[[4,6-*O*-[(1*R*)-ethane-1,1-diyl]-β-D-glucopyranosyl]oxy]-5-[4-[[(5*R*,5a*R*,8a*R*,9*R*)-5-(4-hydroxy-3,5-dimethoxyphenyl)-6-oxo-5,5a,6,8,8a,9-hexahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-9-yl]oxy]-3,5-dimethoxyphenyl]-5,8,8a,9-tetrahydro[2]benzofuro[5,6-*f*][1,3]benzodioxol-6(5a*H*)-one.

**01/2008:1100**
**corrected 10.0**

# EUGENOL

## Eugenolum

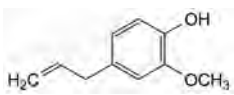

$C_{10}H_{12}O_2$ $M_r$ 164.2
[97-53-0]

DEFINITION

2-Methoxy-4-(prop-2-enyl)phenol.

CHARACTERS

*Appearance*: colourless or pale yellow, clear liquid, darkening on exposure to air.

It has a strong odour of clove.

*Solubility*: practically insoluble in water, freely soluble in ethanol (70 per cent *V/V*), practically insoluble in glycerol, miscible with ethanol (96 per cent), with glacial acetic acid, with methylene chloride and with fatty oils.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Refractive index (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *eugenol CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 µL of the substance to be examined in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

*Reference solution.* Dissolve 50 µL of *eugenol CRS* in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (10:90 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of cold air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.05 mL in 2 mL of *ethanol (96 per cent) R* and add 0.1 mL of *ferric chloride solution R1*. A dark green colour is produced which changes to yellowish-green within 10 min.

TESTS

**Relative density** (*2.2.5*): 1.066 to 1.070.

**Refractive index** (*2.2.6*): 1.540 to 1.542.

**Dimeric and oligomeric compounds**. Dissolve 0.150 g in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. The absorbance (*2.2.25*) of the solution at 330 nm is not greater than 0.25.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 1.00 g of the substance to be examined in *anhydrous ethanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *anhydrous ethanol R.*

*Reference solution (b).* Dissolve 50 mg of *vanillin R* (impurity H) in 1 mL of the test solution and dilute to 5 mL with *anhydrous ethanol R.*

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 80 |
| | 2 - 27 | 80 → 280 |
| | 27 - 47 | 280 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

– *relative retention* with reference to eugenol: impurity H = minimum 1.1.

*Limits*:

– *any impurity*: for each impurity, maximum 0.5 per cent;
– *sum of impurities with a relative retention greater than 2.0 with reference to eugenol*: maximum 1.0 per cent;
– *total*: maximum 3.0 per cent;
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Hydrocarbons**. Dissolve 1 mL in 5 mL of *dilute sodium hydroxide solution R* and add 30 mL of *water R* in a stoppered test-tube. Examined immediately, the solution is yellow and clear (*2.2.1*).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

In a well-filled container, protected from light.

IMPURITIES

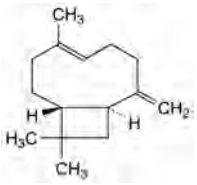

A. (1*R*,4*E*,9*S*)-4,11,11-trimethyl-8-methylenebicyclo[7.2.0]-undec-4-ene (β-caryophyllene),

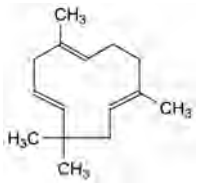

B. (1*E*,4*E*,8*E*)-2,6,6,9-tetramethylcycloundeca-1,4,8-triene (α-humulene, α-caryophyllene),

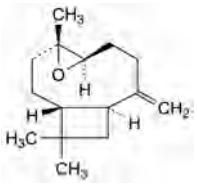

C. (1*R*,4*R*,6*R*,10*S*)-4,12,12-trimethyl-9-methylene-5-oxatricyclo[8.2.0.0$^{4,6}$]dodecane (β-caryophyllene oxide),

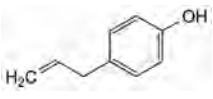

D. 4-(prop-2-enyl)phenol,

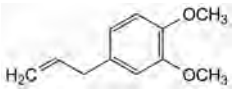

E. 1,2-dimethoxy-4-(prop-2-enyl)benzene (eugenol methyl ether),

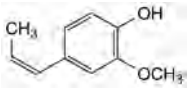

F. 2-methoxy-4-[(Z)-prop-1-enyl]phenol (*cis*-isoeugenol),

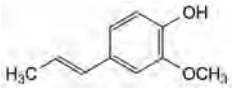

G. 2-methoxy-4-[(E)-prop-1-enyl]phenol (*trans*-isoeugenol),

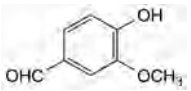

H. 4-hydroxy-3-methoxybenzaldehyde (vanillin),

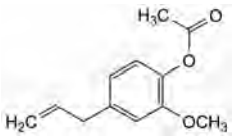

I. 2-methoxy-4-(prop-2-enyl)phenyl acetate (acetyleugenol),

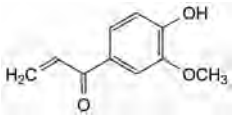

J. 1-(4-hydroxy-3-methoxyphenyl)prop-2-enone,

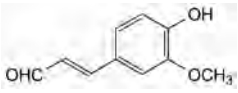

K. (*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enal (*trans*-coniferyl aldehyde),

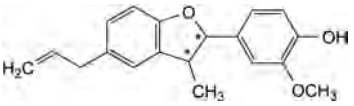

L. 2-methoxy-4-[3-methyl-5-(prop-2-enyl)-2,3-dihydrobenzofuran-2-yl]phenol (dehydrodi-isoeugenol),

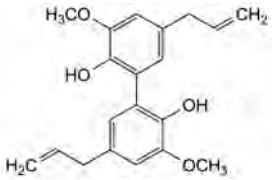

M. 3,3′-dimethoxy-5,5′-bis(prop-2-enyl)biphenyl-2,2′-diol (dehydrodieugenol),

N. O. 2 further unknown dimeric compounds,

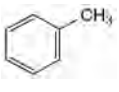

P. toluene.

**01/2020:2104**

# EVENING PRIMROSE OIL, REFINED

## Oenotherae oleum raffinatum

DEFINITION

Fatty oil obtained from seeds of *Oenothera biennis* L. or *Oenothera lamarckiana* L. by extraction and/or expression. It is then refined. A suitable antioxidant may be added.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, light yellow or yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

*Relative density*: about 0.923.

*Refractive index*: about 1.478.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5, or maximum 0.3 if intended for use in the manufacture of parenteral preparations.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 2.5 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *saturated fatty acids of chain length less than $C_{16}$*: maximum 0.3 per cent;
- *palmitic acid*: 4.0 per cent to 10.0 per cent;
- *stearic acid*: 1.0 per cent to 4.0 per cent;
- *oleic acid*: 5.0 per cent to 12.0 per cent;
- *linoleic acid*: 65.0 per cent to 85.0 per cent;
- *gamma-linolenic acid*: 7.0 per cent to 14.0 per cent;
- *alpha-linolenic acid*: maximum 0.5 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

Under an inert gas, in a well-filled, airtight container, protected from light.

LABELLING

The label states:

- where applicable, that the oil is suitable for use in the manufacture of parenteral preparations;
- the name of the inert gas.

**04/2019:2918**
**corrected 10.0**

# EVEROLIMUS

## Everolimusum

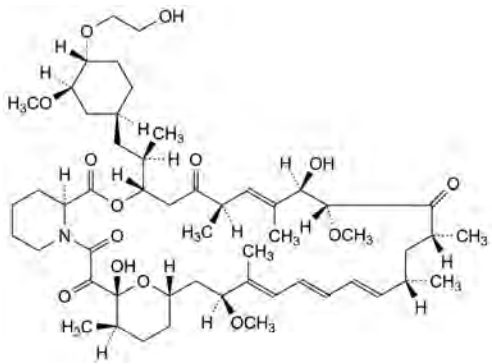

$C_{53}H_{83}NO_{14}$ $M_r$ 958

[159351-69-6]

(1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*,21*R*,23*S*,24*E*,26*E*,28*E*,30*S*,32*S*,-35*R*)-1,18-Dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.0$^{4,9}$]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone.

Semi-synthetic product derived from a fermentation product. A suitable antioxidant may be added.

DEFINITION

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

A reversible isomerisation to everolimus tautomer takes place in solution. The activity is due to both compounds.

CHARACTERS

*Appearance*: white or faintly yellow, hygroscopic powder.

*Solubility*: practically insoluble in water, very soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *everolimus CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): – 153.0 to – 141.0 (anhydrous substance).

Dissolve 0.100 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Impurity A**. Liquid chromatography (*2.2.29*). *Protect the solutions from light. Use plastic labware to minimise the formation of everolimus tautomer.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 1 mg of *sirolimus R* (impurity A) in 5 mL of *acetonitrile R*. Dilute 0.8 mL of the solution to 10 mL with the test solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.7 µm);

Monographs E

– *temperature*: 60 °C.

*Mobile phase*:

– *mobile phase A*: *anhydrous formic acid R*, *methanol R*, 0.70 g/L solution of *ammonium formate R*, *acetonitrile R* (0.05:100:450:450 *V/V/V/V*);

– *mobile phase B*: *anhydrous formic acid R*, *methanol R*, 0.96 g/L solution of *ammonium formate R*, *acetonitrile R* (0.05:100:330:570 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 100 | 0 |
| 14 - 23 | 100 → 0 | 0 → 100 |
| 23 - 28 | 0 | 100 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Autosampler*: set at 6 °C.

*Injection*: 3.5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to everolimus (retention time = about 16 min): impurity A = about 0.9; everolimus tautomer = about 1.1.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to everolimus.

*Calculation of percentage content*:

– for impurity A, use the concentration of everolimus (and everolimus tautomer if present) in reference solution (b).

*Limit*:

– *impurity A*: maximum 0.8 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *everolimus for system suitability CRS* (containing impurities D, E, F, H, I and J) in 1 mL of *acetonitrile R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 25.0 mg of *everolimus CRS* in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (d).* Dissolve 5 mg of *everolimus for impurity C identification CRS* in 1 mL of *acetonitrile R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 3.0 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, 0.27 g/L solution of *potassium dihydrogen phosphate R* (40:60 *V/V*);

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 7 | 100 → 72 | 0 → 28 |
| 7 - 25 | 72 → 60 | 28 → 40 |
| 25 - 33 | 60 → 0 | 40 → 100 |
| 33 - 43 | 0 | 100 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *everolimus for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities D, E, F, H, I and J; use the chromatogram supplied with *everolimus for impurity C identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to everolimus (retention time = about 15 min): impurity H = about 0.78; impurity I = about 0.81; impurity C = about 0.87; impurity D = about 0.92; everolimus tautomer = about 1.1; impurity E = about 1.34; impurity J = about 1.38; impurity F = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity D and everolimus; minimum 1.5 between the peaks due to everolimus and everolimus tautomer.

*Calculation of percentage contents*:

– for each impurity, use the sum of the concentrations of everolimus and everolimus tautomer in reference solution (b).

*Limits*:

– *impurities C, D, I*: for each impurity, maximum 0.8 per cent;

– *impurity H*: maximum 0.4 per cent;

– *sum of impurities E and J*: maximum 0.3 per cent;

– *impurity F*: maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.15 per cent;

– *total*: maximum 2.5 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak due to everolimus tautomer.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.400 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 275 nm.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{53}H_{83}NO_{14}$ taking into account the assigned content of *everolimus CRS* and, if present, the peak due to everolimus tautomer and by subtracting the content of impurity A obtained in the corresponding test.

STORAGE

Under an inert gas, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, C, D, E, F, H, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

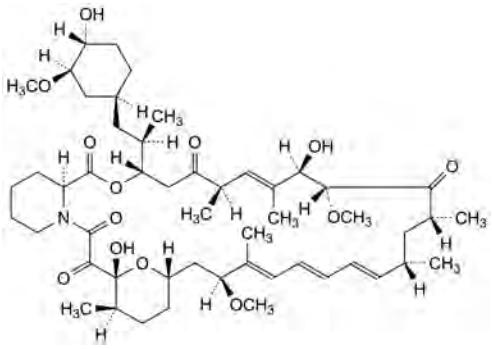

A. (1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*,21*R*,23*S*,24*E*,26*E*,28*E*,-30*S*,32*S*,35*R*)-1,18-dihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.0$^{4,9}$]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (sirolimus; rapamycin),

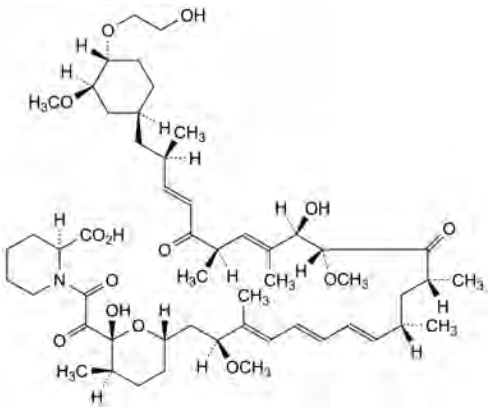

B. (2*S*)-1-[[(2*R*,3*R*,6*S*)-2-hydroxy-6-[(2*S*,3*E*,5*E*,7*E*,9*S*,11*R*,13*R*,14*R*,15*E*,17*R*,19*E*,21*R*)-14-hydroxy-22-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-2,13-dimethoxy-3,9,11,15,17,21-hexamethyl-12,18-dioxodocosa-3,5,7,15,19-pentaen-1-yl]-3-methyloxan-2-yl](oxo)acetyl]piperidine-2-carboxylic acid,

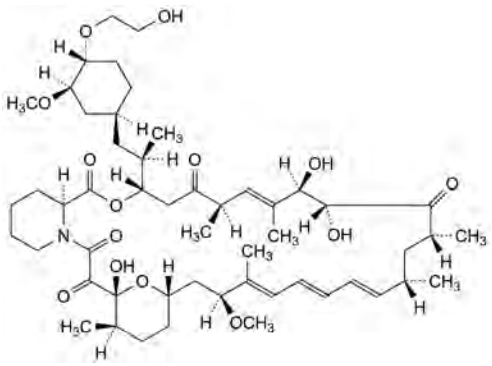

C. (1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*,21*R*,23*S*,24*E*,26*E*,28*E*,30*S*,-32*S*,35*R*)-1,18,19-trihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-30-methoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.04,9]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (19-*O*-demethyl everolimus),

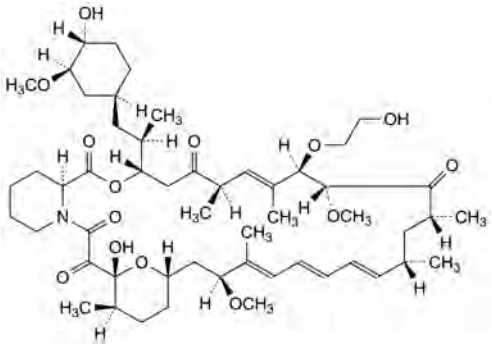

D. (1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*,21*R*,23*S*,24*E*,26*E*,28*E*,30*S*,-32*S*,35*R*)-1-hydroxy-18-(2-hydroxyethoxy)-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36-dioxa-4-azatricyclo[30.3.1.0$^{4,9}$]hexatriaconta-16,24,26,28-tetraene-2,3,10,14,20-pentone (18-*O*-hydroxyethyl sirolimus),

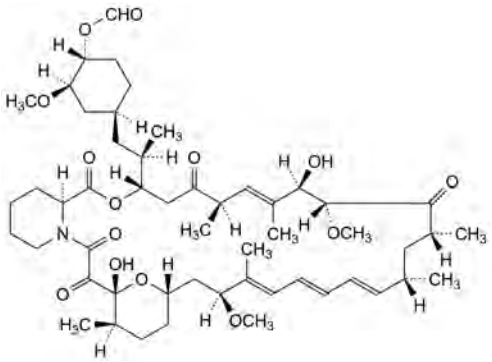

E. (1*R*,2*R*,4*S*)-4-[(2*R*)-2-[(1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*R*,21*R*,23*S*,24*E*,26*E*,28*E*,30*S*,-32*S*,35*R*)-1,18-dihydroxy-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-2,3,10,14,20-pentaoxo-11,36-dioxa-4-azatricyclo[30.3.1.0$^{4,9}$]hexatriaconta-16,24,26,28-tetraen-12-yl]propyl]-2-methoxycyclohexyl formate,

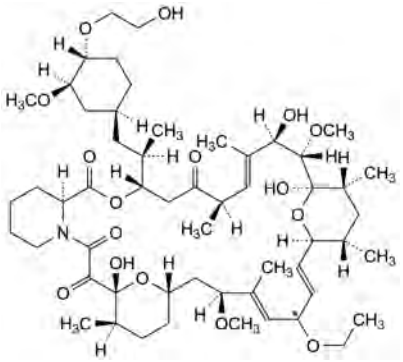

F. (1*R*,9*S*,12*S*,15*R*,16*E*,18*R*,19*S*,20*R*,21*R*,23*S*,24*S*,25*E*,27*Ξ*,-28*E*,30*S*,32*S*,35*R*)-27-ethoxy-1,18,20-trihydroxy-12-[(2*R*)-1-[(1*S*,3*R*,4*R*)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]propan-2-yl]-19,30-dimethoxy-15,17,21,23,29,35-hexamethyl-11,36,37-trioxa-4-azatetracyclo[30.3.1.1$^{20,24}$.0$^{4,9}$]heptatriaconta-16,25,28-triene-2,3,10,14-tetrone,

H. unknown structure,

I. unknown structure,

J. unknown structure.

01/2016:2766

# EXEMESTANE

## Exemestanum

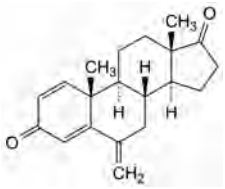

$C_{20}H_{24}O_2$ $M_r$ 296.4
[107868-30-4]

DEFINITION

6-Methylideneandrosta-1,4-diene-3,17-dione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *exemestane CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 290 to + 298 (dried substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Store the solutions protected from light.*

*Solvent mixture*: *water R*, *acetonitrile R* (25:75 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *exemestane for system suitability CRS* (containing impurity G) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 25.0 mg of *exemestane CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 75 | 25 |
| 5 - 35 | 75 → 55 | 25 → 45 |
| 35 - 45 | 55 → 5 | 45 → 95 |
| 45 - 50 | 5 | 95 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 247 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *exemestane for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity G.

*Relative retention* with reference to exemestane (retention time = about 27 min): impurity G = about 1.03.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to exemestane.

*Calculation of percentage contents*:

- for each impurity, use the concentration of exemestane in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{20}H_{24}O_2$ taking into account the assigned content of *exemestane CRS*.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G*.

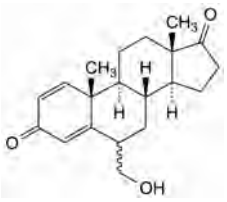

B. 6ξ-(hydroxymethyl)androsta-1,4-diene-3,17-dione,

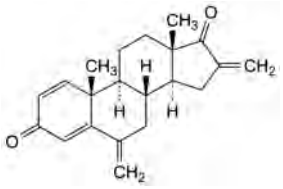

C. 6,16-dimethylideneandrosta-1,4-diene-3,17-dione,

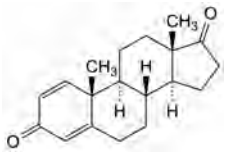

D. androsta-1,4-diene-3,17-dione,

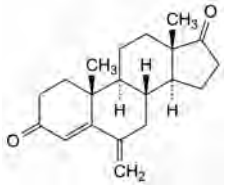

E. 6-methylideneandrost-4-ene-3,17-dione,

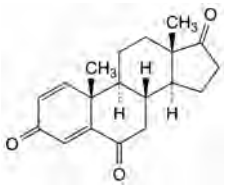

F. androsta-1,4-diene-3,6,17-trione,

G. unknown structure.

# F

Famotidine.... 2609
Febantel for veterinary use.. .... 2610
Felbinac.. .... 2611
Felodipine.. .... 2612
Felypressin.. .... 2614
Fenbendazole for veterinary use.. .... 2615
Fenbufen.. .... 2616
Fenofibrate.. .... 2617
Fenoterol hydrobromide.. .... 2618
Fentanyl.... 2619
Fentanyl citrate.... 2621
Fenticonazole nitrate.. .... 2622
Ferric chloride hexahydrate.... 2624
Ferrous fumarate.... 2624
Ferrous gluconate.. .... 2625
Ferrous sulfate, dried.... 2626
Ferrous sulfate heptahydrate.. .... 2627
Fexofenadine hydrochloride.... 2628
Fibrin sealant kit.. .... 2630
Filgrastim concentrated solution.. .... 2631
Filgrastim injection.. .... 2634
Finasteride.. .... 2636
Fingolimod hydrochloride.... 2637
Fipronil for veterinary use.... 2639
Fish oil, rich in omega-3 acids.. .... 2639
Flavoxate hydrochloride.... 2642
Flecainide acetate.. .... 2643
Flubendazole.. .... 2644
Flucloxacillin magnesium octahydrate.. .... 2645
Flucloxacillin sodium.. .... 2647
Fluconazole.... 2649
Flucytosine.... 2650
Fludarabine phosphate.... 2652
Fludrocortisone acetate.... 2654
Flumazenil.. .... 2655
Flumequine.... 2657
Flumetasone pivalate.... 2658
Flunarizine dihydrochloride.... 2659
Flunitrazepam.. .... 2660
Flunixin meglumine for veterinary use.. .... 2661
Fluocinolone acetonide.. .... 2662
Fluocortolone pivalate.... 2665
Fluorescein.. .... 2666
Fluorescein sodium.. .... 2667
Fluorouracil.. .... 2669
Fluoxetine hydrochloride.... 2670
Flupentixol dihydrochloride.... 2672
Fluphenazine decanoate.... 2674
Fluphenazine dihydrochloride.. .... 2675
Fluphenazine enantate.. .... 2677
Flurazepam monohydrochloride.. .... 2679
Flurbiprofen.... 2680
Fluspirilene.. .... 2681
Flutamide.. .... 2682
Fluticasone propionate.. .... 2683
Flutrimazole.. .... 2685
Fluvastatin sodium.. .... 2686
Fluvoxamine maleate.... 2688
Folic acid hydrate.... 2690
Follitropin.. .... 2691
Follitropin concentrated solution.. .... 2697
Formaldehyde solution (35 per cent).. .... 2703
Formic acid.. .... 2703
Formoterol fumarate dihydrate.... 2704
Foscarnet sodium hexahydrate.. .... 2706
Fosfomycin calcium.. .... 2708
Fosfomycin sodium.. .... 2709
Fosfomycin trometamol.. .... 2710
Fosinopril sodium.. .... 2711
Framycetin sulfate.... 2714
Fructose.... 2715
Fulvestrant.. .... 2716
Furosemide.. .... 2718
Fusidic acid.. .... 2719

**01/2017:1012**

# FAMOTIDINE

## Famotidinum

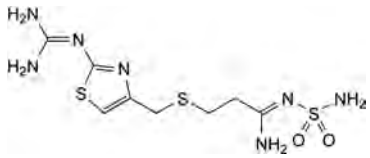

$C_8H_{15}N_7O_2S_3$ $M_r$ 337.4
[76824-35-6]

DEFINITION

3-[[[2-[(Diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]-*N*′-sulfamoylpropanimidamide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder or crystals.

*Solubility*: very slightly soluble in water, freely soluble in glacial acetic acid, very slightly soluble in anhydrous ethanol, practically insoluble in ethyl acetate. It dissolves in dilute mineral acids.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *famotidine CRS*.

If the spectra obtained show differences, suspend 0.10 g of the substance to be examined and 0.10 g of the reference substance separately in 5 mL of *water R*. Heat to boiling and allow to cool, scratching the wall of the tube with a glass rod to initiate crystallisation. Filter, wash the crystals with 2 mL of iced *water R* and dry in an oven at 80 °C at a pressure not exceeding 670 Pa for 1 h. Record new spectra using the residues.

TESTS

**Appearance of solution**. Dissolve 0.20 g in a 50 g/L solution of *hydrochloric acid R*, heating to 40 °C if necessary, and dilute to 20 mL with the same acid. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 12.5 mg of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2.5 mg of *famotidine impurity D CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution add 0.50 mL of the test solution and dilute to 100.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5.0 mg of *famotidine for system suitability CRS* (containing impurities A, B, C, D, F and G) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: mix 6 volumes of *methanol R*, 94 volumes of *acetonitrile R* and 900 volumes of a 1.882 g/L solution of *sodium hexanesulfonate R* previously adjusted to pH 3.5 with *acetic acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 23 | 100 → 96 | 0 → 4 | 1 |
| 23 - 27 | 96 | 4 | 1 → 2 |
| 27 - 47 | 96 → 78 | 4 → 22 | 2 |

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *famotidine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, F and G; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to famotidine (retention time = about 21 min): impurity D = about 1.1; impurity C = about 1.2; impurity G = about 1.4; impurity F = about 1.5; impurity A = about 1.6; impurity B = about 2.0.

*System suitability*:

- *retention time*: famotidine = 19-23 min in all the chromatograms;
- *resolution*: minimum 3.5 between the peaks due to famotidine and impurity D in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity B = 2.5; impurity C = 1.9; impurity F = 1.7; impurity G = 1.4;
- *impurities C, D*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, B, F, G*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 670 Pa for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.87 mg of $C_8H_{15}N_7O_2S_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, H, I, J.*

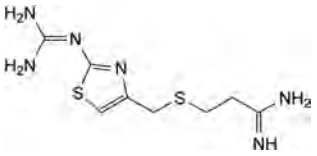

A. 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]propanimidamide,

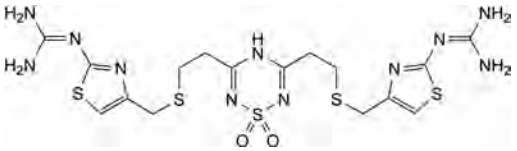

B. 3,5-bis[2-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]ethyl]-4*H*-1,2,4,6-thiatriazine 1,1-dioxide,

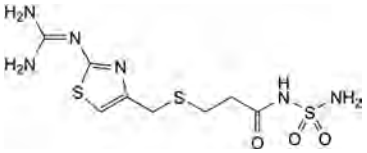

C. 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]-*N*-sulfamoylpropanamide,

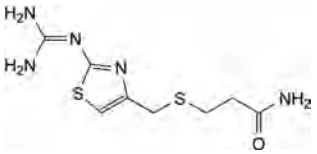

D. 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]propanamide,

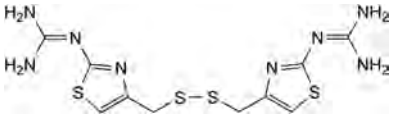

E. 2,2′-[disulfanediylbis(methylenethiazole-4,2-diyl)]diguanidine,

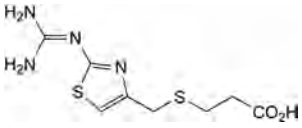

F. 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]propanoic acid,

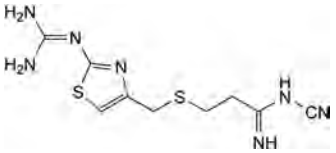

G. *N*-cyano-3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]propanimidamide,

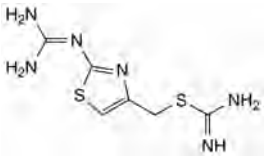

H. [2-[(diaminomethylidene)amino]thiazol-4-yl]methyl carbamimidothioate,

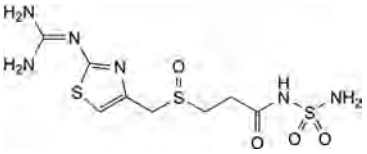

I. 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfinyl]-*N*-sulfamoylpropanamide,

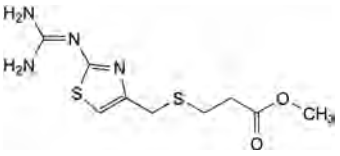

J. methyl 3-[[[2-[(diaminomethylidene)amino]thiazol-4-yl]methyl]sulfanyl]propanoate.

**01/2008:2176**
**corrected 6.0**

# FEBANTEL FOR VETERINARY USE

## Febantelum ad usum veterinarium

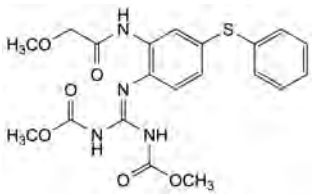

$C_{20}H_{22}N_4O_6S$ $M_r$ 446.5
[58306-30-2]

DEFINITION

Dimethyl *N*,*N*′-[[[2-[(methoxyacetyl)amino]-4-(phenylsulfanyl)phenyl]imino]methylene]dicarbamate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *febantel CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *tetrahydrofuran R* (50:50 *V/V*).

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 50.0 mg of *febantel CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *febantel for system suitability CRS* (containing impurities A, B and C) in 1.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.0 mm;

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm).

*Mobile phase*: dissolve 6.8 g of *potassium dihydrogen phosphate R* in 1000 mL of *water for chromatography R*. Mix 350 mL of *acetonitrile R* with 650 mL of this solution.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (c).

*Run time*: 1.5 times the retention time of febantel.

*Elution order*: impurity A, impurity B, impurity C, febantel.

*Retention time*: febantel = about 32 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to impurities A and B and minimum 4.0 between the peaks due to impurities B and C.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

1.0 g complies with test F. Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_{20}H_{22}N_4O_6S$ from the declared content of *febantel CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

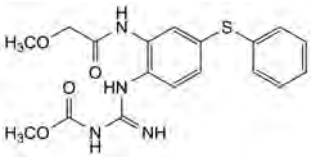

A. methyl [[2-[(methoxyacetyl)amino]-4-(phenylsulfanyl)-phenyl]carbamimidoyl]carbamate,

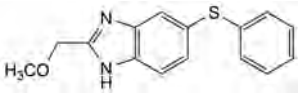

B. 2-(methoxymethyl)-5-(phenylsulfanyl)-1*H*-benzimidazole,

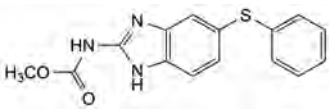

C. methyl [5-(phenylsulfanyl)-1*H*-benzimidazol-2-yl]carbamate (fenbendazole).

**01/2008:2304**

# FELBINAC

## Felbinacum

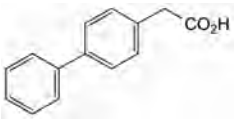

$C_{14}H_{12}O_2$ $M_r$ 212.2
[5728-52-9]

DEFINITION

(Biphenyl-4-yl)acetic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent).

mp: about 164 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *felbinac CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light and inject within 20 min of preparation.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 5.0 mg of *felbinac impurity A CRS* and 5.0 mg of *biphenyl R* (impurity B) in *methanol R*, add 0.5 mL of the test solution and dilute to 50.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 45 volumes of a 0.1 per cent *V/V* solution of *glacial acetic acid R* and 55 volumes of *methanol R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of felbinac.

*Relative retention* with reference to felbinac (retention time = about 15 min): impurity A = about 1.3; impurity B = about 2.8.

*System suitability*: reference solution:

– *resolution*: minimum 3.0 between the peaks due to felbinac and impurity A.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent);
– *impurity B*: not more than the area of the peak due to felbinac in the chromatogram obtained with the reference solution (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the peak due to felbinac in the chromatogram obtained with the reference solution (0.10 per cent);
– *total*: not more than twice the area of the peak due to felbinac in the chromatogram obtained with the reference solution (0.2 per cent);
– *disregard limit*: 0.5 times the area of the peak due to felbinac in the chromatogram obtained with the reference solution (0.05 per cent).

**Chlorides**: maximum 110 ppm.

Dissolve 1.0 g in 40 mL of *acetone R*, add 6 mL of a 10 per cent *V/V* solution of *nitric acid R*, dilute to 50.0 mL with *water R* and mix. Pour 15.0 mL of this solution as a single addition into 1 mL of *0.1 M silver nitrate* and allow to stand for 5 min protected from light. When viewed horizontally against a black background, any opalescence produced is not more intense than that obtained by treating in the same manner 15.0 mL of a mixture of 1.5 mL of *0.002 M hydrochloric acid*, 40 mL of *acetone R*, 6 mL of 10 per cent *V/V* solution of *nitric acid R*, diluted to 50.0 mL with *water R*.

**Sulfates**: maximum 130 ppm.

Dissolve 1.5 g in 40 mL of *dimethylformamide R*, add 1 mL of a 10 per cent *V/V* solution of *hydrochloric acid R*, dilute to 50.0 mL with *dimethylformamide R* and mix. To 15.0 mL of this solution add 2.0 mL of a 120 g/L solution of *barium chloride R* and allow to stand for 5 min. Any opalescence produced is not more intense than that of a standard prepared in the same manner but using 2.0 mL of *0.001 M sulfuric acid* instead of the substance to be examined.

**Loss on drying** (*2.2.32*) : maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 mL of *methanol R*. Titrate with *0.1 M alcoholic potassium hydroxide* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M alcoholic potassium hydroxide* is equivalent to 21.23 mg of $C_{14}H_{12}O_2$.

IMPURITIES

*Specified impurities: A, B.*

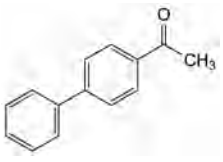


A. 4-acetyl biphenyl,

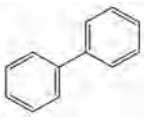

B. biphenyl.

**01/2008:1013**
**corrected 6.0**

# FELODIPINE

## Felodipinum

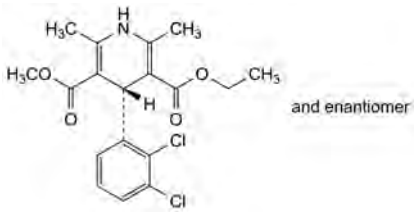


$C_{18}H_{19}Cl_2NO_4$ $M_r$ 384.3
[72509-76-3]

DEFINITION

Ethyl methyl (4*RS*)-4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or light yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol, in methanol and in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *methanol R* and dilute to 100 mL with the same solvent. Dilute 3 mL of this solution to 100 mL with *methanol R*.

*Spectral range*: 220-400 nm.

*Absorption maxima*: at 238 nm and 361 nm.

*Absorbance ratio*: $A_{361}$ / $A_{238}$ = 0.34 to 0.36.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *felodipine CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *felodipine CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *nifedipine CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (40:60 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.150 g in a mixture of 25 mL of *2-methyl-2-propanol R* and 25 mL of *perchloric acid solution R*. Add 10 mL of *0.1 M cerium sulfate*, allow to stand for 15 min, add 3.5 mL of *strong sodium hydroxide solution R* and neutralise with *dilute sodium hydroxide solution R*. Shake with 25 mL of *methylene chloride R*. Evaporate the lower layer to dryness on a water-bath under nitrogen (the residue is also used in the test for related substances). Dissolve about 20 mg of the residue in *methanol R* and dilute to 50 mL with the same solvent. Dilute 2 mL of this solution to 50 mL with *methanol R*.

*Spectral range*: 220-400 nm.

*Absorption maximum*: at 273 nm.

TESTS

**Solution S**. Dissolve 1.00 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Absorbance** (*2.2.25*): maximum 0.10, determined at 440 nm on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of the residue obtained in identification test D (impurity A) and 25.0 mg of *felodipine CRS* in the mobile phase, then dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125-0.15 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *methanol R*, 40 volumes of *acetonitrile R* and 40 volumes of a phosphate buffer solution pH 3.0 containing 0.8 g/L of *phosphoric acid R* and 8 g/L of *sodium dihydrogen phosphate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of felodipine.

*Elution order*: impurity B, impurity A, felodipine, impurity C.

*Retention time*: felodipine = about 12 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to impurity A and felodipine.

*Limits*:

– *sum of impurities B and C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *sum of impurities other than B and C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in a mixture of 25 mL of *2-methyl-2-propanol R* and 25 mL of *perchloric acid solution R*. Add 0.05 mL of *ferroin R*. Titrate with *0.1 M cerium sulfate* until the pink colour disappears. Titrate slowly towards the end of the titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 19.21 mg of $C_{18}H_{19}Cl_2NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

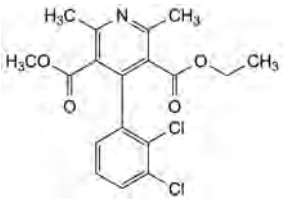

A. ethyl methyl 4-(2,3-dichlorophenyl)-2,6-dimethylpyridine-3,5-dicarboxylate,

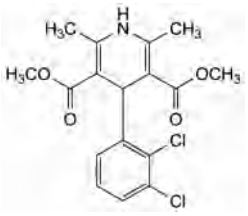

B. dimethyl 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

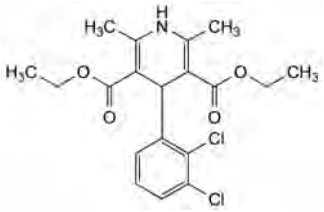

C. diethyl 4-(2,3-dichlorophenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

**01/2008:1634**
**corrected 10.0**

# FELYPRESSIN

## Felypressinum

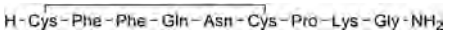


$C_{46}H_{65}N_{13}O_{11}S_2$ $M_r$ 1039
[56-59-7]

DEFINITION

L-Cysteinyl-L-phenylalanyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-lysylglycinamide cyclic (1,6)-disulfide.

Synthetic nonapeptide having a vasoconstricting activity. It is available as an acetate.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white, powder or flakes.

*Solubility*: freely soluble in water, practically insoluble in acetone and ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of amino acids, taking one-seventh of the sum of the number of moles of glutamic acid, aspartic acid, proline, lysine, glycine and phenylalanine as equal to one. The values fall within the following limits: aspartic acid: 0.9 to 1.1; glutamic acid: 0.9 to 1.1; proline: 0.9 to 1.1; glycine: 0.9 to 1.1; phenylalanine: 1.8 to 2.2; half-cystine: 1.8 to 2.2; lysine: 0.9 to 1.1.

TESTS

**Specific optical rotation** (*2.2.7*): − 35 to − 29, determined at 25 °C (anhydrous and acetic acid-free substance).

Dissolve 20.0 mg in a 1 per cent *V/V* solution of *glacial acetic acid R* and dilute to 10.0 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*); use the normalisation procedure. *The solutions are stable for 24 h at room temperature or for 1 week at 2-8 °C.*

*Test solution (a).* Dissolve 5.0 mg of the substance to be examined in 5.0 mL of mobile phase A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 5.0 mL with mobile phase A.

*Reference solution.* Dissolve the contents of a vial of *felypressin CRS* in mobile phase A to obtain a concentration of 0.2 mg/mL.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.62 g of *tetramethylammonium hydroxide R* in 900 mL *water R*; adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: dissolve 1.81 g of *tetramethylammonium hydroxide R* in 450 mL of a 50 per cent *V/V* solution of *acetonitrile for chromatography R*; adjust to pH 2.5 with *phosphoric acid R* and dilute to 500 mL with a 50 per cent *V/V* solution of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 80 → 50 | 20 → 50 |
| 20 - 25 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of test solution (a) and 50 µL of the reference solution.

*Identification of impurities*: use the chromatogram supplied with *felypressin CRS* to identify the peaks due to impurities A to F.

*Relative retention* with reference to felypressin: impurity A = about 0.9; impurity B = about 1.1; impurity F = about 1.2; impurity C = about 1.3; impurity D = about 1.4; impurity E = about 2.1.

*System suitability*: reference solution:

- *retention time*: felypressin = about 7.5 min;
- *resolution*: minimum 1.5 between the peaks due to impurity C and impurity D.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, maximum 0.5 per cent,
- *any other impurity*: for each impurity, maximum 0.1 per cent,
- *total*: maximum 3.0 per cent,
- *disregard limit*: 0.05 per cent.

**Acetic acid** (*2.5.34*): 9.0 per cent to 13.0 per cent.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 7.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 100 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and of the reference solution.

Calculate the content of felypressin ($C_{46}H_{65}N_{13}O_{11}S_2$) from the areas of the peaks and the declared content of $C_{46}H_{65}N_{13}O_{11}S_2$ in *felypressin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the mass of peptide in the container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

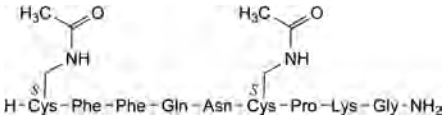

A. $S^1$,$S^6$-bis[(acetylamino)methyl]-(reduced felypressin),

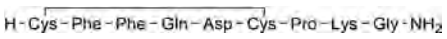

B. [5-aspartic acid]felypressin,

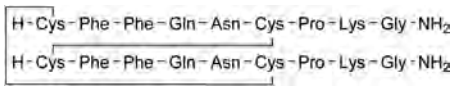

C. bis(reduced felypressin) (1,6′),(1′,6)-bis(disulfide),

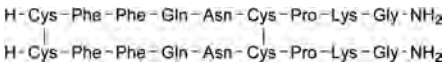

D. bis(reduced felypressin) (1,1′),(6,6′)-bis(disulfide),

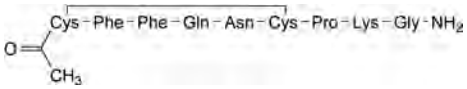

E. $N^1$-acetylfelypressin,

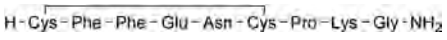

F. [4-glutamic acid]felypressin.

**01/2014:1208**

# FENBENDAZOLE FOR VETERINARY USE

## Fenbendazolum ad usum veterinarium

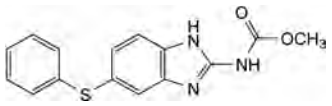

$C_{15}H_{13}N_3O_2S$ $M_r$ 299.4
[43210-67-9]

DEFINITION

Methyl [5-(phenylsulfanyl)-1*H*-benzimidazol-2-yl]carbamate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in dimethylformamide, very slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fenbendazole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use. Keep the temperature of the autosampler at 10 °C.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 10.0 mL of *hydrochloric methanol R*.

*Reference solution (a).* Dissolve 50.0 mg of *fenbendazole CRS* in 10.0 mL of *hydrochloric methanol R*. Dilute 1.0 mL of the solution to 200.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 10.0 mL with *hydrochloric methanol R*.

*Reference solution (b).* Dissolve 10.0 mg of *fenbendazole impurity A CRS* in 100.0 mL of *methanol R*. Dilute 1.0 mL of the solution to 10.0 mL with *hydrochloric methanol R*.

*Reference solution (c).* Dissolve 10.0 mg of *fenbendazole impurity B CRS* in 100.0 mL of *methanol R*. Dilute 1.0 mL of the solution to 10.0 mL with *hydrochloric methanol R*.

*Reference solution (d).* Dissolve 10.0 mg of *fenbendazole CRS* and 10.0 mg of *mebendazole CRS* in 100.0 mL of *methanol R*. Dilute 1.0 mL of the solution to 10.0 mL with *hydrochloric methanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *anhydrous acetic acid R*, *methanol R*, *water R* (1:30:70 *V/V/V*);
– *mobile phase B*: *anhydrous acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 → 0 | 0 → 100 |
| 10 - 40 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to mebendazole.

*Relative retention* with reference to fenbendazole (retention time = about 18 min): impurity A = about 0.2; impurity B = about 0.6; mebendazole = about 0.8.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to mebendazole and fenbendazole.

*Limits*:

– *impurity A*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *impurity B*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *total*: maximum 1.0 per cent;
– *disregard limit*: 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

1.0 g complies with test C. Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 mL of *anhydrous acetic acid R*, warming gently if necessary. Cool and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.94 mg of $C_{15}H_{13}N_3O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

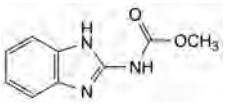

A. methyl (1*H*-benzimidazol-2-yl)carbamate,

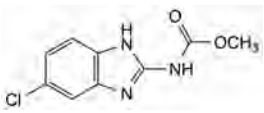

B. methyl (5-chloro-1*H*-benzimidazol-2-yl)carbamate.

**01/2017:1209**

# FENBUFEN

## Fenbufenum

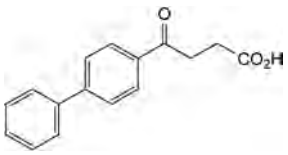

$C_{16}H_{14}O_3$ $M_r$ 254.3

[36330-85-5]

DEFINITION

4-(Biphenyl-4-yl)-4-oxobutanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 186 °C to 189 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fenbufen CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *fenbufen CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *ketoprofen CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. To 5 mL of this solution, add 5 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *hexane R* (5:25:75 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *dimethylformamide R*, mobile phase A (40:60 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 0.5 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 25 mg of *fenbufen CRS* and 6 mg of *ketoprofen CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 1 mL of this solution to 100 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 32 volumes of *acetonitrile R* and 68 volumes of a mixture of 1 volume of *glacial acetic acid R* and 55 volumes of *water R*;

– *mobile phase B*: mix 45 volumes of *acetonitrile R* and 55 volumes of a mixture of 1 volume of *glacial acetic acid R* and 55 volumes of *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 – 15 | 100 | 0 |
| 15 – 20 | 100 → 0 | 0 → 100 |
| 20 – 35 | 0 | 100 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to ketoprofen and fenbufen.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 75 mL of *acetone R* previously neutralised with *phenolphthalein solution R1* and add 50 mL of *water R*. Add 0.2 mL of *phenolphthalein solution R1* and titrate with *0.1 M sodium hydroxide.* Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 25.43 mg of $C_{16}H_{14}O_3$.

IMPURITIES

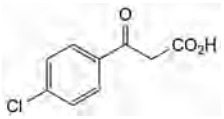

A. 3-(4-chlorophenyl)-3-oxopropanoic acid,

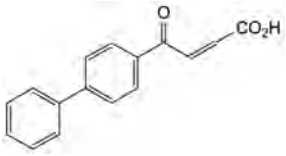

B. 4-(biphenyl-4-yl)-4-oxobut-2-enoic acid,

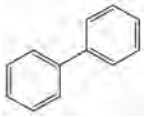

C. biphenyl,

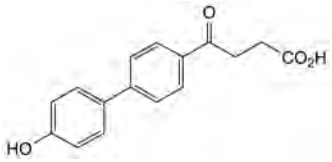

D. 4-(4′-hydroxybiphenyl-4-yl)-4-oxobutanoic acid.

**01/2017:1322**

# FENOFIBRATE

## Fenofibratum

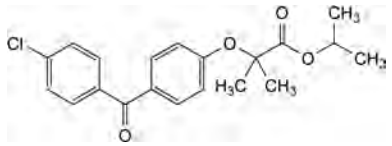

$C_{20}H_{21}ClO_4$ $M_r$ 360.8
[49562-28-9]

DEFINITION

1-Methylethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 79 °C to 82 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fenofibrate CRS*.

TESTS

**Solution S**. To 5.0 g, add 25 mL of *distilled water R* and heat at 50 °C for 10 min. Cool and dilute to 50.0 mL with *distilled water R*. Filter. Use the filtrate as solution S.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.50 g in *acetone R* and dilute to 10.0 mL with the same solvent.

**Acidity**. Dissolve 1.0 g in 50 mL of *ethanol (96 per cent) R* previously neutralised using 0.2 mL of *phenolphthalein solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *fenofibrate CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *fenofibrate CRS*, 5.0 mg of *fenofibrate impurity A CRS*, 5.0 mg of *fenofibrate impurity B CRS* and 10.0 mg of *fenofibrate impurity G CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *water R* acidified to pH 2.5 with *phosphoric acid R* and 70 volumes of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 286 nm.

*Injection*: 20 µL of the test solution and reference solution (b).

*Run time*: twice the retention time of fenofibrate.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and G.

*Relative retention* with reference to fenofibrate (retention time = about 10 min): impurity A = about 0.34; impurity B = about 0.36; impurity G = about 1.35.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and B.

*Limits*:

- *impurities A, B*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *impurity G*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to fenofibrate in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the peak due to fenofibrate in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the peak due to fenofibrate in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Halides expressed as chlorides** (*2.4.4*): maximum 100 ppm.

To 5 mL of solution S add 10 mL of *distilled water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: 5 µL of the test solution and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.0 per cent determined on 6 injections.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F.*

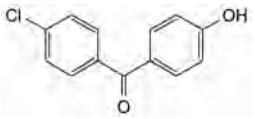

A. (4-chlorophenyl)(4-hydroxyphenyl)methanone,

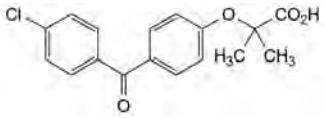

B. 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid (fenofibric acid),

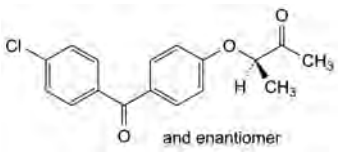


C. (3*RS*)-3-[4-(4-chlorobenzoyl)phenoxy]butan-2-one,

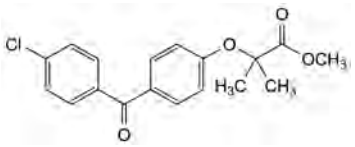

D. methyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate,

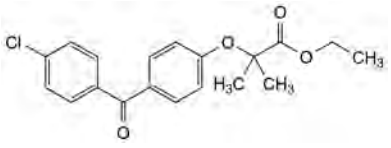

E. ethyl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate,

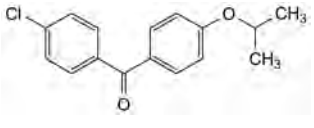

F. (4-chlorophenyl)[4-(1-methylethoxy)phenyl]methanone,

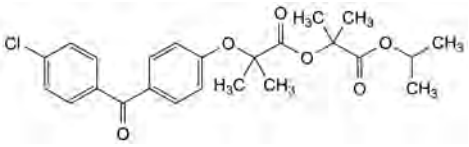

G. 1-methylethyl 2-[[2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoyl]oxy]-2-methylpropanoate.

**01/2020:0901**

# FENOTEROL HYDROBROMIDE

## Fenoteroli hydrobromidum

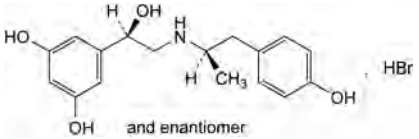


$C_{17}H_{22}BrNO_4$ $M_r$ 384.3

[1944-12-3]

DEFINITION

5-[(1*RS*)-2-[(1*RS*)-2-(4-Hydroxyphenyl)-1-methylethyl]-amino-1-hydroxyethyl]benzene-1,3-diol hydrobromide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fenoterol hydrobromide CRS.*

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.00 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.2 to 5.2 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 6.0 mg of the substance to be examined in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 25.0 mL with *water R.*

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 100.0 mL with *water R.*

*Reference solution (c).* Dissolve the contents of a vial of *fenoterol for system suitability CRS* (containing impurities A, B and C) in 1 mL of *water R.*

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Dissolve 24 g of *anhydrous disodium hydrogen phosphate R* in 1000 mL of *water for chromatography R*. Mix 69 volumes of the solution and 1 volume of a 9 g/L solution of *potassium dihydrogen phosphate R*, adjust to pH 8.5 with *phosphoric acid R* and add 35 volumes of *methanol R2.*

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of fenoterol.

*Identification of impurities*: use the chromatogram supplied with *fenoterol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to fenoterol (retention time = about 7 min): impurity A = about 1.3; impurity B = about 2.0; impurity C = about 2.2.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to fenoterol and impurity A; minimum 1.5 between the peaks due to impurities B and C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.6;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent);
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Iron** (*2.4.9*): maximum 10 ppm.

Dissolve the residue obtained in the test for sulfated ash in 2.5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.600 g in 50 mL of *water R* and add 5 mL of *dilute nitric acid R*, 25.0 mL of *0.1 M silver nitrate* and 2 mL of *ferric ammonium sulfate solution R2*. Shake and titrate with *0.1 M ammonium thiocyanate* until an orange colour is obtained. Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 38.43 mg of $C_{17}H_{22}BrNO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

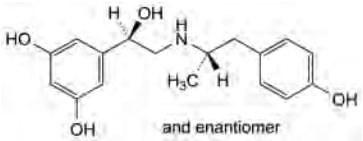


A. 5-[(1*RS*)-2-[(1*SR*)-2-(4-hydroxyphenyl)-1-methylethyl]amino-1-hydroxyethyl]benzene-1,3-diol,

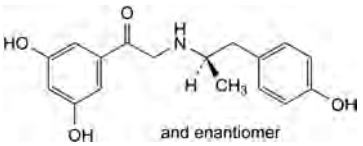


B. 1-(3,5-dihydroxyphenyl)-2-[[(1*RS*)-2-(4-hydroxyphenyl)-1-methylethyl]amino]ethan-1-one,

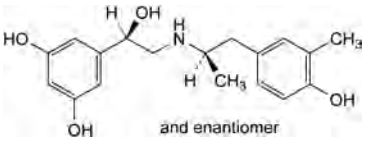


C. 5-[(1*RS*)-2-[(1*RS*)-2-(4-hydroxy-3-methylphenyl)-1-methylethyl]amino-1-hydroxyethyl]benzene-1,3-diol.

**04/2018:1210**

# FENTANYL

## Fentanylum

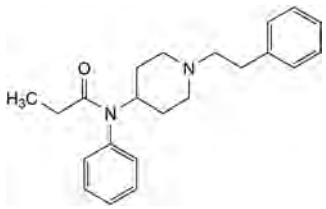


$C_{22}H_{28}N_2O$ $M_r$ 336.5

[437-38-7]

DEFINITION

*N*-Phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fentanyl CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *anhydrous ethanol R*, evaporate to dryness at room temperature in a current of air and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 5.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dissolve the contents of a vial of *fentanyl impurity mixture CRS* (impurities C and D) in 1.0 mL of this solution, using sonication for 1 min if necessary.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;

– *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
– *temperature*: 45 °C.

*Mobile phase*:
– *mobile phase A*: dissolve 0.8 g of *ammonium acetate R* in *water for chromatography R*, add 2 mL of *trifluoroacetic acid R* and dilute to 1000.0 mL with *water for chromatography R*;
– *mobile phase B*: add 2 mL of *trifluoroacetic acid R* to 950 mL of *acetonitrile R1* and shake to homogenise; dissolve 0.8 g of *ammonium acetate R* in this solution and shake until complete dissolution, then dilute to 1000.0 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 98 | 2 |
| 1 - 5 | 98 → 90 | 2 → 10 |
| 5 - 17 | 90 → 70 | 10 → 30 |
| 17 - 30 | 70 → 5 | 30 → 95 |
| 30 - 31 | 5 | 95 |

*Flow rate*: 0.35 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *fentanyl impurity mixture CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and D.

*Relative retention* with reference to fentanyl (retention time = about 19 min): impurity D = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (a):
– *resolution*: minimum 5.0 between the peaks due to impurity C and fentanyl.

*Calculation of percentage contents*:
– for each impurity, use the concentration of fentanyl in reference solution (b).

*Limits*:
– *impurities C, D*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C.

ASSAY

Dissolve 0.200 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.65 mg of $C_{22}H_{28}N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F, G, H, I, J, K, L.*

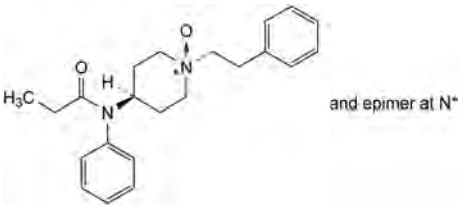


A. (1*rs*,4*rs*)-1-(2-phenylethyl)-4-(*N*-phenylpropanamido)piperidine *N*-oxide,

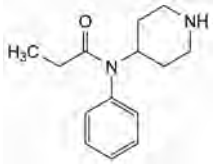

B. *N*-phenyl-*N*-(piperidin-4-yl)propanamide,

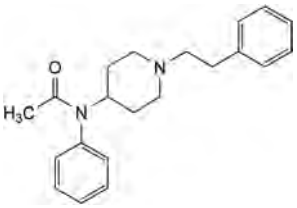

C. *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide,

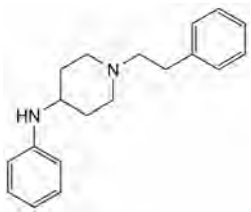

D. *N*-phenyl-1-(2-phenylethyl)piperidin-4-amine,

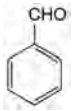

E. benzaldehyde,

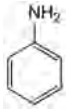

F. aniline (phenylamine),

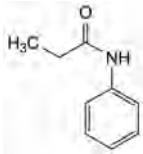

G. *N*-phenylpropanamide,

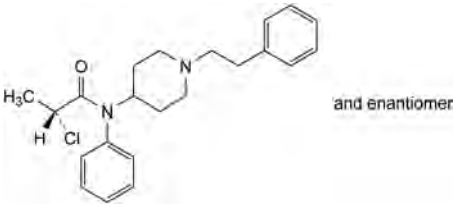


H. (2*RS*)-2-chloro-*N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide,

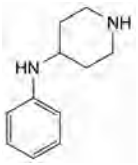

I. *N*-phenylpiperidin-4-amine,

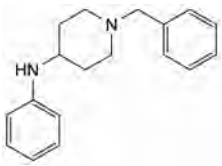

J. 1-benzyl-*N*-phenylpiperidin-4-amine,

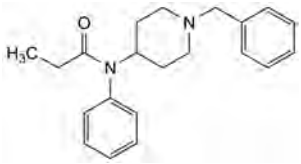

K. *N*-(1-benzylpiperidin-4-yl)-*N*-phenylpropanamide,

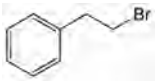

L. (2-bromoethyl)benzene.

**04/2018:1103**
**corrected 10.0**

# FENTANYL CITRATE

## Fentanyli citras

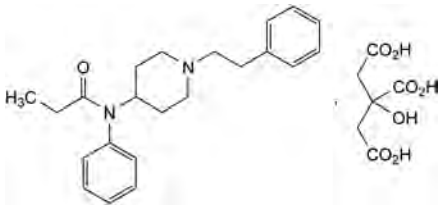

$C_{28}H_{36}N_2O_8$ $M_r$ 528.6
[990-73-8]

DEFINITION

*N*-Phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]propanamide dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, freely soluble in methanol, sparingly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

mp: about 152 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fentanyl citrate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g of the substance to be examined in *water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 39.3 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 5.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dissolve the contents of a vial of *fentanyl impurity mixture CRS* (impurities C and D) in 1.0 mL of this solution, using sonication for 1 min if necessary.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.8 g of *ammonium acetate R* in *water for chromatography R*, add 2 mL of *trifluoroacetic acid R* and dilute to 1000.0 mL with *water for chromatography R*;
- *mobile phase B*: add 2 mL of *trifluoroacetic acid R* to 950 mL of *acetonitrile R1* and shake to homogenise; dissolve 0.8 g of *ammonium acetate R* in this solution and shake until complete dissolution, then dilute to 1000.0 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 98 | 2 |
| 1 - 5 | 98 → 90 | 2 → 10 |
| 5 - 17 | 90 → 70 | 10 → 30 |
| 17 - 30 | 70 → 5 | 30 → 95 |
| 30 - 31 | 5 | 95 |

*Flow rate*: 0.35 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *fentanyl impurity mixture CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and D.

*Relative retention* with reference to fentanyl (retention time = about 19 min): impurity D = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity C and fentanyl.

*Calculation of percentage contents*:

- for each impurity, use the concentration of fentanyl citrate in reference solution (b).

*Limits*:

- *impurities C, D*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.4 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

Monographs F

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 52.86 mg of $C_{28}H_{36}N_2O_8$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F, G, H, I.*

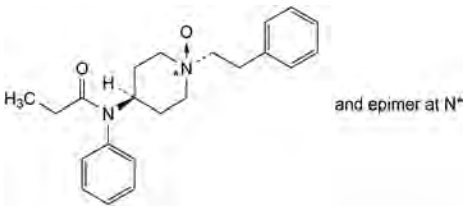


A. (1*rs*,4*rs*)-1-(2-phenylethyl)-4-(*N*-phenylpropanamido)-piperidine *N*-oxide,

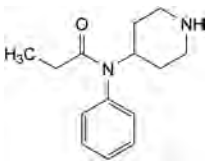

B. *N*-phenyl-*N*-(piperidin-4-yl)propanamide,

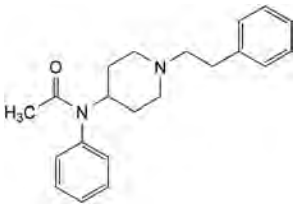

C. *N*-phenyl-*N*-[1-(2-phenylethyl)piperidin-4-yl]acetamide,

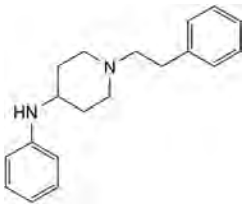

D. *N*-phenyl-1-(2-phenylethyl)piperidin-4-amine,

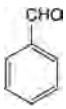

E. benzaldehyde,

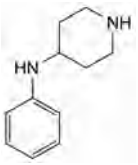

F. *N*-phenylpiperidin-4-amine,

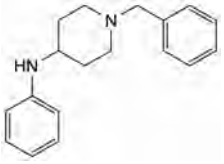

G. 1-benzyl-*N*-phenylpiperidin-4-amine,

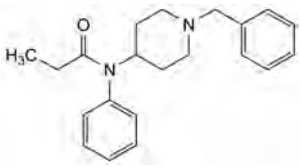

H. *N*-(1-benzylpiperidin-4-yl)-*N*-phenylpropanamide,

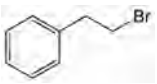

I. (2-bromoethyl)benzene.

**07/2014:1211**
**corrected 10.0**

# FENTICONAZOLE NITRATE

## Fenticonazoli nitras

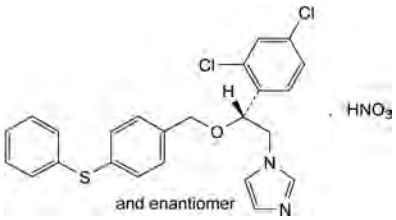


$C_{24}H_{21}Cl_2N_3O_4S$ $M_r$ 518.4
[73151-29-8]

DEFINITION

1-[(2*RS*)-2-(2,4-Dichlorophenyl)-2-[[4-(phenylsulfanyl)-benzyl]oxy]ethyl]-1*H*-imidazole nitrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide and in methanol, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 134 °C to 137 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 252 nm.

*Shoulder*: at about 270 nm.

*Absorption minimum*: at 236 nm.

*Specific absorbance at the absorption maximum*: 260 to 280.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fenticonazole nitrate CRS*.

D. It gives the reaction of nitrates (*2.3.1*).

TESTS

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 0.10 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b).* Dilute 10.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dissolve the contents of a vial of *fenticonazole impurity D CRS* in 1.0 mL of this solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: mix 70 volumes of *acetonitrile R1* and 30 volumes of a phosphate buffer solution prepared by dissolving 3.4 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjusting to pH 3.0 with *phosphoric acid R* and diluting to 1000 mL with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 10 µL.

*Run time*: 5.5 times the retention time of fenticonazole.

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to impurity D and fenticonazole in the chromatogram obtained with reference solution (d);

– *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peak due to the nitric ion (which corresponds to the dead volume of the column).

**Toluene**. Head-space gas chromatography (*2.2.28*): use the standard additions method.

*Test solution.* Disperse 0.2 g of the substance to be examined in a 10 mL vial with 5 mL of *water R*.

*Reference solution.* Mix 4 mg of *toluene R* with *water R* and dilute to 1000 mL with the same solvent. Place 5 mL of this solution in a 10 mL vial.

*Column*:

– *size*: *l* = 25 m, Ø = 0.32 mm;

– *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 1.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:25.

*Column head pressure*: 40 kPa.

*Static head-space conditions which may be used*:

– *equilibration temperature*: 90 °C;

– *equilibration time*: 1 h.

*Temperature*:

– *column*: 80 °C;

– *injection port*: 180 °C;

– *detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the gaseous phase.

*Limit*:

– *toluene*: maximum 100 ppm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.450 g in 50 mL of a mixture of equal volumes of *anhydrous acetic acid R* and *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 51.84 mg of $C_{24}H_{21}Cl_2N_3O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

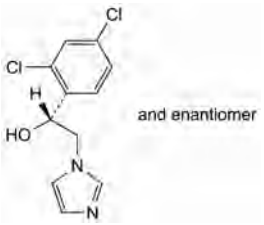


A. (*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

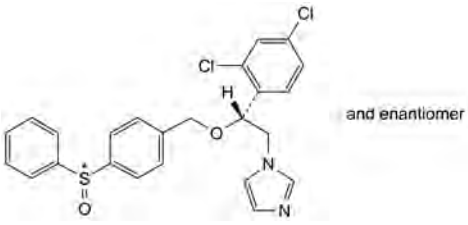


B. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[4-(benzenesulfinyl)-benzyl]oxy]ethyl]-1*H*-imidazole,

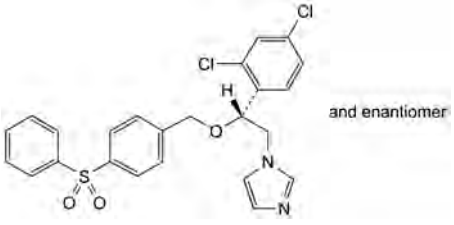


C. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[4-(benzenesulfonyl)-benzyl]oxy]ethyl]-1*H*-imidazole,

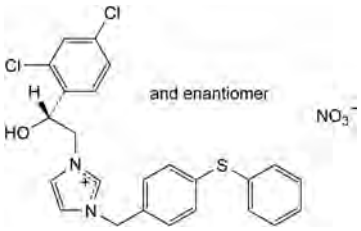


D. (*RS*)-1-[2-(2,4-dichlorophenyl)-2-hydroxyethyl]-3-[4-(phenylsulfanyl)benzyl]imidazolium nitrate,

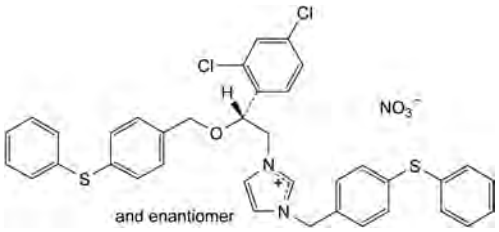


E. (*RS*)-1-[2-(2,4-dichlorophenyl)-2-[4-(phenylsulfanyl)-benzyloxy]ethyl]-3-[4-(phenylsulfanyl)benzyl]imidazolium nitrate.

**01/2017:1515**

# FERRIC CHLORIDE HEXAHYDRATE

## Ferri chloridum hexahydricum

$FeCl_3,6H_2O$ $M_r$ 270.3
[10025-77-1]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: crystalline mass or orange-yellow or brownish-yellow crystals, very hygroscopic.

*Solubility*: very soluble in water and in ethanol (96 per cent), freely soluble in glycerol.

IDENTIFICATION

A. It gives reaction (a) of chlorides (*2.3.1*).

B. It gives reaction (c) of iron (*2.3.1*).

TESTS

**Solution S**. Dissolve 10 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Acidity**. In a suitable polyethylene container, dissolve 3.0 g of *potassium fluoride R* in 15 mL of *water R*. Titrate with *0.1 M sodium hydroxide* using 0.1 mL of *phenolphthalein solution R* as indicator until a pink colour is obtained. Add 10 mL of solution S and allow to stand for 3 h. Filter and use 12.5 mL of the filtrate. Not more than 0.30 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Free chlorine**. Heat 5 mL of solution S. The vapour does not turn *starch iodide paper R* blue.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Heat 15 mL of solution S on a water-bath and add 5 mL of *strong sodium hydroxide solution R*. Allow to cool and filter. Neutralise the filtrate to *blue litmus paper R* using *hydrochloric acid R1* and evaporate to 15 mL.

**Ferrous ions**: maximum 50 ppm.

To 10 mL of solution S, add 1 mL of *water R*, and 0.05 mL of *potassium ferricyanide solution R* followed by 4 mL of *phosphoric acid R*. After 10 min, any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of *water R* and 1 mL of a freshly prepared 0.250 g/L solution of *ferrous sulfate R*.

ASSAY

In a conical flask with a ground-glass stopper, dissolve 0.200 g in 20 mL of *water R*. Add 10 mL of *dilute hydrochloric acid R* and 2 g of *potassium iodide R*. Allow the stoppered flask to stand for 1 h protected from light. Titrate with *0.1 M sodium thiosulfate*, adding 5 mL of *starch solution R* towards the end of the titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 27.03 mg of $FeCl_3,6H_2O$.

STORAGE

In an airtight container, protected from light.

**01/2008:0902**
**corrected 7.0**

# FERROUS FUMARATE

## Ferrosi fumaras

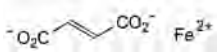


$C_4H_2FeO_4$ $M_r$ 169.9
[141-01-5]

DEFINITION

Iron(II) (*E*)-butenedioate.

*Content*: 93.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: fine, reddish-orange or reddish-brown powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1.0 g add 25 mL of a mixture of equal volumes of *hydrochloric acid R* and *water R* and heat on a water-bath for 15 min. Cool and filter. Use the filtrate for identification test C. Wash the residue with 50 mL of a mixture of 1 volume of *dilute hydrochloric acid R* and 9 volumes of *water R* and discard the washings. Dry the residue at 100-105 °C. Dissolve 20 mg of the residue in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *fumaric acid CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *methylene chloride R*, *butanol R*, *heptane R* (12:16:32:44 *V/V/V/V*).

*Application*: 5 µL.

*Development*: in an unsaturated tank, over a path of 10 cm.

*Drying*: at 105 °C for 15 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

B. Mix 0.5 g with 1 g of *resorcinol R*. To 0.5 g of the mixture in a crucible add 0.15 mL of *sulfuric acid R* and heat gently. A dark red semi-solid mass is formed. Add the mass, with care, to 100 mL of *water R*. An orange-yellow colour develops and the solution shows no fluorescence.

C. The filtrate obtained during preparation of the test solution in identification test A gives reaction (a) of iron (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in a mixture of 10 mL of *lead-free hydrochloric acid R* and 80 mL of *water R*, heating slightly if necessary. Allow to cool, filter if necessary and dilute to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Heat 0.15 g with 8 mL of *dilute hydrochloric acid R* and 20 mL of *distilled water R*. Cool in iced water, filter and dilute to 30 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm.

Mix 1.0 g with 15 mL of *water R* and 15 mL of *sulfuric acid R*. Warm to precipitate the fumaric acid completely. Cool and add 30 mL of *water R*. Filter. Wash the precipitate with *water R*. Dilute the combined filtrate and washings to 125 mL with *water R*. 25 mL of the solution complies with the test.

**Ferric ion**: maximum 2.0 per cent.

In a flask with a ground-glass stopper, dissolve 3.0 g in a mixture of 10 mL of *hydrochloric acid R* and 100 mL of *water R* by heating rapidly to boiling. Boil for 15 s. Cool rapidly, add 3 g of *potassium iodide R*, stopper the flask and allow to stand protected from light for 15 min. Add 2 mL of *starch solution R* as indicator. Titrate the liberated iodine with *0.1 M sodium thiosulfate*. Carry out a blank test. The difference between the volumes used in the 2 titrations corresponds to the amount of iodine liberated by ferric ion.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 5.585 mg of ferric ion.

**Cadmium**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a 10 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

**Chromium**: maximum 200 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *chromium standard solution (0.1 per cent Cr) R* and diluting with a 10 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: chromium hollow-cathode lamp.

*Wavelength*: 357.9 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 20 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (10 ppm Pb) R* and diluting with a 10 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Atomisation device*: air-acetylene flame.

**Mercury**: maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *mercury standard solution (10 ppm Hg) R* and diluting with a 25 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: mercury hollow-cathode lamp.

*Wavelength*: 253.7 nm.

Following the recommendations of the manufacturer, introduce 5 mL of solution S or 5 mL of the reference solutions into the reaction vessel of the cold-vapour mercury assay accessory, add 10 mL of *water R* and 1 mL of *stannous chloride solution R1*.

**Nickel**: maximum 200 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R* and diluting with a 10 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: nickel hollow-cathode lamp.

*Wavelength*: 232 nm.

*Atomisation device*: air-acetylene flame.

**Zinc**: maximum 500 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Solution S diluted to 10 volumes.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R* and diluting with a 1 per cent *V/V* solution of *lead-free hydrochloric acid R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve with slight heating 0.150 g in 7.5 mL of *dilute sulfuric acid R*. Cool and add 25 mL of *water R*. Add 0.1 mL of *ferroin R*. Titrate immediately with *0.1 M cerium sulfate* until the colour changes from orange to light bluish-green.

1 mL of *0.1 M cerium sulfate* is equivalent to 16.99 mg of $C_4H_2FeO_4$.

STORAGE

In an airtight container, protected from light.

**01/2017:0493**

# FERROUS GLUCONATE

## Ferrosi gluconas

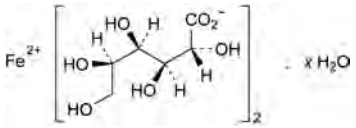

$C_{12}H_{22}FeO_{14},xH_2O$ $M_r$ 446.1 (anhydrous substance)

DEFINITION

Iron(II) bis[(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanoate] (iron(II) di(D-gluconate)).

*Content*: 11.8 per cent to 12.5 per cent of iron(II) (dried substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: greenish-yellow or grey powder or granules.

*Solubility*: freely but slowly soluble in water giving a greenish-brown solution, more readily soluble in hot water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 2 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Reference solution.* Dissolve 20 mg of *ferrous gluconate CRS* in 2 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 105 °C for 20 min; allow to cool.

*Detection*: spray with a solution containing 10 g/L of *cerium sulfate R* and 25 g/L of *ammonium molybdate R* in *dilute sulfuric acid R* and heat at 105 °C for about 10 min.

*Results*: after 5 min, the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. 1 mL of solution S (see Tests) gives reaction (a) of iron (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and heated to about 60 °C, allow to cool and dilute to 50 mL with *carbon dioxide-free water R* prepared from *distilled water R*.

**Appearance of solution**. The solution is clear (*2.2.1*).

Dilute 2 mL of solution S to 10 mL with *water R*. Examine the solution against the light.

**pH** (*2.2.3*): 4.0 to 5.5 for solution S, measured 3-4 h after preparation.

**Sucrose and reducing sugars**. Dissolve 0.5 g in 10 mL of warm *water R* and add 1 mL of *dilute ammonia R1*. Pass *hydrogen sulfide R* through the solution and allow to stand for 30 min. Filter and wash the precipitate with 2 quantities, each of 5 mL, of *water R*. Acidify the combined filtrate and washings to *blue litmus paper R* with *dilute hydrochloric acid R* and add 2 mL in excess. Boil until the vapour no longer darkens *lead acetate paper R* and continue boiling, if necessary, until the volume is reduced to about 10 mL. Cool, add 15 mL of *sodium carbonate solution R*, allow to stand for 5 min and filter. Dilute the filtrate to 100 mL with *water R*. To 5 mL of this solution add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 1 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 0.06 per cent.

Dilute 0.8 mL of solution S to 15 mL with *water R*.

**Oxalates**. Dissolve 5.0 g in a mixture of 10 mL of *dilute sulfuric acid R* and 40 mL of *water R*. Shake the solution with 50 mL of *ether R* for 5 min. Separate the aqueous layer and shake it with 20 mL of *ether R* for 5 min. Combine the ether layers, evaporate to dryness and dissolve the residue in 15 mL of *water R*. Filter, boil the filtrate until the volume is reduced to 5 mL and add 1 mL of *dilute acetic acid R* and 1.5 mL of *calcium chloride solution R*. Allow to stand for 30 min. No precipitate is formed.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 3.0 mL of solution S add 3 mL of *acetic acid R* and dilute to 15 mL with *distilled water R*. Examine the solutions against the light.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Barium**. Dilute 10 mL of solution S to 50 mL with *distilled water R* and add 5 mL of *dilute sulfuric acid R*. Allow to stand for 5 min. Any opalescence in the solution is not more intense than that in a mixture of 10 mL of solution S and 45 mL of *distilled water R*.

**Ferric ions**: maximum 1.0 per cent.

In a ground-glass-stoppered flask, dissolve 5.00 g in a mixture of 10 mL of *hydrochloric acid R* and 100 mL of *carbon dioxide-free water R*. Add 3 g of *potassium iodide R*, close the flask and allow to stand protected from light for 5 min. Titrate with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration. Not more than 9.0 mL of *0.1 M sodium thiosulfate* is used.

**Loss on drying** (*2.2.32*): 5.0 per cent to 10.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 5 h.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.5 g of *sodium hydrogen carbonate R* in a mixture of 30 mL of *dilute sulfuric acid R* and 70 mL of *water R*. When the effervescence stops, dissolve 1.00 g of the substance to be examined with gentle shaking. Using 0.1 mL of *ferroin R* as indicator, titrate with *0.1 M ammonium and cerium nitrate* until the red colour disappears.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 5.585 mg of iron(II).

STORAGE

Protected from light.

**01/2008:2340**
**corrected 7.2**

# FERROUS SULFATE, DRIED

## Ferrosi sulfas desiccatus

$FeSO_4$ $M_r$ 151.9

DEFINITION

Hydrated ferrous sulfate from which part of the water of hydration has been removed by drying.

*Content*: 86.0 per cent to 90.0 per cent.

CHARACTERS

*Appearance*: greyish-white powder.

*Solubility*: slowly but freely soluble in water, very soluble in boiling water, practically insoluble in ethanol (96 per cent).

It is oxidised in moist air, becoming brown.

IDENTIFICATION

A. It gives the reactions of sulfates (*2.3.1*).

B. It gives reaction (a) of iron (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.00 g in a 5 per cent *V/V* solution of *lead-free nitric acid R* and dilute to 100.0 mL with the same acid.

**pH** (*2.2.3*): 3.0 to 4.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dissolve 2.5 g in *water R*, add 0.5 mL of *dilute sulfuric acid R* and dilute to 50 mL with *water R*. Dilute 3.3 mL of this solution to 10 mL with *water R* and add 5 mL of *dilute nitric acid R*. Prepare the standard using a mixture of 10 mL of *chloride standard solution (5 ppm Cl) R* and 5 mL of *dilute nitric acid R*. Use 0.15 mL of *silver nitrate solution R2* in this test.

**Chromium**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluted as necessary with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: chromium hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 357.9 nm.

*Atomisation device*: air-acetylene flame.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R*, diluted as necessary with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Ferric ions**: maximum 0.5 per cent.

In a ground-glass-stoppered flask, dissolve 5.00 g in a mixture of 10 mL of *hydrochloric acid R* and 100 mL of *carbon dioxide-free water R*. Add 3 g of *potassium iodide R*, close the flask and allow to stand in the dark for 5 min. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of titration, as indicator. Carry out a blank test in the same conditions. Not more than 4.5 mL of *0.1 M sodium thiosulfate* is used.

**Manganese**: maximum 0.1 per cent.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dilute 1.0 mL of solution S to 20.0 mL with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *manganese standard solution (1000 ppm Mn) R*, diluted as necessary with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: manganese hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 279.5 nm.

*Atomisation device*: air-acetylene flame.

**Nickel**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluted as necessary with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: nickel hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 232.0 nm.

*Atomisation device*: air-acetylene flame.

**Zinc**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: zinc hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Dissolve 2.5 g of *sodium hydrogen carbonate R* in a mixture of 150 mL of *water R* and 10 mL of *sulfuric acid R*. When the effervescence ceases, add to the solution 0.140 g of the substance to be examined and dissolve with gentle shaking. Add 0.1 mL of *ferroin R* and titrate with *0.1 M ammonium and cerium nitrate* until the red colour disappears.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 15.19 mg of $FeSO_4$.

STORAGE

In an airtight container.

**01/2010:0083**
**corrected 7.2**

# FERROUS SULFATE HEPTAHYDRATE

## Ferrosi sulfas heptahydricus

$FeSO_4,7H_2O$ $M_r$ 278.0
[7782-63-0]

DEFINITION

*Content*: 98.0 per cent to 105.0 per cent.

CHARACTERS

*Appearance*: light green, crystalline powder or bluish-green crystals, efflorescent in air.

*Solubility*: freely soluble in water, very soluble in boiling water, practically insoluble in ethanol (96 per cent).

Ferrous sulfate heptahydrate is oxidised in moist air, becoming brown.

IDENTIFICATION

A. It gives the reactions of sulfates (*2.3.1*).

B. It gives reaction (a) of iron (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 4.0 g in a 5 per cent *V/V* solution of *lead-free nitric acid R* and dilute to 100.0 mL with the same solution.

**pH** (*2.2.3*): 3.0 to 4.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 10 mL with *water R* and add 5 mL of *dilute nitric acid R*. Prepare the standard with a mixture of 2 mL of *water R*, 5 mL of *dilute nitric acid R* and 8 mL of *chloride standard solution (5 ppm Cl) R*. Use 0.15 mL of *silver nitrate solution R2* in this test.

**Chromium**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: chromium hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 357.9 nm.

*Atomisation device*: air-acetylene flame.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (0.1 per cent Cu) R*, diluting with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 324.7 nm.

*Atomisation device*: air-acetylene flame.

**Ferric ions**: maximum 0.3 per cent.

In a ground-glass-stoppered flask, dissolve 5.00 g in a mixture of 10 mL of *hydrochloric acid R* and 100 mL of *carbon dioxide-free water R*. Add 3 g of *potassium iodide R*, close the flask and allow to stand in the dark for 5 min. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank test in the same conditions. Not more than 2.7 mL of *0.1 M sodium thiosulfate* is used, taking into account the blank titration.

**Manganese**: maximum 0.1 per cent.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dilute 1.0 mL of solution S to 20.0 mL with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Reference solutions.* Prepare the reference solutions using *manganese standard solution (1000 ppm Mn) R*, diluting with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: manganese hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 279.5 nm.

*Atomisation device*: air-acetylene flame.

**Nickel**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluting with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: nickel hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 232.0 nm.

*Atomisation device*: air-acetylene flame.

**Zinc**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Solution S.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with a 5 per cent *V/V* solution of *lead-free nitric acid R*.

*Source*: zinc hollow-cathode lamp using a transmission band preferably of 1 nm.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Dissolve 2.5 g of *sodium hydrogen carbonate R* in a mixture of 150 mL of *water R* and 10 mL of *sulfuric acid R*. When the effervescence ceases add to the solution 0.500 g of the substance to be examined and dissolve with gentle swirling. Add 0.1 mL of *ferroin R* and titrate with *0.1 M ammonium and cerium nitrate* until the red colour disappears.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 27.80 mg of $FeSO_4,7H_2O$.

STORAGE

In an airtight container.

**01/2017:2280**

# FEXOFENADINE HYDROCHLORIDE

## Fexofenadini hydrochloridum

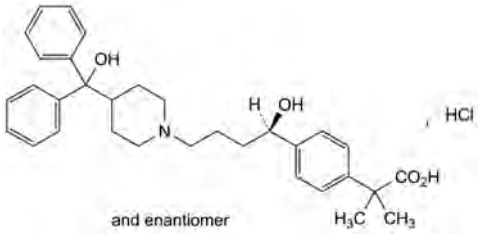


$C_{32}H_{40}ClNO_4$ $M_r$ 538.1

[153439-40-8]

DEFINITION

2-[4-[(1*RS*)-1-Hydroxy-4-[4-(hydroxydiphenylmethyl)-piperidin-1-yl]butyl]phenyl]-2-methylpropanoic acid hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, very slightly soluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fexofenadine hydrochloride CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve 30 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R*; sonicate if necessary and dilute to 2 mL with the same mixture of solvents. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve the contents of a vial of *fexofenadine impurity B CRS* in the test solution and dilute to 2.0 mL with the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel BC for chiral chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile for chromatography R* and 80 volumes of a buffer solution prepared as follows: to 1.15 mL of *glacial acetic acid R* add *water for chromatography R*, adjust to pH 4.0 ± 0.1 with *dilute ammonia R1* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 1.2 times the retention time of fexofenadine.

*Relative retention* with reference to fexofenadine (retention time = about 20 min): impurity B = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to fexofenadine and impurity B.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.3;

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 6.64 g of *sodium dihydrogen phosphate monohydrate R* and 0.84 g of *sodium perchlorate R* in *water for chromatography R*, adjust to pH 2.0 ± 0.1 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Solvent mixture*. Mix equal volumes of *acetonitrile for chromatography R* and the buffer solution.

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in 25.0 mL of the solvent mixture.

*Test solution (b)*. Dilute 3.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *fexofenadine hydrochloride CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 3.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 1 mg each of *fexofenadine impurity A CRS* and *fexofenadine impurity C CRS* in 20 mL of reference solution (a) and dilute to 200.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 350 volumes of *acetonitrile for chromatography R* and 650 volumes of the buffer solution; add 3 volumes of *triethylamine R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Relative retention* with reference to fexofenadine (retention time = about 9 min): impurity A = about 1.7; impurity D = about 2.3; impurity C = about 3.2.

*Run time*: 6 times the retention time of fexofenadine for test solution (a) and reference solution (c), twice the retention time of fexofenadine for reference solution (b).

*System suitability*: reference solution (c):

– *resolution*: minimum 10 between the peaks due to fexofenadine and impurity A.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;

– *impurities A, C, D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent.

Dissolve 1.000 g in *anhydrous methanol R* and dilute to 5.0 mL with the same solvent. Use 1.0 mL of this solution.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*Run time*: twice the retention time of fexofenadine.

Calculate the percentage content of fexofenadine hydrochloride from the declared content of *fexofenadine hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G.*

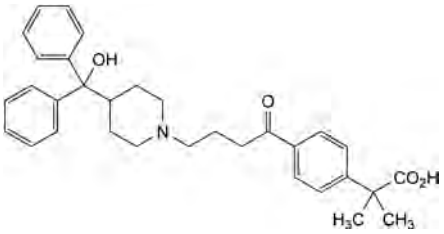


A. 2-[4-[4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butanoyl]phenyl]-2-methylpropanoic acid,

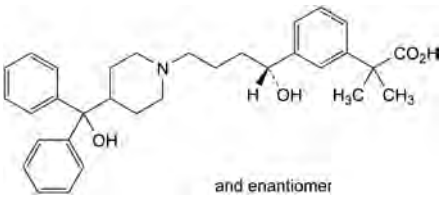


B. 2-[3-[(1*RS*)-1-hydroxy-4-[4-(hydroxydiphenylmethyl)-piperidin-1-yl]butyl]phenyl]-2-methylpropanoic acid,

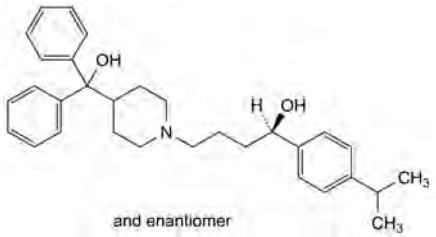


C. (1*RS*)-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]-1-[4-(1-methylethyl)phenyl]butan-1-ol,

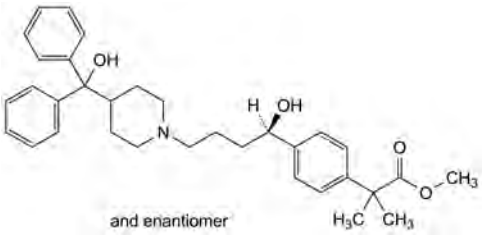


D. methyl 2-[4-[(1*RS*)-1-hydroxy-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butyl]phenyl]-2-methylpropanoate,

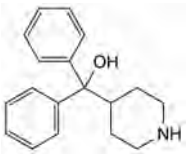


E. diphenyl(piperidin-4-yl)methanol,

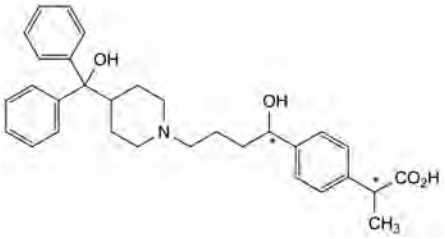


F. 2-[4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butyl]phenyl]propanoic acid,

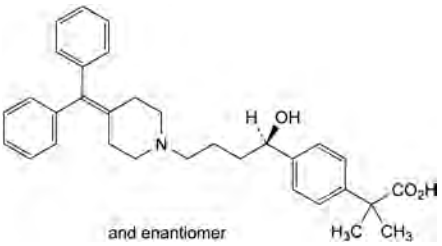


G. 2-[4-[(1*RS*)-4-[4-(diphenylmethylidene)piperidin-1-yl]-1-hydroxybutyl]phenyl]-2-methylpropanoic acid.

**04/2019:0903**

# FIBRIN SEALANT KIT

## Fibrini glutinum

DEFINITION

Sterile, freeze-dried, frozen or liquid preparation of plasma protein fractions containing essentially 2 components, namely fibrinogen concentrate (component 1), a protein fraction containing human fibrinogen, and a preparation containing human thrombin (component 2). A fibrin clot is rapidly formed when the 2 thawed or reconstituted components are mixed. Other ingredients (for example, human coagulation factor XIII, a fibrinolysis inhibitor or calcium ions) and stabilisers (for example, *Human albumin solution (0255)*) may be added.

Human constituents are obtained from plasma that complies with the monograph *Human plasma for fractionation (0853)*.

When thawed or reconstituted as stated on the label, component 1 contains not less than 40 g/L of clottable protein; the thrombin activity of component 2 varies over a wide range (approximately 4-1000 IU/mL).

PRODUCTION

The method of preparation is designed to maintain functional integrity of the components. It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

The production method is shown to yield consistent levels of human coagulation factor XIII.

The constituents or mixtures of constituents are dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. Constituents or mixtures of constituents are passed through a bacteria-retentive filter and distributed aseptically into sterile containers. Containers of freeze-dried constituents are closed under vacuum or filled with a suitable inert gas, such as oxygen-free nitrogen, before being closed.

If the label states that human coagulation factor XIII is an active substance in component 1, the assay of human coagulation factor XIII is carried out.

CHARACTERS

*Appearance*:

- *freeze-dried constituents*: white or pale yellow, hygroscopic powder or friable solid;
- *frozen constituents*: colourless or pale yellow, opaque solid;
- *liquid constituents*: colourless or pale yellow liquid.

*For the freeze-dried or frozen constituents, reconstitute or thaw as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.*

## Component 1 (fibrinogen concentrate)

IDENTIFICATION

A. It complies with the limits of the assay of fibrinogen.

B. It complies with the limits of the assay of human coagulation factor XIII (where its content is stated on the label).

TESTS

**Solubility**. Freeze-dried concentrates dissolve within 20 min in the volume of liquid and at the temperature stated on the label, forming an almost colourless, clear or slightly turbid solution.

**pH** (*2.2.3*): 6.5 to 8.0.

**Stability of solution**. No gel formation appears at room temperature during 120 min following thawing or reconstitution.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

ASSAY

**Fibrinogen (clottable protein)**. Mix 0.2 mL of the reconstituted concentrate with 2 mL of a suitable buffer solution (pH 6.6-7.4) containing sufficient *human thrombin R* (approximately 3 IU/mL) and calcium (0.05 mol/L). Maintain at 37 °C for 20 min, separate the precipitate by centrifugation at 5000 *g* for 20 min, wash thoroughly with a 9 g/L solution of *sodium chloride R* and determine the protein as nitrogen by sulfuric acid digestion (*2.5.9*). Calculate the clottable protein content by multiplying the result by 6.0. The estimated content in milligrams of clottable protein is not less than 70 per cent and not more than 130 per cent of the stated content. If for a particular preparation this method cannot be applied, use another validated method for determination of fibrinogen.

**Human coagulation factor XIII**. Use a reference plasma calibrated against the International Standard for blood coagulation factor XIII in plasma. If the label states that human coagulation factor XIII is present as an active substance, the estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency if the factor XIII potency is stated as a single value, or is within the stated range if the factor XIII potency is stated as a range.

Make at least 3 suitable dilutions of thawed or reconstituted concentrate and of the reference preparation using human coagulation factor XIII-deficient plasma or another suitable diluent. *Coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation. Add to each dilution suitable amounts of the following reagents:

- activator reagent, containing bovine or human thrombin, a suitable buffer, calcium chloride and a suitable inhibitor such as Gly-Pro-Arg-Pro-Ala-$NH_2$ which inhibits clotting of the sample but does not prevent human coagulation factor XIII activation by thrombin;
- detection reagent, containing a suitable factor XIIIa-specific peptide substrate, such as Leu-Gly-Pro-Gly-Glu-Ser-Lys-Val-Ile-Gly-$NH_2$ and glycine ethyl ester as 2nd substrate in a suitable buffer solution;
- NADH reagent, containing glutamate dehydrogenase, α-ketoglutarate and NADH in a suitable buffer solution.

After mixing, the absorbance changes (Δ*A*/min) are measured at a wavelength of 340 nm, after the linear phase of the reaction is reached.

Calculate the potency of the test preparation by the usual statistical methods (*5.3*, for example). The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

## Component 2 (thrombin preparation)

IDENTIFICATION

It complies with the limits of the assay of thrombin.

TESTS

**Solubility**. Freeze-dried preparations dissolve within 5 min in the volume of liquid stated on the label, forming a colourless, clear or slightly turbid solution.

**pH** (*2.2.3*): 5.0 to 8.0.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

ASSAY

**Thrombin**. If necessary, dilute the reconstituted preparation to be examined to approximately 2-20 IU of thrombin per millilitre using as diluent a suitable buffer solution (pH 7.3-7.5), such as *imidazole buffer solution pH 7.3 R* containing 10 g/L of *human albumin R* or *bovine albumin R*. To a suitable volume of the dilution, add a suitable volume of fibrinogen solution (1 g/L of clottable protein) warmed to 37 °C and start measurement of the clotting time immediately. Repeat the procedure with each of at least 3 dilutions, in the range stated above, of a reference preparation of thrombin, calibrated in International Units.

Calculate the activity of the test preparation by the usual statistical methods (*5.3*, for example). The estimated activity is not less than 80 per cent and not more than 125 per cent of the stated activity. For a component with a low thrombin concentration and a nominal value of approximately 4 IU/mL, the estimated activity is not less than 50 per cent and not more than 150 per cent of the stated activity. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated activity.

*The following sections apply to 2-component fibrin sealant.*

STORAGE

Protected from light and, for freeze-dried components, in an airtight container.

LABELLING

The label states:

- the amount of fibrinogen (milligrams of clottable protein) and thrombin (International Units) per container, and the content of human coagulation factor XIII (International Units per millilitre), if the latter is present as an active substance;
- where applicable, the name and volume of the liquid to be used to reconstitute the components.

**07/2019:2206**
**corrected 10.0**

# FILGRASTIM CONCENTRATED SOLUTION

## Filgrastimi solutio concentrata

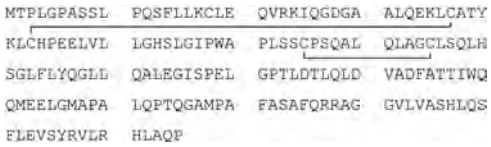


$C_{845}H_{1339}N_{223}O_{243}S_9$ $M_r$ 18 799
[121181-53-1]

DEFINITION

Solution of a protein having the primary structure of the 174-amino-acid isoform of human granulocyte colony-stimulating factor (huG-CSF) plus 1 additional amino acid, an *N*-terminal methionine. In contrast to its natural counterpart, the protein is not glycosylated. huG-CSF is produced and secreted by endothelial cells, monocytes and other immune cells. The protein stimulates the differentiation and proliferation of leucocyte stem cells into mature granulocytes.

*Content*: minimum 0.9 mg of protein per millilitre.

*Potency*: minimum $0.9 \times 10^8$ IU per milligram of protein.

PRODUCTION

Filgrastim concentrated solution is produced by a method based on recombinant DNA (rDNA) technology, using bacteria as host cells.

Prior to release, the following tests are carried out on each batch of filgrastim concentrated solution, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- or vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Examine the electropherograms obtained in the test for impurities with charges differing from that of filgrastim.

*Results*: the principal band in the electropherogram obtained with the test solution is similar in position to the principal band in the electropherogram obtained with reference solution (a).

C. Examine the chromatograms obtained in the test for impurities with molecular masses higher than that of filgrastim.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

D. Examine the electropherograms obtained under both reducing and non-reducing conditions in the test for impurities with molecular masses differing from that of filgrastim.

*Results*: the principal band in the electropherogram obtained with test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (b).

E. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and shape to the principal peak in the chromatogram obtained with the reference solution.

F. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Introduce a volume of the preparation to be examined corresponding to 25 µg of protein into a polypropylene tube. Add 25 µL of a 0.1 mg/mL solution of *glutamyl endopeptidase for peptide mapping R*. Dilute to 100 µL with *0.02 M sodium phosphate buffer solution pH 8.0 R*, stopper the tube and incubate at about 37 °C for 17 h. Cool to 2-8 °C until analysis.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *filgrastim CRS* instead of the preparation to be examined.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm) with a pore size of 20 nm;
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: dilute 0.5 mL of *trifluoroacetic acid R* in 950 mL of *water for chromatography R*, add 50 mL of *acetonitrile for chromatography R* and mix;
- *mobile phase B*: dilute 0.5 mL of *trifluoroacetic acid R* in 50 mL of *water for chromatography R*, add 950 mL of *acetonitrile for chromatography R* and mix;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 97 → 94 | 3 → 6 |
| 8 - 25 | 94 → 66 | 6 → 34 |
| 25 - 40 | 66 → 10 | 34 → 90 |
| 40 - 45 | 10 | 90 |

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of filgrastim digest supplied with *filgrastim CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

**Impurities with molecular masses higher than that of filgrastim**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Solution A*. Dissolve 4.1 g of *sodium acetate R* in 400 mL of *water R*, adjust to pH 4.0 with *acetic acid R* and dilute to 500 mL with *water R*.

*Test solution.* Dilute the preparation to be examined with solution A to obtain a concentration of 0.4 mg/mL.

*Reference solution.* Dilute *filgrastim CRS* with solution A to obtain a concentration of 0.4 mg/mL.

*Resolution solution.* Mix a sample of the reference solution for about 30 s using a vortex mixer.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000;
- *temperature*: 30 °C.

*Mobile phase*: dissolve 7.9 g of *ammonium hydrogen carbonate R* in 1000 mL of *water for chromatography R* and adjust to pH 7.0 with *phosphoric acid R*; dilute to 2000 mL with *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Relative retention* with reference to the filgrastim monomer (retention time = about 19 min): aggregates = about 0.60; filgrastim oligomer 1 = about 0.75; filgrastim oligomer 2 = about 0.80; filgrastim dimer = about 0.85.

*System suitability*: resolution solution:

- *retention time*: filgrastim monomer = 17 min to 20 min;
- *resolution*: minimum 3 between the peaks due to the filgrastim dimer and the filgrastim monomer.

Calculate the percentage content of the dimer, oligomers and aggregates.

*Limits*:

- *impurities with molecular masses higher than that of filgrastim, other than the dimer*: maximum 0.5 per cent;
- *total of impurities with molecular masses higher than that of filgrastim*: maximum 2 per cent.

**Impurities with molecular masses differing from that of filgrastim**. Polyacrylamide gel electrophoresis (*2.2.31*) under both reducing and non-reducing conditions.

*Gel dimensions*: 1 mm thick.

*Resolving gel*: 13 per cent acrylamide.

*Sample buffer (non-reducing conditions).* Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer R*.

*Sample buffer (reducing conditions).* Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer for reducing conditions R* containing 2-mercaptoethanol as the reducing agent.

*Test solution (a).* Dilute the preparation to be examined with sample buffer to obtain a concentration of 100 µg/mL.

*Test solution (b).* To 0.20 mL of test solution (a) add 0.20 mL of sample buffer.

*Test solution (c).* Dilute 0.20 mL of test solution (b) to 1 mL with sample buffer.

*Test solution (d).* Dilute 0.20 mL of test solution (c) to 1 mL with sample buffer.

*Test solution (e).* To 0.20 mL of test solution (d) add 0.20 mL of sample buffer.

*Reference solution (a).* Solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

*Reference solution (b).* Dilute *filgrastim CRS* with sample buffer to obtain a concentration of 100 µg/mL.

*Sample treatment*: boil for 5 min.

*Application*: 20 µL.

*Detection*: by silver staining.

*System suitability*:

- reference solution (a): the validation criteria are met;
- a band is seen in the electropherogram obtained with test solution (e);
- a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (a) to (e).

*Limit*: test solution (a):

- *impurities with molecular masses lower or higher than that of filgrastim*: no band is more intense than the principal band in the electropherogram obtained with test solution (d) (2.0 per cent).

**Impurities with charges differing from that of filgrastim**. Isoelectric focusing (*2.2.54*).

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of 0.3 mg/mL.

*Reference solution (a).* Dilute *filgrastim CRS* with *water R* to obtain a concentration of 0.3 mg/mL.

*Reference solution (b).* Dilute *filgrastim CRS* with *water R* to obtain a concentration of 0.03 mg/mL.

*Reference solution (c).* Use an isoelectric point (pI) calibration solution, in the pI range of 2.5-6.5, prepared according to the manufacturer's instructions.

*Focusing*:

- *pH gradient*: 4.5-8.0;
- *catholyte*: 1 M solution of *sodium hydroxide R*;
- *anolyte*: 0.04 M solution of *glutamic acid R* in a 0.0025 per cent *V/V* solution of *phosphoric acid R*;
- *application*: 20 µL.

*Detection*: as described in *2.2.54*.

*System suitability*:

- in the electropherogram obtained with reference solution (c), the relevant isoelectric point markers are distributed along the entire length of the gel;
- in the electropherogram obtained with reference solution (a), the pI of the principal band is 5.7 to 6.3.

*Limit*:

- *any impurity*: no band is more intense than the principal band in the electropherogram obtained with reference solution (b) (10 per cent).

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined with *water for chromatography R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (a).* Dilute the content of a vial of *filgrastim CRS* with *water for chromatography R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (b).* To 250 µL of reference solution (a), add 2.5 µL of a 4.5 g/L solution of hydrogen peroxide. Mix and incubate at 25 ± 2 °C for 30 min, then add 1.9 mg of *L-methionine R*.

*Reference solution (c).* To 250 µL of reference solution (a), add 0.25 mg of *dithiothreitol R*. Mix and incubate at 35 ± 2 °C for 60 min.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* in 1000 mL of *water for chromatography R*;
- *mobile phase B*: dilute 1 mL of *trifluoroacetic acid R* in 100 mL of *water for chromatography R*, then add 900 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 60 → 20 | 40 → 80 |
| 30 - 35 | 20 | 80 |
| 35 - 45 | 20 → 60 | 80 → 40 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Relative retention* with reference to filgrastim (retention time = about 23 min): oxidised filgrastim (form 1) = about 0.84; oxidised filgrastim (form 2) = about 0.98; reduced filgrastim = about 1.04.

*System suitability*: reference solution (b):

- *symmetry factor*: maximum 1.8 for the peak due to filgrastim;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to oxidised filgrastim (form 2) and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to filgrastim.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to filgrastim and reduced filgrastim;
- *symmetry factor*: maximum 1.8 for the peak due to filgrastim.

*Limits*:

- *any impurity*: for each impurity, maximum 1.0 per cent;
- *total*: maximum 2.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 2 IU in the volume that contains 1.0 mg of protein.

ASSAY

**Protein**. Liquid chromatography (*2.2.29*) as described in the test for related proteins with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the content of filgrastim ($C_{845}H_{1339}N_{223}O_{243}S_9$) taking into account the assigned content of $C_{845}H_{1339}N_{223}O_{243}S_9$ in *filgrastim CRS*.

**Potency**. The potency of the preparation to be examined is determined by comparison of the dilutions of the test preparation with the dilutions of the International Standard of filgrastim or with a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay using a suitable method such as the following, which uses the conversion of a tetrazolium salt (MTS) as a staining method. Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence, have also been found suitable, and may be used as the assay readout, subject to appropriate validation. The assay conditions (for example, cell concentration, incubation time and dilution steps) are then adapted accordingly.

Use an established cell line responsive to filgrastim. M-NFS-60 cells (ATCC No. CRL-1838) that have been made sensitive to G-CSF have been found suitable. Incubate with varying dilutions of test and reference preparations of filgrastim. Then incubate with a solution of *tetrazolium salt R*. This cytochemical stain is converted by cellular dehydrogenases to a coloured formazan product. The formazan is then measured spectrophotometrically.

Add 50 µL of dilution medium to all wells of a 96-well microtitre plate. Add an additional 50 µL of this solution to the wells designed for the blanks. Add 50 µL of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 800 IU/mL, plus a series of 10 twofold dilutions to obtain a standard curve). Prepare a suspension of M-NFS-60 cells containing $7 \times 10^5$ cells per millilitre. Immediately before use, add 2-mercaptoethanol to a final concentration of 0.1 mM, and add 50 µL of the prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 36.0-38.0 °C for 44-48 h in a humidified incubator using 6 ± 1 per cent $CO_2$. Add 20 µL of a 5.0 g/L sterile solution of *tetrazolium salt R* to each well and reincubate for 4 h. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Calculate the potency of the preparation to be examined using a suitable statistical method, for example the parallel line assay (*5.3*).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 74 per cent and not more than 136 per cent of the estimated potency.

LABELLING

The label states:

- the content, in milligrams of protein per millilitre;
- the potency, in International Units per milligram of protein.

**07/2019:2848**
**corrected 10.0**

# FILGRASTIM INJECTION

## Filgrastimi solutio iniectabilis

DEFINITION

Sterile solution for injection of a protein having the primary structure of the 174-amino-acid isoform of human granulocyte colony-stimulating factor (huG-CSF) plus 1 additional amino acid, an *N*-terminal methionine. In contrast to its natural counterpart, the protein is not glycosylated.

*Content*: 90.0 per cent to 110.0 per cent of the amount of filgrastim stated on the label.

*Potency*: 80 per cent to 125 per cent of the potency stated on the label when determined using the conditions described under Assay.

PRODUCTION

Filgrastim injection is prepared from *Filgrastim concentrated solution (2206)*.

The concentrated solution is diluted with a formulation buffer (acetate or glutamate) containing sodium hydroxide, sorbitol, and polysorbate 80 as excipients. This preparation is passed through a bacteria-retentive filter and distributed aseptically into the final containers. Where excipients or formulation buffer other than those mentioned above are used, their compatibility with the tests described hereafter must be confirmed.

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and shape to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): as approved by the competent authority.

**Impurities with molecular masses higher than that of filgrastim**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Solution A*. Dissolve 4.1 g of *sodium acetate R* in 400 mL of *water R*, adjust to pH 4.0 with *acetic acid R* and dilute to 500 mL with *water R*.

*Test solution*. Dilute the preparation to be examined with solution A to obtain a concentration of 0.4 mg/mL.

*Reference solution*. Dilute *filgrastim CRS* with solution A to obtain a concentration of 0.4 mg/mL.

*Resolution solution*. Mix a sample of the reference solution for about 30 s using a vortex mixer.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000;
- *temperature*: 30 °C.

*Mobile phase*: dissolve 7.9 g of *ammonium hydrogen carbonate R* in 1000 mL of *water for chromatography R* and adjust to pH 7.0 with *phosphoric acid R*; dilute to 2000 mL with *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Relative retention* with reference to the filgrastim monomer (retention time = about 19 min): aggregates = about 0.60; filgrastim oligomer 1 = about 0.75; filgrastim oligomer 2 = about 0.80; filgrastim dimer = about 0.85.

*System suitability*: resolution solution:

- *retention time*: filgrastim monomer = 17 min to 20 min;
- *resolution*: minimum 3.0 between the peaks due to the filgrastim dimer and the filgrastim monomer.

Calculate the percentage content of the dimer, oligomers and aggregates.

*Limits*:

- *impurities with molecular masses higher than that of filgrastim, other than the dimer*: maximum 0.5 per cent;
- *total of impurities with molecular masses higher than that of filgrastim*: maximum 1.0 per cent.

**Impurities with charges differing from that of filgrastim**. Isoelectric focusing (*2.2.54*) with the following modifications. *Prepare reference solutions (a), (b) and (c) immediately before loading on the gel.*

*Gel concentration*: 5 per cent; commercially available gels may be used.

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of 0.6 mg/mL.

*Reference solution (a)*. Dilute *filgrastim CRS* with *water R* to obtain a concentration of 0.6 mg/mL.

*Reference solution (b)*. To 10 µL of reference solution (a) add 190 µL of *water R* to obtain a concentration of 0.03 mg/mL.

*Reference solution (c).* To 10 µL of reference solution (a) add 490 µL of *water R* to obtain a concentration of 0.012 mg/mL.

*Reference solution (d).* Use an isoelectric point (pI) calibration solution, in the pI range of 3-10, prepared according to the manufacturer's instructions.

*Focusing*:

- *pH gradient*: a combination of ampholytes giving a functional separation in the pI range of 5.0-7.0;
- *catholyte*: 20 g/L solution of *sodium hydroxide R*;
- *anolyte*: 30 g/L solution of *glacial acetic acid R*;
- *application*: 15 µL.

Proceed with the isoelectric focusing by applying the electrical parameters according to the manufacturer's instructions. The following parameters have been found suitable for gels of dimensions 250 mm × 110 mm × 0.43 mm:

- *pre-focusing*: 700 V, 12 mA, 8 W, 20 min;
- *post-application*: 500 V, 8 mA, 8 W, 20 min;
- *focusing*: 2000 V, 14 mA, 14 W, 90 min;
- *final focusing*: 2500 V, 14 mA, 14 W, 10 min.

*Detection*: silver staining.

*Fixing solution*: a solution containing 36 g/L of *sulfosalicylic acid R* and 116 g/L of *trichloroacetic acid R*.

*Sensitising solution.* Dissolve 17 g of *sodium acetate R* in about 100 mL of *water R*. Add 75 mL of *ethanol (96 per cent) R*, 10 mL of a 50 g/L solution of *anhydrous sodium thiosulfate R* and 1.25 mL of a 250 g/L solution of *glutaraldehyde R* and dilute to 250 mL with *water R*.

*Silver solution.* Mix 100 µL of *formaldehyde solution R1* and 25 mL of a 25 g/L solution of *silver nitrate R* and dilute to 250 mL with *water R*.

*Developing solution.* Dissolve 6.25 g of *sodium carbonate R* in about 200 mL of *water R*, add 50 µL of *formaldehyde solution R1* and dilute to 250 mL with *water R*.

*Stopping solution.* Dissolve 3.65 g of *sodium edetate R* in 250 mL of *water R*.

Alternatively, commercially available staining kits may be used.

*System suitability*:

- in the electropherogram obtained with reference solution (d), the relevant isoelectric point markers are distributed along the entire length of the gel;
- in the electropherogram obtained with reference solution (c), the principal band is clearly visible.

*Limits*:

- maximum 7 bands of an intensity between that of the principal band in the electropherogram obtained with reference solution (c) (2 per cent) and that of the principal band in the electropherogram obtained with reference solution (b) (5 per cent) are present;
- no band has an intensity greater than that of the principal band in the electropherogram obtained with reference solution (b) (5 per cent).

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (a).* Dilute the contents of a vial of *filgrastim CRS* with *water R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (b).* To 250 µL of reference solution (a), add 2.5 µL of a 4.5 g/L solution of hydrogen peroxide. Mix and incubate at 25 ± 2 °C for 30 min, then add 1.9 mg of *L-methionine R*.

*Reference solution (c).* To 250 µL of reference solution (a), add 0.25 mg of *dithiothreitol R*. Mix and incubate at 35 ± 2 °C for 60 min.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* in 1000 mL of *water for chromatography R*;
- *mobile phase B*: dilute 1 mL of *trifluoroacetic acid R* in 100 mL of *water for chromatography R*, then add 900 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 60 → 20 | 40 → 80 |
| 30 - 35 | 20 | 80 |
| 35 - 45 | 20 → 60 | 80 → 40 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Relative retention* with reference to filgrastim (retention time = about 23 min): oxidised filgrastim (form 1) = about 0.84; oxidised filgrastim (form 2) = about 0.98; reduced filgrastim = about 1.04.

*System suitability*: reference solution (b):

- *symmetry factor*: maximum 1.8 for the peak due to filgrastim;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to oxidised filgrastim (form 2) and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to filgrastim.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to filgrastim and reduced filgrastim;
- *symmetry factor*: maximum 1.8 for the peak due to filgrastim.

*Limits*:

- *any impurity*: for each impurity, maximum 3.0 per cent;
- *total*: maximum 6.5 per cent.

**Osmolality** (*2.2.35*): as approved by the competent authority.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU in the volume that contains 1.0 mg of protein.

ASSAY

**Protein**. Liquid chromatography (*2.2.29*) as described in the test for related proteins with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the content of filgrastim ($C_{845}H_{1339}N_{223}O_{243}S_9$) taking into account the assigned content of $C_{845}H_{1339}N_{223}O_{243}S_9$ in *filgrastim CRS*.

**Potency**. The potency of the preparation to be examined is determined by comparison of the dilutions of the test preparation with the dilutions of the International Standard of filgrastim or with a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay using a suitable method such as the following, which uses the conversion of a tetrazolium salt (MTS) as a staining method. Alternative methods of quantifying cell proliferation, such as measurement of intracellular ATP by luciferase bioluminescence, have also been found suitable, and may be used as the assay readout,

subject to appropriate validation. The assay conditions (for example, cell concentration, incubation time and dilution steps) are then adapted accordingly.

Use an established cell line responsive to filgrastim. M-NFS-60 cells (ATCC No. CRL-1838) that have been made sensitive to G-CSF have been found suitable. Incubate with varying dilutions of test and reference preparations of filgrastim. Then incubate with a solution of *tetrazolium salt R*. This cytochemical stain is converted by cellular dehydrogenases to a coloured formazan product. The formazan is then measured spectrophotometrically.

Add 50 µL of dilution medium to each well of a 96-well microtitre plate. Add an additional 50 µL of this solution to the wells designed for the blanks. Add 50 µL of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 800 IU/mL, plus a series of 10 twofold dilutions to obtain a standard curve). Prepare a suspension of M-NFS-60 cells containing $7 \times 10^5$ cells per millilitre. Immediately before use, add 2-mercaptoethanol to a final concentration of 0.1 mM, and add 50 µL of the prepared cell suspension to each well, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 36.0-38.0 °C for 44-48 h in a humidified incubator using 6 ± 1 per cent $CO_2$. Add 20 µL of a 5.0 g/L sterile solution of *tetrazolium salt R* to each well and reincubate for 4 h. Estimate the quantity of formazan produced using a microtitre well plate reader at 490 nm.

Calculate the potency of the preparation to be examined using a suitable statistical method, for example the parallel line assay (*5.3*).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 74 per cent and not more than 136 per cent of the estimated potency.

STORAGE

In a sterile, airtight, tamper-evident container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2014:1615**

# FINASTERIDE

## Finasteridum

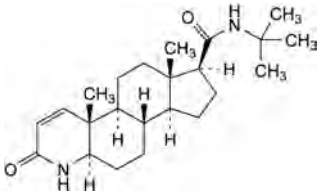

$C_{23}H_{36}N_2O_2$ $M_r$ 372.6

[98319-26-7]

DEFINITION

*N*-(1,1-Dimethylethyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *finasteride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): + 12.0 to + 14.0 (dried substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 100.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *finasteride CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *finasteride for peak identification CRS* (containing impurities A and C) in 1.0 mL of the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*: *acetonitrile R1*, *tetrahydrofuran R*, *water for chromatography R* (10:10:80 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 15 µL of test solution (b) and reference solutions (b) and (c).

*Run time*: twice the retention time of finasteride.

*Identification of impurities*: use the chromatogram supplied with *finasteride for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C.

*Relative retention* with reference to finasteride (retention time = about 28 min): impurity A = about 0.9; impurity C = about 1.3.

*System suitability*:

- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (c);
- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to finasteride in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity A by 2.4;
- for each impurity, use the concentration of finasteride in reference solution (c).

*Limits*:

- *impurities A, C*: for each impurity, maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage content of $C_{23}H_{36}N_2O_2$ taking into account the assigned content of *finasteride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

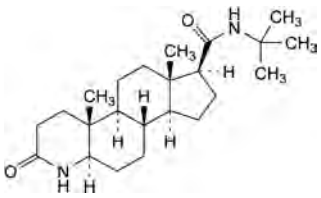

A. *N*-(1,1-dimethylethyl)-3-oxo-4-aza-5α-androstane-17β-carboxamide (dihydrofinasteride),

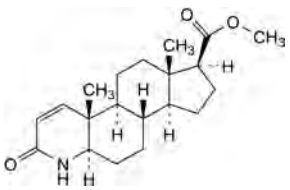

B. methyl 3-oxo-4-aza-5α-androst-1-ene-17β-carboxylate,

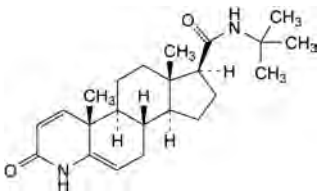

C. *N*-(1,1-dimethylethyl)-3-oxo-4-azaandrosta-1,5-diene-17β-carboxamide (Δ5-finasteride).

**07/2019:2988**

# FINGOLIMOD HYDROCHLORIDE

## Fingolimodi hydrochloridum

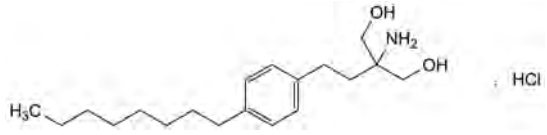

$C_{19}H_{34}ClNO_2$ $M_r$ 343.9
[162359-56-0]

DEFINITION

2-Amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: mulls in *liquid paraffin R* if recording by transmission.

*Comparison*: *fingolimod hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

If precipitation is observed after addition of *dilute nitric acid R*, centrifuge and use the supernatant in the remainder of the test.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, 0.1 per cent *V/V* solution of *phosphoric acid R* (50:50 *V/V*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 30.0 mg of *fingolimod hydrochloride CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 3 mg of *fingolimod for system suitability CRS* (containing impurities C and G) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with embedded polar groups R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 22 | 80 → 5 | 20 → 95 |
| 22 - 25 | 5 | 95 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *fingolimod for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and G.

*Relative retention* with reference to fingolimod (retention time = about 6.5 min): impurity G = about 1.10; impurity C = about 1.13.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities G and C.

*Calculation of percentage contents*:

– for each impurity, use the concentration of fingolimod hydrochloride in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 50.0 mg by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{19}H_{34}ClNO_2$ taking into account the assigned content of *fingolimod hydrochloride CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I.*

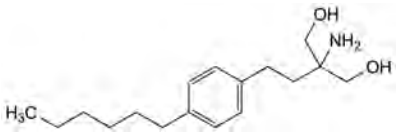

A. 2-amino-2-[2-(4-hexylphenyl)ethyl]propane-1,3-diol,

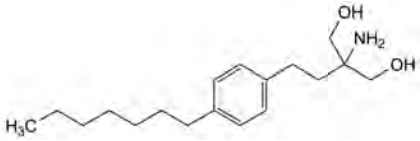

B. 2-amino-2-[2-(4-heptylphenyl)ethyl]propane-1,3-diol,

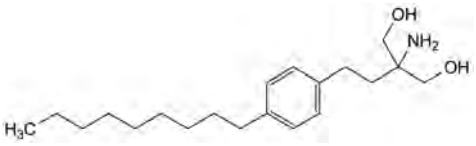

C. 2-amino-2-[2-(4-nonylphenyl)ethyl]propane-1,3-diol,

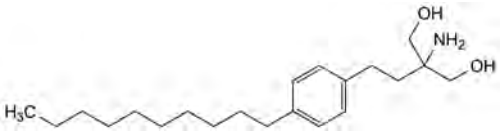

D. 2-amino-2-[2-(4-decylphenyl)ethyl]propane-1,3-diol,

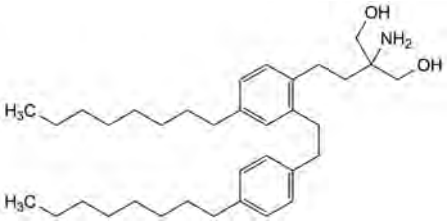

E. 2-amino-2-[2-[4-octyl-2-[2-(4-octylphenyl)ethyl]-phenyl]ethyl]propane-1,3-diol,

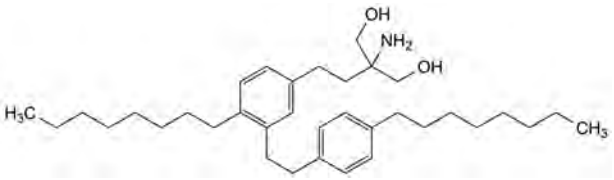

F. 2-amino-2-[2-[4-octyl-3-[2-(4-octylphenyl)ethyl]-phenyl]ethyl]propane-1,3-diol,

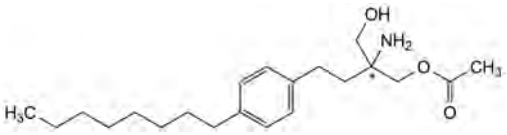

G. (2Ξ)-2-amino-2-(hydroxymethyl)-4-(4-octylphenyl)butyl acetate,

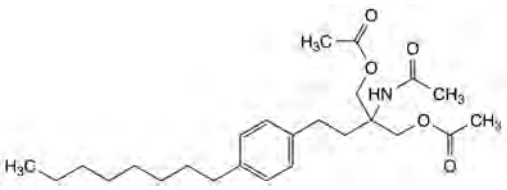

H. 2-acetamido-2-[(acetyloxy)methyl]-4-(4-octylphenyl)butyl acetate,

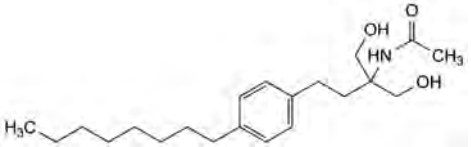

I. *N*-[1-hydroxy-2-(hydroxymethyl)-4-(4-octylphenyl)-butan-2-yl]acetamide.

**07/2018:2869**

# FIPRONIL FOR VETERINARY USE

## Fipronilum ad usum veterinarium

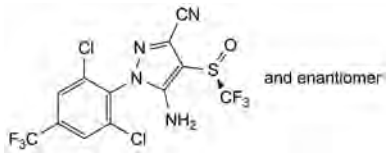


$C_{12}H_4Cl_2F_6N_4OS$ $M_r$ 437.1
[120068-37-3]

DEFINITION

5-Amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(*RS*)-(trifluoromethyl)sulfinyl]-1*H*-pyrazole-3-carbonitrile.

*Content*: 95.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white or yellowish powder.

*Solubility*: practically insoluble in water, soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fipronil CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *methanol R*, *water R*, *acetonitrile R* (30:30:40 *V/V/V*).

*Test solution (a)*. Dissolve 35.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 3.0 mL of test solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 1.5 mg of *fipronil for system suitability CRS* (containing impurities A and B) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 35.0 mg of *fipronil CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 3.0 mL of the solution to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm).

*Mobile phase*: *tetrahydrofuran R*, *methanol R2*, *water for chromatography R*, *acetonitrile R1* (0.5:30:30:40 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: twice the retention time of fipronil.

*Identification of impurities*: use the chromatogram supplied with *fipronil for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to fipronil (retention time = about 6 min): impurity A = about 1.3; impurity B = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of fipronil in reference solution (b).

*Limits*:

- *impurity B*: maximum 3.5 per cent;
- *impurity A*: maximum 1.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 4.5 per cent;
- *reporting threshold*: 0.10 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{12}H_4Cl_2F_6N_4OS$ taking into account the assigned content of *fipronil CRS*.

IMPURITIES

*Specified impurities: A, B.*

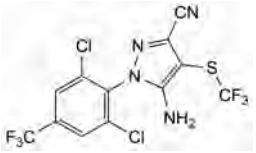


A. 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfanyl]-1*H*-pyrazole-3-carbonitrile,

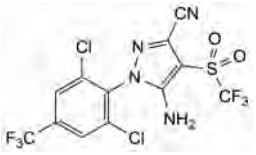


B. 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfonyl]-1*H*-pyrazole-3-carbonitrile.

**01/2020:1912**

# FISH OIL, RICH IN OMEGA-3 ACIDS

## Piscis oleum omega-3 acidis abundans

DEFINITION

Purified, winterised and deodorised fatty oil obtained from fish of families such as *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Scombridae* (except the genera *Thunnus* and *Sarda*) and *Ammodytidae* (type I), or from the genera *Thunnus* and *Sarda* within the family *Scombridae* (type II). The omega-3

acids are defined as the following acids: *alpha*-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA).

*Content*:

| | Type I | Type II |
|---|---|---|
| *EPA, expressed as triglycerides* | minimum 13 per cent | 4 per cent to 12 per cent |
| *DHA, expressed as triglycerides* | minimum 9 per cent | minimum 20 per cent |
| *Total omega-3 acids, expressed as triglycerides* | minimum 28 per cent | minimum 28 per cent |

A suitable antioxidant may be added.

PRODUCTION

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: pale yellow liquid.

*Solubility*: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay for EPA and DHA.

*Results*: the peaks due to eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester in the chromatogram obtained with test solution (b) are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solutions ($a_1$) and ($a_2$).

B. It complies with the limits of the assay for EPA (type I or II).

TESTS

**Appearance**. The substance to be examined is not more intensely coloured than a reference solution prepared as follows: to 3.0 mL of red primary solution add 25.0 mL of yellow primary solution and dilute to 50.0 mL with a 10 g/L solution of *hydrochloric acid R* (*2.2.2, Method II*).

**Absorbance** (*2.2.25*): maximum 0.70 (type I) or maximum 0.50 (type II), at 233 nm.

Dilute 0.300 g of the substance to be examined to 50.0 mL with *trimethylpentane R*. Dilute 2.0 mL of the solution to 50.0 mL with *trimethylpentane R*.

**Acid value** (*2.5.1*): maximum 0.5, determined on 20.0 g.

**Anisidine value** (*2.5.36*): maximum 30.0 (type I) or maximum 15.0 (type II).

**Peroxide value** (*2.5.5, Method A*): maximum 10.0 (type I) or maximum 5.0 (type II).

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Stearin**. Immerse at least 10 mL of the substance to be examined previously at room temperature, for 3 h in a bath of iced water or a thermostatically controlled bath at 0 ± 0.5 °C. The sample remains clear.

**Oligomers**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dilute 50.0 mg of the substance to be examined to 10.0 mL with *tetrahydrofuran R*.

*Reference solution*. In a 100 mL volumetric flask dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent.

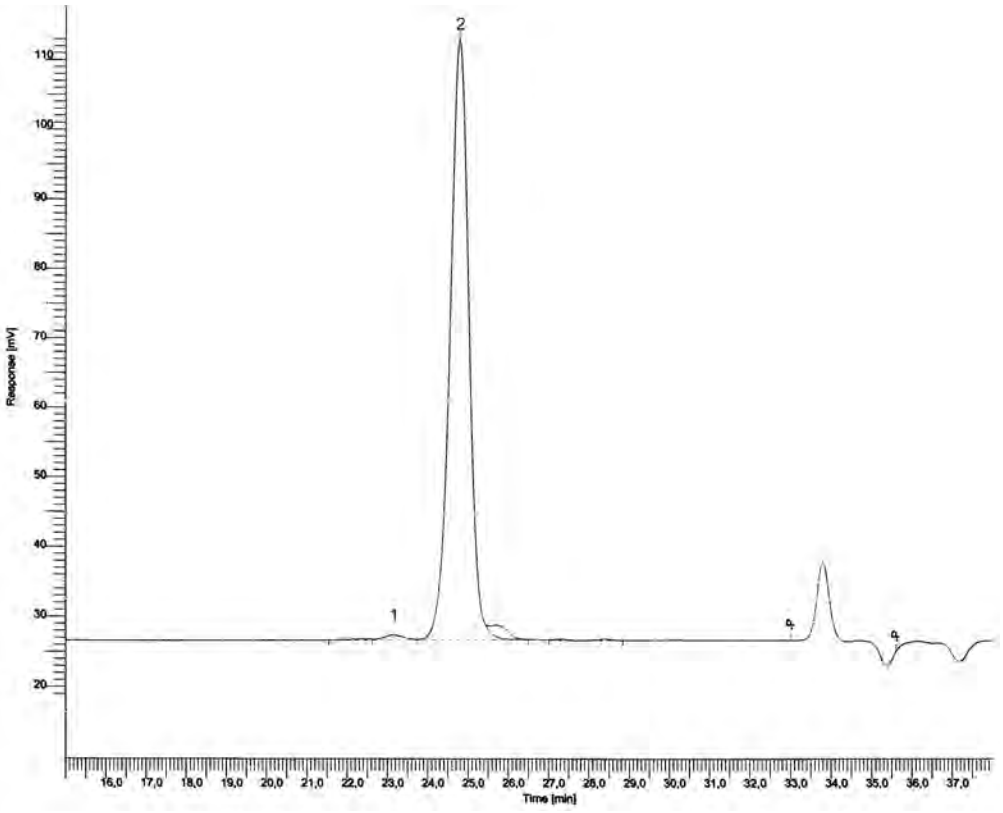


1. oligomers 2. triglycerides

Figure 1912.-1. – *Chromatogram for the test for oligomers in fish oil rich in omega-3 acids*

*Column*: 3 columns to be connected in series:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with the following pore sizes:
  - *column 1*: 50 nm;
  - *column 2*: 10 nm;
  - *column 3*: 5 nm;
- *connection sequence*: injector – column 1 – column 2 – column 3 – detector.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 0.8 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*System suitability*: reference solution:

- *elution order*: tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin;
- *resolution*: minimum 2.0 between the peaks due to didocosahexaenoin and monodocosahexaenoin and minimum 1.0 between the peaks due to tridocosahexaenoin and didocosahexaenoin.

Identify the peaks from the chromatogram (Figure 1912.-1). Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

*A* = sum of the areas of all the peaks in the chromatogram;

*B* = area of the peak with a retention time less than the retention time of the triglyceride peak.

*Limit*:

- *oligomers*: maximum 1.5 per cent.

ASSAY

**EPA and DHA** (*2.4.29*). For identification of the peaks, see Figure 1912.-2.

**Total omega-3 acids** (*2.4.29*). See Figure 1912.-2.

STORAGE

Under an inert gas, in a well-filled, airtight container, protected from light.

LABELLING

The label states:

- the concentration of EPA, DHA and total omega-3 acids, expressed as triglycerides;
- the type of fish oil rich in omega-3 acids (type I or II).

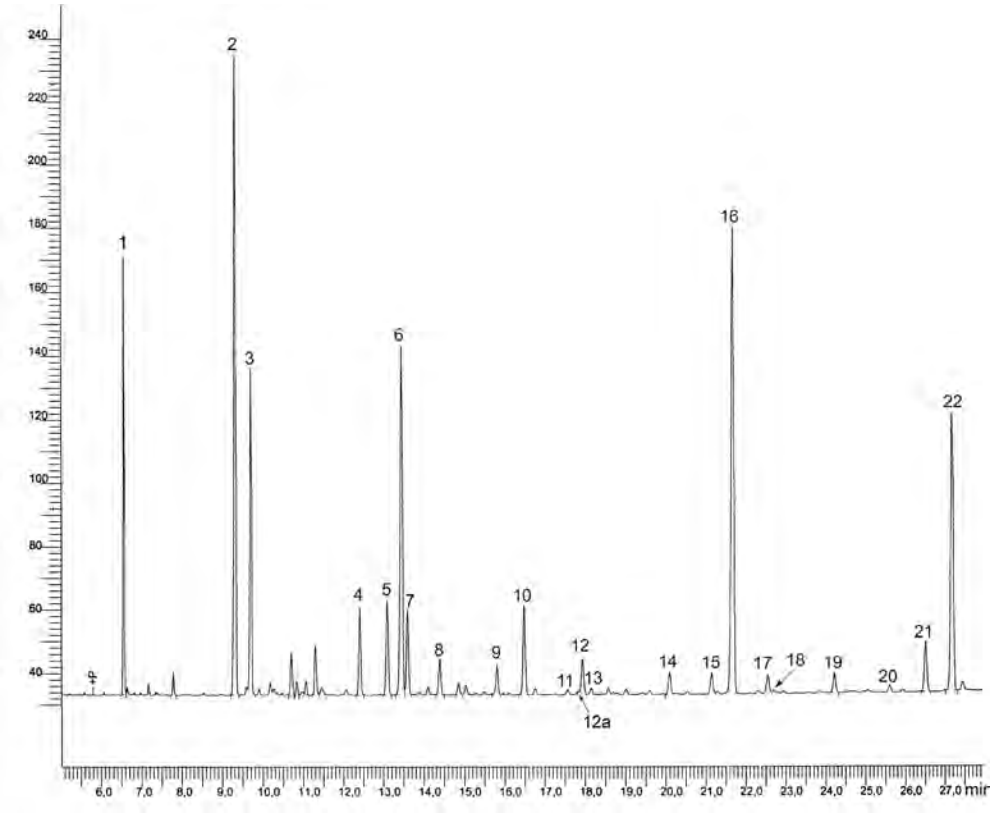


| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 1. C14:0 | 4. C16:4 n-1 | 7. C18:1 n-7 | 10. C18:4 n-3 | 12a. C20:1 n-11 | 15. C20:4 n-3 | 18. C22:1 n-9 | 21. C22:5 n-3 |
| 2. C16:0 | 5. C18:0 | 8. C18:2 n-6 | 11. C20:0 | 13. C20:1 n-7 | 16. C20:5 n-3 | 19. C21:5 n-3 | 22. C22:6 n-3 |
| 3. C16:1 n-7 | 6. C18:1 n-9 | 9. C18:3 n-3 | 12. C20:1 n-9 | 14. C20:4 n-6 | 17. C22:1 n-11 | 20. C22:5 n-6 | |

Figure 1912.-2. – *Chromatogram for the assay of total omega-3 acids in fish oil rich in omega-3 acids*

01/2017:1692

# FLAVOXATE HYDROCHLORIDE

## Flavoxati hydrochloridum

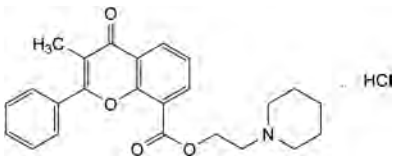

$C_{24}H_{26}ClNO_4$ $M_r$ 427.9
[3717-88-2]

DEFINITION

2-(Piperidin-1-yl)ethyl 3-methyl-4-oxo-2-phenyl-4*H*-1-benzopyran-8-carboxylate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *flavoxate hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Solvent mixture.* Mix 20 volumes of a 0.4 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R* and 80 volumes of *acetonitrile R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 6.0 mg of *flavoxate impurity A CRS* and 3.0 mg of *flavoxate impurity B CRS* in the solvent mixture, add 2.0 mL of the test solution and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 0.435 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 7.5 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 20 | 80 |
| 10 - 20 | 20 → 10 | 80 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Relative retention* with reference to flavoxate (retention time = about 10 min): impurity A = about 0.2; impurity B = about 0.8.

*System suitability*: reference solution (c):
- *resolution*: minimum 4.0 between the peaks due to impurity B and flavoxate.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total of unspecified impurities*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.350 g in 10 mL of *anhydrous formic acid R* and add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 42.79 mg of $C_{24}H_{26}ClNO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

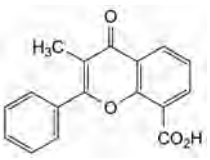

A. 3-methyl-4-oxo-2-phenyl-4*H*-1-benzopyran-8-carboxylic acid,

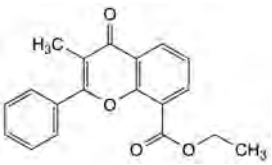

B. ethyl 3-methyl-4-oxo-2-phenyl-4*H*-1-benzopyran-8-carboxylate,

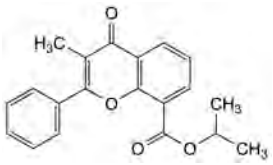

C. 1-methylethyl 3-methyl-4-oxo-2-phenyl-4*H*-1-benzopyran-8-carboxylate.

**01/2017:1324**

# FLECAINIDE ACETATE

## Flecainidi acetas

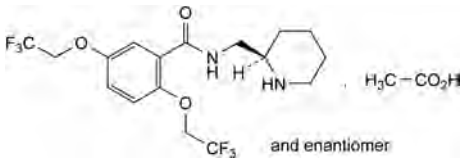


$C_{19}H_{24}F_6N_2O_5$ $M_r$ 474.4
[54143-56-5]

DEFINITION

*N*-[(*RS*)-(Piperidin-2-ylmethyl)]-2,5-bis(2,2,2-trifluoroethoxy)benzamide acetate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic, crystalline powder.

*Solubility*: soluble in water and in anhydrous ethanol. It is freely soluble in dilute acetic acid and practically insoluble in dilute hydrochloric acid.

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *A, B, D.*

A. Melting point (*2.2.14*): 146 °C to 152 °C, with a melting range not greater than 3 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 298 nm.

*Specific absorbance at the absorption maximum*: 61 to 65.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flecainide acetate CRS*.

D. It gives reaction (b) of acetates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.50 g in *water R*, add 0.1 mL of *glacial acetic acid R* and dilute to 20 mL with *water R*.

**pH** (*2.2.3*): 6.7 to 7.1.

Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *flecainide impurity B CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 0.10 g of the substance to be examined in reference solution (a) and dilute to 2.0 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: freshly prepared mixture of 5 volumes of *concentrated ammonia R* and 95 volumes of *acetone R*.

*Application*: 5 µL.

*Development*: over 1/2 of the plate.

*Drying*: at 100-105 °C until the ammonia has evaporated.

*Detection*: examine in ultraviolet light at 254 nm to establish the position of the flecainide spot, then spray with a freshly prepared 2 g/L solution of *ninhydrin R* in *methanol R* and heat at 100-110 °C for 2-5 min; examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.25 g of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *flecainide impurity A CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (c).* Dissolve 5 mg of *flecainide for system suitability CRS* (containing impurities C, D and E) in 1.0 mL of *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 2 mL of *concentrated ammonia R*, 4 mL of *triethylamine R* and 985 mL of *water R*; add 6 mL of *phosphoric acid R* and adjust to pH 2.8 with *concentrated ammonia R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 17 | 90 → 30 | 10 → 70 |
| 17 - 22 | 30 | 70 |

If a suitable baseline cannot be obtained, use another grade of triethylamine.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *flecainide for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C, D, and E; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to flecainide (retention time = about 11 min): impurity C = about 0.9; impurity A = about 1.1; impurity E = about 1.28; impurity D = about 1.32.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities E and D.

*Limits*:

– *impurities A, C, D, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.6 kPa for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.400 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.44 mg of $C_{19}H_{24}F_6N_2O_5$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

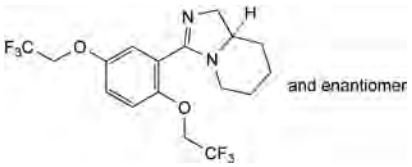


A. (8a*RS*)-3-[2,5-bis(2,2,2-trifluoroethoxy)phenyl]-1,5,6,7,8,8a-hexahydroimidazo[1,5-*a*]pyridine,

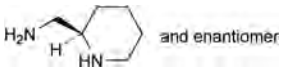


B. (*RS*)-(piperidin-2-yl)methanamine,

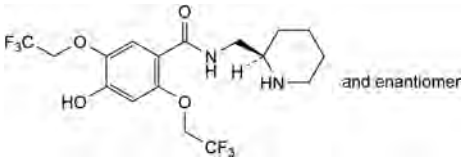


C. (*RS*)-4-hydroxy-*N*-(piperidin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy)benzamide,

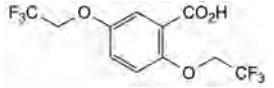


D. 2,5-bis(2,2,2-trifluoroethoxy)benzoic acid,

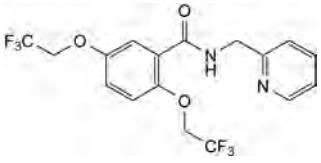


E. *N*-(pyridin-2-ylmethyl)-2,5-bis(2,2,2-trifluoroethoxy) benzamide.

**01/2008:1721**
**corrected 7.0**

# FLUBENDAZOLE

## Flubendazolum

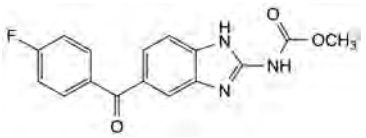


$C_{16}H_{12}FN_3O_3$ $M_r$ 313.3
[31430-15-6]

DEFINITION

Methyl [5-(4-fluorobenzoyl)-1*H*-benzimidazol-2-yl]carbamate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, in alcohol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*), without recrystallisation.

*Comparison*: *flubendazole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *flubendazole for system suitability CRS* in *dimethylformamide R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm,
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: 7.5 g/L solution of *ammonium acetate R*,
– *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 75 | 10 → 25 |
| 15 - 30 | 75 → 45 | 25 → 55 |
| 30 - 32 | 45 → 10 | 55 → 90 |
| 32 - 37 | 10 | 90 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (a):

– the chromatogram obtained is similar to the chromatogram supplied with *flubendazole for system suitability CRS*.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity C = 1.3; impurity D = 1.3; impurity G = 1.4,
– *impurities A, B, C, D, E, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
– *impurity F*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *any other impurity with a relative retention between 1.2 and 1.3*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),
– *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C, for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 3 mL of *anhydrous formic acid R* and add 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.33 mg of $C_{16}H_{12}FN_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

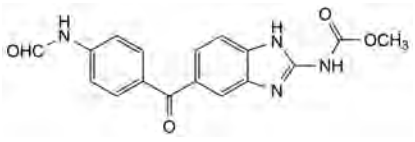

A. methyl [5-[4-(formylamino)benzoyl]-1*H*-benzimidazol-2-yl]carbamate,

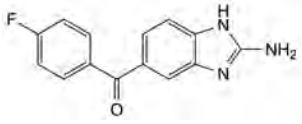

B. (2-amino-1*H*-benzimidazol-5-yl)(4-fluorophenyl)-methanone,

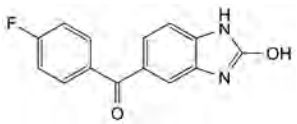

C. (4-fluorophenyl)(2-hydroxy-1*H*-benzimidazol-5-yl)methanone,

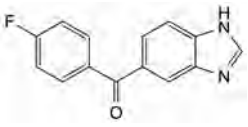

D. (1*H*-benzimidazol-5-yl)(4-fluorophenyl)methanone,

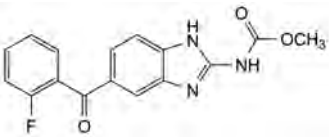

E. methyl [5-(2-fluorobenzoyl)-1*H*-benzimidazol-2-yl]carbamate,

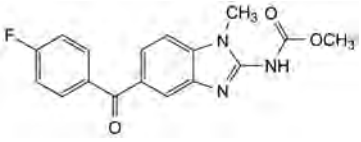

F. methyl [5-(4-fluorobenzoyl)-1-methyl-1*H*-benzimidazol-2-yl]carbamate,

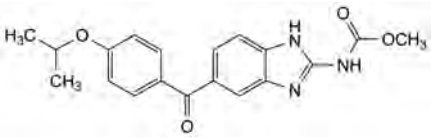

G. methyl [5-[4-(1-methylethoxy)benzoyl]-1*H*-benzimidazol-2-yl]carbamate.

**07/2008:2346**

# FLUCLOXACILLIN MAGNESIUM OCTAHYDRATE

## Flucloxacillinum magnesicum octahydricum

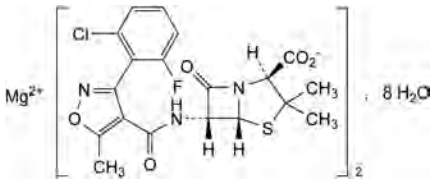

$C_{38}H_{32}Cl_2F_2MgN_6O_{10}S_2,8H_2O$ $M_r$ 1074

[58486-36-5]

DEFINITION

Magnesium bis[(2*S*,5*R*,6*R*)-6-[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate] octahydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *flucloxacillin magnesium octahydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in 5 mL of *water R*.

*Reference solution (a).* Dissolve 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R*.

*Reference solution (b).* Dissolve 25 mg of *cloxacillin sodium CRS*, 25 mg of *dicloxacillin sodium CRS* and 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R*.

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour until the spots appear.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives the reaction of magnesium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 163 to + 175 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *flucloxacillin sodium CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of test solution (b) to 50.0 mL with the mobile phase.

*Reference solution (c).* In order to prepare impurity A *in situ*, add 1 mL of *sodium carbonate solution R* to 10 mg of the substance to be examined, dilute to 25 mL with *water R* and place in an oven at 70 °C for 20 min.

*Reference solution (d).* Dilute 1 mL of reference solution (c) to 10 mL with a 27 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*.

*Reference solution (e).* In order to prepare impurity B *in situ*, add 5 mL of *dilute hydrochloric acid R* to 10 mL of reference solution (c), dilute to 25 mL with *water R* and place in an oven at 70 °C for 1 h. Dilute 1 mL of this solution to 5 mL with a 27 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 7.0 with *phosphoric acid R*.

*Reference solution (f).* Dilute 2 mL of reference solution (a) to 10 mL with reference solution (e).

*Reference solution (g).* Dissolve 1.5 mg of *flucloxacillin impurity C CRS* in 1 mL of the mobile phase and dilute to 50 mL with the mobile phase.

*Reference solution (h).* Dissolve 1 mg of *flucloxacillin impurity D CRS* in 100 mL of the mobile phase.

*Reference solution (i).* Dissolve 1 mg of *flucloxacillin impurity E CRS* in 100 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 25 volumes of *acetonitrile R1* and 75 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.0 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (d), (e), (f), (g), (h) and (i).

*Run time*: 7 times the retention time of flucloxacillin.

*Identification of impurities*: use the chromatograms obtained with reference solutions (d), (e), (g), (h) and (i) to identify the peaks due to impurities A, B, C, D and E respectively.

*Relative retention* with reference to flucloxacillin (retention time = about 8 min): impurity C = about 0.2; impurity A (isomer 1) = about 0.3; impurity A (isomer 2) = about 0.5; impurity D = about 0.6; impurity B (isomer 1) = about 0.8; impurity B (isomer 2) = about 0.9; impurity E = about 6.

*System suitability*: reference solution (f):

– *resolution*: minimum 2.0 between the 2nd peak due to impurity B (isomer 2) and the peak due to flucloxacillin.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 3.3;

– *impurity A* (sum of the 2 isomers): the sum of the areas of the 2 peaks is not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *impurity B* (sum of the 2 isomers): the sum of the areas of the 2 peaks is not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurities D, E*: for each impurity, not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *any other impurity*: for each impurity, not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): 12.0 per cent to 15.0 per cent, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{38}H_{32}Cl_2F_2MgN_6O_{10}S_2$ from the declared content of *flucloxacillin sodium CRS*, multiplying by 0.9773.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

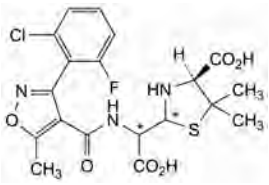

A. (4*S*)-2-[carboxy[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of flucloxacillin),

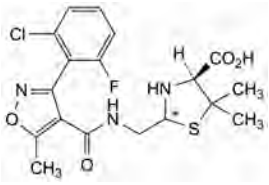

B. (2*RS*,4*S*)-2-[[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of flucloxacillin),

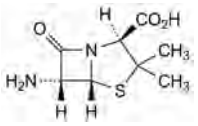

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

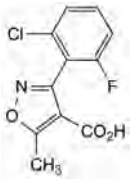

D. 3-(2-chloro-6-fluorophenyl)-5-methylisoxazole-4-carboxylic acid,

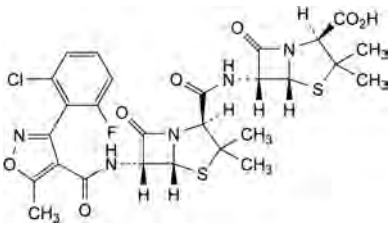

E. (2*S*,5*R*,6*R*)-6-[[[(2*S*,5*R*,6*R*)-6-[[[3-(2-chloro-6-fluorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA flucloxacillin amide).

**01/2008:0668**
**corrected 10.0**

# FLUCLOXACILLIN SODIUM

## Flucloxacillinum natricum

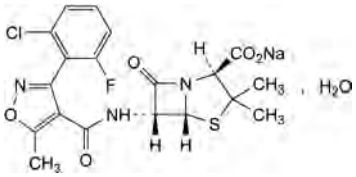

$C_{19}H_{16}ClFN_3NaO_5S,H_2O$ $M_r$ 493.9

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[[3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flucloxacillin sodium CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *water R.*

*Reference solution (a).* Dissolve 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R.*

*Reference solution (b).* Dissolve 25 mg of *cloxacillin sodium CRS*, 25 mg of *dicloxacillin sodium CRS* and 25 mg of *flucloxacillin sodium CRS* in 5 mL of *water R.*

*Plate*: *TLC silanised silica gel plate R.*

*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R.*

*Application*: 1 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R.* Mix the contents of the tube by swirling; the colour of the solution is slightly greenish-yellow. Place the test-tube in a water-bath for 1 min; the solution becomes yellow.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.04.

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 158 to + 168 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *flucloxacillin sodium CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *flucloxacillin sodium CRS* and 5 mg of *cloxacillin sodium CRS* in the mobile phase, then dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R1* and 75 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 6 times the retention time of flucloxacillin.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to cloxacillin (1st peak) and flucloxacillin (2nd peak).

*Limits*:

- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent *m/m*.

**Water** (*2.5.12*): 3.0 per cent to 4.5 per cent, determined on 0.300 g.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test. Inject per kilogram of the rabbit's mass 1 mL of a solution in *water for injections R* containing 20 mg of the substance to be examined per millilitre.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{19}H_{16}ClFN_3NaO_5S$ from the declared content of *flucloxacillin sodium CRS*.

STORAGE

In an airtight container, at a temperature not exceeding 25 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

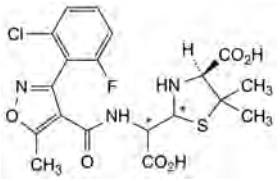

A. (4*S*)-2-[carboxy[[[3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of flucloxacillin),

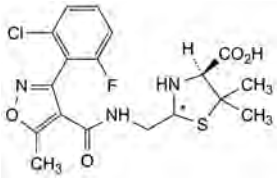

B. (2*RS*,4*S*)-2-[[[[3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penilloic acids of flucloxacillin),

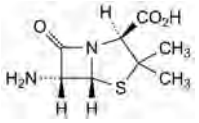

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

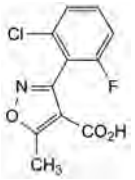

D. 3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazole-4-carboxylic acid,

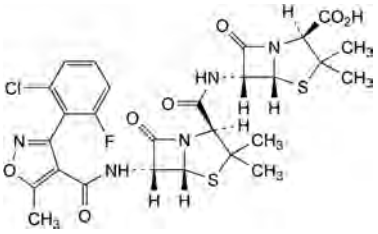

E. (2*S*,5*R*,6*R*)-6-[[[(2*S*,5*R*,6*R*)-6-[[[3-(2-chloro-6-fluorophenyl)-5-methyl-1,2-oxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

**01/2017:2287**

# FLUCONAZOLE

## Fluconazolum

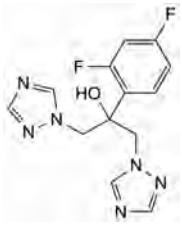

$C_{13}H_{12}F_2N_6O$ $M_r$ 306.3
[86386-73-4]

DEFINITION

2-(2,4-Difluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, soluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluconazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase, sonicate if necessary, and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *fluconazole for peak identification CRS* (containing impurity A) in the mobile phase, sonicate if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (c)*. Dissolve 3.0 mg of *fluconazole impurity B CRS* in the mobile phase, sonicate if necessary, and dilute to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 3.0 mg of *fluconazole impurity C CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (e)*. To 1.0 mL of reference solution (d) add 1.0 mL of the test solution and dilute to 10.0 mL with the mobile phase.

*Reference solution (f)*. Dilute 1.0 mL of reference solution (d) to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, 0.63 g/L solution of *ammonium formate R* (14:86 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b), (c), (e) and (f).

*Run time*: 3.5 times the retention time of fluconazole.

*Identification of impurities*: use the chromatogram supplied with *fluconazole for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B and the chromatogram obtained with reference solution (f) to identify the peak due to impurity C.

*Relative retention* with reference to fluconazole (retention time = about 11 min): impurity B = about 0.4; impurity A = about 0.5; impurity C = about 0.8.

*System suitability*: reference solution (e):
- *resolution*: minimum 3.0 between the peaks due to impurity C and fluconazole.

*Limits*:
- *impurity A*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 0.6 per cent;
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.125 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 15.32 mg of $C_{13}H_{12}F_2N_6O$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G, H, I.*

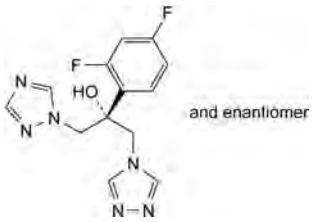


A. (2*RS*)-2-(2,4-difluorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)-3-(4*H*-1,2,4-triazol-4-yl)propan-2-ol,

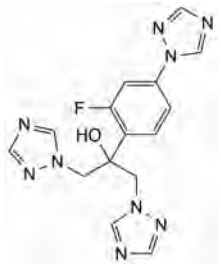

B. 2-[2-fluoro-4-(1*H*-1,2,4-triazol-1-yl)phenyl]-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol,

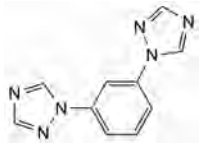

C. 1,1′-(1,3-phenylene)di-1*H*-1,2,4-triazole,

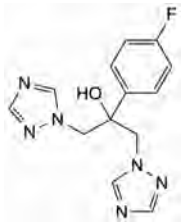

D. 2-(4-fluorophenyl)-1,3-bis(1*H*-1,2,4-triazol-1-yl)propan-2-ol,

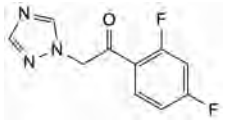

E. 1-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanone,

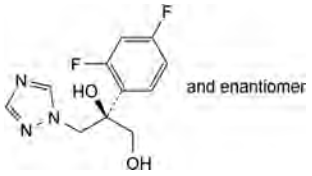


F. (2*RS*)-2-(2,4-difluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propane-1,2-diol,

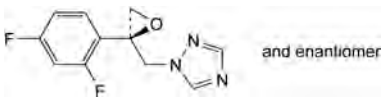


G. 1-[[(2*RS*)-2-(2,4-difluorophenyl)oxiran-2-yl]methyl]-1*H*-1,2,4-triazole,

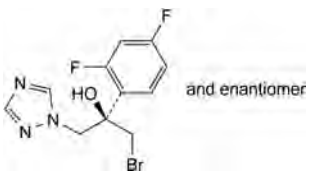


H. (2*RS*)-1-bromo-2-(2,4-difluorophenyl)-3-(1*H*-1,2,4-triazol-1-yl)propan-2-ol,

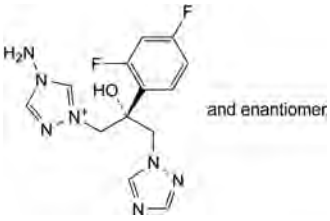


I. 4-amino-1-[(2*RS*)-2-(2,4-difluorophenyl)-2-hydroxy-3(1*H*-1,2,4-triazol-1-yl)propyl]-4*H*-1,2,4-triazolium.

**01/2017:0766**

# FLUCYTOSINE

## Flucytosinum

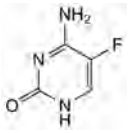

$C_4H_4FN_3O$ $M_r$ 129.1
[2022-85-7]

DEFINITION

4-Amino-5-fluoropyrimidin-2(1*H*)-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flucytosine CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (10:15 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution.* Dissolve 10 mg of *flucytosine CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Plate*: *TLC silica gel* $F_{254}$ *plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *ethyl acetate R* (1:15:25:60 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate in an unsaturated tank with the mobile phase. Then allow the solvents to evaporate.

*Detection*: at the bottom of a chromatography tank place an evaporating dish containing a mixture of 1 volume of *hydrochloric acid R1*, 1 volume of *water R* and 2 volumes of a 15 g/L solution of *potassium permanganate R*. Close the tank and allow to stand for 15 min. Place the dried plate in the tank and close the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of the coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Spray with *potassium iodide and starch solution R*. Examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the solution changes from red to yellow.

D. To 5 mL of solution S (see Tests) add 0.15 mL of *bromine water R* and shake. The colour of the solution is discharged.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ or $Y_7$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Dissolve 13.6 g of *potassium dihydrogen phosphate R* in 950 mL of *water R*. Add 50 mL of *methanol R*. Mix thoroughly.

*Test solution*. Dissolve 15.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Mix well. Sonicate for 5 min. Mix thoroughly. Sonicate the solution for 5 min. Mix thoroughly.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 15.0 mg of *fluorouracil CRS* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Mix well. Sonicate for 5 min. Mix thoroughly. Sonicate the solution for 5 min. Mix thoroughly. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *flucytosine for system suitability CRS* (containing impurity B) in 0.5 mL of the solvent mixture and add 0.5 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 13.6 g of *potassium dihydrogen phosphate R* in 950 mL of *water R*. Filter through a membrane filter (nominal pore size 0.45 µm). Adjust to pH 2.0 by adding *phosphoric acid R* and add 50 mL of *methanol R*. Mix thoroughly.

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Run time*: 15 times the retention time of flucytosine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to flucytosine (retention time = about 2 min): impurity A = about 1.7; impurity B = about 13.3.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to flucytosine and impurity A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 50 for the peak due to impurity B in the chromatogram obtained with reference solution (c);
- *symmetry factor*: maximum 2.0 for the peak due to flucytosine in the chromatogram obtained with reference solution (a).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.6;
- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *impurity B*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Fluorides**: maximum 200 ppm.

Potentiometry (*2.2.36, Method I*). *Prepare and store all solutions in plastic containers.*

*Buffer solution*. Dissolve 58 g of *sodium chloride R* in 500 mL of *water R*. Add 57 mL of *glacial acetic acid R* and 200 mL of a 100 g/L solution of *cyclohexylenedinitrilotetra-acetic acid R* in *1 M sodium hydroxide*. Adjust the pH to 5.0-5.5 with a 200 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R*.

*Test solution*. Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Dissolve 4.42 g of *sodium fluoride R*, previously dried at 120 °C for 2 h, in 300 mL of *water R* and dilute to 1000.0 mL with the same solvent (solution (a): 1.9 g/L of fluoride). Prepare 3 reference solutions by dilution of solution (a) 1 in 100, 1 in 1000 and 1 in 10 000 respectively.

*Indicator electrode*: fluoride selective.

*Reference electrode*: silver-silver chloride.

To 20.0 mL of the test solution and each reference solution, add 10.0 mL of the buffer solution and stir with a magnetic stirrer. Introduce the electrodes into the solution and allow to stand for 5 min with constant stirring.

Calculate the concentration of fluorides using the calibration curve.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.100 g in 40 mL of *anhydrous acetic acid R* and add 100 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 12.91 mg of $C_4H_4FN_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

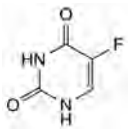

A. 5-fluoropyrimidine-2,4(1*H*,3*H*)-dione (fluorouracil),

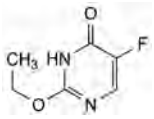

B. 2-ethoxy-5-fluoropyrimidin-4(3*H*)-one.

**01/2017:1781**

# FLUDARABINE PHOSPHATE

## Fludarabini phosphas

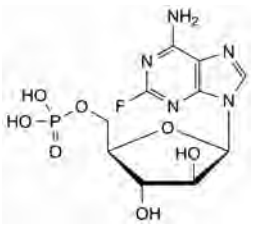

$C_{10}H_{13}FN_5O_7P$ $M_r$ 365.2
[75607-67-9]

DEFINITION

2-Fluoro-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in dimethylformamide, very slightly soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fludarabine phosphate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 50 mg in 5.0 mL of *dimethylformamide R* with the aid of ultrasound.

**Specific optical rotation** (*2.2.7*): + 10.0 to + 14.0 (anhydrous substance).

Dissolve 0.100 g in *water R* with the aid of ultrasound and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 20 mg of the substance to be examined in 50 mL of *water R* with the aid of ultrasound and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *fludarabine phosphate CRS* in 50 mL of *water R* with the aid of ultrasound and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of the substance to be examined in 20 mL of *0.1 M hydrochloric acid* with the aid of ultrasound. Heat in a water-bath at 80 °C for 15 min, cool to room temperature, mix and dilute to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

*Reference solution (d).* Dissolve 5 mg of *fludarabine for system suitability CRS* (containing impurities D, E and F) in 10 mL of *water R* with the aid of ultrasound and dilute to 25.0 mL with the same solvent.

*Blank solution*: *0.02 M hydrochloric acid*.

A. Early eluting impurities.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 60 volumes of *methanol R* and 940 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm and at 292 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b) and (c).

*Run time*: 4.5 times the retention time of the principal peak in the chromatogram obtained with the test solution.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) at 292 nm to identify the peaks due to impurities A and B, the response at 292 nm being much higher than at 260 nm; use the chromatogram supplied with *fludarabine phosphate CRS* and the chromatogram obtained with reference solution (a) at 260 nm to identify impurity C.

*Relative retention* with reference to fludarabine phosphate (retention time = about 9 min): impurity A = about 0.26; impurity B = about 0.34; impurity C = about 0.42.

*System suitability*: reference solution (b) at 292 nm:

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*: at 260 nm:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 4.0; impurity B = 2.5; impurity C = 1.9;
- *impurity A*: maximum 0.8 per cent;
- *impurity C*: maximum 0.4 per cent;
- *impurity B*: maximum 0.2 per cent;
- *unspecified impurities eluting before fludarabine phosphate*: for each impurity, maximum 0.10 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak eluting after fludarabine phosphate.

B. Late eluting impurities.

Conditions as described under Test A with the following modifications.

*Mobile phase*: mix 200 volumes of *methanol R* and 800 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R*.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL of the test solution and reference solutions (c) and (d).

*Run time*: 8 times the retention time of the principal peak in the chromatogram obtained with the test solution.

*Identification of impurities*: use the chromatogram supplied with *fludarabine for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities D, E and F.

*Relative retention* with reference to fludarabine phosphate (retention time = about 2.5 min): impurity D = about 1.5; impurity E = about 1.9; impurity F = about 2.5.

*System suitability*: reference solution (d):

– *resolution*: minimum 5.0 between the peaks due to fludarabine phosphate and impurity D.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.5; impurity E = 0.6; impurity F = 1.8;
– *impurity E*: maximum 0.2 per cent;
– *impurity F*: maximum 0.2 per cent;
– *impurity D*: maximum 0.15 per cent;
– *unspecified impurities eluting after fludarabine phosphate*: for each impurity, maximum 0.10 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak eluting before fludarabine phosphate.

*Total of impurities eluting before fludarabine phosphate in test A, apart from impurities A, B and C, and after fludarabine phosphate in test B, apart from impurities D, E and F*: maximum 0.5 per cent.

*Total of all impurities eluting before fludarabine phosphate in test A and after fludarabine phosphate in test B*: maximum 2.0 per cent.

**Ethanol** (*2.4.24, System A*): maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.200 g (ground to a very fine powder). Stir the substance in 15 mL of *anhydrous methanol R* for about 15 s before titrating.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Test solution*. Dissolve 24.0 mg of the substance to be examined in 50 mL of *water R* with the aid of ultrasound and dilute to 100.0 mL with the same solvent. Dilute 25.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution*. Dissolve 24.0 mg of *fludarabine phosphate CRS* in 50 mL of *water R* with the aid of ultrasound and dilute to 100.0 mL with the same solvent. Dilute 25.0 mL of the solution to 100.0 mL with the mobile phase.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

Calculate the percentage content of $C_{10}H_{13}FN_5O_7P$ taking into account the assigned content of *fludarabine phosphate CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, J.*

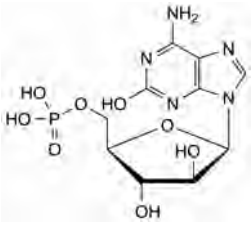

A. 6-amino-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-2-ol,

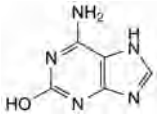

B. 6-amino-7*H*-purin-2-ol,

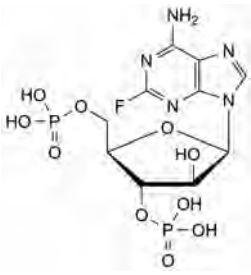

C. 9-(3,5-di-*O*-phosphono-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

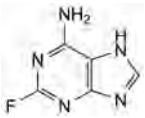

D. 2-fluoro-7*H*-purin-6-amine,

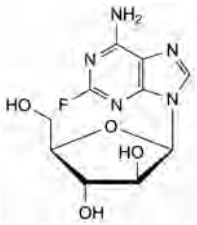

E. 9-β-D-arabinofuranosyl-2-fluoro-9*H*-purin-6-amine,

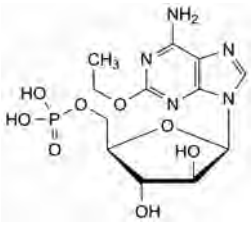

F. 2-ethoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine,

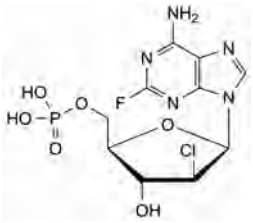

G. 9-(2-chloro-2-deoxy-5-*O*-phosphono-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

H. 9-(2,5-anhydro-β-D-arabinofuranosyl)-2-fluoro-9*H*-purin-6-amine,

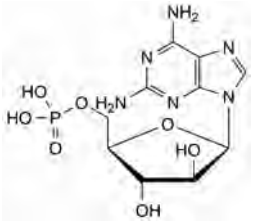

I. 9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purine-2,6-diamine,

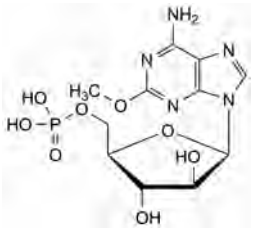

J. 2-methoxy-9-(5-*O*-phosphono-β-D-arabinofuranosyl)-9*H*-purin-6-amine.

**01/2008:0767**
**corrected 8.0**

# FLUDROCORTISONE ACETATE

## Fludrocortisoni acetas

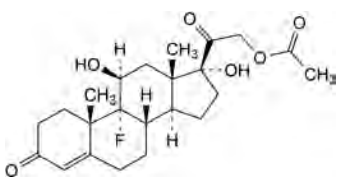

$C_{23}H_{31}FO_6$ $M_r$ 422.5
[514-36-3]

DEFINITION

9-Fluoro-11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fludrocortisone acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *fludrocortisone acetate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *cortisone acetate CRS* in 5 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent (solution A). Dilute 2 mL of the solution to 10 mL with *methylene chloride R*.

*Test solution (b).* Transfer 2 mL of solution A to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat on a water-bath at 45 °C protected from light for 2.5 h. Allow to cool.

*Reference solution (a).* Dissolve 25 mg of *fludrocortisone acetate CRS* in *methanol R* and dilute to 5 mL with the same solvent (solution B). Dilute 2 mL of the solution to 10 mL with *methylene chloride R*.

*Reference solution (b).* Transfer 2 mL of solution B to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and

immediately pass a stream of *nitrogen R* through the solution for 5 min. Stopper the tube. Heat on a water bath at 45 °C protected from light for 2.5 h. Allow to cool.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have $R_F$ values distinctly lower than those of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The colour of the solution to be examined changes from red to yellow.

E. About 10 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 148 to + 156 (dried substance).

Dissolve 0.250 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *fludrocortisone acetate CRS* and 2.0 mg of *hydrocortisone acetate CRS* in the mobile phase, then dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.2 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *tetrahydrofuran R*, *water R* (35:65 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL.

*Run time*: twice the retention time of fludrocortisone acetate.

*Retention time*: hydrocortisone acetate = about 8.5 min; fludrocortisone acetate = about 10 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.0 between the peaks due to hydrocortisone acetate and fludrocortisone acetate; if necessary, adjust slightly the concentration of tetrahydrofuran in the mobile phase (an increase in the concentration of tetrahydrofuran reduces the retention times).

*Limits*:

- *any impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *total*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 10.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 238 nm.

Calculate the content of $C_{23}H_{31}FO_6$ taking the specific absorbance to be 405.

**01/2008:1326**
**corrected 6.0**

# FLUMAZENIL

## Flumazenilum

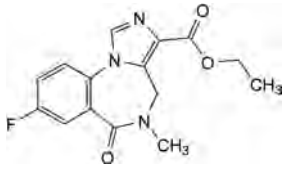

$C_{15}H_{14}FN_3O_3$ $M_r$ 303.3
[78755-81-4]

DEFINITION

Ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine-3-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in methanol.

mp: 198 °C to 202 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of flumazenil.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and is not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 0.10 g in *methanol R* and dilute to 10 mL with the same solvent.

**Impurity C**: maximum 1 per cent.

Dissolve 0.10 g in 0.5 mL of *methylene chloride R* and dilute to 10 mL with *butanol R*. To 5.0 mL of this solution add 2.0 mL of *ninhydrin solution R* and heat in a water-bath at 95 °C for 15 min. Any blue-purple colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 5.0 mL of a 0.1 g/L solution of *dimethylformamide diethylacetal R* in *butanol R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *flumazenil impurity B CRS* and 2.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 10.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: to 800 mL of *water R* adjusted to pH 2.0 with *phosphoric acid R*, add 130 mL of *methanol R* and 70 mL of *tetrahydrofuran R* and mix.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of flumazenil.

*Relative retention* with reference to flumazenil (retention time = about 14 min): impurity A = about 0.4; impurity D = about 0.5; impurity E = about 0.6; impurity B = about 0.7; impurity F = about 2.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity B and flumazenil.

*Limits*:

– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent),

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.250 g in 50 mL of a mixture of 2 volumes of *acetic anhydride R* and 3 volumes of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.33 mg of $C_{15}H_{14}FN_3O_3$.

IMPURITIES

*Specified impurities*: *B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F.*

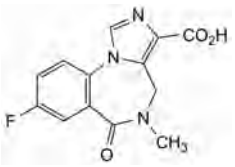

A. 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid,

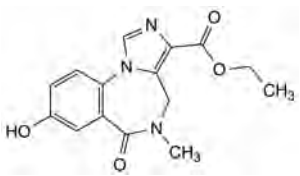

B. ethyl 8-hydroxy-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,

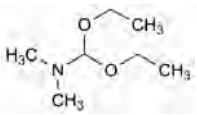

C. diethoxy-*N*,*N*-dimethylmethanamine,

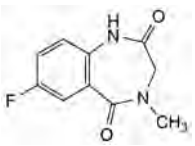

D. 7-fluoro-4-methyl-3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione,

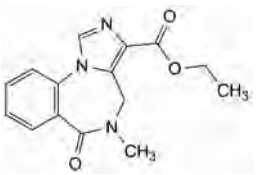

E. ethyl 5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate,

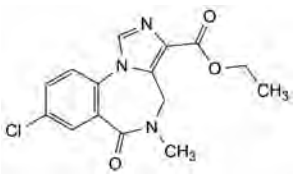

F. ethyl 8-chloro-5-methyl-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate.

**01/2017:1517**

# FLUMEQUINE

## Flumequinum

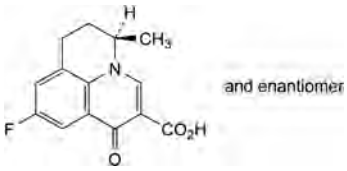


$C_{14}H_{12}FNO_3$ $M_r$ 261.3
[42835-25-6]

DEFINITION

(*RS*)-9-Fluoro-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo[*i,j*]-quinolizine-2-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, microcrystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble in methanol. It is freely soluble in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *flumequine CRS.*

B. Optical rotation (see Tests).

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 5 mg of the substance to be examined in 10 mL of *methylene chloride R.*
*Reference solution.* Dissolve 5 mg of *flumequine CRS* in 10 mL of *methylene chloride R.*
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *ammonia R, water R, ethanol (96 per cent) R* (10:10:90 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 2 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution changes from red to yellow and the blank remains red.

TESTS

**Solution S**. Dissolve 5.00 g in *0.5 M sodium hydroxide* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 35.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *flumequine impurity B CRS* in 2.0 mL of a 50 µg/mL solution of *flumequine CRS* in *dimethylformamide R.*

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with *dimethylformamide R.*

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 1.36 g/L solution of *potassium dihydrogen phosphate R* (49:51 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 313 nm.

*Injection*: 10 µL; inject *dimethylformamide R* as a blank.

*Run time*: 3 times the retention time of flumequine.

*Relative retention* with reference to flumequine (retention time = about 13 min): impurity A = about 0.67; impurity B = about 0.85.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurity B and flumequine.

*Limits*:
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.500 g in 50 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 26.13 mg of $C_{14}H_{12}FNO_3$.

IMPURITIES

*Specified impurities: A, B.*

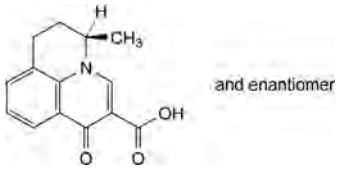


A. (*RS*)-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo-[*i,j*]quinolizine-2-carboxylic acid (defluoroflumequine),

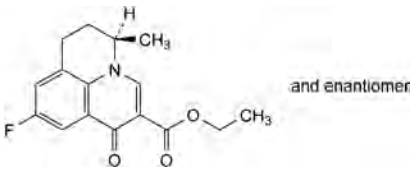


B. ethyl (*RS*)-9-fluoro-5-methyl-1-oxo-6,7-dihydro-1*H*,5*H*-benzo[*i,j*]quinolizine-2-carboxylate (flumequine ethyl ester).

**01/2008:1327**
**corrected 6.0**

# FLUMETASONE PIVALATE

## Flumetasoni pivalas

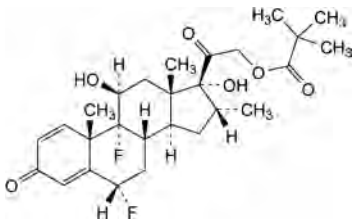


$C_{27}H_{36}F_2O_6$ $M_r$ 494.6
[2002-29-1]

DEFINITION

6α,9-Difluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flumetasone pivalate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *flumetasone pivalate CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *desoxycortone acetate CRS* in *acetone R* and dilute to 10 mL with the same solvent. Dilute 5 mL of this solution to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

C. Add about 2 mg to 2 mL of a mixture of 0.5 mL of *water R* and 1.5 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a pink colour develops. Add this solution to 10 mL of *water R* and mix. The colour fades and a clear solution remains.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R* add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Solution S**. Dissolve 0.50 g in *acetone R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 69 to + 77 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10 mg of *dexamethasone pivalate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. To 5.0 mL of this solution, add 5.0 mL of the test solution, mix and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *tetrahydrofuran R, acetonitrile R, water R, methanol R* (5:30:30:35 *V/V/V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of flumetasone pivalate.

*Relative retention* with reference to flumetasone pivalate: impurity C = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.8 between the peaks due to flumetasone pivalate and impurity C; if necessary, adjust the concentration of tetrahydrofuran in the mobile phase.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent);

– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 4 h.

ASSAY

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 239 nm.

Calculate the content of $C_{27}H_{36}F_2O_6$ taking the specific absorbance to be 336.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D*.

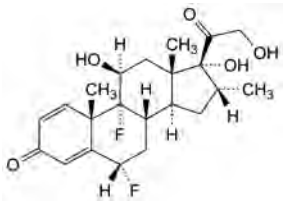

A. 6α,9-difluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione (flumetasone),

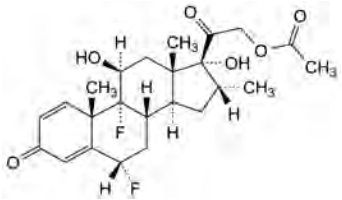

B. 6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (flumetasone acetate),

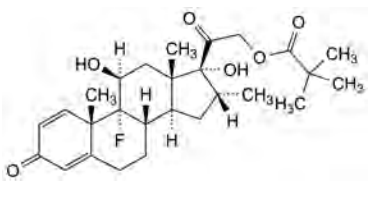

C. 9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate (dexamethasone pivalate),

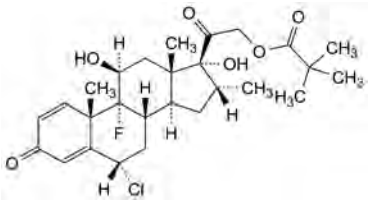

D. 6α-chloro-9-fluoro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate (chlordexamethasone pivalate).

**01/2008:1722**
**corrected 7.0**

# FLUNARIZINE DIHYDROCHLORIDE

## Flunarizini dihydrochloridum

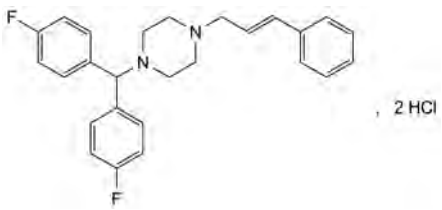

$C_{26}H_{28}Cl_2F_2N_2$ $M_r$ 477.4
[30484-77-6]

DEFINITION

1-[Bis(4-fluorophenyl)methyl]-4-[(2*E*)-3-phenylprop-2-enyl]piperazine dihydrochloride.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: slightly soluble in water, sparingly soluble in methanol, slightly soluble in alcohol and in methylene chloride.

mp: about 208 °C, with decomposition.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of flunarizine dihydrochloride.*

B. Dissolve 25 mg in 2 mL of *methanol R* and add 0.5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *flunarizine dihydrochloride for system suitability CRS* in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 20.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm,

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: solution containing 23.8 g/L of *tetrabutylammonium hydrogen sulfate R* and 7 g/L of *ammonium acetate R*,

– *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 80 → 40 | 20 → 60 |
| 12 - 15 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to flunarizine,

– the chromatogram obtained is concordant with the chromatogram supplied with *flunarizine dihydrochloride for system suitability CRS*.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.5,

– *impurities A, D*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),

– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent),

– *any other impurity*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in 70 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added at the second point of inflexion. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 23.87 mg of $C_{26}H_{28}Cl_2F_2N_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

A. 1-[bis(4-fluorophenyl)methyl]piperazine,

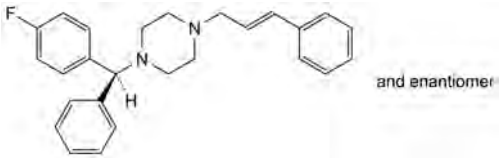


B. 1-[(*RS*)-(4-fluorophenyl)phenylmethyl]-4-[(2*E*)-3-phenylprop-2-enyl]piperazine,

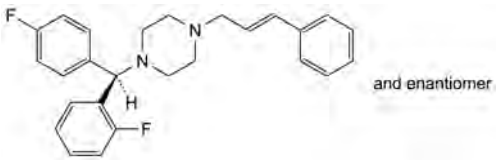


C. 1-[(*RS*)-(2-fluorophenyl)(4-fluorophenyl)methyl]-4-[(2*E*)-3-phenylprop-2-enyl]piperazine,

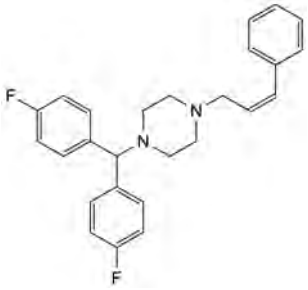

D. 1-[bis(4-fluorophenyl)methyl]-4-[(2*Z*)-3-phenylprop-2-enyl]piperazine.

**01/2008:0717**
**corrected 6.0**

# FLUNITRAZEPAM

## Flunitrazepamum

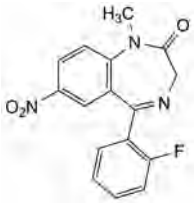

$C_{16}H_{12}FN_3O_3$ $M_r$ 313.3
[1622-62-4]

DEFINITION

5-(2-Fluorophenyl)-1-methyl-7-nitro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, slightly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of flunitrazepam*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use*.

*Test solution*. Dissolve 100.0 mg of the substance to be examined in 10 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 4 mg of the substance to be examined and 4 mg of *nitrazepam R* in 5 mL of *acetonitrile R* and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *acetonitrile R*, *water R* (50:305:645 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of flunitrazepam.

*Relative retention* with reference to flunitrazepam (retention time = about 11 min): impurity A = about 0.2; impurity B = about 0.6; impurity C = about 2.3; impurity D = about 4.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to nitrazepam and flunitrazepam.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2.44,

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 20 mL of *anhydrous acetic acid R* and add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.33 mg of $C_{16}H_{12}FN_3O_3$.

STORAGE

Protected from light.

IMPURITIES

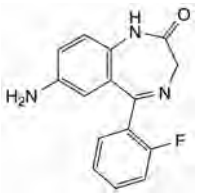

A. 7-amino-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (7-aminodemethylflunitrazepam),

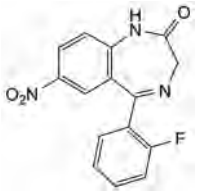

B. 5-(2-fluorophenyl)-7-nitro-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (demethylflunitrazepam),

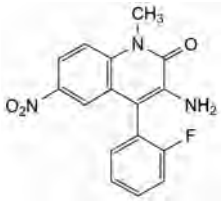

C. 3-amino-4-(2-fluorophenyl)-1-methyl-6-nitroquinolin-2(1*H*)-one,

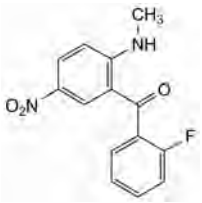

D. (2-fluorophenyl)[2-(methylamino)-5-nitrophenyl]-methanone.

**01/2008:1696**
**corrected 6.0**

# FLUNIXIN MEGLUMINE FOR VETERINARY USE

## Flunixini megluminum ad usum veterinarium

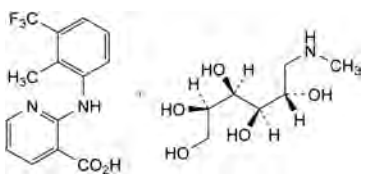

$C_{21}H_{28}F_3N_3O_7$ $M_r$ 491.5
[42461-84-7]

DEFINITION

2-[[2-Methyl-3-(trifluoromethyl)phenyl]amino]pyridine-3-carboxylic acid, 1-deoxy-1-(methylamino)-D-glucitol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): − 12.0 to − 9.0 (dried substance), determined on solution S (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *flunixin meglumine CRS*.

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.0 to 9.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *flunixin impurity B CRS* in 1.0 mL of the test solution and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *2-chloronicotinic acid R* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase. To 2.0 mL of this solution add 2.0 mL of reference solution (a) and dilute to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 50 mg of *flunixin impurity C CRS* in the mobile phase and dilute to 100 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 300 volumes of *water R* and 700 volumes of *acetonitrile R*, and add 0.25 volumes of *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of flunixin.

*Relative retention* with reference to flunixin (retention time = about 3.1 min): impurity A = about 0.4; impurity C = about 0.6; impurity B = about 0.7; impurity D = about 4.2.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.5 between the peaks due to impurity B and flunixin.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.9,
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *impurities C, D*: for each impurity, not more than the area of the peak due to flunixin in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *any other impurity*: for each impurity, not more than the area of the peak due to flunixin in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *total*: not more than 2.5 times the area of the peak due to flunixin in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.25 times the area of the peak due to flunixin in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.175 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.57 mg of $C_{21}H_{28}F_3N_3O_7$.

IMPURITIES

*Specified impurities: A, B, C, D.*

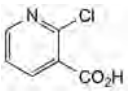

A. 2-chloropyridine-3-carboxylic acid,

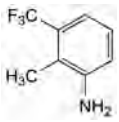

B. 2-methyl-3-(trifluoromethyl)aniline,

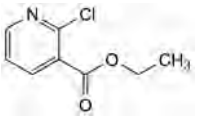

C. ethyl 2-chloropyridine-3-carboxylate,

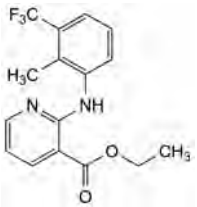

D. ethyl 2-[[2-methyl-3-(trifluoromethyl)phenyl]amino]-pyridine-3-carboxylate.

**04/2015:0494**

# FLUOCINOLONE ACETONIDE

## Fluocinoloni acetonidum

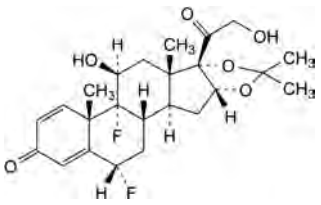

$C_{24}H_{30}F_2O_6$ $M_r$ 452.5
[67-73-2]

DEFINITION

6α,9-Difluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluocinolone acetonide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**Specific optical rotation** (*2.2.7*): + 100 to + 104 (dried substance).

Dissolve 0.100 g in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (20:80 *V/V*).

*Buffer solution*. Dissolve 5.68 g of *anhydrous disodium hydrogen phosphate R* and 3.63 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in 20 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *fluocinolone acetonide for system suitability CRS* (containing impurities H, J and K) in 2 mL of *acetonitrile R* and dilute to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 2 mg of *fluocinolone acetonide for peak identification CRS* (containing impurities D and I) in 2 mL of *acetonitrile R* and dilute to 10.0 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 20.0 mg of *fluocinolone acetonide CRS* in 20 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, buffer solution (28:72 *V/V*);
- *mobile phase B*: *water R*, *acetonitrile R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 42 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 40 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *fluocinolone acetonide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities H, J and K; use the chromatogram supplied with *fluocinolone acetonide for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities D and I.

*Relative retention* with reference to fluocinolone acetonide (retention time = about 18 min): impurity D = about 0.8; impurity H = about 0.90; impurity I = about 0.94; impurity J = about 1.05; impurity K = about 1.2.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to fluocinolone acetonide.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity K by 1.3;
- for each impurity, use the concentration of fluocinolone acetonide in reference solution (c).

*Limits*:

- *impurity K*: maximum 0.3 per cent;
- *impurities D, J*: for each impurity, maximum 0.2 per cent;
- *impurities H, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.7 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of test solution (b) and reference solution (d).

Calculate the percentage content of $C_{24}H_{30}F_2O_6$ taking into account the assigned content of *fluocinolone acetonide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: D, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F, G, L, M.*

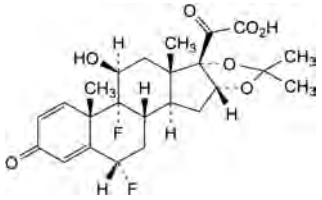

A. 6α,9-difluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-oic acid,

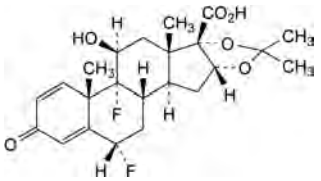

B. 6α,9-difluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3-oxoandrosta-1,4-diene-17β-carboxylic acid,

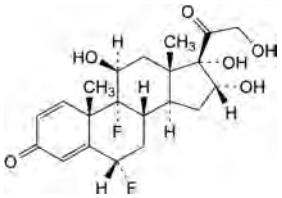

C. 6α,9-difluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (fluocinolone),

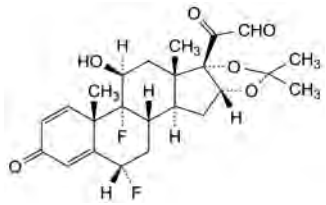

D. 6α,9-difluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-al,

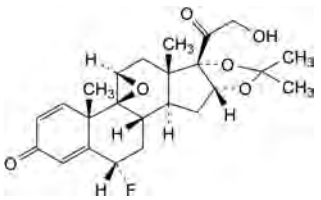

E. 9,11β-epoxy-6α-fluoro-21-hydroxy-16α,17-(1-methylethylidenedioxy)-9β-pregna-1,4-diene-3,20-dione,

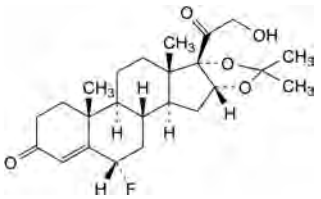

F. 6α-fluoro-21-hydroxy-16α,17-(1-methylethylidenedioxy)pregn-4-ene-3,20-dione,

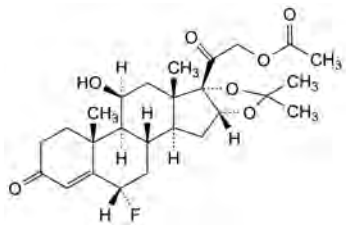

G. 6α-fluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregn-4-en-21-yl acetate,

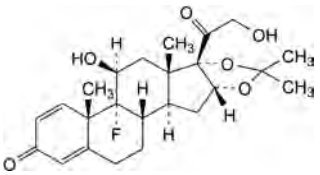

H. 9-fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione,

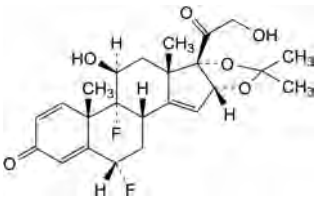

I. 6α,9-difluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4,14-triene-3,20-dione,

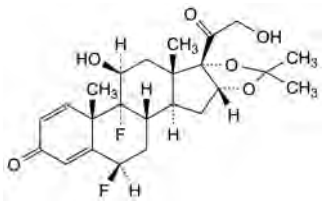

J. 6β,9-difluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione,

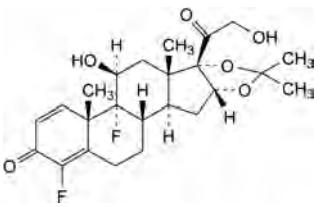

K. 4,9-difluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione,

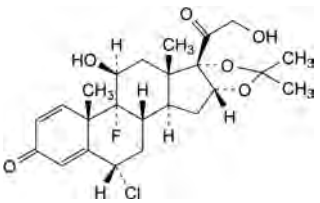

L. 6α-chloro-9-fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione,

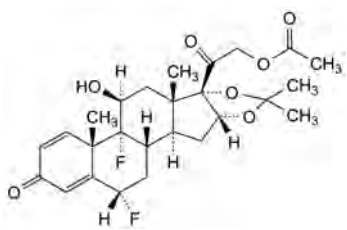

M. 6α,9-difluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-yl acetate.

**01/2008:1212**
**corrected 6.0**

# FLUOCORTOLONE PIVALATE

## Fluocortoloni pivalas

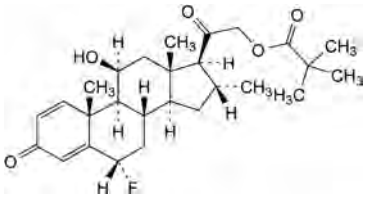

$C_{27}H_{37}FO_5$ $M_r$ 460.6
[29205-06-9]

DEFINITION

6α-Fluoro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride and in dioxan, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fluocortolone pivalate CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *methanol R, methylene chloride R* (1:9 *V/V*).
*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Reference solution (a).* Dissolve 20 mg of *fluocortolone pivalate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.
*Reference solution (b).* Dissolve 10 mg of *norethisterone CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*
*Application*: 5 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
*Detection B*: spray with *alcoholic solution of sulfuric acid R.* Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.
*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.

C. To about 1 mg add 2 mL of a mixture of 2 volumes of *glacial acetic acid R* and 3 volumes of *sulfuric acid R* and heat for 1 min on a water-bath. A red colour is produced. Add 5 mL of *water R*, the colour changes to violet-red.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Specific optical rotation** (*2.2.7*): + 100 to + 105 (dried substance).

Dissolve 0.25 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R.*

*Reference solution (b).* Dissolve 2 mg of *fluocortolone pivalate CRS* and 2 mg of *prednisolone hexanoate CRS* in *acetonitrile R*, then dilute to 100 mL with the same solvent.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *acetonitrile R*, *water R* (25:30:32 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 243 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of fluocortolone pivalate.

*System suitability*: reference solution (b):
– *resolution*: minimum 5.0 between the peaks due to fluocortolone pivalate and prednisolone hexanoate.

*Limits*:
– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2 per cent);
– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 30.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *anhydrous ethanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 242 nm.

Calculate the content of $C_{27}H_{37}FO_5$ taking the specific absorbance to be 350.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

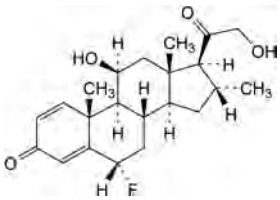

A. 6α-fluoro-11β,21-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (fluocortolone),

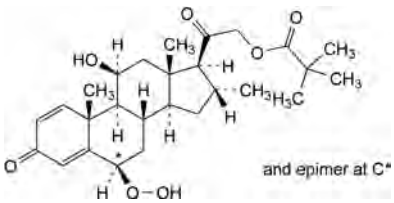


B. 6-hydroperoxy-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate,

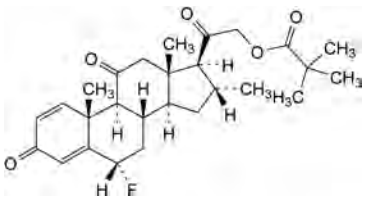

C. 6α-fluoro-16α-methyl-3,11,20-trioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate,

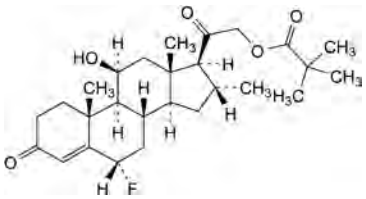

D. 6α-fluoro-11β-hydroxy-16α-methyl-3,20-dioxopregna-4-en-21-yl 2,2-dimethylpropanoate.

**07/2012:2348**

# FLUORESCEIN

## Fluoresceinum

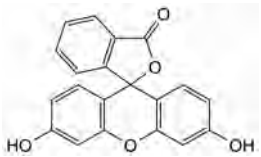

$C_{20}H_{12}O_5$ $M_r$ 332.3

[2321-07-5]

DEFINITION

3′,6′-Dihydroxy-3*H*-spiro[isobenzofuran-1,9′-xanthen]-3-one.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: orange-red, fine powder.

*Solubility*: practically insoluble in water, soluble in hot ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluorescein CRS*.

Dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness and record the spectra using the residues.

B. Dilute 0.1 mL of solution S (see Tests) to 10 mL with *water R*. The solution shows a yellowish-green fluorescence. The fluorescence disappears on addition of 0.1 mL of *dilute hydrochloric acid R* and reappears on addition of 0.2 mL of *dilute sodium hydroxide solution R*.

C. The absorption by a piece of filter paper of 0.05 mL of the solution prepared for identification B (before the addition of *dilute hydrochloric acid R*) colours the paper yellow. On exposing the moist paper to bromine vapour for 1 min and then to ammonia vapour, the colour becomes deep pink.

D. Suspend 0.5 g in 50 mL of *water R* and shake for 10 min. The substance does not completely dissolve.

TESTS

**Solution S**. Suspend 1.0 g in 35.0 mL of *water R* and add dropwise with shaking 4.5 mL of *1 M sodium hydroxide*. Adjust to pH 8.5-9.0 with *1 M sodium hydroxide* and dilute to 50.0 mL with *water R* to obtain a clear solution.

**Appearance of solution**. Solution S is clear (*2.2.1*) and orange-yellow with yellowish-green fluorescence.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile for chromatography R*, mobile phase A (30:70 *V/V*).

*Test solution (a).* Disperse 50.0 mg of the substance to be examined in 15.0 mL of *ethanol (96 per cent) R*. Sonicate and dilute to 50.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 250.0 mL with the solvent mixture.

*Reference solution (a).* Disperse 50.0 mg of *fluorescein CRS* in 15.0 mL of *ethanol (96 per cent) R*. Sonicate and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 250.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10.0 mg of *phthalic acid CRS* (impurity B) and 10.0 mg of *resorcinol CRS* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 5.0 mL of test solution (b) to 20.0 mL with the solvent mixture.

*Reference solution (d).* Dilute 10.0 mL of reference solution (c) to 100.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve the contents of a vial of *fluorescein impurity C CRS* in 1 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R3* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.610 g of *potassium dihydrogen phosphate R* in *water for chromatography R*, adjust to pH 2.0 with *phosphoric acid R* and dilute to 1000.0 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 85 → 20 | 15 → 80 |
| 20 - 29 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c), (d) and (e).

*Identification of impurity C*: use the chromatogram obtained with reference solution (e) to identify the peak due to impurity C.

*Relative retention* with reference to fluorescein (retention time = about 15 min): impurity A = about 0.42; impurity B = about 0.48; impurity C = about 0.86.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.9;
– *impurity C*: not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent);
– *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *sum of impurities other than A, B and C*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 0.25 per cent.

To 10.0 mL of solution S add 90.0 mL of *water R* and 3.0 mL of *dilute nitric acid R*, wait for at least 30 min and filter. Dilute 10.0 mL of the filtrate to 15.0 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{20}H_{12}O_5$ taking into account the assigned content of *fluorescein CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

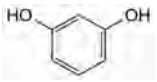


A. benzene-1,3-diol (resorcinol),

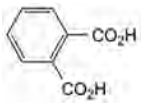


B. benzene-1,2-dicarboxylic acid (phthalic acid),

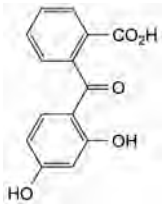


C. 2-(2,4-dihydroxybenzoyl)benzoic acid.

**01/2008:1213**
**corrected 6.0**

# FLUORESCEIN SODIUM

## Fluoresceinum natricum

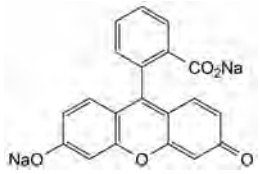


$C_{20}H_{10}Na_2O_5$ $M_r$ 376.3
[518-47-8]

DEFINITION

Disodium 2-(6-oxido-3-oxo-3*H*-xanthen-9-yl)benzoate.

*Content*: 95.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: orange-red, fine powder, hygroscopic.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), practically insoluble in hexane and in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Dilute 0.1 mL of solution S (see Tests) to 10 mL with *water R*. The solution shows yellowish-green fluorescence. The fluorescence disappears on addition of 0.1 mL of *dilute hydrochloric acid R* and reappears on addition of 0.2 mL of *dilute sodium hydroxide solution R*.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *Ph. Eur. reference spectrum of fluorescein sodium*.

C. The absorption by a piece of filter paper of 0.05 mL of the solution prepared for identification A (before the addition of *dilute hydrochloric acid R*) colours the paper yellow. On exposing the moist paper to bromine vapour for 1 min and then to ammonia vapour, the colour becomes deep pink.

D. Ignite 0.1 g in a porcelain crucible. Dissolve the residue in 5 mL of *water R* and filter. 2 mL of the filtrate gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and orange-yellow with yellowish-green fluorescence.

**pH** (*2.2.3*): 7.0 to 9.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A and dilute to 100.0 mL with the same mixture of solvents.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 250.0 mL with a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A.

*Reference solution (a).* Dissolve 55.0 mg of *diacetylfluorescein CRS* in a mixture of 1 mL of *2.5 M sodium hydroxide* and 5 mL of *ethanol (96 per cent) R*, heat on a water-bath for 20 min mixing frequently, cool and dilute to 50.0 mL with *water R*. Dilute 5.0 mL of the solution to 250.0 mL with a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A.

*Reference solution (b).* Dissolve 10.0 mg of *phthalic acid R* (impurity B) and 10.0 mg of *resorcinol R* (impurity A) in a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A and dilute to 100.0 mL with the same mixture of solvents. Dilute 5.0 mL of the solution to 100.0 mL with a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A.

*Reference solution (c).* Dilute 5.0 mL of test solution (b) to 20.0 mL with a mixture of 30 volumes of *acetonitrile R* and 70 volumes of mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.610 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent; adjust to pH 2.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 85 → 20 | 15 → 80 |
| 20 - 29 | 20 | 80 |
| 29 - 30 | 20 → 85 | 80 → 15 |
| 30 - 35 | 85 | 15 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Relative retention* with reference to fluorescein (retention time = about 15 min): impurity A = about 0.4; impurity B = about 0.5; impurity C = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.6;
- *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *sum of impurities other than A, B, C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 0.25 per cent.

To 10 mL of solution S add 90 mL of *water R* and 1 mL of *dilute nitric acid R*, wait for at least 10 min and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1.0 per cent.

To 5 mL of solution S add 90 mL of *distilled water R*, 2.5 mL of *dilute hydrochloric acid R* and dilute to 100 mL with *distilled water R*. Filter.

**Zinc**. Dilute 5 mL of solution S to 10 mL with *water R*. Add 2 mL of *hydrochloric acid R1*, filter and add 0.1 mL of *potassium ferrocyanide solution R*. No turbidity or precipitate is formed immediately.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{20}H_{10}Na_2O_5$ using the chromatogram obtained with reference solution (a) and the declared content of *diacetylfluorescein CRS*.

1 mg of *diacetylfluorescein CRS* is equivalent to 0.9037 mg of $C_{20}H_{10}Na_2O_5$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

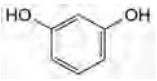


A. benzene-1,3-diol (resorcinol),

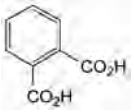


B. benzene-1,2-dicarboxylic acid (phthalic acid),

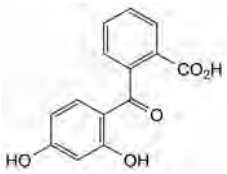


C. 2-(2,4-dihydroxybenzoyl)benzoic acid.

**01/2017:0611**
**corrected 9.2**

# FLUOROURACIL

## Fluorouracilum

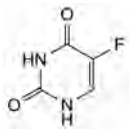

$C_4H_3FN_2O_2$ $M_r$ 130.1
[51-21-8]

DEFINITION

5-Fluoropyrimidine-2,4(1*H*,3*H*)-dione.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluorouracil CRS*.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ or $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.0 for solution S.

**Impurities F and G**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 5.0 mg of *fluorouracil impurity F CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 200.0 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 20.0 mg of *urea R* (impurity G) in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *methanol R*, *water R*, *ethyl acetate R* (15:15:70 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 2/3 of the plate.

*Drying*: in air.

*Detection*:

- *impurity F*: examine in ultraviolet light at 254 nm;
- *impurity G*: spray with a mixture of 200 mL of a 10 g/L solution of *dimethylaminobenzaldehyde R* in *anhydrous ethanol R* and 20 mL of *hydrochloric acid R*; dry in an oven at 80 °C for 3-4 min, then examine in daylight (impurity G produces a yellow spot and fluorouracil is not detected by the spray).

*System suitability*: the chromatogram shows 2 clearly separated spots after both detections.

*Limits*:

- *impurity F*: any spot due to impurity F is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.25 per cent);
- *impurity G*: any spot due to impurity G is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *fluorouracil impurity C CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *fluorouracil impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5.0 mg of *fluorouracil impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (e).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (f).* To 1 mL of reference solution (a) add 1 mL of the test solution and dilute to 10 mL with the mobile phase.

*Reference solution (g).* Dissolve the contents of a vial of *fluorouracil impurity mixture CRS* (containing impurities D and E) in 1.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.805 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 5.7 ± 0.1 with a 5 M potassium hydroxide solution prepared from *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of fluorouracil.

*Identification of impurities*: use the chromatogram supplied with *fluorouracil impurity mixture CRS* and the chromatogram obtained with reference solution (g) to identify the peaks due to impurities D and E.

*Relative retention* with reference to fluorouracil (retention time = about 6 min): impurity A = about 0.5; impurity B = about 0.7; impurity C = about 0.9; impurity D = about 1.6; impurity E = about 1.9.

*System suitability*: reference solution (f):

- *resolution*: minimum 2 between the peaks due to impurity C and fluorouracil.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.5; impurity E = 1.3;

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *impurities D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.100 g in 80 mL of *dimethylformamide R*, warming gently. Cool and titrate with *0.1 M tetrabutylammonium hydroxide*, using 0.25 mL of a 10 g/L solution of *thymol blue R* in *dimethylformamide R* as indicator. Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 13.01 mg of $C_4H_3FN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

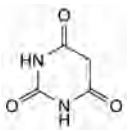

A. pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (barbituric acid),

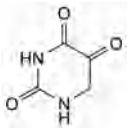

B. dihydropyrimidine-2,4,5(3*H*)-trione (isobarbituric acid or 5-hydroxyuracil),

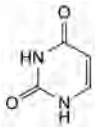

C. pyrimidine-2,4(1*H*,3*H*)-dione (uracil),

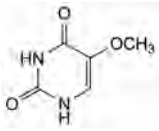

D. 5-methoxypyrimidine-2,4(1*H*,3*H*)-dione (5-methoxyuracil),

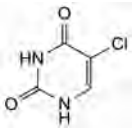

E. 5-chloropyrimidine-2,4(1*H*,3*H*)-dione (5-chlorouracil),

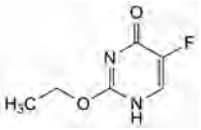

F. 2-ethoxy-5-fluoropyrimidin-4(1*H*)-one (2-ethoxy-5-fluorouracil),

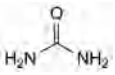

G. carbamide (urea).

**01/2017:1104**

# FLUOXETINE HYDROCHLORIDE

## Fluoxetini hydrochloridum

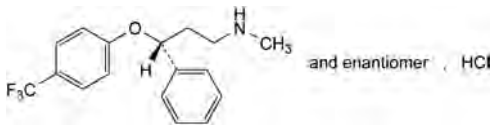


$C_{17}H_{19}ClF_3NO$ $M_r$ 345.8
[56296-78-7]

DEFINITION

(3*RS*)-*N*-Methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]-propan-1-amine hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, sparingly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fluoxetine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *methanol R*, then dilute to 100.0 mL with the same mixture of solvents.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.05° to + 0.05°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 55 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Test solution (b)*. Dilute 2.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution.* Dissolve 22 mg of *fluoxetine hydrochloride CRS* in 10.0 mL of *0.5 M sulfuric acid*. Heat at about 85 °C for 3 h. Allow to cool. The resulting solution contains considerable quantities of impurity A and usually also contains 4-trifluoromethylphenol. To 0.4 mL of this solution add 28.0 mg of *fluoxetine hydrochloride CRS*, about 1 mg of *fluoxetine impurity B CRS* and about 1 mg of *fluoxetine impurity C CRS*, then dilute to 25.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 8 volumes of *methanol R*, 30 volumes of *tetrahydrofuran R* and 62 volumes of a solution of *triethylamine R* prepared as follows: to 10 mL of *triethylamine R*, add 980 mL of *water R*, mix and adjust to pH 6.0 with *phosphoric acid R* (about 4.5 mL) and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of fluoxetine.

*Identification of impurities*: use the chromatogram obtained with the reference solution to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to fluoxetine: impurity A = about 0.24; impurity B = about 0.27; impurity C = about 0.9.

*System suitability*: reference solution:

– *retention time*: fluoxetine = 10 min to 18 min; 4-trifluoromethylphenol: maximum 35 min; if no peak due to 4-trifluoromethylphenol is observed, inject a 0.02 per cent solution of *4-trifluoromethylphenol R* in the mobile phase;
– *peak-to-valley ratio*: minimum 11, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to fluoxetine. If necessary, reduce the volume of methanol and increase the volume of the solution of triethylamine in the mobile phase.

*Limit*: test solution (b):

– *impurity C*: not more than 0.0015 times the area of the principal peak (0.15 per cent).

*Limits*: test solution (a):

– *impurities A, B*: for each impurity, not more than 0.0125 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.25 per cent);
– *unspecified impurities*: for each impurity, not more than 0.005 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.10 per cent);
– *total*: not more than 0.025 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.5 per cent);
– *disregard limit*: 0.0025 times the area of the principal peak in the chromatogram obtained with test solution (b) (0.05 per cent).

**Acetonitrile**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *dimethylformamide R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* To 1.0 g of *acetonitrile R*, add *dimethylformamide R*, mix and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 1000.0 mL with *dimethylformamide R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 10 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 35 |
| | 2 - 14.33 | 35 → 220 |
| | 14.33 - 24.33 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL; inject *dimethylformamide R* as a blank.

In the chromatogram obtained with *dimethylformamide R*, verify that there is no peak with the same retention time as acetonitrile.

*Limit*:

– *acetonitrile*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 55.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution.* Dissolve 55.0 mg of *fluoxetine hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Detection*: spectrophotometer at 227 nm.

*Retention time*: fluoxetine = 10 min to 18 min; if necessary, adjust the volumes of methanol and of the solution of triethylamine in the mobile phase.

*System suitability*: reference solution:

– *symmetry factor*: maximum 2.0 calculated at 10 per cent of the height of the peak due to fluoxetine.

Calculate the content of $C_{17}H_{19}ClF_3NO$ from the declared content of *fluoxetine hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

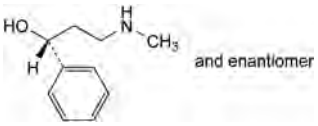


A. (1*RS*)-3-(methylamino)-1-phenylpropan-1-ol,

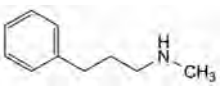


B. *N*-methyl-3-phenylpropan-1-amine,

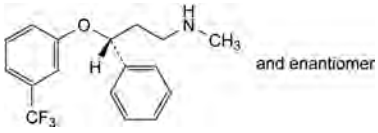


C. (3*RS*)-*N*-methyl-3-phenyl-3-[3-(trifluoromethyl)phenoxy]-propan-1-amine.

**07/2019:1693**

# FLUPENTIXOL DIHYDROCHLORIDE

## Flupentixoli dihydrochloridum

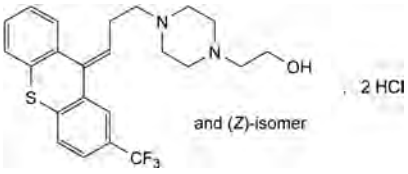


$C_{23}H_{27}Cl_2F_3N_2OS$ $M_r$ 507.4
[2413-38-9]

DEFINITION

2-[4-[3-[(*EZ*)-2-(Trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethan-1-ol dihydrochloride.

*Content*:

– *flupentixol dihydrochloride*: 99.0 per cent to 101.0 per cent (dried substance);
– *(Z)-isomer*: 42.0 per cent to 52.0 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *flupentixol dihydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution*. Dissolve 20 mg of *flupentixol dihydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *water R*, *diethylamine R*, *methyl ethyl ketone R* (1:4:95 *V/V/V*).
*Application*: 2 µL.
*Development*: twice over 3/4 of the plate.
*Drying*: in air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Doubling of the spot may be observed in both chromatograms.
*Detection B*: spray with *alcoholic solution of sulfuric acid R*; heat at 110 °C for 5 min and allow to cool; examine in ultraviolet light at 365 nm.
*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution. Doubling of the spot may be observed in both chromatograms.

C. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter and add to the filtrate a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow. The blank is red.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 410 nm is not greater than 0.125.

Dissolve 0.500 g in 5.0 mL of *water R*.

**pH** (*2.2.3*): 2.0 to 3.0.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Buffer solution*. Dissolve 6.3 g of *ammonium formate R* in about 900 mL of *water for chromatography R*, adjust to pH 8.2 with *concentrated ammonia R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 58.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *flupentixol for system suitability CRS* (containing impurities A, C, H and I) in 1 mL of mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: buffer solution, *acetonitrile for chromatography R*, *water for chromatography R* (58:420:522 *V/V/V*);
– *mobile phase B*: buffer solution, *acetonitrile for chromatography R* (100:900 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 90 → 5 | 10 → 95 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *flupentixol for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to both isomers of flupentixol and impurities A, C + I, and H.

*Relative retention* with reference to flupentixol (*Z*)-isomer (retention time = about 11 min): impurity A = about 0.83; impurity H = about 0.90; impurities C and I = about 0.97; flupentixol (*E*)-isomer = about 1.04.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurities A and H;
- *peak-to-valley ratio*: minimum 10.0, where $H_p$ = height above the baseline of the peak due to impurities C+I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to flupentixol (*Z*)-isomer.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurities C and I = 2.0; impurity H = 2.0;
- for each impurity, use the concentration of flupentixol dihydrochloride (both isomers) in reference solution (a).

*Limits*:

- *impurity H*: maximum 0.5 per cent;
- *sum of impurities C and I*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

**Flupentixol dihydrochloride**. Dissolve 0.200 g in 30 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 50.74 mg of $C_{23}H_{27}Cl_2F_3N_2OS$.

**(*Z*)-Isomer**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution*. Dissolve 20.0 mg of *flupentixol dihydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *concentrated ammonia R*, *2-propanol R*, *heptane R* (2:4:150:850 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of flupentixol (*Z*)-isomer.

*Identification of peaks*: use the chromatogram supplied with *flupentixol dihydrochloride CRS* and the chromatogram obtained with the reference solution to identify the peaks due to both isomers of flupentixol.

*Relative retention* with reference to flupentixol (*Z*)-isomer (retention time = about 6 min): flupentixol (*E*)-isomer = about 1.2.

*System suitability*: reference solution:

- *resolution*: minimum 3.0 between the peaks due to flupentixol (*Z*)-isomer and flupentixol (*E*)-isomer.

Calculate the percentage content of the (*Z*)-isomer of $C_{23}H_{27}Cl_2F_3N_2OS$ taking into account the assigned content of flupentixol (*Z*)-isomer in *flupentixol dihydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, G.*

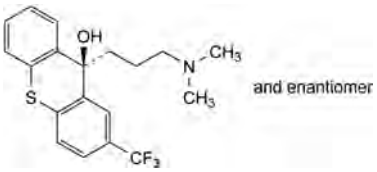


A. (9*RS*)-9-[3-(dimethylamino)propyl]-2-(trifluoromethyl)-9*H*-thioxanthen-9-ol,

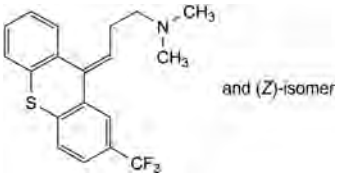


B. *N*,*N*-dimethyl-3-[(*EZ*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propan-1-amine,

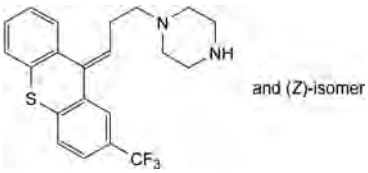


C. 1-[3-[(*EZ*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]piperazine,

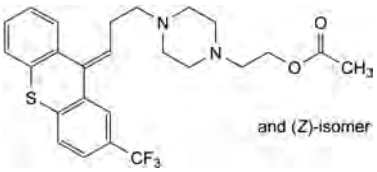


E. 2-[4-[3-[(*EZ*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]piperazin-1-yl]ethyl acetate,

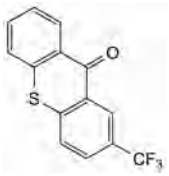


G. 2-(trifluoromethyl)-9*H*-thioxanthen-9-one,

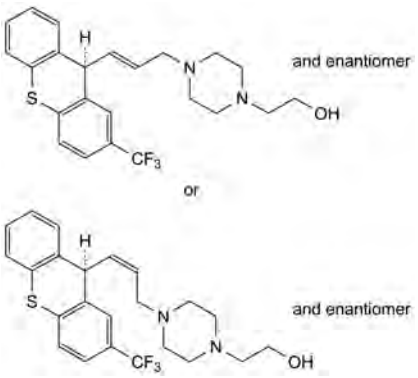


H. either 2-[4-[(*E*)-3-[(9*RS*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-yl]prop-2-en-1-yl]piperazin-1-yl]ethan-1-ol or 2-[4-[(*Z*)-3-[(9*RS*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-yl]prop-2-en-1-yl]piperazin-1-yl]ethan-1-ol,

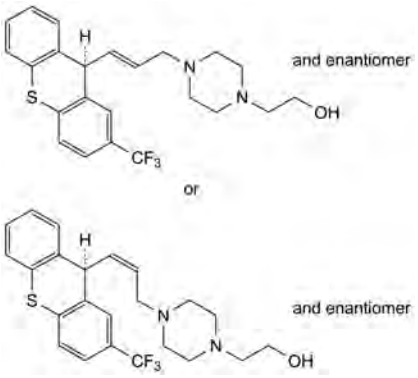


I. either 2-[4-[(*E*)-3-[(9*RS*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-yl]prop-2-en-1-yl]piperazin-1-yl]ethan-1-ol or 2-[4-[(*Z*)-3-[(9*RS*)-2-(trifluoromethyl)-9*H*-thioxanthen-9-yl]prop-2-en-1-yl]piperazin-1-yl]ethan-1-ol.

**01/2017:1014**

# FLUPHENAZINE DECANOATE

## Fluphenazini decanoas

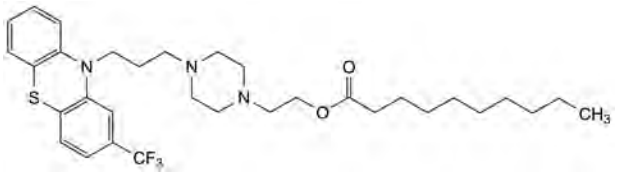

$C_{32}H_{44}F_3N_3O_2S$ $M_r$ 591.8
[5002-47-1]

DEFINITION

2-[4-[3-[2-(Trifluoromethyl)-10*H*-phenothiazin-10-yl]propyl]piperazin-1-yl]ethyl decanoate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow, viscous liquid or yellow solid.

*Solubility*: practically insoluble in water, very soluble in ethanol and in methylene chloride, freely soluble in methanol.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C.*

A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 50.0 mL with *methanol R*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 260 nm and a broad absorption maximum at about 310 nm. The specific absorbance at the maximum at 260 nm is 570 to 630.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: apply 50 µL of a 25 g/L solution in *methylene chloride R* to a disc of *potassium bromide R*. Dry the discs at 60 °C for 1 h before use.

*Comparison*: *fluphenazine decanoate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *fluphenazine decanoate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *fluphenazine enantate CRS* in reference solution (a) and dilute to 5 mL with the same solution.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R1*, *water R*, *methanol R* (1:4:95 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 8 cm.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Test solution*. Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *fluphenazine octanoate CRS* and 5 mg of *fluphenazine enantate CRS* in *acetonitrile R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B.

*Reference solution (c)*. Dissolve 11.7 mg of *fluphenazine dihydrochloride CRS* and 5.0 mg of *fluphenazine sulfoxide CRS* in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 1.0 mL to 50.0 mL with a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 10 g/L solution of *ammonium carbonate R* adjusted to pH 7.5 with *dilute hydrochloric acid R*,
– *mobile phase B*: mobile phase A, *acetonitrile R*, *methanol R* (7.5:45:45 *V/V/V*),

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 20 | 80 |
| 7 - 17 | 20 → 0 | 80 → 100 |
| 17 - 80 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Relative retention* with reference to fluphenazine decanoate (retention time = about 34 min): impurity A = about 0.13; impurity B = about 0.33; impurity C = about 0.76; impurity D = about 0.82.

*System suitability*: reference solution (a):

– *resolution*: minimum 6 between the peaks due to impurity C and impurity D.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *total*: not more than 2.0 per cent,
– *disregard limit for any other impurity*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.250 g in 30 mL of *glacial acetic acid R*. Using 0.05 mL of *crystal violet solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from violet to green.

1 mL of *0.1 M perchloric acid* is equivalent to 29.59 mg of $C_{32}H_{44}F_3N_3O_2S$.

STORAGE

Protected from light.

IMPURITIES

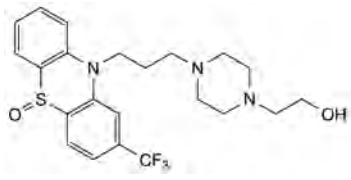

A. fluphenazine *S*-oxide,

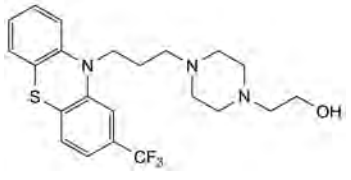

B. fluphenazine,

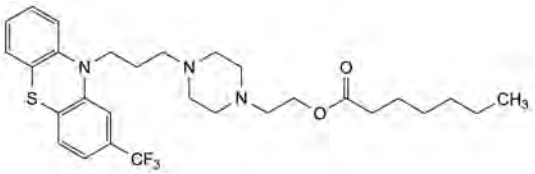

C. fluphenazine enantate,

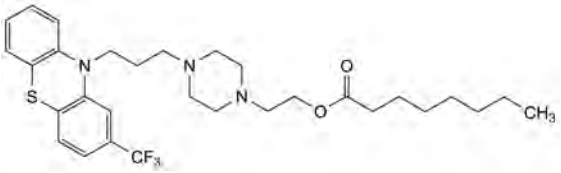

D. fluphenazine octanoate,

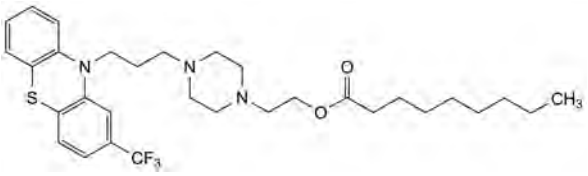

E. fluphenazine nonanoate,

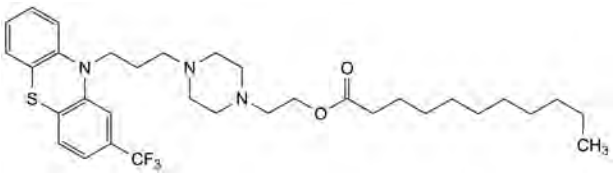

F. fluphenazine undecanoate,

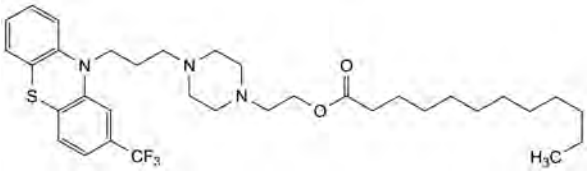

G. fluphenazine dodecanoate.

**01/2017:0904**

# FLUPHENAZINE DIHYDROCHLORIDE

## Fluphenazini dihydrochloridum

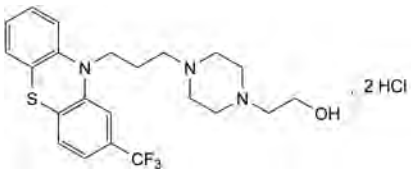

$C_{22}H_{28}Cl_2F_3N_3OS$ $M_r$ 510.4
[146-56-5]

DEFINITION

2-[4-[3-[2-(Trifluoromethyl)-10*H*-phenothiazin-10-yl]-propyl]piperazin-1-yl]ethanol dihydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 260 nm and at about 310 nm (broad band).

*Specific absorbance at the absorption maximum at 260 nm*: 630 to 700.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluphenazine dihydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *fluphenazine dihydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *perphenazine CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R1*, *water R*, *methanol R* (1:4:95 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 1.9 to 2.4.

Dissolve 0.5 g in 10 mL of *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solution A*: mix 4 mL of *acetic acid R* and 996 mL of a 4.33 g/L solution of *sodium octanesulfonate R*.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 25.0 mL with mobile phase A.

*Reference solution (b).* Dissolve the contents of a vial of *fluphenazine impurity mixture CRS* (impurities A, B, C and D) in 5 mL of the test solution and sonicate for 1 min. Mix 1.0 mL of this solution and 1.0 mL of the test solution.

*Reference solution (c).* Dissolve 5.0 mg of *fluphenazine sulfoxide CRS* (impurity A) in mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetic acid R*, *methanol R*, *acetonitrile R*, solution A (0.2:15:40:45 *V/V/V/V*);
– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 35 | 100 → 30 | 0 → 70 |
| 35 - 50 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm and at 274 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *fluphenazine impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to fluphenazine (retention time = about 19 min): impurity A = about 0.2; impurity B = about 0.3; impurity D = about 2.0; impurity C = about 2.1.

*System suitability*: reference solution (b):

– *resolution at 274 nm*: minimum 2.5 between the peaks due to impurities A and B.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.3; impurity C = 0.6;
– *impurity A at 274 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *impurity B at 274 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurities C, D at 260 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities at 260 nm*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of the impurities other than A and B at 260 nm and impurities A and B at 274 nm*: not more than 1.0 per cent;
– *disregard limit at 260 nm*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 65 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.220 g in a mixture of 10 mL of *anhydrous formic acid R* and 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.52 mg of $C_{22}H_{28}Cl_2F_3N_3OS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

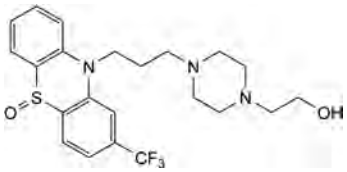

A. 2-[4-[3-[5-oxo-2-(trifluoromethyl)-10*H*-5λ⁴-phenothiazin-10-yl]propyl]piperazin-1-yl]ethanol (fluphenazine *S*-oxide),

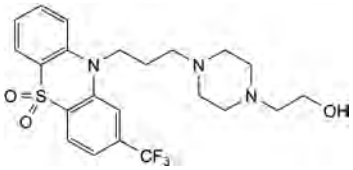

B. 2-[4-[3-[5,5-dioxo-2-(trifluoromethyl)-10*H*-5λ⁶-phenothiazin-10-yl]propyl]piperazin-1-yl]ethanol (fluphenazine *S,S*-dioxide),

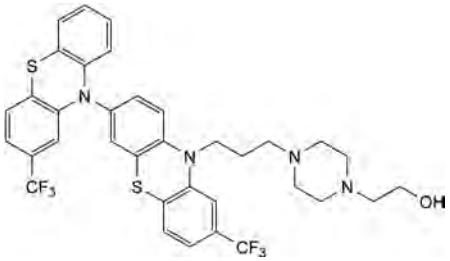

C. 2-[4-[3-[2′,8-bis(trifluoromethyl)-10*H*-3,10′-biphenothiazin-10-yl]propyl]piperazin-1-yl]ethanol,

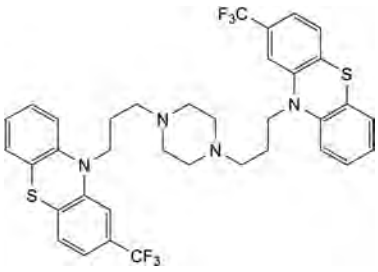

D. 10,10′-[piperazine-1,4-diylbis(propane-3,1-diyl)]bis[2-(trifluoromethyl)-10*H*-phenothiazine],

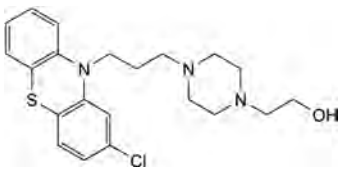

E. 2-[4-[3-[2-chloro-10*H*-phenothiazin-10-yl]propyl]-piperazin-1-yl]ethanol (perphenazine),

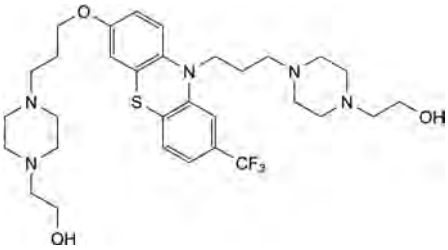

F. 2-[4-[3-[7-[3-[4-(2-hydroxyethyl)piperazin-1-yl]propoxy]-2-(trifluoromethyl)-10*H*-phenothiazin-10-yl]propyl]-piperazin-1-yl)ethanol.

**01/2017:1015**

# FLUPHENAZINE ENANTATE

## Fluphenazini enantas

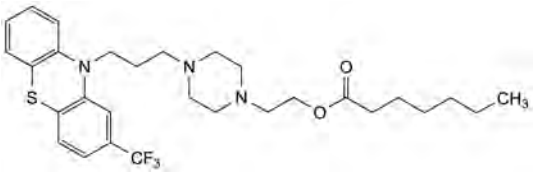

$C_{29}H_{38}F_3N_3O_2S$ $M_r$ 549.7
[2746-81-8]

DEFINITION

2-[4-[3-[2-(Trifluoromethyl)-10*H*-phenothiazin-10-yl]propyl]piperazin-1-yl]ethyl heptanoate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow, viscous liquid or yellow solid.

*Solubility*: practically insoluble in water, very soluble in ethanol and in methylene chloride, freely soluble in methanol.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C.*

A. Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 50.0 mL with *methanol R*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 260 nm and a broad absorption maximum at about 310 nm. The specific absorbance at the maximum at 260 nm is 610 to 670.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: apply 50 µL of a 25 g/L solution in *methylene chloride R* to a disc of *potassium bromide R*. Dry the discs at 60 °C for 1 h before use.

*Comparison*: *fluphenazine enantate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *fluphenazine enantate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *fluphenazine decanoate CRS* in reference solution (a) and dilute to 5 mL with the same solution.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R1*, *water R*, *methanol R* (1:4:95 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 8 cm.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *fluphenazine octanoate CRS* and 5 mg of *fluphenazine enantate CRS* in *acetonitrile R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 5 volumes of mobile phase A and 95 volumes of mobile phase B.

*Reference solution (c).* Dissolve 5.0 mg of *fluphenazine sulfoxide CRS* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 50.0 mL with *acetonitrile R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 10 g/L solution of *ammonium carbonate R* adjusted to pH 7.5 with *dilute hydrochloric acid R*,
– *mobile phase B*: mobile phase A, *acetonitrile R*, *methanol R* (7.5:45:45 *V/V/V*),

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 20 | 80 |
| 7 - 17 | 20 → 0 | 80 → 100 |
| 17 - 80 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Relative retention* with reference to fluphenazine enantate (retention time = about 25 min): impurity A = about 0.2; impurity D = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 6 between the peaks due to fluphenazine enantate and impurity D.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
– *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *total*: not more than 1.6 per cent,
– *disregard limit for any other impurity*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.250 g in 30 mL of *glacial acetic acid R*. Using 0.05 mL of *crystal violet solution R* as indicator titrate with *0.1 M perchloric acid* until the colour changes from violet to green.

1 mL of *0.1 M perchloric acid* is equivalent to 27.49 mg of $C_{29}H_{38}F_3N_3O_2S$.

STORAGE

Protected from light.

IMPURITIES

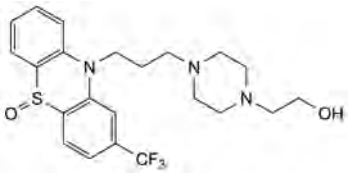

A. fluphenazine *S*-oxide,

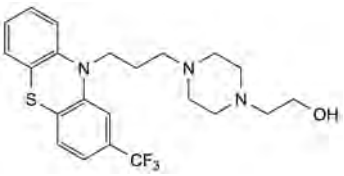

B. fluphenazine,

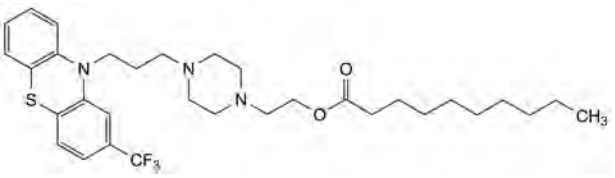

C. fluphenazine decanoate,

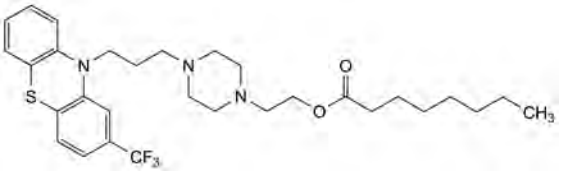

D. fluphenazine octanoate,

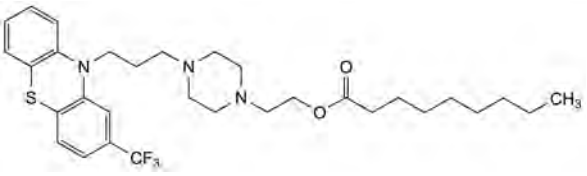

E. fluphenazine nonanoate,

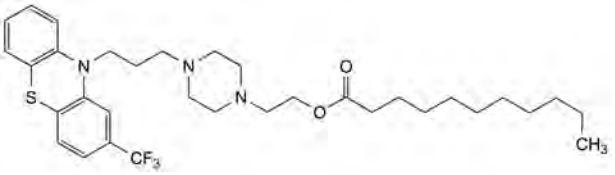

F. fluphenazine undecanoate,

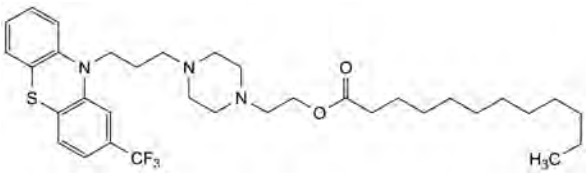

G. fluphenazine dodecanoate.

**01/2008:0905**
**corrected 6.0**

# FLURAZEPAM MONOHYDROCHLORIDE

## Flurazepami monohydrochloridum

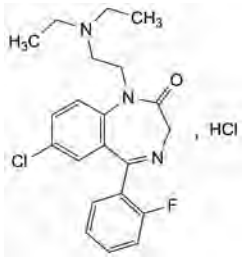

$C_{21}H_{24}Cl_2FN_3O$ $M_r$ 424.3
[36105-20-1]

DEFINITION

7-Chloro-1-[2-(diethylamino)ethyl]-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one monohydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of flurazepam monohydrochloride.*

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 6.0.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *oxazepam R* in 10 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 350 volumes of *acetonitrile R* and 650 volumes of a 10.5 g/L solution of *potassium dihydrogen phosphate R* and ajust to pH 6.1 with a 40 g/L solution of *sodium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 239 nm.

*Injection*: 20 µL.

Run time: 6 times the retention time of flurazepam.

*Relative retention* with reference to flurazepam (retention time = about 7 min): impurity C = about 1.5; impurity B = about 1.9; impurity A = about 2.4.

*System suitability*: reference solution (b):

– *resolution*: minimum of 4.5 between the peaks due to flurazepam and to oxazepam.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.61; impurity C = 0.65,
– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Fluorides** (*2.4.5*): maximum 500 ppm.

0.10 g complies with the limit test for fluorides.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in a mixture of 1.0 mL of *0.1 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 42.43 mg of $C_{21}H_{24}Cl_2FN_3O$.

STORAGE

Protected from light.

IMPURITIES

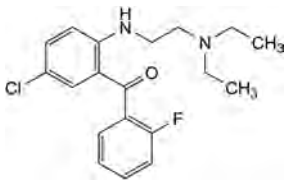

A. [5-chloro-2-[[2-(diethylamino)ethyl]amino]phenyl](2-fluorophenyl)methanone,

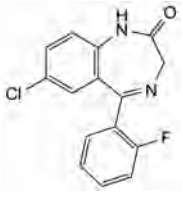

B. 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

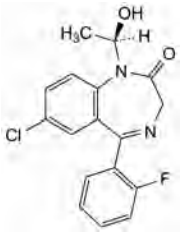

C. 7-chloro-5-(2-fluorophenyl)-1-[(1*RS*)-1-hydroxyethyl]-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

**01/2017:1519**

# FLURBIPROFEN

## Flurbiprofenum

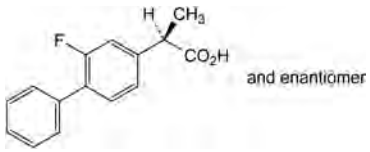


$C_{15}H_{13}FO_2$ $M_r$ 244.3
[5104-49-4]

DEFINITION

(2*RS*)-2-(2-Fluorobiphenyl-4-yl)propanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride. It dissolves in aqueous solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 114 °C to 117 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 0.10 g in *0.1 M sodium hydroxide* and dilute to 100.0 mL with the same alkaline solution. Dilute 1.0 mL of this solution to 100.0 mL with *0.1 M sodium hydroxide*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 247 nm.

*Specific absorbance at the absorption maximum*: 780 to 820.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flurbiprofen CRS*.

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R* add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

Dissolve 1.0 g in *methanol R* and dilute to 10 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.1° to + 0.1°.

Dissolve 0.50 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (45:55 *V/V*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10.0 mg of *flurbiprofen impurity A CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 10.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with reference solution (b).

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (5:35:60 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of flurbiprofen.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity A and flurbiprofen.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *impurities B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *sum of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in 50 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.43 mg of $C_{15}H_{13}FO_2$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

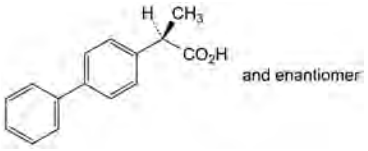


A. (2*RS*)-2-(biphenyl-4-yl)propanoic acid,

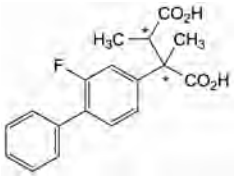


B. 2-(2-fluorobiphenyl-4-yl)-2,3-dimethylbutanedioic acid,

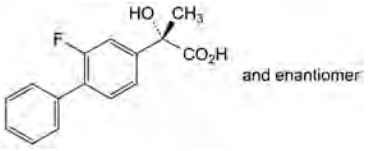


C. (2*RS*)-2-(2-fluorobiphenyl-4-yl)-2-hydroxypropanoic acid,

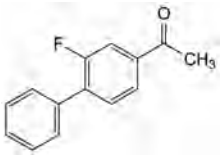


D. 1-(2-fluorobiphenyl-4-yl)ethanone,

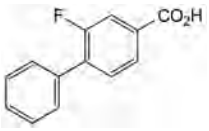


E. 2-fluorobiphenyl-4-carboxylic acid.

**01/2011:1723**

# FLUSPIRILENE

## Fluspirilenum

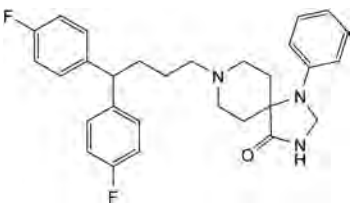


$C_{29}H_{31}F_2N_3O$ $M_r$ 475.6
[1841-19-6]

DEFINITION

8-[4,4-bis(4-Fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluspirilene CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, gently evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.25 g in 25 mL of *methylene chloride R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *fluspirilene impurity C CRS* in *dimethylformamide R*, add 0.5 mL of the test solution and dilute to 100.0 mL with *dimethylformamide R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *dimethylformamide R*. Dilute 1.0 mL of this solution to 25.0 mL with *dimethylformamide R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: 13.6 g/L solution of *tetrabutylammonium hydrogen sulfate R*,
- *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 75 → 70 | 25 → 30 |
| 15 - 20 | 70 | 30 |
| 20 - 22 | 70 → 0 | 30 → 100 |
| 22 - 30 | 0 | 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to fluspirilene (retention time = about 15 min): impurity A = about 0.8; impurity B = about 0.93; impurity C = about 0.97.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.2 between the peaks due to impurity C and fluspirilene.

*Limits*:

- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent),
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.350 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 47.56 mg of $C_{29}H_{31}F_2N_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

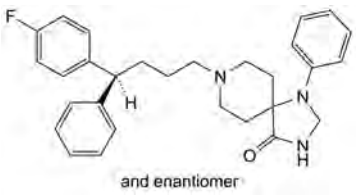


A. 8-[(4*RS*)-4-(4-fluorophenyl)-4-phenylbutyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one,

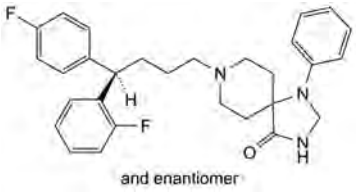


B. 8-[(4*RS*)-4-(2-fluorophenyl)-4-(4-fluorophenyl)butyl]-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one,

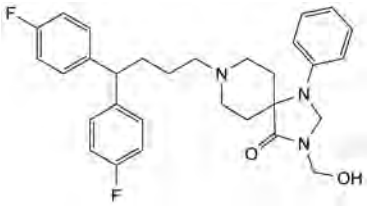


C. 8-[4,4-bis(4-fluorophenyl)butyl]-3-(hydroxymethyl)-1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one.

**01/2017:1423**

# FLUTAMIDE

## Flutamidum

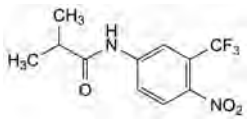


$C_{11}H_{11}F_3N_2O_3$ $M_r$ 276.2
[13311-84-7]

DEFINITION

2-Methyl-*N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent), practically insoluble in heptane.

mp: about 112 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *flutamide CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *flutamide for system suitability CRS* (containing impurities A, B and C) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 20.0 mg of *flutamide CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 1.5 times the retention time of flutamide.

*Identification of impurities*: use the chromatogram supplied with *flutamide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to flutamide (retention time = about 19 min): impurity B = about 0.5; impurity A = about 0.6; impurity C = about 0.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurities B and A.

*Calculation of percentage contents*:

- for each impurity, use the concentration of flutamide in reference solution (b).

*Limits*:

- *impurities A, B, C*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{11}H_{11}F_3N_2O_3$ taking into account the assigned content of *flutamide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F.*

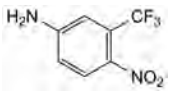

A. 4-nitro-3-(trifluoromethyl)aniline,

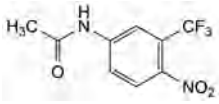

B. *N*-[4-nitro-3-(trifluoromethyl)phenyl]acetamide,

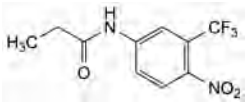

C. *N*-[4-nitro-3-(trifluoromethyl)phenyl]propanamide,

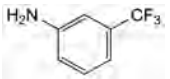

D. 3-(trifluoromethyl)aniline,

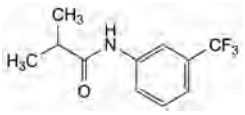

E. 2-methyl-*N*-[3-(trifluoromethyl)phenyl]propanamide,

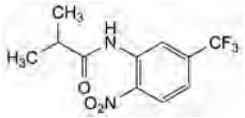

F. 2-methyl-*N*-[2-nitro-5-(trifluoromethyl)-phenyl]propanamide.

**01/2016:1750**

# FLUTICASONE PROPIONATE

## Fluticasoni propionas

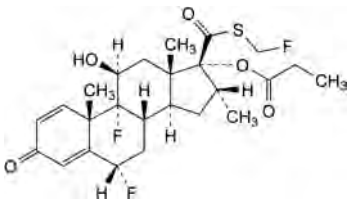

$C_{25}H_{31}F_3O_5S$ $M_r$ 500.6
[80474-14-2]

DEFINITION

6α,9-Difluoro-17-[[(fluoromethyl)sulfanyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl propanoate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluticasone propionate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 32 to + 36 (anhydrous substance).

Dissolve 0.25 g in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution.* Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 1 mg of *fluticasone impurity D CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 25.0 mL with the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *fluticasone propionate for impurity G identification CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2 mg of *fluticasone propionate for impurity C identification CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: solution containing 0.05 per cent *V/V* of *phosphoric acid R* and 3.0 per cent *V/V* of *methanol R* in *water R*;
- *mobile phase B*: solution containing 0.05 per cent *V/V* of *phosphoric acid R* and 3.0 per cent *V/V* of *methanol R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 57 | 43 |
| 2 - 42 | 57 → 45 | 43 → 55 |
| 42 - 62 | 45 → 10 | 55 → 90 |
| 62 - 72 | 10 | 90 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 239 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D; use the chromatogram supplied with *fluticasone propionate for impurity G identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity G; use the chromatogram supplied with *fluticasone propionate for impurity C identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to fluticasone propionate (retention time = about 32 min): impurity C = about 0.8; impurity D = about 0.95; impurity G = about 1.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity D and fluticasone propionate.

*Limits*:

– *impurities D, G*: for each impurity, maximum 0.3 per cent;
– *impurity C*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.8 per cent;
– *reporting threshold*: 0.05 per cent (reference solution (b)).

**Acetone**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 0.5 mL of *tetrahydrofuran R* to 1000 mL with *dimethylformamide R*.

*Test solution.* Dissolve 0.50 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the internal standard solution.

*Reference solution.* Dilute 0.40 g of *acetone R* to 100.0 mL with the internal standard solution. Dilute 1.0 mL of this solution to 10.0 mL with the internal standard solution.

*Column*:

– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.53 mm;
– *stationary phase*: cross-linked *macrogol 20 000 R* (film thickness 2 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 5.5 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3.5 | 60 |
| | 3.5 - 7.5 | 60 → 180 |
| | 7.5 - 10.5 | 180 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.1 µL.

*Limit*:

– *acetone*: maximum 1.0 per cent *m/m*.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g using the evaporation technique:

– *temperature*: 160 °C;
– *heating time*: 3 min.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve, using sonication, 20.0 mg of the substance to be examined in the mobile phase and dilute to 200.0 mL with the mobile phase. Dilute 4.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve, using sonication, 20.0 mg of *fluticasone propionate CRS* in the mobile phase and dilute to 200.0 mL with the mobile phase.

*Reference solution (b).* Dilute 4.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 1 mg of *fluticasone impurity D CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. To 1.0 mL of the solution add 2.0 mL of reference solution (a) and dilute to 5.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 15 volumes of *acetonitrile R*, 35 volumes of a 1.15 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 3.5 and 50 volumes of *methanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 239 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of fluticasone propionate.

*Relative retention* with reference to fluticasone propionate (retention time = about 6 min): impurity D = about 1.1.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to fluticasone propionate and impurity D; if necessary, adjust the ratio of acetonitrile to methanol in the mobile phase.

Calculate the percentage content of $C_{25}H_{31}F_3O_5S$ using the chromatogram obtained with reference solution (b) and taking into account the assigned content of *fluticasone propionate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F, H, I.*

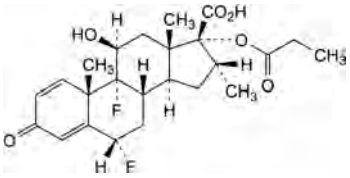

A. 6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxo-17-(propanoyloxy)androsta-1,4-diene-17β-carboxylic acid,

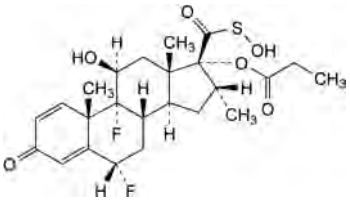

B. 6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxo-17-(propanoyloxy)androsta-1,4-dien-17β-carbo(thioperoxoic) *SO*-acid,

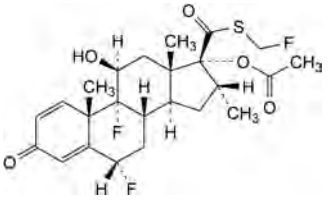

C. 6α,9-difluoro-17-[[(fluoromethyl)sulfanyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl acetate,

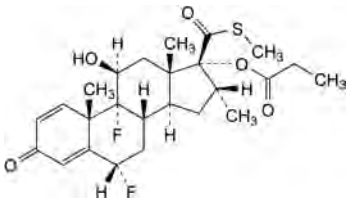

D. 6α,9-difluoro-11β-hydroxy-16α-methyl-17-[(methylsulfanyl)carbonyl]-3-oxoandrosta-1,4-dien-17α-yl propanoate,

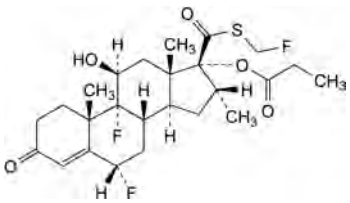

E. 6α,9-difluoro-17-[[(fluoromethyl)sulfanyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrost-4-en-17α-yl propanoate,

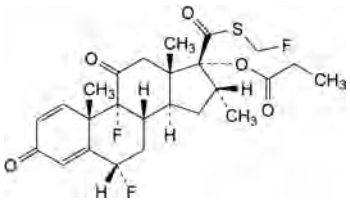

F. 6α,9-difluoro-17-[[(fluoromethyl)sulfanyl]carbonyl]-16α-methyl-3,11-dioxoandrosta-1,4-dien-17α-yl propanoate,

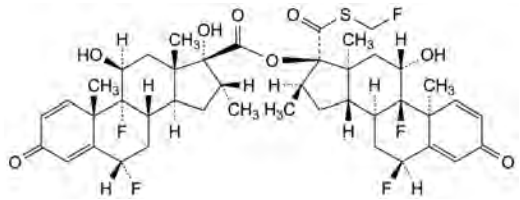

G. 6α,9-difluoro-17-[[(fluoromethyl)sulfanyl]carbonyl]-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl 6α,9-difluoro-11β,17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylate,

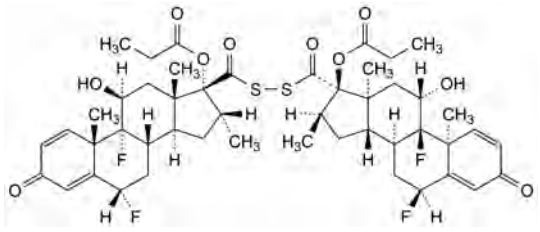

H. 17,17′-(disulfanediyldicarbonyl)bis(6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl) dipropanoate,

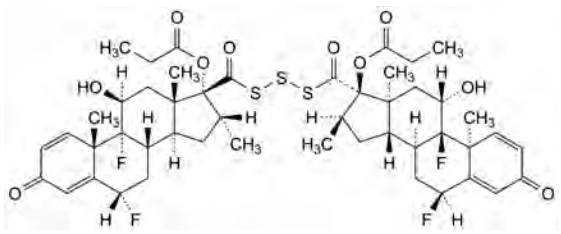

I. 17,17′-(trisulfanediyldicarbonyl)bis(6α,9-difluoro-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl) dipropanoate.

**01/2017:1424**

# FLUTRIMAZOLE

## Flutrimazolum

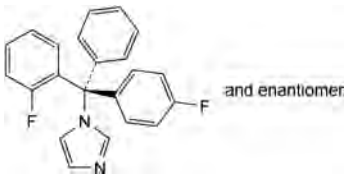


$C_{22}H_{16}F_2N_2$ $M_r$ 346.4
[119006-77-8]

DEFINITION

(*RS*)-1-[(2-Fluorophenyl)(4-fluorophenyl)phenylmethyl]-1*H*-imidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in tetrahydrofuran, soluble in methanol.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Melting point (*2.2.14*): 161 °C to 166 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *flutrimazole CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (a).* Dissolve 20 mg of *flutrimazole CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 20 mg of *flutrimazole CRS* and 10 mg of *metronidazole benzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Pretreatment*: heat the plate at 110 °C for 1 h.
*Mobile phase*: *2-propanol R*, *ethyl acetate R* (10:90 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Solution S**. Dissolve 1.00 g in *methanol R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.05° to + 0.05°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *imidazole CRS* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 30.0 mg of *flutrimazole impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Mix 2.0 mL of reference solution (a) and 2.0 mL of reference solution (b) and dilute to 50.0 mL with the mobile phase.

*Reference solution (d).* Dilute 10.0 mL of reference solution (c) to 50.0 mL with the mobile phase.

*Reference solution (e).* Mix 2.0 mL of the test solution and 10.0 mL of reference solution (c) and dilute to 50.0 mL with the mobile phase.

*Reference solution (f).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.2 m, Ø = 4.6 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *0.03 M phosphate buffer solution pH 7.0 R*, *acetonitrile R* (40:60 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of flutrimazole.

*System suitability*: reference solution (e):

– *resolution*: minimum 2.0 between the peaks due to impurity A (1st peak) and impurity B (2nd peak); minimum 1.5 between the peaks due to impurity B and flutrimazole (3rd peak);
– *symmetry factors*: maximum 2.0 for the peaks due to impurities A and B.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (f) (0.10 per cent);
– *sum of impurities other than B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (f) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (f) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.64 mg of $C_{22}H_{16}F_2N_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

A. imidazole,

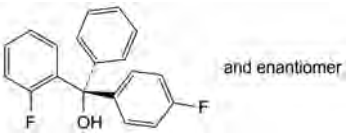


B. (*RS*)-(2-fluorophenyl)(4-fluorophenyl)phenylmethanol,

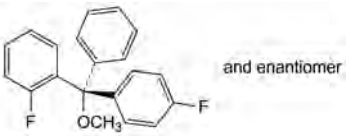


C. (*RS*)-(2-fluorophenyl)(4-fluorophenyl)methoxyphenylmethane.

**01/2017:2333**

# FLUVASTATIN SODIUM

## Fluvastatinum natricum

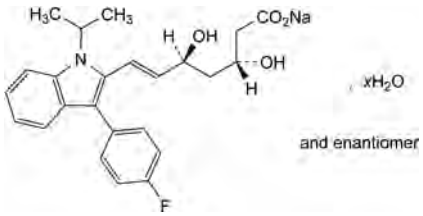


$C_{24}H_{25}FNNaO_4,xH_2O$ $M_r$ 433.5 (anhydrous substance)
Anhydrous fluvastatin sodium: [93957-55-2]

DEFINITION

Sodium (3*RS*,5*SR*,6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxyhept-6-enoate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

It may be anhydrous or contain a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white, or pale yellow or pale reddish-yellow, very hygroscopic, amorphous or crystalline powder.

*Solubility*: soluble in water, freely soluble in methanol, practically insoluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluvastatin sodium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness on a steam bath, protecting the solutions from light, and dry at 105 °C for 30 min. Cool and keep in a desiccator. Record new spectra using the residues.

B. 0.5 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 8.0 to 10.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution*. Dissolve 25 mg of the substance to be examined in 20 mL of mobile phase B and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve the contents of a vial of *fluvastatin for system suitability CRS* (containing impurities A, B and D) in 1.0 mL of a mixture of equal volumes of mobile phase A and mobile phase B.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: to 880 mL of *water R* add 20 mL of a 250 g/L solution of *tetramethylammonium hydroxide R* and adjust quickly to pH 7.2 with *phosphoric acid R*; mix with 100 mL of a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *methanol R*;
- *mobile phase B*: to 80 mL of *water R* add 20 mL of a 250 g/L solution of *tetramethylammonium hydroxide R* and adjust quickly to pH 7.2 with *phosphoric acid R*; mix with 900 mL of a mixture of 40 volumes of *acetonitrile R* and 60 volumes of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 70 | 30 |
| 3 - 23 | 70 → 10 | 30 → 90 |
| 23 - 28 | 10 | 90 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 305 nm and at 365 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *fluvastatin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to fluvastatin (retention time = about 14 min): impurity A = about 1.05; impurity D = about 1.1; impurity B = about 1.6.

*System suitability*: reference solution (b) at 305 nm:

- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to fluvastatin.

*Limits*:

- *impurity A at 305 nm*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *impurity B at 305 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D at 365 nm*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (a) at 305 nm (0.15 per cent);
- *unspecified impurities at 305 nm*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities at 305 nm*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit at 305 nm*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 12.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.325 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 43.35 mg of $C_{24}H_{25}FNNaO_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, G.*

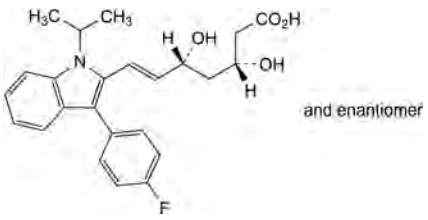


A. (3*RS*,5*RS*,6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxyhept-6-enoic acid,

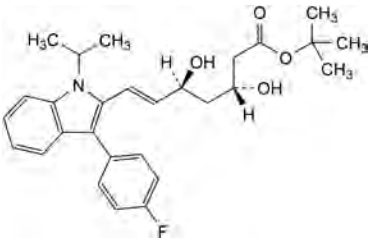

B. 1,1-dimethylethyl (3*R*,5*S*,6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxyhept-6-enoate,

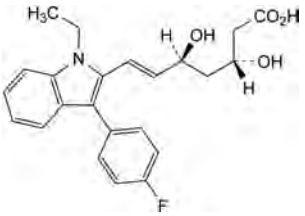

C. (3*R*,5*S*,6*E*)-7-[1-ethyl-3-(4-fluorophenyl)-1*H*-indol-2-yl]-3,5-dihydroxyhept-6-enoic acid,

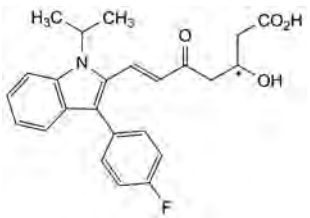

D. (6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3-hydroxy-5-oxohept-6-enoic acid,

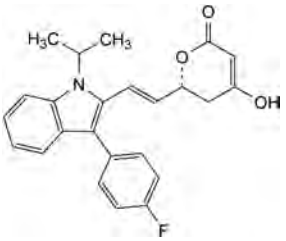

E. (6*R*)-6-[(*E*)-2-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]ethenyl]-4-hydroxy-5,6-dihydro-2*H*-pyran-2-one,

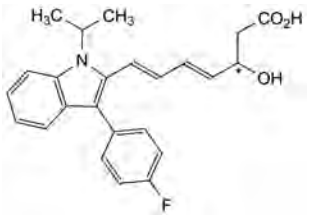

F. (4*E*,6*E*)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3-hydroxyhepta-4,6-dienoic acid,

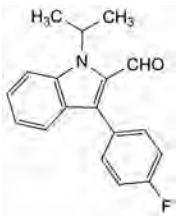

G. 3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indole-2-carbaldehyde.

**01/2017:1977**

# FLUVOXAMINE MALEATE

## Fluvoxamini maleas

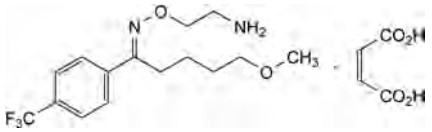

$C_{19}H_{25}F_3N_2O_6$ $M_r$ 434.4
[61718-82-9]

DEFINITION

2-[[[(1*E*)-5-Methoxy-1-[4-(trifluoromethyl)phenyl]pentylidene]amino]oxy]ethanamine (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

The production method must be evaluated to determine the potential for formation of aziridine. Where necessary, a validated test for the substance is carried out or the production method is validated to demonstrate acceptable clearance.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fluvoxamine maleate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test solution immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 25 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *fluvoxamine for system suitability CRS* (containing impurities A, B, C and F) in 1.0 mL of the mobile phase.

*Reference solution (c).* Dissolve 3.0 mg of *fluvoxamine impurity D CRS* in 5 mL of the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 370 volumes of *acetonitrile R1* and 630 volumes of a buffer solution containing 1.1 g/L of *potassium dihydrogen phosphate R* and 1.9 g/L of *sodium pentanesulfonate R* in *water R*, previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 234 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of fluvoxamine.

*Identification of impurities*: use the chromatogram supplied with *fluvoxamine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and F.

*Relative retention* with reference to fluvoxamine (retention time = about 15 min): maleic acid = about 0.15; impurities F and G = about 0.5; impurity C = about 0.6; impurity B = about 0.8; impurity A = about 2.5; impurity D = about 5.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities F and C.

*Limits*:

- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *sum of impurities F and G*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 43.44 mg of $C_{19}H_{25}F_3N_2O_6$.

IMPURITIES

*Specified impurities: A, B, C, D, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, I, J.*

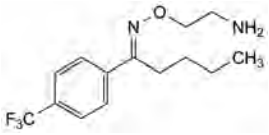

A. 2-[[[(1*E*)-1-[4-(trifluoromethyl)phenyl]pentylidene]-amino]oxy]ethanamine,

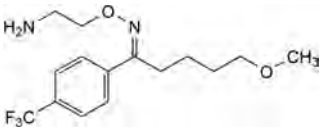

B. 2-[[[(1*Z*)-5-methoxy-1-[4-(trifluoromethyl)phenyl]-pentylidene]amino]oxy]ethanamine,

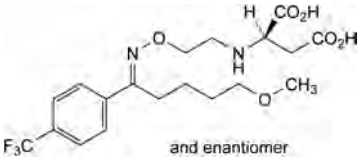


C. (2*RS*)-2-[[2-[[[(1*E*)-5-methoxy-1-[4-(trifluoromethyl)-phenyl]pentylidene]amino]oxy]ethyl]amino]butanedioic acid,

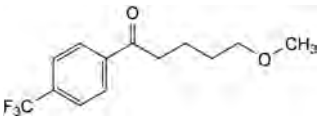

D. 5-methoxy-1-[4-(trifluoromethyl)phenyl]pentan-1-one,

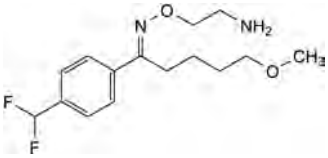

E. 2-[[[(1*E*)-1-[4-(difluoromethyl)phenyl]-5-methoxypentylidene]amino]oxy]ethanamine,

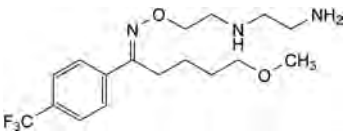

F. *N*-[2-[[[(1*E*)-5-methoxy-1-[4-(trifluoromethyl)phenyl]-pentylidene]amino]oxy]ethyl]ethane-1,2-diamine,

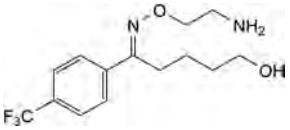

G. (5*E*)-5-[(2-aminoethoxy)imino]-5-[4-(trifluoromethyl)-phenyl]pentan-1-ol,

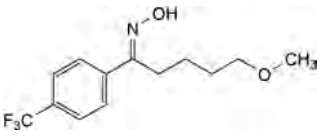

I. (*E*)-*N*-[5-methoxy-1-[4-(trifluoromethyl)phenyl]-pentylidene]hydroxylamine,

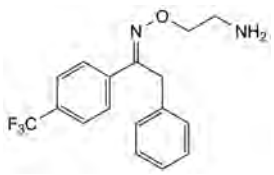

J. 2-[[[(1*E*)-2-phenyl-1-[4-(trifluoromethyl)phenyl]-ethylidene]amino]oxy]ethanamine.

07/2018:0067
corrected 10.0

# FOLIC ACID HYDRATE

## Acidum folicum hydricum

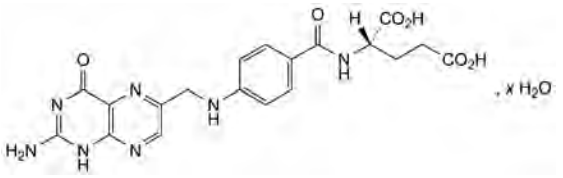

$C_{19}H_{19}N_7O_6,xH_2O$ $M_r$ 441.4 (anhydrous substance)
Anhydrous folic acid: [59-30-3]

DEFINITION

(2*S*)-2-[4-[[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzamido]pentanedioic acid hydrate.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: yellowish or orange, crystalline powder.

*Solubility*: practically insoluble in water and in most organic solvents. It dissolves in dilute acids and in alkaline solutions.

IDENTIFICATION

*First identification*: *A, B, D.*

*Second identification*: *A, C.*

A. Specific optical rotation (*2.2.7*): + 18 to + 22 (anhydrous substance).
Dissolve 0.25 g in a 4.2 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with the same solution.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *folic acid CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 50 mg of the substance to be examined in a mixture of 2 volumes of *concentrated ammonia R* and 9 volumes of *methanol R*, and dilute to 100 mL with the same mixture of solvents.
*Reference solution*. Dissolve 50 mg of *folic acid CRS* in a mixture of 2 volumes of *concentrated ammonia R* and 9 volumes of *methanol R*, and dilute to 100 mL with the same mixture of solvents.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *propanol R*, *ethanol (96 per cent) R* (20:20:60 *V/V/V*).
*Application*: 2 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 365 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the principal spot in the chromatogram obtained with the reference solution.

D. Water (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 28.6 g/L solution of *sodium carbonate R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of solution A and dilute to 50.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 50.0 mg of *folic acid CRS* in 2.5 mL of solution A and dilute to 50.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *folic acid for system suitability CRS* (containing impurities C, E, G and H) in 1 mL of solution A and dilute to 25 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 10.0 mg of *folic acid impurity A CRS* in 1 mL of solution A and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (e)*. Dissolve 4.0 mg of *folic acid impurity D CRS* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (f)*. Dissolve 5 mg of *folic acid for impurity I identification CRS* in 1 mL of solution A and dilute to 25 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 12 volumes of *methanol R* and 88 volumes of a solution containing 11.16 g/L of *potassium dihydrogen phosphate R* and 5.50 g/L of *dipotassium hydrogen phosphate R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 5 µL of the test solution and reference solutions (b), (c), (d), (e) and (f).

*Run time*: 3.3 times the retention time of folic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A; use the chromatogram supplied with *folic acid for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C, E, G and H; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity D; use the chromatogram supplied with *folic acid for impurity I identification CRS* and the chromatogram obtained with reference solution (f) to identify the peak due to impurity I.

*Relative retention* with reference to folic acid (retention time = about 8.5 min): impurity A = about 0.5; impurity C = about 0.9; impurity E = about 1.3; impurity D = about 1.5; impurity I = about 2.15; impurity G = about 2.4; impurity H = about 2.5.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to folic acid and impurity E;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to folic acid; minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity H.

*Calculation of percentage contents*:
- for impurity A, use the concentration of impurity A in reference solution (d);
- for impurity D, use the concentration of impurity D in reference solution (e);
- for impurities other than A and D, use the concentration of folic acid hydrate in reference solution (c).

*Limits*:
- *impurity A*: maximum 0.5 per cent;

- *impurity D*: maximum 0.4 per cent;
- *impurities C, E, G*: for each impurity, maximum 0.3 per cent;
- *impurities H, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 5.0 per cent to 8.5 per cent, determined on 0.150 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

STORAGE

Protected from light, under inert gas.

IMPURITIES

*Specified impurities: A, C, D, E, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, F.*

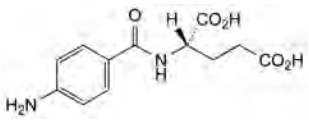

A. (2*S*)-2-(4-aminobenzamido)pentanedioic acid (*N*-(4-aminobenzoyl)-L-glutamic acid),

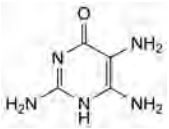

B. 2,5,6-triaminopyrimidin-4(1*H*)-one,

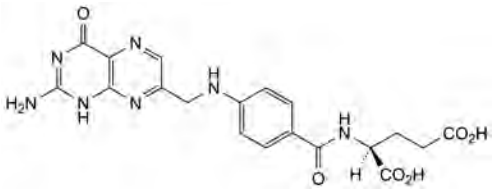

C. (2*S*)-2-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-7-yl)-methyl]amino]benzamido]pentanedioic acid (isofolic acid),

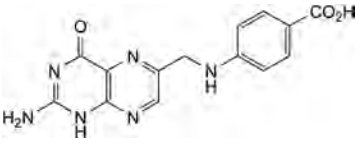

D. 4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)-methyl]amino]benzoic acid (pteroic acid),

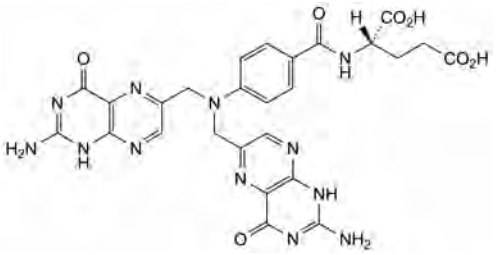

E. (2*S*)-2-[4-[bis[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)-methyl]amino]benzamido]pentanedioic acid (6-pterinylfolic acid),

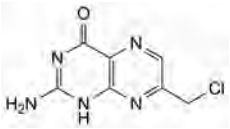

F. 2-amino-7-(chloromethyl)pteridin-4(1*H*)-one,

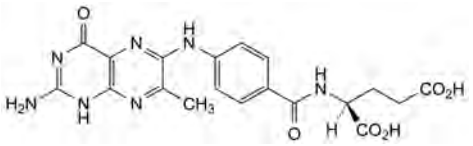

G. (2*S*)-2-[4-[(2-amino-7-methyl-4-oxo-1,4-dihydro-pteridin-6-yl)amino]benzamido]pentanedioic acid,

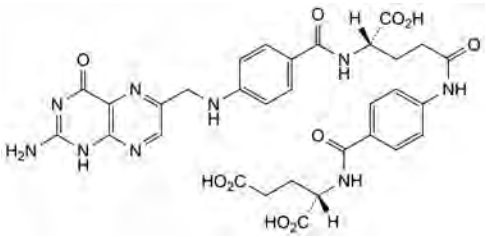

H. (2*S*)-2-[4-[(4*S*)-4-[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzamido]-4-carboxybutanamido]benzamido]pentanedioic acid,

I. unknown structure.

**01/2020:2285**

# FOLLITROPIN

## Follitropinum

α-subunit

```
APDVQDCPEC TLQENPFFSQ PGAPILQCMG CCFSRAYPTP   40
LRSKKTMLVQ KNVTSESTCC VAKSYNRVTV MGGFKVENHT   80
ACHCSTCYYH KS                                 92
```

β-subunit

```
NSCELTNITI AIEKEECRFC ISINTTWCAG YCYTRDLVYK   40*
DPARPKIQKT CTFKELVYET VRVPGCAHHA DSLYTYPVAT   80*
QCHCGKCDSD STDCTVRGLG PSYCSFGEMK E           111*
```

glycosylation sites:
Asn-52, Asn-78, Asn-7*, Asn-24*

disulfide bridges:
7-31, 10-60, 28-82, 32-84, 59-87, 3*-51*, 17*-66*, 20*-104*, 28*-82*, 32*-84*, 87*-94*

$M_r$ approx. 30 000 - 40 000

DEFINITION

Freeze-dried preparation of a heterodimeric glycoprotein having the structure of human follicle-stimulating hormone (FSH). It consists of 2 subunits: a 92-amino-acid α-chain common to other glycoprotein hormones and a specific 111-amino-acid β-chain.

*Potency*: 9000 IU to 17 000 IU per milligram of protein.

PRODUCTION

Follitropin is produced in mammalian cells by a method based on recombinant DNA (rDNA) technology.

Follitropin complies with the following requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: white or almost white powder.

IDENTIFICATION

A. It complies with the requirements described under Assay.

B. Isoelectric focusing (*2.2.54*).

*Test solution.* Dissolve the substance to be examined in *water R* to obtain a concentration of about 2 mg/mL, then desalt and concentrate using a suitably validated procedure. Reconstitute the recovered material in *water R* to obtain a concentration of 5 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 5 mg/mL.

*Focusing*:

- *pH gradient*: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point (pI) range of 3.5-5.5 is selected, as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunction; otherwise, suitable dilute mineral or organic acids and bases are employed at pH levels respectively lower and higher than the functional range of the ampholytes;
- *catholyte*: 20.0 g/L solution of *glycine R*;
- *anolyte*: solution containing 3.4 g/L of *aspartic acid R* and 3.6 g/L of *glutamic acid R*, adjusted to pH 2.8-3.8;
- *application*: 10 µL.

*Detection*: as described in *2.2.54*.

*System suitability*:

- in the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with *follitropin CRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin CRS*.

*Results*: examine the electropherogram obtained with the test solution; identify the bands observed by comparison with the electropherogram obtained with the reference solution; the pattern of bands is qualitatively similar to that seen with the reference solution.

C. Examine the chromatograms obtained in the test for follitropin oligomers.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

D. Peptide mapping (*2.2.55*).

*SEPARATION OF THE α- AND β-SUBUNITS.* Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve the substance to be examined in mobile phase A to obtain a concentration of about 0.4 mg/mL.

*Reference solution.* Dilute *follitropin for peptide mapping and glycan analysis CRS* with mobile phase A to obtain a concentration of about 0.4 mg/mL.

*Precolumn*:

- *size*: *l* = 0.012 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *trifluoroacetic acid R, water for chromatography R, acetonitrile for chromatography R* (0.9:50:950 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 8 | 100 → 76 | 0 → 24 |
| 8 - 17 | 76 | 24 |
| 17 - 36 | 76 → 70 | 24 → 30 |
| 36 - 41 | 70 → 25 | 30 → 75 |
| 41 - 46 | 25 | 75 |
| 46 - 47 | 25 → 100 | 75 → 0 |
| 47 - 57 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 800 µL.

*Retention time*: β-subunit = about 14 min; α-subunit = about 30 min.

Collect the fractions containing the α- and β-subunits and freeze-dry them.

*REDUCTION, MODIFICATION AND DESALTING OF THE PURIFIED SUBUNITS*

**Reduction and modification**

*Solution A*. Dilute 10 µL of *tributylphosphine R* to 2 mL with *propanol R*. Saturate with nitrogen.

*Solution B*. Dilute 20 µL of *4-vinylpyridine R* to 200 µL with *propanol R*. Saturate with nitrogen.

*Test solutions.* Dissolve each of the α- and β-subunit fractions obtained from the test solution in the previous step in 300 µL of *guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.5 R* and incubate at 37 °C for 60 min in a thermostatically controlled water-bath. Add 100 µL of solution A, mix and saturate with nitrogen. Incubate at 37 °C for 90 min. Add 10 µL of solution B, mix and saturate with nitrogen. Incubate at 37 °C for 45 min. Add 100 µL of a 10 per cent *V/V* solution of *trifluoroacetic acid R* and mix.

*Reference solutions.* Prepare at the same time and in the same manner as for the test solutions but using the α- and β-subunit fractions obtained from the reference solution in the previous step.

**Desalting**

Dilute the α- and β-subunit test and reference solutions to 840 µL with mobile phase A.

*Column*:

– *size*: *l* = 0.02 m, Ø = 4.6 mm;

– *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;

– *mobile phase B*: *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile for chromatography R* (1:300:700 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 100 | 0 |
| 7 - 27 | 100 → 0 | 0 → 100 |
| 27 - 27.01 | 0 → 100 | 100 → 0 |
| 27.01 - 32 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 800 µL.

For each solution the chromatogram shows a principal peak due to the monovinylpyridine-modified subunit and several minor peaks due to the di- and oligovinylpyridine-modified subunits. Only the fraction containing the monovinylpyridine-modified subunit is used for digestion in the following step.

*Retention time*: *α-subunit solution*: monovinylpyridine-modified α-subunit = about 15 min; *β-subunit solution*: monovinylpyridine-modified β-subunit = about 16 min.

Collect the fractions containing the monovinylpyridine-modified subunits and freeze-dry them.

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Solution C* (8 M urea solution). Dissolve 480 g of *urea R* in 600 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent. Add about 3-5 g of mixed-bed resin and stir for about 1 h. Filter through a glass filter before use.

*Solution D*. Dissolve 15.8 g of *ammonium hydrogen carbonate R* and 8.3 g of *sodium edetate R* in 800 mL of *water for chromatography R*. Adjust to pH 7.8 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water for chromatography R*.

*Test solutions*. Dissolve each of the modified α- and β-subunits obtained from the test solutions in the previous step in 42.5 µL of solution C and incubate at room temperature for 30 min. Add 42.5 µL of solution D and mix. To 42.5 µL of these solutions add 35 µL of a solution containing about 23 mU/µL of endoproteinase Lys-C and mix. Incubate at 37 °C for 4 h, then add 35 µL of the same endoproteinase Lys-C solution and mix. Incubate at 37 °C overnight, then dilute to 420 µL with mobile phase A.

*Reference solutions*. Prepare at the same time and in the same manner as for the test solutions but using the fractions obtained from the reference solutions in the previous step.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Precolumn*:

– *size*: *l* = 0.02 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;

– *mobile phase B*: *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile R1* (1:300:700 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 100 | 0 |
| 7 - 77 | 100 → 30 | 0 → 70 |
| 77 - 82 | 30 → 0 | 70 → 100 |
| 82 - 87 | 0 | 100 |
| 87 - 92 | 0 → 100 | 100 → 0 |
| 92 - 107 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 400 µL.

*System suitability*:

*α-subunit*:

– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin α-subunit digest supplied with *follitropin for peptide mapping and glycan analysis CRS*; both chromatograms show peaks due to the L4, L6, L3, L5 and L1-2/L1 fragments;

– retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragments L4, L6 and L3, not more than 3 per cent for fragment L5 and not more than 2 per cent for fragments L1-2/L1;

*β-subunit*:

– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin β-subunit digest supplied with *follitropin for peptide mapping and glycan analysis CRS*; both chromatograms show peaks due to the L5, L7, L6, and L1-4 fragments;

– retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragment L5, not more than 2 per cent for fragments L7 and L6 and not more than 1 per cent for fragments L1-4.

*Results*: for each subunit, the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the corresponding reference solution.

E. Glycan analysis (*2.2.59*). Carry out either method A or method B.

METHOD A

*PROTEIN DENATURATION*

*Test solution*. Dissolve 500 µg of the substance to be examined in 60 µL of *0.05 M phosphate buffer solution pH 7.5 R*. Add 6 µL of a 10 mg/mL solution of *sodium dodecyl sulfate R* and 35 µL of a 1 per cent *V/V* solution of *2-mercaptoethanol R*. Mix using a vortex mixer, centrifuge and incubate at 37 °C for 15 min.

*Reference solution*. Freeze dry a sample of *follitropin for peptide mapping and glycan analysis CRS* that contains 500 µg of follitropin. Dissolve in 60 µL of *0.05 M phosphate buffer solution pH 7.5 R* and continue as for the test solution.

*SELECTIVE RELEASE OF THE GLYCANS*

*Test solution*. To the test solution obtained in the previous step add 0.75 µL of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F R*, mix using a vortex mixer and centrifuge. Incubate at 37 °C for 24 h. Remove the protein fraction using a suitable, validated procedure. The following method has been found

to be appropriate. Add 600 µL of *anhydrous ethanol R*, previously cooled at – 20 °C for 45 min. Mix using a vortex mixer and centrifuge. Precipitate the proteins at – 20 °C for 15 min, then centrifuge at 10 600 *g* at 4 °C for 5 min. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 min. Add 1 mL of *particle-free water R* and resume evaporating until the remaining volume is about 500-800 µL, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimised and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labelled glycans from the reaction. Recover the sample in 1.5 mL of *particle-free water R*.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using the reference solution obtained in the previous step.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.075 m, Ø = 7.5 mm;

– *stationary phase*: *weak anion-exchange resin R* (10 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*;

– *mobile phase B*: *0.5 M ammonium acetate buffer solution pH 4.5 R*; filter through a membrane filter (nominal pore size 0.22 µm);

– *mobile phase C*: *particle-free water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 5 | 20 | 0 | 80 |
| 5 - 21 | 20 | 0 → 4 | 80 → 76 |
| 21 - 61 | 20 | 4 → 25 | 76 → 55 |
| 61 - 62 | 20 | 25 → 50 | 55 → 30 |
| 62 - 71 | 20 | 50 | 30 |
| 71 - 72 | 20 | 50 → 0 | 30 → 80 |
| 72 - 117 | 20 | 0 | 80 |

*Flow rate*: 0.4 mL/min.

*Detection*: fluorimeter at 330 nm for excitation and at 420 nm for emission.

*Injection*: 50 µL.

*System suitability*: reference solution:

– the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*;

– by comparison with the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms; determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

$A_0$ = peak area percentage due to the neutral form;

$A_1$ = peak area percentage due to the mono-sialylated form;

$A_2$ = peak area percentage due to the di-sialylated form;

$A_3$ = peak area percentage due to the tri-sialylated form;

$A_4$ = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for the reference solution is in the range 177-233.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

*Result*: *Z* = 177-233.

METHOD B

*PROTEIN DENATURATION*

*Solution A.* To 1.952 g of *2-[N-morpholino]ethanesulfonic acid R* and 57.32 g of *guanidine hydrochloride R*, add 1 mL of a 15.4 g/L solution of *dithiothreitol R*, 10 mL of an 18.61 g/L solution of *sodium edetate R* and 20 mL of *water for chromatography R*. Maintain in a water-bath at about 37 °C for 1 min to dissolve the components. Adjust to pH 8.1 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with *water for chromatography R*. Mix.

*Solution B.* Dissolve 37 mg of *iodoacetamide R* in 1 mL of *water for chromatography R* and mix. Protect from light.

*Solution C.* Dissolve 26.7 g of *disodium hydrogen phosphate dihydrate R* and 11.2 g of *sodium edetate R* in 3000 mL of *water for chromatography R* and mix. Adjust to pH 7.5 (*2.2.3*) with a 40 g/L solution of *sodium hydroxide R*.

*Test solution.* Dissolve 1 mg of the substance to be examined in 0.2 mL of solution A and incubate in a water-bath at 37 ± 1 °C for 2 h. Add 20 µL of freshly prepared solution B, mix and incubate at 37 ± 1 °C for a further 2 h, protected from light. Add 10 µL of *2-mercaptoethanol R* and mix. Dialyse against 1000 mL of solution C. Add 200 µL of solution C and mix. Determine the protein content of the solution.

*Reference solution (a).* To a volume of *follitropin for peptide mapping and glycan analysis CRS* that contains 1 mg of follitropin, add 0.2 mL of solution A. Incubate in a water-bath at 37 ± 1 °C for 2 h. Continue as for the test solution. Determine the protein content of the solution.

*Reference solution (b).* Prepare at the same time and in the same manner as for the test solution but using fetuin instead of the substance to be examined. Determine the protein content of the solution.

*SELECTIVE RELEASE OF THE GLYCANS*

*Test solution.* Dilute the test solution obtained in the previous step with solution C to obtain a concentration of 1.1 g/L. Add 1 U of *peptide N-glycosidase F R* to 500 µg of the solution, mix and incubate at 37 ± 1 °C for 24 h. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol R* and allow to stand in ice for 10 min. Centrifuge at 16 000 *g* for about 5 min and transfer the supernatant to a separate tube. Add 3 µL of a 1 µg/µL solution of *maltotriose R*, then freeze-dry. Dissolve in 100 µL of *water for chromatography R*.

*Reference solution (a).* Prepare in the same manner as for the test solution but using the reference solution obtained with *follitropin for peptide mapping and glycan analysis CRS* in the previous step.

*Reference solution (b).* Prepare in the same manner as for the test solution but using the reference solution obtained with fetuin in the previous step.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Precolumn*:

- *size*: *l* = 0.05 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*;

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R.*

*Mobile phase*:

- *mobile phase A*: 20 g/L solution of *sodium hydroxide R*; maintain under helium;
- *mobile phase B*: *water for chromatography R*; maintain under helium;
- *mobile phase C*: dissolve 41 g of *anhydrous sodium acetate R* in 800 mL of *water for chromatography R*, dilute to 1000 mL with the same solvent, then mix; filter through a membrane filter (nominal pore size 0.45 µm); maintain under helium;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 0.2 | 20 | 80 | 0 |
| 0.2 - 94.0 | 20 | 80 → 34 | 0 → 46 |
| 94.0 - 97.0 | 20 | 34 | 46 |
| 97.0 - 97.1 | 20 | 34 → 80 | 46 → 0 |
| 97.1 - 115.0 | 20 | 80 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and − 0.80 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 45 µL.

*System suitability*:

- the chromatogram obtained with reference solution (b) is qualitatively similar to the chromatogram for fetuin supplied with *follitropin for peptide mapping and glycan analysis CRS*;
- the chromatograms obtained with the test solution and reference solution (a) are qualitatively similar to the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*;
- by comparison with the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms in the chromatogram obtained with reference solution (b); determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

$A_0$ = peak area percentage due to the neutral form;

$A_1$ = peak area percentage due to the mono-sialylated form;

$A_2$ = peak area percentage due to the di-sialylated form;

$A_3$ = peak area percentage due to the tri-sialylated form;

$A_4$ = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for reference solution (b) is in the range 290-325.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

*Result*: *Z* = 178-274.

TESTS

**Follitropin oligomers**. Size-exclusion chromatography (*2.2.30*). Use the normalisation procedure.

*Solution A*. Dissolve 118 mg of *sodium dihydrogen phosphate R*, 1.65 g of *disodium hydrogen phosphate dihydrate R* and 30.0 g of *sucrose R* in 40 mL of *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Solution B*. Dissolve 2.0 mg of *bovine albumin R* in 30 mL of solution A.

*Test solution.* Dissolve the substance to be examined in solution A to obtain a concentration of 0.25 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *follitropin CRS* in solution A to obtain a concentration of 0.5 mg/mL and mix equal volumes of this solution and solution B to obtain a concentration of 0.25 mg/mL.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 300 000 (5 µm).

*Mobile phase*: dissolve 28.4 g of *anhydrous sodium sulfate R* in 2000 mL of *0.1 M phosphate buffer solution pH 6.7 R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 100 µL.

*Retention time*: follitropin = 14-16 min.

*System suitability*: reference solution:

- *resolution*: minimum 1.2 between the peaks due to bovine albumin and follitropin;
- no peak is detected between 5 min and 16 min in blank injections.

*Limit*:

- *sum of the peaks with a retention time less than that of the principal peak*: maximum 0.5 per cent.

**Free subunits**. Polyacrylamide gel electrophoresis (*2.2.31*) under non-reducing conditions.

*Gel dimensions*: 1.5 mm thick.

*Resolving gel*: 12 per cent acrylamide.

*Sample buffer. Concentrated SDS-PAGE sample buffer R.*

*Test solution.* Dissolve the substance to be examined in *water R* to obtain a concentration of 2 µg/µL. To 55 µL of the solution add 55 µL of the sample buffer. Allow to stand for 4 h at room temperature.

Monographs F

*Reference solution (a).* Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 2 µg/µL. To 25 µL of the solution add 25 µL of the sample buffer. To 40 µL of this solution add 180 µL of the sample buffer and 180 µL of *water R*. Allow to stand for 4 h at room temperature, then boil for 5 min.

*Reference solution (b).* A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

*Application*:

| Well | Solution(s) | Volume (µL) |
|---|---|---|
| 1 | Reference solution (a) | 40 |
| 2 | Reference solution (a) | 30 |
| 3 | Reference solution (a) | 20 |
| 4 | Reference solution (a) | 15 |
| 5 | Reference solution (a) | 10 |
| 6 | Reference solution (a) | 5 |
| 7 | Test solution | 50 |
| 8 | Test solution + reference solution (a) | 50 + 25 |
| 9 | Reference solution (b) | 10 |

*Detection*: by Coomassie staining.

*System suitability*:

- reference solution (b): the validation criteria are met (*2.2.31*);
- test solution + reference solution (a): the bands corresponding to the follitropin heterodimer and subunits are clearly separated;
- reference solution (a): no bands corresponding to the follitropin heterodimer are seen.

*Limit*:

- *free subunits*: maximum 3 per cent.

**Oxidised follitropin**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve about 3.3 mg of *2,4-dichlorobenzoic acid R* in 10.0 mL of *ethanol (96 per cent) R*.

*Test solution.* Dissolve the substance to be examined in *water for chromatography R* to obtain a concentration of 300 µg/mL.

*Reference solution (a).* Dissolve the contents of a vial of *follitropin CRS* in *water for chromatography R* to obtain a concentration of 300 µg/mL.

*Reference solution (b).* Dilute 0.1 mL of *strong hydrogen peroxide solution R* to 30 mL with *water for chromatography R*. Dissolve the contents of a vial of *follitropin CRS* in this solution to obtain a concentration of 300 µg/mL. Incubate for 30-45 min. Add solution A to obtain a concentration in 2,4-dichlorobenzoic acid of about 17 µg/mL in the total volume and inject immediately.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *0.2 M phosphate buffer solution pH 2.5 R*;
- *mobile phase B*: *water for chromatography R, acetonitrile R1* (40:60 *V/V*);
- *mobile phase C*: *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 8.4 | 50 | 25 → 39 | 25 → 11 |
| 8.4 - 8.5 | 50 | 39 → 45 | 11 → 5 |
| 8.5 - 15 | 50 | 45 | 5 |
| 15 - 15.1 | 50 | 45 → 25 | 5 → 25 |
| 15.1 - 25 | 50 | 25 | 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 25 µL.

*System suitability*: reference solution (b):

- the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid;
- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin CRS*.

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

$A_1$ = area of the peak due to the follitropin α-subunit;

$A_2$ = area of the peaks due to the oxidised follitropin α-subunit;

$A_3$ = area of the peak due to the follitropin β-subunit;

$A_4$ = area of the peak due to the oxidised follitropin β-subunit.

*Limit*:

- *total oxidised forms*: maximum 6 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 0.1 IU per International Unit of follitropin activity, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

**Protein**. Size-exclusion chromatography (*2.2.30*).

*Solution A*. Dissolve 100 mg of *poloxamer 188 R* in 900 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent.

*Test solution.* Dissolve the substance to be examined in solution A to obtain a concentration of about 0.03 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *follitropin CRS* in solution A to obtain a concentration of about 0.03 mg/mL.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 300 000 (5 µm).

*Mobile phase*: mix 6.74 mL of *phosphoric acid R*, 14.2 g of *anhydrous sodium sulfate R* and 900 mL of *water for chromatography R*, adjust to pH 6.7 (*2.2.3*) with a 0.5 g/mL solution of *sodium hydroxide R* and dilute to 1000 mL with *water for chromatography R*; filter through a membrane filter (nominal pore size 0.45 µm).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*System suitability*: reference solution:

- *number of theoretical plates*: minimum 1300, calculated for the peak due to follitropin.

Calculate the content of follitropin taking into account the assigned content of *follitropin CRS*.

**Potency**

The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 mL. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g/L of phenol or 0.02 g/L of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 h and 48 h after the 1st injection. About 24 h after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods (for example, *5.3*), using the mass of the 2 combined ovaries as the response (a suitable correction of the organ mass with reference to the mass of the rat from which it was taken may be applied; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, at a temperature not exceeding – 20 °C.

LABELLING

The label states:

- the potency in International Units per milligram of protein;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2020:2286**

# FOLLITROPIN CONCENTRATED SOLUTION

## Follitropini solutio concentrata

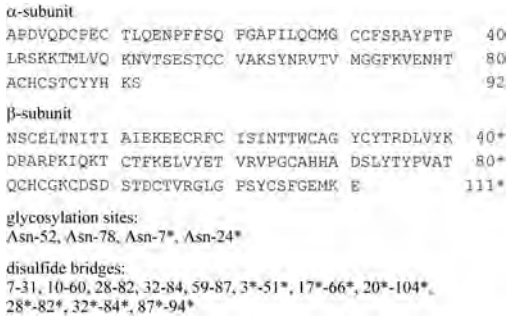


$M_r$ approx. 30 000 - 40 000

DEFINITION

Solution of a heterodimeric glycoprotein having the structure of human follicle-stimulating hormone (FSH). It consists of 2 subunits: a 92-amino-acid α-chain common to other glycoprotein hormones and a specific 111-amino-acid β-chain.

*Content*: 0.4 mg to 0.8 mg of protein per millilitre.

*Potency*: 9000 IU to 17 000 IU per milligram of protein.

PRODUCTION

Follitropin is produced in mammalian cells by a method based on recombinant DNA (rDNA) technology.

Follitropin complies with the following requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: clear or slightly turbid, colourless liquid.

IDENTIFICATION

A. It complies with the requirements described under Assay.

B. Isoelectric focusing (*2.2.54*).

*Test solution.* Desalt and concentrate the preparation to be examined using a suitably validated procedure. Reconstitute the recovered material in *water R* to obtain a concentration of 5 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 5 mg/mL.

*Focusing*:

- *pH gradient*: a combination of ampholytes and electrode buffers giving a functional separation in the isoelectric point (pI) range of 3.5-5.5 is selected, as defined by the system suitability criteria; where pre-cast gels are employed, proprietary electrode solutions may be used in conjunction; otherwise, suitable dilute mineral or

Monographs F

organic acids and bases are employed at pH levels respectively lower and higher than the functional range of the ampholytes;

- *catholyte*: 20.0 g/L solution of *glycine R*;
- *anolyte*: solution containing 3.4 g/L of *aspartic acid R* and 3.6 g/L of *glutamic acid R*, adjusted to pH 2.8-3.8;
- *application*: 10 µL.

*Detection*: as described in *2.2.54*.

*System suitability*:

- in the electropherogram obtained with the reference solution, the number of bands seen in the pI region 3.5-5.5 corresponds to that shown in the electropherogram supplied with *follitropin CRS*; the distribution of bands in the pI region 3.5-5.5 is qualitatively similar to that shown in the electropherogram supplied with *follitropin CRS*.

*Results*: examine the electropherogram obtained with the test solution; identify the bands observed by comparison with the electropherogram obtained with the reference solution; the pattern of bands is qualitatively similar to that seen with the reference solution.

C. Examine the chromatograms obtained in the test for follitropin oligomers.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

D. Peptide mapping (*2.2.55*).

*SEPARATION OF THE α- AND β-SUBUNITS*. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the preparation to be examined with mobile phase A to obtain a concentration of about 0.4 mg/mL.

*Reference solution*. Dilute *follitropin for peptide mapping and glycan analysis CRS* with mobile phase A to obtain a concentration of about 0.4 mg/mL

*Precolumn*:

- *size*: *l* = 0.012 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile for chromatography R* (0.9:50:950 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 8 | 100 → 76 | 0 → 24 |
| 8 - 17 | 76 | 24 |
| 17 - 36 | 76 → 70 | 24 → 30 |
| 36 - 41 | 70 → 25 | 30 → 75 |
| 41 - 46 | 25 | 75 |
| 46 - 47 | 25 → 100 | 75 → 0 |
| 47 - 57 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 800 µL.

*Retention time*: β-subunit = about 14 min; α-subunit = about 30 min.

Collect the fractions containing the α- and β-subunits and freeze-dry them.

*REDUCTION, MODIFICATION AND DESALTING OF THE PURIFIED SUBUNITS*

**Reduction and modification**

*Solution A*. Dilute 10 µL of *tributylphosphine R* to 2 mL with *propanol R*. Saturate with nitrogen.

*Solution B*. Dilute 20 µL of *4-vinylpyridine R* to 200 µL with *propanol R*. Saturate with nitrogen.

*Test solutions*. Dissolve each of the α- and β-subunit fractions obtained from the test solution in the previous step in 300 µL of *guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.5 R* and incubate at 37 °C for 60 min in a thermostatically controlled water-bath. Add 100 µL of solution A, mix and saturate with nitrogen. Incubate at 37 °C for 90 min. Add 10 µL of solution B, mix and saturate with nitrogen. Incubate at 37 °C for 45 min. Add 100 µL of a 10 per cent *V/V* solution of *trifluoroacetic acid R* and mix.

*Reference solutions*. Prepare at the same time and in the same manner as for the test solutions but using the α- and β-subunit fractions obtained from the reference solution in the previous step.

**Desalting**

Dilute the α- and β-subunit test and reference solutions to 840 µL with mobile phase A.

*Column*:

- *size*: *l* = 0.02 m, Ø = 4.6 mm;
- *stationary phase*: *butylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile for chromatography R* (1:300:700 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 100 | 0 |
| 7 - 27 | 100 → 0 | 0 → 100 |
| 27 - 27.01 | 0 → 100 | 100 → 0 |
| 27.01 - 32 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 800 µL.

For each solution the chromatogram shows a principal peak due to the monovinylpyridine-modified subunit and several minor peaks due to the di- and oligovinylpyridine-modified subunits. Only the fraction containing the monovinylpyridine-modified subunit is used for digestion in the following step.

*Retention time*: *α-subunit solution*: monovinylpyridine-modified α-subunit = about 15 min; *β-subunit solution*: monovinylpyridine-modified β-subunit = about 16 min.

Collect the fractions containing the monovinylpyridine-modified subunits and freeze-dry them.

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Solution C* (8 M urea solution). Dissolve 480 g of *urea R* in 600 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent. Add about 3-5 g of mixed-bed resin and stir for about 1 h. Filter through a glass filter before use.

*Solution D*. Dissolve 15.8 g of *ammonium hydrogen carbonate R* and 8.3 g of *sodium edetate R* in 800 mL of *water for chromatography R*. Adjust to pH 7.8 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water for chromatography R*.

*Test solutions*. Dissolve each of the modified α- and β-subunits obtained from the test solutions in the previous step in 42.5 µL of solution C and incubate at room temperature for 30 min. Add 42.5 µL of solution D and mix. To 42.5 µL of these solutions add 35 µL of a solution containing about 23 mU/µL of endoproteinase Lys-C and mix. Incubate at 37 °C for 4 h, then add 35 µL of the same endoproteinase Lys-C solution and mix. Incubate at 37 °C overnight, then dilute to 420 µL with mobile phase A.

*Reference solutions*. Prepare at the same time and in the same manner as for the test solutions but using the fractions obtained from the reference solutions in the previous step.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Precolumn*:

- *size*: *l* = 0.02 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile R1* (1:300:700 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 100 | 0 |
| 7 - 77 | 100 → 30 | 0 → 70 |
| 77 - 82 | 30 → 0 | 70 → 100 |
| 82 - 87 | 0 | 100 |
| 87 - 92 | 0 → 100 | 100 → 0 |
| 92 - 107 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 400 µL.

*System suitability*:

*α-subunit*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of follitropin α-subunit digest supplied with *follitropin for peptide mapping and glycan analysis CRS*; both chromatograms show peaks due to the L4, L6, L3, L5 and L1-2/L1 fragments;
- retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragments L4, L6 and L3, not more than 3 per cent for fragment L5 and not more than 2 per cent for fragments L1-2/L1;

*β-subunit*:

- the chromatogram obtained with the reference solutions is qualitatively similar to the chromatogram of follitropin β-subunit digest supplied with *follitropin for peptide mapping and glycan analysis CRS*; both chromatograms show peaks due to the L5, L7, L6, and L1-4 fragments;
- retention times obtained with the test and reference solutions differ by not more than 5 per cent for fragment L5, not more than 2 per cent for fragments L7 and L6 and not more than 1 per cent for fragments L1-4.

*Results*: for each subunit, the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the corresponding reference solution.

E. Glycan analysis (*2.2.59*). Carry out either method A or method B.

METHOD A

*PROTEIN DENATURATION*

*Test solution*. Freeze-dry a sample of the preparation to be examined that contains 500 µg of follitropin. Dissolve in 60 µL of *0.05 M phosphate buffer solution pH 7.5 R*. Add 6 µL of a 10 mg/mL solution of *sodium dodecyl sulfate R* and 35 µL of a 1 per cent *V/V* solution of *2-mercaptoethanol R*. Mix using a vortex mixer, centrifuge and incubate at 37 °C for 15 min.

*Reference solution*. Prepare at the same time and in the same manner as for the test solution but using *follitropin for peptide mapping and glycan analysis CRS* instead of the freeze-dried preparation to be examined.

*SELECTIVE RELEASE OF THE GLYCANS*

*Test solution*. To the test solution obtained in the previous step add 0.75 µL of octylphenyl-polyethylene glycol and mix using a vortex mixer. Add 25 mU of *peptide N-glycosidase F R*, mix using a vortex mixer and centrifuge. Incubate at 37 °C for 24 h. Remove the protein fraction using a suitable, validated procedure. The following method has been found to be appropriate. Add 600 µL of *anhydrous ethanol R*, previously cooled at − 20 °C for 45 min. Mix using a vortex mixer and centrifuge. Precipitate the proteins at − 20 °C for 15 min, then centrifuge at 10 600 *g* at 4 °C for 5 min. Transfer the supernatant to a separate tube and evaporate the ethanol for 15 min. Add 1 mL of *particle-free water R* and resume evaporating until the remaining volume is about 500-800 µL, then freeze-dry.

Label the liberated glycans contained in the sample with 2-aminobenzamide. The procedure employs a combination of reagents optimised and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labelled glycans from the reaction. Recover the sample in 1.5 mL of *particle-free water R*.

*Reference solution*. Prepare at the same time and in the same manner as for the test solution but using the reference solution obtained in the previous step.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.075 m, Ø = 7.5 mm;
- *stationary phase*: *weak anion-exchange resin R* (10 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*;
- *mobile phase B*: *0.5 M ammonium acetate buffer solution pH 4.5 R*; filter through a membrane filter (nominal pore size 0.22 µm);
- *mobile phase C*: *particle-free water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 5 | 20 | 0 | 80 |
| 5 - 21 | 20 | 0 → 4 | 80 → 76 |
| 21 - 61 | 20 | 4 → 25 | 76 → 55 |
| 61 - 62 | 20 | 25 → 50 | 55 → 30 |
| 62 - 71 | 20 | 50 | 30 |
| 71 - 72 | 20 | 50 → 0 | 30 → 80 |
| 72 - 117 | 20 | 0 | 80 |

*Flow rate*: 0.4 mL/min.

*Detection*: fluorimeter at 330 nm for excitation and at 420 nm for emission.

*Injection*: 50 µL.

*System suitability*: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*;
- by comparison with the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms; determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

$A_0$ = peak area percentage due to the neutral form;

$A_1$ = peak area percentage due to the mono-sialylated form;

$A_2$ = peak area percentage due to the di-sialylated form;

$A_3$ = peak area percentage due to the tri-sialylated form;

$A_4$ = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for the reference solution is in the range 177-233.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

*Result*: *Z* = 177-233.

METHOD B

*PROTEIN DENATURATION*

*Solution A*. To 1.952 g of *2-[N-morpholino]ethanesulfonic acid R* and 57.32 g of *guanidine hydrochloride R*, add 1 mL of a 15.4 g/L solution of *dithiothreitol R*, 10 mL of an 18.61 g/L solution of *sodium edetate R* and 20 mL of *water for chromatography R*. Maintain in a water-bath at about 37 °C for 1 min to dissolve the components. Adjust to pH 8.1 (*2.2.3*) with an 80 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with *water for chromatography R*. Mix.

*Solution B*. Dissolve 37 mg of *iodoacetamide R* in 1 mL of *water for chromatography R* and mix. Protect from light.

*Solution C*. Dissolve 26.7 g of *disodium hydrogen phosphate dihydrate R* and 11.2 g of *sodium edetate R* in 3000 mL of *water for chromatography R* and mix. Adjust to pH 7.5 (*2.2.3*) with a 40 g/L solution of *sodium hydroxide R*.

*Test solution*. To a volume of the preparation to be examined that contains 1 mg of follitropin add 0.2 mL of solution A and incubate in a water-bath at 37 ± 1 °C for 2 h. Add 20 µL of freshly prepared solution B, mix and incubate at 37 ± 1 °C for a further 2 h, protected from light. Add 10 µL of *2-mercaptoethanol R* and mix. Dialyse against 1000 mL of solution C. Add 200 µL of solution C and mix. Determine the protein content of the solution.

*Reference solution (a)*. Prepare at the same time and in the same manner as for the test solution but using *follitropin for peptide mapping and glycan analysis CRS* instead of the preparation to be examined. Determine the protein content of the solution.

*Reference solution (b)*. Prepare at the same time and in the same manner as for the test solution but using fetuin instead of the preparation to be examined. Determine the protein content of the solution.

*SELECTIVE RELEASE OF THE GLYCANS*

*Test solution*. Dilute the test solution obtained in the previous step with solution C to obtain a concentration of 1.1 g/L. Add 1 U of *peptide N-glycosidase F R* to 500 µg of the solution, mix and incubate at 37 ± 1 °C for 24 h. Place the solution in ice. Precipitate the protein and salts with 3 volumes of ice-cold *anhydrous ethanol R* and allow to stand in ice for 10 min. Centrifuge at 16 000 *g* for about 5 min and transfer the supernatant to a separate tube. Add 3 µL of a 1 µg/µL solution of *maltotriose R* then freeze-dry. Dissolve in 100 µL of *water for chromatography R*.

*Reference solution (a)*. Prepare in the same manner as for the test solution but using the reference solution obtained with *follitropin for peptide mapping and glycan analysis CRS* in the previous step.

*Reference solution (b)*. Prepare in the same manner as for the test solution but using the reference solution obtained with fetuin in the previous step.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Precolumn*:

- *size*: *l* = 0.05 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*.

*Mobile phase*:

- *mobile phase A*: 20 g/L solution of *sodium hydroxide R*; maintain under helium;
- *mobile phase B*: *water for chromatography R*; maintain under helium;
- *mobile phase C*: dissolve 41 g of *anhydrous sodium acetate R* in 800 mL of *water for chromatography R*, dilute to 1000 mL with the same solvent, then mix; filter through a membrane filter (nominal pore size 0.45 µm); maintain under helium;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 0.2 | 20 | 80 | 0 |
| 0.2 - 94.0 | 20 | 80 → 34 | 0 → 46 |
| 94.0 - 97.0 | 20 | 34 | 46 |
| 97.0 - 97.1 | 20 | 34 → 80 | 46 → 0 |
| 97.1 - 115.0 | 20 | 80 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and − 0.80 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 45 µL.

*System suitability*:

- the chromatogram obtained with reference solution (b) is qualitatively similar to the chromatogram for fetuin supplied with *follitropin for peptide mapping and glycan analysis CRS*;
- the chromatograms obtained with the test solution and reference solution (a) are qualitatively similar to the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*;
- by comparison with the chromatogram supplied with *follitropin for peptide mapping and glycan analysis CRS*, identify the peaks due to neutral, mono-, di-, tri- and tetra-sialylated forms in the chromatogram obtained with reference solution (b); determine the area of each peak and express it as a percentage of the total; calculate the *Z* number using the following expression:

$$(A_0 \times 0) + (A_1 \times 1) + (A_2 \times 2) + (A_3 \times 3) + (A_4 \times 4)$$

$A_0$ = peak area percentage due to the neutral form;

$A_1$ = peak area percentage due to the mono-sialylated form;

$A_2$ = peak area percentage due to the di-sialylated form;

$A_3$ = peak area percentage due to the tri-sialylated form;

$A_4$ = peak area percentage due to the tetra-sialylated form.

The *Z* number obtained for reference solution (b) is in the range 290-325.

Examine the chromatogram obtained with the test solution and calculate the *Z* number as described above.

*Result*: *Z* = 178-274.

TESTS

**Follitropin oligomers**. Size-exclusion chromatography (*2.2.30*). Use the normalisation procedure.

*Solution A*. Dissolve 118 mg of *sodium dihydrogen phosphate R*, 1.65 g of *disodium hydrogen phosphate dihydrate R*, and 30.0 g of *sucrose R* in 40 mL of *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Solution B*. Dissolve 2.0 mg of *bovine albumin R* in 30 mL of solution A.

*Test solution*. Dilute the preparation to be examined with solution A to obtain a concentration of 0.25 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *follitropin CRS* in solution A to obtain a concentration of 0.5 mg/mL and mix equal volumes of this solution and solution B to obtain a concentration of 0.25 mg/mL.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 300 000 (5 µm).

*Mobile phase*: dissolve 28.4 g of *anhydrous sodium sulfate R* in 2000 mL of *0.1 M phosphate buffer solution pH 6.7 R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 100 µL.

*Retention time*: follitropin = 14-16 min.

*System suitability*: reference solution:

- *resolution*: minimum 1.2 between the peaks due to bovine albumin and follitropin;
- no peak is detected between 5 min and 16 min in blank injections.

*Limit*:

- *sum of the peaks with a retention time less than that of the principal peak*: maximum 0.5 per cent.

**Free subunits**. Polyacrylamide gel electrophoresis (*2.2.31*) under non-reducing conditions.

*Gel dimensions*: 1.5 mm thick.

*Resolving gel*: 12 per cent acrylamide.

*Sample buffer. Concentrated SDS-PAGE sample buffer R.*

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of 2 µg/µL. To 55 µL of the solution add 55 µL of the sample buffer. Allow to stand for 4 h at room temperature.

*Reference solution (a)*. Dissolve the contents of a vial of *follitropin CRS* in *water R* to obtain a concentration of 2 µg/µL. To 25 µL of the solution add 25 µL of the sample buffer. To 40 µL of this solution add 180 µL of the sample buffer and 180 µL of *water R*. Allow to stand for 4 h at room temperature, then boil for 5 min.

*Reference solution (b)*. A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 14.4-94 kDa.

*Application*:

| Well | Solution(s) | Volume (µL) |
|---|---|---|
| 1 | Reference solution (a) | 40 |
| 2 | Reference solution (a) | 30 |
| 3 | Reference solution (a) | 20 |
| 4 | Reference solution (a) | 15 |
| 5 | Reference solution (a) | 10 |
| 6 | Reference solution (a) | 5 |
| 7 | Test solution | 50 |
| 8 | Test solution + reference solution (a) | 50 + 25 |
| 9 | Reference solution (b) | 10 |

*Detection*: by Coomassie staining.

*System suitability*:

- reference solution (b): the validation criteria are met (*2.2.31*);
- test solution + reference solution (a): the bands corresponding to the follitropin heterodimer and subunits are clearly separated;
- reference solution (a): no bands corresponding to the follitropin heterodimer are seen.

*Limit*:

- *free subunits*: maximum 3 per cent.

**Oxidised follitropin**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve about 3.3 mg of *2,4-dichlorobenzoic acid R* in 10.0 mL of *ethanol (96 per cent) R*.

*Test solution*. Dilute the preparation to be examined in *water for chromatography R* to obtain a concentration of 300 µg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *follitropin CRS* in *water for chromatography R* to obtain a concentration of 300 µg/mL.

*Reference solution (b)*. Dilute 0.1 mL of *strong hydrogen peroxide solution R* to 30 mL with *water for chromatography R*. Dissolve the contents of a vial of *follitropin CRS* in this solution to obtain a concentration of 300 µg/mL. Incubate

for 30-45 min. Add solution A to obtain a concentration in 2,4-dichlorobenzoic acid of about 17 µg/mL in the total volume and inject immediately.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *0.2 M phosphate buffer solution pH 2.5 R*;

– *mobile phase B*: *water for chromatography R*, *acetonitrile R1* (40:60 *V/V*);

– *mobile phase C*: *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 8.4 | 50 | 25 → 39 | 25 → 11 |
| 8.4 - 8.5 | 50 | 39 → 45 | 11 → 5 |
| 8.5 - 15 | 50 | 45 | 5 |
| 15 - 15.1 | 50 | 45 → 25 | 5 → 25 |
| 15.1 - 25 | 50 | 25 | 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 25 µL.

*System suitability*: reference solution (b):

– the peaks due to the oxidised follitropin α- and β-subunits are separated from the peaks due to the non-oxidised follitropin subunits and from the peak due to 2,4-dichlorobenzoic acid;

– the chromatogram obtained is qualitatively similar to the chromatogram supplied with *follitropin CRS*.

Calculate the percentage of oxidation of the follitropin subunits using the following expression:

$$\frac{(A_2 + A_4) \times 100}{A_1 + A_2 + A_3 + A_4}$$

$A_1$ = area of the peak due to the follitropin α-subunit;

$A_2$ = area of the peaks due to the oxidised follitropin α-subunit;

$A_3$ = area of the peak due to the follitropin β-subunit;

$A_4$ = area of the peak due to the oxidised follitropin β-subunit.

*Limit*:

– *total oxidised forms*: maximum 6 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 0.1 IU per International Unit of follitropin activity, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

**Protein**. Size-exclusion chromatography (*2.2.30*).

*Solution A*. Dissolve 100 mg of *poloxamer 188 R* in 900 mL of *water for chromatography R* and dilute to 1000 mL with the same solvent.

*Test solution*. Dilute the preparation to be examined with solution A to obtain a concentration of about 0.03 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *follitropin CRS* in solution A to obtain a concentration of about 0.03 mg/mL.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 300 000 (5 µm).

*Mobile phase*: mix 6.74 mL of *phosphoric acid R*, 14.2 g of *anhydrous sodium sulfate R* and 900 mL of *water for chromatography R*, adjust to pH 6.7 (*2.2.3*) with a 0.5 g/mL solution of *sodium hydroxide R* and dilute to 1000 mL with *water for chromatography R*; filter through a membrane filter (nominal pore size 0.45 µm).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*System suitability*: reference solution:

– *number of theoretical plates*: minimum 1300, calculated for the peak due to follitropin.

Calculate the content of follitropin taking into account the assigned content of *follitropin CRS*.

**Potency**

The follicle-stimulating activity of follitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human recombinant follicle-stimulating hormone or of a reference preparation calibrated in International Units. The International Unit of FSH is the activity contained in stated amounts of the International Standard of human recombinant follicle-stimulating hormone. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 rats. If sets of 6 litter mates are available, assign 1 litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all of the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried, although the dose will depend on the sensitivity of the rats used, which may vary widely.

Dilute and dissolve respectively the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 mL. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g/L of phenol or 0.02 g/L of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 h and 48 h after the 1st injection. About 24 h after the last injection, euthanise the rats and remove the ovaries from each rat. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each rat immediately. Calculate the results by the usual statistical methods (for example, *5.3*), using the mass of the 2 combined ovaries as the response (a suitable correction of the organ mass with reference to the mass of the rat from which it was taken may be applied; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, at a temperature not exceeding − 20 °C.

LABELLING

The label states:

- the content of protein in milligrams per millilitre;
- the potency in International Units per milligram of protein;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2008:0826**

# FORMALDEHYDE SOLUTION (35 PER CENT)

## Formaldehydi solutio (35 per centum)

[50-00-0]

DEFINITION

*Content*: 34.5 per cent *m/m* to 38.0 per cent *m/m* of formaldehyde ($CH_2O$; $M_r$ 30.03).

It contains methanol as stabiliser.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

It may be cloudy after storage.

IDENTIFICATION

A. Dilute 1 mL of solution S (see Tests) to 10 mL with *water R*. To 0.05 mL of the solution add 1 mL of a 15 g/L solution of *chromotropic acid, sodium salt R*, 2 mL of *water R* and 8 mL of *sulfuric acid R*. A violet-blue or violet-red colour develops within 5 min.

B. To 0.1 mL of solution S add 10 mL of *water R*. Add 2 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R*, prepared immediately before use, 1 mL of *potassium ferricyanide solution R* and 5 mL of *hydrochloric acid R*. An intense red colour is formed.

C. Mix 0.5 mL with 2 mL of *water R* and 2 mL of *silver nitrate solution R2* in a test-tube. Add *dilute ammonia R2* until slightly alkaline. Heat on a water-bath. A grey precipitate or a silver mirror is formed.

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dilute 10 mL, filtered if necessary, to 50 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S add 1 mL of *phenolphthalein solution R*. Not more than 0.4 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to red.

**Methanol**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dilute 10 mL of *ethanol R1* to 100 mL with *water R*.

*Test solution*. To 10.0 mL of the solution to be examined add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Reference solution*. To 1.0 mL of *methanol R* add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Column*:

- *material*: glass,
- *size*: *l* = 1.5-2.0 m, Ø = 2-4 mm,
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (150-180 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30-40 mL/min.

*Temperature*:

- *column*: 120 °C,
- *injection port and detector*: 150 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and the reference solution.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to methanol and ethanol.

*Limit*:

- *methanol*: 9.0 per cent *V/V* to 15.0 per cent *V/V*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Into a 100 mL volumetric flask containing 2.5 mL of *water R* and 1 mL of *dilute sodium hydroxide solution R*, introduce 1.000 g of the solution to be examined, shake and dilute to 100.0 mL with *water R*. To 10.0 mL of the solution add 30.0 mL of *0.05 M iodine*. Mix and add 10 mL of *dilute sodium hydroxide solution R*. After 15 min, add 25 mL of *dilute sulfuric acid R* and 2 mL of *starch solution R*. Titrate with *0.1 M sodium thiosulfate*.

1 mL of *0.05 M iodine* is equivalent to 1.501 mg of $CH_2O$.

STORAGE

Protected from light, at a temperature of 15 °C to 25 °C.

**04/2018:2809**

# FORMIC ACID

## Acidum formicum

$CH_2O_2$ $M_r$ 46.03

[64-18-6]

DEFINITION

*Content*: 98.0 per cent *m/m* to 100.5 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless, volatile liquid.

*Solubility*: miscible with water, with ethanol (96 per cent) and with methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: films.

*Comparison*: *formic acid CRS*.

B. A 100 g/L solution is strongly acid (*2.2.4*).

C. To 2.0 mL of *dilute sodium hydroxide solution R* add 0.05 mL of *phenolphthalein solution R*. Mix, and add dropwise the substance to be examined until the solution becomes colourless. To 1 mL of this solution add 0.5 mL of *silver nitrate solution R2* and mix. Initially no change occurs. Slowly (quicker upon heating), the solution turns greyish and turbid due to the precipitation of metallic silver.

D. To the remainder of the neutralised solution obtained in identification test C, add 0.5 mL of *ferric chloride solution R1*. The solution turns orange-red or brownish-red.

TESTS

**Solution S**. Extract the residue obtained in the test for residue on evaporation by heating with 2 quantities, each of 15 mL, of *distilled water R*. After cooling, dilute the combined extracts to 50.0 mL with *distilled water R*.

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 1.217 to 1.223.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute 0.500 g of the substance to be examined to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 0.500 g of *anhydrous acetic acid R* (impurity A) to 100.0 mL with the mobile phase. Mix 2.0 mL of the solution and 1.0 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (4 µm).

*Mobile phase*: 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.9 with *dilute phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of formic acid.

*Relative retention* with reference to formic acid (retention time = about 3 min): impurity A = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to formic acid and impurity A.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 10 ppm.

Dilute 12.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 50 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Sulfites**: maximum 300 ppm.

To 0.5 mL add 12.0 mL of a 42 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R* and mix. Upon addition of 0.5 mL of *iodine solution R1* and 0.2 mL of *starch solution R* the solution remains blue.

**Residue on evaporation**: maximum 0.01 per cent.

Condensing the vapour where possible, evaporate 20.0 g to dryness on a water-bath and dry at 105 °C. The residue weighs a maximum of 2.0 mg. The residue is used for the preparation of solution S.

ASSAY

Weigh accurately a conical flask with a ground-glass stopper containing 20 mL of *water R*. Add 1.0 mL of the substance to be examined and weigh again accurately. Titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 46.03 mg of $CH_2O_2$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

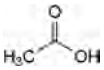

A. acetic acid.

**01/2008:1724**
**corrected 8.4**

# FORMOTEROL FUMARATE DIHYDRATE

## Formoteroli fumaras dihydricus

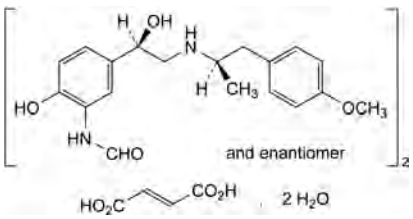


$C_{42}H_{52}N_4O_{12},2H_2O$ $M_r$ 841

DEFINITION

*N*-[2-Hydroxy-5-[(1*RS*)-1-hydroxy-2-[[(1*RS*)-2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide (*E*)-butenedioate dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or slightly yellow powder.

*Solubility*: slightly soluble in water, soluble in methanol, slightly soluble in 2-propanol, practically insoluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *formoterol fumarate dihydrate CRS*.

TESTS

**pH** (*2.2.3*): 5.5 to 6.5.

Dissolve 20 mg in *carbon dioxide-free water R* while heating to about 40 °C, allow to cool and dilute to 20 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 0.25 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 6.10 g of *sodium dihydrogen phosphate monohydrate R* and 1.03 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1000 mL with the same solvent. The pH is 6.0 ± 0.1.

*Solvent mixture*: *acetonitrile R*, solution A (16:84 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture. *Inject within 4 h of preparation, or within 24 h if stored protected from light at 4 °C.*

*Reference solution (a).* Dissolve 5 mg of *formoterol fumarate for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 25.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R3* (5 µm) with a pore size of 8 nm.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*;
- *mobile phase B*: dissolve 3.73 g of *sodium dihydrogen phosphate monohydrate R* and 0.35 g of *phosphoric acid R* in *water R* and dilute to 1000 mL with the same solvent; the pH is 3.1 ± 0.1;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 16 | 84 |
| 10 - 37 | 16 → 70 | 84 → 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL; inject the solvent mixture until a repeatable profile is obtained.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *formoterol fumarate for system suitability CRS* to identify the peaks.

*Relative retention* with reference to formoterol (retention time = about 12 min): impurity G = about 0.4; impurity A = about 0.5; impurity B = about 0.7; impurity C = about 1.2; impurity D = about 1.3; impurity E = about 1.8; impurity F = about 2.0; impurity H = about 2.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity G and impurity A.
- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to formoterol.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.75;
- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities B, C, D, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity E*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity I**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Sonicate if necessary.

*Reference solution (a).* Dissolve 5.0 mg of *formoterol for impurity I identification CRS* in *water R* and dilute to 50.0 mL with the same solvent. Sonicate if necessary.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with *water R*. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecyl vinyl polymer for chromatography R*.

*Mobile phase*: mix 12 volumes of *acetonitrile R1* with 88 volumes of a 5.3 g/L solution of *tripotassium phosphate trihydrate R* previously adjusted to pH 12.0 ± 0.1 with a 280 g/L solution of *potassium hydroxide R* or *phosphoric acid R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL.

*Elution order*: formoterol, impurity I.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to formoterol.

*Limit*:

- *impurity I*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

**Water** (*2.5.12*): 4.0 per cent to 5.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 40.24 mg of $C_{42}H_{52}N_4O_{12}$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H.*

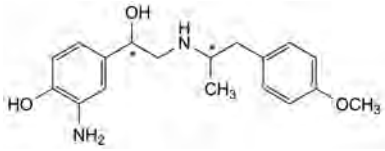

A. 1-(3-amino-4-hydroxyphenyl)-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethanol,

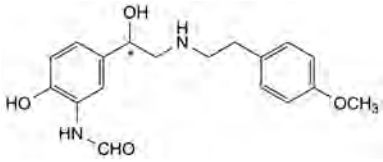

B. *N*-[2-hydroxy-5-[(1*RS*)-1-hydroxy-2-[[2-(4-methoxyphenyl)ethyl]amino]ethyl]phenyl]formamide,

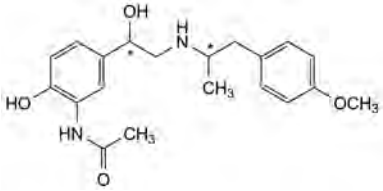

C. *N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]acetamide,

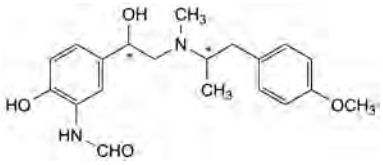

D. *N*-[2-hydroxy-5-[1-hydroxy-2-[methyl[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide,

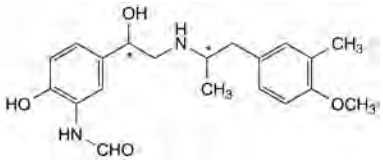

E. *N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxy-3-methylphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide,

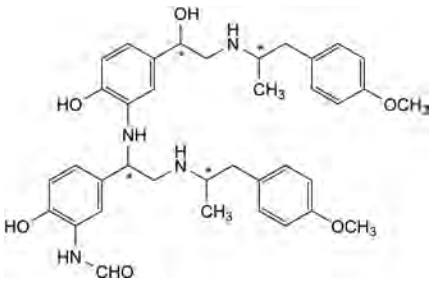

F. *N*-[2-hydroxy-5-[1-[[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]-amino]-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]-ethyl]phenyl]formamide,

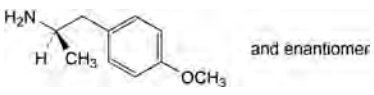


G. (2*RS*)-1-(4-methoxyphenyl)propan-2-amine,

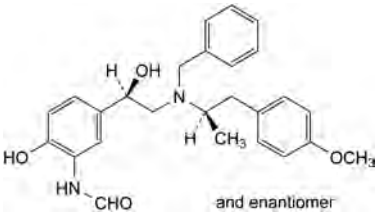


H. *N*-[5-[(1*RS*)-2-[benzyl[(1*RS*)-2-(4-methoxyphenyl)-1-methylethyl]amino]-1-hydroxyethyl]-2-hydroxyphenyl]formamide (monobenzyl analogue),

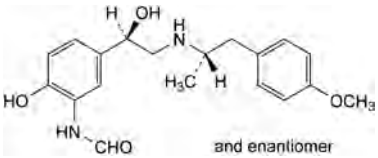


I. *N*-[2-hydroxy-5-[(1*RS*)-1-hydroxy-2-[[(1*SR*)-2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]-formamide (diastereoisomer).

**01/2017:1520**
**corrected 10.0**

# FOSCARNET SODIUM HEXAHYDRATE

## Foscarnetum natricum hexahydricum

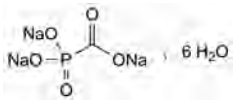

$CNa_3O_5P,6H_2O$ $M_r$ 300.0
[34156-56-4]

DEFINITION

Trisodium phosphonatoformate hexahydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *foscarnet sodium hexahydrate CRS.*

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension I (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.0 to 11.0 for solution S.

**Impurity D**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in 9.0 mL of a 6 g/L solution of *glacial acetic acid R* using a magnetic stirrer. Add 1.0 mL of *anhydrous ethanol R* and mix.

*Reference solution.* Dissolve 25.0 mg of *foscarnet impurity D CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 25 m, Ø = 0.31 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 8 | 100 → 180 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 3 µL

*Limit*:

– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *foscarnet impurity B CRS* in the mobile phase, add 2 mL of the test solution and dilute to 50 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *foscarnet impurity mixture CRS* (impurities A and C) in 1 mL of mobile phase.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 3.22 g of *sodium sulfate decahydrate R* in *water for chromatography R*, add 3 mL of *glacial acetic acid R* and 6 mL of a 44.61 g/L solution of *sodium pyrophosphate R* and dilute to 1000 mL with *water for chromatography R* (solution A); dissolve 3.22 g of *sodium sulfate decahydrate R* in *water for chromatography R*, add 6.8 g of *sodium acetate R* and 6 mL of a 44.61 g/L solution of *sodium pyrophosphate R* and dilute to 1000 mL with *water for chromatography R* (solution B). Mix about 700 mL of solution A and about 300 mL of solution B to obtain a solution of pH 4.4. To 1000 mL of this solution, add 0.25 g of *tetrahexylammonium hydrogen sulfate R* and 100 mL of *methanol R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 40 µL.

*Run time*: 2.5 times the retention time of foscarnet.

*Identification of impurities*: use the chromatogram supplied with *foscarnet impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and C; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to foscarnet (retention time = about 5 min): impurity A = about 0.7; impurity B = about 1.5; impurity C = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 7.0 between the peaks due to foscarnet and impurity B.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.04 per cent); disregard any peak with a relative retention less than 0.6.

**Phosphate and phosphite**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 60.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 28 mg of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (b).* Dissolve 43 mg of *sodium phosphite pentahydrate R* in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) to 25 mL with *water R*.

*Reference solution (d).* Dilute 3 mL of reference solution (a) and 3 mL of reference solution (b) to 25 mL with *water R*.

*Column*:

– *size*: *l* = 0.05 m, Ø = 4.6 mm;

– *stationary phase*: *anion-exchange resin R*.

*Mobile phase*: dissolve 0.102 g of *potassium hydrogen phthalate R* in *water for chromatography R*, add 2.5 mL of *1 M nitric acid* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 290 nm (indirect detection).

*Injection*: 20 µL of the test solution and reference solutions (c) and (d).

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to phosphate (1st peak) and phosphite;

– *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limits*:

– *phosphate*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent);

– *phosphite*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent).

**Loss on drying** (*2.2.32*): 35.0 per cent to 37.0 per cent, determined on 1.000 g by drying in an oven at 150 °C.

**Bacterial endotoxins** (*2.6.14*): less than 83.3 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.200 g in 50 mL of *water R*. Titrate with *0.05 M sulfuric acid*, determining the end-point potentiometrically (*2.2.20*) at the 1st point of inflexion.

1 mL of *0.05 M sulfuric acid* is equivalent to 19.20 mg of $CNa_3O_5P$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

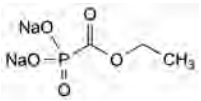

A. disodium (ethoxycarbonyl)phosphonate,

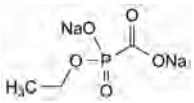

B. disodium (ethoxyoxydophosphanyl)formate,

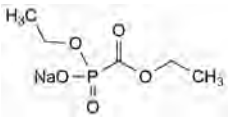

C. ethyl sodium (ethoxycarbonyl)phosphonate,

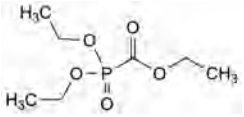

D. ethyl (diethoxyphosphoryl)formate.

**01/2017:1328**

# FOSFOMYCIN CALCIUM

## Fosfomycinum calcicum

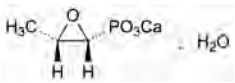

$C_3H_5CaO_4P,H_2O$ $M_r$ 194.1

[26469-67-0]

DEFINITION

Calcium (2*R*,3*S*)-(3-methyloxiran-2-yl)phosphonate monohydrate.

Substance produced by certain strains of *Streptomyces fradiae* or obtained by any other means.

*Content*: 95.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, practically insoluble in acetone, in methanol and in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fosfomycin calcium CRS*.

B. Dissolve about 0.1 g in 3 mL of a 25 per cent *V/V* solution of *perchloric acid R*. Add 1 mL of *0.1 M sodium periodate* and heat on a water-bath for 30 min. Allow to cool and add 50 mL of *water R*. Neutralise with a saturated solution of *sodium hydrogen carbonate R* and add 1 mL of a freshly prepared 400 g/L solution of *potassium iodide R*. Prepare a blank at the same time and in the same manner. The test solution remains colourless and the blank is orange.

C. To about 8 mg add 2 mL of *water R*, 1 mL of *perchloric acid R* and 2 mL of *0.1 M sodium periodate*. Heat on a water-bath for 10 min and add, without cooling, 1 mL of *ammonium molybdate solution R5* and 1 mL of *aminohydroxynaphthalenesulfonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

D. It gives reaction (a) of calcium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 8.1 to 9.6.

Dissolve 20 mg in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 11.0 to − 13.0 (anhydrous substance), determined at 405 nm using a mercury lamp.

Dissolve 2.5 g in a 125 g/L solution of *sodium edetate R* previously adjusted to pH 8.5 with *strong sodium hydroxide solution R*, and dilute to 50.0 mL with the same solution.

**Impurity A**: maximum 1.5 per cent.

In a glass-stoppered flask, dissolve 0.200 g in 100.0 mL of *water R*. Add 50 mL of *0.5 M phthalate buffer solution pH 6.4 R* and 5.0 mL of *0.005 M sodium periodate*, close and shake. Allow to stand protected from light for 90 min. Add 10 mL of a freshly prepared 400 g/L solution of *potassium iodide R*, close and shake for 2 min. Titrate with *0.0025 M sodium arsenite* until the yellow colour almost disappears. Add 2 mL of *starch solution R* and slowly continue the titration until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_7CaO_5P$ using the following expression:

$$\frac{(n_1 - n_2) \times c \times 97 \times 100}{m(100 - H)} \times 100$$

$m$ = mass of the substance to be examined, in milligrams;

$n_1$ = volume of *0.0025 M sodium arsenite* used in the blank titration, in millilitres;

$n_2$ = volume of *0.0025 M sodium arsenite* used in the titration of the test solution, in millilitres;

$c$ = molarity of the sodium arsenite solution;

$H$ = percentage content of water.

**Chlorides** (*2.4.4*): maximum 0.2 per cent.

Dissolve 0.500 g in *water R*, add 2 mL of *nitric acid R* and dilute to 50 mL with the same acid. To 2.5 mL of this solution add 12.5 mL of *water R*.

**Water** (*2.5.12*): 8.5 per cent to 11.5 per cent, determined on 0.250 g. Use as the solvent a mixture of 1 volume of *pyridine R* and 3 volumes of *ethylene glycol R*.

ASSAY

In a glass-stoppered flask, dissolve 0.120 g in 20.0 mL of *0.1 M sodium periodate*. Add 5 mL of a 50 per cent *V/V* solution of *perchloric acid R* and shake. Heat in a water-bath at 37 °C for 105 min. Add 50 mL of *water R* and immediately adjust to pH 6.4 with a saturated solution of *sodium hydrogen carbonate R*. Add 10 mL of a freshly prepared 400 g/L solution of *potassium iodide R*, close and allow to stand for 2 min. Titrate with *0.1 M sodium arsenite* until the yellow colour almost disappears. Add 2 mL of *starch solution R* and slowly continue the titration until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_5CaO_4P$ using the following expression:

$$\frac{(n_1 - n_2) \times c \times 88 \times 100}{m(100 - H)} \times (100 - G)$$

$m$ = mass of the substance to be examined, in milligrams;

$n_1$ = volume of *0.1 M sodium arsenite* used in the blank titration, in millilitres;

$n_2$ = volume of *0.1 M sodium arsenite* used in the titration of the test solution, in millilitres;

$c$ = molarity of the sodium arsenite solution;

$G$ = percentage content of impurity A;

$H$ = percentage content of water.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A*.

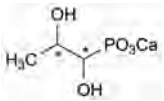


A. calcium (1,2-dihydroxypropyl)phosphonate.

**01/2017:1329**
**corrected 10.0**

# FOSFOMYCIN SODIUM

## Fosfomycinum natricum

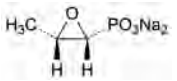


$C_3H_5Na_2O_4P$ $M_r$ 182.0
[26016-99-9]

DEFINITION

Disodium (2*R*,3*S*)-(3-methyloxiran-2-yl)phosphonate.

Substance produced by certain strains of *Streptomyces fradiae* or obtained by any other means.

*Content*: 95.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: very soluble in water, sparingly soluble in methanol, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *Ph. Eur. reference spectrum of fosfomycin sodium*.

B. Dissolve about 0.1 g in 3 mL of a 25 per cent *V/V* solution of *perchloric acid R*. Add 1 mL of *0.1 M sodium periodate* and heat on a water-bath for 30 min. Allow to cool and add 50 mL of *water R*. Neutralise with a saturated solution of *sodium hydrogen carbonate R* and add 1 mL of a freshly prepared 400 g/L solution of *potassium iodide R*. Prepare a blank at the same time and in the same manner. The test solution remains colourless and the blank is orange.

C. To about 8 mg add 2 mL of *water R*, 1 mL of *perchloric acid R* and 2 mL of *0.1 M sodium periodate*. Heat on a water-bath for 10 min and add, without cooling, 1 mL of *ammonium molybdate solution R5* and 1 mL of *aminohydroxynaphthalenesulfonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_9$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.0 to 10.5.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 13.0 to − 15.0 (anhydrous substance), determined at 405 nm using a mercury lamp.

Dissolve 2.5 g in *water R* and dilute to 50.0 mL with the same solvent.

**Impurity A**: maximum 1.0 per cent.

In a glass-stoppered flask, dissolve 0.200 g in 100.0 mL of *water R*. Add 50 mL of *0.5 M phthalate buffer solution pH 6.4 R* and 5.0 mL of *0.005 M sodium periodate*, close and shake. Allow to stand protected from light for 90 min. Add 10 mL of a freshly prepared 400 g/L solution of *potassium iodide R*, close and shake for 2 min. Titrate with *0.0025 M sodium arsenite* until the yellow colour almost disappears. Add 2 mL of *starch solution R* and slowly continue the titration until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_7Na_2O_5P$ using the following expression:

$$\frac{(n_1 - n_2) \times c \times 100 \times 100}{m(100 - H)} \times 100$$

$m$ = mass of the substance to be examined, in milligrams;

$n_1$ = volume of *0.0025 M sodium arsenite* used in the blank titration, in millilitres;

$n_2$ = volume of *0.0025 M sodium arsenite* used in the titration of the test solution, in millilitres;

$c$ = molarity of the sodium arsenite solution;

$H$ = percentage content of water.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g. Use as the solvent a mixture of 1 volume of *pyridine R* and 3 volumes of *ethylene glycol R*.

**Bacterial endotoxins** (*2.6.14*): less than 0.083 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

In a glass-stoppered flask, dissolve 0.120 g in 20.0 mL of *0.1 M sodium periodate*. Add 5 mL of a 50 per cent *V/V* solution of *perchloric acid R* and shake. Heat in a water-bath at 37 °C for 105 min. Add 50 mL of *water R* and immediately adjust to pH 6.4 with a saturated solution of *sodium hydrogen carbonate R*. Add 10 mL of a freshly prepared 400 g/L solution of *potassium iodide R*, close and allow to stand for 2 min. Titrate with *0.1 M sodium arsenite* until the yellow

colour almost disappears. Add 2 mL of *starch solution R* and slowly continue the titration until the colour is completely discharged. Carry out a blank test under the same conditions.

Calculate the percentage content of $C_3H_5Na_2O_4P$ using the following expression:

$$\frac{(n_1 - n_2) \times c \times 91 \times 100}{m(100 - H)} \times (100 - G)$$

$m$ = mass of the substance to be examined, in milligrams;

$n_1$ = volume of *0.1 M sodium arsenite* used in the blank titration, in millilitres;

$n_2$ = volume of *0.1 M sodium arsenite* used in the titration of the test solution, in millilitres;

$c$ = molarity of the sodium arsenite solution;

$G$ = percentage content of impurity A;

$H$ = percentage content of water.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A*.

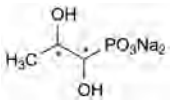


A. disodium (1,2-dihydroxypropyl)phosphonate.

**01/2017:1425**

# FOSFOMYCIN TROMETAMOL

## Fosfomycinum trometamolum

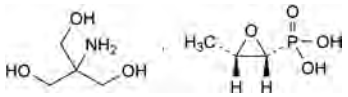


$C_7H_{18}NO_7P$ $M_r$ 259.2

[78964-85-9]

DEFINITION

2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrogen (2*R*,3*S*)-(3-methyloxiran-2-yl)phosphonate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent) and in methanol, practically insoluble in acetone.

IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B, C*.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fosfomycin trometamol CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *fosfomycin trometamol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: *concentrated ammonia R*, *water R*, *2-propanol R* (10:20:70 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of warm air.

*Detection*: expose to iodine vapour until the spots appear.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 15 mg add 2 mL of *water R*, 1 mL of *perchloric acid R* and 2 mL of *0.1 M sodium periodate*. Heat on a water-bath for 10 min and add, without cooling, 1 mL of *ammonium molybdate solution R5* and 1 mL of *aminohydroxynaphthalenesulfonic acid solution R*. Allow to stand for 30 min. A blue colour develops.

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): – 13.5 to – 12.5 (anhydrous substance), determined on solution S at 365 nm using a mercury lamp.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.600 g of the substance to be examined in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 0.600 g of *fosfomycin trometamol CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 3.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Wet 0.3 g of the substance to be examined with 60 µL of *water R* and heat in an oven at 60 °C for 24 h. Dissolve the residue in the mobile phase and dilute to 20.0 mL with the mobile phase (solution A). Dissolve 0.6 g of the substance to be examined in solution A and dilute to 5.0 mL with the same solution (*in situ* degradation to obtain impurities A, B, C and D).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 10.89 g/L solution of *potassium dihydrogen phosphate R* in *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: differential refractometer at 35 °C.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of fosfomycin.

*Relative retention* with reference to fosfomycin (retention time = about 9 min): trometamol (2 peaks) = about 0.3; impurity B = about 0.48; impurity C = about 0.54; impurity A = about 0.88; impurity D = about 1.27.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity A and fosfomycin,

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the peak due to fosfomycin in the chromatogram obtained with reference solution (b) (0.3 per cent),
– *impurities C, D*: for each impurity, not more than 0.33 times the area of the peak due to fosfomycin in the chromatogram obtained with reference solution (b) (0.1 per cent),
– *unspecified impurities*: for each impurity, not more than 0.33 times the area of the peak due to fosfomycin in the chromatogram obtained with reference solution (b) (0.1 per cent),
– *total*: not more than 1.67 times the area of the peak due to fosfomycin in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.17 times the area of the peak due to fosfomycin in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the 2 peaks due to trometamol.

**Phosphates**: maximum 500 ppm.

Dissolve 0.1 g in 3 mL of *dilute nitric acid R* and dilute to 10 mL with *water R*. To 5 mL of this solution add 5 mL of *water R* and 5 mL of *molybdovanadic reagent R*. Shake vigorously. After 5 min, any colour in the test solution is not more intense than that in a standard prepared at the same time in the same manner, using 5 mL of *phosphate standard solution (5 ppm $PO_4$) R*.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 5 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_7H_{18}NO_7P$ from the areas of the peaks due to fosfomycin and the declared content of *fosfomycin trometamol CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D.*

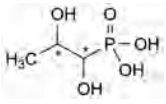

A. (1,2-dihydroxypropyl)phosphonic acid,

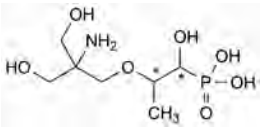

B. [2-[2-amino-3-hydroxy-2-(hydroxymethyl)propoxy]-1-hydroxypropyl]phosphonic acid,

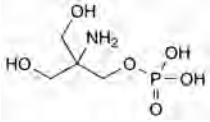

C. 2-amino-3-hydroxy-2-(hydroxymethyl)propyl dihydrogen phosphate (trometamol phosphoric acid monoester),

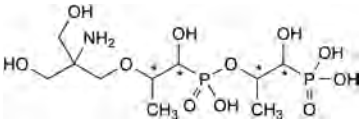

D. [2-[[[2-[2-amino-3-hydroxy-2-(hydroxymethyl)propoxy]-1-hydroxypropyl]hydroxyphosphoryl]oxy]-1-hydroxypropyl]phosphonic acid (trometamoyloxy fosfomycin dimer).

**01/2017:1751**

# FOSINOPRIL SODIUM

## Fosinoprilum natricum

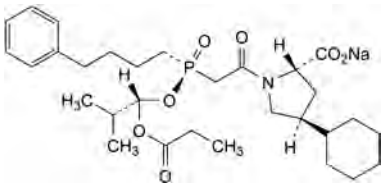

$C_{30}H_{45}NNaO_7P$ $M_r$ 585.7
[88889-14-9]

DEFINITION

Sodium (2*S*,4*S*)-4-cyclohexyl-1-[[(*R*)-[(1*S*)-2-methyl-1-(1-oxopropoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]-pyrrolidine-2-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in anhydrous ethanol, practically insoluble in hexane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 6.7 to – 4.7 (anhydrous substance).
Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fosinopril sodium CRS*.
If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in a 2 per cent *V/V* solution of *water R* in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Related substances**

A. Liquid chromatography (*2.2.29*).
*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Sonicate until dissolution is complete.
*Reference solution (a)*. Dissolve 2 mg of the substance to be examined, 2 mg of *fosinopril impurity A CRS*, 2 mg of *fosinopril impurity B CRS*, 2 mg of *fosinopril impurity I CRS* and 2 mg of *fosinopril impurity K CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.
*Reference solution (b)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm);
- *temperature*: 33 °C.

*Mobile phase*: *phosphoric acid R*, *water R*, *acetonitrile R1* (0.05:0.35:100 *V/V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of fosinopril.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, I and K.

*Relative retention* with reference to fosinopril (retention time = about 5 min): impurity K = about 0.3; impurity I = about 0.5; impurities B, E and H = about 0.7; impurity A = about 2.0.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity B and fosinopril in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity I by 1.3;
- *sum of impurities B, E and H*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities I, K*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent).

B. Impurities C and D. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Sonicate until dissolution is complete.

*Reference solution (a).* Dissolve 5 mg of the substance to be examined and 5.0 mg of *fosinopril impurity C CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *fosinopril impurity D CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: *phosphoric acid R*, *water R*, *acetonitrile R1* (0.2:1.5:400 *V/V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of fosinopril.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to fosinopril (retention time = about 10 min): impurity C = about 1.2; impurity D = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to fosinopril and impurity C.

*Limits*:

- *impurity C*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity D*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent).

C. Impurities E and F. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *fosinopril impurity mixture CRS* (containing impurities E and F) in 1.0 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: 0.2 per cent *V/V* solution of *phosphoric acid R*, *acetonitrile R1* (44:56 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of fosinopril.

*Identification of impurities*: use the chromatogram supplied with *fosinopril impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E and F.

*Relative retention* with reference to fosinopril (retention time = about 8 min): impurity E = about 0.8; impurity F = about 0.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity F and fosinopril.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity E by 0.7;
- *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.2 per cent *m/m*.

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 1.00 g.

ASSAY

Dissolve 0.450 g in 50 mL of *water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 58.57 mg of $C_{30}H_{45}NNaO_7P$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, H, I, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *N.*

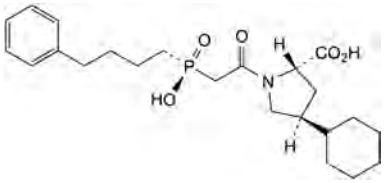

A. (2*S*,4*S*)-4-cyclohexyl-1-[[(*R*)-hydroxy(4-phenylbutyl)-phosphoryl]acetyl]pyrrolidine-2-carboxylic acid,

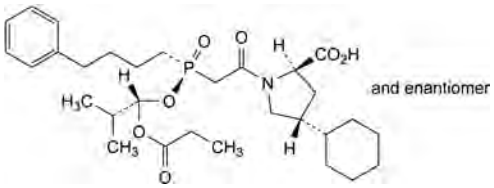


B. (2*RS*,4*RS*)-4-cyclohexyl-1-[[(*RS*)-[(1*SR*)-2-methyl-1-(1-oxopropoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]-pyrrolidine-2-carboxylic acid,

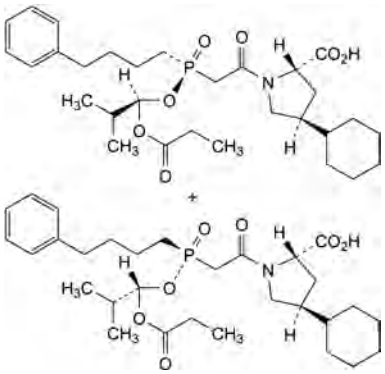

C. mixture of (2*S*,4*S*)-4-cyclohexyl-1-[[(*S*)-[(1*S*)-2-methyl-1-(1-oxopropoxy)propoxy](4-phenylbutyl)-phosphoryl]acetyl]pyrrolidine-2-carboxylic acid and (2*S*,4*S*)-4-cyclohexyl-1-[[(*R*)-[(1*R*)-2-methyl-1-(1-oxo-propoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]-pyrrolidine-2-carboxylic acid,

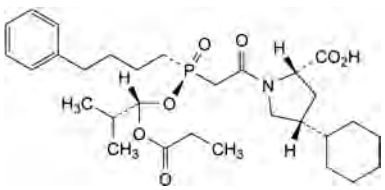

D. (2*S*,4*R*)-4-cyclohexyl-1-[[(*R*)-[(1*S*)-2-methyl-1-(1-oxo-propoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]-pyrrolidine-2-carboxylic acid,

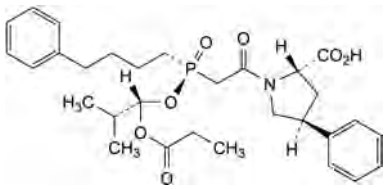

E. (2*S*,4*S*)-1-[[(*R*)-[(1*S*)-2-methyl-1-(1-oxopropoxy)propoxy]-(4-phenylbutyl)phosphoryl]acetyl]-4-phenylpyrrolidine-2-carboxylic acid (phenylfosinopril),

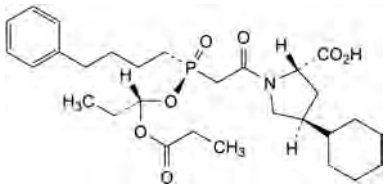

F. (2*S*,4*S*)-4-cyclohexyl-1-[[(*R*)-(4-phenylbutyl)[(1*S*)-1-(1-oxopropoxy)propoxy]phosphoryl]acetyl]pyrrolidine-2-carboxylic acid,

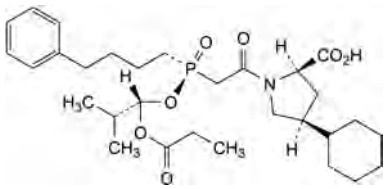

H. (2*R*,4*S*)-4-cyclohexyl-1-[[(*R*)-[(1*S*)-2-methyl-1-(1-oxo-propoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]-pyrrolidine-2-carboxylic acid,

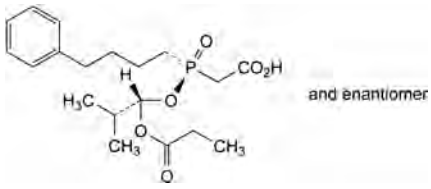


I. [(*RS*)-[(1*SR*)-2-methyl-1-(1-oxopropoxy)propoxy]-(4-phenylbutyl)phosphoryl]acetic acid,

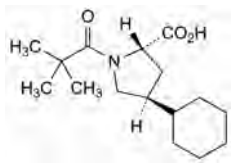

K. (2*S*,4*S*)-4-cyclohexyl-1-(2,2-dimethyl-1-oxopropyl)-pyrrolidine-2-carboxylic acid,

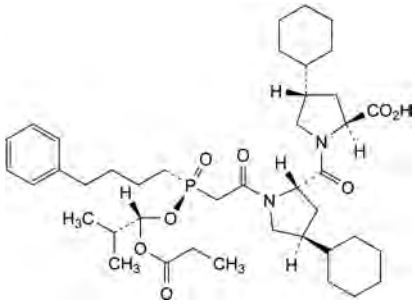

N. (2*S*,4*S*)-4-cyclohexyl-1-[[(2*S*,4*S*)-4-cyclohexyl-1-[[(*R*)-[(1*S*)-2-methyl-1-(1-oxopropoxy)propoxy](4-phenylbutyl)phosphoryl]acetyl]pyrrolidin-2-yl]-carbonyl]pyrrolidine-2-carboxylic acid.

01/2008:0180
corrected 10.0

# FRAMYCETIN SULFATE

## Framycetini sulfas

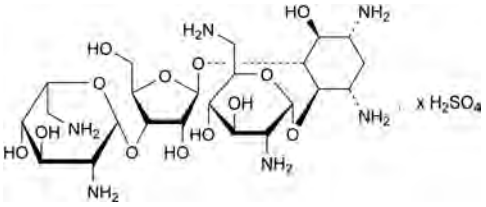


$C_{23}H_{46}N_6O_{13},xH_2SO_4$ $M_r$ 615 (base)
[4146-30-9]

DEFINITION

Sulfate of 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B).

Substance produced by the growth of selected strains of *Streptomyces fradiae* or *Streptomyces decaris* or obtained by any other means.

*Content*: minimum of 630 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: freely soluble in water, very slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

*Results*:

- the retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with reference solution (a),
- it complies with the limit given for impurity C.

B. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 6.0 to 7.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 52.5 to + 55.5 (dried substance).

Dissolve 1.00 g in *water R* and dilute to 10.0 mL with the same solvent

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve the contents of a vial of *framycetin sulfate CRS* in the mobile phase and dilute with the mobile phase to obtain a solution containing 0.5 mg/mL.

*Reference solution (b).* Dilute 3.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (d).* Dissolve the contents of a vial of *neamine CRS* (corresponding to 0.5 mg) in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 10 mg of *neomycin sulfate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 25 °C.

*Mobile phase*: mix 20.0 mL of *trifluoroacetic acid R*, 6.0 mL of *carbonate-free sodium hydroxide solution R* and 500 mL of *water R*, allow to equilibrate, dilute to 1000 mL with *water R* and degas.

*Flow rate*: 0.7 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted 1 in 25 previously degassed, which is added pulse-less to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate*: 0.5 mL/min.

*Detection*: pulsed amperometric detector with a gold working electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively 0.00 V detection, + 0.80 V oxidation and – 0.60 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of neomycin B.

*Relative retention* with reference to neomycin B (retention time = about 10 min): impurity A = about 0.65; impurity C = about 0.9; impurity G = about 1.1.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity C and to neomycin B in the chromatogram obtained with reference solution (e); if necessary, adjust the volume of the carbonate-free sodium hydroxide solution in the mobile phase,
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) and taking into account the declared content of *neamine CRS* (1.0 per cent),
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- *total of other impurities*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

**Sulfate**: 27.0 per cent to 31.0 per cent (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *alcohol R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

**Sterility** (*2.6.1*). If intended for introduction into body cavities without a further appropriate sterilisation procedure, it complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14, Method D*): less than 1.3 IU/mg if intended for introduction into body cavities without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *framycetin sulfate CRS* as the reference substance.

STORAGE

In an airtight container, protected from light. If the substance is intended for introduction into body cavities, store in a sterile, tamper-evident container.

IMPURITIES

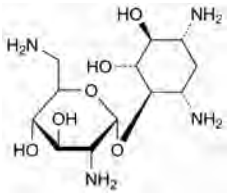

A. 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (neamine or neomycin A-LP),

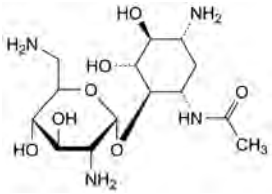

B. 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (3-acetylneamine),

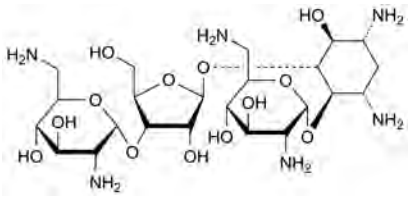

C. 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin C),

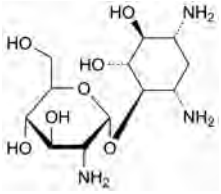

D. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine (paromamine or neomycin D),

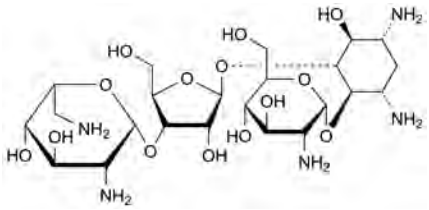

E. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin I or neomycin E),

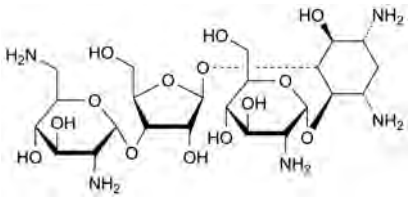

F. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin II or neomycin F),

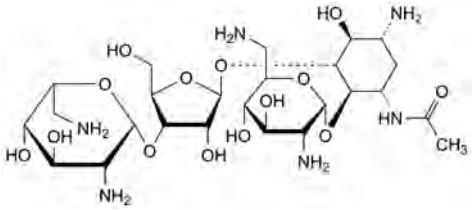

G. 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B-LP).

**07/2019:0188**

# FRUCTOSE

## Fructosum

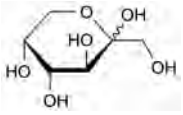

$C_6H_{12}O_6$ $M_r$ 180.2

[57-48-7]

DEFINITION

D-*arabino*-Hex-2-ulopyranose (levulose).

The substance described in this monograph is not necessarily suitable for parenteral administration.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (2:3 *V/V*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *fructose CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *fructose R*, 10 mg of *glucose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*). Measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 2 µL; thoroughly dry the points of application.

*Development A*: over 3/4 of the plate.

*Drying A*: in a current of warm air.

*Development B*: immediately, over 3/4 of the plate, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*. Heat at 130 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 4 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

B. Dissolve 0.1 g in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. A red precipitate is formed.

C. To 1 mL of solution S (see Tests) add 9 mL of *water R*. To 1 mL of the solution add 5 mL of *hydrochloric acid R* and heat to 70 °C. A brown colour develops.

D. Dissolve 5 g in *water R* and dilute to 10 mL with the same solvent. To 0.5 mL of the solution add 0.2 g of *resorcinol R* and 9 mL of *dilute hydrochloric acid R* and heat on a water-bath for 2 min. A red colour develops.

TESTS

**Solution S**. Dissolve 10.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Dissolve 5.0 g in *water R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*). Add 10 mL of *water R*. The solution is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 6.0 g in 25 mL of *carbon dioxide-free water R* and add 0.3 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.15 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Specific optical rotation** (*2.2.7*): – 93.5 to – 91.0 (anhydrous substance).

Dissolve 10.0 g in 80 mL of *water R*, add 0.2 mL of *dilute ammonia R1*, allow to stand for 30 min and dilute to 100.0 mL with *water R*.

**Foreign sugars**. Dissolve 5.0 g in *water R* and dilute to 10 mL with the same solvent. To 1 mL of the solution add 9 mL of *ethanol (96 per cent) R*. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of the initial solution and 9 mL of *water R*.

**5-Hydroxymethylfurfural and related compounds**. To 5 mL of solution S add 5 mL of *water R*. The absorbance (*2.2.25*) measured at 284 nm is not greater than 0.32.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** : maximum 0.1 per cent.

Dissolve 5.0 g in 10 mL of *water R*, add 2 mL of *sulfuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass.

**01/2017:2443**
**corrected 10.0**

# FULVESTRANT

## Fulvestrantum

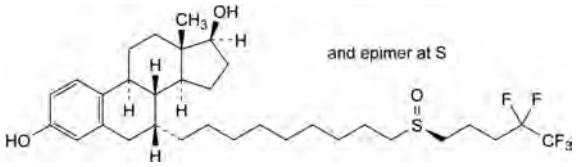


$C_{32}H_{47}F_5O_3S$ $M_r$ 607
[129453-61-8]

DEFINITION

7α-[9-[(*RS*)-(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17β-diol.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): + 108 to + 115 (anhydrous substance), measured at 365 nm at a temperature of 25 °C.
Dissolve 0.50 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *fulvestrant CRS*.

C. Stereochemical purity (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).
Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *methanol R1* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *fulvestrant CRS* in *methanol R1* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *fulvestrant for system suitability CRS* (containing impurities A, B, C, D and F) in 1.0 mL of *methanol R1*.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R1*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R2*, *acetonitrile R1*, *water for chromatography R* (27:32:41 *V/V/V*);
– *mobile phase B*: *water for chromatography R*, *methanol R2*, *acetonitrile R1* (10:41:49 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 55 | 100 → 0 | 0 → 100 |
| 55 - 65 | 0 | 100 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *fulvestrant for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and F.

*Relative retention* with reference to fulvestrant (retention time = about 23 min): impurity F = about 0.4; impurity A = about 1.1; impurity B = about 1.2; impurity C = about 1.7; impurity D = about 1.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to fulvestrant and impurity A.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.7; impurity F = 0.3;
– for each impurity, use the concentration of fulvestrant in reference solution (c).

*Limits*:

– *impurity D*: maximum 0.6 per cent;
– *impurity C*: maximum 0.3 per cent;
– *impurity B*: maximum 0.2 per cent;
– *impurity F*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Stereochemical purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution.* Dissolve 5 mg of *fulvestrant CRS* in the mobile phase and dilute to 5 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm);
– *temperature*: 25 °C.

*Mobile phase*: *propanol R1*, *heptane R* (15:85 *V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of fulvestrant epimer B.

*Identification of peaks*: use the chromatogram supplied with *fulvestrant CRS* and the chromatogram obtained with the reference solution to identify the peaks due to fulvestrant epimers A and B.

*Relative retention* with reference to fulvestrant epimer B (retention time = about 18 min): fulvestrant epimer A = about 1.2.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to fulvestrant epimer B and fulvestrant epimer A.

*Limit*:

– *fulvestrant epimer A*: 42 per cent to 48 per cent;
– *fulvestrant epimer B*: 52 per cent to 58 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 50 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

**Bacterial endotoxins** (*2.6.14*): less than 1.25 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

*Test solution.* Dissolve 0.1 g of the substance to be examined in 1 mL of *ethanol (96 per cent) R* and dilute to 80 mL with water for BET.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{32}H_{47}F_5O_3S$ taking into account the assigned content of *fulvestrant CRS*.

STORAGE

Protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: B, C, D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, E.*

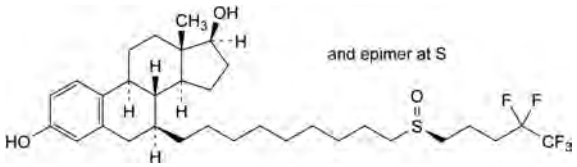


A. 7β-[9-[(*RS*)-(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol (7β-fulvestrant),

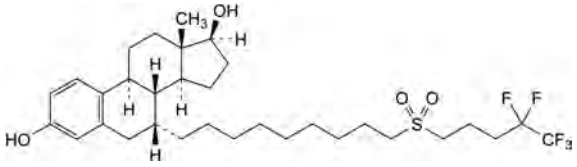


B. 7α-[9-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]nonyl]estra-1,3,5(10)-triene-3,17β-diol,

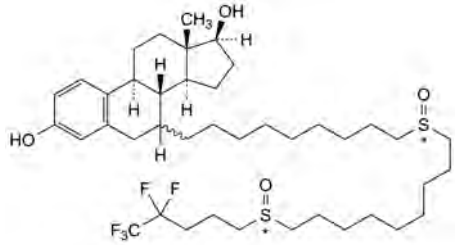


C. 7ξ-[9-[[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]-sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17β-diol,

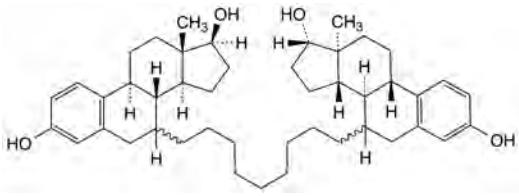

D. 7ξ,7′ξ-nonane-1,9-diylbis[estra-1,3,5(10)-triene-3,17β-diol],

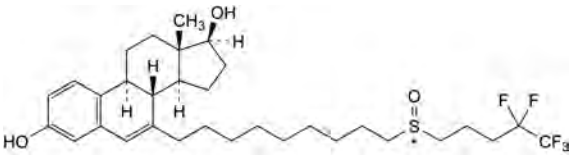

E. 7-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10),6-tetraene-3,17β-diol (Δ6-fulvestrant),

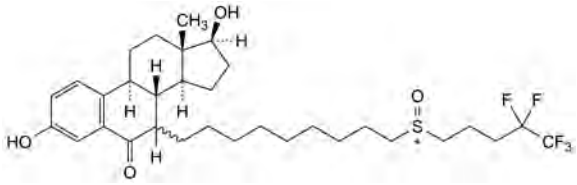

F. 3,17β-dihydroxy-7ξ-[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-trien-6-one (6-keto-fulvestrant).

**04/2018:0391**

# FUROSEMIDE

## Furosemidum

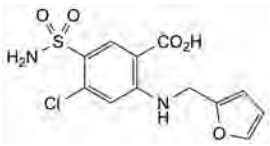

$C_{12}H_{11}ClN_2O_5S$ $M_r$ 330.7
[54-31-9]

DEFINITION

4-Chloro-2-[[(furan-2-yl)methyl]amino]-5-sulfamoylbenzoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A*.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *methanol R*.

*Reference solution*. Dissolve 20 mg of *furosemide CRS* in 1 mL of *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *toluene R* (5:45:50 *V/V/V*).

*Application*: 2 µL; the volume may be adapted according to the type of plate used.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Detection B*: treat with *ninhydrin solution R*, dry at 100-105 °C until the spots appear and examine in daylight.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *furosemide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *0.5 M sodium hydroxide* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *furosemide impurity A CRS* in the mobile phase, add 2.0 mL of the test solution and dilute to 20.0 mL with the mobile phase. Dilute 0.5 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 2 mg of *furosemide for peak identification CRS* (containing impurities C and D) in 2.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.0 g of *potassium dihydrogen phosphate R* and 2.5 g of *cetrimide R* in 700 mL of *water for chromatography R*, adjust to pH 7.0 with *ammonia R* and add 300 mL of *propanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of furosemide.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *furosemide for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and D.

*Relative retention* with reference to furosemide (retention time = about 9 min): impurity C = about 0.5; impurity A = about 0.8; impurity D = about 1.5.

*System suitability*:

- *resolution*: minimum 4.0 between the peaks due to impurity A and furosemide in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.4; impurity D = 2.0;
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 0.5 g add a mixture of 0.2 mL of *nitric acid R* and 30 mL of *water R* and shake for 5 min. Allow to stand for 15 min and filter.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 1.0 g add a mixture of 0.2 mL of *acetic acid R* and 30 mL of *distilled water R* and shake for 5 min. Allow to stand for 15 min and filter.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 40 mL of *dimethylformamide R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.07 mg of $C_{12}H_{11}ClN_2O_5S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F.*

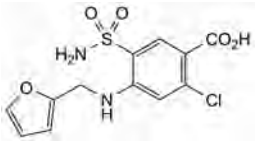

A. 2-chloro-4-[[(furan-2-yl)methyl]amino]-5-sulfamoylbenzoic acid,

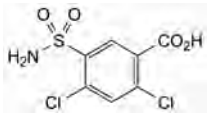

B. 2,4-dichloro-5-sulfamoylbenzoic acid,

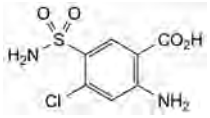

C. 2-amino-4-chloro-5-sulfamoylbenzoic acid,

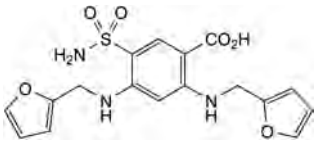

D. 2,4-bis[[(furan-2-yl)methyl]amino]-5-sulfamoylbenzoic acid,

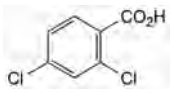

E. 2,4-dichlorobenzoic acid,

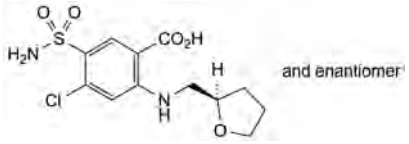


F. 4-chloro-5-sulfamoyl-2-[[[(2*RS*)-oxolan-2-yl]methyl]amino]benzoic acid.

**01/2012:0798**

# FUSIDIC ACID

## Acidum fusidicum

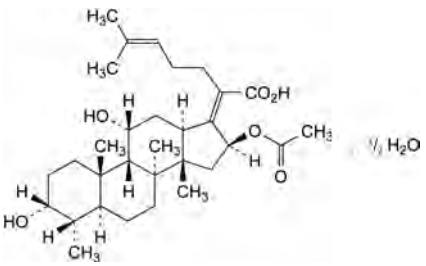

$C_{31}H_{48}O_6,½H_2O$ $M_r$ 525.7

[6990-06-3]

DEFINITION

*ent*-(17*Z*)-16α-(Acetyloxy)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid hemihydrate.

Antimicrobial substance produced by fermentation of certain strains of *Fusidium coccineum* or by any other means.

*Content*: 97.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *fusidic acid CRS*.

B. Ignite 1 g. The residue does not give reaction (a) of sodium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *methanol R*, 5 g/L solution of *phosphoric acid R*, *acetonitrile R* (10:40:50 *V/V/V*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *fusidic acid for peak identification CRS* (containing impurities A, B, C, D, F, G, H and N) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve the contents of a vial of *fusidic acid impurity mixture CRS* (containing impurities I, K, L and M) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, *acetonitrile R*, 5 g/L solution of *phosphoric acid R* (20:40:40 *V/V/V*);
- *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *methanol R*, *acetonitrile R* (10:20:70 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 28 | 100 → 0 | 0 → 100 |
| 28 - 33 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *fusidic acid for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, F, G, H and N; use the chromatogram supplied with *fusidic acid impurity mixture CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities I, K, L and M.

*Relative retention* with reference to fusidic acid (retention time = about 18 min): impurity A = about 0.4; impurity B = about 0.5; impurity C = about 0.6; impurity D = about 0.63; impurity N = about 0.65; impurity F = about 0.7; impurity G = about 0.82; impurity H = about 0.85; impurity I = about 0.96; impurity K = about 1.18; impurity L = about 1.23; impurity M = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurities G and H.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity D = 0.7; impurity F = 0.3; impurity I = 0.6; impurity K = 0.6;
- *impurity M*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity G*: not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
- *impurity L*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.4 per cent);
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurities C, D, F, I, K, N*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): 1.4 per cent to 2.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 10 mL of *ethanol (96 per cent) R*. Add 0.5 mL of *phenolphthalein solution R*. Titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 51.67 mg of $C_{31}H_{48}O_6$.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, F, G, I, K, L, M, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, H, J, O.*

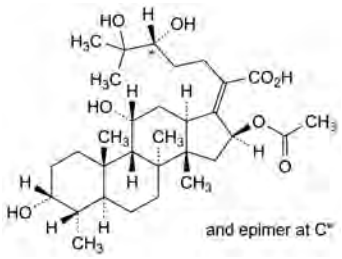


A. *ent*-(24*SR*,17*Z*)-16α-(acetyloxy)-3β,11β,24,25-tetrahydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholest-17(20)-en-21-oic acid (24,25-dihydro-24,25-dihydroxyfusidic acid),

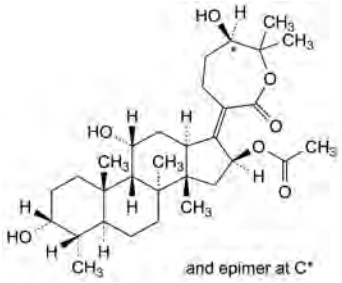


B. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*SR*)-6-hydroxy-7,7-dimethyl-2-oxooxepan-3-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate (24,25-dihydro-24,25-dihydroxyfusidic acid 21,25-lactone),

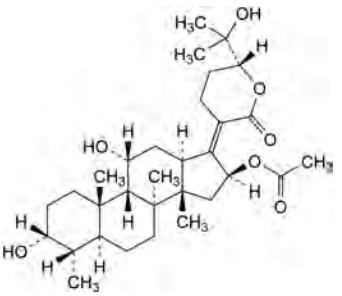

C. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*S*)-6-(1-hydroxy-1-methylethyl)-2-oxodihydro-2*H*-pyran-3(4*H*)-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate ((24*R*)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone),

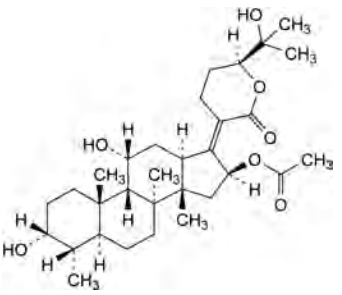

D. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*R*)-6-(1-hydroxy-1-methylethyl)-2-oxodihydro-2*H*-pyran-3(4*H*)-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate ((24*S*)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone),

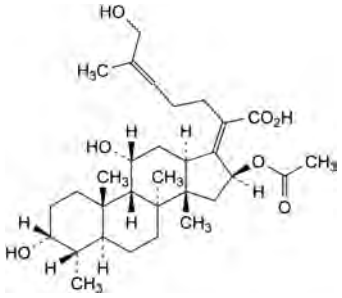

E. *ent*-(17*Z*,24*EZ*)-16α-(acetyloxy)-3β,11β,26-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (26-hydroxyfusidic acid),

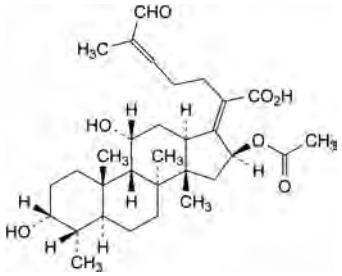

F. *ent*-(17*Z*,24*EZ*)-16α-(acetyloxy)-3β,11β-dihydroxy-4β,8,14-trimethyl-26-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (26-oxofusidic acid),

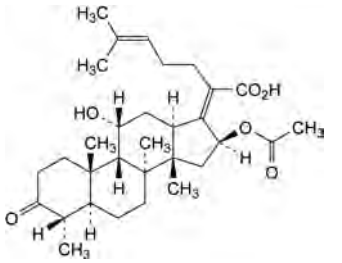

G. *ent*-(17*Z*)-16α-(acetyloxy)-11β-hydroxy-4β,8,14-trimethyl-3-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (3-didehydrofusidic acid),

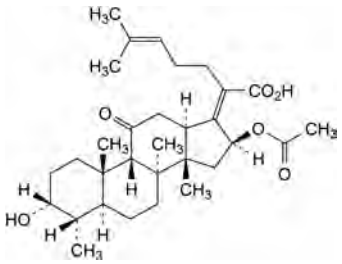

H. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-11-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (11-didehydrofusidic acid),

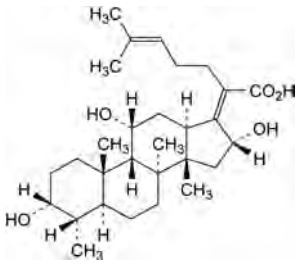

I. *ent*-(17*Z*)-3β,11β,16β-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (16-*epi*-deacetylfusidic acid),

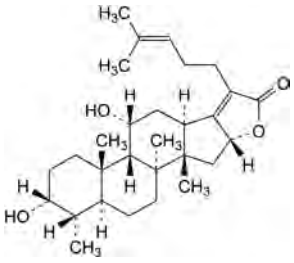

J. *ent*-(17*Z*)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dieno-21(16β)-lactone (16-*epi*-deacetylfusidic acid 21,16-lactone),

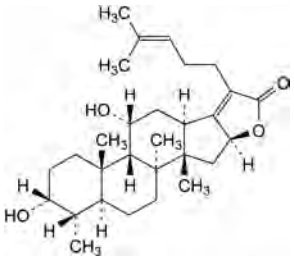

K. *ent*-(17*Z*)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dieno-21(16α)-lactone (deacetylfusidic acid 21,16-lactone),

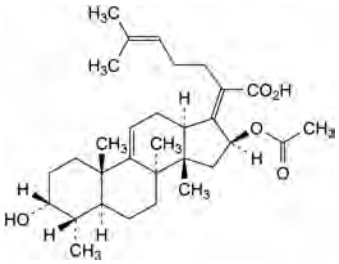

L. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-9(11),17(20),24-trien-21-oic acid (9,11-anhydrofusidic acid),

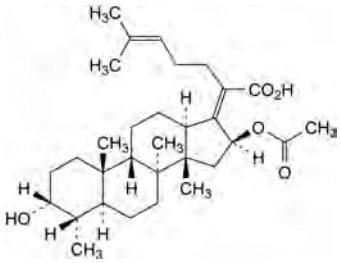

M. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (11-deoxyfusidic acid),

N. unknown structure,

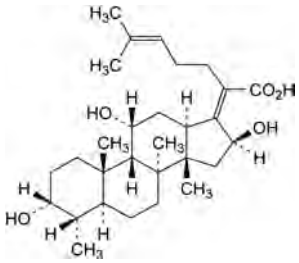

O. *ent*-(17*Z*)-3β,11β,16α-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (deacetylfusidic acid).

# G

Gabapentin.. 2725
Gadobutrol monohydrate.. 2726
Gadodiamide hydrate.. 2728
Galactose.. 2730
Galantamine hydrobromide.. 2731
Gammadex.. 2734
Ganciclovir.. 2735
Gefitinib.. 2737
Gelatin.. 2738
Gemcitabine hydrochloride.. 2739
Gemfibrozil.. 2740
Gentamicin sulfate.. 2742
Gestodene.. 2744
Glibenclamide.. 2746
Gliclazide.. 2747
Glimepiride.. 2749
Glipizide.. 2751
Glucagon, human.. 2753
Glucosamine hydrochloride.. 2754
Glucosamine sulfate potassium chloride.. 2755
Glucosamine sulfate sodium chloride.. 2757
Glucose.. 2758
Glucose, liquid.. 2759
Glucose, liquid, spray-dried.. 2760
Glucose monohydrate.. 2761
Glutamic acid.. 2762
Glutathione.. 2763
Glycerol.. 2765
Glycerol (85 per cent).. 2766
Glycerol dibehenate.. 2767
Glycerol distearate.. 2768
Glycerol formal.. 2769
Glycerol monocaprylate.. 2770
Glycerol monocaprylocaprate.. 2771
Glycerol monolinoleate.. 2772
Glycerol mono-oleate.. 2773
Glycerol monostearate 40-55.. 2774
Glyceryl trinitrate solution.. 2775
Glycine.. 2777
Glycopyrronium bromide.. 2779
Gonadorelin acetate.. 2781
Gonadotrophin, chorionic.. 2782
Gonadotrophin, equine serum, for veterinary use.. 2783
Goserelin.. 2783
Gramicidin.. 2785
Granisetron hydrochloride.. 2787
Griseofulvin.. 2788
Guaiacol.. 2789
Guaifenesin.. 2791
Guanethidine monosulfate.. 2792
Guar galactomannan.. 2793

**01/2017:2173**
**corrected 9.4**

# GABAPENTIN

## Gabapentinum

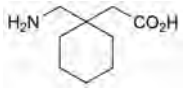


$C_9H_{17}NO_2$ $M_r$ 171.2
[60142-96-3]

DEFINITION

[1-(Aminomethyl)cyclohexyl]acetic acid.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute acids and dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *gabapentin CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.50 g in a mixture of 0.5 mL of *acetic acid R*, 19.5 mL of *methanol R* and 30 mL of *water R*.

**pH** (*2.2.3*): 6.5 to 7.5.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**

A. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 2.32 g of *ammonium dihydrogen phosphate R* in 950 mL of *water R*, adjust to pH 2.0 with *phosphoric acid R*, and dilute to 1000 mL with *water R*.

*Buffer solution*. Dissolve 0.58 g of *ammonium dihydrogen phosphate R* and 1.83 g of *sodium perchlorate R* in 950 mL of *water for chromatography R*, adjust to pH 1.8 with *perchloric acid R*, and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 0.140 g of the substance to be examined in solution A and dilute to 10.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 7.0 mg of *gabapentin impurity A CRS* and 10 mg of *gabapentin impurity B CRS* in *methanol R1* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with solution A.

*Reference solution (c)*. Dissolve 0.140 g of *gabapentin CRS* in solution A and dilute to 10.0 mL with solution A.

*Reference solution (d)*. Dissolve 7.0 mg of *gabapentin impurity D CRS* in 25 mL of *methanol R1* and dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R1*, buffer solution (24:76 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: 4 times the retention time of gabapentin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to gabapentin (retention time = about 4 min): impurity A = about 2.4; impurity B = about 2.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.3 between the peaks due to impurities A and B.

To avoid memory effects between 2 chromatograms, the column may be washed using *acetonitrile R1*.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply for this test.

B. Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Mobile phase*: *methanol R2*, *acetonitrile R1*, buffer solution (30:35:35 *V/V/V*).

*Injection*: 20 µL of the test solution and reference solution (d).

*Run time*: 1.2 times the retention time of impurity D.

*Retention time*: impurity D = about 10 min.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent);
- *disregard limit*: 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.03 per cent); disregard any peak with a relative retention of not more than 0.4 with reference to impurity D.

*Limit*:

- *total for tests A and B*: maximum 0.5 per cent.

**Chlorides**: maximum 100 ppm.

Dissolve 1.5 g in a mixture of 0.5 mL of *acetic acid R*, 19.5 mL of *methanol R* and 30 mL of *water R*. Titrate with *0.001 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.001 M silver nitrate* is equivalent to 0.03545 mg of chlorides.

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: 20 µL of the test solution and reference solution (c).

*System suitability*: reference solution (c):

– *symmetry factor*: maximum 5.0 for the peak due to gabapentin.

Calculate the percentage content of $C_9H_{17}NO_2$ taking into account the assigned content of *gabapentin CRS*.

IMPURITIES

**Test A for related substances**: A, B, E, G.

**Test B for related substances**: D.

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, G.*

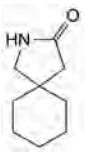

A. 2-azaspiro[4.5]decan-3-one,

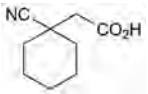

B. (1-cyanocyclohexyl)acetic acid,

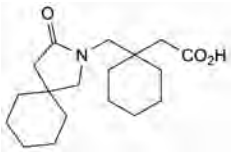

D. [1-[(3-oxo-2-azaspiro[4.5]dec-2-yl)methyl]cyclohexyl]-acetic acid,

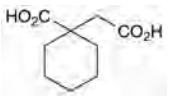

E. 1-(carboxymethyl)cyclohexanecarboxylic acid,

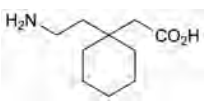

G. [1-(2-aminoethyl)cyclohexyl]acetic acid.

**04/2016:2735**
**corrected 10.0**

# GADOBUTROL MONOHYDRATE

## Gadobutrolum monohydricum

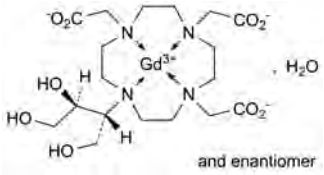


$C_{18}H_{31}GdN_4O_9,H_2O$ $M_r$ 623
[198637-52-4]

DEFINITION

Gadolinium 2,2′,2″-[10-[(1*RS*,2*SR*)-2,3-dihydroxy-1-(hydroxymethyl)propyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triyl]triacetate monohydrate.

*Content*:

– *gadobutrol*: 97.5 per cent to 102.5 per cent (anhydrous substance);
– *gadolinium*: 98.0 per cent to 102.0 per cent, calculated as gadobutrol (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol and in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *gadobutrol monohydrate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in 0.5 mL of *water R*, then add 5 mL of *anhydrous ethanol R*; evaporate to dryness and record new spectra using the residues.

B. Inductively coupled plasma-atomic emission spectrometry (*2.2.57*) as described in the assay for gadolinium.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 6.0 g in *water R* and dilute to 10.0 mL with the same solvent. Sonicate if necessary.

**Specific optical rotation** (*2.2.7*): – 0.05 to + 0.05 (anhydrous substance), using an instrument with a minimum sensitivity of ± 0.002°.

Dissolve 6.00 g in *water R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Use suitable plastic labware for the preparation of the solutions and plastic vials. Store the solutions at 10 °C and use them within 42 h.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 10 mg of *gadobutrol for peak identification CRS* (containing impurity C) in 1 mL of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 20.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 50.0 mg of *gadobutrol monohydrate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped phenylhexylsilyl silica gel for chromatography R* (3 µm) with a pore size of 10 nm.

*Temperature*:

– *autosampler*: 10 °C;

– *column*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 5 volumes of *acetonitrile R1* and 995 volumes of *water for chromatography R* previously adjusted to pH 3.6 with a 50 per cent *V/V* solution of *anhydrous formic acid R* in *water for chromatography R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 30 | 100 → 75 | 0 → 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: charged aerosol detector at 100 pA.

*Injection*: 40 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *gadobutrol for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to gadobutrol (retention time = about 11 min): impurity C = about 1.2.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to gadobutrol.

*Calculation of percentage contents*:

– for each impurity, use the concentration of gadobutrol monohydrate in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.05 per cent;

– *total*: maximum 0.3 per cent;

– *reporting threshold*: 0.03 per cent.

**Free gadolinium**: maximum 0.01 per cent (anhydrous substance).

*Solution A*. To 30.0 mL of *sodium acetate buffer solution pH 5.0 R* add 3.0 mL of *xylenol orange solution R* and dilute to 200.0 mL with *water R*.

*Gadolinium sulfate solution.* Dissolve 93.35 mg of *gadolinium sulfate octahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Test solution.* Dissolve 0.250 g of the substance to be examined in 5.0 mL of the gadolinium sulfate solution and 30 mL of *water R* with the aid of ultrasound. Add 10.0 mL of solution A and adjust to pH 5.0 with a 10.3 g/L solution of *hydrochloric acid R*. Titrate with *0.00025 M sodium edetate*, determining the end-point photometrically using a suitable autotitrator equipped with a photometric sensor at a wavelength of 570-574 nm ($V_1$ mL).

*Standard solution.* Prepare a solution in the same manner as for the test solution but omitting the substance to be examined ($V_2$ mL).

Determine the free gadolinium content from the difference in the volumes of titrant consumed ($V_1$ - $V_2$).

1 mL of *0.00025 M sodium edetate* is equivalent to 0.03931 mg of gadolinium.

**Water** (*2.5.32*): 2.0 per cent to 7.0 per cent, determined on 0.100 g using the evaporation technique at 220 °C.

ASSAY

**Gadobutrol**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 195 nm.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{31}GdN_4O_9$ taking into account the assigned content of *gadobutrol monohydrate CRS*.

**Gadolinium**. Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Yttrium standard solution.* To 100.0 mL of a certified reference solution containing 1000 mg/L of Y add 50 mL of *nitric acid R* and dilute to 1000.0 mL with *water R*.

*Zero solution.* To 1.5 mL of the yttrium standard solution add 0.5 mL of *nitric acid R* and dilute to 50.0 mL with *water R*.

*Test solution.* Dissolve 50.0 mg in 150.0 mL of *water R*, add 7.5 mL of yttrium standard solution and 2.0 mL of *nitric acid R* and dilute to 250.0 mL with *water R*.

*Reference solutions.* Into 3 volumetric flasks introduce respectively 2.0 mL, 2.5 mL and 3.0 mL of a certified reference solution containing 1000 mg/L of Gd. To each flask add 1.5 mL of the yttrium standard solution and 0.5 mL of *nitric acid R* and dilute to 50.0 mL with *water R*.

*Wavelengths*: 217.069 nm, 217.774 nm, 219.103 nm, 226.109 nm (gadolinium), 224.306 nm (yttrium).

From the calibration curve obtained with the reference solutions, calculate the percentage content of gadolinium in the substance to be examined, using the mean of the results obtained with the different wavelengths and applying a conversion factor of 3.846.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

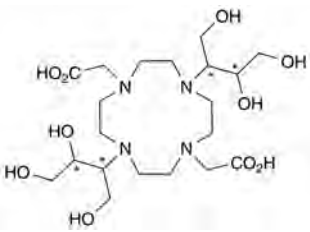


A. 2,2′-[4,10-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]-1,4,7,10-tetraazacyclododecane-1,7-diyl]diacetic acid,

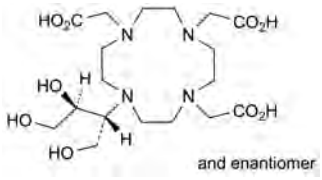


B. 2,2′,2″-[10-[(1*RS*,2*SR*)-2,3-dihydroxy-1-(hydroxymethyl)-propyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triyl]triacetic acid,

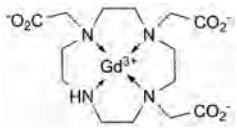


C. gadolinium 2,2′,2″-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate.

**04/2017:2225**
**corrected 10.0**

# GADODIAMIDE HYDRATE

## Gadodiamidum hydricum

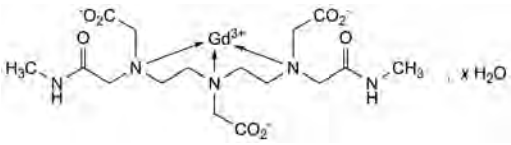


$C_{16}H_{26}GdN_5O_8,xH_2O$ with $x$ = 2 to 5 — $M_r$ 573.7 (anhydrous substance)

Anhydrous gadodiamide: [131410-48-5]

DEFINITION

6-(Carboxylatomethyl)-3,9-bis[(methylcarbamoyl)methyl]-3,6,9-triazaundecanedioato-κ$^3N^3$,$N^6$,$N^9$-gadolinium(III) hydrate (Gd-DTPA-BMA).

*Content*:

– *gadolinium* (Gd; $M_r$ 157.3): 26.0 per cent to 29.0 per cent (anhydrous substance);
– *gadodiamide* ($C_{16}H_{26}GdN_5O_8$; $M_r$ 573.7): 96.5 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white powder, slightly hygroscopic.

*Solubility*: freely soluble in water and in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *gadodiamide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Gadolinium (see Assay).

*Results*: the test solution exhibits the characteristic emission of gadolinium at 342.247 nm.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*). If this is not the case, heat the solution at 60-70 °C for 2-3 min, cool to room temperature and compare the solutions again.

Dissolve 7.5 g (anhydrous substance) in *water R* and dilute to 25 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 6.5.

Dissolve 0.50 g (anhydrous substance) in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dissolve 60.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *gadodiamide impurity A CRS* and 5.0 mg of *gadodiamide impurity B CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *gadodiamide CRS* in *water R*, add 0.5 mL of reference solution (a) and dilute to 10 mL with *water R*.

*Reference solution (c)*. Dissolve 60.0 mg of *gadodiamide CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (e)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: to 980 mL of *water for chromatography R* add 287 µL of *glacial acetic acid R* and 700 µL of *triethylamine R*, adjust to pH 6.8 with a 0.6 g/L solution of *acetic acid R* or with a 42 g/L solution of *sodium hydroxide R*, then dilute to 1000 mL with *water for chromatography R*; use within 2 days.

*Flow rate*: 1.5 mL/min.

*Post-column solution*: dissolve 0.120 g of *arsenazo III R* in about 400 mL of *water for chromatography R*, add 0.650 g of *urea R* and mix to dissolve; add 6.3 mL of *nitric acid R* and sonicate for 15 min, filter through a membrane filter (nominal pore size 0.45 µm) and wash the filter with about 550 mL of *water for chromatography R*; dilute the filtrate and washings to 1000 mL with *water for chromatography R*; use within 1 week.

*Flow rate of post-column solution*: 1.5 mL/min.

*Detection*: spectrophotometer at 658 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b), (d) and (e).

*Run time*: 5 times the retention time of gadodiamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to gadodiamide (retention time = about 5 min): impurity A = about 2.6; impurity B = about 4.0. The peak due to gadodiamide shows a shoulder due to an isomer that is to be integrated with the main peak.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to gadodiamide and impurity A; minimum 2.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

- for impurities A and B, use the concentration of each substance in reference solution (e);
- for impurities other than A and B, use the concentration of gadodiamide hydrate in reference solution (d).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurity B*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *sum of unspecified impurities*: maximum 0.3 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.10 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Excess of gadolinium or DTPA-BMA**: maximum 20 ppm (anhydrous substance) for the excess of gadolinium and maximum 0.1 per cent (anhydrous substance) for the excess of diethylenetriaminepentaacetic acid bis-(methylamide) (DTPA-BMA).

*Test solution.* Dissolve 2.0 g of the substance to be examined in 25.0 mL of *1 M morpholinoethanesulfonate buffer solution pH 6.0 R*. Add 50 µL of a 1.5 g/L solution of *arsenazo III R*.

*Blank solution.* To 25.0 mL of *1 M morpholinoethanesulfonate buffer solution pH 6.0 R*, add 50 µL of a 1.5 g/L solution of *arsenazo III R*. The solution is violet-pink.

*Standard solution.* Dissolve 18.6 g of *gadolinium chloride hexahydrate R* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 1000.0 mL with the same acid solution. Dilute 20.0 mL of this solution to 1000.0 mL with *water R* (0.001 M solution). Standardise 10.0 mL of *0.001 M sodium edetate*, using a mixture of 25.0 mL of *1 M morpholinoethanesulfonate buffer solution pH 6.0 R* and 50 µL of a 1.5 g/L solution of *arsenazo III R*. Titrate with the standard solution until a greenish-blue colour is obtained.

*Excess of DTPA-BMA*. If the test solution is violet-pink, there is no excess of gadolinium. Determine the excess of DTPA-BMA by titrating the test solution and the blank solution with the standard solution until a greenish-blue colour is obtained. Calculate the excess of DTPA-BMA, in per cent, using the following expression:

$$\frac{(V_1 - V_0) \times M_1 \times 419.43 \times 100}{m}$$

$V_0$ = volume of standard solution used in the blank titration, in millilitres;

$V_1$ = volume of standard solution used in the titration of the test solution, in millilitres;

$M_1$ = molarity of the gadolinium solution, in moles per litre;

$m$ = mass of the substance to be examined (anhydrous substance) used to prepare the test solution, in milligrams.

*Excess of gadolinium.* If the test solution is greenish-blue, there is no excess of DTPA-BMA. Determine the excess of gadolinium by titrating the test solution with *0.001 M sodium edetate* until a violet-pink colour is obtained. Calculate the excess of gadolinium, in parts per million, using the following expression:

$$\frac{V_2 \times M_2 \times 157.25 \times 10^6}{m}$$

$V_2$ = volume of *0.001 M sodium edetate* used in the titration of the test solution, in millilitres;

$M_2$ = molarity of the sodium edetate solution, in moles per litre;

$m$ = mass of the substance to be examined (anhydrous substance) used to prepare the test solution, in milligrams.

**Impurity C**: maximum 0.02 per cent (anhydrous substance).

*Phthalaldehyde solution.* Introduce 0.100 g of *phthalaldehyde R* into a brown-glass vessel and dissolve in 3 mL of *methanol R*. Add 220 mL of *borate buffer solution pH 10.0 R* and 100 µL of *2-mercaptoethanol R* and mix. Keep refrigerated and use within 24 h.

*Test solution.* Dissolve 1.5 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solutions.* Prepare a series of reference solutions for calibration in the range of 1 µg/mL to 35 µg/mL of methylamine (impurity C) from a 0.220 g/L solution of *methylamine hydrochloride R*. Calculate the concentrations of methylamine by multiplying the concentrations of methylamine hydrochloride by 0.46.

*Blank solution.* A recently prepared mixture of 3.0 mL of phthalaldehyde solution and 3.0 mL of *water R*.

Introduce 3.0 mL of the test solution and of each reference solution into separate test tubes. Add 3.0 mL of phthalaldehyde solution to each tube and mix. Immediately transfer each solution to a cell and immediately measure the absorbance (*2.2.25*) at 335 nm using the blank solution as compensation liquid. Calculate the linear regression curve from the absorbances obtained with the reference solutions: the coefficient of determination $r^2$ is not less than 0.99. If the absorbance obtained with the test solution is outside the calibration range, dilute the test solution with *water R* and repeat the procedure.

**Water** (*2.5.12*): 6.0 per cent to 13.0 per cent, determined on 0.150 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*): less than 3.50 IU/g.

ASSAY

**Gadolinium**. Inductively coupled plasma-atomic emission spectrometry (*2.2.57*). An external calibration curve is used for quantification.

*Test solution.* Dissolve 0.600 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with a 20 g/L solution of *nitric acid R*.

*Reference solutions.* Into 3 volumetric flasks, introduce 10.0 mL, 15.0 mL and 20.0 mL of a certified reference solution containing 1000 µg/mL of gadolinium. Dilute each flask to 100.0 mL with a 20 g/L solution of *nitric acid R*. Mix to obtain reference solutions containing respectively 100 µg/mL, 150 µg/mL and 200 µg/mL of gadolinium.

The standard operating conditions prescribed by the manufacturer of the apparatus are to be followed. The following operating conditions are cited as an example of conditions found suitable for a given apparatus:

- *flush time*: 15 s;
- *pure gas ($N_2$) flow*: 1 mL/min;
- *plasma flow*: 15 L/min;
- *auxiliary flow*: 1.0 mL/min;
- *nebuliser flow*: 1.0 mL/min;
- *pump rate*: 1.0 mL/min;
- *power*: 1160 W;
- *read delay*: 25 s;
- *wavelength*: 342.247 nm;

– *viewing height*: 14.0 mm;
– *background correction*: none;
– *nebuliser*: cross-flow nebuliser.

*System suitability*:
– the correlation coefficient of the calibration curve is not less than 0.999.

**Gadodiamide**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{16}H_{26}GdN_5O_8$ taking into account the assigned content of *gadodiamide CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A, B, C.*

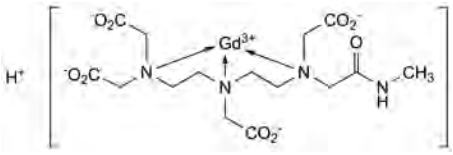

A. hydrogen[3,6-bis(carboxylatomethyl)-9-[(methylcarbamoyl)methyl]-3,6,9-triazaundecanedioato-$\kappa^3N^3,N^6,N^9$-gadolinate](1-) (Gd-DTPA-MMA),

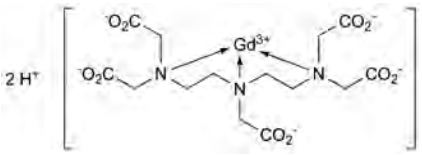

B. dihydrogen[3,6,9-tris(carboxylatomethyl)-3,6,9-triazaundecanedioato-$\kappa^3N^3,N^6,N^9$-gadolinate](2-) (Gd-DTPA),

C. methanamine (methylamine).

**01/2020:1215**

# GALACTOSE

## Galactosum

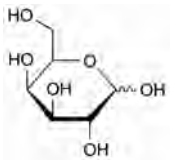

$C_6H_{12}O_6$ $M_r$ 180.2
[59-23-4]

DEFINITION

D-Galactopyranose.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or finely granulated powder.

*Solubility*: freely soluble or soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *galactose CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.
*Reference solution (a)*. Dissolve 10 mg of *galactose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.
*Reference solution (b)*. Dissolve 10 mg each of *galactose R*, *glucose R* and *lactose monohydrate R* (impurity A) in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *water R*, *propanol R* (15:85 *V/V*).
*Application*: 2 µL; thoroughly dry the points of application.
*Development*: in an unsaturated tank over 3/4 of the plate.
*Drying*: in a current of warm air.
*Detection*: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*. Heat in an oven at 130 °C for 10 min.
*System suitability*: reference solution (b):
– the chromatogram shows 3 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. An orange or red precipitate is formed.

TESTS

**Solution S**. Dissolve, with heating in a water-bath at 50 °C, 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 30 mL of solution S add 0.3 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Proteins**: maximum 0.1 mg/mL.

Dissolve 1.000 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent. Measure the absorbance (*2.2.25*) of the solution at 260 nm and at 280 nm and calculate the protein content, in milligrams per millilitre, using the following expression:

$$(A_{280} \times 1.45) - (A_{260} \times 0.74)$$

$A_{280}$ = absorbance at 280 nm;

$A_{260}$ = absorbance at 260 nm.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 3 mg each of *1,6-galactosylgalactose R* (impurity B), *galacturonic acid R* (impurity C) and *lactose monohydrate R* (impurity A) in *water R* and dilute to 10 mL with the same solvent. To 100 µL of the solution add 900 µL of test solution (a).

*Reference solution (c).* Dissolve 25.0 mg of *galactose CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Column*: 2 columns to be connected in series:

- *size*: *l* = 0.30 m, Ø = 6.5 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (10 µm);
- *temperature*: 75 °C.

*Mobile phase*: dissolve 50 mg of *sodium calcium edetate R* in 900 mL of *water for chromatography R*, add 1.0 mL of *sulfuric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 0.4 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (about 40 °C).

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: twice the retention time of galactose.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A + B and C.

*Relative retention* with reference to galactose (retention time = about 24 min): impurities A and B = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity C and galactose.

*Calculation of percentage contents*:

- for each impurity, use the concentration of galactose in reference solution (a).

*Limits*:

- *sum of impurities A and B*: maximum 1.0 per cent;
- *unspecified impurities*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.2 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Sulfated ash**: maximum 0.1 per cent.

To 5 mL of solution S add 2 mL of *sulfuric acid R*, evaporate to dryness on a water-bath and ignite to constant mass. The residue weighs a maximum of 1 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_6H_{12}O_6$ taking into account the assigned content of *galactose CRS*.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E.*

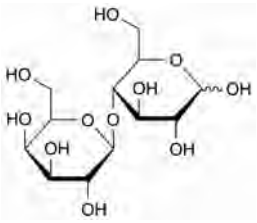

A. 4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose),

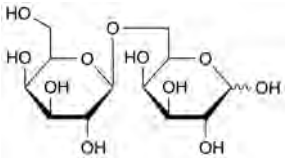

B. 6-*O*-β-D-galactopyranosyl-D-galactopyranose (1,6-galactosylgalactose),

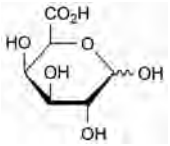

C. D-galactopyranuronic acid (galacturonic acid),

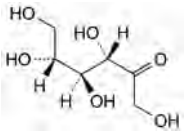

D. D-tagatose,

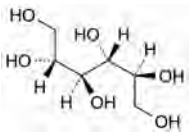

E. galactitol (dulcitol).

**01/2020:2366**

# GALANTAMINE HYDROBROMIDE

## Galantamini hydrobromidum

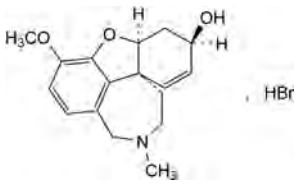

$C_{17}H_{22}BrNO_3$ $M_r$ 368.3

[1953-04-4]

DEFINITION

(4a*S*,6*R*,8a*S*)-3-Methoxy-11-methyl-5,6,9,10,11,12-hexahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-ol hydrobromide.

It is isolated from natural sources or produced by a synthetic process.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or amorphous powder.

*Solubility*: sparingly soluble in water, very slightly soluble in anhydrous ethanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *galantamine hydrobromide CRS*.

B. Specific optical rotation or enantiomeric purity (see Tests).

C. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.60 g in *carbon dioxide-free water R* and dilute to 30.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*) for galantamine from natural sources: − 100 to − 90 (dried substance), determined on Solution S.

**Enantiomeric purity** for galantamine produced by a synthetic process. Capillary electrophoresis (*2.2.47*). *Prepare the solutions immediately before use.*

*Buffer electrolyte*: 8.9 g/L solution of *disodium hydrogen phosphate dihydrate R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 50.0 mL of *water R* and filter through a membrane filter (nominal pore size 0.22 µm).

*Reference solution (a)*. Dissolve 5 mg of *galantamine racemic mixture CRS* in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to 100 mL with *water R* and filter through a membrane filter (nominal pore size 0.22 µm).

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R* and filter through a membrane filter (nominal pore size 0.22 µm).

*Blank solution*. Filter *water R* through a membrane filter (nominal pore size 0.22 µm).

*Capillary*:

– *material*: uncoated fused silica;

– *size*: effective length = about 0.50 m, Ø = 75 µm.

*Temperature*: 20 °C.

*CZE buffer*. Dissolve 0.196 g of *α-cyclodextrin R* in 10.0 mL of buffer electrolyte and filter through a membrane filter (nominal pore size 0.22 µm).

*Detection*: spectrophotometer at 214 nm.

*Preconditioning of the capillary*: at 137.9 kPa, rinse the capillary for 5 min with *water R* and for 5 min with CZE buffer.

*Injection*: under pressure (3.45 kPa) for 4 s.

*Migration*: apply a voltage of 15kV.

*Run time*: 35 min.

*Relative migration times* with reference to galantamine (migration time = about 18 min): impurity F = about 1.05.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to galantamine and impurity F.

*Limit*:

– *impurity F*: not more than 1.5 times the area of the principal peak in the electropherogram obtained with reference solution (b) (0.15 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

A. Galantamine from natural sources

*Solvent mixture*: mobile phase B, mobile phase A (10:90 *V/V*).

*Test solution*. Dissolve 12 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *galantamine natural for system suitability CRS* (containing impurities A and E) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 3.15 g of *ammonium formate R* in 900 mL of *water for chromatography R*, adjust to pH 3.8 with *anhydrous formic acid R* and dilute to 1000 mL with *water for chromatography R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 20 | 95 → 80 | 5 → 20 |
| 20 - 23 | 80 → 50 | 20 → 50 |
| 23 - 31 | 50 → 20 | 50 → 80 |
| 31 - 35 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 287 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *galantamine natural for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and E.

*Relative retention* with reference to galantamine (retention time = about 12 min): impurity E = about 0.8; impurity A = about 1.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurity E and galantamine.

*Limits*:

– *impurity E*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);

– *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

B. Galantamine produced by a synthetic process

*Solvent mixture*. Dilute 50 mL of *methanol R* to 1000 mL with *water R*.

*Test solution*. Dissolve 0.10 g of the substance to be examined in 50.0 mL of the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2.5 mg of *galantamine synthetic for system suitability CRS* (containing impurities C and D) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (3.5 µm);
- *temperature*: 55 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.79 g of *disodium hydrogen phosphate dihydrate R* and 2.46 g of *sodium dihydrogen phosphate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; to 950 mL of the solution add 50 mL of *methanol R1*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 100 | 0 |
| 6 - 20 | 100 → 95 | 0 → 5 |
| 20 - 35 | 95 → 85 | 5 → 15 |
| 35 - 50 | 85 → 80 | 15 → 20 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *galantamine synthetic for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to galantamine (retention time = about 16 min): impurity C = about 0.8; impurity D = about 2.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.5 between the peaks due to impurity C and galantamine.

*Limits*:

- *impurities C, D*: for each impurity, not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.275 g in 40 mL of *water R*. Add 40 mL of *ethanol (96 per cent) R*. Add 5 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.83 mg of $C_{17}H_{22}BrNO_3$.

LABELLING

The label states the origin of the substance:

- isolated from natural sources;
- produced by a synthetic process.

IMPURITIES

**Test A for related substances**: A, B, E.

*Specified impurities: A, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

**Test B for related substances**: A, B, C, D, E, F.

*Specified impurities: C, D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E.*

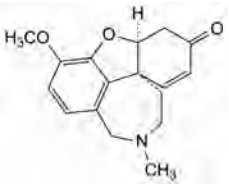

A. (4a*S*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-one (narwedine),

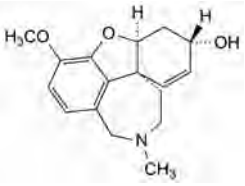

B. (4a*S*,6*S*,8a*S*)-3-methoxy-11-methyl-5,6,9,10,11,12-hexahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-ol (*epi*-galantamine),

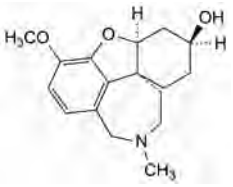

C. (4a*S*,6*S*,8a*R*)-3-methoxy-11-methyl-5,6,7,8,9,10,11,12-octahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-ol (dihydrogalantamine),

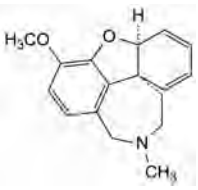

D. (4a*S*,8a*S*)-3-methoxy-11-methyl-9,10,11,12-tetrahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepine (anhydrogalantamine),

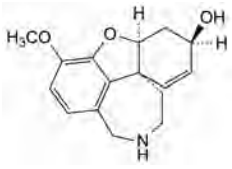

E. (4aS,6*R*,8a*S*)-3-methoxy-5,6,9,10,11,12-hexahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-ol (*N*-demethylgalantamine),

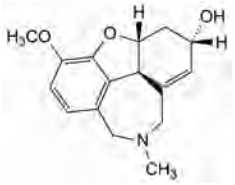

F. (4a*R*,6*S*,8a*R*)-3-methoxy-11-methyl-5,6,9,10,11,12-hexahydro-4a*H*-[1]benzofuro[3a,3,2-*ef*][2]benzazepin-6-ol (*ent*-galantamine).

**04/2018:2769**

# GAMMADEX

## Gammadexum

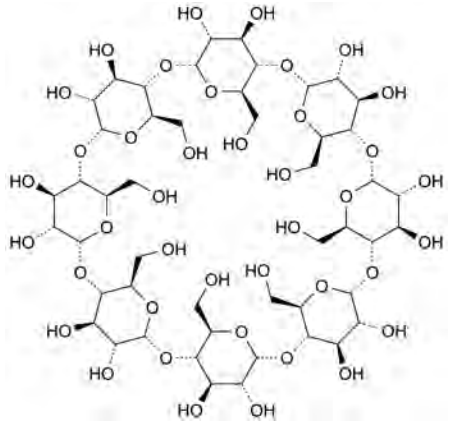

$[C_6H_{10}O_5]_8$ $M_r$ 1297
[17465-86-0]

DEFINITION

Cyclooctakis-(1→4)-(α-D-glucopyranosyl) (cyclomaltooctaose or γ-cyclodextrin).

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline, hygroscopic powder.

*Solubility*: freely soluble in water, very slightly soluble in propylene glycol, practically insoluble in anhydrous ethanol and in methylene chloride. When dissolved in water, it forms a colloidal dispersion over time.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *gammacyclodextrin CRS*.

TESTS

**Solution S**. Dissolve 1.000 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**pH** (*2.2.3*): 5.0 to 8.0.
Mix 1 mL of a 223.6 g/L solution of *potassium chloride R* and 30 mL of solution S.

**Specific optical rotation** (*2.2.7*): + 174 to + 180 (dried substance), determined on solution S after filtration through a membrane filter (nominal pore size 0.45 µm).

**Reducing sugars**: maximum 0.2 per cent.

*Test solution*. To 1 mL of solution S add 1 mL of *cupri-tartaric solution R4*. Heat on a water-bath for 10 min and allow to cool to room temperature. Add 10 mL of *ammonium molybdate reagent R1* and allow to stand for 15 min.

*Reference solution*. Prepare at the same time and in the same manner as for the test solution, using 1 mL of a 0.02 g/L solution of *glucose R*.

Measure the absorbances (*2.2.25*) of the 2 solutions at the absorption maximum at 740 nm using *water R* as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Light-absorbing impurities**. Examine solution S after filtration through a membrane filter (nominal pore size 0.45 µm). The absorbance (*2.2.25*) is not greater than 0.10 between 230 nm and 350 nm and not greater than 0.05 between 350 nm and 750 nm.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.250 g of the substance to be examined in *water R* with heating, cool and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 25.0 mg of *alfadex CRS* (impurity A), 25.0 mg of *betadex CRS* (impurity B) and 25.0 mg of *gammacyclodextrin CRS* (gammadex) in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 50.0 mL with *water R*.

*Reference solution (c)*. Dissolve 25.0 mg of *gammacyclodextrin CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *methanol R*, *water for chromatography R* (5:95 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Equilibration*: with the mobile phase for about 3 h.

*Injection*: 50 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of gammadex.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to gammadex (retention time = about 7 min): impurity A = about 1.5; impurity B = about 3.2.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.5 between the peaks due to gammadex and impurity A;
- *symmetry factor*: 0.8 to 1.8 for the peak due to gammadex.

*Calculation of percentage contents*:
- for impurities A and B, use the concentration of the corresponding impurity in reference solution (b);

– for impurities other than A and B, use the concentration of gammadex in reference solution (b).

*Limits*:

– *impurities A, B*: for each impurity, maximum 0.5 per cent;

– *sum of impurities other than A and B*: maximum 0.5 per cent;

– *reporting threshold*: 0.15 per cent.

**Loss on drying** (*2.2.32*): maximum 11.0 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (c).

*System suitability*: reference solution (c):

– *symmetry factor*: 0.8 to 1.8 for the peak due to gammadex;

– *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 5 injections.

Calculate the percentage content of $[C_6H_{10}O_5]_8$ taking into account the assigned content of *gammacyclodextrin CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

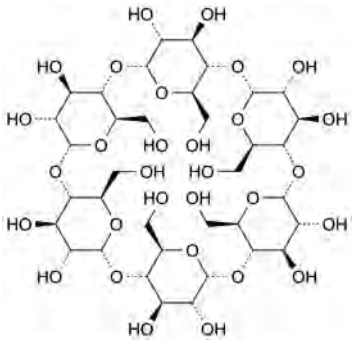

A. cyclohexakis-(1→4)-(α-D-glucopyranosyl) (alfadex or cyclomaltohexaose or α-cyclodextrin),

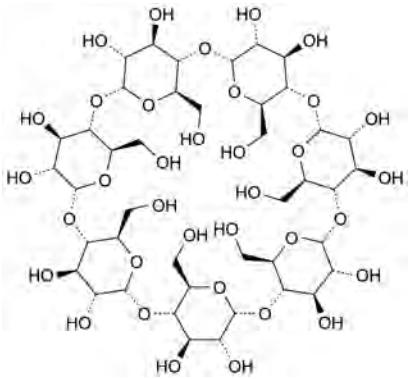

B. cycloheptakis-(1→4)-(α-D-glucopyranosyl) (betadex or cyclomaltoheptaose or β-cyclodextrin).

**01/2017:1752**

# GANCICLOVIR

## Ganciclovirum

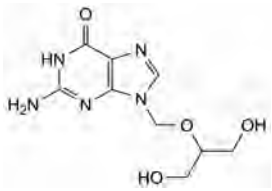

$C_9H_{13}N_5O_4$ $M_r$ 255.2

[82410-32-0]

DEFINITION

2-Amino-9-[[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl]-1,9-dihydro-6*H*-purin-6-one.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of mineral acids and alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ganciclovir CRS*.

If the spectra obtained in the solid state show differences, dissolve 0.10 g of the substance to be examined and the reference substance separately in about 3.6 mL of *water R* at 80 °C. Allow to cool in an ice-bath and filter the precipitate. Dry in an oven at 105 °C for 3 h and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.25 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 25 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30 mg of the substance to be examined in the mobile phase with the aid of ultrasound and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 3 mg of *ganciclovir CRS* in the mobile phase with the aid of ultrasound and dilute to 5.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve the contents of a vial of *ganciclovir impurity mixture CRS* (impurities A, B, C, D, E and F) in 1.0 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (10 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix equal volumes of *acetonitrile R* and a 0.05 per cent *V/V* solution of *trifluoroacetic acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of ganciclovir.

*Identification of impurities*: use the chromatogram supplied with *ganciclovir impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to ganciclovir (retention time = about 14 min): impurity A = about 0.6; impurity B = about 0.67; impurity C = about 0.71; impurity D = about 0.8; impurity E = about 0.9; impurity F = about 2.0.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ganciclovir.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.3; impurity F = 0.7;
- *impurity F*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities A, C, D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g. Use *methanol R* as solvent. The substance to be examined has limited solubility in methanol. The sample will appear as a slurry. Replace the solvent after each titration.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.84 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.200 g in 10 mL of *anhydrous formic acid R* and dilute to 60 mL with *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.52 mg of $C_9H_{13}N_5O_4$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I, J.*

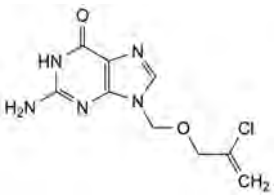

A. 2-amino-9-[[(2-chloroprop-2-en-1-yl)oxy]methyl]-1,9-dihydro-6*H*-purin-6-one,

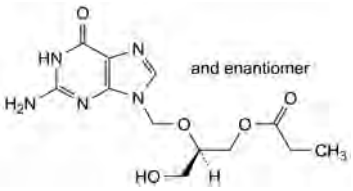


B. (2*RS*)-2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]-3-hydroxypropyl propionate,

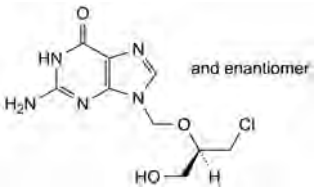


C. 2-amino-9-[[(1*RS*)-2-chloro-1-(hydroxymethyl)ethoxy]-methyl]-1,9-dihydro-6*H*-purin-6-one,

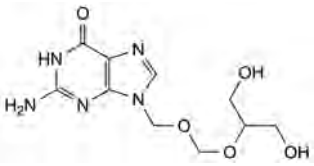

D. 2-amino-9-[[[2-hydroxy-1-(hydroxymethyl)ethoxy]-methoxy]methyl]-1,9-dihydro-6*H*-purin-6-one,

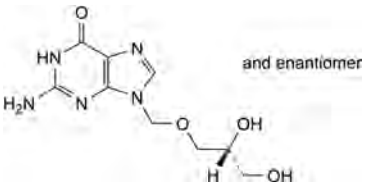


E. 2-amino-9-[[(2*RS*)-2,3-dihydroxypropoxy]methyl]-1,9-dihydro-6*H*-purin-6-one,

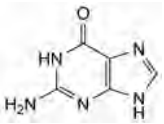

F. 2-amino-1,9-dihydro-6*H*-purin-6-one (guanine),

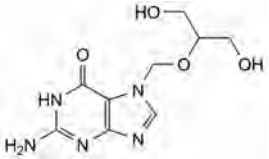

H. 2-amino-7-[[2-hydroxy-1-(hydroxymethyl)ethoxy]-methyl]-1,7-dihydro-6*H*-purin-6-one,

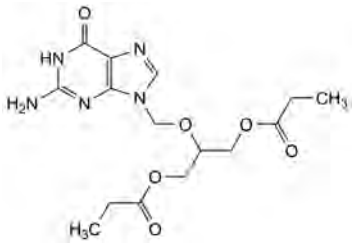

I. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]propane-1,3-diyl dipropanoate,

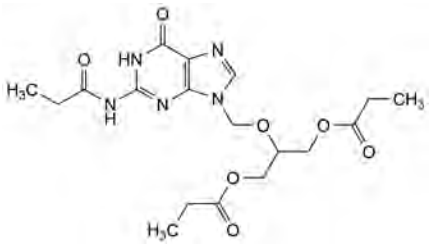

J. 2-[2-(propanoylamino)-6-oxo-1,6-dihydro-9*H*-purin-9-yl]methoxy]propane-1,3-diyl dipropanoate.

**01/2018:2866**

# GEFITINIB

## Gefitinibum

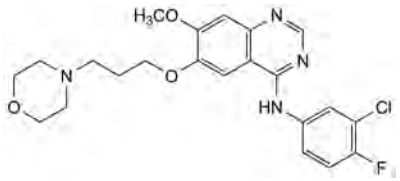

$C_{22}H_{24}ClFN_4O_3$ $M_r$ 446.9
[184475-35-2]

DEFINITION

*N*-(3-Chloro-4-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy]quinazolin-4-amine.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *gefitinib CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 0.2 per cent *V/V* solution of *trifluoroacetic acid R* (40:60 *V/V*).

*Test solution.* Dissolve 35.0 mg of the substance to be examined in 85 mL of the solvent mixture, with the aid of ultrasound, and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 35.0 mg of *gefitinib CRS* in 85 mL of the solvent mixture, with the aid of ultrasound, and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *gefitinib for system suitability CRS* (containing impurity B) in 1.0 mL of the solvent mixture with the aid of ultrasound.

*Column*:

– *size*: *l* = 0.10 m, Ø = 3 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 60 °C.

*Mobile phase*: *acetonitrile R*, 9.68 g/L solution of *ammonium acetate R* (38:62 *V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 247 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Run time*: 5 times the retention time of gefitinib.

*Identification of impurities*: use the chromatogram supplied with *gefitinib for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*Relative retention* with reference to gefitinib (retention time = about 5.5 min): impurity B = about 1.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to gefitinib and impurity B.

*Calculation of percentage contents*:

– for each impurity, use the concentration of gefitinib in reference solution (b).

*Limits*:

– *impurity B*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}ClFN_4O_3$ taking into account the assigned content of *gefitinib CRS*.

IMPURITIES

*Specified impurities*: *B*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

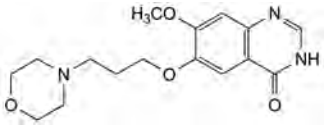

A. 7-methoxy-6-[3-(morpholin-4-yl)propoxy]quinazolin-4(3*H*)-one,

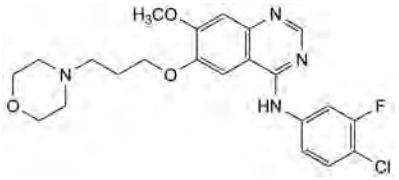

B. *N*-(4-chloro-3-fluorophenyl)-7-methoxy-6-[3-(morpholin-4-yl)propoxy]quinazolin-4-amine.

01/2020:0330

# GELATIN(1)

## Gelatina

DEFINITION

Purified protein obtained from collagen of animals by partial alkaline and/or acid hydrolysis♦ and/or enzymatic hydrolysis,♦ or by thermal hydrolysis.

The hydrolysis leads to gelling or non-gelling product grades. This monograph covers both grades.

♦CHARACTERS

*Appearance*:

- *gelling grade*: faintly yellow or light yellowish-brown solid, usually occurring as translucent sheets, shreds, granules or powder;
- *non-gelling grade*: faintly yellow or white granules or powder.

*Solubility*:

- *gelling grade*: practically insoluble in common organic solvents; gelling grades swell in cold water and give on heating a colloidal solution which on cooling forms a more or less firm gel;
- *non-gelling grade*: soluble in cold or warm water, practically insoluble in common organic solvents.

Different gelatins form aqueous solutions that vary in clarity and colour. For a particular application, a suitable specification for clarity and colour may be required.♦

IDENTIFICATION

*Gelling grade: A, B.*

*Non-gelling grade: A, B, C.*

A. To 2 mL of solution S (see Tests) add 0.05 mL of *copper sulfate solution R*. Mix and add 0.5 mL of *dilute sodium hydroxide solution R*. A violet colour is produced.

B. In a test-tube about 15 mm in internal diameter, place 0.5 g of the substance to be examined and add 10 mL of *water R*. Allow to stand for 10 min, heat at 60 °C for 15 min and keep the tube upright at 2-8 °C for 6 h. Invert the tube; the contents flow out immediately for non-gelling grades and do not flow out immediately for gelling grades.

C. To 0.5 g in a 250 mL bottle, add 10 mL of *water R* and 5 mL of *sulfuric acid R*. Place the bottle, partly but not completely closed (for example, using a watch glass), in an oven at 105 °C for 4 h. Allow to cool and add 200 mL of *water R*. Adjust to pH 6.0-8.0 using a 200 g/L solution of *sodium hydroxide R*. Place 2 mL of the solution in a test-tube and add 2 mL of a solution prepared immediately before use containing 14 g/L of *chloramine R* in *phosphate buffer solution pH 6.8 R*. Mix and allow to stand for 20 min. Add 2 mL of *dimethylaminobenzaldehyde solution R9*. Mix and place in a water-bath at 60 °C for 15 min. A red to violet colour develops.

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* at about 55 °C, dilute to 100 mL with the same solvent and keep the solution at this temperature to carry out the tests.

**pH** (*2.2.3*): 3.8 to 7.6 for solution S, measured at 55 °C.

**Conductivity** (*2.2.38*): maximum 1 $mS \cdot cm^{-1}$, determined on a 10.0 g/L solution at 30 ± 1.0 °C (without the use of a temperature compensation device).

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Peroxides**: maximum 10 ppm, determined using *peroxide test strips R*.

Peroxidase transfers oxygen from peroxides to an organic redox indicator which is converted to a blue oxidation product. The intensity of the colour obtained is proportional to the quantity of peroxide and can be compared with a colour scale provided with the test strips, to determine the peroxide concentration.

*Suitability test.* Dip a test strip for 1 s into *hydrogen peroxide standard solution (2 ppm $H_2O_2$) R*, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid and after 15 s compare the reaction zone with the colour scale provided. The test strips are suitable if the colour matches that of the 2 ppm concentration.

*Test.* Weigh 20.0 ± 0.1 g of the substance to be examined in a beaker and add 80.0 ± 0.2 mL of *water R*. Stir to moisten all the gelatin and allow the sample to stand at room temperature for 1-3 h. Cover the beaker with a watch-glass. If dissolution is not complete, place the beaker for 20 ± 5 min in a water-bath at 65 ± 2 °C to dissolve the sample. Stir the contents of the beaker with a glass rod to achieve a homogeneous solution. Dip a test strip for 1 s into the test solution, such that the reaction zone is properly wetted. Remove the test strip, shake off excess liquid and after 15 s compare the reaction zone with the colour scale provided. Multiply the concentration read from the colour scale by a factor of 5 to calculate the concentration in parts per million of peroxide in the substance to be examined.

**Gel strength (Bloom value)**: 80 per cent to 120 per cent of the nominal value stated on the label for the gelling grade.

The gel strength is expressed as the mass in grams necessary to produce the force which, applied to a plunger 12.7 mm in diameter, makes a depression 4 mm deep in a gel having a concentration of 6.67 per cent *m/m* and matured at 10 °C.

*Apparatus.* Texture analyser or gelometer with:

- a cylindrical piston 12.7 ± 0.1 mm in diameter with a plane pressure surface with a sharp bottom edge;
- a bottle 59 ± 1 mm in internal diameter and 85 mm high.

Adjust the apparatus according to the manufacturer's manual. Settings are: distance 4 mm, test speed 0.5 mm/s.

*Method.* Place 7.5 g of the substance to be examined in a bottle. Add 105 mL of *water R*, close the bottle and allow to stand for 1-4 h. Heat in a water-bath at 65 ± 2 °C for 15 min. While heating, stir gently with a glass rod. Ensure that the solution is uniform and that any condensed water on the inner walls of the bottle is incorporated. Allow to cool at room temperature for 15 min and transfer the bottle to a thermostatically controlled bath at 10.0 ± 0.1 °C, fitted with a device to ensure that the platform on which the bottle stands is perfectly horizontal. Close the bottle with a rubber stopper and allow to stand for 17 ± 1 h. Remove the bottle from the bath and quickly wipe the water from the exterior of the bottle. Centre the bottle on the platform of the apparatus so that the plunger contacts the sample as near to its midpoint as possible and start the measurement.

**Iron**: maximum 30 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*Test solution.* To 5.00 g of the substance to be examined, in a conical flask, add 10 mL of *hydrochloric acid R*. Close the flask and place in a water-bath at 75-80 °C for 2 h (if necessary for proper solubilisation, the gelatin may be allowed to swell after addition of the acid and before heating, the heating time may be prolonged, and a higher temperature may be used). Allow to cool and adjust the contents of the flask to 100.0 g with *water R*.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (8 ppm Fe) R*, diluting with *water R*.

*Wavelength*: 248.3 nm.

**Chromium**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Test solution described in the test for iron.

*Reference solutions.* Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with *water R*.

*Wavelength*: 357.9 nm.

**Zinc**: maximum 30 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Test solution described in the test for iron.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *water R*.

*Wavelength*: 213.9 nm.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 5.000 g by drying in an oven at 105 °C for 16 h.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

STORAGE

Protected from heat and moisture.

LABELLING

The label states the gel strength (Bloom value) or that it is a non-gelling grade.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for gelling grade gelatin used as viscosity-increasing agent, binder or used for microencapsulation.*

**Gel strength (Bloom value)** (see Tests).

**01/2017:2306**
**corrected 10.0**

# GEMCITABINE HYDROCHLORIDE

## Gemcitabini hydrochloridum

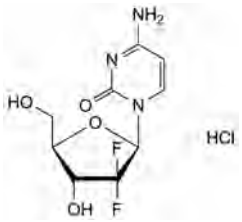


$C_9H_{12}ClF_2N_3O_4$ $M_r$ 299.7
[122111-03-9]

DEFINITION

4-Amino-1-(2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranosyl)pyrimidin-2(1*H*)-one hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, slightly soluble in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *gemcitabine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.0 to 3.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 43.0 to + 50.0, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 200.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of the substance to be examined and 10.0 mg of *gemcitabine impurity A CRS* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 20.0 mg of *gemcitabine hydrochloride CRS* in *water R* and dilute to 200.0 mL with the same solvent.

*Reference solution (c).* Place 10 mg of the substance to be examined in a small vial. Add 4 mL of a 168 g/L solution of *potassium hydroxide R* in *methanol R*, sonicate for 5 min then seal with a cap. The mixture may be cloudy. Heat at 55 °C for a minimum of 6 h to produce impurity B. Allow to cool, then transfer the entire contents of the vial to a 100 mL volumetric flask by successively washing with a 1 per cent *V/V* solution of *phosphoric acid R*. Dilute to 100 mL with a 1 per cent *V/V* solution of *phosphoric acid R* and mix.

*Reference solution (d).* Dissolve 2 mg of *gemcitabine impurity C CRS* in *water R* and dilute to 5.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 13.8 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 2.5 ± 0.1 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 97 | 3 |
| 8 - 13 | 97 → 50 | 3 → 50 |
| 13 - 20 | 50 | 50 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity C.

*Relative retention* with reference to gemcitabine (retention time = about 7 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 1.7.

*System suitability*: reference solution (c):
- *resolution*: minimum 8.0 between the peaks due to impurity B and gemcitabine.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2.4;
- *impurity C*: not more than twice the area of the peak due to gemcitabine in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to gemcitabine in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 4 times the area of the peak due to gemcitabine in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *disregard limit*: 0.5 times the area of the peak due to gemcitabine in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

**Bacterial endotoxins** (*2.6.14*): less than 0.05 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *methanol R*, mobile phase A (3:97 *V/V*).

*Injection*: test solution (b) and reference solutions (b) and (c).

*Relative retention* with reference to gemcitabine (retention time = about 8 min): impurity B = about 0.5.

*System suitability*: reference solution (c):
- *resolution*: minimum 8.0 between the peaks due to impurity B and gemcitabine.

Calculate the percentage content of $C_9H_{12}ClF_2N_3O_4$ taking into account the assigned content of *gemcitabine hydrochloride CRS*.

STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

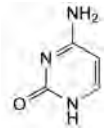

A. 4-aminopyrimidin-2(1*H*)-one (cytosine),

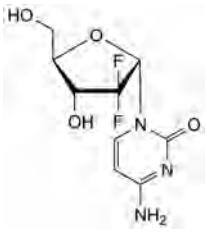

B. 4-amino-1-(2-deoxy-2,2-difluoro-α-D-*erythro*-pentofuranosyl)pyrimidin-2(1*H*)-one (gemcitabine α-anomer),

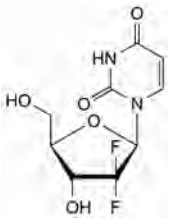

C. 1-(2-deoxy-2,2-difluoro-β-D-*erythro*-pentofuranosyl)pyrimidin-2,4(1*H*,3*H*)-dione (2′-deoxy-2′,2′-difluorouridine) (β-uridine).

**07/2018:1694**

# GEMFIBROZIL

## Gemfibrozilum

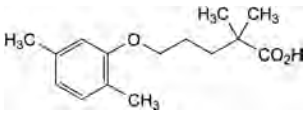

$C_{15}H_{22}O_3$ $M_r$ 250.3

[25812-30-0]

DEFINITION

5-(2,5-Dimethylphenoxy)-2,2-dimethylpentanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, waxy, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, freely soluble in anhydrous ethanol and in methanol.

IDENTIFICATION

A. Melting point (*2.2.14*): 58 °C to 61 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *gemfibrozil CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve the contents of a vial of *gemfibrozil for system suitability CRS* (containing impurities C, D and E) in 2.0 mL of *acetonitrile R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of *2,5-dimethylphenol R* (impurity A) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 0.49 g of *potassium acetate R* in 400 mL of *water for chromatography R*, adjust to pH 4.0 with *phosphoric acid R* and add 600 mL of *acetonitrile R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *gemfibrozil for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C, D and E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to gemfibrozil (retention time = about 7 min): impurity A = about 0.4; impurity C = about 1.3; impurity D = about 1.5; impurity E = about 1.7; impurity I = about 2.0; impurity H = about 2.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 6.0 between the peaks due to gemfibrozil and impurity C, and minimum 2.0 between the peaks due to impurities D and E.

*Limits*:

- *correction factors*: for the calculation of content multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.5; impurity D = 1.8; impurity E = 0.2; impurity H = 0.6;
- *impurities E, I*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities A, D, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.25 per cent, determined on 2.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g. Allow to stand for 1 h after the first moistening before heating.

ASSAY

Dissolve 0.200 g in 40 mL of *methanol R*. Add 10 mL of *water R* and 1 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 25.03 mg of $C_{15}H_{22}O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F, G.*

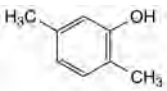

A. 2,5-dimethylphenol (*p*-xylenol),

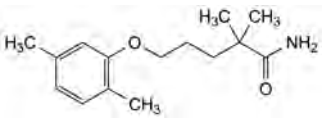

B. 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanamide,

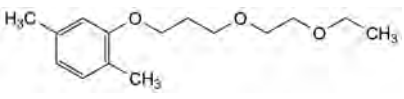

C. 2-[3-(2-ethoxyethoxy)propoxy]-1,4-dimethylbenzene,

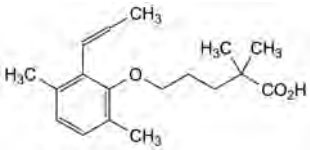

D. 5-[3,6-dimethyl-2-(prop-1-en-1-yl)phenoxy]-2,2-dimethylpentanoic acid,

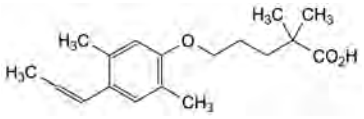

E. 5-[2,5-dimethyl-4-(prop-1-en-1-yl)phenoxy]-2,2-dimethylpentanoic acid,

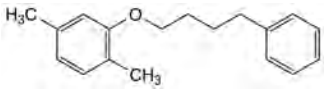

F. 1,4-dimethyl-2-(4-phenylbutoxy)benzene,

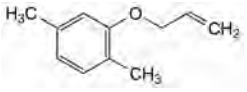

G. 1,4-dimethyl-2-(prop-2-en-1-yloxy)benzene,

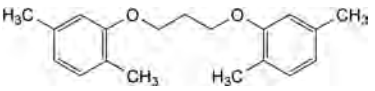

H. 1,1′-[propane-1,3-diylbis(oxy)]bis(2,5-dimethylbenzene),

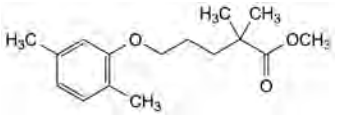

I. methyl 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoate.

**07/2012:0331**
**corrected 10.0**

# GENTAMICIN SULFATE

## Gentamicini sulfas

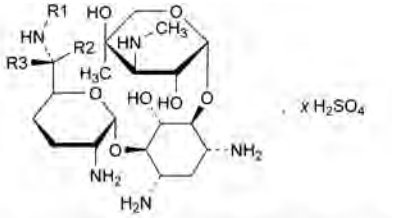

| Gentamicin | Mol. Formula | R1 | R2 | R3 |
|---|---|---|---|---|
| C1 | $C_{21}H_{43}N_5O_7$ | $CH_3$ | $CH_3$ | H |
| C1a | $C_{19}H_{39}N_5O_7$ | H | H | H |
| C2 | $C_{20}H_{41}N_5O_7$ | H | $CH_3$ | H |
| C2a | $C_{20}H_{41}N_5O_7$ | H | H | $CH_3$ |
| C2b | $C_{20}H_{41}N_5O_7$ | $CH_3$ | H | H |

[1405-41-0]

DEFINITION

Mixture of the sulfates of antimicrobial substances produced by *Micromonospora purpurea*, the main components being gentamicins C1, C1a, C2, C2a and C2b.

*Content*: minimum 590 IU/mg (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: B, C.

*Second identification*: A, C.

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution*. Dissolve the contents of a vial of *gentamicin sulfate CRS* in *water R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: the lower layer of a mixture of equal volumes of *concentrated ammonia R*, *methanol R* and *methylene chloride R*.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*Results*: the 3 principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the 3 principal spots in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the test for composition.

*Results*: the chromatogram obtained with test solution (b) shows 5 principal peaks having the same retention times as the 5 principal peaks in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.8 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 107 to + 121 (anhydrous substance).

Dissolve 2.5 g in *water R* and dilute to 25.0 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure taking into account only the peaks due to gentamicins C1, C1a, C2, C2a and C2b.

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *gentamicin for peak identification CRS* (containing impurity B) in the mobile phase and dilute to 25 mL with the mobile phase.

*Reference solution (b)*. Dissolve 20.0 mg of *sisomicin sulfate CRS* (impurity A) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Reference solution (d)*. To 1 mL of reference solution (b), add 5 mL of test solution (a) and dilute to 50 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*. To 900 mL of *carbon dioxide-free water R*, add 7.0 mL of *trifluoroacetic acid R*, 250.0 µL of *pentafluoropropanoic acid R* and 4.0 mL of *carbonate-free*

*sodium hydroxide solution R*, allow to equilibrate and adjust to pH 2.6 using *carbonate-free sodium hydroxide solution R* diluted 1 to 25. Add 15 mL of *acetonitrile R* and dilute to 1000.0 mL with *carbon dioxide-free water R*.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted 1 to 25, previously degassed, which is added pulse-less to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate of post-column solution*: 0.3 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode, and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and – 0.15 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 20 µL of test solution (b) and reference solutions (a), (c) and (d).

*Run time*: 1.2 times the retention time of gentamicin C1.

*Identification of peaks*: use the chromatogram supplied with *gentamicin for peak identification CRS* to identify the peaks due to gentamicins C1, C1a, C2, C2a and C2b.

*Relative retention* with reference to impurity A (retention time = about 23 min): gentamicin C1a = about 1.1; gentamicin C2 = about 1.8; gentamicin C2b = about 2.0; gentamicin C2a = about 2.3; gentamicin C1 = about 3.0.

*System suitability*:

- *resolution*: minimum 1.2 between the peaks due to impurity A and gentamicin C1a and minimum 1.5 between the peaks due to gentamicin C2 and gentamicin C2b in the chromatogram obtained with reference solution (d); if necessary, adjust the volume of *acetonitrile R* in the mobile phase, a total volume of up to 50 mL may be added per litre of mobile phase;
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *gentamicin C1*: 25.0 per cent to 45.0 per cent;
- *gentamicin C1a*: 10.0 per cent to 30.0 per cent;
- *sum of gentamicins C2, C2a and C2b*: 35.0 per cent to 55.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition with the following modifications; use reference solution (c) to calculate the percentage content of each impurity.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram supplied with *gentamicin for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Limits*:

- *impurities A, B*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *any other impurity*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (10 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Methanol** (*2.4.24, System B*): maximum 1.0 per cent.

**Sulfate**: 32.0 per cent to 35.0 per cent (anhydrous substance).

Dissolve 0.250 g in 100 mL of *distilled water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*, adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Water** (*2.5.12*): maximum 15.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 0.50 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.71 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

## ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

## STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

## IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E.*

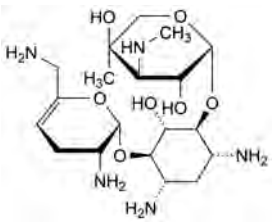

A. 2-deoxy-4-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-6-*O*-(2,6-diamino-2,3,4,6-tetradeoxy-α-D-*glycero*-hex-4-enopyranosyl)-L-streptamine (sisomicin),

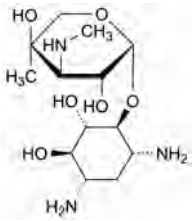

B. 2-deoxy-4-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-L-streptamine (garamine),

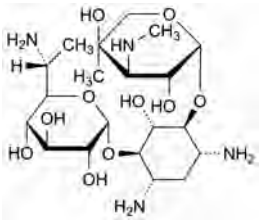

C. 4-*O*-(6-amino-6,7-dideoxy-D-*glycero*-α-D-*gluco*-heptopyranosyl)-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-D-streptamine (gentamicin $B_1$),

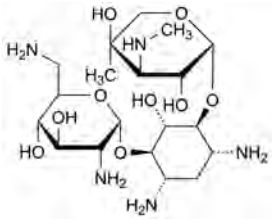

D. 2-deoxy-4-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-6-*O*-(2,6-diamino-2,6-dideoxy-α-D-*gluco*-hexopyranosyl)-L-streptamine,

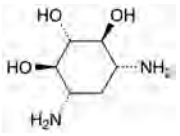

E. 2-deoxystreptamine.

**07/2009:1726**
**corrected 6.8**

# GESTODENE

## Gestodenum

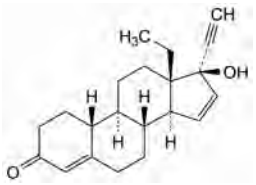

$C_{21}H_{26}O_2$ $M_r$ 310.4
[60282-87-3]

DEFINITION

13-Ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *gestodene CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): − 188 to − 198 (dried substance).

Dissolve 0.100 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in 5 mL of *acetonitrile R1* and dilute to 10.0 mL with *water R*.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 3 mg of *gestodene for system suitability CRS* (containing impurities A, B, C and L) in 0.5 mL of *acetonitrile R1* and dilute to 1.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 30.0 mg of *gestodene CRS* in 5 mL of *acetonitrile R1* and dilute to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve the contents of a vial of *gestodene impurity I CRS* in 1.0 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:
- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 62 | 38 |
| 2 - 20 | 62 → 58 | 38 → 42 |
| 20 - 24 | 58 → 30 | 42 → 70 |
| 24 - 32 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm and at 254 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *gestodene for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and L; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity I.

*Relative retention* with reference to gestodene (retention time = about 12.5 min): impurity A = about 0.9; impurity C = about 1.1; impurity I = about 1.2; impurity L = about 1.46; impurity B = about 1.53.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurity A and gestodene.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.2; impurity I = 1.3;

- *impurity A at 254 nm*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity B at 205 nm*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity C at 254 nm*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities I, L at 205 nm*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities at 254 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total at 254 nm*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit at 254 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (c).

*Detection*: spectrophotometer at 254 nm.

Calculate the percentage content of $C_{21}H_{26}O_2$ from the declared content of *gestodene CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, I, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*):

- *at 205 nm*: *G, J, K*;
- *at 254 nm*: *D, E, F, H.*

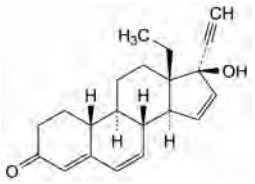

A. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,6,15-trien-20-yn-3-one (Δ6-gestodene),

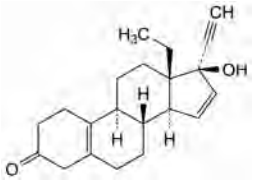

B. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-5(10),15-dien-20-yn-3-one (Δ5(10)-gestodene),

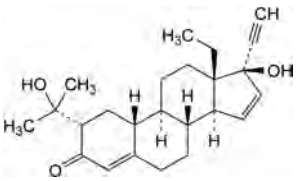

C. 13-ethyl-17-hydroxy-2α-(1-hydroxy-1-methylethyl)-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one (2-isopropanol-gestodene),

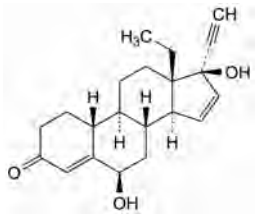

D. 13-ethyl-6β,17-dihydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one (6β-hydroxy-gestodene),

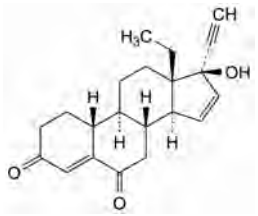

E. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,15-dien-20-yne-3,6-dione (6-keto-gestodene),

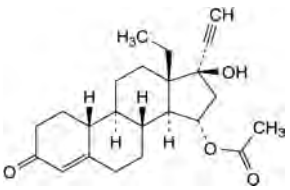

F. 13-ethyl-17-hydroxy-3-oxo-18,19-dinor-17α-pregn-4-en-20-yn-15α-yl acetate (15α-acetoxy-gestodene),

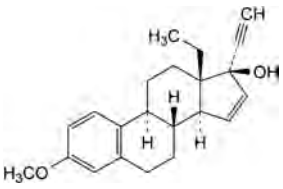

G. 13-ethyl-3-methoxy-18,19-dinor-17α-pregna-1,3,5(10),15-tetraen-20-yn-17-o1 (*A*-aromatic-gestodene),

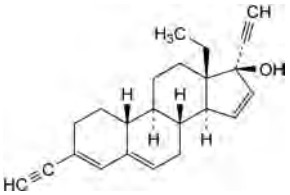

H. 13-ethyl-3-ethynyl-18,19-dinor-17α-pregna-3,5,15-trien-20-yn-17-o1 (diethynyl-gestodene),

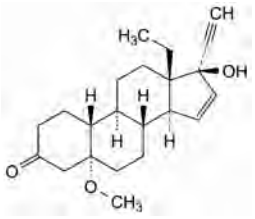

I. 13-ethyl-17-hydroxy-5-methoxy-18,19-dinor-5α,17α-pregn-15-en-20-yn-3-one (5-methoxy-gestodene),

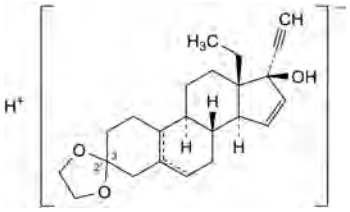

J. 13-ethylspiro(18,19-dinor-17α-pregna-5,15-dien-20-yne-3,2′-[1,3]dioxolan)-17-ol and 13-ethylspiro(18,19-dinor-17α-pregna-5(10),15-dien-20-yne-3,2′-[1,3]dioxolan)-17-ol (gestodene ketal),

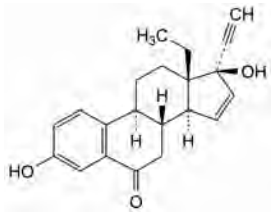

K. 13-ethyl-3,17-dihydroxy-18,19-dinor-17α-pregna-1,3,5(10),15-tetraen-20-yn-6-one (aromatic 6-keto-gestodene),

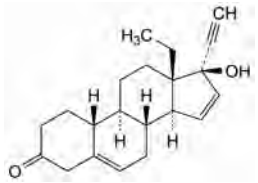

L. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-5,15-dien-20-yn-3-one (Δ5(6)-gestodene).

**01/2017:0718**

# GLIBENCLAMIDE

## Glibenclamidum

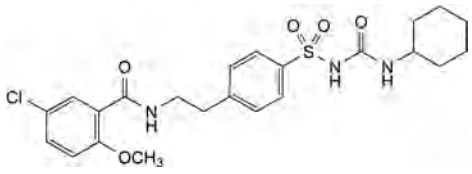

$C_{23}H_{28}ClN_3O_5S$ $M_r$ 494.0
[10238-21-8]

DEFINITION

1-[[4-[2-[(5-Chloro-2-methoxybenzoyl)amino]ethyl]phenyl]-sulfonyl]-3-cyclohexylurea.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent) and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 169 °C to 174 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *methanol R*, with the aid of ultrasound if necessary, and dilute to 50.0 mL with the same solvent. To 10.0 mL of the solution add 1.0 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 300 nm and a less intense maximum at 275 nm.

*Specific absorbance at the absorption maxima*:
- at 300 nm: 61 to 65;
- at 275 nm: 27 to 32.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *glibenclamide CRS*.

If the spectra obtained show differences, moisten separately the substance to be examined and the reference substance with *methanol R*, triturate, dry at 100-105 °C and record the spectra again.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution.* Dissolve 10 mg of *glibenclamide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *ethanol (96 per cent) R*, *glacial acetic acid R*, *cyclohexane R*, *methylene chloride R* (5:5:45:45 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve 20 mg in 2 mL of *sulfuric acid R*. The solution is colourless and shows blue fluorescence in ultraviolet light at 365 nm. Dissolve 0.1 g of *chloral hydrate R* in the solution. After about 5 min, the colour changes to deep yellow and, after about 20 min, develops a brownish tinge.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 5 °C for not more than 40 h.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R*, with the aid of ultrasound if necessary, and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 3.0 mg of *glibenclamide impurity A CRS* and 3 mg of *glibenclamide impurity B CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 20.0 mL with *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 12.5 mg of *glibenclamide for peak identification CRS* (containing impurity C) in *methanol R*, with the aid of ultrasound if necessary, and dilute to 5.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: spherical *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 mL of a 100.0 g/L solution of *triethylamine R2* previously adjusted to pH 3.0 using *phosphoric acid R*, and 50 mL of *acetonitrile R*; dilute to 1000 mL with *water R*;
- *mobile phase B*: mobile phase A, *water R*, *acetonitrile R* (2:6.5:91.5 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 45 | 55 |
| 15 - 30 | 45 → 5 | 55 → 95 |
| 30 - 40 | 5 | 95 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B; use the chromatogram supplied with *glibenclamide for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Relative retention* with reference to glibenclamide (retention time = about 5 min): impurity A = about 0.5; impurity B = about 0.6; impurity C = about 0.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.8;
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: 0.8 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g with heating in 100 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 49.40 mg of $C_{23}H_{28}ClN_3O_5S$.

IMPURITIES

*Specified impurities*: *A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E.*

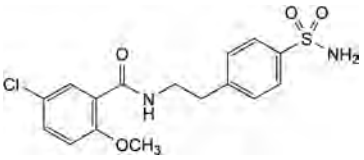

A. 5-chloro-2-methoxy-*N*-[2-(4-sulfamoylphenyl)ethyl]-benzamide,

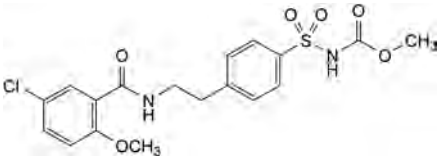

B. methyl [[4-[2-[(5-chloro-2-methoxybenzoyl)amino]-ethyl]phenyl]sulfonyl]carbamate,

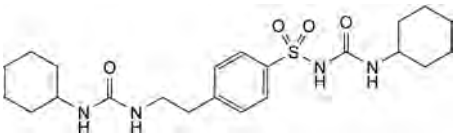

C. 1-cyclohexyl-3-[[4-[2-[(cyclohexylcarbamoyl)amino]-ethyl]phenyl]sulfonyl]urea,

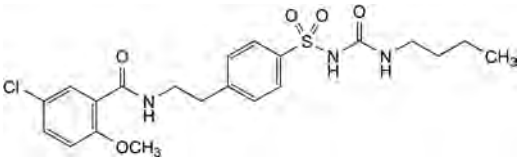

D. 1-butyl-3-[[4-[2-[(5-chloro-2-methoxybenzoyl)amino]-ethyl]phenyl]sulfonyl]urea,

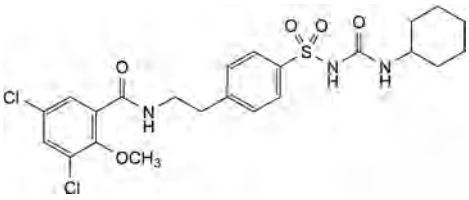

E. 1-cyclohexyl-3-[[4-[2-[(3,5-dichloro-2-methoxybenzoyl)-amino]ethyl]phenyl]sulfonyl]urea.

**01/2017:1524**

# GLICLAZIDE

## Gliclazidum

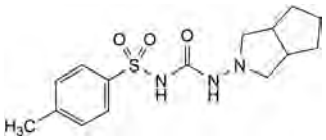

$C_{15}H_{21}N_3O_3S$ $M_r$ 323.4

[21187-98-4]

DEFINITION

1-(Hexahydrocyclopenta[*c*]pyrrol-2(1*H*)-yl)-3-[(4-methylphenyl)sulfonyl]urea.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *gliclazide CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (45:55 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 23 mL of *acetonitrile R* and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 10.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 15 mg of *gliclazide impurity F CRS* in 23 mL of *acetonitrile R* and dilute to 50 mL with *water R*. Dilute 1 mL of the solution to 20 mL with the solvent mixture.

*Reference solution (c).* Dissolve 15.0 mg of *gliclazide impurity F CRS* in 45 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *triethylamine R*, *trifluoroacetic acid R*, *acetonitrile R*, *water R* (0.1:0.1:45:55 *V/V/V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of gliclazide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity F.

*Relative retention* with reference to gliclazide (retention time = about 16 min): impurity F = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to impurity F and gliclazide.

*Limits*:

- *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than F*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity B**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 0.400 g of the substance to be examined in 2.5 mL of *dimethyl sulfoxide R* and dilute to 10.0 mL with *water R*. Stir for 10 min, store at 4 °C for 30 min and filter.

*Reference solution.* Dissolve 20.0 mg of *gliclazide impurity B CRS* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. To 1.0 mL of the solution, add 12 mL of *dimethyl sulfoxide R* and dilute to 50.0 mL with *water R*. To 1.0 mL of this solution, add 12 mL of *dimethyl sulfoxide R* and dilute to 50.0 mL with *water R*.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram obtained with the reference solution to identify the peak due to impurity B.

*Retention time*: impurity B = about 8 min.

*Limit*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (2 ppm).

**Loss on drying** (*2.2.32*): maximum 0.25 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.34 mg of $C_{15}H_{21}N_3O_3S$.

IMPURITIES

*Specified impurities: B, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, G.*

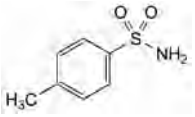

A. 4-methylbenzenesulfonamide,

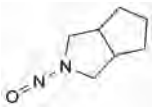

B. 2-nitroso-octahydrocyclopenta[*c*]pyrrole,

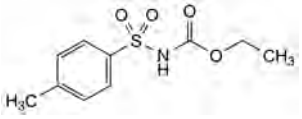

C. ethyl [(4-methylphenyl)sulfonyl]carbamate,

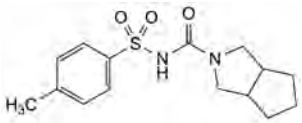

D. *N*-[(4-methylphenyl)sulfonyl]hexahydrocyclopenta[*c*]pyrrol-2(1*H*)-carboxamide,

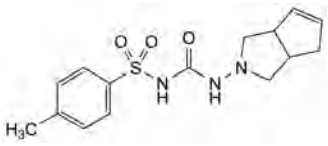

E. 1-[(4-methylphenyl)sulfonyl]-3-(3,3a,4,6a-tetrahydrocyclopenta[*c*]pyrrol-2(1*H*)-yl)urea,

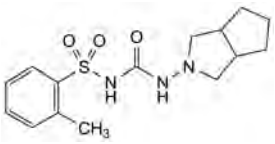

F. 1-(hexahydrocyclopenta[*c*]pyrrol-2(1*H*)-yl)-3-[(2-methylphenyl)sulfonyl]urea,

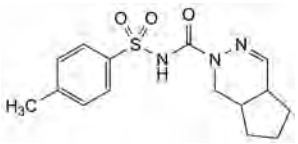

G. *N*-[(4-methylphenyl)sulfonyl]-1,4a,5,6,7,7a-hexahydro-2*H*-cyclopenta[*d*]pyridazine-2-carboxamide.

**01/2016:2223**

# GLIMEPIRIDE

## Glimepiridum

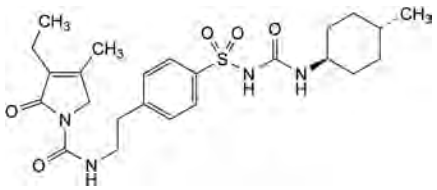

$C_{24}H_{34}N_4O_5S$ $M_r$ 490.6
[93479-97-1]

DEFINITION

1-[[4-[2-(3-Ethyl-4-methyl-2-oxo-3-pyrroline-1-carboxamido)ethyl]phenyl]sulfonyl]-3-*trans*-(4-methylcyclohexyl)urea.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, slightly soluble in methylene chloride, very slightly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *glimepiride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *dimethylformamide R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Store the solutions at a temperature not exceeding 12 °C and for not more than 15 h.*

*Solvent mixture*: *water for chromatography R*, *acetonitrile for chromatography R* (1:4 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *glimepiride for system suitability CRS* (containing impurities B, C and D) in 2.0 mL of the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20.0 mg of *glimepiride CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*: dissolve 0.5 g of *sodium dihydrogen phosphate R* in 500 mL of *water for chromatography R* and adjust to pH 2.5 with *phosphoric acid R*. Add 500 mL of *acetonitrile for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 228 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of glimepiride.

*Relative retention* with reference to glimepiride (retention time = about 17 min): impurity B = about 0.2; impurity C = about 0.3; impurity D = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to impurities B and C.

*Limits*:

– *impurity B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent),
– *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent),
– *sum of impurities other than B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity A**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 5 mL of *methylene chloride R* and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 0.8 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.0 mg of *glimepiride for impurity A identification CRS* in 1 mL of *methylene chloride R* and dilute to 4.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3 mm;
– *stationary phase*: *diol silica gel for chromatography R* (5 µm).

Monographs G

*Mobile phase*: *anhydrous acetic acid R, 2-propanol R, heptane R* (1:100:899 *V/V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 228 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of glimepiride.

*Identification of impurities*: use the chromatogram supplied with *glimepiride for impurity A identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to glimepiride (retention time = about 14 min): impurity A = about 0.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to glimepiride.

*Limit*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent.

Dissolve 0.250 g in *dimethylformamide R* and dilute to 5.0 mL with the same solvent. Carry out the test on 1.0 mL of solution. Carry out a blank test.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{24}H_{34}N_4O_5S$ from the areas of the peaks and the assigned content of *glimepiride CRS*.

IMPURITIES

*Specified impurities*: *A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, G, H, I, J.*

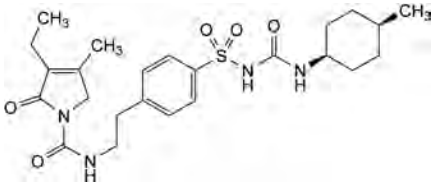

A. 1-[[4-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-3-(*cis*-4-methylcyclohexyl)urea,

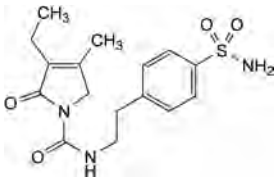

B. 3-ethyl-4-methyl-2-oxo-*N*-[2-(4-sulfamoylphenyl)ethyl]-2,3-dihydro-1*H*-pyrrole-1-carboxamide,

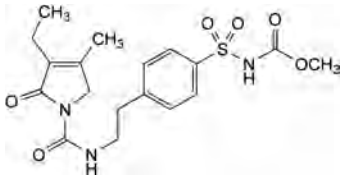

C. methyl [[4-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-carbamate,

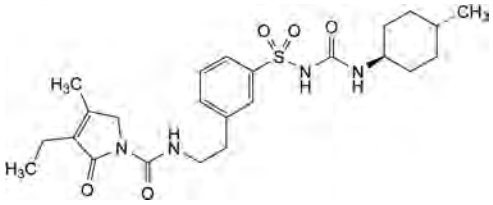

D. 1-[[3-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea,

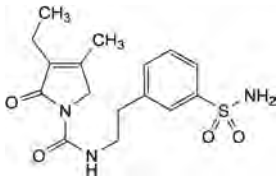

E. 3-ethyl-4-methyl-2-oxo-*N*-[2-(3-sulfamoylphenyl)ethyl]-2,3-dihydro-1*H*-pyrrole-1-carboxamide,

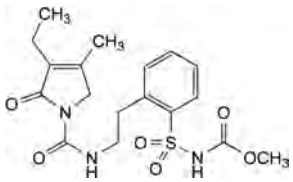

F. methyl [[2-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-carbamate,

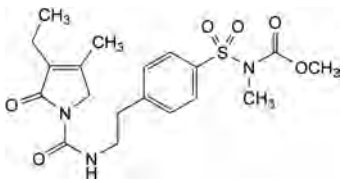

G. methyl [[4-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-methylcarbamate,

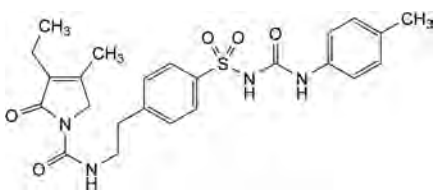

H. 1-[[4-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-3-(4-methylphenyl)urea,

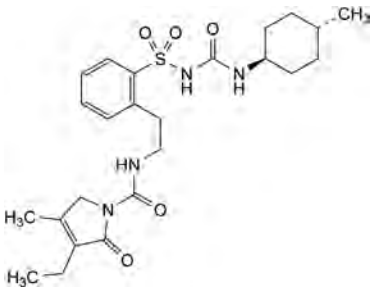

I. 1-[[2-[2-[[(3-ethyl-4-methyl-2-oxo-2,3-dihydro-1*H*-pyrrol-1-yl)carbonyl]amino]ethyl]phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea,

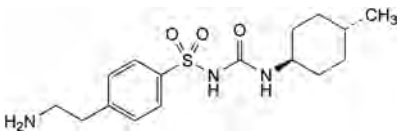

J. 1-[[4-(2-aminoethyl)phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea.

**04/2019:0906**
**corrected 10.0**

# GLIPIZIDE

## Glipizidum

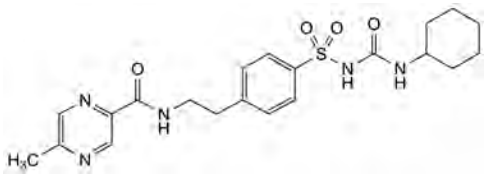

$C_{21}H_{27}N_5O_4S$ $M_r$ 445.5
[29094-61-9]

DEFINITION

*N*-[2-[4-[(Cyclohexylcarbamoyl)sulfamoyl]phenyl]ethyl]-5-methylpyrazine-2-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very slightly soluble in acetone and in methylene chloride, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve about 2 mg in *methanol R* and dilute to 100 mL with the same solvent.

*Spectral range*: 220-350 nm.

*Absorption maxima*: 226 nm and 274 nm.

*Absorbance ratio*: $A_{226}/A_{274}$ = 2.0 to 2.4.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *glipizide CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution.* Dissolve 10 mg of *glipizide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *ethyl acetate R*, *methylene chloride R* (25:25:50 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 40 volumes of *acetonitrile R* and 60 volumes of *water R* previously adjusted to pH 3.5 with *acetic acid R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 20.0 mL of *methanol R* using sonication and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 6.0 mg of *glipizide impurity A CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *glipizide impurity C CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 100 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2 mg of *glipizide impurity D CRS* in the solvent mixture and dilute to 250 mL with the solvent mixture. Dilute 1 mL of the solution to 20 mL with reference solution (b).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water for chromatography R* adjusted to pH 3.5 with *acetic acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 75 | 25 |
| 5 - 12 | 75 → 65 | 25 → 35 |
| 12 - 20 | 65 | 35 |
| 20 - 25 | 65 → 50 | 35 → 50 |
| 25 - 30 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram obtained with reference

Monographs G

solution (c) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention* with reference to glipizide (retention time = about 22 min): impurity A = about 0.25; impurity D = about 0.27; impurity C = about 1.2.

*System suitability*: reference solution (d):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 1.7;
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity B**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 25.0 mg of *decane R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *methylene chloride R*.

*Test solution (a)*. Dissolve 1.000 g of the substance to be examined in 50 mL of a 12 g/L solution of *sodium hydroxide R* and shake with 2 quantities, each of 5.0 mL, of *methylene chloride R*. Use the combined lower layers.

*Test solution (b)*. Dissolve 1.000 g of the substance to be examined in 50 mL of a 12 g/L solution of *sodium hydroxide R* and shake with 2 quantities, each of 5.0 mL, of the internal standard solution. Use the combined lower layers.

*Reference solution*. Dissolve 10.0 mg of *cyclohexylamine R* (impurity B) in a 17.5 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. To 1.0 mL of this solution add 50 mL of a 12 g/L solution of *sodium hydroxide R* and shake with 2 quantities, each of 5.0 mL, of the internal standard solution. Use the combined lower layers.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.8 mL/min.

*Split ratio*: 1:11.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 40 |
| | 4 - 20 | 40 → 200 |
| | 20 - 25 | 200 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: impurity B, decane.

*System suitability*:

- *resolution*: minimum 7 between the peaks due to impurity B and the internal standard in the chromatogram obtained with the reference solution;
- there is no peak with the same retention time as that of the internal standard in the chromatogram obtained with test solution (a).

Calculate the ratio (*R*) of the area of the peak due to impurity B to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with test solution (b), calculate the ratio of the area of any peak due to impurity B to the area of the peak due to the internal standard.

*Limit*:

- *impurity B*: not more than *R* (100 ppm).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *dimethylformamide R*. Add 0.2 mL of *quinaldine red solution R*. Titrate with *0.1 M lithium methoxide* until the colour changes from red to colourless.

1 mL of *0.1 M lithium methoxide* is equivalent to 44.55 mg of $C_{21}H_{27}N_5O_4S$.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G, H, I, J.*

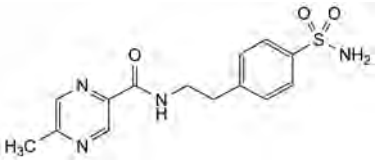

A. 5-methyl-*N*-[2-(4-sulfamoylphenyl)ethyl]pyrazine-2-carboxamide,

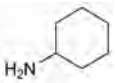

B. cyclohexanamine (cyclohexylamine),

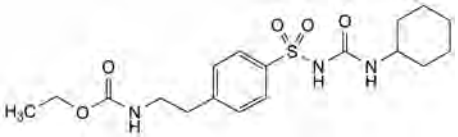

C. ethyl [2-[4-[(cyclohexylcarbamoyl)sulfamoyl]phenyl]-ethyl]carbamate,

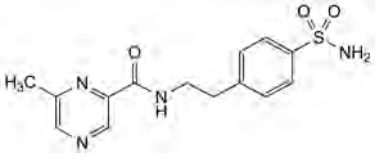

D. 6-methyl-*N*-[2-(4-sulfamoylphenyl)ethyl]pyrazine-2-carboxamide,

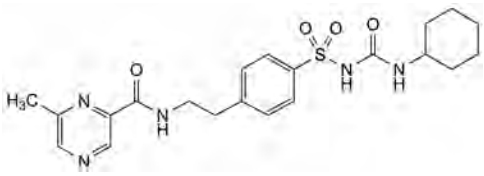

E. *N*-[2-[4-[(cyclohexylcarbamoyl)sulfamoyl]phenyl]ethyl]-6-methylpyrazine-2-carboxamide,

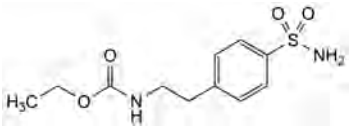

F. ethyl [2-(4-sulfamoylphenyl)ethyl]carbamate,

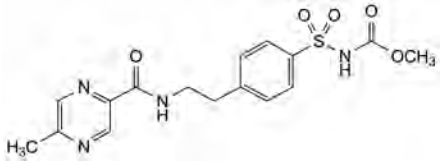

G. methyl [4-[2-(5-methylpyrazine-2-carboxamido)ethyl]-benzene-1-sulfonyl]carbamate,

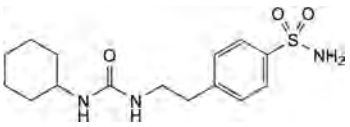

H. 4-[2-[(cyclohexylcarbamoyl)amino]ethyl]benzene-1-sulfonamide,

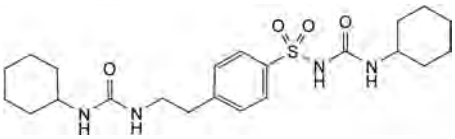

I. *N*-(cyclohexylcarbamoyl)-4-[2-[(cyclohexylcarbamoyl)-amino]ethyl]benzene-1-sulfonamide,

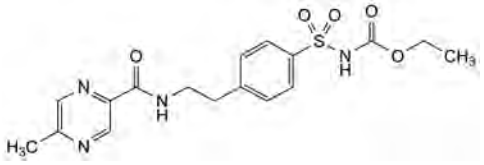

J. ethyl [4-[2-(5-methylpyrazine-2-carboxamido)ethyl]-benzene-1-sulfonyl]carbamate.

**07/2013:1635**
**corrected 10.0**

# GLUCAGON, HUMAN

## Glucagonum humanum

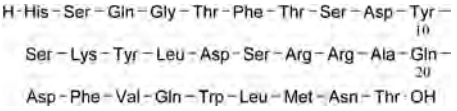


$C_{153}H_{225}N_{43}O_{49}S$ $M_r$ 3483

DEFINITION

Polypeptide having the same structure (29 amino acids) as the hormone produced by the α-cells of the human pancreas, which increases the blood-glucose concentration by promoting rapid breakdown of liver glycogen.

*Content*: 92.5 per cent to 105.0 per cent (anhydrous substance).

PRODUCTION

Human glucagon is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 1 IU/mg using a suitable validated bioassay.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in most organic solvents. It is soluble in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Peptide mapping. Liquid chromatography (*2.2.29*).

*Test solution*. Prepare a 5 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid*. Mix 200 µL of this solution with 800 µL of *0.1 M ammonium carbonate buffer solution pH 10.3 R* (diluted stock solution). Prepare a 2 mg/mL solution of *α-chymotrypsin for peptide mapping R* in *0.1 M ammonium carbonate buffer solution pH 10.3 R* and add 25 µL of this solution to the diluted stock solution. Place the solution in a closed vial at 37 °C for 2 h. Remove the vial and stop the reaction immediately by adding 120 µL of *glacial acetic acid R*.

*Reference solution*. Prepare a 1 mg/mL solution of *human glucagon CRS* in *0.1 M ammonium carbonate buffer solution pH 10.3 R* (diluted stock solution) and continue as described for the test solution.

*Column*:

– *size*: *l* = 0.05 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 500 µL of *trifluoroacetic acid R* and 1000 mL of *water R*;
– *mobile phase B*: mix 500 µL of *trifluoroacetic acid R* with 600 mL of *anhydrous ethanol R* and add 400 mL of *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 100 → 53 | 0 → 47 |
| 35 - 45 | 53 → 0 | 47 → 100 |
| 45 - 46 | 0 → 100 | 100 → 0 |
| 46 - 75 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Equilibration*: with mobile phase A for at least 15 min.

*Injection*: 20 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human glucagon CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Monographs G

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related proteins and deamidated forms**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve the substance to be examined in *0.01 M hydrochloric acid* to obtain a concentration of 0.5 mg/mL. *Maintain the solution at 2-8 °C.*

*Reference solution (a)*. Dissolve the contents of a vial of *human glucagon CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 0.5 mg/mL. *Maintain the solution at 2-8 °C.*

*Reference solution (b)*. Dissolve the substance to be examined in *0.01 M hydrochloric acid* to obtain a concentration of about 0.5 mg/mL. Heat at 50 °C for 48 h (*in situ* preparation of all 4 deamidated forms of glucagon at a total concentration of not less than 7 per cent).

*Column*:

- *size*: *l* = 0.15 m, Ø = 3 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 16.3 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust to pH 2.7 with *phosphoric acid R* and add 200 mL of *acetonitrile for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*, *water R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 61 | 39 |
| 25 - 29 | 61 → 12 | 39 → 88 |
| 29 - 30 | 12 | 88 |
| 30 - 31 | 12 → 61 | 88 → 39 |

*NOTE*: the end time of the isocratic elution may be adjusted so that the gradient begins after elution of the peak due to deamidated glucagon 4 (see relative retention below).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 15 µL.

*Relative retention* with reference to glucagon (retention time = about 21 min): deamidated glucagon 1 = about 1.1; deamidated glucagon 4 = about 1.4.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to glucagon and deamidated glucagon 1 in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.8 for the peak due to glucagon in the chromatogram obtained with reference solution (a);
- *repeatability*: maximum relative standard deviation of 2.0 per cent after 5 injections of reference solution (a);
- 4 peaks eluting after the principal peak, that correspond to the deamidated forms, are clearly visible in the chromatogram obtained with reference solution (b).

*Limits*:

- *deamidated forms*: maximum 0.8 per cent;
- *total*: maximum 3.0 per cent.

**Water** (*2.5.32*): maximum 10 per cent, determined on 50 mg.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related proteins and deamidated forms with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of human glucagon ($C_{153}H_{225}N_{43}O_{49}S$) taking into account the assigned content of $C_{153}H_{225}N_{43}O_{49}S$ in *human glucagon CRS*.

STORAGE

In an airtight container, protected from light, at a temperature lower than – 15 °C.

**07/2018:2446**

# GLUCOSAMINE HYDROCHLORIDE

## Glucosamini hydrochloridum

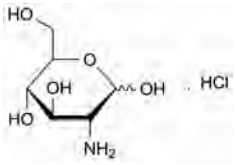


$C_6H_{14}ClNO_5$ $M_r$ 215.6
[66-84-2]

DEFINITION

2-Amino-2-deoxy-D-glucopyranose hydrochloride.

Isolated from natural sources or produced by fermentation.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

The animals from which glucosamine hydrochloride is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *glucosamine hydrochloride CRS*.

B. 1 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

C. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 5.0 mL of solution S to 25.0 mL with *water R*.

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 70.0 to + 74.0 (dried substance), determined on solution S.

Examine 3 h after preparation of solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 0.300 g of the substance to be examined add 80 mL of the mobile phase and sonicate for 10 min. Cool to room temperature and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *2-methylpyrazine CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *glucosamine for system suitability CRS* (containing impurities B and C) in 1.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 30 °C.

*Mobile phase*: dissolve 0.5 g of *sodium heptanesulfonate R* in *water for chromatography R*, add 0.5 mL of *phosphoric acid R* and 4 mL of a 56 g/L solution of *potassium hydroxide R* and dilute to 1000 mL with *water for chromatography R*; to 1000 mL of this solution add 50 mL of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 195 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of 2-methylpyrazine.

*Retention time*: 2-methylpyrazine = about 9 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities B and C.

*Limits*:

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Dissolve 0.200 g in 50 mL of *water R* and add 1.0 mL of *0.1 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 21.56 mg of $C_6H_{14}ClNO_5$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E.*

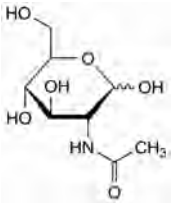

A. 2-(acetylamino)-2-deoxy-D-glucopyranose (*N*-acetylglucosamine),

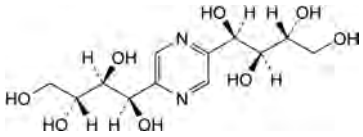

B. (1*R*,1′*R*,2*S*,2′*S*,3*R*,3′*R*)-1,1′-pyrazine-2,5-diylbis(butane-1,2,3,4-tetrol) (fructosazine),

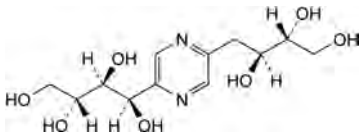

C. (1*R*,2*S*,3*R*)-1-[5-[(2*S*,3*R*)-2,3,4-trihydroxybutyl]pyrazin-2-yl]butane-1,2,3,4-tetrol (deoxyfructosazine),

E. 5-(hydroxymethyl)furan-2-carbaldehyde (5-hydroxymethylfurfural).

**07/2019:2708**

# GLUCOSAMINE SULFATE POTASSIUM CHLORIDE

## Glucosamini sulfas kalii chloridum

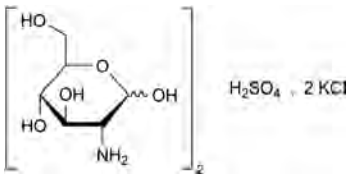

$C_{12}H_{28}Cl_2K_2N_2O_{14}S$ $M_r$ 606

[216699-44-4]

DEFINITION

Bis(2-amino-2-deoxy-D-glucopyranose) sulfate bis(potassium chloride).

Substance prepared from glucosamine hydrochloride isolated from natural sources or produced by fermentation, and potassium sulfate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

The animals from which glucosamine sulfate potassium chloride is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol, practically insoluble in acetone.

Monographs G

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *glucosamine sulfate potassium chloride CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

D. It gives reaction (a) of sulfates (*2.3.1*).

E. 1 mL of solution S (see Tests) gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 5.0 mL of solution S to 25.0 mL with *water R*.

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 47.0 to + 53.0 (dried substance), determined on solution S.

Examine 3 h after preparation of solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 0.42 g of the substance to be examined add 80 mL of the mobile phase and sonicate for 10 min. Cool to room temperature and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *2-methylpyrazine CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *glucosamine for system suitability CRS* (containing impurities B and C) in 1 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm) (3 µm);

– *temperature*: 30 °C.

*Mobile phase*: dissolve 0.5 g of *sodium heptanesulfonate R* in *water for chromatography R*, add 0.5 mL of *phosphoric acid R*, 4 mL of a 56 g/L solution of *potassium hydroxide R* and dilute to 1000 mL with *water for chromatography R*, then add 50 mL of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 195 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of 2-methylpyrazine.

*Retention time*: 2-methylpyrazine = about 9 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities B and C.

*Calculation of percentage contents*:

– for each impurity, use the concentration of 2-methylpyrazine in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.05 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): 27.0 per cent to 31.0 per cent, determined on 1.0 g.

Ignite first at 600 ± 50 °C for 2 h. Do not repeat the moistening with *sulfuric acid R* between any re-ignition.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Dissolve 0.280 g in 50 mL of *water R* and add 1.0 mL of *0.1 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.28 mg of $C_{12}H_{28}Cl_2K_2N_2O_{14}S$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E*.

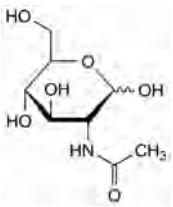

A. 2-(acetylamino)-2-deoxy-D-glucopyranose (*N*-acetylglucosamine),

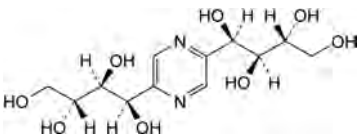

B. (1*R*,1′*R*,2*S*,2′*S*,3*R*,3′*R*)-1,1′-pyrazine-2,5-diylbis (butane-1,2,3,4-tetrol) (fructosazine),

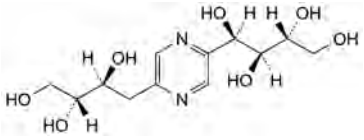

C. (1*R*,2*S*,3*R*)-1-[5-[(2*S*,3*R*)-2,3,4-trihydroxybutyl]pyrazin-2-yl]butane-1,2,3,4-tetrol (deoxyfructosazine),

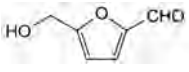

E. 5-(hydroxymethyl)furan-2-carbaldehyde (5-hydroxymethylfurfural).

**07/2019:2447**

# GLUCOSAMINE SULFATE SODIUM CHLORIDE

## Glucosamini sulfas natrii chloridum

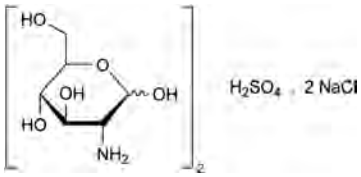


$C_{12}H_{28}Cl_2N_2Na_2O_{14}S$ $M_r$ 573.3
[216447-62-0]

DEFINITION

Bis(2-amino-2-deoxy-D-glucopyranose) sulfate bis(sodium chloride).

Substance prepared from glucosamine hydrochloride isolated from natural sources or produced by fermentation, and sodium sulfate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

PRODUCTION

The animals from which glucosamine sulfate sodium chloride is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *glucosamine sulfate sodium chloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

C. 1 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

D. It gives reaction (a) of sulfates (*2.3.1*).

E. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 5.0 mL of solution S to 25.0 mL with *water R*.

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 50.0 to + 55.0 (dried substance), determined on solution S.

Examine 3 h after preparation of solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 0.40 g of the substance to be examined add 80 mL of the mobile phase and sonicate for 10 min. Cool to room temperature and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25.0 mg of *2-methylpyrazine CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *glucosamine for system suitability CRS* (containing impurities B and C) in 1 mL of the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*: dissolve 0.5 g of *sodium heptanesulfonate R* in *water for chromatography R*, add 0.5 mL of *phosphoric acid R* and 4 mL of a 56 g/L solution of *potassium hydroxide R* and dilute to 1000 mL with *water for chromatography R*; to 1000 mL of this solution add 50 mL of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 195 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of 2-methylpyrazine.

*Retention time*: 2-methylpyrazine = about 9 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities B and C.

*Limits*:
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): 23.5 per cent to 26.0 per cent, determined on 1.0 g.

Ignite first at 600 ± 50 °C for 2 h. Do not repeat the moistening with *sulfuric acid R* between any re-ignition.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Dissolve 0.250 g in 50 mL of *water R* and add 1.0 mL of *0.1 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.67 mg of $C_{12}H_{28}Cl_2N_2Na_2O_{14}S$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E*.

Monographs G

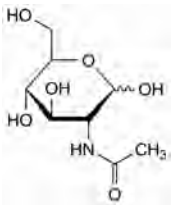

A. 2-(acetylamino)-2-deoxy-D-glucopyranose (*N*-acetylglucosamine),

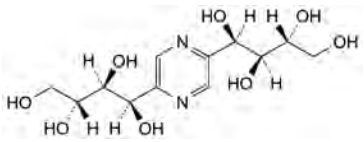

B. (1*R*,1′*R*,2*S*,2′*S*,3*R*,3′*R*)-1,1′-pyrazine-2,5-diylbis (butane-1,2,3,4-tetrol) (fructosazine),

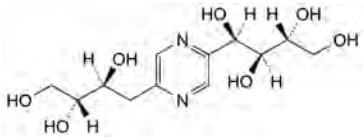

C. (1*R*,2*S*,3*R*)-1-[5-[(2*S*,3*R*)-2,3,4-trihydroxybutyl] pyrazin-2-yl]butane-1,2,3,4-tetrol (deoxyfructosazine),

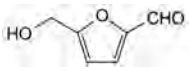

E. 5-(hydroxymethyl)furan-2-carbaldehyde (5-hydroxymethylfurfural).

**01/2017:0177**
**corrected 9.1**

# GLUCOSE(2)

## Glucosum

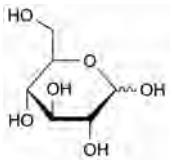

$C_6H_{12}O_6$ $M_r$ 180.2
[50-99-7]

DEFINITION

D-Glucopyranose.

It is derived from starch.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

◆CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).◆

IDENTIFICATION

*First identification: ◊A◊, B, E.*

*◊Second identification: C, D.◊*

◊A. Specific optical rotation (*2.2.7*): + 52.5 to + 53.3 (anhydrous substance).

Dissolve 10.0 g in 80 mL of *water R*, add 0.2 mL of *dilute ammonia R1*, allow to stand for 30 min and dilute to 100.0 mL with *water R*.◊

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

◊C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (2:3 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *glucose monohydrate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg each of *fructose R*, *glucose R*, *lactose monohydrate R* and *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 2 µL; thoroughly dry the points of application.

*Development A*: over 3/4 of the plate.

*Drying A*: in a current of warm air.

*Development B*: immediately, over 3/4 of the plate, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: treat with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*; heat at 130 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 4 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.1 g in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. A red precipitate is formed.◊

E. Water (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 10.0 g in 15 mL of *water R*, heating on a water-bath.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.300 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.330 g of *glucose monohydrate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 250.0 mL with *water R*.

*Reference solution (c).* Dilute 25.0 mL of reference solution (b) to 200.0 mL with *water R*.

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8 Pharmacopoeial harmonisation*.

*Reference solution (d).* Dissolve 5 mg of *fructose R* (impurity D), 5 mg of *maltose monohydrate R* (impurity A) and 5 mg of *maltotriose R* (impurity C) in *water R* and dilute to 50 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;
– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
– *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water R*.

*Flow rate*: 0.3 mL/min.

*Detection*: refractometer maintained at a constant temperature (40 °C for example).

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 1.5 times the retention time of glucose.

*Relative retention* with reference to glucose (retention time = about 21 min): impurity C = about 0.7; impurities A and B = about 0.8; impurity D = about 1.3.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.3 between the peaks due to impurities C and A.

*Limits*:

– *sum of impurities A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
– *impurity C*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurity D*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Dextrin**. To 1 g of the finely powdered substance to be examined add 20 mL of *ethanol (96 per cent) R* and heat under a reflux condenser. The substance dissolves completely.

**Soluble starch, sulfite**: maximum 15 ppm.

Dissolve 6.7 g in 15.0 mL of *water R*, heating on a water-bath. Allow to cool and add 25 µL of *iodine solution R5*. The solution is yellow.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

◊**Pyrogens** (*2.6.8*). If intended for use in the manufacture of large-volume parental preparations without a further appropriate procedure for the removal of pyrogens, the competent authority may require that it comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of a solution in *water for injections R* containing 50 mg of the substance to be examined per millilitre.◊

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_6H_{12}O_6$ taking into account the assigned content of *glucose monohydrate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D.*

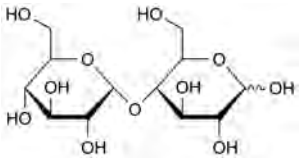

A. 4-*O*-α-D-glucopyranosyl-D-glucopyranose (maltose),

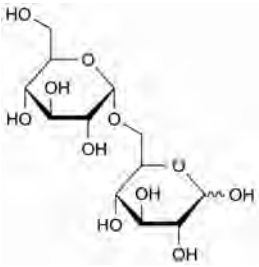

B. 6-*O*-α-D-glucopyranosyl-D-glucopyranose (isomaltose),

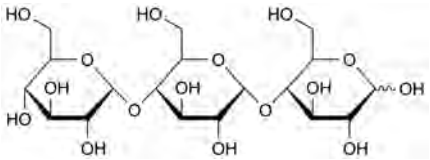

C. α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucopyranose (maltotriose),

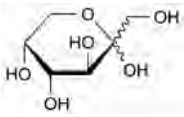

D. D-*arabino*-hex-2-ulopyranose (fructose).

**01/2017:1330**

# GLUCOSE, LIQUID

## Glucosum liquidum

DEFINITION

Aqueous solution containing a mixture of glucose, oligosaccharides and polysaccharides obtained by hydrolysis of starch.

It contains a minimum of 70.0 per cent dry matter.

The degree of hydrolysis, expressed as dextrose equivalent (DE), is not less than 20 (nominal value).

CHARACTERS

*Appearance*: clear, colourless or brown, viscous liquid.

*Solubility*: miscible with water.

It may partly or totally solidify at room temperature and liquefies again when heated to 50 °C.

IDENTIFICATION

A. Dissolve 0.1 g in 2.5 mL of *water R* and heat with 2.5 mL of *cupri-tartaric solution R*. A red precipitate is formed.
B. Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 5 g/L solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s the colour changes from yellow to green or blue.

C. It is a clear, colourless or brown, viscous liquid, miscible with water. The substance may partly or totally solidify at room temperature and liquefies again when heated to 50 °C.

D. Dextrose equivalent (see Tests).

TESTS

**Solution S**. Dissolve 25.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 6.0.

Mix 1 mL of a 223.6 g/L solution of *potassium chloride R* and 30 mL of solution S.

**Sulfur dioxide** (*2.5.29*): maximum 20 ppm; maximum 400 ppm if intended for the production of lozenges or pastilles obtained by high boiling techniques, provided that the final product contains maximum 50 ppm of sulfur dioxide.

**Loss on drying** (*2.2.32*): maximum 30.0 per cent, determined on 1.000 g. Triturate the sample with 3.000 g of *kieselguhr G R*, previously dried at 80 °C under high vacuum for 2 h, and dry at 80 °C under high vacuum for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Dextrose equivalent** (DE): within 10 per cent of the nominal value.

Weigh an amount of the substance to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 mL volumetric flask. Dissolve in *water R* and dilute to 500.0 mL with the same solvent. Transfer the solution to a 50 mL burette.

Pipette 25.0 mL of *cupri-tartaric solution R* into a 250 mL flask and add 18.5 mL of the test solution from the burette, mix and add a few glass beads. Place the flask on a hot plate, previously adjusted so that the solution begins to boil after 2 min ± 15 s. Allow to boil for exactly 120 s, add 1 mL of a 1 g/L solution of *methylene blue R* and titrate with the test solution ($V_1$) until the blue colour disappears. Maintain the solution at boiling throughout the titration.

Standardise the cupri-tartaric solution using a 6.00 g/L solution of *glucose R* ($V_0$).

Calculate the dextrose equivalent using the following expression:

$$\frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

$V_0$ = total volume of glucose standard solution, in millilitres,

$V_1$ = total volume of test solution, in millilitres,

$M$ = mass of the sample, in grams,

$D$ = percentage content of dry matter in the substance.

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

**01/2017:1525**

# GLUCOSE, LIQUID, SPRAY-DRIED

## Glucosum liquidum dispersione desiccatum

DEFINITION

Mixture of glucose, oligosaccharides and polysaccharides, obtained by the partial hydrolysis of starch.

The degree of hydrolysis, expressed as dextrose equivalent (DE), is not less than 20 (nominal value).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder or granules.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. Dissolve 0.1 g in 2.5 mL of *water R* and heat with 2.5 mL of *cupri-tartaric solution R*. A red precipitate is formed.

B. Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 5 g/L solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s the colour changes from yellow to green or blue.

C. It is a powder or granules.

D. Dextrose equivalent (see Tests).

TESTS

**Solution S**. Dissolve 12.5 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 7.0.

Mix 1 mL of a 223.6 g/L solution of *potassium chloride R* and 30 mL of solution S.

**Sulfur dioxide** (*2.5.29*): maximum 20 ppm.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 10.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Dextrose equivalent** (DE): within 10 per cent of the nominal value.

Weigh an amount of the substance to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 mL volumetric flask. Dissolve in *water R* and dilute to 500.0 mL with the same solvent. Transfer the solution to a 50 mL burette.

Pipette 25.0 mL of *cupri-tartaric solution R* into a 250 mL flask and add 18.5 mL of the test solution from the burette, mix and add a few glass beads. Place the flask on a hot plate, previously adjusted so that the solution begins to boil after 2 min ± 15 s. Allow to boil for exactly 120 s, add 1 mL of a 1 g/L solution of *methylene blue R* and titrate with the test solution ($V_1$) until the blue colour disappears. Maintain the solution at boiling throughout the titration.

Standardise the cupri-tartaric solution using a 6.00 g/L solution of *glucose R* ($V_0$).

Calculate the dextrose equivalent using the following expression:

$$\frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

$V_0$ = total volume of glucose standard solution, in millilitres;

$V_1$ = total volume of test solution, in millilitres;

$M$ = mass of the sample, in grams;

$D$ = percentage content of dry matter in the substance.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for spray-dried liquid glucose used as filler or binder for wet granulation.*

**Dextrose equivalent** (see Tests).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**01/2016:0178**
**corrected 9.1**

# GLUCOSE MONOHYDRATE(3)

## Glucosum monohydricum

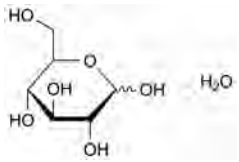


$C_6H_{12}O_6,H_2O$ $M_r$ 198.2
[77938-63-7]

DEFINITION

D-Glucopyranose monohydrate.

It is derived from starch.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

◆CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).◆

IDENTIFICATION

*First identification: ◊A◊, B, E.*

*◊Second identification: C, D.◊*

◊A. Specific optical rotation (*2.2.7*): + 52.5 to + 53.3 (anhydrous substance).

Dissolve 10.0 g in 80 mL of *water R*, add 0.2 mL of *dilute ammonia R1*, allow to stand for 30 min and dilute to 100.0 mL with *water R*.◊

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

◊C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (2:3 *V/V*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *glucose monohydrate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg each of *fructose R*, *glucose R*, *lactose monohydrate R* and *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 2 µL; thoroughly dry the points of application.

*Development A*: over 3/4 of the plate.

*Drying A*: in a current of warm air.

*Development B*: immediately, over 3/4 of the plate, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: treat with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*; heat at 130 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 4 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.1 g in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. A red precipitate is formed.◊

E. Water (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 10.0 g in 15 mL of *water R*.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.330 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.330 g of *glucose monohydrate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 250.0 mL with *water R*.

*Reference solution (c)*. Dilute 25.0 mL of reference solution (b) to 200.0 mL with *water R*.

*Reference solution (d)*. Dissolve 5 mg of *fructose R* (impurity D), 5 mg of *maltose monohydrate R* (impurity A) and 5 mg of *maltotriose R* (impurity C) in *water R* and dilute to 50 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;
– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
– *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water R*.

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8 Pharmacopoeial harmonisation*.

*Flow rate*: 0.3 mL/min.

*Detection*: refractometer maintained at a constant temperature (40 °C for example).

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 1.5 times the retention time of glucose.

*Relative retention* with reference to glucose (retention time = about 21 min): impurity C = about 0.7; impurities A and B = about 0.8; impurity D = about 1.3.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.3 between the peaks due to impurities C and A.

*Limits*:

– *sum of impurities A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
– *impurity C*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurity D*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Dextrin**. To 1 g of the finely powdered substance to be examined add 20 mL of *ethanol (96 per cent) R* and heat under a reflux condenser. The substance dissolves completely.

**Soluble starch, sulfite**: maximum 15 ppm.

Dissolve 7.4 g in 15.0 mL of *water R*, heating on a water-bath. Allow to cool and add 25 µL of *iodine solution R5*. The solution is yellow.

**Water** (*2.5.12*): 7.5 per cent to 9.5 per cent, determined on 0.25 g.

◊**Pyrogens** (*2.6.8*). If intended for use in the manufacture of large-volume parenteral preparations without a further appropriate procedure for the removal of pyrogens, the competent authority may require that it comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of a solution in *water for injections R* containing 50 mg of the substance to be examined per millilitre.◊

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_6H_{12}O_6$ taking into account the assigned content of *glucose monohydrate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D.*

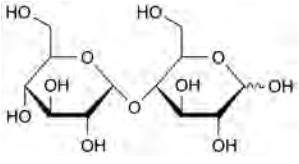

A. 4-*O*-α-D-glucopyranosyl-D-glucopyranose (maltose),

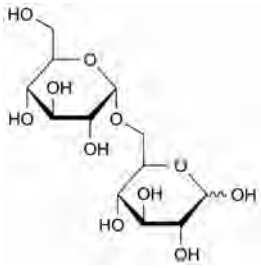

B. 6-*O*-α-D-glucopyranosyl-D-glucopyranose (isomaltose),

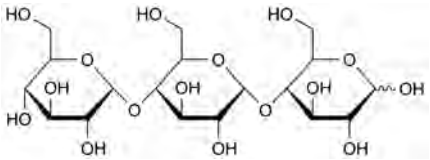

C. α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucopyranose (maltotriose),

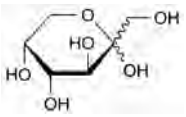

D. D-*arabino*-hex-2-ulopyranose (fructose).

**01/2017:0750**

# GLUTAMIC ACID

## Acidum glutamicum

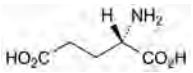

$C_5H_9NO_4$ $M_r$ 147.1

[56-86-0]

DEFINITION

Glutamic acid contains not less than 98.5 per cent and not more than the equivalent of 100.5 per cent of (2*S*)-2-aminopentanedioic acid, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, freely soluble in boiling water, slightly soluble in cold water, practically insoluble in acetic acid, in acetone and in alcohol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *glutamic acid CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum quantity of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 2.0 mL of solution S (see Tests) add 0.1 mL of *phenolphthalein solution R* and 3.0 mL to 3.5 mL of *1 M sodium hydroxide* to change the colour of the indicator to red. Add a mixture of 3 mL of *formaldehyde solution R*, 3 mL of *carbon dioxide-free water R* and 0.1 mL of *phenolphthalein solution R*, to which sufficient *1 M sodium hydroxide* has been added to produce a pink colour. The solution is decolourised. Add *1 M sodium hydroxide* until a red colour is produced. The total volume of *1 M sodium hydroxide* used is 4.0 mL to 4.7 mL.

TESTS

**Solution S**. Dissolve 5.00 g in *1 M hydrochloric acid* with gentle heating, and dilute to 50.0 mL with the same acid.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 30.5 to + 32.5, determined on solution S and calculated with reference to the dried substance.

**Ninhydrin-positive substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R*.

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in 5 mL of *dilute ammonia R2* and dilute to 10 mL with *water R*.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 50 mL with *water R*.

*Reference solution (a)*. Dissolve 10 mg of *glutamic acid CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dilute 5 mL of test solution (b) to 20 mL with *water R*.

*Reference solution (c)*. Dissolve 10 mg of *glutamic acid CRS* and 10 mg of *aspartic acid CRS* in *water R* and dilute to 25 mL with the same solvent.

Apply to the plate 5 µL of each solution. Dry the plate in a current of air for 15 min. Develop over a path of 15 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air, spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

**Chlorides** (*2.4.4*). Dissolve 0.25 g in 3 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution, to which 1 mL of *water R* is added instead of *dilute nitric acid R*, complies with the limit test for chlorides (200 ppm).

**Sulfates** (*2.4.13*). Dilute 5 mL of solution S to 15 mL with *distilled water R*. The solution complies with the limit test for sulfates (300 ppm).

**Ammonium** (*2.4.1*). 50 mg complies with limit test B for ammonium (200 ppm). Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*). In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron (10 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 50 mL of *carbon dioxide-free water R* with gentle heating. Cool. Using 0.1 mL of *bromothymol blue solution R1* as indicator, titrate with *0.1 M sodium hydroxide* until the colour changes from yellow to blue.

1 mL of *0.1 M sodium hydroxide* is equivalent to 14.71 mg of $C_5H_9NO_4$.

STORAGE

Protected from light.

**01/2017:1670**

# GLUTATHIONE

## Glutathionum

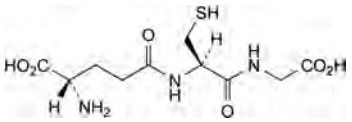

$C_{10}H_{17}N_3O_6S$ $M_r$ 307.3
[70-18-8]

DEFINITION

L-γ-Glutamyl-L-cysteinylglycine.

Fermentation product.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *glutathione CRS*.

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 17.5 to − 15.5 (dried substance).

Dissolve 1.0 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Capillary electrophoresis (*2.2.47*).
*Prepare the solutions immediately before use.*

*Internal standard solution (a)*. Dissolve 0.100 g of *phenylalanine R* in the electrolyte solution and dilute to 50.0 mL with the same solution.

*Internal standard solution (b)*. Dilute 10.0 mL of internal standard solution (a) to 100.0 mL with the electrolyte solution.

*Test solution (a)*. Dissolve 0.200 g of the substance to be examined in the electrolyte solution and dilute to 10.0 mL with the same solution.

*Test solution (b)*. Dissolve 0.200 g of the substance to be examined in internal standard solution (b) and dilute to 10.0 mL with the same solution.

*Reference solution (a)*. Dissolve 20.0 mg of the substance to be examined in internal standard solution (a) and dilute to 10.0 mL with the same solution.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the electrolyte solution.

*Reference solution (c).* Dissolve 0.200 g of the substance to be examined in 5 mL of the electrolyte solution. Add 1.0 mL of internal standard solution (a), 0.5 mL of a 2 mg/mL solution of *L-cysteine R* (impurity B) in the electrolyte solution, 0.5 mL of a 2 mg/mL solution of *oxidised L-glutathione R* (impurity C) in the electrolyte solution and 0.5 mL of a 2 mg/mL solution of *L-γ-glutamyl-L-cysteine R* (impurity D) in the electrolyte solution. Dilute to 10.0 mL with the electrolyte solution.

*Capillary*:

- *material*: uncoated fused silica;
- *size*: length to the detector cell = 0.5 m; total length = 0.6 m; Ø = 75 µm.

*Temperature*: 25 °C.

*Electrolyte solution.* Dissolve 1.50 g of *anhydrous sodium dihydrogen phosphate R* in 230 mL of *water R* and adjust to pH 1.80 with *phosphoric acid R*. Dilute to 250.0 mL with *water R*. Check the pH and, if necessary, adjust with *phosphoric acid R* or *dilute sodium hydroxide solution R*.

*Detection*: spectrophotometer at 200 nm.

*Preconditioning of a new capillary*: rinse the new capillary before the first injection with *0.1 M hydrochloric acid* at 138 kPa for 20 min and with *water R* at 138 kPa for 10 min; for complete equilibration, condition the capillary with the electrolyte solution at 350 kPa for 40 min, and subsequently at a voltage of 20 kV for 60 min.

*Preconditioning of the capillary*: rinse the capillary with the electrolyte solution at 138 kPa for 40 min.

*Between-run rinsing*: rinse the capillary with *water R* at 138 kPa for 1 min, with *0.1 M sodium hydroxide* at 138 kPa for 2 min, with *water R* at 138 kPa for 1 min, with *0.1 M hydrochloric acid* at 138 kPa for 3 min and with the electrolyte solution at 138 kPa for 10 min.

*Injection*: test solutions (a) and (b), reference solutions (b) and (c) and the electrolyte solution (blank): under pressure (3.45 kPa) for 5 s.

*Migration*: apply a voltage of 20 kV.

*Run time*: 45 min.

*Relative migration* with reference to the internal standard (about 14 min): impurity A = about 0.77; impurity B = about 1.04; impurity E = about 1.2; impurity C = about 1.26; impurity D = about 1.3.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to the internal standard and impurity B in the electropherogram obtained with reference solution (c); if necessary, increase the pH with *dilute sodium hydroxide solution R*;
- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to glutathione in the electropherogram obtained with reference solution (c); if necessary, lower the pH with *phosphoric acid R*;
- check that in the electropherogram obtained with test solution (a) there is no peak with the same migration time as the internal standard (in such case correct the area of the phenylalanine peak).

*Limits*: test solution (b):

- *corrected areas*: divide all the peak areas by the corresponding migration times;
- *correction factors*: for the calculation of content, multiply the ratio of time-corrected peak areas of impurity and the internal standard by the corresponding correction factor: impurity B = 3.0; impurity D = 1.4;
- *impurity C*: not more than 1.5 times the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (1.5 per cent);
- *impurity D*: not more than the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (1.0 per cent);
- *impurities A, B, E*: for each impurity, not more than 0.5 times the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (0.5 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than 2.5 times the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (2.5 per cent);
- *disregard limit*: 0.05 times the ratio of the area of the peak due to glutathione to the area of the peak due to the internal standard in the electropherogram obtained with reference solution (b) (0.05 per cent).

**Chlorides**: maximum 200 ppm.
Dissolve 0.5 g in 5 mL of *dilute nitric acid R* and dilute to 10 mL with the same solvent. Add 10 mL of *strong hydrogen peroxide solution R* and heat on a water-bath for 30 min. Cool and dilute to 50 mL with *water R*. Add 1 mL of *silver nitrate solution R2* and mix. Allow to stand for 5 min protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 mL of *chloride standard solution (50 ppm Cl) R*. Examine the tubes laterally against a black background.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers, add 10 mL of *water R* and shake for 3 min. The aqueous layer complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a ground-glass-stoppered flask, dissolve 0.500 g of the substance to be examined and 2 g of *potassium iodide R* in 50 mL of *water R*. Cool the solution in iced water and add 10 mL of *hydrochloric acid R1* and 20.0 mL of *0.05 M iodine*. Stopper the flask and allow to stand in the dark for 15 min. Titrate with *0.1 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 30.73 mg of $C_{10}H_{17}N_3O_6S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

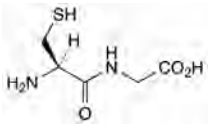

A. L-cysteinylglycine,

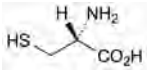

B. (2*R*)-2-amino-3-sulfanylpropanoic acid (cysteine),

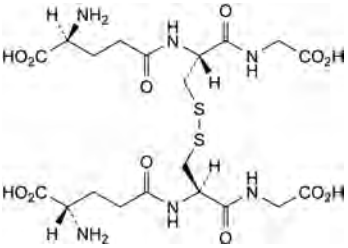

C. bis(L-γ-glutamyl-L-cysteinylglycine) disulfide (L-glutathione oxidised),

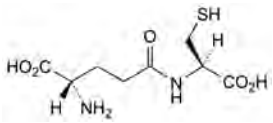

D. L-γ-glutamyl-L-cysteine,

E. unknown structure (product of degradation).

**01/2019:0496**
**corrected 10.0**

# GLYCEROL

## Glycerolum

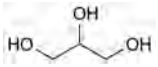

$C_3H_8O_3$ $M_r$ 92.1
[56-81-5]

DEFINITION

Propane-1,2,3-triol.

*Content*: 98.0 per cent *m/m* to 101.0 per cent *m/m* (anhydrous substance).

CHARACTERS

*Aspect*: syrupy liquid, unctuous to the touch, colourless or almost colourless, clear, very hygroscopic.

*Solubility*: miscible with water and with ethanol (96 per cent), slightly soluble in acetone, practically insoluble in fatty oils and in essential oils.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Refractive index (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: to 5 mL add 1 mL of *water R* and mix carefully.
*Comparison*: *Ph. Eur. reference spectrum of glycerol (85 per cent)*.

C. Relative density (*2.2.5*): 1.258 to 1.268.

TESTS

**Solution S**. Dilute 100.0 g to 200.0 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*). Dilute 10 mL of solution S to 25 mL with *water R*. The solution is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.5 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Refractive index** (*2.2.6*): 1.470 to 1.475.

**Aldehydes**: maximum 10 ppm.

Place 7.5 mL of solution S in a ground-glass-stoppered flask and add 7.5 mL of *water R* and 1.0 mL of *decolorised pararosaniline solution R*. Close the flask and allow to stand for 1 h at a temperature of 25 ± 1 °C. The absorbance (*2.2.25*) of the solution measured at 552 nm is not greater than that of a standard prepared at the same time and in the same manner using 7.5 mL of *formaldehyde standard solution (5 ppm $CH_2O$) R* and 7.5 mL of *water R*. The test is not valid unless the standard is pink.

**Esters**. Add 10.0 mL of *0.1 M sodium hydroxide* to the final solution obtained in the test for acidity or alkalinity. Boil under a reflux condenser for 5 min. Cool. Add 0.5 mL of *phenolphthalein solution R* and titrate with *0.1 M hydrochloric acid*. Not less than 8.0 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Impurity A and related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dilute 10.0 mL of solution S to 100.0 mL with *water R*.

*Reference solution (a)*. Dilute 10.0 g of *glycerol R1* to 20.0 mL with *water R*. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 1.000 g of *diethylene glycol R* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with reference solution (a). Dilute 1.0 mL of this solution to 20.0 mL with reference solution (a).

*Reference solution (d)*. Mix 1.0 mL of the test solution and 5.0 mL of reference solution (b) and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (e)*. Dilute 5.0 mL of reference solution (b) to 100.0 mL with *water R*.

*Column:*

– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R*.

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:10.

*Linear velocity*: 38 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 100 |
| | 0 - 16 | 100 → 220 |
| | 16 - 20 | 220 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Elution order*: impurity A, glycerol.

*System suitability*: reference solution (d):

– *resolution*: minimum 7.0 between the peaks due to impurity A and glycerol.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *any other impurity with a retention time less than the retention time of glycerol*: not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total of all impurities with retention times greater than the retention time of glycerol*: not more than 5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Halogenated compounds**: maximum 35 ppm.

To 10 mL of solution S add 1 mL of *dilute sodium hydroxide solution R*, 5 mL of *water R* and 50 mg of *halogen-free nickel-aluminium alloy R*. Heat on a water-bath for 10 min, allow to cool and filter. Rinse the flask and the filter with *water R* until 25 mL of filtrate is obtained. To 5 mL of the filtrate add 4 mL of *ethanol (96 per cent) R*, 2.5 mL of *water R*, 0.5 mL of *nitric acid R* and 0.05 mL of *silver nitrate solution R2* and mix. Allow to stand for 2 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 7.0 mL of *chloride standard solution (5 ppm Cl) R*, 4 mL of *ethanol (96 per cent) R*, 0.5 mL of *water R*, 0.5 mL of *nitric acid R* and 0.05 mL of *silver nitrate solution R2*.

**Sugars**. To 10 mL of solution S add 1 mL of *dilute sulfuric acid R* and heat on a water-bath for 5 min. Add 3 mL of carbonate-free *dilute sodium hydroxide solution R* (prepared by the method described for carbonate-free *1 M sodium hydroxide*), mix and add dropwise 1 mL of freshly prepared *copper sulfate solution R*. The solution is clear and blue. Continue heating on the water-bath for 5 min. The solution remains blue and no precipitate is formed.

**Chlorides** (*2.4.4*): maximum 10 ppm.

Dilute 1 mL of solution S to 15 mL with *water R*. Prepare the standard using 1 mL of *chloride standard solution (5 ppm Cl) R* diluted to 15 mL with *water R*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.01 per cent, determined on 5.0 g after heating to boiling and ignition.

ASSAY

Thoroughly mix 0.075 g with 45 mL of *water R*. Add 25.0 mL of a mixture of 1 volume of *0.1 M sulfuric acid* and 20 volumes of *0.1 M sodium periodate*. Allow to stand protected from light for 15 min. Add 5.0 mL of a 500 g/L solution of *ethylene glycol R* and allow to stand protected from light for 20 min. Using 0.5 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide*. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 9.21 mg of $C_3H_8O_3$.

STORAGE

In an airtight container.

IMPURITIES

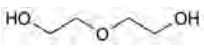


A. 2,2′-oxydi(ethan-1-ol) (diethylene glycol),

B. ethane-1,2-diol (ethylene glycol),

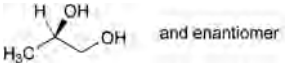


C. (2*RS*)-propane-1,2-diol (propylene glycol).

**01/2019:0497**
**corrected 10.0**

# GLYCEROL (85 PER CENT)

## Glycerolum (85 per centum)

DEFINITION

Aqueous solution of propane-1,2,3-triol.

*Content*: 83.5 per cent *m/m* to 88.5 per cent *m/m* of propane-1,2,3-triol ($C_3H_8O_3$; $M_r$ 92.1).

CHARACTERS

*Aspect*: syrupy liquid, unctuous to the touch, colourless or almost colourless, clear, very hygroscopic.

*Solubility*: miscible with water and with ethanol (96 per cent), slightly soluble in acetone, practically insoluble in fatty oils and in essential oils.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Refractive index (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of glycerol (85 per cent)*.

C. Relative density (*2.2.5*): 1.221 to 1.232.

TESTS

**Solution S**. Dilute 117.6 g to 200.0 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*). Dilute 10 mL of solution S to 25 mL with *water R*. The solution is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.5 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Refractive index** (*2.2.6*): 1.449 to 1.455.

**Aldehydes**: maximum 10 ppm.

Place 7.5 mL of solution S in a ground-glass-stoppered flask and add 7.5 mL of *water R* and 1.0 mL of *decolorised pararosaniline solution R*. Close the flask and allow to stand for 1 h at a temperature of 25 ± 1 °C. The absorbance (*2.2.25*) of the solution measured at 552 nm is not greater than that of a standard prepared at the same time and in the same manner using 7.5 mL of *formaldehyde standard solution (5 ppm $CH_2O$) R* and 7.5 mL of *water R*. The test is not valid unless the standard is pink.

**Esters**. Add 10.0 mL of *0.1 M sodium hydroxide* to the final solution obtained in the test for acidity or alkalinity. Boil under a reflux condenser for 5 min. Cool. Add 0.5 mL of *phenolphthalein solution R* and titrate with *0.1 M hydrochloric acid*. Not less than 8.0 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Impurity A and related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dilute 10.0 mL of solution S to 100.0 mL with *water R*.

*Reference solution (a).* Dilute 11.8 g of *glycerol (85 per cent) R1* to 20.0 mL with *water R*. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 1.000 g of *diethylene glycol R* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with reference solution (a). Dilute 1.0 mL of this solution to 20.0 mL with reference solution (a).

*Reference solution (d).* Mix 1.0 mL of the test solution and 5.0 mL of reference solution (b) and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (e).* Dilute 5.0 mL of reference solution (b) to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R*.

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:10.

*Linear velocity*: 38 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 100 |
| | 0 - 16 | 100 → 220 |
| | 16 - 20 | 220 |
| Injection port | | 220 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Elution order*: impurity A, glycerol.

*System suitability*: reference solution (d):

- *resolution*: minimum 7.0 between the peaks due to impurity A and glycerol.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *any other impurity with a retention time less than the retention time of glycerol*: not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *total of all impurities with retention times greater than the retention time of glycerol*: not more than 5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.05 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Halogenated compounds**: maximum 30 ppm.

To 10 mL of solution S add 1 mL of *dilute sodium hydroxide solution R*, 5 mL of *water R* and 50 mg of *halogen-free nickel-aluminium alloy R*. Heat on a water-bath for 10 min, allow to cool and filter. Rinse the flask and the filter with *water R* until 25 mL of filtrate is obtained. To 5 mL of the filtrate add 4 mL of *ethanol (96 per cent) R*, 2.5 mL of *water R*, 0.5 mL of *nitric acid R* and 0.05 mL of *silver nitrate solution R2* and mix. Allow to stand for 2 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 7.0 mL of *chloride standard solution (5 ppm Cl) R*, 4 mL of *ethanol (96 per cent) R*, 0.5 mL of *water R*, 0.5 mL of *nitric acid R* and 0.05 mL of *silver nitrate solution R2*.

**Sugars**. To 10 mL of solution S add 1 mL of *dilute sulfuric acid R* and heat on a water-bath for 5 min. Add 3 mL of carbonate-free *dilute sodium hydroxide solution R* (prepared by the method described for carbonate-free *1 M sodium hydroxide*), mix and add dropwise 1 mL of freshly prepared *copper sulfate solution R*. The solution is clear and blue. Continue heating on the water-bath for 5 min. The solution remains blue and no precipitate is formed.

**Chlorides** (*2.4.4*): maximum 10 ppm.

Dilute 1 mL of solution S to 15 mL with *water R*. Prepare the standard using 1 mL of *chloride standard solution (5 ppm Cl) R* diluted to 15 mL with *water R*.

**Water** (*2.5.12*): 12.0 per cent to 16.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.01 per cent, determined on 5.0 g after heating to boiling and ignition.

ASSAY

Thoroughly mix 0.075 g with 45 mL of *water R*. Add 25.0 mL of a mixture of 1 volume of *0.1 M sulfuric acid* and 20 volumes of *0.1 M sodium periodate*. Allow to stand protected from light for 15 min. Add 5.0 mL of a 500 g/L solution of *ethylene glycol R* and allow to stand protected from light for 20 min. Using 0.5 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide*. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 9.21 mg of $C_3H_8O_3$.

STORAGE

In an airtight container.

IMPURITIES

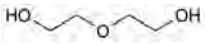

A. 2,2′-oxydi(ethan-1-ol) (diethylene glycol),

B. ethane-1,2-diol (ethylene glycol),

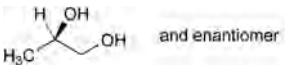


C. (2*RS*)-propane-1,2-diol (propylene glycol).

**01/2020:1427**

# GLYCEROL DIBEHENATE

## Glyceroli dibehenas

DEFINITION

Mixture of diacylglycerols, mainly dibehenoylglycerol, together with variable quantities of mono- and triacylglycerols, obtained by esterification of *Glycerol (0496)* with behenic (docosanoic) acid.

*Content*:

- *monoacylglycerols*: 15.0 per cent to 23.0 per cent;
- *diacylglycerols*: 40.0 per cent to 60.0 per cent;
- *triacylglycerols*: 21.0 per cent to 35.0 per cent.

CHARACTERS

*Appearance*: hard, waxy mass, or powder or white or almost white, unctuous flakes.

*Solubility*: practically insoluble in water, soluble in methylene chloride, partly soluble in hot ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 65 °C to 77 °C.

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of diacylglycerols).

TESTS

**Acid value** (*2.5.1*): maximum 4.0, determined on 1.0 g using a mixture of equal volumes of *ethanol (96 per cent) R* and *toluene R* as solvent and with gentle heating.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Saponification value** (*2.5.6*): 145 to 165.

Carry out the titration with heating.

**Free glycerol**: maximum 1.0 per cent, determined as described under Assay.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*) with the following modifications. Raise the temperature of the column to 240 °C and use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty acid fraction of the substance*:

- *palmitic acid*: maximum 3.0 per cent;
- *stearic acid*: maximum 5.0 per cent;
- *arachidic acid*: maximum 10.0 per cent;
- *behenic acid*: minimum 83.0 per cent;
- *erucic acid*: maximum 3.0 per cent;
- *lignoceric acid*: maximum 3.0 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use *pyridine R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.00 g.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Stock solution*. Place 0.100 g of *glycerol R* in a flask and dilute to 25.0 mL with *tetrahydrofuran R*.

*Test solution*. In a 15 mL flask, weigh 0.200 g ($m$) of the substance to be examined and add 5.0 mL of *tetrahydrofuran R*. Heat gently, at about 35 °C, and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance ($M$); use immediately.

*Reference solutions*. Into four 15 mL flasks, introduce respectively 0.25 mL, 0.5 mL, 1.0 mL and 2.5 mL of the stock solution and add 5.0 mL of *tetrahydrofuran R*. Weigh each flask and calculate the concentration of glycerol in milligrams per gram of each reference solution.

*Column*:

- *size*: $l$ = 0.6 m, Ø = 7 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractive index.

*Injection*: 40 µL; when injecting the test solution, maintain the flask at about 35 °C to avoid precipitation.

*Relative retention* with reference to glycerol (retention time = about 15 min): triacylglycerols = about 0.73; diacylglycerols = about 0.76; monoacylglycerols = about 0.82.

*Calculations*:

- *free glycerol*: from the calibration curve obtained with the reference solutions, determine the concentration ($C$) in milligrams per gram in the test solution and calculate the percentage content ($A$) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *free fatty acids*: calculate the percentage content of free fatty acids ($D$) using the following expression:

$$\frac{I_A \times 340}{561.1}$$

$I_A$ = acid value;

- *monoacylglycerols*: calculate the percentage content of monoacylglycerols using the following expression:

$$\left[\frac{X}{X+Y+Z}(100 - A - B)\right] - D$$

$A$ = percentage content of free glycerol (see Tests);

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoacylglycerols;

$Y$ = area of the peak due to diacylglycerols;

$Z$ = area of the peak due to triacylglycerols;

- *diacylglycerols*: calculate the percentage content of diacylglycerols using the following expression:

$$\frac{Y}{X+Y+Z}(100 - A - B)$$

- *triacylglycerols*: calculate the percentage content of triacylglycerols using the following expression:

$$\frac{Z}{X+Y+Z}(100 - A - B)$$

**01/2020:1428**

# GLYCEROL DISTEARATE

## Glyceroli distearas

DEFINITION

Mixture of diacylglycerols, mainly distearoylglycerol, together with variable quantities of mono- and triacylglycerols. It is obtained by partial glycerolysis of vegetable oils containing triacylglycerols of palmitic (hexadecanoic) and stearic (octadecanoic) acids or by esterification of glycerol with stearic acid. The fatty acids may be of vegetable or animal origin.

*Content*:

- *monoacylglycerols*: 8.0 per cent to 22.0 per cent;
- *diacylglycerols*: 40.0 per cent to 60.0 per cent;
- *triacylglycerols*: 25.0 per cent to 35.0 per cent.

CHARACTERS

*Appearance*: hard, waxy mass or powder, or white or almost white, unctuous flakes.

*Solubility*: practically insoluble in water, soluble in methylene chloride, partly soluble in hot ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 50 °C to 60 °C (types I and II), 50 °C to 70 °C (type III).

B. Composition of fatty acids (see Tests) according to the type stated on the label.

C. It complies with the limits of the assay (diacylglycerol content).

TESTS

**Acid value** (*2.5.1*): maximum 6.0, determined on 1.0 g.

Use a mixture of equal volumes of *ethanol (96 per cent) R* and *toluene R* as solvent and heat gently.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Saponification value** (*2.5.6*): 165 to 195, determined on 2.0 g.

Carry out the titration with heating.

**Free glycerol**: maximum 1.0 per cent, determined as described under Assay.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance:*

| Glycerol distearate | Composition of fatty acids |
|---|---|
| Type I | *Stearic acid*: 40.0 per cent to 60.0 per cent |
| | *Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent |
| Type II | *Stearic acid*: 60.0 per cent to 80.0 per cent |
| | *Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent |
| Type III | *Stearic acid*: 80.0 per cent to 99.0 per cent |
| | *Sum of the contents of palmitic and stearic acids*: minimum 96.0 per cent |

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use *pyridine R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* Into a 15 mL flask, weigh 0.200 g (*m*) of the substance to be examined. Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* Into three 15 mL flasks, respectively weigh 2.0 mg, 5.0 mg and 10.0 mg of *glycerol R* and add 5.0 mL of *tetrahydrofuran R* to each flask. Into a 4th flask, weigh 2.0 mg of *glycerol R* and add 10.0 mL of *tetrahydrofuran R*. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to glycerol (retention time = about 15 min): triacylglycerols = about 0.75; diacylglycerols = about 0.80; monoacylglycerols and free fatty acids = about 0.85.

*Calculations*:

- *free glycerol*: from the calibration curve obtained with the reference solutions, determine the concentration (*C*) in milligrams per gram in the test solution and calculate the percentage content (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 340}{561.1}$$

$I_A$ = acid value;

- *monoacylglycerols*: calculate the percentage content of monoacylglycerols using the following expression:

$$\left[\frac{X}{X+Y+Z}(100-A-B)\right]-D$$

$B$ = percentage content of water (see Tests);

$X$ = area of the peak due to monoacylglycerols and free fatty acids;

$Y$ = area of the peak due to diacylglycerols;

$Z$ = area of the peak due to triacylglycerols;

- *diacylglycerols*: calculate the percentage content of diacylglycerols using the following expression:

$$\frac{Y}{X+Y+Z}(100-A-B)$$

- *triacylglycerols*: calculate the percentage content of triacylglycerols using the following expression:

$$\frac{Z}{X+Y+Z}(100-A-B)$$

LABELLING

The label states the type of glycerol distearate.

**01/2017:1671**

# GLYCEROL FORMAL

## Glycerol-formalum

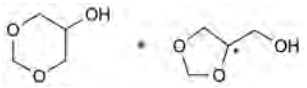

$C_4H_8O_3$ $M_r$ 104.1

DEFINITION

Mixture of 1,3-dioxan-5-ol and (1,3-dioxolan-4-yl)methanol.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. Relative density (see Tests).

B. Refractive index (see Tests).

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *glycerol formal CRS*.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.5.

Dilute 1 mL to 10 mL with *carbon dioxide-free water R*.

**Relative density** (*2.2.5*): 1.210 to 1.220.

**Refractive index** (*2.2.6*): 1.445 to 1.455.

**Peroxide value** (*2.5.5*): maximum 15.

**Formaldehyde**: maximum 200 ppm.

Dilute 0.250 g to 10 mL with *water R*. Add 2.0 mL of *acetylacetone reagent R2*, mix and heat on a water-bath at 60 °C for 20 min. Cool and dilute to 20.0 mL with *water R*. The absorbance (*2.2.25*) of the solution measured at 412 nm is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a dilution of *formaldehyde solution R* containing 10 µg of formaldehyde ($CH_2O$) per millilitre.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 5.000 g.

IMPURITIES

*Specified impurities: A*.

Monographs G

A. formaldehyde.

STORAGE

Under nitrogen, in an airtight container.

**01/2008:2213**
**corrected 10.0**

# GLYCEROL MONOCAPRYLATE

## Glyceroli monocaprylas

DEFINITION

Mixture of monoacylglycerols, mainly mono-*O*-octanoylglycerol, containing variable quantities of di- and triacylglycerols, obtained by direct esterification of glycerol with caprylic (octanoic) acid, followed by a distillation step in the case of glycerol monocaprylate (type II).

*Content*:

- *glycerol monocaprylate (type I)*:
  - *monoacylglycerols*: 45.0 per cent to 75.0 per cent;
  - *diacylglycerols*: 20.0 per cent to 50.0 per cent;
  - *triacylglycerols*: maximum 10.0 per cent;
- *glycerol monocaprylate (type II)*:
  - *monoacylglycerols*: minimum 80.0 per cent;
  - *diacylglycerols*: maximum 20.0 per cent;
  - *triacylglycerols*: maximum 5.0 per cent.

CHARACTERS

*Appearance*: colourless or slightly yellow, oily liquid or soft mass.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and freely soluble in methylene chloride.

IDENTIFICATION

A. Composition of fatty acids (see Tests).

B. It complies with the limits of the assay (monoacylglycerols).

TESTS

**Acid value** (*2.5.1*): maximum 3.0.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-2.

*Composition of the fatty acid fraction of the substance*:

- *caproic acid*: maximum 1.0 per cent;
- *caprylic acid*: minimum 90.0 per cent;
- *capric acid*: maximum 10.0 per cent;
- *lauric acid*: maximum 1.0 per cent;
- *myristic acid*: maximum 0.5 per cent.

**Free glycerol**: maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat to about 50 °C then allow to cool. Add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake again and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R* and allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate with *0.1 M sodium thiosulfate* until the aqueous phase becomes colourless. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* To 0.25 g of the substance to be examined, add 5.0 mL of *tetrahydrofuran R* and shake to dissolve.

*Reference solution (a).* To 0.25 g of *glycerol monocaprylate CRS*, add 5.0 mL of *tetrahydrofuran R* and shake to dissolve.

*Reference solution (b).* To 50 mg of *glycerol 1-octanoate R* and 50 mg of *glycerol 1-decanoate R*, add 2.5 mL of *tetrahydrofuran R* and shake to dissolve.

*Column*:

- *size*: *l* = 10 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 60 |
| | 3 - 38 | 60 → 340 |
| | 38 - 50 | 340 |
| Injection port | | 350 |
| Detector | | 370 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *glycerol monocaprylate CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to mono-, di- and triacylglycerols.

*System suitability*: reference solution (b):

- *resolution*: minimum 5 between the peaks due to glycerol 1-octanoate and glycerol 1-decanoate.

For the calculation of the contents of mono-, di- and triacylglycerols, disregard the peaks with a retention time less than that of the monoacylglycerols, which are due to impurities of the solvent and to the free fatty acids.

Calculate the percentage content of free fatty acids (*C*) using the following expression:

$$\frac{I_A \times 144}{561.1}$$

$I_A$ = acid value of glycerol monocaprylate.

Calculate the content of mono-, di- and triacylglycerols using the following equations:

$$\text{Monoacylglycerols content} = \frac{X \times (100 - A - B - C)}{100}$$

$$\text{Diacylglycerols content} = \frac{Y \times (100 - A - B - C)}{100}$$

$$\text{Triacylglycerols content} = \frac{Z \times (100 - A - B - C)}{100}$$

$A$ = percentage content of free glycerol (see Tests);

$B$ = percentage content of water;

$X$ = monoacylglycerols content obtained by normalisation;

$Y$ = diacylglycerols content obtained by normalisation;

$Z$ = triacylglycerols content obtained by normalisation.

LABELLING

The label states the type of glycerol monocaprylate (type I or II).

**01/2011:2392**
**corrected 10.0**

# GLYCEROL MONOCAPRYLOCAPRATE

## Glyceroli monocaprylocapras

DEFINITION

Mixture of monoacylglycerols, mainly mono-*O*-octanoylglycerol and mono-*O*-decanoylglycerol, containing variable quantities of di- and triacylglycerols, obtained by direct esterification of glycerol with caprylic (octanoic) and capric (decanoic) acids, followed by a distillation step in the case of glycerol monocaprylocaprate (type II).

*Content*:

- *glycerol monocaprylocaprate (type I)*:
  - *monoacylglycerols*: 45.0 per cent to 75.0 per cent;
  - *diacylglycerols*: 20.0 per cent to 50.0 per cent;
  - *triacylglycerols*: maximum 10.0 per cent;
- *glycerol monocaprylocaprate (type II)*:
  - *monoacylglycerols*: minimum 80.0 per cent;
  - *diacylglycerols*: maximum 20.0 per cent;
  - *triacylglycerols*: maximum 5.0 per cent.

CHARACTERS

*Appearance*: colourless or slightly yellow, oily liquid or soft mass.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and freely soluble in methylene chloride.

IDENTIFICATION

A. Composition of fatty acids (see Tests).

B. It complies with the limits of the assay (monoacylglycerols).

TESTS

**Acid value** (*2.5.1*): maximum 3.0.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-2.

*Composition of the fatty acid fraction of the substance*:

- *caproic acid*: maximum 3.0 per cent;
- *caprylic acid*: 50.0 per cent to 90.0 per cent;
- *capric acid*: 10.0 per cent to 50.0 per cent;
- *lauric acid*: maximum 3.0 per cent;
- *myristic acid*: maximum 1.0 per cent.

**Free glycerol** : maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat to about 50 °C and allow to cool. Add 100 mL of *water R*, shake and add 25.0 mL of *periodic acetic acid solution R*. Shake again and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R* and allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate with *0.1 M sodium thiosulfate* until the aqueous phase becomes colourless. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* To 0.25 g of the substance to be examined, add 5.0 mL of *tetrahydrofuran R* and shake to dissolve.

*Reference solution (a).* To 0.25 g of *glycerol monocaprylocaprate CRS*, add 5.0 mL of *tetrahydrofuran R* and shake to dissolve.

*Reference solution (b).* To 50 mg of *glycerol 1-octanoate R* and 50 mg of *glycerol 1-decanoate R*, add 2.5 mL of *tetrahydrofuran R* and shake to dissolve.

*Column*:

- *size*: *l* = 10 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.3 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 60 |
| | 3 - 38 | 60 → 340 |
| | 38 - 50 | 340 |
| Injection port | | 350 |
| Detector | | 370 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *glycerol monocaprylocaprate CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to mono-, di- and triacylglycerols.

*System suitability*: reference solution (b):

- *resolution*: minimum 5 between the peaks due to glycerol 1-octanoate and glycerol 1-decanoate.

For the calculation of the contents of mono-, di- and triacylglycerols, disregard the peaks with a retention time less than that of the monoacylglycerols, which are due to the impurities of the solvent and to the free fatty acids.

Calculate the percentage content of free fatty acids *(C)* using the following equations:

$$\frac{I_A \times 144}{561.1}$$

$I_A$ = acid value of the glycerol monocaprylocaprate.

Calculate the content of mono-, di- and triacylglycerols using the following equations:

$$\text{Monoacylglycerols content} = \frac{X \times (100 - A - B - C)}{X + Y + Z}$$

$$\text{Diacylglycerols content} = \frac{Y \times (100 - A - B - C)}{X + Y + Z}$$

$$\text{Triacylglycerols content} = \frac{Z \times (100 - A - B - C)}{X + Y + Z}$$

$A$ = percentage content of free glycerol (see Tests);

$B$ = percentage content of water;

$X$ = area of the peak due to monoacylglycerols;

$Y$ = area of the peak due to diacylglycerols;

$Z$ = area of the peak due to triacylglycerols.

LABELLING

The labelling states the type of glycerol monocaprylocaprate (type I or II).

01/2017:1429

# GLYCEROL MONOLINOLEATE

## Glyceroli monolinoleas

DEFINITION

Mixture of monoacylglycerols, mainly mono-oleoyl- and monolinoleoylglycerol, together with variable quantities of di- and triacylglycerols, obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of linoleic ((9*Z*,12*Z*)-octadeca-9,12-dienoic) acid. A suitable antioxidant may be added.

*Content*:

– *monoacylglycerols*: 32.0 per cent to 52.0 per cent;
– *diacylglycerols*: 40.0 per cent to 55.0 per cent;
– *triacylglycerols*: 5.0 per cent to 20.0 per cent.

CHARACTERS

*Appearance*: amber, oily liquid which may be partially solidified at room temperature.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride.

IDENTIFICATION

A. Iodine value (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution.* Dissolve 1.0 g of *glycerol monolinoleate CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 6.0, determined on 1.0 g.

**Iodine value** (*2.5.4, Method A*): 100 to 140.

**Peroxide value** (*2.5.5, Method A*): maximum 12.0, determined on 2.0 g.

**Saponification value** (*2.5.6*): 160 to 180, determined on 2.0 g.

**Free glycerol**: maximum 6.0 per cent, determined as described in the assay.

**Composition of fatty acids** (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance:*

– *palmitic acid*: 4.0 per cent to 20.0 per cent;
– *stearic acid*: maximum 6.0 per cent;
– *oleic acid*: 10.0 per cent to 35.0 per cent;
– *linoleic acid*: minimum 50.0 per cent;
– *linolenic acid*: maximum 2.0 per cent;
– *arachidic acid*: maximum 1.0 per cent;
– *eicosenoic acid*: maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* Into a 15 mL flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* Into four 15 mL flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 mL of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

*Column*:

– *size*: *l* = 0.6 m, Ø = 7 mm,
– *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to glycerol (retention time = about 15.6 min): triacylglycerols = about 0.76; diacylglycerols = about 0.80; monoacylglycerols = about 0.86.

*Calculations*:

– *free glycerol*: from the calibration curve obtained with the reference solutions, determine the concentration of glycerol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free glycerol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

– *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 280}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);
280 = rounded molar mass of linoleic acid, in grams per mole;
56.11 = molar mass of potassium hydroxide, in grams per mole.

– *monoacylglycerols*: calculate the percentage content of monoacylglycerols using the following expression:

$$\left[\frac{X}{X+Y+Z}\ (100 - A - B)\right] - D$$

$A$ = percentage content of free glycerol;
$B$ = percentage content of water (see Tests);
$D$ = percentage content of free fatty acids;
$X$ = area of the peak due to monoacylglycerols and free fatty acids;
$Y$ = area of the peak due to diacylglycerols;
$Z$ = area of the peak due to triacylglycerols.

– *diacylglycerols*: calculate the percentage content of diacylglycerols using the following expression:

$$\frac{Y}{X+Y+Z}\ (100 - A - B)$$

– *triacylglycerols*: calculate the percentage content of triacylglycerols using the following expression:

$$\frac{Z}{X+Y+Z}\ (100 - A - B)$$

STORAGE

In an airtight container, protected from light.

**01/2017:1430**

# GLYCEROL MONO-OLEATE

## Glyceroli mono-oleas

DEFINITION

Mixture of monoacylglycerols, mainly mono-oleoylglycerol, together with variable quantities of di- and triacylglycerols. It is defined by the nominal content of monoacylglycerols and obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of oleic ((9*Z*)-octadec-9-enoic) acid or by esterification of glycerol by oleic acid, this fatty acid being of vegetable or animal origin. A suitable antioxidant may be added.

*Content*:

| | Nominal content of acylglycerol (per cent) | | |
|---|---|---|---|
| | **40** | **60** | **90** |
| Monoacylglycerols | 32.0 - 52.0 | 55.0 - 65.0 | 90.0 - 101.0 |
| Diacylglycerols | 30.0 - 50.0 | 15.0 - 35.0 | < 10.0 |
| Triacylglycerols | 5.0 - 20.0 | 2.0 - 10.0 | < 2.0 |

CHARACTERS

*Appearance*: amber, oily liquid which may be partially solidified at room temperature.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride.

IDENTIFICATION

A. Iodine value (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution.* Dissolve 1.0 g of *glycerol mono-oleate CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. It complies with the limits of the assay (monoacylglycerol content).

TESTS

**Acid value** (*2.5.1*): maximum 6.0, determined on 1.0 g.

**Iodine value** (*2.5.4, Method A*): 65.0 to 95.0.

**Peroxide value** (*2.5.5, Method A*): maximum 12.0, determined on 2.0 g.

**Saponification value** (*2.5.6*): 150 to 175, determined on 2.0 g.

**Free glycerol**: maximum 6.0 per cent, determined as described in the assay.

**Composition of fatty acids** (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance:*

– *palmitic acid*: maximum 12.0 per cent;
– *stearic acid*: maximum 6.0 per cent;
– *oleic acid*: minimum 60.0 per cent;
– *linoleic acid*: maximum 35.0 per cent;
– *linolenic acid*: maximum 2.0 per cent;
– *arachidic acid*: maximum 2.0 per cent;
– *eicosenoic acid*: maximum 2.0 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* Into a 15 mL flask, weigh about 0.2 g (*m*), to the nearest 0.1 mg. Add 5 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* Into four 15 mL flasks, respectively weigh, to the nearest 0.1 mg, about 2.5 mg, 5 mg, 10 mg and 20 mg of *glycerol R*. Add 5 mL of *tetrahydrofuran R* and shake until well mixed. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

*Column*:

– *size*: *l* = 0.6 m, Ø = 7 mm;
– *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to glycerol (retention time = about 15.6 min): triacylglycerols = about 0.76; diacylglycerols = about 0.79; monoacylglycerols = about 0.85.

*Calculations*:

– *free glycerol*: from the calibration curve obtained with the reference solutions determine the concentration of glycerol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free glycerol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

– *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 282}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

282 = rounded molar mass of oleic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

Monographs G

– *monoacylglycerols*: calculate the percentage content of monoacylglycerols using the following expression:

$$\left[\frac{X}{X+Y+Z}\ (100-A-B)\right]-D$$

$A$ = percentage content of free glycerol;

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoacylglycerols and free fatty acids;

$Y$ = area of the peak due to diacylglycerols;

$Z$ = area of the peak due to triacylglycerols.

– *diacylglycerols*: calculate the percentage content of diacylglycerols using the following expression:

$$\frac{Y}{X+Y+Z}\ (100-A-B)$$

– *triacylglycerols*: calculate the percentage content of triacylglycerols using the following expression:

$$\frac{Z}{X+Y+Z}\ (100-A-B)$$

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the nominal content of monoacylglycerol.

**01/2020:0495**

# GLYCEROL MONOSTEARATE 40-55

## Glyceroli monostearas 40-55

DEFINITION

Mixture of monoacylglycerols, mainly monostearoylglycerol, together with variable quantities of di- and triacylglycerols. It is obtained by partial glycerolysis of vegetable oils mainly containing triacylglycerols of palmitic (hexadecanoic) or stearic (octadecanoic) acid or by esterification of glycerol with stearic acid. The fatty acids may be of vegetable or animal origin.

*Content*:

– *monoacylglycerols*: 40.0 per cent to 55.0 per cent;

– *diacylglycerols*: 30.0 per cent to 45.0 per cent;

– *triacylglycerols*: 5.0 per cent to 15.0 per cent.

CHARACTERS

*Appearance*: hard, waxy mass or unctuous powder or flakes, white or almost white.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) at 60 °C.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B.*

A. Melting point (*2.2.15*): 54 °C to 66 °C.

Introduce the melted substance into the capillary tubes and allow to stand for 24 h in a well-closed container.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.5 g of the substance to be examined in *methylene chloride R*, with gentle heating, and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.5 g of *glycerol monostearate 40-55 CRS* in *methylene chloride R*, with gentle heating, and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Suitability system*: reference solution:

– the chromatogram shows 4 clearly separated spots.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

C. Composition of fatty acids (see Tests) according to the type stated on the label.

D. It complies with the limits of the assay (monoacylglycerol content).

TESTS

**Acid value** (*2.5.1*): maximum 3.0, determined on 1.0 g.

Use a mixture of equal volumes of *ethanol (96 per cent) R* and *toluene R* as solvent and heat gently.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Saponification value** (*2.5.6*): 158 to 177, determined on 2.0 g. Carry out the titration with heating.

**Free glycerol**: maximum 6.0 per cent, determined as described under Assay.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance*:

| Glycerol monostearate 40-55 | Composition of fatty acids |
|---|---|
| Type I | *Stearic acid*: 40.0 per cent to 60.0 per cent<br>*Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent |
| Type II | *Stearic acid*: 60.0 per cent to 80.0 per cent<br>*Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent |
| Type III | *Stearic acid*: 80.0 per cent to 99.0 per cent<br>*Sum of the contents of palmitic and stearic acids*: minimum 96.0 per cent |

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use *pyridine R* as the solvent and heat gently.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* Into a 15 mL flask, weigh 0.200 g (*m*). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* Into four 15 mL flasks, respectively weigh 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *glycerol R*, and add 5.0 mL of *tetrahydrofuran R* to each flask. Weigh the flasks again and calculate the concentration of glycerol in milligrams per gram for each reference solution.

*Column*:

– *size*: *l* = 0.6 m, Ø = 7 mm;

– *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to glycerol (retention time = about 15 min): triacylglycerols = about 0.75; diacylglycerols = about 0.80; monoacylglycerols = about 0.85.

*Calculations*:

– *free glycerol*: from the calibration curve obtained with the reference solutions, determine the concentration of glycerol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free glycerol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

– *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 270}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

270 = average rounded molar mass of stearic acid and palmitic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole;

– *monoacylglycerols*: calculate the percentage content of monoacylglycerols using the following expression:

$$\left[\frac{X}{X+Y+Z}\ (100 - A - B)\right] - D$$

*A* = percentage content of free glycerol;

*B* = percentage content of water (see Tests);

*D* = percentage content of free fatty acids;

*X* = area of the peak due to monoacylglycerols and free fatty acids;

*Y* = area of the peak due to diacylglycerols;

*Z* = area of the peak due to triacylglycerols;

– *diacylglycerols*: calculate the percentage content of diacylglycerols using the following expression:

$$\frac{Y}{X+Y+Z}\ (100 - A - B)$$

– *triacylglycerols*: calculate the percentage content of triacylglycerols using the following expression:

$$\frac{Z}{X+Y+Z}\ (100 - A - B)$$

LABELLING

The label states the type of glycerol monostearate 40-55.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for glycerol monostearate 40-55 used as matrix former in prolonged-release oral solid dosage forms.*

**Composition of fatty acids** (see Tests).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**Thermal analysis** (*2.2.34*). The melting behaviour of the substance as is and after melting followed by solidification when cooling may be considered.

*The following characteristics may be relevant for glycerol monostearate 40-55 used as consistency agent in dosage forms for cutaneous application.*

**Composition of fatty acids** (see Tests).

**Thermal analysis** (*2.2.34*). The melting behaviour of the substance as is and after melting followed by solidification when cooling may be considered.

**04/2016:1331**

# GLYCERYL TRINITRATE SOLUTION

## Glyceroli trinitratis solutio

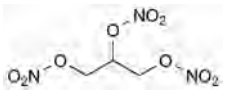

$C_3H_5N_3O_9$ $M_r$ 227.1

DEFINITION

Ethanolic solution of glyceryl trinitrate containing 1 per cent *m/m* to 10 per cent *m/m* of propane-1,2,3-triyl trinitrate.

*Content*: 96.5 per cent to 102.5 per cent of the declared content of glyceryl trinitrate stated on the label.

CHARACTERS

*Appearance*: clear, colourless or slightly yellow solution.

*Solubility*: miscible with acetone and with anhydrous ethanol.

*Solubility of pure glyceryl trinitrate*: practically insoluble in water, freely soluble in anhydrous ethanol, miscible with acetone.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

*Upon diluting glyceryl trinitrate solution, care must be taken to always use anhydrous ethanol, otherwise droplets of pure glyceryl trinitrate may precipitate from the solution.*

*After examination, the residues and the solutions obtained in both the identification and the test sections must be heated on a water-bath for 5 min with dilute sodium hydroxide solution R.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: place 50 µL of a solution diluted, if necessary, with *anhydrous ethanol R*, to contain 10 g/L of glyceryl trinitrate, on a disc of *potassium bromide R* and evaporate the solvent *in vacuo*.

*Comparison*: *Ph. Eur. reference spectrum of glyceryl trinitrate*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute a quantity of the substance to be examined corresponding to 50 mg of glyceryl trinitrate to 100 mL with *acetone R*.

Monographs G

*Reference solution.* Dilute 0.05 mL of *glyceryl trinitrate solution CRS* to 1 mL with *acetone R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R, toluene R* (20:80 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with freshly prepared *potassium iodide and starch solution R*; expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It complies with the limits of the assay.

TESTS

*Upon diluting glyceryl trinitrate solution, care must be taken to always use anhydrous ethanol, otherwise droplets of pure glyceryl trinitrate may precipitate from the solution.*

*After examination, the residues and the solutions obtained in both the identification and the test sections must be heated on a water-bath for 5 min with dilute sodium hydroxide solution R.*

**Appearance of solution**. If necessary, dilute the solution to be examined to a concentration of 10 g/L with *anhydrous ethanol R*. The solution is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Impurity A (inorganic nitrates)**. Thin-layer chromatography (*2.2.27*).

*Test solution.* If necessary, dilute the solution to be examined to a concentration of 10 g/L with *anhydrous ethanol R.*

*Reference solution.* Dissolve 5 mg of *potassium nitrate R* in 1 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R.*

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R, acetone R, toluene R* (15:30:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of air until the acetic acid is completely removed.

*Detection*: spray intensively with freshly prepared *potassium iodide and starch solution R*; expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Limit*:

– *nitrate ion*: any spot due to the nitrate ion in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent of the content of glyceryl trinitrate calculated as potassium nitrate).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dilute a quantity of the substance to be examined equivalent to 5.0 mg of glyceryl trinitrate to 10.0 mL with *water for chromatography R. NOTE*: for glyceryl trinitrate solutions of more than 1 per cent *m/m*, dilute first to a 1 per cent solution with *anhydrous ethanol R.*

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water for chromatography R.*

*Reference solution (b).* Dilute 25 µL of *glyceryl trinitrate for system suitability CRS* (containing impurities B, C, D and E) to 0.5 mL with *water for chromatography R.*

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;
– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 10 | 90 → 50 | 10 → 50 |
| 10 - 20 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *glyceryl trinitrate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D and E.

*Relative retention* with reference to glyceryl trinitrate (retention time = about 15.5 min): impurity C = about 0.19; impurity B = about 0.21; impurity E = about 0.63; impurity D = about 0.65.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities C and B; minimum 1.5 between the peaks due to impurities E and D.

*Calculation of percentage contents*:

– for each impurity, use the concentration of glyceryl trinitrate in reference solution (a).

*Limits*:

– *impurities B, C, D, E*: for each impurity, maximum 0.5 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.5 per cent;
– *reporting threshold*: 0.05 per cent.

ASSAY

*Test solution.* Prepare a solution containing 1.0 mg of glyceryl trinitrate in 250.0 mL of *methanol R.*

*Reference solution.* Dissolve 70.0 mg of *sodium nitrite R* in *methanol R* and dilute to 250.0 mL with the same solvent. Dilute 5.0 mL of the solution to 500.0 mL with *methanol R.*

Into three 50 mL volumetric flasks introduce 10.0 mL of the test solution, 10.0 mL of the reference solution and 10 mL of *methanol R* as a blank. To each flask add 5 mL of *dilute sodium hydroxide solution R*, close the flask, mix and allow to stand at room temperature for 30 min. Add 10 mL of *sulfanilic acid solution R* and 10 mL of *dilute hydrochloric acid R* and mix. After exactly 4 min, add 10 mL of *naphthylethylenediamine dihydrochloride solution R*, dilute to volume with *water R* and mix. After 10 min read the absorbance (*2.2.25*) of the test solution and the reference solution at 540 nm using the blank solution as the compensation liquid.

Calculate the percentage content of glyceryl trinitrate using the following expression:

$$\frac{A_T \times m_S \times C}{A_R \times m_T \times 60.8}$$

$A_T$ = absorption of the test solution;

$m_S$ = mass of sodium nitrite, in milligrams.

$C$ = percentage content of sodium nitrite used as reference;

$A_R$ = absorption of the reference solution;

$m_T$ = mass of the substance to be examined, in milligrams;

STORAGE

Store the diluted solutions (1 per cent *m/m*) protected from light, at a temperature of 2 °C to 15 °C.

Store the other solutions protected from light, at a temperature of 15 °C to 20 °C.

LABELLING

The label states the declared content of glyceryl trinitrate.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

A. inorganic nitrates,

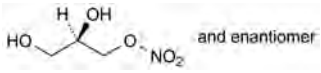


B. (2*RS*)-2,3-dihydroxypropyl nitrate,

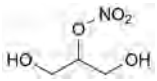


C. 2-hydroxy-1-(hydroxymethyl)ethyl nitrate,

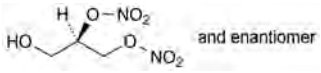


D. (2*RS*)-3-hydroxypropane-1,2-diyl dinitrate,

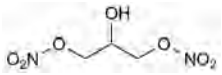


E. 2-hydroxypropane-1,3-diyl dinitrate.

**01/2017:0614**

# GLYCINE

## Glycinum

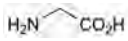


$C_2H_5NO_2$ $M_r$ 75.1
[56-40-6]

DEFINITION

2-Aminoacetic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *glycine CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (60 per cent V/V) R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *glycine CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 80 °C for 30 min.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in 5 mL of *water R*, add 1 mL of *strong sodium hypochlorite solution R* and boil for 2 min. Add 1 mL of *hydrochloric acid R* and boil for 4-5 min. Add 2 mL of *hydrochloric acid R* and 1 mL of a 20 g/L solution of *resorcinol R*, boil for 1 min and cool. Add 10 mL of *water R* and mix. To 5 mL of the solution add 6 mL of *dilute sodium hydroxide solution R*. The solution is violet with greenish-yellow fluorescence. After a few minutes, the colour becomes orange and then yellow and an intense fluorescence remains.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.9 to 6.4.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* To 500 mL of the mobile phase add 1.5 mL of *phosphoric acid R*.

*Test solution.* Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 80 mg of *iminodiacetic acid R* (impurity A) and 80 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *glycine anhydride R* (impurity B), 50.0 mg of *diglycine R* (impurity H) and 50.0 mg of *triglycine R* (impurity I) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: dissolve 1.4 g of *sodium pentanesulfonate R* in 900 mL of *water R*, adjust to pH 2.2 with *phosphoric acid R* and dilute to 1 L with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of glycine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, H and I.

*Relative retention* with reference to glycine (retention time = about 5.5 min): impurity A = about 0.7; impurity B = about 0.75; impurity H = about 1.7; impurity I = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurity A and glycine.

*Calculation of percentage contents*:

– for each impurity, use the concentration of glycine in reference solution (a);
– for impurities B, H and I, use the concentration of the corresponding impurity in reference solution (c).

*Limits*:

– *impurities B, H, I*: for each impurity, maximum 0.10 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.2 per cent;
– *reporting threshold*: 0.05 per cent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions (a), (b) and (d) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of glycine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 75 ppm.

Dissolve 0.67 g in *water R* and dilute to 15 mL with the same solvent.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 70.0 mg in 5 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Immediately after dissolution, titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 7.51 mg of $C_2H_5NO_2$.

IMPURITIES

*Specified impurities: B, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F, G.*

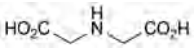

A. 2,2′-iminodiacetic acid,

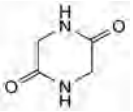

B. piperazine-2,5-dione (glycine anhydride),

C. 2-chloroacetic acid,

D. 1,3,5,7-tetraazatricyclo[3.3.1.1$^{3,7}$]decane,

E. 3-aminopropanoic acid (β-alanine),

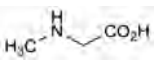

F. 2-(methylamino)acetic acid (sarcosine),

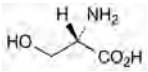

G. (2*S*)-2-amino-3-hydroxypropanoic acid (serine),

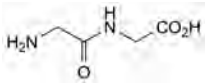

H. 2-[(2-aminoacetyl)amino]acetic acid (diglycine),

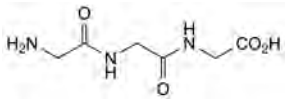

I. 2-[[2-[(2-aminoacetyl)amino]acetyl]amino]acetic acid (triglycine).

**04/2011:1783**

# GLYCOPYRRONIUM BROMIDE

## Glycopyrronii bromidum

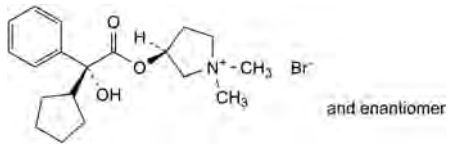


$C_{19}H_{28}BrNO_3$ $M_r$ 398.3
[51186-83-5]

DEFINITION

(3*RS*)-3-[(2*SR*)-(2-Cyclopentyl-2-hydroxy-2-phenylacetyl)oxy]-1,1-dimethylpyrrolidinium bromide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *glycopyrronium bromide CRS*.

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink. Add 0.4 mL of *0.01 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The solution is red or orange.

**Impurity N**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 3.2 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of *water R*, adjust to pH 6.5 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.0 mg of *glycopyrronium impurity N CRS* in 10.0 mL of the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution and 5.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *silica gel BC for chiral chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, solution A, *methanol R2* (10:40:50 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 222 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.5 times the retention time of glycopyrronium.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity N.

*Relative retention* with reference to glycopyrronium (retention time = about 30 min): impurity N = about 0.9.

*System suitability*:
- *resolution*: minimum 1.25 between the peaks due to impurity N and glycopyrronium in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 5 for the peak due to impurity N in the chromatogram obtained with reference solution (b).

*Limit*:
- *impurity N*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 5 mg of *glycopyrronium for peak identification CRS* (containing impurities E and I) in 5.0 mL of mobile phase A.

*Reference solution (c)*. Dissolve 10 mg of *benzaldehyde R* (impurity F) in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution and 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 0.25 g of *sodium heptanesulfonate R* in 615 mL of a 1.63 g/L solution of *anhydrous sodium sulfate R*; add 3 mL of a 5.15 g/L solution of *sulfuric acid R*, 150 mL of *methanol R2* and 235 mL of *acetonitrile R1*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 30 | 100 → 50 | 0 → 50 |
| 30 - 45 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

Monographs G

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *glycopyrronium for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities E and I; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to glycopyrronium (retention time = about 11 min): impurity E = about 0.7; impurity F = about 0.8; impurity I = about 2.3.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to impurity F and glycopyrronium.

*Limits*:

- *impurity I*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to the bromide ion appearing close to the peak due to the solvent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *anhydrous acetic acid R* and 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 39.83 mg of $C_{19}H_{28}BrNO_3$.

IMPURITIES

*Specified impurities: E, I, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, J, K, L, M, O.*

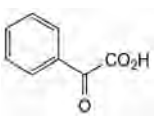

B. oxophenylacetic acid (benzoylformic acid),

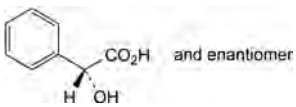

C. (2*RS*)-2-hydroxy-2-phenylacetic acid (mandelic acid),

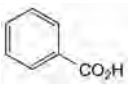

D. benzoic acid,

E. unknown structure,

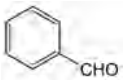

F. benzaldehyde,

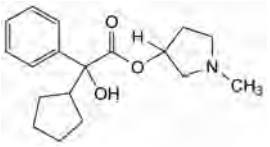

G. 1-methylpyrrolidin-3-yl 2-cyclopentyl-2-hydroxy-2-phenylacetate,

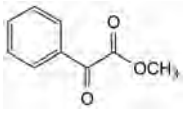

H. methyl 2-oxo-2-phenylacetate,

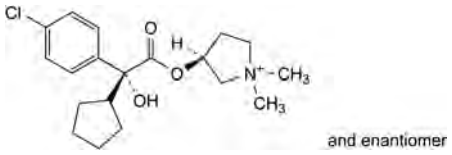

I. (3*RS*)-3-[(2*SR*)-(2-(4-chlorophenyl)-2-cyclopentyl-2-hydroxyacetyl)oxy]-1,1-dimethylpyrrolidinium,

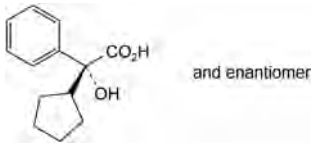

J. (2*RS*)-2-cyclopentyl-2-hydroxy-2-phenylacetic acid,

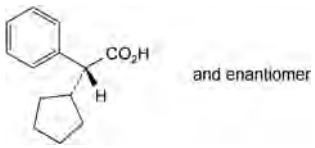

K. (2*RS*)-2-cyclopentyl-2-phenylacetic acid,

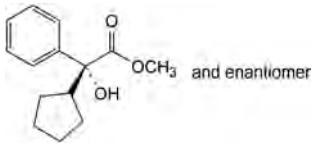

L. methyl (2*RS*)-2-cyclopentyl-2-hydroxy-2-phenylacetate,

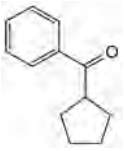

M. cyclopentylphenylmethanone,

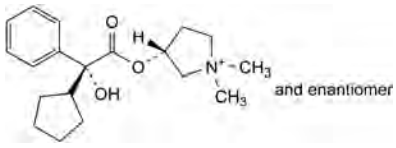

N. (3*RS*)-3-[(2*RS*)-(2-cyclopentyl-2-hydroxy-2-phenylacetyl)oxy]-1,1-dimethylpyrrolidinium,

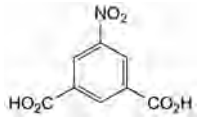

O. 5-nitroisophtalic acid.

**04/2018:0827**
**corrected 10.0**

# GONADORELIN ACETATE

## Gonadorelini acetas

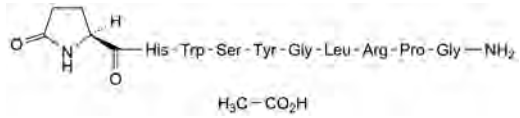

$C_{57}H_{79}N_{17}O_{15}$ $M_r$ 1242
[499785-55-6]

DEFINITION

5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosylglycyl-L-leucyl-L-arginyl-L-prolylglycinamide acetate.

Acetate form of a synthetic hypothalamic peptide that stimulates the release of follicle-stimulating hormone and luteinising hormone from the pituitary gland.

*Content*: 95.0 per cent to 102.0 per cent of the peptide $C_{55}H_{75}N_{17}O_{13}$ (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder, hygroscopic.

*Solubility*: soluble in water, sparingly soluble in methanol. It dissolves in a 1 per cent *V/V* solution of glacial acetic acid.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Nuclear magnetic resonance spectrometry (*2.2.64*).

*Preparation*: 4 mg/mL solution in a mixture of 20 volumes of *deuterated acetic acid R* and 80 volumes of *deuterium oxide R*.

*Comparison*: dissolve the contents of a vial of *gonadorelin for NMR identification CRS* in a mixture of 20 volumes of *deuterated acetic acid R* and 80 volumes of *deuterium oxide R* to obtain a concentration of 4 mg/mL.

*Operating conditions*:

– *field strength*: minimum 300 MHz;
– *temperature*: 27 °C.

*Results*: examine the $^1$H NMR spectrum from 0 to 9 ppm. The $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *gonadorelin for NMR identification CRS*.

C. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/8 of the sum of the number of moles of histidine, glutamic acid, leucine, proline, glycine, tyrosine and arginine as equal to 1. The values fall within the following limits: serine and tyrosine 0.7 to 1.05; glutamic acid, proline, leucine, histidine and arginine 0.9 to 1.1; glycine 1.8 to 2.2. Lysine and isoleucine are absent, and not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): – 65 to – 58 (anhydrous and acetic acid-free substance).

Dissolve 10.0 mg in 1.0 mL of a 1 per cent *V/V* solution of *glacial acetic acid R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *gonadorelin CRS* in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dissolve the contents of a vial of *gonadorelin for system suitability CRS* (containing impurities C, D, E, F and G) in 1.0 mL of *water R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 13 volumes of *acetonitrile R1* and 87 volumes of a 1.18 per cent *V/V* solution of *phosphoric acid R* previously adjusted to pH 2.3 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of gonadorelin.

*Relative retention* with reference to gonadorelin (retention time = about 12-16 min): impurities C and D = about 0.7; impurity E = about 0.8; impurities F and G = about 1.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities C + D, and impurity E.

*Limits*:

– *impurity E*: maximum 2.0 per cent;
– *sum of impurities F and G*: maximum 1.5 per cent;
– *sum of impurities C and D*: maximum 1.0 per cent;
– *unspecified impurities*: for each impurity, maximum 0.5 per cent;
– *total*: maximum 5.0 per cent;
– *reporting threshold*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 4.0 per cent to 7.5 per cent.

*Test solution*. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of solvents.

**Water** (*2.5.32*): maximum 7.0 per cent, determined on 20.0 mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of gonadorelin ($C_{55}H_{75}N_{17}O_{13}$) taking into account the assigned content of $C_{55}H_{75}N_{17}O_{13}$ in *gonadorelin CRS*.

STORAGE

In an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states:

- the gonadorelin peptide content;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: C, D, E, F, G.*

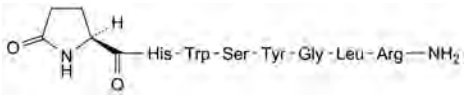


C. des-9-L-proline,10-L-glycine-gonadorelin,

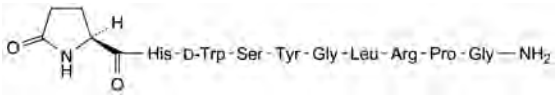


D. [3-D-tryptophan]gonadorelin,

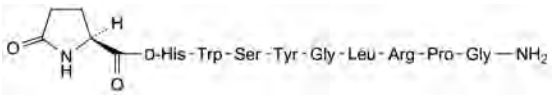


E. [2-D-histidine]gonadorelin,

F. [10-glycine]gonadorelin,

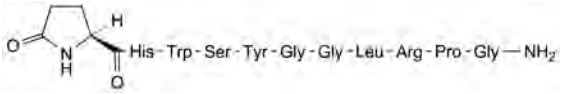


G. endo-5a-glycine-gonadorelin.

**01/2020:0498**

# GONADOTROPHIN, CHORIONIC

## Gonadotrophinum chorionicum

DEFINITION

Dried preparation containing placental glycoproteins obtained from the urine of pregnant women. It has luteinising activity.

*Potency*: minimum 2500 IU/mg.

PRODUCTION

It is prepared by suitable collection and extraction procedures followed by purification steps.

The method of preparation includes steps that have been shown to remove and/or inactivate extraneous agents. In addition, the process is designed to minimise microbial contamination.

It is either dried under reduced pressure or freeze-dried.

CHARACTERS

*Appearance*: white or yellowish-white, amorphous powder.

*Solubility*: soluble in water.

IDENTIFICATION

When administered to immature rats as prescribed in the assay, it causes an increase in the mass of the seminal vesicles and of the prostate gland.

TESTS

**Water** (*2.5.32*): maximum 5.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 0.02 IU per International Unit of chorionic gonadotrophin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The potency of chorionic gonadotrophin is estimated by comparing under given conditions its effect in increasing the mass of the seminal vesicles (or the prostate gland) of immature rats with the same effect of the International Standard of chorionic gonadotrophin or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a mixture of a freeze-dried extract of chorionic gonadotrophin from the urine of pregnant women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature male rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having body masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose is sufficient to produce a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression and as an initial approximation total doses of 4 IU, 8 IU and 16 IU may be tried although the dose will depend on the sensitivity of the animals used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 mL. Add a suitable antimicrobial preservative such as 4 g/L of phenol or 0.02 g/L of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group, on 4 consecutive days at the same time each day. On the 5$^{th}$ day, about 24 h after the last injection, euthanise the rats and remove the seminal vesicles. Remove any extraneous fluid and tissue and weigh the vesicles immediately. Calculate the results by the usual statistical methods (for example, *5.3*) using the mass of the vesicles as the response (a suitable correction of the organ mass with reference to the body mass of the animal from which it was taken may be applied; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the number of International Units per container;
- the potency, in International Units per milligram.

**01/2008:0719**
**corrected 10.0**

# GONADOTROPHIN, EQUINE SERUM, FOR VETERINARY USE

## Gonadotropinum sericum equinum ad usum veterinarium

DEFINITION

Equine serum gonadotrophin for veterinary use is a dry preparation of a glycoprotein fraction obtained from the serum or plasma of pregnant mares. It has follicle-stimulating and luteinising activities. The potency is not less than 1000 IU of gonadotrophin activity per milligram, calculated with reference to the anhydrous substance.

PRODUCTION

Equine serum gonadotrophin may be prepared by precipitation with alcohol (70 per cent *V/V*) and further purification by a suitable form of chromatography. It is prepared in conditions designed to minimise microbial contamination.

CHARACTERS

*Appearance*: white or pale grey, amorphous powder.

*Solubility*: soluble in water.

IDENTIFICATION

When administered as prescribed in the assay it causes an increase in the mass of the ovaries of immature female rats.

TESTS

**Water** (*2.5.12*): maximum 10.0 per cent, determined on 80 mg.

**Bacterial endotoxins** (*2.6.14, method C*): less than 0.035 IU per IU of equine serum gonadotrophin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The potency of equine serum gonadotrophin is estimated by comparing under given conditions its effect of increasing the mass of the ovaries of immature female rats with the same effect of the International Standard of equine serum gonadotrophin or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a mixture of a freeze-dried extract of equine serum gonadotrophin from the serum of pregnant mares with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 21 to 28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of not fewer than 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose is sufficient to produce a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression: as an initial approximation total doses of 8 IU, 12 IU and 18 IU may be tried, although the dose will depend on the sensitivity of the animals used and may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the doses to be used in sufficient of a sterile 9 g/L solution of *sodium chloride R* containing 1 mg/mL of *bovine albumin R* such that each single dose is administered in a volume of about 0.2 mL. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the dose allocated to its group. Repeat the injections 18 h, 21 h, 24 h, 42 h and 48 h after the first injection. Not less than 40 h and not more than 72 h after the last injection, euthanise the rats and remove the ovaries. Remove any extraneous fluid and tissue and weigh the 2 ovaries immediately. Calculate the results by the usual statistical methods, using the combined mass of the 2 ovaries of each animal as the response.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the potency in International Units per milligram.

**01/2013:1636**
**corrected 9.6**

# GOSERELIN

## Goserelinum

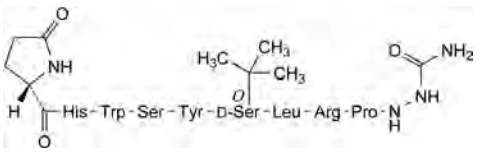


$C_{59}H_{84}N_{18}O_{14}$ $M_r$ 1269
[65807-02-5]

DEFINITION

1-Carbamoyl-2-[5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-*O*-(1,1-dimethylethyl)-D-seryl-L-leucyl-L-arginyl-L-prolyl]hydrazine.

Synthetic nonapeptide analogue of the hypothalamic decapeptide gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

*Content*: 94.5 per cent to 103.0 per cent of the peptide $C_{59}H_{84}N_{18}O_{14}$ (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, freely soluble in glacial acetic acid. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

Carry out either tests A and B or tests B and C.

A. Nuclear magnetic resonance spectrometry (*2.2.64*).
*Preparation*: 13 mg/mL solution in *0.2 M deuterated sodium phosphate buffer solution pH 5.0 R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R*.

*Comparison*: 13 mg/mL solution of *goserelin for NMR identification CRS* in *0.2 M deuterated sodium phosphate buffer solution pH 5.0 R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R* (dissolve the contents of a vial of *goserelin for NMR identification CRS* in this solvent to obtain the desired concentration).

*Operating conditions*:

– *field strength*: minimum 300 MHz;

– *temperature*: 25 °C.

*Results*: examine the $^1$H NMR spectrum from 0 ppm to 9 ppm; the $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *goserelin for NMR identification CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking 1/6 of the sum of the number of moles of glutamic acid, histidine, tyrosine, leucine, arginine, proline as equal to 1. The values fall within the following limits: glutamic acid, histidine, tyrosine, leucine, arginine and proline 0.9 to 1.1; serine 1.6 to 2.2. Not more than traces of other amino acids are present, with the exception of tryptophan.

TESTS

**Specific optical rotation** (*2.2.7*): – 56 to – 52 (anhydrous and acetic acid-free substance).

Dissolve the substance to be examined in *water R* to obtain a concentration of 2 mg/mL.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *goserelin CRS* in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 10.0 mL with *water R*.

*Resolution solution (a)*. Dissolve the contents of a vial of *4-D-Ser-goserelin CRS* in *water R* to obtain a concentration of 0.1 mg/mL. Mix equal volumes of this solution and reference solution (c).

*Resolution solution (b)*. Dissolve the contents of a vial of *goserelin validation mixture CRS* in 1.0 mL of *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (3.5 µm) with a pore size of 12.5 nm;

– *temperature*: 50-55 °C.

*Mobile phase*: *trifluoroacetic acid R*, *acetonitrile for chromatography R*, *water for chromatography R* (0.05:20:80 *V/V/V*).

*Flow rate*: 0.7-1.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution, reference solution (b) and resolution solutions (a) and (b).

*Run time*: 90 min.

*Relative retention* with reference to goserelin: impurity A = about 0.67; impurity C = about 0.78; impurity B = about 0.79; impurity D = about 0.85; impurity E = about 0.89; impurity F = about 0.92; impurity G = about 0.94; impurity H = about 0.98; impurity I = about 1.43; impurity J = about 1.53; impurity K = about 1.67; impurity L = about 1.77.

*System suitability*:

– *retention time*: goserelin = 40 min to 50 min in the chromatogram obtained with resolution solution (b); adjust the flow rate of the mobile phase if necessary; if adjusting the flow rate does not result in a correct retention time of the principal peak, change the proportion of acetonitrile in the mobile phase to obtain the requested retention time for goserelin;

– *resolution*: minimum 7.0 between the peaks due to impurity A and goserelin in the chromatogram obtained with resolution solution (a);

– *symmetry factor*: 0.8 to 2.5 for the peaks due to impurity A and goserelin in the chromatogram obtained with resolution solution (a);

– the chromatogram obtained with resolution solution (b) is similar to the chromatogram supplied with *goserelin validation mixture CRS*; 2 peaks eluting prior to the principal peak and corresponding to impurities E and G are clearly visible.

*Limits*:

– *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Acetic acid** (*2.5.34*): 4.5 per cent to 15.0 per cent.

*Test solution*. Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 10.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 16 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*Run time*: 60 min.

Calculate the content of goserelin ($C_{59}H_{84}N_{18}O_{14}$) taking into account the assigned content of $C_{59}H_{84}N_{18}O_{14}$ in *goserelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

– the mass of peptide in the container;

– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L.*

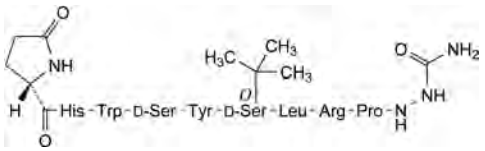

A. [4-D-serine]goserelin,

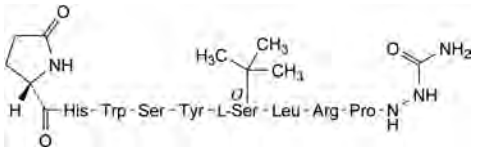

B. [6-[*O*-(1,1-dimethylethyl)-L-serine]]goserelin,

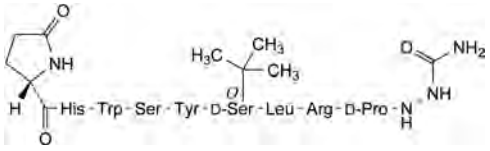

C. [9-D-proline]goserelin,

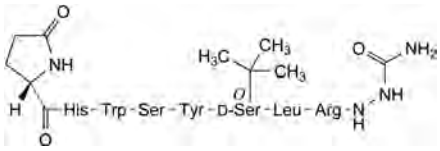

D. des-9-L-proline-goserelin,

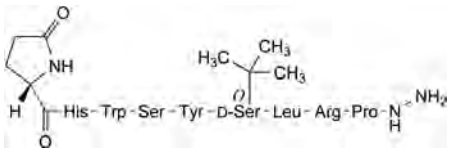

E. goserelin-(1-8)-peptidyl-L-prolinohydrazide,

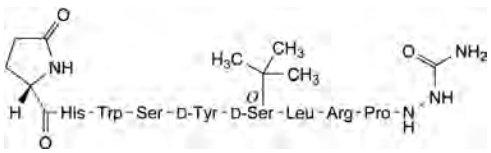

F. [5-D-tyrosine]goserelin,

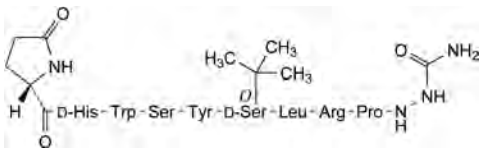

G. [2-D-histidine]goserelin,

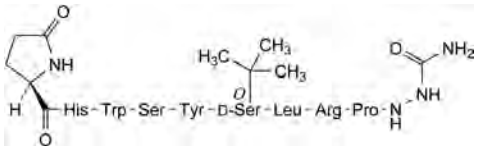

H. [1-(5-oxo-D-proline)]goserelin,

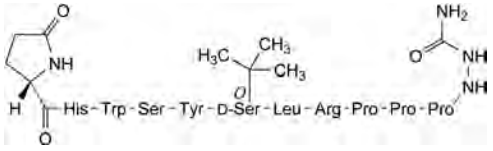

I. endo-8a,8b-di-L-proline-goserelin,

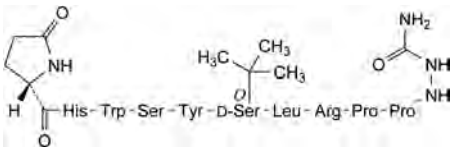

J. endo-8a-L-proline-goserelin,

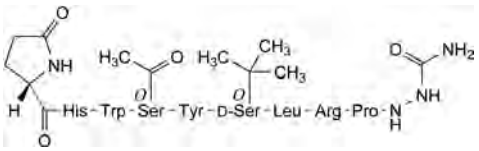

K. [4-(*O*-acetyl-L-serine)]goserelin,

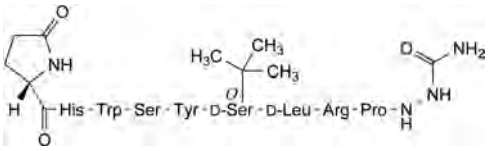

L. [7-D-leucine]goserelin.

**01/2008:0907**
**corrected 10.0**

# GRAMICIDIN

## Gramicidinum

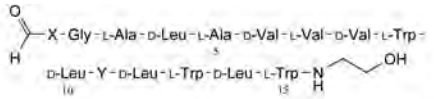

| Gramicidin | X | Y | Mol. formula | $M_r$ |
|---|---|---|---|---|
| A1 | L-Val | L-Trp | $C_{99}H_{140}N_{20}O_{17}$ | 1882 |
| A2 | L-Ile | L-Trp | $C_{100}H_{142}N_{20}O_{17}$ | 1896 |
| B1 | L-Val | L-Phe | $C_{97}H_{139}N_{19}O_{17}$ | 1843 |
| C1 | L-Val | L-Tyr | $C_{97}H_{139}N_{19}O_{18}$ | 1859 |
| C2 | L-Ile | L-Tyr | $C_{98}H_{141}N_{19}O_{18}$ | 1873 |

DEFINITION

Gramicidin consists of a family of antimicrobial linear polypeptides, usually obtained by extraction from tyrothricin, the complex isolated from the fermentation broth of *Brevibacillus brevis* Dubos. The main component is gramicidin A1, together with gramicidins A2, B1, C1 and C2 in particular.

*Content*: minimum 900 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, soluble in methanol, sparingly soluble in alcohol.

mp: about 230 °C.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Dissolve 0.100 g in *alcohol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *alcohol R*. Examined between 240 nm and 320 nm (*2.2.25*), the solution shows 2 absorption maxima,

Monographs G

at 282 nm and 290 nm, a shoulder at about 275 nm and an absorption minimum at 247 nm. The specific absorbance at the maximum at 282 nm is 105 to 125.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in 6.0 mL of *alcohol R*.

*Reference solution (a)*. Dissolve 5 mg of *gramicidin CRS* in 6.0 mL of *alcohol R*.

*Reference solution (b)*. Dissolve 5 mg of *tyrothricin CRS* in 6.0 mL of *alcohol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *butanol R*, *water R*, *glacial acetic acid R*, *butyl acetate R* (3:9:15:24:49 *V/V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: dip the plate into *dimethylaminobenzaldehyde solution R2*. Heat at 90 °C until the spots appear.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots or 2 clearly separated groups of spots.

*Results*: the principal spot or group of principal spots in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot or group of principal spots in the chromatogram obtained with reference solution (a) and to the spot or group of spots with the highest $R_F$ value in the chromatogram obtained with reference solution (b).

C. Examine the chromatograms obtained in the test for composition.

*Results*: the 3 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 3 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *methanol R* and dilute to 25 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25 mg of *gramicidin CRS* in 10 mL of *methanol R* and dilute to 25 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 50 °C.

*Mobile phase*: *water R*, *methanol R* (29:71 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of gramicidin A1.

*Relative retention* with reference to gramicidin A1 (retention time = about 22 min): gramicidin C1 = about 0.7; gramicidin C2 = about 0.8; gramicidin A2 = about 1.2; gramicidin B1 = about 1.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to gramicidin A1 and gramicidin A2,
- the chromatogram obtained is concordant with the chromatogram supplied with *gramicidin CRS*.

*Limits*:

- *sum of gramicidins A1, A2, B1, C1 and C2*: minimum 95.0 per cent,
- *ratio of gramicidin A1 to the sum of gramicidins A1, A2, B1, C1 and C2*: minimum 0.60,
- *disregard limit*: the area of the peak due to gramicidin A1 in the chromatogram obtained with reference solution (b).

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition.

*Limit*:

- *any impurity*: maximum 2.0 per cent and not more than 1 peak is more than 1.0 per cent; disregard the peaks due to gramicidins A1, A2, B1, C1 and C2.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.1 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*), using the turbidimetric method. Use *gramicidin CRS* as the reference substance.

STORAGE

In an airtight container, protected from light.

IMPURITIES

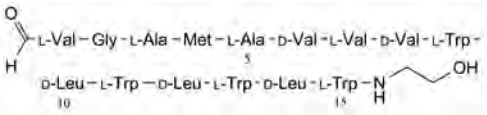


A. [4-methionine]gramicidin A1,

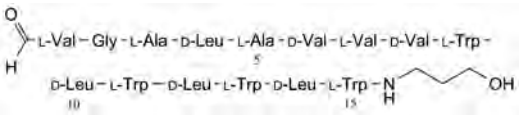


B. gramicidin A1 3-hydroxypropyl,

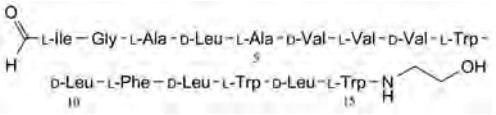


C. gramicidin B2,

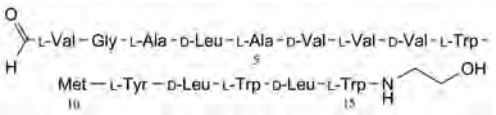


D. [10-methionine]gramicidin C1,

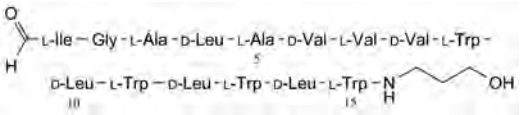


E. gramicidin A2 3-hydroxypropyl.

**01/2019:1695**

# GRANISETRON HYDROCHLORIDE

## Granisetroni hydrochloridum

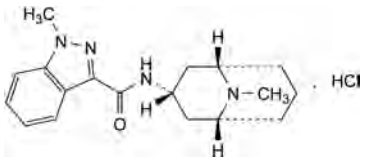

$C_{18}H_{25}ClN_4O$ $M_r$ 348.9
[107007-99-8]

DEFINITION

1-Methyl-*N*-[(1*R*,3*r*,5*S*)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1*H*-indazole-3-carboxamide hydrochloride.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, sparingly soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *granisetron hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.5 for solution S.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *acetonitrile R* (20:80 *V/V*).

*Test solution*. Dissolve 0.25 g of the substance to be examined in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution*. Dissolve 5.0 mg of *granisetron impurity E CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R*, *ethyl acetate R* (6.5:30:50 *V/V/V*).

*Application*: 2 µL.

*Development*: over half of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour for 30 min.

*Limit*:

– *impurity E*: any spot due to impurity E is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Transfer 2 mL of the test solution to a colourless glass vial, stopper and expose the solution either to sunlight for 4 h or place under a UV lamp for 16 h (partial degradation of granisetron to impurity C). A degradation of at least about 0.3 per cent of granisetron to impurity C must be obtained as shown by appearance of a corresponding peak in the chromatogram. If not, expose the solution once again to sunlight or place under a UV lamp.

*Reference solution (c)*. Dissolve 50.0 mg of *granisetron hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve the contents of a vial of *granisetron impurity A CRS* in 1 mL of the mobile phase.

*Reference solution (e)*. Dissolve the contents of a vial of *granisetron impurity B CRS* in 1 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: spherical *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: dilute 1.6 mL of *phosphoric acid R* to 800 mL with *water for chromatography R*, add 200 mL of *acetonitrile R* and mix. Add 1.0 mL of *hexylamine R* and mix. Adjust to pH 7.5 ± 0.05 with freshly distilled *triethylamine R* (about 4 mL).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 305 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b), (d) and (e).

*Run time*: twice the retention time of granisetron.

*Relative retention* with reference to granisetron (retention time = about 7 min): impurity D = about 0.4; impurity B = about 0.5; impurity A = about 0.7; impurity C = about 0.8.

*System suitability*:

– *resolution*: minimum 3.5 between the peaks due to impurity C and granisetron in the chromatogram obtained with reference solution (b);
– *symmetry factor*: maximum 2.0 for the peak due to granisetron in the chromatogram obtained with reference solution (a).

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.7;
– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity C*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *impurity D*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

Monographs G

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{18}H_{25}ClN_4O$ taking into account the assigned content of *granisetron hydrochloride CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G, H, I.*

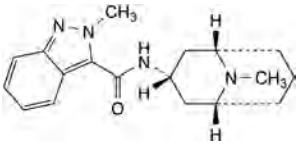

A. 2-methyl-*N*-[(1*R*,3*r*,5*S*)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-2*H*-indazole-3-carboxamide,

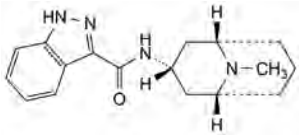

B. *N*-[(1*R*,3*r*,5*S*)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1*H*-indazole-3-carboxamide,

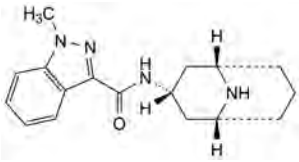

C. *N*-[(1*R*,3*r*,5*S*)-9-azabicyclo[3.3.1]non-3-yl]-1-methyl-1*H*-indazole-3-carboxamide,

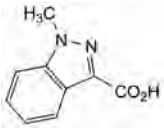

D. 1-methyl-1*H*-indazole-3-carboxylic acid,

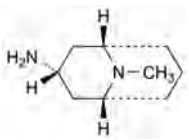

E. (1*R*,3*r*,5*S*)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine,

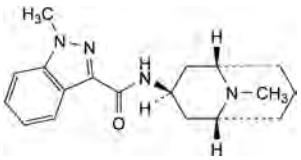

F. 1-methyl-*N*-[(1*R*,3*s*,5*S*)-9-methyl-9-azabicyclo[3.3.1]non-3-yl]-1*H*-indazole-3-carboxamide (*exo*-granisetron),

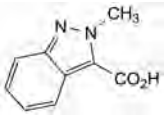

G. 2-methyl-2*H*-indazole-3-carboxylic acid,

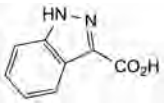

H. 1*H*-indazole-3-carboxylic acid,

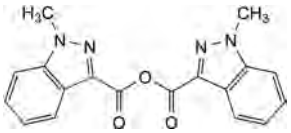

I. 1-methyl-1*H*-indazole-3-carboxylic anhydride.

**04/2019:0182**

# GRISEOFULVIN

## Griseofulvinum

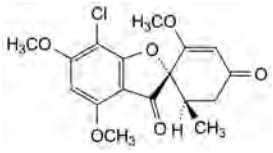

$C_{17}H_{17}ClO_6$ $M_r$ 352.8

[126-07-8]

DEFINITION

(1′*S*,6′*R*)-7-Chloro-2′,4,6-trimethoxy-6′-methyl-3*H*-spiro[1-benzofuran-2,1′-cyclohex[2]ene]-3,4′-dione.

Substance produced by the growth of certain strains of *Penicillium griseofulvum*.

*Content*: 94.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in anhydrous ethanol and in methanol.

It shows polymorphism (*5.9*).

mp: about 220 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *griseofulvin for LC assay and identification CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_4$ (*2.2.2, Method II*).

Dissolve 0.75 g in *dimethylformamide R* and dilute to 10 mL with the same solvent.

**Acidity**. Suspend 0.25 g in 20 mL of *ethanol (96 per cent) R* and add 0.1 mL of *phenolphthalein solution R*. Not more than 1.0 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): + 354 to + 364 (dried substance).

Dissolve 0.250 g in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in mobile phase B and dilute to 50.0 mL with mobile phase B.

*Reference solution (a)*. Dissolve 25.0 mg of *griseofulvin for LC assay and identification CRS* in mobile phase B and dilute to 50.0 mL with mobile phase B.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B.

*Reference solution (c)*. Dissolve 5 mg of *griseofulvin for system suitability CRS* (containing impurities A, B and C) in mobile phase B and dilute to 10 mL with mobile phase B.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of a 0.1 per cent *V/V* solution of *anhydrous formic acid R* previously adjusted to pH 4.5 with *dilute ammonia R2* and 80 volumes of *water for chromatography R*;
- *mobile phase B*: mix 15 volumes of *water for chromatography R*, 20 volumes of a 0.1 per cent *V/V* solution of *anhydrous formic acid R* previously adjusted to pH 4.5 with *dilute ammonia R2* and 65 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 50 | 50 |
| 3 - 13 | 50 → 40 | 50 → 60 |
| 13 - 16 | 40 → 10 | 60 → 90 |
| 16 - 24 | 10 | 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to griseofulvin (retention time = about 16 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 1.1.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to griseofulvin.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity A by 0.6;
- for each impurity, use the concentration of griseofulvin in reference solution (b).

*Limits*:

- *impurity B*: maximum 3.0 per cent;
- *impurity A*: maximum 2.0 per cent;
- *impurity C*: maximum 0.75 per cent;
- *unspecified impurities*: for each impurity, maximum 0.15 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.10 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections.

Calculate the percentage content of $C_{17}H_{17}ClO_6$ taking into account the assigned content of *griseofulvin for LC assay and identification CRS*.

IMPURITIES

*Specified impurities*: *A, B, C*.

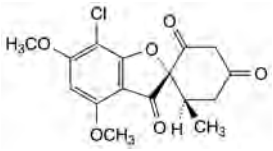

A. (1′*S*,6′*R*)-7-chloro-4,6-dimethoxy-6′-methyl-3*H*-spiro[1-benzofuran-2,1′-cyclohexane]-2′,3,4′-trione,

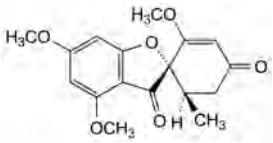

B. (1′*S*,6′*R*)-2′,4,6-trimethoxy-6′-methyl-3*H*-spiro[1-benzofuran-2,1′-cyclohex[2]ene]-3,4′-dione,

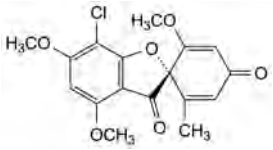

C. (1′*S*)-7-chloro-2′,4,6-trimethoxy-6′-methyl-3*H*-spiro[1-benzofuran-2,1′-cyclohexa[2,5]diene]-3,4′-dione.

**07/2009:1978**
**corrected 10.0**

# GUAIACOL

## Guaiacolum

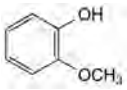

$C_7H_8O_2$ $M_r$ 124.1
[90-05-1]

DEFINITION

2-Methoxyphenol.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: crystalline mass or colourless or yellowish liquid, hygroscopic.

*Solubility*: sparingly soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

mp: about 28 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *guaiacol CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 25 mL with the same solvent.
*Reference solution.* Dissolve 0.5 g of *guaiacol CRS* in *methanol R* and dilute to 25 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *anhydrous acetic acid R*, *methanol R*, *toluene R* (6:14:80 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ferric chloride solution R1*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 1.00 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method I*).

**Acidity or alkalinity**. To 5.0 mL of solution S, add 10 mL of *carbon dioxide-free water R* and 0.1 mL of *methyl red mixed solution R*. Not more than 0.05 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *phosphoric acid R*, *water R*, *methanol R* (1:499:500 *V/V/V*).

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 0.20 g of *pyrocatechol R* (impurity A) and 0.20 g of *phenol R* (impurity B) in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of this solution to 10 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20.0 mg of *guaiacol CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *methanol R*, *water R* (1:150:849 *V/V/V*);
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 28 | 100 | 0 |
| 28 - 30 | 100 → 35 | 0 → 65 |
| 30 - 40 | 35 | 65 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Retention time*: guaiacol = about 20 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to impurities A (1st peak) and B (2nd peak).

*Limit*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 1.00 g of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.20 g of *phenol R* (impurity B) and 0.40 g of *methyl benzoate R* (impurity E) in *acetonitrile R* and dilute to 50 mL with the same solvent. Dilute 1 mL of this solution to 20 mL with *acetonitrile R*.

*Reference solution (b).* Dilute 0.5 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 10 mg of *veratrole R* (impurity C) in *acetonitrile R* and dilute to 10 mL with the same solvent.

*Column*:
- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 5 mL/min.

*Split ratio*: 1:5.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 90 |
| | 15 - 45 | 90 → 180 |
| Injection port | | 200 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to guaiacol (retention time = about 25 min): impurity E = about 0.88; impurity B = about 0.92; impurity C = about 1.1.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurities E (1st peak) and B (2nd peak).

*Limits*:
- *impurity C*: maximum 0.4 per cent;
- *impurity E*: maximum 0.2 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.000 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurity A with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_7H_8O_2$ from the declared content of *guaiacol CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, G, H.*

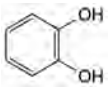

A. benzene-1,2-diol (pyrocatechol),

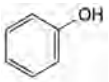

B. phenol,

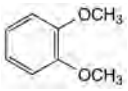

C. 1,2-dimethoxybenzene (veratrole),

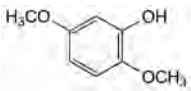

D. 2,5-dimethoxyphenol,

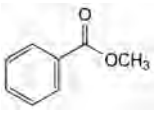

E. methyl benzoate,

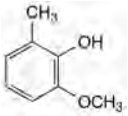

F. 2-methoxy-6-methylphenol (6-methylguaiacol),

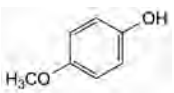

G. 4-methoxyphenol,

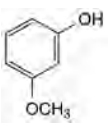

H. 3-methoxyphenol.

**04/2018:0615**
**corrected 9.6**

# GUAIFENESIN

## Guaifenesinum

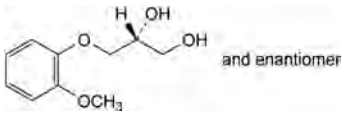


$C_{10}H_{14}O_4$ $M_r$ 198.2
[93-14-1]

DEFINITION

(2*RS*)-3-(2-Methoxyphenoxy)propane-1,2-diol.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 79 °C to 83 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *guaifenesin CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution*. Dissolve 30 mg of *guaifenesin CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *methylene chloride R*, *2-propanol R* (20:80 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with a solution, prepared immediately before use, containing 0.4 g of *dimethylaminobenzaldehyde R* in an ice-cooled mixture of 10 mL of *water R* and 10 mL of *hydrochloric acid R*; allow to dry in air and heat at 105 °C for 10 min; examine under white light.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R*, heating gently if necessary, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *phenolphthalein solution R1*. Not more than 0.1 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator. To 10 mL of solution S add 0.15 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 50.0 mg of *guaiacol R* (impurity A) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 10.0 mL with test solution (a).

*Reference solution (c).* Dissolve 5.0 mg of *guaifenesin for peak identification CRS* (containing impurities B, C and D) in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (d).* Dissolve 25.0 mg of *guaifenesin CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *glacial acetic acid R*, *water for chromatography R* (1:99 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 34 | 80 → 50 | 20 → 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C and D.

*Relative retention* with reference to guaifenesin (retention time = about 8 min): impurity B = about 0.9; impurity A = about 1.4; impurity C = about 3.1; impurity D = about 3.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to guaifenesin and impurity A.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity D by 0.7;
- for each impurity, use the concentration of guaifenesin in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.5 per cent;
- *impurities C, D*: for each impurity, maximum 0.1 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total:* maximum 0.7 per cent;
- *reporting threshold*: 0.03 per cent.

**Chlorides and monochlorhydrins**: maximum 250 ppm.

To 10 mL of solution S add 2 mL of *dilute sodium hydroxide solution R* and heat on a water-bath for 5 min. Cool and add 3 mL of *dilute nitric acid R*. The resulting solution complies with the limit test for chlorides (*2.4.4*).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{10}H_{14}O_4$ taking into account the assigned content of *guaifenesin CRS*.

IMPURITIES

*Specified impurities: B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

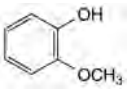

A. 2-methoxyphenol (guaiacol),

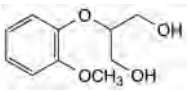

B. 2-(2-methoxyphenoxy)propane-1,3-diol (B-isomer),

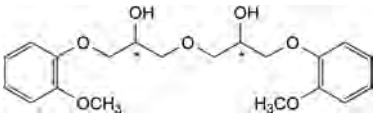

C. 1,1′-oxybis[3-(2-methoxyphenoxy)propan-2-ol] (bisether),

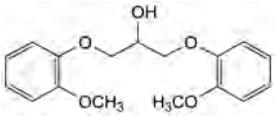

D. 1,3-bis(2-methoxyphenoxy)propan-2-ol.

**01/2017:0027**

# GUANETHIDINE MONOSULFATE

## Guanethidini monosulfas

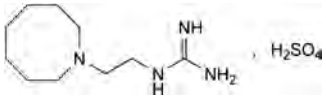

$C_{10}H_{24}N_4O_4S$ $M_r$ 296.4

[645-43-2]

DEFINITION

1-[2-(Hexahydroazocin-1(2*H*)-yl)ethyl]guanidine monosulfate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

mp: about 250 °C, with decomposition.

IDENTIFICATION

A. Dissolve about 25 mg in 25 mL of *water R*, add 20 mL of *picric acid solution R* and filter. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at about 154 °C.

B. Dissolve about 25 mg in 5 mL of *water R*. Add 1 mL of *strong sodium hydroxide solution R*, 1 mL of *α-naphthol solution R* and, dropwise with shaking, 0.5 mL of *strong sodium hypochlorite solution R*. A bright pink precipitate is formed and becomes violet-red on standing.

C. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.4 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.7 to 5.5 for solution S.

**Oxidisable substances**. In a conical, ground-glass-stoppered flask, dissolve 1.0 g in 25 mL of *water R* and add 25 mL of *dilute sodium hydroxide solution R*. Allow to stand for 10 min and add 1 g of *potassium bromide R* and 1 mL of *0.0083 M potassium bromate*. Acidify with 30 mL of *dilute hydrochloric acid R*. Mix and allow to stand in the dark for 5 min. Add 2 g of *potassium iodide R* and shake. Allow to stand for 2 min and titrate the liberated iodine with *0.05 M sodium thiosulfate*, using *starch solution R* as indicator. Not less than 0.3 mL of *0.05 M sodium thiosulfate* is required to decolorise the solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g, warming if necessary, in 30 mL of *anhydrous acetic acid R* and add 15 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.64 mg of $C_{10}H_{24}N_4O_4S$.

STORAGE

Protected from light.

**01/2010:0908**
**corrected 9.0**

# GUAR GALACTOMANNAN

## Guar galactomannanum

DEFINITION

Guar galactomannan is obtained from the seeds of *Cyamopsis tetragonolobus* (L.) Taub. by grinding of the endosperms and subsequent partial hydrolysis. The main components are polysaccharides composed of D-galactose and D-mannose at molar ratios of 1:1.4 to 1:2. The molecules consist of a linear main chain of β-(1→4)-glycosidically linked mannopyranoses and single α-(1→6)-glycosidically linked galactopyranoses.

CHARACTERS

*Appearance*: yellowish-white powder.

*Solubility*: soluble in cold water and in hot water, practically insoluble in organic solvents.

IDENTIFICATION

A. Mix 5 g of solution S (see Tests) with 0.5 mL of a 10 g/L solution of *disodium tetraborate R*. A gel forms within a short time.

B. Heat 20 g of solution S in a water-bath for 10 min. Allow to cool and adjust to the original mass with *water R*. The solution does not gel.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 10 mg of the substance to be examined in a thick-walled centrifuge tube add 2 mL of a 230 g/L solution of *trifluoroacetic acid R*, shake vigorously to dissolve the forming gel, stopper the tube and heat the mixture at 120 °C for 1 h. Centrifuge the hydrolysate, transfer the clear supernatant carefully into a 50 mL flask, add 10 mL of *water R* and evaporate the solution to dryness under reduced pressure. Take up the residue in 10 mL of *water R* and evaporate again to dryness under reduced pressure. To the resulting clear film, which has no odour of acetic acid, add 0.1 mL of *water R* and 1 mL of *methanol R*. Centrifuge to separate the amorphous precipitate. Dilute the supernatant, if necessary, to 1 mL with *methanol R*.

*Reference solution.* Dissolve 10 mg of *galactose R* and 10 mg of *mannose R* in 2 mL of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Application*: 5 µL, as bands of 20 mm by 3 mm.

*Development*: over a path of 15 cm.

*Detection*: spray with *aminohippuric acid reagent R* and heat at 120 °C for 5 min.

*Results*: the chromatogram obtained with the reference solution shows in the lower part 2 clearly separated brownish zones due to galactose and mannose in order of increasing $R_F$ value; the chromatogram obtained with the test solution shows 2 zones due to galactose and mannose.

TESTS

**Solution S**. Moisten 1.0 g with 2 mL of *2-propanol R*. While stirring, dilute to 100 g with *water R* and stir until the substance is uniformly dispersed. Allow to stand for at least 1 h. If the apparent viscosity is below 200 mPa·s, use 3.0 g of substance instead of 1.0 g.

**pH** (*2.2.3*): 5.5 to 7.5 for solution S.

**Apparent viscosity** (*2.2.10*): 75 per cent to 140 per cent of the value stated on the label.

Moisten a quantity of the substance to be examined equivalent to 2.00 g of the dried substance with 2.5 mL of *2-propanol R* and, while stirring, dilute to 100.0 mL with *water R*. After 1 h, determine the viscosity at 20 °C using a rotating viscometer and a shear rate of 100 $s^{-1}$.

**Insoluble matter**: maximum 7.0 per cent.

In a 250 mL flask disperse, while stirring, 1.50 g in a mixture of 1.6 mL of *sulfuric acid R* and 150 mL of *water R* and weigh. Immerse the flask in a water-bath and heat under a reflux condenser for 6 h. Adjust to the original mass with *water R*. Filter the hot solution through a tared, sintered-glass filter (160) (*2.1.2*). Rinse the filter with hot *water R* and dry at 100-105 °C. The residue weighs a maximum of 105 mg.

**Protein**: maximum 5.0 per cent.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.400 g. Multiply the result by 6.25.

**Tragacanth, sterculia gum, agar, alginates and carrageenan**. To a small amount of the substance to be examined add 0.2 mL of freshly prepared *ruthenium red solution R*. Examined under a microscope, none of the structures are red.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Total ash** (*2.4.16*): maximum 1.8 per cent, determined on 1.00 g after wetting with 10 mL of *water R*.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the apparent viscosity in millipascal seconds for a 20 g/L solution.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for guar galactomannan used as viscosity-increasing agent or binder.*

**Apparent viscosity**: see Tests.

# H

Haemodialysis solutions, concentrated, water for diluting.... 2797
Haemodialysis, solutions for.. .... 2798
Haemofiltration and haemodiafiltration, concentrated solutions for.. .... 2801
Haemofiltration and haemodiafiltration, solutions for.. .... 2803
Halofantrine hydrochloride.... 2805
Haloperidol.... 2806
Haloperidol decanoate.. .... 2808
Halothane.. .... 2809
Hard fat.. .... 2811
Hard fat with additives.... 2812
Helium.. .... 2814
Heparin calcium.. .... 2814
Heparin sodium.. .... 2816
Heparins, low-molecular-mass.. .... 2818
Heptaminol hydrochloride.. .... 2821
Hexamidine diisetionate.. .... 2822
Hexetidine.. .... 2823
Hexylresorcinol.. .... 2824
Histamine dihydrochloride.. .... 2825
Histidine.. .... 2826
Histidine hydrochloride monohydrate.. .... 2827
Homatropine hydrobromide.. .... 2829
Homatropine methylbromide.. .... 2830
Honey.. .... 2831
Human albumin solution.. .... 2832
Human anti-D immunoglobulin.. .... 2834
Human anti-D immunoglobulin for intravenous administration.... 2835
Human antithrombin III concentrate.. .... 2835
Human C1-esterase inhibitor.. .... 2837
Human coagulation factor VII.... 2838
Human coagulation factor VIIa (rDNA) concentrated solution.. .... 2840
Human coagulation factor VIII.. .... 2844
Human coagulation factor VIII (rDNA).. .... 2845
Human coagulation factor IX.. .... 2846
Human coagulation factor IX (rDNA) concentrated solution.. .... 2848
Human coagulation factor IX (rDNA) powder for solution for injection.. .... 2853
Human coagulation factor XI.. .... 2855
Human fibrinogen.. .... 2856
Human haematopoietic stem cells.... 2857
Human hepatitis A immunoglobulin.... 2858
Human hepatitis B immunoglobulin.. .... 2858
Human hepatitis B immunoglobulin for intravenous administration.... 2859
Human measles immunoglobulin.. .... 2859
Human normal immunoglobulin for intramuscular administration.... 2860
Human normal immunoglobulin for intravenous administration.... 2862
Human normal immunoglobulin for subcutaneous administration.... 2864
Human plasma for fractionation.. .... 2865
Human plasma (pooled and treated for virus inactivation).. .... 2867
Human α-1-proteinase inhibitor.... 2869
Human prothrombin complex.. .... 2870
Human rabies immunoglobulin.. .... 2871
Human rubella immunoglobulin.. .... 2872
Human tetanus immunoglobulin.. .... 2873
Human varicella immunoglobulin.. .... 2875
Human varicella immunoglobulin for intravenous administration.... 2875
Human von Willebrand factor.. .... 2875
Hyaluronidase .. .... 2877
Hydralazine hydrochloride.... 2878
Hydrochloric acid, concentrated.. .... 2879
Hydrochloric acid, dilute.. .... 2879
Hydrochlorothiazide.. .... 2879
Hydrocodone hydrogen tartrate 2.5-hydrate.. .... 2881
Hydrocortisone.. .... 2883
Hydrocortisone acetate.. .... 2886
Hydrocortisone hydrogen succinate.... 2888
Hydrogen peroxide solution (3 per cent).. .... 2889
Hydrogen peroxide solution (30 per cent).. .... 2890
Hydromorphone hydrochloride.... 2890
Hydroxocobalamin acetate.. .... 2892
Hydroxocobalamin chloride.... 2893
Hydroxocobalamin sulfate.... 2894
Hydroxycarbamide.. .... 2895
Hydroxychloroquine sulfate.. .... 2896
Hydroxyethyl salicylate.. .... 2897
Hydroxyethylcellulose.. .... 2898
Hydroxypropylbetadex.... 2901
Hydroxypropylcellulose.. .... 2903
Hydroxypropylcellulose, low-substituted.. .... 2904
Hydroxyzine hydrochloride.... 2906
Hymecromone.... 2907
Hymenoptera venoms for allergen products.... 2908
Hyoscine.. .... 2909
Hyoscine butylbromide.... 2910
Hyoscine hydrobromide.. .... 2911
Hyoscyamine sulfate.... 2913
Hypromellose.. .... 2914
Hypromellose phthalate.... 2916

**01/2009:1167**
**corrected 7.0**

# HAEMODIALYSIS SOLUTIONS, CONCENTRATED, WATER FOR DILUTING

## Aqua ad dilutionem solutionum concentratarum ad haemodialysim

*The following monograph is given for information.*

*The analytical methods described and the limits proposed are intended to be used for validating the procedure for obtaining the water.*

DEFINITION

Water for diluting concentrated haemodialysis solutions is obtained from potable water by distillation, by reverse osmosis, by ion exchange or by any other suitable method. The conditions of preparation, transfer and storage are designed to minimise the risk of chemical and microbial contamination.

When water obtained by one of the methods described above is not available, potable water may be used for home dialysis. Because the chemical composition of potable water varies considerably from one locality to another, consideration must be given to its chemical composition to enable adjustments to be made to the content of ions so that the concentrations in the diluted solution correspond to the intended use.

Attention has also to be paid to the possible presence of residues from water treatment (for example, chloramines) and volatile halogenated hydrocarbons.

For the surveillance of the quality of water for diluting concentrated haemodialysis solutions, the following methods may be used to determine the chemical composition and/or to detect the presence of possible contaminants together with suggested limits to be obtained.

CHARACTERS

Clear, colourless, liquid.

TESTS

**Acidity or alkalinity**. To 10 mL of the water to be examined, freshly boiled and cooled in a borosilicate glass flask, add 0.05 mL of *methyl red solution R*. The solution is not red. To 10 mL of the water to be examined add 0.1 mL of *bromothymol blue solution R1*. The solution is not blue.

**Oxidisable substances**. To 100 mL of the water to be examined add 10 mL of *dilute sulfuric acid R* and 0.1 mL of *0.02 M potassium permanganate* and boil for 5 min. The solution remains faintly pink.

**Total available chlorine**: maximum 0.1 ppm.

In a 125 mL test-tube (A), place successively 5 mL of *buffer solution pH 6.5 R*, 5 mL of *diethylphenylenediamine sulfate solution R* and 1 g of *potassium iodide R*. In a second 125 mL test-tube (B), place successively 5 mL of *buffer solution pH 6.5 R* and 5 mL of *diethylphenylenediamine sulfate solution R*. Add as simultaneously as possible to tube A 100 mL of the water to be examined and to tube B a reference solution prepared as follows: to 1 mL of a 10 mg/L solution of *potassium iodate R*, add 1 g of *potassium iodide R* and 1 mL of *dilute sulfuric acid R*; allow to stand for 1 min, add 1 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*. Any colour in the mixture obtained with the water to be examined is not more intense than that in the mixture obtained with the reference solution.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 1 mL of the water to be examined to 15 mL with *water R*.

**Fluorides**: maximum 0.2 ppm.

Potentiometry (*2.2.36, Method I*): use as indicator electrode a fluoride-selective solid-membrane electrode and as reference electrode a silver-silver chloride electrode.

*Test solution*. The water to be examined.

*Reference solutions*. Dilute 2.0 mL, 4.0 mL and 10.0 mL of *fluoride standard solution (1 ppm F) R* respectively to 20.0 mL with *total-ionic-strength-adjustment buffer R1*.

Carry out the measurement of each solution.

**Nitrates**: maximum 2 ppm.

Dilute 2 mL of the water to be examined to 100 mL with *nitrate-free water R*. Place 5 mL of the dilution in a test-tube immersed in iced water, add 0.4 mL of a 100 g/L solution of *potassium chloride R* and 0.1 mL of *diphenylamine solution R* and then, dropwise and with shaking, 5 mL of *sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. Allow to stand for 15 min. Any blue colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 0.1 mL of *nitrate standard solution (2 ppm $NO_3$) R* and 4.9 mL of *nitrate-free water R*.

**Sulfates** (*2.4.13*): maximum 50 ppm.

Dilute 3 mL of the water to be examined to 15 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 10 µg/L.

*Prescribed solution*. To 400 mL of the water to be examined add 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Ammonium**: maximum 0.2 ppm.

To 20 mL of the water to be examined in a flat-bottomed and transparent tube, add 1 mL of *alkaline potassium tetraiodomercurate solution R*. Allow to stand for 5 min. The solution is not more intensely coloured than a standard prepared at the same time and in the same manner using a mixture of 4 mL of *ammonium standard solution (1 ppm $NH_4$) R* and 16 mL of *ammonium-free water R*. Examine the solutions along the vertical axis of the tube.

**Calcium**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. The water to be examined.

*Reference solutions*. Prepare reference solutions (1 ppm to 5 ppm) using *calcium standard solution (400 ppm Ca) R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: oxidising air-acetylene flame.

**Magnesium**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dilute 10 mL of the water to be examined to 100 mL with *distilled water R*.

*Reference solutions*. Prepare reference solutions (0.1 ppm to 0.5 ppm) using *magnesium standard solution (100 ppm Mg) R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: oxidising air-acetylene flame.

**Mercury**: maximum 0.001 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Add 5 mL of *nitric acid R* per litre of the water to be examined. In a 50 mL borosilicate glass flask with a ground-glass-stopper, place 20 mL of the water to be examined and add 1 mL of *dilute nitric acid R* and shake. Add 0.3 mL of *bromine water R1*. Stopper the flask, shake and heat the stoppered flask at 45 °C for 4 h. Allow to cool. If the solution

does not become yellow, add 0.3 mL of *bromine water R1* and re-heat at 45 °C for 4 h. Add 0.5 mL of a freshly prepared 10 g/L solution of *hydroxylamine hydrochloride R*. Shake. Allow to stand for 20 min.

*Reference solutions.* Use freshly prepared reference solutions (0.0005 ppm to 0.002 ppm) obtained by diluting *mercury standard solution (1000 ppm Hg) R* with a 5 per cent *V/V* solution of *dilute nitric acid R* and treat as described for the test solution.

To a volume of solution suitable for the instrument to be used, add *stannous chloride solution R2* equal to 1/5 of this volume. Fit immediately the device for the entrainment of the mercury vapour. Wait 20 s and pass through the device a stream of *nitrogen R* as the carrier gas.

*Source*: mercury hollow-cathode tube or a discharge lamp.

*Wavelength*: 253.7 nm.

*Atomisation device*: flameless system whereby the mercury can be entrained in the form of cold vapour.

**Potassium**: maximum 2 ppm.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution (a).* Dilute 50.0 mL of the water to be examined to 100 mL with *distilled water R*. Carry out a determination using this solution. If the potassium content is more than 0.75 mg/L, further dilute the water to be examined with *distilled water R*.

*Test solution (b).* Take 50.0 mL of the water to be examined or, if necessary, the water to be examined diluted as described in the preparation of test solution (a). Add 1.25 mL of *potassium standard solution (20 ppm K) R* and dilute to 100.0 mL with *distilled water R*.

*Reference solutions.* Prepare reference solutions (0 ppm; 0.25 ppm; 0.50 ppm; 0.75 ppm; 1 ppm) using *potassium standard solution (20 ppm K) R*.

*Wavelength*: 766.5 nm.

Calculate the potassium content of the water to be examined in parts per million from the expression:

$$\frac{p \times n_1 \times 0.5}{n_2 - n_1}$$

$p$ = dilution factor used for the preparation of test solution (a);

$n_1$ = measured value of test solution (a);

$n_2$ = measured value of test solution (b).

**Sodium**: maximum 50 ppm.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* The water to be examined. If the sodium content is more than 10 mg/L, dilute with *distilled water R* to obtain a concentration suitable for the apparatus used.

*Reference solutions.* Prepare reference solutions (0 ppm; 2.5 ppm; 5.0 ppm; 7.5 ppm; 10 ppm) using *sodium standard solution (200 ppm Na) R*.

*Wavelength*: 589 nm.

**Zinc**: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*): use sampling and analytical equipment free from zinc or not liable to yield zinc under the conditions of use.

*Test solution.* The water to be examined.

*Reference solutions.* Prepare reference solutions (0.05 ppm to 0.15 ppm) using *zinc standard solution (100 ppm Zn) R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: oxidising air-acetylene flame.

**Heavy metals** (*2.4.8*): maximum 0.1 ppm.

Heat 200 mL of the water to be examined in a glass evaporating dish on a water-bath until the volume is reduced to 20 mL. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mL.

**01/2019:0128**

# HAEMODIALYSIS, SOLUTIONS FOR

## Solutiones ad haemodialysem

DEFINITION

Solutions of electrolytes with a concentration close to the electrolytic composition of plasma. Glucose may be included in the formulation.

Because of the large volumes used, haemodialysis solutions are usually prepared by diluting a concentrated solution with water of suitable quality (see the monograph *Haemodialysis solutions, concentrated, water for diluting (1167)*), using for example an automatic dosing device.

## Concentrated solutions for haemodialysis

Concentrated haemodialysis solutions are prepared and stored using materials and methods designed to produce solutions having as low a degree of microbial contamination as possible. In certain circumstances, it may be necessary to use sterile solutions.

During dilution and use, precautions are taken to avoid microbial contamination. Diluted solutions are to be used immediately after preparation.

Concentrated solutions for haemodialysis are supplied in:

- rigid, semi-rigid or flexible plastic containers;
- glass containers.

3 types of concentrated solutions are used:

**1. Concentrated solutions with acetate or lactate**

Several formulations of concentrated solutions are used. The concentrations of the components in the solutions are such that, after dilution to the stated volume, the concentrations of the components per litre are usually in the following ranges (see Table 0128.-1):

Table 0128.-1.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 130 - 145 | 130 - 145 |
| Potassium | 0 - 3.0 | 0 - 3.0 |
| Calcium | 0 - 2.0 | 0 - 4.0 |
| Magnesium | 0 - 1.2 | 0 - 2.4 |
| Acetate or lactate | 32 - 45 | 32 - 45 |
| Chloride | 90 - 120 | 90 - 120 |
| Glucose | 0 - 12.0 | |

Concentrated solutions with acetate or lactate are diluted before use.

**2. Concentrated acid solutions**

Several formulations of concentrated solutions are used. The concentrations of the components in the solutions are such that, after dilution to the stated volume and before neutralisation with sodium hydrogen carbonate, the

concentrations of the components per litre are usually in the following ranges (see Table 0128.-2):

Table 0128.-2.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 80 - 110 | 80 - 110 |
| Potassium | 0 - 3.0 | 0 - 3.0 |
| Calcium | 0 - 2.0 | 0 - 4.0 |
| Magnesium | 0 - 1.2 | 0 - 2.4 |
| Acetic acid | 2.5 - 10 | 2.5 - 10 |
| Chloride | 90 - 120 | 90 - 120 |
| Glucose | 0 - 12.0 | |

Sodium hydrogen carbonate must be added immediately before use to a final concentration of not more than 45 mmol/L. The concentrated solution of sodium hydrogen carbonate is supplied in a separate container. The concentrated acid solutions and the concentrated solutions of sodium hydrogen carbonate are diluted and mixed immediately before use using a suitable device. Alternatively, sodium hydrogen carbonate in powder form may be used to prepare the solution.

**3. Concentrated solutions without buffer**

Several formulations of concentrated solutions without buffer are used. The concentrations of the components in the solutions are such that, after dilution to the stated volume, the concentrations of the components per litre are usually in the following ranges (see Table 0128.-3):

Table 0128.-3.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 130 - 145 | 130 - 145 |
| Potassium | 0 - 3.0 | 0 - 3.0 |
| Calcium | 0 - 2.0 | 0 - 4.0 |
| Magnesium | 0 - 1.2 | 0 - 2.4 |
| Chloride | 130 - 155 | 130 - 155 |
| Glucose | 0 - 12.0 | |

Concentrated solutions without buffer are used together with parenteral administration of suitable hydrogen carbonate solutions.

*The following tests are carried out on the diluted, ready-to-use solutions.*

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (*2.3.1*):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- lactates;
- carbonates and hydrogen carbonates;
- acetates:
  - if the solution is free from glucose, use reaction (b);
  - if the solution contains glucose, use the following method: to 5 mL of the solution to be examined add 1 mL of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- magnesium: to 0.1 mL of *titan yellow solution R* add 10 mL of *water R*, 2 mL of the solution to be examined and 1 mL of a 4.2 g/L solution of *sodium hydroxide R*; a pink colour is produced;
- glucose: to 5 mL of the solution to be examined add 2 mL of *dilute sodium hydroxide solution R* and 0.05 mL of *copper sulfate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

**Appearance of solution**. The solution to be examined is clear (*2.2.1*). If it does not contain glucose, it is colourless (*2.2.2, Method I*). If it contains glucose, it is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**Aluminium**: maximum 10 µg/L.

Atomic absorption spectrometry (*2.2.23, Method I or II*). Use a matrix modifier (for example, *nitric acid R* and *magnesium nitrate R* in *water R*) in the same quantity for the test solution, the reference solutions and the blank solution.

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions. Method I – direct calibration.*

Prepare the reference solutions by diluting, for example *aluminium standard solution (10 ppm Al) R* with acidified *water R*.

*Reference solutions. Method II – standard additions.*

Prepare at least 3 reference solutions in the test solution, in a range spanning the expected aluminium concentration of the test solution, for example by diluting *aluminium standard solution (10 ppm Al) R* with the test solution.

*Blank solution*: *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: graphite furnace.

**Extractable volume** (*2.9.17*). The volume measured is not less than the nominal volume stated on the label.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/mL (*2.6.12*).

**Sterility** (*2.6.1*). If the label states that the concentrated haemodialysis solution is sterile, it complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mL in the solution diluted for use.

**Pyrogens** (*2.6.8*). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Dilute the solution to be examined with *water for injections R* to the concentration prescribed for use. Inject 10 mL of the solution per kilogram of the rabbit's mass.

ASSAY

*Determine the density (2.2.5) of the concentrated solution and calculate the content in grams per litre and in millimoles per litre.*

**Sodium**: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*.

*Wavelengths*: 589.0 nm or 589.6 nm (sodium emits as a doublet).

**Potassium**: 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute with *water R* an accurately weighed quantity of the solution to be examined to a concentration suitable for the instrument to be used. To 100 mL of this solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Reference solutions.* Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 mL of each reference solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Source*: potassium hollow-cathode lamp.

*Wavelength*: 766.5 nm.

*Atomisation device*: air-acetylene flame.

**Calcium**: 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 5.0 mL of the solution to be examined to 100.0 mL with *water R*. To 3.0 mL of this solution add 5 mL of *lanthanum chloride solution R* and dilute to 50.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks each containing 5 mL of *lanthanum chloride solution R*, introduce respectively 2.5 mL, 5.0 mL, 7.0 mL and 10.0 mL of *calcium standard solution (10 ppm Ca) R* and dilute to 50.0 mL with *water R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: air-acetylene flame.

**Magnesium**: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 5.0 mL of the solution to be examined to 100.0 mL with *water R*. To 2.0 mL of this solution add 5 mL of *lanthanum chloride solution R* and dilute to 50.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks each containing 5 mL of *lanthanum chloride solution R*, introduce respectively 1.0 mL, 2.0 mL, 3.0 mL and 4.0 mL of *magnesium standard solution (10 ppm Mg) R* and dilute to 50.0 mL with *water R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-acetylene flame.

**Total chloride**: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label.

Dilute an accurately measured volume of the solution to be examined containing the equivalent of about 0.68 mEq of chloride with an appropriate volume of *water R* in order to immerse the electrode. Carry out a potentiometric titration (*2.2.20*), using *0.1 M silver nitrate*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Acetate**: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of acetate.

**Lactate**: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 mL of *0.1 M hydrochloric acid*. Then add 50 mL of *acetonitrile R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of lactate.

**Sodium hydrogen carbonate**: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label.

Titrate with *0.1 M hydrochloric acid* a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 8.40 mg of $NaHCO_3$.

**Reducing sugars** (expressed as glucose): 95.0 per cent to 105.0 per cent of the content of glucose stated on the label.

Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 mL conical flask with a ground-glass neck and add 25.0 mL of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and maintain boiling for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Carefully add, in small amounts, 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 mL of *water R*.

Calculate the content of reducing sugars, expressed as glucose ($C_6H_{12}O_6$), using Table 0128.-4.

Table 0128.-4.

| Volume of *0.1 M sodium thiosulfate* (mL) | Glucose (mg) |
|---|---|
| 8 | 19.8 |
| 9 | 22.4 |
| 10 | 25.0 |
| 11 | 27.6 |
| 12 | 30.3 |
| 13 | 33.0 |
| 14 | 35.7 |
| 15 | 38.5 |
| 16 | 41.3 |

STORAGE

At 4 °C or above.

LABELLING

The label states:

- the formula of the concentrated solution for haemodialysis expressed in grams per litre and in millimoles per litre;
- the nominal volume of the solution in the container;
- where applicable, that the concentrated solution is sterile;
- the storage conditions;
- that the concentrated solution is to be diluted immediately before use;
- the dilution to be made;
- that the volume taken for use is to be measured accurately;
- the ionic formula for the diluted solution ready for use in millimoles per litre;
- that any unused portion of solution is to be discarded;
- where applicable, that sodium hydrogen carbonate is to be added before use;
- that the solution is not to be used for intraveneous infusion.

**01/2019:2770**

# HAEMOFILTRATION AND HAEMODIAFILTRATION, CONCENTRATED SOLUTIONS FOR

## Solutiones concentratae ad haemocolaturam haemodiacolaturamque

DEFINITION

Concentrated solutions containing electrolytes that are diluted to the required concentration prior to administration. Dilutions are performed with specialist equipment using an automatic dosing device equipped with ultrafilters. The final solution is used for parenteral administration and contains electrolytes with a concentration close to the electrolytic composition of plasma. Glucose may be included in the formulation.

Concentrated solutions for haemofiltration and haemodiafiltration are supplied in rigid, semi-rigid or flexible plastic containers.

The containers and closures comply with the requirements for containers for preparations for parenteral administration (*3.2*).

Several formulations of concentrated solutions are used and the concentrations of the components per litre of solution are typically in the following ranges (see Table 2770.-1):

Table 2770.-1.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 0 - 4900 | 0 - 4900 |
| Potassium | 0 - 400 | 0 - 400 |
| Calcium | 35 - 300 | 70 - 600 |
| Magnesium | 8.75 - 100 | 17.5 - 200 |
| Chloride | 325 - 5180 | 325 - 5180 |
| Glucose | 0 - 1100 | - |
| Acetate | 0 - 600 | 0 - 600 |
| Citrate | 0 - 45 | 0 - 135 |

The concentrated solution is diluted according to the manufacturer's instructions.

The concentrations of components in the solutions are such that, after dilution to the stated volume and before neutralisation with sodium hydrogen carbonate, the concentrations of the components per litre are compliant with the monograph *Haemofiltration and haemodiafiltration, solutions for (0861)*.

When hydrogen carbonate is used or is required to be present, sodium hydrogen carbonate must be added immediately before use in accordance with the manufacturer's instructions.

Antioxidants are not added to the solutions.

IDENTIFICATION

According to the stated composition, and after dilution where applicable, the solution to be examined gives the following identification reactions (*2.3.1*):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- acetates:
  - if the solution is free from glucose, use reaction (b);
  - if the solution contains glucose, use the following method: to 5 mL of the solution to be examined add 1 mL of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- citrates;
- carbonates and hydrogen carbonates;
- magnesium: to 0.1 mL of *titan yellow solution R* add 10 mL of *water R*, 2 mL of the solution to be examined and 1 mL of *1 M sodium hydroxide*; a pink colour is produced;
- glucose: to 5 mL of the solution to be examined, add 2 mL of *dilute sodium hydroxide solution R* and 0.05 mL of *copper sulfate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

**Appearance of solution**. The solution to be examined is clear (*2.2.1*). If it does not contain glucose, it is colourless (*2.2.2, Method I*). If it contains glucose, dilute the solution to be examined with *water R* to the concentration prescribed for use. The diluted solution is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 5.0 to 7.5. If the solution to be examined contains glucose, the pH is 4.5 to 6.5.

**Hydroxymethylfurfural**. Carry out the test only if glucose is added to the preparation. To a volume of the solution to be examined containing the equivalent of 25 mg of glucose, add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R*, then add 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*), determined at 550 nm after allowing the mixture to stand for 2-3 min, is not greater than that of a standard prepared at the same time and in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the solution to be examined (400 ppm expressed with reference to the glucose concentration). If the solution contains hydrogen carbonate, use as the standard a solution containing 20 µg of *hydroxymethylfurfural R* (800 ppm expressed with reference to the glucose concentration).

**Aluminium**: maximum 10 µg/L.

Atomic absorption spectrometry (*2.2.23, Method I or II*). Use a matrix modifier (for example, *nitric acid R* and *magnesium nitrate R* in *water R*) in the same quantity for the test solution, the reference solutions and the blank solution.

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions. Method I – direct calibration*.

Prepare the reference solutions by diluting, for example *aluminium standard solution (10 ppm Al) R* with acidified *water R*.

*Reference solutions. Method II – standard additions*.

Prepare at least 3 reference solutions in the test solution, in a range spanning the expected aluminium concentration of the test solution, for example by diluting *aluminium standard solution (10 ppm Al) R* with the test solution.

*Blank solution*: *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: graphite furnace.

**Particulate contamination** (*2.9.19, Method I*). Dilute the solution to be examined with *particle-free water R* to the concentration prescribed for use. Use 50 mL of the diluted solution to be examined.

**Extractable volume** (*2.9.17*). The solution complies with the test prescribed for parenteral infusions.

**Sterility** (*2.6.1*). If the label states that the concentrated solution for haemofiltration or haemodiafiltration is sterile, it complies with the test for sterility.

ASSAY

**Sodium**: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*.

*Wavelengths*: 589.0 nm or 589.6 nm (sodium emits as a doublet).

**Potassium**: 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used. To 100 mL of the solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Reference solutions*. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 mL of each reference solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Source*: potassium hollow-cathode lamp.

*Wavelength*: 766.5 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Calcium**: 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Magnesium**: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *magnesium standard solution (100 ppm Mg) R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Total chloride**: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label.

Dilute an accurately measured volume of the solution to be examined containing the equivalent of about 0.68 mEq of chloride with an appropriate volume of *water R* to immerse the electrode. Carry out a potentiometric titration (*2.2.20*), using *0.1 M silver nitrate*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Acetate**: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of acetate.

**Citrate**: 95.0 per cent to 105.0 per cent of the content of citrate stated on the label.

*Test solution*. Use the preparation to be examined, diluted if necessary.

Using 0.2 mL of *phenolphthalein solution R* as indicator, titrate 10 mL of the test solution with *0.1 M sodium hydroxide* until the colour changes from colourless to pink.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.33 mmol of citrate.

**Reducing sugars** (expressed as glucose): 95.0 per cent to 105.0 per cent of the content of glucose stated on the label.

Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 mL conical flask with a ground-glass neck and add 25.0 mL of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Carefully add, in small amounts, 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 mL of *water R*.

Calculate the content of reducing sugars expressed as glucose ($C_6H_{12}O_6$), using Table 2770.-2.

Table 2770.-2.

| Volume of *0.1 M sodium thiosulfate* (mL) | Glucose (mg) |
|---|---|
| 8 | 19.8 |
| 9 | 22.4 |
| 10 | 25.0 |
| 11 | 27.6 |
| 12 | 30.3 |
| 13 | 33.0 |
| 14 | 35.7 |
| 15 | 38.5 |
| 16 | 41.3 |

STORAGE

At 4 °C or above.

LABELLING

The label states:

- the formula of the concentrated solution for haemofiltration or haemodiafiltration, expressed in grams per litre and in millimoles per litre;
- the nominal volume of the concentrated solution for haemofiltration or haemodiafiltration in the container;
- where applicable, that the concentrated solution is sterile;
- that the concentrated solution is to be diluted immediately before use;
- that the volume taken for use is to be measured accurately;
- the dilution to be made;
- the ionic formula for the diluted solution ready for use in millimoles per litre;
- the calculated osmolarity, expressed in milliosmoles per litre;
- the storage conditions;
- that any unused portion of solution is to be discarded;

– where applicable, that sodium hydrogen carbonate is to be added before use.

**01/2019:0861**

# HAEMOFILTRATION AND HAEMODIAFILTRATION, SOLUTIONS FOR

## Solutiones ad haemocolaturam haemodiacolaturamque

DEFINITION

Preparations for parenteral administration containing electrolytes with a concentration close to the electrolytic composition of plasma. Glucose may be included in the formulation.

Solutions for haemofiltration and haemodiafiltration are supplied in:

– rigid or semi-rigid plastic containers;
– flexible plastic containers inside closed protective envelopes;
– glass containers.

The containers and closures comply with the requirements for containers for preparations for parenteral administration (*3.2*).

In haemofiltration and haemodiafiltration, the following formulations are used. The concentrations of the components per litre of solution are usually in the following range (see Table 0861.-1):

Table 0861.-1.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 125 - 150 | 125 - 150 |
| Potassium | 0 - 4.5 | 0 - 4.5 |
| Calcium | 1.0 - 2.5 | 2.0 - 5.0 |
| Magnesium | 0.25 - 1.5 | 0.50 - 3.0 |
| Acetate and/or lactate and/or hydrogen carbonate | 30 - 60 | 30 - 60 |
| Chloride | 90 - 120 | 90 - 120 |
| Glucose | 0 - 25 | |

When hydrogen carbonate is present, the solution of sodium hydrogen carbonate is supplied in a container or a separate compartment and is added to the electrolyte solution immediately before use.

In haemofiltration and haemodiafiltration, the following formulations may also be used (see Table 0861.-2):

Table 0861.-2.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 130 - 167 | 130 - 167 |
| Potassium | 0 - 4.0 | 0 - 4.0 |
| Hydrogen carbonate | 20 - 167 | 20 - 167 |
| Chloride | 0 - 147 | 0 - 147 |

Antioxidants are not added to the solutions.

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (*2.3.1*):

– potassium: reaction (b);
– calcium: reaction (a);
– sodium: reaction (b);
– chlorides: reaction (a);
– acetates:
  – if the solution is free from glucose, use reaction (b);
  – if the solution contains glucose, use the following method: to 5 mL of the solution to be examined add 1 mL of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
– lactates;
– carbonates and hydrogen carbonates;
– magnesium: to 0.1 mL of *titan yellow solution R* add 10 mL of *water R*, 2 mL of the solution to be examined and 1 mL of *1 M sodium hydroxide*; a pink colour is produced;
– glucose: to 5 mL of the solution to be examined, add 2 mL of *dilute sodium hydroxide solution R* and 0.05 mL of *copper sulfate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*). If it does not contain glucose, it is colourless (*2.2.2, Method I*). If it contains glucose, it is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 5.0 to 7.5. If the solution contains glucose, the pH is 4.5 to 6.5. If the solution contains hydrogen carbonate, the pH is 7.0 to 8.5.

**Hydroxymethylfurfural**. Carry out the test only if glucose is added to the preparation. To a volume of the solution to be examined containing the equivalent of 25 mg of glucose, add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R*, then add 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*), determined at 550 nm after allowing the mixture to stand for 2-3 min, is not greater than that of a standard prepared at the same time and in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the solution to be examined (400 ppm expressed with reference to the glucose concentration). If the solution contains hydrogen carbonate, use as the standard a solution containing 20 µg of *hydroxymethylfurfural R* (800 ppm expressed with reference to the glucose concentration).

**Aluminium**: maximum 10 µg/L.

Atomic absorption spectrometry (*2.2.23, Method I or II*). Use a matrix modifier (for example, *nitric acid R* and *magnesium nitrate R* in *water R*) in the same quantity for the test solution, the reference solutions and the blank solution.

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions. Method I – direct calibration.*

Prepare the reference solutions by diluting, for example *aluminium standard solution (10 ppm Al) R* with acidified *water R*.

*Reference solutions. Method II – standard additions.*

Prepare at least 3 reference solutions in the test solution, in a range spanning the expected aluminium concentration of the test solution, for example by diluting *aluminium standard solution (10 ppm Al) R* with the test solution.

*Blank solution*: *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: graphite furnace.

**Particulate contamination** (*2.9.19, Method I*). Use 50 mL of the solution to be examined.

**Extractable volume** (*2.9.17*). The solution complies with the test prescribed for parenteral infusions.

**Sterility** (*2.6.1*). The solution complies with the test.

**Bacterial endotoxins** (*2.6.14*): less than 0.05 IU/mL.

**Pyrogens** (*2.6.8*). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the solution.

ASSAY

**Sodium**: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*.

*Wavelengths*: 589.0 nm or 589.6 nm (sodium emits as a doublet).

**Potassium**: 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used. To 100 mL of the solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Reference solutions*. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 mL of each reference solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Source*: potassium hollow-cathode lamp.

*Wavelength*: 766.5 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Calcium**: 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Magnesium**: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions*. Prepare the reference solutions using *magnesium standard solution (100 ppm Mg) R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Total chloride**: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label.

Dilute an accurately measured volume of the solution to be examined containing the equivalent of about 0.68 mEq of chloride with an appropriate volume of *water R* to immerse the electrode. Carry out a potentiometric titration (*2.2.20*), using *0.1 M silver nitrate*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Acetate**: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of acetate.

**Lactate**: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 mL of *0.1 M hydrochloric acid*. Add 50 mL of *acetonitrile R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of lactate.

**Sodium hydrogen carbonate**: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label.

Titrate with *0.1 M hydrochloric acid*, a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 8.40 mg of $NaHCO_3$.

**Lactate and hydrogen carbonate**: 95.0 per cent to 105.0 per cent of the content of lactates and hydrogen carbonates stated on the label.

Liquid chromatography (*2.2.29*).

*Test solution*. The solution to be examined.

*Reference solution*. Dissolve in 100 mL of *water for chromatography R* quantities of lactates and hydrogen carbonates, accurately weighed, in order to obtain solutions having concentrations representing about 90 per cent, 100 per cent and 110 per cent of the concentrations stated on the label.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *cation-exchange resin R* (9 µm);
- *temperature*: 60 °C.

*Mobile phase*: *0.005 M sulfuric acid* previously degassed with *helium for chromatography R*.

*Flow rate*: 0.6 mL/min.

*Detection*: differential refractometer.

*Injection*: 20 µL, twice.

*Order of elution*: lactates, hydrogen carbonates.

Determine the concentration of lactates and hydrogen carbonates in the test solution by interpolating the peak area for lactate and the peak height for hydrogen carbonate from the linear regression curve obtained with the reference solutions.

**Reducing sugars** (expressed as glucose): 95.0 per cent to 105.0 per cent of the content of glucose stated on the label.

Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 mL conical flask with a ground-glass neck and add 25.0 mL of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Carefully add, in small amounts, 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 mL of *water R*.

Calculate the content of reducing sugars expressed as glucose ($C_6H_{12}O_6$), using Table 0861.-3.

Table 0861.-3.

| Volume of *0.1 M sodium thiosulfate* (mL) | Glucose (mg) |
|---|---|
| 8 | 19.8 |
| 9 | 22.4 |
| 10 | 25.0 |
| 11 | 27.6 |
| 12 | 30.3 |
| 13 | 33.0 |
| 14 | 35.7 |
| 15 | 38.5 |
| 16 | 41.3 |

STORAGE

At 4 °C or above.

LABELLING

The label states:

- the formula of the solution for haemofiltration or haemodiafiltration, expressed in grams per litre and in millimoles per litre;
- the calculated osmolarity, expressed in milliosmoles per litre;
- the nominal volume of the solution for haemofiltration or haemodiafiltration in the container;
- that the solution is free from bacterial endotoxins, or where applicable, that it is apyrogenic;
- the storage conditions;
- that any unused portion of solution is to be discarded.

**01/2017:1979**

# HALOFANTRINE HYDROCHLORIDE

## Halofantrini hydrochloridum

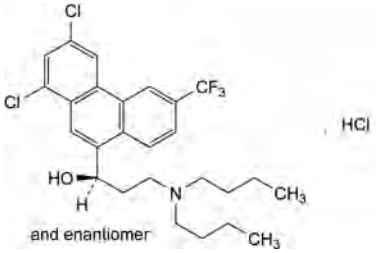


$C_{26}H_{31}Cl_3F_3NO$ $M_r$ 536.9

[36167-63-2]

DEFINITION

(1*RS*)-3-(Dibutylamino)-1-[1,3-dichloro-6-(trifluoromethyl)-phenanthren-9-yl]propan-1-ol hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol, sparingly soluble in alcohol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *halofantrine hydrochloride CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methyl ethyl ketone R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 1.00 g in *alcohol R* and dilute to 100.0 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.085 at 450 nm.

Dissolve 0.200 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 40.0 mg of *halofantrine hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 10.0 mg of *halofantrine impurity C CRS* in the mobile phase and dilute to 25 mL with the mobile phase. To 5.0 mL of the solution, add 5.0 mL of reference solution (a) and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.30 m, Ø = 3.9 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm) of irregular type, with a specific surface of 330 $m^2$/g, a pore size of 12.5 nm and a carbon loading of 9.8 per cent .

*Mobile phase*: mix 250 mL of a 2.0 g/L solution of *sodium hydroxide R*, previously adjusted to pH 2.5 with *perchloric acid R* and 750 mL of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL; inject the test solution (a) and reference solutions (c) and (d).

*Run time*: 5 times the retention time of halofantrine which is about 6 min.

*System suitability*:

- *resolution*: minimum 3.3 between the peaks due to halofantrine and impurity C in the chromatogram obtained with reference solution (d).

*Limits*:

- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of halofantrine hydrochloride.

STORAGE

Protected from light.

IMPURITIES

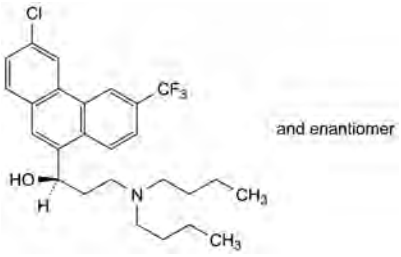


A. (1*RS*)-1-[3-chloro-6-(trifluoromethyl)phenanthren-9-yl]-3-(dibutylamino)propan-1-ol (1-dechlorohalofantrine),

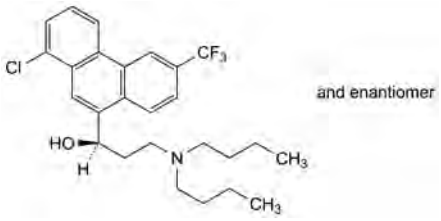


B. (1*RS*)-1-[1-chloro-6-(trifluoromethyl)phenanthren-9-yl]-3-(dibutylamino)propan-1-ol (3-dechlorohalofantrine),

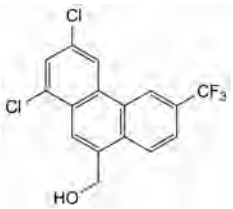


C. [1,3-dichloro-6-(trifluoromethyl)phenanthren-9-yl]methanol.

**01/2013:0616**

# HALOPERIDOL

## Haloperidolum

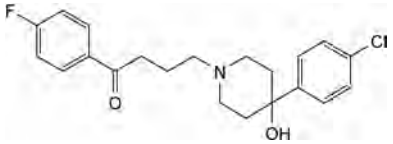


$C_{21}H_{23}ClFNO_2$ $M_r$ 375.9

[52-86-8]

DEFINITION

4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 150 °C to 153 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *haloperidol CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (a).* Dissolve 10 mg of *haloperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *haloperidol CRS* and 10 mg of *bromperidol CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC octadecylsilyl silica gel plate R.*
*Mobile phase*: *tetrahydrofuran R*, *methanol R*, 58 g/L solution of *sodium chloride R* (10:45:45 *V/V/V*).
*Application*: 1 µL.
*Development*: in an unsaturated tank, over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 spots which may, however, not be completely separated.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in 5 mL of *anhydrous ethanol R*. Add 0.5 mL of *dinitrobenzene solution R* and 0.5 mL of *2 M alcoholic potassium hydroxide R*. A violet colour is produced and becomes brownish-red after 20 min.

E. To 0.1 g in a platinum crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 0.2 g in 20 mL of a 1 per cent *V/V* solution of *lactic acid R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *haloperidol for system suitability CRS* (containing impurities B and D) in 1.0 mL of *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 10 mg of *haloperidol for peak identification CRS* (containing impurities G and H) in 1.0 mL of *methanol R*.

*Column*:
– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 17 g/L solution of *tetrabutylammonium hydrogen sulfate R1*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 17 | 90 → 50 | 10 → 50 |
| 17 - 22 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *haloperidol for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and D; use the chromatogram supplied with *haloperidol for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities G and H.

*Relative retention* with reference to haloperidol (retention time = about 8 min): impurity B = about 0.9; impurity D = about 1.6; impurity G = about 1.8; impurity H = about 2.0.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity B and haloperidol.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.7;

– *impurity D*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *impurities G, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 37.59 mg of $C_{21}H_{23}ClFNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, D, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F.*

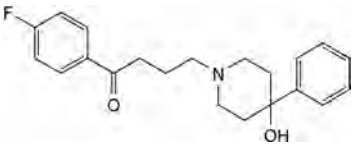

A. 1-(4-fluorophenyl)-4-(4-hydroxy-4-phenylpiperidin-1-yl)butan-1-one,

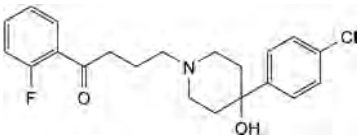

B. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(2-fluorophenyl)butan-1-one,

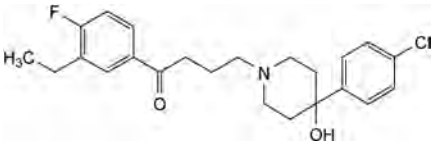

C. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(3-ethyl-4-fluorophenyl)butan-1-one,

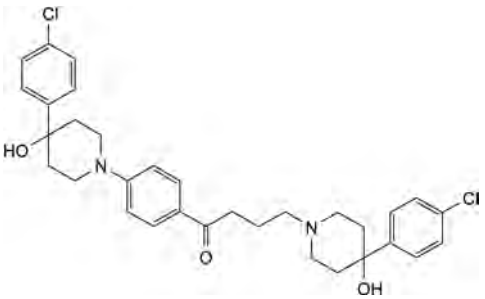

D. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-[4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]phenyl]butan-1-one,

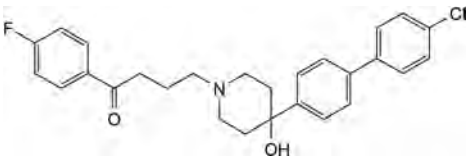

E. 4-[4-(4′-chlorobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one,

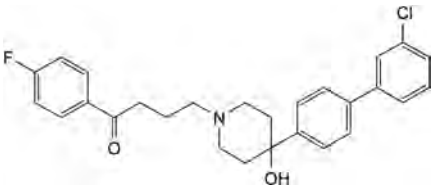

F. 4-[4-(3′-chlorobiphenyl-4-yl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one,

G. unknown structure,

H. unknown structure.

07/2011:1431

# HALOPERIDOL DECANOATE

## Haloperidoli decanoas

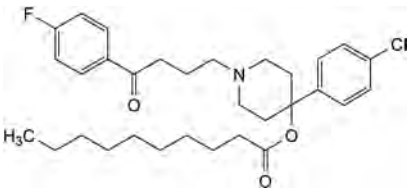

$C_{31}H_{41}ClFNO_3$ $M_r$ 530.1
[74050-97-8]

DEFINITION

4-(4-Chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent), in methanol and in methylene chloride.

mp: about 42 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *haloperidol decanoate CRS*.

B. To 0.1 g in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methylene chloride R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.5 mg of *bromperidol decanoate CRS* and 2.5 mg of *haloperidol decanoate CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 27 g/L solution of *tetrabutylammonium hydrogen sulfate R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 80 → 40 | 20 → 60 |
| 30 - 35 | 40 | 60 |
| 35 - 40 | 40 → 80 | 60 → 20 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Relative retention* with reference to haloperidol decanoate (retention time = about 24 min): impurity G = about 0.1; impurity L = about 0.2; impurity H = about 0.8; impurity I = about 0.88; impurity A = about 0.9; impurity B = about 0.98; bromperidol decanoate = about 1.02; impurity J = about 1.1; impurity C = about 1.15; impurity D = about 1.2; impurity K = about 1.22; impurity F = about 1.26; impurity E = about 1.28.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to haloperidol decanoate and bromperidol decanoate.

*Limits*:

– *impurities A, B, C, D, E, F, G, H, I, J, K*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 30 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.425 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 53.01 mg of $C_{31}H_{41}ClFNO_3$.

STORAGE

Protected from light, at a temperature below 25 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *L.*

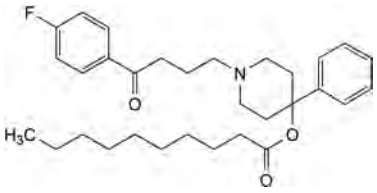

A. 1-[4-(4-fluorophenyl)-4-oxobutyl]-4-phenylpiperidin-4-yl decanoate,

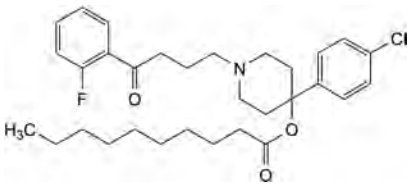

B. 4-(4-chlorophenyl)-1-[4-(2-fluorophenyl)-4-oxobutyl]-piperidin-4-yl decanoate,

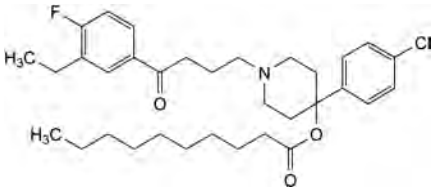

C. 4-(4-chlorophenyl)-1-[4-(3-ethyl-4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

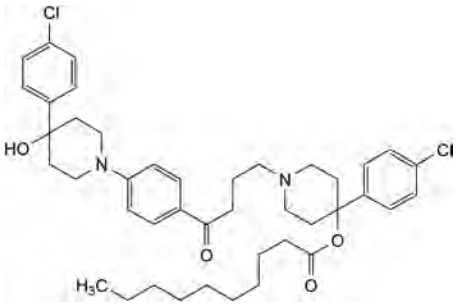

D. 4-(4-chlorophenyl)-1-[4-[4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]phenyl]-4-oxobutyl]piperidin-4-yl decanoate,

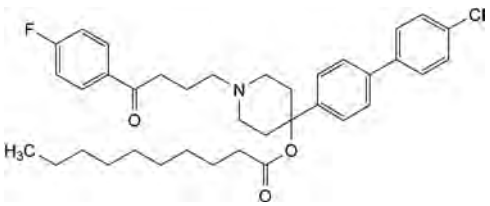

E. 4-(4′-chlorobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

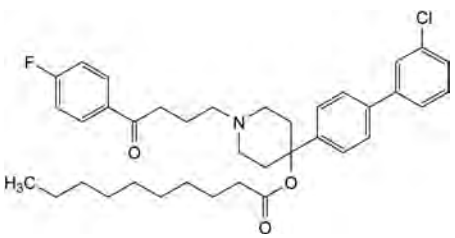

F. 4-(3′-chlorobiphenyl-4-yl)-1-[4-(4-fluorophenyl)-4-oxobutyl]piperidin-4-yl decanoate,

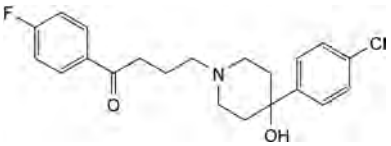

G. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl)butan-1-one (haloperidol),

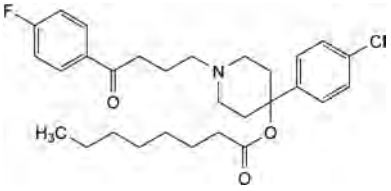

H. 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl octanoate,

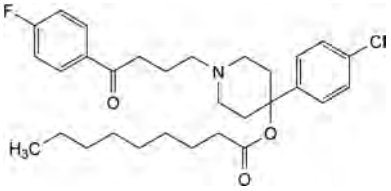

I. 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl nonanoate,

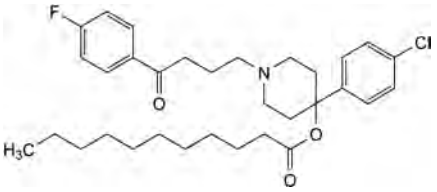

J. 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl undecanoate,

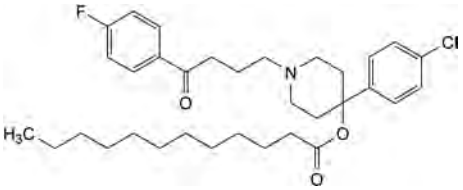

K. 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-piperidin-4-yl dodecanoate,

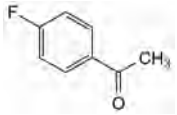

L. 1-(4-fluorophenyl)ethanone.

**04/2016:0393**
**corrected 10.0**

# HALOTHANE

## Halothanum

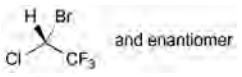


$C_2HBrClF_3$ $M_r$ 197.4
[151-67-7]

DEFINITION

(*RS*)-2-Bromo-2-chloro-1,1,1-trifluoroethane to which 0.01 per cent *m/m* of thymol has been added.

CHARACTERS

*Appearance*: clear, colourless, mobile, heavy, non-flammable liquid.

*Solubility*: slightly soluble in water, miscible with anhydrous ethanol.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Distillation range (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the substance in a 0.1 mm cell.

*Comparison*: *Ph. Eur. reference spectrum of halothane.*

C. Add 0.1 mL to 2 mL of *2-methyl-2-propanol R* in a test-tube. Add 1 mL of *copper edetate solution R*, 0.5 mL of *concentrated ammonia R* and a mixture of 0.4 mL of *strong hydrogen peroxide solution R* and 1.6 mL of *water R* (solution A). Prepare a blank at the same time (solution B). Place both tubes in a water-bath at 50 °C for 15 min, cool and add 0.3 mL of *glacial acetic acid R*. To 1 mL of each of solutions A and B add 0.5 mL of a mixture of equal volumes of freshly prepared *alizarin S solution R* and *zirconyl nitrate solution R*. Solution A is yellow and solution B is red.

To 1 mL of each of solutions A and B add 1 mL of *buffer solution pH 5.2 R*, 1 mL of *phenol red solution R* diluted 1 to 10 with *water R* and 0.1 mL of *chloramine solution R*. Solution A is bluish-violet and solution B is yellow.

To 2 mL of each of solutions A and B add 0.5 mL of a mixture of 25 volumes of *sulfuric acid R* and 75 volumes of *water R*, 0.5 mL of *acetone R* and 0.2 mL of a 50 g/L solution of *potassium bromate R* and shake. Warm the tubes in a water-bath at 50 °C for 2 min, cool and add 0.5 mL of a mixture of equal volumes of *nitric acid R* and *water R* and 0.5 mL of *silver nitrate solution R2*. Solution A is opalescent and a white precipitate is formed after a few minutes; solution B remains clear.

TESTS

**Acidity or alkalinity**. To 20 mL add 20 mL of *carbon dioxide-free water R*, shake for 3 min and allow to stand. Separate the aqueous layer and add 0.2 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.01 M sodium hydroxide* or 0.6 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Relative density** (*2.2.5*): 1.872 to 1.877.

**Distillation range** (*2.2.11*): it distils completely between 49.0 °C and 51.0 °C and 95 per cent distills within a range of 1.0 °C.

**Volatile related substances**. Gas chromatography (*2.2.28*).

*Internal standard*: *trichlorotrifluoroethane CRS*.

*Test solution (a).* The substance to be examined.

*Test solution (b).* Dilute 1.0 mL of *trichlorotrifluoroethane CRS* to 20.0 mL with the substance to be examined. Dilute 1.0 mL of the solution to 100.0 mL with the substance to be examined. Dilute 1.0 mL of this solution to 10.0 mL with the substance to be examined.

*Column*:

- *size*: *l* = 2.75 m, Ø = 5 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (180-250 µm), the first 1.8 m being impregnated with 30 per cent *m/m* of *macrogol 400 R* and the remainder with 30 per cent *m/m* of *dinonyl phthalate R*;
- *temperature*: 50 °C.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Detection*: flame ionisation.

*Injection*: 5 µL.

*Limit*: test solution (b):

- *total*: not more than the area of the peak due to the internal standard, corrected if necessary for any impurity with the same retention time as the internal standard (0.005 per cent).

**Thymol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.10 g of *menthol R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Test solution.* To 20.0 mL of the substance to be examined add 5.0 mL of the internal standard solution.

*Reference solution.* Dissolve 20.0 mg of *thymol R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent. To 20.0 mL of this solution, add 5.0 mL of the internal standard solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1.5 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 15 mL/min.

*Temperature*:

- *column*: 150 °C;
- *injection port*: 170 °C;
- *detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Limit*:

- *thymol*: 0.75 times to 1.15 times the area of the corresponding peak in the chromatogram obtained with the reference solution (0.008 per cent *m/m* to 0.012 per cent *m/m*).

**Bromides and chlorides**. To 10 mL add 20 mL of *water R* and shake for 3 min. To 5 mL of the aqueous layer add 5 mL of *water R*, 0.05 mL of *nitric acid R* and 0.2 mL of *silver nitrate solution R1*. The solution is not more opalescent than a mixture of 5 mL of the aqueous layer and 5 mL of *water R*.

**Bromine and chlorine**. To 10 mL of the aqueous layer obtained in the test for bromides and chlorides add 1 mL of *potassium iodide and starch solution R*. No blue colour is produced.

**Non-volatile matter**: maximum 20 mg/L.

Evaporate 50 mL to dryness on a water-bath and dry the residue in an oven at 100-105 °C for 2 h. The residue weighs a maximum of 1 mg.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 25 °C. The choice of material for the container is made taking into account the particular reactivity of halothane with certain metals.

IMPURITIES

A. (*E*)-1,1,1,4,4,4-hexafluorobut-2-ene,

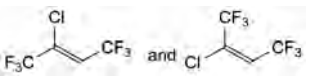

B. (*EZ*)-2-chloro-1,1,1,4,4,4-hexafluorobut-2-ene (*cis* and *trans*),

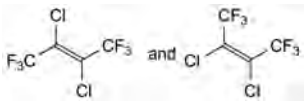

C. (*EZ*)-2,3-dichloro-1,1,1,4,4,4-hexafluorobut-2-ene (*cis* and *trans*),

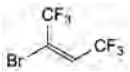

D. (*E*)-2-bromo-1,1,1,4,4,4-hexafluorobut-2-ene,

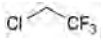

E. 2-chloro-1,1,1-trifluoroethane,

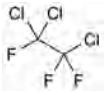

F. 1,1,2-trichloro-1,2,2-trifluoroethane,

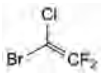

G. 1-bromo-1-chloro-2,2-difluoroethene,

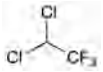

H. 2,2-dichloro-1,1,1-trifluoroethane,

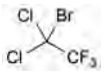

I. 1-bromo-1,1-dichloro-2,2,2-trifluoroethane,

J. 1,2-dichloro-1,1-difluoroethane.

**01/2020:0462**

# HARD FAT

## Adeps solidus

DEFINITION

Mixture of triglycerides, diglycerides and monoglycerides, which may be obtained either by esterification of hydrogenated fatty acids of vegetable origin with glycerol or by interesterification of hydrogenated vegetable oils.

Each type of hard fat is characterised by its melting point, its hydroxyl value and its saponification value.

It does not contain additives.

CHARACTERS

*Appearance*: white or almost white, waxy, brittle mass.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol and in methylene chloride.

When heated to 50 °C it melts, giving a colourless or slightly yellowish liquid.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ether R*, *methylene chloride R* (10:90 *V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.7 ($R_{st}$ 1) and may show spots due to 1,3-diglycerides ($R_{st}$ 0.6), to 1,2-diglycerides ($R_{st}$ 0.4) and to 1-monoglycerides ($R_{st}$ 0.07). For substances with a low hydroxyl value, the spots due to monoglycerides and diglycerides may be faint or absent. In this case, the test for hydroxyl value (see Tests) is also carried out to confirm identification.

B. Soya bean lecithin, macrogol cetostearyl ether and polysorbate 65. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 0.100 g of *soya bean lecithin R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.100 g of *macrogol cetostearyl ether R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dissolve 0.100 g of *polysorbate 65 R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plates*: *TLC silica gel G plate R* (2 plates).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (4:25:65 *V/V/V*).

Plate 1

*Application*: 4 µL of the test solution and reference solution (a).

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*Results*:

– the chromatogram obtained with the test solution shows no spots corresponding to those of soya bean lecithin ($R_F$ = about 0.3 and 0.5) in the chromatogram obtained with reference solution (a);

– the chromatogram obtained with the test solution shows a spot due to triglycerides with an $R_F$ value of about 1.0.

Plate 2

*Application*: 4 µL of the test solution and reference solutions (b) and (c).

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R4* and examine in daylight.

*Results*:

– the chromatogram obtained with the test solution shows no orange spot corresponding to that of macrogol cetostearyl ether ($R_F$ = about 0.85) in the chromatogram obtained with reference solution (b);

– the chromatogram obtained with the test solution shows no orange spot corresponding to that of polysorbate 65 ($R_F$ = about 1.0) in the chromatogram obtained with reference solution (c).

C. Beeswax. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 8 mL with the same solvent.

*Reference solution.* Dissolve 0.100 g of *white beeswax R* in *methylene chloride R* and dilute to 8 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with a freshly prepared 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 120 °C for 3 min; examine in daylight.

*Results*: the chromatogram obtained with the test solution shows no black spot corresponding to that of white beeswax ($R_F$ = about 0.9) in the chromatogram obtained with the reference solution.

TESTS

**Alkaline impurities**. Melt 10.00 g and while maintaining the temperature at about 50 °C, dissolve the melted mass in 40.0 mL of *ethanol (96 per cent) R*, mix and add 0.05 mL of *bromophenol blue solution R*. Not more than 0.75 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Melting point** (*2.2.15*): 30 °C to 45 °C, and within 2 °C of the nominal value.

Introduce the melted substance into a capillary tube and allow to stand at a temperature below 10 °C for 24 h.

**Acid value** (*2.5.1*): maximum 0.5.

Melt 5.0 g and dissolve in 20 mL of the prescribed mixture of solvents.

**Hydroxyl value** (*2.5.3, Method A*): maximum 50, and within 5 units of the nominal value; maximum 5 if the nominal value is less than 5.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Peroxide value** (*2.5.5, Method A*): maximum 3.0.

**Saponification value** (*2.5.6*): 210 to 260, and within 5 per cent of the nominal value, determined on 2.0 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.0 g in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

Protected from light, at a temperature at least 5 °C below the nominal melting point.

LABELLING

The label states:

- the nominal melting point;
- the nominal hydroxyl value;
- the nominal saponification value.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hard fat used as basis for solid dosage forms.*

**Melting point** (see Tests).

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**01/2020:2731**

# HARD FAT WITH ADDITIVES

## Adeps solidus cum additamentis

DEFINITION

Mixture of triglycerides, diglycerides and monoglycerides, which may be obtained either by esterification of hydrogenated fatty acids of vegetable origin with glycerol or by interesterification of hydrogenated vegetable oils.

Each type of hard fat with additives is characterised by its melting point, its hydroxyl value and its saponification value.

It contains additives such as lecithin, surfactants, beeswax or a mixture thereof.

CHARACTERS

*Appearance*: white or pale yellow, waxy, brittle mass.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol and in methylene chloride.

When heated to 50 °C it melts, giving a colourless or slightly yellowish liquid.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ether R*, *methylene chloride R* (10:90 *V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.7 ($R_{st}$ 1) and may show spots due to 1,3-diglycerides ($R_{st}$ 0.6), to 1,2-diglycerides ($R_{st}$ 0.4) and to 1-monoglycerides ($R_{st}$ 0.07). For substances with a low hydroxyl value, the spots due to monoglycerides and diglycerides may be faint or absent. In this case, the test for hydroxyl value (see Tests) is also carried out to confirm identification.

B. Soya bean lecithin, macrogol cetostearyl ether and polysorbate 65. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 0.100 g of *soya bean lecithin R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.100 g of *macrogol cetostearyl ether R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dissolve 0.100 g of *polysorbate 65 R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plates*: *TLC silica gel G plate R* (2 plates).

*Mobile phase*: *water R*, *methanol R*, *methylene chloride R* (4:25:65 *V/V/V*).

Plate 1

*Application*: 4 µL of the test solution and reference solution (a).

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*Results*:

– in the presence of soya bean lecithin, the chromatogram obtained with the test solution shows 2 spots ($R_F$ = about 0.3 and 0.5) similar in position to the spots in the chromatogram obtained with reference solution (a);

– the chromatogram obtained with the test solution shows a spot due to triglycerides with an $R_F$ value of about 1.0.

Plate 2

*Application*: 4 µL of the test solution and reference solutions (b) and (c).

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R4* and examine in daylight.

*Results*:

– in the presence of macrogol cetostearyl ether, the chromatogram obtained with the test solution shows an orange spot ($R_F$ = about 0.85) similar in position to the spot in the chromatogram obtained with reference solution (b);

– in the presence of polysorbate 65, the chromatogram obtained with the test solution shows an orange spot ($R_F$ = about 1.0) similar in position to the spot in the chromatogram obtained with reference solution (c).

C. Beeswax. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 8 mL with the same solvent.

*Reference solution.* Dissolve 0.100 g of *white beeswax R* in *methylene chloride R* and dilute to 8 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with a freshly prepared 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 120 °C for 3 min; examine in daylight.

*Results*: in the presence of beeswax, the chromatogram obtained with the test solution shows a black spot ($R_F$ = about 0.9) similar in position to the spot in the chromatogram obtained with the reference solution.

TESTS

**Alkaline impurities**. Melt 10.00 g and while maintaining the temperature at about 50 °C, dissolve the melted mass in 40.0 mL of *ethanol (96 per cent) R*, mix and add 0.05 mL of *bromophenol blue solution R*. Not more than 0.75 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

If the substance to be examined contains lecithins, not more than 3.75 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Melting point** (*2.2.15*): 30 °C to 45 °C, and within 2 °C of the nominal value.

Introduce the melted substance into a capillary tube and allow to stand at a temperature below 10 °C for 24 h.

**Acid value** (*2.5.1*): maximum 1.0.

Melt 5.0 g and dissolve in 20 mL of the prescribed mixture of solvents.

**Hydroxyl value** (*2.5.3, Method A*): maximum 70, and within 5 units of the nominal value.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Peroxide value** (*2.5.5, Method A*): maximum 3.0.

**Saponification value** (*2.5.6*): 210 to 260, and within 5 per cent of the nominal value, determined on 2.0 g.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.0 g in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

Protected from light, at a temperature at least 5 °C below the nominal melting point.

LABELLING

The label states:

– the nominal melting point;

– the nominal hydroxyl value;

– the nominal saponification value.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hard fat with additives used as basis for solid dosage forms.*

**Melting point** (see Tests).

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

01/2008:2155

# HELIUM

## Helium

He $A_r$ 4.00

DEFINITION

*Content*: minimum 99.5 per cent *V/V* of He.

This monograph applies to helium obtained by separation from natural gas and intended for medicinal use.

CHARACTERS

*Appearance*: colourless, inert gas.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The retention time of the principal peak in the chromatogram obtained with the substance to be examined is approximately the same as that of the principal peak in the chromatogram obtained with the reference gas.

TESTS

**Methane**: maximum 50.0 ppm *V/V*.

Infrared analyser.

*Gas to be examined*. The substance to be examined. It must be filtered to avoid stray light phenomena (3 µm filter).

*Reference gas (a)*: *helium for chromatography R*.

*Reference gas (b)*: mixture containing 50.0 ppm *V/V* of *methane R* in *helium for chromatography R*.

The infrared analyser generally comprises an infrared source emitting broadband infrared radiation, an optical device, a sample cell, a detector and in some analysers a reference cell. The optical device may be positioned either before or after the sample cell. It consists of one or more optical filters, through which the broadband radiation is passed. The optical device is selected for methane determination. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature. When methane is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law, and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of methane. The generated signal is linearised in order to determine the methane content.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the methane content in the gas to be examined.

**Oxygen**: maximum 50.0 ppm *V/V*, determined using an oxygen analyser equipped with an electrochemical cell and a detector scale ranging from 0-100 ppm *V/V*.

The gas to be examined passes through a detection cell containing an aqueous solution of an electrolyte, generally potassium hydroxide. The presence of oxygen in the gas to be examined produces a variation in the electric signal recorded at the outlet of the cell that is proportional to the oxygen content.

Calibrate the analyser according to the instructions of the manufacturer. Pass the gas to be examined through the analyser using a suitable pressure regulator and airtight metal tubes and operating at the prescribed flow rates until constant readings are obtained.

**Water** (*2.5.28*): maximum 67 ppm *V/V*.

ASSAY

Gas chromatography (*2.2.28*).

*Gas to be examined*. The substance to be examined.

*Reference gas*: *helium for chromatography R*.

*Column*:

- *size*: *l* = 2 m, Ø = 4.5 mm;
- *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *argon for chromatography R*.

*Flow rate*: 60 mL/min.

*Temperature*:

- *column*: 50 °C;
- *detector*: 150 °C.

*Detection*: thermal conductivity.

*Injection*: 0.5 mL.

Inject the reference gas. Adjust the injected volumes and operating conditions so that the height of the peak due to helium in the chromatogram obtained is at least 35 per cent of the full scale of the recorder.

*System suitability*: reference gas:

- *symmetry factor*: minimum 0.6.

Calculate the content of He in the gas to be examined.

STORAGE

As compressed gas or liquid at cryogenic temperature, in appropriate containers, complying with the legal regulations.

IMPURITIES

*Specified impurities*: *A, B, C.*

A. $CH_4$: methane,

B. $O_2$: oxygen,

C. $H_2O$: water.

01/2020:0332

# HEPARIN CALCIUM

## Heparinum calcicum

DEFINITION

Preparation containing the calcium salt of a sulfated glycosaminoglycan present in mammalian tissues. It is prepared from the intestinal mucosae of pigs. On complete hydrolysis, it liberates D-glucosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulfuric acid. It has the property of delaying the clotting of blood by catalysing the inhibition of thrombin and factor Xa by antithrombin.

*Potency*: minimum 180 IU/mg (dried substance), determined by the assay of anti-factor IIa activity as described under Assay.

PRODUCTION

The animals from which heparin calcium is derived must fulfil the requirements for the health of animals suitable for human consumption. All stages of production and sourcing are subjected to a suitable quality management system. The identity of the source species and the absence of material from possible contaminant species such as cattle, sheep and goats, is verified by appropriate testing during production. The method used to confirm identity of the source species, and the point of application in the process, have been validated and shown to be capable of identifying the presence of material of other species at the level of 0.1 per cent (*m/m*). Species verification by methods based on polymerase chain reaction (PCR) amplification of species-specific DNA sequences has been widely shown to be an appropriate surrogate. If such a method

has been chosen, it is also used to test for porcine DNA and to determine that it is present at a consistent level, in line with the manufacturing process used.

It is produced by methods of manufacturing designed to minimise or eliminate substances lowering blood pressure.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. It complies with the requirements described under Assay.

B. Carry out the assay of anti-factor Xa activity of heparin (*2.7.5*). The ratio of anti-factor Xa activity to anti-factor IIa activity determined as described under Assay, ranges between 0.9 and 1.1.

C. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Preparation*: dissolve 20 mg of the substance to be examined in 0.7 mL of a 20 µg/mL solution of *deuterated sodium trimethylsilylpropionate R* in *deuterium oxide R*.

*Comparison*: dissolve 20 mg of *heparin calcium for NMR identification CRS* in 0.7 mL of a 20 µg/mL solution of *deuterated sodium trimethylsilylpropionate R* in *deuterium oxide R*.

*Apparatus*: spectrometer operating at minimum 300 MHz.

*Acquisition of $^1$H-NMR spectra*:

- *number of transients*: minimum 16; it is adjusted until the signal-to-noise ratio is at least 1000:1 for the heparin methyl signal at 2.04 ppm;
- *temperature*: about 25 °C; test sample and reference spectra have to be obtained at the same temperature;
- *acquisition time*: minimum 2 s;
- *repetition time* (acquisition time plus delay): minimum 4 s;
- *spectral width*: 10-12 ppm, centred at around 4.5 ppm;
- *pulse width*: to give a flip angle between 30° and 90°.

*Processing*:

- *exponential line-broadening window function*: 0.3 Hz;
- Fourier transformation;
- trimethylsilylpropionate reference signal set at 0.00 ppm.

*Results*:

- the large heparin calcium signals must be present: 2.05 ppm, 3.29 ppm (doublet), 4.37 ppm, 5.35 ppm and 5.43 ppm, all within ± 0.03 ppm;
- the $^1$H-NMR spectrum obtained with the test sample and that obtained with *heparin calcium for NMR identification CRS* are compared qualitatively after the 2 spectra have been normalised so as to have a similar intensity; dermatan sulfate with a methyl signal at 2.08 ± 0.02 ppm may be observed; no unidentified signals larger than 4 per cent compared to the height of the heparin signal at 5.43 ppm are present in the ranges 0.10-2.00 ppm, 2.10-3.10 ppm and 5.70-8.00 ppm; signals from the solvent or process-related substances may be present and have to be identified to be accepted.

D. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (c).

*Relative retention* with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the peak due to dermatan sulfate and chondroitin sulfate and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to heparin.

*Results*: the principal peak in the chromatogram obtained with test solution (a) is similar in retention time and shape to the principal peak in the chromatogram obtained with reference solution (c).

E. It gives the reactions of calcium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve a quantity equivalent to 50 000 IU in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 8.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Nucleotidic impurities**. Dissolve 40 mg in 10 mL of *water R*. The absorbance (*2.2.25*) measured at 260 nm is not greater than 0.15.

**Protein**: maximum 0.5 per cent (dried substance).

*Solution A*. Mix 2 volumes of a 10 g/L solution of *sodium hydroxide R* and 2 volumes of a 50 g/L solution of *sodium carbonate R* and dilute to 5 volumes with *water R*.

*Solution B*. Mix 2 volumes of a 12.5 g/L solution of *copper sulfate pentahydrate R* and 2 volumes of a 29.8 g/L solution of *sodium tartrate R* and dilute to 5 volumes with *water R*.

*Solution C*. Mix 1 volume of solution B and 50 volumes of solution A.

*Solution D*. Dilute a phosphomolybdotungstic reagent in *water R*. Suitable dilutions produce solutions of pH 10.25 ± 0.25 after addition of solutions C and D to the test and reference solutions.

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 5 mg/mL.

*Reference solutions*. Dissolve *bovine albumin R1* in *water R* to obtain a concentration of 100 mg/mL. Prepare dilutions of the solution in *water R* as prescribed in general chapter *2.5.33, method 2*.

*Blank*: *water R*.

*Procedure*. To 1 mL of each reference solution, of the test solution and of the blank, add 5 mL of solution C. Allow to stand for 10 min. Add 0.5 mL of solution D, mix and allow to stand at room temperature for 30 min. Filter through a membrane filter (nominal pore size 0.45 µm). Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution prepared from the blank as compensation liquid.

*Calculations*. As prescribed in general chapter *2.5.33, method 2*.

**Related substances**. Liquid chromatography (*2.2.29*).
*Reference solutions are stable at room temperature for 24 h.*

*Test solution (a)*. Dissolve an accurately weighed quantity of about 50 mg of the substance to be examined in 5.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete.

*Test solution (b)*. Dissolve an accurately weighed quantity of about 0.1 g of the substance to be examined in 1.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete. Mix 500 µL of the solution and 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Reference solution (a)*. Dissolve 250 mg of *heparin for physico-chemical analysis CRS* in *water for chromatography R* and dilute to 2.0 mL with the same solvent. Mix using a vortex mixer until dissolution is complete.

*Reference solution (b).* Add 1200 µL of reference solution (a) to 300 µL of *dermatan sulfate and over-sulfated chondroitin sulfate CRS*. Mix using a vortex mixer to homogenise.

*Reference solution (c).* Add 100 µL of reference solution (b) to 900 µL of *water for chromatography R*. Mix using a vortex mixer to homogenise.

*Reference solution (d).* Add 400 µL of reference solution (a) to 100 µL of *water for chromatography R* and mix using a vortex mixer. Add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Reference solution (e).* To 500 µL of reference solution (b), add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Precolumn*:
- *size*: *l* = 0.05 m, Ø = 2 mm;
- *stationary phase*: *anion-exchange resin R* (13 µm).

*Column*:
- *size*: *l* = 0.25 m, Ø = 2 mm;
- *stationary phase*: *anion-exchange resin R* (9 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1000 mL of *water for chromatography R* and adjust to pH 3.0 with *dilute phosphoric acid R*;
- *mobile phase B*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1000 mL of *water for chromatography R*, add 140 g of *sodium perchlorate R* and adjust to pH 3.0 with *dilute phosphoric acid R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 75 | 25 |
| 10 - 35 | 75 → 0 | 25 → 100 |
| 35 - 40 | 0 | 100 |

*Flow rate*: 0.22 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Equilibration*: at least 15 min.

*Injection*: 20 µL of test solution (b) and reference solutions (d) and (e).

*Relative retention* with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

*System suitability*:
- the chromatogram obtained with reference solution (d) shows no peak at the retention time of heparin;
- *resolution*: minimum 3.0 between the peak due to dermatan sulfate and chondroitin sulfate and the peak due to over-sulfated chondroitin sulfate in the chromatogram obtained with reference solution (e).

*Limits*:
- *sum of dermatan sulfate and chondroitin sulfate*: not more than the area of the peak due to dermatan sulfate and chondroitin sulfate in the chromatogram obtained with reference solution (e) (2.0 per cent);
- *any other impurity*: no peak with an area greater than 0.01 times the area of the peak due to dermatan sulfate and chondroitin sulfate in the chromatogram obtained with reference solution (e) is detected (corresponding to a disregard limit of 0.02 per cent). Disregard any peaks that appear during the initial isocratic step.

**Nitrogen** (*2.5.9*): 1.5 per cent to 2.5 per cent (dried substance), determined on 0.100 g.

**Calcium**: 9.5 per cent to 11.5 per cent (dried substance), determined on 0.200 g by complexometric titration (*2.5.11*).

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.01 IU per International Unit of heparin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. The addition of divalent cations may be necessary in order to fulfil the validation criteria.

ASSAY

Carry out the assay of anti-factor IIa activity of heparin (*2.7.5*). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency (*P* = 0.95) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states the number of International Units per milligram.

**01/2020:0333**

# HEPARIN SODIUM

## Heparinum natricum

DEFINITION

Preparation containing the sodium salt of a sulfated glycosaminoglycan present in mammalian tissues. It is prepared from the intestinal mucosae of pigs. On complete hydrolysis, it liberates D-glucosamine, D-glucuronic acid, L-iduronic acid, acetic acid and sulfuric acid. It has the property of delaying the clotting of blood by catalysing the inhibition of thrombin and factor Xa by antithrombin.

*Potency*: minimum 180 IU/mg (dried substance), determined by the assay of anti-factor IIa activity as described under Assay.

PRODUCTION

The animals from which heparin sodium is derived must fulfil the requirements for the health of animals suitable for human consumption. All stages of production and sourcing are subjected to a suitable quality management system. The identity of the source species and the absence of material from possible contaminant species such as cattle, sheep and goats, is verified by appropriate testing during production. The method used to confirm identity of the source species, and the point of application in the process, have been validated and shown to be capable of identifying the presence of material of other species at the level of 0.1 per cent (*m/m*). Species verification by methods based on polymerase chain reaction (PCR) amplification of species-specific DNA sequences has been widely shown to be an appropriate surrogate. If such a method has been chosen, it is also used to test for porcine DNA and to determine that it is present at a consistent level, in line with the manufacturing process used.

It is produced by methods of manufacturing designed to minimise or eliminate substances lowering blood pressure.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. It complies with the requirements described under Assay.

B. Carry out the assay of anti-factor Xa activity of heparin (*2.7.5*). The ratio of anti-factor Xa activity to anti-factor IIa activity determined as described under Assay, ranges between 0.9 and 1.1.

C. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Solution A*. A solution in *deuterium oxide R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R* and if the signal at 5.22 ppm is smaller than 80 per cent of the signal at 5.44 ppm, 12 µg/mL of *sodium edetate R*.

*Preparation*: dissolve 20 mg of the substance to be examined in 0.7 mL of solution A.

*Comparison*: dissolve 20 mg of *heparin sodium for NMR identification CRS* in 0.7 mL of solution A.

*If stored, the sodium edetate and deuterated sodium trimethylsilylpropionate solutions must be kept in high-density, natural polyethylene bottles.*

*Apparatus*: spectrometer operating at minimum 300 MHz.

*Acquisition of $^1$H-NMR spectra*:

- *number of transients*: minimum 16; it is adjusted until the signal-to-noise ratio is at least 1000:1 for the heparin methyl signal at 2.04 ppm;
- *temperature*: about 25 °C; test sample and reference spectra have to be obtained at the same temperature;
- *acquisition time*: minimum 2 s;
- *repetition time* (acquisition time plus delay): minimum 4 s;
- *spectral width*: 10-12 ppm, centred at around 4.5 ppm;
- *pulse width*: to give a flip angle between 30° and 90°.

*Processing*:

- *exponential line-broadening window function*: 0.3 Hz;
- Fourier transformation;
- trimethylsilylpropionate reference signal set at 0.00 ppm.

*Results*:

- the large heparin sodium signals must be present: 2.04 ppm, 3.27 ppm (doublet), 4.34 ppm, 5.22 ppm and 5.42 ppm, all within ± 0.03 ppm;
- the $^1$H-NMR spectrum obtained with the test sample and that obtained with *heparin sodium for NMR identification CRS* are compared qualitatively after the 2 spectra have been normalised so as to have a similar intensity; dermatan sulfate with a methyl signal at 2.08 ± 0.02 ppm may be observed; no unidentified signals larger than 4 per cent compared to the height of the heparin signal at 5.42 ppm are present in the ranges 0.10-2.00 ppm, 2.10-3.10 ppm and 5.70-8.00 ppm; signals from the solvent or process-related substances may be present and have to be identified to be accepted; variations in the intensity of some signal regions of the spectrum of heparin may occur: the intensity-variable regions are between 3.35 ppm and 4.55 ppm, where the signal pattern is approximately kept but intensity varies.

D. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (c).

*Relative retention* with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the peak due to dermatan sulfate and chondroitin sulfate and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to heparin.

*Results*: the principal peak in the chromatogram obtained with test solution (a) is similar in retention time and shape to the principal peak in the chromatogram obtained with reference solution (c).

E. It complies with the test for sodium (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve a quantity equivalent to 50 000 IU in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 8.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Nucleotidic impurities**. Dissolve 40 mg in 10 mL of *water R*. The absorbance (*2.2.25*) measured at 260 nm is not greater than 0.15.

**Protein**: maximum 0.5 per cent (dried substance).

*Solution A*. Mix 2 volumes of a 10 g/L solution of *sodium hydroxide R* and 2 volumes of a 50 g/L solution of *sodium carbonate R* and dilute to 5 volumes with *water R*.

*Solution B*. Mix 2 volumes of a 12.5 g/L solution of *copper sulfate pentahydrate R* and 2 volumes of a 29.8 g/L solution of *sodium tartrate R* and dilute to 5 volumes with *water R*.

*Solution C*. Mix 1 volume of solution B and 50 volumes of solution A.

*Solution D*. Dilute a phosphomolybdotungstic reagent in *water R*. Suitable dilutions produce solutions of pH 10.25 ± 0.25 after addition of solutions C and D to the test and reference solutions.

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 5 mg/mL.

*Reference solutions*. Dissolve *bovine albumin R1* in *water R* to obtain a concentration of 100 mg/mL. Prepare dilutions of the solution in *water R* as prescribed in general chapter *2.5.33, method 2*.

*Blank*: *water R*.

*Procedure*. To 1 mL of each reference solution, of the test solution and of the blank, add 5 mL of solution C. Allow to stand for 10 min. Add 0.5 mL of solution D, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution prepared from the blank as compensation liquid.

*Calculations*. As prescribed in general chapter *2.5.33, method 2*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Reference solutions are stable at room temperature for 24 h.*

*Test solution (a)*. Dissolve an accurately weighed quantity of about 50 mg of the substance to be examined in 5.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete.

*Test solution (b)*. Dissolve an accurately weighed quantity of about 0.1 g of the substance to be examined in 1.0 mL of *water for chromatography R*. Mix using a vortex mixer until dissolution is complete. Mix 500 µL of the solution and 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Reference solution (a)*. Dissolve 250 mg of *heparin for physico-chemical analysis CRS* in *water for chromatography R* and dilute to 2.0 mL with the same solvent. Mix using a vortex mixer until dissolution is complete.

*Reference solution (b)*. Add 1200 µL of reference solution (a) to 300 µL of *dermatan sulfate and over-sulfated chondroitin sulfate CRS*. Mix using a vortex mixer to homogenise.

*Reference solution (c).* Add 100 µL of reference solution (b) to 900 µL of *water for chromatography R*. Mix using a vortex mixer to homogenise.

*Reference solution (d).* Add 400 µL of reference solution (a) to 100 µL of *water for chromatography R* and mix using a vortex mixer. Add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Reference solution (e).* To 500 µL of reference solution (b), add 250 µL of *1 M hydrochloric acid*, then add 50 µL of a 250 mg/mL solution of *sodium nitrite R*. Mix gently and allow to stand at room temperature for 40 min before adding 200 µL of *1 M sodium hydroxide* to stop the reaction.

*Precolumn*:

- *size*: *l* = 0.05 m, Ø = 2 mm;
- *stationary phase*: *anion-exchange resin R* (13 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 2 mm;
- *stationary phase*: *anion-exchange resin R* (9 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1000 mL of *water for chromatography R* and adjust to pH 3.0 with *dilute phosphoric acid R*;
- *mobile phase B*: dissolve 0.40 g of *sodium dihydrogen phosphate R* in 1000 mL of *water for chromatography R*, add 140 g of *sodium perchlorate R* and adjust to pH 3.0 with *dilute phosphoric acid R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 75 | 25 |
| 10 - 35 | 75 → 0 | 25 → 100 |
| 35 - 40 | 0 | 100 |

*Flow rate*: 0.22 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Equilibration*: at least 15 min.

*Injection*: 20 µL of test solution (b) and reference solutions (d) and (e).

*Relative retention* with reference to heparin (retention time = about 26 min): dermatan sulfate and chondroitin sulfate = about 0.9; over-sulfated chondroitin sulfate = about 1.3.

*System suitability*:

- the chromatogram obtained with reference solution (d) shows no peak at the retention time of heparin;
- *resolution*: minimum 3.0 between the peak due to dermatan sulfate and chondroitin sulfate and the peak due to over-sulfated chondroitin sulfate in the chromatogram obtained with reference solution (e).

*Limits*:

- *sum of dermatan sulfate and chondroitin sulfate*: not more than the area of the peak due to dermatan sulfate and chondroitin sulfate in the chromatogram obtained with reference solution (e) (2.0 per cent);
- *any other impurity*: no peak with an area greater than 0.01 times the area of the peak due to dermatan sulfate and chondroitin sulfate in the chromatogram obtained with reference solution (e) is detected (corresponding to a disregard limit of 0.02 per cent). Disregard any peaks that appear during the initial isocratic step.

**Nitrogen** (*2.5.9*): 1.5 per cent to 2.5 per cent (dried substance), determined on 0.100 g.

**Sodium**: 10.5 per cent to 13.5 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 50 mg of the substance to be examined in a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare reference solutions containing 25 ppm, 50 ppm and 75 ppm of Na, using *sodium standard solution (200 ppm Na) R* diluted with a 1.27 mg/mL solution of *caesium chloride R* in *0.1 M hydrochloric acid*.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 330.3 nm.

*Atomisation device*: flame of suitable composition (for example 11 L of air and 2 L of acetylene per minute).

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.01 IU per International Unit of heparin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Carry out the assay of anti-factor IIa activity of heparin (*2.7.5*). The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits of the estimated potency (*P* = 0.95) are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states the number of International Units per milligram.

**07/2019:0828**
**corrected 10.0**

# HEPARINS, LOW-MOLECULAR-MASS

## Heparina massae molecularis minoris

DEFINITION

Salts of sulfated glycosaminoglycans having a mass-average relative molecular mass less than 8000 and for which at least 60 per cent of the total mass has a relative molecular mass less than 8000. Low-molecular-mass heparins display different chemical structures at the reducing, or the non-reducing end of the polysaccharide chains.

The potency is not less than 70 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

PRODUCTION

Low-molecular-mass heparins are obtained by fractionation or depolymerisation of heparin of natural origin that complies with the monograph *Heparin sodium (0333)* or *Heparin calcium (0332)*, whichever is appropriate, unless otherwise justified and authorised. For each type of low-molecular-mass heparin the batch-to-batch consistency is ensured by demonstrating, for example, that the mass-average relative molecular mass and the mass percentage within defined relative molecular-mass ranges lower than 8000 are not less than 75 per cent and not more than 125 per cent of the mean value stated as type specification. The same limits apply also to the ratio of anti-factor Xa activity to anti-factor IIa activity.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Preparation*: dissolve 0.200 g of the substance to be examined in a mixture of 0.2 mL of *deuterium oxide R* and 0.8 mL of *water R*.

*Comparison*: dissolve 0.200 g of the appropriate specific low-molecular-mass heparin reference standard in a mixture of 0.2 mL of *deuterium oxide R* and 0.8 mL of *water R*.

*Operating conditions*:

- *field strength*: 75 MHz;
- *temperature*: 40 °C;
- *cell diameter*: 5 mm.

*Processing*:

- Fourier transformation;
- deuterated methanol reference signal set at 50.0 ppm.

*Results*: the $^{13}C$ NMR spectrum obtained is similar to that obtained with the appropriate specific low-molecular-mass heparin reference standard.

B. The ratio of anti-factor Xa activity to anti-factor IIa activity, determined as described under Assay, is not less than 1.5.

C. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 2 mL of the mobile phase.

*Reference solution*. Dissolve 20 mg of *heparin low-molecular-mass for calibration CRS* in 2 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: appropriate porous silica beads (5 µm) with a fractionation range for proteins of approximately 15 000 to 100 000;
- *number of theoretical plates*: minimum of 20 000 per metre.

*Mobile phase*: 7.7 g/L solution of *ammonium acetate R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer.

*Injection*: 25 µL.

*Calibration*. Inject 25 µL of the reference solution and record the chromatogram for a period of time, ensuring complete elution of sample, salt and solvent peaks.

Calculate the total area under the RI curve (designated ΣRI) by numerical integration over the range of interest (i.e. excluding salt and solvent peaks at the end of the chromatogram as well as the artefact peak (designated * on the chromatogram), that if present elutes at the same retention time as the peak that would be obtained after injection of sodium sulfate) as shown in Figure 0828.-1.

Calculate the area under the curve up to each point as a percentage of the total area.

Using the Broad Standard Table provided in the leaflet supplied with *heparin low-molecular-mass for calibration CRS*, identify those points in the chromatogram for which the percentage of the total area under the curve is closest to the percentage mass fractions listed in the Broad Standard Table, and assign the molecular mass in the table to the corresponding retention time in the chromatogram. A calibration curve for the chromatographic system is derived by fitting a suitable mathematical relationship to the set of retention times and logarithms of the molecular masses. A polynomial of the 3rd degree is recommended.

Inject 25 µL of the test solution and record the chromatogram for a period of time, ensuring complete elution of sample, salt and solvent peaks.

The mass-average relative molecular mass is defined by the following expression:

$$\frac{\sum(RI_i M_i)}{\sum RI_i}$$

$RI_i$ = refractive index of the substance eluting in the fraction *i* (proportional to the mass of the substance);

$M_i$ = relative molecular mass corresponding to fraction *i*.

Any low-molecular-mass heparin covered by an individual monograph complies with the requirements for molecular mass parameters assessed in identification test C in the corresponding monograph.

Where no individual monograph exists for the low-molecular-mass heparin to be examined, the mass-average relative molecular mass is not greater than 8000 and at least 60 per cent of the total mass has a relative molecular mass lower than 8000. In addition, the molecular mass parameters (mass-average molecular mass and mass percentages of chains comprised between specified values) correspond to those of the manufacturer's reference preparation.

D. It gives reaction (a) of sodium or the reactions of calcium (as appropriate) (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.5 to 8.0.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

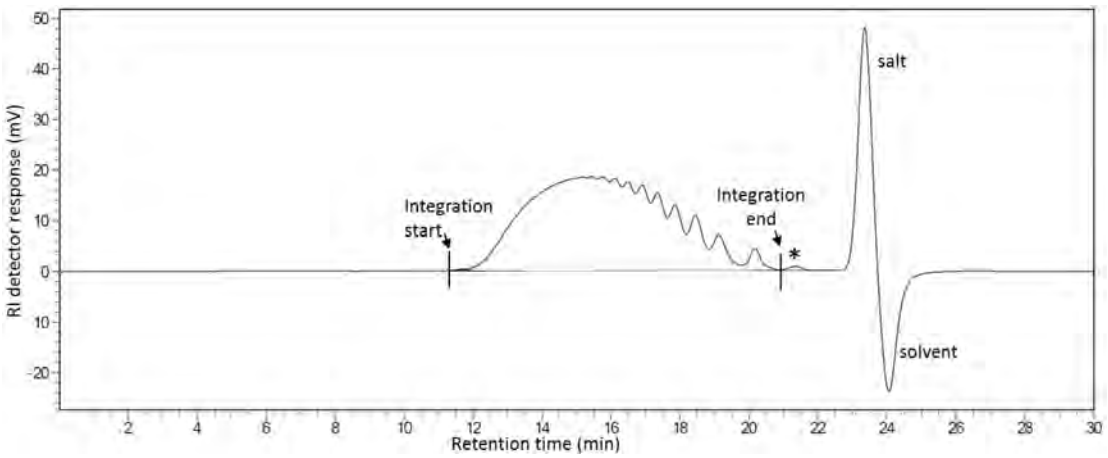


Figure 0828.-1. – *Example chromatogram of a low-molecular-mass heparin sample analysed using the method described in identification test C indicating peak integration*

**Nitrogen** (*2.5.9*): 1.5 per cent to 2.5 per cent (dried substance).

**Calcium** (*2.5.11*): 9.5 per cent to 11.5 per cent (dried substance), if prepared from heparin complying with the monograph *Heparin calcium (0332)*. Use 0.200 g.

**Sodium**: 10.5 per cent to 13.5 per cent (dried substance), if prepared from heparin complying with the monograph *Heparin sodium (0333)*.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dissolve 50 mg in *0.1 M hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Prepare reference solutions (25 ppm, 50 ppm and 75 ppm) using *sodium standard solution (200 ppm Na) R* diluted with *0.1 M hydrochloric acid* containing 1.27 mg of *caesium chloride R* per millilitre.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 330.3 nm.

*Atomisation device*: flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Molar ratio of sulfate ions to carboxylate ions** (*2.2.38*): minimum 1.8.

*The sample of heparin used in this titration must be free from ionisable impurities, particularly salts*.

Weigh 0.100 g of the substance to be examined taking the necessary measures to avoid the problems linked to hygroscopicity.

Take up into about 20 mL of double-glass-distilled *water R*. Cool to 4 °C and apply 2.0 mL of this solution to a pre-cooled column (approximately 10 × 1 cm), packed with a suitable *cation-exchange resin R*. Wash through with double-glass-distilled *water R* into the titration vessel up to a final volume of about 10-15 mL (*the titration vessel must be just large enough to hold the electrodes from the conductivity meter, a small stirrer bar and a fine flexible tube from the outlet of a 2 mL burette)*. Stir magnetically. When the conductivity reading is constant, note it and titrate with *0.05 M sodium hydroxide* added in approximately 50 µL portions. Record the burette level and the conductivity meter reading a few seconds after each addition until the end-point is reached.

For each measured figure, calculate the number of milliequivalents of sodium hydroxide added from the volume and the known concentration of the sodium hydroxide solution. Plot on a graph the figures for conductivity (as *y*-axis) against the figures of milliequivalent of sodium hydroxide (as *x*-axis). The graph will have 3 approximately linear sections: an initial steep downward slope, a middle slight rise and a final steep rise. Estimate the best straight lines through these 3 parts of the graph. At the points where the 1st and 2nd lines intersect, and where the 2nd and 3rd lines intersect, draw perpendiculars to the *x*-axis to estimate the milliequivalents of sodium hydroxide taken up by the sample at those points. The point where the 1st and 2nd lines intersect will give the number of milliequivalents of sodium hydroxide taken up by the sulfate groups, and the point where the 2nd and 3rd lines intersect will give the number of milliequivalents taken up by the sulfate and carboxylate groups together. The difference between the 2 will therefore give the number of milliequivalents taken up by the carboxylate groups.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.67 kPa for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.01 IU per International Unit of anti-Xa activity, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. The addition of divalent cations may be necessary to fulfil the validation criteria.

ASSAY

The anticoagulant activity of low-molecular-mass heparins is determined *in vitro* by 2 assays which determine its ability to accelerate the inhibition of factor Xa (anti-Xa assay) and thrombin, factor IIa (anti-IIa assay), by antithrombin III.

The International Units for anti-Xa and anti-IIa activity are the activities contained in a stated amount of the International Standard for low-molecular-mass heparin.

*Heparin low-molecular-mass for assay BRP*, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as reference preparation.

*ANTI-FACTOR Xa ACTIVITY*

**Reference and test solutions**

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low-molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*; the concentration range should be within 0.025 IU to 0.2 IU of anti-factor Xa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

**Procedure**

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_1$, $T_2$, $T_3$, $T_4$ for each of the 4 series of dilutions of the substance to be examined and $S_1$, $S_2$, $S_3$, $S_4$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R1* and 50 µL of the appropriate dilution of the substance to be examined, or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_1$, $S_2$, $S_3$, $S_4$, $T_1$, $T_2$, $T_3$, $T_4$, $T_1$, $T_2$, $T_3$, $T_4$, $S_1$, $S_2$, $S_3$, $S_4$, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *bovine factor Xa solution R*. Incubate for exactly 1 min and add 250 µL of *chromogenic substrate R1*. Stop the reaction after exactly 4 min by adding 375 µL of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins and calculate the potency of the substance to be examined in International Units of anti-factor Xa activity per millilitre using the usual statistical methods for parallel-line assays.

*ANTI-FACTOR IIa ACTIVITY*

**Reference and test solutions**

Prepare 4 independent series of 4 dilutions each, of the substance to be examined and of the reference preparation of low molecular-mass heparin in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*; the concentration range should be within 0.015 IU to 0.075 IU of anti-factor IIa activity per millilitre and the dilutions chosen should give a linear response when results are plotted as absorbance against log concentration.

**Procedure**

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: $T_1$, $T_2$, $T_3$, $T_4$ for each of the 4 series of dilutions of the substance to be examined and $S_1$, $S_2$, $S_3$, $S_4$ for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R2* and 50 µL of the appropriate dilution of the substance to be examined or the reference

preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order $S_1$, $S_2$, $S_3$, $S_4$, $T_1$, $T_2$, $T_3$, $T_4$, $T_1$, $T_2$, $T_3$, $T_4$, $S_1$, $S_2$, $S_3$, $S_4$, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *human thrombin solution R*. Incubate for exactly 1 min and add 250 µL of *chromogenic substrate R2*. Stop the reaction after exactly 4 min by adding 375 µL of *acetic acid R*. Transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm. Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* instead of the reference and test solutions; the 2 blank values do not differ significantly. Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of the reference preparation of low-molecular-mass heparins, and calculate the potency of the substance to be examined in International Units of anti-factor IIa activity per millilitre using the usual statistical methods for parallel-line assays.

LABELLING

The label states:

- the number of International Units of anti-factor Xa activity per milligram;
- the number of International Units of anti-factor IIa activity per milligram;
- the mass-average molecular mass and the percentage of molecules within defined molecular mass ranges;
- where applicable, that the contents are the sodium salt;
- where applicable, that the contents are the calcium salt.

STORAGE

In an airtight tamper-evident container. If the product is sterile and free of bacterial endotoxins, store in a sterile and apyrogenic container.

**01/2017:1980**
**corrected 10.0**

# HEPTAMINOL HYDROCHLORIDE

## Heptaminoli hydrochloridum

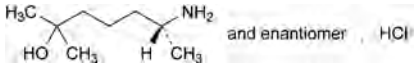


$C_8H_{20}ClNO$ $M_r$ 181.7
[543-15-7]

DEFINITION

(6*RS*)-6-Amino-2-methylheptan-2-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. To 1 mL of solution S (see Tests) add 4 mL of *water R* and 2 mL of a 200 g/L solution of *ammonium and cerium nitrate R* in *dilute nitric acid R1*. An orange-brown colour develops.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *heptaminol hydrochloride CRS*.

C. Examine the chromatograms obtained in the test for related substances.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (b).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.3 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.6 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a)*. Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a)*. Dilute 3.0 mL of test solution (a) to 10.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 50.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 0.10 g of *heptaminol hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (c)*. Dissolve 10.0 mg of *heptaminol impurity A CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (c) to 10.0 mL with *methanol R*.

*Reference solution (e)*. To 2.5 mL of reference solution (c) add 0.5 mL of test solution (b) and dilute to 5 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *dioxan R*, *2-propanol R* (10:50:50 *V/V/V*).

*Application*: 10 µL; apply test solutions (a) and (b) and reference solutions (a), (b), (d) and (e).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour for at least 15 h.

*System suitability*: the chromatogram obtained with reference solution (e) shows 2 clearly separated principal spots and the chromatogram obtained with reference solution (a) shows a single principal spot.

*Limits*: in the chromatogram obtained with test solution (a):

- *impurity A*: any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.2 per cent),
- *any other impurity*: any spot, apart from the principal spot and any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.6 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 50 mL of *alcohol R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.17 mg of $C_8H_{20}ClNO$.

IMPURITIES

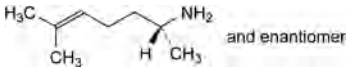


A. (2*RS*)-6-methylhept-5-en-2-amine.

**04/2016:1436**

# HEXAMIDINE DIISETHIONATE

## Hexamidini diisetionas

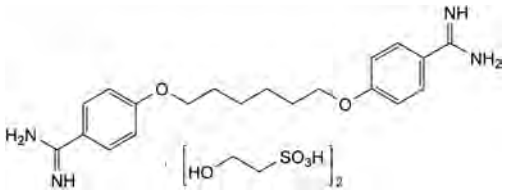


$C_{24}H_{38}N_4O_{10}S_2$ $M_r$ 607
[659-40-5]

DEFINITION

4,4′-[Hexane-1,6-diylbis(oxy)]dibenzimidamide bis(2-hydroxyethanesulfonate).

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

PRODUCTION

It is considered that alkyl 2-hydroxyethanesulfonate esters are potential impurities in hexamidine diisetionate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation including, where necessary, demonstration that alkyl 2-hydroxyethanesulfonate esters are not detectable in the final product.

CHARACTERS

*Appearance*: white or slightly yellow powder, hygroscopic.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *hexamidine diisetionate CRS*.

B. Dissolve about 40 mg in 5 mL of *water R* and add dropwise with shaking 1 mL of a 100 g/L solution of *sodium chloride R*. Allow to stand for 5 min. An abundant, shimmering white precipitate is slowly formed.

TESTS

**Appearance of solution**. Dissolve 0.50 g in *carbon dioxide-free water R*, heating at about 70 °C and dilute to 10 mL with the same solvent. Allow to cool to room temperature for 10-15 min. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 2.0 g in *water R* heating at about 50 °C and dilute to 20 mL with *water R* heating at about 50 °C. Allow to cool to about 35 °C, add 0.1 mL of *methyl red solution R*. Not more than 0.25 mL of *0.05 M hydrochloric acid* or *0.05 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 5 mg of the substance to be examined and 5 mg of *pentamidine diisetionate CRS* in mobile phase A and dilute to 100 mL with mobile phase A. Dilute 2 mL of the solution to 5 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm).

*Mobile phase*:
- *mobile phase A*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*,
- *mobile phase B*: mix equal volumes of *acetonitrile R* and of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 0 | 0 → 100 |
| 30 - 35 | 0 | 100 |
| 35 - 40 | 0 → 100 | 100 → 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 263 nm.

*Injection*: 20 µL.

*Relative retention* with reference to hexamidine (retention time = about 6 min): impurity B = about 1.7; impurity A = about 2.0; impurity C = about 3.7; impurity D = about 4.7.

*System suitability*: reference solution (c):
- *resolution*: minimum 5.0 between the peaks due to hexamidine and pentamidine.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide* under a current of *nitrogen R*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 30.35 mg of $C_{24}H_{38}N_4O_{10}S_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D.*

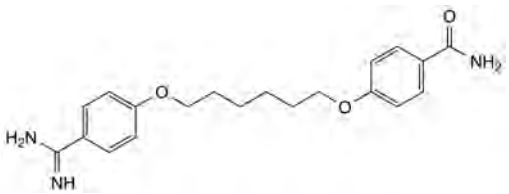

A. 4-[[6-(4-carbamimidoylphenoxy)hexyl]oxy]benzamide,

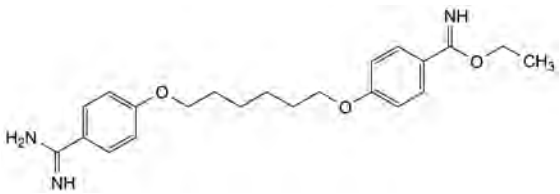

B. ethyl 4-[[6-(4-carbamimidoylphenoxy)hexyl]oxy]-benzimidoate,

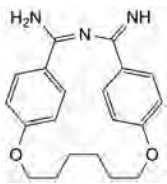

C. 4-imino-9,16-dioxa-3-azatricyclo[15.2.2.2$^{5,8}$]tricosa-1(19),2,5,7,17,20,22-heptaen-2-amine,

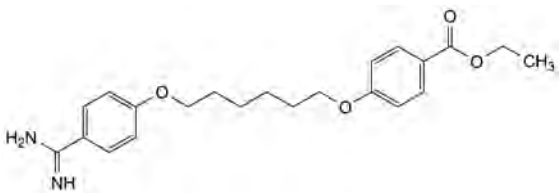

D. ethyl 4-[[6-(4-carbamimidoylphenoxy)hexyl]oxy]benzoate.

**01/2017:1221**

# HEXETIDINE

## Hexetidinum

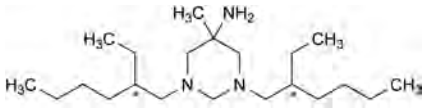

$C_{21}H_{45}N_3$ $M_r$ 339.6
[141-94-6]

DEFINITION

Hexetidine contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1,3-bis(2-ethylhexyl)]-5-methylhexahydropyrimidin-5-amine.

CHARACTERS

An oily liquid, colourless or slightly yellow, very slightly soluble in water, very soluble in acetone, in alcohol and in methylene chloride. It dissolves in dilute mineral acids.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *hexetidine CRS*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 0.2 mL add 2 mL of *sulfuric acid R* and 2 mg of *chromotropic acid, sodium salt R*. Heat in a water-bath at 60 °C. A violet colour develops.

D. Dissolve 0.2 mL in 1 mL of *methylene chloride R*. Add 0.5 mL of *copper sulfate solution R*, 0.05 mL of *0.25 M alcoholic sulfuric acid R* and 5 mL of *water R*. Shake, then allow to stand. The lower layer becomes deep blue.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*)and not more intensely coloured than reference solution $Y_5$ or reference solution $GY_5$ (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 0.864 to 0.870.

**Refractive index** (*2.2.6*): 1.461 to 1.467.

**Optical rotation** (*2.2.7*). Dissolve 1.0 g in *ethanol R* and dilute to 10.0 mL with the same solvent. The angle of optical rotation is − 0.10° to + 0.10°.

**Absorbance** (*2.2.25*). Dissolve 0.50 g in *heptane R* and dilute to 50.0 mL with the same solvent. At wavelengths from 270 nm to 350 nm, the absorbance of the solution is not greater than 0.1.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel H R* as the coating substance. *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 2.0 g of the substance to be examined in *heptane R* and dilute to 20 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *heptane R*.

*Reference solution (a).* Dissolve 20 mg of *hexetidine CRS* in *heptane R* and dilute to 2 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (a) to 100 mL with *heptane R*.

*Reference solution (c).* Dilute 5 mL of reference solution (b) to 10 mL with *heptane R*.

*Reference solution (d).* Dissolve 10 mg of *dehydrohexetidine CRS* in test solution (a) and dilute to 10 mL with the same solution.

Apply separately to the plate 1 µL of each solution. At the bottom of a chromatographic tank, place an evaporating dish containing *concentrated ammonia R1*. Place the dried plate in the tank and close the tank. Leave the plate in contact with the ammonia vapour for 15 min. Withdraw the plate and place it in a current of air to remove the ammonia vapour. Develop over a path of 15 cm using a mixture of 20 volumes of *methanol R* and 80 volumes of *toluene R*. Allow the plate to dry in air. Expose the plate to iodine vapour for 30 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.98 mg of $C_{21}H_{45}N_3$.

STORAGE

Store protected from light.

IMPURITIES

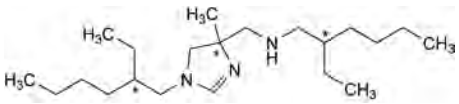

A. 2-ethyl-*N*-[[1-(2-ethylhexyl)-4-methyl-4,5-dihydro-1*H*-imidazol-4-yl]methyl]hexan-1-amine (dehydrohexetidine),

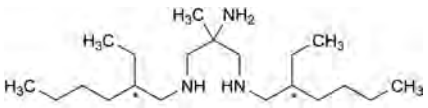

B. $N^1$,$N^3$-bis(2-ethylhexyl)-2-methylpropane-1,2,3-triamine (triamine),

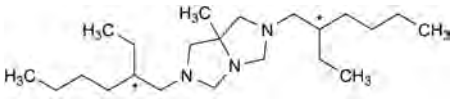

C. 2,6-bis(2-ethylhexyl)-7a-methylhexahydro-1*H*-imidazo-[1,5-*c*]imidazole (hexedine),

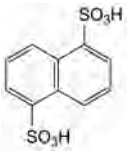

D. naphthalene-1,5-disulfonic acid.

**01/2016:1437**

# HEXYLRESORCINOL

## Hexylresorcinolum

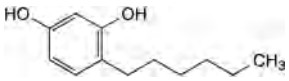

$C_{12}H_{18}O_2$ $M_r$ 194.3
[136-77-6]

DEFINITION

4-Hexylbenzene-1,3-diol.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: colourless, yellowish or reddish, crystalline powder or needles, turning brownish-pink on exposure to light or air.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 66 °C to 68 °C, melting may occur at about 60 °C, followed by solidification and a second melting between 66 °C and 68 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hexylresorcinol CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 0.1 mL of solution S (see Tests) to 10 mL with *ethanol (96 per cent) R*.

*Reference solution (a).* Dissolve 10 mg of *hexylresorcinol CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *hexylresorcinol CRS* and 10 mg of *resorcinol R* in *ethanol (96 per cent) R*, then dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *methyl ethyl ketone R*, *pentane R* (50:50 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 5 min.

*Detection*: spray with 3 mL of *anisaldehyde solution R* and heat at 100-105 °C for 5 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve 0.1 g in 1 mL of *ethanol (96 per cent) R*. Add one drop of *ferric chloride solution R1*. A green colour is produced. Add *dilute ammonia R1*. The solution changes to brown.

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Acidity**. Dissolve 0.5 g in a mixture of 25 mL of *carbon dioxide-free water R* and 25 mL of *ether R* previously neutralised to *phenolphthalein solution R1* and titrate with *0.1 M sodium hydroxide*, shaking vigorously after each addition. Not more than 0.4 mL is required to change the colour of the solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *hexylresorcinol for system suitability CRS* (containing impurities C and D) in 1.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 30 volumes of a 3.0 g/L solution of *glacial acetic acid R* previously adjusted to pH 5.9 with *dilute ammonia R1*, and 70 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 281 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of hexylresorcinol.

*Identification of impurities*: use the chromatogram supplied with *hexylresorcinol for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to hexylresorcinol (retention time = about 10 min): impurity C = about 0.7; impurity D = about 1.1.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to hexylresorcinol.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity D by 0.3;
- for each impurity, use the concentration of hexylresorcinol in reference solution (a).

*Limits*:

- *impurities C, D*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 10 mL of *methanol R* in a ground-glass-stoppered flask, add 30.0 mL of *0.0167 M potassium bromate* and 2 g of *potassium bromide R*. Shake to dissolve the substance and add 15 mL of *dilute sulfuric acid R*. Stopper the flask, shake and allow to stand in the dark for 15 min, stirring continuously. Add 5 mL of *methylene chloride R* and a solution of 1 g of *potassium iodide R* in 10 mL of *water R*, allow to stand in the dark for 15 min, stirring continuously. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R*, shaking thoroughly. Carry out a blank titration under the same conditions.

1 mL of *0.0167 M potassium bromate* is equivalent to 4.857 mg of $C_{12}H_{18}O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

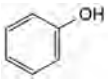

A. phenol,

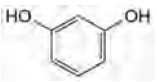

B. benzene-1,3-diol (resorcinol),

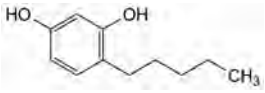

C. 4-pentylbenzene-1,3-diol,

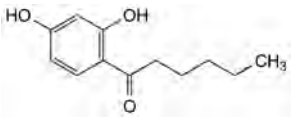

D. 1-(2,4-dihydroxyphenyl)hexan-1-one.

**01/2008:0143**
**corrected 6.0**

# HISTAMINE DIHYDROCHLORIDE

## Histamini dihydrochloridum

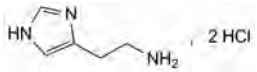

$C_5H_{11}Cl_2N_3$ $M_r$ 184.1
[56-92-8]

DEFINITION

Histamine dihydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-(1*H*-imidazol-4-yl)ethan-1-amine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, soluble in alcohol.

IDENTIFICATION

*First identification: A, D.*

*Second identification*: *B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *histamine dihydrochloride CRS*. Examine as discs prepared using 1 mg of substance.

B. Examine the chromatograms obtained in the test for histidine. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 7 mL of *water R* and add 3 mL of a 200 g/L solution of *sodium hydroxide R*. Dissolve 50 mg of *sulfanilic acid R* in a mixture of 0.1 mL of *hydrochloric acid R* and 10 mL of *water R* and add 0.1 mL of *sodium nitrite solution R*. Add the second solution to the first and mix. A red colour is produced.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 2.85 to 3.60.

**Histidine**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel G plate R*.

*Test solution (a).* Dissolve 0.5 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 2 mL of test solution (a) to 10 mL with *water R*.

*Reference solution (a).* Dissolve 0.1 g of *histamine dihydrochloride CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 50 mg of *histidine monohydrochloride R* in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (c).* Mix 1 mL of test solution (a) and 1 mL of reference solution (b).

Apply to the plate 1 µL of test solution (a), 1 µL of test solution (b), 1 µL of reference solution (a), 1 µL of reference solution (b) and 2 µL of reference solution (c). Develop over a path of 15 cm using a mixture of 5 volumes of *concentrated ammonia R*, 20 volumes of *water R* and 75 volumes of *acetonitrile R*. Dry the plate in a current of air. Repeat the development in the same direction, dry the plate in a current of air and spray with *ninhydrin solution R1*. Heat the plate at 110 °C for 10 min. Any spot corresponding to histidine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

**Sulfates** (*2.4.13*). 3 mL of solution S diluted to 15 mL with *distilled water R* complies with the limit test for sulfates (0.1 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 0.20 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.080 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the first and third points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 9.203 mg of $C_5H_{11}Cl_2N_3$.

STORAGE

Store in an airtight container, protected from light.

**01/2017:0911**

# HISTIDINE

## Histidinum

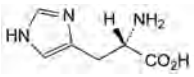

$C_6H_9N_3O_2$ $M_r$ 155.2

[71-00-1]

DEFINITION

(2*S*)-2-Amino-3-(1*H*-imidazol-4-yl)propanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *histidine CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *histidine CRS* in *water R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.1 g in 7 mL of *water R* and add 3 mL of a 200 g/L solution of *sodium hydroxide R*. Dissolve 50 mg of *sulfanilic acid R* in a mixture of 0.1 mL of *hydrochloric acid R* and 10 mL of *water R* and add 0.1 mL of *sodium nitrite solution R*. Add the second solution to the first and mix. An orange-red colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R*, heating in a water-bath, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 11.4 to + 12.4 (dried substance).

Dissolve 2.75 g in 12.0 mL of *hydrochloric acid R1* and dilute to 25.0 mL with *water R*.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of histidine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 50 mL of *water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 15.52 mg of $C_6H_9N_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

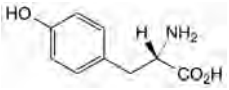


A. (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid (tyrosine).

**01/2017:0910**
**corrected 10.0**

# HISTIDINE HYDROCHLORIDE MONOHYDRATE

## Histidini hydrochloridum monohydricum

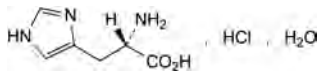


$C_6H_{10}ClN_3O_2,H_2O$ $M_r$ 209.6
[5934-29-2]

DEFINITION

(2*S*)-2-Amino-3-(1*H*-imidazol-4-yl)propanoic acid hydrochloride monohydrate.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B, C, F.*

*Second identification*: *A, B, D, E, F.*

A. Specific optical rotation (see Tests).

B. pH (see Tests).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *histidine hydrochloride monohydrate CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *histidine hydrochloride monohydrate CRS* in *water R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve 0.1 g in 7 mL of *water R* and add 3 mL of a 200 g/L solution of *sodium hydroxide R*. Dissolve 50 mg of *sulfanilic acid R* in a mixture of 0.1 mL of *hydrochloric acid R* and 10 mL of *water R* and add 0.1 mL of *sodium nitrite solution R*. Add the second solution to the first and mix. An orange-red colour develops.

F. About 20 mg gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 9.2 to + 10.6 (dried substance).

Dissolve 2.75 g in 12.0 mL of *hydrochloric acid R1* and dilute to 25.0 mL with *water R*.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50 mL with solution A. Dilute 1 mL of the solution to 200 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of histidine hydrochloride monohydrate in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): 7.0 per cent to 10.0 per cent, determined on 1.000 g by drying in an oven at 145-150 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 19.16 mg of $C_6H_{10}ClN_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

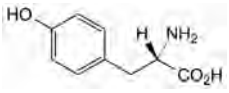

A. (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid (tyrosine).

**01/2008:0500**
**corrected 6.0**

# HOMATROPINE HYDROBROMIDE

## Homatropini hydrobromidum

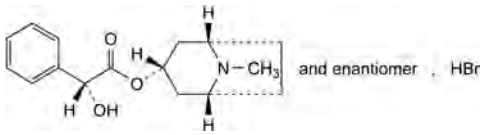


$C_{16}H_{22}BrNO_3$ $M_r$ 356.3
[51-56-9]

DEFINITION

(1*R*,3*r*,5*S*)-8-Methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-2-hydroxy-2-phenylacetate hydrobromide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, sparingly soluble in alcohol.

mp: about 215 °C, with decomposition.

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *homatropine hydrobromide CRS.*

B. Dissolve 50 mg in 1 mL of *water R* and add 2 mL of *dilute acetic acid R*. Heat and add 4 mL of *picric acid solution R*. Allow to cool, shaking occasionally. Collect the crystals, wash with 2 quantities, each of 3 mL, of iced *water R* and dry at 100-105 °C. The crystals melt (*2.2.14*) at 182 °C to 186 °C.

C. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5.0 mg of *hyoscine hydrobromide CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10.0 mL of this solution add 0.5 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.1 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm),
- *temperature*: 40 °C.

*Mobile phase*: mix 33 volumes of *methanol R2* and 67 volumes of a solution prepared as follows: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 7.0 g of *sodium heptanesulfonate monohydrate R* in 1000 mL of *water R* and adjust to pH 2.7 with a 330 g/L solution of *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of homatropine.

*Relative retention* with reference to homatropine (retention time = about 6.8 min): impurity C = about 0.2; impurity A = about 0.9; impurity B = about 1.1; impurity D = about 1.9.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to homatropine and impurity B,
- *symmetry factor*: maximum 2.5 for the peak due to homatropine.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *impurities B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); disregard the peak due to the bromide ion which appears close to the peak due to the solvent,
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.63 mg of $C_{16}H_{22}BrNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

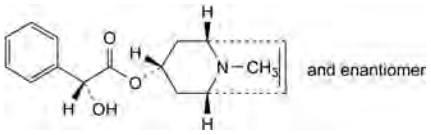


A. (1*R*,3*s*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-6-en-3-yl (2*RS*)-2-hydroxy-2-phenylacetate (dehydrohomatropine),

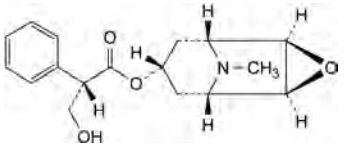

B. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo-[3.3.1.$0^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscine),

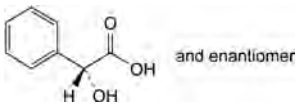


C. (2*RS*)-2-hydroxy-2-phenylacetic acid (mandelic acid),

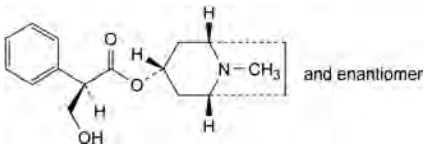


D. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate (atropine).

**01/2012:0720**

# HOMATROPINE METHYLBROMIDE

## Homatropini methylbromidum

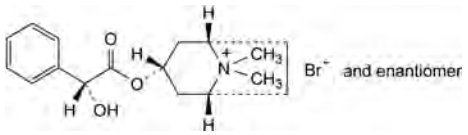


$C_{17}H_{24}BrNO_3$ $M_r$ 370.3

[80-49-9]

DEFINITION

(1*R*,3*r*,5*S*)-3-[[(2*RS*)-2-Hydroxy-2-phenylacetyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane bromide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, soluble in ethanol 96 per cent.

mp: about 190 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *homatropine methylbromide CRS*.

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, mobile phase A (9:41 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5.0 mg of *homatropine hydrobromide CRS* (impurity B) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. To 10.0 mL of the solution add 0.5 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 2.0 mg of *homatropine methylbromide for system suitability CRS* (containing impurity A) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.4 g of *potassium dihydrogen phosphate R* and 5.0 g of *sodium pentanesulfonate monohydrate R* in 980 mL of *water for chromatography R*, adjust to pH 3.0 with a 330 g/L solution of *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: mix 400 mL of mobile phase A and 600 mL of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 70 | 30 |
| 2 - 15 | 70 → 30 | 30 → 70 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Relative retention* with reference to homatropine methylbromide (retention time = about 5 min): impurity A = about 0.9; impurity B = about 1.2.

*Identification of impurities*: use the chromatogram supplied with *homatropine methylbromide for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*System suitability*:

- *resolution*: minimum 2.5 between the peaks due to homatropine methylbromide and impurity B in the chromatogram obtained with reference solution (c);
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to homatropine methylbromide in the chromatogram obtained with reference solution (d).

*Limits*:

- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent); disregard the peak due to the bromide ion which appears close to the peak due to the solvent;
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 10 mL of *water R*. Titrate with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*), using a silver indicator electrode and a silver-silver chloride reference electrode.

1 mL of *0.1 M silver nitrate* is equivalent to 37.03 mg of $C_{17}H_{24}BrNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F.*

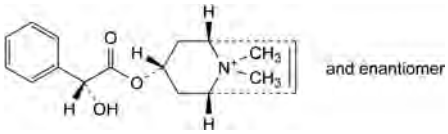


A. (1*R*,3*s*,5*S*)-3-[[(2*RS*)-2-hydroxy-2-phenylacetyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]oct-6-ene (methyldehydrohomatropine),

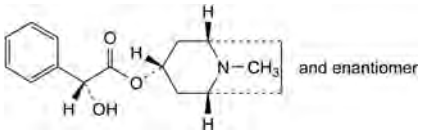


B. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-2-hydroxy-2-phenylacetate (homatropine),

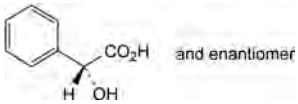


C. (2*RS*)-2-hydroxy-2-phenylacetic acid (mandelic acid),

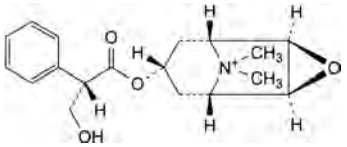

D. (1*R*,2*R*,4*S*,5*S*,7*s*)-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]-oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane (methylhyoscine),

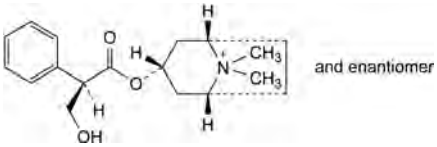


E. (1*R*,3*r*,5*S*)-3-[[(2*RS*)-3-hydroxy-2-phenylpropanoyl]oxy]-8,8-dimethyl-8-azoniabicyclo[3.2.1]octane (methylatropine),

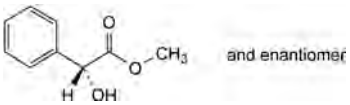


F. methyl (2*RS*)-2-hydroxy-2-phenylacetate (methyl mandelate).

**01/2008:2051**

# HONEY

## Mel

DEFINITION

Honey is produced by bees (*Apis mellifera* L.) from the nectar of plants or from secretions of living parts of plants which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature.

PRODUCTION

If the bee has been exposed to treatment to prevent or cure diseases or to any substance intended for preventing, destroying or controlling any pest, unwanted species of plants or animals, appropriate measures are taken to ensure that the levels of residues are as low as possible.

CHARACTERS

*Appearance*: viscous liquid which may be partly crystalline, almost white to dark brown.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.6 g of the substance to be examined in 50 mL of *ethanol (30 per cent V/V) R*.

*Reference solution.* Dissolve 0.5 g of *fructose R*, 0.5 g of *glucose R* and 0.1 g of *sucrose R* in 100 mL of *ethanol (30 per cent V/V) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (13:87 *V/V*).

*Application*: 5 µL as bands.

*Development*: 3 times over a path of 15 cm.

*Drying*: in warm air.

*Detection*: spray with a solution prepared as follows: dissolve 2 g of *diphenylamine R* and 2 mL of *aniline R* in 100 mL of *acetone R*. Add a 850 g/L solution of *phosphoric acid R* until the precipitate formed dissolves again (about 15-20 mL). Examine in daylight after heating at 100-105 °C for 5-10 min.

*Results*: see below the sequence of the zones present in the chromatograms obtained with the reference solution and the test solution. Furthermore, the weak brown zone due to sucrose in the chromatogram obtained with the reference solution may be present in the chromatogram obtained with the test solution. One or more other weak zones may be present in the chromatogram obtained with the test solution.

| Top of the plate | |
|---|---|
| ——— | ——— |
| Fructose: an intense brown zone | An intense brown zone (fructose) |
| Glucose: an intense greyish-blue zone | An intense greyish-blue zone (glucose) |
| ——— | ——— |
| Sucrose: a brown zone | |
| | 2 to 3 brownish-grey zones |
| **Reference solution** | **Test solution** |

TESTS

**Refractive index** (*2.2.6*): minimum 1.487 (equivalent to a maximum water content of 20 per cent).

Homogenise 100 g and transfer into a flask. Close tightly and place in a water-bath at 50 ± 0.2 °C until all sugar crystals have dissolved. Cool the solution to 20 °C and rehomogenise. Immediately after rehomogenisation, cover the surface of the refractometer prism evenly with the sample. Determine the refractive index after 2 min if using an Abbe refractometer and after 4 min if using a digital refractometer. Use the average value of 2 determinations.

**Conductivity** (*2.2.38*): maximum 800 $\mu S \cdot cm^{-1}$.

Using the value obtained for the refractive index, determine the water content of the substance to be examined from Table 2051.-1. Using this information, dissolve an amount of the substance to be examined equivalent to 20.0 g of honey dry solids, in *water R* to produce 100.0 mL.

**Optical rotation** (*2.2.7*): maximum + 0.6°.

Using the value obtained for the refractive index, determine the water content of the substance to be examined from Table 2051.-1. Using this information, dissolve an amount of the substance to be examined, equivalent to 20.0 g of honey dry solids, in 50 mL of *water R*. Add 0.2 mL of *concentrated ammonia R* and dilute to 100.0 mL with *water R*. If necessary decolourise the solution with *activated charcoal R*.

Table 2051.-1. – *Relationship of water content of honey to refractive index*

| Water content (per cent *m/m*) | Refractive index at 20 °C |
|---|---|
| 15.0 | 1.4992 |
| 15.2 | 1.4987 |
| 15.4 | 1.4982 |
| 15.6 | 1.4976 |
| 15.8 | 1.4971 |
| 16.0 | 1.4966 |
| 16.2 | 1.4961 |
| 16.4 | 1.4956 |
| 16.6 | 1.4951 |
| 16.8 | 1.4946 |
| 17.0 | 1.4940 |
| 17.2 | 1.4935 |
| 17.4 | 1.4930 |
| 17.6 | 1.4925 |
| 17.8 | 1.4920 |
| 18.0 | 1.4915 |
| 18.2 | 1.4910 |
| 18.4 | 1.4905 |
| 18.6 | 1.4900 |
| 18.8 | 1.4895 |
| 19.0 | 1.4890 |
| 19.2 | 1.4885 |
| 19.4 | 1.4880 |
| 19.6 | 1.4875 |
| 19.8 | 1.4870 |
| 20.0 | 1.4865 |

**5-Hydroxymethylfurfural**: maximum 80 ppm, calculated on dry solids.

Using the value obtained for the refractive index, determine the water content of the substance to be examined from Table 2051.-1. Using this information, dissolve an amount of the substance to be examined, equivalent to 5.0 g of honey dry solids, in 25 mL of *water R* and transfer to a 50.0 mL volumetric flask with the same solvent. Add 0.5 mL of a 150 g/L solution of *potassium ferrocyanide R* and mix. Add 0.5 mL of a 300 g/L solution of *zinc acetate R*, mix and dilute to 50.0 mL with *water R* (a drop of *anhydrous ethanol R* may be added to avoid foaming). Filter. Transfer 5.0 mL of the filtered solution into each of 2 tubes. To one tube add 5.0 mL of *water R* (test solution). To the other tube add 5.0 mL of a 2.0 g/L solution of *sodium hydrogensulfite R* (reference solution). Determine the absorbance (*2.2.25*) of the test solution against the reference solution at 284 nm and 336 nm within 60 min. If the absorbance at 284 nm is greater than 0.8, dilute to the same extent the test solution with *water R* and the reference solution with a 2.0 g/L solution of *sodium hydrogensulfite R* so as to obtain an absorbance of less than 0.8.

Calculate the content of 5-hydroxymethylfurfural from the expression:

$$(A_1 - A_2) \times D \times 149.7$$

$A_1$ = absorbance at 284 nm,

$A_2$ = absorbance at 336 nm,

$D$ = dilution factor, where applicable.

**Chlorides** (*2.4.4*): maximum 350 ppm, determined on 15 mL of a 10 g/L solution.

**Sulfates** (*2.4.13*): maximum 250 ppm, determined on 15 mL of a 40 g/L solution.

**01/2013:0255**
**corrected 8.0**

# HUMAN ALBUMIN SOLUTION

## Albumini humani solutio

DEFINITION

Sterile liquid preparation of a plasma protein fraction containing human albumin. It is obtained from plasma that complies with the monograph *Human plasma for fractionation (0853)*. The preparation may contain excipients such as sodium caprylate (sodium octanoate) or *N*-acetyltryptophan or a combination of the two.

PRODUCTION

Separation of the albumin is carried out under controlled conditions, particularly of pH, ionic strength and temperature so that in the final product not less than 95 per cent of the total protein is albumin. Human albumin solution is prepared as a concentrated solution containing 150-250 g/L of total protein or as an isotonic solution containing 35-50 g/L of total protein. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter and distributed aseptically into sterile containers which are then closed so as to prevent contamination. The solution in its final container is heated to 60 ± 1.0 °C and maintained at this temperature for not less than 10 h. The containers are then incubated at 30-32 °C for not less than 14 days or at 20-25 °C for not less than 4 weeks and examined visually for evidence of microbial contamination.

CHARACTERS

*Appearance*: clear, slightly viscous liquid, almost colourless, yellow, amber or green.

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/L of protein. The main component of the preparation to be examined corresponds to the main component of normal human serum. The preparation may show the presence of small quantities of other plasma proteins.

TESTS

**pH** (*2.2.3*): 6.7 to 7.3.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing 10 g/L of protein.

**Total protein**. If necessary, dilute an accurately measured volume of the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of this solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the quantity of protein by multiplying by 6.25. The protein content is not less than 95 per cent and not more than 105 per cent of the stated content.

**Protein composition**. Zone electrophoresis (*2.2.31*).

Use strips of suitable cellulose acetate gel or agarose gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

If cellulose acetate is the supporting material, the method described below can be used. If agarose gels are used, and because they are normally part of an automated system, the manufacturer's instructions are followed instead.

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a protein concentration of 20 g/L.

*Reference solution*. Dilute *human albumin for electrophoresis BRP* with a 9 g/L solution of *sodium chloride R* to a protein concentration of 20 g/L.

To a strip apply 2.5 µL of the test solution as a 10 mm band or apply 0.25 µL per millimetre if a narrower strip is used. To another strip, apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the most rapid band migrates at least 30 mm. Treat the strips with *amido black 10B solution R* for 5 min. Decolorise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* until the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

*System suitability*: in the electropherogram obtained with the reference solution on cellulose acetate or on agarose gels, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

*Results*: in the electropherogram obtained with the test solution on cellulose acetate or on agarose gels, not more than 5 per cent of the protein has a mobility different from that of the principal band.

**Molecular-size distribution**. Size exclusion chromatography (*2.2.30*).

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range of 4-12 g/L and injection of 50-600 µg of protein are usually suitable.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7.5 mm, or *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

*Mobile phase*: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 L of *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

The peak due to polymers and aggregates is located in the part of the chromatogram representing the void volume. Disregard the peak due to the stabiliser. The area of the peak due to polymers and aggregates is not greater than 10 per cent of the total area of the chromatogram. This represents not more than 5 per cent when expressed in percentage of protein considering the difference in response factor between the albumin monomer and the polymers and aggregates.

**Haem**. Dilute the preparation to be examined using a 9 g/L solution of *sodium chloride R* to obtain a solution containing 10 g/L of protein. The absorbance (*2.2.25*) of the solution measured at 403 nm using *water R* as the compensation liquid is not greater than 0.15.

**Prekallikrein activator** (*2.6.15*): maximum 35 IU/mL.

**Aluminium**: maximum 200 µg/L.

Atomic absorption spectrometry (*2.2.23, Method I or II*).

Use a furnace as atomic generator.

*Use plastic containers for preparation of the solutions and use plastic equipment where possible. Wash glassware (or equipment) in nitric acid (200 g/L $HNO_3$) before use.*

*Test solution*. Use the preparation to be examined, diluted if necessary.

*Reference solutions*. Prepare at least 3 reference solutions in a range spanning the expected aluminium concentration of the test solution, for example by diluting *aluminium standard solution (10 ppm Al) R* with a 1 g/L solution of *octoxinol 10 R*.

*Monitor solution*. Add *aluminium standard solution (10 ppm Al) R* or a suitable certified reference material to the test solution in a sufficient amount to increase the aluminium concentration by 20 µg/L.

*Blank solution*. 1 g/L solution of *octoxinol 10 R*.

*Wavelength*: 309.3 nm or other suitable wavelength.

*Slit width*: 0.5 nm.

*Tube*: pyrolytically coated, with integrated platform.

*Background corrector*: off.

*Atomisation device*: furnace; fire between readings.

The operating conditions in Table 0255.-1 are cited as an example of conditions found suitable for a given apparatus; they may be modified to obtain optimum conditions.

Table 0255.-1. – *Operating conditions found suitable, cited as an example*

| Step | Final temperature (°C) | Ramp time (s) | Hold time (s) | Gas |
|---|---|---|---|---|
| 1 | 120 | 10 | 80 | argon |
| 2 | 200 | 5 | 20 | argon |
| 3 | 650 | 5 | 10 | argon |
| 4 | 1300 | 5 | 10 | argon |
| 5 | 1300 | 1 | 10 | no gas |
| 6 | 2500 | 0.7 | 4 | no gas |
| 7 | 2600 | 0.5 | 3 | argon |
| 8 | 20 | 12.9 | 3 | no gas |

*Injection*: each of the following solutions 3 times: blank solution, reference solutions, test solution and monitor solution.

*System suitability*:

– the recovery of aluminium added in preparation of the monitor solution is within the range 80-120 per cent.

Prepare a calibration curve from the mean of the readings obtained with the reference solutions and determine the aluminium content of the preparation to be examined using the calibration curve.

**Potassium**: maximum 0.05 mmol of K per gram of protein.

Atomic emission spectrometry (*2.2.22, Method I*).

*Wavelength*: 766.5 nm.

**Sodium**: maximum 160 mmol/L and 95 per cent to 105 per cent of the content of Na stated on the label.

Atomic emission spectrometry (*2.2.22, Method I*).

*Wavelength*: 589 nm.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens (*2.6.8*) or Bacterial endotoxins (*2.6.14*)**. It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, for a solution with a protein content of 35-50 g/L, inject 10 mL per kilogram of the rabbit's mass; for a solution with a protein content of 150-250 g/L, inject 5 mL per kilogram of the rabbit's mass.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.5 IU of endotoxin per millilitre for solutions with a protein content not greater than 50 g/L, less than 1.3 IU of endotoxin per millilitre for solutions with a protein content greater than 50 g/L but not greater than 200 g/L, and less than 1.7 IU of endotoxin per millilitre for solutions with a protein content greater than 200 g/L but not greater than 250 g/L.

STORAGE

Protected from light.

LABELLING

The label states:

– the name of the preparation;
– the volume of the preparation;
– the content of protein expressed in grams per litre;
– the content of sodium expressed in millimoles per litre;
– that the product is not to be used if it is cloudy or if a deposit has formed;
– the name and quantity of any added substance.

**01/2015:0557**

# HUMAN ANTI-D IMMUNOGLOBULIN

## Immunoglobulinum humanum anti-D

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. *Human normal immunoglobulin for intramuscular administration (0338)* and/or *Human albumin solution (0255)* may be added.

It complies with the monograph *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

The assay of human anti-D immunoglobulin (*2.7.13*) is carried out, as prescribed below under Potency.

For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D, where authorised, the test for antibodies to hepatitis B surface antigen is not required.

PRODUCTION

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

*ERYTHROCYTE DONORS*

Erythrocyte donors comply with the requirements for donors prescribed in the monograph *Human plasma for fractionation (0853)*.

*IMMUNISATION*

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organization (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

*POOLED PLASMA*

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (*2.6.21*).

**B19 virus DNA**: maximum 10.0 IU/µL.

A positive control with 10.0 IU of B19 virus DNA per microlitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

*B19 virus DNA for NAT testing BRP* is suitable for use as a positive control.

If *Human normal immunoglobulin for intramuscular administration (0338)* and/or *Human albumin solution (0255)* are added to the preparation, the plasma pool or pools from which they are derived comply with the above requirement for B19 virus DNA.

POTENCY

**Human anti-D immunoglobulin** (*2.7.13, Method A*). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (*2.7.13*) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2008:1527**
**corrected 7.6**

# HUMAN ANTI-D IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

## Immunoglobulinum humanum anti-D ad usum intravenosum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It contains specific antibodies against erythrocyte D-antigen and may also contain small quantities of other blood-group antibodies. *Human normal immunoglobulin for intravenous administration (0918)* and/or *Human albumin solution (0255)* may be added.

It complies with the monograph *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content, the limit for osmolality and the limit for prekallikrein activator.

The test for anti-D antibodies (*2.6.26*) prescribed in the monograph *Human normal immunoglobulin for intravenous administration (0918)* is not carried out, since it is replaced by the assay of human anti-D immunoglobulin (*2.7.13*) as prescribed below under Potency.

For products prepared by a method that eliminates immunoglobulins with specificities other than anti-D, where authorised, the test for antibodies to hepatitis B surface antigen is not required; a suitable test for Fc function is carried out instead of that described in general chapter *2.7.9*, which is not applicable to such a product.

PRODUCTION

Human anti-D immunoglobulin is preferably obtained from the plasma of donors with a sufficient titre of previously acquired anti-D antibodies. Where necessary, in order to ensure an adequate supply of human anti-D immunoglobulin, it is obtained from plasma derived from donors immunised with D-positive erythrocytes that are compatible in relevant blood group systems in order to avoid formation of undesirable antibodies.

*ERYTHROCYTE DONORS*

Erythrocyte donors comply with the requirements for donors prescribed in the monograph *Human plasma for fractionation (0853)*.

*IMMUNISATION*

Immunisation of the plasma donor is carried out under proper medical supervision. Recommendations concerning donor immunisation, including testing of erythrocyte donors, have been formulated by the World Health Organization (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

*POOLED PLASMA*

To limit the potential B19 virus burden in plasma pools used for the manufacture of anti-D immunoglobulin, the plasma pool is tested for B19 virus using validated nucleic acid amplification techniques (*2.6.21*).

**B19 virus DNA**: maximum 10.0 IU/µL.

A positive control with 10.0 IU of B19 virus DNA per microlitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

*B19 virus DNA for NAT testing BRP* is suitable for use as a positive control.

If *Human normal immunoglobulin for intravenous administration (0918)* and/or *Human albumin solution (0255)* are added to the preparation, the plasma pool or pools from which they are derived comply with the above requirement for B19 virus DNA.

ASSAY

**Human anti-D immunoglobulin** (*2.7.13, Method A*). The estimated potency is not less than 90 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

Method B or C (*2.7.13*) may be used for potency determination if a satisfactory correlation with the results obtained by Method A has been established for the particular product.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the number of International Units per container.

**01/2012:0878**
**corrected 8.6**

# HUMAN ANTITHROMBIN III CONCENTRATE

## Antithrombinum III humanum densatum

DEFINITION

Sterile, freeze-dried preparation of a plasma glycoprotein fraction that inactivates thrombin in the presence of an excess of heparin. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers.

When reconstituted in the volume of solvent stated on the label, the potency is not less than 25 IU of antithrombin III per millilitre.

PRODUCTION

The method of preparation is designed to maintain functional integrity of antithrombin III. It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 3 IU of antithrombin III per milligram of total protein, excluding albumin.

The antithrombin III is purified and concentrated. No antimicrobial preservative or antibiotic is added. The antithrombin III concentrate is passed through a bacteria-retentive filter, distributed aseptically into its final, sterile containers and immediately frozen. It is then freeze-dried and the containers are closed under vacuum or in an atmosphere of inert gas.

It shall be demonstrated that the manufacturing process yields a product with a consistent fraction of antithrombin III able to bind to heparin. It is evaluated by a suitable analytical procedure which is determined during process development, such as:

*Heparin-binding fraction*. Examine by agarose gel electrophoresis (*2.2.31*). Prepare a 10 g/L solution of *agarose for electrophoresis R* containing 15 IU of *heparin R* per millilitre in *barbital buffer solution pH 8.4 R*. Pour 5 mL of this solution onto a glass plate 5 cm square. Cool at 4 °C for 30 min. Cut 2 wells 2 mm in diameter 1 cm and 4 cm from the side of the plate and 1 cm from the cathode. Introduce into one well 5 µL of the preparation to be examined, diluted to an activity of about 1 IU of antithrombin III per millilitre. Introduce into the other well 5 µL of a solution of a marker dye such as *bromophenol blue R*. Allow the electrophoresis to proceed at 4 °C, using a constant electric field of 7 V/cm, until the dye reaches the anode.

Cut across the agarose gel 1.5 cm from that side of the plate on which the preparation to be examined was applied and remove the larger portion of the gel leaving a band 1.5 cm wide containing the material to be examined. Replace the removed portion with an even layer consisting of 3.5 mL of a 10 g/L solution of *agarose for electrophoresis R* in *barbital buffer solution pH 8.4 R*, containing a rabbit anti-human antithrombin III antiserum at a suitable concentration, previously determined, to give adequate peak heights of at least 1.5 cm. Place the plate with the original gel at the cathode so that a 2nd electrophoretic migration can occur at right angles to the 1st. Allow this 2nd electrophoresis to proceed using a constant electric field of 2 V/cm for 16 h. Cover the plates with filter paper and several layers of thick lint soaked in a 9 g/L solution of *sodium chloride R* and compress for 2 h, renewing the saline several times. Rinse with *water R*, dry the plates and stain with *acid blue 92 solution R*.

Calculate the fraction of antithrombin III bound to heparin, which is the peak closest to the anode, with respect to the total amount of antithrombin III, by measuring the area defined by the 2 precipitation peaks.

The fraction of antithrombin III able to bind to heparin is not less than 60 per cent.

CHARACTERS

*Appearance*: white or almost white, hygroscopic, friable solid or powder.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility, total protein and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Solubility**. To a container of the preparation to be examined add the volume of liquid stated on the label at the recommended temperature. The preparation dissolves completely under gentle swirling within 10 min in the volume of the solvent stated on the label, forming a clear or slightly turbid, colourless or almost colourless solution.

**pH** (*2.2.3*): 6.0 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of the solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25.

**Heparin**: maximum 0.1 IU of heparin per International Unit of antithrombin III.

The anticoagulant activity of heparin is determined *in vitro* by comparing its ability in given conditions to delay the clotting of recalcified citrated sheep plasma with the same ability of a reference preparation of heparin calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a quantity of freeze-dried heparin sodium from pork intestinal mucosa. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Heparin sodium BRP* is calibrated in International Units by comparison with the International Standard by means of the assay given below.

Carry out the assay using one of the following methods for determining the onset of clotting and using tubes and other equipment appropriate to the chosen method:

a) direct visual inspection, preferably using indirect illumination and viewing against a matt black background;
b) spectrophotometric recording of the change in optical density at a wavelength of approximately 600 nm;
c) visual detection of the change in fluidity on manual tilting of the tubes;
d) mechanical recording of the change in fluidity on stirring, care being taken to cause the minimum disturbance of the solution during the earliest phase of clotting.

It is necessary to validate the method for assay of heparin for each preparation to be examined to allow for interference by antithrombin III.

ASSAY PROCEDURE

*The volumes are given as examples and may be adapted to the apparatus used provided that the ratios between the different volumes are respected.*

Dilute *heparin sodium BRP* with a 9 g/L solution of *sodium chloride R* to contain a precisely known number of International Units per millilitre and prepare a similar solution of the preparation to be examined which is expected to have the same activity. Using a 9 g/L solution of *sodium chloride R*, prepare from each solution a series of dilutions in geometric progression such that the clotting time obtained with the lowest concentration is not less than 1.5 times the blank recalcification time, and that obtained with the highest concentration is such as to give a satisfactory log dose-response curve, as determined in a preliminary test.

Place 12 tubes in a bath of iced water, labelling them in duplicate: $T_1$, $T_2$ and $T_3$ for the dilutions of the preparation to be examined and $S_1$, $S_2$ and $S_3$ for the dilutions of the reference preparation. To each tube add 1.0 mL of thawed *plasma substrate R1* and 1.0 mL of the appropriate dilution of the preparation to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in the order $S_1$, $S_2$, $S_3$, $T_1$, $T_2$, $T_3$, transfer each tube to a water-bath at 37 °C, allow to equilibrate at 37 °C for about 15 min and add to each tube 1 mL of a suitable APTT (Activated Partial Thromboplastin Time) reagent containing phospholipid and a contact activator, at a dilution giving a suitable blank recalcification time not exceeding 60 s. After exactly 2 min add 1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C and record as the clotting time the interval in seconds between this last addition and the onset of clotting determined by the chosen technique. Determine the blank recalcification time at the beginning and at the end of the procedure in a similar manner, using 1 mL of a 9 g/L solution of *sodium chloride R* in place of one of the heparin dilutions; the 2 blank values obtained should not differ significantly. Transform the clotting times to logarithms, using the mean value for the duplicate tubes. Repeat the procedure using fresh dilutions and carrying out the incubation in the order $T_1$, $T_2$, $T_3$, $S_1$, $S_2$, $S_3$. Calculate the results by the usual statistical methods (*5.3*).

Carry out not fewer than 3 independent assays. For each such assay prepare fresh solutions of the reference preparation and the preparation to be examined and use another, freshly thawed portion of plasma substrate.

Calculate the potency of the preparation to be examined, combining the results of these assays, by the usual statistical methods (*5.3*). When the variance due to differences between assays is significant at $P = 0.01$, a combined estimate of potency may be obtained by calculating the non-weighted mean of potency estimates.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to 50 IU of antithrombin III.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.1 IU of endotoxin per International Unit of antithrombin III.

ASSAY

**Human antithrombin III** (*2.7.17*). The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 90 per cent and not more than 110 per cent of the estimated potency.

STORAGE

Protected from light, in an airtight container.

LABELLING

The label states:

- the number of International Units of antithrombin III in the container;
- the name and volume of the liquid to be used for reconstitution;
- where applicable, the amount of albumin added as a stabiliser.

**04/2016:2818**

# HUMAN C1-ESTERASE INHIBITOR

## C1-esterasi inhibitor humanus

DEFINITION

Plasma protein fraction containing mainly human C1-esterase inhibitor (also known as C1-inhibitor or C1-INH). Human C1-esterase inhibitor is a soluble, single-chain glycoprotein containing 478 amino-acid residues. Human C1-esterase inhibitor belongs to the group of serine protease inhibitors. It is obtained from human plasma that complies with the monograph *Human plasma for fractionation (0853)*, using a suitable fractionation process and further purification steps. Other plasma proteins may be present.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

Human C1-esterase inhibitor is purified. The method of preparation is designed to maintain the functional integrity of human C1-esterase inhibitor. Buffers and other auxiliary substances such as a stabiliser may be included. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas. No antimicrobial preservative is added at any stage of production.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

It shall be demonstrated that the manufacturing process yields a product having a consistent composition. This is evaluated by suitable analytical procedures that are determined during process development and that normally include:

- assay of human C1-esterase inhibitor (*2.7.34*);
- determination of specific human C1-esterase inhibitor activity, expressed as the ratio of active human C1-esterase inhibitor content to total protein content;
- determination of molecular-size distribution by size-exclusion chromatography (*2.2.30*);
- molecular identification of human C1-esterase inhibitor, characterisation of accompanying plasma proteins that might be present, by a set of suitable methods such as SDS-PAGE, cellulose acetate electrophoresis or capillary zone electrophoresis (*2.2.31*, *2.2.47*) and quantitative determination of relevant accompanying plasma proteins.

CHARACTERS

*Appearance*: white, pale yellow, pale blue or greenish, hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Solubility**. To the contents of a container of the preparation to be examined add the volume of the solvent stated on the label at the recommended temperature. The preparation dissolves

completely with gentle swirling within the limits approved by the competent authority, giving a clear, colourless, pale yellow, pale blue or greenish solution.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 200 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the protein content by multiplying the result by 6.25. The protein content is within the limits approved by the competent authority.

**Specific activity**: minimum 4 IU/mg.

Calculate the ratio of active human C1-esterase inhibitor content (as determined under Assay) to total protein content.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to 50 IU of human C1-esterase inhibitor.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.25 IU of endotoxin per International Unit of human C1-esterase inhibitor.

ASSAY

Carry out the assay of human C1-esterase inhibitor (*2.7.34*).

The estimated potency is not less than 75 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

In an airtight and sterile container, protected from light, at a temperature not exceeding 25 °C, unless otherwise justified and authorised.

LABELLING

The label states:

- the number of International Units of human C1-esterase inhibitor per container;
- the quantity of protein in the container;
- the name and quantity of any added substance;
- the name and volume of the solvent to be used for reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2011:1224**
**corrected 7.6**

# HUMAN COAGULATION FACTOR VII

## Factor VII coagulationis humanus

DEFINITION

Sterile, liquid or freeze-dried preparation of a plasma protein fraction containing the single-chain glycoprotein human coagulation factor VII and may also contain small amounts of the activated form, the 2-chain derivative human coagulation factor VIIa. It may also contain human coagulation factors II, IX and X, protein C and protein S. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers, heparin and antithrombin.

The potency of the preparation, reconstituted as stated on the label, is not less than 15 IU of human coagulation factor VII per millilitre.

PRODUCTION

*GENERAL PROVISIONS*

The method of preparation is designed to maintain functional integrity of human coagulation factor VII and to minimise activation of any coagulation factor (to minimise potential thrombogenicity). It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 2 IU of human coagulation factor VII per milligram of total protein, before the addition of any protein stabiliser.

The human coagulation factor VII fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

It shall be demonstrated that the manufacturing process yields a product with consistent activities of human coagulation factors II, IX and X, expressed in International Units relative to the activity of human coagulation factor VII. This is evaluated by suitable analytical procedure(s) that is (are) determined during process development.

It shall be demonstrated that the manufacturing process yields a product with a consistent activity of human coagulation factor VIIa. This is evaluated by suitable analytical procedure(s) that is (are) determined during process development.

**Activity of human coagulation factor VIIa**. It may be determined, for example, using a recombinant soluble tissue factor that does not activate human coagulation factor VII but possesses a cofactor function specific for human coagulation factor VIIa; after incubation of a mixture of the recombinant soluble tissue factor with phospholipids reagent and the dilution of the test sample in human coagulation factor VII-deficient plasma, calcium chloride is added and the clotting time determined; the clotting time is inversely related to the human coagulation factor VIIa activity of the test sample.

## CHARACTERS

*Appearance*: white or almost white, pale yellow, green or blue, hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

## IDENTIFICATION

It complies with the limits of the assay.

## TESTS

**Solubility**. To a container of the preparation to be examined add the volume of liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent solution that may be coloured.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of the solution in a round-bottomed centrifuge tube, add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25.

**Activated coagulation factors** (*2.6.22*). For each of the dilutions, the coagulation time is not less than 150 s.

**Heparin** (*2.7.12*). If heparin has been added, the preparation to be examined contains not more than the amount of heparin stated on the label and in any case not more than 0.5 IU of heparin per International Unit of human coagulation factor VII.

**Thrombin**. If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralise the heparin by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and of a 3 g/L solution of *fibrinogen R*. Keep one of the tubes at 37 °C for 6 h and the other at room temperature for 24 h. In a 3rd tube, mix equal volumes of the fibrinogen solution and of a solution of *human thrombin R* (1 IU/mL) and place the tube in a water-bath at 37 °C. No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 s in the tube containing thrombin.

**Human coagulation factor II** (*2.7.18*). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P$ = 0.95) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

**Human coagulation factor IX** (*2.7.11*). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

**Human coagulation factor X** (*2.7.19*). The estimated content is not more than 125 per cent of the stated content. The confidence limits ($P$ = 0.95) are not less than 90 per cent and not more than 111 per cent of the estimated potency.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectrometry (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the test for bacterial endotoxins.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 30 IU of human coagulation factor VII.

Where the test for bacterial endotoxins is used, the preparation to be examined contains less than 0.1 IU of endotoxin per International Unit of human coagulation factor VII.

## ASSAY

**Human coagulation factor VII** (*2.7.10*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

## STORAGE

In an airtight container, protected from light.

## LABELLING

The label states:

- the number of International Units of human coagulation factor VII per container;
- the maximum content of human coagulation factor II, human coagulation factor IX and human coagulation factor X per container, in International Units;
- the amount of protein per container;
- the name and quantity of any added substances, including, where applicable, heparin;
- the name and volume of the liquid to be used for reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2015:2534**
**corrected 10.0**

# HUMAN COAGULATION FACTOR VIIa (rDNA) CONCENTRATED SOLUTION

## Factoris VIIa coagulationis humani (ADNr) solutio concentrata

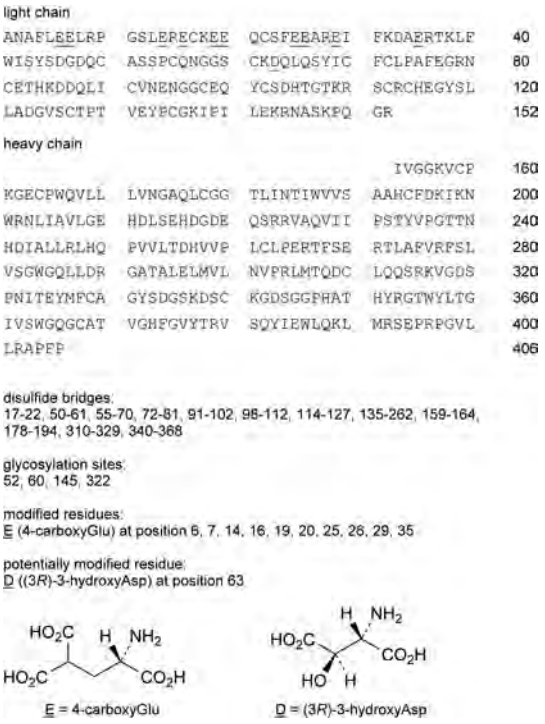


$C_{1982}H_{3054}N_{560}O_{618}S_{28}$ $M_r$ approx. 50 000

DEFINITION

Solution containing closely related glycoproteins, which have the same amino acid sequence (406 amino acids) and disulfide bridges as the naturally occurring analogue (plasma-derived activated coagulation factor VII). Human coagulation factor VIIa (rDNA) (eptacog alfa, activated) is a 2-chain molecule, obtained by proteolytic cleavage of the peptide bond between Arg 152 and Ile 153 of single-chain coagulation factor VII, consisting of a 20 kDa light chain (*N*-terminal) and a 30 kDa heavy chain (*C*-terminal) connected by a disulfide bond.

Human coagulation factor VIIa (rDNA) is distinguishable from the naturally occurring analogue in terms of its post-translational modifications, including glycosylation pattern.

*Content*: 1.11 mg to 1.78 mg of protein per millilitre.

*Potency*: 44 000 IU to 64 000 IU per milligram of protein.

PRODUCTION

Human coagulation factor VIIa (rDNA) is produced in mammalian cells by a method based on recombinant DNA technology (rDNA).

Prior to release, the following tests are carried out on each batch of human coagulation factor VIIa (rDNA) concentrated solution, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

**Glycan analysis**.

Use a suitable method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*.

Glycan analysis includes the following steps:

- after desalting, release of the glycans (see *2.2.59* section 2-3);
- labelling of the glycans with a suitable fluorescent label (Table 2.2.59.-2);
- analysis of the labelled glycans by liquid chromatography (*2.2.29*) with fluorometric detection.

The following procedures may be used.

*Test solution*. Dilute the preparation to be examined in *water R* to obtain a concentration of about 1.5 mg/mL.

*Reference solution*. Dissolve *human coagulation factor VIIa (rDNA) CRS* in *water R* to obtain a concentration of 1.5 mg/mL.

*DESALTING*

Desalt the test solution and the reference solution as described under Identification B. The buffer used for desalting and elution is a 1.21 g/L solution of *tris(hydroxymethyl)aminomethane R*, adjusted to pH 7.5 with *hydrochloric acid R*. After desalting, the concentration of the solutions is about 1.0 mg/mL.

*SELECTIVE RELEASE OF GLYCANS*

Transfer 500 µL of the desalted test solution and 500 µL of the desalted reference solution to separate centrifuge tubes, and add 10 µL of a 200 U/mL solution of *peptide N-glycosidase F R*. Cap the tubes and incubate for 16-24 h at 37 °C. Remove the protein fraction by adding 1.5 mL of *ethanol (96 per cent) R* at − 20 °C to the tubes. Mix and allow to stand at − 20 °C for 20-30 min. Centrifuge the tubes at 10 000 r/min for 10 min. Collect the supernatant and evaporate to dryness, using for example a rotary evaporator.

*LABELLING OF GLYCANS*

Label the liberated glycans with 2-aminobenzamide using a suitable procedure. The procedure employs a combination of reagents optimised and validated for the efficient labelling of glycans, and for the subsequent extraction and recovery of the labelled glycans from the reaction.

*LIQUID CHROMATOGRAPHY (2.2.29)*

*Precolumn*:

- *size*: *l* = 0.05m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R*;
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 6 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: solution containing 6 g/L of *sodium hydroxide R* and 40.8 g/L of *sodium acetate R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 52 | 100 → 35 | 0 → 65 |
| 52.0 - 52.1 | 35 → 0 | 65 → 100 |
| 52.1 - 65 | 0 | 100 |
| 65 - 65.1 | 0 → 100 | 100 → 0 |
| 65.1 - 90 | 100 | 0 |

*Flow rate*: 0.5 mL/min.

*Detection*: fluorimeter at 330 nm for excitation and 420 nm for emission.

*Injection*: 100 µL, using an automatic injector maintained at 2-8 °C.

*System suitability*: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram shown in Figure 2534.-1; peaks 1 to 12 are clearly visible;
- *peak width at half-height*: maximum 30 s for peak 8.

Calculate the percentage content of charged glycans in the reference solution using the following expression:

$$\frac{A}{A+B} \times 100$$

$A$ = sum of the areas of the peaks due to charged glycans (peaks 6 to 12);

$B$ = sum of the areas of the peaks due to uncharged glycans (peaks 1 to 5).

Calculate the percentage content of charged glycans in the test solution accordingly.

*Limit*:

- *percentage of charged glycans*: as authorised by the competent authority.

CHARACTERS

*Appearance*: colourless liquid.

IDENTIFICATION

A. It forms a clot when examined in the conditions described under Assay (Potency).

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Solution A*. Dissolve 0.74 g of *calcium chloride R* and 6.06 g of *tris(hydroxymethyl)aminomethane R* in 1000 mL of *water R* and adjust to pH 7.5 with *hydrochloric acid R*.

*Test solution*. Dilute the preparation to be examined with solution A to obtain a concentration of about 1.5 mg/mL. Desalt a volume of this solution by a suitable method (for example using a suitable centrifugal filter unit or gel-filtration column with solution A as elution buffer). After desalting, the concentration should be about 1.0 mg/mL. Transfer the desalted solution to a polypropylene tube. Prepare a 1 mg/mL solution of *trypsin for peptide mapping R* and add 10 µL to 1 mL of the desalted solution. Cap the tube and mix gently by inversion. Incubate at 37 °C for 24 h. At time 5.5 h, add 10 µL of the trypsin solution. Remove the sample from the incubator, place it at room temperature, add 9 µL of *glacial acetic acid R* and mix by inversion. Maintain the solution at − 15 °C or below until chromatographic separation; if analysed immediately using an automatic injector, maintain at 2-8 °C.

*Reference solution*. Dissolve *human coagulation factor VIIa (rDNA) CRS* in solution A to obtain a concentration of 1.5 mg/mL. Desalt and digest at the same time and in the same manner as for the test solution.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 30 °C.

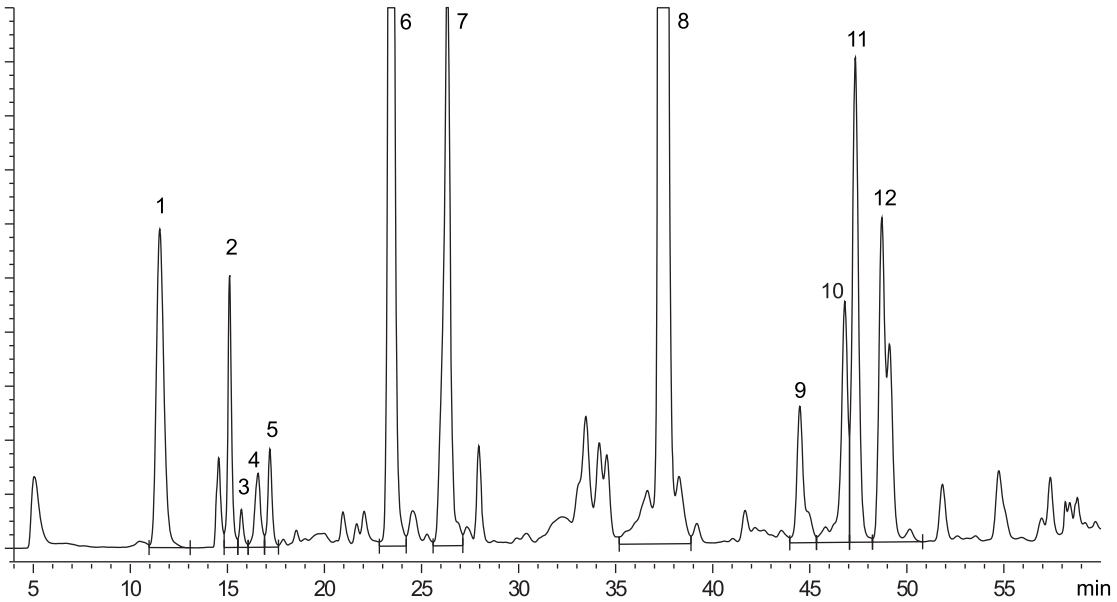


| Peak | Charged | Structure | Peak | Charged | Structure |
|---|---|---|---|---|---|
| 1. | No | Core fucosylated biantennary – non sialylated (2 *N*-acetylgalactosamine terminals) | 7. | Yes | Core fucosylated biantennary – monosialylated (and 1 galactose terminal) |
| 2. | No | Core fucosylated biantennary – non sialylated (*N*-acetylgalactosamine and galactose terminals) | 8. | Yes | Core fucosylated biantennary – bisialylated |
| 3. | No | Structure not determined | 9. | Yes | High-mannose structure with 1 phosphate group |
| 4. | No | Core fucosylated biantennary – non sialylated (galactose and *N*-acetylglucosamine terminals) | 10. | Yes | Core fucosylated triantennary – trisialylated |
| 5. | No | Core fucosylated biantennary – non sialylated (2 galactose terminals) | 11. | Yes | Core fucosylated triantennary – trisialylated |
| 6. | Yes | Core fucosylated biantennary – monosialylated (and 1 *N*-acetylgalactosamine terminal) | 12. | Yes | Structure not determined |

Figure 2534.-1. – *Chromatogram for the test for glycan analysis of human coagulation factor VIIa (rDNA): reference solution*

*Mobile phase*:

– *mobile phase A*: add 0.65 mL of *trifluoroacetic acid R* to 1000 mL of *water for chromatography R* and degas;

– *mobile phase B*: mix 0.5 mL of *trifluoroacetic acid R*, 100 mL of *water for chromatography R* and 900 mL of *acetonitrile R1* and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 100 | 100 → 50 | 0 → 50 |
| 100 - 105 | 50 → 0 | 50 → 100 |
| 105 - 110 | 0 | 100 |
| 110 - 110.1 | 0 → 100 | 100 → 0 |
| 110.1 - 125 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 25 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human coagulation factor VIIa (rDNA) CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution:

– all major peaks identified in the chromatogram obtained with the reference solution are present in the chromatogram obtained with the test solution;

– no new major peaks are observed in the chromatogram obtained with the test solution in comparison with the chromatogram obtained with the reference solution.

C. Examine the chromatograms obtained in the test for glycan analysis.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

**Degraded heavy chain and oxidised forms of human coagulation factor VIIa (rDNA)**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of about 1.5 mg/mL.

*Reference solution.* Dissolve *human coagulation factor VIIa (rDNA) CRS* in *water R* to obtain a concentration of 1.5 mg/mL.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;

– *temperature*: 60-70 °C.

*Mobile phase*:

– *mobile phase A*: mix 1 mL of *trifluoroacetic acid R* and 999 mL of *water for chromatography R* and degas;

– *mobile phase B*: mix 1 mL of *trifluoroacetic acid R*, 200 mL of *water for chromatography R* and 800 mL of *acetonitrile R1* and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 54 → 41 | 46 → 59 |
| 30 - 33 | 41 → 0 | 59 → 100 |
| 33 - 38 | 0 | 100 |
| 38 - 40 | 0 → 54 | 100 → 46 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: about 20 µL, using an automatic injector maintained at 2-8 °C.

*Retention time*: human coagulation factor VIIa (rDNA) = about 26 min.

*System suitability*:

– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram shown in Figure 2534.-2; peaks 1 to 10 are clearly visible;

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of peak 6 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from peak 7.

*Results*:

– the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Calculate the individual percentage area (relative to the total peak area) of the peaks due to the degraded heavy chain human coagulation factor VIIa (rDNA) (peaks 1, 2 and 6) and oxidised forms of human coagulation factor VIIa (rDNA) (peaks 3, 4 and 5).

*Limits*:

– *sum of degraded heavy chain forms*: maximum 11 per cent;

– *sum of oxidised forms*: maximum 2.2 per cent.

**Gla-domainless human coagulation factor VIIa (rDNA) (gamma-carboxylation)**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of about 1.5 mg/mL.

*Reference solution.* Dissolve *human coagulation factor VIIa (rDNA) CRS* in *water R* to obtain a concentration of 1.5 mg/mL.

*Precolumn*:

– *stationary phase*: *styrene-divinylbenzene copolymer R* with iminodiacetic groups, for removal of calcium.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R1*;

– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: solution containing 1.2 g/L of *tris(hydroxymethyl)aminomethane R* and 2.8 g/L of *bis-tris propane R*, adjusted to pH 9.4 with *glacial acetic acid R* and degassed;

– *mobile phase B*: solution containing 1.2 g/L of *tris(hydroxymethyl)aminomethane R*, 2.8 g/L of *bis-tris propane R* and 107.9 g/L of *ammonium acetate R*, adjusted to pH 9.4 with *concentrated ammonia R* and degassed;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.5 | 100 | 0 |
| 2.5 - 27.5 | 100 → 0 | 0 → 100 |
| 27.5 - 30.5 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: about 100 µL, using an automatic injector maintained at 2-8 °C.

*Relative retention* with reference to human coagulation factor VIIa (rDNA) (retention time = about 14 min): Gla-domainless human coagulation factor VIIa (rDNA) = about 0.7.

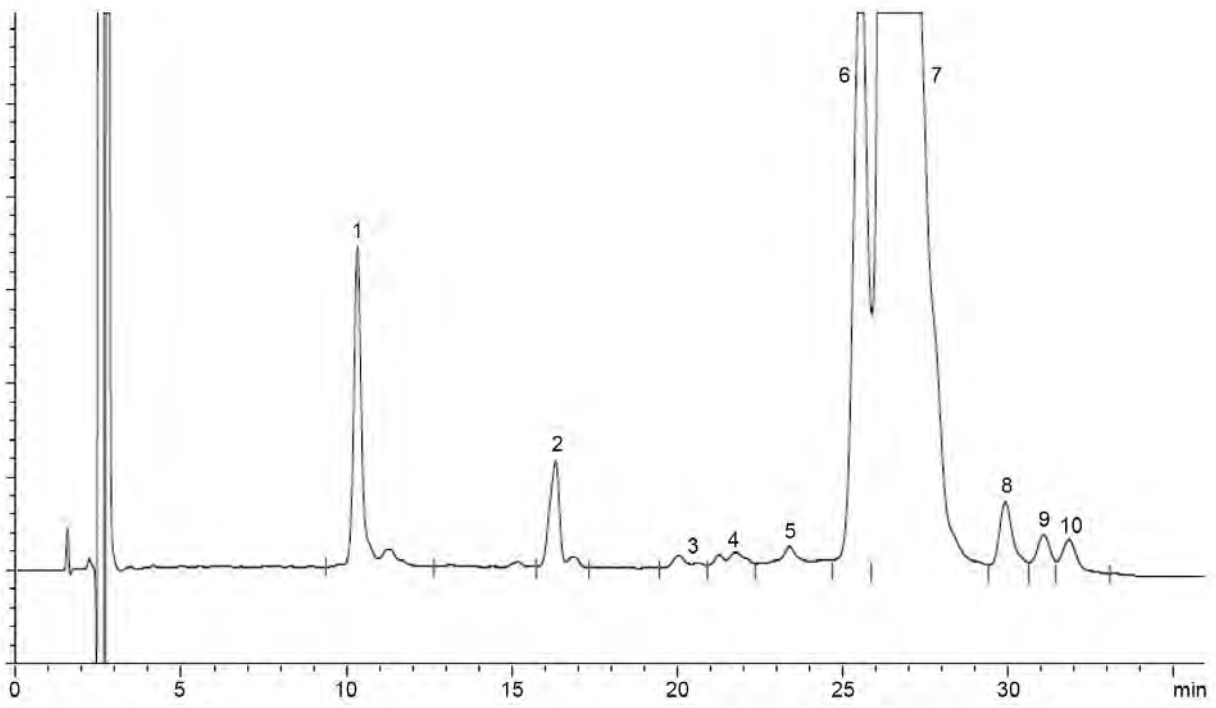


1. degraded heavy chain
2. degraded heavy chain
3. oxidised form
4. oxidised form
5. oxidised form
6. degraded heavy chain
7. human coagulation factor VIIa (rDNA)
8. unknown
9. unknown
10. unknown

Figure 2534.-2. – *Chromatogram for the test for degraded heavy chain and oxidised forms of human coagulation factor VIIa (rDNA): reference solution*

*System suitability*: reference solution:

– *resolution*: baseline separation between the peak due to Gla-domainless human coagulation factor VIIa (rDNA) and the peak cluster due to human coagulation factor VIIa (rDNA).

*Limit*:

– *Gla-domainless human coagulation factor VIIa (rDNA)*: maximum 6.1 per cent.

Integrate the peak cluster to baseline.

**Dimers and related substances of higher molecular mass**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of about 1.5 mg/mL.

*Reference solution.* Dissolve *human coagulation factor VIIa (rDNA) CRS* in *water R* to obtain a concentration of 1.5 mg/mL.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.5 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R* (10 µm) of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000;

– *temperature*: 21-25 °C.

*Mobile phase.* Dissolve 26.4 g of *ammonium sulfate R* in approximately 900 mL of *water for chromatography R*. Adjust first to pH 2.5 with *phosphoric acid R* and then to pH 7.0 with *triethylamine R*. Add 50 mL of *2-propanol R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL, using an automatic injector maintained at 2-8 °C.

*System suitability*: reference solution:

– the chromatogram obtained is qualitatively similar to the chromatogram supplied with *human coagulation factor VIIa (rDNA) CRS*;

– *symmetry factor*: maximum 1.3 for the peak due to the monomer;

– *peak-to-valley ratio*: minimum 1.1, where $H_p$ = height above the baseline of the peak due to dimers and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limit*:

– *sum of the areas of the peaks with a retention time less than that of the monomer*: maximum 2.7 per cent.

**Non-activated single-chain factor VII (rDNA)**. Polyacrylamide gel electrophoresis (*2.2.31*): use the normalisation procedure.

*Gel dimensions*: 1 mm thick.

*Resolving gel*: 12 per cent acrylamide.

*Sample buffer (reducing conditions)*: *concentrated SDS-PAGE sample buffer for reducing conditions R* containing *dithiothreitol R* as the reducing agent.

*Test solution.* Dilute the preparation to be examined in *water R* to obtain a concentration of about 800 µg/mL. Mix equal volumes of this solution and the sample buffer (reducing conditions).

*Reference solution (a).* Dissolve *human coagulation factor VIIa (rDNA) CRS* in *water R* to obtain a concentration of about 800 µg/mL. Mix equal volumes of this solution and the sample buffer (reducing conditions).

*Reference solution (b).* Solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 10-70 kDa.

*Sample treatment*: boil for 5 min or heat at 73 ± 3 °C for 10 min.

*Application*: 10 µL.

*Detection*: by Coomassie staining.

*Quantification*: integrating densitometer.

*System suitability*:

- the principal bands in the electropherogram obtained with the test solution correspond in position to the principal bands in the electropherogram obtained with reference solution (a) (30 kDa, heavy chain and 20-25 kDa, light chain);
- reference solution (b): the validation criteria are met (*2.2.31*);
- a band corresponding to non-activated single-chain factor VII (rDNA) (molecular mass of 51 kDa) is visible in the electropherogram obtained with reference solution (a).

*Limit*:

- *non-activated single-chain factor VII (rDNA)*: maximum 3 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mL.

ASSAY

**Protein**. Size-exclusion chromatography (*2.2.30*) as described in the test for dimers and related substances of higher molecular mass with the following modifications.

*Injection*: 10 µL, 20 µL and 30 µL of the reference solution.

Plot peak areas against injected protein content and perform a linear regression to create a standard curve.

Calculate the content of human coagulation factor VIIa (rDNA) using the monomer peak area in the chromatogram obtained with the test solution and taking into account the assigned content of *human coagulation factor VIIa (rDNA) CRS*.

*System suitability*:

- *repeatability*: maximum relative standard deviation of 2.0 per cent after 5 injections of 20 µL of the reference solution;
- the correlation coefficient calculated for the standard curve ($r^2$) is not less than 0.990.

**Potency**.

The principle of the assay is to measure the ability of a factor VIIa preparation to reduce the prolonged coagulation time of factor VII-deficient plasma.

The biological activity is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference preparation calibrated in International Units. The International Unit is the activity contained in a stated amount of the International Reference Preparation.

The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

*Method*.

*Use a suitable coagulation analyser or carry out the assay with incubation tubes and reagents maintained in a water-bath at 37 °C.*

*Solution A*. Prepare a solution containing 15.12 g/L of *1,4-piperazinediethanesulfonic acid R*, 5.73 g/L of *sodium chloride R*, 0.74 g/L of *sodium edetate R* and 10 g/L of *bovine albumin R*; adjust to pH 7.2 with *sodium hydroxide R*.

Prepare 3 different solutions of the preparation to be examined and of the reference preparation, by diluting with solution A, to obtain concentrations within the linearity range (0.002-0.15 IU/mL). Prepare in duplicate and use the solutions immediately.

To 40 µL of each solution, add 40 µL of *factor VII-deficient plasma R*, incubate for an appropriate time at 37 °C, and add 40 µL of *human tissue factor solution R*.

Measure the coagulation time, i.e. the interval between the addition of the human tissue factor solution and the first indication of the formation of fibrin.

*The volumes given above and sequence of reagents may be adapted to the human tissue factor solution and apparatus used.*

Calculate the activity in International Units per millilitre using an appropriate statistical method, for example the parallel-line assay (*5.3*).

The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

LABELLING

The label states:

- the content of human coagulation factor VIIa (rDNA), in milligrams per millilitre;
- the specific activity, in International Units per milligram of protein.

**07/2013:0275**

# HUMAN COAGULATION FACTOR VIII

## Factor VIII coagulationis humanus

DEFINITION

Sterile, freeze-dried preparation of a plasma protein fraction containing the glycoprotein human coagulation factor VIII together with varying amounts of human von Willebrand factor, depending on the method of preparation. It is prepared from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers.

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of factor VIII:C per millilitre.

PRODUCTION

*GENERAL PROVISIONS*

The method of preparation is designed to maintain functional integrity of human coagulation factor VIII and to minimise potential neoantigenicity. It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for the inactivation of viruses, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 1 IU of factor VIII:C per milligram of total protein before the addition of any protein stabiliser.

The human coagulation factor VIII fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

*Products stated to have human von Willebrand factor activity (products intended for treatment of von Willebrand's disease).* It shall be demonstrated by suitable analytical procedures determined during process development that the manufacturing process yields a product with a consistent composition with respect to human von Willebrand factor. This composition may be characterised in a number of ways. For example, the distribution of the different human von Willebrand factor multimers may be determined by sodium dodecyl sulfate (SDS) agarose gel electrophoresis (about 1 per cent agarose) with or without Western blot analysis, using a normal human plasma pool as reference. Visualisation of the multimeric pattern may be performed using, for example, an immunoenzymatic technique and quantitative evaluation may be carried out by densitometric analysis.

*Products that show flakes or particles after reconstitution for use*. If a few small flakes or particles remain when the preparation is reconstituted, it shall be demonstrated during validation studies that the potency is not significantly affected after passage of the preparation through the filter provided.

CHARACTERS

*Appearance*: white or pale yellow, hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Solubility**. To a container of the preparation to be examined, add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent, colourless or slightly yellow solution.

Where the label states that the product may show a few small flakes or particles after reconstitution, reconstitute the preparation as described on the label and pass it through the filter provided: the filtered solution is clear or slightly opalescent.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25. *For some products, especially those without a protein stabiliser such as albumin, this method may not be applicable and another validated method for protein determination must therefore be performed.*

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method A*). The 1 to 64 dilution does not show agglutination. Dilute the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to contain 3 IU of factor VIII:C per millilitre.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the test for bacterial endotoxins.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 50 IU of factor VIII:C.

Where the test for bacterial endotoxins is used, the preparation to be examined contains less than 0.03 IU of endotoxin per International Unit of factor VIII:C.

ASSAY

**Human coagulation factor VIII** (*2.7.4*). The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Human von Willebrand factor** (*2.7.21*). If preparations are intended for the treatment of von Willebrand's disease, the estimated potency is not less than 60 per cent and not more than 140 per cent of the stated potency.

*Pending the availability of an International Standard for human von Willebrand factor concentrate calibrated for use in the collagen-binding assay, only the ristocetin cofactor assay may be used.*

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of factor VIII:C and, where applicable, of human von Willebrand factor in the container;
- the amount of protein in the container;
- the name and quantity of any added substance;
- the name and volume of the liquid to be used for reconstitution;
- where applicable, that the preparation may show the presence of a few small flakes or particles after reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2008:1643**

# HUMAN COAGULATION FACTOR VIII (rDNA)

## Factor VIII coagulationis humanus (ADNr)

DEFINITION

Human coagulation factor VIII (rDNA) is a freeze-dried preparation of glycoproteins having the same activity as coagulation factor VIII in human plasma. It acts as a cofactor of the activation of factor X in the presence of factor IXa, phospholipids and calcium ions.

Human coagulation factor VIII circulates in plasma mainly as a two-chain glycosylated protein with 1 heavy (relative molecular mass of about 200 000) and 1 light (relative molecular mass 80 000) chain held together by divalent metal ions. Human coagulation factor VIII (rDNA) is prepared as full-length factor VIII (octocog alfa), or as a shortened two-chain structure (relative molecular mass 90 000 and 80 000), in which the B-domain has been deleted from the heavy chain (moroctocog alfa).

Full-length human rDNA coagulation factor VIII contains 25 potential *N*-glycosylation sites, 19 in the B domain of the heavy chain, 3 in the remaining part of the heavy chain (relative molecular mass 90 000) and 3 in the light chain (relative molecular mass 80 000). The different products are characterised by their molecular size and post-translational modification and/or other modifications.

PRODUCTION

Human coagulation factor VIII (rDNA) is produced by recombinant DNA technology in mammalian cell culture. It is produced under conditions designed to minimise microbial contamination.

Purified bulk factor VIII (rDNA) may contain added human albumin and/or other stabilising agents, as well as other auxiliary substances to provide, for example, correct pH and osmolality.

The specific activity is not less than 2000 IU of factor VIII:C per milligram of total protein before the addition of any protein stabiliser, and varies depending on purity and the type of modification of molecular structure of factor VIII.

The quality of the bulk preparation is controlled using one or more manufacturer's reference preparations as reference.

*MANUFACTURER'S REFERENCE PREPARATIONS*

During development, reference preparations are established for subsequent verification of batch consistency during production, and for control of bulk and final preparation. They are derived from representative batches of purified bulk factor VIII (rDNA) that are extensively characterised by tests including those described below and whose procoagulant and other relevant functional properties have been ascertained and compared, wherever possible, with the International Standard for factor VIII concentrate. The reference preparations are suitably characterised for their intended purpose and are stored in suitably sized aliquots under conditions ensuring their stability.

*PURIFIED BULK FACTOR VIII (rDNA)*

*The purified bulk complies with a suitable combination of the following tests for characterisation of integrity of the factor VIII (rDNA). Where any substance added during preparation of the purified bulk interferes with a test, the test is carried out before addition of that substance. Where applicable, the characterisation tests may alternatively be carried out on the finished product.*

**Specific biological activity or ratio of factor VIII activity to factor VIII antigen**. Carry out the assay of human coagulation factor VIII (*2.7.4*). The protein content, or where a protein stabiliser is present, the factor VIII antigen content, is determined by a suitable method and the specific biological activity or the ratio of factor VIII activity to factor VIII antigen is calculated.

**Protein composition**. The protein composition is determined by a selection of appropriate characterisation techniques which may include peptide mapping, Western blots, HPLC, gel electrophoresis, capillary electrophoresis, mass spectrometry or other techniques to monitor integrity and purity. The protein composition is comparable to that of the manufacturer's reference preparation.

**Molecular size distribution**. Using size-exclusion chromatography (*2.2.30*), the molecular size distribution is comparable to that of the manufacturer's reference preparation.

**Peptide mapping** (*2.2.55*). There is no significant difference between the test protein and the manufacturer's reference preparation.

**Carbohydrates/sialic acid**. To monitor batch-to-batch consistency, the monosaccharide content and the degree of sialylation or the oligosaccharide profile are monitored and correspond to those of the manufacturer's reference preparation.

*FINAL LOT*

It complies with the requirements under Identification, Tests and Assay.

**Excipients**: 80 per cent to 120 per cent of the stated content, determined by a suitable method, where applicable.

CHARACTERS

*Appearance*: white or slightly yellow powder or friable mass.

IDENTIFICATION

A. It complies with the limits of the assay.

B. The distribution of characteristic peptide bands corresponds with that of the manufacturer's reference preparation (SDS-PAGE or Western blot).

TESTS

*Reconstitute the preparation as stated on the label immediately before carrying out the tests (except those for solubility and water) and assay.*

**Solubility**. It dissolves within 5 min at 20-25 °C, giving a clear or slightly opalescent solution.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 3 IU in the volume that contains 100 IU of factor VIII activity.

ASSAY

Carry out the assay of human coagulation factor VIII (*2.7.4*).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

Protected from light.

LABELLING

The label states:

- the factor VIII content in International Units,
- the name and amount of any excipient,
- the composition and volume of the liquid to be used for reconstitution.

**01/2011:1223**
**corrected 7.6**

# HUMAN COAGULATION FACTOR IX

## Factor IX coagulationis humanus

DEFINITION

Sterile freeze-dried preparation of a plasma protein fraction containing coagulation factor IX. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*, by a method that effectively separates human coagulation factor IX from other prothrombin complex factors (human coagulation factors II, VII and X). The preparation may contain excipients such stabilisers, heparin and antithrombin.

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of human coagulation factor IX per millilitre.

PRODUCTION

*GENERAL PROVISIONS*

The method of preparation is designed to maintain functional integrity of human coagulation factor IX and to minimise activation of any coagulation factor (to minimise potential thrombogenicity). It includes a step or steps that have been shown to remove or to inactivate known agents of infection;

if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 50 IU of human coagulation factor IX per milligram of total protein, before the addition of any protein stabiliser.

The human coagulation factor IX fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

It shall be demonstrated that the manufacturing process yields a product having a consistent composition. This is evaluated by suitable analytical procedures that are determined during process development and that normally include:

- assay of human coagulation factor IX;
- determination of activated coagulation factors;
- determination of activities of human coagulation factors II, VII and X, which shall be shown to be not more than 5 per cent of the activity of human coagulation factor IX.

## CHARACTERS

*Appearance*: white or pale yellow, hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

## IDENTIFICATION

It complies with the limits of the assay.

## TESTS

**Solubility**. To a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear or slightly opalescent, colourless solution.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of the solution in a round-bottomed centrifuge tube, add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25. *For some products, especially those without a protein stabiliser such as albumin, this method may not be applicable. Another validated method for protein determination must therefore be performed.*

**Activated coagulation factors** (*2.6.22*). If necessary, dilute the reconstituted preparation to contain 20 IU of human coagulation factor IX per millilitre. For each of the dilutions, the coagulation time is not less than 150 s.

**Heparin** (*2.7.12*). If heparin has been added, the preparation to be examined contains not more than the amount of heparin stated on the label and in all cases not more than 0.5 IU of heparin per International Unit of human coagulation factor IX.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the test for bacterial endotoxins.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 50 IU of human coagulation factor IX.

Where the test for bacterial endotoxins is used, the preparation to be examined contains less than 0.03 IU of endotoxin per International Unit of human coagulation factor IX.

## ASSAY

**Human coagulation factor IX** (*2.7.11*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

## STORAGE

In an airtight container, protected from light.

## LABELLING

The label states:

- the number of International Units of human coagulation factor IX per container;
- the amount of protein per container;
- the name and quantity of any added substances including, where applicable, heparin;
- the name and volume of the liquid to be used for reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

01/2018:2522
corrected 10.0

# HUMAN COAGULATION FACTOR IX (rDNA) CONCENTRATED SOLUTION

## Factoris IX coagulationis humani (ADNr) solutio concentrata

| | | | | |
|---|---|---|---|---|
| YNSGKLEEFV | QGNLERECME | EKCSFEEARE | VFENTERTTE | 40 |
| FWKQYVDGDQ | CESNPCLNGG | SCKDDINSYE | CWCPFGFEGK | 80 |
| NCELDVTCNI | KNGRCEQFCK | NSADNKVVCS | CTEGYRLAEN | 120 |
| QKSCEPAVPF | PCGRVSVSQT | SKLTRAEAVF | PDVDYVNSTE | 160 |
| AETILDNITQ | STQSFNDFTR | VVGGEDAKPG | QFPWQVVLNG | 200 |
| KVDAFCGGSI | VNEKWIVTAA | HCVETGVKIT | VVAGEHNIEE | 240 |
| TEHTEQKRNV | IRIIPHHNYN | AAINKYNHDI | ALLELDEPLV | 280 |
| LNSYVTPICI | ADKEYTNIFL | KFGSGYVSGW | GRVFHKGRSA | 320 |
| LVLQYLRVPL | VDRATCLRST | KFTIYNNMFC | AGFHEGGRDS | 360 |
| CQGDSGGPHV | TEVEGTSFLT | GIISWGEECA | MKGKYGIYTK | 400 |
| VSRYVNWIKE | KTKLT | | | 415 |

disulfide bridges:
18-23, 51-62, 56-71, 73-82, 88-99, 95-109, 111-124, 132-289, 206-222, 336-350, 361-389

glycosylation sites:
Ser-53, Ser-61, Asn-157, Thr-159, Asn-167, Thr-169

modified residues:
E (4-carboxyGlu): 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36, 40
D ((3*R*)-3-hydroxyAsp): 64
S ($O^3$-phosphoSer): 68, 158
Y ($O^4$-sulfoTyr): 155

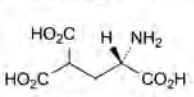

E = 4-carboxyGlu

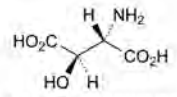

D = (3*R*)-3-hydroxyAsp

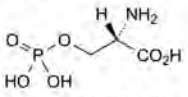

S = ($O^3$-phosphoSer)

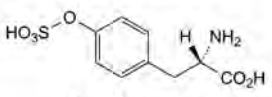

Y = ($O^4$-sulfoTyr)

$C_{2053}H_{3116}N_{558}O_{674}P_2S_{26}$ $M_r$ approx. 55 000

DEFINITION

Solution containing closely related glycoproteins, which have the same amino acid sequence (415 amino acids) as the naturally occurring Ala 148 allelic form analogue (plasma-derived coagulation factor IX). It is a single-chain glycoprotein with structural and functional characteristics similar to those of the endogenous factor IX. It may contain buffer salts and/or non-proteinaceous stabilisers.

*Content*: minimum 150 IU per millilitre.

*Potency*: 200 to 360 IU per milligram of protein.

PRODUCTION

Human coagulation factor IX (rDNA) is produced in mammalian cells by a method based on recombinant DNA technology (rDNA). The method of preparation is designed to maintain the functional integrity of factor IX, and to minimise the activation of human coagulation factor IX (rDNA) (to minimise potential thrombogenicity).

No antibiotic or antimicrobial preservative is added.

Prior to release, the following tests are carried out on each batch of human coagulation factor IX (rDNA) concentrated solution, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority

**Glycan analysis**. Use a suitable method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*, Section 2-3.

- Release the glycans using one of the agents described in Table *2.2.59.*-1, for example peptide *N*-glycosidase F (PNGase F).
- Label the released glycans with one of the fluorescent labelling agents described in Table *2.2.59.*-2, for example 2-aminobenzamide.
- Analyse the labelled glycans by liquid chromatography (*2.2.29*) using a high-pH-resistant column with fluorescence detection.

*The following procedure is given as an example.*

*Test solution.* Dilute the preparation to be examined with the formulation buffer (see Tests) to obtain a concentration of about 2 mg/mL. Use 50 µL of this solution to proceed to glycan release and labelling. Resuspend or dilute the labelled glycans in 200 µL of *water R*.

*Reference solution (a).* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer (see Tests) to obtain a concentration of about 2 mg/mL. Use 50 µL of this solution to proceed to glycan release and labelling. Resuspend or dilute the labelled glycans in 200 µL of *water R*.

*Reference solution (b).* Use a suitable human coagulation factor IX (rDNA) in-house reference preparation shown to be representative of batches tested clinically and batches used to demonstrate consistency of production. Dilute with the formulation buffer to obtain a concentration of about 2 mg/mL. Use 50 µL of this solution to proceed to glycan release and labelling. Resuspend or dilute the labelled glycans in 200 µL of *water R*.

*Blank solution.* Use 50 µL of the formulation buffer to proceed to glycan release and labelling.

Analyse the labelled glycans by liquid chromatography (*2.2.29*).

*Precolumn*:

- *size*: *l* = 0.01 m, Ø = 4.6 mm;
- *stationary phase*: *polyamine grafted poly(vinyl alcohol) copolymer R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *polyamine grafted poly(vinyl alcohol) copolymer R* (5 µm).
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *water R*, *glacial acetic acid R*, *acetonitrile R* (1:2:97 *V/V/V*);
- *mobile phase B*: *concentrated ammonia R*, *glacial acetic acid R*, *water R* (1:3:96 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 70 | 30 |
| 2 - 67 | 70 → 0 | 30 → 100 |
| 67 - 70 | 0 | 100 |
| 70 - 70.1 | 0 → 70 | 100 → 30 |
| 70.1 - 95 | 70 | 30 |

*Flow rate*: 0.5 mL/min.

*Detection*: fluorimeter at 330 nm for excitation and 420 nm for emission.

*Injection*: 20 µL, using an automatic injector maintained at 2-8 °C.

If carry-over of material is observed, running a blank gradient after each injection may be appropriate.

*Identification of peak groups*: use the chromatogram in Figure 2522.-1 to identify the 5 groups of oligosaccharides corresponding to P0 neutral, P1 mono-, P2 di-, P3 tri- and P4 tetrasialylated oligosaccharides. Record the retention times of the most prominent peaks in groups P0 to P4. Calculate the relative retentions of the most prominent peaks in groups P0 to P3 with reference to the most prominent peak in group P4.

Calculate the tetrasialylated peak area ratio for the test solution using the following expression:

$$\frac{A_{P4}}{\Sigma_{i=0}^{3} A_{Pi}}$$

$A_{P4}$ = peak area of group P4;

$A_{Pi}$ = peak area of groups P0 to P3.

*System suitability*:

- the chromatogram obtained with reference solution (a) is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*; 5 groups of oligosaccharide peaks corresponding to P0 neutral, P1 mono-, P2 di-, P3 tri- and P4 tetrasialylated oligosaccharides are present; group P4 includes the highest peak, and P3 the second-highest peak;
- no significant peaks are observed in regions P0 to P4 in the chromatogram obtained with the blank solution.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution (b);
- the relative retentions of the most prominent peaks in groups P0 to P3 in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with reference solution (b);
- the tetrasialylated peak area ratio for the test solution is within the limits authorised by the competent authority.

CHARACTERS

*Appearance*: clear, colourless liquid.

IDENTIFICATION

A. It forms a clot when examined in the conditions described under Assay (Potency).

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Solution A*. Dissolve 143.3 g of *guanidine hydrochloride R*, 9.086 g of *tris(hydroxymethyl)aminomethane R* and 0.931 g of *sodium edetate R* in 250 mL of *water R* and adjust to pH 8.0 ± 0.1 with *hydrochloric acid R*.

*Test solution*. Dilute the preparation to be examined with the formulation buffer (see Tests) to obtain a concentration of about 1.5 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer (see Tests) to obtain a concentration of about 1.5 mg/mL.

*Reduction and alkylation*. To 67 µL of the test solution add 28 µL of *water R*, 100 µL of solution A, then 5 µL of a 30.85 g/L solution of *dithiothreitol R*, mix well and centrifuge briefly. Overlay with nitrogen. Incubate in a water-bath at 40 °C for 1 h. Add 6.6 µL of a freshly prepared 115.04 g/L solution of *iodoacetic acid R*, mix well and centrifuge briefly. Overlay with nitrogen. Incubate at room temperature for 1 h protected from light. Add 5.3 µL of a 30.85 g/L of solution of *dithiothreitol R* and mix well. Add 188.1 µL of *water R*.

*Digestion*. To the reduced solution prepared previously, add 10 µL of a freshly prepared 3.4 U/mL solution of *lysyl endopeptidase R*, mix well and centrifuge briefly. Overlay with nitrogen. Incubate at 30 °C for 4 h. Mix 90 µL of the digested solution and 180 µL of a 33.22 g/L solution of *sodium edetate R*.

Carry out the reduction/alkylation and digestion steps for the reference solution in the same manner as for the test solution.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

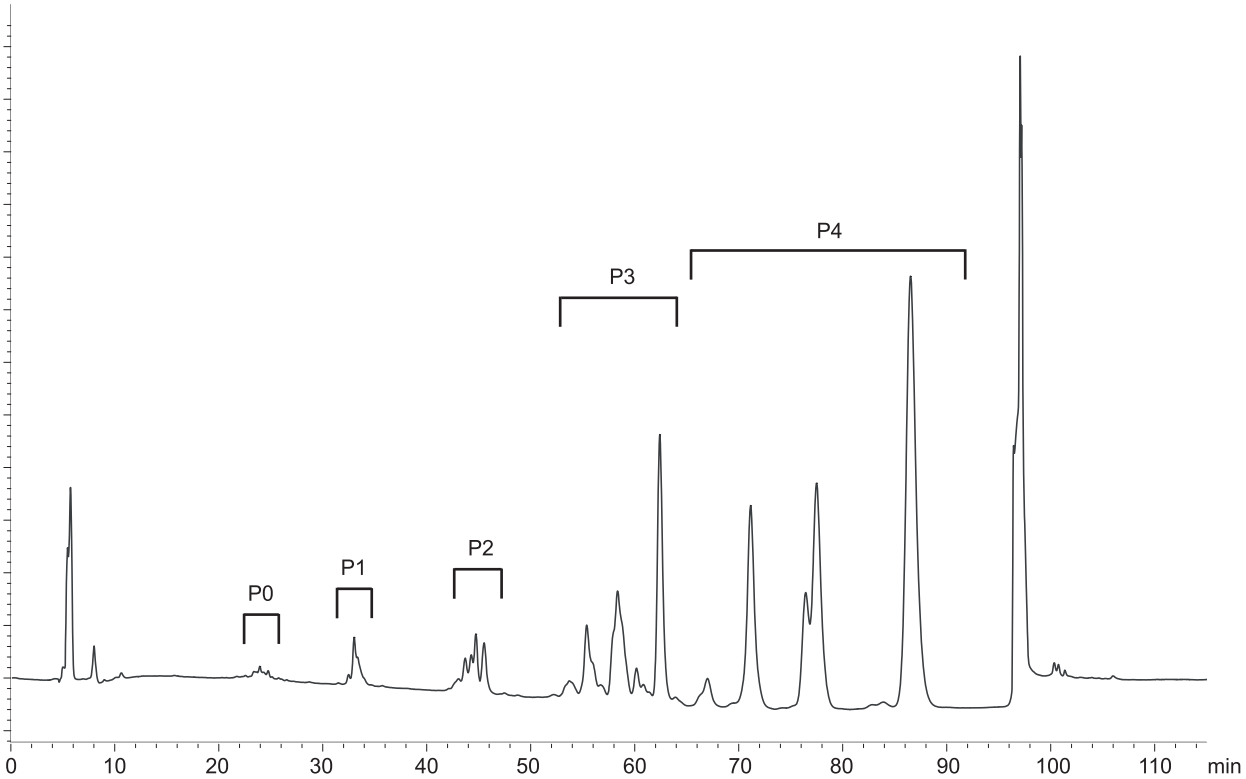


Figure 2522.-1. – *Chromatogram for the test for glycan analysis of human coagulation factor IX (rDNA)*

*Column*:
- *size*: *l* = 0.25 m, Ø = 2.1 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: add 0.5 mL of *trifluoroacetic acid R* to 1000 mL of *water R* and degas;
- *mobile phase B*: mix 0.5 mL of *trifluoroacetic acid R*, 50 mL of *water R* and 950 mL of *acetonitrile for chromatography R* and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 97 | 3 |
| 5 - 35 | 97 → 85 | 3 → 15 |
| 35 - 60 | 85 → 81 | 15 → 19 |
| 60 - 81 | 81 → 74 | 19 → 26 |
| 81 - 101 | 74 → 71 | 26 → 29 |
| 101 - 135 | 71 → 60 | 29 → 40 |
| 135 - 140 | 60 → 0 | 40 → 100 |
| 140 - 150 | 0 | 100 |
| 150 - 150.01 | 0 → 97 | 100 → 3 |
| 150.01 - 190 | 97 | 3 |
| 190 - 191 | 97 → 50 | 3 → 50 |
| 191 - 251 | 50 | 50 |

*Flow rate*: 0.25 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 240 µL, using an automatic injector maintained at 2-8 °C.

*System suitability*:
- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- all peaks identified in the chromatogram supplied with *human coagulation factor IX (rDNA) CRS* are visible in the chromatogram obtained with the reference solution.

*Results*:
- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution;
- no new major peaks are observed in the chromatogram obtained with the test solution in comparison to the chromatogram obtained with the reference solution.

C. Polyacrylamide gel electrophoresis (*2.2.31*).

Examine the electropherograms obtained in the test for impurities with molecular masses differing from that of human coagulation factor IX (rDNA).

Calculate the relative mobility (in per cent) of the main band in the electropherogram obtained with the test solution with reference to the mobility of the main band in the electropherogram obtained with reference solution (a), using the following expression:

$$\frac{M_1 - M_2}{M_2} \times 100$$

$M_1$ = molecular mass of the main band in the electropherogram obtained with the test solution;

$M_2$ = molecular mass of the main band in the electropherogram obtained with reference solution (a).

*Results*:
- the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);
- the mobility of the main band in the electropherogram obtained with the test solution is within 10 per cent of that of the main band in the electropherogram obtained with reference solution (a).

TESTS

**Formulation buffer**. Dissolve 19.53 g of *glycine R*, 1.55 g of *histidine R* and 10.00 g of *sucrose R* in 1000 mL of *water R*. Add 50 µL of *polysorbate 80 R* and adjust to pH 6.8 with *hydrochloric acid R*.

**Gamma-carboxyglutamic acid (Gla)**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined with the formulation buffer to obtain a concentration of about 1 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 1 mg/mL.

*Blank solution*. The formulation buffer.

*Column*:
- *size*: *l* = 0.05 m, Ø = 5 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (10 µm).

*Mobile phase*:
- *mobile phase A*: solution containing 2.42 g/L of *tris(hydroxymethyl)aminomethane R*, adjusted to pH 9.0 with *hydrochloric acid R*;
- *mobile phase B*: solution containing 2.42 g/L of *tris(hydroxymethyl)aminomethane R* and 58.45 g/L of *sodium chloride R*, adjusted to pH 9.0 with *hydrochloric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 70 → 60 | 30 → 40 |
| 40 - 49 | 60 → 0 | 40 → 100 |
| 49 - 50 | 0 → 70 | 100 → 30 |
| 50 - 71 | 70 | 30 |

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 50 µL; perform at least 3 injections using an automatic injector maintained at 2-8 °C.

*Relative retention* with reference to human coagulation factor IX (rDNA) containing 12 Gla residues per molecule (12 Gla, retention time = about 25 min): 9Gla = 0.60; 10Gla = 0.75; 11Gla = 0.85. *NOTE*: molecular species containing 9 or fewer Gla residues per molecule of human coagulation factor IX (rDNA) may not be present in the preparation.

*System suitability*: reference solution:
- *repeatability*: maximum relative standard deviation of 3 per cent for the total area of the peak due to human coagulation factor IX (rDNA), determined on 3 injections performed immediately before the run;
- the 10Gla peak is visible and is similar to the corresponding peak in the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to 11Gla, and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to 12Gla.

*Results*:

- *repeatability*: maximum relative standard deviation of 3 per cent for the total area of the peak due to human coagulation factor IX (rDNA), determined on 3 injections of the test solution;
- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

Calculate the total Gla content using the following expression:

$$\sum_{i=9}^{12} \frac{A_{Pi}}{total\ peak\ area} \times i\ Gla.mol^{-1}$$

- $A_{Pi}$: area of the concerned peak (9Gla, 10Gla, 11Gla or 12Gla); any shoulder appearing on the descending part of the 12Gla peak is included in the area of the 12Gla peak;
- *total peak area*: sum of the areas of peaks 9Gla to 12Gla;
- *i Gla.mol*$^{-1}$: 9, 10, 11 or 12, corresponding to the theoretical number of Gla residues per mole of human coagulation factor IX (rDNA) for the concerned peak.

*Limit*:

- 11.0 to 12.0 moles of Gla per mole of human coagulation factor IX (rDNA).

**Related proteins and impurities**. Liquid chromatography (*2.2.29*).

*Test solution.* Dilute the preparation to be examined with the formulation buffer to obtain a concentration of about 0.5 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 0.5 mg/mL.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (10 µm) with a pore size of 400 nm;
- *temperature*: 37 °C.

*Mobile phase*:

- *mobile phase A*: add 1 mL of *trifluoroacetic acid R* to 1000 mL of *water R*;
- *mobile phase B*: mix 1 mL of *trifluoroacetic acid R*, 200 mL of *water R* and 800 mL of *acetonitrile for chromatography R*.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 75 | 25 |
| 0.5 - 30 | 75 → 20 | 25 → 80 |
| 30 - 31 | 20 → 0 | 80 → 100 |
| 31 - 33 | 0 | 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL; perform at least 3 injections using an automatic injector.

*Relative retention* with reference to the 2nd peak of the double peak due to human coagulation factor IX (rDNA) (retention time = about 12-14 min): related protein A = about 0.75; related protein B = about 0.78; related protein C = about 0.80; related protein D = about 0.85; related protein E = about 0.93.

*System suitability*: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- *repeatability*: maximum relative standard deviation of 3 per cent for the total area of the peak due to human coagulation factor IX (rDNA) after 3 injections performed immediately before the run;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to related protein E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA).

Report individual relative peak areas considering the peak area of the entire chromatogram. Individual relative per cent peak areas are calculated as the average of the 3 injections of the test solution.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution, except for minor peaks due to impurities, that may be absent in the chromatogram obtained with the test solution.

*Limits*:

- *related protein C*: maximum 0.6 per cent;
- *total impurities (all peaks not eluted at the positions expected for human coagulation factor IX (rDNA) and its related proteins)*: maximum 1.0 per cent.

**Impurities with molecular masses differing from that of human coagulation factor IX (rDNA)**. Polyacrylamide gel electrophoresis (*2.2.31*) using a gradient gel with the following modifications.

*Gel dimensions*: 1.0 mm thick.

*Resolving gel*: 3-15 per cent acrylamide gradient gel.

*Acrylamide solution*: *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*.

To prepare a 15 per cent acrylamide solution, add *sucrose R* to obtain a concentration of 142 g/L.

The following elements are provided as examples for loading the gel forming apparatus to prepare the stacking gel.

*Stacking gel.* The stacking gel solution is prepared by mixing:

- 100 volumes of *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*;
- 125 volumes of *1 M tris-hydrochloride buffer solution pH 6.8 R*;
- 740.5 volumes of *water R*;
- 1.5 volumes of *tetramethylethylenediamine R*;
- 33 volumes of a 150 mg/mL solution of *ammonium persulfate R*.

Load the compartments of the gradient-forming apparatus with the acrylamide solutions and proceed as per the instructions of the equipment supplier to obtain the polymerised gradient gel.

After polymerisation is completed, rinse the gradient gel with *water R*. Remove any excess liquid. Pour the stacking gel solution into the equipment, insert a clean comb and allow for polymerisation.

Alternatively, commercially available gradient gels may be used.

*Test solution.* Dilute the preparation to be examined with the formulation buffer to obtain a concentration of about 1 mg/mL.

*Reference solution (a).* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 1 mg/mL.

*Reference solution (b).* 0.01 mg/mL solution of *bovine albumin R* in the formulation buffer.

*Reference solution (c).* A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 5-200 kDa.

*Sample buffer. Concentrated SDS-PAGE sample buffer for reducing conditions R* containing dithiothreitol as the reducing agent.

*Sample treatment*: incubate in a water-bath for 5 min.

*Application*: 35 µL.

Use *SDS-PAGE running buffer R* for running the gel.

For each gel, run 1 lane with reference solution (b), 2 lanes with reference solution (c), 2 lanes with the incubated reducing buffer (as blank) and at least 1 lane with reduced reference solution (a); use the remaining lanes for reduced test solutions.

*Detection*: by Coomassie staining.

*Identification of bands*: human coagulation factor IX (rDNA) has an approximate molecular mass of 55 kDa, and related protein bands with molecular masses of approximately 54 kDa, 44 kDa, 29-32 kDa, 27 kDa and 14 kDa are present.

*System suitability*:

- a clear background is obtained after destaining;
- the band in the electropherogram obtained with reference solution (b) is clearly visible;
- all expected bands in the electropherogram obtained with reference solution (c) are visible;
- the bands in the electropherogram obtained with reference solution (c) are clearly separated;
- no band is visible in the blank lanes.

*Results*:

- the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);
- no new band in the electropherogram obtained with the test solution has an intensity greater than that of the band in the electropherogram obtained with reference solution (b).

**Impurities with molecular masses greater than that of human coagulation factor IX (rDNA)**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined with the formulation buffer to obtain a concentration of about 400 µg/mL.

*Reference solution.* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL.

*Resolution solution.* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL. Desalt and concentrate the preparation to be examined using a suitably validated procedure. Reconstitute the recovered material in *0.1 M phosphate buffer solution pH 8.0 R* to obtain a concentration of 400 µg/mL. To 500 µL of this solution add 1.4 µL of a 250 mg/L solution of *glutaraldehyde R*. Mix and incubate at 37 °C for 120 min.

*Blank solution.* The formulation buffer.

*Precolumn*:

- *size*: *l* = 0.04 m, Ø = 6 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

*Mobile phase.* Dissolve 7.10 g of *anhydrous disodium hydrogen phosphate R* and 8.77 g of *sodium chloride R* in 1 L of *water R*. Adjust to pH 7.00 ± 0.05 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 50 µL; perform 3 injections using an automatic injector maintained at 2-8 °C.

*Retention time*: human coagulation factor IX (rDNA) = about 9 min.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the high molecular mass species and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA) in the chromatogram obtained with the resolution solution.

Calculate the relative area (in per cent) of the sum of the peaks with retention times less than that of human coagulation factor IX (rDNA), with reference to the area of the peak due to human coagulation factor IX (rDNA). Any shoulder appearing on the descending part of the peak due to human coagulation factor IX (rDNA) is included in its area.

*Result*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*Limit*:

- *sum of the peaks eluted before the principal peak*: maximum 1.3 per cent.

**Microbial contamination** (*2.6.12*): maximum 10 CFU/mL.

**Bacterial endotoxins** (*2.6.14*): less than 1 IU per 100 IU of factor IX activity.

ASSAY

The specific biological activity of the substance is determined before the addition of any protein stabiliser.

**Protein**. Size-exclusion chromatography (*2.2.30*) as described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA) with the following modifications.

Prepare triplicate dilutions of the test solution.

*Reference solutions.* Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of 1 mg/mL. Further dilute this solution to prepare a standard curve with concentrations in the range of 100-800 µg/mL (5 concentrations, typically 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL).

Plot peak areas versus injected protein content and perform linear regression to create a standard curve.

*System suitability* (in addition to those described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA)):

- the correlation coefficient ($r^2$) calculated for the standard curve is not less than 0.995.

Calculate the protein concentration of each replicate of the preparation to be examined using the standard curve and the assigned content in *human coagulation factor IX (rDNA) CRS*.

**Potency**. Assay of human coagulation factor IX (*2.7.11*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

*Human coagulation factor IX concentrate BRP* is suitable for use as a reference preparation.

STORAGE

In an airtight container, under approved conditions.

LABELLING

The label states the factor IX content in International Units per millilitre and in International Units per milligram of protein.

**01/2018:2994**

# HUMAN COAGULATION FACTOR IX (rDNA) POWDER FOR SOLUTION FOR INJECTION

## Factoris IX coagulationis humani (ADNr) pulvis ad solutionem iniectabilem

DEFINITION

Sterile, freeze-dried preparation of closely related glycoproteins, which have the same amino acid sequence (415 amino acids) as the naturally occurring Ala 148 allelic form analogue (plasma-derived coagulation factor IX). The preparation is intended for intravenous injection.

*Potency*: 80 per cent to 125 per cent of the potency stated on the label when determined using the conditions described under Assay.

*Human coagulation factor IX (rDNA) powder for solution for injection complies with the monograph on Parenteral preparations (0520) and with the following additional requirements.*

PRODUCTION

Human coagulation factor IX (rDNA) powder for solution for injection is prepared from *Human coagulation factor IX (rDNA) concentrated solution (2522)*. The concentrated solution is diluted with a formulation buffer containing excipients such as histidine, sucrose, glycine and polysorbate 80. This preparation is passed through a bacteria-retentive filter, distributed aseptically into the final containers and freeze-dried. The containers are closed under vacuum or under an inert gas. Where excipients other than those mentioned above are used, their compatibility with the tests described hereafter must be confirmed.

CHARACTERS

*Appearance* (before reconstitution): white, hygroscopic powder or friable solid, free from visible foreign matter.

IDENTIFICATION

A. It complies with the limits of the assay.

B. Polyacrylamide gel electrophoresis (*2.2.31*).

Examine the electropherograms obtained in the test for impurities with molecular masses differing from that of human coagulation factor IX (rDNA).

Calculate the relative mobility (in per cent) of the main band in the electropherogram obtained with the test solution with reference to the mobility of the main band in the electropherogram obtained with reference solution (a), using the following expression:

$$\frac{M_1 - M_2}{M_2} \times 100$$

$M_1$ = molecular mass of the main band in the electropherogram obtained with the test solution;

$M_2$ = molecular mass of the main band in the electropherogram obtained with reference solution (a).

*Results*:

- the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);
- the mobility of the main band in the electropherogram obtained with the test solution is within 10 per cent of that of the main band in the electropherogram obtained with reference solution (a).

TESTS

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, test (except those for solubility and water) and assay.*

**Formulation buffer**. Dissolve 19.53 g of *glycine R*, 1.55 g of *histidine R* and 10.00 g of *sucrose R* in 1000 mL of *water R*. Add 50 µL of *polysorbate 80 R* and adjust to pH 6.8 with *hydrochloric acid R*.

**Appearance of solution**. After reconstitution, the solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*), without visible particles.

**Solubility**. To a vial of the preparation to be examined add the volume of the diluent stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 5 min.

**pH**(*2.2.3*). The limit is approved by the competent authority.

**Related proteins**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of 0.5 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 0.5 mg/mL.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (10 µm) with a pore size of 400 nm;
- *temperature*: 37 °C.

*Mobile phase*:

- *mobile phase A*: add 1 mL of *trifluoroacetic acid R* to 1000 mL of *water for chromatography R*;
- *mobile phase B*: mix 1 mL of *trifluoroacetic acid R*, 200 mL of *water for chromatography R* and 800 mL of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 75 | 25 |
| 0.5 - 30 | 75 → 20 | 25 → 80 |
| 30 - 31 | 20 → 0 | 80 → 100 |
| 31 - 33 | 0 | 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL; perform at least 3 injections using an automatic injector.

*Relative retention* with reference to the 2nd peak of the double peak due to human coagulation factor IX (rDNA) (retention time = about 12-14 min): related protein A = about 0.75; related protein B = about 0.78; related protein C = about 0.80; related protein D = about 0.85; related protein E = about 0.93.

*System suitability*: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- *repeatability*: maximum relative standard deviation of 3 per cent for the total area of the peak due to human coagulation factor IX (rDNA), determined on 3 injections performed immediately before the run;

Monographs H

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to related protein E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA).

Report individual relative peak areas considering the peak area of the entire chromatogram. Individual relative per cent peak areas are calculated as the average of the 3 injections of the test solution.

Peaks due to related proteins A and D are not always present.

*Results*:

– the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution, except for minor peaks due to impurities, that may be absent in the chromatogram obtained with the test solution.

*Limits*:

– *related protein C*: maximum 0.6 per cent.

**Impurities with molecular masses differing from that of human coagulation factor IX (rDNA)**. Polyacrylamide gel electrophoresis (*2.2.31*) using a gradient gel with the following modifications.

*Gel dimensions*: 1.0 mm thick.

*Resolving gel*: 3-15 per cent acrylamide gradient gel.

*Acrylamide solution*: *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*.

To prepare a 15 per cent acrylamide solution, add *sucrose R* to obtain a concentration of 142 g/L.

The following elements are provided as examples for loading the gel-forming apparatus to prepare the stacking gel.

*Stacking gel*. The stacking gel solution is prepared by mixing:

– 100 volumes of *30 per cent acrylamide/bisacrylamide (36.5:1) solution R*;
– 125 volumes of *1 M tris-hydrochloride buffer solution pH 6.8 R*;
– 740.5 volumes of *water R*;
– 1.5 volumes of *tetramethylethylenediamine R*;
– 33 volumes of a 150 mg/mL solution of *ammonium persulfate R*.

Load the compartments of the gradient-forming apparatus with the acrylamide solutions and proceed as per the instructions of the equipment supplier to obtain the polymerised gradient gel.

After polymerisation is completed, rinse the gradient gel with *water R*. Remove any excess liquid. Pour the stacking gel solution into the equipment, insert a clean comb and allow for polymerisation.

Alternatively, commercially available gradient gels may be used.

*Test solution*. Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of about 1 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 1 mg/mL.

*Reference solution (b)*. A 0.01 mg/mL solution of *bovine albumin R* in the formulation buffer.

*Reference solution (c)*. A solution of molecular mass markers suitable for calibrating SDS-polyacrylamide gels in the range of 5-200 kDa.

*Sample buffer*. *Concentrated SDS-PAGE sample buffer for reducing conditions R* containing dithiothreitol as the reducing agent.

*Sample treatment*: incubate in a water-bath for 5 min.

*Application*: 35 µL.

Use *SDS-PAGE running buffer R* for running the gel.

For each gel, run 1 lane with reference solution (b), 2 lanes with reference solution (c), 2 lanes with the incubated reducing buffer (as blank) and at least 1 lane with reduced reference solution (a); use the remaining lanes for reduced test solutions.

*Detection*: by Coomassie staining.

*Identification of bands*: human coagulation factor IX (rDNA) has an approximate molecular mass of 55 kDa, and related protein bands with molecular masses of approximately 54 kDa, 44 kDa, 29-32 kDa, 27 kDa and 14 kDa are present.

*System suitability*:

– a clear background is obtained after destaining;
– the band in the electropherogram obtained with reference solution (b) is clearly visible;
– all expected bands in the electropherogram obtained with reference solution (c) are visible;
– the bands in the electropherogram obtained with reference solution (c) are clearly separated;
– no band is visible in the blank lanes.

*Results*:

– the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (a);
– no new band in the electropherogram obtained with the test solution has an intensity greater than that of the band in the electropherogram obtained with reference solution (b).

**Impurities with molecular masses greater than that of human coagulation factor IX (rDNA)**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Dilute the reconstituted preparation to be examined with the formulation buffer to obtain a concentration of 400 µg/mL.

*Reference solution*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL.

*Resolution solution*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of about 400 µg/mL. Desalt and concentrate the preparation to be examined using a suitably validated procedure. Reconstitute the recovered material in *0.1 M phosphate buffer solution pH 8.0 R* to obtain a concentration of 400 µg/mL. To 500 µL of this solution add 1.4 µL of a 250 mg/L solution of *glutaraldehyde R*. Mix and incubate at 37 °C for 120 min.

*Blank solution*. The formulation buffer.

*Precolumn*:

– *size*: *l* = 0.04 m, Ø = 6 mm;
– *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) of a grade suitable for the fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

*Mobile phase*. Dissolve 7.10 g of *anhydrous disodium hydrogen phosphate R* and 8.77 g of *sodium chloride R* in 1 L of *water for chromatography R*. Adjust to pH 7.00 ± 0.05 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 50 µL; perform 3 injections using an automatic injector maintained at 2-8 °C.

*Retention time*: human coagulation factor IX (rDNA) = about 9 min.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram supplied with *human coagulation factor IX (rDNA) CRS*;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the high molecular mass species and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to human coagulation factor IX (rDNA) in the chromatogram obtained with the resolution solution.

Calculate the relative area (in per cent) of the sum of the peaks with retention times less than that of human coagulation factor IX (rDNA), with reference to the area of the peak due to human coagulation factor IX (rDNA). Any shoulder appearing on the descending part of the peak due to human coagulation factor IX (rDNA) is included in its area.

*Result*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*Limit*:

- *sum of the peaks eluted before the principal peak*: maximum 1.8 per cent.

**Activated human coagulation factor IX (rDNA)** (*2.6.22*). Dilute the reconstituted preparation to be examined with *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R* to obtain a concentration of human coagulation factor IX (rDNA) of 20 IU/mL. For each of the dilutions, the coagulation time is not less than 150 s.

**Osmolality** (*2.2.35*). The limit is approved by the competent authority.

**Water** (*2.5.32*): maximum 2.0 per cent.

**Sterility** (*2.6.1*). It complies with the test.

**Bacterial endotoxins** (*2.6.14*): less than 1 IU per 100 IU of factor IX activity.

ASSAY

**Protein**. Size-exclusion chromatography (*2.2.30*) as described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA) with the following modifications.

Prepare triplicate dilutions of the test solution.

*Reference solutions*. Dissolve the contents of a vial of *human coagulation factor IX (rDNA) CRS* in the formulation buffer to obtain a concentration of 1 mg/mL. Further dilute the solution to prepare a standard curve with concentrations in the range of 100-800 µg/mL (5 concentrations, typically 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL and 800 µg/mL).

Plot peak areas versus injected protein content and perform linear regression to create a standard curve.

*System suitability* (in addition to those described in the test for impurities with molecular masses greater than that of human coagulation factor IX (rDNA)):

- the correlation coefficient ($r^2$) calculated for the standard curve is not less than 0.995.

Calculate the protein concentration of each replicate of the preparation to be examined using the standard curve and the assigned content in *human coagulation factor IX (rDNA) CRS*.

**Potency**. Assay of human coagulation factor IX (*2.7.11*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

*Human coagulation factor IX concentrate BRP* is suitable for use as a reference preparation.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the factor IX content in International Units;
- the name of any excipient and added substance;
- the composition and volume of the liquid to be used for reconstitution.

**04/2016:1644**

# HUMAN COAGULATION FACTOR XI

## Factor XI coagulationis humanus

DEFINITION

Sterile plasma protein fraction containing coagulation factor XI. It is prepared from *Human plasma for fractionation (0853)*. The preparation may contain excipients such as heparin, C1-esterase inhibitor and antithrombin III.

The potency of the preparation, reconstituted as stated on the label, is not less than 50 units per millilitre.

PRODUCTION

The method of preparation is designed to maintain functional integrity of human coagulation factor XI and to minimise activation of any coagulation factor (to minimise potential thrombogenicity). It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

After preparation, the factor XI fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is distributed into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under inert gas.

CHARACTERS

*Appearance*: white or almost white powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Solubility**. To a container of the preparation to be examined, add the volume of liquid stated on the label at room temperature. The preparation dissolves completely with gentle swirling within 10 min.

**pH** (*2.2.3*): 6.8 to 7.4.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube

to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25.

**Activated coagulation factors** (*2.6.22*). For each of the dilutions, the coagulation time is not less than 150 s.

**Heparin** (*2.7.12*). If heparin has been added, the preparation to be examined contains not more than the amount of heparin stated on the label and in all cases not more than 0.5 IU of heparin per unit of factor XI.

**Antithrombin III** (*2.7.17*). If antithrombin III has been added, the preparation to be examined contains not more than the amount of antithrombin III stated on the label.

**C1-esterase inhibitor** (*2.7.34*). If C1-esterase inhibitor has been added, the preparation to be examined contains not more than the amount of C1-esterase inhibitor stated on the label.

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method A*). The 1 to 64 dilution does not show agglutination.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to 100 IU of factor XI.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.1 IU of endotoxin per International Unit of factor XI.

ASSAY

Carry out the assay of human coagulation factor XI (*2.7.22*).

The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the number of units per container;
- the maximum amount of protein per container;
- where applicable, the amount of heparin per container;
- where applicable, the amount of antithrombin III per container;
- where applicable, the amount of C1-esterase inhibitor per container;
- the name and volume of the liquid to be used for reconstitution.

**01/2011:0024**
**corrected 7.6**

# HUMAN FIBRINOGEN

## Fibrinogenum humanum

DEFINITION

Sterile, freeze-dried preparation of a plasma protein fraction containing the soluble constituent of human plasma that is transformed to fibrin on the addition of thrombin. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as salts, buffers and stabilisers.

When reconstituted as stated on the label, the solution contains not less than 10 g/L of fibrinogen.

PRODUCTION

The method of preparation is designed to maintain functional integrity of human fibrinogen. It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and any residues are such as not to compromise the safety of the preparation for patients.

The specific activity (fibrinogen content with respect to total protein content) is not less than 80 per cent before addition of any protein stabiliser. The fibrinogen content is determined by a suitable method such as that described under Assay, and the total protein content is determined by a suitable method such as that described under Total protein in *Human albumin solution (0255)*. Albumin may also be obtained with fibrinogen during fractionation, in which case a specific determination of albumin is carried out by a suitable immunochemical method (*2.7.1*) and the quantity of albumin determined is subtracted from the total protein content for the calculation of the specific activity.

The protein fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

CHARACTERS

*Appearance*: white or pale yellow, hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Solubility**. To a container of the preparation to be examined add the volume of liquid stated on the label at the recommended temperature. The preparation dissolves within 30 min at 20-25 °C, forming an almost colourless, slightly opalescent solution.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Stability of solution**. No gel formation appears at 20-25 °C within 60 min following reconstitution.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the test for bacterial endotoxins.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 30 mg of fibrinogen.

Where the test for bacterial endotoxins is used, the preparation to be examined contains less than 0.03 IU of endotoxin per milligram of fibrinogen.

ASSAY

Mix 0.2 mL of the reconstituted preparation with 2 mL of a suitable buffer solution (pH 6.6-6.8) containing sufficient thrombin (approximately 3 IU/mL) and calcium (0.05 mol/L). Maintain at 37 °C for 20 min, separate the precipitate by centrifugation (5000 *g*, 20 min) and wash thoroughly with a 9 g/L solution of *sodium chloride R*. Determine the nitrogen content by sulfuric acid digestion (*2.5.9*) and calculate the fibrinogen (clottable protein) content by multiplying the result by 6.0. The content is not less than 70 per cent and not more than 130 per cent of the stated content of fibrinogen.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the content of fibrinogen in the container;
- the name and volume of the liquid to be used for reconstitution;
- where applicable, the name and amount of protein stabiliser added in the preparation.

**04/2019:2323**

# HUMAN HAEMATOPOIETIC STEM CELLS

## Cellulae stirpes haematopoieticae humanae

*This monograph provides a standard for the preparation and control of human haematopoietic stem cells for use in therapy. It does not exclude the use of alternative preparation and control methods that are acceptable to the competent authority.*

DEFINITION

Human haematopoietic stem cells are primitive multipotent cells capable of self-renewal as well as differentiation and maturation into all haematopoietic lineages. They are found in small numbers in bone marrow, in the mononuclear cell fraction of circulating blood and in umbilical cord blood. The preparation also contains haematopoietic progenitor cells, which are capable of differentiation but not self-renewal. The numbers of haematopoietic stem cells and haematopoietic progenitor cells are correlated.

This monograph applies to haematopoietic stem cells that have not undergone expansion or genetic modification, and that are intended to provide a successful engraftment leading to a permanent restoration of all lineages of blood cell production to a sufficient level and function in a recipient whose haematopoiesis has been compromised by, for example, disease or high doses of chemotherapy and/or radiation therapy, or has to be replaced in certain congenital diseases. The infused haematopoietic stem cells can originate from the recipient (autologous) or from another individual (allogeneic).

Haematopoietic stem cells are recognised by their ability to reconstitute human haematopoiesis *in vivo*. They also have the capacity to differentiate into colony-forming cells, which are able to give rise to colonies in the presence of various growth factors. The membrane marker CD34 is commonly used for the successful isolation/purification of haematopoietic stem cells from crude preparations and as an indicator of haematopoietic stem cell content in routine quality control.

PRODUCTION

*DONORS*

Where allogeneic cells are used, they are derived from carefully selected donors in accordance with donor selection criteria. Directive 2004/23/EC of the European Union deals with the criteria for donor selection.

*COLLECTION*

*Peripheral blood stem cells*. These are collected by cytapheresis after mobilisation from the bone marrow by administration of growth factors and/or treatment of autologous donors with cytotoxic substances. The cells may be processed to select a population of interest and may be cryopreserved.

*Bone marrow*. Bone marrow is harvested by aspirating the cells from the cavities of hollow bones, then removing bone fragments by filtration and, if necessary, separating the buffy coat cells after centrifugation or with commercial kits based on the cytapheresis principle. The cells may be processed to select a population of interest and may be cryopreserved.

*Umbilical cord blood*. Placental blood haematopoietic cells are collected from placentae via the vein of the umbilical cord. The cells are then cryopreserved.

*CRYOPRESERVATION*

Cryopreservation allows storage for long periods. The cells are suspended in a validated medium containing a suitable cryoprotectant (for example, dimethyl sulfoxide) and macromolecules (for example, autologous plasma/albumin) and are frozen in cryobags in a manner designed to maintain viability of the cells by controlled cooling according to a validated method. They are stored at a temperature of – 140 °C or lower. Where cryobags are stored under other conditions of temperature and duration, the functionality of the preparation must be validated. Cryobags from donors that test positive for any infectious disease marker must be stored in such a way as to avoid cross-contamination.

*SUBSTANCES USED IN PRODUCTION*

The quality of substances used in production may be critical with respect to the quality, safety and efficacy of the final product, particularly for substances of biological origin. This is of particular importance for:

- proteins, including enzymes and antibodies;
- cryopreservation reagents;
- purification reagents.

**Quality assurance**. All substances must be produced within a recognised quality management system using suitable production facilities.

**Quality specifications**. A suitable quality specification must be presented for each substance, including notably:

- identity;
- potency (where applicable);
- purity;
- determination of bacterial endotoxins (*2.6.14*) (where applicable);
- microbiological quality (total viable count, tests for specified micro-organisms);
- sterility (*2.6.1*) (where applicable).

**Viral safety**. The requirements of chapter *5.1.7* apply.

**Transmissible spongiform encephalopathies** (*5.2.8*). A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out, and suitable measures are taken to minimise any such risk.

**Water**. Water used in the preparation of cellular products complies with the relevant monograph (*Water for injections (0169)*, *Purified water (0008)*). Water incorporated into the final product complies with the section on Water for injections in bulk in the monograph *Water for injections (0169)*, and in addition is sterile.

TESTS

*Target specifications are established for the different tests, but these are not used as rigid acceptance criteria.*

Tests carried out include the following (further tests, such as purging, cell depletion, allogeneic application, may be necessary depending on any treatment applied to the cells and on the intended recipient):

**Nucleated cell count** (*2.7.29*).

**Viability** (*2.7.29*). Viability is assessed for products that are not infused within 24 h of collection.

**CD34+ cell count**. For peripheral blood stem cells, CD34+ cell count is determined using a validated automated apparatus to analyse cells labelled with anti-CD34 antibodies. The apparatus and method employed must be able to determine the number of CD34+ cells with a sensitivity, accuracy and reproducibility comparable with those of immunophenotyping (*2.7.23*), where cells are labelled using anti-CD34 and anti-CD45 antibodies conjugated to a fluorochrome and analysed by flow cytometry (*2.7.24*).

**Colony-forming cell (CFC) assay** (*2.7.28*). Proliferative capacity is established by a suitable assay. The test is not necessarily carried out on each unit. The correlation between the dose of CD34 and the number of CFCs in a given situation (pathology, packaging, mobilisation) is determined. The CFC assay is carried out periodically; whenever a change that could affect the quality of CD34+ cells is made to the protocol for packaging or mobilisation, it is carried out on a suitable number of units.

**Microbiological control**. Examine as prescribed in general chapter *2.6.27. Microbiological examination of cell-based preparations*. Where justified, the product may be released before completion of the test.



**01/2015:0769**

# HUMAN HEPATITIS A IMMUNOGLOBULIN

## Immunoglobulinum humanum hepatitidis A

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected donors having antibodies against hepatitis A virus. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (*2.7.1*).

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis A immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human hepatitis A immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 600 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2015:0722**

# HUMAN HEPATITIS B IMMUNOGLOBULIN

## Immunoglobulinum humanum hepatitidis B

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular or subcutaneous administration. It is obtained from plasma from selected and/or immunised donors having antibodies against hepatitis B surface antigen. *Human normal immunoglobulin for intramuscular administration (0338)* or *Human normal immunoglobulin for subcutaneous administration (2788)* may be added.

Depending on the route of administration, it complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)* or *Human normal immunoglobulin for subcutaneous administration (2788)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (*2.7.1*).

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis B immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human hepatitis B immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 100 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)* or *Human normal immunoglobulin for subcutaneous administration (2788)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)* or *Human normal immunoglobulin for subcutaneous administration (2788)*.

The label states the number of International Units per container.

**01/2015:1016**

# HUMAN HEPATITIS B IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

## Immunoglobulinum humanum hepatitidis B ad usum intravenosum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It is obtained from plasma from selected and/or immunised donors having antibodies against hepatitis B surface antigen. *Human normal immunoglobulin for intravenous administration (0918)* may be added.

It complies with the monograph *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content and the limit for osmolality.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay (*2.7.1*) of suitable sensitivity and specificity.

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis B immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human hepatitis B immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 50 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the minimum number of International Units of hepatitis B immunoglobulin per container.

**01/2020:0397**

# HUMAN MEASLES IMMUNOGLOBULIN

## Immunoglobulinum humanum morbillicum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against measles virus. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency of the liquid preparation and of the freeze-dried preparation after reconstitution as stated on the label is not less than 50 IU per millilitre of neutralising antibody against measles virus.

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using a challenge dose of measles virus in a suitable cell culture system. An alternative method may be used providing that the competent authority is satisfied that it correlates with the determination of neutralising activity for the measles virus by comparison with the reference preparation.

The International Unit is the specific neutralising activity for measles virus contained in a stated amount of the International Standard for human anti-measles serum. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

*Method*

Prepare serial 2-fold dilutions of the immunoglobulin to be examined and of the reference preparation. Mix each dilution with an equal volume of a suspension of measles virus containing about 100 $CCID_{50}$ in 0.1 mL and incubate protected from light at 37 °C for 2 h. Using not fewer than 6 cell cultures per mixture, inoculate 0.2 mL of each mixture into each of the cell cultures allocated to that mixture and incubate for not less than 10 days. Examine the cultures for viral activity and compare the dilution containing the smallest quantity of the immunoglobulin which neutralises the virus with that of the corresponding dilution of the reference preparation.

Calculate the potency of the immunoglobulin to be examined in International Units per millilitre of neutralising antibody against measles virus.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2015:0338**

# HUMAN NORMAL IMMUNOGLOBULIN FOR INTRAMUSCULAR ADMINISTRATION

## Immunoglobulinum humanum normale ad usum intramusculum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for intramuscular administration contains the IgG antibodies of normal subjects and is intended for intramuscular administration. The preparation may contain excipients such as stabilisers. Multidose preparations contain an antimicrobial preservative.

This monograph does not apply to products intentionally prepared to contain fragments of IgG or chemically modified IgG.

Human normal immunoglobulin for intramuscular administration is obtained from plasma that complies with the monograph *Human plasma for fractionation (0853)*.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intramuscularly. Any antimicrobial preservative or stabilising agent used shall have been shown to have no deleterious effect on the final product in the amount present.

Human normal immunoglobulin for intramuscular administration is prepared from pooled material from not fewer than 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection;
- at a protein concentration of 50 g/L, contains at least 2 antibodies (1 viral and 1 bacterial) for which an International Standard or reference preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material;
- has a defined distribution of IgG subclasses.

Human normal immunoglobulin for intramuscular administration is prepared as a stabilised solution, for example in a 9 g/L solution of sodium chloride, a 22.5 g/L solution of glycine or, if the preparation is to be freeze-dried, a 60 g/L solution of glycine. No antibiotic is added to the plasma used. Single-dose preparations do not contain an antimicrobial preservative. The solution is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

*Appearance*:

- *liquid preparation*: clear or slightly opalescent, colourless or pale-yellow or light-brown liquid; during storage it may show formation of slight turbidity or a small amount of visible particulate matter;
- *freeze-dried preparation*: white or slightly yellow powder or solid friable mass, hygroscopic.

*For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.*

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to obtain a protein concentration of 10 g/L. The main component of the preparation to be examined corresponds to the IgG component of normal human serum. The preparation to be examined may show the presence of small quantities of other plasma proteins; if human albumin has been added as a stabiliser, it may be seen as a component.

TESTS

**Solubility**. For the freeze-dried preparation, to a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely within 20 min at 20-25 °C.

**pH** (*2.2.3*): 5.0 to 7.2.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 10 g/L.

**Total protein**. The preparation has a protein concentration of not less than 100 g/L and not more than 180 g/L and contains not less than 90 per cent and not more than 110 per cent of the quantity of protein stated on the label.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the centrifugation residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying the result by 6.25.

**Protein composition**. Zone electrophoresis (*2.2.31*).

Use strips of suitable cellulose acetate gel or suitable agarose gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

If cellulose acetate is the supporting material, the method described below can be used. If agarose gels are used, and because they are normally part of an automated system, the manufacturer's instructions are followed instead.

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 30 g/L.

*Reference solution*. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 30 g/L.

To a strip apply 4.0 µL of the test solution as a 10 mm band or apply 0.4 µL per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strip with *amido black 10B solution R* for 5 min. Decolourise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a

mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

*System suitability*: in the electropherogram obtained with the reference solution, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

*Results*: in the electropherogram obtained with the test solution, not more than 10 per cent of protein has a mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabiliser; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabiliser.

**Molecular-size distribution**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range of 4-12 g/L and injection of 50-600 µg of protein are usually suitable.

*Reference solution.* Dilute *human immunoglobulin (molecular size) BRP* with a 9 g/L solution of *sodium chloride R* to the same protein concentration as the test solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7.5 mm [or *l* = 0.3 m, Ø = 7.8 mm];
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

*Mobile phase*: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 L of *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Identification of peaks*: in the chromatogram obtained with the reference solution, the principal peak corresponds to the IgG monomer and there is a peak corresponding to the dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time less than that of the dimer corresponds to polymers and aggregates.

*Results*: in the chromatogram obtained with the test solution:

- *retention time*: for the monomer and for the dimer, the retention time relative to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02;
- *peak area*: the sum of the peak areas of the monomer and the dimer represent not less than 85 per cent of the total area of the chromatogram and the sum of the peak areas of polymers and aggregates represents not more than 10 per cent of the total area of the chromatogram. This requirement is not applicable if albumin has been added as a stabiliser; for such preparations, a test for molecular-size distribution is carried out during manufacture before addition of the stabiliser.

**Antibody to hepatitis B surface antigen**: minimum 0.5 IU per gram of immunoglobulin, determined by a suitable immunochemical method (*2.7.1*).

**Antibody to hepatitis A virus**. If intended for use in the prophylaxis of hepatitis A, it complies with the following additional requirement.

Determine the antibody content by comparison with a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (*2.7.1*).

The International Unit is the activity contained in a stated amount of the International Standard for anti-hepatitis A immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human hepatitis A immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

The stated potency is not less than 100 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

**Immunoglobulin A**. As determined by a suitable immunochemical method (*2.7.1*), the content of immunoglobulin A is not greater than the maximum content stated on the label.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject 1 mL per kilogram of the rabbit's mass.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 5 IU of endotoxin per millilitre.

STORAGE

In an airtight, colourless glass container, protected from light, at the temperature stated on the label.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre;
- for freeze-dried preparations:
  - the quantity of protein in the container;
  - the name or composition and the volume of the reconstituting liquid to be added;
- the route of administration;
- the distribution of subclasses of IgG present in the preparation;
- where applicable, that the preparation is suitable for use in the prophylaxis of hepatitis A infection;
- where applicable, the anti-hepatitis A virus activity in International Units per millilitre;
- where applicable, the amount of albumin added as a stabiliser;
- where applicable, the name and amount of antimicrobial preservative in the preparation;
- the maximum content of immunoglobulin A.

**01/2012:0918**

# HUMAN NORMAL IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

## Immunoglobulinum humanum normale ad usum intravenosum

DEFINITION

Human normal immunoglobulin for intravenous administration is a sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for intravenous administration contains the IgG antibodies of normal subjects. This monograph does not apply to products intentionally prepared to contain fragments or chemically modified IgG.

Human normal immunoglobulin for intravenous administration is obtained from plasma that complies with the monograph *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin. The method of preparation also includes a step or steps that have been shown to remove thrombosis-generating agents. Emphasis is given to the identification of activated coagulation factors and their zymogens and process steps that may cause their activation. Consideration is also to be given to other procoagulant agents that could be introduced by the manufacturing process.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered intravenously.

Human normal immunoglobulin for intravenous administration is prepared from pooled material from not fewer than 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection;
- at an immunoglobulin concentration of 50 g/L, contains antibodies for at least 2 of which (1 viral and 1 bacterial) an International Standard or Reference Preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material;
- has a defined distribution of immunoglobulin G subclasses;
- complies with the test for Fc function of immunoglobulin (*2.7.9*);
- does not exhibit thrombogenic (procoagulant) activity.

Human normal immunoglobulin for intravenous administration is prepared as a stabilised solution or as a freeze-dried preparation. In both cases the preparation is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas. No antibiotic is added to the plasma used. No antimicrobial preservative is added either during fractionation or at the stage of the final bulk solution.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

*Appearance*:

- *liquid preparation*: clear or slightly opalescent, colourless or pale yellow liquid;
- *freeze-dried preparation*: hygroscopic, white or slightly yellow powder or solid friable mass.

*For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.*

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to contain 10 g/L of protein. The main component of the preparation to be examined corresponds to the IgG component of normal human serum. The preparation to be examined may show the presence of small quantities of other plasma proteins; if human albumin has been added as a stabiliser, it may be seen as a major component.

TESTS

**Solubility**. For the freeze-dried preparation, add to the container the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely within 30 min at 20-25 °C.

**pH** (*2.2.3*): 4.0 to 7.4.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing 10 g/L of protein.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. The preparation contains not less than 30 g/L and between 90 per cent and 110 per cent of the quantity of protein stated on the label.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of this solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the centrifugation residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying the result by 6.25.

**Protein composition**. Zone electrophoresis (*2.2.31*).

Use strips of suitable cellulose acetate gel or suitable agarose gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

If cellulose acetate is the supporting material, the method described below can be used. If agarose gels are used, and because they are normally part of an automated system, the manufacturer's instructions are followed instead.

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to an immunoglobulin concentration of 30 g/L.

*Reference solution*. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/L solution of *sodium chloride R* to a protein concentration of 30 g/L.

To a strip apply 4.0 µL of the test solution as a 10 mm band or apply 0.4 µL per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strips with *amido black 10B solution R* for 5 min. Decolourise with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a

mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

*System suitability*: in the electropherogram obtained with the reference solution, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

*Results*: in the electropherogram obtained with the test solution, not more than 5 per cent of protein has a mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabiliser; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabiliser.

**Molecular size distribution**. Size exclusion chromatography (*2.2.30*).

*Test solution.* Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range of 4-12 g/L and injection of 50-600 µg of protein are usually suitable.

*Reference solution.* Dilute *human immunoglobulin (molecular size) BRP* with a 9 g/L solution of *sodium chloride R* to the same protein concentration as the test solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7.5 mm, or *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

*Mobile phase*: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 L of *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Identification of peaks*: in the chromatogram obtained with the reference solution, the principal peak corresponds to the IgG monomer and there is a peak corresponding to the dimer with a relative retention to the principal peak of about 0.85; identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time shorter than that of the dimer corresponds to polymers and aggregates.

*Results*: in the chromatogram obtained with the test solution:

- *retention time*: for the monomer and for the dimer, the retention time relative to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02;
- *peak area*: the sum of the peak areas of the monomer and the dimer represent not less than 90 per cent of the total area of the chromatogram and the sum of the peak areas of polymers and aggregates represents not more than 3 per cent of the total area of the chromatogram. This requirement does not apply to products where albumin has been added as a stabiliser; for products stabilised with albumin, a test for distribution of molecular size is carried out during manufacture before addition of the stabiliser.

**Anticomplementary activity** (*2.6.17*). The consumption of complement is not greater than 50 per cent (1 $CH_{50}$ per milligram of immunoglobulin).

**Prekallikrein activator** (*2.6.15*): maximum 35 IU/mL, calculated with reference to a dilution of the preparation to be examined containing 30 g/L of immunoglobulin.

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method B*). It complies with the test for anti-A and anti-B haemagglutinins (direct method).

**Anti-D antibodies** (*2.6.26*). It complies with the test for anti-D antibodies in human immunoglobulin.

**Antibody to hepatitis B surface antigen**: minimum 0.5 IU per gram of immunoglobulin, determined by a suitable immunochemical method (*2.7.1*).

**Immunoglobulin A.** As determined by a suitable immunochemical method (*2.7.1*), the content of immunoglobulin A is not greater than the maximum content stated on the label.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to 0.5 g of immunoglobulin, but not more than 10 mL per kilogram of the rabbit's mass.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.5 IU of endotoxin per millilitre for solutions with a protein content not greater than 50 g/L, and less than 1.0 IU of endotoxin per millilitre for solutions with a protein content greater than 50 g/L but not greater than 100 g/L.

## STORAGE

Liquid preparation: in a colourless glass container, protected from light, at the temperature stated on the label.

Freeze-dried preparation: in an airtight colourless glass container, protected from light, at a temperature not exceeding 25 °C.

## LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre;
- for freeze-dried preparations:
  - the quantity of protein in the container;
  - the name or composition and the volume of the reconstituting liquid to be added;
- the amount of immunoglobulin in the container;
- the route of administration;
- the distribution of subclasses of immunoglobulin G present in the preparation;
- where applicable, the amount of albumin added as a stabiliser;
- the maximum content of immunoglobulin A.

**01/2015:2788**

# HUMAN NORMAL IMMUNOGLOBULIN FOR SUBCUTANEOUS ADMINISTRATION

## Immunoglobulinum humanum normale ad usum subdermicum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G (IgG). Other proteins may be present. Human normal immunoglobulin for subcutaneous administration contains the IgG antibodies of normal subjects and is intended for subcutaneous administration. The preparation may contain excipients such as stabilisers.

This monograph does not apply to products intentionally prepared to contain fragments of IgG or chemically modified IgG.

Human normal immunoglobulin for subcutaneous administration is obtained from plasma that complies with the monograph *Human plasma for fractionation (0853)*.

PRODUCTION

The method of preparation includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses, it shall have been shown that any residues present in the final product have no adverse effects on the patients treated with the immunoglobulin.

The method of preparation also includes a step or steps that have been shown to remove thrombosis-generating agents. Emphasis is given to the identification of activated coagulation factors and their zymogens and process steps that may cause their activation. Consideration is also to be given to other procoagulant agents that could be introduced by the manufacturing process.

The product shall have been shown, by suitable tests in animals and evaluation during clinical trials, to be well tolerated when administered subcutaneously. Any stabilising agent used shall have been shown to have no deleterious effect on the final product in the amount present.

Human normal immunoglobulin for subcutaneous administration is prepared from pooled material from not fewer than 1000 donors by a method that has been shown to yield a product that:

- does not transmit infection;
- at a protein concentration of 50 g/L, contains at least 2 antibodies (1 viral and 1 bacterial) for which an International Standard or reference preparation is available, the concentration of such antibodies being at least 3 times that in the initial pooled material;
- has a defined distribution of IgG subclasses;
- complies with the test for Fc function of immunoglobulin (*2.7.9*);
- does not exhibit thrombogenic (procoagulant) activity.

Human normal immunoglobulin for subcutaneous administration is prepared as a stabilised solution, for example in a 9 g/L solution of sodium chloride, a 22.5 g/L solution of glycine or, if the preparation is to be freeze-dried, a 60 g/L solution of glycine. No antibiotic is added to the plasma used. Preparations do not contain an antimicrobial preservative. The solution is passed through a bacteria-retentive filter. The preparation may subsequently be freeze-dried and the containers closed under vacuum or under an inert gas.

The stability of the preparation is demonstrated by suitable tests carried out during development studies.

CHARACTERS

*Appearance*:

- *liquid preparation*: clear or slightly opalescent, colourless or pale-yellow or light-brown liquid; during storage it may show formation of slight turbidity or a small amount of visible particulate matter;
- *freeze-dried preparation*: white or slightly yellow powder or solid friable mass, hygroscopic.

*For the freeze-dried preparation, reconstitute as stated on the label immediately before carrying out the identification and the tests, except those for solubility and water.*

IDENTIFICATION

Examine by a suitable immunoelectrophoresis technique. Using antiserum to normal human serum, compare normal human serum and the preparation to be examined, both diluted to obtain a protein concentration of 10 g/L. The main component of the preparation to be examined corresponds to the IgG component of normal human serum. The preparation to be examined may show the presence of small quantities of other plasma proteins; if human albumin has been added as a stabiliser, it may be seen as a component.

TESTS

**Solubility**. For the freeze-dried preparation, to a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely within 20 min at 20-25 °C.

**pH** (*2.2.3*): 4.6 to 7.2.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 10 g/L.

**Total protein**. The preparation has a protein concentration of not less than 100 g/L and not more than 220 g/L and contains not less than 90 per cent and not more than 110 per cent of the quantity of protein stated on the label.

Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the centrifugation residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the content of protein by multiplying the result by 6.25.

**Protein composition**. Zone electrophoresis (*2.2.31*).

Use strips of suitable cellulose acetate gel or suitable agarose gel as the supporting medium and *barbital buffer solution pH 8.6 R1* as the electrolyte solution.

If cellulose acetate is the supporting material, the method described below can be used. If agarose gels are used, and because they are normally part of an automated system, the manufacturer's instructions are followed instead.

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 30 g/L.

*Reference solution*. Reconstitute *human immunoglobulin for electrophoresis BRP* and dilute with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of 30 g/L.

To a strip apply 4.0 µL of the test solution as a 10 mm band or apply 0.4 µL per millimetre if a narrower strip is used. To another strip apply in the same manner the same volume of the reference solution. Apply a suitable electric field such that the albumin band of normal human serum applied on a control strip migrates at least 30 mm. Stain the strip with *amido black 10B solution R* for 5 min. Decolourise

with a mixture of 10 volumes of *glacial acetic acid R* and 90 volumes of *methanol R* so that the background is just free of colour. Develop the transparency of the strips with a mixture of 19 volumes of *glacial acetic acid R* and 81 volumes of *methanol R*. Measure the absorbance of the bands at 600 nm in an instrument having a linear response over the range of measurement. Calculate the result as the mean of 3 measurements of each strip.

*System suitability*: in the electropherogram obtained with the reference solution, the proportion of protein in the principal band is within the limits stated in the leaflet accompanying the reference preparation.

*Results*: in the electropherogram obtained with the test solution, not more than 10 per cent of protein has a mobility different from that of the principal band. This limit is not applicable if albumin has been added to the preparation as a stabiliser; for such preparations, a test for protein composition is carried out during manufacture before addition of the stabiliser.

**Molecular-size distribution**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to a concentration suitable for the chromatographic system used. A concentration in the range of 4-12 g/L and injection of 50-600 µg of protein are usually suitable.

*Reference solution*. Dilute *human immunoglobulin (molecular size) BRP* with a 9 g/L solution of *sodium chloride R* to the same protein concentration as the test solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7.5 mm [or *l* = 0.3 m, Ø = 7.8 mm];
- *stationary phase*: *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins with relative molecular masses in the range 10 000 to 500 000.

*Mobile phase*: dissolve 4.873 g of *disodium hydrogen phosphate dihydrate R*, 1.741 g of *sodium dihydrogen phosphate monohydrate R*, 11.688 g of *sodium chloride R* and 50 mg of *sodium azide R* in 1 L of *water R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Identification of peaks*: in the chromatogram obtained with the reference solution, the principal peak corresponds to the IgG monomer and there is a peak corresponding to the dimer with a relative retention to the principal peak of about 0.85. Identify the peaks in the chromatogram obtained with the test solution by comparison with the chromatogram obtained with the reference solution; any peak with a retention time less than that of the dimer corresponds to polymers and aggregates.

*Results*: in the chromatogram obtained with the test solution:

- *retention time*: for the monomer and for the dimer, the retention time relative to the corresponding peak in the chromatogram obtained with the reference solution is 1 ± 0.02;
- *peak area*: the sum of the peak areas of the monomer and the dimer represent not less than 85 per cent of the total area of the chromatogram and the sum of the peak areas of polymers and aggregates represents not more than 10 per cent of the total area of the chromatogram. This requirement is not applicable if albumin has been added as a stabiliser; for such preparations, a test for molecular-size distribution is carried out during manufacture before addition of the stabiliser.

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method B*). It complies with the test.

**Anti-D antibodies** (*2.6.26*). It complies with the test.

**Antibody to hepatitis B surface antigen**: minimum 0.5 IU per gram of immunoglobulin, determined by a suitable immunochemical method (*2.7.1*).

**Immunoglobulin A**. As determined by a suitable immunochemical method (*2.7.1*), the content of immunoglobulin A is not greater than the maximum content stated on the label.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject 1 mL per kilogram of the rabbit's mass.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 5 IU of endotoxin per millilitre.

STORAGE

In an airtight, colourless glass container, protected from light, at the temperature stated on the label.

LABELLING

The label states:

- for liquid preparations, the volume of the preparation in the container and the protein content expressed in grams per litre;
- for freeze-dried preparations:
  - the quantity of protein in the container;
  - the name or composition and the volume of the reconstituting liquid to be added;
- the route of administration;
- the distribution of subclasses of IgG present in the preparation;
- where applicable, the amount of albumin added as a stabiliser;
- the maximum content of immunoglobulin A.

**01/2020:0853**

# HUMAN PLASMA FOR FRACTIONATION

## Plasma humanum ad separationem

DEFINITION

Liquid part of human blood remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure; it is intended for the manufacture of plasma-derived products.

PRODUCTION

*DONORS*

Only a carefully selected, healthy donor who, as far as can be ascertained after medical examination, laboratory blood tests and a study of the donor's medical history, is free from detectable agents of infection transmissible by plasma-derived products may be used. Recommendations in this field are made by the Council of Europe [*Recommendation No. R (95) 15 on the preparation, use and quality assurance of blood components,* or subsequent revision]; a directive of the European Union also deals with the matter: *Commission Directive 2004/33/EC of 22 March 2004 implementing*

*Directive 2002/98/EC of the European Parliament and of the Council as regards certain technical requirements for blood and blood components.*

**Immunisation of donors**. Immunisation of donors to obtain immunoglobulins with specific activities may be carried out when sufficient supplies of material of suitable quality cannot be obtained from naturally immunised donors. Recommendations for such immunisations are formulated by the World Health Organization (*Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives*, WHO Technical Report Series, No. 840, 1994 or subsequent revision).

**Records**. Records of donors and donations made are kept in such a way that, while maintaining the required degree of confidentiality concerning the donor's identity, the origin of each donation in a plasma pool and the results of the corresponding acceptance procedures and laboratory tests can be traced.

**Laboratory tests**. Laboratory tests are carried out for each donation to detect the following viral markers:

1. antibodies against human immunodeficiency virus 1 (anti-HIV-1);
2. antibodies against human immunodeficiency virus 2 (anti-HIV-2);
3. hepatitis B surface antigen (HBsAg);
4. antibodies against hepatitis C virus (anti-HCV).

The test methods used are of suitable sensitivity and specificity and comply with the regulations in force. If a repeat-reactive result is found in any of these tests, the donation is not accepted.

*INDIVIDUAL PLASMA UNITS*

The plasma is prepared by a method that removes cells and cell debris as completely as possible. Whether prepared from whole blood or by plasmapheresis, the plasma is separated from the cells by a method designed to prevent the introduction of micro-organisms. No antibacterial or antifungal agent is added to the plasma. The containers comply with the requirements for glass containers (*3.2.1*) or for plastic containers for blood and blood components (*3.3.4*). The containers are closed so as to prevent any possibility of contamination.

If 2 or more units are pooled prior to freezing, the operations are carried out using sterile connecting devices or under aseptic conditions and using containers that have not previously been used.

When obtained by plasmapheresis or from whole blood (after separation from cellular elements), plasma intended for the recovery of proteins that are labile in plasma is frozen within 24 h of collection by cooling rapidly in conditions validated to ensure that a temperature of – 25 °C or below is attained at the core of each plasma unit within 12 h of placing in the freezing apparatus.

When obtained by plasmapheresis, plasma intended solely for the recovery of proteins that are not labile in plasma is frozen by cooling rapidly in a chamber at – 20 °C or below within 24 h of collection.

When obtained from whole blood, plasma intended solely for the recovery of proteins that are not labile in plasma is separated from cellular elements and frozen in a chamber at – 20 °C or below within 72 h of collection.

*It is not intended that the determination of total protein and human coagulation factor VIII shown below be carried out on each unit of plasma. They are rather given as guidelines for good manufacturing practice, the test for human coagulation factor VIII being relevant for plasma intended for use in the preparation of concentrates of labile proteins.*

*The total protein content of a unit of plasma depends on the serum protein content of the donor and the degree of dilution inherent in the donation procedure. When plasma is obtained from a suitable donor and using the intended proportion of anticoagulant solution, a total protein content complying with the limit of 50 g/L is obtained. If a volume of blood or plasma smaller than intended is collected into the anticoagulant solution, the resulting plasma is not necessarily unsuitable for pooling for fractionation. The aim of good manufacturing practice must be to achieve the prescribed limit for all normal donations.*

*Preservation of human coagulation factor VIII in the donation depends on the collection procedure and the subsequent handling of the blood and plasma. With good practice, 0.7 IU/mL can usually be achieved, but units of plasma with a lower activity may still be suitable for use in the production of coagulation factor concentrates. The aim of all steps taken during production of plasma is to obtain plasma of the intended quality and to conserve labile proteins as much as possible.*

**Total protein**. Carry out the test using a pool of not fewer than 10 units. Dilute an appropriate volume of the preparation with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of this solution in a round-bottomed centrifuge tube, add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the protein content by multiplying the quantity of nitrogen by 6.25. The total protein content is not less than 50 g/L.

**Human coagulation factor VIII** (*2.7.4*). Carry out the test using a pool of not fewer than 10 units. Thaw the samples to be examined, if necessary, at 37 °C. Carry out the assay using a reference plasma calibrated against the International Standard for human coagulation factor VIII in plasma. The activity is not less than 0.7 IU/mL.

*STORAGE AND TRANSPORT*

Frozen plasma is stored and transported in conditions designed to maintain the temperature at or below – 20 °C; for accidental reasons, the storage temperature may rise above – 20 °C on one or more occasions during storage and transport but the plasma is nevertheless considered suitable for fractionation if all the following conditions are fulfilled:

- the total period of time during which the temperature exceeds – 20 °C does not exceed 72 h;
- the temperature does not exceed – 15 °C on more than 1 occasion;
- the temperature at no time exceeds – 5 °C.

*POOLED PLASMA*

During the manufacture of plasma products, the first homogeneous pool of plasma (for example, after removal of cryoprecipitate) is tested for HBsAg and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

The plasma pool is also tested for hepatitis C virus RNA using a validated nucleic acid amplification technique (*2.6.21*). A positive control with 100 IU/mL of hepatitis C virus RNA and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The plasma pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

*Hepatitis C virus RNA for NAT testing BRP* is suitable for use as a positive control.

CHARACTERS

Before freezing: clear or slightly turbid liquid without visible signs of haemolysis; it may vary in colour from light yellow to green.

LABELLING

The label enables each individual unit to be traced to a specific donor.

**01/2020:1646**

# HUMAN PLASMA (POOLED AND TREATED FOR VIRUS INACTIVATION)

## Plasma humanum coagmentatum conditumque ad exstinguendum virum

DEFINITION

Sterile, frozen or freeze-dried, non-pyrogenic preparation obtained from human plasma derived from donors belonging to the same ABO blood group. The preparation is thawed or reconstituted before use to give a solution for infusion.

The human plasma used complies with the monograph *Human plasma for fractionation (0853)*.

PRODUCTION

The units of plasma to be used are cooled to − 30 °C or lower within 6 h of separation of cells and always within 24 h of collection.

The pool is prepared by mixing units of plasma belonging to the same ABO blood group.

*PLASMA POOL TESTS*

The pool of plasma is tested for hepatitis B surface antigen (HBsAg) and for HIV antibodies using test methods of suitable sensitivity and specificity; the pool must give negative results in these tests.

**Hepatitis A virus RNA**. The plasma pool is tested using a validated nucleic acid amplification technique (*2.6.21*). A positive control with $1.0 \times 10^2$ IU of hepatitis A virus RNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis A virus RNA.

*Hepatitis A virus RNA for NAT testing BRP* is suitable for use as a positive control.

**Hepatitis C virus RNA**. The plasma pool is tested using a validated nucleic acid amplification technique (*2.6.21*). A positive control with $1.0 \times 10^2$ IU of hepatitis C virus RNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis C virus RNA.

*Hepatitis C virus RNA for NAT testing BRP* is suitable for use as a positive control.

**Hepatitis E virus RNA**. The plasma pool is tested using a validated nucleic acid amplification technique (*2.6.21*). A positive control with $3.2 \times 10^2$ IU of hepatitis E virus RNA per millilitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors. The pool complies with the test if it is found non-reactive for hepatitis E virus RNA.

*Hepatitis E virus RNA for NAT testing BRP* is suitable for use as a positive control.

**B19 virus DNA**. The plasma pool contains not more than 10.0 IU/µL.

To limit the potential burden of B19 virus in plasma pools, the plasma pool is also tested for B19 virus using a validated nucleic acid amplification technique (*2.6.21*). A positive control with 10.0 IU of B19 virus DNA per microlitre and, to test for inhibitors, an internal control prepared by addition of a suitable marker to a sample of the plasma pool are included in the test. The test is invalid if the positive control is non-reactive or if the result obtained with the internal control indicates the presence of inhibitors.

*B19 virus DNA for NAT testing BRP* is suitable for use as a positive control.

*METHOD OF PREPARATION*

The method of preparation is designed to minimise activation of any coagulation factor (to minimise potential thrombogenicity) and includes a step or steps that have been shown to inactivate known agents of infection; if substances are used for the inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

**Inactivation process**. The solvent-detergent process, which is one of the methods used to inactivate enveloped viruses, uses treatment with a combination of tributyl phosphate and octoxinol 10; these reagents are subsequently removed by oil extraction or by solid phase extraction so that the amount in the final product is less than 2 µg/mL for tributyl phosphate and less than 5 µg/mL for octoxinol 10.

No antimicrobial preservative is added.

The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen; it may subsequently be freeze-dried.

Plastic containers comply with the requirements for sterile plastic containers for human blood and blood components (*3.3.4*).

Glass containers comply with the requirements for glass containers for pharmaceutical use (*3.2.1*).

CHARACTERS

*Appearance*:

- *frozen preparation*: clear or slightly opalescent liquid, free from solid and gelatinous particles after thawing;
- *freeze-dried preparation*: almost white or slightly yellow powder or friable solid mass.

*Thaw or reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests and assay.*

IDENTIFICATION

A. Examine by electrophoresis (*2.2.31*) comparing with normal human plasma. The electropherograms show the same bands.

B. It complies with the test for anti-A and anti-B haemagglutinins (see Tests).

TESTS

**pH** (*2.2.3*): 6.5 to 7.6.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**: minimum 45 g/L.

Dilute if necessary with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube

and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the quantity of protein by multiplying the result by 6.25.

**Activated coagulation factors** (*2.6.22*). It complies with the test for activated coagulation factors. Carry out the test with 0.1 mL of the preparation to be examined instead of 10-fold and 100-fold dilutions. The coagulation time for the preparation to be examined is not less than 150 s.

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method A*). The presence of haemagglutinins (anti-A or anti-B) corresponds to the blood group stated on the label.

**Hepatitis A virus antibodies**: minimum 0.3 IU/mL, determined by a suitable immunochemical method (*2.7.1*).

*Human hepatitis A immunoglobulin BRP* is suitable for use as a reference preparation.

**Irregular erythrocyte antibodies**. The preparation to be examined does not show the presence of irregular erythrocyte antibodies when examined without dilution by an indirect antiglobulin test.

**Citrate**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute the preparation to be examined with an equal volume of a 9 g/L solution of *sodium chloride R*. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution*. Dissolve 0.300 g of *sodium citrate R* in *water R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;

– *stationary phase*: *cation-exchange resin R* (9 µm).

*Mobile phase*: 0.51 g/L solution of *sulfuric acid R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Equilibration*: 15 min.

*Injection*: 10 µL.

*Retention time*: citrate = about 10 min.

*Limit*:

– *citrate*: maximum 25 mmol/L.

**Calcium**: maximum 5.0 mmol/L.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Source*: calcium hollow-cathode lamp using a transmission band preferably of 0.5 nm.

*Wavelength*: 622 nm.

*Atomisation device*: air-acetylene or acetylene-propane flame.

**Potassium**: maximum 5.0 mmol/L.

Atomic emission spectrometry (*2.2.22, Method I*).

*Wavelength*: 766.5 nm.

**Sodium**: maximum 200 mmol/L.

Atomic emission spectrometry (*2.2.22, Method I*).

*Wavelength*: 589 nm.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority (freeze-dried product).

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject 3 mL per kilogram of the rabbit's mass.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.1 IU of endotoxin per millilitre.

ASSAY

**Assay of human coagulation factor VIII** (*2.7.4*). Use a reference plasma calibrated against the International Standard for blood coagulation factor VIII in plasma.

The estimated potency is not less than 0.5 IU/mL. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Assay of human coagulation factor V**. Carry out the assay of human coagulation factor V described below using a reference plasma calibrated against the International Standard for blood coagulation factor V in plasma.

Using *imidazole buffer solution pH 7.3 R*, prepare at least 3 twofold dilutions of the preparation to be examined, preferably in duplicate, from 1 in 10 to 1 in 40. Test each dilution as follows: mix 1 volume of *plasma substrate deficient in factor V R*, 1 volume of the dilution to be examined, 1 volume of *thromboplastin R* and 1 volume of a 3.5 g/L solution of *calcium chloride R*; measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the 1st indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time of 4 twofold dilutions (1 in 10 to 1 in 80) of human normal plasma in *imidazole buffer solution pH 7.3 R*.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

The estimated potency is not less than 0.5 IU/mL. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Assay of human coagulation factor XI** (*2.7.22*). Use a reference plasma calibrated against the International Standard for blood coagulation factor XI in plasma.

The estimated potency is not less than 0.5 IU/mL. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

*Coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation in the above assays.

**Assay of human protein C** (*2.7.30*). Use a reference plasma calibrated against the International Standard for human protein C in plasma.

The estimated potency is not less than 0.7 IU/mL. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Assay of human protein S** (*2.7.31*). Use a reference plasma calibrated against the International Standard for human protein S in plasma.

The estimated potency is within the limits approved for the particular product. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Assay of human plasmin inhibitor** (*2.7.25*) **($\alpha_2$-antiplasmin)**. Use a reference plasma calibrated against human normal plasma.

1 unit of human plasmin inhibitor is equal to the activity of 1 mL of human normal plasma. Human normal plasma is prepared by pooling plasma units from not fewer than 30 donors and storing at − 30 °C or lower.

The estimated potency is not less than 0.2 units/mL. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

**Activated partial thromboplastin time (APTT)**. Use an apparatus suitable for measurement of coagulation times or perform the assay with incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 mL of the preparation to be examined and 0.1 mL of a suitable APTT reagent (containing phospholipid and contact activator), both previously heated to 37 °C, and incubate the mixture for a recommended time at 37 °C. To each tube add 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the 1st indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus. The volumes given above may be adapted to the APTT reagent and apparatus used. The coagulation time complies with the approved specification for the product.

LABELLING

The label states:

- the ABO blood group;
- the method used for virus inactivation.

**01/2014:2387**

# HUMAN α-1-PROTEINASE INHIBITOR

## α-1-Proteinasi inhibitor humanum

DEFINITION

Sterile liquid or freeze-dried preparation of a plasma protein fraction containing mainly human α-1-proteinase inhibitor (also known as human α-1-antitrypsin or α-1-antiproteinase). Human α-1-proteinase inhibitor is a glycoprotein existing in isoforms with different isoelectric points and is the most abundant multifunctional serine proteinase inhibitor in human plasma.

It is obtained from human plasma that complies with the monograph *Human plasma for fractionation (0853)*, using a suitable fractionation process and further purification steps. Other plasma proteins may be present. The preparation may contain excipients such as stabilisers.

PRODUCTION

*GENERAL PROVISIONS*

The method of preparation is designed to maintain functional integrity of α-1-proteinase inhibitor. It includes a step or steps that have been shown to remove or to inactivate known agents of infection. The subsequent purification procedure must be validated to demonstrate that the concentration of any substances used for inactivation of viruses during production is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 0.35 mg of active human α-1-proteinase inhibitor per milligram of total protein. The ratio of human α-1 proteinase inhibitor activity to human α-1-proteinase inhibitor antigen is not less than 0.7.

No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter and distributed aseptically into the final containers. It may be subsequently freeze-dried.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

It shall be demonstrated that the manufacturing process yields a product with a consistent composition. It is evaluated by suitable analytical procedures that are determined during process development, and which include:

- assay of human α-1-proteinase inhibitor activity;
- determination of specific human α-1-proteinase inhibitor activity, expressed as the ratio of active human α-1-proteinase inhibitor to total protein;
- characterisation of isoform composition and protein structure by suitable methods such as isoelectric focusing (*2.2.54*), spectrometric methods (for example, mass spectrometry) or capillary electrophoresis (*2.2.47*);
- determination of the ratio of human α-1-proteinase inhibitor activity to human α-1-proteinase inhibitor antigen;
- characterisation of accompanying plasma proteins that might be present, by a set of suitable methods such as SDS-PAGE, cellulose acetate electrophoresis or capillary zone electrophoresis (*2.2.31*) and quantitative determination of relevant accompanying plasma proteins;
- determination of molecular-size distribution, used to quantify the polymeric forms of human α-1-proteinase inhibitor; consideration is given to the potential presence of accompanying proteins that might affect the results.

CHARACTERS

*Appearance*:

- *liquid preparations*: clear or slightly opalescent, colourless or pale yellow or pale green or pale brown;
- *freeze-dried preparations*: powders or solid friable masses, hygroscopic, white or pale yellow or pale brown.

*If the preparation to be examined is freeze-dried, reconstitute it as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**pH** (*2.2.3*): 6.5 to 7.8.

**Solubility**. For freeze-dried preparations, add to a container of the preparation to be examined the volume of the liquid stated on the label at room temperature. The preparation dissolves completely, giving a clear, colourless or pale green or pale yellow or pale brown solution.

**Osmolality** (*2.2.35*): minimum 210 mosmol/kg.

**Total protein**. Dilute the preparation to be examined with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. To 2.0 mL of this solution in a round-bottomed centrifuge tube, add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the protein content by multiplying by 6.25.

**Water**. Determined by a suitable method, such as the semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 60 mg of human α-1-proteinase inhibitor.
Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.08 IU of endotoxin per milligram of human α-1-proteinase inhibitor.

ASSAY

**Assay of human α-1-proteinase inhibitor** (*2.7.32*). The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

STORAGE

In an airtight and sterile container, at a temperature not exceeding 25 °C, unless otherwise justified and authorised.

LABELLING

The label states:

- the potency of active (functional) human α-1-proteinase inhibitor per container;
- the name and quantity of any added substances;
- the quantity of protein in the container;
- the route of administration;
- where applicable, the name and volume of the liquid to be used for reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2011:0554**
**corrected 7.6**

# HUMAN PROTHROMBIN COMPLEX

## Prothrombinum multiplex humanum

DEFINITION

Sterile plasma protein fraction containing human coagulation factor IX together with variable amounts of human coagulation factors II, VII and X; the presence and proportion of these additional factors depends on the method of fractionation. It is obtained from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers, heparin and antithrombin.

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of human coagulation factor IX per millilitre.

If the content of any of the factors is stated as a single value, the estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency; if the content of any of the factors is stated as a range, the estimated potency is not less than the lower limit and not greater than the upper limit of the stated range.

PRODUCTION

The method of preparation is designed to maintain functional integrity of the relevant coagulation factors it contains and to minimise activation of any coagulation factor (to minimise potential thrombogenicity). It includes a step or steps that have been shown to remove or to inactivate known agents of infection; if substances are used for inactivation of viruses during production, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients.

The specific activity is not less than 0.6 IU of human coagulation factor IX per milligram of total protein, before the addition of any protein stabiliser.

The prothrombin complex fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

CHARACTERS

*Appearance*: white or slightly coloured, very hygroscopic powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

IDENTIFICATION

It complies with the limits of the assays for human coagulation factors IX and II and, where applicable, those for human coagulation factors VII and X.

TESTS

**Solubility**. To a container of the preparation to be examined add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, giving a clear solution that may be coloured.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to obtain a solution containing about 15 mg of protein in 2 mL. To 2.0 mL of the solution in a round-bottomed centrifuge tube add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25.

**Activated coagulation factors** (*2.6.22*). If necessary, dilute the reconstituted preparation to contain 20 IU of human coagulation factor IX per millilitre. For each of the dilutions, the coagulation time is not less than 150 s.

**Heparin** (*2.7.12*). If heparin has been added during preparation, the preparation to be examined contains not more than the amount of heparin stated on the label and in all cases not more than 0.5 IU of heparin per International Unit of human coagulation factor IX.

**Thrombin**. If the preparation to be examined contains heparin, determine the amount present as described in the test for heparin and neutralise it by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). In each of 2 test-tubes, mix equal volumes of the reconstituted preparation and of a 3 g/L solution of *fibrinogen R*. Keep one of the tubes at 37 °C for 6 h and the other at room temperature for 24 h. In a 3rd tube, mix equal volumes of the fibrinogen solution and of a solution of *human thrombin R* (1 IU/mL) and place the tube in a water-bath at 37 °C. No coagulation occurs in the tubes containing the preparation to be examined. Coagulation occurs within 30 s in the tube containing thrombin.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectrometry (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the bacterial endotoxin test.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 30 IU of human coagulation factor IX.

Where the bacterial endotoxin test is used, the preparation to be examined contains less than 0.05 IU of endotoxin per International Unit of human coagulation factor IX.

ASSAY

**Human coagulation factor IX** (*2.7.11*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P$ = 0.95) is not greater than 80 per cent to 125 per cent of the estimated potency.

**Human coagulation factor II** (*2.7.18*). The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P$ = 0.95) is not greater than 90 per cent to 111 per cent of the estimated potency.

The estimated human coagulation factor II potency is not less than 70 per cent and not more than 165 per cent of the estimated human coagulation factor IX potency.

**Human coagulation factor VII** (*2.7.10*). If the label states that the preparation contains human coagulation factor VII, the estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P$ = 0.95) is not greater than 80 per cent to 125 per cent of the estimated potency.

**Human coagulation factor X** (*2.7.19*). If the label states that the preparation contains human coagulation factor X, the estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence interval ($P$ = 0.95) is not greater than 90 per cent to 111 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of human coagulation factor IX, and the number or range of International Units of human coagulation factor II per container;
- where applicable, the number or range of International Units of human coagulation factor VII and human coagulation factor X per container;
- the amount of protein per container;
- the name and quantity of any added substances, including, where applicable, heparin and antithrombin;
- the name and quantity of the liquid to be used for reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2015:0723**

# HUMAN RABIES IMMUNOGLOBULIN

## Immunoglobulinum humanum rabicum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from donors immunised against rabies. It contains specific antibodies neutralising the rabies virus. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the dose of immunoglobulin required to neutralise the infectivity of a rabies virus suspension with the dose of a reference preparation, calibrated in International Units, required to produce the same degree of neutralisation (*2.7.1*). The test is performed in sensitive cell cultures and the presence of unneutralised virus is revealed by immunofluorescence.

The International Unit is the specific neutralising activity for rabies virus in a stated amount of the International Standard for anti-rabies immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human rabies immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

*Method*

Carry out the test in suitable sensitive cells. It is usual to use the BHK-21 cell line, grown in the medium described below, between the 18$^{th}$ and 30$^{th}$ passage levels counted from the ATCC seed lot. Harvest the cells after 2 to 4 days of growth, treat with trypsin and prepare a suspension containing 500 000 cells per millilitre (cell suspension). 10 min before using this suspension add 10 µg of *diethylaminoethyldextran R* per millilitre, if necessary, to increase the sensitivity of the cells.

Use a fixed virus strain grown in sensitive cells, such as the CVS strain of rabies virus adapted to growth in the BHK-21 cell line (seed virus suspension). Estimate the titre of the seed virus suspension as follows.

Prepare a series of dilutions of the viral suspension. In the chambers of cell-culture slides (8 chambers per slide), place 0.1 mL of each dilution and 0.1 mL of medium and add 0.2 mL of the cell suspension. Incubate in an atmosphere of carbon dioxide at 37 °C for 24 h. Carry out fixation, immunofluorescence staining and evaluation as described below. Determine the end-point titre of the seed virus suspension and prepare the working virus dilution corresponding to 100 $CCID_{50}$ per 0.1 mL.

For each assay, check the amount of virus used by performing a control titration: from the dilution corresponding to 100 $CCID_{50}$ per 0.1 mL, make 3 tenfold dilutions. Add 0.1 mL of each dilution to 4 chambers containing 0.1 mL of medium and add 0.2 mL of the cell suspension. The test is not valid unless the titre lies between 30 $CCID_{50}$ and 300 $CCID_{50}$.

Dilute the reference preparation to a concentration of 2 IU/mL using non-supplemented culture medium (stock reference dilution, stored below – 80 °C). Prepare 2 suitable predilutions (1:8 and 1:10) of the stock reference dilution so

that the dilution of the reference preparation that reduces the number of fluorescent fields by 50 per cent lies within the 4 dilutions of the cell-culture slide. Add 0.1 mL of the medium to each chamber, except the first in each of 2 rows, to which add respectively 0.2 mL of the 2 predilutions of the stock reference dilution transferring successively 0.1 mL to the other chambers.

Dilute the preparation to be examined 1 in 100 using non-supplemented medium (stock immunoglobulin dilution) – to reduce to a minimum errors due to viscosity of the undiluted preparation – and make 3 suitable predilutions so that the dilution of the preparation to be examined that reduces the number of fluorescent fields by 50 per cent lies within the 4 dilutions of the cell-culture slide. Add 0.1 mL of the medium to all the chambers except the first in each of 3 rows, to which add respectively 0.2 mL of the 3 predilutions of the stock immunoglobulin dilution. Prepare a series of 2-fold dilutions transferring successively 0.1 mL to the other chambers.

To all the chambers containing the dilutions of the reference preparation and the dilutions of the preparation to be examined, add 0.1 mL of the virus suspension corresponding to 100 $CCID_{50}$ per 0.1 mL (working virus dilution), shake manually, allow to stand in an atmosphere of carbon dioxide at 37 °C for 90 min, add 0.2 mL of the cell suspension, shake manually and allow to stand in an atmosphere of carbon dioxide at 37 °C for 24 h.

After 24 h, discard the medium and remove the plastic walls. Wash the cell monolayer with *phosphate buffered saline pH 7.4 R* and then with a mixture of 20 volumes of *water R* and 80 volumes of *acetone R* and fix in a mixture of 20 volumes of *water R* and 80 volumes of *acetone R* at − 20 °C for 3 min. Spread on the slides *fluorescein-conjugated rabies antiserum R* ready for use. Allow to stand in an atmosphere with a high level of moisture at 37 °C for 30 min. Wash with *phosphate buffered saline pH 7.4 R* and dry. Examine 20 fields in each chamber at a magnification of 250 ×, using a microscope equipped for fluorescence readings. Note the number of fields with at least 1 fluorescent cell. Check the test dose used in the virus titration slide and determine the dilution of the reference preparation and the dilution of the preparation to be examined that reduce the number of fluorescent fields by 50 per cent, calculating the 2 or 3 dilutions together using probit analysis. The test is not valid unless the statistical analysis shows a significant slope of the dose-response curve and no evidence of deviation from linearity or parallelism.

The stated potency is not less than 150 IU/mL. The estimated potency is not less than the stated potency and is not greater than twice the stated potency. The confidence limits ($P = 0.95$) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

CULTURE MEDIUM FOR GROWTH OF BHK-21 CELLS

*Commercially available media that have a slightly different composition from that shown below may also be used.*

| | |
|---|---|
| Sodium chloride | 6.4 g |
| Potassium chloride | 0.40 g |
| Calcium chloride, anhydrous | 0.20 g |
| Magnesium sulfate, heptahydrate | 0.20 g |
| Sodium dihydrogen phosphate, monohydrate | 0.124 g |
| Glucose monohydrate | 4.5 g |
| Ferric nitrate, nonahydrate | 0.10 mg |
| L-Arginine hydrochloride | 42.0 mg |
| L-Cystine | 24.0 mg |
| L-Histidine | 16.0 mg |
| L-Isoleucine | 52.0 mg |
| L-Leucine | 52.0 mg |
| L-Lysine hydrochloride | 74.0 mg |
| L-Phenylalanine | 33.0 mg |
| L-Threonine | 48.0 mg |
| L-Tryptophan | 8.0 mg |
| L-Tyrosine | 36.0 mg |
| L-Valine | 47.0 mg |
| L-Methionine | 15.0 mg |
| L-Glutamine | 0.292 g |
| *i*-Inositol | 3.60 mg |
| Choline chloride | 2.0 mg |
| Folic acid | 2.0 mg |
| Nicotinamide | 2.0 mg |
| Calcium pantothenate | 2.0 mg |
| Pyridoxal hydrochloride | 2.0 mg |
| Thiamine hydrochloride | 2.0 mg |
| Riboflavine | 0.2 mg |
| Phenol red | 15.0 mg |
| Sodium hydrogen carbonate | 2.75 g |
| Water | to 1000 mL |

The medium is supplemented with:

| | |
|---|---|
| Foetal calf serum (heated at 56 °C for 30 min) | 10 per cent |
| Tryptose phosphate broth | 10 per cent |
| Benzylpenicillin sodium | 60 mg/L |
| Streptomycin | 0.1 g/L |

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2015:0617**

# HUMAN RUBELLA IMMUNOGLOBULIN

## Immunoglobulinum humanum rubellae

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against rubella virus. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

POTENCY

The potency is determined by comparing the activity of the preparation to be examined in a suitable haemagglutination-inhibition test with that of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard for anti-rubella immunoglobulin. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

The estimated potency is not less than 4500 IU/mL. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 50 per cent and not more than 200 per cent of the stated potency.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per millilitre.

**01/2015:0398**

# HUMAN TETANUS IMMUNOGLOBULIN

## Immunoglobulinum humanum tetanicum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma containing specific antibodies against the toxin of *Clostridium tetani*. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph *Human normal immunoglobulin for intramuscular administration (0338)*, except for the minimum number of donors and the minimum total protein content.

PRODUCTION

During development, a satisfactory relationship shall be established between the potency determined by immunoassay as described under Potency and that determined by means of the following test for toxin-neutralising capacity in mice.

*Toxin-neutralising capacity in mice*. The potency is determined by comparing the quantity necessary to protect mice against the paralytic effects of a fixed quantity of tetanus toxin with the quantity of a reference preparation of human tetanus immunoglobulin, calibrated in International Units, necessary to give the same protection.

The International Unit of antitoxin is the specific neutralising activity for tetanus toxin contained in a stated amount of the International Standard, which consists of freeze-dried human immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human tetanus immunoglobulin BRP* is calibrated in International Units by comparison with the International Standard.

**Method**

*Selection of animals*. Use mice weighing 16-20 g.

*Preparation of the test toxin*. Prepare the test toxin by a suitable method from the sterile filtrate of a culture in liquid medium of *C. tetani*. The 2 methods shown below are given as examples and any other suitable method may be used.

(1) To the filtrate of an approximately 9-day culture, add 1-2 volumes of *glycerol R* and store the mixture in the liquid state at a temperature slightly below 0 °C.

(2) Precipitate the toxin by addition to the filtrate of *ammonium sulfate R*, dry the precipitate *in vacuo* over *diphosphorus pentoxide R*, reduce to a powder and store dry, either in sealed ampoules or *in vacuo* over *diphosphorus pentoxide R*.

*Determination of test dose of toxin (Lp/10 dose)*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre. If the test toxin is stored dry, reconstitute it using a suitable liquid. Prepare mixtures of the solution of the reference preparation and the test toxin such that each contains 2.0 mL of the solution of the reference preparation, one of a graded series of volumes of the test toxin and sufficient of a suitable liquid to bring the volume to 5.0 mL. Allow the mixtures to stand, protected from light, for 60 min. Using 6 mice for each mixture, inject a dose of 0.5 mL subcutaneously into each mouse. Observe the mice for 96 h. Mice that become paralysed may be euthanised. The test dose of toxin is the quantity in 0.5 mL of the mixture made with the smallest amount of toxin capable of causing, despite partial neutralisation by the reference preparation, paralysis in all 6 mice injected with the mixture, within the observation period.

*Determination of potency of the immunoglobulin*. Prepare a solution of the reference preparation in a suitable liquid such that it contains 0.5 IU of antitoxin per millilitre. Prepare a solution of the test toxin in a suitable liquid such that it contains 5 test doses per millilitre. Prepare mixtures of the solution of the test toxin and the immunoglobulin to be examined such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the immunoglobulin to be examined and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Also prepare mixtures of the solution of the test toxin and the solution of the reference preparation such that each contains 2.0 mL of the solution of the test toxin, one of a graded series of volumes of the solution of the reference preparation centred on that volume (2.0 mL) that contains 1 IU and sufficient of a suitable liquid to bring the total volume to 5.0 mL. Allow the mixtures to stand, protected from light, for 60 min. Using 6 mice for each mixture, inject subcutaneously a dose of 0.5 mL into each mouse. Observe the mice for 96 h. Mice that become paralysed may be euthanised. The mixture that contains the largest volume of immunoglobulin that fails to protect the mice from paralysis contains 1 IU. This quantity is used to calculate the potency of the immunoglobulin in International Units per millilitre.

The test is not valid unless all the mice injected with mixtures containing 2.0 mL or less of the solution of the reference preparation show paralysis and all those injected with mixtures containing more do not.

POTENCY

The potency is determined by comparing the antibody titre of the preparation to be examined with that of a reference preparation calibrated in International Units, using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA) or toxoid inhibition assay (TIA).

The International Unit is the activity contained in a stated amount of the International Standard for anti-tetanus immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Human tetanus immunoglobulin BRP* is calibrated in International Units and is suitable for use as a reference preparation.

The stated potency is not less than 100 IU/mL of tetanus antitoxin. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

The description of methods A and B below are provided as examples.

**Method A: direct enzyme immunoassay**

The amount of tetanus immunoglobulin bound to tetanus toxoid, which is coated to a microtitre plate, is determined by means of a peroxidase-conjugated polyclonal anti-human IgG antibody.

*Materials*

- *Phosphate-buffered saline pH 7.1 (PBS).* Dissolve 0.2 g of *potassium chloride R*, 0.2 g of *potassium dihydrogen phosphate R*, 1.15 g of *anhydrous disodium hydrogen phosphate R* and 8.0 g of *sodium chloride R* in *water R* and adjust the pH (*2.2.3*) if necessary. Dilute to 1000 mL with *water R*.
- *PBS-T.* PBS containing 0.05 per cent *V/V* of *polysorbate 20 R*.
- *Carbonate buffer pH 9.6.* Dissolve 1.4 g of *anhydrous sodium carbonate R* and 3.0 g of *sodium hydrogen carbonate R* in *water R* and adjust the pH (*2.2.3*) if necessary. Dilute to 1000 mL with *water R*.
- *Tetanus toxoid.* Purified and chemically inactivated tetanus toxin.
- *Microtitre plate.* Use a flat-bottomed microtitre plate with high protein-binding capacity.

*Method*

Distribute 100 µL of a 0.2 Lf/mL solution of tetanus toxoid in carbonate buffer pH 9.6 into each of the wells of the microtitre plate. Incubate at 4 °C for approximately 18 h. Wash the plate 5 times with PBS-T. To block unbound binding sites add 200 µL of PBS containing 5 g/L of *bovine albumin R* to each of the wells and incubate for 1 h at 37 °C on a plate shaker set at 120 r/min. Wash 5 times with PBS-T.

Reconstitute the reference preparation and the preparation to be examined according to the instructions. For each preparation, prepare 2 independent predilutions of 0.004 IU/mL in PBS by applying several dilution steps. Using PBS, prepare from each predilution 5 serial dilutions with a dilution factor of 1.5 resulting in a dilution series of 6 dilutions in the range of 0.0005-0.004 IU/mL. Depending on the reagents used, a small modification of the dilution series might be necessary to meet the conditions of the statistical model used.

Apply 100 µL of each of the samples of the dilution series to the plate. Incubate for 2 h at 37 °C on a plate shaker set at 120 r/min and wash the plate 5 times with PBS-T. Apply 100 µL of a peroxidase-conjugated anti-human IgG antibody diluted to a suitable concentration with PBS-T containing 5 g/L of *bovine albumin R* to each of the wells and incubate for 1 h at 37 °C on a plate shaker set at 120 r/min. Wash the plate 5 times with PBS-T and apply 100 µL of a suitable 3,3′,5,5′-tetramethylbenzidine (TMB) substrate to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 100 µL of a 196.2 g/L solution of *sulfuric acid R* to each of the wells. Measure the absorbances at 450 nm and at the reference wavelength of 630 nm. Calculate the potencies of the preparations by the usual statistical methods (*5.3*).

**Method B: indirect determination by toxoid-binding inhibition assay**

The amount of unbound toxoid in a mixture of toxoid and tetanus immunoglobulin is determined by an enzyme immunoassay and is inversely proportional to the amount of tetanus immunoglobulin present. The method is performed over 2 consecutive days.

*Materials*

- *Phosphate-buffered saline pH 7.1 (PBS).* See under Method A.
- *PBS-T.* See under Method A.
- *Carbonate buffer pH 9.6.* See under Method A.
- *Tetanus toxoid.* See under Method A.
- *Mab.* Mouse monoclonal tetanus toxoid antibody. Use according to the instructions. Prepare a suitable dilution of Mab, e.g. 1/5000, in PBS.
- *Peroxidase-conjugated antibody.* Peroxidase-conjugated anti-mouse IgG (H+L) antibody, affinity-purified F(ab)2 fragment without cross-reactivity to human serum proteins. Use according to the instructions. Prepare a suitable dilution of the peroxidase-conjugated antibody in PBS-T containing 5 g/L of *bovine albumin R*.
- *Microtitre plate.* Use a round-bottomed microtitre plate with medium protein-binding capacity.
- *ELISA plate.* Use a flat-bottomed microtitre plate with high protein-binding capacity.

*Method*

Day 1

To block the protein-binding sites of the microtitre plate, add 200 µL of PBS containing 5 g/L of *bovine albumin R* to each of the wells of the microtitre plate and incubate for 1 h at 37 °C on a plate shaker set at 120 r/min. Wash the plate 5 times with PBS-T.

Reconstitute the reference preparation and the preparation to be examined according to the instructions. For each preparation, prepare 2 independent predilutions of 0.4 IU/mL in PBS by applying several dilution steps. Prepare from each predilution a dilution series of dilutions containing 0.04 IU/mL, 0.10 IU/mL, 0.12 IU/mL, 0.14 IU/mL, 0.16 IU/mL, 0.18 IU/mL and 0.20 IU/mL. Prepare each dilution directly from the 0.4 IU/mL predilution.

Transfer 100 µL of each dilution of the dilution series to a well of the blocked plate and add 50 µL of a 0.2 Lf/mL solution of tetanus toxoid in carbonate buffer pH 9.6 into each of the wells. Incubate for approximately 18 h at 37 °C on a plate shaker set at 120 r/min.

To coat the ELISA plate, distribute 100 µL of a solution of a human tetanus immunoglobulin diluted to 1 IU/mL in carbonate buffer pH 9.6 into each of the wells of the ELISA plate. Incubate for approximately 18 h at 37 °C on a plate shaker set at 120 r/min.

Day 2

Wash the coated ELISA plate 5 times with PBS-T. To block unbound binding sites add 200 µL of PBS containing 5 g/L of *bovine albumin R* to each of the wells and incubate for 1 h at 37 °C on a plate shaker set at 120 r/min. Wash the plate 5 times with PBS-T. Transfer 100 µL of each mixture of toxoid and tetanus immunoglobulin from the microtitre plate to the coated ELISA plate and incubate for 2 hours at 37 °C on a plate shaker set at 120 r/min. Wash the plate 5 times with PBS-T. Add 100 µL of diluted Mab to each of the wells, incubate the plate for 1 h at 37 °C on a plate shaker set at 120 r/min and wash the plate 5 times with PBS-T. Add 100 µL of the diluted peroxidase-conjugated antibody to each of the wells, incubate the plate for 1 h at 37 °C on a plate shaker set at 120 r/min and wash the plate 5 times with PBS-T. Apply 100 µL of a suitable 3,3′,5,5′-tetramethylbenzidine (TMB) substrate to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 100 µL of a 196.2 g/L solution of *sulfuric acid R* to each of the wells. Measure the absorbances at 450 nm and at the reference wavelength of 630 nm. Calculate the potencies of the preparations by the usual statistical methods (*5.3*).

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2015:0724**

## HUMAN VARICELLA IMMUNOGLOBULIN

### Immunoglobulinum humanum varicellae

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. The preparation is intended for intramuscular administration. It is obtained from plasma from selected donors having antibodies against *Herpesvirus varicellae*. *Human normal immunoglobulin for intramuscular administration (0338)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intramuscular administration (0338)* except for the minimum number of donors, the minimum total protein content and, where authorised, the test for antibody to hepatitis B surface antigen.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (*2.7.1*).

The International Unit is the activity contained in a stated amount of the International Standard for anti varicella-zoster. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The stated potency is not less than 100 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intramuscular administration (0338)*.

LABELLING

See *Human normal immunoglobulin for intramuscular administration (0338)*.

The label states the number of International Units per container.

**01/2008:1528 corrected 7.6**

## HUMAN VARICELLA IMMUNOGLOBULIN FOR INTRAVENOUS ADMINISTRATION

### Immunoglobulinum humanum varicellae ad usum intravenosum

DEFINITION

Sterile liquid or freeze-dried preparation containing immunoglobulins, mainly immunoglobulin G. It is obtained from plasma from selected donors having antibodies against human herpesvirus 3 (varicella-zoster virus 1). *Human normal immunoglobulin for intravenous administration (0918)* may be added.

It complies with the monograph on *Human normal immunoglobulin for intravenous administration (0918)*, except for the minimum number of donors, the minimum total protein content and the limit for osmolality.

POTENCY

The potency is determined by comparing the antibody titre of the immunoglobulin to be examined with that of a reference preparation calibrated in International Units, using an immunoassay of suitable sensitivity and specificity (*2.7.1*).

The International Unit is the activity contained in a stated amount of the International Standard for anti varicella-zoster immunoglobulin. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The stated potency is not less than 25 IU/mL. The estimated potency is not less than the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

See *Human normal immunoglobulin for intravenous administration (0918)*.

LABELLING

See *Human normal immunoglobulin for intravenous administration (0918)*.

The label states the number of International Units per container.

**07/2013:2298**

## HUMAN VON WILLEBRAND FACTOR

### Factor humanus von Willebrandi

DEFINITION

Sterile, freeze-dried preparation of a plasma protein fraction containing the glycoprotein human von Willebrand factor with varying amounts of human coagulation factor VIII, depending on the method of preparation. It is prepared from human plasma that complies with the monograph on *Human plasma for fractionation (0853)*. The preparation may contain excipients such as stabilisers.

This monograph applies to preparations formulated according to the human von Willebrand factor activity.

The potency of the preparation, reconstituted as stated on the label, is not less than 20 IU of human von Willebrand factor per millilitre.

## PRODUCTION

*GENERAL PROVISIONS*

The method of preparation is designed to maintain functional integrity of human von Willebrand factor. It includes steps that have been shown to remove or to inactivate known agents of infection; if substances are used for the inactivation of viruses, the subsequent purification procedure must be validated to demonstrate that the concentration of these substances is reduced to a suitable level and that any residues are such as not to compromise the safety of the preparation for patients. The specific activity is not less than 1 IU of human von Willebrand factor per milligram of total protein, before the addition of any protein stabiliser.

The human von Willebrand factor fraction is dissolved in a suitable liquid. No antimicrobial preservative or antibiotic is added. The solution is passed through a bacteria-retentive filter, distributed aseptically into the final containers and immediately frozen. It is subsequently freeze-dried and the containers are closed under vacuum or under an inert gas.

*CONSISTENCY OF THE METHOD OF PRODUCTION*

It shall be demonstrated that the manufacturing process yields a product having a consistent composition with respect to human von Willebrand factor, human coagulation factor VIII and the proportions of human von Willebrand factor and human coagulation factor VIII. This is evaluated by suitable analytical procedures that are determined during process development, and that include the following checks:

**Human von Willebrand factor multimers**. The distribution of the different human von Willebrand factor multimers is determined by a suitable method such as sodium dodecyl sulfate (SDS) agarose gel electrophoresis with or without Western blot analysis, using a suitable normal human plasma as standard. Visualisation of the multimeric pattern may be performed using, for example, an immunoenzymatic technique and quantitative evaluation may be carried out by densitometric analysis.

**Human von Willebrand factor activity** (*2.7.21*). The human von Willebrand factor activity is estimated by determining the ristocetin cofactor activity and by one or more other suitable assays such as determination of collagen-binding activity using a suitable reference preparation.

**Human von Willebrand factor activity/antigen ratio**. Consistency of the manufacturing process with respect to the ratio of human von Willebrand factor activity to human von Willebrand factor antigen content is demonstrated.

*PRODUCTS THAT SHOW PARTICLES AFTER RECONSTITUTION.* If a few particles remain when the preparation is reconstituted, it shall be demonstrated during validation studies that the potency is not significantly affected after passage of the preparation through the filter to be provided with the preparation.

## CHARACTERS

*Appearance*: hygroscopic, white or pale yellow, powder or friable solid.

*Reconstitute the preparation to be examined as stated on the label immediately before carrying out the identification, tests (except those for solubility and water) and assay.*

## IDENTIFICATION

It complies with the limits of the assay.

## TESTS

**Solubility**. To a container of the preparation to be examined, add the volume of the liquid stated on the label at the recommended temperature. The preparation dissolves completely with gentle swirling within 10 min, forming a clear or slightly opalescent, colourless or slightly yellow solution.

In addition, where the label states that the product may show a few particles after reconstitution, reconstitute the preparation as described on the label and pass it through the filter provided: the filtered solution is clear or slightly opalescent.

**pH** (*2.2.3*): 6.5 to 7.5.

**Osmolality** (*2.2.35*): minimum 240 mosmol/kg.

**Total protein**. If necessary, dilute an accurately measured volume of the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to obtain a protein concentration of about 7.5 mg/mL. Place 2.0 mL of this solution in a round-bottomed centrifuge tube and add 2 mL of a 75 g/L solution of *sodium molybdate R* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid R* and 30 volumes of *water R*. Shake, centrifuge for 5 min, decant the supernatant and allow the inverted tube to drain on filter paper. Determine the nitrogen in the residue by the method of sulfuric acid digestion (*2.5.9*) and calculate the amount of protein by multiplying the result by 6.25. *For some products, especially those without a protein stabiliser, this method may not be applicable. Another validated method for protein determination must therefore be performed.*

**Anti-A and anti-B haemagglutinins** (*2.6.20, Method A*). The 1 to 64 dilution does not show agglutination. Dilute the reconstituted preparation with a 9 g/L solution of *sodium chloride R* to contain 6 IU of human von Willebrand factor activity per millilitre.

**Water**. Determined by a suitable method, such as semi-micro determination of water (*2.5.12*), loss on drying (*2.2.32*) or near-infrared spectroscopy (*2.2.40*), the water content is within the limits approved by the competent authority.

**Sterility** (*2.6.1*). It complies with the test.

**Pyrogens** (*2.6.8*) **or Bacterial endotoxins** (*2.6.14*). It complies with the test for pyrogens or, preferably and where justified and authorised, with a validated *in vitro* test such as the test for bacterial endotoxins.

For the pyrogen test, inject per kilogram of the rabbit's mass a volume equivalent to not less than 100 IU of human von Willebrand factor.

Where the test for bacterial endotoxins is used, the preparation to be examined contains less than 0.05 IU of endotoxin per International Unit of human von Willebrand factor.

## ASSAY

**Human von Willebrand factor** (*2.7.21*). The estimated potency is not less than 80 per cent and not more than 120 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

*Pending the availability of an International Standard for human von Willebrand factor concentrate calibrated for use in the collagen-binding assay, only the ristocetin cofactor assay may be used.*

**Human coagulation factor VIII** (*2.7.4*). The assay is carried out where the human coagulation factor VIII content is greater than 10 IU of human coagulation factor VIII per 100 IU of human von Willebrand factor activity. The estimated potency is not less than 60 per cent and not more than 140 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 120 per cent of the estimated potency.

## STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

- the number of International Units of human von Willebrand factor in the container;
- the number of International Units of human coagulation factor VIII in the container, or that the content of human coagulation factor VIII is less than or equal to 10 IU of human coagulation factor VIII per 100 IU of human von Willebrand factor activity;
- the amount of protein in the container;
- the name and quantity of any added substance;
- the name and volume of the liquid to be used for reconstitution;
- where applicable, that the preparation may show the presence of a few particles after reconstitution;
- that the transmission of infectious agents cannot be totally excluded when medicinal products prepared from human blood or plasma are administered.

**01/2008:0912**
**corrected 10.0**

# HYALURONIDASE

## Hyaluronidasum

[9001-54-1]

DEFINITION

Enzyme extracted from mammalian testes (for example bovine testes) and capable of hydrolysing mucopolysaccharides of the hyaluronic acid type. It may contain a suitable stabiliser.

*Potency*: minimum 300 IU of hyaluronidase activity per milligram (dried substance).

PRODUCTION

The animals from which hyaluronidase is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or yellowish-white, amorphous powder.

*Solubility*: soluble in water, practically insoluble in acetone and in anhydrous ethanol.

IDENTIFICATION

A solution containing the equivalent of 100 IU of hyaluronidase in 1 mL of a 9 g/L solution of *sodium chloride R* depolymerises an equal volume of a 10 g/L solution of *sodium hyaluronate BRP* in 1 min at 20 °C as shown by a pronounced decrease in viscosity. This action is destroyed by heating the hyaluronidase at 100 °C for 30 min.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.10 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 7.5.

Dissolve 30 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying at 60 °C at a pressure not exceeding 670 Pa for 2 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.2 IU per IU of hyaluronidase.

ASSAY

The activity of hyaluronidase is determined by comparing the rate at which it hydrolyses *sodium hyaluronate BRP* with the rate obtained with the International Standard, or a reference preparation calibrated in International Units, using a slope-ratio assay.

*Substrate solution*. To 0.10 g of *sodium hyaluronate BRP* in a 25 mL conical flask add slowly 20.0 mL of *water R* at 4 °C. The rate of addition must be slow enough to allow the substrate particles to swell (about 5 min). Maintain at 4 °C and stir for at least 12 h. Store at 4 °C and use within 4 days.

*For the test solution and the reference solution, prepare the solution and carry out the dilution at 0 °C to 4 °C.*

*Test solution*. Dissolve a suitable amount of the substance to be examined in *hyaluronidase diluent R* so as to obtain a solution containing 0.6 ± 0.3 IU of hyaluronidase per millilitre.

*Reference solution*. Dissolve a suitable amount of *hyaluronidase BRP* in *hyaluronidase diluent R* so as to obtain a solution containing 0.6 IU of hyaluronidase per millilitre.

In a reaction vessel, mix 1.50 mL of *phosphate buffer solution pH 6.4 R* and 1.0 mL of the substrate solution and equilibrate at 37 ± 0.1 °C. At time $t_1 = 0$ (first chronometer) add 0.50 mL of the test solution containing $E_t$ mg of the enzyme to be examined, mix, measure the viscosity of the solution using a suitable viscometer maintained at 37 ± 0.1 °C and record the outflow time $t_2$ using a second chronometer (graduated in 0.1 second intervals), several times during about 20 min (read on the first chronometer). The following viscometer has been found suitable: Ubbelohde microviscometer (DIN 51 562, Part 2), capillary type MII, viscometer constant about 0.1 $mm^2/s^2$.

Repeat the procedure using 0.50 mL of the reference solution containing $E_r$ mg of *hyaluronidase BRP*.

Calculate the viscosity ratio from the expression:

$$\eta_r = \frac{k \times t_2}{0.6915}$$

$k$ = the viscometer constant in $mm^2/s^2$ (indicated on the viscometer);

$t_2$ = the outflow time (in seconds) of the solution;

0.6915 = the kinematic viscosity in $mm^2/s$ of the buffer solution at 37 °C.

Since the enzymatic reaction continues during the outflow time measurements, the real reaction time equals $t_1 + t_2/2$, half of the outflow time ($t_2/2$) for which a certain measurement is valid being added to the time $t_1$ at which the measurement is started. Plot $(\ln \eta_r)^{-1}$ as a function of the reaction time ($t_1 + t_2/2$) in seconds. A linear relationship is obtained. Calculate the slope for the substance to be examined ($b_t$) and the reference preparation ($b_r$).

Calculate the specific activity in International Units per milligram from the expression:

$$\frac{b_t}{b_r} \times \frac{E_r}{E_t} \times A$$

$A$ = the specific activity of *hyaluronidase BRP* in International Units per milligram.

Carry out the complete procedure at least three times and calculate the average activity of the substance to be examined.

STORAGE

Store in an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, tamper-evident container.

LABELLING

The label states the activity in International Units per milligram.

01/2017:0829
corrected 9.2

# HYDRALAZINE HYDROCHLORIDE

## Hydralazini hydrochloridum

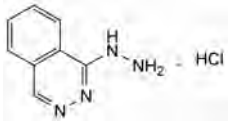

$C_8H_9ClN_4$ $M_r$ 196.6
[304-20-1]

DEFINITION

1-Hydrazinophthalazine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

mp: about 275 °C, with decomposition.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *water R* and dilute to 100 mL with the same solvent. Dilute 2 mL of this solution to 100 mL with *water R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 240 nm, 260 nm, 303 nm and 315 nm.

*Absorbance ratio*: $A_{240}/A_{303}$ = 2.0 to 2.2.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *hydralazine hydrochloride CRS*.

C. Dissolve 0.5 g in a mixture of 8 mL of *dilute hydrochloric acid R* and 100 mL of *water R*. Add 2 mL of *sodium nitrite solution R*, allow to stand for 10 min and filter. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 209 °C to 212 °C.

D. Dissolve about 10 mg in 2 mL of *water R*. Add 2 mL of a 20 g/L solution of *nitrobenzaldehyde R* in *ethanol (96 per cent) R*. An orange precipitate is formed.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

Dilute 4 mL of solution S to 20 mL with *water R*.

**pH** (*2.2.3*): 3.5 to 4.2 for solution S.

**Hydrazine**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.12 g of the substance to be examined in 4 mL of *water R* and add 4 mL of a 150 g/L solution of *salicylaldehyde R* in *methanol R* and 0.2 mL of *hydrochloric acid R*. Mix and keep at a temperature not exceeding 25 °C for 2-4 h, until the precipitate formed has sedimented. Add 4 mL of *toluene R*, shake vigorously and centrifuge. Transfer the clear supernatant to a 100 mL separating funnel and shake vigorously, each time for 3 min, with 2 quantities, each of 20 mL, of a 200 g/L solution of *sodium metabisulfite R* and with 2 quantities, each of 50 mL, of *water R*. Separate the upper toluene layer which is the test solution.

*Reference solution (a).* Dissolve 12 mg of *hydrazine sulfate R* in *dilute hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 1.0 mL of this solution to 100.0 mL with *dilute hydrochloric acid R*.

*Reference solution (b).* Prepare the solution at the same time and in the same manner as for the test solution, using 1.0 mL of reference solution (a) and 3 mL of *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ethanol (96 per cent) R*, *toluene R* (10:90 *V/V*).

*Application*: 20 µL of the test solution and reference solution (b).

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Limit*:

– *hydrazine*: any yellow fluorescent spot due to hydrazine is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (10 ppm).

**Related substances**. Liquid chromatography (*2.2.29*). *The solutions must be injected within one working day.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 10.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25.0 mg of *phthalazine R* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 4.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (d).* Dilute a mixture of 4.0 mL of the test solution and 10.0 mL of reference solution (c) to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *nitrile silica gel for chromatography R1* (10 µm).

*Mobile phase*: mix 22 volumes of *acetonitrile R* and 78 volumes of a solution containing 1.44 g/L of *sodium laurilsulfate R* and 0.75 g/L of *tetrabutylammonium bromide R*, then adjust to pH 3.0 with *dilute sulfuric acid R1*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of hydralazine.

*Retention time*: hydralazine = about 10 min to 12 min; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*System suitability*:

– the chromatogram obtained with reference solution (d) shows 2 principal peaks;
– *resolution*: minimum 2.5 between the peaks due to hydralazine and phtalazine in the chromatogram obtained with reference solution (d);
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (b).

*Limit*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 25 mL of *water R*. Add 35 mL of *hydrochloric acid R* and titrate with *0.05 M potassium iodate*, determining the end-point potentiometrically (*2.2.20*) using a platinum indicator electrode.

1 mL of *0.05 M potassium iodate* is equivalent to 9.832 mg of $C_8H_9ClN_4$.

STORAGE

Protected from light.

**01/2017:0002**

# HYDROCHLORIC ACID, CONCENTRATED

## Acidum hydrochloridum concentratum

HCl $M_r$ 36.46
[7647-01-0 ]

DEFINITION

*Content*: 35.0 per cent *m/m* to 39.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless, fuming liquid.

*Solubility*: miscible with water.

*Relative density*: about 1.18.

IDENTIFICATION

A. Dilute with *water R*. The solution is strongly acid (*2.2.4*).

B. It gives the reactions of chlorides (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Appearance of solution**. To 2 mL add 8 mL of *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Free chlorine**: maximum 4 ppm.

To 15 mL add 100 mL of *carbon dioxide-free water R*, 1 mL of a 100 g/L solution of *potassium iodide R* and 0.5 mL of *iodide-free starch solution R*. Allow to stand in the dark for 2 min. Any blue colour disappears on the addition of 0.2 mL of *0.01 M sodium thiosulfate*.

**Sulfates** (*2.4.13*): maximum 20 ppm.

To 6.4 mL add 10 mg of *sodium hydrogen carbonate R* and evaporate to dryness on a water-bath. Dissolve the residue in 15 mL of *distilled water R*.

**Residue on evaporation**: maximum 0.01 per cent.

Evaporate 100.0 g to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 10 mg.

ASSAY

Weigh accurately a ground-glass-stoppered flask containing 30 mL of *water R*. Introduce 1.5 mL of the acid to be examined and weigh again. Titrate with *1 M sodium hydroxide*, using *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 36.46 mg of HCl.

STORAGE

In a stoppered container made of glass or another inert material, at a temperature not exceeding 30 °C.

**01/2017:0003**

# HYDROCHLORIC ACID, DILUTE

## Acidum hydrochloridum dilutum

DEFINITION

*Content*: 9.5 per cent *m/m* to 10.5 per cent *m/m* of HCl ($M_r$ 36.46).

PREPARATION

To 726 g of *water R* add 274 g of concentrated hydrochloric acid and mix.

IDENTIFICATION

A. It is strongly acid (*2.2.4*).

B. It gives the reactions of chlorides (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Free chlorine**: maximum 1 ppm.

To 60 mL add 50 mL of *carbon dioxide-free water R*, 1 mL of a 100 g/L solution of *potassium iodide R* and 0.5 mL of *iodide-free starch solution R*. Allow to stand in the dark for 2 min. Any blue colour disappears on the addition of 0.2 mL of *0.01 M sodium thiosulfate*.

**Sulfates** (*2.4.13*): maximum 5 ppm.

To 26 mL add 10 mg of *sodium hydrogen carbonate R* and evaporate to dryness on a water-bath. Dissolve the residue in 15 mL of *distilled water R*.

**Residue on evaporation**: maximum 0.01 per cent.

Evaporate 100.0 g to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 10 mg.

ASSAY

To 6.00 g add 30 mL of *water R*. Titrate with *1 M sodium hydroxide*, using *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 36.46 mg of HCl.

**01/2013:0394**

# HYDROCHLOROTHIAZIDE

## Hydrochlorothiazidum

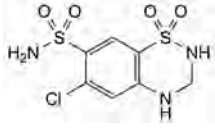

$C_7H_8ClN_3O_4S_2$ $M_r$ 297.7
[58-93-5]

DEFINITION

6-Chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in 10 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 100.0 mL with *0.01 M sodium hydroxide*.

*Spectral range*: 250-350 nm.

*Absorption maxima*: at 273 nm and 323 nm.

*Absorbance ratio*: $A_{273}/A_{323}$ = 5.4 to 5.7.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydrochlorothiazide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol R1*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 50 mg of *hydrochlorothiazide CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 25 mg of *chlorothiazide R* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R.*

*Application*: 2 µL.

*Development*: over 1/2 of the plate.

*Drying*: in a current of air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Gently heat about 1 mg with 2 mL of a freshly prepared 0.5 g/L solution of *chromotropic acid, sodium salt R* in a cooled mixture of 35 volumes of *water R* and 65 volumes of *sulfuric acid R*. A violet colour develops.

TESTS

**Acidity or alkalinity**. Shake 0.5 g of the powdered substance to be examined with 25 mL of *water R* for 2 min and filter. To 10 mL of the filtrate, add 0.2 mL of *0.01 M sodium hydroxide* and 0.15 mL of *methyl red solution R*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Dilute 50.0 mL of a mixture of equal volumes of *acetonitrile R1* and *methanol R2* to 200.0 mL with *phosphate buffer solution pH 3.2 R1*.

*Test solution (a).* Dissolve 30.0 mg of the substance to be examined in 5 mL of a mixture of equal volumes of *acetonitrile R1* and *methanol R2*, using sonication if necessary, and dilute to 20.0 mL with *phosphate buffer solution pH 3.2 R1*.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20.0 mL with *phosphate buffer solution pH 3.2 R1*.

*Reference solution (a).* Dissolve 3 mg of *chlorothiazide CRS* (impurity A) and 3 mg of *hydrochlorothiazide CRS* in 5 mL of a mixture of equal volumes of *acetonitrile R1* and *methanol R2*, using sonication if necessary, and dilute to 20.0 mL with *phosphate buffer solution pH 3.2 R1*. Dilute 5.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 30.0 mg of *hydrochlorothiazide CRS* in 5 mL of a mixture of equal volumes of *acetonitrile R1* and *methanol R2*, using sonication if necessary, and dilute to 20.0 mL with *phosphate buffer solution pH 3.2 R1*. Dilute 1.0 mL of this solution to 20.0 mL with *phosphate buffer solution pH 3.2 R1*.

*Reference solution (d).* Dissolve 3 mg of *hydrochlorothiazide for peak identification CRS* (containing impurities B and C) in 0.5 mL of a mixture of equal volumes of *acetonitrile R1* and *methanol R2*, using sonication if necessary, and dilute to 2.0 mL with *phosphate buffer solution pH 3.2 R1*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: to 940 mL of *phosphate buffer solution pH 3.2 R1* add 60.0 mL of *methanol R2* and 10.0 mL of *tetrahydrofuran R* and mix;
– *mobile phase B*: to a mixture of 500 mL of *methanol R2* and 500 mL of *phosphate buffer solution pH 3.2 R1* add 50.0 mL of *tetrahydrofuran R* and mix;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 17 | 100 → 55 | 0 → 45 |
| 17 - 30 | 55 | 45 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 224 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *hydrochlorothiazide for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and C.

*Relative retention* with reference to hydrochlorothiazide (retention time = about 8 min): impurity B = about 0.7; impurity A = about 0.9; impurity C = about 2.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to impurity A and hydrochlorothiazide.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 1.0 g in 25 mL of *acetone R* and dilute to 30 mL with *water R*. Prepare the standard using 5 mL of *acetone R* containing 15 per cent *V/V* of *water R* and 10 mL of *chloride standard solution (5 ppm Cl) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 80 | 20 |
| 4 - 10 | 80 → 20 | 20 → 80 |

*Flow rate*: 1.6 mL/min.

*Injection*: test solution (b) and reference solutions (a) and (c).

*Relative retention* with reference to hydrochlorothiazide (retention time = about 2.2 min): impurity A = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity A and hydrochlorothiazide.

Calculate the percentage content of $C_7H_8ClN_3O_4S_2$ taking into account the assigned content of *hydrochlorothiazide CRS*.

IMPURITIES

*Specified impurities*: *A, B, C.*

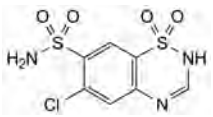

A. 6-chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (chlorothiazide),

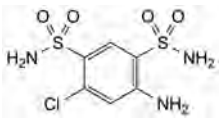

B. 4-amino-6-chlorobenzene-1,3-disulfonamide (salamide),

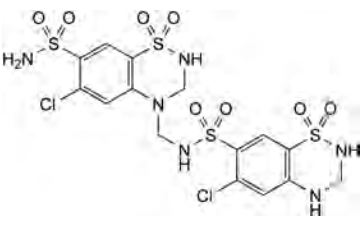

C. 6-chloro-*N*-[(6-chloro-7-sulfamoyl-2,3-dihydro-4*H*-1,2,4-benzothiadiazin-4-yl 1,1-dioxide)methyl]-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide.

**04/2009:1784 corrected 7.0**

# HYDROCODONE HYDROGEN TARTRATE 2.5-HYDRATE

## Hydrocodoni hydrogenotartras 2.5-hydricus

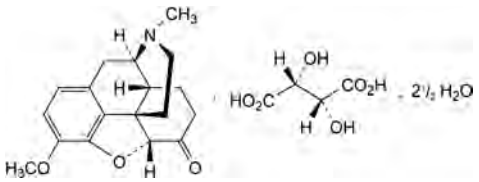

$C_{22}H_{27}NO_9,2.5H_2O$ $M_r$ 494.5

DEFINITION

4,5α-Epoxy-3-methoxy-17-methylmorphinan-6-one hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate 2.5-hydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble or soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydrocodone hydrogen tartrate 2.5-hydrate CRS*.

If the spectra obtained in the solid state show differences, dry the substance to be examined and the reference substance at 105 °C and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 3.2 to 3.8.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 87 to – 91 (anhydrous substance).

Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 5 mg of *oxycodone hydrochloride CRS* (impurity D) in mobile phase A, add 0.5 mL of the test solution and dilute to 5.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 20 mg of *benzophenone CRS* (impurity H) in 50.0 mL of *methanol R*. Dilute 1.0 mL of this solution to 20.0 mL with mobile phase A.

*Reference solution (d)*. Dissolve the contents of a vial of *hydrocodone for peak identification CRS* (containing impurities B, C, D, E, F and I) in 1.0 mL of mobile phase A.

*Reference solution (e)*. Dissolve 5 mg of *morphine sulfate CRS* (impurity A) in 5 mL of mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 1.08 g of *sodium octanesulfonate R* in *water R*, adjust to pH 2.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 80 | 20 |
| 15 - 30 | 80 → 70 | 20 → 30 |
| 30 - 40 | 70 → 40 | 30 → 60 |
| 40 - 42 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *hydrocodone for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B, C, D, E, F and I; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity A.

*Relative retention* with reference to hydrocodone (retention time = about 14 min): impurity A = about 0.3; impurity K = about 0.43; impurity B = about 0.57; impurity C = about 0.61; impurity D = about 0.9; impurity E = about 1.1; impurity F = about 1.5; impurity I = about 2.0; impurity H = about 2.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity D and hydrocodone.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity I by 0.2;

– *impurity I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *impurity H*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);

– *impurities A, B, C, D, E, F, K*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 7.0 per cent to 12.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 44.95 mg of $C_{22}H_{27}NO_9$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, H, I, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, J.*

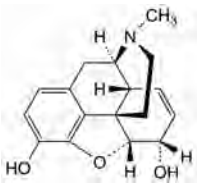

A. morphine,

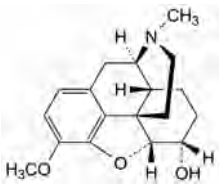

B. 4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (dihydrocodeine),

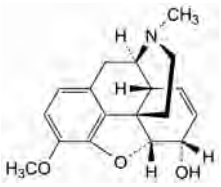

C. codeine,

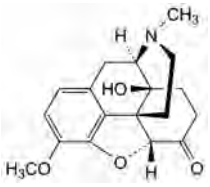

D. 4,5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one (oxycodone),

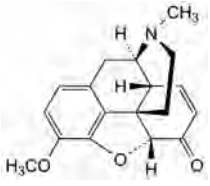

E. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6-one (codeinone),

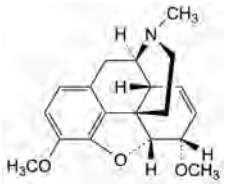

F. 7,8-didehydro-4,5α-epoxy-3,6α-dimethoxy-17-methylmorphinan (methylcodeine),

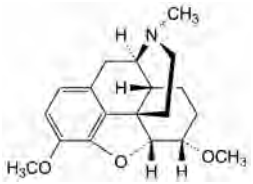

G. 4,5α-epoxy-3,6α-dimethoxy-17-methylmorphinan (tetrahydrothebaine),

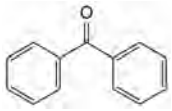

H. diphenylmethanone (benzophenone),

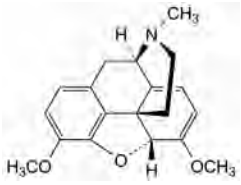

I. 6,7,8,14-tetradehydro-4,5α-epoxy-3,6-dimethoxy-17-methylmorphinan (thebaine),

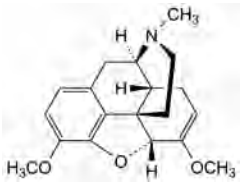

J. 6,7-didehydro-4,5α-epoxy-3,6-dimethoxy-17-methylmorphinan,

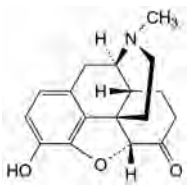

K. 4,5α-epoxy-3-hydroxy-17-methylmorphinan-6-one.

**01/2011:0335**

# HYDROCORTISONE

## Hydrocortisonum

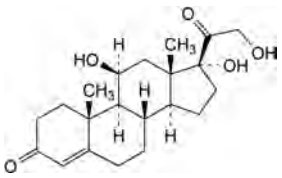

$C_{21}H_{30}O_5$ $M_r$ 362.5
[50-23-7]

DEFINITION

11β,17,21-Trihydroxypregn-4-ene-3,20-dione.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone and in ethanol (96 per cent), slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydrocortisone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Thin-layer chromatography (*2.2.27*).

*Solution A*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Solution B*. Dissolve 25 mg of *hydrocortisone CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Test solution (a).* Dilute 2 mL of solution A to 10 mL with *methylene chloride R*.

*Test solution (b).* Transfer 0.4 mL of solution A to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap. Evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker, protected from light, for 1 h. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 mL separating funnel, washing the filter with 2 quantities, each of 5 mL, of *water R*. Shake the clear filtrate with 10 mL of *methylene chloride R*. Wash the organic layer with 5 mL of *1 M sodium hydroxide* and then with 2 quantities, each of 5 mL, of *water R*. Dry over *anhydrous sodium sulfate R*.

*Reference solution (a).* Dilute 2 mL of solution B to 10 mL with *methylene chloride R*.

*Reference solution (b).* Transfer 0.4 mL of solution B to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap. Evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker, protected from light, for 1 h. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 mL separating funnel, washing the filter with 2 quantities, each of 5 mL, of *water R*. Shake the clear filtrate with 10 mL of *methylene chloride R*. Wash the organic layer with 5 mL of *1 M sodium hydroxide* and then with 2 quantities, each of 5 mL, of *water R*. Dry over *anhydrous sodium sulfate R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase A*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Mobile phase B*: *butanol R* saturated with *water R*, *toluene R*, *ether R* (5:15:80 *V/V/V*).

*Application*: 5 µL of test solution (a) and reference solution (a), 25 µL of test solution (b) and reference solution (b), applying the latter 2 in small quantities to obtain small spots.

*Development*: over a path of 15 cm with mobile phase A, and then over a path of 15 cm with mobile phase B.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with test solutions (a) and (b) is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 10 min or until the spots appear; allow to cool, and examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with test solutions (a) and (b) is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution; the principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an $R_F$ value distinctly higher than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, an intense brownish-red colour develops with a green fluorescence that is particularly intense when examined in ultraviolet light at 365 nm. Add the solution to 10 mL of *water R* and mix. The colour fades and a clear solution remains. The fluorescence in ultraviolet light does not disappear.

TESTS

**Specific optical rotation** (*2.2.7*): + 162 to + 168 (dried substance).

Dissolve 0.200 g in *methanol R*, dilute to 25.0 mL with the same solvent and sonicate for 10 min.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution.* Dissolve 20 mg of the substance to be examined in the solvent mixture, dilute to 10.0 mL with the solvent mixture and sonicate for 10 min.

*Reference solution (a).* Dissolve 4 mg of *prednisolone CRS* (impurity A), 2 mg of *cortisone R* (impurity B), 8 mg of *hydrocortisone acetate CRS* (impurity C) and 6 mg of *Reichstein's substance S R* (impurity F) in 40 mL of *acetonitrile R* and dilute to 100.0 mL with *water R*. Dilute 0.5 mL of the solution to 5.0 mL with the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *hydrocortisone CRS* in 1.0 mL of the solvent mixture and sonicate for 10 min.

*Reference solution (d).* Dissolve 2 mg of *hydrocortisone for peak identification CRS* (containing impurities D, E, G, H, I and N) in 1.0 mL of the solvent mixture and sonicate for 10 min.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 18 | 74 | 26 |
| 18 - 32 | 74 → 55 | 26 → 45 |
| 32 - 48 | 55 → 30 | 45 → 70 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *hydrocortisone for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities D, E, G, H, I and N; use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and F.

*Relative retention* with reference to hydrocortisone (retention time = about 24 min): impurity D = about 0.2; impurity H = about 0.3; impurity I = about 0.5; impurity G = about 0.8; impurity E = about 0.86; impurity A = about 0.96; impurity B = about 1.1; impurity F = about 1.4; impurity C = about 1.5; impurity N = about 1.7.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to hydrocortisone.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.8; impurity E = 2.7;
- *impurities C, D, E, I*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity G*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities H, N*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 20 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241.5 nm.

Calculate the content of $C_{21}H_{30}O_5$ taking the specific absorbance to be 440.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L, M, O.*

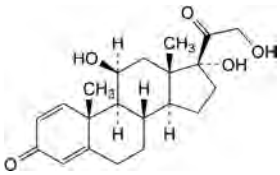

A. 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone),

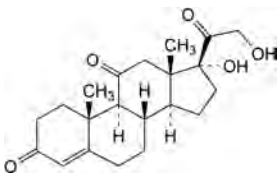

B. 17,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone),

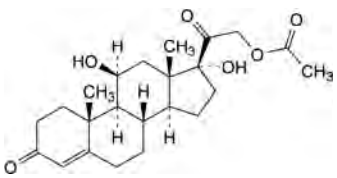

C. 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (hydrocortisone acetate),

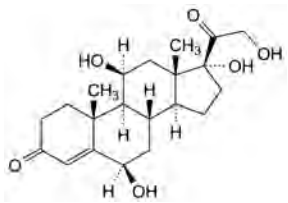

D. 6β,11β,17,21-tetrahydroxypregn-4-ene-3,20-dione (6β-hydroxyhydrocortisone),

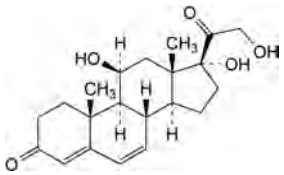

E. 11β,17,21-trihydroxypregna-4,6-diene-3,20-dione (Δ6-hydrocortisone),

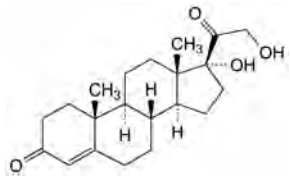

F. 17,21-dihydroxypregn-4-ene-3,20-dione (Reichstein's substance S),

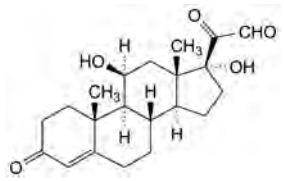

G. 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-al (hydrocortisone-21-aldehyde),

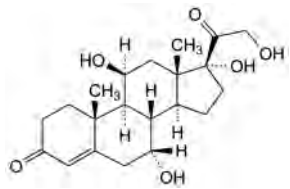

H. 7α,11β,17,21-tetrahydroxypregn-4-ene-3,20-dione (7α-hydroxyhydrocortisone),

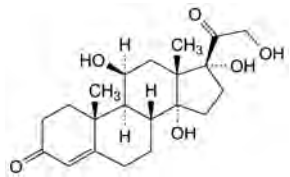

I. 11β,14,17,21-tetrahydroxypregn-4-ene-3,20-dione (14α-hydroxyhydrocortisone),

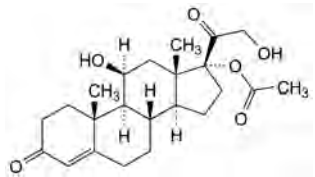

J. 11β,21-dihydroxy-3,20-dioxopregn-4-en-17-yl acetate (hydrocortisone-17-acetate),

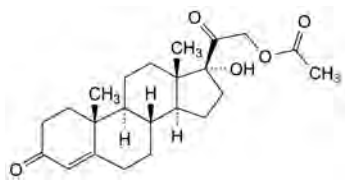

K. 17-hydroxy-3,20-dioxopregn-4-en-21-yl acetate (Reichstein's substance S-21-acetate),

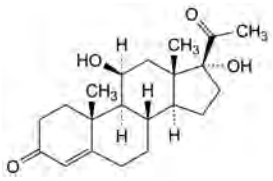

L. 11β,17-dihydroxypregn-4-ene-3,20-dione (oxenol),

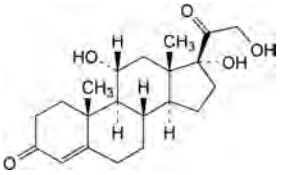

M. 11α,17,21-trihydroxypregn-4-ene-3,20-dione (*epi*-hydrocortisone),

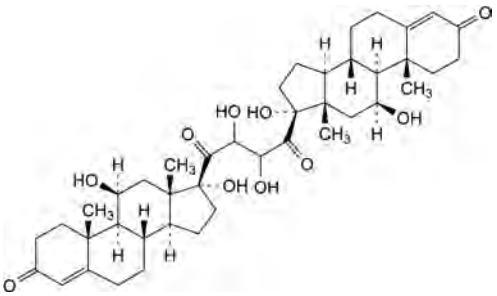

N. 11β,17,21-trihydroxy-21-(11β,17,21-trihydroxy-3,20-dioxopregn-4-en-21-yl)pregn-4-ene-3,20-dione (hydrocortisone dimer),

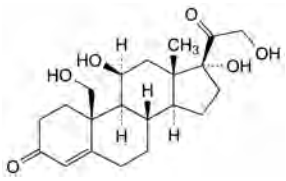

O. 11β,17,19,21-tetrahydroxypregn-4-ene-3,20-dione (19-hydroxyhydrocortisone).

**07/2019:0334**

# HYDROCORTISONE ACETATE

## Hydrocortisoni acetas

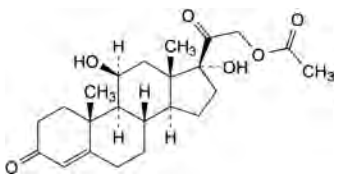

$C_{23}H_{32}O_6$ $M_r$ 404.5
[50-03-3]

DEFINITION

11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl acetate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydrocortisone acetate CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent (solution A). Dilute 2 mL of the solution to 10 mL with *methylene chloride R*.

*Test solution (b).* Transfer 2 mL of solution A to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

*Reference solution (a).* Dissolve 25 mg of *hydrocortisone acetate CRS* in *methanol R* and dilute to 5 mL with the same solvent (solution B). Dilute 2 mL of the solution to 10 mL with *methylene chloride R*.

*Reference solution (b).* Transfer 2 mL of solution B to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of *saturated methanolic potassium hydrogen carbonate solution R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C protected from light for 2 h 30 min. Allow to cool.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 10 min or until the spots appear and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spots in the chromatograms obtained with test solution (b) and reference solution (b) have an $R_F$ value distinctly lower than that of the principal spots in the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min an intense brownish-red colour develops with a green fluorescence which is particularly intense when viewed in ultraviolet light at 365 nm. Add this solution to 10 mL of *water R* and mix. The colour fades and the fluorescence in ultraviolet light does not disappear.

E. About 10 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 158 to + 167 (dried substance).

Dissolve 0.250 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetic acid R*, *water R*, *methanol R* (1:10:90 *V/V/V*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2 mg of *hydrocortisone acetate CRS* and 2 mg of *prednisolone acetate CRS* (impurity C) in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *hydrocortisone acetate for peak identification CRS* (containing impurities A, B, D, E and G) in 5 mL of the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *hydrocortisone acetate CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 400 mL of *acetonitrile R* with 550 mL of *water for chromatography R* and allow to equilibrate; dilute to 1000 mL with *water for chromatography R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: 4 times the retention time of hydrocortisone acetate.

*Identification of impurities*: use the chromatogram supplied with *hydrocortisone acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, D, E and G; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to hydrocortisone acetate (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 0.9; impurity D = about 1.2; impurity G = about 1.8; impurity E = about 2.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity C and hydrocortisone acetate.

*Limits*:

- *impurity C*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, D, E*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (d).

*Run time*: 1.5 times the retention time of hydrocortisone acetate.

*Retention time*: hydrocortisone acetate = about 10 min.

Calculate the percentage content of $C_{23}H_{32}O_6$ taking into account the assigned content of *hydrocortisone acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

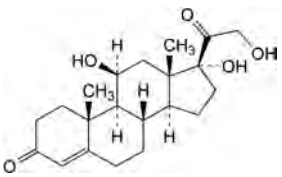

A. 11β,17,21-trihydroxypregn-4-ene-3,20-dione (hydrocortisone),

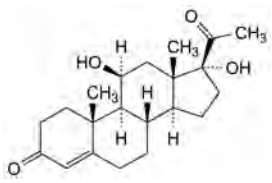

B. 11β,17-dihydroxypregn-4-ene-3,20-dione (oxenol),

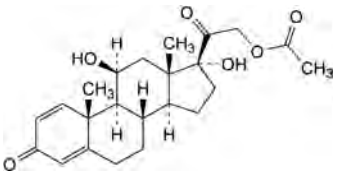

C. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (prednisolone acetate),

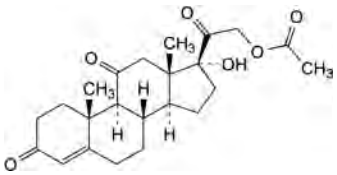

D. 17-hydroxy-3,11,20-trioxopregn-4-en-21-yl acetate (cortisone acetate),

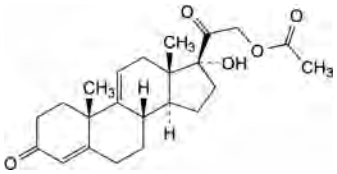

E. 17-hydroxy-3,20-dioxopregna-4,9(11)-dien-21-yl acetate,

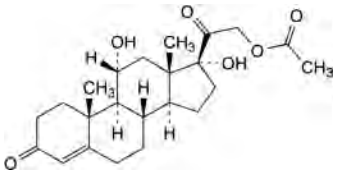

F. 11α,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (*epi*-hydrocortisone acetate),

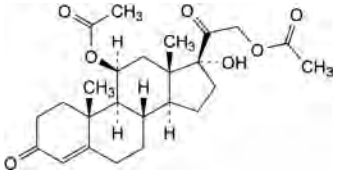

G. 17-hydroxy-3,20-dioxopregn-4-ene-11β,21-diyl diacetate.

**01/2008:0768**
**corrected 6.0**

# HYDROCORTISONE HYDROGEN SUCCINATE

## Hydrocortisoni hydrogenosuccinas

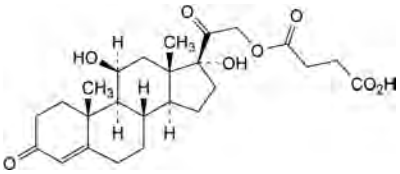

$C_{25}H_{34}O_8$ $M_r$ 462.5
[2203-97-6]

DEFINITION

11β,17-Dihydroxy-3,20-dioxopregn-4-en-21-yl hydrogen butanedioate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in anhydrous ethanol. It dissolves in dilute solutions of alkali carbonates and alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substances before use at 100-105 °C for 3 h.

*Comparison*: *hydrocortisone hydrogen succinate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20 mg of *hydrocortisone hydrogen succinate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *methylprednisolone hydrogen succinate CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous formic acid R*, *anhydrous ethanol R*, *methylene chloride R* (0.1:1:15 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which may, however, not be completely separated.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 25 mg of the substance to be examined in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution A). Dilute 2 mL of this solution to 10 mL with *methylene chloride R*.

*Test solution (b).* Transfer 2 mL of solution A to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of a 0.8 g/L solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

*Reference solution (a).* Dissolve 25 mg of *hydrocortisone hydrogen succinate CRS* in *methanol R* with gentle heating and dilute to 5 mL with the same solvent (solution B). Dilute 2 mL of this solution to 10 mL with *methylene chloride R*.

*Reference solution (b).* Transfer 2 mL of solution B to a 15 mL glass tube with a ground-glass stopper or a polytetrafluoroethylene cap. Add 10 mL of a 0.8 g/L solution of *sodium hydroxide R* in *methanol R* and immediately pass a stream of *nitrogen R* briskly through the solution for 5 min. Stopper the tube. Heat in a water-bath at 45 °C, protected from light, for 30 min. Allow to cool.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Developpement*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) has an $R_F$ value distinctly higher than that of the principal spot in each of the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, an intense brownish-red colour develops with a green fluorescence which is particularly intense when viewed in ultraviolet light at 365 nm. Add this solution to 10 mL of *water R* and mix. The colour fades and a clear solution remains. The fluorescence in ultraviolet light does not disappear.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.10 g in 5 mL of *sodium hydrogen carbonate solution R*.

**Specific optical rotation** (*2.2.7*): + 147 to + 153 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 2 mg of *hydrocortisone hydrogen succinate CRS* and 2 mg of *dexamethasone CRS* in 50 mL of *acetonitrile R*, then dilute to 100.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: in a 1000 mL volumetric flask mix 330 mL of *acetonitrile R* with 600 mL of *water R* and 1.0 mL of *phosphoric acid R*, then allow to equilibrate; dilute to 1000 mL with *water R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL.

*Run time*: twice the retention time of hydrocortisone hydrogen succinate.

*Retention time*: dexamethasone = about 12.5 min; hydrocortisone hydrogen succinate = about 15 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to dexamethasone and hydrocortisone hydrogen succinate; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurities A, B*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *total*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241.5 nm.

Calculate the content of $C_{25}H_{34}O_8$ taking the specific absorbance to be 353.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B*.

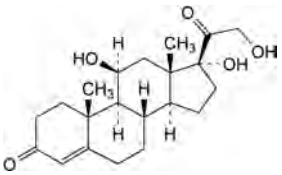

A. 11β,17,21-trihydroxypregn-4-ene-3,20-dione (hydrocortisone),

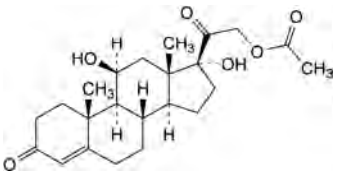

B. 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (hydrocortisone acetate).

**01/2013:0395**

# HYDROGEN PEROXIDE SOLUTION (3 PER CENT)

## Hydrogenii peroxidum 3 per centum

DEFINITION

*Content*: 2.5 per cent *m/m* to 3.5 per cent *m/m* of $H_2O_2$ ($M_r$ 34.01).

1 volume of hydrogen peroxide solution (3 per cent) corresponds to about 10 times its volume of oxygen. A suitable stabiliser may be added.

CHARACTERS

*Appearance*: colourless, clear liquid.

IDENTIFICATION

A. To 2 mL, add 0.2 mL of *dilute sulfuric acid R* and 0.2 mL of *0.02 M potassium permanganate*. The solution becomes colourless or slightly pink within 2 min.

B. To 1 mL, add 0.1 mL of *dilute hydrochloric acid R* and 0.1 mL of *potassium iodide solution R*. A brown colour appears. Black particles may be formed.

C. It complies with the requirement for the content of $H_2O_2$.

TESTS

**Acidity**. To 10 mL, add 20 mL of *water R* and 0.25 mL of *methyl red solution R*. Not less than 0.05 mL and not more than 1.0 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Organic stabilisers**: maximum 250 ppm.

Shake 20 mL with 10 mL of *chloroform R* and then with 2 quantities, each of 5 mL, of *chloroform R*. Evaporate the combined chloroform layers under reduced pressure at a temperature not exceeding 25 °C and dry in a desiccator. The residue weighs a maximum of 5 mg.

**Non-volatile residue**: maximum 2 g/L.

Allow 10 mL to stand in a platinum dish until all effervescence has ceased. Evaporate to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 20 mg.

ASSAY

Dilute 10.0 g to 100.0 mL with *water R*. To 10.0 mL of this solution add 20 mL of *dilute sulfuric acid R*. Titrate with *0.02 M potassium permanganate* until a pink colour is obtained.

1 mL of *0.02 M potassium permanganate* is equivalent to 1.701 mg of $H_2O_2$ or 0.56 mL of oxygen.

STORAGE

Protected from light, and if the solution does not contain a stabiliser, at a temperature below 15 °C.

LABELLING

If the solution contains a stabiliser, the label states that the contents are stabilised. The competent authority may require that the name of the stabiliser be stated on the label.

CAUTION

It decomposes in contact with oxidisable organic matter and with certain metals and if allowed to become alkaline.

**01/2013:0396**

# HYDROGEN PEROXIDE SOLUTION (30 PER CENT)

## Hydrogenii peroxidum 30 per centum

[7722-84-1]

DEFINITION

*Content*: 29.0 per cent *m/m* to 31.0 per cent *m/m* of $H_2O_2$ ($M_r$ 34.01).

1 volume of hydrogen peroxide solution (30 per cent) corresponds to about 110 times its volume of oxygen. A suitable stabiliser may be added.

CHARACTERS

*Appearance*: colourless, clear liquid.

IDENTIFICATION

A. To 1 mL, add 0.2 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate*. The solution becomes colourless with evolution of gas.

B. To 1 mL, add 0.1 mL of *dilute hydrochloric acid R* and 0.1 mL of *potassium iodide solution R*. A brown colour appears. Black particles may be formed.

C. It complies with the requirement for the content of $H_2O_2$.

TESTS

**Acidity**. To 10 mL, add 100 mL of *water R* and 0.25 mL of *methyl red solution R*. Not less than 0.05 mL and not more than 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Organic stabilisers**: maximum 500 ppm.

Shake 20 mL with 10 mL of *chloroform R* and then with 2 quantities, each of 5 mL, of *chloroform R*. Evaporate the combined chloroform layers under reduced pressure at a temperature not exceeding 25 °C and dry in a desiccator. The residue weighs a maximum of 10 mg.

**Non-volatile residue**: maximum 2 g/L.

Allow 10 mL to stand in a platinum dish until all effervescence has ceased, cooling if necessary. Evaporate to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 20 mg.

ASSAY

Dilute 1.00 g to 100.0 mL with *water R*. To 10.0 mL of this solution add 20 mL of *dilute sulfuric acid R*. Titrate with *0.02 M potassium permanganate* until a pink colour is obtained.

1 mL of *0.02 M potassium permanganate* is equivalent to 1.701 mg of $H_2O_2$ or 0.56 mL of oxygen.

STORAGE

Protected from light, and if the solution does not contain a stabiliser, at a temperature below 15 °C.

LABELLING

If the solution contains a stabiliser, the label states that the contents are stabilised. The competent authority may require that the name of the stabiliser be stated on the label.

CAUTION

It decomposes vigorously in contact with oxidisable organic matter and with certain metals and if allowed to become alkaline.

**01/2008:2099**
**corrected 6.0**

# HYDROMORPHONE HYDROCHLORIDE

## Hydromorphoni hydrochloridum

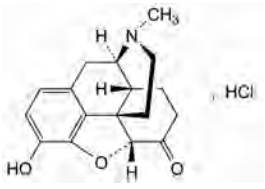


$C_{17}H_{20}ClNO_3$ $M_r$ 321.8
[71-68-1]

DEFINITION

4,5α-Epoxy-3-hydroxy-17-methylmorphinan-6-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *hydromorphone hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.250 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 2 mL of solution S add 0.1 mL of *methyl red solution R*. The solution is not yellow. To 2 mL of solution S add 0.05 mL of *bromocresol green solution R*. The solution is not yellow.

**Specific optical rotation** (*2.2.7*): − 136 to − 140 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water R*, sonicating if necessary and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. To 5 mL of the test solution add 5 mg of *naloxone hydrochloride dihydrate CRS* and dilute to 50 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 18.29 g of *diethylamine R* and 2.88 g of *sodium laurilsulfate R* in *water R* and dilute to 1000 mL with the same solvent. Adjust 800 mL of this solution to pH 3.0 with *phosphoric acid R*. Add 100 mL of *acetonitrile R* and 100 mL of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 284 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of hydromorphone.

*Relative retention* with reference to hydromorphone (retention time = about 9 min): impurity D = about 0.72; impurity B = about 0.77; impurity C = about 0.82; impurity A = about 3.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to hydromorphone and naloxone.

*Limits*:

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities B, C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.18 mg of $C_{17}H_{20}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

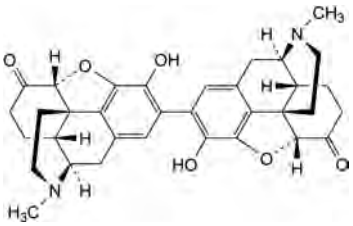

A. 4,5α:4′,5′α-diepoxy-3,3′-dihydroxy-17,17′-dimethyl-2,2′-bimorphinanyl-6,6′-dione (pseudohydromorphone),

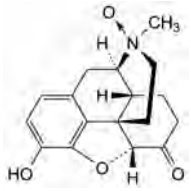

B. 4,5α-epoxy-3-hydroxy-17-methylmorphinan-6-one 17-oxide (hydromorphone *N*-oxide),

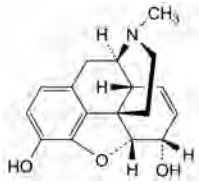

C. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol (morphine),

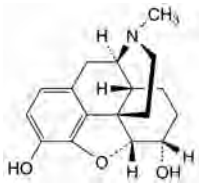

D. 4,5α-epoxy-17-methylmorphinan-3,6α-diol (dihydromorphine).

01/2008:0913
corrected 6.0

# HYDROXOCOBALAMIN ACETATE

## Hydroxocobalamini acetas

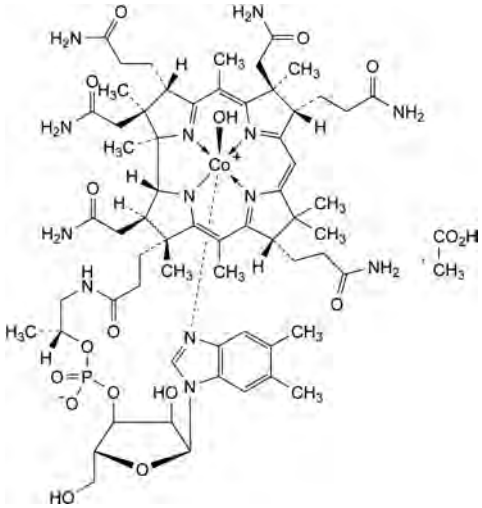

$C_{64}H_{93}CoN_{13}O_{17}P$ $M_r$ 1406
[22465-48-1]

DEFINITION

Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-hydroxocobamide acetate.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

This monograph applies to hydroxocobalamin acetate produced by fermentation.

CHARACTERS

*Appearance*: dark red, crystalline powder or dark red crystals, very hygroscopic.

*Solubility*: soluble in water.

Some decomposition may occur on drying.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 2.5 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R*, then dilute to 100 mL with the same solution.

*Spectral range*: 260-610 nm.

*Absorption maxima*: at 274 nm, 351 nm and 525 nm.

*Absorbance ratio*:

- $A_{274}/A_{351}$ = 0.75 to 0.83;
- $A_{525}/A_{351}$ = 0.31 to 0.35.

B. Thin-layer chromatography (*2.2.27*). *Carry out the test protected from light.*

*Test solution.* Dissolve 2 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Reference solution.* Dissolve 2 mg of *hydroxocobalamin CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *dilute ammonia R1*, *methanol R* (25:75 *V/V*).

*Application*: 10 µL.

*Development*: in an unlined tank, over a path of 12 cm.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of acetates (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and protect them from bright light.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25 mg of the substance to be examined in 10 mL of *water R*, warming if necessary. Allow to cool and add 1 mL of a 20 g/L solution of *chloramine R* and 0.5 mL of *0.05 M hydrochloric acid*. Dilute this solution to 25 mL with *water R*. Shake and allow to stand for 5 min. Inject immediately.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 19.5 volumes of *methanol R* and 80.5 volumes of a solution containing 15 g/L of *citric acid monohydrate R* and 8.1 g/L of *disodium hydrogen phosphate dodecahydrate R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 351 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of hydroxocobalamine.

*System suitability*:

- the chromatogram obtained with reference solution (c) shows 3 principal peaks;
- *resolution*: minimum 3.0 between each pair of adjacent peaks in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (5 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): 8.0 per cent to 12.0 per cent, determined on 0.400 g by drying at 105 °C at a pressure not exceeding 0.7 kPa.

ASSAY

*Protect the solutions from light throughout the assay.* Dissolve 25.0 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R*, then dilute to 1000.0 mL with the same solution. Measure the absorbance (*2.2.25*) at the absorption maximum at 351 nm.

Calculate the content of $C_{64}H_{93}CoN_{13}O_{17}P$ taking the specific absorbance to be 187.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2008:0914**
**corrected 6.0**

# HYDROXOCOBALAMIN CHLORIDE

## Hydroxocobalamini chloridum

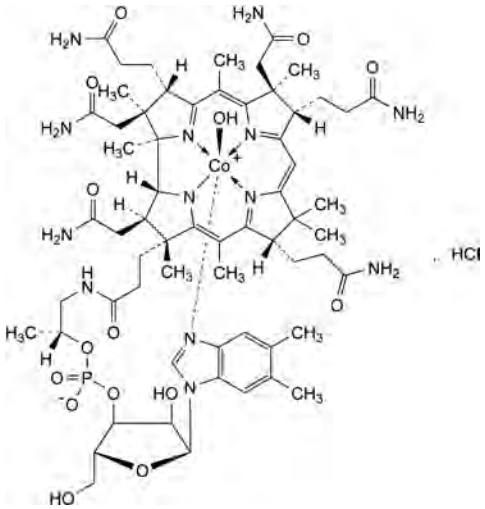

$C_{62}H_{90}ClCoN_{13}O_{15}P$ $M_r$ 1383
[58288-50-9]

DEFINITION

Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-hydroxocobamide chloride.

Fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: dark red crystalline powder or dark red crystals, very hygroscopic.

*Solubility*: soluble in water.

Some decomposition may occur on drying.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 2.5 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R*, then dilute to 100 mL with the same solution.

*Spectral range*: 260-610 nm.

*Absorption maxima*: at 274 nm, 351 nm and 525 nm.

*Absorbance ratio*:

- $A_{274}/A_{351}$ = 0.75 to 0.83;
- $A_{525}/A_{351}$ = 0.31 to 0.35.

B. Thin-layer chromatography (*2.2.27*). *Carry out the identification test protected from light.*

*Test solution*. Dissolve 2 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Reference solution*. Dissolve 2 mg of *hydroxocobalamin CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *dilute ammonia R1*, *methanol R* (25:75 *V/V*).

*Application*: 10 µL.

*Development*: in an unlined tank, over a path of 12 cm.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and protect them from bright light.*

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 25 mg of the substance to be examined in 10 mL of *water R*, warming if necessary. Allow to cool and add 1 mL of a 20 g/L solution of *chloramine R* and 0.5 mL of *0.05 M hydrochloric acid*. Dilute to 25 mL with *water R*. Shake and allow to stand for 5 min. Inject immediately.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 19.5 volumes of *methanol R* and 80.5 volumes of a solution containing 15 g/L of *citric acid monohydrate R* and 8.1 g/L of *disodium hydrogen phosphate dodecahydrate R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 351 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of hydroxocobalamin.

*System suitability*:

- the chromatogram obtained with reference solution (c) shows 3 principal peaks;
- *resolution*: minimum 3.0 between each pair of adjacent peaks in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): 8.0 per cent to 12.0 per cent, determined on 0.400 g by drying at 105 °C at a pressure not exceeding 0.7 kPa.

ASSAY

*Protect the solutions from light throughout the assay.* Dissolve 25.0 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R*, then dilute to 1000.0 mL with the same solution. Measure the absorbance (*2.2.25*) at the absorption maximum at 351 nm.

Calculate the content of $C_{62}H_{90}ClCoN_{13}O_{15}P$ taking the specific absorbance to be 190.

STORAGE

In an airtight container protected from light, at a temperature of 2 °C to 8 °C.

01/2008:0915
corrected 6.0

# HYDROXOCOBALAMIN SULFATE

## Hydroxocobalamini sulfas

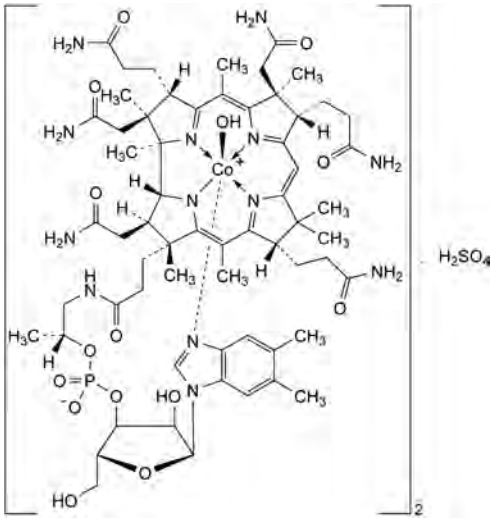

$C_{124}H_{180}Co_2N_{26}O_{34}P_2S$ $M_r$ 2791

DEFINITION

Di-(Coα-[α-(5,6-dimethylbenzimidazolyl)]-Coβ-hydroxocobamide) sulfate.

Fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: dark red crystalline powder or dark red crystals, very hygroscopic.

*Solubility*: soluble in water.

Some decomposition may occur on drying.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 2.5 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R*, then dilute to 100 mL with the same solution.

*Spectral range*: 260-610 nm.

*Absorption maxima*: at 274 nm, 351 nm and 525 nm.

*Absorbance ratios*:

- $A_{274}/A_{351}$ = 0.75 to 0.83;
- $A_{525}/A_{351}$ = 0.31 to 0.35.

B. Thin-layer chromatography (*2.2.27*). *Carry out the test protected from light.*

*Test solution*. Dissolve 2 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Reference solution*. Dissolve 2 mg of *hydroxocobalamin CRS* in 1 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *dilute ammonia R1*, *methanol R* (25:75 *V/V*).

*Application*: 10 µL.

*Development*: in an unlined tank, over a path of 12 cm.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and protect them from bright light.*

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 25 mg of the substance to be examined in 10 mL of *water R*, warming if necessary. Allow to cool and add 1 mL of a 20 g/L solution of *chloramine R* and 0.5 mL of *0.05 M hydrochloric acid*. Dilute to 25 mL with *water R*. Shake and allow to stand for 5 min. Inject immediately.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 19.5 volumes of *methanol R* and 80.5 volumes of a solution containing 15 g/L of *citric acid monohydrate R* and 8.1 g/L of *disodium hydrogen phosphate dodecahydrate R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 351 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of hydroxocobalamin.

*System suitability*:

- the chromatogram obtained with reference solution (c) shows 3 principal peaks;
- *resolution*: minimum 3.0 between each pair of adjacent peaks in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Loss on drying** (*2.2.32*): 8.0 per cent to 16.0 per cent, determined on 0.400 g by drying at 105 °C at a pressure not exceeding 0.7 kPa.

ASSAY

*Protect the solutions from light throughout the assay.* Dissolve 25.0 mg in a solution containing 0.8 per cent *V/V* of *glacial acetic acid R* and 10.9 g/L of *sodium acetate R* and dilute to 1000.0 mL with the same solution. Measure the absorbance (*2.2.25*) at the absorption maximum at 351 nm.

Calculate the content of $C_{124}H_{180}Co_2N_{26}O_{34}P_2S$ taking the specific absorbance to be 188.

STORAGE

In an airtight container protected from light, at a temperature of 2 °C to 8 °C.

**01/2017:1616**

# HYDROXYCARBAMIDE

## Hydroxycarbamidum

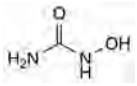

$CH_4N_2O_2$ $M_r$ 76.1
[127-07-1]

DEFINITION

*N*-Hydroxyurea.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydroxycarbamide CRS*.

If the spectra obtained in the solid state show differences dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

B. Examine the chromatograms obtained in the test for urea.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).

TESTS

**Urea**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *water R* and dilute to 1.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 12.5 mg of *urea R* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *urea R* in *water R* and dilute to 20 mL with the same solvent.

*Reference solution (c)*. Dissolve 50 mg of *hydroxycarbamide CRS* in *water R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *pyridine R*, *water R*, *ethyl acetate R* (2:2:10 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 10 g/L solution of *dimethylaminobenzaldehyde R* in *1 M hydrochloric acid*.

*System suitability*: the test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Limit*:

– *urea*: any spot corresponding to urea in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the same mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 0.100 g of *hydroxylamine hydrochloride R* and 5 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase. Prepare immediately before use.

*Reference solution (b)*. Dilute 0.1 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 0.100 g of *hydroxycarbamide CRS* in the mobile phase and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *water R* (5:95 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL; inject test solution (a) and reference solutions (a) and (b).

*Run time*: 3 times the retention time of hydroxycarbamide which is about 5 min.

*System suitability*: reference solution (a):

– *resolution*: minimum of 1.0 between the peaks due to impurity A and to hydroxycarbamide.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *total*: not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dissolve 1.0 g in *water R* and dilute to 15 mL with the same solvent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution (b) and reference solution (c).

STORAGE

In an airtight container, protected from light.

IMPURITIES

A. $H_2N$-OH: hydroxylamine.

Monographs H

01/2017:2849
corrected 10.0

# HYDROXYCHLOROQUINE SULFATE

## Hydroxychloroquini sulfas

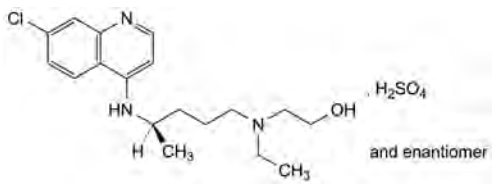


$C_{18}H_{28}ClN_3O_5S$ $M_r$ 434.0
[747-36-4]

DEFINITION

2-[[(4*RS*)-4-[(7-Chloroquinolin-4-yl)amino]pentyl]-(ethyl)amino]ethan-1-ol sulfate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *hydroxychloroquine sulfate CRS*.

B. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

Dissolve 1.0 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Mix 500 mL of *water R*, 500 mL of *methanol R* and 4 mL of a 10 per cent *V/V* solution of *sulfuric acid R*.

*Buffer solution*. Dissolve 1.36 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*. Add 0.15 g of *sodium heptanesulfonate R*, adjust to pH 7.0 with *triethylamine R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *hydroxychloroquine for system suitability CRS* (containing impurities B and C) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 25.0 mg of *hydroxychloroquine sulfate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.05 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *methanol R*, buffer solution (10:90 *V/V*);
- *mobile phase B*: buffer solution, *methanol R* (15:85 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 1 - 11 | 100 → 0 | 0 → 100 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 4 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *hydroxychloroquine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to hydroxychloroquine (retention time = about 6 min): impurity B = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.0 between the peaks due to impurity C and hydroxychloroquine; minimum 3.0 between the peaks due to impurities B and C.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity B by 1.6;
- for each impurity, use the concentration of hydroxychloroquine sulfate in reference solution (b).

*Limits*:
- *impurity C*: maximum 0.4 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 4 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{28}ClN_3O_5S$ taking into account the assigned content of *hydroxychloroquine sulfate CRS*.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F, G.*

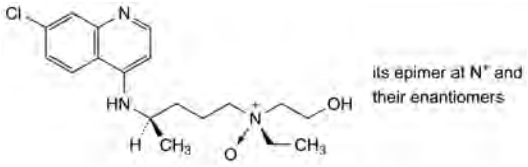


A. mixture of diastereoisomers of 4-[(7-chloroquinolin-4-yl)amino]-*N*-ethyl-*N*-(hydroxyethyl)pentan-1-amine *N*-oxide,

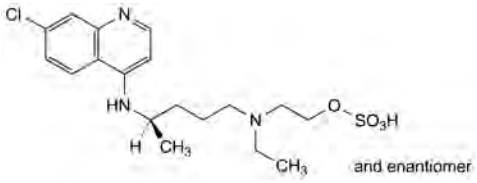


B. 2-[[(4*RS*)-4-[(7-chloroquinolin-4-yl)amino]pentyl]-(ethyl)amino]ethyl hydrogen sulfate,

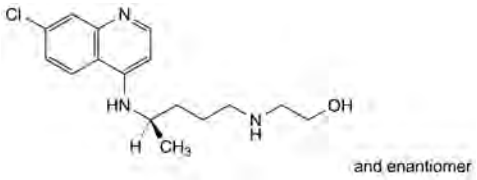


C. 2-[[(4*RS*)-4-[(7-chloroquinolin-4-yl)amino]pentyl]-amino]ethan-1-ol,

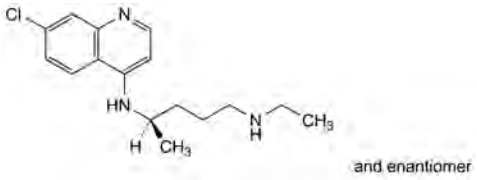


D. (4*RS*)-$N^4$-(7-chloroquinolin-4-yl)-$N^1$-ethylpentane-1,4-diamine,

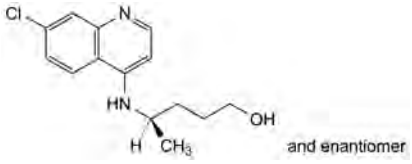


E. (4*RS*)-4-[(7-chloroquinolin-4-yl)amino]pentan-1-ol,

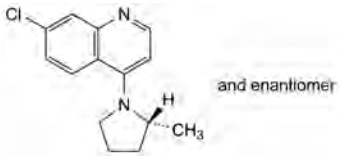


F. 7-chloro-4-[(2*RS*)-2-methylpyrrolidin-1-yl]quinoline,

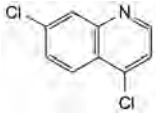

G. 4,7-dichloroquinoline.

**01/2008:1225**

# HYDROXYETHYL SALICYLATE

## Hydroxyethylis salicylas

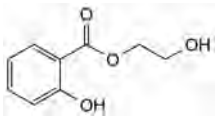

$C_9H_{10}O_4$ $M_r$ 182.2
[87-28-5]

DEFINITION

2-Hydroxyethyl 2-hydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: oily, colourless or almost colourless liquid, or colourless crystals.

*Solubility*: sparingly soluble in water, very soluble in acetone and in methylene chloride, freely soluble in ethanol (96 per cent).

Mp: about 21 °C.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D, E.*

A. Refractive index (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: thin films.

*Comparison*: *hydroxyethyl salicylate CRS.*

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 mL of solution S (see Tests), add 1 mL of *water R* and 0.2 mL of *ferric chloride solution R2*. A violet-red colour appears which disappears immediately after the addition of 2 mL of *dilute acetic acid R*. A very faint violet colour may remain.

E. In a test tube 160 mm long, mix 1.0 g with 2.0 g of finely powdered *manganese sulfate R*. Insert 2 cm into the test-tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent *V/V* solution of *diethanolamine R* and 11 volumes of a 50 g/L solution of *sodium nitroprusside R* adjusted to pH 9.8 with *1 M hydrochloric acid*. Heat the test-tube over a naked flame for 1-2 min. The filter paper becomes blue.

TESTS

**Solution S**. Dissolve 2.5 g in 40 mL of *ethanol (96 per cent) R* and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 2 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is red.

**Relative density** (*2.2.5*): 1.252 to 1.257.

**Refractive index** (*2.2.6*): 1.548 to 1.551.

Monographs H

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a)*. Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 2 mL of test solution (a) to 50 mL with *methanol R*.

*Reference solution (a)*. Dissolve 50.0 mg of *hydroxyethyl salicylate CRS* in *methanol R* and dilute to 25 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of test solution (b) to 10 mL with *methanol R*.

*Reference solution (c)*. Dissolve 0.10 g of *ethylene glycol R* in *methanol R* and dilute to 50 mL with the same solvent. Dilute 1.25 mL of the solution to 10 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ethyl acetate R*, *glacial acetic acid R*, *cyclohexane R* (20:20:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of cold air.

*Detection A*: in ultraviolet light at 254 nm.

*Limits A*:

– *any impurity*: any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent).

*Detection B*: spray the plate with *ammonium vanadate solution R* and heat at 100 °C for 10 min. Allow to cool for 10 min and examine in daylight.

*Limits B*: in the chromatogram obtained with test solution (a):

– *impurity B*: any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *any other impurity*: any spot, apart from the principal spot and any spot corresponding to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (1 per cent).

*System suitability*: the chromatogram obtained with reference solution (c) shows a clearly visible spot.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 250 ppm.

Dilute 12 mL of solution S to 15 mL with *distilled water R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a flask with a ground-glass stopper, dissolve 0.125 g in 30 mL of *glacial acetic acid R*. Add 10 mL of *dilute sulfuric acid R*, 1.5 g of *potassium bromide R* and 50.0 mL of *0.0167 M potassium bromate*. Immediately close the flask and allow to stand protected from light for 15 min. Add 1.5 g of *potassium iodide R* immediately after removing the stopper and titrate with *0.1 M sodium thiosulfate*, adding 1 mL of *starch solution R* towards the end of the titration. Carry out a blank titration.

1 mL of *0.0167 M potassium bromate* is equivalent to 4.555 mg of $C_9H_{10}O_4$.

STORAGE

Protected from light.

IMPURITIES

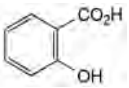


A. 2-hydroxybenzenecarboxylic acid (salicylic acid),

B. ethane-1,2-diol (ethylene glycol).

**01/2018:0336**
**corrected 10.0**

# HYDROXYETHYLCELLULOSE(1)

## Hydroxyethylcellulosum

[9004-62-0]

DEFINITION

Partly *O*-(2-hydroxyethylated) cellulose. Suitable pH-stabilisers, such as phosphates, may be added.

*Content*: 30.0 per cent to 70.0 per cent of hydroxyethoxy ($-OC_2H_4OH$) groups (dried substance).

◆CHARACTERS

*Appearance*: white, yellowish-white or greyish-white, hygroscopic powder or granules.

*Solubility*: soluble in hot and cold water giving a colloidal solution, practically insoluble in acetone, in ethanol (96 per cent) and in toluene.◆

IDENTIFICATION

*First identification: A, B.*

◊*Second identification: B, C, D, E.*◊

A. Infrared absorption spectrophotometry (*2.2.24*). Examine by attenuated total reflectance (ATR).
*Comparison*: *hydroxyethylcellulose CRS*.

B. Heat 10 mL of solution S (see Tests) to boiling. The solution remains clear.

◊C. To 10 mL of solution S add 0.3 mL of *dilute acetic acid R* and 2.5 mL of a 100 g/L solution of *tannic acid R*. A yellowish-white, flocculent precipitate is formed which dissolves in *dilute ammonia R1*.

D. In a test-tube about 160 mm in length, thoroughly mix 1 g with 2 g of finely powdered *manganese sulfate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 200 g/L solution of *diethanolamine R* and 11 volumes of a 50 g/L solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with *1 M hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath and heat at 190-200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.

E. Dissolve 0.2 g completely, without heating, in 15 mL of a 700 g/L solution of *sulfuric acid R*. Pour the solution with stirring into 100 mL of iced *water R* and dilute to 250 mL with iced *water R*. In a test-tube, mix thoroughly while cooling in iced water 1 mL of the solution with 8 mL of *sulfuric acid R*, added dropwise. Heat on a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 mL of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and does not become violet within 100 min.◊

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

TESTS

**Solution S**. Disperse 1.0 g (dried substance) in 50 mL of *carbon dioxide-free water R*. After 10 min, dilute to 100 mL with *carbon dioxide-free water R* and stir until dissolution is complete.

**pH** (*2.2.3*): 5.5 to 8.5 for solution S.

♦**Viscosity** (*2.2.10*): 75 per cent to 140 per cent of the value stated on the label.

While stirring, introduce a quantity of the substance to be examined equivalent to 2.00 g of the dried substance into 50 g of *water R*. Dilute to 100.0 g with *water R* and stir until dissolution is complete. Determine the viscosity using a rotating viscometer at 25 °C and at a shear rate of 100 $s^{-1}$ for substances with an expected viscosity up to 100 mPa·s, at a shear rate of 10 $s^{-1}$ for substances with an expected viscosity between 100 mPa·s and 20 000 mPa·s and at a shear rate of 1 $s^{-1}$ for substances with an expected viscosity above 20 000 mPa·s. If it is impossible to obtain a shear rate of exactly 1 $s^{-1}$, 10 $s^{-1}$ or 100 $s^{-1}$ respectively, use a rate slightly higher and a rate slightly lower and interpolate.♦

**Chlorides** (*2.4.4*): maximum 1.0 per cent.

Dilute 1 mL of solution S to 30 mL with *water R*.

**Nitrates**: maximum 3.0 per cent (dried substance) if hydroxyethylcellulose has a viscosity of 1000 mPa·s or less and maximum 0.2 per cent (dried substance) if hydroxyethylcellulose has a viscosity greater than 1000 mPa·s.

Determine potentiometrically (*2.2.36, Method I*) using as indicator a nitrate-selective electrode and a silver-silver chloride electrode with a 13.2 g/L solution of *ammonium sulfate R* as reference electrolyte.

*Prepare the solutions immediately before use.*

*Buffer solution.* To a mixture of 50 mL of *dilute sulfuric acid R* and 800 mL of *water R*, add 135 g of *potassium dihydrogen phosphate R* and dilute to 1000 mL with *water R*.

*Buffered water.* Dilute 80 mL of the buffer solution to 2000 mL with *water R*.

*Nitrate standard solution (500 ppm $NO_3$).* Dissolve 0.8154 g of *potassium nitrate R* in 500 mL of the buffered water and dilute to 1000.0 mL with the same solvent.

*Test solution.* Dissolve 0.50 g of the substance to be examined in the buffered water and dilute to 100.0 mL with the same solvent.

*Reference solutions.* If hydroxyethylcellulose has a viscosity of 1000 mPa·s or less, dilute 10.0 mL, 20.0 mL and 40.0 mL of the nitrate standard solution (500 ppm $NO_3$) to 100.0 mL with the buffered water and mix.

If hydroxyethylcellulose has a viscosity greater than 1000 mPa·s, dilute 1.0 mL, 2.0 mL and 4.0 mL of the nitrate standard solution (500 ppm $NO_3$) to 100.0 mL with the buffered water and mix.

Carry out the measurements for each solution. Calculate the concentration of nitrates using the calibration curve.

**Aldehydes**: maximum 20 ppm, expressed as glyoxal.

Introduce 1.0 g into a test-tube with a ground-glass stopper and add 10.0 mL of *anhydrous ethanol R*. Stopper the tube and stir mechanically for 30 min. Centrifuge. To 2.0 mL of the supernatant add 5.0 mL of a 4 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* in an 80 per cent *V/V* solution of *glacial acetic acid R*. Shake to homogenise. After 2 h, the solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 2.0 mL of *glyoxal standard solution (2 ppm $C_2H_2O_2$) R* instead of the 2.0 mL of supernatant.

◊**Ethylene oxide**. Head-space gas chromatography (*2.4.25*).

*Test preparation.* Place 1.00 g of the substance to be examined in a 5 mL vial (other sizes may be used depending on the operating conditions) and add 1 mL of *water R*. It swells in water but does not dissolve.

*Reference preparation (a).* Place 1.00 g of the substance to be examined in an identical 5 mL vial. Add 0.1 mL of cooled *ethylene oxide solution R2* and 0.9 mL of *water R*. It swells in water but does not dissolve.

*Reference preparation (b).* To 0.1 mL of *ethylene oxide solution R2* in a 5 mL vial add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R*.

*Close the vials immediately with a butyl rubber membrane stopper, coated with aluminium or polytetrafluoroethylene and secured with an aluminium crimped cap.*

*Limit*:

– *ethylene oxide*: maximum 1 ppm.

**2-Chloroethanol**. Head-space gas chromatography (*2.2.28*).

*Test preparation.* To 50 mg of the substance to be examined in a 10 mL vial (other sizes may be used depending on the operating conditions), add 2 µL of *2-propanol R*. Seal the flask and mix.

*Reference preparation (a).* Dissolve 0.125 g of *2-chloroethanol R* and dilute to 50.0 mL with *2-propanol R*. Dilute 1.0 mL of the solution to 10.0 mL with *2-propanol R*.

*Reference preparation (b).* To 50 mg of the substance to be examined in an identical 10 mL vial, add 2 µL of reference solution (a). Seal the flask and mix.

*Close the vials immediately with a butyl rubber membrane stopper, coated with aluminium or polytetrafluoroethylene and secured with an aluminium crimped cap.*

*Column*:

– *size*: *l* = 50 m, Ø = 0.32 mm,

– *stationary phase*: *methylpolysiloxane R* (1.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 25-35 cm/s.

*Split ratio*: 1:10.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 110 °C,

– *equilibration time*: 20 min,

– *temperature of injection system*: 115 °C.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 60 |
| | 6 - 16 | 60 → 110 |
| | 16 - 31 | 110 → 230 |
| | 31 - 36 | 230 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 mL.

*Retention time*: 2-chloroethanol = about 7.8 min.

*Limit*:

– *2-chloroethanol*: not more than 0.5 times the area of the peak due to 2-chloroethanol in the chromatogram obtained with reference solution (b) (10 ppm).◊

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 4.0 per cent if hydroxyethylcellulose has a viscosity of 1000 mPa·s or less and maximum 1.0 per cent if hydroxyethylcellulose has a viscosity greater than 1000 mPa·s, determined on 1.0 g.

ASSAY

**Gas chromatography** (*2.2.28*).

*Internal standard solution.* To 10 mL of *o-xylene R* add 0.5 mL of *octane R* and dilute to 100.0 mL with *o-xylene R*.

*Test solution.* To 30.0 mg (dried substance) add 60 mg of *adipic acid R* in a 5 mL pressure-tight reaction vial equipped with a pressure-tight membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap or another sealing system providing a sufficient air-tightness. Add 2.00 mL of the internal standard solution and 1.0 mL of *hydriodic acid R* and close immediately. Accurately weigh the vial (total mass before heating). Do not mix the contents of the vial by hand before placing in the oven or the heater. Place the vial in an oven or heat in a suitable heater with continuous mechanical agitation, maintaining an internal temperature of the vial of 165 ± 2 °C for 2.5 h. Allow to cool and weigh accurately the vial (total mass after heating). If the difference between the total mass before heating and the total mass after heating is more than 10 mg, prepare a new test solution. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper layer as test solution.

*Reference solution.* Place 60 mg of *adipic acid R* and 2.00 mL of the internal standard solution in another 5 mL reaction vial, add 1.0 mL of *hydriodic acid R* and close immediately with a septum. Weigh accurately the vial then inject 55 µL of *iodoethane R* through the septum in the vial, weigh again accurately and mix. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper layer as reference solution.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.53 mm;

– *stationary phase*: *methylpolysiloxane R* (3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.2 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 50 |
| | 3 - 8 | 50 → 100 |
| | 8 - 12 | 100 → 250 |
| | 12 - 20 | 250 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to octane (retention time = about 10 min): iodoethane = about 0.6.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to iodoethane and octane;

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the response factor of the principal peak determined on 6 injections.

Calculate the response factor (*R*) using the following expression:

$$\frac{A_1 \times m_1 \times C}{A_2 \times 100}$$

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to iodoethane in the chromatogram obtained with the reference solution;

$m_1$ = mass of *iodoethane R* in the reference solution, in milligrams;

$C$ = percentage content of *iodoethane R*.

Calculate the percentage content *m/m* of the hydroxyethoxy groups using the following expression:

$$\frac{A_4 \times R \times M_1 \times 100}{A_3 \times m_2 \times M_2}$$

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to iodoethane in the chromatogram obtained with the test solution;

$R$ = response factor;

$M_1$ = molar mass of hydroxyethoxy group (61.1);

$M_2$ = molar mass of iodoethane (156.0);

$m_2$ = mass of the sample (dried substance) in the test solution, in milligrams.

♦LABELLING

The label states:

– the viscosity, in millipascal seconds for a 2 per cent *m/m* solution;

– the name and concentration of any added pH-stabiliser. ♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hydroxyethylcellulose used as viscosity-increasing agent.*

**Viscosity** (see Tests).

**Degree of substitution** (see Assay).

**01/2017:1804**

# HYDROXYPROPYLBETADEX

## Hydroxypropylbetadexum

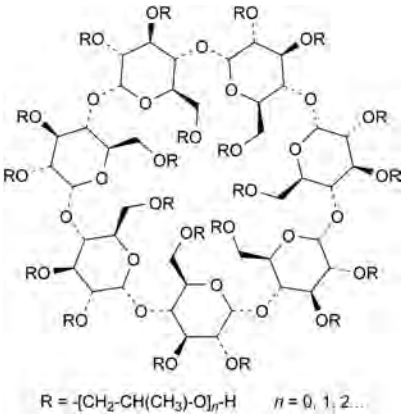


$C_{42}H_{70}O_{35}(C_3H_6O)_x$ with $x = 7$ *MS*

DEFINITION

Hydroxypropylbetadex (β-cyclodextrin, 2-hydroxypropyl ether) is a partially substituted poly(hydroxypropyl) ether of betadex.

*Content*:

– *hydroxypropyl groups per anhydroglucose unit, expressed as molar substitution (MS)*: 0.40 to 1.50 and content within 10 per cent of the value stated on the label.

CHARACTERS

*Appearance*: white or almost white, amorphous or crystalline powder.

*Solubility*: freely soluble in water and in propylene glycol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydroxypropylbetadex CRS*.

*Results*: the spectrum obtained with the substance to be examined shows the same absorption bands as the spectrum obtained with *hydroxypropylbetadex CRS*. Due to differences in the substitution of the substance, the intensity of some absorption bands can vary.

B. Appearance of solution (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*), and remains so after cooling to room temperature.

Dissolve 5.0 g in 10.0 mL of *water R*, with heating.

**Conductivity** (*2.2.38*): maximum 200 µS·cm$^{-1}$.

Measure the conductivity of solution S, while gently stirring with a magnetic stirrer.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.600 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 60.0 mg of *betadex CRS* (impurity A) in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solutions (b), (c), (d), (e), (f)*. Dilute reference solution (a) with *water R* to obtain 5 reference solutions containing respectively 0.03 mg/mL, 0.09 mg/mL, 0.45 mg/mL, 0.90 mg/mL and 1.20 mg/mL of *betadex CRS*.

*Reference solution (g)*. Dissolve 0.15 g of *hydroxypropylbetadex CRS* (containing impurity A) in *water R* and dilute to 10 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *4-nitrophenylcarbamidesilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *water for chromatography R*;

– *mobile phase B*: *water for chromatography R*, *methanol R* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 52 | 48 |
| 5 - 15 | 52 → 0 | 48 → 100 |
| 15 - 20 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria. The use of a 2-port/6-way valve is advisable for 'heart-cutting' hydroxypropylbetadex peaks to save the detector from the huge amount of injected hydroxypropylbetadex:

– *carrier gas*: *nitrogen R*;

– *flow rate*: 1.5 L/min;

– *evaporator temperature*: 70 °C.

*Injection*: 20 µL.

*Retention time*: impurity A = about 4.2 min.

Hydroxypropylbetadex elutes as a very wide peak or as several peaks after impurity A. Other typical impurities elute together as a wide peak or as a group of several peaks before impurity A.

*System suitability*:

– *resolution*: minimum 2.0 between the peak due to impurity A and the 1st peak due to hydroxypropylbetadex in the chromatogram obtained with reference solution (g); if necessary, adjust the column temperature (decreasing the temperature improves the resolution);

– plot a curve representing the logarithm of the concentration of impurity A in reference solutions (b), (c), (d), (e) and (f) as the abscissa and the logarithm of the corresponding peak areas as ordinates taking the assigned content of *betadex CRS* into account; the coefficient of correlation is not less than 0.950.

Calculate the percentage content of impurities with reference to the dried substance using the curve.

*Limits*:

– *impurity A*: maximum 1.5 per cent;

– *sum of impurities other than A*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak eluting after impurity A.

**Impurity B**. Gas chromatography (*2.2.28*).

*Internal standard solution*. To 62.5 mg of *ethylene glycol R*, add *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *ethanol (96 per cent) R*.

*Reference solution.* Dissolve 62.5 mg of *propylene glycol CRS* (impurity B) in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *ethanol (96 per cent) R*. To 1.0 mL of this solution, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *ethanol (96 per cent) R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.4 mL/min.

*Split ratio*: 1:35.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 150 → 200 |
| | 10 - 11 | 200 → 240 |
| Injection port | | 220 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 2 µL; wash the syringe thoroughly with *ethanol (96 per cent) R* to avoid occlusion in the needle.

*Relative retention* with reference to ethylene glycol (retention time = about 7.5 min): impurity B = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 4.0 between the peaks due to impurity B and ethylene glycol;
- *symmetry factor*: maximum 2.0 for the peak due to propylene glycol.

*Calculation of percentage contents*: use the internal standard method.

*Limit*:

- *impurity B*: maximum 2.5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 120 °C for 2 h.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
- TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
- absence of *Escherichia coli* (*2.6.13*);
- absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Nuclear magnetic resonance spectrometry (*2.2.33*).

The molar substitution (*MS*) is calculated from the ratio between the signal from the 3 protons of the methyl group that is part of the hydroxypropyl group and the signal from the proton attached to the C1 carbon (glycosidic proton) of the anhydroglucose units.

*Test solution.* Introduce not less than the equivalent of 10.0 mg of the substance to be examined, previously dried, into a 5 mm NMR tube equipped with a spinner in order to record the spectrum in rotation. Add approximately 0.75 mL of *deuterium oxide R1*. Cap the tube, mix thoroughly and adapt the spinner.

*Apparatus*: FT-NMR spectrometer operating at minimum 250 MHz, suited to record a proton spectrum and to carry out quantitative analysis, at a temperature of at least 25 °C.

*Acquisition of $^1$H NMR spectra.* Use the appropriate instrument settings (frequency, gain, digital resolution, sample rotation, shims, probe tuning, resolution/data point, receiver gain, etc.) so as to obtain a suitable spectrum for quantitative analysis (good FID (Free Induction Decay), no distortion of the spectrum after Fourier transform and phase corrections). The relaxation delay must be adapted to the pulse angle in order to have sufficient relaxation of the protons of interest between 2 pulses (for example: 10 s for a 90° pulse).

Record the FID signal with at least 8 scans so as to obtain a spectral window comprised, at least, between 0 ppm and + 6.2 ppm, referring to the signal of exchangeable protons (solvent) at + 4.8 ppm (25 °C).

Make a zero filling at least 3-fold in size relative to the acquisition data file and transform the FID to the spectrum without any correction of Gaussian broadening factor (GB = 0) and with a line broadening factor not greater than 0.2 Hz (LB ≤ 0.2).

Call the integration sub-routine after phase corrections and baseline correction between + 0.5 ppm and + 6.2 ppm.

Measure the peak areas of the doublet from the methyl groups at + 1.2 ppm ($A_1$), and of the signals of the glycosidic protons between + 5 ppm and + 5.4 ppm ($A_2$).

Calculate the molar substitution (*MS*) using the following expression:

$$\frac{A_1}{(3 \times A_2)}$$

$A_1$ = area of the signal due to the 3 protons of the methyl groups that are part of the hydroxypropyl groups;

$A_2$ = area of the signals due to the glycosidic protons (protons attached to the C1 carbon) of the anhydroglucose units.

The degree of substitution is the number of hydroxypropyl groups per molecule of β-cyclodextrin and is obtained by multiplying the *MS* by 7.

LABELLING

The label states:

- the molar substitution (*MS*);
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

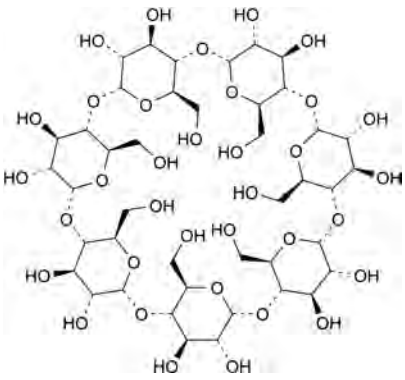

A. cycloheptakis-(1→4)-(α-D-glucopyranosyl) (betadex or cyclomaltoheptaose or β-cyclodextrin),

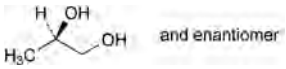


B. (2*RS*)-propane-1,2-diol (propylene glycol).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for hydroxypropylbetadex used as solubility-increasing agent.*

**Degree of substitution** (see Assay).

**01/2017:0337**
**corrected 10.0**

# HYDROXYPROPYLCELLULOSE(2)

## Hydroxypropylcellulosum

[9004-64-2]

DEFINITION

Partly *O*-(2-hydroxypropylated) cellulose.

*Content*: 53.4 per cent to 80.5 per cent of hydroxypropoxy groups (dried substance).

It may contain suitable anticaking agents, such as silica.

♦CHARACTERS

*Appearance*: white or yellowish-white powder or granules, slightly hygroscopic.

*Solubility*: soluble in cold water, in ethanol (96 per cent) and in propylene glycol giving colloidal solutions, practically insoluble in hot water.♦

IDENTIFICATION

A. Dissolve 1 g in *water R* and dilute to 100 mL with the same solvent. Place 1 mL of this solution on a glass plate. After evaporation of the water a thin film is formed.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydroxypropylcellulose CRS*.

Disregard any peak at about 1719 $cm^{-1}$.

◊C. Evenly distribute 1.0 g into 100 mL of boiling *water R*, and stir the mixture using a magnetic stirrer with a bar about 25 mm long. Add 50 mL of this solution to 50 mL of *water R* in a beaker. Insert a thermometer into the solution. Stir the solution using a magnetic stirrer on a hot plate and heat at a rate of 2-5 °C/min. At a temperature above 40 °C the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.◊

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

Evenly distribute 1.0 g into 100 mL of boiling *carbon dioxide-free water R*, and stir the mixture using a magnetic stirrer.

♦**Viscosity** (*2.2.10*): 75 per cent to 140 per cent of the value stated on the label.

While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced water, continue the stirring and allow to remain in the bath of iced water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity with a rotating viscometer at 20 °C and a shear rate of 10 $s^{-1}$.

For a product of low viscosity, use a quantity of the substance to be examined sufficient to prepare a solution of the concentration stated on the label.♦

**Silica**: maximum 0.6 per cent.

If the addition of silica is stated on the label and if more than 0.2 per cent is found in the test for sulfated ash, moisten the residue obtained in the test for sulfated ash with *water R* and add 5 mL of *hydrofluoric acid R* in small portions. Evaporate to dryness at 95-105 °C, taking care to avoid loss from sputtering. Cool and rinse the wall of the platinum crucible with 5 mL of *hydrofluoric acid R*. Add 0.5 mL of *sulfuric acid R* and evaporate to dryness. Progressively increase the temperature until all the acids have been volatised and ignite at 1000 ± 25 °C. Allow to cool in a desiccator and weigh. The difference between the mass of the residue obtained in the test for sulfated ash and the mass of the final residue is equal to the amount of silica in the substance to be examined.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.8 per cent, determined on 1.0 g using a platinum crucible.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* To 10 mL of *o-xylene R* add 1.0 mL of *methylcyclohexane R* and dilute to 50.0 mL with *o-xylene R*.

*Test solution.* To 30.0 mg (dried substance) add 60 mg of *adipic acid R* in a 5.0 mL reaction vial. Add 2.00 mL of the internal standard solution and 1.0 mL of *hydriodic acid R*. Close with a septum and weigh accurately the reaction vial (total mass before heating). Place the vial in an oven or heat in a suitable heater capable of maintaining an internal temperature of 115 ± 2 °C for 70 min with continuous stirring. Allow the vial to cool and weigh accurately the reaction vial (total mass after heating). If the difference of the total mass before heating to the total mass after heating is more than 10 mg, prepare a new solution. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper phase as the test solution.

*Reference solution.* Place 60 mg of *adipic acid R* and 2.00 mL of the internal standard solution in a 5.0 mL reaction vial. Add 1.0 mL of *hydriodic acid R*, close with a septum and weigh accurately. Inject 25 µL of *isopropyl iodide R* through the septum and weigh again accurately. Mix well. After phase separation, pierce through the septum of the vial with a cooled syringe and withdraw a sufficient volume of the upper phase as the reference solution.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter 5.8. *Pharmacopoeial harmonisation.*

Monographs H

– *stationary phase*: *methylpolysiloxane R* (3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 7 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 40 |
| | 3 - 9 | 40 → 100 |
| | 9 - 12 | 100 → 250 |
| | 12 - 15 | 250 |
| Injection port | | 180 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Relative retention* with reference to methylcyclohexane (retention time = about 8 min): isopropyl iodide = about 0.8.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to isopropyl iodide and methylcyclohexane in the chromatogram obtained with the reference solution;

– *repeatability*: maximum relative standard deviation of 2.0 per cent of the response factor of the principal peak determined on 6 injections.

Calculate the response factor $R$ using the following expression:

$$\frac{A_1 \times W_1 \times C}{A_2 \times 100}$$

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with the reference solution;

$A_2$ = area of the peak due to isopropyl iodide in the chromatogram obtained with the reference solution;

$W_1$ = mass of *isopropyl iodide R* in the reference solution, in milligrams;

$C$ = percentage content of *isopropyl iodide R*.

Calculate the percentage content *m/m* of the hydroxypropoxy groups using the following expression:

$$\frac{1.15 \times A_4 \times R \times M_1 \times 100}{A_3 \times W_2 \times M_2}$$

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$A_4$ = area of the peak due to isopropyl iodide in the chromatogram obtained with the test solution;

$R$ = response factor;

$M_1$ = molar mass of hydroxypropoxy group (75.1);

$M_2$ = molar mass of isopropyl iodide (170.0);

$W_2$ = mass of sample (dried substance) in the test solution, in milligrams;

1.15 = conversion factor.

♦LABELLING

The label states:

– the viscosity in millipascal seconds for a 2 per cent *m/m* solution;

– for a product of low viscosity, the concentration of the solution to be used and the viscosity in millipascal seconds;

– where applicable, that the substance contains silica.♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hydroxypropylcellulose used as binder, viscosity-increasing agent or film former:*

**Viscosity** (see Tests).

**Degree of substitution** (see Assay).

*The following characteristics may be relevant for hydroxypropylcellulose used as matrix former in prolonged-release tablets.*

**Viscosity** (see Tests).

**Degree of substitution** (see Assay).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**07/2018:2083**
**corrected 10.0**

# HYDROXYPROPYLCELLULOSE, LOW-SUBSTITUTED(3)

## Hydroxypropylcellulosum substitutum humile

[9004-64-2]

DEFINITION

Low-substituted *O*-(2-hydroxypropylated) cellulose.

*Content*: 5.0 per cent to 16.0 per cent of hydroxypropoxy groups (dried substance).

♦CHARACTERS

*Appearance*: white or yellowish-white, hygroscopic powder or granules.

*Solubility*: practically insoluble in ethanol (96 per cent). It dissolves in a dilute solution of sodium hydroxide producing a viscous solution. It swells in water, in a 106 g/L solution of sodium carbonate and in a 206 g/L solution of *hydrochloric acid R*.♦

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *low-substituted hydroxypropylcellulose CRS*.

B. Shake thoroughly 0.1 g with 10 mL of *water R*. It does not dissolve.

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

C. To the suspension obtained in Identification B add 1 g of *sodium hydroxide R* and shake until it becomes homogeneous. Transfer 5 mL of the solution to a suitable container, add 10 mL of a mixture of 1 volume of *methanol R* and 4 volumes of *acetone R* and shake; a white, flocculent precipitate is formed.

TESTS

**pH** (*2.2.3*): 5.0 to 7.5.

Evenly distribute 1.0 g onto the surface of 100 mL of *carbon dioxide-free water R* and stir using a magnetic stirrer.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): not more than 0.8 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Apparatus*:

- *reaction vial*: a 5 mL pressure-tight vial, 50 mm in height, 20 mm in external diameter and 13 mm in internal diameter at the mouth, equipped with a pressure-tight butyl rubber membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap or another sealing system providing a sufficient air-tightness;
- *heater*: a heating module with a square aluminium block having holes 20 mm in diameter and 32 mm in depth, so that the reaction vials fit; mixing of the contents of the vial is effected using a magnetic stirrer equipped in the heating module or using a reciprocal shaker that performs approximately 100 cycles/min.

*Internal standard solution.* 30 g/L solution of *octane R* in *o-xylene R*.

*Test solution.* Weigh 65.0 mg of the substance to be examined, place in a reaction vial, add 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R*, immediately cap and seal the vial, and weigh accurately. Mix the contents of the vial continuously for 60 min while heating the block so that the temperature of the contents is maintained at 130 ± 2 °C. If a reciprocal shaker or magnetic stirrer cannot be used, shake the vial thoroughly by hand at 5 min intervals during the initial 30 min of the heating time. Allow the vial to cool, and again weigh accurately. If the loss of mass is less than 26 mg and there is no evidence of a leak, use the upper layer of the mixture as the test solution.

*Reference solution.* Place 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R* in another reaction vial, cap and seal the vial, and weigh accurately. Add 15-22 µL of *isopropyl iodide R* through the septum with a syringe, and weigh accurately. Shake the reaction vial thoroughly and use the upper layer as the reference solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (3 µm).

Use a precolumn if needed.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.3 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 50 |
| | 3 - 8 | 50 → 100 |
| | 8 - 12 | 100 → 250 |
| | 12 - 20 | 250 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation or thermal conductivity.

*Injection*: 1-2 µL of the test solution and the reference solution.

*Relative retention* with reference to octane (retention time = about 8 min): isopropyl iodide = about 0.8.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to isopropyl iodide and octane;
- *repeatability*: maximum relative standard deviation of 2.0 per cent for the ratio of the area of the peak due to isopropyl iodide to that due to octane determined on 6 injections.

Calculate the ratio ($Q$) of the area of the peak due to isopropyl iodide to the area of the peak due to the internal standard from the chromatogram obtained with the test solution, and the ratio ($Q_1$) of the area of the peak due to isopropyl iodide to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution.

Calculate the percentage content of hydroxypropoxy groups using the following expression:

$$\frac{Q \times m_1}{Q_1 \times m} \times \frac{M_1}{M_2} \times 100$$

$m_1$ = mass of isopropyl iodide in the reference solution, in milligrams;

$m$ = mass of the sample (dried substance), in milligrams;

$M_1$ = molar mass of hydroxypropoxy group (75.1);

$M_2$ = molar mass of isopropyl iodide (170.0).

STORAGE

In an airtight container.

FUNCTIONALITY-RELATED CHARACTERISTICS

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*The following characteristics may be relevant for low-substituted hydroxypropylcellulose used as disintegrant.*

**Settling volume**: 20.0 mL to 35.0 mL.

To 20 mL of *2-propanol R* in a 100 mL graduated cylinder add 5.0 g of the substance to be examined and shake vigorously. Dilute to 30 mL with *2-propanol R*, then dilute to 50 mL with *water R* and shake vigorously. Within 15 min, repeat the

shaking 3 times. Seal the cylinder to avoid evaporation of the solvent. Allow to stand for 2 h and determine the volume of the settled mass.

**Degree of substitution** (see Assay).

**Particle-size distribution** (*2.9.31*).

**01/2017:0916**
**corrected 9.5**

# HYDROXYZINE HYDROCHLORIDE

## Hydroxyzini hydrochloridum

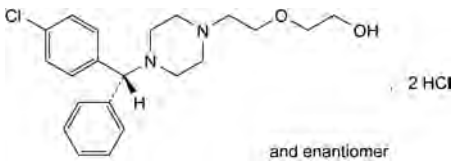


$C_{21}H_{29}Cl_3N_2O_2$ $M_r$ 447.8
[2192-20-3]

DEFINITION

(*RS*)-2-[2-[4-[(4-Chlorophenyl)phenylmethyl]piperazin-1-yl]ethoxy]ethanol dihydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), very slightly soluble in acetone.

mp: about 200 °C, with decomposition.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hydroxyzine hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R, methylene chloride R* (50:50 *V/V*).

*Test solution.* Dissolve 0.50 g of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 0.50 g of *hydroxyzine hydrochloride CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 0.50 g of *meclozine dihydrochloride R* in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 1 mL of the solution to 2 mL with reference solution (a).

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R, ethanol (96 per cent) R, toluene R* (1:24:75 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R2.*

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 15 mL with the same solvent. Add 15 mL of a saturated solution of *picric acid R* in *ethanol (96 per cent) R*. Allow to stand for 15 min. A precipitate is formed. Filter. Recrystallise from *ethanol (96 per cent) R*. Initiate crystallisation, if necessary, by scratching the wall of the tube with a glass rod. The crystals melt (*2.2.14*) at 189 °C to 192 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *hydroxyzine hydrochloride CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 3.0 mL of the test solution to 200.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 25.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 0.5 g of *sodium methanesulfonate R* in a mixture of 14 mL of *triethylamine R*, 300 mL of *acetonitrile for chromatography R* and 686 mL of *water for chromatography R*, then adjust to pH 2.7 with *sulfuric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of hydroxyzine.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak immediately before the peak due to hydroxyzine and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to hydroxyzine.

*Limits*:

– *any impurity*: for each impurity, not more than 1/3 of the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 10 mL of *anhydrous acetic acid R*. Add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.39 mg of $C_{21}H_{29}Cl_3N_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

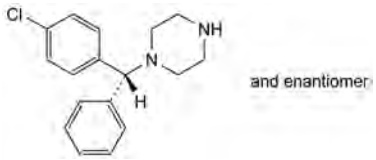


A. (*RS*)-1-[(4-chlorophenyl)phenylmethyl]piperazine,

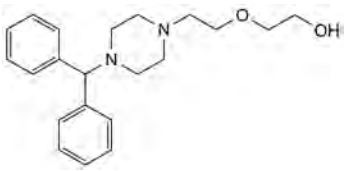


B. 2-[2-[4-(diphenylmethyl)piperazin-1-yl]ethoxy]ethanol (decloxizine).

**01/2017:1786**

# HYMECROMONE

## Hymecromonum

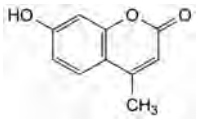


$C_{10}H_8O_3$ $M_r$ 176.2
[90-33-5]

DEFINITION

7-Hydroxy-4-methyl-2*H*-1-benzopyran-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white crystalline powder.

*Solubility*: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride. It dissolves in dilute solutions of ammonia.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hymecromone CRS*.

TESTS

**Absorbance** (*2.2.25*). Dissolve 50 mg in 10 mL of *ammonium chloride buffer solution pH 10.4 R* and dilute to 100.0 mL with *water R*. To 1.0 mL of the solution, add 10 mL of *ammonium chloride buffer solution pH 10.4 R* and dilute to 100.0 mL with *water R*. Examined between 200 nm and 400 nm, the solution shows 2 absorption maxima, at 229 nm and 360 nm, and an absorption minimum at 276 nm. The specific absorbance at the maximum at 360 nm is 1020 to 1120.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. To 280 mL of a 1.56 g/L solution of *sodium dihydrogen phosphate R*, add 720 mL of a 3.58 g/L solution of *disodium hydrogen phosphate dodecahydrate R*. Adjust to pH 7 with a 100 g/L solution of *phosphoric acid R*.

*Test solution*. Dissolve 10 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20 mg of *hymecromone CRS*, 10 mg of *hymecromone impurity A CRS* and 10 mg of *hymecromone impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 200.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm,
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: *methanol R*, buffer solution (465:535 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of hymecromone.

*Relative retention* with reference to hymecromone (retention time = about 6 min): impurity A = about 0.5; impurity B = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum of 2 between the peaks due to impurity A and to impurity B and minimum of 3 between the peaks due to impurity B and to hymecromone.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
– *unspecified impurities*: for each impurity, not more than the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.10 per cent),
– *total*: not more than twice the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.2 per cent),
– *disregard limit*: 0.1 times the area of the peak due to hymecromone in the chromatogram obtained with reference solution (b) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 80 mL of *2-propanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide in 2-propanol* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 17.62 mg of $C_{10}H_8O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

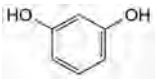

A. benzene-1,3-diol (resorcinol),

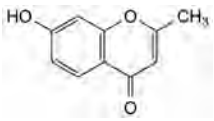

B. 7-hydroxy-2-methyl-4*H*-1-benzopyran-4-one.

**01/2017:2623**

# HYMENOPTERA VENOMS FOR ALLERGEN PRODUCTS

## Hymenopteri venena ad producta allergenica

DEFINITION

Hymenoptera venoms for allergen products are obtained from stinging insects mainly belonging to the families Apidae or Vespidae. They contain soluble substances, including proteins from which they derive their antigenic and allergenic properties, e.g. phospholipase and hyaluronidase.

Hymenoptera venoms for allergen products are unprocessed liquid venom, the venom sac, or venom processed (e.g. by dissolution, filtration and/or drying) using qualified methods.

PRODUCTION

The species of Hymenoptera from which the venom is extracted is identified and specified e.g. by morphological features. The origin, quality and traceability of the source material must be demonstrated. The methods of insect collection and venom extraction are described and must ensure that the venom is of appropriate quality. Electrostimulation may be used to collect the venom.

Pesticides and/or chemical attractants are not used in the collection of insects.

Where major changes to the production of the Hymenoptera venom source material take place (e.g. when a new process or supplier of Hymenoptera venom source material is introduced), such changes are qualified for each Hymenoptera venom source material.

Microbial contamination of the Hymenoptera venom source material may be unavoidable and should be monitored on a representative number of batches of venom source material according to a justified sampling plan and each time a new supplier and/or a new process for the venom source material production is introduced; if a determination of microbial contamination is not applicable (e.g. when the source material is bactericidal), this must be justified. Microbial contamination values and potential increases in microbial contamination are monitored during stability studies, in order to assess this aspect along with the venom source material characteristics upon storage.

Control methods and acceptance criteria relating to identity and purity (e.g. absence of foreign matter) of the Hymenoptera venom source material are established. The acceptance criteria must ensure the consistency of the venom source material from a qualitative and quantitative point of view. The venom source material is stored under controlled conditions justified by stability data. The collection and production, as well as the handling of the venom source material, are such that consistent composition is ensured from batch to batch.

*HYMENOPTERA VENOMS FOR ALLERGEN PRODUCTS REFERENCE BATCH*

An appropriate reference batch is established for each Hymenoptera venom source material. The nature of the reference batch depends on the testing approach to verify batch-to-batch consistency and to establish acceptable quality. The reference batch may be, for example, an internal reference preparation (if available), a venom or venom sac source material, or a sample of a production batch. Its characterisation must be described. The extent of characterisation of the reference batch depends on the nature of the venom source material. The reference batch is stored under controlled conditions ensuring its stability.

*BATCH-TO-BATCH CONSISTENCY*

To establish batch-to-batch consistency, one or more of the following tests are performed on each batch. The choice of tests and the production step at which they are carried out must be justified.

**Total protein** (*2.5.33*).

**Protein profile**. Determined by using suitable electrophoresis methods (*2.2.31*, *2.2.54*).

**Enzyme activity** (e.g. hyaluronidase or phospholipase activity, as appropriate). Determined by using a suitable assay.

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific antibodies.

**Major allergen content**. Determined by using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

**Total allergenic activity**. Determined by testing inhibition of the binding capacity of specific immunoglobulin E antibodies or by a suitable equivalent *in vitro* method.

CHARACTERS

Hymenoptera venoms for allergen products are colourless or yellowish sacs or liquid, or white to yellowish powders.

IDENTIFICATION

Where possible, tests on identity are performed on each individual batch of venom using adequate methods, e.g. polyacrylamide gel electrophoresis analysis (*2.2.31*), liquid chromatography (*2.2.29*) and/or enzyme activity.

TESTS

**Foreign matter**. Foreign matter is defined as any particles that are not part of the venom or venom sac. Foreign matter is detected using appropriate methods (e.g. microscopic methods); a limit for the amount of foreign matter has to be established based on historical data.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*). The water content of dried material is determined; specification limits must be supported by batch analysis and stability data.

STORAGE

The Hymenoptera venom source material is stored under controlled conditions justified by stability data.

LABELLING

The label states the species of the source insect(s).

**01/2008:2167**

# HYOSCINE

## Hyoscinum

## Scopolaminum

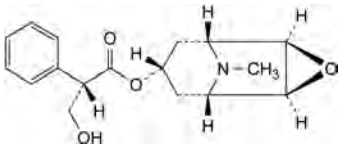

$C_{17}H_{21}NO_4$ $M_r$ 303.4
[51-34-3]

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*)-9-Methyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent).

mp: 66 °C to 70 °C.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *hyoscine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 33 to − 39 (anhydrous substance).

Dissolve 1.00 g in *dilute hydrochloric acid R* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *hyoscine impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 5.0 mL of reference solution (b) to 25.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (d)*. Mix 2.0 mL of reference solution (b) and 1.0 mL of the test solution and dilute to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: mix 33 volumes of *acetonitrile R* and 67 volumes of a 2.5 g/L solution of *sodium dodecyl sulfate R* previously adjusted to pH 2.5 with a 346 g/L solution of *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 3 times the retention time of hyoscine.

*Relative retention* with reference to hyoscine (retention time = about 5 min): impurity C = about 0.2; impurity A = about 0.9; impurity D = about 1.3; impurity B = about 2.5.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to impurity A and hyoscine.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.6; impurity C = 0.3;
- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurities B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.34 mg of $C_{17}H_{21}NO_4$.

IMPURITIES

*Specified impurities: A, B, C, D.*

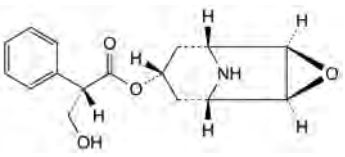

A. (1*R*,2*R*,4*S*,5*S*,7*s*)-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (norhyoscine),

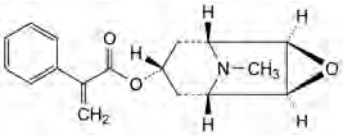

B. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]-non-7-yl 2-phenylprop-2-enoate (apohyoscine),

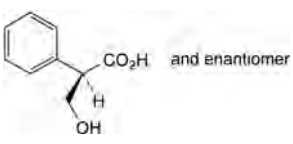


C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

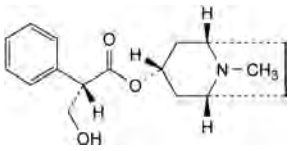

D. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscyamine).

**07/2018:0737**
**corrected 9.6**

# HYOSCINE BUTYLBROMIDE

## Hyoscini butylbromidum

## Scopolamini butylbromidum

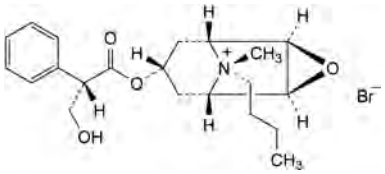

$C_{21}H_{30}BrNO_4$ $M_r$ 440.4
[149-64-4]

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*,9*r*)-9-Butyl-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]nonan-9-ium bromide.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methylene chloride, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A, C, F.*

*Second identification: A, B, D, E, F.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 139 °C to 141 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *hyoscine butylbromide CRS*.

D. To about 1 mg add 0.2 mL of *nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 mL of *acetone R* and add 0.1 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. To 5 mL of solution S (see Tests) add 2 mL of *dilute sodium hydroxide solution R*. No precipitate is formed.

F. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.25 mL of *0.01 M sodium hydroxide* and 0.2 mL of *methyl red mixed solution R*. The solution is green. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to reddish-violet.

**Specific optical rotation** (*2.2.7*): – 20 to – 18 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in mobile phase B and dilute to 10.0 mL with mobile phase B.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (b)*. Dissolve 5 mg of *hyoscine butylbromide for system suitability CRS* (containing impurities A and B) in mobile phase B and dilute to 10.0 mL with mobile phase B.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases R* (1.8 µm);
- *temperature*: 50 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, 0.2 per cent *V/V* solution of *perchloric acid R* (5:95 *V/V*);
- *mobile phase B*: 0.2 per cent *V/V* solution of *perchloric acid R*, *acetonitrile R1* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 91 | 9 |
| 1 - 4.2 | 91 → 75 | 9 → 25 |
| 4.2 - 5.5 | 75 → 66 | 25 → 34 |
| 5.5 - 10 | 66 → 15 | 34 → 85 |
| 10 - 11 | 15 | 85 |

*Flow rate*: 2.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 2 µL.

*Identification of impurities*: use the chromatogram supplied with *hyoscine butylbromide for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to hyoscine butylbromide (retention time = about 6 min): bromide = about 0.1; impurity B = about 0.28; impurity A = about 0.37.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurities B and A.

*Calculation of percentage contents*:
- for each impurity, use the concentration of hyoscine butylbromide in reference solution (a).

*Limits*:
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.4 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to bromide.

**Loss on drying** (*2.2.32*): maximum 2.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.400 g in 50 mL of *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*) using a silver indicator electrode and a silver-silver chloride reference electrode.

1 mL of *0.1 M silver nitrate* is equivalent to 44.04 mg of $C_{21}H_{30}BrNO_4$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H.*

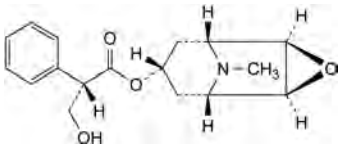

A. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]-nonan-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscine),

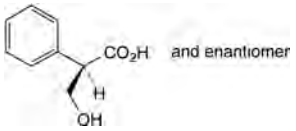


B. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

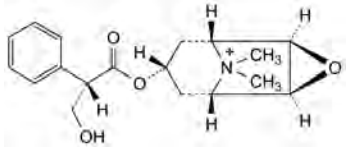

C. (1*R*,2*R*,4*S*,5*S*,7s)-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]-oxy]-9,9-dimethyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]nonan-9-ium (methylhyoscine),

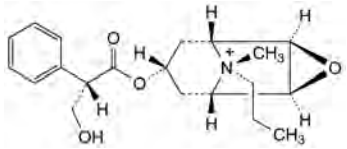

D. (1*R*,2*R*,4*S*,5*S*,7*s*,9*r*)-7-[[(2*S*)-3-hydroxy-2-phenyl-propanoyl]oxy]-9-methyl-9-propyl-3-oxa-9-azatricyclo-[3.3.1.0$^{2,4}$]nonan-9-ium (propylhyoscine),

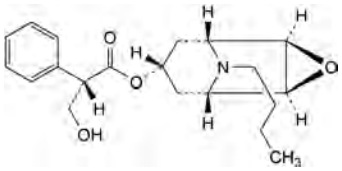

E. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-butyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]-nonan-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (*N*-butylnorhyoscine),

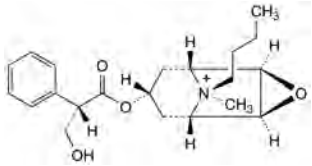

F. (1*R*,2*R*,4*S*,5*S*,7*s*,9*s*)-9-butyl-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]nonan-9-ium,

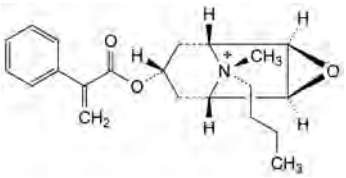

G. (1*R*,2*R*,4*S*,5*S*,7*s*,9*r*)-9-butyl-9-methyl-7-[(2-phenylprop-2-enoyl)oxy]-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]nonan-9-ium (apo-*N*-butylhyoscine),

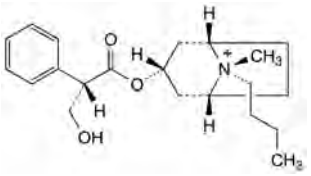

H. (1*R*,3*r*,5*S*,8*s*)-8-butyl-3-[[(2*S*)-3-hydroxy-2-phenyl-propanoyl]oxy]-8-methyl-8-azabicyclo[3.2.1]octan-8-ium (*N*-butylhyoscyamine).

**01/2008:0106**

# HYOSCINE HYDROBROMIDE

## Hyoscini hydrobromidum

## Scopolamini hydrobromidum

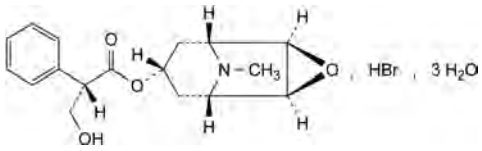

$C_{17}H_{22}BrNO_4,3H_2O$ $M_r$ 438.3

[6533-68-2]

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*)-9-Methyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate hydrobromide trihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, efflorescent.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hyoscine hydrobromide CRS.*

If the spectra obtained in the solid state show differences, proceed as follows: dissolve 3 mg of the substance to be examined in 1 mL of *ethanol (96 per cent) R* and evaporate to dryness on a water-bath; dissolve the residue in 0.5 mL of *methylene chloride R* and add 0.2 g of *potassium bromide R* and 15 mL of *ether R*; allow to stand for 5 min shaking frequently; decant; dry the residue on a water-bath until the solvents have evaporated; using the residue prepare a disc and dry at 100-105 °C for 3 h. Repeat the procedure with *hyoscine hydrobromide CRS* and record the spectra.

C. Dissolve about 50 mg in 5 mL of *water R* and add 5 mL of *picric acid solution R* dropwise and with shaking. The precipitate, washed with *water R* and dried at 100-105 °C for 2 h, melts (*2.2.14*) at 188 °C to 193 °C.

D. To about 1 mg add 0.2 mL of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 mL of *acetone R* and add 0.1 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): − 24 to − 27 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 70.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *hyoscine hydrobromide impurity B CRS* in the mobile phase, add 5.0 mL of the test solution and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm,

– *stationary phase*: *octylsilyl silica gel for chromatography R* (3 µm),

– *temperature*: 25 ± 1 °C.

*Mobile phase*: mix 330 mL of *acetonitrile R* with 670 mL of a 2.5 g/L solution of *sodium dodecyl sulfate R* previously adjusted to pH 2.5 with a 345 g/L solution of *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 3 times the retention time of hyoscine.

*Relative retention* with reference to hyoscine (retention time = about 5.0 min): impurity D = about 0.2; impurity B = about 0.9; impurity A = about 1.3; impurity C = about 2.4.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity B and hyoscine,

– *symmetry factor*: maximum 2.5 for the peak due to hyoscine.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.3; impurity C = 0.6;

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *impurities A, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *total*: not more than 1.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent); disregard any peak due to the bromide ion which appears close to the solvent peak;

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 10.0 per cent to 13.0 per cent, determined on 0.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide* free from carbonate. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 38.43 mg of $C_{17}H_{22}BrNO_4$.

STORAGE

In a well-filled, airtight container of small capacity, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

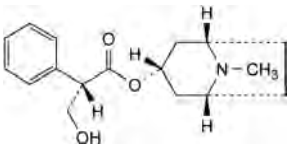


A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscyamine),

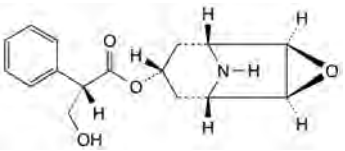


B. (1*R*,2*R*,4*S*,5*S*,7*s*)-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (norhyoscine),

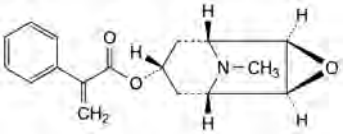


C. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]-non-7-yl 2-phenylprop-2-enoate (apohyoscine),

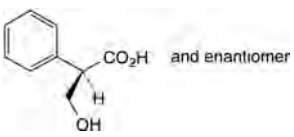


D. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid).

**01/2008:0501**
**corrected 10.0**

# HYOSCYAMINE SULFATE

## Hyoscyamini sulfas

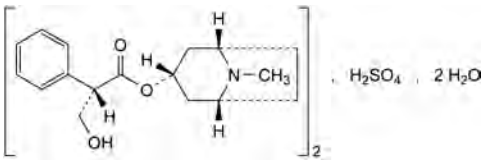

$C_{34}H_{48}N_2O_{10}S,2H_2O$ $M_r$ 713
[6835-16-1]

DEFINITION

Bis[(1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[*3.2.1*]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate] sulfate dihydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless needles.

*Solubility*: very soluble in water, sparingly soluble or soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *hyoscyamine sulfate CRS*.

C. To 0.5 mL of solution S (see Tests) add 2 mL of *dilute acetic acid R* and heat. To the hot solution add 4 mL of *picric acid solution R*. Allow to cool, shaking occasionally. Collect the crystals, wash with 2 quantities, each of 3 mL, of iced *water R* and dry at 100-105 °C. The crystals melt (*2.2.14*) at 164 °C to 168 °C.

D. To about 1 mg add 0.2 mL of *fuming nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 mL of *acetone R* and add 0.2 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.2.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 29 to – 24 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 60.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 10.0 mL of the solution to 50.0 mL with mobile phase A.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 25.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 5 mg of *hyoscyamine impurity E CRS* in the test solution and dilute to 20 mL with the test solution. Dilute 5 mL of this solution to 25 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 25 ± 1 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 3.5 g of *sodium dodecyl sulfate R* in 606 mL of a 7.0 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.3 with a 0.58 g/L solution of *phosphoric acid R* and mix with 320 mL of *acetonitrile R1*;
– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.0 | 95 | 5 |
| 2.0 - 20.0 | 95 → 70 | 5 → 30 |
| 20.0 - 20.1 | 70 → 95 | 30 → 5 |
| 20.1 - 25.0 | 95 | 5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Relative retention* with reference to hyoscyamine (retention time = about 10.5 min): impurity A = about 0.2; impurity B = about 0.67; impurity C = about 0.72; impurity D = about 0.8; impurity E = about 0.9; impurity F = about 1.1; impurity G = about 1.8.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to hyoscyamine and impurity E;
– *symmetry factor*: maximum 2.5 for the peak due to hyoscyamine.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.3; impurity G = 0.6;
– *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *impurities A, B, C, D, F, G*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 2.0 per cent to 5.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 67.7 mg of $C_{34}H_{48}N_2O_{10}S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

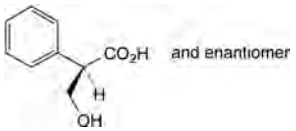


A. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

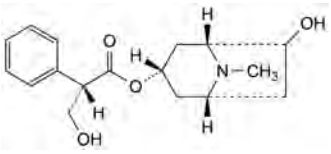


B. (1*R*,3*S*,5*R*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo-[*3.2.1*]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (7-hydroxyhyoscyamine),

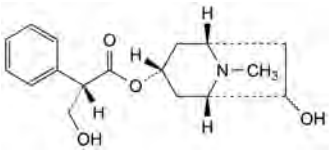


C. (1*S*,3*R*,5*S*,6*RS*)-6-hydroxy-8-methyl-8-azabicyclo-[*3.2.1*]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (6-hydroxyhyoscyamine),

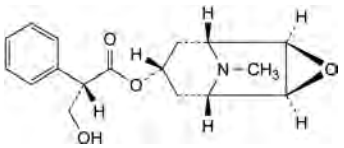


D. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-methyl-3-oxa-9-azatricyclo-[3.3.1.0$^{2,4}$]non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (hyoscine),

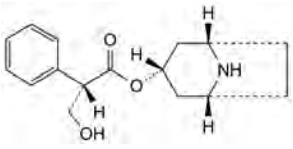


E. (1*R*,3*r*,5*S*)-8-azabicyclo[*3.2.1*]oct-3-yl (2*S*)-3-hydroxy-2-phenylpropanoate (norhyoscyamine),

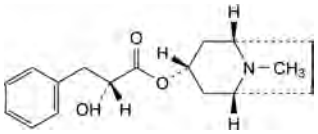


F. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[*3.2.1*]oct-3-yl (2*R*)-2-hydroxy-3-phenylpropanoate (littorine),

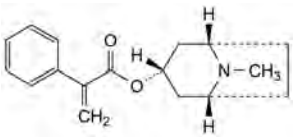


G. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[*3.2.1*]oct-3-yl 2-phenylprop-2-enoate (apoatropine).

**01/2019:0348**
**corrected 10.0**

# HYPROMELLOSE(4)

## Hypromellosum

[9004-65-3]

DEFINITION

Hydroxypropylmethylcellulose. Cellulose, 2-hydroxypropyl-methyl ether.

Partly *O*-methylated and *O*-(2-hydroxypropylated) cellulose.

*Content*: methoxy (-$OCH_3$; $M_r$ 31.03) and hydroxypropoxy (-$OC_3H_6OH$; $M_r$ 75.09) groups (dried substance) conforming to the types of hypromellose set forth in the accompanying table.

| Substitution type | Methoxy (per cent) | Hydroxypropoxy (per cent) |
|---|---|---|
| 1828 | 16.5 to 20.0 | 23.0 to 32.0 |
| 2208 | 19.0 to 24.0 | 4.0 to 12.0 |
| 2906 | 27.0 to 30.0 | 4.0 to 7.5 |
| 2910 | 28.0 to 30.0 | 7.0 to 12.0 |

♦CHARACTERS

*Appearance*: white, yellowish-white or greyish-white powder or granules, hygroscopic after drying.

*Solubility*: practically insoluble in hot water, in acetone, in anhydrous ethanol and in toluene. It dissolves in cold water giving a colloidal solution.♦

IDENTIFICATION

A. Evenly distribute 1.0 g onto the surface of 100 mL of *water R* in a beaker, tapping the top of the beaker gently if necessary to ensure a uniform layer on the surface. Allow to stand for 1-2 min: the powdered material aggregates on the surface.

B. Evenly distribute 1.0 g into 100 mL of boiling *water R*, and stir the mixture using a magnetic stirrer with a bar 25 mm long: a slurry is formed and the particles do not dissolve. Allow the slurry to cool to 10 °C and stir using a magnetic stirrer: a clear or slightly turbid solution occurs with its thickness dependent on the viscosity grade.

C. To 0.1 mL of the solution obtained in identification test B add 9 mL of a 90 per cent *V/V* solution of *sulfuric acid R*, shake, heat on a water-bath for exactly 3 min, immediately cool in an ice-bath, carefully add 0.6 mL of a 20 g/L solution of *ninhydrin R*, shake and allow to stand at 25 °C: a red colour develops at first and changes to purple within 100 min.

D. Place 2-3 mL of the solution obtained in identification test B onto a glass slide as a thin film and allow the water to evaporate: a coherent, clear film forms on the glass slide.

(4) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

E. Add 50.0 mL of the solution obtained in identification test B to 50.0 mL of *water R* in a beaker. Insert a thermometer into the solution. Stir the solution on a magnetic stirrer/hot plate and begin heating, increasing the temperature at a rate of 2-5 °C per minute. Determine the temperature at which a turbidity increase begins to occur and designate the temperature as the flocculation temperature: the flocculation temperature is higher than 50 °C.

TESTS

◊**Appearance of solution**. The solution is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete.◊

**pH** (*2.2.3*): 5.0 to 8.0 for the solution prepared as described under Viscosity.

Read the indicated pH value after the probe has been immersed for 5 ± 0.5 min.

**Viscosity**: 80 per cent to 120 per cent of the nominal value for samples with a viscosity less than 600 mPa·s (Method 1); 75 per cent to 140 per cent of the nominal value for samples with a viscosity of 600 mPa·s or higher (Method 2).

*Method 1, to be applied to samples with a viscosity of less than 600 mPa·s.* Weigh a quantity of the substance to be examined equivalent to 4.000 g of the dried substance. Transfer into a wide-mouthed bottle, and adjust the total mass of the sample and the water to 200.0 g with hot *water R* (90-99 °C). Capping the bottle, stir by mechanical means at 400 ± 50 r/min for 10-20 min until the particles are thoroughly dispersed and wetted. Scrape down the insides of the bottle with a spatula if necessary, to ensure that there is no undissolved material on the sides of the bottle, and continue the stirring in a cooling water-bath maintained at a temperature below 10 °C for another 20-40 min. Adjust the solution mass if necessary to 200.0 g using cold *water R*. Centrifuge the solution if necessary to expel any entrapped air bubbles. Using a spatula, remove any foam. Determine the kinematic viscosity ($\nu$) of this solution using the capillary viscometer method (*2.2.9*). Separately determine the density ($\rho$) (*2.2.5*) of the solution and calculate the dynamic viscosity ($\eta$), as $\eta = \rho\nu$.

*Method 2, to be applied to samples with a viscosity of 600 mPa·s or higher.* Weigh a quantity of the substance to be examined equivalent to 10.00 g of the dried substance. Transfer into a wide-mouthed bottle, and adjust the total mass of the sample and the water to 500.0 g with hot *water R* (90-99 °C). Capping the bottle, stir by mechanical means at 400 ± 50 r/min for 10-20 min until the particles are thoroughly dispersed and wetted. Scrape down the insides of the bottle with a spatula if necessary, to ensure that there is no undissolved material on the sides of the bottle, and continue the stirring in a cooling water-bath maintained at a temperature below 10 °C for another 20-40 min. Adjust the solution mass if necessary to 500.0 g using cold *water R*. Centrifuge the solution if necessary to expel any entrapped air bubbles. Using a spatula, remove any foam. Determine the viscosity (*2.2.10*) of this solution at 20 ± 0.1 °C using a rotating viscometer.

*Apparatus*: single-cylinder type spindle viscometer.

*Rotor number, revolution and calculation multiplier*: apply the conditions specified in Table 0348.-1.

Table 0348.-1.

| Nominal viscosity* (mPa·s) | Rotor number | Revolution (r/min) | Calculation multiplier |
|---|---|---|---|
| 600 to less than 1400 | 3 | 60 | 20 |
| 1400 to less than 3500 | 3 | 12 | 100 |
| 3500 to less than 9500 | 4 | 60 | 100 |
| 9500 to less than 99 500 | 4 | 6 | 1000 |
| 99 500 or more | 4 | 3 | 2000 |

* the nominal viscosity is based on the manufacturer's specifications.

Allow the spindle to rotate for 2 min before taking the measurement. Allow a rest period of at least 2 min between subsequent measurements. Repeat the measurement twice and determine the mean of the 3 readings.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Apparatus*:

- *reaction vial*: a 5 mL pressure-tight vial, 50 mm in height, 20 mm in external diameter and 13 mm in internal diameter at the mouth, equipped with a pressure-tight butyl rubber membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap or another sealing system providing a sufficient air-tightness;
- *heater*: a heating module with a square aluminium block having holes 20 mm in diameter and 32 mm in depth, so that the reaction vials fit; mixing of the contents of the vial is effected using a magnetic stirrer equipped in the heating module or using a reciprocal shaker that performs approximately 100 cycles/min.

*Internal standard solution*: 30 g/L solution of *octane R* in *o-xylene R*.

*Test solution*. Weigh 65.0 mg of the substance to be examined, place in a reaction vial, add 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R*, immediately cap and seal the vial, and weigh accurately. Mix the contents of the vial continuously for 60 min while heating the block so that the temperature of the contents is maintained at 130 ± 2 °C. If a reciprocal shaker or magnetic stirrer cannot be used, shake the vial thoroughly by hand at 5 min intervals during the initial 30 min of the heating time. Allow the vial to cool, and again weigh accurately. If the loss of mass is less than 26 mg and there is no evidence of a leak, use the upper layer of the mixture as the test solution.

*Reference solution*. Place 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R* in another reaction vial, cap and seal the vial, and weigh accurately. Add 15-22 µL of *isopropyl iodide R* through the septum with a syringe, weigh accurately, add 45 µL of *methyl iodide R* in the same manner, and weigh accurately. Shake the reaction vial thoroughly and use the upper layer as the reference solution.

Use a precolumn if needed.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.3 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 50 |
| | 3 - 8 | 50 → 100 |
| | 8 - 12.3 | 100 → 250 |
| | 12.3 - 20.3 | 250 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation or thermal conductivity.

*Injection*: 1-2 µL.

*Relative retention* with reference to octane (retention time = about 10 min): methyl iodide = about 0.4; isopropyl iodide = about 0.7.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to methyl iodide and isopropyl iodide and between the peaks due to isopropyl iodide and octane;
- *repeatability*: maximum relative standard deviation of 2.0 per cent for the ratios of the areas of the peaks respectively due to methyl iodide and isopropyl iodide to the area of the peak due to octane, determined on 6 injections.

Calculate the ratios ($Q_1$ and $Q_2$) of the areas of the peaks due to methyl iodide and isopropyl iodide to the area of the peak due to the internal standard in the chromatogram obtained with the test solution, and the ratios ($Q_3$ and $Q_4$) of the areas of the peaks due to methyl iodide and isopropyl iodide to the area of the peak due to the internal standard in the chromatogram obtained with the reference solution.

Calculate the percentage content of methoxy groups using the following expression:

$$\frac{Q_1}{Q_3} \times \frac{m_1}{m} \times 21.864$$

Calculate the percentage content of hydroxypropoxy groups using the following expression:

$$\frac{Q_2}{Q_4} \times \frac{m_2}{m} \times 44.17$$

$m_1$ = mass of methyl iodide in the reference solution, in milligrams;

$m_2$ = mass of isopropyl iodide in the reference solution, in milligrams;

$m$ = mass of the sample (dried substance), in milligrams.

LABELLING

The label states:

- the nominal viscosity in millipascal seconds (mPa·s);
- the substitution type.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hypromellose used as binder, viscosity-increasing agent or film former.*

**Viscosity**: see Tests.

**Degree of substitution**: see Assay.

*The following characteristics may be relevant for hypromellose used as matrix former in prolonged-release tablets.*

**Viscosity**: see Tests.

**Degree of substitution**: see Assay.

**Molecular mass distribution** (*2.2.30*).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2017:0347**

# HYPROMELLOSE PHTHALATE

## Hypromellosi phthalas

DEFINITION

Hydroxypropylmethylcellulose phthalate.

Monophthalic acid ester of hypromellose, containing methoxy (-$OCH_3$), 2-hydroxypropoxy (-$OCH_2CHOHCH_3$) and phthaloyl (*o*-carboxybenzoyl $C_8H_5O_3$) groups.

CHARACTERS

*Appearance*: white or almost white, free-flowing flakes or granular powder.

*Solubility*: practically insoluble in water, soluble in a mixture of equal volumes of acetone and methanol and in a mixture of equal volumes of methanol and methylene chloride, very slightly soluble in acetone and in toluene, practically insoluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 40 mg in 1 mL of a mixture of equal volumes of *methanol R* and *methylene chloride R*; spread 2 drops of this solution between 2 sodium chloride plates, then remove one of the plates to evaporate the solvent.

*Comparison*: *hypromellose phthalate CRS*.

TESTS

**Free phthalic acid**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in about 50 mL of *acetonitrile R* with the aid of ultrasound. Add 10 mL of *water R*, cool to room temperature, dilute to 100.0 mL with *acetonitrile R* and mix.

*Reference solution.* Dissolve 12.5 mg of *phthalic acid R* in 125 mL of *acetonitrile R*. Add 25 mL of *water R*, dilute to 250.0 mL with *acetonitrile R* and mix.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: *acetonitrile R*, 1 g/L solution of *trifluoroacetic acid R* (1:9 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 2 injections.

*Limit*:

– *phthalic acid*: not more than 0.4 times the area of the corresponding peak in the chromatogram obtained with the reference solution (1.0 per cent).

**Chlorides**: maximum 0.07 per cent.

Dissolve 1.0 g in 40 mL of *0.2 M sodium hydroxide*, add 0.05 mL of *phenolphthalein solution R* and add *dilute nitric acid R* dropwise, with stirring, until the red colour disappears. Add an additional 20 mL of *dilute nitric acid R* with stirring. Heat on a water-bath with stirring until the gel-like precipitate formed becomes granular. Cool and centrifuge. Separate the liquid phase and wash the residue with 3 quantities, each of 20 mL, of *water R*, separating the washings by centrifugation. Combine the liquid phases, dilute to 200 mL with *water R*, mix and filter. To 50 mL of this solution, add 1 mL of *0.1 M silver nitrate*. The solution is not more opalescent than a standard prepared by mixing 0.5 mL of *0.01 M hydrochloric acid* with 10 mL of *0.2 M sodium hydroxide*, adding 7 mL of *dilute nitric acid R* and 1 mL of *0.1 M silver nitrate*, and diluting to 50 mL with *water R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for hypromellose phthalate used as a gastro-resistant coating agent.*

**Apparent viscosity** (*2.2.9*): 80 per cent to 120 per cent of the nominal value.

Dissolve 10 g, previously dried at 105 °C for 1 h, in 90 g of a mixture of equal masses of *methanol R* and *methylene chloride R* by mixing and shaking.

**Solubility**. 0.2 g does not dissolve in *0.1 M hydrochloric acid* but dissolves quickly and completely in 100 mL of *phosphate buffer solution pH 6.8 R* with stirring.

**Phthaloyl groups**: typically 21.0 per cent to 35.0 per cent (anhydrous substance).

Dissolve 1.000 g in 50 mL of a mixture of 1 volume of *water R*, 2 volumes of *acetone R* and 2 volumes of *ethanol (96 per cent) R*. Add 0.1 mL of *phenolphthalein solution R* and titrate with *0.1 M sodium hydroxide* until a faint pink colour is obtained. Carry out a blank titration.

Calculate the percentage content of phthaloyl groups using the following expression:

$$\frac{149n}{(100 - a)m} - 1.795S$$

$a$ = percentage content of water;

$m$ = mass of the substance to be examined, in grams;

$n$ = volume of *0.1 M sodium hydroxide* used, in millilitres;

$S$ = percentage content of free phthalic acid (see Tests).

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**01/2017:0721**
**corrected 9.6**

# IBUPROFEN

## Ibuprofenum

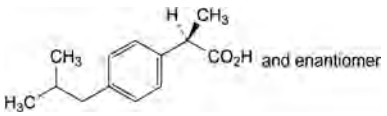


$C_{13}H_{18}O_2$ $M_r$ 206.3
[15687-27-1]

DEFINITION

(2*RS*)-2-[4-(2-Methylpropyl)phenyl]propanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, freely soluble in acetone, in methanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *A, B, D.*

A. Melting point (*2.2.14*): 75 °C to 78 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in a 4 g/L solution of *sodium hydroxide R* and dilute to 100.0 mL with the same alkaline solution.

*Spectral range*: 240-300 nm, using a spectrophotometer with a band width of 1.0 nm and a scan speed of not more than 50 nm/min.

*Absorption maxima*: at 264 nm and 272 nm.

*Shoulder*: at 258 nm.

*Absorbance ratio*:

- $A_{264} / A_{258}$ = 1.20 to 1.30;
- $A_{272} / A_{258}$ = 1.00 to 1.10.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ibuprofen CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *ibuprofen CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *anhydrous acetic acid R*, *ethyl acetate R*, *hexane R* (5:24:71 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: at 120 °C for 30 min.

*Detection*: lightly spray with a 10 g/L solution of *potassium permanganate R* in *dilute sulfuric acid R* and heat at 120 °C for 20 min; examine in ultraviolet light at 365 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.05° to + 0.05°.

Dissolve 0.50 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 2 mL of *acetonitrile R* and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of *ibuprofen impurity B CRS* to 10.0 mL with *acetonitrile R* (solution A). Dissolve 20 mg of *ibuprofen CRS* in 2 mL of *acetonitrile R*, add 1.0 mL of solution A and dilute to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve the contents of a vial of *ibuprofen for peak identification CRS* (mixture of impurities A, J and N) in 1 mL of *acetonitrile R* and dilute to 5 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 0.5 volumes of *phosphoric acid R*, 340 volumes of *acetonitrile R1* and 600 volumes of *water for chromatography R*; allow to equilibrate and dilute to 1000 volumes with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 55 | 100 → 15 | 0 → 85 |
| 55 - 70 | 15 | 85 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *ibuprofen for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, J and N.

*Relative retention* with reference to ibuprofen (retention time = about 21 min): impurity J = about 0.2; impurity N = about 0.3; impurity A = about 0.9; impurity B = about 1.1.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity B, and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ibuprofen. If necessary, adjust the concentration of acetonitrile in mobile phase A.

*Limits*:

- *impurities A, J, N*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Impurity F**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Methylating solution*. Dilute 1 mL of *N,N-dimethylformamide dimethylacetal R* and 1 mL of *pyridine R* to 10 mL with *ethyl acetate R*.

*Test solution*. Weigh about 50.0 mg of the substance to be examined into a sealable vial, dissolve in 1.0 mL of *ethyl acetate R*, add 1 mL of the methylating solution, seal and heat at 100 °C in a block heater for 20 min. Allow to cool. Remove the reagents under a stream of nitrogen at room temperature. Dissolve the residue in 5 mL of *ethyl acetate R*.

*Reference solution (a)*. Dissolve 0.5 mg of *ibuprofen impurity F CRS* in *ethyl acetate R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Weigh about 50.0 mg of *ibuprofen CRS* into a sealable vial, dissolve in 1.0 mL of reference solution (a), add 1 mL of the methylating solution, seal and heat at 100 °C in a block heater for 20 min. Allow to cool. Remove the reagents under a stream of nitrogen at room temperature. Dissolve the residue in 5 mL of *ethyl acetate R*.

*Column*:
– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.53 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 5.0 mL/min.

*Temperature*:
– *column*: 150 °C;
– *injection port*: 200 °C;
– *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solution (b).

*Run time*: twice the retention time of ibuprofen.

*System suitability*:
– *relative retention* with reference to ibuprofen (retention time = about 17 min): impurity F = about 1.5.

*Limit*:
– *impurity F*: maximum 0.1 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.450 g in 50 mL of *methanol R*. Add 0.4 mL of *phenolphthalein solution R1*. Titrate with *0.1 M sodium hydroxide* until a red colour is obtained. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.63 mg of $C_{13}H_{18}O_2$.

IMPURITIES

*Specified impurities: A, F, J, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, G, H, I, K, L, M, O, P, Q, R.*

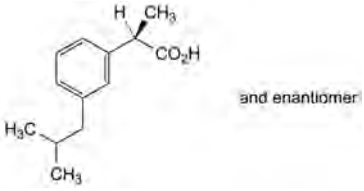


A. (2*RS*)-2-[3-(2-methylpropyl)phenyl]propanoic acid,

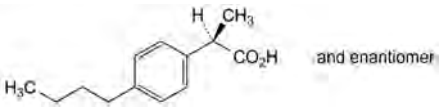


B. (2*RS*)-2-(4-butylphenyl)propanoic acid,

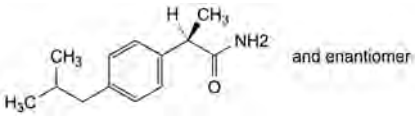


C. (2*RS*)-2-[4-(2-methylpropyl)phenyl]propanamide,

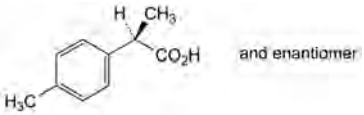


D. (2*RS*)-2-(4-methylphenyl)propanoic acid,

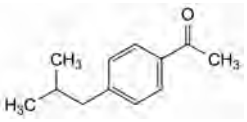


E. 1-[4-(2-methylpropyl)phenyl]ethanone,

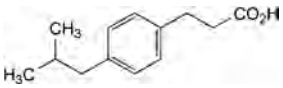


F. 3-[4-(2-methylpropyl)phenyl]propanoic acid,

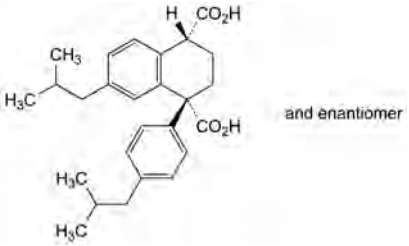


G. (1*RS*,4*RS*)-7-(2-methylpropyl)-1-[4-(2-methylpropyl)-phenyl]-1,2,3,4-tetrahydronaphthalene-1,4-dicarboxylic acid,

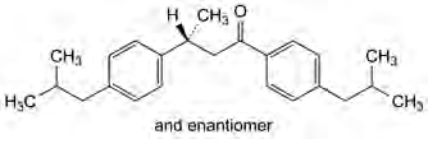


H. (3*RS*)-1,3-bis[4-(2-methylpropyl)phenyl]butan-1-one,

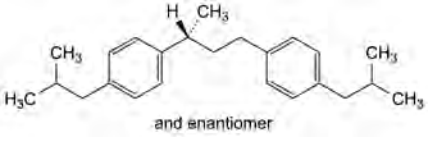


I. 1-(2-methylpropyl)-4-[(3*RS*)-3-[4-(2-methylpropyl)-phenyl]butyl]benzene,

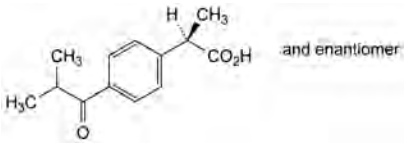


J. (2*RS*)-2-[4-(2-methylpropanoyl)phenyl]propanoic acid,

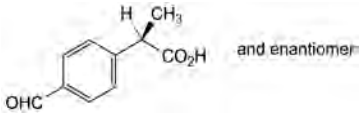


K. (2*RS*)-2-(4-formylphenyl)propanoic acid,

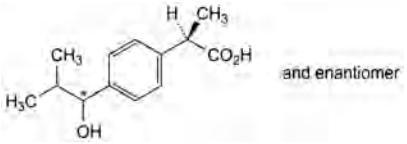


L. (2*RS*)-2-[4-(1-hydroxy-2-methylpropyl)phenyl]propanoic acid,

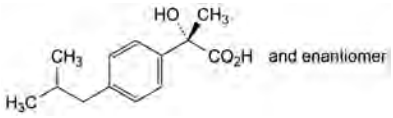


M. (2*RS*)-2-hydroxy-2-[4-(2-methylpropyl)phenyl]propanoic acid,

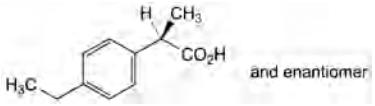


N. (2*RS*)-2-(4-ethylphenyl)propanoic acid,

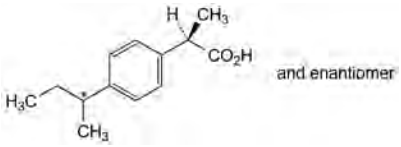


O. (2*RS*)-2-[4-(1-methylpropyl)phenyl]propanoic acid,

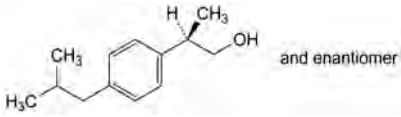


P. (2*RS*)-2-[4-(2-methylpropyl)phenyl]propan-1-ol,

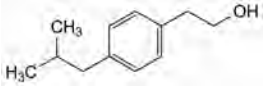


Q. 2-[4-(2-methylpropyl)phenyl]ethanol,

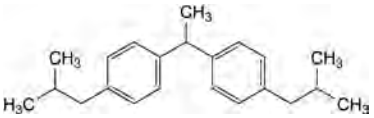


R. 1,1′-(ethane-1,1-diyl)-4,4′-(2-methylpropyl)dibenzene.

**01/2008:0917**
**corrected 6.3**

# ICHTHAMMOL

## Ichthammolum

DEFINITION

Ichthammol is obtained by distillation from certain bituminous schists, sulfonation of the distillate and neutralisation of the product with ammonia.

*Content*:

- *dry matter*: 50.0 per cent *m/m* to 56.0 per cent *m/m*;
- *total ammonia* ($NH_3$; $M_r$ 17.03): 4.5 per cent *m/m* to 7.0 per cent *m/m* (dried substance);
- *organically combined sulfur*: minimum 10.5 per cent *m/m* (dried substance);
- *sulfur in the form of sulfate*: maximum 20.0 per cent *m/m* of the total sulfur.

CHARACTERS

*Appearance*: dense, blackish-brown liquid.

*Solubility*: miscible with water and with glycerol, slightly soluble in ethanol (96 per cent), in fatty oils and in liquid paraffin. It forms homogeneous mixtures with wool fat and soft paraffin.

IDENTIFICATION

A. Dissolve 1.5 g in 15 mL of *water R* (solution A). To 2 mL of solution A add 2 mL of *hydrochloric acid R*. A resinous precipitate is formed. Decant the supernatant. The precipitate is partly soluble in *ether R*.

B. 2 mL of solution A, obtained in identification test A, gives the reaction of ammonium salts and salts of volatile bases (*2.3.1*).

C. Evaporate and ignite the mixture of solution A and *dilute sodium hydroxide solution R* obtained in identification test B. Take up the residue with 5 mL of *dilute hydrochloric acid R*. Gas is evolved which turns *lead acetate paper R* brown or black. Filter the solution. The filtrate gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Acidity or alkalinity**. To 10.0 mL of the clear filtrate obtained in the assay of total ammonia add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M hydrochloric acid* or *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Relative density** (*2.2.5*): 1.040 to 1.085, determined on a mixture of equal volumes of the substance to be examined and *water R*.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.00 g.

ASSAY

**Dry matter**. Weigh 1.000 g in a tared flask containing 2 g of *sand R*, previously dried to constant mass, and a small glass rod. Heat on a water-bath for 2 h with frequent stirring and dry in an oven at 100-105 °C until 2 consecutive weighings do not differ by more than 2.0 mg; the 2nd weighing is carried out after drying again for 1 h.

**Total ammonia**. Dissolve 2.50 g in 25 mL of warm *water R*. Rinse the solution into a 250 mL volumetric flask, add 200 mL of *sodium chloride solution R* and dilute to 250.0 mL with *water R*. Filter the solution, discarding the first 20 mL of filtrate. To 100.0 mL of the clear filtrate add 25 mL of

Monographs 1

*formaldehyde solution R*, neutralised to *phenolphthalein solution R1*. Titrate with *0.1 M sodium hydroxide* until a faint pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 1.703 mg of $NH_3$.

**Organically combined sulfur**. Mix 0.500 g with 4 g of *anhydrous sodium carbonate R* and 3 mL of *methylene chloride R* in a porcelain crucible of about 50 mL capacity, warm and stir until all the methylene chloride has evaporated. Add 10 g of coarsely powdered *copper nitrate R*, mix thoroughly and heat the mixture very gently using a small flame. When the initial reaction has subsided, increase the temperature slightly until most of the material has blackened. Cool, place the crucible in a large beaker, add 20 mL of *hydrochloric acid R* and, when the reaction has ceased, add 100 mL of *water R* and boil until all the copper oxide has dissolved. Filter the solution, add 400 mL of *water R*, heat to boiling and add 20 mL of *barium chloride solution R1*. Allow to stand for 2 h, filter, wash with *water R*, dry and ignite at about 600 ± 50 °C until 2 successive weighings do not differ by more than 0.2 per cent of the mass of the residue.

1 g of residue is equivalent to 0.1374 g of total sulfur.

Calculate the percentage content of total sulfur and subtract the percentage content of sulfur in the form of sulfate.

**Sulfur in the form of sulfate**. Dissolve 2.000 g in 100 mL of *water R*, add 2 g of *cupric chloride R* dissolved in 80 mL of *water R* and dilute to 200.0 mL with *water R*. Shake and filter. Heat 100.0 mL of the filtrate almost to boiling, add 1 mL of *hydrochloric acid R* and 5 mL of *barium chloride solution R1* dropwise and heat on a water-bath. Filter, wash the precipitate with *water R*, dry and ignite at about 600 ± 50 °C until 2 successive weighings do not differ by more than 0.2 per cent of the mass of the residue.

1 g of residue is equivalent to 0.1374 g of sulfur present in the form of sulfate.

Calculate the percentage content of sulfur in the form of sulfate.

**01/2008:0669**

# IDOXURIDINE

## Idoxuridinum

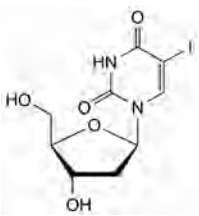

$C_9H_{11}IN_2O_5$ $M_r$ 354.1

[54-42-2]

DEFINITION

Idoxuridine contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 5-iodo-1-(2-deoxy-β-D-*erythro*-pentofuranosyl)pyrimidine-2,4(1*H*,3*H*)-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

It melts at about 180 °C, with decomposition.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *idoxuridine CRS*. Examine the substances as discs prepared using 1 mg of the substance to be examined and of the reference substance each in 0.3 g of *potassium bromide R*.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (c).

C. Heat about 5 mg in a test-tube over a naked flame. Violet vapour is evolved.

D. Disperse about 2 mg in 1 mL of *water R* and add 2 mL of *diphenylamine solution R2*. Heat in a water-bath for 10 min. A persistent light-blue colour develops.

TESTS

**Solution S**. Dissolve 0.500 g in *1 M sodium hydroxide* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent. The pH of the solution is 5.5 to 6.5.

**Specific optical rotation** (*2.2.7*): + 28 to + 32, determined on solution S and calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using as coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *5-iodouracil R*, 20 mg of *2′-deoxyuridine R* and 20 mg of *5-bromo-2′-deoxyuridine R* in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 0.20 g of the substance to be examined in 5 mL of reference solution (a).

*Reference solution (c).* Dissolve 20 mg of *idoxuridine CRS* in a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (d).* Dilute 1 mL of test solution (b) to 20 mL with a mixture of 1 volume of *concentrated ammonia R* and 5 volumes of *methanol R*.

Apply separately to the plate 5 µL of each solution. Develop twice over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 40 volumes of *chloroform R* and 50 volumes of *2-propanol R*, drying the plate in a current of cold air after each development. Examine in ultraviolet light at 254 nm. In the chromatogram obtained with test solution (a): any spots corresponding to 5-iodouracil, 2′-deoxyuridine and 5-bromo-2′-deoxyuridine are not more intense than the corresponding spots in the chromatogram obtained with reference solution (a) (0.5 per cent); any spot, apart from the principal spot and the spots corresponding to 5-iodouracil, 2′-deoxyuridine and 5-bromo-2′-deoxyuridine, is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows four clearly separated spots.

**Iodide**. Dissolve 0.25 g in 25 mL of *0.1 M sodium hydroxide*, add 5 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Allow to stand for 10 min and filter. To 25 mL of the filtrate add 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of *chloroform R* and shake. Any pink colour in the organic layer is not more intense than that in a standard prepared at the same time in the same manner using 1 mL of a 0.33 g/L solution of *potassium iodide R* instead of the substance to be examined (0.1 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 20 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 35.41 mg of $C_9H_{11}IN_2O_5$.

STORAGE

Store protected from light.

**01/2017:1529**

# IFOSFAMIDE

## Ifosfamidum

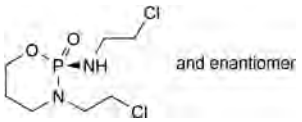


$C_7H_{15}Cl_2N_2O_2P$ $M_r$ 261.1
[3778-73-2]

DEFINITION

Ifosfamide contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (*RS*)-*N*,3-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, fine, crystalline powder, hygroscopic, soluble in water, freely soluble in methylene chloride.

IDENTIFICATION

Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the *Ph. Eur. reference spectrum of ifosfamide*. Examine the substance prepared as a disc.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 5 mL of solution S to 50 mL with *carbon dioxide-free water R*. To 10 mL of this solution add 0.1 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red. To another 10 mL of the solution add 0.1 mL of *phenolphthalein solution R*. Not more than 0.3 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Optical rotation** (*2.2.7*). The angle of optical rotation, determined on solution S, is − 0.10° to + 0.10°.

**Related substances**

A. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R*.

*Test solution*. Dissolve 1.00 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 25 mg of *ifosfamide impurity A CRS* and 25 mg of *chloroethylamine hydrochloride R* (impurity C) ina mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (b)*. Dissolve 15 mg of *ifosfamide impurity B CRS* ina mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (c)*. Dissolve 5 mg of *ethanolamine R* (impurity D), 20 mg of *ifosfamide impurity A CRS* and 80 mg of *chloroethylamine hydrochloride R* (impurity C) in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same mixture of solvents.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *methylene chloride R*. Dry the plate at 115 °C for 45 min. At the bottom of a chromatographic tank, place an evaporating dish containing a 3.2 g/L solution of *potassium permanganate R* and add an equal volume of *dilute hydrochloric acid R*, close the tank and allow to stand for 10 min. Place the plate whilst still hot in the tank, avoiding contact of the stationary phase with the solution, and close the tank. Leave the plate in contact with the chlorine vapour for 20 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed (about 20 min) and an area of coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R*. Avoid prolonged exposure to cold air. Immerse the plate in a 1 g/L solution of *tetramethylbenzidine R* in *alcohol R* for 5 s. Allow the plate to dry and examine. In the chromatogram obtained with the test solution: any spot corresponding to impurity A or impurity C is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.25 per cent); any spot corresponding to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.15 per cent); any other spot is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.15 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows 3 clearly separated spots.

B. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R*.

*Test solution*. Dissolve 0.200 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 5 mg of *ifosfamide impurity E CRS* and 5 mg of *ifosfamide impurity F CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (b)*. Dissolve 10 mg of *ifosfamide impurity E CRS* and 10 mg of *ifosfamide CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 100 mL with the same mixture of solvents.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *methylene chloride R* and 10 volumes of *acetone R*. Dry the plate at 115 °C for 45 min. Proceed as described in test A for

related substances. Any spot corresponding to impurity E or impurity F in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

**Chlorides** (*2.4.4*). Dilute 5 mL of solution S to 15 mL with *water R*. The freshly prepared solution complies with the limit test for chlorides (100 ppm).

**Water** (*2.5.12*). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

ASSAY

Examine by liquid chromatography (*2.2.29*). *Use the solutions within 24 h.*

*Solution A*. Dissolve 50.0 mg of *ethyl parahydroxybenzoate R* in 25 mL of *alcohol R*, dilute to 100.0 mL with *water R* and mix.

*Test solution*. To 0.150 g of the substance to be examined add 10.0 mL of solution A and dilute to 250.0 mL with *water R*.

*Reference solution*. To 15.0 mg of *ifosfamide CRS* add 1.0 mL of solution A and dilute to 25.0 mL with *water R*.

The chromatography may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
– as mobile phase at a flow rate of 1.5 mL/min a mixture of 30 volumes of *acetonitrile R* and 70 volumes of *water R*,
– as detector a spectrophotometer set at 195 nm.

Inject 1 µL of the reference solution six times. The assay is not valid unless the resolution between the peaks due to ifosfamide and to ethyl parahydroxybenzoate is not less than 6.0 and the relative standard deviation of the peak area for ifosfamide is at most 2.0 per cent.

Inject 1 µL of the test solution. Calculate the percentage content of $C_7H_{15}Cl_2N_2O_2P$ from the area of the corresponding peak in the chromatogram obtained and the declared content of *ifosfamide CRS*.

STORAGE

Store in an airtight container.

IMPURITIES

**Test A for related substances**: A, B, C, D.

**Test B for related substances**: E, F.

*Specified impurities: A, B, C, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

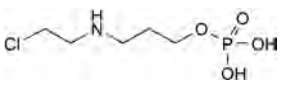

A. 3-[(2-chloroethyl)amino]propyl dihydrogen phosphate,

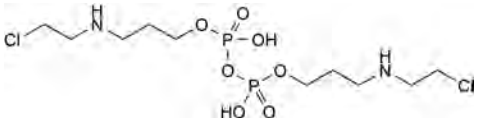

B. bis[3-[(2-chloroethyl)amino]propyl] dihydrogen diphosphate,

C. 2-chloroethanamine,

D. 2-aminoethanol.

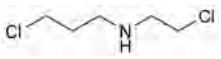

E. 3-chloro-*N*-(2-chloroethyl)propan-1-amine,

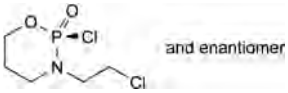


F. (*RS*)-2-chloro-3-(2-chloroethyl)-1,3,2-oxazaphosphinane 2-oxide.

**07/2017:2736**
**corrected 9.6**

# IMATINIB MESILATE

## Imatinibi mesilas

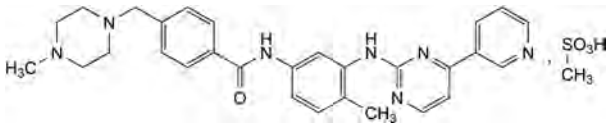

$C_{30}H_{35}N_7SO_4$ $M_r$ 589.7
[220127-57-1]

DEFINITION

4-[(4-Methylpiperazin-1-yl)methyl]-*N*-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide methanesulfonate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in imatinib mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, slightly brownish or yellowish powder; yellow or pale yellow, very hygroscopic, for the amorphous form.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *imatinib mesilate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Impurity F**. Liquid chromatography (*2.2.29*) coupled with mass spectrometry (*2.2.43*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (30:70 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution.* Dissolve 2.0 mg of *imatinib impurity F CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 200.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 1.26 g/L solution of *ammonium formate R* in *water for chromatography R* adjusted to pH 3.4-3.5 with *anhydrous formic acid R*;
- *mobile phase B*: 0.05 per cent *V/V* solution of *anhydrous formic acid R* in *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 80 | 20 |
| 6 - 10 | 80 → 20 | 20 → 80 |
| 10 - 15 | 20 | 80 |

*NOTE: MS acquisition can be started at 3.5 min and stopped at 6 min; during non-acquisition the eluent is directed to waste.*

*Flow rate*: 0.5 mL/min.

*Detection*: mass detector: the following settings have been found to be suitable and are given as examples; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:

- *ionisation*: ESI-positive;
- *detection m/z (SIM)*: 278.2;
- *gas temperature*: 350 °C;
- *drying gas flow*: 12 L/min;
- *nebuliser pressure*: 414 kPa;
- *capillary voltage (Vcap)*: 3 kV.

*Injection*: 10 µL.

*System suitability*: reference solution:

- *signal-to-noise ratio*: minimum 20 for the principal peak;
- *repeatability*: maximum relative standard deviation of 10 per cent determined on 6 injections.

*Calculation of percentage content*:

- for impurity F, use the concentration of impurity F in the reference solution.

*Limit*:

- *impurity F*: maximum 20 ppm.

**Impurity H**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (30:70 *V/V*).

*Test solution.* Dissolve 75.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *imatinib impurity A CRS* in 1.0 mL of the solvent mixture.

*Reference solution (b).* Dissolve 60.0 mg of *imatinib impurity H CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 0.150 g of the substance to be examined in the solvent mixture, add 1.0 mL each of reference solutions (a) and (b) and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.3 g of *sodium octanesulfonate monohydrate R* in 700 mL of *water for chromatography R* and add 300 mL of *acetonitrile R1* and 1.2 mL of *dilute phosphoric acid R*;
- *mobile phase B*: dissolve 2.3 g of *sodium octanesulfonate monohydrate R* in 100 mL of *water for chromatography R* and add 900 mL of *acetonitrile R1* and 1.2 mL of *dilute phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 98 | 2 |
| 6 - 8 | 98 → 20 | 2 → 80 |
| 8 - 10 | 20 | 80 |

*Flow rate*: 2.3 mL/min.

*Detection*: spectrophotometer at 227 nm.

*Injection*: 10 µL of the test solution and reference solutions (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A and H.

*Relative retention* with reference to imatinib (retention time = about 8 min): impurity A = about 0.17; impurity H = about 0.2.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurities A and H.

*Calculation of percentage content*:

- for impurity H, use the concentration of impurity H in reference solution (c).

*Limit*:

- *impurity H*: maximum 0.02 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (30:70 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 1 mg of *imatinib for system suitability CRS* (containing impurities A, B, C, D and J) in the solvent mixture and dilute to 2 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25.0 mg of *imatinib mesilate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.3 g of *sodium octanesulfonate monohydrate R* in 700 mL of *water for chromatography R* and add 300 mL of *acetonitrile R1* and 1.2 mL of *dilute phosphoric acid R*;
- *mobile phase B*: dissolve 2.3 g of *sodium octanesulfonate monohydrate R* in 100 mL of *water for chromatography R* and add 900 mL of *acetonitrile R1* and 1.2 mL of *dilute phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16 | 98 | 2 |
| 16 - 30 | 98 → 50 | 2 → 50 |

*Flow rate*: 2.3 mL/min.

*Detection*: spectrophotometer at 267 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *imatinib for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and J.

*Relative retention* with reference to imatinib (retention time = about 11 min): impurity A = about 0.2; impurity B = about 0.6; impurity J = about 0.9; impurity C = about 1.2; impurity D = about 2.3.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to imatinib and impurity C in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 45 for the principal peak in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to imatinib in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.2; impurity B = 2.0;
- for each impurity, use the concentration of imatinib mesilate in reference solution (a).

*Limits*:

- *impurity C*: maximum 0.3 per cent;
- *impurity D*: maximum 0.2 per cent;
- *impurities A, B*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.8 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{30}H_{35}N_7SO_4$ taking into account the assigned content of *imatinib mesilate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, F, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J.*

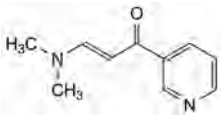

A. (2*E*)-3-(dimethylamino)-1-(pyridin-3-yl)prop-2-en-1-one,

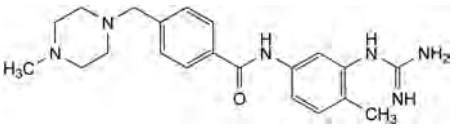

B. *N*-(3-carbamimidamido-4-methylphenyl)-4-[(4-methylpiperazin-1-yl)methyl]benzamide,

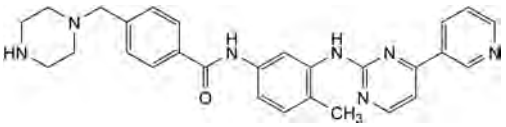

C. *N*-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]-4-(piperazin-1-ylmethyl)benzamide (desmethylimatinib),

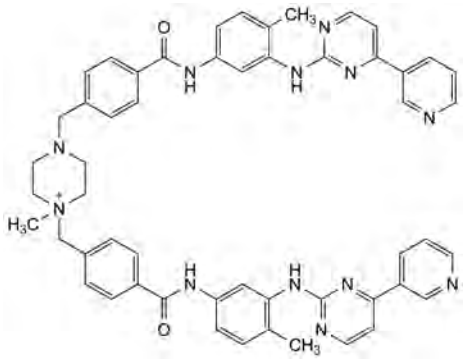

D. 1-methyl-1,4-bis[4-[[4-methyl-3-[[4-(pyridin-3-yl)-pyrimidin-2-yl]amino]phenyl]carbamoyl]benzyl]-piperazin-1-ium (imatinib dimer),

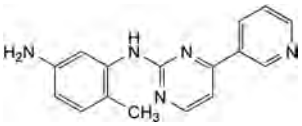

F. 4-methyl-$N^3$-[4-(pyridin-3-yl)pyrimidin-2-yl]benzene-1,3-diamine,

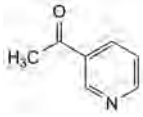

H. 1-(pyridin-3-yl)ethan-1-one,

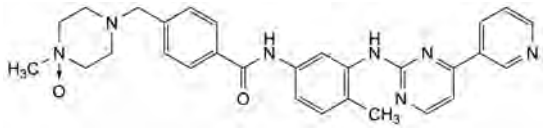

J. 4-[(4-methyl-4-oxidopiperazin-1-yl)methyl]-*N*-[4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]phenyl]benzamide.

**01/2020:2924**

# IMIDACLOPRID FOR VETERINARY USE

## Imidaclopridum ad usum veterinarium

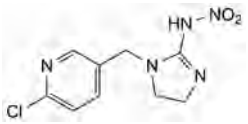

$C_9H_{10}ClN_5O_2$ $M_r$ 255.7
[138261-41-3]

DEFINITION

*N*-[1-[(6-Chloropyridin-3-yl)methyl]-4,5-dihydro-1*H*-imidazol-2-yl]nitramide.

*Content*: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *imidacloprid CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetonitrile R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*. Dissolve 7.5 g of *sodium hexanesulfonate R* in 900 mL of *water for chromatography R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution (a).* Dissolve 40.0 mg of the substance to be examined in 2 mL of *acetonitrile R* and dilute to 20.0 mL with solution A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with solution A.

*Reference solution (a).* Dissolve 40.0 mg of *imidacloprid CRS* in 2 mL of *acetonitrile R* and dilute to 20.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dilute 1.0 mL of test solution (b) to 50.0 mL with solution A.

*Reference solution (c).* Dissolve 10 mg of *imidacloprid for system suitability CRS* (containing impurities A, B and D) in 0.5 mL of *acetonitrile R* and dilute to 5 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: solution A;
- *mobile phase B*: solution A, *acetonitrile R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 3 | 90 | 10 | 1.0 |
| 3 - 9 | 90 → 80 | 10 → 20 | 1.0 |
| 9 - 9.5 | 80 | 20 | 1.5 |
| 9.5 - 15 | 80 → 55 | 20 → 45 | 1.5 |
| 15 - 21 | 55 → 40 | 45 → 60 | 1.5 |
| 21 - 28 | 40 → 0 | 60 → 100 | 1.5 |

*Detection*: spectrophotometer at 268 nm.

*Autosampler*: set at 25 °C.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *imidacloprid for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to imidacloprid (retention time = about 17 min): impurity A = about 0.19; impurity B = about 0.22; impurity D = 1.04.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to imidacloprid.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.5; impurity B = 0.4.
- for each impurity, use the concentration of imidacloprid in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.7 per cent;
- *impurity B*: maximum 0.25 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.10 per cent.

**Chlorides**: maximum 0.5 per cent.

Dissolve 0.500 g in 30 mL of *acetone R*. Add 20 mL of *water R* and 2 mL of *dilute nitric acid R*.

Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and reference solution (a).

Calculate the percentage content of $C_9H_{10}ClN_5O_2$ taking into account the assigned content of *imidacloprid CRS*.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G.*

Monographs

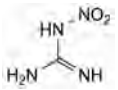

A. *N*-nitroguanidine,

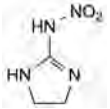

B. *N*-[4,5-dihydro-1*H*-imidazol-2-yl]nitramide,

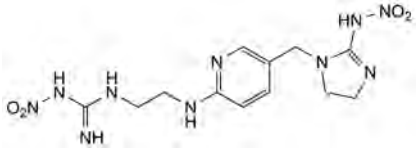

C. *N*-[2-[[5-[(2-nitroamido-4,5-dihydro-1*H*-imidazol-1-yl)methyl]pyridin-2-yl]amino]ethyl]-*N*′-nitroguanidine,

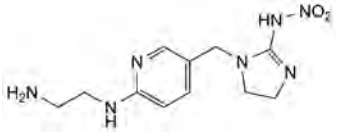

D. *N*-[1-[[6-[(2-aminoethyl)amino]pyridin-3-yl]methyl]-4,5-dihydro-1*H*-imidazol-2-yl]nitramide,

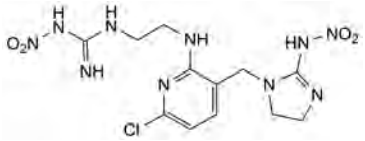

E. *N*-[2-[(6-chloro-3-[[2-nitroamido-4,5-dihydro-1*H*-imidazol-1-yl]methyl]pyridin-2-yl)amino]ethyl]-*N*′-nitroguanidine,

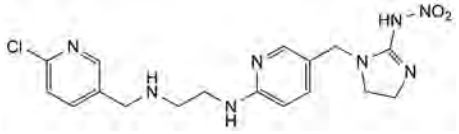

F. *N*-[1-[[6-[[2-[[(6-chloropyridin-3-yl)methyl]amino]-ethyl]amino]pyridin-3-yl]methyl]-4,5-dihydro-1*H*-imidazol-2-yl]nitramide,

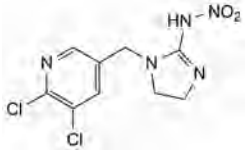

G. *N*-[1-[(5,6-dichloropyridin-3-yl)methyl]-4,5-dihydro-1*H*-imidazol-2-yl]nitramide.

**04/2018:1226**
**corrected 10.0**

# IMIPENEM MONOHYDRATE

## Imipenemum monohydricum

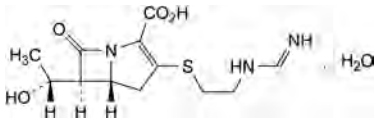

$C_{12}H_{17}N_3O_4S,H_2O$ $M_r$ 317.4
[74431-23-5]

DEFINITION

(5*R*,6*S*)-6-[(*R*)-1-Hydroxyethyl]-3-[[2-[(iminomethyl)amino]-ethyl]sulfanyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product or obtained by any other means.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or pale yellow powder, slightly hygroscopic.

*Solubility*: slightly soluble in water and in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *imipenem CRS*.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 6 of the range of the reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 0.500 g in *phosphate buffer solution pH 7.0 R3* and dilute to 50 mL with the same solution.

**pH** (*2.2.3*): 4.5 to 7.5.

Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 90 to + 95 (anhydrous substance), measured at 25 °C. *Prepare the solutions immediately before use.*

Dissolve 0.125 g in *phosphate buffer solution pH 7.0 R3* and dilute to 25.0 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Buffer solution A*. Dissolve 0.32 g of *anhydrous sodium dihydrogen phosphate R* and 1.04 g of *anhydrous disodium hydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust to pH 7.3 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Buffer solution B*. Dissolve 0.11 g of *anhydrous disodium hydrogen phosphate R* in 900 mL of *water R*. Adjust to pH 6.8 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Solvent mixture*: *acetonitrile R*, buffer solution B (0.7:99.3 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *imipenem CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of the substance to be examined in 8 mL of a mixture of 1 volume of *dilute sulfuric acid R* and 200 volumes of *water R*. After 5 min, add 10 mg of *sodium carbonate R* and dilute to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution A (0.7:99.3 *V/V*);
- *mobile phase B*: *acetonitrile R1*, buffer solution A (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 100 | 0 |
| 9 - 24 | 100 → 68 | 0 → 32 |
| 24 - 24.5 | 68 → 50 | 32 → 50 |
| 24.5 - 29 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 5 °C.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to the epimers of impurity B.

*Relative retention* with reference to imipenem (retention time = about 8 min): epimer I of impurity B = about 0.33; epimer II of impurity B = about 0.35; impurity A = about 0.8.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to epimer I of impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to epimer II of impurity B.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity A by 2.4;
- for each impurity, use the concentration of imipenem monohydrate in reference solution (b).

*Limits*:

- *impurity A*: maximum 1.0 per cent;
- *impurity B*: for each epimer, maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 5.0 per cent to 8.0 per cent, determined on 0.100 g. Use an iodosulfurous reagent containing imidazole instead of pyridine and a clean container for each determination.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{17}N_3O_4S$ taking into account the assigned content of *imipenem CRS*.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B.*

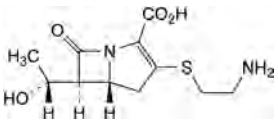

A. (5*R*,6*S*)-3-[(2-aminoethyl)sulfanyl]-6-[(*R*)-1-hydroxyethyl]-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid (thienamycin),

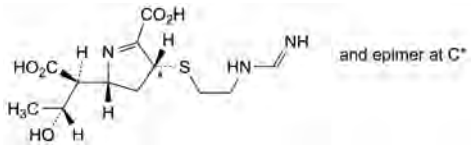


B. (2*R*,4*RS*)-2-[(1*S*,2*R*)-1-carboxy-2-hydroxypropyl]-4-[[2-[(iminomethyl)amino]ethyl]sulfanyl]-3,4-dihydro-2*H*-pyrrole-5-carboxylic acid (imipenemoic acid).

**01/2017:0029**

# IMIPRAMINE HYDROCHLORIDE

## Imipramini hydrochloridum

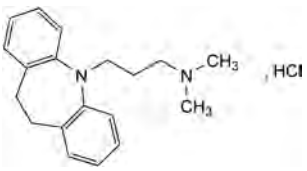

$C_{19}H_{25}ClN_2$ $M_r$ 316.9

[113-52-0]

DEFINITION

3-(10,11-Dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 170 °C to 174 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *imipramine hydrochloride CRS*.

C. Dissolve about 5 mg in 2 mL of *nitric acid R*. An intense blue colour develops.

D. About 20 mg gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. To 3.0 g add 20 mL of *carbon dioxide-free water R*, dissolve rapidly by shaking and triturating with a glass rod and dilute to 30 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*). Immediately after preparation, dilute solution S with an equal volume of *water R*. This solution is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.6 to 5.0 for solution S, measured immediately after preparation.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *imipramine for system suitability CRS* (containing impurity B) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 40 volumes of *acetonitrile R1* with 60 volumes of a 5.2 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 7.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of imipramine.

*Relative retention* with reference to imipramine (retention time = about 7 min): impurity B = about 0.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity B and imipramine.

*Limits*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to imipramine in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the peak due to imipramine in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the peak due to imipramine in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.69 mg of $C_{19}H_{25}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

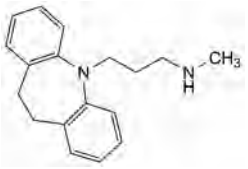

A. 3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*-methylpropan-1-amine (desipramine),

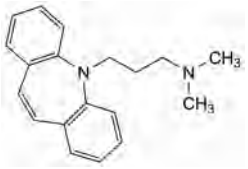

B. 3-(5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*-dimethylpropan-1-amine (depramine),

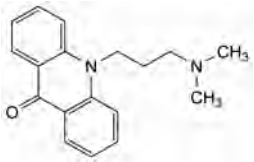

C. 10-[3-(dimethylamino)propyl]acridin-9(10*H*)-one.

**01/2017:1108**

# INDAPAMIDE

## Indapamidum

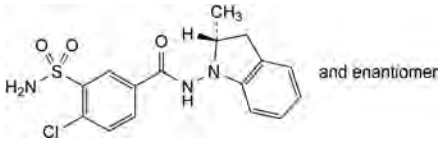


$C_{16}H_{16}ClN_3O_3S$ $M_r$ 365.8

[26807-65-8]

DEFINITION

4-Chloro-*N*-[(2*RS*)-2-methyl-2,3-dihydro-1*H*-indol-1-yl]-3-sulfamoylbenzamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 242 nm.

*Shoulders*: at 279 nm and 287 nm.

*Specific absorbance at the absorption maximum*: 590 to 630.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *indapamide CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *indapamide CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *indometacin R* in 5 mL of reference solution (a) and dilute to 10 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *toluene R* (1:20:79 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Optical rotation** (*2.2.7*): − 0.02° to + 0.02°.

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use or maintain them at 4 °C.*

*Solvent mixture*: *acetonitrile R*, *methanol R* (50:50 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 7 mL of the solvent mixture and dilute to 20.0 mL with a 0.2 g/L solution of *sodium edetate R*.

*Reference solution (a).* Dissolve 3.0 mg of *indapamide impurity B CRS* in 3.5 mL of the solvent mixture and dilute to 10.0 mL with a 0.2 g/L solution of *sodium edetate R*. To 1.0 mL of the solution add 35 mL of the solvent mixture and dilute to 100.0 mL with a 0.2 g/L solution of *sodium edetate R*.

*Reference solution (b).* To 1.0 mL of the test solution add 17.5 mL of the solvent mixture and dilute to 50.0 mL with a 0.2 g/L solution of *sodium edetate R*. To 1.0 mL of this solution add 7 mL of the solvent mixture and dilute to 20.0 mL with a 0.2 g/L solution of *sodium edetate R*.

*Reference solution (c).* Dissolve 20.0 mg of *indapamide CRS* in 7 mL of the solvent mixture and dilute to 20.0 mL with a 0.2 g/L solution of *sodium edetate R*.

*Reference solution (d).* Dissolve 25.0 mg of *indapamide CRS* and 45.0 mg of *methylnitrosoindoline CRS* (impurity A) in 17.5 mL of the solvent mixture and dilute to 50.0 mL with a 0.2 g/L solution of *sodium edetate R*.

*Column*:

– *size*: *l* = 0.20 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *methanol R*, 0.2 g/L solution of *sodium edetate R* (0.1:17.5:17.5:65 *V/V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b) and (d).

*Run time*: 2.5 times the retention time of indapamide.

*Retention time*: indapamide = about 11 min.

*System suitability*:

– *resolution*: minimum 4.0 between the peaks due to indapamide and impurity A in the chromatogram obtained with reference solution (d).

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 1 mL of *acetonitrile R* and dilute to 10.0 mL with *water R*. Shake for 15 min. Allow to stand at 4 °C for 1 h and filter.

*Reference solution.* Dissolve 25.0 mg of the substance to be examined in 1.0 mL of a 0.125 mg/L solution of *methylnitrosoindoline CRS* (impurity A) in *acetonitrile R* and dilute to 10.0 mL with *water R*. Shake for 15 min. Allow to stand at 4 °C for 1 h and filter.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: mix 7 volumes of *acetonitrile R*, 20 volumes of *tetrahydrofuran R* and 73 volumes of a 1.5 g/L solution of *triethylamine R* adjusted to pH 2.8 with *phosphoric acid R*.

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 305 nm.

*Injection*: 0.1 mL.

*System suitability*: reference solution:

– *signal-to-noise ratio*: minimum 3 for the peak due to impurity A appearing just before the peak due to indapamide;
– *peak-to-valley-ratio*: minimum 6.7, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to indapamide.

*Limit*:

– *impurity A*: not more than the difference between the areas of the peaks due to impurity A in the chromatograms obtained with the reference solution and the test solution (5 ppm).

**Impurity C**. Liquid chromatography (*2.2.29*). *Maintain the solutions at 10 °C after preparation.*

*Solution A*. Dissolve 0.20 g of *sodium edetate R* in *water R*, add 1.5 mL of *anhydrous acetic acid R* and dilute to 1 L with *water R*.

*Test solution.* Dissolve 75.0 mg of the substance to be examined in 7.5 mL of *acetonitrile for chromatography R* and dilute to 25.0 mL with *water R*.

*Reference solution (a).* Dissolve 9.0 mg of *indapamide impurity C CRS* in 1.0 mL of *water R*, add 6.0 mL of *acetonitrile for chromatography R* and dilute to 20.0 mL with *water R*. To 1.0 mL of the solution add 7.5 mL of *acetonitrile for chromatography R* and dilute to 25.0 mL with *water R*.

*Reference solution (b).* To 1.0 mL of reference solution (a) add 3.0 mL of *acetonitrile for chromatography R* and dilute to 10.0 mL with *water R*.

*Reference solution (c).* To 1.0 mL of reference solution (a) add 3.0 mL of *acetonitrile for chromatography R* and dilute to 10.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.05 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm);
- *temperature*: 50 °C.

*Mobile phase*: *acetonitrile for chromatography R*, solution A (30:70 *V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 2 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of indapamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to indapamide (retention time = about 1.3 min): impurity C = about 0.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 4.0 between the peaks due to impurity C and indapamide;
- *signal-to-noise ratio*: minimum 20 for the peak due to impurity C.

*Calculation of content*:

- for impurity C, use the concentration of impurity C in reference solution (b).

*Limit*:

- *impurity C*: maximum 600 ppm.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{16}H_{16}ClN_3O_3S$ taking into account the assigned content of *indapamide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

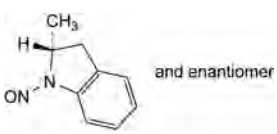


A. (2*RS*)-2-methyl-1-nitroso-2,3-dihydro-1*H*-indole,

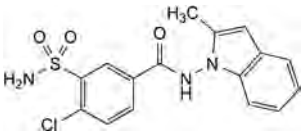

B. 4-chloro-*N*-(2-methyl-1*H*-indol-1-yl)-3-sulfamoylbenzamide,

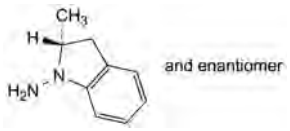


C. (2*RS*)-2-methyl-2,3-dihydro-1*H*-indol-1-amine.

**01/2017:2214**

# INDINAVIR SULFATE

## Indinaviri sulfas

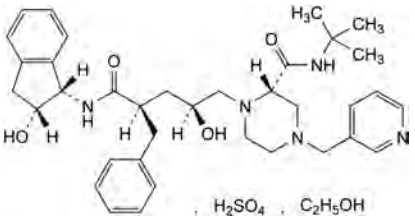


$C_{36}H_{49}N_5O_8S,C_2H_6O$ $M_r$ 758
[157810-81-6]

DEFINITION

(2*S*)-1-[(2*S*,4*R*)-4-Benzyl-2-hydroxy-5-[[(1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl]amino]-5-oxopentyl]-*N*-(1,1-dimethylethyl)-4-(pyridin-3-ylmethyl)piperazine-2-carboxamide sulfate ethanolate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous and ethanol-free substance).

PRODUCTION

A test for enantiomeric purity is carried out unless it has been demonstrated that the manufacturing process is enantioselective for the substance.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, soluble in methanol, practically insoluble in heptane.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 122 to + 129 (anhydrous and ethanol-free substance), determined at 365 nm and at 25 °C.

Dissolve 0.500 g in *water R* and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of indinavir sulfate.*

C. It gives reaction (a) of sulfates (*2.3.1*).

D. Ethanol (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Thoroughly mix equal volumes of mobile phase A and *acetonitrile R1*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 100.0 mL with the same solution.

*Reference solution (a).* Dissolve 4 mg of *indinavir for system suitability CRS* (containing impurities B, C and E) in solution A and dilute to 10 mL with the same solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c).* Dissolve 5.0 mg of *cis-aminoindanol R* (impurity A) in solution A and dilute to 10.0 mL with the same solution. Dilute 1.0 mL of the solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (d).* To 30 mg of the substance to be examined add 0.25 mL of *2 M hydrochloric acid R* and allow to stand at room temperature for 1 h. Dilute to 100 mL with a mixture of 2 volumes of *acetonitrile R1* and 3 volumes of mobile phase A and mix (*in situ* degradation to obtain impurity D).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: solution containing 0.27 g/L of *potassium dihydrogen phosphate R* and 1.40 g/L of *dipotassium hydrogen phosphate R*; filter and degas;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 40 | 80 → 30 | 20 → 70 |
| 40 - 45 | 30 | 70 |
| 45 - 47 | 30 → 80 | 70 → 20 |
| 47 - 52 | 80 | 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *indinavir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C and E; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention* with reference to indinavir (retention time = about 25 min): impurity A = about 0.2; impurity B = about 0.8; impurity C = about 0.98; impurity D = about 1.1; impurity E = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.8 between the peaks due to impurity C and indinavir.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 1.8;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities B, C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Ethanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *propanol R* to 200.0 mL with *water R*.

*Test solution.* Dissolve 0.400 g of the substance to be examined in 50.0 mL of *water R*, add 8.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Reference solution.* Dilute 1.0 mL of *anhydrous ethanol R* to 200.0 mL. Dilute 2.0 mL of this solution and 2.0 mL of the internal standard solution to 25.0 mL with *water R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 10 mL/min.

*Split ratio*: 1:10.

*Temperature*:

- *column*: 35 °C;
- *injection port*: 140 °C;
- *detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*System suitability*: reference solution:

- *retention time*: ethanol = 2 min to 4 min;
- *resolution*: minimum 5.0 between the peaks due to ethanol and propanol.

Calculate the percentage content of ethanol taking the density (*2.2.5*) to be 0.790 g/mL.

*Limit*:

- *ethanol*: 5.0 per cent to 8.0 per cent *m/m*.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution B.* Add 20 mL of *dibutylammonium phosphate for ion-pairing R* to 1000 mL of *water R*. Adjust to pH 6.5 with *1 M sodium hydroxide*.

*Test solution.* Dissolve 60.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution.* Dissolve 50.0 mg of *indinavir CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, solution B (45:55 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of indinavir.

*Retention time*: indinavir = about 10 min.

Calculate the percentage content of $C_{36}H_{49}N_5O_8S$ using the declared content of *indinavir CRS* and multiplying by a correction factor of 1.1598.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

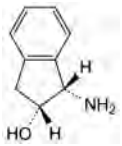

A. (1*S*,2*R*)-1-amino-2,3-dihydro-1*H*-inden-2-ol (*cis*-aminoindanol),

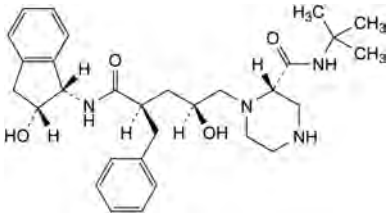

B. (2*S*)-1-[(2*S*,4*R*)-4-benzyl-2-hydroxy-5-[[(1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl]amino]-5-oxopentyl]-*N*-(1,1-dimethylethyl)piperazine-2-carboxamide,

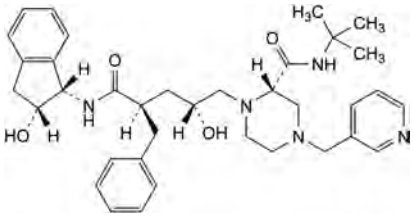

C. (2*S*)-1-[(2*R*,4*R*)-4-benzyl-2-hydroxy-5-[[(1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl]amino]-5-oxopentyl]-*N*-(1,1-dimethylethyl)-4-(pyridin-3-ylmethyl)piperazine-2-carboxamide,

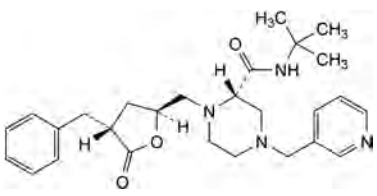

D. (3*R*,5*S*)-3-benzyl-5-[[(2*S*)-2-[(1,1-dimethylethyl)-carbamoyl]-4-(pyridin-3-ylmethyl)piperazin-1-yl]methyl]-4,5-dihydrofuran-2(3*H*)-one,

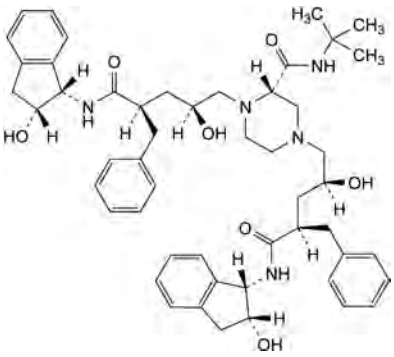

E. (2*S*)-1,4-bis[(2*S*,4*R*)-4-benzyl-2-hydroxy-5-[[(1*S*,2*R*)-2-hydroxy-2,3-dihydro-1*H*-inden-1-yl]amino]-5-oxopentyl]-*N*-(1,1-dimethylethyl)piperazine-2-carboxamide,

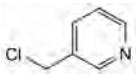

F. 3-(chloromethyl)pyridine (nicotinyl chloride).

**04/2019:0092**

# INDOMETACIN

## Indometacinum

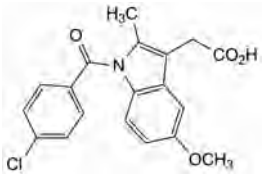

$C_{19}H_{16}ClNO_4$ $M_r$ 357.8

[53-86-1]

DEFINITION

[1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*). The acceptable crystalline form corresponds to *indometacin CRS*.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 158 °C to 162 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25 mg in a mixture of 1 volume of a 103.0 g/L solution of *hydrochloric acid R* and 9 volumes of *methanol R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 10.0 mL of the solution to 100.0 mL with a mixture of 1 volume of a 103.0 g/L solution of *hydrochloric acid R* and 9 volumes of *methanol R*.

*Spectral range*: 300-350 nm.

*Absorption maximum*: at 318 nm.

*Specific absorbance at the absorption maximum*: 170 to 190.

C. Infrared absorption spectrophotometry (*2.2.24*), without recrystallisation.

*Comparison*: *indometacin CRS*.

D. Dissolve 0.1 g in 10 mL of *ethanol (96 per cent) R*, heating slightly if necessary. To 0.1 mL of the solution add 2 mL of a freshly prepared mixture of 1 volume of a 250 g/L solution of *hydroxylamine hydrochloride R* and 3 volumes of *dilute sodium hydroxide solution R*. Add 2 mL of *dilute hydrochloric acid R* and 1 mL of *ferric chloride solution R2* and mix. A violet-pink colour develops.

E. To 0.5 mL of the solution in ethanol (96 per cent) prepared in identification test D, add 0.5 mL of *dimethylaminobenzaldehyde solution R2*. A precipitate is formed that dissolves on shaking. Heat on a water-bath. A bluish-green colour is produced. Continue to heat for 5 min and cool in iced water for 2 min. A precipitate is formed and the colour changes to light greyish-green. Add 3 mL of *ethanol (96 per cent) R*. The solution is clear and violet-pink in colour.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture, using sonication if necessary, and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *indometacin impurity mixture CRS* (impurities I and J) in 1 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: 5 g/L solution of *anhydrous formic acid R*;
- *mobile phase B*: 5 g/L solution of *anhydrous formic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5.5 | 60 | 40 |
| 5.5 - 5.6 | 60 → 30 | 40 → 70 |
| 5.6 - 9 | 30 | 70 |

*Flow rate*: 0.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 1.4 µL.

*Identification of impurities*: use the chromatogram supplied with *indometacin impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities I and J.

*Relative retention* with reference to indometacin (retention time = about 6 min): impurity I = about 1.3; impurity J = about 1.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurities I and J.

*Calculation of percentage contents*:

- for each impurity, use the concentration of indometacin in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution.* Dissolve 25.0 mg of *indometacin CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.6 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: 10 g/L solution of *acetic acid R*;
- *mobile phase B*: 10 g/L solution of *acetic acid R* in *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 50 | 50 |
| 5 - 5.5 | 50 → 0 | 50 → 100 |
| 5.5 - 8 | 0 | 100 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Retention time*: indometacin = about 4 min.

Calculate the percentage content of $C_{19}H_{16}ClNO_4$ taking into account the assigned content of *indometacin CRS*.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J.*

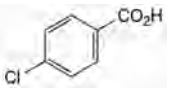


A. 4-chlorobenzoic acid,

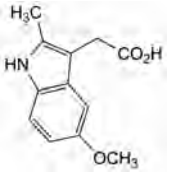


B. (5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid,

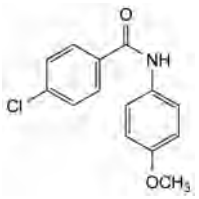

C. 4-chloro-*N*-(4-methoxyphenyl)benzamide,

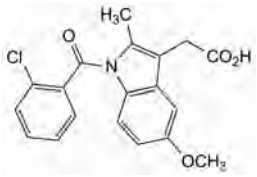

D. [1-(2-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid,

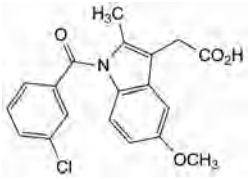

E. [1-(3-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid,

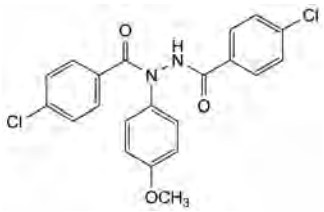

F. 4-chloro-*N*′-(4-chlorobenzoyl)-*N*-(4-methoxyphenyl)-benzohydrazide,

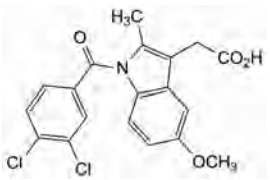

G. [1-(3,4-dichlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid,

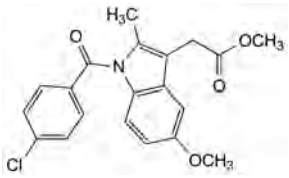

H. methyl [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetate,

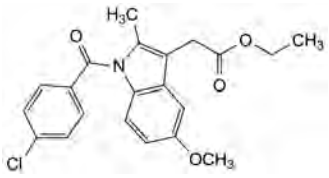

I. ethyl [1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetate,

J. 4-chloro-*N*′-[[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetyl]-*N*-(4-methoxyphenyl)-benzohydrazide.

**01/2020:2928**

# INFLIXIMAB CONCENTRATED SOLUTION

## Infliximabum solutio concentrata

heavy chain

```
EVKLEESGGG  LVQPGGSMKL  SCVASGFIFS  NHWMNWVRQS   40
PEKGLEWVAE  IRSKSINSAT  HYAESVKGRF  TISRDDSKSA   80
VYLQMTDLRT  EDTGVYYCSR  NYYGSTYDYW  GQGTTLTVSS  120
ASTKGPSVFP  LAPSSKSTSG  GTAALGCLVK  DYFPEPVTVS  160
WNSGALTSGV  HTFPAVLQSS  GLYSLSSVVT  VPSSSLGTQT  200
YICNVNHKPS  NTKVDKKVEP  KSCDKTHTCP  PCPAPELLGG  240
PSVFLFPPKP  KDTLMISRTP  EVTCVVVDVS  HEDPEVKFNW  280
YVDGVEVHNA  KTKPREEQYN  STYRVVSVLT  VLHQDWLNGK  320
EYKCKVSNKA  LPAPIEKTIS  KAKGQPREPQ  VYTLPPSRDE  360
LTKNQVSLTC  LVKGFYPSDI  AVEWESNGQP  ENNYKTTPPV  400
LDSDGSFFLY  SKLTVDKSRW  QQGNVFSCSV  MHEALHNHYT  440
QKSLSLSPGK                                    450
```

light chain

```
DILLTQSPAT  LSVSPGERVS  FSCRASQFVG  SSIHWYQQRT   40
NGSPRLLIKY  ASESMSGIPS  RFSGSGSGTD  FTLSINTVES   80
EDIADYYCQQ  SHSWPFTFGS  GTNLEVKRTV  AAPSVFIFPP  120
SDEQLKSGTA  SVVCLLNNFY  PREAKVQWKV  DNALQSGNSQ  160
ESVTEQDSKD  STYSLSSTLT  LSKADYEKHK  VYACEVTHQG  200
LSSPVTKSFN  RGEC                              214
```

disulfide bridges:

| | | | | |
|---|---|---|---|---|
| Intra-H | 22-98 | 147-203 | 264-324 | 370-428 |
| | 22''-98'' | 147''-203'' | 264''-324'' | 370''-428'' |
| Intra-L | 23'-88' | 134'-194' | | |
| | 23'''-88''' | 134'''-194''' | | |
| Inter-H-L | 223-214' | | | |
| | 223''-214''' | | | |
| Inter-H-H | 229-229'' | 232-232'' | | |

*N*-glycosylation sites:
300, 300''

$C_{6462}H_{9960}N_{1728}O_{2036}S_{44}$ (dimer without glycosylation)

$M_r$ approx. 145 kDa
(dimer without glycosylation)

DEFINITION

Solution of a monoclonal antibody consisting of a bisdisulfide dimer of 1328 amino acid residues with a molecular weight of approximately 145 kDa, which binds with high affinity to both soluble and transmembrane forms of TNF-α.

Infliximab is a chimeric human-murine IgG1 kappa monoclonal antibody representing a glycosylated immunoglobulin with 1 *N*-linked glycosylation site (Asn 300) in the CH2 domain of each heavy chain. The detected oligosaccharides are mostly G0F (absence of terminal galactose) and G1F (1 terminal galactose) structures. Each heavy chain consists of 450 amino acids with 11 cysteine residues, and each light chain consists of 214 amino acids with 5 cysteine residues. All cysteine residues in heavy and light chains are involved in either intra- or inter-disulfide bonding.

*Content (milligrams of protein per millilitre)*: as approved by the competent authority.

*Potency*: $8 \times 10^3$ to $12 \times 10^3$ IU per milligram of protein.

PRODUCTION

Infliximab is produced in a suitable mammalian cell expression system by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process consistently produces a product with the expected *N*-glycan occupancy and Fc-effector functions (antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC)) using suitably qualified assay(s).

*Prior to release, the following tests are carried out on each batch of infliximab concentrated solution, unless an exemption has been granted by the competent authority.*

**Host-cell-derived proteins** (*2.6.34*). The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

**Residual Protein A**. Use a suitable immunochemical method (*2.7.1*) based on an enzyme-linked immunosorbent assay (ELISA). To determine residual Protein A, the preparation to be examined is transferred to an ELISA microplate coated with an anti-protein A capture antibody. Peroxidase-conjugated polyclonal anti-protein A antibody is added followed by a peroxidase substrate. Optical density is measured and the amount of residual Protein A is calculated using a standard curve and the usual statistical methods (for example, *5.3*).

*Limit*: as approved by the competent authority.

**Glycan analysis**. Use a suitable method developed according to general chapter *2.2.59. Glycan analysis of glycoproteins*, section 2-3:

- after desalting, release the glycans using 1 of the agents described in Table *2.2.59.*-1, for example peptide *N*-glycosidase F (PNGase F);
- if needed, label the released glycans with 1 of the fluorescent labelling agents described in Table *2.2.59.*-2;
- analyse the labelled or unlabelled glycans using a suitable technique.

*The following procedure is given as an example.*

*Solution A*. Dissolve 0.17 g of *anhydrous sodium dihydrogen phosphate R* and 0.53 g of *anhydrous disodium hydrogen phosphate R* in *water R*. Adjust to pH 7.6 with *sodium hydroxide solution R* or *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

*Test solution*. Desalt a volume of the preparation to be examined by a suitable method (for example, using a suitable centrifugal filter with *water for chromatography R* as elution buffer), and dilute with solution A to obtain a concentration of about 1 mg/mL. To 200 µL of this solution add 0.3 µL of a 500 000 U/mL solution of *peptide N-glycosidase F R*. Incubate at 37 °C for at least 16 hours.

*Reference solution (a)*. Dissolve the contents of a vial of *infliximab CRS* in *water R*. Desalt a volume of this preparation and carry out the glycan release at the same time and in the same manner as for the test solution.

*Reference solution (b)*. Use a suitable infliximab in-house reference preparation shown to be representative of batches tested clinically and batches used to demonstrate consistency of production. Desalt a volume of this preparation and carry out the glycan release at the same time and in the same manner as for the test solution.

*Blank solution*. Use 200 µL of solution A to proceed to glycan release.

Analyse the native glycans by liquid chromatography (*2.2.29*).

*Precolumn*:

- *size*: *l* = 0.05 m, Ø = 3.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R2* (5.5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R2* (5.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 2 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: solution containing 2 g/L of *sodium hydroxide R* and 10.25 g/L of *anhydrous sodium acetate R*;
- *mobile phase C*: solution containing 2 g/L of *sodium hydroxide R* and 41 g/L of *anhydrous sodium acetate R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|---|
| 0 - 5 | 99.2 | 0.8 | 0 | 0.35 |
| 5 - 45 | 99.2 → 95.2 | 0.8 → 4.8 | 0 | 0.35 |
| 45 - 50 | 95.2 → 72 | 4.8 → 28 | 0 | 0.35 |
| 50 - 77 | 72 → 4 | 28 → 96 | 0 | 0.35 |
| 77 - 77.1 | 4 → 0 | 96 → 0 | 0 → 100 | 0.35 |
| 77.1 - 87 | 0 | 0 | 100 | 0.40 |

*Detection*: electrochemical detector (pulsed amperometry).

*Autosampler*: set at 2-8 °C.

*Injection*: 25 µL.

*Identification of peaks*: use the chromatogram in Figure 2928.-1 to identify the 7 peaks corresponding to fucosylated (peaks 1, 4 and 5), afucosylated (peaks 2 and 3) and sialylated (peaks 6 and 7) glycans; record the retention time of each peak.

*System suitability*:

- the chromatogram obtained with reference solution (a) is qualitatively similar to the chromatogram supplied with *infliximab CRS* and peaks 1 to 7 are clearly visible;
- no significant peaks are observed in the chromatogram obtained with the blank solution.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution (b);
- the retention times of the peaks in the chromatogram obtained with the test solution correspond to those in the chromatogram obtained with reference solution (b);
- no additional peaks are observed in the chromatogram obtained with the test solution in comparison with the chromatogram obtained with reference solution (b).

Calculate the relative peak areas of the individual peaks corresponding to fucosylated, afucosylated and sialylated glycans with reference to the sum of the areas of all retained glycan peaks.

Calculate the percentage contents of fucosylated, afucosylated and sialylated glycans, using the following expressions:

$$\frac{A}{A+B+C} \times 100$$

$$\frac{B}{A+B+C} \times 100$$

$$\frac{C}{A+B+C} \times 100$$

$A$ = sum of the areas of the peaks due to fucosylated glycans;

$B$ = sum of the areas of the peaks due to afucosylated glycans;

$C$ = sum of the areas of the peaks due to sialylated glycans.

*NOTE: sialylated glycans elute as peak clusters and are integrated as such.*

*Limits*:

- *percentage of fucosylated glycans*: as authorised by the competent authority;
- *percentage of afucosylated glycans*: as authorised by the competent authority;
- *percentage of sialylated glycans*: as authorised by the competent authority.

**Charged variants**.

A. Isoelectric focusing (*2.2.54*): use suitable agarose gels.

*The following procedure is given as an example.*

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *infliximab CRS* in *water R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (b)*. Use a suitable infliximab in-house reference preparation shown to be representative of batches tested clinically and batches used to demonstrate consistency of production. Dilute with *water R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (c)*. Use an isoelectric point (pI) calibration solution, in the pI range of 3-10, prepared according to the manufacturer's instructions. Adjust the concentration to allow detection of all marker proteins.

*Focusing*:

- *pH gradient*: 3-10;
- *catholyte*: 40 g/L solution of *sodium hydroxide R*;
- *anolyte*: 2.87 per cent *V/V* solution of *glacial acetic acid R*;
- *application*: 15 µL.

For each gel, run 2 lanes with reference solution (a), 2 lanes with reference solution (b) and 2 lanes with reference solution (c); use the remaining lanes for the test solution. Proceed with the isoelectric focusing by applying the electrical parameters according to the manufacturer's instructions (the following parameters have been found suitable: 1500 V, 7 mA, 25 W and 80 min).

*Detection*: as described in general chapter *2.2.54*, with the following modifications.

Immerse the gel in a solution containing 36 g/L of *sulfosalicylic acid R*, 60 g/L of *trichloroacetic acid R* and 285 g/L of *methanol R*. Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and transfer the gel to a mixture of 80 volumes of *glacial acetic acid R*, 250 volumes of *ethanol R* and 670 volumes of *water R* (mixture A); while shaking, rinse for 10 min at room temperature. Immerse the gel in a staining solution (1 g/L solution of *acid blue 83 R* in mixture A) and incubate for 20 min at room temperature. Destain the gel by passive diffusion (for at least 6 h) with mixture A until the bands are well visualised against a clear background. Soak and wash the gel for 1 h with *water R*.

*System suitability*:

- in the electropherogram obtained with reference solution (a), 7 bands (4 major and 3 minor) in the pI region 7.35-8.30 are clearly visible;
- in the electropherogram obtained with reference solution (c), the relevant pI markers are distributed along the entire length of the gel.

*Results*:

- the electropherogram obtained with the test solution is similar to the electropherogram obtained with reference solution (b). Plot the migration distances of the relevant pI markers versus their pI and determine the isoelectric points of the principal components of the test solution and reference solution (b); they do not differ by more than 0.05 pI units;
- no additional bands are observed in the electropherogram obtained with the test solution in comparison to the electropherogram obtained with reference solution (b).

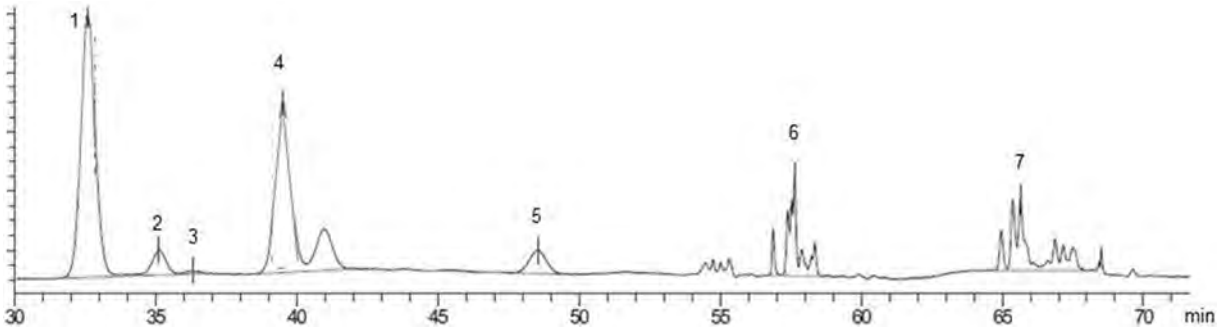


| *Peak* | *Charged* | *Glycoform* | *Peak* | *Charged* | *Glycoform* |
|---|---|---|---|---|---|
| 1. | No | Fucosylated G0F (absence of terminal galactose) | 5. | No | Fucosylated G2F (2 terminal galactoses) |
| 2. | No | Afucosylated Man5 (addition of 2 mannoses instead of terminal *N*-acetylglucosamine) | 6. | Yes | Sialylated SA1 (addition of 1 sialic acid) |
| 3. | No | Afucosylated G0 (absence of fucose) | 7. | Yes | Sialylated SA2 (addition of 2 sialic acids) |
| 4. | No | Fucosylated G1F (1 terminal galactose) | | | |

Figure 2928.-1. – *Chromatogram for glycan analysis of infliximab*

Alternatively, use a suitable capillary isoelectric focusing method developed according to general chapter *2.2.47. Capillary electrophoresis*.

B. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined with mobile phase A to obtain a concentration of about 1 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *infliximab CRS* in mobile phase A to obtain a concentration of about 1 mg/mL.

*Reference solution (b)*. Use a suitable infliximab in-house reference preparation shown to be representative of batches tested clinically and batches used to demonstrate consistency of production. Dilute with mobile phase A to obtain a concentration of about 1 mg/mL.

*Precolumn*:

- *size*: *l* = 0.05 m, Ø = 4 mm;
- *stationary phase*: *weak cation-exchange resin R* (10 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *weak cation-exchange resin R* (10 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 0.56 g of *sodium dihydrogen phosphate R* and 1.14 g of *disodium hydrogen phosphate dihydrate R* in 800 mL of *water for chromatography R*, and adjust to pH 7.25, if necessary, with *sodium hydroxide solution R* or *hydrochloric acid R*; dilute to 1000.0 mL with *water for chromatography R* and degas;
- *mobile phase B*: dissolve 0.56 g of *sodium dihydrogen phosphate R*, 1.14 g of *disodium hydrogen phosphate dihydrate R* and 58.44 g of *sodium chloride R* in 800 mL of *water for chromatography R*, and adjust to pH 7.25, if necessary, with *sodium hydroxide solution R* or *hydrochloric acid R*; dilute to 1000.0 mL with *water for chromatography R* and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 → 92 | 0 → 8 |
| 15 - 16 | 92 → 60 | 8 → 40 |
| 16 - 21 | 60 | 40 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 10 °C.

*Injection*: 50 µL.

*Relative retention* with reference to the peak due to isoform 6 (retention time = about 9.2 min): isoform 1 = 0.68; isoform 2 = 0.74; isoform 3 = 0.80; isoform 4 = 0.87; isoform 5 = 0.95.

*System suitability*: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *infliximab CRS*;
- *resolution*: minimum 1.5 between the peaks due to isoforms 3 and 4.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with reference solution (b);
- the relative retentions of the peaks due to isoforms 2, 3 and 4 in the chromatogram obtained with the test solution are within 1 per cent of those of the corresponding peaks in the chromatogram obtained with reference solution (b).

Calculate the relative peak areas of the individual peaks due to isoforms with reference to the total area of all peaks eluting between 3 min and 11 min.

*Limits*:

- *sum of isoforms 1 and 2*: as authorised by the competent authority;
- *sum of isoforms 3, 4 and 6*: as authorised by the competent authority;
- *isoform 5*: as authorised by the competent authority.

CHARACTERS

*Appearance*: opalescent or slightly opalescent, colourless or light yellow liquid.

IDENTIFICATION

A. It complies with the limits of the assay (potency).

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Dilution buffer*. Dissolve 50.00 g of *sucrose R*, 0.22 g of *sodium dihydrogen phosphate monohydrate R*, 0.61 g of *disodium hydrogen phosphate dihydrate R* and 0.05 g of *polysorbate 80 R* in *water R*. Adjust to pH 7.2, if necessary, and dilute to 500.0 mL with *water R*.

*Test solution*. Dilute the preparation to be examined with the dilution buffer to obtain a concentration of about 5 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *infliximab CRS* in the dilution buffer to obtain a concentration of about 5 mg/mL.

*Reduction and alkylation*. Dilute the test solution with *guanidine-tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.6 R* to obtain a concentration of 2 mg/mL. To 1 mL of this solution add 10 µL of a 154 g/L solution of *dithiothreitol R* and incubate at 37 °C for 1 h. Add 20 µL of a freshly prepared 185 g/L solution of *iodoacetamide R* and incubate at room temperature for 15 min, protected from light. Add 10 µL of a 154 g/L of solution of *dithiothreitol R* and mix well.

*Digestion*. Desalt a volume of the reduced solution prepared previously by a suitable method (for example, using a suitable centrifugal filter unit with *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R1* as elution buffer), and adjust the concentration to 1 mg/mL with *tris(hydroxymethyl)aminomethane buffer solution pH 7.5 R1*. Prepare a 0.5 mg/mL solution of *trypsin for peptide mapping R*, and add 10 µL of the solution to 100 µL of the desalted solution. Incubate at 37 °C for 16 h. Add 2 µL of a 150 g/L solution of *trifluoroacetic acid R* and mix gently using a vortex mixer.

*NOTE: a protease/protein ratio of 1:20 (w/w) is recommended.*

Carry out the reduction/alkylation and digestion steps for the reference solution in the same manner as for the test solution.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm;
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: add 0.6 mL of *trifluoroacetic acid R* to 1000 mL of *water for chromatography R*; degas;
- *mobile phase B*: add 0.6 mL of *trifluoroacetic acid R* to a mixture of 100 mL of *water for chromatography R* and 900 mL of *acetonitrile R1*; degas;

Monographs 1

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 115 | 100 → 50 | 0 → 50 |
| 115 - 115.5 | 50 → 10 | 50 → 90 |
| 115.5 - 135 | 10 | 90 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 80 µL.

*Identification of peaks*: use the chromatogram supplied with *infliximab CRS* to identify peaks 1 to 20.

*System suitability*: reference solution:

- the chromatogram obtained is qualitatively similar to the chromatogram supplied with *infliximab CRS* and peaks 1 to 20 are clearly visible;
- peaks 5 and 6 are separated as shown in the chromatogram supplied with *infliximab CRS*.

*Results*:

- the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution;
- no additional peak in the chromatogram obtained with the test solution has an area greater than 0.5 per cent of the sum of the areas of peaks 1 to 20.

TESTS

**pH** (*2.2.3*). As approved by the competent authority.

**Related proteins**. Capillary electrophoresis (*2.2.47*) under both reducing and non-reducing conditions.

*Sample buffer*. Dissolve 1 g of *sodium dodecyl sulfate R* in *tris(hydroxymethyl)aminomethane buffer solution pH 9.0 R1* and dilute to 100.0 mL with the same solution.

*Test solution*. Dilute the preparation to be examined with *water R* to obtain a concentration of 2 mg/mL. Mix 27 µL of this solution and 30 µL of sample buffer.

- *reducing conditions*: add 3 µL of *2-mercaptoethanol R* and incubate at 80 °C for 10 min; allow to cool for 5 min and transfer 60 µL to the autosampler vial;
- *non-reducing conditions*: add 3 µL of a 46.3 g/L solution of *iodoacetamide R* and incubate at 60-65 °C for 5 min; allow to cool for 5 min and transfer 60 µL to the autosampler vial.

*Reference solution*. Dissolve the contents of a vial of *infliximab CRS* in *water R* to obtain a concentration of 2 mg/mL. Mix 27 µL of the solution and 30 µL of sample buffer. Proceed at the same time and in the same manner as for the test solution.

*Capillary*:

- *material*: uncoated fused silica;
- *size*: total length = about 30 cm, effective length = 20 cm, Ø = 50 µm.

*Temperature*: 25 °C.

*Gel buffer*: use a formulation suitable for a sieving range of approximately 10-225 kDa.

*Acidic wash solution*: *dilute hydrochloric acid R3*.

*Basic wash solution*: 4 g/L solution of *sodium hydroxide R*.

*Detection*: spectrophotometer at 220 nm.

*Autosampler*: set at 25 °C.

*Preconditioning of the capillary*: rinse the capillary with the basic wash solution for 10 min at 138 kPa, with the acidic wash solution for 5 min at 138 kPa, with *water R* for 2 min at 138 kPa and with the gel buffer for 10 min at 483 kPa. Apply voltage for 10 min (15 kV reversed polarity).

*Between-run rinsing*: rinse the capillary with the basic wash solution for 3 min at 483 kPa, with the acidic wash solution for 1 min at 483 kPa, with *water R* for 1 min at 483 kPa and with the gel buffer for 10 min at 483 kPa.

*Injection*: electrokinetically at 5 kV reversed polarity for 20 s.

*Migration*: apply a voltage of 15 kV reversed polarity for 35 min using the gel buffer as the electrolyte in both buffer reservoirs.

*Migration time*:

- *reducing conditions*: light chain = 14 min to 17 min; non-glycosylated heavy chain = 17 min to 20 min; heavy chain = 18 min to 21 min;
- *non-reducing conditions*: intact IgG = 26 min to 32 min.

Calculate corrected areas of all peaks with a migration time greater than 11 min, using the following expression:

$$\frac{L_d \times A}{t}$$

$L_d$ = capillary length to detector;

$A$ = uncorrected peak area;

$t$ = migration time.

*System suitability*: reference solution:

- *reducing conditions*: the electropherogram obtained is qualitatively similar to the electropherogram supplied with *infliximab CRS*;
- *non-reducing conditions*: the electropherogram obtained is qualitatively similar to the electropherogram supplied with *infliximab CRS*.

*Result*:

- the profile of the electropherogram obtained with the test solution corresponds to that of the electropherogram obtained with the reference solution, except for minor peaks, that may be absent in the electropherogram obtained with the test solution.

Calculate individual peak areas expressed as a percentage of the sum of all corrected peak areas with a migration time greater than 11 min.

*Limits*:

*Reducing conditions*:

- *sum of all peaks other than heavy chain and light chain*: maximum 2 per cent;

*Non-reducing conditions*:

- *sum of all peaks other than the principal peak*: maximum 8 per cent.

**Impurities with molecular masses differing from that of infliximab**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined with the mobile phase to obtain a concentration of 8 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *infliximab CRS* in the mobile phase to obtain a concentration of 8 mg/mL.

*Reference solution (b)*. Reconstitute a mixture of thyroglobulin, gamma-globulin, ovalbumin, myoglobin and vitamin $B_{12}$ in *water R* to obtain an 18 mg/mL solution of molecular mass markers suitable for calibration in the range of 1350-670 000 Da. Further dilute 10 µL of the solution with *water for chromatography R* to obtain a concentration of 0.9 mg/mL.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5 µm) with a pore size of 25 nm and of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 10 000 to 500 000.

*Mobile phase*. Dissolve 0.78 g of *anhydrous sodium dihydrogen phosphate R*, 1.92 g of *anhydrous disodium hydrogen phosphate R* and 8.77 g of *sodium chloride R* in 900 mL of *water for chromatography R* and dilute to 1000.0 mL with *water for chromatography R*; filter and degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 10 °C.

*Injection*: 10 µL.

*Relative retention* with reference to infliximab monomer (retention time = about 8 min): high molecular weight species = about 0.88; low molecular weight species = about 1.28.

Calculate individual peak areas expressed as a percentage relative to the sum of all peak areas eluting between 5 min and 11 min. Individual relative per cent peak areas are calculated as the average of 3 injections.

*NOTE*: protein species that elute between 5 min and the monomer peak are classified as high molecular weight species, while those that elute after the monomer peak and before 11 min are classified as low molecular weight species.

*System suitability*:

- the chromatogram obtained with reference solution (a) is qualitatively similar to the chromatogram supplied with *infliximab CRS*;
- *resolution*: minimum 1.2 between the peaks due to gamma-globulin and ovalbumin in the chromatogram obtained with reference solution (b).

*Result*:

- the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (a).

*Limit*:

- *sum of all peaks other than the monomer peak*: maximum 2 per cent.

ASSAY

**Protein** (*2.5.33, Method 1*).

*Test solution*. Dilute the preparation to be examined with a suitable buffer to obtain a concentration of about 1 mg/mL. Prepare and analyse each preparation in duplicate.

Record the UV spectrum between 280 nm and 350 nm. Measure the value at the absorbance maximum of 280 nm, after correction for any light scattering measured up to 350 nm. Calculate the protein content, taking the specific absorbance to be 14.5.

**Potency**. The potency of infliximab is determined by comparison of dilutions of the test preparation with dilutions of *infliximab BRP* using a suitable cell-based assay based on the inhibitory action of infliximab on the biological activity of TNF-α with a suitable readout for assessing this inhibitory effect.

*The following procedure is given as an example.*

Carry out a cell proliferation assay based on the ability of infliximab to block TNF-induced inhibition of murine fibrosarcoma WEHI-164 cell proliferation. The WEHI-164 cells (ATCC No. CRL-1751) are incubated with varying dilutions of test and reference preparations of infliximab in the presence of TNF-α. Cell growth is assessed by a staining method using the tetrazolium salt WST-8 ([2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt]), which is converted by cellular dehydrogenases to a coloured formazan product. The amount of released formazan is then measured spectrophotometrically and is directly proportional to the number of living cells.

*Assay medium*. RPMI 1640 containing 4.5 g/L *glucose R*, 1.5 g/L *sodium hydrogen carbonate R*, 0.11 g/L *sodium pyruvate R*, 2.4 g/L *HEPES R* (10 mM), 300 g/L *L-glutamine R*, heat-inactivated foetal bovine serum (10 per cent *V/V*) and a penicillin/streptomycin solution (1 per cent *V/V*) containing 10 000 U/mL of *benzylpenicillin sodium R* and 10 000 µg/mL of *streptomycin sulfate R* in an 8.5 g/L solution of *sodium chloride R*.

*Test solution*. Dilute the preparation to be examined with assay medium to obtain a concentration of about 640 ng/mL. Analyse in duplicate.

*Reference solution*. Reconstitute the contents of 1 ampoule of *infliximab BRP* with sterilised *water for injections R* to obtain a concentration of 500 IU/mL. Further dilute with assay medium to obtain a concentration of 6.4 IU/mL. Analyse in duplicate.

*TNF-α working solutions*. Dissolve the contents of a vial of TNF-α according to the supplier's instructions. Further dilute with assay medium to obtain 2 suitable working concentrations in the range of 400-50 000 pg/mL (working solution A and working solution B (dilution of A)). As the biological activity of TNF-α is likely to vary between different suppliers and also between different batches from the same supplier, this should be controlled by use of an appropriate standard (e.g. WHO International Standard for TNF-α).

*Method*.

*Plate preparation*. Add 150 µL of assay medium to the wells designated for 'cell only control' (columns 1-6, row H) and for blanks (columns 2-12, row A) on a 96-well microplate. Add 187.5 µL of TNF-α working solution A (column 1, row A) and further perform 5-fold dilutions (columns 2-12, row A) to generate the 'TNF-α control curve'. Add 100 µL of assay medium and 50 µL of TNF-α working solution B to the wells designated for 'cell + TNF-α control' (columns 7-12, row H). Add 100 µL of assay medium to the sample wells (columns 2-12, rows B-G) and 200 µL of the test or reference solutions (column 1, rows B-G). Further prepare a series of 2-fold dilutions (columns 2-12, rows B-G). Then, add 50 µL of TNF-α working solution B (columns 1-12, rows B-G). Incubate at 36.0-38.0 °C for 1 h in an incubator using 5 ± 2 per cent $CO_2$.

*Cell preparation*. Prepare a suspension of WEHI-164 cells containing $1 \times 10^6$ cells per millilitre, using assay medium containing 2 µg/mL of actinomycin D.

*Plating cells*. Add 50 µL of the cell suspension to each well maintaining the cells in a uniform suspension during addition. Incubate at 36.0-38.0 °C for 20-24 h in an incubator using 5 ± 2 per cent $CO_2$.

*Addition of tetrazolium salt*. Remove 100 µL of medium from each well, add 10 µL of reconstituted WST-8 mixture to each well and reincubate for 3-4 h. Estimate the quantity of formazan produced using a microtitre well plate reader at 450 nm and 650 nm. Subtract the reading at 650 nm from the reading at 450 nm.

Calculate the potency of the preparation to be examined using the four-parameter logistic curve model (see general chapter *5.3. Statistical analysis*).

*System suitability*:

- the 'TNF-α control curve' corresponds to a sigmoid curve;
- the coefficient of determination calculated for the 'TNF-α control curve' ($r^2$) is not less than 0.97;
- the standard curve corresponds to a sigmoid curve with upper and lower plateaus corresponding to 'cell only control' and 'cell + TNF-α control' respectively;
- the coefficient of determination calculated for the standard curve ($r^2$) is not less than 0.97;
- *maximum value (cell only) to minimum value (TNF-α control) ratio*: minimum 3.0.

Calculate the potency of the preparation to be examined using a suitable statistical method (see general chapter *5.3. Statistical analysis*).

*Result*: the estimated potency is not less than 80 per cent and not more than 120 per cent relative to the reference solution. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

In an airtight container, under approved conditions.

LABELLING

The label states the content in milligrams of protein per millilitre.

**07/2019:1805**

# *myo*-INOSITOL

## *myo*-Inositolum

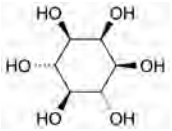

$C_6H_{12}O_6$ $M_r$ 180.2
[87-89-8]

DEFINITION

Cyclohexane-1,2,3,5/4,6-hexol.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *myo-inositol CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 10.0 g in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Conductivity** (*2.2.38*): maximum 30 µS·cm$^{-1}$.

Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, with gentle warming if necessary, and dilute to 50.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.500 g of *myo-inositol CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dissolve 0.5 g of *myo-inositol R* and 0.5 g of *mannitol R* in *water R* and dilute to 10 mL with the same solvent.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 85 ± 2 °C.

*Mobile phase*: *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of *myo*-inositol.

*Relative retention* with reference to *myo*-inositol (retention time = about 17.5 min): impurity A = about 1.3; impurity B = about 1.4.

*System suitability*: reference solution (c):

- *resolution*: minimum 4.0 between the peaks due to *myo*-inositol and impurity A.

*Limits*:

- *impurities A, B*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_6H_{12}O_6$ taking into account the assigned content of *myo-inositol CRS*.

IMPURITIES

*Specified impurities: A, B.*

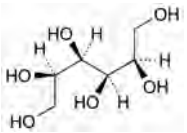

A. D-mannitol,

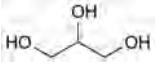

B. propane-1,2,3-triol (glycerol).

**01/2008:2084**
**corrected 10.0**

# INSULIN ASPART

## Insulinum aspartum

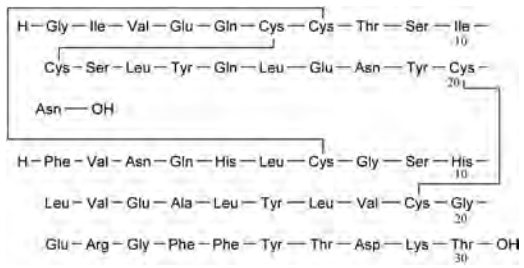


$C_{256}H_{381}N_{65}O_{79}S_6$ $M_r$ 5826

DEFINITION

$28^B$-L-Aspartate insulin (human).

Insulin aspart is a 2-chain peptide containing 51 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. It is identical in primary structure to human insulin, except that it has aspartic acid instead of proline at position 28 of the B-chain. As in human insulin, insulin aspart contains 2 interchain disulfide bonds and 1 intrachain disulfide bond.

*Content*: 90.0 per cent to 104.0 per cent of insulin aspart $C_{256}H_{381}N_{65}O_{79}S_6$ plus A21Asp insulin aspart, B3Asp insulin aspart, B3isoAsp insulin aspart and B28isoAsp insulin aspart (dried substance).

By convention, for the purpose of labelling insulin aspart preparations, 0.0350 mg of insulin aspart is equivalent to 1 unit.

PRODUCTION

Insulin aspart is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Prior to release, the following tests are carried out on each batch of insulin aspart, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Single-chain precursor**. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in ethanol (96 per cent), in methanol and in aqueous solutions with a pH around 5.1. In aqueous solutions below pH 3.5 or above pH 6.5, the solubility is greater than or equal to 25 mg/mL.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 25 µL of this solution to a clean tube. Add 100 µL of *HEPES buffer solution pH 7.5 R* and 20 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 145 µL of *sulfate buffer solution pH 2.0 R*.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution, but using *insulin aspart CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm) with a pore size of 8 nm,
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 100 mL of *acetonitrile for chromatography R*, 200 mL of *sulfate buffer solution pH 2.0 R* and 700 mL of *water R*; filter and degas;
- *mobile phase B*: mix 200 mL of *sulfate buffer solution pH 2.0 R*, 400 mL of *acetonitrile for chromatography R* and 400 mL of *water R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 90 → 30 | 10 → 70 |
| 60 - 65 | 30 → 0 | 70 → 100 |
| 65 - 70 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 50 µL.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of insulin aspart digest supplied with *insulin aspart CRS*,
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III:

  *symmetry factor*: maximum 1.5, for the peaks due to fragments II and III,

  *resolution* minimum 8.0, between the peaks due to fragments II and III.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*NOTE: the retention times of fragments I, II and IV are the same as for human insulin. The retention time of fragment III differs from human insulin due to substitution of proline by aspartic acid.*

TESTS

**Impurities with molecular masses greater than that of insulin aspart**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Prepare a solution containing 4 mg/mL of the substance to be examined in *0.01 M hydrochloric acid*. Maintain the solution at 2-8 °C and use within 48 h.

*Resolution solution.* Use a solution of insulin (about 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid* may be used. Insulin containing the indicated percentage of

Monographs

high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2-8 °C and use within 7 days.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm,

– *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile for chromatography R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Equilibration*: at least 3 injections of the resolution solution; the column is equilibrated when repeatable results are obtained from 2 subsequent injections.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention time*: insulin aspart polymers = 13-17 min; insulin aspart dimer = about 17.5 min; insulin aspart monomer = about 20 min; salts = about 22 min.

*System suitability*: resolution solution:

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limits*: the sum of the areas of the peaks with a retention time less than that of the principal peak is not more than 0.5 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin aspart monomer.

**Related proteins**. Liquid chromatography (*2.2.29*) as described under Assay: use the normalisation procedure.

*Limits*:

– *B28isoAsp insulin aspart*: maximum 1.0 per cent,

– *total of the peaks due to A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart*: maximum 2.0 per cent,

– *total of other impurities*: maximum 1.5 per cent.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C for 24 h.

**Sulfated ash** (*2.4.14*): maximum 6.0 per cent, determined on 0.200 g (dried substance).

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve the substance to be examined in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL. Maintain the solution at 2-8 °C and use within 24 h.

*Reference solution*. Dissolve the contents of a vial of *insulin aspart CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL. Maintain the solution at 2-8 °C and use within 48 h.

*Resolution solution*. Use an appropriate solution with a content of B3Asp insulin aspart and A21Asp insulin aspart of not less than 1 per cent. This may be achieved by storing reference solution at room temperature for about 1-3 days. Maintain the solution at 2-8 °C and use within 72 h.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 142.0 g of *anhydrous sodium sulfate R* in *water R*; add 13.5 mL of *phosphoric acid R* and dilute to 5000 mL with *water R*; adjust to pH 3.6, if necessary, with *strong sodium hydroxide solution R*; filter and degas; mix 9 volumes of the solution with 1 volume of *acetonitrile for chromatography R*; filter and degas;

– *mobile phase B*: mix equal volumes of *water R* and *acetonitrile for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 58 | 42 |
| 35 - 40 | 58 → 20 | 42 → 80 |
| 40 - 45 | 20 | 80 |
| 45 - 46 | 20 → 58 | 80 → 42 |
| 46 - 60 | 58 | 42 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL.

*Relative retention* with reference to insulin aspart (retention time = 20-24 min): B28isoAsp insulin aspart = about 0.9; B3Asp insulin aspart plus A21Asp insulin aspart (generally coeluted) = about 1.3; B3isoAsp insulin aspart = about 1.5.

*System suitability*: resolution solution:

– *resolution*: minimum 2.0 between the peak due to insulin aspart and the peak due to A21Asp insulin aspart and to B3Asp insulin aspart.

Calculate the content of insulin aspart $C_{256}H_{381}N_{65}O_{79}S_6$, plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution and the declared content of insulin aspart plus B28isoAsp insulin aspart, A21Asp insulin aspart, B3Asp insulin aspart and B3isoAsp insulin aspart in *insulin aspart CRS*.

STORAGE

In an airtight container, protected from light, at or below − 18 °C until released by the manufacturer. When thawed, insulin aspart is stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin aspart must be at room temperature before opening the container.

**01/2008:1637**
**corrected 10.0**

# INSULIN, BOVINE

## Insulinum bovinum

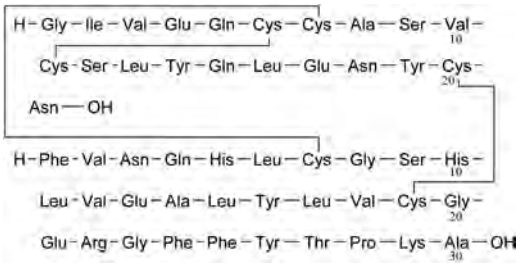


$C_{254}H_{377}N_{65}O_{75}S_6$ $M_r$ 5734

DEFINITION

Bovine insulin is the natural antidiabetic principle obtained from beef pancreas and purified.

*Content*:

– *sum of bovine insulin ($C_{254}H_{377}N_{65}O_{75}S_6$) and A21 desamido bovine insulin*: 93.0 per cent to 105.0 per cent (dried substance).

By convention, for the purpose of labelling insulin preparations, 0.0342 mg of bovine insulin is equivalent to 1 IU of insulin.

PRODUCTION

The animals from which bovine insulin is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (c).

B. Peptide mapping

*Test solution.* Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 500 µL of this solution to a clean tube. Add 2.0 mL of *HEPES buffer solution pH 7.5 R* and 400 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 mL of *sulfate buffer solution pH 2.0 R*.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *bovine insulin CRS* instead of the substance to be examined.

Examine the digests by liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 100 mL of *acetonitrile for chromatography R*, 700 mL of *water for chromatography R* and 200 mL of *sulfate buffer solution pH 2.0 R*; filter and degas;

– *mobile phase B*: mix 400 mL of *acetonitrile for chromatography R*, 400 mL of *water for chromatography R* and 200 mL of *sulfate buffer solution pH 2.0 R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 90 → 30 | 10 → 70 |
| 60 - 65 | 30 → 0 | 70 → 100 |
| 65 - 70 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 50 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of bovine insulin digest supplied with *bovine insulin CRS*. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III. The symmetry factor of the peaks due to fragments II and III is not greater than 1.5, and the resolution between the 2 peaks is at least 1.9.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*NOTE: The retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.*

TESTS

**Impurities with molecular masses greater than that of insulin**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure. *Maintain the solutions at 2-10 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-10 °C.*

*Test solution.* Dissolve 4 mg of the substance to be examined in 1.0 mL of *0.01 M hydrochloric acid*.

*Resolution solution.* Use a solution of insulin (approximately 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

*Column*:

– *size*: *l* = 0.3 m, Ø = at least 7.5 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm), of a grade suitable for the separation of insulin monomer from dimer and polymers.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Equilibration*: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention times*: polymeric insulin complexes = 13 min to 17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

*System suitability*: resolution solution:

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limits*: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks; disregard any peak with a retention time greater than that of the insulin peak.

**Related proteins**. Liquid chromatography (*2.2.29*) as described under Assay, following the elution conditions as described in the table below.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 42 | 58 |
| 30 - 44 | 42 → 11 | 58 → 89 |
| 44 - 50 | 11 | 89 |

*Maintain the solutions at 2-10 °C and use within 24 h.* Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µL of reference solution (c) and 20 µL of the test solution. If necessary, adjust the injection volume to between 10 µL and 20 µL in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (c), A21 desamido bovine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido bovine insulin is not greater than 3.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to bovine insulin and A21 desamido bovine insulin, is not greater than 3.0 per cent of the total area of the peaks.

**Bovine proinsulin-like immunoreactivity (PLI)**: maximum 10 ppm (dried substance).

Use a suitably sensitive immunochemical method (*2.7.1*) such as radio-immunoassay, using the International Reference Reagent for bovine proinsulin to calibrate the method.

**Zinc**: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid*.

*Reference solutions.* Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Flame*: air-acetylene flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C for 24 h.

**Sulfated ash** (*2.4.14*): maximum 2.5 per cent, determined on 0.200 g (dried substance).

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve a suitable amount of the substance to be examined in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (a).* Dissolve the contents of a vial of *human insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (b).* Dissolve the contents of a vial of *insulin porcine for system suitability CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4 mg/mL.

*Reference solution (c).* Dissolve the contents of a vial of *bovine insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 10.0 mL with *0.01 M hydrochloric acid*.

*Resolution solution.* Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b).

Maintain the solutions at 2-10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2-10 °C.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- *mobile phase A*: dissolve 28.4 g of *anhydrous sodium sulfate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; add 2.7 mL of *phosphoric acid R*; adjust to pH 2.3, if necessary, with *ethanolamine R*; filter and degas;
- *mobile phase B*: mix 550 mL of mobile phase A with 450 mL of *acetonitrile R*; warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*System suitability*:

- *resolution*: inject 20 µL of the resolution solution and 20 µL of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved;
- *linearity*: inject 20 µL each of reference solutions (c) and (d). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (c) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d). If this test fails, adjust the injection volume to between 10 µL and 20 µL, in order that the responses are within the linearity range of the detector.

*Injection*: 20 µL of the test solution.

Calculate the content of bovine insulin $C_{254}H_{377}N_{65}O_{75}S_6$ plus A21 desamido bovine insulin from the area of the principal peak and the area of the peak due to A21 desamido bovine insulin in the chromatograms obtained with the test solution and reference solution (c) and the declared content of bovine insulin plus A21 desamido bovine insulin in *bovine insulin CRS*.

STORAGE

In an airtight container, protected from light, at – 20 °C until released by the manufacturer. When thawed, insulin may be stored at 5 ± 3 °C and used for manufacturing preparations

within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

**07/2018:2571**
**corrected 10.0**

# INSULIN GLARGINE

## Insulinum glarginum

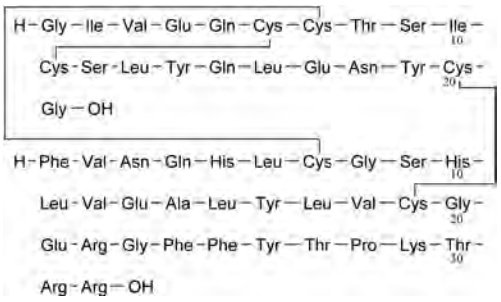


$C_{267}H_{404}N_{72}O_{78}S_6$ $M_r$ 6063

DEFINITION

21$^A$-Glycine-30$^B$a-L-arginine-30$^B$b-L-arginine-insulin (human).

2-chain peptide containing 53 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 32 amino acids. It is identical in primary structure to human insulin, only differing in amino acid sequence at position 21 in the A-chain and at the C-terminal end of the B-chain where it contains 2 additional amino acids. Human insulin is Asn(A21), whereas insulin glargine is Gly(A21), Arg(B31), Arg(B32). As in human insulin, insulin glargine contains 2 interchain disulfide bonds and 1 intrachain disulfide bond.

*Content*: 94.0 per cent to 105.0 per cent (anhydrous substance).

By convention, for the purpose of labelling insulin glargine preparations, 0.0364 mg of insulin glargine is equivalent to 1 unit.

PRODUCTION

Insulin glargine is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Prior to release, the following tests are carried out on each batch of insulin glargine, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Single-chain precursor**. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water and in anhydrous ethanol, soluble in dilute mineral acids.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Prepare a 10.0 mg/mL solution of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and transfer 5 µL of the solution to a clean tube. Add 1.0 mL of *1 M tris-hydrochloride buffer solution pH 7.5 R* and 100 µL of a 20 U/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R* in *1 M tris-hydrochloride buffer solution pH 7.5 R*. Mix and incubate at 45 °C for about 2 h. Stop the reaction by adding 2 µL of *phosphoric acid R*.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *insulin glargine CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 11.6 g of *phosphoric acid R* and 42.1 g of *sodium perchlorate R* in 1600 mL of *water for chromatography R*, adjust to pH 2.3 with *triethylamine R* and dilute to 2000 mL with *water for chromatography R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 3.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution (7:93 *V/V*);
- *mobile phase B*: buffer solution, *acetonitrile R1* (43:57 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 90 → 20 | 10 → 80 |
| 30 - 35 | 20 | 80 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min.

*Injection*: 50 µL.

*Retention time*: insulin glargine fragment II = about 14 min; insulin glargine fragment III = about 15 min.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of insulin glargine digest supplied with *insulin glargine CRS*;
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments II and III:

  *symmetry factor*: maximum 1.5 for the peaks due to fragments II and III;

  *resolution*: minimum 3.4 between the peaks due to fragments II and III.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*NOTE: the retention times of fragments I and IV are the same as for human insulin; the retention times of fragments II and III differ from human insulin due to the difference in the sequence at position 21 of the A-chain and to the 2 additional amino acids of the B-chain.*

TESTS

**Impurities with molecular masses greater than that of insulin glargine**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Prepare a 4 mg/mL solution of the substance to be examined in a 1 g/L solution of *hydrochloric acid R*.

*Resolution solution.* Dry about 200 mg of the substance to be examined in an oven at 100 °C for 1.5-3 h. Dissolve 15.0 mg of the dried substance in 1.5 mL of a 1 g/L solution of *hydrochloric acid R* and dilute to 10.0 mL with *water R*.

*Reference solution.* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 3.0 mL of this solution to 20.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (10 µm) with a pore size of 12.5 nm, of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention time*: insulin glargine = about 18 min.

*System suitability*:

- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with the reference solution;
- *symmetry factor*: maximum 2.0 for the peak due to insulin glargine in the chromatogram obtained with the resolution solution;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to high molecular mass proteins and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine in the chromatogram obtained with the resolution solution.

*Limits*:

- *total of impurities with a retention time less than that of insulin glargine*: maximum 0.3 per cent of the total area of the peaks; disregard any peak with a retention time greater than that of the peak due to insulin glargine.

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Maintain the solutions at 2-8 °C.*

*Test solution.* Dissolve 15.0 mg of the substance to be examined in 1.5 mL of a 1 g/L solution of *hydrochloric acid R* and dilute to 10.0 mL with *water R*.

*Reference solution.* Dissolve the contents of a vial of *insulin glargine CRS* in 1.5 mL of a 1 g/L solution of *hydrochloric acid R*, transfer the solution with *water R* to a 10 mL volumetric flask and dilute to 10.0 mL with *water R*.

*Resolution solution.* Dissolve the contents of a vial of *insulin glargine for peak identification CRS* (containing $0^A$-Arg-insulin glargine) in 0.3 mL of a 1 g/L solution of *hydrochloric acid R* and add 1.7 mL of *water R*.

*Buffer solution.* Dissolve 20.7 g of *anhydrous sodium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 3.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 18.4 g of *sodium chloride R* in 250 mL of the buffer solution, add 250 mL of *acetonitrile R1* and mix; dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: dissolve 3.2 g of *sodium chloride R* in 250 mL of the buffer solution, add 650 mL of *acetonitrile R1* and mix; dilute to 1000 mL with *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 96 → 83 | 4 → 17 |
| 20 - 30 | 83 → 63 | 17 → 37 |
| 30 - 33 | 63 → 96 | 37 → 4 |
| 33 - 40 | 96 | 4 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 5 µL of the test solution and the resolution solution.

*Retention time*: insulin glargine = about 20 min.

*System suitability*: resolution solution:

- *peak-to-valley ratio*: minimum 2, where $H_p$ = height above the baseline of the peak due to $0^A$-Arg-insulin glargine and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to insulin glargine.

*Limits*:

- *any impurity*: for each impurity, maximum 0.4 per cent;
- *total*: maximum 1.0 per cent.

**Zinc**: maximum 0.80 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 45.0 mg of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solution. Dilute 10.0 mL of the solution to 100.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Reference solutions.* Prepare reference solutions containing 0.2 µg, 0.4 µg and 0.6 µg of zinc per millilitre by diluting *zinc standard solution (10 ppm Zn) R* with a 1 g/L solution of *hydrochloric acid R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Water** (*2.5.32*): maximum 8.0 per cent, determined on 30.0 mg.

**Bacterial endotoxins** (*2.6.14, Method D*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related proteins with the following modification.

*Injection*: 5 µL of the test solution and the reference solution.

Calculate the content of insulin glargine ($C_{267}H_{404}N_{72}O_{78}S_6$) taking into account the assigned content of *insulin glargine CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of − 20 ± 5 °C.

**01/2011:0838**
**corrected 10.0**

# INSULIN, HUMAN

## Insulinum humanum

$C_{257}H_{383}N_{65}O_{77}S_6$ $M_r$ 5808

DEFINITION

Human insulin is a 2-chain peptide having the structure of the antidiabetic hormone produced by the human pancreas.

*Content*: 95.0 per cent to 105.0 per cent of human insulin $C_{257}H_{383}N_{65}O_{77}S_6$ plus A21 desamido human insulin (dried substance).

By convention, for the purpose of labelling insulin preparations, 0.0347 mg of human insulin is equivalent to 1 IU of insulin.

PRODUCTION

Human insulin is produced either by enzymatic modification and suitable purification of insulin obtained from the pancreas of the pig or by a method based on recombinant DNA (rDNA) technology.

Where applicable, the animals from which human insulin is derived must fulfil the requirements for the health of animals suitable for human consumption.

Human insulin is produced under conditions designed to minimise the degree of microbial contamination.

For human insulin produced by enzymatic modification of insulin obtained from the pancreas of the pig, the manufacturing process is validated to demonstrate removal of any residual proteolytic activity. The competent authority may require additional tests.

For human insulin produced by a method based on rDNA technology, prior to release the following tests are carried out on each batch of human insulin, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Single chain precursor**. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. Peptide mapping (*2.2.55*).
*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*
*Test solution.* Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 500 µL of this solution to a clean tube. Add 2.0 mL of *HEPES buffer solution pH 7.5 R* and 400 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 mL of *sulfate buffer solution pH 2.0 R*.
*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *human insulin CRS* instead of the substance to be examined.
*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).
*Column*:
– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm) with a pore size of 8 nm;
– *temperature*: 40 °C.
*Mobile phase*:
– *mobile phase A*: mix 100 mL of *acetonitrile for chromatography R*, 200 mL of *sulfate buffer solution pH 2.0 R* and 700 mL of *water R*; filter and degas;
– *mobile phase B*: mix 200 mL of *sulfate buffer solution pH 2.0 R*, 400 mL of *acetonitrile for chromatography R* and 400 mL of *water R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 90 → 30 | 10 → 70 |
| 60 - 65 | 30 → 0 | 70 → 100 |
| 65 - 70 | 0 | 100 |

*Flow rate*: 1 mL/min.
*Detection*: spectrophotometer at 214 nm.
*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.
*Injection*: 50 µL.
*System suitability*:
– the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of human insulin digest supplied with *human insulin CRS*;
– in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III;
*symmetry factor*: maximum 1.5 for the peaks due to fragments II and III;
*resolution*: minimum 3.4 between the peaks due to fragments II and III;
*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.
*NOTE: the retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.*

TESTS

**Impurities with molecular masses greater than that of insulin**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.
*Test solution.* Prepare a solution containing 4 mg/mL of the substance to be examined in *0.01 M hydrochloric acid*.
*Resolution solution.* Use a solution of insulin (about 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated

Monographs 1

percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

Maintain the solutions at 2-8 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-8 °C.

*Column*:

- *size*: *l* = 0.3 m, Ø = minimum 7.5 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Equilibration*: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention time*: polymeric insulin complexes = 13-17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

*System suitability*: resolution solution:

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limits*: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin.

**Related proteins**. Liquid chromatography (*2.2.29*) as described under Assay, following the elution conditions as described below:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 42 | 58 |
| 30 - 44 | 42 → 11 | 58 → 89 |
| 44 - 50 | 11 | 89 |

Maintain the solutions at 2-8 °C and use within 24 h. Perform a system suitability test (resolution, linearity) as described in the assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µL of reference solution (a), 20 µL of reference solution (b), 20 µL of reference solution (c) and 20 µL of the test solution. If necessary, adjust the injection volume to a volume between 10 µL and 20 µL in accordance with the results obtained in the test for linearity as described in the assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (a), A21 desamido human insulin appears as a small peak after the principal peak and has a retention time of about 1.3 relative to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido human insulin is not greater than 2.0 per cent of the total area of the peaks; the sum of the areas of all peaks, apart from those due to human insulin and that due to A21 desamido human insulin, is not greater than 2.0 per cent of the total area of the peaks. For semi-synthetic human insulin only: in the chromatogram obtained with the test solution, the area of any peak corresponding to the peak due to porcine insulin in the chromatogram obtained with reference solution (c) is not greater than 1.0 per cent of the total area of the peaks.

*The following test applies only to human insulin produced by enzymatic modification of porcine insulin.*

**Proinsulin-like immunoreactivity (PLI)**: maximum 10 ppm, calculated with reference to the dried substance and determined by a suitably sensitive immunochemical method (*2.7.1*) such as radio-immunoassay. Use the International Reference Reagent for porcine proinsulin to calibrate the method.

**Zinc**: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4-1.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid*.

*Reference solutions.* Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C for 24 h.

**Sulfated ash** (*2.4.14*): maximum 2.5 per cent, determined on 0.200 g (dried substance).

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *human insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (b).* Dissolve the contents of a vial of *insulin porcine for system suitability CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4 mg/mL.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 50.0 mL with *0.01 M hydrochloric acid.* To 1.0 mL of this solution add 1.0 mL of reference solution (a).

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *0.01 M hydrochloric acid.*

*Resolution solution.* Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b).

Maintain the solutions at 2-8 °C and use within 48 h. If an automatic injector is used, maintain at 2-8 °C.

*Column*:

- *size*: *l* = 0.25, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

– *mobile phase A*: dissolve 28.4 g of *anhydrous sodium sulfate R* in *water R* and dilute to 1000 mL with the same solvent; add 2.7 mL of *phosphoric acid R*; adjust to pH 2.3, if necessary, with *ethanolamine R*; filter and degas;
– *mobile phase B*: mix 550 mL of mobile phase A with 450 mL of *acetonitrile R*. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*System suitability*:

– *resolution*: inject 20 µL of the resolution solution and 20 µL of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
– *linearity*: inject 20 µL each of reference solutions (a) and (d). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (a) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d). If this test fails, adjust the injection volume to between 10 µL and 20 µL, in order that the responses are within the linearity range of the detector.

*Injection*: 20 µL of the test solution and reference solution (a).

Calculate the content of human insulin $C_{257}H_{383}N_{65}O_{77}S_6$ plus A21 desamido human insulin using the areas of the corresponding peaks in the chromatograms obtained with the test solution and reference solution (a) and the declared content of human insulin plus A21 desamido human insulin in *human insulin CRS*.

STORAGE

In an airtight container, protected from light, at – 18 °C or below, until released by the manufacturer. When thawed, insulin is stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

LABELLING

The label states whether the substance is produced by enzymatic modification of porcine insulin or by rDNA technology.

**01/2008:0831**
**corrected 6.0**

# INSULIN INJECTION, BIPHASIC

## Insulinum biphasicum iniectabile

*Biphasic insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.*

DEFINITION

Biphasic insulin injection is a sterile suspension of crystals containing bovine insulin in a solution of porcine insulin.

CHARACTERS

A white or almost white suspension. When examined under a microscope, the majority of the particles are seen to be rhombohedral crystals, with a maximum dimension measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The position of the peaks due to the two insulins in the chromatogram obtained with the test solution correspond to those of the principal peaks in the chromatogram obtained with the appropriate reference solution.

TESTS

**pH** (*2.2.3*). The pH of the suspension to be examined is 6.6 to 7.2.

**Insulin in the supernatant**: 22.0 per cent to 28.0 per cent of insulin in solution. Determine by the method described in the test for insulin in the supernatant in the monograph on *Insulin preparations, injectable (0854)*.

**Total zinc**: 26.0 µg to 37.5 µg per 100 IU of insulin. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

**01/2008:0832**
**corrected 6.0**

# INSULIN INJECTION, BIPHASIC ISOPHANE

## Insulinum isophanum biphasicum iniectabile

*Biphasic isophane insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the exception of the test for Insulin in the supernatant and with the amendments prescribed below for the other tests.*

DEFINITION

Biphasic isophane insulin injection is a sterile buffered suspension of either porcine or human insulin, complexed with protamine sulfate or another suitable protamine, in a solution of insulin of the same species.

PRODUCTION

Biphasic isophane insulin injection is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

Biphasic isophane insulin injection is produced by mixing, in defined ratios, soluble insulin injection and isophane insulin injection. The defined ratios shall be demonstrated by a test method which has been approved by the competent authority to comply with the label claim.

CHARACTERS

A white or almost white suspension which on standing deposits a white or almost white sediment and leaves a colourless or almost colourless supernatant; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak obtained with the appropriate reference solution.

TESTS

**Total zinc**. Not more than 40.0 µg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

LABELLING

The label states in addition to the indications mentioned in the monograph on *Insulin preparations, injectable (0854)* the ratio of soluble insulin injection to isophane insulin injection used in the manufacturing process of biphasic isophane insulin injection.

**01/2008:0833**
**corrected 6.0**

# INSULIN INJECTION, ISOPHANE

## Insulinum isophanum iniectabile

*Isophane insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the modifications prescribed below.*

DEFINITION

Isophane insulin injection is a sterile suspension of bovine, porcine or human insulin, complexed with protamine sulfate or another suitable protamine.

PRODUCTION

Isophane insulin injection is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

The amount of protamine is based on the known isophane ratio and is not less than the equivalent of 0.3 mg and not more than the equivalent of 0.6 mg of protamine sulfate for each 100 IU of insulin in the insulin-protamine complex.

CHARACTERS

A white or almost white suspension which on standing deposits a white or almost white sediment and leaves a colourless or almost colourless supernatant; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rod-shaped crystals, the majority with a maximum dimension greater than 1 µm but rarely exceeding 60 µm, free from large aggregates.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

**Total zinc**. Not more than 40.0 µg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

**01/2008:0834**

# INSULIN INJECTION, SOLUBLE

## Insulinum solubile iniectabile

*Soluble insulin injection complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.*

DEFINITION

Soluble insulin injection is a neutral, sterile solution of bovine, porcine or human insulin.

CHARACTERS

A colourless liquid, free from turbidity and foreign matter; during storage, traces of a very fine sediment may be deposited.

IDENTIFICATION

Examine the chromatograms obtained in the assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak obtained with the appropriate reference solution.

TESTS

**Total zinc**. Not more than 40.0 µg per 100 IU of insulin.

Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

*Use the following test solution.*

*Test solution.* Dilute a volume of the gently shaken preparation containing 200 IU to 25.0 mL with *water R*. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with *water R*.

**01/2008:2085**
**corrected 10.0**

# INSULIN LISPRO

## Insulinum lisprum

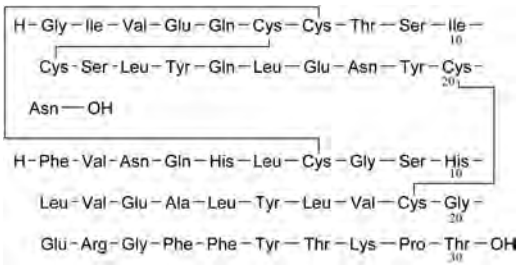


$C_{257}H_{383}N_{65}O_{77}S_6$ $M_r$ 5808

DEFINITION

$28^B$-L-Lysine-$29^B$-L-proline insulin (human).

Insulin lispro is a 2-chain peptide containing 51 amino acids. The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. It is identical in primary structure to human insulin, only differing in amino acid sequence at positions 28 and 29 of the B-chain. Human insulin is Pro(B28), Lys(B29), whereas insulin lispro is Lys(B28), Pro(B29). As in human insulin, insulin lispro contains 2 interchain disulfide bonds and 1 intrachain disulfide bond.

*Content*: 94.0 per cent to 104.0 per cent (dried substance).

By convention, for the purpose of labelling insulin lispro preparations, 0.0347 mg of insulin lispro is equivalent to 1 unit.

PRODUCTION

Insulin lispro is produced by a method based on recombinant DNA (rDNA) technology under conditions designed to minimise the degree of microbial contamination.

Prior to release, the following tests are carried out on each batch of insulin lispro, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Single-chain precursor**. The limit is approved by the competent authority. Use a suitably sensitive method.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

B. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution*. Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 500 µL of this solution to a clean tube. Add 2.0 mL of *HEPES buffer solution pH 7.5 R* and 400 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 mL of *sulfate buffer solution pH 2.0 R*.

*Reference solution*. Prepare at the same time and in the same manner as for the test solution but using *insulin lispro CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm) with a pore size of 8 nm,
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 100 mL of *acetonitrile for chromatography R*, 200 mL of *sulfate buffer solution pH 2.0 R* and 700 mL of *water R*; filter and degas;
- *mobile phase B*: mix 200 mL of *sulfate buffer solution pH 2.0 R*, 400 mL of *acetonitrile for chromatography R* and 400 mL of *water R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 90 → 30 | 10 → 70 |
| 60 - 65 | 30 → 0 | 70 → 100 |
| 65 - 70 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 50 µL.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of insulin lispro digest supplied with *insulin lispro CRS*,
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III:

  *symmetry factor*: maximum 1.5 for the peaks due to fragments II and III,

  *resolution*: minimum 8.0 between the peaks due to fragments II and III.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*NOTE: the retention times of fragments I, II and IV are the same as for human insulin. The retention time of fragment III differs from human insulin due to differences in sequence at positions 28 and 29 of the B-chain.*

TESTS

**Impurities with molecular masses greater than that of insulin lispro**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Prepare a solution containing 4 mg/mL of the substance to be examined in *0.01 M hydrochloric acid*. Maintain the solution at 2-8 °C and use within 48 h.

*Resolution solution*. Use a solution of insulin (about 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days. Maintain the solution at 2-8 °C and use within 8 days.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm,
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm) with a pore size of 12-12.5 nm, of a grade suitable for the separation of insulin monomer from dimer and polymers.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile for chromatography R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Equilibration*: at least 3 injections of the resolution solution; the column is equilibrated when repeatable results are obtained for 2 subsequent injections.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention time*: insulin lispro polymers = 13-17 min; insulin lispro dimer = about 17.5 min; insulin lispro monomer = about 20 min; salts = about 22 min.

*System suitability*: resolution solution:

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer,
- *symmetry factor*: maximum 2.0 for the peak due to insulin lispro.

*Limits*: the sum of the areas of the peaks with a retention time less than that of the principal peak is not more than 0.25 per cent of the total area of the peaks. Disregard any peak with a retention time greater than that of the peak due to insulin lispro monomer.

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 3.5 mg of the substance to be examined in 1.0 mL of *0.01 M hydrochloric acid*. Maintain the solution at 2-8 °C and use within 56 h.

*Resolution solution.* Dissolve 3.5 mg of the substance to be examined in 1.0 mL of *0.01 M hydrochloric acid*. Allow to stand at room temperature to obtain a solution containing between 0.8 per cent and 11 per cent of A21 desamido insulin lispro.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm,
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 82 volumes of a 28.4 g/L solution of *anhydrous sodium sulfate R* adjusted to pH 2.3 with *phosphoric acid R* and 18 volumes of *acetonitrile for chromatography R*; filter and degas;
- *mobile phase B*: mix equal volumes of a 28.4 g/L solution of *anhydrous sodium sulfate R* adjusted to pH 2.3 with *phosphoric acid R* and *acetonitrile for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 81 | 19 |
| 60 - 83 | 81 → 51 | 19 → 49 |
| 83 - 84 | 51 → 81 | 49 → 19 |
| 84 - 94 | 81 | 19 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Retention time*: adjust the mobile phase composition to obtain a retention time of about 41 min for insulin lispro; A21 desamido insulin lispro elutes near the start of the gradient elution.

*System suitability*: resolution solution:

- *resolution*: minimum 1.5 between the 1st peak (insulin lispro) and the 2nd peak (A21 desamido insulin lispro),
- *symmetry factor*: maximum 2.0 for the peak due to insulin lispro.

*Limits*:

- *A21 desamido insulin lispro*: maximum 1.0 per cent,
- *any other impurity*: maximum 0.50 per cent,
- *total (excluding A21)*: maximum 2.0 per cent.

**Zinc**: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve at least 50 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25 mL with the same acid. Dilute if necessary to a suitable concentration (for example 0.4-0.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid*.

*Reference solutions.* Use solutions of concentrations which bracket the expected zinc concentration of the samples, for example, 0.2-0.8 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C for 16 h.

**Sulfated ash** (*2.4.14*): maximum 2.5 per cent, determined on 0.200 g (dried substance).

**Bacterial endotoxins** (*2.6.14, Method D*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve the substance to be examined in *0.01 M hydrochloric acid* to obtain a concentration of 0.8 mg/mL. Maintain the solution at 2-8 °C and use within 48 h.

*Reference solution.* Dissolve the contents of a vial of *insulin lispro CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 0.8 mg/mL. Maintain the solution at 2-8 °C and use within 48 h.

*Resolution solution.* Dissolve about 10 mg of the substance to be examined in 10 mL of *0.01 M hydrochloric acid*. Allow to stand at room temperature to obtain a solution containing between 0.8 per cent and 11 per cent of A21 desamido insulin lispro. Maintain the solution at 2-8 °C and use within 14 days.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm) with a pore size of 8 nm,
- *temperature*: 40 °C.

*Mobile phase*: mix 745 volumes of a 28.4 g/L solution of *anhydrous sodium sulfate R* adjusted to pH 2.3 with *phosphoric acid R* and 255 volumes of *acetonitrile for chromatography R*; filter and degas.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Retention time*: insulin lispro = about 24 min.

*System suitability*:

- *resolution*: minimum 1.8 between the 1st peak (insulin lispro) and the 2nd peak (A21 desamido insulin lispro), in the chromatogram obtained with the resolution solution,
- *repeatability*: maximum relative standard deviation of 1.1 per cent after 3 injections of the reference solution.

Calculate the content of insulin lispro $C_{257}H_{383}N_{65}O_{77}S_6$ using the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{257}H_{383}N_{65}O_{77}S_6$ in *insulin lispro CRS*.

STORAGE

In an airtight container, protected from light, at or below – 18 °C. When thawed, insulin lispro is stored and weighed under conditions defined by the manufacturer to maintain the quality attributes of the drug substance and is used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, insulin lispro must be at room temperature before opening the container.

**01/2008:1638**
**corrected 10.0**

# INSULIN, PORCINE

## Insulinum porcinum

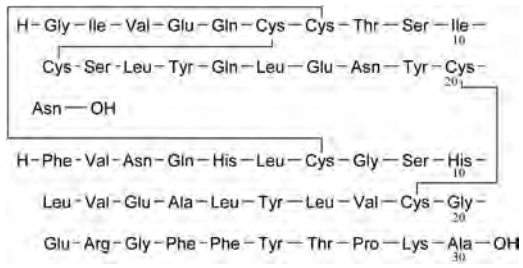


$C_{256}H_{381}N_{65}O_{76}S_6$ $M_r$ 5778

DEFINITION

Porcine insulin is the natural antidiabetic principle obtained from pork pancreas and purified.

*Content*:

– *sum of porcine insulin ($C_{256}H_{381}N_{65}O_{76}S_6$) and A21 desamido porcine insulin*: 95.0 per cent to 105.0 per cent (dried substance).

By convention, for the purpose of labelling insulin preparations, 0.0345 mg of porcine insulin is equivalent to 1 IU of insulin.

PRODUCTION

The animals from which porcine insulin is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids and with decomposition in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the retention time of the principal peak in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with reference solution (b).

B. Peptide mapping.

*Test solution.* Prepare a 2.0 mg/mL solution of the substance to be examined in *0.01 M hydrochloric acid* and transfer 500 µL of this solution to a clean tube. Add 2.0 mL of *HEPES buffer solution pH 7.5 R* and 400 µL of a 1 mg/mL solution of *Staphylococcus aureus strain V8 protease, type XVII-B R*. Cap the tube and incubate at 25 °C for 6 h. Stop the reaction by adding 2.9 mL of *sulfate buffer solution pH 2.0 R*.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *insulin porcine for system suitability CRS* instead of the substance to be examined.

Examine the digests by liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 100 mL of *acetonitrile for chromatography R*, 700 mL of *water for chromatography R* and 200 mL of *sulfate buffer solution pH 2.0 R*; filter and degas;

– *mobile phase B*: mix 400 mL of *acetonitrile for chromatography R*, 400 mL of *water for chromatography R* and 200 mL of *sulfate buffer solution pH 2.0 R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 90 → 30 | 10 → 70 |
| 60 - 65 | 30 → 0 | 70 → 100 |
| 65 - 70 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 15 min. Carry out a blank run using the above-mentioned gradient.

*Injection*: 50 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of porcine insulin digest supplied with *insulin porcine for system suitability CRS*. In the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II and III. The symmetry factor of the peaks due to fragments II and III is not greater than 1.5, and the resolution between the 2 peaks is at least 1.9.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*NOTE: the retention time of fragment I is the same for porcine insulin and for human insulin. The retention times of fragments II and IV are the same for all insulins. The retention time of fragment III is the same for bovine insulin and for porcine insulin.*

TESTS

**Impurities with molecular masses greater than that of insulin**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure. *Maintain the solutions at 2-10 °C and use within 7 days. If an automatic injector is used, maintain the temperature at 2-10 °C.*

*Test solution.* Dissolve 4 mg of the substance to be examined in 1.0 mL of *0.01 M hydrochloric acid*.

*Resolution solution.* Use a solution of insulin (approximately 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about 10 days.

*Column*:

– *size*: *l* = 0.3 m, Ø = at least 7.5 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm), of a grade suitable for the separation of insulin monomer from dimer and polymers.

*Mobile phase*: mix 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Equilibration*: before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least 3 injections of the resolution solution. The column is equilibrated when repeatable results are obtained from 2 subsequent injections.

*Injection*: 100 µL.

*Run time*: about 35 min.

*Retention times*: polymeric insulin complexes = 13 min to 17 min; covalent insulin dimer = about 17.5 min; insulin monomer = about 20 min; salts = about 22 min.

*System suitability*: resolution solution:

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limits*: the sum of the areas of any peaks with a retention time less than that of the principal peak is not greater than 1.0 per cent of the total area of the peaks; disregard any peak with a retention time greater than that of the insulin peak.

**Related proteins**. Liquid chromatography (*2.2.29*) as described under Assay, following the elution conditions as described in the table below.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 42 | 58 |
| 30 - 44 | 42 → 11 | 58 → 89 |
| 44 - 50 | 11 | 89 |

*Maintain the solutions at 2-10 °C and use within 24 h.*

Perform a system suitability test (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µL of reference solution (b) and 20 µL of the test solution. If necessary, adjust the injection volume to between 10 µL and 20 µL in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (b), A21 desamido porcine insulin appears as a small peak after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. In the chromatogram obtained with the test solution, the area of the peak due to A21 desamido porcine insulin is not greater than 2.0 per cent of the total area of the peaks; the sum of the areas of all the peaks, apart from those due to porcine insulin and A21 desamido porcine insulin, is not greater than 2.0 per cent of the total area of the peaks.

**Porcine proinsulin-like immunoreactivity (PLI)**: maximum 10 ppm (dried substance).

Use a suitably sensitive immunochemical method (*2.7.1*) such as radio-immunoassay, using the International Reference Reagent for porcine proinsulin to calibrate the method.

**Zinc**: maximum 1.0 per cent (dried substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4 µg to 1.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid*.

*Reference solutions*. Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Flame*: air-acetylene flame of suitable composition (for example, 11 L of air and 2 L of acetylene per minute).

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 0.200 g by drying in an oven at 105 °C for 24 h.

**Sulfated ash** (*2.4.14*): maximum 2.5 per cent, determined on 0.200 g (dried substance).

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve the contents of a vial of *human insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (b)*. Dissolve the contents of a vial of *porcine insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with *0.01 M hydrochloric acid*.

*Resolution solution*. Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b).

Maintain the solutions at 2-10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2-10 °C.

*Column*:

- *size*: *l* = 0.25, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Prepare and maintain the following solutions at a temperature of at least 20 °C:

- *mobile phase A*: dissolve 28.4 g of *anhydrous sodium sulfate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; add 2.7 mL of *phosphoric acid R*; adjust to pH 2.3, if necessary, with *ethanolamine R*; filter and degas;
- *mobile phase B*: mix 550 mL of mobile phase A with 450 mL of *acetonitrile R*; warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*System suitability*:

- *resolution*: inject 20 µL of the resolution solution and 20 µL of reference solution (b). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (b) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.
- *linearity*: inject 20 µL each of reference solutions (b) and (c). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (b) is 10 ± 0.5 times the area of the principal peak

Monographs I

in the chromatogram obtained with reference solution (c). If this test fails, adjust the injection volume to between 10 µL and 20 µL, in order that the responses are within the linearity range of the detector.

*Injection*: 20 µL of the test solution.

Calculate the content of porcine insulin $C_{256}H_{381}N_{65}O_{76}S_6$ plus A21 desamido porcine insulin from the area of the principal peak and the area of the peak due to A21 desamido porcine insulin in the chromatograms obtained with the test solution and reference solution (b) and the declared content of porcine insulin plus A21 desamido porcine insulin in *porcine insulin CRS*.

STORAGE

In an airtight container, protected from light, at − 20 °C until released by the manufacturer. When thawed, insulin may be stored at 5 ± 3 °C and used for manufacturing preparations within a short period of time. To avoid absorption of humidity from the air during weighing, the insulin must be at room temperature.

**01/2008:0854**
**corrected 10.0**

# INSULIN PREPARATIONS, INJECTABLE

## Praeparationes insulini iniectabiles

*Injectable insulin preparations comply with the requirements for Injections prescribed in the monograph on Parenteral preparations (0520).*

DEFINITION

Injectable insulin preparations are sterile preparations of *Insulin, human (0838)*, *Insulin, bovine (1637)* or *Insulin, porcine (1638)*. They contain not less than 90.0 per cent and not more than the equivalent of 110.0 per cent of the amount of insulin stated on the label. They are either solutions or suspensions or they are prepared by combining solutions and suspensions.

PRODUCTION

The methods of preparation are designed to confer suitable properties with respect to the onset and duration of therapeutic action.

The following procedures are carried out in a suitable sequence, depending on the method of preparation:

- addition of suitable antimicrobial preservatives;
- addition of a suitable substance or substances to render the preparation isotonic with blood;
- addition of a suitable substance or substances to adjust the pH to the appropriate value;
- determination of the strength of the insulin-containing component or components followed, where necessary, by adjustment so that the final preparation contains the requisite number of International Units per millilitre;
- sterilisation by filtration of the insulin-containing component or components; once this procedure has been carried out all subsequent procedures are carried out aseptically using materials that have been sterilised by a suitable method.

In addition, where appropriate, suitable excipients are added and suitable procedures carried out to confer the appropriate physical form on the insulin-containing component or components. The final preparation is distributed aseptically into sterile containers which are closed so as to exclude microbial contamination.

TESTS

**pH** (*2.2.3*). The pH of the solution or suspension is 6.9 to 7.8, unless otherwise prescribed in the specific monograph.

**Insulin in the supernatant**. For injectable insulin preparations that are suspensions, not more than 2.5 per cent of the total insulin content, unless otherwise stated. Centrifuge 10 mL of the suspension at 1500 *g* for 10 min and carefully separate the supernatant and the residue. Determine the insulin content of the supernatant (*S*) by a suitable method, for example using the chromatographic conditions described under Assay. Calculate the percentage of insulin in solution using the following expression:

$$\frac{100S}{T}$$

where *T* is the total insulin content determined as described under the Assay.

**Impurities with molecular masses greater than that of insulin**. Examine by size-exclusion chromatography (*2.2.30*).

*Test solution*. Add 4 µL of *6 M hydrochloric acid R* per millilitre of the preparation to be examined, whether a suspension or a solution, to obtain a clear acid insulin solution. When sampling a suspension, agitate the material prior to sampling in order to obtain a homogeneous sample. If a suspension does not turn clear within 5 min of the initial addition of hydrochloric acid, add small aliquots of acid (less than 4 µL per millilitre) until a solution is obtained. Preparations with concentrations higher than 100 IU/mL need to be diluted with *0.01 M hydrochloric acid* to avoid overloading the column with insulin monomer.

*Resolution solution*. Use a solution of insulin (approximately 4 mg/mL), containing more than 0.4 per cent of high molecular mass proteins. An injectable insulin preparation, whether a solution or a suspension, that has been clarified with a sufficient amount of *6 M hydrochloric acid R*, containing the indicated percentage of high molecular mass proteins, or a solution prepared from insulin, dissolved in *0.01 M hydrochloric acid*, may be used. Insulin containing the indicated percentage of high molecular mass proteins may be prepared by allowing insulin powder to stand at room temperature for about ten days.

Maintain the solutions at 2 °C to 10 °C and use within 30 h (soluble insulin injection) or 7 days (other insulin preparations). If an automatic injector is used, maintain the temperature at 2 °C to 10 °C.

The chromatographic procedure may be carried out using:

- a column 0.3 m long and at least 7.5 mm in internal diameter packed with *hydrophilic silica gel for chromatography R* (5 µm to 10 µm), of a grade suitable for the separation of insulin monomer from dimers and polymers;
- as mobile phase at a flow rate of 0.5 mL/min a mixture consisting of 15 volumes of *glacial acetic acid R*, 20 volumes of *acetonitrile R* and 65 volumes of a 1.0 g/L solution of *arginine R*; filter and degas;
- as detector a spectrophotometer set at 276 nm.

*Equilibration of the column*. Before using a new column for chromatographic analysis, equilibrate by repeated injections of an insulin solution containing high molecular mass proteins. This can be done by at least three injections of the resolution solution. The column is equilibrated when repeatable results are obtained from two subsequent injections. If protamine-containing samples are to be analysed, the equilibration of the column is performed using a solution containing protamine.

Inject 100 µL of the resolution solution. When the chromatograms are recorded under the prescribed conditions, the retention times are: polymeric insulin complexes or covalent insulin-protamine complex: about 13 min to 17 min, covalent insulin dimer: about 17.5 min, insulin monomer: about 20 min, salts: about 22 min. If the sample solution

contains preservatives, for example methyl paraben, *m*-cresol or phenol, these compounds elute later. The test is not valid unless the resolution, defined by the ratio of the height of the dimer peak to the height above the baseline of the valley separating the monomer and dimer peaks, is at least 2.0.

Inject 100 µL of the test solution. Record the chromatogram for approximately 35 min. In the chromatogram obtained, the sum of the areas of any peak with a retention time less than that of the insulin peak is not greater than 3.0 per cent (protamine containing preparations) or 2.0 per cent (non-protamine containing preparations) of the total area of the peaks. Disregard any peak with a retention time greater than that of the insulin peak.

**Related proteins**. Examine by liquid chromatography (*2.2.29*) as described under Assay, following the elution conditions as described in the table below:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Comment |
|---|---|---|---|
| 0 - 30 | 42 | 58 | isocratic |
| 30 - 44 | 42 → 11 | 58 → 89 | linear gradient |
| 44 - 50 | 11 | 89 | isocratic |

Maintain the solutions at 2 °C to 10 °C and use within 24 h. Perform a system suitability check (resolution, linearity) as described under Assay. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Inject 20 µL of the test solution and 20 µL of either reference solution (a), for insulin preparations containing 100 IU/mL, or reference solution (b), for insulin preparations containing 40 IU/mL. If necessary, adjust the injection volume to a volume between 10 µL and 20 µL in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. If necessary, make further adjustments to the mobile phase in order to ensure that the antimicrobial preservatives present in the test solution are well separated from the insulin and show a shorter retention time. A small reduction in the concentration of acetonitrile increases the retention time of the insulin peaks relatively more than those of the preservatives. In the chromatogram obtained with either reference solution (a), or reference solution (b), as appropriate, A21 desamido insulin appears as a small peak after the principal peak and has a retention time of about 1.3 relative to the principal peak, due to insulin. In the chromatogram obtained with the test solution the area of the peak due to A21 desamido insulin is not greater than 5.0 per cent of the total area of the peaks; the sum of the areas of any other peaks, apart from those due to insulin and A21 desamido insulin is not greater than 6.0 per cent of the total area of the peaks. Disregard the peaks due to the preservatives and protamine (early eluting peaks).

**Total zinc**. Not more than the amount stated in the individual monograph, determined by atomic absorption spectrometry (*2.2.23, Method I*).

*Use the following method, unless otherwise prescribed in the specific monograph.*

*Test solution.* Shake the preparation gently and dilute a volume containing 200 IU of insulin to 25.0 mL with *0.01 M hydrochloric acid*. Dilute if necessary to a suitable concentration of zinc (for example 0.4 µg to 1.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid*.

*Reference solutions.* Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 L of air and 2 L of acetylene per minute).

**Zinc in solution**. Where applicable, not more than the amount stated in the individual monograph, determined by atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Centrifuge the preparation to be examined and dilute 1 mL of the clear supernatant obtained to 25.0 mL with *water R*. Dilute if necessary to a suitable concentration of zinc (for example 0.4 µg to 1.6 µg of Zn per millilitre) with *water R*.

*Reference solutions.* Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

Measure the absorbance at 213.9 nm using a zinc hollow-cathode lamp as source of radiation and an air-acetylene flame of suitable composition (for example 11 L of air and 2 L of acetylene per minute).

**Bacterial endotoxins** (*2.6.14*): less than 80 IU per 100 IU of insulin.

ASSAY

Examine by liquid chromatography (*2.2.29*).

*Test solution*. Add 4 µL of *6 M hydrochloric acid R* per millilitre of the preparation to be examined, whether a suspension or a solution, to obtain a clear solution. When sampling a suspension, shake the material prior to sampling in order to obtain a homogeneous sample. If a suspension does not turn clear within 5 min of the initial addition of acid, add small aliquots of acid (less than 4 µL per millilitre) until a solution is obtained. For a preparation containing more than 100 IU mL, an additional dilution with *0.01 M hydrochloric acid* is necessary to avoid overloading the column.

*Reference solution (a).* For a preparation containing a single species of insulin, dissolve in *0.01 M hydrochloric acid*, as appropriate, the contents of a vial of *human insulin CRS*, *porcine insulin CRS* or *bovine insulin CRS* to obtain a concentration of 4.0 mg/mL. For a preparation containing both bovine and porcine insulins, mix 1.0 mL of a solution containing 4.0 mg of *bovine insulin CRS* per millilitre of *0.01 M hydrochloric acid* and 1.0 mL of a solution containing 4.0 mg of *porcine insulin CRS* per millilitre of *0.01 M hydrochloric acid*. *Reference solution (a) is used for the assay of insulin preparations containing 100 IU/mL.*

*Reference solution (b).* Dilute 4.0 mL of reference solution (a) to 10.0 mL with *0.01 M hydrochloric acid*. *Reference solution (b) is used for the assay of insulin preparations containing 40 IU/mL.*

*Reference solution (c).* Dissolve the contents of a vial of *human insulin CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4.0 mg/mL.

*Reference solution (d).* Dissolve the contents of a vial of *insulin porcine for system suitability CRS* in *0.01 M hydrochloric acid* to obtain a concentration of 4 mg/mL.

*Reference solution (e).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *0.01 M hydrochloric acid*.

*Reference solution (f).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with *0.01 M hydrochloric acid*.

*Resolution solution.* Mix 1.0 mL of reference solution (c) and 1.0 mL of reference solution (d).

Maintain the solutions at 2 °C to 10 °C and use within 48 h. If an automatic injector is used, maintain the temperature at 2 °C to 10 °C.

The chromatographic procedure may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm);

– as mobile phase at a flow rate of 1 mL/min the following solutions prepared and maintained at a temperature not lower than 20 °C:

*Mobile phase A.* Dissolve 28.4 g of *anhydrous sodium sulfate R* in *water R* and dilute to 1000 mL with the same solvent; add 2.7 mL of *phosphoric acid R*; adjust the pH to 2.3, if necessary, with *ethanolamine R*; filter and degas;

*Mobile phase B.* Mix 550 mL of mobile phase A with 450 mL of *acetonitrile R*. Warm the solution to a temperature not lower than 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas;

– as detector a spectrophotometer set at 214 nm;

maintaining the temperature of the column at 40 °C.

Elute with a mixture of 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusted if necessary.

Inject 20 µL of the resolution solution and 20 µL of reference solution (d). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (d) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks due to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.

Inject 20 µL of the test solution and 20 µL of either reference solutions (a) and (e), for insulin preparations containing 100 IU/mL, or 20 µL of reference solutions (b) and (f), for insulin preparations containing 40 IU/mL. If necessary, make further adjustments of the mobile phase in order to ensure that the antimicrobial preservatives present in the test solution are well separated from the insulin and show shorter retention times. A small reduction in the concentration of acetonitrile increases the retention time of the insulin peaks relatively more than those of the preservatives. If necessary, after having carried out the chromatography of a solution wash the column with a mixture of equal volumes of *acetonitrile R* and *water R* for a sufficient time to ensure elution of any interfering substances before injecting the next solution. The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (a) or (b) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) or (f). If this test fails, adjust the injection volume between 10 µL and 20 µL, in order to be in the linearity range of the detector.

Calculate the content of insulin plus A21 desamido insulin from the area of the peak due to the bovine, porcine or human insulin and that of any peak due to the A21 desamido insulin, using the declared content of insulin plus A21 desamido insulin in *bovine insulin CRS*, *porcine insulin CRS* or *human insulin CRS*, as appropriate. For preparations containing both bovine and porcine insulin use the sum of the areas of both the bovine and porcine insulin peaks and of the peaks due to the A21 desamido insulin[(1)] derivatives.

STORAGE

Unless otherwise prescribed, store in a sterile, airtight, tamper-evident container, protected from light, at a temperature of 2 °C to 8 °C. Insulin preparations are not to be frozen.

LABELLING

The label states:

– the potency in International Units per millilitre;
– the concentration in terms of the number of milligrams of insulin per millilitre (for preparations containing both bovine insulin and porcine insulin the concentration is stated as the combined amount of both insulins);
– where applicable, that the substance is produced by enzymatic modification of porcine insulin;
– where applicable, that the substance is produced by recombinant DNA technology;
– where applicable, the animal species of origin;
– that the preparation must not be frozen;
– where applicable, that the preparation must be resuspended before use.

**01/2008:0837**
**corrected 6.0**

# INSULIN ZINC INJECTABLE SUSPENSION

## Insulini zinci suspensio iniectabilis

*Insulin zinc injectable suspension complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.*

DEFINITION

Insulin zinc injectable suspension is a sterile neutral suspension of bovine insulin and/or porcine insulin or of human insulin with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

PRODUCTION

Insulin zinc injectable suspension is prepared by carrying out the procedures described in the monograph on *Insulin preparations, injectable (0854)*.

Insulin zinc injectable suspension is produced by mixing insulin zinc injectable suspension (crystalline) and insulin zinc injectable suspension (amorphous) in a ratio of 7 to 3.

CHARACTERS

A white or almost white suspension which on standing deposits a white or almost white sediment and leaves a colourless or almost colourless supernatant; the sediment is readily resuspended by gently shaking. When examined under a microscope, the majority of the particles are seen to be rhombohedral crystals with a maximum dimension when measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm; a considerable proportion of the particles are seen to have no uniform shape and a maximum dimension rarely exceeding 2 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay.

For preparations made from a single species of insulin (bovine, porcine or human), the position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution. For preparations made from a mixture of bovine and porcine insulin, the positions of the peaks due to the two insulins in the chromatogram obtained with the test solution correspond to those of the principal peaks in the chromatogram obtained with the appropriate reference solution.

(1) 100 IU are equivalent to 3.47 mg of human insulin, to 3.45 mg of porcine insulin and to 3.42 mg of bovine insulin.

Monographs

TESTS

**Insulin not extractable with buffered acetone solution**: 63 per cent to 77 per cent of the total insulin content. Centrifuge a volume of the substance to be examined containing 200 IU of insulin and discard the supernatant. Suspend the residue in 1.65 mL of *water R*, add 3.3 mL of *buffered acetone solution R*, stir for 3 min, again centrifuge, discard the supernatant and repeat all the operations with the residue. Dissolve the residue using a suitable procedure, for example dissolve in *0.1 M hydrochloric acid* to give a final volume of 2.0 mL. Determine the insulin content of the residue (*R*) and determine the total insulin content (*T*) of an equal volume of the suspension by a suitable method. Calculate the percentage of insulin not extractable with buffered acetone solution from the expression:

$$\frac{100R}{T}$$

**Total zinc**: 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

**Zinc in solution**: 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

**01/2008:0835**
**corrected 6.0**

# INSULIN ZINC INJECTABLE SUSPENSION (AMORPHOUS)

## Insulini zinci amorphi suspensio iniectabilis

*Insulin zinc injectable suspension (amorphous) complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.*

DEFINITION

Insulin zinc injectable suspension (amorphous) is a sterile neutral suspension of bovine, porcine or human insulin complexed with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

CHARACTERS

A white or almost white suspension which on standing deposits a white or almost white sediment and leaves a colourless or almost colourless supernatant; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to have no uniform shape and a maximum dimension rarely exceeding 2 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

**Total zinc**. 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

**Zinc in solution**. 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

**01/2008:0836**
**corrected 6.0**

# INSULIN ZINC INJECTABLE SUSPENSION (CRYSTALLINE)

## Insulini zinci cristallini suspensio iniectabilis

*Insulin zinc injectable suspension (crystalline) complies with the monograph on Insulin preparations, injectable (0854) with the amendments prescribed below.*

DEFINITION

Insulin zinc injectable suspension (crystalline) is a sterile neutral suspension of bovine, porcine or human insulin, complexed with a suitable zinc salt; the insulin is in a form which is practically insoluble in water.

CHARACTERS

A white or almost white suspension which on standing deposits a white or almost white sediment and leaves a colourless or almost colourless supernatant; the sediment is readily resuspended by gently shaking. When examined under a microscope, the particles are seen to be rhombohedral crystals, the majority having a maximum dimension when measured from corner to corner through the crystal greater than 10 µm but rarely exceeding 40 µm.

IDENTIFICATION

Examine the chromatograms obtained in the Assay. The position of the peak due to insulin in the chromatogram obtained with the test solution corresponds to that of the principal peak in the chromatogram obtained with the appropriate reference solution.

TESTS

**Insulin not extractable with buffered acetone solution**. Not less than 90 per cent of the total insulin content. Centrifuge a volume of the substance to be examined containing 200 IU of insulin and discard the supernatant. Suspend the residue in 1.65 mL of *water R*, add 3.3 mL of *buffered acetone solution R*, stir for 3 min, again centrifuge, discard the supernatant and repeat all the operations with the residue. Dissolve the residue using a suitable procedure, for example dissolve in *0.1 M hydrochloric acid* to give a final volume of 2.0 mL. Determine the insulin content of the residue (*R*) and determine the total insulin content (*T*) of an equal volume of the suspension by a suitable method. Calculate the percentage of insulin not extractable with buffered acetone solution from the expression:

$$\frac{100R}{T}$$

**Total zinc**: 0.12 mg to 0.25 mg per 100 IU of insulin, determined as described in the monograph on *Insulin preparations, injectable (0854)*.

**Zinc in solution**: 20 per cent to 65 per cent of the total zinc is in the form of zinc in solution. Determine by the method described in the monograph on *Insulin preparations, injectable (0854)*.

**07/2015:1110**
**corrected 10.0**

# INTERFERON ALFA-2 CONCENTRATED SOLUTION

## Interferoni alfa-2 solutio concentrata

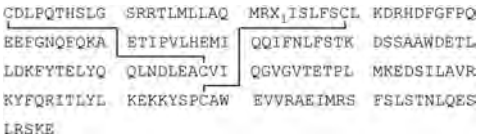


DEFINITION

Interferon alfa-2 concentrated solution is a solution of a protein that is produced according to the information coded by the alfa-2 sub-species of interferon alfa gene and that exerts non-specific antiviral activity, at least in homologous cells, through cellular metabolic processes involving synthesis of both ribonucleic acid and protein. Interferon alfa-2 concentrated solution also exerts antiproliferative activity. Different types of alfa-2 interferon, varying in the amino acid residue at position 23, are designated by a letter in lower case.

| Designation | Residue at position 23 ($X_1$) |
|---|---|
| alfa-2a | Lys |
| alfa-2b | Arg |

This monograph applies to interferon alfa-2a and -2b concentrated solutions.

The potency of interferon alfa-2 concentrated solution is not less than $1.4 \times 10^8$ IU per milligram of protein. Interferon alfa-2 concentrated solution contains not less than $2 \times 10^8$ IU of interferon alfa-2 per millilitre.

PRODUCTION

Interferon alfa-2 concentrated solution is produced by a method based on recombinant DNA (rDNA) technology using bacteria as host cells. It is produced under conditions designed to minimise microbial contamination of the product.

Interferon alfa-2 concentrated solution complies with the following additional requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- or vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

A clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Examine by isoelectric focusing.

*Test solution.* Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/mL.

*Reference solution.* Prepare a 1 mg/mL solution of *interferon alfa-2b CRS* in *water R*.

*Isoelectric point calibration solution pI range 3.0 to 10.0.* Prepare and use according to the manufacturer's instructions.

Use a suitable apparatus connected with a recirculating temperature controlled water-bath set at 10 °C and gels for isoelectric focusing with a pH gradient from 3.5 to 9.5. Operate the apparatus in accordance with the manufacturer's instructions. Use as the anode solution *phosphoric acid R* (98 g/L $H_3PO_4$) and as the cathode solution *1 M sodium hydroxide*. Samples are applied to the gel by filter papers. Place sample application filters on the gel close to the cathode.

Apply 15 µL of the test solution and 15 µL of the reference solution. Start the isoelectric focusing at 1500 V and 50 mA. Turn off the power after 30 min, remove the application filters and reconnect the power supply for 1 h. Keep the power constant during the focusing process. After focusing, immerse the gel in a suitable volume of a solution containing 115 g/L of *trichloroacetic acid R* and 34.5 g/L of *sulfosalicylic acid R* in *water R* and agitate the container gently for 60 min. Transfer the gel to a mixture of 32 volumes of *glacial acetic acid R*, 100 volumes of *anhydrous ethanol R* and 268 volumes of *water R*, and soak for 5 min. Immerse the gel for 10 min in a staining solution prewarmed to 60 °C in which 1.2 g/L of *acid blue 83 R* has been added to the previous mixture of glacial acetic acid, ethanol and water. Wash the gel in several containers with the previous mixture of glacial acetic acid, ethanol and water and keep the gel in this mixture until the background is clear (12 h to 24 h). After adequate destaining, soak the gel for 1 h in a 10 per cent *V/V* solution of *glycerol R* in the previous mixture of glacial acetic acid, ethanol and water.

The principal bands of the electropherogram obtained with the test solution correspond in position to the principal bands of the electropherogram obtained with the reference solution. Plot the migration distances of the isoelectric point markers versus their isoelectric points and determine the isoelectric points of the principal components of the test solution and the reference solution. They do not differ by more than 0.2 pI units. The test is not valid unless the isoelectric point markers are distributed along the entire length of the gel and the isoelectric points of the principal bands in the electropherogram obtained with the reference solution are between 5.8 and 6.3.

C. Examine the electropherograms obtained under reducing conditions in the test for impurities of molecular masses differing from that of interferon alfa-2. The principal band in the electropherogram obtained with test solution (a) corresponds in position to the principal band in the electropherogram obtained with reference solution (a).

D. Examine by peptide mapping.

*Test solution.* Dilute the preparation to be examined in *water R* to a protein concentration of 1.5 mg/mL. Transfer 25 µL to a polypropylene or glass tube of 1.5 mL capacity. Add 1.6 µL of *1 M phosphate buffer solution pH 8.0 R*, 2.8 µL of a freshly prepared 1.0 mg/mL solution of *trypsin for peptide mapping R* in *water R* and 3.6 µL of *water R* and mix vigorously. Cap the tube and place it in a water-bath at 37 °C for 18 h, then add 100 µL of a 573 g/L solution of *guanidine hydrochloride R* and mix well. Add 7 µL of 154.2 g/L solution of *dithiothreitol R* and mix well. Place the capped tube in boiling water for 1 min. Cool to room temperature.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but use a 1.5 mg/mL solution of *interferon alfa-2b CRS* in *water R*.

Examine by liquid chromatography (*2.2.29*).

The chromatographic procedure may be carried out using:

– a stainless steel column 0.10 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm,

– as mobile phase at a flow rate of 1.0 mL/min:

*Mobile phase A*. Dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water R*,

*Mobile phase B*. To 100 mL of *water R* add 1 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile for chromatography R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Comment |
|---|---|---|---|
| 0 - 8 | 100 | 0 | isocratic |
| 8 - 68 | 100 → 40 | 0 → 60 | linear gradient |
| 68 - 72 | 40 | 60 | isocratic |
| 72 - 75 | 40 → 100 | 60 → 0 | linear gradient |
| 75 - 80 | 100 | 0 | re-equilibration |

– as detector a spectrophotometer set at 214 nm,

maintaining the temperature of the column at 30 °C.

Equilibrate the column with mobile phase A for at least 15 min.

Inject 100 µL of the test solution and 100 µL of the reference solution.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of interferon alfa-2 digest supplied with *interferon alfa-2b CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

**Impurities of molecular masses differing from that of interferon alfa-2**. Examine by SDS polyacrylamide gel electrophoresis (*2.2.31*). The test is performed under both reducing and non-reducing conditions, using resolving gels of 14 per cent acrylamide and silver staining as the detection method.

*Sample buffer (non-reducing conditions).* Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer R*.

*Sample buffer (reducing conditions).* Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer for reducing conditions R* containing 2-mercaptoethanol as the reducing agent.

*Test solution (a).* Dilute the preparation to be examined in sample buffer to a protein concentration of 0.5 mg/mL.

*Test solution (b).* Dilute 0.20 mL of test solution (a) to 1 mL with sample buffer.

*Reference solution (a).* Prepare a 0.625 mg/mL solution of *interferon alfa-2b CRS* in sample buffer.

*Reference solution (b).* Dilute 0.20 mL of reference solution (a) to 1 mL with sample buffer.

*Reference solution (c).* Dilute 0.20 mL of reference solution (b) to 1 mL with sample buffer.

*Reference solution (d).* Dilute 0.20 mL of reference solution (c) to 1 mL with sample buffer.

*Reference solution (e).* Dilute 0.20 mL of reference solution (d) to 1 mL with sample buffer.

*Reference solution (f).* Use a solution of molecular mass standards suitable for calibrating SDS-PAGE gels in the range 15 kDa to 67 kDa.

Place test and reference solutions, contained in covered test-tubes, on a water-bath for 2 min.

Apply 10 µL of reference solution (f) and 50 µL of each of the other solutions to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless: the validation criteria are met (*2.2.31*); a band is seen in the electropherogram obtained with reference solution (e); and a gradation of intensity of staining is seen in the electropherograms obtained, respectively, with test solution (a) and test solution (b) and with reference solutions (a) to (e).

The electropherogram obtained with test solution (a) under reducing conditions may show, in addition to the principal band, less intense bands with molecular masses lower than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) (1.0 per cent) and not more than 3 such bands are more intense than the principal band in the electropherogram obtained with reference solution (e) (0.2 per cent).

The electropherogram obtained with test solution (a) under non-reducing conditions may show, in addition to the principal band, less intense bands with molecular masses higher than the principal band. No such band is more intense than the principal band in the electropherogram obtained with reference solution (d) (1.0 per cent) and not more than 3 such bands are more intense than the principal band in the electropherogram obtained with reference solution (e) (0.2 per cent).

**Related proteins**. Examine by liquid chromatography (*2.2.29*).

*Test solution.* Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/mL.

*0.25 per cent m/m hydrogen peroxide solution.* Dilute *dilute hydrogen peroxide solution R* in *water R* in order to obtain a 0.25 per cent *m/m* solution.

*Reference solution.* To a volume of the test solution, add a suitable volume of 0.25 per cent *m/m* hydrogen peroxide solution to give a final hydrogen peroxide concentration of 0.005 per cent *m/m*, and allow to stand at room temperature for 1 h, or for the length of time that will generate about 5 per cent oxidised interferon. Add 12.5 mg of *L-methionine R* per millilitre of solution. Allow to stand at room temperature for 1 h. Store the solutions for not longer than 24 h at a temperature of 2-8 °C.

The chromatographic procedure may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm,

– as mobile phase at a flow rate of 1.0 mL/min:

*Mobile phase A*. To 700 mL of *water R* add 2 mL of *trifluoroacetic acid R* and 300 mL of *acetonitrile for chromatography R*,

*Mobile phase B*. To 200 mL of *water R* add 2 mL of *trifluoroacetic acid R* and 800 mL of *acetonitrile for chromatography R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Comment |
|---|---|---|---|
| 0 - 1 | 72 | 28 | isocratic |
| 1 - 5 | 72 → 67 | 28 → 33 | linear gradient |
| 5 - 20 | 67 → 63 | 33 → 37 | linear gradient |
| 20 - 30 | 63 → 57 | 37 → 43 | linear gradient |
| 30 - 40 | 57 → 40 | 43 → 60 | linear gradient |
| 40 - 42 | 40 | 60 | isocratic |
| 42 - 50 | 40 → 72 | 60 → 28 | linear gradient |
| 50 - 60 | 72 | 28 | re-equilibration |

– as detector a spectrophotometer set at 210 nm.

Equilibrate the column with the mobile phases in the initial gradient ratio for at least 15 min. Inject 50 µL of each solution.

In the chromatograms obtained, interferon alfa-2 elutes at a retention time of about 20 min. In the chromatogram obtained with the reference solution a peak related to oxidised interferon appears at a relative retention of about 0.9 with reference to the principal peak. The test is not valid unless the resolution between the peaks due to oxidised interferon and interferon is at least 1.0. Consider only the peaks whose relative retention is 0.7 to 1.4 with reference to the principal

peak. In the chromatogram obtained with the test solution, the area of any peak, apart from the principal peak, is not greater than 3.0 per cent of the total area of all of the peaks. The sum of the areas of any peaks other than the principal peak is not greater than 5.0 per cent of the total area of all of the peaks.

**Bacterial endotoxins** (*2.6.14*): less than 100 IU in the volume that contains 1.0 mg of protein.

ASSAY

**Protein**

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of about 0.5 mg/mL of interferon alfa-2.

*Reference solutions.* Prepare a stock solution of 0.5 mg/mL of *bovine albumin R*. Prepare 8 dilutions of the stock solution containing between 3 µg/mL and 30 µg/mL of *bovine albumin R*.

Prepare 30-fold and 50-fold dilutions of the test solution. Add 1.25 mL of a mixture prepared the same day by combining 2.0 mL of a 20 g/L solution of *copper sulfate pentahydrate R* in *water R*, 2.0 mL of a 40 g/L solution of *sodium tartrate R* in *water R* and 96.0 mL of a 40 g/L solution of *sodium carbonate R* in *0.2 M sodium hydroxide* to test-tubes containing 1.5 mL of *water R* (blank), 1.5 mL of the different dilutions of the test solution or 1.5 mL of the reference solutions. Mix after each addition. After approximately 10 min, add to each test-tube 0.25 mL of a mixture of equal volumes of *water R* and *phosphomolybdotungstic reagent R*. Mix after each addition. After approximately 30 min, measure the absorbance (*2.2.25*) of each solution at 750 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbances of the 8 reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

**Potency**

The potency of interferon alfa-2 is estimated by comparing its effect to protect cells against a viral cytopathic effect with the same effect of the appropriate International Standard of human recombinant interferon alfa-2 or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay by a suitable method, based on the following design.

Use, in standard culture conditions, an established cell line sensitive to the cytopathic effect of a suitable virus (a human diploid fibroblast cell line, free of microbial contamination, responsive to interferon and sensitive to encephalomyocarditis virus, is suitable).

The following cell cultures and virus have been shown to be suitable: MDBK cells (ATCC No. CCL22), or Mouse L cells (NCTC clone 929; ATCC No. CCL 1) as the cell culture and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as the infective agent; or A-549 cells (ATCC No. CCL-185) responsive to interferon as the cell culture, and encephalomyocarditis virus (ATCC No. VR-129B) as the infective agent.

Incubate in at least 4 series, cells with 3 or more different concentrations of the preparation to be examined and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect. Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells. Determine the cytopathic effect of virus quantitatively with a suitable method. Calculate the potency of the preparation to be examined by the usual statistical methods for a parallel line assay.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits of the estimated potency ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store in an airtight container, protected from light, at or below – 20 °C.

LABELLING

The label states:

- the type of interferon (alfa-2a or alfa-2b),
- the type of production.

**01/2009:1639**
**corrected 10.0**

# INTERFERON BETA-1a CONCENTRATED SOLUTION

## Interferoni beta-1a solutio concentrata

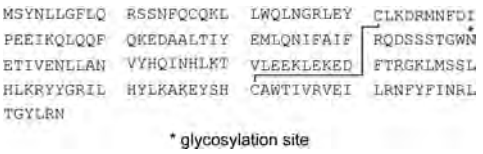


$C_{908}H_{1406}N_{246}O_{252}S_7$ $M_r$ approx. 22 500

DEFINITION

Solution of a glycosylated protein having the same amino acid sequence and disulfide bridge and a similar glycosylation pattern as interferon beta produced by human diploid fibroblasts in response to viral infections and various other inducers. It exerts antiviral, antiproliferative and immunomodulatory activity.

*Content*: minimum 0.20 mg of protein per millilitre.

*Potency*: minimum $1.5 \times 10^8$ IU per milligram of protein.

It may contain buffer salts.

PRODUCTION

Interferon beta-1a concentrated solution is produced by a method based on recombinant DNA (rDNA) technology, using mammalian cells in culture.

Prior to release, the following tests are carried out on each batch of interferon beta-1a concentrated solution, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell or vector-derived DNA**. The limit is approved by the competent authority.

***N*-terminal truncated forms**. Examination for specific *N*-terminal truncated forms should be performed using a suitable technique such as *N*-terminal sequence determination. The limits are approved by the competent authority.

**Dimer and related substances of higher molecular mass**: not more than the amount approved by the competent authority, using an appropriate validated liquid chromatography method.

CHARACTERS

*Appearance*: clear or slightly opalescent, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Isoform distribution. Mass spectrometry (*2.2.43*).

*Introduction of the sample*: direct inflow of a desalted preparation to be examined or liquid chromatography-mass spectrometry combination.

*Mode of ionisation*: electrospray.

*Signal acquisition*: complete spectrum mode from 1100 to 2400.

*Calibration*: use myoglobin in the *m/z* range of 600-2400; set the instrument within validated instrumental settings and analyse the sample; the deviation of the measured mass does not exceed 0.02 per cent of the reported mass.

*Interpretation of results*: a typical spectrum consists of 6 major glycoforms (A to F), which differ in their degree of sialylation and/or antennarity type as shown in Table 1639.-1.

Table 1639.-1.

| MS peak | Glycoform* | Expected $M_r$ | Sialylation level |
|---|---|---|---|
| A | 2A2S1F | 22 375 | Disialylated |
| B | 2A1S1F | 22 084 | Monosialylated |
| C | 3A2S1F and/or 2A2S1F + 1 HexNacHex repeat | 22 739 | Disialylated |
| D | 3A3S1F | 23 031 | Trisialylated |
| E | 4A3S1F and/or 3A3S1F + 1 HexNacHex repeat | 23 400 | Trisialylated |
| F | 2A0S1F | 21 793 | Non-sialylated |

* 2A = biantennary complex type oligosaccharide; 3A = triantennary complex type oligosaccharide; 4A = tetraantennary complex type oligosaccharide; 0S = non-sialylated; 1S = monosialylated; 2S = disialylated; 3S = trisialylated; 1F = fucosylated.

*Results*: the mass spectrum obtained with the preparation to be examined corresponds, with respect to the 6 major peaks, to the mass spectrum obtained with *interferon beta-1a CRS*.

C. Peptide mapping (*2.2.55*) and liquid chromatography (*2.2.29*).

*Test solution.* Add 5 µL of a 242 g/L solution of *tris(hydroxymethyl)aminomethane R* and a volume of the preparation to be examined containing 20 µg of protein to a polypropylene tube of 0.5 mL capacity. Add 4 µL of a 1 mg/mL solution of *endoprotease LysC R* in *0.05 M tris-hydrochloride buffer solution pH 9.0 R*. Mix gently and incubate at 30 °C for 2 h. Add 10 µL of a 15.4 g/L solution of *dithiothreitol R*. Dilute the solution with the same volume of a 573 g/L solution of *guanidine hydrochloride R*. Incubate at 4 °C for 3-4 h.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but using *interferon beta-1a CRS* instead of the preparation to be examined.

*Precolumn*:

– *size*: *l* = 0.02 m, Ø = 2.1 mm;

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Column*:

– *size*: *l* = 0.25 m, Ø = 2.1 mm;

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;

– *mobile phase B*: dilute 1 mL of *trifluoroacetic acid R* in 700 mL of *acetonitrile R1*, then dilute to 1000 mL with *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 64 | 0 → 36 |
| 30 - 45 | 64 → 55 | 36 → 45 |
| 45 - 50 | 55 → 40 | 45 → 60 |
| 50 - 70 | 40 → 0 | 60 → 100 |
| 70 - 83 | 0 | 100 |
| 83 - 85 | 0 → 100 | 100 → 0 |

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: volume that contains 20 µg of digested protein.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of interferon beta-1a digest supplied with *interferon beta-1a CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

TESTS

**Impurities of molecular masses differing from that of interferon beta-1a**. Polyacrylamide gel electrophoresis (*2.2.31*) under reducing conditions.

*Resolving gel*: 12 per cent acrylamide.

*Concentrated sample buffer*: *concentrated SDS-PAGE sample buffer for reducing conditions R* containing 2-mercaptoethanol as the reducing agent.

*Sample buffer*: mixture of equal volumes of *concentrated SDS-PAGE sample buffer for reducing conditions R* and *water R*.

*Test solution (a).* Concentrate the preparation to be examined using a suitable method to obtain a protein concentration of 1.5 mg/mL.

*Test solution (b)*: mixture of equal volumes of test solution (a) and the concentrated sample buffer.

*Test solution (c).* Dilute test solution (a) to obtain a protein concentration of 0.6 mg/mL. Mix equal volumes of this solution and the concentrated sample buffer.

*Test solution (d).* Mix 8 µL of test solution (c) and 40 µL of the sample buffer.

*Test solution (e).* Mix 15 µL of test solution (d) and 35 µL of the sample buffer.

*Test solution (f).* Mix 18 µL of test solution (e) and 18 µL of the sample buffer.

*Test solution (g).* Mix 12 µL of test solution (f) and 12 µL of the sample buffer.

*Reference solution.* Solution of relative molecular mass markers suitable for calibrating SDS-PAGE gels in the range of 15-67 kDa. Dissolve in the sample buffer.

*Sample treatment*: boil for 3 min.

*Application*: 20 µL of test solutions (b) to (g) and the reference solution.

*Detection*: Coomassie staining, carried out as follows: immerse the gel in *Coomassie staining solution R1* at 33-37 °C for 90 min with gentle shaking, then remove the staining solution; destain the gel with a large excess of a mixture of 1 volume of *glacial acetic acid R*, 1 volume of *2-propanol R* and 8 volumes of *water R*.

*Apparent molecular masses*: interferon beta-1a = about 23 000; underglycosylated interferon beta-1a = about 21 000; deglycosylated interferon beta-1a = about 20 000; interferon beta-1a dimer = about 46 000.

*Identification of bands*: use the electropherogram provided with *interferon beta-1a CRS*.

*System suitability*:

- the validation criteria are met (*2.2.31*);
- a band is seen in the electropherogram obtained with test solution (g);
- a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (b) to (g).

*Limits*:

- in the electropherogram obtained with test solution (c), the band corresponding to underglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (5 per cent);
- in the electropherogram obtained with test solution (b), the band corresponding to deglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (e) (2 per cent); any other band corresponding to an impurity of a molecular mass lower than that of interferon beta-1a, apart from the band corresponding to underglycosylated interferon beta-1a is not more intense than the principal band in the electropherogram obtained with test solution (f) (1 per cent).

**Oxidised interferon beta-1a**: maximum 6 per cent.

Use the chromatogram obtained with the test solution in identification C. Locate the peaks due to the peptide fragment comprising amino acids 34-45 and its oxidised form using the chromatogram of oxidised interferon beta-1a digest supplied with *interferon beta-1a CRS*.

Calculate the percentage of oxidation of interferon beta-1a using the following expression:

$$\frac{A_{34-45ox}}{A_{34-45} + A_{34-45ox}} \times 100$$

$A_{34\text{-}45ox}$ = area of the peak due to the oxidised peptide fragment 34-45;

$A_{34\text{-}45}$ = area of the peak due to the peptide fragment 34-45.

**Bacterial endotoxins** (*2.6.14*): less than 0.7 IU in the volume that contains $1 \times 10^6$ IU of interferon beta-1a, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

**Protein**. Liquid chromatography (*2.2.29*). Prepare 3 independent dilutions for each solution.

*Test solution*. Dilute the preparation to be examined to obtain a concentration of 100 µg/mL.

*Reference solution*. Dissolve the contents of a vial of *interferon beta-1a CRS* to obtain a concentration of 100 µg/mL.

*Precolumn*:

- *size*: *l* = 0.02 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Column*:

- *size*: *l* = 0.25 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: to 300 mL of *water for chromatography R*, add 1 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |
| 25 - 26 | 0 → 100 | 100 → 0 |
| 26 - 40 | 100 | 0 |

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 50 µL.

*Retention time*: interferon beta-1a = about 20 min.

*System suitability*: reference solution:

- *symmetry factor*: 0.8 to 2.0 for the peak due to interferon beta-1a;
- *repeatability*: maximum relative standard deviation of 3.0 per cent between the peak areas obtained after injection of the 3 independent dilutions.

Calculate the content of interferon beta-1a ($C_{908}H_{1406}N_{246}O_{252}S_7$) taking into account the assigned content of $C_{908}H_{1406}N_{246}O_{252}S_7$ in *interferon beta-1a CRS*.

**Potency**

The potency of interferon beta-1a is estimated by comparing its ability to protect cells against a viral cytopathic effect with the same ability of the appropriate International Standard of human recombinant interferon beta-1a or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay using a suitable method, based on the following design.

Use, in standard culture conditions, an established cell line sensitive to the cytopathic effect of a suitable virus and responsive to interferon. The cell cultures and viruses that have been shown to be suitable include the following:

- WISH cells (ATCC No. CCL-25) and vesicular stomatitis virus VSV, Indiana strain (ATCC No. VR-158) as infective agent;
- A549 cells (ATCC No. CCL-185) and encephalomyocarditis virus EMC (ATCC No. VR-129B) as infective agent.

Incubate in at least 4 series, cells with 3 or more different concentrations of the preparation to be examined and the reference preparation in a microtitre plate and include in each series appropriate controls of untreated cells. Choose the concentrations of the preparations such that the lowest concentration produces some protection and the largest concentration produces less than maximal protection against the viral cytopathic effect. Add at a suitable time the cytopathic virus to all wells with the exception of a sufficient number of wells in all series, which are left with uninfected control cells. Determine the cytopathic effect of the virus quantitatively with a suitable method. Calculate the potency of the preparation to be examined by the usual statistical methods (for example, *5.3*).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 64 per cent and not more than 156 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light, at a temperature below – 70 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the interferon beta-1a content, in milligrams per millilitre;

Monographs 1

– the antiviral activity, in International Units per millilitre;
– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**07/2014:1440**
**corrected 10.0**

# INTERFERON GAMMA-1b CONCENTRATED SOLUTION

## Interferoni gamma-1b solutio concentrata

$C_{734}H_{1166}N_{204}O_{216}S_5$ $M_r$ 16 465

DEFINITION

Interferon gamma-1b concentrated solution is a solution of the *N*-terminal methionyl form of interferon gamma, a protein which is produced and secreted by human antigen-stimulated T lymphocytes in response to viral infections and various other inducers. It has specific immunomodulatory properties, such as potent phagocyte-activating effects. The protein consists of non-covalent dimers of two identical monomers. The formula of the monomer is as follows:

```
                                                  M
QDPYVKEAEN  LKKYFNAGHS  DVADNGTLFL  GILKNWKEES
DRKIMQSQIV  SFYFKLFKNF  KDDQSIQKSV  ETIKEDMNVK
FFNSNKKKRD  DFEKLTNYSV  TDLNVQRKAI  HELIQVMAEL
SPAAKTGKRK  RSQMLFRGR
```

The potency of interferon gamma-1b is not less than $20 \times 10^6$ IU per milligram of protein. Interferon gamma-1b concentrated solution contains not less than $30 \times 10^6$ IU of interferon gamma-1b per millilitre.

PRODUCTION

Interferon gamma-1b concentrated solution is produced by a method based on recombinant DNA technology, using bacteria as host-cells. It is produced under conditions designed to minimise microbial contamination.

Interferon gamma-1b concentrated solution complies with the following additional requirements.

**Host-cell derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

A clear, colourless or slightly yellowish liquid.

IDENTIFICATION

A. It shows the expected biological activity when tested as prescribed in the assay.

B. Examine the electropherograms obtained in the test for impurities of molecular masses differing from that of interferon gamma-1b. The principal bands in the electropherogram obtained with the test solution correspond in position to the principal bands in the electropherogram obtained with reference solution (a).

C. Examine by peptide mapping.

*Solution A.* Prepare a solution containing 1.2 g/L of *tris(hydroxymethyl)aminomethane R*, 8.2 g/L of *anhydrous sodium acetate R*, 0.02 g/L of *calcium chloride R* and adjust to pH 8.3 with *dilute acetic acid R*. Add *polysorbate 20 R* to a concentration of 0.1 per cent *V/V*.

*Test solution.* Desalt a volume of the preparation to be examined containing 1 mg of protein by a suitable procedure. For example, filter in a microcentrifuge tube and reconstitute with 500 µL of solution A. Add 10 µL of a freshly prepared 1 mg/mL solution of *trypsin for peptide mapping R* in *water R* and mix gently by inversion. Incubate at 30 °C to 37 °C for 24 h, add 100 µL of *phosphoric acid R* per millilitre of digested sample and mix by inversion.

*Reference solution.* Dilute *interferon gamma-1b CRS* in *water R* to obtain a concentration of 1 mg/mL. Prepare as for the test solution, ensuring that all procedures are carried out simultaneously and under identical conditions.

Examine by liquid chromatography (*2.2.29*).

The chromatographic procedure may be carried out using:
– a stainless steel column, 0.15 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (10 µm),
– as mobile phase at a flow rate of 1.0 mL/min:

*Mobile phase A* (*0.05 M sodium phosphate buffer solution pH 3.3*). Solution I: dissolve 7.80 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Solution II: dilute 0.33 mL of *phosphoric acid R* to 100.0 mL with *water R*. Mix 920 mL of solution I and 80 mL of solution II. Adjust the pH if necessary,

*Mobile phase B. Acetonitrile for chromatography R*, with the following elution conditions (if necessary, the gradient may be modified to improve the separation of the digest):

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 80 | 0 → 20 |
| 30 - 50 | 80 → 60 | 20 → 40 |
| 50 - 51 | 60 → 30 | 40 → 70 |
| 51 - 59 | 30 | 70 |

– as detector a spectrophotometer set at 214 nm,

maintaining the temperature of the column at 40 °C.

Equilibrate the column for at least 15 min at the initial elution composition. Carry out a blank run using the above-mentioned gradient.

Inject 100 µL of the test solution and 100 µL of the reference solution.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of interferon gamma-1b digest supplied with *interferon gamma-1b CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine by *N*-terminal sequence analysis.

Use an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Equilibrate by a suitable procedure the equivalent of 100 µg of interferon gamma-1b in a 10 g/L solution of *ammonium hydrogen carbonate R*, pH 9.0.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino acids.

The separation procedure is calibrated using:
– the mixture of PTH-amino acids provided by the manufacturer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids,
– a sample from a blank sequencing cycle, obtained as recommended by the equipment manufacturer.

The first fifteen amino acids are:

Met-Gln-Asp-Pro-Tyr-Val-Lys-Glu-Ala-Glu-Asn-Leu-Lys-Lys-Tyr.

TESTS

**Appearance**. The preparation to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of the preparation to be examined is 4.5 to 5.5.

**Covalent dimers and oligomers**. Not greater than 2 per cent, determined by size-exclusion chromatography (*2.2.30*).

*Test solution.* Dilute the preparation to be examined with the mobile phase to a protein concentration of 0.1 mg/mL.

*Reference solution (a).* Dilute *interferon gamma-1b CRS* with the mobile phase to a protein concentration of 0.1 mg/mL.

*Reference solution (b).* Prepare a mixture of the following molecular mass standards: bovine albumin, ovalbumin, trypsinogen, lysozyme, at a concentration of 0.1 mg/mL to 0.2 mg/mL for each standard.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 to 500 000 (5 µm),
- as mobile phase at a flow rate of 1.0 mL/min a mixture prepared as follows (0.2 M sodium phosphate buffer solution pH 6.8). Solution I: dissolve 31.2 g of *sodium dihydrogen phosphate R* and 1.0 g of *sodium dodecyl sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Solution II: dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 1.0 g of *sodium dodecyl sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Mix 450 mL of solution I and 550 mL of solution II. Adjust the pH if necessary,
- as detector a spectrophotometer set at 210 nm to 214 nm.

Inject 200 µL of each solution. The test is not valid unless: the molecular mass standards in reference solution (b) are well separated; the retention time of the principal peak in the chromatogram obtained with reference solution (a) is between the retention time of trypsinogen and lysozyme in the chromatogram obtained with reference solution (b).

Compare the chromatograms obtained with the test solution and with reference solution (a). There are no additional shoulders or peaks in the chromatogram obtained with the test solution compared with the chromatogram obtained with reference solution (a).

Calculate the percentage content of covalent dimers and oligomers.

**Monomer and aggregates**. Examine by size-exclusion chromatography (*2.2.30*). The content of monomer and aggregates is not greater than 2 per cent.

*Solution A.* Prepare a solution of the following composition: 0.59 g/L of *succinic acid R* and 40 g/L of *mannitol R*, adjusted to pH 5.0 with *sodium hydroxide solution R*.

*Test solution.* Dilute the preparation to be examined with solution A to a protein concentration of 1 mg/mL.

*Reference solution.* Dilute *interferon gamma-1b CRS* with solution A to a protein concentration of 1 mg/mL.

*Resolution solution.* Prepare 500 µL of a mixture consisting of 0.04 mg/mL of *bovine albumin R* and 0.2 mg/mL of *interferon gamma-1b CRS* in solution A. Use this solution within 24 h of preparation.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.3 m long and 7.8 mm in internal diameter packed with *hydrophilic silica gel for chromatography R*, of a grade suitable for fractionation of globular proteins in the molecular weight range of 10 000 - 300 000 (5 µm),
- as mobile phase at a flow rate of 0.8 mL/min a 89.5 g/L solution of *potassium chloride R* (1.2 M),
- as detector a spectrophotometer set at 214 nm.

Inject 20 µL of the resolution solution. In the chromatogram obtained, the retention time of the principal peak, corresponding to the native interferon gamma-1b dimer, is about 10 min. Bovine albumin elutes at a relative retention of about 0.85 with reference to the main peak. The test is not valid unless the resolution between the peaks due to bovine albumin and interferon gamma-1b is at least 1.5.

Inject 20 µL of the test solution and 20 µL of the reference solution. The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of monomer and aggregates from the peak area of the monomer peak and of peaks which elute prior to the native interferon gamma-1b peak in the chromatogram obtained with the test solution, by the normalisation procedure, disregarding any peak due to the solvent.

**Deamidated and oxidised forms and heterodimers**. Examine by liquid chromatography (*2.2.29*). The content of deamidated and oxidised forms is not greater than 10 per cent. The content of heterodimers is not greater than 3 per cent.

*Test solution.* Dilute the preparation to be examined with *water R* to a protein concentration of 1 mg/mL.

*Reference solution (a).* Dilute *interferon gamma-1b CRS* with *water R* to a protein concentration of 1 mg/mL.

*Reference solution (b).* Dissolve the contents of a vial of *interferon gamma-1b for system suitability CRS* in *water R* to obtain a protein concentration of about 1 mg/mL.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.075 m long and 7.5 mm in internal diameter packed with an appropriate hydrophilic polymethacrylate, strong cation-exchange gel (10 µm, 100 nm),
- as mobile phase at a flow rate of 1.2 mL/min:

  *Mobile phase A* (*0.05 M ammonium acetate buffer pH 6.5*). A 3.86 g/L solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

  *Mobile phase B* (*1.2 M ammonium acetate buffer pH 6.5*). A 92.5 g/L solution of *ammonium acetate R*, adjusted to pH 6.5 with *dilute acetic acid R*,

  with the following elution conditions (if necessary, the slope of the gradient may be modified to improve the separation).

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 100 | 0 |
| 2 - 30 | 100 → 0 | 0 → 100 |
| 31 - 35 | 0 | 100 |

- as detector a spectrophotometer set at 280 nm,

maintaining the temperature of the column at 35 °C.

Inject 25 µL of reference solution (b). In the chromatogram obtained, the retention time of the principal peak is about 26 min. Deamidated and oxidised forms co-elute at a relative retention of about 0.95 with reference to the principal peak. The test is not valid unless the resolution, defined by the ratio of the height of the peak corresponding to the deamidated and oxidised forms to the height above the baseline of the valley separating the two peaks, is at least 1.2.

Inject 25 µL of the test solution and 25 µL of reference solution (a). The chromatograms obtained show principal peaks with identical retention times. Calculate the percentage content of deamidated and oxidised interferon gamma-1b as a percentage of the area of the main peak. Heterodimers have relative retentions of 0.7 and 0.85 with reference to the main peak. Calculate the percentage of heterodimers as a percentage of the sum of the areas of all peaks.

**Impurities of molecular masses differing from that of interferon gamma-1b**. Examine by polyacrylamide gel electrophoresis (*2.2.31*). The test is performed under both

reducing and non-reducing conditions, using resolving gels of 15 per cent acrylamide and silver staining as the detection method.

*Sample buffer (non-reducing conditions).* Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 80 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

*Sample buffer (reducing conditions).* Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 0.100 g of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 80 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R* and dilute to 100 mL with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 250 mM.

*Test solution.* Dilute the preparation to be examined in *water R* to a protein concentration of 1 mg/mL. Dilute 150 µL of the solution with 38 µL of sample buffer.

*Reference solution (a).* Prepare in the same manner as for the test solution, but using *interferon gamma-1b CRS* instead of the preparation to be examined.

*Reference solution (b) (5 ng control).* Mix 50 µL of a 0.01 mg/mL solution of *bovine albumin R* with 2000 µL of *water R* and 450 µL of sample buffer.

*Reference solution (c) (2 ng control).* Mix 20 µL of a 0.01 mg/mL solution of *bovine albumin R* with 2000 µL of *water R* and 450 µL of sample buffer.

*Reference solution (d).* Use a solution of molecular mass standards suitable for calibrating SDS-polyacrylamide gels in the range of 10 kDa to 70 kDa.

Leave each solution, contained in a test tube, at ambient temperature for 15 min, then store on ice.

Apply 25 µL of each solution to the stacking gel wells. Perform the electrophoresis under the conditions recommended by the manufacturer of the equipment. Detect proteins in the gel by silver staining.

The test is not valid unless: the validation criteria are met (*2.2.31*); a band is seen in the electropherograms obtained with reference solutions (b) and (c).

The principal band in the electropherogram obtained with the test solution is similar in intensity to the principal band in the electropherogram obtained with reference solution (a). In the electropherogram obtained with the test solution, no significant bands are observed that are not present in the electropherogram obtained with reference solution (a) (0.01 per cent). A significant band is defined as any band whose intensity is greater than or equal to that of the band in the electropherogram obtained with reference solution (c).

**Norleucine**. Not more than 0.2 mole of norleucine per mole of interferon gamma-1b, determined by amino acid analysis.

*Test solution.* Add 2.5 mL of the preparation to be examined onto a column suitable for the desalting of proteins previously equilibrated with 25 mL of a 10 per cent *V/V* solution of *acetic acid R*. Elute the sample with another 2.5 mL of a 10 per cent *V/V* solution of *acetic acid R*. Determine the protein content by measuring the absorbance of this solution as described under Protein, in the Assay section. Pipette a volume containing the equivalent of 100 µg of interferon gamma-1b into each of three reaction vials. Evaporate to dryness under reduced pressure.

Perform the hydrolysis of the three samples as follows. Add to each reaction vial 200 µL of a 50 per cent *V/V* solution of *hydrochloric acid R* containing 1 per cent *V/V* of *phenol R*, evacuate the samples, purge with nitrogen and hydrolyse in the gas phase. Heat the reaction vials at 110 °C for 22 h. After hydrolysis evaporate to dryness under reduced pressure.

Perform the derivatisation of the samples as follows. Prepare immediately before use a mixture consisting of two volumes of *ethanol R*, one volume of *water R* and one volume of *triethylamine R*. Add 50 µL of this solution to each reaction vial and shake lightly. Evaporate to dryness under reduced pressure. Add to each vial 50 µL of a mixture consisting of 7 volumes of *ethanol R*, one volume of *water R*, one volume of *triethylamine R* and one volume of *phenyl isothiocyanate R*. Shake lightly and allow to stand at room temperature for about 15 min. Evaporate to dryness under reduced pressure. Reconstitute the samples in 250 µL of mobile phase A.

*Norleucine stock solution.* Prepare a 250 nmol/mL solution of *DL-norleucine R* in *0.01 M hydrochloric acid. This solution may be kept for two months at 4 °C.*

*Leucine stock solution.* Prepare a 250 nmol/mL solution of *leucine R* in *0.01 M hydrochloric acid. This solution may be kept at 4 °C for two months.*

*Reference solution.* Mix 10 µL of norleucine stock solution with 100 µL of leucine stock solution in each of the three reaction vials. Evaporate to dryness under reduced pressure. Perform the derivatisation of the samples as described for the preparation of the test solution.

Examine by liquid chromatography (*2.2.29*).

The chromatographic procedure may be carried out using:

- a stainless steel column 0.15 m long and 3.9 mm in diameter packed with *octadecylsilyl silica gel for chromatography R* (4 µm),
- as mobile phase at a flow rate of 1.0 mL/min:

  *Mobile phase A*. Mix 70 volumes of a 19 g/L solution of *sodium acetate R* containing 0.05 per cent *V/V* of *triethylamine R* and adjusted to pH 6.4 with *dilute acetic acid R* and 30 volumes of mobile phase B,

  *Mobile phase B*. Mix 40 volumes of *water R* and 60 volumes of *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Comment |
|---|---|---|---|
| 0 - 7 | 100 | 0 | isocratic |
| 7 - 7.1 | 100 → 0 | 0 → 100 | linear gradient |
| 7.1 - 10 | 0 | 100 | washing step |
| 10 - 10.1 | 0 → 100 | 100 → 0 | linear gradient |
| 10.1 - 15 | 100 | 0 | re-equilibration |

- as detector a spectrophotometer set at 254 nm,

maintaining the temperature of the column at 43 °C.

Inject 50 µL of each solution.

In the chromatograms obtained with the test solution, identify the peaks corresponding to leucine and norleucine. The retention time of norleucine is 6.2 min to 7 min.

Calculate the content of norleucine (in moles of norleucine per mole of interferon gamma-1b) from the peak areas of leucine and norleucine in the chromatograms obtained with the reference and test solutions, considering that there are 10 moles of leucine per mole of interferon gamma-1b.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU in the volume that contains $20 \times 10^6$ IU of interferon gamma-1b.

ASSAY

**Protein** (*2.2.25*). Dilute the substance to be examined in *water R* to obtain a concentration of 1 mg/mL. Record the absorbance spectrum between 220 nm and 340 nm. Measure the value at the absorbance maximum of 280 nm, after correction for any light scattering due to turbidity measured at 316 nm. Calculate the concentration of interferon gamma-1b using a specific absorbance value of 7.5.

**Potency**. The potency of interferon gamma-1b is estimated by evaluating the increase of the expression of human-leukocyte-antigen-DR (HLA-DR) due to the interferon gamma-1b present in test solutions during cultivation of the cells, and comparing this increase with the same effect of the

appropriate International Standard of human recombinant interferon gamma or of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Carry out the assay by a suitable method, based on the following design.

Use COLO 205 cells under standard culture conditions. Trypsinise a 3- to 5-day-old flask of COLO 205 cells and prepare a cell suspension at a concentration of $1.0 \times 10^6$ cells/mL.

Add 100 µL of the dilution medium to all wells of a 96-well microtitre plate. Add an additional 100 µL of this solution to the wells designed for the blanks. Add 100 µL of each solution to be tested onto the plate and carry out a series of twofold dilution steps in order to obtain a standard curve. Then add 100 µL of the cell suspension to all wells and incubate the plate under appropriate conditions for cell cultivation.

After cultivation remove the growth medium and wash and fix cells to the plate. Add an antibody able to detect HLA-DR expressed due to the presence of interferon gamma-1b and incubate under appropriate conditions. After washing the plate, incubate with an antibody conjugated to a marker enzyme which is able to detect the anti-HLA-DR antibody. After this incubation step, wash the plate and add an appropriate substrate solution. Stop the reaction. Measure the absorbance of the solution and calculate the potency of the preparation to be examined by the usual statistical methods.

The estimated specific activity is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P = 0.95$) are not less than 70 per cent and not more than 140 per cent of the estimated potency.

STORAGE

Store in an airtight container, protected from light and at a temperature of − 70 °C.

**01/2008:0031**

# IODINE

## Iodum

$I_2$ $M_r$ 253.8
[7553-56-2]

DEFINITION

*Content*: 99.5 per cent to 100.5 per cent of I.

CHARACTERS

*Appearance*: greyish-violet, brittle plates or fine crystals with a metallic sheen.

*Solubility*: very slightly soluble in water, very soluble in concentrated solutions of iodides, soluble in ethanol (96 per cent), slightly soluble in glycerol.

It volatilises slowly at room temperature.

IDENTIFICATION

A. Heat a few fragments in a test-tube. Violet vapour is evolved and a bluish-black crystalline sublimate is formed.

B. To a saturated solution add *starch solution R*. A blue colour is produced. Heat until decolourised. On cooling, the colour reappears.

TESTS

**Solution S**. Triturate 3.0 g with 20 mL of *water R*, filter, wash the filter with *water R* and dilute the filtrate to 30 mL with the same solvent. To the solution add 1 g of *zinc powder R*. When the solution is decolourised, filter, wash the filter with *water R* and dilute to 40 mL with the same solvent.

**Bromides and chlorides**: maximum 250 ppm.

To 10 mL of solution S add 3 mL of *ammonia R* and 6 mL of *silver nitrate solution R2*. Filter, wash the filter with *water R* and dilute the filtrate to 20 mL with the same solvent. To 10 mL of the solution add 1.5 mL of *nitric acid R*. After 1 min, any opalescence in the solution is not more intense than that in a standard prepared at the same time by mixing 10.75 mL of *water R*, 0.25 mL of *0.01 M hydrochloric acid*, 0.2 mL of *dilute nitric acid R* and 0.3 mL of *silver nitrate solution R2*.

**Non-volatile substances**: maximum 0.1 per cent.

Heat 1.00 g in a porcelain dish on a water-bath until the iodine has volatilised. Dry the residue at 100-105 °C. The residue weighs a maximum of 1 mg.

ASSAY

Introduce 0.200 g into a flask containing 1 g of *potassium iodide R* and 2 mL of *water R* and add 1 mL of *dilute acetic acid R*. When dissolution is complete, add 50 mL of *water R* and titrate with *0.1 M sodium thiosulfate*, using *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 12.69 mg of I.

**01/2017:2215**

# IODIXANOL

## Iodixanolum

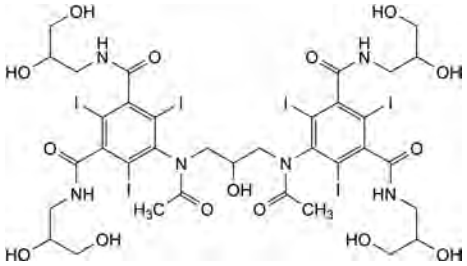

$C_{35}H_{44}I_6N_6O_{15}$ $M_r$ 1550
[92339-11-2]

DEFINITION

Mixture of stereoisomers of 5,5′-[(2-hydroxypropane-1,3-diyl)bis(acetylimino)]bis[*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide].

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *iodixanol CRS*.

B. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.
*Injection*: test solution and reference solution (b).

*Results*: the 3 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 3 principal peaks in the chromatogram obtained with reference solution (b).

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Heat solution S at about 98 °C for 30 min without boiling then allow to cool to room temperature. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Impurities E and H**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 5 mg of *iodixanol impurity E CRS* and 5 mg of *iodixanol impurity H CRS* in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (c)*. Mix 5.0 mL of the test solution with 5.0 mL of reference solution (b) and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, *water R* (50:50 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 30 | 70 |
| 2 - 27 | 30 → 68 | 70 → 32 |

*Flow rate*: 1.7 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E and H.

*Relative retention* with reference to iodixanol (1st peak) (retention time = about 16 min): impurity E (1st peak) = about 0.7; impurity E (2nd peak) = about 0.8; impurity H = about 1.4.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the 1st peak due to impurity E and the 1st peak due to iodixanol.

*Limits*:

- *correction factor*: for the calculation of total content of impurity E, multiply the peak area of the 1st peak due to impurity E by 1.7;
- *impurity H*: not more than 0.6 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *impurity E*: not more than 0.3 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 25 mg of *iodixanol CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 5 mg of *iodixanol impurity C CRS* and 5 mg of *iopentol CRS* in *water R* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (d)*. Mix 5.0 mL of the test solution with 5.0 mL of reference solution (c) and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*, *water R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 94 | 6 |
| 2 - 32 | 94 → 80 | 6 → 20 |
| 32 - 72 | 80 → 0 | 20 → 100 |
| 72 - 82 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurity C and iopentol.

*Relative retention* with reference to iodixanol (1st peak) (retention time = about 27 min): iopentol (1st peak) = about 0.8; iopentol (2nd peak) = about 0.9; impurity C (1st peak) = about 1.04; overalkylated impurities (a group of peaks) = 1.33-1.70.

*System suitability*: reference solution (d):

- *resolution*: baseline separation between the 2 peaks due to iopentol;
- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the 1st peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the 1st peak due to iodixanol.

*Limits*:

- *correction factor*: for the calculation of total content of impurity C, multiply the peak area of the 1st peak due to impurity C by 1.3;
- *impurity C*: not more than 0.4 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *overalkylated impurities (such as impurity I)*: not more than the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.5 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.05 times the sum of the areas of the 2 principal peaks in the chromatogram obtained with reference solution (a) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Free aromatic amine**: maximum 500 ppm.

*Test solution.* Transfer 0.200 g of the substance to be examined to a 25 mL volumetric flask and dissolve in 15.0 mL of *water R*.

*Reference solution.* Dissolve 5.0 mg of *iohexol impurity J CRS* in *water R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Mix 10.0 mL of this solution with 5.0 mL of *water R* in a 25 mL volumetric flask.

*Blank solution.* Transfer 15.0 mL of *water R* to a 25 mL volumetric flask.

*In conducting the following steps, keep the flasks in iced water and protected as much as possible from light until all the reagents have been added.*

Place the 3 flasks containing respectively the test solution, the reference solution and the blank solution in iced water, protected from light, for 5 min. Add 1.5 mL of *hydrochloric acid R1* and mix by swirling. Add 1.0 mL of a 20 g/L solution of *sodium nitrite R*, mix and allow to stand for 4 min. Add 1.0 mL of a 40 g/L solution of *sulfamic acid R*, swirl gently until gas liberation has ceased and allow to stand for 1 min. (*CAUTION: considerable pressure is produced*). Add 1.0 mL of a freshly prepared 3 g/L solution of *naphthylethylenediamine dihydrochloride R* in a mixture of 30 volumes of *water R* and 70 volumes of *propylene glycol R* and mix. Remove the flasks from the iced water, dilute to 25.0 mL with *water R*, mix and examine the solutions after 5 min. The solution obtained from the test solution is less coloured than the solution obtained from the reference solution. If the solution obtained from the test solution is about the same colour or darker than the solution obtained from the reference solution, proceed as follows. Concomitantly determine the absorbance (*2.2.25*) at 495 nm of the solution obtained from the test solution and the reference solution in 5 cm cells, using the blank solution as the compensation liquid. The absorbance of the solution obtained from the test solution is not greater than that of the solution obtained from the reference solution.

**Free iodine**. Transfer 2.0 g to a glass-stoppered tube, add 20 mL of *water R*, 5 mL of *toluene R* and 5 mL of *dilute sulfuric acid R*, shake vigorously and allow the phases to separate: the toluene layer shows no red or pink colour.

**Iodide**: maximum 10 ppm.

Dissolve 5.000 g in *water R* and dilute to 20.0 mL with the same solvent. Titrate with *0.001 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*) using a silver indicator electrode and an appropriate reference electrode.

1 mL of *0.001 M silver nitrate* is equivalent to 126.9 µg of iodide.

**Ionic compounds** (*2.2.38*): maximum 0.02 per cent *m/m* calculated as sodium chloride.

*Rinse all glassware with distilled water R 5 times before use.*

*Test solution.* Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dissolve 20.0 mg of *sodium chloride R* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

Measure the specific conductivity of the test solution and the reference solution using a suitable conductivity meter. The specific conductivity of the test solution is not greater than that of the reference solution.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

In a 125 mL round-bottomed flask, dissolve 0.200 g in 25 mL of a 50 g/L solution of *sodium hydroxide R*, add 0.5 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 1 h. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (40) (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 5 mL of *glacial acetic acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 25.84 mg of $C_{35}H_{44}I_6N_6O_{15}$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: C, E, H, overalkylated impurities.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use: A, B, F, G.*

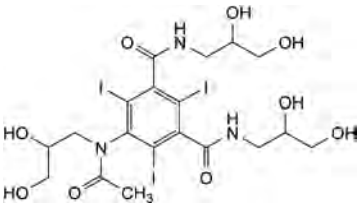

A. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide (iohexol),

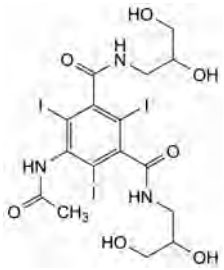

B. 5-acetamido-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

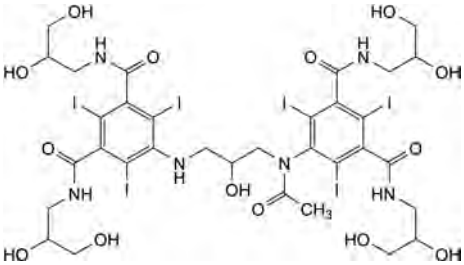

C. 5-[acetyl[3-[[3,5-bis[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropyl]amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

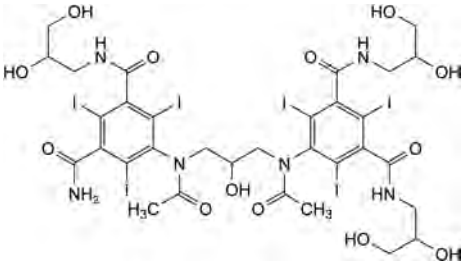

E. 5-[acetyl[3-[acetyl[3-carbamoyl-5-[(2,3-dihydroxypropyl)-carbamoyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropyl]-amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

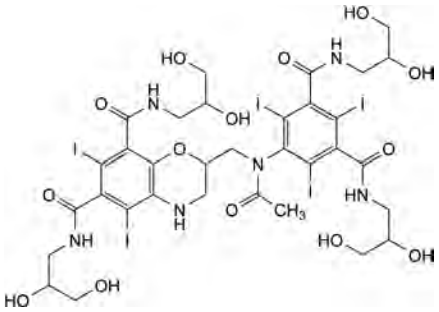

F. 2-[[acetyl[3,5-bis[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl]amino]methyl]-*N*,*N*′-bis(2,3-dihydroxypropyl)-5,7-diiodo-3,4-dihydro-2*H*-1,4-benzoxazine-6,8-dicarboxamide,

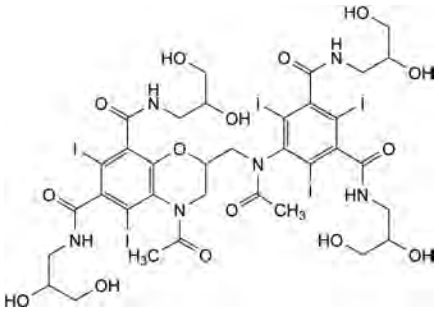

G. 4-acetyl-2-[[acetyl[3,5-bis[(2,3-dihydroxypropyl)-carbamoyl]-2,4,6-triiodophenyl]amino]methyl]-*N*,*N*′-bis(2,3-dihydroxypropyl)-5,7-diiodo-3,4-dihydro-2*H*-1,4-benzoxazine-6,8-dicarboxamide,

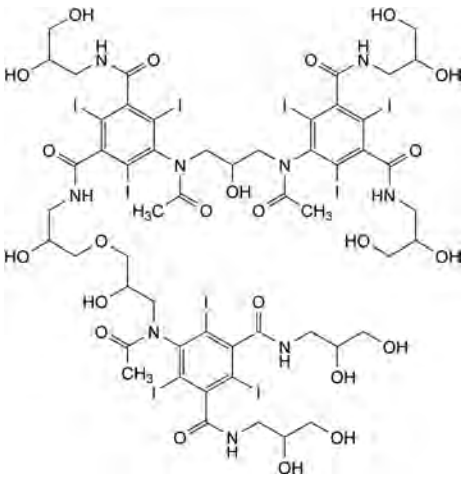

H. 5-[acetyl[3-[acetyl[3-[[3-[3-[acetyl[3,5-bis[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropoxy]-2-hydroxypropyl]carbamoyl]-5-[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropyl]amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide.

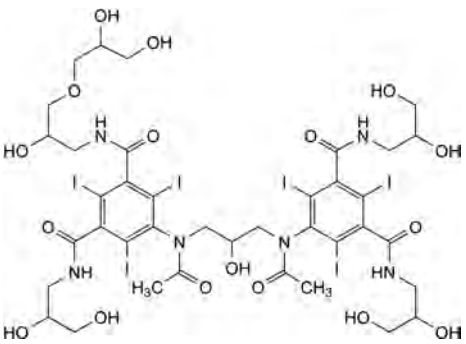

I. overalkylated impurities (an example): 5-[acetyl[3-[acetyl[3,5-bis[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodophenyl]amino]-2-hydroxypropyl]amino]-*N*-[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide.

**01/2017:1114**
**corrected 10.0**

# IOHEXOL

## Iohexolum

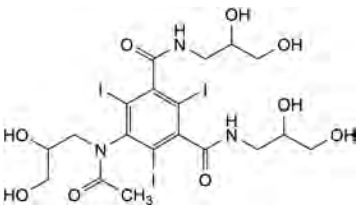

$C_{19}H_{26}I_3N_3O_9$ $M_r$ 821
[66108-95-0]

DEFINITION

5-[Acetyl(2,3-dihydroxypropyl)amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide.

The substance is a mixture of diastereoisomers and atropisomers.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or greyish-white, hygroscopic powder.

*Solubility*: very soluble in water, freely soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *iohexol CRS*.

B. Examine the chromatograms obtained in test A for related substances.
*Results*: the principal peaks in the chromatogram obtained with reference solution (b) are similar in retention time and size to the peaks due to iohexol in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Related substances**

A. Liquid chromatography (*2.2.29*).

*NOTE: iohexol gives rise to 2 non-resolved peaks in the chromatogram due to endo-exo isomerism. In addition, a small peak (also due to iohexol) usually appears at the leading edge of the 1st principal peak. This small peak has a retention time about 1.2 min less than the 1st principal peak.*

*Test solution.* Dissolve 0.150 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 15.0 mg of *iohexol CRS* and 15.0 mg of *iohexol impurity A CRS* in a mixture of 0.05-0.1 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 5.0 mg of *iohexol for peak identification CRS* (containing impurities B, C, D and E) in *water R* and dilute to 5.0 mL with the same solvent.

*Blank solution*: *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 99 → 87 | 1 → 13 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Retention time*: impurities A and H = about 17 min; iohexol (peaks corresponding to *endo-exo* isomerism) = about 20 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peak due to impurity A and the 2nd and greater peak due to iohexol.

*Limits*:

- *sum of impurities B, C, D and E* (relative retention with reference to the 2nd and greater peak due to iohexol between 1.1 and 1.4): not more than 0.6 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.6 per cent); use the chromatogram obtained with reference solution (c) to identify the corresponding peaks;
- *sum of impurities A and H*: not more than 0.5 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (1.5 per cent);
- *disregard limit*: 0.03 times the total area of the principal peaks in the chromatogram obtained with reference solution (b) (0.03 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50 mg of *iohexol impurity J CRS* and 50 mg of *iohexol CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 50.0 mL with *water R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Pretreatment*: wash the plate with the mobile phase, dry at room temperature for 30 min, then at 90 °C for 1 h.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *2-propanol R*, *acetone R* (16:16:28:40 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (a):

- the chromatogram shows 2 clearly separated spots.

*Limits*:

- *any impurity*: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**3-Chloropropane-1,2-diol**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in 1.0 mL of *water R*. Shake with 4 quantities, each of 2 mL, of *methyl acetate R*. Dry the combined upper layers over *anhydrous sodium sulfate R*. Filter and concentrate to about 0.7 mL using a warm water-bath at 60 °C and a stream of nitrogen and dilute to 1.0 mL with *methyl acetate R*.

*Reference solution.* Dissolve 0.25 g of *3-chloropropane-1,2-diol R* in 100.0 mL of *methyl acetate R*. Dilute 1.0 mL of this solution to 100.0 mL with *methyl acetate R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.33 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 80 |
| | 2 - 8 | 80 → 170 |
| | 8 - 10 | 170 |
| Injection port | | 230 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 µL (splitless for 30 s).

*System suitability*: reference solution:

- *retention time*: 3-chloropropane-1,2-diol = about 8 min.

*Limit*:

- *3-chloropropane-1,2-diol*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (25 ppm).

**Free aromatic amine**: maximum 500 ppm.

*Test solution.* Transfer 0.200 g of the substance to be examined to a 25 mL volumetric flask and dissolve in 15.0 mL of *water R*.

*Reference solution.* Dissolve 5.0 mg of *iohexol impurity J CRS* in *water R* and dilute to 5.0 mL with *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. Mix 10.0 mL of this solution with 5.0 mL of *water R* in a 25 mL volumetric flask.

*Blank solution.* Transfer 15.0 mL of *water R* to a 25 mL volumetric flask.

*In conducting the following steps, keep the flasks in iced water and protected as much as possible from light until all of the reagents have been added.*

Place the 3 flasks containing respectively the test solution, the reference solution and the blank solution in iced water, protected from light, for 5 min. Add 1.5 mL of *hydrochloric acid R1* and mix by swirling. Add 1.0 mL of a 20 g/L solution of *sodium nitrite R*, mix and allow to stand for 4 min. Add 1.0 mL of a 40 g/L solution of *sulfamic acid R*, swirl gently until gas liberation has ceased and allow to stand for 1 min. *(CAUTION: considerable pressure is produced).* Add 1.0 mL of a freshly prepared 3 g/L solution of *naphthylethylenediamine dihydrochloride R* in a mixture of 30 volumes of *water R* and 70 volumes of *propylene glycol R* and mix. Remove the flasks from the iced water, dilute to 25.0 mL with *water R*, mix and allow to stand for 5 min. Simultaneously determine the absorbance (*2.2.25*) at 495 nm of the solutions obtained from the test solution and the reference solution in 5 cm cells, using the blank as the compensation liquid. The absorbance of the test solution is not greater than that of the reference solution.

**Iodide**: maximum 10 ppm.

Dissolve 6.000 g in *water R* and dilute to 20 mL with the same solvent. Add 2.0 mL of *0.001 M potassium iodide*. Titrate with *0.001 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*), using a silver indicator electrode and an appropriate reference electrode. Subtract the volume of titrant corresponding to the 2.0 mL of *0.001 M potassium iodide*, determined by titrating a blank to which is added 2.0 mL of *0.001 M potassium iodide* and use the residual value to calculate the iodide content.

1 mL of *0.001 M silver nitrate* is equivalent to 126.9 µg of $I^-$.

**Ionic compounds** (*2.2.38*): maximum 0.01 per cent *m/m* calculated as sodium chloride.

*Rinse all glassware with distilled water R 5 times before use.*

*Test solution.* Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dissolve 20.0 mg of *sodium chloride R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

Measure the conductivity of the test solution and the reference solution using a suitable conductivity meter. The conductivity of the test solution is not greater than that of the reference solution.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 1.00 g.

ASSAY

To 0.500 g in a 125 mL round-bottomed flask add 25 mL of a 50 g/L solution of *sodium hydroxide R*, 0.5 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 5 mL of *glacial acetic acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 27.37 mg of $C_{19}H_{26}I_3N_3O_9$.

STORAGE

In an airtight container, protected from light and moisture.

IMPURITIES

*Specified impurities: A, B, C, D, E, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G, I, J, K, L, M, N, O, P, Q.*

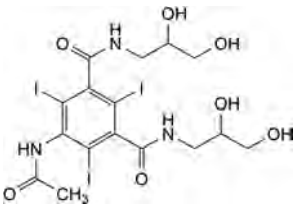

A. 5-(acetylamino)-*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

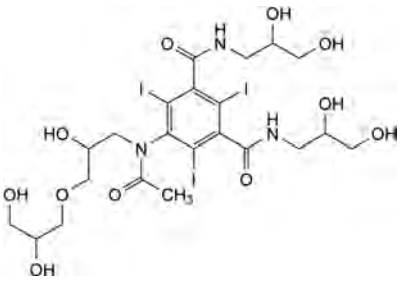

B. 5-[acetyl[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-amino]-*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

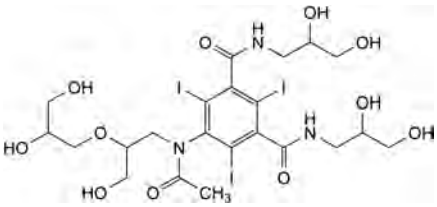

C. 5-[acetyl[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]-amino]-*N,N′*-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

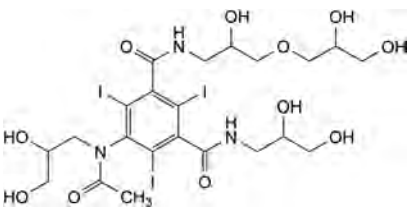

D. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*-[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-*N′*-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

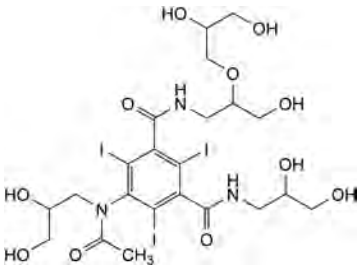

E. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*-[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

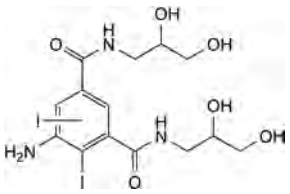

F. 5-amino-*N*,*N*′-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,

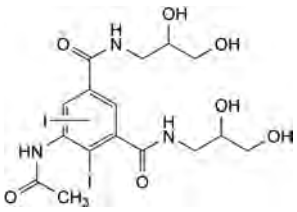

G. 5-(acetylamino)-*N*,*N*′-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,

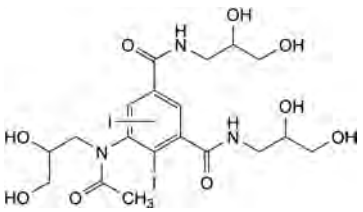

H. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*,*N*′-bis(2,3-dihydroxypropyl)diiodobenzene-1,3-dicarboxamide,

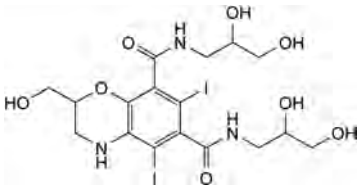

I. *N*,*N*′-bis(2,3-dihydroxypropyl)-2-(hydroxymethyl)-5,7-diiodo-3,4-dihydro-2*H*-1,4-benzoxazine-6,8-dicarboxamide,

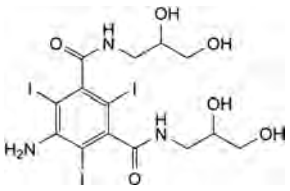

J. 5-amino-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

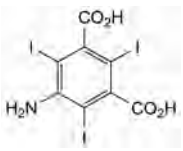

K. 5-amino-2,4,6-triiodobenzene-1,3-dicarboxylic acid,

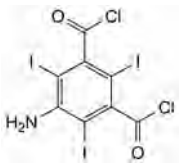

L. 3,5-bis(chlorocarbonyl)-2,4,6-triiodobenzenamine,

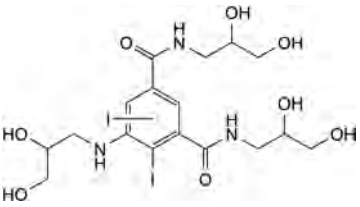

M. *N*,*N*′-bis(2,3-dihydroxypropyl)-5-[(2,3-dihydroxypropyl)amino]diiodobenzene-1,3-dicarboxamide,

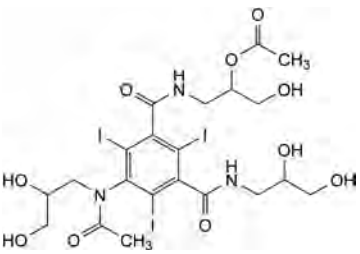

N. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*-[2-(acetyloxy)-3-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

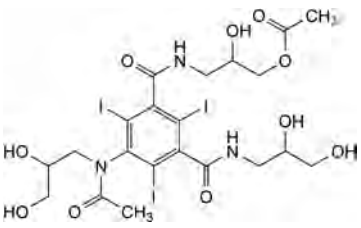

O. 5-[acetyl(2,3-dihydroxypropyl)amino]-*N*-[3-(acetyloxy)-2-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

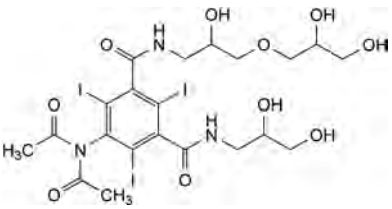

P. 5-(diacetylamino)-*N*-[3-(2,3-dihydroxypropoxy)-2-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

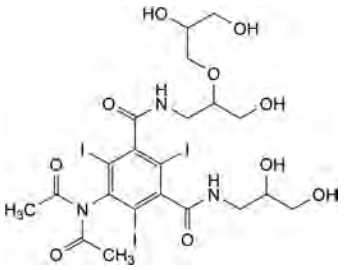

Q. 5-(diacetylamino)-*N*-[2-(2,3-dihydroxypropoxy)-3-hydroxypropyl]-*N*′-(2,3-dihydroxypropyl)-2,4,6-triiodobenzene-1,3-dicarboxamide.

**01/2017:1115**
**corrected 10.0**

# IOPAMIDOL

## Iopamidolum

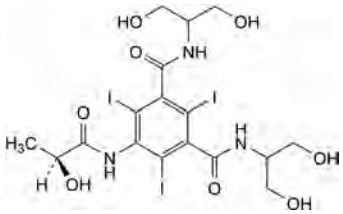

$C_{17}H_{22}I_3N_3O_8$ $M_r$ 777
[60166-93-0]

DEFINITION

*N*,*N*′-Bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzene-1,3-dicarboxamide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in methanol, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *iopamidol CRS*.

B. Loss on drying (see Tests).

C. Specific optical rotation (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1 g in *water R* and dilute to 50 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent. Not more than 0.75 mL of *0.01 M hydrochloric acid* or 1.4 mL of *0.01 M sodium hydroxide* is required to adjust to pH 7.0 (*2.2.3*).

**Specific optical rotation** (*2.2.7*): − 4.6 to − 5.2 (dried substance), determined at 436 nm.

Dissolve 10.0 g, with heating if necessary, in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *iopamidol impurity H CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 20.0 mL with *water R*. Dilute 1.0 mL of this solution to 50.0 mL with *water R*.

*Reference solution (c).* Add 0.1 mL of the test solution to 20 mL of reference solution (a) and dilute to 50 mL with *water R*.

*Column*: 2 columns coupled in series,

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: *water R*,
- *mobile phase B*: *acetonitrile R*, *water R* (50:50 *V/V*),

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 18 | 100 | 0 |
| 18 - 40 | 100 - 62 | 0 - 38 |
| 40 - 45 | 62 - 50 | 38 - 50 |
| 45 - 50 | 50 - 100 | 50 - 0 |
| 50 - 60 | 100 | 0 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Relative retention* with reference to iopamidol (retention time = about 14.6 min): impurity D = about 0.1; impurity B = about 0.6; impurities I and H = about 0.9; impurity G = about 1.1; impurity K = about 1.2; impurity C = about 1.3; impurity J = about 1.5; impurity A = about 1.8; impurity E = about 2.2; impurity F = about 2.3.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to impurity H and iopamidol.

*Limits*:

- *sum of impurities H and I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *impurities A, B, C, D, E, F, G, J, K*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *sum of impurities other than H and I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

**Free aromatic amines**: maximum 200 ppm.

*Keep the solutions and reagents in iced water, protected from bright light.*

*Test solution.* In a 25 mL volumetric flask, dissolve 0.500 g of the substance to be examined in 20.0 mL of *water R*.

*Reference solution.* In a 25 mL volumetric flask, mix 4.0 mL of a 25.0 mg/L solution of *iopamidol impurity A CRS* with 16.0 mL of *water R*.

*Blank solution.* Place 20.0 mL of *water R* in a 25 mL volumetric flask.

Place the flasks in iced water, protected from light, for 5 min. Add 1.0 mL of *hydrochloric acid R* to each flask, mix and allow to stand for 5 min. Add 1.0 mL of a 20 g/L solution of *sodium nitrite R* prepared immediately before use, mix and allow to stand for 5 min. Add 1.0 mL of a 120 g/L solution of *ammonium sulfamate R*, swirl gently until gas liberation has ceased, and allow to stand for 5 min. *(CAUTION: considerable pressure is produced).* Add 1.0 mL of a freshly prepared 1 g/L solution of *naphthylethylenediamine dihydrochloride R* and mix. Remove the flasks from the iced water and allow to stand for 10 min. Dilute to 25.0 mL with *water R* and mix. Measure immediately the absorbance (*2.2.25*) at 500 nm of the solutions obtained from the test solution and the reference solution using, as the compensation liquid, the solution obtained from the blank solution.

The absorbance of the test solution is not greater than that of the reference solution.

**Free iodine**: maximum 10 ppm.

Dissolve 2.0 g in 25 mL of *water R* in a ground-glass stoppered centrifuge tube. Add 5 mL of *toluene R* and 5 mL of *dilute sulfuric acid R*. Shake and centrifuge. Any red colour of the upper layer is not more intense than that of the upper phase obtained in the same way from 22 mL of *water R*, 2 mL of *iodide standard solution (10 ppm I) R*, 5 mL of *dilute sulfuric acid R*, 1 mL of *strong hydrogen peroxide solution R* and 5 mL of *toluene R*.

**Iodide**: maximum 10 ppm.

Dissolve 6.000 g in *water R* and dilute to 20 mL with the same solvent. Add 2.0 mL of *0.001 M potassium iodide*. Carry out a potentiometric titration (*2.2.20*) with *0.001 M silver nitrate* using a silver indicator electrode and an appropriate reference electrode. Subtract the volume of titrant corresponding to the 2.0 mL of *0.001 M potassium iodide*, determined by titrating a blank to which is added 2.0 mL of *0.001 M potassium iodide* and use the residual value to calculate the iodide content.

1 mL of *0.001 M silver nitrate* is equivalent to 126.9 µg of iodide.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.4 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

To 0.300 g in a 250 mL round-bottomed flask add 5 mL of *strong sodium hydroxide solution R*, 20 mL of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 5 mL of *glacial acetic acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*) using a suitable electrode system such as silver-silver chloride.

l mL of *0.1 M silver nitrate* is equivalent to 25.90 mg of $C_{17}H_{22}I_3N_3O_8$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K.*

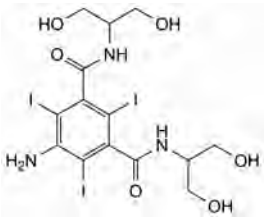

A. 5-amino-*N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,

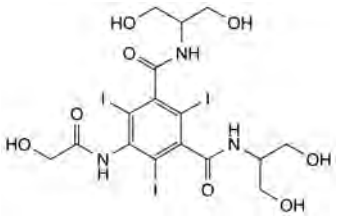

B. 5-[(hydroxyacetyl)amino]-*N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,

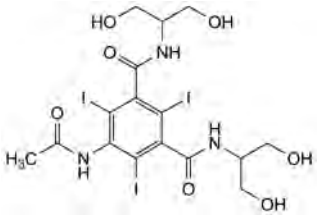

C. 5-(acetylamino)-*N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,

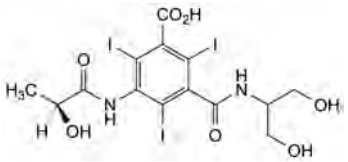

D. 3-[[2-hydroxy-1-(hydroxymethyl)ethyl]carbamoyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzoic acid,

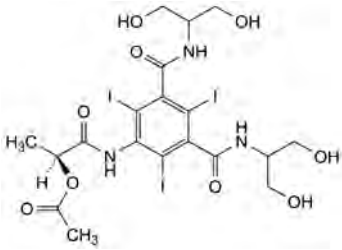

E. (1*S*)-2-[[3,5-bis[[2-hydroxy-1-(hydroxymethyl)ethyl]-carbamoyl]-2,4,6-triiodophenyl]amino]-1-methyl-2-oxoethyl acetate,

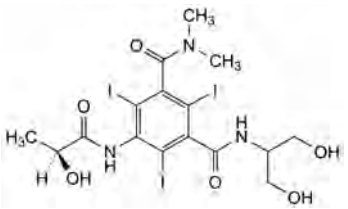

F. *N*'-[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodo-*N*,*N*-dimethylbenzene-1,3-dicarboxamide,

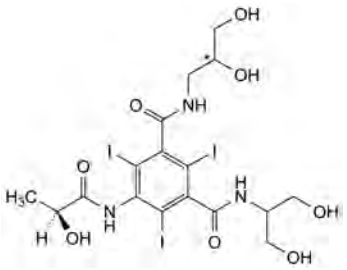

G. *N*-(2,3-dihydroxypropyl)-*N*'-[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]-amino]-2,4,6-triiodobenzene-1,3-dicarboxamide,

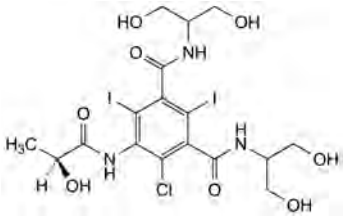

H. 4-chloro-*N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,6-diiodobenzene-1,3-dicarboxamide,

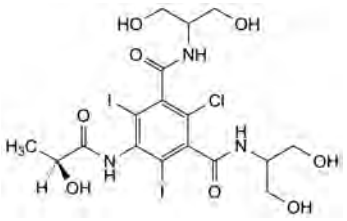

I. 2-chloro-*N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-4,6-diiodobenzene-1,3-dicarboxamide,

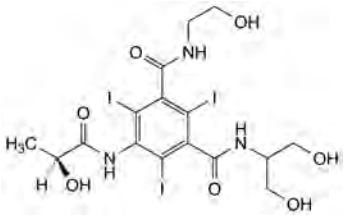

J. *N*-(2-hydroxyethyl)-*N*'-[2-hydroxy-1-(hydroxymethyl)-ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,4,6-triiodobenzene-1,3-dicarboxamide,

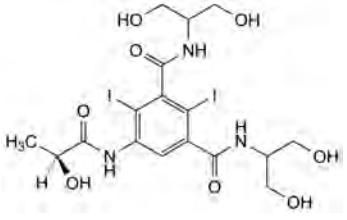

K. *N*,*N*'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5-[[(2*S*)-2-hydroxypropanoyl]amino]-2,4-diiodobenzene-1,3-dicarboxamide.

**01/2016:0700**

# IOPANOIC ACID

## Acidum iopanoicum

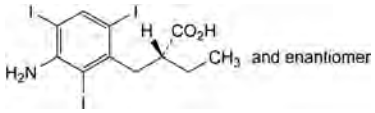

$C_{11}H_{12}I_3NO_2$ $M_r$ 571

[96-83-3]

DEFINITION

Iopanoic acid contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (*RS*)-2-(3-amino-2,4,6-tri-iodobenzyl)butanoic acid, calculated with reference to the dried substance.

CHARACTERS

A white or yellowish-white powder, practically insoluble in water, soluble in anhydrous ethanol and in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): about 155 °C, with decomposition.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *iopanoic acid CRS*.

C. Examine the chromatograms obtained in the test for related substances (see Tests). Spray the plate with a 1 g/L solution of *4-dimethylaminocinnamaldehyde R* in a mixture of 1 volume of *hydrochloric acid R* and 99 volumes of *ethanol (96 per cent) R*. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Heat 50 mg carefully in a small porcelain dish over a flame. Violet vapour is evolved.

TESTS

**Appearance of solution**. Dissolve 1.0 g in *1 M sodium hydroxide* and dilute to 20 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R*.

*Reference solution (a).* Dissolve 50 mg of *iopanoic acid CRS* in a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 50 mL with a mixture of 3 volumes of *ammonia R* and 97 volumes of *methanol R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *concentrated ammonia R*, 20 volumes of *methanol R*, 20 volumes of *toluene R* and 50 volumes of *dioxan R*. Examine

in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Halides**. To 0.46 g add 10 mL of *nitric acid R* and 15 mL of *water R*. Shake for 5 min and filter. 15 mL of the filtrate complies with the limit test for chlorides (*2.4.4*) (180 ppm, expressed as chloride).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.150 g in a 250 mL round-bottomed flask add 5 mL of *strong sodium hydroxide solution R*, 20 mL of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 60 min. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 mL of *dilute sulfuric acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 19.03 mg of $C_{11}H_{12}I_3NO_2$.

STORAGE

Store protected from light.

**01/2017:1753**

# IOPROMIDE

## Iopromidum

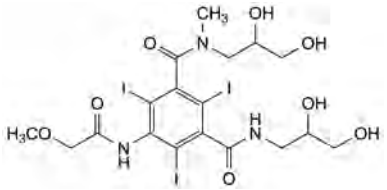

$C_{18}H_{24}I_3N_3O_8$ $M_r$ 791
[73334-07-3]

DEFINITION

*N*,*N*′-Bis(2,3-dihydroxypropyl)-2,4,6-triiodo-5-[(methoxyacetyl)amino]-*N*-methylbenzene-1,3-dicarboxamide.

Mixture of diastereoisomers and atropisomers.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder.

*Solubility*: freely soluble in water and in dimethyl sulfoxide, practically insoluble in ethanol (96 per cent) and in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *iopromide CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solutions $BY_6$, $B_6$ and $Y_6$ (*2.2.2, Method I*).

Dissolve 16.5 g in 20 mL of *carbon dioxide-free water R* while heating on a water-bath at a temperature not exceeding 70 °C. Allow to cool to room temperature.

**Conductivity** (*2.2.38*): maximum 50 µS·cm$^{-1}$.

Dissolve 1.000 g in *water R* and dilute to 50.0 mL with the same solvent.

**Impurity A and related primary aromatic amines**: maximum 0.01 per cent.

*Protect the solutions from light throughout the test. All given times are critical for the test results. The test solution, reference solution and blank solution must be processed in parallel.*

*Test solution*. Dissolve 0.500 g of the substance to be examined in 20.0 mL of *water R* in a 25 mL volumetric flask.

*Reference solution*. Dissolve the contents of a vial of *iopromide impurity A CRS* in 5.0 mL of *water R*. Transfer 2.0 mL of this solution to a 25 mL volumetric flask and add 18.0 mL of *water R*.

*Blank solution*. Place 20.0 mL of *water R* in a 25 mL volumetric flask.

Cool the test solution, reference solution and blank solution in a bath of iced water for 5 min. Add 1.0 mL of *hydrochloric acid R1* to each solution and cool again for 5 min in a bath of iced water. Add 1.0 mL of a 20 g/L solution of *sodium nitrite R*, shake vigorously and cool for another 5 min in a bath of iced water. To each solution add 0.50 mL of an 80 g/L solution of *sulfamic acid R*. Over the next 5 min, shake vigorously several times, raising the stoppers to vent the gas that evolves. Afterwards, add to each solution 1.0 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* in a mixture of 300 volumes of *water R* and 700 volumes of *propylene glycol R*, shake, allow to cool to room temperature for 10 min and dilute to 25.0 mL with *water R*. Degas the solutions in an ultrasonic bath for 1 min and measure the absorbance (*2.2.25*) of the test solution and the reference solution at 495 nm against the blank, within 5 min. The test is not valid unless the absorbance of the reference solution is at least 0.08. The absorbance of the test solution is not greater than the absorbance of the reference solution.

**Impurity B**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 40.0 mg of *iopromide CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b)*. Introduce several millilitres of reference solution (a) into a vial sealed with a crimp-top. Heat at 121 °C for 15 min.

*Reference solution (c)*. Dilute 1.5 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*: mix 6 g of *chloroform R* with 59 g of *methanol R*. Add 900 g of *water for chromatography R* in small portions to the chloroform/methanol mixture and stir for at least 2 h to obtain a homogeneous solution.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 50 min.

*Identification of impurities*: use the chromatogram supplied with *iopromide CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurity B isomers $Y_1$ and $Y_2$.

*Relative retention* with reference to iopromide isomer $Z_2$ (retention time = about 34 min): impurity B isomer $Y_1$ = about 0.28; impurity B isomer $Y_2$ = about 0.31.

*System suitability*: reference solution (a):

– the chromatogram obtained shows 2 peaks due to impurity B isomers $Y_1$ and $Y_2$.

*Limit*:

– *sum of impurity B isomers $Y_1$ and $Y_2$*: not more than the sum of the areas of the 2 principal peaks due to the iopromide in the chromatogram obtained with reference solution (c) (1.5 per cent).

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 1.0 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (e)*. Dissolve the contents of a vial of *iopromide for system suitability 1 CRS* (containing impurities B and E) in 50 µL of the solvent mixture.

*Reference solution (f)*. Dissolve the contents of a vial of *iopromide for system suitability 2 CRS* (containing impurities B, C, D and F) in 50 µL of the solvent mixture.

*Plates*: *TLC silica gel $F_{254}$ plate R* (2 plates).

A. *Mobile phase*: *concentrated ammonia R*, *water R*, *dioxan R* (4:15:85 *V/V/V*).

*Application*: 2 µL of the test solution and reference solutions (b), (d) and (e).

*Development*: over 3/4 of the plate.

*Drying*: in a current of air, until complete evaporation of the solvents, then at 120 °C for 30 min.

*Detection*: examine immediately in ultraviolet light at 254 nm; expose to ultraviolet light for 2-5 min until the principal spots appear clearly as yellow spots, then spray with *ferric chloride-ferricyanide-arsenite reagent R* and examine immediately in daylight.

*Retardation factors*: impurity B = about 0.26; iopromide = about 0.34; impurity E = about 0.41.

*System suitability*: reference solution (e):

– the chromatogram shows 3 clearly separated spots.

*Limits*:

– *impurity E*: any spot due to impurity E is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *unspecified impurities*: any other spot is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.10 per cent); disregard any spot due to impurity B.

B. *Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *chloroform R* (2:6:32:62 *V/V/V/V*).

*Application*: 2 µL of the test solution and reference solutions (a), (b) ,(c), (d) and (f).

*Development*: over 3/4 of the plate.

*Drying*: in a current of air, until complete evaporation of the solvents, then at 120 °C for 30 min.

*Detection*: examine immediately in ultraviolet light at 254 nm; expose to an ammonia vapour for 30 min, dry in a current of air for 10 min, then expose to ultraviolet light for 2-5 min until the principal spots appear clearly as yellow spots, then spray with *ferric chloride-ferricyanide-arsenite reagent R* and examine immediately in daylight.

*Retardation factors*: impurity C = about 0.23; impurity D = about 0.29; impurity B = about 0.36; iopromide = about 0.43; impurity F = about 0.71.

*System suitability*: reference solution (f):

– the chromatogram shows 5 clearly separated spots.

*Limits*:

– *impurity D*: any spot due to impurity D is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *impurity C*: any spot due to impurity C is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurity F*: any spot due to impurity F is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: any other spot is not more intense than the principal spot in the chromatogram obtained with reference solution (d) (0.10 per cent); disregard any spot due to impurity B.

**Isomer distribution**. Liquid chromatography (*2.2.29*) as described in the test for impurity B with the following modifications.

Calculate the percentage content of the isomer groups with reference to the total area of all the peaks due to the 4 iopromide isomers, using the chromatogram obtained with the test solution.

*Limits*:

– *sum of iopromide isomers $E_1$ and $Z_1$*: 40.0 per cent to 51.0 per cent;

– *sum of iopromide isomers $E_2$ and $Z_2$*: 49.0 per cent to 60.0 per cent.

**Free iodine**. Dissolve 2.0 g in 20 mL of *water R* in a glass-stoppered test tube. Add 2 mL of *dilute sulfuric acid R* and 2 mL of *toluene R*, close and shake vigorously. The upper layer remains colourless (*2.2.2, Method II*).

**Iodide**: maximum 2 ppm.

Dissolve 10.0 g in 50 mL of *carbon dioxide-free water R*. Adjust to pH 3-4 adding about 0.15 mL of *0.1 M sulfuric acid*. Titrate with *0.001 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*) using a combined metal electrode. Not more than 0.15 mL of *0.001 M silver nitrate* is required to reach the end-point.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.0 IU/g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurity B with the following modifications.

*Injection*: test solution and reference solutions (a) and (b).

*Identification of the isomers*: the 2 principal peaks in the chromatogram obtained with reference solution (a) are due to iopromide isomers $Z_1$ and $Z_2$ . The 2 peaks that have an increased size in the chromatogram obtained with reference solution (b) in comparison to the chromatogram obtained with reference solution (a), are due to iopromide isomers $E_1$ and $E_2$.

*Relative retention* with reference to iopromide isomer $Z_2$ (retention time = about 34 min): iopromide isomer $E_1$ = about 0.70; iopromide isomer $E_2$ = about 0.75; iopromide isomer $Z_1$ = about 0.85.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to iopromide isomers $Z_1$ and $Z_2$.

Calculate the percentage content of iopromide from the declared content of *iopromide CRS* and from the sum of the areas of all of the peaks due to isomer groups E and Z.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H.*

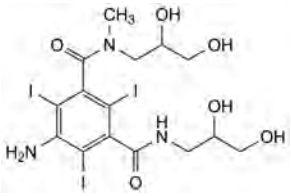

A. 5-amino-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-*N*-methylbenzene-1,3-dicarboxamide,

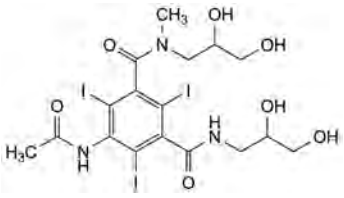

B. 5-(acetylamino)-*N*,*N*′-bis(2,3-dihydroxypropyl)-2,4,6-triiodo-*N*-methylbenzene-1,3-dicarboxamide,

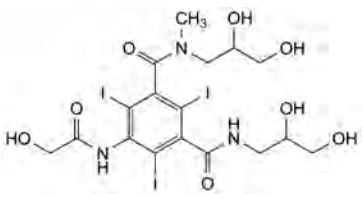

C. *N*,*N*′-bis(2,3-dihydroxypropyl)-5-[(hydroxyacetyl)amino]-2,4,6-triiodo-*N*-methylbenzene-1,3-dicarboxamide,

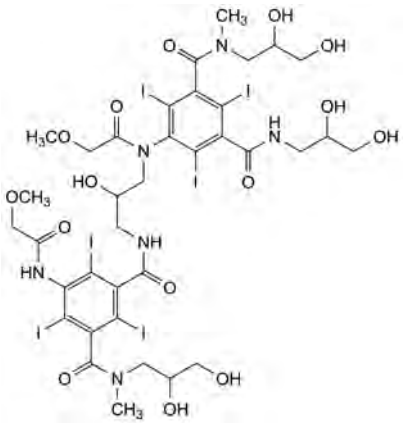

D. *N*-(2,3-dihydroxypropyl)-*N*′-[3-[[3-[(2,3-dihydroxypropyl)carbamoyl]-5-[(2,3-dihydroxypropyl)methylcarbamoyl]-2,4,6-triiodophenyl](methoxyacetyl)amino]-2-hydroxypropyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]-*N*-methylbenzene-1,3-dicarboxamide,

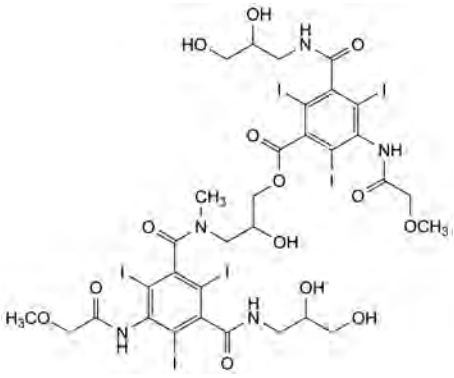

E. 3-[[3-[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]benzoyl]methylamino]-2-hydroxypropyl 3-[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]benzoate,

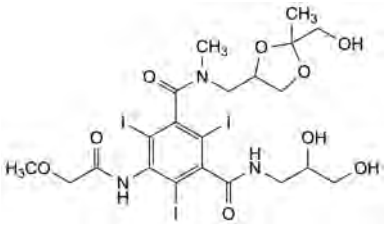

F. *N*′-(2,3-dihydroxypropyl)-*N*-[[2-(hydroxymethyl)-2-methyl-1,3-dioxolan-4-yl]methyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]-*N*-methylbenzene-1,3-dicarboxamide,

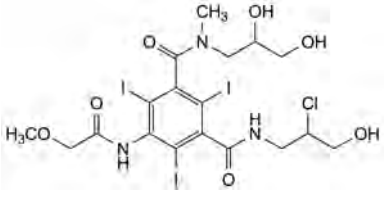

G. *N*′-(2-chloro-3-hydroxypropyl)-*N*-(2,3-dihydroxypropyl)-2,4,6-triiodo-5-[(methoxyacetyl)amino]-*N*-methylbenzene-1,3-dicarboxamide,

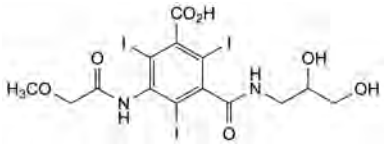

H. 3-[(2,3-dihydroxypropyl)carbamoyl]-2,4,6-triiodo-5-[(methoxyacetyl)amino]benzoic acid.

**01/2017:1754**

# IOTROLAN

## Iotrolanum

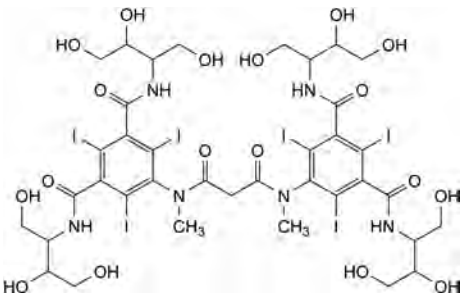

$C_{37}H_{48}I_6N_6O_{18}$ $M_r$ 1626

[79770-24-4]

DEFINITION

Mixture of stereoisomers of 5,5′-[propanedioylbis(methylimino)]bis[*N*,*N*′-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]2,4,6-triiodobenzene-1,3-dicarboxamide].

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: very soluble in water, freely soluble in dimethyl sulfoxide, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *iotrolan CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 18.0 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 25 µS·cm$^{-1}$.

Dissolve 1.000 g in *water R* and dilute to 50.0 mL with the same solvent.

**Primary aromatic amines**. *Protect the solutions from light throughout the test. All given times are critical for the test results. The test solution, the reference solution and the blank solution must be processed in parallel.*

*Test solution.* Dissolve 0.500 g of the substance to be examined in 20.0 mL of *water R* in a 25 mL volumetric flask.

*Reference solution.* Dissolve 5.0 mg of *iopamidol impurity A CRS* in *water R* and dilute to 20.0 mL with the same solvent. Transfer 1.0 mL of this solution to a 25 mL volumetric flask and add 19.0 mL of *water R*.

*Blank solution.* Place 20.0 mL of *water R* in a 25 mL volumetric flask.

*Procedure.* Cool the test solution, reference solution and blank solution in a bath of iced water for 5 min. Add 1.0 mL of *hydrochloric acid R1* to each solution and cool again for 5 min in a bath of iced water. Add 1.0 mL of a 20 g/L solution of *sodium nitrite R*, shake vigorously and cool for another 5 min in a bath of iced water. To each solution add 0.50 mL of an 80 g/L solution of *sulfamic acid R*. Over the next 5 min, shake vigorously several times, raising the stoppers to vent the gas that evolves. Afterwards add to each solution 1.0 mL of a 1 g/L solution of *naphthylethylenediamine dihydrochloride R* in a mixture of 300 volumes of *water R* and 700 volumes of *propylene glycol R*, shake, allow to cool to room temperature for 10 min and dilute to 25.0 mL with *water R*. Degas the solutions in an ultrasonic bath for 1 min and measure the absorbance (*2.2.25*) of the test solution and the reference solution at 495 nm against the blank, within 5 min.

*System suitability*:

– *absorbance of the reference solution*: minimum 0.40.

*Limit*:

– *absorbance of the test solution*: not more than the absorbance of the reference solution (0.05 per cent).

**Related substances**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 1.0 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution (b).* Dilute 2.0 mL of reference solution (a) to 10.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution (c).* Dissolve the contents of a vial of *iotrolan for system suitability CRS* (containing about 0.05 per cent of each of impurities A and B) in 50 µL of a mixture of equal volumes of *methanol R* and *water R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Pretreatment*: over 3/4 of the plate with *methylene chloride R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *dioxan R* (4:20:80 *V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in a current of air until the solvents have evaporated.

*Detection*: examine in ultraviolet light at 254 nm. Expose the plate to the ultraviolet light for 2-5 min until the principal spots appear clearly as yellow spots. Spray with *ferric chloride-ferricyanide-arsenite reagent R* and examine in daylight.

*$R_F$ values*: iotrolan = about 0.25; impurity A = about 0.4; impurity B = about 0.5.

*System suitability*: reference solution (c):

– the chromatogram shows 3 clearly separated spots.

*Limits*:

– *impurities A, B*: any spot due to impurity A or B is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *unspecified impurities*: any other spot is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.10 per cent).

**Isomer distribution**. Liquid chromatography (*2.2.29*) as described under Assay. Use the normalisation procedure.

*Identification of peaks*: use the chromatogram supplied with *iotrolan CRS* and the chromatogram obtained with the reference solution to identify the peaks due to the 3 isomer groups.

Calculate the percentage content of each of the isomer groups G1, G2 and G3, with reference to the total area of all of the peaks due to the 3 isomer groups, using the chromatogram obtained with the test solution.

*Limits*:

- *isomer group G1*: 53.0 per cent to 70.0 per cent;
- *isomer group G2*: 3.0 per cent to 11.0 per cent;
- *isomer group G3*: 25.0 per cent to 39.0 per cent.

**Free iodine**. Dissolve 0.20 g in 1 mL of *water R* in a glass-stoppered test tube. Add 4 mL of a 370 g/L solution of *sulfuric acid R* and 5 mL of *toluene R*, close and shake vigorously. The upper layer remains colourless (*2.2.2, Method II*).

**Iodide**: maximum 20 ppm.

Dissolve 10.0 g in 50 mL of *carbon dioxide-free water R*. Adjust to pH 3-4 adding about 0.15 mL of *dilute sulfuric acid R*. Titrate with *0.001 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Not more than 1.5 mL of *0.001 M silver nitrate* is required to reach the end-point.

**Water** (*2.5.12*): maximum 3.5 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.7 IU/g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution.* Dissolve 40.0 mg of *iotrolan CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *methanol R*, *water for chromatography R* (10:90 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 40 min.

*Retention time*: isomer group G1 = about 8 min to 12 min; isomer group G2 = about 15 min to 22 min; isomer group G3 = about 22 min to 32 min.

*System suitability*: reference solution:

- the chromatogram obtained is similar to the chromatogram supplied with *iotrolan CRS*.

Calculate the percentage content of iotrolan from the total area of all of the peaks of the 3 isomer groups G1, G2 and G3 and the declared content of *iotrolan CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G, H, I, J.*

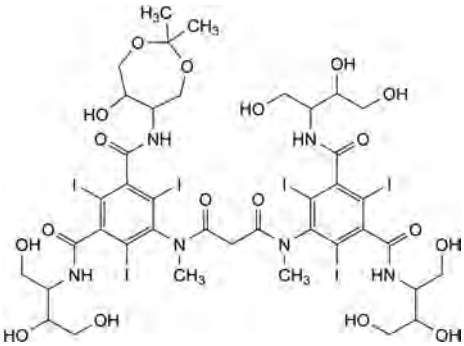

A. *N*,*N*′-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]-5-[[3-[[3-[[2,3-dihydroxy-1-(hydroxymethyl)propyl]carbamoyl]-5-[(6-hydroxy-2,2-dimethyl-1,3-dioxepan-5-yl)carbamoyl]-2,4,6-triiodophenyl]methylamino]-3-oxopropanoyl]methylamino]-2,4,6-triiodobenzene-1,3-dicarboxamide,

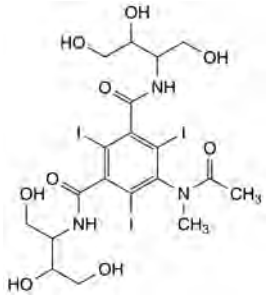

B. 5-(acetylmethylamino)-*N*,*N*′-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]-2,4,6-triiodobenzene-1,3-dicarboxamide,

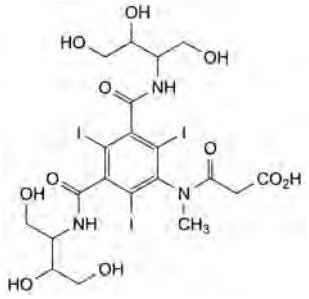

C. 3-[[3,5-bis[[2,3-dihydroxy-1-(hydroxymethyl)propyl]-carbamoyl]-2,4,6-triiodophenyl]methylamino]-3-oxopropanoic acid,

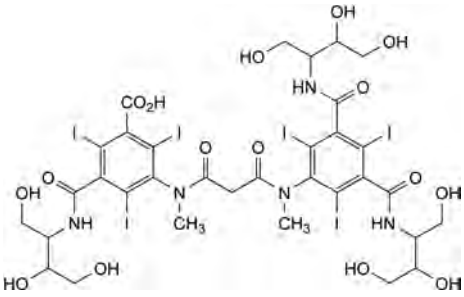

D. 3-[[3-[[3,5-bis[[2,3-dihydroxy-1-(hydroxymethyl)-propyl]carbamoyl]-2,4,6-triiodophenyl]methylamino]-3-oxopropanoyl]methylamino]-5-[[2,3-dihydroxy-1-(hydroxymethyl)propyl]carbamoyl]-2,4,6-triiodobenzoic acid,

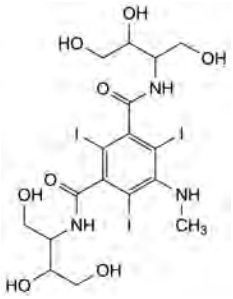

E. *N*,*N*′-bis[2,3-dihydroxy-1-(hydroxymethyl)propyl]-2,4,6-triiodo-5-(methylamino)benzene-1,3-dicarboxamide,

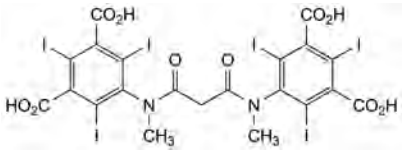

F. 5,5′-[propanedioylbis(methylimino)]bis[2,4,6-triiodobenzene-1,3-dicarboxylic] acid,

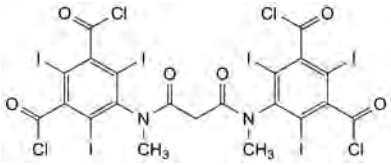

G. 5,5′-[propanedioylbis(methylimino)]bis[2,4,6-triiodobenzene-1,3-dicarbonyl] tetrachloride,

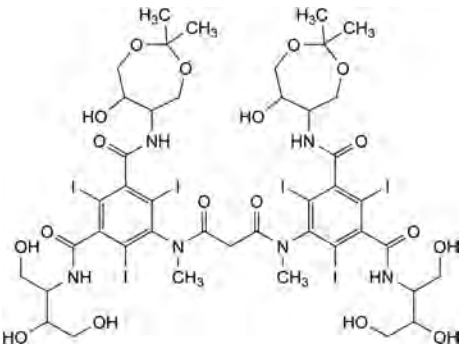

H. 5,5′-[propanedioylbis(methylimino)]bis[*N*-[2,3-dihydroxy-1-(hydroxymethyl)propyl]-*N*′-(6-hydroxy-2,2-dimethyl-1,3-dioxepan-5-yl)-2,4,6-triiodobenzene-1,3-dicarboxamide],

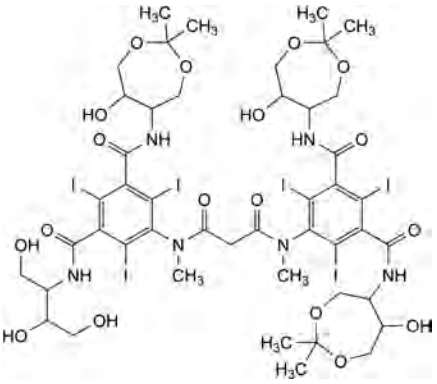

I. 5-[[3-[[3-[[2,3-dihydroxy-1-(hydroxymethyl)propyl]-carbamoyl]-5-[(6-hydroxy-2,2-dimethyl-1,3-dioxepan-5-yl)carbamoyl]-2,4,6-triiodophenyl]methylamino]-3-oxopropanoyl]methylamino]-*N*,*N*′-bis(6-hydroxy-2,2-dimethyl-1,3-dioxepan-5-yl)-2,4,6-triiodobenzene-1,3-dicarboxamide,

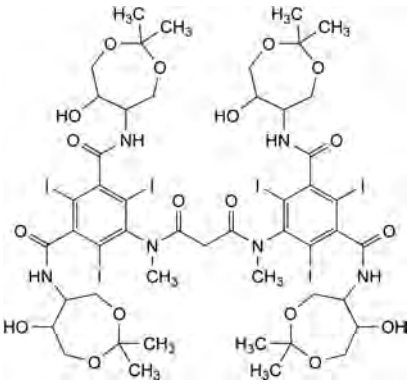

J. 5,5′-[propanedioylbis(methylimino)]bis[*N*,*N*′-bis(6-hydroxy-2,2-dimethyl-1,3-dioxepan-5-yl)-2,4,6-triiodobenzene-1,3-dicarboxamide].

**01/2017:2009**

# IOXAGLIC ACID

## Acidum ioxaglicum

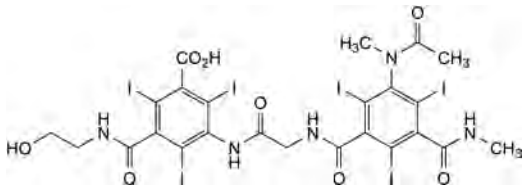

$C_{24}H_{21}I_6N_5O_8$ $M_r$ 1269
[59017-64-0]

DEFINITION

3-[[[[3-(Acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ioxaglic acid CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 1.0 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 20 mL with the same solution.

**Absorbance** (*2.2.25*): maximum 0.18, calculated for a solution containing 40 per cent of anhydrous ioxaglic acid.

Dissolve 10.0 g in about 8 mL of a 40 g/L solution of *sodium hydroxide R*. Adjust to pH 7.2-7.6 with a 40 g/L solution of *sodium hydroxide R* or *1 M hydrochloric acid*. Dilute to 25 mL with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm). Measure the absorbance at 450 nm using *water R* as the compensation liquid.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: *acetonitrile R*, *water R* (5:95 *V/V*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in about 40 mL of the solvent mixture. Add 0.5 ± 0.1 mL of a 4 g/L solution of *sodium hydroxide R* and dilute to 50.0 mL with the solvent mixture. Shake until dissolution is complete, using ultrasound if necessary.

*Reference solution (a).* Dissolve 0.10 g of *ioxaglic acid CRS* in about 40 mL of the solvent mixture. Add 0.5 ± 0.1 mL of a 4 g/L solution of *sodium hydroxide R* and dilute to 50.0 mL with the solvent mixture. Shake until dissolution is complete, using ultrasound if necessary.

*Reference solution (b).* Dissolve 5 mg of *ioxaglic acid impurity A CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 0.136 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 → 90 | 5 → 10 |
| 5 - 40 | 90 | 10 |
| 40 - 85 | 90 → 70 | 10 → 30 |
| 85 - 115 | 70 | 30 |
| 115 - 120 | 70 → 50 | 30 → 50 |
| 120 - 125 | 50 | 50 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *ioxaglic acid CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C, D1, D2, D3, D4, E and F; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to ioxaglic acid (retention time = about 65 min): impurity A = about 0.3; impurity B = about 0.7; impurity C = about 0.9; impurity D1 = about 1.09; impurity E = about 1.12; impurity D2 = about 1.20; impurity D3 = about 1.26; impurity D4 = about 1.28; impurity F = about 1.6.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ioxaglic acid.

*Limits*:

- *impurity D (sum of the peaks due to impurities D1, D2, D3 and D4)*: maximum 0.7 per cent;
- *impurity E*: maximum 0.7 per cent;
- *impurity F*: maximum 0.4 per cent;
- *impurity B*: maximum 0.3 per cent;
- *impurity C*: maximum 0.3 per cent;
- *impurity A*: maximum 0.1 per cent;
- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard any peak with a retention time greater than 125 min.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Iodides**: maximum 50 ppm.

Disperse 10.0 g in 50 mL of *water R*. Add 8 mL of *1 M sodium hydroxide*. After dissolution and homogenisation, add 1.0 mL of *glacial acetic acid R*. Immediately titrate with *0.001 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*), using a silver indicator electrode and a suitable reference electrode.

1 mL of *0.001 M silver nitrate* is equivalent to 0.1269 mg of iodides.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

In a round-bottomed flask place 0.100 g of the substance to be examined and add 5 mL of *strong sodium hydroxide solution R*, 20 mL of *water R*, 1 g of *zinc powder R* and a few glass beads. Fit the flask with a reflux condenser and boil for 30 min. Cool and rinse the condenser with 20 mL of *water R*. Add the rinsings to the contents of the flask. Filter, wash the filter with 3 quantities, each of 15 mL, of *water R* and add the washings to the filtrate. Add 40 mL of *dilute sulfuric acid R* and titrate immediately with *0.05 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.05 M silver nitrate* is equivalent to 10.58 mg of $C_{24}H_{21}I_6N_5O_8$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H.*

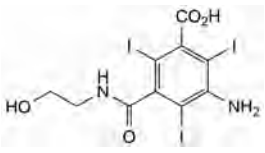


A. 3-amino-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

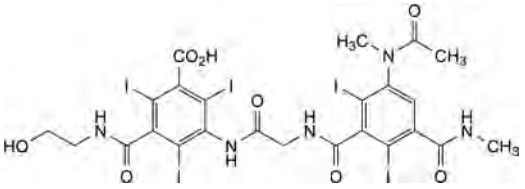


B. 3-[[[[3-(acetylmethylamino)-2,6-diiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

C. unknown structure,

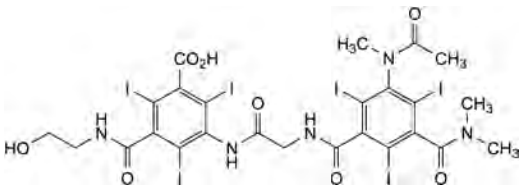

D. D1, D2, D3 and D4: 3-[[[[3-(acetylmethylamino)-5-(dimethylcarbamoyl)-2,4,6-triiodobenzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

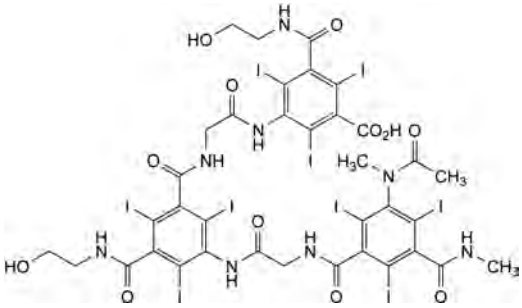

E. 3-[[[[3-[[[[3-(acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoyl]amino]acetyl]amino]-5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic acid,

F. unknown structure,

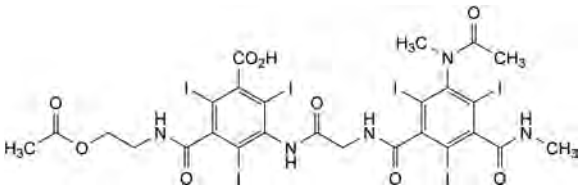

G. 3-[[[[3-(acetylmethylamino)-2,4,6-triiodo-5-(methylcarbamoyl)benzoyl]amino]acetyl]amino]-5-[[2-(acetyloxy)ethyl]carbamoyl]-2,4,6-triiodobenzoic acid,

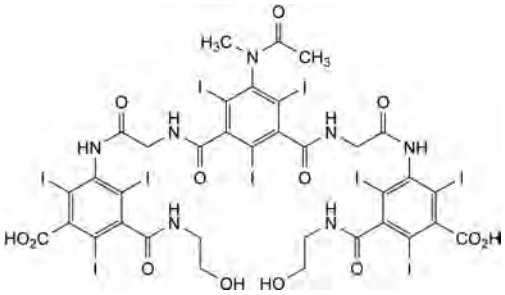

H. 3,3′-[[5-(acetylmethylamino)-2,4,6-triiodo-1,3-phenylene]bis(carbonyliminomethylenecarbonylimino)]bis[5-[(2-hydroxyethyl)carbamoyl]-2,4,6-triiodobenzoic] acid.

**01/2008:0919**
**corrected 9.6**

# IPRATROPIUM BROMIDE

## Ipratropii bromidum

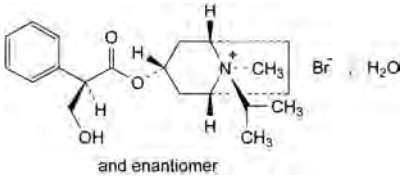


$C_{20}H_{30}BrNO_3,H_2O$ $M_r$ 430.4
[66985-17-9]

DEFINITION

(1*R*,3*r*,5*S*,8*r*)-3-[[(2*RS*)-3-Hydroxy-2-phenylpropanoyl]oxy]-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane bromide monohydrate.

*Content*: 99.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in methanol, slightly soluble in ethanol (96 per cent).

mp: about 230 °C, with decomposition.

IDENTIFICATION

*First identification: A, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ipratropium bromide CRS*.

B. Examine the chromatograms obtained in the test for impurity A.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 5 mL of solution S (see Tests), add 2 mL of *dilute sodium hydroxide solution R*. No precipitate is formed.

D. To about 1 mg add 0.2 mL of *nitric acid R* and evaporate to dryness on a water-bath. Dissolve the residue in 2 mL of *acetone R* and add 0.1 mL of a 30 g/L solution of *potassium hydroxide R* in *methanol R*. A violet colour develops.

E. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.5 for solution S.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *ipratropium bromide CRS* in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *methylatropine bromide CRS* in 1.0 mL of reference solution (a).

*Reference solution (c).* Dissolve 5 mg of *ipratropium impurity A CRS* in 100.0 mL of *methanol R*. Dilute 2.0 mL of the solution to 5.0 mL with *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *anhydrous formic acid R*, *water R*, *ethanol (96 per cent) R*, *methylene chloride R* (1:3:18:18 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over a path of 6 cm.

*Drying*: at 60 °C for 15 min.

*Detection*: spray with *potassium iodobismuthate solution R5*, allow the plate to dry in air, spray with a 50 g/L solution of *sodium nitrite R* and protect immediately with a sheet of glass.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated principal spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10.0 mg of *ipratropium bromide CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *ipratropium bromide CRS* and 5 mg of *ipratropium impurity B CRS* in 1 mL of *methanol R* and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: dissolve 12.4 g of *sodium dihydrogen phosphate R* and 1.7 g of *tetrapropylammonium chloride R* in 870 mL of *water for chromatography R*; adjust to pH 5.5 with a 180 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and add 130 mL of *methanol R1*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 5 µL.

*Run time*: 6 times the retention time of ipratropium.

*Relative retention* with reference to ipratropium (retention time = about 4.9 min): impurity C = about 0.7; impurity B = about 1.2; impurity D = about 1.8; impurity E = about 2.3; impurity F = about 5.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity B and ipratropium;
– *symmetry factor*: maximum 2.5 for the principal peak.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.3; impurity D = 0.2; impurity F = 0.5;
– *impurity D*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
– *impurities B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
– *disregard limit*: one-third of the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent); disregard the peak due to the bromide ion.

**Water** (*2.5.12*): 3.9 per cent to 4.4 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *water R* and add 3 mL of *dilute nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 41.24 mg of $C_{20}H_{30}BrNO_3$.

IMPURITIES

*Specified impurities*: *A, B, C, D*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F*.

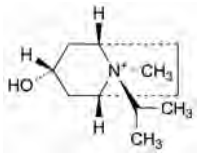

A. (1*R*,3*r*,5*S*,8*r*)-3-hydroxy-8-methyl-8-(1-methylethyl)-8-azoniabicyclo[3.2.1]octane,

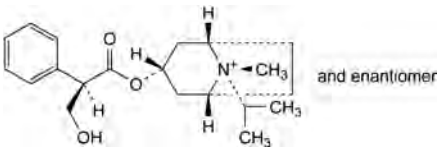


B. (1*R*,3*r*,5*S*,8*s*)-3-[[(2*RS*)-3-hydroxy-2-phenylpropanoyl]-oxy]-8-methyl-8-(1-methylethyl)-8-azoniabicyclo-[3.2.1]octane,

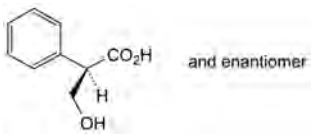


C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (DL-tropic acid),

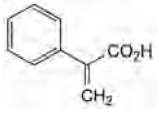

D. 2-phenylpropenoic acid (atropic acid),

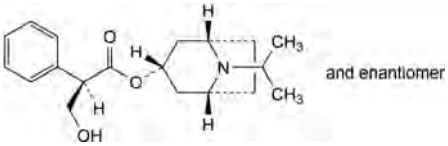


E. (1*R*,3*r*,5*S*)-8-(1-methylethyl)-8-azabicyclo[3.2.1]oct-3-yl (2*RS*)-3-hydroxy-2-phenylpropanoate,

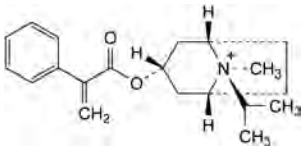

F. (1*R*,3*r*,5*S*,8*r*)-8-methyl-8-(1-methylethyl)-3-[(2-phenylpropenoyl)oxy]-8-azoniabicyclo[3.2.1]octane.

**01/2020:2465**

# IRBESARTAN

## Irbesartanum

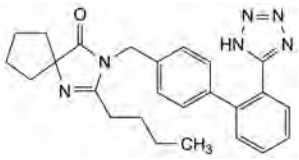

$C_{25}H_{28}N_6O$ $M_r$ 428.5
[138402-11-6]

DEFINITION

2-Butyl-3-[[2′-(1*H*-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]-1,3-diazaspiro[4.4]non-1-en-4-one.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

PRODUCTION

As *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are classified as probable human carcinogens, manufacturers must ensure that their manufacturing process does not generate such impurities and develop appropriate control strategies. To allow manufacturers to make the necessary changes to their process, a transition period has been agreed by competent authorities and strict temporary limits on levels of these impurities introduced in the Test section.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *irbesartan CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness at 60 °C and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 0.50 g in a mixture of 1 volume of *2 M sodium hydroxide R* and 9 volumes of *methanol R2* and dilute to 10 mL with the same mixture of solvents.

**Impurity B**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution*. Dissolve 25.0 mg of *sodium azide R* (sodium salt of impurity B) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 0.25 mL of the solution to 200.0 mL with the mobile phase.

*Precolumn* (used to prevent saturation of the column with irbesartan):

- *size*: *l* = 0.05 m, Ø = 4 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (8.5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (8.5 µm).

*Mobile phase*: 4.2 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: conductivity detector with a sensitivity of 3 µS; use a self-regenerating anion suppressor.

*Neutralisation of the eluent*: either chemical or electrochemical:

- *chemical*: by continuous countercurrent circulation in a neutralising micromembrane, performed before detection:
  - *neutralising solvent*: *0.025 M sulfuric acid*;
  - *flow rate*: 10 mL/min;
  - *pressure*: about 100 kPa.
- *electrochemical*: 300 mA (for example).

*Injection*: 200 µL; after each injection of the test solution, rinse the precolumn with a mixture of mobile phase and *methanol R* (40:60 *V/V*) for 10 min; equilibrate to initial conditions as necessary; a switch valve can be used to avoid disconnecting the precolumn from the column.

*Run time*: 25 min.

*Retention time*: impurity B = about 14 min.

*System suitability*: reference solution:

- *signal-to-noise ratio*: minimum 10 for the peak due to impurity B.

*Limit*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 3.2*. Mix 5.5 mL of *phosphoric acid R* and 950 mL of *water for chromatography R* and adjust to pH 3.2 with *triethylamine R*.

*Test solution*. Dissolve 50 mg of the substance to be examined in *methanol R2* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R2*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R2*.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *irbesartan impurity A CRS* in *methanol R2* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *methanol R2*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, buffer solution pH 3.2 (33:67 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 1.4 times the retention time of irbesartan.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to irbesartan (retention time = about 23 min): impurity A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity A and irbesartan.

*Limits*:

– *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Nitrosamines**. Carry out the test by a suitable method.

The substance to be examined does not contain either NDMA or NDEA above the limits provided below or both impurities at whatever level:

– *N-nitrosodimethylamine (NDMA)*: maximum 0.320 ppm;
– *N-nitrosodiethylamine (NDEA)*: maximum 0.088 ppm.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 42.85 mg of $C_{25}H_{28}N_6O$.

IMPURITIES

*Specified impurities*: *A, B*.

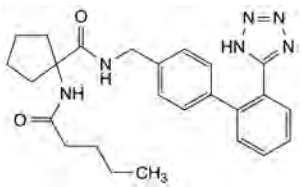

A. 1-(pentanoylamino)-*N*-[[2′-(1*H*-tetrazol-5-yl)[1,1′-biphenyl]-4-yl]methyl]cyclopentane-1-carboxamide,

B. $N_3^-$: trinitride (azide).

**01/2017:2675**
**corrected 10.0**

# IRINOTECAN HYDROCHLORIDE TRIHYDRATE

## Irinotecani hydrochloridum trihydricum

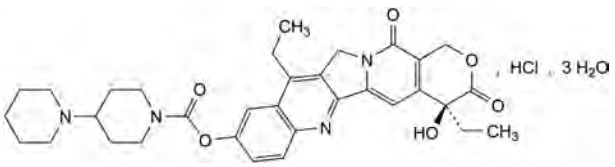

$C_{33}H_{39}ClN_4O_6,3H_2O$ $M_r$ 677
[136572-09-3]

DEFINITION

(4*S*)-4,11-Diethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate hydrochloride trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: pale yellow or yellow, crystalline powder.

*Solubility*: sparingly soluble in water, in ethanol (96 per cent) and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *irinotecan hydrochloride trihydrate CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Water (see Tests).

C. It gives reaction (a) of chlorides (*2.3.1*).
Dissolve 0.10 g in *water R* and dilute to 50 mL with the same solvent.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_2$ (*2.2.2, Method II*).

Dissolve 0.200 g in *water R* with heating at 80 °C and dilute to 20 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *diethylamine R*, *anhydrous ethanol R* (0.4:100 *V/V*).

*Test solution*. Dissolve 15.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *irinotecan for system suitability 1 CRS* (containing impurity L) in 1 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *diethylamine R*, *anhydrous ethanol R*, *hexane R* (0.2:50:50 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 370 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of irinotecan.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity L.

*Relative retention* with reference to irinotecan (retention time = about 15 min): impurity L = about 0.7.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to impurity L and irinotecan in the chromatogram obtained with reference solution (b).

*Calculation of percentage content*:

– for impurity L, use the concentration of irinotecan hydrochloride trihydrate in reference solution (a).

*Limit*:

– *impurity L*: maximum 0.15 per cent.

Monographs 1

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R*, *methanol R*, mobile phase A (25:25:50 *V/V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 50.0 mg of *irinotecan hydrochloride trihydrate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *irinotecan for peak identification CRS* (containing impurities C and E) in 1 mL of the solvent mixture.

*Reference solution (d).* Dissolve 5 mg of *irinotecan for system suitability 2 CRS* (containing impurity M) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 2.72 g of *potassium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 3.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R2*, *acetonitrile R1* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 42 | 80 → 30 | 20 → 70 |
| 42 - 47 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *irinotecan for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and E; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity M.

*Relative retention* with reference to irinotecan (retention time = about 17 min): impurity M = about 0.9; impurity C = about 1.3; impurity E = about 1.5.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity M and irinotecan in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity M by 1.3;
- for each impurity, use the concentration of irinotecan hydrochloride trihydrate in reference solution (b).

*Limits*:

- *impurities C, E, M*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 7.0 per cent to 9.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{33}H_{39}ClN_4O_6$ taking into account the assigned content of *irinotecan hydrochloride trihydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: C, E, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, F, G, H, K.*

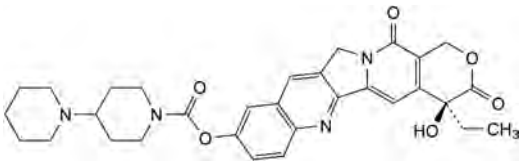

A. (4*S*)-4-ethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate (11-desethyl irinotecan),

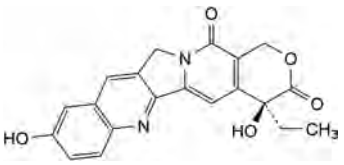

B. (4*S*)-4-ethyl-4,9-dihydroxy-1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione (9-hydroxycamptothecin),

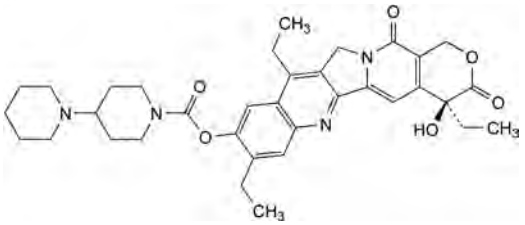

C. (4*S*)-4,8,11-triethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate (8-ethyl irinotecan),

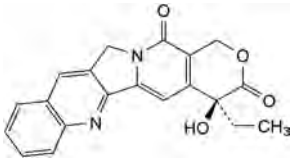

D. (4*S*)-4-ethyl-4-hydroxy-1*H*-pyrano[3′,4′:6,7]indolizino-[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione (camptothecin),

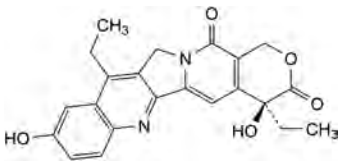

E. (4*S*)-4,11-diethyl-4,9-dihydroxy-1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione (11-ethyl-9-hydroxycamptothecin),

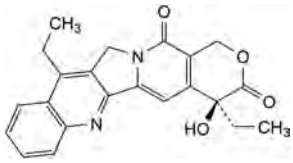

F. (4*S*)-4,11-diethyl-4-hydroxy-1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione (11-ethylcamptothecin),

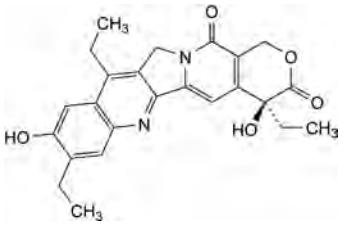

G. (4*S*)-4,8,11-triethyl-4,9-dihydroxy-1*H*-pyrano[3′,4′:6,7]-indolizino[1,2-*b*]quinoline-3,14(4*H*,12*H*)-dione (8,11-diethyl-9-hydroxycamptothecin),

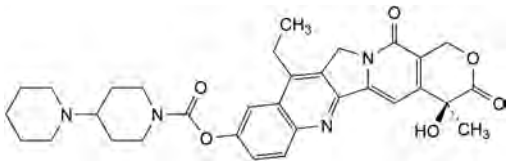

H. (4*S*)-11-ethyl-4-hydroxy-4-methyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate (4-methyl irinotecan analogue),

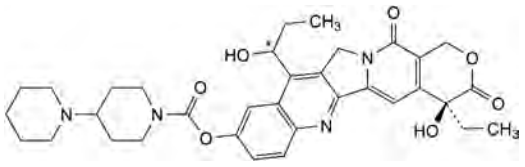

K. (4*S*)-4-ethyl-4-hydroxy-11-(1-hydroxypropyl)-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino-[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate,

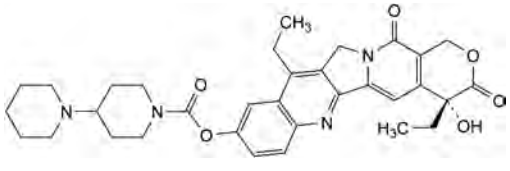

L. (4*R*)-4,11-diethyl-4-hydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate (irinotecan enantiomer).

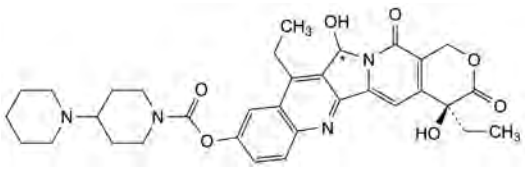

M. (4*S*)-4,11-diethyl-4,12-dihydroxy-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3′,4′:6,7]indolizino[1,2-*b*]quinolin-9-yl 1,4′-bipiperidine-1′-carboxylate (12-hydroxy irinotecan).

**01/2008:1018**
**corrected 6.0**

# ISOCONAZOLE

## Isoconazolum

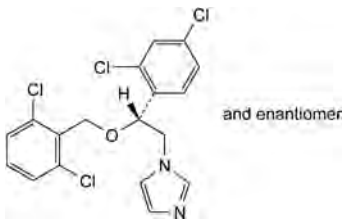


$C_{18}H_{14}Cl_4N_2O$ $M_r$ 416.1
[27523-40-6]

DEFINITION

1-[(2*RS*)-2-[(2,6-Dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in methanol, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 111 °C to 115 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *isoconazole CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 30 mg of *isoconazole CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 30 mg of *isoconazole CRS* and 30 mg of *econazole nitrate CRS* in *methanol R*, then dilute to 5 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R.*

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

Monographs 1

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.20 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in 3.2 mL of *methanol R*. Add 3.0 mL of *acetonitrile R* and dilute to 10.0 mL with a solution of *ammonium acetate R* (6.0 g in 380 mL of *water R*).

*Reference solution (a)*. Dissolve 2.5 mg of *isoconazole CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase, then dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 6.0 g of *ammonium acetate R* in a mixture of 300 mL of *acetonitrile R*, 320 mL of *methanol R* and 380 mL of *water R*.

*Flow rate*: 2 ml/min.

*Detection*: spectrophotometer at 235 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of isoconazole.

*Retention time*: econazole = about 10 min; isoconazole = about 14 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to econazole and isoconazole; if necessary, adjust the composition of the mobile phase.

*Limits*:

– *impurities B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from orange-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 41.61 mg of $C_{18}H_{14}Cl_4N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, C, D*.

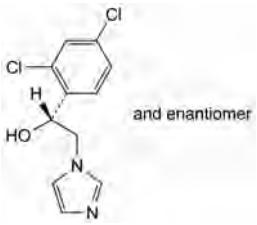


B. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

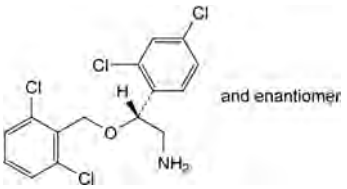


C. (2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)-ethanamine,

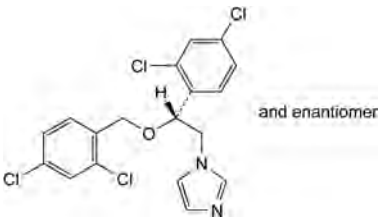


D. 1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

**01/2008:1017**
**corrected 6.7**

# ISOCONAZOLE NITRATE

## Isoconazoli nitras

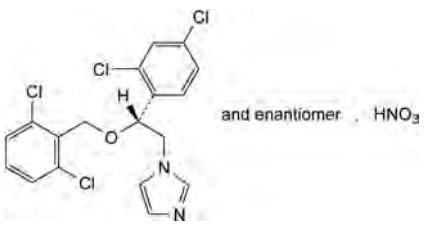


$C_{18}H_{15}Cl_4N_3O_4$ $M_r$ 479.1
[24168-96-5]

DEFINITION

1-[(2*RS*)-2-[(2,6-Dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole nitrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 178 °C to 182 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *isoconazole nitrate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a)*. Dissolve 30 mg of *isoconazole nitrate CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dissolve 30 mg of *isoconazole nitrate CRS* and 30 mg of *econazole nitrate CRS* in *methanol R*, then dilute to 5 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives the reaction of nitrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.20 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.5 mg of *isoconazole nitrate CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase, then dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 6.0 g of *ammonium acetate R* in a mixture of 300 mL of *acetonitrile R*, 320 mL of *methanol R* and 380 mL of *water R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of isoconazole.

*Retention time*: econazole = about 10 min; isoconazole = about 14 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to econazole and isoconazole; if necessary, adjust the composition of the mobile phase.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to the nitrate ion.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 75 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.91 mg of $C_{18}H_{15}Cl_4N_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

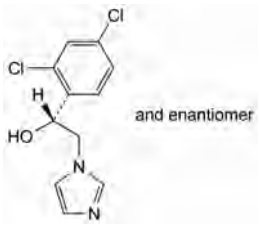


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

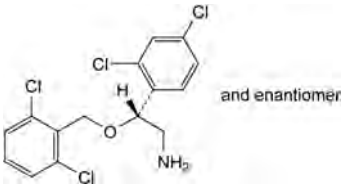


B. (2*RS*)-2-[(2,6-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethanamine,

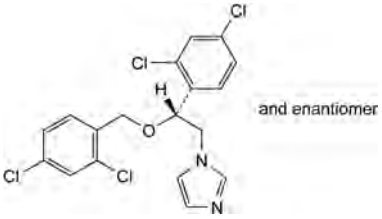


C. 1-[(2*RS*)-2-[(2,4-dichlorobenzyl)oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

**01/2008:1673**
**corrected 9.0**

# ISOFLURANE

## Isofluranum

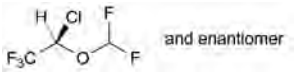


$C_3H_2ClF_5O$ $M_r$ 184.5
[26675-46-7]

DEFINITION

(2*RS*)-2-Chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane.

CHARACTERS

*Appearance*: clear, colourless, mobile, heavy liquid.

*Solubility*: practically insoluble in water, miscible with ethanol.

bp: about 48 °C.

It is non-flammable.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the substance in the gaseous state.

*Comparison*: *Ph. Eur. reference spectrum of isoflurane.*

TESTS

**Acidity or alkalinity**. To 20 mL add 20 mL of *carbon dioxide-free water R*, shake for 3 min and allow to stand. Collect the upper layer and add 0.2 mL of *bromocresol purple solution R*. Not more than 0.1 mL of *0.01 M sodium hydroxide* or 0.6 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Reference solution*. To 80 mL of *anhydrous ethanol R*, add 1.0 mL of the substance to be examined and 1.0 mL of *acetone R*, avoiding loss by evaporation. Dilute to 100.0 mL with *anhydrous ethanol R*. Dilute 1.0 mL of the solution to 100.0 mL with *anhydrous ethanol R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:25.

*Temperature*:

- *column*: 35 °C,
- *injection port*: 150 °C,
- *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL of each solution and 1.0 µL of *anhydrous ethanol R* as a blank.

*Run time*: until elution of the ethanol peak in the chromatogram obtained with the reference solution.

*Relative retention* with reference to isoflurane (retention time = about 3.8 min): acetone = about 0.75.

*System suitability*: reference solution:

- *resolution*: minimum of 5 between the peaks due to acetone and to isoflurane,
- *repeatability*: maximum relative standard deviation 15.0 per cent for the peak due to isoflurane after 3 injections.

*Limits*:

- *acetone*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.01 per cent),
- *any other impurity*: not more than the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.01 per cent),
- *total*: not more than 3 times the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.03 per cent),
- *disregard limit*: 0.1 times the area of the peak due to isoflurane in the chromatogram obtained with the reference solution (0.001 per cent).

**Chlorides** (*2.4.4*): maximum 10 ppm.

To 10 mL add 10 mL of *0.01 M sodium hydroxide* and shake for 3 min. To 5 mL of the upper layer add 10 mL of *water R*.

**Fluorides**: maximum 10 ppm.

Determine by potentiometry (*2.2.36, Method I*) using a fluoride-selective indicator-electrode and a silver-silver chloride reference electrode.

*Test solution*. To 10.0 mL in a separating funnel, add 10 mL of a mixture of 30.0 mL of *dilute ammonia R2* and 70.0 mL of *distilled water R*. Shake for 1 min and collect the upper layer. Repeat this extraction procedure twice collecting the upper layer each time. Adjust the combined upper layers to pH 5.2 using *dilute hydrochloric acid R*. Add 5.0 mL of *fluoride standard solution (1 ppm F) R* and dilute to 50.0 mL with *distilled water R*. To 20.0 mL of the solution add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

*Reference solutions*. To each of 5.0 mL, 4.0 mL, 3.0 mL, 2.0 mL and 1.0 mL of *fluoride standard solution (10 ppm F) R* add 20.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50.0 mL with *distilled water R*.

Carry out the measurements on 20 mL of each solution. Calculate the concentration of fluorides using the calibration curve, taking into account the addition of fluoride to the test solution.

**Non-volatile matter**: maximum 200 mg/L.

Evaporate 10.0 mL to dryness with the aid of a stream of cold air and dry the residue at 50 °C for 2 h. The residue weighs a maximum of 2.0 mg.

**Water** (*2.5.12*): maximum 1.0 mg/mL, determined on 10.0 mL.

STORAGE

In an airtight container, protected from light.

IMPURITIES

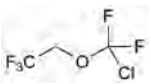


A. 2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane,

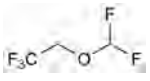


B. 2-(difluoromethoxy)-1,1,1-trifluoroethane,

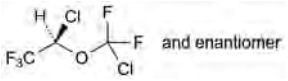


C. (2*RS*)-2-chloro-2-(chlorodifluoromethoxy)-1,1,1-trifluoroethane,

Monographs I

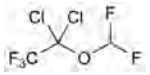

D. 1,1-dichloro-1-(difluoromethoxy)-2,2,2-trifluoroethane,

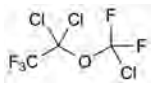

E. 1,1-dichloro-1-(chlorodifluoromethoxy)-2,2,2-trifluoroethane,

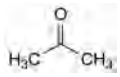

F. propanone (acetone).

**01/2017:0770**

# ISOLEUCINE

## Isoleucinum

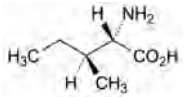

$C_6H_{13}NO_2$ $M_r$ 131.2
[73-32-5]

DEFINITION

(2*S*,3*S*)-2-Amino-3-methylpentanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or flakes.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *isoleucine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Reference solution.* Dissolve 10 mg of *isoleucine CRS* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

**Specific optical rotation** (*2.2.7*): + 40.0 to + 43.0 (dried substance).

Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *valine R* (impurity A) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (d).* Dissolve 30.0 mg of *leucine R* (impurity C) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (e).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (f).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* (impurity C) in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test solution, blank solution and reference solutions (a), (b), (c), (d) and (f) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (f):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and impurity C.

*Calculation of percentage contents*:

– for impurity A, use the concentration of impurity A in reference solution (b);
– for impurity C, use the concentration of impurity C in reference solution (d);
– for any ninhydrin-positive substance detected at 570 nm, use the concentration of isoleucine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (c); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *impurities A and C at 570 nm*: for each impurity, maximum 0.3 per cent;
– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related Substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in *water R* and dilute to 15 mL with the same solvent.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in 3 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (e) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.12 mg of $C_6H_{13}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D.*

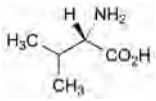

A. (2*S*)-2-amino-3-methylbutanoic acid (valine),

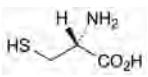

B. (2*R*)-2-amino-3-sulfanylpropanoic acid (cysteine),

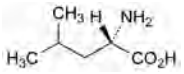

C. (2*S*)-2-amino-4-methylpentanoic acid (leucine),

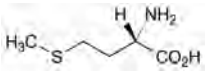

D. (2*S*)-2-amino-4-(methylsulfanyl)butanoic acid (methionine).

**07/2019:1531**

# ISOMALT[(2)]

## Isomaltum

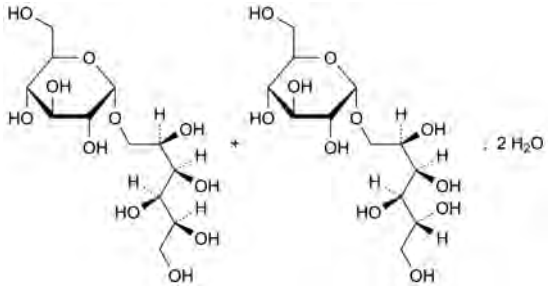

$C_{12}H_{24}O_{11}$ $M_r$ 344.3

$C_{12}H_{24}O_{11},2H_2O$ $M_r$ 380.3

Anhydrous isomalt: [64519-82-0]

DEFINITION

Mixture of 6-*O*-α-D-glucopyranosyl-D-glucitol (6-*O*-α-D-glucopyranosyl-D-sorbitol; 1,6-GPS) and 1-*O*-α-D-glucopyranosyl-D-mannitol (1,1-GPM).

*Content*: 98.0 per cent to 102.0 per cent for the mixture of 1,6-GPS and 1,1-GPM and neither of the 2 components is less than 3.0 per cent (anhydrous substance).

◆ CHARACTERS

*Appearance*: white or almost white powder or granules.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol. ◆

IDENTIFICATION

*First identification: A.*

◊*Second identification: B, C.*◊

A. Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatogram obtained with reference solution (a).

◊B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *isomalt CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetic acid R*, *propionic acid R*, *water R*, *ethyl acetate R*, *pyridine R* (5:5:10:50:50 *V/V/V/V/V*).

*Application*: 1 µL; thoroughly dry the points of application in warm air.

*Development*: over 1/2 of the plate.

*Drying*: in a current of warm air.

*Detection*: dip for 3 s in a 1 g/L solution of *sodium periodate R* and dry in a current of hot air; dip for 3 s in a mixture of 1 volume of *acetic acid R*, 1 volume of

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

*anisaldehyde R*, 5 volumes of *sulfuric acid R* and 90 volumes of *anhydrous ethanol R*; dry in a current of hot air until coloured spots become visible; the background colour may be brightened in warm steam; examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows 2 blue-grey spots with $R_F$ values of about 0.13 (1,6-GPS) and 0.16 (1,1-GPM). The chromatogram obtained with the test solution shows principal spots similar in position and colour to the principal spots in the chromatogram obtained with the reference solution.

C. To 3 mL of a freshly prepared 100 g/L solution of *pyrocatechol R* add 6 mL of *sulfuric acid R* while cooling in iced water. To 3 mL of the cooled mixture add 0.3 mL of a 100 g/L solution of the substance to be examined. Heat gently over a naked flame for about 30 s. A pink colour develops.◊

TESTS

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* with gentle heating (40-50 °C) and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.3 per cent, expressed as glucose equivalent.

Dissolve 3.3 g in 10 mL of *water R* with gentle heating. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R*. When the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R* as indicator, added towards the end of the titration. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in 4 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.200 g of *isomalt CRS* in 4 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10.0 mg of *sorbitol CRS* (impurity C) and 10.0 mg of *mannitol CRS* (impurity B) in 20 mL of *water R* and dilute to 100.0 mL with the same solvent.

*Precolumn*:

- *size*: *l* = 30 mm, Ø = 4.6 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 80 ± 3 °C.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 80 ± 3 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 40 °C).

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of 1,1-GPM.

*Relative retention* with reference to 1,1-GPM (retention time = about 14 min): 1,6-GPS = about 1.2; impurity B = about 1.6; impurity C = about 2.0.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to 1,1-GPM and 1,6-GPS.

*Limits*:

- *impurities B, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to impurity C in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *total*: not more than 4 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.2 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Water** (*2.5.12*): maximum 7.0 per cent, determined on 0.300 g. As solvent, use a mixture of 20 mL of *anhydrous methanol R* and 20 mL of *formamide R1* at 50 ± 5 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of isomalt (1,1-GPM and 1,6-GPS) taking into account the assigned contents of 1,1-GPM and 1,6-GPS in *isomalt CRS*.

LABELLING

The label states the percentage contents of 1,1-GPM and 1,6-GPS.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D.*

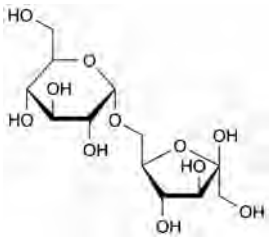

A. 6-*O*-α-D-glucopyranosyl-β-D-*arabino*-hex-2-ulofuranose (isomaltulose),

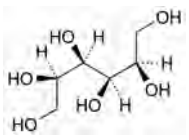

B. D-mannitol,

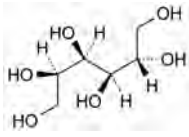

C. D-glucitol (D-sorbitol),

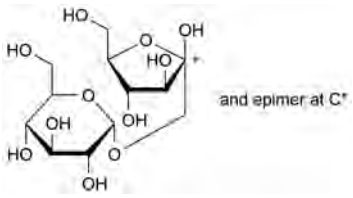


D. 1-*O*-α-D-glucopyranosyl-D-*arabino*-hex-2-ulofuranose (trehalulose).

**07/2018:0146**

# ISONIAZID

## Isoniazidum

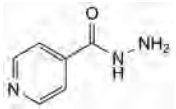


$C_6H_7N_3O$ $M_r$ 137.1
[54-85-3]

DEFINITION

Pyridine-4-carbohydrazide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 170 °C to 174 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *isoniazid CRS*.

C. Dissolve 0.1 g in 2 mL of *water R* and add 10 mL of a warm 10 g/L solution of *vanillin R*. Allow to stand and scratch the wall of the test tube with a glass rod. A yellow precipitate is formed, which, after recrystallisation from 5 mL of *ethanol (70 per cent V/V) R* and drying at 100-105 °C, melts (*2.2.14*) at 226 °C to 231 °C.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.0 for solution S.

**Impurity E**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Solution A*. To 2.0 mL of *benzaldehyde R* add *acetonitrile R* and dilute to 100.0 mL with the same solvent. Use the solution within 4 h.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 1.0 mL of *water R*, add 5.0 mL of solution A and shake well. Wait for 45 min for completion of derivatisation and dilute to 10.0 mL with the solvent mixture.

*Reference solution.* Dissolve 20.0 mg of *hydrazine sulfate R* (equivalent to 4.925 mg of impurity E) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 2.5 mL of the solution to 100.0 mL with *water R*. To 1.0 mL of this solution add 2.5 mL of solution A and shake well. Wait for 45 min for completion of derivatisation and dilute to 25.0 mL with the solvent mixture. Dilute 7.5 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R* (40:60 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of benzaldehyde azine.

*Retention time*: benzaldehyde azine = about 13 min.

*System suitability*: reference solution:
- *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections.

*Calculation of percentage content*:
- for impurity E, use the concentration of hydrazine sulfate in the reference solution.

*Limit*:
- *impurity E*: maximum 15 ppm.

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *isonicotinic acid R* (impurity A), 5 mg of *isonicotinamide R* (impurity B) and 5 mg of *nicotinoyl hydrazide R* (impurity D) in mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: mix 3 volumes of *methanol R* and 97 volumes of a buffer solution prepared as follows: dissolve 13.6 g of *potassium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 6.9 with *strong sodium hydroxide solution R*, add 30 mg of *triethanolamine R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 100 | 0 |
| 12 - 20 | 100 → 85 | 0 → 15 |
| 20 - 28 | 85 | 15 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to isoniazid (retention time = about 8.4 min): impurity A = about 0.5; impurity D = about 1.15; impurity B = about 1.4.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.8, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to isoniazid.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity B = 1.5;
- for each impurity, use the concentration of isoniazid in reference solution (a).

*Limits*:

- *impurities A, B*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in *water R* and dilute to 100.0 mL with the same solvent. To 20.0 mL of the solution add 100 mL of *water R*, 20 mL of *hydrochloric acid R*, 0.2 g of *potassium bromide R* and 0.05 mL of *methyl red solution R*. Titrate dropwise with *0.0167 M potassium bromate*, shaking continuously, until the red colour disappears.

1 mL of *0.0167 M potassium bromate* is equivalent to 3.429 mg of $C_6H_7N_3O$.

IMPURITIES

*Specified impurities: A, B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

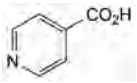

A. pyridine-4-carboxylic acid (isonicotinic acid),

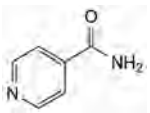

B. pyridine-4-carboxamide (isonicotinamide),

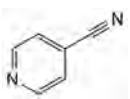

C. pyridine-4-carbonitrile (isonicotinonitrile),

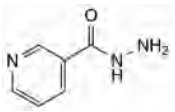

D. pyridine-3-carbohydrazide (nicotinoyl hydrazide),

E. hydrazine.

**07/2013:1332**

# ISOPRENALINE HYDROCHLORIDE

## Isoprenalini hydrochloridum

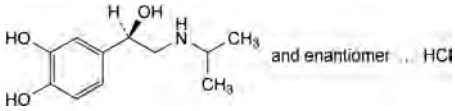


$C_{11}H_{18}ClNO_3$ $M_r$ 247.7

[51-30-9]

DEFINITION

(1*RS*)-1-(3,4-Dihydroxyphenyl)-2-[(1-methylethyl)amino]-ethanol hydrochloride.

*Content*: 98.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, C, E.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 165 °C to 170 °C, with decomposition.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *isoprenaline hydrochloride CRS.*

C. Optical rotation (see Tests).

D. To 0.1 mL of solution S (see Tests) add 0.05 mL of *ferric chloride solution R1* and 0.9 mL of *water R*. A green colour is produced. Add dropwise *sodium hydrogen carbonate solution R*. The colour becomes blue and then red.

E. To 0.5 mL of solution S add 1.5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

*Prepare the solutions immediately before use.*

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ or $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.3 to 5.5.

Mix 5 mL of solution S and 5 mL of *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.5 mg of *orciprenaline sulfate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

Monographs

*Reference solution (c)*. To 5.0 mL of reference solution (a) add 5.0 mL of reference solution (b).

*Reference solution (d)*. Dissolve 6.0 mg of *isoprenaline impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 11.5 g/L solution of *phosphoric acid R* (5:95 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of isoprenaline.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to orciprenaline.

*Relative retention* with reference to isoprenaline (retention time = about 3 min): orciprenaline = about 1.5; impurity A = about 1.8. If necessary, adjust the concentration of methanol in the mobile phase.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to isoprenaline and orciprenaline.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 1.0 per cent;
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 15-25 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.150 g in 10 mL of *anhydrous formic acid R* and add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.77 mg of $C_{11}H_{18}ClNO_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

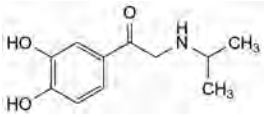

A. 1-(3,4-dihydroxyphenyl)-2-[(1-methylethyl)amino]-ethanone.

**01/2008:0502**
**corrected 10.0**

# ISOPRENALINE SULFATE

## Isoprenalini sulfas

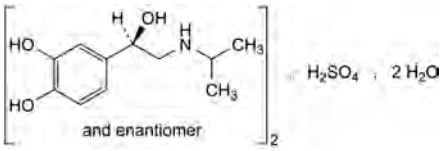


$C_{22}H_{36}N_2O_{10}S,2H_2O$ $M_r$ 556.6
[6700-39-6]

DEFINITION

Bis[(1*RS*)-1-(3,4-dihydroxyphenyl)-2-[(1-methylethyl)-amino]ethanol] sulfate dihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

mp: about 128 °C, with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

Dissolve 0.5 g in 1.5 mL of *water R* and add 3.5 mL of *2-propanol R*. Scratch the wall of the tube with a glass rod to initiate crystallisation. Collect the crystals and dry *in vacuo* (*2.2.32*) at 60 °C.

*Comparison*: repeat the operations using 0.5 g of *isoprenaline sulfate CRS*.

B. To 0.1 mL of solution S (see Tests) add 0.9 mL of *water R* and 0.05 mL of *ferric chloride solution R1*. A green colour is produced. Add dropwise *sodium hydrogen carbonate solution R*. The colour becomes blue and then red.

C. Dilute 1 mL of solution S to 10 mL with *water R* and add 0.25 mL of *silver nitrate solution R1*. A shining, grey, fine precipitate is formed within 10 min and the solution becomes pink.

D. Solution S gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent. Use within 2 h of preparation.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.3 to 5.5.

Dilute 5 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Isoprenalone**: the absorbance (*2.2.25*) is not greater than 0.20 at 310 nm.

Dissolve 0.20 g in *0.005 M sulfuric acid* and dilute to 100.0 mL with the same acid.

**Water** (*2.5.12*): 5.0 per cent to 7.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 20 mL of *anhydrous acetic acid R*, warming gently if necessary and add 20 mL of *methyl isobutyl ketone R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 52.06 mg of $C_{22}H_{36}N_2O_{10}S$.

STORAGE

In an airtight container, protected from light.

**07/2015:0970**
**corrected 10.0**

# ISOPROPYL ALCOHOL

## Alcohol isopropylicus

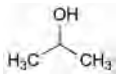

$C_3H_8O$ $M_r$ 60.1
[67-63-0]

DEFINITION

Propan-2-ol.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B, D.*

A. Relative density (*2.2.5*): 0.785 to 0.789.

B. Refractive index (*2.2.6*): 1.376 to 1.379.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *isopropyl alcohol CRS*.

D. To 1 mL add 4 mL of *water R* and mix. Carefully add 2 mL of a 10 g/L solution of *dimethylaminobenzaldehyde R* in *sulfuric acid R*, ensuring that the liquids do not mix; a bright reddish-violet ring forms immediately at the junction of the 2 liquids. After 2-5 min, the entire sulfuric acid layer turns violet.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*). Dilute 1 mL to 20 mL with *water R*. After 5 min, the solution is clear (*2.2.1*).

**Acidity or alkalinity**. Gently boil 25 mL for 5 min. Add 25 mL of *carbon dioxide-free water R* and allow to cool protected from carbon dioxide in the air. Add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.6 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pale pink.

**Absorbance** (*2.2.25*): maximum 0.30 at 230 nm, 0.10 at 250 nm, 0.03 at 270 nm, 0.02 at 290 nm and 0.01 at 310 nm.

The absorbance is measured between 230 nm and 310 nm using *water R* as the compensation liquid. The spectrum shows a steadily descending curve with no observable peaks or shoulders.

**Benzene and related substances**. Gas chromatography (*2.2.28*).

*Test solution (a).* The substance to be examined.

*Test solution (b).* Dilute 1.0 mL of *2-butanol R1* to 50.0 mL with test solution (a). Dilute 5.0 mL of this solution to 100.0 mL with test solution (a).

*Reference solution (a).* Dilute 0.5 mL of *2-butanol R1* and 0.5 mL of *propanol R* to 50.0 mL with test solution (a). Dilute 5.0 mL of this solution to 50.0 mL with test solution (a).

*Reference solution (b).* Dilute 100 µL of *benzene R* to 100.0 mL with test solution (a). Dilute 0.20 mL of this solution to 100.0 mL with test solution (a).

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *helium for chromatography R*.

*Auxiliary gas*: *nitrogen for chromatography R* or *helium for chromatography R*.

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:5.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 40 |
| | 12 - 32 | 40 → 240 |
| | 32 - 42 | 240 |
| Injection port | | 280 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Retention time*: benzene = about 10 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 10 between the 1st peak (propanol) and the 2nd peak (2-butanol).

*Limits*:

- *benzene* (test solution (a)): not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (2 ppm), after the sensitivity has been adjusted so that the height of the peak due to benzene in the chromatogram obtained with reference solution (b) represents at least 10 per cent of the full scale of the recorder;
- *total of impurities apart from 2-butanol* (test solution (b)): not more than 3 times the area of the peak due to 2-butanol in the chromatogram obtained with test solution (b) (0.3 per cent), after the sensitivity has been adjusted so that the height of the 2 peaks following the principal peak in the chromatogram obtained with reference solution (a) represents at least 50 per cent of the full scale of the recorder.

**Peroxides**. In a 12 mL test-tube with a ground-glass stopper and a diameter of about 15 mm, introduce 8 mL of *potassium iodide and starch solution R*. Fill completely with the substance to be examined. Shake vigorously and allow to stand protected from light for 30 min. No colour develops.

**Non-volatile substances**: maximum 20 ppm.

Evaporate 100 g to dryness on a water-bath *after having verified that it complies with the test for peroxides* and dry in an oven at 100-105 °C. The residue weighs a maximum of 2 mg.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 5.0 g.

STORAGE

Protected from light.

Monographs 1

IMPURITIES

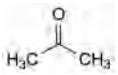

A. propanone (acetone),

B. benzene,

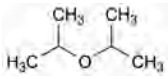

C. 2-(1-methylethoxy)propane (diisopropyl ether),

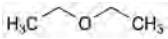

D. ethoxyethane (diethyl ether),

E. methanol,

F. propan-1-ol (*n*-propanol).

**07/2017:2867**

# ISOPROPYL ISOSTEARATE

## Isopropylis isostearas

DEFINITION

Product obtained by esterification of isostearic acid (regioisomers mainly of octadecanoic acid, a complex mixture of branched and straight-chain fatty acids) with isopropyl alcohol. The monograph applies to isopropyl isostearate for cutaneous use.

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent) and with fatty oils.

*Viscosity*: about 11 mPa·s at 20 °C.

*Relative density*: about 0.866 at 20 °C.

*Refractive index*: about 1.447 at 20 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *isopropyl isostearate CRS*.
An additional weak absorption band at 1780 $cm^{-1}$ may be present.

B. Saponification value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).
Dissolve 10.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 1.0, determined on 20.0 g.

**Iodine value** (*2.5.4, Method B*): maximum 3.0, determined on 3.0 g.

**Peroxide value** (*2.5.5, Method B*): maximum 5.0.

**Saponification value** (*2.5.6*): 165 to 180, determined on 2.0 g.

**Composition of fatty acids** (*2.4.22, Method B*). Use the mixture of calibrating substances in Table 2.4.22.-1.
*Composition of the fatty-acid fraction of the substance*:
– *sum of the contents of the fatty acids eluting between palmitic acid and stearic acid*: minimum 65.0 per cent;
– *sum of the contents of myristic acid, palmitic acid and stearic acid*: maximum 11.0 per cent.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 5.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

**01/2008:0725**
**corrected 10.0**

# ISOPROPYL MYRISTATE

## Isopropylis myristas

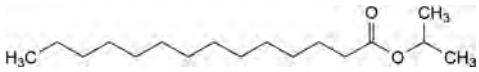

$C_{17}H_{34}O_2$ $M_r$ 270.5

DEFINITION

1-Methylethyl tetradecanoate together with variable amounts of other fatty acid isopropyl esters.
*Content*: minimum 90.0 per cent of $C_{17}H_{34}O_2$.

CHARACTERS

*Appearance*: clear, colourless, oily liquid.

*Solubility*: immiscible with water, miscible with ethanol (96 per cent), with methylene chloride, with fatty oils and with liquid paraffin.

*Relative density*: about 0.853.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Saponification value (see Tests).

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

C. Superpose 2 mL of a 1 g/L solution in *ethanol (96 per cent) R* on a freshly prepared solution of 20 mg of *dimethylaminobenzaldehyde R* in 2 mL of *sulfuric acid R*. After 2 min, a yellowish-red colour appears at the junction of the 2 liquids and gradually becomes red.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).
Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Refractive index** (*2.2.6*): 1.434 to 1.437.

**Viscosity** (*2.2.9*): 5 mPa·s to 6 mPa·s.

**Acid value** (*2.5.1*): maximum 1.0.

**Iodine value** (*2.5.4*): maximum 1.0.

**Saponification value** (*2.5.6*): 202 to 212.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 5.0 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 50.0 mg of *tricosane R* in *heptane R* and dilute to 250.0 mL with the same solvent.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the internal standard solution and dilute to 100.0 mL with the same solution.

*Reference solution.* Dissolve 20.0 mg of *isopropyl tetradecanoate CRS* in the internal standard solution and dilute to 100.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.2 mm;
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 125 → 185 |
| | 6 - 16 | 185 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

Calculate the percentage content of $C_{17}H_{34}O_2$ in the substance to be examined.

STORAGE

Protected from light.

**01/2008:0839**
**corrected 10.0**

# ISOPROPYL PALMITATE

## Isopropylis palmitas

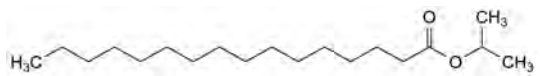

$C_{19}H_{38}O_2$ $M_r$ 298.5

DEFINITION

1-Methylethyl hexadecanoate together with varying amounts of other fatty acid isopropyl esters.

*Content*: minimum 90.0 per cent of $C_{19}H_{38}O_2$.

CHARACTERS

*Appearance*: clear, colourless, oily liquid.

*Solubility*: immiscible with water, miscible with ethanol (96 per cent), with methylene chloride, with fatty oils and with liquid paraffin.

*Relative density*: about 0.854.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C.*

A. Saponification value (see Tests).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

C. Superpose 2 mL of a 1 g/L solution in *ethanol (96 per cent) R* on a freshly prepared solution of 20 mg of *dimethylaminobenzaldehyde R* in 2 mL of *sulfuric acid R*. After 2 min, a yellowish-red colour appears at the junction of the 2 liquids which gradually becomes red.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Refractive index** (*2.2.6*): 1.436 to 1.440.

**Viscosity** (*2.2.9*): 5 mPa·s to 10 mPa·s.

**Acid value** (*2.5.1*): maximum 1.0.

**Iodine value** (*2.5.4*): maximum 1.0.

**Saponification value** (*2.5.6*): 183 to 193.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 5.0 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 50.0 mg of *tricosane R* in *heptane R* and dilute to 250.0 mL with the same solvent.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the internal standard solution and dilute to 100.0 mL with the same solution.

*Reference solution.* Dissolve 20.0 mg of *isopropyl hexadecanoate CRS* in the internal standard solution and dilute to 100.0 mL with the same solution.

*Column*:

- *material*: fused silica,
- *size*: *l* = 50 m, Ø = 0.2 mm,
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 125 → 185 |
| | 6 - 16 | 185 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

Calculate the percentage content of $C_{19}H_{38}O_2$ in the substance to be examined.

STORAGE

Protected from light.

Monographs 1

**01/2008:1117**
**corrected 10.0**

# ISOSORBIDE DINITRATE, DILUTED

## Isosorbidi dinitras dilutus

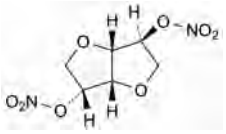

$C_6H_8N_2O_8$ $M_r$ 236.1

DEFINITION

Dry mixture of isosorbide dinitrate and *Lactose monohydrate (0187)* or *Mannitol (0559)*.

*Content*: 95.0 per cent *m/m* to 105.0 per cent *m/m* of the content of 1,4:3,6-dianhydro-D-glucitol 2,5-dinitrate stated on the label.

*CAUTION: undiluted isosorbide dinitrate may explode if subjected to percussion or excessive heat. Appropriate precautions must be taken and only very small quantities handled.*

CHARACTERS

*Appearance*: undiluted isosorbide dinitrate is a fine, white or almost white, crystalline powder.

*Solubility*: undiluted isosorbide dinitrate is very slightly soluble in water, very soluble in acetone, sparingly soluble in ethanol (96 per cent).

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs prepared with the residue obtained in identification test D.

*Comparison*: *isosorbide dinitrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 10 mg of isosorbide dinitrate with 10 mL of *ethanol (96 per cent) R* for 5 min and filter.

*Reference solution.* Shake a quantity of *isosorbide dinitrate CRS* corresponding to 10 mg of isosorbide dinitrate with 10 mL of *ethanol (96 per cent) R* for 5 min and filter.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *methanol R, methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air.

*Detection*: spray with freshly prepared *potassium iodide and starch solution R*; expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 mL of *water R*. Filter if necessary.

*Reference solution (a).* Dissolve 0.10 g of *lactose monohydrate R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Mix equal volumes of reference solutions (a) and (b).

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R, methanol R, anhydrous acetic acid R, ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 1 µL; thoroughly dry the points of application.

*Development A*: over a path of 15 cm.

*Drying A*: in a current of warm air.

*Development B*: immediately, over a path of 15 cm, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: spray with *4-aminobenzoic acid solution R*, dry in a current of cold air until the acetone is removed, then heat at 100 °C for 15 min; allow to cool, spray with a 2 g/L solution of *sodium periodate R*, dry in a current of cold air, and heat at 100 °C for 15 min.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol.

D. Shake a quantity of the substance to be examined corresponding to 25 mg of isosorbide dinitrate with 10 mL of *acetone R* for 5 min. Filter, evaporate to dryness at a temperature below 40 °C and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. The melting point (*2.2.14*) of the residue is 69 °C to 72 °C.

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of isosorbide dinitrate with 5 mL of *ethanol (96 per cent) R* and filter.

*Reference solution.* Dissolve 10 mg of *potassium nitrate R* in 1 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R, acetone R, toluene R* (15:30:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air until the acetic acid is completely removed.

*Detection*: spray copiously with freshly prepared *potassium iodide and starch solution R*; expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

**Impurities B and C**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Sonicate a quantity of the substance to be examined corresponding to 25.0 mg of isosorbide dinitrate with 20 mL of the mobile phase for 15 min and dilute to 25.0 mL with the mobile phase. Filter the solution through a suitable membrane filter.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Sonicate a quantity of *isosorbide dinitrate CRS* corresponding to 25.0 mg of isosorbide dinitrate with 20 mL of the mobile phase for 15 min and dilute to 25.0 mL with the mobile phase. Filter the solution through a suitable membrane filter.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10.0 mg of *isosorbide-2-nitrate CRS* (impurity B) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 0.1 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 10.0 mg of *isosorbide mononitrate CRS* (impurity C) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 0.1 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 5 mg of *isosorbide-2-nitrate CRS* (impurity B) in the mobile phase and dilute to 10 mL with the mobile phase. To 1 mL of this solution add 0.5 mL of reference solution (a) and dilute to 10 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *aminopropylmethylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (15:85 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210-215 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (c), (d) and (e).

*Retention time*: isosorbide dinitrate = about 5 min; impurity B = about 8 min; impurity C = about 11 min.

*System suitability*: reference solution (e):

– *resolution*: minimum 6.0 between the peaks due to isosorbide dinitrate and impurity B.

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurities B and C with the following modifications.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (b) and reference solution (b).

If the areas of the peaks from 2 successive injections of reference solution (b) do not agree to within 1.0 per cent, then inject a further 4 times and calculate, for the 6 injections, the relative standard deviation.

*System suitability*: reference solution (b):

– *repeatability*: maximum relative standard deviation of 2.0 per cent after 6 injections.

Calculate the content of isosorbide dinitrate as a percentage of the declared content.

STORAGE

Protected from light.

LABELLING

The label states the percentage content of isosorbide dinitrate.

IMPURITIES

*Specified impurities: A, B, C.*

A. inorganic nitrates,

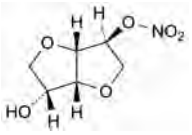

B. 1,4:3,6-dianhydro-D-glucitol 2-nitrate (isosorbide 2-nitrate),

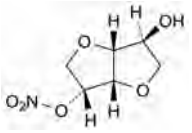

C. 1,4:3,6-dianhydro-D-glucitol 5-nitrate (isosorbide 5-nitrate, isosorbide mononitrate).

**01/2008:1118**
**corrected 6.0**

# ISOSORBIDE MONONITRATE, DILUTED

## Isosorbidi mononitras dilutus

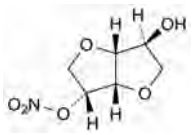

$C_6H_9NO_6$ $M_r$ 191.1

DEFINITION

Dry mixture of isosorbide mononitrate and *Lactose monohydrate (0187)* or *Mannitol (0559)*.

*Content*: 95.0 per cent *m/m* to 105.0 per cent *m/m* of the content of 1,4:3,6-dianhydro-D-glucitol 5-nitrate stated on the label.

CHARACTERS

*Appearance*: undiluted isosorbide mononitrate is a white or almost white, crystalline powder.

*Solubility*: undiluted isosorbide mononitrate is freely soluble in water, in acetone, in ethanol (96 per cent) and in methylene chloride.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs prepared with the residue obtained in identification test D.

*Comparison*: *isosorbide mononitrate CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 10 mg of isosorbide mononitrate with 10 mL of *ethanol (96 per cent) R* for 5 min and filter.

*Reference solution.* Dissolve 10 mg of *isosorbide mononitrate CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *methanol R*, *methylene chloride R* (5:95 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air.

*Detection*: spray with freshly prepared *potassium iodide and starch solution R*. Expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 mL of *water R*; filter if necessary.

*Reference solution (a).* Dissolve 0.10 g of *lactose monohydrate R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Mix equal volumes of reference solutions (a) and (b).

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 1 µL; thoroughly dry the points of application.

*Development A*: over a path of 15 cm.

*Drying A*: in a current of warm air.

*Development B*: immediately, over a path of 15 cm, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: spray with *4-aminobenzoic acid solution R* and dry in a current of cold air until the acetone is removed; heat at 100 °C for 15 min and allow to cool; spray with a 2 g/L solution of *sodium periodate R* and dry in a current of cold air; heat at 100 °C for 15 min.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol.

D. Shake a quantity of the substance to be examined corresponding to 25 mg of isosorbide mononitrate with 10 mL of *acetone R* for 5 min. Filter, evaporate to dryness at a temperature below 40 °C and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. The melting point (*2.2.14*) of the residue is 89 °C to 91 °C.

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of isosorbide mononitrate with 5 mL of *ethanol (96 per cent) R* and filter.

*Reference solution.* Dissolve 10 mg of *potassium nitrate R* in 1 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *toluene R* (15:30:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air until the acetic acid is completely removed.

*Detection*: spray copiously with freshly prepared *potassium iodide and starch solution R*; expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

**Impurities B and C**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Sonicate a quantity of the substance to be examined corresponding to 25.0 mg of isosorbide mononitrate with 20 mL of the mobile phase for 15 min and dilute to 25.0 mL with the mobile phase. Filter the solution through a suitable membrane filter.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *isosorbide mononitrate CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *isosorbide-2-nitrate CRS* (impurity C) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 0.1 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c).* Sonicate a quantity of *isosorbide dinitrate CRS* (impurity B) corresponding to 10.0 mg of isosorbide dinitrate in 15 mL of the mobile phase for 15 min and dilute to 20.0 mL with the mobile phase. Filter the solution through a suitable membrane filter. Dilute 0.1 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *isosorbide mononitrate CRS* and 5 mg of *isosorbide-2-nitrate CRS* (impurity C) in the mobile phase and dilute to 10 mL with the mobile phase. Dilute 1 mL of this solution to 10 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *aminopropylmethylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: *anhydrous ethanol R*, *trimethylpentane R* (15:85 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210-215 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b), (c) and (d).

*Retention time*: impurity B = about 5 min;
impurity C = about 8 min; isosorbide 5-nitrate = about 11 min.

*System suitability*: reference solution (d):

– *resolution*: minimum 4.0 between the peaks due to impurity C and isosorbide 5-nitrate.

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurities B and C with the following modifications.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (b) and reference solution (a).

If the areas of the peaks from 2 successive injections of reference solution (a) do not agree to within 1.0 per cent, then inject a further 4 times and calculate, for the 6 injections, the relative standard deviation.

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 2.0 per cent after 6 injections.

Calculate the content of isosorbide mononitrate as a percentage of the declared content.

STORAGE

Protected from light.

LABELLING

The label states the percentage content of isosorbide mononitrate.

IMPURITIES

*Specified impurities: A, B, C.*

A. inorganic nitrates,

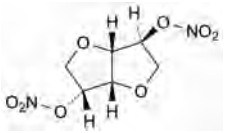

B. 1,4:3,6-dianhydro-D-glucitol 2,5-dinitrate (isosorbide dinitrate),

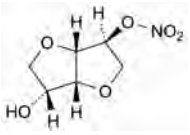

C. 1,4:3,6-dianhydro-D-glucitol 2-nitrate (isosorbide 2-nitrate).

**07/2018:1019**

# ISOTRETINOIN

## Isotretinoinum

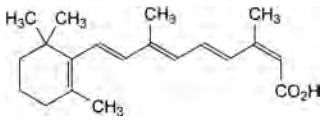

$C_{20}H_{28}O_2$ $M_r$ 300.4

[4759-48-2]

DEFINITION

(2*Z*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or light orange, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

It is sensitive to air, heat and light, especially in solution.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *isotretinoin CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *tretinoin CRS* (impurity A) in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Mix 0.5 mL of the test solution and 1 mL of reference solution (a) and dilute to 25 mL with *methanol R*.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *glacial acetic acid R*, *water for chromatography R*, *methanol R* (5:225:770 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 355 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.6 times the retention time of isotretinoin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to isotretinoin (retention time = about 26 min): impurity A = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to isotretinoin and impurity A.

*Limits*:

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 16 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 mL of *acetone R*. Titrate with *0.1 M tetrabutylammonium hydroxide in 2-propanol*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 30.04 mg of $C_{20}H_{28}O_2$.

STORAGE

Under an inert gas, in an airtight container, protected from light.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of inert gas.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these

impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, I.*

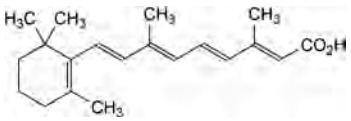

A. (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (tretinoin),

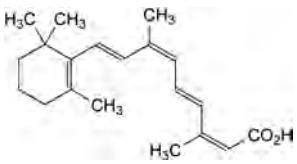

B. (2*Z*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (9,13-di*cis*-retinoic acid),

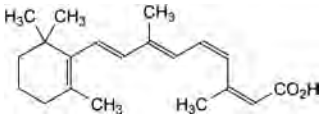

C. (2*Z*,4*Z*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (11,13-di*cis*-retinoic acid),

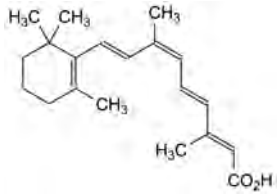

D. (2*E*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (9-*cis*-retinoic acid),

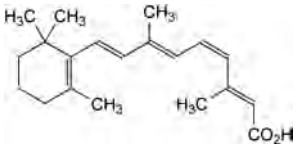

F. (2*E*,4*Z*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (11-*cis*-retinoic acid),

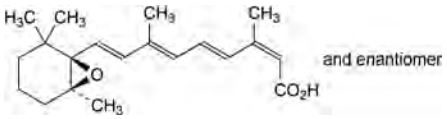


G. (2*Z*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-[(1*RS*,6*SR*)-2,2,6-trimethyl-7-oxabicyclo[4.1.0]heptan-1-yl]nona-2,4,6,8-tetraenoic acid (13-*cis*-5,6-dihydro-5,6-epoxyretinoic acid),

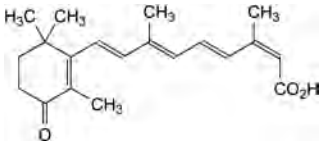

H. (2*Z*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxocyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid (13-*cis*-4-oxoretinoic acid),

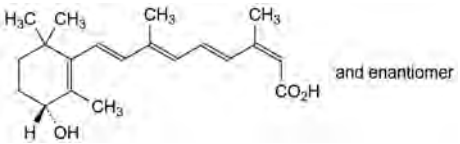


I. (2*Z*,4*E*,6*E*,8*E*)-9-[(3*RS*)-3-hydroxy-2,6,6-trimethylcyclohex-1-en-1-yl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (13-*cis*-4-hydroxyretinoic acid).

**01/2017:1119**
**corrected 10.0**

# ISOXSUPRINE HYDROCHLORIDE

## Isoxsuprini hydrochloridum

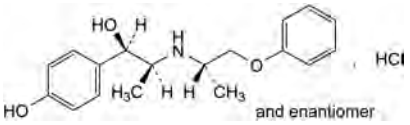


$C_{18}H_{24}ClNO_3$ $M_r$ 337.8
[579-56-6]

DEFINITION

(1*RS*,2*SR*)-1-(4-Hydroxyphenyl)-2-[[(1*SR*)-1-methyl-2-phenoxyethyl]amino]propan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: about 205 °C, with decomposition.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *0.1 M hydrochloric acid* and dilute to 50.0 mL with the same acid. Dilute 10.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 269 nm and 275 nm.

*Resolution* (*2.2.25*): minimum 1.7 for the absorbance ratio.

*Specific absorbance at the absorption maxima*:

– at 269 nm: 71 to 74;
– at 275 nm: 70 to 73.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *isoxsuprine hydrochloride CRS.*

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and of the reference substance separately in 2 mL of *methanol R*, add 15 mL of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *isoxsuprine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (0.25:15:85 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a 10 g/L solution of *potassium permanganate R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 1 mL of solution S (see Tests) add 0.05 mL of *copper sulfate solution R* and 0.5 mL of *strong sodium hydroxide solution R*. The solution becomes blue. Add 1 mL of *ether R* and shake. Allow to separate. The upper layer remains colourless.

E. 2 mL of solution S gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g, with gentle heating if necessary, in *carbon dioxide-free water R*, cool and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Optical rotation** (*2.2.7*): − 0.05° to + 0.05°, determined on solution S.

**Phenones**: maximum 1.0 per cent, calculated as impurity B.

Dissolve 10.0 mg in *water R* and dilute to 100.0 mL with the same solvent. The absorbance (*2.2.25*) of the solution measured at the absorption maximum at 310 nm is not greater than 0.10.

**Related substances**. Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use.*

*Internal standard solution (a).* Dissolve 0.1 g of *hexacosane R* in *trimethylpentane R* and dilute to 20 mL with the same solvent.

*Internal standard solution (b).* Dilute 1 mL of internal standard solution (a) to 50 mL with *trimethylpentane R*.

*Test solution.* To 10.0 mg of the substance to be examined, add 0.5 mL of *N-trimethylsilylimidazole R*. Heat to 65 °C for 10 min. Allow to cool, then add 2.0 mL of the internal standard solution (b) and 2.0 mL of *water R*. Shake. Use the upper layer.

*Reference solution (a).* To 10.0 mg of the substance to be examined, add 0.5 mL of *N-trimethylsilylimidazole R*. Heat to 65 °C for 10 min. Allow to cool, then add 2.0 mL of the internal standard solution (a) and 2.0 mL of *water R*. Shake. Dilute 1.0 mL of the upper layer to 50.0 mL with *trimethylpentane R*.

*Reference solution (b).* To 10.0 mg of the substance to be examined, add 0.5 mL of *N-trimethylsilylimidazole R*. Heat to 65 °C for 10 min. Allow to cool, then add 2.0 mL of *trimethylpentane R* and 2.0 mL of *water R*. Shake. Use the upper layer.

*Column*:

– *material*: glass;

– *size*: *l* = 1.5 m, Ø = 4 mm;

– *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (125-135 µm) impregnated with 3 per cent *m/m* of *methylpolysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 25 | 195 |
| | 25 - 29 | 195 → 215 |
| | 29 - 39 | 215 |
| Injection port | | 225 |
| Detector | | 225 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: isoxsuprine, hexacosane.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to isoxsurpine and hexacosane in the chromatogram obtained with reference solution (a);

– in the chromatogram obtained with reference solution (b), there is no peak with the same retention time as the internal standard.

*Limit*:

– *total*: calculate the ratio (*R*) of the area of the peak due to the trimethylsilyl derivative of isoxsuprine to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than *R* (2.0 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 80 mL of *ethanol (96 per cent) R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.78 mg of $C_{18}H_{24}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

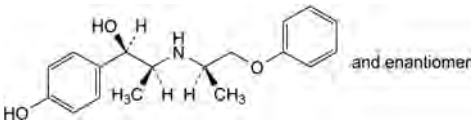


A. (1*RS*,2*SR*)-1-(4-hydroxyphenyl)-2-[[(1*RS*)-1-methyl-2-phenoxyethyl]amino]propan-1-ol,

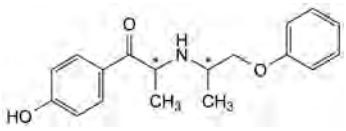

B. 1-(4-hydroxyphenyl)-2-[(1-methyl-2-phenoxyethyl)amino]propan-1-one.

**01/2008:2110**
**corrected 6.0**

# ISRADIPINE

## Isradipinum

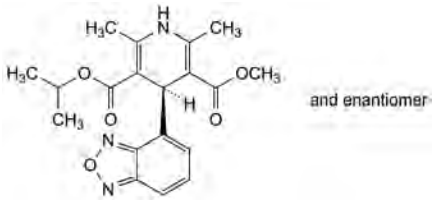


$C_{19}H_{21}N_3O_5$ $M_r$ 371.4
[75695-93-1]

DEFINITION

Methyl 1-methylethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in methanol.

mp: about 168 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *isradipine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in 1 mL of *methanol R*, using an ultrasonic bath if necessary, and dilute to 25.0 mL with the mobile phase.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in 2 mL of *methanol R* and dilute to 250.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of the substance to be examined and 2 mg of *isradipine impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *isradipine CRS* in 2 mL of *methanol R* and dilute to 250.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *tetrahydrofuran R*, *water R* (125:270:625 *V/V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 5 times the retention time of isradipine.

*Identification of impurities*: use the chromatogram supplied with *isradipine CRS* to identify the peaks due to impurities A and B.

*Relative retention* with reference to isradipine (retention time = about 7 min): impurity A = about 0.8; impurity D = about 0.9; impurity B = about 1.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to isradipine and impurity D.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 1.4,
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity B*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent),
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 326 nm.

*Injection*: test solution (b) and reference solution (c).

*Run time*: twice the retention time of isradipine.

Calculate the percentage content of isradipine from the areas of the peaks and the declared content of *isradipine CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034).* It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E.*

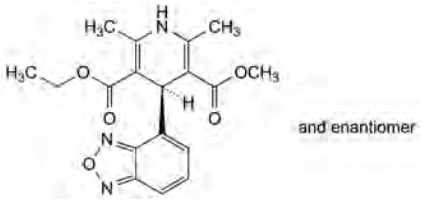


A. ethyl methyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

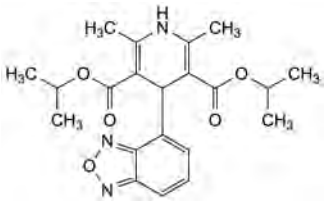

B. bis(1-methylethyl) (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

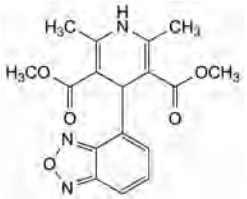

C. dimethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate,

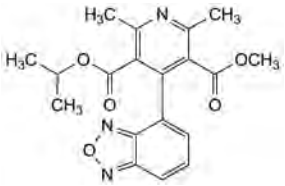

D. methyl 1-methylethyl 4-(2,1,3-benzoxadiazol-4-yl)-2,6-dimethylpyridine-3,5-dicarboxylate,

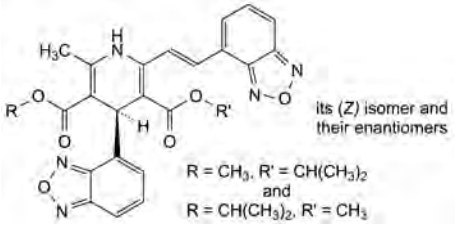


E. methyl 1-methylethyl (4*RS*)-4-(2,1,3-benzoxadiazol-4-yl)-2-[(*EZ*)-2-(2,1,3-benzoxadiazol-4-yl)ethenyl]-6-methyl-1,4-dihydropyridine-3,5-dicarboxylate.

**01/2011:1335**

# ITRACONAZOLE

## Itraconazolum

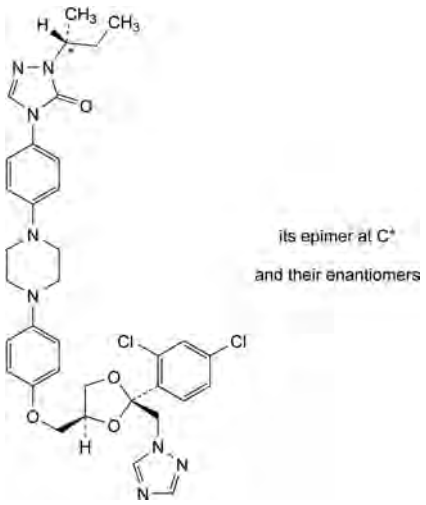


$C_{35}H_{38}Cl_2N_8O_4$ $M_r$ 706
[84625-61-6]

DEFINITION

4-[4-[4-[4-[[*cis*-2-(2,4-Dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *itraconazole CRS*.

TESTS

**Solution S**. Dissolve 2.0 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $R_6$ or $B_6$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanolic hydrochloric acid R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanolic hydrochloric acid R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanolic hydrochloric acid R*.

*Reference solution (b)*. Dissolve 10 mg of *itraconazole for system suitability CRS* (containing impurities B, C, D, E, F and G) in 1.0 mL of *methanolic hydrochloric acid R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm or 3.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: 27.2 g/L solution of *tetrabutylammonium hydrogen sulfate R1*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 22 | 80 → 50 | 20 → 50 |
| 22 - 27 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *itraconazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D, E, F and G.

*Relative retention* with reference to itraconazole (retention time = about 14 min): impurity B = about 0.7; impurities C and D = about 0.8; impurity E = about 0.9; impurity F = about 1.05; impurity G = about 1.3.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to itraconazole.

*Limits*:
- *impurities B, G*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities C and D*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 70 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* by vigorous stirring for at least 10 min. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically at the second point of inflexion (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.3 mg of $C_{35}H_{38}Cl_2N_8O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, F.*

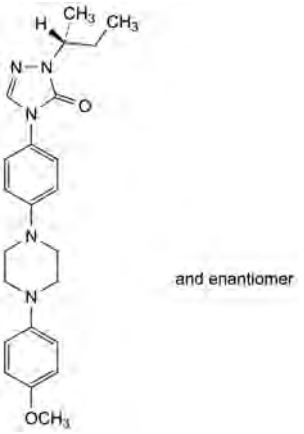


A. 4-[4-[4-(4-methoxyphenyl)piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

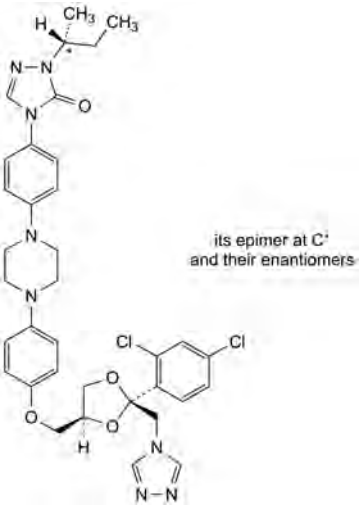


B. 4-[4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(4*H*-1,2,4-triazol-4-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

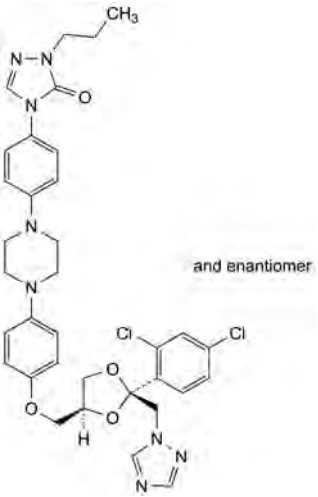


C. 4-[4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-propyl-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

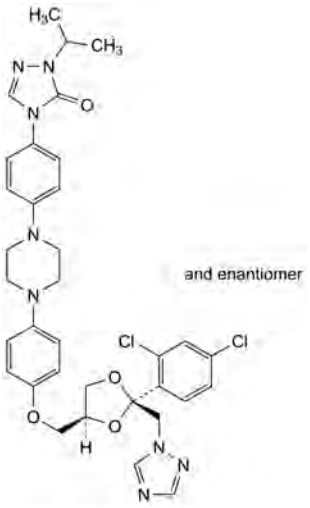


D. 4-[4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-(1-methylethyl)-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

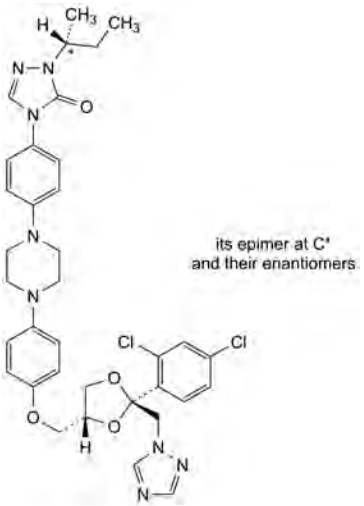


E. 4-[4-[4-[4-[[*trans*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[(1*RS*)-1-methylpropyl]-2,4-dihydro-3*H*-1,2,4-triazol- 3-one,

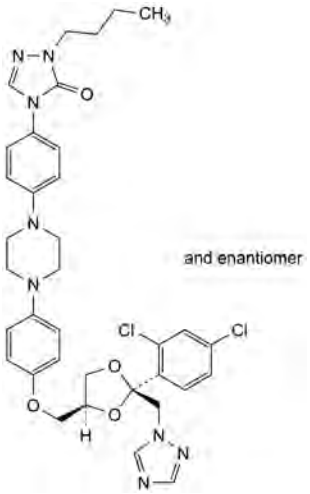


F. 2-butyl-4-[4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one,

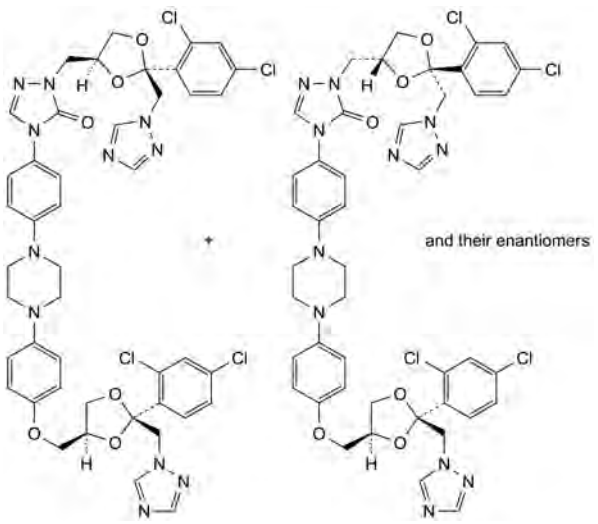


G. 4-[4-[4-[4-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazin-1-yl]phenyl]-2-[[*cis*-2-(2,4-dichlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methyl]-2,4-dihydro-3*H*-1,2,4-triazol-3-one.

**01/2017:1336**

# IVERMECTIN

## Ivermectinum

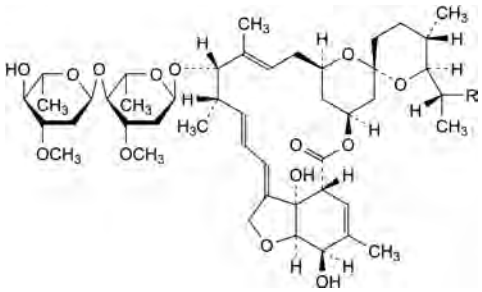

| Component | R | Molecular formula | $M_r$ |
|---|---|---|---|
| $H_2B_{1a}$ | $CH_2$-$CH_3$ | $C_{48}H_{74}O_{14}$ | 875 |
| $H_2B_{1b}$ | $CH_3$ | $C_{47}H_{72}O_{14}$ | 861 |

Ivermectin B1a: [71827-03-7]
Ivermectin B1b: [70209-81-3]

DEFINITION

Mixture of (2a*E*,4*E*,5′*S*,6*S*,6′*R*,7*S*,8*E*,11*R*,13*R*,15*S*,17a*R*,20*R*,-20a*R*,20b*S*)-7-[[2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-3-*O*-methyl-α-L-*arabino*-hexopyranosyl]oxy]-20,20b-dihydroxy-5′,6,8,19-tetramethyl-6′-[(1*S*)-1-methylpropyl]-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradecahydrospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-*pq*][2,6]-benzodioxacyclooctadecene-13,2′-[2*H*]pyran]-17-one (or 5-*O*-demethyl-22,23-dihydroavermectin $A_{1a}$) (component $H_2B_{1a}$) and (2a*E*,4*E*,5′*S*,6*S*,6′*R*,7*S*,8*E*,11*R*,13*R*,15*S*,17a*R*,20*R*,20a*R*,-20b*S*)-7-[[2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-3-*O*-methyl-α-L-*arabino*-hexopyranosyl]oxy]-20,20b-dihydroxy-5′,6,8,19-tetramethyl-6′-(1-methylethyl)-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradecahydrospiro[11,15-methano-2*H*,13*H*,17*H*-furo[4,3,2-*pq*][2,6]benzodioxacyclooctadecene-13,2′-[2*H*]pyran]-17-one (or 5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-22,23-dihydroavermectin $A_{1a}$) (component $H_2B_{1b}$).

Semi-synthetic product derived from a fermentation product.

*Content*:

– *ivermectin* ($H_2B_{1a}$ + $H_2B_{1b}$): 95.0 per cent to 102.0 per cent (anhydrous substance);

– *ratio* $H_2B_{1a}/(H_2B_{1a} + H_2B_{1b})$ (areas by liquid chromatography): minimum 90.0 per cent.

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ivermectin CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 1.0 g in 50 mL of *toluene R*.

**Specific optical rotation** (*2.2.7*): − 20 to − 17 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 40.0 mg of *ivermectin CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *methanol R*.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 100.0 mL with *methanol R*.

*Reference solution (d).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *methanol R*, *acetonitrile R* (15:34:51 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*System suitability*:

– *resolution*: minimum 3.0 between the 1st peak (component $H_2B_{1b}$) and the 2nd peak (component $H_2B_{1a}$) in the chromatogram obtained with reference solution (a);

– *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c);

– *symmetry factor*: maximum 2.5 for the principal peak in the chromatogram obtained with reference solution (a).

*Limits*:

– *impurity with a relative retention of 1.3 to 1.5* with reference to the principal peak: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent);

– *any other impurity* (apart from the 2 principal peaks): not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent);

**Ethanol and formamide**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 0.5 mL of *propanol R* to 100 mL with *water R*.

*Test solution.* In a centrifuge tube, dissolve 0.120 g of the substance to be examined in 2.0 mL of *m-xylene R* (if necessary heat in a water-bath at 40-50 °C). Add 2.0 mL of *water R*, mix thoroughly and centrifuge. Remove the upper layer and extract it with 2.0 mL of *water R*. Discard the upper layer and combine the aqueous layers. Add 1.0 mL of the internal standard solution. Centrifuge and discard any remaining *m*-xylene.

*Reference solution (a).* Dilute 3.0 g of *anhydrous ethanol R* to 100.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 g of *formamide R* to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 5.0 mL of reference solution (a) and 5.0 mL of reference solution (b) to 50.0 mL with *water R*. Introduce 2.0 mL of this solution into a centrifuge tube, add 2.0 mL of *m-xylene R*, mix thoroughly and centrifuge. Remove the upper layer and extract it with 2.0 mL of *water R*. Discard the upper layer and combine the aqueous layers. Add 1.0 mL of the internal standard solution. Centrifuge and discard any remaining *m*-xylene.

*Reference solution (d).* Dilute 10.0 mL of reference solution (a) and 10.0 mL of reference solution (b) to 50.0 mL with *water R*. Treat as prescribed for reference solution (c) (from "Introduce 2.0 mL of this solution...").

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.53 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 7.5 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 50 → 80 |
| | 2 - 8 | 80 → 240 |
| Injection port | | 220 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solutions (c) and (d).

*Limits:*

– *ethanol*: maximum 5.0 per cent;

– *formamide*: maximum 3.0 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: 20 µL of the test solution and reference solutions (a) and (d).

Calculate the percentage content of ivermectin ($H_2B_{1a}$ + $H_2B_{1b}$) and the ratio $H_2B_{1a}$/($H_2B_{1a}$ + $H_2B_{1b}$) taking into account the assigned content of component $H_2B_{1a}$ in *ivermectin CRS*. Determine the content of ivermectin component $H_2B_{1a}$ by comparing with the peak area due to component $H_2B_{1a}$ in the chromatogram obtained with reference solution (a). Determine the content of ivermectin component $H_2B_{1b}$ by comparing with the peak area due to component $H_2B_{1a}$ in the chromatogram obtained with reference solution (d).

STORAGE

In an airtight container.

IMPURITIES

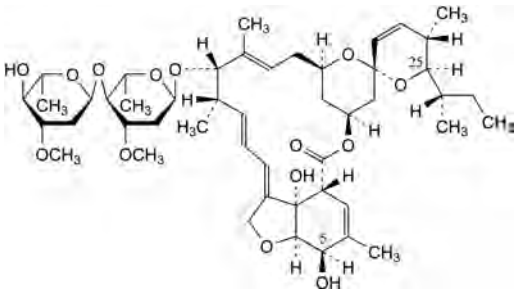

A. 5-*O*-demethylavermectin $A_{1a}$ (avermectin $B_{1a}$),

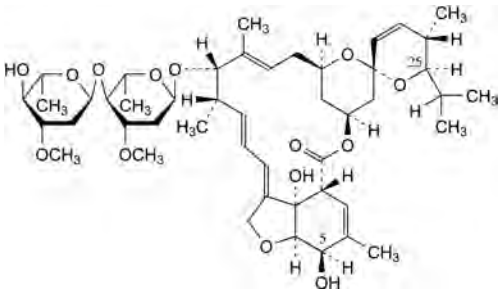

B. 5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)avermectin $A_{1a}$ (avermectin $B_{1b}$),

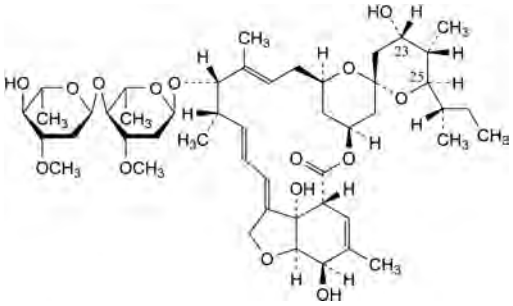

C. (23*S*)-5-*O*-demethyl-23-hydroxy-22,23-dihydroavermectin $A_{1a}$ (avermectin $B_{2a}$),

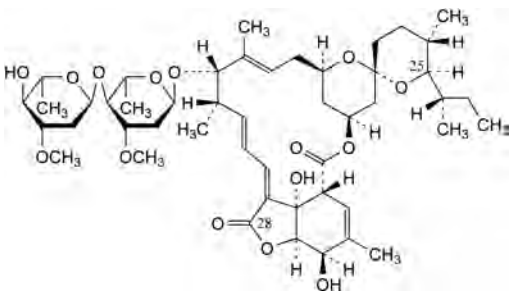

D. 5-*O*-demethyl-28-oxo-22,23-dihydroavermectin $A_{1a}$ (28-oxo$H_2B_{1a}$),

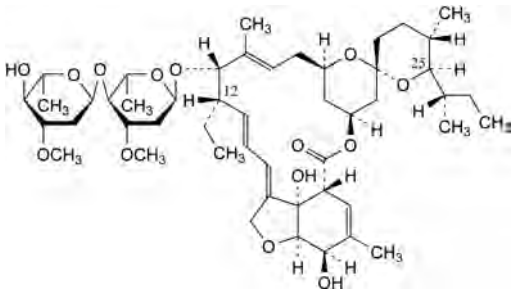

E. 5-*O*,12-didemethyl-12-ethyl-22,23-dihydroavermectin $A_{1a}$ (12-demethyl-12-ethyl-$H_2B_{1a}$),

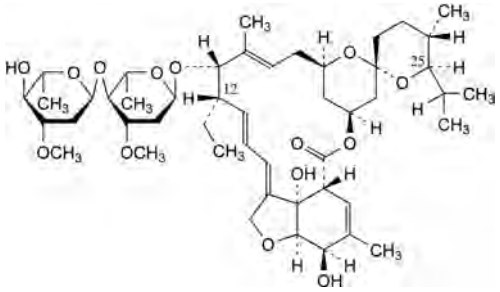

F. 5-*O*,12-didemethyl-25-de(1-methylpropyl)-12-ethyl-25-(1-methylethyl)-22,23-dihydroavermectin $A_{1a}$ (12-demethyl-12-ethyl-$H_2B_{1b}$),

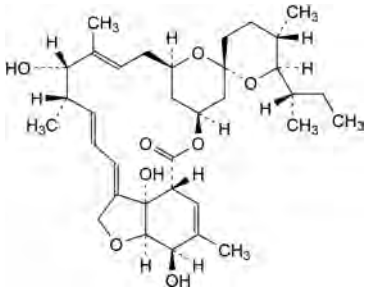

G. (6*R*,13*S*,25*R*)-5-*O*-demethyl-28-deoxy-6,28-epoxy-13-hydroxy-25-[(1*S*)-1-methylpropyl]milbemycin B ($H_2B_{1a}$ aglycone),

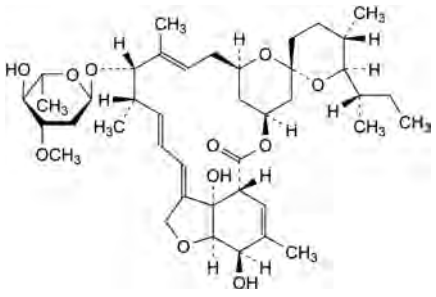

H. 4′-*O*-de(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-5-*O*-demethyl-22,23-dihydroavermectin $A_{1a}$,

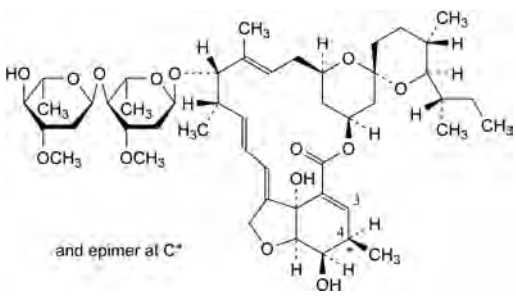


I. 2,3-didehydro-5-*O*-demethyl-3,4,22,23-tetrahydroavermectin $A_{1a}$ ($\Delta^{2,3}H_2B_{1a}$),

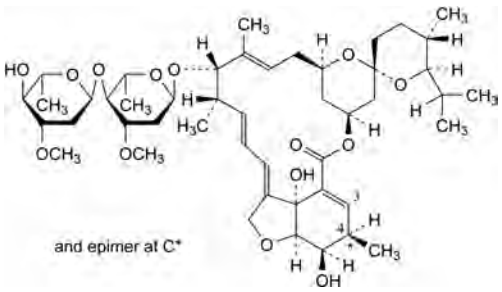


J. 2,3-didehydro-5-*O*-demethyl-25-de(1-methylpropyl)-25-(1-methylethyl)-3,4,22,23-tetrahydroavermectin $A_{1a}$ ($\Delta^{2,3}H_2B_{1b}$),

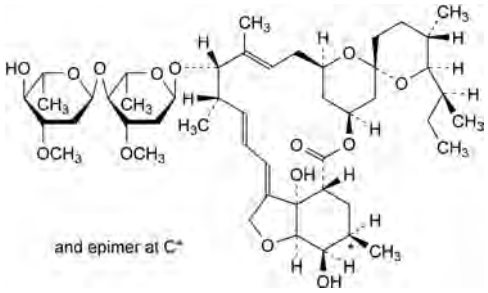


K. (4*R*) and (4*S*)-5-*O*-demethyl-3,4,22,23-tetrahydroavermectin $A_{1a}$ ($H_4B_{1a}$ isomers).

# J

Josamycin..... 3021
Josamycin propionate..... 3023

**01/2017:1983**

# JOSAMYCIN

## Josamycinum

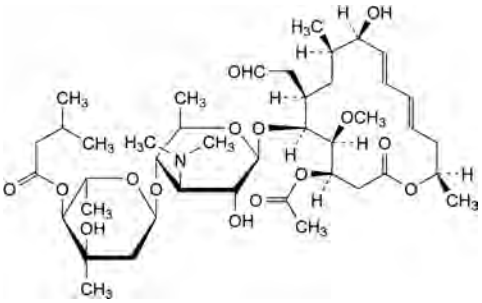

$C_{42}H_{69}NO_{15}$ $M_r$ 828
[16846-24-5]

DEFINITION

Josamycin is a macrolide antibiotic obtained by fermentation using, for example, certain strains of *Streptomyces narbonensis* var. *josamyceticus* var. *nova*. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one.

*Content*: minimum 900 Ph. Eur. U./mg (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder, slightly hygroscopic.

*Solubility*: very slightly soluble in water, freely soluble in methanol and in methylene chloride, soluble in acetone.

IDENTIFICATION

*First identification*: A, C.

*Second identification*: A, B.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 0.10 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 232 nm.

*Specific absorbance at the absorption maximum*: 330 to 370.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in 2.5 mL of *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *josamycin CRS* in 2.5 mL of *methanol R*.

*Reference solution (b)*. Dissolve 10 mg of *josamycin propionate CRS* in 2.5 mL of *methanol R*.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *methanol R*, *acetone R*, *ethyl acetate R*, *toluene R*, *hexane R* (8:10:20:25:30 *V/V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for 10 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and different in position from the principal spot in the chromatogram obtained with reference solution (b).

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in position and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 65 to − 75 (dried substance).

Dissolve 1.000 g in *methanol R* and dilute to 100.0 mL with the same solvent. Allow to stand for 30 min before measuring the angle of rotation.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (30:70 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *josamycin CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 50.0 mL with the solvent mixture.

*Reference solution (d)*. To 10 mL of the test solution add 0.1 mL of *strong hydrogen peroxide solution R* and heat in a water-bath for 10 min. Mix 1.0 mL of this solution and 1.0 mL of the test solution.

*Reference solution (e)*. Dissolve 12.5 mg of *josamycin for peak identification CRS* (containing impurities A, B, C, D and E) in 5 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: mix 3 volumes of a 67.9 g/L solution of *tetrabutylammonium hydrogen sulfate R*, 5 volumes of a 27.6 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 3.0 with *dilute phosphoric acid R*, and 21 volumes of *acetonitrile R*, and dilute to 100 volumes with *water R*;
- *mobile phase B*: mix 5 volumes of a 27.6 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 3.0 with *dilute phosphoric acid R*, and 50 volumes of *acetonitrile R*, and dilute to 100 volumes with *water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 38 | 100 | 0 |
| 38 - 55 | 100 → 0 | 0 → 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *josamycin for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to josamycin (retention time = about 35 min): impurity A = about 0.5; impurity B = about 0.8; impurity C = about 0.9; impurity D = about 1.2; impurity E = about 1.4.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.7 between the 2 peaks due to josamycin and the peak eluted with a relative retention with reference to josamycin of about 1.1;
- *retention time of josamycin*: between 32 min and 38 min.

If necessary, adjust the concentration of acetonitrile in the mobile phases.

*Limits*:

- *impurities A, B, C, D, E* (any shoulder observed on the peak due to impurity A and/or the peak due to impurity B is not to be integrated separately): for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent);
- *any other impurity*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (20.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

## ASSAY

Dissolve 30.0 mg in 5 mL of *methanol R* and dilute to 100.0 mL with *water R*.

Carry out the microbiological assay of antibiotics (*2.7.2*).

## STORAGE

In an airtight container.

## IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G, H, I, J, K.*

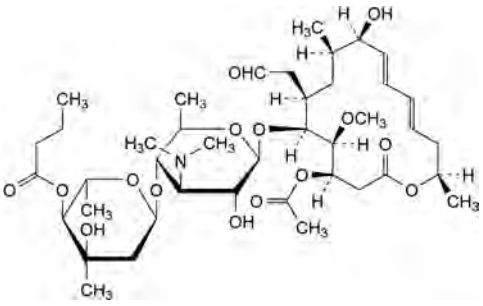

A. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-4-*O*-butanoyl-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

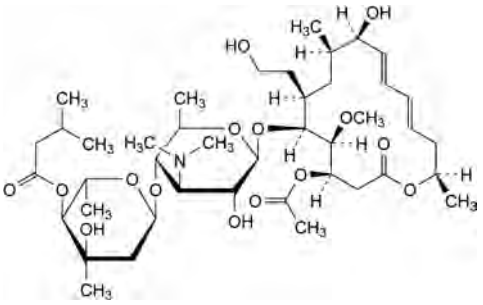

B. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-7-(2-hydroxyethyl)-5-methoxy-9,16-dimethyloxacyclohexadeca-11,13-dien-2-one,

C. unknown structure,

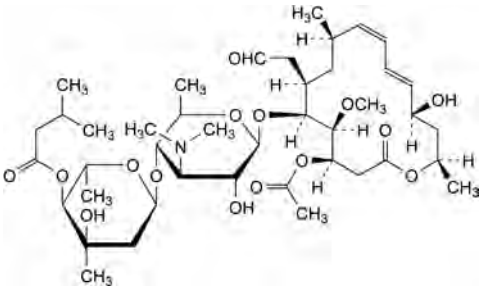

D. (4*R*,5*S*,6*S*,7*R*,9*R*,10*Z*,12*E*,14*R*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-14-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-10,12-dien-2-one (isojosamycin),

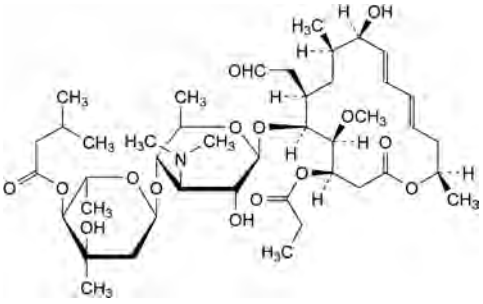

E. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-4-(propanoyloxy)oxacyclohexadeca-11,13-dien-2-one,

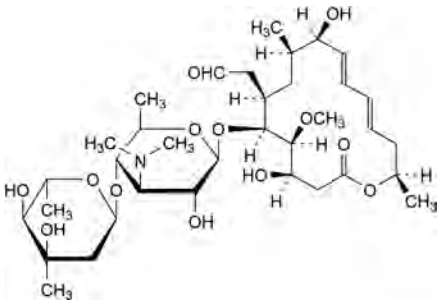

F. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4,10-dihydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

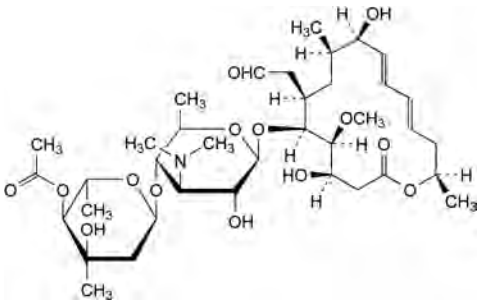

G. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[4-*O*-(4-*O*-acetyl-2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4,10-dihydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

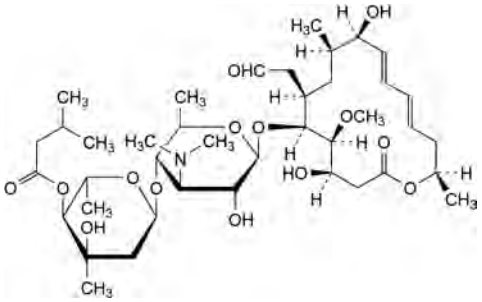

H. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4,10-dihydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

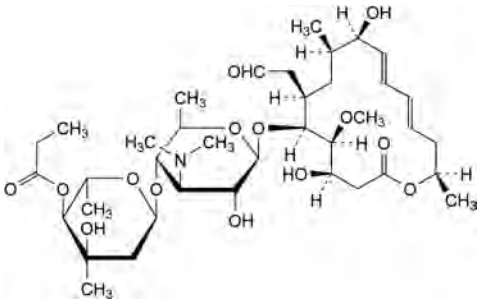

I. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-4-*O*-propanoyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4,10-dihydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

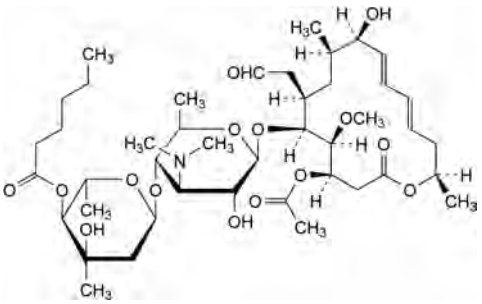

J. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-4-*O*-hexanoyl-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one,

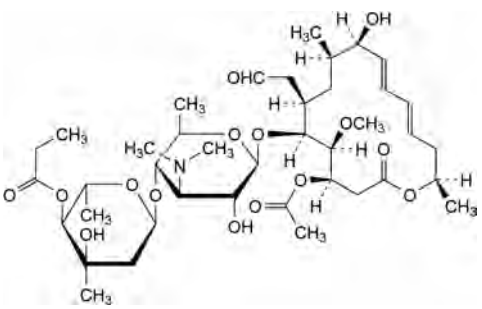

K. (4*R*,5S,6S,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-4-*O*-propanoyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one.

**01/2008:1982**
**corrected 6.0**

# JOSAMYCIN PROPIONATE

## Josamycini propionas

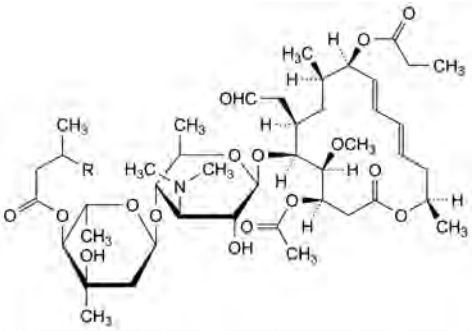

| Leucomycin propionate | R | Mol. Formula | $M_r$ |
|---|---|---|---|
| A3 | $CH_3$ | $C_{45}H_{73}NO_{16}$ | 884 |
| A4 | H | $C_{44}H_{71}NO_{16}$ | 870 |

DEFINITION

Propionyl ester of a macrolide antibiotic produced by certain strains of *Streptomyces narbonensis* var. *josamyceticus* var. *nova*, or obtained by any other means. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-

Monographs J

3-(dimethylamino)-β-D-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-(propanoyloxy)oxacyclohexadeca-11,13-dien-2-one propionate (leucomycin A3 propionate).

Semi-synthetic product derived from a fermentation product.

*Content*:

– minimum 843 Ph. Eur. U./mg (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline, slightly hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in methanol and in methylene chloride, soluble in acetone.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C.*

*Prepare solutions in methanol immediately before use.*

A. Dissolve 0.10 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *methanol R*. Examined between 220 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 231 nm. The specific absorbance at the absorption maximum is 310 to 350.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 1 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *josamycin propionate CRS* in *methanol R* and dilute to 1 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *josamycin CRS* in *methanol R* and dilute to 1 mL with the same solvent.

*Reference solution (c)*. Dissolve 10 mg of *spiramycin CRS* in *methylene chloride R* and dilute to 1 mL with the same solvent.

*Reference solution (d)*. Mix 0.5 mL of reference solution (a) with 0.5 mL of reference solution (b).

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *methanol R*, *acetone R*, *ethyl acetate R*, *toluene R*, *hexane R* (8:10:20:25:30 *V/V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for 10 min.

*Detection*: spray with *dilute sulfuric acid R* and heat at 100 °C for 10 min.

*System suitability*: the chromatogram obtained with reference solution (d) shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) and its position is different from that of the principal spot in the chromatograms obtained with reference solutions (b) and (c).

C. Dissolve about 10 mg in 5 mL of *hydrochloric acid R1* and allow to stand for 10-20 min. A pink colour develops, turning brown.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method II*).

Dissolve 1 g in *methanol R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 65 to – 75 (dried substance).

Dissolve 1.000 g in *methanol R* and dilute to 100.0 mL with the same solvent. Allow to stand for 30 min before measuring the angle of rotation.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *acetonitrile for chromatography R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50.0 mg of *josamycin propionate CRS* in *acetonitrile for chromatography R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined in 10 mL of *methanol R* and add 40 µL of *dilute phosphoric acid R*. Mix, allow to stand for 5 min and inject.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (a) to 100.0 mL with *acetonitrile for chromatography R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm,
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
– *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, a 15.4 g/L solution of *ammonium acetate R* previously adjusted to pH 6.0 with *dilute phosphoric acid R* (60:40 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of leucomycin A3 propionate.

*Relative retention* with reference to leucomycin A3 propionate (retention time = about 18 min): impurity E = about 0.2; impurity A = about 0.3; impurity B = about 0.5; leucomycin A4 propionate = about 0.7; impurity C = about 1.4; impurity D = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the 2 peaks eluting with a relative retention with reference to leucomycin A3 propionate of about 0.5 and 0.7 respectively.

*Limits*:

– *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c),
– *impurities A, B, C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c),
– *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (c),
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 40.0 mg in 20 mL of *methanol R* and dilute to 100.0 mL with *phosphate buffer solution pH 5.6 R*.

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

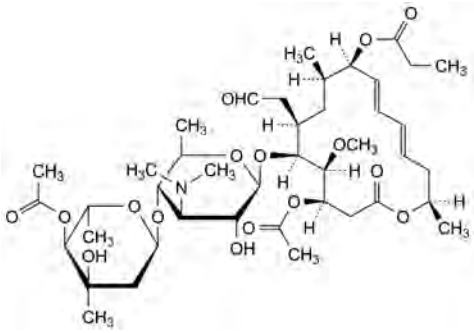

A. leucomycin A8 9-propionate,

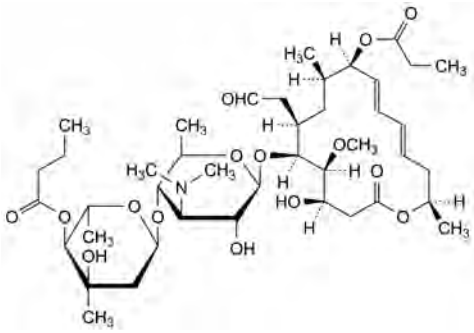

B. leucomycin A5 9-propionate,

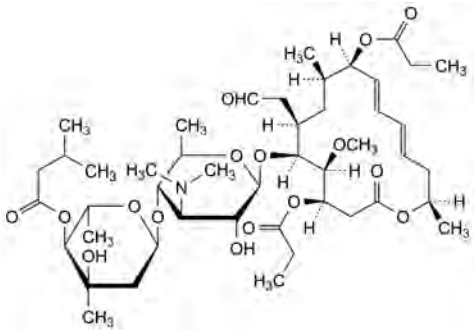

C. platenomycin A1 9-propionate,

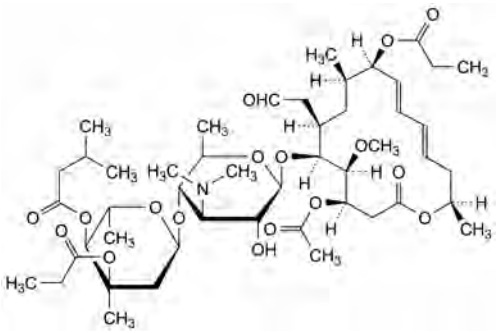

D. leucomycin A3 3″,9-dipropionate,

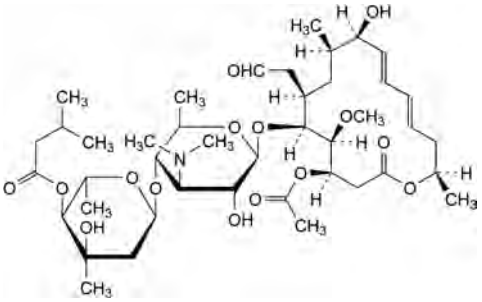

E. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-4-(acetyloxy)-6-[[3,6-dideoxy-4-*O*-[2,6-dideoxy-3-*C*-methyl-4-*O*-(3-methylbutanoyl)-α-L-*ribo*-hexopyranosyl]-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)oxacyclohexadeca-11,13-dien-2-one (josamycin).

# K

Kanamycin acid sulfate.. ..... 3029
Kanamycin monosulfate.. ..... 3030
Kaolin, heavy.. ..... 3031
Ketamine hydrochloride.. ..... 3031
Ketobemidone hydrochloride.. ..... 3032
Ketoconazole.. ..... 3033
Ketoprofen.. ..... 3035
Ketorolac trometamol.. ..... 3037
Ketotifen hydrogen fumarate.. ..... 3038

**01/2019:0033**
**corrected 10.0**

# KANAMYCIN ACID SULFATE

## Kanamycini sulfas acidus

DEFINITION

Kanamycin acid sulfate is a form of kanamycin sulfate prepared by adding sulfuric acid to a solution of kanamycin monosulfate and drying by a suitable method. The potency is not less than 670 IU/mg, calculated with reference to the dried substance.

Fermentation product.

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure.

CHARACTERS

A white or almost white powder, hygroscopic, soluble in about 1 part of water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 mL of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

   Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

   *Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

   *Reference solution (a)*. Dissolve 10 mg of *kanamycin monosulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

   *Reference solution (b)*. Dissolve 10 mg of *kanamycin monosulfate CRS*, 10 mg of *neomycin sulfate CRS* and 10 mg of *streptomycin sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

   Apply separately to the plate 10 µL of each solution. Develop over a path of 12 cm using a 70 g/L solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with a mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 460 g/L solution of *sulfuric acid R*. Heat at 150 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

B. Dissolve 0.5 g in 10 mL of *water R*. Add 10 mL of *picric acid solution R*. Initiate crystallisation if necessary by scratching the wall of the tube with a glass rod and allow to stand. Collect the crystals, wash with 20 mL of *water R* and filter. Dry at 100 °C. The crystals melt (*2.2.14*) at about 235 °C, with decomposition.

C. Dissolve about 50 mg in 2 mL of *water R*. Add 1 mL of a 10 g/L solution of *ninhydrin R* and heat for a few minutes on a water-bath. A violet colour develops.

D. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*). The pH of solution S is 5.5 to 7.5.

**Specific optical rotation** (*2.2.7*). +103 to +115, determined on solution S and calculated with reference to the dried substance.

**Kanamycin B**. Examine by thin-layer chromatography (*2.2.27*), using a plate prepared as prescribed under identification test A.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

*Test solution*. Dissolve 0.11 g of the substance to be examined in *water R* and dilute to 20 mL with the same solvent.

*Reference solution*. Dissolve 4 mg of *kanamycin B sulfate CRS* in *water R* and dilute to 20 mL with the same solvent.

Apply separately to the plate 4 µL of each solution. Develop over a path of 12 cm using a 70 g/L solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air and spray with *ninhydrin and stannous chloride reagent R*. Heat the plate at 110 °C for 15 min. Any spot corresponding to kanamycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (4.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 5.0 per cent, determined on 1.00 g by drying at 60 °C at a pressure not exceeding 670 Pa for 3 h.

**Sulfated ash** (*2.4.14*). Not more than 0.5 per cent, determined on 1.0 g.

**Sulfate**. 23.0 per cent to 26.0 per cent of sulfate ($SO_4$), calculated with reference to the dried substance. Dissolve 0.175 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of sulfate ($SO_4$).

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 mL of a solution in *water for injections R* containing 10 mg per millilitre of the substance to be examined.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *kanamycin monosulfate CRS* as the reference substance.

STORAGE

If the substance is sterile, store in a sterile, tamper-evident container.

**01/2019:0032**
**corrected 10.0**

# KANAMYCIN MONOSULFATE

## Kanamycini monosulfas

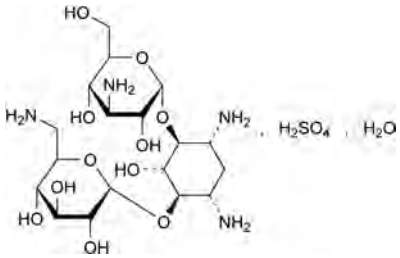

$C_{18}H_{38}N_4O_{15}S,H_2O$ $M_r$ 601
[5965-95-7]

DEFINITION

6-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-4-*O*-(6-amino-6-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine sulfate monohydrate.

Antimicrobial substance produced by the growth of certain strains of *Streptomyces kanamyceticus*.

*Content*: minimum 750 IU/mg (dried substance).

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *kanamycin monosulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *kanamycin monosulfate CRS*, 10 mg of *neomycin sulfate CRS* and 10 mg of *streptomycin sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: suitable plate coated with a 0.75 mm layer of a mixture prepared as follows: mix 0.3 g of *carbomer R* with 240 mL of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

*Pretreatment*: heat the plate at 110 °C for 1 h, allow to cool and use immediately.

*Mobile phase*: 70 g/L solution of *potassium dihydrogen phosphate R*.

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 460 g/L solution of *sulfuric acid R*. Heat at 150 °C for 5 min to 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

B. Dissolve 0.5 g in 10 mL of *water R*. Add 10 mL of *picric acid solution R*. Initiate crystallisation if necessary by scratching the wall of the tube with a glass rod and allow to stand. Collect the crystals, wash with 20 mL of *water R* and filter. Dry at 100 °C. The crystals melt (*2.2.14*) at about 235 °C, with decomposition.

C. Dissolve about 50 mg in 2 mL of *water R*. Add 1 mL of a 10 g/L solution of *ninhydrin R* and heat for a few minutes on a water-bath. A violet colour develops.

D. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 6.5 to 8.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 112 to + 123 (dried substance), determined on solution S.

**Kanamycin B**. Thin-layer chromatography (*2.2.27*) as described under Identification A with the following modifications.

*Test solution*. Dissolve 0.1 g of the substance to be examined in *water R* and dilute to 20 mL with the same solvent.

*Reference solution*. Dissolve 4 mg of *kanamycin B sulfate CRS* in *water R* and dilute to 20 mL with the same solvent.

*Application*: 4 µL.

*Detection*: spray with *ninhydrin and stannous chloride reagent R*. Heat at 110 °C for 15 min.

*Limit*:

– *kanamycin B*: any spot corresponding to kanamycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (4.0 per cent).

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 670 Pa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Sulfate**: 15.0 per cent to 17.0 per cent of sulfate (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 with *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 1 mL of a 10 mg/mL solution of the substance to be examined in *water for injections R*.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

If the substance is sterile, store in a sterile, tamper-evident container.

**01/2017:0503**

# KAOLIN, HEAVY

## Kaolinum ponderosum

DEFINITION

Purified, natural, hydrated aluminium silicate of variable composition.

CHARACTERS

*Appearance*: fine, white or greyish-white, unctuous powder.

*Solubility*: practically insoluble in water and in organic solvents.

IDENTIFICATION

A. To 0.5 g in a metal crucible add 1 g of *potassium nitrate R* and 3 g of *sodium carbonate R* and heat until the mixture melts. Allow to cool. To the residue add 20 mL of boiling *water R*, mix and filter. Wash the residue with 50 mL of *water R*. To the residue add 1 mL of *hydrochloric acid R* and 5 mL of *water R*. Filter. To the filtrate add 1 mL of *strong sodium hydroxide solution R* and filter. To the filtrate add 3 mL of *ammonium chloride solution R*. A gelatinous white precipitate is formed.

B. Add 2.0 g in 20 portions to 100 mL of a 10 g/L solution of *sodium laurilsulfate R* in a 100 mL graduated cylinder about 30 mm in diameter. Allow 2 min between additions for each portion to settle. Allow to stand for 2 h. The apparent volume of the sediment is not greater than 5 mL.

C. 0.25 g gives the reaction of silicates (*2.3.1*).

TESTS

**Solution S**. To 4 g add a mixture of 6 mL of *acetic acid R* and 34 mL of *distilled water R*, shake for 1 min and filter.

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R*, shake for 2 min and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.25 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Organic impurities**. Heat 0.3 g to redness in a calcination tube. The residue is only slightly more coloured than the original substance.

**Adsorption power**. To 1.0 g in a ground-glass-stoppered test-tube add 10.0 mL of a 3.7 g/L solution of *methylene blue R* and shake for 2 min. Allow to settle. Centrifuge and dilute the solution 1 to 100 with *water R*. The solution is not more intensely coloured than a 0.03 g/L solution of *methylene blue R*.

**Swelling power**. Triturate 2 g with 2 mL of *water R*. The mixture does not flow.

**Substances soluble in dilute hydrochloric acid**: maximum 1 per cent.

To 5.0 g add 7.5 mL of *dilute hydrochloric acid R* and 27.5 mL of *water R* and boil for 5 min. Filter, wash the residue on the filter with *water R* and dilute the combined filtrate and washings to 50.0 mL with *water R*. To 10.0 mL of the solution add 1.5 mL of *dilute sulfuric acid R*, evaporate to dryness on a water-bath and ignite. The residue weighs a maximum of 10 mg.

**Chlorides** (*2.4.4*): maximum 250 ppm.

Dilute 2 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 1.5 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 250 ppm.

Dilute 4 mL of solution S to 15 mL with *distilled water R*.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

LABELLING

The label states, where applicable, that the substance is suitable for internal use.

**01/2017:1020**

# KETAMINE HYDROCHLORIDE

## Ketamini hydrochloridum

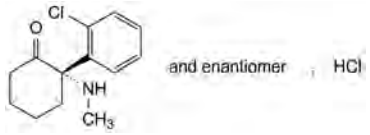


$C_{13}H_{17}Cl_2NO$ $M_r$ 274.2

[1867-66-9]

DEFINITION

(2*RS*)-2-(2-Chlorophenyl)-2-(methylamino)cyclohexanone hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol (96 per cent).

mp: about 260 °C, with decomposition.

IDENTIFICATION

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ketamine hydrochloride CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 4.1.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): − 0.2° to + 0.2°.

Dilute 2.5 mL of solution S to 25.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *ketamine impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase (using ultrasound if necessary). To 1.0 mL of the solution, add 0.5 mL of the test solution and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.95 g of *sodium hexanesulfonate R* in 1 L of a mixture of 25 volumes of *acetonitrile R1* and 75 volumes of *water R* and add 4 mL of *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 10 times the retention time of ketamine.

*Relative retention* with reference to ketamine (retention time = about 3 min): impurity A = about 1.6; impurity B = about 3.3; impurity C = about 4.6.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to ketamine and impurity A.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *methanol R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.42 mg of $C_{13}H_{17}Cl_2NO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

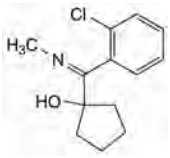


A. 1-(2-chloro-*N*-methylbenzimidoyl)cyclopentanol,

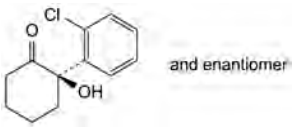


B. (2*RS*)-2-(2-chlorophenyl)-2-hydroxycyclohexanone,

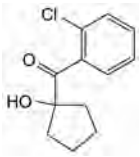


C. (2-chlorophenyl)(1-hydroxycyclopentyl)methanone.

**01/2008:1746**
**corrected 7.0**

# KETOBEMIDONE HYDROCHLORIDE

## Cetobemidoni hydrochloridum

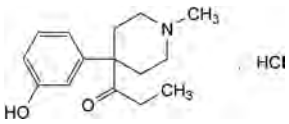


$C_{15}H_{22}ClNO_2$ $M_r$ 283.8
[5965-49-1]

DEFINITION

1-[4-(3-Hydroxyphenyl)-1-methylpiperidin-4-yl]propan-1-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of ketobemidone hydrochloride*.

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 1.54 g/L solution of *ammonium acetate R* adjusted to pH 8.0 with *dilute ammonia R1*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 25.0 mL with the same solution.

*Reference solution (a)*. Dissolve 1 mg of *ketobemidone impurity B CRS* and 1 mg of *ketobemidone impurity C CRS* in solution A and dilute to 25 mL with the same solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 20.0 mL of this solution to 100.0 mL with solution A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *phenylhexylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, solution A (20:80 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL.

*Run time*: 4.5 times the retention time of ketobemidone.

*Relative retention* with reference to ketobemidone (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.6; impurity C = about 0.7; impurity D = about 3.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to impurity B and impurity C.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.38 mg of $C_{15}H_{22}ClNO_2$.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E.*

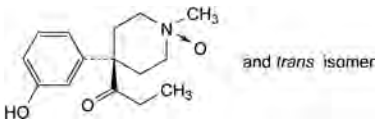


A. 1-[4-(3-hydroxyphenyl)-1-methyl-1-oxidopiperidin-4-yl]propan-1-one (*cis* and *trans* isomers),

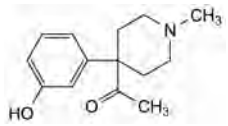

B. 1-[4-(3-hydroxyphenyl)-1-methylpiperidin-4-yl]ethanone,

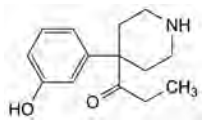

C. 1-[4-(3-hydroxyphenyl)piperidin-4-yl]propan-1-one,

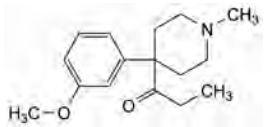

D. 1-[4-(3-methoxyphenyl)-1-methylpiperidin-4-yl]propan-1-one,

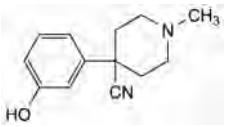

E. 4-(3-hydroxyphenyl)-1-methylpiperidin-4-carbonitrile.

**01/2017:0921**

# KETOCONAZOLE

## Ketoconazolum

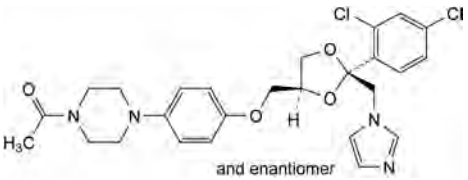


$C_{26}H_{28}Cl_2N_4O_4$ $M_r$ 531.4
[65277-42-1]

DEFINITION

1-Acetyl-4-[4-[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)1,3-dioxolan-4-yl]methoxy]phenyl]-piperazine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 148 °C to 152 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *ketoconazole CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 5 mL with the mobile phase.

*Reference solution (a)*. Dissolve 30 mg of *ketoconazole CRS* in the mobile phase and dilute to 5 mL with the mobile phase.

*Reference solution (b)*. Dissolve 30 mg of *ketoconazole CRS* and 30 mg of *econazole nitrate CRS* in the mobile phase, then dilute to 5 mL with the mobile phase.

*Plate*: *TLC octadecylsilyl silica gel plate R.*

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *methylene chloride R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_4$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 2.5 mg of *ketoconazole CRS* and 2.5 mg of *loperamide hydrochloride CRS* in *methanol R*, then dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, 3.4 g/L solution of *tetrabutylammonium hydrogen sulfate R* (5:95 *V/V*);

– *mobile phase B*: *acetonitrile R1*, 3.4 g/L solution of *tetrabutylammonium hydrogen sulfate R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 → 0 | 0 → 100 |
| 10 - 15 | 0 | 100 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Equilibration*: with *acetonitrile R* for at least 30 min and then with mobile phase A for at least 5 min.

*Injection*: 10 µL; inject *methanol R* as a blank.

*Retention time*: ketoconazole = about 6 min; loperamide = about 8 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 15 between the peaks due to ketoconazole and loperamide; if necessary, adjust the final concentration of acetonitrile in the mobile phase or adjust the time programme for the linear gradient elution.

*Limits*:

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.57 mg of $C_{26}H_{28}Cl_2N_4O_4$.

STORAGE

Protected from light.

IMPURITIES

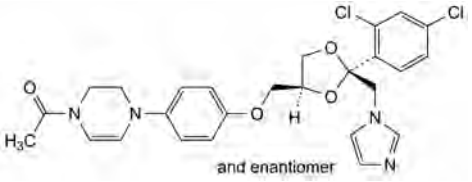


A. 1-acetyl-4-[4-[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1,2,3,4-tetrahydropyrazine,

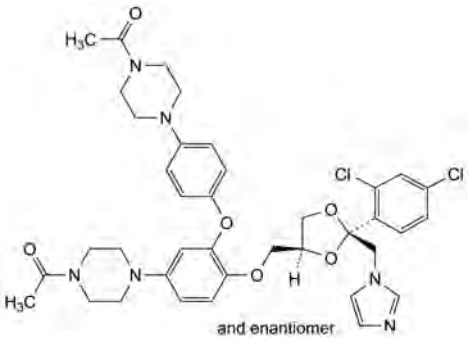


B. 1-acetyl-4-[4-[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]-3-[4-(4-acetylpiperazin-1-yl)phenoxy]phenyl]piperazine,

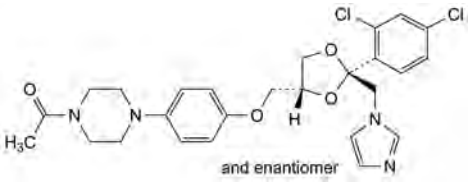


C. 1-acetyl-4-[4-[[(2*RS*,4*RS*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-piperazine,

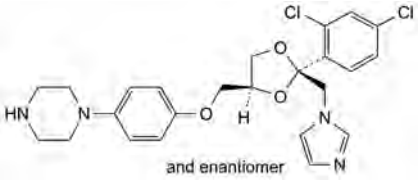


D. 1-[4-[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine,

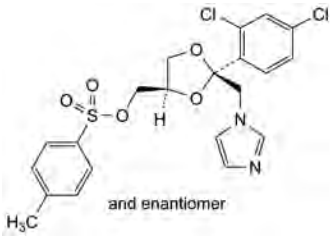


E. [(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methyl 4-methylbenzenesulfonate.

**01/2017:0922**

# KETOPROFEN

## Ketoprofenum

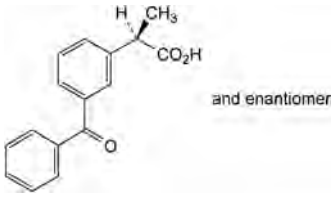


$C_{16}H_{14}O_3$ $M_r$ 254.3
[22071-15-4]

DEFINITION

(2*RS*)-2-(3-Benzoylphenyl)propanoic acid.

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: C.

*Second identification*: A, B, D.

A. Melting point (*2.2.14*): 94 °C to 97 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 255 nm.

*Specific absorbance at the absorption maximum*: 615 to 680.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ketoprofen CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *ketoprofen CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *indometacin CRS* in *acetone R* and dilute to 10 mL with the same solvent. To 1 mL of this solution add 1 mL of reference solution (a).

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *glacial acetic acid R*, *methylene chloride R*, *acetone R* (1:49:50 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in *acetone R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *ketoprofen impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *ketoprofen impurity C CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. To 1.0 mL of this solution add 1.0 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 $m^2/g$ and a pore size of 10 nm.

*Mobile phase*: mix 2 volumes of freshly prepared *phosphate buffer solution pH 3.5 R*, 43 volumes of *acetonitrile R* and 55 volumes of *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 233 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Run time*: 7 times the retention time of ketoprofen.

*Relative retention* with reference to ketoprofen (retention time = about 7 min): impurity C = about 0.3; impurity E = about 0.69; impurity B = about 0.73; impurity D = about 1.35; impurity A = about 1.5; impurity F = about 2.0.

*System suitability*: reference solution (d):

– *resolution*: minimum 7.0 between the peaks due to ketoprofen and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

- *impurities B, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than A and C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.67 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 mL of *ethanol (96 per cent) R*. Add 25 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 25.43 mg of $C_{16}H_{14}O_3$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, J, K, L.*

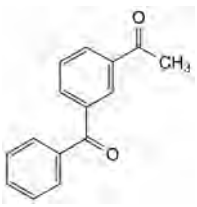

A. 1-(3-benzoylphenyl)ethanone,

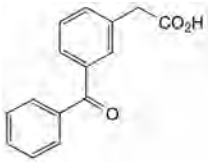

B. (3-benzoylphenyl)acetic acid,

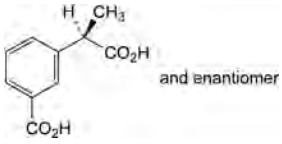

C. 3-[(1*RS*)-1-carboxyethyl]benzoic acid,

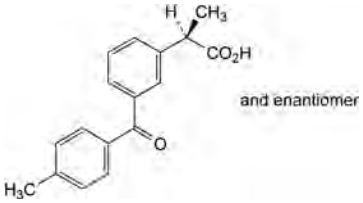

D. (2*RS*)-2-[3-(4-methylbenzoyl)phenyl]propanoic acid,

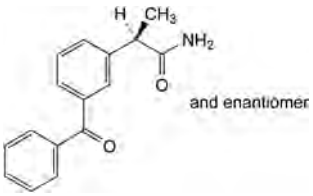

E. (2*RS*)-2-(3-benzoylphenyl)propanamide,

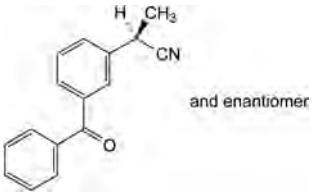

F. (2*RS*)-2-(3-benzoylphenyl)propanenitrile,

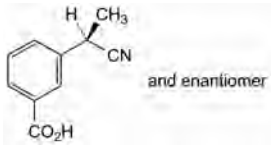

G. 3-[(1*RS*)-1-cyanoethyl]benzoic acid,

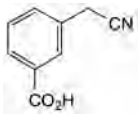

H. 3-(cyanomethyl)benzoic acid,

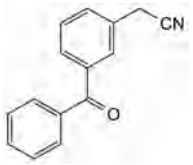

I. (3-benzoylphenyl)ethanenitrile,

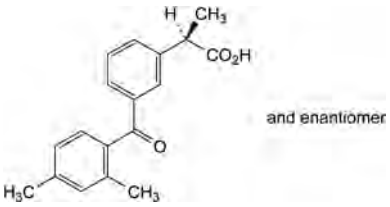

J. (2*RS*)-2-[3-(2,4-dimethylbenzoyl)phenyl]propanoic acid,

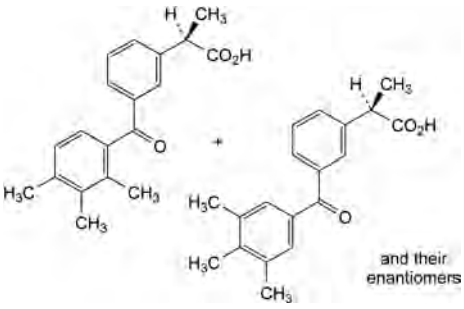

K. mixture of (2*RS*)-2-[3-(2,3,4-trimethylbenzoyl)phenyl]-propanoic acid and (2*RS*)-2-[3-(3,4,5-trimethylbenzoyl)-phenyl]propanoic acid,

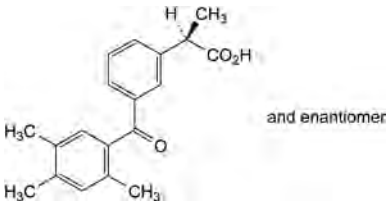


L. (2*RS*)-2-[3-(2,4,5-trimethylbenzoyl)phenyl]propanoic acid.

**01/2017:1755**

# KETOROLAC TROMETAMOL

## Ketorolacum trometamolum

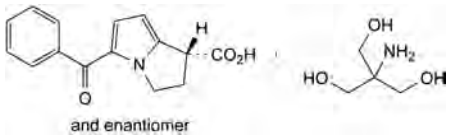


$C_{19}H_{24}N_2O_6$ $M_r$ 376.4
[74103-07-4]

DEFINITION

2-Amino-2-(hydroxymethyl)propane-1,3-diol (1*RS*)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ketorolac trometamol CRS*.

TESTS

**Solution S**. Dissolve 0.75 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 5.7 to 6.7.

Dilute 5 mL of solution S to 15 mL with *carbon dioxide-free water R*.

**Absorbance** (*2.2.25*): maximum 0.10, determined at 430 nm for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from bright light.*

*Solvent mixture*: *tetrahydrofuran R*, *water R* (30:70 *V/V*).

*Test solution.* Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2 mg of *ketorolac trometamol for peak identification CRS* (containing impurities A, B, C and D) in 5 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 30 volumes of *tetrahydrofuran R* with 70 volumes of a solution prepared as follows: dissolve 5.75 g of *ammonium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 313 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of ketorolac.

*Identification of impurities*: use the chromatogram supplied with *ketorolac trometamol for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to ketorolac (retention time = about 10 min): impurity C = about 0.5; impurity A = about 0.6; impurity D = about 0.7; impurity B = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity B and ketorolac.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.67; impurity B = 0.52; impurity C = 2.2;
- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.64 mg of $C_{19}H_{24}N_2O_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G, H, I, J.*

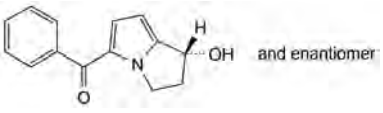


A. (1*RS*)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizin-1-ol,

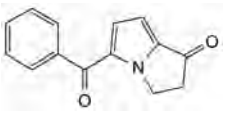

B. 5-benzoyl-2,3-dihydro-1*H*-pyrrolizin-1-one,

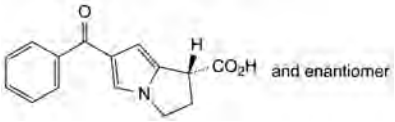


C. (1*RS*)-6-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid,

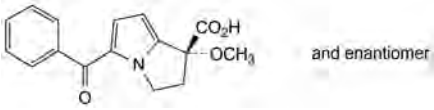


D. (1*RS*)-5-benzoyl-1-methoxy-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid,

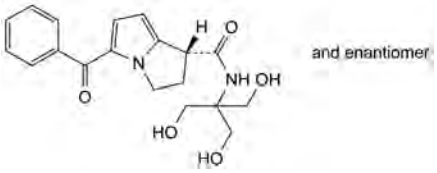


E. (1*RS*)-5-benzoyl-*N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]-2,3-dihydro-1*H*-pyrrolizine-1-carboxamide,

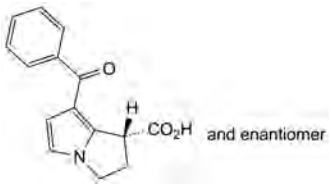


F. (1*RS*)-7-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid,

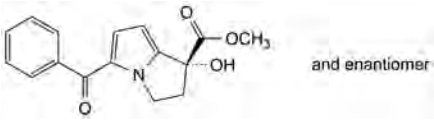


G. methyl (1*RS*)-5-benzoyl-1-hydroxy2,3-dihydro-1*H*-pyrrolizine-1-carboxylate,

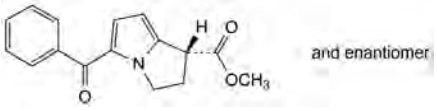


H. methyl (1*RS*)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate,

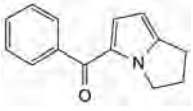

I. phenyl(2,3-dihydro-1*H*-pyrrolizin-5-yl)methanone,

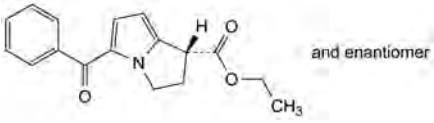


J. ethyl (1*RS*)-5-benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylate.

**04/2014:1592**

# KETOTIFEN HYDROGEN FUMARATE

## Ketotifeni hydrogenofumaras

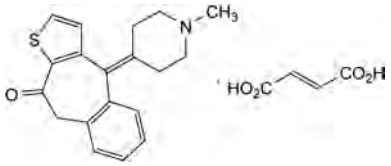

$C_{23}H_{23}NO_5S$ $M_r$ 425.5
[34580-14-8]

DEFINITION

4-(1-Methylpiperidin-4-ylidene)-4,9-dihydro-10*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10-one hydrogen (*E*)-butenedioate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or brownish-yellow, fine, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in methanol, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ketotifen hydrogen fumarate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_4$, $BY_4$ or $B_4$ (*2.2.2, Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *methanol R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *ketotifen impurity G CRS* in 1.0 mL of a solution prepared as follows: mix 1.0 mL of the test solution with 9.0 mL of the solvent mixture. Sonicate until dissolution of impurity G is complete.

*Reference solution (c)*. Dissolve 5 mg of *ketotifen for peak identification CRS* (containing impurity A) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 175 µL of *triethylamine R* and 500 mL of *water R*;
- *mobile phase B*: mix 175 µL of *triethylamine R* and 500 mL of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 40 | 60 |
| 12 - 20 | 40 → 10 | 60 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 297 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *ketotifen for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to ketotifen (retention time = about 11 min): fumaric acid = about 0.1; impurity G = about 0.8; impurity A = about 1.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity G and ketotifen.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity G by 1.4;

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to fumaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in a mixture of 30 mL of *acetic anhydride R* and 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 42.55 mg of $C_{23}H_{23}NO_5S$.

IMPURITIES

*Specified impurities: A, G.*

*Other detectable impurities (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph Substances for pharmaceutical use (2034). It is therefore not necessary to identify these impurities for demonstration of compliance. See also 5.10. Control of impurities in substances for pharmaceutical use): B, C, D, E, F.*

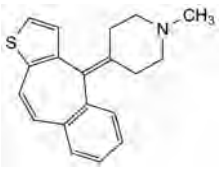

A. 4-(4*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-4-ylidene)-1-methylpiperidine,

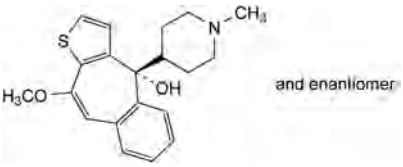


B. (4*RS*)-10-methoxy-4-(1-methylpiperidin-4-yl)-4*H*-benzo-[4,5]cyclohepta[1,2-*b*]thiophen-4-ol,

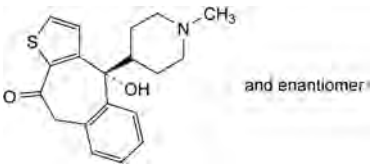


C. (4*RS*)-4-hydroxy-4-(1-methylpiperidin-4-yl)-4,9-dihydro-10*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10-one,

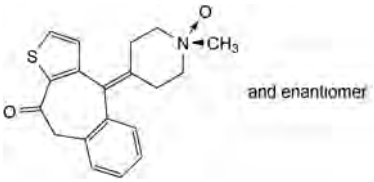


D. 4-[($R_aS_a$)-1-methylpiperidin-4-ylidene]-4,9-dihydro-10*H*-benzo[4,5]cyclohepta[1,2-*b*]thiophen-10-one *N*-oxide (ketotifen *N*-oxide),

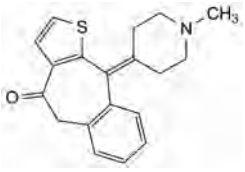

E. 10-(1-methylpiperidin-4-ylidene)-5,10-dihydro-4*H*-benzo-[5,6]cyclohepta[1,2-*b*]thiophen-4-one,

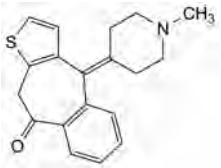

F. 4-(1-methylpiperidin-4-ylidene)-4,10-dihydro-9*H*-benzo-[4,5]cyclohepta[1,2-*b*]thiophen-9-one,

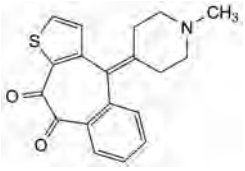

G. 4-(1-methylpiperidin-4-ylidene)-4*H*-benzo[4,5]cyclo-hepta[1,2-*b*]thiophen-9,10-dione.

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# EUROPEAN PHARMACOPOEIA – 10th EDITION

published July 2019

replaces the 9th Edition on 1 January 2020

## BOOK

Volumes I, II and III of this publication 10.0 constitute the 10th Edition of the European Pharmacopoeia. They will be complemented by **non-cumulative supplements** (see the publication schedule opposite) that are to be kept for the duration of the 10th Edition. Each new edition or supplement of the European Pharmacopoeia is usually published 6 months before its implementation date.

To use the 10th Edition correctly, make sure that you have all the published supplements and consult the index of the most recent supplement to ensure that you use the latest versions of the monographs and general chapters.

A cumulative list of reagents will be published in supplements 10.4 and 10.7. A list of new reagents published during the course of the 10th Edition is available under 'Useful information' in Pharmeuropa Online.

## ELECTRONIC VERSION

The 10th Edition is also available in an electronic format containing all of the monographs and general chapters found in the book. With the publication of each supplement the electronic version is replaced by a new, fully updated, cumulative version. Each update of the electronic version is usually available 6 months before its implementation date. A list of new reagents published during the course of the 10th Edition is available under 'Useful information' in Pharmeuropa Online.

## CORRECTIONS

Corrections are to be taken into account as soon as possible and not later than the end of the month following the month of publication (see the publication schedule opposite).

## PHARMACOPOEIAL HARMONISATION

See the information given in general chapter *5.8. Pharmacopoeial harmonisation*.

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# COMBISTATS

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# KEY TO MONOGRAPHS



*Version date of the text*

**01/2012:0884**
**corrected 10.0**

*Text reference number*

*Modification to be taken into account as soon as possible and not later than the end of the month following the month of publication of Ph. Eur. 10.0 (see section IV. Contents of the 10th Edition)*

*Link to further information on the text (e.g. Knowledge database) for smartphones/tablets with camera and barcode reader app*

## CARBIMAZOLE(1)

Carbimazolum

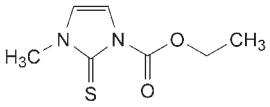

$C_7H_{10}N_2O_2S$

[22232-54-8] $M_r$ 186.2

*CAS number*

DEFINITION

Ethyl 3-methyl-2-thioxo-2,3-dihydro-1*H*-imidazole-1-carboxylate.

*Chemical name in accordance with IUPAC nomenclature rules*

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

*For the meaning of black and white diamonds see chapter 5.8. Pharmacopoeial harmonisation*

♦CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in acetone and in ethanol (96 per cent). ♦

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

*Application of the first and second identification is defined in the General Notices (chapter 1)*

◊A. Melting point (*2.2.14*): 122 °C to 125 °C.◊

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *carbimazole CRS*.

*Reference standard available from the EDQM (see http://crs.edqm.eu)*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reagent described in chapter 4*

*Reference solution*. Dissolve 10 mg of *carbimazole CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel GF254 plate R*.

*Further information on certain reagents available in the Knowledge database (http://go.edqm.eu/knowledge)*

*Mobile phase*: *acetone R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air for 30 min.

*Vertical line in the margin indicating where the text has been modified*

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Horizontal line in the margin indicating where part of the text has been deleted*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Reference to a general chapter*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in 10.0 mL of a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Use this solution within 5 min of preparation.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter 5.8. *Pharmacopoeial harmonisation*.

SPECIMEN

*IMPORTANT NOTICE*

# GENERAL MONOGRAPHS

The European Pharmacopoeia contains a number of general monographs covering classes of products. These general monographs give requirements that are applicable to all products in the given class or, in some cases, to any product in the given class for which there is a specific monograph in the Pharmacopoeia (see *1. General Notices*, General monographs). Where no restriction on scope of a general monograph is given in a preamble, it is applicable to all products in the class defined, irrespective of whether there is an individual monograph for the product in the Pharmacopoeia.

Whenever a monograph is used, it is essential to ascertain whether there is a general monograph applicable to the product in question. The general monographs listed below are published in the General Monographs section (unless otherwise stated). This list is updated where necessary and republished in each supplement.

Allergen products (1063)

Chemical precursors for radiopharmaceutical preparations (2902)

Dosage Forms monographs
*(published in the Dosage Forms section or the Homoeopathic Preparations section, as appropriate)*

Essential oils (2098)

Herbal drug extracts (0765)

Herbal drug preparations (1434)

Herbal drugs (1433)

Herbal drugs for homoeopathic preparations (2045)
*(published in the Homoeopathic Preparations section)*

Herbal teas (1435)

Herbal teas, instant (2620)

Homoeopathic preparations (1038)
*(published in the Homoeopathic Preparations section)*

Immunosera for human use, animal (0084)

Immunosera for veterinary use (0030)

Live biotherapeutic products for human use (3053)

Methods of preparation of homoeopathic stocks and potentisation (2371)
*(published in the Homoeopathic Preparations section)*

Monoclonal antibodies for human use (2031)

Mother tinctures for homoeopathic preparations (2029)
*(published in the Homoeopathic Preparations section)*

Pharmaceutical preparations (2619)

Products of fermentation (1468)

Products with risk of transmitting agents of animal spongiform encephalopathies (1483)

Radiopharmaceutical preparations (0125)

Recombinant DNA technology, products of (0784)

Substances for pharmaceutical use (2034)

Vaccines for human use (0153)

Vaccines for veterinary use (0062)

Vegetable fatty oils (1579)

# EUROPEAN PHARMACOPOEIA

## TENTH EDITION

## Volume III

*Published in accordance with the*
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**01/2017:0923**
**corrected 10.0**

# LABETALOL HYDROCHLORIDE

## Labetaloli hydrochloridum

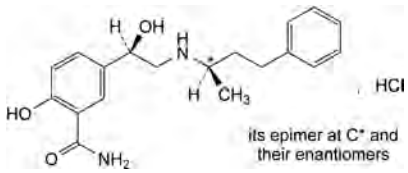


$C_{19}H_{25}ClN_2O_3$ $M_r$ 364.9
[32780-64-6]

DEFINITION

Mixture of 4 stereoisomers of 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]benzamide hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: *A, C, E.*

*Second identification*: *A, B, D, E.*

A. Optical rotation (*2.2.7*): – 0.05° to + 0.05°, determined on solution S (see Tests).

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in *0.1 M hydrochloric acid* and dilute to 250.0 mL with the same acid.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 302 nm.

*Specific absorbance at the absorption maximum*: 83 to 88.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *labetalol hydrochloride CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 1 mL of *ethanol (96 per cent) R*.

*Reference solution (a).* Dissolve 10 mg of *labetalol hydrochloride CRS* in 1 mL of *ethanol (96 per cent) R*.

*Reference solution (b).* Dissolve 10 mg of *labetalol hydrochloride CRS* and 10 mg of *propranolol hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: *perchloric acid R*, *water R*, *methanol R* (0.5:50:80 *V/V/V*).

*Application*: 2 µL.

*Development*: place the plate in a chromatographic tank immediately after the addition of the mobile phase, close the tank and develop over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent. *Solution S must be freshly prepared.*

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Diastereoisomer ratio**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 2.0 mg of the substance to be examined in 1.0 mL of a 12.0 g/L solution of *butylboronic acid R* in *anhydrous pyridine R* and allow to stand for 20 min.

*Column*:

– *material*: glass;
– *size*: *l* = 1.5 m, Ø = 4 mm;
– *stationary phase*: *silanised diatomaceous earth for gas chromatography R* (125-150 µm) impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*.

*Carrier gas*: *nitrogen for chromatography R.*

*Flow rate*: 40 mL/min.

*Temperature*:

– *column, injection port and detector*: 300 °C.

*Detection*: flame ionisation.

*Injection*: 2 µL.

*System suitability*:

– the height of the trough separating the 2 peaks due to the pairs of diastereoisomers is less than 5 per cent of the full scale of the recorder.

*Limit*:

– *each pair of diastereoisomers*: for the area of each peak, 45 per cent to 55 per cent of the total area of the 2 peaks.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 50.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A. Dissolve 5 mg of *labetalol impurity A CRS* in this solution.

*Reference solution (c).* Dissolve 25.0 mg of *labetalol hydrochloride CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 50.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *water for chromatography R* (0.1:99.9 *V/V*);
– *mobile phase B*: *acetonitrile for chromatography R*, mobile phase A (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 40 | 100 → 0 | 0 → 100 |
| 40 - 45 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to labetalol (retention time = about 22 min): impurity A = about 1.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to labetalol and impurity A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of labetalol in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.05 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A, mobile phase B (45:55 *V/V*).

*Injection*: test solution (b) and reference solution (c).

*Run time*: twice the retention time of labetalol.

*Retention time*: labetalol = about 2 min.

Calculate the percentage content of $C_{19}H_{25}ClN_2O_3$ taking into account the assigned content of *labetalol hydrochloride CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

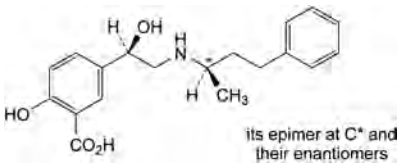


A. mixture of 4 stereoisomers of 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]benzoic acid,

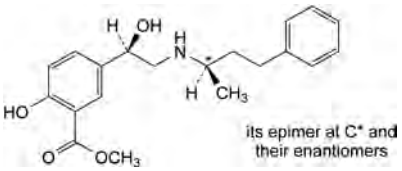


B. mixture of 4 stereoisomers of methyl 2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]-ethyl]benzoate,

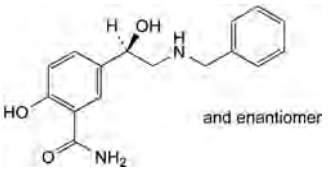


C. 5-[(1*RS*)-2-(benzylamino)-1-hydroxyethyl]-2-hydroxybenzamide,

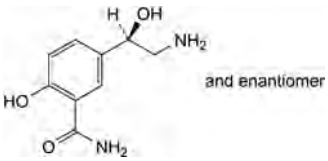


D. 5-[(1*RS*)-2-amino-1-hydroxyethyl]-2-hydroxybenzamide,

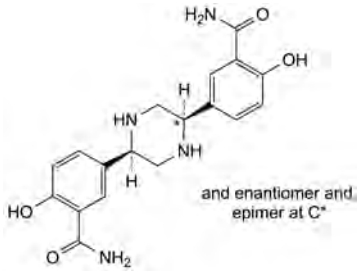


E. mixture of 3 stereoisomers of 5,5′-piperazine-2,5-diylbis(2-hydroxybenzamide),

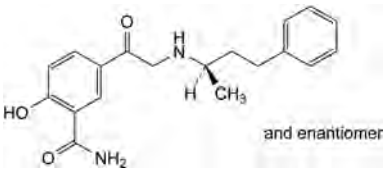


F. 2-hydroxy-5-[2-[[(1*RS*)-1-methyl-3-phenylpropyl]amino]-acetyl]benzamide,

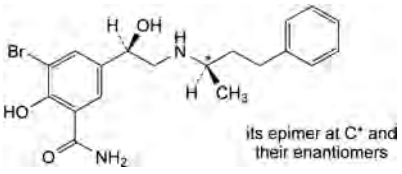


G. mixture of 4 stereoisomers of 3-bromo-2-hydroxy-5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]-ethyl]benzamide.

**07/2018:2992**

# LACOSAMIDE

## Lacosamidum

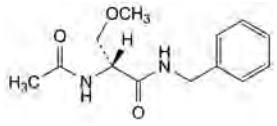

$C_{13}H_{18}N_2O_3$ $M_r$ 250.3
[175481-36-4]

DEFINITION

(2*R*)-2-Acetamido-*N*-benzyl-3-methoxypropanamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or light yellow powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): + 14 to + 18 (anhydrous substance), measured at 25 °C.

Dissolve 0.100 g in *methanol R* and dilute to 10.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lacosamide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Enantiomeric purity (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.500 g in *water R* and dilute to 50.0 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1 mg of *lacosamide impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 20 mg of the substance to be examined in the mobile phase, add 1.0 mL of reference solution (a) and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *water for chromatography R*, *2-propanol R*, *heptane R* (3:100:900 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.7 times the retention time of lacosamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to lacosamide (retention time = about 25 min): impurity A = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity A and lacosamide.

*Limit*:

– *impurity A*: maximum 0.15 per cent;
– *reporting threshold*: 0.05 per cent (reference solution (c)).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 1 mL of *methanol R* and dilute to 10.0 mL with *water R*.

*Reference solution (a).* Dissolve 50.0 mg of *lacosamide CRS* in 1 mL of *methanol R* and dilute to 10.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of 10 volumes of *methanol R* and 90 volumes of *water R*. Dilute 1.0 mL of this solution to 10.0 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 5 mg of *lacosamide for system suitability CRS* (containing impurities B, C, G and I) in 0.1 mL of *methanol R* and dilute to 1.0 mL with *water R*.

*Reference solution (d).* Dissolve 1 mg of *lacosamide impurity F CRS* in 2 mL of *methanol R* and dilute to 10.0 mL with *water R*. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

– *mobile phase A*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R*;
– *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R*, *methanol R* (0.3:500:500 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 89 | 11 |
| 2 - 14.2 | 89 → 69 | 11 → 31 |
| 14.2 - 19.5 | 69 → 23 | 31 → 77 |
| 19.5 - 20 | 23 → 0 | 77 → 100 |
| 20 - 21 | 0 | 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 258 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *lacosamide for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C, G and I; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity F.

*Relative retention* with reference to lacosamide (retention time = about 12 min): impurity F = about 0.7; impurity G = about 0.9; impurity B = about 1.2; impurity C = about 1.3; impurity I = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to impurity G and lacosamide; minimum 4.5 between the peaks due to lacosamide and impurity B.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 0.7; impurity I = 0.7;
- for each impurity, use the concentration of lacosamide in reference solution (b).

*Limits*:

- *impurities B, C, F, G, I*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 0.150 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 2.4 for the peak due to lacosamide.

Calculate the percentage content of $C_{13}H_{18}N_2O_3$ taking into account the assigned content of *lacosamide CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, F, G, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, H, J, K.*

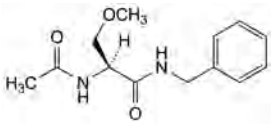

A. (2*S*)-2-acetamido-*N*-benzyl-3-methoxypropanamide,

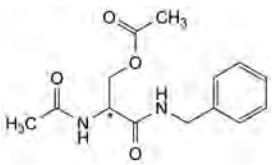

B. (2*Ξ*)-2-acetamido-3-(benzylamino)-3-oxopropyl acetate,

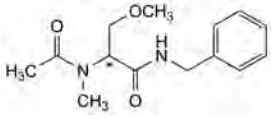

C. (2*Ξ*)-*N*-benzyl-3-methoxy-2-(*N*-methylacetamido)-propanamide,

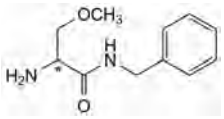

D. (2*Ξ*)-2-amino-*N*-benzyl-3-methoxypropanamide,

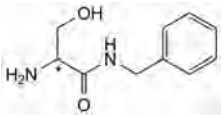

E. (2*Ξ*)-2-amino-*N*-benzyl-3-hydroxypropanamide,

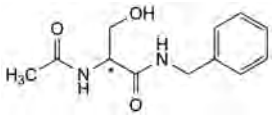

F. (2*Ξ*)-2-acetamido-*N*-benzyl-3-hydroxypropanamide,

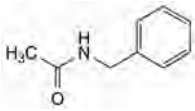

G. *N*-benzylacetamide,

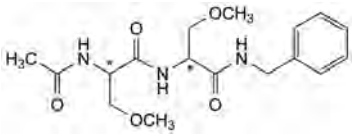

H. (2*Ξ*)-2-acetamido-*N*-[(2*Ξ*)-1-(benzylamino)-3-methoxy-1-oxopropan-2-yl]-3-methoxypropanamide,

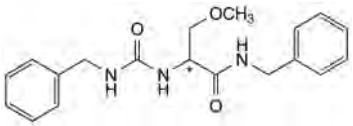

I. (2*Ξ*)-*N*-benzyl-2-[(benzylcarbamoyl)amino]-3-methoxypropanamide,

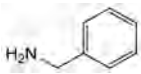

J. phenylmethanamine,

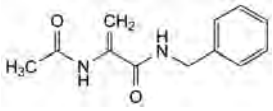

K. 2-acetamido-*N*-benzylprop-2-enamide.

**04/2019:2991**

# LACOSAMIDE INFUSION

## Lacosamidi praeparatio ad infusionem

DEFINITION

Sterile solution for infusion of *Lacosamide (2992)*.

*It complies with the monograph Parenteral preparations (0520) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of lacosamide ($C_{13}H_{18}N_2O_3$) stated on the label.

IDENTIFICATION

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay with a diode array detector in the range of 210-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (13:87 *V/V*).

*Test solution*. Mix the contents of 10 vials of the preparation to be examined and dilute a suitable volume of the solution with the solvent mixture to obtain a concentration of lacosamide of 1.0 mg/mL.

*Reference solution (a)*. Dissolve 20.0 mg of *lacosamide CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *lacosamide impurity D CRS* and 3 mg of *lacosamide impurity F CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *methanesulfonic acid R*, *acetonitrile R1*, *water for chromatography R* (0.75:130:870 *V/V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of lacosamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and F.

*Relative retention* with reference to lacosamide (retention time = about 6 min): impurity D = about 0.4; impurity F = about 0.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurities D and F.

*Calculation of percentage contents*:

- for each impurity, use the concentration of lacosamide in reference solution (b).

*Limits*:

- *impurity D*: maximum 0.8 per cent;
- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.1 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of lacosamide ($C_{13}H_{18}N_2O_3$) taking into account the assigned content of *lacosamide CRS*.

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph)*: B, C, E, F, J, K.*

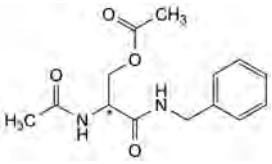

B. (2*Ξ*)-2-acetamido-3-(benzylamino)-3-oxopropyl acetate,

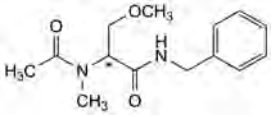

C. (2*Ξ*)-*N*-benzyl-3-methoxy-2-(*N*-methylacetamido)-propanamide,

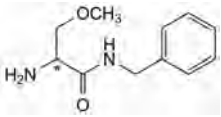

D. (2*Ξ*)-2-amino-*N*-benzyl-3-methoxypropanamide,

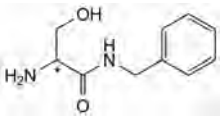

E. (2*Ξ*)-2-amino-*N*-benzyl-3-hydroxypropanamide,

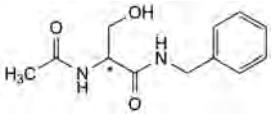

F. (2*Ξ*)-2-acetamido-*N*-benzyl-3-hydroxypropanamide,

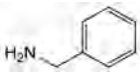

J. phenylmethanamine,

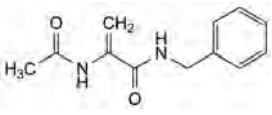

K. 2-acetamido-*N*-benzylprop-2-enamide.

**04/2019:2990**

# LACOSAMIDE ORAL SOLUTION

## Lacosamidi solutio peroralis

DEFINITION

Oral solution of *Lacosamide (2992)*.

*It complies with the monograph Liquid preparations for oral use (0672) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of lacosamide ($C_{13}H_{18}N_2O_3$) stated on the label.

IDENTIFICATION

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay, with a diode array detector in the range of 210-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (13:87 *V/V*).

*Test solution*. Dilute a suitable volume of the preparation to be examined with the solvent mixture to obtain a concentration of lacosamide of 1.0 mg/mL.

*Reference solution (a)*. Dissolve 20.0 mg of *lacosamide CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *lacosamide impurity D CRS* and 3 mg of *lacosamide impurity F CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *trifluoroacetic acid R*, *acetonitrile R1*, *water for chromatography R* (0.56:100:900 *V/V/V*);
- *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R1* (0.5:1000 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 31 | 100 | 0 |
| 31 - 33 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and F; impurities D and F may be inverted in the elution order, but the peak area of each impurity is different, so a clear identification of the impurities is possible.

*Relative retention* with reference to lacosamide (retention time = about 27 min): impurity D = about 0.4; impurity F = about 0.5 (D and F may be inverted).

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to impurities D and F.

*Calculation of percentage contents*:

- for each impurity, use the concentration of lacosamide in reference solution (b).

*Limits*:

- *impurity D*: maximum 0.6 per cent;
- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 2.0 per cent;
- *reporting threshold*: 0.1 per cent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

- *mobile phase A*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R1* (0.5:1000 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 75 | 25 |
| 9 - 12.5 | 45 | 55 |

*Injection*: 4 µL of the test solution and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of lacosamide ($C_{13}H_{18}N_2O_3$) taking into account the assigned content of *lacosamide CRS*.

IMPURITIES

*Specified impurities*: *D*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph): *E, F, G, J, K*.

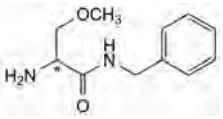


D. (2*Ξ*)-2-amino-*N*-benzyl-3-methoxypropanamide,

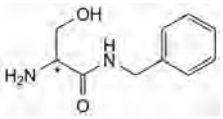


E. (2*Ξ*)-2-amino-*N*-benzyl-3-hydroxypropanamide,

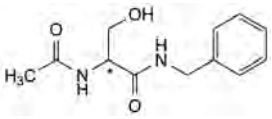


F. (2*Ξ*)-2-acetamido-*N*-benzyl-3-hydroxypropanamide,

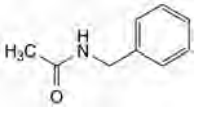


G. *N*-benzylacetamide,

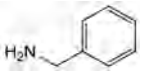


J. phenylmethanamine,

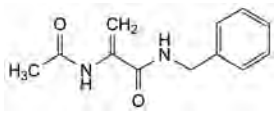

K. 2-acetamido-*N*-benzylprop-2-enamide.

**07/2019:2989**

# LACOSAMIDE TABLETS

## Lacosamidi compressi

DEFINITION

Tablets containing *Lacosamide (2992)*.

*They comply with the monograph Tablets (0478) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of lacosamide ($C_{13}H_{18}N_2O_3$) stated on the label.

IDENTIFICATION

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay, with a diode array detector in the range of 210-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (13:87 *V/V*).

*Test solution.* To 10 tablets add a suitable volume of the solvent mixture to obtain a concentration of lacosamide of 2-4 mg/mL. Shake vigorously for 30 min, sonicate for 10 min and allow to stand for 30 min. Dilute a suitable volume of the supernatant with the solvent mixture to obtain a concentration of lacosamide of 1.0 mg/mL.

*Reference solution (a).* Dissolve 20.0 mg of *lacosamide CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *lacosamide impurity D CRS* and 3 mg of *lacosamide impurity F CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *methanesulfonic acid R*, *acetonitrile R1*, *water for chromatography R* (0.75:130:870 *V/V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of lacosamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and F.

*Relative retention* with reference to lacosamide (retention time = about 6 min): impurity D = about 0.4; impurity F = about 0.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurities D and F.

*Calculation of percentage contents*:

- for each impurity, use the concentration of lacosamide in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.1 per cent.

**Dissolution** (*2.9.3*, Apparatus 2).

*The tablets comply with the test and the acceptance criterion described below, unless otherwise justified and authorised.*

*Dissolution medium*: 10.3 g/L solution of *hydrochloric acid R*. Use 900 mL of the medium.

*Rotation speed*: 50 r/min.

*Time*: 30 min.

*Analysis.* Liquid chromatography (*2.2.29*).

*Test solutions.* Samples withdrawn from the dissolution vessel and filtered.

*Reference solution.* Using sonication, dissolve a suitable quantity of *lacosamide CRS* in a suitable volume of the dissolution medium to obtain a concentration of lacosamide corresponding to the theoretical concentration of lacosamide in the test solution, based on the labelled content of the tablets.

*Column*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*: *trifluoroacetic acid R*, *acetonitrile R1*, *water for chromatography R* (1:300:700 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 2 µL.

*Run time*: 2.5 min.

*System suitability*: reference solution:

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the amount of dissolved lacosamide ($C_{13}H_{18}N_2O_3$), expressed as a percentage of the content stated on the label, taking into account the assigned content of *lacosamide CRS*.

*Acceptance criterion*:

- *Q* = 80 per cent after 30 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of lacosamide ($C_{13}H_{18}N_2O_3$) taking into account the assigned content of *lacosamide CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph): *B, C, D, E, F, J, K.*

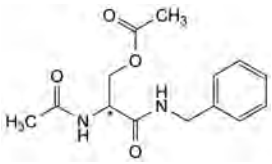

B. (2Ξ)-2-acetamido-3-(benzylamino)-3-oxopropyl acetate,

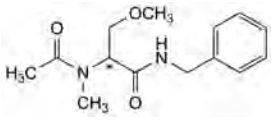

C. (2Ξ)-*N*-benzyl-3-methoxy-2-(*N*-methylacetamido)-propanamide,

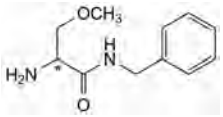

D. (2Ξ)-2-amino-*N*-benzyl-3-methoxypropanamide,

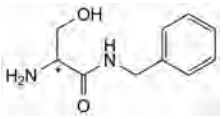

E. (2Ξ)-2-amino-*N*-benzyl-3-hydroxypropanamide,

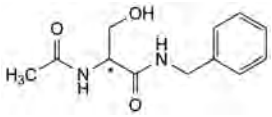

F. (2Ξ)-2-acetamido-*N*-benzyl-3-hydroxypropanamide,

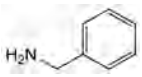

J. phenylmethanamine,

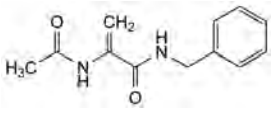

K. 2-acetamido-*N*-benzylprop-2-enamide.

**01/2017:0458**

# LACTIC ACID

## Acidum lacticum

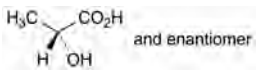


$C_3H_6O_3$ $M_r$ 90.1

DEFINITION

Mixture of 2-hydroxypropanoic acid, its condensation products, such as lactoyl-lactic acid and polylactic acids, and water. The equilibrium between lactic acid and polylactic acids depends on the concentration and temperature. It is usually the racemate ((*RS*)-lactic acid).

*Content*: 88.0 per cent *m/m* to 92.0 per cent *m/m* of $C_3H_6O_3$.

CHARACTERS

*Appearance*: colourless or slightly yellow, syrupy liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in 10 mL of *water R*. The solution is strongly acidic (*2.2.4*).

B. Relative density (*2.2.5*): 1.20 to 1.21.

C. It gives the reaction of lactates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 42 mL of *1 M sodium hydroxide* and dilute to 50 mL with *distilled water R*.

**Appearance**. The substance to be examined is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Ether-insoluble substances**. Dissolve 1.0 g in 25 mL of *ether R*. The solution is not more opalescent than the solvent used for the test.

**Sugars and other reducing substances**. To 1 mL of solution S add 1 mL of *1 M hydrochloric acid*, heat to boiling, allow to cool and add 1.5 mL of *1 M sodium hydroxide* and 2 mL of *cupri-tartaric solution R*. Heat to boiling. No red or greenish precipitate is formed.

**Methanol** (*2.4.24*): maximum 50 ppm, if intended for use in the manufacture of parenteral preparations.

**Citric, oxalic and phosphoric acids**. To 5 mL of solution S add *dilute ammonia R1* until slightly alkaline (*2.2.4*). Add 1 mL of *calcium chloride solution R*. Heat on a water-bath for 5 min. Both before and after heating, any opalescence in the solution is not more intense than that in a mixture of 1 mL of *water R* and 5 mL of solution S.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Before use, neutralise the test solution to pH 7.0-7.5 with *strong sodium hydroxide solution R* and shake vigorously.

ASSAY

Place 1.000 g in a ground-glass-stoppered flask and add 10 mL of *water R* and 20.0 mL of *1 M sodium hydroxide*. Close the flask and allow to stand for 30 min. Using 0.5 mL of *phenolphthalein solution R* as indicator, titrate with *1 M hydrochloric acid* until the pink colour is discharged.

1 mL of *1 M sodium hydroxide* is equivalent to 90.1 mg of $C_3H_6O_3$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:1771**

# (*S*)-LACTIC ACID

## Acidum (*S*)-lacticum

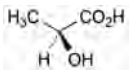

$C_3H_6O_3$ $M_r$ 90.1

DEFINITION

Mixture of (*S*)-2-hydroxypropanoic acid, its condensation products, such as lactoyl-lactic acid and polylactic acids, and water. The equilibrium between lactic acid and polylactic acids depends on the concentration and temperature.

*Content*: 88.0 per cent *m/m* to 92.0 per cent *m/m* of $C_3H_6O_3$, not less than 95.0 per cent of which is the (*S*)-enantiomer.

CHARACTERS

*Appearance*: colourless or slightly yellow, syrupy liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in 10 mL of *water R*. The solution is strongly acidic (*2.2.4*).

B. Relative density (*2.2.5*): 1.20 to 1.21.

C. It gives the reaction of lactates (*2.3.1*).

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 5.0 g in 42 mL of *1 M sodium hydroxide* and dilute to 50 mL with *distilled water R*.

**Appearance**. The substance to be examined is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Ether-insoluble substances**. Dissolve 1.0 g in 25 mL of *ether R*. The solution is not more opalescent than the solvent used for the test.

**Sugars and other reducing substances**. To 1 mL of solution S add 1 mL of *1 M hydrochloric acid*, heat to boiling, allow to cool and add 1.5 mL of *1 M sodium hydroxide* and 2 mL of *cupri-tartaric solution R*. Heat to boiling. No red or greenish precipitate is formed.

**Methanol** (*2.4.24*): maximum 50 ppm, if intended for use in the manufacture of parenteral preparations.

**Citric, oxalic and phosphoric acids**. To 5 mL of solution S add *dilute ammonia R1* until slightly alkaline (*2.2.4*). Add 1 mL of *calcium chloride solution R*. Heat on a water-bath for 5 min. Both before and after heating, any opalescence in the solution is not more intense than that in a mixture of 1 mL of *water R* and 5 mL of solution S.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/g if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Before use, neutralise the test solution to pH 7.0-7.5 with *strong sodium hydroxide solution R* and shake vigorously.

ASSAY

Place 1.000 g in a ground-glass-stoppered flask and add 10 mL of *water R* and 20.0 mL of *1 M sodium hydroxide*. Close the flask and allow to stand for 30 min. Using 0.5 mL of *phenolphthalein solution R* as indicator, titrate with *1 M hydrochloric acid* until the pink colour is discharged.

1 mL of *1 M sodium hydroxide* is equivalent to 90.1 mg of $C_3H_6O_3$.

**(*S*)-enantiomer**

Transfer an amount of the substance to be examined equivalent to 2.00 g of lactic acid into a round-bottomed flask, add 25 mL of *1 M sodium hydroxide* and boil gently for 15 min. Cool down and adjust to pH 7.0 using *1 M hydrochloric acid*. Add 5.0 g of *ammonium molybdate R*, dissolve and dilute to 50.0 mL with *water R*. Filter and measure the angle of optical rotation (*2.2.7*). Calculate the percentage content of (*S*)-enantiomer using the expression:

$$50 + \left(24.18 \times \alpha \times \frac{2.222}{m} \times \frac{90}{c}\right)$$

$\alpha$ = angle of optical rotation (absolute value),

$m$ = mass of the substance to be examined, in grams,

$c$ = percentage content of $C_3H_6O_3$ in the substance to be examined.

The complex of (*S*)-lactic acid formed under these test conditions is laevorotatory.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**07/2019:1337**

# LACTITOL MONOHYDRATE

## Lactitolum monohydricum

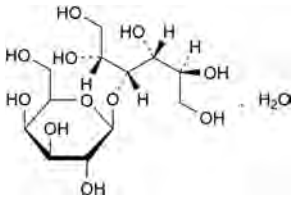

$C_{12}H_{24}O_{11},H_2O$ $M_r$ 362.3

[81025-04-9]

DEFINITION

4-*O*-β-D-Galactopyranosyl-D-glucitol monohydrate.

*Content*: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lactitol monohydrate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *lactitol monohydrate CRS* in *methanol R* and dilute to 2 mL with the same solvent.

*Reference solution (b).* Dissolve 2.5 mg of *sorbitol CRS* (impurity E) in 1 mL of reference solution (a) and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *acetonitrile R* (25:75 *V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *4-aminobenzoic acid solution R* and dry in a current of cold air until the acetone is removed; heat at 100 °C for 15 min and allow to cool; spray with a 2 g/L solution of *sodium periodate R* and dry in a current of cold air; heat at 100 °C for 15 min.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 5.000 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 10 mL of *carbon dioxide-free water R*. To 10 mL of this solution add 0.05 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink. To a further 10 mL of the solution add 0.05 mL of *methyl red solution R*. Not more than 0.3 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Specific optical rotation**(*2.2.7*): + 13.5 to + 15.5 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dilute 2.0 mL of test solution (a) to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 5.0 mg of *lactitol monohydrate CRS* and 5 mg of *glycerol R* in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 2.5 mL of reference solution (a) to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R*;
- *temperature*: 60 °C.

*Mobile phase*: *water for chromatography R*.

*Flow rate*: 0.6 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 100 µL; inject test solution (a) and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of lactitol.

*Relative retention* with reference to lactitol (retention time = about 13 min): impurity A = about 0.7; impurity B = about 0.8; glycerol = about 1.3; impurity C = about 1.5; impurity D = about 1.8; impurity E = about 1.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to lactitol and glycerol.

*Limits*:

- *impurity B*: not more than the area of the peak due to lactitol in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *total of other impurities*: not more than the area of the peak due to lactitol in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Reducing sugars**: maximum 0.2 per cent.

Dissolve 5.0 g in 3 mL of *water R* with gentle heating. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R*. When the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R* added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

Absence of *Pseudomonas aeruginosa* (*2.6.13*).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{12}H_{24}O_{11}$ taking into account the assigned content of *lactitol monohydrate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

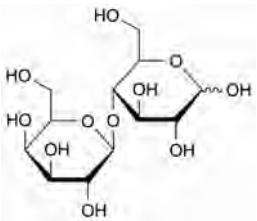


A. 4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose),

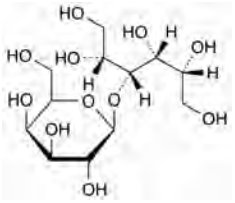

B. 3-*O*-β-D-galactopyranosyl-D-mannitol (lactulitol),

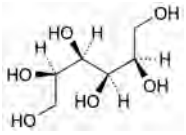

C. D-mannitol,

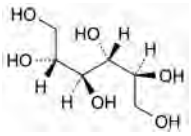

D. galactitol (dulcitol),

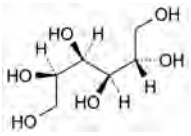

E. D-glucitol (D-sorbitol).

**01/2017:1647**

# LACTOBIONIC ACID

## Acidum lactobionicum

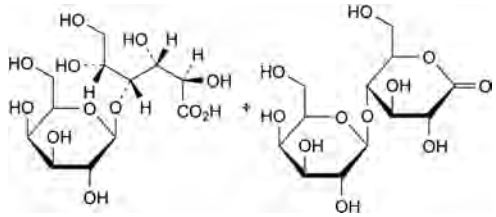

$C_{12}H_{22}O_{12}$ (acid form) $M_r$ 358.3
[96-82-2]

$C_{12}H_{20}O_{11}$ (δ-lactone) $M_r$ 340.3
[5965-65-1]

DEFINITION

Mixture in variable proportions of 4-*O*-β-D-galactopyranosyl-D-gluconic acid and 4-*O*-β-D-galactopyranosyl-D-glucono-1,5-lactone.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in glacial acetic acid, in anhydrous ethanol and in methanol.

mp: about 125 °C with decomposition.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lactobionic acid CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *water R*, dry at 105 °C and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 1 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *lactobionic acid CRS* in *water R* and dilute to 1 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R1*, *ethyl acetate R*, *water R*, *methanol R* (2:2:2:4 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Detection*: spray 3 times with *ammonium molybdate solution R6* and heat in an oven at 110 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 3.0 g in 25 mL of *water R*.

**Specific optical rotation** (*2.2.7*): + 23.0 to + 29.0 (anhydrous substance).

Dissolve 1.0 g in 80 mL of *water R* and dilute to 100.0 mL with the same solvent. Allow to stand for 24 h.

**Reducing sugars**: maximum 0.2 per cent, calculated as glucose.

Dissolve 5.0 g in 25 mL of *water R* with the aid of gentle heat. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.50 g.

Use a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as solvent.

**Total ash** (*2.4.16*): maximum 0.2 per cent.

ASSAY

Dissolve 0.350 g in 50 mL of *carbon dioxide-free water R*, previously heated to 30 °C. Immediately titrate with *0.1 M sodium hydroxide* and determine the 2 equivalence points potentiometrically (*2.2.20*).

The first equivalence point ($V_1$) corresponds to the acid form of lactobionic acid and the second equivalence point ($V_2 - V_1$) corresponds to the δ-lactone form.

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.83 mg of $C_{12}H_{22}O_{12}$.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.03 mg of $C_{12}H_{20}O_{11}$.

The sum of the 2 results is expressed as a percentage content of lactobionic acid.

**01/2018:1061 corrected 10.0**

# LACTOSE[(1)]

## Lactosum

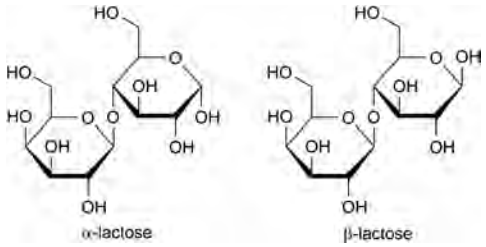


$C_{12}H_{22}O_{11}$ $M_r$ 342.3
[63-42-3]

DEFINITION

*O*-β-D-Galactopyranosyl-(1→4)-β-D-glucopyranose or mixture of *O*-β-D-galactopyranosyl-(1→4)-α-D-glucopyranose and *O*-β-D-galactopyranosyl-(1→4)-β-D-glucopyranose.

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).♦

IDENTIFICATION

*First identification: A, ◊D.*

*Second identification: B, C, D.◊*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anhydrous lactose CRS*.

◊B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *methanol R* (40:60 *V/V*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *anhydrous lactose CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *fructose R*, 10 mg of *glucose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *methanol R*, *glacial acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately, as a slight excess of water produces cloudiness.

*Application*: 2 µL; thoroughly dry the points of application.

*Development A*: over 3/4 of the plate.

*Drying A*: in a current of warm air.

*Development B*: immediately, over 3/4 of the plate, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*; heat at 130 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 4 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.25 g in 5 mL of *water R*. Add 5 mL of *ammonia R* and heat in a water-bath at 80 °C for 10 min. A red colour develops.

D. Water (see Tests).◊

TESTS

**Solution S**. Dissolve 1.0 g in boiling *water R*, allow to cool and dilute to 10.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 6.0 g by heating in 25 mL of *carbon dioxide-free water R*, cool and add 0.3 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.4 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink or red.

**Specific optical rotation** (*2.2.7*): + 54.4 to + 55.9 (anhydrous substance).

Dissolve 10.0 g in 80 mL of *water R* with heating at 50 °C. Allow to cool and add 0.2 mL of *dilute ammonia R1*. Allow to stand for 30 min and dilute to 100.0 mL with *water R*.

**Absorbance: proteins and light-absorbing impurities** (*2.2.25*).

*Test solution (a)*. Solution S.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *water R*.

*Spectral range*: 400 nm for test solution (a) and 210-300 nm for test solution (b).

*Results*:

– at 400 nm: maximum 0.04 for test solution (a);
– from 210 nm to 220 nm: maximum 0.25 for test solution (b);
– from 270 nm to 300 nm: maximum 0.07 for test solution (b).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g, using a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as solvent.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for lactose used as filler/diluent in solid dosage forms (compressed and powder).*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Bulk and tapped density** (*2.9.34*). Determine the bulk density and the tapped density. Calculate the Hausner ratio.

**α-Lactose and β-lactose**. Gas chromatography (*2.2.28*).

*Silylation reagent*: *dimethyl sulfoxide R*, *N-trimethylsilylimidazole R*, *pyridine R* (19.5:22:58.5 *V/V/V*).

*Test solution*. Introduce 10 mg of the substance to be examined into a vial with a screw cap and add 4 mL of the silylation reagent. Sonicate for 20 min at room temperature, allow to cool and transfer 400 µL to an injection vial. Add 1 mL of *pyridine R*, close the vial and mix well.

*Reference solution*. Prepare a mixture of *α-lactose monohydrate R* and *β-lactose R* to obtain an anomeric ratio of about 1:1 based on the labelled anomeric contents of the α-lactose monohydrate and the β-lactose. Introduce 10 mg of the mixture into a vial with a screw cap and add 4 mL of the silylation reagent. Sonicate for 20 min at room temperature, allow to cool, and transfer 400 µL to an injection vial. Add 1 mL of *pyridine R*, close the vial and mix well.

*Precolumn*:

– *material*: intermediate-polarity deactivated fused silica;
– *size*: *l* = 2 m, Ø = 0.53 mm.

*Column*:

– *material*: fused silica;
– *size*: *l* = 15 m, Ø = 0.25 mm;
– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.8 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 80 |
| | 1 - 3 | 80 → 150 |
| | 3 - 15.5 | 150 → 300 |
| | 15.5 - 17.5 | 300 |
| Injection port | | 275 or use cold on-column injection |
| Detector | | 325 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL, splitless or by cold on-column injection.

*Relative retention* with reference to β-lactose (retention time = about 12 min): α-lactose = about 0.9.

*System suitability*: reference solution:

– *resolution*: minimum 3.0 between the peaks due to α-lactose and β-lactose.

Calculate the percentage content of α-lactose using the following expression:

$$\frac{100 S_a}{S_a + S_b}$$

Calculate the percentage content of β-lactose using the following expression:

$$\frac{100 S_b}{S_a + S_b}$$

$S_a$ = area of the peak due to α-lactose;

$S_b$ = area of the peak due to β-lactose.

**Loss on drying** (*2.2.32*). Determine on 1.000 g by drying in an oven at 80 °C for 2 h.

**01/2018:0187**

# LACTOSE MONOHYDRATE(2)

## Lactosum monohydricum

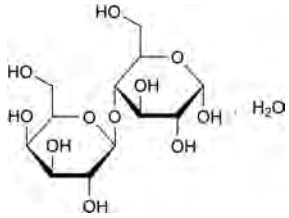

$C_{12}H_{22}O_{11},H_2O$ $M_r$ 360.3

DEFINITION

*O*-β-D-Galactopyranosyl-(1→4)-α-D-glucopyranose monohydrate.

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).♦

IDENTIFICATION

*First identification: A, ◊D.*

*Second identification: B, C, D.◊*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lactose monohydrate CRS*.

◊B. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *water R*, *methanol R* (40:60 *V/V*).
*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.
*Reference solution (a)*. Dissolve 10 mg of *lactose monohydrate CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.
*Reference solution (b)*. Dissolve 10 mg of *fructose R*, 10 mg of *glucose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *water R*, *methanol R*, *glacial acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately, as a slight excess of water produces cloudiness.
*Application*: 2 µL; thoroughly dry the points of application.
*Development A*: over 3/4 of the plate.
*Drying A*: in a current of warm air.
*Development B*: immediately, over 3/4 of the plate, after renewing the mobile phase.
*Drying B*: in a current of warm air.
*Detection*: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*; heat at 130 °C for 10 min.
*System suitability*: reference solution (b):
– the chromatogram shows 4 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

C. Dissolve 0.25 g in 5 mL of *water R*. Add 5 mL of *ammonia R* and heat in a water-bath at 80 °C for 10 min. A red colour develops.

D. Water (see Tests).◊

TESTS

**Solution S**. Dissolve 1.0 g in boiling *water R*, allow to cool and dilute to 10.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) ◊and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*)◊.

**Acidity or alkalinity**. Dissolve 6.0 g by heating in 25 mL of *carbon dioxide-free water R*, cool and add 0.3 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.4 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink or red.

**Specific optical rotation** (*2.2.7*): + 54.4 to + 55.9 (anhydrous substance).

Dissolve 10.0 g in 80 mL of *water R* with heating at 50 °C. Allow to cool and add 0.2 mL of *dilute ammonia R1*. Allow to stand for 30 min and dilute to 100.0 mL with *water R*.

**Absorbance: proteins and light-absorbing impurities** (*2.2.25*).

*Test solution (a)*. Solution S.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *water R*.

*Spectral range*: 400 nm for test solution (a) and 210-300 nm for test solution (b).

*Results*:

- at 400 nm: maximum 0.04 for test solution (a);
- from 210 nm to 220 nm: maximum 0.25 for test solution (b);
- from 270 nm to 300 nm: maximum 0.07 for test solution (b).

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.50 g, using a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as solvent.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**♦Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for lactose monohydrate used as a filler/diluent in solid dosage forms (compressed and powder).*

**Particle size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*). Determine the bulk density and the tapped density. Calculate the Hausner ratio.

**07/2019:1230**

# LACTULOSE

## Lactulosum

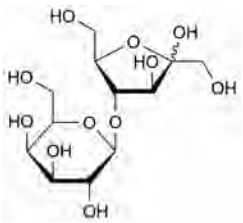

$C_{12}H_{22}O_{11}$ $M_r$ 342.3

[4618-18-2]

DEFINITION

4-*O*-β-D-Galactopyranosyl-D-*arabino*-hex-2-ulofuranose.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol, practically insoluble in toluene.

mp: about 168 °C.

IDENTIFICATION

*First identification: B, C, D, E.*

*Second identification: A, C, D, E.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 50.0 mg of *lactulose CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, 50 g/L solution of *boric acid R*, *methanol R*, *ethyl acetate R* (10:15:20:55 *V/V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C for 5 min; allow to cool.

*Detection*: spray with a 1.0 g/L solution of *1,3-dihydroxynaphthalene R* in a mixture of 10 volumes of *sulfuric acid R* and 90 volumes of *methanol R*; heat at 110 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

C. Dissolve 50 mg in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. A red precipitate is formed.

D. Dissolve 0.125 g in 5 mL of *water R*. Add 5 mL of *ammonia R*. Heat in a water-bath at 80 °C for 10 min. A red colour develops.

E. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 7.0.

To 10 mL of solution S add 0.1 mL of a saturated solution of *potassium chloride R*.

**Specific optical rotation** (*2.2.7*): − 50.0 to − 46.0 (anhydrous substance).

Dissolve 1.25 g in *water R*, add 0.2 mL of *concentrated ammonia R* and dilute to 25.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in 10 mL of *water R*. Add 12.5 mL of *acetonitrile R* with gentle heating and dilute to 25.0 mL with *water R*.

*Reference solution (a).* To 3.0 mL of the test solution add 48.5 mL of *acetonitrile R* with gentle heating and dilute to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 1.00 g of *lactulose CRS* in 10 mL of *water R*. Add 12.5 mL of *acetonitrile R* with gentle heating and dilute to 25.0 mL with *water R*.

*Reference solution (c).* Dissolve 10 mg of *lactulose R*, 10 mg of *epilactose R* (impurity A) and 10 mg of *lactose monohydrate R* (impurity C) in 2 mL of *water R*. Add 2.5 mL of *acetonitrile R* with gentle heating and dilute to 5 mL with *water R*.

*Reference solution (d).* To 5.0 mL of the test solution add 47.5 mL of *acetonitrile R* with gentle heating and dilute to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Column 1*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 38 ± 1 °C.

*Column 2*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 38 ± 1 °C.

Columns 1 and 2 are coupled in series.

*Mobile phase*: dissolve 0.253 g of *sodium dihydrogen phosphate R* in 200 mL of *water for chromatography R* and dilute to 1000 mL with *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and reference solutions (a), (c) and (d).

*Run time*: twice the retention time of lactulose.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and C.

*Relative retention* with reference to lactulose (retention time = about 18 min): impurity A = about 0.9; impurity C = about 1.2.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lactulose.

*Limits*:

- *impurity C*: not more than the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (3.0 per cent);
- *unspecified impurities*: for each impurity, not more than twice the area of the peak due to lactulose in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *total*: not more than the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (3.0 per cent);
- *disregard limit*: the area of the peak due to lactulose in the chromatogram obtained with reference solution (d) (0.25 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Methanol**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Mix 0.5 mL of *propanol R* and 100.0 mL of *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 50.0 mL with *water R*.

*Test solution.* To 79 mg of the substance to be examined in a 20 mL vial add 1.0 mL of the internal standard solution and 5 µL of a 0.1 per cent *V/V* solution of *methanol R*.

*Reference solution.* To 1.0 mL of the internal standard solution in a 20 mL vial add 5 µL of a 0.1 per cent *V/V* solution of *methanol R*.

*Column*:

- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (180 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 30 mL/min.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 1 h;
- *pressurisation time*: 1 min.

*Temperature*:

- *column*: 140 °C;
- *injection port*: 200 °C;
- *detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the gaseous phase.

Calculate the content of methanol, taking its density (*2.2.5*) at 20 °C to be 0.79 g/mL.

*Limit*:

- *methanol*: calculate the ratio (*R*) of the area of the peak due to methanol to the area of the peak due to the internal standard in the chromatogram obtained with the reference solution; calculate the ratio of the area of the peak due to methanol to the area of the peak due to the internal standard in the chromatogram obtained with the test solution: this ratio is not greater than 2*R* (50 ppm).

**Boron**: maximum 9 ppm.

*Avoid where possible the use of glassware.*

*Reference solution.* Dissolve 50.0 mg of *boric acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. *Keep in a well-closed polyethylene container.*

In 4 polyethylene 25 mL flasks, place separately:

- 0.50 g of the substance to be examined dissolved in 2.0 mL of *water R* (solution A);
- 0.50 g of the substance to be examined dissolved in 1.0 mL of the reference solution and 1.0 mL of *water R* (solution B);

– 1.0 mL of the reference solution and 1.0 mL of *water R* (solution C);
– 2.0 mL of *water R* (solution D).

To each flask add 4.0 mL of *acetate-edetate buffer solution pH 5.5 R*. Mix and add 4.0 mL of freshly prepared *azomethine H solution R*. Mix and allow to stand for 1 h. Measure the absorbance (*2.2.25*) of solutions A, B and C at 420 nm, using solution D as the compensation liquid. The test is not valid unless the absorbance of solution C is at least 0.25. The absorbance of solution B is not less than twice that of solution A.

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (b).

*System suitability*: reference solution (b):

– *symmetry factor*: 0.6 to 2.0 for the principal peak.

Calculate the percentage content of $C_{12}H_{22}O_{11}$ taking into account the assigned content of *lactulose CRS*.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E.*

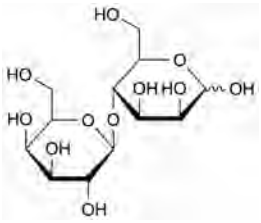

A. 4-*O*-β-D-galactopyranosyl-D-mannopyranose (epilactose),

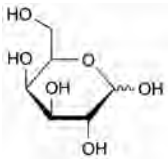

B. D-galactopyranose (galactose),

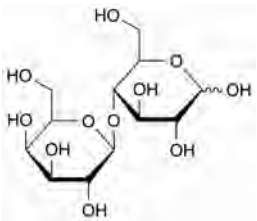

C. 4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose),

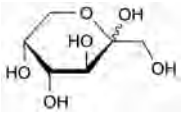

D. D-*arabino*-hex-2-ulopyranose (fructose),

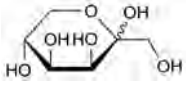

E. D-*lyxo*-hex-2-ulopyranose (tagatose).

**07/2019:0924**

# LACTULOSE, LIQUID

## Lactulosum liquidum

DEFINITION

Aqueous solution of 4-*O*-β-D-galactopyranosyl-D-*arabino*-hex-2-ulofuranose normally prepared by alkaline isomerisation of lactose. It may contain other sugars including lactose, epilactose, galactose, tagatose and fructose.

*Content*: minimum 620 g/L of lactulose ($C_{12}H_{22}O_{11}$; $M_r$ 342.3) and 95.0 per cent to 105.0 per cent of the content of lactulose stated on the label.

It may contain a suitable antimicrobial preservative.

CHARACTERS

*Appearance*: clear, viscous liquid, colourless or pale brownish-yellow.

*Solubility*: miscible with water. It may be a supersaturated solution or may contain crystals that disappear on heating.

A 10 per cent *V/V* solution is laevorotatory.

IDENTIFICATION

*First identification: B, C, D.*

*Second identification: A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dilute 0.50 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution*. Dissolve 60.0 mg of *lactulose CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, 50 g/L solution of *boric acid R*, *methanol R*, *ethyl acetate R* (10:15:20:55 *V/V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C for 5 min and allow to cool.

*Detection*: spray with a 1.0 g/L solution of *1,3-dihydroxynaphthalene R* in a mixture of 10 volumes of *sulfuric acid R* and 90 volumes of *methanol R*; heat at 110 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (b).

C. To 0.1 g add 10 mL of *water R* and 3 mL of *cupri-tartaric solution R* and heat. A red precipitate is formed.

D. To 0.25 g add 5 mL of *water R* and 5 mL of *ammonia R*. Heat in a water-bath at 80 °C for 10 min. A red colour develops.

TESTS

**Solution S**. Mix 10 g with *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 7.0.

To 10 mL of solution S add 0.1 mL of a saturated solution of *potassium chloride R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Mix 4.00 g of the substance to be examined and 20 mL of *water R*. Add 25.0 mL of *acetonitrile R* with gentle heating and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. To 5.0 mL of the test solution add 47.5 mL of *acetonitrile R* with gentle heating and dilute to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 2.00 g of *lactulose CRS* in 20 mL of *water R*. Add 25.0 mL of *acetonitrile R* with gentle heating and dilute to 50.0 mL with *water R*.

*Reference solution (c)*. Dissolve 65 mg of *fructose CRS* (impurity D) in a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (d)*. Dissolve 1 g of *lactulose for peak identification CRS* (containing impurities A, B, C, E, F, G and H) in reference solution (c) and dilute to 25 mL with reference solution (c).

*Reference solution (e)*. Dilute 5.0 mL of reference solution (a) to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Column 1*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 38 ± 1 °C.

*Column 2*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 38 ± 1 °C.

Columns 1 and 2 are coupled in series.

*Mobile phase*: dissolve 0.253 g of *sodium dihydrogen phosphate R* in 200 mL of *water for chromatography R* and dilute to 1000 mL with *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and of reference solutions (a), (d) and (e).

*Run time*: twice the retention time of lactulose.

*Identification of impurities*: use the chromatogram supplied with *lactulose for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, E, F, G and H.

*Relative retention* with reference to lactulose (retention time = about 18 min): impurity F = about 0.2; impurity E = about 0.38; impurity D = about 0.42; impurity B = about 0.6; impurity G = about 0.8; impurity A = about 0.9; impurity C = about 1.2; impurity H = about 1.5.

*System suitability*: reference solution (d):

- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lactulose.

*Limits*:

- *impurity B*: not more than 3 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (15.0 per cent);
- *impurities A, C*: for each impurity, not more than twice the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (10.0 per cent);
- *impurities E, F*: for each impurity, not more than 0.8 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (4.0 per cent);
- *impurities G, H*: for each impurity, not more than 0.3 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *impurity D*: not more than 0.2 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *sum of impurities eluting after impurity H*: not more than 0.26 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (1.3 per cent);
- *total (excluding impurities B and C)*: not more than 2.4 times the area of the peak due to lactulose in the chromatogram obtained with reference solution (a) (12.0 per cent);
- *disregard limit*: not more than the area of the peak due to lactulose in the chromatogram obtained with reference solution (e) (0.25 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Methanol**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution*. Mix 0.5 mL of *propanol R* and 100.0 mL of *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 50.0 mL with *water R*.

*Test solution*. To 0.13 g of the substance to be examined in a 20 mL vial add 1.0 mL of the internal standard solution and 5 µL of a 0.1 per cent *V/V* solution of *methanol R*.

*Reference solution*. To 1.0 mL of the internal standard solution in a 20 mL vial add 5 µL of a 0.1 per cent *V/V* solution of *methanol R*.

*Column*:

- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (180 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 30 mL/min.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 1 h;
- *pressurisation time*: 1 min.

*Temperature*:

- *column*: 140 °C;
- *injection port*: 200 °C;
- *detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1 mL of the gaseous phase.

Calculate the content of methanol, taking its density (*2.2.5*) at 20 °C to be 0.79 g/mL.

*Limit*:

– *methanol*: calculate the ratio (*R*) of the area of the peak due to methanol to the area of the peak due to the internal standard in the chromatogram obtained with the reference solution; calculate the ratio of the area of the peak due to methanol to the area of the peak due to the internal standard in the chromatogram obtained with the test solution: this ratio is not greater than 2*R* (30 ppm).

**Sulfites**: maximum 30 ppm.

Mix 5.0 g with 40 mL of *water R*, add 2.0 mL of *0.1 M sodium hydroxide* and dilute to 100 mL with *water R*. To 10.0 mL of this solution, add 1.0 mL of *hydrochloric acid R1*, 2.0 mL of *decolorised fuchsin solution R1* and 2.0 mL of a 0.5 per cent *V/V* solution of *formaldehyde R*. Allow to stand for 30 min and measure the absorbance (*2.2.25*) at 583 nm using as the compensation liquid a solution prepared at the same time and in the same manner with 10.0 mL of *water R* instead of the solution of the substance to be examined. The absorbance is not greater than that of a reference solution prepared at the same time and in the same manner using 10.0 mL of *sulfite standard solution (1.5 ppm $SO_2$) R* instead of the solution of the substance to be examined.

**Boron**: maximum 5 ppm.

*Avoid where possible the use of glassware.*

*Reference solution.* Dissolve 56.0 mg of *boric acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*. *Keep in a well-closed polyethylene container.*

In 4 polyethylene 25 mL flasks, place separately:

– 1.00 g of the substance to be examined and 1 mL of *water R* (solution A);
– 1.00 g of the substance to be examined and 1 mL of the reference solution (solution B);
– 1 mL of the reference solution and 1 mL of *water R* (solution C);
– 2 mL of *water R* (solution D).

To each flask, add 4.0 mL of *acetate-edetate buffer solution pH 5.5 R*. Mix and add 4.0 mL of freshly prepared *azomethine H solution R*. Mix and allow to stand for 1 h. Measure the absorbance (*2.2.25*) of solutions A, B and C at 420 nm, using solution D as the compensation liquid. The test is not valid unless the absorbance of solution C is at least 0.25. The absorbance of solution B is not less than twice that of solution A.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.5 g and calculated with reference to the declared content of lactulose.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^1$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (b).

*System suitability*: reference solution (b):

– *symmetry factor*: 0.6 to 2.0 for the principal peak.

Calculate the percentage content of $C_{12}H_{22}O_{11}$ taking into account the assigned content of *lactulose CRS*.

LABELLING

The label states the declared content of lactulose.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

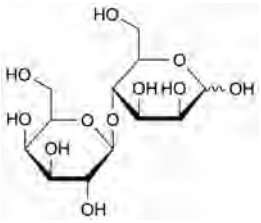

A. 4-*O*-β-D-galactopyranosyl-D-mannopyranose (epilactose),

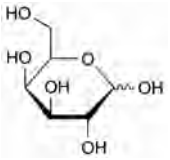

B. D-galactopyranose (galactose),

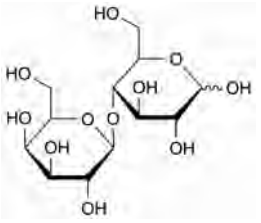

C. 4-*O*-β-D-galactopyranosyl-D-glucopyranose (lactose),

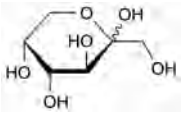

D. D-*arabino*-hex-2-ulopyranose (fructose),

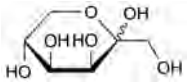

E. D-*lyxo*-hex-2-ulopyranose (tagatose),

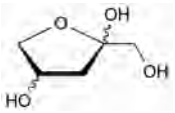

F. (2Ξ,4Ξ)-2-(hydroxymethyl)oxolane-2,4-diol,

G. unknown structure,

H. unknown structure.

**01/2017:2217**

# LAMIVUDINE

## Lamivudinum

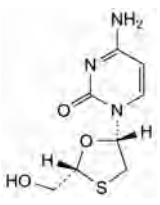

$C_8H_{11}N_3O_3S$ $M_r$ 229.3

[134678-17-4]

DEFINITION

4-Amino-1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B.*

A. Specific optical rotation (*2.2.7*): – 99 to – 97 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lamivudine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Enantiomeric purity (see Tests).

TESTS

**Absorbance** (*2.2.25*): maximum 0.3 at 440 nm, using a path length of 4 cm.

Dissolve 1.00 g in *water R*, using sonication if necessary, and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *salicylic acid R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *lamivudine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *cytosine R* and 5 mg of *uracil R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 5 mg of *lamivudine for system suitability 1 CRS* (containing impurities A and B) in 2 mL of the mobile phase. Add 1.0 mL of reference solution (d) and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 5 volumes of *methanol R* and 95 volumes of a 1.9 g/L solution of *ammonium acetate R*, previously adjusted to pH 3.8 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 277 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of lamivudine.

*Identification of impurities*: use the chromatograms obtained with reference solutions (b) and (e) to identify the peaks due to impurities A, B, E, F and C.

*Relative retention* with reference to lamivudine (retention time = about 9 min): impurity E = about 0.28; impurity F = about 0.32; impurity A = about 0.36; impurity B = about 0.91; impurity J = about 1.45; impurity C = about 2.32.

*System suitability*: reference solution (e):

- *resolution*: minimum 1.5 between the peaks due to impurities F and A; minimum 1.5 between the peaks due to impurity B and lamivudine.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.6; impurity F = 2.2; impurity J = 2.2;
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution.* Dissolve the contents of a vial of *lamivudine for system suitability 2 CRS* (containing impurity D) in 1.0 mL of *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel BC for chiral chromatography R* (5 µm);
- *temperature*: maintain at constant temperature between 15 °C and 30 °C; the temperature may be adjusted to optimise the resolution between lamivudine and impurity D; a lower temperature favours improved resolution.

*Mobile phase*: mix 5 volumes of *methanol R* and 95 volumes of a 7.7 g/L solution of *ammonium acetate R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of lamivudine.

*Relative retention* with reference to lamivudine (retention time = about 8 min): impurity D = about 1.2; impurity B and enantiomer = about 1.3 and 1.5.

*System suitability*: reference solution:

- *peak-to-valley-ratio*: minimum 15, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lamivudine.

Calculate the sum of the percentage contents of all impurity peaks with a relative retention from 1.2 to 1.5. Subtract the percentage content of impurity B as obtained in the test for related substances.

*Limit*:

– *impurity D*: maximum 0.3 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_8H_{11}N_3O_3S$ using the chromatograms obtained with the test solution and reference solution (c) and the declared content of $C_8H_{11}N_3O_3S$ in *lamivudine CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G, H, I, J.*

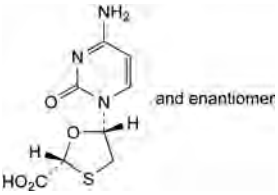


A. (2*RS*,5*SR*)-5-(4-amino-2-oxopyrimidin-1(2*H*)-yl)-1,3-oxathiolane-2-carboxylic acid,

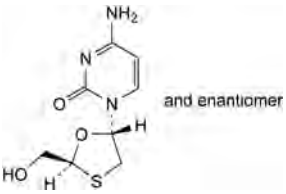


B. 4-amino-1-[(2*RS*,5*RS*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one ((±)-*trans*-lamivudine),

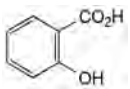


C. 2-hydroxybenzenecarboxylic acid (salicylic acid),

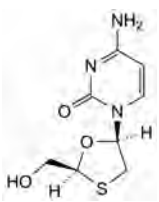


D. 4-amino-1-[(2*S*,5*R*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one,

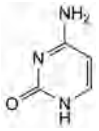


E. 4-aminopyrimidin-2(1*H*)-one (cytosine),

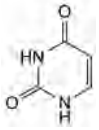


F. pyrimidine-2,4(1*H*,3*H*)-dione (uracil),

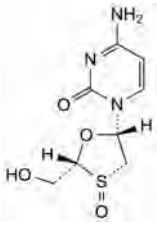


G. 4-amino-1-[(2*R*,3*S*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one *S*-oxide,

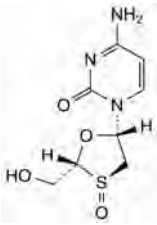


H. 4-amino-1-[(2*R*,3*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidin-2(1*H*)-one *S*-oxide,

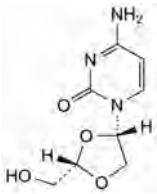


I. 4-amino-1-[(2*S*,4*S*)-2-(hydroxymethyl)-1,3-dioxolan-4-yl]pyrimidin-2(1*H*)-one,

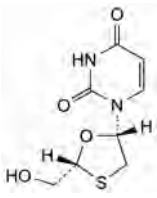


J. 1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidine-2,4(1*H*,3*H*)-dione.

**01/2017:1756**

# LAMOTRIGINE

## Lamotriginum

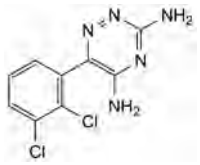

$C_9H_7Cl_2N_5$ $M_r$ 256.1
[84057-84-1]

DEFINITION

6-(2,3-Dichlorophenyl)-1,2,4-triazine-3,5-diamine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, slightly soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lamotrigine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (a).* Dissolve 5 mg of *lamotrigine for system suitability CRS* (containing impurity G) in 2.5 mL of *methanol R* and dilute to 25.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. Dilute 2.0 mL of this solution to 10.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (c).* Dissolve 5.0 mg of *lamotrigine impurity E CRS* in a mixture of 0.25 mL of *hydrochloric acid R* and 45 mL of *methanol R* and dilute to 50.0 mL with *methanol R*. Dilute 5.0 mL of the solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. To 4.0 mL of this solution add 5 mL of *methanol R* and dilute to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (d).* Dissolve 10 mg of *lamotrigine for peak identification CRS* (containing impurities A, E and F) in 2.5 mL of *methanol R* and dilute to 50.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Blank solution.* Mix 5 volumes of *methanol R* and 95 volumes of a 10.3 g/L solution of *hydrochloric acid R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 1 volume of *triethylamine R* and 150 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R*; adjust to pH 2.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 85 | 15 |
| 4 - 14 | 85 → 20 | 15 → 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL of the test solution, reference solutions (a), (b) and (d) and the blank solution.

*Identification of impurities*: use the chromatogram supplied with *lamotrigine for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, E and F; use the chromatogram supplied with *lamotrigine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity G.

*Relative retention* with reference to lamotrigine (retention time = about 7 min): impurity G = about 1.1; impurity A = about 1.3; impurity E = about 1.7; impurity F = about 1.8.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lamotrigine.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 1.3;
- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities A, G*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to impurity E.

**Impurity E**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *acetonitrile for chromatography R*, mobile phase A (35:65 *V/V*).

*Detection*: spectrophotometer at 210 nm.

*Injection*: test solution and reference solutions (d) and (c).

*Run time*: 10 min.

*Retention time*: impurity E = about 5.5 min; impurity F = about 8.5 min.

*System suitability*: reference solution (d):

- the chromatogram obtained is similar to the chromatogram supplied with *lamotrigine for peak identification CRS*.

*Limit*:

- *impurity E*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 2.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.200 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 25.61 mg of $C_9H_7Cl_2N_5$.

IMPURITIES

*Specified impurities: A, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

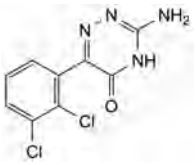

A. 3-amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-5(4*H*)-one,

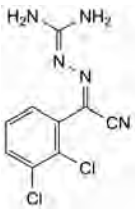

B. (2*E*)-[2-(diaminomethylidene)diazanylidene](2,3-dichlorophenyl)acetonitrile,

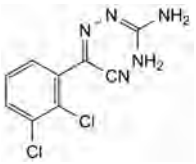

C. (2*Z*)-[2-(diaminomethylidene)diazanylidene](2,3-dichlorophenyl)acetonitrile,

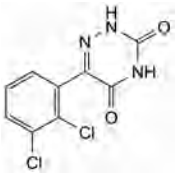

D. 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione,

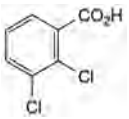

E. 2,3-dichlorobenzoic acid,

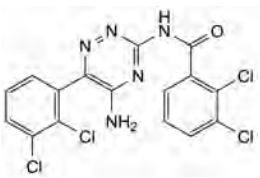

F. *N*-[5-amino-6-(2,3-dichlorophenyl)-1,2,4-triazin-3-yl]-2,3-dichlorobenzamide,

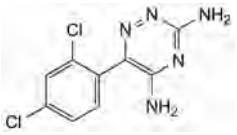

G. 6-(2,4-dichlorophenyl)-1,2,4-triazine-3,5-diamine.

**01/2010:2219**
**corrected 9.3**

# LANSOPRAZOLE

## Lansoprazolum

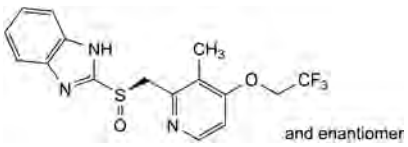


$C_{16}H_{14}F_3N_3O_2S$ $M_r$ 369.4
[103577-45-3]

DEFINITION

2-[(*RS*)-[[3-Methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl]methyl]sulfinyl]-1*H*-benzimidazole.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or brownish powder.

*Solubility*: practically insoluble in water, soluble in anhydrous ethanol, very slightly soluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lansoprazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_2$ or $BY_2$ (*2.2.2, Method II*).

Dissolve 1.0 g in *dimethylformamide R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect them from light.*

*Solvent mixture*: mix 1 volume of *triethylamine R* and 60 volumes of *water R* and adjust to pH 10.5 with *phosphoric acid R*; mix this solution with 40 volumes of *acetonitrile R1*.

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *lansoprazole for peak identification CRS* (containing impurities A and B) in 1.0 mL of the solvent mixture.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *2-hydroxybenzimidazole R* (impurity D) and 5 mg of *2-mercaptobenzimidazole R* (impurity E) in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of this solution to 10 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 1 volume of *triethylamine R* and 60 volumes of *water R* and adjust to pH 6.2 with *phosphoric acid R*; mix this solution with 40 volumes of *acetonitrile R1*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of lansoprazole.

*Identification of impurities*: use the chromatogram supplied with *lansoprazole for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B; use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and E.

*Relative retention* with reference to lansoprazole (retention time = about 7 min): impurity D = about 0.4; impurity A = about 0.5; impurity E = about 0.6; impurity B = about 1.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to lansoprazole and impurity B.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity E by 0.4;
– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
– *impurities A, D, E*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 0.150-0.200 g using the evaporation technique:

– *temperature*: 50-70 °C;
– *heating time*: 15 min;
– *flow rate*: 150 mL/min.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 40 mL of *ethanol (96 per cent) R* and dilute to 50 mL with *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.94 mg of $C_{16}H_{14}F_3N_3O_2S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, F.*

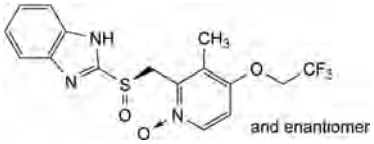


A. 2-[(*RS*)-[[3-methyl-1-oxido-4-(2,2,2-trifluoroethoxy)-pyridin-2-yl]methyl]sulfinyl]-1*H*-benzimidazole,

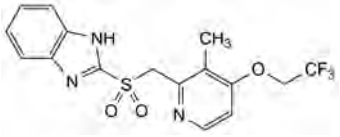


B. 2-[[[3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl]methyl]sulfonyl]-1*H*-benzimidazole,

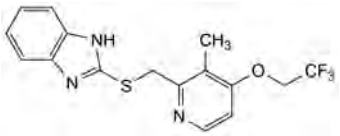


C. 2-[[[3-methyl-4-(2,2,2-trifluoroethoxy)pyridin-2-yl]methyl]sulfanyl]-1*H*-benzimidazole,

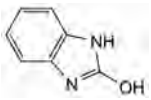


D. 1*H*-benzimidazol-2-ol,

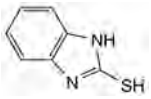


E. 1*H*-benzimidazole-2-thiol,

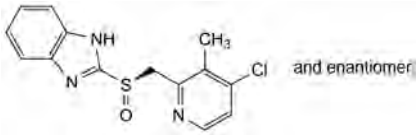


F. 2-[(*RS*)-[(4-chloro-3-methylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole.

**01/2020:2046**

# LAUROMACROGOL 400

## Lauromacrogolum 400

DEFINITION

Mixture of lauryl alcohol (dodecanol) monoethers of mixed macrogols. It may contain some free macrogols and it contains various amounts of free lauryl alcohol. The number of moles of ethylene oxide reacted per mole of lauryl alcohol is 9. The name of the substance is followed by a number (400) corresponding approximately to the average molecular mass of the macrogol portion.

This monograph applies to lauromacrogol 400 used as active substance.

CHARACTERS

*Appearance*: white or almost white, unctuous and hygroscopic mass, melting at 24 °C into a colourless or yellowish, viscous liquid.

*Solubility*: freely soluble in water, very soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Saponification value (see Tests).

C. Warm the substance to be examined in an incubator at 50 °C for 1 h until fully molten and clear. Transfer 50 mL to a warmed cloud-point tube (flat-bottomed glass tube 30-33.5 mm in internal diameter and 115-125 mm high). Insert the tube into a cooling bath that allows the outer surface of the tube to be in contact with chilled air, contained within a cylindrical metal container (internal diameter 9.5-12.5 mm greater than the external diameter of the sample tube, 115 mm high) that is surrounded by iced water. The base of the glass tube rests on a 6 mm thick cork disc, which prevents direct thermal contact with the cooled metal cylinder. Stir the substance to be examined continuously with a thermometer so that the temperature is constant throughout the substance. Periodically lift the tube out of the cooling bath to check for signs of cloudiness at the bottom of the tube. Examine the tube against a bright light source. When cloudiness is first observed, check more frequently until the substance becomes completely cloudy and the thermometer, suspended in the centre of the substance, is only just visible when viewed horizontally. Record the temperature. It is 20 °C to 25 °C.

TESTS

**Appearance**. The molten substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method I*).

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *carbon dioxide-free water R* and 10 mL of *ethanol (96 per cent) R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 90 to 105, determined on 2.0 g.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Peroxide value**: maximum 5.0.

Introduce 10.0 g into a 100 mL beaker, dissolve with *glacial acetic acid R* and dilute to 20 mL with the same solvent. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R*. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution, in moles per litre;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): maximum 3.0.

**Free lauryl alcohol (dodecanol)**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 2.00 g of *lauryl alcohol R* in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *acetone R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 50:1.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 120 |
| | 1 - 23 | 120 → 350 |
| | 23 - 33 | 350 |
| Injection port | | 300 |
| Detector | | 350 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Retention time*: lauryl alcohol = about 5 min.

*Limit*:

- *free lauryl alcohol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (2.0 per cent).

**Free macrogols**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dissolve 5.0 g of the substance to be examined in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve about 0.4 g of *macrogol 1000 R* in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 50.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Precolumns* (2):

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydroxylated polymethacrylate gel R* (6 µm) with a pore size of 12 nm.

Connect both precolumns to the column using a 3-way valve and switch the mobile phase flow according to the following programme:

- 0-114 s: precolumn 1 and column;
- 115 s to the end: precolumn 2 and column;
- 115 s to 8 min: flow back of precolumn 1.

*Mobile phase*: *water R*, *methanol R* (2:8 *V/V*).

*Flow rate*: 1.1 mL/min.

*Detection*: refractometer.

*Injection*: 20 µL.

Calculate the percentage content of free macrogols using the following expression:

$$\frac{A_1 \times m_2 \times 200}{m_1 \times (A_2 + 2A_3)}$$

$m_1$ = mass of the substance to be examined in the test solution, in grams;

$m_2$ = mass of *macrogol 1000 R* in reference solution (a), in grams;

$A_1$ = area of the peak due to free macrogols in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to macrogol 1000 in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to macrogol 1000 in the chromatogram obtained with reference solution (b).

*Limit*:

– *free macrogols*: maximum 3.0 per cent.

**Average chain length of the fatty alcohol and average number of moles of ethylene oxide**. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Test solution.* If the substance is in the solid state at room temperature, heat gently before sampling. Dissolve 0.4 mL of the substance to be examined in 0.3 mL of a mixture of 1 volume of *deuterated methanol R* and 2 volumes of *deuterated chloroform R*, containing 0.1 mol/L of *chromium(III) acetylacetonate R* as a relaxation aid.

*Apparatus*: high resolution FT-NMR spectrometer operating at minimum 300 MHz.

*Acquisition of $^{13}C$ NMR spectra.* The following parameters may be used:

– *sweep width*: 250 ppm (– 15 ppm to 235 ppm);
– *irradiation frequency offset*: 110 ppm;
– *time domain*: 64 K;
– *pulse delay*: 3 s;
– *pulse program*: zgig 30 (inverse gated, 30° excitation pulse);
– *dummy scans*: 4;
– *number of scans*: 2048.

*Processing and plotting.* The following parameters may be used:

– *size*: 64 K (zero-filling);
– *window multiplication*: exponential;
– *Lorentzian broadening factor*: 1 Hz.

Use the $CD_3OD$ signal for shift referencing. The shift of the central peak of the multiplet is set to 49.0 ppm.

Plot the spectral region δ 0.0-80.0 ppm. Compare the spectrum with the spectrum in Figure 2046.-1. The shift values lie near the values given in Table 2046.-1.

Table 2046.-1. – *Shift values*

| Signal | Shift (ppm) | Normalised integrals |
|---|---|---|
| $CH_3$ | 14.4 | 0.989 |
| $CH_2$ (alkyl chain) | 23.2 | 1.000 |
| $CH_2$ (alkyl chain) | 25.5 | 1.001 |
| $CH_2$'s (alkyl chain) | 30 | 7.410 |
| $CH_2$ (alkyl chain) | 32.5 | 0.963 |
| $CH_2$ (-$CH_2$-OH) (end $CH_2$-group of macrogol) | 61.6 | 1.001 |
| $CH_2$'s (macrogol) | 70.7 | 16.25 |
| $CH_2$ (R-CH-O-macrogol) ($CH_2$ in alpha position) | 72.6 | 0.998 |
| $CH_2$ (macrogol) | 73.1 | 0.929 |

*System suitability*:

– *signal-to-noise ratio*: minimum 150, for the smallest relevant peak ($CH_2$ at 73.1 ppm);
– *peak width at half-height*: maximum 0.05 ppm, for the central $CDCl_3$ signal (at δ 78.6 ppm).

*Calculation of the average chain length of the fatty alcohol and the average number of moles of ethylene oxide*: define the signal at 23.2 ppm as 1.000 and normalise the integrals of the other signals listed in Table 2046.-1.

The average chain length of the fatty alcohol is calculated using the following expression:

$$\Sigma_{14-33} I_{n,i} + I_{n,72.6}$$

$\Sigma_{14-33} I_{n,i}$ = sum of the normalised integrals of the signals from 14 ppm to 33 ppm;

$I_{n,72.6}$ = normalised integral of the signal at 72.6 ppm.

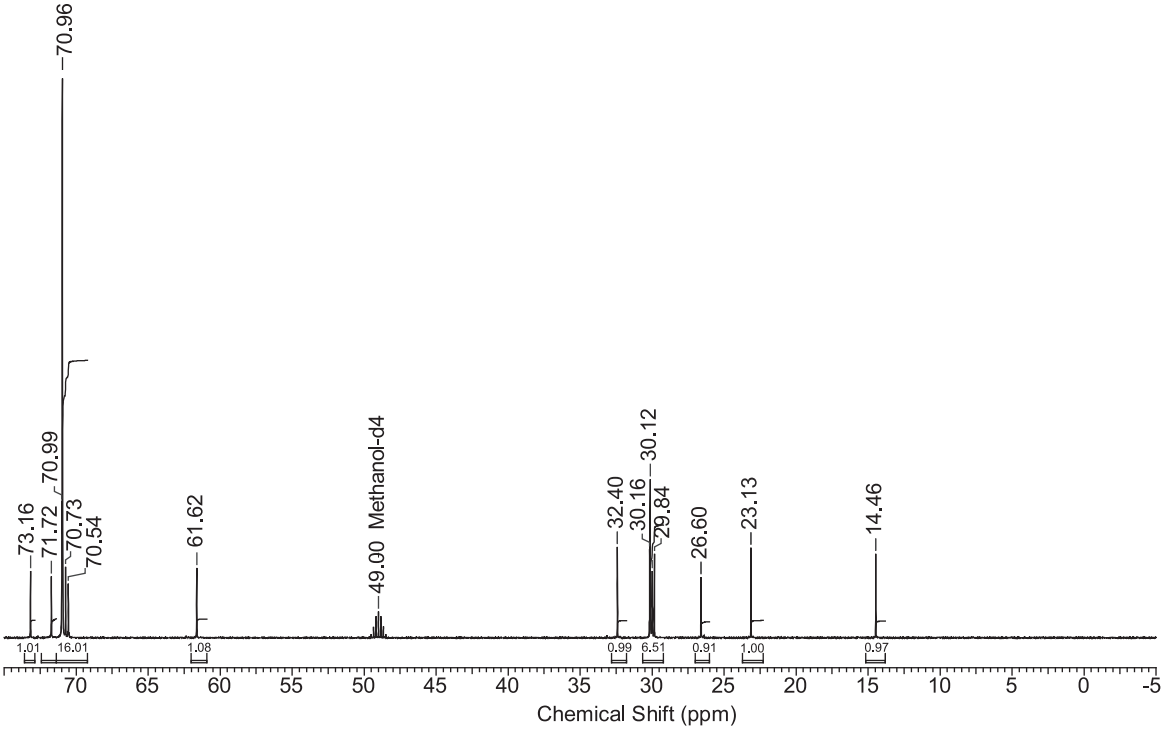


Figure 2046.-1. – *$^{13}C$ NMR spectrum of lauromacrogol 400*

The average number of moles of ethylene oxide is calculated using the following expression:

$$0.5 \times (I_{n,62} + I_{n,71} + I_{n,73})$$

$I_{n,62}$, $I_{n,71}$, $I_{n,73}$ = normalised integral of the signals at 62 ppm, 71 ppm and 73 ppm respectively.

The sum of the normalised integrals of the signals at 62 ppm, 71 ppm and 73 ppm corresponds to the average number of methylene groups in the macrogol part of lauromacrogol 400.

*Limits*:

- *average chain length of the fatty alcohol*: 10.0 to 14.0;
- *average number of moles of ethylene oxide*: 7.0 to 11.0.

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent, determined on 2.0 g.

**04/2017:1231**

# LAUROYL MACROGOLGLYCERIDES

## Macrogolglyceridorum laurates

DEFINITION

Mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 300 and 1500.

They are obtained by partial alcoholysis of saturated oils mainly containing triglycerides of lauric (dodecanoic) acid, using macrogol, or by esterification of glycerol and macrogol with saturated fatty acids, or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of these hydrogenated oils.

CHARACTERS

*Appearance*: pale yellow waxy solid.

*Solubility*: dispersible in hot water, freely soluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.9 ($R_{st}$ 1) and spots due to 1,3-diglycerides ($R_{st}$ 0.7), to 1,2-diglycerides ($R_{st}$ 0.6), to monoglycerides ($R_{st}$ 0.1) and to esters of macrogol ($R_{st}$ 0).

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Drop point** (*2.2.17*). Introduce into the cup the substance to be examined, which has been melted by heating for 1 h in an oven at 100 ± 2 °C, and allow to stand for 5 h at about 5 °C.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Drop point |
|---|---|---|
| 6 | 300 | 33 - 38 |
| 8 | 400 | 36 - 41 |
| 12 | 600 | 38 - 43 |
| 32 | 1500 | 42.5 - 47.5 |

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*). Use 1.0 g.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Hydroxyl value |
|---|---|---|
| 6 | 300 | 65 - 85 |
| 8 | 400 | 60 - 80 |
| 12 | 600 | 50 - 70 |
| 32 | 1500 | 36 - 56 |

**Peroxide value** (*2.5.5, Method A*): maximum 6.0, determined on 2.0 g.

**Saponification value** (*2.5.6*). Use 2.0 g.

| Ethylene oxide units per molecule (nominal value) | Type of macrogol | Saponification value |
|---|---|---|
| 6 | 300 | 190 - 204 |
| 8 | 400 | 170 - 190 |
| 12 | 600 | 150 - 170 |
| 32 | 1500 | 79 - 93 |

**Alkaline impurities**. Introduce 5.0 g into a test tube and carefully add a mixture, neutralised if necessary with *0.01 M hydrochloric acid* or with *0.01 M sodium hydroxide*, of 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*, 0.3 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Shake and allow to stand. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

**Free glycerol**: maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat if necessary. After cooling, add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate the iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

- *caprylic acid*: maximum 15.0 per cent;
- *capric acid*: maximum 12.0 per cent;
- *lauric acid*: 30.0 per cent to 50.0 per cent;
- *myristic acid*: 5.0 per cent to 25.0 per cent;
- *palmitic acid*: 4.0 per cent to 25.0 per cent;
- *stearic acid*: 5.0 per cent to 35.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for lauroyl macrogolglycerides used as self-emulsifying agents, solubilisers, modified-release agents and wetting agents for powders and tablets.*

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**Composition of fatty acids** (see Tests).

**01/2017:2330**

# LEFLUNOMIDE

## Leflunomidum

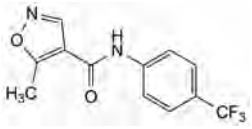

$C_{12}H_9F_3N_2O_2$ $M_r$ 270.2

DEFINITION

5-Methyl-*N*-[4-(trifluoromethyl)phenyl]isoxazole-4-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol, sparingly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: heat the substance to be examined and the reference substance at 130 °C for 10 min.

*Comparison*: *leflunomide CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Store all solutions protected from light.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 5 mL of *acetonitrile for chromatography R* and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dissolve 0.125 g of the substance to be examined in 5 mL of *acetonitrile for chromatography R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 12.5 mg of *leflunomide impurity A CRS* in 5 mL of *acetonitrile for chromatography R* and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25.0 mg of *leflunomide CRS* in 5 mL of *acetonitrile for chromatography R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (d).* Dissolve the contents of 1 vial of *leflunomide for peak identification CRS* (containing impurities B and C) in 2.0 mL of the mobile phase and sonicate for 10 min.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 5 volumes of *triethylamine R* with 650 volumes of *water for chromatography R*, adjust to pH 3.4 ± 0.1 with *phosphoric acid R* and add 350 volumes of *acetonitrile for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of test solutions (a) and (b) and reference solutions (a), (b) and (d).

*Run time*: twice the retention time of leflunomide.

*Identification of impurities*: use the chromatogram supplied with *leflunomide for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and C.

*Relative retention* with reference to leflunomide (retention time = about 25 min): impurity B = about 0.2; impurity A = about 0.4; impurity C = about 0.9.

*System suitability*: reference solution (d):

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to leflunomide.

*Limits*: test solution (a):

– *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *sum of impurities C and E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities other than B*: not more twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

*Limit*: test solution (b):

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.3 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (c).

Calculate the percentage content of $C_{12}H_9F_3N_2O_2$ from the declared content of *leflunomide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G, H.*

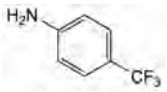

A. 4-(trifluoromethyl)aniline,

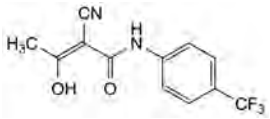

B. (2*Z*)-2-cyano-3-hydroxy-*N*-[4-(trifluoromethyl)phenyl]-but-2-enamide (teriflunomide),

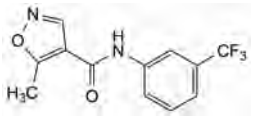

C. 5-methyl-*N*-[3-(trifluoromethyl)phenyl]isoxazole-4-carboxamide,

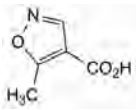

D. 5-methylisoxazole-4-carboxylic acid,

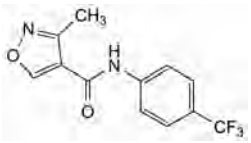

E. 3-methyl-*N*-[4-(trifluoromethyl)phenyl]isoxazole-4-carboxamide,

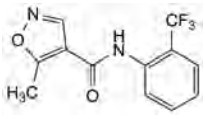

F. 5-methyl-*N*-[2-(trifluoromethyl)phenyl]isoxazole-4-carboxamide,

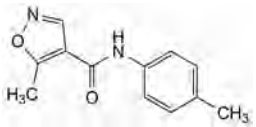

G. 5-methyl-*N*-(4-methylphenyl)isoxazole-4-carboxamide,

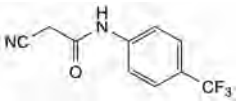

H. 2-cyano-*N*-[4-(trifluoromethyl)phenyl]acetamide.

**01/2008:2334**

# LETROZOLE

## Letrozolum

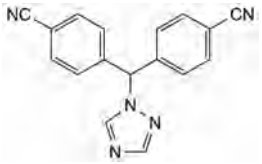

$C_{17}H_{11}N_5$ $M_r$ 285.3

[112809-51-5]

DEFINITION

4,4′-(1*H*-1,2,4-Triazol-1-ylmethylene)dibenzonitrile.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *letrozole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 15 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Test solution (b).* To 2.0 mL of test solution (a) add 30 mL of *acetonitrile R1* and dilute to 100.0 mL with *water R*.

*Reference solution (a).* Dissolve 5.0 mg of *letrozole CRS* (containing impurities A and B) in 3 mL of *acetonitrile R1* and dilute to 10.0 mL with *water R*.

*Reference solution (b).* To 2.0 mL of test solution (a) add 30 mL of *acetonitrile R1* and dilute to 100.0 mL with *water R*. To 1.0 mL of this solution add 6 mL of *acetonitrile R1* and dilute to 20.0 mL with *water R*.

*Reference solution (c).* Dissolve 25.0 mg of *letrozole CRS* in 15 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*. To 2.0 mL of this solution add 30 mL of *acetonitrile R1* and dilute to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 70 | 30 |
| 4 - 29 | 70 → 30 | 30 → 70 |
| 29 - 30 | 30 → 70 | 70 → 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Relative retention* with reference to letrozole (retention time = about 13 min): impurity A = about 0.6; impurity B = about 1.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 5 between the peaks due to impurity A and letrozole;
- the chromatogram obtained is similar to the chromatogram supplied with *letrozole CRS*.

*Limits*:

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.3 per cent, determined on 1.000 g. Use a validated pyridine-free iodosulfurous reagent.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (c).

*System suitability*: reference solution (c):

- *symmetry factor*: maximum 1.7 for the peak due to letrozole.

Calculate the percentage content of $C_{17}H_{11}N_5$ from the declared content of *letrozole CRS*.

IMPURITIES

*Specified impurities: A, B.*

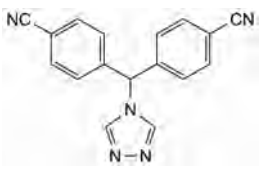

A. 4,4′-(4*H*-1,2,4-triazol-4-ylmethylene)dibenzonitrile,

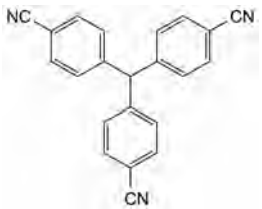

B. 4,4′,4″-methanetriyltribenzonitrile.

**01/2017:0771**

# LEUCINE

## Leucinum

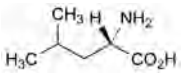

$C_6H_{13}NO_2$ $M_r$ 131.2

[61-90-5]

DEFINITION

(2*S*)-2-Amino-4-methylpentanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or shiny flakes.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*)

*Comparison*: *leucine CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.

*Reference solution.* Dissolve 10 mg of *leucine CRS* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

**Specific optical rotation** (*2.2.7*): + 14.5 to + 16.5 (dried substance).

Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solutions and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are

adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 25.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *isoleucine R* (impurity A) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (d)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (e)*. Dissolve 30 mg of *isoleucine R* (impurity A) and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test solutions, blank solution and reference solutions (a), (b), (c) and (e) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (e):

– *resolution*: minimum 1.5 between the peaks due to impurity A and leucine.

*Calculation of percentage contents*:

– for impurity A in test solution (b), use the concentration of impurity A in reference solution (b);
– for any ninhydrin-positive substance detected at 570 nm in test solution (a), use the concentration of leucine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm in test solution (a), use the concentration of proline in reference solution (c); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *impurity A at 570 nm*: maximum 0.8 per cent;
– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in *water R* and dilute to 15 mL with the same solvent.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in 3 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution (a), reference solution (d) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.12 mg of $C_6H_{13}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E*.

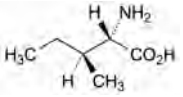

A. (2*S*,3*S*)-2-amino-3-methylpentanoic acid (isoleucine),

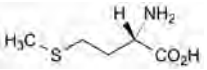

B. (2*S*)-2-amino-4-(methylsulfanyl)butanoic acid (methionine),

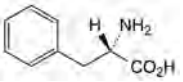

C. (2*S*)-2-amino-3-phenylpropanoic acid (phenylalanine),

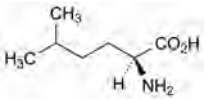

D. (2*S*)-2-amino-5-methylhexanoic acid (5-methyl-norleucine),

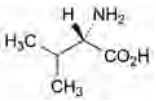

E. (2*S*)-2-amino-3-methylbutanoic acid (valine).

**01/2008:1442**
**corrected 10.0**

# LEUPRORELIN

## Leuprorelinum

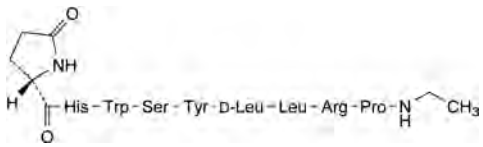


$C_{59}H_{84}N_{16}O_{12}$ $M_r$ 1209
[53714-56-0]

DEFINITION

5-Oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-*N*-ethyl-L-prolinamide.

Synthetic nonapeptide analogue of the hypothalamic peptide, gonadorelin. It is obtained by chemical synthesis and is available as an acetate.

*Content*: 97.0 per cent to 103.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: hygroscopic, white or almost white powder.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *Ph. Eur. reference spectrum of leuprorelin*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (b).

C. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.
Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking one seventh of the sum of the number of moles of histidine, glutamic acid, leucine, proline, tyrosine and arginine as equal to 1. The values fall within the following limits: serine present; glutamic acid = 0.85 to 1.1; proline = 0.85 to 1.1; leucine = 1.8 to 2.2; tyrosine = 0.85 to 1.1; histidine = 0.85 to 1.1 and arginine = 0.85 to 1.1. Not more than traces of other amino acids are present, with the exception of tryptophan.

TESTS

**Specific optical rotation** (*2.2.7*): − 38.0 to − 42.0 (anhydrous and acetic acid-free substance).

Dissolve the substance to be examined in a 1 per cent *V/V* solution of *glacial acetic acid R* to obtain a concentration of 10.0 mg/mL.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution (a).* Dissolve the substance to be examined in the mobile phase to obtain a concentration of 1.0 mg/mL.

*Test solution (b).* Dilute 0.5 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve *leuprorelin CRS* in the mobile phase to obtain a concentration of 1.0 mg/mL.

*Reference solution (b).* Dilute 0.5 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Resolution solution.* Dilute 5.0 mL of reference solution (a) to 50.0 mL with *water R*. To 5 mL of the solution add 100 µL of *1 M sodium hydroxide* and shake vigorously. Heat in an oven at 100 °C for 60 min, cool immediately and add 50 µL of *dilute phosphoric acid R*. Shake vigorously.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve about 15.2 g of *triethylamine R* in 800 mL of *water R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water R*. Add 850 mL of this solution to 150 mL of a mixture of 2 volumes of *propanol R* and 3 volumes of *acetonitrile R*.

*Flow rate*: 1.0-1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and the resolution solution.

*Run time*: 90 min.

*Relative retention* with reference to leuprorelin (retention time = 41-49 min): impurity E = about 0.7; impurity F = about 0.7; impurity H = about 0.78; impurity A = about 0.8; impurity B = about 0.9; impurity I = about 0.94; impurity J = about 1.09; impurity C = about 1.2; impurity G = about 1.3; impurity K = about 1.31; impurity D = about 1.5.

*System suitability*: resolution solution:
- *resolution*: minimum 1.5 between the peaks due to impurity B and leuprorelin.

*Limits*:
- *impurity D*: maximum 1.0 per cent;
- *impurities A, B, C*: for each impurity, maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.5 per cent;
- *total*: maximum 2.5 per cent;
- *disregard limit*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 4.7 per cent to 9.0 per cent.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 5.0 per cent.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent.

**Bacterial endotoxins** (*2.6.14, Method D*): less than 16.7 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Run time*: 60 min.

*Injection*: 20 µL of test solution (b) and reference solution (b).

Calculate the content of leuprorelin ($C_{59}H_{84}N_{16}O_{12}$) using the areas of the peaks and the declared content of $C_{59}H_{84}N_{16}O_{12}$ in *leuprorelin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 30 °C.

If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the mass of peptide in the container.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G, H, I, J, K.*

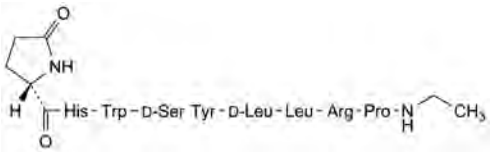


A. [4-D-serine]leuprorelin,

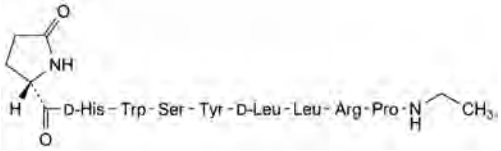


B. [2-D-histidine]leuprorelin,

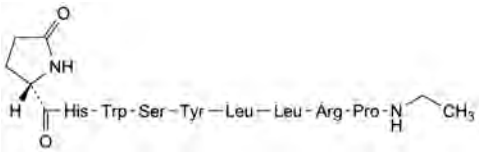


C. [6-L-leucine]leuprorelin,

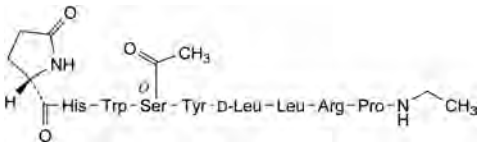


D. [4-(*O*-acetyl-L-serine)]leuprorelin,

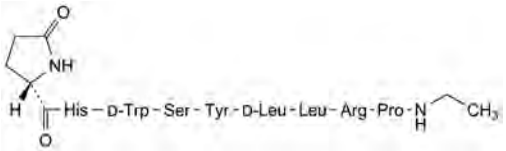


E. [3-D-tryptophane]leuprorelin,

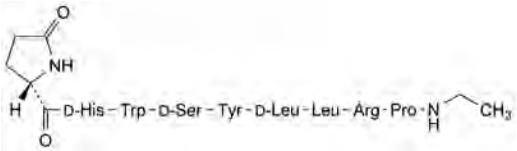


F. [2-D-histidine,4-D-serine]leuprorelin,

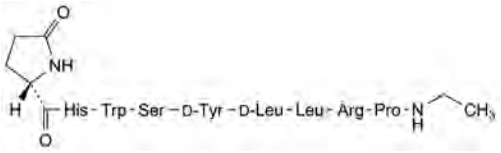


G. [5-D-tyrosine]leuprorelin,

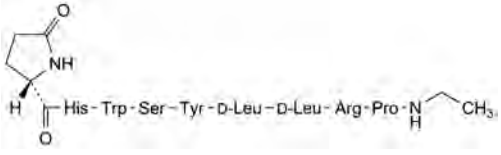


H. [7-D-leucine]leuprorelin,

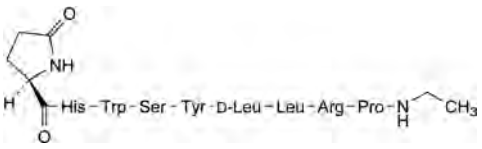


I. [1-(5-oxo-D-proline)]leuprorelin,

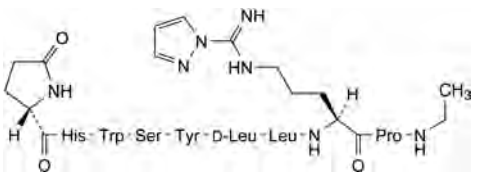


J. [8-[5-*N*-[imino(1*H*-pyrazol-1-yl)methyl]-L-ornithine]]leuprorelin,

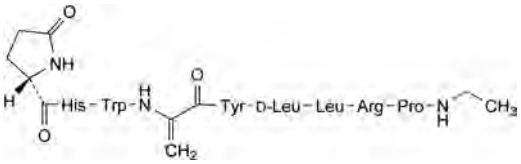


K. [4-dehydroalanine]leuprorelin.

**04/2015:1728**

# LEVAMISOLE FOR VETERINARY USE

## Levamisolum ad usum veterinarium

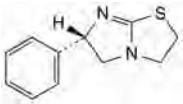

$C_{11}H_{12}N_2S$ $M_r$ 204.3
[14769-73-4]

DEFINITION

(6*S*)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of levamisole.*

If the spectra show differences, dissolve the substance to be examined in *methylene chloride R*, evaporate to dryness and record a new spectrum using the residue.

TESTS

**Solution S**. Dissolve 2.50 g in *ethanol R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 89 to − 85 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, protect from light and keep below 25 °C.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *levamisole hydrochloride for system suitability CRS* (containing impurities A, B, C, D and E) in *methanol R*, add 0.1 mL of *concentrated ammonia R* and dilute to 1.0 mL with *methanol R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of the solution to 25.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 0.5 g of *ammonium dihydrogen phosphate R* in 90 mL of *water R*; adjust to pH 6.5 with a 40 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 90 → 30 | 10 → 70 |
| 8 - 10 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *levamisole hydrochloride for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to levamisole (retention time = about 3 min): impurity A = about 0.9; impurity B = about 1.4; impurity C = about 1.5; impurity D = about 1.6; impurity E = about 2.0.

*System suitability*: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *levamisole hydrochloride for system suitability CRS*.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.0; impurity B = 1.7; impurity C = 2.9; impurity D = 1.3; impurity E = 2.7;
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *any other impurity*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 20.43 mg of $C_{11}H_{12}N_2S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

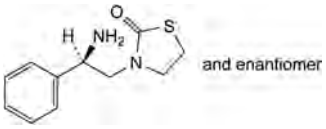


A. 3-[(2*RS*)-2-amino-2-phenylethyl]thiazolidin-2-one,

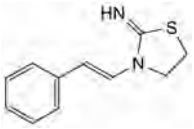


B. 3-[(*E*)-2-phenylethenyl]thiazolidin-2-imine,

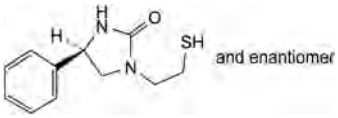


C. (4*RS*)-4-phenyl-1-(2-sulfanylethyl)imidazolidin-2-one,

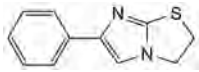


D. 6-phenyl-2,3-dihydroimidazo[2,1-*b*]thiazole,

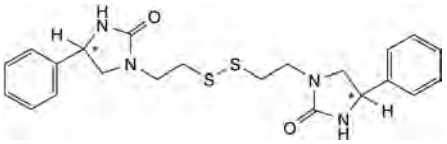


E. 1,1′-[(disulfane-1,2-diyl)bis(ethylene)]bis[(4*RS*)-4-phenylimidazolidin-2-one].

**01/2017:0726**

# LEVAMISOLE HYDROCHLORIDE

## Levamisoli hydrochloridum

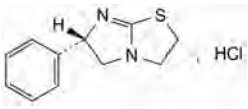


$C_{11}H_{13}ClN_2S$ $M_r$ 240.8
[16595-80-5]

DEFINITION

(6*S*)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levamisole hydrochloride CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 4.5 for solution S.

**Specific optical rotation** (*2.2.7*): − 128 to − 121 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use, protect from light and keep below 25 °C.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R*, add 1.0 mL of *concentrated ammonia R* and dilute to 10.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 10 mg of *levamisole hydrochloride for system suitability CRS* (containing impurities A, B, C, D and E) in *methanol R*, add 0.1 mL of *concentrated ammonia R* and dilute to 1.0 mL with *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 25.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 0.5 g of *ammonium dihydrogen phosphate R* in 90 mL of *water R*, adjust to pH 6.5 with a 40 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 90 → 30 | 10 → 70 |
| 8 - 10 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Equilibration*: at least 4 min with the mobile phase at the initial composition.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *levamisole hydrochloride for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to levamisole (retention time = about 3 min): impurity A = about 0.9; impurity B = about 1.4; impurity C = about 1.5; impurity D = about 1.6; impurity E = about 2.0.

*System suitability*:
- the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *levamisole hydrochloride for system suitability CRS*;
- *symmetry factor*: maximum 3.5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.0; impurity B = 1.7; impurity C = 2.9; impurity D = 1.3; impurity E = 2.7;
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.08 mg of $C_{11}H_{13}ClN_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

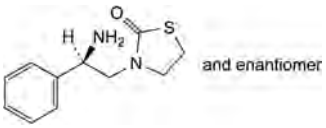


A. 3-[(2*RS*)-2-amino-2-phenylethyl]thiazolidin-2-one,

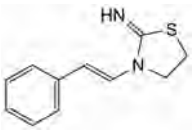


B. 3-[(*E*)-2-phenylethenyl]thiazolidin-2-imine,

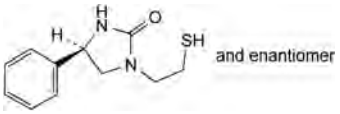


C. (4*RS*)-4-phenyl-1-(2-sulfanylethyl)imidazolidin-2-one,

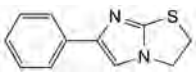


D. 6-phenyl-2,3-dihydroimidazo[2,1-*b*]thiazole,

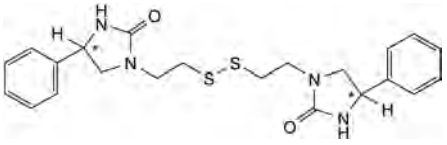


E. 1,1′-[(disulfane-1,2-diyl)bis(ethylene)]bis[(4*RS*)-4-phenylimidazolidin-2-one].

**04/2018:2535**

# LEVETIRACETAM

## Levetiracetamum

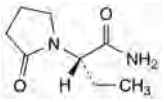

$C_8H_{14}N_2O_2$ $M_r$ 170.2
[102767-28-2]

DEFINITION

(2*S*)-2-(2-Oxopyrrolidin-1-yl)butanamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, soluble in acetonitrile, practically insoluble in heptane.

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): − 82 to − 76.
Dissolve 0.500 g in *water R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levetiracetam CRS*.

C. Enantiomeric purity (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10.0 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 0.200 g of the substance to be examined in *2-propanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of the substance to be examined and 5 mg of *levetiracetam impurity D CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *2-propanol R*, *heptane R* (18:82 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Run time*: 1.4 times the retention time of levetiracetam.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to levetiracetam (retention time = about 12 min): impurity D = about 0.8.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity D and levetiracetam;
- *symmetry factor*: maximum 2.4 for the peak due to levetiracetam.

*Limit*:
- *impurity D*: maximum 0.8 per cent.
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Impurity G**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 1.22 g of *sodium decanesulfonate R* in 850 mL of *water for chromatography R*, add 1.3 mL of *phosphoric acid R*, adjust to pH 3.0 with a 200 g/L solution of *potassium hydroxide R* and dilute to 1000.0 mL with *water for chromatography R*.

*Test solution.* Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *levetiracetam impurity G CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Reference solution (c).* To 1.0 mL of reference solution (a) add 1.0 mL of the test solution and dilute to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 27 °C.

*Mobile phase*: *acetonitrile R1*, buffer solution (15:85 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Run time*: 5 times the retention time of levetiracetam.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to levetiracetam (retention time = about 4 min): impurity G = about 3.8.

*System suitability*: reference solution (c):
- *resolution*: minimum 5.0 between the peaks due to levetiracetam and impurity G.

*Calculation of percentage content*:
- for impurity G, use the concentration of impurity G in reference solution (b).

*Limit*:
- *impurity G*: maximum 0.05 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *levetiracetam impurity A CRS* and 5 mg of *levetiracetam impurity E CRS* in mobile phase A, add 1.0 mL of test solution (a) and dilute to 100.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 20.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 5.0 mg of *levetiracetam impurity C CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 40.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 50.0 mg of *levetiracetam CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 50.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, *phosphate buffer solution pH 5.5 R* (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 20 | 100 → 71 | 0 → 29 |
| 20 - 25 | 71 | 29 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Relative retention* with reference to levetiracetam (retention time = about 11 min): impurity C = about 0.5; impurity A = about 0.7; impurity E = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.5 between the peaks due to impurity E and levetiracetam.

*Calculation of percentage contents*:

- for impurity C, use the concentration of impurity C in reference solution (c);
- for impurities other than C, use the concentration of levetiracetam in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.3 per cent;
- *impurity C*: maximum 250 ppm;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.4 per cent;
- *reporting threshold*: 0.03 per cent, except for impurity C.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_8H_{14}N_2O_2$ taking into account the assigned content of *levetiracetam CRS*.

IMPURITIES

*Specified impurities: A, C, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E.*

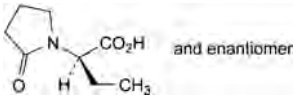


A. (2*RS*)-2-(2-oxopyrrolidin-1-yl)butanoic acid,

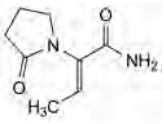


B. (2*Z*)-2-(2-oxopyrrolidin-1-yl)but-2-enamide,

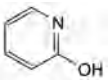


C. pyridin-2-ol,

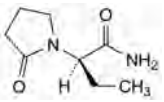


D. (2*R*)-2-(2-oxopyrrolidin-1-yl)butanamide ((*R*)-etiracetam),

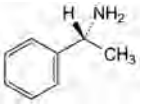


E. (1*R*)-1-phenylethan-1-amine,

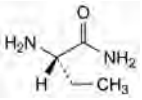


G. (2*S*)-2-aminobutanamide.

**07/2014:1484**
**corrected 10.0**

# LEVOCABASTINE HYDROCHLORIDE

## Levocabastini hydrochloridum

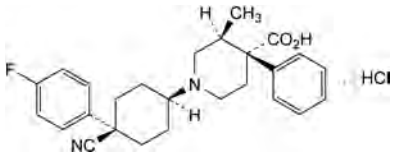


$C_{26}H_{30}ClFN_2O_2$ $M_r$ 457.0
[79547-78-7]

DEFINITION

(3*S*,4*R*)-1-[*cis*-4-Cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent) and in a 2 g/L solution of sodium hydroxide.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levocabastine hydrochloride CRS*.

C. Dissolve 50 mg in a mixture of 0.4 mL of *ammonia R* and 2 mL of *water R*. Mix, allow to stand for 5 min and filter. Acidify the filtrate with *dilute nitric acid R*. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 106 to − 102 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *levocabastine for system suitability 1 CRS* (containing impurities A, B, E, J and K) in 1.0 mL of *methanol R1.*

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R1.* Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1.*

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: 17 g/L solution of *tetrabutylammonium hydrogen sulfate R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 95 | 5 |
| 0.5 - 3.5 | 95 → 90 | 5 → 10 |
| 3.5 - 6.0 | 90 → 85 | 10 → 15 |
| 6.0 - 11.0 | 85 → 70 | 15 → 30 |
| 11.0 - 14.5 | 70 → 20 | 30 → 80 |
| 14.5 - 15.5 | 20 | 80 |

*Flow rate*: 0.45 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 2.0 µL.

*Identification of impurities*: use the chromatogram supplied with *levocabastine for system suitability 1 CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, E, J and K.

*Relative retention* with reference to levocabastine (retention time = about 6.5 min): impurity A = about 0.85; impurity J = about 0.86; impurity B = about 0.90; impurity E = about 0.94; impurity K = about 1.07.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.9, where $H_p$ = height above the baseline of the peak due to impurity K and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to levocabastine; minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

- for each impurity, use the concentration of levocabastine hydrochloride in reference solution (b).

*Limits*:

- *impurity E*: maximum 0.4 per cent;
- *impurity A*: maximum 0.2 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurity C**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *levocabastine for system suitability 2 CRS* (containing impurity C) in 1.0 mL of *methanol R1.*

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R1.* Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1.*

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 17 g/L solution of *tetrabutylammonium hydrogen sulfate R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 90 | 10 |
| 0.5 - 15.5 | 90 → 80 | 10 → 20 |
| 15.5 - 20.5 | 80 → 50 | 20 → 50 |

*Flow rate*: 0.30 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 2.0 µL.

*Identification of impurities*: use the chromatogram supplied with *levocabastine for system suitability 2 CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to levocabastine (retention time = about 16 min): impurity C = about 0.98.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 10.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to levocabastine.

*Calculation of percentage content*:

- for impurity C, use the concentration of levocabastine hydrochloride in reference solution (b).

*Limit*:

- *impurity C*: maximum 0.3 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.175 g in 50 mL of *ethanol (96 per cent) R*, previously neutralised to *phenol red solution R*, and add 5.0 mL of *water R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide.* Read the volume at the 2nd point of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 22.85 mg of $C_{26}H_{30}ClFN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, G, H, I, J, K, L.*

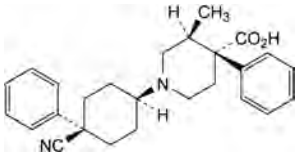

A. (3*S*,4*R*)-1-(*cis*-4-cyano-4-phenylcyclohexyl)-3-methyl-4-phenylpiperidine-4-carboxylic acid,

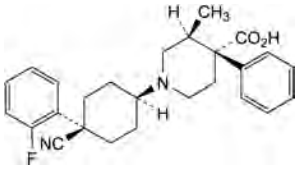

B. (3*S*,4*R*)-1-[*cis*-4-cyano-4-(2-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

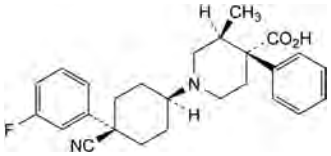

C. (3*S*,4*R*)-1-[*cis*-4-cyano-4-(3-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

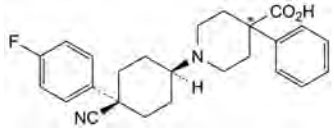

D. 1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-4-phenylpiperidine-4-carboxylic acid,

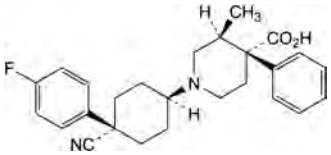

E. (3*S*,4*R*)-1-[*trans*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

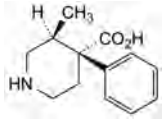

F. (3*S*,4*R*)-3-methyl-4-phenylpiperidine-4-carboxylic acid,

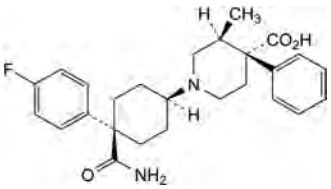

G. (3*S*,4*R*)-1-[*cis*-4-carbamoyl-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

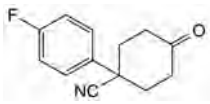

H. 1-(4-fluorophenyl)-4-oxocyclohexanecarbonitrile,

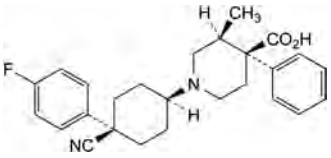

I. (3*S*,4*S*)-1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid,

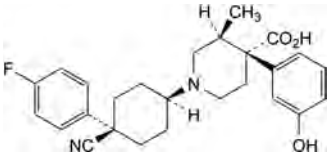

J. (3*S*,4*R*)-1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-4-(3-hydroxyphenyl)-3-methylpiperidine-4-carboxylic acid,

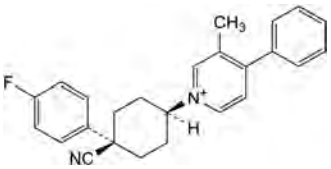

K. 1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpyridinium,

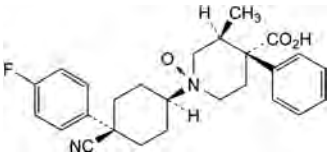

L. (3*S*,4*R*)-1-[*cis*-4-cyano-4-(4-fluorophenyl)cyclohexyl]-3-methyl-4-phenylpiperidine-4-carboxylic acid 1-oxide.

**01/2019:1339**

# LEVOCARNITINE

## Levocarnitinum

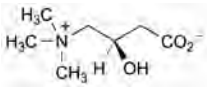

$C_7H_{15}NO_3$ $M_r$ 161.2

[541-15-1]

DEFINITION

(3*R*)-3-Hydroxy-4-(trimethylazaniumyl)butanoate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: freely soluble in water, soluble in warm ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs, prepared using substance previously dried *in vacuo* at 50 °C for 5 h.

*Comparison*: *levocarnitine CRS*.

C. To 1 mL of solution S (see Tests) add 9 mL of *water R*, 10 mL of *dilute sulfuric acid R* and 30 mL of *ammonium reineckate solution R*. A pink precipitate is formed. Allow to stand for 30 min. Filter and wash with *water R*, with *ethanol (96 per cent) R* and then with *acetone R* and dry at 80 °C. The precipitate melts (*2.2.14*) at 147 °C to 150 °C.

TESTS

**Solution S**. Dissolve 5.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 8.5.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): – 29.0 to – 32.0 (anhydrous substance), determined on solution S at 25 °C.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 12.5 mg of *levocarnitine impurity A CRS* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50 mg of the substance to be examined in 10 mL of reference solution (b).

*Column*:

– *size*: *l* = 0.30 m, Ø = 3.9 mm;
– *stationary phase*: *aminopropylmethylsilyl silica gel for chromatography R* (10 µm);
– *temperature*: 30 °C.

*Mobile phase*: mix 35 volumes of a 6.81 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 4.7 with *dilute sodium hydroxide solution R*, and 65 volumes of *acetonitrile R1*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 25 µL.

*Run time*: twice the retention time of levocarnitine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to levocarnitine (retention time = about 10 min): impurity A = about 1.1.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to levocarnitine and impurity A.

*Limits*:

– *impurity A*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total (excluding impurity A)*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.125 g in a mixture of 3 volumes of *anhydrous formic acid R* and 50 volumes of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.12 mg of $C_7H_{15}NO_3$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

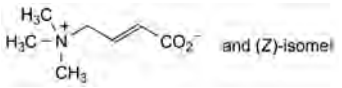


A. (2*EZ*)-4-(trimethylazaniumyl)but-2-enoate,

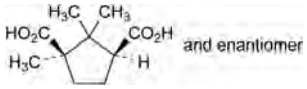


B. (1*RS*,3*SR*)-1,2,2-trimethylcyclopentane-1,3-dicarboxylic acid (camphoric acid),

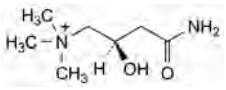

C. (2*R*)-4-amino-2-hydroxy-*N*,*N*,*N*-trimethyl-4-oxobutan-1-aminium (carnitinamide),

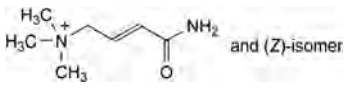


D. (2*EZ*)-4-amino-*N*,*N*,*N*-trimethyl-4-oxobut-2-en-1-aminium.

01/2017:0038

# LEVODOPA

## Levodopum

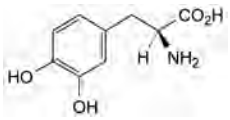

$C_9H_{11}NO_4$ $M_r$ 197.2
[59-92-7]

DEFINITION

(2*S*)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent). It is freely soluble in *1 M hydrochloric acid* and sparingly soluble in *0.1 M hydrochloric acid*.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *levodopa CRS*.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 25 mL with the same solution.

**pH** (*2.2.3*): 4.5 to 7.0.

Shake 0.10 g with 10 mL of *carbon dioxide-free water R* for 15 min.

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Solution A*. 10.3 g/L solution of *hydrochloric acid R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in solution A and dilute to 25 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with solution A. Dilute 5.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (b).* Dissolve 8 mg of *tyrosine R* (impurity B) and 4 mg of *3-methoxy-L-tyrosine R* (L-isomer of impurity C) in 2 mL of the test solution and dilute to 50 mL with solution A. Dilute 5 mL of this solution to 100 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *di-isobutyloctadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 8 nm.

*Mobile phase*:

- *mobile phase A*: *0.1 M phosphate buffer solution pH 3.0 R*;
- *mobile phase B*: *methanol R, 0.1 M phosphate buffer solution pH 3.0 R* (18:85 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 18 | 90 | 10 |
| 18 - 22 | 90 → 0 | 10 → 100 |
| 22 - 35 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to levodopa (retention time = about 6 min): impurity A = about 0.7; impurity B = about 2; impurity C = about 3.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 10 between the peaks due to levodopa and impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 2.2;
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions*.

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *D-dopa R* (impurity D) in 10 mL of the test solution. Dilute 1 mL of this solution to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve separately 200 mg of *copper acetate R* and 387 mg of *N,N-dimethyl-L-phenylalanine R* in 250 mL of *water R*; mix the 2 solutions and adjust immediately to pH 4.0 with *acetic acid R*; add 50 mL of *methanol R* and dilute to 1000 mL with *water R*; mix and filter.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of levodopa.

*Relative retention* with reference to levodopa (retention time = about 7 min): impurity D = about 0.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 5 between the peaks due to impurity D and levodopa.

*Limit*:

- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g, heating if necessary, in 5 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.72 mg of $C_9H_{11}NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

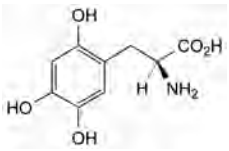

A. (2*S*)-2-amino-3-(2,4,5-trihydroxyphenyl)propanoic acid,

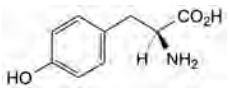

B. (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid (tyrosine),

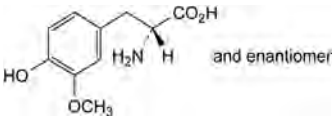


C. (2*RS*)-2-amino-3-(4-hydroxy-3-methoxyphenyl)propanoic acid (3-methoxy-DL-tyrosine),

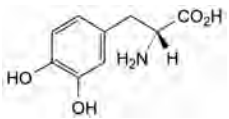

D. (2*R*)-2-amino-3-(3,4-dihydroxyphenyl)propanoic acid (D-dopa).

**01/2020:1535**

# LEVODROPROPIZINE

## Levodropropizinum

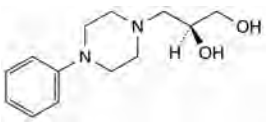

$C_{13}H_{20}N_2O_2$ $M_r$ 236.3
[99291-25-5]

DEFINITION

(2*S*)-3-(4-Phenylpiperazin-1-yl)propane-1,2-diol.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in dilute acetic acid and in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): − 33.5 to − 30.0 (dried substance).
Dissolve 1.50 g in a 21 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same acid.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levodropropizine CRS*.

C. Enantiomeric purity (see Tests).

TESTS

**pH** (*2.2.3*): 9.2 to 10.2.

Suspend 2.5 g in *carbon dioxide-free water R*, heat to dissolve, cool to room temperature and dilute to 100 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *levodropropizine impurity B CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Mix 1 mL of the test solution with 1 mL of reference solution (a).

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 12 volumes of *methanol R* and 88 volumes of a 6.81 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of levodropropizine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to levodropropizine (retention time = about 7 min): impurity B = about 1.2.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to levodropropizine and impurity B.

*Limits*:
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity C**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use. Use vials sealed with a crimp-top to prevent evaporation of the solvent.*

*Test solution.* Dissolve 50 mg of *sodium diethyldithiocarbamate R* and 1.0 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent. Heat at 60 °C for 20 min and then cool to room temperature. Add 5 mL of *water R* and 0.5 mL of *phosphoric acid R*. Extract with 5 mL of *methylene chloride R*.

*Reference solution (a).* Dissolve 0.100 g of *levodropropizine impurity C CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 50 mg of *sodium diethyldithiocarbamate R* in reference solution (a) and dilute to 5.0 mL with reference solution (a). Heat at 60 °C for 20 min and then cool to room temperature. Add 5 mL of *water R* and 0.5 mL of *phosphoric acid R*. Extract with 5 mL of *methylene chloride R*.

*Reference solution (c).* Dissolve 50 mg of *sodium diethyldithiocarbamate R* and 1.0 g of the substance to be examined in reference solution (a) and dilute to 5.0 mL with reference solution (a). Heat at 60 °C for 20 min and then cool to room temperature. Add 5 mL of *water R* and 0.5 mL of *phosphoric acid R*. Extract with 5 mL of *methylene chloride R*.

*Blank solution.* Dissolve 50 mg of *sodium diethyldithiocarbamate R* in *methanol R* and dilute to 5.0 mL with the same solvent. Heat at 60 °C for 20 min and then cool to room temperature. Add 5 mL of *water R* and 0.5 mL of *phosphoric acid R*. Extract with 5 mL of *methylene chloride R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *tetrahydrofuran R*, *heptane R* (15:15:70 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL of the blank solution, the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of impurity C.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Retention time*: impurity C = about 8 min.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 50 for the peak due to impurity C.

Calculate the content of impurity C in parts per million using the following expression:

$$\frac{A}{B-A} \times 5$$

*A* = area of the peak due to impurity C in the chromatogram obtained with the test solution;

*B* = area of the peak due to impurity C in the chromatogram obtained with reference solution (c).

*Limit*:

- *impurity C*: maximum 5 ppm.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *anhydrous ethanol R*, *hexane R* (40:60 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 10.0 mL of the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *levodropropizine CRS* in 10.0 mL of the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10.0 mg of *levodropropizine impurity A CRS* in 10 mL of the solvent mixture. Dilute 1 mL of this solution to 50 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dilute 0.5 mL of reference solution (b) to 25 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *diethylamine R*, *anhydrous ethanol R*, *hexane R* (0.2:5:95 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to levodropropizine (retention time = about 28 min): impurity A = about 0.9.

*System suitability*:

- *retention times*: the retention times of the principal peaks in the chromatograms obtained with the test solution and reference solution (a) are similar;
- *resolution*: minimum 1.3 between the peaks due to impurity A and levodropropizine in the chromatogram obtained with reference solution (d).

*Limit*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (2 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C at a pressure of 0.15-0.25 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *anhydrous acetic acid R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M perchloric acid*. Read the volume added at the 2nd point of inflexion.

1 mL of *0.1 M perchloric acid* is equivalent to 11.82 mg of $C_{13}H_{20}N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

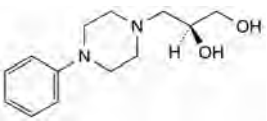


A. (2*R*)-3-(4-phenylpiperazin-1-yl)propane-1,2-diol (dextrodropropizine),

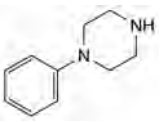


B. 1-phenylpiperazine,

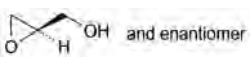


C. [(2*RS*)-oxiran-2-yl]methanol (glycidol).

**07/2019:2598**

# LEVOFLOXACIN HEMIHYDRATE

## Levofloxacinum hemihydricum

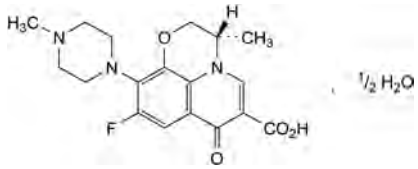

$C_{18}H_{20}FN_3O_4,½H_2O$ $M_r$ 370.4
[138199-71-0]

DEFINITION

(3*S*)-9-Fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid hemihydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: light yellowish-white or light yellow, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in acetic acid, sparingly soluble in methanol, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levofloxacin hemihydrate CRS*.

B. Examine the chromatograms obtained in the test for related substances.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_3$ (*2.2.2, Method II*).

Dissolve 0.100 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*: solution containing 1.25 g/L of *copper sulfate pentahydrate R*, 1.3 g/L of *isoleucine R* and 8.5 g/L of *ammonium acetate R* in *water for chromatography R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *levofloxacin for system suitability CRS* (containing impurities A, B and G) in 1 mL of the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: *methanol R*, buffer solution (30:70 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 360 nm.

*Injection*: 25 µL.

*Run time*: 3 times the retention time of levofloxacin.

*Identification of impurities*: use the chromatogram supplied with *levofloxacin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and G.

*Relative retention* with reference to levofloxacin (retention time = about 20 min): impurity B = about 0.50; impurity G = about 0.56; impurity A = about 1.22.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities B and G.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity B by 1.3;
- for each impurity, use the concentration of levofloxacin hemihydrate in reference solution (a).

*Limits*:
- *impurity A*: maximum 0.5 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurity F**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *levofloxacin impurity F CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 4 mL of reference solution (a) to 10 mL with the mobile phase. Dilute 1 mL of this solution to 10 mL with the test solution.

*Mobile phase*: *methanol R*, buffer solution (50:50 *V/V*).

*Detection*: spectrophotometer at 320 nm.

*Injection*: test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity F.

*Relative retention* with reference to levofloxacin (retention time = about 6 min): impurity F = about 1.8.

*System suitability*: reference solution (c):
- *resolution*: minimum 5.0 between the peaks due to levofloxacin and impurity F.

*Calculation of percentage content*:
- for impurity F, use the concentration of impurity F in reference solution (b).

*Limit*:
- *impurity F*: maximum 0.2 per cent.

**Water** (*2.5.12*): 2.0 per cent to 3.0 per cent, determined on 0.500 g using a mixture of equal volumes of *formamide R* and *methanol R* as solvent.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 100 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.14 mg of $C_{18}H_{20}FN_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, G, H, I.*

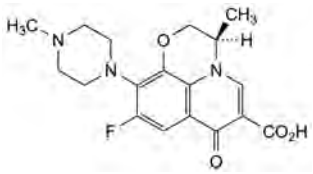

A. (3*R*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid (enantiomer of levofloxacin),

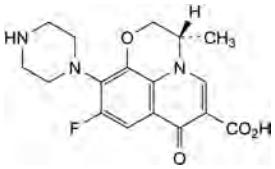

B. (3*S*)-9-fluoro-3-methyl-7-oxo-10-(piperazin-1-yl)-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid,

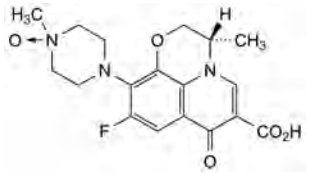

C. 4-[(3*S*)-6-carboxy-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazin-10-yl]-1-methylpiperazine 1-oxide,

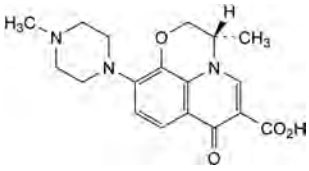

D. (3*S*)-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid,

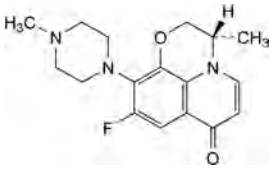

E. (3*S*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazin-7-one,

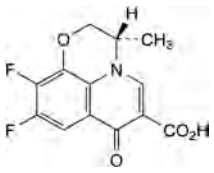

F. (3*S*)-9,10-difluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid,

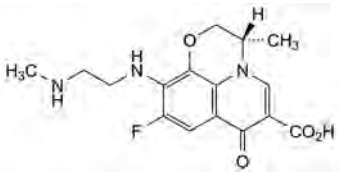

G. (3*S*)-9-fluoro-3-methyl-10-[[2-(methylamino)ethyl]-amino]-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]-benzoxazine-6-carboxylic acid,

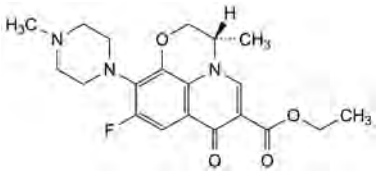

H. ethyl (3*S*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylate,

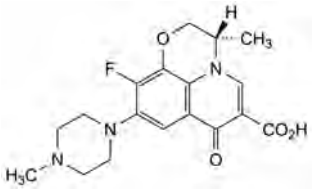

I. (3*S*)-10-fluoro-3-methyl-9-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*][1,4]benzoxazine-6-carboxylic acid.

**01/2008:0619**

# LEVOMENTHOL

## Levomentholum

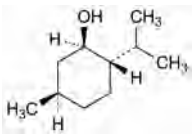

$C_{10}H_{20}O$ $M_r$ 156.3

[2216-51-5]

DEFINITION

(1*R*,2*S*,5*R*)-5-Methyl-2-(1-methylethyl)cyclohexanol.

CHARACTERS

*Appearance*: prismatic or acicular, colourless, shiny crystals.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and in light petroleum, freely soluble in fatty oils and in liquid paraffin, very slightly soluble in glycerol.

mp: about 43 °C.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, D.*

A. Specific optical rotation (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution*. Dissolve 25 mg of *menthol CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: in air, until the solvents have evaporated.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in position and approximate dimensions to the principal peak in the chromatogram obtained with reference solution (c).

D. Dissolve 0.20 g in 0.5 mL of *anhydrous pyridine R*. Add 3 mL of a 150 g/L solution of *dinitrobenzoyl chloride R* in *anhydrous pyridine R*. Heat on a water-bath for 10 min. Add 7.0 mL of *water R* in small quantities with stirring and allow to stand in iced water for 30 min. A precipitate is formed. Allow to stand and decant the supernatant. Wash the precipitate with 2 quantities, each of 5 mL, of iced *water R*, recrystallise from 10 mL of *acetone R*, wash with iced *acetone R* and dry at 75 °C at a pressure not exceeding 2.7 kPa for 30 min. The crystals melt (*2.2.14*) at 154 °C to 157 °C.

TESTS

**Solution S**. Dissolve 2.50 g in 10 mL of *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Specific optical rotation** (*2.2.7*): − 48 to − 51, determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution (a)*. Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *methylene chloride R*.

*Reference solution (a)*. Dissolve 40.0 mg of the substance to be examined and 40.0 mg of *isomenthol R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dilute 0.10 mL of test solution (a) to 100.0 mL with *methylene chloride R*.

*Reference solution (c)*. Dissolve 40.0 mg of *menthol CRS* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *material*: glass;

– *size*: *l* = 2.0 m, Ø = 2 mm;

– *stationary phase*: *diatomaceous earth for gas chromatography R* impregnated with 15 per cent *m/m* of *macrogol 1500 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 120 °C;

– *injection port*: 150 °C;

– *detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: twice the retention time of menthol.

*System suitability*:

– *resolution*: minimum 1.4 between the peaks due to menthol and isomenthol in the chromatogram obtained with reference solution (a);

– *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*: test solution (a):

– *total*: not more than 1 per cent of the area of the principal peak;

– *disregard limit*: 0.05 per cent of the area of the principal peak.

**Residue on evaporation**: maximum 0.05 per cent.

Evaporate 2.00 g on a water-bath and heat in an oven at 100-105 °C for 1 h. The residue weighs not more than 1.0 mg.

**01/2008:0505**
**corrected 6.0**

# LEVOMEPROMAZINE HYDROCHLORIDE

## Levomepromazini hydrochloridum

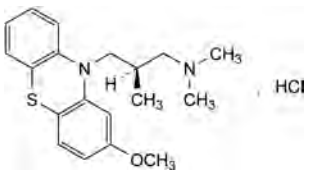


$C_{19}H_{25}ClN_2OS$ $M_r$ 364.9
[1236-99-3]

DEFINITION

Levomepromazine hydrochloride contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-3-(2-methoxy-10*H*-phenothiazin-10-yl)-*N*,*N*,2-trimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or very slightly yellow, crystalline powder, slightly hygroscopic, freely soluble in water and in alcohol. It deteriorates when exposed to air and light. It exists in two forms, one melting at about 142 °C and the other at about 162 °C.

IDENTIFICATION

A. *Prepare the solution protected from bright light and carry out the measurements immediately*. Dissolve 50.0 mg in *water R* and dilute to 500.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *water R*. Examined between 230 nm and 340 nm (*2.2.25*), the

solution shows two absorption maxima, at 250 nm and 302 nm. The specific absorbance at the maximum at 250 nm is 640 to 700.

B. It complies with the identification test for phenothiazines by thin-layer chromatography (*2.3.3*): use *levomepromazine hydrochloride CRS* to prepare the reference solution.

C. Introduce 0.2 g into a 100 mL separating funnel. Add 5 mL of *water R* and 0.5 mL of *strong sodium hydroxide solution R*. Shake vigorously with two quantities, each of 10 mL, of *ether R*. Combine the ether layers, dry over *anhydrous sodium sulfate R* and evaporate to dryness. Keep the residue at 100 °C to 105 °C for 15 min and allow to crystallise in iced water. Initiate crystallisation if necessary by scratching the wall of the flask with a glass rod. Dry the crystals at 60 °C for 2 h. The crystals melt (*2.2.14*) at 122 °C to 128 °C.

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromocresol green solution R*. Not more than 0.5 mL of *0.01 M sodium hydroxide* or 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): + 9.5 to + 11.5, determined on solution S and calculated with reference to the dried substance.

**Related substances**. *Carry out the test protected from bright light*. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents. Prepare immediately before use.

*Reference solution*. Dilute 0.5 mL of the test solution to 100 mL with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 5 mL of *water R* and add 50 mL of *2-propanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.49 mg of $C_{19}H_{25}ClN_2OS$.

STORAGE

Store in an airtight container, protected from light.

**01/2008:0925**
**corrected 6.0**

# LEVOMEPROMAZINE MALEATE

## Levomepromazini maleas

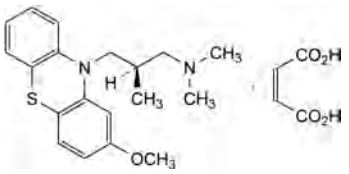

$C_{23}H_{28}N_2O_5S$ $M_r$ 444.6
[7104-38-3]

DEFINITION

Levomepromazine maleate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (2*R*)-3-(2-methoxy-10*H*-phenothiazin-10-yl)-*N*,*N*,2-trimethylpropan-1-amine (*Z*)-butenedioate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, slightly soluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol. It deteriorates when exposed to air and light.

It melts at about 186 °C, with decomposition.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *levomepromazine maleate CRS*. Examine the substances prepared as discs.

C. Identification test for phenothiazines by thin-layer chromatography (*2.3.3*): use *levomepromazine maleate CRS* to prepare the reference solution.

D. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.20 g of the substance to be examined in a mixture of 10 volumes of *water R* and 90 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution*. Dissolve 50 mg of *maleic acid CRS* in a mixture of 10 volumes of *water R* and 90 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents.

Apply separately to the plate as bands 10 mm by 2 mm 5 µL of each solution. Develop over a path of 12 cm using a mixture of 3 volumes of *water R*, 7 volumes of *anhydrous formic acid R* and 90 volumes of *di-isopropyl ether R*. Dry the plate at 120 °C for 10 min and examine in ultraviolet light at 254 nm. The chromatogram obtained with the test solution shows a zone at the point of application and another zone similar in position and size to the principal zone in the chromatogram obtained with the reference solution.

TESTS

**pH** (*2.2.3*). *Carry out the test protected from bright light.* Introduce 0.50 g into a conical flask and add 25.0 mL of *carbon dioxide-free water R*. Shake and allow the solids to settle. The pH of the supernatant solution is 3.5 to 5.5.

**Specific optical rotation** (*2.2.7*). Dissolve 1.25 g in *dimethylformamide R* and dilute to 25.0 mL with the same solvent. The specific optical rotation is − 7.0 to − 8.5, calculated with reference to the dried substance.

**Related substances**. *Carry out the test protected from bright light and prepare the solutions immediately before use.* Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.20 g of the substance to be examined in a mixture of 10 volumes of *water R* and 90 volumes of *acetone R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution*. Dilute 0.5 mL of the test solution to 100 mL with a mixture of 10 volumes of *water R* and 90 volumes of *acetone R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 44.46 mg of $C_{23}H_{28}N_2O_5S$.

STORAGE

Store protected from light.

IMPURITIES

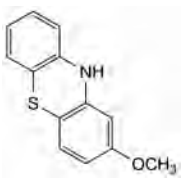

A. 2-methoxyphenothiazine,

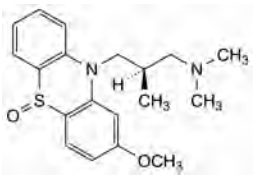

B. 10-[(2*R*)-3-(dimethylamino)-2-methylpropyl]-2-methoxy-10*H*-phenothiazine 5-oxide.

**01/2008:1787**
**corrected 6.5**

# LEVOMETHADONE HYDROCHLORIDE

## Levomethadoni hydrochloridum

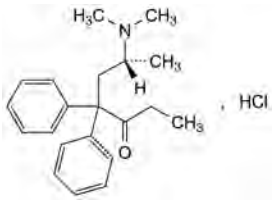

$C_{21}H_{28}ClNO$ $M_r$ 345.9
[5967-73-7]

DEFINITION

(6*R*)-6-(Dimethylamino)-4,4-diphenylheptan-3-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: A, B, D.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 239 °C to 242 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of methadone hydrochloride.*

D. Dilute 1 mL of solution S (see Tests) to 5 mL with *water R* and add 1 mL of *dilute ammonia R1*. Mix, allow to stand for 5 min and filter. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 10 mL of solution S to 25 mL with *carbon dioxide-free water R*. To 10 mL of the solution add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Specific optical rotation** (*2.2.7*): − 125 to − 135 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 12.0 mg of *imipramine hydrochloride CRS* in the mobile phase and dilute to 10 mL with the mobile phase. To 1 mL of the solution add 5 mL of the test solution and dilute to 10 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of an 11.5 g/L solution of *phosphoric acid R* adjusted to pH 3.6 with *tetraethylammonium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Equilibration*: about 30 min.

*Injection*: 10 µL.

*Run time*: 7 times the retention time of levomethadone.

*Retention time*: levomethadone = about 5 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.5 between the peaks due to imipramine and levomethadone.

*Limits*:
- *any impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Dextromethadone**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *2-hydroxypropylbetadex for chromatography R* (5 µm);
- *temperature*: 10 °C.

*Mobile phase*: mix 1 volume of *triethylamine R* adjusted to pH 4.0 with *phosphoric acid R*, 15 volumes of *acetonitrile R* and 85 volumes of a 13.6 g/L solution of *potassium dihydrogen phosphate R*.

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Equilibration*: about 30 min.

*Injection*: 10 µL.

*Relative retention* with reference to levomethadone: dextromethadone = about 1.4.

*System suitability*: test solution:
- *number of theoretical plates*: minimum 2000, calculated for the peak due to levomethadone;
- *tailing factor*: maximum 3 for the peak due to levomethadone.

*Limit*:
- *dextromethadone*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 40 mL of *water R* and 5 mL of *acetic acid R*. Titrate with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*), using a silver electrode.

1 mL of *0.1 M silver nitrate* is equivalent to 34.59 mg of $C_{21}H_{28}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

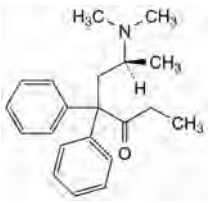

A. (6*S*)-6-(dimethylamino)-4,4-diphenylheptan-3-one,

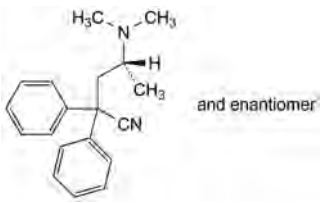


B. (4*RS*)-4-(dimethylamino)-2,2-diphenylpentanenitrile,

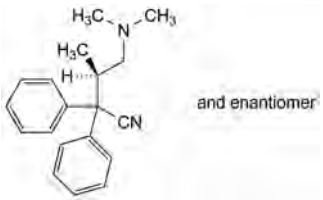


C. (3*RS*)-4-(dimethylamino)-3-methyl-2,2-diphenylbutanenitrile,

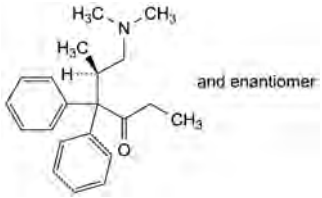


D. (5*RS*)-6-(dimethylamino)-5-methyl-4,4-diphenylhexan-3-one,

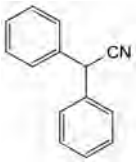

E. diphenylacetonitrile,

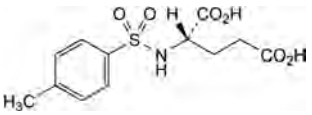

F. (2*S*)-2-[[(4-methylphenyl)sulfonyl]amino]pentanedioic acid (*N*-*p*-tosyl-L-glutamic acid).

**01/2014:0926**

# LEVONORGESTREL

## Levonorgestrelum

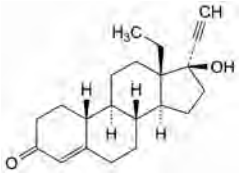

$C_{21}H_{28}O_2$ $M_r$ 312.5
[797-63-7]

DEFINITION

13-Ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *levonorgestrel CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 35 to − 30.

Dissolve 0.200 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

**Related substances**

A. Impurities A, B, H, K, M, O, S, U. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *acetonitrile R1* (30:70 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 7 mL of *acetonitrile R1* using sonication and dilute to 10.0 mL with *water for chromatography R*.

*Reference solution (a).* Dissolve 5 mg of *levonorgestrel for system suitability 1 CRS* (containing impurities A, H, K, M, O and S) in 3.5 mL of *acetonitrile R1* using sonication and dilute to 5.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5.0 mg of *levonorgestrel impurity B CRS* in 35 mL of *acetonitrile R1* and dilute to 50.0 mL with *water for chromatography R*. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5.0 mg of *norethisterone CRS* (impurity U) in 35 mL of *acetonitrile R1* and dilute to 50.0 mL with *water for chromatography R*. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, *water for chromatography R* (40:60 *V/V*);

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 → 20 | 0 → 80 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 215 nm and, for impurity O, at 200 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatograms supplied with *levonorgestrel for system suitability 1 CRS* and the chromatograms obtained with reference solution (a) at 215 nm to identify the peaks due to impurities A, H, K, M and S, and at 200 nm to identify the peak due to impurity O; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity U.

*Relative retention* with reference to levonorgestrel (retention time = about 20 min): impurity H = about 0.5; impurity U = about 0.8; impurity K = about 0.85; impurity A = about 0.91; impurity M = about 0.95; impurity O = about 1.16; impurity B = about 1.26; impurity S = about 1.9.

*System suitability*:

– *signal-to-noise ratio*: minimum 60 for the principal peak in the chromatogram obtained with reference solution (b);

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity M and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.4; impurity M = 3.1; impurity O = 2.6;

– for impurity B, use the concentration of impurity B in reference solution (c);

– for impurity U, use the concentration of impurity U in reference solution (d);

– for impurities other than B and U, use the concentration of levonorgestrel in reference solution (b).

*Limits*:

– *impurities A, B, K*: for each impurity, maximum 0.3 per cent;

– *impurity O at 200 nm*: maximum 0.3 per cent;

– *impurities M, S, U*: for each impurity, maximum 0.2 per cent;

– *impurity H*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *sum of impurities other than O*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent.

B. Impurities V and W. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *acetonitrile R1* (30:70 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 7 mL of *acetonitrile R1* using sonication and dilute to 10.0 mL with *water for chromatography R*.

*Reference solution (a).* Dissolve 5 mg of *levonorgestrel for system suitability 2 CRS* (containing impurities V and W) in 3.5 mL of *acetonitrile R1* using sonication and dilute to 5.0 mL with *water for chromatography R*.

*Reference solution (b).* Dissolve 5.0 mg of *ethinylestradiol CRS* in 35 mL of *acetonitrile R1* using sonication and dilute to 50.0 mL with *water for chromatography R*. Dilute 3.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, *water for chromatography R* (40:60 *V/V*);
- *mobile phase B*: *water for chromatography R*, *acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 92 | 8 |
| 1 - 3 | 92 → 82 | 8 → 18 |
| 3 - 6 | 82 | 18 |
| 6 - 16 | 82 → 60 | 18 → 40 |
| 16 - 21 | 60 → 0 | 40 → 100 |
| 21 - 32 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram supplied with *levonorgestrel for system suitability 2 CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities V and W.

*Relative retention* with reference to levonorgestrel (retention time = about 12 min): impurity W = about 0.9; impurity V = about 1.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.8 between the peaks due to impurity W and levonorgestrel.

*Calculation of percentage contents*:

- for each impurity, use the concentration of ethinylestradiol in reference solution (b).

*Limits*:

- *impurity W*: maximum 0.3 per cent;
- *impurity V*: maximum 0.15 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 45 mL of *tetrahydrofuran R*. Add 10 mL of a 100 g/L solution of *silver nitrate R*. After 1 min, titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.25 mg of $C_{21}H_{28}O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, H, K, M, O, S, U, V, W.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, G, I, J, L, N, P, Q, R, T.*

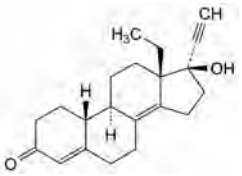

A. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,8(14)-dien-20-yn-3-one,

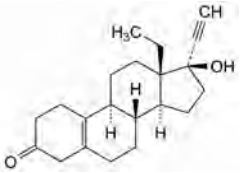

B. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-5(10)-en-20-yn-3-one,

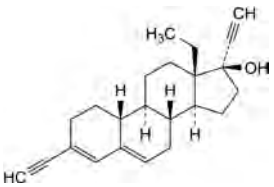

C. 13-ethyl-3-ethynyl-18,19-dinor-17α-pregna-3,5-dien-20-yn-17-ol,

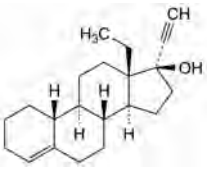

D. 13-ethyl-18,19-dinor-17α-pregn-4-en-20-yn-17-ol (3-deoxolevonorgestrel),

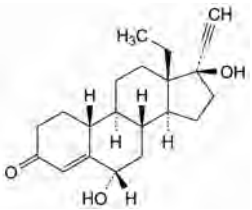

G. 13-ethyl-6α,17-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (6α-hydroxylevonorgestrel),

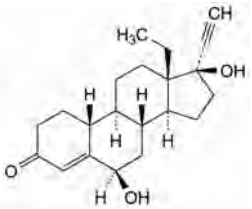

H. 13-ethyl-6β,17-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (6β-hydroxylevonorgestrel),

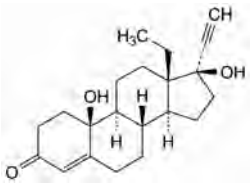

I. 13-ethyl-10,17-dihydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (10-hydroxylevonorgestrel),

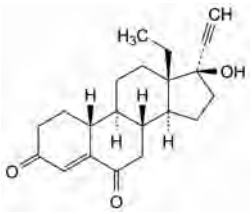

J. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yne-3,6-dione (6-oxolevonorgestrel),

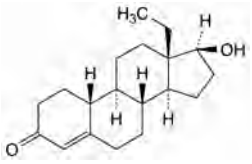

K. 13-ethyl-17β-hydroxygon-4-en-3-one (18-methylnandrolone),

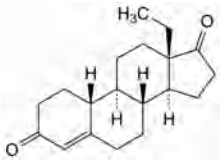

L. 13-ethylgon-4-ene-3,17-dione (levodione),

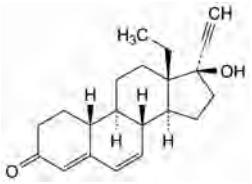

M. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,6-dien-20-yn-3-one (Δ6-levonorgestrel),

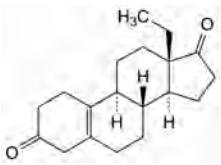

N. 13-ethylgon-5(10)-ene-3,17-dione (Δ5(10)-levodione),

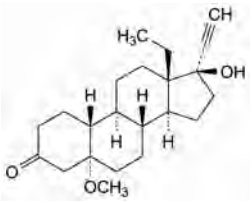

O. 13-ethyl-17-hydroxy-5α-methoxy-18,19-dinor-17α-pregn-20-yn-3-one (4,5-dihydro-5α-methoxylevonorgestrel),

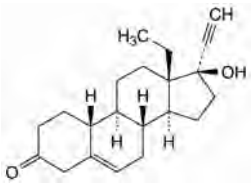

P. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-5-en-20-yn-3-one (Δ5-levonorgestrel),

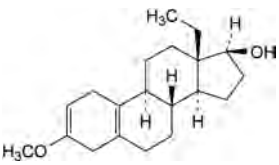

Q. 13-ethyl-3-methoxygona-2,5(10)-dien-17β-ol,

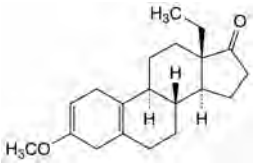

R. 13-ethyl-3-methoxygona-2,5(10)-dien-17-one,

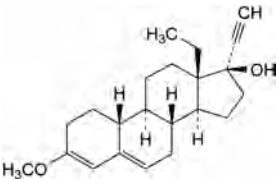

S. 13-ethyl-3-methoxy-18,19-dinor-17α-pregna-3,5-dien-20-yn-17-ol,

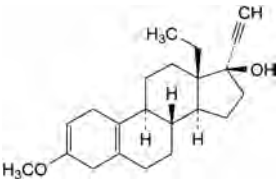

T. 13-ethyl-3-methoxy-18,19-dinor-17α-pregna-2,5(10)-dien-20-yn-17-ol,

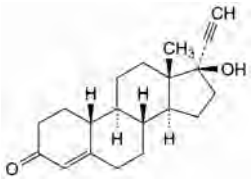

U. 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethisterone),

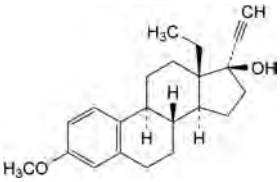

V. 13-ethyl-3-methoxy-18,19-dinor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol,

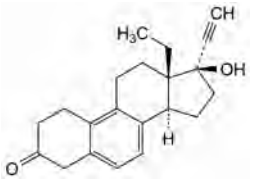

W. 13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-5,7,9-trien-20-yn-3-one.

**01/2013:0401**

# LEVOTHYROXINE SODIUM

## Levothyroxinum natricum

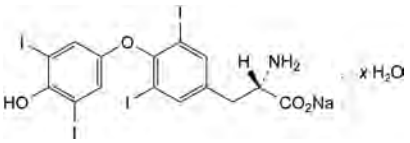

$C_{15}H_{10}I_4NNaO_4,xH_2O$ ($x \approx 5$) $M_r$ 799 (anhydrous substance) [25416-65-3]

DEFINITION

Sodium (2*S*)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]propanoate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: almost white or slightly brownish-yellow, fine, slightly hygroscopic, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *levothyroxine sodium CRS*.

B. To 200 mg add 2 mL of *dilute sulfuric acid R*. Heat on a water-bath and then carefully over a naked flame, increasing the temperature gradually up to 600 ± 50 °C. Continue the ignition until most of the black particles have disappeared. Dissolve the residue in 2 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in 23 mL of a gently boiling mixture of 1 volume of *1 M hydrochloric acid* and 4 volumes of *ethanol (96 per cent) R*. Cool and dilute to 25.0 mL with the same mixture of solvents.

**Appearance of solution**. Freshly prepared solution S is not more intensely coloured than reference solution $BY_3$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 16 to + 20 (anhydrous substance), determined on freshly prepared solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: mobile phase A, *ethanol (96 per cent) R* (1:2 *V*/*V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2.5 mg of *levothyroxine sodium CRS* and 2.5 mg of *liothyronine sodium CRS* (impurity A) in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25.0 mg of *levothyroxine sodium CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 25.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 2.0 mg of *levothyroxine for peak identification CRS* (containing impurities F and G) in 10.0 mL of the solvent mixture and sonicate for 10 min.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 1.97 g of *phosphoric acid R* in *water R* and dilute to 2 L with the same solvent;
- *mobile phase B*: dissolve 1.97 g of *phosphoric acid R* in *acetonitrile R1* and dilute to 2 L with the same solvent;

| Time (min) | Mobile phase A (per cent *V*/*V*) | Mobile phase B (per cent *V*/*V*) |
|---|---|---|
| 0 - 10 | 70 | 30 |
| 10 - 40 | 70 → 20 | 30 → 80 |
| 40 - 50 | 20 | 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 25 µL of the test solution and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram supplied with *levothyroxine for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities F and G.

*Relative retention* with reference to levothyroxine (retention time = about 11 min): impurity A = about 0.5; impurity F = about 2.0; impurity G = about 2.4.

*System suitability*: reference solution (a):
- *resolution*: minimum 5.0 between the peaks due to impurity A and levothyroxine.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurity F*: not more than 5 times the area of the peak due to levothyroxine in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity G*: not more than 3 times the area of the peak due to levothyroxine in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than twice the area of the peak due to levothyroxine in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: maximum 2.0 per cent;
- *disregard limit*: 0.5 times the area of the peak due to levothyroxine in the chromatogram obtained with reference solution (b) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Water** (*2.5.32*): 6.0 per cent to 12.0 per cent, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{15}H_{10}I_4NNaO_4$ taking into account the assigned content of *levothyroxine sodium CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities*: *A, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, H, I, J, K.*

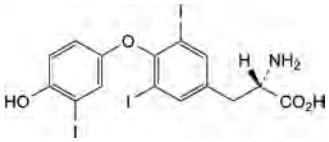

A. (2*S*)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propanoic acid (liothyronine),

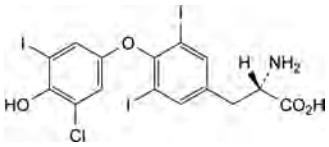

B. (2*S*)-2-amino-3-[4-(3-chloro-4-hydroxy-5-iodophenoxy)-3,5-diiodophenyl]propanoic acid,

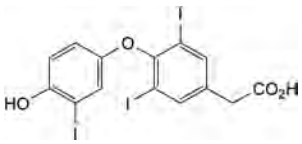

C. [4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]acetic acid (triiodothyroacetic acid),

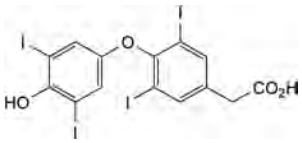

D. [4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-acetic acid (tetraiodothyroacetic acid),

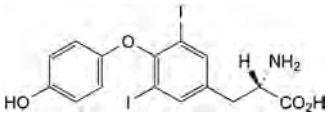

E. (2*S*)-2-amino-3-[4-(4-hydroxyphenoxy)-3,5-diiodophenyl]propanoic acid (diiodothyronine),

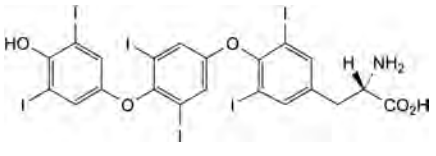

F. (2*S*)-2-amino-3-[4-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenoxy]-3,5-diiodophenyl]propanoic acid,

G. unknown structure,

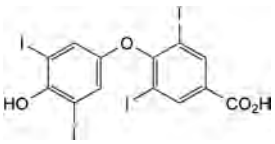

H. 4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodobenzoic acid,

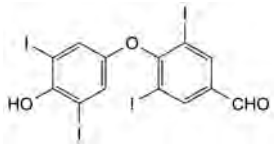

I. 4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodo-benzaldehyde,

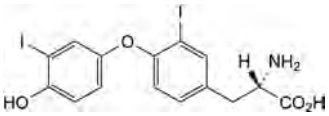

J. (2*S*)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3-iodophenyl]propanoic acid,

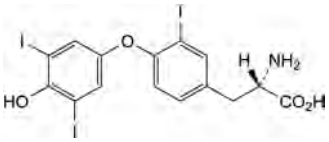

K. (2*S*)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3-iodophenyl]propanoic acid.

**04/2008:0727**

# LIDOCAINE

## Lidocainum

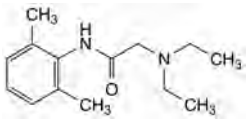

$C_{14}H_{22}N_2O$ $M_r$ 234.3

[137-58-6]

DEFINITION

2-(Diethylamino)-*N*-(2,6-dimethylphenyl)acetamide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lidocaine CRS*.

B. Melting point (*2.2.14*): 66 °C to 70 °C, determined without previous drying.

C. To about 5 mg add 0.5 mL of *fuming nitric acid R*. Evaporate to dryness on a water-bath, cool, and dissolve the residue in 5 mL of *acetone R*. Add 0.2 mL of *alcoholic potassium hydroxide solution R*. A green colour develops.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *2,6-dimethylaniline R* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *2-chloro-N-(2,6-dimethylphenyl)acetamide R* (impurity H) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Mix 1.0 mL of reference solution (a), 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c), then dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 30 volumes of *acetonitrile for chromatography R* and 70 volumes of a 4.85 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 8.0 with *strong sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of lidocaine.

*Relative retention* with reference to lidocaine (retention time = about 17 min): impurity H = about 0.37; impurity A = about 0.40.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurities H and A.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.01 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: not more than 5 times the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 35 ppm.

Dissolve 1.4 g in a mixture of 3 mL of *dilute nitric acid R* and 12 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dissolve 0.2 g in 5 mL of *ethanol (96 per cent) R* and dilute to 20 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.200 g add 50 mL of *anhydrous acetic acid R* and stir until dissolution is complete. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.43 mg of $C_{14}H_{22}N_2O$.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, J*.

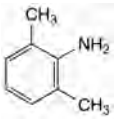

A. 2,6-dimethylaniline,

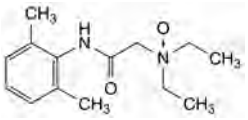

B. 2-(diethylazinoyl)-*N*-(2,6-dimethylphenyl)acetamide (lidocaine *N*-oxide),

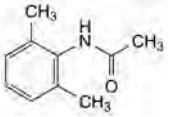

C. *N*-(2,6-dimethylphenyl)acetamide,

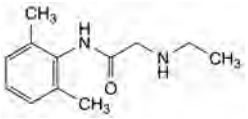

D. *N*-(2,6-dimethylphenyl)-2-(ethylamino)acetamide,

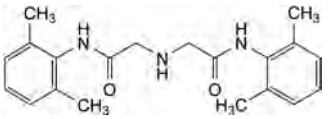

E. 2,2′-iminobis(*N*-(2,6-dimethylphenyl)acetamide),

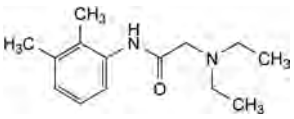

F. 2-(diethylamino)-*N*-(2,3-dimethylphenyl)acetamide,

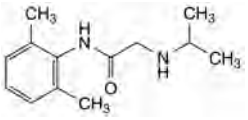

G. *N*-(2,6-dimethylphenyl)-2-((1-methylethyl)amino) acetamide,

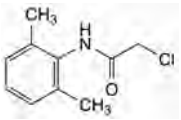

H. 2-chloro-*N*-(2,6-dimethylphenyl)acetamide,

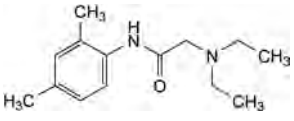

I. 2-(diethylamino)-*N*-(2,4-dimethylphenyl)acetamide,

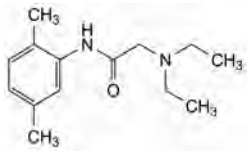

J. 2-(diethylamino)-*N*-(2,5-dimethylphenyl)acetamide.

**01/2019:0227**

# LIDOCAINE HYDROCHLORIDE MONOHYDRATE

## Lidocaini hydrochloridum monohydricum

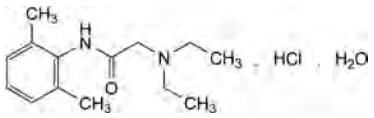

$C_{14}H_{23}ClN_2O,H_2O$ $M_r$ 288.8
[6108-05-0]

DEFINITION

2-(Diethylamino)-*N*-(2,6-dimethylphenyl)acetamide hydrochloride monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 74 °C to 79 °C, determined without previous drying.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lidocaine hydrochloride CRS*.

C. To about 5 mg add 0.5 mL of *fuming nitric acid R*. Evaporate to dryness on a water-bath, cool and dissolve the residue in 5 mL of *acetone R*. Add 0.2 mL of *alcoholic potassium hydroxide solution R*. A green colour is produced.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.5.
Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *2,6-dimethylaniline R* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *2-chloro-N-(2,6-dimethylphenyl)acetamide R* (impurity H) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Mix 1.0 mL of reference solution (a), 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c), and dilute to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 30 volumes of *acetonitrile for chromatography R* and 70 volumes of a 4.85 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 8.0 with *strong sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Run time*: 3.5 times the retention time of lidocaine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity H.

*Relative retention* with reference to lidocaine (retention time = about 17 min): impurity H = about 0.37; impurity A = about 0.40.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to impurities H and A.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.01 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: not more than 5 times the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the peak due to lidocaine in the chromatogram obtained with reference solution (d) (0.05 per cent); do not disregard the peak due to impurity A .

**Water** (*2.5.12*): 5.5 per cent to 7.0 per cent, determined on 0.25 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.220 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.08 mg of $C_{14}H_{23}ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these

impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, J, K.*

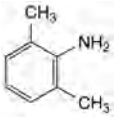

A. 2,6-dimethylaniline,

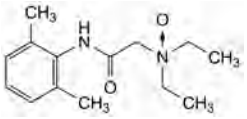

B. 2-(diethylazinoyl)-*N*-(2,6-dimethylphenyl)acetamide (lidocaine $N^2$-oxide),

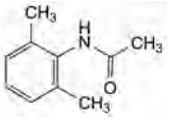

C. *N*-(2,6-dimethylphenyl)acetamide,

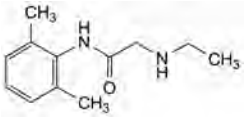

D. *N*-(2,6-dimethylphenyl)-2-(ethylamino)acetamide,

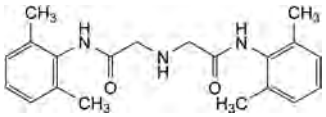

E. 2-2′-(azanediyl)bis[*N*-(2,6-dimethylphenyl)acetamide],

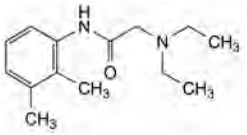

F. 2-(diethylamino)-*N*-(2,3-dimethylphenyl)acetamide,

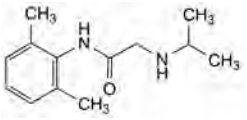

G. *N*-(2,6-dimethylphenyl)-2-[(1-methylethyl)amino]-acetamide,

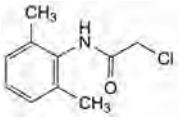

H. 2-chloro-*N*-(2,6-dimethylphenyl)acetamide,

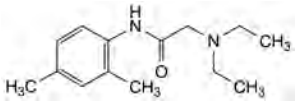

I. 2-(diethylamino)-*N*-(2,4-dimethylphenyl)acetamide,

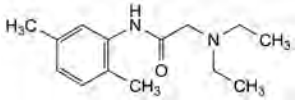

J. 2-(diethylamino)-*N*-(2,5-dimethylphenyl)acetamide,

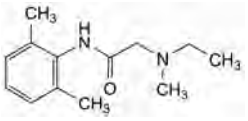

K. *N*-(2,6-dimethylphenyl)-2-(ethylmethylamino)acetamide.

**01/2017:0583**
**corrected 10.0**

# LINCOMYCIN HYDROCHLORIDE

## Lincomycini hydrochloridum

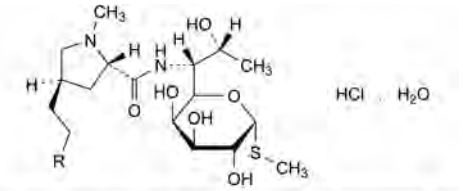

| Compound | R | Molecular formula | $M_r$ |
|---|---|---|---|
| Lincomycin | $CH_3$ | $C_{18}H_{35}ClN_2O_6S,H_2O$ | 461.0 |
| Lincomycin B | H | $C_{17}H_{33}ClN_2O_6S,H_2O$ | 447.0 |

Lincomycin hydrochloride monohydrate: [7179-49-9]

DEFINITION

Mixture of antibiotics produced by *Streptomyces lincolnensis* var. *lincolnensis* or obtained by any other means, the main component being methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (lincomycin) hydrochloride monohydrate.

*Content*:

– *sum of the contents of lincomycin hydrochloride and lincomycin B hydrochloride*: 96.0 per cent to 102.0 per cent (anhydrous substance);
– *lincomycin B hydrochloride*: maximum 5.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lincomycin hydrochloride CRS*.

B. Dissolve 0.1 g in *water R* and dilute to 10 mL with the same solvent. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 135 to + 150 (anhydrous substance).

Dissolve 1.000 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *lincomycin hydrochloride CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *lincomycin hydrochloride for system suitability CRS* (containing impurities A, B and C) in 2 mL of the mobile phase.

*Reference solution (c).* Dilute 2.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Buffer solution pH 6.1*: dissolve 34 g of *phosphoric acid R* in 900 mL of *water for chromatography R*, adjust to pH 6.1 with *concentrated ammonia R* and dilute to 1000 mL with *water for chromatography R*.

*Mobile phase*: *methanol R*, *acetonitrile R1*, buffer solution pH 6.1 (8:17:75 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 5.5 times the retention time of lincomycin.

*Relative retention* with reference to lincomycin (retention time = about 10 min): impurity C = about 0.4; lincomycin B = about 0.5; impurity A = about 0.7; impurity B = about 1.2 and 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peak due to lincomycin and the 1st peak due to impurity B.

*Limits*:

- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *sum of the areas of the peaks due to impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Water** (*2.5.12*): 3.1 per cent to 4.6 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solutions (a) and (c).

Calculate the percentage content of $C_{18}H_{35}ClN_2O_6S$ (lincomycin) and $C_{17}H_{33}ClN_2O_6S$ (lincomycin B) taking into account the assigned content of $C_{18}H_{35}ClN_2O_6S$ in *lincomycin hydrochloride CRS*. Determine the content of lincomycin by comparing with the area of the peak due to lincomycin in the chromatogram obtained with reference solution (a). Determine the content of lincomycin B by comparing with the area of the peak due to lincomycin in the chromatogram obtained with reference solution (c).

STORAGE

At a temperature not exceeding 30 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F.*

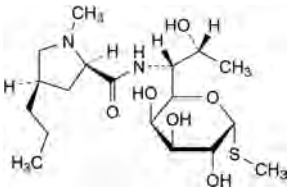

A. methyl 6,8-dideoxy-6-[[[(2*R*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (α-amide epimer),

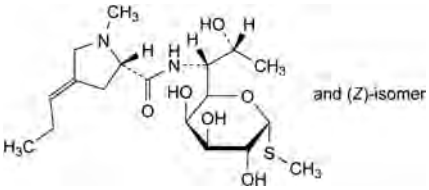


B. methyl 6,8-dideoxy-6-[[[(2*S*,4*EZ*)-1-methyl-4-propylidenepyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (propylidene analogues),

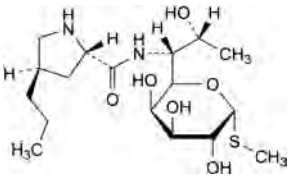

C. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (*N*-desmethyl lincomycin),

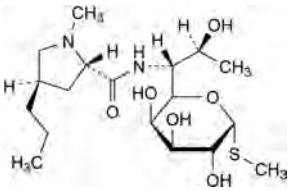

D. methyl 6,8-dideoxy-6-[[[(2*S*,4*R*)-1-methyl-4-propylpyrrolidin-2-yl]carbonyl]amino]-1-thio-L-*threo*-α-D-*galacto*-octopyranoside (7-*epi*-lincomycin),

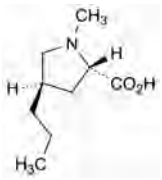

E. (2*S*,4*R*)-1-methyl-4-propylpyrrolidine-2-carboxylic acid (4-propyl hygric acid),

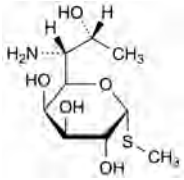

F. methyl 6-amino-6,8-dideoxy-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside (methyl-1-thiolincosaminide).

**04/2017:1232**

# LINOLEOYL MACROGOLGLYCERIDES

## Macrogolglyceridorum linoleates

DEFINITION

Mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols.

They are obtained by partial alcoholysis of an unsaturated oil mainly containing triglycerides of linoleic ((9*Z*,12*Z*)-octadeca-9,12-dienoic) acid, using macrogol with a mean relative molecular mass between 300 and 400, or by esterification of glycerol and macrogol with unsaturated fatty acids, or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of this unsaturated oil.

CHARACTERS

*Appearance*: amber, oily liquid which may give rise to a deposit after prolonged periods at 20 °C.

*Solubility*: practically insoluble but dispersible in water, freely soluble in methylene chloride.

*Relative density*: about 0.95 at 20 °C.

*Refractive index*: about 1.47 at 20 °C.

*Viscosity*: about 35 mPa·s at 40 °C.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.9 ($R_{st}$ 1) and spots due to 1,3-diglycerides ($R_{st}$ 0.7), to 1,2-diglycerides ($R_{st}$ 0.6), to monoglycerides ($R_{st}$ 0.1) and to esters of macrogol ($R_{st}$ 0).

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 45 to 65, determined on 1.0 g.

**Iodine value** (*2.5.4, Method A*): 90 to 110.

**Peroxide value** (*2.5.5, Method A*): maximum 12.0, determined on 2.0 g.

**Saponification value** (*2.5.6*): 150 to 170, determined on 2.0 g.

**Alkaline impurities**. Introduce 5.0 g into a test tube and carefully add a mixture, neutralised if necessary with *0.01 M hydrochloric acid* or with *0.01 M sodium hydroxide*, of 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*, 0.3 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Shake and allow to stand. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

**Free glycerol**: maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat if necessary. After cooling, add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate the iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance:*

- *palmitic acid*: 4.0 per cent to 20.0 per cent;
- *stearic acid*: maximum 6.0 per cent;
- *oleic acid*: 20.0 per cent to 35.0 per cent;
- *linoleic acid*: 50.0 per cent to 65.0 per cent;
- *linolenic acid*: maximum 2.0 per cent;
- *arachidic acid*: maximum 1.0 per cent;
- *eicosenoic acid*: maximum 1.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

STORAGE

Protected from light.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for linoleoyl macrogolglycerides used as self-emulsifying agents and solubilisers.*

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**Composition of fatty acids** (see Tests).

**07/2014:1908**

# LINSEED OIL, VIRGIN

## Lini oleum virginale

DEFINITION

Fatty oil obtained by cold expression from ripe seeds of *Linum usitatissimum* L. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, yellow or brownish-yellow liquid, on exposure to air turning dark and gradually thickening. When cooled, it becomes a soft mass at about – 20 °C.

*Solubility*: very slightly soluble in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.931.

*Refractive index*: about 1.480.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Iodine value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 4.5.

**Iodine value** (*2.5.4*): 160 to 200.

**Peroxide value** (*2.5.5, Method A*): maximum 15.0.

**Saponification value** (*2.5.6*): 188 to 195; carry out the saponification for 1 h.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*). Use the calibration mixture in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *fatty acids with a chain length less than $C_{16}$*: maximum 1.0 per cent,
- *palmitic acid*: 3.0 per cent to 8.0 per cent,
- *palmitoleic acid*: maximum 1.0 per cent,
- *stearic acid*: 2.0 per cent to 8.0 per cent,
- *oleic acid*: 11.0 per cent to 35.0 per cent,
- *linoleic acid*: 11.0 per cent to 24.0 per cent,
- *linolenic acid*: 35.0 per cent to 65.0 per cent,
- *arachidic acid*: maximum 1.0 per cent.

**Cadmium**: maximum 0.5 ppm, determined as described in general method *2.4.27. Heavy metals in herbal drugs and herbal drug preparations.*

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In an airtight container, protected from light.

**04/2008:0728**
**corrected 6.4**

# LIOTHYRONINE SODIUM

## Liothyroninum natricum

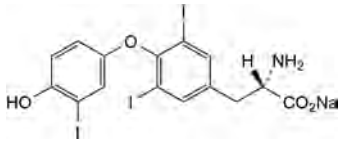


$C_{15}H_{11}I_3NNaO_4$ $M_r$ 673

[55-06-1]

DEFINITION

Sodium (2*S*)-2-amino-3-[4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]propanoate.

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly coloured, hygroscopic powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, C, E.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in *0.1 M sodium hydroxide* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 319 nm.

*Specific absorbance at the absorption maximum*: 63 to 69 (dried substance).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *liothyronine sodium CRS*.

D. To about 50 mg in a porcelain dish add a few drops of *sulfuric acid R* and heat. Violet vapour is evolved.

E. To 200 mg add 2 mL of *dilute sulfuric acid R*. Heat on a water-bath and then carefully over a naked flame, increasing the temperature gradually up to about 600 °C. Continue the ignition until most of the particles have disappeared. Dissolve the residue in 2 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 18.0 to + 22.0 (dried substance).

Dissolve 0.200 g in a mixture of 1 volume of *1 M hydrochloric acid* and 4 volumes of *ethanol (96 per cent) R* and dilute to 20.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light throughout the test.*

*Solution A*. Mix 10 volumes of mobile phase A with 90 volumes of *methanol R*.

*Solution B*. Mix 30 volumes of mobile phase B and 70 volumes of mobile phase A. Mix equal volumes of this solution with solution A.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 20 mL of solution A. Dilute 4.0 mL of this solution to 20.0 mL with solution B.

*Reference solution (a).* Dissolve 2.5 mg of *levothyroxine sodium CRS* (impurity A) and 2.5 mg of *liothyronine sodium CRS* in solution A and dilute to 25 mL with the same solution. Dilute 1.0 mL of this solution to 50.0 mL with solution B.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with solution B.

*Reference solution (c).* Dissolve the contents of a vial of *liothyronine for peak identification CRS* (containing impurities A, B, C, D and E) in solution B and dilute to 1.0 mL with the same solution.

*Reference solution (d).* Dissolve 20.0 mg of *liothyronine sodium CRS* in 20 mL of solution A. Dilute 4.0 mL of this solution to 20.0 mL with solution B.

*Blank solution*: solution B.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 9.7 g of *sulfamic acid R* in *water R* and dilute to 2000 mL with the same solvent; add 1.5 g of *sodium hydroxide R* and adjust to pH 2.0 with *2 M sodium hydroxide*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 4 | 75 → 70 | 25 → 30 |
| 4 - 14 | 70 | 30 |
| 14 - 44 | 70 → 20 | 30 → 80 |
| 44 - 54 | 20 | 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 25 µL of the test solution and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D and E.

- *relative retention* with reference to liothyronine (retention time = about 14 min): impurity B = about 0.2; impurity E = about 0.5; impurity A = about 1.4; impurity C = about 2; impurity D = about 2.4.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to impurity A and liothyronine in the chromatogram obtained with reference solution (a).

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurity E*: not more than 5 times the area of the peak due to liothyronine in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, C*: for each impurity, not more than 3 times the area of the peak due to liothyronine in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity D*: not more than twice the area of the peak due to liothyronine in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to liothyronine in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the peak due to liothyronine in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the peak due to liothyronine in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides**: maximum 2.0 per cent, expressed as NaCl (dried substance).

Dissolve 0.500 g in a 2 g/L solution of *sodium hydroxide R* and dilute to 100 mL with the same solvent. Add 15 mL of *dilute nitric acid R* and titrate with *0.05 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.05 M silver nitrate* is equivalent to 2.93 mg of NaCl.

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (d).

Calculate the percentage content of $C_{15}H_{11}I_3NNaO_4$ from the declared content of *liothyronine sodium CRS*.

STORAGE

In an airtight container, protected from light, at a temperature between 2 °C and 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

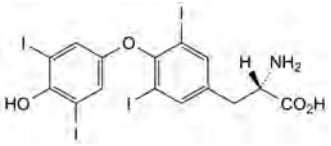

A. (2*S*)-2-amino-3-[4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)propanoic acid (levothyroxine),

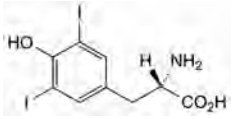

B. (2*S*)-2-amino-3-(4-hydroxy-3,5-diiodophenyl)propanoic acid (diiodotyrosine),

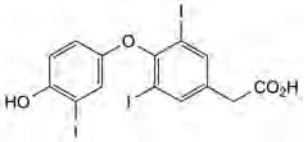

C. [4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]acetic acid (triiodothyroacetic acid),

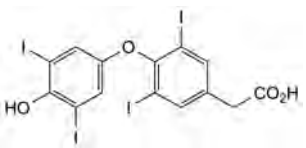

D. [4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl]-acetic acid (tetraiodothyroacetic acid),

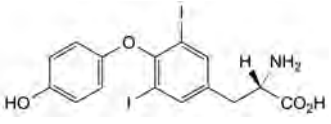

E. (2*S*)-2-amino-3-[4-(4-hydroxyphenoxy)-3,5-diiodophenyl]propanoic acid (diiodothyronine).

**04/2015:1120**

# LISINOPRIL DIHYDRATE

## Lisinoprilum dihydricum

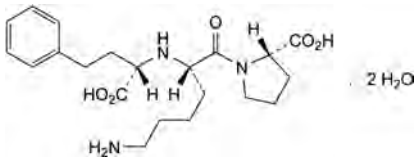

$C_{21}H_{31}N_3O_5,2H_2O$ $M_r$ 441.5
[83915-83-7]

DEFINITION

(2*S*)-1-[(2*S*)-6-Amino-2-[[(1*S*)-1-carboxy-3-phenylpropyl]-amino]hexanoyl]pyrrolidine-2-carboxylic acid dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in anhydrous ethanol and in heptane.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 47 to – 43 (anhydrous substance).

Dissolve 0.5 g in *zinc acetate solution R* and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lisinopril dihydrate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *lisinopril for system suitability A CRS* (containing impurities A and E) in mobile phase A and dilute to 1.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve the contents of a vial of *lisinopril impurity F CRS* in 1.0 mL of mobile phase A.

*Reference solution (d).* Dissolve 5 mg of *lisinopril for peak identification CRS* (containing impurity G) in mobile phase A and dilute to 1.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 3 volumes of *acetonitrile R1* and 97 volumes of a 3.12 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 3.8 with *dilute phosphoric acid R*;
– *mobile phase B*: mix 20.5 volumes of *acetonitrile R1* and 79.5 volumes of a 3.12 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 37 | 100 → 0 | 0 → 100 |
| 37 - 62 | 0 | 100 |

*Flow rate*: 1.6 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram supplied with *lisinopril for system suitability A CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity F; use the chromatogram supplied with *lisinopril for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to lisinopril (retention time = about 14 min): impurity A = about 0.7; impurity E = about 1.2; impurity F = about 1.9; impurity G = about 2.9.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to lisinopril and impurity E in the chromatogram obtained with reference solution (a);
– *signal-to-noise ratio*: minimum 45 for the principal peak in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents:*

– *correction factor*: multiply the peak area of impurity F by 2.1;
– for each impurity, use the concentration of lisinopril in reference solution (b).

*Limits*:

– *impurities A, E, F*: for each impurity, maximum 0.2 per cent;
– *impurity G*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent; disregard any peak with a retention time less than 3 min.

**Water** (*2.5.12*): 8.0 per cent to 9.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 40.55 mg of $C_{21}H_{31}N_3O_5$.

IMPURITIES

*Specified impurities: A, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, H, I.*

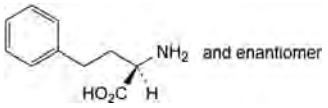


A. (2*RS*)-2-amino-4-phenylbutanoic acid,

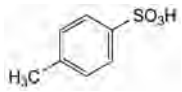


B. 4-methylbenzenesulfonic acid,

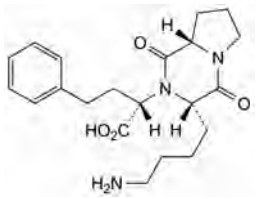

C. (2*S*)-2-[(3*S*,8a*S*)-3-(4-aminobutyl)-1,4-dioxohexahydropyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoic acid (*S*,*S*,*S*-diketopiperazine),

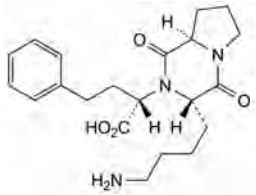

D. (2*S*)-2-[(3*S*,8a*R*)-3-(4-aminobutyl)-1,4-dioxohexahydropyrrolo[1,2-*a*]pyrazin-2(1*H*)-yl]-4-phenylbutanoic acid (*R*,*S*,*S*-diketopiperazine),

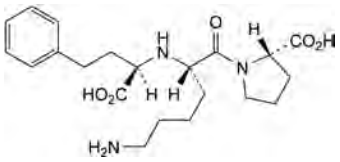

E. (2*S*)-1-[(2*S*)-6-amino-2-[[(1*R*)-1-carboxy-3-phenylpropyl]amino]hexanoyl]pyrrolidine-2-carboxylic acid (lisinopril *R*,*S*,*S*-isomer),

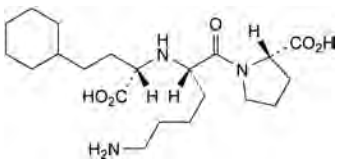

F. (2*S*)-1-[(2*S*)-6-amino-2-[[(1*S*)-1-carboxy-3-cyclohexylpropyl]amino]hexanoyl]pyrrolidine-2-carboxylic acid (cyclohexyl analogue),

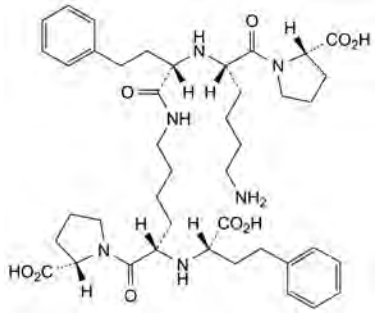

G. (2*S*)-1-[(2*S*)-6-amino-2-[[(2*S*)-1-[[[(5*S*)-5-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]-6-[(2*S*)-2-carboxypyrrolidin-1-yl]-6-oxohexyl]amino]carbonyl]-3-phenylpropyl]amino]hexanoyl]pyrrolidine-2-carboxylic acid (lisinopril dimer),

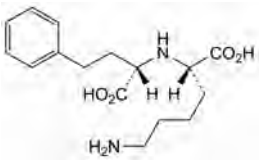

H. (2*S*)-6-amino-2-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]-hexanoic acid,

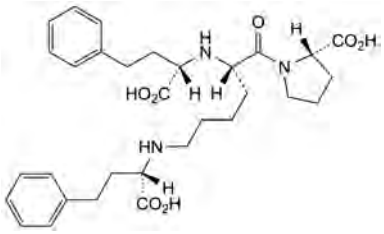

I. (2*S*)-1-[(2*S*)-2,6-bis[[(1*S*)-1-carboxy-3-phenylpropyl]-amino]hexanoyl]pyrrolidine-2-carboxylic acid.

**01/2017:0228**

# LITHIUM CARBONATE

## Lithii carbonas

$Li_2CO_3$ $M_r$ 73.9
[554-13-2]

DEFINITION

*Content*: 98.5 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. When moistened with *hydrochloric acid R*, it gives a red colour to a non-luminous flame.

B. Dissolve 0.2 g in 1 mL of *hydrochloric acid R*. Evaporate to dryness on a water-bath. The residue dissolves in 3 mL of *ethanol (96 per cent) R*.

C. It gives the reaction of carbonates (*2.3.1*).

TESTS

**Solution S**. Suspend 10.0 g in 30 mL of *distilled water R* and dissolve by the addition of 22 mL of *nitric acid R*. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 100 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Disperse 1.25 g in 5 mL of *distilled water R* and dissolve by adding 5 mL of *hydrochloric acid R1*. Boil for 2 min. Cool and add *dilute sodium hydroxide solution R* until neutral. Dilute to 25 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium** (*2.4.6*): maximum 150 ppm.

Dilute 1 mL of solution S to 10 mL with *water R*. Dilute 6.7 mL of this solution to 10 mL with *water R*.

**Potassium**: maximum 300 ppm.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. Dissolve 1.0 g in 10 mL of *hydrochloric acid R1* and dilute to 50.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using a solution of *potassium chloride R* containing 500 µg of K per millilitre, diluted as necessary with *water R*.

*Wavelength*: 766.5 nm.

**Sodium**: maximum 300 ppm.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. Dissolve 1.0 g in 10 mL of *hydrochloric acid R1* and dilute to 50.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using a solution of *sodium chloride R* containing 500 µg of Na per millilitre, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

ASSAY

Dissolve 0.250 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection.

1 mL of *1 M hydrochloric acid* is equivalent to 36.95 mg of $Li_2CO_3$.

**01/2017:0621**

# LITHIUM CITRATE

## Lithii citras

$C_6H_5Li_3O_7,4H_2O$ $M_r$ 282.0

DEFINITION

Trilithium 2-hydroxypropane-1,2,3-tricarboxylate tetrahydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, fine crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. When moistened with *hydrochloric acid R*, it gives a red colour to a non-luminous flame.

B. Dilute 3 mL of solution S (see Tests) to 10 mL with *water R*. Add 3 mL of *potassium ferriperiodate solution R*. A white or yellowish-white precipitate is formed.

C. To 1 mL of solution S add 4 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Readily carbonisable substances**. To 0.20 g of the powdered substance to be examined add 10 mL of *sulfuric acid R* and heat in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution $Y_2$ or $GY_2$ (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Oxalates**: maximum 300 ppm, calculated as anhydrous oxalate ion.

Dissolve 0.50 g in 4 mL of *water R*, add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 4 mL of a 0.05 g/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 3 mL of solution S add 2 mL of *hydrochloric acid R1* and dilute to 17 mL with *distilled water R*. Prepare the reference solution using 15 mL of a mixture of 2 mL of *hydrochloric acid R1* and 15 mL of *sulfate standard solution (10 ppm $SO_4$) R* and compare the opalescence after 15 min.

**Water** (*2.5.12*): 24.0 per cent to 27.0 per cent, determined on 0.100 g. After adding the substance to be examined, stir for 15 min before titrating. Carry out a blank titration.

ASSAY

Dissolve 80.0 mg in 50 mL of *anhydrous acetic acid R*, heating to about 50 °C. Allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 mL of *naphtholbenzein solution R* as indicator, until the colour changes from yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 7.00 mg of $C_6H_5Li_3O_7$.

STORAGE

In an airtight container.

**01/2008:1988**

# LOBELINE HYDROCHLORIDE

## Lobelini hydrochloridum

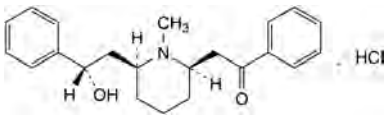


$C_{22}H_{28}ClNO_2$ $M_r$ 373.9

[134-63-4]

DEFINITION

2-[(2*R*,6*S*)-6-[(2*S*)-2-Hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, microcrystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent), soluble in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification*: *B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lobeline hydrochloride CRS.*

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

C. Examine the chromatograms obtained in the test for foreign alkaloids.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

TESTS

**Solution S**. Dissolve 0.250 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.6 to 6.4 for solution S.

**Specific optical rotation** (*2.2.7*): – 55 to – 59 (dried substance), determined on solution S.

**Foreign alkaloids**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dilute 0.1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (b).* Dissolve 10 mg of *lobeline hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *diethylamine R*, *cyclohexane R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 120 °C.

*Detection*: examine in ultraviolet light at 254 nm.

*Limits*: in the chromatogram obtained with test solution (a):

– *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *phenytoin CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. To 1 mL of the solution add 0.1 mL of the test solution and dilute to 25 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm,

– *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.0 g of *sodium methanesulfonate R* and 2.50 g of *disodium hydrogen phosphate dihydrate R* in a mixture of 3 volumes of a 6.7 per cent *V/V* solution of *phosphoric acid R*, 29 volumes of *acetonitrile R* and 70 volumes of *water R* and dilute to 1000 mL with the same mixture of solvents.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: 2 times the retention time of lobeline which is about 17 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to phenytoin and to lobeline.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: maximum of 2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

– *disregard level*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 0.1 per cent, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.300 g in 50 mL of *ethanol (96 per cent) R*. Add 5 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.39 mg of $C_{22}H_{28}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

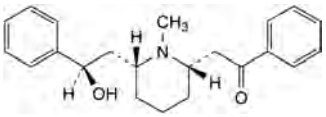


A. 2-[(2*S*,6*R*)-6-[(2*R*)-2-hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone ((+)-lobeline),

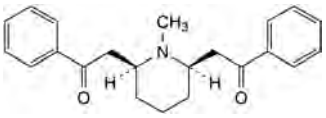


B. 2,2′-[(2*R*,6*S*)-1-methylpiperidine-2,6-diyl]bis(1-phenylethanone) (lobelanine),

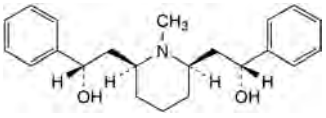


C. *meso*-(1*R*,1′*S*)-2,2′-[(2*R*,6*S*)-1-methylpiperidine-2,6-diyl]bis(1-phenylethanol) (lobelanidine),

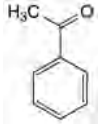


D. acetophenone.

**01/2014:0928**
**corrected 10.0**

# LOMUSTINE

## Lomustinum

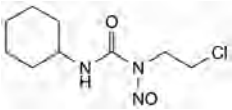

$C_9H_{16}ClN_3O_2$ $M_r$ 233.7
[13010-47-4]

DEFINITION

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, soluble in ethanol (96 per cent).

*Carry out the tests protected from light and prepare all the solutions immediately before use.*

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lomustine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Phosphate buffer solution.* Dissolve 1.36 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *dicyclohexylurea R* (impurity C) in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *acetonitrile R1*. Mix 1.0 mL of this solution and 1.0 mL of the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, phosphate buffer solution (20:80 *V/V*);
- *mobile phase B*: phosphate buffer solution, *acetonitrile R1* (24:76 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 75 | 25 |
| 2 - 17 | 75 → 40 | 25 → 60 |
| 17 - 34 | 40 → 30 | 60 → 70 |
| 34 - 42 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to lomustine (retention time = about 23 min): impurity C = about 0.7.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity C and lomustine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of lomustine in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dissolve 0.24 g in 4 mL of *methanol R* and add 20 mL of *water R*. Allow to stand for 20 min and filter. To 10 mL of the filtrate, add 5 mL of *methanol R*. When preparing the standard, replace the 5 mL of *water R* with 5 mL of *methanol R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

ASSAY

Dissolve 0.200 g in about 3 mL of *ethanol (96 per cent) R* and add 20 mL of a 200 g/L solution of *potassium hydroxide R* and boil under a reflux condenser for 2 h. Add 75 mL of *water R* and 4 mL of *nitric acid R*. Cool and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 23.37 mg of $C_9H_{16}ClN_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

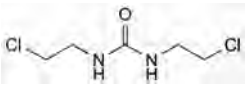

A. 1,3-bis(2-chloroethyl)urea,

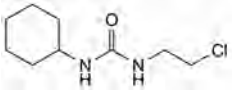

B. 1-(2-chloroethyl)-3-cyclohexylurea,

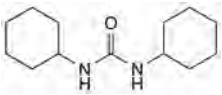

C. 1,3-dicyclohexylurea.

07/2019:0929

# LOPERAMIDE HYDROCHLORIDE

## Loperamidi hydrochloridum

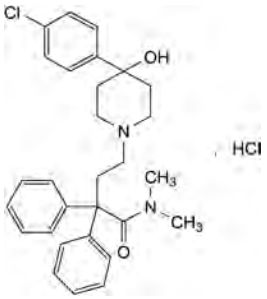

$C_{29}H_{34}Cl_2N_2O_2$ $M_r$ 513.5
[34552-83-5]

DEFINITION

4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *loperamide hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10.0 mg of *loperamide for system suitability CRS* (containing impurities A, B, D, E, G and H) in *methanol R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 17.0 g/L solution of *tetrabutylammonium hydrogen sulfate R1*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 30 | 10 → 70 |
| 15 - 17 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *loperamide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, D, E, G and H.

*Relative retention* with reference to loperamide (retention time = about 6 min): impurity D = about 0.8; impurity G = about 1.20; impurity H = about 1.25; impurity E = about 1.45; impurity A = about 1.50; impurity B = about 1.9.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity H; minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity D by 1.7;
- for each impurity, use the concentration of loperamide hydrochloride in reference solution (b).

*Limits*:

- *impurities B, D*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 51.35 mg of $C_{29}H_{34}Cl_2N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F, G, H*.

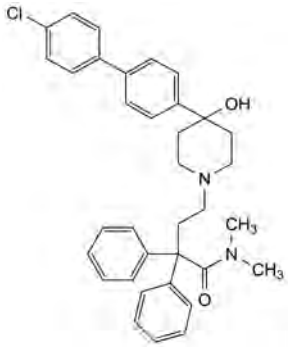

A. 4-[4-(4′-chloro-[1,1′-biphenyl]-4-yl)-4-hydroxypiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide,

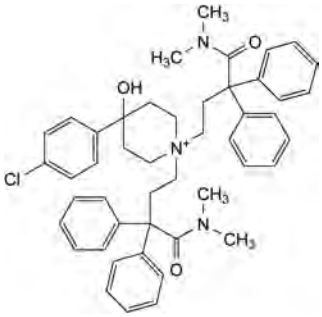

B. 4-(4-chlorophenyl)-1,1-bis[4-(dimethylamino)-4-oxo-3,3-diphenylbutyl]-4-hydroxypiperidin-1-ium,

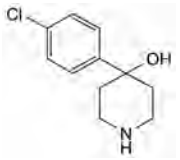

C. 4-(4-chlorophenyl)piperidin-4-ol,

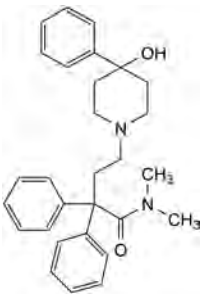

D. 4-(4-hydroxy-4-phenylpiperidin-1-yl)-*N*,*N*-dimethyl-2,2-diphenylbutanamide,

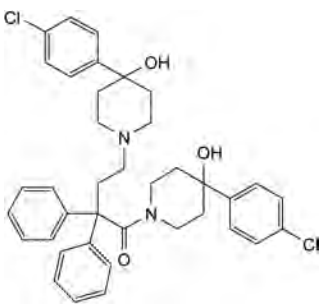

E. 1,4-bis[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-2,2-diphenylbutan-1-one,

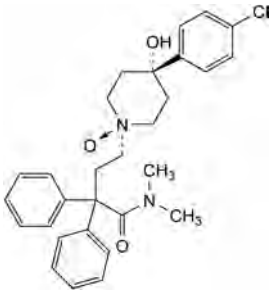

F. (1*r*,4*r*)-4-(4-chlorophenyl)-1-[4-(*N*,*N*-dimethylamino)-3,3-diphenyl-4-oxobutyl]-4-hydroxypiperazine 1-oxide (loperamide oxide),

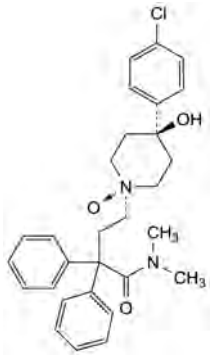

G. (1*s*,4*s*)-4-(4-chlorophenyl)-1-[4-(*N*,*N*-dimethylamino)-3,3-diphenyl-4-oxobutyl]-4-hydroxypiperazine 1-oxide,

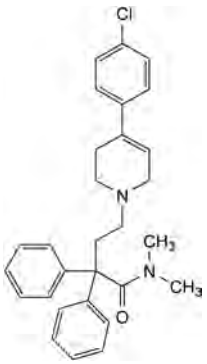

H. 4-[4-(4-chlorophenyl)-3,6-dihydropyridin-1(2*H*)-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide.

**01/2008:1729**
**corrected 7.0**

# LOPERAMIDE OXIDE MONOHYDRATE

## Loperamidi oxidum monohydricum

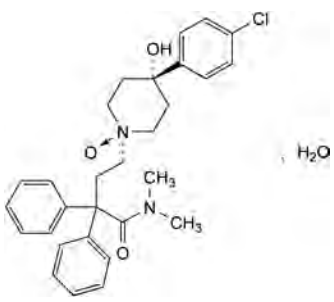

$C_{29}H_{33}ClN_2O_3,H_2O$ $M_r$ 511.1

DEFINITION

4-[*trans*-4-(4-Chlorophenyl)-4-hydroxy-1-oxidopiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, slightly hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in alcohol and in methylene chloride.

mp: about 152 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *loperamide oxide monohydrate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *loperamide hydrochloride CRS* in *methanol R*, add 0.5 mL of the test solution and dilute to 100.0 mL with *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 25.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm),
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 17.0 g/L solution of *tetrabutylammonium hydrogen sulfate R1*,
- *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 30 | 10 → 70 |
| 15 - 17 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to loperamide oxide (retention time = about 7 min): impurity A = about 0.9; impurity B = about 1.11; impurity C = about 1.13.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.8 between the peaks due to loperamide oxide and impurity A.

*Limits*:

- *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent),
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 3.4 per cent to 4.2 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid* using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 49.30 mg of $C_{29}H_{33}ClN_2O_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

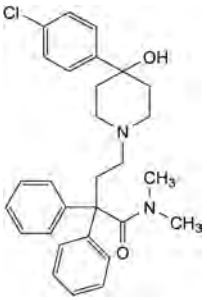


A. 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide (loperamide),

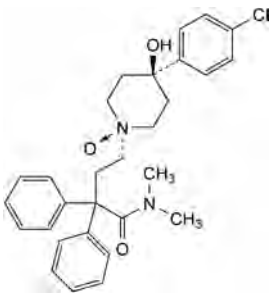


B. 4-[*cis*-4-(4-chlorophenyl)-4-hydroxy-1-oxidopiperidin-1-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide,

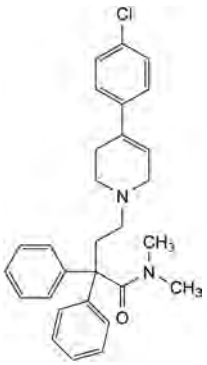


C. 4-[4-(4-chlorophenyl)-3,6-dihydropyridin-1(2*H*)-yl]-*N*,*N*-dimethyl-2,2-diphenylbutanamide.

**01/2017:2615**

# LOPINAVIR

## Lopinavirum

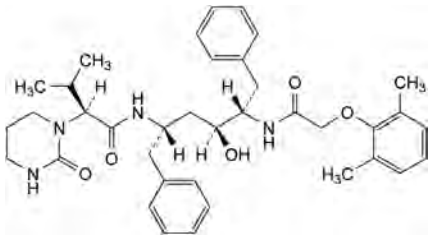

$C_{37}H_{48}N_4O_5$ $M_r$ 629
[192725-17-0]

DEFINITION

(2*S*)-*N*-[(1*S*,3*S*,4*S*)-1-Benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxo-tetrahydropyrimidin-1(2*H*)-yl]butanamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, slightly hygroscopic powder.

*Solubility*: practically insoluble in water, very soluble in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lopinavir CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): − 27.0 to − 22.0 (anhydrous substance).

Dissolve 0.200 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (50:50 *V/V*).

*Phosphate buffer solution*. Dissolve 0.9 g of *dipotassium hydrogen phosphate R* and 2.7 g of *potassium dihydrogen phosphate R* in 900 mL of *water R* and mix well. Adjust to pH 6.0 with *phosphoric acid R*, dilute to 1000 mL with *water R* and filter.

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *lopinavir CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of test solution (b) to 250.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2.5 mg of *lopinavir for system suitability CRS* (containing impurities A, B, C, F, G, I, N, Q, R, S and T) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 2.5 mg of *lopinavir for peak identification CRS* (containing impurities D and O) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, phosphate buffer solution (45:55 *V/V*);
– *mobile phase B*: phosphate buffer solution, *acetonitrile R1* (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 100 | 0 |
| 60 - 61 | 100 → 0 | 0 → 100 |
| 61 - 81 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *lopinavir for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, F, G, I and N; use the chromatogram supplied with *lopinavir for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention r* (not $r_G$) with reference to lopinavir (retention time = about 37 min): impurity A = about 0.03; impurity B = about 0.07; impurity C = about 0.10; impurity D = about 0.13; impurity F = about 0.59; impurity G = about 0.62; impurity I = about 1.1; impurity N = about 1.4.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities F and G.

*Calculation of percentage contents*:

– for impurity A, multiply the peak area by the correction factor 1.6;
– for impurity B, multiply the peak area by the correction factor 1.3;
– for impurity C, multiply the peak area by the correction factor 1.5;
– for impurity D, multiply the peak area by the correction factor 1.3;
– for each impurity, use the concentration of lopinavir in reference solution (b).

*Limits*:

– *impurities B, I*: for each impurity, maximum 0.2 per cent;
– *impurities A, C, D, F, G*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *reporting threshold*: 0.05 per cent; disregard any peak eluting after impurity N.

B. Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Mobile phase*: mobile phase A, mobile phase B (30:70 *V/V*).

*Run time*: 8.3 times the retention time of lopinavir.

*Identification of impurities*: use the chromatogram supplied with *lopinavir for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities Q, R, S and T; use the chromatogram supplied with *lopinavir for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity O.

*Relative retention r* (not $r_G$) with reference to lopinavir (retention time = about 6 min): impurity N = about 1.4; impurity O = about 1.5; impurity Q = about 4.4; impurity R = about 6.0; impurity S = about 7.1; impurity T = about 8.5.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to impurities S and T.

*Calculation of percentage contents*:

– for impurity O, multiply the peak area by the correction factor 1.3;

– for impurity Q, multiply the peak area by the correction factor 0.7;

– for each impurity, use the concentration of lopinavir in reference solution (b).

*Limits*:

– *impurities O, Q, R, T*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak eluting before and including impurity N;

– *total of all impurities eluting before and including impurity N in test A and after impurity N in test B*: maximum 0.7 per cent.

**Water** (*2.5.12*): maximum 4.4 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Mobile phase*: mobile phase A.

*Injection*: test solution (b) and reference solution (a).

*Run time*: 1.6 times the retention time of lopinavir.

Calculate the percentage content of $C_{37}H_{48}N_4O_5$ taking into account the assigned content of *lopinavir CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, F, G, I, O, Q, R, T.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, H, J, K, L, M, N, P, S.*

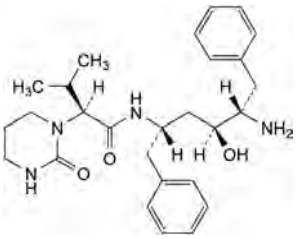

A. (2*S*)-*N*-[(1*S*,3*S*,4*S*)-1-benzyl-4-amino-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

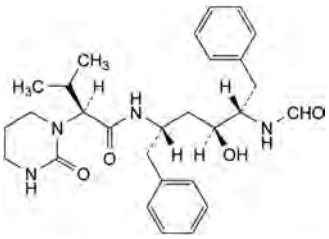

B. (2*S*)-*N*-[(1*S*,3*S*,4*S*)-1-benzyl-4-(formylamino)-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

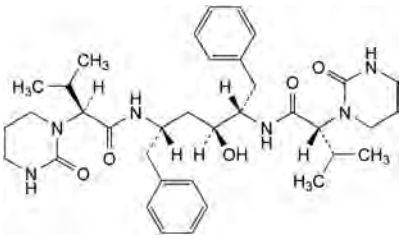

C. (2*R*)-*N*-[(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanoyl]amino]-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

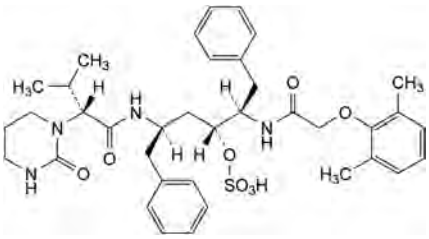

D. (1*R*,3*R*)-1-[(1*R*)-1-[[2-(2,6-dimethylphenoxy)acetyl]-amino]-2-phenylethyl]-3-[[(2*R*)-3-methyl-2-[2-oxo-tetrahydropyrimidin-1(2*H*)-yl]butanoyl]amino]-4-phenylbutyl hydrogen sulfate,

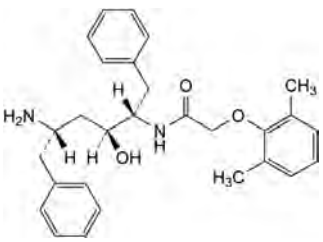

E. *N*-[(1*S*,2*S*,4*S*)-4-amino-1-benzyl-2-hydroxy-5-phenylpentyl]-2-(2,6-dimethylphenoxy)acetamide,

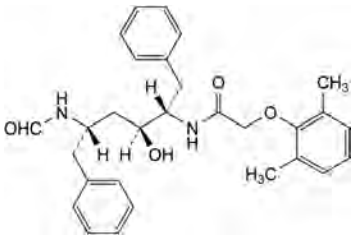

F. *N*-[(1*S*,2*S*,4*S*)-1-benzyl-4-(formylamino)-2-hydroxy-5-phenylpentyl]-2-(2,6-dimethylphenoxy)acetamide,

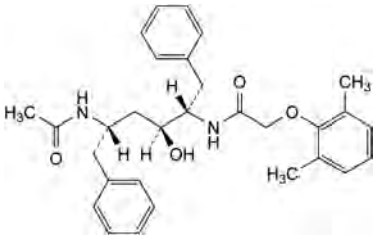

G. *N*-[(1*S*,2*S*,4*S*)-(4-acetylamino)-1-benzyl-2-hydroxy-5-phenylpentyl]-2-(2,6-dimethylphenoxy)acetamide,

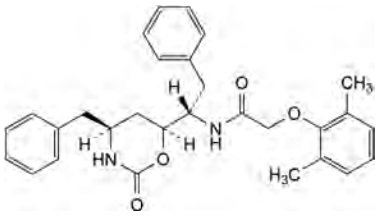

H. *N*-[(1*S*)-1-[(4*S*,6*S*)-4-benzyl-2-oxo-1,3-oxazinan-6-yl]-2-phenylethyl]-2-(2,6-dimethylphenoxy)acetamide,

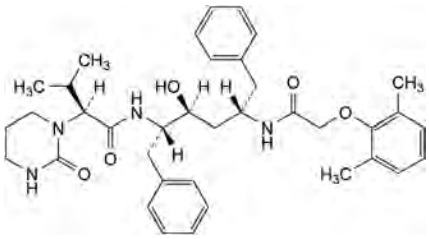

I. (2*S*)-*N*-[(1*S*,2*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-2-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

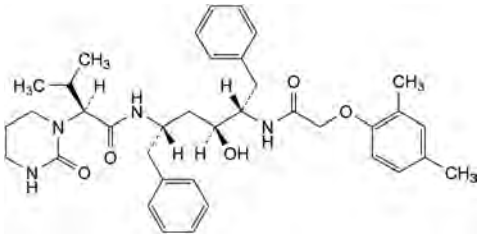

J. (2*S*)-*N*-[(1*S*,3*S*,4*S*)-1-benzyl-4-[[2-(2,4-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

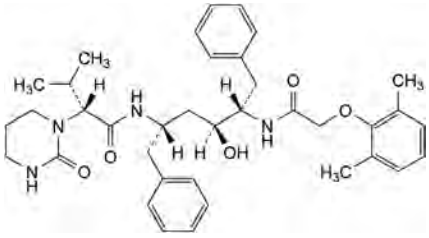

K. (2*R*)-*N*-[(1*S*,3*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

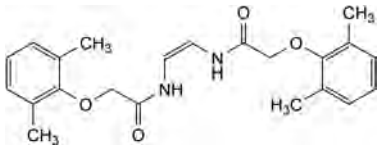

L. *N*,*N*′-(*Z*)-ethene-1,2-diylbis[2-(2,6-dimethylphenoxy)-acetamide],

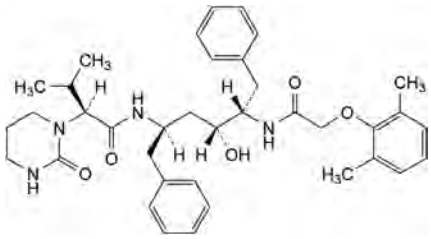

M. (2*S*)-*N*-[(1*R*,3*R*,4*S*)-1-benzyl-4-[[2-(2,6-dimethyl-phenoxy)acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]-butanamide,

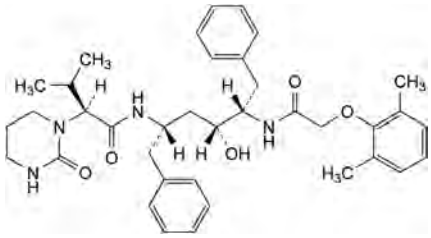

N. (2*S*)-*N*-[(1*S*,3*R*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

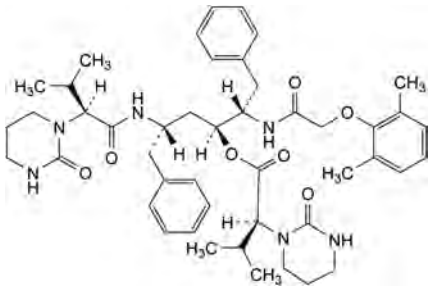

O. (1*S*,3*S*)-1-[(1*S*)-1-[[2-(2,6-dimethylphenoxy)acetyl]-amino]-2-phenylethyl]-3-[[(2*S*)-3-methyl-2-[2-oxo-tetrahydropyrimidin-1(2*H*)-yl]butanoyl]amino]-4-phenylbutyl (2*S*)-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanoate,

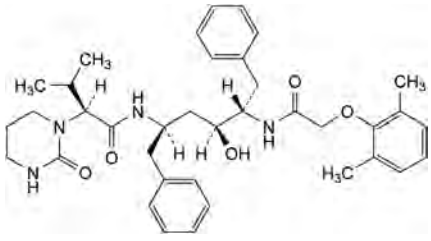

P. (2*S*)-*N*-[(1*R*,3*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-3-methyl-2-[2-oxotetrahydropyrimidin-1(2*H*)-yl]butanamide,

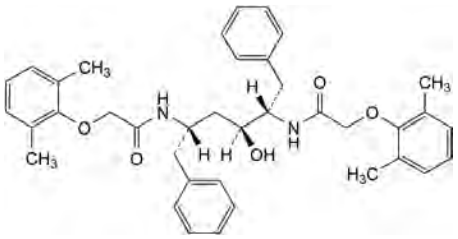

Q. *N*-[(1*S*,2*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-2-hydroxy-5-phenylpentyl]-2-(2,6-dimethylphenoxy)acetamide,

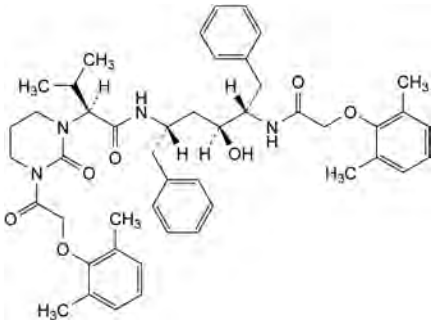

R. (2*S*)-*N*-[(1*S*,3*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethylphenoxy)-acetyl]amino]-3-hydroxy-5-phenylpentyl]-2-[3-[2-(2,6-dimethylphenoxy)acetyl]-2-oxotetrahydropyrimidin-1(2*H*)-yl]-3-methylbutanamide,

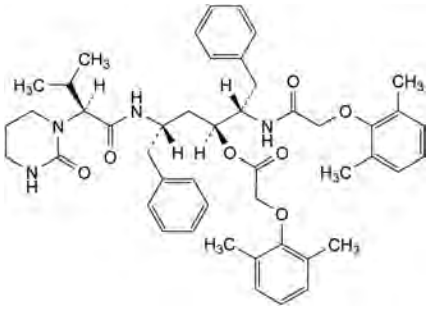

S. (1*S*,3*S*)-1-[(1*S*)-1-[[2-(2,6-dimethylphenoxy)acetyl]-amino]-2-phenylethyl]-3-[[(2*S*)-3-methyl-2-[2-oxo-tetrahydropyrimidin-1(2*H*)-yl]butanoyl]amino]-4-phenylbutyl 2-(2,6-dimethylphenoxy)acetate,

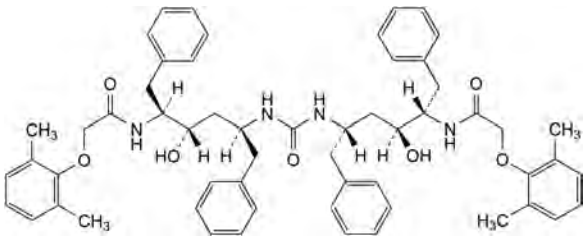

T. *N*,*N*′-bis[(1*S*,3*S*,4*S*)-1-benzyl-4-[[2-(2,6-dimethyl-phenoxy)acetyl]amino]-3-hydroxy-5-phenylpentyl]urea.

**01/2010:2124**
**corrected 10.0**

# LORATADINE

## Loratadinum

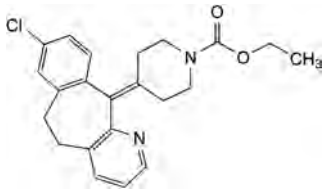

$C_{22}H_{23}ClN_2O_2$ $M_r$ 382.9
[79794-75-5]

DEFINITION

Ethyl 4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta-[1,2-*b*]pyridin-11-ylidene)piperidine-1-carboxylate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *loratadine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Impurity H**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 25 mg of *isoamyl benzoate R* in *methylene chloride R* and dilute to 100 mL with the same solvent. Dilute 5.0 mL of this solution to 50 mL with *methylene chloride R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in *methylene chloride R*, add 1.0 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 5.0 mL with *methylene chloride R*.

*Reference solution (a)*. Dissolve 25.0 mg of *loratadine impurity H CRS* in *methylene chloride R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with *methylene chloride R*.

*Reference solution (b)*. To 1.0 mL of reference solution (a) add 1.0 mL of the internal standard solution and dilute to 5.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.52 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 80 |
| | 1 - 23 | 80 → 300 |
| | 23 - 33 | 300 |
| Injection port | | 260 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solution (b).

*Relative retention* with reference to loratadine (retention time = about 32 min): impurity H = about 0.33; isoamyl benzoate = about 0.37.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity H and isoamyl benzoate;
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity H.

*Limit*:

- *impurity H*: calculate the ratio (*R*) of the area of the peak due to impurity H to the area of the peak due to isoamyl benzoate from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of the peak due to impurity H to the area of the peak due to isoamyl benzoate: this ratio is not greater than twice *R* (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *loratadine impurity F CRS* in the mobile phase and dilute to 25 mL with the mobile phase. Dilute 1 mL of this solution to 10 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *loratadine for system suitability CRS* (containing impurities A and E) in the mobile phase, add 0.5 mL of reference solution (a) and dilute to 5 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with very low silanol activity;
- *temperature*: 40 °C.

*Mobile phase*: mix 30 volumes of *methanol R*, 35 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.80 ± 0.05 with *phosphoric acid R* and 40 volumes of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 5 times the retention time of loratadine.

*Identification of impurities*: use the chromatogram supplied with *loratadine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and E.

*Relative retention* with reference to loratadine (retention time = about 12 min): impurity D = about 0.2; impurity B = about 0.4; impurity F = about 0.9; impurity E = about 1.1; impurity A = about 2.4; impurity C = about 2.7.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to loratadine.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.7; impurity F = 1.6; impurity E = 1.9;
- *impurity F*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 150 ppm.

Ignite 1.33 g at 800 ± 25 °C and take up the residue with 20 mL of *distilled water R*. Filter, if necessary, through paper free from sulfates. Repeat the filtration with new paper filters until the filtrate is no longer turbid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 38.29 mg of $C_{22}H_{23}ClN_2O_2$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

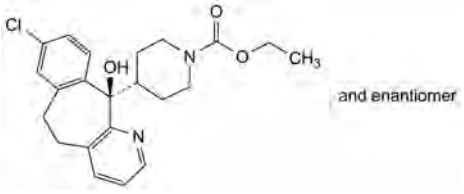


A. ethyl 4-[(11*RS*)-8-chloro-11-hydroxy-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-yl]piperidine-1-carboxylate,

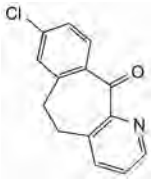

B. 8-chloro-5,6-dihydro-11*H*-benzo[5,6]cyclohepta-[1,2-*b*]pyridin-11-one,

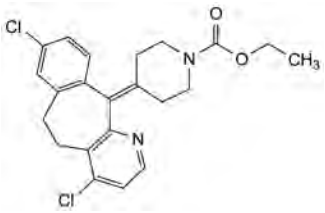

C. ethyl 4-(4,8-dichloro-5,6-dihydro-11*H*-benzo[5,6]cyclo-hepta[1,2-*b*]pyridin-11-ylidene)piperidine-1-carboxylate,

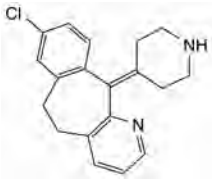

D. 8-chloro-11-(piperidin-4-ylidene)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine,

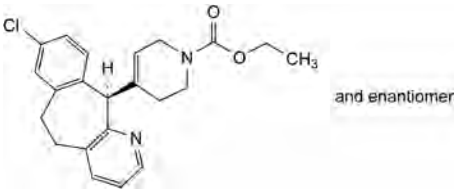


E. ethyl 4-[(11*RS*)-8-chloro-6,11-dihydro-5*H*-benzo-[5,6]cyclohepta[1,2-*b*]pyridin-11-yl]-3,6-dihydropyridine-1(2*H*)-carboxylate,

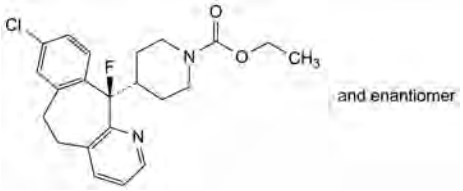


F. ethyl 4-[(11*RS*)-8-chloro-11-fluoro-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridin-11-yl]piperidine-1-carboxylate,

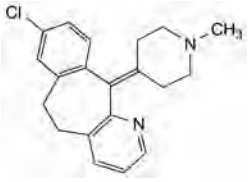

G. 8-chloro-11-(1-methylpiperidin-4-ylidene)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine,

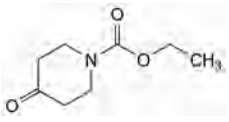

H. ethyl 4-oxopiperidine-1-carboxylate.

**01/2008:1121**
**corrected 6.0**

# LORAZEPAM

## Lorazepamum

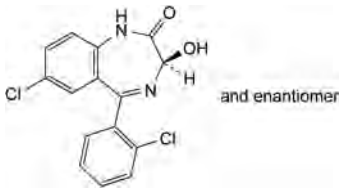


$C_{15}H_{10}Cl_2N_2O_2$ $M_r$ 321.2
[846-49-1]

DEFINITION

(3*RS*)-7-Chloro-5-(2-chlorophenyl)-3-hydroxy-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 98.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent), sparingly soluble or slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Spectral range*: 600-2000 $cm^{-1}$.

*Comparison*: *lorazepam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 40.0 mg of the substance to be examined in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (b).* Dissolve the contents of a vial of *lorazepam for system suitability CRS* (containing impurities B and D) in 1.0 mL of a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Reference solution (c).* Dissolve 4.0 mg of *lorazepam impurity D CRS* in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R1* and *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) resistant to bases up to pH 11.5.

*Mobile phase*:

- *mobile phase A*: dissolve 3.48 g of *dipotassium hydrogen phosphate R* in a mixture of 50 mL of *acetonitrile R1* and 850 mL of *water R*; adjust the apparent pH to 10.5 with a 40 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 35 | 80 → 30 | 20 → 70 |
| 35 - 50 | 30 | 70 |
| 50 - 60 | 30 → 80 | 70 → 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Relative retention* with reference to lorazepam (retention time = about 17 min): impurity D = about 0.9; impurity B = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.5 between the peaks due to impurity D and lorazepam;
- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lorazepam.

*Limits*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g under high vacuum at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Protect the solution from atmospheric carbon dioxide throughout the titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 32.12 mg of $C_{15}H_{10}Cl_2N_2O_2$.

IMPURITIES

*Specified impurities: B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E.*

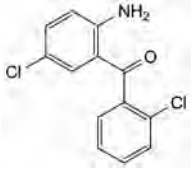

A. (2-amino-5-chlorophenyl)(2-chlorophenyl)methanone,

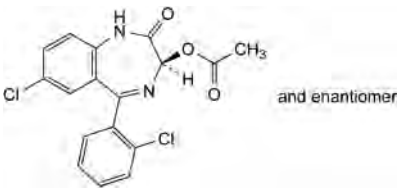

B. (3*RS*)-7-chloro-5-(2-chlorophenyl)-2-oxo-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl acetate,

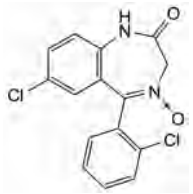

C. 7-chloro-5-(2-chlorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide,

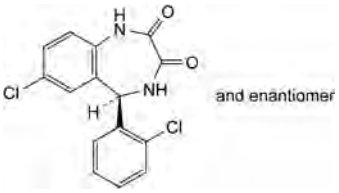

D. (5*RS*)-7-chloro-5-(2-chlorophenyl)-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione,

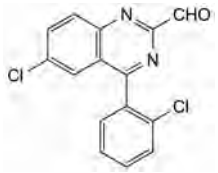

E. 6-chloro-4-(2-chlorophenyl)quinazoline-2-carbaldehyde.

**01/2020:2232**

# LOSARTAN POTASSIUM

## Losartanum kalicum

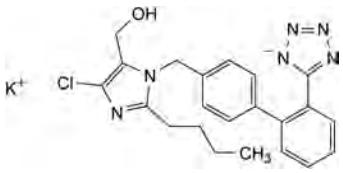

$C_{22}H_{22}ClKN_6O$ $M_r$ 461.0

[124750-99-8]

DEFINITION

Potassium 5-[4′-[[2-butyl-4-chloro-5-(hydroxymethyl)-1*H*-imidazol-1-yl]methyl]biphenyl-2-yl]tetrazol-1-ide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

PRODUCTION

As *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are classified as probable human carcinogens, manufacturers must ensure that their manufacturing process does not generate such impurities and develop appropriate control strategies. To allow manufacturers to make the necessary changes to their process, a transition period has

been agreed by competent authorities and strict temporary limits on levels of these impurities introduced in the Test section.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water and in methanol, slightly soluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *losartan potassium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve 25 mg in 3 mL of *water R*. The solution gives reaction (a) of potassium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 30.0 mg of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 6 mg of *triphenylmethanol R* (impurity G) in 100 mL of *methanol R*. Dilute 1 mL of the solution to 100 mL with *methanol R*. Use 1 mL of this solution to dissolve the contents of a vial of *losartan for system suitability CRS* (containing impurities J, K, L and M) and sonicate for 5 min.

*Reference solution (c).* Dissolve 3.0 mg of *losartan impurity D CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.5 mL of this solution to 100.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1.0 mL of *phosphoric acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 75 | 25 |
| 5 - 30 | 75 → 10 | 25 → 90 |
| 30 - 40 | 10 | 90 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *losartan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities G, J, K, L and M; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to losartan (retention time = about 14 min): impurity D = about 0.9; impurity J = about 1.4; impurity K = about 1.5; impurity L = about 1.6; impurity M = about 1.75; impurity G = about 1.8.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity M and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity G.

*Limits*:

- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *impurities J, K, L, M*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Nitrosamines**. Carry out the test by a suitable method.

The substance to be examined does not contain either NDMA or NDEA above the limits provided below or both impurities at whatever level:

- *N-nitrosodimethylamine (NDMA)*: maximum 0.640 ppm;
- *N-nitrosodiethylamine (NDEA)*: maximum 0.177 ppm.

ASSAY

Dissolve 0.200 g in 75 mL of *anhydrous acetic acid R* and sonicate for 10 min. Carry out a potentiometric titration (*2.2.20*) using *0.1 M perchloric acid*.

1 mL of *0.1 M perchloric acid* is equivalent to 23.05 mg of $C_{22}H_{22}ClKN_6O$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *D, J, K, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, F, G, H, I.*

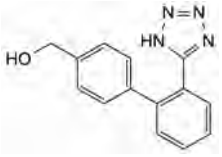


B. [2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methanol,

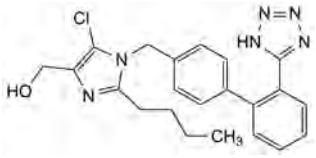

C. [2-butyl-5-chloro-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-4-yl]methanol,

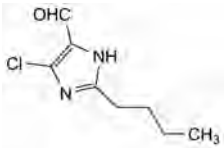

D. 2-butyl-4-chloro-1*H*-imidazole-5-carbaldehyde,

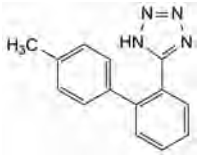

E. 5-(4′-methylbiphenyl-2-yl)-1*H*-tetrazole,

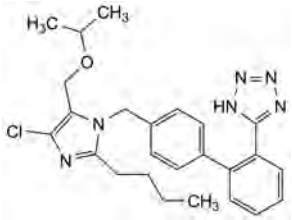

F. 5-[4′-[[2-butyl-4-chloro-5-[[(1-methylethyl)oxy]methyl]-1*H*-imidazol-1-yl]methyl]biphenyl-2-yl]-1*H*-tetrazole,

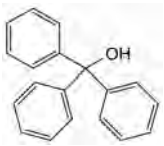

G. triphenylmethanol,

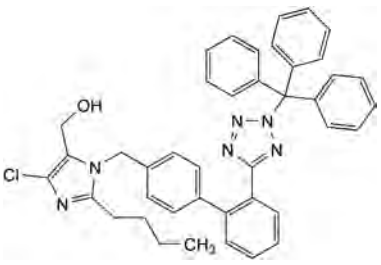

H. [2-butyl-4-chloro-1-[[2′-[2-(triphenylmethyl)-2*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-1*H*-imidazol-5-yl]methanol,

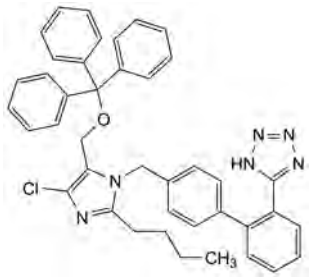

I. 5-[4′-[[2-butyl-4-chloro-5-[[(triphenylmethyl]oxy]-methyl]-1*H*-imidazol-1-yl]methyl]biphenyl-2-yl]-1*H*-tetrazole,

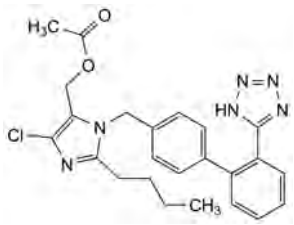

J. [2-butyl-4-chloro-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-5-yl]methyl acetate,

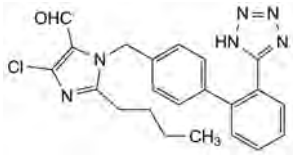

K. 2-butyl-4-chloro-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-5-carbaldehyde,

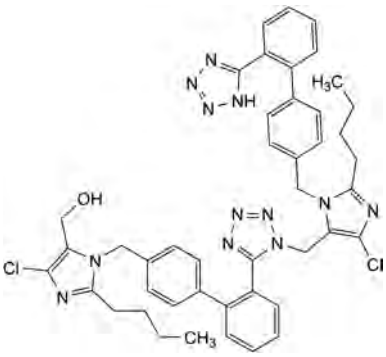

L. [2-butyl-1-[[2′-[1-[[2-butyl-4-chloro-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-5-yl]methyl]-1*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-4-chloro-1*H*-imidazol-5-yl]methanol,

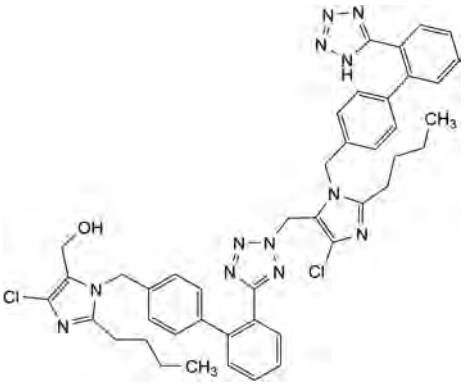

M. [2-butyl-1-[[2′-[2-[[2-butyl-4-chloro-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazol-5-yl]methyl]-2*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-4-chloro-1*H*-imidazol-5-yl]methanol.

01/2017:1538

# LOVASTATIN

## Lovastatinum

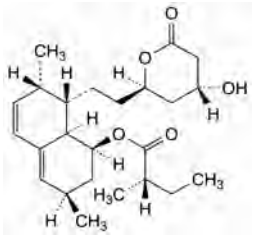

$C_{24}H_{36}O_5$ $M_r$ 404.5
[75330-75-5]

DEFINITION

(1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-Hydroxy-6-oxo-tetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *lovastatin CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 325 to + 340 (dried substance).

Dissolve 0.125 g in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

**Impurity E**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *acetonitrile R1* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with *acetonitrile R1*. Dilute 5.0 mL of this solution to 50.0 mL with *acetonitrile R1*.

*Reference solution (b)*. Dissolve 4 mg of *lovastatin for peak identification CRS* (containing impurities A, B, C, D, E and F) in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 7 volumes of a 1.1 g/L solution of *phosphoric acid R* and 13 volumes of *acetonitrile R1*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of lovastatin.

*Identification of impurities*: use the chromatogram supplied with *lovastatin for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to lovastatin (retention time = about 5 min): impurity E = about 1.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to lovastatin and impurity E.

*Limit*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity E by 1.6;
- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20.0 mg of *lovastatin CRS* in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 5.0 mL of this solution to 50.0 mL with *acetonitrile R*.

*Reference solution (c)*. Dissolve 4 mg of *lovastatin for peak identification CRS* (containing impurities A, B, C, D, E and F) in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 40 | 60 |
| 7 - 9 | 40 → 35 | 60 → 65 |
| 9 - 15 | 35 → 10 | 65 → 90 |
| 15 - 20 | 10 | 90 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *lovastatin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D and F.

*Relative retention* with reference to lovastatin (retention time = about 7 min): impurity B = about 0.6; impurity A = about 0.8; impurity F = about 0.9; impurity C = about 1.6; impurity D = about 2.3.

*System suitability*: reference solution (c):
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lovastatin.

*Limits*:
- *impurities A, B, C, D*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity F*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator under high vacuum at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the content of $C_{24}H_{36}O_5$ from the declared content of *lovastatin CRS*.

STORAGE

Under nitrogen, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

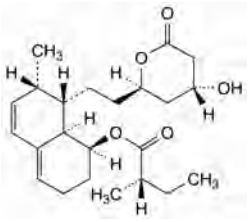

A. (1*S*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (mevastatin),

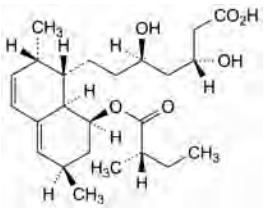

B. (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-2,6-dimethyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid (hydroxyacid lovastatin),

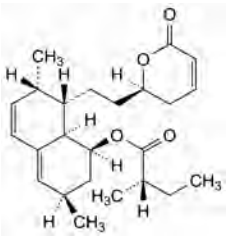

C. (1*S*,3*R*,7*S*,8*S*,8a*R*)-3,7-dimethyl-8-[2-[(2*R*)-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]ethyl]-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (dehydrolovastatin),

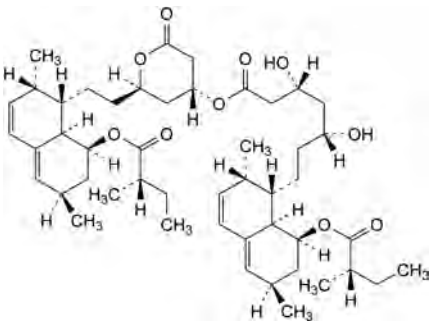

D. (2*R*,4*R*)-2-[2-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-2,6-dimethyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]ethyl]-6-oxotetrahydro-2*H*-pyran-4-yl (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-2,6-dimethyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoate (lovastatin dimer),

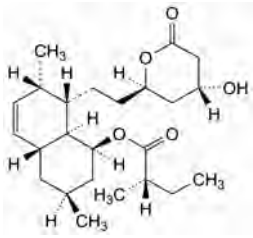

E. (1*S*,3*S*,4a*R*,7*S*,8*S*,8a*S*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (4,4a-dihydrolovastatin),

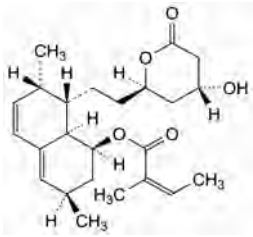

F. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*Z*)-2-methylbut-2-enoate.

**01/2017:2177**

# LUFENURON FOR VETERINARY USE

## Lufenuronum ad usum veterinarium

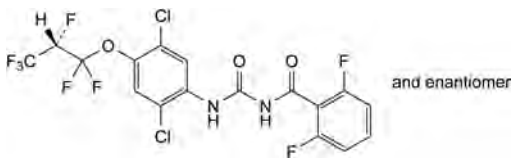


$C_{17}H_8Cl_2F_8N_2O_3$ $M_r$ 511.2
[103055-07-8]

DEFINITION

1-[2,5-Dichloro-4-[(2*RS*)-1,1,2,3,3,3-hexafluoropropoxy]-phenyl]-3-(2,6-difluorobenzoyl)urea.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow powder.

*Solubility*: practically insoluble in water, freely soluble in acetonitrile, soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

mp: about 172 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lufenuron CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R* (30:70 *V/V*).

*Test solution (a)*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture by sonicating for about 10 min and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 7 mg of *lufenuron impurity G CRS* in test solution (a) and dilute to 50.0 mL with test solution (a).

*Reference solution (c)*. Dissolve the contents of a vial of *lufenuron for peak identification CRS* (containing impurities B and C) in 1.0 mL of the solvent mixture.

*Reference solution (d)*. Dissolve 40.0 mg of *lufenuron CRS* in the solvent mixture by sonicating for about 10 min and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 0.01 per cent *V/V* of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 30 | 70 |
| 5 - 15 | 30 → 10 | 70 → 90 |
| 15 - 17 | 10 | 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 255 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *lufenuron for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B and C.

*Relative retention* with reference to lufenuron (retention time = about 9 min): impurity B = about 0.3; impurity C = about 0.7; impurity G = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity G and lufenuron.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.3; impurity C = 1.3;
- *impurity C*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

Dissolve 1.0 g in 20 mL of a mixture of 15 volumes of *water R* and 85 volumes of *dioxan R*. 12 mL of the solution complies with test B. Prepare the reference solution using lead standard solution (1 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *dioxan R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{17}H_8Cl_2F_8N_2O_3$ from the declared content of *lufenuron CRS*.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F, G, H.*

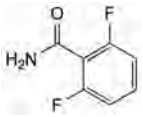

A. 2,6-difluorobenzamide,

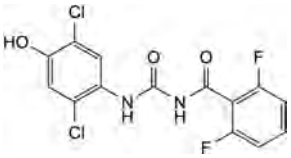

B. 1-(2,5-dichloro-4-hydroxyphenyl)-3-(2,6-difluorobenzoyl)urea,

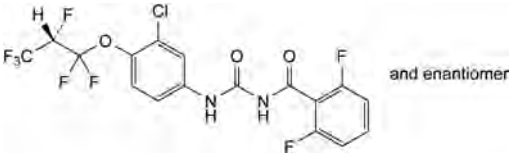


C. 1-[3-chloro-4-[(2*RS*)-1,1,2,3,3,3-hexafluoropropoxy]-phenyl]-3-(2,6-difluorobenzoyl)urea,

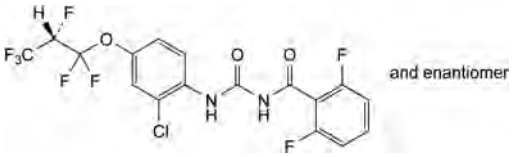


D. 1-[2-chloro-4-[(2*RS*)-1,1,2,3,3,3-hexafluoropropoxy]-phenyl]-3-(2,6-difluorobenzoyl)urea,

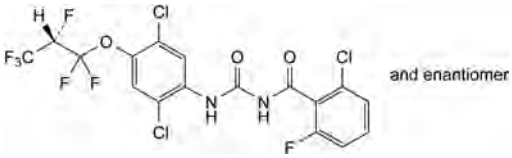


E. 1-(2-chloro-6-fluorobenzoyl)-3-[2,5-dichloro-4-[(2*RS*)-1,1,2,3,3,3-hexafluoropropoxy]phenyl]urea,

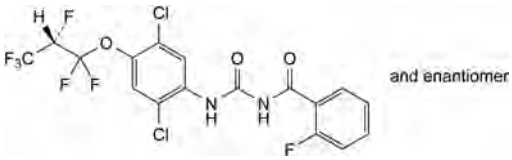


F. 1-[2,5-dichloro-4-[(2*RS*)-1,1,2,3,3,3-hexafluoropropoxy]-phenyl]-3-(2-fluorobenzoyl)urea,

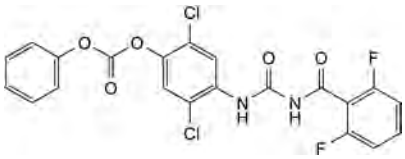

G. 2,5-dichloro-4-[[[(2,6-difluorophenyl)carbonyl]-carbamoyl]amino]phenyl phenyl carbonate,

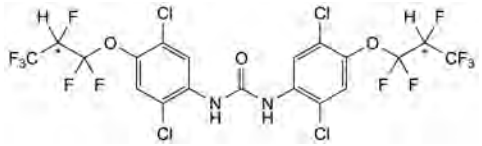

H. 1,3-bis[2,5-dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)-phenyl]urea.

**04/2008:1654**

# LYMECYCLINE

## Lymecyclinum

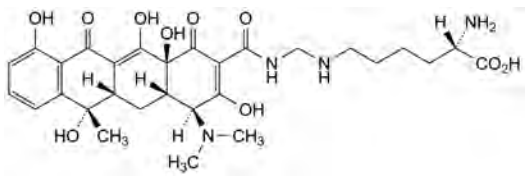

$C_{29}H_{38}N_4O_{10}$ $M_r$ 603

[992-21-2]

DEFINITION

(2*S*)-2-Amino-6-[[[[[(4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracen-2-yl]carbonyl]amino]-methyl]amino]hexanoic acid (reaction product of formaldehyde, lysine and tetracycline).

Semi-synthetic product derived from a fermentation product.

*Content*: 81.0 per cent to 102.0 per cent (equivalent to 60.0 per cent to 75.0 per cent of tetracycline) (anhydrous substance).

CHARACTERS

*Appearance*: yellow, hygroscopic powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *tetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *oxytetracycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2.0 with *concentrated ammonia R*.

*Application*: 2 µL.

*Development*: over half of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 50 mL of *water R*.

*Reference solution (a)*. Dissolve 10 mg of *lysine hydrochloride CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *arginine CRS* and 10 mg of *lysine hydrochloride CRS* in *water R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C until the ammonia disappears completely.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.2 g in 5 mL of *water R*, add 0.3 mL of *phosphoric acid R* and distil. To 1 mL of the distillate add 10 mL of *chromotropic acid-sulfuric acid solution R*. A violet colour is produced.

D. Specific optical rotation (see Tests).

TESTS

**pH** (*2.2.3*): 7.8 to 8.2.

Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): – 180 to – 210 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 50.0 mL with the same solvent.

**Free tetracycline (impurity H)**: maximum 2.5 per cent (anhydrous and methanol-free substance).

To 0.5 g add 50 mL of *butyl acetate R* and allow to stand at 25 °C for 1 h. Filter and extract the filtrate with 2 quantities, each of 25 mL, of *0.1 M hydrochloric acid*. Combine the extracts and dilute to 50.0 mL with *0.1 M hydrochloric acid*. Dilute 10.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*. The absorbance (*2.2.25*) measured at 355 nm is not greater than 0.64.

**Light-absorbing impurities**: the absorbance (*2.2.25*) is not greater than 0.50 at 430 nm (anhydrous and methanol-free substance).

Dissolve 25.0 mg in *0.01 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 0.125 g of the substance to be examined in 5.0 mL of *water R*. Add 1.0 mL of a 40 g/L solution of *sodium metabisulfite R* and allow to stand in the dark at 20-25 °C for 16-24 h, without stirring. Add 50 mL of *0.05 M hydrochloric acid*, shake to dissolve the precipitate and dilute to 100.0 mL with *water R*.

*Reference solution (a)*. Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (b)*. Dissolve 12.5 mg of *4-epitetracycline hydrochloride CRS* (impurity A) in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (c)*. Dissolve 10.0 mg of *anhydrotetracycline hydrochloride CRS* (impurity C) in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

*Reference solution (d)*. Dissolve 10.0 mg of *4-epianhydrotetracycline hydrochloride CRS* (impurity D) in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (e)*. Mix 1 mL of reference solution (a), 2 mL of reference solution (b) and 5 mL of reference solution (d) and dilute to 25 mL with *0.01 M hydrochloric acid*.

*Reference solution (f)*. Mix 40.0 mL of reference solution (b), 20.0 mL of reference solution (c) and 5.0 mL of reference solution (d) and dilute to 200.0 mL with *0.01 M hydrochloric acid*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm) with a pore size of 10 nm;
- *temperature*: 60 °C.

*Mobile phase*: weigh 80.0 g of *2-methyl-2-propanol R* and transfer to a 1000 mL volumetric flask with the aid of 200 mL of *water R*; add 100 mL of a 35 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 8.0 with *dilute phosphoric acid R*, 200 mL of a 10 g/L solution of *tetrabutylammonium hydrogen sulfate R* adjusted to pH 8.0 with *dilute sodium hydroxide solution R*, and 10 mL of a 40 g/L solution of *sodium edetate R* adjusted to pH 8.0 with *dilute sodium hydroxide solution R*; dilute to 1000.0 mL with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (e) and (f).

*Run time*: 5 times the retention time of the principal peak in the chromatogram obtained with the test solution.

*Relative retention* with reference to tetracycline (retention time = about 8 min): impurity E = about 0.50; impurity A = about 0.6; impurity F = about 0.68; impurity B (eluting on the tail of the principal peak) = about 1.2; impurity D = about 1.45; impurity G = about 1.45; impurity C = about 2.95.

*System suitability*: reference solution (e):

- *resolution*: minimum 3.0 between the 1st peak (impurity A) and the 2nd peak (tetracycline) and minimum 5.0 between the 2nd peak and the 3rd peak (impurity D); adjust the concentration of 2-methyl-2-propanol in the mobile phase if necessary;
- *symmetry factor*: maximum 1.25 for the peak due to tetracycline.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (5.0 per cent),
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (1.0 per cent),
- *impurities B, E, F*: for each impurity, not more than 0.1 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.5 per cent),
- *sum of impurities D and G*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent),
- *any other impurity*: for each impurity, not more than 0.04 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.2 per cent),
- *total*: not more than 1.6 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (8.0 per cent),
- *disregard limit*: 0.02 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.1 per cent).

**Methanol** (*2.4.24, System A*): maximum 1.5 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*:

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

Calculate the percentage content of tetracycline and multiply it by 1.356 to obtain the percentage content of lymecycline.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H.*

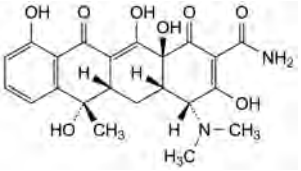


A. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epitetracycline),

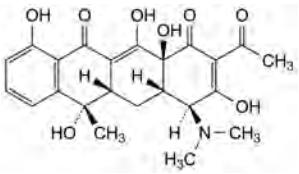

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyltetracycline),

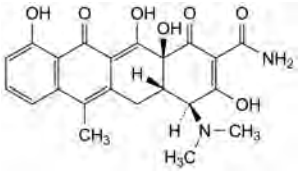

C. (4*S*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

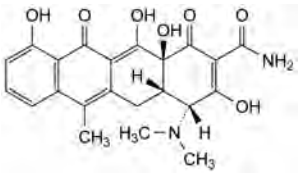

D. (4*R*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline),

E. unknown structure,

F. unknown structure,

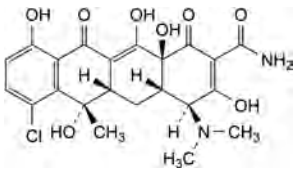

G. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-7-chloro-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (chlortetracycline),

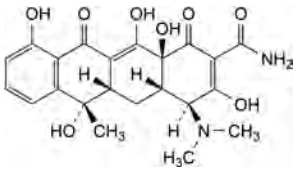

H. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (tetracycline).

**01/2009:0558**
**corrected 10.0**

# LYNESTRENOL

## Lynestrenolum

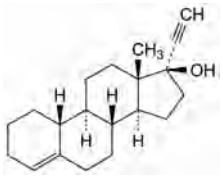

$C_{20}H_{28}O$ $M_r$ 284.4
[52-76-6]

DEFINITION

19-Nor-17α-pregn-4-en-20-yn-17-ol.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lynestrenol CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 9.5 to – 11 (dried substance).

Dissolve 0.900 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in *ethyl acetate R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *ethyl acetate R*. Dilute 1.0 mL of this solution to 10.0 mL with *ethyl acetate R*.

*Reference solution (b)*. Dissolve 10 mg of *lynestrenol for peak identification CRS* (containing impurities A, B and C) in 1.0 mL of *ethyl acetate R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3.0 mL/min.

*Split ratio*: 1:34.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 30 | 80 → 230 |
| | 30 - 32 | 230 → 310 |
| | 32 - 42 | 310 |
| Injection port | | 150 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Identification of impurities*: use the chromatogram supplied with *lynestrenol for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to lynestrenol (retention time = about 38 min): artefact degradation peak = about 0.97; impurity A = about 0.99; impurity B = about 1.005; impurity C = about 1.01.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to lynestrenol.

*Limits*:

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent). Disregard the artefact peak, which may be generated in the injection system.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.150 g in 40 mL of *tetrahydrofuran R* and add 5.0 mL of a 100 g/L solution of *silver nitrate R*. Titrate with *0.1 M sodium hydroxide*. Determine the end-point potentiometrically (*2.2.20*), using a glass indicator electrode and as comparison electrode a silver-silver chloride double-junction electrode with a saturated solution of *potassium nitrate R* as junction liquid. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.44 mg of $C_{20}H_{28}O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

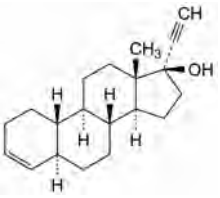

A. 19-nor-5α,17α-pregn-3-en-20-yn-17-ol,

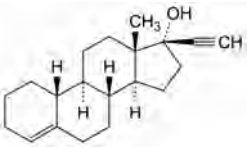

B. 19-norpregn-4-en-20-yn-17-ol,

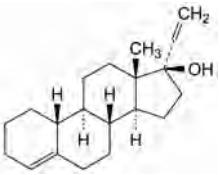

C. 19-nor-17α-pregna-4,20-dien-17-ol.

**01/2017:2114**

# LYSINE ACETATE

## Lysini acetas

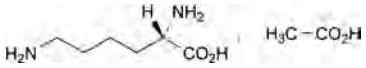

$C_8H_{18}N_2O_4$ $M_r$ 206.2

[57282-49-2]

DEFINITION

(2*S*)-2,6-Diaminohexanoic acid acetate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lysine acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.1 mL of solution S (see Tests) add 2 mL of *water R* and 1 mL of a 50 g/L solution of *phosphomolybdic acid R*. A yellowish-white precipitate is formed.

E. It gives reaction (a) of acetates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 8.5 to + 10.0 (dried substance), determined on solution S.

**Ninhydrin-positive substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 50 mL with *water R*.

*Reference solution (a)*. Dissolve 10 mg of *lysine acetate CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b)*. Dilute 5 mL of test solution (b) to 20 mL with *water R*.

*Reference solution (c)*. Dissolve 10 mg of *lysine acetate CRS* and 10 mg of *arginine CRS* in *water R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C until the ammonia has evaporated.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Limits*: test solution (a):

– *any impurity*: any spot, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 30 ppm.

In a separating funnel, dissolve 0.33 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. The aqueous layer complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 3 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 10.31 mg of $C_8H_{18}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

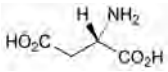

A. (2*S*)-2-aminobutanedioic acid (aspartic acid),

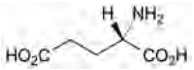

B. (2*S*)-2-aminopentanedioic acid (glutamic acid),

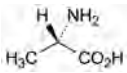

C. (*S*)-2-aminopropanoic acid (alanine),

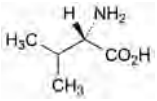

D. (*S*)-2-amino-3-methylbutanoic acid (valine),

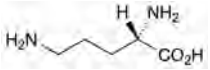

E. (2*S*)-2,5-diaminopentanoic acid (ornithine),

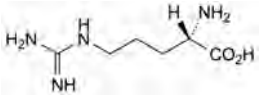

F. (*S*)-2-amino-5-guanidinopentanoic acid (arginine).

**01/2017:0930**

# LYSINE HYDROCHLORIDE

## Lysini hydrochloridum

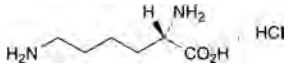

$C_6H_{15}ClN_2O_2$ $M_r$ 182.7

[657-27-2]

DEFINITION

(2*S*)-2,6-diaminohexanoic acid hydrochloride.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B, E.*

*Second identification*: *A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *lysine hydrochloride CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *lysine hydrochloride CRS* in *water R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *concentrated ammonia R*, *2-propanol R* (30:70 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 105 °C until the ammonia disappears completely.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.1 mL of solution S (see Tests) add 2 mL of *water R* and 1 mL of a 50 g/L solution of *phosphomolybdic acid R*. A yellowish-white precipitate is formed.

E. To 0.1 mL of solution S add 2 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ or $GY_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 21.0 to + 22.5 (dried substance).

Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *water R* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* (impurity A) in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test solution, blank solution and reference solutions (a), (b) and (d) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and impurity A.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of lysine hydrochloride in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 30 ppm.

In a separating funnel, dissolve 0.33 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.27 mg of $C_6H_{15}ClN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It

is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

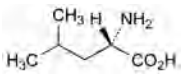


A. (2*S*)-2-amino-4-methylpentanoic acid (leucine),

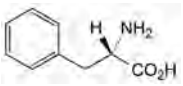


B. (2*S*)-2-amino-3-phenylpropanoic acid (phenylalanine),

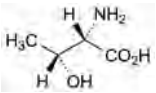


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**01/2008:1443 corrected 6.0**

# MACROGOL 6 GLYCEROL CAPRYLOCAPRATE

## Macrogol 6 glyceroli caprylocapras

DEFINITION

Mixture of mainly mono- and diesters of polyoxyethylene glycerol ethers mainly with caprylic (octanoic) and capric (decanoic) acids. The average number of moles of ethylene oxide reacted per mole of substance is 6.

Macrogol 6 glycerol caprylocaprate may be obtained by ethoxylation of glycerol and esterification with distilled coconut or palm kernel fatty acids, or by ethoxylation of mono- and diglycerides of caprylic and capric acids.

CHARACTERS

*Appearance*: pale yellow liquid.

*Solubility*: partly soluble in water, freely soluble in castor oil, in glycerol, in isopropanol and in propylene glycol.

*Viscosity*: about 145 mPa·s.

IDENTIFICATION

A. Dissolve 1.0 g in 99 g of a mixture of 10 volumes of *2-propanol R* and 90 volumes of *water R*. Heat the solution obtained to about 40 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 15 °C and 35 °C.

B. Saponification value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_2$ (*2.2.2, Method I*).

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 5.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 165 to 225.

**Saponification value** (*2.5.6*): 85 to 105, determined on 2.0 g.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

– *caproic acid*: maximum 2.0 per cent;

– *caprylic acid*: 50.0 per cent to 80.0 per cent;

– *capric acid*: 20.0 per cent to 50.0 per cent;

– *lauric acid*: maximum 3.0 per cent;

– *myristic acid*: maximum 1.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.3 per cent.

**01/2020:2052**

# MACROGOL 15 HYDROXYSTEARATE

## Macrogoli 15 hydroxystearas

DEFINITION

Mixture of mainly monoesters and diesters of 12-hydroxystearic ((12Ξ)-hydroxyoctadecanoic) acid and macrogols obtained by ethoxylation of 12-hydroxystearic acid. The number of moles of ethylene oxide reacted per mole of 12-hydroxystearic acid is 15 (nominal value). It contains free macrogols.

CHARACTERS

*Appearance*: yellowish, waxy mass.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), insoluble in liquid paraffin.

It solidifies at about 25 °C.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1.0 g add 100 mL of a 100 g/L solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Acidify the warm solution with 20 mL of *hydrochloric acid R* and cool to room temperature. Shake the mixture with 50 mL of *ether R* and allow to stand until a separation of the layers is visible. Separate the clear upper layer, add 5 g of *anhydrous sodium sulfate R*, wait for 30 min, filter and evaporate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 mL of *ether R*.

*Reference solution*. Dissolve 50 mg of *12-hydroxystearic acid R* in 25 mL of *methylene chloride R*.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: spray with a 80 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for 1-2 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve 15.0 g in 50 mL of *water R*. The viscosity (*2.2.9*) has a maximum of 20 mPa·s.

C. Free macrogols (see Tests).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $B_6$ or $BY_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 20 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 1.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 90 to 110.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Saponification value** (*2.5.6*): 53 to 63.

**Free macrogols**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dissolve 1.20 g of the substance to be examined in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution (a).* Dissolve about 0.4 g of *macrogol 1000 R* in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution (b).* Dilute 50.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Precolumns* (2):

- *size*: $l$ = 0.125 m, Ø = 4 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm.

*Column*:

- *size*: $l$ = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydroxylated polymethacrylate gel R* (6 µm) with a pore size of 12 nm.

Connect both precolumns to the column using a 3-way valve and switch the mobile phase flow according to the following programme:

- 0-114 s: precolumn 1 and column;
- 115 s to the end: precolumn 2 and column;
- 115 s to 7 min: flow back of precolumn 1.

*Mobile phase*: *water for chromatography R*, *methanol R* (20:80 *V/V*).

*Flow rate*: 1.1 mL/min.

*Detection*: refractometer.

*Injection*: 50 µL.

Calculate the percentage content of free macrogols using the following expression:

$$\frac{A_1 \times m_2 \times 200}{m_1 \times (A_2 + 2A_3)}$$

$m_1$ = mass of the substance to be examined in the test solution, in grams;

$m_2$ = mass of *macrogol 1000 R* in reference solution (a), in grams;

$A_1$ = area of the peak due to free macrogols in the substance to be examined in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to macrogol 1000 in the chromatogram obtained with reference solution (a);

$A_3$ = area of the peak due to macrogol 1000 in the chromatogram obtained with reference solution (b).

*Limit*:

- *free macrogols*: 27.0 per cent to 39.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 50 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 2.00 g.

**Total ash** (*2.4.16*): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

**01/2017:2044**

# MACROGOL 20 GLYCEROL MONOSTEARATE

## Macrogol 20 glyceroli monostearas

DEFINITION

Macrogol 20 glycerol monostearate is obtained by ethoxylation with ethylene oxide of different types of glycerol stearates, mainly *Glycerol monostearate 40-55 (0495)*. The number of moles of ethylene oxide reacted per mole of glycerol stearate is 20 (nominal value).

CHARACTERS

*Appearance*: pale yellow, oily liquid or gel.

*Solubility*: soluble in water at 40 °C and above and in ethanol (96 per cent), practically insoluble in light liquid paraffin and in fatty oils.

*Relative density*: about 1.07.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Saponification value (see Tests).

C. Composition of fatty acids (see Tests).

D. Place 1 g in a test tube and add 0.1 mL of *sulfuric acid R*. Heat the tube until white fumes appear. The fumes turn filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

TESTS

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 65 to 85, determined on 0.350 g.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Peroxide value** (*2.5.5, Method A*): maximum 6.0.

**Saponification value** (*2.5.6*): 40 to 60.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty-acid fraction of the substance:*

| Type of macrogol 20 glycerol monostearate | Type of glycerol stearate used | Composition of fatty acids |
|---|---|---|
| Type I | Type I (obtained using stearic acid 50) | *Stearic acid*: 40.0 per cent to 60.0 per cent, *Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent. |
| Type II | Type II (obtained using stearic acid 70) | *Stearic acid*: 60.0 per cent to 80.0 per cent, *Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent. |
| Type III | Type III (obtained using stearic acid 95) | *Stearic acid*: 90.0 per cent to 99.0 per cent, *Sum of the contents of palmitic and stearic acids*: minimum 96.0 per cent. |

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent.

STORAGE

Protected from light.

LABELLING

The label states the type of macrogol 20 glycerol monostearate.

**01/2020:2584**

# MACROGOL 30 DIPOLYHYDROXYSTEARATE

## Macrogoli 30 dipolyhydroxystearas

DEFINITION

Mixture of mainly diesters of polymerised 12-hydroxystearic ((12Ξ)-hydroxyoctadecanoic) acid and *Macrogols (1444)* obtained by esterification of macrogol with 12-hydroxystearic acid. The average number of moles of ethylene oxide reacted per mole of substance is 30.

CHARACTERS

*Appearance*: brownish-red, waxy mass.

*Solubility*: practically insoluble in water, very soluble in methylene chloride and soluble in most aliphatic and aromatic hydrocarbons.

mp: 30 °C to 40 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *macrogol 30 dipolyhydroxystearate CRS*.

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 10.0.

**Hydroxyl value** (*2.5.3, Method A*): 12 to 30.

**Iodine value** (*2.5.4, Method A*): maximum 10.0.

**Peroxide value** (*2.5.5*): maximum 5.0.

**Saponification value** (*2.5.6*): 125 to 145, determined on 2.0 g.

Use 30.0 mL of *0.5 M alcoholic potassium hydroxide*, heat under reflux for 60 min and add 50 mL of *anhydrous ethanol R* before carrying out the titration.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.0 g in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C, until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

In an airtight container.

**01/2008:2396 corrected 7.0**

# MACROGOL 40 SORBITOL HEPTAOLEATE

## Macrogol 40 sorbitoli heptaoleas

DEFINITION

Mixture of esters of fatty acids, mainly *Oleic acid (0799)*, and sorbitol ethoxylated with approximately 40 moles of ethylene oxide for each mole of sorbitol. 7 moles of oleic acid are used for each mole of sorbitol. It also contains macrogol fatty acid esters.

CHARACTERS

*Appearance*: clear or slightly opalescent, yellowish, viscous, hygroscopic liquid.

*Solubility*: dispersible in water, soluble in isopropyl myristate, in isopropyl palmitate, in mineral oils and in vegetable fatty oils.

*Relative density*: about 1.0.

*Viscosity* (*2.2.9*): about 175 mPa·s at 25 °C.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *macrogol 40 sorbitol heptaoleate CRS*.

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 12.0, determined on 3.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 22 to 55.

**Peroxide value**: maximum 10.0.

Introduce 10.0 g into a 100 mL beaker and dissolve with 20 mL of *glacial acetic acid R*. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the titration of the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): 90 to 110, determined on 4.0 g.

Use 30.0 mL of *0.5 M alcoholic potassium hydroxide*, heat under reflux for 60 min and add 50 mL of *anhydrous ethanol R* before carrying out the titration.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the substance*:

– *myristic acid*: maximum 5.0 per cent;

– *palmitic acid*: maximum 16.0 per cent;

– *palmitoleic acid*: maximum 8.0 per cent;
– *stearic acid*: maximum 6.0 per cent;
– *oleic acid*: minimum 58.0 per cent;
– *linoleic acid*: maximum 18.0 per cent;
– *linolenic acid*: maximum 4.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.50 g.

**Sulfated ash**: maximum 0.25 per cent.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.0 g of the substance to be examined in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C, until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

In an airtight container, protected from light.

**01/2008:1123**

# MACROGOL CETOSTEARYL ETHER

## Macrogoli aether cetostearylicus

DEFINITION

Mixture of ethers of mixed macrogols with linear fatty alcohols, mainly cetostearyl alcohol. It may contain some free macrogols and it contains various amounts of free cetostearyl alcohol. The number of moles of ethylene oxide reacted per mole of cetostearyl alcohol is 2 to 33 (nominal value).

CHARACTERS

*Appearance*: white or yellowish-white, waxy, unctuous mass, pellets, microbeads or flakes.

*Solubility*:

– macrogol cetostearyl ether with low numbers of moles of ethylene oxide reacted per mole: practically insoluble in water, soluble in ethanol (96 per cent) and in methylene chloride;
– macrogol cetostearyl ether with higher numbers of moles of ethylene oxide reacted per mole: dispersible or soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

It solidifies at 32 °C to 52 °C.

IDENTIFICATION

A. Hydroxyl value (see Tests).
B. Iodine value (see Tests).
C. Saponification value (see Tests).
D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve the prescribed amount of substance to be examined (see table below) in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 75 mL with the same mixture of solvents.

| Number of moles of ethylene oxide reacted per mole | Amount to be dissolved (g) |
|---|---|
| 2 - 6 | 5.0 |
| 10 - 22 | 10.0 |
| 25 - 33 | 15.0 |

Add 60 mL of *hexane R* and shake for 3 min. The formation of foam can be reduced by the addition of some drops of *ethanol (96 per cent) R*. Filter the upper layer through *anhydrous sodium sulfate R*, wash the filter with 3 quantities, each of 10 mL, of *hexane R* and evaporate the combined filtrates to dryness. Dissolve 0.05 g of the residue in 10 mL of *methanol R* (the solution may be opalescent).

*Reference solution.* Dissolve 25 mg of *stearyl alcohol CRS* in *methanol R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*.

*Application*: 20 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with vanillin-sulfuric acid reagent prepared as follows: dissolve 0.5 g of *vanillin R* in 50 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *sulfuric acid R*; allow to dry in air; heat at about 130 °C for 15 min and allow to cool in air.

*Results*: the chromatogram obtained with the test solution shows several spots; one of these spots corresponds to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve or disperse 0.1 g in 5 mL of *ethanol (96 per cent) R*, add 2 mL of *water R*, 10 mL of *dilute hydrochloric acid R*, 10 mL of *barium chloride solution R1* and 10 mL of a 100 g/L solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 5.0 g in *ethanol (96 per cent) R* and dilute to 50 mL with the same solvent.

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*).

| Number of moles of ethylene oxide reacted per mole (nominal value) | Hydroxyl value |
|---|---|
| 2 | 150 - 180 |
| 3 | 135 - 155 |
| 5 - 6 | 100 - 134 |
| 10 | 75 - 90 |
| 12 | 67 - 77 |
| 15 | 58 - 67 |
| 20 - 22 | 40 - 55 |
| 25 | 36 - 46 |
| 30 - 33 | 32 - 40 |

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Saponification value** (*2.5.6*): maximum 3.0, determined on 10.0 g.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.00 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent, determined on 2.0 g.

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of cetostearyl alcohol (nominal value).

**07/2015:2730**
**corrected 10.0**

# MACROGOL ISOTRIDECYL ETHER

## Macrogoli aether isotridecylicus

DEFINITION

Mixture of ethers of mixed macrogols with linear and branched fatty alcohols, mainly $C_{13}H_{28}O$. It contains a variable quantity of free $C_{13}H_{28}O$ and it may contain free macrogols. The number of moles of ethylene oxide reacted per mole of branched $C_{13}H_{28}O$ is 3 or 4 (nominal value).

CHARACTERS

*Appearance*: colourless liquid.

*Solubility*: practically insoluble in water, soluble or dispersible in ethanol (96 per cent), practically insoluble in light petroleum.

IDENTIFICATION

A. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 0.35 g of *lauryl alcohol R*, 0.35 g of *tridecyl alcohol R*, 0.15 g of *myristyl alcohol R* and 0.15 g of *ethylene glycol monododecyl ether R* in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetone R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 120 |
| | 1 - 24 | 120 → 350 |
| | 24 - 34 | 350 |
| Injection port | | 300 |
| Detector | | 350 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Retention time*: lauryl alcohol = about 6.7 min; tridecyl alcohol = about 7.8 min; myristyl alcohol = about 9 min; ethylene glycol monododecyl ether = about 9.3 min.

*System suitability*: reference solution:

- *resolution*: minimum 5 between the peaks due to myristyl alcohol and ethylene glycol monododecyl ether.

*Results*: the sum of the areas of the peaks eluting between the peak due to lauryl alcohol and the peak due to tridecyl alcohol is greater than the area of the peak due to tridecyl alcohol and the area of the peak due to lauryl alcohol.

B. Hydroxyl value (see Tests).

C. Iodine value (see Tests).

D. Saponification value (see Tests).

E. Dissolve or disperse 0.1 g in 5 mL of *ethanol (96 per cent) R*. Add 10 mL of *dilute hydrochloric acid R*, 10 mL of *barium chloride solution R1* and 10 mL of a 100 g/L solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 5.0 g in *ethanol (96 per cent) R* and dilute to 50 mL with the same solvent.

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*). See Table 2730.-1.

Table 2730.-1

| Ethylene oxide units per molecule (nominal value) | Hydroxyl value |
|---|---|
| 3 | 165 - 185 |
| 4 | 145 - 160 |

**Iodine value** (*2.5.4*): maximum 2.0.

**Saponification value** (*2.5.6*): maximum 3.0, determined on 10.0 g.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent.

Ignite a silica crucible at 600 ± 50 °C for 30 min, allow to cool in a desiccator over a suitable desiccant and weigh. Evenly distribute 1.0 g in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of $C_{13}H_{28}O$ (nominal value).

**07/2019:1124**
**corrected 10.0**

# MACROGOL LAURYL ETHER

## Macrogoli aether laurilicus

DEFINITION

Mixture of ethers of mixed macrogols with linear fatty alcohols, mainly $C_{12}H_{26}O$. It contains a variable quantity of free $C_{12}H_{26}O$ and may contain free macrogols. The number of moles of ethylene oxide reacted per mole of $C_{12}H_{26}O$ is 3 to 23 (nominal value).

CHARACTERS

- Macrogol lauryl ethers with 3 to 5 units of ethylene oxide per molecule.

*Appearance*: colourless liquid.

*Solubility*: practically insoluble in water, soluble or dispersible in ethanol (96 per cent), practically insoluble in light petroleum.

– Macrogol lauryl ethers with 9 units of ethylene oxide per molecule.

*Appearance*: white or almost white, unctuous, hygroscopic mass, melting at 24 °C into a colourless or yellowish, viscous liquid.

*Solubility*: freely soluble in water, soluble or dispersible in ethanol (96 per cent), practically insoluble in light petroleum.

– Macrogol lauryl ethers with 10 to 23 units of ethylene oxide per molecule.

*Appearance*: white or almost white, waxy mass.

*Solubility*: soluble or dispersible in water, soluble in ethanol (96 per cent), practically insoluble in light petroleum.

IDENTIFICATION

Carry out tests A, B, C, D, E for macrogol lauryl ethers with 3 to 5 units of ethylene oxide per molecule.

Carry out tests A, B, C, D, F for macrogol lauryl ethers with 9 to 23 units of ethylene oxide per molecule.

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Saponification value (see Tests).

D. Dissolve or disperse 0.1 g in 5 mL of *ethanol (96 per cent) R*, add 10 mL of *dilute hydrochloric acid R*, 10 mL of *barium chloride solution R1* and 10 mL of a 100 g/L solution of *phosphomolybdic acid R*. A precipitate is formed.

E. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 0.15 g of *ethylene glycol monododecyl ether R*, 0.15 g of *lauryl alcohol R* and 0.15 g of *myristyl alcohol R* in *acetone R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.25 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R* or *hydrogen for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 120 |
| | 1 - 24 | 120 → 350 |
| | 24 - 34 | 350 |
| Injection port | | 300 |
| Detector | | 350 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the peaks due to ethylene glycol monododecyl ether, lauryl alcohol and myristyl alcohol.

*Retention time*: lauryl alcohol = about 6.7 min; myristyl alcohol = about 8.9 min; ethylene glycol monododecyl ether = about 9.3 min.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to myristyl alcohol and ethylene glycol monododecyl ether.

*Results*: the sum of the areas of the peaks eluted before the peak due to lauryl alcohol is less than 10 per cent of the area of the peak due to lauryl alcohol; disregard the peak due to the solvent.

F. Gas chromatography (*2.2.28*).

*Test solution.* Into a 50 mL round-bottomed flask fitted with a reflux condenser introduce 0.300 g of the substance to be examined, 15 mL of *hydriodic acid R*, 2.5 mL of a 603 g/L solution of *phosphorous acid R* and 2 or 3 anti-bumping granules. Heat to 140 °C using an oil bath or an electric heating mantle, then boil for 2 h. Allow to cool to room temperature and rinse the condenser with 5 mL of *ethanol (96 per cent) R*. Add the rinsings to the flask and transfer to a separating funnel, rinsing the flask with 2 quantities, each of 5 mL, of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*.

Extract with 15 mL of *heptane R* and wash the upper layer with 5 mL portions of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* until the pH of the lower layer is greater than 3 using a *pH indicator strip R*.

Transfer the upper layer into a 20 mL glass vial with a screw cap and shake with 5 g of *anhydrous sodium sulfate R*. Allow to settle and transfer about 1 mL of the clear supernatant to an autosampler vial.

*Reference solution.* Prepare as described for the test solution using 0.150 g of *2-butyloctanol R*, 0.150 g of *lauryl alcohol R* and 0.150 g of *myristyl alcohol R* instead of the substance to be examined.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R* or *hydrogen for chromatography R*.

*Flow rate*: 3 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 100 |
| | 5 - 55 | 100 → 300 |
| | 55 - 70 | 300 |
| Injection port | | 250 |
| Detector | | 310 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the peaks due to 2-butyloctyl iodide, lauryl iodide and myristyl iodide.

*Retention time*: 2-butyloctyl iodide = about 22.7 min; lauryl iodide = about 25.6 min; myristyl iodide = about 31.1 min.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to 2-butyloctyl iodide and lauryl iodide.

*Results*: the sum of the areas of the peaks eluted before the peak due to lauryl iodide is less than 10 per cent of the area of the peak due to lauryl iodide; disregard the peak due to the solvent.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 5.0 g in *ethanol (96 per cent) R* and dilute to 50 mL with the same solvent.

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*).

| Ethylene oxide units per molecule (nominal value) | Hydroxyl value |
|---|---|
| 3 | 165 - 180 |
| 4 | 145 - 165 |
| 5 | 130 - 140 |
| 9 | 90 - 100 |
| 10 | 85 - 95 |
| 12 | 73 - 83 |
| 15 | 64 - 74 |
| 20 - 23 | 40 - 60 |

**Iodine value** (*2.5.4*): maximum 2.0.

**Saponification value** (*2.5.6*): maximum 3.0, determined on 10.0 g.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent.

Ignite a silica crucible at 600 ± 50 °C for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.0 g in the crucible and weigh again. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C until the substance is thoroughly charred. Carry out the test for sulfated ash on the residue obtained, starting from "Moisten the substance to be examined...".

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of $C_{12}H_{26}O$ (nominal value).

**01/2008:1618**

# MACROGOL OLEATE

## Macrogoli oleas

DEFINITION

A mixture of monoesters and diesters of mainly oleic (*cis*-9-octadecenoic) acid and macrogols. It may be obtained by ethoxylation of *Oleic acid (0799)* or by esterification of macrogols with oleic acid of animal or vegetable origin. It may contain free macrogols. The average polymer length is equivalent to 5-6 or 10 moles of ethylene oxide per mole (nominal value). A suitable antioxidant may be added.

CHARACTERS

*Appearance*: slightly yellowish, viscous liquid.

*Solubility*: dispersible in water, soluble in ethanol (96 per cent) and in 2-propanol, dispersible in oils, miscible with fatty oils and with waxes.

*Refractive index*: about 1.466.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Saponification value (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 20 mg add 10 mL of *methylene chloride R* and mix.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: 25 per cent *V/V* solution of *concentrated ammonia R*, *2-propanol R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *potassium iodobismuthate solution R4*; examine the plate about 10 min later.

*Results*: the chromatogram obtained shows 3 principal spots, corresponding, in order of increasing $R_F$ value, to free macrogol, macrogol mono-oleate and macrogol dioleate.

C. Composition of fatty acids (see Tests).

TESTS

**Alkalinity**. Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent. To 2 mL of this solution add 0.05 mL of *phenol red solution R*. The solution is not red.

**Acid value** (*2.5.1*): maximum 2.0.

**Hydroxyl value** (*2.5.3, Method A*): see Table 1618.-1.

**Iodine value** (*2.5.4, Method A*): see Table 1618.-1.

**Peroxide value** (*2.5.5, Method A*): maximum 12.0.

**Saponification value** (*2.5.6*): see Table 1618.-1.

Table 1618.-1

| | 5-6 moles of ethylene oxide | 10 moles of ethylene oxide |
|---|---|---|
| Hydroxyl value | 50 - 70 | 65 - 90 |
| Iodine value | 50 - 60 | 27 - 34 |
| Saponification value | 105 - 120 | 68 - 85 |

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

- *myristic acid*: maximum 5.0 per cent;
- *stearic acid*: maximum 6.0 per cent;
- *palmitic acid*: maximum 16.0 per cent;
- *palmitoleic acid*: maximum 8.0 per cent;
- *oleic acid*: 65.0 per cent to 88.0 per cent;
- *linoleic acid*: maximum 18.0 per cent;
- *linolenic acid*: maximum 4.0 per cent;
- *fatty acids with a chain length greater than $C_{18}$*: maximum 4.0 per cent.

**Residual ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 1.00 g using *anhydrous methanol R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide per mole (nominal value).

**01/2008:1125**

# MACROGOL OLEYL ETHER

## Macrogoli aether oleicus

DEFINITION

Mixture of ethers of mixed macrogols with linear fatty alcohols, mainly oleyl alcohol. It contains a variable quantity of free oleyl alcohol and it may contain free macrogols. The number of moles of ethylene oxide reacted per mole of oleyl alcohol is 2 to 20 (nominal value). A suitable antioxidant may be added.

CHARACTERS

– Macrogol oleyl ether with 2 to 5 units of ethylene oxide per molecule.

*Appearance*: yellow liquid.

*Solubility*: practically insoluble in water, soluble in alcohol, practically insoluble in light petroleum.

– Macrogol oleyl ether with 10 to 20 units of ethylene oxide per molecule.

*Appearance*: yellowish-white waxy mass.

*Solubility*: dispersible or soluble in water, soluble in alcohol, practically insoluble in light petroleum.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Saponification value (see Tests).

D. Dissolve or disperse 0.1 g in 5 mL of *alcohol R*, add 2 mL of *water R*, 10 mL of *dilute hydrochloric acid R*, 10 mL of *barium chloride solution R1* and 10 mL of a 100 g/L solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 5.0 g in *alcohol R* and dilute to 50 mL with the same solvent.

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *water R* and 10 mL of *alcohol R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*). See Table 1125.-1.

**Iodine value** (*2.5.4*). See Table 1125.-1.

Table 1125.-1

| Ethylene oxide units per molecule (nominal value) | Hydroxyl value | Iodine value |
|---|---|---|
| 2 | 158 - 178 | 48 - 74* |
| 5 | 110 - 125 | 48 - 56 |
| 10 | 75 - 95 | 24 - 38 |
| 20 | 40 - 65 | 14 - 24 |

* This broad range is needed since 2 different grades of oleyl alcohol may be used for the synthesis. The iodine value does not differ by more than 5 units from the nominal iodine value and is within the limits stated in the table.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Saponification value** (*2.5.6*): maximum 3.0.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.00 g.

**Total ash** (*2.4.16*): maximum 0.2 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states:

– the number of moles of ethylene oxide reacted per mole of oleyl alcohol (nominal value),

– the nominal iodine value for the type with 2 units of ethylene oxide per molecule.

**01/2013:2523**
**corrected 10.0**

# MACROGOL POLY(VINYL ALCOHOL) GRAFTED COPOLYMER

## Copolymerum macrogolo et alcoholi poly(vinylico) constatum

DEFINITION

Grafted copolymer of macrogol and poly(vinyl alcohol), having a mean relative molecular mass of about 45 000.

It consists of about 75 per cent of poly(vinyl alcohol) units and 25 per cent of macrogol units. It may contain *Anhydrous colloidal silica (0434)* to improve flowability.

CHARACTERS

*Appearance*: white or slightly yellowish powder; opalescent solutions may be obtained during testing due to the presence of anhydrous colloidal silica.

*Solubility*: very soluble in water, practically insoluble in anhydrous ethanol and in acetone. It dissolves in dilute acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *macrogol poly(vinyl alcohol) grafted copolymer CRS*.

*Preparation*: dissolve 0.2 g in 20 mL of *water R*, spread a few drops of the solution on a thallium bromoiodide plate and evaporate the solvent at 110 °C for 30 min.

B. Dissolve 0.4 g in 2 mL of *water R*. Place 1 mL of the solution on a glass plate and allow to dry. A transparent film is formed.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Ester value** : 10 to 75.

Determine the acid value ($I_A$) as follows. Dissolve 5.00 g in 100 mL of *distilled water R* while stirring with a magnetic stirrer. Titrate with *0.01 M alcoholic potassium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank test under the same conditions.

$$I_A = \frac{0.561(n_1 - n_2)}{m}$$

$n_1$ = volume of titrant used in the test, in millilitres;

$n_2$ = volume of titrant used in the blank test, in millilitres;

$m$ = mass of the sample, in grams.

Determine the saponification value ($I_S$) (*2.5.6*) on 5.00 g, using 50.0 mL of *0.5 M alcoholic potassium hydroxide* and stirring vigorously with a magnetic stirrer.

The ester value ($I_E$) is calculated from the saponification value ($I_S$) and the acid value ($I_A$):

$$I_E = I_S - I_A$$

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 0.250 g of the substance to be examined into a 10 mL volumetric flask and add about 1 mL of *methanol R*. Sonicate. Add about 8 mL of *water R* and dilute to 10.0 mL with the same solvent. Filter.

*Reference solution (a).* Dissolve 5.0 mg of *vinyl acetate CRS* (impurity A) in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 5 mg of *vinyl acetate R* (impurity A) and 5 mg of *1-vinylpyrrolidin-2-one R* in 10 mL of *methanol R* and dilute to 50 mL with *water R*. Dilute 1 mL of the solution to 20 mL with *water R*.

A precolumn containing *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) may be used if a matrix effect is observed.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, *methanol R2*, *water for chromatography R* (5:5:90 *V/V/V*);
- *mobile phase B*: *methanol R2*, *acetonitrile R1*, *water for chromatography R* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 40 | 100 → 85 | 0 → 15 |
| 40 - 42 | 85 → 0 | 15 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL.

*Retention time*: impurity A = about 19 min; 1-vinylpyrrolidin-2-one = about 25 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to impurity A and 1-vinylpyrrolidin-2-one.

*Limit*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (100 ppm).

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* Mix 0.200 g of the substance to be examined with *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 30 mg of *citric acid monohydrate R* and 0.100 g of *acetic acid R* (impurity B) in the mobile phase. Shake gently to dissolve and dilute to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 0.50 g/L solution of *sulfuric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL. After each injection, rinse the column with a mixture of equal volumes of *acetonitrile R1* and a 0.50 g/L solution of *sulfuric acid R*.

*Retention time*: impurity B = about 5 min; citric acid = about 7 min.

*System suitability*: reference solution:
- *resolution*: minimum 2.0 between the peaks due to impurity B and citric acid.

*Limit*:
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (1.5 per cent).

**Sulfated ash** (*2.4.14*): maximum 3.0 per cent, determined on 5.0 g.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C.

IMPURITIES

*Specified impurities: A, B.*

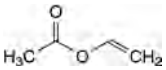

A. ethenyl acetate,

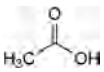

B. acetic acid.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are*

*recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for macrogol poly(vinyl alcohol) grafted copolymer used as film former in film-coated tablets.*

**Viscosity** (*2.2.10*): typically less than 250 mPa·s, determined on a 20 per cent *m/m* solution, using a rotating viscometer at 25 °C and rotation speed of 100 r/min.

**07/2019:1234**

# MACROGOL STEARATE

## Macrogoli stearas

DEFINITION

Mixture of monoesters and diesters of mainly stearic (octadecanoic) acid and/or palmitic (hexadecanoic) acid and macrogols. It may be obtained by ethoxylation or by esterification of macrogols with stearic acid 50 (type I) or stearic acid 95 (type II) (see *Stearic acid (1474)*). It may contain free macrogols. The average polymer length is equivalent to 6 to 100 ethylene oxide units per molecule (nominal value).

CHARACTERS

*Appearance*: white or slightly yellowish, waxy mass.

*Solubility*: soluble in ethanol (96 per cent) and in 2-propanol. Macrogol stearate corresponding to a product with 6-9 units of ethylene oxide per molecule is practically insoluble, but freely dispersible in water and miscible with fatty oils and with waxes. Macrogol stearate corresponding to a product with 20-100 units of ethylene oxide per molecule is soluble in water and practically insoluble in fatty oils and in waxes.

IDENTIFICATION

A. Saponification value (see Tests).

B. Composition of fatty acids (see Tests).

TESTS

**Alkalinity**. Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent. To 2 mL of this solution add 0.05 mL of *phenol red solution R*. The solution is not red.

**Melting point** (*2.2.15*). See Table 1234.-1.

Melt about 10 g at 80-90 °C. Introduce a sufficient amount of the substance into the tube by capillary action to form a column of the prescribed height. Allow to stand at 0 °C for 2 h.

**Acid value** (*2.5.1*): maximum 4.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*). See Table 1234.-1.

**Iodine value** (*2.5.4*): maximum 2.0.

**Saponification value** (*2.5.6*). See Table 1234.-1.

Table 1234.-1

| Ethylene oxide units per molecule (nominal value) | Melting point (°C) | Hydroxyl value | Saponification value |
|---|---|---|---|
| 6 | 26 - 37 | 80 - 110 | 85 - 115 |
| 8 - 9 | 26 - 35 | 80 - 105 | 88 - 100 |
| 20 | 33 - 40 | 50 - 62 | 46 - 56 |
| 32 | 46 - 50 | 20 - 40 | 30 - 45 |
| 40 - 50 | 38 - 52 | 23 - 40 | 20 - 35 |
| 100 | 48 - 60 | 15 - 30 | 5 - 20 |

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance:*

| | Type of fatty acid used | Composition of fatty acids |
|---|---|---|
| Macrogol stearate type I | Stearic acid 50 | *Stearic acid*: 40.0 per cent to 60.0 per cent,<br>*Sum of the contents of palmitic and stearic acids*: not less than 90.0 per cent. |
| Macrogol stearate type II | Stearic acid 95 | *Stearic acid*: 90.0 per cent to 99.0 per cent,<br>*Sum of the contents of palmitic and stearic acids*: not less than 96.0 per cent. |

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.50 g. Use as the solvent a mixture of equal volumes of *anhydrous methanol R* and *methylene chloride R*.

**Total ash** (*2.4.16*): maximum 0.3 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the number of ethylene oxide units per molecule (nominal value);
- the type of macrogol stearate.

**01/2008:1340**

# MACROGOL STEARYL ETHER

## Macrogoli aether stearylicus

DEFINITION

Mixture of ethers obtained by ethoxylation of stearyl alcohol. It may contain some free macrogols and various amounts of free stearyl alcohol. The number of moles of ethylene oxide reacted per mole of stearyl alcohol is 2 to 20 (nominal value).

CHARACTERS

*Appearance*: white or yellowish-white, waxy, unctuous mass, pellets, microbeads or flakes.

*Solubility*:

- macrogol stearyl ether with 2 moles of ethylene oxide reacted per mole: practically insoluble in water, soluble in ethanol (96 per cent) with heating and in methylene chloride;
- macrogol stearyl ether with 10 moles of ethylene oxide reacted per mole: soluble in water and in ethanol (96 per cent);
- macrogol stearyl ether with 20 moles of ethylene oxide reacted per mole: soluble in water, in ethanol (96 per cent) and in methylene chloride.

After melting, it solidifies at about 45 °C.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Saponification value (see Tests).

D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10.0 g in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 75 mL with the same mixture of solvents. Add 60 mL of *heptane R* and shake for 3 min. The formation of foam can be reduced by the addition of a few drops of *ethanol (96 per cent) R*. Filter the upper layer through *anhydrous sodium sulfate R*, wash the filter with 3 quantities, each of 10 mL, of *heptane R* and evaporate the combined filtrates to dryness. Dissolve 50 mg of the residue in 10 mL of *methanol R* (the solution may be opalescent).

*Reference solution*. Dissolve 25 mg of *stearyl alcohol CRS* in *methanol R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*.

*Application*: 20 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with vanillin-sulfuric acid reagent prepared as follows: dissolve 0.5 g of *vanillin R* in 50 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *sulfuric acid R*; allow to dry in air; heat at about 130 °C for 15 min and allow to cool in air.

*Results*: the chromatogram obtained with the test solution shows several spots; one of these spots corresponds to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve or disperse 0.1 g in 5 mL of *ethanol (96 per cent) R*, add 2 mL of *water R*, 10 mL of *dilute hydrochloric acid R*, 10 mL of *barium chloride solution R1* and 10 mL of a 100 g/L solution of *phosphomolybdic acid R*. A precipitate is formed.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 5.0 g in *ethanol (96 per cent) R* and dilute to 50 mL with the same solvent.

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*).

| Number of moles of ethylene oxide reacted per mole (nominal value) | Hydroxyl value |
|---|---|
| 2 | 150 - 180 |
| 10 | 75 - 90 |
| 20 | 40 - 60 |

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

**Saponification value** (*2.5.6*): maximum 3.0, determined on 10.0 g.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of stearyl alcohol (nominal value).

**01/2008:1122**
**corrected 6.0**

# MACROGOLGLYCEROL COCOATES

## Macrogolglyceroli cocoates

DEFINITION

Mixtures of mono-, di- and triesters of ethoxylated glycerol with fatty acids of vegetable origin having a composition corresponding to the fatty acid composition of the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. The average number of moles of ethylene oxide reacted per mole of substance (nominal value) is either 7 (macrogol 7 glycerol cocoate) or 23 (macrogol 23 glycerol cocoate).

CHARACTERS

*Appearance*: clear, yellowish, oily liquid.

*Solubility*: soluble in water and in ethanol (96 per cent) and practically insoluble in light petroleum (bp: 50-70 °C) for macrogol 7 glycerol cocoate and macrogol 23 glycerol cocoate.

*Relative density*: about 1.05 for macrogol 7 glycerol cocoate; about 1.09 for macrogol 23 glycerol cocoate.

IDENTIFICATION

A. Dissolve 1.0 g of macrogol 7 glycerol cocoate in 99 g of a mixture of 10 volumes of *2-propanol R* and 90 volumes of *water R*. Heat the solution to about 65 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 35 °C and 54 °C.

Heat a 10 g/L solution of macrogol 23 glycerol cocoate in a 100 g/L solution of *sodium chloride R* to about 90 °C. A turbidity is produced. Allow to cool until the turbidity disappears. The cloud point is between 65 °C and 85 °C.

B. Iodine value (see Tests).

C. Saponification value (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_2$ (*2.2.2, Method I*).

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *ethanol (96 per cent) R* and 10 mL of *water R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 5.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): see Table 1122.-1.

**Saponification value** (*2.5.6*): see Table 1122.-1.

Table 1122.-1

| Number of moles of ethylene oxide reacted per mole (nominal value) | Hydroxyl value | Saponification value (determined on 2.0 g) |
|---|---|---|
| 7 | 170 - 210 | 85 - 105 |
| 23 | 80 - 100 | 40 - 50 |

**Iodine value** (*2.5.4, Method A*): maximum 5.0.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

- *caproic acid*: maximum 1.0 per cent;
- *caprylic acid*: 5.0 per cent to 10.0 per cent;
- *capric acid*: 4.0 per cent to 10.0 per cent;

– *lauric acid*: 40.0 per cent to 55.0 per cent;
– *myristic acid*: 14.0 per cent to 23.0 per cent;
– *palmitic acid*: 8.0 per cent to 12.0 per cent;
– *stearic acid*: 1.0 per cent to 5.0 per cent;
– *oleic acid*: 5.0 per cent to 10.0 per cent;
– *linoleic acid*: maximum 3.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.0 g.

**Total ash** (*2.4.16*): maximum 0.3 per cent.

LABELLING

The label states the number of moles of ethylene oxide reacted per mole of substance (nominal value).

**01/2017:1083**

# MACROGOLGLYCEROL HYDROXYSTEARATE

## Macrogolglyceroli hydroxystearas

DEFINITION

Contains mainly tris(12-hydroxystearyl) glycerol ethoxylated with 7 to 60 molecules of ethylene oxide (nominal value), with small amounts of macrogol hydroxystearate and of the corresponding free glycols. It results from the reaction of hydrogenated castor oil with ethylene oxide.

CHARACTERS

*Appearance*:
– if less than 10 units of ethylene oxide per molecule: yellowish, turbid, viscous liquid;
– if more than 20 units of ethylene oxide per molecule: white or yellowish semi-liquid or pasty mass.

*Solubility*:
– if less than 10 units of ethylene oxide per molecule: practically insoluble in water, soluble in acetone, dispersible in ethanol (96 per cent);
– if more than 20 units of ethylene oxide per molecule: freely soluble in water, in acetone and in ethanol (96 per cent), practically insoluble in light petroleum.

IDENTIFICATION

A. Iodine value (see Tests).
B. Saponification value (see Tests).
C. Thin-layer chromatography (*2.2.27*).

*Test solution.* To 1 g of the substance to be examined, add 100 mL of a 100 g/L solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Allow to cool. Acidify the solution with 20 mL of *hydrochloric acid R*. Shake the mixture with 50 mL of *ether R* and allow to stand until separation of the layers is obtained. Transfer the clear upper layer to a suitable tube, add 5 g of *anhydrous sodium sulfate R*, close the tube and allow to stand for 30 min. Filter and evaporate the filtrate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 mL of *ether R*.

*Reference solution.* Dissolve 50 mg of *12-hydroxystearic acid R* in *methylene chloride R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R.*

*Mobile phase*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 8 cm.

*Drying*: in a current of cold air.

*Detection*: spray with a 80 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for about 1-2 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

D. Place about 2 g in a test-tube and add 0.2 mL of *sulfuric acid R*. Close the tube using a stopper fitted with a glass tube bent twice at right angles. Heat the tube until white fumes appear. Collect the fumes in 1 mL of *mercuric chloride solution R*. A white precipitate is formed and the fumes turn a filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

TESTS

**Solution S**. Dissolve 5.0 g of macrogolglycerol hydroxystearate with less than 40 units of ethylene oxide per molecule in a mixture of 50 volumes of *acetone R* and 50 volumes of *anhydrous ethanol R* and dilute to 50 mL with the same mixture of solvents.

Dissolve 5.0 g of macrogolglycerol hydroxystearate with 40 units or more of ethylene oxide per molecule in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Alkalinity**. To 2 mL of solution S add 0.5 mL of *bromothymol blue solution R1*. The solution is not blue.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*). See Table 1083.-1.

**Iodine value** (*2.5.4*): maximum 5.0.

**Saponification value** (*2.5.6*). See Table 1083.-1.

Table 1083.-1

| Ethylene oxide units per molecule (nominal value) | Hydroxyl value | Saponification value |
|---|---|---|
| 7 | 115 - 135 | 125 - 140 |
| 25 | 70 - 90 | 70 - 90 |
| 40 | 57 - 80 | 45 - 69 |
| 60 | 45 - 67 | 40 - 51 |

**Residual ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.000 g.

**Total ash** (*2.4.16*): maximum 0.3 per cent, determined on 2.0 g.

LABELLING

The label states the number of ethylene oxide units per molecule (nominal value).

**01/2017:1082**

# MACROGOLGLYCEROL RICINOLEATE

## Macrogolglyceroli ricinoleas

DEFINITION

Contains mainly ricinoleyl glycerol ethoxylated with 30-50 molecules of ethylene oxide (nominal value), with small amounts of macrogol ricinoleate and of the corresponding free glycols. It results from the reaction of castor oil with ethylene oxide.

CHARACTERS

*Appearance*: clear, yellow viscous liquid or semi-solid.

*Solubility*: freely soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

*Relative density*: about 1.05.

*Viscosity*: 500 mPa·s to 800 mPa·s at 25 °C.

IDENTIFICATION

A. Iodine value (see Tests).

B. Saponification value (see Tests).

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. To 1 g of the substance to be examined add 100 mL of a 100 g/L solution of *potassium hydroxide R* and boil under a reflux condenser for 30 min. Allow to cool. Acidify the solution with 20 mL of *hydrochloric acid R*. Shake the mixture with 50 mL of *ether R* and allow to stand until separation of the layers is obtained. Transfer the clear upper layer to a suitable tube, add 5 g of *anhydrous sodium sulfate R*, close the tube and allow to stand for 30 min. Filter and evaporate the filtrate to dryness on a water-bath. Dissolve 50 mg of the residue in 25 mL of *ether R*.

*Reference solution*. Dissolve 50 mg of *ricinoleic acid R* in *methylene chloride R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (10:40:50 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 8 cm.

*Drying*: in a current of cold air.

*Detection*: spray with an 80 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 120 °C for 1-2 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with the reference solution.

D. Place about 2 g of the substance to be examined in a test-tube and add 0.2 mL of *sulfuric acid R*. Close the tube using a stopper fitted with a glass tube bent twice at right angles. Heat the tube until white fumes appear. Collect the fumes in 1 mL of *mercuric chloride solution R*. A white precipitate is formed and the fumes turn a filter paper impregnated with *alkaline potassium tetraiodomercurate solution R* black.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*). If intended for use in the manufacture of parenteral preparations, solution S is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Alkalinity**. Dissolve 2.0 g in a hot mixture of 10 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*). See Table 1082.-1.

**Iodine value** (*2.5.4*): 25 to 35.

**Saponification value** (*2.5.6*). See Table 1082.-1.

Table 1082.-1

| Ethylene oxide units per molecule (nominal value) | Hydroxyl value | Saponification value |
|---|---|---|
| 30 - 35 | 65 - 82 | 60 - 75 |
| 50 | 48 - 68 | 38 - 52 |

**Residual ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of residual ethylene oxide and 10 ppm of residual dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 2.000 g.

**Total ash** (*2.4.16*): maximum 0.3 per cent, determined on 2.0 g.

STORAGE

Protected from light.

LABELLING

The label states:

- the amount of ethylene oxide reacted with castor oil (nominal value),
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:1444**

# MACROGOLS

## Macrogola

DEFINITION

Mixtures of polymers with the general formula $H-[OCH_2-CH_2]_n-OH$ where *n* represents the average number of oxyethylene groups. The type of macrogol is defined by a number that indicates the average relative molecular mass. A suitable stabiliser may be added.

CHARACTERS

| Type of macrogol | Appearance | Solubility |
|---|---|---|
| 300<br>400<br>600 | clear, viscous, colourless or almost colourless, hygroscopic liquid | miscible with water, very soluble in acetone, in alcohol and in methylene chloride, practically insoluble in fatty oils and in mineral oils |
| 1000 | white or almost white, hygroscopic solid with a waxy or paraffin-like appearance | very soluble in water, freely soluble in alcohol and in methylene chloride, practically insoluble in fatty oils and in mineral oils |
| 1500 | white or almost white solid with a waxy or paraffin-like appearance | very soluble in water and in methylene chloride, freely soluble in alcohol, practically insoluble in fatty oils and in mineral oils |
| 3000<br>3350 | white or almost white solid with a waxy or paraffin-like appearance | very soluble in water and in methylene chloride, very slightly soluble in alcohol, practically insoluble in fatty oils and in mineral oils |
| 4000<br>6000<br>8000 | white or almost white solid with a waxy or paraffin-like appearance | very soluble in water and in methylene chloride, practically insoluble in alcohol, in fatty oils and in mineral oils |
| 20 000<br>35 000 | white or almost white solid with a waxy or paraffin-like appearance | very soluble in water, soluble in methylene chloride, practically insoluble in alcohol, in fatty oils and in mineral oils |

IDENTIFICATION

A. Viscosity (see Tests).

B. To 1 g in a test-tube add 0.5 mL of *sulfuric acid R*, close the test-tube with a stopper fitted with a bent delivery tube and heat until white fumes are evolved. Collect the fumes via the delivery tube into 1 mL of *mercuric chloride solution R*. An abundant, white, crystalline precipitate is formed.

C. To 0.1 g add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R* and mix thoroughly with a glass rod. Add 5 mL of *methylene chloride R* and shake. The liquid phase becomes blue.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 12.5 g in *water R* and dilute to 50 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 5.0 g in 50 mL of *carbon dioxide-free water R* and add 0.15 mL of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Viscosity** (*2.2.9*). The viscosity is calculated using a density given in Table 1444.-1.

For macrogols with a relative molecular mass greater than 400, determine the viscosity on a 50 per cent *m/m* solution of the substance to be examined.

**Freezing point** (*2.2.18*): see Table 1444.-2.

**Hydroxyl value**. Introduce *m* g (see Table 1444.-3) into a dry conical flask fitted with a reflux condenser. Add 25.0 mL of *phthalic anhydride solution R*, swirl to dissolve and boil under a reflux condenser on a hot plate for 60 min. Allow to cool. Rinse the condenser first with 25 mL of *pyridine R* and then with 25 mL of *water R*, add 1.5 mL of *phenolphthalein solution R* and titrate with *1 M sodium hydroxide* until a faint pink colour is obtained ($n_1$ mL). Carry out a blank test ($n_2$ mL). Calculate the hydroxyl value using the following expression:

$$\frac{56.1 \times (n_2 - n_1)}{m}$$

For macrogols with a relative molecular mass greater than 1000, if the water content is more than 0.5 per cent, dry a sample of suitable mass at 100-105 °C for 2 h and carry out the determination of the hydroxyl value on the dried sample.

Table 1444.-1

| Type of macrogol | Kinematic viscosity ($mm^2 \cdot s^{-1}$) | Dynamic viscosity (mPa·s) | Density* (g/mL) |
|---|---|---|---|
| 300 | 71 - 94 | 80 - 105 | 1.120 |
| 400 | 94 - 116 | 105 - 130 | 1.120 |
| 600 | 13.9 - 18.5 | 15 - 20 | 1.080 |
| 1000 | 20.4 - 27.7 | 22 - 30 | 1.080 |
| 1500 | 31 - 46 | 34 - 50 | 1.080 |
| 3000 | 69 - 93 | 75 - 100 | 1.080 |
| 3350 | 76 - 110 | 83 - 120 | 1.080 |
| 4000 | 102 - 158 | 110 - 170 | 1.080 |
| 6000 | 185 - 250 | 200 - 270 | 1.080 |
| 8000 | 240 - 472 | 260 - 510 | 1.080 |
| 20 000 | 2500 - 3200 | 2700 - 3500 | 1.080 |
| 35 000 | 10 000 - 13 000 | 11 000 - 14 000 | 1.080 |

*Density of the substance for macrogols 300 and 400. Density of the 50 per cent *m/m* solution for the other macrogols.

Table 1444.-2

| Type of macrogol | Freezing point (°C) |
|---|---|
| 600 | 15 - 25 |
| 1000 | 35 - 40 |
| 1500 | 42 - 48 |
| 3000 | 50 - 56 |
| 3350 | 53 - 57 |
| 4000 | 53 - 59 |
| 6000 | 55 - 61 |
| 8000 | 55 - 62 |
| 20 000 | minimum 57 |
| 35 000 | minimum 57 |

Table 1444.-3

| Type of macrogol | Hydroxyl value | *m* (g) |
|---|---|---|
| 300 | 340 - 394 | 1.5 |
| 400 | 264 - 300 | 1.9 |
| 600 | 178 - 197 | 3.5 |
| 1000 | 107 - 118 | 5.0 |
| 1500 | 70 - 80 | 7.0 |
| 3000 | 34 - 42 | 12.0 |
| 3350 | 30 - 38 | 12.0 |
| 4000 | 25 - 32 | 14.0 |
| 6000 | 16 - 22 | 18.0 |
| 8000 | 12 - 16 | 24.0 |
| 20 000 | - | - |
| 35 000 | - | - |

**Reducing substances**. Dissolve 1 g in 1 mL of a 10 g/L solution of *resorcinol R* and warm gently if necessary. Add 2 mL of *hydrochloric acid R*. After 5 min the solution is not more intensely coloured than reference solution $R_3$ (*2.2.2, Method I*).

**Formaldehyde**: maximum 30 ppm.

*Test solution.* To 1.00 g add 0.25 mL of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 mL of *sulfuric acid R*. Allow to stand for 15 min and dilute slowly to 10 mL with *water R*.

*Reference solution.* Dilute 0.860 g of *formaldehyde solution R* to 100 mL with *water R*. Dilute 1.0 mL of this solution to 100 mL with *water R*. In a 10 mL flask, mix 1.00 mL of this solution and 0.25 mL of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 mL of *sulfuric acid R*. Allow to stand for 15 min and dilute slowly to 10 mL with *water R*.

*Blank solution.* In a 10 mL flask mix 1.00 mL of *water R* and 0.25 mL of *chromotropic acid, sodium salt solution R*, cool in iced water and add 5.0 mL of *sulfuric acid R*. Dilute slowly to 10 mL with *water R*.

Determine the absorbance (*2.2.25*) of the test solution at 567 nm, against the blank solution. It is not higher than that of the reference solution.

If the use of macrogols with a higher content of formaldehyde may have adverse effects, the competent authority may impose a limit of not more than 15 ppm.

**Ethylene glycol and diethylene glycol**: *carry out this test only if the macrogol has a relative molecular mass below 1000.*

Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 5.00 g of the substance to be examined in *acetone R* and dilute to 100.0 mL with the same solvent.

*Reference solution*. Dissolve 0.10 g of *ethylene glycol R* and 0.50 g of *diethylene glycol R* in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetone R*.

*Column*:

– *material*: glass;

– *size*: *l* = 1.8 m, Ø = 2 mm;

– *stationary phase*: *silanised diatomaceous earth for gas chromatography R*, impregnated with 5 per cent *m/m* of *macrogol 20 000 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: if necessary, precondition the column by heating at 200 °C for about 15 h; adjust the initial temperature of the column to obtain a retention time of 14-16 min for diethylene glycol; raise the temperature of the column by about 30 °C at a rate of 2 °C/min but without exceeding 170 °C;

– *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 2 µL.

Carry out 5 replicate injections to check the repeatability of the response.

*Limit*: maximum 0.4 per cent, calculated as the sum of the contents of ethylene glycol and diethylene glycol.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 2.0 per cent for macrogols with a relative molecular mass not greater than 1000 and maximum 1.0 per cent for macrogols with a relative molecular mass greater than 1000, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

– the type of macrogol;

– the content of formaldehyde.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for macrogols used as solvent.*

**Viscosity** (see Tests).

*The following characteristics may be relevant for macrogols used as suspension stabiliser and thickener.*

**Viscosity** (see Tests).

*The following characteristic may be relevant for macrogols used as lubricant in tablets.*

**Particle-size distribution** (*2.9.31*).

*The following characteristics may be relevant for macrogols used as suppository base and for macrogols used in hydrophilic ointments.*

**Viscosity** (see Tests).

**Melting point** (*2.2.15*).

**01/2015:2444**

# MACROGOLS, HIGH-MOLECULAR-MASS

## Macrogola massae molecularis magnae

DEFINITION

Mixtures of high-molecular-mass macrogols (also known as polyethylene oxides). The type of high-molecular-mass macrogol is defined by a number that indicates the nominal average molecular mass in the range of 100 000 to 7 000 000. A suitable stabiliser (e.g. butylhydroxytoluene) and a suitable flowability agent (e.g. silicon dioxide) may be added.

CHARACTERS

*Appearance*: white or almost white, free-flowing powder.

*Solubility*: freely soluble in water.

mp: minimum 65 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *high-molecular-mass macrogol CRS*.

B. Viscosity (see Tests).

TESTS

**Solution S**. Prepare a solution of the substance to be examined in a mixture of *carbon dioxide-free water R* and *2-propanol R* according to Table 2444.-1.

**pH** (*2.2.3*): 6.0 to 10.0 for solution S.

**Viscosity** (*2.2.10*). Determine the dynamic viscosity of solution S at 25 °C using a rotating viscometer and a suitable spindle.

Table 2444.-1. – *Solution S and viscosity*

| Nominal average molecular mass | Composition of solution S (g of substance / mL of *carbon dioxide-free water R* + mL of *2-propanol R*) | Dynamic viscosity (mPa·s) | Rotation speed (r/min) |
|---|---|---|---|
| 100 000 | 30 g / 570 + 125 | 12 - 50 | 50 |
| 200 000 | 30 g / 570 + 125 | 65 - 115 | 50 |
| 300 000 | 30 g / 570 + 125 | 600 - 1200 | 10 |
| 400 000 | 30 g / 570 + 125 | 2250 - 4500 | 2 |
| 600 000 | 30 g / 570 + 125 | 4500 - 8800 | 2 |
| 900 000 | 30 g / 570 + 125 | 8800 - 17 600 | 2 |
| 1 000 000 | 12 g / 588 + 125 | 400 - 800 | 10 |
| 2 000 000 | 12 g / 588 + 125 | 2000 - 4000 | 10 |
| 4 000 000 | 6 g / 594 + 125 | 1650 - 5500 | 2 |
| 5 000 000 | 6 g / 594 + 125 | 5500 - 7500 | 2 |
| 7 000 000 | 6 g / 594 + 125 | 7500 - 10 000 | 2 |

**Ethylene oxide** (*2.4.25, Method A*): maximum 1 ppm.

Carry out the test with the following modifications.

*Stock solution.* Dilute 0.5 mL of *ethylene oxide stock solution R2* to 10.0 mL with *propanol R*. Dilute 1.0 mL of this solution to 50.0 mL with *propanol R*.

*Test solution.* Weigh 0.50 g ($M_T$) of the substance to be examined into a 10 mL vial (other sizes may be used depending on the operating conditions). Close and allow to stand at 70 °C for 30 min.

*Reference solution (a).* Weigh 0.50 g ($M_R$) of the substance to be examined into an identical 10 mL vial and add 10 µL of the stock solution. Close and homogenise, and allow to stand at 70 °C for 30 min.

*Reference solution (b).* To 0.50 mL of *ethylene oxide solution R3* in a 10 mL vial add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R*. Close and homogenise, and allow to stand at 70 °C for 30 min.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 45 min.

**Sulfated ash**: maximum 5.0 per cent, determined on 1.00 g.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.00 g of the substance to be examined in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C, until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined…".

STORAGE

In an airtight container.

LABELLING

The label states the type of high-molecular-mass macrogol.

**01/2017:1539**

# MAGALDRATE

## Magaldratum

$Al_5Mg_{10}(OH)_{31}(SO_4)_2,xH_2O$ $M_r$ 1097 (anhydrous substance)
[74978-16-8]

DEFINITION

Magaldrate is composed of aluminium and magnesium hydroxides and sulfates. Its composition corresponds approximately to the formula $Al_5Mg_{10}(OH)_{31}(SO_4)_2,xH_2O$.

*Content*: 90.0 per cent to 105.0 per cent (dried substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It is soluble in dilute mineral acids.

IDENTIFICATION

A. Dissolve 0.6 g in 20 mL of *3 M hydrochloric acid R*, add about 30 mL of *water R* and heat to boiling. Adjust to pH 6.2 with *dilute ammonia R1*, continue boiling for a further 2 min, filter and retain the precipitate and the filtrate. To 2 mL of the filtrate add 2 mL of *ammonium chloride solution R* and neutralise with a solution prepared by dissolving 2 g of *ammonium carbonate R* and 2 mL of *dilute ammonia R1* in 20 mL of *water R*; no precipitate is produced. Add *disodium hydrogen phosphate solution R*; a white, crystalline precipitate is produced which does not dissolve in *dilute ammonia R1*.

B. The precipitate retained in identification test A gives the reaction of aluminium (*2.3.1*).

C. The filtrate retained in identification test A gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Soluble chlorides**: maximum 3.5 per cent.

To 0.5 g add 25 mL of *dilute nitric acid R* and shake until completely dissolved. Add 10.0 mL of *0.1 M silver nitrate* and 2 mL of *ferric ammonium sulfate solution R2* as indicator. Titrate with *0.1 M ammonium thiocyanate*, shaking vigorously until a persistent brownish-red colour is obtained.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Soluble sulfates**: maximum 1.9 per cent.

Disperse 0.5 g in 25 mL of *water R*, boil for 5 min, cool, dilute to 25.0 mL with *water R*, mix and filter. To 2.5 mL of the filtrate, add 30 mL of *water R*, neutralise to *blue litmus paper R* with *hydrochloric acid R*, add 3 mL of *1 M hydrochloric acid*, 3 mL of a 120 g/L solution of *barium chloride R* and dilute to 50 mL with *water R*. Mix and allow to stand for 10 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner using 1 mL of *0.01 M sulfuric acid* instead of 2.5 mL of filtrate.

**Sulfates**: 16.0 per cent to 21.0 per cent (dried substance).

Dissolve 0.875 g in a mixture of 5 mL of *glacial acetic acid R* and 10 mL of *water R* and dilute to 25.0 mL with *water R*. Prepare a chromatographic column of 1 cm in internal diameter containing 15 mL of *cation-exchange resin R* (150-300 µm), previously washed with 30 mL of *water R*. Transfer 5.0 mL of the solution to be examined to the column and elute with 15 mL of *water R*. To the eluate add 5 mL of a 53.6 g/L solution of *magnesium acetate R*, 32 mL of *methanol R* and 0.2 mL of *alizarin S solution R*. Add from a burette about 4.0 mL of *0.05 M barium chloride*, add a further 0.2 mL of

*alizarin S solution R* and slowly complete the titration until the yellow colour disappears and a violet-red tinge is visible.

1 mL of *0.05 M barium chloride* is equivalent to 4.803 mg of $SO_4$.

**Aluminium hydroxide**: 32.1 per cent to 45.9 per cent (dried substance).

Dissolve 0.800 g in 10 mL of *dilute hydrochloric acid R*, heating on a water-bath. Cool and dilute to 50.0 mL with *water R*. To 10.0 mL of this solution, add *dilute ammonia R1* until a precipitate begins to appear. Add the smallest quantity of *dilute hydrochloric acid R* needed to dissolve the precipitate and dilute to 20 mL with *water R*. Carry out the complexometric titration of aluminium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 7.80 mg of $Al(OH)_3$.

**Magnesium hydroxide**: 49.2 per cent to 66.6 per cent (dried substance).

Dissolve 0.100 g in 2 mL of *dilute hydrochloric acid R* and transfer to a 500 mL conical flask with the aid of *water R*. Dilute to 200 mL with *water R*, add 20 mL of *triethanolamine R* with shaking, 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 5.832 mg of $Mg(OH)_2$.

**Sodium**: maximum 0.10 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Weigh 2.00 g into a 100 mL volumetric flask, place in an ice-bath, add 5 mL of *nitric acid R* and swirl to mix. Allow to warm to room temperature and dilute to 100 mL with *water R*. Filter, if necessary, to obtain a clear solution. Dilute 10.0 mL of the filtrate to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *dilute nitric acid R*.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 589 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): 10.0 per cent to 20.0 per cent, determined on 1.000 g by drying in an oven at 200 °C for 4 h.

ASSAY

To 1.500 g add 50.0 mL of *1 M hydrochloric acid*. Titrate the excess hydrochloric acid with *1 M sodium hydroxide* to pH 3.0, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *1 M hydrochloric acid* is equivalent to 35.40 mg of $Al_5Mg_{10}(OH)_{31}(SO_4)_2$.

**01/2017:2035**

# MAGNESIUM ACETATE TETRAHYDRATE

## Magnesii acetas tetrahydricus

$Mg(CH_3COO)_2,4H_2O$ $M_r$ 214.5
[16674-78-5]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent of magnesium acetate (anhydrous substance).

CHARACTERS

*Appearance*: colourless crystals or white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve about 100 mg in 2 mL of *water R*. Add 1 mL of *dilute ammonia R1* and heat. A white precipitate is formed that dissolves slowly on addition of 5 mL of *ammonium chloride solution R*. Add 1 mL of *disodium hydrogen phosphate solution R*. A white crystalline precipitate is formed.

B. It gives reaction (b) of acetates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 7.5 to 8.5.

Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dissolve 1.0 g in *water R* and dilute to 100 mL with the same solvent.

**Nitrates**: maximum 3 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 10 mL with the same solvent, add 5 mg of *sodium chloride R*, 0.05 mL of *indigo carmine solution R* and while stirring, 10 mL of *nitrogen-free sulfuric acid R*. A blue colour is produced which persists for at least 10 min.

**Sulfates** (*2.4.13*): maximum 600 ppm.

Dissolve 0.25 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Aluminium** (*2.4.17*): maximum 1 ppm.

*Prescribed solution.* Dissolve 4.0 g in *water R* and dilute to 100 mL with the same solvent. Add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution.* Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution.* Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Calcium** (*2.4.3*): maximum 100 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Potassium**: maximum 0.1 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* Dissolve 0.5 g in *water R* and dilute to 100 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *potassium standard solution (600 ppm K) R*, diluted as necessary with *water R*.

*Wavelength*: 766.5 nm.

**Sodium**: maximum 0.5 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* Dissolve 1.0 g in *water R* and dilute to 100 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589.0 nm.

**Readily oxidisable substances**. Dissolve 2.0 g in 100 mL of boiling *water R*, add 6 mL of a 150 g/L solution of *sulfuric acid R* and 0.3 mL of *0.02 M potassium permanganate*. Mix and boil gently for 5 min. The pink colour is not completely discharged.

**Water** (*2.5.12*): 33.0 per cent to 35.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.150 g in 300 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 14.24 mg of $C_4H_6MgO_4$.

**04/2019:2854**

# MAGNESIUM ALUMINOMETASILICATE

## Magnesii aluminometasilicas

DEFINITION

Magnesium aluminometasilicate of synthetic origin exists in 2 forms (type A and type B), which differ in their apparent pH. It contains a variable quantity of water.

*Content (type A and type B)*:

- *silicon dioxide* ($SiO_2$; $M_r$ 60.1): 29.2 per cent to 35.6 per cent (dried substance);
- *aluminium oxide* ($Al_2O_3$; $M_r$ 102.0): 29.1 per cent to 35.5 per cent (dried substance);
- *magnesium oxide* (MgO; $M_r$ 40.30): 11.4 per cent to 14.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder or granules.

*Solubility*: practically insoluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. To 0.5 g add 5 mL of a mixture of 1 volume of *sulfuric acid R* and 2 volumes of *water R* and heat until white fumes evolve. Cool, add 20 mL of *water R* and filter. Use the filtrate for identification tests B and C. Wash the residue with *water R*. The residue gives the reaction of silicates (*2.3.1*).

B. Neutralise the filtrate obtained in identification test A with *ammonia R*. A white, gelatinous precipitate is formed. Centrifuge and keep the supernatant for identification test C. Dissolve the precipitate in *dilute hydrochloric acid R*. Add dropwise *dilute sodium hydroxide solution R*. A white, gelatinous precipitate is formed. Filter and add a few drops of *phenolphthalein solution R* to the residue. The residue turns pink. Wash the residue with *water R* until the pink colour is completely discharged and the residue remains white upon addition of a drop of *phenolphthalein solution R*. Sprinkle a few crystals of *sodium fluoride R* on the residue. The residue, in contact with the crystals, turns pink again in a short time.

C. To 2 mL of the supernatant obtained after centrifugation in identification test B, add 1 mL of *dilute ammonia R1* and 1 mL of *ammonium chloride solution R*. Upon the addition of *dilute ammonia R1* a white precipitate may form, which dissolves after addition of the *ammonium chloride solution R*. Add 1 mL of *disodium hydrogen phosphate solution R*. A white precipitate is formed.

TESTS

**Solution S**. Disperse 10.0 g in 100.0 mL of *water R* and boil gently for 15 min with shaking. After cooling, dilute to 100.0 mL with *water R* and centrifuge. Dilute 50.0 mL of the clear supernatant to 100.0 mL with *water R*.

**pH** (*2.2.3*): 6.0 to 8.5 for type A; 8.5 to 10.5 for type B.

Disperse 2.0 g in 50 mL of *carbon dioxide-free water R*. Read the pH after the electrode has been immersed in the suspension for 2 min.

**Water-soluble salts**: maximum 1.5 per cent.

Evaporate 25 mL of solution S to dryness on a water-bath and heat at 700 °C for 2 h. The residue weighs a maximum of 19 mg.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 2 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 10 mL of solution S to 100 mL with *distilled water R*. Dilute 6 mL of the solution to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 300 ppm.

Disperse 0.10 g in 8 mL of *dilute nitric acid R*, boil for 1 min, cool and dilute to 30.0 mL with *water R*. Centrifuge and use 10 mL of the clear supernatant.

**Loss on drying** (*2.2.32*): maximum 20.0 per cent, determined on 1.000 g by drying in an oven at 110 °C for 7 h.

**Neutralising capacity**: minimum 210 mL of *0.1 M hydrochloric acid* per gram of dried substance.

To 0.200 g in a glass-stoppered flask add 100.0 mL of *0.1 M hydrochloric acid*. Stopper the flask tightly, shake at 37 ± 2 °C for 1 h and filter. Titrate 50.0 mL of the filtrate with *0.1 M sodium hydroxide* to pH 3.5 while stirring thoroughly. Carry out a blank determination.

ASSAY

**Silicon dioxide**. In a suitable container, disperse 1.000 g (*m*) in 30 mL of *dilute hydrochloric acid R* and evaporate to dryness on a water-bath. Moisten the residue with *hydrochloric acid R* and evaporate to dryness on a water-bath. To the residue add 8 mL of *hydrochloric acid R*, stir, add 25 mL of boiling *water R* and stir again. Allow to stand, then filter the supernatant through filter paper. To the residue in the container add 10 mL of boiling *water R*, stir, allow to stand, then filter the supernatant through the filter paper. Wash the residue in the container with 3 quantities, each of 10 mL, of boiling *water R*, each time filtering the washings through the filter paper. Add 50 mL of *water R* to the residue in the container, heat on a water-bath for 15 min and filter through the filter paper. Wash the residue on the filter paper with boiling *water R* until no further precipitate is formed when 1 mL of *silver nitrate solution R1* is added to 5 mL of the washings. Transfer the residue and the filter paper to a platinum crucible that has been weighed prior to the transfer (*a*). Heat strongly to incinerate and continue to heat at 775-825 °C for 1 h. Cool and weigh (*b*).

Calculate the percentage content of silicon dioxide ($SiO_2$) using the following expression:

$$\frac{b-a}{m} \times 100$$

**Aluminium oxide**. In a conical flask disperse 1.250 g in 15 mL of *dilute hydrochloric acid R* and 50 mL of *water R* and heat on a water-bath for 15 min. To this solution add 8 mL of *hydrochloric acid R* and heat on a water-bath for 10 min. After cooling, transfer the solution to a 250 mL volumetric flask, rinse the conical flask with *water R* and add the washings to the volumetric flask. Dilute to volume with *water R*. Centrifuge and use the supernatant as the test solution. Retain a portion for use in the assay for magnesium oxide.

Carry out the complexometric titration of aluminium (*2.5.11*). Carry out a blank determination.

1 mL of *0.1 M sodium edetate* is equivalent to 5.098 mg of $Al_2O_3$.

**Magnesium oxide**. Transfer 100.0 mL of the test solution obtained in the assay for aluminium oxide to a conical flask, add 25 mL of a 50 per cent *V/V* solution of *triethanolamine R* and shake. Add 25 mL of *ammonium chloride buffer solution*

*pH 10.7 R* and 40 mg of *mordant black 11 triturate R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

LABELLING

The label states the type of magnesium aluminometasilicate.

STORAGE

In an airtight container.

**01/2018:1445**
**corrected 10.0**

# MAGNESIUM ASPARTATE DIHYDRATE

## Magnesii aspartas dihydricus

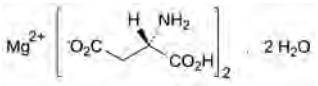

$C_8H_{12}MgN_2O_8,2H_2O$ $M_r$ 324.5
[215533-00-9]

DEFINITION

Magnesium bis[(3*S*)-3-amino-3-carboxypropanoate] dihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water.

IDENTIFICATION

Carry out either tests A, C, D, E or tests B, C, D, E.

A. Specific optical rotation (*2.2.7*): + 22.0 to + 24.0 (anhydrous substance).

Dissolve 0.50 g in a 515 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid.

B. Enantiomeric purity (see Tests).

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 50 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *magnesium aspartate dihydrate CRS* in *water R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Ignite about 15 mg until a white residue is obtained. Dissolve the residue in 1 mL of *dilute hydrochloric acid R*, neutralise to *red litmus paper R* by adding *dilute sodium hydroxide solution R* and filter if necessary. The solution gives the reaction of magnesium (*2.3.1*).

E. Water (see Tests).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.0 for solution S.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.120 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.100 g of *D-aspartic acid R* (impurity A) in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 0.120 g of the substance to be examined in 90 mL of *water R*, add 0.3 mL of reference solution (a) and dilute to 100 mL with *water R*.

*Reference solution (c)*. Dilute 0.3 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *L-penicillamine coated silica gel for chiral separations R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: *2-propanol R*, 0.5 g/L solution of *copper sulfate pentahydrate R* (5:95 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to magnesium aspartate (retention time = about 12 min): impurity A = about 0.85.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and magnesium aspartate.

*Calculation of percentage content*:

– for impurity A, use the concentration of impurity A in reference solution (c).

*Limit*:

– *impurity A*: maximum 0.3 per cent.

**Other dicarboxylic acids**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.600 g of the substance to be examined in 2.0 mL of a 618 g/L solution of *hydrochloric acid R* and dilute to 10.0 mL with *water R*.

*Reference solution (a)*. Dissolve 20.0 mg of *malic acid R* (impurity B) in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 10.0 mg of *maleic acid R* (impurity I) in *water R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Reference solution (c)*. Dilute 1 mL of reference solution (b) to 10 mL with reference solution (a).

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R*.

*Reference solution (e)*. Dissolve 10 mg of *fumaric acid R* (impurity C) in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of the solution to 100 mL with *water R*.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *cation-exchange resin R* (9 µm);
– *temperature*: 30 °C.

*Mobile phase*: 0.39 g/L solution of *sulfuric acid R*.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of impurity I.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B and I; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity C.

*Relative retention* with reference to impurity I (retention time = about 7.5 min): impurity B = about 1.2; impurity C = about 2.0.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities I and B.

*Calculation of percentage contents*:

– for impurities C and I, use the concentration of impurity I in reference solution (b).

– for impurities other than C and I, use the concentration of impurity B in reference solution (d).

*Limits*:

– *impurity C*: maximum 0.10 per cent;

– *impurity I*: maximum 0.10 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– *reporting threshold*: 0.05 per cent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test and reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 35.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50 mL with solution A. Dilute 1 mL of the solution to 200 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test solution, blank solution and reference solutions (a), (b) and (d) into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of magnesium aspartate dihydrate in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 12 mL of solution S to 15 mL with *distilled water R*. Carry out the evaluation of the test after 30 min.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modification.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.04 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 50 ppm.

In a separating funnel, dissolve 0.20 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Water** (*2.5.12*): 10.0 per cent to 16.0 per cent, determined on 0.100 g.

Dissolve the substance to be examined in a mixture of 2 mL of *anhydrous methanol R* and 10 mL of *formamide R1* at 50 °C protected from moisture. Allow to cool. Carry out a blank determination.

ASSAY

Dissolve 0.260 g in 10 mL of *water R* and carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 28.85 mg of $C_8H_{12}MgN_2O_8$.

IMPURITIES

*Specified impurities: A, C, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, G, H.*

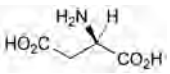


A. (2*R*)-2-aminobutanedioic acid (D-aspartic acid),

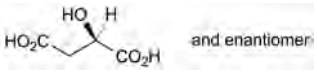


B. (2*RS*)-2-hydroxybutanedioic acid (malic acid),

C. (2*E*)-but-2-enedioic acid (fumaric acid),

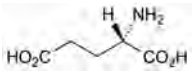

D. (2*S*)-2-aminopentanedioic acid (glutamic acid),

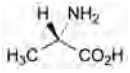

E. (2*S*)-2-aminopropanoic acid (alanine),

F. butanedioic acid (succinic acid),

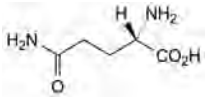

G. (2*S*)-2,5-diamino-5-oxopentanoic acid (L-glutamine),

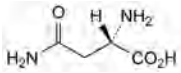

H. (2*S*)-2,4-diamino-4-oxobutanoic acid (asparagine),

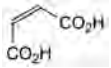

I. (2*Z*)-but-2-enedioic acid (maleic acid).

**01/2017:0043**

# MAGNESIUM CARBONATE, HEAVY

## Magnesii subcarbonas ponderosus

DEFINITION

Hydrated basic magnesium carbonate.

*Content*: 40.0 per cent to 45.0 per cent, calculated as MgO ($M_r$ 40.30).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water. It dissolves in dilute acids with effervescence.

IDENTIFICATION

A. Bulk density (*2.9.34*): minimum 0.25 g/mL.

B. It gives the reaction of carbonates (*2.3.1*).

C. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 100 mL of *dilute acetic acid R*. When the effervescence has ceased, boil for 2 min, allow to cool and dilute to 100 mL with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $B_4$ (*2.2.2, Method II*).

**Soluble substances**: maximum 1.0 per cent.

Mix 2.00 g with 100 mL of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *water R*. Evaporate 50 mL of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 10 mg.

**Substances insoluble in acetic acid**: maximum 0.05 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 ± 50 °C, weighs not more than 2.5 mg.

**Chlorides** (*2.4.4*): maximum 700 ppm.

Dilute 1.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.6 per cent.

Dilute 0.5 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 10 mL of solution S.

**Calcium** (*2.4.3*): maximum 0.75 per cent.

Dilute 2.6 mL of solution S to 150 mL with *distilled water R*. 15 mL of the solution complies with the test.

**Iron** (*2.4.9*): maximum 400 ppm.

Dissolve 0.1 g in 3 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Dilute 2.5 mL of the solution to 10 mL with *water R*.

ASSAY

Dissolve 0.150 g in a mixture of 2 mL of *dilute hydrochloric acid R* and 20 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for heavy magnesium carbonate used as filler in tablets.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**01/2017:0042**

# MAGNESIUM CARBONATE, LIGHT

## Magnesii subcarbonas levis

DEFINITION

Hydrated basic magnesium carbonate.

*Content*: 40.0 per cent to 45.0 per cent, calculated as MgO ($M_r$ 40.30).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water. It dissolves in dilute acids with effervescence.

IDENTIFICATION

A. Bulk density (*2.9.34*): maximum 0.15 g/mL.

B. It gives the reaction of carbonates (*2.3.1*).

C. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 100 mL of *dilute acetic acid R*. When the effervescence has ceased, boil for 2 min, allow to cool and dilute to 100 mL with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $B_4$ (*2.2.2, Method II*).

**Soluble substances**: maximum 1.0 per cent.

Mix 2.00 g with 100 mL of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *water R*. Evaporate 50 mL of the filtrate to dryness and dry at 100-105 °C. The residue weighs a maximum of 10 mg.

**Substances insoluble in acetic acid**: maximum 0.05 per cent.

Any residue obtained during the preparation of solution S, washed, dried and ignited at 600 ± 50 °C, weighs a maximum of 2.5 mg.

**Chlorides** (*2.4.4*): maximum 700 ppm.

Dilute 1.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.3 per cent.

Dilute 1 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 10 mL of solution S.

**Calcium** (*2.4.3*): maximum 0.75 per cent.

Dilute 2.6 mL of solution S to 150 mL with *distilled water R*. 15 mL of the solution complies with the test.

**Iron** (*2.4.9*): maximum 400 ppm.

Dissolve 0.1 g in 3 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Dilute 2.5 mL of this solution to 10 mL with *water R*.

ASSAY

Dissolve 0.150 g in a mixture of 2 mL of *dilute hydrochloric acid R* and 20 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for light magnesium carbonate used as filler in oral solid dosage forms.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**01/2017:1341**

# MAGNESIUM CHLORIDE 4.5-HYDRATE

## Magnesii chloridum 4.5-hydricum

$MgCl_2,xH_2O$ with $x \approx 4.5$ $M_r$ 95.21 (anhydrous substance)

DEFINITION

*Content*: 52.5 per cent to 55.5 per cent (calculated on an as-is basis, without allowing for the results of the test for water).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, granular powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Water (see Tests).

B. It gives reaction (a) of chlorides (*2.3.1*).

C. It gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S add 0.05 mL of *phenol red solution R*. Not more than 0.3 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromides**: maximum 500 ppm.

Dilute 2.0 mL of solution S to 10.0 mL with *water R*. To 1.0 mL of the solution add 4.0 mL of *water R*, 2.0 mL of *phenol red solution R3* and 1.0 mL of *chloramine solution R2* and mix immediately. After exactly 2 min, add 0.30 mL of *0.1 M sodium thiosulfate*, mix and dilute to 10.0 mL with *water R*. The absorbance (*2.2.25*) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 3 mg/L solution of *potassium bromide R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Aluminium** (*2.4.17*): maximum 1 ppm, if intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions, or haemofiltration solutions.

*Prescribed solution*. Dissolve 4 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Calcium** (*2.4.3*): maximum 0.1 per cent.

Dilute 1 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Potassium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using the following solution, diluted as necessary with *water R*: dissolve 1.144 g of *potassium chloride R*, previously dried at 100-105 °C for 3 h, in *water R* and dilute to 1000.0 mL with the same solvent (600 µg of K per millilitre).

*Wavelength*: 766.5 nm.

**Water** (*2.5.12*): 44.0 per cent to 48.0 per cent, determined on 50.0 mg.

ASSAY

Dissolve 0.250 g in 50 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 9.521 mg of $MgCl_2$.

STORAGE

In an airtight container.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:0402**

# MAGNESIUM CHLORIDE HEXAHYDRATE

## Magnesii chloridum hexahydricum

$MgCl_2,6H_2O$ $M_r$ 203.3
[7791-18-6]

DEFINITION

*Content*: 98.0 per cent to 101.0 per cent of $MgCl_2,6H_2O$.

CHARACTERS

*Appearance*: colourless crystals, hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Water (see Tests).

B. It gives reaction (a) of chlorides (*2.3.1*).

C. It gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S add 0.05 mL of *phenol red solution R*. Not more than 0.3 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromides**: maximum 500 ppm.

Dilute 2.0 mL of solution S to 10.0 mL with *water R*. To 1.0 mL of this solution add 4.0 mL of *water R*, 2.0 mL of *phenol red solution R3* and 1.0 mL of *chloramine solution R2* and mix immediately. After exactly 2 min, add 0.30 mL of *0.1 M sodium thiosulfate*, mix and dilute to 10.0 mL with *water R*. The absorbance (*2.2.25*) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 3 mg/L solution of *potassium bromide R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Aluminium** (*2.4.17*): maximum 1 ppm, if intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions, or haemofiltration solutions.

*Prescribed solution.* Dissolve 4 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution.* Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution.* Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Calcium** (*2.4.3*): maximum 0.1 per cent.

Dilute 1 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Potassium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using the following solution, diluted as necessary with *water R*: dissolve 1.144 g of *potassium chloride R*, previously dried at 100-105 °C for 3 h in *water R* and dilute to 1000.0 mL with the same solvent (600 µg of K per millilitre).

*Wavelength*: 766.5 nm.

**Water** (*2.5.12*): 51.0 per cent to 55.0 per cent, determined on 50.0 mg.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 20.33 mg of $MgCl_2,6H_2O$.

STORAGE

In an airtight container.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:2339**

# MAGNESIUM CITRATE

## Magnesii citras

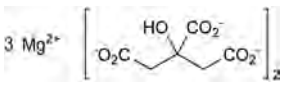


$Mg_3(C_6H_5O_7)_2$ $M_r$ 451.1
[3344-18-1]

DEFINITION

Trimagnesium bis(2-hydroxypropane-1,2,3-tricarboxylate).

*Content*: 15.0 per cent to 16.5 per cent of Mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine, slightly hygroscopic powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute hydrochloric acid.

IDENTIFICATION

A. It gives the reaction of citrates (*2.3.1*).

B. It gives the reaction of magnesium (*2.3.1*).

C. pH (see Tests).

D. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R*, heating at 60 °C, cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solutions $Y_7$ or $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.5 for solution S.

**Oxalates**: maximum 280 ppm.

Dissolve 0.50 g in 4 mL of *water R*. Add 3 mL of *hydrochloric acid R* and 1 g of *activated zinc R*. Allow to stand for 5 min. Transfer the liquid to a tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R*. Heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 4 mL of a 50 mg/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Dilute 1.5 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 0.2 per cent.

Dilute 1.0 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2.0 mL of solution S to 10 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 3.5 per cent, determined on 1.000 g by drying in an oven at 180 ± 10 °C for 5 h.

ASSAY

Dissolve 0.150 g in 50 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

STORAGE

In a non-metallic, airtight container.

**01/2017:2401**

# MAGNESIUM CITRATE DODECAHYDRATE

## Magnesii citras dodecahydricus

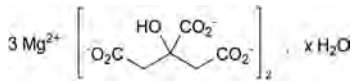

$Mg_3(C_6H_5O_7)_2,xH_2O$ $M_r$ 451.1 (anhydrous substance)
with $x \approx 12$

DEFINITION

Trimagnesium bis(2-hydroxypropane-1,2,3-tricarboxylate) dodecahydrate.

*Content*: 15.0 per cent to 16.5 per cent of Mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute hydrochloric acid.

IDENTIFICATION

A. It gives the reaction of citrates (*2.3.1*).

B. It gives the reaction of magnesium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.5 g in 15 mL of *dilute hydrochloric acid R* with heating. Cool and dilute to 100 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.5.

Disperse 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent. Centrifuge and measure the pH of the clear supernatant.

**Oxalates**: maximum 280 ppm.

Dissolve 0.50 g in a mixture of 3 mL of *hydrochloric acid R* and 4 mL of *water R* and add 1 g of *activated zinc R*. Allow to stand for 5 min. Transfer the liquid to a tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R*. Heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 4 mL of a 50 mg/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Dilute 3.0 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 0.2 per cent.

To a mixture of 2 mL of solution S and 8 mL of *distilled water R*, add about 0.2 mL of *ammonia R* and dilute to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 4.0 mL of solution S to 10 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): 29.0 per cent to 36.0 per cent, determined on 1.000 g by drying in an oven at 180 ± 10 °C for 5 h.

ASSAY

Dissolve 0.200 g in 5 mL of *dilute hydrochloric acid R* with heating. Cool and add 50 mL of *water R*. Adjust to pH 7.0 with *ammonia R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

**01/2017:2402**

# MAGNESIUM CITRATE NONAHYDRATE

## Magnesii citras nonahydricus

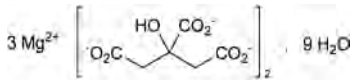

$Mg_3(C_6H_5O_7)_2,9H_2O$ $M_r$ 613

[153531-96-5]

DEFINITION

Trimagnesium bis(2-hydroxypropane-1,2,3-tricarboxylate) nonahydrate.

*Content*: 15.0 per cent to 16.5 per cent of Mg (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute hydrochloric acid.

IDENTIFICATION

A. It gives the reaction of citrates (*2.3.1*).

B. It gives the reaction of magnesium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.5 g in 15 mL of *dilute hydrochloric acid R* with heating. Cool and dilute to 100 mL with *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.5.

Disperse 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent. Centrifuge and measure the pH of the clear supernatant.

**Oxalates**: maximum 280 ppm.

Dissolve 0.50 g in a mixture of 3 mL of *hydrochloric acid R* and 4 mL of *water R* and add 1 g of *activated zinc R*. Allow to stand for 5 min. Transfer the liquid to a tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R*. Heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 4 mL of a 50 mg/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Dilute 3.0 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 0.2 per cent.

To a mixture of 2 mL of solution S and 8 mL of *distilled water R*, add about 0.2 mL of *ammonia R* and dilute to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 4.0 mL of solution S to 10 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): 24.0 to 28.0 per cent, determined on 1.000 g by drying in an oven at 180 ± 10 °C for 5 h.

ASSAY

Dissolve 0.200 g in 5 mL of *dilute hydrochloric acid R* with heating. Cool and add 50 mL of *water R*. Adjust to pH 7.0 with *ammonia R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

**01/2017:2161**

# MAGNESIUM GLUCONATE

## Magnesii gluconas

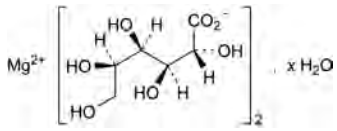

$C_{12}H_{22}MgO_{14},xH_2O$ $M_r$ 414.6 (anhydrous substance)

DEFINITION

Anhydrous or hydrated magnesium bis[(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanoate] (anhydrous or hydrated magnesium di(D-gluconate)).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, amorphous, hygroscopic, crystalline or granular powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *water R*.

*Reference solution*. Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 105 °C for 20 min, then allow to cool to room temperature.

*Detection*: spray with a solution containing 25 g/L of *ammonium molybdate R* and 10 g/L of *cerium sulfate R* in *dilute sulfuric acid R*, then heat at 105 °C for about 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. To 10 mL of solution S (see Tests) add 3 mL of *ammonium chloride solution R*. A slight opalescence may be observed. Add 10 mL of *disodium hydrogen phosphate solution R*. A white precipitate is formed that does not dissolve upon the addition of 2 mL of *dilute ammonia R1*.

TESTS

**Solution S**. Dissolve 1.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dissolve 2.0 g in a mixture of 10 mL of *acetic acid R* and 90 mL of *distilled water R*.

**Water** (*2.5.32*): maximum 12.0 per cent, determined on 80 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.350 g in 100 mL of *water R* and carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 41.46 mg of $C_{12}H_{22}MgO_{14}$.

STORAGE

In an airtight container.

**01/2017:1446**

# MAGNESIUM GLYCEROPHOSPHATE

## Magnesii glycerophosphas

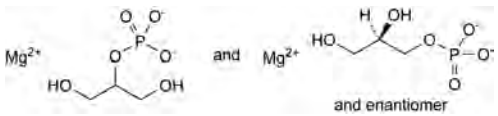


$C_3H_7MgO_6P$ $M_r$ 194.4

DEFINITION

Mixture, in variable proportions, of magnesium salts of (*RS*)-2,3-dihydroxypropyl phosphate and 2-hydroxy-1-(hydroxymethyl)ethyl phosphate, which may be hydrated.

*Content*: 11.0 per cent to 12.5 per cent of Mg (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of acids.

IDENTIFICATION

A. Mix 1 g with 1 g of *potassium hydrogen sulfate R* in a test tube fitted with a glass tube. Heat strongly and direct the white vapour towards a piece of filter paper impregnated with a freshly prepared 10 g/L solution of *sodium nitroprusside R*. The filter paper develops a blue colour in contact with *piperidine R*.

B. Ignite 0.1 g in a crucible. Take up the residue with 5 mL of *nitric acid R* and heat on a water-bath for 1 min. Filter. The filtrate gives reaction (b) of phosphates (*2.3.1*).

C. It gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*).

**Acidity**. Dissolve 1.0 g in 100 mL of *carbon dioxide-free water R*. Add 0.1 mL of *phenolphthalein solution R*. Not more than 1.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Glycerol and ethanol (96 per cent)-soluble substances**: maximum 1.5 per cent.

Shake 1.0 g with 25 mL of *ethanol (96 per cent) R* for 2 min. Filter and wash the residue with 5 mL of *ethanol (96 per cent) R*. Combine the filtrate and the washings, evaporate to dryness on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs a maximum of 15 mg.

**Chlorides** (*2.4.4*): maximum 0.15 per cent.

Dissolve 1.0 g in *water R* and dilute to 100 mL with the same solvent. Dilute 3.5 mL of this solution to 15 mL with *water R*.

**Phosphates** (*2.4.11*): maximum 0.5 per cent.

Dilute 4 mL of solution S to 100 mL with *water R*. Dilute 1 mL of this solution to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 3 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 150 ppm.

Dissolve 67 mg in *water R* and dilute to 10 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g by drying in an oven at 150 °C for 4 h.

ASSAY

Dissolve 0.200 g in 40 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

STORAGE

In an airtight container.

**01/2017:0039**

# MAGNESIUM HYDROXIDE

## Magnesii hydroxidum

$Mg(OH)_2$ $M_r$ 58.32

[1309-42-8]

DEFINITION

*Content*: 95.0 per cent to 100.5 per cent of $Mg(OH)_2$.

CHARACTERS

*Appearance*: white or almost white, fine, amorphous powder.

*Solubility*: practically insoluble in water. It dissolves in dilute acids.

IDENTIFICATION

A. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

B. Loss on ignition (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in a mixture of 50 mL of *acetic acid R* and 50 mL of *distilled water R*. Not more than slight effervescence is produced. Boil for 2 min, cool and dilute to 100 mL with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $B_3$ (*2.2.2, Method II*).

**Soluble substances**: maximum 2.0 per cent.

Mix 2.00 g with 100 mL of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *water R*. Evaporate 50 mL of the filtrate to dryness and dry at 100-105 °C. The residue weighs not more than 20 mg.

**Substances insoluble in acetic acid**: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 ± 50 °C, weighs not more than 5 mg.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dilute 1 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1.0 per cent.

Dilute 0.3 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Calcium** (*2.4.3*): maximum 1.5 per cent.

Dilute 1.3 mL of solution S to 150 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 0.07 per cent.

Dissolve 0.15 g in 5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Dilute 1 mL of the solution to 10 mL with *water R*.

**Loss on ignition**: 29.0 per cent to 32.5 per cent.

Heat 0.5 g gradually to 900 ± 50 °C and ignite to constant mass.

ASSAY

Dissolve 0.100 g in a mixture of 2 mL of *dilute hydrochloric acid R* and 20 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 5.832 mg of $Mg(OH)_2$.

**01/2017:2160**

# MAGNESIUM LACTATE DIHYDRATE

## Magnesii lactas dihydricus

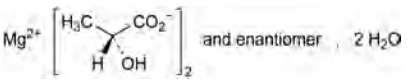


$C_6H_{10}MgO_6,2H_2O$ $M_r$ 238.5

DEFINITION

Magnesium bis(2-hydroxypropanoate) or mixture of magnesium (2*R*)-, (2*S*)- and (2*RS*)-2-hydroxypropanoate dihydrate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder.

*Solubility*: slightly soluble in water, soluble in boiling water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives the reaction of lactates (*2.3.1*).

B. It gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g with heating in *carbon dioxide-free water R* prepared from *distilled water R*, allow to cool and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 8.5 for solution S.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): 14.0 per cent to 17.0 per cent, determined on 0.500 g by drying in an oven at 125 °C.

ASSAY

Dissolve 0.180 g in *water R* and dilute to 300 mL with the same solvent. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 20.25 mg of $C_6H_{10}MgO_6$.

**01/2017:0041**

# MAGNESIUM OXIDE, HEAVY

## Magnesii oxidum ponderosum

MgO $M_r$ 40.30
[1309-48-4]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent of MgO (ignited substance).

CHARACTERS

*Appearance*: fine, white or almost white powder.

*Solubility*: practically insoluble in water. It dissolves in dilute acids with at most slight effervescence.

IDENTIFICATION

A. Bulk density (*2.9.34*): minimum 0.25 g/mL.

B. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

C. Loss on ignition (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in a mixture of 30 mL of *distilled water R* and 70 mL of *acetic acid R*, boil for 2 min, cool and dilute to 100 mL with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $B_3$ (*2.2.2, Method II*).

**Soluble substances**: maximum 2.0 per cent.

To 2.00 g add 100 mL of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *water R*. Evaporate 50 mL

of the filtrate to dryness and dry at 100-105 °C. The residue weighs a maximum of 20 mg.

**Substances insoluble in acetic acid**: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S, washed, dried and ignited at 600 ± 50 °C, weighs a maximum of 5 mg.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dilute 1 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1.0 per cent.

Dilute 0.3 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Calcium** (*2.4.3*): maximum 1.5 per cent.

Dilute 1.3 mL of solution S to 150 mL with *distilled water R*. 15 mL of the solution complies with the test.

**Iron** (*2.4.9*): maximum 0.07 per cent.

Dissolve 0.15 g in 5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Dilute 1 mL of the solution to 10 mL with *water R*.

**Loss on ignition**: maximum 8.0 per cent, determined on 1.00 g at 900 ± 25 °C.

ASSAY

Dissolve 0.320 g in 20 mL of *dilute hydrochloric acid R* and dilute to 100.0 mL with *water R*. Using 20.0 mL of the solution, carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for heavy magnesium oxide used as filler in oral solid dosage forms.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**01/2017:0040**

# MAGNESIUM OXIDE, LIGHT

## Magnesii oxidum leve

MgO $M_r$ 40.30
[1309-48-4]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent of MgO (ignited substance).

CHARACTERS

*Appearance*: fine, white or almost white, amorphous powder.

*Solubility*: practically insoluble in water. It dissolves in dilute acids with at most slight effervescence.

IDENTIFICATION

A. Bulk density (*2.9.34*): maximum 0.15 g/mL.

B. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

C. Loss on ignition (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in a mixture of 30 mL of *distilled water R* and 70 mL of *acetic acid R*, boil for 2 min, allow to cool and dilute to 100 mL with *dilute acetic acid R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of a suitable porosity to give a clear filtrate.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $B_2$ (*2.2.2, Method II*).

**Soluble substances**: maximum 2.0 per cent.

To 2.00 g add 100 mL of *water R* and boil for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *water R*. Evaporate 50 mL of the filtrate to dryness and dry at 100-105 °C. The residue weighs a maximum of 20 mg.

**Substances insoluble in acetic acid**: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S, washed, dried, and ignited at 600 ± 50 °C, weighs a maximum of 5 mg.

**Chlorides** (*2.4.4*): maximum 0.15 per cent.

Dilute 0.7 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1.0 per cent.

Dilute 0.3 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S.

**Calcium** (*2.4.3*): maximum 1.5 per cent.

Dilute 1.3 mL of solution S to 150 mL with *distilled water R*. 15 mL of this solution complies with the test.

**Iron** (*2.4.9*): maximum 0.1 per cent.

Dissolve 50 mg in 5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Dilute 2 mL of this solution to 10 mL with *water R*.

**Loss on ignition**: maximum 8.0 per cent, determined on 1.00 g at 900 ± 25 °C.

ASSAY

Dissolve 0.320 g in 20 mL of *dilute hydrochloric acid R* and dilute to 100.0 mL with *water R*. Using 20.0 mL of this solution, carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are*

*recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for light magnesium oxide used as filler in oral solid dosage forms.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Bulk and tapped density** (*2.9.34*).

**01/2017:1540**

# MAGNESIUM PEROXIDE

## Magnesii peroxidum

DEFINITION

Mixture of magnesium peroxide and magnesium oxide.

*Content*: 22.0 per cent to 28.0 per cent of $MgO_2$ ($M_r$ 56.30).

CHARACTERS

*Appearance*: white or slightly yellow, amorphous, light powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids.

IDENTIFICATION

A. Dissolve about 15 mg in 2 mL of *dilute nitric acid R* and neutralise with *dilute sodium hydroxide solution R*. The solution gives the reaction of magnesium (*2.3.1*).

B. Dissolve 50 mg in 2 mL of *dilute sulfuric acid R*. Add 2 mL of a 5 g/L solution of *potassium permanganate R* and shake. The solution becomes colourless with evolution of gas.

TESTS

**Solution S1**. Dissolve cautiously 5.0 g in 40 mL of *hydrochloric acid R1*. Cautiously evaporate the solution to 10 mL and dilute to 100 mL with a mixture of equal volumes of *acetic acid R* and *distilled water R*. Filter, if necessary, through a previously ignited and tared porcelain or silica filter crucible of suitable porosity to give a clear filtrate. Keep the residue for the test for acid insoluble substances.

**Solution S2**. Dilute 5 mL of solution S1 to 25 mL with *distilled water R*.

**Appearance of solution**. Solution S1 is not more intensely coloured than reference solution $B_4$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 2.0 g add 100 mL of *carbon dioxide-free water R* and heat to boiling for 5 min. Filter whilst hot through a sintered-glass filter (40) (*2.1.2*), allow to cool and dilute to 100 mL with *carbon dioxide-free water R*. To 15 mL of the filtrate, add 0.1 mL of *phenolphthalein solution R*. The solution is red. Not more than 0.2 mL of *0.1 M hydrochloric acid* is necessary to change the colour of the indicator. Keep the filtrate for the test for soluble substances.

**Acid insoluble substances**: maximum 0.1 per cent.

Any residue obtained during the preparation of solution S1, washed, dried and ignited at 600 ± 50 °C, weighs a maximum of 5 mg.

**Soluble substances**: maximum 1.5 per cent.

Take 50 mL of the filtrate obtained in the test for acidity or alkalinity, evaporate to dryness and dry at 100-105 °C. The residue weighs a maximum of 15 mg.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Dissolve 50 mg in 5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 3 mL of solution S2 to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 4 ppm, determined on 5 mL of solution S1.

**Calcium** (*2.4.3*): maximum 1.0 per cent.

Dilute 1 mL of solution S2 to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 500 ppm.

Dilute 2 mL of solution S2 to 10 mL with *water R*.

ASSAY

Dissolve 80.0 mg, shaking cautiously, in a mixture, previously cooled to 20 °C, of 10 mL of *sulfuric acid R* and 90 mL of *water R*. Titrate with *0.02 M potassium permanganate* until a pink colour is obtained.

1 mL of *0.02 M potassium permanganate* is equivalent to 2.815 mg of $MgO_2$.

STORAGE

Protected from light.

**07/2019:1619**

# MAGNESIUM PIDOLATE

## Magnesii pidolas

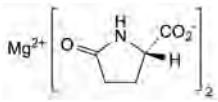

$C_{10}H_{12}MgN_2O_6$ $M_r$ 280.5

[62003-27-4]

DEFINITION

Magnesium bis[(2*S*)-5-oxopyrrolidine-2-carboxylate].

*Content*: 8.49 per cent to 8.84 per cent of Mg ($A_r$ = 24.31) (anhydrous substance).

CHARACTERS

*Appearance*: amorphous, white or almost white powder, hygroscopic.

*Solubility*: very soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 60 mg of the substance to be examined in 2 mL of *water R* and dilute to 10 mL with *methanol R*.

*Reference solution*. Dissolve 55 mg of *pidolic acid CRS* in 2 mL of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *glacial acetic acid R*, *methylene chloride R* (15:20:65 *V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 15 min.

*Detection*: spray with *strong sodium hypochlorite solution R*. Allow to stand for 10 min and spray abundantly with *glacial acetic acid R*. Allow to stand again for 10 min and dry at 100-105 °C for 2 min. Spray with *potassium iodide and starch solution R* until spots appear.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution. The chromatogram obtained with the test solution may show 2 faint secondary spots.

B. To 0.15 mL of solution S (see Tests) add 1.8 mL of *water R*. The solution gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 5.5 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): − 26.5 to − 23.3 (anhydrous substance), determined on solution S.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in 4 mL of *water R* and dilute to 50.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 60.0 mg of *glutamic acid R* in 50 mL of *water R* and dilute to 100.0 mL with *methanol R*. Dilute 1.0 mL of the solution to 20.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 10 mg of *aspartic acid R* and 10 mg of *glutamic acid R* in *water R* and dilute to 25 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.6 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50.0 mg of *pidolate impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 10.0 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dilute 1.0 mL of *nitrate standard solution (100 ppm $NO_3$) R* to 100.0 mL with the mobile phase.

*Reference solution (e)*. Dilute 6.0 mL of reference solution (a) to 10.0 mL with reference solution (b).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1.56 g of *sodium dihydrogen phosphate R* in 1000 mL of *water for chromatography R* and adjust to pH 2.5 with a 10 per cent *V/V* solution of *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Run time*: 4 times the retention time of pidolic acid.

*Retention times*: pidolic acid = about 4.5 min; impurity B = about 7.5 min.

*System suitability*: reference solution (e):

– *resolution*: minimum 10 between the peaks due to pidolic acid and impurity B.

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total of other impurities*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to the nitrate ion ($NO_3^-$).

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 1.0 mL of solution S to 15.0 mL with *water R*.

**Nitrates**. Examine the chromatogram obtained with the test solution in the test for related substances.

*Limit*:

– *nitrates*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (200 ppm).

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 1.5 mL of solution S to 15.0 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 200 ppm.

Dilute 0.5 mL of solution S to 10 mL with *water R*.

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

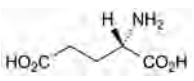


A. (2*S*)-2-aminopentanedioic acid (glutamic acid),

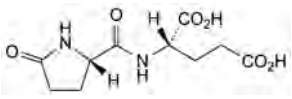


B. (2*S*)-2-[(2*S*)-5-oxopyrrolidine-2-carboxamido]pentanedioic acid.

**01/2015:0229**
**corrected 10.0**

# MAGNESIUM STEARATE(1)

## Magnesii stearas

DEFINITION

Compound of magnesium with a mixture of solid organic acids and consisting mainly of variable proportions of magnesium stearate and magnesium palmitate obtained from sources of vegetable or animal origin.

*Content*:

- *magnesium* (Mg; $A_r$ 24.305): 4.0 per cent to 5.0 per cent (dried substance);
- *stearic acid in the fatty acid fraction*: minimum 40.0 per cent;
- *sum of stearic acid and palmitic acid in the fatty acid fraction*: minimum 90.0 per cent.

♦CHARACTERS

*Appearance*: white or almost white, very fine, light powder, greasy to the touch.

*Solubility*: practically insoluble in water and in anhydrous ethanol.♦

IDENTIFICATION

*First identification: C, D.*

◊*Second identification: A, B, D.*

A. Freezing point (*2.2.18*): minimum 53 °C, determined on the residue obtained in the preparation of solution S (see Tests).

B. Acid value (*2.5.1*): 195 to 210.

Dissolve 0.200 g of the residue obtained in the preparation of solution S in 25 mL of the prescribed mixture of solvents.◊

C. Examine the chromatograms obtained in the assay of stearic acid and palmitic acid.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time to the 2 principal peaks in the chromatogram obtained with the reference solution.

D. To 1 mL of solution S add 1 mL of *dilute ammonia R1*; a white precipitate is formed that dissolves on addition of 1 mL of *ammonium chloride solution R*. Add 1 mL of a 120 g/L solution of *disodium hydrogen phosphate dodecahydrate R*; a white crystalline precipitate is formed.

TESTS

**Solution S**. To 5.0 g add 50 mL of *peroxide-free ether R*, 20 mL of *dilute nitric acid R* and 20 mL of *water R* and heat under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 4 mL, of *water R*. Combine the aqueous layers, wash with 15 mL of *peroxide-free ether R* and dilute to 50.0 mL with *water R* (solution S). Evaporate the organic layer to dryness and dry the residue at 100-105 °C. Keep the residue for identification tests A and B.

**Acidity or alkalinity**. To 1.0 g add 20 mL of *carbon dioxide-free water R* and boil for 1 min with continuous shaking. Cool and filter. To 10 mL of the filtrate add 0.05 mL of *bromothymol blue solution R4*. Not more than 0.05 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides**: maximum 0.1 per cent.

Dilute 10.0 mL of solution S to 40 mL with *water R*. Neutralise if necessary with *nitric acid R* using *litmus R* as indicator. Add 1 mL of *nitric acid R* and 1 mL of *0.1 M silver nitrate* and dilute to 50 mL with *water R*. Mix and allow to stand for 5 min protected from light. The turbidity, if any, is not greater than that produced in a solution containing 1.4 mL of *0.02 M hydrochloric acid*.

**Sulfates**: maximum 1.0 per cent.

Dilute 6.0 mL of solution S to 40 mL with *water R*. Neutralise if necessary with *hydrochloric acid R* using *litmus R* as indicator. Add 1 mL of *3 M hydrochloric acid R* and 3 mL of a 120 g/L solution of *barium chloride R* and dilute to 50 mL with *water R*. Mix and allow to stand for 10 min. The turbidity, if any, is not greater than that produced in a solution containing 3.0 mL of *0.02 M sulfuric acid*.

**Cadmium**: maximum 3 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution.* Dilute 25 mL of *cadmium- and lead-free nitric acid R* to 100.0 mL with *water R*.

*Modifier solution.* Dissolve 20 g of *ammonium dihydrogen phosphate R* and 1 g of *magnesium nitrate R* in *water R* and dilute to 100 mL with the same solvent. Alternatively, use an appropriate matrix modifier as recommended by the graphite furnace atomic absorption (GFAA) spectrometer manufacturer.

*Test solution.* Place 0.100 g of the substance to be examined in a polytetrafluoroethylene digestion bomb and add 2.5 mL of *cadmium- and lead-free nitric acid R*. Close and seal the bomb according to the manufacturer's operating instructions *(when using a digestion bomb, be thoroughly familiar with the safety and operating instructions. Carefully follow the bomb manufacturer's instructions regarding care and maintenance of these digestion bombs. Do not use metal jacketed bombs or liners which have been used with hydrochloric acid due to contamination from corrosion of the metal jacket by hydrochloric acid).* Heat the bomb in an oven at 170 °C for 3 h. Cool the bomb slowly in air to room temperature according to the bomb manufacturer's instructions. Place the bomb in a fume cupboard and open carefully as corrosive gases may be expelled. Dissolve the residue in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Prepare a solution of 0.0030 µg/mL of Cd by suitable dilutions of a 0.00825 µg/mL solution of *cadmium nitrate tetrahydrate R* in the blank solution.

Dilute 1.0 mL of the test solution to 10.0 mL with the blank solution. Prepare mixtures of this solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:1.0:0 *V/V/V*). To each mixture add 50 µL of modifier solution and mix. These solutions contain respectively 0 µg, 0.00075 µg and 0.0015 µg of cadmium per millilitre from the reference solution (keep the remaining test solution for use in the test for lead and nickel).

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*Operating conditions*: use the temperature programme recommended for cadmium by the GFAA spectrometer manufacturer. An example of temperature parameters for GFAA analysis of cadmium is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 600 | 10 | 30 |
| Atomisation | 1800 | 0 | 5 |

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution.* Use the solution described in the test for cadmium.

*Modifier solution.* Use the solution described in the test for cadmium.

*Test solution.* Use the solution described in the test for cadmium.

*Reference solution.* Prepare a solution of 0.100 µg/mL of Pb by suitable dilutions of *lead standard solution (100 ppm Pb) R* with the blank solution.

Prepare mixtures of the test solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:1.0:0 *V/V/V*). To each mixture add 50 µL of modifier solution and mix. These solutions contain respectively 0 µg, 0.025 µg and 0.05 µg of lead per millilitre from the reference solution.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

*Operating conditions*: use the temperature programme recommended for lead by the GFAA spectrometer manufacturer. An example of temperature parameters for GFAA analysis of lead is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 450 | 10 | 30 |
| Atomisation | 2000 | 0 | 5 |

**Nickel**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*For the preparation of all aqueous solutions and for the rinsing of glassware before use, employ water that has been passed through a strong-acid, strong-base, mixed-bed ion-exchange resin before use. Select all reagents to have as low a content of cadmium, lead and nickel as practicable and store all reagent solutions in containers of borosilicate glass. Clean glassware before use by soaking in a warm 773 g/L solution of nitric acid R for 30 min and by rinsing with deionised water.*

*Blank solution.* Use the solution described in the test for cadmium.

*Modifier solution.* Dissolve 20 g of *ammonium dihydrogen phosphate R* in *water R* and dilute to 100 mL with the same solvent. Alternatively, use an appropriate matrix modifier as recommended by the GFAA spectrometer manufacturer.

*Test solution.* Use the solution described in the test for cadmium.

*Reference solution.* Prepare a solution of 0.050 µg/mL of Ni by suitable dilutions of a 0.2477 µg/mL solution of *nickel nitrate hexahydrate R* in the blank solution.

Prepare mixtures of the test solution, the reference solution and the blank solution in the following proportions: (1.0:0:1.0 *V/V/V*), (1.0:0.5:0.5 *V/V/V*), (1.0:1.0:0 *V/V/V*). To each mixture add 50 µL of matrix modifier solution and mix. These reference solutions contain respectively 0 µg, 0.0125 µg and 0.025 µg of nickel per millilitre from the reference solution.

*Source*: nickel hollow-cathode lamp.

*Wavelength*: 232.0 nm.

*Atomisation device*: furnace.

*Platform*: pyrolytically coated with integrated tube.

*Operating conditions*: use the temperature programme recommended for nickel by the GFAA spectrometer manufacturer. An example of temperature parameters for GFAA analysis of nickel is shown below.

| Stage | Final temperature (°C) | Ramp time (s) | Hold time (s) |
|---|---|---|---|
| Drying | 110 | 10 | 20 |
| Ashing | 1000 | 20 | 30 |
| Atomisation | 2300 | 0 | 5 |

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

♦ **Microbial contamination**.

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*). ♦

ASSAY

**Magnesium**. To 0.500 g in a 250 mL conical flask add 50 mL of a mixture of equal volumes of *anhydrous ethanol R* and *butanol R*, 5 mL of *concentrated ammonia R*, 3 mL of *ammonium chloride buffer solution pH 10.0 R*, 30.0 mL of *0.1 M sodium edetate* and 15 mg of *mordant black 11 triturate R*. Heat at 45-50 °C until the solution is clear and titrate with *0.1 M zinc sulfate* until the colour changes from blue to violet. Carry out a blank titration.

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

**Stearic acid and palmitic acid**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 mL of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 mL of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 mL of *saturated sodium chloride solution R*. Shake and allow the layers to separate. Dry the organic layer over 0.1 g of *anhydrous sodium sulfate R* (previously washed with *heptane R*). Dilute 1.0 mL of the solution to 10.0 mL with *heptane R*.

*Reference solution.* Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of the substance to be examined.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 70 |
| | 2 - 36 | 70 → 240 |
| | 36 - 41 | 240 |
| Injection port | | 220 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to methyl stearate: methyl palmitate = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to methyl palmitate and methyl stearate;
- *relative standard deviation*: maximum 3.0 per cent for the areas of the peaks due to methyl palmitate and methyl stearate, determined on 6 injections; maximum 1.0 per cent for the ratio of the areas of the peaks due to methyl palmitate to the areas of the peaks due to methyl stearate, determined on 6 injections.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for magnesium stearate used as lubricant in tablets and capsules.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26, Method I*). Determine the specific surface area in the $P/P_o$ range of 0.05 to 0.15.

*Sample outgassing*: 2 h at 40 °C.

**Thermogravimetry** (*2.2.34*).

**01/2017:0044**

# MAGNESIUM SULFATE HEPTAHYDRATE

## Magnesii sulfas heptahydricus

$MgSO_4,7H_2O$ $M_r$ 246.5
[10034-99-8]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or brilliant, colourless crystals.

*Solubility*: freely soluble in water, very soluble in boiling water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives the reactions of sulfates (*2.3.1*).

B. It gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *phenol red solution R*. Not more than 0.2 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dilute 1.7 mL of solution S to 15 mL with *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): 48.0 per cent to 52.0 per cent, determined on 0.500 g by drying in an oven at 110-120 °C for 1 h and then at 400 °C to constant mass.

ASSAY

Dissolve 0.450 g in 100 mL of *water R* and carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 12.04 mg of $MgSO_4$.

**01/2017:0403**

# MAGNESIUM TRISILICATE

## Magnesii trisilicas

DEFINITION

It has a variable composition corresponding approximately to $Mg_2Si_3O_8,xH_2O$.

*Content*:

- *magnesium oxide* (MgO; $M_r$ 40.30): minimum 29.0 per cent (ignited substance),
- *silicon dioxide* ($SiO_2$; $M_r$ 60.1): minimum 65.0 per cent (ignited substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. 0.25 g gives the reaction of silicates (*2.3.1*).

B. 1 mL of solution S (see Tests) neutralised with *dilute sodium hydroxide solution R* gives the reaction of magnesium (*2.3.1*).

TESTS

**Solution S**. To 2.0 g add a mixture of 4 mL of *nitric acid R* and 4 mL of *distilled water R*. Heat to boiling with frequent shaking. Add 12 mL of *distilled water R* and allow to cool. Filter or centrifuge to obtain a clear solution and dilute to 20 mL with *distilled water R*.

**Alkalinity**. To 10.0 g in a 200 mL conical flask, add 100.0 g of *water R* and heat on a water-bath for 30 min. Allow to cool and make up to the initial mass with *water R*. Allow to stand and filter or centrifuge until a clear liquid is obtained. To 10 mL of this liquid add 0.1 mL of *phenolphthalein solution R*. Not more than 1.0 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Water-soluble salts**: maximum 1.5 per cent.

In a platinum dish, evaporate to dryness on a water-bath 20.0 mL of the liquid obtained in the test for alkalinity. The residue, ignited to constant mass at 900 ± 50 °C, weighs a maximum of 30 mg.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 0.5 mL of solution S to 15 mL with *water R*. Prepare the standard using a mixture of 5 mL of *chloride standard solution (5 ppm Cl) R* and 10 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 0.5 per cent.

Dilute 0.3 mL of solution S to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, method A*): maximum 4 ppm, determined on 2.5 mL of solution S.

**Loss on ignition**: 17 per cent to 34 per cent, determined on 0.5 g by ignition to constant mass at 900 ± 50 °C in a platinum crucible.

**Acid-absorbing capacity**. Suspend 0.25 g in *0.1 M hydrochloric acid*, dilute to 100.0 mL with the same acid and allow to stand for 2 h in a water-bath at 37 ± 0.5 °C, with frequent shaking. Allow to cool. To 20.0 mL of the supernatant solution add 0.1 mL of *bromophenol blue solution R* and titrate with *0.1 M sodium hydroxide* until a blue colour is obtained. The acid-absorbing capacity is not less than 100.0 mL of *0.1 M hydrochloric acid* per gram.

ASSAY

**Magnesium oxide**. To 1.000 g in a 200 mL conical flask, add 35 mL of *hydrochloric acid R* and 60 mL of *water R* and heat in a water-bath for 15 min. Allow to cool, filter, wash the conical flask and the residue with *water R* and dilute the combined filtrate and washings to 250.0 mL with *water R*. Neutralise 50.0 mL of the solution with *strong sodium hydroxide solution R* (about 8 mL). Carry out the complexometric titration of magnesium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 4.030 mg of MgO.

**Silicon dioxide**. To 0.700 g add 10 mL of *dilute sulfuric acid R* and 10 mL of *water R*. Heat for 90 min on a water-bath with frequent shaking, replacing the evaporated water. Allow to cool and decant onto an ashless filter paper (diameter 7 cm). Wash the precipitate by decantation with 3 quantities, each of 5 mL, of hot *water R*, transfer it to the filter and wash it with hot *water R* until 1 mL of the filtrate remains clear after the addition of 0.05 mL of *dilute hydrochloric acid R* and 2 mL of *barium chloride solution R1*. Incinerate the filter and its contents in a platinum crucible, then ignite the residue ($SiO_2$) at 900 ± 50 °C to constant mass.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for magnesium trisilicate used as a lubricant in tablets and capsules.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26, Method I*).

**01/2010:1342**
**corrected 6.8**

# MAIZE OIL, REFINED

## Maydis oleum raffinatum

DEFINITION

Fatty oil obtained from the seeds of *Zea mays* L. by expression or by extraction. It is then refined.

CHARACTERS

*Appearance*: clear, light yellow or yellow oil.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C) and with methylene chloride.

*Relative density*: about 0.920.

*Refractive index*: about 1.474.

IDENTIFICATION

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained with the test solution is similar to the chromatogram obtained with the reference solution.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5, or maximum 0.3 if intended for use in the manufacture of parenteral preparations, determined on 10.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 2.8 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil:*

- *fatty acids of chain length less than $C_{16}$*: maximum 0.6 per cent;
- *palmitic acid*: 8.6 per cent to 16.5 per cent;
- *stearic acid*: maximum 3.3 per cent;
- *oleic acid*: 20.0 per cent to 42.2 per cent;
- *linoleic acid*: 39.4 per cent to 65.6 per cent;
- *linolenic acid*: 0.5 per cent to 1.5 per cent;
- *arachidic acid*: maximum 0.8 per cent;
- *eicosenoic acid*: maximum 0.5 per cent;
- *behenic acid*: maximum 0.5 per cent;
- *other fatty acids*: maximum 0.5 per cent.

**Sterols** (*2.4.23*): maximum 0.3 per cent of brassicasterol in the sterol fraction of the oil.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

Protected from light, at a temperature not exceeding 25 °C.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- whether the oil is obtained by mechanical expression or by extraction.

**01/2014:0344**
**corrected 10.0**

# MAIZE STARCH(2)

## Maydis amylum

DEFINITION

Maize starch is obtained from the caryopsis of *Zea mays* L.

♦CHARACTERS

*Appearance*: matt, white to slightly yellowish, very fine powder that creaks when pressed between the fingers.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

The presence of granules with cracks or irregularities on the edge is exceptional.♦

IDENTIFICATION

A. Microscopic examination (*2.8.23*), using a 50 per cent *V/V* solution of *glycerol R*. It appears as either angular polyhedral granules of irregular sizes with diameters ranging from about 2 µm to about 23 µm or as rounded or spheroidal granules of irregular sizes with diameters ranging from about 25 µm to about 35 µm (Figure 0344.-1). The central hilum consists of a distinct cavity or 2- to 5-rayed cleft and there are no concentric striations. Between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.

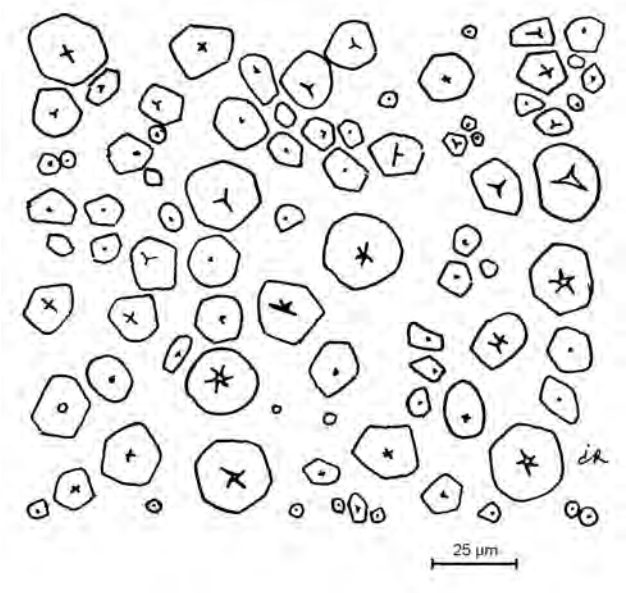


Figure 0344.-1. – *Illustration for identification test A of maize starch*

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B add 0.05 mL of *iodine solution R1*. An orange-red to dark blue colour is produced, which disappears on heating.

TESTS

**pH** (*2.2.3*): 4.0 to 7.0.

To 5.0 g add 25.0 mL of *carbon dioxide-free water R*. Agitate continuously at a moderate rate for 60 s. Stop the agitation and allow to stand for 15 min.

◊**Foreign matter**. Examined under a microscope using a 50 per cent *V/V* solution of *glycerol R*, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.◊

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 10 ppm.

Shake 1.5 g with 15 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

◊Absence of *Salmonella* (*2.6.13*).◊

**01/2008:1343**

# MALATHION

## Malathionum

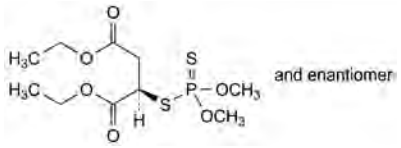


$C_{10}H_{19}O_6PS_2$ $M_r$ 330.4
[121-75-5]

DEFINITION

Diethyl (2*RS*)-2-(dimethoxyphosphinodithioyl)butanedioate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless or slightly yellowish liquid.

*Solubility*: slightly soluble in water, miscible with acetone, with cyclohexane, with ethanol (96 per cent) and with vegetable oils.

It solidifies at about 3 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *malathion CRS*.

TESTS

**Relative density** (*2.2.5*): 1.220 to 1.240.

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

**Optical rotation** (*2.2.7*): – 0.1° to + 0.1°.

Dissolve 2.50 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R* (1:3 *V/V*).

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 0.100 g of *malathion CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 0.5 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5.0 mg of *malathion impurity A CRS* and 5.0 mg of *malathion impurity B CRS* in the solvent mixture, then dilute 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dilute 2.0 mL of reference solution (c) to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm);
- *temperature*: 35 °C.

*Mobile phase*: *acetonitrile R*, *water R* (45:55 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Retention time*: impurity B = about 3.5 min;
impurity A = about 5 min; malathion = about 16 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to impurities B and A.

*Limits*:

- *impurity A*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *sum of impurities other than A and B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 2.000 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{10}H_{19}O_6PS_2$ from the declared content of *malathion CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

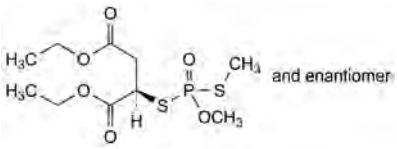


A. diethyl (2*RS*)-2-[(methoxy)(methylsulfanyl)-*S*-phosphinothioyl]butanedioate (isomalathion),

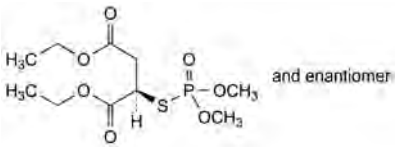


B. diethyl (2*RS*)-2-(dimethoxy-*S*-phosphinothioyl)-butanedioate (maloxon),

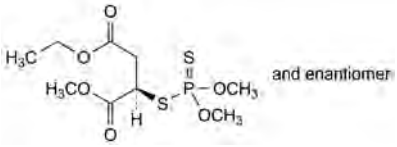


C. ethyl and methyl (2*RS*)-2-(dimethoxyphosphino-dithioyl)butanedioate (methyl analogue).

**01/2017:0365**

# MALEIC ACID

## Acidum maleicum

$C_4H_4O_4$ $M_r$ 116.1
[110-16-7]

DEFINITION

Maleic acid contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (*Z*)-butenedioic acid, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, freely soluble in water and in alcohol.

IDENTIFICATION

A. Dilute 5 mL of solution S (see Tests) to 10 mL with *water R*. The pH of the dilution is less than 2.

B. Examine the chromatograms obtained in the test for fumaric acid. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 10 mL of *water R* (solution a). To 0.3 mL of solution (a) add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min; no colour develops. To 3 mL of solution (a) add 1 mL of *bromine water R*. Heat on a water-bath to remove the

bromine (15 min), heat to boiling and cool. To 0.2 mL of this solution add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min. A violet-pink colour develops.

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Fumaric acid**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.5 g of the substance to be examined in *acetone R* and dilute to 5 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 50 mL with *acetone R*.

*Reference solution (a).* Dissolve 20 mg of *maleic acid CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 15 mg of *fumaric acid CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Mix 5 mL of reference solution (a) and 5 mL of reference solution (b).

Apply separately to the plate 5 µL of test solutions (a) and (b), 5 µL of reference solutions (a) and (b) and 10 µL of reference solution (c). Develop in an unsaturated tank over a path of 10 cm using a mixture of 12 volumes of *anhydrous formic acid R*, 16 volumes of *chloroform R*, 32 volumes of *butanol R* and 44 volumes of *heptane R*. Dry the plate at 100 °C for 15 min and examine in ultraviolet light at 254 nm. Any spot corresponding to fumaric acid in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

**Iron**. To 10 mL of solution S add 2 mL of *dilute hydrochloric acid R* and 0.05 mL of *bromine water R*. After 5 min, remove the excess of bromine by passing a current of air and add 3 mL of *potassium thiocyanate solution R*. Shake. Prepare a standard at the same time and in the same manner, using a mixture of 5 mL of *iron standard solution (1 ppm Fe) R*, 1 mL of *dilute hydrochloric acid R*, 6 mL of *water R* and 0.05 mL of *bromine water R*. Allow both solutions to stand for 5 min. Any red colour in the test solution is not more intense than that in the standard (5 ppm).

**Water** (*2.5.12*). Not more than 2.0 per cent, determined on 1.00 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide* using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 58.04 mg of $C_4H_4O_4$.

STORAGE

Store in a glass container, protected from light.

**01/2017:2080**

# MALIC ACID

## Acidum malicum

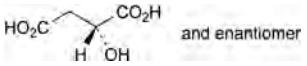


$C_4H_6O_5$ $M_r$ 134.1
[6915-15-7]

DEFINITION

(2*RS*)-2-Hydroxybutanedioic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in alcohol, sparingly soluble in acetone.

IDENTIFICATION

A. Melting point (*2.2.14*): 128 °C to 132 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of malic acid.*

TESTS

**Solution S**. Dissolve 5.00 g in *water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Water-insoluble substances**: maximum 0.1 per cent.

Dissolve 25.0 g in 100 mL of *water R*, filter the solution through a tared sintered-glass filter (16) (*2.1.2*), wash the filter with hot *water R* and dry at 100-105 °C to constant weight. The residue weighs a maximum of 25 mg.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 100.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *fumaric acid R* and 4.0 mg of *maleic acid R* in 25 mL of the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 2.5 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 20.0 mg of the substance to be examined in the mobile phase, add 1.0 mL of reference solution (a) and dilute to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.30 m, Ø = 7.8 mm,
- *stationary phase*: *ion-exclusion resin for chromatography R* (9 µm),
- *temperature*: 37 °C.

*Mobile phase*: *0.005 M sulfuric acid.*

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of the principal peak in the chromatogram obtained with the test solution.

*Relative retention* with reference to malic acid (retention time = about 10 min): impurity B = about 0.8; impurity A = about 1.5.

*System suitability*: reference solution (c):
- *resolution*: minimum 2.5 between the peaks due to impurity B and malic acid.

*Limits*:
- *impurity A*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *impurity B*: not more than 0.25 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent),
- *any other impurity*: for each impurity, not more than 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total of other impurities*: not more than 2.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 67.05 mg of $C_4H_6O_5$.

IMPURITIES

*Specified impurities: A, B.*

A. (*E*)-butenedioic acid (fumaric acid),

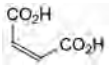

B. (*Z*)-butenedioic acid (maleic acid).

**07/2019:1235**

# MALTITOL

## Maltitolum

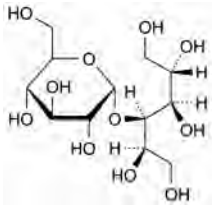

$C_{12}H_{24}O_{11}$ $M_r$ 344.3
[585-88-6]

DEFINITION

4-*O*-α-D-Glucopyranosyl-D-glucitol (D-maltitol).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, practically insoluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *maltitol CRS*.

B. Melting point (*2.2.14*): 148 °C to 151 °C.

C. Specific optical rotation (*2.2.7*): + 105.5 to + 108.5 (anhydrous substance).
Dissolve 5.00 g in *water R* and dilute to 100.0 mL with the same solvent.

D. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.
*Reference solution (a)*. Dissolve 25 mg of *maltitol CRS* in *water R* and dilute to 10 mL with the same solvent.
*Reference solution (b)*. Dissolve 25 mg of *maltitol CRS* and 25 mg of *sorbitol CRS* in *water R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel G plate R*.
*Mobile phase*: *water R*, *ethyl acetate R*, *propanol R* (10:20:70 *V/V/V*).
*Application*: 2 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air.
*Detection*: spray with *4-aminobenzoic acid solution R*. Dry in a current of cold air until the acetone is removed. Heat at 100-105 °C for 15 min. Allow to cool and spray with a 2 g/L solution of *sodium periodate R*. Dry in a current of cold air. Heat at 100 °C for 15 min.
*System suitability*: reference solution (b):
- the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution, while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent, expressed as glucose equivalent.

Dissolve 5.0 g in 6 mL of *water R* with the aid of gentle heat. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 5.0 g of the substance to be examined in 20 mL of *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.50 g of *maltitol CRS* in 2.0 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 10.0 mL of reference solution (b) to 100.0 mL with *water R*.

*Reference solution (d).* Dissolve 0.5 g of *maltitol R* and 0.5 g of *sorbitol R* in 5 mL of *water R* and dilute to 10 mL with the same solvent.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of maltitol.

*Relative retention* with reference to maltitol (retention time = about 16 min): impurity B = about 0.8; impurity A = about 1.8.

*System suitability*: reference solution (d):

- *resolution*: minimum 2.0 between the peaks due to maltitol and impurity A.

*Limits*:

- *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion: $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
- TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
- absence of *Escherichia coli* (*2.6.13*);
- absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins:

- less than 4 IU/g for parenteral preparations having a concentration of less than 100 g/L of maltitol;
- less than 2.5 IU/g for parenteral preparations having a concentration of 100 g/L or more of maltitol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{24}O_{11}$ taking into account the assigned content of *maltitol CRS*.

LABELLING

The label states:

- where applicable, the maximum concentration of bacterial endotoxins;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

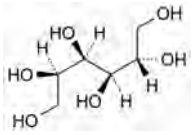

A. D-glucitol (D-sorbitol),

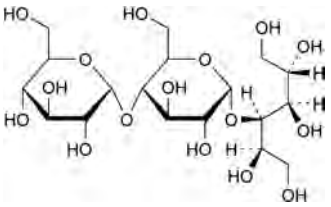

B. α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucitol (maltotriitol).

**07/2019:1236**

# MALTITOL, LIQUID

## Maltitolum liquidum

DEFINITION

Aqueous solution of a hydrogenated, partly hydrolysed starch, composed of a mixture of mainly 4-*O*-α-D-glucopyranosyl-D-glucitol (D-maltitol) with D-glucitol (D-sorbitol) and hydrogenated oligo- and polysaccharides.

*Content*:

- *D-maltitol* ($C_{12}H_{24}O_{11}$): minimum 50.0 per cent *m/m* (anhydrous substance) and 95.0 per cent to 105.0 per cent of the content stated on the label;
- *D-sorbitol* ($C_6H_{14}O_6$): maximum 8.0 per cent *m/m* (anhydrous substance);
- *anhydrous substance*: 68.0 per cent *m/m* to 85.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless, syrupy liquid.

*Solubility*: miscible with water and with glycerol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dilute 0.35 g of the substance to be examined to 100 mL with *water R*.

*Reference solution (a).* Dissolve 20 mg of *maltitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *maltitol CRS* and 20 mg of *sorbitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *water R*, *ethyl acetate R*, *propanol R* (10:20:70 *V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *4-aminobenzoic acid solution R*. Dry in a current of cold air until the acetone is removed. Heat at 100-105 °C for 15 min. Allow to cool and spray with a 2 g/L solution of *sodium periodate R*. Dry in a current of cold air. Heat at 100 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and colour to the principal spot in the chromatogram obtained with reference solution (a).

C. To 3 mL of a freshly prepared 100 g/L solution of *pyrocatechol R*, add 6 mL of *sulfuric acid R* while cooling in iced water. To 3 mL of the cooled mixture, add 0.3 mL of solution S (see Tests). Heat gently over a naked-flame for about 30 s. A pink colour develops.

TESTS

**Solution S**. Dilute 7.0 g to 50 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Conductivity** (*2.2.38*): maximum 10 µS·cm$^{-1}$, measured on undiluted liquid maltitol while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent, calculated as glucose equivalent.

To 5.0 g add 6 mL of *water R*, 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): 15.0 per cent *m/m* to 32.0 per cent *m/m*, determined on 0.100 g. Use as solvent a mixture of equal volumes of *anhydrous methanol R* and *formamide R1*. Carry out the titration at about 50 °C.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Mix 1.00 g of the solution to be examined with 20 mL of *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50.0 mg of *maltitol CRS* in 2 mL of *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 8.0 mg of *sorbitol CRS* in 2 mL of *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 50 mg of *maltitol R* and 50 mg of *sorbitol R* in 2 mL of *water R* and dilute to 5 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;

– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);

– *temperature*: 85 ± 2 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL.

*Run time*: 3 times the retention time of maltitol.

*Relative retention* with reference to maltitol (retention time = about 16 min): sorbitol = about 1.8.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the peaks due to maltitol and sorbitol.

Calculate the percentage contents of D-maltitol ($C_{12}H_{24}O_{11}$) and D-sorbitol ($C_6H_{14}O_6$) taking into account the assigned contents of *maltitol CRS* and *sorbitol CRS*.

LABELLING

The label states the content of D-maltitol.

**01/2017:1542**

# MALTODEXTRIN

## Maltodextrinum

DEFINITION

Mixture of glucose, disaccharides and polysaccharides, obtained by the partial hydrolysis of starch.

The degree of hydrolysis, expressed as dextrose equivalent (DE), is less than 20 (nominal value).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder or granules.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. Dissolve 0.1 g in 2.5 mL of *water R* and heat with 2.5 mL of *cupri-tartaric solution R*. A red precipitate is formed.

B. Dip, for 1 s, a suitable stick with a reactive pad containing glucose-oxidase, peroxidase and a hydrogen-donating substance, such as tetramethylbenzidine, in a 100 g/L solution of the substance to be examined. Observe the colour of the reactive pad; within 60 s a colour change is observed, characteristic of the hydrogen-donating substance used (from yellow to green or blue if tetramethylbenzidine is used).

C. It is a powder or granules.

D. Dextrose equivalent (see Tests).

TESTS

**Solution S**. Dissolve 12.5 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 7.0.

Mix 1 mL of a 223.6 g/L solution of *potassium chloride R* and 30 mL of solution S.

**Sulfur dioxide** (*2.5.29*): maximum 20 ppm.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 10.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Dextrose equivalent** (DE): within 2 DE units of the nominal value.

Weigh an amount of the substance to be examined equivalent to 2.85-3.15 g of reducing carbohydrates, calculated as dextrose equivalent, into a 500 mL volumetric flask. Dissolve in *water R* and dilute to 500.0 mL with the same solvent. Transfer the solution to a 50 mL burette.

Pipette 25.0 mL of *cupri-tartaric solution R* into a 250 mL flask and add 18.5 mL of the test solution from the burette, mix and add a few glass beads. Place the flask on a hot plate, previously adjusted so that the solution begins to boil within 2 min ± 15 s. Allow to boil for exactly 120 s, add 1 mL of a

1 g/L solution of *methylene blue R* and titrate with the test solution ($V_1$) until the blue colour disappears. Maintain the solution at boiling throughout the titration.

Standardise the cupri-tartaric solution using a 6.00 g/L solution of *glucose R* ($V_0$).

Calculate the dextrose equivalent using the following expression:

$$\frac{300 \times V_0 \times 100}{V_1 \times M \times D}$$

$V_0$ = total volume of glucose standard solution, in millilitres;

$V_1$ = total volume of test solution, in millilitres;

$M$ = sample mass, in grams;

$D$ = percentage content of dry matter in the substance.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the dextrose equivalent (DE) (= nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for maltodextrin used as filler and binder in tablets and capsules.*

**Dextrose equivalent** (see Tests).

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2017:2162**

# MANGANESE GLUCONATE

## Mangani gluconas

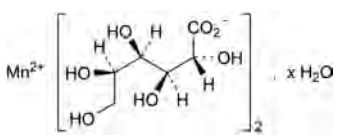


$C_{12}H_{22}MnO_{14},xH_2O$ $M_r$ 445.2 (anhydrous substance)

DEFINITION

Anhydrous or hydrated manganese(II) bis[(2*R*,3*S*,4*R*,5*R*)-2,3,4,5,6-pentahydroxyhexanoate] (anhydrous or hydrated manganese(II) di(D-gluconate)).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale pink, slightly hygroscopic, crystalline powder.

*Solubility*: soluble in water, practically insoluble in anhydrous ethanol, insoluble in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 1 mL of *water R*.

*Reference solution.* Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 105 °C for 20 min, then allow to cool to room temperature.

*Detection*: spray with a solution containing 25 g/L of *ammonium molybdate R* and 10 g/L of *cerium sulfate R* in *dilute sulfuric acid R*, and heat at 105 °C for about 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve 50 mg in 5 mL of *water R*. Add 0.5 mL of *ammonium sulfide solution R*. A pale pink precipitate is formed that dissolves upon the addition of 1 mL of *glacial acetic acid R*.

TESTS

**Solution S**. Dissolve 1.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dissolve 2.0 g in a mixture of 10 mL of *acetic acid R* and 90 mL of *distilled water R*.

**Zinc**: maximum 50 ppm.

To 10 mL of solution S add 1 mL of *sulfuric acid R* and 0.1 mL of *potassium ferrocyanide solution R*. After 30 s, any opalescence in the solution is not more intense than that in a mixture of 1.0 mL of *zinc standard solution (10 ppm Zn) R*, 9 mL of *water R*, 1 mL of *sulfuric acid R* and 0.1 mL of *potassium ferrocyanide solution R*.

**Water** (*2.5.32*): maximum 9.0 per cent, determined on 80 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.400 g in 50 mL of *water R*. Add 10 mg of *ascorbic acid R*, 20 mL of *ammonium chloride buffer solution pH 10.0 R* and 0.2 mL of a 2 g/L solution of *mordant black 11 R* in *triethanolamine R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 44.52 mg of $C_{12}H_{22}MnO_{14}$.

STORAGE

In a non-metallic, airtight container.

**01/2017:2163**

# MANGANESE GLYCEROPHOSPHATE, HYDRATED

## Mangani glycerophosphas hydricus

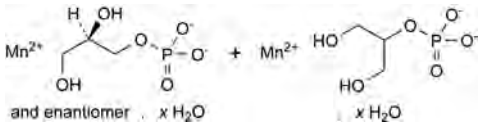


$C_3H_7MnO_6P,xH_2O$ $M_r$ 225.0 (anhydrous substance)

DEFINITION

Mixture of variable proportions of hydrated manganese(II) (2*RS*)-2,3-dihydroxypropyl phosphate and hydrated manganese(II) 2-hydroxy-1-(hydroxymethyl)ethyl phosphate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale pink, hygroscopic powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It is freely soluble in dilute mineral acids.

IDENTIFICATION

A. Mix 1 g with 1 g of *potassium hydrogen sulfate R* in a test tube fitted with a delivery tube. Heat strongly and direct the white vapour towards a piece of filter paper impregnated with a freshly prepared 10 g/L solution of *sodium nitroprusside R*. The filter paper develops a blue colour in contact with *piperidine R*.

B. Disperse 50 mg in 5 mL of *water R*. Add 0.5 mL of *ammonium sulfide solution R*. A pale pink precipitate is formed that dissolves on the addition of 1 mL of *acetic acid R*.

C. Ignite 0.1 g in a crucible. Take up the residue with 5 mL of *nitric acid R* and heat on a water-bath for 1 min. Filter. The filtrate gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 20 mL of *dilute hydrochloric acid R*. Filter if necessary. Add *dilute ammonia R1* until a precipitate is formed. Dissolve the precipitate by adding the minimum quantity needed of *dilute hydrochloric acid R* and dilute to 100 mL with *distilled water R*.

**Glycerol and ethanol (96 per cent)-soluble substances**: maximum 1.0 per cent.

Shake 1.00 g with 25 mL of *ethanol (96 per cent) R* for 1 min. Filter. Evaporate the filtrate to dryness on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs a maximum of 10 mg.

**Chlorides** (*2.4.4*): maximum 0.15 per cent.

Dissolve 0.22 g in a mixture of 1 mL of *nitric acid R* and 10 mL of *water R* and dilute to 100 mL with *water R*.

**Phosphates** (*2.4.11*): maximum 0.3 per cent.

Dilute 1.0 mL of solution S to 100.0 mL with *water R*. To 10 mL of this solution add 140 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 0.2 per cent.

Dilute 5 mL of solution S to 50 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 4 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

ASSAY

To 0.200 g add 1.5 mL of *1 M hydrochloric acid*, 50 mL of *water R*, 10 mg of *ascorbic acid R* and 20 mL of *ammonium chloride buffer solution pH 10.0 R*. Stir until dissolution. Immediately add 0.3 mL of a 2 g/L solution of *mordant black 11 R* in *triethanolamine R* and titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 22.50 mg of $C_3H_7MnO_6P$.

STORAGE

In an airtight container.

**07/2017:1543**

# MANGANESE SULFATE MONOHYDRATE

## Mangani sulfas monohydricus

$MnSO_4,H_2O$ $M_r$ 169.0

[10034-96-5]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent (ignited substance).

CHARACTERS

*Appearance*: pale pink crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

B. Dissolve 50 mg in 5 mL of *water R*. Add 0.5 mL of *sodium sulfide solution R*. A pale pink precipitate is formed which dissolves on the addition of 1 mL of *anhydrous acetic acid R*.

C. Loss on ignition (see Tests).

TESTS

**Solution S**. Dissolve 10.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Zinc**: maximum 50 ppm.

To 10 mL of solution S add 1 mL of *sulfuric acid R* and 0.1 mL of *potassium ferrocyanide solution R*. After 30 s, any

opalescence in the solution is not more intense than that in a mixture of 5 mL of *zinc standard solution (10 ppm Zn) R*, 5 mL of *water R*, 1 mL of *sulfuric acid R* and 0.1 mL of *potassium ferrocyanide solution R*.

**Loss on ignition**: 10.0 per cent to 12.0 per cent, determined on 1.00 g at 500 ± 50 °C.

ASSAY

Dissolve 0.150 g in 50 mL of *water R*. Add 10 mg of *ascorbic acid R*, 20 mL of *ammonium chloride buffer solution pH 10.0 R* and 0.2 mL of a 2 g/L solution of *mordant black 11 R* in *triethanolamine R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to pure blue.

1 mL of *0.1 M sodium edetate* is equivalent to 15.10 mg of $MnSO_4$.

**07/2019:0559**

# MANNITOL[3]

## Mannitolum

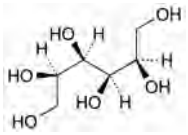

$C_6H_{14}O_6$ $M_r$ 182.2
[69-65-8]

DEFINITION

D-Mannitol.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

◆CHARACTERS

*Appearance*: white or almost white crystals or powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).◆

IDENTIFICATION

*First identification: C.*

◊*Second identification: A, B, D.*

A. Specific optical rotation (*2.2.7*): + 23 to + 25 (dried substance).

Dissolve 2.00 g of the substance to be examined and 2.6 g of *disodium tetraborate R* in about 20 mL of *water R* at 30 °C; shake continuously for 15-30 min without further heating. Dilute the resulting clear solution to 25.0 mL with *water R*.

B. Melting point (see Tests).◊

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mannitol CRS*.

If the spectra obtained in the solid state show differences, dissolve separately in 2 glass vials 25 mg of the substance to be examined and 25 mg of the reference substance in 0.25 mL of *distilled water R* without heating. The solutions obtained are clear. Evaporate to dryness by heating in a microwave oven with a power range of 600-700 W for 20 min or by heating in an oven at 100 °C for 1 h then gradually applying vacuum until a dry residue is obtained. Non-sticky, white or slightly yellowish powders are obtained. Record new spectra using the residues.

◊D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 25 mg of *mannitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 25 mg of *mannitol R* and 25 mg of *sorbitol R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *ethyl acetate R*, *propanol R* (10:20:70 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *4-aminobenzoic acid solution R* and dry in a current of cold air until the acetone is removed; heat at 100 °C for 15 min, allow to cool then spray with a 2 g/L solution of *sodium periodate R*; dry in a current of cold air and heat at 100 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).◊

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* by heating at 40-50 °C and dilute to 100.0 mL with the same solvent. After cooling, measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Melting point** (*2.2.14*): 165 °C to 170 °C.

**Reducing sugars**: maximum 0.1 per cent (calculated as glucose equivalent).

To 7.0 g add 13 mL of *water R*. Boil gently with 40 mL of *cupri-tartaric solution R* for 3 min, and allow to stand for 2 min. A precipitate is formed. Filter through a sintered-glass filter (16) (*2.1.2*) coated with *diatomaceous earth R* or a sintered-glass filter (10) (*2.1.2*). Wash the precipitate with hot *water R* (about 50-60 °C) until the washing is no longer alkaline, and filter the washings through the same sintered-glass filter. Discard the filtrate. Immediately dissolve the precipitate in 20 mL of *ferric sulfate solution R*, filter through the same sintered-glass filter, and wash the filter with 15-20 mL of *water R*. Combine the washings and the filtrate, heat to 80 °C, and titrate with *0.02 M potassium permanganate*. Not more than 3.2 mL is required to change the colour of the solution from green to pink so that the colour persists for at least 10 s.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in 2.5 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.50 g of *mannitol CRS* in 2.5 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dilute 0.5 mL of reference solution (b) to 20.0 mL with *water R*.

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*Reference solution (d).* Dissolve 0.25 g of *mannitol R* and 0.25 g of *sorbitol R* (impurity A) in 5 mL of *water R* and dilute to 10 mL with the same solvent.

*Reference solution (e).* Dissolve 0.5 g of *maltitol R* (impurity B) and 0.5 g of *isomalt R* (impurity C) in 5 mL of *water R* and dilute to 100 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *water R*.

*Column*:
- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 85 ± 2 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 40 °C).

*Injection*: 20 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Run time*: 1.5 times the retention time of mannitol.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities B and C.

*Relative retention* with reference to mannitol (retention time = about 20 min): impurity C (1st peak) = about 0.6; impurity B = about 0.7; impurity C (2nd peak) = about 0.73; impurity A = about 1.2. Impurity C elutes in 2 peaks. Coelution of impurity B and the 2nd peak due to impurity C may be observed.

*System suitability*: reference solution (d):
- *resolution*: minimum 2.0 between the peaks due to mannitol and impurity A.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *sum of impurities B and C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Microbial contamination**. If intended for use in the manufacture of parenteral preparations:
- TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:
- TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
- TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
- absence of *Escherichia coli* (*2.6.13*);
- ◊– absence of *Salmonella* (*2.6.13*).◊

♦**Bacterial endotoxins** (*2.6.14*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins:
- less than 4 IU/g for parenteral preparations having a concentration of 100 g/L or less of mannitol;
- less than 2.5 IU/g for parenteral preparations having a concentration of more than 100 g/L of mannitol.♦

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_6H_{14}O_6$ taking into account the assigned content of *mannitol CRS*.

LABELLING

The label states:
- where applicable, the maximum concentration of bacterial endotoxins;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C.*

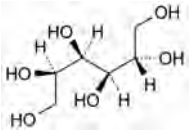

A. D-glucitol (D-sorbitol),

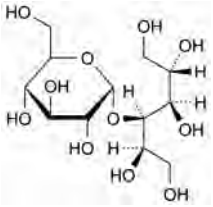

B. 4-*O*-α-D-glucopyranosyl-D-glucitol (D-maltitol),

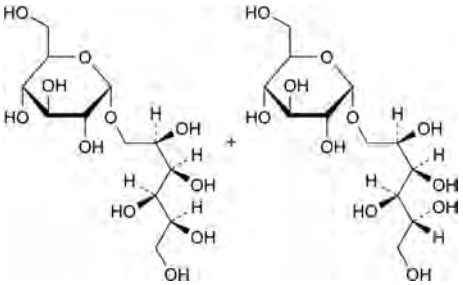

C. mixture of 6-*O*-α-D-glucopyranosyl-D-glucitol and 1-*O*-α-D-glucopyranosyl-D-mannitol (isomalt).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for mannitol used as filler in tablets and capsules.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**07/2010:1237**

# MAPROTILINE HYDROCHLORIDE

## Maprotilini hydrochloridum

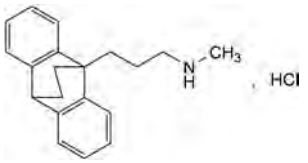

$C_{20}H_{24}ClN$ $M_r$ 313.9
[10347-81-6]

DEFINITION

3-(9,10-Ethanoanthracen-9(10*H*)-yl)-*N*-methylpropan-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent), sparingly soluble in methylene chloride, very slightly soluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10 mg in *1 M hydrochloric acid* and dilute to 100 mL with the same acid.

*Spectral range*: 250-300 nm.

*Absorption maxima*: at 265 nm and 272 nm.

*Absorption minimum*: at 268 nm.

*Absorbance ratio*: $A_{272}/A_{265}$ = 1.1 to 1.3.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *maprotiline hydrochloride CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *maprotiline hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *maprotiline impurity D CRS* in reference solution (a) and dilute to 2 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R*, *dilute ammonia R1*, *2-butanol R1* (4:5:14 *V/V/V*).

*Application*: 5 µL.

*Development*: over half of the plate.

*Drying*: in a current of warm air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dilute 0.5 mL of solution S (see Tests) to 2 mL with *methanol R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 1.0 mg of *maprotiline impurity D CRS* in the test solution and dilute to 10.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve about 0.580 g of *ammonium acetate R* in 200 mL of *water R* and add 2 mL of a 70 g/L solution of *concentrated ammonia R*; add 150 mL of *2-propanol R* and 650 mL of *methanol R*; the resulting apparent pH value is between 8.2 and 8.4.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of maprotiline.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to maprotiline (retention time = about 10 min): impurity A = about 0.3; impurity B = about 0.5; impurity C = about 0.7; impurity D = about 0.8; impurity E = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: 1.8 to 3.2 between the peaks due to impurity D and maprotiline; if necessary, adjust the pH of the mobile phase, in steps of 0.1 pH unit, by adding a 50 per cent *V/V* solution of *acetic acid R* if the resolution is less than 1.8, or by adding a 70 g/L solution of *concentrated ammonia R* if the resolution is greater than 3.2.

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 2.5 kPa for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5 mL of *0.1 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.39 mg of $C_{20}H_{24}ClN$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

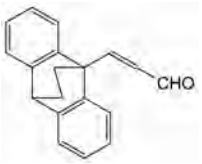


A. 3-(9,10-ethanoanthracen-9(10*H*)-yl)prop-2-enal,

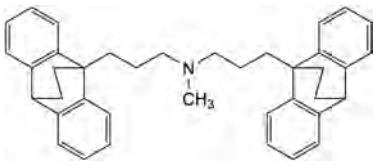


B. 3-(9,10-ethanoanthracen-9(10*H*)-yl)-*N*-[3-(9,10-ethanoanthracen-9(10*H*)-yl)propyl]-*N*-methylpropan-1-amine,

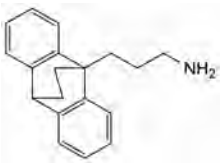


C. 3-(9,10-ethanoanthracen-9(10*H*)-yl)propan-1-amine,

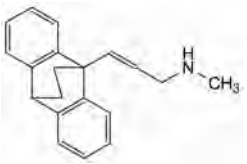


D. 3-(9,10-ethanoanthracen-9(10*H*)-yl)-*N*-methylprop-2-en-1-amine (dehydromaprotiline),

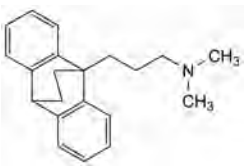


E. 3-(9,10-ethanoanthracen-9(10*H*)-yl)-*N*,*N*-dimethylpropan-1-amine.

**04/2015:2233**

# MARBOFLOXACIN FOR VETERINARY USE

## Marbofloxacinum ad usum veterinarium

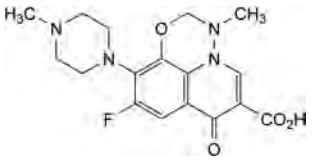


$C_{17}H_{19}FN_4O_4$ $M_r$ 362.4
[115550-35-1]

DEFINITION

9-Fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[3,2,1-*ij*][4,1,2]benzoxadiazine-6-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: light yellow, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble or slightly soluble in methylene chloride, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *marbofloxacin CRS*.

TESTS

**Absorbance** (*2.2.25*): maximum 0.20, determined at 450 nm. *Prepare the solution immediately before use.*

Dissolve 0.400 g in *0.1 M ammonium carbonate buffer solution pH 10.3 R* using sonication and dilute to 10.0 mL with the same buffer solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *methanol R*, *water R* (23:77 *V/V*).

*Test solution.* To 0.100 g of the substance to be examined add 80 mL of the solvent mixture, sonicate until dissolution and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *marbofloxacin for peak identification CRS* (containing impurities A, B, C, D and E) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 230 volumes of *methanol R* and 5 volumes of *glacial acetic acid R* with 770 volumes of a 2.70 g/L solution of *sodium dihydrogen phosphate R* containing 3.50 g/L of *sodium octanesulfonate R* and previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 315 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of marbofloxacin.

*Identification of impurities*: use the chromatogram supplied with *marbofloxacin for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to marbofloxacin (retention time = about 33 min): impurity B = about 0.5; impurity A = about 0.7; impurity C = about 0.9; impurity D = about 1.3; impurity E = about 1.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity C and marbofloxacin, and minimum 4.0 between the peaks due to marbofloxacin and impurity D.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity E by 1.5;

– *impurities C, D, E*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

Dissolve 0.5 g in *dilute acetic acid R* and dilute to 30 mL with the same solvent. Adding 2 mL of *water R* instead of 2 mL of *buffer solution pH 3.5 R*, the filtrate complies with test E. Prepare the reference solution using 5 mL of *lead standard solution (2 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

## ASSAY

Dissolve 0.300 g in 80 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.24 mg of $C_{17}H_{19}FN_4O_4$.

## STORAGE

Protected from light.

## IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

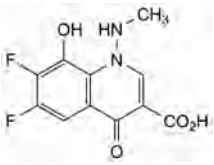

A. 6,7-difluoro-8-hydroxy-1-(methylamino)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

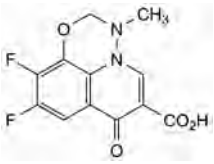

B. 9,10-difluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[3,2,1-*ij*][4,1,2]benzoxadiazine-6-carboxylic acid,

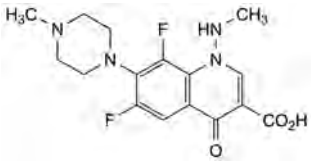

C. 6,8-difluoro-1-(methylamino)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

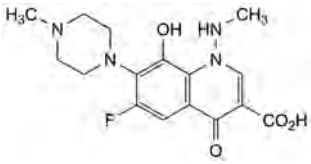

D. 6-fluoro-8-hydroxy-1-(methylamino)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

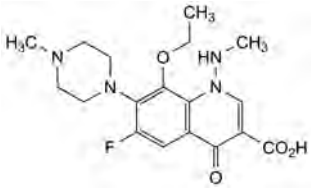

E. 8-ethoxy-6-fluoro-1-(methylamino)-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

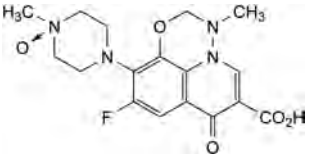

F. 4-[6-carboxy-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[3,2,1-*ij*][4,1,2]benzoxadiazin-10-yl]-1-methylpiperazine 1-oxide.

04/2013:0845

# MEBENDAZOLE

## Mebendazolum

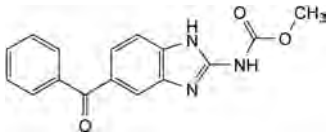

$C_{16}H_{13}N_3O_3$ $M_r$ 295.3
[31431-39-7]

DEFINITION

Methyl (5-benzoyl-1*H*-benzimidazol-2-yl)carbamate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*). The acceptable crystalline form corresponds to *mebendazole CRS*.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the substances without prior treatment.

*Comparison*: *mebendazole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *mebendazole for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in *dimethylformamide R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *dimethylformamide R*. Dilute 5.0 mL of this solution to 20.0 mL with *dimethylformamide R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 7.5 g/L solution of *ammonium acetate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 80 → 70 | 20 → 30 |
| 15 - 20 | 70 → 10 | 30 → 90 |
| 20 - 25 | 10 | 90 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *mebendazole for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to mebendazole (retention time = about 12 min): impurity A = about 0.4; impurity B = about 0.5; impurity C = about 0.7; impurity D = about 1.1; impurity E = about 1.3; impurity F = about 1.4; impurity G = about 1.6.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to mebendazole.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity G by 1.4;
- *impurity G*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 3 mL of *anhydrous formic acid R* and add 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.53 mg of $C_{16}H_{13}N_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

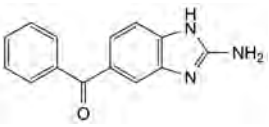

A. (2-amino-1*H*-benzimidazol-5-yl)phenylmethanone,

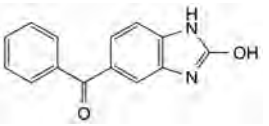

B. (2-hydroxy-1*H*-benzimidazol-5-yl)phenylmethanone,

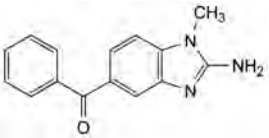

C. (2-amino-1-methyl-1*H*-benzimidazol-5-yl)phenylmethanone,

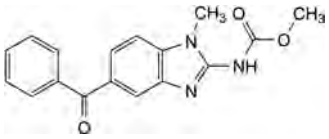

D. methyl (5-benzoyl-1-methyl-1*H*-benzimidazol-2-yl)carbamate,

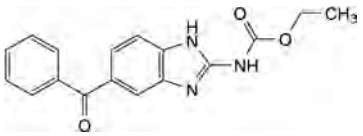

E. ethyl (5-benzoyl-1*H*-benzimidazol-2-yl)carbamate,

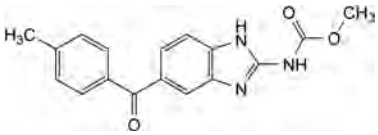

F. methyl [5-(4-methylbenzoyl)-1*H*-benzimidazol-2-yl]carbamate,

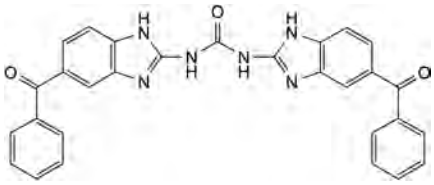

G. *N*,*N*′-bis(5-benzoyl-1*H*,-benzimidazol-2-yl)urea.

**07/2019:2097**

# MEBEVERINE HYDROCHLORIDE

## Mebeverini hydrochloridum

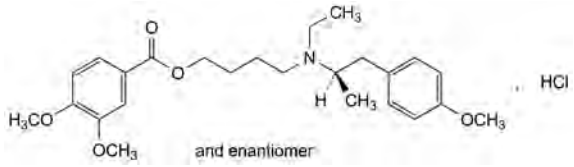

$C_{25}H_{36}ClNO_5$ $M_r$ 466.0
[2753-45-9]

DEFINITION

4-[Ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butyl 3,4-dimethoxybenzoate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water and in methylene chloride, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *mebeverine hydrochloride CRS*.

B. Dissolve 25 mg in 2 mL of *water R*, acidify with *dilute nitric acid R* and centrifuge. The supernatant gives reaction (a) of chlorides (*2.3.1*) starting from 'add 0.4 mL of *silver nitrate solution R1*'.

TESTS

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *mebeverine for system suitability CRS* (containing impurity J) in 1 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: mix 1 volume of *triethylamine R* and 100 volumes of *water for chromatography R* and adjust to pH 5.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 75 | 25 |
| 6 - 20 | 75 → 30 | 25 → 70 |
| 20 - 40 | 30 | 70 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 2 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity J.

*Relative retention* with reference to mebeverine (retention time = about 15 min): impurity J = about 0.82.

*System suitability*:
- *resolution*: minimum 3.5 between the peaks due to impurity J and mebeverine in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 70 for the principal peak in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity J by 3.5;
- for each impurity, use the concentration of mebeverine hydrochloride in reference solution (a).

*Limits*:
- *impurity J*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in a mixture of 20 mL of *acetic anhydride R* and 30 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 46.60 mg of $C_{25}H_{36}ClNO_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, K, N, O.*

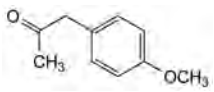

A. 1-(4-methoxyphenyl)propan-2-one,

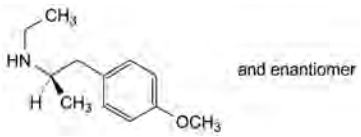


B. (2*RS*)-*N*-ethyl-1-(4-methoxyphenyl)propan-2-amine,

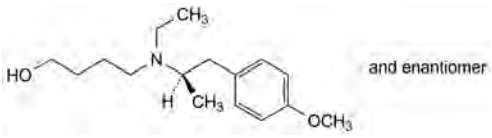


C. 4-[ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butan-1-ol,

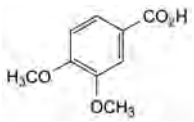

D. 3,4-dimethoxybenzoic acid,

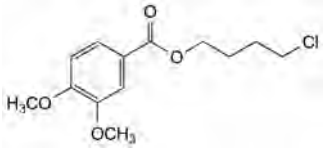

E. 4-chlorobutyl 3,4-dimethoxybenzoate,

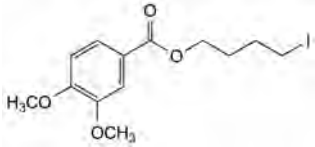

F. 4-iodobutyl 3,4-dimethoxybenzoate,

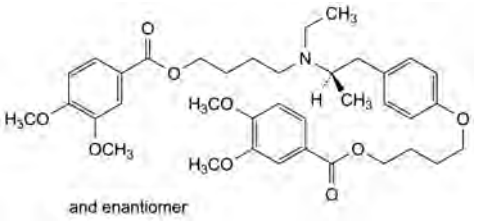


G. 4-[[(2*RS*)-1-[4-[4-[(3,4-dimethoxybenzoyl)oxy]-butoxy]phenyl]propan-2-yl](ethyl)amino]butyl 3,4-dimethoxybenzoate,

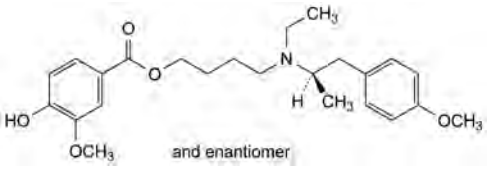


H. 4-[ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butyl 4-hydroxy-3-methoxybenzoate,

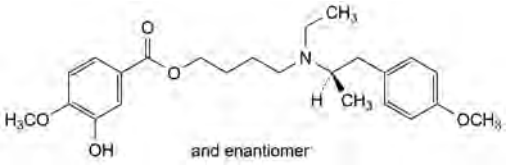


I. 4-[ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butyl 3-hydroxy-4-methoxybenzoate,

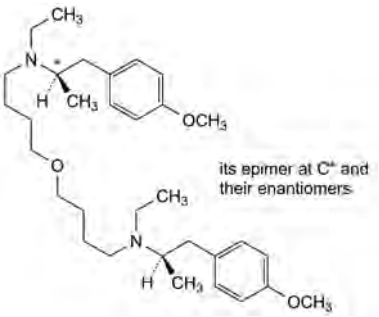


J. 4,4′-oxybis[*N*-ethyl-*N*-[(2*RS*)-1-(4-methoxyphenyl)-propan-2-yl]butan-1-amine],

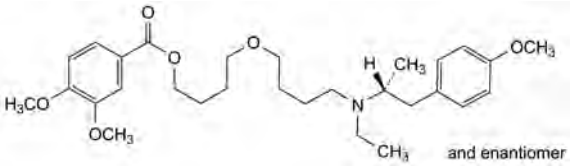


K. 4-[4-[ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butoxy]butyl 3,4-dimethoxybenzoate,

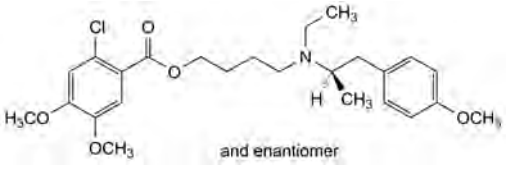


N. 4-[ethyl[(2*RS*)-1-(4-methoxyphenyl)propan-2-yl]amino]butyl 2-chloro-4,5-dimethoxybenzoate,

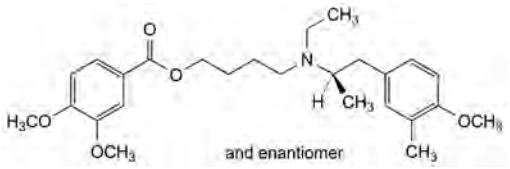


O. 4-[ethyl[(2*RS*)-1-(4-methoxy-3-methylphenyl)propan-2-yl]amino]butyl 3,4-dimethoxybenzoate.

**01/2011:0622**
**corrected 9.4**

# MECLOZINE DIHYDROCHLORIDE

## Meclozini dihydrochloridum

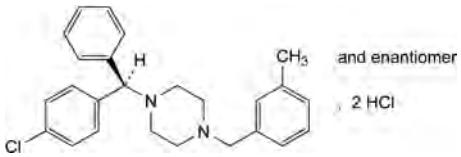


$C_{25}H_{29}Cl_3N_2$ $M_r$ 463.9
[1104-22-9]

DEFINITION

1-[(*RS*)-(4-Chlorophenyl)phenylmethyl]-4-[(3-methylphenyl)methyl]piperazine dihydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, slightly hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 15.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid.*

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 232 nm.

*Specific absorbance at the absorption maximum*: 345 to 380 (anhydrous substance).

The solution also shows a weak absorbance without a defined maximum between 260 nm and 300 nm.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *meclozine dihydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *meclozine dihydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *diethylamine R, toluene R, cyclohexane R* (10:15:75 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air for 5 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 15 mg in 2 mL of *ethanol (96 per cent) R.* The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Acidity or alkalinity**. Calculate the acidity or alkalinity from the titration volumes obtained in the assay using the following equation:

$$A = V_2 - 2V_1$$

$V_1$ = volume of *0.1 M sodium hydroxide* added at the 1st point of inflexion;

$V_2$ = volume of *0.1 M sodium hydroxide* added at the 2nd point of inflexion.

*A* is not less than − 0.3 mL and not more than 0.3 mL for 0.350 g of the substance to be examined.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 7.5 mg of *meclozine impurity B CRS* and 7.5 mg of *meclozine impurity H CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *concentrated ammonia R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 60 | 40 |
| 3 - 13 | 60 → 15 | 40 → 85 |
| 13 - 23 | 15 → 5 | 85 → 95 |
| 23 - 33 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and H.

*Relative retention* with reference to meclozine (retention time = about 18 min): impurity B = about 0.45; impurity H = about 0.49.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities B and H.

*Limits*:

- *impurity B*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 46.39 mg of $C_{25}H_{29}Cl_3N_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, H.*

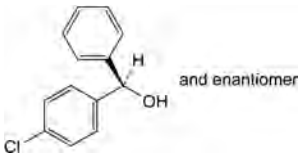


B. (*RS*)-(4-chlorophenyl)phenylmethanol,

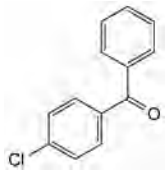


C. (4-chlorophenyl)phenylmethanone (4-chlorobenzophenone),

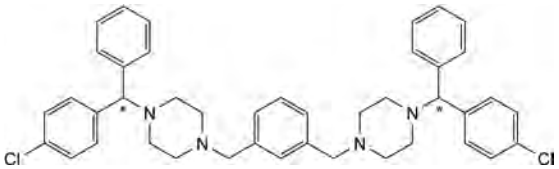


D. 1,1′-(1,3-phenylenebismethylene)bis[4-[(4-chlorophenyl)phenylmethyl]piperazine],

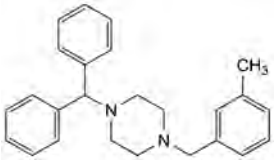


E. 1-(diphenylmethyl)-4-[(3-methylphenyl)methyl]-piperazine,

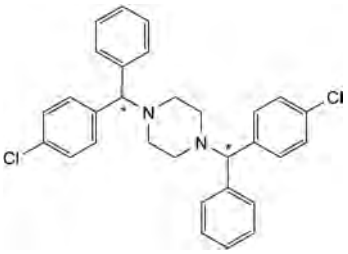


F. 1,4-bis[(4-chlorophenyl)phenylmethyl]piperazine,

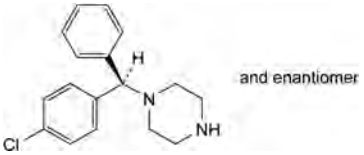


H. 1-[(*RS*)-(4-chlorophenyl)phenylmethyl]piperazine.

**01/2013:0673**

# MEDROXYPROGESTERONE ACETATE

## Medroxyprogesteroni acetas

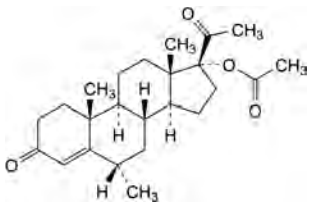


$C_{24}H_{34}O_4$ $M_r$ 386.5
[71-58-9]

DEFINITION

6α-Methyl-3,20-dioxopregn-4-en-17-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *medroxyprogesterone acetate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 47 to + 53 (dried substance).

Dissolve 0.250 g in *acetone R* and dilute to 25.0 mL with the same solvent.

**Impurity F**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 5.0 mL of *acetonitrile R1* and dilute to 10.0 mL with *water for chromatography R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *medroxyprogesterone acetate for peak identification CRS* (containing impurity F) in 3.0 mL of *acetonitrile R1* and dilute to 5.0 mL with *water for chromatography R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R1* (44:56 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *medroxyprogesterone acetate for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity F.

*Relative retention* with reference to medroxyprogesterone acetate (retention time = about 8 min): impurity F = about 1.8.

*Limit*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity F by 1.8;

– *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 4 mg of *medroxyprogesterone acetate for system suitability CRS* (containing impurities A, B, C, D, E, G and I) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 3.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 60 °C.

*Mobile phase*: *tetrahydrofuran R*, *acetonitrile R*, *water R* (12:23:65 *V/V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of medroxyprogesterone acetate.

*Identification of impurities*: use the chromatogram supplied with *medroxyprogesterone acetate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, G and I.

*Relative retention* with reference to medroxyprogesterone acetate (retention time = about 20 min): impurity A = about 0.3; impurity I = about 0.5; impurity H = about 0.65; impurity B = about 0.7; impurity C = about 0.8; impurity G = about 0.85; impurity D = about 0.9; impurity E = about 0.95.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to medroxyprogesterone acetate.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity G = 2.6;

– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurity B*: not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);

– *impurities C, E, G, I*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

## ASSAY

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241 nm.

Calculate the content of $C_{24}H_{34}O_4$ taking the specific absorbance to be 420.

## STORAGE

Protected from light.

## IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H.*

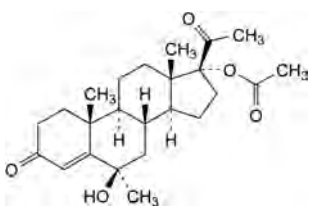

A. 6β-hydroxy-6-methyl-3,20-dioxopregn-4-en-17-yl acetate (6-hydroxymedroxyprogesterone acetate),

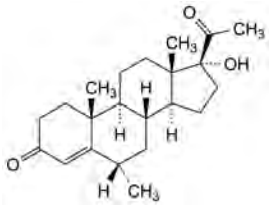

B. 17-hydroxy-6α-methylpregn-4-ene-3,20-dione (medroxyprogesterone),

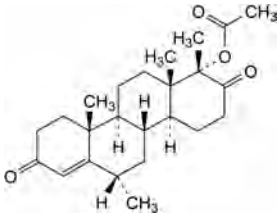

C. 6α,17a-dimethyl-3,17-dioxo-*D*-homoandrost-4-en-17aα-yl acetate,

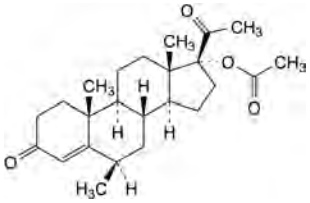

D. 6β-methyl-3,20-dioxopregn-4-en-17-yl acetate (6-epimedroxyprogesterone acetate),

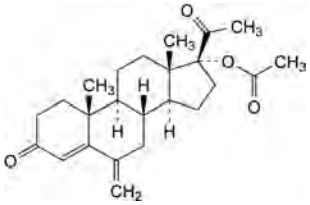

E. 6-methylidene-3,20-dioxopregn-4-en-17-yl acetate (6-methylenehydroxyprogesterone acetate),

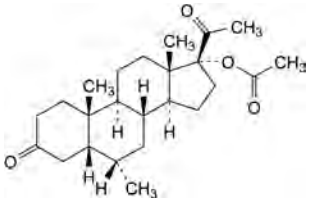

F. 6α-methyl-3,20-dioxo-5β-pregnan-17-yl acetate (4,5-dihydromedroxyprogesterone acetate),

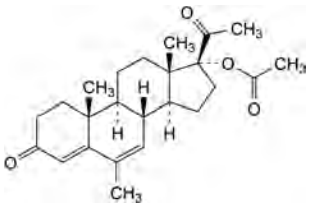

G. 6-methyl-3,20-dioxopregna-4,6-dien-17-yl acetate (megestrol acetate),

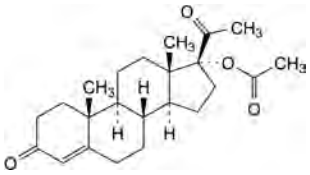

H. 3,20-dioxopregn-4-en-17-yl acetate (hydroxyprogesterone acetate),

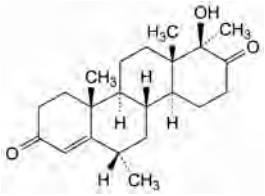

I. 17aβ-hydroxy-6,17a-dimethyl-*D*-homoandrost-4-ene-3,17-dione.

**07/2019:1240**

# MEFENAMIC ACID

## Acidum mefenamicum

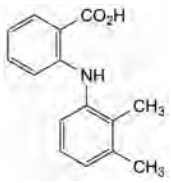

$C_{15}H_{15}NO_2$ $M_r$ 241.3
[61-68-7]

DEFINITION

2-(2,3-Dimethylanilino)benzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, microcrystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mefenamic acid CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50 mg of *2-chlorobenzoic acid R* (impurity C) and 50 mg of *benzoic acid R* (impurity D) in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 1 mL of the solution to 100 mL with the mobile phase.

*Reference solution (c)*. Dissolve 10.0 mg of *mefenamic acid impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 20.0 mg of *benzoic acid R* in the mobile phase and dilute to 1000.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 14 volumes of *tetrahydrofuran R*, 40 volumes of a 5.75 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 5.0 with *dilute ammonia R2*, and 46 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of mefenamic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to mefenamic acid (retention time = about 8 min): impurity C = about 0.3; impurity D = about 0.35; impurity A = about 0.5.

*System suitability*:
- *resolution*: minimum 3.0 between the peaks due to impurities C and D in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (d).

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 5.9; impurity D = 4.0;
- *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (100 ppm);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to impurity A.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve with the aid of ultrasound 0.200 g in 100 mL of warm *anhydrous ethanol R*, previously neutralised to *phenol red solution R*. Add 0.1 mL of *phenol red solution R* and titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.13 mg of $C_{15}H_{15}NO_2$.

IMPURITIES

*Specified impurities: A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E.*

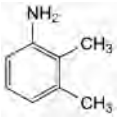

A. 2,3-dimethylaniline,

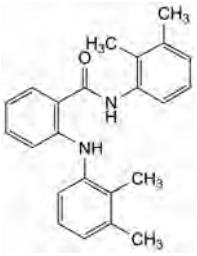

B. 2-(2,3-dimethylanilino)-*N*-(2,3-dimethylphenyl)benzamide,

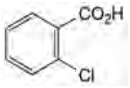

C. 2-chlorobenzoic acid,

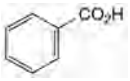

D. benzoic acid,

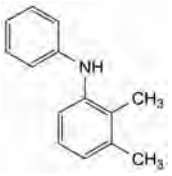

E. 2,3-dimethyl-*N*-phenylaniline.

**01/2017:1241**

# MEFLOQUINE HYDROCHLORIDE

## Mefloquini hydrochloridum

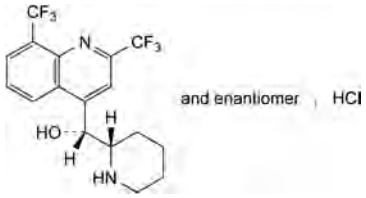


$C_{17}H_{17}ClF_6N_2O$ $M_r$ 414.8
[51773-92-3]

DEFINITION

(*RS*)-[2,8-Bis(trifluoromethyl)quinolin-4-yl][(2*SR*)-piperidin-2-yl]methanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

mp: about 260 °C, with decomposition.

IDENTIFICATION

*First identification: A, E.*

*Second identification*: *B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mefloquine hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 8 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a)*. Dissolve 8 mg of *mefloquine hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of the test solution to 100 mL with *methanol R*.

*Reference solution (c)*. To 1 mL of reference solution (b) add 1 mL of a 0.016 g/L solution of *quinidine sulfate R* in *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Pretreatment*: develop the plate with a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R*, and dry at 100-105 °C for 15 min before use.

*Mobile phase*: *anhydrous acetic acid R*, *methanol R*, *methylene chloride R* (10:10:80 *V/V/V*).

*Application*: 20 µL.

*Development*: over a path of 10 cm.

*Drying*: in a current of warm air for 15 min.

*Detection*: examine in ultraviolet light at 254 nm; lightly spray with a mixture prepared immediately before use of 1 volume of *sulfuric acid R* and 40 volumes of *iodoplatinate reagent R*; spray with *strong hydrogen peroxide solution R*.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Mix about 10 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until a practically white residue is obtained. Allow to cool, then add 2 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to make the solution colourless. Filter. To the filtrate add a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with a blank prepared in the same manner. The test solution is yellow and the blank is red.

D. To about 20 mg add 0.2 mL of *sulfuric acid R*. Blue fluorescence appears in ultraviolet light at 365 nm.

E. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *methanol R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method I*).

**Optical rotation** (*2.2.7*): − 0.2° to + 0.2°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 8 mg of *mefloquine hydrochloride CRS* and 8 mg of *quinidine sulfate R* in the mobile phase, then dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Precolumn*:

– *size*: *l* = 0.025 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 1 g of *tetraheptylammonium bromide R* in a mixture of 200 volumes of *methanol R*, 400 volumes of a 1.5 g/L solution of *sodium hydrogen sulfate R* and 400 volumes of *acetonitrile R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Equilibration*: with the mobile phase at a flow rate of 2 mL/min for about 30 min.

*Injection*: 20 µL.

*Run time*: 10 times the retention time of mefloquine.

*Retention time*: quinidine = about 2 min; mefloquine = about 4 min; impurity B = about 15 min; impurity A = about 36 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 8.5 between the peaks due to quinidine and mefloquine.

*Limits*:

– *impurity with a relative retention with reference to mefloquine of about 0.7*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *sum of impurities other than the impurity with a relative retention with reference to mefloquine of about 0.7*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 15 mL of *anhydrous formic acid R* and add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.48 mg of $C_{17}H_{17}ClF_6N_2O$.

STORAGE

Protected from light.

IMPURITIES

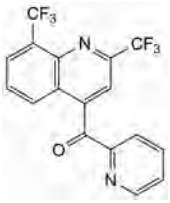

A. [2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanone,

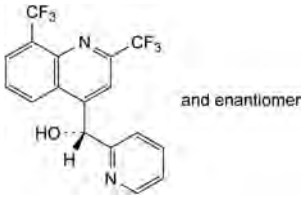


B. (*RS*)-[2,8-bis(trifluoromethyl)quinolin-4-yl](pyridin-2-yl)methanol,

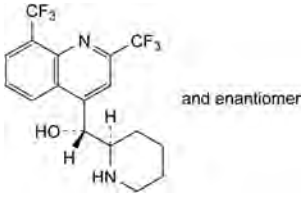


C. (*RS*)-[2,8-bis(trifluoromethyl)quinolin-4-yl][(2*RS*)-piperidin-2-yl]methanol.

**01/2014:1593**

# MEGESTROL ACETATE

## Megestroli acetas

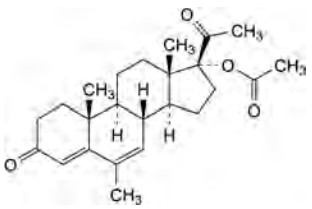

$C_{24}H_{32}O_4$ $M_r$ 384.5
[595-33-5]

DEFINITION

6-Methyl-3,20-dioxopregna-4,6-dien-17-yl acetate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent).

mp: about 217 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *megestrol acetate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 14.0 to + 17.0 (dried substance).

Dissolve 2.50 g in *methylene chloride R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetic acid R*, *water R*, *acetonitrile R1* (0.1:20:80 *V/V/V*).

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *megestrol acetate for system suitability CRS* (containing impurities A, D, G, H, I, J and L) in 1.0 mL of the solvent mixture.

*Reference solution (c).* Dissolve 10 mg of *megestrol acetate for peak identification CRS* (containing impurities B, C and E) in 1.0 mL of the solvent mixture.

*Reference solution (d).* Dissolve 50.0 mg of *megestrol acetate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve the contents of a vial of *megestrol acetate for impurity K identification CRS* in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m; Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *tetrahydrofuran R*, *acetonitrile R1*, *water R* (7.5:12.5:80 *V/V/V*);
- *mobile phase B*: *water R*, *tetrahydrofuran R*, *acetonitrile R1* (20:30:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16 | 70 | 30 |
| 16 - 42 | 70 → 30 | 30 → 70 |
| 42 - 49 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 245 nm and, for impurity J, at 210 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (c) and (e).

*Identification of impurities*: use the chromatogram supplied with *megestrol acetate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, D, G, H, I, J and L; use the chromatogram supplied with *megestrol acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, C and E; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity K.

*Relative retention* with reference to megestrol acetate (retention time = about 22 min): impurity B = about 0.75; impurity E = about 0.80; impurity K = about 0.83; impurity C = about 0.9; impurity D = about 1.11; impurity A = about 1.14; impurity I = about 1.2; impurity G = about 1.3; impurity J = about 1.4; impurity H = about 1.5; impurity L = about 1.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.2; impurity D = 0.4; impurity E = 0.4; impurity I = 0.5; impurity K = 0.2; impurity L = 0.6;
– *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurities D, H*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *impurity J at 210 nm*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) at 210 nm (0.3 per cent);
– *impurity G*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurities B, C, E, I, K, L*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities other than J*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{24}H_{32}O_4$ taking into account the assigned content of *megestrol acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, G, H, I, J, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

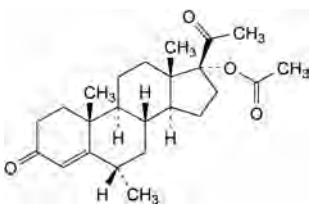

A. 6α-methyl-3,20-dioxopregn-4-en-17-yl acetate (medroxyprogesterone acetate),

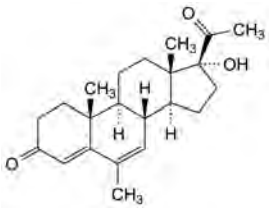

B. 6-methyl-17-hydroxypregna-4,6-diene-3,20-dione (megestrol),

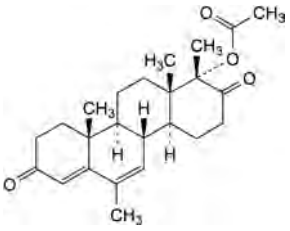

C. 6,17a-dimethyl-3,17-dioxo-*D*-homoandrosta-4,6-dien-17aα-yl acetate (*D-homo* megestrol acetate),

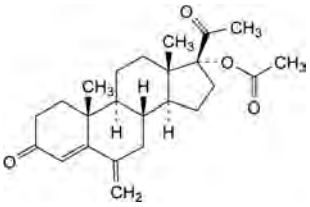

D. 6-methylene-3,20-dioxopregn-4-en-17-yl acetate (6-methylene hydroxyprogesterone acetate),

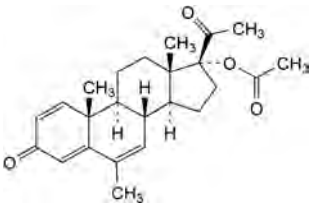

E. 6-methyl-3,20-dioxopregna-1,4,6-trien-17-yl acetate,

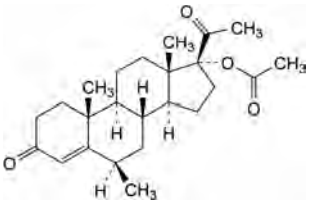

F. 6β-methyl-3,20-dioxopregn-4-en-17-yl acetate,

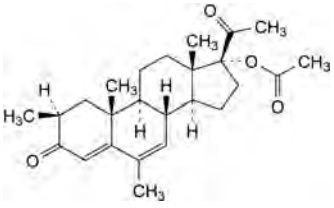

G. 2β,6-dimethyl-3,20-dioxopregna-4,6-dien-17-yl acetate,

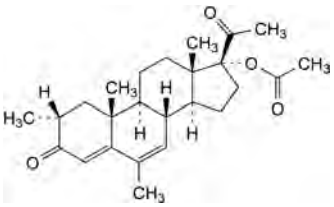

H. 2α,6-dimethyl-3,20-dioxopregna-4,6-dien-17-yl acetate,

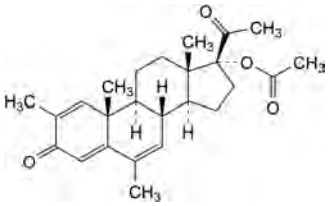

I. 2,6-dimethyl-3,20-dioxopregna-1,4,6-trien-17-yl acetate,

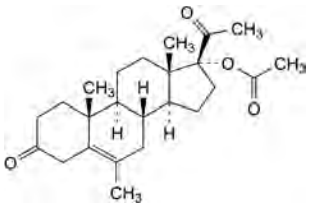

J. 6-methyl-3,20-dioxopregn-5-en-17-yl acetate,

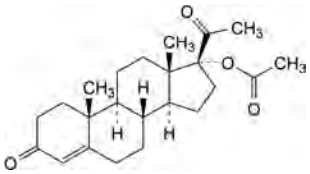

K. 3,20-dioxopregn-4-en-17-yl acetate,

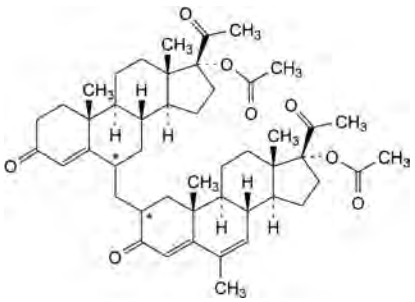

L. 2ξ-[[17-(acetyloxy)-3,20-dioxopregn-4-en-6ξ-yl]methyl]-6-methyl-3,20-dioxopregna-4,6-dien-17-yl acetate (megestrol acetate dimer).

**07/2019:2055**

# MEGLUMINE

## Megluminum

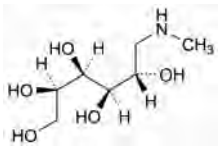

$C_7H_{17}NO_5$ $M_r$ 195.2
[6284-40-8]

DEFINITION

1-Deoxy-1-(methylamino)-D-glucitol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: about 128 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *meglumine CRS*.

TESTS

**Solution S**. Dissolve 20.0 g in *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm is not greater than 0.03.

Dissolve the residue obtained in the test for loss on drying in *water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 17.0 to − 16.0 (dried substance).

Dilute 12.5 mL of solution S to 25.0 mL with *water R*.

**Reducing substances**: maximum 0.2 per cent, expressed as glucose.

Dilute 1.25 mL of solution S to 2.5 mL with *water R*, add 2 mL of *cupri-tartaric solution R* and heat on a water-bath for 10 min. Cool under running water for 1 min, then sonicate for 20 s. Immediately filter through a membrane filter 25 mm in diameter (nominal pore size 0.5 µm). Rinse with 10 mL of *water R*. Prepare a standard in the same manner using 2.5 mL of a solution obtained by dissolving 20 mg of *glucose R* in *water R* and diluting to 100 mL with the same solvent. Any precipitate on the membrane filter obtained with the test solution is not more intensely coloured than the precipitate obtained with the standard.

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 2.5 mL of solution S add 12.5 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

To 5 mL of solution S add 10 mL of *distilled water R*.

**Aluminium**: maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) (*2.2.57*).

*Test solution*. Dissolve 5.00 g in 30 mL of *water R*, add 10.0 mL of *lead-free hydrochloric acid R* and dilute to 50.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *aluminium standard solution (10 ppm Al) R*, diluted as necessary with *water R*.

*Wavelength*: 396.153 nm.

**Iron**: maximum 10 ppm.

To 10 mL of solution S add about 0.8 mL of *hydrochloric acid R* and 0.05 mL of *bromine water R*. Allow to stand for 5 min, evaporate the excess of bromine in a current of air and add 3 mL of *potassium thiocyanate solution R*. Prepare a reference solution at the same time and in the same manner using 10 mL of *iron standard solution (2 ppm Fe) R*, to which 2 mL of *hydrochloric acid R* has been added. After 5 min, any red colour in the test solution is not more intense than that in the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.5 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.180 g in 30 mL of *water R*. Titrate with *0.05 M sulfuric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.05 M sulfuric acid* is equivalent to 19.52 mg of $C_7H_{17}NO_5$.

01/2015:2624
corrected 10.0

# MELDONIUM DIHYDRATE

## Meldonium dihydricum

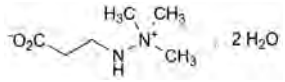

$C_6H_{14}N_2O_2,2H_2O$ $M_r$ 182.2
[86426-17-7]

DEFINITION

3-(2,2,2-Trimethylhydrazin-2-ium-1-yl)propanoate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white crystals or crystalline powder, deliquescent.

*Solubility*: very soluble in water, freely soluble in methanol, practically insoluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *meldonium dihydrate CRS*.

TESTS

**Solution S**. Dissolve 10.0 g in 50 mL of *distilled water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension I (*2.2.1*) and not more intensely coloured than reference solution $B_9$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*) coupled with mass spectrometry (*2.2.43*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 10.0 mg of *meldonium impurity A CRS*, 10.0 mg of *meldonium impurity B CRS*, 10.0 mg of *meldonium impurity C CRS*, 10.0 mg of *meldonium impurity D CRS*, 10.0 mg of *meldonium impurity E CRS* and 10.0 mg of *meldonium impurity F CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.5 mL of reference solution (a) to 100.0 mL with mobile phase A.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *heptafluorobutyric acid R* in *water for chromatography R*;
- *mobile phase B*: 0.1 per cent *V/V* solution of *heptafluorobutyric acid R* in *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 90 → 65 | 10 → 35 |
| 7 - 12 | 65 → 40 | 35 → 60 |
| 12 - 18 | 40 → 25 | 60 → 75 |

*Flow rate*: 0.2 mL/min.

*Detection*:

- triple-quadrupole mass spectrometer; the following settings have been found to be suitable and are given as examples; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria:
  - *ionisation*: ESI-positive;
  - *detection*:
    - multiple reaction monitoring (MRM) mode;
    - scanning mode: 50 - 300 *m*/*z*;
  - *ion source temperature*: 110 °C;
  - *desolvation temperature*: 220 °C;
  - *cone voltage*: 15 V;
  - *capillary voltage (Vcap)*: 3 kV;
  - *CID gas*: argon;
  - *CID gas pressure*: $2.7 \times 10^{-3}$ mbar.
- MRM mode parameters:

| Substance | MRM channels | Collision energy (eV) for the interval 0-25 min |
|---|---|---|
| Meldonium | 147.00 → 59.00 | 18.0 |
| Impurity A | 59.97 → 44.98 | 18.0 |
| Impurity B | 74.99 → 59.04 | 15.0 |
| Impurity F | 115.19 → 71.92 | 19.0 |
| Impurity C | 161.19 → 59.00 | 23.0 |
| Impurity D | 175.19 → 58.07 | 23.0 |
| Impurity E | 189.26 → 58.01 | 22.0 |

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Relative retention* with reference to meldonium (retention time = about 9 min): impurity F = about 0.7; impurity B = about 0.8; impurity A = about 0.9; impurity C = about 1.5; impurity D = about 1.8; impurity E = about 2.0.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: impurity A = minimum 10; impurity B = minimum 50; impurity C = minimum 200; impurity D = minimum 1000; impurity E = minimum 1000; impurity F = minimum 50;
- *repeatability*: maximum relative standard deviation of 10 per cent after 6 injections.

*Calculation of percentage contents*:

- for impurities A, B, C, D, E and F (MRM mode), use the concentration of the corresponding impurity in reference solution (b);
- for impurities other than A, B, C, D, E and F (complete spectrum mode), use the concentration of meldonium dihydrate in reference solution (c).

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 30 mL with *distilled water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dilute 10 mL of solution S to 20 mL with *distilled water R*.

**Water** (*2.5.12*): 19.7 per cent to 21.0 per cent, determined on 0.120 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 40 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.62 mg of $C_6H_{14}N_2O_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

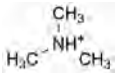

A. *N*,*N*-dimethylmethanaminium,

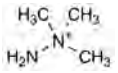

B. 1,1,1-trimethylhydrazin-1-ium,

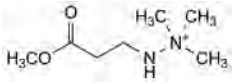

C. 2-(3-methoxy-3-oxopropyl)-1,1,1-trimethylhydrazin-1-ium,

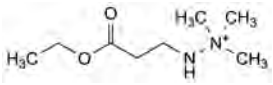

D. 2-(3-ethoxy-3-oxopropyl)-1,1,1-trimethylhydrazin-1-ium,

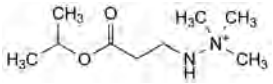

E. 1,1,1-trimethyl-2-[3-(1-methylethoxy)-3-oxopropyl]hydrazin-1-ium,

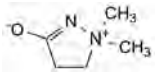

F. 1,1-dimethyl-4,5-dihydro-1*H*-pyrazol-1-ium-3-olate.

**01/2017:2373**

# MELOXICAM

## Meloxicamum

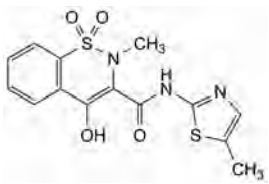

$C_{14}H_{13}N_3O_4S_2$ $M_r$ 351.4

[71125-38-7]

DEFINITION

4-Hydroxy-2-methyl-*N*-(5-methylthiazol-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, very slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *meloxicam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40 mg of the substance to be examined in a mixture of 5 mL of *methanol R* and 0.3 mL of *1 M sodium hydroxide* and dilute to 20.0 mL with *methanol R*.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 2 mg of the substance to be examined, 2 mg of *meloxicam impurity A CRS*, 2 mg of *meloxicam impurity B CRS*, 2 mg of *meloxicam impurity C CRS* and 2 mg of *meloxicam impurity D CRS* in a mixture of 5 mL of *methanol R* and 0.3 mL of *1 M sodium hydroxide* and dilute to 25 mL with *methanol R*. If complete dissolution cannot be achieved, filter the solution through a membrane filter (nominal pore size 0.45 µm) before injection.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: 1 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 6.0 with *1 M sodium hydroxide*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 60 | 40 |
| 2 - 10 | 60 → 30 | 40 → 70 |
| 10 - 15 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 260 nm and 350 nm.

*Injection*: 10 µL.

*Relative retention* with reference to meloxicam (retention time = about 7 min): impurity B = about 0.5; impurity A = about 1.4; impurity C = about 1.7; impurity D = about 1.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to meloxicam and impurity A at 350 nm; minimum 3.0 between the peaks due to impurity B and meloxicam at 260 nm.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2.0;
- *impurity A at 350 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) at 350 nm (0.1 per cent);
- *impurity B at 260 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) at 350 nm (0.1 per cent);

– *impurities C, D at 350 nm*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) at 350 nm (0.05 per cent);
– *unspecified impurities*: for each impurity, at the wavelength giving the higher value for the impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) at the same wavelength (0.10 per cent);
– *total*: not more than 0.3 per cent;
– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) at the same wavelength (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.250 g in a mixture of 5 mL of *anhydrous formic acid R* and 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.14 mg of $C_{14}H_{13}N_3O_4S_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

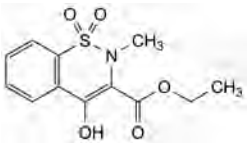

A. ethyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

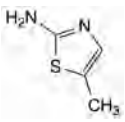

B. 5-methylthiazol-2-amine,

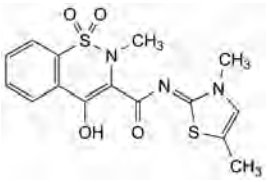

C. *N*-[(2*Z*)-3,5-dimethylthiazol-2(3*H*)-ylidene]-4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide,

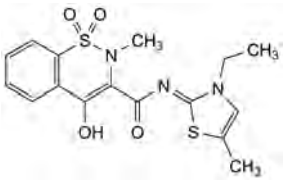

D. *N*-[(2*Z*)-3-ethyl-5-methylthiazol-2(3*H*)-ylidene]-4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide,

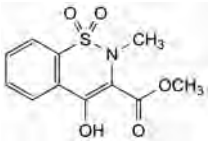

E. methyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

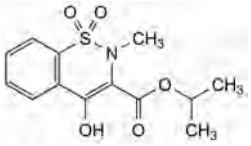

F. isopropyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide.

**07/2012:1698**

# MELPHALAN

## Melphalanum

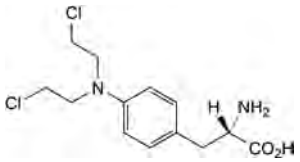

$C_{13}H_{18}Cl_2N_2O_2$ $M_r$ 305.2
[148-82-3]

DEFINITION

4-[Bis(2-chloroethyl)amino]-L-phenylalanine.

*Content*: 94.0 per cent to 102.0 per cent (anhydrous and diethylamine-free substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water, slightly soluble in methanol. It dissolves in dilute mineral acids.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of melphalan.*

TESTS

**Appearance of solution**. If intended for use in the manufacture of parenteral preparations, the solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.25 g in *dilute hydrochloric acid R* and dilute to 25 mL with the same acid.

**Specific optical rotation** (*2.2.7*): – 36.0 to – 30.0 (anhydrous and diethylamine-free substance).

Dissolve 0.175 g at 45 °C for 10 min in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and protect from light.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in *methanol R1* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *methanol R1*.

*Reference solution (a).* Dissolve 50.0 mg of *melphalan hydrochloride CRS* in *methanol R1* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R1*.

*Reference solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with *methanol R1*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 100.0 mL with *methanol R1*.

*Reference solution (d).* Dilute 5.0 mL of reference solution (b) to 100.0 mL with *methanol R1*.

*Reference solution (e).* In order to prepare impurity I *in situ*, dissolve 5 mg of *melphalan for system suitability CRS* (containing impurities B, D, G, H and J) in *methanol R1*, dilute to 5.0 mL with the same solvent and heat at 60 °C for 15 min.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mixture of 5 volumes of *acetonitrile for chromatography R* and 95 volumes of *water R* containing 0.01 per cent *V/V* of *triethylamine R*, 0.05 per cent *m/m* of *ammonium acetate R* and 0.05 per cent *V/V* of *glacial acetic acid R*;
- *mobile phase B*: mixture of 40 volumes of *water R* containing 0.01 per cent *V/V* of *triethylamine R*, 0.05 per cent *m/m* of *ammonium acetate R* and 0.05 per cent *V/V* of *glacial acetic acid R*, and 60 volumes of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *melphalan for system suitability CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities B, D, G, H, I and J.

*Relative retention* with reference to melphalan (retention time = about 10 min): impurity B = about 0.3; impurity D = about 0.6; impurity I = about 0.8; impurity J = about 1.04; impurity G = about 1.4; impurity H = about 1.5.

*System suitability*: reference solution (e):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to melphalan.

*Limits*:

- *impurity D*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (d) (3.0 per cent);
- *impurity G*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent);
- *impurities J, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity I*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 11 times the area of the principal peak in the chromatogram obtained with reference solution (d) (5.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Impurity K (diethylamine)**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.125 g of substance to be examined in 0.15 mL of *hydrochloric acid R* and dilute to 5.0 mL with *dimethyl sulfoxide R*.

*Reference solution.* Dilute 1 mL of *methanol R* and 0.125 g of *diethylamine R1* (impurity K) to 10.0 mL with *dimethyl sulfoxide R*. Dilute 1.0 mL of the solution to 100.0 mL with *dimethyl sulfoxide R*.

*Column*:

- *material*: glass;
- *size*: *l* = 1.6 m, Ø = 4 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* coated with potassium carbonate (149-177 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 42.5 mL/min.

*Temperature*:

- *column*: 170 °C;
- *injection port*: 190 °C;
- *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: methanol, impurity K.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to methanol and impurity K.

*Limit*:

- *impurity K*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.5 per cent).

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{13}H_{18}Cl_2N_2O_2$ taking into account the assigned content of *melphalan hydrochloride CRS* and a conversion factor of 0.8933.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *B, D, G, H, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F.*

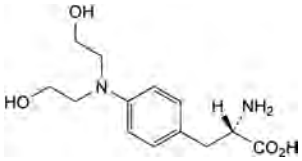

A. 4-[bis(2-hydroxyethyl)amino]-L-phenylalanine,

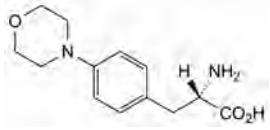

B. 4-morpholin-4-yl-L-phenylalanine,

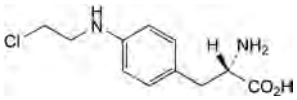

C. 4-[(2-chloroethyl)amino]-L-phenylalanine,

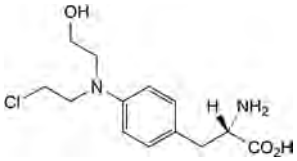

D. 4-[(2-chloroethyl)(2-hydroxyethyl)amino]-L-phenylalanine,

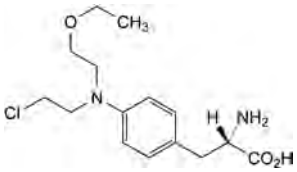

E. 4-[(2-chloroethyl)(2-ethoxyethyl)amino]-L-phenylalanine,

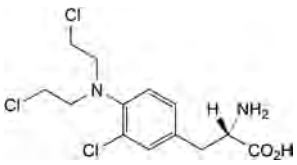

F. 4-[bis(2-chloroethyl)amino]-3-chloro-L-phenylalanine (3-chloromelphalan),

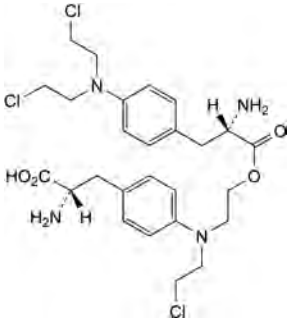

G. 4-[[2-[[4-[bis(2-chloroethyl)amino]-L-phenylalanyl]-oxy]ethyl](2-chloroethyl)amino]-L-phenylalanine (melphalan dimer),

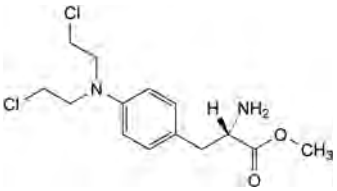

H. methyl 4-[bis(2-chloroethyl)amino]-L-phenylalaninate,

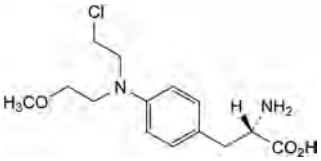

I. 4-[(2-chloroethyl)(2-methoxyethyl)amino]-L-phenylalanine,

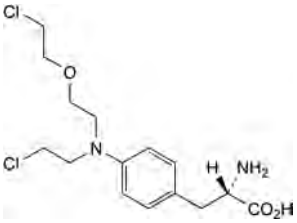

J. 4-[[2-(2-chloroethoxy)ethyl](2-chloroethyl)amino]-L-phenylalanine,

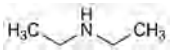

K. *N*-ethylethanamine (diethylamine).

**01/2008:0507**
**corrected 10.0**

# MENADIONE

## Menadionum

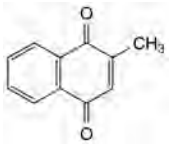

$C_{11}H_8O_2$ $M_r$ 172.2
[58-27-5]

DEFINITION

Menadione contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 2-methylnaphthalene-1,4-dione, calculated with reference to the dried substance.

CHARACTERS

A pale-yellow, crystalline powder, practically insoluble in water, freely soluble in toluene, sparingly soluble in alcohol and in methanol. It is unstable in light.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 105 °C to 108 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *menadione CRS*.

C. Dissolve about 1 mg in 5 mL of *alcohol R*, add 2 mL of *ammonia R* and 0.2 mL of *ethyl cyanoacetate R*. An intense bluish-violet colour develops. Add 2 mL of *hydrochloric acid R*. The colour disappears.

D. Dissolve about 10 mg in 1 mL of *alcohol R*, add 1 mL of *hydrochloric acid R* and heat in a water-bath. A red colour develops.

TESTS

**Related substances**. *Carry out the test protected from bright light.* Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.2 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dilute 0.5 mL of the test solution to 100 mL with *acetone R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *nitromethane R*, 2 volumes of *acetone R*, 5 volumes of *ethylene chloride R* and 90 volumes of *cyclohexane R*. Dry the plate in a current of hot air. Repeat the development and drying a further two times. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 15 mL of *glacial acetic acid R* in a flask with a stopper fitted with a valve. Add 15 mL of *dilute hydrochloric acid R* and 1 g of *zinc powder R*. Close the flask. Allow the mixture to stand for 60 min, protected from light, with occasional shaking. Filter the solution over a cotton wad, wash with three quantities, each of 10 mL, of *carbon dioxide-free water R*. Add 0.1 mL of *ferroin R* and immediately titrate the combined filtrate and washings with *0.1 M ammonium and cerium nitrate*.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 8.61 mg of $C_{11}H_8O_2$.

STORAGE

Store protected from light.

**01/2008:0623**
**corrected 7.0**

# MENTHOL, RACEMIC

## Mentholum racemicum

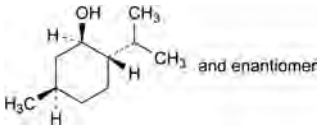


$C_{10}H_{20}O$ $M_r$ 156.3
[89-78-1]

DEFINITION

Mixture of equal parts of (1*RS*,2*SR*,5*RS*)-5-methyl-2-(1-methylethyl)cyclohexanol.

CHARACTERS

*Appearance*: free-flowing or agglomerated, crystalline powder or prismatic or acicular, colourless, shiny crystals.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and in light petroleum, freely soluble in fatty oils and in liquid paraffin, very slightly soluble in glycerol.

mp: about 34 °C.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, D.*

A. Optical rotation (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution.* Dissolve 25 mg of *menthol CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: in air, until the solvents have evaporated.

*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C for 5-10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in position and approximate dimensions to the principal peak in the chromatogram obtained with reference solution (c).

D. Dissolve 0.20 g in 0.5 mL of *anhydrous pyridine R*. Add 3 mL of a 150 g/L solution of *dinitrobenzoyl chloride R* in *anhydrous pyridine R*. Heat on a water-bath for 10 min. Add 7.0 mL of *water R* in small quantities with stirring and allow to stand in iced water for 30 min. A precipitate is formed. Allow to stand and decant the supernatant. Wash the precipitate with 2 quantities, each of 5 mL, of iced *water R*, recrystallise from 10 mL of *acetone R*, wash with iced *acetone R* and dry at 75 °C at a pressure not exceeding 2.7 kPa for 30 min. The crystals melt (*2.2.14*) at 130 °C to 131 °C.

TESTS

**Solution S**. Dissolve 2.50 g in 10 mL of *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Optical rotation** (*2.2.7*): − 0.2° to + 0.2°, determined on solution S.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *methylene chloride R*.

*Reference solution (a).* Dissolve 40.0 mg of the substance to be examined and 40.0 mg of *isomenthol R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 0.10 mL of test solution (a) to 100.0 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve 40.0 mg of *menthol CRS* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Column*:
- *material*: glass;
- *size*: *l* = 2.0 m, Ø = 2 mm;
- *stationary phase*: *diatomaceous earth for gas chromatography R* impregnated with 15 per cent *m/m* of *macrogol 1500 R*.

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:
- *column*: 120 °C;
- *injection port*: 150 °C;
- *detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: twice the retention time of menthol.

*System suitability*:
- *resolution*: minimum 1.4 between the peaks due to menthol and isomenthol in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*: test solution (a):
- *total*: not more than 1 per cent of the area of the principal peak;
- *disregard limit*: 0.05 per cent of the area of the principal peak.

**Residue on evaporation**: maximum 0.05 per cent.

Evaporate 2.00 g on a water-bath and heat in an oven at 100-105 °C for 1 h. The residue weighs not more than 1.0 mg.

**01/2019:1242**
**corrected 10.0**

# MEPIVACAINE HYDROCHLORIDE

## Mepivacaini hydrochloridum

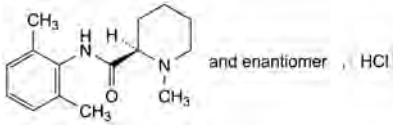


$C_{15}H_{23}ClN_2O$ $M_r$ 282.8
[1722-62-9]

DEFINITION

(2*RS*)-*N*-(2,6-Dimethylphenyl)-1-methylpiperidine-2-carboxamide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), very slightly soluble in methylene chloride.

mp: about 260 °C, with decomposition.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mepivacaine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and dry in an oven at 80 °C for 45 min. Record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *mepivacaine hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *mepivacaine hydrochloride CRS* and 20 mg of *lidocaine hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *1,1-dimethylethyl methyl ether R* (1:5:100 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):
- the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.500 g in *carbon dioxide-free water R* and dilute to 30.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0.

Dilute 2 mL of solution S to 5 mL with *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 20.0 mg of the substance to be examined and 30.0 mg of *mepivacaine impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 2.25 g/L solution of *phosphoric acid R*, previously adjusted to pH 7.6 with *strong sodium hydroxide solution R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of mepivacaine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to mepivacaine (retention time = about 7 min): impurity B = about 0.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to impurity B and mepivacaine.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity A**. Head-space gas chromatography (*2.2.28*).

*Test solution.* Introduce 60.0 mg of the substance to be examined into a 20 mL vial. Add 2.0 mL of a 103.0 g/L solution of *hydrochloric acid R* and 1.0 mL of a 126.0 g/L solution of *sodium hydroxide R*, and close the vial immediately.

*Reference solution.* Dissolve 6.0 mg of *bupivacaine impurity F CRS* (impurity A) in a 103.0 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid solution. Dilute 1.0 mL of this solution to 100.0 mL with a 103.0 g/L solution of *hydrochloric acid R*. Introduce 2.0 mL of this solution into a 20 mL vial, add 1.0 mL of a 126.0 g/L solution of *sodium hydroxide R*, and close the vial immediately.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.0 mL/min.

*Split ratio*: 1:1.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 90 °C;
– *equilibration time*: 25 min;
– *pressurisation time*: 2 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 130 → 230 |
| | 10 - 15 | 230 |
| Injection port | | 225 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Retention time*: impurity A = about 6 min.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 15.0 per cent determined on 6 injections.

*Calculation of content*:

– for impurity A, use the concentration of impurity A in the reference solution.

*Limit*:

– *impurity A*: maximum 20 ppm.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.28 mg of $C_{15}H_{23}ClN_2O$.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

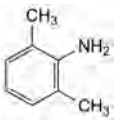

A. 2,6-dimethylaniline,

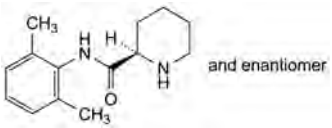


B. (2*RS*)-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide,

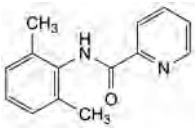

C. *N*-(2,6-dimethylphenyl)pyridine-2-carboxamide,

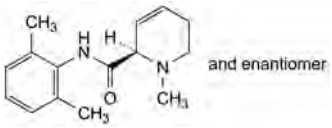


D. (2*RS*)-*N*-(2,6-dimethylphenyl)-1-methyl-1,2,5,6-tetrahydropyridine-2-carboxamide,

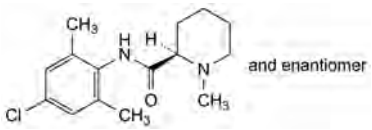


E. (2*RS*)-*N*-(4-chloro-2,6-dimethylphenyl)-1-methylpiperidine-2-carboxamide.

01/2017:0407

# MEPROBAMATE

## Meprobamatum

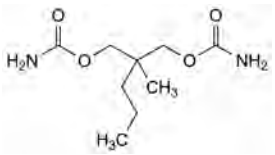

$C_9H_{18}N_2O_4$ $M_r$ 218.3
[57-53-4]

DEFINITION

Meprobamate contains not less than 97.0 per cent and not more than the equivalent of 101.0 per cent of 2-methyl-2-propylpropane-1,3-diyl dicarbamate, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, amorphous or crystalline powder, slightly soluble in water, freely soluble in alcohol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 104 °C to 108 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *meprobamate CRS*.

C. To 0.5 g add 1 mL of *acetic anhydride R* and 0.05 mL of *sulfuric acid R*, mix and allow to stand for 30 min, shaking frequently. Pour the solution dropwise into 50 mL of *water R*, mix and allow to stand. Initiate crystallisation by scratching the wall of the tube with a glass rod. Collect the precipitate by filtration, wash and dry at 60 °C. The precipitate melts (*2.2.14*) at 124 °C to 128 °C.

D. Dissolve 0.2 g in 15 mL of *0.5 M alcoholic potassium hydroxide* and boil under a reflux condenser for 15 min. Add 0.5 mL of *glacial acetic acid R* and 1 mL of a 50 g/L solution of *cobalt nitrate R* in *ethanol R*. A deep-blue colour develops.

TESTS

**Appearance of solution**. Dissolve 1.0 g in 20 mL of *ethanol R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution.* Dissolve 0.20 g of the substance to be examined in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dilute 0.1 mL of the test solution to 10 mL with *alcohol R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *pyridine R*, 30 volumes of *acetone R* and 70 volumes of *hexane R*. Dry the plate at 120 °C for 30 min, allow to cool and spray with a solution of 0.25 g of *vanillin R* in a cooled mixture of 10 mL of *alcohol R* and 40 mL of *sulfuric acid R* and heat at 100 °C to 105 °C for 30 min. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1000 g in 15 mL of a 25 per cent *V/V* solution of *sulfuric acid R* and boil under a reflux condenser for 3 h. Cool, dissolve by cautiously adding 30 mL of *water R*, cool again and place in a steam-distillation apparatus. Add 40 mL of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate into 40 mL of a 40 g/L solution of *boric acid R* until the total volume in the receiver reaches about 200 mL. Add 0.25 mL of *methyl red mixed solution R*. Titrate with *0.1 M hydrochloric acid* until the colour changes from green to violet. Carry out a blank titration.

1 mL of *0.1 M hydrochloric acid* is equivalent to 10.91 mg of $C_9H_{18}N_2O_4$.

01/2017:0278
corrected 10.0

# MEPYRAMINE MALEATE

## Mepyramini maleas

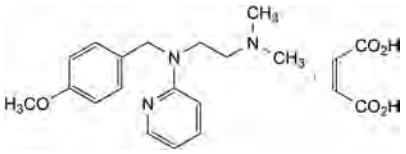

$C_{21}H_{27}N_3O_5$ $M_r$ 401.5
[59-33-6]

DEFINITION

*N*-(4-Methoxybenzyl)-*N*′,*N*′-dimethyl-*N*-(pyridin-2-yl)ethane-1,2-diamine (2*Z*)-but-2-enedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 99 °C to 103 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: 50 g/L solutions in *methylene chloride R* using a 0.1 mm cell.

*Comparison*: *mepyramine maleate CRS.*

C. Dissolve 0.100 g in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 1.0 mL of this solution to 100.0 mL with *0.01 M hydrochloric acid.* Examined between 220 nm and 350 nm (*2.2.25*), the solution shows 2 absorption maxima, at 239 nm and 316 nm. The specific absorbances at the absorption maxima are 431 to 477 and 196 to 220, respectively.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 40 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 40 mg of *mepyramine maleate CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R.*

*Mobile phase*: *diethylamine R*, *ethyl acetate R* (2:100 *V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. Triturate 0.1 g with 3 mL of *water R* and 1 mL of *strong sodium hydroxide solution R*. Shake with 3 quantities, each of 5 mL, of *ether R*. To 0.1 mL of the aqueous layer add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min; no colour develops. To the rest of the aqueous layer add 1 mL of *bromine water R*. Heat on a water-bath for 15 min and then heat to boiling and cool. To 0.2 mL of this solution add a solution of 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min; a violet-pink colour develops.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dilute 5 mL of solution S to 25 mL with *carbon dioxide-free water R*.

**pH** (*2.2.3*): 4.9 to 5.2.

Dilute 1.0 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *anisaldehyde R* (impurity B), 5.0 mg of *mepyramine impurity A CRS* and 5.0 mg of *mepyramine impurity C CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 0.1 volumes of *triethylamine R*, 40 volumes of a 0.771 g/L solution of *ammonium acetate R* and 60 volumes of *methanol R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of mepyramine.

*Relative retention* with reference to mepyramine (retention time = about 13 min): maleic acid = about 0.2; impurity C = about 0.3; impurity B = about 0.4; impurity A = about 0.5.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurities C and B.

*Limits*:

- *impurities A, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to maleic acid.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.25 per cent, determined on 1.000 g by drying in an oven at 80 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.150 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.07 mg of $C_{21}H_{27}N_3O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

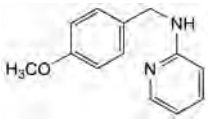


A. *N*-(4-methoxybenzyl)pyridin-2-amine,

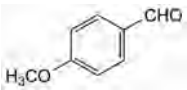


B. 4-methoxybenzaldehyde (anisaldehyde),

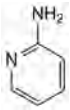


C. pyridin-2-amine.

**01/2008:0096**

# MERCAPTOPURINE

## Mercaptopurinum

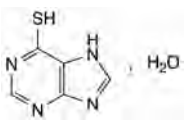


$C_5H_4N_4S,H_2O$ $M_r$ 170.2

[6112-76-1]

DEFINITION

7*H*-Purine-6-thiol monohydrate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

A. Dissolve 20 mg in 5 mL of *dimethyl sulfoxide R* and dilute to 100 mL with *0.1 M hydrochloric acid*. Dilute 5 mL of this solution to 200 mL with *0.1 M hydrochloric acid*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows only 1 absorption maximum, at 325 nm.

B. Dissolve about 20 mg in 20 mL of *ethanol (96 per cent) R* heated to 60 °C and add 1 mL of a saturated solution of *mercuric acetate R* in *ethanol (96 per cent) R*. A white precipitate is formed.

C. Dissolve about 20 mg in 20 mL of *ethanol (96 per cent) R* heated to 60 °C and add 1 mL of a 10 g/L solution of *lead acetate R* in *ethanol (96 per cent) R*. A yellow precipitate is formed.

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 1 mL of *dimethyl sulfoxide R* and dilute to 10 mL with *methanol R*.

*Reference solution*. Dissolve 10 mg of *hypoxanthine R* in 10 mL of *dimethyl sulfoxide R* and dilute to 100 mL with *methanol R*.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *acetone R* (3:7:90 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Limit*:

– *impurity A*: any spot corresponding to hypoxanthine in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (2.0 per cent).

**Water** (*2.5.12*): 10.0 per cent to 12.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 15.22 mg of $C_5H_4N_4S$.

STORAGE

Protected from light.

IMPURITIES

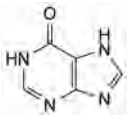

A. 1,7-dihydro-6*H*-purin-6-one (hypoxanthine).

**01/2008:0120**
**corrected 6.0**

# MERCURIC CHLORIDE

## Hydrargyri dichloridum

$HgCl_2$ $M_r$ 271.5
[7487-94-7]

DEFINITION

*Content*: 99.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless or white or almost white crystals or heavy crystalline masses.

*Solubility*: soluble in water and in glycerol, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives the reactions of chlorides (*2.3.1*).

B. Solution S (see Tests) gives the reactions of mercury (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R*. The solution is red. Add 0.5 g of *sodium chloride R*. The solution becomes yellow. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour to red.

**Mercurous chloride**. Dissolve 1.0 g in 30 mL of *ether R*. The solution shows no opalescence.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 2.00 g by drying *in vacuo* for 24 h.

ASSAY

Dissolve 0.500 g in 100 mL of *water R*. Add 20.0 mL of *0.1 M sodium edetate* and 5 mL of *buffer solution pH 10.9 R*. Allow to stand for 15 min. Add 0.1 g of *mordant black 11 triturate R* and titrate with *0.1 M zinc sulfate* until the colour changes to purple. Add 3 g of *potassium iodide R*, allow to stand for 2 min, add a further 0.1 g of *mordant black 11 triturate R* and titrate with *0.1 M zinc sulfate*.

1 mL of *0.1 M zinc sulfate* used in the second titration is equivalent to 27.15 mg of $HgCl_2$.

STORAGE

Protected from light.

**01/2017:2234**
**corrected 10.0**

# MEROPENEM TRIHYDRATE

## Meropenemum trihydricum

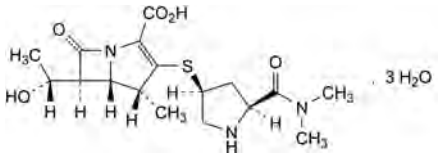

$C_{17}H_{25}N_3O_5S,3H_2O$ $M_r$ 437.5
[119478-56-7]

DEFINITION

(4*R*,5*S*,6*S*)-3-[[(3*S*,5*S*)-5-[(Dimethylamino)carbonyl]-pyrrolidin-3-yl]sulfanyl]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid trihydrate.

Semi-synthetic product derived from a fermentation product, or synthetic product.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or light yellow, crystalline powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *meropenem trihydrate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in 20 mL of a 50 g/L solution of *sodium hydrogen carbonate R*.

**pH** (*2.2.3*): 4.0 to 6.0.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 21 to − 17 (anhydrous substance).

Dissolve 0.125 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare test solutions (a) and (b) and reference solution (c) immediately before use. Prepare and store reference solution (a) at 4 °C and use within 6 h.*

*Solvent mixture*. To 1.0 mL of *triethylamine R* add 900 mL of *water for chromatography R*. Adjust to pH 5.0 with *dilute phosphoric acid R* and dilute to 1000.0 mL with *water for chromatography R*.

*Test solution (a)*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. In order to prepare impurities A and B *in-situ*, heat 10 mL of test solution (a) to 60 °C for about 20 min or, alternatively, allow 10 mL of test solution (a) to stand at room temperature for about 8 h.

*Reference solution (c)*. Dissolve 50.0 mg of *meropenem trihydrate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R1*, solvent mixture (7:100 *V/V*).

*Flow rate*: 1.6 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of meropenem.

*Relative retention* with reference to meropenem (retention time = about 7 min): impurity A = about 0.5; impurity B = about 2.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and meropenem.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.6;
- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than A and B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

*For meropenem trihydrate produced by a fully synthetic process*:

- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Water** (*2.5.12*): 11.4 per cent to 13.4 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.125 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{17}H_{25}N_3O_5S$ taking into account the assigned content of *meropenem trihydrate CRS*.

STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B.*

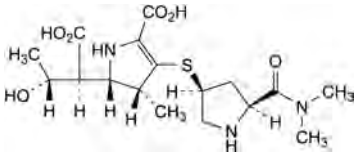

A. (4*R*,5*S*)-5-[(1*S*,2*R*)-1-carboxy-2-hydroxypropyl]-3-[[(3*S*,5*S*)-5-[(dimethylamino)carbonyl]pyrrolidin-3-yl]sulfanyl]-4-methyl-4,5-dihydro-1*H*-pyrrole-2-carboxylic acid,

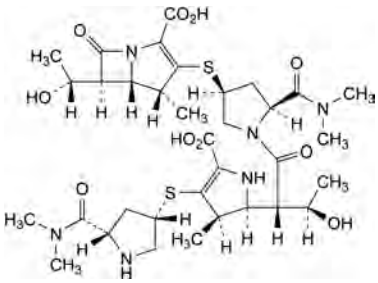

B. (4*R*,5*S*,6*S*)-3-[[(3*S*,5*S*)-1-[(2*S*,3*R*)-2-[(2*S*,3*R*)-5-carboxy-4-[[(3*S*,5*S*)-5-[(dimethylamino)-carbonyl]pyrrolidin-3-yl]sulfanyl]-3-methyl-2,3-dihydro-1*H*-pyrrol-2-yl]-3-hydroxybutanoyl]-5-[(dimethylamino)carbonyl]pyrrolidin-3-yl]sulfanyl]-6-[(1*R*)-1-hydroxyethyl]-4-methyl-7-oxo-1-azabicyclo-[3.2.0]hept-2-ene-2-carboxylic acid.

**07/2019:1699**

# MESALAZINE

## Mesalazinum

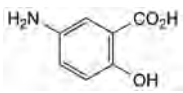

$C_7H_7NO_3$ $M_r$ 153.1

[89-57-6]

DEFINITION

5-Amino-2-hydroxybenzoic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or light grey or light pink powder or crystals.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides and in dilute hydrochloric acid.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in 10 mL of a 10.3 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of this solution to 200.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 210-250 nm.

*Absorption maximum*: at about 230 nm.

*Specific absorbance at the absorption maximum*: 430 to 450.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mesalazine CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 10.0 mL with *methanol R*.

*Reference solution.* Dissolve 25 mg of *mesalazine CRS* in 5 mL of a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 10.0 mL with *methanol R*.

*Plate*: a suitable silica gel as the coating substance.

*Mobile phase*: *glacial acetic acid R*, *methanol R*, *methyl isobutyl ketone R* (10:40:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. *Maintain the solutions at 40 °C during preparation and measurements.* Dissolve 0.5 g in a 103.0 g/L solution of *hydrochloric acid R* and dilute to 20 mL with the same solution. The solution is clear (*2.2.1*). Immediately measure the absorbance (*2.2.25*) of the solution at 440 nm and 650 nm. The absorbance is not greater than 0.15 at 440 nm and 0.10 at 650 nm.

**Reducing substances**. Dissolve 0.10 g in *dilute hydrochloric acid R* and dilute to 25 mL with the same solvent. Add 0.2 mL of *starch solution R* and 0.25 mL of *0.01 M iodine*. Allow to stand for 2 min. The solution is blue or violet-brown.

**Impurities A and C**. Liquid chromatography (*2.2.29*). *Prepare the solutions and mobile phases immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5.0 mg of *mesalazine impurity C CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 25.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 5.0 mg of *mesalazine impurity A CRS* in mobile phase A and dilute to 250.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A.

*Reference solution (d).* Dilute 1 mL of the test solution to 200 mL with mobile phase A. To 5 mL of this solution add 5 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *phosphoric acid R* and 2.2 g of *perchloric acid R* in *water for chromatography R* and dilute to 1000 mL with the same solvent;

– *mobile phase B*: dissolve 1.0 g of *phosphoric acid R* and 1.7 g of *perchloric acid R* in *acetonitrile R1* and dilute to 1000 mL with the same solvent;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 100 | 0 |
| 8 - 25 | 100 → 40 | 0 → 60 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to mesalazine (retention time = about 9 min): impurity A = about 0.5; impurity C = about 0.9.

*System suitability*: reference solution (d):

– *resolution*: minimum 3.0 between the peaks due to impurity C and mesalazine.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (200 ppm);

– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (200 ppm).

**Impurity K**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution.* Dissolve 27.8 mg of *aniline hydrochloride R* (equivalent to 20.0 mg of impurity K) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 0.20 mL of the solution to 20.0 mL with the mobile phase. Dilute 0.20 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 15 volumes of *methanol R2* and 85 volumes of a solution containing 0.47 g/L of *disodium hydrogen phosphate dihydrate R* and 1.41 g/L of *potassium dihydrogen phosphate R* previously adjusted to pH 8.0 with a 42 g/L solution of *sodium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 50 µL.

*Run time*: 1.5 times the retention time of impurity K.

*Retention time*: impurity K = about 14 min.

*System suitability*: reference solution:

– *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limit*:

– *impurity K*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (10 ppm).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*: 1.03 g/L solution of *hydrochloric acid R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in solution A using sonication and dilute to 10.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 5 mg of *mesalazine for system suitability CRS* (containing impurities F, J and P) in solution A and dilute to 5 mL with solution A.

*Reference solution (c).* Dissolve 5 mg of *4-aminosalicylic acid R* (impurity E), 5 mg of *2,5-dihydroxybenzoic acid R* (impurity G), 15 mg of *salicylic acid R* (impurity H), 5 mg of *2-chlorobenzoic acid R* (impurity L), 5 mg of *2-chloro-5-nitrobenzoic acid R* (impurity M), 10 mg of *sulfanilic acid R* (impurity O) and 5 mg of *3-nitrosalicylic acid R* (impurity R) in solution A and dilute to 100 mL with solution A. Dilute 1 mL of the solution to 50 mL with solution A.

*Reference solution (d).* Dissolve 3.0 mg of *2-chlorobenzoic acid R* (impurity L) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 100.0 mL with solution A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 6.9 g of *sodium dihydrogen phosphate monohydrate R* in about 950 mL of *water for chromatography R*, adjust to pH 6.2 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*;

– *mobile phase B*: *acetonitrile for chromatography R*, mobile phase A (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 100 | 0 |
| 8 - 20 | 100 → 85 | 0 → 15 |
| 20 - 40 | 85 → 25 | 15 → 75 |
| 40 - 60 | 25 → 0 | 75 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *mesalazine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities F, J and P; use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E, G, H, L, M, O and R.

*Relative retention* with reference to mesalazine (retention time = about 6 min): impurity O = about 0.5; impurity J = about 0.6; impurity E = about 0.8; impurity F = about 1.36; impurity G = about 1.44; impurity P = about 1.5; impurity L = about 2.0; impurity M = about 3.3; impurity H = about 3.5; impurity R = about 5.1.

*System suitability*:

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to mesalazine in the chromatogram obtained with reference solution (b);

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity L in the chromatogram obtained with reference solution (d).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 1.3; impurity G = 1.4; impurity H = 1.4; impurity J = 2.0; impurity L = 4.5; impurity M = 1.7; impurity O = 0.6; impurity P = 0.6; impurity R = 1.3;
- *impurity H*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities F, J, O, P*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurities E, G, L, M, R*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Chlorides**: maximum 0.1 per cent.

Dissolve 1.50 g in 50 mL of *anhydrous formic acid R*. Add 100 mL of *water R* and 5 mL of *dilute nitric acid R*. Titrate with *0.005 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.005 M silver nitrate* is equivalent to 0.1773 mg of Cl.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Shake 1.0 g with 20 mL of *distilled water R* for 1 min and filter. 15 mL of the filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in 100 mL of boiling *water R*. Cool rapidly to room temperature and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.31 mg of $C_7H_7NO_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, C, E, F, G, H, J, K, L, M, O, P, R.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, I, N, Q, S.*

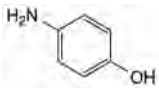

A. 4-aminophenol,

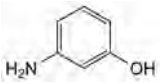

B. 3-aminophenol,

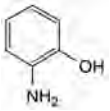

C. 2-aminophenol,

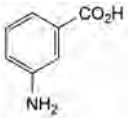

D. 3-aminobenzoic acid,

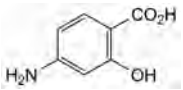

E. 4-amino-2-hydroxybenzoic acid (4-aminosalicylic acid),

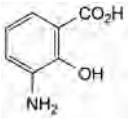

F. 3-amino-2-hydroxybenzoic acid (3-aminosalicylic acid),

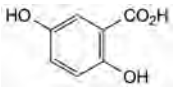

G. 2,5-dihydroxybenzoic acid,

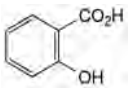

H. 2-hydroxybenzoic acid (salicylic acid),

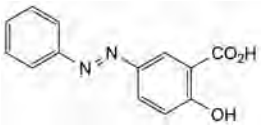

I. 2-hydroxy-5-(phenyldiazenyl)benzoic acid (phenylazosalicylic acid),

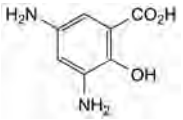

J. 3,5-diamino-2-hydroxybenzoic acid (3,5-diaminosalicylic acid),

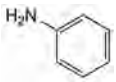

K. aniline,

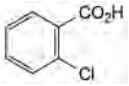

L. 2-chlorobenzoic acid,

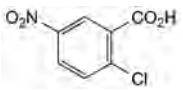

M. 2-chloro-5-nitrobenzoic acid,

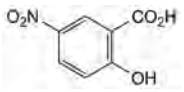

N. 2-hydroxy-5-nitrobenzoic acid (5-nitrosalicylic acid),

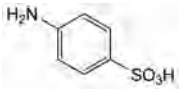

O. 4-aminobenzenesulfonic acid (sulfanilic acid),

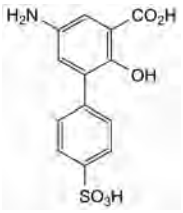

P. 5-amino-2-hydroxy-3-(4-sulfophenyl)benzoic acid (3-(4-sulfophenyl)-5-aminosalicylic acid),

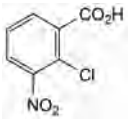

Q. 2-chloro-3-nitrobenzoic acid,

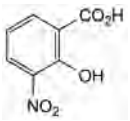

R. 2-hydroxy-3-nitrobenzoic acid (3-nitrosalicylic acid),

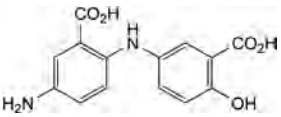

S. 2-hydroxy-5-[(2-carboxy-4-aminophenyl)amino]benzoic acid.

**01/2017:1674**
**corrected 9.2**

# MESNA

## Mesnum

$C_2H_5NaO_3S_2$ $M_r$ 164.2
[19767-45-4]

DEFINITION

Sodium 2-sulfanylethanesulfonate.

*Content*: 96.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of mesna.*

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 4.0 mg of *mesna impurity C CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 6.0 mg of *mesna impurity D CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 3.0 mL of the test solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 100.0 mL with the mobile phase.

*Reference solution (e).* Dilute 6.0 mL of reference solution (c) to 20.0 mL with the mobile phase. To 10 mL of the solution add 10 mL of reference solution (a).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: dissolve 2.94 g of *potassium dihydrogen phosphate R*, 2.94 g of *dipotassium hydrogen phosphate R* and 2.6 g of *tetrabutylammonium hydrogen sulfate R* in about 600 mL of *water R*. Adjust to pH 2.3 with *phosphoric acid R*, add 335 mL of *methanol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of mesna.

*Relative retention* with reference to mesna (retention time = about 4.8 min): impurities A and B = about 0.6; impurity E = about 0.8; impurity C = about 1.4; impurity D = about 2.3.

*System suitability*: reference solution (e):
- *resolution*: minimum 3.0 between the peaks due to mesna and impurity C.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of impurities A, B and E by 0.01,
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- *impurities A, B, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent),
- *any other impurity*: for each impurity, not more than one third of the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent),
- *sum of other impurities*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.3 per cent),

– *disregard limit*: 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.045 per cent).

**Chlorides** (*2.4.4*): maximum 250 ppm.

Dilute 1 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 30 mL with *distilled water R*. 15 mL of the solution complies with the test.

**Disodium edetate**: maximum 500 ppm.

Dissolve 4.000 g in 90 mL of *water R* and adjust to pH 4.5 using *0.1 M hydrochloric acid*. Add 10 mL of *acetate buffer solution pH 4.5 R* and 50 mL of *2-propanol R*. Add 2 mL of a 0.25 g/L solution of *dithizone R* in *2-propanol R*. Titrate with *0.01 M zinc sulfate* until the colour changes from bluish-grey to pink.

1 mL of *0.01 M zinc sulfate* is equivalent to 3.72 mg of $C_{10}H_{14}N_2Na_2O_8,2H_2O$.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g under high vacuum at 60 °C for 2 h.

ASSAY

Dissolve 0.120 g in 10 mL of *water R*. Add 10 mL of *dilute sulfuric acid R* and 10.0 mL of *0.1 M iodine*. Titrate with *0.1 M sodium thiosulfate* adding 1 mL of *starch solution R* near the endpoint. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 16.42 mg of $C_2H_5NaO_3S_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

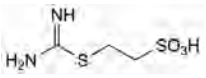

A. 2-(carbamimidoylsulfanyl)ethanesulfonic acid,

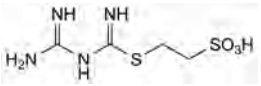

B. 2-[[(guanidino)(imino)methyl]sulfanyl]ethanesulfonic acid,

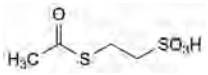

C. 2-(acetylsulfanyl)ethanesulfonic acid,

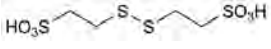

D. 2,2′-(disulfanediyl)bis(ethanesulfonic acid),

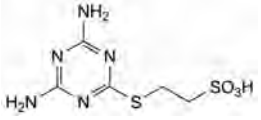

E. 2-(4,6-diamino-1,3,5-triazin-2-yl)sulfanylethanesulfonic acid.

**01/2020:1730**

# MESTEROLONE

## Mesterolonum

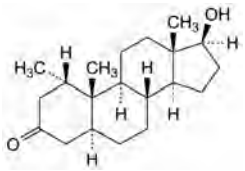

$C_{20}H_{32}O_2$ $M_r$ 304.5

[1424-00-6]

DEFINITION

17β-Hydroxy-1α-methyl-5α-androstan-3-one.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone, in ethyl acetate and in methanol.

IDENTIFICATION

A. Melting point (*2.2.14*): 206 °C to 211 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mesterolone CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 20 to + 24 (dried substance).

Dissolve 0.200 g in *methylene chloride R* and dilute to 10.0 mL with the same solvent.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (50:50 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 200.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *mesterolone impurity A CRS* in reference solution (a) and dilute to 100 mL with the same solution.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *acetone R*, *toluene R* (2:15:85 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 366 nm; spray with a 200 g/L solution of *toluenesulfonic acid R* in *ethanol (96 per cent) R* and heat at 120 °C for 10 min.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots (blue spot due to mesterolone and yellow spot due to impurity A).

*Limit*:

– *impurity B*: any blue spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R* (20:80 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 50.0 mg of *mesterolone CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *mesterolone for system suitability CRS* (containing impurity C) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *acetonitrile R1*, *water for chromatography R*, *methanol R2* (20:40:60 *V/V/V*).

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of mesterolone.

*Identification of impurities*: use the chromatogram supplied with *mesterolone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to mesterolone (retention time = about 22 min): impurity C = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity C and mesterolone.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity C by 0.2;
- for each impurity, use the concentration of mesterolone in reference solution (c).

*Limits*:

- *impurity C*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of $C_{20}H_{32}O_2$ taking into account the assigned content of *mesterolone CRS*.

IMPURITIES

*Specified impurities*: *B*, *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

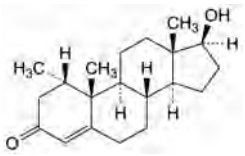

A. 17β-hydroxy-1α-methylandrost-4-en-3-one,

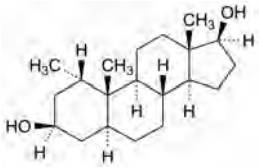

B. 1α-methyl-5α-androstane-3β,17β-diol,

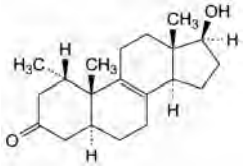

C. 17β-hydroxy-1α-methyl-5α-androst-8-en-3-one.

**01/2008:0509**
**corrected 6.0**

# MESTRANOL

## Mestranolum

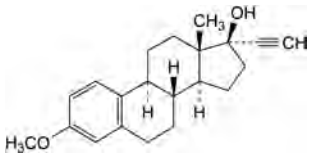

$C_{21}H_{26}O_2$ $M_r$ 310.4
[72-33-3]

DEFINITION

Mestranol contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3-methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in alcohol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 150 °C to 154 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *mestranol CRS*.

C. Examine the chromatograms obtained in the test for related substances in daylight and in ultraviolet light at 365 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour, fluorescence and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in 1 mL of *sulfuric acid R*. A red colour develops with a greenish-yellow fluorescence in ultraviolet light at 365 nm. Add the solution to 10 mL of *water R* and mix. The solution becomes pink and a pink to violet precipitate is formed on standing.

TESTS

**Specific optical rotation** (*2.2.7*). Dissolve 0.100 g in *anhydrous pyridine R* and dilute to 10.0 mL with the same solvent. The specific optical rotation is – 20 to – 24, calculated with reference to the dried substance.

**Absorbance** (*2.2.25*). Dissolve 25.0 mg in *alcohol R* and dilute to 25.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *alcohol R*. Examined between 260 nm and 310 nm, the solution shows two absorption maxima, at 279 nm and 288 nm, and a minimum at 286 nm. The specific absorbances at the maxima are 62 to 68 and 59 to 64, respectively.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *chloroform R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *chloroform R*.

*Reference solution (a).* Dissolve 10 mg of *mestranol CRS* in *chloroform R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 10 mL with *chloroform R*.

*Reference solution (c).* Dilute 5 mL of reference solution (b) to 10 mL with *chloroform R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *alcohol R* and 90 volumes of *toluene R*. Allow the plate to dry in air until the solvent has evaporated. Heat at 110 °C for 10 min. Spray the hot plate with *alcoholic solution of sulfuric acid R*. Heat again at 110 °C for 10 min. Examine in daylight and in ultraviolet light at 365 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.200 g in 40 mL of *tetrahydrofuran R* and add 5 mL of a 100 g/L solution of *silver nitrate R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.04 mg of $C_{21}H_{26}O_2$.

STORAGE

Store protected from light.

**01/2008:2077**
**corrected 10.0**

# METACRESOL

## Metacresolum

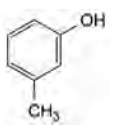


$C_7H_8O$ $M_r$ 108.1
[108-39-4]

DEFINITION

3-Methylphenol.

CHARACTERS

*Appearance*: colourless or yellowish liquid.

*Solubility*: sparingly soluble in water, miscible with ethanol (96 per cent) and with methylene chloride.

*Relative density*: about 1.03.

mp: about 11 °C.

bp: about 202 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of metacresol.*

TESTS

**Solution S**. Dissolve 1.5 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Freshly prepared solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Acidity**. To 25 mL of solution S add 0.15 mL of *methyl red solution R*. The solution is red. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 1.00 g of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.10 g of *cresol R*, 0.10 g of *p-cresol R* and 0.10 g of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 20.0 mL with *methanol R*.

*Column*:

- *material*: fused silica,
- *size*: *l* = 25 m, Ø = 0.25 mm,
- *stationary phase*: *cyanopropyl(25)phenyl(25)methyl(50)polysiloxane R* (0.2 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.8 mL/min.

*Split ratio*: 1:30.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 35 | 100 |
| | 35 - 40 | 100 → 150 |
| | 40 - 50 | 150 |
| Injection port | | 200 |
| Detector | | 200 |

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Relative retention* with reference to metacresol (retention time = about 28 min): impurity B = about 0.75; impurity C = about 0.98.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.4 between the peaks due to impurity C and metacresol.

*Limits*:

- *impurities B, C*: for each impurity, not more than 0.5 per cent,

– *any other impurity*: for each impurity, not more than 0.1 per cent,
– *total*: not more than 1.0 per cent.
– *disregard limit*: the area of the peak due to metacresol in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Residue on evaporation**: maximum 0.1 per cent.

Evaporate 2.0 g to dryness on a water-bath in a fume cupboard and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2 mg.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F, G, H, I, J, K, L, M.*

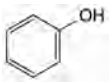

A. phenol,

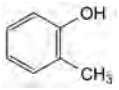

B. 2-methylphenol (*o*-cresol, cresol),

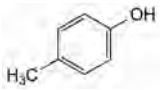

C. 4-methylphenol (*p*-cresol),

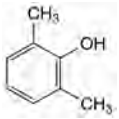

D. 2,6-dimethylphenol (2,6-xylenol),

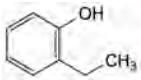

E. 2-ethylphenol (*o*-ethylphenol),

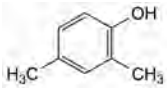

F. 2,4-dimethylphenol (2,4-xylenol),

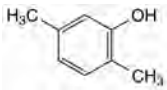

G. 2,5-dimethylphenol (2,5-xylenol),

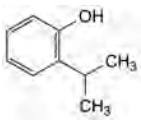

H. 2-(1-methylethyl)phenol,

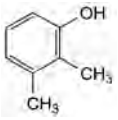

I. 2,3-dimethylphenol (2,3-xylenol),

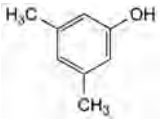

J. 3,5-dimethylphenol (3,5-xylenol),

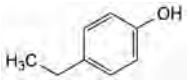

K. 4-ethylphenol (*p*-ethylphenol),

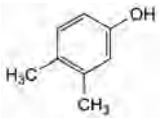

L. 3,4-dimethylphenol (3,4-xylenol),

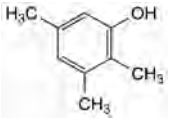

M. 2,3,5-trimethylphenol.

**01/2017:1346**

# METAMIZOLE SODIUM MONOHYDRATE

## Metamizolum natricum monohydricum

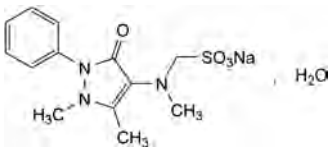

$C_{13}H_{16}N_3NaO_4S,H_2O$ $M_r$ 351.4
[5907-38-0]

DEFINITION

Sodium [(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)(methyl)amino]methanesulfonate monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *metamizole sodium CRS.*

B. Dissolve 50 mg in 1 mL of *strong hydrogen peroxide solution R*. A blue colour is produced which fades rapidly and turns to intense red in a few minutes.

C. Place 0.10 g in a test tube, add some glass beads and dissolve the substance in 1.5 mL of *water R*. Add 1.5 mL of *dilute hydrochloric acid R* and place a filter paper wetted with a solution of 20 mg of *potassium iodate R* in 2 mL of *starch solution R* at the open end of the test tube. Heat gently, the evolving vapour of sulfur dioxide colours the filter paper blue. After heating gently for 1 min, take a glass rod with a drop of a 10 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* and place in the opening of the tube. Within 10 min, a blue-violet colour develops in the drop of the reagent.

D. 0.5 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 40 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and, immediately after preparation, not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method I*).

**Acidity or alkalinity**. To 5 mL of solution S, add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *metamizole impurity A CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase. Use 1.0 mL of this solution to dissolve the contents of a vial of *metamizole impurity E CRS*.

*Reference solution (c).* In order to prepare impurity C *in situ*, dissolve 40 mg of the substance to be examined in *methanol R*, dilute to 20 mL with the same solvent and boil under reflux for 10 min. Allow to cool to room temperature and dilute to 20 mL with *methanol R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm).

*Mobile phase*: mix 28 volumes of *methanol R* and 72 volumes of a buffer solution prepared as follows: mix 1000 volumes of a 6.0 g/L solution of *sodium dihydrogen phosphate R* and 1 volume of *triethylamine R*, then adjust to pH 7.0 with *strong sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 4.5 times the retention time of metamizole.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Relative retention* with reference to metamizole (retention time = about 2 min): impurity A = about 0.7; impurity E = about 0.8; impurity C = about 2.5.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity E.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity E by 1.5;
- *impurity C*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *impurity E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.03 per cent).

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dissolve 0.150 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Loss on drying** (*2.2.32*): 4.9 per cent to 5.3 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.200 g in 10 mL of *0.01 M hydrochloric acid* previously cooled in iced water and titrate immediately, dropwise, with *0.05 M iodine*. Before each addition of *0.05 M iodine* dissolve the precipitate by swirling. At the end of the titration, add 2 mL of *starch solution R* and titrate until the blue colour of the solution persists for at least 2 min. The temperature of the solution during the titration must not exceed 10 °C.

1 mL of *0.05 M iodine* is equivalent to 16.67 mg of $C_{13}H_{16}N_3NaO_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D.*

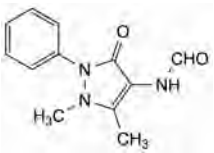


A. 4-(formylamino)-1,5-dimethyl-2-phenyl-2,3-dihydro-1*H*-pyrazol-3-one,

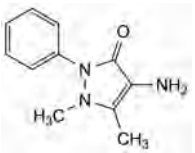


B. 4-amino-1,5-dimethyl-2-phenyl-2,3-dihydro-1*H*-pyrazol-3-one,

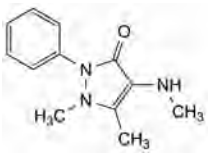

C. 1,5-dimethyl-4-(methylamino)-2-phenyl-2,3-dihydro-1*H*-pyrazol-3-one,

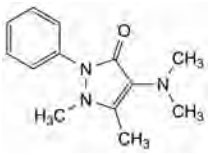

D. 1,5-dimethyl-4-(dimethylamino)-2-phenyl-2,3-dihydro-1*H*-pyrazol-3-one,

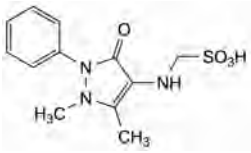

E. [(1,5-dimethyl-3-oxo-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)amino]methanesulfonic acid (4-*N*-desmethylmetamizole).

**01/2017:0931**

# METFORMIN HYDROCHLORIDE

## Metformini hydrochloridum

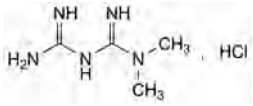

$C_4H_{12}ClN_5$ $M_r$ 165.6
[1115-70-4]

DEFINITION

1,1-Dimethylbiguanide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone and in methylene chloride.

IDENTIFICATION

*First identification*: B, E.

*Second identification*: A, C, D, E.

A. Melting point (*2.2.14*): 222 °C to 226 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *metformin hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.
*Reference solution*. Dissolve 20 mg of *metformin hydrochloride CRS* in *water R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel G plate R*.
*Mobile phase*: *glacial acetic acid R*, *butanol R*, *water R* (10:40:50 *V/V/V*); use the upper layer.
*Application*: 5 µL.
*Development*: over 3/4 of the plate.
*Drying*: at 100-105 °C for 15 min.
*Detection*: spray with a mixture of equal volumes of a 100 g/L solution of *sodium nitroprusside R*, a 100 g/L solution of *potassium ferricyanide R* and a 100 g/L solution of *sodium hydroxide R*, prepared 20 min before use.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in *water R* and dilute to 100 mL with the same solvent. To 2 mL of the solution add 0.25 mL of *strong sodium hydroxide solution R* and 0.10 mL of *α-naphthol solution R*. Mix and allow to stand in iced water for 15 min. Add 0.5 mL of *sodium hypobromite solution R* and mix. A pink colour develops.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*). Heat the solution to 50 °C and cool to room temperature.

**Impurity F**. Liquid chromatography (*2.2.29*).

*Derivatisation solution. Prepare the solution immediately before use.* Dissolve 1 mL of *fluorodinitrobenzene R* in 100.0 mL of *acetonitrile for chromatography R*.

*Blank solution.* To 5.0 mL of *acetonitrile for chromatography R* add 100 µL of *triethylamine R1* and 1.0 mL of the derivatisation solution. Shake well and heat at 60 °C for 30 min. After cooling, dilute to 10.0 mL with *acetonitrile for chromatography R*.

*Test solution. Prepare the solution immediately before use.* Suspend 10.0 mg of the substance to be examined in 5.0 mL of *acetonitrile for chromatography R* and sonicate for 5 min. Add 100 µL of *triethylamine R1* and 1.0 mL of the derivatisation solution. Shake well and heat at 60 °C for 30 min. After cooling, dilute to 10.0 mL with *acetonitrile for chromatography R*. Filter or centrifuge at 800 *g* for 5 min before use.

*Reference solution.* Dissolve 1.0 mL of *metformin impurity F CRS* in 100.0 mL of *acetonitrile for chromatography R*. Dilute 2.5 mL of the solution to 100.0 mL with *acetonitrile for chromatography R*. To 1.0 mL of this solution add successively 5.0 mL of *acetonitrile for chromatography R*, 100 µL of *triethylamine R1* and 1.0 mL of the derivatisation solution. Shake well and heat at 60 °C for 30 min. After cooling, dilute to 10.0 mL with *acetonitrile for chromatography R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 3 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *water R* (0.1:99.9 *V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 60 → 45 | 40 → 55 |
| 10 - 11 | 45 → 25 | 55 → 75 |
| 11 - 15 | 25 | 75 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 380 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatograms obtained with the blank solution and the reference solution to identify the peak due to the impurity F derivative.

*Retention time*: impurity F derivative = about 4 min.

*System suitability*: reference solution:

– *resolution*: minimum 3.0 between the peak due to the impurity F derivative and the nearby eluting peaks due to the derivatisation reagent.

*Limit*:

– *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 20.0 mg of *metformin impurity A CRS* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 200.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 10 mg of *melamine R* (impurity D) in about 90 mL of *water R*. Add 5 mL of the test solution and dilute to 100 mL with *water R*. Dilute 1 mL of this solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *strong cation-exchange silica gel for chromatography R* (10 µm).

*Mobile phase*: 17 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 218 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of metformin.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to metformin (retention time = about 10 min): impurity A = about 0.2; impurity D = about 0.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 10 between the peaks due to impurity D and metformin.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.02 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);

– *total*: maximum 0.2 per cent;

– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent); do not disregard the peak due to impurity A.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 4 mL of *anhydrous formic acid R*. Add 80 mL of *acetonitrile R*. Carry out the titration immediately. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.56 mg of $C_4H_{12}ClN_5$.

IMPURITIES

*Specified impurities: A, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

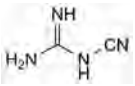

A. cyanoguanidine,

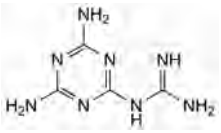

B. (4,6-diamino-1,3,5-triazin-2-yl)guanidine,

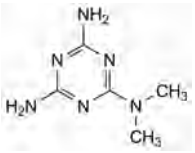

C. $N^2$,$N^2$-dimethyl-1,3,5-triazine-2,4,6-triamine (*N*,*N*-dimethylmelamine),

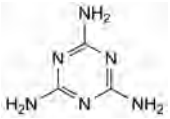

D. 1,3,5-triazine-2,4,6-triamine (melamine),

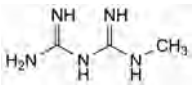

E. 1-methylbiguanide,

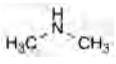

F. *N*-methylmethanamine (dimethylamine).

**01/2019:1128**

# METHACRYLIC ACID - ETHYL ACRYLATE COPOLYMER (1:1)

## Acidi methacrylici et ethylis acrylatis polymerisatum 1:1

DEFINITION

Copolymer of methacrylic acid and ethyl acrylate having a mean relative molecular mass of about 250 000. The ratio of carboxylic groups to ester groups is about 1:1. The substance is

in the acid form (type A) or partially neutralised using sodium hydroxide (type B). It may contain suitable surface-active agents such as sodium dodecyl sulfate and polysorbate 80.

*Content*:

– *type A*: 46.0 per cent to 50.6 per cent of methacrylic acid units (dried substance);

– *type B*: 43.0 per cent to 48.0 per cent of methacrylic acid units (dried substance).

CHARACTERS

*Appearance*: white or almost white, free-flowing powder.

*Solubility*: practically insoluble in water (type A) or dispersible in water (type B), freely soluble in anhydrous ethanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/L solution of sodium hydroxide.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.1 g of the substance to be examined in 1 mL of *ethanol (90 per cent V/V) R* and place 2 drops of the solution on a sodium chloride plate; dry to allow the formation of a film and cover with another sodium chloride plate.

*Comparison*: *methacrylic acid - ethyl acrylate copolymer (1:1) - type A CRS* or *methacrylic acid - ethyl acrylate copolymer (1:1) - type B CRS*.

B. It complies with the limits of the assay.

C. Sulfated ash (see Tests).

TESTS

**Viscosity** (*2.2.10*).

– *Type A*: 100 mPa·s to 200 mPa·s.

Dissolve a quantity of the substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity at 20 °C using a rotating viscometer at a shear rate of 10 $s^{-1}$.

– *Type B*: maximum 100 mPa·s.

Disperse a quantity of the substance to be examined corresponding to 80.0 g of the dried substance in *water R* and make up to 400 g with the same solvent. Stir for 3 h and determine the viscosity at 23 °C using a rotating viscometer and a spindle rotating at 100 r/min.

*Dimensions of the spindle*: diameter = 47.0 mm; height = 27.0 mm; shaft diameter = 3.18 mm.

**Appearance of a film**. Spread 1 mL of the solution (type A) or dispersion (type B) prepared for the test for viscosity on a glass plate and allow to dry. A clear, brittle film is formed.

**Ethyl acrylate and methacrylic acid**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 3.5 g of *sodium perchlorate R* in *water R* and dilute to 100.0 mL with the same solvent.

*Blank solution*: *methanol R*, solution A (50:50 *V/V*).

*Test solution*. Dissolve 3.0 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent. To 5.0 mL of solution A add 5.0 mL of the solution dropwise whilst stirring continuously. Centrifuge until a clear supernatant is obtained. Use the clear supernatant as the test solution.

*Reference solution*. Dissolve 50.0 mg of *ethyl acrylate R* and 50.0 mg of *methacrylic acid R* in 5 mL of *butanol R* and dilute to 100.0 mL with *methanol R*. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R*. Mix 5.0 mL of this solution and 5.0 mL of solution A.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R2* and 80 volumes of *water for chromatography R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 20 µL.

*Retention time*: methacrylic acid = about 3 min; ethyl acrylate = about 9 min.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to methacrylic acid and ethyl acrylate in the chromatogram obtained with the reference solution;

– the chromatogram obtained with the blank solution does not show peaks with the same retention times as ethyl acrylate or methacrylic acid.

*Calculation of percentage contents*:

– for ethyl acrylate and methacrylic acid, use the respective concentration of these substances in the reference solution.

*Limit*:

– *sum of the contents of ethyl acrylate and methacrylic acid*: maximum 0.01 per cent;

– *reporting threshold*: 1 ppm.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent (type A) or 0.5 per cent to 3.0 per cent (type B), determined on 1.0 g.

ASSAY

Dissolve 1.000 g in a mixture of 40 mL of *water R* and 60 mL of *2-propanol R*. Titrate slowly while stirring with *0.5 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *0.5 M sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

LABELLING

The label states the type (type A or type B).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for methacrylic acid - ethyl acrylate copolymer (1:1) used as gastro-resistant coating agent.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take a piece of the film obtained in the test for appearance of a film (see Tests), place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* and stir; it does not dissolve within 2 h. Take another piece of the film, place it in a flask containing *phosphate buffer solution pH 6.0 R* and stir; it dissolves within 1 h.

07/2016:1129

# METHACRYLIC ACID - ETHYL ACRYLATE COPOLYMER (1:1) DISPERSION 30 PER CENT

## Acidi methacrylici et ethylis acrylatis polymerisati 1:1 dispersio 30 per centum

DEFINITION

Dispersion in water of a copolymer of methacrylic acid and ethyl acrylate having a mean relative molecular mass of about 250 000. The ratio of carboxylic groups to ester groups is about 1:1.

*Content*: 46.0 per cent to 50.6 per cent of methacrylic acid units (residue on evaporation).

It may contain suitable surface-active agents such as sodium dodecyl sulfate and polysorbate 80.

CHARACTERS

*Appearance*: opaque, white or almost white, slightly viscous liquid.

*Solubility*: miscible with water. On addition of solvents such as acetone, anhydrous ethanol or 2-propanol, a precipitate is formed which dissolves on addition of excess solvent. It is miscible with a 40 g/L solution of sodium hydroxide.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of methacrylic acid - ethyl acrylate copolymer (1:1) dispersion 30 per cent*.

B. It complies with the limits of the assay.

TESTS

**Viscosity** (*2.2.10*): maximum 15 mPa·s, determined using a rotating viscometer at 20 °C and at a shear rate of 50 $s^{-1}$.

**Appearance of a film**. Place 1 mL on a glass plate and allow to dry. A clear, brittle film is formed.

**Particulate matter**. Filter 100.0 g through a tared stainless steel sieve (90). Rinse with *water R* until a clear filtrate is obtained and dry at 100-105 °C. The residue weighs a maximum of 1.00 g.

**Ethyl acrylate and methacrylic acid**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 3.5 g of *sodium perchlorate R* in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Blank solution*: solution A, *methanol R1* (1:2 *V/V*).

*Test solution*. Dissolve 10.0 g of the dispersion to be examined in *methanol R1* and dilute to 50.0 mL with the same solvent. To 5.0 mL of solution A add 10.0 mL of the solution dropwise whilst stirring continuously. Centrifuge until a clear supernatant is obtained. Use the clear supernatant as the test solution.

*Reference solution*. Dissolve 50.0 mg of *ethyl acrylate R* and 50.0 mg of *methacrylic acid R* in 5 mL of *butanol R* and dilute to 50.0 mL with *methanol R1*. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R1*. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R1*. Mix 10.0 mL of this solution and 5.0 mL of solution A.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R1* and 80 volumes of *water for chromatography R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 20 µL.

*Retention time*: methacrylic acid = about 3 min, ethyl acrylate = about 8 min.

*System suitability*:
- *resolution*: minimum 5.0 between the peaks due to methacrylic acid and ethyl acrylate in the chromatogram obtained with the reference solution;
- the chromatogram obtained with the blank solution does not show peaks with the same retention times as ethyl acrylate or methacrylic acid.

*Calculation of percentage contents*:
- for ethyl acrylate and methacrylic acid, use the respective concentration of these substances in the reference solution.

*Limit*:
- *sum of the contents of ethyl acrylate and methacrylic acid*: maximum 0.01 per cent;
- *reporting threshold*: 1 ppm.

**Residue on evaporation**: 28.5 per cent to 31.5 per cent.

Dry 1.000 g at 110 °C for 5 h. The residue weighs not less than 0.285 g and not more than 0.315 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 1.500 g in a mixture of 40 mL of *water R* and 60 mL of *2-propanol R*. Titrate slowly while stirring with *0.5 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *0.5 M sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

STORAGE

At a temperature of 5 °C to 25 °C, protected from freezing.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for methacrylic acid-ethyl acrylate copolymer (1:1) dispersion 30 per cent used as a gastro-resistant coating agent.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take a piece of the film obtained in the test for appearance of a film (see Tests), place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* and stir;

it does not dissolve within 2 h. Take another piece of the film, place it in a flask containing *phosphate buffer solution pH 6.0 R* and stir; it dissolves within 1 h.

**07/2016:1127**

# METHACRYLIC ACID - METHYL METHACRYLATE COPOLYMER (1:1)

## Acidi methacrylici et methylis methacrylatis polymerisatum 1:1

DEFINITION

Copolymer of methacrylic acid and methyl methacrylate having a mean relative molecular mass of about 135 000. The ratio of carboxylic groups to ester groups is about 1:1.

*Content*: 46.0 per cent to 50.6 per cent of methacrylic acid units (dried substance).

CHARACTERS

*Appearance*: white or almost white, free-flowing powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in 2-propanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/L solution of sodium hydroxide.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.1 g of the substance to be examined in 1 mL of *2-propanol R* and place 2 drops of the solution on a sodium chloride plate; dry to allow the formation of a film and cover with another sodium chloride plate.

*Comparison*: *methacrylic acid - methyl methacrylate copolymer (1:1) CRS*.

B. It complies with the limits of the assay.

TESTS

**Viscosity** (*2.2.10*): 50 mPa·s to 200 mPa·s.

Dissolve a quantity of the substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity using a rotating viscometer at 20 °C and at a shear rate of 10 $s^{-1}$.

**Appearance of a film**. Place 1 mL of the solution prepared in the test for viscosity on a glass plate and allow to dry. A clear, brittle film is formed.

**Methyl methacrylate and methacrylic acid**. Liquid chromatography (*2.2.29*).

*Blank solution*. Mix 3.0 mL of *methanol R1* and 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R*.

*Test solution*. Dissolve 1.00 g of the substance to be examined in *methanol R1* and dilute to 50.0 mL with the same solvent. To 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R* add 3.0 mL of the solution dropwise whilst stirring continuously. Centrifuge until a clear supernatant is obtained. Use the clear supernatant as the test solution.

*Reference solution*. Dissolve 50.0 mg of *methacrylic acid R* and 50.0 mg of *methyl methacrylate R* in 5 mL of *butanol R* and dilute to 100.0 mL with *methanol R1*. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R1*. Mix 3.0 mL of this solution and 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R1* and 80 volumes of *water for chromatography R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 20 µL.

*Retention time*: methacrylic acid = about 3 min; methyl methacrylate = about 9 min.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to methacrylic acid and methyl methacrylate in the chromatogram obtained with the reference solution;
– the chromatogram obtained with the blank solution does not show peaks with the same retention times as methacrylic acid or methyl methacrylate.

*Calculation of percentage contents*:

– for methacrylic acid and methyl methacrylate, use the respective concentration of these substances in the reference solution.

*Limits*:

– *methacrylic acid*: maximum 0.03 per cent;
– *methyl methacrylate*: maximum 0.02 per cent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 1.000 g in a mixture of 40 mL of *water R* and 60 mL of *2-propanol R*. Titrate slowly while stirring with *0.5 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *0.5 M sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for methacrylic acid-methyl methacrylate copolymer (1:1) used as gastro-resistant coating agent.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take a piece of the film obtained in the test for appearance of a film (see Tests), place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* and stir; it does not dissolve within 2 h. Take another piece of the film, place it in a flask containing *phosphate buffer solution pH 6.8 R* and stir; it dissolves within 1 h.

07/2016:1130

# METHACRYLIC ACID - METHYL METHACRYLATE COPOLYMER (1:2)

## Acidi methacrylici et methylis methacrylatis polymerisatum 1:2

DEFINITION

Copolymer of methacrylic acid and methyl methacrylate having a mean relative molecular mass of about 135 000. The ratio of carboxylic groups to ester groups is about 1:2.

*Content*: 27.6 per cent to 30.7 per cent of methacrylic acid units (dried substance).

CHARACTERS

*Appearance*: white or almost white, free-flowing powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in 2-propanol, practically insoluble in ethyl acetate. It is freely soluble in a 40 g/L solution of sodium hydroxide.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.1 g of the substance to be examined in 1 mL of *2-propanol R* and place 2 drops of the solution on a sodium chloride plate; dry to allow the formation of a film and cover with another sodium chloride plate.

*Comparison*: *methacrylic acid - methyl methacrylate copolymer (1:2) CRS*.

B. It complies with the limits of the assay.

TESTS

**Viscosity** (*2.2.10*): 50 mPa·s to 200 mPa·s.

Dissolve a quantity of the substance to be examined corresponding to 37.5 g of the dried substance in a mixture of 7.9 g of *water R* and 254.6 g of *2-propanol R*. Determine the viscosity using a rotating viscometer at 20 °C and at a shear rate of 10 $s^{-1}$.

**Appearance of a film**. Place 1 mL of the solution prepared in the test for viscosity on a glass plate and allow to dry. A clear, brittle film is formed.

**Methyl methacrylate and methacrylic acid**. Liquid chromatography (*2.2.29*).

*Blank solution*. Mix 3.0 mL of *methanol R1* and 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R*.

*Test solution*. Dissolve 1.00 g of the substance to be examined in *methanol R1* and dilute to 50.0 mL with the same solvent. To 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R* add 3.0 mL of the solution dropwise whilst stirring continuously. Centrifuge until a clear supernatant is obtained. Use the clear supernatant as the test solution.

*Reference solution*. Dissolve 50.0 mg of *methacrylic acid R* and 50.0 mg of *methyl methacrylate R* in 5 mL of *butanol R* and dilute to 100.0 mL with *methanol R1*. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R1*. Mix 3.0 mL of this solution and 10.0 mL of *0.125 M phosphate buffer solution pH 2.0 R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 20 volumes of *methanol R1* and 80 volumes of *water for chromatography R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 202 nm.

*Injection*: 20 µL.

*Retention time*: methacrylic acid = about 3 min; methyl methacrylate = about 9 min.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to methacrylic acid and methyl methacrylate in the chromatogram obtained with the reference solution;
- the chromatogram obtained with the blank solution does not show peaks with the same retention times as methacrylic acid or methyl methacrylate.

*Calculation of percentage contents*:

- for methacrylic acid and methyl methacrylate, use the respective concentration of these substances in the reference solution.

*Limits*:

- *methacrylic acid*: maximum 0.03 per cent;
- *methyl methacrylate*: maximum 0.02 per cent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 1.000 g in a mixture of 40 mL of *water R* and 60 mL of *2-propanol R*. Titrate slowly while stirring with *0.5 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *0.5 M sodium hydroxide* is equivalent to 43.05 mg of $C_4H_6O_2$ (methacrylic acid units).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for methacrylic acid-methyl methacrylate copolymer (1:2) used as gastro-resistant coating agent.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take a piece of the film obtained in the test for appearance of a film (see Tests), place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* and stir; it does not dissolve within 2 h. Take another piece of the film, place it in a flask containing *phosphate buffer solution pH 6.8 R* and stir; it does not dissolve within 2 h. Take another piece of the film, place it in a flask containing *0.2 M phosphate buffer solution pH 7.5 R* and stir; it dissolves within 1 h.

**01/2008:0408**
**corrected 10.0**

# METHADONE HYDROCHLORIDE

## Methadoni hydrochloridum

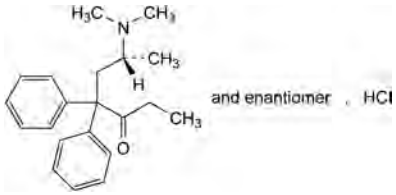


$C_{21}H_{28}ClNO$ $M_r$ 345.9
[1095-90-5]

DEFINITION

(6*RS*)-6-(Dimethylamino)-4,4-diphenylheptan-3-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, C, D.*

*Second identification*: *A, B, D.*

A. Optical rotation (see Tests).

B. Melting point (*2.2.14*): 233 °C to 236 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of methadone hydrochloride.*

D. Dilute 1 mL of solution S (see Tests) to 5 mL with *water R* and add 1 mL of *dilute ammonia R1*. Mix, allow to stand for 5 min and filter. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 10 mL of solution S to 25 mL with *carbon dioxide-free water R*. To 10 mL of the solution add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Optical rotation** (*2.2.7*): – 0.05° to + 0.05°, determined on solution S in a 2 dm tube.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of *imipramine hydrochloride CRS* and 5 mg of *cyclobenzaprine hydrochloride CRS* in 100.0 mL of *methanol R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 50 m, Ø = 0.32 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.05 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.2 mL/min.

*Injection liner*: packed with deactivated glass wool to wipe the needle.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 150 → 250 |
| | 4 - 35 | 250 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Run time*: 1.5 times the retention time of methadone.

*Relative retention* with reference to methadone (retention time = about 25 min): impurity E = about 0.44; impurity C = about 0.81; impurity B = about 0.89; impurity D = about 0.98; impurity A = about 1.14; imipramine = about 1.19; cyclobenzaprine = about 1.24.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to imipramine and cyclobenzaprine.

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *anhydrous ethanol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.59 mg of $C_{21}H_{28}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

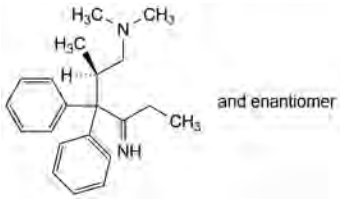


A. (2*RS*)-4-imino-*N*,*N*,2-trimethyl-3,3-diphenylhexan-1-amine (isomethadone ketimine),

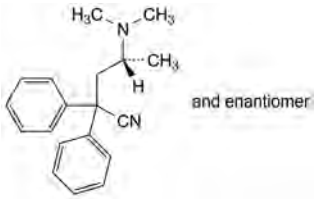


B. (4*RS*)-4-(dimethylamino)-2,2-diphenylpentanenitrile (didiavalo),

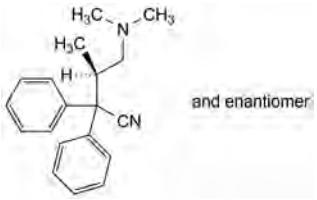


C. (3*RS*)-4-(dimethylamino)-3-methyl-2,2-diphenylbutanenitrile (isodidiavalo),

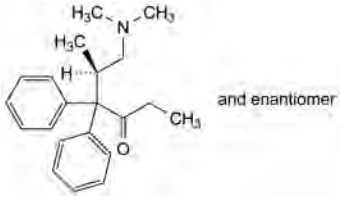


D. (5*RS*)-6-(dimethylamino)-5-methyl-4,4-diphenylhexan-3-one (isomethadone),

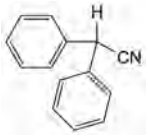

E. diphenylacetonitrile.

**01/2015:2413**

# METHANE

## Methanum

$CH_4$ $M_r$ 16.04
[74-82-8]

DEFINITION

*Content*: minimum 99.5 per cent *V/V* of $CH_4$.

This monograph applies to methane obtained from natural gas and intended for medicinal use.

CHARACTERS

*Appearance*: colourless gas, flammable in air.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 27 volumes of water.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with reference gas (b).

TESTS

**Nitrogen**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Mixture containing 500 ppm *V/V* of *nitrogen R1* in *methane R1*.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 30 mL/min.

*Temperature*:

- *column*: 80 °C;
- *detector*: 150 °C.

*Detection*: thermal conductivity.

*Injection*: 1 mL.

*Relative retention* with reference to methane (retention time = about 3 min): nitrogen = about 0.7.

*System suitability*: reference gas:

- *resolution*: minimum 1.5 between the peaks due to nitrogen and methane.

*Calculation of percentage content*:

- use the concentration of nitrogen in the reference gas.

*Limit*:

- maximum 500 ppm *V/V*.

**$C_2$-$C_4$ Hydrocarbons**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Use the following mixture of gases in *methane R*: *ethane R* (impurity C) (100 ppm *V/V*), *propane R* (impurity D) (100 ppm *V/V*), *i-butane R* (impurity E) (100 ppm *V/V*) and *n-butane R* (impurity F) (100 ppm *V/V*).

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *deactivated aluminium oxide for chromatography R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 113 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 30 |
| | 1 - 3.4 | 30 → 150 |
| | 3.4 - 4.5 | 150 |
| Detector | | 300 |

*Detection*: flame ionisation. Use appropriate fuel gases: *hydrocarbon-free air R* and *hydrogen for chromatography R.*

*Injection*: 0.1 mL.

*Relative retention* with reference to methane (retention time = about 1.2 min): impurity C = about 1.2; impurity D = about 1.8; impurity E = about 2.5; impurity F = about 2.6.

*System suitability*: reference gas:

– *resolution*: minimum 1.5 between the peaks due to methane and impurity C; minimum 1.5 between the peaks due to impurity E and impurity F.

*Calculation of percentage contents*:

– for each of the impurities C, D, E and F, use the corresponding concentration in the reference gas.

*Limit*:

– *total* (sum of impurities C, D, E and F): maximum 100 ppm *V/V*.

**Water** (*2.5.28*): maximum 10.0 ppm *V/V*.

ASSAY

Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a). Methane R1.*

*Reference gas (b).* Mixture containing 1 per cent *V/V* of *nitrogen R1* in *methane R1*.

*Column*:

– *material*: stainless steel;

– *size*: *l* = 2 m, Ø = 2 mm;

– *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 80 °C;

– *detector*: 150 °C.

*Detection*: thermal conductivity.

*Injection*: 10 µL.

*Relative retention* with reference to methane (retention time = about 3 min): nitrogen = about 0.7.

*System suitability*: reference gas (b):

– *resolution*: minimum 1.5 between the peaks due to nitrogen and methane.

Calculate the percentage content of $CH_4$, using the chromatogram obtained with reference gas (a).

STORAGE

As a compressed gas, in appropriate containers complying with the legal regulations.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

A. $N_2$: nitrogen,

B. $H_2O$: water,

C. ethane,

D. propane,

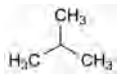

E. 2-methylpropane (*i*-butane),

F. butane (*n*-butane).

**01/2018:2905**

# METHANE INTERMIX (2 PER CENT) IN NITROGEN

## Methanum (2 per centum) in nitrogenio intermixtum

DEFINITION

A mixture containing 2 per cent *V/V* of *Methane (2413)* in *Low-oxygen nitrogen (1685)*.

*Content*: 1.9 per cent *V/V* to 2.1 per cent *V/V* of methane ($CH_4$) in nitrogen ($N_2$).

This monograph applies to methane intermix (2 per cent) in nitrogen used in the preparation of lung function test gas mixtures for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

IDENTIFICATION

A. It complies with the limits of the assay.

B. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas. Nitrogen R1.*

*Column*:

– *material*: stainless steel;

– *size*: *l* = 2 m, Ø = 2 mm;

– *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 20 mL/min.

*Temperature*:

– *column*: 80 °C;

– *detector*: 130 °C.

*Detection*: thermal conductivity.

*Injection*: 10 µL.

*Retention time*: nitrogen = about 2 min.

*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with the reference gas.

TESTS

**Water** (*2.5.28*): maximum 10 ppm *V/V*.

ASSAY

Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* Mixture containing 2.0 per cent *V/V* of *methane R1* in *nitrogen R1*.

*Column*:

– *material*: stainless steel;

– *size*: *l* = 2 m, Ø = 2 mm;

– *stationary phase*: 3 per cent *squalane R* on alumina.

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 20 mL/min.

*Temperature*:

– *column*: 100 °C;

– *detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 100 µL.

*Retention time*: methane = about 0.5 min.

Calculate the percentage content of $CH_4$.

STORAGE

As a compressed gas, in appropriate high-pressure cylinders complying with the legal regulations.

LABELLING

The label states the nominal content, in per cent *V/V*, of methane in nitrogen.

IMPURITIES

*Specified impurities: A.*

A. water.

**01/2008:1989**
**corrected 10.0**

# METHANOL

## Methanolum



$CH_4O$ $M_r$ 32.04
[67-56-1]

DEFINITION

Methyl alcohol.

CHARACTERS

*Appearance*: clear, colourless, volatile, hygroscopic liquid.

*Solubility*: miscible with water and with methylene chloride.

bp: about 64 °C.

It is flammable.

IDENTIFICATION

A. Refractive index (*2.2.6*): 1.328 to 1.330.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of methanol.*

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 25 mL add 25 mL of *water R* and 0.25 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.9 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Relative density** (*2.2.5*): 0.791 to 0.793.

**Absorbance** (*2.2.25*): maximum 0.15 at 230 nm, maximum 0.05 at 250 nm, maximum 0.02 at 270 nm and maximum 0.01 at 290 nm.

Examine between 230 nm and 290 nm using *water R* as the compensation liquid. The absorption curve is smooth.

**Impurity A**. Gas chromatography (*2.2.28*).

*Test solution (a).* The substance to be examined.

*Test solution (b).* Dilute 1.0 mL of *4-methylpentan-2-ol R* to 50.0 mL with test solution (a). Dilute 5.0 mL of this solution to 100.0 mL with test solution (a).

*Reference solution (a).* To 50 µL of *anhydrous ethanol R* add 50 µL of *acetone R* and dilute to 50.0 mL with test solution (a). Dilute 100 µL of this solution to 10.0 mL with test solution (a).

*Reference solution (b).* Dilute 100 µL of *benzene R* to 100.0 mL with test solution (a). Dilute 0.20 mL to 100.0 mL with test solution (a).

*Column*:
- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 40 |
| | 12 - 32 | 40 → 240 |
| | 32 - 42 | 240 |
| Injection port | | 200 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (a):
- *resolution*: minimum 4.0 between the peaks due to impurity B (1st peak) and impurity C (2nd peak).

*Limit*:
- *impurity A*: maximum 2 ppm *V/V*.
  Calculate the content of impurity A in parts per million *V/V* using the following expression:

$$\frac{2 \times A_1}{A_2 - A_1}$$

$A_1$ = area of the peak due to impurity A in the chromatogram obtained with test solution (a),

$A_2$ = area of the peak due to impurity A in the chromatogram obtained with reference solution (b).

If necessary, the identity of impurity A can be confirmed using another suitable chromatographic system (stationary phase with a different polarity).

**Related substances**. Gas chromatography (*2.2.28*) as described in the test for impurity A.

*Limits*:
- *any impurity*: for each impurity, not more than the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (0.1 per cent),
- *total*: not more than 3 times the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (0.3 per cent),
- *disregard limit*: 0.05 times the area of the peak due to 4-methylpentan-2-ol in the chromatogram obtained with test solution (b) (50 ppm).

**Reducing substances**. To 20 mL add 0.1 mL of *0.02 M potassium permanganate*. The pink colour is not completely discharged within 5 min.

**Residue on evaporation**: maximum 10 ppm.

Evaporate 100 g to dryness on a water bath and dry in an oven at 100-105 °C. The residue weighs a maximum of 1 mg.

**Water** (*2.5.12*): maximum 0.10 per cent, determined on 10.0 g.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

A. benzene,

B. ethanol,

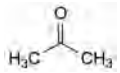


C. propanone (acetone).

**01/2017:1545**

# METHENAMINE

## Methenaminum

$C_6H_{12}N_4$ $M_r$ 140.2
[100-97-0]

DEFINITION

1,3,5,7-Tetraazotricyclo[3.3.1.1$^{3,7}$]decane.

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methenamine CRS.*

B. To 1 mL of solution S (see Tests) add 1 mL of *sulfuric acid R* and immediately heat to boiling. Allow to cool. To 1 mL of the solution add 4 mL of *water R* and 5 mL of *acetylacetone reagent R1*. Heat on a water-bath for 5 min. An intense yellow colour develops.

C. To 1 mL of solution S add 1 mL of *dilute sulfuric acid R* and immediately heat to boiling. The solution gives the reaction of ammonium salts and salts of volatile bases (*2.3.1*).

D. Dissolve 10 mg in 5 mL of *water R* and acidify with *dilute hydrochloric acid R*. Add 1 mL of *potassium iodobismuthate solution R*. An orange precipitate is formed immediately.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Free formaldehyde**: maximum 50 ppm.

Dissolve 0.8 g in *water R* and dilute to 8 mL with the same solvent. Add 2 mL of *ammoniacal silver nitrate solution R*. After 5 min, any grey colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner with a mixture of 8 mL of freshly prepared *formaldehyde standard solution (5 ppm $CH_2O$) R* and 2 mL of *ammoniacal silver nitrate solution R*.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Ammonium** (*2.4.1*): maximum 50 ppm.

Dilute 2 mL of freshly prepared solution S to 13 mL with *water R*. Add 2 mL of *dilute sodium hydroxide solution R*.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in a desiccator.

ASSAY

Dissolve 0.100 g in 30 mL of *methanol R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.02 mg of $C_6H_{12}N_4$.

STORAGE

Protected from light.

**01/2017:1027**

# METHIONINE

## Methioninum

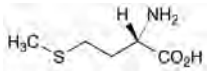


$C_5H_{11}NO_2S$ $M_r$ 149.2
[63-68-3]

DEFINITION

(2*S*)-2-Amino-4-(methylsulfanyl)butanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison: methionine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *dilute hydrochloric acid R* and dilute to 50 mL with the same acid.

*Reference solution.* Dissolve 10 mg of *methionine CRS* in *dilute hydrochloric acid R* and dilute to 50 mL with the same acid.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 0.1 g of the substance to be examined and 0.1 g of *glycine R* in 4.5 mL of *dilute sodium hydroxide solution R*. Add 1 mL of a 25 g/L solution of *sodium nitroprusside R*. Heat to 40 °C for 10 min. Allow to cool and add 2 mL of a mixture of 1 volume of *phosphoric acid R* and 9 volumes of *hydrochloric acid R*. A dark red colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 6.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 22.5 to + 24.0 (dried substance).

Dissolve 1.00 g in *hydrochloric acid R1* and dilute to 50.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.30 g of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 10 mg of *L-methionine sulfoxide R* (impurity A) in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, 23 g/L solution of *phosphoric acid R*, *water for chromatography R* (0.5:12.5:87 *V/V/V*);
- *mobile phase B*: 23 g/L solution of *phosphoric acid R*, *water for chromatography R*, *acetonitrile R1* (12.5:40:47.5 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 100 | 0 |
| 6 - 50 | 100 → 0 | 0 → 100 |
| 50 - 60 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 50 µL.

*Relative retention* with reference to methionine (retention time = about 6 min): impurity A = about 0.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and methionine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of methionine in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Chlorides**: maximum 200 ppm.

Dissolve 0.5 g of the substance to be examined in 5 mL of *dilute nitric acid R* and dilute to 10 mL with the same acid. Add 10 mL of *strong hydrogen peroxide solution R* and heat on a water bath for 30 min. Cool and dilute to 50 mL with *water R*. Add 1 mL of *silver nitrate solution R2* and mix. Allow to stand protected from light for 5 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 2 mL of *chloride standard solution (50 ppm Cl) R*. Examine the tubes laterally against a black background.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in 3 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 0.10 g.

Prepare the standard using 0.2 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined upper layers, add 10 mL of *water R* and shake for 3 min. Use the lower layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.125 g in 5 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

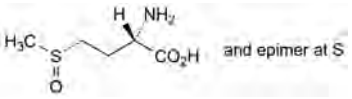


A. (2*S*)-2-amino-4-[(*RS*)-methylsulfinyl]butanoic acid (L-methionine sulfoxide),

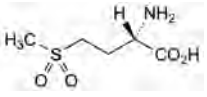


B. (2*S*)-2-amino-4-(methylsulfonyl)butanoic acid,

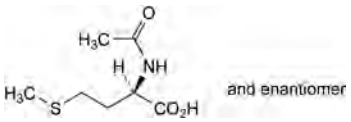


C. (2*RS*)-2-(acetylamino)-4-(methylsulfanyl)butanoic acid,

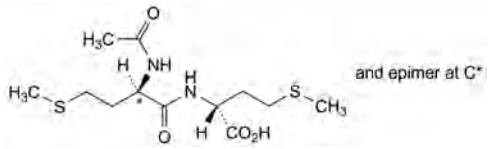


D. (2*R*)-2-[[(2*RS*)-2-(acetylamino)-4-(methylsulfanyl)-butanoyl]amino]-4-(methylsulfanyl)butanoic acid,

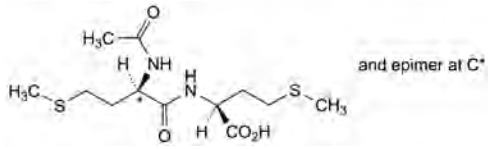


E. (2*S*)-2-[[(2*RS*)-2-(acetylamino)-4-(methylsulfanyl)-butanoyl]amino]-4-(methylsulfanyl)butanoic acid.

**01/2017:0624**

# DL-METHIONINE

## DL-Methioninum

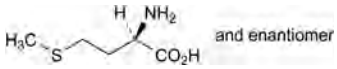


$C_5H_{11}NO_2S$ $M_r$ 149.2
[59-51-8]

DEFINITION

DL-Methionine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-2-amino-4-(methylsulfanyl)butanoic acid, calculated with reference to the dried substance.

CHARACTERS

Almost white, crystalline powder or small flakes, sparingly soluble in water, very slightly soluble in alcohol. It dissolves in dilute acids and in dilute solutions of the alkali hydroxides.

It melts at about 270 °C (instantaneous method).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *DL-methionine CRS*. Dry the substances at 105 °C.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 2.50 g in *1 M hydrochloric acid* and dilute to 50.0 mL with the same acid. The angle of optical rotation (*2.2.7*) is − 0.05° to + 0.05°.

D. Dissolve 0.1 g of the substance to be examined and 0.1 g of *glycine R* in 4.5 mL of *dilute sodium hydroxide solution R*. Add 1 mL of a 25 g/L solution of *sodium nitroprusside R*. Heat to 40 °C for 10 min. Allow to cool and add 2 mL of a mixture of 1 volume of *phosphoric acid R* and 9 volumes of *hydrochloric acid R*. A deep-red colour develops.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 5.4 to 6.1.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.2 g in *water R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 50 mL with *water R*.

*Reference solution (a).* Dissolve 20 mg of *DL-methionine CRS* in *water R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of reference solution (a) to 10 mL with *water R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 10 cm using a mixture of 20 volumes of *glacial acetic acid R*, 20 volumes of *water R* and 60 volumes of *butanol R*. Allow the plate to dry in air and spray with *ninhydrin solution R*. Heat the plate at 100 °C to 105 °C for 15 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Chlorides**. Dissolve 0.25 g in 35 mL of *water R*. Add 5 mL of *dilute nitric acid R* and 10 mL of *silver nitrate solution R2*. Allow to stand protected from light for 5 min. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 10 mL of *chloride standard solution (5 ppm Cl) R* and 25 mL of *water R* (200 ppm). Examine the tubes laterally against a black background.

**Sulfates** (*2.4.13*). Dissolve 1.0 g in 20 mL of *distilled water R*, heating to 60 °C. Cool to 10 °C and filter. 15 mL of the solution complies with the limit test for sulfates (200 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Immediately after dissolution, titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.

STORAGE

Store protected from light.

**01/2017:0560**

# METHOTREXATE

## Methotrexatum

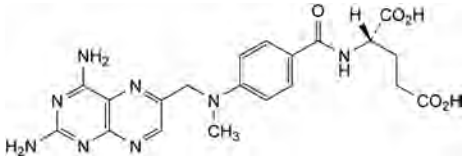

$C_{20}H_{22}N_8O_5$ $M_r$ 454.4
[59-05-2]

DEFINITION

(2*S*)-2-[[4-[[(2,4-Diaminopteridin-6-yl)methyl]methylamino]benzoyl]amino]pentanedioic acid.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow or orange, crystalline, hygroscopic powder.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methotrexate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 40.0 mg of the substance to be examined in a mixture of 0.5 mL of *dilute ammonia R1* and 5 mL of mobile phase A and dilute to 100.0 mL with mobile phase A.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in a mixture of 0.5 mL of *dilute ammonia R1* and 5 mL of mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 25.0 mg of *methotrexate CRS* in a mixture of 0.5 mL of *dilute ammonia R1* and 5 mL of mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 25.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 5 mg of the substance to be examined, 5 mg of *4-aminofolic acid R* (impurity B), 5 mg of *methotrexate impurity C CRS*, 5 mg of *methotrexate impurity D CRS* and 5 mg of *methotrexate impurity E CRS* in a mixture of 0.5 mL of *dilute ammonia R1* and 5 mL of mobile phase A and dilute to 100 mL with mobile phase A.

*Reference solution (e).* Dissolve 8 mg of *methotrexate for peak identification CRS* (containing impurities H and I) in a mixture of 0.1 mL of *dilute ammonia R1* and 1 mL of mobile phase A and dilute to 20 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *acetonitrile for chromatography R* and 95 volumes of a 3.4 g/L solution of *anhydrous sodium dihydrogen phosphate R* previously adjusted to pH 6.0 with a 42 g/L solution of *sodium hydroxide R*;
- *mobile phase B*: mix 50 volumes of *acetonitrile for chromatography R* and 50 volumes of a 3.4 g/L solution of *anhydrous sodium dihydrogen phosphate R* previously adjusted to pH 6.0 with a 42 g/L solution of *sodium hydroxide R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 20 | 100 → 95 | 0 → 5 |
| 20 - 28 | 95 → 50 | 5 → 50 |
| 28 - 37 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *methotrexate for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities H and I; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B, C and E.

*Relative retention* with reference to methotrexate (retention time = about 18 min): impurity B = about 0.3; impurity C = about 0.4; impurity D = about 0.9; impurity E = about 1.4; impurity I = about 1.5; impurity H = about 1.6.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurities B and C and minimum 1.5 between the peaks due to impurity D and methotrexate, in the chromatogram obtained with reference solution (d); minimum 1.5 between the peaks due to impurities I and H in the chromatogram obtained with reference solution (e); if the resolution between impurity D and methotrexate does not comply, increase the flow rate to meet the requirement.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.8; impurity I = 1.4;
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities B, E*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities H, I*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent);
- *sum of impurities other than B, C and E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.03 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4.0 mg of *methotrexate for system suitability CRS* (containing impurity F) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *bovine albumin R* bound to *silica gel for chromatography R* (7 µm) with a pore size of 30 nm.

*Mobile phase*: add 500 mL of a 7.1 g/L solution of *anhydrous disodium hydrogen phosphate R* to 600 mL of a 6.9 g/L solution of *sodium dihydrogen phosphate monohydrate R*, mix, and adjust to pH 6.9 with *dilute sodium hydroxide solution R*; to 920 mL of this mixture add 80 mL of *propanol R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 302 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity F.

*Relative retention* with reference to methotrexate (retention time = about 4 min): impurity F = about 1.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to methotrexate and impurity F.

*Limit*:

– *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent).

**Water** (*2.5.12*): maximum 13.0 per cent, determined on 0.10 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{20}H_{22}N_8O_5$ taking into account the assigned content of *methotrexate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C, E, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, G, J, K, L.*

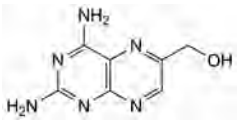

A. (2,4-diaminopteridin-6-yl)methanol,

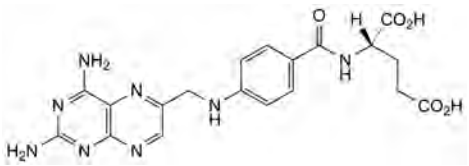

B. (2*S*)-2-[[4-[[(2,4-diaminopteridin-6-yl)methyl]amino]-benzoyl]amino]pentanedioic acid (4-aminofolic acid, aminopterin),

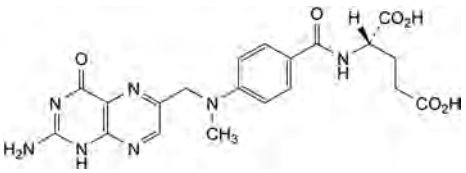

C. (2*S*)-2-[[4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)-methyl]methylamino]benzoyl]amino]pentanedioic acid (*N*-methylfolic acid, methopterin),

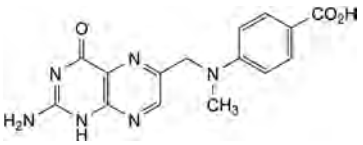

D. 4-[[(2-amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]-methylamino]benzoic acid ($N^{10}$-methylpteroic acid),

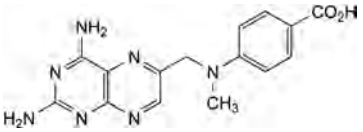

E. 4-[[(2,4-diaminopteridin-6-yl)methyl]methylamino]-benzoic acid (4-amino-$N^{10}$-methylpteroic acid, APA),

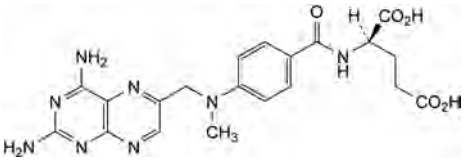

F. (2*R*)-2-[[4-[[(2,4-diaminopteridin-6-yl)methyl]methyl-amino]benzoyl]amino]pentanedioic acid ((*R*)-methotrexate),

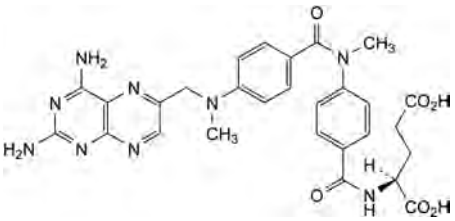

G. (2*S*)-2-[[4-[[4-[[(2,4-diaminopteridin-6-yl)methyl]-methylamino]benzoyl]methylamino]benzoyl]amino]-pentanedioic acid,

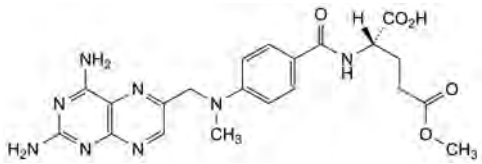

H. (2*S*)-2-[[4-[[(2,4-diaminopteridin-6-yl)methyl]methyl-amino]benzoyl]amino]-5-methoxy-5-oxopentanoic acid (methotrexate 5-methyl ester),

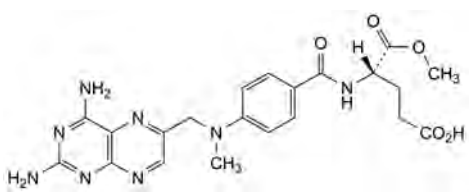

I. (4*S*)-4-[[4-[[(2,4-diaminopteridin-6-yl)methyl]methyl-amino]benzoyl]amino]-5-methoxy-5-oxopentanoic acid (methotrexate 1-methyl ester),

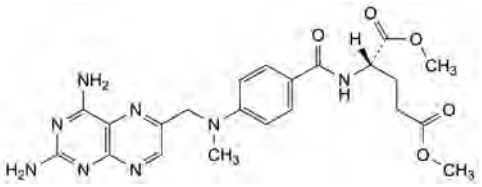

J. dimethyl (2*S*)-2-[[4-[[(2,4-diaminopteridin-6-yl)methyl]-methylamino]benzoyl]amino]pentanedioate (methotrexate dimethyl ester),

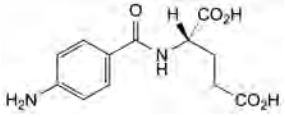

K. (2*S*)-2-[(4-aminobenzoyl)amino]pentanedioic acid,

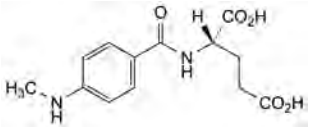

L. (2*S*)-2-[[4-(methylamino)benzoyl]amino]pentanedioic acid.

**01/2008:2129**

# METHYL NICOTINATE

## Methylis nicotinas

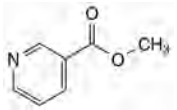

$C_7H_7NO_2$ $M_r$ 137.1
[93-60-7]

DEFINITION

Methyl pyridine-3-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Melting point (*2.2.14*): 40 °C to 42 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methyl nicotinate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 2 mL with the same solvent.
*Reference solution*. Dissolve 10 mg of *methyl nicotinate CRS* in *methanol R* and dilute to 2 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *methanol R*, *toluene R* (10:90 *V/V*).
*Application*: 2 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 0.5 g add 0.1 g of *citric acid monohydrate R* and 0.2 mL of *acetic anhydride R*. Heat cautiously for 1 min. A yellow colour is produced which turns first to orange, then to red and then to violet.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25 mg of *nicotinic acid R* in the mobile phase and dilute to 25.0 mL with the mobile phase. To 0.5 mL of this solution add 0.5 mL of the test solution and dilute to 100 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetic acid R*, *water R*, *acetonitrile R* (1:29:70 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 261 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of methyl nicotinate.

*Retention time*: methyl nicotinate = about 3.3 min.

*System suitability*: reference solution (a):
- *resolution*: minimum 2 between the peaks due to impurity A and methyl nicotinate.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in *water R* and dilute to 15 mL with the same solvent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.71 mg of $C_7H_7NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: A.

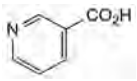

A. pyridine-3-carboxylic acid (nicotinic acid).

**07/2010:0409**

# METHYL PARAHYDROXYBENZOATE

## Methylis parahydroxybenzoas

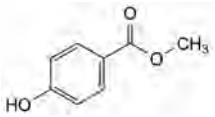

$C_8H_8O_3$ $M_r$ 152.1
[99-76-3]

DEFINITION

Methyl 4-hydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 125 °C to 128 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.
*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.
*Reference solution (a).* Dissolve 10 mg of *methyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).
*Application*: 2 µL of test solution (b) and reference solutions (a) and (b).
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated principal spots.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. To 2 mL of solution S add 3 mL of *ethanol (96 per cent) R*, 5 mL of *carbon dioxide-free water R* and 0.1 mL of *bromocresol green solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution.* Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.
*Reference solution (a).* Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.
*Reference solution (b).* Dissolve 50.0 mg of *methyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.
*Reference solution (c).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.
*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).
*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).
*Flow rate*: 1.3 mL/min.
*Detection*: spectrophotometer at 272 nm.
*Injection*: 10 µL of the test solution and reference solutions (a) and (c).
*Run time*: 5 times the retention time of methyl parahydroxybenzoate.
*Relative retention* with reference to methyl parahydroxybenzoate (retention time = about 2.3 min): impurity A = about 0.6.
*System suitability*: reference solution (a):
– *resolution*: minimum 2.0 between the peaks due to impurity A and methyl parahydroxybenzoate.
*Limits*:
– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_8H_8O_3$ from the declared content of *methyl parahydroxybenzoate CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

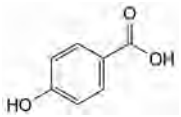

A. 4-hydroxybenzoic acid,

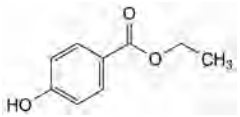

B. ethyl 4-hydroxybenzoate (ethyl parahydroxybenzoate),

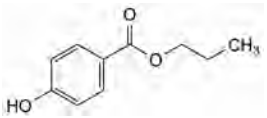

C. propyl 4-hydroxybenzoate (propyl parahydroxybenzoate),

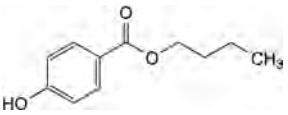

D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

**07/2019:0230**
**corrected 10.0**

# METHYL SALICYLATE

## Methylis salicylas

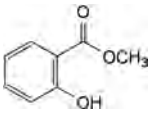

$C_8H_8O_3$ $M_r$ 152.1

[119-36-8]

DEFINITION

Methyl 2-hydroxybenzoate.

*Content*: 99.0 per cent *m/m* to 101.0 per cent *m/m*.

CHARACTERS

*Appearance*: colourless or slightly yellow liquid.

*Solubility*: very slightly soluble in water, miscible with ethanol (96 per cent) and with fatty and essential oils.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methyl salicylate CRS.*

B. Heat 0.25 mL with 2 mL of *dilute sodium hydroxide solution R* on a water-bath for 5 min. Add 3 mL of *dilute sulfuric acid R*. A crystalline precipitate is formed. Filter. The precipitate, washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 156 °C to 161 °C.

C. To 10 mL of a saturated solution add 0.05 mL of *ferric chloride solution R1*. A violet colour develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

To 2 mL add 10 mL of *ethanol (96 per cent) R.*

**Acidity**. Dissolve 5.0 g in a mixture of 0.2 mL of *bromocresol green solution R* and 50 mL of *ethanol (96 per cent) R* previously neutralised to a blue colour by addition of *0.1 M sodium hydroxide*. Not more than 0.4 mL of *0.1 M sodium hydroxide* is required to restore the blue colour.

**Relative density** (*2.2.5*): 1.182 to 1.188.

**Refractive index** (*2.2.6*): 1.535 to 1.538.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution.* The substance to be examined.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetone R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetone R*.

*Reference solution (b).* Dissolve 59.0 mg of *phenol R* (impurity B) in *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetone R*.

*Reference solution (c).* Dissolve 5 mg of *methyl 2-methoxybenzoate R* (impurity M) and 5 mg of *methyl 4-methoxybenzoate R* (impurity D) in *acetone R* and dilute to 100 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 5.0 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 32.5 | 100 → 230 |
| | 32.5 - 40.5 | 230 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and M.

*Relative retention* with reference to methyl salicylate (retention time = about 9 min): methanol = about 0.15; anisole = about 0.37; impurity B = about 0.47; impurity M = about 1.56; impurity D = about 1.59.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurities M and D.

*Calculation of contents*:

– for impurity B, use the concentration of impurity B in reference solution (b);

– for impurities other than B, use the concentration of methyl salicylate in reference solution (a).

*Limits*:

– *impurity B*: maximum 50 ppm;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent, except for impurity B; disregard the peaks due to anisole and methanol.

ASSAY

Dissolve 0.500 g in 25 mL of *ethanol (96 per cent) R*. Add 0.05 mL of *phenol red solution R* and neutralise with *0.1 M sodium hydroxide*. To the neutralised solution add 50.0 mL of *0.1 M sodium hydroxide* and heat under a reflux condenser on a water-bath for 30 min. Cool and titrate with *0.1 M hydrochloric acid*. Calculate the volume of *0.1 M sodium hydroxide* used in the saponification. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.21 mg of $C_8H_8O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, I, J, K, L, M.*

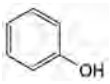


B. phenol,

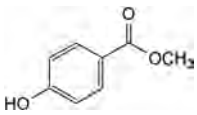


C. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

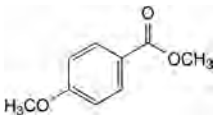


D. methyl 4-methoxybenzoate,

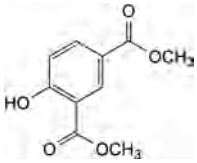


E. dimethyl 4-hydroxybenzene-1,3-dicarboxylate,

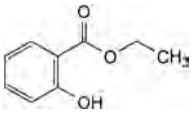


F. ethyl 2-hydroxybenzoate,

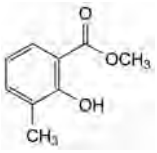


I. methyl 2-hydroxy-3-methylbenzoate,

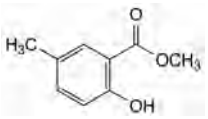


J. methyl 2-hydroxy-5-methylbenzoate,

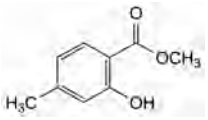


K. methyl 2-hydroxy-4-methylbenzoate,

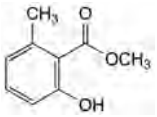


L. methyl 2-hydroxy-6-methylbenzoate,

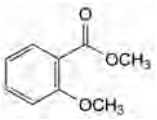


M. methyl 2-methoxybenzoate.

**01/2019:0345**
**corrected 10.0**

# METHYLCELLULOSE(4)

## Methylcellulosum

[9004-67-5]

DEFINITION

Partly *O*-methylated cellulose. Methyl ether of cellulose.

*Content*: 26.0 per cent to 33.0 per cent of methoxy groups (-$OCH_3$; $M_r$ 31.03) (dried substance).

◆CHARACTERS

*Appearance*: white, yellowish-white or greyish-white powder or granules, hygroscopic after drying.

*Solubility*: practically insoluble in hot water, in acetone, in anhydrous ethanol and in toluene. It dissolves in cold water giving a colloidal solution.◆

IDENTIFICATION

A. Evenly distribute 1.0 g onto the surface of 100 mL of *water R* in a beaker, tapping the top of the beaker gently if necessary to ensure a uniform layer on the surface. Allow to stand for 1-2 min: the powdered material aggregates on the surface.

(4) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

B. Evenly distribute 1.0 g into 100 mL of boiling *water R*, and stir the mixture using a magnetic stirrer with a bar 25 mm long: a slurry is formed and the particles do not dissolve. Allow the slurry to cool to 5 °C and stir using a magnetic stirrer: a clear or slightly turbid solution occurs with its thickness dependent on the viscosity grade.

C. To 0.1 mL of the solution obtained in identification test B add 9 mL of a 90 per cent *V/V* solution of *sulfuric acid R*, shake, heat on a water-bath for exactly 3 min, immediately cool in an ice-bath, carefully add 0.6 mL of a 20 g/L solution of *ninhydrin R*, shake and allow to stand at 25 °C: a red colour develops and does not change to purple within 100 min.

D. Place 2-3 mL of the solution obtained in identification test B on a glass slide as a thin film and allow the water to evaporate: a coherent, clear film forms on the glass slide.

E. Add 50.0 mL of the solution obtained in identification test B to 50.0 mL of *water R* in a beaker. Insert a thermometer into the solution. Stir the solution on a magnetic stirrer/hot plate and begin heating, increasing the temperature at a rate of 2-5 °C per minute. Determine the temperature at which a turbidity increase begins to occur and designate the temperature as the flocculation temperature: the flocculation temperature is higher than 50 °C.

TESTS

◊**Appearance of solution**. The solution is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete. Allow to stand at 2-8 °C for 1 h before carrying out the test.◊

**pH** (*2.2.3*): 5.0 to 8.0 for the solution prepared as described under Viscosity.

Read the pH after the probe has been immersed for 5 ± 0.5 min.

**Viscosity**: 80 per cent to 120 per cent of the nominal value for samples with a viscosity of less than 600 mPa·s (Method 1); 75 per cent to 140 per cent of the nominal value for samples with a viscosity of 600 mPa·s or higher (Method 2).

*Method 1, to be applied to samples with a viscosity of less than 600 mPa·s.* Weigh a quantity of the substance to be examined equivalent to 4.000 g of the dried substance. Transfer into a wide-mouthed bottle, and adjust the total mass of the sample and the water to 200.0 g with hot *water R* (90-99 °C). Capping the bottle, stir by mechanical means at 400 ± 50 r/min for 10-20 min until the particles are thoroughly dispersed and wetted. Scrape down the insides of the bottle with a spatula if necessary, to ensure that there is no undissolved material on the sides of the bottle, and continue the stirring in a cooling water-bath maintained at a temperature below 5 °C for another 20-40 min. Adjust the solution mass if necessary to 200.0 g using cold *water R*. Centrifuge the solution if necessary to expel any entrapped air bubbles. Using a spatula, remove any foam. Determine the kinematic viscosity ($\nu$) of this solution using the capillary viscometer method (*2.2.9*). Separately, determine the density ($\rho$) (*2.2.5*) of the solution and calculate the dynamic viscosity ($\eta$), as $\eta = \rho\nu$.

*Method 2, to be applied to samples with a viscosity of 600 mPa·s or higher.* Weigh a quantity of the substance to be examined equivalent to 10.00 g of the dried substance. Transfer into a wide-mouthed bottle, and adjust the total mass of the sample and the water to 500.0 g with hot *water R* (90-99 °C). Capping the bottle, stir by mechanical means at 400 ± 50 r/min for 10-20 min until the particles are thoroughly dispersed and wetted. Scrape down the insides of the bottle with a spatula if necessary, to ensure that there is no undissolved material on the sides of the bottle, and continue the stirring in a cooling water-bath maintained at a temperature below 5 °C for another 20-40 min. Adjust the solution mass if necessary to 500.0 g using cold *water R*. Centrifuge the solution if necessary to expel any entrapped air bubbles. Using a spatula, remove any foam. Determine the viscosity (*2.2.10*) of this solution at 20 ± 0.1 °C using a rotating viscometer.

*Apparatus*: single-cylinder type spindle viscometer.

*Rotor number, revolution and calculation multiplier*: apply the conditions specified in Table 0345.-1.

Table 0345.-1.

| Nominal viscosity* (mPa·s) | Rotor number | Revolution (r/min) | Calculation multiplier |
|---|---|---|---|
| 600 to less than 1400 | 3 | 60 | 20 |
| 1400 to less than 3500 | 3 | 12 | 100 |
| 3500 to less than 9500 | 4 | 60 | 100 |
| 9500 to less than 99 500 | 4 | 6 | 1000 |
| 99 500 or more | 4 | 3 | 2000 |

*the nominal viscosity is based on the manufacturer's specifications.

Allow the spindle to rotate for 2 min before taking the measurement. Allow a rest period of at least 2 min between subsequent measurements. Repeat the measurement twice and determine the mean of the 3 readings.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Apparatus*:

- *reaction vial*: a 5 mL pressure-tight vial, 50 mm in height, 20 mm in external diameter and 13 mm in internal diameter at the mouth, equipped with a pressure-tight butyl rubber membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap or another sealing system providing a sufficient air-tightness;
- *heater*: a heating module with a square aluminium block having holes 20 mm in diameter and 32 mm in depth, so that the reaction vials fit; mixing of the contents of the vial is effected using a magnetic stirrer equipped in the heating module or using a reciprocal shaker that performs approximately 100 cycles/min.

*Internal standard solution*: 30 g/L solution of *octane R* in *o-xylene R*.

*Test solution*. Weigh 65.0 mg of the substance to be examined, place in a reaction vial, add 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R*, immediately cap and seal the vial, and weigh accurately. Mix the contents of the vial continuously for 60 min while heating the block so that the temperature of the contents is maintained at 130 ± 2 °C. If a reciprocal shaker or magnetic stirrer cannot be used, shake the vial thoroughly by hand at 5 min intervals during the initial 30 min of the heating time. Allow the vial to cool, and again weigh accurately. If the loss of mass is less than 26 mg and there is no evidence of a leak, use the upper layer of the mixture as the test solution.

*Reference solution*. Place 0.06-0.10 g of *adipic acid R*, 2.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R* in another reaction vial, cap and seal the vial, and weigh

accurately. Add 45 µL of *methyl iodide R* through the septum with a syringe, and weigh accurately. Shake the reaction vial thoroughly and use the upper layer as the reference solution.

Use a precolumn if needed.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *methylpolysiloxane R* (3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.3 mL/min.

*Split ratio*: 1:40.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 50 |
| | 3 - 8 | 50 → 100 |
| | 8 - 12.3 | 100 → 250 |
| | 12.3 - 20.3 | 250 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation or thermal conductivity.

*Injection*: 1-2 µL.

*Relative retention* with reference to octane (retention time = about 10 min): methyl iodide = about 0.4.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to methyl iodide and octane;
– *repeatability*: maximum relative standard deviation of 2.0 per cent for the ratio of the area of the peak due to methyl iodide to that due to octane, determined on 6 injections.

Calculate the ratio ($Q$) of the area of the peak due to methyl iodide to the area of the peak due to the internal standard in the chromatogram obtained with the test solution, and the ratio ($Q_1$) of the area of the peak due to methyl iodide to the area of the peak due to the internal standard in the chromatogram obtained with the reference solution.

Calculate the percentage content of methoxy groups using the following expression:

$$\frac{Q \times m_1}{Q_1 \times m} \times 21.864$$

$m_1$ = mass of methyl iodide in the reference solution, in milligrams;

$m$ = mass of the sample (dried substance), in milligrams.

LABELLING

The label states the viscosity in millipascal seconds.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for methylcellulose used as binder, viscosity-enhancing agent or film former.*

**Viscosity**: see Tests.

**Degree of substitution**: see Assay.



**01/2017:0045**

# METHYLDOPA

## Methyldopum

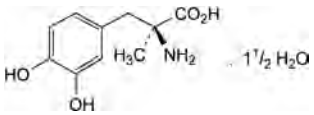


$C_{10}H_{13}NO_4,1½H_2O$ $M_r$ 238.2

[41372-08-1]

DEFINITION

(2*S*)-2-Amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid sesquihydrate (L-methyldopa sesquihydrate).

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder or colourless or almost colourless crystals.

*Solubility*: slightly soluble in water, very slightly soluble in ethanol (96 per cent). It is freely soluble in dilute mineral acids.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methyldopa CRS*.

B. Enantiomeric purity (see Tests).

C. Specific optical rotation (*2.2.7*): – 28.0 to – 25.0.
Dissolve a quantity equivalent to 2.20 g of the anhydrous substance in *aluminium chloride solution R* and dilute to 50.0 mL with the same solution.

TESTS

**Appearance of solution**. Dissolve 1.0 g in *1 M hydrochloric acid* and dilute to 25 mL with the same solvent. The solution is not more intensely coloured than reference solution $BY_6$ or $B_6$ (*2.2.2, Method II*).

**Acidity**. Dissolve 1.0 g with heating in 100 mL of *carbon dioxide-free water R*. Add 0.1 mL of *methyl red solution R*. Not more than 0.5 mL of *0.1 M sodium hydroxide* is required to produce the pure yellow colour of the indicator.

**Absorbance** (*2.2.25*).

*Test solution*. Dissolve 40.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 280 nm.

*Specific absorbance at the absorption maximum*: 122 to 137 (anhydrous substance).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *racemic methyldopa CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve separately 0.200 g of *copper acetate R* and 0.387 g of *N,N-dimethyl-L-phenylalanine R* in *water R*; mix the 2 solutions and adjust immediately to pH 4.3 with *acetic acid R*; add 50 mL of *methanol R* and dilute to 1000 mL with *water R*; mix and filter.

Equilibrate the column with the mobile phase for about 2 h.

If necessary, decrease the concentration of *methanol R* so the peak corresponding to D-methyldopa is clearly separated from the negative system peak that appears at about 6 min.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of L-methyldopa.

*Relative retention* with reference to L-methyldopa (retention time = about 14 min): D-methyldopa = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to D-methyldopa and L-methyldopa.

*Limit*:

- *D-methyldopa (impurity D)*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with *0.1 M hydrochloric acid.* Dilute 5.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid.*

*Reference solution (b).* Dissolve the contents of a vial of *methyldopa for system suitability CRS* (containing impurities A, B and C) in 1.0 mL of *0.1 M hydrochloric acid.*

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *di-isobutyloctadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 8 nm.

*Mobile phase*: *methanol R, 0.1 M phosphate buffer solution pH 3.0 R* (15:85 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of methyldopa.

*Identification of impurities*: use the chromatogram supplied with *methyldopa for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to methyldopa (retention time = about 5 min): impurity A = about 1.9; impurity B = about 4.3; impurity C = about 4.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities B and C.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 2.6; impurity C = 1.3;
- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Water** (*2.5.12*): 10.0 per cent to 13.0 per cent, determined on 0.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g, heating if necessary, in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.12 mg of $C_{10}H_{13}NO_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

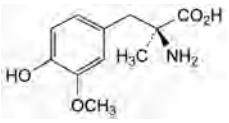


A. (2*S*)-2-amino-3-(4-hydroxy-3-methoxyphenyl)-2-methylpropanoic acid (3-methoxymethyldopa),

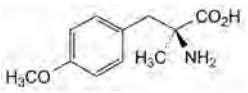


B. (2*S*)-2-amino-3-(4-methoxyphenyl)-2-methylpropanoic acid,

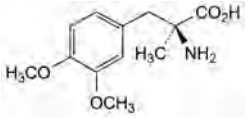


C. (2*S*)-2-amino-3-(3,4-dimethoxyphenyl)-2-methylpropanoic acid,

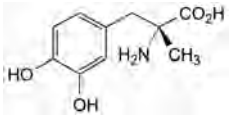


D. (2*R*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acid (D-methyldopa).

**01/2017:0932**
**corrected 10.0**

# METHYLENE CHLORIDE

## Methyleni chloridum

$CH_2Cl_2$ $M_r$ 84.9
[75-09-2]

DEFINITION

Dichloromethane.

It may contain maximum 2.0 per cent *V/V* of anhydrous ethanol and/or maximum 0.03 per cent *V/V* of 2-methylbut-2-ene as stabiliser.

CHARACTERS

*Appearance*: clear, colourless, volatile liquid.

*Solubility*: sparingly soluble in water, miscible with ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, D, E.*

A. Relative density (see Tests).

B. Refractive index (see Tests).

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: films.
*Comparison*: *methylene chloride CRS*.

D. Heat 2 mL with 2 g of *potassium hydroxide R* and 20 mL of *ethanol (96 per cent) R* under a reflux condenser for 30 min. Allow to cool. Add 15 mL of *dilute sulfuric acid R* and filter. To 1 mL of the filtrate add 1 mL of a 15 g/L solution of *chromotropic acid, sodium salt R*, 2 mL of *water R* and 8 mL of *sulfuric acid R*. A violet colour is produced.

E. 2 mL of the filtrate obtained in identification test D gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 50 mL of *methanol R* previously neutralised to 0.1 mL of *bromothymol blue solution R1*, add 50 g of the substance to be examined. Not more than 0.15 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Relative density** (*2.2.5*): 1.320 to 1.332.

**Refractive index** (*2.2.6*): 1.423 to 1.425.

**Ethanol, 2-methylbut-2-ene and volatile impurities**. Gas chromatography (*2.2.28*).

*Test solution*. The substance to be examined.

*Reference solution (a)*. Dilute 100 µL of *carbon tetrachloride R* (impurity A), 500 µL of *chloroform R* (impurity B), 3.0 mL of *2-methylbut-2-ene R* and 5.0 mL of *methanol R* (impurity D) to 100.0 mL with the test solution.

*Reference solution (b)*. Dilute 2.0 mL of *anhydrous ethanol R* and 1.0 mL of reference solution (a) to 100.0 mL with the test solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm).

*Carrier gas*: *nitrogen for chromatography R*.
*Flow rate*: 1.0 mL/min, constant flow.
*Split ratio*: 1:40.
*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 40 |
| | 5 - 12.5 | 40 → 55 |
| | 12.5 - 18 | 55 → 100 |
| | 18 - 20 | 100 |
| Injection port | | 260 |
| Detector | | 300 |

*Detection*: flame ionisation; make-up gas flow rate: 25 mL/min.

*Injection*: 2 µL of the test solution and reference solution (b).

*Relative retention* with reference to methylene chloride (retention time = about 7 min): impurity D = about 0.6; ethanol = about 0.8; 2-methylbut-2-ene = about 0.9; impurity B = about 1.7; impurity A = about 1.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.0 between the peaks due to ethanol and 2-methylbut-2-ene;
- *signal-to-noise ratio*: minimum 5 for the peak due to impurity A.

*Calculation of percentage contents*:
- for ethanol, 2-methylbut-2-ene and impurities A and B, use the respective concentration of these substances in reference solution (b); correct the areas of the peaks in the chromatogram obtained with reference solution (b) by subtracting the area of the corresponding peak in the chromatogram obtained with the test solution;
- for any other impurity, use the concentration of impurity D in reference solution (b); correct the area of the peak due to impurity D in the chromatogram obtained with reference solution (b) by subtracting the area of the corresponding peak in the chromatogram obtained with the test solution.

*Limits*:
- *ethanol*: maximum 2.0 per cent *V/V*;
- *2-methylbut-2-ene*: maximum 300 ppm *V/V*;
- *impurity A*: maximum 10 ppm *V/V*;
- *impurity B*: maximum 50 ppm *V/V*;
- *sum of impurities other than ethanol and 2-methylbut-2-ene*: maximum 0.1 per cent *V/V*;
- *reporting threshold*: 50 ppm *V/V*; the reporting threshold does not apply to impurity A.

**Free chlorine**. Place 5 mL in a ground-glass-stoppered tube. Add 5 mL of a 100 g/L solution of *potassium iodide R* and 0.2 g of *soluble starch R*. Shake for 30 s and allow to stand for 5 min. No blue colour develops.

**Residue on evaporation**: maximum 20 ppm.

Evaporate 50.0 g to dryness on a water-bath and dry at 100-105 °C for 30 min. The residue weighs a maximum of 1 mg.

**Water** (*2.5.32*): maximum 0.02 per cent *m/m*, determined on 10.00 g.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the name and concentration of any stabilisers.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

A. carbon tetrachloride,

B. trichloromethane (chloroform),

D. methanol.

**07/2009:1788**

# METHYLERGOMETRINE MALEATE

## Methylergometrini maleas

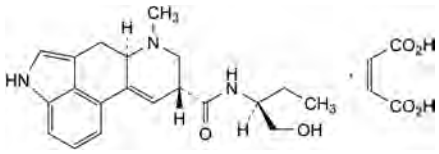

$C_{24}H_{29}N_3O_6$ $M_r$ 455.5
[57432-61-8]

DEFINITION

(6a*R*,9*R*)-*N*-[(1*S*)-1-(Hydroxymethyl)propyl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: soluble in water, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methylergometrine maleate CRS*.

TESTS

**Solution S**. Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 4.4 to 5.2.

Dilute 2.0 mL of solution S to 50.0 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 44.0 to + 50.0 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25 mg of the substance to be examined in 15 mL of mobile phase B and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve the contents of a vial of *methylergometrine for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H and I) in 1.0 mL of a mixture of 30 volumes of mobile phase B and 70 volumes of *water R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

– *mobile phase A*: 2 g/L solution of *ammonium carbamate R*;
– *mobile phase B*: *acetonitrile R*, *water R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 7 | 85 → 65 | 15 → 35 |
| 7 - 12 | 65 | 35 |
| 12 - 17 | 65 → 20 | 35 → 80 |
| 17 - 19 | 20 | 80 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 310 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *methylergometrine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to methylergometrine (retention time = about 12 min): impurity A = about 0.2; impurity B = about 0.5; impurity C = about 0.6; impurity D = about 0.7; impurity I = about 1.10; impurity E = about 1.14; impurity F = about 1.2; impurity G = about 1.3; impurity H = about 1.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to methylergometrine and impurity I; minimum 1.5 between the peaks due to impurities I and E.

*Limits*:

– *impurity I*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *impurities A, B, D, E, F, G, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 45.55 mg of $C_{24}H_{29}N_3O_6$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

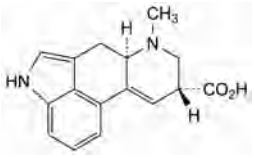

A. (6a*R*,9*R*)-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxylic acid,

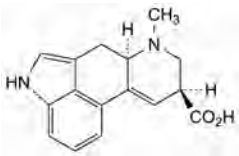

B. (6a*R*,9*S*)-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxylic acid,

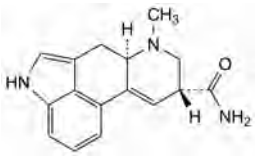

C. (6a*R*,9*R*)-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide,

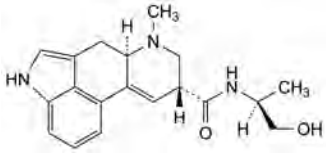

D. (6a*R*,9*R*)-*N*-[(1*S*)-2-hydroxy-1-methylethyl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergometrine),

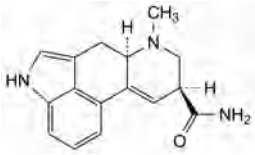

E. (6a*R*,9*S*)-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide,

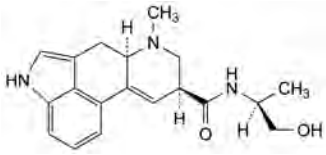

F. (6a*R*,9*S*)-*N*-[(1*S*)-2-hydroxy-1-methylethyl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (ergometrinine),

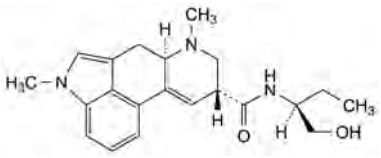

G. (6a*R*,9*R*)-*N*-[(1*S*)-1-(hydroxymethyl)propyl]-4,7-dimethyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (methysergide),

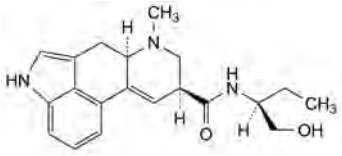

H. (6a*R*,9*S*)-*N*-[(1*S*)-1-(hydroxymethyl)propyl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (methylergometrinine),

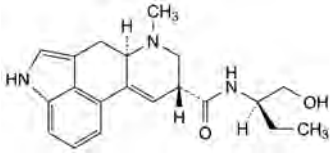

I. (6a*R*,9*R*)-*N*-[(1*R*)-1-(hydroxymethyl)propyl]-7-methyl-4,6,6a,7,8,9-hexahydroindolo[4,3-*fg*]quinoline-9-carboxamide (1′-*epi*-methylergometrine).

**01/2017:0346**

# METHYLHYDROXYETHYLCELLULOSE

## Methylhydroxyethylcellulosum

DEFINITION

Partly *O*-methylated and *O*-(2-hydroxyethylated) cellulose.

CHARACTERS

*Appearance*: white, yellowish-white or greyish-white powder or granules, hygroscopic after drying.

*Solubility*: practically insoluble in hot water, in acetone, in anhydrous ethanol and in toluene. It dissolves in cold water giving a colloidal solution.

IDENTIFICATION

A. Heat 10 mL of solution S (see Tests) in a water-bath while stirring. At a temperature above 50 °C, the solution becomes cloudy or a flocculent precipitate is formed. The solution becomes clear again on cooling.

B. To 10 mL of solution S add 0.3 mL of *dilute acetic acid R* and 2.5 mL of a 100 g/L solution of *tannic acid R*. A yellowish-white flocculent precipitate is formed which dissolves in *dilute ammonia R1*.

C. In a test-tube about 160 mm long, thoroughly mix 1 g with 2 g of finely powdered *manganese sulfate R*. Introduce to a depth of 2 cm into the upper part of the tube a strip of filter paper impregnated with a freshly prepared mixture of 1 volume of a 20 per cent *V/V* solution of *diethanolamine R* and 11 volumes of a 50 g/L solution of *sodium nitroprusside R*, adjusted to about pH 9.8 with *1 M hydrochloric acid*. Insert the tube 8 cm into a silicone-oil bath at 190-200 °C. The filter paper becomes blue within 10 min. Carry out a blank test.

D. Dissolve completely 0.2 g without heating in 15 mL of a 70 per cent *m/m* solution of *sulfuric acid R*. Pour the solution with stirring into 100 mL of iced *water R* and dilute to 250 mL with iced *water R*. In a test-tube, mix thoroughly

while cooling in iced water 1 mL of this solution with 8 mL of *sulfuric acid R* added dropwise. Heat in a water-bath for exactly 3 min and immediately cool in iced water. While the mixture is cold, carefully add 0.6 mL of *ninhydrin solution R2* and mix well. Allow to stand at 25 °C. A pink colour is produced immediately and does not become violet within 100 min.

E. Place 1 mL of solution S on a glass plate. After evaporation of the water a thin film is formed.

TESTS

**Solution S**. While stirring, introduce a quantity of the substance to be examined equivalent to 1.0 g of the dried substance into 50 g of *carbon dioxide-free water R* heated to 90 °C. Allow to cool, adjust the mass of the solution to 100 g with *carbon dioxide-free water R* and stir until dissolution is complete.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 8.0 for solution S.

**Apparent viscosity** (*2.2.10*): 75 per cent to 140 per cent of the value stated on the label.

While stirring, introduce a quantity of the substance to be examined equivalent to 6.00 g of the dried substance into 150 g of *water R* heated to 90 °C. Stir with a propeller-type stirrer for 10 min, place the flask in a bath of iced water, continue the stirring and allow to remain in the bath of iced water for 40 min to ensure that dissolution is complete. Adjust the mass of the solution to 300 g and centrifuge the solution to expel any entrapped air. Adjust the temperature of the solution to 20 ± 0.1 °C. Determine the viscosity with a rotating viscometer at 20 °C and a shear rate of 10 $s^{-1}$.

**Chlorides** (*2.4.4*): maximum 0.5 per cent.

Dilute 1 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.000 g.

LABELLING

The label states the apparent viscosity in millipascal seconds for a 2 per cent *m/m* solution.

**01/2017:2235**

# METHYLPHENIDATE HYDROCHLORIDE

## Methylphenidati hydrochloridum

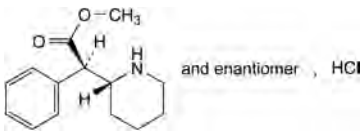


$C_{14}H_{20}ClNO_2$ $M_r$ 269.8
[298-59-9]

DEFINITION

Methyl (2*RS*)-phenyl[(2*RS*)-piperidin-2-yl]acetate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methylphenidate hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in 1.0 mL of *methanol R.*

*Reference solution.* Dissolve 5 mg of *methylphenidate hydrochloride CRS* in 1.0 mL of *methanol R.*

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:4:95 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 60 °C for 5 min.

*Detection*: spray with a freshly prepared 5 g/L solution of *fast blue B salt R*; heat to 60 °C for 1 min.

*Result*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 20 volumes of *acetonitrile R1* and 80 volumes of a solution prepared as follows: dissolve 1.36 g of *sodium octanesulfonate R* in 950 mL of *water for chromatography R*, add 1.0 mL of *triethylamine R2*, adjust to pH 2.7 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R.*

*Test solution.* Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2 mg of *methylphenidate impurity C CRS* in 100.0 mL of the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *methylphenidate impurity mixture CRS* (impurities A and B) in 1.0 mL of reference solution (a).

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.075 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.16 g of *sodium octanesulfonate R* in 950 mL of *water for chromatography R*, add 1.0 mL of *triethylamine R2*, adjust to pH 2.7 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 80 | 20 |
| 15 - 35 | 80 → 60 | 20 → 40 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *methylphenidate impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to methylphenidate (retention time = about 20 min): impurity A = about 0.35; impurity C = about 0.40; impurity B = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities A and C.

*Limits*:

– *impurities A, B*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide* and an electrode for non-aqueous acid-base titrations. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 26.98 mg of $C_{14}H_{20}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F.*

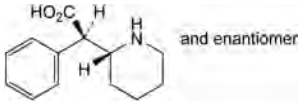


A. (2*RS*)-phenyl[(2*RS*)-piperidin-2-yl]acetic acid,

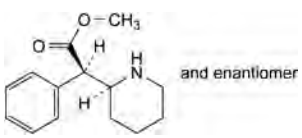


B. methyl (2*RS*)-phenyl[(2*SR*)-piperidin-2-yl]acetate,

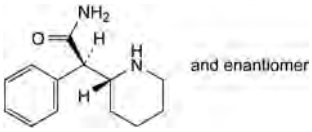


C. (2*RS*)-2-phenyl-2-[(2*RS*)-piperidin-2-yl]acetamide,

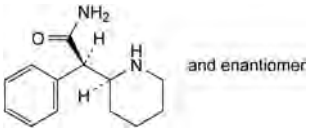


D. (2*RS*)-2-phenyl-2-[(2*SR*)-piperidin-2-yl]acetamide,

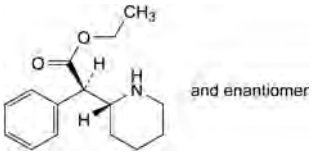


E. ethyl (2*RS*)-phenyl[(2*RS*)-piperidin-2-yl]acetate,

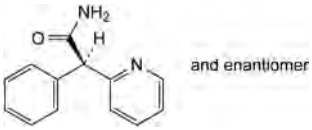


F. (2*RS*)-2-phenyl-2-(pyridin-2-yl)acetamide.

**01/2011:0189**

# METHYLPHENOBARBITAL

## Methylphenobarbitalum

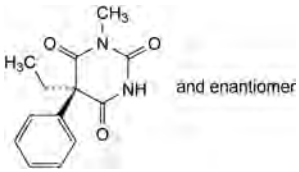


$C_{13}H_{14}N_2O_3$ $M_r$ 246.3
[115-38-8]

DEFINITION

(5*RS*)-5-Ethyl-1-methyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, very slightly soluble in ethanol (96 per cent).

It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *methylphenobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 178 °C) is not greater than 2 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methylphenobarbital CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *methylphenobarbital CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine immediately in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To about 10 mg add 0.2 mL of *sulfuric acid R* and 0.1 mL of *nitric acid R*. Heat on a water-bath for 10 min. Cool in iced water and add 5 mL of *water R* and 5 mL of *strong sodium hydroxide solution R*. Add 5 mL of *acetone R*, shake and allow to stand. A dark-red colour develops in the upper layer.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g, with gentle heating, in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 6 mL of *water R*.

**Acidity**. Boil 1.0 g with 50 mL of *water R* for 2 min, allow to cool and filter. To 10 mL of the filtrate add 0.15 mL of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to produce a pure yellow colour.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 10.0 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *phenobarbital CRS* (impurity A) in 1.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*. Dissolve 6.60 g of *sodium acetate R* in 900 mL of *water R*, add 3 mL of *glacial acetic acid R*, adjust to pH 4.5 with *glacial acetic acid R* and dilute to 1000 mL with *water R*. Mix 40 volumes of this solution with 60 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of methylphenobarbital.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to methylphenobarbital (retention time = about 7 min): impurity A = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and methylphenobarbital.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 70 mL of *ethanol (96 per cent) R* and add 20 mL of *water R*. Stir with a mechanical stirrer for about 30 min and sonicate to achieve complete dissolution. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.63 mg of $C_{13}H_{14}N_2O_3$.

IMPURITIES

*Specified impurities: A.*

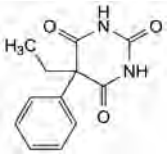

A. 5-ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (phenobarbital).

**04/2016:0561**

# METHYLPREDNISOLONE

## Methylprednisolonum

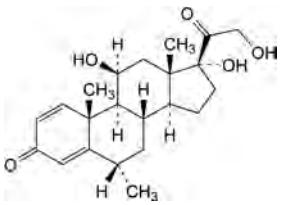

$C_{22}H_{30}O_5$ $M_r$ 374.5
[83-43-2]

DEFINITION

11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent), slightly soluble in acetone and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methylprednisolone CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 2 mL with the same solvent (solution A). Dilute 1 mL of solution A to 5 mL with *methylene chloride R*.

*Test solution (b).* Transfer 0.4 mL of solution A to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker protected from light for 1 h. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 mL separating funnel, washing the filter with 2 quantities, each of 5 mL, of *water R*. Shake the clear filtrate with 10 mL of *methylene chloride R*. Wash the organic layer with 5 mL of *1 M sodium hydroxide* and 2 quantities, each of 5 mL, of *water R*. Dry over *anhydrous sodium sulfate R*.

*Reference solution (a).* Dissolve 10 mg of *methylprednisolone CRS* in *methanol R* and dilute to 2 mL with the same solvent (solution B). Dilute 1 mL of solution B to 5 mL with *methylene chloride R*.

*Reference solution (b).* Transfer 0.4 mL of solution B to a glass tube 100 mm long and 20 mm in diameter and fitted with a ground-glass stopper or a polytetrafluoroethylene cap and evaporate the solvent with gentle heating under a stream of *nitrogen R*. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and 50 mg of *sodium bismuthate R*. Stopper the tube and shake the suspension in a mechanical shaker protected from light for 1 h. Add 2 mL of a 15 per cent *V/V* solution of *glacial acetic acid R* and filter into a 50 mL separating funnel, washing the filter with 2 quantities, each of 5 mL, of *water R*. Shake the clear filtrate with 10 mL of *methylene chloride R*. Wash the organic layer with 5 mL of *1 M sodium hydroxide* and 2 quantities, each of 5 mL, of *water R*. Dry over *anhydrous sodium sulfate R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *butanol R* saturated with *water R*, *toluene R*, *ether R* (5:10:85 *V/V/V*).

*Application*: 5 µL of test solution (a) and reference solution (a), 10 µL of test solution (b) and reference solution (b), applying the latter 2 in small quantities in order to obtain small spots.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position and size to the principal spot in the chromatogram obtained with the corresponding reference solution.

*Detection B*: spray with *alcoholic solution of sulfuric acid R* and heat at 120 °C for 15 min. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in each of the chromatograms obtained with the test solutions is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with the corresponding reference solution. The principal spot in each of the chromatograms obtained with test solution (b) and reference solution (b) have an $R_F$ value distinctly higher than that of the principal spot in each of the chromatograms obtained with test solution (a) and reference solution (a).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, brownish-red fluorescence is seen. Add this solution to 10 mL of *water R* and mix. The colour fades and there is a yellowish-green fluorescence in ultraviolet light at 365 nm.

TESTS

**Specific optical rotation** (*2.2.7*): + 97.0 to + 103.0 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *phosphoric acid R*, *acetonitrile R*, *water R* (0.1:50:50 *V/V/V*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 3 mg of *methylprednisolone for system suitability A CRS* (containing impurities A, B, C, D, E, F, G, H and I) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 30.0 mg of *methylprednisolone CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R*, *tetrahydrofuran R*, *acetonitrile R*, *water R* (0.1:1.5:10:90 *V/V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *tetrahydrofuran R*, *acetonitrile R* (0.1:1.5:100 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 83 | 17 |
| 14 - 30 | 83 → 52 | 17 → 48 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 247 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *methylprednisolone for system suitability A CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F, G, H and I.

*Relative retention* with reference to methylprednisolone (retention time = about 12 min): impurity B = about 0.85; impurity H = about 0.88; impurity A = about 0.92; impurity F = about 1.1; impurities G and I = about 1.54; impurity C = about 1.7; impurity E = about 1.9; impurity D (isomer 1) = about 2.10; impurity D (isomer 2) = about 2.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.7 between the peaks due to impurity A and methylprednisolone;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to methylprednisolone.

*Limits*:

- *impurity D*: for the sum of the areas of the 2 isomer peaks, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity A*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *sum of impurities G and I*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities B, H*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities C, E, F*: for each impurity, not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

## ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{22}H_{30}O_5$ taking into account the assigned content of *methylprednisolone CRS*.

## STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

## IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L.*

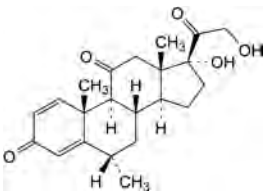

A. 17,21-dihydroxy-6α-methylpregna-1,4-diene-3,11,20-trione,

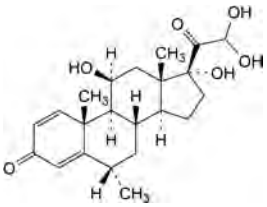

B. 11β,17,21,21-tetrahydroxy-6α-methylpregna-1,4-diene-3,20-dione,

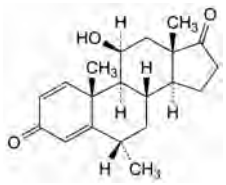

C. 11β-hydroxy-6α-methylandrosta-1,4-diene-3,17-dione,

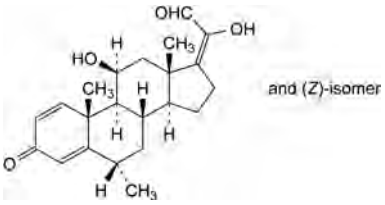


D. (*EZ*)-11β,20-dihydroxy-6α-methylpregna-1,4,17(20)-triene-3,21-dione,

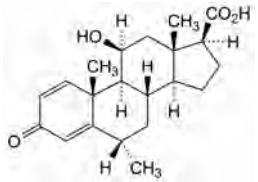

E. 11β-hydroxy-6α-methyl-3-oxoandrosta-1,4-diene-17β-carboxylic acid,

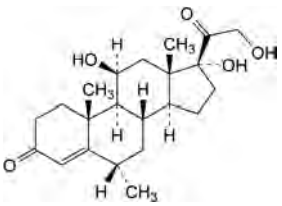

F. 11β,17,21-trihydroxy-6α-methylpregn-4-ene-3,20-dione,

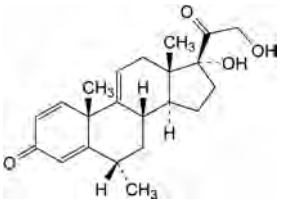

G. 17,21-dihydroxy-6α-methylpregna-1,4,9(11)-triene-3,20-dione,

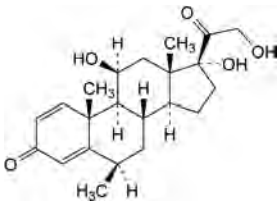

H. 11β,17,21-trihydroxy-6β-methylpregna-1,4-diene-3,20-dione,

I. unknown structure,

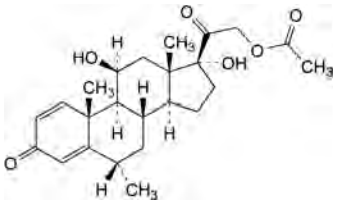

J. 11β,17-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (methylprednisolone acetate),

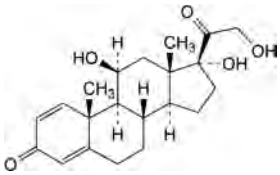

K. 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone),

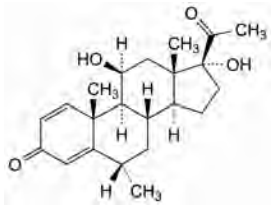

L. 11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione.

**04/2018:0933**

# METHYLPREDNISOLONE ACETATE

## Methylprednisoloni acetas

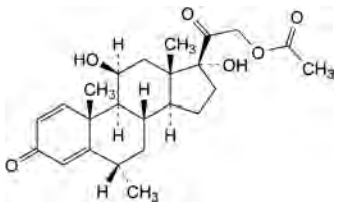

$C_{24}H_{32}O_6$ $M_r$ 416.5
[53-36-1]

DEFINITION

11β,17-Dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone and in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methylprednisolone acetate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness and record new spectra using the residues.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**Specific optical rotation** (*2.2.7*): + 107 to + 113 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Mix 20 mL of a 103 g/L solution of *hydrochloric acid R*, 50 mL of a 68 g/L solution of *sodium acetate R* and 150 mL of a 37.3 g/L solution of *potassium chloride R* and dilute to 1.0 L with *water R*.

*Solvent mixture*: *acetonitrile R*, buffer solution (50:50 *V/V*).

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *methylprednisolone acetate for system suitability CRS* (containing impurities E, G and J) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *methylprednisolone acetate for peak identification CRS* (impurities A and K) in 1.0 mL of the solvent mixture.

*Reference solution (d)*. Dissolve 20.0 mg of *methylprednisolone acetate CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R, water for chromatography R* (28:72 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 95 | 5 |
| 15 - 25 | 95 → 50 | 5 → 50 |
| 25 - 28 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *methylprednisolone acetate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities E, G and J; use the chromatogram supplied with *methylprednisolone acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and K.

*Relative retention* with reference to methylprednisolone acetate (retention time = about 15 min): impurity A = about 0.4; impurity E = about 0.6; impurity J = about 0.9; impurity G = about 1.1; impurity K = about 1.2.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to methylprednisolone acetate.

*Calculation of percentage contents*:

- for each impurity, use the concentration of methylprednisolone acetate in reference solution (b).

*Limits*:

- *impurity J*: maximum 0.3 per cent;
- *impurities A, G*: for each impurity, maximum 0.2 per cent;
- *impurities E, K*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *acetonitrile R*, *water for chromatography R* (32:68 *V/V*).

*Injection*: test solution (b) and reference solution (d).

*Run time*: 1.5 times the retention time of methylprednisolone acetate.

*Retention time*: methylprednisolone acetate = about 15 min.

Calculate the percentage content of $C_{24}H_{32}O_6$ taking into account the assigned content of *methylprednisolone acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, E, G, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, H, I.*

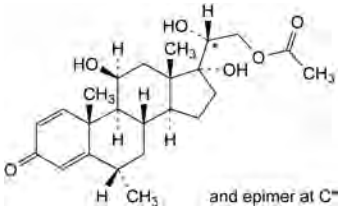


A. (20*RS*)-11β,17,20-trihydroxy-6α-methyl-3-oxopregna-1,4-dien-21-yl acetate,

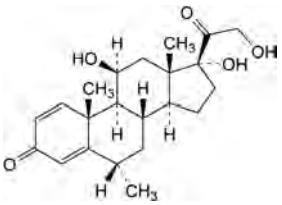

B. 11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione (methylprednisolone),

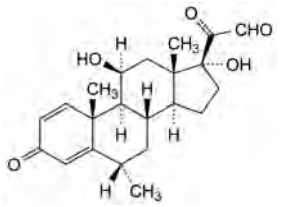

C. 11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20,21-trione,

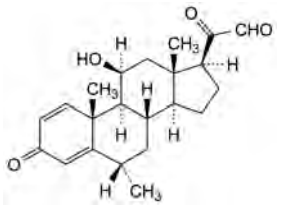

D. 11β-hydroxy-6α-methylpregna-1,4-diene-3,20,21-trione,

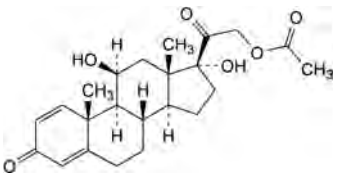

E. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (prednisolone acetate),

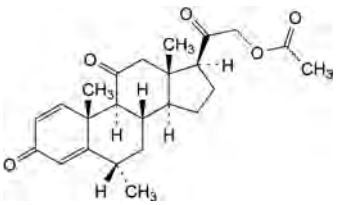

F. 6α-methyl-3,11,20-trioxopregna-1,4-dien-21-yl acetate,

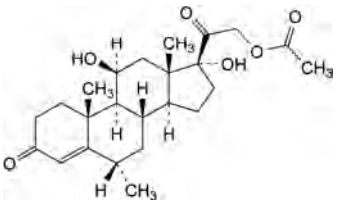

G. 11β,17-dihydroxy-6α-methyl-3,20-dioxopregn-4-en-21-yl acetate,

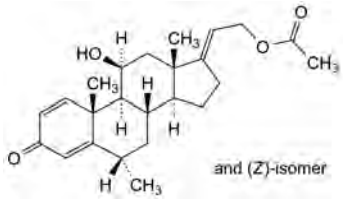


H. (*EZ*)-11β-hydroxy-6α-methyl-3-oxopregna-1,4,17(20)-trien-21-yl acetate,

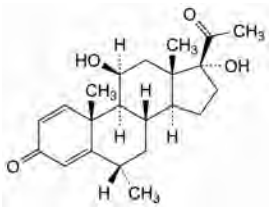

I. 11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione,

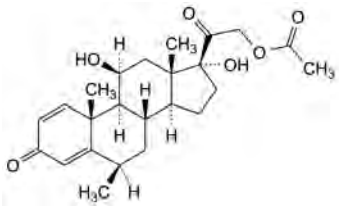

J. 11β,17-dihydroxy-6β-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

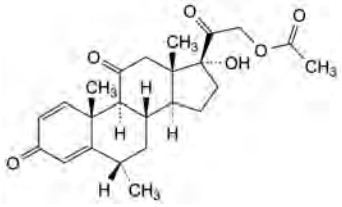

K. 17-hydroxy-6α-methyl-3,11,20-trioxopregna-1,4-dien-21-yl acetate.

**01/2016:1131**

# METHYLPREDNISOLONE HYDROGEN SUCCINATE

## Methylprednisoloni hydrogenosuccinas

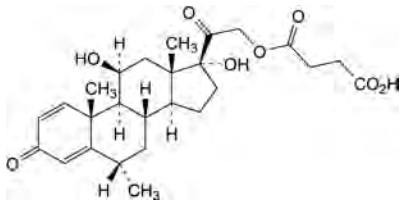

$C_{26}H_{34}O_8$ $M_r$ 474.5
[2921-57-5]

DEFINITION

4-[(11β,17-Dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl)oxy]-4-oxobutanoic acid.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water, slightly soluble in acetone and in anhydrous ethanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *methylprednisolone hydrogen succinate CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (e).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.100 g in 5 mL of *sodium hydrogen carbonate solution R*.

**Specific optical rotation** (*2.2.7*): + 97 to + 104 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in 1 mL of *tetrahydrofuran R* and dilute to 20.0 mL with the mobile phase.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *methylprednisolone hydrogen succinate for performance test CRS* (containing impurity D) in 0.25 mL of *tetrahydrofuran R* and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *methylprednisolone hydrogen succinate for peak identification CRS* (containing impurity F) in 0.25 mL of *tetrahydrofuran R* and dilute to 5.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 5 mg of *methylprednisolone R* (impurity A) in 0.25 mL of *tetrahydrofuran R* and dilute to 5.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 20.0 mg of *methylprednisolone hydrogen succinate CRS* in 1 mL of *tetrahydrofuran R* and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (2:31:67 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (c) and (d).

*Run time*: twice the retention time of methylprednisolone hydrogen succinate.

*Identification of impurities*: use the chromatogram supplied with *methylprednisolone hydrogen succinate for performance test CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity D; use the chromatogram supplied with *methylprednisolone hydrogen succinate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity F; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to methylprednisolone hydrogen succinate (retention time = about 21 min): impurity A = about 0.5; impurity F = about 0.9; impurity D = about 1.1.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to methylprednisolone hydrogen succinate.

*Calculation of percentage contents*:

– for each impurity, use the concentration of methylprednisolone hydrogen succinate in reference solution (b).

*Limits*:

– *impurity A*: maximum 0.3 per cent;
– *impurity F*: maximum 0.2 per cent;
– *impurity D*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (e).

Calculate the percentage content of $C_{26}H_{34}O_8$ taking into account the assigned content of *methylprednisolone hydrogen succinate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, G.*

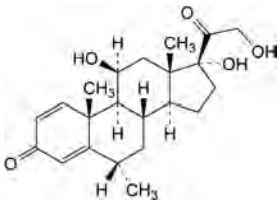

A. 11β,17,21-trihydroxy-6α-methylpregna-1,4-diene-3,20-dione (methylprednisolone),

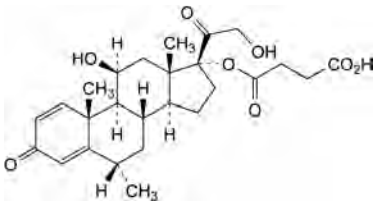

B. 4-[(11β,21-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-17-yl)oxy]-4-oxobutanoic acid (methylprednisolone 17-(hydrogen succinate)),

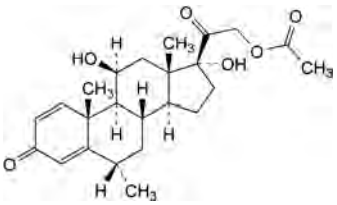

C. 11β,17-dihydroxy-6α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate (methylprednisolone acetate),

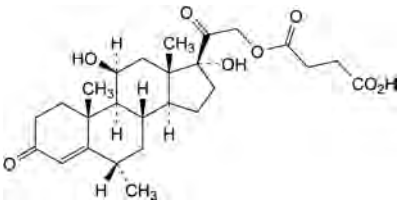

D. 4-[(11β,17-dihydroxy-6α-methyl-3,20-dioxopregn-4-en-21-yl)oxy]-4-oxobutanoic acid (methylhydrocortisone 21-(hydrogen succinate)),

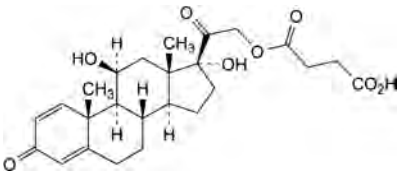

E. 4-[(11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl)oxy]-4-oxobutanoic acid,

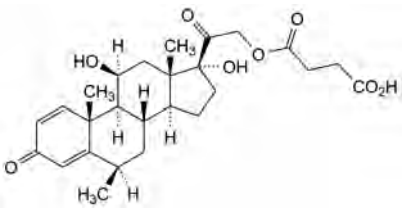

F. 4-[(11β,17-dihydroxy-6β-methyl-3,20-dioxopregna-1,4-dien-21-yl)oxy]-4-oxobutanoic acid,

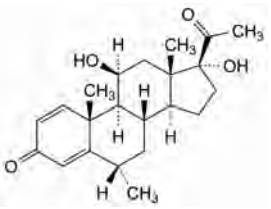

G. 11β,17-dihydroxy-6α-methylpregna-1,4-diene-3,20-dione.

**01/2017:1675**
**corrected 10.0**

# *N*-METHYLPYRROLIDONE

## *N*-Methylpyrrolidonum

$C_5H_9NO$ $M_r$ 99.1
[872-50-4]

DEFINITION

1-Methylpyrrolidin-2-one.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Solubility*: miscible with water and with alcohol.

bp: about 204 °C.

*Relative density*: about 1.034.

*Refractive index*: about 1.469.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: films.

*Comparison*: *Ph. Eur. reference spectrum of N-methylpyrrolidone.*

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Alkalinity**. Dissolve 50 mL of the substance to be examined in 50 mL of *water R* previously adjusted with *0.02 M potassium hydroxide* or *0.02 M hydrochloric acid* until a yellow colour is obtained using 0.5 mL of *bromothymol blue solution R1* as indicator. Titrate with *0.02 M hydrochloric acid* to the initial coloration. Not more than 8.0 mL of *0.02 M hydrochloric acid* is required.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution.* To 1 mL of the substance to be examined, add 1 mL of *2-pyrrolidone R* and dilute to 20 mL with *methylene chloride R*.

*Column*:

– *material*: fused silica,

– *size*: *l* = 30 m, Ø = 0.32 mm,

– *stationary phase*: *methylpolysiloxane R* (5 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Linear velocity*: 20 cm/s.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 100 |
| | 0 - 23.3 | 100 → 170 |
| | 23.3 - 53 | 170 |
| Injection port | | 280 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to *N*-methylpyrrolidone and impurity G.

*Limits*:

– *any impurity*: maximum 0.1 per cent,

– *total*: maximum 0.3 per cent,

– *disregard limit*: 0.02 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.000 g.

STORAGE

Protected from light.

IMPURITIES

A. methanamine (methylamine),

B. dihydrofuran-2(3*H*)-one (γ-butyrolactone),

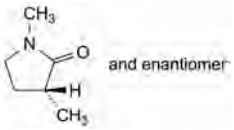

C. (3*RS*)-1,3-dimethylpyrrolidin-2-one,

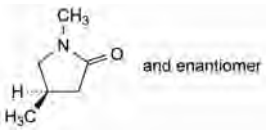

D. (4*RS*)-1,4-dimethylpyrrolidin-2-one,

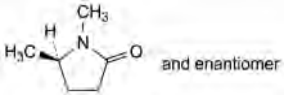

E. (5*RS*)-1,5-dimethylpyrrolidin-2-one,

F. butane-1,4-diol,

G. pyrrolidin-2-one (2-pyrrolidone),

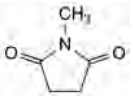

H. 1-methylpyrrolidine-2,5-dione (*N*-methylsuccinimide),

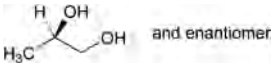

I. (*RS*)-propane-1,2-diol (propylene glycol).

**04/2018:1990**

# METHYLROSANILINIUM CHLORIDE

## Methylrosanilinii chloridum

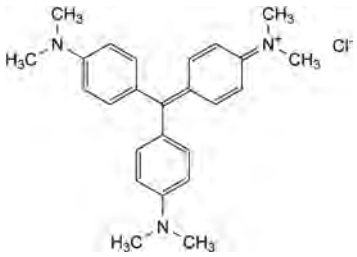

$C_{25}H_{30}ClN_3$ $M_r$ 408.0

[548-62-9]

DEFINITION

4-[Bis[4-(dimethylamino)phenyl]methylidene]-*N*,*N*-dimethylcyclohexa-2,5-dien-1-iminium chloride (hexamethyl-*p*-rosanilinium chloride). It is also known as crystal violet or gentian violet.

*Content*: 95.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: dark green, shiny powder, hygroscopic.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: A.

*Second identification*: B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methylrosanilinium chloride CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *methylrosanilinium chloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (17:17:66 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution. In the chromatogram obtained with the test solution, a secondary spot may be observed.

C. Dissolve 50 mg in *water R* and dilute to 5 mL with the same solvent; add 3 mL of *dilute sulfuric acid R*, 1 g of *zinc powder R* and heat gently. The mixture decolourises. Filter. To 3 mL of the filtrate add 0.5 mL of *silver nitrate solution R1*. A white turbidity is produced which slowly forms a dark, coagulating precipitate.

TESTS

***N*,*N*-Dimethylaniline** (*2.4.26, Method A*): maximum 100 ppm.

*Test solution*. Dissolve in a ground-glass-stoppered tube 0.50 g of the substance to be examined in 30.0 mL of *water R*. Add 1.0 mL of the internal standard solution. Adjust the solution to 26-28 °C. Add 1.0 mL of *strong sodium hydroxide solution R* and mix for 2 min. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and centrifuge. Use the upper layer.

*Reference solution*. Dissolve 50.0 mg of *N,N-dimethylaniline R* in 4.0 mL of *0.1 M hydrochloric acid* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. To 0.50 g of the substance to be examined add 5.0 mL of this solution and dilute to 30.0 mL with *water R*. Add 1.0 mL of the internal standard solution and 1.0 mL of *strong sodium hydroxide solution R*. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and centrifuge. Use the upper layer.

*Limit*:

– calculate the ratio (*R*) of the area of the peak due to *N*,*N*-dimethylaniline to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the area of the peak due to *N*,*N*-dimethylaniline to the area of the peak due to the internal standard: this ratio is not greater than 0.5 *R*.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 3 mg of *methylrosanilinium for system suitability CRS* (containing impurities A and B) in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *ethanol (96 per cent) R*. Dilute 1.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: spherical *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *water for chromatography R*, *methanol R* (10:190:800 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 589 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of methylrosanilinium.

*Identification of impurities*: use the chromatogram supplied with *methylrosanilinium for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to methylrosanilinium (retention time = about 23 min): impurity A = about 0.71; impurity B = about 0.85.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity B and methylrosanilinium.

*Limits*:

– *impurity B*: maximum 10.0 per cent;

– *impurity A*: maximum 1.0 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *sum of impurities other than A and B*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent (peak due to methylrosanilinium in the chromatogram obtained with reference solution (b)).

**Substances insoluble in ethanol (90 per cent *V/V*)**: maximum 0.5 per cent.

In a conical flask introduce 1.0 g and add 50 mL of *ethanol (90 per cent V/V) R*. Boil under a reflux condenser for 1 h. Filter the warm liquid through a weighed sintered glass filter (16) (*2.1.2*) previously dried at 100-105 °C. Wash with hot *ethanol (90 per cent V/V) R* until a colourless filtrate is obtained. Dry at 100-105 °C until constant weight.

**Water** (*2.5.12*): maximum 10.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 250.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the maximum at 589 nm.

Calculate the content of $C_{25}H_{30}ClN_3$ taking the specific absorbance to be 2605.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: A, B.

A. unknown structure,

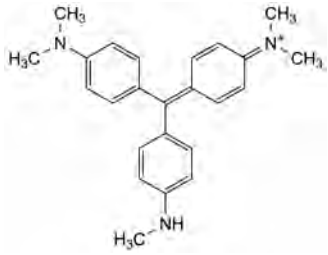

B. 4-[[4-(dimethylamino)phenyl][4-(methylamino)phenyl]methylidene]-*N*,*N*-dimethylcyclohexa-2,5-dien-1-iminium.

**04/2016:0410**

# METHYLTESTOSTERONE

## Methyltestosteronum

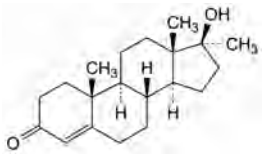

$C_{20}H_{30}O_2$ $M_r$ 302.5
[58-18-4]

DEFINITION

17β-Hydroxy-17-methylandrost-4-en-3-one.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methyltestosterone CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 79 to + 85 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 0.5 mL of the test solution to 100.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 5 mg of *methyltestosterone for system suitability CRS* (containing impurity A) in *methanol R* and dilute to 10 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water R*;
– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 30 | 70 |
| 15 - 45 | 30 → 0 | 70 → 100 |
| 45 - 50 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *methyltestosterone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to methyltestosterone (retention time = about 8 min): impurity A = about 1.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 5 between the peaks due to methyltestosterone and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 2 h.

ASSAY

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*. Dilute 10.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241 nm.

Calculate the content of $C_{20}H_{30}O_2$ taking the specific absorbance to be 540.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A*.

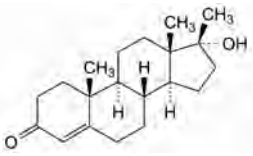

A. 17α-hydroxy-17-methylandrost-4-en-3-one.

07/2019:1132

# METHYLTHIONINIUM CHLORIDE HYDRATE

## Methylthioninii chloridum hydricum

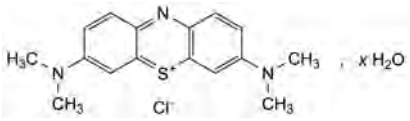

$C_{16}H_{18}ClN_3S,xH_2O$ $M_r$ 319.9 (anhydrous substance)
[122965-43-9]

DEFINITION

3,7-Bis(dimethylamino)phenothiazin-5-ylium chloride (methylene blue) hydrate.

*Content*: 93.0 per cent to 102.0 per cent (dried substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: dark blue or dark green, hygroscopic, crystalline powder with a metallic sheen.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *methylthioninium chloride CRS*.

B. Ignite 50 mg with 0.5 g of *anhydrous sodium carbonate R*. Cool and dissolve the residue in 10 mL of *dilute nitric acid R*. Filter. The filtrate, without further addition of *dilute nitric acid R*, gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, mobile phase A (30:70 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Sonicate for 5 min.

*Reference solution (a)*. Dissolve 50.0 mg of *methylthioninium chloride CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Sonicate for 5 min.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10 mg of *methylthioninium for system suitability CRS* (containing impurity A) in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 25 | 80 → 30 | 20 → 70 |
| 25 - 32 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 246 nm.

*Injection*: 5 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *methylthioninium for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to methylthioninium (retention time = about 11 min): impurity A = about 0.8.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.5 between the peaks due to impurity A and methylthioninium.

*Calculation of percentage contents*:

- for impurity A, use the concentration of methylthioninium chloride hydrate in reference solution (b);
- for impurities other than A, use the concentration of methylthioninium chloride hydrate in reference solution (d).

*Limits*:

- *impurity A*: maximum 5.0 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *sum of impurities other than A*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Elemental impurities**. Any method that fulfils the requirements of general chapter *2.4.20. Determination of elemental impurities* may be used.

| Element | Maximum content (ppm) |
|---|---|
| Aluminium | 100 |
| Cadmium | 1 |
| Chromium | 100 |
| Copper | 300 |
| Iron | 200 |
| Lead | 10 |
| Manganese | 10 |
| Mercury | 1 |
| Molybdenum | 10 |
| Nickel | 10 |
| Tin | 10 |
| Zinc | 100 |

**Loss on drying** (*2.2.32*): 8.0 per cent to 24.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.25 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *symmetry factor*: maximum 3.0 for the principal peak.

Calculate the percentage content of $C_{16}H_{18}ClN_3S$ taking into account the assigned content of *methylthioninium chloride CRS*.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 30 °C.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

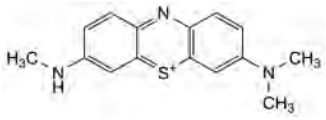

A. 3-(dimethylamino)-7-(methylamino)phenothiazin-5-ylium (azure B),

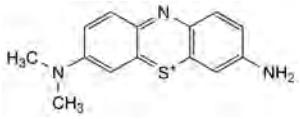

B. 3-amino-7-(dimethylamino)phenothiazin-5-ylium (azure A),

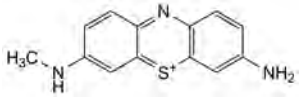

C. 3-amino-7-(methylamino)phenothiazin-5-ylium (azure C).

**01/2008:1347**

# METIXENE HYDROCHLORIDE

## Metixeni hydrochloridum

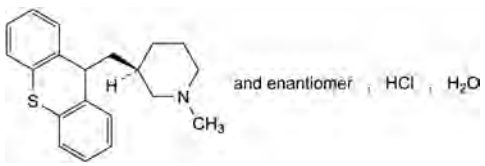


$C_{20}H_{24}ClNS,H_2O$ $M_r$ 363.9
[7081-40-5]

DEFINITION

Metixene hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of (*RS*)-1-methyl-3-[(9*H*-thioxanthen-9-yl)methyl]piperidine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline or fine crystalline powder, soluble in water, soluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *metixene hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 0.40 g in *methanol R* and dilute to 20.0 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

**pH** (*2.2.3*). Dissolve 0.18 g in *carbon dioxide-free water R* heating if necessary at about 50 °C, cool and dilute to 10.0 mL with the same solvent. The pH of the solution, measured immediately, is 4.4 to 5.8.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R. Carry out the test rapidly and protected from light.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *metixene hydrochloride CRS* in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *thioxanthene CRS* in 50 mL of *methylene chloride R*. Dilute 1.0 mL of the solution to 20.0 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve 5 mg of *thioxanthone CRS* in 50 mL of *methylene chloride R*. Dilute 1.0 mL of the solution to 20.0 mL with *methylene chloride R*.

*Reference solution (d).* Dilute 4 mL of reference solution (a) to 10.0 mL with *methylene chloride R*.

Apply to the plate as narrow bands 5 µL of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *glacial acetic acid R*, 10 volumes of *methanol R* and 80 volumes of *methylene chloride R*. Dry the plate in a stream of cold air. Spray with a mixture of 1 volume of *sulfuric acid R* and 9 volumes of *alcohol R* and heat at 100 °C for 10 min. Allow the plate to cool and examine in ultraviolet light at 365 nm. Thioxanthene shows orange fluorescence and thioxanthone shows greenish-blue fluorescence. Any band corresponding to thioxanthene in the chromatogram obtained with the test solution is not more intense than the band in the chromatogram obtained with reference solution (b) (0.2 per cent); any band corresponding to thioxanthone in the chromatogram obtained with the test solution is not more intense than the band in the chromatogram obtained with reference solution (c) (0.05 per cent); any band, apart from the principal band and the bands corresponding to thioxanthene and thioxanthone, is not more intense than the band in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most one such band is more intense than the band in the chromatogram obtained with reference solution (d) (0.2 per cent). The test is not valid unless the bands in the chromatograms obtained with reference solutions (b) and (c) are clearly visible and differentiated.

**Loss on drying** (*2.2.32*). Not less than 4.0 per cent and not more than 6.0 per cent, determined on 0.500 g by drying in an oven at 138-142 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.59 mg of $C_{20}H_{24}ClNS$.

STORAGE

Store protected from light.

IMPURITIES

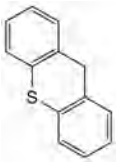

A. 9*H*-thioxanthene,

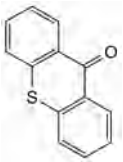

B. 9*H*-thioxanthen-9-one (thioxanthone).

**04/2018:1348**

# METOCLOPRAMIDE

## Metoclopramidum

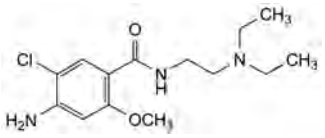

$C_{14}H_{22}ClN_3O_2$ $M_r$ 299.8
[364-62-5]

DEFINITION

4-Amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: practically insoluble in water, sparingly soluble or slightly soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 145 °C to 149 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *metoclopramide CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness at room temperature then heat at 50 °C for about 1 h. Record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*) as described in the test for impurity E with the following modifications.

*Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *metoclopramide CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Detection*: examine in ultraviolet light at 254 nm then spray with *dimethylaminobenzaldehyde solution R1*, allow to dry in air and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution.

TESTS

**Appearance of solution**. The freshly prepared solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 2.5 g in 25 mL of a 103.0 g/L solution of *hydrochloric acid R*.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.160 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *metoclopramide CRS* and 10 mg of *sulpiride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *N,N-diethylethane-1,2-diamine R* (impurity E) in *methanol R* and dilute to 50 mL with the same solvent. Dilute 2 mL of the solution to 25 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *dioxan R*, *methanol R*, *methylene chloride R* (2:10:14:90 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *dimethylaminobenzaldehyde solution R1*, allow to dry in air and examine in daylight.

*Retardation factors*: impurity E = about 0.2; metoclopramide = about 0.6.

*System suitability*: reference solution (a):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity E*: any spot due to impurity E in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (5:95 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *metoclopramide impurity A CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Mix 1 mL of the solution with 0.1 mL of the test solution and dilute to 10 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
– *temperature*: 37 °C.

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium acetate R* in *water for chromatography R* adjusted to pH 7.0 with *ammonia R* or *acetic acid R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1.5 | 95 | 5 |
| 1.5 - 16.5 | 95 → 42.5 | 5 → 57.5 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to metoclopramide (retention time = about 9 min): impurity A = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and metoclopramide.

*Calculation of percentage contents*:

– for each impurity, use the concentration of metoclopramide in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.2 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R* and add 5 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.98 mg of $C_{14}H_{22}ClN_3O_2$.

IMPURITIES

*Specified impurities: E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, G, H.*

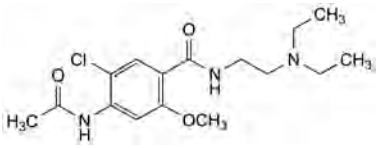

A. 4-acetamido-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide,

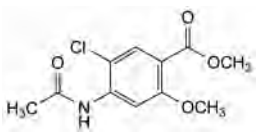

B. methyl 4-acetamido-5-chloro-2-methoxybenzoate,

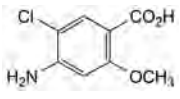

C. 4-amino-5-chloro-2-methoxybenzoic acid,

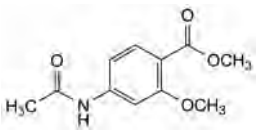

D. methyl 4-acetamido-2-methoxybenzoate,

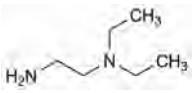

E. *N*,*N*-diethylethane-1,2-diamine,

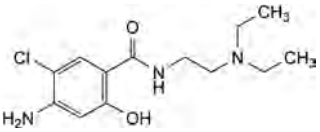

F. 4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-hydroxybenzamide,

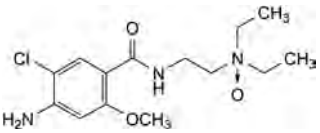

G. 2-(4-amino-5-chloro-2-methoxybenzamido)-*N*,*N*-diethylethan-1-amine N-oxide,

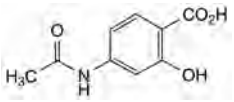

H. 4-acetamido-2-hydroxybenzoic acid.

**04/2018:0674**
**corrected 10.0**

# METOCLOPRAMIDE HYDROCHLORIDE MONOHYDRATE

## Metoclopramidi hydrochloridum monohydricum

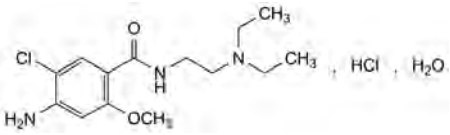

$C_{14}H_{23}Cl_2N_3O_2,H_2O$ $M_r$ 354.3
[54143-57-6]

DEFINITION

4-Amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide hydrochloride monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), sparingly soluble in methylene chloride.

mp: about 183 °C, with decomposition.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D, E.*

A. pH (*2.2.3*): 4.5 to 6.0 for solution S (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *metoclopramide hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*) as described in the test for impurity E with the following modifications.

*Test solution*. Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 20 mg of *metoclopramide hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Detection*: examine in ultraviolet light at 254 nm then spray with *dimethylaminobenzaldehyde solution R1*, allow to dry in air and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dilute 1 mL of solution S to 2 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

E. Dissolve about 2 mg in 2 mL of *water R*. The solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.40 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *N,N-diethylethane-1,2-diamine R* (impurity E) in *methanol R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *dioxan R*, *methanol R*, *methylene chloride R* (2:10:14:90 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *dimethylaminobenzaldehyde solution R1*, allow to dry in air and examine in daylight.

*Retardation factors*: impurity E = about 0.2; metoclopramide = about 0.6.

*Limit*:

– *impurity E*: any spot due to impurity E in the chromatogram obtained with the test solution is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (5:95 *V/V*).

*Test solution*. Dissolve 24.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *metoclopramide impurity A CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture. Mix 1 mL of the solution with 0.1 mL of the test solution and dilute to 10 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);

– *temperature*: 37 °C.

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium acetate R* in *water for chromatography R* adjusted to pH 7.0 with *ammonia R* or *acetic acid R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1.5 | 95 | 5 |
| 1.5 - 16.5 | 95 → 42.5 | 5 → 57.5 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to metoclopramide (retention time = about 9 min): impurity A = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and metoclopramide.

*Calculation of percentage contents*:

– for each impurity, use the concentration of metoclopramide hydrochloride monohydrate in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 4.5 per cent to 5.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 33.63 mg of $C_{14}H_{23}Cl_2N_3O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *E*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, G, H*.

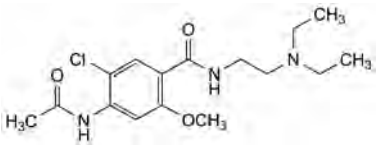


A. 4-acetamido-5-chloro-*N*-[2-(diethylamino)ethyl]-2-methoxybenzamide,

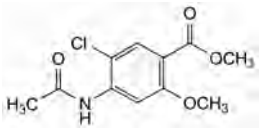

B. methyl 4-acetamido-5-chloro-2-methoxybenzoate,

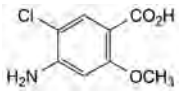

C. 4-amino-5-chloro-2-methoxybenzoic acid,

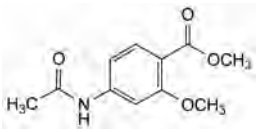

D. methyl 4-acetamido-2-methoxybenzoate,

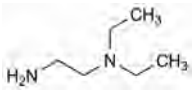

E. *N*,*N*-diethylethane-1,2-diamine,

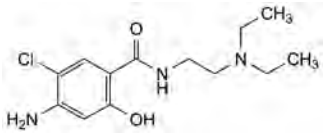

F. 4-amino-5-chloro-*N*-[2-(diethylamino)ethyl]-2-hydroxybenzamide,

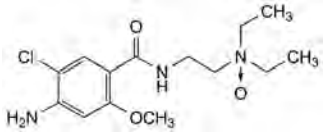

G. 2-(4-amino-5-chloro-2-methoxybenzamido)-*N*,*N*-diethylethan-1-amine *N*-oxide,

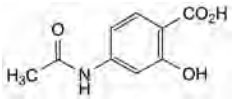

H. 4-acetamido-2-hydroxybenzoic acid.

**01/2017:1757**

# METOLAZONE

## Metolazonum

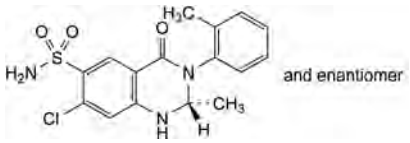


$C_{16}H_{16}ClN_3O_3S$ $M_r$ 365.8

DEFINITION

(2*RS*)-7-Chloro-2-methyl-3-(2-methylphenyl)-4-oxo-1,2,3,4-tetrahydroquinazoline-6-sulfonamide.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethyl acetate, very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *metolazone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Test solution (b)*. Dilute 2.0 mL of test solution (a) to 100.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 3.0 mg of *metolazone for system suitability CRS* (containing impurities A, B, C, D and E) in 1 mL of *methanol R*.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 30.0 mg of *metolazone CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 5.44 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 70 | 30 |
| 5 - 25 | 70 → 50 | 30 → 50 |
| 25 - 35 | 50 | 50 |
| 35 - 38 | 50 → 70 | 50 → 30 |
| 38 - 48 | 70 | 30 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *metolazone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D and E.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.6 between the peaks due to impurities E and C and minimum 1.5 between the peaks due to impurities A and B.

*Limits*:

- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{16}H_{16}ClN_3O_3S$ from the declared content of *metolazone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

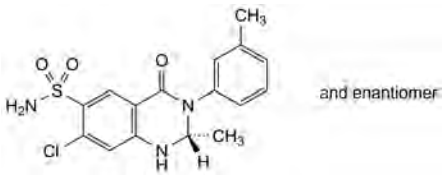


A. (2*RS*)-7-chloro-2-methyl-3-(3-methylphenyl)-4-oxo-1,2,3,4-tetrahydroquinazoline-6-sulfonamide,

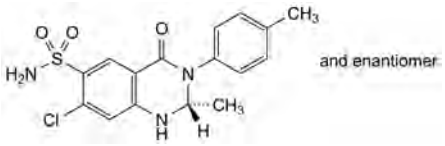


B. (2*RS*)-7-chloro-2-methyl-3-(4-methylphenyl)-4-oxo-1,2,3,4-tetrahydroquinazoline-6-sulfonamide,

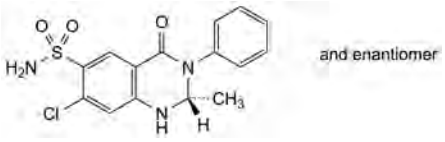


C. (2*RS*)-7-chloro-2-methyl-4-oxo-3-phenyl-1,2,3,4-tetrahydroquinazoline-6-sulfonamide,

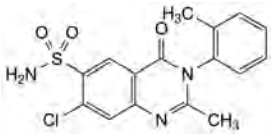


D. 7-chloro-2-methyl-3-(2-methylphenyl)-4-oxo-3,4-dihydroquinazoline-6-sulfonamide,

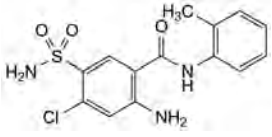


E. 2-amino-4-chloro-*N*-(2-methylphenyl)-5-sulfamoylbenzamide.

**01/2017:1448**

# METOPROLOL SUCCINATE

## Metoprololi succinas

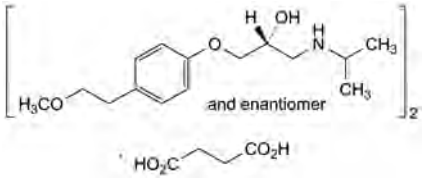


$C_{34}H_{56}N_2O_{10}$ $M_r$ 653
[98418-47-4]

DEFINITION

Bis[(2*RS*)-1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol] butanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in methanol, slightly soluble in ethanol (96 per cent), very slightly soluble in ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of metoprolol succinate.*

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.0 to 7.6 for solution S.

**Impurities M, N, O**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dilute 1 mL of the test solution to 50 mL with *methanol R*. Dilute 5 mL of this solution to 50 mL with *methanol R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: place 2 beakers, each containing 30 volumes of *concentrated ammonia R*, at the bottom of a chromatographic tank containing a mixture of 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*.

*Application*: 10 µL.

*Development*: over 2/3 of the plate in a tank saturated for at least 1 h.

*Drying*: in air for at least 3 h.

*Detection*: expose the plate to iodine vapour for at least 15 h.

*Limits*:

– *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent);
– *disregard* any spot on the line of application.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1.5 mg of *metoprolol impurity A CRS* and 2.5 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.9 g of *ammonium acetate R* in 810 mL of *water R*, add 2.0 mL of *triethylamine R*, 3.0 mL of *phosphoric acid R*, 10.0 mL of *glacial acetic acid R* and 146 mL of *acetonitrile R* and mix.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of metoprolol.

*Relative retention* with reference to metoprolol (retention time = about 7 min): impurity C = about 0.4; impurity A = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 6.0 between the peaks due to impurity A and metoprolol.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.1;
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to succinic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.64 mg of $C_{34}H_{56}N_2O_{10}$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, M, N, O.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F, G, H, J.*

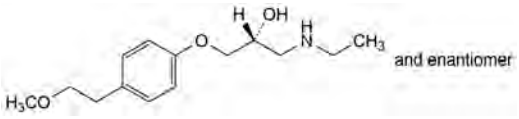


A. (2*RS*)-1-(ethylamino)-3-[4-(2-methoxyethyl)phenoxy]-propan-2-ol,

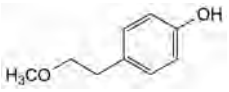

B. 4-(2-methoxyethyl)phenol,

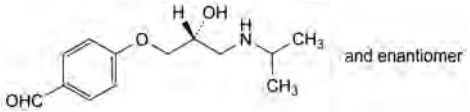


C. 4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]-propoxy]benzaldehyde,

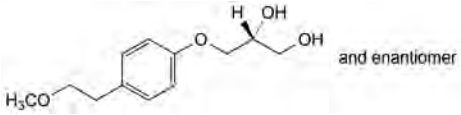


D. (2*RS*)-3-[4-(2-methoxyethyl)phenoxy]propane-1,2-diol,

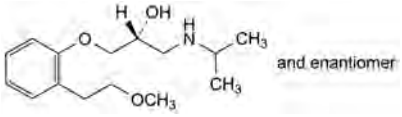


E. (2*RS*)-1-[2-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol,

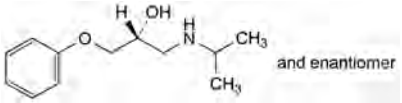


F. (2*RS*)-1-[(1-methylethyl)amino]-3-phenoxypropan-2-ol,

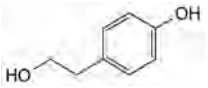

G. 2-(4-hydroxyphenyl)ethanol,

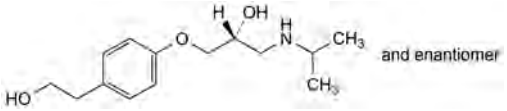


H. (2*RS*)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol,

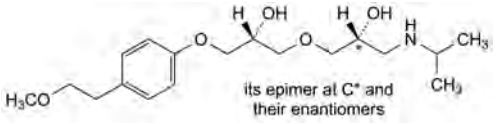


J. mixture of the 4 stereoisomers of 1-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,

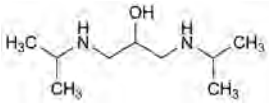

M. 1,3-bis[(1-methylethyl)amino]propan-2-ol,

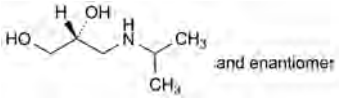


N. (2*RS*)-3-[(1-methylethyl)amino]propane-1,2-diol,

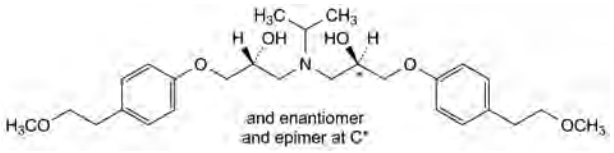


O. mixture of the 3 stereoisomers of 1,1′-[(1-methylethyl)imino]bis[3-[4-(2-methoxyethyl)-phenoxy]propan-2-ol].

**01/2017:1028**

# METOPROLOL TARTRATE

## Metoprololi tartras

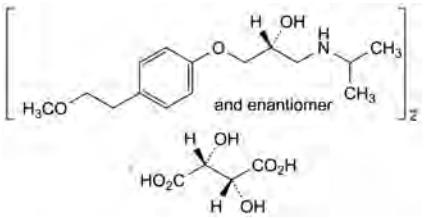


$C_{34}H_{56}N_2O_{12}$ $M_r$ 685
[56392-17-7]

DEFINITION

Bis[(2*RS*)-1-[4-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)amino]propan-2-ol] (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *metoprolol tartrate CRS*.

If the spectra obtained in the solid state show differences, record further spectra using discs prepared by placing 25 µL of a 100 g/L solution in *methylene chloride R* on a disc of *potassium bromide R* and evaporating the solvent. Examine immediately.

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 7.0 to + 10.0 (dried substance), determined on solution S.

**Impurities M, N, O**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dilute 1 mL of the test solution to 20 mL with *methanol R*. Dilute 5 mL of this solution to 50 mL with *methanol R*.

*Reference solution (b)*. Dilute 4 mL of reference solution (a) to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: place 2 beakers, each containing 30 volumes of *concentrated ammonia R*, at the bottom of a chromatographic tank containing a mixture of 20 volumes of *methanol R* and 80 volumes of *ethyl acetate R*.

*Application*: 5 µL.

*Development*: over 2/3 of the plate in a tank saturated for at least 1 h.

*Drying*: in air for at least 3 h.

*Detection*: expose the plate to iodine vapour for at least 15 h.

*Limits*:

– *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *disregard* any spot on the line of application.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 1.5 mg of *metoprolol impurity A CRS* and 2.5 mg of *metoprolol tartrate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.9 g of *ammonium acetate R* in 810 mL of *water R*, add 2.0 mL of *triethylamine R*, 3.0 mL of *phosphoric acid R*, 10.0 mL of *glacial acetic acid R* and 146 mL of *acetonitrile R* and mix.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of metoprolol.

*Relative retention* with reference to metoprolol (retention time = about 7 min): impurity H = about 0.3; impurity C = about 0.4; impurity G = about 0.45; impurity F = about 0.7; impurity A = about 0.8; impurity J = about 1.4; impurity D = about 1.6; impurity E = about 1.8; impurity B = about 2.

*System suitability*: reference solution (a):

– *resolution*: minimum 6.0 between the peaks due to impurity A and metoprolol.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.1;
– *impurities A, B, C, D, E, F, G, H, J*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to tartaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* over *anhydrous calcium chloride R* for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.24 mg of $C_{34}H_{56}N_2O_{12}$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, J, M, N, O.*

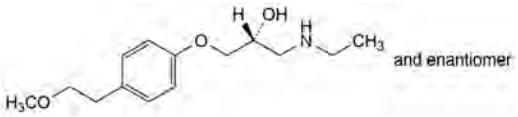


A. (2*RS*)-1-(ethylamino)-3-[4-(2-methoxyethyl)phenoxy]-propan-2-ol,

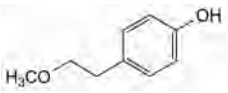

B. 4-(2-methoxyethyl)phenol,

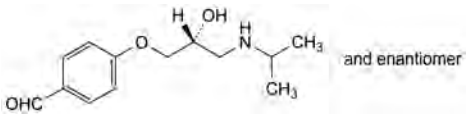


C. 4-[(2*RS*)-2-hydroxy-3-[(1-methylethyl)amino]propoxy]-benzaldehyde,

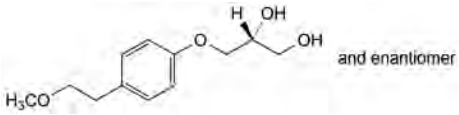


D. (2*RS*)-3-[4-(2-methoxyethyl)phenoxy]propane-1,2-diol,

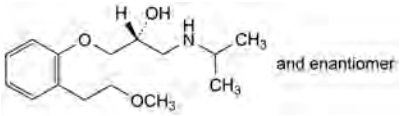


E. (2*RS*)-1-[2-(2-methoxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol,

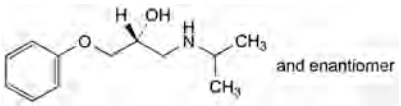


F. (2*RS*)-1-[(1-methylethyl)amino]-3-phenoxypropan-2-ol,

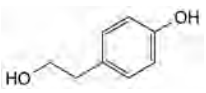

G. 2-(4-hydroxyphenyl)ethanol,

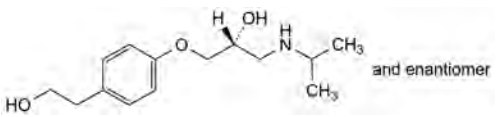


H. (2*RS*)-1-[4-(2-hydroxyethyl)phenoxy]-3-[(1-methylethyl)-amino]propan-2-ol,

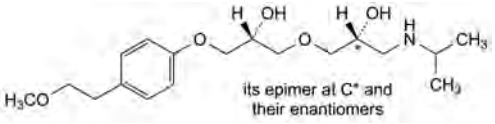


J. mixture of the 4 stereoisomers of 1-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-3-[4-(2-methoxyethyl)phenoxy]propan-2-ol,

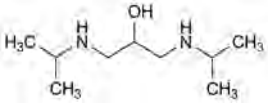

M. 1,3-bis[(1-methylethyl)amino]propan-2-ol,

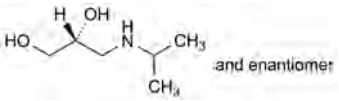


N. (2*RS*)-3-[(1-methylethyl)amino]propane-1,2-diol,

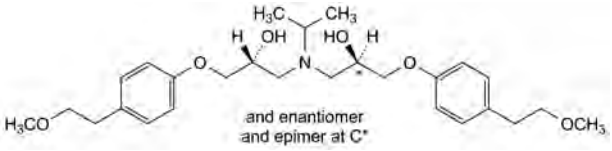


O. mixture of the 3 stereoisomers of 1,1′-[(1-methylethyl)imino]bis[3-[4-(2-methoxyethyl)-phenoxy]propan-2-ol].

**01/2017:1133**

# METRIFONATE

## Metrifonatum

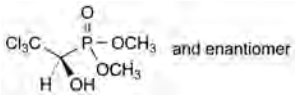


$C_4H_8Cl_3O_4P$ $M_r$ 257.4
[52-68-6]

DEFINITION

Dimethyl (*RS*)-(2,2,2-trichloro-1-hydroxyethyl)phosphonate.

*Content*: 98.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very soluble in methylene chloride, freely soluble in acetone and in ethanol (96 per cent).

mp: 76 °C to 81 °C.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *metrifonate CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *metrifonate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *dioxan R*, *toluene R* (5:25:70 *V/V/V*).

*Application*: 10 µL.

*Development*: in an unsaturated tank over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 50 g/L solution of *4-(4-nitrobenzyl)pyridine R* in *acetone R* and heat at 120 °C for 15 min; spray the still-warm plate with a 100 g/L solution of *tetraethylene pentamine R* in *acetone R* and examine immediately.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 20 mg in 1 mL of *dilute sodium hydroxide solution R*. Add 1 mL of *pyridine R*. Shake and heat on a water-bath for 2 min. A red colour develops in the upper layer.

D. To 0.1 g add 0.5 mL of *nitric acid R*, 0.5 mL of a 500 g/L solution of *ammonium nitrate R1* and 0.1 mL of *strong hydrogen peroxide solution R*. Heat on a water-bath for 10 min. Heat to boiling and add 1 mL of *ammonium molybdate solution R*. A yellow colour is produced or a yellow precipitate is formed.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 5.0 g in 20 mL of *methanol R*.

**Acidity**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent. Add 0.1 mL of *methyl red solution R*. Not more than 1.0 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (10:90 *V/V*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a). Use a freshly prepared solution.* Dissolve 10.0 mg of *desmethylmetrifonate CRS* (impurity A) in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 5.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 0.10 g of *dichlorvos R* (impurity B) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 10.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (d). Use a freshly prepared solution.* Mix 1.0 mL of reference solution (a), 1.0 mL of reference solution (b) and 0.025 mL of the test solution.

*Reference solution (e).* Dilute 4.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 2.9 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 25 | 90 → 85 | 10 → 15 |
| 25 - end | 85 → 45 | 15 → 55 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Run time*: 3 times the retention time of metrifonate.

*Elution order*: impurity A, metrifonate, impurity B.

*System suitability*: reference solution (d):

- *resolution*: minimum 3.0 between the peaks due to impurity A and metrifonate and minimum 4.5 between the peaks due to metrifonate and impurity B.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *sum of impurities other than A and B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.04 per cent).

**Chlorides**: maximum 500 ppm.

Dissolve 5.00 g in 30 mL of *ethanol (96 per cent) R* and add a mixture of 15 mL of *nitric acid R* and 100 mL of *water R*. Titrate with *0.01 M silver nitrate* determining the end-point potentiometrically (*2.2.20*), using a silver electrode.

1 mL of *0.01 M silver nitrate* is equivalent to 0.3546 mg of Cl.

**Water** (*2.5.12*): maximum 0.3 per cent, determined on 3.000 g.

ASSAY

Dissolve 0.300 g in 30 mL of *ethanol (96 per cent) R*. Add 10 mL of *ethanolamine R* and allow to stand for 1 h at 20-22 °C. Add a chilled mixture of 15 mL of *nitric acid R* and 100 mL of *water R* maintaining the temperature of the mixture at 20-22 °C. Maintain at that temperature and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*), using a silver electrode.

Calculate the percentage content of $C_4H_8Cl_3O_4P$, taking into account the content of chloride and using the following expression:

$$\left[\frac{V_P}{M_P} - \frac{V_{Cl} \times 0.1}{M_{Cl}}\right] \times 25.74 \times 0.1$$

$V_P$ = volume of silver nitrate used in the assay, in millilitres;

$M_P$ = mass of substance used in the assay, in grams;

$V_{Cl}$ = volume of silver nitrate used in the test for chlorides, in millilitres;

$M_{Cl}$ = mass of substance used in the test for chlorides, in grams.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

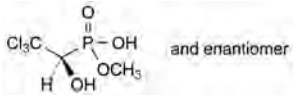


A. methyl (*RS*)-(2,2,2-trichloro-1-hydroxyethyl)phosphonate acid (desmethylmetrifonate),

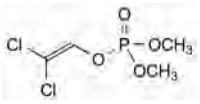


B. 2,2-dichloroethenyl dimethyl phosphate (dichlorvos).

**01/2017:0675**

# METRONIDAZOLE

## Metronidazolum

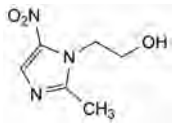


$C_6H_9N_3O_3$ $M_r$ 171.2

[443-48-1]

DEFINITION

2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethanol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: slightly soluble in water, in acetone, in alcohol and in methylene chloride.

IDENTIFICATION

*First identification: C.*

*Second identification*: *A, B, D.*

A. Melting point (*2.2.14*): 159 °C to 163 °C.

B. Dissolve 40.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of the solution to 100.0 mL with *0.1 M hydrochloric acid*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 277 nm and a minimum at 240 nm. The specific absorbance at the maximum is 365 to 395.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *metronidazole CRS*.

D. To about 10 mg add about 10 mg of *zinc powder R*, 1 mL of *water R* and 0.25 mL of *dilute hydrochloric acid R*. Heat on a water-bath for 5 min. Cool. The solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in *1 M hydrochloric acid* and dilute to 20 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution.* Dissolve 0.05 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase and dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *metronidazole impurity A CRS* in the mobile phase, add 10.0 mL of the test solution and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *methanol R* and 70 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R*,

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 315 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of metronidazole.

*Relative retention* with reference to metronidazole (retention time = about 7 min): impurity A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum of 2.0 between the peaks due to metronidazole and to impurity A.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.12 mg of $C_6H_9N_3O_3$.

STORAGE

Protected from light.

IMPURITIES

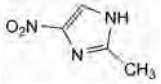


A. 2-methyl-4-nitroimidazole,

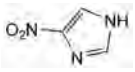


B. 4-nitroimidazole,

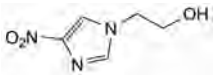


C. 2-(4-nitro-1*H*-imidazol-1-yl)ethanol,

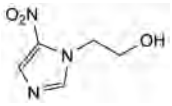

D. 2-(5-nitro-1*H*-imidazol-1-yl)ethanol,

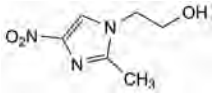

E. 2-(2-methyl-4-nitro-1*H*-imidazol-1-yl)ethanol,

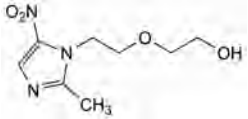

F. 2-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy]ethanol,

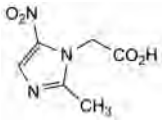

G. 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)acetic acid.

**01/2017:0934**

# METRONIDAZOLE BENZOATE

## Metronidazoli benzoas

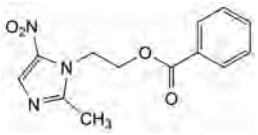

$C_{13}H_{13}N_3O_4$ $M_r$ 275.3
[13182-89-3]

DEFINITION

2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethyl benzoate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder or flakes.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, slightly soluble in alcohol.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 99 °C to 102 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.100 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 1.0 mL of the solution to 100.0 mL with a 103 g/L solution of *hydrochloric acid R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 232 nm and 275 nm.

*Specific absorbance at the absorption maximum at 232 nm*: 525 to 575.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of metronidazole benzoate.*

D. To about 10 mg add about 10 mg of *zinc powder R*, 1 mL of *water R* and 0.3 mL of *hydrochloric acid R*. Heat on a water-bath for 5 min and cool. The solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_3$ (*2.2.2, Method II*).

Dissolve 1.0 g in *dimethylformamide R* and dilute to 10 mL with the same solvent.

**Acidity**. Dissolve 2.0 g in a mixture of 20 mL of *dimethylformamide R* and 20 mL of *water R*, previously neutralised with *0.02 M hydrochloric acid* or *0.02 M sodium hydroxide* using 0.2 mL of *methyl red solution R*. Not more than 0.25 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (45:55 *V/V*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *metronidazole CRS* (impurity A), 5.0 mg of *metronidazole impurity A CRS* (impurity B) and 5.0 mg of *benzoic acid CRS* (impurity C) in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *di-isobutyloctadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 180 $m^2$/g, a pore size of 8 nm and a carbon loading of 10 per cent.

*Mobile phase*:

- *mobile phase A*: 1.5 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.2 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 15 | 80 → 55 | 20 → 45 |
| 15 - 40 | 55 | 45 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*Relative retention* with reference to metronidazole benzoate (retention time = about 20 min): impurity B = about 0.17; impurity A = about 0.20; impurity C = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

- *impurities A, B, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.53 mg of $C_{13}H_{13}N_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

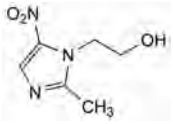

A. 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (metronidazole),

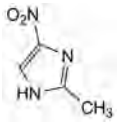

B. 2-methyl-4-nitroimidazole,

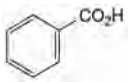

C. benzenecarboxylic acid (benzoic acid).

**01/2017:1029**

# MEXILETINE HYDROCHLORIDE

## Mexiletini hydrochloridum

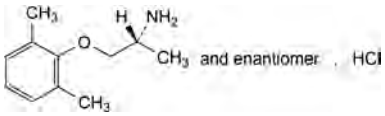


$C_{11}H_{18}ClNO$ $M_r$ 215.7
[5370-01-4]

DEFINITION

(2*RS*)-1-(2,6-Dimethylphenoxy)propan-2-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, sparingly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *mexiletine hydrochloride CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Dilute 1.5 mL of solution S (see Tests) to 15 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 5 mL of solution S to 10 mL with *water R*.

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a)*. Dissolve the contents of a vial of *mexiletine impurity D CRS* in 4.0 mL of *methanol R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 5.0 mL with *methanol R*.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 5.0 mL with reference solution (b).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R, ethanol (96 per cent) R, acetone R, toluene R* (3:7:45:45 *V/V/V/V*).

*Application*: 5 µL of the test solution and reference solutions (c) and (d).

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R3* and heat at 100-105 °C for 15 min or until the spots appear.

*System suitability*: the chromatogram obtained with reference solution (d) shows 2 clearly separated spots.

*Limit*:

– *impurity D*: any spot corresponding to impurity D in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *mexiletine impurity C CRS* in the mobile phase and transfer the solution quantitatively to a volumetric flask containing 16.0 mg of *2,6-dimethylphenol R* (impurity A). Dilute to 20.0 mL with the mobile phase. Mix 1.0 mL of this solution with 2.0 mL of reference solution (a) and dilute the mixture to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 65 volumes of *methanol R2* and 35 volumes of a solution prepared as follows: dissolve 11.5 g of *anhydrous sodium acetate R* in 500 mL of *water R*, add 3.2 mL of *glacial acetic acid R*, mix and allow to cool; adjust to pH 4.8 with *glacial acetic acid R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 262 nm.

*Injection*: 20 µL.

*Run time*: 5.5 times the retention time of mexiletine.

*Relative retention* with reference to mexiletine (retention time = about 4 min): impurity C = about 0.7; impurity A = about 1.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurity C and mexiletine.

*Limits*:

– *impurity A*: not more than 2.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *impurity C*: not more than 20 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *any other impurity*: for each impurity, not more than 0.5 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *total*: not more than 2.5 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.25 times the area of the peak due to mexiletine in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*) and completing the titration within 2 min.

1 mL of *0.1 M perchloric acid* is equivalent to 21.57 mg of $C_{11}H_{18}ClNO$.

IMPURITIES

*Specified impurities*: *A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

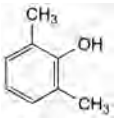

A. 2,6-dimethylphenol,

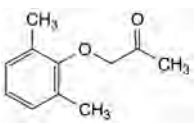

B. 1-(2,6-dimethylphenoxy)propan-2-one,

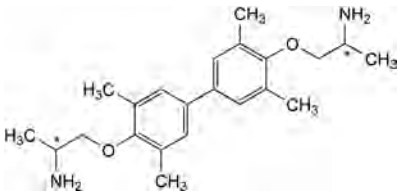

C. 1,1′-[(3,3′,5,5′-tetramethylbiphenyl-4,4′-diyl)bisoxy]-dipropan-2-amine,

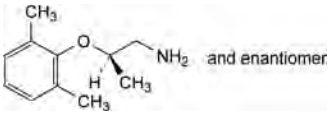


D. (2*RS*)-2-(2,6-dimethylphenoxy)propan-1-amine.

**07/2015:0846**
**corrected 10.0**

# MIANSERIN HYDROCHLORIDE

## Mianserini hydrochloridum

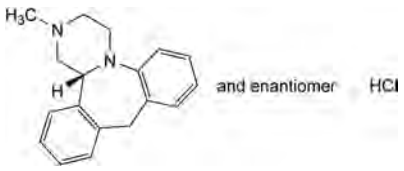


$C_{18}H_{21}ClN_2$ $M_r$ 300.8
[21535-47-7]

DEFINITION

(14b*RS*)-2-Methyl-1,2,3,4,10,14b-hexahydrodibenzo-[*c,f*]pyrazino[1,2-*a*]azepine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or crystals.

*Solubility*: sparingly soluble in water and in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *water R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *water R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 279 nm.

*Specific absorbance at the absorption maximum*: 64 to 72.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mianserin hydrochloride CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *mianserin hydrochloride CRS* in *methylene chloride R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *mianserin hydrochloride CRS* and 10 mg of *cyproheptadine hydrochloride CRS* in *methylene chloride R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R.*

*Mobile phase*: *diethylamine R, ether R, cyclohexane R* (5:20:75 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.0 to 5.5.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 3.0.* Dissolve 5.0 g of *sodium octanesulfonate R* in *water R* and dilute to 350 mL with the same solvent. Stir until complete dissolution. Adjust to pH 3.0 with a mixture of 1 volume of *phosphoric acid R* and 3 volumes of *water R*. Dilute to 400 mL with *water R*.

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *mianserin for system suitability CRS* (containing impurities A, D and E) in 1.0 mL of the mobile phase.

*Reference solution (c).* Dissolve 5.0 mg of *mianserin impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: buffer solution pH 3.0, *methanol R* (37:63 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of mianserin.

*Identification of impurities*: use the chromatogram supplied with *mianserin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, D and E.

*Relative retention* with reference to mianserin (retention time = about 18 min): impurity B = about 0.2; impurity A = about 0.5; impurity D = about 0.7; impurity E = about 1.1.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to mianserin.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.4; impurity D = 2.1;
– *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
– *impurities A, D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 65 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.08 mg of $C_{18}H_{21}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, F.*

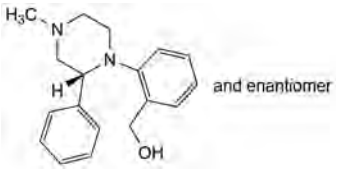


A. [2-[(2*RS*)-4-methyl-2-phenylpiperazin-1-yl]phenyl]-methanol,

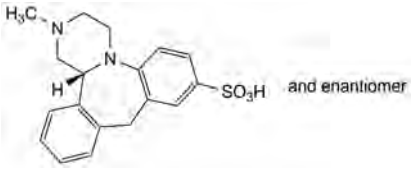


B. (14b*RS*)-2-methyl-1,2,3,4,10,14b-hexahydrodibenzo-[*c*,*f*]pyrazino[1,2-*a*]azepine-8-sulfonic acid,

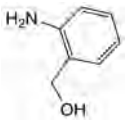


C. (2-aminophenyl)methanol,

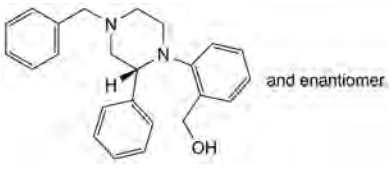


D. [2-[(2*RS*)-4-benzyl-2-phenylpiperazin-1-yl]phenyl]methanol,

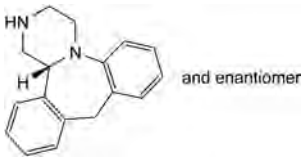


E. (14b*RS*)-1,2,3,4,10,14b-hexahydrodibenzo-[*c*,*f*]pyrazino[1,2-*a*]azepine,

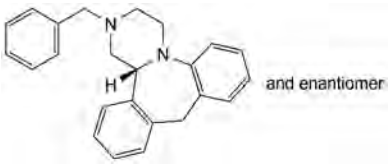


F. (14b*RS*)-2-benzyl-1,2,3,4,10,14b-hexahydrodibenzo-[*c*,*f*]pyrazino[1,2-*a*]azepine.

**04/2019:0935**

# MICONAZOLE

## Miconazolum

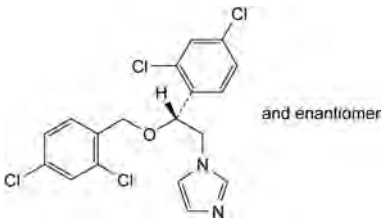


$C_{18}H_{14}Cl_4N_2O$ $M_r$ 416.1
[22916-47-8]

DEFINITION

1-[(2*RS*)-2-(2,4-Dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D.*

A. Melting point (*2.2.14*): 83 °C to 87 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *miconazole CRS*.

C. Melting point (*2.2.14*).
*Determination A*: determine the melting point of the substance to be examined.
*Result A*: 83 °C to 87 °C.
*Determination B*: mix equal parts of the substance to be examined and *miconazole CRS*, and determine the melting point of the mixture.
*Result B*: the absolute difference between the melting point of the mixture and the value obtained in determination A is not greater than 2 °C.

D. To 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.1 g in *methanol R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 0.100 g of the substance to be examined add 3.0 mL of *acetonitrile R* and 3.2 mL of *methanol R* and shake until complete dissolution. Further dilute to 10.0 mL with a 15.8 g/L solution of *ammonium acetate R*.

*Reference solution (a)*. Dissolve 2.5 mg of *miconazole CRS* and 2.5 mg of *econazole nitrate CRS* in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 6.0 g of *ammonium acetate R* in a mixture of 300 mL of *acetonitrile for chromatography R*, 320 mL of *methanol R1* and 380 mL of *water for chromatography R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of miconazole.

*Retention time*: econazole = about 10 min; miconazole = about 20 min.

*System suitability*: reference solution (a):
- *resolution*: minimum 10 between the peaks due to econazole and miconazole; if necessary, adjust the composition of the mobile phase.

*Limits*:
- *impurities A, B, C, D, E, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Using 0.2 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until the colour changes from orange-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 41.61 mg of $C_{18}H_{14}Cl_4N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I.*

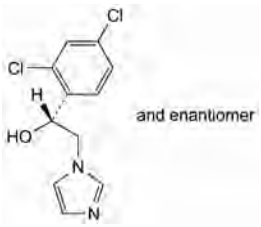


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethan-1-ol,

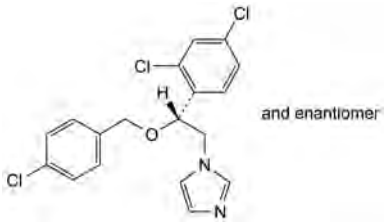


B. 1-[(2*RS*)-2-[[(4-chlorophenyl)methyl]oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole (econazole),

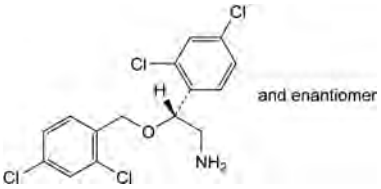


C. (2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethan-1-amine,

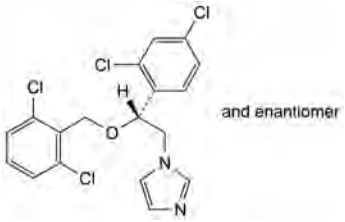


D. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,6-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole (isoconazole),

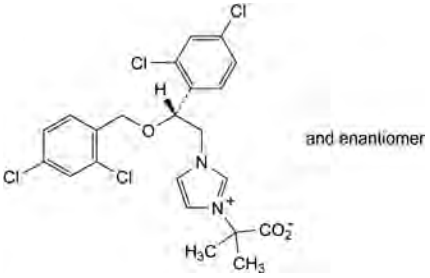


E. 2-[1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazol-3-ium-3-yl]-2-methylpropanoate,

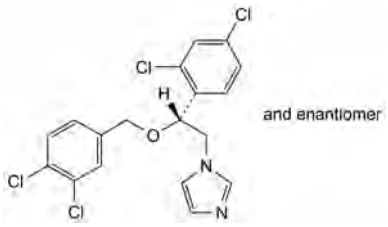


F. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(3,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole,

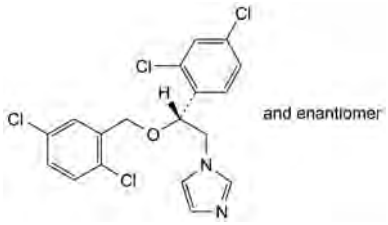


G. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,5-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole,

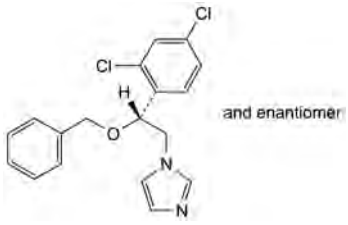


H. 1-[(2*RS*)-2-(benzyloxy)-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

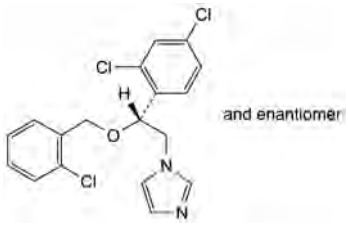


I. 1-[(2*RS*)-2-[[(2-chlorophenyl)methyl]oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

**04/2019:0513**

# MICONAZOLE NITRATE

## Miconazoli nitras

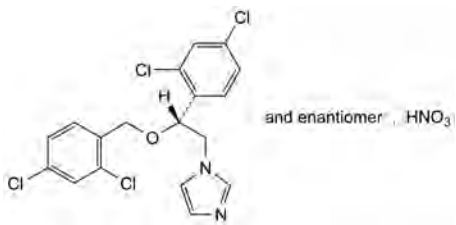


$C_{18}H_{15}Cl_4N_3O_4$ $M_r$ 479.1

[22832-87-7]

DEFINITION

1-[(2*RS*)-2-(2,4-Dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole nitrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: C, D.*

A. Melting point (*2.2.14*): 178 °C to 184 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *miconazole nitrate CRS*.

C. Melting point (*2.2.14*).
*Determination A*: determine the melting point of the substance to be examined.
*Result A*: 178 °C to 184 °C.
*Determination B*: mix equal parts of the substance to be examined and *miconazole nitrate CRS*, and determine the melting point of the mixture.
*Result B*: the absolute difference between the melting point of the mixture and the value obtained in determination A is not greater than 2 °C.

D. It gives the reaction of nitrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.1 g in *methanol R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.5 mg of *miconazole nitrate CRS* and 2.5 mg of *econazole nitrate CRS* (impurity B) in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: dissolve 6.0 g of *ammonium acetate R* in a mixture of 300 mL of *acetonitrile for chromatography R*, 320 mL of *methanol R1* and 380 mL of *water for chromatography R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of miconazole.

*Relative retention* with reference to miconazole (retention time = about 20 min): impurity A = about 0.1; impurity E = about 0.3; impurity C = about 0.4; impurity B = about 0.6; impurity D = about 0.75; impurity F = about 0.85; impurity G = about 0.9.

*System suitability*: reference solution (a):
- *resolution*: minimum 10 between the peaks due to impurity B and miconazole.

*Limits*:
- *impurities A, B, C, D, E, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to the nitrate ion.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 75 mL of *anhydrous acetic acid R*, with slight heating if necessary. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 47.91 mg of $C_{18}H_{15}Cl_4N_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I.*

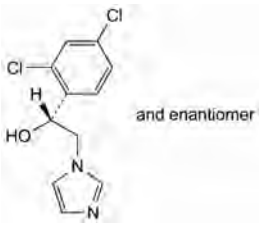


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethan-1-ol,

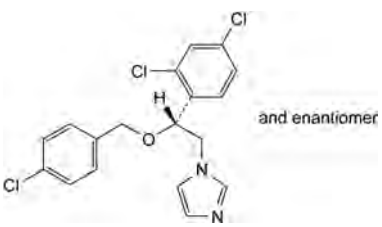


B. 1-[(2*RS*)-2-[[(4-chlorophenyl)methyl]oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole (econazole),

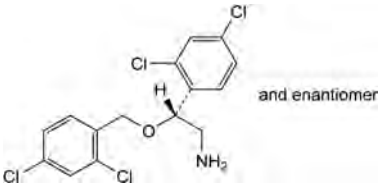


C. (2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethan-1-amine,

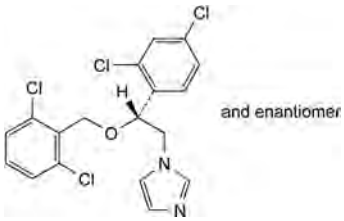


D. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,6-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole (isoconazole),

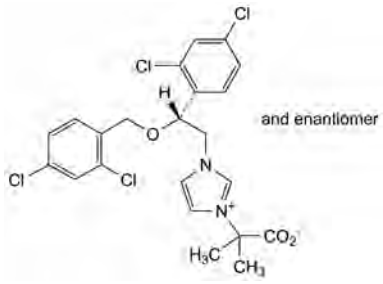


E. 2-[1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazol-3-ium-3-yl]-2-methylpropanoate,

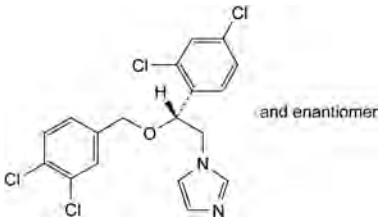


F. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(3,4-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole,

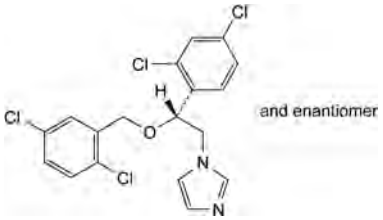


G. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[[(2,5-dichlorophenyl)methyl]oxy]ethyl]-1*H*-imidazole,

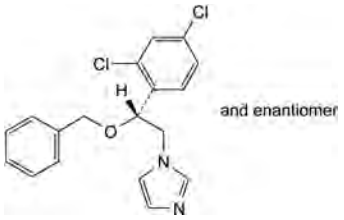


H. 1-[(2*RS*)-2-(benzyloxy)-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

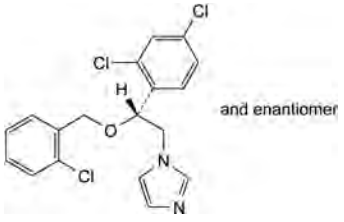


I. 1-[(2*RS*)-2-[[(2-chlorophenyl)methyl]oxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

**04/2016:2050**

# MICROCRYSTALLINE CELLULOSE AND CARMELLOSE SODIUM

## Cellulosum microcristallinum et carmellosum natricum

DEFINITION

Colloid-forming, powdered mixture of *Microcrystalline cellulose (0316)* with 5 per cent to 22 per cent of *Carmellose sodium (0472)*.

*Content*: 75.0 per cent to 125.0 per cent of the nominal content of carmellose sodium (dried substance).

CHARACTERS

*Appearance*: white or off-white, coarse or fine, hygroscopic powder.

*Solubility*: dispersible in water producing a white, opaque colloidal dispersion; practically insoluble in organic solvents and in dilute acids.

IDENTIFICATION

A. Mix 6 g with 300 mL of *water R* and stir at 18 000 r/min for 5 min. A white opaque dispersion is obtained which does not produce a supernatant.

B. Add several drops of the dispersion obtained in identification A to a 100 g/L solution of *aluminium chloride R*. Each drop forms a white, opaque globule which does not disperse on standing.

C. Add 2 mL of *iodinated potassium iodide solution R* to the dispersion obtained in test A. No blue or purplish colour is produced.

D. It complies with the limits of the assay.

TESTS

**Solubility**. Add 50 mg to 10 mL of *ammoniacal solution of copper tetrammine R* and shake. It dissolves completely, leaving no residue.

**pH** (*2.2.3*): 6.0 to 8.0 for the dispersion obtained in identification A.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 7.4 per cent, determined on 2.0 g.

ASSAY

Heat 2.00 g with 75 mL of *anhydrous acetic acid R* under a reflux condenser for 2 h, cool and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.6 mg of carmellose sodium.

LABELLING

The label states the nominal content of carmellose sodium in per cent *m/m*.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section.*

*Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for microcrystalline cellulose and carmellose sodium used as a suspending agent.*

**Viscosity** (*2.2.10*): 60 per cent to 140 per cent of the nominal value.

Calculate the quantity (*x* g) needed to prepare exactly 600 g of a dispersion of the stated percentage *m/m* (dried substance). To (600 - *x*) g of *water R* at 23-25 °C contained in a 1000 mL high-speed blender bowl, add *x* g of the substance to be examined and stir at reduced speed, taking care to avoid contacting the sides of the bowl with the powder. Continue stirring at low speed for 15 s after the addition of the powder and then stir at 18 000 r/min for exactly 2 min.

Determine the viscosity with a suitable relative rotational viscometer under the following conditions:

- spindle: as appropriate;
- speed: 20 r/min.

Immerse the spindle into the suspension immediately after preparation, switch on the rotation spindle after 30 s; after a further 30 s, take scale readings and calculate the viscosity according to the viscometer manual.

**04/2016:0936**

# MIDAZOLAM

## Midazolamum

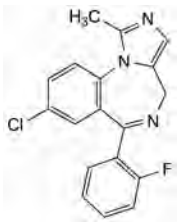


$C_{18}H_{13}ClFN_3$ $M_r$ 325.8
[59467-70-8]

DEFINITION

8-Chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent), soluble in methanol.

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A, C, D, E*.

A. Melting point (*2.2.14*): 161 °C to 164 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *midazolam CRS*.

C. Examine the chromatograms obtained in the test for impurity C.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

D. Mix 90 mg with 0.30 g of *anhydrous sodium carbonate R* and ignite in a crucible until an almost white residue is obtained (normally in less than 5 min). Allow to cool and dissolve the residue in 5 mL of *dilute nitric acid R*. Filter (the filtrate is also used in identification test E). Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank solution is red.

E. To 1 mL of the filtrate obtained in identification test D add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.1 g in *0.1 M hydrochloric acid* and dilute to 10 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *midazolam for system suitability CRS* (containing impurities A, B, E, G and H) in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: prepare a solution containing 7.7 g/L of *ammonium acetate R* and 10 mL/L of *tetrabutylammonium hydroxide solution (400 g/L) R* and adjust to pH 5.3 with *glacial acetic acid R*. Mix 44 volumes of this solution with 56 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of midazolam.

*Identification of impurities*: use the chromatogram supplied with *midazolam for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, E, G and H.

*Relative retention* with reference to midazolam (retention time = about 17 min): impurity E = about 0.5; impurity A = about 0.9; impurity G = about 1.2; impurity H = about 1.9; impurity B = about 2.2.

*System suitability*:

- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to midazolam in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.0; impurity E = 2.0; impurity H = 1.7;
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities A, E, G, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 50 mL with *ethanol (96 per cent) R*.

*Reference solution (a).* Dissolve the contents of a vial of *midazolam impurity C CRS* in 1.0 mL of *methanol R*. Dilute 0.5 mL of the solution to 1.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 8 mg of *midazolam CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dissolve 40 mg of the substance to be examined in 1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R*, *ethyl acetate R* (2:15:20:80 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (c):

- the chromatogram shows 2 clearly separated spots.

*Limit*:

- *impurity C*: any spot due to impurity C in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.120 g in 30 mL of *anhydrous acetic acid R* and add 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid* to the 2nd point of inflexion, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.29 mg of $C_{18}H_{13}ClFN_3$.

IMPURITIES

*Specified impurities*: *A, B, C, E, G, H*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, I, J*.

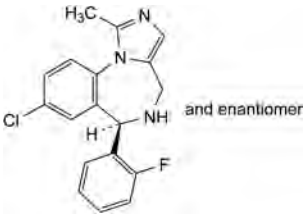


A. (6*RS*)-8-chloro-6-(2-fluorophenyl)-1-methyl-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine,

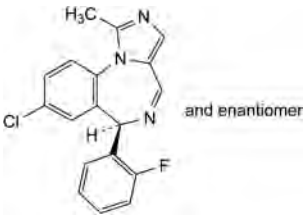


B. (6*RS*)-8-chloro-6-(2-fluorophenyl)-1-methyl-6*H*-imidazo-[1,5-*a*][1,4]benzodiazepine,

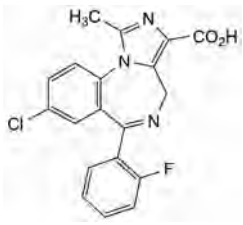


C. 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid,

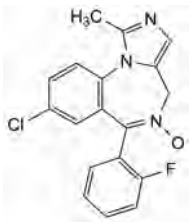


D. 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine 5-oxide,

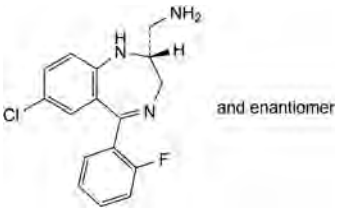


E. [(2*RS*)-7-chloro-5-(2-fluorophenyl)-2,3-dihydro-1*H*-1,4-benzodiazepin-2-yl]methanamine,

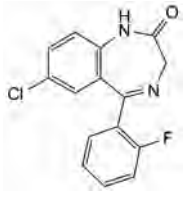


F. 7-chloro-5-(2-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (1-des[(diethylamino)ethyl]flurazepam),

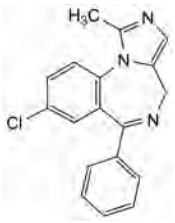

G. 8-chloro-1-methyl-6-phenyl-4*H*-imidazo-[1,5-*a*][1,4]benzodiazepine (desfluoromidazolam),

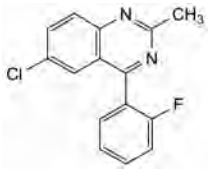

H. 6-chloro-4-(2-fluorophenyl)-2-methylquinazoline,

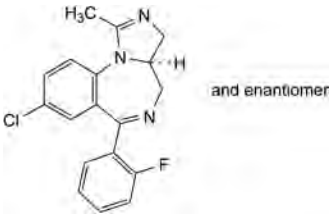

I. (3a*RS*)-8-chloro-6-(2-fluorophenyl)-1-methyl-3a,4-dihydro-3*H*-imidazo[1,5-*a*][1,4]benzodiazepine,

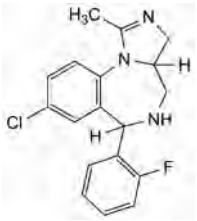

J. 8-chloro-6-(2-fluorophenyl)-1-methyl-3a,4,5,6-tetrahydro-3*H*-imidazo[1,5-*a*][1,4]benzodiazepine.

**07/2017:2536**
**corrected 9.6**

# MILBEMYCIN OXIME FOR VETERINARY USE

## Milbemycinum oximum ad usum veterinarium

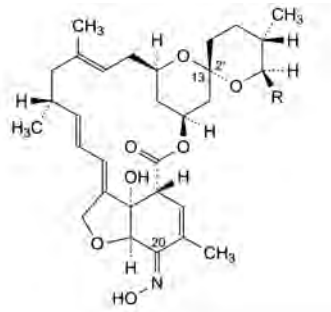

| Compound | R | Mol. formula | $M_r$ |
|---|---|---|---|
| Milbemycin $A_4$ oxime | $C_2H_5$ | $C_{32}H_{45}NO_7$ | 555.7 |
| Milbemycin $A_3$ oxime | $CH_3$ | $C_{31}H_{43}NO_7$ | 541.7 |

[129496-10-2]

DEFINITION

Mixture of (2a*E*,2′*R*,2a$^1$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*)-6′-ethyl-2a$^1$-hydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-2a$^1$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]-benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin $A_4$ oxime) and (2a*E*,2′*R*,2a$^1$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*)-2a$^1$-hydroxy-20-(hydroxyimino)-5′,6,6′,8,19-pentamethyl-2a$^1$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]-benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin $A_3$ oxime).

Semi-synthetic product derived from a fermentation product.

*Content*:

– *milbemycin oxime* ($A_4$ + $A_3$): 95.0 per cent to 102.0 per cent (anhydrous substance);
– *ratio* $A_4$ / ($A_3$ + $A_4$): minimum 0.80.

CHARACTERS

*Appearance*: white or almost white or light yellow, amorphous powder.

*Solubility*: practically insoluble in water, very soluble in acetonitrile, in anhydrous ethanol and in ethyl acetate.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *milbemycin oxime CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Mix 25 volumes of a 0.5 g/L solution of *phosphoric acid R* and 75 volumes of *acetonitrile R*.

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 200.0 mL with the solvent mixture. NOTE: this solution is stable for 72 h if stored at 2-8 °C protected from light.

*Reference solution (a).* Dissolve 40.0 mg of *milbemycin oxime CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2 mg of *milbemycin oxime for system suitability CRS* (containing impurities E, F, G, H and I) in the solvent mixure and dilute to 10 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.1 m, Ø = 3.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 26.0 | 74.0 |
| 2 - 27 | 26.0 → 24.5 | 74.0 → 75.5 |
| 27 - 47 | 24.5 | 75.5 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *milbemycin oxime for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities E, F, G, H and I.

*Relative retention* with reference to milbemycin $A_3$ oxime (retention time = about 20 min): impurity B = about 0.89; impurity F = about 1.09; impurity D = about 1.13; impurity H = about 1.17; impurity A = about 1.25; impurity I = about 1.33; milbemycin $A_4$ oxime = about 1.43; impurity E = about 1.51; impurity C = about 1.90; impurity G = about 2.18.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to milbemycin $A_3$ oxime; minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to milbemycin $A_4$ oxime.

*Calculation of percentage contents*:

- for each impurity, use the concentration of milbemycin oxime and the sum of the peak areas due to milbemycin $A_3$ oxime and milbemycin $A_4$ oxime in reference solution (c).

*Limits*:

- *impurity G*: maximum 2.0 per cent;
- *impurity E*: maximum 0.7 per cent;
- *impurities H, I*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 3.5 per cent;
- *reporting threshold*: 0.10 per cent.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the percentage content of milbemycin oxime ($A_3$ + $A_4$) and the ratio $A_4$ / ($A_3$ + $A_4$) using the chromatogram obtained with reference solution (a) and taking into account the assigned content of milbemycin $A_3$ oxime and milbemycin $A_4$ oxime in *milbemycin oxime CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: E, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F.*

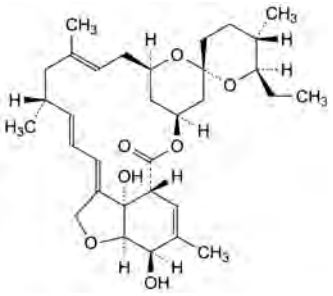

A. (2a*E*,2′*R*,2a$^1$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*)-6′-ethyl-2a$^1$,20-dihydroxy-5′,6,8,19-tetramethyl-2a$^1$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin $A_4$),

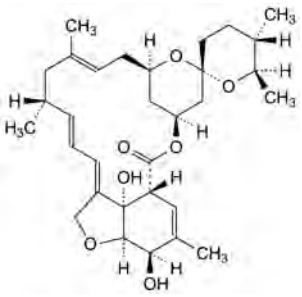

B. (2a*E*,2′*R*,2a$^1$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*)-2a$^1$,20-dihydroxy-5′,6,6′,8,19-pentamethyl-2a$^1$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin $A_3$),

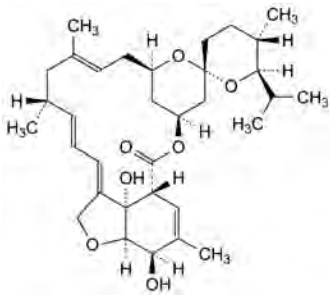

C. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*)-2a$^{1}$,20-dihydroxy-5′,6,8,19-tetramethyl-6′-(1-methylethyl)- 2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin D),

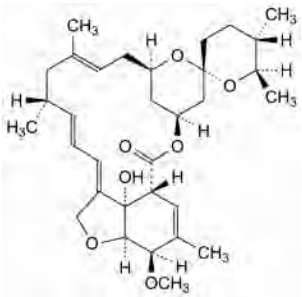

D. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*)-2a$^{1}$-hydroxy-20-methoxy-5′,6,6′,8,19-pentamethyl-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin $B_2$),

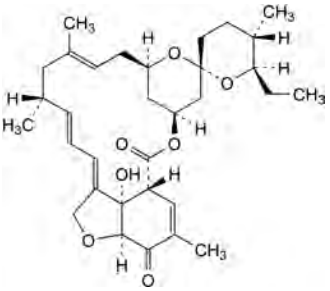

E. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20a*S*)-6′-ethyl-2a$^{1}$-hydroxy-5′,6,8,19-tetramethyl-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17′-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17,20-dione (20-oxomilbemycin $A_4$),

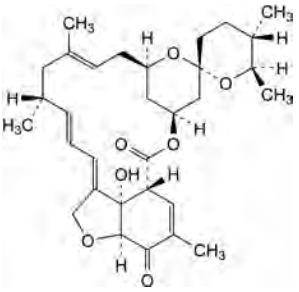

F. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20a*S*)-2a$^{1}$-hydroxy-5′,6,6′,8,19-pentamethyl-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17,20-dione (20-oxomilbemycin $A_3$),

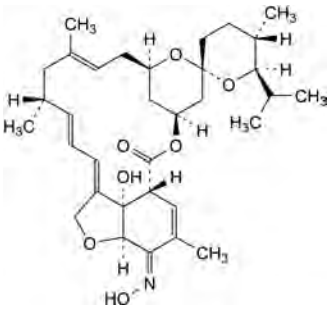

G. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*)-2a$^{1}$-hydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-6′-(1-methylethyl)-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (milbemycin D oxime),

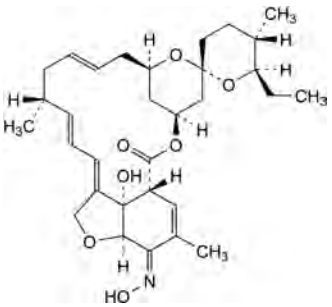

H. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*)-6′-ethyl-2a$^{1}$-hydroxy-20-(hydroxyimino)-5′,6,19-trimethyl-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one (8-desmethylmilbemycin $A_4$ oxime),

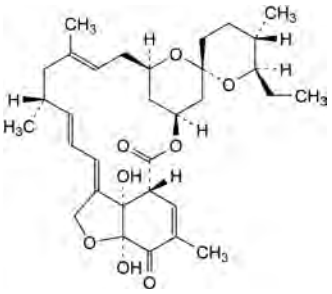

I. (2a*E*,2′*R*,2a$^{1}$*S*,4*E*,5′*S*,6*R*,6′*R*,8*E*,11*R*,15*S*,17a*R*,20a*R*)-6′-ethyl-2a$^{1}$,20a-dihydroxy-5′,6,8,19-tetramethyl-2a$^{1}$,3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17,20-dione (20a-hydroxy-20-oxomilbemycin $A_4$).

**07/2017:1030**
**corrected 10.0**

# MINOCYCLINE HYDROCHLORIDE DIHYDRATE

## Minocyclini hydrochloridum dihydricum

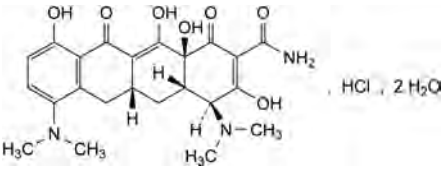

$C_{23}H_{28}ClN_3O_7,2H_2O$ $M_r$ 530.0
[128420-71-3]

DEFINITION

(4*S*,4a*S*,5a*R*,12a*S*)-4,7-Bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride dihydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 94.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, hygroscopic, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *minocycline hydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (a)*. Dissolve 5 mg of *minocycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (b)*. Dissolve 5 mg of *minocycline hydrochloride CRS* and 5 mg of *oxytetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.
*Application*: 1 µL
*Development*: over 3/4 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 450 nm using a 1 cm cell is not greater than 0.23.

Dilute 1.0 mL of solution S to 10.0 mL with *water R*.

**pH** (*2.2.3*): 3.5 to 4.5 for solution S.

**Light-absorbing impurities**. *Carry out the measurement within 1 h of preparing solution S.*

The absorbance (*2.2.25*) of solution S measured at 560 nm is not greater than 0.06.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Store the solutions at 2-8 °C and use them within 3 h of preparation.*

*Solution A*. Mix 18 volumes of a 3.75 g/L solution of *sodium edetate R* and 60 volumes of a 28.3 g/L solution of *ammonium oxalate R* and adjust to pH 7.2 with *dilute ammonia R2*.

*Test solution*. Dissolve 24.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b)*. Dissolve 2 mg of *minocycline for system suitability CRS* (containing impurities A, B, C, E, F, G and H) in *water R* and dilute to 5 mL with the same solvent.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: *tetrahydrofuran R*, *dimethylformamide R*, solution A (8:12:78 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: 3 times the retention time of minocycline.

*Identification of impurities*: use the chromatogram supplied with *minocycline for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, E, F, G and H.

*Relative retention* with reference to minocycline (retention time = about 11 min): impurity C = about 0.52; impurity H = about 0.55; impurity B = about 0.66; impurity A = about 0.74; impurity G = about 0.79; impurity F = about 0.92; impurity E = about 2.1.

*System suitability*: reference solution (b):
– *resolution*: minimum 1.5 between the peaks due to impurities C and H; minimum 1.5 between the peaks due to impurities A and G; minimum 1.5 between the peaks due to impurity F and minocycline.

*Calculation of percentage contents*:
– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 1.6; impurity F = 1.6; impurity G = 1.4;
– for each impurity, use the concentration of minocycline hydrochloride dihydrate in reference solution (a).

*Limits*:
– *impurity A*: maximum 1.2 per cent;
– *impurity B*: maximum 0.8 per cent;
– *impurities C, E*: for each impurity, maximum 0.6 per cent;
– *impurities F, G*: for each impurity, maximum 0.5 per cent;
– *impurity H*: maximum 0.3 per cent;

– *any other impurity*: for each impurity, maximum 0.15 per cent;

– *total*: maximum 3.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 5.0 per cent to 8.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 1.25 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dissolve 30.0 mg of *minocycline hydrochloride CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Injection*: test solution and reference solution.

Calculate the percentage content of $C_{23}H_{28}ClN_3O_7$ taking into account the assigned content of *minocycline hydrochloride CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, I.*

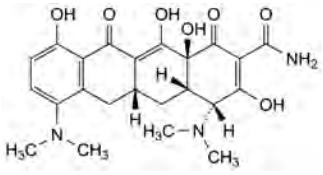

A. (4*R*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epiminocycline),

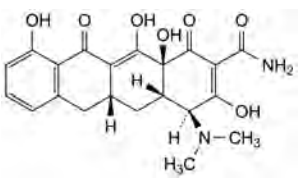

B. (4*S*,4a*S*,5a*R*,12a*S*)-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (sancycline),

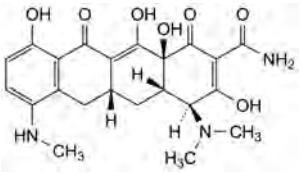

C. (4*S*,4a*S*,5a*R*,12a*S*)-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-7-(methylamino)-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (7-monodemethylminocycline),

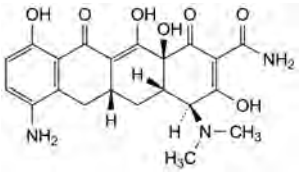

D. (4*S*,4a*S*,5a*R*,12a*S*)-7-amino-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (7-aminosancycline),

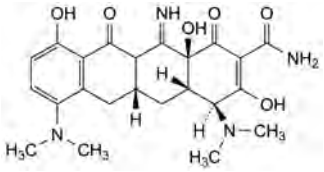

E. (4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-3,10,12a-trihydroxy-12-imino-1,11-dioxo-1,4,4a,5,5a,6,11,11a,12,12a-decahydrotetracene-2-carboxamide,

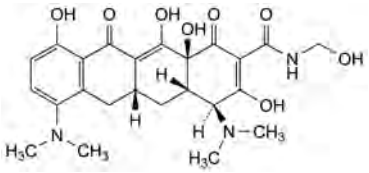

F. (4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-*N*-(hydroxymethyl)-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide,

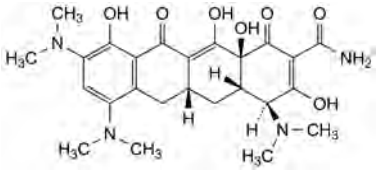

G. (4*S*,4a*S*,5a*R*,12a*S*)-4,7,9-tris(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide,

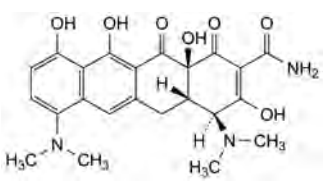

H. (4*S*,4a*S*,12a*S*)-4,7-bis(dimethylamino)-3,10,11,12a-tetrahydroxy-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide,

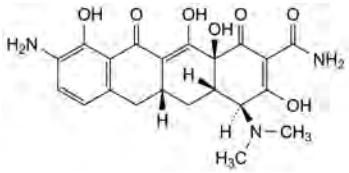

I. (4*S*,4a*S*,5a*R*,12a*S*)-9-amino-4-(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (9-aminosancycline).

**01/2017:0937**

# MINOXIDIL

## Minoxidilum

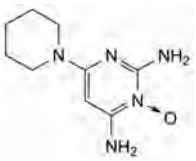

$C_9H_{15}N_5O$ $M_r$ 209.3
[38304-91-5]

DEFINITION

6-(Piperidin-1-yl)pyrimidine-2,4-diamine 3-oxide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol and in propylene glycol.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 20.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid (solution A). Dilute 2.0 mL of solution A to 100.0 mL with *0.1 M hydrochloric acid*.

*Test solution (b).* Dilute 2.0 mL of solution A to 100.0 mL with *0.1 M sodium hydroxide*.

*Spectral range*: 200-350 nm.

*Absorption maxima*: at 230 nm and 281 nm for test solution (a); at 230 nm, 262 nm and 288 nm for test solution (b).

*Specific absorbances at the absorption maxima*:

- at 230 nm: 1015 to 1120 for test solution (a); 1525 to 1685 for test solution (b);
- at 262 nm: 485 to 535 for test solution (b);
- at 281 nm: 1060 to 1170 for test solution (a);
- at 288 nm: 555 to 605 for test solution (b).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *minoxidil CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *minoxidil CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R* (1.5:100 *V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 1 mL of *methanol R*. Add 0.1 mL of *copper sulfate solution R*. A green colour develops. The solution becomes greenish-yellow on the addition of 0.1 mL of *dilute hydrochloric acid R*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 12.5 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *minoxidil for system suitability CRS* (containing impurities A, B and E) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (2.6 µm);
- *temperature*: 40 °C.

*Mobile phase*: solution containing 0.1 per cent *V/V* of *trifluoroacetic acid R* and 2 g/L of *sodium heptanesulfonate R* in a mixture of 45 volumes of *methanol R* and 55 volumes of *water for chromatography R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of minoxidil.

*Identification of impurities*: use the chromatogram supplied with *minoxidil for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and E.

*Relative retention* with reference to minoxidil (retention time = about 5 min): impurity A = about 0.3; impurity B = about 0.4; impurity E = about 1.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and B; minimum 2.0 between the peaks due to minoxidil and impurity E.

*Calculation of percentage contents*:

- for impurity B, multiply the peak area by the correction factor 1.6;
- for each impurity, use the concentration of minoxidil in reference solution (a).

*Limits*:

- *impurity E*: maximum 0.2 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 20.93 mg of $C_9H_{15}N_5O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

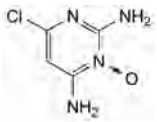

A. 6-chloropyrimidine-2,4-diamine 3-oxide,

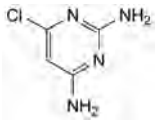

B. 6-chloropyrimidine-2,4-diamine,

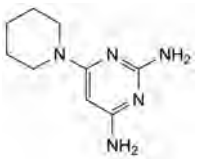

E. 6-(piperidin-1-yl)pyrimidine-2,4-diamine (deoxyminoxidil).

**07/2009:2338**
**corrected 9.0**

# MIRTAZAPINE

## Mirtazapinum

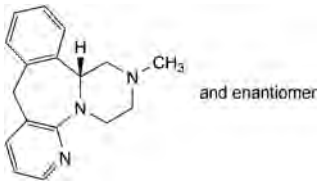


$C_{17}H_{19}N_3$ $M_r$ 265.4
[85650-52-8]

DEFINITION

(14b*RS*)-2-Methyl-1,2,3,4,10,14b-hexahydropyrazino[2,1-*a*]pyrido[2,3-*c*][2]benzazepine.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, slightly hygroscopic to hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mirtazapine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10° (anhydrous substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Buffer solution*. Dissolve 18.0 g of *tetramethylammonium hydroxide R* in 950 mL of *water R*. While stirring, adjust to pH 7.4 with *phosphoric acid R*, then dilute to 1000 mL with *water R* and mix.

*Test solution*. Dissolve 30 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 3 mg of *mirtazapine for system suitability CRS* (containing impurities A, B, C, D, E and F) in 2 mL of the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *tetrahydrofuran for chromatography R*, *methanol R*, *acetonitrile R*, buffer solution (7.5:12.5:15:65 *V/V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of mirtazapine.

*Identification of impurities*: use the chromatogram supplied with *mirtazapine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to mirtazapine (retention time = about 25 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.35; impurity D = about 0.4; impurity E = about 1.3; impurity F = about 1.35.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurities E and F in the chromatogram obtained with reference solution (a);
- *symmetry factor*: 0.8 to 2.0 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.3; impurity B = 1.3; impurity F = 0.2;
- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 3.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 35 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 13.27 mg of $C_{17}H_{19}N_3$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

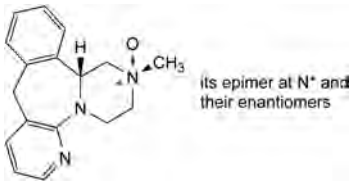


A. (14b*RS*)-2-methyl-1,2,3,4,10,14b-hexahydropyrazino[2,1-*a*]pyrido[2,3-*c*][2]benzazepine 2-oxide,

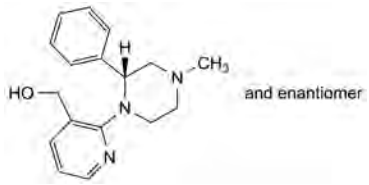


B. [2-[(2*RS*)-4-methyl-2-phenylpiperazin-1-yl]pyridin-3-yl]methanol,

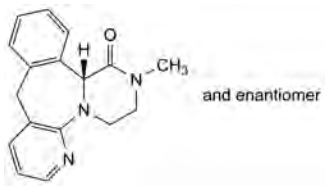


C. (14b*RS*)-2-methyl-3,4,10,14b-tetrahydropyrazino[2,1-*a*]pyrido[2,3-*c*][2]benzazepin-1(2*H*)-one,

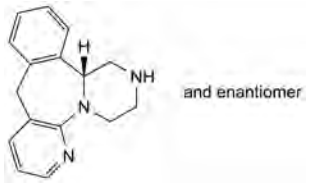


D. (14b*RS*)-1,2,3,4,10,14b-hexahydropyrazino[2,1-*a*]pyrido[2,3-*c*][2]benzazepine,

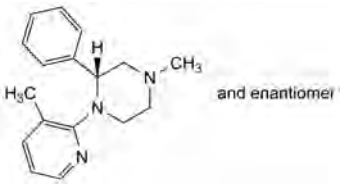


E. (2*RS*)-4-methyl-1-(3-methylpyridin-2-yl)-2-phenylpiperazine,

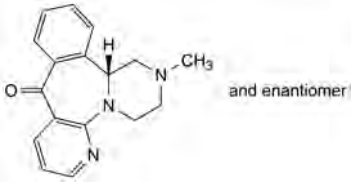


F. (14b*RS*)-2-methyl-1,3,4,14b-tetrahydropyrazino[2,1-*a*]pyrido[2,3-*c*][2]benzazepin-10(2*H*)-one.

**04/2010:1731**

# MISOPROSTOL

## Misoprostolum

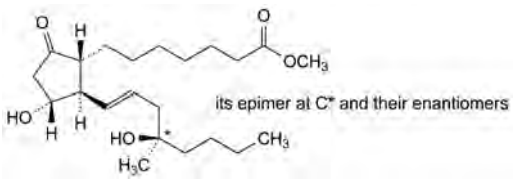


$C_{22}H_{38}O_5$ $M_r$ 382.5
[59122-46-2]

DEFINITION

Mixture of methyl 7-[(1*RS*,2*RS*,3*RS*)-3-hydroxy-2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate and methyl 7-[(1*RS*,2*RS*,3*RS*)-3-hydroxy-2-[(1*E*,4*SR*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate.

The 4 stereoisomers are present in approximately equal proportions.

*Content*: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless or yellowish, oily liquid, hygroscopic.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent), sparingly soluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *misoprostol CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *misoprostol CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *misoprostol for system suitability CRS* (containing impurities A, B and C) in 1 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 5 volumes of *acetonitrile R1*, 215 volumes of *dioxan R*, 780 volumes of *heptane R* and sonicate for 10 min.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.5 times the retention time of misoprostol.

*Identification of impurities*: use the chromatogram supplied with *misoprostol for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to misoprostol (retention time = about 18 min): impurity C = about 0.2; impurity A = about 0.7; impurity B (1st peak) = about 0.85; impurity B (2nd peak) = about 0.91.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.2 between the peaks due to impurity B (2nd peak) and misoprostol.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.13;

– *impurity B* (sum of 1st and 2nd peaks): not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Diastereoisomers**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 20 volumes of *2-propanol R*, 40 volumes of *anhydrous ethanol R*, 940 volumes of *heptane R* and sonicate for 10 min.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of the 1st peak of misoprostol.

*Retention time*: misoprostol 1st peak = about 19 min; misoprostol 2nd peak = about 21 min.

*System suitability*: test solution:

– *resolution*: minimum 2.0 between the 1st and 2nd peaks of misoprostol.

*Limit*:

– *1st peak of misoprostol*: 45 per cent to 55 per cent of the sum of the areas of the 2 peaks due to misoprostol.

**Water** (*2.5.32*): maximum 1.0 per cent.

Use 1.0 mL of a 10 mg/mL solution of the substance to be examined in *methanol R*.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: 20 µL of the test solution and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 3.7 for the peak due to misoprostol.

Calculate the percentage content of $C_{22}H_{38}O_5$ using the declared content of *misoprostol CRS*.

STORAGE

In an airtight container, at – 20 °C.

IMPURITIES

*Specified impurities*: *A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F.*

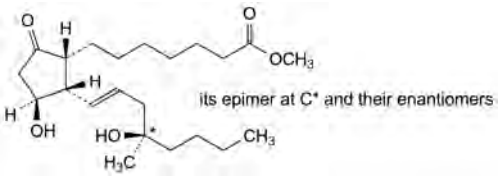


A. mixture of methyl 7-[(1*RS*,2*SR*,3*SR*)-3-hydroxy-2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate and methyl 7-[(1*RS*,2*SR*,3*SR*)-3-hydroxy-2-[(1E,4*SR*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate (8-epimisoprostol),

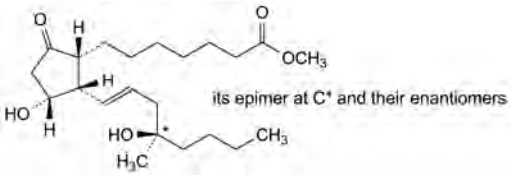


B. mixture of methyl 7-[(1*RS*,2*SR*,3*RS*)-3-hydroxy-2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate and methyl 7-[(1*RS*,2*SR*,3*RS*)-3-hydroxy-2-[(1*E*,4*SR*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate (12-epimisoprostol),

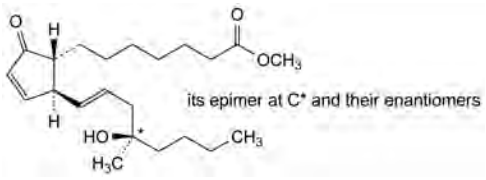


C. mixture of methyl 7-[(1*RS*,2*SR*)-2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopent-3-enyl]heptanoate and methyl 7-[(1*RS*,2*SR*)-2-[(1*E*,4*SR*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopent-3-enyl]heptanoate (misoprostol A),

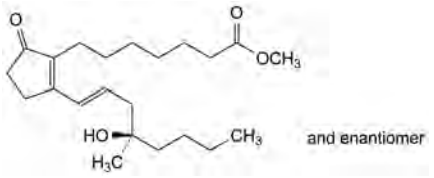


D. methyl 7-[2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopent-1-enyl]heptanoate (misoprostol B),

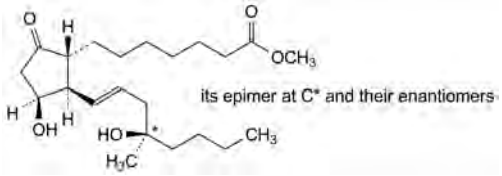


E. mixture of methyl 7-[(1*RS*,2*RS*,3*SR*)-3-hydroxy-2-[(1*E*,4*RS*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate and methyl 7-[(1*RS*,2*RS*,3*SR*)-3-hydroxy-2-[(1*E*,4*SR*)-4-hydroxy-4-methyloct-1-enyl]-5-oxocyclopentyl]heptanoate (11-epi misoprostol),

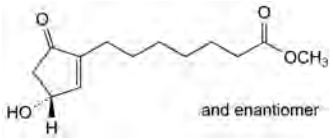


F. methyl 7-[(3*RS*)-3-hydroxy-5-oxocyclopent-1-enyl]heptanoate.

**01/2017:2625**

# MITES FOR ALLERGEN PRODUCTS

## Acari ad producta allergenica

DEFINITION

Mites for allergen products are inactivated mite cultures that may consist of faecal particles, mite bodies, mite parts, eggs, larvae and residues from the growth medium. They contain soluble substances, including proteins, whose functional activity is unrelated to any antigenic and allergenic properties they may have.

PRODUCTION

Mites for allergen products are obtained from cultivation techniques. The method of mite cultivation is described and must ensure the quality, homogeneity and traceability of the mites. Critical cultivation parameters such as temperature and humidity are controlled and monitored. Unless otherwise justified, the culture medium is selected to avoid the presence of materials with potential allergenicity. Appropriate measures are taken to avoid contamination by foreign mite cultures. Mite cultures are macroscopically free of moulds.

After cultivation, the mite culture is inactivated by methods whose capability to maintain allergenic properties are qualified.

Certain mite fractions (e.g. mite bodies or mite faeces) may be purified or enriched before the initiation of the active substance manufacture. In these cases, the process must be performed using qualified methods.

Where major changes to the production of the mites take place (e.g. when a new process or supplier of mites is introduced), such changes are qualified.

Microbial contamination of the mite culture may be unavoidable and should be monitored on a representative number of batches of mites according to a justified sampling plan, and each time a new supplier and/or a new process for the mites production is introduced; if a determination of microbial contamination is not applicable, this must be justified. Microbial contamination values and potential increases in microbial contamination are monitored during stability studies, in order to assess this aspect along with the mites for allergen products characteristics upon storage.

Control methods and acceptance criteria relating to identity and purity of the mites are established. The acceptance criteria must ensure the consistency of the mite source material from a qualitative and quantitative point of view. The mite source material is stored under controlled conditions justified by stability data. The collection and production, as well as the handling of the source material, are such that consistent composition is ensured from batch to batch.

*MITES FOR ALLERGEN PRODUCTS REFERENCE BATCH*

An appropriate reference batch is established for each species. The nature of the reference batch depends on the testing approach to verify batch-to-batch consistency and to establish acceptable quality. The reference batch may be, for example, an internal reference preparation (if available), a source material extract or a sample of a production batch. Its characterisation must be described. The extent of characterisation of the reference batch depends on the mite source material, knowledge of the allergenic components and availability of suitable reagents. The reference batch is stored under controlled conditions ensuring its stability.

*BATCH-TO-BATCH CONSISTENCY*

To establish batch-to-batch consistency, one or more of the following tests are performed on each batch. The choice of tests must be justified.

**Total protein** (*2.5.33*).

**Protein profile**. Determined by using suitable electrophoresis methods (*2.2.31*, *2.2.54*).

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific antibodies.

**Major allergen content**. Determined by using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

**Total allergenic activity**. Determined by testing inhibition of the binding capacity of specific immunoglobulin E antibodies or by a suitable equivalent *in vitro* method.

CHARACTERS

Mites for allergen products are supplied as yellow to brown powders, which may contain one or more of the following: mite bodies, parts, eggs, larvae and faecal particles. The mite source material may contain residual amounts of medium components.

IDENTIFICATION

The identity of the mite species is confirmed by their relevant macroscopic (e.g. colour, appearance) and microscopic (e.g. shape of the mites, size of their legs, number and distribution of hairs and if necessary the geometry of dorsal striations)

morphological characteristics in comparison to those of a reference batch or reference documents. Identity may also be confirmed using protein analysis methods (e.g. protein profile, individual allergen test) or by genetic identification, if performed by generally accepted methods.

TESTS

**Foreign matter**. Foreign matter is defined as any particles that are not part of the mite culture, i.e. any particulate matter not arising from the medium or the mites. Foreign matter is typically detected using a suitable microscopic method; a limit for the amount of foreign matter has to be established based on historical data.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*). The water content of dried material is determined; specification limits must be supported by batch analysis and stability data.

**Purity**. When the source material is a purified fraction of the mite culture (e.g. mite bodies), the purity of the fraction needs to be evaluated and results are within a predefined limit.

STORAGE

The mite source material is stored under controlled conditions justified by stability data.

LABELLING

The label states:
- the species of the mite;
- the fraction of mite culture, where applicable.

**04/2016:1655**

# MITOMYCIN

## Mitomycinum

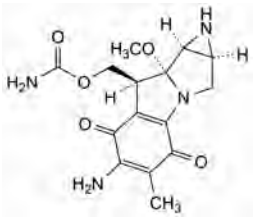

$C_{15}H_{18}N_4O_5$ $M_r$ 334.3
[50-07-7]

DEFINITION

[(1a*S*,8*S*,8a*R*,8b*S*)-6-Amino-8a-methoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2′,3′:3,4]pyrrolo[1,2-*a*]-indol-8-yl]methyl carbamate (mitomycin C).

Substance produced by a strain of *Streptomyces caespitosus*.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: blue-violet crystals or crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in dimethylacetamide, sparingly soluble in methanol, slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *mitomycin CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**pH** (*2.2.3*): 5.5 to 7.5.

Dissolve 10 mg in 10 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 10 mg of *cinnamamide R* (impurity A) in *methanol R* and dilute to 10 mL with the same solvent. Mix 2 mL of this solution and 1 mL of the test solution and dilute to 10 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: *methanol R*, 0.77 g/L solution of *ammonium acetate R* (20:80 *V/V*);
- *mobile phase B*: *methanol R*, 0.77 g/L solution of *ammonium acetate R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 30 | 100 → 0 | 0 → 100 |
| 30 - 45 | 0 | 100 |
| 45 - 50 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Relative retention* with reference to mitomycin (retention time = about 21 min): impurity D = about 0.6; impurity C = about 1.2; impurity A = about 1.3; impurity B = about 1.6.

*System suitability*: reference solution (b):
- *resolution*: minimum 15.0 between the peaks due to mitomycin and impurity A.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.35;
- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.30 g.

**Bacterial endotoxins** (*2.6.14, Method B*): less than 10 IU/ mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *dimethylacetamide R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 25.0 mg of *mitomycin CRS* in *dimethylacetamide R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *cinnamamide R* (impurity A) in *methanol R* and dilute to 20 mL with the same solvent. Mix 2 mL of this solution with 2 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.30 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped phenylsilyl silica gel for chromatography R* (10 µm) with a specific surface area of 330 $m^2$/g, a carbon loading of 8 per cent and a pore size of 12.5 nm.

*Mobile phase*: mix 23 volumes of *methanol R*, 77 volumes of a solution containing 2.05 g/L of *ammonium acetate R* and 2.8 mL/L of *dilute acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 365 nm and 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of mitomycin.

*Relative retention* with reference to mitomycin (retention time = about 8 min): impurity A = about 1.2.

*System suitability*:

- *resolution*: minimum 1.8 between the peaks due to mitomycin and impurity A in the chromatogram obtained with reference solution (b) at 254 nm,
- *symmetry factor*: maximum 1.3 for the principal peak in the chromatogram obtained with reference solution (a) at 365 nm.

Calculate the percentage content of $C_{15}H_{18}N_4O_5$, using the chromatograms obtained with the test solution and reference solution (a) at 365 nm and taking into account the assigned content of *mitomycin CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

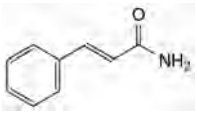

A. (*E*)-3-phenylprop-2-enamide (cinnamamide),

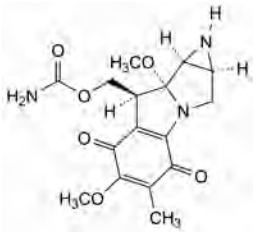

B. [(1a*S*,8*S*,8a*R*,8b*S*)-6,8a-dimethoxy-5-methyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2′,3′:3,4]pyrrolo[1,2-*a*]indol-8-yl]methyl carbamate (mitomycin A),

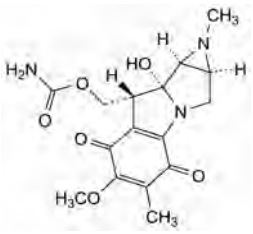

C. [(1a*S*,8*R*,8a*R*,8b*S*)-8a-hydroxy-6-methoxy-1,5-dimethyl-4,7-dioxo-1,1a,2,4,7,8,8a,8b-octahydroazirino[2′,3′:3,4]pyrrolo[1,2-*a*]indol-8-yl]methyl carbamate (mitomycin B),

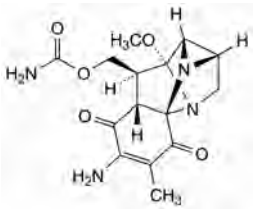

D. [(1*S*,2*S*,4*S*,5*R*,6*S*,6a*R*,10a*S*,11*S*)-8-amino-5-methoxy-9-methyl-7,10-dioxo-2,3,6,6a,7,10-hexahydro-1,2,5-metheno-1*H*,5*H*-imidazo[2,1-*i*]indol-6-yl]methyl carbamate (albomitomycin C).

**01/2008:1243**

# MITOXANTRONE HYDROCHLORIDE

## Mitoxantroni hydrochloridum

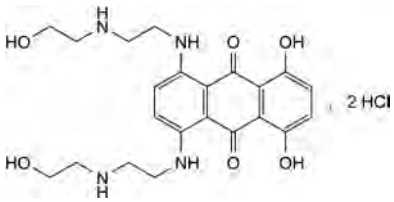

$C_{22}H_{30}Cl_2N_4O_6$ $M_r$ 517.4
[70476-82-3]

DEFINITION

1,4-Dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]-amino]anthracene-9,10-dione dihydrochloride.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: dark blue, electrostatic, hygroscopic powder.

*Solubility*: sparingly soluble in water, slightly soluble in methanol, practically insoluble in acetone.

*CAUTION: mitoxantrone hydrochloride and impurity A are electrostatic; the use of an antistatic gun or other suitable method to discharge the solids before weighing or transfer is recommended.*

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 2-3 mg in 1 mL of *methanol R* by warming in a water-bath at 40-50 °C. Evaporate to dryness under a stream of dry nitrogen, warming gently if necessary. Examine the residue.

*Comparison*: *mitoxantrone hydrochloride CRS*.

B. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in about 40 mL of the mobile phase, sonicating if necessary, and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 20.0 mg of *mitoxantrone hydrochloride CRS* in about 40 mL of the mobile phase, sonicating if necessary, and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1 mL of the test solution to 100 mL with the mobile phase.

*Reference solution (c).* Dissolve 2.0 mg of *mitoxantrone impurity A CRS* in 1.0 mL of reference solution (a).

*Reference solution (d).* Dilute 1 mL of reference solution (b) to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.30 m, Ø = 3.0 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 750 volumes of *water R*, 250 volumes of *acetonitrile R* and 25 volumes of a solution prepared as follows: dissolve 22.0 g of *sodium heptanesulfonate R* in about 150 mL of *water R* and filter through a 0.45 µm filter; wash the filter with *water R* and combine the filtrate and washings; add 32.0 mL of *glacial acetic acid R* and dilute to 250 mL with *water R*.

*Flow rate*: 3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 50 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of mitoxantrone.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to mitoxantrone and impurity A.

*Limits*:

- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

**Ethanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 2.0 mL of *propanol R* to 100 mL with *water R*. Dilute 5.0 mL of this solution to 100 mL with *water R*.

*Test solution.* Mix 0.100 g of the substance to be examined with 2.0 mL of the internal standard solution and dilute to 5.0 mL with *water R*. Place the flask in an ultrasonic bath for 2 min, then shake the flask for 2 min. If necessary, repeat the sonication and shaking until dissolution is complete.

*Reference solution.* Dilute 2.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Dilute 10.0 mL of this solution and 10.0 mL of the internal standard solution to 25.0 mL with *water R*.

*Column*:

- *size*: *l* = 2 m, Ø = 3 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R*.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 19 mL/min.

*Temperature*:

- *column*: 120 °C;
- *injection port*: 175 °C;
- *detector*: 210 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Retention time*: ethanol = about 1 min; propanol = about 2 min.

*System suitability*: reference solution:

- *resolution*: minimum 6 between the peaks due to ethanol and propanol.

Calculate the content of ethanol, taking its density (*2.2.5*) to be 0.790 g/mL at 20 °C.

*Limit*:

- *ethanol*: maximum 1.6 per cent *m/m*.

**Water** (*2.5.12*): maximum 6.0 per cent, determined on 0.300 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{30}Cl_2N_4O_6$ from the declared content of *mitoxantrone hydrochloride CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D.*

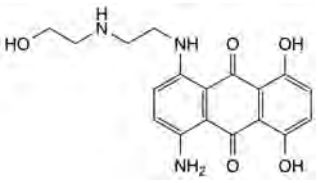

A. 1-amino-5,8-dihydroxy-4-[[2-[(2-hydroxyethyl)amino]-ethyl]amino]anthracene-9,10-dione,

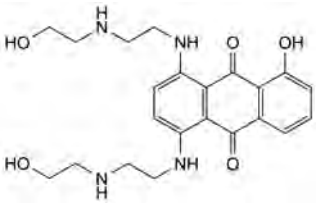

B. 5-hydroxy-1,4-bis[[2-[(2-hydroxyethyl)amino]ethyl]-amino]anthracene-9,10-dione,

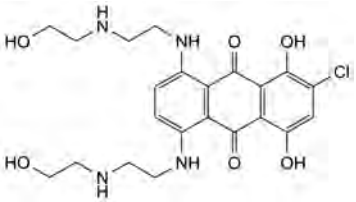

C. 2-chloro-1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]ethyl]amino]anthracene-9,10-dione,

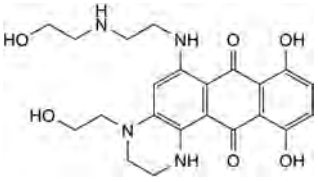

D. 8,11-dihydroxy-4-(2-hydroxyethyl)-6-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-1,2,3,4-tetrahydronaphtho[2,3-*f*]quinoxaline-7,12-dione.

**01/2017:2307**

# MODAFINIL

## Modafinilum

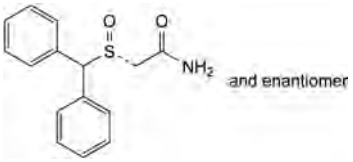


$C_{15}H_{15}NO_2S$ $M_r$ 273.4
[68693-11-8]

DEFINITION

2-[(*RS*)-(Diphenylmethyl)sulfinyl]acetamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble or practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *modafinil CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (35:65 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 35 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 50.0 mg of *modafinil CRS* in 35 mL of *acetonitrile R1* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c)*. Add 2.0 mL of the solvent mixture to a vial of *modafinil for system suitability CRS* (containing impurities A, B and C) and sonicate for 10 min.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.3 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 4 times the retention time of modafinil.

*Identification of impurities*: use the chromatogram supplied with *modafinil for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to modafinil (retention time = about 4 min): impurity A = about 1.3; impurity B = about 1.8; impurity C = about 3.0.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to modafinil.

*Limits*:

- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities B, C*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of modafinil from the declared content of $C_{15}H_{15}NO_2S$ in *modafinil CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

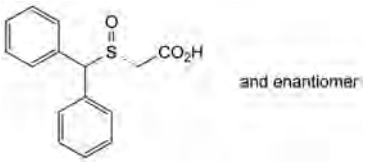


A. [(*RS*)-(diphenylmethyl)sulfinyl]acetic acid,

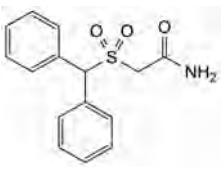


B. 2-[(diphenylmethyl)sulfonyl]acetamide,

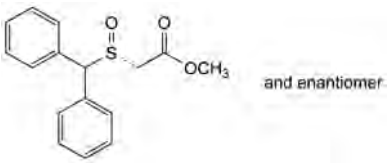


C. methyl [(*RS*)-(diphenylmethyl)sulfinyl]acetate.

01/2008:1641
corrected 10.0

# MOLGRAMOSTIM CONCENTRATED SOLUTION

## Molgramostimi solutio concentrata

APARSPSPST QPWEHVNAIQ EARRLLNLSR
DTAAEMNETV EVISEMFDLQ EPTCLQTRLE
LYKQGLRGSL TKLKGPLTMM ASHYKQHCPP
TPETSCATQI ITFESFKENL KDFLLVIPFD
CWEPVQE

$C_{639}H_{1007}N_{171}O_{196}S_8$ $M_r$ 14 477

DEFINITION

Solution of a protein having the structure of the granulocyte macrophage colony stimulating factor which is produced and secreted by various human blood cell types. The protein stimulates the differentiation and proliferation of leucocyte stem cells into mature granulocytes and macrophages.

*Content*: minimum 2.0 mg of protein per millilitre.

*Potency*: minimum $0.7 \times 10^7$ IU per milligram of protein.

PRODUCTION

Molgramostim concentrated solution is produced by a method based on recombinant DNA (rDNA) technology, using bacteria as host cells. It is produced under conditions designed to minimise microbial contamination of the product.

Prior to release, the following tests are carried out on each batch of molgramostim concentrated solution, unless exemption has been granted by the competent authority.

**Host-cell derived proteins**: the limit is approved by the competent authority.

**Host-cell or vector derived DNA**: the limit is approved by the competent authority.

CHARACTERS

*Appearance*: clear, colourless liquid.

IDENTIFICATION

A. It shows the expected biological activity (see Assay).

B. Isoelectric focusing (*2.2.54*).

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of 0.25 mg/mL.

*Reference solution (a).* Dilute *molgramostim CRS* with *water R* to obtain a concentration of 0.25 mg/mL.

*Reference solution (b).* Use an isoelectric point (pI) calibration solution, in the pI range of 2.5-6.5, prepared according to the manufacturer's instructions.

*Focusing*:

- *pH gradient*: 4.0-6.5;
- *catholyte*: 8.91 g/L (0.1 M) solution of *3-aminopropionic acid R*;
- *anolyte*: 14.7 g/L (0.1 M) solution of *glutamic acid R* in a 50 per cent *V/V* solution of *dilute phosphoric acid R* (0.5 M);
- *application*: 20 µL.

*Detection*: immerse the gel in a suitable volume of a solution containing 115 g/L of *trichloroacetic acid R* and 34.5 g/L of *sulfosalicylic acid R* and shake the container gently for 30 min. Transfer the gel to a mixture of 32 volumes of *glacial acetic acid R*, 100 volumes of *anhydrous ethanol R* and 268 volumes of *water R* (mixture A) and rinse for 5 min. Immerse the gel for 10 min in a staining solution prewarmed to 60 °C and prepared by adding *acid blue 83 R* at a concentration of 1.2 g/L to mixture A. Wash the gel in several containers with mixture A and keep the gel in this mixture until the background is clear (12-24 h). After adequate destaining, soak the gel for 1 h in a 10 per cent *V/V* solution of *glycerol R* in mixture A.

*System suitability*:

- in the electropherogram obtained with reference solution (b), the relevant isoelectric point markers are distributed along the entire length of the gel;
- in the electropherogram obtained with reference solution (a), the pI of the principal band is 4.9 to 5.4.

*Results*: the principal band in the electropherogram obtained with the test solution corresponds in position to the principal band in the electropherogram obtained with reference solution (a). Plot the migration distances of the relevant pI markers versus their pI and determine the isoelectric points of the principal component of each of the test solution and reference solution (a). They do not differ by more than 0.2 pI units.

C. Examine the electropherograms obtained under reducing conditions in the test for impurities with molecular masses differing from that of molgramostim. The principal band in the electropherogram obtained with test solution (a) is similar in position to the principal band in the electropherogram obtained with reference solution (a).

D. Peptide mapping (*2.2.55*).

*Test solution.* Introduce 50 µL of *tris-hydrochloride buffer solution pH 8.0 R* and 50 µL of the preparation to be examined at a concentration of 2 mg/mL into a polypropylene tube of 0.5 mL capacity. Add 4 µL of a 1 mg/mL solution of *trypsin for peptide mapping R* in a 0.01 per cent *V/V* solution of *trifluoroacetic acid R*, cap tightly and mix well. Incubate at about 37 °C for 18 h. Add 125 µL of a 764 g/L (8 M) solution of *guanidine hydrochloride R* and mix well. Add 10 µL of a 154.2 g/L (1 M) solution of *dithiothreitol R* and mix well. Place the capped tube in boiling water for 1 min. Cool to room temperature.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution but use *molgramostim CRS* instead of the preparation to be examined.

Examine the 2 tryptic digests by liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* in 1000 mL of *water for chromatography R*;
- *mobile phase B*: dilute 1 mL of *trifluoroacetic acid R* in 100 mL of *water for chromatography R*; add 900 mL of *acetonitrile R1* and mix;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35.0 | 100 → 65 | 0 → 35 |
| 35.0 - 105.0 | 65 → 35 | 35 → 65 |
| 105.0 - 107.5 | 35 → 100 | 65 → 0 |
| 107.5 - 120.0 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Equilibration*: at initial conditions for at least 12 min.

*Injection*: 200 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the *Ph. Eur. reference chromatogram of molgramostim digest*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

E. *N*-Terminal sequence analysis.

Perform the Edman degradation using an automated solid-phase sequencer, operated in accordance with the manufacturer's instructions.

Load about 1 nmol of the test preparation to a sequencing cartridge using the protocol provided by the manufacturer. Run 16 sequencing cycles, noting, if appropriate, the presence of proline at positions 2, 6, 8 and 12.

Identify the phenylthiohydantoin (PTH)-amino acids released at each sequencing cycle by reverse-phase liquid chromatography. The procedure may be carried out using the column and reagents recommended by the manufacturer of the sequencing equipment for the separation of PTH-amino acids.

The separation procedure is calibrated using:

- the mixture of PTH-amino acids provided by the manufacturer of the sequencer, with the gradient conditions adjusted as indicated to achieve optimum resolution of all amino acids;
- a sample obtained from a blank sequencing cycle obtained as recommended by the equipment manufacturer.

*Results*: the first 16 amino acids are: Ala-Pro-Ala-Arg-Ser-Pro-Ser-Pro-Ser-Thr-Gln-Pro-Trp-Glu-His-Val.

TESTS

**Impurities with molecular masses differing from that of molgramostim**. Polyacrylamide gel electrophoresis (*2.2.31*) under both reducing and non-reducing conditions.

*Gel dimensions*: 0.75 mm thick.

*Resolving gel*: 14 per cent acrylamide.

*Sample buffer A*. Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer R*.

*Sample buffer B (reducing conditions)*. Mix equal volumes of *water R* and *concentrated SDS-PAGE sample buffer for reducing conditions R*.

*Test solution (a)*. Dilute the preparation to be examined in *water R* to obtain a concentration of 1.0 mg/mL. To 1 volume of this solution add 1 volume of *concentrated SDS-PAGE sample buffer R*.

*Test solution (b)* (2 per cent control). Dilute 0.020 mL of test solution (a) to 1.0 mL with sample buffer A.

*Test solution (c)* (1 per cent control). To 0.20 mL of test solution (b) add 0.20 mL of sample buffer A.

*Test solution (d)* (0.5 per cent control). To 0.20 mL of test solution (c) add 0.20 mL of sample buffer A.

*Test solution (e)* (0.25 per cent control). To 0.20 mL of test solution (d) add 0.20 mL of sample buffer A.

*Test solution (f)* (0.1 per cent control). To 0.20 mL of test solution (e) add 0.30 mL of sample buffer A.

*Test solution (g)* (0.05 per cent control). To 0.20 mL of test solution (f) add 0.20 mL of sample buffer A.

*Test solution (h)* (0.025 per cent control). To 0.20 mL of test solution (g) add 0.20 mL of sample buffer A.

*Test solution (i)*. Prepare as for test solution (a), but using *concentrated SDS-PAGE sample buffer for reducing conditions R*.

*Test solutions (j)-(p)*. Prepare as for test solutions (b)-(h), but using sample buffer B.

*Reference solution (a)*. Dilute *molgramostim CRS* in *water R* to obtain a concentration of 0.02 mg/mL. Mix 1 volume of this solution with 1 volume of *concentrated SDS-PAGE sample buffer R*.

*Reference solution (b)*. Prepare as for reference solution (a), but using *concentrated SDS-PAGE sample buffer for reducing conditions R*.

*Reference solution (c)*. Use a solution of molecular mass markers suitable for calibrating SDS-PAGE gels in the range of 14 400-94 000. Dissolve in sample buffer or sample buffer (reducing conditions), as appropriate.

*Sample treatment*: boil for 3 min.

*Application*: 50 µL; apply reduced and non-reduced solutions to separate gels.

*Detection*: silver staining as described below.

Immerse the gel overnight in a mixture of 10 volumes of *acetic acid R*, 40 volumes of *water R* and 50 volumes of *methanol R*. Transfer the gel to a 100 g/L solution of *glutaraldehyde R* and shake for about 30 min. Replace the glutaraldehyde solution with *water R*, and keep the gel in *water R* for 20 min. Repeat this washing-step twice. Transfer the gel to a mixture containing 0.75 g/L of *sodium hydroxide R*, 14 g/L of *concentrated ammonia R* and 8 g/L of *silver nitrate R*. This solution is prepared immediately before use. Place the gel on a shaker in the dark for 5 min. Wash the gel for 30 s in each of 3 containers with *water R* and shake the gel in a mixture consisting of 0.05 g/L of *citric acid monohydrate R*, 0.05 per cent *V/V* of *formaldehyde R* and 0.005 per cent *V/V* of *methanol R* in *water R*. Protein bands become visible during this step. Keep the gel in the solution until sufficiently stained and then rinse the gel repeatedly with *water R* in a shaking water bath. Soak gels in a solution consisting of 10 per cent *V/V* of *acetic acid R* and 1 per cent *V/V* of *glycerol R*.

*System suitability*:

- the validation criteria are met (*2.2.31*);
- a band is seen in the electropherogram obtained with test solution (h);
- a gradation of intensity of staining is seen in the electropherograms obtained with test solutions (a)-(h) and (i)-(p);
- the molecular mass of the principal band in the electropherogram obtained with reference solution (a) or (b) is within the range of 15 100 to 17 100.

*Limits*: compare the staining intensity of each non-molgramostim band observed in the electropherogram obtained with test solution (a) to the staining intensity of the principal band in the electropherograms obtained with test solutions (b)-(h). Proceed similarly with the electropherograms obtained with test solutions (i)-(p). The impurity level is estimated as the dilution, in percentage, of the solution giving the electropherogram with the closest intensity of staining.

Reducing conditions:

- *impurity with an apparent molecular mass of 20 000*: maximum 1 per cent;
- *impurity with an apparent molecular mass of 25 000*: maximum 0.1 per cent;
- *impurity with an apparent molecular mass of 30 000*: maximum 0.3 per cent;
- *total*: maximum 2 per cent.

Non-reducing conditions:

- *total of all impurities of molecular masses higher than 30 000*: maximum 1 per cent.

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution (a)*. Dilute the preparation to be examined with *0.05 M phosphate buffer solution pH 7.0 R* to obtain a concentration of 0.5 mg/mL.

*Test solution (b).* Mix 1 volume of test solution (a) with 4 volumes of a 0.125 mg/mL solution of *human albumin R* or *bovine albumin R* in *0.05 M phosphate buffer solution pH 7.0 R*.

*Reference solution (a).* Dilute *molgramostim CRS* with *0.05 M phosphate buffer solution pH 7.0 R* to obtain a concentration of 0.5 mg/mL.

*Reference solution (b).* Mix 1 volume of reference solution (a) with 4 volumes of a 0.125 mg/mL solution of *human albumin R* or *bovine albumin R* in *0.05 M phosphate buffer solution pH 7.0 R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm.

*Mobile phase*:

- *mobile phase A*: to about 800 mL of *water for chromatography R* add 1.0 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: to 100 mL of *water for chromatography R* add 1.0 mL of *trifluoroacetic acid R* and 900 mL of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 64 → 44 | 36 → 56 |
| 30 - 35 | 44 → 0 | 56 → 100 |
| 35 - 45 | 0 | 100 |
| 45 - 50 | 0 → 64 | 100 → 36 |
| 50 - 60 | 64 | 36 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL of test solution (a), reference solutions (a) and (b).

*System suitability*: reference solution (b):

- *retention time*: molgramostin = about 22 min,
- *repeatability*: maximum relative standard deviation of 5.0 per cent after 4 injections,
- *resolution*: minimum 2 between the peaks due to albumin and molgramostim.

*Limits*:

- *any impurity*: for each impurity, maximum 1.5 per cent,
- *total of impurities eluting between 5 min and 30 min*: maximum 4 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU in the volume that contains 1.0 mg of protein.

ASSAY

**Protein**. Liquid chromatography (*2.2.29*) as described in the test for related proteins.

*Injection*: 150 µL of test solution (b) and reference solution (b).

Calculate the content of molgramostim using the declared content of molgramostim in *molgramostim CRS*.

**Potency**. Determination of the biological activity of molgramostim concentrated solution based on the stimulation of proliferation of TF-1 cells by molgramostim.

The following method uses the conversion of tetrazolium bromide (MTT) as a staining method. Validated alternative stains such as Almar blue have also been found suitable.

TF-1 cells are incubated with varying dilutions of test and reference preparations of molgramostim. They are then incubated with a solution of MTT. This cytochemical stain is converted by cellular dehydrogenases to a purple formazan product. The formazan is then measured spectrophotometrically. The potency of the preparation to be examined is determined by comparison of the dilutions of the test preparation with the dilutions of the appropriate International Standard of molgramostim or with a reference preparation calibrated in International Units, which yield the same response (50 per cent maximal stimulation).

The International Unit is the activity contained in a stated amount of the appropriate International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Add 50 µL of dilution medium to all wells of a 96-well microtitre plate. Add an additional 50 µL of this solution to the wells designed for the blanks. Add 50 µL of each solution to be tested in triplicate (test preparation and reference preparation at a concentration of about 65 IU/mL, plus a series of 10 twofold dilutions to obtain a standard curve). Then add to each well 50 µL of a TF-1 cell suspension containing $3 \times 10^5$ cells per millilitre, maintaining the cells in a uniform suspension during addition.

Incubate the plate at 36.0-38.0 °C for a minimum of 24 h in a humidified incubator using 6 ± 1 per cent $CO_2$. Add 25 µL of a 5.0 g/L sterile solution of *tetrazolium bromide R* to each well. Reincubate for 5 h. Remove the plates from the incubator and add to each well 100 µL of a 240 g/L solution of *sodium dodecyl sulfate R* previously adjusted to pH 2.7 with hydrochloric acid. Reincubate overnight.

Determine the relative quantity of purple formazan product formed in each well by measuring the absorbance (*2.2.25*) using a 96-well microtitre plate reader. Read each plate at 570 nm and at 690 nm. Subtract the reading at 690 nm from the reading at 570 nm. Analyse the data by fitting a sigmoidal dose-response curve to the data obtained and by using a suitable statistical method, for example the 4-parameter model (see *5.3*).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 74 per cent and not more than 136 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature below – 65 °C.

LABELLING

The label states:

- the content, in milligrams of protein per millilitre,
- the potency, in International Units per milligram of protein.

**01/2017:1701**

# MOLSIDOMINE

## Molsidominum

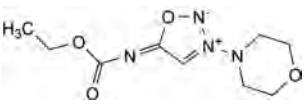

$C_9H_{14}N_4O_4$ $M_r$ 242.2

[25717-80-0]

DEFINITION

*N*-(Ethoxycarbonyl)-3-(morpholin-4-yl)sydnonimine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in anhydrous ethanol and in methylene chloride.

mp: about 142 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *molsidomine CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 1.0 g in *anhydrous ethanol R* by heating at about 50 °C for about 5 min and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 7.5.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Impurity B**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of test solution (a) and reference solution (b).

*Relative retention* with reference to molsidomine (retention time = about 9 min): impurity B = about 0.43.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 20 for the principal peak.

*Limit*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (3 ppm).

**Impurity E**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 50.0 mg of *morpholine for chromatography R* in 500.0 mL of *water for chromatography R*. Dilute 20.0 mL of the solution to 500.0 mL with *water for chromatography R*. Dilute 5.0 mL of this solution to 100.0 mL with *water for chromatography R*.

*Reference solution (b)*. Mix 10.0 mL of the test solution with 10.0 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *resin for reversed-phase ion chromatography R*;

– *temperature*: 25 °C.

*Mobile phase*: mix 3.0 mL of *methanesulfonic acid R* and 75 mL of *acetonitrile R* in *water for chromatography R* and dilute to 5000 mL with *water for chromatography R*.

*Suppressor regenerant*: *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Expected background conductivity*: less than 0.5 µS.

*Detection*: conductivity detector at 10 µS.

*Injection*: 50 µL.

*Run time*: 20 min.

*Relative retention* with reference to molsidomine (retention time = about 3 min): impurity E = about 2.4.

*System suitability*: reference solution (b):

– *signal-to-noise ratio*: minimum 6 for the peak due to impurity E.

*Limit*:

– *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Solvent mixture*: *methanol R*, mobile phase A (10:90 *V/V*).

*Test solution (a)*. Dissolve 0.200 g of the substance to be examined in 2.5 mL of *methanol R* and dilute to 5.0 mL with mobile phase A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2.4 mg of *molsidomine impurity B CRS* in 80 mL of *methanol R* and dilute to 100.0 mL with *methanol R*. Dilute 2.0 mL of the solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10 mg of *linsidomine hydrochloride R* (impurity A) and 5 mg of *molsidomine impurity D CRS* in 10 mL of *methanol R* and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 4.0 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent;

– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 90 | 10 |
| 3 - 10 | 90 → 20 | 10 → 80 |
| 10 - 13 | 20 | 80 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (a) and (c).

*Relative retention* with reference to molsidomine (retention time = about 9 min): impurity A = about 0.2; impurity D = about 0.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.5 between the peaks due to impurities A and D.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the peak due to molsidomine in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the peak due to molsidomine in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the peak due to molsidomine in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5 mL of *acetic anhydride R* and 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.22 mg of $C_9H_{14}N_4O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D.*

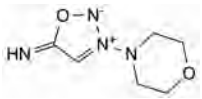

A. 3-(morpholin-4-yl)sydnonimine (linsidomine),

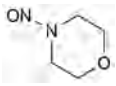

B. 4-nitrosomorpholine,

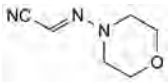

C. (2*E*)-(morpholin-4-ylimino)acetonitrile,

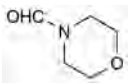

D. morpholine-4-carbaldehyde,

E. morpholine.

**07/2018:1449**

# MOMETASONE FUROATE

## Mometasoni furoas

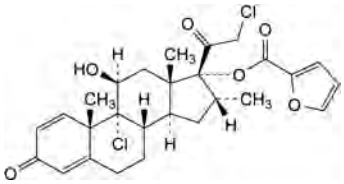

$C_{27}H_{30}Cl_2O_6$ $M_r$ 521.4

[83919-23-7]

DEFINITION

9,21-Dichloro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, E, F.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mometasone furoate CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *mometasone furoate CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *anhydrous beclometasone dipropionate CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R.* Heat at 120 °C for 10 min or until the spots appear. Allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 spots which, when examined in ultraviolet light at 365 nm, may not be completely separated.

C. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 15 min a light yellow colour develops. When examined in ultraviolet light at 365 nm, no fluorescence is seen. Add this solution to 10 mL of *water R* and mix. The colour fades and there is no fluorescence.

D. Mix 80 mg with 0.30 g of *anhydrous sodium carbonate R* and ignite in a crucible until an almost white residue is obtained. Allow to cool and dissolve the residue in 5 mL of *dilute nitric acid R*; filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

E. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

F. Loss on drying (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 50 to + 55 (dried substance).

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 200 mL of *acetonitrile R* and 200 mL of *water R*, then add 0.4 mL of *acetic acid R.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in 15 mL of *acetonitrile R* and dilute to 50.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *mometasone furoate for system suitability CRS* (containing impurities C and J) in 3 mL of *acetonitrile R* and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25.0 mg of *mometasone furoate monohydrate CRS* in 15 mL of *acetonitrile R* and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water for chromatography R* (50:50 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of mometasone furoate.

*Identification of impurities*: use the chromatogram supplied with *mometasone furoate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and J.

*Relative retention* with reference to mometasone furoate (retention time = about 24 min): impurity C = about 0.9; impurity J = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to impurity C and mometasone furoate.

*Limits*:

– *impurity J*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{30}Cl_2O_6$ taking into account the assigned content of *mometasone furoate monohydrate CRS*.

IMPURITIES

*Specified impurities*: *J*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, K, L, M, N, O, P, Q, R, S, T, U.*

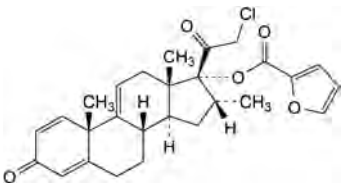

A. 21-chloro-16α-methyl-3,20-dioxopregna-1,4,9(11)-trien-17-yl furan-2-carboxylate,

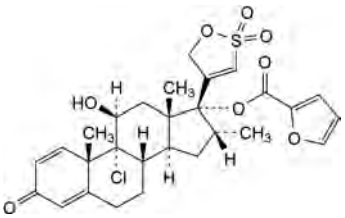

B. 9-chloro-17β-(2,2-dioxo-2,5-dihydro-1,2λ⁶-oxathiol-4-yl)-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl furan-2-carboxylate,

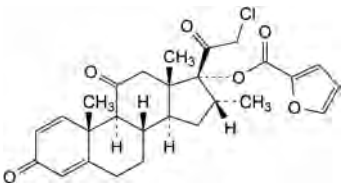

C. 21-chloro-16α-methyl-3,11,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,

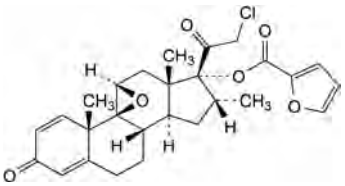

D. 21-chloro-9,11β-epoxy-16α-methyl-3,20-dioxo-9β-pregna-1,4-dien-17-yl furan-2-carboxylate,

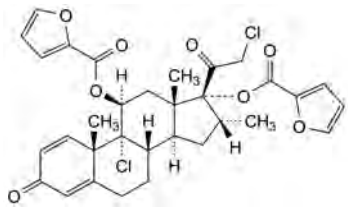

E. 9,21-dichloro-16α-methyl-3,20-dioxopregna-1,4-diene-11β,17-diyl di(furan-2-carboxylate),

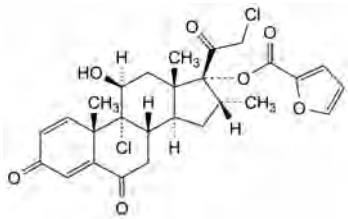

F. 9,21-dichloro-11β-hydroxy-16α-methyl-3,6,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,

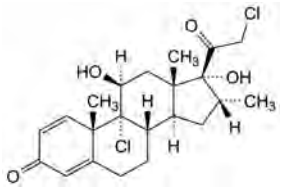

G. 9,21-dichloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (mometasone),

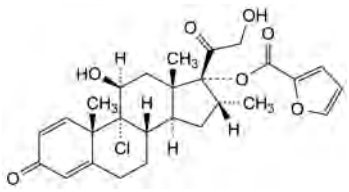

H. 9-chloro-11β,21-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

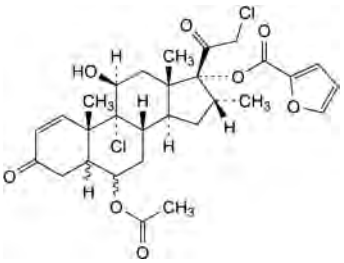

I. 6ξ-acetoxy-9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxo-5ξ-pregn-1-en-17-yl furan-2-carboxylate,

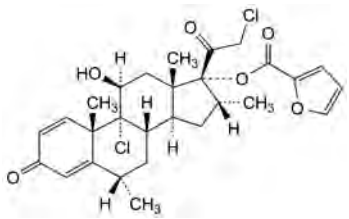

J. 9,21-dichloro-11β-hydroxy-6α,16α-dimethyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

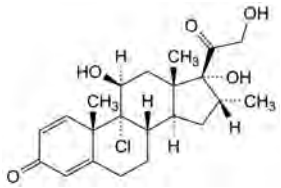

K. 9-chloro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

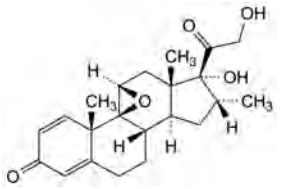

L. 9,11β-epoxy-17,21-dihydroxy-16α-methyl-9β-pregna-1,4-diene-3,20-dione,

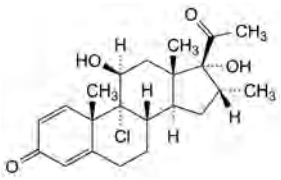

M. 9-chloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

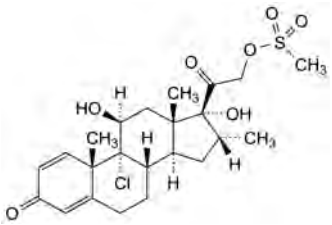

N. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl methanesulfonate,

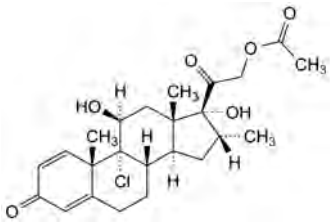

O. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

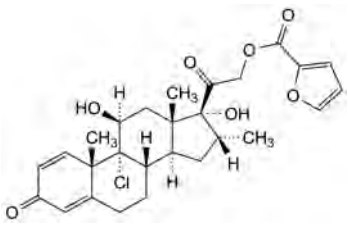

P. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl furan-2-carboxylate,

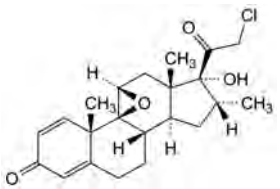

Q. 21-chloro-9,11β-epoxy-17-hydroxy-16α-methyl-9β-pregna-1,4-diene-3,20-dione,

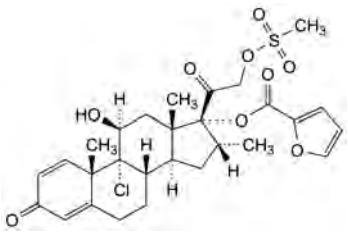

R. 9-chloro-11β-hydroxy-16α-methyl-21-[(methylsulfonyl)oxy]-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

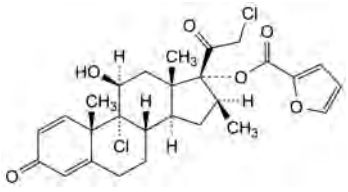

S. 9,21-dichloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

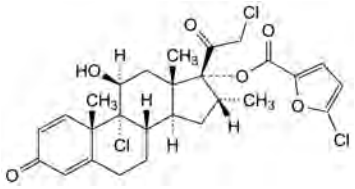

T. 9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl 5-chlorofuran-2-carboxylate,

U. unknown structure.

**07/2018:2858**

# MOMETASONE FUROATE MONOHYDRATE

## Mometasoni furoas monohydricus

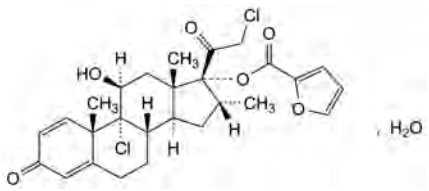

$C_{27}H_{30}Cl_2O_6,H_2O$ $M_r$ 539.4
[141646-00-6]

DEFINITION

9,21-Dichloro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methylene chloride, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *mometasone furoate monohydrate CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Water (see Tests).

TESTS

**Specific optical rotation** (*2.2.7*): + 50 to + 55 (anhydrous substance).

Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*. Mix 200 mL of *acetonitrile R* and 200 mL of *water R*, then add 0.4 mL of *acetic acid R*.

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in 15 mL of *acetonitrile R* and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *mometasone furoate for system suitability CRS* (containing impurity C) in 3 mL of *acetonitrile R* and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 25.0 mg of *mometasone furoate monohydrate CRS* in 15 mL of *acetonitrile R* and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 25.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water for chromatography R* (50:50 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3.5 times the retention time of mometasone furoate.

*Identification of impurities*: use the chromatogram supplied with *mometasone furoate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to mometasone furoate (retention time = about 24 min): impurity C = about 0.9.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.5 between the peaks due to impurity C and mometasone furoate.

*Limits*:
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 2.5 per cent to 4.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{30}Cl_2O_6$ taking into account the assigned content of *mometasone furoate monohydrate CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these

impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U.*

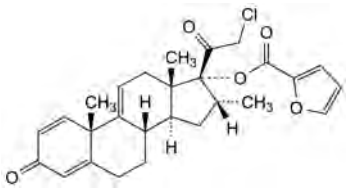

A. 21-chloro-16α-methyl-3,20-dioxopregna-1,4,9(11)-trien-17-yl furan-2-carboxylate,

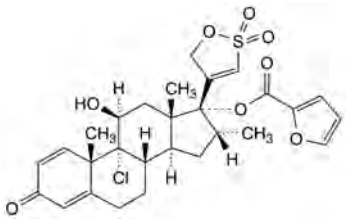

B. 9-chloro-17β-(2,2-dioxo-2,5-dihydro-1,2λ$^6$-oxathiol-4-yl)-11β-hydroxy-16α-methyl-3-oxoandrosta-1,4-dien-17α-yl furan-2-carboxylate,

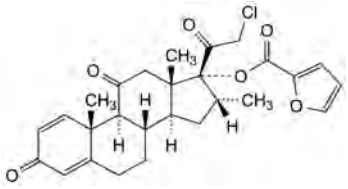

C. 21-chloro-16α-methyl-3,11,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,

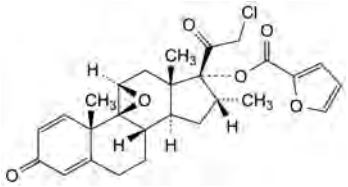

D. 21-chloro-9,11β-epoxy-16α-methyl-3,20-dioxo-9β-pregna-1,4-dien-17-yl furan-2-carboxylate,

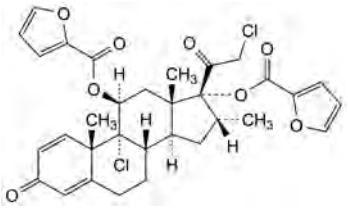

E. 9,21-dichloro-16α-methyl-3,20-dioxopregna-1,4-diene-11β,17-diyl di(furan-2-carboxylate),

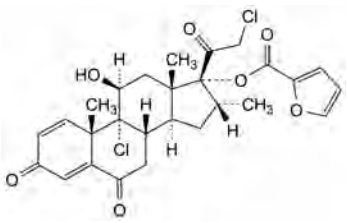

F. 9,21-dichloro-11β-hydroxy-16α-methyl-3,6,20-trioxopregna-1,4-dien-17-yl furan-2-carboxylate,

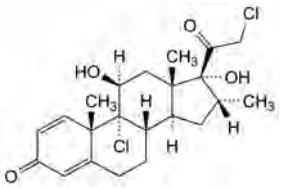

G. 9,21-dichloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione (mometasone),

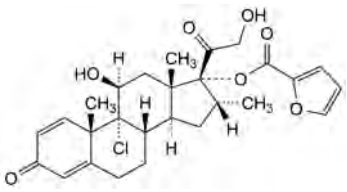

H. 9-chloro-11β,21-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

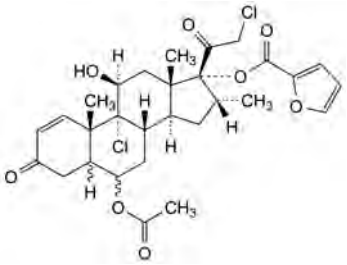

I. 6ξ-acetoxy-9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxo-5ξ-pregn-1-en-17-yl furan-2-carboxylate,

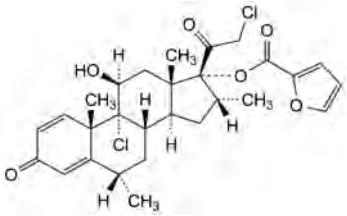

J. 9,21-dichloro-11β-hydroxy-6α,16α-dimethyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

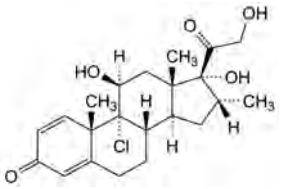

K. 9-chloro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

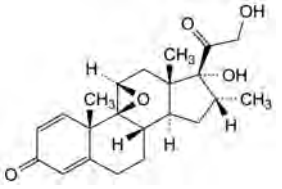

L. 9,11β-epoxy-17,21-dihydroxy-16α-methyl-9β-pregna-1,4-diene-3,20-dione,

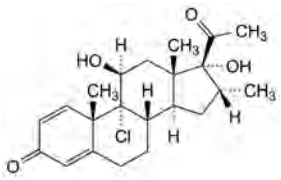

M. 9-chloro-11β,17-dihydroxy-16α-methylpregna-1,4-diene-3,20-dione,

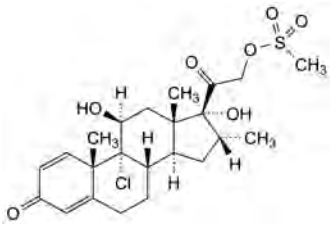

N. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl methanesulfonate,

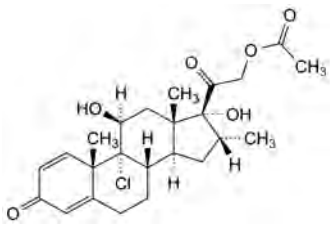

O. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl acetate,

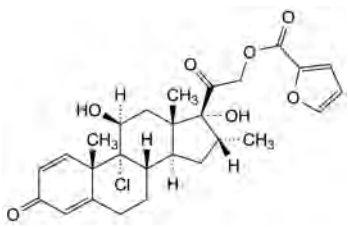

P. 9-chloro-11β,17-dihydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-21-yl furan-2-carboxylate,

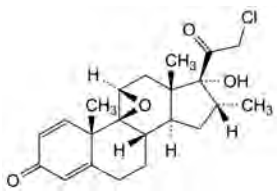

Q. 21-chloro-9,11β-epoxy-17-hydroxy-16α-methyl-9β-pregna-1,4-diene-3,20-dione,

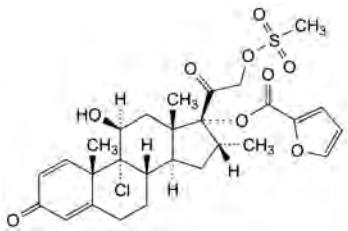

R. 9-chloro-11β-hydroxy-16α-methyl-21-[(methylsulfonyl)oxy]-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

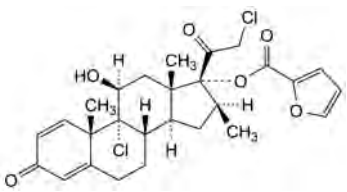

S. 9,21-dichloro-11β-hydroxy-16β-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate,

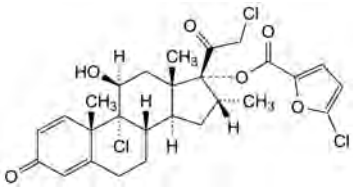

T. 9,21-dichloro-11β-hydroxy-16α-methyl-3,20-dioxopregna-1,4-dien-17-yl 5-chlorofuran-2-carboxylate,

U. unknown structure.

**04/2017:2583**

# MONTELUKAST SODIUM

## Montelukastum natricum

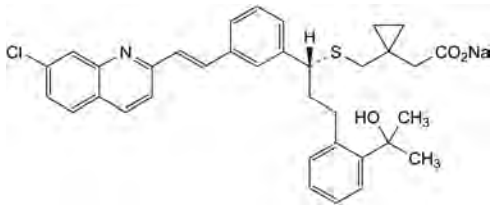

$C_{35}H_{35}ClNNaO_3S$ $M_r$ 608
[151767-02-1]

DEFINITION

Sodium [1-[[[(1*R*)-1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)-ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]-propyl]sulfanyl]methyl]cyclopropyl]acetate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water and in methylene chloride, freely soluble to very soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *montelukast sodium CRS*.

B. Enantiomeric purity (see Tests).

C. Ignite 0.1 g in a crucible until an almost white residue is obtained. Take up the residue in 2 mL of *water R* and filter. The filtrate gives reaction (a) of sodium (*2.3.1*).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Prepare the solutions in amber flasks.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *montelukast racemate CRS* in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: 2.3 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *glacial acetic acid R*;
- *mobile phase B*: *acetonitrile R*, *methanol R* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 70 → 60 | 30 → 40 |
| 30 - 35 | 60 | 40 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Relative retention* with reference to montelukast (retention time = about 25 min): impurity A = about 0.7.

*System suitability*:

- *resolution*: minimum 2.9 between the peaks due to impurity A and montelukast in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (a).

Calculate the percentage content of impurity A using the following expression:

$$100\left(\frac{r_1}{r_2}\right)$$

$r_1$ = area of the peak due to impurity A in the chromatogram obtained with the test solution;

$r_2$ = sum of the areas of the peaks due to montelukast and impurity A in the chromatogram obtained with the test solution.

*Limit*:

- *impurity A*: maximum 0.2 per cent.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Carry out the test protected from light. Prepare the solutions in amber flasks.*

*Solvent mixture*: *water R*, *methanol R* (10:90 *V/V*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *montelukast for peak identification CRS* (containing impurities B, C, D, E and F) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c).* In order to prepare impurity G *in situ*, transfer 1 mL of reference solution (b) to a colourless glass vial and expose to ambient light for about 20 min.

*Reference solution (d).* Dissolve 65.0 mg of *montelukast dicyclohexylamine CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.05 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (1.8 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 1.5 mL of *trifluoroacetic acid R* and 1000 mL of *water R*;
- *mobile phase B*: mix 1.5 mL of *trifluoroacetic acid R* and 1000 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 60 | 40 |
| 3 - 16 | 60 → 49 | 40 → 51 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (c); 20 µL of reference solution (b).

*Identification of impurities*: use the chromatogram supplied with *montelukast for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D, E and F; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity G.

*Relative retention* with reference to montelukast (retention time = about 7 min): impurity C = about 0.4; impurity G = about 0.8; impurities D and E = about 0.9; impurity F = about 1.2; impurity B = about 1.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to impurity G and montelukast; minimum 1.5 between the peaks due to montelukast and impurity F.

*Limits*:

- *impurity B*: maximum 0.3 per cent;
- *impurity C*: maximum 0.2 per cent;
- *impurities F, G*: for each impurity, maximum 0.15 per cent;
- *sum of impurities D and E*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{35}H_{35}ClNNaO_3S$ using the following expression:

$$\frac{A_1 \times m_2 \times 79.24 \times p}{A_2 \times m_1 \times (100 - a)}$$

$A_1$ = area of the principal peak in the chromatogram obtained with test solution (b);

$A_2$ = area of the principal peak in the chromatogram obtained with reference solution (d);

$m_1$ = mass of the substance to be examined used to prepare test solution (a), in milligrams;

$m_2$ = mass of *montelukast dicyclohexylamine CRS* used to prepare reference solution (d), in milligrams;

$p$ = declared percentage content of *montelukast dicyclohexylamine CRS*;

$a$ = percentage content of water in the substance to be examined.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, I.*

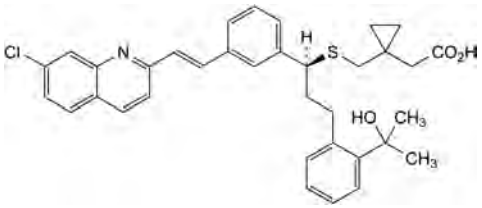

A. [1-[[[(1*S*)-1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)ethenyl]-phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]-sulfanyl]methyl]cyclopropyl]acetic acid,

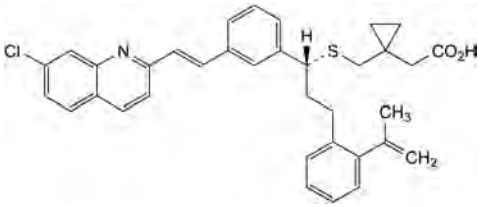

B. [1-[[[(1*R*)-1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)ethenyl]-phenyl]-3-[2-(1-methylethenyl)phenyl]propyl]sulfanyl]-methyl]cyclopropyl]acetic acid,

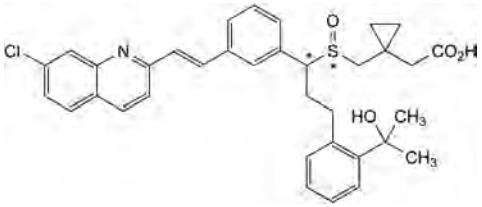

C. [1-[[[1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)ethenyl]-phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]-sulfinyl]methyl]cyclopropyl]acetic acid,

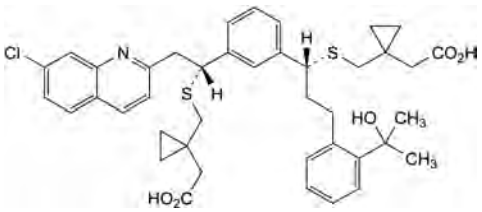

D. 1-[[[(1*R*)-1-[3-[(1*R*)-1-[[[1-(carboxymethyl)cyclopropyl]-methyl]sulfanyl]-2-(7-chloroquinolin-2-yl)ethyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]sulfanyl]-methyl]cyclopropyl]acetic acid,

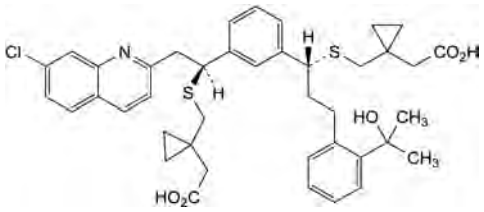

E. 1-[[[(1*R*)-1-[3-[(1*S*)-1-[[[1-(carboxymethyl)cyclopropyl]-methyl]sulfanyl]-2-(7-chloroquinolin-2-yl)ethyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]sulfanyl]-methyl]cyclopropyl]acetic acid,

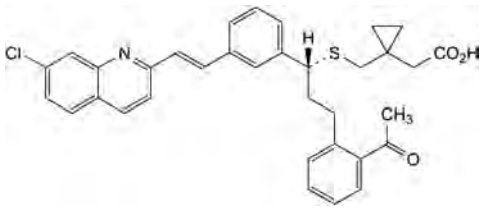

F. [1-[[[(1*R*)-3-(2-acetylphenyl)-1-[3-[(*E*)-2-(7-chloro-quinolin-2-yl)ethenyl]phenyl]propyl]sulfanyl]methyl]-cyclopropyl]acetic acid,

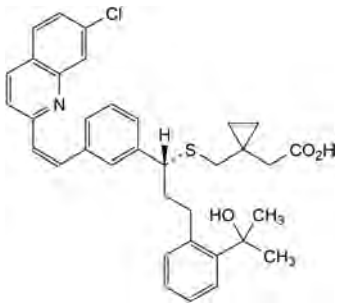

G. [1-[[[(1*R*)-1-[3-[(*Z*)-2-(7-chloroquinolin-2-yl)ethenyl]-phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]-sulfanyl]methyl]cyclopropyl]acetic acid,

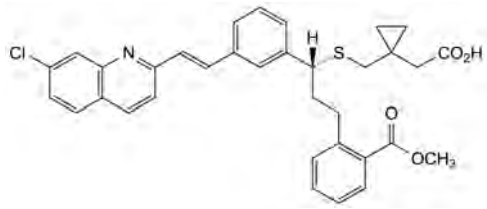

H. [1-[[[(1*R*)-1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)ethenyl]-phenyl]-3-[2-(methoxycarbonyl)phenyl]propyl]sulfanyl]-methyl]cyclopropyl]acetic acid,

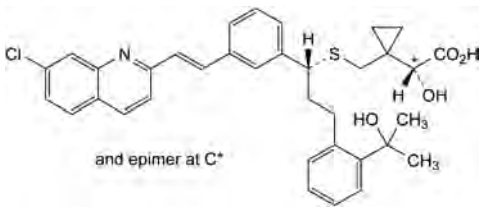


I. (2*RS*)-[1-[[[(1*R*)-1-[3-[(*E*)-2-(7-chloroquinolin-2-yl)-ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]-propyl]sulfanyl]methyl]cyclopropyl](hydroxy)acetic acid.

01/2008:1546
corrected 6.0



# MORANTEL HYDROGEN TARTRATE FOR VETERINARY USE

## Moranteli hydrogenotartras ad usum veterinarium

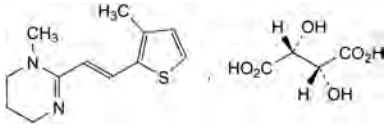

$C_{16}H_{22}N_2O_6S$ $M_r$ 370.4
[26155-31-7]

DEFINITION

1-Methyl-2-[(*E*)-2-(3-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine hydrogen tartrate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder.

*Solubility*: very soluble in water and in ethanol (96 per cent), practically insoluble in ethyl acetate.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 167 °C to 172 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *morantel hydrogen tartrate CRS.*

C. Dissolve about 10 mg in 1 mL of a 5 g/L solution of *ammonium vanadate R*. Evaporate to dryness. Add 0.1 mL of *sulfuric acid R*. A purple colour is produced.

D. Dissolve about 10 mg in 1 mL of *0.1 M sodium hydroxide*. Transfer to a separating funnel and shake with 5 mL of *methylene chloride R*. Discard the organic layer. Neutralise the aqueous layer with a few drops of *dilute hydrochloric acid R*. The solution gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ or $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.3 to 3.9 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 2.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Expose 10 mL of reference solution (a) to daylight for 15 min before injection.

*Reference solution (d).* Dissolve 15.0 mg of *tartaric acid R* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: to a mixture of 0.35 volumes of *triethylamine R* and 85 volumes of *water R* adjusted to pH 2.5 with *phosphoric acid R*, add 5 volumes of *tetrahydrofuran R* and 10 volumes of *methanol R*.

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 226 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of morantel.

*System suitability*: reference solution (c):
- *resolution*: minimum of 2 between the principal peak and the preceding peak ((*Z*)-isomer).

*Limits*:
- *any impurity apart from the peak due to tartaric acid*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

1.0 g complies with test C. Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R.*

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.280 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.04 mg of $C_{16}H_{22}N_2O_6S$.

STORAGE

Protected from light.

IMPURITIES

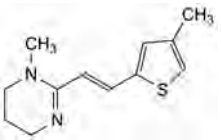

A. 1-methyl-2-[(*E*)-2-(4-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine,

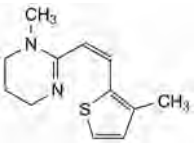

B. 1-methyl-2-[(*Z*)-2-(3-methylthiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine,

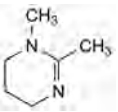

C. 1,2-dimethyl-1,4,5,6-tetrahydropyrimidine,

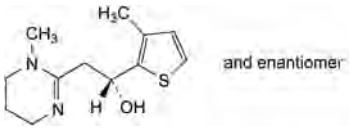


D. (1*RS*)-2-(1-methyl-1,4,5,6-tetrahydropyrimidin-2-yl)-1-(3-methylthiophen-2-yl)ethanol,

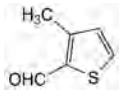


E. 3-methylthiophene-2-carbaldehyde.

**04/2008:0097**
**corrected 7.1**

# MORPHINE HYDROCHLORIDE

## Morphini hydrochloridum

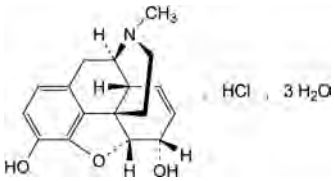


$C_{17}H_{20}ClNO_3,3H_2O$ $M_r$ 375.8
[6055-06-7]

DEFINITION

7,8-Didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol hydrochloride trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless, silky needles or cubical masses, efflorescent in a dry atmosphere.

*Solubility*: soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in toluene.

IDENTIFICATION

*First identification*: *A, E.*

*Second identification*: *B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *morphine hydrochloride trihydrate CRS.*

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).
*Solution A*. Dissolve 25.0 mg in *water R* and dilute to 25.0 mL with the same solvent.
*Test solution (a)*. Dilute 10.0 mL of solution A to 100.0 mL with *water R*.
*Test solution (b)*. Dilute 10.0 mL of solution A to 100.0 mL with *0.1 M sodium hydroxide*.
*Spectral range*: 250-350 nm for test solutions (a) and (b).
*Absorption maximum*: at 285 nm for test solution (a); at 298 nm for test solution (b).
*Specific absorbance at the absorption maximum*: 37 to 43 for test solution (a); 64 to 72 for test solution (b).

C. To about 1 mg of powdered substance in a porcelain dish add 0.5 mL of *sulfuric acid-formaldehyde reagent R*. A purple colour develops and becomes violet.

D. It gives the reaction of alkaloids (*2.3.1*).

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): − 110 to − 115 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in a 1 per cent *V/V* solution of *acetic acid R* and dilute to 50 mL with the same solution.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with a 1 per cent *V/V* solution of *acetic acid R*. Dilute 2.0 mL of this solution to 10.0 mL with a 1 per cent *V/V* solution of *acetic acid R*.

*Reference solution (b)*. Dissolve 5 mg of *morphine for system suitability CRS* (containing impurities B, C, E and F) in a 1 per cent *V/V* solution of *acetic acid R* and dilute to 2 mL with the same solution.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: 1.01 g/L solution of *sodium heptanesulfonate R* adjusted to pH 2.6 with a 50 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 35 | 85 → 50 | 15 → 50 |
| 35 - 40 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *morphine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, E and F.

*Relative retention* with reference to morphine (retention time = about 12.5 min): impurity F = about 0.95; impurity E = about 1.1; impurity C = about 1.6; impurity B = about 1.9.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 2, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to morphine.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.25; impurity C = 0.4; impurity E = 0.5;
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *impurities C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Water** (*2.5.12*): 12.5 per cent to 15.5 per cent, determined on 0.10 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5 mL of *0.01 M hydrochloric acid* and 30 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.18 mg of $C_{17}H_{20}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, F.*

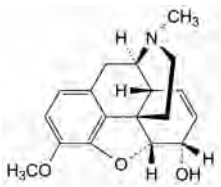

A. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (codeine),

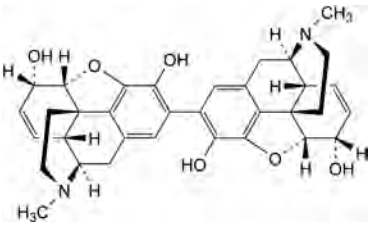

B. 7,7′,8,8′-tetradehydro-4,5α:4′,5′α-diepoxy-17,17′-dimethyl-2,2′-bimorphinanyl-3,3′,6α,6′α-tetrol (2,2′-bimorphine),

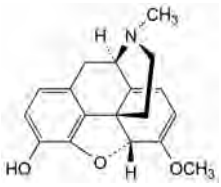

C. 6,7,8,14-tetradehydro-4,5α-epoxy-6-methoxy-17-methylmorphinan-3-ol (oripavine),

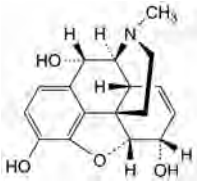

D. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α,10α-triol (10*S*-hydroxymorphine),

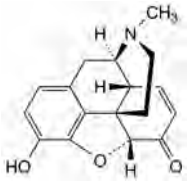

E. 7,8-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan-6-one (morphinone),

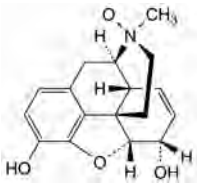

F. (17*S*)-7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol 17-oxide (morphine *N*-oxide).

**04/2008:1244**
**corrected 6.7**

# MORPHINE SULFATE

## Morphini sulfas

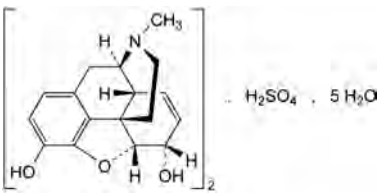

$C_{34}H_{40}N_2O_{10}S,5H_2O$ $M_r$ 759
[6211-15-0]

DEFINITION

Di(7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol) sulfate pentahydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in toluene.

IDENTIFICATION

*First identification: A, E.*

*Second identification*: *B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 20 mg in 1 mL of *water R*, add 0.05 mL of *1 M sodium hydroxide* and shake. A precipitate is formed. Filter, wash with 2 quantities, each of 0.5 mL, of *water R* and dry the precipitate at 145 °C for 1 h. Prepare discs using the dried precipitate.

*Comparison*: repeat the operations using 20 mg of *morphine sulfate CRS*.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Solution A*. Dissolve 25.0 mg in *water R* and dilute to 25.0 mL with the same solvent.

*Test solution (a)*. Dilute 10.0 mL of solution A to 100.0 mL with *water R*.

*Test solution (b)*. Dilute 10.0 mL of solution A to 100.0 mL with *0.1 M sodium hydroxide*.

*Spectral range*: 250-350 nm for test solutions (a) and (b).

*Absorption maximum*: at 285 nm for test solution (a); at 298 nm for test solution (b).

*Specific absorbance at the absorption maximum*: 37 to 43 for test solution (a); 64 to 72 for test solution (b).

C. To about 1 mg of powdered substance in a porcelain dish add 0.5 mL of *sulfuric acid-formaldehyde reagent R*. A purple colour develops and becomes violet.

D. It gives the reaction of alkaloids (*2.3.1*).

E. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): – 107 to – 110 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in a 1 per cent *V/V* solution of *acetic acid R* and dilute to 50 mL with the same solution.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with a 1 per cent *V/V* solution of *acetic acid R*. Dilute 2.0 mL of this solution to 10.0 mL with a 1 per cent *V/V* solution of *acetic acid R*.

*Reference solution (b)*. Dissolve 5 mg of *morphine for system suitability CRS* (containing impurities B, C, E and F) in a 1 per cent *V/V* solution of *acetic acid R* and dilute to 2 mL with the same solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: 1.01 g/L solution of *sodium heptanesulfonate R* adjusted to pH 2.6 with a 50 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 35 | 85 → 50 | 15 → 50 |
| 35 - 40 | 50 | 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *morphine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, E and F.

*Relative retention* with reference to morphine (retention time = about 12.5 min): impurity F = about 0.95; impurity E = about 1.1; impurity C = about 1.6; impurity B = about 1.9.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to morphine.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.25; impurity C = 0.4; impurity E = 0.5;
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurities C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Iron** (*2.4.9*): maximum 5 ppm.

Dissolve the residue from the test for sulfated ash in *water R* and dilute to 10.0 mL with the same solvent.

**Water** (*2.5.12*): 10.4 per cent to 13.4 per cent, determined on 0.10 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.500 g in 120 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 66.88 mg of $C_{34}H_{40}N_2O_{10}S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, F.*

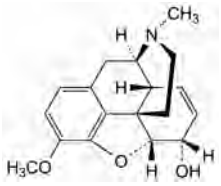

A. 7,8-didehydro-4,5α-epoxy-3-methoxy-17-methylmorphinan-6α-ol (codeine),

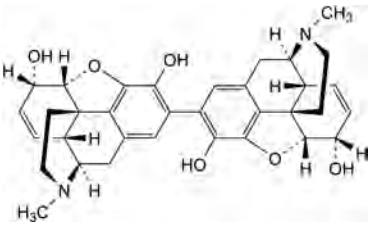

B. 7,7′,8,8′-tetradehydro-4,5α:4′,5′α-diepoxy-17,17′-dimethyl-2,2′-bimorphinanyl-3,3′,6α,6′α-tetrol (2,2′-bimorphine),

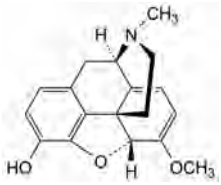

C. 6,7,8,14-tetradehydro-4,5α-epoxy-6-methoxy-17-methylmorphinan-3-ol (oripavine),

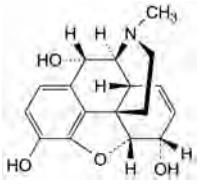

D. 7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α,10α-triol (10*S*-hydroxymorphine),

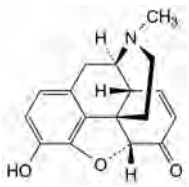

E. 7,8-didehydro-4,5α-epoxy-3-hydroxy-17-methylmorphinan-6-one (morphinone),

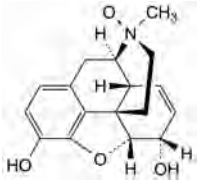

F. (17*S*)-7,8-didehydro-4,5α-epoxy-17-methylmorphinan-3,6α-diol 17-oxide (morphine *N*-oxide).

**01/2017:2626**

# MOULDS FOR ALLERGEN PRODUCTS

## Mucores ad producta allergenica

DEFINITION

Moulds for allergen products primarily consist of inactivated mould cultures, which may contain mycelia and/or spores, and may also contain allergenic constituents released into the culture medium. Moulds are microscopic filamentous fungi belonging mainly to the genera *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium*. This monograph also applies to yeast cells, e.g. *Candida*.

PRODUCTION

The method of cultivation, inactivation, harvesting and post-harvesting is described and must ensure the quality, homogeneity and traceability of the mould or yeast. The type of source material harvested (mycelium and/or spores or yeast cells) is specified. Appropriate measures are taken to avoid contamination by foreign species.

Critical cultivation parameters such as temperature and humidity are controlled and monitored. Unless otherwise justified, the culture medium is selected to avoid the presence of materials with potential allergenicity.

Where major changes to the production of the source material take place (e.g. when a new process or supplier of source materials is introduced), such changes are qualified for each species.

Microbial contamination of the mould or yeast should be monitored on a representative number of batches of source material according to a justified sampling plan and each time a new supplier and/or a new process for the source material production is introduced; if a determination of microbial contamination is not applicable, this must be justified. Microbial contamination values and potential increases in microbial contamination are monitored during stability studies, in order to assess this aspect along with the source material characteristics upon storage.

Control methods and acceptance criteria relating to identity and purity of the mould or yeast are established. The acceptance criteria must ensure the consistency of the source material from a qualitative and quantitative point of view. The source material is stored under controlled conditions justified by stability data. The production, as well as the handling of the source material, are such that consistent composition is ensured from batch to batch.

*SOURCE MATERIAL REFERENCE BATCH*

An appropriate reference batch is established for each species. The nature of the reference batch depends on the testing approach to verify batch-to-batch consistency and to establish acceptable quality. The reference batch may be, for example, an internal reference preparation (if available), a source material extract or a sample of a production batch. Its characterisation must be described. The extent of characterisation of the reference batch depends on the species, knowledge of the allergenic components and availability of suitable reagents. The reference batch is stored under controlled conditions ensuring its stability.

*BATCH-TO-BATCH CONSISTENCY*

To establish batch-to-batch consistency, one or more of the following tests are performed on each batch. The choice of tests must be justified.

**Total protein** (*2.5.33*).

**Protein profile**. Determined by using suitable electrophoresis methods (*2.2.31*, *2.2.54*).

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific antibodies.

**Major allergen content**. Determined by using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

**Total allergenic activity**. Determined by testing inhibition of the binding capacity of specific immunoglobulin E antibodies or by a suitable equivalent *in vitro* method.

CHARACTERS

Moulds or yeasts for allergen products are supplied as coloured powders or liquid suspensions.

IDENTIFICATION

The identity of the mould or yeast is confirmed by its relevant macroscopic (e.g. colour, appearance) and microscopic (e.g. appearance of the mycelium, spores, specialised cells) morphological characteristics specific to each species, in comparison to those of a reference batch or reference documents. Identity may also be confirmed using protein analysis methods (e.g. protein profile, individual allergen test) or genetic identification, if performed by generally accepted methods.

TESTS

**Foreign species**. The absence of foreign species is determined by macroscopic and microscopic examination.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*). The water content of dried material is determined; specification limits must be supported by batch analysis and stability data.

**Mycotoxins**. For moulds, the content of mycotoxin is determined, unless otherwise justified.

STORAGE

Moulds or yeasts for allergen products are stored under controlled conditions justified by stability data.

LABELLING

The label states the species of the mould or yeast.

**01/2008:1656**
**corrected 6.5**

# MOXIDECTIN FOR VETERINARY USE

## Moxidectinum ad usum veterinarium

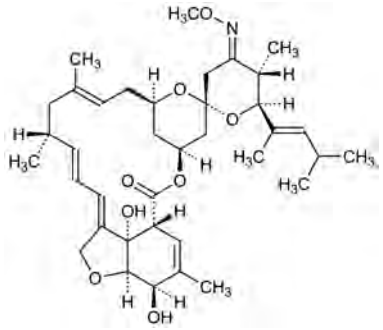

$C_{37}H_{53}NO_8$ $M_r$ 640
[113507-06-5]

DEFINITION

(2a*E*,2′*R*,4*E*,4′*E*,5′*S*,6*R*,6′*S*,8*E*,11*R*,15*S*,17a*R*,20*R*,20a*R*,20b*S*)-6′-[(1*E*)-1,3-Dimethylbut-1-enyl]-20,20b-dihydroxy-4′-(methoxyimino)-5′,6,8,19-tetramethyl-3′,4′,5′,6,6′,7,10,11,14,-15,17a,20,20a,20b-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((6*R*,23*E*,25*S*)-5*O*-demethyl-28-deoxy-25-[(1*E*)-1,3-dimethylbut-1-enyl]-6,28-epoxy-23-(methoxyimino)milbemycin B).

Semi-synthetic product derived from a fermentation product.

It may contain suitable stabilisers such as antioxidants.

*Content*: 92.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, amorphous powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent), slightly soluble in hexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *moxidectin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

Dissolve 0.40 g in *benzyl alcohol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

A. *Test solution.* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL *acetonitrile R*.

*Reference solution (b).* Dissolve 5 mg of *moxidectin for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H, I, J and K) in 5 mL of *acetonitrile R*.

*Reference solution (c).* Dissolve 25.0 mg of *moxidectin CRS* in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase: end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 50 °C.

*Mobile phase*: dissolve 7.7 g of *ammonium acetate R* in 400 mL of *water R*, adjust to pH 4.8 with *glacial acetic acid R* and add 600 mL of *acetonitrile R*.

*Flow rate*: 2.5 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Run time*: 2 times the retention time of moxidectin.

*Identification of impurities*: use the chromatogram supplied with *moxidectin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D, E + F and G.

*Relative retention* with reference to moxidectin (retention time = about 12 min): impurity A = about 0.5; impurity B = about 0.7; impurity C = about 0.75; impurity D = about 0.94; impurities E and F = about 1.3-1.5; impurity G = about 1.6.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to moxidectin.

*Limits*:

- *impurity D*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent);
- *sum of impurities E and F*: not more than 1.7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.7 per cent);
- *impurities A, C, G*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *impurity B*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *any other impurity eluting before impurity G*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard the peak due to the stabiliser (identify this peak, where applicable, by injecting a suitable reference solution).

B. *Test solution.* Dissolve 75.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 5 mg of *moxidectin for system suitability CRS* (containing impurities A, B, C, D, E, F, G, H, I, J and K) in 5 mL of *acetonitrile R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 35 °C.

*Mobile phase*: dissolve 3.8 g of *ammonium acetate R* in 250 mL of *water R*, adjust to pH 4.2 with *acetic acid R* and add 750 mL of *acetonitrile R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL.

*Run time*: 10 times the retention time of moxidectin.

*Identification of impurities*: use the chromatogram supplied with *moxidectin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities H + I, J and K.

*Relative retention* with reference to moxidectin (retention time = about 4 min): impurity G = about 1.4; impurities H and I = about 2.0; impurity J = about 2.2; impurity K = about 3.4.

*System suitability*: reference solution (b):

- *resolution*: baseline separation between the peaks due to impurities H + I and J.

*Limits*:

- *sum of impurities H and I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *impurities J, K*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *any other impurity eluting after impurity G*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard the peak due to the stabiliser (identify this peak, where applicable, by injecting a suitable reference solution).

*Total of all impurities.* Calculate the sum of the impurities eluting from the start of the run to impurity G in test A, and from impurities H + I to the end of the run in test B. The total of all impurities is not more than 7.0 per cent.

**Heavy metals** (*2.4.8*): maximum 20 ppm.

It complies with test A with the following modifications.

*Prescribed solution.* Dissolve 0.50 g in 20 mL of *ethanol (96 per cent) R*.

*Test solution.* 12 mL of the prescribed solution.

*Reference solution.* A mixture of 2 mL of the prescribed solution, 4 mL of *water R* and 6 mL of *lead standard solution (1 ppm Pb) R*.

*Blank solution.* A mixture of 2 mL of the prescribed solution and 10 mL of *ethanol (96 per cent) R*.

Use a membrane filter (nominal pore size 0.45 µm).

**Water** (*2.5.12*): maximum 1.3 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{37}H_{53}NO_8$ using the declared content of *moxidectin CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K.*

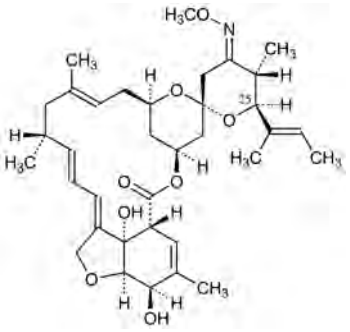

A. 25-des[(1*E*)-1,3-dimethylbut-1-enyl]-25-[(1*E*)-1-methylprop-1-enyl]moxidectin,

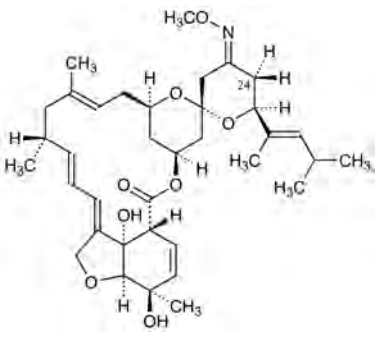

B. 24-desmethylmoxidectin,

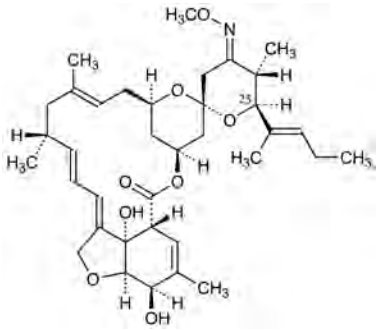

C. 25-des[(1*E*)-1,3-dimethylbut-1-enyl]-25-[(1*E*)-1-methylbut-1-enyl]moxidectin,

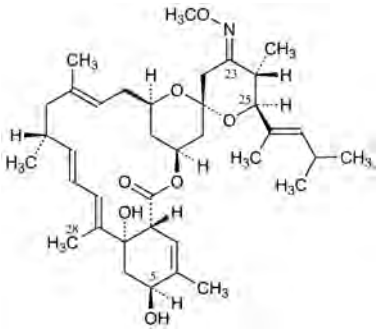

G. (23*E*,25*S*)-5*O*-desmethyl-28-deoxy-25-[(1*E*)-1,3-dimethylbut-1-enyl]-23-(methoxyimino)milbemycin B,

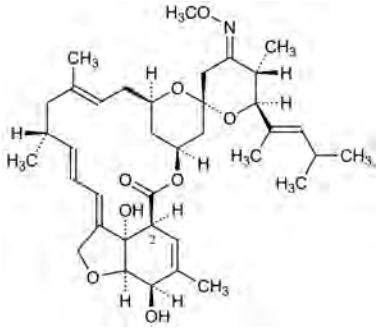

D. 2-*epi*-moxidectin,

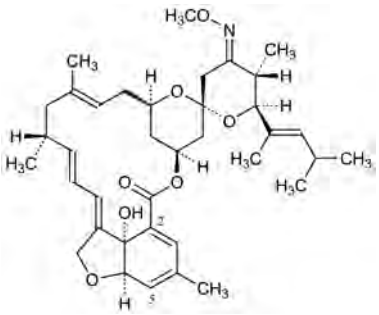

H. 2,5-didehydro-5-deoxymoxidectin,

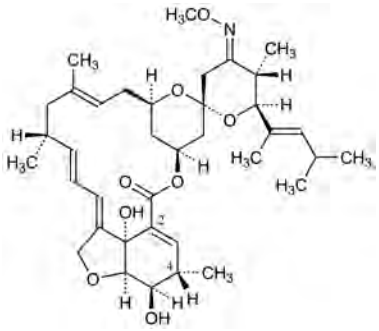

E. (4*S*)-2-dehydro-4-hydromoxidectin,

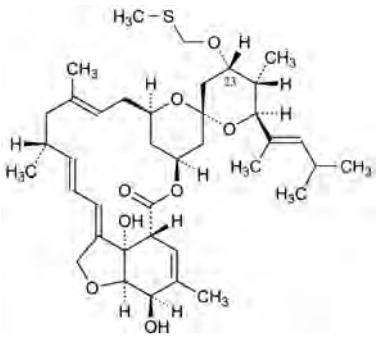

I. (23*S*)-23-des(methoxyimino)-23-[(methylsulfanyl)-methoxy]moxidectin,

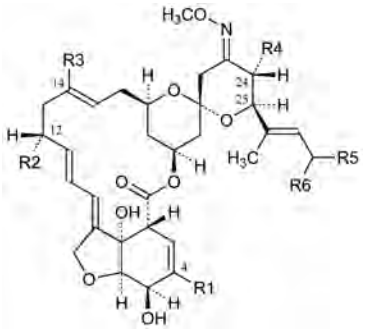

F. one of groups R1 to R6 is $C_2H_5$, the others are $CH_3$: x-desmethyl-x-ethylmoxidectin,

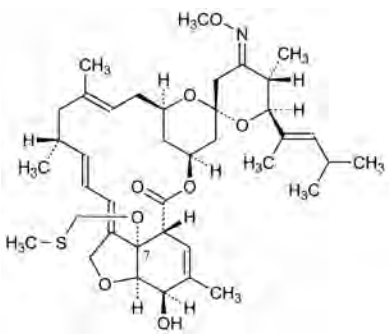

J. 7-*O*-[(methylsulfanyl)methyl]moxidectin,

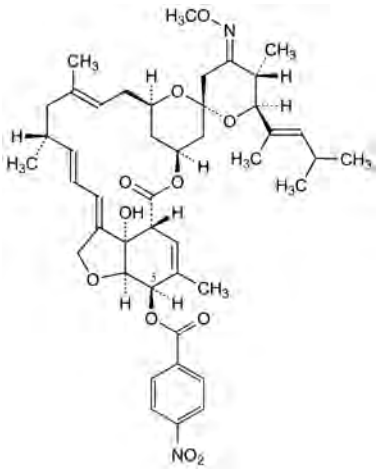

K. 5-*O*-(4-nitrobenzoyl)moxidectin,

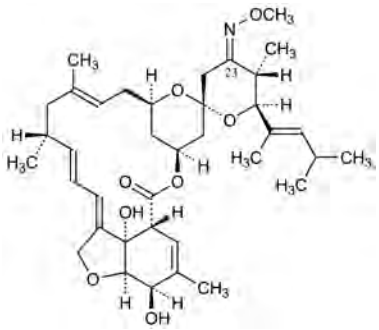

L. (23*Z*)-moxidectin.

**07/2019:2254**

# MOXIFLOXACIN HYDROCHLORIDE

## Moxifloxacini hydrochloridum

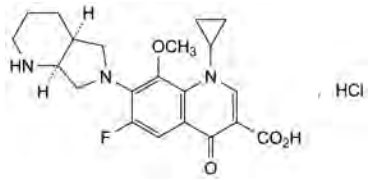

$C_{21}H_{25}ClFN_3O_4$ $M_r$ 437.9
[186826-86-8]

DEFINITION

1-Cyclopropyl-6-fluoro-8-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: light yellow or yellow, slightly hygroscopic powder or crystals.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *moxifloxacin hydrochloride CRS*.

B. Enantiomeric purity (see Tests).

C. Dissolve 50 mg in 5 mL of *water R*, add 1 mL of *dilute nitric acid R*, mix, allow to stand for 5 min and filter. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_2$ (*2.2.2, Method II*). If intended for use in the manufacture of parenteral preparations, the solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_2$ (*2.2.2, Method II*).

Dissolve 1.0 g in 20 mL of *dilute sodium hydroxide solution R*.

**pH** (*2.2.3*): 3.9 to 4.6.

Dissolve 0.10 g in 50 mL of *carbon dioxide-free water R*.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 2.49 g of *anhydrous copper sulfate R* and 2.6 g of *isoleucine R* in 1000 mL of *water for chromatography R*.

*Test solution*. Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a)*. Dilute 3.0 mL of the test solution to 200.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 5 mg of *moxifloxacin for system suitability CRS* (containing impurity G) in 5 mL of *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, buffer solution (25:75 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 293 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of moxifloxacin.

*Identification of impurities*: use the chromatogram supplied with *moxifloxacin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to moxifloxacin (retention time = about 16 min): impurity G = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurity G and moxifloxacin.

*Calculation of percentage content*:
- for impurity G, use the concentration of moxifloxacin hydrochloride in reference solution (a).

*Limit*:
- *impurity G*: maximum 0.15 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solution A*. Dissolve 0.50 g of *tetrabutylammonium hydrogen sulfate R* and 1.0 g of *potassium dihydrogen phosphate R* in about 500 mL of *water R*. Add 2 mL of *phosphoric acid R* and 0.050 g of *anhydrous sodium sulfite R* and dilute to 1000 mL with *water R*.

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 50.0 mg of *moxifloxacin hydrochloride CRS* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 10.0 mL with solution A.

*Reference solution (c).* Dissolve 5 mg of *moxifloxacin for peak identification A CRS* (containing impurities A, B and E) in 5 mL of solution A.

*Reference solution (d).* Dissolve 2 mg of *moxifloxacin for peak identification B CRS* (containing impurity F) in 2 mL of solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: mix 28 volumes of *methanol R* and 72 volumes of a solution containing 0.5 g/L of *tetrabutylammonium hydrogen sulfate R*, 1.0 g/L of *potassium dihydrogen phosphate R* and 3.4 g/L of *phosphoric acid R*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 293 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (c) and (d).

*Run time*: 2.5 times the retention time of moxifloxacin.

*Identification of impurities*: use the chromatogram supplied with *moxifloxacin for peak identification A CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and E; use the chromatogram supplied with *moxifloxacin for peak identification B CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity F.

*Relative retention* with reference to moxifloxacin (retention time = about 11 min): impurity F = about 0.9; impurity A = about 1.1; impurity B = about 1.3; impurity E = about 1.7.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to moxifloxacin and impurity A.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.4; impurity E = 3.5;
- *impurities B, E, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 4.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_{21}H_{25}ClFN_3O_4$ taking into account the assigned content of *moxifloxacin hydrochloride CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: B, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, H.*

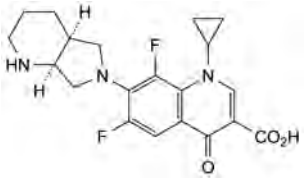

A. 1-cyclopropyl-6,8-difluoro-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

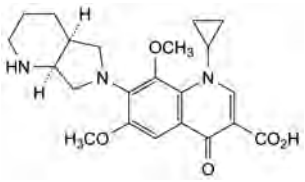

B. 1-cyclopropyl-6,8-dimethoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

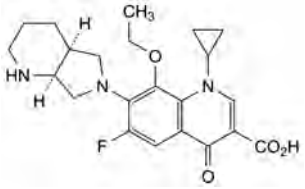

C. 1-cyclopropyl-8-ethoxy-6-fluoro-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

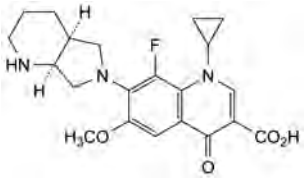

D. 1-cyclopropyl-8-fluoro-6-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

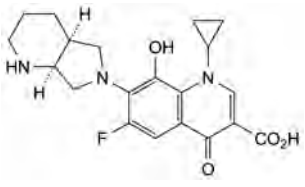

E. 1-cyclopropyl-6-fluoro-8-hydroxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

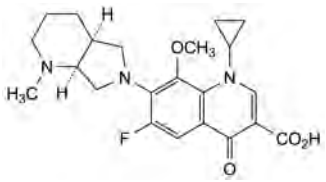

F. 1-cyclopropyl-6-fluoro-8-methoxy-7-[(4a*S*,7a*S*)-1-methyloctahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

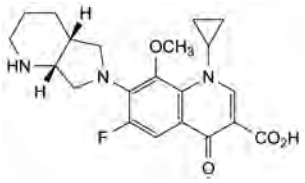

G. 1-cyclopropyl-6-fluoro-8-methoxy-7-[(4a*R*,7a*R*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

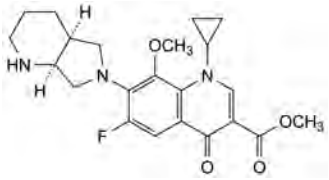

H. methyl 1-cyclopropyl-6-fluoro-8-methoxy-7-[(4a*S*,7a*S*)-octahydro-6*H*-pyrrolo[3,4-*b*]pyridin-6-yl]-4-oxo-1,4-dihydroquinoline-3-carboxylate.

**01/2008:1758**
**corrected 6.0**

# MOXONIDINE

## Moxonidinum

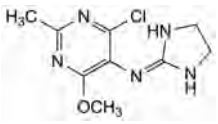

$C_9H_{12}ClN_5O$ $M_r$ 241.7
[75438-57-2]

DEFINITION

4-Chloro-*N*-(imidazolidin-2-ylidene)-6-methoxy-2-methylpyrimidin-5-amine.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in methanol, slightly soluble in methylene chloride, very slightly soluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *moxonidine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 10.0 mg of *moxonidine CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with a mixture of equal volumes of *methanol R* and *water R*. Dilute 2.0 mL of this solution to 20.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution (c)*. Dissolve 5.0 mg of *moxonidine impurity A CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (d)*. Dilute 6.0 mL of reference solution (c) to 100.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution (e)*. Dilute 2.5 mL of reference solution (a) to 50.0 mL with reference solution (c).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 136 volumes of *acetonitrile R* with 1000 volumes of a 3.48 g/L solution of *sodium pentanesulfonate R* previously adjusted to pH 3.5 with *dilute sulfuric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (d) and (e).

*Run time*: twice the retention time of moxonidine.

*Relative retentions* with reference to moxonidine (retention time = about 11.6 min): impurity A = about 0.9; impurity B = about 1.7.

*System suitability*: reference solution (e):

- *resolution*: minimum of 2 between the peaks due to impurity A and moxonidine.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurity B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_9H_{12}ClN_5O$ from the areas of the peaks and the declared content of *moxonidine CRS*.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

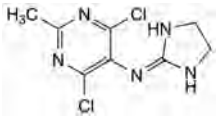

A. 4,6-dichloro-*N*-(imidazolidin-2-ylidene)-2-methylpyrimidin-5-amine (6-chloromoxonidine),

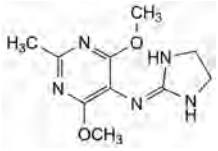

B. *N*-(imidazolidin-2-ylidene)-4,6-dimethoxy-2-methylpyrimidin-5-amine (4-methoxymoxonidine),

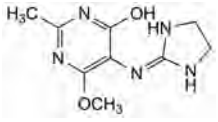

C. 5-[(imidazolidin-2-ylidene)amino]-6-methoxy-2-methylpyrimidin-4-ol (4-hydroxymoxonidine),

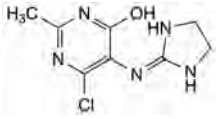

D. 6-chloro-5-[(imidazolidin-2-ylidene)amino]-2-methylpyrimidin-4-ol (6-desmethylmoxonidine).

**04/2019:1450**

# MUPIROCIN

## Mupirocinum

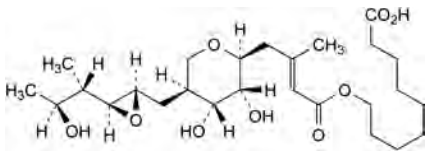

$C_{26}H_{44}O_9$ $M_r$ 500.6
[12650-69-0]

DEFINITION

9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-Dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid.

Substance produced by the growth of certain strains of *Pseudomonas fluorescens* or obtained by any other means.

*Content*: 93.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in acetone, in anhydrous ethanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of mupirocin.*

TESTS

**pH** (*2.2.3*): 3.5 to 4.0 for a freshly prepared saturated solution (about 10 g/L) in *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): – 17 to – 21 (anhydrous substance).

Dissolve 0.50 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 50 volumes of *methanol R* and 50 volumes of a 13.6 g/L solution of *sodium acetate R* adjusted to pH 4.0 with *acetic acid R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Adjust 10 mL of reference solution (a) to pH 2.0 with *hydrochloric acid R* and allow to stand for 20 h.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *water for chromatography R*, 30 volumes of *tetrahydrofuran R* and 50 volumes of a 10.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of mupirocin.

*Relative retention* with reference to mupirocin: impurity C = about 0.75.

*System suitability*: reference solution (b):

- *resolution*: minimum 7.0 between the 2nd of the 2 peaks due to hydrolysis products and the peak due to mupirocin.

*Limits*:

- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent);
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (6 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 200.0 mL with a 7.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Reference solution (a).* Dissolve 25.0 mg of *mupirocin lithium CRS* in 5 mL of *methanol R* and dilute to 200.0 mL with a 7.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Reference solution (b).* Adjust 10 mL of the test solution to pH 2.0 with *hydrochloric acid R* and allow to stand for 20 h.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 19 volumes of *water for chromatography R*, 32 volumes of *tetrahydrofuran R* and 49 volumes of a 10.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*System suitability*:

– *resolution*: minimum 7.0 between the 2nd of the 2 peaks due to hydrolysis products and the peak due to mupirocin in the chromatogram obtained with reference solution (b);

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

Calculate the percentage content of $C_{26}H_{44}O_9$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *mupirocin lithium CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F*.

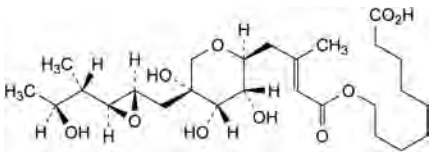

A. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*R*)-3,4,5-trihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]-nonanoic acid (pseudomonic acid B),

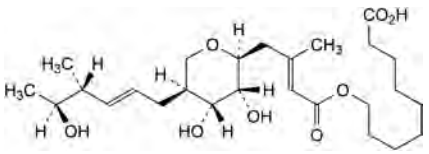

B. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[(2*E*,4*R*,5*S*)-5-hydroxy-4-methylhex-2-enyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid (pseudomonic acid C),

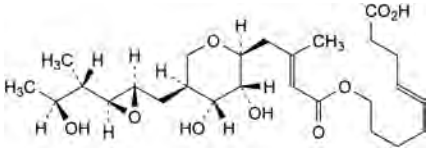

C. (4*E*)-9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]-non-4-enoic acid (pseudomonic acid D),

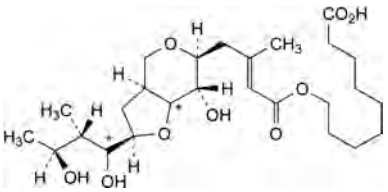

D. 9-[[(2*E*)-4-[(2*R*,3a*S*,6*S*,7*S*)-2-[(2*S*,3*S*)-1,3-dihydroxy-2-methylbutyl]-7-hydroxyhexahydro-4*H*-furo[3,2-*c*]pyran-6-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

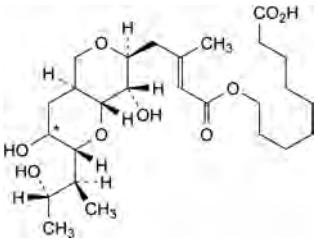

E. 9-[[(2*E*)-4-[(2*R*,3*RS*,4a*S*,7*S*,8*S*,8a*R*)-3,8-dihydroxy-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]hexahydro-2*H*,5*H*-pyrano[4,3-*b*]pyran-7-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

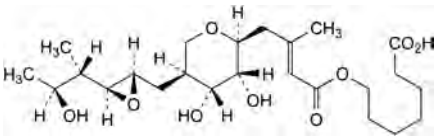

F. 7-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]heptanoic acid.

**04/2019:1451**

# MUPIROCIN CALCIUM

## Mupirocinum calcicum

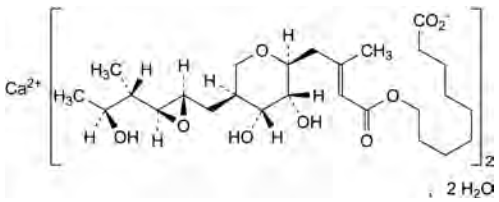

$C_{52}H_{86}CaO_{18},2H_2O$ $M_r$ 1075

[115074-43-6]

DEFINITION

Calcium bis[9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1methylpropyl]oxiranyl]-methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]-oxy]nonanoate] dihydrate.

Substance produced by the growth of certain strains of *Pseudomonas fluorescens* or obtained by any other means.

*Content*: 93.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of mupirocin calcium.*

B. It gives reaction (a) of calcium (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): − 16 to − 20 (anhydrous substance).

Dissolve 0.50 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 50 volumes of *methanol R* and 50 volumes of a 13.6 g/L solution of *sodium acetate R* adjusted to pH 4.0 with *acetic acid R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Adjust 10 mL of reference solution (a) to pH 2.0 with *hydrochloric acid R* and allow to stand for 20 h.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *water for chromatography R*, 30 volumes of *tetrahydrofuran R* and 50 volumes of a 10.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of mupirocin.

*Relative retention* with reference to mupirocin: impurity C = about 0.75.

*System suitability*: reference solution (b):

- *resolution*: minimum 7.0 between the 2nd of the 2 peaks due to hydrolysis products and the peak due to mupirocin.

*Limits*:

- *impurity C*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent);
- *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
- *total*: not more than 2.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 0.5 per cent.

Dissolve 10.0 mg in a mixture of 1 mL of *dilute nitric acid R* and 15 mL of *methanol R*.

**Water** (*2.5.12*): 3.0 per cent to 4.5 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 200.0 mL with a 7.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Reference solution (a).* Dissolve 25.0 mg of *mupirocin lithium CRS* in 5 mL of *methanol R* and dilute to 200.0 mL with a 7.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Reference solution (b).* Adjust 10 mL of the test solution to pH 2.0 with *hydrochloric acid R* and allow to stand for 20 h.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 19 volumes of *water for chromatography R*, 32 volumes of *tetrahydrofuran R* and 49 volumes of a 10.5 g/L solution of *ammonium acetate R* adjusted to pH 5.7 with *acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*System suitability*:

- *resolution*: minimum 7.0 between the 2nd of the 2 peaks due to hydrolysis products and the peak due to mupirocin in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

Calculate the percentage content of $C_{52}H_{86}CaO_{18}$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *mupirocin lithium CRS* and a conversion factor of 1.038.

IMPURITIES

*Specified impurities*: *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F, G, H, I.*

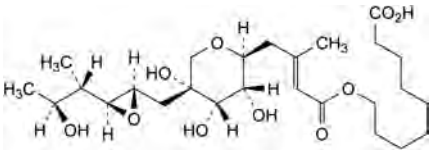

A. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*R*)-3,4,5-trihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]-nonanoic acid (pseudomonic acid B),

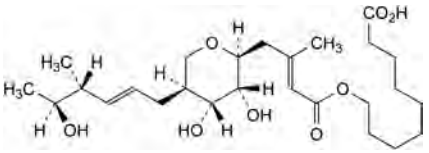

B. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[(2*E*,4*R*,5*S*)-5-hydroxy-4-methylhex-2-enyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid (pseudomonic acid C),

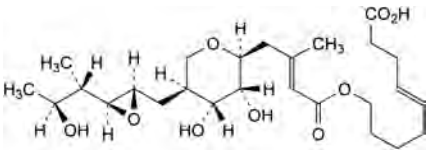

C. (4*E*)-9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]-tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]-non-4-enoic acid (pseudomonic acid D),

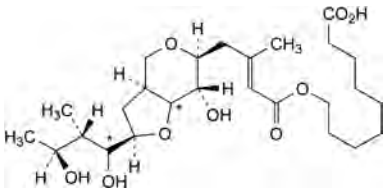

D. 9-[[(2*E*)-4-[(2*R*,3a*S*,6*S*,7*S*)-2-[(2*S*,3*S*)-1,3-dihydroxy-2-methylbutyl]-7-hydroxyhexahydro-4*H*-furo[3,2-*c*]pyran-6-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

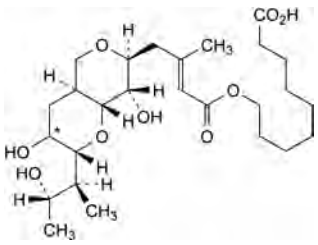

E. 9-[[(2*E*)-4-[(2*R*,3*RS*,4a*S*,7*S*,8*S*,8a*R*)-3,8-dihydroxy-2-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]hexahydro-2*H*,5*H*-pyrano[4,3-*b*]pyran-7-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

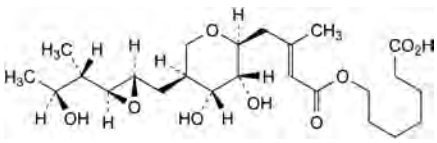

F. 7-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[[(2*S*,3*S*)-3-[(1*S*,2*S*)-2-hydroxy-1-methylpropyl]oxiranyl]methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]heptanoic acid,

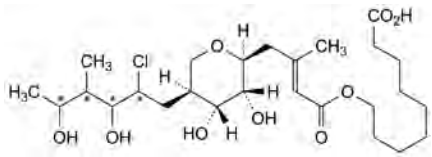

G. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-5-(2-chloro-3,5-dihydroxy-4-methylhexyl)-3,4-dihydroxytetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

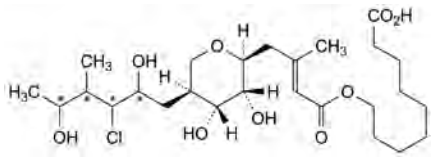

H. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-5-(3-chloro-2,5-dihydroxy-4-methylhexyl)-3,4-dihydroxytetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid,

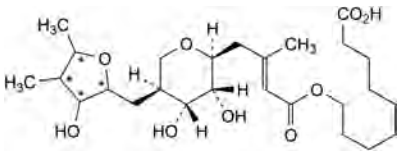

I. 9-[[(2*E*)-4-[(2*S*,3*R*,4*R*,5*S*)-3,4-dihydroxy-5-[(3-hydroxy-4,5-dimethyltetrahydrofuran-2-yl)methyl]tetrahydro-2*H*-pyran-2-yl]-3-methylbut-2-enoyl]oxy]nonanoic acid.

**01/2017:1700**

# MYCOPHENOLATE MOFETIL

## Mycophenolas mofetil

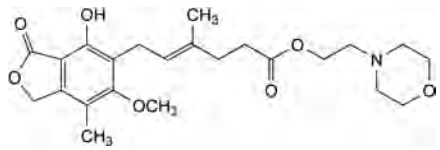

$C_{23}H_{31}NO_7$ $M_r$ 433.5
[128794-94-5]

DEFINITION

2-(Morpholin-4-yl)ethyl (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in anhydrous ethanol.

mp: about 96 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mycophenolate mofetil CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.10 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light. Prepare the solutions immediately before use, or store them at 4-8 °C. Keep the temperature of the autosampler at 10 °C, allow the temperature of the solutions to equilibrate in the vials for 15 min before injection.*

*Test solution.* Dissolve 20 mg of the substance to be examined in *acetonitrile R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 5 mg of *mycophenolate mofetil for peak identification CRS* (mycophenolate mofetil with impurities A, B, D, E, F, G and H) in *acetonitrile R* and dilute to 2.5 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 45 °C.

*Mobile phase*: mix 350 mL of *acetonitrile R* with a mixture of 650 mL of *water R* and 2.0 mL of *triethylamine R* previously adjusted to pH 5.3 with *dilute phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of mycophenolate mofetil.

*Relative retention* with reference to mycophenolate mofetil (retention time = about 22 min): impurity F = about 0.3; impurity A = about 0.4; impurity H = about 0.5; impurity G = about 0.6; impurity B = about 0.8; impurity D = about 1.2; impurity E = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity A and impurity H,
- the chromatogram obtained is similar to the chromatogram supplied with *mycophenolate mofetil for peak identification CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 2.1,
- *impurity F*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *impurities A, D, E, G, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent),
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 43.35 mg of $C_{23}H_{31}NO_7$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, D, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

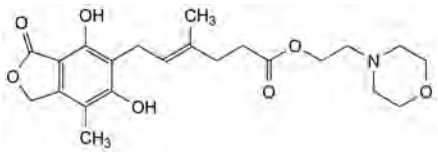

A. 2-(morpholin-4-yl)ethyl (4*E*)-6-(4,6-dihydroxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate,

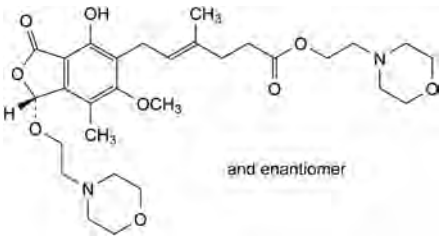


B. 2-(morpholin-4-yl)ethyl (4*E*)-6-[(1*RS*)-4-hydroxy-6-methoxy-7-methyl-1-[2-(morpholin-4-yl)ethoxy]-3-oxo-1,3-dihydroisobenzofuran-5-yl]-4-methylhex-4-enoate,

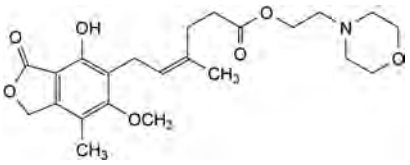

C. 2-(morpholin-4-yl)ethyl (4*Z*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate,

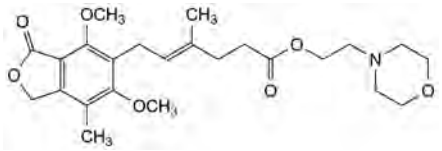

D. 2-(morpholin-4-yl)ethyl (4*E*)-6-(4,6-dimethoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate,

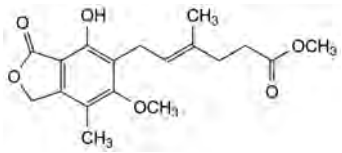

E. methyl (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate,

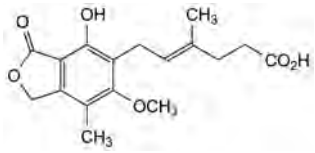

F. (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoic acid (mycophenolic acid),

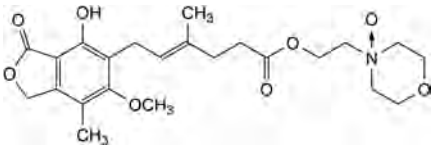

G. 2-(morpholin-4-yl)ethyl (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydroisobenzofuran-5-yl)-4-methylhex-4-enoate *N*-oxide,

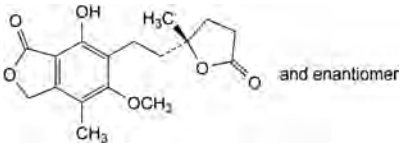


H. 7-hydroxy-5-methoxy-4-methyl-6-[2-[(2*RS*)-2-methyl-5-oxotetrahydrofuran-2-yl]ethyl]isobenzofuran-1(3*H*)-one.

07/2016:2813

# MYCOPHENOLATE SODIUM

## Mycophenolatum natricum

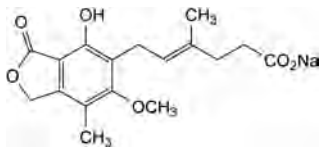

$C_{17}H_{19}NaO_6$ $M_r$ 342.3
[37415-62-6]

DEFINITION

Sodium (4*E*)-6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-1,3-dihydro-2-benzofuran-5-yl)-4-methylhex-4-enoate.

Semi-synthetic product derived from a fermentation product.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in anhydrous ethanol, very slightly soluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *mycophenolate sodium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R2*, *water for chromatography R* (10:90 *V/V*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 40.0 mg of *mycophenolate sodium CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c).* In order to prepare impurity A *in situ*, dissolve 2 mg of the substance to be examined in *water R* and dilute to 25 mL with the same solvent. Transfer 5 mL of this solution to a vial, seal the vial and heat at about 80 °C for 2-6 h.

*Column*:

– *size*: *l* = 0.25 m, Ø = 3.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: *phosphoric acid R*, *acetonitrile R1*, *water for chromatography R* (0.2:100:900 *V/V/V*);

– *mobile phase B*: *phosphoric acid R*, *water for chromatography R*, *acetonitrile R1* (0.2:200:800 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 37 | 90 → 10 | 10 → 90 |
| 37 - 42 | 10 | 90 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 216 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to mycophenolic acid (retention time = about 19 min): impurity A = about 0.9.

*System suitability*:

– *resolution*: minimum 3.0 between the peaks due to impurity A and mycophenolic acid in the chromatogram obtained with reference solution (c);

– *signal-to-noise ratio*: minimum 25 for the principal peak in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity A by 1.4;

– for each impurity, use the concentration of mycophenolate sodium in reference solution (b).

*Limits*:

– *impurity A*: maximum 0.1 per cent;

– *unspecified impurities*: for each impurity, maximum 0.05 per cent;

– *total*: maximum 0.1 per cent;

– *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{17}H_{19}NaO_6$ taking into account the assigned content of *mycophenolate sodium CRS*.

IMPURITIES

*Specified impurities*: *A*.

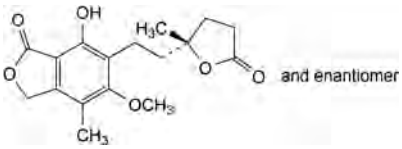


A. 7-hydroxy-5-methoxy-4-methyl-6-[2-[(2*RS*)-2-methyl-5-oxotetrahydrofuran-2-yl]ethyl]-2-benzofuran-1(3*H*)-one.

# N

Nabumetone.. 3317
Nadolol.. 3318
Nadroparin calcium.. 3319
Naftidrofuryl hydrogen oxalate.. 3322
Nalidixic acid.. 3323
Naloxone hydrochloride dihydrate.. 3325
Naltrexone hydrochloride.. 3327
Nandrolone decanoate.. 3328
Naphazoline hydrochloride.. 3330
Naphazoline nitrate.. 3331
Naproxen.. 3333
Naproxen sodium.. 3335
Nateglinide.. 3337
Neohesperidin-dihydrochalcone.. 3339
Neomycin sulfate.. 3340
Neostigmine bromide.. 3342
Neostigmine metilsulfate.. 3343
Netilmicin sulfate.. 3344
Nevirapine.. 3346
Nevirapine hemihydrate.. 3347
Nicardipine hydrochloride.. 3348
Nicergoline.. 3349
Niclosamide.. 3351
Niclosamide monohydrate.. 3352
Nicorandil.. 3353
Nicotinamide.. 3354
Nicotine.. 3355
Nicotine ditartrate dihydrate.. 3356
Nicotine resinate.. 3357
Nicotinic acid.. 3359
Nifedipine.. 3360
Niflumic acid.. 3362
Nifuroxazide.. 3363
Nikethamide.. 3364
Nilotinib hydrochloride monohydrate.. 3365
Nilutamide.. 3367
Nimesulide.. 3368
Nimodipine.. 3369
Nitrazepam.. 3371
Nitrendipine.. 3372
Nitric acid.. 3373
Nitric oxide.. 3373
Nitrofural.. 3374
Nitrofurantoin.. 3375
Nitrogen.. 3376
Nitrogen, low-oxygen.. 3377
Nitrous oxide.. 3377
Nizatidine.. 3379
Nomegestrol acetate.. 3381
Nonoxinol 9.. 3381
Noradrenaline hydrochloride.. 3382
Noradrenaline tartrate.. 3384
Norethisterone.. 3385
Norethisterone acetate.. 3387
Norfloxacin.. 3388
Norflurane.. 3390
Norgestimate.. 3396
Norgestrel.. 3397
Nortriptyline hydrochloride.. 3397
Noscapine.. 3399
Noscapine hydrochloride hydrate.. 3400
Nystatin.. 3401

**01/2017:1350**

# NABUMETONE

## Nabumetonum

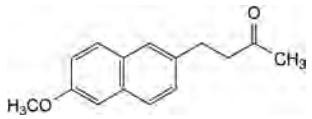

$C_{15}H_{16}O_2$ $M_r$ 228.3
[42924-53-8]

DEFINITION

4-(6-Methoxynaphthalen-2-yl)butan-2-one.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nabumetone CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 25.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 5.0 mL with *acetonitrile R*.

*Reference solution (a)*. Dissolve 20.0 mg of *nabumetone CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with *acetonitrile R*.

*Reference solution (b)*. Dilute 0.5 mL of test solution (a) to 100.0 mL with *acetonitrile R*.

*Reference solution (c)*. Dissolve 1.5 mg of *nabumetone impurity F CRS* in *acetonitrile R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d)*. Dissolve 4 mg of *nabumetone impurity D CRS* in *acetonitrile R* and dilute to 100 mL with the same solvent. To 5 mL of this solution, add 5 mL of test solution (b).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 12 volumes of *tetrahydrofuran R*, 28 volumes of *acetonitrile for chromatography R* and 60 volumes of a 0.1 per cent *V/V* solution of *glacial acetic acid R* in *carbon dioxide-free water R* prepared from *distilled water R*;
- *mobile phase B*: mix 24 volumes of *tetrahydrofuran R*, 56 volumes of *acetonitrile for chromatography R* and 20 volumes of a 0.1 per cent *V/V* solution of *glacial acetic acid R* in *carbon dioxide-free water R* prepared from *distilled water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 100 | 0 |
| 12 - 28 | 100 → 0 | 0 → 100 |
| 28 - 33 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Retention time*: nabumetone = about 11 min.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to nabumetone and impurity D.

*Limits*:

- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{15}H_{16}O_2$ from the declared content of *nabumetone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *F*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E*.

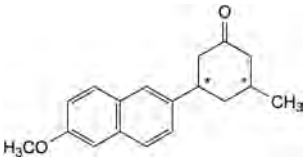

A. 3-(6-methoxynaphthalen-2-yl)-5-methylcyclohexanone,

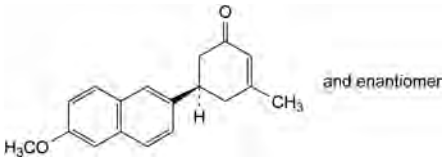


B. (5*RS*)-5-(6-methoxynaphthalen-2-yl)-3-methylcyclohex-2-enone,

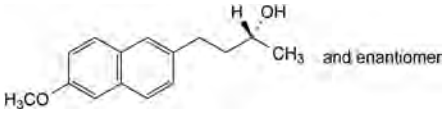


C. (2*RS*)-4-(6-methoxynaphthalen-2-yl)butan-2-ol,

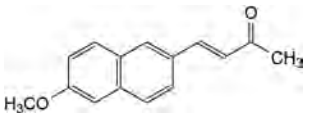


D. (*E*)-4-(6-methoxynaphthalen-2-yl)but-3-en-2-one,

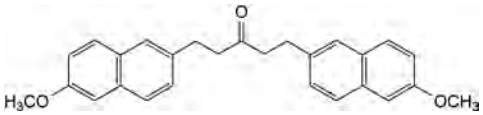


E. 1,5-bis(6-methoxynaphthalen-2-yl)pentan-3-one,

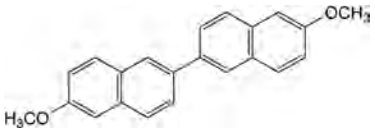


F. 6,6′-dimethoxy-2,2′-binaphthalenyl.

**01/2017:1789**

# NADOLOL

## Nadololum

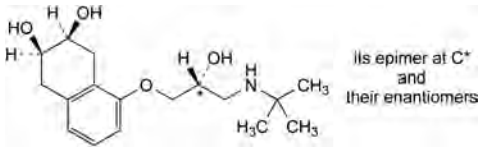


$C_{17}H_{27}NO_4$ $M_r$ 309.4
[42200-33-9]

DEFINITION

*cis*-5-[(2*RS*)-3-[(1,1-Dimethylethyl)amino]-2-hydroxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol.

It consists of 2 pairs of enantiomers that are present as 2 racemic compounds: racemate A and racemate B.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nadolol CRS*.

TESTS

**Racemate content**. Infrared absorption spectrophotometry (*2.2.24*).

Prepare a mull in *liquid paraffin R* of the substance to be examined (dried substance), adjusting the thickness of the mull to give an absorbance reading of 0.6 ± 0.1 at 1587 $cm^{-1}$. Record the spectrum from 1667 to 1111 $cm^{-1}$, using *liquid paraffin R* as reference. Measure the absorbance $A_a$, corresponding to racemate A, at the maximum at 1266 $cm^{-1}$ and the absorbance $A_b$, corresponding to racemate B, at the maximum at 1250 $cm^{-1}$. The ratio $A_a/A_b$ is 0.72 to 1.08 (corresponding to racemate A content of between 40 per cent and 60 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R1*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in 4.0 mL of the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *nadolol impurity mixture CRS* (impurities A and D) in 1.0 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 5.6 g/L solution of *sodium octanesulfonate R* adjusted to pH 3.5 with a 300 g/L solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 77 | 23 |
| 7 - 30 | 77 → 65 | 23 → 35 |
| 30 - 35 | 65 → 55 | 35 → 45 |
| 35 - 55 | 55 | 45 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 206 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *nadolol impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and D.

*Relative retention* with reference to nadolol (retention time = about 15 min): impurity A = about 0.2; impurity C (doublet) = about 0.47 and 0.53; impurity D = about 1.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 8.0 between the peaks due to nadolol and impurity D.

*Limits*:

- *correction factor*: for the calculation of content, multiply the sum of the 2 peak areas of impurity C by 0.7;
- *impurities A, C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 100 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.94 mg of $C_{17}H_{27}NO_4$.

IMPURITIES

*Specified impurities: A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E, F, G.*

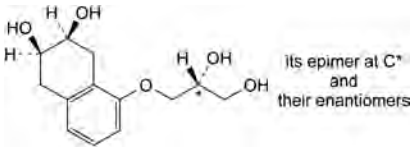


A. *cis*-5-[(2*RS*)-2,3-dihydroxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol (tetraol),

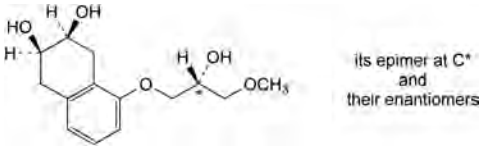


B. *cis*-5-[(2*RS*)-2-hydroxy-3-methoxypropoxy]-1,2,3,4-tetrahydronaphthalene-2,3-diol,

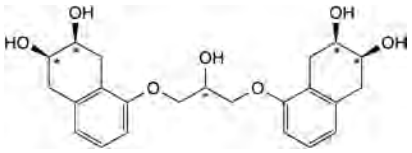

C. 5,5′-[(2*rs*)-2-hydroxypropane-1,3-diylbis(oxy)]bis-(*cis*-1,2,3,4-tetrahydronaphthalene-2,3-diol) (3 diastereoisomers),

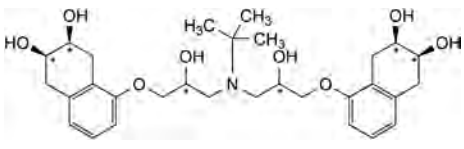

D. 5,5′-[[(1,1-dimethylethyl)imino]bis[(2-hydroxypropane-1,3-diyl)oxy]]bis(*cis*-1,2,3,4-tetrahydronaphthalene-2,3-diol) (10 stereoisomers),

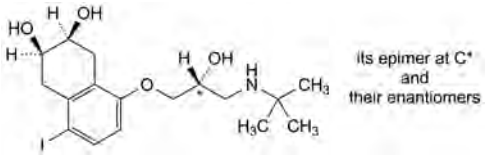


E. *cis*-5-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-8-iodo-1,2,3,4-tetrahydronaphthalene-2,3-diol,

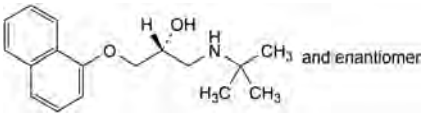


F. (2*RS*)-1-[(1,1-dimethylethyl)amino]-3-(naphthalen-1-yloxy)propan-2-ol,

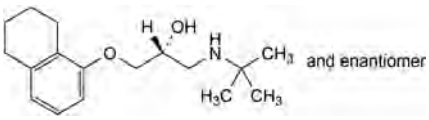


G. (2*RS*)-1-[(1,1-dimethylethyl)amino]-3-[(5,6,7,8-tetrahydronaphthalen-1-yl)oxy]propan-2-ol.

**01/2008:1134**
**corrected 9.5**

# NADROPARIN CALCIUM

## Nadroparinum calcicum

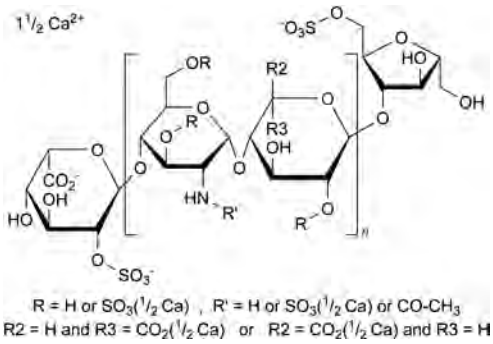


DEFINITION

Calcium salt of low-molecular-mass heparin obtained by nitrous acid depolymerisation of heparin from pork intestinal mucosa, followed by fractionation to eliminate selectively most of the chains with a molecular mass lower than 2000. The majority of the components have a 2-*O*-sulfo-α-L-idopyranosuronic acid structure at the non-reducing end and a 6-*O*-sulfo-2,5-anhydro-D-mannitol structure at the reducing end of their chain.

*Nadroparin calcium complies with the monograph Low-molecular-mass heparins (0828) with the modifications and additional requirements below.*

The mass-average relative molecular mass ranges between 3600 and 5000 with a characteristic value of about 4300.

The degree of sulfatation is about 2 per disaccharide unit.

The potency is not less than 95 IU and not more than 130 IU of anti-factor Xa activity per milligram, calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 2.5 and 4.0.

IDENTIFICATION

Carry out identification test A as described in the monograph *Low-molecular-mass heparins (0828)* using *nadroparin calcium CRS*.

Carry out identification test C as described in the monograph *Low-molecular-mass heparins (0828)*. The following requirements apply.

The mass-average relative molecular mass ranges between 3600 and 5000. The mass percentage of chains lower than 2000 is not more than 15 per cent. The mass percentage of chains between 2000 and 8000 ranges between 75 per cent and 95 per cent. The mass percentage of chains between 2000 and 4000 ranges between 35 per cent and 55 per cent.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent.

**Ethanol**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1.0 mL of *2-propanol R* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Blank solution*. 1.0 mL of *water R*.

*Test solution (a)*. To 10.0 mg of the substance to be examined, add 1.0 mL of *water R*.

*Test solution (b)*. To 10.0 mg of the substance to be examined, add 0.50 mL of *water R* and 0.50 mL of the internal standard solution.

*Reference solution (a)*. Dilute 1.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 0.5 mL of the solution to 20.0 mL with *water R*.

*Reference solution (b)*. To 0.50 mL of reference solution (a), add 0.50 mL of the internal standard solution.

*Column*:

- *material*: nickel;
- *size*: *l* = 1.5 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (150-180 µm).

*Carrier gas*: *helium for chromatography R* or *nitrogen for chromatography R*.

*Flow rate*: 30 mL/min.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 90 °C;
- *equilibration time*: 15 min;
- *pressurisation time*: 1 min.

*Temperature*:

- *column*: 150 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Identification of peaks*: use the chromatogram obtained with reference solution (b) to identify the peaks due to ethanol and 2-propanol.

*Retention time*: ethanol = about 2.5 min; 2-propanol = about 4 min.

Calculate the percentage content *m/m* of ethanol taking its density at 20 °C to be 0.792 g/mL.

*Limit*:

- *ethanol*: maximum 1.0 per cent *m/m*.

**N-NO groups**: maximum 0.25 ppm.

The content of N-NO-groups is determined by cleavage of the N-NO bond with hydrobromic acid in ethyl acetate under a reflux condenser and detection of the released NO by chemiluminescence.

*Description of the apparatus* (Figure 1134.-1). Use a 500 mL borosilicate glass round-bottomed flask, above which is attached a condenser which is equipped with:

- on one side, a torion joint through which a stream of *argon R* can be introduced via a cannula;
- on the other side, a screw joint with a piston equipped with a septum through which the reference solution and test solution will be injected.

The round-bottomed flask is connected in series to 3 bubble traps which are themselves connected to 2 cold traps, which are in turn connected to a chemiluminescence detector. Suitable tubing ensures the junctions are leak-free.

*Preparation of the chemiluminescence detector.* Switch on the chemiluminescence detector 48 h before use and start the vacuum pump. The vacuum must be less than 0.5 mm Hg. 1 h before use, open the oxygen valve at a pressure of 0.2 MPa and a flow rate of 9.4 mL/min.

*Preparation of the bubble trap*. In each bubble trap, place 30 mL of a 300 g/L solution of *sodium hydroxide R* in *water R*.

*Preparation of the cold traps.*

- Trap at − 120 °C: Slowly add liquid nitrogen to an isothermic flask containing 250 mL of *anhydrous ethanol R* whilst stirring with a wooden spatula until a paste is obtained. Place the cold trap in the isothermic flask prepared as described.
- Trap at − 160 °C: Slowly add liquid nitrogen to an isothermic flask containing 250 mL of *2-methylbutane R* whilst stirring with a wooden spatula until a paste is obtained. Place the cold trap in the isothermic flask prepared as described.

*Drying of the 500 mL borosilicate-glass round-bottomed flask and condenser.* Boil 50 mL of *ethyl acetate R* under reflux for 1 h under *argon R* without connecting the system to the chemiluminescence detector.

*Test solution.* Dry the substance to be examined for 12 h over *diphosphorus pentoxide R* at 60 °C under vacuum. Dissolve 0.10 g of the treated substance to be examined in 1.0 mL of *treated formamide R*. Shake the solution obtained for 30 min.

*Reference solution.* Dilute 0.1 mL of *nitrosodipropylamine solution R* in 6.0 mL of *anhydrous ethanol R*. Dilute 0.1 mL of the solution obtained in 1.0 mL of *treated formamide R*. (This solution is equivalent to 0.05 ppm of N-NO groups).

Place 50 mL of *treated ethyl acetate R* in the dry 500 mL borosilicate glass round-bottomed flask equipped with a septum. Connect the round-bottomed flask to the condenser which has been previously cooled to − 15 °C for 2 h.

Connect the *argon R* cannula and adjust the flow rate to 0.1 L/min. Check that the system is leak-free. Only the connector to the chemiluminescence detector remains open in order to avoid excess pressure.

Heat the *treated ethyl acetate R* to boiling.

Evacuate the system by slowly turning the valve of the chemiluminescence detector. At the same time tighten the inlet on the chemiluminescence detector.

When the system is equilibrated, the vacuum reaches 4 mm Hg.

The signal of the zero adjuster on the chemiluminescence detector is set to 10 per cent of the full scale of the recorder.

Through the septum of the 500 mL borosilicate glass round-bottomed flask, sequentially inject 0.5 mL of *water R*, 2.0 mL of *dilute hydrobromic acid R* and then another 2.0 mL of *dilute hydrobromic acid R*, making sure that the recorder pen has returned to the baseline between each injection.

Inject 50.0 µL of the reference solution, then 50.0 µL of the test solution after the recorder pen has returned to the baseline.

Calculate the content of N-NO groups of the substance to be examined.

**Free sulfates**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

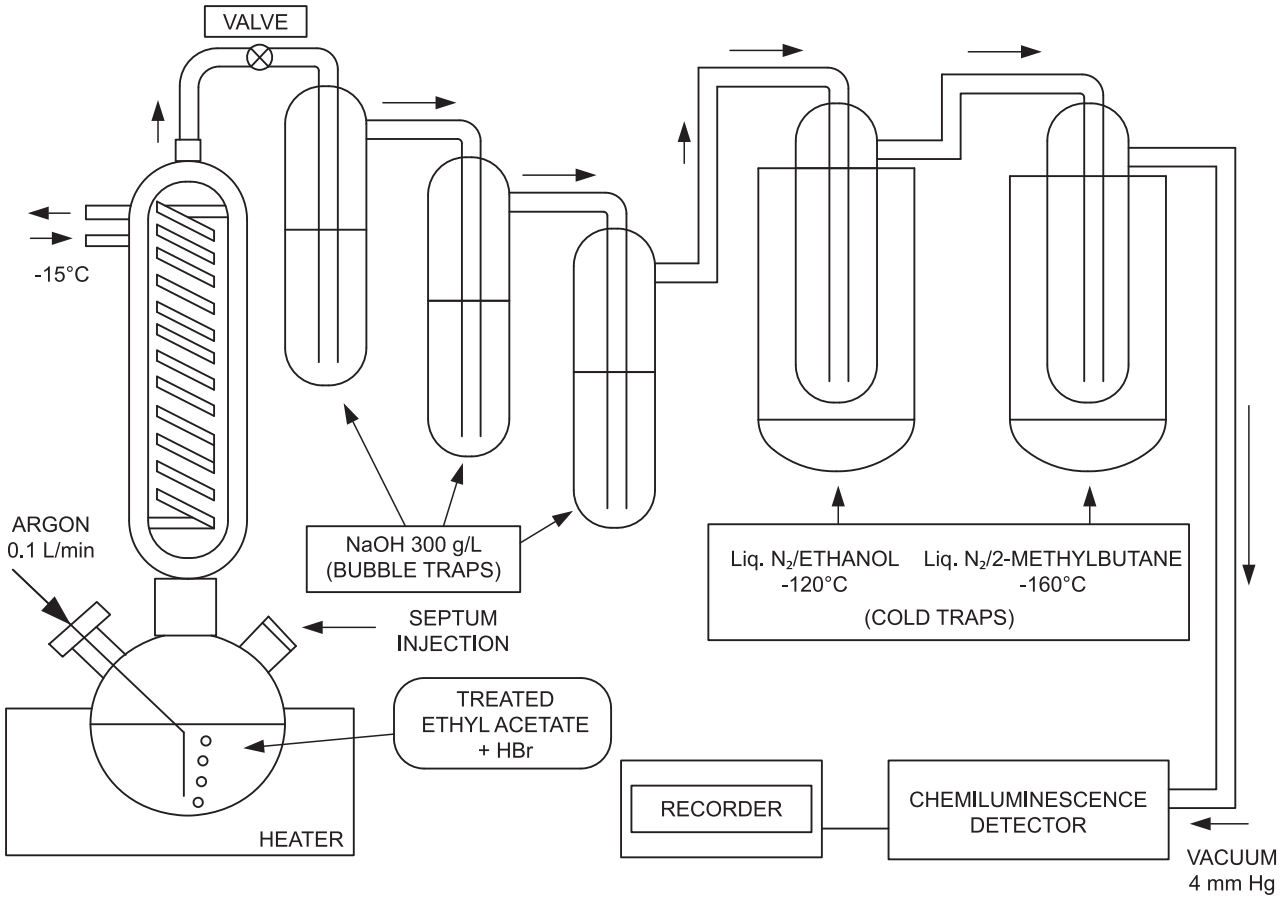


*Flask*: round-bottom borosilicate glass flask equipped with a central rodavis joint, a torion joint on the left neck and a 15 mm screw joint on the right neck; septum: silicone material, diameter 14 mm and thickness 3.5 mm.

*Condenser*: height 21 cm and internal diameter 3 cm, with a lower rodavis joint and an upper torion joint.

*Bubble traps*: height 24 cm and internal diameter 2.5 cm; internal tubing: length 23 cm and internal diameter 0.5 cm. Equipped with a centrally positioned rotulex mounting with torion joints on the inlet and outlet.

*Cold traps*: height 16.5 cm and internal diameter 4 cm; internal tubing: length 14 cm and internal diameter 1.3 cm. Equipped with torion joints on the inlet and outlet and placed in an isothermic flask: internal depth 22 cm and internal diameter 8 cm.

*Tubing*: fluorinated ethylene propylene material, internal diameter 3.2 mm and thickness 0.8 mm.

Figure 1134.-1. – *Apparatus used for the assay of N-NO groups*

*Reference solution.* Dissolve 1.4787 g of *anhydrous sodium sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 1.0 mL of the solution to 200.0 mL with *distilled water R* (5 ppm of sulfate ions).

*Column*:

– *size*: *l* = 50 mm, Ø = 4.6 mm;

– *stationary phase*: anion-exchange resin.

*Chemical neutralisation system*: neutralisation micromembrane in line with the mobile phase for anion detection; continuously pump in counter-flow with a 2.45 g/L solution of *sulfuric acid R*, at a flow rate of 4 mL/min.

*Mobile phase*:

– *mobile phase A*: 1.91 g/L solution of *disodium tetraborate R*;

– *mobile phase B*: *0.1 M sodium hydroxide*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 15.5 | 100 → 0 | 0 → 100 |
| 15.5 - 25.5 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: conductivity detector with a sensitivity of 30 µS.

*Injection*: 50 µL.

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the principal peak due to the sulfate ion.

*Retention time*: sulfate ion = about 7.5 min. Change the composition of the mobile phase, if necessary, to obtain the prescribed retention time.

*Limit*:

– *free sulfates*: maximum 0.5 per cent.

01/2017:1594
corrected 10.0

# NAFTIDROFURYL HYDROGEN OXALATE

## Naftidrofuryli hydrogenooxalas

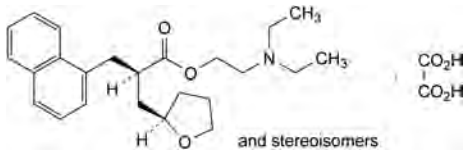


$C_{26}H_{35}NO_7$ $M_r$ 473.6
[3200-06-4]

DEFINITION

Mixture of 4 stereoisomers of 2-(diethylamino)ethyl 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate hydrogen oxalate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, freely soluble or soluble in ethanol (96 per cent), slightly or sparingly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 1.0 g in *water R* and dilute to 50 mL with the same solvent. Add 2 mL of *concentrated ammonia R* and shake with 3 quantities, each of 10 mL, of *methylene chloride R*. To the combined lower layers, add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means at a temperature not exceeding 30 °C. Use the residue obtained.

*Comparison*: *Ph. Eur. reference spectrum of naftidrofuryl*.

B. Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent. Add 2.0 mL of *calcium chloride solution R*. A white precipitate is formed. The precipitate dissolves after the addition of 3.0 mL of *hydrochloric acid R*.

TESTS

**Absorbance** (*2.2.25*): maximum 0.1 at 430 nm.

Dissolve 1.5 g in *water R* and dilute to 10 mL with the same solvent. If necessary use an ultrasonic bath.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 80.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase. Sonicate for 10 s. A precipitate is formed. Filter through a membrane filter (nominal pore size 0.45 µm), discarding the first 5 mL. *Use a freshly prepared solution.*

*Reference solution (a)*. Dissolve 5.0 mg of *naftidrofuryl impurity A CRS* in *acetonitrile R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *naftidrofuryl impurity B CRS* and 5 mg of the substance to be examined in *acetonitrile R* and dilute to 50 mL with the same solvent. Dilute 1 mL of the solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 60 mL of *methanol R* with 150 mL of *tetrabutylammonium buffer solution pH 7.0 R* and dilute to 1000 mL with *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 20 µL.

*Run time*: 2.3 times the retention time of naftidrofuryl.

*Relative retention* with reference to naftidrofuryl (retention time = about 7 min): impurity A = about 0.5; impurity B = about 0.8; impurity C = about 1.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity B and naftidrofuryl.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

B. Gas chromatography (*2.2.28*).

*Test solution (a)*. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 50 mL with the same solvent. Add 2 mL of *concentrated ammonia R* and shake with 3 quantities, each of 10 mL, of *methylene chloride R*. To the combined lower layers, add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means at a temperature not exceeding 30 °C. Take up the residue with *methylene chloride R* and dilute to 20.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *methylene chloride R*.

*Reference solution*. Dissolve 5 mg of *naftidrofuryl impurity F CRS* in *methylene chloride R* and dilute to 50 mL with the same solvent.

*Column*:

– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.32 mm;
– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.45 µm).

*Carrier gas*: *helium for chromatography R*.

*Splitter flow rate*: 25 mL/min.

*Flow rate*: 2.9 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 210 |
| | 4 - 8 | 210 → 230 |
| | 8 - 18 | 230 → 260 |
| | 18 - 30 | 260 |
| Injection port | | 290 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to the second eluting peak of naftidrofuryl: impurity D = about 0.14; impurity B = about 0.55 (for the second eluting peak); impurity E = about 0.86; impurity F = about 1.04 (for the second eluting peak).

*System suitability*: test solution (b):

– *resolution*: minimum 1.0 between the 2 peaks due to the diastereoisomers of naftidrofuryl.

*Limits*: test solution (a):

– *impurity F*: for the sum of the areas of the 2 peaks, maximum 0.20 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.20 per cent);
– *impurity E*: maximum 0.20 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.20 per cent);
– *impurity D*: maximum 0.10 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.10 per cent);
– *any other impurity*: for each impurity, maximum 0.10 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.10 per cent);
– *total*: maximum 0.50 per cent of the sum of the areas of the 2 peaks due to naftidroduryl (0.50 per cent);
– *disregard limit*: 0.02 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl (0.02 per cent); disregard any peaks due to impurity B.

**Diastereoisomer ratio**. Gas chromatography (*2.2.28*) as described in test B for related substances.

*Limits*: test solution (b):

– *first eluting naftidrofuryl diastereoisomer*: minimum 30 per cent of the sum of the areas of the 2 peaks due to naftidrofuryl.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.36 mg of $C_{26}H_{35}NO_7$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

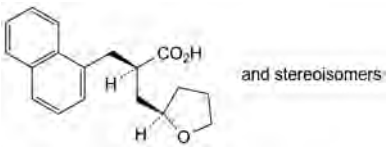


A. 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoic acid,

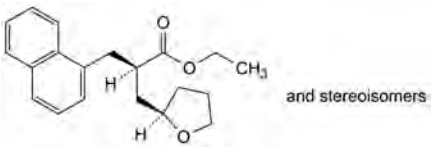


B. ethyl 2-[(naphthalen-1-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate,

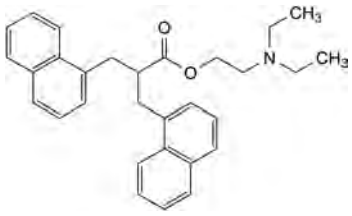


C. 2-(diethylamino)ethyl 3-(naphthalen-1-yl)-2-[(naphthalen-1-yl)methyl]propanoate,

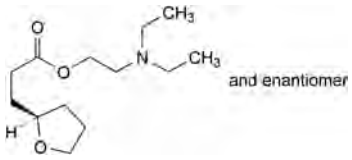


D. 2-(diethylamino)ethyl 3-[(2*RS*)-tetrahydrofuran-2-yl]propanoate,

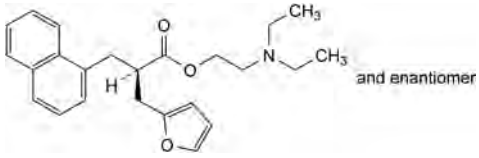


E. 2-(diethylamino)ethyl (2*RS*)-2-[(furan-2-yl)methyl]-3-(naphthalen-1-yl)propanoate,

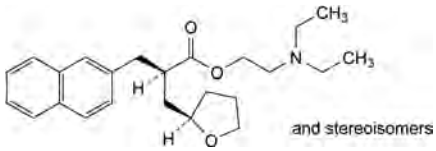


F. 2-(diethylamino)ethyl 2-[(naphthalen-2-yl)methyl]-3-(tetrahydrofuran-2-yl)propanoate.

**01/2017:0701**

# NALIDIXIC ACID

## Acidum nalidixicum

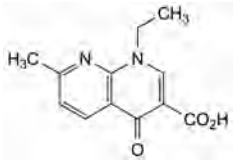


$C_{12}H_{12}N_2O_3$ $M_r$ 232.2
[389-08-2]

DEFINITION

1-Ethyl-7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

mp: about 230 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nalidixic acid CRS*.

TESTS

**Absorbance** (*2.2.25*): maximum 0.10 at 420 nm.

Dissolve 1.50 g in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *methanol R* (50:50 *V/V*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *nalidixic acid impurity C CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Mix 1.0 mL of this solution and 10.0 mL of the test solution and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: solution containing 0.2 g/L of *sodium heptanesulfonate monohydrate R* and 2.3 g/L of *ammonium dihydrogen phosphate R*, adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*, *methanol R* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 → 70 | 20 → 30 |
| 10 - 20 | 70 → 65 | 30 → 35 |
| 20 - 35 | 65 | 35 |
| 35 - 45 | 65 → 30 | 35 → 70 |
| 45 - 62 | 30 | 70 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to nalidixic acid (retention time = about 40 min): impurity C = about 1.02.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to nalidixic acid and impurity C.

*Calculation of percentage contents*:

- for impurity C, use the concentration of impurity C in reference solution (b);
- for impurities other than C, use the concentration of nalidixic acid in reference solution (a).

*Limits*:

- *impurity C*: maximum 0.05 per cent;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 10 mL of *methylene chloride R* and add 30 mL of *2-propanol R* and 10 mL of *carbon dioxide-free water R*. Keep the titration vessel covered and pass *nitrogen R* through the solution throughout the titration. Keep the temperature of the solution between 15 °C and 20 °C. Titrate with *0.1 M ethanolic sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*) using a silver-silver chloride comparison electrode with a sleeve diaphragm or a capillary tip, filled with a saturated solution of *lithium chloride R* in *anhydrous ethanol R*, and a glass indicator electrode.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 23.22 mg of $C_{12}H_{12}N_2O_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D.*

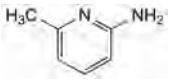


A. 6-methylpyridin-2-amine,

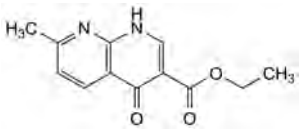


B. ethyl 7-methyl-4-oxo-1,4-dihydro-1,8-naphthyridine-3-carboxylate,

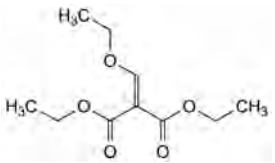


C. diethyl (ethoxymethylidene)propanedioate,

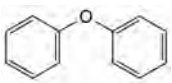


D. 1,1′-oxydibenzene.

**01/2018:0729**

# NALOXONE HYDROCHLORIDE DIHYDRATE

## Naloxoni hydrochloridum dihydricum

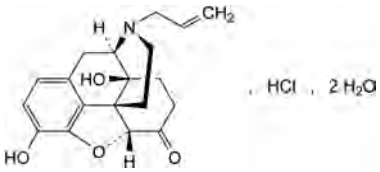

$C_{19}H_{22}ClNO_4,2H_2O$ $M_r$ 399.9
[51481-60-8]

DEFINITION

4,5α-Epoxy-3,14-dihydroxy-17-(prop-2-enyl)morphinan-6-one hydrochloride dihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent), practically insoluble in toluene.

IDENTIFICATION

*First identification*: A, C.

*Second identification*: B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *naloxone hydrochloride dihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 8 mg of the substance to be examined in 0.5 mL of *water R* and dilute to 1 mL with *methanol R*.

*Reference solution*. Dissolve 8 mg of *naloxone hydrochloride dihydrate CRS* in 0.5 mL of *water R* and dilute to 1 mL with *methanol R*.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: mix 5 volumes of *methanol R* and 95 volumes of the upper layer from a mixture of 60 mL of *dilute ammonia R2* and 100 mL of *butanol R*.

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a freshly prepared 5 g/L solution of *potassium ferricyanide R* in *ferric chloride solution R1*; examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10.0 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): – 181 to – 170 (anhydrous substance), determined on solution S.

**Impurity D**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 1.58 g of *ammonium hydrogen carbonate R* in 950 mL of *water for chromatography R*, adjust to pH 9.0 with *concentrated ammonia R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 0.500 g of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 20.0 mL with the same solution.

*Reference solution (a)*. Dissolve 10.0 mg of *naloxone impurity D CRS* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 20.0 mL with the same solution. Dilute 5.0 mL of this solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (c)*. To 4.0 mL of the test solution add 2.0 mL of reference solution (a) and dilute to 20.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, solution A (20:80 *V/V*);
- *mobile phase B*: *acetonitrile R1*, solution A (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 | 0 |
| 50 - 51 | 100 → 0 | 0 → 100 |
| 51 - 60 | 0 | 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Relative retention* with reference to naloxone (retention time = about 50 min): impurity D = about 0.8.

*System suitability*: reference solution (c):

- *symmetry factor*: maximum 1.8 for the peak due to impurity D.

*Limit*:

- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (75 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 1.10 g of *sodium octanesulfonate R* in 950 mL of *water for chromatography R*, adjust to pH 2.0 with a 50 per cent *V/V* solution of *phosphoric acid R*, filter and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 0.125 g of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Reference solution (a)*. Dissolve the contents of a vial of *naloxone for peak identification CRS* (containing impurities A, B, C, D, E and F) in 1 mL of a 10.3 g/L solution of *hydrochloric acid R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. Dilute 1.0 mL of this solution to 25.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, *tetrahydrofuran R*, solution A (2:4:94 *V/V/V*);

– *mobile phase B*: *tetrahydrofuran R*, *acetonitrile R*, solution A (4:17:79 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 100 → 0 | 0 → 100 |
| 40 - 50 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to naloxone (retention time = about 11 min): impurity C = about 0.6; impurity A = about 0.8; impurity F = about 0.9; impurity D = about 1.1; impurity E = about 3.0; impurity B = about 3.2.

*Identification of impurities*: use the chomatogram supplied with *naloxone for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E and F.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to naloxone.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity E by 0.5;

– *impurities A, B, C, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 7.5 per cent to 11.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 0.50 g.

ASSAY

Dissolve 0.300 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M ethanolic sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 36.38 mg of $C_{19}H_{22}ClNO_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

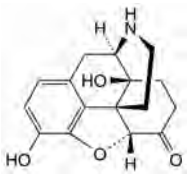

A. 4,5α-epoxy-3,14-dihydroxymorphinan-6-one (noroxymorphone),

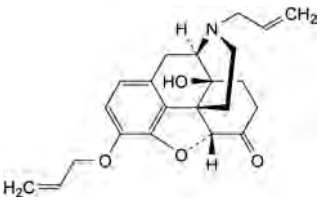

B. 4,5α-epoxy-14-hydroxy-17-(prop-2-enyl)-3-(prop-2-enyloxy)morphinan-6-one (3-*O*-allylnaloxone),

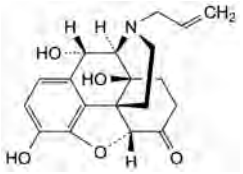

C. 4,5α-epoxy-3,10α,14-trihydroxy-17-(prop-2-enyl)morphinan-6-one (10α-hydroxynaloxone),

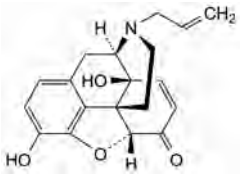

D. 7,8-didehydro-4,5α-epoxy-3,14-dihydroxy-17-(prop-2-enyl)morphinan-6-one (7,8-didehydronaloxone),

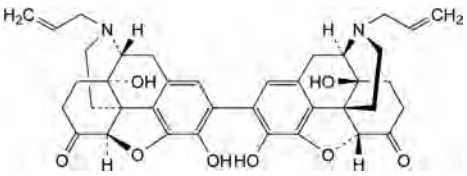

E. 4,5α:4′,5′α-diepoxy-3,3′,14,14′-tetrahydroxy-17,17′-bis(prop-2-enyl)-2,2′-bimorphinanyl-6,6′-dione (2,2′-binaloxone),

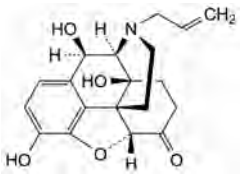

F. 4,5α-epoxy-3,10β,14-trihydroxy-17-(prop-2-enyl)morphinan-6-one (10β-hydroxynaloxone),

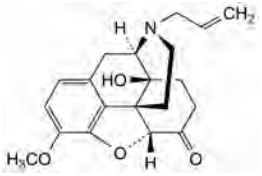

G. 4,5α-epoxy-14-hydroxy-3-methoxy-17-(prop-2-enyl)morphinan-6-one (3-*O*-methylnaloxone).

**01/2008:1790**

# NALTREXONE HYDROCHLORIDE

## Naltrexoni hydrochloridum

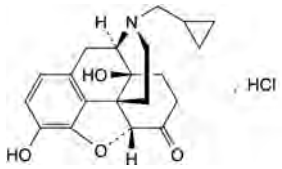

$C_{20}H_{24}ClNO_4$ $M_r$ 377.9

DEFINITION

17-(Cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxy-morphinan-6-one hydrochloride. It may be anhydrous, a monohydrate or a dihydrate, a mixture or a solvate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, very hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

Dissolve 20 mg in *water R* and dilute to 5 mL with the same solvent. Make alkaline with *dilute ammonia R1*. Shake with 10 mL of *methylene chloride R*, separate the organic layer and evaporate the solvent. Dry the residue obtained *in vacuo*.

*Comparison*: *naltrexone hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.40 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $B_6$ (*2.2.2, Method II*).

**Acidity and alkalinity**. To 10 mL of solution S, add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): – 187 to – 195 (anhydrous substance).

Dissolve 0.40 g in *water R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in *0.1 M hydrochloric acid* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *naltrexone impurity C CRS* in *0.1 M hydrochloric acid* and dilute to 2.5 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution and 1.0 mL of reference solution (a) to 100.0 mL with *0.1 M hydrochloric acid*. Dilute 1.0 mL of this solution to 10.0 mL with *0.1 M hydrochloric acid*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 1.1 g/L solution of *sodium octanesulfonate R* adjusted to pH 2.3 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 45 | 90 → 55 | 10 → 45 |
| 45 - 47 | 55 → 90 | 45 → 10 |
| 47 - 55 | 90 | 10 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Equilibration*: 8 min.

*Injection*: 10 µL.

*Relative retention* with reference to naltrexone (retention time = about 16 min): impurity A = about 0.4; impurity B = about 0.7; impurity F = about 0.8; impurity G = about 0.9; impurity C = about 1.05; impurity H = about 1.1; impurity I = about 1.2; impurity J = about 1.3; impurity D = about 1.4; impurity E = 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to naltrexone and impurity C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.4;
- *impurities C, D, E, F, G*: for each impurity, not more than twice the area of the peak due to naltrexone in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities A, B, H, I, J*: for each impurity, not more than the area of the peak due to naltrexone in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the peak due to naltrexone in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than 10 times the area of the peak due to naltrexone in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the peak due to naltrexone in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethanol** (*2.4.24, System A*): maximum 3.0 per cent.

*Test solution*. Dissolve 0.25 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dilute 0.750 g of *anhydrous ethanol R* to 1000.0 mL with *water R*.

**Water** (*2.5.12*): maximum 10.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 60 mL of *ethanol (96 per cent) R*, add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. The curve shows 3 points of inflexion. Read the volume added between the first 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.79 mg of $C_{20}H_{24}ClNO_4$.

STORAGE

In an airtight container. Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

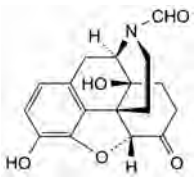

A. 17-formyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one,

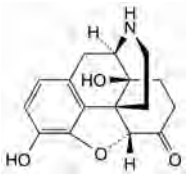

B. 4,5α-epoxy-3,14-dihydroxymorphinan-6-one (noroxymorphone),

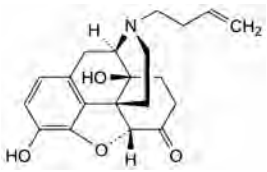

C. 17-but-3-enyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one,

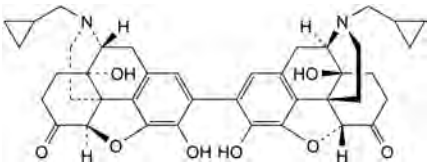

D. 17,17′-bis(cyclopropylmethyl)-4,5α:4′,5′α-diepoxy-3,3′,14,14′-tetrahydroxy-2,2′-bimorphinanyl-6,6′-dione (pseudonaltrexone),

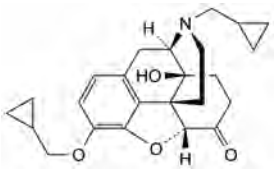

E. 3-(cyclopropylmethoxy)-17-(cyclopropylmethyl)-4,5α-epoxy-14-hydroxymorphinan-6-one,

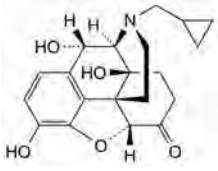

F. 17-(cyclopropylmethyl)-4,5α-epoxy-3,10α,14-trihydroxymorphinan-6-one,

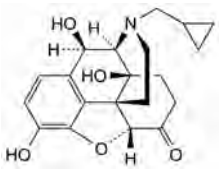

G. 17-(cyclopropylmethyl)-4,5α-epoxy-3,10β,14-trihydroxymorphinan-6-one,

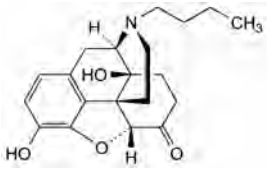

H. 17-butyl-4,5α-epoxy-3,14-dihydroxymorphinan-6-one,

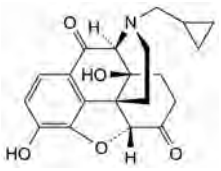

I. 17-(cyclopropylmethyl)-4,5α-epoxy-3,14-dihydroxymorphinan-6,10-dione,

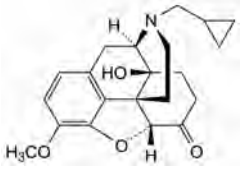

J. 17-(cyclopropylmethyl)-4,5α-epoxy-14-hydroxy-3-methoxymorphinan-6-one.

**01/2008:1992**
**corrected 10.0**

# NANDROLONE DECANOATE

## Nandroloni decanoas

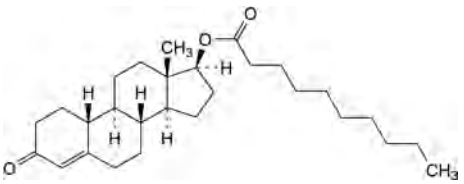

$C_{28}H_{44}O_3$ $M_r$ 428.7
[360-70-3]

DEFINITION

3-Oxoestr-4-en-17β-yl decanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Melting point (*2.2.14*): 34 °C to 38 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nandrolone decanoate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in 10 mL of *methanol R*.

**Specific optical rotation** (*2.2.7*): + 35.0 to + 40.0 (dried substance).

Dissolve 0.200 g in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent.

**Impurities A, B, C**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *methylene chloride R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with *methylene chloride R*.

*Reference solution (d).* Dissolve 5 mg of *nandrolone decanoate for system suitability CRS* (containing impurities A, B, C) in 0.5 mL of *methylene chloride R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetone R*, *heptane R* (30:70 *V/V*).

*Application*: 10 µL of the test solution and reference solutions (b), (c) and (d).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: treat with *alcoholic solution of sulfuric acid R* and heat at 130 °C until the spots appear. Examine in ultraviolet light at 366 nm.

*Retardation factors*: nandrolone decanoate = about 0.37; impurity A = about 0.45; impurity B = about 0.55; impurity C = about 0.58.

*System suitability*: reference solution (d):

– the chromatogram shows 4 clearly separated spots.

*Limits*:

– *impurity A*: any spot due to impurity A is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurities B, C*: any spot due to impurity B or C is not more intense than the principal spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *nandrolone decanoate for peak identification CRS* (containing impurities D, F, G, H, I, K, L) in *methanol R* and dilute to 2.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water R*,

– *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 35 | 65 |
| 5 - 40 | 35 → 0 | 65 → 100 |
| 40 - 75 | 0 | 100 |
| 75 - 80 | 0 → 35 | 100 → 65 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Relative retention* with reference to nandrolone decanoate (retention time = about 30 min): impurity D = about 0.05; impurity F = about 0.6; impurity K = about 0.7; impurity L = about 0.9; impurity G = about 0.97; impurity H = about 1.1; impurity I = about 1.2.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nandrolone decanoate.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.5; impurity F = 0.6; impurity H = 1.1; impurity I = 1.3; impurity K = 0.8;

– *impurities D, F, G, H, I, K, L*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at a pressure not exceeding 0.7 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with *anhydrous ethanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 240 nm. Calculate the content of $C_{28}H_{44}O_3$ taking the specific absorbance to be 407.

STORAGE

Under nitrogen, protected from light and at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, F, G, H, I, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, J.*

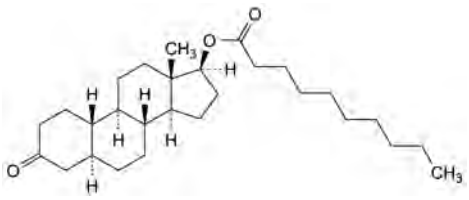

A. 3-oxo-5α-estran-17β-yl decanoate,

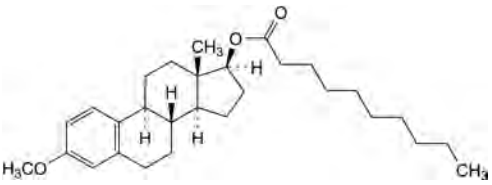

B. 3-methoxyestra-1,3,5(10)-trien-17β-yl decanoate,

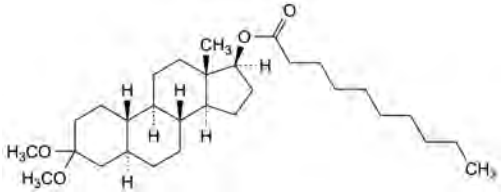

C. 3,3-dimethoxy-5α-estran-17β-yl decanoate,

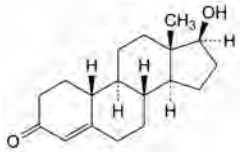

D. 17β-hydroxyestr-4-en-3-one,

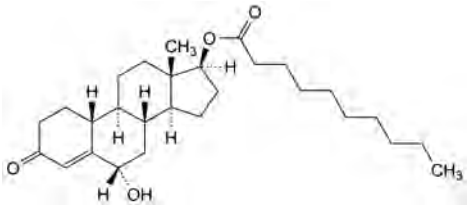

E. 6α-hydroxy-3-oxoestr-4-en-17β-yl decanoate,

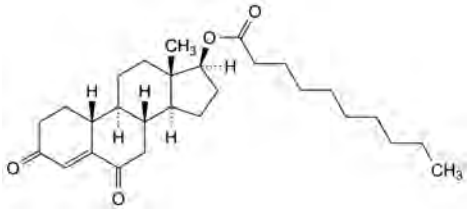

F. 3,6-dioxoestr-4-en-17β-yl decanoate,

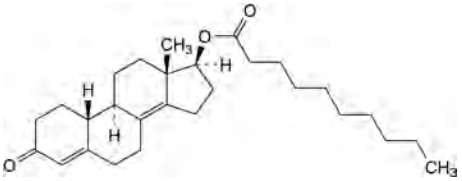

G. 3-oxoestra-4,8(14)-dien-17β-yl decanoate,

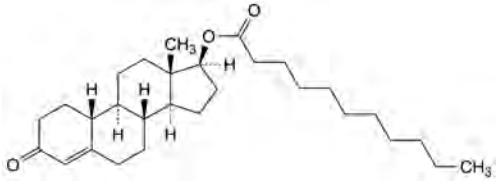

H. 3-oxoestr-4-en-17β-yl undecanoate,

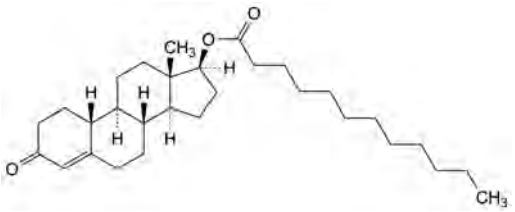

I. 3-oxoestr-4-en-17β-yl dodecanoate,

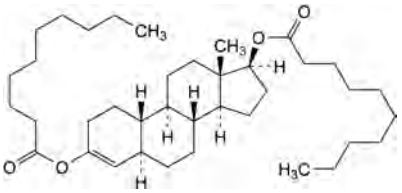

J. 5α-estr-3-ene-3,17β-diyl didecanoate,

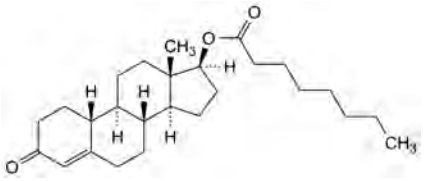

K. 3-oxoestr-4-en-17β-yl octanoate,

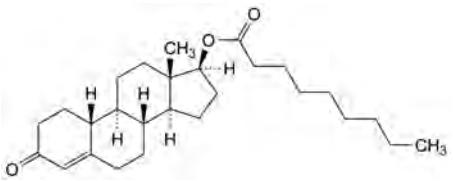

L. 3-oxoestr-4-en-17β-yl nonanoate.

**01/2009:0730**
**corrected 10.0**

# NAPHAZOLINE HYDROCHLORIDE

## Naphazolini hydrochloridum

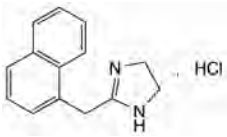

$C_{14}H_{15}ClN_2$ $M_r$ 246.7
[550-99-2]

DEFINITION

2-(Naphthalen-1-ylmethyl)-4,5-dihydro-1*H*-imidazole hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

mp: about 259 °C, with decomposition.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Dissolve 50.0 mg in *0.01 M hydrochloric acid* and dilute to 250.0 mL with the same acid. Dilute 25.0 mL of the solution to 100.0 mL with *0.01 M hydrochloric acid*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows 4 absorption maxima, at 270 nm, 280 nm, 287 nm and 291 nm. The ratios of the absorbances measured at the maxima at 270 nm, 287 nm and 291 nm to that measured at the maximum at 280 nm are 0.82 to 0.86, 0.67 to 0.70 and 0.65 to 0.69, respectively.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *naphazoline hydrochloride CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 20 mL of solution S add 0.2 mL of *0.01 M sodium hydroxide* and 0.1 mL of *methyl red solution R*. The solution is yellow. Not more than 0.6 mL of *0.01 M hydrochloric acid* is required to change the colour of the solution to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *1-naphthylacetic acid R* in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *naphazoline impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (4 µm) with a pore size of 6 nm.

*Mobile phase*: dissolve 1.1 g of *sodium octanesulfonate R* in a mixture of 5 mL of *glacial acetic acid R*, 300 mL of *acetonitrile R* and 700 mL of *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of naphazoline.

*Retention time*: naphazoline = about 14 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to naphazoline and impurity B.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.67 mg of $C_{14}H_{15}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities: B, C, D.*

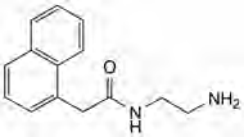


A. *N*-(2-aminoethyl)-2-(naphthalen-1-yl)acetamide (naphthylacetylethylenediamine),

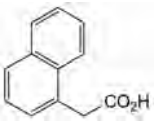


B. (naphthalen-1-yl)acetic acid (1-naphthylacetic acid),

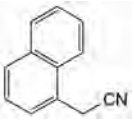


C. (naphthalen-1-yl)acetonitrile (1-naphthylacetonitrile),

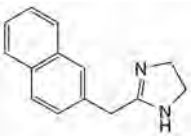


D. 2-(naphthalen-2-ylmethyl)-4,5-dihydro-1*H*-imidazole (β-naphazoline).

**01/2008:0147**
**corrected 10.0**

# NAPHAZOLINE NITRATE

## Naphazolini nitras

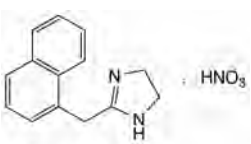


$C_{14}H_{15}N_3O_3$ $M_r$ 273.3
[5144-52-5]

DEFINITION

2-(Naphthalen-1-ylmethyl)-4,5-dihydro-1*H*-imidazole nitrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 167 °C to 170 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *0.01 M hydrochloric acid* and dilute to 250.0 mL with the same acid. Dilute 25.0 mL of the solution to 100.0 mL with *0.01 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 270 nm, 280 nm, 287 nm and 291 nm.

*Absorbance ratio*:

- $A_{270}/A_{280}$ = 0.82 to 0.86,
- $A_{291}/A_{280}$ = 0.65 to 0.69.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *naphazoline nitrate CRS*.

D. Dissolve 45 mg of the substance to be examined in 2 mL of *water R*. Add 1 mL of *sulfuric acid R*. Shake carefully and allow to cool. Add 1 mL of *ferrous sulfate solution R2* dropwise along the walls of the container. At the junction of the 2 liquids, a brown colour develops.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R*, warming gently, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *1-naphthylacetic acid R* in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *naphazoline impurity A CRS* in the mobile phase and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 2.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (4 µm) with a pore size of 6 nm.

*Mobile phase*: dissolve 1.1 g of *sodium octanesulfonate R* in a mixture of 5 mL of *glacial acetic acid R*, 300 mL of *acetonitrile R* and 700 mL of *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of naphazoline.

*Relative retention* with reference to naphazoline (retention time = about 14 min): impurity A = about 0.76; impurity D = about 1.24; impurity B = about 1.27; impurity C = about 2.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to naphazoline and impurity B.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peak due to the nitrate ion.

**Chlorides** (*2.4.4*): maximum 330 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.33 mg of $C_{14}H_{15}N_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

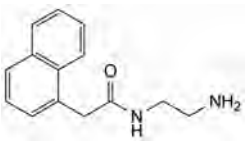


A. *N*-(2-aminoethyl)-2-(naphthalen-1-yl)acetamide (naphthylacetylethylenediamine),

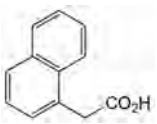


B. (naphthalen-1-yl)acetic acid (1-naphthylacetic acid),

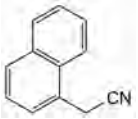


C. (naphthalen-1-yl)acetonitrile (1-naphthylacetonitrile),

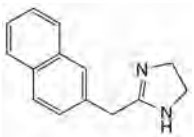

D. 2-(naphthalen-2-ylmethyl)-4,5-dihydro-1*H*-imidazole (β-naphazoline).

**01/2017:0731**

# NAPROXEN

## Naproxenum

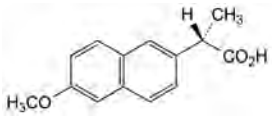

$C_{14}H_{14}O_3$ $M_r$ 230.3
[22204-53-1]

DEFINITION

(2*S*)-2-(6-Methoxynaphthalen-2-yl)propanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *A, B, C.*

A. Specific optical rotation (*2.2.7*): + 59 to + 62 (dried substance).
Dissolve 0.50 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

B. Melting point (*2.2.14*): 154 °C to 158 °C.

C. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).
*Test solution.* Dissolve 40.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *methanol R*.
*Spectral range*: 230-350 nm.
*Absorption maxima*: at 262 nm, 271 nm, 316 nm and 331 nm.
*Specific absorbances at the absorption maxima*:
– at 262 nm: 216 to 238;
– at 271 nm: 219 to 241;
– at 316 nm: 61 to 69;
– at 331 nm: 79 to 87.

D. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *naproxen CRS.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 1.25 g in *methanol R* and dilute to 25 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.5 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *racemic naproxen CRS* in 10.0 mL of *tetrahydrofuran R* and dilute to 100.0 mL with the mobile phase.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel π-acceptor/π-donor for chiral separations R* (5 µm) (*S,S*);
– *temperature*: 25 °C.

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *2-propanol R*, *hexane R* (0.5:5:10:84.5 *V/V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 263 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of naproxen (retention time = about 5 min).

*System suitability*: reference solution (b):
– *resolution*: minimum 3 between the peaks due to impurity G and naproxen.

*Limit*:
– *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Dissolve 12 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 6 mg of *bromomethoxynaphthalene R* (impurity N), 6.0 mg of *naproxen impurity L CRS*, 6 mg of *6-methoxy-2-naphthoic acid R* (impurity O) and 6 mg of *(1RS)-1-(6-methoxynaphthalen-2-yl)ethanol R* (impurity K) in *acetonitrile R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution add 1.0 mL of the test solution and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 50 °C.

*Mobile phase*: mix 42 volumes of *acetonitrile R* and 58 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of impurity N.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities K, L, N and O.

*Relative retention* with reference to naproxen (retention time = about 2.5 min): impurity O = about 0.8; impurity K = about 0.9; impurity L = about 1.4; impurity N = about 5.3.

*System suitability*: reference solution (b):
– *resolution*: minimum 2.2 between the peaks due to impurity K and naproxen.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity O by 2.0;
- *impurity O*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity L*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 25 mL of *water R* and 75 mL of *methanol R*. Titrate with *0.1 M sodium hydroxide*, using 1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 23.03 mg of $C_{14}H_{14}O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: G, L, O.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, H, I, J, K, M, N.*

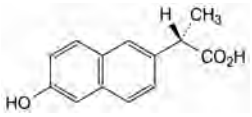

A. (2*S*)-2-(6-hydroxynaphthalen-2-yl)propanoic acid,

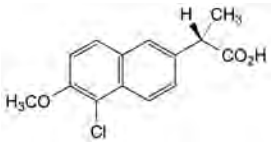

B. (2*S*)-2-(5-chloro-6-methoxynaphthalen-2-yl)propanoic acid,

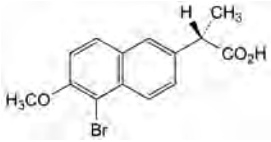

C. (2*S*)-2-(5-bromo-6-methoxynaphthalen-2-yl)propanoic acid,

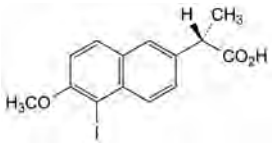

D. (2*S*)-2-(5-iodo-6-methoxynaphthalen-2-yl)propanoic acid,

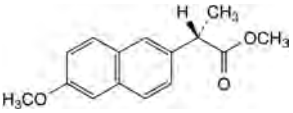

E. methyl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate,

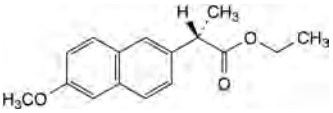

F. ethyl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate,

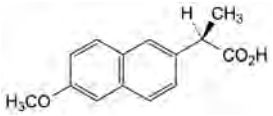

G. (2*R*)-2-(6-methoxynaphthalen-2-yl)propanoic acid ((*R*)-enantiomer),

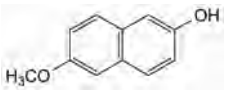

H. 6-methoxynaphthalen-2-ol,

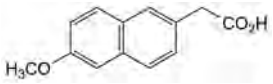

I. (6-methoxynaphthalen-2-yl)acetic acid,

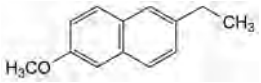

J. 2-ethyl-6-methoxynaphthalene,

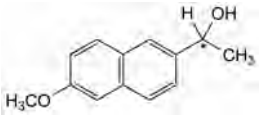

K. (1*RS*)-1-(6-methoxynaphthalen-2-yl)ethanol,

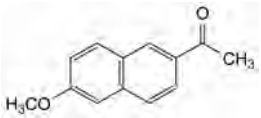

L. 1-(6-methoxynaphthalen-2-yl)ethanone,

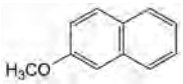

M. 2-methoxynaphthalene (nerolin),

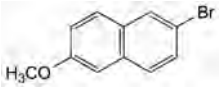

N. 2-bromo-6-methoxynaphthalene,

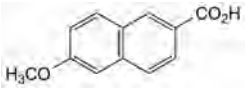

O. 6-methoxynaphthalene-2-carboxylic acid (6-methoxy-2-naphthoic acid).

**01/2017:1702**

# NAPROXEN SODIUM

## Naproxenum natricum

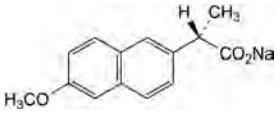


$C_{14}H_{13}O_3Na$ $M_r$ 252.2
[26159-34-2]

DEFINITION

Sodium (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, freely soluble or soluble in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: A, B, D.*

A. Specific optical rotation (*2.2.7*): – 17.0 to – 14.7 (dried substance).

Dissolve 0.50 g in a 4.2 g/L solution of *sodium hydroxide R* and dilute to 25.0 mL with the same solution.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 40.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 262 nm, 271 nm, 316 nm and 331 nm.

*Specific absorbance at the absorption maxima*:

- at 262 nm: 207 to 227;
- at 271 nm: 200 to 220;
- at 316 nm: 56 to 68;
- at 331 nm: 72 to 84.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation.* Dissolve 50 mg in 5 mL of *water R*. Add 1 mL of *dilute sulfuric acid R* and 5 mL of *ethyl acetate R*. Shake vigorously. Allow the 2 layers to separate. Evaporate the upper layer to dryness and subsequently dry at 60 °C for 15 min. Record the spectrum using the residue.

*Comparison*: *naproxen CRS.*

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 1.25 g in *water R* and dilute to 25 mL with the same solvent.

**pH** (*2.2.3*): 7.0 to 9.8.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 15 mL of *water R* and add 1 mL of *hydrochloric acid R*. Shake with 2 quantities, each of 10 mL, of *ethyl acetate R*, combine the upper layers and evaporate to dryness under reduced pressure. Dissolve the residue in 50.0 mL of *tetrahydrofuran R*. Dilute 2.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.5 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *racemic naproxen CRS* in 10 mL of *tetrahydrofuran R* and dilute to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel π-acceptor/π-donor for chiral separations R* (5 µm) (*S,S*);
- *temperature*: 25 °C.

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *2-propanol R*, *hexane R* (5:50:100:845 *V/V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 263 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of naproxen (retention time = about 5 min).

*System suitability*: reference solution (b):

- *resolution*: minimum 3 between the peaks due to impurity G and naproxen.

*Limit*:

- *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Dissolve 12 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 6 mg of *bromomethoxynaphthalene R* (impurity N), 6.0 mg of *naproxen impurity L CRS* and 6 mg of *(1RS)-1-(6-methoxynaphthalen-2-yl)ethanol R* (impurity K) in *acetonitrile R* and dilute to 10 mL with the same solvent. To 1 mL of the solution add 1 mL of the test solution and dilute to 50 mL with the mobile phase. Dilute 1 mL of this solution to 20 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*: mix 42 volumes of *acetonitrile R* and 58 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of impurity N.

*Relative retention* with reference to naproxen (retention time = about 2.5 min): impurity K = about 0.9; impurity L = about 1.4; impurity N = about 5.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.2 between the peaks due to impurity K and naproxen.

*Limits*:

- *impurity L*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.22 mg of $C_{14}H_{13}O_3Na$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: G, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, H, I, J, K, M, N.*

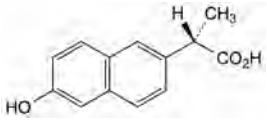


A. (2*S*)-2-(6-hydroxynaphthalen-2-yl)propanoic acid,

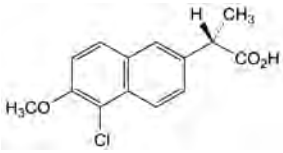


B. (2*S*)-2-(5-chloro-6-methoxynaphthalen-2-yl)propanoic acid,

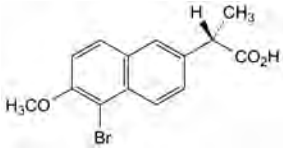


C. (2*S*)-2-(5-bromo-6-methoxynaphthalen-2-yl)propanoic acid,

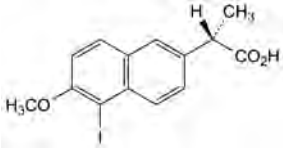


D. (2*S*)-2-(5-iodo-6-methoxynaphthalen-2-yl)propanoic acid,

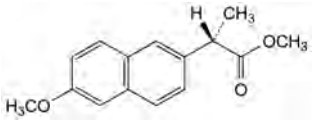


E. methyl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate,

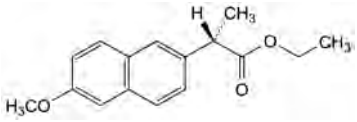


F. ethyl (2*S*)-2-(6-methoxynaphthalen-2-yl)propanoate,

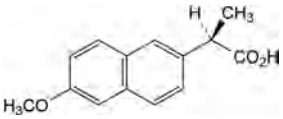


G. (2*R*)-2-(6-methoxynaphthalen-2-yl)propanoic acid,

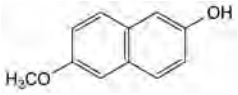


H. 6-methoxynaphthalen-2-ol,

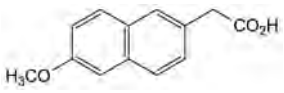


I. (6-methoxynaphthalen-2-yl)acetic acid,

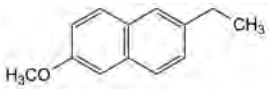


J. 2-ethyl-6-methoxynaphthalene,

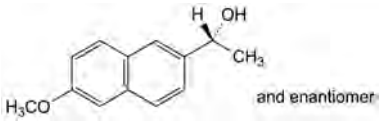


K. (1*RS*)-1-(6-methoxynaphthalen-2-yl)ethanol,

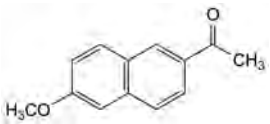


L. 1-(6-methoxynaphthalen-2-yl)ethanone,

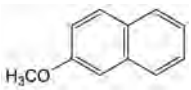


M. 2-methoxynaphthalene (nerolin),

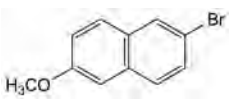


N. 2-bromo-6-methoxynaphthalene.

**01/2017:2575**

# NATEGLINIDE

## Nateglinidum

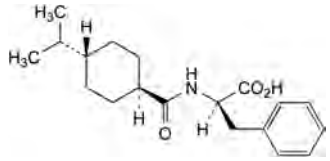

$C_{19}H_{27}NO_3$ $M_r$ 317.4
[105816-04-4]

DEFINITION

*N*-[[*trans*-4-(1-Methylethyl)cyclohexyl]carbonyl]-D-phenylalanine.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): − 40.0 to − 36.5 (dried substance).

Dissolve 0.200 g in a 4 g/L solution of *sodium hydroxide R* and dilute to 20.0 mL with the same solution.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nateglinide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Test B for related substances (see Tests).

TESTS

**Related substances**

A. Impurity A and unspecified impurities. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 60.0 mg of the substance to be examined in 1 mL of *acetonitrile R1* and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 3.0 mg of *nateglinide impurity A CRS* in 1 mL of *acetonitrile R1* and dilute to 25.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 3 mg of the substance to be examined in 1 mL of *acetonitrile R1*, add 4.0 mL of reference solution (a) and dilute to 10 mL with the mobile phase.

*Reference solution (d)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.05 m, Ø = 3.9 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 7.8 g/L solution of *sodium dihydrogen phosphate monohydrate R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 100 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 5 times the retention time of nateglinide.

*Relative retention* with reference to nateglinide (retention time = about 7 min): impurity A = about 0.5.

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to impurity A and nateglinide.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *sum of unspecified impurities*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent).

B. Impurity B. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *methanol R2* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *nateglinide impurity B CRS* in *methanol R2* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 0.10 g of the substance to be examined in *methanol R2*. Add 1.0 mL of reference solution (a) and dilute to 10.0 mL with *methanol R2*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *urea type silica gel for chiral chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: dissolve 0.77 g of *ammonium acetate R* in *methanol R2* and dilute to 1000 mL with the same solvent.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.5 times the retention time of nateglinide.

*Relative retention* with reference to nateglinide (retention time = about 21 min): impurity B = about 0.9.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nateglinide.

*Limit*:

- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

C. Impurities C and D. Liquid chromatography (*2.2.29*).

*Sodium phosphate buffer.* Dissolve 8.5 g of *anhydrous disodium hydrogen phosphate R* in 950 mL of *water R*. Adjust to pH 7.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 25 mL of *methanol R2* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *phenylalanine CRS* (impurity D) and 5 mg of *nateglinide impurity C CRS* in *methanol R2* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 20 mg of the substance to be examined in 10 mL of *methanol R2*, add 1.0 mL of reference solution (a) and dilute to 20.0 mL with sodium phosphate buffer.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 50.0 mg of *nateglinide CRS* in 25 mL of *methanol R2* and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 6.0 mm;
– *stationary phase*: *polymethacrylate gel R* (6 µm);
– *temperature*: 30 °C.

*Mobile phase*: *methanol R2*, sodium phosphate buffer (45:55 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 1.4 times the retention time of nateglinide.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and D.

*Relative retention* with reference to nateglinide (retention time = about 18 min): impurity D = about 0.2; impurity C = about 0.9.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nateglinide.

*Limits*:

– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.2 per cent).

*Limits*:

– *total for impurities A, B, C, D and sum of unspecified impurities*: maximum 0.5 per cent;
– *disregard limit for impurities A, B, C and D*: 0.05 per cent for each impurity.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test C for related substances with the following modification.

*Injection*: test solution and reference solution (e).

Calculate the percentage content of $C_{19}H_{27}NO_3$ taking into account the assigned content of *nateglinide CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G.*

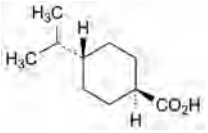

A. *trans*-4-(1-methylethyl)cyclohexanecarboxylic acid,

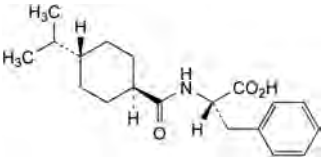

B. *N*-[[*trans*-4-(1-methylethyl)cyclohexyl]carbonyl]-L-phenylalanine (L-phenylalanine isomer),

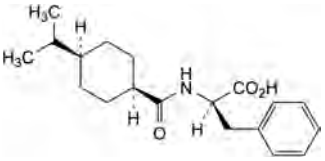

C. *N*-[[*cis*-4-(1-methylethyl)cyclohexyl]carbonyl]-D-phenylalanine (*cis*-isomer),

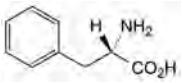

D. (2*S*)-2-amino-3-phenylpropanoic acid (phenylalanine),

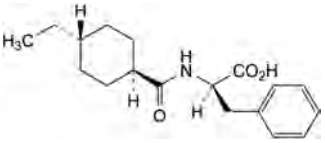

E. *N*-[(*trans*-4-ethylcyclohexyl)carbonyl]-D-phenylalanine,

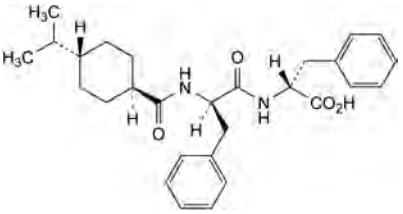

F. *N*-[[*trans*-4-(1-methylethyl)cyclohexyl]carbonyl]-D-phenylalanyl-D-phenylalanine,

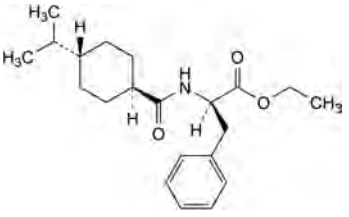

G. ethyl *N*-[[*trans*-4-(1-methylethyl)cyclohexyl]carbonyl]-D-phenylalaninate.

**01/2017:1547**

# NEOHESPERIDIN-DIHYDROCHALCONE

## Neohesperidin-dihydrochalconum

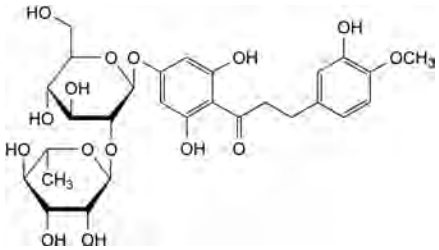

$C_{28}H_{36}O_{15}$ $M_r$ 613
[20702-77-6]

DEFINITION

1-[4-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one.

*Content*: 96.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: practically insoluble in water, freely soluble in dimethyl sulfoxide, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *neohesperidin-dihydrochalcone CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_4$ (*2.2.2, Method II*).

Dissolve 0.25 g in *methanol R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *dimethyl sulfoxide R* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 20.0 mL with *dimethyl sulfoxide R*.

*Reference solution (a).* Dissolve 50.0 mg of *neohesperidin-dihydrochalcone CRS* in *dimethyl sulfoxide R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dissolve 4.0 mg of *neohesperidin-dihydrochalcone impurity B CRS* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *dimethyl sulfoxide R*.

*Reference solution (d).* In order to prepare *in situ* impurity F and impurity G, suspend 0.10 g of the substance to be examined in 10.0 mL of a 100 g/L solution of *sulfuric acid R*. Heat the sample for 5 min on a water-bath. Dilute immediately 1.0 mL of the resulting solution to 50.0 mL with *dimethyl sulfoxide R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (4 µm) with a carbon loading of 7 per cent,
- *temperature*: 30 °C.

*Mobile phase*: mix 20 volumes of *acetonitrile R* and 80 volumes of a solution prepared by adding 5.0 mL of *glacial acetic acid R* to 1000.0 mL of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 10 µL; inject test solution (a) and reference solutions (a), (b), (c) and (d).

*Run time*: 5 times the retention time of neohesperidin-dihydrochalcone which is about 10 min.

*Relative retention* with reference to neohesperidin-dihydrochalcone: impurity B = about 0.4; impurity D = about 0.7; impurity F = about 1.2; impurity G = about 3.7.

*System suitability*:
- *resolution*: minimum of 2.5 between the first peak (neohesperidin-dihydrochalcone) and the second peak (impurity F) in the chromatogram obtained with reference solution (d),
- chromatogram obtained with reference solution (a) is similar to the chromatogram provided with *neohesperidin-dihydrochalcone CRS*.

*Limits*:
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (2 per cent),
- *any other impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent),
- *total of all impurities apart from impurity B*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (2.5 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water**(*2.5.12*): maximum 12.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: 10 µL; inject test solution (b) and reference solutions (a) and (d).

*System suitability*:
- *resolution*: minimum of 2.5 between the first peak (neohesperidin-dihydrochalcone) and the second peak (impurity F) in the chromatogram obtained with reference solution (d),
- *repeatability*: reference solution (a).

Calculate the percentage content of $C_{28}H_{36}O_{15}$ using the chromatogram obtained with reference solution (a) and the stated content of $C_{28}H_{36}O_{15}$ in *neohesperidin-dihydrochalcone CRS*, correcting for the water content of the substance to be examined.

STORAGE

Protected from light.

IMPURITIES

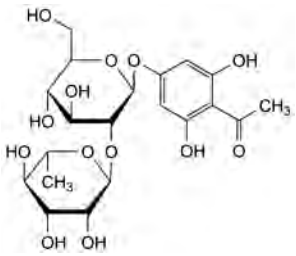

A. 1-[4-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]ethanone (phloroacetophenone neohesperidoside),

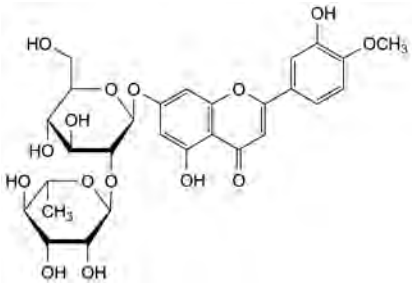

B. 7-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one (neodiosmin),

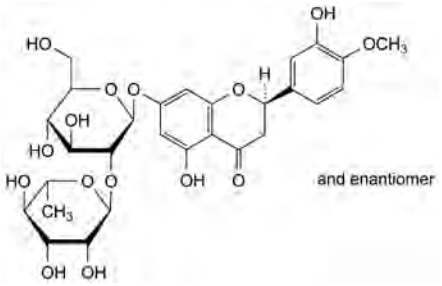


C. (2*RS*)-7-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one (neohesperidin),

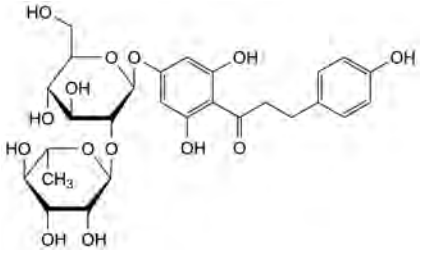

D. 1-[4-[[2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)propan-1-one (naringin-dihydrochalcone),

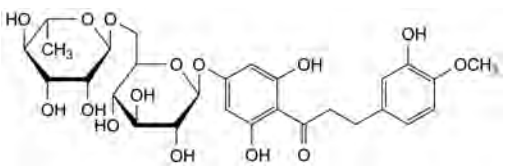

E. 1-[4-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperidin-dihydrochalcone),

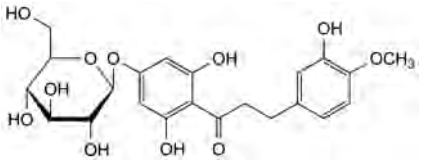

F. 1-[4-(β-D-glucopyranosyloxy)-2,6-dihydroxyphenyl]-3-(3-hydroxy-4-methoxyphenyl)propan-1-one (hesperetin-dihydrochalcone 7′-glucoside),

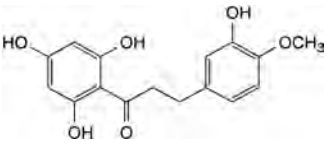

G. 3-(3-hydroxy-4-methoxyphenyl)-1-(2,4,6-trihydroxyphenyl)propan-1-one (hesperetin-dihydrochalcone).

**01/2008:0197**
**corrected 10.0**

# NEOMYCIN SULFATE

## Neomycini sulfas

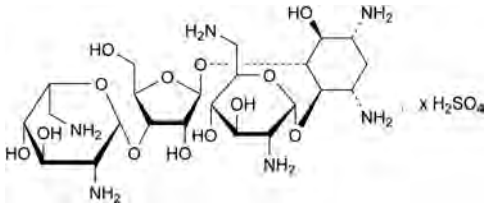


$C_{23}H_{46}N_6O_{13},xH_2SO_4$ $M_r$ 615 (base)

DEFINITION

Mixture of sulfates of substances produced by the growth of certain selected strains of *Streptomyces fradiae*, the main component being the sulfate of 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B).

*Content*: minimum of 680 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: very soluble in water, very slightly soluble in alcohol, practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

*Results*:

- the retention time of the principal peak in the chromatogram obtained with the test solution is approximately the same as that of the principal peak in the chromatogram obtained with reference solution (e),
- it complies with the limits given for impurity C.

B. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 7.5.

Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 53.5 to + 59.0 (dried substance).

Dissolve 1.00 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *framycetin sulfate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (d).* Dissolve the contents of a vial of *neamine CRS* (corresponding to 0.5 mg) in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 10 mg of *neomycin sulfate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 25 °C.

*Mobile phase*: mix 20.0 mL of *trifluoroacetic acid R*, 6.0 mL of *carbonate-free sodium hydroxide solution R* and 500 mL of *water R*, allow to equilibrate, dilute to 1000 mL with *water R* and degas.

*Flow rate*: 0.7 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted 1 in 25 previously degassed, which is added pulse-less to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate*: 0.5 mL/min.

*Detection*: pulsed amperometric detector with a gold indicator electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively 0.00 V detection, + 0.80 V oxidation and − 0.60 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 10 µL; inject the test solution and the reference solutions (b), (c), (d) and (e).

*Run time*: 1.5 times the retention time of neomycin B.

*Relative retention* with reference to neomycin B (retention time = about 10 min): impurity A = about 0.65; impurity C = about 0.9; impurity G = about 1.1.

*System suitability*:

- *resolution*: minimum of 2.0 between the peaks due to impurity C and to neomycin B in the chromatogram obtained with reference solution (e); if necessary, adjust the volume of the carbonate-free sodium hydroxide solution in the mobile phase,
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (2.0 per cent),
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (15.0 per cent) and not less than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent),
- *total of other impurities*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (15.0 per cent),
- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent).

**Sulfate**: 27.0 per cent to 31.0 per cent (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *alcohol R* when the colour of the solution begins to change, continuing the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light.

IMPURITIES

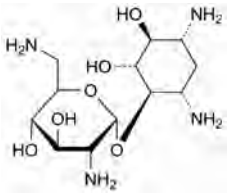

A. 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (neamine or neomycin A-LP),

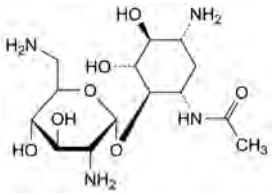

B. 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (3-acetylneamine),

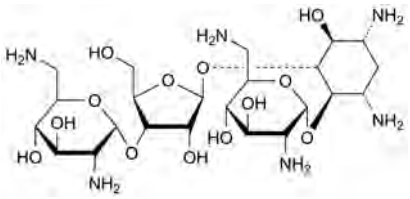

C. 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin C),

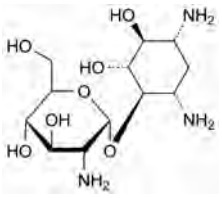

D. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-D-streptamine (paromamine or neomycin D),

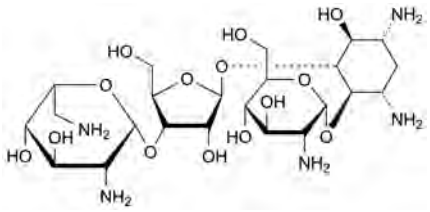

E. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin I or neomycin E),

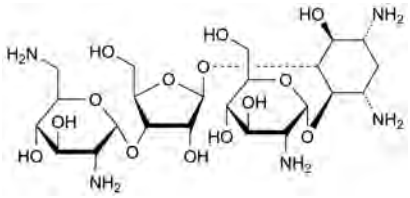

F. 4-*O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-2-deoxy-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-β-D-ribofuranosyl]-D-streptamine (paromomycin II or neomycin F),

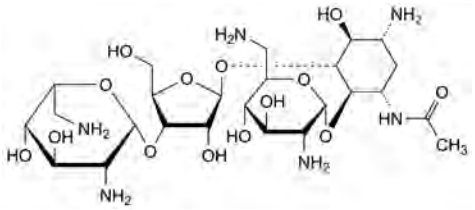

G. 3-*N*-acetyl-2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-5-*O*-[3-*O*-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B-LP).

**01/2015:0046**
**corrected 10.0**

# NEOSTIGMINE BROMIDE

## Neostigmini bromidum

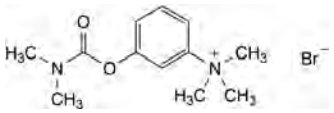

$C_{12}H_{19}BrN_2O_2$ $M_r$ 303.2
[114-80-7]

DEFINITION

3-[(Dimethylcarbamoyl)oxy]-*N*,*N*,*N*-trimethylanilinium bromide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20 mg in *0.5 M sulfuric acid* and dilute to 100 mL with the same acid.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 260 nm and 266 nm.

*Specific absorbance at the absorption maxima*:

– at 260 nm: about 16;
– at 266 nm: about 14.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *neostigmine bromide CRS.*

C. To 50 mg add 0.4 g of *potassium hydroxide R* and 2 mL of *ethanol (96 per cent) R* and heat on a water-bath for 3 min, replacing the evaporated ethanol (96 per cent). Cool and add 2 mL of *water R* and 2 mL of *diazobenzenesulfonic acid solution R1.* An orange-red colour develops.

D. It gives the reactions of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *3-dimethylaminophenol R* (impurity B) in 50.0 mL of the mobile phase. Dilute 1.0 mL of the solution to 200.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *neostigmine impurity A CRS* in 1.0 mL of reference solution (b).

*Reference solution (d).* Mix 1.0 mL of the mobile phase and 1.0 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: to 710 mL of a 3.6 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 3.2 with *phosphoric acid R*, add 4.3 g of *sodium dodecyl sulfate R* and 290 mL of *acetonitrile R1.*

*Flow rate*: 1.6 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL of the test solution and reference solutions (a), (c) and (d).

*Run time*: twice the retention time of neostigmine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to neostigmine (retention time = about 20 min): impurity B = about 0.56; impurity A = about 0.61.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurities B and A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 25 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- for each impurity, use the concentration of neostigmine bromide in reference solution (a);
- *correction factor*: multiply the peak area of impurity B by 0.5.

*Limits*:

- *impurity B*: maximum 0.01 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to impurity B.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.225 g in 2 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.32 mg of $C_{12}H_{19}BrN_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

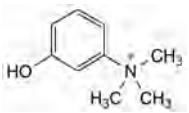

A. 3-hydroxy-*N*,*N*,*N*-trimethylanilinium,

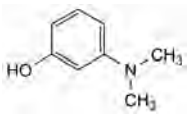

B. 3-(dimethylamino)phenol,

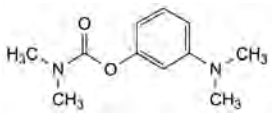

C. 3-(dimethylamino)phenyl dimethylcarbamate.

**07/2018:0626**
**corrected 10.0**

# NEOSTIGMINE METILSULFATE

## Neostigmini metilsulfas

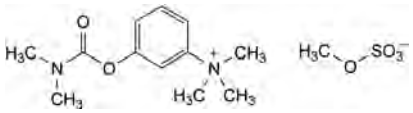

$C_{13}H_{22}N_2O_6S$ $M_r$ 334.4
[51-60-5]

DEFINITION

3-[(Dimethylcarbamoyl)oxy]-*N*,*N*,*N*-trimethylanilinium methyl sulfate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 144 °C to 149 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *0.5 M sulfuric acid* and dilute to 100 mL with the same acid.

*Spectral range*: 230-350 nm.

*Absorption maxima*: 261 nm and 267 nm.

*Resolution* (*2.2.25*): minimum 1.9 for the absorbance ratio.

*Absorbance ratio*: $A_{267}/A_{261}$ = 0.84 to 0.87.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *neostigmine metilsulfate CRS.*

D. To 50 mg add 0.4 g of *potassium hydroxide R* and 2 mL of *ethanol (96 per cent) R* and heat on a water-bath for 3 min, replacing the evaporated ethanol (96 per cent). Cool and add 2 mL of *water R* and 2 mL of *diazobenzenesulfonic acid solution R1.* An orange-red colour develops.

E. Dissolve 0.1 g in 5 mL of *distilled water R* and add 1 mL of *barium chloride solution R1.* No precipitate is formed. Add 2 mL of *hydrochloric acid R* and heat in a water-bath for 10 min. A fine, white precipitate is formed.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 4.0 mL of solution S add 6.0 mL of *water R* and 0.1 mL of *phenolphthalein solution R1.* The solution is colourless. Add 0.3 mL of *0.01 M sodium hydroxide*; the solution becomes red. Add 0.4 mL of *0.01 M hydrochloric acid*; the solution becomes colourless. Add 0.1 mL of *methyl red solution R*; the solution becomes red or yellowish-red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *3-dimethylaminophenol R* (impurity B) in 50.0 mL of the mobile phase. Dilute 1.0 mL of the solution to 200.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *neostigmine impurity A CRS* in 1.0 mL of reference solution (b).

*Reference solution (d).* Mix 1.0 mL of the mobile phase and 1.0 mL of reference solution (a).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: to 710 mL of a 3.6 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 3.2 with *phosphoric acid R*, add 4.3 g of *sodium dodecyl sulfate R* and 290 mL of *acetonitrile for chromatography R*.

*Flow rate*: 1.6 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL of the test solution and reference solutions (a), (c) and (d).

*Run time*: twice the retention time of neostigmine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to neostigmine (retention time = about 20 min): impurity B = about 0.56; impurity A = about 0.61.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to impurities B and A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 25 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity B by 0.5;
- for each impurity, use the concentration of neostigmine metilsulfate in reference solution (a).

*Limits*:
- *impurity B*: maximum 0.01 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent, except for impurity B.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 150 mL of *water R* and add 100 mL of *dilute sodium hydroxide solution R*. Distil, collecting the distillate in 40 mL of a 40 g/L solution of *boric acid R* until the total volume in the collecting vessel is about 250 mL. Titrate the solution in the collecting vessel with *0.1 M hydrochloric acid*, using 0.25 mL of *methyl red mixed solution R* as indicator. Carry out a blank test.

1 mL of *0.1 M hydrochloric acid* is equivalent to 33.44 mg of $C_{13}H_{22}N_2O_6S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

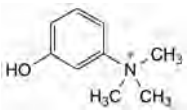

A. 3-hydroxy-*N*,*N*,*N*-trimethylanilinium,

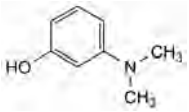

B. 3-(dimethylamino)phenol,

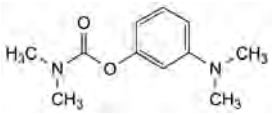

C. 3-(dimethylamino)phenyl dimethylcarbamate.

**07/2017:1351**
**corrected 10.0**

# NETILMICIN SULFATE

## Netilmicini sulfas

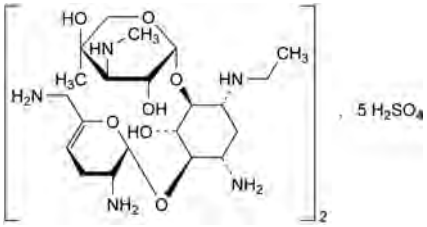

$C_{42}H_{92}N_{10}O_{34}S_5$ $M_r$ 1442

[56391-57-2]

DEFINITION

Bis[2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-4-*O*-(2,6-diamino-2,3,4,6-tetradeoxy-α-D-*glycero*-hex-4-enopyranosyl)-1-*N*-ethyl-D-streptamine] pentasulfate.

Substance obtained by synthesis from sisomicin.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, very hygroscopic powder.

*Solubility*: very soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.80 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 400 nm is not greater than 0.08.

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Perform weighing steps as quickly as possible and immediately after opening the sample container.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve the contents of a vial of *netilmicin sulfate for LC assay CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 25.0 mg of *sisomicin sulfate CRS* (impurity A) in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25.0 mg of *1-N-ethylgaramine sulfate CRS* (impurity B) in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (d).* Dilute 0.6 mL of reference solution (a), 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c) to 50.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 5.0 mg of *netilmicin for peak identification CRS* (containing impurities E and F) in the mobile phase and dilute to 2.5 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*: solution in *carbon dioxide-free water R* containing 0.3 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 20 mL/L of *tetrahydrofuran R* and 50 mL/L of *0.2 M potassium dihydrogen phosphate R*, adjusted to pH 3.0 with a 22.5 g/L solution of *phosphoric acid R*; degas.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: mixture of 1 volume of *carbonate-free sodium hydroxide solution R* and 24 volumes of previously degassed *carbon dioxide-free water R*, which is added pulse-free to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate of post-column solution*: 0.3 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold indicator electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and – 0.15 V reduction potentials, with pulse durations according to the instrument used.

*Injection*: 20 µL of the test solution and reference solutions (d) and (e).

*Run time*: 3 times the retention time of netilmicin.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A and B; use the chromatogram supplied with *netilmicin for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities E and F.

*Relative retention* with reference to netilmicin (retention time = about 16 min): impurity B = about 0.4; impurity A = about 0.7; impurity E = about 1.9; impurity F = about 2.1.

*System suitability*: reference solution (d):

- *resolution*: minimum 10.0 between the peaks due to impurities B and A; minimum 6.0 between the peaks due to impurity A and netilmicin; if necessary, adjust the concentration of sodium octanesulfonate in the mobile phase.

*Calculation of percentage contents*:

- for impurities A and B, use the concentration of each impurity in reference solution (d);
- for impurities other than A and B, use the concentration of netilmicin sulfate in reference solution (d).

*Limits*:

- *impurities A, B, E, F*: for each impurity, maximum 1.0 per cent;
- *any other impurity*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 3.0 per cent;
- *reporting threshold*: 0.1 per cent.

**Sulfate**: 31.5 per cent to 35.0 per cent (dried substance).

Dissolve 0.12 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 30.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 0.500 g by drying at 110 °C under high vacuum for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 0.5 g.

ASSAY

Liquid chromatography (*2.2.29*). *Perform weighing steps as quickly as possible and immediately after opening the sample container.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution.* Dissolve the contents of a vial of *netilmicin sulfate for LC assay CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*: solution in *carbon dioxide-free water R* containing 0.15 g/L of *sodium octanesulfonate R*, 20 g/L of *anhydrous sodium sulfate R1*, 20 mL/L of *2-methyl-2-propanol R* and 50 mL/L of *0.2 M potassium dihydrogen phosphate R*, adjusted to pH 3.0 with *dilute phosphoric acid R*; degas.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL.

*Run time*: 1.3 times the retention time of netilmicin.

*Retention time*: netilmicin = about 4 min.

Calculate the percentage content of $C_{42}H_{92}N_{10}O_{34}S_5$ taking into account the assigned content of *netilmicin sulfate for LC assay CRS*.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities*: *A, B, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

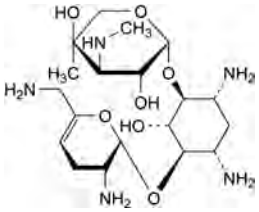

A. 2-deoxy-4-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-6-*O*-(2,6-diamino-2,3,4,6-tetradeoxy-α-D-*glycero*-hex-4-enopyranosyl)-L-streptamine (sisomicin),

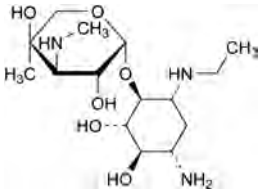

B. 2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (1-*N*-ethylgaramine),

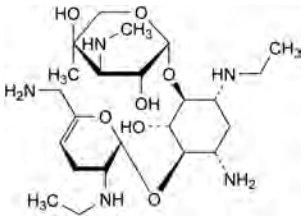

C. 4-*O*-[6-amino-2,3,4,6-tetradeoxy-2-(ethylamino)-α-D-*glycero*-hex-4-enopyranosyl]-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (2′-*N*-ethylnetilmicin),

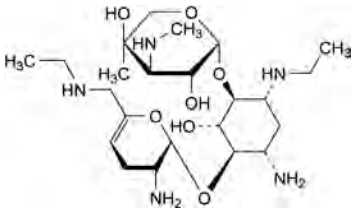

D. 4-*O*-[2-amino-2,3,4,6-tetradeoxy-6-(ethylamino)-α-D-*glycero*-hex-4-enopyranosyl]-2-deoxy-6-*O*-[3-deoxy-4-*C*-methyl-3-(methylamino)-β-L-arabinopyranosyl]-1-*N*-ethyl-D-streptamine (6′-*N*-ethylnetilmicin),

E. unknown structure,

F. unknown structure.

**01/2017:2255**
**corrected 9.3**

# NEVIRAPINE

## Nevirapinum

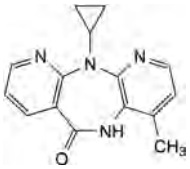

$C_{15}H_{14}N_4O$ $M_r$ 266.3
[129618-40-2]

DEFINITION

11-Cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, sparingly soluble or slightly soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *anhydrous nevirapine CRS.*

B. Loss on drying (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 24.0 mg of the substance to be examined in a mixture of 4 mL of *acetonitrile R* and 80 mL of the mobile phase and sonicate until dissolution is complete. Dilute to 100.0 mL with the mobile phase.

*Test solution (b).* Dilute 3.0 mL of test solution (a) to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Add 2.0 mL of the mobile phase to a vial of *nevirapine for peak identification CRS* (containing impurities A, B and C), mix and sonicate for 1 min.

*Reference solution (c).* Dissolve 24.0 mg of *anhydrous nevirapine CRS* in a mixture of 4 mL of *acetonitrile R* and 80 mL of the mobile phase and sonicate until complete dissolution. Dilute to 100.0 mL with the mobile phase. Dilute 3.0 mL of this solution to 25.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 2.88 g/L solution of *ammonium dihydrogen phosphate R*, previously adjusted to pH 5.0 using *dilute sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 10 times the retention time of nevirapine.

*Identification of impurities*: use the chromatogram supplied with *nevirapine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to nevirapine (retention time = about 8 min): impurity B = 0.7; impurity A = 1.5; impurity C = 2.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 5 between the peaks due to impurity B and nevirapine.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 25 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{15}H_{14}N_4O$ from the declared content of *anhydrous nevirapine CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

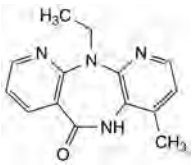

A. 11-ethyl-4-methyl-5,11-dihydro-6*H*-dipyrido-[3,2-b:2′,3′-*e*][1,4]diazepin-6-one,

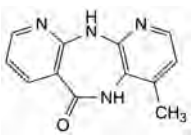

B. 4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-b:2′,3′-*e*]-[1,4]diazepin-6-one,

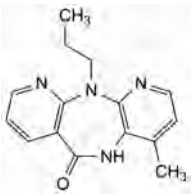

C. 4-methyl-11-propyl-5,11-dihydro-6*H*-dipyrido[3,2-b:2′,3′-*e*][1,4]diazepin-6-one.

**01/2017:2479**
**corrected 10.0**

# NEVIRAPINE HEMIHYDRATE

## Nevirapinum hemihydricum

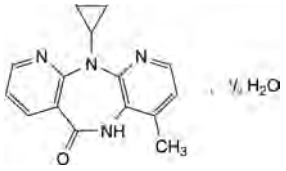

$C_{15}H_{14}N_4O,½H_2O$ $M_r$ 275.3
[220988-26-1]

DEFINITION

11-Cyclopropyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one hemihydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, slightly soluble in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nevirapine hemihydrate CRS*.

B. Water (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *methanol R* and sonicate until dissolution is complete. Dilute to 50.0 mL with *methanol R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Add 1 mL of *methanol R* to a vial of *nevirapine for peak identification CRS* (containing impurities A, B and C), mix and sonicate for 1 min.

*Reference solution (c).* Dissolve 20.0 mg of *anhydrous nevirapine CRS* in *methanol R* and sonicate until dissolution is complete. Dilute to 50.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 50 mm, Ø = 2.1 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (1.8 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 0.77 g of *ammonium acetate R* in 900 mL of *water for chromatography R*, adjust to pH 5.6 with *acetic acid R* and dilute to 1000 mL with *water for chromatography R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 – 1.35 | 90 | 10 |
| 1.35 – 3.85 | 90 → 67 | 10 → 33 |
| 3.85 – 6.70 | 67 → 60 | 33 → 40 |
| 6.70 – 7.65 | 60 | 40 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 2.0 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *nevirapine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to nevirapine (retention time = about 3 min): impurity B = about 0.9; impurity A = about 1.2; impurity C = about 1.3.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to impurity B and nevirapine and minimum 5.0 between the peaks due to nevirapine and impurity A in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.7 for the peak due to nevirapine in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

- for each impurity, use the concentration of nevirapine hemihydrate in reference solution (a).

*Limits*:

- *impurities A, B, C*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.1 per cent to 3.9 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 2.0 µL of the test solution and reference solution (c).

Calculate the percentage content of $C_{15}H_{14}N_4O$ taking into account the assigned content of *anhydrous nevirapine CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

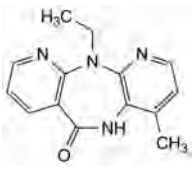

A. 11-ethyl-4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one,

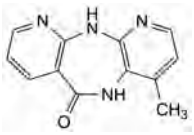

B. 4-methyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one,

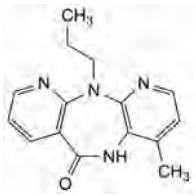

C. 4-methyl-11-propyl-5,11-dihydro-6*H*-dipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepin-6-one,

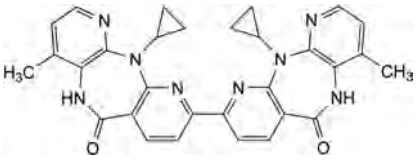

D. 11,11′-dicyclopropyl-4,4′-dimethyl-5,5′,11,11′-tetrahydro-6*H*,6′*H*-9,9′-bidipyrido[3,2-*b*:2′,3′-*e*][1,4]diazepine-6,6′-dione.

**01/2018:2776**
**corrected 10.0**

# NICARDIPINE HYDROCHLORIDE

## Nicardipini hydrochloridum

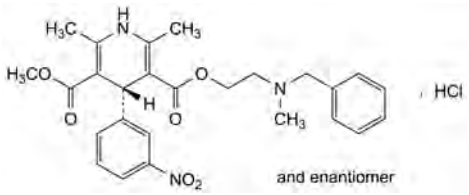


$C_{26}H_{30}ClN_3O_6$ $M_r$ 516.0
[54527-84-3]

DEFINITION

2-[Benzyl(methyl)amino]ethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow or pale greenish-yellow, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nicardipine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve 30 mg in 10 mL of *water R* and shake vigorously. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *nicardipine for system suitability CRS* (containing impurities A, B and C) in 2 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.5 g/L solution of *perchloric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of nicardipine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to nicardipine (retention time = about 8 min): impurity B = about 0.5; impurity A = about 0.8; impurity C = about 2.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity A and nicardipine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of nicardipine hydrochloride in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.5 per cent;
- *impurities A, C*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 51.60 mg of $C_{26}H_{30}ClN_3O_6$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

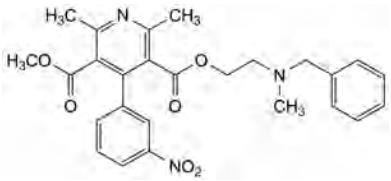

A. 2-[benzyl(methyl)amino]ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate,

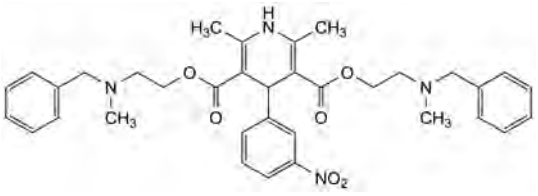

B. bis[2-[benzyl(methyl)amino]ethyl] 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

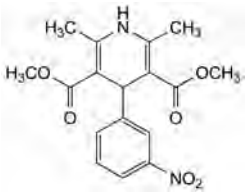

C. dimethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

**04/2019:1998**

# NICERGOLINE

## Nicergolinum

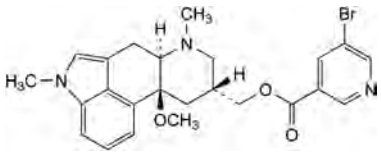

$C_{24}H_{26}BrN_3O_3$ $M_r$ 484.4

[27848-84-6]

DEFINITION

[(6a*R*,9*R*,10a*S*)-10a-Methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: fine to granular, white or yellowish powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Specific optical rotation (*2.2.7*): + 4.8 to + 5.8 (anhydrous substance).

Dissolve 0.50 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *ethanol (96 per cent) R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 288 nm.

*Absorption minimum*: at 251 nm.

*Specific absorbance at the absorption maximum*: 175 to 185 (anhydrous substance).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nicergoline CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

D. Dissolve 2 mg in 2 mL of *sulfuric acid R*. A blue colour develops.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 0.5 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 2.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 2 mg of *nicergoline for system suitability CRS* (containing impurities A, B, C, D, F and H) in *acetonitrile R* and dilute to 2 mL with the same solvent.

*Reference solution (c).* Dissolve 5.0 mg of *nicergoline impurity D CRS* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 50.0 mL with *acetonitrile R*.

*Reference solution (d).* Dissolve the contents of a vial of *nicergoline for peak identification CRS* (containing impurity I) in 1 mL of *acetonitrile R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *solution A*: dissolve 34.02 g of *potassium dihydrogen phosphate R* in 930 mL of *water for chromatography R* and dilute to 1000 mL with *water for chromatography R* (buffer solution); dissolve 21.21 g of *tetrabutylammonium hydrogen sulfate R* in 225 mL of the buffer solution and dilute to 250.0 mL with the same solution; adjust to pH 7.5 with a 300 g/L solution of *potassium hydroxide R*;
- *mobile phase A*: mix 2.0 mL of solution A with 300 mL of *acetonitrile R* and 700 mL of *water for chromatography R*;
- *mobile phase B*: mix 2.0 mL of solution A with 300 mL of *water for chromatography R* and 700 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 30 | 100 → 70 | 0 → 30 |
| 30 - 40 | 70 → 0 | 30 → 100 |
| 40 - 50 | 0 | 100 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *nicergoline for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, F and H; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity I.

*Relative retention* with reference to nicergoline (retention time = about 34 min): impurity D = about 0.06; impurity C = about 0.1; impurity B = about 0.6; impurity H = about 0.8; impurity A = about 0.96; impurity F = about 1.1; impurity I = about 1.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity A and nicergoline.

*Calculation of percentage contents*:

- for impurity D, use the concentration of impurity D in reference solution (c);
- for impurities other than D, use the concentration of nicergoline in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.8 per cent;
- *impurity A*: maximum 0.5 per cent;
- *impurity H*: maximum 0.3 per cent;
- *impurities C, D, F, I*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *acetone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Titrate to the 1st point of inflexion.

1 mL of *0.1 M perchloric acid* is equivalent to 48.44 mg of $C_{24}H_{26}BrN_3O_3$.

IMPURITIES

*Specified impurities: A, B, C, D, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, G, J.*

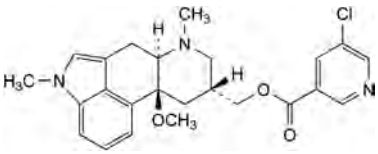


A. [(6a*R*,9*R*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-chloropyridine-3-carboxylate (chloronicergoline),

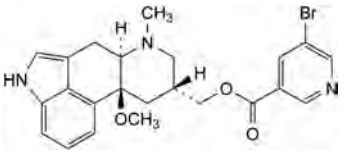


B. [(6a*R*,9*R*,10a*S*)-10a-methoxy-7-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate (1-desmethylnicergoline),

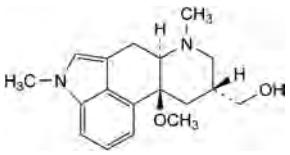

C. [(6a*R*,9*R*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methanol,

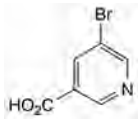

D. 5-bromopyridine-3-carboxylic acid,

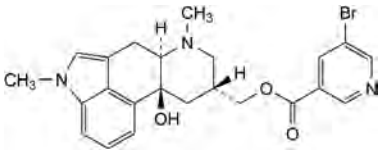

E. [(6a*R*,9*R*,10a*S*)-10a-hydroxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate,

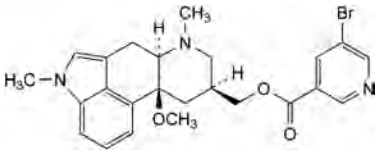

F. [(6a*R*,9*S*,10a*S*)-10a-methoxy-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate (isonicergoline),

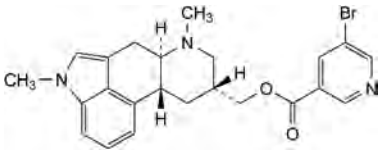

G. [(6a*R*,9*R*,10a*R*)-4,7-dimethyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate,

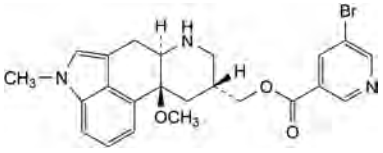

H. [(6a*R*,9*R*,10a*S*)-10a-methoxy-4-methyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinolin-9-yl]methyl 5-bromopyridine-3-carboxylate (6-desmethylnicergoline),

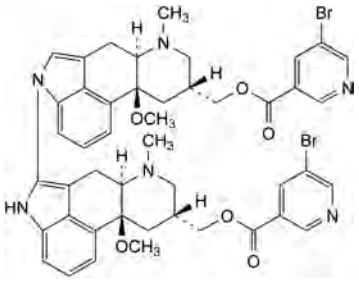

I. [(6a*R*,6a′*R*,9*R*,9′*R*,10a*S*,10a′*S*)-9′-[[[(5-bromopyridin-3-yl)carbonyl]oxy]methyl]-10a,10a′-dimethoxy-7,7′-dimethyl-4′,6′,6a,6a′,7,7′,8,8′,9,9′,10,10′,10a,10a′-tetradecahydro-6*H*-4,5′-biindolo[4,3-*fg*]quinoline-9-yl]methyl 5-bromopyridine-3-carboxylate,

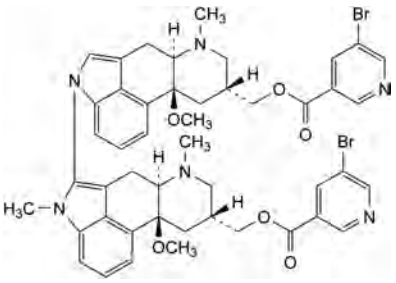

J. [(6a*R*,6a′*R*,9*R*,9′*R*,10a*S*,10a′*S*)-9′-[[[(5-bromopyridin-3-yl)carbonyl]oxy]methyl]-10a,10a′-dimethoxy-4′,7,7′-trimethyl-4′,6′,6a,6a′,7,7′,8,8′,9,9′,10,10′,10a,10a′-tetradecahydro-6*H*-4,5′-biindolo[4,3-*fg*]quinoline-9-yl]methyl 5-bromopyridine-3-carboxylate.

**01/2017:0679**

# NICLOSAMIDE

## Niclosamidum

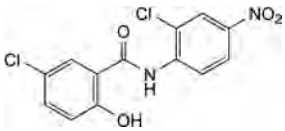

$C_{13}H_8Cl_2N_2O_4$ $M_r$ 327.1
[50-65-7]

DEFINITION

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish-white or yellowish, fine crystals.

*Solubility*: practically insoluble in water, sparingly soluble in acetone, slightly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Melting point (*2.2.14*): 227 °C to 232 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs prepared using about 0.5 mg of substance and 0.3 g of *potassium bromide R*.
*Comparison*: *anhydrous niclosamide CRS*.

C. To 50 mg add 5 mL of *1 M hydrochloric acid* and 0.1 g of *zinc powder R*, heat in a water-bath for 10 min, cool and filter. To the filtrate add 1 mL of a 5 g/L solution of *sodium nitrite R* and allow to stand for 3 min; add 2 mL of a 20 g/L solution of *ammonium sulfamate R*, shake, allow to stand for 3 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. A violet colour is produced.

D. Heat the substance on a copper wire in a non-luminous flame. The flame becomes green.

E. Loss on drying (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in *methanol R*, heating gently, cool and dilute to 50.0 mL with the same solvent.

*Reference solution*. Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 20.0 mL with *acetonitrile R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mixture of equal volumes of *acetonitrile R* and a solution containing 2 g/L of *potassium dihydrogen phosphate R*, 1 g/L of *disodium hydrogen phosphate dodecahydrate R* and 2 g/L of *tetrabutylammonium hydrogen sulfate R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of niclosamide.

*Limits*:
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.005 per cent).

**5-Chlorosalicylic acid**: maximum 60 ppm.

*Test solution.* To 1.0 g add 15 mL of *water R*, boil for 2 min, cool, filter through a membrane filter (nominal pore size 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20.0 mL with *water R*.

*Reference solution.* Dissolve 30 mg of *5-chlorosalicylic acid R* in 20 mL of *methanol R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

To 10.0 mL of the test solution and to 10.0 mL of the reference solution add separately 0.1 mL of *ferric chloride solution R2*. Any violet colour in the test solution is not more intense than that in the reference solution.

**2-Chloro-4-nitroaniline**: maximum 100 ppm.

*Test solution.* To 0.250 g add 5 mL of *methanol R*, heat to boiling, cool, add 45 mL of *1 M hydrochloric acid*, heat again to boiling, cool, filter and dilute the filtrate to 50.0 mL with *1 M hydrochloric acid*.

*Reference solution.* Dissolve 50 mg of *2-chloro-4-nitroaniline R* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 20.0 mL with *1 M hydrochloric acid*.

To 10.0 mL of the test solution and to 10.0 mL of the reference solution add separately 0.5 mL of a 5 g/L solution of *sodium nitrite R* and allow to stand for 3 min. Add 1 mL of a 20 g/L solution of *ammonium sulfamate R*, shake, allow to stand for 3 min and add 1 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. Any pinkish-violet colour in the test solution is not more intense than that in the reference solution.

**Chlorides** (*2.4.4*): maximum 500 ppm.

To 2 g add a mixture of 1.2 mL of *acetic acid R* and 40 mL of *water R*, boil for 2 min, cool and filter. Dilute 2 mL of the filtrate to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 80 mL of a mixture of equal volumes of *acetone R* and *methanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 32.71 mg of $C_{13}H_8Cl_2N_2O_4$.

STORAGE

In an airtight container, protected from light.

**01/2008:0680**
**corrected 6.0**

# NICLOSAMIDE MONOHYDRATE

## Niclosamidum monohydricum

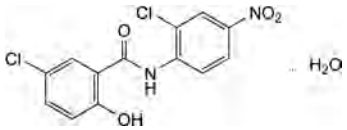


$C_{13}H_8Cl_2N_2O_4,H_2O$ $M_r$ 345.1

DEFINITION

5-Chloro-*N*-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide monohydrate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish, fine crystals.

*Solubility*: practically insoluble in water, sparingly soluble in acetone, slightly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 227 °C to 232 °C, determined after drying at 100-105 °C for 4 h.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: dry the substance to be examined at 100-105 °C for 4 h and examine as discs prepared using about 0.5 mg of substance and 0.3 g of *potassium bromide R*.
*Comparison*: *anhydrous niclosamide CRS*.

C. To 50 mg add 5 mL of *1 M hydrochloric acid* and 0.1 g of *zinc powder R*, heat in a water-bath for 10 min, cool and filter. To the filtrate add 1 mL of a 5 g/L solution of *sodium nitrite R* and allow to stand for 3 min; add 2 mL of a 20 g/L solution of *ammonium sulfamate R*, shake, allow to stand for 3 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. A violet colour is produced.

D. Heat the substance on a copper wire in a non-luminous flame. The flame becomes green.

E. Loss on drying (see Tests).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R*, heating gently, cool and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 20.0 mL with *acetonitrile R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mixture of equal volumes of *acetonitrile R* and a solution containing 2 g/L of *potassium dihydrogen phosphate R*, 1 g/L of *disodium hydrogen phosphate dodecahydrate R* and 2 g/L of *tetrabutylammonium hydrogen sulfate R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of niclosamide.

*Limits*:

- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with the reference solution (0.005 per cent).

**5-Chlorosalicylic acid**: maximum 60 ppm.

*Test solution.* To 1.0 g add 15 mL of *water R*, boil for 2 min, cool, filter through a membrane filter (nominal pore size 0.45 µm), wash the filter and dilute the combined filtrate and washings to 20.0 mL with *water R*.

*Reference solution.* Dissolve 30 mg of *5-chlorosalicylic acid R* in 20 mL of *methanol R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

To 10.0 mL of the test solution and to 10.0 mL of the reference solution add separately 0.1 mL of *ferric chloride solution R2*. Any violet colour produced in the test solution is not more intense than that in the reference solution.

**2-Chloro-4-nitroaniline**: maximum 100 ppm.

*Test solution.* To 0.250 g add 5 mL of *methanol R*, heat to boiling, cool, add 45 mL of *1 M hydrochloric acid*, heat again to boiling, cool, filter and dilute the filtrate to 50.0 mL with *1 M hydrochloric acid*.

*Reference solution.* Dissolve 50 mg of *2-chloro-4-nitroaniline R* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 20.0 mL with *1 M hydrochloric acid*.

To 10.0 mL of the test solution and to 10.0 mL of the reference solution add separately 0.5 mL of a 5 g/L solution of *sodium nitrite R* and allow to stand for 3 min. Add 1 mL of a 20 g/L solution of *ammonium sulfamate R*, shake, allow to stand for 3 min and add 1 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. Any pinkish-violet colour produced in the test solution is not more intense than that in the reference solution.

**Chlorides** (*2.4.4*): maximum 500 ppm.

To 2 g add a mixture of 1.2 mL of *acetic acid R* and 40 mL of *water R*, boil for 2 min, cool and filter. Dilute 2 mL of the filtrate to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): 4.5 per cent to 6.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.3000 g in 80 mL of a mixture of equal volumes of *acetone R* and *methanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 32.71 mg of $C_{13}H_8Cl_2N_2O_4$.

STORAGE

Protected from light.

**01/2016:2332**

# NICORANDIL

## Nicorandilum

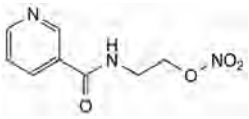

$C_8H_9N_3O_4$ $M_r$ 211.2
[65141-46-0]

DEFINITION

2-[(Pyridin-3-ylcarbonyl)amino]ethyl nitrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nicorandil CRS*.

TESTS

**pH** (*2.2.3*): 5.5 to 7.0.

Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *nicorandil impurity A CRS* in the mobile phase using sonication and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the test solution.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Mobile phase*: *trifluoroacetic acid R*, *triethylamine R*, *tetrahydrofuran R*, *water R* (3:5:10:982 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of nicorandil.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to nicorandil (retention time = about 19 min): impurity A = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity A and nicorandil.

*Calculation of percentage contents*:

- for each impurity, use the concentration of nicorandil in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dissolve 5.000 g in 30 mL of *ethanol (50 per cent V/V) R*, add 1 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *distilled water R*.

**Water** (*2.5.32*): maximum 0.5 per cent.

Dissolve 0.300 g in a suitable solvent and dilute to 1.0 mL with the same solvent. Inject 0.400 mL of the solution through the septum.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.12 mg of $C_8H_9N_3O_4$.

STORAGE

At a temperature of 2 °C to 8 °C.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

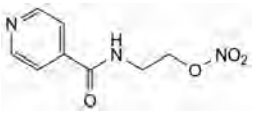

A. 2-[(pyridin-4-ylcarbonyl)amino]ethyl nitrate,

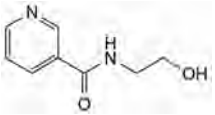

B. *N*-(2-hydroxyethyl)pyridine-3-carboxamide,

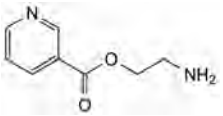

C. 2-aminoethyl pyridine-3-carboxylate,

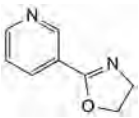

D. 3-(4,5-dihydro-1,3-oxazol-2-yl)pyridine.

**01/2017:0047**

# NICOTINAMIDE

## Nicotinamidum

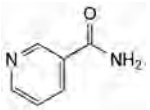

$C_6H_6N_2O$ $M_r$ 122.1
[98-92-0]

DEFINITION

Pyridine-3-carboxamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water and in anhydrous ethanol, slightly soluble in methylene chloride.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 128 °C to 131 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nicotinamide CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 5 mg of the substance to be examined in a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and dilute to 5.0 mL with the same mixture of solvents.
*Reference solution.* Dissolve 5 mg of *nicotinamide CRS* in a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and dilute to 5.0 mL with the same mixture of solvents.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *water R*, *ethanol (96 per cent) R*, *methylene chloride R* (4:45:48 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 7.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.150 g of the substance to be examined in mobile phase A and dilute to 150.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *isonicotinamide R* (impurity D) in the test solution and dilute to 100.0 mL with the test solution. Dilute 1.0 mL of this solution to 25.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: mix 5.0 mL of *dilute acetic acid R* and 900 mL of *water for chromatography R*, then add 30 mL of *dilute ammonia R3* and 15 mL of *acetonitrile R*; dilute to 1 L with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*, mobile phase A (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 98 | 2 |
| 2 - 16 | 98 → 0 | 2 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to nicotinamide (retention time = about 7 min): impurity D = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity D and nicotinamide.

*Calculation of percentage contents*:

- for each impurity, use the concentration of nicotinamide in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 18 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 12.21 mg of $C_6H_6N_2O$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

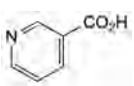

A. pyridine-3-carboxylic acid (nicotinic acid),

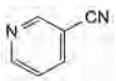

B. pyridine-3-carbonitrile,

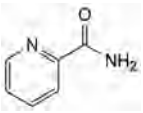

C. pyridine-2-carboxamide (picolinamide),

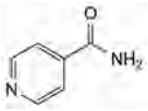

D. pyridine-4-carboxamide (isonicotinamide),

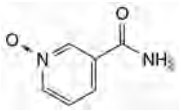

E. pyridine-3-carboxamide 1-oxide (nicotinamide *N*-oxide).

**01/2009:1452**
**corrected 6.6**

# NICOTINE

## Nicotinum

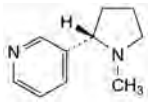

$C_{10}H_{14}N_2$ $M_r$ 162.2
[54-11-5]

DEFINITION

3-[(2*S*)-1-Methylpyrrolidin-2-yl]pyridine.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: colourless or brownish viscous liquid, volatile, hygroscopic.

*Solubility*: soluble in water, miscible with anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of nicotine.*

TESTS

**Appearance of solution**. Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$, $BY_5$ or $R_5$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 140 to − 152.

Dissolve 1.00 g in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *nicotine for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in 1.0 mL of *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: to 900 mL of *water R*, add 25 mL of a 60 g/L solution of *acetic acid R*, then add 6 mL of *concentrated ammonia R1*. Adjust to pH 10.0 with *dilute ammonia R2* or *dilute acetic acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 3.01 | 100 → 95 | 0 → 5 |
| 3.01 - 28 | 95 → 74 | 5 → 26 |
| 28 - 32 | 74 → 60 | 26 → 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *nicotine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to nicotine (retention time = about 17.8 min): impurity E = about 0.3; impurity C = about 0.55; impurity F = about 0.7; impurity A = about 0.8; impurity D = about 0.86; impurity G = about 0.9; impurity B = about 1.6.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity G and nicotine.

*Limits*:

- *impurities A, B, C, D, E, F, G*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

ASSAY

Dissolve 60.0 mg in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 8.11 mg of $C_{10}H_{14}N_2$.

STORAGE

Under nitrogen, in an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

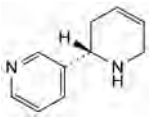

A. (2*S*)-1,2,3,6-tetrahydro-2,3′-bipyridyl (anatabine),

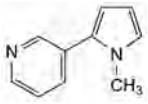

B. 3-(1-methyl-1*H*-pyrrol-2-yl)pyridine (β-nicotyrine),

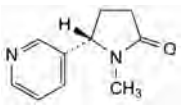

C. (5*S*)-1-methyl-5-(pyridin-3-yl)pyrrolidin-2-one (cotinine),

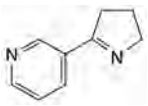

D. 3-(4,5-dihydro-3*H*-pyrrol-2-yl)pyridine (myosmine),

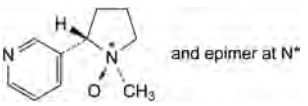


E. (1*RS*,2*S*)-1-methyl-2-(pyridin-3-yl)pyrrolidine 1-oxide (nicotine *N*′-oxide),

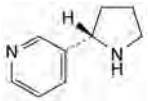

F. 3-[(2*S*)-pyrrolidin-2-yl]pyridine (nornicotine),

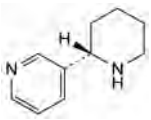

G. 3-[(2*S*)-piperidin-2-yl]pyridine (anabasine).

**01/2014:2599**

# NICOTINE DITARTRATE DIHYDRATE

## Nicotini ditartras dihydricus

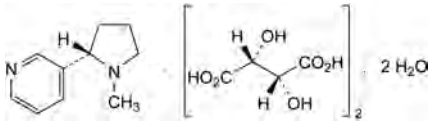

$C_{18}H_{26}N_2O_{12},2H_2O$ $M_r$ 498.4

[6019-06-3]

DEFINITION

3-[(2*S*)-1-Methylpyrrolidin-2-yl]pyridine bis[(2*R*,3*R*)-2,3-dihydroxybutanedioate] dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nicotine ditartrate dihydrate CRS*.

TESTS

**pH** (*2.2.3*): 3.0 to 3.4.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 21.0 to + 23.0.

Dissolve 0.25 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 60 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve the contents of a vial of *nicotine for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in 1.0 mL of *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: to 900 mL of *water R* add 25 mL of a 60 g/L solution of *acetic acid R* and 6 mL of *concentrated ammonia R1*; adjust to pH 10.0 with *dilute ammonia R2* or *dilute acetic acid R* and dilute to 1000.0 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 3.01 | 100 → 95 | 0 → 5 |
| 3.01 - 28 | 95 → 74 | 5 → 26 |
| 28 - 32 | 74 → 60 | 26 → 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *nicotine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to nicotine (retention time = about 17.8 min): impurity E = about 0.3; impurity C = about 0.55; impurity F = about 0.7; impurity A = about 0.8; impurity D = about 0.86; impurity G = about 0.9; impurity B = about 1.6.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity G and nicotine.

*Limits*:

- *impurities A, B, C, D, E, F, G*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 6.5 per cent to 8.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.12 mg of $C_{18}H_{26}N_2O_{12}$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

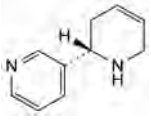

A. (2*S*)-1,2,3,6-tetrahydro-2,3′-bipyridyl (anatabine),

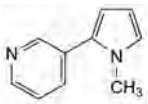

B. 3-(1-methyl-1*H*-pyrrol-2-yl)pyridine (β-nicotyrine),

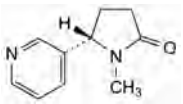

C. (5*S*)-1-methyl-5-(pyridin-3-yl)pyrrolidin-2-one (cotinine),

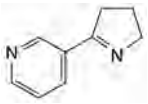

D. 3-(4,5-dihydro-3*H*-pyrrol-2-yl)pyridine (myosmine),

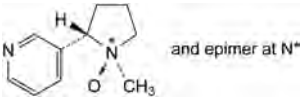


E. (1*RS*,2*S*)-1-methyl-2-(pyridin-3-yl)pyrrolidine 1-oxide (nicotine *N*′-oxide),

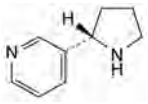

F. 3-[(2*S*)-pyrrolidin-2-yl]pyridine (nornicotine),

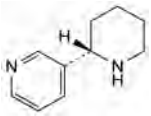

G. 3-[(2*S*)-piperidin-2-yl]pyridine (anabasine).

**01/2015:1792**

# NICOTINE RESINATE

## Nicotini resinas

[96055-45-7]

DEFINITION

Complex of nicotine (3-[(2*S*)-1-methylpyrrolidin-2-yl]-pyridine) with a weak cationic exchange resin.

*Content*: 95.0 per cent to 115.0 per cent of the declared content of nicotine ($C_{10}H_{14}N_2$) stated on the label (dried substance).

It may contain glycerol.

CHARACTERS

*Appearance*: white or slightly yellowish powder, hygroscopic.

*Solubility*: practically insoluble in water.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: shake a quantity of the substance to be examined equivalent to 100 mg of nicotine with a mixture of 10 mL of *dilute ammonia R2*, 10 mL of *water R*, 5 mL of *strong sodium hydroxide solution R* and 20 mL of *hexane R* for 5 min. Transfer the upper layer to a beaker and evaporate to produce an oily residue. Record the spectrum of the oily residue as a thin film between *sodium chloride R* plates.

*Comparison*: *Ph. Eur. reference spectrum of nicotine.*

B. Nicotine release (see Tests).

TESTS

**Nicotine release**: minimum 70 per cent of the content determined under Assay in 10 min.

Transfer an accurately weighed quantity of the substance to be examined, equivalent to about 4 mg of nicotine, to a glass-stoppered test-tube, add 10.0 mL of a 9 g/L solution of *sodium chloride R* previously heated to 37 °C and shake vigorously for 10 min. Immediately filter the liquid through a dry filter paper discarding the 1st millilitre of filtrate. Transfer 1.0 mL of the filtrate to a 20 mL volumetric flask, dilute to 20 mL with *0.1 M hydrochloric acid* and mix. Determine the absorbance (*2.2.25*) at the minima at about 236 nm and 282 nm and at the maximum at 259 nm using 1.0 mL of a 9 g/L solution of *sodium chloride R* diluted to 20 mL with *0.1 M hydrochloric acid* as compensation liquid.

Calculate the percentage of nicotine release using the following expression:

$$\frac{20 \times 10^6 \times (A_{259} - 0.5A_{236} - 0.5A_{282})}{323 \times C \times m}$$

| | | |
|---|---|---|
| 323 | = | specific absorbance of nicotine at 259 nm; |
| $C$ | = | percentage of nicotine in the substance to be examined determined in the assay; |
| $m$ | = | mass of the substance to be examined, in milligrams; |
| $A_{236}$, $A_{259}$, $A_{282}$ | = | absorbances of the solution at the wavelength indicated by the subscript. |

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Weigh a quantity of the substance to be examined, equivalent to 30.0 mg of nicotine, into a glass-stoppered test-tube, add 10.0 mL of *dilute ammonia R2* and shake vigorously for 10 min. Centrifuge for 20 min at about 3000 r/min. To 5.0 mL of the clear solution, add 5 mL of a 60 g/L solution of *acetic acid R* and dilute to 25.0 mL with *water R*.

*Reference solution (a).* Dissolve the contents of a vial of *nicotine for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in 1.0 mL of *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 46.0 mg of *nicotine ditartrate CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

- *mobile phase A*: to 900 mL of *water R*, add 25 mL of a 60 g/L solution of *acetic acid R*, then add 6 mL of *concentrated ammonia R1*; adjust to pH 10.0 with *dilute ammonia R2* or *dilute acetic acid R* and dilute to 1 L with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 3.01 | 100 → 95 | 0 → 5 |
| 3.01 - 28 | 95 → 74 | 5 → 26 |
| 28 - 32 | 74 → 60 | 26 → 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *nicotine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to nicotine (retention time = about 18 min): impurity E = about 0.3; impurity C = about 0.55; impurity F = about 0.7; impurity A = about 0.8; impurity D = about 0.86; impurity G = about 0.9; impurity B = about 1.6.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity G and nicotine.

*Limits*:

- *impurities A, B, C, D, E, F, G*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of nicotine ($C_{10}H_{14}N_2$) taking into account the assigned content of $C_{10}H_{14}N_2$ in *nicotine ditartrate CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the content of nicotine.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

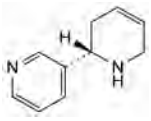

A. (2*S*)-1,2,3,6-tetrahydro-2,3′-bipyridine (anatabine),

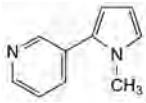

B. 3-(1-methyl-1*H*-pyrrol-2-yl)pyridine (β-nicotyrine),

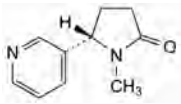

C. (5*S*)-1-methyl-5-(pyridin-3-yl)pyrrolidin-2-one (cotinine),

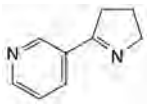

D. 3-(4,5-dihydro-3*H*-pyrrol-2-yl)pyridine (myosmine),

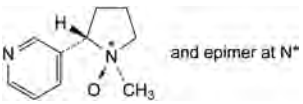


E. (1*RS*,2*S*)-1-methyl-2-(pyridin-3-yl)pyrrolidine 1-oxide (nicotine *N*-oxide),

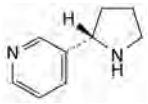

F. 3-[(2*S*)-pyrrolidin-2-yl]pyridine (nornicotine),

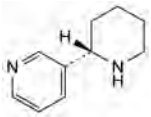

G. 3-[(2*S*)-piperidin-2-yl]pyridine (anabasine).

**01/2017:0459**
**corrected 9.3**

# NICOTINIC ACID

## Acidum nicotinicum

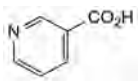

$C_6H_5NO_2$ $M_r$ 123.1
[59-67-6]

DEFINITION

Pyridine-3-carboxylic acid.

*Content*: 99.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in boiling water and in boiling ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 234 °C to 240 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nicotinic acid CRS.*

C. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).
*Solvent mixture.* Dissolve 6.8 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.0 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water R.*
*Test solution.* Dissolve 50 mg in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 25.0 mL with the solvent mixture.
*Spectral range*: 237-262 nm.
*Absorption maximum*: at 262 nm.
*Absorption minimum*: at 237 nm.
*Absorbance ratio*: $A_{237}/A_{262}$ = 0.46 to 0.50.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.120 g of the substance to be examined in 200 µL of *dilute ammonia R1* and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve the contents of a vial of *nicotinic acid impurity mixture CRS* (impurities A and B) in 1.0 mL of mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (4 µm);
- *temperature*: 15 °C.

*Mobile phase*:
- *mobile phase A*: dilute 2 mL of *acetic acid R* in 950 mL of *water for chromatography R*, adjust to pH 5.6 with *dilute ammonia R1* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*, *methanol R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 30 | 100→20 | 0→80 |
| 30 - 35 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *nicotinic acid impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to nicotinic acid (retention time = about 6 min): impurity A = about 2.7; impurity B = about 2.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities A and B.

*Limits*:

- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in *water R*, heating on a water-bath, and dilute to 15 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *water R*. Add 0.25 mL of *phenolphthalein solution R*. Titrate with *0.1 M sodium hydroxide* until a pink colour is obtained. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 12.31 mg of $C_6H_5NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I.*

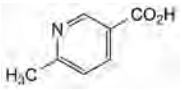

A. 6-methylpyridine-3-carboxylic acid (6-methylnicotinic acid),

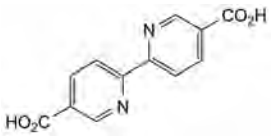

B. 2,2′-bipyridine-5,5′-dicarboxylic acid (6,6′-dinicotinic acid),

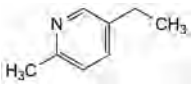

C. 5-ethyl-2-methylpyridine,

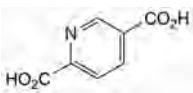

D. pyridine-2,5-dicarboxylic acid,

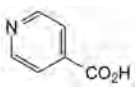

E. pyridine-4-carboxylic acid (isonicotinic acid),

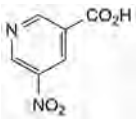

F. 5-nitropyridine-3-carboxylic acid (5-nitronicotinic acid),

G. pyridine,

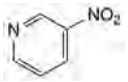

H. 3-nitropyridine,

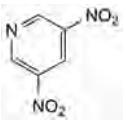

I. 3,5-dinitropyridine.

**01/2008:0627**
**corrected 6.0**

# NIFEDIPINE

## Nifedipinum

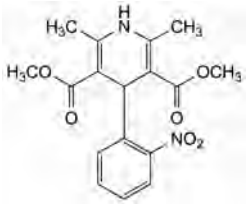

$C_{17}H_{18}N_2O_6$ $M_r$ 346.3
[21829-25-4]

DEFINITION

Dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol.

When exposed to daylight and to artificial light of certain wavelengths, it readily converts to a nitrosophenylpyridine derivative. Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative.

*Prepare solutions immediately before use in the dark or under long-wavelength light (> 420 nm) and protect them from light.*

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 171 °C to 175 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nifedipine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *nifedipine CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (40:60 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, appearance at 254 nm and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 25 mg in a test tube, add 10 mL of a mixture of 1.5 volumes of *hydrochloric acid R*, 3.5 volumes of *water R* and 5 volumes of *alcohol R* and dissolve with gentle heating. Add 0.5 g of *zinc R* in granules and allow to stand for 5 min with occasional swirling. Filter into a second test tube, add 5 mL of a 10 g/L solution of *sodium nitrite R* to the filtrate and allow to stand for 2 min. Add 2 mL of a 50 g/L solution of *ammonium sulfamate R*, shake vigorously with care and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. An intense red colour develops which persists for not less than 5 min.

TESTS

**Impurity D and other basic impurities**. Transfer 4 g to a 250 mL conical flask and dissolve in 160 mL of *glacial acetic acid R* using an ultrasonic bath. Titrate with *0.1 M perchloric acid* using 0.25 mL of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green. Not more than 0.48 mL of *0.1 M perchloric acid* is required (0.14 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in 20 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10 mg of *nifedipine impurity A CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *nifedipine impurity B CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (c).* Mix 1.0 mL of reference solution (a), 1.0 mL of reference solution (b) and 0.1 mL of the test solution and dilute to 20.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *methanol R*, *water R* (9:36:55 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL; inject the test solution and reference solution (c).

*Run time*: twice the retention time of nifedipine.

*Elution order*: impurity A, impurity B, nifedipine.

*Retention time*: nifedipine = about 15.5 min.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B and minimum 1.5 between the peaks due to impurity B and nifedipine.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *any other impurity*: not more than the area of the peak due to nifedipine in the chromatogram obtained with reference solution (c) (0.1 per cent),
- *total*: not more than 0.3 per cent,
- *disregard limit*: 0.1 times the area of the peak due to nifedipine in the chromatogram obtained with reference solution (c) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1300 g in a mixture of 25 mL of *2-methyl-2-propanol R* and 25 mL of *perchloric acid solution R*. Titrate with *0.1 M cerium sulfate* using 0.1 mL of *ferroin R* as indicator, until the pink colour disappears. Titrate slowly towards the end of the titration. Carry out a blank titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 17.32 mg of $C_{17}H_{18}N_2O_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

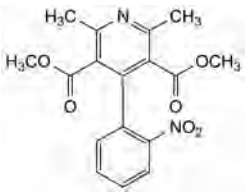


A. dimethyl 2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate (nitrophenylpyridine analogue),

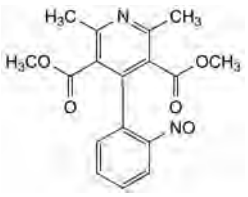


B. dimethyl 2,6-dimethyl-4-(2-nitrosophenyl)pyridine-3,5-dicarboxylate (nitrosophenylpyridine analogue),

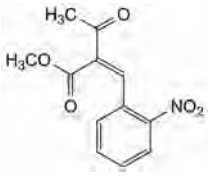


C. methyl 2-(2-nitrobenzylidene)-3-oxobutanoate,

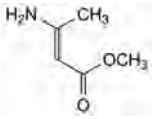


D. methyl 3-aminobut-2-enoate.

01/2017:2115

# NIFLUMIC ACID

## Acidum niflumicum

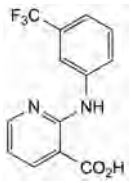

$C_{13}H_9F_3N_2O_2$ $M_r$ 282.2
[4394-00-7]

DEFINITION

2-[[3-(Trifluoromethyl)phenyl]amino]pyridine-3-carboxylic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent) and in methanol.

mp: about 204 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *niflumic acid CRS*.

TESTS

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.50 g of the substance to be examined in 5 mL of *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 25 mg of *3-trifluoromethylaniline R* (impurity C) in 20 mL of *methanol R* and dilute to 100 mL with the same solvent. Dilute 1.0 mL of this solution to 100 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetic acid R*, *ethyl acetate R*, *toluene R* (5:25:90 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air, until the solvents have evaporated.

*Detection*: spray with *4-dimethylaminocinnamaldehyde solution R* and heat at 60 °C for 10 min.

*Limit*:

– *impurity C*: any spot due to impurity C is not more intense than the principal spot in the chromatogram obtained with the reference solution (50 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in 10 mL of *acetonitrile R* and dilute to 20.0 mL with *water R*.

*Reference solution*. Dissolve 5.0 mg of *niflumic acid impurity A CRS*, 5.0 mg of *niflumic acid impurity B CRS* and 6.0 mg of *niflumic acid impurity E CRS* in 20 mL of *acetonitrile R*, add 5.0 mL of the test solution and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 25 °C.

*Mobile phase*: *phosphoric acid R*, *acetonitrile R*, *water R* (2.5:500:500 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 267 nm.

*Injection*: 10 µL.

*Run time*: 4 times the retention time of niflumic acid.

*Relative retention* with reference to niflumic acid (retention time = about 5.5 min): impurity A = about 0.25; impurity B = about 0.57; impurity E = about 0.64.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to impurities B and E.

*Limits*:

– *impurity B*: not more than 4 times the area of the corresponding peak in the chromatogram obtained with the reference solution (0.4 per cent);
– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the peak due to niflumic acid in the chromatogram obtained with the reference solution (0.10 per cent);
– *sum of impurities other than B*: not more than twice the area of the peak due to niflumic acid in the chromatogram obtained with the reference solution (0.2 per cent);
– *disregard limit*: 0.5 times the area of the peak due to niflumic acid in the chromatogram obtained with the reference solution (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.5 g in a mixture of 1 mL of *nitric acid R* and 10 mL of *methanol R*, and dilute to 20 mL with *water R*. To 10 mL of this solution add 5 mL of *water R*.

**Phosphates** (*2.4.11*): maximum 100 ppm.

Dilute 1.0 mL of the test solution prepared as described in general chapter *2.4.8* (method C) to 100 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.3 per cent, determined on 2.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in a mixture of 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.22 mg of $C_{13}H_9F_3N_2O_2$.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

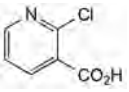

A. 2-chloropyridine-3-carboxylic acid,

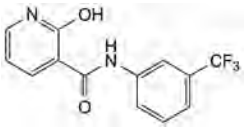

B. 2-hydroxy-*N*-[3-(trifluoromethyl)phenyl]pyridine-3-carboxamide,

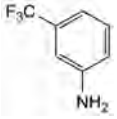

C. 3-(trifluoromethyl)aniline,

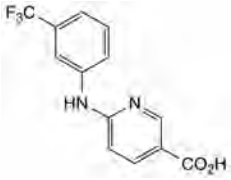

E. 6-[[3-(trifluoromethyl)phenyl]amino]pyridine-3-carboxylic acid,

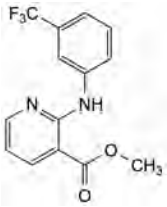

F. methyl 2-[[3-(trifluoromethyl)phenyl]amino]pyridine-3-carboxylate.

**01/2017:1999**

# NIFUROXAZIDE

## Nifuroxazidum

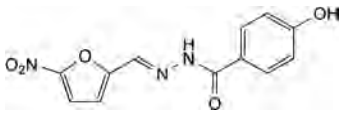

$C_{12}H_9N_3O_5$ $M_r$ 275.2
[965-52-6]

DEFINITION

(*E*)-4-Hydroxy-*N*′-[(5-nitrofuran-2-yl)methylidene]-benzohydrazide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: bright yellow, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nifuroxazide CRS*.

TESTS

**Specific absorbance** (*2.2.25*): 940 to 1000 at the absorption maximum at 367 nm.

Protected from light, dissolve 10.0 mg in 10 mL of *ethylene glycol monomethyl ether R* and dilute to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 100.0 mL with *methanol R*.

**Impurity A**: maximum 0.05 per cent.

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in *dimethyl sulfoxide R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* To 5.5 mL of test solution (a) add 50.0 mL of *water R* while stirring. Allow to stand for 15 min and filter.

*Reference solution.* To 0.5 mL of test solution (a) add 5.0 mL of a 50 mg/L solution of *4-hydroxybenzohydrazide R* (impurity A) in *dimethyl sulfoxide R*. Add 50.0 mL of *water R* while stirring. Allow to stand for 15 min and filter.

Add 0.5 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *sodium carbonate solution R* separately to 10.0 mL of test solution (b) and to 10.0 mL of the reference solution. Allow to stand for 1 h. Examine the 2 solutions at 750 nm. The absorbance (*2.2.25*) of the solution obtained with test solution (b) is not greater than that obtained with the reference solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Use amber volumetric flasks, unless otherwise specified.*

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture, using sonication for not more than 5 min, and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* In order to prepare impurity E *in situ*, dissolve 5 mg of the substance to be examined in the solvent mixture in a colourless volumetric flask, using sonication for 5 min, and dilute to 50 mL with the solvent mixture. Allow to stand in ambient light for 1 h.

*Reference solution (c).* Dissolve 5.0 mg of *methyl parahydroxybenzoate CRS* (impurity B) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 10 °C.

*Mobile phase*:

- *mobile phase A*: *tetrahydrofuran R*, *water R* (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 67 | 33 |
| 10 - 30 | 67 → 43 | 33 → 57 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 50 µL.

*Relative retention* with reference to nifuroxazide (retention time = about 8 min): impurity A (keto-enol tautomers) = about 0.36 and 0.39; impurity E = about 0.9; impurity B = about 1.2; impurity C = about 2.6; impurity D = about 3.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity E and nifuroxazide.

*Limits*:

- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

- *impurities B, C, D*: for each impurity, not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent), and not more than 1 such peak has an area greater than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peaks due to impurity A.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g, with heating if necessary, in 30 mL of *dimethylformamide R* and add 20 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.52 mg of $C_{12}H_9N_3O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

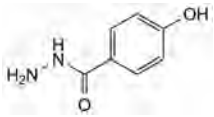

A. 4-hydroxybenzohydrazide (*p*-hydroxybenzohydrazide),

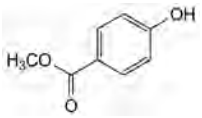

B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

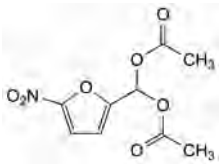

C. (5-nitrofuran-2-yl)methylidene diacetate,

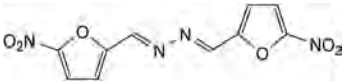

D. (*E,E*)-*N,N′*-bis[(5-nitrofuran-2-yl)methylidene]hydrazine (5-nitrofurfural azine),

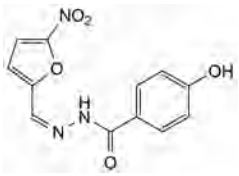

E. (*Z*)-4-hydroxy-*N′*-[(5-nitrofuran-2-yl)methylidene]-benzohydrazide.

**01/2017:0233**

# NIKETHAMIDE

## Nicethamidum

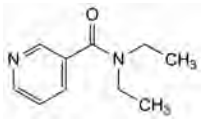

$C_{10}H_{14}N_2O$ $M_r$ 178.2
[59-26-7]

DEFINITION

Nikethamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of *N,N*-diethylpyridine-3-carboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

An oily liquid or a crystalline mass, colourless or slightly yellowish, miscible with water and with alcohol.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Dissolve 0.15 g in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 1.0 mL of this solution to 100.0 mL with *0.01 M hydrochloric acid*. Examined between 230 nm and 350 nm (*2.2.25*) in a 2 cm cell, the solution shows a single absorption maximum, at 263 nm. The specific absorbance at the maximum is about 285.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *nikethamide CRS*.

C. Heat 0.1 g with 1 mL of *dilute sodium hydroxide solution R*. Diethylamine is evolved progressively and is recognisable by its characteristic odour and by its turning *red litmus paper R* blue.

D. Dilute 1 mL of solution S (see Tests) to 250 mL with *water R*. To 2 mL of this solution add 2 mL of *cyanogen bromide solution R*. Add 3 mL of a 25 g/L solution of *aniline R* and shake. A yellow colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Appearance**. The substance to be examined, in liquid form or liquefied by slight heating, is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 6.0 to 7.8.

**Refractive index** (*2.2.6*). 1.524 to 1.526.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.4 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 40 mg of *ethylnicotinamide CRS* in *methanol R* and dilute to 100 mL with the same solvent.

*Reference solution (b)*. Dilute 1 mL of reference solution (a) to 10 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *propanol R* and 75 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. In the chromatogram obtained with the test solution,

any spot corresponding to ethylnicotinamide is not more intense than the spot in the chromatogram obtained with reference solution (a) (1.0 per cent) and any spot, apart from the principal spot and the spot corresponding to ethylnicotinamide, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Water** (*2.5.12*). Not more than 0.3 per cent, determined on 2.00 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 5 mL of *acetic anhydride R* and 20 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.82 mg of $C_{10}H_{14}N_2O$.

**07/2019:2993**

# NILOTINIB HYDROCHLORIDE MONOHYDRATE

## Nilotinibi hydrochloridum monohydricum

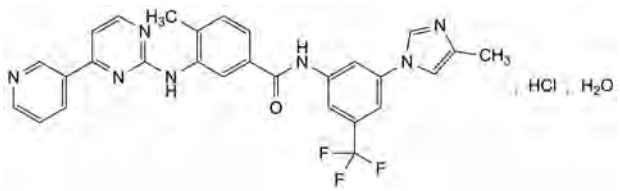

$C_{28}H_{23}ClF_3N_7O,H_2O$ $M_r$ 584.0
[923288-90-8]

DEFINITION

4-Methyl-*N*-[3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzamide hydrochloride monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish or slightly greenish-yellow, hygroscopic, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in anhydrous ethanol, very slightly soluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nilotinib hydrochloride monohydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (b) of chlorides (*2.3.1*).

C. Water (see Tests).

TESTS

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *dimethyl sulfoxide R*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 0.300 g of the substance to be examined in 2 mL of *dimethyl sulfoxide R*, add 7 mL of *water R*, allow to equilibrate at room temperature without shaking to avoid foam formation, and then dilute to 10.0 mL with *water R*. Shake well, allow the substance to be examined to precipitate for about 2 h in the dark and filter the supernatant through a 0.45 µm filter.

*Reference solution (a)*. Dissolve 7.5 mg of *nilotinib impurity A CRS* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *dimethyl sulfoxide R*. Dilute 2.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 7.5 mg of *nilotinib impurity B CRS* and 7.5 mg of *nilotinib impurity C CRS* in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *dimethyl sulfoxide R*. Dilute 2.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *encapsulated polar-embedded octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: mobile phase A, *acetonitrile R1* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 10 | 85 → 80 | 15 → 20 |
| 10 - 12 | 80 → 75 | 20 → 25 |
| 12 - 18 | 75 → 10 | 25 → 90 |
| 18 - 19 | 10 | 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 207 nm.

*Injection*: 20 µL of the test solution and reference solution (a).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to nilotinib (retention time = about 17 min): impurity A = about 0.3.

*System suitability*:

- *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution (a).

*Calculation of content*:

- for impurity A, use the concentration of impurity A in reference solution (a).

*Limit*:

- *impurity A*: maximum 3 ppm.

**Impurities B and C**. Liquid chromatography (*2.2.29*) as described in the test for impurity A with the following modifications.

*Detection*: spectrophotometer at 225 nm.

*Injection*: test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to nilotinib (retention time = about 17 min): impurity C = about 0.2; impurity B = about 0.6.

*System suitability*:

– *signal-to-noise ratio*: minimum 10 for the peaks due to impurities B and C in the chromatogram obtained with reference solution (c);

– *repeatability*: maximum relative standard deviation of 5.0 per cent for the areas of the peaks due to impurities B and C, determined on 6 injections of reference solution (b).

*Calculation of contents*:

– for impurities B and C, use the concentration of the corresponding impurity in reference solution (b).

*Limits*:

– *impurities B, C*: for each impurity, maximum 2 ppm.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *anhydrous ethanol R*, *water R* (50:50 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *nilotinib hydrochloride monohydrate CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *nilotinib for system suitability CRS* (containing impurities E, F and G) in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;

– *mobile phase B*: mobile phase A, *acetonitrile for chromatography R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 16 | 90 → 10 | 10 → 90 |
| 16 - 17 | 10 | 90 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 5 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *nilotinib for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities E, F and G.

*Relative retention* with reference to nilotinib (retention time = about 12 min): impurity E = about 1.03; impurity F = about 1.07; impurity G = about 1.09.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurities F and G;

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nilotinib.

*Calculation of percentage contents*:

– for each impurity, use the concentration of nilotinib hydrochloride monohydrate in reference solution (b).

*Limits*:

– *impurity F*: maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.40 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.0 per cent to 5.0 per cent, determined on 0.150 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{28}H_{23}ClF_3N_7O$ taking into account the assigned content of *nilotinib hydrochloride monohydrate CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, G, H.*

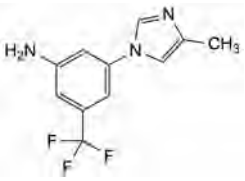

A. 3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)aniline,

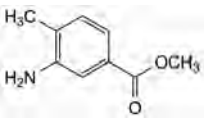

B. methyl 3-amino-4-methylbenzoate,

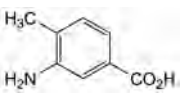

C. 3-amino-4-methylbenzoic acid,

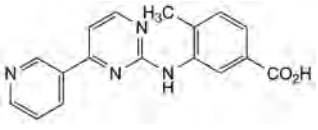

D. 4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzoic acid,

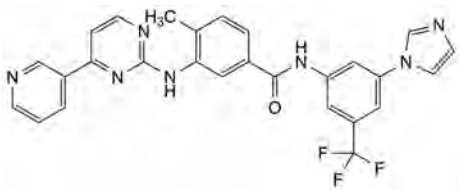

E. *N*-[3-(1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzamide,

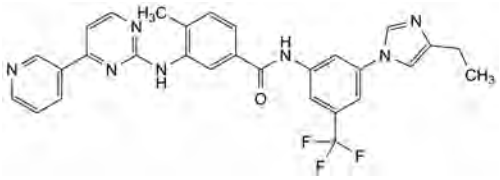

F. *N*-[3-(4-ethyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)-phenyl]-4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzamide,

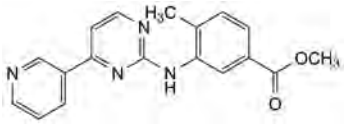

G. methyl 4-methyl-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzoate,

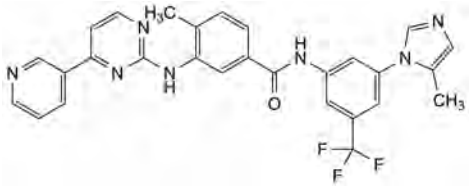

H. 4-methyl-*N*-[3-(5-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl]-3-[[4-(pyridin-3-yl)pyrimidin-2-yl]amino]benzamide.

**01/2017:2256**

# NILUTAMIDE

## Nilutamidum

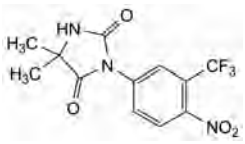

$C_{12}H_{10}F_3N_3O_4$ $M_r$ 317.2
[63612-50-0]

DEFINITION

5,5-Dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl]-imidazolidine-2,4-dione.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone, soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nilutamide CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile for chromatography R*, *water R* (35:65 *V/V*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 100 mL with the solvent mixture.

*Reference solution (a).* Dilute 20.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2 mg of the substance to be examined and 2 mg of *nilutamide impurity B CRS* in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 2.0 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 7.5 with *1 M sodium hydroxide*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 55 | 45 |
| 8 - 30 | 55 → 30 | 45 → 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to nilutamide (retention time = about 5.3 min): impurity B = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity B and nilutamide.

*Limits*:

- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*). *The solutions are stable for 24 h at room temperature and in daylight.*

*Solvent mixture*: *acetonitrile for chromatography R*, *water R* (35:65 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution.* Dissolve 50.0 mg of *nilutamide CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 40 volumes of *acetonitrile R* and 60 volumes of a 2.0 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 7.5 with *1 M sodium hydroxide*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 267 nm.

*Injection*: 20 µL.

*Retention time*: about 9 min.

Calculate the percentage content of $C_{12}H_{10}F_3N_3O_4$ from the declared content of *nilutamide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

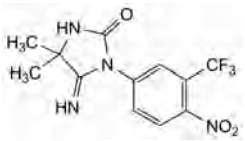

A. 5-imino-4,4-dimethyl-1-[4-nitro-3-(trifluoromethyl)-phenyl]imidazolidin-2-one,

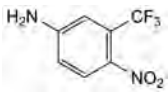

B. 4-nitro-3-(trifluoromethyl)aniline (nifeline),

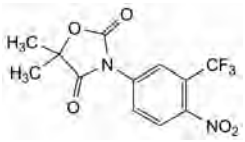

C. 5,5-dimethyl-3-[4-nitro-3-(trifluoromethyl)phenyl]-oxazolidine-2,4-dione,

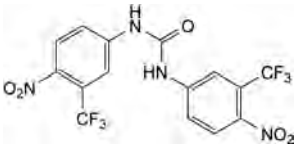

D. 1,3-bis[4-nitro-3-(trifluoromethyl)phenyl]urea.

**01/2017:1548**

# NIMESULIDE

## Nimesulidum

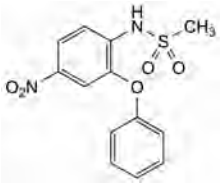

$C_{13}H_{12}N_2O_5S$ $M_r$ 308.3

[51803-78-2]

DEFINITION

*N*-(4-Nitro-2-phenoxyphenyl)methanesulfonamide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, slightly soluble in anhydrous ethanol.

mp: about 149 °C.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nimesulide CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Absorbance** (*2.2.25*): maximum 0.50 at 450 nm.

Dissolve 1.0 g in *acetone R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 8 mL of *acetonitrile R* and dilute to 20.0 mL with *water R*.

*Reference solution (a).* Dissolve 5 mg of *2-phenoxyaniline R* (impurity C) in 10 mL of *acetonitrile R* and dilute to 25.0 mL with *water R*. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase. Mix 1.0 mL of this solution with the contents of a vial of *nimesulide impurity D CRS* previously dissolved in 1.0 mL of *acetonitrile R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 4 mg of *nimesulide for peak identification CRS* (containing impurities A, B, E and F) in 4.0 mL of *acetonitrile R* and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.15 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 7.0 with *ammonia R*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of nimesulide.

*Identification of impurities*: use the chromatogram supplied with *nimesulide for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, E and F; use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and D.

*Relative retention* with reference to nimesulide (retention time = about 5 min): impurity A = about 0.3; impurity B = about 2.4; impurity C = about 3.2; impurity D = about 3.7; impurity E = about 4.2; impurity F = about 6.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurities C and D.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity E = 1.4;
- *impurity E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities A, B, C, D, F*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.240 g in 30 mL of previously neutralised *acetone R* and add 20 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.83 mg of $C_{13}H_{12}N_2O_5S$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): G.

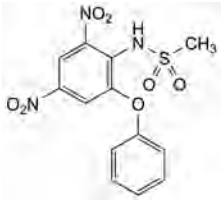

A. *N*-(2,4-dinitro-6-phenoxyphenyl)methanesulfonamide,

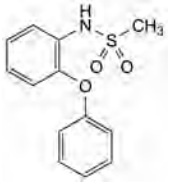

B. *N*-(2-phenoxyphenyl)methanesulfonamide,

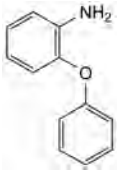

C. 2-phenoxyaniline,

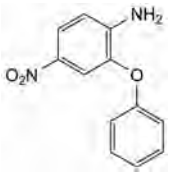

D. 4-nitro-2-phenoxyaniline,

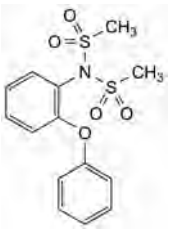

E. *N*,*N*-bis(methylsulfonyl)-2-phenoxyaniline,

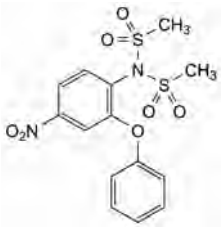

F. *N*,*N*-bis(methylsulfonyl)-4-nitro-2-phenoxyaniline,

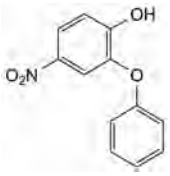

G. 4-nitro-2-phenoxyphenol.

**04/2016:1245**

# NIMODIPINE

## Nimodipinum

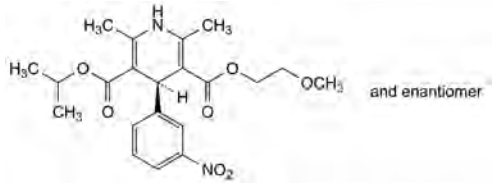


$C_{21}H_{26}N_2O_7$ $M_r$ 418.4
[66085-59-4]

DEFINITION

2-Methoxyethyl 1-methylethyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: light yellow or yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethyl acetate, sparingly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative.

IDENTIFICATION

A. Optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nimodipine CRS*.

If the spectra obtained in the solid state show differences, record new spectra using 20 g/L solutions in *methylene chloride R* and a 0.2 mm cell.

TESTS

*Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).*

**Solution S**. Dissolve 1.0 g in *acetone R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40 mg of the substance to be examined in 2.5 mL of *tetrahydrofuran R* and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4 mg of *nimodipine for peak identification CRS* (containing impurity C) in 0.25 mL of *tetrahydrofuran R* and dilute to 2.5 mL with the mobile phase.

*Reference solution (c).* Dilute 0.5 mL of the test solution to 25.0 mL with the mobile phase. Mix 0.5 mL of this solution with 0.5 mL of *nimodipine impurity A CRS* and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: *methanol R*, *tetrahydrofuran R*, *water R* (20:20:60 *V/V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Run time*: 4.5 times the retention time of nimodipine.

*Identification of impurities*: use the chromatogram supplied with *nimodipine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to nimodipine (retention time = about 7 min): impurity C = about 0.5; impurity A = about 0.9.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nimodipine.

*Limits*:

– *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities* : for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).*

Dissolve with gentle heating 0.180 g in a mixture of 25 mL of *2-methyl-2-propanol R* and 25 mL of *perchloric acid solution R*. Add 0.1 mL of *ferroin R*. Titrate with *0.1 M cerium sulfate*. Titrate slowly towards the end of the titration. Carry out a blank titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 20.92 mg of $C_{21}H_{26}N_2O_7$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph Substances for pharmaceutical use (2034). It is therefore not necessary to identify these impurities for demonstration of compliance. See also 5.10. Control of impurities in substances for pharmaceutical use): A, B.*

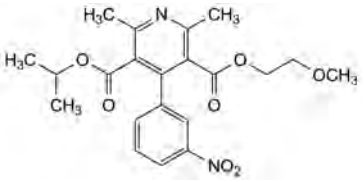

A. 2-methoxyethyl 1-methylethyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate,

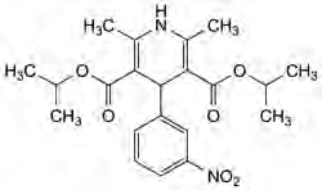

B. bis(1-methylethyl) 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

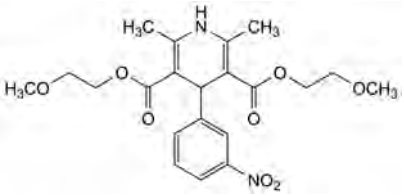

C. bis(2-methoxyethyl) 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

**04/2010:0415**

# NITRAZEPAM

## Nitrazepamum

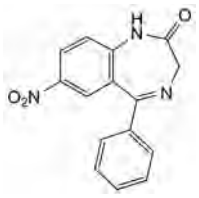

$C_{15}H_{11}N_3O_3$ $M_r$ 281.3
[146-22-5]

DEFINITION

7-Nitro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellow, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nitrazepam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *acetonitrile R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 2 mg of *clonazepam CRS* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 65 | 35 |
| 3 - 10 | 65 → 50 | 35 → 50 |
| 10 - 20 | 50 | 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*Relative retention* with reference to nitrazepam (retention time = about 9 min): clonazepam = about 1.1.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to clonazepam and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nitrazepam.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.13 mg of $C_{15}H_{11}N_3O_3$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

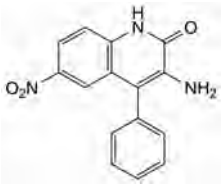

A. 3-amino-6-nitro-4-phenylquinolin-2(1*H*)-one,

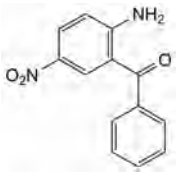

B. (2-amino-5-nitrophenyl)phenylmethanone,

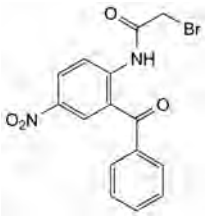

C. 2-bromo-*N*-[4-nitro-2-(phenylcarbonyl)phenyl]acetamide,

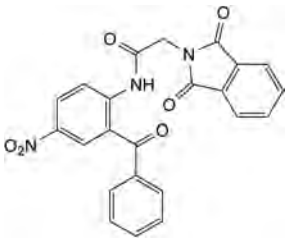

D. 2-(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-*N*-[4-nitro-2-(phenylcarbonyl)phenyl]acetamide.



**07/2012:1246**
**corrected 8.0**

# NITRENDIPINE

## Nitrendipinum

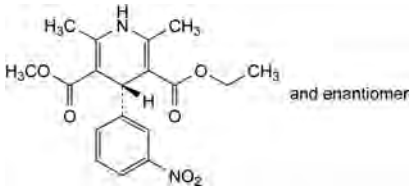


$C_{18}H_{20}N_2O_6$ $M_r$ 360.4
[39562-70-4]

DEFINITION

Ethyl methyl (4*RS*)-2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethyl acetate, sparingly soluble in anhydrous ethanol and in methanol.

It shows polymorphism (*5.9*).

Exposure to ultraviolet light leads to the formation of a nitrophenylpyridine derivative.

*Prepare solutions immediately before use either protected from light or under long-wavelength light (> 420 nm).*

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nitrendipine CRS*.

If the spectra obtained in the solid state show differences, record new spectra using 20 g/L solutions in *methylene chloride R* and a 0.2 mm cell.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in 2.5 mL of *tetrahydrofuran R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 15.0 mg of *nitrendipine impurity A CRS* in 2.5 mL of *tetrahydrofuran R* and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 0.5 mL of the test solution to 20.0 mL with the mobile phase.

*Reference solution (d).* Mix 1.0 mL of reference solution (b) and 1.0 mL of reference solution (c), then dilute to 25.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 2 mg of *nitrendipine for peak identification CRS* (containing impurities B and C) in 0.5 mL of *tetrahydrofuran R* and dilute to 1.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: irregular *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *tetrahydrofuran R*, *water R* (14:22:64 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (d) and (e).

*Run time*: 5 times the retention time of nitrendipine.

*Identification of impurities*: use the chromatogram supplied with *nitrendipine for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities B and C; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to nitrendipine (retention time = about 9 min): impurity B = about 0.7; impurity A = about 0.8; impurity C = about 1.4.

*System suitability*: reference solution (d):

- *resolution*: minimum 2.0 between the peaks due to impurity A and nitrendipine.

*Limits*:

- *impurities B, C*: for each impurity, not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 0.7 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g with gentle heating if necessary in a mixture of 25 mL of *2-methyl-2-propanol R* and 25 mL of *perchloric acid solution R*. Titrate with *0.1 M cerium sulfate*, using 0.1 mL of *ferroin R* as indicator. Titrate slowly towards the end of the titration. Carry out a blank titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 18.02 mg of $C_{18}H_{20}N_2O_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

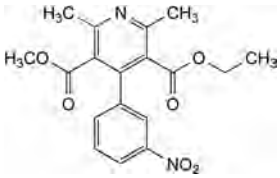

A. ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate,

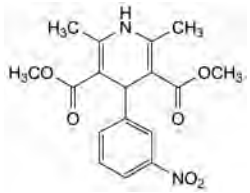

B. dimethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate,

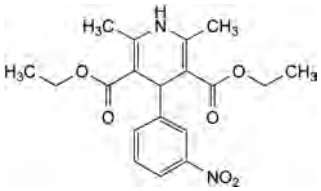

C. diethyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate.

**01/2017:1549**

# NITRIC ACID

## Acidum nitricum

$HNO_3$ $M_r$ 63.0
[7697-37-2]

DEFINITION

*Content*: 68.0 per cent *m/m* to 70.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless or almost colourless liquid.

*Solubility*: miscible with water.

*Relative density*: about 1.41.

IDENTIFICATION

A. Dilute 1 mL to 100 mL with *water R*. The solution is strongly acid (*2.2.4*).

B. 0.2 mL of the solution obtained in identification test A gives the reaction of nitrates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dilute 2 mL to 10 mL with *water R*.

**Chlorides** (*2.4.4*): maximum 0.5 ppm.

To 5 g add 10 mL of *water R* and 0.3 mL of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared at the same time in the same manner using 13 mL of *water R*, 0.5 mL of *nitric acid R*, 0.5 mL of *chloride standard solution (5 ppm Cl) R* and 0.3 mL of *silver nitrate solution R2*.

**Sulfates** (*2.4.13*): maximum 10 ppm.

To 15 g add 0.2 g of *sodium carbonate R*. After carbon dioxide has evolved, evaporate to dryness. Dissolve the residue in 15 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm.

Dissolve the residue obtained in the test for sulfated ash in 1 mL of *dilute hydrochloric acid R* and dilute to 20 mL with *water R*. Dilute 1 mL of this solution to 10 mL with *water R*.

**Sulfated ash**: maximum 0.01 per cent.

Carefully evaporate 20.00 g to dryness. Moisten the residue with a few drops of *sulfuric acid R* and ignite to dull red.

ASSAY

To 0.750 g add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 63.0 mg of $HNO_3$.

STORAGE

Protected from light.

**01/2008:1550**

# NITRIC OXIDE

## Nitrogenii oxidum

NO $M_r$ 30.01
[10102-43-9]

DEFINITION

*Content*: minimum 99.0 per cent *V/V* of NO.

This monograph applies to nitric oxide for medicinal use.

CHARACTERS

*Appearance*: colourless gas which turns brown when exposed to air.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 21 volumes of water.

PRODUCTION

**Carbon dioxide**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas*: mixture containing 3000 ppm *V/V* of *carbon dioxide R1* in *nitrogen R*.

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3.5 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R*;
- *temperature*: 50 °C.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 15 mL/min.

*Detection*: thermal conductivity.

*Injection*: loop injector.

*System suitability*:

- the chromatograms obtained show a clear separation of carbon dioxide from nitric oxide.

*Limit*:

- *carbon dioxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (3000 ppm *V/V*).

**Nitrogen**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas*: mixture containing 3000 ppm *V/V* of *nitrogen R* in *helium for chromatography R*.

*Column*:

– *material*: stainless steel;
– *size*: *l* = 3.5 m, Ø = 2 mm;
– *stationary phase*: *molecular sieve for chromatography R* (0.5 nm);
– *temperature*: 50 °C.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 15 mL/min.

*Detection*: thermal conductivity.

*Injection*: loop injector.

*System suitability*:

– the chromatograms obtained show a clear separation of nitrogen from nitric oxide.

*Limit*:

– *nitrogen*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (3000 ppm *V/V*).

**Nitrogen dioxide**: maximum 400 ppm *V/V*.

Ultraviolet absorption spectrophotometry analyser.

*Gas to be examined.* The substance to be examined.

*Reference gas (a)*: *nitrogen R1*.

*Reference gas (b)*: mixture containing 400 ppm *V/V* of *nitrogen dioxide R* in *nitrogen R*.

*Apparatus*:

– an ultraviolet-visible light source (analytical wavelength about 400 nm);
– a sample gas cell through which the feed gas flows;
– a closed reference gas cell containing *nitrogen R1* in parallel with the sample gas cell;
– a rotating chopper which feeds light alternately through the reference gas cell and the sample gas cell;
– a semiconductor detector which generates a frequency modulated output whose amplitude is a measure of the difference of absorption of the sample gas and the reference gas.

*Analysis*:

– set the zero of the instrument using reference gas (a) through the sample gas cell at a flow rate of 1 L/min;
– adjust the span while feeding reference gas (b) through the sample gas cell at a flow rate of 1 L/min;
– feed the gas to be examined through the sample gas cell at a flow rate of 1 L/min, read the value from the instrument output and calculate, if necessary, the concentration of nitrogen dioxide.

**Nitrous oxide**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas*: mixture containing 3000 ppm *V/V* of *nitrous oxide R* in *nitrogen R*.

*Column*:

– *material*: stainless steel;
– *size*: *l* = 3.5 m, Ø = 2 mm;
– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R*;
– *temperature*: 50 °C.

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 15 mL/min.

*Detection*: thermal conductivity.

*Injection*: loop injector.

*System suitability*:

– the chromatograms obtained show a clear separation of nitrous oxide from nitric oxide.

*Limit*:

– *nitrous oxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (3000 ppm *V/V*).

**Water** (*2.5.28*): maximum 100 ppm *V/V*.

**Assay**. Determine the content of nitric oxide by difference using the mass balance equation after determining the sum of the impurities described under Production.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of nitric oxide*.

STORAGE

Compressed at a pressure not exceeding 2.5 MPa (25 bar) measured at 15 °C, in suitable containers complying with the legal regulations.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

A. $CO_2$: carbon dioxide,

B. $N_2$: nitrogen,

C. $NO_2$: nitrogen dioxide,

D. $N_2O$: nitrous oxide,

E. $H_2O$: water.

**04/2013:1135**

# NITROFURAL

## Nitrofuralum

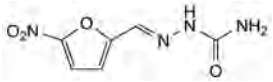

$C_6H_6N_4O_4$ $M_r$ 198.1

[59-87-0]

DEFINITION

2-[(5-Nitrofuran-2-yl)methylene]diazanecarboxamide.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or brownish-yellow, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*). *Carry out the test protected from bright light.*
*Test solution.* Use the solution prepared for the assay.
*Spectral range*: 220-400 nm.
*Absorption maxima*: at 260 nm and 375 nm.
*Absorbance ratio*: $A_{375}/A_{260}$ = 1.15 to 1.30.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nitrofural CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *nitrofural CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *methanol R*, *nitromethane R* (10:90 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *phenylhydrazine hydrochloride solution R.*

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 1 mg in 1 mL of *dimethylformamide R* and add 0.1 mL of *alcoholic potassium hydroxide solution R*. A violet-red colour is produced.

TESTS

**pH** (*2.2.3*): 5.0 to 7.0.

To 1.0 g add 100 mL of *carbon dioxide-free water R*. Shake and filter.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10.0 mg of *nitrofural impurity B CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of the substance to be examined and 10 mg of *nitrofurantoin R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve with the aid of ultrasound the contents of a vial of *nitrofural for peak identification CRS* (containing impurities A and B) in 1.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 310 nm.

*Injection*: 20 µL.

*Run time*: 10 times the retention time of nitrofural.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to nitrofural (retention time = about 4 min): nitrofurantoin = about 1.2; impurity B = about 4.0; impurity A = about 7.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to nitrofural and nitrofurantoin.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*Carry out the assay protected from bright light.*

Dissolve 60.0 mg in 20 mL of *dimethylformamide R* and dilute to 500.0 mL with *water R*. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Prepare a reference solution in the same manner using 60.0 mg of *nitrofural CRS*. Measure the absorbances (*2.2.25*) of the 2 solutions at the absorption maximum at 375 nm.

Calculate the content of $C_6H_6N_4O_4$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

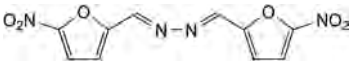

A. 1,2-bis[(5-nitrofuran-2-yl)methylidene]diazane,

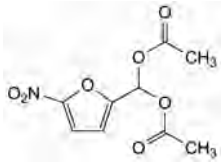

B. (5-nitrofuran-2-yl)methylene diacetate.

**01/2008:0101**
**corrected 7.0**

# NITROFURANTOIN

## Nitrofurantoinum

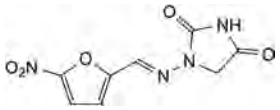

$C_8H_6N_4O_5$ $M_r$ 238.2
[67-20-9]

DEFINITION

Nitrofurantoin contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 1-[[(5-nitrofuran-2-yl)methylene]amino]imidazolidine-2,4-dione, calculated with reference to the dried substance.

CHARACTERS

A yellow, crystalline powder or yellow crystals, very slightly soluble in water and in ethanol (96 per cent), soluble in dimethylformamide.

IDENTIFICATION

A. *Carry out the test protected from bright light.* Use the solution prepared for the assay. Examined between 220 nm and 400 nm (*2.2.25*), the solution shows two absorption maxima, at 266 nm and 367 nm. The ratio of the absorbance at the maximum at 367 nm to that at the maximum at 266 nm is 1.36 to 1.42.

B. Dissolve about 10 mg in 10 mL of *dimethylformamide R*. To 1 mL of the solution add 0.1 mL of *0.5 M alcoholic potassium hydroxide*. A brown colour develops.

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $HF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.25 g of the substance to be examined in a minimum of *dimethylformamide R* and dilute to 10 mL with *acetone R*.

*Reference solution.* Dilute 1 mL of the test solution to 100 mL with *acetone R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *methanol R* and 90 volumes of *nitromethane R*. Allow the plate to dry in air and heat at 100 °C to 105 °C for 5 min. Examine in ultraviolet light at 254 nm. Spray with *phenylhydrazine hydrochloride solution R*. Heat the plate at 100 °C to 105 °C for a further 10 min. When examined in ultraviolet light and after spraying, any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

*Carry out the assay protected from bright light.* Dissolve 0.120 g in 50 mL of *dimethylformamide R* and dilute to 1000.0 mL with *water R*. Dilute 5.0 mL of the solution to 100.0 mL with a solution containing 18 g/L of *sodium acetate R* and 0.14 per cent *V/V* of *glacial acetic acid R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 367 nm, using the sodium acetate solution described above as compensation liquid.

Calculate the content of $C_8H_6N_4O_5$, taking the specific absorbance to be 765.

STORAGE

Store protected from light, at a temperature below 25 °C.

**01/2008:1247**

# NITROGEN

## Nitrogenium

$N_2$ $M_r$ 28.01

[7727-37-9]

DEFINITION

*Content*: minimum 99.5 per cent *V/V* of $N_2$.

This monograph applies to nitrogen for medicinal use.

CHARACTERS

*Appearance*: colourless, odourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 62 volumes of water and about 10 volumes of ethanol (96 per cent).

PRODUCTION

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using an infrared analyser (*2.5.24*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Nitrogen R1.*

*Reference gas (b).* Mixture containing 300 ppm *V/V* of *carbon dioxide R1* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using an infrared analyser (*2.5.25*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Nitrogen R1.*

*Reference gas (b).* Mixture containing 5 ppm *V/V* of *carbon monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

**Oxygen**: maximum 50 ppm *V/V*, determined using an oxygen analyser with a detector scale ranging from 0-100 ppm *V/V* and equipped with an electrochemical cell.

The gas to be examined passes through a detection cell containing an aqueous solution of an electrolyte, generally potassium hydroxide. The presence of oxygen in the gas to be examined produces variation in the electric signal recorded at the outlet of the cell that is proportional to the oxygen content.

Calibrate the analyser according to the instructions of the manufacturer. Pass the gas to be examined through the analyser using a suitable pressure regulator and airtight metal tubes and operating at the prescribed flow-rates until constant readings are obtained.

**Water** (*2.5.28*): maximum 67 ppm *V/V*.

**Assay**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a).* Ambient air.

*Reference gas (b). Nitrogen R1.*

*Column*:

- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 2 mm;
- *stationary phase*: *molecular sieve for chromatography R* (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 40 mL/min.

*Temperature*:

- *column*: 50 °C;
- *detection*: 130 °C.

*Detection*: thermal conductivity.

*Injection*: loop injector.

Inject reference gas (a). Adjust the injected volumes and operating conditions so that the height of the peak due to nitrogen in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

*System suitability*:

- the chromatograms obtained show a clear separation of oxygen and nitrogen.

Calculate the content of $N_2$ in the gas to be examined.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine the chromatograms obtained in the assay (see Production).

*Results*: the principal peak in the chromatogram obtained with the substance to be examined is similar in retention time to the principal peak in the chromatogram obtained with reference gas (b).

B. In a 250 mL conical flask replace the air by the substance to be examined. Place a burning or glowing splinter of wood in the flask. The splinter is extinguished.

C. In a suitable test tube, place 0.1 g of *magnesium R* in turnings. Close the tube with a two-hole stopper fitted with a glass tube reaching about 1 cm above the turnings. Pass the substance to be examined through the glass tube for 1 min without heating, then for 15 min while heating the test tube to a red glow. After cooling, add 5 mL of *dilute sodium hydroxide solution R*. The evolving vapours change the colour of moistened *red litmus paper R* blue.

TESTS

**Carbon dioxide** (*2.1.6*): maximum 300 ppm *V/V*, determined using a carbon dioxide detector tube.

**Carbon monoxide** (*2.1.6*): maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube.

**Water vapour** (*2.1.6*): maximum 67 ppm *V/V*, determined using a water vapour detector tube.

STORAGE

As a compressed gas or a liquid in appropriate containers complying with the legal regulations.

IMPURITIES

*Specified impurities: A, B, C, D.*

A. $CO_2$: carbon dioxide,

B. CO: carbon monoxide,

C. $O_2$: oxygen,

D. $H_2O$: water.

**01/2008:1685**

# NITROGEN, LOW-OXYGEN

## Nitrogenium oxygenio depletum

$N_2$ $M_r$ 28.01

DEFINITION

This monograph applies to nitrogen which is used for inerting finished medicinal products which are particularly sensitive to degradation by oxygen. It does not necessarily apply to nitrogen used in earlier production steps.

*Content*: minimum 99.5 per cent *V/V* of $N_2$, calculated by deduction of the sum of impurities found when performing the test for impurities.

CHARACTERS

Colourless and odourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 62 volumes of water and about 10 volumes of alcohol.

PRODUCTION

**Oxygen**: maximum 5 ppm *V/V*, determined using an oxygen analyser with a detector scale ranging from 0 ppm *V/V* to 100 ppm *V/V* and equipped with an electrochemical cell.

The gas to be examined passes through a detection cell containing an aqueous solution of an electrolyte, generally potassium hydroxide. The presence of oxygen in the gas to be examined produces variation in the electric signal recorded at the outlet of the cell that is proportional to the oxygen content.

Calibrate the analyser according to the manufacturer's instructions. Pass the gas to be examined through the analyser using a suitable pressure regulator and airtight metal tubes and operating at the prescribed flow rates until constant readings are obtained.

**Impurities**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a).* Use ambient air.

*Reference gas (b).* Use *nitrogen R1*.

*Column*:

– *material*: stainless steel,
– *size*: *l* = 2 m, Ø = 2 mm,
– *stationary phase*: appropriate molecular sieve for chromatography (0.5 nm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 40 mL/min.

*Temperature*:

– *column*: 50 °C,
– *detector*: 130 °C.

*Detection*: thermal conductivity.

*System suitability*: reference gas (a): adjust the injected volumes and operating conditions so that the height of the peak due to nitrogen in the chromatogram obtained is at least 35 per cent of the full scale of the recorder:

– the chromatogram obtained shows a clear separation of oxygen and nitrogen.

*Limit*:

– *total*: not more than 0.5 per cent of the sum of the areas of all the peaks (0.5 per cent *V/V*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine the chromatograms obtained in the test for impurities (see Production).

*Results*: the principal peak in the chromatogram obtained with the gas to be examined is similar in retention time to the principal peak in the chromatogram obtained with reference gas (b).

B. In a 250 mL conical flask replace the air by the gas to be examined. Place a burning or glowing splinter of wood in the flask. The splinter is extinguished.

C. In a suitable test tube, place 0.1 g of *magnesium R* in turnings. Close the tube with a two-hole stopper fitted with a glass tube reaching about 1 cm above the turnings. Pass the gas to be examined through the glass tube for 1 min without heating, then for 15 min while heating the test tube to a red glow. After cooling, add 5 mL of *dilute sodium hydroxide solution R*. The evolving vapours turn the colour of moistened *red litmus paper R* blue.

STORAGE

Where the gas has to be stored, store as a compressed gas or a liquid in appropriate containers complying with the legal regulations.

IMPURITIES

A. $O_2$: oxygen,

B. Ar: argon.

**01/2008:0416**

# NITROUS OXIDE

## Dinitrogenii oxidum

$N_2O$ $M_r$ 44.01
[10024-97-2]

DEFINITION

*Content*: minimum 98.0 per cent *V/V* of $N_2O$ in the gaseous phase, when sampled at 15 °C.

This monograph applies to nitrous oxide for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 1.5 volumes of water.

PRODUCTION

Nitrous oxide is produced from ammonium nitrate by thermic decomposition.

*Examine the gaseous phase.*

*If the test is performed on a cylinder, keep the cylinder at room temperature for at least 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.*

**Carbon dioxide**. Gas chromatography (*2.2.28*).

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 300 ppm *V/V* of *carbon dioxide R1* in *nitrous oxide R.*

*Column*:

- *material*: stainless steel;
- *size*: *l* = 3.5 m, Ø = 2 mm;
- *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R.*

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 15 mL/min.

*Temperature*:

- *column*: 40 °C;
- *detector*: 90 °C.

*Detection*: thermal conductivity.

*Injection*: loop injector.

Adjust the injected volumes and operating conditions so that the height of the peak due to carbon dioxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder. The test is not valid unless the chromatograms obtained show a clear separation of carbon dioxide from nitrous oxide.

*Limit*:

- *carbon dioxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (300 ppm *V/V*).

**Carbon monoxide**. Gas chromatography (*2.2.28*). *When the test is carried out on a cylinder, use the first portion of gas to be withdrawn.*

*Gas to be examined.* The substance to be examined.

*Reference gas.* A mixture containing 5 ppm *V/V* of *carbon monoxide R* in *nitrous oxide R.*

*Column*:

- *material*: stainless steel;
- *size*: *l* = 2 m, Ø = 4 mm;
- *stationary phase*: suitable molecular sieve for chromatography (0.5 nm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 60 mL/min.

*Temperature*:

- *column*: 50 °C;
- *injection port and detector*: 130 °C.

*Detection*: flame ionisation with methaniser.

*Injection*: loop injector.

Adjust the injected volumes and the operating conditions so that the height of the peak due to carbon monoxide in the chromatogram obtained with the reference gas is at least 35 per cent of the full scale of the recorder.

*Limit*:

- *carbon monoxide*: not more than the area of the corresponding peak in the chromatogram obtained with the reference gas (5 ppm *V/V*).

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total in the gaseous and liquid phases, determined using a chemiluminescence analyser (*2.5.26*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a). Nitrous oxide R.*

*Reference gas (b).* A mixture containing 2 ppm *V/V* of *nitrogen monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide, separately examining the samples collected from the gaseous phase and the liquid phase of the gas to be examined.

Multiply the result obtained by the quenching correction factor in order to correct the quenching effect on the analyser response caused by the nitrous oxide matrix effect.

The quenching correction factor is determined by applying a known reference mixture of nitrogen monoxide in nitrous oxide and comparing the actual content with the content indicated by the analyser which has been calibrated with an $NO/N_2$ reference mixture.

$$\text{Quenching correction factor} = \frac{\text{actual nitrogen monoxide content}}{\text{indicated nitrogen monoxide content}}$$

**Water**: maximum 67 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

**Assay**. Infrared analyser (*2.5.35*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Nitrous oxide R.*

*Reference gas (b).* A mixture containing 5.0 per cent *V/V* of *nitrogen R1* and 95.0 per cent *V/V* of *nitrous oxide R.*

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrous oxide in the gas to be examined.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. It complies with the limits of the assay.

B. Place a glowing splinter of wood in the substance to be examined. The splinter bursts into flame.

C. Introduce the substance to be examined into *alkaline pyrogallol solution R.* A brown colour does not develop.

TESTS

*Examine the gaseous phase.*

*If the test is performed on a cylinder, keep the cylinder of the substance to be examined at room temperature for at least 6 h before carrying out the tests. Keep the cylinder in the vertical position with the outlet valve uppermost.*

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using a carbon dioxide detector tube (*2.1.6*).

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube (*2.1.6*). *When the test is carried out on a cylinder, use the first portion of the gas to be withdrawn.*

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V*, determined using a nitrogen monoxide and nitrogen dioxide detector tube (*2.1.6*).

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

STORAGE

Store liquefied under pressure in suitable containers complying with the legal regulations. The taps and valves are not greased or oiled.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

A. $CO_2$: carbon dioxide,

B. CO: carbon monoxide,

C. NO: nitrogen monoxide,

D. $NO_2$: nitrogen dioxide,

E. $H_2O$: water.

**01/2017:1453**

# NIZATIDINE

## Nizatidinum

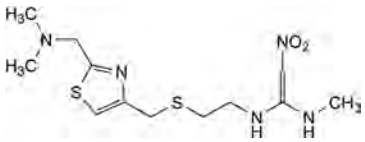

$C_{12}H_{21}N_5O_2S_2$ $M_r$ 331.5
[76963-41-2]

DEFINITION

(*EZ*)-*N*-[2-[[[2-[(Dimethylamino)methyl]thiazol-4-yl]methyl]sulfanyl]ethyl]-*N'*-methyl-2-nitroethene-1,1-diamine.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or slightly brownish, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in methanol.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 131 °C to 134 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.10 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 242 nm and 325 nm.

*Absorbance ratio*: $A_{325}/A_{242}$ = 2.2 to 2.5.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nizatidine CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 50 mg of *nizatidine CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 50 mg of *nizatidine CRS* and 50 mg of *ranitidine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *water R*, *concentrated ammonia R1*, *2-propanol R*, *ethyl acetate R* (4:8:30:50 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots are clearly visible. Examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 0.2 g in a 10 g/L solution of *hydrochloric acid R* and dilute to 20 mL with the same solution.

**pH** (*2.2.3*): 8.5 to 10.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (15:85 *V/V*).

*Test solution (a).* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 15.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 15.0 mg of *nizatidine CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of the substance to be examined and 0.5 mg of *nizatidine impurity F CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5 mg of *2-(dimethylamino)thioacetamide hydrochloride R* (impurity H hydrochloride) in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture. Use 1.0 mL of this solution to dissolve 5 mg of *nizatidine for system suitability CRS* (containing impurities A, B, C, D, G, J and K).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: dissolve 5.9 g of *ammonium acetate R* in 760 mL of *water R*, add 1 mL of *diethylamine R*, and adjust to pH 7.5 with *acetic acid R*;

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 85 | 15 |
| 3 - 20 | 85 → 50 | 15 → 50 |
| 20 - 45 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *nizatidine for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A, B, C, D, G, H, J and K; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity F.

*Relative retention* with reference to nizatidine (retention time = about 18 min): impurity A = about 0.19; impurity K = about 0.21; impurity H = about 0.5; impurity B = about 0.6; impurity C = about 0.66; impurity J = about 0.7; impurity D = about 0.75; impurity F = about 1.03; impurity G = about 1.5.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to nizatidine and impurity F in the chromatogram obtained with reference solution (c); minimum 1.5 between the peaks due to impurities A and K in the chromatogram obtained with reference solution (d).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.7; impurity D = 2.3; impurity H = 0.5;
- *impurities A, B, C, D, F, G, H, J, K*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (35:65 *V/V*).

*Injection*: test solution (b) and reference solution (b).

*Retention time*: nizatidine = 9 min.

Calculate the percentage content of $C_{12}H_{21}N_5O_2S_2$ taking into account the assigned content of *nizatidine CRS*.

IMPURITIES

*Specified impurities*: *A, B, C, D, F, G, H, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, I.*

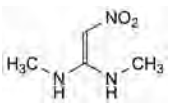

A. *N*,*N*′-dimethyl-2-nitroethene-1,1-diamine,

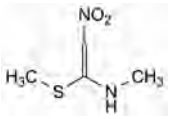

B. (*EZ*)-*N*-methyl-1-(methylsulfanyl)-2-nitroethen-1-amine,

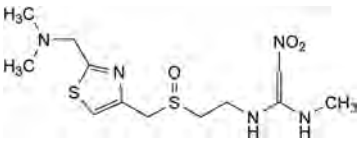

C. (*EZ*)-*N*-[2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulfinyl]ethyl]-*N*′-methyl-2-nitroethene-1,1-diamine,

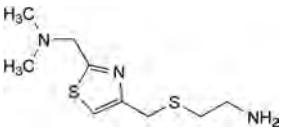

D. 2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]-methyl]sulfanyl]ethanamine,

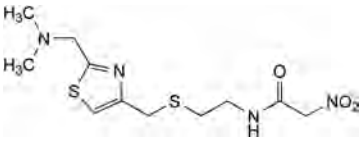

E. *N*-[2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]-methyl]sulfanyl]ethyl]-2-nitroacetamide,

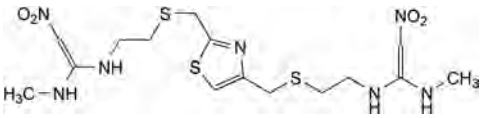

F. (*EZ*)-*N*-methyl-*N*′-[2-[[[4-[[[2-[[(*EZ*)-1-(methylamino)-2-nitroethenyl]amino]ethyl]sulfanyl]methyl]thiazol-2-yl]methyl]sulfanyl]ethyl]-2-nitroethene-1,1-diamine,

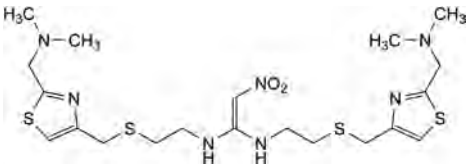

G. *N*,*N*′-bis[2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulfanyl]ethyl]-2-nitroethene-1,1-diamine,

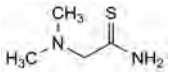

H. 2-(dimethylamino)thioacetamide,

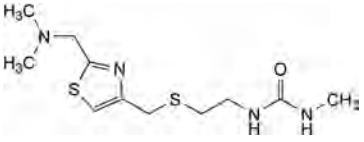

I. *N*-[2-[[[2-[(dimethylamino)methyl]thiazol-4-yl]methyl]sulfanyl]ethyl]-*N*′-methylurea,

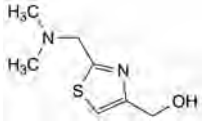

J. [2-[(dimethylamino)methyl]thiazol-4-yl]methanol,

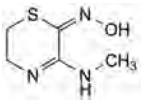

K. 3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-one oxime.

**01/2008:1551**
**corrected 6.0**

# NOMEGESTROL ACETATE

## Nomegestroli acetas

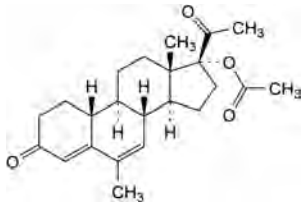

$C_{23}H_{30}O_4$ $M_r$ 370.5
[58652-20-3]

DEFINITION

6-Methyl-3,20-dioxo-19-norpregna-4,6-dien-17-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nomegestrol acetate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methylene chloride R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 60.0 to – 64.0 (dried substance).

Dissolve 0.500 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 25.0 mg of *nomegestrol acetate impurity A CRS* in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (c).* Dissolve 25.0 mg of *nomegestrol acetate CRS* in 20 mL of *methanol R*, add 0.25 mL of reference solution (b) and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *methanol R*, *water R* (24:38:38 *V/V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 245 nm and at 290 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of nomegestrol acetate.

*Retention time* at 245 nm: nomegestrol acetate = about 17 min; impurity A = about 18.5 min.

*System suitability*: reference solution (c) at 245 nm:

– *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to nomegestrol acetate.

*Limits*:

– *impurity A at 245 nm*: not more than 0.4 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *unspecified impurities at 245 nm*: for each impurity, not more than 0.2 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *unspecified impurities at 290 nm*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities other than A at 290 nm and 245 nm*: maximum 0.3 per cent;
– *disregard limit at 245 nm*: 0.1 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (c) (0.05 per cent);
– *disregard limit at 290 nm*: 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 50.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *anhydrous ethanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 287 nm.

Calculate the content of $C_{23}H_{30}O_4$ taking the specific absorbance to be 685.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

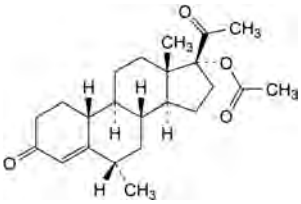

A. 6α-methyl-3,20-dioxo-19-norpregn-4-en-17-yl acetate.

**01/2017:1454**

# NONOXINOL 9

## Nonoxinolum 9

DEFINITION

α-(4-Nonylphenyl)-ω-hydroxynona(oxyethylene).

Mixture consisting mainly of monononylphenyl ethers of macrogols corresponding to the formula: $C_9H_{19}C_6H_4$-$[OCH_2$-$CH_2]_n$-OH where the average value of *n* is 9. It may contain free macrogols.

CHARACTERS

*Appearance*: clear, colourless or light yellow, viscous liquid.

*Solubility*: miscible with water, with ethanol (96 per cent) and with vegetable oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nonoxinol 9 CRS*.
*Preparation*: film between *sodium chloride R* plates.

B. Cloud point (see Tests).

TESTS

**Acidity or alkalinity**. Boil 1.0 g with 20 mL of *carbon dioxide-free water R* for 1 min, with constant stirring. Cool and filter. To 10 mL of the filtrate, add 0.05 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Hydroxyl value** (*2.5.3, Method A*): 84 to 94.

**Cloud point**: 52 °C to 58 °C.

Dissolve 1.0 g in 99 g of *water R*. Transfer about 30 mL of this solution into a test-tube, heat on a water-bath and stir continuously until the solution becomes cloudy. Remove the test-tube from the water-bath (ensuring that the temperature does not increase to more than 2 °C) and continue to stir. The cloud point is the temperature at which the solution becomes sufficiently clear that the entire thermometer bulb is plainly seen.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Total ash** (*2.4.16*): maximum 0.4 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

**01/2008:0732**
**corrected 9.3**

# NORADRENALINE HYDROCHLORIDE

## Noradrenalini hydrochloridum

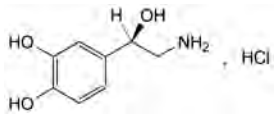


$C_8H_{12}ClNO_3$ $M_r$ 205.6
[329-56-6]

DEFINITION

(1*R*)-2-Amino-1-(3,4-dihydroxyphenyl)ethanol hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or brownish-white, crystalline powder.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent).

It becomes coloured on exposure to air and light.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of noradrenaline base prepared as follows. Dissolve 2 g in 20 mL of a 5 g/L solution of *sodium metabisulfite R* and make alkaline by addition of *ammonia R*. Keep in iced water for 1 h and filter. Wash the precipitate with 3 quantities, each of 2 mL, of *water R*, then with 5 mL of *ethanol (96 per cent) R* and finally with 5 mL of *methylene chloride R* and dry *in vacuo* for 3 h.
*Comparison*: use noradrenaline base prepared as above from a suitable amount of *noradrenaline tartrate CRS*.

C. 0.2 mL of solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than a mixture of 0.2 mL of blue primary solution, 0.4 mL of yellow primary solution, 0.4 mL of red primary solution and 9 mL of a 13.7 per cent *V/V* solution of *dilute hydrochloric acid R* (*2.2.2, Method II*).

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Examine the solution immediately.

**pH** (*2.2.3*): 3.5 to 4.5 for solution S.

**Specific optical rotation** (*2.2.7*): − 37 to − 41 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from air. Remove oxygen from the mobile phases with nitrogen R immediately before use. Fill up the flasks.*

*Test solution*. Dissolve 0.125 g of the substance to be examined in mobile phase A and dilute to 50 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 10 mg of the substance to be examined in 5 mL of *0.1 M hydrochloric acid*. To 1 mL of this solution add 0.1 mL of *strong hydrogen peroxide solution R* and expose to UV light at 254 nm for 90 min. Dilute to 10 mL with mobile phase A. The degradation of noradrenaline produces 2 peaks, one with a relative retention of about 1.2 (unidentified compound) and the other with a relative retention of about 1.5 (impurity B). Use this solution to identify the peak due to impurity B.

*Reference solution (c)*. Dissolve 7.5 mg of *noradrenaline impurity D CRS* and 5 mg of *noradrenaline impurity E CRS* in mobile phase A and dilute to 100 mL with mobile phase A.

*Reference solution (d)*. Dissolve 5 mg of *noradrenaline impurity F CRS* in mobile phase A and dilute to 10 mL with mobile phase A. To 1 mL of this solution, add 1 mL of reference solution (c) and dilute to 20 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*;
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 0.50 g of *sodium heptanesulfonate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; adjust to pH 2.2 with *phosphoric acid R*;
- *mobile phase B*: dissolve 0.25 g of *sodium heptanesulfonate R* in *water for chromatography R* and dilute to 500 mL with the same solvent; add 500 mL of *acetonitrile for chromatography R* and adjust the apparent pH to 2.4 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 2.0 | 98 | 2 | 1.5 |
| 2.0 - 17.0 | 98 → 70 | 2 → 30 | 1.5 |
| 17.0 - 24.0 | 70 → 50 | 30 → 50 | 1.5 |
| 24.0 - 24.1 | 50 → 0 | 50 → 100 | 1.5 → 4.0 |
| 24.1 - 28.0 | 0 | 100 | 4.0 |
| 28.0 - 28.1 | 0 → 98 | 100 → 2 | 4.0 |
| 28.1 - 30.0 | 98 | 2 | 4.0 → 1.5 |

*Detection*: spectrophotometer at 280 nm, except for impurity F: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Relative retention* with reference to noradrenaline (retention time = about 3 min): impurity B = about 1.5; impurity D = about 2.8; impurity E = about 3.0; impurity F = about 6.9.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to impurities D and E.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.3; impurity E = 0.3; impurity F = 1.5;
– *impurity D at 280 nm*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity F at 254 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *impurities B, E at 280 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *unspecified impurities at 280 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities other than D at 280 nm*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *sum of the impurities at 280 nm and impurity F at 254 nm*: maximum 0.7 per cent;
– *disregard limit at 280 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

## ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.180 g in 50 mL of *acetic anhydride R* and add 10 mL of *anhydrous formic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.56 mg of $C_8H_{12}ClNO_3$.

## STORAGE

In an airtight container, or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

## IMPURITIES

*Specified impurities: B, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, G.*

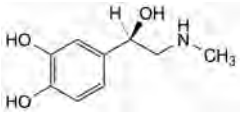

A. 4-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol (adrenaline),

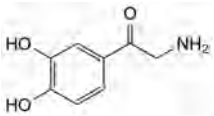

B. 2-amino-1-(3,4-dihydroxyphenyl)ethanone (noradrenalone),

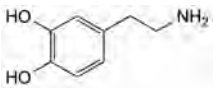

C. 4-(2-aminoethyl)benzene-1,2-diol (dopamine),

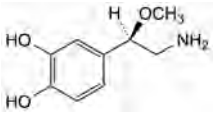

D. 4-[(1*R*)-2-amino-1-methoxyethyl]benzene-1,2-diol (noradrenaline methyl ether),

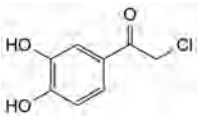

E. 2-chloro-1-(3,4-dihydroxyphenyl)ethanone,

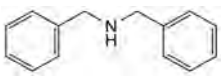

F. *N*-benzyl-1-phenylmethanamine,

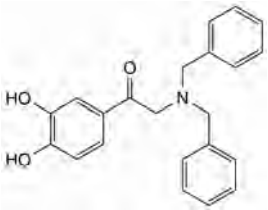

G. 2-(dibenzylamino)-1-(3,4-dihydroxyphenyl)ethanone.

01/2008:0285
corrected 9.3

# NORADRENALINE TARTRATE

## Noradrenalini tartras

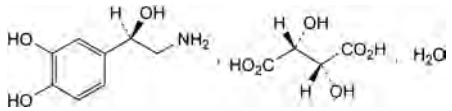

$C_{12}H_{17}NO_9,H_2O$ $M_r$ 337.3
[108341-18-0]

DEFINITION

(1*R*)-2-Amino-1-(3,4-dihydroxyphenyl)ethanol hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate monohydrate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 2 g in 20 mL of a 5 g/L solution of *sodium metabisulfite R* and make alkaline by addition of *ammonia R*. Keep in iced water for 1 h and filter. Reserve the filtrate for identification test C. Wash the precipitate with 3 quantities, each of 2 mL, of *water R*, then with 5 mL of *ethanol (96 per cent) R* and finally with 5 mL of *methylene chloride R* and dry *in vacuo* for 3 h. The specific optical rotation (*2.2.7*) of the precipitate (noradrenaline base) is − 48 to − 44, determined using a 20.0 g/L solution in *0.5 M hydrochloric acid*.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of noradrenaline base prepared as described in identification test A.

*Comparison*: use noradrenaline base prepared as described in identification test A from a suitable amount of *noradrenaline tartrate CRS*.

C. 0.2 mL of the filtrate obtained in identification test A gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.2 g in *water R* and dilute to 10 mL with the same solvent. Examine the solution immediately.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from air. Remove oxygen from the mobile phases with nitrogen R immediately before use. Fill up the flasks.*

*Test solution.* Dissolve 0.20 g of the substance to be examined in mobile phase A and dilute to 50 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined in 5 mL of *0.1 M hydrochloric acid*. To 1 mL of this solution add 0.1 mL of *strong hydrogen peroxide solution R* and expose to UV light at 254 nm for 90 min. Dilute to 10 mL with mobile phase A. The degradation of noradrenaline produces 2 peaks, one with a relative retention of about 1.2 (unidentified compound) and the other with a relative retention of about 1.5 (impurity B). Use this solution to identify the peak due to impurity B.

*Reference solution (c).* Dissolve 7.5 mg of *noradrenaline impurity D CRS* and 5 mg of *noradrenaline impurity E CRS* in mobile phase A and dilute to 100 mL with mobile phase A.

*Reference solution (d).* Dissolve 5 mg of *noradrenaline impurity F CRS* in mobile phase A and dilute to 10 mL with mobile phase A. To 1 mL of this solution, add 1 mL of reference solution (c) and dilute to 20 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*;
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 0.50 g of *sodium heptanesulfonate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; adjust to pH 2.2 with *phosphoric acid R*;
- *mobile phase B*: dissolve 0.25 g of *sodium heptanesulfonate R* in *water for chromatography R* and dilute to 500 mL with the same solvent; add 500 mL of *acetonitrile for chromatography R* and adjust the apparent pH to 2.4 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 2.0 | 98 | 2 | 1.5 |
| 2.0 - 17.0 | 98 → 70 | 2 → 30 | 1.5 |
| 17.0 - 24.0 | 70 → 50 | 30 → 50 | 1.5 |
| 24.0 - 24.1 | 50 → 0 | 50 → 100 | 1.5 → 4.0 |
| 24.1 - 28.0 | 0 | 100 | 4.0 |
| 28.0 - 28.1 | 0 → 98 | 100 → 2 | 4.0 |
| 28.1 - 30.0 | 98 | 2 | 4.0 → 1.5 |

*Detection*: spectrophotometer at 280 nm, except for impurity F: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Relative retention* with reference to noradrenaline (retention time = about 3 min): impurity B = about 1.5; impurity D = about 2.8; impurity E = about 3.0; impurity F = about 6.9.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurities D and E.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.3; impurity E = 0.3; impurity F = 1.5;
- *impurity F at 254 nm*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurities B, D, E at 280 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities at 280 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of the impurities at 280 nm and impurity F at 254 nm*: maximum 0.3 per cent;
- *disregard limit at 280 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 4.5 per cent to 5.8 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*, heating gently if necessary. Titrate with *0.1 M perchloric acid* using 0.1 mL of *crystal violet solution R* as indicator, until a bluish-green colour is obtained.

1 mL of *0.1 M perchloric acid* is equivalent to 31.93 mg of $C_{12}H_{17}NO_9$.

STORAGE

In an airtight container or preferably in a sealed tube under vacuum or under an inert gas, protected from light.

IMPURITIES

*Specified impurities*: *B, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, G.*

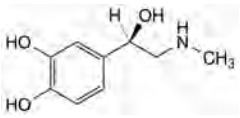

A. 4-[(1*R*)-1-hydroxy-2-(methylamino)ethyl]benzene-1,2-diol (adrenaline),

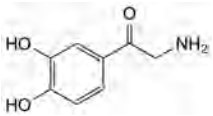

B. 2-amino-1-(3,4-dihydroxyphenyl)ethanone (noradrenalone),

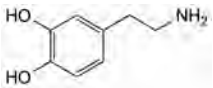

C. 4-(2-aminoethyl)benzene-1,2-diol (dopamine),

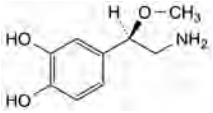

D. 4[(1*R*)-2-amino-1-methoxyethyl]benzene-1,2-diol (noradrenaline methyl ether),

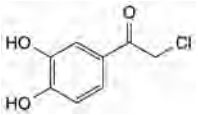

E. 2-chloro-1-(3,4-dihydroxyphenyl)ethanone,

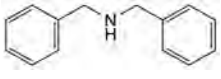

F. *N*-benzyl-1-phenylmethanamine,

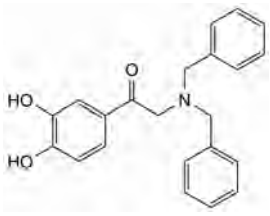

G. 2-(dibenzylamino)-1-(3,4-dihydroxyphenyl)ethanone.

**01/2008:0234**
**corrected 7.0**



# NORETHISTERONE

## Norethisteronum

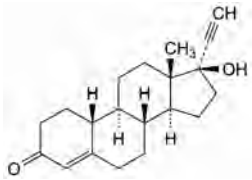

$C_{20}H_{26}O_2$ $M_r$ 298.4
[68-22-4]

DEFINITION

17-Hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, sparingly soluble in acetone and in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *norethisterone CRS.*

TESTS

**Specific optical rotation** (*2.2.7*): − 32.0 to − 37.0 (dried substance).

Dissolve 0.250 g in *acetone R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in a mixture of 40 volumes of *water R* and 60 volumes of *acetonitrile R1* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 5 mg of *norethisterone for system suitability CRS* (containing impurities A, B, C, D, E, F, G and H) in a mixture of 40 volumes of *water R* and 60 volumes of *acetonitrile R1* and dilute to 2.0 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of 40 volumes of *water R* and 60 volumes of *acetonitrile R1.* Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 40 volumes of *water R* and 60 volumes of *acetonitrile R1*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,
– *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *water R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 63 | 37 |
| 20 - 25 | 63 → 20 | 37 → 80 |
| 25 - 35 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: variable wavelength spectrophotometer capable of operating at 254 nm and at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *norethisterone for system suitability CRS* to identify the peaks due to the impurities A, B, C, D, E, F, G and H.

*Relative retention at 254 nm* with reference to norethisterone (retention time = about 10 min): impurity H = about 0.3; impurity A = about 0.8; impurity B = about 0.9; impurity G = about 1.5; impurity C (at 210 nm) = about 1.6; impurity D (at 210 nm) = about 1.7; impurity E = about 2.3; impurity F = about 2.4.

*System suitability*: reference solution (a) at 254 nm:

– *resolution*: baseline separation between the peaks due to impurity B and norethisterone;
– *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Limits*: spectrophotometer at 254 nm:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.5; impurity E = 0.7; impurity F = 1.4; impurity H = 1.7;
– *impurities E, G, H*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurities A, B, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

*Limits*: spectrophotometer at 210 nm:

– *impurities C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.200 g in 40 mL of *tetrahydrofuran R*. Add 10 mL of a 100 g/L solution of *silver nitrate R* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Rinse the electrode with *acetone R* after each titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 29.84 mg of $C_{20}H_{26}O_2$.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H.*

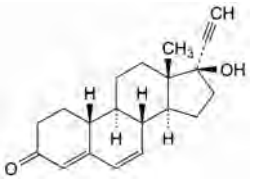

A. 17-hydroxy-19-nor-17α-pregna-4,6-dien-20-yn-3-one,

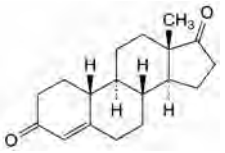

B. estr-4-ene-3,17-dione (norandrostenedione),

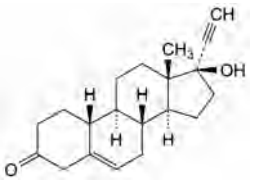

C. 17-hydroxy-19-nor-17α-pregn-5-en-20-yn-3-one,

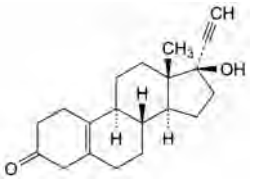

D. 17-hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one,

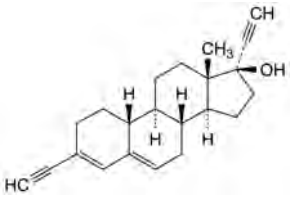

E. 3-ethynyl-19-nor-17α-pregna-3,5-dien-20-yn-17-ol,

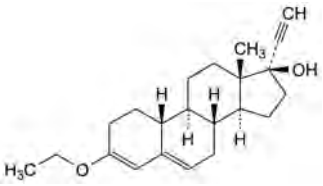

F. 3-ethoxy-19-nor-17α-pregna-3,5-dien-20-yn-17-ol,

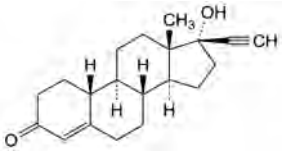

G. 17-hydroxy-19-norpregn-4-en-20-yn-3-one (17-*epi*-norethisterone),

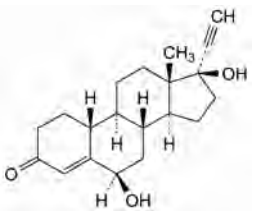

H. 6β,17-dihydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (6β-hydroxynorethisterone).

**01/2017:0850**

# NORETHISTERONE ACETATE

## Norethisteroni acetas

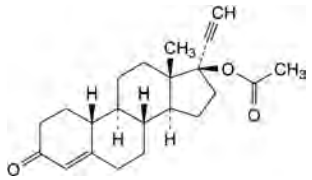

$C_{22}H_{28}O_3$ $M_r$ 340.5
[51-98-9]

DEFINITION

3-Oxo-19-nor-17α-pregn-4-en-20-yn-17-yl acetate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *norethisterone acetate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): – 35 to – 30 (dried substance).

Dissolve 0.500 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with mobile phase A.

*Reference solution (a).* Dissolve the contents of a vial of *norethisterone acetate for system suitability CRS* (containing impurities B, C, D, E, F, G and H) in 1.0 mL of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c).* Dissolve 25.0 mg of *norethisterone acetate CRS* in mobile phase A and dilute to 10.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 μm).

*Mobile phase*:

– *mobile phase A*: *water for chromatography R, acetonitrile R1* (40:60 *V/V*);
– *mobile phase B*: *water for chromatography R, acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 17 | 100 | 0 |
| 17 - 20 | 100 → 0 | 0 → 100 |
| 20 - 39 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm and, for impurities B and C, at 210 nm.

*Injection*: 20 μL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatograms supplied with *norethisterone acetate for system suitability CRS* and the chromatograms obtained with reference solution (a) to identify the peaks due to impurities B, C, D + G, E, F and H.

*Relative retention* with reference to norethisterone acetate (retention time = about 12 min): impurity F = about 0.4; impurities D and G = about 0.6; impurity E = about 0.8; impurity C = about 1.5; impurity B = about 1.6; impurity H = about 2.8.

*System suitability*: reference solution (a) at 210 nm:

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.3; impurity F = 1.7;
– for each impurity, use the concentration of norethisterone acetate in reference solution (b).

*Limits*:

– *impurities B, C at 210 nm*: for each impurity, maximum 0.3 per cent;
– *impurities F, H*: for each impurity, maximum 0.3 per cent;
– *impurity E*: maximum 0.2 per cent;
– *sum of impurities D and G*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{22}H_{28}O_3$ taking into account the assigned content of *norethisterone acetate CRS*.

IMPURITIES

*Specified impurities: B, C, D, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, I, J.*

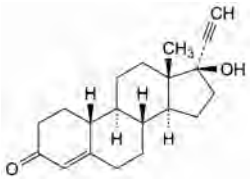

A. 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethisterone),

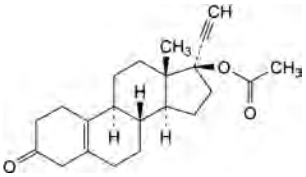

B. 3-oxo-19-nor-17α-pregn-5(10)-en-20-yn-17-yl acetate,

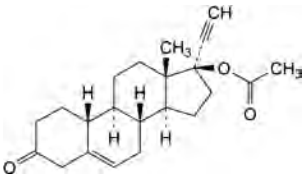

C. 3-oxo-19-nor-17α-pregn-5-en-20-yn-17-yl acetate,

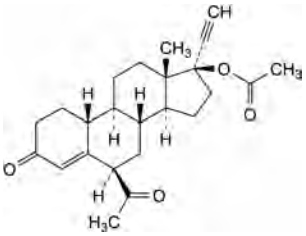

D. 6β-acetyl-3-oxo-19-nor-17α-pregn-4-en-20-yn-17-yl acetate,

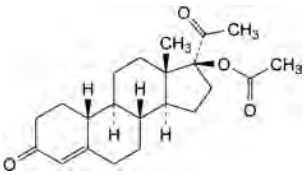

E. 3,20-dioxo-19-nor-17α-pregn-4-en-17-yl acetate,

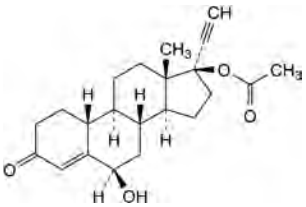

F. 6β-hydroxy-3-oxo-19-nor-17α-pregn-4-en-20-yn-17-yl acetate,

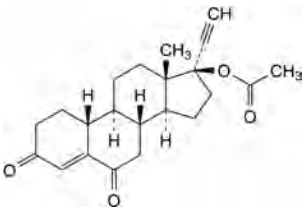

G. 3,6-dioxo-19-nor-17α-pregn-4-en-20-yn-17-yl acetate,

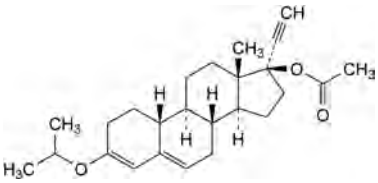

H. 3-(propan-2-yloxy)-19-nor-17α-pregna-3,5-dien-20-yn-17-yl acetate,

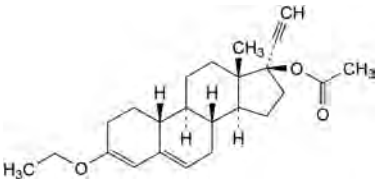

I. 3-ethoxy-19-nor-17α-pregna-3,5-dien-20-yn-17-yl acetate,

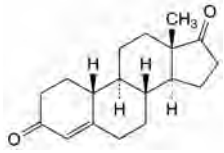

J. estr-4-ene-3,17-dione (norandrostenedione).

**04/2019:1248**

# NORFLOXACIN

## Norfloxacinum

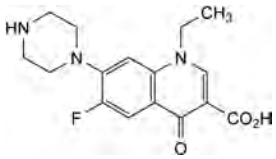

$C_{16}H_{18}FN_3O_3$ $M_r$ 319.3
[70458-96-7]

DEFINITION

1-Ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, hygroscopic, photosensitive, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in acetone and in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *norfloxacin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness in a water-bath at 45 °C, dry in an oven at 105 °C for about 1 h and record new spectra using the residues.

TESTS

**Appearance of solution**. Dissolve 0.5 g in a previously filtered 4 g/L solution of *sodium hydroxide R* in *methanol R* and dilute to 50 mL with the same solution. The solution

is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 5 volumes of *acetonitrile R* and 95 volumes of *water R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 25 mL of the solvent mixture using sonication, and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 4 mg of *norfloxacin for system suitability CRS* (containing impurities A, E and H) in 5 mL of the solvent mixture using sonication, and dilute to 10 mL with the solvent mixture.

*Reference solution (c).* Dissolve 4 mg of *norfloxacin for peak identification CRS* (containing impurity K) in 5 mL of the solvent mixture using sonication, and dilute to 10 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped amidohexadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R* adjusted to pH 2.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 7 | 95 → 93 | 5 → 7 |
| 7 - 10 | 93 → 87 | 7 → 13 |
| 10 - 15 | 87 → 47 | 13 → 53 |
| 15 - 20 | 47 → 10 | 53 → 90 |

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *norfloxacin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, E and H; use the chromatogram supplied with *norfloxacin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity K.

*Relative retention* with reference to norfloxacin (retention time = about 11 min): impurity K = about 0.6; impurity E = about 0.97; impurity A = about 1.5; impurity H = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurities A and H;
- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to norfloxacin.

*Limits*:

- *impurities E, K*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying under high vacuum at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.240 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.93 mg of $C_{16}H_{18}FN_3O_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: E, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, G, H, I, J.*

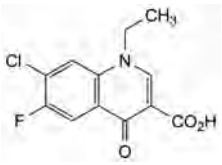


A. 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

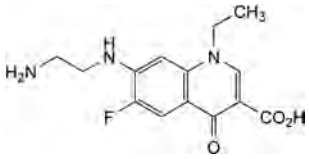


B. 7-[(2-aminoethyl)amino]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

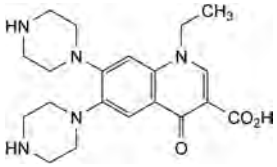


C. 1-ethyl-4-oxo-6,7-di(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

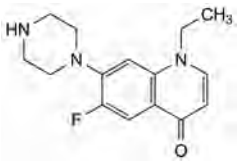


D. 1-ethyl-6-fluoro-7-(piperazin-1-yl)quinolin-4(1*H*)-one,

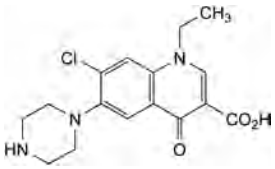

E. 7-chloro-1-ethyl-4-oxo-6-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

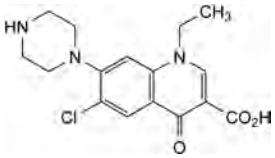

F. 6-chloro-1-ethyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid,

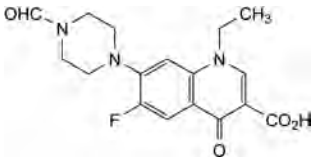

G. 1-ethyl-6-fluoro-7-(4-formylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

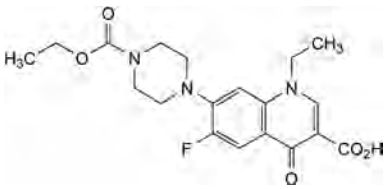

H. 7-[4-(ethoxycarbonyl)piperazin-1-yl]-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

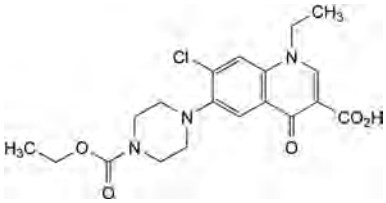

I. 7-chloro-6-[4-(ethoxycarbonyl)piperazin-1-yl]-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

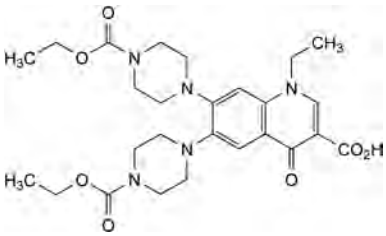

J. 6,7-bis[4-(ethoxycarbonyl)piperazin-1-yl]-1-ethyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

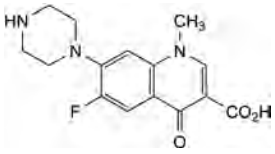

K. 6-fluoro-1-methyl-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid.

**04/2013:2257**
**corrected 10.0**

# NORFLURANE

## Norfluranum

$C_2H_2F_4$ $M_r$ 102.0
[811-97-2]

DEFINITION

1,1,1,2-Tetrafluoroethane (HFC 134a).

CHARACTERS

*Appearance*: clear, colourless gas, liquid under pressure.

*Solubility*: at 20 °C and at a pressure of 101 kPa, slightly soluble in water, freely soluble in ethanol (96 per cent).

*Relative density*: about 1.23 at 20 °C, for the gas in the liquid phase.

*bp*: about − 26 °C.

It is a hygroscopic, non-flammable gas.

IDENTIFICATION

Carry out either test A or test B.

A. Infrared absorption spectrophotometry (*2.2.24*). *Carry out the test at atmospheric pressure.*

*Preparation*: dilute the gas to be examined in *nitrogen R* (approximately 20:80 *V/V* depending on the sensitivity of the spectrophotometer).

*Comparison*: *Ph. Eur. reference spectrum of norflurane.*

B. Mass spectrometry (*2.2.43*).

*Results*: the mass spectrum obtained with the gas to be examined is similar to the mass spectrum of norflurane shown in Figure 2257.-1.

TESTS

**Acidity**: maximum 0.1 ppm, expressed as HCl.

Transfer 200 mL of deionised *water R* previously neutralised to *bromocresol purple solution R* to a glass washing bottle fitted with a distribution tube with a sintered-glass disc. Pass 750 g of the gas to be examined through the water, at a rate of about 60 L/h. Titrate with *0.02 M sodium hydroxide* using *bromocresol purple solution R* as indicator until the colour changes from yellow to bluish-violet. Carry out a blank titration using deionised *water R*.

1 mL of *0.02 M sodium hydroxide* is equivalent to 0.729 mg of HCl.

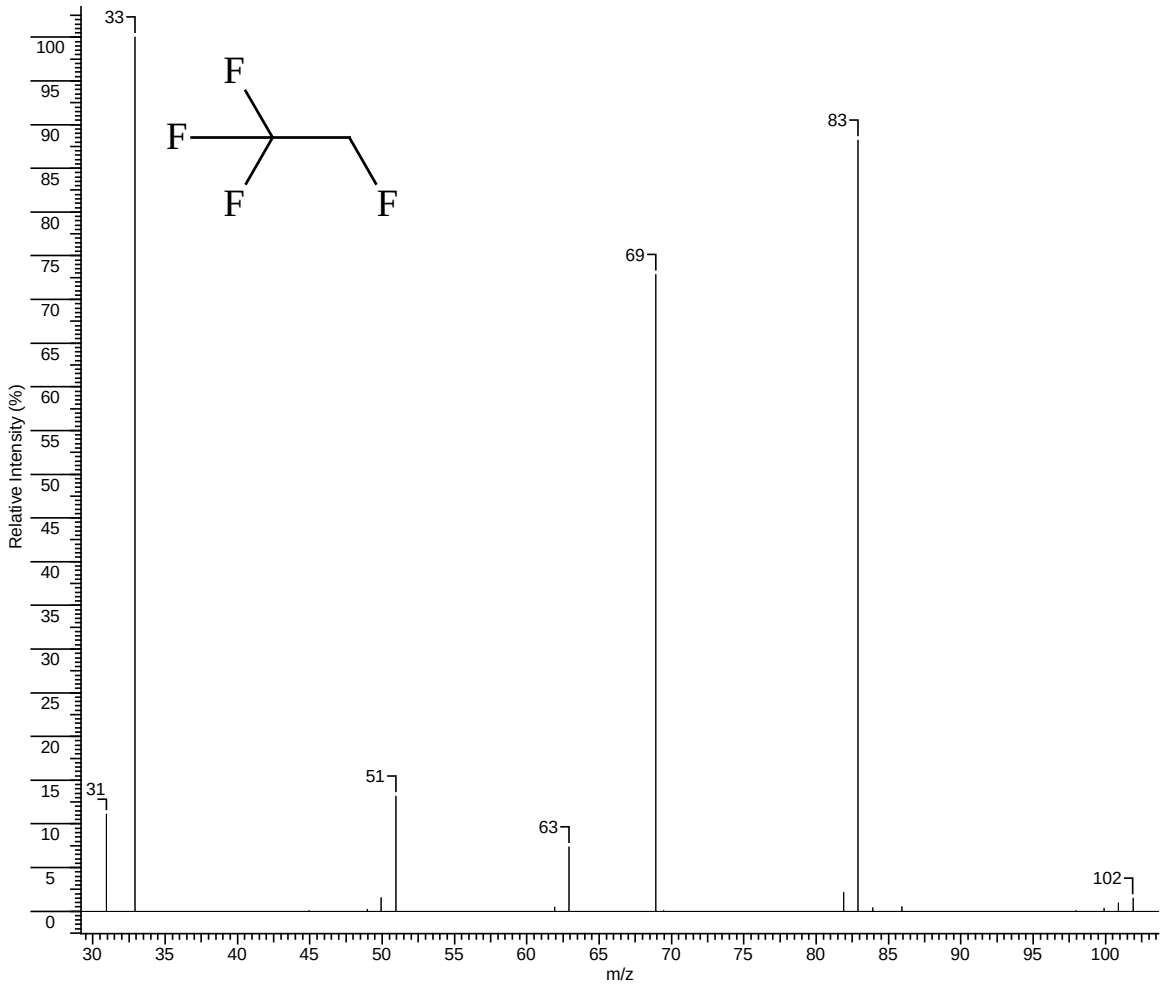


| *m/z* | RI (%) | ion | *m/z* | RI (%) | ion |
|---|---|---|---|---|---|
| 31 | 11.1 | $[CF]^+$ | 82 | 2.1 | $[CF_2\text{-}CHF]^{+\cdot}$ |
| 33 | 100.0 | $[CH_2F]^+$ | 83 | 88.2 | $[CF_2\text{-}CH_2F]^+$ |
| 50 | 1.5 | $[CF_2]^{+\cdot}$ | 100 | 0.3 | $[CF_3\text{-}CF]^{+\cdot}$ |
| 51 | 13.2 | $[CHF_2]^+$ | 101 | 0.9 | $[CF_3\text{-}CHF]^+$ |
| 63 | 7.4 | $[CF{=}CHF]^+$ | 102 | 1.5 | $[CF_3\text{-}CH_2F]^{+\cdot}$ |
| 69 | 72.9 | $[CF_3]^+$ | | | |

Figure 2257.-1. – *Mass spectrum of norflurane*

**Non-volatile matter**: maximum 50 ppm.

Carry out the test using a glass double-wall vessel (see Figure 2257.-2).

Dry the removable part in an oven at 105 ± 2 °C for 30 min. Allow to cool in a desiccator and weigh to the nearest 0.1 mg. Connect it to the vessel.

Weigh the gas cylinder to the nearest 1 g. Fill the vessel with about 500 mL of liquefied gas and weigh the gas cylinder again. Determine the mass of the sample by weight difference. Using a suitable heating device such as a water-bath, heat the removable part such that the sample evaporates in about 2 h.

Dry the removable part in an oven at 105 ± 2 °C for 30 min. Allow to cool in a desiccator and weigh to the nearest 0.1 mg. Determine the mass of the residue by weight difference.

Calculate the content of non-volatile matter in the gas using the following expression:

$$\frac{10^3 \times m}{M}$$

$m$ = mass of residue, in milligrams;

$M$ = mass of sample, in grams.

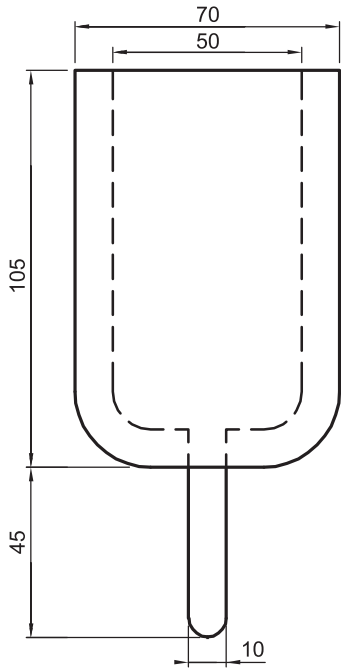


Figure 2257.-2. – *Glass double-wall vessel*
*Dimensions in millimetres*

**Non-condensable gases**: maximum 1.5 per cent *V/V*.

Gas chromatography (*2.2.28*).

*Gas to be examined.* The sample is taken from the vapour phase maintaining the cylinder in an upright position. Evacuate the gas loop using a multiway tap and fill cautiously with the gas to be examined.

*Reference gases.* Mixtures of ambient air in *helium for chromatography R* covering a concentration range of 0.5 per cent to 2.0 per cent.

*Column*:

– *material*: stainless steel;
– *size*: *l* = 5 m, Ø = 2 mm;
– *stationary phase*: *oxypropionitrilsilyl silica gel for chromatography R* (150-180 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 21 mL/min.

*Temperature*:

– *column*: 80 °C;
– *injection port*: 150 °C;
– *detector*: 180 °C.

*Detection*: thermal conductivity.

*Injection*: 150 µL loop injector.

*Run time*: 10 min.

*Relative retention* with reference to norflurane (retention time = about 4 min): non-condensable gases = about 0.4.

Determine the concentration (*V/V*) of non-condensable gases in the gas to be examined using the calibration curve obtained with the reference gases.

**Related substances**. Gas chromatography (*2.2.28*), equipped with a gas valve sampling system and a cryogenic unit, coupled with mass spectrometry (*2.2.43*).

*Gas to be examined.* Connect the cylinder to the gas valve sampling system and sample from the liquid phase. Then evacuate the loop including the transfer line using a multiway tap and a vacuum pump. Open the valves of the cylinder and fill the loop cautiously with the gas to be examined.

*Reference gas (a).* Prepare a mixture in *helium for chromatography R* of the impurities expected in the gas to be examined (see Table 2257.-1) at a concentration of 2-6 ppm each, always including impurity G.

*Reference gas (b).* Prepare a mixture of FC 1318my/c (impurity S) and FC 1318my/t (impurity T) in *helium for chromatography R* at a combined concentration of approximately 20 ppm.

*Reference gas (c).* Prepare a mixture of CFC 114 (impurity L) and HCC 40 (impurity W) in *helium for chromatography R* at a concentration of approximately 1 ppm each.

*Reference gas (d).* Prepare a 5-fold dilution of reference gas (a) in *helium for chromatography R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 60 m, Ø = 0.18 mm;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.1 mL/min.

*Split ratio*: 1:75.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | – 25 |
| | 2 - 7.2 | – 25 → – 12 |
| | 7.2 - 14 | – 12 → 15 |
| | 14 - 18.7 | 15 → 250 |
| | 18.7 - 21.2 | 250 |
| Injection port | | 150 |

*Detection*: mass spectrometer; the following settings were found to be suitable and are given as examples; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria:

– *ionisation mode*: electron impact (70 eV);
– *trap current*: 0.2 mA;
– *mass range*: 30-300 Da;
– *scan rate*: ≤ 0.2 s/scan;
– *temperature*: ion source: 160 °C; transfer line: 200 °C.

*Injection*: 500 µL.

*Identification of impurities*: use the reconstructed ion chromatogram obtained with reference gas (a) and the information supplied in Table 2257.-1.

*System suitability*:

– *resolution*: minimum 1.4 between the peaks due to FC 1318my/c (impurity S) and FC 1318my/t (impurity T) in the chromatogram obtained with reference gas (b);
– *signal-to-noise ratio*: minimum 10 each for CFC 114 (impurity L) and HCC 40 (impurity W) in the chromatogram obtained with reference gas (c).

*Selectivity – co-elution*: several impurities elute with a resolution of less than 1; close inspection of the individual ion traces is necessary to detect and quantify potential co-eluting compounds; the relative retentions of these compounds are indicated in italics in Table 2257.-1.

Before assessing the presence of individual impurities in the reconstructed ion chromatogram, a suitable background subtraction may be necessary. Two potential sources of background have to be taken into account: use of cryo-cooling (liquid carbon dioxide, *m/z* 44) and possible bleeding of the column (siloxanes, several ions at *m/z* greater than 200).

If a peak is observed at a retention time where co-elution is possible, the identification and quantification are applied to each of the co-eluting compounds.

*Calculation of parts per million contents:*

– for impurities A, B, C, D, E, F, G, H and I, use the concentration of the corresponding impurity in reference gas (a);

– for the other impurities, use the concentration of impurity G in reference gas (a);
– for the reporting threshold, use the concentration of impurity G in reference gas (d).

*Limits*:

***Saturated impurities***:
– *HFC 134 (impurity C)*: maximum 1000 ppm;
– *HFC 152a (impurity E)*: maximum 500 ppm;
– *CFC 12 (impurity A), HCFC 124 (impurity B)*: for each impurity, maximum 100 ppm;
– *HFC 143a (impurity D)*: maximum 50 ppm.

***Unsaturated impurities***:
– *CFC 1112a (impurity F), HCFC 1122 (impurity G), HFC 1225ye/c (impurity H), HFC 1243zf (impurity I)*: for each impurity, maximum 5 ppm.

***Other detectable impurities***:
– *other saturated or unsaturated impurities or unknow impurities*: for each impurity, maximum 5 ppm;
– *sum of other detectable impurities*: maximum 10 ppm.

***Total***: maximum 1000 ppm.

***Reporting threshold***: 1 ppm.

**Water** (*2.5.32*): maximum 10 ppm, determined on 30.0 g. *Take care to avoid uptake of water by the gas to be examined during the test.*

Transfer the gas to be examined in liquid phase from the inverted steel cylinder to an evacuated sample can. To transfer the sample, connect a metal tube with fittings at one end to the cylinder valve, and at the other end to the needle valve on the sample can.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, AA, BB, CC, DD, EE, FF, GG, HH, II, JJ, KK, LL, MM, NN, OO, PP, QQ, RR, SS.*

$CCl_2F_2$

A. dichlorodifluoromethane (CFC 12 [75-71-8]),

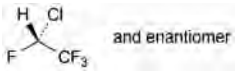


B. (2*RS*)-2-chloro-1,1,1,2-tetrafluoroethane (HCFC 124 [2837-89-0]),

C. 1,1,2,2-tetrafluoroethane (HFC 134 [359-35-3]),

$H_3C-CF_3$

D. 1,1,1-trifluoroethane (HFC 143a [420-46-2]),

E. 1,1-difluoroethane (HFC 152a [75-37-6]),

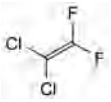


F. 1,1-dichloro-2,2-difluoroethene (CFC 1112a [79-35-6]),

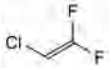


G. 1-chloro-2,2-difluoroethene (HCFC 1122 [359-10-4]),

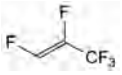


H. (1*Z*)-1,2,3,3,3-pentafluoroprop-1-ene (HFC 1225ye/c [5528-43-8]),

I. 3,3,3-trifluoroprop-1-ene (HFC 1243zf [677-21-4]),

$CCl_3F$

J. trichlorofluoromethane (CFC 11 [75-69-4]),

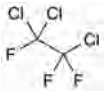


K. 1,1,2-trichloro-1,2,2-trifluoroethane (CFC 113 [76-13-1]),

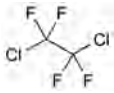


L. 1,2-dichloro-1,1,2,2-tetrafluoroethane (CFC 114 [76-14-2]),

M. 1,1-dichloro-1,2,2,2-tetrafluoroethane (CFC 114a [374-07-2]),

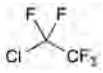


N. 1-chloro-1,1,2,2,2-pentafluoroethane (CFC 115 [76-15-3]),

$CBrClF_2$

O. bromochlorodifluoromethane (CFC 12B1 [353-59-3]),

$CClF_3$

P. chlorotrifluoromethane (CFC 13 [75-72-9]),

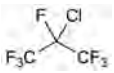


Q. 2-chloro-1,1,1,2,3,3,3-heptafluoropropane (CFC 217ba [76-18-6]),

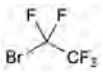


R. 1-bromo-1,1,2,2,2-pentafluoroethane (FC 115B1 [354-55-2]),

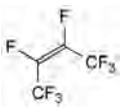


S. (2*Z*)-1,1,1,2,3,4,4,4-octafluorobut-2-ene (FC 1318my/c [1516-65-0]),

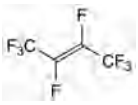

T. (2*E*)-1,1,1,2,3,4,4,4-octafluorobut-2-ene (FC 1318my/t [1516-64-9]),

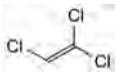

U. 1,1,2-trichloroethene (trichloroethylene, HCC 1120 [79-01-6]),

$CH_2Cl_2$

V. dichloromethane (methylene chloride, HCC 30 [75-09-2]),

$CH_3Cl$

W. chloromethane (methyl chloride, HCC 40 [74-87-3]),

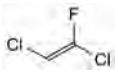

X. (*E*)-1,2-dichloro-1-fluoroethene (HCFC 1121/t),

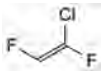

Y. (*Z*)-1-chloro-1,2-difluoroethene (HCFC 1122a/c [359-04-6]),

Z. (*Z*)-1-chloro-2-fluoroethene (HCFC 1131/c [2268-31-7]),

AA. (*E*)-1-chloro-2-fluoroethene (HCFC 1131/t [2268-32-8]),

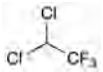

BB. 1,1-dichloro-2,2,2-trifluoroethane (HCFC 123 [306-83-2]),

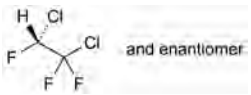


CC. (2*RS*)-1,2-dichloro-1,1,2-trifluoroethane (HCFC 123a [354-23-4]),

DD. 1-chloro-1,1,2,2-tetrafluoroethane (HCFC 124a [354-25-6]),

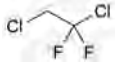

EE. 1,2-dichloro-1,1-difluoroethane (HCFC 132b [1649-08-7]),

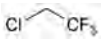

FF. 2-chloro-1,1,1-trifluoroethane (HCFC 133a [75-88-7]),

$CHClF_2$

GG. chlorodifluoromethane (HCFC 22 [75-45-6]),

$CH_2ClF$

HH. chlorofluoromethane (HCFC 31 [593-70-4]),

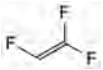

II. 1,1,2-trifluoroethene (HFC 1123 [359-11-5]),

JJ. 1,1-difluoroethene (HFC 1132a [75-38-7]),

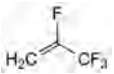

KK. 2,3,3,3-tetrafluoroprop-1-ene (HFC 1234yf [754-12-1]),

LL. (1*E*)-1,3,3,3-tetrafluoroprop-1-ene (HFC 1234ze [1645-83-6]),

MM. 1,1,1,2,2-pentafluoroethane (HFC 125 [354-33-6]),

NN. (2*Z*)-1,1,1,4,4,4-hexafluorobut-2-ene (HFC 1336mzz/c [692-49-9]),

OO. 1,2-difluoroethane (HFC 152 [624-72-6]),

PP. fluoroethane (HFC 161 [353-36-6]),

$CHF_3$

QQ. trifluoromethane (HFC 23 [75-46-7]),

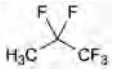

RR. 1,1,1,2,2-pentafluoropropane (HFC 245cb [1814-88-6]),

$CH_2F_2$

SS. difluoromethane (HFC 32 [75-10-5]).

Table 2257.-1. – *Norflurane impurities: quantification ions and relative retentions*

| Impurity | Code | Structure | $M_r$ | Quantification ion *(m/z)* | Relative retention |
|---|---|---|---|---|---|
| P | CFC 13 | $CClF_3$ | 104 | 69 | 0.71 |
| QQ | HFC 23 | $CHF_3$ | 70 | 51 | *0.73* |
| JJ | HFC 1132a | $CH_2=CF_2$ | 64 | 64 | *0.73* |
| N | CFC 115 | $CClF_2$-$CF_3$ | 154 | 85 | 0.77 |
| D | HFC 143a | $CH_3$-$CF_3$ | 84 | 65/69* | *0.81* |
| SS | HFC 32 | $CH_2F_2$ | 52 | 51/33* | *0.81* |
| II | HFC 1123 | $CHF=CF_2$ | 82 | 63 | *0.82* |
| MM | HFC 125 | $CHF_2$-$CF_3$ | 120 | 101 | *0.84* |
| T | FC 1318my/t | $CF_3$-CF=CF-$CF_3$ | 200 | 131 | *0.84* |
| S | FC 1318my/c | $CF_3$-CF=CF-$CF_3$ | 200 | 131 | 0.87 |
| Q | CFC 217ba | $CF_3$-$CClF$-$CF_3$ | 204 | 85 | 0.93 |
| KK | HFC 1234yf | $CH_2=CF$-$CF_3$ | 114 | 114 | 0.96 |
| RR | HFC 245cb | $CH_3$-$CF_2$-$CF_3$ | 134 | 65 | 0.98 |
| Norflurane | **HFC 134a** | **$CH_2F$-$CF_3$** | **102** | **83** | **1** |
| R | FC 115B1 | $CBrF_2$-$CF_3$ | 198 | 119 | 1.03 |
| H | HFC 1225ye/c | $CHF=CF$-$CF_3$ | 132 | 113 | *1.10* |
| E | HFC 152a | $CH_3$-$CHF_2$ | 66 | 65 | *1.11* |
| PP | HFC 161 | $CH_3$-$CH_2F$ | 48 | 33/47* | *1.11* |
| I | HFC 1243zf | $CH_2=CH$-$CF_3$ | 96 | 96 | *1.11* |
| LL | HFC 1234ze | $CHF=CH$-$CF_3$ | 114 | 114 | 1.14 |
| A | CFC 12 | $CCl_2F_2$ | 120 | 85 | 1.17 |
| C | HFC 134 | $CHF_2$-$CHF_2$ | 102 | 51/83* | 1.21 |
| NN | HFC 1336mzz/c | $CF_3$-$CH=CH$-$CF_3$ | 164 | 95 | *1.30* |
| GG | HCFC 22 | $CHClF_2$ | 86 | 51 | *1.32* |
| L | CFC 114 | $CClF_2$-$CClF_2$ | 170 | 85 | *1.63* |
| M | CFC 114a | $CCl_2F$-$CF_3$ | 170 | 101/103* | *1.64* |
| W | HCC 40 | $CH_3Cl$ | 50 | 52 | 1.67 |
| G | HCFC 1122 | $CHCl=CF_2$ | 98 | 98 | 1.72 |
| DD | HCFC 124a | $CHF_2$-$CClF_2$ | 136 | 101 | 1.77 |
| B | HCFC 124 | $CHClF$-$CF_3$ | 136 | 67 | 1.87 |
| HH | HCFC 31 | $CH_2ClF$ | 68 | 68 | 1.97 |
| Y | HCFC 1122a/c | $CHF=CClF$ | 98 | 98 | 2.03 |
| O | CFC 12B1 | $CBrClF_2$ | 164 | 85 | 2.08 |
| AA | HCFC 1131/t | $CHCl=CHF$ | 80 | 80 | 2.19 |
| OO | HFC 152 | $CH_2F$-$CH_2F$ | 66 | 33 | 2.41 |
| FF | HCFC 133a | $CH_2Cl$-$CF_3$ | 118 | 118 | 2.47 |
| F | CFC 1112a | $CCl_2=CF_2$ | 132 | 132 | 2.74 |
| Z | HCFC 1131/c | $CHCl=CHF$ | 80 | 80 | 2.84 |
| J | CFC 11 | $CCl_3F$ | 136 | 101 | 2.97 |
| CC | HCFC 123a | $CHClF$-$CClF_2$ | 152 | 67 | 3.15 |
| BB | HCFC 123 | $CHCl_2$-$CF_3$ | 152 | 83 | *3.18* |
| K | CFC 113 | $CCl_2F$-$CClF_2$ | 186 | 151 | *3.18* |
| X | HCFC 1121/t | $CHCl=CClF$ | 114 | 114 | 3.25 |
| V | HCC 30 | $CH_2Cl_2$ | 84 | 49 | 3.29 |
| EE | HCFC 132b | $CClF_2$-$CH_2Cl$ | 134 | 99 | 3.32 |
| U | HCC 1120 | $CHCl=CCl_2$ | 130 | 95 | 3.59 |

* Depending on the actual chromatographic resolution and potentially overlapping compounds, it may be necessary to select a different quantification ion.

01/2008:1732

# NORGESTIMATE

## Norgestimatum

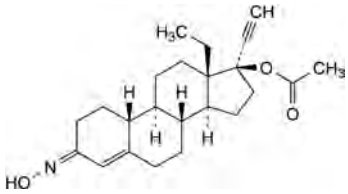

$C_{23}H_{31}NO_3$ $M_r$ 369.5
[35189-28-7]

DEFINITION

(3*EZ*)-13β-Ethyl-3-(hydroxyimino)-18,19-dinor-17α-pregn-4-en-20-yn-17-yl acetate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *norgestimate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 42.0 to + 50.0 (dried substance).

Dissolve 0.200 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *methanol R* (1:4 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2 mg of *norgestimate for system suitability CRS* (containing impurity A) in 4 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *tetrahydrofuran for chromatography R*, *water R* (18:22:60 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 244 nm.

*Injection*: 25 µL.

*Run time*: twice the retention time of the (*E*)-isomer of norgestimate.

*Identification of impurities*: use the chromatogram supplied with *norgestimate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to the (*E*)-isomer of norgestimate (retention time = about 14 min): impurity A = about 0.7; (*Z*)-isomer of norgestimate = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to the (*E*)- and (*Z*)-isomers of norgestimate.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of the (*Z*)-isomer of norgestimate by 1.33;
- *impurity A*: not more than twice the sum of the areas of the peaks due to the (*E*)- and (*Z*)-isomers of norgestimate in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the sum of the areas of the peaks due to the (*E*)- and (*Z*)-isomers of norgestimate in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the sum of the areas of the peaks due to the (*E*)- and (*Z*)-isomers of norgestimate in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the sum of the areas of the peaks due to the (*E*)- and (*Z*)-isomers of norgestimate in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Ratio of (*E*)- to (*Z*)-isomers**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution.

Calculate the (*E*)- to (*Z*)-isomer ratio by dividing the area of the peak due to the (*E*)-isomer by 1.33 times the area of the peak due to the (*Z*)-isomer. The ratio is 1.27 to 1.78.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.300 g in 40 mL of *tetrahydrofuran R*. Add 10 mL of a 100 g/L solution of *silver nitrate R* and titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Rinse the electrode with *acetone R* after each titration.

If necessary, after several titrations re-equilibrate the electrode in *water R* for 15 min to obtain sharper titration curves.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.95 mg of $C_{23}H_{31}NO_3$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

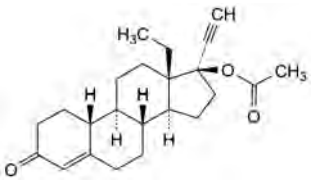

A. 13β-ethyl-3-oxo-18,19-dinor-17α-pregn-4-en-20-yn-17-yl acetate (levonorgestrel acetate),

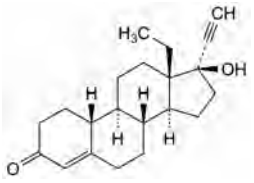

B. 13β-ethyl-17β-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one (levonorgestrel),

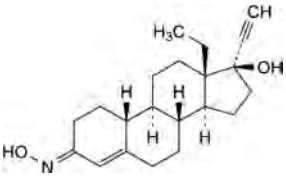

C. (3*E*)-13β-ethyl-3-(hydroxyimino)-18,19-dinor-17α-pregn-4-en-20-yn-17-ol ((*E*)-norelgestromin),

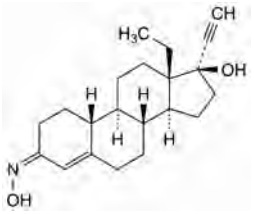

D. (3*Z*)-13β-ethyl-3-(hydroxyimino)-18,19-dinor-17α-pregn-4-en-20-yn-17-ol ((*Z*)-norelgestromin).

**01/2008:0940**
**corrected 6.0**

# NORGESTREL

## Norgestrelum

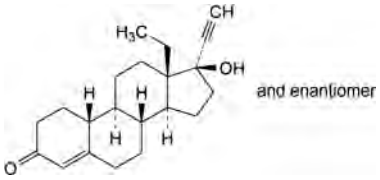


$C_{21}H_{28}O_2$ $M_r$ 312.5
[6533-00-2]

DEFINITION

Norgestrel contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of *rac*-13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in alcohol.

IDENTIFICATION

A. Dissolve 0.5 g in *methylene chloride R* and dilute to 10.0 mL with the same solvent. The angle of optical rotation (*2.2.7*) is + 0.05° to − 0.05°.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *norgestrel CRS*.

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. Dissolve 0.2 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dilute 1 mL of the test solution to 10 mL with *methylene chloride R*. Dilute 1 mL of this solution to 20 mL with *methylene chloride R*.

*Reference solution (b)*. Dilute 4 mL of reference solution (a) to 10 mL with *methylene chloride R*.

*Reference solution (c)*. Dissolve 5 mg of *norgestrel CRS* and 5 mg of *ethinylestradiol CRS* in *methylene chloride R* and dilute to 50 mL with the same solvent.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *ethyl acetate R* and 80 volumes of *methylene chloride R*. Allow the plate to dry in air, spray with a 100 g/L solution of *phosphomolybdic acid R* in *alcohol R*, heat at 100-105 °C for 15 min and examine immediately. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and at most two such spots are more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash**(*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 45 mL of *tetrahydrofuran R*. Add 10 mL of a 100 g/L solution of *silver nitrate R*. After 1 min, titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.25 mg of $C_{21}H_{28}O_2$.

STORAGE

Store protected from light.

**04/2019:0941**

# NORTRIPTYLINE HYDROCHLORIDE

## Nortriptylini hydrochloridum

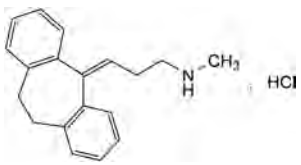

$C_{19}H_{22}ClN$ $M_r$ 299.8
[894-71-3]

DEFINITION

3-(10,11-Dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-ylidene)-*N*-methylpropan-1-amine hydrochloride.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *nortriptyline hydrochloride CRS*.

B. Dissolve 20 mg in 2 mL of *water R*, acidify with 1 drop of *dilute sulfuric acid R* and add 1 mL of a 6.25 g/L solution of *silver sulfate R*. Shake and allow to stand. A curdled, white precipitate is formed. Centrifuge and wash the precipitate with 3 quantities, each of 1 mL, of a 0.5 per cent *V/V* solution of *sulfuric acid R*. Carry out this operation rapidly in subdued light, disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in *water R* and add *ammonia R*. Shake vigorously for at least 1 min. The precipitate dissolves easily with the possible exception of a few large particles which dissolve slowly.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* with gentle heating and dilute to 25 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.2 g with gentle heating in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *dibenzosuberone CRS* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 20 mg of *norcyclobenzaprine CRS* (impurity B) in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 1 mL of the solution to 10 mL with the mobile phase.

*Reference solution (d).* Dissolve 10 mg of *nortriptyline for system suitability CRS* (containing impurity D) in the mobile phase, add 1 mL of reference solution (c) and dilute to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: mix 70 volumes of *methanol R1* and 30 volumes of a solution prepared as follows: mix 3.25 mL of *tetrabutylammonium hydroxide solution (400 g/L) R*, 0.68 g of *potassium dihydrogen phosphate R* and about 900 mL of *water for chromatography R*, then adjust to pH 7.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of nortriptyline.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram supplied with *nortriptyline for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity D.

*Relative retention* with reference to nortriptyline (retention time = about 13 min): impurity A = about 0.5; impurity D = about 0.8; impurity B = about 0.9.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.4 between the peaks due to impurities D and B; minimum 2.0 between the peaks due to impurity B and nortriptyline.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 1.7;
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *ethanol (96 per cent) R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 29.98 mg of $C_{19}H_{22}ClN$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E, F, G, H, I, J.*

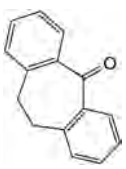


A. 10,11-dihydro-5*H*-dibenzo[*a,d*][7]annulen-5-one (dibenzosuberone),

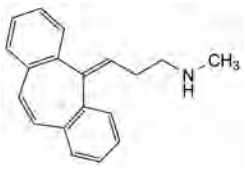

B. 3-(5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*-methylpropan-1-amine (norcyclobenzaprine),

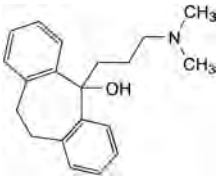

D. 5-[3-(dimethylamino)propyl]-10,11-dihydro-5*H*-dibenzo-[*a*,*d*][7]annulen-5-ol,

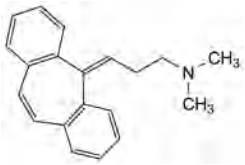

E. 3-(5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*,*N*-dimethylpropan-1-amine (cyclobenzaprine),

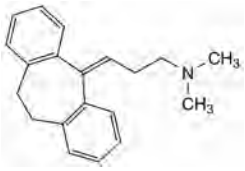

F. 3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-*N*,*N*-dimethylpropan-1-amine (amitriptyline),

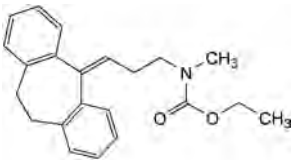

G. ethyl *N*-[3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)propyl]-*N*-methylcarbamate,

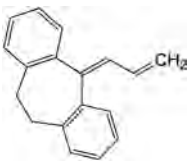

H. 5-prop-2-en-1-ylidene-10,11-dihydro-5*H*-dibenzo-[*a*,*d*][7]annulene,

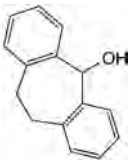

I. 10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ol (dibenzosuberol),

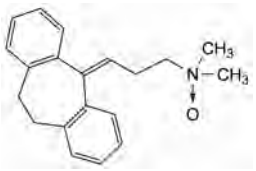

J. [3-(10,11-dihydro-5*H*-dibenzo[*a*,*d*][7]annulen-5-ylidene)-propyl]dimethylamine *N*-oxide (amitriptyline-*N*-oxide).

**04/2011:0516**

# NOSCAPINE

## Noscapinum

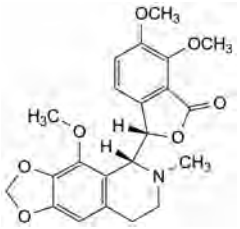

$C_{22}H_{23}NO_7$ $M_r$ 413.4
[128-62-1]

DEFINITION

(3*S*)-6,7-Dimethoxy-3-[(5*R*)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in strong acids; on dilution of the solution with water, the base may be precipitated.

IDENTIFICATION

*First identification: C, E.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 174 °C to 177 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *noscapine CRS*.

D. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in *acetone R* and dilute to 100 mL with the same solvent.
*Reference solution*. Dissolve 25 mg of *noscapine CRS* in *acetone R* and dilute to 100 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (1:3:20:20 *V/V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *dilute potassium iodobismuthate solution R*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. To 20 mg add 10 mL of *water R* and shake. It does not dissolve.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.2 g in *acetone R* and dilute to 10 mL with the same solvent. Examine immediately after dissolution.

**Specific optical rotation** (*2.2.7*): + 42 to + 48 (dried substance).

Dissolve 0.500 g in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in 8 mL of *methanol R*, with the aid of ultrasound, and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *papaverine hydrochloride R* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 1.5 mg of *papaverine hydrochloride R* in 10 mL of the test solution and dilute to 25 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *nitrile silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *phosphate buffer solution pH 6.0 R1* (350:650 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of noscapine.

*Relative retention* with reference to noscapine (retention time = about 10 min): impurity A = about 1.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 2 between the peaks due to noscapine and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *any other impurity*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *sum of impurities other than A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 40 mL of *anhydrous acetic acid R*, warming gently. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.34 mg of $C_{22}H_{23}NO_7$.

STORAGE

Protected from light.

IMPURITIES

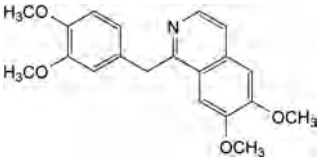

A. 1-(3,4-dimethoxybenzyl)-6,7-dimethoxyisoquinoline (papaverine).

**07/2015:0515**

# NOSCAPINE HYDROCHLORIDE HYDRATE

## Noscapini hydrochloridum hydricum

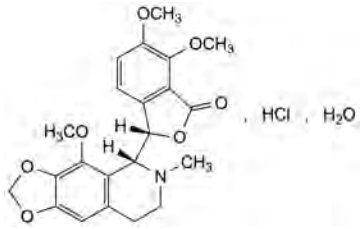

$C_{22}H_{24}ClNO_7,H_2O$ $M_r$ 467.9

DEFINITION

(3*S*)-6,7-Dimethoxy-3-[(5*R*)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]-2-benzofuran-1(3*H*)-one hydrochloride hydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: freely soluble in water and in ethanol (96 per cent). Aqueous solutions are slightly acid; the base may be precipitated when the solutions are allowed to stand.

mp: about 200 °C, with decomposition.

IDENTIFICATION

*First identification: C, E.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*) of the precipitate obtained in identification test E: 174 °C to 177 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the precipitate obtained in identification test E.

*Comparison*: *noscapine CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent.

*Reference solution.* Dissolve 22 mg of *noscapine CRS* in *acetone R* and dilute to 100 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *acetone R*, *toluene R* (1:3:20:20 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *dilute potassium iodobismuthate solution R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

E. Dissolve about 40 mg in a mixture of 2 mL of *water R* and 3 mL of *ethanol (96 per cent) R* and add 1 mL of *dilute ammonia R2*. Heat until dissolution is complete. Allow to cool, scratching the wall of the tube with a glass rod. Filter. The filtrate gives reaction (a) of chlorides (*2.3.1*). Wash the precipitate with *water R*, dry at 100-105 °C and reserve for identification tests B and C.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R*, add 0.3 mL of *0.1 M hydrochloric acid* and dilute to 25 mL with *water R*.

**pH** (*2.2.3*): minimum 3.0.

Dissolve 0.2 g in 10 mL of *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 38.5 to + 44.0 (dried substance).

Dissolve 0.500 g in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *methanol R*, with the aid of ultrasound, and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5.0 mg of *papaverine hydrochloride CRS* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase (solution A). Dilute 1.0 mL of solution A to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 6.0 mL of solution A to 10.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *cyanosilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *phosphate buffer solution pH 6.0 R1* (35:65 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of noscapine.

*Relative retention* with reference to noscapine (retention time = about 10 min): impurity A = about 1.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 2 between the peaks due to noscapine and impurity A.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *any other impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *sum of impurities other than A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): 2.5 per cent to 6.5 per cent, determined on 0.200 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid over heating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.400 g in a mixture of 3 mL of *anhydrous formic acid R* and 30 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 44.99 mg of $C_{22}H_{24}ClNO_7$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

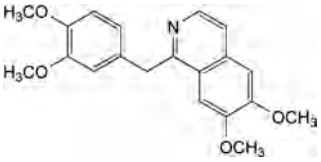

A. 1-[(3,4-dimethoxyphenyl)methyl]-6,7-dimethoxyisoquinoline (papaverine).

**01/2019:0517**
**corrected 10.0**

# NYSTATIN

## Nystatinum

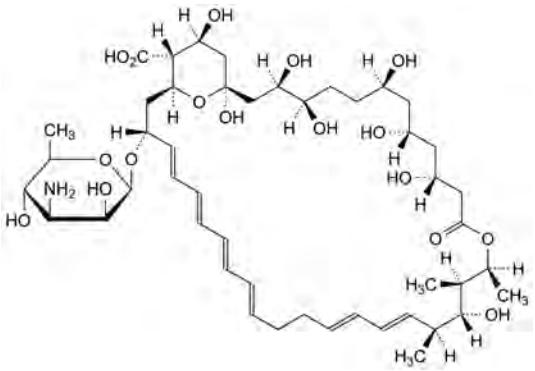

$C_{47}H_{75}NO_{17}$ $M_r$ 926

DEFINITION

Antifungal substance obtained by fermentation using certain strains of *Streptomyces noursei* as the production micro-organism. It contains mainly tetraenes, the principal component being (1*S*,3*R*,4*R*,7*R*,9*R*,11*R*,15*S*,16*R*,17*R*,18*S*,19*E*,21*E*,25*E*,27*E*,29*E*,31*E*,33*R*,35*S*,36*R*,37*S*)-33-[(3-amino-3,6-dideoxy-β-D-mannopyranosyl)oxy]-1,3,4,7,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo-[33.3.1]nonatriaconta-19,21,25,27,29,31-hexaene-36-carboxylic acid (nystatin A1).

*Content*: minimum 4400 IU/mg (dried substance) and minimum 5000 IU/mg (dried substance) if intended for oral administration.

CHARACTERS

*Appearance*: yellow or slightly brownish powder, hygroscopic.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide and in dimethyl sulfoxide, slightly soluble in methanol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D.*

A. Examine the solution prepared in the test for absorbance between 220 nm and 350 nm (*2.2.25*). The solution shows 4 absorption maxima at 230 nm, 291 nm, 305 nm and 319 nm, and a shoulder at 280 nm. The ratios of the absorbances at the absorption maxima at 291 nm and 319 nm to the absorbance at the absorption maximum at 305 nm are 0.61 to 0.73 and 0.83 to 0.96, respectively. The ratio of the absorbance measured at the absorption maximum at 230 nm to that measured at the shoulder at 280 nm is 0.83 to 1.25.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *nystatin CRS*.

C. To about 2 mg add 0.1 mL of *hydrochloric acid R*. A brown colour develops.

D. To about 2 mg add 0.1 mL of *sulfuric acid R*. A brown colour develops that becomes violet on standing.

E. Examine the chromatograms obtained in the test for composition.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Absorbance** (*2.2.25*). Dissolve 0.10 g in a mixture of 5.0 mL of *glacial acetic acid R* and 50 mL of *methanol R* and dilute to 100.0 mL with *methanol R*. Dilute 1.0 mL of the solution to 100.0 mL with *methanol R*. Determined at the maximum at 305 nm within 30 min of preparation of the solution, the absorbance is not less than 0.60.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Carry out the test protected from light.*

*Test solution.* Dissolve 20 mg of the substance to be examined in *dimethyl sulfoxide R* and dilute to 50 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *nystatin CRS* in *dimethyl sulfoxide R* and dilute to 50 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of the substance to be examined in 25 mL of *methanol R* and dilute to 50 mL with *water R*. To 10.0 mL of the solution add 2.0 mL of *dilute hydrochloric acid R*. Allow to stand at room temperature for 1 h.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *dimethyl sulfoxide R*. Dilute 1.0 mL of this solution to 10.0 mL with *dimethyl sulfoxide R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase: base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R*, 3.85 g/L solution of *ammonium acetate R* (29:71 *V/V*),
- *mobile phase B*: 3.85 g/L solution of *ammonium acetate R*, *acetonitrile R* (40:60 *V/V*),

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 35 | 100 → 0 | 0 → 100 |
| 35 - 45 | 0 | 100 |
| 45 - 50 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 305 nm.

*Injection*: 20 µL

*Retention time*: nystatin A1 = about 14 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.5 between the 2 principal peaks (retention time = about 13 min and 19 min).

*Composition*:
- *nystatin A1*: minimum 85.0 per cent,
- *any other compound*: maximum 4.0 per cent,
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c); disregard any peak with a retention time of less than 2 min.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.1 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 3.5 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). *Protect the solutions from light throughout the assay.*

Dissolve the substance to be examined and *nystatin CRS* separately in *dimethylformamide R* and dilute with a mixture of 5 volumes of *dimethylformamide R* and 95 volumes of buffer solution pH 6.0.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states where applicable, that the substance is only for cutaneous use.

# O

Octoxinol 10.. 3405
Octreotide.. 3405
Octyl gallate.. 3407
Octyldodecanol.. 3407
Ofloxacin 3408
Olanzapine.. 3410
Oleic acid.. 3411
Oleoyl macrogolglycerides.. 3411
Oleyl alcohol 3412
Olive oil, refined.. 3413
Olive oil, virgin.. 3414
Olmesartan medoxomil.. 3415
Olsalazine sodium.. 3417
Omega-3-acid ethyl esters 60.. 3419
Omega-3-acid ethyl esters 90.. 3421
Omega-3-acid triglycerides.. 3423
Omeprazole.. 3425
Omeprazole magnesium.. 3427
Omeprazole sodium.. 3428
Ondansetron hydrochloride dihydrate.. 3430
Orbifloxacin for veterinary use.. 3431
Orciprenaline sulfate.. 3433
Orphenadrine citrate.. 3434
Orphenadrine hydrochloride.. 3436
Oseltamivir phosphate.. 3437
Ouabain.. 3439
Oxacillin sodium monohydrate.. 3440
Oxaliplatin.. 3442
Oxazepam.. 3445
Oxcarbazepine 3446
Oxeladin hydrogen citrate.. 3448
Oxfendazole for veterinary use.. 3449
Oxitropium bromide.. 3450
Oxolinic acid.. 3451
Oxybuprocaine hydrochloride.. 3453
Oxybutynin hydrochloride.. 3454
Oxycodone hydrochloride.. 3455
Oxygen.. 3456
Oxygen (93 per cent) 3457
Oxymetazoline hydrochloride 3459
Oxytetracycline dihydrate 3460
Oxytetracycline hydrochloride 3462
Oxytocin.. 3464
Oxytocin concentrated solution 3465

**01/2017:1553**

# OCTOXINOL 10

## Octoxinolum 10

DEFINITION

α-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-ω-hydroxydeca(oxyethylene).

Mixture consisting mainly of mono-octylphenyl ethers of macrogols corresponding to the formula $C_8H_{17}C_6H_4$-$[OCH_2$-$CH_2]_n$-OH where the average value of *n* is 10. It may contain free macrogols.

CHARACTERS

*Appearance*: clear, colourless or light yellow, viscous liquid.

*Solubility*: miscible with water, with ethanol (96 per cent) and with vegetable oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *octoxinol 10 CRS*.

*Preparation*: film between *sodium chloride R* plates.

B. Cloud point (see Tests).

TESTS

**Acidity or alkalinity**. Boil 1.0 g with 20 mL of *carbon dioxide-free water R* for 1 min, with constant stirring. Cool and filter. To 10 mL of the filtrate, add 0.05 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Hydroxyl value** (*2.5.3, Method A*): 85 to 101.

**Cloud point**: 63 °C to 70 °C.

Dissolve 1.0 g in 99 g of *water R*. Transfer about 30 mL of this solution to a test-tube, heat on a water-bath and stir continuously until the solution becomes cloudy. Remove the test-tube from the water-bath (ensuring that the temperature does not increase more than 2 °C), and continue to stir. The cloud point is the temperature at which the solution becomes sufficiently clear that the entire thermometer bulb is plainly seen.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Total ash** (*2.4.16*): maximum 0.4 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

**01/2020:2414**

# OCTREOTIDE

## Octreotidum

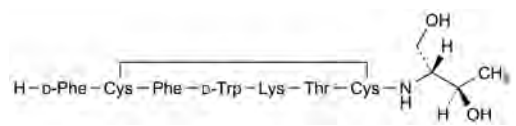


$C_{49}H_{66}N_{10}O_{10}S_2$ $M_r$ 1019

[83150-76-9]

DEFINITION

D-Phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-L-cysteinyl-L-threoninol cyclic (2→7)-disulfide.

Synthetic octapeptide analogue of the natural hormone somatostatin. It is available as an acetate.

*Content*: 95.0 per cent to 103.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water, in acetic acid and in methanol.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

B. Nuclear magnetic resonance spectrometry (*2.2.64*).

*Preparation*: 2 mg/mL solution in a mixture of 10 volumes of *deuterated acetic acid R* and 90 volumes of *deuterium oxide R* containing 30 µg/mL of *deuterated sodium trimethylsilylpropionate R*.

*Comparison*: 2 mg/mL solution of *octreotide for NMR identification CRS* in a mixture of 10 volumes of *deuterated acetic acid R* and 90 volumes of *deuterium oxide R* containing 30 µg/mL of *deuterated sodium trimethylsilylpropionate R*.

*Operating conditions*:

– *field strength*: minimum 300 MHz;

– *temperature*: 25 °C.

*Results*: examine the $^1$H NMR spectrum from 0 to 8 ppm. The $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *octreotide for NMR identification CRS*.

C. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking 1/4 of the sum of the number of moles of phenylalanine, threonine and lysine as equal to 1. The values fall within the following limits: threonine: 0.7 to 1.1; threoninol: 0.7 to 1.2; lysine: 0.9 to 1.3; half-cystine: 1.0 to 2.2; phenylalanine: 1.8 to 2.2. Not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): – 18.5 to – 14.5 (anhydrous and acetic acid-free substance).

Dissolve the substance to be examined in a 1 per cent *V/V* solution of *glacial acetic acid R* to obtain a concentration of 2.0 mg/mL.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*. Mix 10 volumes of *acetonitrile R* and 90 volumes of *water R*, then adjust to pH 3.5 with *acetic acid R*.

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution*. Dissolve the contents of a vial of *octreotide CRS* in the solvent mixture to obtain a concentration of 1.0 mg/ml.

*Resolution solution*. Dissolve the contents of a vial of *octreotide impurity mixture CRS* (containing impurities F and G) in 1.0 mL of the reference solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 4.5 g of *tetramethylammonium hydroxide R* in 800 mL of *water for chromatography R* and adjust to pH 2.0 with *phosphoric acid R*; dilute to 900 mL with *water for chromatography R* and add 100 mL of *acetonitrile R1*;

– *mobile phase B*: dissolve 4.5 g of *tetramethylammonium hydroxide R* in 300 mL of *water for chromatography R* and adjust to pH 2.0 with *phosphoric acid R*; dilute to 400 mL with *water for chromatography R* and add 600 mL of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 80 | 10 → 20 |
| 15 - 40 | 80 → 55 | 20 → 45 |
| 40 - 45 | 55 → 30 | 45 → 70 |

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *octreotide impurity mixture CRS* and the chromatogram obtained with the resolution solution to identify the peaks due to impurities F and G.

*Relative retention* with reference to octreotide (retention time = about 20 min): impurity A = about 0.76; impurity B = about 0.89; impurity C = about 0.94; impurity E = about 1.13; impurity F = about 1.30; impurity G = about 1.33; impurity H = about 1.66; impurity I = about 1.88.

*System suitability*: resolution solution:

– *resolution*: minimum 2.0 between the peaks due to impurities F and G.

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.5 per cent;

– *total*: maximum 2.0 per cent;

– *reporting threshold*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 5.0 per cent to 12.8 per cent.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 10.0 per cent, determined on 20.0 mg.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution.

Calculate the percentage content of octreotide ($C_{49}H_{66}N_{10}O_{10}S_2$) taking into account the assigned content of $C_{49}H_{66}N_{10}O_{10}S_2$ in *octreotide CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states the octreotide content ($C_{49}H_{66}N_{10}O_{10}S_2$).

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F, G, H, I.*

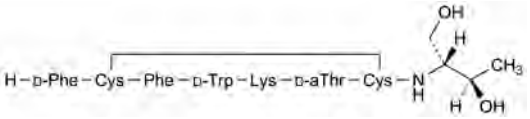


A. [6-D-allothreonine]octreotide,

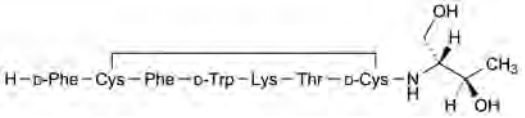


B. [7-D-cysteine]octreotide,

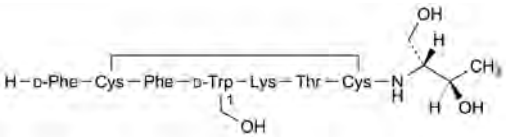


C. $N^{1.4}$-(hydroxymethyl)octreotide,

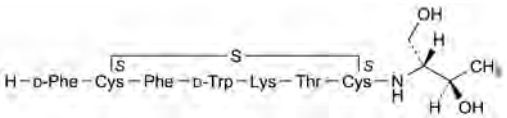


E. D-phenylalanyl-*S*-sulfanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-L-cysteinyl-L-threoninol cyclic (2 → 7)-trisulfide,

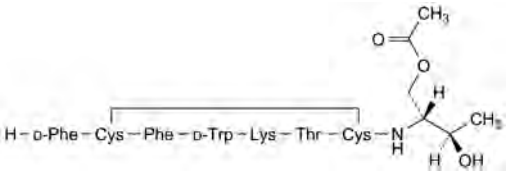


F. $O^{1.8}$-acetyloctreotide,

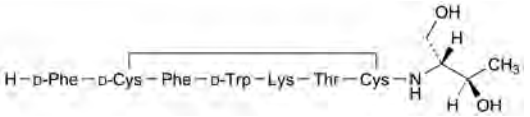


G. [2-D-cysteine]octreotide,

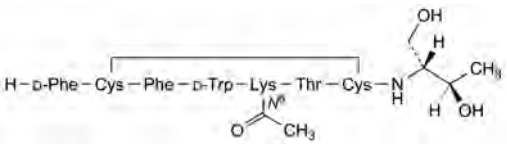


H. $N^{6.5}$-acetyloctreotide,

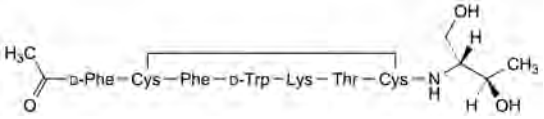


I. $N^{2.1}$-acetyloctreotide.

**01/2017:2057**

# OCTYL GALLATE

## Octylis gallas

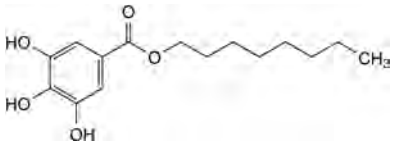

$C_{15}H_{22}O_5$ $M_r$ 282.3
[1034-01-1]

DEFINITION

Octyl 3,4,5-trihydroxybenzoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Melting point (*2.2.14*).
Determine the melting point of the substance to be examined. Mix equal parts of the substance to be examined and *octyl gallate CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 101 °C) is not greater than 2 °C.

B. Examine the chromatograms obtained in the test for impurity A.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20 mL with *acetone R*.

*Reference solution (a).* Dissolve 10 mg of *octyl gallate CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *gallic acid R* in *acetone R* and dilute to 20 mL with the same solvent.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10 mL with *acetone R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (b) to 5 mL with test solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *anhydrous formic acid R*, *ethyl formate R*, *toluene R* (10:40:50 *V/V/V*).

*Application*: 5 µL of test solutions (a) and (b) and reference solutions (a), (c) and (d).

*Development*: over 2/3 of the plate.

*Drying*: in air for 10 min.

*Detection*: spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol (96 per cent) R*.

*System suitability*: reference solution (d):
– the chromatogram shows 2 clearly separated principal spots.

*Limit*: test solution (a):
– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.5 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.
To 1.65 g add 50 mL of *water R*. Shake for 5 min. Filter. 15 mL of the filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 70 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 250.0 mL with the same solvent. Dilute 5.0 mL of the solution to 200.0 mL with *methanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 275 nm.
Calculate the content of $C_{15}H_{22}O_5$ taking the specific absorbance to be 387.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

*Specified impurities: A.*

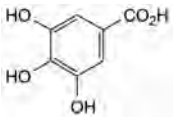

A. 3,4,5-trihydroxybenzoic acid (gallic acid).

**01/2017:1136**
**corrected 10.0**

# OCTYLDODECANOL

## Octyldodecanolum

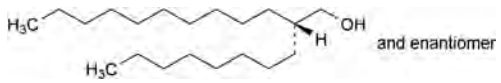


[5333-42-6]

DEFINITION

Condensation product of saturated liquid fatty alcohols.

*Content*: minimum 90 per cent of (2*RS*)-2-octyldodecan-1-ol ($C_{20}H_{42}O$; $M_r$ 298.6), the remainder consisting mainly of related alcohols.

CHARACTERS

*Appearance*: clear, colourless or yellowish, oily liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent).

*Relative density*: about 0.840.

*Refractive index*: about 1.455.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 0.20 g of the substance to be examined in *toluene R* and dilute to 20 mL with the same solvent.
*Reference solution.* Dissolve 0.20 g of *octyldodecanol CRS* in *toluene R* and dilute to 20 mL with the same solvent.

*Plate*: suitable silica gel plate.

*Mobile phase*: *ethyl acetate R*, *toluene R* (5:95 *V/V*).

*Application*: 2 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: spray with about 7 mL of a mixture of 1 volume of a 25 g/L solution of *vanillin R* in *ethanol (96 per cent) R* and 4 volumes of *sulfuric acid R* and heat at 130 °C for 5-10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Acidity or alkalinity**. Mix 5.0 g thoroughly for 1 min with a mixture of 0.1 mL of *bromothymol blue solution R1*, 2 mL of *heptane R* and 10 mL of *water R*. If the aqueous layer is blue, not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow. If the aqueous layer is yellow, add 0.45 mL of *0.01 M sodium hydroxide* and shake vigorously. After standing to ensure complete separation, the aqueous layer is blue.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 2.50 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

**Hydroxyl value** (*2.5.3, Method A*): 175 to 190.

**Iodine value** (*2.5.4, Method A*): maximum 8.0.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Saponification value** (*2.5.6*): maximum 5.0.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.4 g of *tetradecane R* in *hexane R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the same solution.

*Reference solution.* Dissolve 0.100 g of *octyldodecanol CRS* in the internal standard solution and dilute to 10.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.68 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 180 |
| | 2 - 22 | 180 → 280 |
| | 22 - 52 | 280 |
| Injection port | | 290 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

Calculate the content of $C_{20}H_{42}O$ in the substance to be examined.

STORAGE

Protected from light.

**01/2017:1455**

# OFLOXACIN

## Ofloxacinum

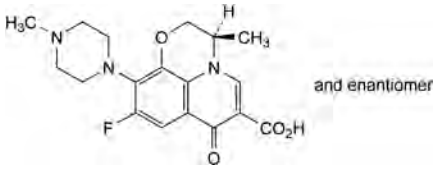


$C_{18}H_{20}FN_3O_4$ $M_r$ 361.4

[82419-36-1]

DEFINITION

(3*RS*)-9-Fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow or bright yellow, crystalline powder.

*Solubility*: slightly soluble in water, soluble in glacial acetic acid, slightly soluble or soluble in methylene chloride, slightly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ofloxacin CRS*.

TESTS

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 0.300 g in a mixture of 10 volumes of *methanol R* and 40 volumes of *methylene chloride R* and dilute to 10.0 mL with the same mixture of solvents.

**Absorbance** (*2.2.25*): maximum 0.25 at 440 nm.

Dissolve 0.5 g in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (10:40 *V/V*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution.* Dissolve 10.0 mg of *ofloxacin impurity A CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Plate*: *TLC silica gel $GF_{254}$ plate R* (2-10 µm).

*Mobile phase*: *glacial acetic acid R*, *water R*, *ethyl acetate R* (10:10:20 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Limit*:

- *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (10:60 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *ofloxacin impurity E CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Mix 10 mL of the solution and 5 mL of the test solution and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: dissolve 4.0 g of *ammonium acetate R* and 7.0 g of *sodium perchlorate R* in 1300 mL of *water R*; adjust to pH 2.2 with *phosphoric acid R* and add 240 mL of *acetonitrile R*.

*Flow rate*: adjust so that a retention time of about 20 min is obtained for ofloxacin.

*Detection*: spectrophotometer at 294 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of ofloxacin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to ofloxacin (retention time = about 20 min): impurity B = about 0.3; impurity C = about 0.5; impurity D = about 0.7; impurity E = about 0.9; impurity F = about 1.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity E and ofloxacin.

*Limits*:

- *impurities B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 100 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.14 mg of $C_{18}H_{20}FN_3O_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

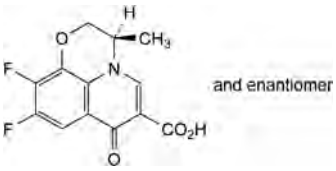


A. (*3RS*)-9,10-difluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid (FPA),

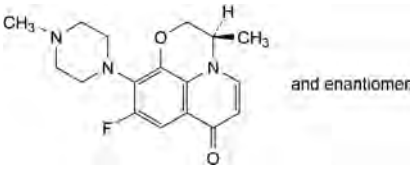


B. (3*RS*)-9-fluoro-3-methyl-10-(4-methylpiperazin-1-yl)-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazin-7-one,

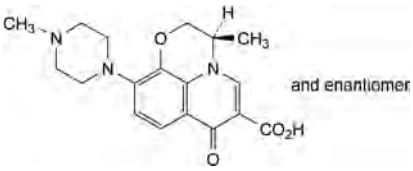


C. (3*RS*)-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid,

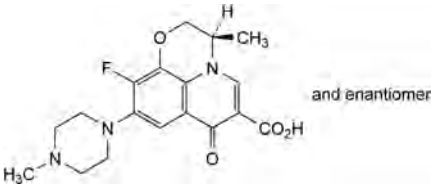


D. (3*RS*)-10-fluoro-3-methyl-9-(4-methylpiperazin-1-yl)-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid,

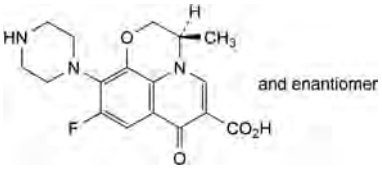


E. (3*RS*)-9-fluoro-3-methyl-7-oxo-10-(piperazin-1-yl)-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazine-6-carboxylic acid,

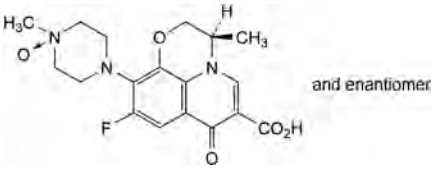


F. 4-[(3*RS*)-6-carboxy-9-fluoro-3-methyl-7-oxo-2,3-dihydro-7*H*-pyrido[1,2,3-*de*]-1,4-benzoxazin-10-yl]-1-methylpiperazine 1-oxide.

01/2017:2258

# OLANZAPINE

## Olanzapinum

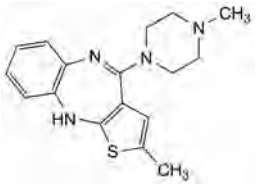

$C_{17}H_{20}N_4S$ $M_r$ 312.4
[132539-06-1]

DEFINITION

2-Methyl-4-(4-methylpiperazin-1-yl)-10*H*-thieno[2,3-*b*]-[1,5]benzodiazepine.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *olanzapine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethyl acetate R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test and reference solutions immediately before use or keep them refrigerated and inject within 20 h of preparation.*

*Solution A*. Dissolve 13 g of *sodium dodecyl sulfate R* in about 1450 mL of *water R*, add 5 mL of *phosphoric acid R* and adjust to pH 2.5 by slowly adding *strong sodium hydroxide solution R*. If a precipitate is formed, this precipitate has to be re-dissolved prior to final pH adjustment. Dilute to 1500 mL with *water R*.

*Solvent mixture*. Mix 4 volumes of *acetonitrile R1* with 6 volumes of a 37 mg/L solution of *sodium edetate R* in solution A.

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 4 mg of *olanzapine for system suitability CRS* (containing impurities B, C and D) in 10.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, solution A (48:52 *V/V*);
- *mobile phase B*: solution A, *acetonitrile R1* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 20 | 100 → 0 | 0 → 100 |
| 20 - 25 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *olanzapine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C and D.

*Relative retention* with reference to olanzapine (retention time = about 13 min): impurity B = about 0.3; impurity D = about 0.9; impurity C = about 1.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity D and olanzapine.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.4;
- *impurities B, C, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 50.0 mg of *olanzapine CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of the substance to be examined and 1 mg of *olanzapine impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*. Mix 1 volume of *acetonitrile R* with 1 volume of a 6.9 g/L solution of *sodium dihydrogen phosphate monohydrate R* adjusted to pH 2.5 with *phosphoric acid R* and containing 12 g/L of *sodium dodecyl sulfate R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: 1.2 times the retention time of olanzapine.

*Relative retention* with reference to olanzapine (retention time = about 7 min): impurity A = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and olanzapine.

Calculate the percentage content of $C_{17}H_{20}N_4S$ using the chromatogram obtained with reference solution (a) and the declared content of *olanzapine CRS*.

IMPURITIES

*Specified impurities: B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

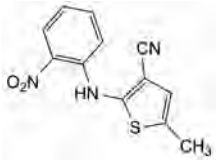

A. 5-methyl-2-[(2-nitrophenyl)amino]thiophene-3-carbonitrile,

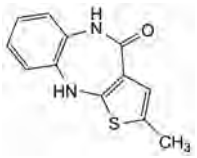

B. 2-methyl-5,10-dihydro-4*H*-thieno[2,3-*b*]-[1,5]benzodiazepin-4-one,

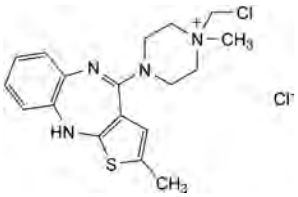

C. 1-(chloromethyl)-1-methyl-4-(2-methyl-10*H*-thieno[2,3-*b*][1,5]benzodiazepin-4-yl)piperazin-1-ium chloride,

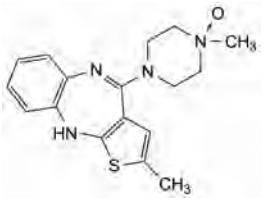

D. 1-methyl-4-(2-methyl-10*H*-thieno[2,3-*b*][1,5]benzodiazepin-4-yl)piperazin-1-oxide.

**01/2008:0799**

# OLEIC ACID

## Acidum oleicum

[112-80-1]

DEFINITION

(*Z*)-Octadec-9-enoic acid ($C_{18}H_{34}O_2$; $M_r$ 282.5), together with varying amounts of saturated and other unsaturated fatty acids. A suitable antioxidant may be added.

*Content*: 65.0 per cent to 88.0 per cent of $C_{18}H_{34}O_2$.

CHARACTERS

*Appearance*: clear, yellowish or brownish, oily liquid.

*Solubility*: practically insoluble in water, miscible with alcohol and with methylene chloride.

*Relative density*: about 0.892.

IDENTIFICATION

A. Acid value (see Tests).

B. Iodine value (see Tests).

C. Composition of fatty acids (see Tests).

*Margaric acid*: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

**Appearance**. The substance to be examined is not more intensely coloured than reference solution $Y_1$ or $BY_1$ (*2.2.2, Method I*).

**Acid value** (*2.5.1*): 195 to 204, determined on 0.5 g.

**Iodine value** (*2.5.4*): 89 to 105.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Test solution*. Prepare as described in the method but omitting the initial hydrolysis.

*Composition of the fatty acid fraction of the substance*:

– *myristic acid*: maximum 5.0 per cent,

– *palmitic acid*: maximum 16.0 per cent,

– *palmitoleic acid*: maximum 8.0 per cent,

– *stearic acid*: maximum 6.0 per cent,

– *oleic acid*: 65.0 per cent to 88.0 per cent,

– *linoleic acid*: maximum 18.0 per cent,

– *linolenic acid*: maximum 4.0 per cent,

– *fatty acids of chain length greater than $C_{18}$*: maximum 4.0 per cent.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 2.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states the origin of oleic acid (animal or vegetable).

**04/2017:1249**

# OLEOYL MACROGOLGLYCERIDES

## Macrogolglyceridorum oleates

DEFINITION

Mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols.

They are obtained by partial alcoholysis of an unsaturated oil mainly containing triglycerides of oleic ((9*Z*)-octadec-9-enoic) acid, using macrogol with a mean relative molecular mass between 300 and 400, or by esterification of glycerol and macrogol with unsaturated fatty acids, or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of this unsaturated oil.

CHARACTERS

*Appearance*: amber oily liquid, which may give rise to a deposit after prolonged periods at 20 °C.

*Solubility*: practically insoluble but dispersible in water, freely soluble in methylene chloride.

*Relative density*: about 0.95 at 20 °C.

*Refractive index*: about 1.47 at 20 °C.

*Viscosity*: about 35 mPa·s at 40 °C.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.9 ($R_{st}$ 1) and spots due to 1,3-diglycerides ($R_{st}$ 0.7), to 1,2-diglycerides ($R_{st}$ 0.6), to monoglycerides ($R_{st}$ 0.1) and to esters of macrogol ($R_{st}$ 0).

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 45 to 65, determined on 1.0 g.

**Iodine value** (*2.5.4, Method A*): 75 to 95.

**Peroxide value** (*2.5.5, Method A*): maximum 12.0, determined on 2.0 g.

**Saponification value** (*2.5.6*): 150 to 170, determined on 2.0 g.

**Alkaline impurities**. Introduce 5.0 g into a test tube and carefully add a mixture, neutralised if necessary with *0.01 M hydrochloric acid* or with *0.01 M sodium hydroxide*, of 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*, 0.3 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Shake and allow to stand. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

**Free glycerol**: maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat if necessary. After cooling, add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate the iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

- *palmitic acid*: 4.0 per cent to 9.0 per cent;
- *stearic acid*: maximum 6.0 per cent;
- *oleic acid*: 58.0 per cent to 80.0 per cent;
- *linoleic acid*: 15.0 per cent to 35.0 per cent;
- *linolenic acid*: maximum 2.0 per cent;
- *arachidic acid*: maximum 2.0 per cent;
- *eicosenoic acid*: maximum 2.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

STORAGE

Protected from light.

LABELLING

The label states the type of macrogol used (mean relative molecular mass) or the number of units of ethylene oxide per molecule (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for oleoyl macrogolglycerides used as self-emulsifying agents and solubilisers.*

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**Composition of fatty acids** (see Tests).

**04/2011:2073**
**corrected 10.0**

# OLEYL ALCOHOL

## Alcohol oleicus

DEFINITION

Mixture of unsaturated and saturated long-chain fatty alcohols consisting mainly of octadec-9-enol (oleyl alcohol and elaidyl alcohol; $C_{18}H_{36}O$; $M_r$ 268.5). It may be of vegetable or animal origin.

CHARACTERS

*Appearance*: colourless or light yellow liquid.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Composition of fatty alcohols (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Refractive index** (*2.2.6*): 1.458 to 1.461, determined at 25 °C.

**Cloud point**: maximum 10 °C.

Introduce about 60 g into a cylindrical flat-bottomed container, 30-33.5 mm in internal diameter and 115-125 mm

high. Heat to 30 °C, cool, and immerse the container in iced water with the surfaces of the water and the sample at the same level. Insert a thermometer and, using it as a stirring rod, begin stirring rapidly and steadily when the temperature falls below 20 °C. Keep the thermometer immersed throughout the test, and remove and examine the container at regular intervals. The cloud point is the temperature at which the immersed portion of the thermometer, positioned vertically in the centre of the container, is no longer visible when viewed horizontally through the container and sample.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 205 to 215.

**Saponification value** (*2.5.6*): maximum 2.0.

**Composition of fatty alcohols**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Mix 25 mg of the substance to be examined with 1.0 mL of *methylene chloride R*.

*Reference solution (a)*. Dissolve 25 mg of each of *arachidyl alcohol R*, *linolenyl alcohol R*, *linoleyl alcohol R*, *oleyl alcohol R*, *palmityl alcohol R* and *stearyl alcohol R* in *methylene chloride R* and dilute to 5 mL with the same solvent. Dilute 1 mL of this solution to 5 mL with *methylene chloride R*.

*Reference solution (b)*. Dissolve 10 mg of *linoleyl alcohol R* and 1 g of *oleyl alcohol R* in *methylene chloride R* and dilute to 40 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:11.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 170 |
| | 1 - 9 | 170 → 210 |
| | 9 - 65 | 210 |
| Injection port | | 270 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

Identify the peaks using the chromatogram obtained with reference solution (a).

*Relative retention* with reference to oleyl alcohol (retention time = about 30 min): palmityl alcohol = about 0.6; linolenyl alcohol = about 0.8; linoleyl alcohol = about 0.9; stearyl alcohol = about 1.1; arachidyl alcohol = about 1.9 (elaidyl alcohol co-elutes with oleyl alcohol).

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to linoleyl alcohol and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to oleyl alcohol.

*Limits*:

- *palmityl alcohol*: maximum 8.0 per cent;
- *stearyl alcohol*: maximum 5.0 per cent;
- *oleyl alcohol* (sum of oleyl and elaidyl alcohols): minimum 80.0 per cent;
- *linoleyl alcohol*: maximum 3.0 per cent;
- *linolenyl alcohol*: maximum 0.5 per cent;
- *arachidyl alcohol*: maximum 0.3 per cent.

**01/2018:1456**

# OLIVE OIL, REFINED

## Olivae oleum raffinatum

DEFINITION

Fatty oil obtained by refining of crude olive oil, obtained by cold expression or other suitable mechanical means from the ripe drupes of *Olea europaea* L. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, colourless or greenish-yellow transparent liquid.

*Solubility*: practically insoluble in ethanol (96 per cent), miscible with light petroleum (bp: 50-70 °C).

When cooled, it begins to become cloudy at 10 °C and becomes a butter-like mass at about 0 °C.

*Relative density*: about 0.913.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B.*

A. Acid value (see Tests).

B. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1. For certain types of olive oil, the difference in the size of spots E and F is less pronounced than in the corresponding chromatogram shown in Figure 2.3.2.-1.

C. Composition of fatty acids (see Tests).

TESTS

**Specific absorbance** (*2.2.25*): maximum 1.20, determined at the absorption maximum at 270 nm.

To 1.00 g add *cyclohexane R* and dilute to 100.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 0.3, determined on 10.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter**: maximum 1.5 per cent.

Place 5.0 g (*m* g) in a 150 mL flask fitted with a reflux condenser. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, shaking frequently. Add 50 mL of *water R* through the top of the condenser, shake, allow to cool and transfer the contents of the flask to a separating funnel. Rinse the flask with several portions totalling 50 mL of *light petroleum R1* and add the rinsings to the separating funnel. Shake vigorously for 1 min. Allow to separate and transfer the aqueous layer to a 2nd separating funnel. If an emulsion forms, add small quantities of *ethanol (96 per cent) R* or a concentrated solution of *potassium hydroxide R*. Shake the aqueous layer with 2 quantities, each of 50 mL, of *light petroleum R1*. Combine the light petroleum layers in a 3rd separating funnel and wash with 3 quantities, each of 50 mL, of *ethanol (50 per cent V/V) R*. Transfer the light petroleum layer to a tared 250 mL flask. Rinse the separating funnel with small quantities of *light petroleum R1* and add to the flask. Evaporate the light petroleum on a water-bath and dry the residue at 100-105 °C for 15 min, keeping the flask horizontal. Allow to cool in a desiccator and weigh (*a* g). Repeat the drying for successive periods of 15 min until the loss of mass between 2 successive weighings does not exceed 0.1 per cent. Dissolve the residue in 20 mL

of *ethanol (96 per cent) R*, previously neutralised to 0.1 mL of *bromophenol blue solution R*. If necessary, titrate with *0.1 M hydrochloric acid* (*b* mL).

Calculate the percentage content of unsaponifiable matter using the following expression:

$$\frac{100(a - 0.032b)}{m}$$

If 0.032*b* is greater than 5 per cent of *a*, the test is not valid and must be repeated.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil:*

- *saturated fatty acids of chain length less than $C_{16}$*: maximum 0.1 per cent;
- *palmitic acid*: 7.5 per cent to 20.0 per cent;
- *palmitoleic acid*: maximum 3.5 per cent;
- *stearic acid*: 0.5 per cent to 5.0 per cent;
- *oleic acid and isomer*: 56.0 per cent to 85.0 per cent;
- *linoleic acid*: 3.5 per cent to 20.0 per cent;
- *linolenic acid*: maximum 1.2 per cent;
- *arachidic acid*: maximum 0.7 per cent;
- *eicosenoic acid*: maximum 0.4 per cent;
- *behenic acid*: maximum 0.2 per cent;
- *lignoceric acid*: maximum 0.2 per cent.

**Sterols** (*2.4.23, Method B*).

*Composition of the sterol fraction of the oil:*

- *cholesterol*: maximum 0.5 per cent;
- *campesterol*: maximum 4.0 per cent;
- *Δ7-stigmastenol*: maximum 0.5 per cent;
- *sum of contents of Δ5,23-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol*: minimum 93.0 per cent.

The content of stigmasterol is not greater than that of campesterol.

**Sesame oil**. In a ground-glass-stoppered cylinder shake 10 mL for about 1 min with a mixture of 0.5 mL of a 0.35 per cent *V/V* solution of *furfural R* in *acetic anhydride R* and 4.5 mL of *acetic anhydride R*. Filter through a filter paper impregnated with *acetic anhydride R*. To the filtrate add 0.2 mL of *sulfuric acid R*. No bluish-green colour develops.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light, at a temperature not exceeding 25 °C. If intended for use in the manufacture of parenteral preparations, store under an inert gas.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations and the name of the inert gas.

**01/2018:0518**

# OLIVE OIL, VIRGIN

## Olivae oleum virginale

DEFINITION

Fatty oil obtained by cold expression or other suitable mechanical means from the ripe drupes of *Olea europaea* L.

CHARACTERS

*Appearance*: clear, transparent, yellow or greenish-yellow liquid.

*Solubility*: practically insoluble in ethanol (96 per cent), miscible with light petroleum (bp: 50-70 °C).

When cooled, it begins to become cloudy at 10 °C and becomes a butter-like mass at about 0 °C.

*Relative density*: about 0.913.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1. For certain types of olive oil, the difference in the size of spots E and F is less pronounced than in the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Absorbance** (*2.2.25*): maximum 0.20 at 270 nm. The ratio of the absorbance at 232 nm to that at 270 nm is greater than 8.

To 1.00 g add *cyclohexane R* and dilute to 100.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 20.0.

**Unsaponifiable matter**: maximum 1.5 per cent.

Place 5.0 g (*m* g) in a 150 mL flask fitted with a reflux condenser. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, shaking frequently. Add 50 mL of *water R* through the top of the condenser, shake, allow to cool and transfer the contents of the flask to a separating funnel. Rinse the flask with several portions totalling 50 mL of *light petroleum R1* and add the rinsings to the separating funnel. Shake vigorously for 1 min. Allow to separate and transfer the aqueous layer to a 2nd separating funnel. If an emulsion forms, add small quantities of *ethanol (96 per cent) R* or a concentrated solution of *potassium hydroxide R*. Shake the aqueous layer with 2 quantities, each of 50 mL, of *light petroleum R1*. Combine the light petroleum layers in a 3rd separating funnel and wash with 3 quantities, each of 50 mL, of *ethanol (50 per cent V/V) R*. Transfer the light petroleum layer to a tared 250 mL flask. Rinse the separating funnel with small quantities of *light petroleum R1* and add to the tared flask. Evaporate the light petroleum on a water-bath and dry the residue at 100-105 °C for 15 min, keeping the flask horizontal. Allow to cool in a desiccator and weigh (*a* g). Repeat the drying for successive periods of 15 min until the loss of mass between 2 successive weighings does not exceed 0.1 per cent. Dissolve the residue in 20 mL of *ethanol (96 per cent) R*, previously neutralised to 0.1 mL of *bromophenol blue solution R*. If necessary, titrate with *0.1 M hydrochloric acid* (*b* mL).

Calculate the percentage content of unsaponifiable matter using the following expression:

$$\frac{100(a - 0.032b)}{m}$$

If 0.032*b* is greater than 5 per cent of *a*, the test is not valid and must be repeated.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil:*

- *saturated fatty acids of chain length less than $C_{16}$*: maximum 0.1 per cent;
- *palmitic acid*: 7.5 per cent to 20.0 per cent;

- *palmitoleic acid*: maximum 3.5 per cent;
- *stearic acid*: 0.5 per cent to 5.0 per cent;
- *oleic acid and isomer*: 56.0 per cent to 85.0 per cent;
- *linoleic acid*: 3.5 per cent to 20.0 per cent;
- *linolenic acid*: maximum 1.2 per cent;
- *arachidic acid*: maximum 0.7 per cent;
- *eicosenoic acid*: maximum 0.4 per cent;
- *behenic acid*: maximum 0.2 per cent;
- *lignoceric acid*: maximum 0.2 per cent.

**Sterols** (*2.4.23, Method B*).

*Composition of the sterol fraction of the oil*:

- *cholesterol*: maximum 0.5 per cent;
- *campesterol*: maximum 4.0 per cent;
- *Δ7-stigmasterol*: maximum 0.5 per cent;
- *sum of contents of Δ5,23-stigmastadienol, clerosterol, β-sitosterol, sitostanol, Δ5-avenasterol and Δ5,24-stigmastadienol*: minimum 93.0 per cent.

The content of stigmasterol is not greater than that of campesterol.

**Sesame oil**. In a ground-glass-stoppered cylinder shake 10 mL for about 1 min with a mixture of 0.5 mL of a 0.35 per cent *V/V* solution of *furfural R* in *acetic anhydride R* and 4.5 mL of *acetic anhydride R*. Filter through a filter paper impregnated with *acetic anhydride R*. To the filtrate add 0.2 mL of *sulfuric acid R*. No bluish-green colour develops.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light, at a temperature not exceeding 25 °C.

**01/2020:2600**

# OLMESARTAN MEDOXOMIL

## Olmesartanum medoxomilum

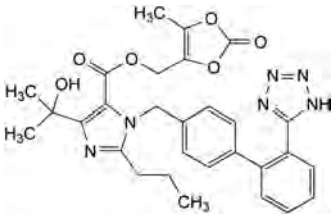

$C_{29}H_{30}N_6O_6$ $M_r$ 558.6

[144689-63-4]

DEFINITION

(5-Methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

As *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are classified as probable human carcinogens, manufacturers must ensure that their manufacturing process does not generate such impurities and develop appropriate control strategies. To allow manufacturers to make the necessary changes to their process, a transition period has been agreed by competent authorities and strict temporary limits on levels of these impurities introduced in the Test section.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *olmesartan medoxomil CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *olmesartan medoxomil for system suitability CRS* (containing impurities A, B and C) in *acetonitrile R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 50.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 25.0 mg of *olmesartan medoxomil CRS* in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 2.04 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.4 with a 1.73 g/L solution of *phosphoric acid R*;
- *mobile phase B*: mix 20 volumes of a 2.04 g/L solution of *potassium dihydrogen phosphate R*, previously adjusted to pH 3.4 with a 1.73 g/L solution of *phosphoric acid R*, and 80 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 75 | 25 |
| 10 - 35 | 75 → 0 | 25 → 100 |
| 35 - 45 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *olmesartan medoxomil for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to olmesartan medoxomil (retention time = about 10 min): impurity A = about 0.2; impurity B = about 0.7; impurity C = about 1.5.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.5 between the peaks due to impurity B and olmesartan medoxomil.

*Limits*:

- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Acetone**. Head-space gas chromatography (*2.2.28*): use the direct calibration method.

*Internal standard solution*. Dilute 1.0 mL of *butanol R* to 100.0 mL with *dimethyl sulfoxide R*.

*Test solution*. Dissolve 0.250 g of the substance to be examined in *dimethyl sulfoxide R*, add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *dimethyl sulfoxide R*.

*Reference solution*. Dilute 0.50 mL of *acetone R* to 200.0 mL with *dimethyl sulfoxide R*. Dilute 15.0 mL of the solution to 100.0 mL with *dimethyl sulfoxide R*. To 25.0 mL of this solution add 10.0 mL of the internal standard solution and dilute to 50.0 mL with *dimethyl sulfoxide R*.

*Column*:
– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.53 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

*Carrier gas*: *nitrogen for chromatography R* or *helium for chromatography R*.

*Flow rate*: 4.0 mL/min.

*Split ratio*: 1:5.

*Static head-space conditions that may be used*:
– *equilibration temperature*: 80 °C;
– *equilibration time*: 30 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 5 | 50 |
| | 5 - 18 | 50 → 180 |
| | 18 - 23 | 180 |
| Injection port | | 200 |
| Detection | | 200 |

*Detection*: flame ionisation.

*Injection*: 1 mL.

Calculate the content of acetone, taking its relative density to be 0.79 at 20 °C.

*Limit*:
– *acetone*: maximum 0.6 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Nitrosamines**. Carry out the test by a suitable method.

The substance to be examined does not contain either NDMA or NDEA above the limits provided below or both impurities at whatever level:
– *N-nitrosodimethylamine (NDMA)*: maximum 2.400 ppm;
– *N-nitrosodiethylamine (NDEA)*: maximum 0.663 ppm.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (25:75 *V/V*).

*Injection*: test solution (b) and reference solution (c).

*Retention time*: olmesartan medoxomil = about 10 min.

*Run time*: 1.5 times the retention time of olmesartan medoxomil.

Calculate the percentage content of $C_{29}H_{30}N_6O_6$ taking into account the assigned content of *olmesartan medoxomil CRS*.

IMPURITIES

*Specified impurities*: *A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D.*

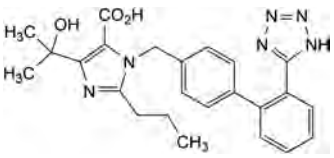

A. 4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylic acid (olmesartan),

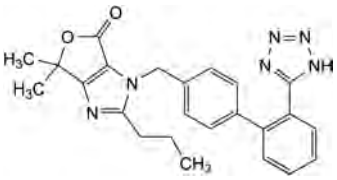

B. 6,6-dimethyl-2-propyl-3-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-3,6-dihydro-4*H*-furo[3,4-*d*]imidazol-4-one,

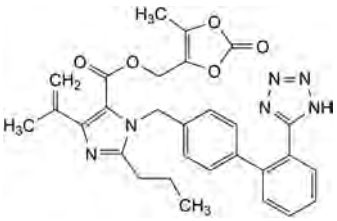

C. (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(1-methylethenyl)-2-propyl-1-[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylate,

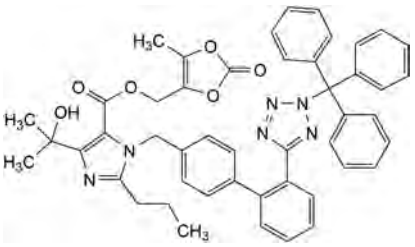

D. (5-methyl-2-oxo-1,3-dioxol-4-yl)methyl 4-(1-hydroxy-1-methylethyl)-2-propyl-1-[[2′-[(2-triphenylmethyl)-2*H*-tetrazol-5-yl]biphenyl-4-yl]methyl]-1*H*-imidazole-5-carboxylate.

**01/2017:1457**
**corrected 10.0**

# OLSALAZINE SODIUM

## Olsalazinum natricum

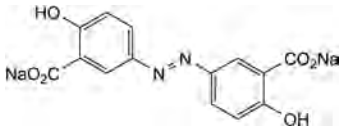


$C_{14}H_8N_2Na_2O_6$ $M_r$ 346.2
[6054-98-4]

DEFINITION

Disodium 3,3′-diazenediylbis(6-hydroxybenzoate).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, fine, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in dimethyl sulfoxide, very slightly soluble in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 40.0 mg in 5 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with a 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 7.2 with *strong sodium hydroxide solution R* (buffer solution). Dilute 2.0 mL of the solution to 100.0 mL with the buffer solution.

*Spectral range*: 240 nm to 400 nm.

*Absorption maxima*: at 255 nm and 362 nm.

*Absorbance ratio*: $A_{255}/A_{362}$ = 0.53 to 0.56.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *olsalazine sodium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *dilute ammonia R2* and 4 volumes of *ethanol (96 per cent) R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 10 mg of *olsalazine sodium CRS* in a mixture of 1 volume of *dilute ammonia R2* and 4 volumes of *ethanol (96 per cent) R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 5 mg of *sulfasalazine CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous formic acid R*, *acetone R*, *methylene chloride R* (5:50:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 0.5 g add 2 mL of *sulfuric acid R*. Progressively heat to ignition and continue heating until an almost white or at most greyish residue is obtained. Carry out the ignition at a temperature up to 800 ± 50 °C. Dissolve the residue in 10 mL of boiling *water R* and filter. 2 mL of the filtrate gives reaction (a) of sodium (*2.3.1*).

TESTS

**Acetate**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.125 g of the substance to be examined in 25.0 mL of *water R* and add 1.0 mL of *dilute hydrochloric acid R*. Centrifuge and then filter the solution through a 0.45 µm filter and also through an appropriate filter for removal of chlorides.

*Reference solution (a).* Dissolve 0.140 g of *sodium acetate R*, 0.150 g of *sodium formate R* and 0.180 g of *dipotassium sulfate R* in 100.0 mL of *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (b).* Use suitable amounts of *sodium acetate R* to prepare not fewer than 5 reference solutions containing 10-50 µg/mL of acetate.

*Column*:

– *size*: l = 0.25 m, Ø = 9 mm;
– *stationary phase*: *ion-exclusion resin for chromatography R* with a capacity of about 27 meq/column.

*Suppressor column.*

*Mobile phase*: *0.0001 M hydrochloric acid.*

*Flow rate*: 0.9 mL/min.

*Detection*: conductivity detector at 10 µs·cm$^{-1}$.

*Injection*: 0.1 mL.

*System suitability*: reference solution (a):

– the chromatogram shows 3 separated peaks.

Determine the concentration of acetate in the test solution using the calibration curve generated by the average of the readings obtained with the reference solutions. Measure the peak area for acetate. Calculate the percentage content of acetate using the following expression:

$$\frac{2.6\ c}{m}$$

$c$ = concentration of acetate in the test solution, in micrograms per millilitre, determined by linear interpolation of the standard curve for reference solution (b);

$ym$ = mass of sample, in milligrams.

*Limit*:

– *acetate*: maximum 1.0 per cent.

**Methanesulfonic acid**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.25 g of the substance to be examined in 20 mL of *water R*, add 1.0 mL of *dilute hydrochloric acid R* and dilute to 25.0 mL with *water R*. Centrifuge and then filter the solution through a 0.45 µm filter and also through an appropriate filter for removal of chlorides.

*Reference solution (a).* Dissolve 0.25 g of *methanesulfonic acid R* in 50 mL of *water R*. Add 0.58 g of *sodium acetate R* and 0.08 g of *sodium chloride R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 0.10 g of *methanesulfonic acid R* in *water R* and dilute to 100.0 mL with *water R*. Dilute 3.0 mL of this solution to 100.0 mL with *water R*.

*Precolumn*:

– *size*: *l* = 0.035 m, Ø = 4 mm;

– *stationary phase*: *resin for reversed-phase ion chromatography R* (10 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *resin for reversed-phase ion chromatography R* (10 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile for chromatography R* and 990 volumes of a solution containing 1.6 g/L of *tetrabutylammonium hydroxide R* and 0.053 g/L of *anhydrous sodium carbonate R*.

*Flow rate*: 1.0 mL/min.

*Detection*: conductivity detector at 50 µS·cm$^{-1}$.

*Injection*: 100 µL.

*System suitability*: reference solution (a):

– the chromatogram shows 3 separated peaks.

*Limit*:

– *methanesulfonic acid*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a).* Dilute 0.5 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 20.0 mg of *olsalazine sodium for performance test CRS* in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 2.38 g of *tetrabutylammonium hydrogen sulfate R* and 3.6 g of *disodium hydrogen phosphate dihydrate R* in 900 mL of *water R*, adjust to pH 7.6 with *dilute sodium hydroxide solution R* and dilute to 1000.0 mL with *water R*; mix 700 mL of this buffer solution with 300 mL of *methanol R*;

– *mobile phase B*: dissolve 4.75 g of *tetrabutylammonium hydrogen sulfate R* and 3.6 g of *disodium hydrogen phosphate dihydrate R* in 900 mL of *water R*, adjust to pH 7.6 with *dilute sodium hydroxide solution R* and dilute to 1000.0 mL with *water R*; mix 350 mL of this buffer solution with 650 mL of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 55 | 45 |
| 15 - 45 | 55 → 0 | 45 → 100 |
| 45 - 50 | 0 → 55 | 100 → 45 |
| 50 - 65 | 55 | 45 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 360 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– the chromatogram is similar to the chromatogram obtained with *olsalazine sodium for performance test CRS*.

*Limits*:

– *impurities A, B, C, D, E, F, G, H, I*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent), and not more than one of the peaks has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 150 °C.

ASSAY

Dissolve 0.100 g in 15 mL of *ethylene glycol R*. Add 40 mL of *dioxan R* and 0.2 mL of a 224 g/L solution of *potassium chloride R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

Correct the volume consumed for the content of acetate, taking the molecular mass of acetate to be 59.0.

1 mL of *0.1 M hydrochloric acid* is equivalent to 17.31 mg of $C_{14}H_8N_2Na_2O_6$.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

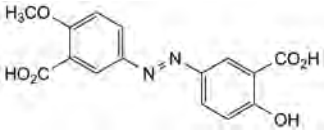

A. 6-hydroxy-6′-methoxy-3,3′-diazenediyldibenzoic acid,

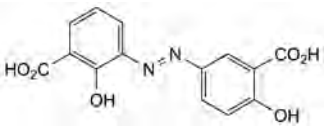

B. 2,6′-dihydroxy-3,3′-diazenediyldibenzoic acid,

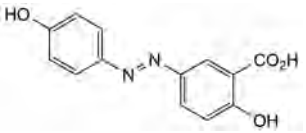

C. 2-hydroxy-5-[(4-hydroxyphenyl)diazenyl]benzoic acid,

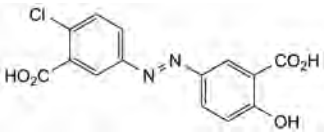

D. 6-chloro-6′-hydroxy-3,3′-diazenediyldibenzoic acid,

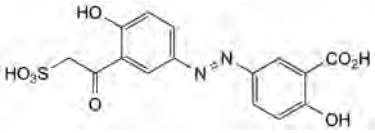

E. 2-hydroxy-5-[[4-hydroxy-3-(sulfoacetyl)phenyl]-diazenyl]benzoic acid,

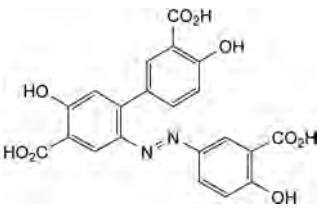

F. 2′-[(3-carboxy-4-hydroxyphenyl)diazenyl]-4,5′-dihydroxybiphenyl-3,4′-dicarboxylic acid,

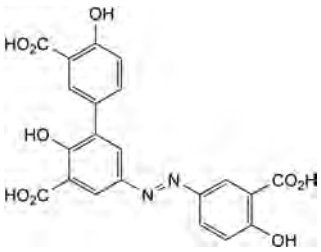

G. 5-[(3-carboxy-4-hydroxyphenyl)diazenyl]-2,4′-dihydroxybiphenyl-3,3′-dicarboxylic acid,

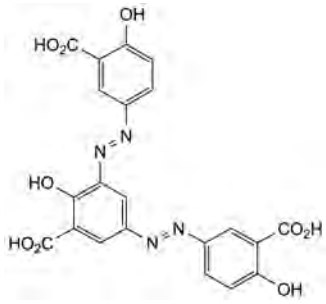

H. 3,3′-[5-carboxy-4-hydroxy-1,3-phenylene-bis(diazenediyl)]bis(6-hydroxybenzoic) acid,

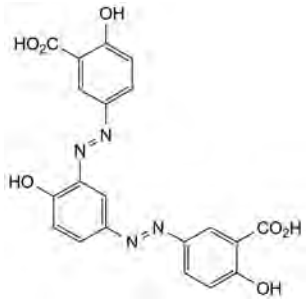

I. 3,3′-[4-hydroxy-1,3-phenylenebis(diazenediyl)]-bis(6-hydroxybenzoic) acid.

**07/2012:2063**

# OMEGA-3-ACID ETHYL ESTERS 60

## Omega-3 acidorum esteri ethylici 60

DEFINITION

Ethyl esters of *alpha*-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA). Omega-3-acid ethyl esters 60 are obtained by transesterification of the body oil obtained from fish of families such as *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae* or from animals of the class *Cephalopoda* and subsequent physico-chemical purification processes, including molecular distillation. The minimum content of total omega-3-acid ethyl esters and the minimum content of the omega-3-acids EPA and DHA ethyl esters are indicated in Table 2063.-1.

Table 2063.-1

| Total omega-3-acid ethyl esters | EPA and DHA ethyl esters | EPA ethyl esters | DHA ethyl esters |
|---|---|---|---|
| | Minimum content (per cent) | | |
| 65 | 50 | 25 | 20 |
| 60 | 50 | - | 40 |
| 55 | 50 | 40 | - |

A suitable antioxidant may be added.

PRODUCTION

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: light yellow liquid.

Slight fish-like odour.

*Solubility*: practically insoluble in water, very soluble in acetone, in ethanol (96 per cent), in heptane and in methanol.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay for EPA and DHA ethyl esters.

*Results*: the peaks due to eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester in the chromatogram obtained with test solution (b) are similar in retention time to the corresponding peaks in the chromatograms obtained with reference solutions ($a_1$) and ($a_2$).

B. It complies with the limits of the assay for total omega-3-acid ethyl esters.

TESTS

**Absorbance** (*2.2.25*): maximum 0.60 at 233 nm.

Dilute 0.300 g to 50.0 mL with *trimethylpentane R*. Dilute 2.0 mL of the solution to 50.0 mL with *trimethylpentane R*.

**Acid value** (*2.5.1*): maximum 2.0, determined on 10 g in 50 mL of the prescribed mixture of solvents.

**Anisidine value** (*2.5.36*): maximum 20.0.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Oligomers and partial glycerides**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dilute 50.0 mg of the substance to be examined to 10.0 mL with *tetrahydrofuran R*.

*Reference solution*. Dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent.

*Column*: 3 columns to be connected in series:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with the following pore sizes:
  - *column 1*: 50 nm;
  - *column 2*: 10 nm;
  - *column 3*: 5 nm;
- *connection sequence*: injector – column 1 – column 2 – column 3 – detector.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 0.8 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*System suitability*: reference solution:

- *elution order*: tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin;

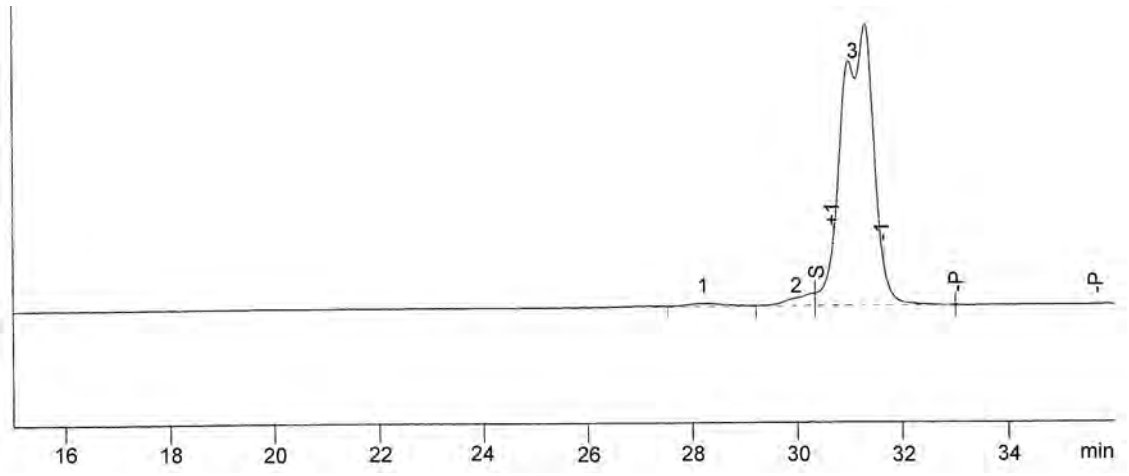


1. oligomers 2. monoglycerides 3. fatty acid ethyl esters

Figure 2063.-1. – *Chromatogram for the test for oligomers and partial glycerides in omega-3-acid ethyl esters 60*

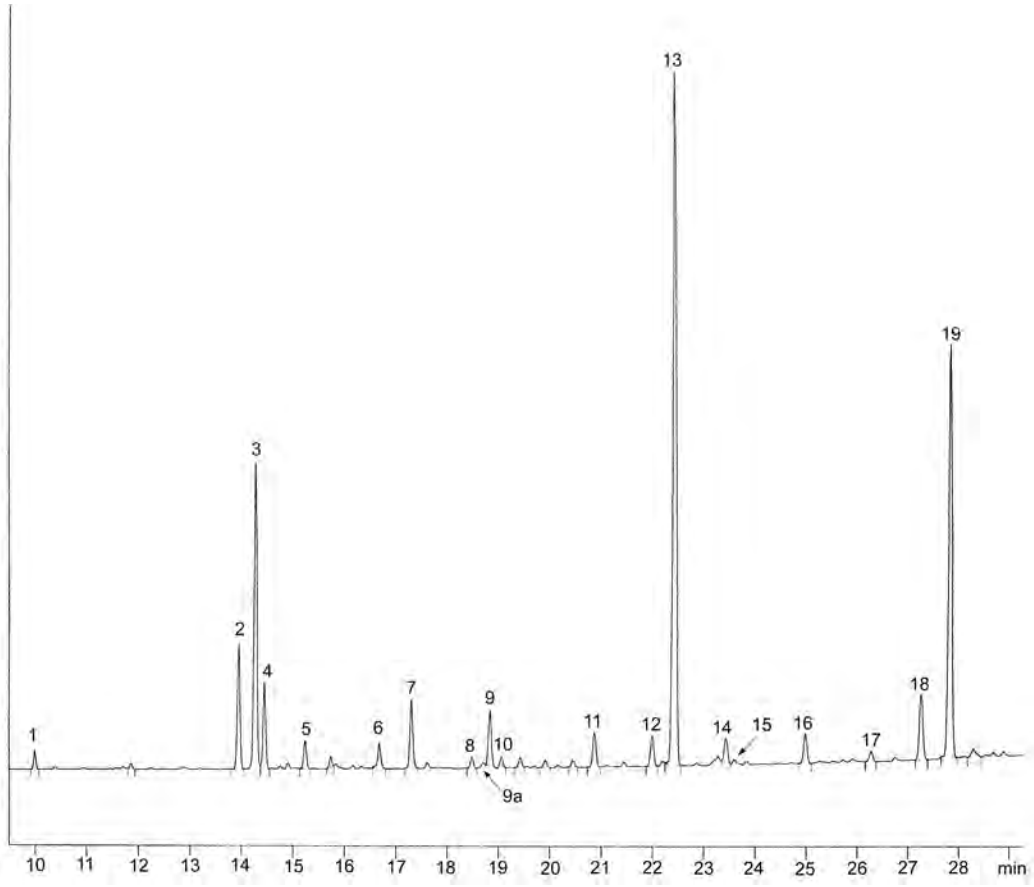


| | | | | | | |
|---|---|---|---|---|---|---|
| 1. C16:0 | 4. C18:1 n-7 | 7. C18:4 n-3 | 9a. C20:1 n-11 | 12. C20:4 n-3 | 15. C22:1 n-9 | 18. C22:5 n-3 |
| 2. C18:0 | 5. C18:2 n-6 | 8. C20:0 | 10. C20:1 n-7 | 13. EPA | 16. C21:5 n-3 | 19. DHA |
| 3. C18:1 n-9 | 6. C18:3 n-3 | 9. C20:1 n-9 | 11. C20:4 n-6 | 14. C22:1 n-11 | 17. C22:5 n-6 | |

Figure 2063.-2. – *Chromatogram for the assays of omega-3-acid ethyl esters 60*

– *resolution*: minimum 2.0 between the peaks due to didocosahexaenoin and monodocosahexaenoin; minimum 1.0 between the peaks due to tridocosahexaenoin and didocosahexaenoin.

Calculate the percentage content of oligomers plus partial glycerides using the following expression:

$$\frac{B}{A} \times 100$$

$A$ = sum of the areas of all the peaks in the chromatogram;

$B$ = sum of the areas of the peaks with a retention time less than the retention time of the peaks due to ethyl esters.

The ethyl ester peaks, which may be present in the form of an unresolved double peak, are identified as the major peaks in the chromatogram (see Figure 2063.-1).

*Limit*:

– *sum of oligomers and partial glycerides*: maximum 7.0 per cent.

ASSAY

**EPA and DHA ethyl esters** (*2.4.29*). For identification of the peaks, see Figure 2063.-2.

**Total omega-3-acid ethyl esters** (*2.4.29*). See Figure 2063.-2.

STORAGE

Under an inert gas, in an airtight container, protected from light.

LABELLING

The label states:

– the content of total omega-3-acid ethyl esters;

– the content of EPA ethyl ester and DHA ethyl ester.

**07/2017:1250**

# OMEGA-3-ACID ETHYL ESTERS 90

## Omega-3 acidorum esteri ethylici 90

DEFINITION

Ethyl esters of *alpha*-linolenic acid (C18:3 n-3), moroctic (stearidonic) acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic (omega-3 docosapentaenoic) acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA). Omega-3-acid ethyl esters 90 are obtained by transesterification of the body oil obtained from fish of families such as *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae* or from animals of the class *Cephalopoda* and subsequent physico-chemical purification processes, including urea fractionation followed by molecular distillation.

*Content*:

– *EPA and DHA ethyl esters*: minimum 80.0 per cent and maximum 88.2 per cent, with minimum 43.0 per cent and maximum 49.5 per cent of EPA ethyl esters and minimum 34.7 per cent and maximum 40.3 per cent of DHA ethyl esters;

– *total omega-3-acid ethyl esters (corresponding to the sum of the ethyl esters listed under Definition)*: minimum 90 per cent.

A suitable antioxidant may be added.

PRODUCTION

The content of polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (PCDD/PCDF), dioxin-like polychlorinated biphenyls (DLPCBs), non-dioxin-like polychlorinated biphenyls (NDLPCBs; 7PCB) and polybrominated diphenyl ethers (PBDEs) is controlled using suitable and validated methods, for example as outlined in Commission Regulation (EU) No 589/2014, Annex III, and in scientific publications of the European Food Safety Authority [EFSA, 2011].

| Persistent organic pollutants | Maximum content |
|---|---|
| *Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDF)** | 1 pg/g (WHO-TEQ***) |
| *Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDF) and dioxin-like polychlorinated biphenyls (DLPCBs)** | 5 pg/g (WHO-TEQ) |
| *Non-dioxin-like polychlorinated biphenyls (NDLPCBs; 7PCB) (sum of congeners 28, 52, 101, 118, 138, 153 and 180)** | 60 ng/g |
| *Polybrominated diphenyl ethers (PBDEs) (sum of congeners 28, 47, 49, 99, 100, 153 and 154)*** | 3 ng/g |

*Commission Regulation (EU) No 589/2014, Annex III.

**_EFSA Journal_ 2011;9(5):2156, [274 pp.].

***WHO-TEQ = 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) toxic equivalent (TEQ) as agreed by WHO.

Calculate the upper-bound contents on the assumption that all values of the different congeners below the limit of quantification are equal to the limit of quantification.

CHARACTERS

*Appearance*: light yellow liquid.

*Solubility*: practically insoluble in water, very soluble in acetone, in ethanol (96 per cent), in heptane and in methanol.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay for EPA and DHA ethyl esters.

*Results*: the peaks due to eicosapentaenoic acid ethyl ester and docosahexaenoic acid ethyl ester in the chromatogram obtained with test solution (b) are similar in retention time to the corresponding peaks in the chromatograms obtained with reference solutions ($a_1$) and ($a_2$).

B. It complies with the limits of the assay for total omega-3-acid ethyl esters.

TESTS

**Absorbance** (*2.2.25*): maximum 0.55 at 233 nm.

Dilute 0.300 g to 50.0 mL with *trimethylpentane R*. Dilute 2.0 mL of the solution to 50.0 mL with *trimethylpentane R*.

**Acid value** (*2.5.1*): maximum 2.0, determined on 10 g in 50 mL of the prescribed mixture of solvents.

**Anisidine value** (*2.5.36*): maximum 20.0.

**Peroxide value** (*2.5.5, Method B*): maximum 10.0.

**Unidentified fatty acid ethyl esters** (*2.4.29*).

Identify the peaks in the chromatogram obtained with test solution (b) in the assay, using Figure 1250.-2, integrating up to 1.3 times the retention time of DHA ethyl ester. Disregard any peak with an area less than 0.05 per cent of the total area.

Calculate the percentage content of the fatty acid ethyl ester corresponding to the largest single unidentified peak, using the following expression:

$$\frac{100 \times B}{A}$$

A = sum of the areas of all peaks, excluding those due to solvents, butylhydroxytoluene and the internal standard;

B = area of the largest single unidentified peak, excluding those due to solvents, butylhydroxytoluene and the internal standard.

Calculate the percentage content of total unidentified fatty acid ethyl esters, using the following expression:

$$100 - \frac{100 \times C}{A}$$

C = sum of the areas of the peaks due to ethyl esters identified in Figure 1250.-2.

*Limits*:

- *fatty acid ethyl ester corresponding to the largest single unidentified peak*: maximum 0.5 per cent;
- *total unidentified fatty acid ethyl esters*: maximum 2 per cent.

**Cholesterol** (*2.4.32*): maximum 3.0 mg/g.

**Oligomers**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dilute 50.0 mg of the substance to be examined to 10.0 mL with *tetrahydrofuran R*.

*Reference solution.* Dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent.

*Column*: 3 columns to be connected in series:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with the following pore sizes:
  - *column 1*: 50 nm;
  - *column 2*: 10 nm;
  - *column 3*: 5 nm;
- *connection sequence*: injector – column 1 – column 2 – column 3 – detector.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 0.8 mL/min.

*Run time*: 38 min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*System suitability*: reference solution:

- *elution order*: tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin;
- *resolution*: minimum 2.0 between the peaks due to didocosahexaenoin and monodocosahexaenoin; minimum 1.0 between the peaks due to tridocosahexaenoin and didocosahexaenoin.

Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

*A* = sum of the areas of all the peaks in the chromatogram;

*B* = sum of the areas of the peaks with a retention time less than the retention time of the peaks due to ethyl esters.

The ethyl ester peaks, which may be present in the form of an unresolved double peak, are identified as the major peaks in the chromatogram (see Figure 1250.-1).

Where the result obtained exceeds the limit due to the presence of monoglycerides, the following procedure is carried out.

*Test solution.* Weigh 50.0 mg of the substance to be examined into a quartz tube. Add 1.5 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R*, cover with *nitrogen R*, cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Allow to cool. Add 2 mL of *boron trichloride-methanol solution R*, cover with *nitrogen R*, cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 mL of *trimethylpentane R*, cap and shake vigorously for at least 30 s. Immediately add 5 mL of *saturated sodium chloride solution R*, cover with *nitrogen R*, cap and shake thoroughly for at least 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer once more with 1 mL of *trimethylpentane R*. Wash the combined trimethylpentane extracts with 2 quantities, each of 1 mL, of *water R*. Carefully evaporate the solvent under a current of *nitrogen R* then add 10.0 mL of *tetrahydrofuran R* to the residue. Add a small amount of *anhydrous sodium sulfate R* and filter.

Calculate the percentage content of oligomers using the following expression:

$$\frac{B'}{A} \times 100$$

*A* = sum of the areas of all the peaks in the chromatogram;

*B′* = sum of the areas of the peaks with a retention time less than the retention time of the peaks due to methyl esters.

*Limit*:

- *oligomers*: maximum 1.0 per cent.

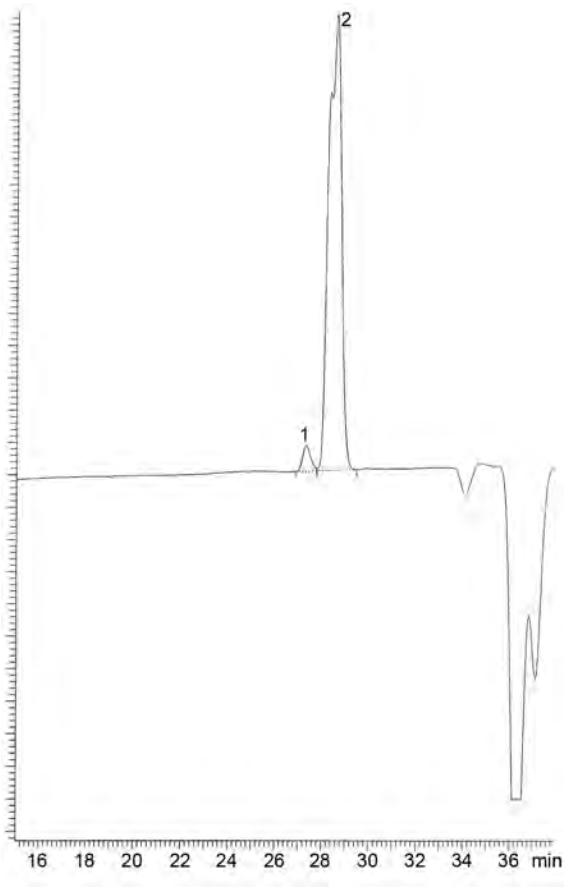


1. oligomers 2. ethyl esters

Figure 1250.-1. – *Chromatogram for the test for oligomers in omega-3-acid ethyl esters 90: spiked sample*

ASSAY

**EPA and DHA ethyl esters** (*2.4.29*). For identification of the peaks, see Figure 1250.-2.

**Total omega-3-acid ethyl esters** (*2.4.29*). See Figure 1250.-2.

STORAGE

Under an inert gas, in an airtight container, protected from light.

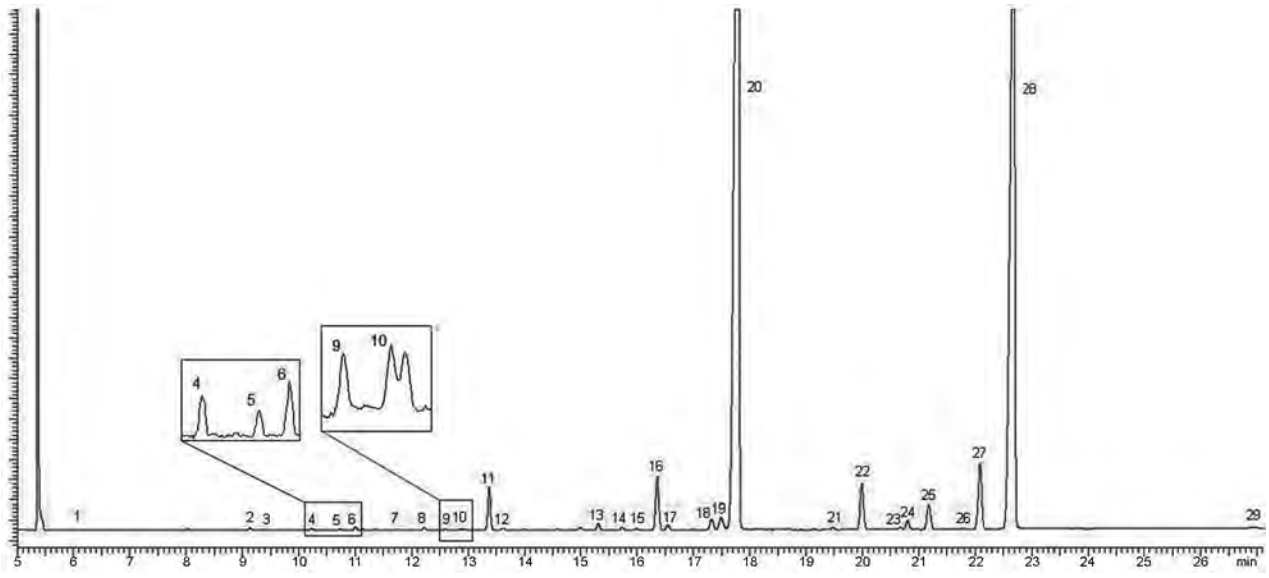


| Fatty acid | *r* | Fatty acid | *r* | Fatty acid | *r* | Fatty acid | *r* |
|---|---|---|---|---|---|---|---|
| 1. C14:0 | 0.278 | 8. C18:3 *n*-6 | 0.557 | 15. C20:3 *n*-6 | 0.720 | 22. C21:5 *n*-3 | 0.889 |
| 2. Phytanic acid | 0.416 | 9. C18:3 *n*-4 | 0.574 | 16. C20:4 *n*-6 | 0.736 | 23. C22:4 *n*-6 | 0.917 |
| 3. C16:3 *n*-4 | 0.431 | 10. C18:3 *n*-3 | 0.585 | 17. Furan acid 7 | 0.744 | 24. Furan acid 10 | 0.922 |
| 4. C16:4 *n*-1 | 0.468 | 11. C18:4 *n*-3 | 0.608 | 18. C20:4 *n*-3 | 0.777 | 25. C22:5 *n*-6 | 0.939 |
| 5. C18:0 | 0.488 | 12. C18:4 *n*-1 | 0.618 | 19. Furan acid 8 | 0.783 | 26. Furan acid 11 | 0.963 |
| 6. C18:1 *n*-9 | 0.501 | 13. Furan acid 5 | 0.691 | 20. EPA | 0.796 | 27. C22:5 *n*-3 | 0.977 |
| 7. C18:2 *n*-6 | 0.535 | 14. C19:5 | 0.710 | 21. Furan acid 9 | 0.867 | 28. DHA | 1.000 |
| | | | | | | 29. C24:6 | 1.183 |

*r: relative retention with reference to DHA ethyl ester (retention time = about 23 min).*

Figure 1250.-2. – *Chromatogram for the assay of omega-3-acid ethyl esters 90 and the test for unidentified fatty acid ethyl esters*

**07/2012:1352**

# OMEGA-3-ACID TRIGLYCERIDES

## Omega-3 acidorum triglycerida

DEFINITION

Mixture of mono-, di- and triesters of omega-3 acids with glycerol, containing mainly triesters and obtained either by esterification of concentrated and purified omega-3 acids with glycerol or by transesterification of the omega-3 acid ethyl esters with glycerol. The origin of the omega-3 acids is the body oil obtained from fish of families such as *Engraulidae*, *Carangidae*, *Clupeidae*, *Osmeridae*, *Salmonidae* and *Scombridae* or from animals of the class *Cephalopoda*. The omega-3 acids are identified as the following acids: *alpha*-linolenic acid (C18:3 n-3), moroctic acid (C18:4 n-3), eicosatetraenoic acid (C20:4 n-3), timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA), heneicosapentaenoic acid (C21:5 n-3), clupanodonic acid (C22:5 n-3) and cervonic (docosahexaenoic) acid (C22:6 n-3; DHA).

*Content*:

– *sum of the contents of the omega-3 acids EPA and DHA, expressed as triglycerides*: minimum 45 per cent;

– *total omega-3 acids, expressed as triglycerides*: minimum 60 per cent.

A suitable antioxidant may be added.

PRODUCTION

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

CHARACTERS

*Appearance*: pale yellow liquid.

*Solubility*: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in anhydrous ethanol.

IDENTIFICATION

Examine the chromatograms obtained in the assay for EPA and DHA.

*Results*: the peaks due to eicosapentaenoic acid methyl ester and docosahexaenoic acid methyl ester in the chromatogram obtained with test solution (b) are similar in retention time to the corresponding peaks in the chromatograms obtained with reference solutions ($a_1$) and ($a_2$).

TESTS

**Absorbance** (*2.2.25*): maximum 0.73 at 233 nm.

Dilute 0.300 g to 50.0 mL with *trimethylpentane R*. Dilute 2.0 mL of the solution to 50.0 mL with *trimethylpentane R*.

**Acid value** (*2.5.1*): maximum 3.0, determined on 10.0 g in 50 mL of the prescribed mixture of solvents.

**Anisidine value** (*2.5.36*): maximum 30.0.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Oligomers and partial glycerides**. Size-exclusion chromatography (*2.2.30*).

*Test solution*. Dilute 50.0 mg of the substance to be examined to 10.0 mL with *tetrahydrofuran R*.

*Reference solution*. Dissolve 50 mg of *monodocosahexaenoin R*, 30 mg of *didocosahexaenoin R* and 20 mg of *tridocosahexaenoin R* in *tetrahydrofuran R* and dilute to 100.0 mL with the same solvent.

*Column*: 3 columns to be connected in series:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with the following pore sizes:
  - *column 1*: 50 nm;
  - *column 2*: 10 nm;
  - *column 3*: 5 nm;
- *connection sequence*: injector – column 1 – column 2 – column 3 – detector.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 0.8 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*System suitability*: reference solution:

- *elution order*: tridocosahexaenoin, didocosahexaenoin, monodocosahexaenoin;
- *resolution*: minimum 2.0 between the peaks due to didocosahexaenoin and monodocosahexaenoin; minimum 1.0 between the peaks due to tridocosahexaenoin and didocosahexaenoin.

Identify the peaks using the chromatogram shown in Figure 1352.-1. Calculate the percentage content of oligomers using the following expression:

$$\frac{B}{A} \times 100$$

$A$ = sum of the areas of all the peaks in the chromatogram;

$B$ = area of the peak with a retention time less than the retention time of the peak due to the triglycerides.

Calculate the percentage content of partial glycerides using the following expression:

$$\frac{C}{A} \times 100$$

$A$ = sum of the areas of all the peaks in the chromatogram;

$C$ = (sum of the) area(s) of the peak(s) due to the mono- and diglycerides.

*Limits*:

- *oligomers*: maximum 3.0 per cent;
- *partial glycerides*: maximum 50.0 per cent.

ASSAY

**EPA and DHA** (*2.4.29*). For identification of the peaks, see Figure 1352.-2.

**Total omega-3-acids** (*2.4.29*). See Figure 1352.-2.

STORAGE

Under an inert gas, in a well-filled, airtight container, protected from light.

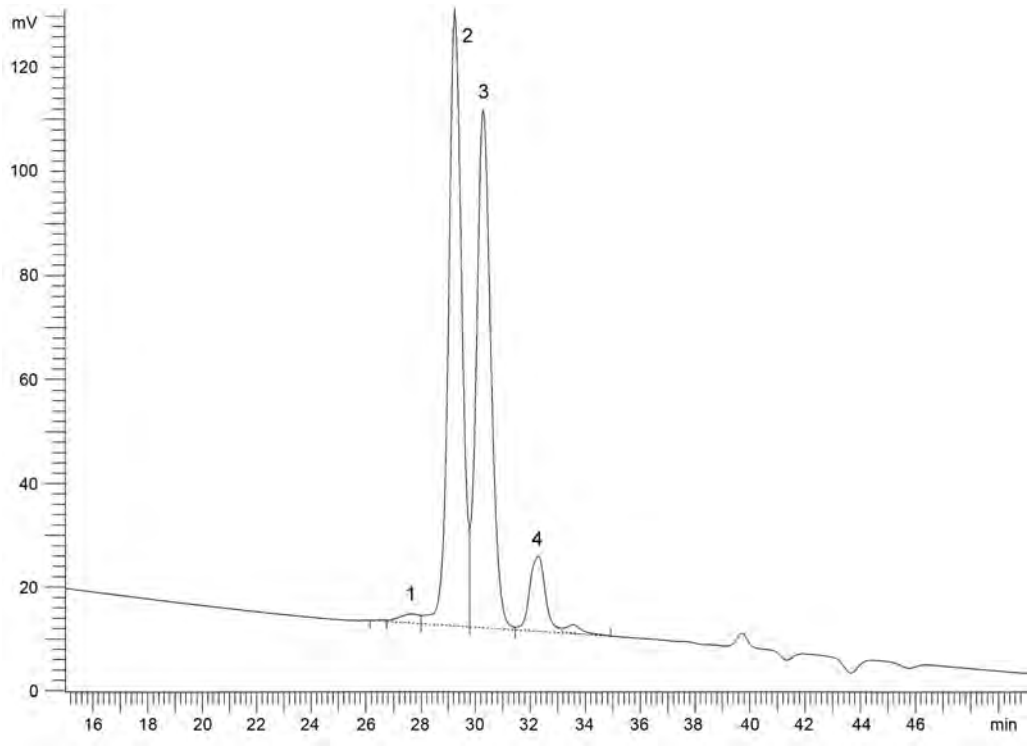


1. oligomers 2. triglycerides 3. diglycerides 4. monoglycerides

Figure 1352.-1. – *Chromatogram for the test for oligomers and partial glycerides in omega-3-acid triglycerides*

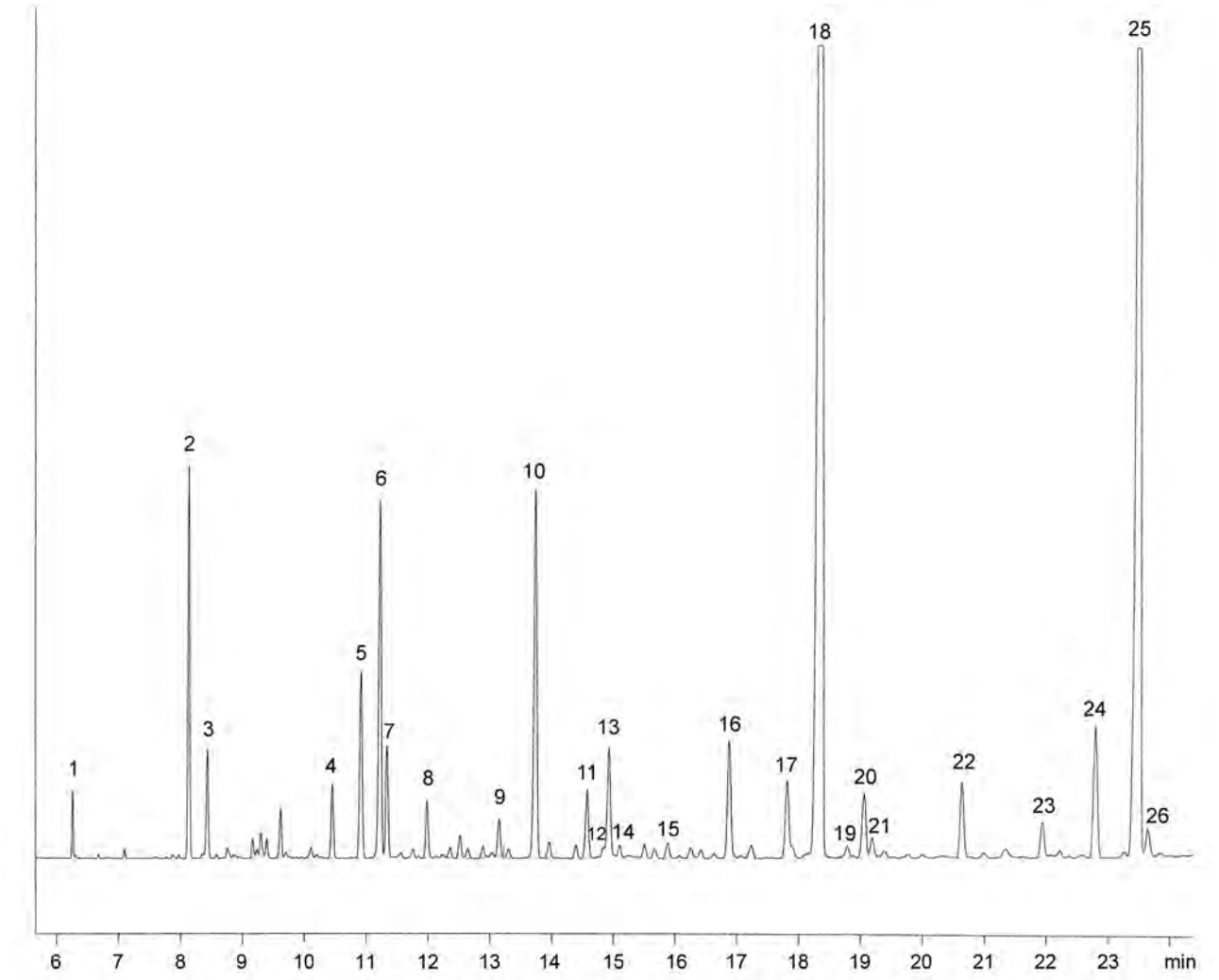


| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1. C14:0 | 4. C16:4 n-1 | 7. C18:1 n-7 | 10. C18:4 n-3 | 13. C20:1 n-9 | 16. C20:4 n-6 | 19. C22:0 | 22. C21:5 n-3 | 25. DHA |
| 2. C16:0 | 5. C18:0 | 8. C18:2 n-6 | 11. C20:0 | 14. C20:1 n-7 | 17. C20:4 n-3 | 20. C22:1 n-11 | 23. C22:5 n-6 | 26. C24:1 n-9 |
| 3. C16:1 n-7 | 6. C18:1 n-9 | 9. C18:3 n-3 | 12. C20:1 n-11 | 15. C20:2 n-6 | 18. EPA | 21. C22:1 n-9 | 24. C22:5 n-3 | |

Figure 1352.-2. – *Chromatogram for the assays of omega-3-acids in omega-3-acid triglycerides*

**04/2013:0942**

# OMEPRAZOLE

## Omeprazolum

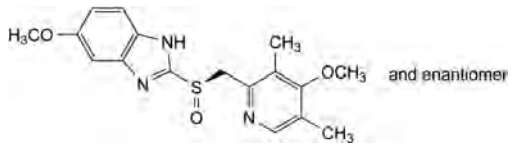


$C_{17}H_{19}N_3O_3S$ $M_r$ 345.4
[73590-58-6]

DEFINITION

5-Methoxy-2-[(*RS*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, soluble in methylene chloride, sparingly soluble in ethanol (96 per cent) and in methanol. It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *omeprazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Solution S**. Dissolve 0.50 g in *methylene chloride R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Impurities F and G**: maximum 350 ppm for the sum of the contents.

The absorbance (*2.2.25*) of solution S determined at 440 nm is not greater than 0.10.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 3 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 3 mg of *omeprazole for peak identification CRS* (containing impurity E) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 40 µL.

*Run time*: 5 times the retention time of omeprazole.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D; use the chromatogram supplied with *omeprazole for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to omeprazole (retention time = about 9 min): impurity E = about 0.6; impurity D = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity D and omeprazole; if necessary, adjust the pH of the aqueous part of the mobile phase or the concentration of *acetonitrile R*; an increase in the pH will improve the resolution.

*Limits*:

- *impurities D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 34.54 mg of $C_{17}H_{19}N_3O_3S$.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, H, I.*

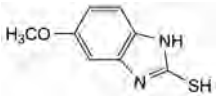

A. 5-methoxy-1*H*-benzimidazole-2-thiol,

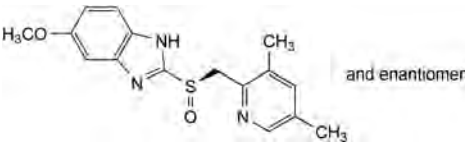


B. 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

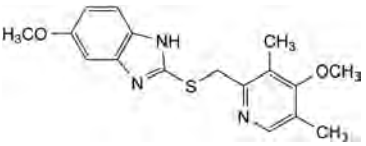

C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole (ufiprazole),

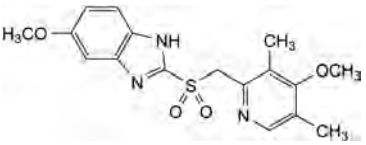

D. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole (omeprazole sulfone),

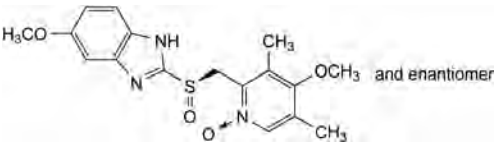


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide,

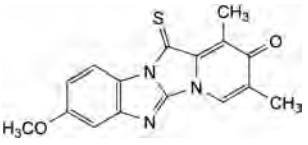

F. 8-methoxy-1,3-dimethyl-12-thioxopyrido[1′,2′:3,4]-imidazo[1,2-*a*]benzimidazol-2(12*H*)-one,

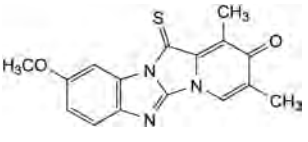

G. 9-methoxy-1,3-dimethyl-12-thioxopyrido[1′,2′:3,4]-imidazo[1,2-*a*]benzimidazol-2(12*H*)-one,

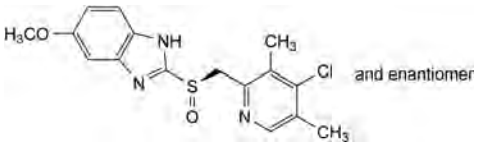


H. 2-[(*RS*)-[(4-chloro-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

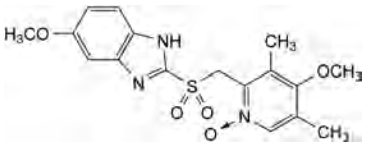


I. 4-methoxy-2-[[(5-methoxy-1*H*-benzimidazol-2-yl)sulfonyl]methyl]-3,5-dimethylpyridine 1-oxide.

**01/2009:2374**
**corrected 6.7**

# OMEPRAZOLE MAGNESIUM

## Omeprazolum magnesicum

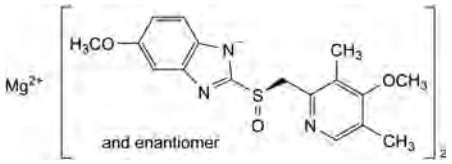


$C_{34}H_{36}MgN_6O_6S_2$ $M_r$ 713
[95382-33-5]

DEFINITION

Magnesium bis[5-methoxy-2-[(*RS*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazol-1-ide]. It contains a variable quantity of water.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very slightly soluble in water, sparingly soluble in methanol, practically insoluble in heptane.

IDENTIFICATION

Carry out either tests A, B, C or tests A, B, D.

A. Optical rotation (*2.2.7*): – 0.10° to + 0.10°.
Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *omeprazole magnesium CRS*.

C. Atomic absorption spectrometry (*2.2.23*) as described in the test for magnesium.
The test solution shows the absorption maximum at 285.2 nm.

D. Ignite about 0.5 g of the substance to be examined according to the procedure for the sulfated ash test (*2.4.14*). Dissolve the residue in 10 mL of *water R*. 2 mL of this solution gives the reaction of magnesium (*2.3.1*).

TESTS

**Absorbance** (*2.2.25*): maximum 0.10 at 440 nm.

Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent. Filter the solution through a membrane filter (nominal pore size 0.45 µm).

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 3.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 3 mg of *omeprazole for peak identification CRS* (containing impurity E) in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 40 µL.

*Run time*: 5 times the retention time of omeprazole.

*Identification of impurities*:
- use the chromatogram supplied with *omeprazole for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity E;
- use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to omeprazole (retention time = about 9 min): impurity E = about 0.6, impurity D = about 0.8.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.0 between the peaks due to impurity D and omeprazole; if necessary, adjust the pH of the aqueous part of the mobile phase or its proportion of acetonitrile; an increase in the pH will improve the resolution.

*Limits*:
- *impurities D, E*: for each impurity, maximum 0.1 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *disregard limit*: half the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Magnesium**: 3.30 per cent to 3.55 per cent (anhydrous substance).

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 0.250 g in 20.0 mL of a 103 g/L solution of *hydrochloric acid R* by slow addition of the acid and dilute to 100.0 mL with *water R*. Dilute 10.0 mL of the solution to 200.0 mL with *water R*. To 10.0 mL of this solution add 4 mL of *lanthanum chloride solution R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *magnesium standard solution (1000 ppm Mg) R*, diluting with a mixture of 1 mL of a 103 g/L solution of *hydrochloric acid R* and 1000.0 mL of *water R*.

*Wavelength*: 285.2 nm.

**Water** (*2.5.12*): 7.0 per cent to 10.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer pH 11.0.* Mix 11 mL of a 95.0 g/L solution of *trisodium phosphate dodecahydrate R* and 22 mL of a 179.1 g/L solution of *disodium hydrogen phosphate dodecahydrate R*. Dilute to 100.0 mL with *water R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in about 10 mL of *methanol R*. Add 10 mL of buffer pH 11.0 and dilute to 200.0 mL with *water R*.

*Reference solution.* Dissolve 10.0 mg of *omeprazole CRS* in about 10 mL of *methanol R*. Add 10 mL of buffer pH 11.0 and dilute to 200.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R* previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of omeprazole.

*Retention time*: omeprazole = about 4 min.

Calculate the percentage content of $C_{34}H_{36}MgN_6O_6S_2$ from the declared content of *omeprazole CRS*.

1 g of omeprazole is equivalent to 1.032 g of omeprazole magnesium.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

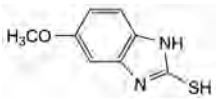

A. 5-methoxy-1*H*-benzimidazole-2-thiol,

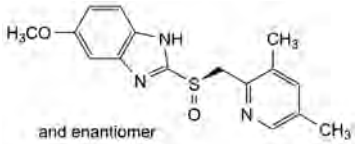


B. 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

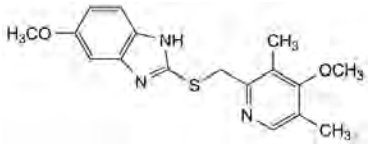

C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole,

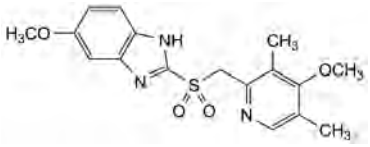

D. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole,

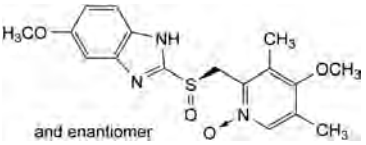


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide.

**01/2017:1032**

# OMEPRAZOLE SODIUM

## Omeprazolum natricum

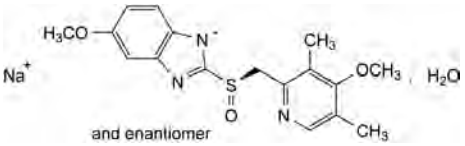


$C_{17}H_{18}N_3NaO_3S,H_2O$ $M_r$ 385.4
[95510-70-6]

DEFINITION

Sodium 5-methoxy-2-[(*RS*)-[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole monohydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), soluble in propylene glycol, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Optical rotation (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.50 g of the substance to be examined in 1.50 mL of *water R*, add 3.0 mL of *methanol R* and stir; while stirring, adjust to pH 8-9 by adding, dropwise, *dilute acetic acid R* (about 0.4 mL); continue stirring until crystallisation and isolate the crystalline precipitate by filtration; wash with 5 mL of *water R*, then 2 mL of *methanol R*, and dry *in vacuo* at 40 °C for 30 min.

*Comparison*: *omeprazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the crystalline precipitate and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Ignite 1 g and cool. Add 1 mL of *water R* to the residue and neutralise with *hydrochloric acid R*. Filter and dilute the filtrate to 4 mL with *water R*. 0.1 mL of the solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 10.3 to 11.3 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare solutions immediately before use.*

*Test solution*. Dissolve 3 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 1 mg of *omeprazole CRS* and 1 mg of *omeprazole impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 3 mg of *omeprazole for peak identification CRS* (containing impurity E) in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 27 volumes of *acetonitrile R* and 73 volumes of a 1.4 g/L solution of *disodium hydrogen phosphate dodecahydrate R*, previously adjusted to pH 7.6 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 40 µL.

*Run time*: 5 times the retention time of omeprazole.

*Identification of impurities*: use the chromatogram supplied with *omeprazole for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to omeprazole (retention time = about 9 min): impurity E = about 0.6; impurity D = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity D and omeprazole; if necessary adjust the pH of the aqueous part of the mobile phase or the concentration of *acetonitrile R*; an increase in the pH will improve the resolution.

*Limits*:

- *impurities D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): 4.5 per cent to 10.0 per cent, determined on 0.300 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* corresponds to 36.74 mg of $C_{17}H_{18}N_3NaO_3S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

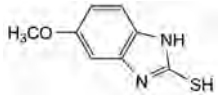


A. 5-methoxy-1*H*-benzimidazole-2-thiol,

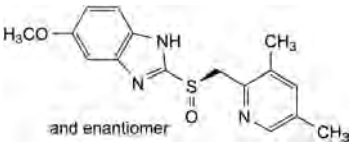


B. 2-[(*RS*)-[(3,5-dimethylpyridin-2-yl)methyl]sulfinyl]-5-methoxy-1*H*-benzimidazole,

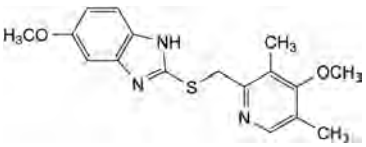


C. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole (ufiprazole),

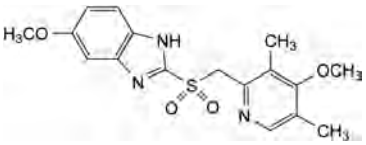


D. 5-methoxy-2-[[(4-methoxy-3,5-dimethylpyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole (omeprazole-sulfone),

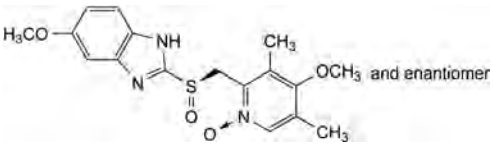


E. 4-methoxy-2-[[(*RS*)-(5-methoxy-1*H*-benzimidazol-2-yl)sulfinyl]methyl]-3,5-dimethylpyridine 1-oxide.

**07/2011:2016**
**corrected 7.4**

# ONDANSETRON HYDROCHLORIDE DIHYDRATE

## Ondansetroni hydrochloridum dihydricum

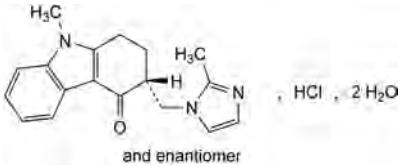


$C_{18}H_{20}ClN_3O,2H_2O$ $M_r$ 365.9
[103639-04-9]

DEFINITION

(3*RS*)-9-Methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4*H*-carbazol-4-one hydrochloride dihydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ondansetron hydrochloride dihydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *methanol R* (0.5:100:100 *V/V/V*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 12.5 mg of *ondansetron for TLC system suitability CRS* (containing impurities A and B) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1 mL of the test solution to 100 mL with the solvent mixture. Dilute 4.0 mL of this solution to 10.0 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *methylene chloride R* (2:40:50:90 *V/V/V/V*).

*Application*: 20 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Retardation factors*: impurity A = about 0.3; impurity B = about 0.4; ondansetron = about 0.6.

*System suitability*: the chromatogram obtained with reference solution (a) shows 3 clearly separated spots.

*Limit*:

– *impurity B*: any spot corresponding to impurity B in the chromatogram obtained with the test solution is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.4 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Test solution (b)*. Dissolve 90.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *ondansetron impurity E CRS* and 5 mg of *ondansetron impurity A CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5 mg of *ondansetron for LC system suitability CRS* (containing impurities C and D) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 5.0 mg of *ondansetron impurity D CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (e)*. Dissolve 90.0 mg of *ondansetron hydrochloride dihydrate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (f)*. Dissolve 5.0 mg of *ondansetron impurity F CRS* and 5 mg of *ondansetron impurity G CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (g)*. To 1.0 mL of reference solution (b) add 1.0 mL of reference solution (f) and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: spherical *nitrile silica gel for chromatography R* (5 µm) with a specific surface area of 220 $m^2$/g and a pore size of 8 nm.

*Mobile phase*: mix 20 volumes of *acetonitrile R1* and 80 volumes of a 2.8 g/L solution of *sodium dihydrogen phosphate monohydrate R* previously adjusted to pH 5.4 with a 40 g/L solution of *sodium hydroxide R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 216 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b), (c), (d), (f) and (g).

*Run time*: 1.5 times the retention time of ondansetron.

*Identification of impurities*:

– use the chromatogram supplied with *ondansetron for LC system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C and D;
– use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and E;
– use the chromatogram obtained with reference solution (f) to identify the peaks due to impurities F and G.

*Relative retention* with reference to ondansetron (retention time = about 18 min): impurity E = about 0.17; impurity F = about 0.20 (E and F may coelute); impurity C = about 0.35; impurity D = about 0.45; impurity A = about 0.80; impurity G = about 0.89 (A and G may coelute or be inverted).

*System suitability*: reference solution (c):

– *resolution*: minimum 2.5 between the peaks due to impurities C and D.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.6;

- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.15 per cent);
- *sum of impurities A and G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities E and F*: not more than the sum of the areas of the corresponding peaks in the chromatogram obtained with reference solution (g) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: maximum 0.4 per cent;
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 9.0 per cent to 10.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (e).

Calculate the percentage content of $C_{18}H_{20}ClN_3O$ from the declared content of *ondansetron hydrochloride dihydrate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H.*

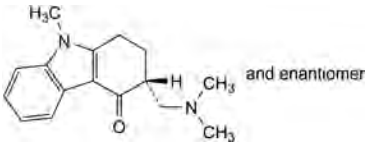


A. (3*RS*)-3-[(dimethylamino)methyl]-9-methyl-1,2,3,9-tetrahydro-4*H*-carbazol-4-one,

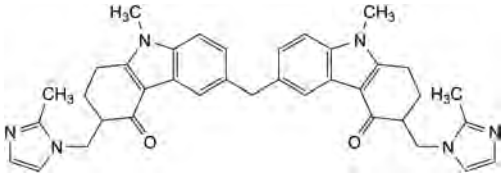

B. 6,6′-methylenebis[(3*RS*)-9-methyl-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4*H*-carbazol-4-one],

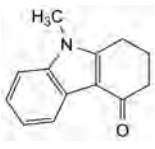

C. 9-methyl-1,2,3,9-tetrahydro-4*H*-carbazol-4-one,

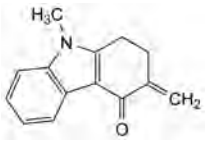

D. 9-methyl-3-methylene-1,2,3,9-tetrahydro-4*H*-carbazol-4-one,

E. 1*H*-imidazole,

F. 2-methyl-1*H*-imidazole,

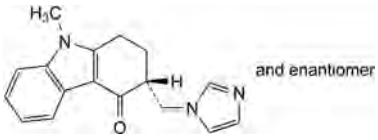


G. (3*RS*)-3-[(1*H*-imidazol-1-yl)methyl]-9-methyl-1,2,3,9-tetrahydro-4*H*-carbazol-4-one (*C*-desmethylondansetron),

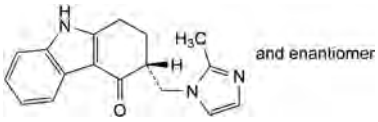


H. (3*RS*)-3-[(2-methyl-1*H*-imidazol-1-yl)methyl]-1,2,3,9-tetrahydro-4*H*-carbazol-4-one (*N*-desmethylondansetron).

**01/2010:2259**
**corrected 7.0**

# ORBIFLOXACIN FOR VETERINARY USE

## Orbifloxacinum ad usum veterinarium

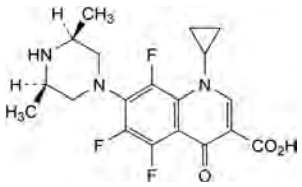

$C_{19}H_{20}F_3N_3O_3$ $M_r$ 395.4
[113617-63-3]

DEFINITION

1-Cyclopropyl-7-[(3*R*,5*S*)-3,5-dimethylpiperazin-1-yl]-5,6,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, crystals or crystalline powder.

*Solubility*: very slightly soluble in water, soluble in glacial acetic acid, practically insoluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *orbifloxacin CRS*.

If the spectra obtained in the solid state show differences, dissolve 0.1 g of the substance to be examined and 0.1 g of the reference substance separately in 12 mL of *methanol R*. Heat to boiling while shaking. Filter the solutions and let them cool slowly to room temperature. Filter under vacuum and wash the residues with cooled *methanol R*. Dry the residues under vacuum and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

Dissolve 0.4 g in a 4 g/L solution of *sodium hydroxide R* and dilute to 20 mL with the same solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 5.9 g of *sodium citrate R* in 800 mL of *water R*, add 90 mL of *glacial acetic acid R* and mix. Adjust to pH 3.5 with a 240 g/L solution of *sodium hydroxide R* in *water R* and dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 10 mg of the substance to be examined in the buffer solution and dilute to 50.0 mL with the buffer solution.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the buffer solution. Dilute 1.0 mL of this solution to 10.0 mL with the buffer solution.

*Reference solution (b)*. Dissolve 10.0 mg of *methyl 4-aminobenzoate R* in the buffer solution and dilute to 100.0 mL with the buffer solution. Mix 10.0 mL of the solution with 5.0 mL of the test solution and dilute to 50.0 mL with the buffer solution. Dilute 1.0 mL of this solution to 50.0 mL with the buffer solution.

*Reference solution (c)*. Dissolve the contents of a vial of *orbifloxacin impurity mixture CRS* (impurities A and D) in 1.0 mL of the buffer solution.

*Reference solution (d)*. Dilute 0.25 mL of reference solution (c) to 1.0 mL of the buffer solution.

*Column*:

- *size*: *l* = 33 mm, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *dioxan R*, *methanol R*, buffer solution (4:11:86 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 10 µL.

*Run time*: 9 times the retention time of orbifloxacin.

*Identification of the impurities*: use the chromatogram supplied with *orbifloxacin impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and D.

*Relative retention* with reference to orbifloxacin (retention time = about 2 min): impurity A = about 0.5; methyl 4-aminobenzoate = about 1.2; impurity D = about 2.5.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to orbifloxacin and methyl 4-aminobenzoate in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity A in the chromatogram obtained with reference solution (d).

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 2.8; impurity D = 1.4;
- *impurities A, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent).

**Water** (*2.5.12*): 1.5 per cent to 2.9 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 100 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 39.54 mg of $C_{19}H_{20}F_3N_3O_3$.

IMPURITIES

*Specified impurities*: *A, D*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, F, G*.

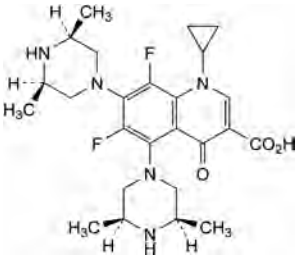

A. 1-cyclopropyl-5,7-bis[(3*R*,5*S*)-3,5-dimethylpiperazin-1-yl]6,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

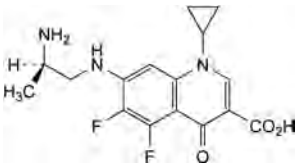

B. 7-[[(2*R*)-2-aminopropyl]amino]-1-cyclopropyl-5,6-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

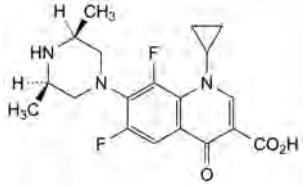

C. 1-cyclopropyl-7-[(3*R*,5*S*)3,5-dimethylpiperazin-1-yl]-6,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

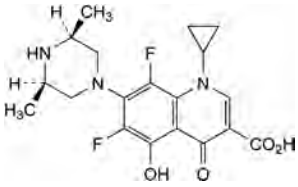

D. 1-cyclopropyl-7-[(3*R*,5*S*)-3,5-dimethylpiperazin-1-yl]-6,8-difluoro-5-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

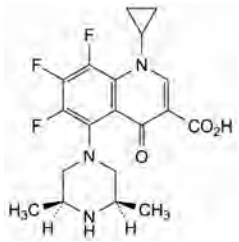

E. 1-cyclopropyl-5-[(3*R*,5*S*)-3,5-dimethylpiperazin-1-yl]-6,7,8-trifluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

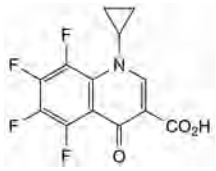

F. 1-cyclopropyl-5,6,7,8-tetrafluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid,

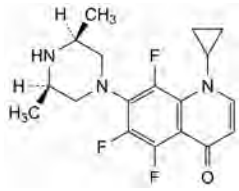

G. 1-cyclopropyl-7-[(3*R*,5*S*)-3,5-dimethylpiperazin-1-yl]-5,6,8-trifluoroquinolin-4(1*H*)-one.

**01/2017:1033**

# ORCIPRENALINE SULFATE

## Orciprenalini sulfas

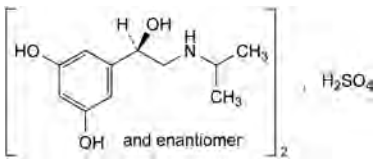

$C_{22}H_{36}N_2O_{10}S$ $M_r$ 520.6
[5874-97-5]

DEFINITION

Bis[5-[(1*RS*)-1-hydroxy-2-[(1-methylethyl)amino]ethyl]-benzene-1,3-diol] sulfate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in a 0.04 per cent *V/V* solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solution. Dilute 5.0 mL of this solution to 50.0 mL with a 0.04 per cent *V/V* solution of *hydrochloric acid R*.

*Spectral range*: 240-350 nm.

*Absorption maximum*: at 278 nm.

*Specific absorbance at the absorption maximum*: 68.5 to 76.0 (anhydrous substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *orciprenaline sulfate CRS*.

If the spectra obtained show differences, dissolve separately, with heating, 50 mg of the substance to be examined and 50 mg of the reference substance, in the minimum volume of *water R*. Add 10 mL of *acetone R* and centrifuge. Dry the precipitates at 40 °C under reduced pressure for 3 h and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *orciprenaline sulfate CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *orciprenaline sulfate CRS* and 10 mg of *salbutamol CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *ammonia R, water R, aldehyde-free methanol R* (1.5:10:90 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 10 g/L solution of *potassium permanganate R*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 20 mg in 2 mL of *ethanol (96 per cent) R*. Add 2 mL of a 1 g/L solution of *dichloroquinonechlorimide R* in *ethanol (96 per cent) R* and 1 mL of *sodium carbonate solution R*. A violet colour is produced, turning to brown.

E. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *orciprenaline for system suitability CRS* (containing impurities A and B) in 2.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase.* Dissolve 9.1 g of *potassium dihydrogen phosphate R* and 4.6 g of *sodium octanesulfonate R* in *water R*, adjust to pH 4.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*. Add 140 mL of *acetonitrile R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of orciprenaline.

*Identification of impurities*: use the chromatogram supplied with *orciprenaline for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to orciprenaline (retention time = about 7 min): impurity A = about 0.9; impurity B = about 1.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity A and orciprenaline.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.3;
- *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Phenone**: maximum 0.1 per cent.

Dissolve 0.50 g in a 0.04 per cent *V/V* solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution. The absorbance (*2.2.25*) of the solution measured at 328 nm is not greater than 0.16.

**Iron** (*2.4.9*): maximum 20 ppm.

The residue obtained in the test for sulfated ash complies with the test. Prepare the reference solution using *iron standard solution (2 ppm Fe) R*.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 5 mL of *anhydrous formic acid R* and add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* using 0.1 mL of *crystal violet solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 52.06 mg of $C_{22}H_{36}N_2O_{10}S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

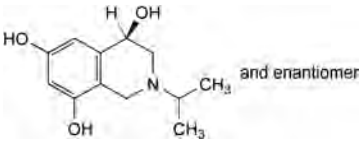


A. (4*RS*)-2-(1-methylethyl)-1,2,3,4-tetrahydroisoquinoline-4,6,8-triol,

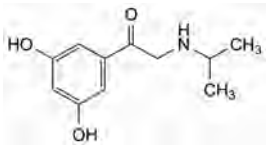


B. 1-(3,5-dihydroxyphenyl)-2-[(1-methylethyl)amino]-ethanone,

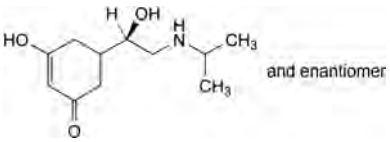


C. 3-hydroxy-5-[(1*RS*)-1-hydroxy-2-[(1-methylethyl)amino]-ethyl]cyclohex-2-enone.

**01/2017:1759**
**corrected 10.0**

# ORPHENADRINE CITRATE

## Orphenadrini citras

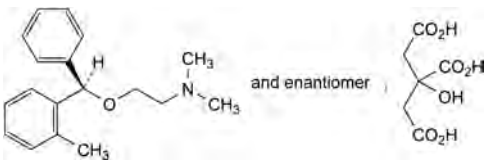


$C_{24}H_{31}NO_8$ $M_r$ 461.5
[4682-36-4]

DEFINITION

(*RS*)-*N,N*-Dimethyl-2-[(2-methylphenyl)phenylmethoxy]-ethanamine dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 137 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *orphenadrine citrate CRS.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 436 nm has a maximum of 0.050.

Dissolve 1.0 g in a 3.6 per cent *V/V* solution of *hydrochloric acid R* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same acid solution.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 50 mL with the same solvent. Add 2 mL of *concentrated ammonia R* and shake with 3 quantities, each of 10 mL, of *toluene R*. To the combined upper layers add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means, at a temperature not exceeding 50 °C. Take up the residue with *toluene R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 30 mg of *orphenadrine citrate CRS* and 30 mg of *orphenadrine impurity E CRS* in 20 mL of *water R*. Add 1 mL of *concentrated ammonia R* and shake with 3 quantities, each of 5 mL, of *toluene R*. To the combined upper layers add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means, at a temperature not exceeding 50 °C. Take up the residue with *toluene R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dissolve the contents of a vial of *orphenadrine for peak identification CRS* (containing impurities A, B, C, D and F) in 1.0 mL of *toluene R*.

*Column*:

- *size*: *l* = 60 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:25.

*Temperature*:

- *column*: 240 °C;
- *injection port and detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Run time*: 1.3 times the retention time of orphenadrine.

*Identification of impurities*: use the chromatogram supplied with *orphenadrine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and F. Use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E.

*Relative retention* with reference to orphenadrine (retention time = about 13 min): impurity B = about 0.5; impurity A = about 0.6; impurity D = about 0.8; impurity C = about 0.9; impurity E = about 0.98; impurity F = about 1.1.

*System suitability*: reference solution (a):

- *resolution*: minimum of 1.5 between the peaks due to impurity E and orphenadrine.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, not more than 0.3 per cent;
- *unspecified impurities*: for each impurity, not more than 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *disregard limit*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 46.15 mg of $C_{24}H_{31}NO_8$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F*.

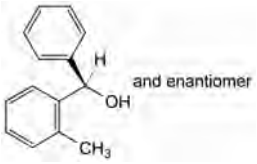


A. (*RS*)-(2-methylphenyl)phenylmethanol (2-methylbenzhydrol),

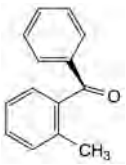


B. (2-methylphenyl)phenylmethanone (2-methylbenzophenone),

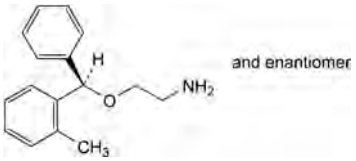


C. (*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,

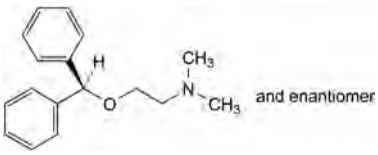


D. 2-(diphenylmethoxy)-*N*,*N*-dimethylethanamine (diphenhydramine),

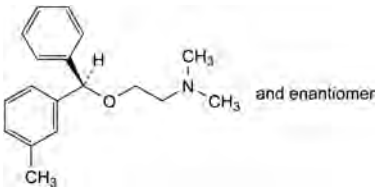


E. (*RS*)-*N*,*N*-dimethyl-2-[(3-methylphenyl)phenylmethoxy]-ethanamine (*meta*-methylbenzyl isomer),

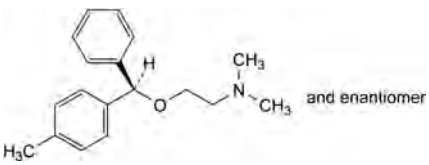


F. (*RS*)-*N*,*N*-dimethyl-2-[(4-methylphenyl)phenylmethoxy]-ethanamine (*para*-methylbenzyl isomer).

**01/2017:1760**
**corrected 10.0**

# ORPHENADRINE HYDROCHLORIDE

## Orphenadrini hydrochloridum

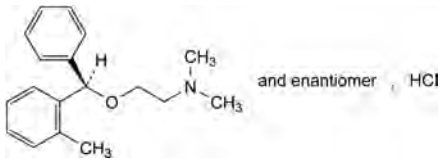


$C_{18}H_{24}ClNO$ $M_r$ 305.9
[341-69-5]



DEFINITION

(*RS*)-*N,N*-Dimethyl-2-[(2-methylphenyl)phenylmethoxy]-ethanamine hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

mp: about 160 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *orphenadrine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 436 nm has a maximum of 0.050.
Dissolve 0.70 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.300 g of the substance to be examined in *water R* and dilute to 50 mL with the same solvent. Add 2 mL of *concentrated ammonia R* and shake with 3 quantities, each of 10 mL, of *toluene R*. To the combined upper layers add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means, at a temperature not exceeding 50 °C. Take up the residue with *toluene R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *orphenadrine hydrochloride CRS* and 20 mg of *orphenadrine impurity E CRS* in 20 mL of *water R*. Add 1 mL of *concentrated ammonia R* and shake with 3 quantities, each of 5 mL, of *toluene R*. To the combined upper layers add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means, at a temperature not exceeding 50 °C. Take up the residue with *toluene R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b)*. Dissolve the contents of a vial of *orphenadrine for peak identification CRS* (containing impurities A, B, C, D and F) in 1.0 mL of *toluene R*.

*Column*:
- *size*: *l* = 60 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:25.

*Temperature*:
- *column*: 240 °C;
- *injection port and detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Run time*: 1.3 times the retention time of orphenadrine.

*Identification of impurities*: use the chromatogram supplied with *orphenadrine for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and F. Use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E.

*Relative retention* with reference to orphenadrine (retention time = about 13 min): impurity B = about 0.5; impurity A = about 0.6; impurity D = about 0.8; impurity C = about 0.9; impurity E = about 0.98; impurity F = about 1.1.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity E and orphenadrine.

*Limits*:
- *impurities A, B, C, D, E, F*: for each impurity, not more than 0.3 per cent;
- *unspecified impurities*: for each impurity, not more than 0.10 per cent;
- *total*: not more than 1.0 per cent;
- *disregard limit*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.59 mg of $C_{18}H_{24}ClNO$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

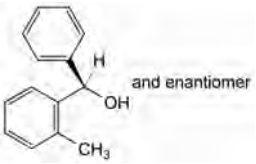


A. (*RS*)-(2-methylphenyl)phenylmethanol (2-methyl-benzhydrol),

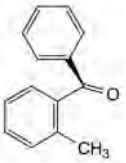


B. (2-methylphenyl)phenylmethanone (2-methyl-benzophenone),

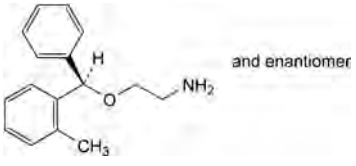


C. (*RS*)-2-[(2-methylphenyl)phenylmethoxy]ethanamine,

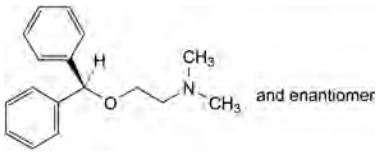


D. 2-(diphenylmethoxy)-*N*,*N*-dimethylethanamine (diphenhydramine),

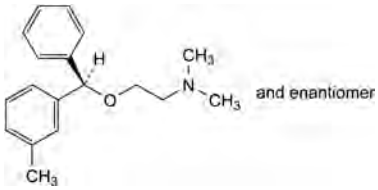


E. (*RS*)-*N*,*N*-dimethyl-2-[(3-methylphenyl)phenylmethoxy]-ethanamine (*meta*-methylbenzyl isomer),

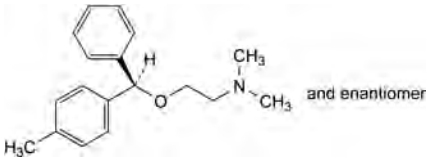


F. (*RS*)-*N*,*N*-dimethyl-2-[(4-methylphenyl)phenylmethoxy]-ethanamine (*para*-methylbenzyl isomer).

**04/2011:2422**
**corrected 10.0**

# OSELTAMIVIR PHOSPHATE

## Oseltamiviri phosphas

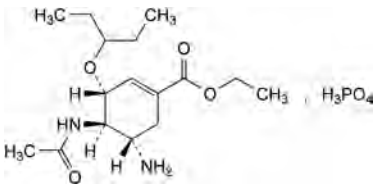


$C_{16}H_{31}N_2O_8P$ $M_r$ 410.4
[204255-11-8]

DEFINITION

Ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylate phosphate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *oseltamivir phosphate (impurity B-free) CRS*.
If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Dissolve 200 mg in 10 mL of *water R*. It gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): – 30.7 to – 32.6 (anhydrous substance), measured at 25 °C.

Dissolve 0.50 g in *water R* and dilute to 50.0 mL with the same solvent.

**Impurity B**. Liquid chromatography (*2.2.29*) coupled with mass spectrometry (*2.2.43*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water for chromatography R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 2.5 mg of *oseltamivir impurity B CRS* in 5.0 mL of *anhydrous ethanol R* and dilute to 50.0 mL with *water for chromatography R*. Dilute 2.0 mL of the solution to 100.0 mL with *water for chromatography R*.

*Reference solution (b)*. Dissolve 50.0 mg of *oseltamivir phosphate (impurity B-free) CRS* in reference solution (a) and dilute to 5.0 mL with the same solution.

*Column*:
- *size*: *l* = 0.05 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 10 volumes of a 1.54 g/L solution of *ammonium acetate R* in *water for chromatography R*, 30 volumes of *acetonitrile R1* and 60 volumes of *water for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Post-column split ratio*: use a split ratio suitable for the mass detector (e.g. 1:3).

*Detection*:
- mass detector: the following settings have been found to be suitable and are given as examples; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:
  - ionisation: ESI-positive;
  - detection *m/z*: 356.2;
  - dwell: 580 ms;
  - gain EMV: 1;
  - fragmentator voltage: 120 V;
  - gas temperature: 350 °C;
  - drying gas flow: 13 L/min,
  - nebuliser pressure: 345 kPa;
  - capillary voltage (Vcap): 3 kV.

*Injection*: 1 µL of the test solution and reference solution (b).

*Run time*: 3 min.

*System suitability*: reference solution (b):
- *repeatability*: maximum relative standard deviation of 15 per cent determined on 6 injections.

*Limit*:
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (100 ppm).

**Impurity H**. Gas chromatography (*2.2.28*).

*Silylation reagent*. Mix 1.0 mL of *chlorotrimethylsilane R*, 2.0 mL of *hexamethyldisilazane R* and 10.0 mL of *anhydrous pyridine R*.

*Test solution*. Introduce 15.0 mg of the substance to be examined into a 2 mL vial and add 1.0 mL of the silylation reagent. Close the vial, shake and heat at 60 °C for 20 min. Centrifuge and discard the precipitate.

*Reference solution*. Introduce 15.0 mg of *oseltamivir impurity H CRS* into a 2 mL vial and add 1.0 mL of *anhydrous pyridine R*. Close the vial and shake (solution A). (Note: impurity H is hygroscopic.) Introduce 15.0 mg of the substance to be examined into another 2 mL vial and add 1.0 mL of the silylation reagent. Close the vial, shake and heat

at 60 °C for 20 min. Centrifuge and discard the precipitate (solution B). Introduce 10.0 µL of solution A and 10.0 µL of solution B into a volumetric flask and dilute to 10.0 mL with *anhydrous pyridine R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.2 mL/min.

*Split ratio*: 1:50.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 180 |
| | 2 - 11 | 180 → 250 |
| | 11 - 21 | 250 |
| Injection port | | 260 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to oseltamivir phosphate (retention time = about 10 min): impurity H = about 0.5.

*System suitability*: reference solution:

- *repeatability*: maximum relative standard deviation of 5 per cent for the peak due to impurity H after 6 injections.

*Limit*:

- *impurity H*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (0.15 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *methanol R2*, *water for chromatography R* (135:245:620 *V/V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *oseltamivir impurity A CRS* and 5.0 mg of *oseltamivir impurity C CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *oseltamivir phosphate (impurity B-free) CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*: mix 135 volumes of *acetonitrile R1*, 245 volumes of *methanol R2* and 620 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* in *water for chromatography R*, adjusted to pH 6.0 with a 1 M potassium hydroxide solution prepared from *potassium hydroxide R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 207 nm.

*Injection*: 15 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of oseltamivir phosphate.

*Relative retention* with reference to oseltamivir phosphate (retention time = about 17 min): impurity A = about 0.16; impurity C = about 0.17.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and C.

*Limits*:

- *impurity C*: not more than 0.3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{16}H_{31}N_2O_8P$ from the declared content of *oseltamivir phosphate (impurity B-free) CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F, G.*

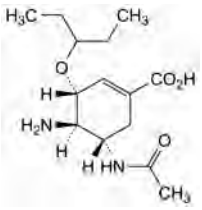

A. (3*R*,4*R*,5*S*)-5-acetamido-4-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylic acid,

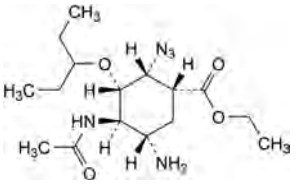

B. ethyl (1*R*,2*R*,3*S*,4*R*,5*S*)-4-acetamido-5-amino-2-azido-3-(1-ethylpropoxy)cyclohexanecarboxylate,

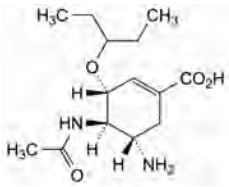

C. (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)-cyclohex-1-ene-1-carboxylic acid,

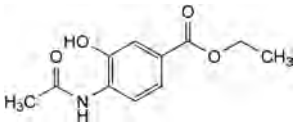

D. ethyl 4-acetamido-3-hydroxybenzoate,

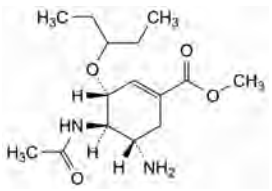

E. methyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-ethylpropoxy)cyclohex-1-ene-1-carboxylate,

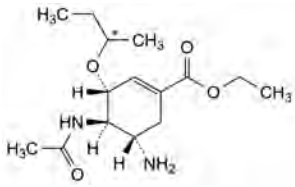

F. ethyl (3*R*,4*R*,5*S*)-4-acetamido-5-amino-3-(1-methylpropoxy)cyclohex-1-ene-1-carboxylate,

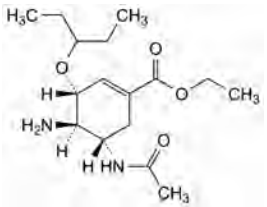

G. ethyl (3*R*,4*R*,5*S*)-5-acetamido-4-amino-3-(1-ethylpropoxy)cyclohex-1-ene-1-carboxylate,

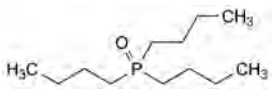

H. tributylphosphane oxide.

**01/2008:0048**
**corrected 6.0**

# OUABAIN

## Ouabainum

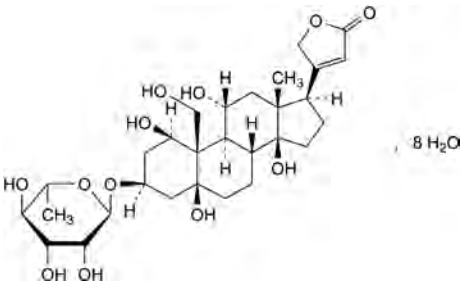

$C_{29}H_{44}O_{12},8H_2O$ $M_r$ 729
[11018-89-6]

DEFINITION

3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-1β,5,11α,14,19-pentahydroxy-5β,14β-card-20(22)-enolide octahydrate.

*Content*: 96.0 per cent to 104.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: sparingly soluble in water and in anhydrous ethanol, practically insoluble in ethyl acetate.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a).

B. Dissolve 2 mg to 3 mg in 2 mL of *sulfuric acid R*; a pink colour develops which quickly changes to red. The solution shows green fluorescence in ultraviolet light.

C. Dissolve about 1 mg in 1 mL of *dinitrobenzene solution R* and add 0.2 mL of *dilute sodium hydroxide solution R*. An intense blue colour develops.

D. Dissolve 0.1 g in 5 mL of a 150 g/L solution of *sulfuric acid R* and boil for a few minutes. The solution becomes yellow and turbid. Filter and add to the filtrate 5 mL of a 120 g/L solution of *sodium hydroxide R* and 3 mL of *cupri-tartaric solution R*. Heat. A red precipitate is formed.

TESTS

**Solution S**. Dissolve 0.20 g in 15 mL of *water R*, heating on a water-bath. Allow to cool and dilute to 20.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): – 33 to – 30 (anhydrous substance), determined on solution S.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *chloroform R*, *methanol R* (16:50:50 *V/V/V*).

*Test solution*. Dissolve a quantity of the substance to be examined corresponding to 20 mg of the anhydrous substance in 1.0 mL of the solvent mixture.

*Reference solution (a)*. Dissolve a quantity of *ouabain CRS* corresponding to 20 mg of the anhydrous substance in 1.0 mL of the solvent mixture.

*Reference solution (b).* Dissolve a quantity of *ouabain CRS* corresponding to 10 mg of the anhydrous substance in the solvent mixture and dilute to 25 mL with the solvent mixture.

*Reference solution (c).* Dilute 2.5 mL of reference solution (b) to 10 mL with the solvent mixture.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R, dimethyl sulfoxide R, methanol R, chloroform R* (4:15:15:70 *V/V/V/V*); homogenise the mixture before use.

*Application*: 5 µL.

*Development*: over a path of 13 cm.

*Drying*: immediately at 140 °C for 30 min in a ventilated oven.

*Detection*: allow to cool, spray with *alcoholic solution of sulfuric acid R* and heat at 140 °C for 15 min.

*System suitability*:

- the principal spot in the chromatogram obtained with the test solution and the principal spot in the chromatogram obtained with reference solution (a) migrate over a distance sufficient to give unequivocal separation of the secondary spots;
- the chromatogram obtained with reference solution (c) shows a clearly visible spot.

*Limit*:

- *any impurity*: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (2.0 per cent).

**Alkaloids and strophanthin-K**. To 5.0 mL of solution S add 0.5 mL of a 100 g/L solution of *tannic acid R*. No precipitate is formed.

**Water** (*2.5.12*): 18.0 per cent to 22.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*Test solution.* Dissolve 40.0 mg in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*.

*Reference solution.* Dissolve 40.0 mg of *ouabain CRS* in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *ethanol (96 per cent) R*.

To 5.0 mL of each solution add 3.0 mL of *alkaline sodium picrate solution R*, allow to stand protected from bright light for 30 min and measure the absorbance (*2.2.25*) of both solutions at the absorption maximum at 495 nm using as the compensation liquid a mixture of 3.0 mL of *alkaline sodium picrate solution R* and 5.0 mL of *ethanol (96 per cent) R* prepared at the same time.

Calculate the percentage content of $C_{29}H_{44}O_{12}$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Protected from light.

**04/2013:2260**
**corrected 10.0**

# OXACILLIN SODIUM MONOHYDRATE

## Oxacillinum natricum monohydricum

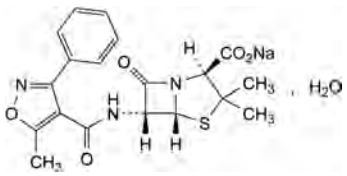

$C_{19}H_{18}N_3NaO_5S,H_2O$ $M_r$ 441.4
[7240-38-2]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-3,3-dimethyl-6-[[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *oxacillin sodium monohydrate CRS.*

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.10.

Dissolve 2.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 7.5.

Dissolve 0.30 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 196 to + 212 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *oxacillin sodium monohydrate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of test solution (b) to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *cloxacillin sodium CRS* (impurity E) and 5 mg of *oxacillin sodium monohydrate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (d).* In order to prepare impurities B and D *in situ*, dissolve 25 mg of the substance to be examined in 1 mL of *0.05 M sodium hydroxide*, allow to stand for 3 min, then dilute to 100 mL with the mobile phase. Inject immediately.

*Reference solution (e).* Dissolve 5 mg of *oxacillin for peak identification CRS* (containing impurities E, F, G, I and J) in 5 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R* and 75 volumes of a 2.7 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.0 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c), (d) and (e).

*Run time*: 7 times the retention time of oxacillin.

*Identification of impurities*:

– in the chromatogram obtained with reference solution (d), the 2 principal peaks eluting before the main peak are due to impurities B and D respectively;

– use the chromatogram supplied with *oxacillin for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities E, F, G, I and J.

*Relative retention* with reference to oxacillin (retention time = about 5 min): impurity A = about 0.3; impurity B (isomer 1) = about 0.4; impurity B (isomer 2) = about 0.5; impurity C = about 0.65; impurity D (2 epimers) = about 0.9; impurity E = about 1.5; impurity F = about 1.9; impurity G = about 2.1; impurity I = about 3.8; impurity J = about 5.8.

*System suitability*:

– *resolution*: minimum 2.5 between the peaks due to oxacillin and impurity E in the chromatogram obtained with reference solution (c);

– the chromatogram obtained with reference solution (e) is similar to the chromatogram supplied with *oxacillin for peak identification CRS*.

*Limits*:

– *impurity B*: for the sum of the areas of the 2 isomer peaks, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurities D (sum of the 2 epimers), F, G, I, J*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *any other impurity*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethyl acetate and butyl acetate**. Head-space gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in 6.0 mL of *water R*.

*Reference solution.* Dissolve 83 mg of *butyl acetate R* and 83 mg of *ethyl acetate R* in *water R* and dilute to 250.0 mL with the same solvent. Use 6.0 mL of this solution.

*Close the vials immediately with a rubber membrane stopper coated with polytetrafluoroethylene and secured with an aluminium crimped cap. Mix to obtain a homogeneous solution.*

*Column*:

– *material*: fused silica;

– *size*: *l* = 50 m, Ø = 0.32 mm;

– *stationary phase*: *methylpolysiloxane R* (film thickness 5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2 mL/min.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 80 °C;

– *equilibration time*: 60 min;

– *transfer-line temperature*: 140 °C;

– *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 70 |
| | 6 - 16 | 70 → 220 |
| | 16 - 18 | 220 |
| Injection port | | 140 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Retention time*: ethyl acetate = about 10 min; butyl acetate = about 15.5 min.

*Limits*:

– *butyl acetate*: maximum 1.0 per cent;

– *ethyl acetate*: maximum 1.0 per cent.

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent.

**Water** (*2.5.12*): 3.5 per cent to 5.0 per cent, determined on 0.300 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.20 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{19}H_{18}N_3NaO_5S$ taking into account the assigned content of *oxacillin sodium monohydrate CRS*.

IMPURITIES

*Specified impurities: B, D, E, F, G, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C*.

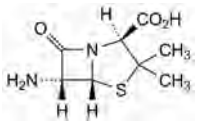

A. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

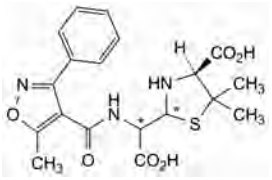

B. (4*S*)-2-[carboxy[[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of oxacillin),

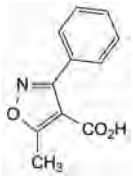

C. 5-methyl-3-phenylisoxazole-4-carboxylic acid,

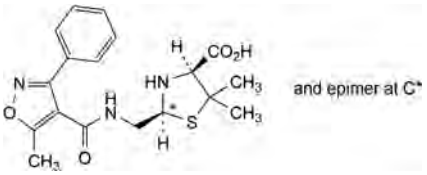


D. (2*RS*,4*S*)-5,5-dimethyl-2-[[[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]methyl]thiazolidine-4-carboxylic acid (penilloic acids of oxacillin),

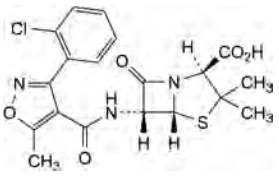

E. (2*S*,5*R*,6*R*)-6-[[[3-(2-chlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (cloxacillin),

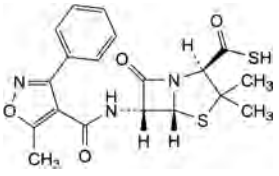

F. (2*R*,5*R*,6*R*)-3,3-dimethyl-6-[[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carbothioic acid (thiooxacillin),

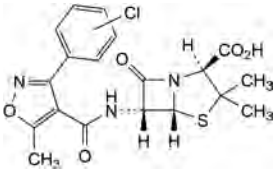

G. (2*S*,5*R*,6*R*)-6-[[[3-(chlorophenyl)-5-methylisoxazol-4-yl]carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (cloxacillin isomer),

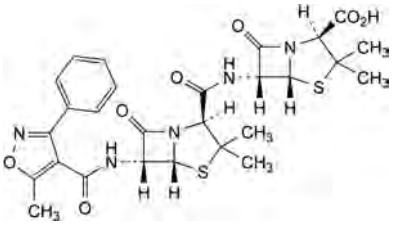

I. (2*S*,5*R*,6*R*)-6-[[(2*S*,5*R*,6*R*)-3,3-dimethyl-6-[[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-APA oxacillin amide),

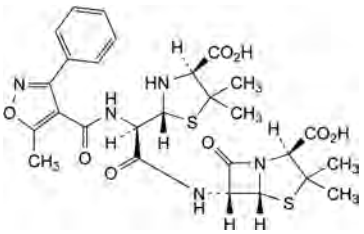

J. (2*S*,5*R*,6*R*)-6-[[(2*R*)-[(2*R*,4*S*)-4-carboxy-5,5-dimethylthiazolidin-2-yl][[(5-methyl-3-phenylisoxazol-4-yl)carbonyl]amino]acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ozolamide of 6-APA dimer).

**07/2019:2017**

# OXALIPLATIN

## Oxaliplatinum

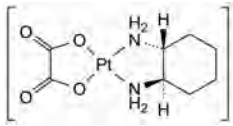

$C_8H_{14}N_2O_4Pt$ $M_r$ 397.3
[61825-94-3]

DEFINITION

(*SP*-4-2)-[(1*R*,2*R*)-Cyclohexane-1,2-diamine-$\kappa^2N,N'$][ethanedioato-$\kappa^2O,O'$(2-)]platinum.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, very slightly soluble in methanol, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *oxaliplatin CRS*.

B. Specific optical rotation (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.10 g in *water R* and dilute to 50 mL with the same solvent.

**Acidity**. Dissolve 0.10 g in *carbon dioxide-free water R*, dilute to 50 mL with the same solvent and add 0.5 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.60 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Specific optical rotation** (*2.2.7*): + 74.5 to + 78.0 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 50.0 mL with the same solvent.

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *oxaliplatin impurity D CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 15.0 mL of reference solution (a) to 50.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 75 mg of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (d).* Dilute 5.0 mL of reference solution (c) to 100.0 mL with *methanol R*.

*Reference solution (e).* To 40 mL of reference solution (c) add 1.0 mL of reference solution (b) and dilute to 50.0 mL with *methanol R*.

*Reference solution (f).* To 4.0 mL of reference solution (a) add 5.0 mL of reference solution (d) and dilute to 50.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *cellulose derivative of silica gel for chiral separation R*;

– *temperature*: 40 °C.

*Mobile phase*: *anhydrous ethanol R*, *methanol R* (30:70 *V/V*).

*Flow rate*: 0.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (e) and (f).

*Run time*: twice the retention time of oxaliplatin.

*Retention time*: oxaliplatin = about 14 min; impurity D = about 16 min.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to oxaliplatin and impurity D in the chromatogram obtained with reference solution (f);

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity D in the chromatogram obtained with reference solution (e).

*Limit*:

– *impurity D*: not more than 3 times the difference between the heights of the peaks due to impurity D in the chromatograms obtained with reference solution (e) and the test solution (0.15 per cent).

**Related substances**

A. Impurity A. Liquid chromatography (*2.2.29*). *Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 14.0 mg of *oxalic acid R* (impurity A) in *water R* and dilute to 250.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 200.0 mL with *water R*.

*Reference solution (c).* Dissolve 12.5 mg of *sodium nitrate R* in *water R* and dilute to 250.0 mL with the same solvent. Dilute a mixture of 2.0 mL of this solution and 25.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 20 volumes of *acetonitrile R1* and 80 volumes of a solution prepared as follows: to 10 mL of a 320 g/L solution of *tetrabutylammonium hydroxide R* add 1.36 g of *potassium dihydrogen phosphate R*, dilute to 1000 mL with *water for chromatography R* and adjust to pH 6.0 with *phosphoric acid R*.

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of impurity A.

*Retention times*: nitrate = about 2.7 min; impurity A = about 4.7 min.

*System suitability*:

– *resolution*: minimum 9 between the peaks due to nitrate and impurity A in the chromatogram obtained with reference solution (c);

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity A in the chromatogram obtained with reference solution (b).

*Limit*:

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent).

B. Impurity B. Liquid chromatography (*2.2.29*). *Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation. Use suitable polypropylene containers for the preparation and injection of all solutions. Glass pipettes may be used for diluting solutions.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Add 5.0 mg of *oxaliplatin impurity B CRS* to 25 mL of *methanol R* and dilute to 100.0 mL with *water R*. Sonicate for about 1.5 h until dissolved (solution A). Dilute 3.0 mL of solution A to 200.0 mL with *water R*.

*Reference solution (b).* In order to prepare impurity E *in situ*, adjust 50.0 mL of solution A to pH 6.0 with a 0.2 g/L solution of *sodium hydroxide R*, heat at 70 °C for 4 h and allow to cool.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 20 volumes of *acetonitrile R1* and 80 volumes of a solution prepared as follows: dissolve 1.36 g of *potassium dihydrogen phosphate R* and 1 g of *sodium heptanesulfonate R* in 1000 mL of *water for chromatography R* and adjust to pH 3.0 ± 0.05 with *phosphoric acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of impurity B.

*Retention time*: impurity B = about 4.3 min; impurity E = about 6.4 min.

*System suitability*:

– *resolution*: minimum 7 between the peaks due to impurities B and E in the chromatogram obtained with reference solution (b);

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity B in the chromatogram obtained with reference solution (a).

*Limit*:

– *impurity B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent).

C. Impurity C and other related substances. Liquid chromatography (*2.2.29*). *Use vigorous shaking and very brief sonication to dissolve the substance to be examined. Inject the test solution within 20 min of preparation.*

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 500.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *oxaliplatin CRS* and 5.0 mg of *oxaliplatin impurity C CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 25.0 mg of *oxaliplatin CRS* in *water R* and dilute to 250.0 mL with the same solvent.

*Reference solution (d).* Dissolve 5.0 mg of *dichlorodiaminocyclohexaneplatinum CRS* in reference solution (c) and dilute to 50.0 mL with reference solution (c).

*Reference solution (e).* Dilute 5 mL of reference solution (d) to 50.0 mL with *water R*.

*Reference solution (f).* To 0.100 g of the substance to be examined add 1.5 mL of reference solution (a) and dilute to 50.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 1 volume of *acetonitrile R1* and 99 volumes of a solution prepared as follows: dilute 0.6 mL of *dilute phosphoric acid R* in 1000 mL of *water for chromatography R* and adjust to pH 3.0 with either *sodium hydroxide solution R* or *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b), (e) and (f).

*Run time*: 3 times the retention time of oxaliplatin.

*Retention time*: impurity C = about 4.4 min; dichlorodiaminocyclohexaneplatinum = about 6.9 min; oxaliplatin = about 8.0 min.

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to dichlorodiaminocyclohexaneplatinum and oxaliplatin in the chromatogram obtained with reference solution (e);

– *signal-to-noise ratio*: minimum 50 for the peak due to impurity C and minimum 10 for the peak due to oxaliplatin in the chromatogram obtained with reference solution (b).

*Limits*:

– *impurity C*: not more than 0.5 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (f) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than twice the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *sum of unspecified impurities*: not more than 3 times the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *disregard limit*: the area of the peak due to oxaliplatin in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak with a retention time less than 2 min.

D. Sum of impurities other than D: maximum 0.30 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Bacterial endotoxins** (*2.6.14*): less than 1.0 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurity C and other related substances with the following modifications.

*Injection*: 20 µL of test solution (b) and reference solutions (c) and (d).

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to dichlorodiaminocyclohexaneplatinum and oxaliplatin in the chromatogram obtained with reference solution (d);

– *repeatability*: reference solution (c).

Calculate the percentage content of oxaliplatin using the chromatogram obtained with reference solution (c).

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E.*

A. oxalic acid,

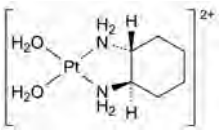

B. (*SP*-4-2)-diaqua[(1*R*,2*R*)-cyclohexane-1,2-diamine-κ²*N*,*N*′]platinum (diaquodiaminocyclohexaneplatinum; supplied as dinitrate),

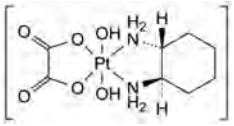

C. (*OC*-6-33)-[(1*R*,2*R*)-cyclohexane-1,2-diamine-κ²*N*,*N*′][ethanedioato-κ²*O*,*O*′(2-)]dihydroxidoplatinum,

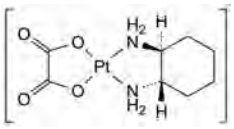

D. (*SP*-4-2)-[(1*S*,2*S*)-cyclohexane-1,2-diamine-κ²*N*,*N*′][ethanedioato-κ²*O*,*O*′(2-)]platinum (*S*,*S*-enantiomer of oxaliplatin),

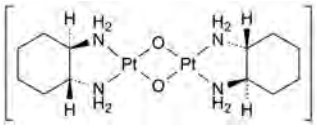

E. (*SP*-4-2)-di-μ-oxidobis[(1*R*,2*R*)-cyclohexane-1,2-diamine-1κ²*N*,2κ²*N*′]diplatinum (diaquodiamino-cyclohexaneplatinum dimer).

**01/2008:0778**
**corrected 6.0**

# OXAZEPAM

## Oxazepamum

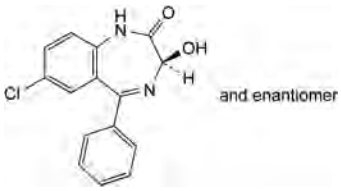


$C_{15}H_{11}ClN_2O_2$ $M_r$ 286.7
[604-75-1]

DEFINITION

(3*RS*)-7-Chloro-3-hydroxy-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxazepam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 40.0 mg of the substance to be examined in 25 mL of a mixture of equal volumes of *acetonitrile R* and *water R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*. Dilute 2.0 mL of this solution to 10.0 mL with a mixture of equal volumes of *acetonitrile R* and *water R*.

*Reference solution (b).* Dissolve the contents of a vial of *oxazepam for peak identification CRS* (containing impurities A, B, C, D and E) in 1.0 mL of the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 μm) resistant to bases up to pH 11.

*Mobile phase*:

– *mobile phase A*: dissolve 3.48 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 10.5 with a 40 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 75 | 25 |
| 4 - 34 | 75 → 25 | 25 → 75 |
| 34 - 45 | 25 | 75 |
| 45 - 50 | 25 → 75 | 75 → 25 |
| 50 - 60 | 75 | 25 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 10 μL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) and the chromatogram supplied with *oxazepam for peak identification CRS* to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to oxazepam (retention time = about 15 min): impurity E = about 0.7; impurity A = about 0.8; impurity B = about 1.2; impurity C = about 1.4; impurity D = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities E and A.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 4.0; impurity B = 1.1;
– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 10 mL of *anhydrous acetic acid R* and 90 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.67 mg of $C_{15}H_{11}ClN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

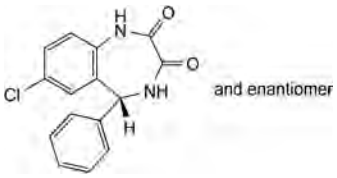


A. (5*RS*)-7-chloro-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione,

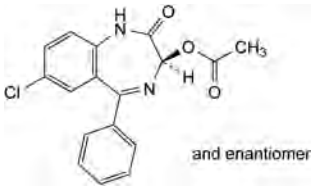


B. (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl acetate,

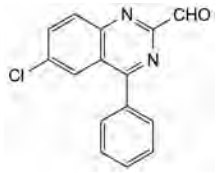

C. 6-chloro-4-phenylquinazoline-2-carbaldehyde,

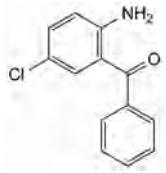

D. (2-amino-5-chlorophenyl)phenylmethanone,

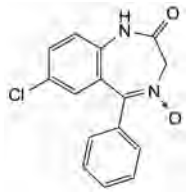

E. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide.

**07/2013:2577**

# OXCARBAZEPINE

## Oxcarbazepinum

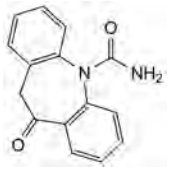

$C_{15}H_{12}N_2O_2$ $M_r$ 252.3

[28721-07-5]

DEFINITION

10-Oxo-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or faintly orange, crystalline powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxcarbazepine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R*, solution A (50:50 *V/V*).

*Phosphate buffer solution.* Dissolve 0.54 g of *potassium dihydrogen phosphate R* and 8.9 g of *disodium hydrogen phosphate dihydrate R* in 1.0 L of *water R*.

*Solution A*: 1.8 g/L solution of *ascorbic acid R*.

*Solution B*: 1.8 g/L solution of *sodium edetate R* in a mixture of equal volumes of the phosphate buffer solution and *water R*.

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in 25 mL of *acetonitrile R*, sonicate for 10 min, cool to room temperature and dilute to 50.0 mL with solution A.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *oxcarbazepine impurity mixture CRS* (impurities A, B, I and K) in 0.5 mL of *acetonitrile R* and dilute to 1.0 mL with solution A.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *oxcarbazepine CRS* in 25 mL of *acetonitrile R*, sonicate for 10 min, cool to room temperature and dilute to 50.0 mL with solution A. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylhexylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, solution B, *tetrahydrofuran R*, *water R* (5:10:10:75 *V/V/V/V*);
- *mobile phase B*: solution B, *tetrahydrofuran R*, *water R*, *acetonitrile R* (10:10:20:60 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 60 | 40 |
| 10 - 20 | 60 → 5 | 40 → 95 |
| 20 - 27 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *oxcarbazepine impurity mixture CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, I and K.

*Relative retention* with reference to oxcarbazepine (retention time = about 6 min): impurity I = about 0.8; impurity A = about 1.3; impurities K and L = about 1.4; impurity B = about 1.6.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurities K and L and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of oxcarbazepine in reference solution (b).

*Limits*:

– *impurities B, I*: for each impurity, maximum 0.1 per cent;
– *sum of impurities K and L*: maximum 0.1 per cent;
– *unspecified impurities*: for each impurity, maximum 0.05 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 60 | 40 |
| 7 - 8 | 60 → 5 | 40 → 95 |
| 8 - 13 | 5 | 95 |

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{15}H_{12}N_2O_2$ taking into account the assigned content of *oxcarbazepine CRS*.

IMPURITIES

*Specified impurities: B, I, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F, G, H, M.*

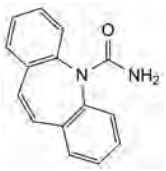

A. 5*H*-dibenzo[*b,f*]azepine-5-carboxamide (carbamazepine),

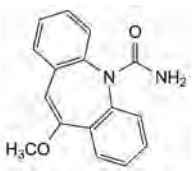

B. 10-methoxy-5*H*-dibenzo[*b,f*]azepine-5-carboxamide (10-methoxycarbamazepine),

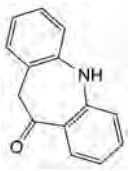

C. 5,11-dihydro-10*H*-dibenzo[*b,f*]azepin-10-one,

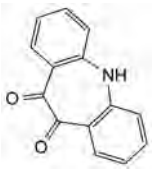

D. 5*H*-dibenzo[*b,f*]azepine-10,11-dione,

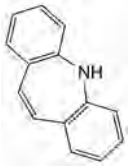

E. 5*H*-dibenzo[*b,f*]azepine,

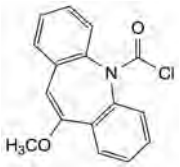

F. 10-methoxy-5*H*-dibenzo[*b,f*]azepine-5-carbonyl chloride,

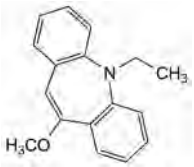

G. 5-ethyl-10-methoxy-5*H*-dibenzo[*b,f*]azepine,

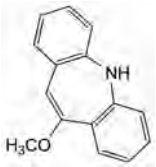

H. 10-methoxy-5*H*-dibenzo[*b,f*]azepine,

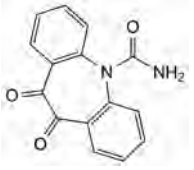

I. 10,11-dioxo-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide,

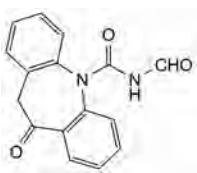

K. *N*-formyl-10-oxo-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide,

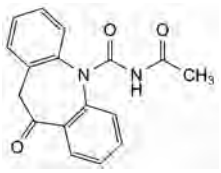

L. *N*-acetyl-10-oxo-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide,

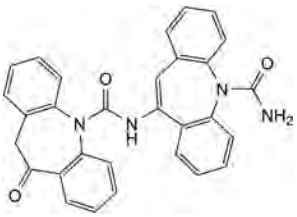

M. 10-[[(10-oxo-10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)carbonyl]amino]-5*H*-dibenzo[*b,f*]azepine-5-carboxamide.



**01/2016:1761**
**corrected 10.0**

# OXELADIN HYDROGEN CITRATE

## Oxeladini hydrogenocitras

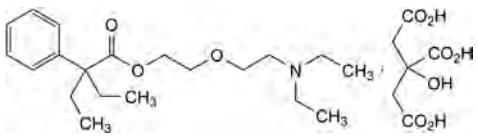

$C_{26}H_{41}NO_{10}$ $M_r$ 527.6
[52432-72-1]

DEFINITION

2-[2-(Diethylamino)ethoxy]ethyl 2-ethyl-2-phenylbutanoate dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble or very slightly soluble in ethyl acetate.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxeladin hydrogen citrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.500 g of the substance to be examined in *water R* and dilute to 50 mL with the same solvent. Add 1 mL of a 10.3 g/L solution of *hydrochloric acid R* and shake with 3 quantities, each of 10 mL, of *methylene chloride R*. Combine the lower layers. Add 5 mL of *concentrated ammonia R* to the aqueous layer and shake with 3 quantities, each of 10 mL, of *methylene chloride R*. Combine the lower layers with the lower layers obtained previously, add *anhydrous sodium sulfate R*, shake, filter and evaporate the filtrate by suitable means at a temperature not exceeding 30 °C. Take up the residue with *methylene chloride R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *oxeladin impurity D CRS* in 10 mL of *water R*, add 0.5 mL of *concentrated ammonia R* and shake with 3 quantities, each of 2 mL, of *methylene chloride R*. To the combined lower layers, add 0.2 mL of the test solution and dilute to 10.0 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 20.0 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve 5 mg of *oxeladin impurity C CRS* in 10 mL of *water R*, add 0.5 mL of *concentrated ammonia R* and shake with 3 quantities, each of 2 mL, of *methylene chloride R*. Combine the lower layers and dilute to 10 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.4 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min; adjust the flow rate if necessary to obtain a retention time of about 13 min for oxeladin.

*Split ratio*: 1:15.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 160 |
| | 4 - 12 | 160 → 240 |
| | 12 - 21 | 240 |
| | 21 - 30 | 240 → 160 |
| Injection port | | 280 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to oxeladin (retention time = about 13 min): impurity A = about 0.2; impurity B = about 0.4; impurity C = about 0.8; impurity D = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 10 between the peaks due to impurity D and oxeladin.

*Limits*:

- *impurity C*: maximum 0.2 per cent;
- *impurity D*: maximum 0.3 per cent;
- *any other impurity*: for each impurity, maximum 0.1 per cent;
- *total*: maximum 1.0 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 52.76 mg of $C_{26}H_{41}NO_{10}$.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

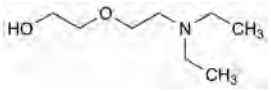

A. 2-[2-(diethylamino)ethoxy]ethanol,

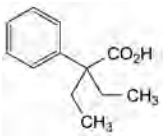

B. 2-ethyl-2-phenylbutanoic acid,

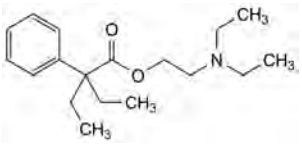

C. 2-(diethylamino)ethyl 2-ethyl-2-phenylbutanoate,

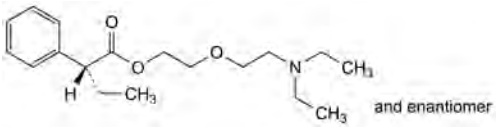


D. 2-[2-(diethylamino)ethoxy]ethyl (2*RS*)-2-phenylbutanoate.

**07/2019:1458**

# OXFENDAZOLE FOR VETERINARY USE

## Oxfendazolum ad usum veterinarium

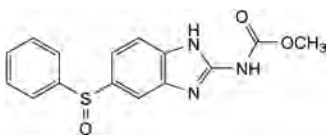

$C_{15}H_{13}N_3O_3S$ $M_r$ 315.3
[53716-50-0]

DEFINITION

Methyl [5-(phenylsulfinyl)-1*H*-benzimidazol-2-yl]carbamate.

*Content*: 97.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxfendazole CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. In order to prepare impurity C *in situ*, add 0.25 mL of *strong hydrogen peroxide solution R* to 10 mL of the test solution, then dilute to 25 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5.0 mg of *fenbendazole CRS* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 10.0 mg of *oxfendazole impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (e)*. Dissolve 5 mg of *oxfendazole with impurity D CRS* in the mobile phase and dilute to 20 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 36 volumes of *acetonitrile R* and 64 volumes of a 2 g/L solution of *sodium pentanesulfonate R* previously adjusted to pH 2.7 with a 2.8 per cent *V/V* solution of *sulfuric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of oxfendazole.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity C; use the chromatogram supplied with *oxfendazole with impurity D CRS* and the chromatogram obtained with reference solution (e) to identify the peak due to impurity D.

*Relative retention* with reference to oxfendazole (retention time = about 7 min): impurity C = about 0.4; impurity B = about 1.9; impurity D = about 2.7; impurity A = about 5.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity C and oxfendazole.

*Limits*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (2.0 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

- *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *unspecified impurities*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);
- *total*: maximum 3.0 per cent;
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 3 mL of *anhydrous formic acid R*. Add 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 31.53 mg of $C_{15}H_{13}N_3O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

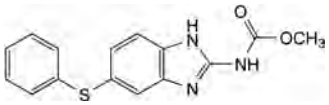

A. methyl [5-(phenylsulfanyl)-1*H*-benzimidazol-2-yl]carbamate (fenbendazole),

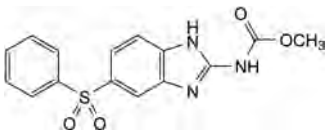

B. methyl [5-(phenylsulfonyl)-1*H*-benzimidazol-2-yl]carbamate,

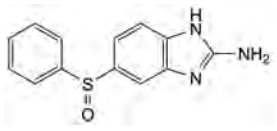

C. 5-(phenylsulfinyl)-1*H*-benzimidazol-2-amine,

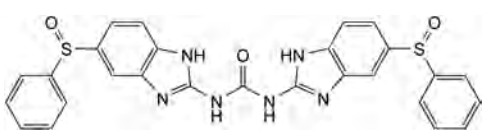

D. *N*,*N*′-bis[5-(phenylsulfinyl)-1*H*-benzimidazol-2-yl]urea.

**01/2008:2170**
**corrected 7.6**

# OXITROPIUM BROMIDE

## Oxitropii bromidum

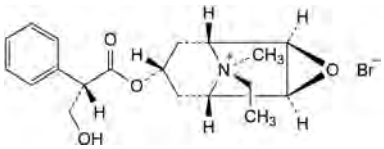

$C_{19}H_{26}BrNO_4$ $M_r$ 412.3
[30286-75-0]

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*,9*s*)-9-Ethyl-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane bromide (ethylhyoscine).

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in methanol, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxitropium bromide CRS*.

If the spectra obtained in the solid state show differences at about 1700 $cm^{-1}$ and about 3300 $cm^{-1}$, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): − 26 to − 24 (dried substance).

Dissolve 1.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 75.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 7.5 mg of *oxitropium bromide impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Mix 5.0 mL of the test solution and 5.0 mL of reference solution (a).

*Reference solution (d).* Dilute 15.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (e).* Dilute 5.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 350 $m^2$/g and a pore size of 6 nm.

*Mobile phase*: *acetonitrile for chromatography R*, 7.8 g/L solution of *sodium dihydrogen phosphate R* (10:100 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Relative retention* with reference to oxitropium (retention time = about 6 min): impurity A = about 0.8; impurity B = about 0.9; impurity C = about 1.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.6 between the peaks due to impurity B and oxitropium.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.1 per cent);
– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.5 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.10 per cent);
– *sum of unspecified impurities*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 75.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 6.0 mg of *oxitropium bromide impurity D CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 200.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* To 5.0 mL of the test solution add 5.0 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;
– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile for chromatography R*, 7.8 g/L solution of *sodium dihydrogen phosphate R* (18.5:100 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to impurity D and oxitropium.

*Limit*:

– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.350 g in 100 mL of *water R* and add 5.0 mL of *dilute nitric acid R*. Titrate with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*) using a silver indicator electrode and a silver-silver chloride reference electrode.

1 mL of *0.1 M silver nitrate* is equivalent to 41.23 mg of $C_{19}H_{26}BrNO_4$.

IMPURITIES

*Specified impurities: A, B, C, D.*

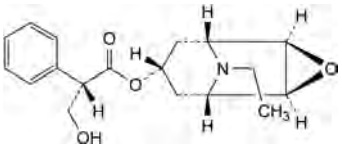

A. (1*R*,2*R*,4*S*,5*S*,7*s*)-9-ethyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]-non-7-yl (2*S*)-3-hydroxy-2-phenylpropanoate (*N*-ethylnorhyoscine),

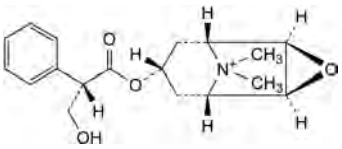

B. (1*R*,2*R*,4*S*,5*S*,7*s*)-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]-oxy]-9,9-dimethyl-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane (methylhyoscine),

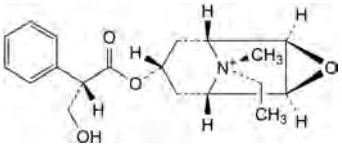

C. (1*R*,2*R*,4*S*,5*S*,7*s*,9*r*)-9-ethyl-7-[[(2*S*)-3-hydroxy-2-phenylpropanoyl]oxy]-9-methyl-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane (pseudo-isomer),

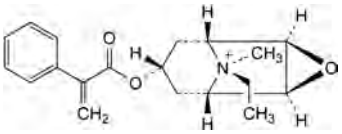

D. (1*R*,2*R*,4*S*,5*S*,7*s*,9*s*)-9-ethyl-9-methyl-7-[(2-phenylacryloyl)oxy]-3-oxa-9-azoniatricyclo[3.3.1.$0^{2,4}$]nonane (apo-*N*-ethylhyoscine).

**01/2017:1353**
**corrected 10.0**

# OXOLINIC ACID

## Acidum oxolinicum

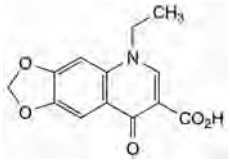

$C_{13}H_{11}NO_5$ $M_r$ 261.2
[14698-29-4]

DEFINITION

5-Ethyl-8-oxo-5,8-dihydro-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, very slightly soluble in methylene chloride, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in 5 mL of *0.1 M sodium hydroxide*, heating on a water-bath. Allow to cool and dilute to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 260 nm, 322 nm and 336 nm.

*Absorbance ratio*: $A_{260}/A_{336}$ = 4.9 to 5.2.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxolinic acid CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 3 mL of *dilute sodium hydroxide solution R* and dilute to 20 mL with *ethanol (96 per cent) R*.

*Reference solution (a).* Dissolve 10 mg of *oxolinic acid CRS* in 3 mL of *dilute sodium hydroxide solution R* and dilute to 20 mL with *ethanol (96 per cent) R*.

*Reference solution (b).* Dissolve 5 mg of *ciprofloxacin hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 2 mL with reference solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *acetonitrile R*, *concentrated ammonia R*, *methanol R*, *methylene chloride R* (10:20:40:40 *V/V/V/V*).

*Application*: 10 µL.

*Development*: at the bottom of a chromatographic tank, place an evaporating dish containing 50 mL of *concentrated ammonia R* and expose the plate to the ammonia vapour for 15 min in the closed tank; withdraw the plate, transfer to a second chromatographic tank and proceed with development over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 0.6 g in 20 mL of a 40 g/L solution of *sodium hydroxide R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in 3 mL of *dilute sodium hydroxide solution R* and dilute to 10 mL with *ethanol (96 per cent) R*.

*Reference solution (a).* Dilute 1 mL of the test solution to 50.0 mL with *ethanol (96 per cent) R*. Dilute 1.0 mL of this solution to 5.0 mL with *ethanol (96 per cent) R*.

*Reference solution (b).* Dissolve 2 mg of *oxolinic acid impurity B CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent. Dilute 1.0 mL of this solution to 10 mL with *ethanol (96 per cent) R*.

*Reference solution (c).* Dissolve 5 mg of the substance to be examined and 5 mg of *oxolinic acid impurity A CRS* in 2 mL of *dilute sodium hydroxide solution R* and dilute to 40 mL with *ethanol (96 per cent) R*.

*Plate*: *cellulose for chromatography R* as the coating substance.

*Mobile phase*: *ammonia R*, *water R*, *propanol R* (15:30:55 *V/V/V*).

*Application*: 5 µL, in sufficiently small portions to obtain small spots.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated principal spots.

*Limits*:

– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurities A, C*: any spot due to impurities A or C is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.4 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by heating in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 150 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Use a glass indicator electrode and a suitable reference electrode.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 26.12 mg of $C_{13}H_{11}NO_5$.

STORAGE

Protected from light.

IMPURITIES

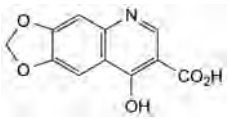

A. 8-hydroxy-1,3-dioxolo[4,5-*g*]quinoline-7-carboxylic acid,

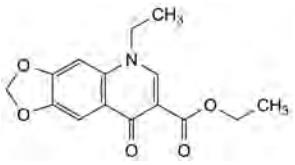

B. ethyl 5-ethyl-8-oxo-5,8-dihydro-1,3-dioxolo-[4,5-*g*]quinoline-7-carboxylate,

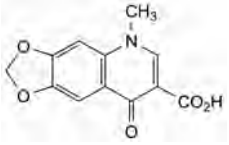

C. 5-methyl-8-oxo-5,8-dihydro-1,3-dioxolo-[4,5-*g*]quinoline-7-carboxylic acid.

**01/2017:1251**

# OXYBUPROCAINE HYDROCHLORIDE

## Oxybuprocaini hydrochloridum

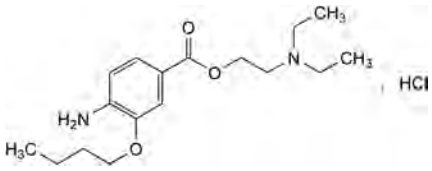

$C_{17}H_{29}ClN_2O_3$ $M_r$ 344.9
[5987-82-6]

DEFINITION

2-(Diethylamino)ethyl 4-amino-3-butoxybenzoate hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 158 °C to 162 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *oxybuprocaine hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 40 mg of *oxybuprocaine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 20 mg of *procaine hydrochloride R* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *methanol R*, *water R*, *ethyl acetate R* (10:15:15:60 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in a current of warm air for 10 min.

*Detection*: spray with *dimethylaminobenzaldehyde solution R7* and examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dilute 0.2 mL of solution S (see Tests) to 2 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution pH 2.5*. Add 6 mL of *perchloric acid solution R* and 12 mL of *dilute phosphoric acid R* to 950 mL of *water R*. Adjust to pH 2.5 with a 40 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R*.

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Mix 1.0 mL of the test solution with 1 mL of a 40 g/L solution of *sodium hydroxide R* and allow to stand for 20 min. Add 1 mL of *dilute phosphoric acid R* and dilute to 100.0 mL with the mobile phase. Dilute 25 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent;
– *temperature*: 35 °C.

*Mobile phase*: *acetonitrile R*, buffer solution pH 2.5 (25:75 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 309 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of oxybuprocaine.

*Retention time*: oxybuprocaine = about 9 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 12 between the peaks due to oxybuprocaine and impurity B (hydrolysis product).

*Limits*:

– *any impurity*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.0125 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 20 mL of *anhydrous acetic acid R* and 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.49 mg of $C_{17}H_{29}ClN_2O_3$.

STORAGE

Protected from light.

IMPURITIES

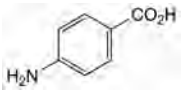

A. 4-aminobenzoic acid,

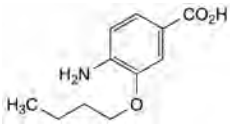

B. 4-amino-3-butoxybenzoic acid,

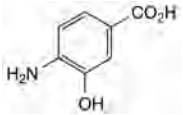

C. 4-amino-3-hydroxybenzoic acid.



**01/2017:1354**

# OXYBUTYNIN HYDROCHLORIDE

## Oxybutynini hydrochloridum

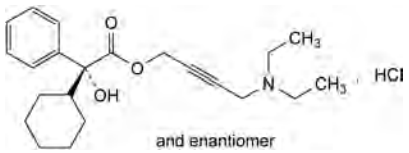


$C_{22}H_{32}ClNO_3$ $M_r$ 394.0
[1508-65-2]

DEFINITION

4-(Diethylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate hydrochloride.

*Content*: 99.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), soluble in acetone, practically insoluble in cyclohexane.

IDENTIFICATION

*First identification: B, D.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 124 °C to 129 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *oxybutynin hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *oxybutynin hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *methanol R.*

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour for 30 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.00 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *oxybutynin hydrochloride CRS* and 5.0 mg of *oxybutynin impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R2* (5 µm).

*Mobile phase*: mix 49 volumes of a solution containing 3.4 g/L of *potassium dihydrogen phosphate R* and 4.36 g/L of *dipotassium hydrogen phosphate R* and 51 volumes of *acetonitrile R1*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of oxybutynin.

*Retention time*: oxybutynin = about 15 min; impurity A = about 24 min.

*System suitability*: reference solution (a):

- *resolution*: minimum 11.0 between the peaks due to oxybutynin and impurity A.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 39.4 mg of $C_{22}H_{32}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E*.

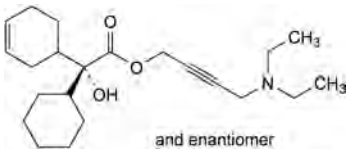


A. 4-(diethylamino)but-2-ynyl (*RS*)-2-(cyclohex-3-enyl)-2-cyclohexyl-2-hydroxyacetate,

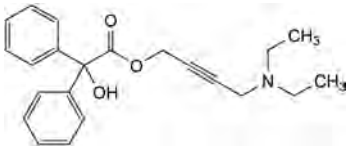


B. 4-(diethylamino)but-2-ynyl 2-hydroxy-2,2-diphenylacetate (diphenyl analogue of oxybutynin),

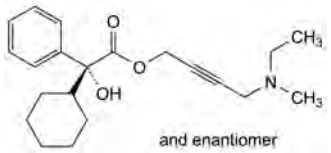


C. 4-(ethylmethylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate (methylethyl analogue of oxybutynin),

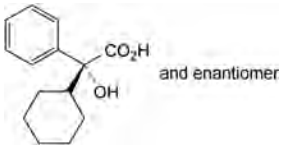


D. (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetic acid (phenylcyclohexylglycolic acid),

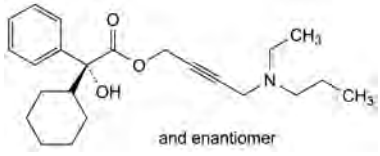


E. 4-(ethylpropylamino)but-2-ynyl (*RS*)-2-cyclohexyl-2-hydroxy-2-phenylacetate (ethylpropyl analogue of oxybutynin).

**01/2008:1793**
**corrected 7.0**

# OXYCODONE HYDROCHLORIDE

## Oxycodoni hydrochloridum

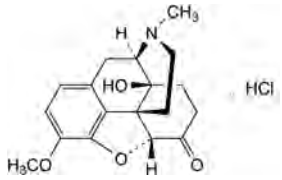


$C_{18}H_{22}ClNO_4$ $M_r$ 351.9
[124-90-3]

DEFINITION

4,5α-Epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in anhydrous ethanol, practically insoluble in toluene.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

Dissolve 50 mg in *water R* and dilute to 5 mL with the same solvent. Render the solution alkaline with *dilute ammonia R1*. Allow the mixture to stand until a precipitate is formed. Filter, wash the precipitate with 10 mL of cold *water R*, and dry for 1 h at 105 °C. Examine the precipitate.

*Comparison*: repeat the operations using 50 mg of *oxycodone hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): − 140 to − 148 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in a 1 per cent *V/V* solution of *dilute acetic acid R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20.0 mg of *oxycodone impurity D CRS* in a 1 per cent *V/V* solution of *dilute acetic acid R* and dilute to 10.0 mL with the same solution.

*Reference solution (b)*. To 1.0 mL of the test solution, add 1.0 mL of reference solution (a) and dilute to 100.0 mL with a 1 per cent *V/V* solution of *dilute acetic acid R*. Dilute 1.0 mL of the solution to 10.0 mL with a 1 per cent *V/V* solution of *dilute acetic acid R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 830 mL of a 1.1 g/L solution of *sodium heptanesulfonate monohydrate R* previously adjusted to pH 2.0 with a mixture of equal volumes of *phosphoric acid R* and *water R*, with 70 mL of *acetonitrile R* and 100 mL of *methanol R*;

– *mobile phase B*: mix 600 mL of a 1.1 g/L solution of *sodium heptanesulfonate monohydrate R* previously adjusted to pH 2.0 with a mixture of equal volumes of *phosphoric acid R* and *water R*, with 150 mL of *acetonitrile R* and 250 mL of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 100 → 50 | 0 → 50 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to oxycodone (retention time = about 24 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 1.14; impurity D = about 1.18; impurity E = about 1.18; impurity F = about 2.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 3 between the peaks due to oxycodone and impurity D.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity F by 0.5;

– *sum of impurities D and E*: not more than 10 times the area of the peak due to oxycodone in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurities A, B, C, F*: for each impurity, not more than the area of the peak due to oxycodone in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *any other impurity*: for each impurity, not more than the area of the peak due to oxycodone in the chromatogram obtained with reference solution (b) (0.1 per cent);

– *total*: not more than 15 times the area of the peak due to oxycodone in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the peak due to oxycodone in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethanol** (*2.4.24, System A*): maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 7.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 60 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M ethanolic sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Measure the volume used between the 2 inflexion points.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 35.19 mg of $C_{18}H_{22}ClNO_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

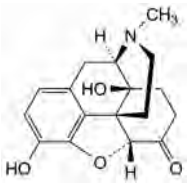

A. 4,5α-epoxy-3,14-dihydroxy-17-methylmorphinan-6-one (oxymorphone),

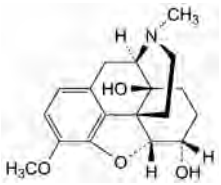

B. 4,5α-epoxy-3-methoxy-17-methylmorphinan-6α,14-diol (7,8-dihydro-14-hydroxycodeine),

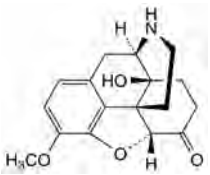

C. 4,5α-epoxy-14-hydroxy-3-methoxymorphinan-6-one (noroxycodone),

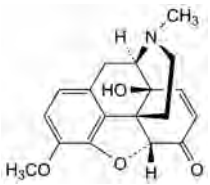

D. 7,8-didehydro-4,5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one (14-hydroxycodeinone),

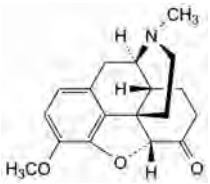

E. 4,5α-epoxy-3-methoxy-17-methylmorphinan-6-one (hydrocodone),

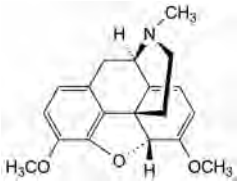

F. 6,7,8,14-tetradehydro-4,5α-epoxy-3-6-dimethoxy-17-methylmorphinan (thebain).

**01/2010:0417**

# OXYGEN

## Oxygenium

$O_2$ $M_r$ 32.00

[7782-44-7]

DEFINITION

*Content*: minimum 99.5 per cent *V/V* of $O_2$.

This monograph applies to oxygen for medicinal use.

CHARACTERS

*Appearance*: colourless gas.

*Solubility*: at 20 °C and at a pressure of 101 kPa, 1 volume dissolves in about 32 volumes of water.

PRODUCTION

Oxygen is produced by a purification process followed by cryodistillation of the ambient air.

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using an infrared analyser (*2.5.24*).

*Gas to be examined.* Filter the substance to be examined to avoid stray light phenomena.

*Reference gas (a). Oxygen R.*

*Reference gas (b).* Mixture containing 300 ppm *V/V* of *carbon dioxide R1* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using an infrared analyser (*2.5.25*).

*Gas to be examined.* Filter the substance to be examined to avoid stray light phenomena.

*Reference gas (a). Oxygen R.*

*Reference gas (b).* Mixture containing 5 ppm *V/V* of *carbon monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

**Water**: maximum 67 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

**Assay**. Determine the concentration of oxygen using a paramagnetic analyser (*2.5.27*).

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using a carbon dioxide detector tube (*2.1.6*).

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube (*2.1.6*).

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

STORAGE

As a compressed gas or liquid in appropriate containers, complying with the legal regulations. Oils and grease are not to be used unless they are oxygen-compatible.

IMPURITIES

*Specified impurities: A, B, C.*

A. $CO_2$: carbon dioxide,

B. CO: carbon monoxide,

C. $H_2O$: water.

**04/2011:2455**

# OXYGEN (93 PER CENT)

## Oxygenium 93 per centum

$O_2$ $M_r$ 32.00

DEFINITION

*Content*: 90.0 per cent *V/V* to 96.0 per cent *V/V* of $O_2$, the remainder mainly consisting of argon and nitrogen.

This monograph applies to oxygen (93 per cent) for medicinal use. It does not apply to gas produced using individual concentrators for domiciliary use.

PRODUCTION

Oxygen (93 per cent) is produced in single-stage concentrators by adsorption purification of ambient air using zeolites. During production, the oxygen content is continuously monitored by means of a paramagnetic analyser (*2.5.27*). Following the design and installation of the concentrator, and after any modification or significant intervention, the gas produced complies with the following requirements.

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using an infrared analyser (*2.5.24*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Oxygen R.*

*Reference gas (b).* A mixture of 7 per cent *V/V* of *nitrogen R1* and 93 per cent *V/V* of *oxygen R*, containing 300 ppm *V/V* of *carbon dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon dioxide in the gas to be examined.

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using an infrared analyser (*2.5.25*).

*Gas to be examined.* The substance to be examined. It must be filtered to avoid stray light phenomena.

*Reference gas (a). Oxygen R.*

*Reference gas (b).* A mixture containing 5 ppm *V/V* of *carbon monoxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of carbon monoxide in the gas to be examined.

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total, determined using a chemiluminescence analyser (*2.5.26*).

*Gas to be examined.* The substance to be examined.

*Reference gas (a).* A mixture of 21 per cent *V/V* of *oxygen R* and 79 per cent *V/V* of *nitrogen R1*, containing less than 0.05 ppm *V/V* of nitrogen monoxide and nitrogen dioxide.

*Reference gas (b).* A mixture containing 2 ppm *V/V* of *nitrogen dioxide R* in *nitrogen R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of nitrogen monoxide and nitrogen dioxide in the gas to be examined.

**Sulfur dioxide**: maximum 1 ppm *V/V*, determined using an ultraviolet fluorescence analyser (Figure 2455.-1.).

The apparatus consists of the following:

– a system generating ultraviolet radiation with a wavelength of 210 nm, made up of an ultraviolet lamp, a collimator, and a selective filter; the beam is blocked periodically by a chopper rotating at high speeds;
– a reaction chamber, through which flows the gas to be examined;

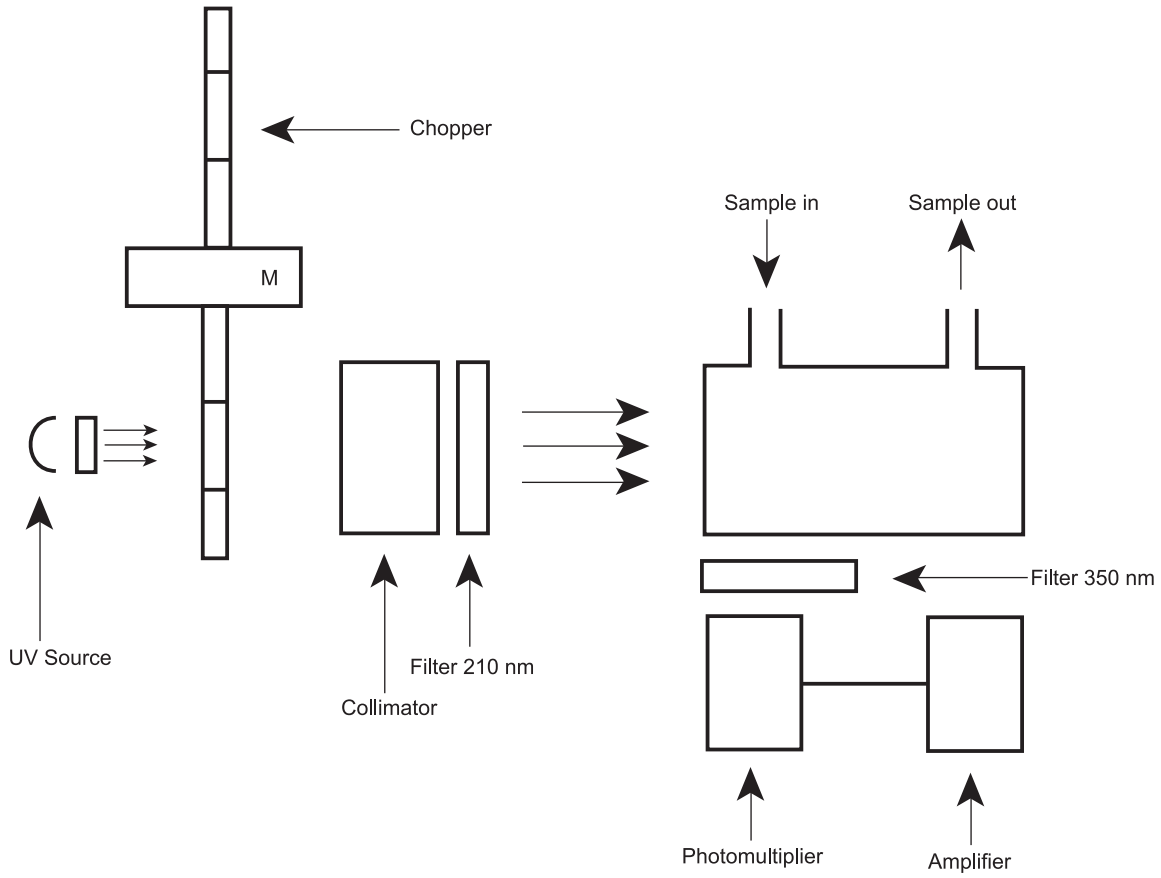


Figure 2455.-1. – *UV fluorescence analyser*

– a system that detects radiation emitted at a wavelength of 350 nm, made up of a selective filter, a photomultiplier tube and an amplifier.

*Gas to be examined*. The substance to be examined. It must be filtered.

*Reference gas (a)*. A mixture of 7 per cent *V/V* of *nitrogen R1* and 93 per cent *V/V* of *oxygen R*.

*Reference gas (b)*. A mixture of 7 per cent *V/V* of *nitrogen R1* and 93 per cent *V/V* of *oxygen R*, containing 0.5 ppm *V/V* to 2 ppm *V/V* of *sulfur dioxide R1*.

Calibrate the apparatus and set the sensitivity using reference gases (a) and (b). Measure the content of sulfur dioxide in the gas to be examined.

**Oil**: maximum 0.1 mg/m$^3$, determined using an oil detector tube (*2.1.6*).

**Water**: maximum 67 ppm *V/V*, determined using an electrolytic hygrometer (*2.5.28*).

**Assay**. Determine the concentration of oxygen using a paramagnetic analyser (*2.5.27*).

CHARACTERS

*Appearance*: colourless gas.

IDENTIFICATION

It complies with the limits of the assay.

TESTS

**Carbon dioxide**: maximum 300 ppm *V/V*, determined using a carbon dioxide detector tube (*2.1.6*).

**Carbon monoxide**: maximum 5 ppm *V/V*, determined using a carbon monoxide detector tube (*2.1.6*).

**Nitrogen monoxide and nitrogen dioxide**: maximum 2 ppm *V/V* in total, determined using a nitrogen monoxide and nitrogen dioxide detector tube (*2.1.6*).

**Sulfur dioxide**: maximum 1 ppm *V/V*, determined using a sulfur dioxide detector tube (*2.1.6*).

**Oil**: maximum 0.1 mg/m$^3$, determined using an oil detector tube (*2.1.6*).

**Water vapour**: maximum 67 ppm *V/V*, determined using a water vapour detector tube (*2.1.6*).

ASSAY

Determine the content of oxygen using a paramagnetic analyser (*2.5.27*).

STORAGE

Oxygen 93 per cent obtained from an oxygen concentrator is normally used on the site where it is produced. It is fed directly into a medicinal gas pipeline or administration system. Where authorised by the competent authority, it may be stored in suitable containers complying with the legal regulations. Oils and grease are not to be used unless they are oxygen-compatible.

IMPURITIES

A. $CO_2$: carbon dioxide,

B. CO: carbon monoxide,

C. $SO_2$: sulfur dioxide,

D. NO and $NO_2$: nitrogen monoxide and nitrogen dioxide,

E. oil,

F. $H_2O$: water.

**01/2013:0943**

# OXYMETAZOLINE HYDROCHLORIDE

## Oxymetazolini hydrochloridum

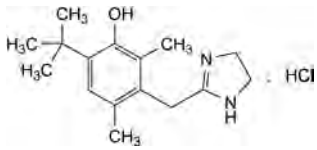

$C_{16}H_{25}ClN_2O$ $M_r$ 296.8
[2315-02-8]

DEFINITION

3-[(4,5-Dihydro-1*H*-imidazol-2-yl)methyl]-6-(1,1-dimethylethyl)-2,4-dimethylphenol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *oxymetazoline hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in a mixture of equal volumes of *ethyl acetate R* and *methanol R* and dilute to 5 mL with the same mixture of solvents.
*Reference solution.* Dissolve 20 mg of *oxymetazoline hydrochloride CRS* in a mixture of equal volumes of *ethyl acetate R* and *methanol R* and dilute to 5 mL with the same mixture of solvents.
*Plate*: *TLC silica gel G plate R.*
*Mobile phase*: *diethylamine R*, *cyclohexane R*, *anhydrous ethanol R* (6:15:79 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of warm air for 5 min, then allow to cool.
*Detection*: spray with a freshly prepared 5.0 g/L solution of *potassium ferricyanide R* in *ferric chloride solution R2*; examine in daylight.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 2 mg in 1 mL of *water R*, then add 0.2 mL of a 50 g/L solution of *sodium nitroprusside R* and 0.2 mL of *dilute sodium hydroxide solution R*. Allow to stand for 10 min. Add 2 mL of *sodium hydrogen carbonate solution R*. A violet colour develops.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 50 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Not more than 0.4 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 5.0 mg of *oxymetazoline impurity A CRS* and 5 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 20.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 70 | 30 |
| 5 - 20 | 70 → 15 | 30 → 85 |
| 20 - 35 | 15 | 85 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to oxymetazoline (retention time = about 5.0 min): impurity A = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to impurity A and oxymetazoline.

*Limits*:
- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.3 per cent, determined on 1.00 g .

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 20 mL of *acetic anhydride R* and 20 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.68 mg of $C_{16}H_{25}ClN_2O$.

IMPURITIES

*Specified impurities*: *A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

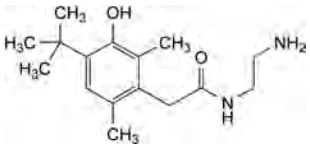

A. *N*-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]acetamide,

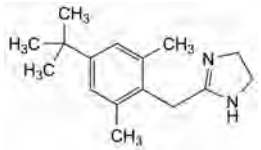

B. 2-[[4-(1,1-dimethylethyl)-2,6-dimethylphenyl]methyl]-4,5-dihydro-1*H*-imidazole (xylometazoline),

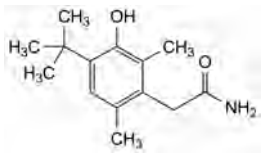

C. 2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetamide,

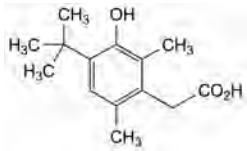

D. 2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetic acid,

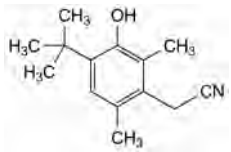

E. 2-[4-(1,1-dimethylethyl)-3-hydroxy-2,6-dimethylphenyl]-acetonitrile.

**01/2017:0199**
**corrected 10.0**

# OXYTETRACYCLINE DIHYDRATE

## Oxytetracyclinum dihydricum

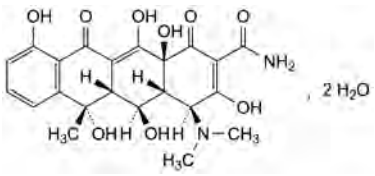

$C_{22}H_{24}N_2O_9,2\ H_2O$ $M_r$ 496.4
[6153-64-6]

DEFINITION

(4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(Dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide dihydrate.

Substance produced by the growth of certain strains of *Streptomyces rimosus*.

*Content*: 94.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder, slightly hygroscopic.

*Solubility*: very slightly soluble in water. It dissolves in dilute acid and alkaline solutions.

IDENTIFICATION

*First identification*: *B, C, D.*

*Second identification*: *A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *oxytetracycline CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 5 mg of *oxytetracycline CRS*, 5 mg of *tetracycline hydrochloride R* and 5 mg of *minocycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. To about 2 mg add 5 mL of *sulfuric acid R*. A deep red colour develops. Add the solution to 2.5 mL of *water R*. The colour becomes yellow.

D. Water (*2.5.12*): 6.0 per cent to 9.0 per cent (see Tests).

TESTS

**pH** (*2.2.3*): 4.5 to 7.5.

Suspend 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Light-absorbing impurities**. *Carry out the measurements within 1 h of preparing the solutions.*

Dissolve 20.0 mg in a mixture of 1 volume of a 103 g/L solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. The absorbance (*2.2.25*), determined at 430 nm has a maximum of 0.25 (anhydrous substance).

Dissolve 0.100 g in a mixture of 1 volume of a 103 g/L solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. The absorbance (*2.2.25*) determined at 490 nm has a maximum of 0.20 (anhydrous substance).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (a).* Dissolve 20.0 mg of *oxytetracycline CRS* in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid. Dilute 5.0 mL of the solution to 50.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL in a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (c).* Dissolve 4.0 mg of *oxytetracycline for system suitability CRS* (containing impurities A, B, C, D and E) in a 1 g/L solution of *hydrochloric acid R* and dilute to 5.0 mL with the same solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*;
– *mobile phase B*: *tetrahydrofuran for chromatography R*, *methanol R1*, *acetonitrile R1* (5:15:80 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 65 | 10 → 35 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *oxytetracycline for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to oxytetracycline (retention time = about 6.5 min): impurity A = about 0.9; impurity B = about 1.2; impurity C = about 1.3; impurity D = about 1.4; impurity E = about 2.3.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline; minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline.

*Calculation of percentage contents*:

– for each impurity, use the concentration of oxytetracycline dihydrate in reference solution (b);
– for impurities D and E multiply the peak areas by the correction factor 0.4.

*Limits*:

– *impurity C*: maximum 2.0 per cent;
– *impurity B*: maximum 1.0 per cent;
– *impurity A*: maximum 0.7 per cent;
– *impurities D, E*: for each impurity, maximum 0.2 per cent;
– *any other impurity*: maximum 0.1 per cent;
– *total*: maximum 3.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 6.0 per cent to 9.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}N_2O_9$ taking into account the assigned content of *oxytetracycline CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F*.

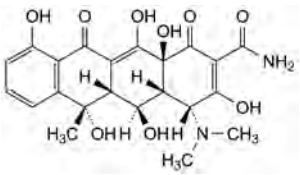

A. (4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epioxytetracycline),

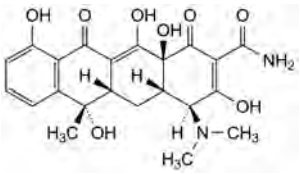

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (tetracycline),

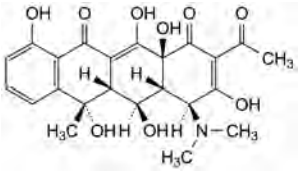

C. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyloxytetracycline),

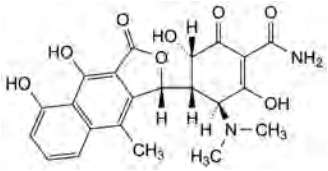

D. (3*S*,4*S*,5*S*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

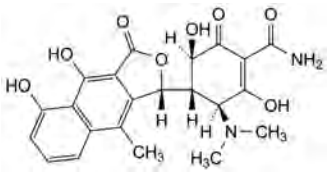

E. (3*S*,4*S*,5*R*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

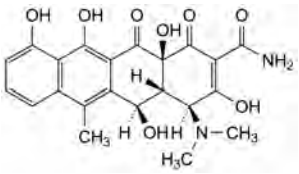

F. (4*S*,4a*R*,5*R*,12a*S*)-4-(dimethylamino)-3,5,10,11,12a-pentahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydro-oxytetracycline).

**01/2020:0198**

# OXYTETRACYCLINE HYDROCHLORIDE

## Oxytetracyclini hydrochloridum

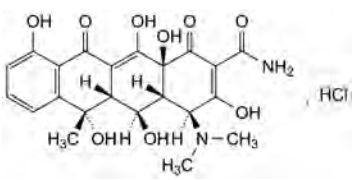

$C_{22}H_{25}ClN_2O_9$ $M_r$ 496.9
[2058-46-0]

DEFINITION

(4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(Dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by the growth of certain strains of *Streptomyces rimosus*.

*Content*: 94.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent). Solutions in water become turbid on standing, owing to the precipitation of oxytetracycline.

IDENTIFICATION

*First identification: B, C, D.*

*Second identification: A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *oxytetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *oxytetracycline hydrochloride CRS*, 5 mg of *tetracycline hydrochloride R* and 5 mg of *minocycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. To about 2 mg add 5 mL of *sulfuric acid R*. A deep red colour develops. Add the solution to 2.5 mL of *water R*. The colour becomes yellow.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 2.3 to 2.9.

Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Light-absorbing impurities**. *Carry out the measurements within 1 h of preparing the solutions.*

Dissolve 20.0 mg in a mixture of 1 volume of a 103 g/L solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. The absorbance (*2.2.25*), determined at 430 nm has a maximum of 0.50 (anhydrous substance).

Dissolve 0.100 g in a mixture of 1 volume of a 103 g/L solution of *hydrochloric acid R* and 99 volumes of *methanol R* and dilute to 10.0 mL with the same mixture of solvents. The absorbance (*2.2.25*) determined at 490 nm has a maximum of 0.20 (anhydrous substance).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (a).* Dissolve 20.0 mg of *oxytetracycline CRS* in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same acid. Dilute 5.0 mL of the solution to 50.0 mL with a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL in a 1 g/L solution of *hydrochloric acid R*.

*Reference solution (c).* Dissolve 4.0 mg of *oxytetracycline for system suitability A CRS* (containing impurities A, B, C, D, E and F) in a 1 g/L solution of *hydrochloric acid R* and dilute to 5.0 mL with the same solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: 0.05 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: *tetrahydrofuran for chromatography R*, *methanol R1*, *acetonitrile R1* (5:15:80 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 65 | 10 → 35 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *oxytetracycline for system suitability A CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to oxytetracycline (retention time = about 6.5 min): impurity A = about 0.9; impurity B = about 1.2; impurity C = about 1.3; impurity D = about 1.4; impurity E = about 2.2; impurity F = about 2.3.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline; minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to oxytetracycline.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 0.4; impurity E = 0.4;
- for each impurity, use the concentration of oxytetracycline hydrochloride in reference solution (b).

*Limits*:

- *impurity C*: maximum 2.0 per cent;
- *impurity B*: maximum 1.0 per cent;
- *impurity A*: maximum 0.5 per cent;
- *impurities D, E*: for each impurity, maximum 0.2 per cent;
- *sum of impurities D, E and F*: maximum 1.0 per cent;
- *any other impurity*: maximum 0.1 per cent;
- *total*: maximum 3.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.4 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{22}H_{25}ClN_2O_9$ taking into account the assigned content of *oxytetracycline CRS* and a conversion factor of 1.079.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

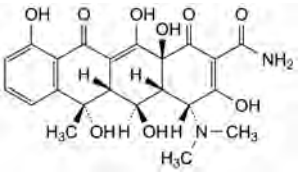

A. (4*R*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epioxytetracycline),

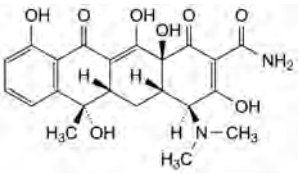

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (tetracycline),

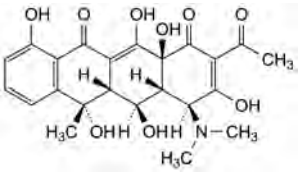

C. (4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,5,6,10,12,12a-hexahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyloxytetracycline),

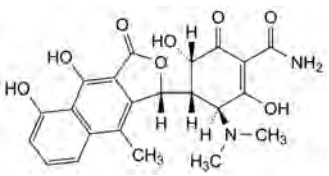

D. (3*S*,4*S*,5*S*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

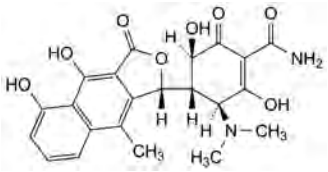

E. (3*S*,4*S*,5*R*)-4-[(1*R*)-4,5-dihydroxy-9-methyl-3-oxo-1,3-dihydronaphtho[2,3-*c*]furan-1-yl]-3-(dimethylamino)-2,5-dihydroxy-6-oxocyclohex-1-ene-1-carboxamide,

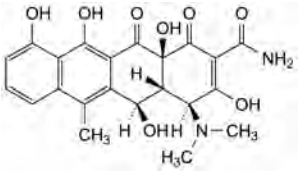

F. (4*S*,4a*R*,5*R*,12a*S*)-4-(dimethylamino)-3,5,10,11,12a-pentahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydro-oxytetracycline).

**01/2008:0780**
**corrected 10.0**

# OXYTOCIN

## Oxytocinum

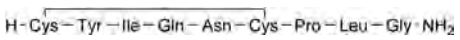

$C_{43}H_{66}N_{12}O_{12}S_2$ $M_r$ 1007
[50-56-6]

DEFINITION

L-Cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide cyclic (1→6)-disulfide.

Synthetic cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in receptive mammals. It is available in the freeze-dried form as an acetate.

*Content*: 93.0 per cent to 102.0 per cent (anhydrous and acetic acid-free substance).

By convention, for the purpose of labelling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}N_{12}O_{12}S_2$) is equivalent to 600 IU of biological activity.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: very soluble in water. It dissolves in dilute solutions of acetic acid and of ethanol (96 per cent).

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/6 of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine as equal to 1. The values fall within the following limits: aspartic acid: 0.90 to 1.10; glutamic acid: 0.90 to 1.10; proline: 0.90 to 1.10; glycine: 0.90 to 1.10; leucine: 0.90 to 1.10; isoleucine: 0.90 to 1.10; tyrosine: 0.7 to 1.05; half-cystine: 1.4 to 2.1. Not more than traces of other amino acids are present.

TESTS

**pH** (*2.2.3*): 3.0 to 6.0.

Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Prepare a 0.25 mg/mL solution of the substance to be examined in a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

*Resolution solution.* Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* in 1 mL of a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 15.6 g/L solution of *sodium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile for chromatography R*, *water R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 70 → 40 | 30 → 60 |
| 30 - 30.1 | 40 → 70 | 60 → 30 |
| 30.1 - 45 | 70 | 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL.

*Retention time*: oxytocin = about 7.5 min; desmopressin = about 10 min.

*System suitability*: resolution solution:
- *resolution*: minimum 5.0 between the peaks due to desmopressin and oxytocin.

*Limits*:
- *any impurity*: maximum 1.5 per cent;
- *total*: maximum 5 per cent;
- *disregard limit*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 6.0 per cent to 10.0 per cent.

*Test solution.* Dissolve 15.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on at least 50 mg.

**Bacterial endotoxins** (*2.6.14*): less than 300 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Reference solution.* Dissolve the contents of a vial of *oxytocin CRS* in a 15.6 g/L solution of *sodium dihydrogen phosphate R* to obtain a concentration of 0.25 mg/mL.

*Injection*: 25 µL.

Calculate the content of oxytocin ($C_{43}H_{66}N_{12}O_{12}S_2$) from the declared content of $C_{43}H_{66}N_{12}O_{12}S_2$ in *oxytocin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the oxytocin peptide content ($C_{43}H_{66}N_{12}O_{12}S_2$).

**01/2008:0779**
**corrected 10.0**

# OXYTOCIN CONCENTRATED SOLUTION

## Oxytocini solutio concentrata

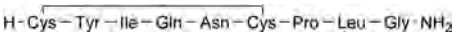


$C_{43}H_{66}N_{12}O_{12}S_2$ $M_r$ 1007

DEFINITION

L-Cysteinyl-L-tyrosyl-L-isoleucyl-L-glutamyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-leucylglycinamide cyclic (1→6)-disulfide.

Solution of oxytocin, a synthetic cyclic nonapeptide having the structure of the hormone produced by the posterior lobe of the pituitary gland that stimulates contraction of the uterus and milk ejection in receptive mammals. It is available as a solution with a stated concentration of not less than 0.25 mg of oxytocin per millilitre, in a solvent that may contain an appropriate antimicrobial preservative.

*Content*: 95.0 per cent to 105.0 per cent of the amount of the peptide stated per millilitre.

By convention, for the purpose of labelling oxytocin preparations, 1 mg of oxytocin peptide ($C_{43}H_{66}N_{12}O_{12}S_2$) is equivalent to 600 IU of biological activity.

CHARACTERS

*Appearance*: clear, colourless liquid.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking 1/6 of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, isoleucine and leucine as equal to 1. The values fall within the following limits: aspartic acid: 0.90 to 1.10; glutamic acid: 0.90 to 1.10; proline: 0.90 to 1.10; glycine: 0.90 to 1.10; leucine: 0.90 to 1.10; isoleucine: 0.90 to 1.10; tyrosine: 0.7 to 1.05; half cystine: 1.4 to 2.1. Not more than traces of other amino acids are present.

TESTS

**pH** (*2.2.3*): 3.0 to 5.0.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* The preparation to be examined.

*Resolution solution.* Dissolve the contents of a vial of *oxytocin/desmopressin validation mixture CRS* in 1 mL of a 15.6 g/L solution of *sodium dihydrogen phosphate R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 15.6 g/L solution of *sodium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile for chromatography R*, *water R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 70 → 40 | 30 → 60 |
| 30 - 30.1 | 40 → 70 | 60 → 30 |
| 30.1 - 45 | 70 | 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL.

*Retention time*: oxytocin = about 7.5 min; desmopressin = about 10 min.

*System suitability*: resolution solution:

- *resolution*: minimum 5.0 between the peaks due to desmopressin and oxytocin.

*Limits*:

- *any impurity*: maximum 1.5 per cent;
- *total*: maximum 5 per cent;
- *disregard limit*: 0.1 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 300 IU in the volume that contains 1 mg of oxytocin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Reference solution.* Dissolve the contents of a vial of *oxytocin CRS* in a 15.6 g/L solution of *sodium dihydrogen phosphate R* to obtain a concentration of 0.25 mg/mL.

*Injection*: 25 µL.

Calculate the content of oxytocin ($C_{43}H_{66}N_{12}O_{12}S_2$) from the declared content of $C_{43}H_{66}N_{12}O_{12}S_2$ in *oxytocin CRS*.

STORAGE

At a temperature of 2 °C to 8 °C, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the oxytocin peptide content in milligrams of $C_{43}H_{66}N_{12}O_{12}S_2$ per millilitre.

# P


Paclitaxel.. 3469
Palmitic acid.. 3473
Pamidronate disodium pentahydrate.. 3474
Pancreas powder.. 3474
Pancuronium bromide.. 3477
Pantoprazole sodium sesquihydrate.. 3478
Papaverine hydrochloride.. 3480
Paracetamol.. 3481
Paraffin, hard.. 3483
Paraffin, light liquid.. 3483
Paraffin, liquid.. 3484
Paraffin, white soft.. 3485
Paraffin, yellow soft.. 3486
Paraldehyde.. 3486
Parnaparin sodium.. 3487
Paroxetine hydrochloride.. 3488
Paroxetine hydrochloride hemihydrate.. 3490
Pea starch.. 3492
Pefloxacin mesilate dihydrate.. 3493
Pemetrexed disodium heptahydrate.. 3494
Penbutolol sulfate.. 3497
Penicillamine.. 3498
Pentaerythrityl tetranitrate, diluted.. 3499
Pentamidine diisetionate.. 3501
Pentazocine.. 3502
Pentazocine hydrochloride.. 3502
Pentazocine lactate.. 3503
Pentobarbital.. 3504
Pentobarbital sodium.. 3505
Pentoxifylline.. 3507
Pentoxyverine hydrogen citrate.. 3509
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Pergolide mesilate.. 3511
Perindopril *tert*-butylamine.. 3512
Peritoneal dialysis, solutions for.. 3515
Permethrin (25:75).. 3517
Perphenazine.. 3519
Pethidine hydrochloride.. 3520
Phenazone.. 3522
Pheniramine maleate.. 3523
Phenobarbital.. 3524
Phenobarbital sodium.. 3525
Phenol.. 3527
Phenolphthalein.. 3527
Phenolsulfonphthalein.. 3528
Phenoxyethanol.. 3529
Phenoxymethylpenicillin.. 3530
Phenoxymethylpenicillin (benzathine) tetrahydrate.. 3531
Phenoxymethylpenicillin potassium.. 3533
Phentolamine mesilate.. 3535
Phenylalanine.. 3536
Phenylbutazone.. 3538
Phenylephrine.. 3539
Phenylephrine hydrochloride.. 3541
Phenylmercuric acetate.. 3542
Phenylmercuric borate.. 3542
Phenylmercuric nitrate.. 3543
Phenylpropanolamine hydrochloride.. 3543
Phenytoin.. 3545
Phenytoin sodium.. 3546
Phloroglucinol.. 3548
Phloroglucinol dihydrate.. 3549
Pholcodine monohydrate.. 3551
Phosphoric acid, concentrated.. 3553
Phosphoric acid, dilute.. 3553
Phthalylsulfathiazole.. 3553
Physostigmine salicylate.. 3554
Phytomenadione, racemic.. 3555
Phytosterol.. 3557
Picotamide monohydrate.. 3558
Pilocarpine hydrochloride.. 3559
Pilocarpine nitrate.. 3560
Pimobendan for veterinary use.. 3561
Pimozide.. 3562
Pindolol.. 3563
Pioglitazone hydrochloride.. 3565
Pipemidic acid trihydrate.. 3566
Piperacillin.. 3567
Piperacillin sodium.. 3568
Piperazine adipate.. 3570
Piperazine citrate.. 3571
Piperazine hydrate.. 3572
Piracetam.. 3573
Pirenzepine dihydrochloride monohydrate.. 3574
Piretanide.. 3575
Pirfenidone.. 3576
Piroxicam.. 3577
Pivampicillin.. 3579
Pivmecillinam hydrochloride.. 3581
Podophyllotoxin.. 3582
Pollens for allergen products.. 3583
Poloxamers.. 3584
Polyacrylate dispersion 30 per cent.. 3586
Polymyxin B sulfate.. 3587
Polyoxypropylene stearyl ether.. 3588
Polysorbate 20.. 3589
Polysorbate 40.. 3590
Polysorbate 60.. 3591
Polysorbate 80.. 3591
Poly(vinyl acetate).. 3593
Poly(vinyl acetate) dispersion 30 per cent.. 3594
Poly(vinyl alcohol).. 3595
Potassium acetate.. 3596
Potassium bromide.. 3597
Potassium carbonate.. 3598
Potassium chloride.. 3598
Potassium citrate.. 3599
Potassium clavulanate.. 3599
Potassium clavulanate, diluted.. 3602
Potassium dihydrogen phosphate.. 3603
Potassium hydrogen aspartate hemihydrate.. 3604
Potassium hydrogen carbonate.. 3605
Potassium hydrogen tartrate.. 3605
Potassium hydroxide.. 3606
Potassium iodide.. 3606
Potassium metabisulfite.. 3607
Potassium nitrate.. 3607
Potassium perchlorate.. 3608
Potassium permanganate.. 3609
Potassium sodium tartrate tetrahydrate.. 3609
Potassium sorbate.. 3610
Potassium sulfate.. 3610
Potato starch.. 3611
Povidone.. 3612
Povidone, iodinated.. 3615
Pramipexole dihydrochloride monohydrate.. 3615
Prasugrel hydrochloride.. 3617
Pravastatin sodium.. 3618
Prazepam.. 3620
Praziquantel.. 3621
Prazosin hydrochloride.. 3622
Prednicarbate.. 3624
Prednisolone.. 3625
Prednisolone acetate.. 3627
Prednisolone pivalate.. 3629
Prednisolone sodium phosphate.. 3630

Prednisone.. 3631
Pregabalin.. 3633
Prilocaine.. 3635
Prilocaine hydrochloride.. 3636
Primaquine diphosphate.. 3637
Primidone.. 3639
Probenecid.. 3640
Procainamide hydrochloride.. 3641
Procaine hydrochloride.. 3642
Prochlorperazine maleate.. 3642
Progesterone.. 3644
Proguanil hydrochloride.. 3646
Proline.. 3647
Promazine hydrochloride.. 3649
Promethazine hydrochloride.. 3649
Propacetamol hydrochloride.. 3651
Propafenone hydrochloride.. 3652
Propanol.. 3654
Propantheline bromide.. 3655
Propofol.. 3656
Propranolol hydrochloride.. 3658
Propyl gallate.. 3659
Propyl parahydroxybenzoate.. 3660
Propylene glycol.. 3661
Propylene glycol dicaprylocaprate.. 3662
Propylene glycol dilaurate.. 3662
Propylene glycol monolaurate.. 3663
Propylene glycol monopalmitostearate.. 3664
Propylthiouracil.. 3665
Propyphenazone.. 3666
Protamine sulfate.. 3667
Protirelin.. 3669
Proxyphylline.. 3670
Pseudoephedrine hydrochloride.. 3671
Pullulan.. 3672
Pyrantel embonate.. 3673
Pyrazinamide.. 3674
Pyridostigmine bromide.. 3675
Pyridoxine hydrochloride.. 3676
Pyrimethamine.. 3677
Pyrrolidone.. 3678

**01/2017:1794**
**corrected 10.0**

# PACLITAXEL

## Paclitaxelum

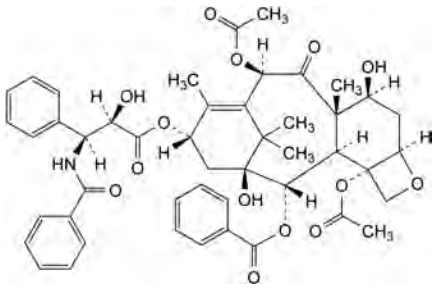

$C_{47}H_{51}NO_{14}$ $M_r$ 854
[33069-62-4]

DEFINITION

4,10β-Bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate.

It is isolated from natural sources or produced by fermentation or by a semi-synthetic process.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methanol and freely soluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *paclitaxel CRS*.

If the spectra obtained in the solid state show differences, dissolve 10 mg of the substance to be examined and the reference substance separately in 0.4 mL of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.1 g in 10 mL of *methanol R*.

**Specific optical rotation** (*2.2.7*): − 55.0 to − 49.0 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

A. Paclitaxel isolated from natural sources or produced by fermentation.

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with *acetonitrile R1*.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 100.0 mL with *acetonitrile R1*.

*Reference solution (b)*. Dissolve 5.0 mg of *paclitaxel CRS* in *acetonitrile R1* and dilute to 5.0 mL with the same solvent. Dilute 2.0 mL of this solution to 20.0 mL with *acetonitrile R1*.

*Reference solution (c)*. Dissolve 2.0 mg of *paclitaxel impurity C CRS* in *acetonitrile R1* and dilute to 20.0 mL with the same solvent.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (c) to 50.0 mL with *acetonitrile R1*.

*Reference solution (e)*. To 1 mL of reference solution (b) add 1 mL of reference solution (c).

*Reference solution (f)*. Dissolve 5 mg of *paclitaxel natural for peak identification CRS* (containing impurities A, B, C, D, E, F, H, O, P, Q and R) in *acetonitrile R1* and dilute to 5 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *diisopropylcyanosilyl silica gel for chromatography R* (5 µm) with a specific surface area of 180 $m^2$/g and a pore size of 8 nm;

– *temperature*: 20 ± 1 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R1*, *water for chromatography R* (20:80 *V/V*);

– *mobile phase B*: *methanol R1*, *acetonitrile for chromatography R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 85 → 56 | 15 → 44 |
| 60 - 61 | 56 → 85 | 44 → 15 |
| 61 - 75 | 85 | 15 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 227 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (d), (e) and (f).

*Identification of impurities*: use the chromatogram supplied with *paclitaxel natural for peak identification CRS* and the chromatogram obtained with reference solution (f) to identify the peaks due to impurities A, B, C, D, E, F, H, O, P, Q and R.

*Relative retention* with reference to paclitaxel (retention time = about 50 min): impurities A and B = about 0.90; impurity R = about 0.93; impurity H = about 0.96; impurities Q and P = about 1.02; impurity C = about 1.05; impurity D = about 1.07; impurities O and E = about 1.15; impurity F = about 1.20.

*System suitability*: reference solution (e):

– *resolution*: minimum 3.5 between the peaks due to paclitaxel and impurity C.

*Limits*:

- *sum of impurities E and O*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity R*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *sum of impurities A and B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurity C*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities P and Q*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

B. Paclitaxel produced by a semi-synthetic process.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 100.0 mL with *acetonitrile R1*.

*Reference solution (b).* Dissolve 5.0 mg of *paclitaxel CRS* in *acetonitrile R1* and dilute to 5.0 mL with the same solvent.

*Reference solution (c).* Dissolve 5 mg of *paclitaxel semi-synthetic for peak identification CRS* (containing impurities A, G, I and L) in *acetonitrile R1* and dilute to 5 mL with the same solvent.

*Reference solution (d).* Dissolve the contents of a vial of *paclitaxel semi-synthetic for system suitability CRS* (containing impurities E, H and N) in 1 mL of *acetonitrile R1*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm) with a specific surface area of 300 $m^2$/g and a pore size of 12 nm;
- temperature: 35 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile for chromatography R*, *water for chromatography R* (40:60 *V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 60 | 100 → 10 | 0 → 90 |
| 60 - 62 | 10 → 100 | 90 → 0 |
| 62 - 70 | 100 | 0 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 227 nm.

*Injection*: 15 µL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *paclitaxel semi-synthetic for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, G, I and L; use the chromatogram supplied with *paclitaxel semi-synthetic for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities E, H and N.

*Relative retention* with reference to paclitaxel (retention time = about 23 min): impurity N = about 0.2; impurity G = about 0.5; impurity A = about 0.8; impurities M, J and H = about 0.9; impurity E = about 1.3; impurity I = about 1.4; impurity L = about 1.5; impurity K = about 2.2.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurity H and paclitaxel.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity N by 1.29;
- *impurity A*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *impurity L*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities E, I*: for each impurity, not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *sum of impurities H, J and M*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
- *impurities G, K, N*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 12 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 3.0 per cent, determined on 0.050 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

**Bacterial endotoxins** (*2.6.14*): less than 0.4 IU/mg.

ASSAY

A. Paclitaxel isolated from natural sources or produced by fermentation.

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_{47}H_{51}NO_{14}$ from the declared content of *paclitaxel CRS*.

B. Paclitaxel produced by a semi-synthetic process.

Liquid chromatography (*2.2.29*) as described in test B for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (b).

Calculate the percentage content of $C_{47}H_{51}NO_{14}$ from the declared content of *paclitaxel CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the origin of the substance:

– isolated from natural sources;

– produced by fermentation;

– produced by a semi-synthetic process.

IMPURITIES

**Test A for related substances**: A, B, C, D, E, F, H, O, P, Q, R.

*Specified impurities: A, B, C, D, E, F, O, P, Q, R.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H.*

**Test B for related substances**: A, E, G, H, I, J, K, L, M, N.

*Specified impurities: A, E, G, H, I, J, K, L, M, N.*

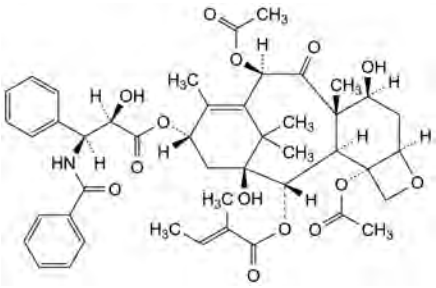

A. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl (2*E*)-2-methylbut-2-enoate (2-*O*-debenzoyl-2-*O*-tigloylpaclitaxel),

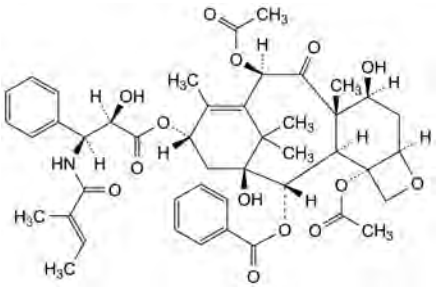

B. 4,10β-bis(acetyloxy)-1,7β-dihydroxy-13α-[[(2*R*,3*S*)-2-hydroxy-3-[[(2*E*)-2-methylbut-2-enoyl]amino]-3-phenylpropanoyl]oxy]-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-tigloylpaclitaxel; cephalomannine),

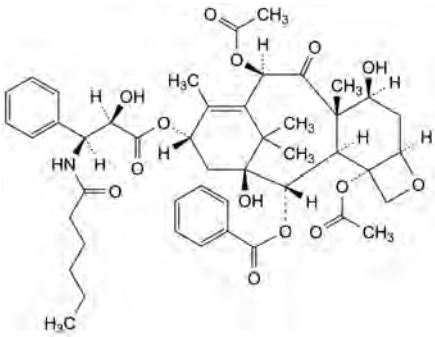

C. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-(hexanoylamino)-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-hexanoylpaclitaxel; paclitaxel C),

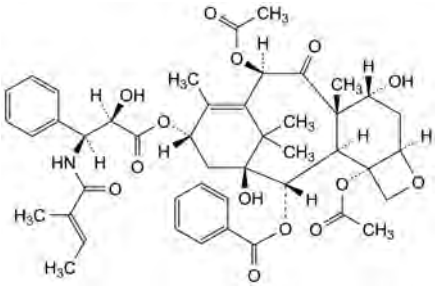

D. 4,10β-bis(acetyloxy)-1,7α-dihydroxy-13α-[[(2*R*,3*S*)-2-hydroxy-3-[[(2*E*)-2-methylbut-2-enoyl]amino]-3-phenylpropanoyl]oxy]-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-tigloyl-7-*epi*-paclitaxel; 7-*epi*-cephalomannine),

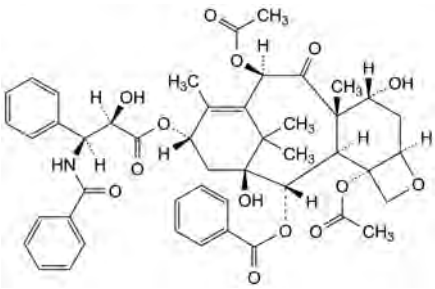

E. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7α-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (7-*epi*-paclitaxel),

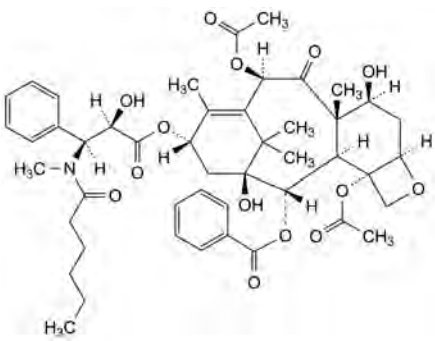

F. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-[hexanoyl(methyl)-amino]-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-hexanoyl-*N*-methylpaclitaxel; *N*-methylpaclitaxel C),

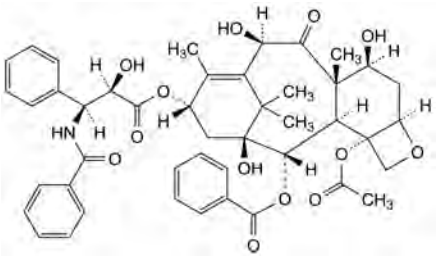

G. 4-(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β,10β-trihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (10-*O*-deacetylpaclitaxel),

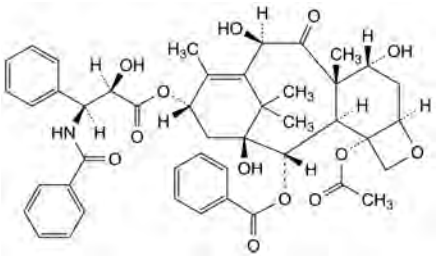

H. 4-(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7α,10β-trihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (10-*O*-deacetyl-7-*epi*-paclitaxel),

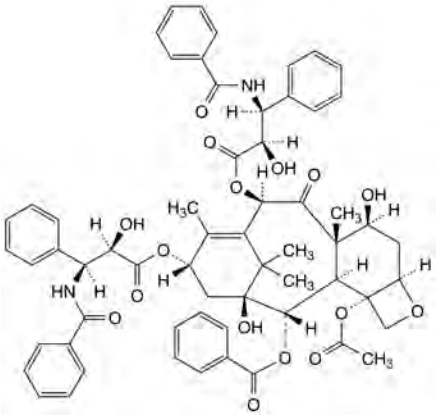

I. 4-(acetyloxy)-10β,13α-bis[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (10-*O*-[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]-10-*O*-deacetylpaclitaxel),

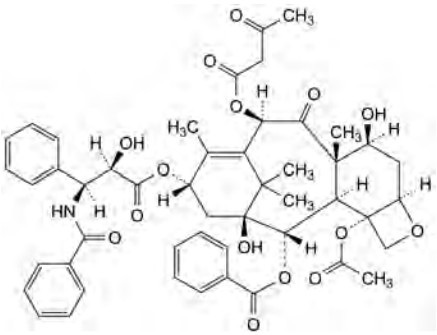

J. 4-(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-10β-[(3-oxobutanoyl)oxy]-5β,20-epoxytax-11-en-2α-yl benzoate (10-*O*-deacetyl-10-*O*-(3-oxobutanoyl)paclitaxel),

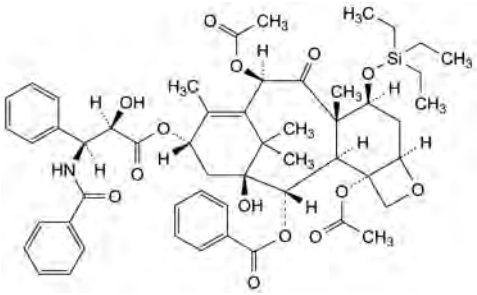

K. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1-hydroxy-9-oxo-7β-[(triethylsilyl)oxy]-5β,20-epoxytax-11-en-2α-yl benzoate (7-*O*-(triethylsilanyl)paclitaxel),

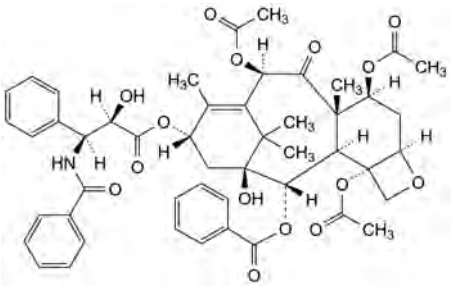

L. 4,7β,10β-tris(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1-hydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (7-*O*-acetylpaclitaxel),

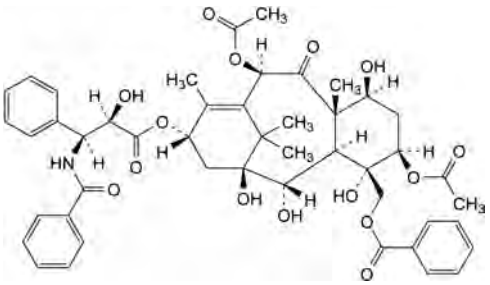

M. 5β,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]oxy]-1,2α,4,7β-tetrahydroxy-9-oxotax-11-en-20-yl benzoate,

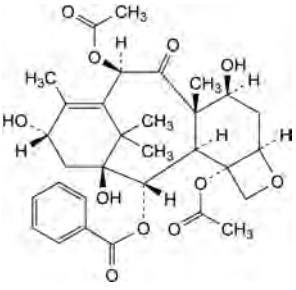

N. 4,10β-bis(acetyloxy)-1,7β,13α-trihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (13-*O*-de[(2*R*,3*S*)-3-benzamido-2-hydroxy-3-phenylpropanoyl]paclitaxel; baccatin III),

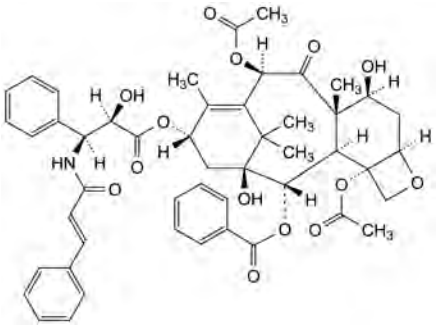

O. 4,10β-bis(acetyloxy)-1,7β-dihydroxy-13α-[[(2*R*,3*S*)-2-hydroxy-3-phenyl-3-[[(2*E*)-3-phenylprop-2-enoyl]amino]propanoyl]oxy]-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-cinnamoyl-*N*-debenzoylpaclitaxel),

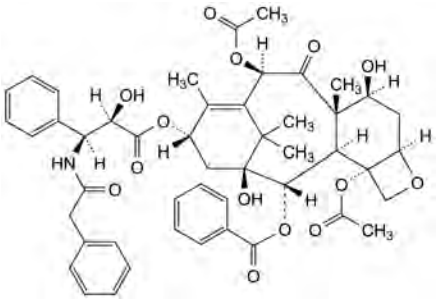

P. 4,10β-bis(acetyloxy)-1,7β-dihydroxy-13α-[[(2*R*,3*S*)-2-hydroxy-3-phenyl-3-(2-phenylacetamido)propanoyl]oxy]-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-(phenylacetyl)paclitaxel),

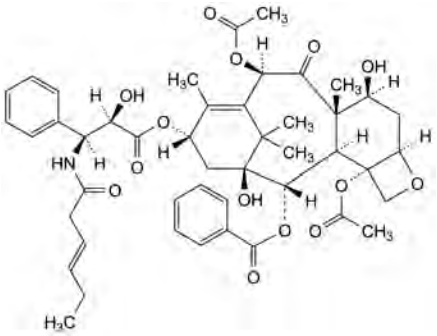

Q. 4,10β-bis(acetyloxy)-13α-[[(2*R*,3*S*)-3-[[(3*E*)-hex-3-enoyl]amino]-2-hydroxy-3-phenylpropanoyl]oxy]-1,7β-dihydroxy-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-[(3*E*)-hex-3-enoyl]paclitaxel),

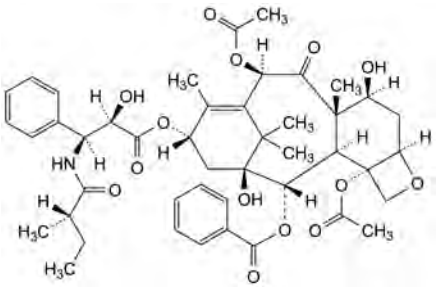

R. 4,10β-bis(acetyloxy)-1,7β-dihydroxy-13α-[[(2*R*,3*S*)-2-hydroxy-3-[[(2*S*)-2-methylbutanoyl]amino]-3-phenylpropanoyl]oxy]-9-oxo-5β,20-epoxytax-11-en-2α-yl benzoate (*N*-debenzoyl-*N*-[(2*S*)-2-methylbutanoyl]paclitaxel).

**01/2020:1904**

# PALMITIC ACID

## Acidum palmiticum

[57-10-3]

DEFINITION

Hexadecanoic acid ($C_{16}H_{32}O_2$; $M_r$ 256.4), obtained from fats or oils of vegetable or animal origin.

*Content*: minimum 92.0 per cent.

CHARACTERS

*Appearance*: white or almost white, waxy solid.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Freezing point (see Tests).

B. Acid value (*2.5.1*): 216 to 220, determined on 0.1 g.

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Appearance**. Heat the substance to be examined to about 75 °C. The resulting liquid is not more intensely coloured than reference solution $Y_7$ or $BY_7$ (*2.2.2, Method I*).

**Acidity**. Melt 5.0 g, stir for 2 min in 10 mL of hot *carbon dioxide-free water R*, cool slowly and filter. To the filtrate add 0.05 mL of *methyl orange solution R*. No red colour develops.

**Freezing point** (*2.2.18*): 60 °C to 66 °C.

**Iodine value** (*2.5.4*): maximum 1.

**Stearic acid**: maximum 6.0 per cent, determined as prescribed in the assay.

ASSAY

Gas chromatography (*2.4.22, Method C*) with the following modifications. Prepare the solutions as described in the method but omitting the initial hydrolysis.

*Reference solution*. Prepare the reference solution in the same manner as the test solution using a mixture of 50 mg of *palmitic acid R* and 50 mg of *stearic acid R* instead of the substance to be examined.

*Relative retention* with reference to methyl stearate: methyl palmitate = about 0.9.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to methyl stearate and methyl palmitate.

**01/2017:1779**

# PAMIDRONATE DISODIUM PENTAHYDRATE

## Dinatrii pamidronas pentahydricus

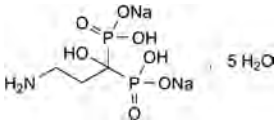

$C_3H_9NNa_2O_7P_2,5H_2O$ $M_r$ 369.1
[109552-15-0]

DEFINITION

Disodium dihydrogen (3-amino-1-hydroxypropylidene)-bisphosphonate pentahydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in methylene chloride. It is sparingly soluble in dilute mineral acids and dissolves in dilute alkaline solutions.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pamidronate disodium pentahydrate CRS*.

B. Dissolve 0.5 g in 10 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 7.8 to 8.8.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 30 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 15 mg of *3-aminopropionic acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *di-isopropyl ether R*, *methanol R* (4:8:9 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: spray with a *ninhydrin solution R*. Heat at 100-105 °C for 15 min.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Impurities B and C**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* To 2.0 mL of a 0.3 g/L solution of *phosphoric acid R* add 2.0 mL of a 0.25 g/L solution of *phosphorous acid R* and dilute to 50.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm,
– *stationary phase*: *anion-exchange resin R* (5 µm),
– *temperature*: 35 °C.

*Mobile phase*: to 0.5 mL of *anhydrous formic acid R* add 2500 mL of *water R*; adjust to pH 3.5 with an 80 g/L solution of *sodium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: refractometer.

*Injection*: 100 µL.

*Relative retention* with reference to pamidronate (retention time = about 13 min): impurity B = about 1.3; impurity C = about 1.6.

*System suitability*: reference solution:

– *resolution*: minimum 2.5 between the peaks due to impurities B and C.

*Limits*:

– *impurities B, C*: for each impurity, not more than the area of the corresponding peaks in the chromatogram obtained with the reference solution (0.5 per cent).

**Water** (*2.5.12*): 23.0 per cent to 27.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.250 g in 70 mL of *water R*. Titrate with *0.1 M hydrochloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 27.91 mg of $C_3H_9NNa_2O_7P_2$.

IMPURITIES

*Specified impurities: A, B, C.*

A. 3-aminopropanoic acid (β-alanine),

B. $H_3PO_4$: phosphoric acid,

C. $H_3PO_3$: phosphorous acid.

**01/2016:0350**

# PANCREAS POWDER

## Pancreatis pulvis

DEFINITION

Pancreas powder is prepared from the fresh or frozen pancreases of mammals. It contains various enzymes having proteolytic, lipolytic and amylolytic activities.

1 mg of pancreas powder contains not less than 1.0 Ph. Eur. U. of total proteolytic activity, 15 Ph. Eur. U. of lipolytic activity and 12 Ph. Eur. U. of amylolytic activity.

PRODUCTION

The animals from which pancreas powder is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: slightly brown, amorphous powder.

*Solubility*: partly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Triturate 0.5 g with 10 mL of *water R* and adjust to pH 8 with *0.1 M sodium hydroxide*, using 0.1 mL of *cresol red solution R* as indicator. Divide the suspension into 2 equal parts (suspension (a) and suspension (b)). Boil suspension (a). To each suspension add 10 mg of *fibrin congo red R*, heat to 38-40 °C and maintain at this temperature for 1 h. Suspension (a) is colourless or slightly pink and suspension (b) is distinctly more red.

B. Triturate 0.25 g with 10 mL of *water R* and adjust to pH 8 with *0.1 M sodium hydroxide*, using 0.1 mL of *cresol red solution R* as indicator. Divide the suspension into 2 equal parts (suspension (a) and suspension (b)). Boil suspension (a). Dissolve 0.1 g of *soluble starch R* in 100 mL of boiling *water R*, boil for 2 min, cool and dilute to 150 mL with *water R*. To 75 mL of the starch solution add suspension (a) and to the remaining 75 mL add suspension (b). Heat each mixture to 38-40 °C and maintain at this temperature for 5 min.

   To 1 mL of each mixture add 10 mL of *iodine solution R2*. The mixture obtained with suspension (a) has an intense blue-violet colour; the mixture obtained with suspension (b) has the colour of the iodine solution.

TESTS

**Fat content**: maximum 5.0 per cent.

In an extraction apparatus, treat 1.0 g with *light petroleum R1* for 3 h. Evaporate the solvent and dry the residue at 100-105 °C for 2 h. The residue weighs a maximum of 50 mg.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.50 g by drying at 60 °C at a pressure not exceeding 670 Pa for 4 h.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

**Total proteolytic activity**. The total proteolytic activity of pancreas powder is determined by comparing the quantity of peptides non-precipitable by a 50 g/L solution of *trichloroacetic acid R* released per minute from a substrate of casein solution with the quantity of such peptides released by *pancreas powder (protease) BRP* from the same substrate in the same conditions.

*Casein solution*. Suspend a quantity of *casein BRP* equivalent to 1.25 g of dried substance in 5 mL of *water R*, add 10 mL of *0.1 M sodium hydroxide* and stir for 1 min. (Determine the water content of *casein BRP* prior to the test by heating at 60 °C *in vacuo* for 4 h.) Add 60 mL of *water R* and stir with a magnetic stirrer until the solution is practically clear. Adjust to pH 8.0 with *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid*. Dilute to 100.0 mL with *water R*. Use the solution on the day of preparation.

*Enterokinase solution*. Dissolve 50 mg of *enterokinase BRP* in *0.02 M calcium chloride solution R* and dilute to 50.0 mL with the same solvent. Use the solution on the day of preparation.

*To avoid absorption of water formed by condensation, allow the preparation to be examined and the reference preparation to reach room temperature before opening the containers.*

*For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0-4 °C.*

*Test suspension.* Triturate 0.100 g of the substance to be examined for 5 min adding gradually 25 mL of *0.02 M calcium chloride solution R*. Transfer completely to a volumetric flask and dilute to 100.0 mL with *0.02 M calcium chloride solution R*. To 10.0 mL of this suspension add 10.0 mL of the enterokinase solution and heat on a water-bath at 35 ± 0.5 °C for 15 min. Cool and dilute with *borate buffer solution pH 7.5 R* at 5 ± 3 °C to a final concentration of about 0.065 Ph. Eur. U. of total proteolytic activity per millilitre calculated on the basis of the stated activity.

*Reference suspension.* Prepare a suspension of *pancreas powder (protease) BRP* as described for the test suspension but without the addition of enterokinase so as to obtain a known final concentration of about 0.065 Ph. Eur. U. per millilitre calculated on the basis of the stated activity.

Designate tubes in duplicate T, $T_b$, $S_1$, $S_{1b}$, $S_2$, $S_{2b}$, $S_3$, $S_{3b}$; designate a tube B.

Add *borate buffer solution pH 7.5 R* to the tubes as follows:

B: 3.0 mL;

$S_1$ and $S_{1b}$: 2.0 mL;

$S_2$, $S_{2b}$, T and $T_b$: 1.0 mL.

Add the reference suspension to the tubes as follows:

$S_1$ and $S_{1b}$: 1.0 mL;

$S_2$ and $S_{2b}$: 2.0 mL;

$S_3$ and $S_{3b}$: 3.0 mL.

Add 2.0 mL of the test suspension to tubes T and $T_b$.

Add 5.0 mL of a 50 g/L solution of *trichloroacetic acid R* to tubes B, $S_{1b}$, $S_{2b}$, $S_{3b}$ and $T_b$. Mix by shaking.

Place the tubes and the casein solution in a water-bath at 35 ± 0.5 °C. Place a glass rod in each tube. When temperature equilibrium is reached, add 2.0 mL of the casein solution to tubes B, $S_{1b}$, $S_{2b}$, $S_{3b}$ and $T_b$. Mix. At time zero, add 2.0 mL of casein solution successively and at intervals of 30 s to tubes $S_1$, $S_2$, $S_3$ and T. Mix immediately after each addition. Exactly 30 min after addition of the casein solution, taking into account the regular interval adopted, add 5.0 mL of a 50 g/L solution of *trichloroacetic acid R* to tubes $S_1$, $S_2$, $S_3$ and T. Mix. Withdraw the tubes from the water-bath and allow to stand at room temperature for 20 min.

Filter the contents of each tube twice through the same suitable filter paper previously washed with a 50 g/L solution of *trichloroacetic acid R*, then with *water R* and dried.

A suitable filter paper complies with the following test: filter 5 mL of a 50 g/L solution of *trichloroacetic acid R* on a 7 cm disc of white filter paper; the absorbance (*2.2.25*) of the filtrate, measured at 275 nm using unfiltered trichloroacetic acid solution as the compensation liquid, is less than 0.04.

A schematic presentation of the above operations is shown in Table 0350.-1.

Table 0350.-1

| | Tubes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $S_1$ | $S_{1b}$ | $S_2$ | $S_{2b}$ | $S_3$ | $S_{3b}$ | T | $T_b$ | B |
| Buffer solution | 2 | 2 | 1 | 1 | | | 1 | 1 | 3 |
| Reference suspension | 1 | 1 | 2 | 2 | 3 | 3 | | | |
| Test suspension | | | | | | | 2 | 2 | |
| Trichloroacetic acid solution | | 5 | | 5 | | 5 | | 5 | 5 |
| Mix | | + | | + | | + | | + | + |
| Water-bath 35 °C | + | + | + | + | + | + | + | + | + |
| Casein solution | | 2 | | 2 | | 2 | | 2 | 2 |
| Mix | | + | | + | | + | | + | + |
| Casein solution | 2 | | 2 | | 2 | | 2 | | |
| Mix | + | | + | | + | | + | | |
| Water-bath 35 °C 30 min | + | + | + | + | + | + | + | + | + |
| Trichloroacetic acid solution | 5 | | 5 | | 5 | | 5 | | |
| Mix | + | | + | | + | | + | | |
| Room temperature 20 min | + | + | + | + | + | + | + | + | + |
| Filter | + | + | + | + | + | + | + | + | + |

Measure the absorbance (*2.2.25*) of the filtrates at 275 nm using the filtrate obtained from tube B as the compensation liquid.

Correct the average absorbance values for the filtrates obtained from tubes $S_1$, $S_2$ and $S_3$ by subtracting the average values obtained for the filtrates from tubes $S_{1b}$, $S_{2b}$ and $S_{3b}$ respectively. Draw a calibration curve of the corrected values against the volume of reference suspension used.

Determine the activity of the substance to be examined using the corrected absorbance for the test suspension ($T - T_b$) and the calibration curve and taking into account the dilution factors.

The test is not valid unless the corrected absorbance values are between 0.15 and 0.60.

**Lipolytic activity**. The lipolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of olive oil emulsion with the rate at which a suspension of *pancreas powder (lipase) BRP* hydrolyses the same substrate under the same conditions. *The test is carried out under nitrogen.*

*Olive oil stock emulsion.* In an 800 mL beaker 9 cm in diameter, place 40 mL of *olive oil R*, 330 mL of *acacia solution R* and 30 mL of *water R*. Place an electric mixer at the bottom of the beaker. Place the beaker in a vessel containing *ethanol (96 per cent) R* and a sufficient quantity of ice as a cooling mixture. Emulsify using the mixer at an average speed of 1000-2000 r/min. Cool to 5-10 °C. Increase the mixing speed to 8000 r/min. Mix for 30 min keeping the temperature below 25 °C by the continuous addition of crushed ice into the cooling mixture. (A mixture of calcium chloride and crushed ice is also suitable). Store the stock emulsion in a refrigerator and use within 14 days. The emulsion must not separate into 2 distinct layers. Check the diameter of the globules of the emulsion under a microscope. At least 90 per cent have a diameter below 3 µm and none has a diameter greater than 10 µm. Shake the emulsion thoroughly before preparing the emulsion substrate.

*Olive oil emulsion.* For 10 determinations, mix the following solutions in the order indicated: 100 mL of the stock emulsion, 80 mL of *tris(hydroxymethyl)aminomethane solution R1*, 20 mL of a freshly prepared 80 g/L of *sodium taurocholate BRP* and 95 mL of *water R*. Use on the day of preparation.

*Apparatus.* Use a reaction vessel of about 50 mL capacity provided with:

- a device that will maintain a temperature of 37 ± 0.5 °C;
- a magnetic stirrer;
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration apparatus may be used. In the latter case, the burette is graduated in 0.005 mL and the pH-meter is provided with a wide reading scale and glass-silver-silver chloride or other suitable electrodes. After each test the reaction vessel is evacuated by suction and washed several times with *water R*, the washings being removed each time by suction.

*To avoid absorption of water formed by condensation, allow the preparation to be examined and the reference preparation to reach room temperature before opening the containers.*

*For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0-4 °C.*

*Test suspension.* In a small mortar cooled to 0-4 °C, triturate carefully a quantity of the substance to be examined equivalent to about 2500 Ph. Eur. U. of lipolytic activity with 1 mL of *maleate buffer solution pH 7.0 R* (lipase solvent) until a very fine suspension is obtained. Dilute the suspension with *maleate buffer solution pH 7.0 R*, transfer quantitatively to a volumetric flask and dilute to 100.0 mL with the buffer solution. Keep the flask containing the test suspension in iced water during the titration.

*Reference suspension.* Prepare a suspension of *pancreas powder (lipase) BRP* as described for the test suspension using a quantity equivalent to about 2500 Ph. Eur. U.

Carry out the titrations immediately after preparation of the test suspension and the reference suspension. Place 29.5 mL of olive oil emulsion in the reaction vessel equilibrated at 37 ± 0.5 °C. Fit the vessel with the electrodes, a stirrer and the burette (the tip being immersed in the olive oil emulsion).

Put the lid in place and switch on the apparatus. Carefully add *0.1 M sodium hydroxide* with stirring to adjust to pH 9.2. Using a rapid-flow graduated pipette transfer about 0.5 mL of the previously homogenised reference suspension, start the chronometer and add continuously *0.1 M sodium hydroxide* to maintain the pH at 9.0. After exactly 1 min, note the volume of *0.1 M sodium hydroxide* used. Carry out the measurement a further 4 times. Discard the first reading and determine the average of the 4 others ($S_1$). Make 2 further determinations ($S_2$ and $S_3$). Calculate the average of the values $S_1$, $S_2$ and $S_3$. The average volume of *0.1 M sodium hydroxide* used should be about 0.12 mL per minute with limits of 0.08 mL to 0.16 mL.

Carry out 3 determinations in the same manner for the test suspension ($T_1$, $T_2$ and $T_3$). If the quantity of *0.1 M sodium hydroxide* used is outside the limits of 0.08 mL to 0.16 mL per minute, the assay is repeated with a quantity of test suspension that is more suitable but situated between 0.4 mL and 0.6 mL. Otherwise the quantity of the substance to be examined is adjusted to comply with the conditions of the test. Calculate the average of the values $T_1$, $T_2$ and $T_3$.

Calculate the activity in European Pharmacopoeia Units per milligram using the following expression:

$$\frac{n \times m_1}{n_1 \times m} \times A$$

$n$ = average volume of *0.1 M sodium hydroxide* used per minute during the titration of the test suspension, in millilitres;

$n_1$ = average volume of *0.1 M sodium hydroxide* used per minute during the titration of the reference suspension, in millilitres;

$m$ = mass of the substance to be examined, in milligrams;

$m_1$ = mass of the reference preparation, in milligrams;

$A$ = activity of *pancreas powder (lipase) BRP*, in European Pharmacopoeia Units per milligram.

**Amylolytic activity**. The amylolytic activity is determined by comparing the rate at which a suspension of pancreas powder hydrolyses a substrate of starch solution with the rate at which a suspension of *pancreas powder (amylase) BRP* hydrolyses the same substrate under the same conditions.

*Starch solution.* To a quantity of *starch BRP* equivalent to 2.0 g of the dried substance add 10 mL of *water R* and mix. (Determine the water content of *starch BRP* prior to the test by heating at 120 °C for 4 h). Add this suspension, whilst stirring continuously, to 160 mL of boiling *water R*. Wash the container several times with successive quantities, each of 10 mL, of *water R* and add the washings to the hot starch solution. Heat to boiling, stirring continuously. Cool to room temperature and dilute to 200 mL with *water R*. Use the solution on the day of preparation.

*To avoid absorption of water formed by condensation, allow the preparation to be examined and the reference preparation to reach room temperature before opening the containers.*

*For the test suspension and the reference suspension, prepare the suspension and carry out the dilution at 0-4 °C.*

*Test suspension.* Triturate a quantity of the substance to be examined equivalent to about 1500 Ph. Eur. U. of amylolytic activity with 60 mL of *phosphate buffer solution pH 6.8 R1* for 15 min. Transfer quantitatively to a volumetric flask and dilute to 100.0 mL with *phosphate buffer solution pH 6.8 R1*.

*Reference suspension.* Prepare a suspension of *pancreas powder (amylase) BRP* as described for the test suspension, using a quantity equivalent to about 1500 Ph. Eur. U.

In a test-tube 200 mm long and 22 mm in diameter, fitted with a ground-glass stopper, place 25.0 mL of starch solution, 10.0 mL of *phosphate buffer solution pH 6.8 R1* and 1.0 mL of an 11.7 g/L solution of *sodium chloride R*. Close the tube, shake and place in a water-bath at 25.0 ± 0.1 °C. When the temperature equilibrium has been reached, add 1.0 mL of the test suspension and start the chronometer. Mix and place the tube in the water-bath. After exactly 10 min, add 2 mL of *1 M hydrochloric acid*. Transfer the mixture quantitatively to a 300 mL conical flask fitted with a ground-glass stopper. Whilst shaking continuously, add 10.0 mL of *0.05 M iodine* immediately followed by 45 mL of *0.1 M sodium hydroxide*. Allow to stand in the dark at a temperature between 15 °C and 25 °C for 15 min. Add 4 mL of a mixture of 1 volume of *sulfuric acid R* and 4 volumes of *water R*. Titrate the excess of iodine with *0.1 M sodium thiosulfate* using a microburette. Carry out a blank titration adding the 2 mL of *1 M hydrochloric acid* before introducing the test suspension. Carry out the titration of the reference suspension in the same manner.

The test is not valid unless both $n'$-$n$ and $n'_1$-$n_1$ are between 1.9 mL and 3.6 mL.

Calculate the amylolytic activity in European Pharmacopoeia Units per milligram using the following expression:

$$\frac{(n' - n)m_1}{(n'_1 - n_1)m} \times A$$

$n$ = volume of *0.1 M sodium thiosulfate* used in the titration of the test suspension, in millilitres;

$n_1$ = volume of *0.1 M sodium thiosulfate* used in the titration of the reference suspension, in millilitres;

$n'$ = volume of *0.1 M sodium thiosulfate* used in the blank titration of the test suspension, in millilitres;

$n'_1$ = volume of *0.1 M sodium thiosulfate* used in the blank titration of the reference suspension, in millilitres;

$m$ = mass of the substance to be examined, in milligrams;

$m_1$ = mass of the reference preparation, in milligrams;

$A$ = activity of *pancreas powder (amylase) BRP*, in European Pharmacopoeia Units per milligram.

STORAGE

In an airtight container.

**01/2008:0681**

# PANCURONIUM BROMIDE

## Pancuronii bromidum

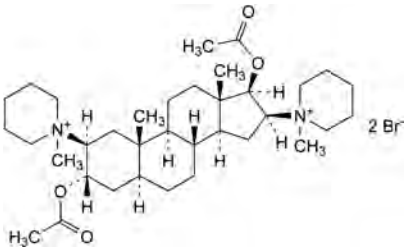


$C_{35}H_{60}Br_2N_2O_4$ $M_r$ 733

[15500-66-0]

DEFINITION

1,1′-[3α,17β-Bis(acetyloxy)-5α-androstane-2β,16β-diyl]bis(1-methylpiperidinium) dibromide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white, yellowish-white or slightly pink, crystalline powder, hygroscopic.

*Solubility*: very soluble or freely soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pancuronium bromide CRS*.

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 50 mg in *water R* and dilute to 25 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 38.0 to + 42.0 (anhydrous substance).

Dissolve 0.75 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 20.0 mL with *methylene chloride R*.

*Reference solution (b).* Dissolve 10.0 mg of *pancuronium bromide for system suitability CRS* (containing 1.0 per cent of impurity D) in 1.0 mL of *methylene chloride R*.

*Plate*: *TLC silica gel plate R* (2-10 μm).

*Mobile phase*: 400 g/L solution of *sodium iodide R*, *acetonitrile R*, *2-propanol R* (5:10:85 *V/V/V*).

*Application*: 5 μL.

*Development*: in an unlined and unsaturated tank over a path of 8 cm.

*Drying*: in a current of air at room temperature.

*Detection*: spray with a 20 g/L solution of *sodium nitrite R* and allow to dry for 5 min. Then spray with *potassium iodobismuthate solution R5*. Cover the plate with a transparent glass cover.

*System suitability*:

- the chromatogram obtained with reference solution (b) shows 2 clearly separated spots due to pancuronium bromide ($R_F$ = about 0.5) and impurity D ($R_F$ = about 0.6);
- the chromatogram obtained with reference solution (a) shows a clearly visible spot.

*Note*: impurity A if present will co-migrate with impurity D.

*Limits*:

- *impurities A, D*: any spot due to impurities A and/or D is not more intense than the spot due to impurity D in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *unspecified impurities*: any other spot is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.10 per cent).

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *acetic anhydride R*, heating if necessary. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.63 mg of $C_{35}H_{60}Br_2N_2O_4$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E.*

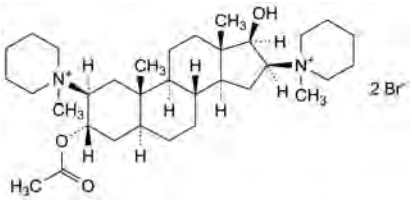

A. 1,1′-[3α-(acetyloxy)-17β-hydroxy-5α-androstane-2β,16β-diyl]bis(1-methylpiperidinium) dibromide (dacuronium bromide),

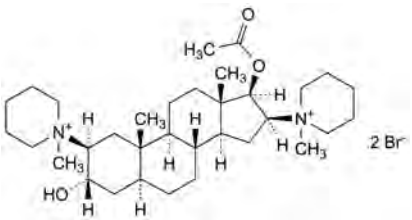

B. 1,1′-[17β-(acetyloxy)-3α-hydroxy-5α-androstane-2β,16β-diyl]bis(1-methylpiperidinium) dibromide,

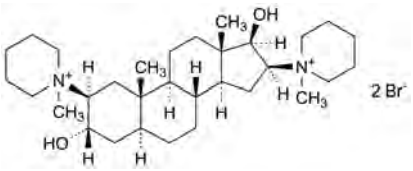

C. 1,1′-(3α,17β-dihydroxy-5α-androstane-2β,16β-diyl)bis(1-methylpiperidinium) dibromide,

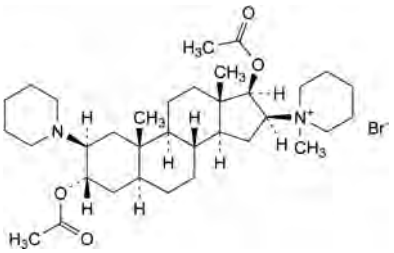

D. 1-[3α,17β-bis(acetyloxy)-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidinium bromide (vecuronium bromide),

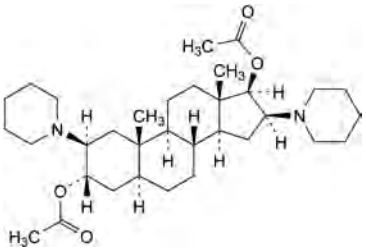

E. 2β,16β-bis(piperidin-1-yl)-5α-androstane-3α,17β-diyl diacetate.

**01/2019:2296**

# PANTOPRAZOLE SODIUM SESQUIHYDRATE

## Pantoprazolum natricum sesquihydricum

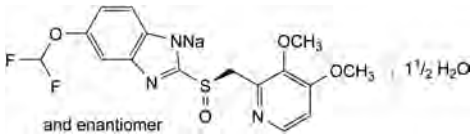


$C_{16}H_{14}F_2N_3NaO_4S,1½H_2O$ $M_r$ 432.4
[164579-32-2]

DEFINITION

Sodium 5-(difluoromethoxy)-2-[(*RS*)-[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]benzimidazol-1-ide sesquihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

PRODUCTION

It is produced by methods of manufacture designed to guarantee the proper hydrate form and it complies, if tested, with a suitable test that demonstrates its sesquihydrate nature (for example near-infrared spectroscopy (*2.2.40*) or X-ray powder diffraction (*2.9.33*)).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in hexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pantoprazole sodium sesquihydrate CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in *water R* and dilute to 20.0 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.4° to + 0.4°.

Dissolve 0.2 g in 10 mL of *water R*. Adjust to pH 11.5-12.0 with an 8 g/L solution of *sodium hydroxide R*. Dilute to 20.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 40 mg/L solution of *sodium hydroxide R* (50:50 *V/V*).

*Test solution*. Dissolve 23 mg of the substance to be examined in the solvent mixture and dilute to 50.0 ml with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2.5 mg of *pantoprazole for system suitability CRS* (containing impurities A, B, C, D and E) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 1.74 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 7.00 ± 0.05 with a 330 g/L solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 80 → 20 | 20 → 80 |
| 40 - 45 | 20 → 80 | 80 → 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 290 nm and, for impurity C, at 305 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *pantoprazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D + F and E.

*Relative retention* with reference to pantoprazole (retention time = about 11 min): impurity C = about 0.6; impurity A = about 0.9; impurities D and F = about 1.2; impurity E = about 1.3; impurity B = about 1.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities E and D + F;
- the chromatogram obtained is similar to the chromatogram supplied with *pantoprazole for system suitability CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.3;
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities D and F*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities B, C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 5.9 per cent to 6.9 per cent, determined on 0.150 g.

ASSAY

Dissolve 0.200 g in 80 mL of *anhydrous acetic acid R*, add 5 mL of *acetic anhydride R* and mix for at least 10 min. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.27 mg of $C_{16}H_{14}F_2N_3NaO_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

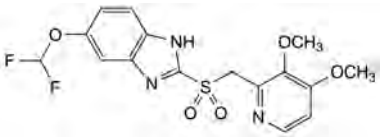

A. 5-(difluoromethoxy)-2-[[(3,4-dimethoxypyridin-2-yl)methyl]sulfonyl]-1*H*-benzimidazole,

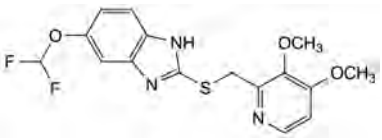

B. 5-(difluoromethoxy)-2-[[(3,4-dimethoxypyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole,

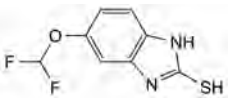

C. 5-(difluoromethoxy)-1*H*-benzimidazole-2-thiol,

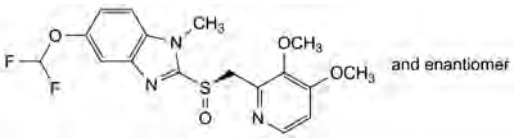


D. 5-(difluoromethoxy)-2-[(*RS*)-[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1-methyl-1*H*-benzimidazole,

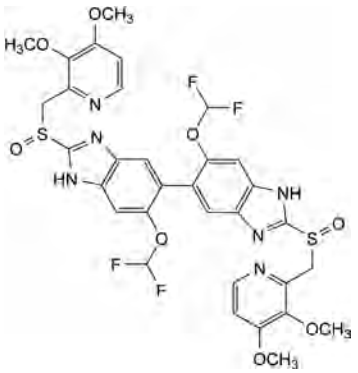

E. mixture of the stereoisomers of 6,6′-bis(difluoromethoxy)-2,2′-bis[[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1*H*,1′*H*-5,5′-bibenzimidazolyl,

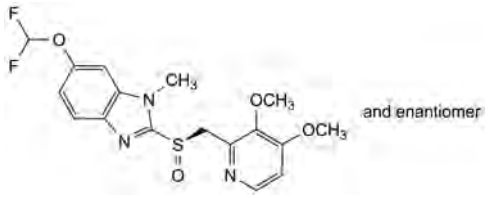


F. 6-(difluoromethoxy)-2-[(*RS*)-[(3,4-dimethoxypyridin-2-yl)methyl]sulfinyl]-1-methyl-1*H*-benzimidazole.

**01/2008:0102**
**corrected 10.0**

# PAPAVERINE HYDROCHLORIDE

## Papaverini hydrochloridum

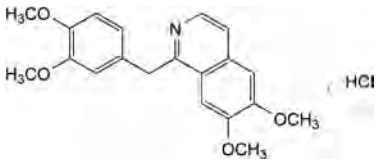

$C_{20}H_{22}ClNO_4$ $M_r$ 375.9
[61-25-6]

DEFINITION

1-(3,4-Dimethoxybenzyl)-6,7-dimethoxyisoquinoline hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, or white or almost white crystals.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *papaverine hydrochloride CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution*. Dissolve 5 mg of *papaverine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $GF_{254}$ plate R*.
*Mobile phase*: *diethylamine R*, *ethyl acetate R*, *toluene R* (10:20:70 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: at 100-105 °C for 2 h.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 10 mL of solution S (see Tests) add 5 mL of *ammonia R* dropwise and allow to stand for 10 min. The precipitate, washed and dried, melts (*2.2.14*) at 146 °C to 149 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.4 g in *carbon dioxide-free water R*, heating gently if necessary, and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.0 to 4.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, mobile phase A (20:80 *V/V*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 12 mg of *noscapine CRS* in 1.0 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 3.4 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *dilute phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;
- *mobile phase C*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 5 | 85 | 5 | 10 |
| 5 - 12 | 85 → 60 | 5 | 10 → 35 |
| 12 - 20 | 60 | 5 | 35 |
| 20 - 24 | 60 → 40 | 5 → 20 | 35 → 40 |
| 24 - 27 | 40 | 20 | 40 |
| 27 - 32 | 40 → 85 | 20 → 5 | 40 → 10 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 10 µL.

*Relative retention* with reference to papaverine (retention time = about 24 min): impurity E = about 0.7; impurity C = about 0.75; impurity B = about 0.8; impurity A = about 0.9; impurity F = about 1.1; impurity D = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity A and papaverine.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 6.2; impurity C = 2.7; impurity D = 0.5;
– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue from the test for loss on drying.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 37.59 mg of $C_{20}H_{22}ClNO_4$.

IMPURITIES

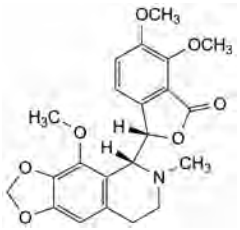

A. (3*S*)-6,7-dimethoxy-3-[(5*R*)-4-methoxy-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one (noscapine),

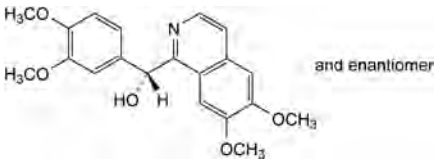


B. (*RS*)-(3,4-dimethoxyphenyl)(6,7-dimethoxyisoquinolin-1-yl)methanol (papaverinol),

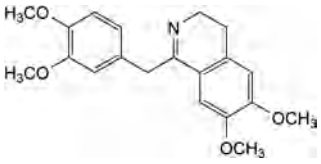

C. 1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-3,4-dihydroisoquinoline (dihydropapaverine),

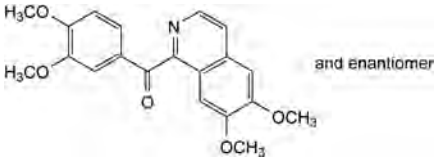


D. (3,4-dimethoxyphenyl)(6,7-dimethoxyisoquinolin-1-yl)methanone (papaveraldine),

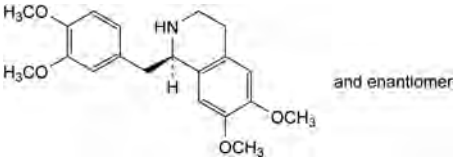


E. (1*RS*)-1-(3,4-dimethoxybenzyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (tetrahydropapaverine),

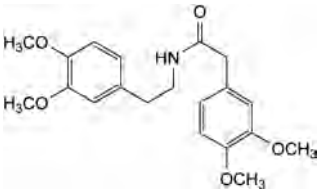

F. 2-(3,4-dimethoxyphenyl)-*N*-[2-(3,4-dimethoxyphenyl)-ethyl]acetamide.

**04/2018:0049**

# PARACETAMOL

## Paracetamolum

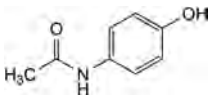

$C_8H_9NO_2$ $M_r$ 151.2
[103-90-2]

DEFINITION

*N*-(4-Hydroxyphenyl)acetamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Melting point (*2.2.14*).

*Determination A*: determine the melting point of the substance to be examined.

*Result A*: 168 °C to 172 °C.

*Determination B*: mix equal parts of the substance to be examined and *paracetamol CRS* and determine the melting point of the mixture.

*Result B*: the absolute difference between the melting point of the mixture and the value obtained in determination A is not greater than 2 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *paracetamol CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (15:85 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b). Prepare immediately before use.* Dissolve 5.0 mg of *paracetamol impurity K CRS* and 5 mg of *paracetamol CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5.0 mg of *paracetamol impurity J CRS* in the solvent mixture and dilute to 250.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 200.0 mL with the solvent mixture.

*Precolumn*:

- *size*: *l* = 0.005 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.7 µm).

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (2.7 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.7 g of *potassium dihydrogen phosphate R* and 1.8 g of *dipotassium hydrogen phosphate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 95 | 5 |
| 1 - 10 | 95 → 90 | 5 → 10 |
| 10 - 20 | 90 | 10 |
| 20 - 40 | 90 → 66 | 10 → 34 |
| 40 - 50 | 66 | 34 |

*Flow rate*: 0.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity K; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity J.

*Relative retention* with reference to paracetamol (retention time = about 4 min): impurity K = about 0.4; impurity J = about 10.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity K and paracetamol.

*Calculation of percentage contents*:

- for impurity J, use the concentration of impurity J in reference solution (c);
- for impurity K, use the concentration of impurity K in reference solution (b);
- for impurities other than J and K, use the concentration of paracetamol in reference solution (a).

*Limits*:

- *impurity K*: maximum 50 ppm;
- *impurity J*: maximum 10 ppm;
- *unspecified impurities*: for each impurity, maximum 0.05 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.03 per cent, except for impurities J and K.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *water R* and 30 mL of *dilute sulfuric acid R*. Boil under a reflux condenser for 1 h, cool and dilute to 100.0 mL with *water R*. To 20.0 mL of the solution add 40 mL of *water R*, 40 g of ice, 15 mL of *dilute hydrochloric acid R* and 0.1 mL of *ferroin R*. Titrate with *0.1 M cerium sulfate* until a greenish-yellow colour is obtained. Carry out a blank titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 7.56 mg of $C_8H_9NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, L, M, N.*

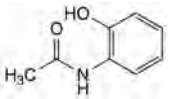

A. *N*-(2-hydroxyphenyl)acetamide,

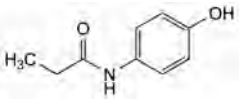

B. *N*-(4-hydroxyphenyl)propanamide,

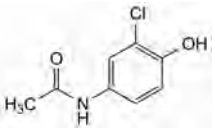

C. *N*-(3-chloro-4-hydroxyphenyl)acetamide,

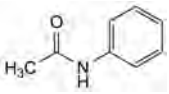

D. *N*-phenylacetamide,

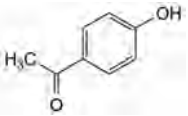

E. 1-(4-hydroxyphenyl)ethan-1-one,

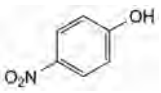

F. 4-nitrophenol,

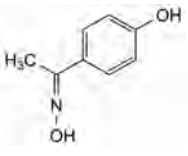

G. [1-(4-hydroxyphenyl)ethylidene]hydroxylamine,

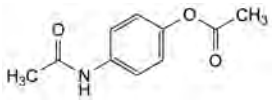

H. 4-acetamidophenyl acetate,

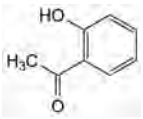

I. 1-(2-hydroxyphenyl)ethan-1-one,

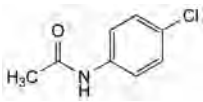

J. *N*-(4-chlorophenyl)acetamide (chloroacetanilide),

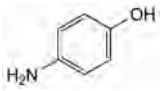

K. 4-aminophenol,

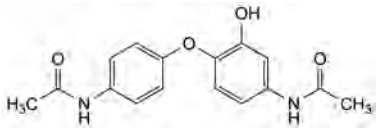

L. *N*-[4-(4-acetamido-2-hydroxyphenoxy)phenyl]acetamide,

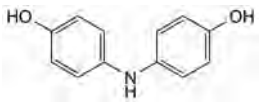

M. 4,4′-azanediyldiphenol,

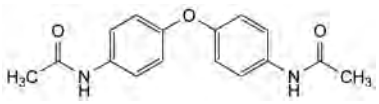

N. *N*,*N*′-[oxydi(4,1-phenylene)]diacetamide.

**01/2008:1034**

# PARAFFIN, HARD

## Paraffinum solidum

DEFINITION

A purified mixture of solid saturated hydrocarbons generally obtained from petroleum. It may contain a suitable antioxidant.

CHARACTERS

*Appearance*: colourless or white or almost white mass; the melted substance is free from fluorescence in daylight.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: place about 2 mg on a sodium chloride plate, heat in an oven at 100 °C for 10 min, spread the melted substance with another sodium chloride plate and remove one of the plates.
*Comparison*: *hard paraffin CRS.*

B. Acidity or alkalinity (see Tests).

C. Melting point (*2.2.16*): 50 °C to 61 °C.

TESTS

**Acidity or alkalinity**. To 15 g add 30 mL of boiling *water R* and shake vigorously for 1 min. Allow to cool and to separate. To 10 mL of the aqueous layer add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to red. To a further 10 mL of the aqueous layer add 0.1 mL of *methyl red solution R*. The solution is yellow. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Polycyclic aromatic hydrocarbons**. *Use reagents for ultraviolet absorption spectrophotometry*. Dissolve 0.50 g in 25 mL of *heptane R* and place in a 125 mL separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 5.0 mL of *dimethyl sulfoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers are formed. Transfer the lower layer to a 2nd separating funnel, add 2 mL of *heptane R* and shake the mixture vigorously. Allow to stand until 2 clear layers are formed. Separate the lower layer and measure its absorbance (*2.2.25*) between 265 nm and 420 nm using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 mL of *dimethyl sulfoxide R* with 25 mL of *heptane R* for 1 min. Prepare a 7.0 mg/L reference solution of *naphthalene R* in *dimethyl sulfoxide R* and measure the absorbance of this solution at the absorption maximum at 278 nm using *dimethyl sulfoxide R* as the compensation liquid. At wavelengths from 265 nm to 420 nm, the absorbance of the test solution is not greater than one-third that of the reference solution at 278 nm.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Introduce 2.0 g of the melted substance to be examined into a 50 mL ground-glass-stoppered separating funnel. Add 30 mL of boiling *distilled water R*, shake vigorously for 1 min and filter.

STORAGE

Protected from light.

**07/2018:0240**

# PARAFFIN, LIGHT LIQUID

## Paraffinum perliquidum

DEFINITION

Purified mixture of liquid saturated hydrocarbons obtained from petroleum.

CHARACTERS

*Appearance*: colourless, transparent, oily liquid, free from fluorescence in daylight.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), miscible with hydrocarbons.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of liquid paraffin.*

B. In a test tube cautiously boil 1 mL with 1 mL of *0.1 M sodium hydroxide*, with continuous shaking, for about 30 s. On cooling to room temperature, 2 phases separate. To the aqueous phase add 0.1 mL of *phenolphthalein solution R*. The solution becomes red.

C. Viscosity (see Tests).

TESTS

**Acidity or alkalinity**. To 10 mL add 20 mL of boiling *water R* and shake vigorously for 1 min. Separate the aqueous layer and filter. To 10 mL of the filtrate, add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Relative density** (*2.2.5*): 0.810 to 0.875.

**Viscosity** (*2.2.9*): 25 mPa·s to 80 mPa·s.

**Polycyclic aromatic hydrocarbons**. *Use reagents for ultraviolet spectrophotometry*.

Introduce 25.0 mL into a 125 mL separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 25 mL of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulfoxide R*. Mix and add 5.0 mL of *dimethyl sulfoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers are formed. Transfer the lower layer to a 2nd separating funnel, add 2 mL of *hexane R* and shake the mixture vigorously. Allow to stand until 2 clear layers are formed. Separate the lower layer and measure its absorbance (*2.2.25*) between 260 nm and 420 nm, using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 mL of *dimethyl sulfoxide R* with 25 mL of *hexane R* for 1 min. Prepare a 7.0 mg/L reference solution of *naphthalene R* in *trimethylpentane R* and measure the absorbance of the solution at the absorption maximum at 275 nm, using *trimethylpentane R* as the compensation liquid. At no wavelength between 260 nm and 420 nm does the absorbance of the test solution exceed one-third that of the reference solution at 275 nm.

**Readily carbonisable substances**. Use a ground-glass-stoppered tube about 125 mm long and 18 mm in internal diameter, graduated at 5 mL and 10 mL; wash with hot *water R* (temperature at least 60 °C), *acetone R*, *heptane R* and finally with *acetone R*, dry at 100-110 °C. Cool in a desiccator. Introduce 5 mL of the substance to be examined and add 5 mL of *nitrogen-free sulfuric acid R1*. Insert the stopper and shake as vigorously as possible, in the longitudinal direction of the tube, for 5 s. Loosen the stopper, immediately place the tube in a water-bath, avoiding contact of the tube with the bottom or side of the bath, and heat for 10 min. After 2 min, 4 min, 6 min and 8 min, remove the tube from the bath and shake as vigorously as possible, in the longitudinal direction of the tube for 5 s. At the end of 10 min of heating, remove the tube from the water-bath and allow to stand for 10 min. Centrifuge at 2000 *g* for 5 min. The lower layer is not more intensely coloured (*2.2.2, Method I*) than a mixture of 0.5 mL of blue primary solution, 1.5 mL of red primary solution, 3.0 mL of yellow primary solution and 2 mL of a 10 g/L solution of *hydrochloric acid R*.

**Solid paraffins**. Dry a suitable quantity of the substance to be examined by heating at 100 °C for 2 h and cool in a desiccator over *sulfuric acid R*. Place in a glass tube with an internal diameter of about 25 mm, close the tube and immerse in a bath of iced water. After 4 h, the liquid is sufficiently clear for a black line, 0.5 mm wide, to be easily seen against a white background held vertically behind the tube.

STORAGE

Protected from light.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for light liquid paraffin used as emollient in ointments, as vehicle in eye preparations or as lubricant in tablets and capsules.*

**Viscosity** (see Tests).

**07/2018:0239**

# PARAFFIN, LIQUID

## Paraffinum liquidum

DEFINITION

Purified mixture of liquid saturated hydrocarbons obtained from petroleum.

CHARACTERS

*Appearance*: colourless, transparent, oily liquid, free from fluorescence in daylight.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), miscible with hydrocarbons.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of liquid paraffin*.

B. In a test tube cautiously boil 1 mL with 1 mL of *0.1 M sodium hydroxide*, with continuous shaking, for about 30 s. On cooling to room temperature, 2 phases separate. To the aqueous phase add 0.1 mL of *phenolphthalein solution R*. The solution becomes red.

C. Viscosity (see Tests).

TESTS

**Acidity or alkalinity**. To 10 mL add 20 mL of boiling *water R* and shake vigorously for 1 min. Separate the aqueous layer and filter. To 10 mL of the filtrate, add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Relative density** (*2.2.5*): 0.827 to 0.890.

**Viscosity** (*2.2.9*): 110 mPa·s to 230 mPa·s.

**Polycyclic aromatic hydrocarbons**. *Use reagents for ultraviolet spectrophotometry*.

Introduce 25.0 mL into a 125 mL separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 25 mL of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulfoxide R*. Mix and add 5.0 mL of *dimethyl sulfoxide R*. Shake vigorously for 1 min

and allow to stand until 2 clear layers are formed. Transfer the lower layer to a 2[d] separating funnel, add 2 mL of *hexane R* and shake the mixture vigorously. Allow to stand until 2 clear layers are formed. Separate the lower layer and measure its absorbance (*2.2.25*) between 260 nm and 420 nm, using as the compensation liquid the clear lower layer obtained by vigorously shaking 5.0 mL of *dimethyl sulfoxide R* with 25 mL of *hexane R* for 1 min. Prepare a 7.0 mg/L reference solution of *naphthalene R* in *trimethylpentane R* and measure the absorbance of the solution at the absorption maximum at 275 nm, using *trimethylpentane R* as the compensation liquid. At no wavelength between 260 nm and 420 nm does the absorbance of the test solution exceed one-third that of the reference solution at 275 nm.

**Readily carbonisable substances**. Use a ground-glass-stoppered tube about 125 mm long and 18 mm in internal diameter, graduated at 5 mL and 10 mL; wash with hot *water R* (temperature at least 60 °C), *acetone R*, *heptane R* and finally with *acetone R*, dry at 100-110 °C. Cool in a desiccator. Introduce 5 mL of the substance to be examined and add 5 mL of *nitrogen-free sulfuric acid R1*. Insert the stopper and shake as vigorously as possible, in the longitudinal direction of the tube, for 5 s. Loosen the stopper, immediately place the tube in a water-bath, avoiding contact of the tube with the bottom or side of the bath, and heat for 10 min. After 2 min, 4 min, 6 min and 8 min, remove the tube from the bath and shake as vigorously as possible, in the longitudinal direction of the tube for 5 s. At the end of 10 min of heating, remove the tube from the water-bath and allow to stand for 10 min. Centrifuge at 2000 *g* for 5 min. The lower layer is not more intensely coloured (*2.2.2, Method I*) than a mixture of 0.5 mL of blue primary solution, 1.5 mL of red primary solution, 3.0 mL of yellow primary solution and 2 mL of a 10 g/L solution of *hydrochloric acid R*.

**Solid paraffins**. Dry a suitable quantity of the substance to be examined by heating at 100 °C for 2 h and cool in a desiccator over *sulfuric acid R*. Place in a glass tube with an internal diameter of about 25 mm, close the tube and immerse in a bath of iced water. After 4 h, the liquid is sufficiently clear for a black line, 0.5 mm wide, to be easily seen against a white background held vertically behind the tube.

STORAGE

Protected from light.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for liquid paraffin used as emollient in ointments or as lubricant in tablets and capsules.*

**Viscosity** (see Tests).

**07/2009:1799**

# PARAFFIN, WHITE SOFT

## Vaselinum album

DEFINITION

Purified and wholly or nearly decolorised mixture of semi-solid hydrocarbons, obtained from petroleum. It may contain a suitable antioxidant. White soft paraffin described in this monograph is not suitable for oral use.

CHARACTERS

*Appearance*: white or almost white, translucent, soft unctuous mass, slightly fluorescent in daylight when melted.

*Solubility*: practically insoluble in water, slightly soluble in methylene chloride, practically insoluble in ethanol (96 per cent) and in glycerol.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. The drop point is between 35 °C and 70 °C and does not differ by more than 5 °C from the value stated on the label, according to method (*2.2.17*) with the following modification to fill the cup: heat the substance to be examined at a temperature not exceeding 80 °C, with stirring to ensure uniformity. Warm the metal cup at a temperature not exceeding 80 °C in an oven, remove it from the oven, place on a clean plate or ceramic tile and pour a sufficient quantity of the melted sample into the cup to fill it completely. Allow the filled cup to cool for 30 min on the plate or the ceramic tile and place it in a water bath at 24-26 °C for 30-40 min. Level the surface of the sample with a single stroke of a knife or razor blade, avoiding compression of the sample.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: place about 2 mg on a *sodium chloride R* plate, spread the substance with another *sodium chloride R* plate and remove 1 of the plates.

*Comparison*: repeat the operations using *white soft paraffin CRS*.

C. Melt 2 g and when a homogeneous phase is obtained, add 2 mL of *water R* and 0.2 mL of *0.05 M iodine*. Shake. Allow to cool. The solid upper layer is violet-pink or brown.

D. Appearance (see Tests).

TESTS

**Appearance**. The substance is white. Melt 12 g on a water-bath. The melted mass is not more intensely coloured than a mixture of 1 volume of yellow primary solution and 9 volumes of a 10 g/L solution of *hydrochloric acid R* (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 g add 20 mL of boiling *water R* and shake vigorously for 1 min. Allow to cool and decant. To 10 mL of the aqueous layer add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to red.

**Consistency** (*2.9.9*): 60 to 300.

**Polycyclic aromatic hydrocarbons**. *Use reagents for ultraviolet spectrophotometry.* Dissolve 1.0 g in 50 mL of *hexane R* which has been previously shaken twice with 10 mL of *dimethyl sulfoxide R*. Transfer the solution to a 125 mL separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 20 mL of *dimethyl sulfoxide R*. Shake vigorously for 1 min and allow to stand until 2 clear layers

are formed. Transfer the lower layer to a second separating funnel. Repeat the extraction with a further 20 mL of *dimethyl sulfoxide R*. Shake vigorously the combined lower layers with 20 mL of *hexane R* for 1 min. Allow to stand until 2 clear layers are formed. Separate the lower layer and dilute to 50.0 mL with *dimethyl sulfoxide R*. Measure the absorbance (*2.2.25*) over the range 260 nm to 420 nm using a path length of 4 cm and as compensation liquid the clear lower layer obtained by vigorously shaking 10 mL of *dimethyl sulfoxide R* with 25 mL of *hexane R* for 1 min. Prepare a reference solution in *dimethyl sulfoxide R* containing 6.0 mg of *naphthalene R* per litre and measure the absorbance of the solution at the maximum at 278 nm using a path length of 4 cm and *dimethyl sulfoxide R* as compensation liquid. At no wavelength in the range 260 nm to 420 nm does the absorbance of the test solution exceed that of the reference solution at 278 nm.

**Sulfated ash** (*2.4.14*): maximum 0.05 per cent, determined on 2.0 g.

STORAGE

Protected from light.

LABELLING

The label states the nominal drop point.



**07/2008:1554**
**corrected 6.8**

# PARAFFIN, YELLOW SOFT

## Vaselinum flavum

DEFINITION

Purified mixture of semi-solid hydrocarbons, obtained from petroleum. It may contain a suitable antioxidant.

CHARACTERS

*Appearance*: yellow, translucent, unctuous mass, slightly fluorescent in daylight when melted.

*Solubility*: practically insoluble in water, slightly soluble in methylene chloride, practically insoluble in ethanol (96 per cent) and in glycerol.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. The drop point (*2.2.17*) is 40 °C to 60 °C and does not differ by more than 5 °C from the value stated on the label, with the following modification to fill the cup: heat the substance to be examined at 118-122 °C, with stirring to ensure uniformity, then cool to 100-107 °C. Warm the metal cup at 103-107 °C in an oven, remove it from the oven, place on a clean plate or ceramic tile and pour a sufficient quantity of the melted sample into the cup to fill it completely. Allow the filled cup to cool for 30 min on the ceramic tile and place it in a water-bath at 24-26 °C for a further 30-40 min. Level the surface of the sample with a single stroke of a knife or razor blade, avoiding compression of the sample.

B. Examine by infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: place about 2 mg on a *sodium chloride R* plate, spread the substance with another *sodium chloride R* plate and remove 1 of the plates.

*Comparison*: repeat the operations using *yellow soft paraffin CRS*.

C. Melt 2 g and when a homogeneous phase is obtained, add 2 mL of *water R* and 0.2 mL of *0.05 M iodine*. Shake. Allow to cool. The solid upper layer is violet-pink or brown.

D. Appearance (see Tests).

TESTS

**Appearance**. The substance is yellow. Melt 12 g on a water-bath. The melted mass is not more intensely coloured than a mixture of 7.6 volumes of yellow primary solution and 2.4 volumes of red primary solution (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 g add 20 mL of boiling *water R* and shake vigorously for 1 min. Allow to cool and decant. To 10 mL of the aqueous layer add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to red.

**Consistency** (*2.9.9*): 100 to 300.

**Polycyclic aromatic hydrocarbons**. *Use reagents for ultraviolet absorption spectrophotometry.* Dissolve 1.0 g in 50 mL of *hexane R* which has been previously shaken twice with one-fifth its volume of *dimethyl sulfoxide R*. Transfer the solution to a 125 mL separating funnel with unlubricated ground-glass parts (stopper, stopcock). Add 20 mL of *dimethyl sulfoxide R*. Shake vigorously for 1 min and allow to stand until two clear layers are formed. Transfer the lower layer to a 2nd separating funnel. Repeat the extraction with a further 20 mL of *dimethyl sulfoxide R*. Shake vigorously the combined lower layers with 20 mL of *hexane R* for 1 min. Allow to stand until 2 clear layers are formed. Separate the lower layer and dilute to 50.0 mL with *dimethyl sulfoxide R*. Measure the absorbance (*2.2.25*) between 260 nm and 420 nm using a path length of 4 cm and using as the compensation liquid the clear lower layer obtained by vigorously shaking 10 mL of *dimethyl sulfoxide R* with 25 mL of *hexane R* for 1 min. Prepare a 9.0 mg/L reference solution of *naphthalene R* in *dimethyl sulfoxide R* and measure the absorbance of this solution at the maximum at 278 nm using a path length of 4 cm and using *dimethyl sulfoxide R* as the compensation liquid. At no wavelength in the range of 260 nm to 420 nm does the absorbance of the test solution exceed that of the reference solution at 278 nm.

**Sulfated ash** (*2.4.14*): not more than 0.05 per cent, determined on 2.0 g.

STORAGE

Store protected from light.

LABELLING

The label states the nominal drop point.

**01/2008:0351**

# PARALDEHYDE

## Paraldehydum

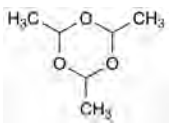

$C_6H_{12}O_3$ $M_r$ 132.2
[123-63-7]

DEFINITION

2,4,6-Trimethyl-1,3,5-trioxane (cyclic trimer of acetaldehyde). It may contain a suitable quantity of an antioxidant.

CHARACTERS

*Appearance*: colourless or slightly yellow, transparent liquid. It solidifies on cooling to form a crystalline mass.

*Solubility*: soluble in water, but less soluble in boiling water, miscible with ethanol (96 per cent) and with essential oils.

IDENTIFICATION

A. Solution S (see Tests) is clear (*2.2.1*) but becomes turbid on warming.

B. To 5 mL add 0.1 mL of *dilute sulfuric acid R* and heat. Acetaldehyde, recognisable by its odour, is evolved.

C. To 5 mL of solution S in a test-tube add 5 mL of *ammoniacal silver nitrate solution R* and heat in a water-bath. Silver is deposited as a mirror on the wall of the tube.

TESTS

**Solution S**. Dissolve 20.0 mL in *carbon dioxide-free water R* and dilute to 200.0 mL with the same solvent.

**Acidity**. To 50.0 mL of solution S add 0.05 mL of *phenolphthalein solution R*. Not more than 1.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Refractive index** (*2.2.6*): 1.403 to 1.406.

**Relative density** (*2.2.5*): 0.991 to 0.996.

**Distillation range** (*2.2.11*): a maximum of 10 per cent distils below 123 °C and a minimum of 95 per cent distils below 126 °C.

**Freezing point** (*2.2.18*): 10 °C to 13 °C.

**Acetaldehyde**. To 5.0 mL add a mixture of 0.2 mL of *methyl orange solution R*, 5 mL of *ethanol (60 per cent V/V) R* and 5 mL of *alcoholic hydroxylamine solution R* and shake. Not more than 0.8 mL of *0.5 M sodium hydroxide* is required to change the colour of the indicator to pure yellow.

**Peroxides**. Place 50.0 mL of solution S in a ground-glass-stoppered flask, add 5 mL of *dilute sulfuric acid R* and 10 mL of *potassium iodide solution R*, close the flask and allow to stand protected from light for 15 min. Titrate with *0.1 M sodium thiosulfate* using 1 mL of *starch solution R* as indicator. Allow to stand for 5 min and, if necessary complete the titration. Not more than 2.0 mL of *0.1 M sodium thiosulfate* is required.

**Non-volatile residue**: maximum 0.6 g/L.

Heat 5.0 mL in a tared evaporating dish on a water-bath and dry at 105 °C for 1 h. The residue weighs a maximum of 3 mg.

STORAGE

In a small, well-filled, airtight container, protected from light. If the substance has solidified the whole contents of the container must be liquefied before use.

**07/2019:1252**

# PARNAPARIN SODIUM

## Parnaparinum natricum

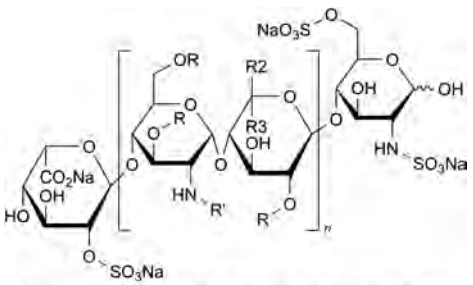


n = 1 to 21 , R = H or $SO_3Na$ , R' = $SO_3Na$ or $CO\text{-}CH_3$
R2 = H and R3 = $CO_2Na$ or R2 = $CO_2Na$ and R3 = H

DEFINITION

Sodium salt of a low-molecular-mass heparin that is obtained by radical-catalysed depolymerisation, with hydrogen peroxide and with a cupric salt, of heparin from porcine intestinal mucosa. The majority of the components have a 2-*O*-sulfo-α-L-idopyranuronic acid structure at the non-reducing end and a 2-*N*,6-*O*-disulfo-D-glucosamine structure at the reducing end of their chain.

*Parnaparin sodium complies with the monograph Low-molecular-mass heparins (0828), with the modifications and additional requirements below.*

The mass-average relative molecular mass ranges between 4000 and 6000 with a characteristic value of about 5000.

The degree of sulfatation is 2.0 to 2.6 per disaccharide unit.

The potency is not less than 75 IU and not more than 110 IU of anti-factor Xa activity per milligram calculated with reference to the dried substance. The ratio of anti-factor Xa activity to anti-factor IIa activity is between 1.5 and 3.0.

IDENTIFICATION

Carry out identification test A as described in the monograph *Low-molecular-mass heparins (0828)* using *parnaparin sodium CRS*.

Carry out identification test C as described in the monograph *Low-molecular-mass heparins (0828)*. In order to verify the suitability of the system in the lower molecular mass ranges (for example $M_r$ 2000), a suitable reference preparation is used. The following requirements apply.

The mass-average relative molecular mass ranges between 4000 and 6000. The mass percentage of chains lower than 3000 is not more than 30 per cent. The mass percentage of chains between 3000 and 8000 ranges between 50 per cent and 60 per cent.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.5 g in 10 mL of *water R*.

Monographs

**01/2017:2283**

# PAROXETINE HYDROCHLORIDE

## Paroxetini hydrochloridum

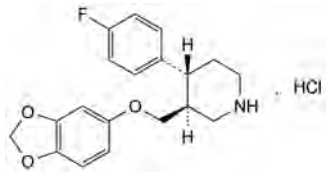

$C_{19}H_{21}ClFNO_3$ $M_r$ 365.8
[78246-49-8]

DEFINITION

(3*S*,4*R*)-3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

**Impurity G**: maximum 1 ppm, determined by a suitable validated method.

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, sparingly soluble in anhydrous ethanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anhydrous paroxetine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, mix 1 part of the substance to be examined and 1 part of the reference substance separately with 30 parts of anhydrous *acetone R* and heat to boiling to dissolve. Recrystallise and record new spectra using the residues.

B. Water (see Tests).

C. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *paroxetine impurity D CRS* in 2 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the test solution.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: dissolve 8.7 g of *dipotassium hydrogen phosphate R* in 1000 mL of *water for chromatography R* and adjust to pH 6.5 with *phosphoric acid R*; mix 930 mL of this solution and 70 mL of *acetonitrile R*.

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of paroxetine which is about 12 min.

*System suitability*:

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to paroxetine in the chromatogram obtained with reference solution (b);
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c);
– *symmetry factor*: the requirements stated in chapter *2.2.46* are not applicable.

*Limit*:

– *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *tetrahydrofuran R*, *water R* (10:90 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *anhydrous paroxetine hydrochloride impurity H CRS* in 25 mL of *tetrahydrofuran R* and dilute to 50.0 mL with *water R*.

*Reference solution (c).* Dissolve 5 mg of *anhydrous paroxetine hydrochloride impurity C CRS* in 25 mL of *tetrahydrofuran R* and dilute to 50.0 mL with *water R*.

*Reference solution (d).* To 5.0 mL of reference solution (a) add 1.0 mL of reference solution (b) and dilute to 100.0 mL with the solvent mixture.

*Reference solution (e).* To 5.0 mL of reference solution (a) add 5.0 mL of reference solution (b) and 5.0 mL of reference solution (c). Dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (f).* Dissolve 2.5 mg of *paroxetine impurity E CRS* in the solvent mixture, add 2.5 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Reference solution (g).* Dissolve 5 mg of *paroxetine impurity A CRS* in the solvent mixture and dilute to 50 mL with the solvent mixture. Use this solution to identify the peak due to impurity A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *tetrahydrofuran R*, *water R* (5:100:900 *V/V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *tetrahydrofuran R*, *acetonitrile R* (5:100:900 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 80 | 20 |
| 30 - 50 | 80 → 20 | 20 → 80 |
| 50 - 55 | 20 | 80 |
| 55 - 60 | 20 → 80 | 80 → 20 |
| 60 - 65 | 80 | 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 20 µL of the test solution and reference solutions (d), (e), (f) and (g).

*Relative retention* with reference to paroxetine (retention time = about 28 min): impurity A = about 0.8; impurity E = about 0.9; impurity C = about 1.5.

*Relative retention* with reference to impurity C: impurity F = about 0.97; impurity J = about 1.02.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to impurity E and paroxetine in the chromatogram obtained with reference solution (f);
- *signal-to-noise ratio*: minimum 3 for the peak due to paroxetine in the chromatogram obtained with reference solution (e).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.6; impurity F = 1.7; impurity J = 1.3;
- *impurity A*: not more than 0.6 times the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurities C, F, J*: for each impurity, not more than 0.2 times the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.10 per cent);
- *total*: not more than the area of the peak due to paroxetine in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *disregard limit*: the area of the peak due to paroxetine in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Impurities H and I**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 263 nm.

*Injection*: test solution and reference solutions (d) and (e).

*Relative retention* with reference to paroxetine (retention time = about 28 min): impurity I = about 0.2; impurity H = about 0.4.

*System suitability*: reference solution (e):

- *signal-to-noise ratio*: minimum 3 for the peak due to impurity H.

*Limits*:

- *impurities H, I*: for each impurity, not more than the area of the peak due to impurity H in the chromatogram obtained with reference solution (d) (0.1 per cent).

**Acetone** (*2.4.24, System B*): maximum 3.5 per cent.

**2-Propanol** (*2.4.24, System B*): maximum 4.3 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 51.2 mg of *paroxetine hydrochloride hemihydrate CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *paroxetine hydrochloride hemihydrate CRS* and 5 mg of *paroxetine impurity A CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *trimethylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.85 g of *ammonium acetate R* in *water R*, adjust to pH 5.5 with *anhydrous acetic acid R* and dilute to 600 mL with *water R*; add 400 mL of *acetonitrile R*; slowly add, with stirring, 10 mL of *triethylamine R* and adjust to pH 5.5 with *anhydrous acetic acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of paroxetine.

*System suitability*: reference solution (b):

- *resolution*: minimum 2 between the peaks due to paroxetine and impurity A.

Calculate the percentage content of $C_{19}H_{21}ClFNO_3$ using the chromatogram obtained with reference solution (a) and the declared content of *paroxetine hydrochloride hemihydrate CRS*.

STORAGE

In an airtight container, at a temperature not exceeding 25 °C.

IMPURITIES

*Specified impurities: A, C, D, F, G, H, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E.*

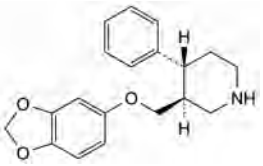

A. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-phenylpiperidine (desfluoroparoxetine),

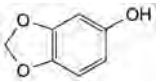

B. 1,3-benzodioxol-5-ol (sesamol),

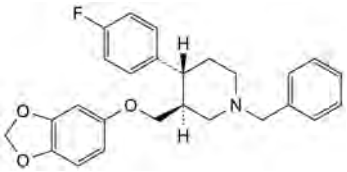

C. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-1-benzyl-4-(4-fluorophenyl)piperidine (*N*-benzylparoxetine),

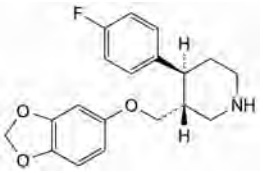

D. (3*R*,4*S*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine ((+)-*trans*-paroxetine),

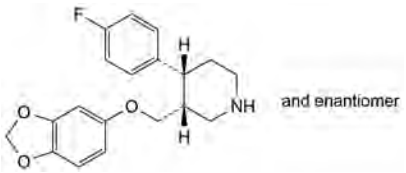


E. (3*RS*,4*RS*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine (*cis*-paroxetine),

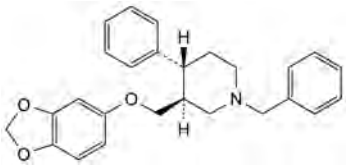


F. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-1-benzyl-4-phenylpiperidine (*N*-benzyldesfluoroparoxetine),

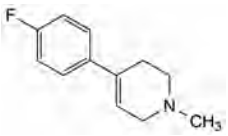


G. 4-(4-fluorophenyl)-1-methyl-1,2,3,6-tetrahydropyridine,

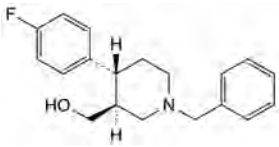


H. [(3*S*,4*R*)-1-benzyl-4-(4-fluorophenyl)piperidin-3-yl]methanol,

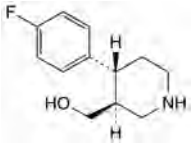


I. [(3*S*,4*R*)-4-(4-fluorophenyl)piperidin-3-yl]methanol,

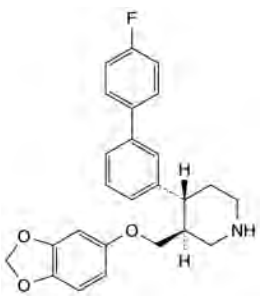


J. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4′-fluorobiphenyl-3-yl)piperidine.

**01/2017:2018**

# PAROXETINE HYDROCHLORIDE HEMIHYDRATE

## Paroxetini hydrochloridum hemihydricum

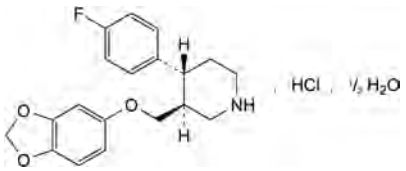


$C_{19}H_{21}ClFNO_3,½H_2O$ $M_r$ 374.8
[110429-35-1]

DEFINITION

(3*S*,4*R*)-3-[(1,3-Benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine hydrochloride hemihydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

**Impurity G**: maximum 1 ppm, determined by a suitable, validated method.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, sparingly soluble in ethanol (96 per cent) and in methylene chloride.

It shows pseudopolymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *paroxetine hydrochloride hemihydrate CRS*.

If the spectra obtained show differences, dissolve 1 part of the substance to be examined and 1 part of the reference substance separately in 10 parts of a mixture of 1 volume of *water R* and 9 volumes of *2-propanol R* and heat to 70 °C to dissolve. Recrystallise and record new spectra using the residues.

B. Examine the chromatograms obtained in the test for impurity D.

*Injection*: test solution and reference solution (c).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Water (see Tests).

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.1000 g of the substance to be examined in 20 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *paroxetine impurity D CRS* and 5 mg of *paroxetine hydrochloride hemihydrate CRS* in 2 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10 mg of *paroxetine hydrochloride hemihydrate CRS* in 2 mL of *methanol R* and dilute to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm).

*Mobile phase*: mix 2 volumes of *methanol R* and 8 volumes of a 5.8 g/L solution of *sodium chloride R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of paroxetine.

*Retention time*: paroxetine = about 30 min.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.2 between the peaks due to impurity D and paroxetine.

*Limit*:
- *impurity D*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Related subtances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *tetrahydrofuran R*, *water R* (1:9 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the same solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 200.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2 mg of *paroxetine for system suitability CRS* (containing impurity C) in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 1 mL of this solution to 10 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *paroxetine impurity A CRS* in the solvent mixture and dilute to 20 mL with the same solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *trifluoroacetic acid R*, *tetrahydrofuran R*, *water R* (5:100:900 *V/V/V*);
- *mobile phase B*: *trifluoroacetic acid R*, *tetrahydrofuran R*, *acetonitrile R* (5:100:900 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 80 | 20 |
| 30 - 50 | 80 → 20 | 20 → 80 |
| 50 - 60 | 20 | 80 |
| 60 - 65 | 20 → 80 | 80 → 20 |
| 65 - 70 | 80 | 20 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 20 µL.

*Relative retention* with reference to paroxetine: impurity A = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.5 between the peaks due to impurity C and paroxetine.

*Limits*:
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 2.2 per cent to 2.7 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50.0 mg of *paroxetine hydrochloride hemihydrate CRS* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5.0 mg of *paroxetine hydrochloride hemihydrate CRS* and 5 mg of *paroxetine impurity A CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *trimethylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 3.85 g of *ammonium acetate R* in *water R*, adjust to pH 5.5 with *anhydrous acetic acid R* and dilute to 600 mL with the same solvent; add 400 mL of *acetonitrile R*; slowly add, with stirring, 10 mL of *triethylamine R* and readjust to pH 5.5 with *anhydrous acetic acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of paroxetine.

*System suitability*: reference solution (b):
- *resolution*: minimum 2 between the peaks due to paroxetine and impurity A.

Calculate the percentage content of paroxetine hydrochloride using the chromatogram obtained with reference solution (a).

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, F.*

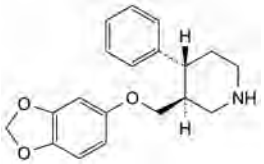


A. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-phenylpiperidine (desfluoroparoxetine),

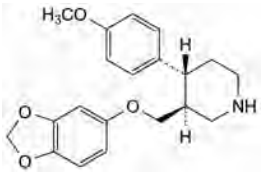

B. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-methoxyphenyl)piperidine,

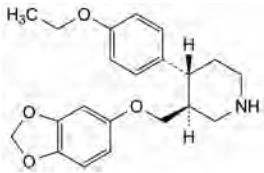

C. (3*S*,4*R*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-ethoxyphenyl)piperidine,

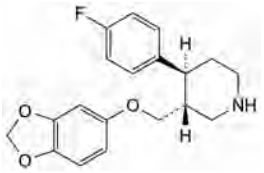

D. (3*R*,4*S*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine ((+)-*trans*-paroxetine),

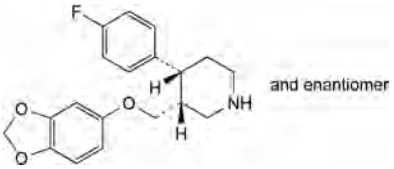


E. (3*RS*,4*RS*)-3-[(1,3-benzodioxol-5-yloxy)methyl]-4-(4-fluorophenyl)piperidine (*cis*-paroxetine),

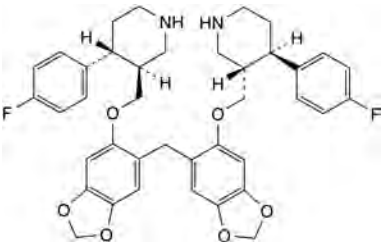

F. 3,3′-[methylenebis(1,3-benzodioxole-6,5-diyloxymethylene)]bis[3*S*,4*R*)4-(4-fluorophenyl)piperidine],

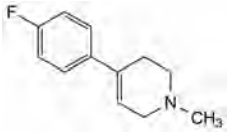

G. 4-(4-fluorophenyl)-1-methyl-1,2,3,6-tetrahydropyridine.

**04/2014:2403**

# PEA STARCH

## Pisi amylum

DEFINITION

Pea starch is obtained from the seeds of *Pisum sativum* L.

CHARACTERS

*Appearance*: white or almost white, very fine powder.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

IDENTIFICATION

A. Microscopic examination (*2.8.23*) using a 50 per cent *V/V* solution of *glycerol R*. It presents a majority of large elliptical granules, 25-45 µm in size, sometimes irregular, or reniform. It also presents a minority of small rounded granules, 5-8 µm in size (Figure 2403.-1). Granules can present cracks or irregularities. Sometimes, granules show barely visible concentric striations. Exceptionally, granules show a slit along the main axis. Between orthogonally oriented polarising plates or prisms, the granules show a distinct black cross.

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B, add 0.05 mL of *iodine solution R1*. A dark blue colour is produced, which disappears on heating.

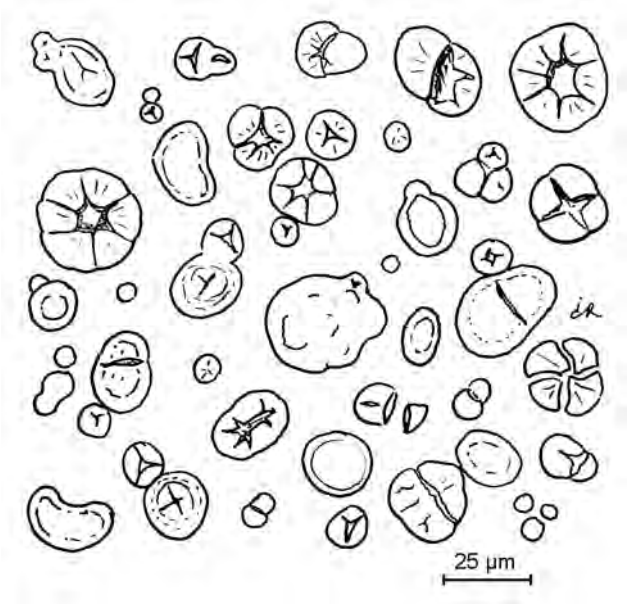


Figure 2403.-1. – *Illustration for identification test A of pea starch*

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

Shake 5.0 g with 25.0 mL of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min and shake again.

**Foreign matter**. Examined under a microscope using a 50 per cent *V/V* solution of *glycerol R*, not more than traces of matter other than starch granules are present. No starch granules of any other origin are present.

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 50 ppm.

Shake 1.0 g with 50 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test for iron.

**Loss on drying** (*2.2.32*): maximum 16.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

**01/2017:1460**

# PEFLOXACIN MESILATE DIHYDRATE

## Pefloxacini mesilas dihydricus

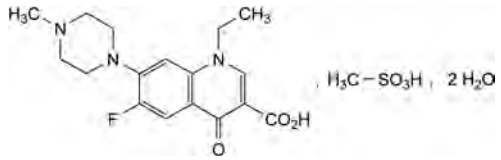

$C_{18}H_{24}FN_3O_6S,2H_2O$ $M_r$ 465.5
[149676-40-4]

DEFINITION

1-Ethyl-6-fluoro-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid methanesulfonate dihydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in pefloxacin mesilate dihydrate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: fine, white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.1 g in 10 mL of *water R*. Add 5 mL of *1 M sodium hydroxide*. Adjust to pH 7.4 ± 0.1 with *phosphoric acid R* and shake with 2 quantities, each of 30 mL, of *methylene chloride R*. Combine the organic layers and dry over *anhydrous sodium sulfate R*. Evaporate to dryness. Examine the residue as a disc of *potassium bromide R*.

*Comparison*: repeat the operations using 0.1 g of *pefloxacin mesilate dihydrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 40 mg in *water R* and dilute to 1 mL with the same solvent.

*Reference solution*. Dissolve 60 mg of *methanesulfonic acid R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *ammonia R*, *butanol R*, *acetone R* (5:10:20:65 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.4 g/L solution of *bromocresol purple R* in *ethanol (50 per cent V/V) R*, adjusted to pH 10 using *1 M sodium hydroxide*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Examined within 1 h after its preparation, solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 3 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 4.5.

Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5.0 mg of *pefloxacin impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. In 2.0 mL of this solution, dissolve the contents of a vial of *pefloxacin impurity C CRS*.

*Reference solution (b)*. Dissolve 10.0 mg of *norfloxacin impurity A CRS* (impurity F) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 6 mm;
- *stationary phase*: *octadecylsilyl vinyl polymer for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *acetonitrile R*, 70 volumes of a solution containing 2.70 g/L of *cetyltrimethylammonium bromide R* and 6.18 g/L of *boric acid R* (exactly adjusted to pH 8.30 with *1 M sodium hydroxide*), and 0.2 volumes of *thiodiethylene glycol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 258 nm and at 273 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of pefloxacin (about 60 min).

*Relative retentions and correction factors*:

| | Approximate relative retention | Correction factor |
|---|---|---|
| Impurity E | 0.2 | – |
| Impurity D | 0.3 | – |
| Impurity A | 0.5 | – |
| Impurity G | 0.8 | 1.4 |
| Pefloxacin | 1 | – |
| Impurity C | 1.7 | 2.4 |
| Impurity B | 1.8 | – |
| Impurity H | 2.4 | 1.8 |
| Impurity F | 3.5 | – |

*System suitability*: reference solution (a) at 273 nm:

- *resolution*: minimum 1.5 between the peaks due to impurities C and B.

From the chromatogram obtained at 258 nm with the test solution, calculate the percentage content of impurities C, F, G and H using the area of the principal peak in the chromatogram obtained at 258 nm with reference solution (b) (external standardisation) taking into account the correction factors indicated in the table.

From the chromatogram obtained at 273 nm with the test solution, calculate the percentage content of impurities A, B, D and E and of any other impurity from the areas of the peaks in the chromatogram obtained with the test solution by the normalisation procedure.

*Limits*:

- *impurities A, B, D, E and any other impurity at 273 nm and impurities C, F, G, H at 258 nm*: for each impurity, maximum 0.5 per cent and not more than 3 impurities have a content between 0.2 per cent and 0.5 per cent;
- *total*: maximum 1.0 per cent;
- *disregard limit at 273 nm*: 0.0005 times the area of the principal peak in the chromatogram obtained with the test solution (0.05 per cent).

**Water** (*2.5.12*): 7.0 per cent to 8.5 per cent, determined on 50.0 mg using a mixture of 10 volumes of *methanol R* and 50 volumes of *methylene chloride R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 15.0 mL of *anhydrous acetic acid R* and add 75.0 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 21.48 mg of $C_{18}H_{24}FN_3O_6S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H.*

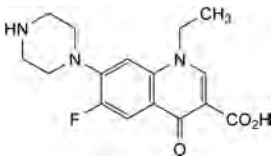

A. 1-ethyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (demethylated pefloxacin or norfloxacin),

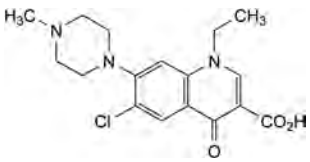

B. 6-chloro-1-ethyl-7-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (chlorinated homologue of pefloxacin),

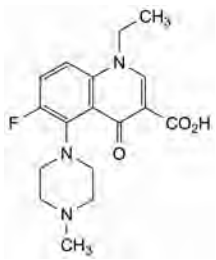

C. 1-ethyl-6-fluoro-5-(4-methylpiperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (isopefloxacin),

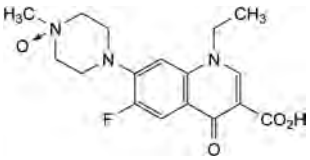

D. 4-(3-carboxy-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinolin-7-yl)-1-methylpiperazine 1-oxide (*N*-oxide of pefloxacin),

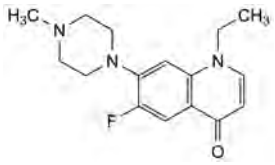

E. 1-ethyl-6-fluoro-7-(4-methylpiperazin-1-yl)quinoline-4(1*H*)-one (decarboxylated pefloxacin),

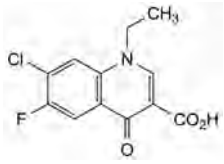

F. 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (*N*-ethyl acid) (norfloxacin impurity A),

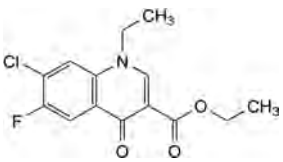

G. ethyl 7-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (*N*-ethyl ester),

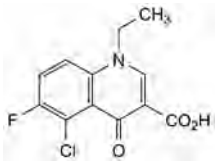

H. 5-chloro-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (iso-*N*-ethyl acid).

**01/2017:2637**
**corrected 10.0**

# PEMETREXED DISODIUM HEPTAHYDRATE

## Pemetrexedum dinatricum heptahydricum

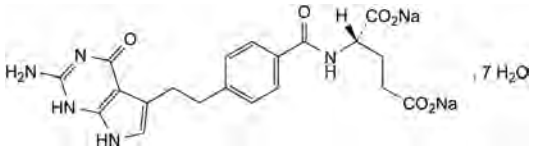

$C_{20}H_{19}N_5Na_2O_6,7H_2O$ $M_r$ 597.5
[357166-29-1]

DEFINITION

Disodium (2*S*)-2-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino]-pentanedioate heptahydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

Carry out either tests A, C, D, E or tests B, C, D, E.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pemetrexed disodium heptahydrate CRS*.

B. Nuclear magnetic resonance spectrometry (*2.2.33*).
*Preparation*: 25-50 mg/mL solution in *deuterium oxide R*.
*Comparison*: solution of equal concentration of *pemetrexed disodium heptahydrate CRS* in *deuterium oxide R*.
*Results*: the $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *pemetrexed disodium heptahydrate CRS*; disregard the peak located at approximately 5.0 ppm for the comparison.

C. It gives reaction (a) of sodium (*2.3.1*).

D. Enantiomeric purity (see Tests).

E. Water (see Tests).

TESTS

**Solution S**. Dissolve 0.56 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ or $Y_4$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 8.4 for solution S.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C for not more than 24 h.*

*Solution A*. Dissolve 8 g of *β-cyclodextrin R* in 900 mL of *water for chromatography R*. Add 15 mL of *triethylamine R* then 6 mL of *phosphoric acid R* and adjust to pH 6.0 with *phosphoric acid R*. Dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 12 mg of the substance to be examined in *water for chromatography R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 6 mg of *pemetrexed for system suitability CRS* (containing impurity E) in *water for chromatography R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water for chromatography R*. Dilute 3.0 mL of this solution to 10.0 mL with *water for chromatography R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 12 nm;
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, solution A (5:95 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 50 µL.

*Run time*: 1.5 times the retention time of pemetrexed.

*Relative retention* with reference to pemetrexed (retention time = about 30 min): impurity E = about 0.94.

*System suitability*:
- *symmetry factor*: maximum 2.0 for the principal peak in the chromatogram obtained with reference solution (b);
- *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to pemetrexed in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:
- for impurity E, use the concentration of pemetrexed disodium heptahydrate in reference solution (b).

*Limit*:
- *impurity E*: maximum 0.3 per cent.

*Column rinse: the following program is given for information only.*

Use a gradient column rinse before column storage or after 30 sample injections to avoid build-up on the column. If a drifting baseline is observed, allow additional time for equilibration with the mobile phase. If a blank chromatogram exhibits broad humps, perform a gradient column rinse.

*Rinsing solution A*: *water for chromatography R*.

*Rinsing solution B*: *acetonitrile R1*.

| Time (min) | Mobile phase (per cent *V/V*) | Rinsing solution A (per cent *V/V*) | Rinsing solution B (per cent *V/V*) |
|---|---|---|---|
| 0 - 4 | 100 → 0 | 0 → 50 | 0 → 50 |
| 4 - 9 | 0 | 50 | 50 |
| 9 - 14 | 0 | 50 → 10 | 50 → 90 |
| 14 - 54 | 0 | 10 | 90 |
| 54 - 69 | 0 | 10 → 95 | 90 → 5 |
| 69 - 100 | 0 | 95 | 5 |

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C for not more than 24 h.*

*Solution A*. 1.45 g/L solution of *ammonium formate R* in *water for chromatography R*, adjusted to pH 3.5 with *anhydrous formic acid R*.

*Test solution.* Dissolve 20 mg of the substance to be examined in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water for chromatography R*. Dilute 1.0 mL of this solution to 10.0 mL with *water for chromatography R*.

*Reference solution (b).* In order to prepare impurities B and C *in situ*, dissolve 30 mg of the substance to be examined in 10.0 mL of a 4.0 g/L solution of *sodium hydroxide R*, heat at 70 °C for 40 min and allow to cool. Dilute 1.0 mL of the solution to 10.0 mL with *water for chromatography R*.

*Reference solution (c).* Dissolve the contents of a vial of *pemetrexed impurity mixture CRS* (impurities A and D) in 1.0 mL of *water for chromatography R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:
- *mobile phase A*: *acetonitrile R*, solution A (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R*, solution A (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 45 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *pemetrexed impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and D; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to pemetrexed (retention time = about 26 min): impurity A = about 0.82; impurity B = about 0.87; impurity C = about 0.88; impurity D = about 0.90.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity C.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pemetrexed disodium heptahydrate in reference solution (a).

*Limits*:

– *impurities A, D*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.6 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 19.5 per cent to 22.1 per cent, determined on 0.050 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.17 IU/mg.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C for not more than 24 h.*

*Acetate buffer.* Mix 1.7 mL of *glacial acetic acid R* and 900 mL of *water for chromatography R*, adjust to pH 5.3 with a 760 g/L solution of *sodium hydroxide R* in *water for chromatography R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in *water for chromatography R* and dilute to 200.0 mL with the same solvent.

*Reference solution.* Dissolve 30.0 mg of *pemetrexed disodium heptahydrate CRS* in *water for chromatography R* and dilute to 200.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (3.5 µm);

– *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, acetate buffer (11:89 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of pemetrexed (retention time = about 3 min).

Calculate the percentage content of $C_{20}H_{19}N_5Na_2O_6$ taking into account the assigned content of *pemetrexed disodium heptahydrate CRS*.

IMPURITIES

*Specified impurities: A, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

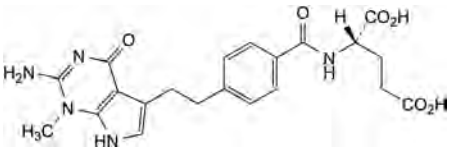

A. (2*S*)-2-[[4-[2-(2-amino-1-methyl-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino]-pentanedioic acid,

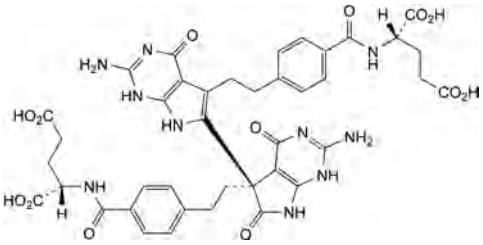

B. (2*S*,2′*S*)-2,2′-[[(5*R*)-2,2′-diamino-4,4′,6-trioxo-1,4,4′,6,7,7′-hexahydro-1′*H*,5*H*-5,6′-bipyrrolo[2,3-*d*]pyrimidine-5,5′-diyl]bis(ethylenebenzene-4,1-diylcarbonylimino)]dipentanedioic acid,

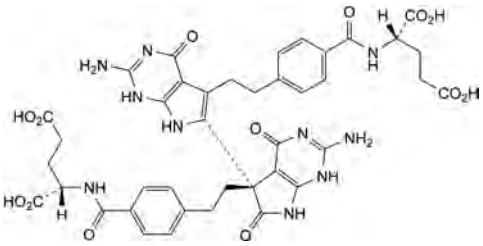

C. (2*S*,2′*S*)-2,2′-[[(5*S*)-2,2′-diamino-4,4′,6-trioxo-1,4,4′,6,7,7′-hexahydro-1′*H*,5*H*-5,6′-bipyrrolo[2,3-*d*]pyrimidine-5,5′-diyl]bis(ethylenebenzene-4,1-diylcarbonylimino)]dipentanedioic acid,

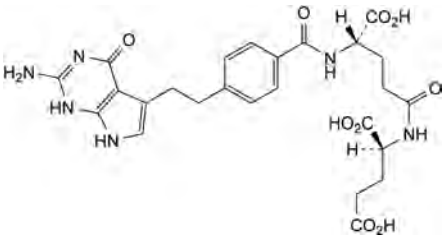

D. (2*S*)-2-[[(4*S*)-4-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino]-4-carboxybutanoyl]amino]pentanedioic acid,

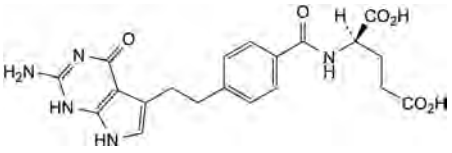

E. (2*R*)-2-[[4-[2-(2-amino-4-oxo-4,7-dihydro-1*H*-pyrrolo-[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]amino]pentanedioic acid.

**01/2017:1461**

# PENBUTOLOL SULFATE

## Penbutololi sulfas

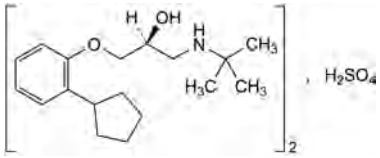

$C_{36}H_{60}N_2O_8S$ $M_r$ 681
[38363-32-5]

DEFINITION

Di[(2*S*)-1-(2-cyclopentylphenoxy)-3-[(1,1-dimethylethyl)-amino]propan-2-ol] sulfate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol, practically insoluble in cyclohexane.

IDENTIFICATION

*First identification*: *A, C, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *penbutolol sulfate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 40 mg of the substance to be examined in 1 mL of *methanol R*.
*Reference solution*. Dissolve 40 mg of *penbutolol sulfate CRS* in 1 mL of *methanol R*.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R*, *ethyl acetate R* (10:20:35:35 *V/V/V/V*).
*Application*: 5 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 50 mg in a mixture of 5 mL of *water R* and 1 mL of *0.1 M hydrochloric acid*. The solution gives reaction (a) of sulfates (*2.3.1*).

D. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 1.00 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Acidity or alkalinity**. To 4 mL of solution S add 4 mL of *carbon dioxide-free water R*. Add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*; the solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid*; the solution is red.

**Specific optical rotation** (*2.2.7*): – 23 to – 25 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (40:60 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 4.0 mg of the substance to be examined and 1.0 mg of *penbutolol impurity A CRS* in 5.0 mL of the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 200.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 5.0 mg of *penbutolol impurity A CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
– *mobile phase A*: *acetonitrile for chromatography R*, *methanol R* (39:61 *V/V*);
– *mobile phase B*: dissolve 11 g of *sodium heptanesulfonate R* in 1000 mL of *water R*, add 5.0 mL of *triethylamine R* and adjust to pH 2.7 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 60 | 40 |
| 15 - 35 | 60 → 80 | 40 → 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (a):
– *resolution*: minimum 3.0 between the 2 principal peaks.

*Limits*:
– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 68.10 mg of $C_{36}H_{60}N_2O_8S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

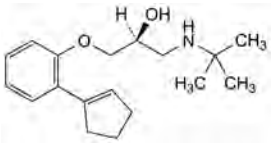

A. (2*S*)-1-[2-(cyclopent-1-enyl)phenoxy]-3-[(1,1-dimethylethyl)amino]propan-2-ol.

**01/2020:0566**

# PENICILLAMINE

## Penicillaminum

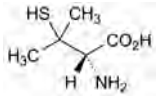

$C_5H_{11}NO_2S$ $M_r$ 149.2
[52-67-5]

DEFINITION

(2*S*)-2-Amino-3-methyl-3-sulfanylbutanoic acid.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B, D.*

*Second identification*: *A, C, D.*

A. Dissolve 0.5 g in a mixture of 0.5 mL of *hydrochloric acid R* and 4 mL of warm *acetone R*, cool in iced water and initiate crystallisation by scratching the wall of the tube with a glass rod. A white precipitate is formed. Filter with the aid of vacuum, wash with *acetone R* and dry with suction. A 10 g/L solution of the precipitate is dextrorotatory.

B. Examine the chromatograms obtained in the test for impurity A.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 4 mL of *water R*.

*Reference solution.* Dissolve 10 mg of *penicillamine CRS* in 4 mL of *water R*.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (18:18:72 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 10 cm.

*Drying*: at 100-105 °C for 5-10 min.

*Detection*: expose to iodine vapour for 5-10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 40 mg in 4 mL of *water R* and add 2 mL of *phosphotungstic acid solution R*. Allow to stand for 5 min. A blue colour develops.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.5.

Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 65.0 to − 61.0 (dried substance).

Dissolve 0.500 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 10.0 mL with the same solution.

**Ultraviolet-absorbing substances**: maximum 0.5 per cent of penilloic acid.

Dissolve 0.100 g in *water R* and dilute to 50.0 mL with the same solvent. The absorbance (*2.2.25*) of the solution at 268 nm is not greater than 0.07.

**Impurity A**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 40 mg of *penicillamine CRS* in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dissolve 20.0 mg of *penicillamine disulfide CRS* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: solution containing 0.1 g/L of *sodium edetate R* and 2 g/L of *methanesulfonic acid R*.

*Flow rate*: 1.7 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of penicillamine.

*Relative retention* with reference to penicillamine (retention time = about 6 min): impurity A = about 1.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to penicillamine and impurity A.

*Limit*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1 per cent).

**Impurity B**: maximum 0.1 ppm.

*Carry out all the operations in a penicillin-free atmosphere and with equipment reserved for this test. Sterilise the equipment at 180 °C for 3 h and the buffer solutions at 121 °C for 20 min before use.*

*Test solution (a).* Dissolve 1.000 g in 8 mL of *buffer solution pH 2.5 R* and add 8 mL of *ether R*. Shake vigorously for 1 min. Repeat the extraction and combine the ether layers. Add 8 mL of *buffer solution pH 2.5 R*. Shake for 1 min, allow to settle and quantitatively separate the upper layer, taking care to eliminate the aqueous phase completely (*penicillin is unstable at pH 2.5; carry out operations at this pH within 6-7 min*). Add 8 mL of *phosphate buffer solution pH 6.0 R2* to the ether phase, shake for 5 min, allow to settle, then separate the aqueous layer and check that the pH is 6.0.

*Test solution (b).* To 2 mL of test solution (a) add 20 µL of *penicillinase solution R* and incubate at 37 °C for 1 h.

*Reference solution (a).* Dissolve 5 mg of *benzylpenicillin sodium R* in 500 mL of *phosphate buffer solution pH 6.0 R2.* Dilute 0.25 mL of the solution to 200.0 mL with *buffer solution pH 2.5 R.* Carry out the extraction using 8 mL of this solution as described for test solution (a).

*Reference solution (b).* To 2 mL of reference solution (a) add 20 µL of *penicillinase solution R* and incubate at 37 °C for 1 h.

*Blank solution.* Prepare the solution as described for test solution (a) but omitting the substance to be examined.

Liquefy a suitable nutrient medium such as that described below and inoculate it at a suitable temperature with a culture of *Kocuria rhizophila* (ATCC 9341), to give $5 \times 10^4$ micro-organisms per millilitre or a different quantity if necessary to obtain the required sensitivity and formation of clearly defined inhibition zones of suitable diameter. Immediately pour the inoculated medium into 5 Petri dishes 10 cm in diameter to give uniform layers 2-5 mm deep. The medium may alternatively consist of 2 layers, only the upper layer being inoculated. Store the dishes so that no appreciable growth or death of the micro-organisms occurs before use and so that the surface of the agar is dry at the time of use. In each dish, place 5 stainless steel hollow cylinders 6 mm in diameter on the surface of the agar evenly spaced on a circle with a radius of about 25 mm and concentric with the dish. For each dish, place in separate cylinders 0.15 mL of test solutions (a) and (b), reference solutions (a) and (b) and the blank solution. Maintain at 30 °C for at least 24 h. Measure the diameters of the inhibition zones to the nearest 0.1 mm. The test is valid if reference solution (a) gives a clear inhibition zone and if reference solution (b) and the blank solution give no inhibition zone. If test solution (a) gives an inhibition zone, this is caused by penicillin if test solution (b) gives no inhibition zone. If this is so, the average diameter of the inhibition zones given by test solution (a) for the 5 Petri dishes is less than the average diameter of the inhibition zones given by reference solution (a) measured in the same conditions.

*Nutrient medium (pH 6.0)*

| | |
|---|---|
| Peptone | 5 g |
| Yeast extract | 1.5 g |
| Meat extract | 1.5 g |
| Sodium chloride | 3.5 g |
| Agar | 15 g |
| *Distilled water R* | 1000 mL |

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.67 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1000 g in 30 mL of *anhydrous acetic acid R.* Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.92 mg of $C_5H_{11}NO_2S$.

IMPURITIES

*Specified impurities: A, B.*

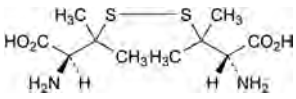

A. 3,3′-(disulfanediyl)bis[(2*S*)-2-amino-3-methylbutanoic] acid (penicillamine disulfide),

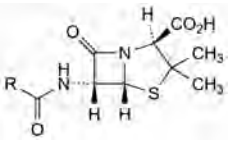

B. penicillin.

**07/2009:1355**

# PENTAERYTHRITYL TETRANITRATE, DILUTED

## Pentaerythrityli tetranitras dilutus

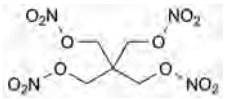

$C_5H_8N_4O_{12}$ $M_r$ 316.1

DEFINITION

Dry mixture of 2,2-bis(hydroxymethyl)propane-1,3-diol tetranitrate (pentaerythrityl tetranitrate) and *Lactose monohydrate (0187)* or *Mannitol (0559).*

*Content*: 95.0 per cent *m/m* to 105.0 per cent *m/m* of the declared content of pentaerythrityl tetranitrate.

*CAUTION: undiluted pentaerythrityl tetranitrate may explode if subjected to percussion or excessive heat. Appropriate precautions must be taken and only very small quantities handled.*

CHARACTERS

*Appearance of pentaerythrityl tetranitrate*: white or slightly yellowish powder.

*Solubility of pentaerythrityl tetranitrate*: practically insoluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent).

The solubility of diluted pentaerythrityl tetranitrate depends on the diluent and its concentration.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: separately shake a quantity of the substance to be examined and a quantity of the reference substance, each corresponding to 25 mg of pentaerythrityl tetranitrate, with 10 mL of *acetone R* for 5 min; filter, evaporate to dryness at a temperature below 40 °C, and dry the residue over *diphosphorus pentoxide R* at a pressure of 0.7 kPa for 16 h. Examine the residues prepared as discs.

*Comparison*: *diluted pentaerythrityl tetranitrate CRS.*

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 10 mg of pentaerythrityl tetranitrate with 10 mL of *ethanol (96 per cent) R* for 5 min and filter.

*Reference solution.* Shake a quantity of *diluted pentaerythrityl tetranitrate CRS* corresponding to 10 mg of pentaerythrityl tetranitrate with 10 mL of *ethanol (96 per cent) R* for 5 min and filter.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *ethyl acetate R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with freshly prepared *potassium iodide and starch solution R*, expose to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of lactose or mannitol with 10 mL of *water R*. Filter if necessary.

*Reference solution (a)* Dissolve 0.10 g of *lactose monohydrate R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.10 g of *mannitol R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Mix equal volumes of reference solutions (a) and (b).

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*). Measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 1 µL; thoroughly dry the points of application.

*Development A*: over 2/3 of the plate.

*Drying A*: in a current of warm air.

*Development B*: immediately, over 2/3 of the plate, after renewing the mobile phase.

*Drying B*: in a current of warm air.

*Detection*: spray with *4-aminobenzoic acid solution R*, dry in a current of cold air until the acetone is removed, then heat at 100 °C for 15 min; allow to cool, spray with a 2 g/L solution of *sodium periodate R*, dry in a current of cold air and heat at 100 °C for 15 min.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a) for lactose or to the principal spot in the chromatogram obtained with reference solution (b) for mannitol.

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Shake a quantity of the substance to be examined corresponding to 0.10 g of pentaerythrityl tetranitrate with 5 mL of *ethanol (96 per cent) R* and filter.

*Reference solution.* Dissolve 10 mg of *potassium nitrate R* in 1 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *toluene R* (15:30:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of air until the acetic acid is completely removed.

*Detection*: spray copiously with freshly prepared *potassium iodide and starch solution R*, expose the plate to ultraviolet light at 254 nm for 15 min and examine in daylight.

*Limit*:

– *nitrate*: any spot due to nitrate is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent, calculated as potassium nitrate).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Sonicate for 15 min a quantity of the substance to be examined corresponding to 25.0 mg of pentaerythrityl tetranitrate in 20 mL of the mobile phase and dilute to 25.0 mL with the mobile phase. Filter.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a).* Sonicate for 15 min a quantity of *diluted pentaerythrityl tetranitrate CRS* corresponding to 25.0 mg of pentaerythrityl tetranitrate in 20 mL of the mobile phase and dilute to 25.0 mL with the mobile phase. Filter.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 0.3 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Reference solution (d).* Dilute 200 µL of *glyceryl trinitrate solution CRS* to 25.0 mL with the mobile phase.

*Reference solution (e).* To 1 mL of reference solution (b) add 1 mL of reference solution (d) and dilute to 10 mL with the mobile phase.

*Reference solution (f).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with the mobile phase. Dilute 0.5 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *acetonitrile R* (35:65 *V/V*).

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (c), (e) and (f).

*Run time*: 5 times the retention time of pentaerythrityl tetranitrate.

*Relative retention* with reference to pentaerythrityl tetranitrate (retention time = about 2.4 min): impurity B = about 0.7; impurity C = about 3.0.

*System suitability*: reference solution (e):

– *resolution*: minimum 3.0 between the peaks due to glyceryl trinitrate and pentaerythrityl tetranitrate.

*Limits*:

– *impurities C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (f) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.6 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (f) (0.05 per cent).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_5H_8N_4O_{12}$ from the declared content of *diluted pentaerythrityl tetranitrate CRS*.

STORAGE

Protected from light and heat.

LABELLING

The label states:

– the percentage content of pentaerythrityl tetranitrate;
– the diluent used.

IMPURITIES

*Specified impurities*: *A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

A. $NO_3^-$: inorganic nitrates,

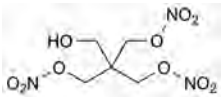

B. 2,2-bis(hydroxymethyl)propane-1,3-diol trinitrate (pentaerythritol trinitrate),

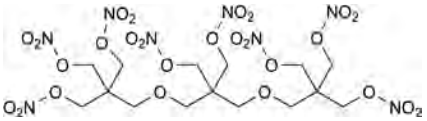

C. 2,2-bis[[3-hydroxy-2,2-bis(hydroxymethyl)propoxy]-methyl]propane-1,3-diol octanitrate (tripentaerythrityl octanitrate),

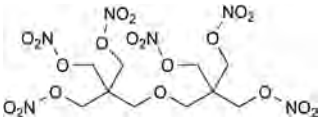

D. 2,2′-(oxybismethylene)bis[2-(hydroxymethyl)propane-1,3-diol] hexanitrate (dipentaerythrityl hexanitrate).

**01/2017:1137**

# PENTAMIDINE DIISETIONATE

## Pentamidini diisetionas

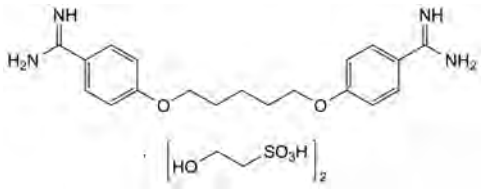

$C_{23}H_{36}N_4O_{10}S_2$ $M_r$ 592.7

[140-64-7]

DEFINITION

4,4′-[Pentane-1,5-diylbis(oxy)]dibenzimidamide bis(2-hydroxyethanesulfonate).

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

PRODUCTION

It is considered that alkyl 2-hydroxyethanesulfonate esters are potential impurities in pentamidine diisetionate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation including, where necessary, demonstration that alkyl 2-hydroxyethanesulfonate esters are not detectable in the final product.

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *pentamidine diisetionate CRS*.

B. Dissolve about 40 mg in 5 mL of *water R* and add dropwise with shaking 1 mL of a 10 g/L solution of *sodium chloride R*. Allow to stand for 5 min. The mixture remains clear.

C. Treat 0.15 g by the oxygen-flask method (*2.5.10*). Use 10 mL of *dilute hydrogen peroxide solution R* to absorb the combustion products. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. To 0.1 g in a conical flask, add 40 mL of *water R* and glass beads. Adjust to pH 10.5 with *dilute sodium hydroxide solution R* and boil under reflux for 20 min. Cool and dilute to 50 mL with *water R*. Dilute 1 mL of the solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 65 volumes of *methanol R* and 35 volumes of a 30 g/L solution of *ammonium acetate R* previously adjusted to pH 7.5 with *triethylamine R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 10 µL.

*Run time*: 3.5 times the retention time of pentamidine.

*System suitability*: reference solution (b):

– the chromatogram obtained shows 2 principal peaks;
– *resolution*: minimum 2.0 between the 2 principal peaks.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *dimethylformamide R*. Add 0.25 mL of *thymol blue solution R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, under a current of *nitrogen R*, until the colour of the indicator changes to blue. Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 29.63 mg of $C_{23}H_{36}N_4O_{10}S_2$.

STORAGE

In an airtight container.

IMPURITIES

A. 4-[[5-(4-amidinophenoxy)pentyl]oxy]benzenecarboxamide.

**01/2008:1462**

# PENTAZOCINE

## Pentazocinum

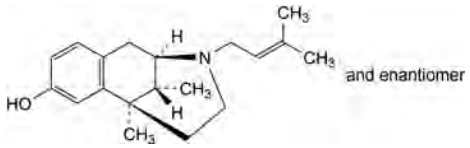


$C_{19}H_{27}NO$ $M_r$ 285.4
[359-83-1]

DEFINITION

Pentazocine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, freely soluble in methylene chloride and soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the *Ph. Eur. reference spectrum for pentazocine (form A)*.

TESTS

**Absorbance** (*2.2.25*). Dissolve 0.100 g in a mixture of 20 mL of *water R* and 1 mL of *1 M hydrochloric acid*, and dilute to 100.0 mL with *water R*. To 10.0 mL add 1 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*. The absorbance at the absorption maximum at 278 nm is 0.67 to 0.71, calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R*.

*Test solution.* Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dilute 1 mL of the test solution to 100 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 5 mL of reference solution (a) to 10 mL with *methylene chloride R*.

*Reference solution (c).* Dilute 5 mL of reference solution (a) to 20 mL with *methylene chloride R*.

Apply to the plate 10 µL of each solution. Develop over a path corresponding to two thirds of the plate height using a mixture of 3 volumes of *isopropylamine R*, 3 volumes of *methanol R* and 94 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Heat the plate at 100-105 °C for 15 min, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. By each method of visualisation: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot obtained with reference solution (a) (1 per cent); not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and not more than 4 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.54 mg of $C_{19}H_{27}NO$.

STORAGE

Store protected from light.

**01/2008:1463**

# PENTAZOCINE HYDROCHLORIDE

## Pentazocini hydrochloridum

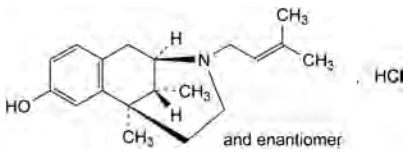


$C_{19}H_{28}ClNO$ $M_r$ 321.9
[64024-15-3]

DEFINITION

Pentazocine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*,6*RS*,11*RS*)-6,11-dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, sparingly soluble in water, soluble in ethanol (96 per cent) and sparingly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the *Ph. Eur. reference spectrum of pentazocine hydrochloride.*

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*). Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*. The pH of the solution is 4.0 to 6.0.

**Absorbance** (*2.2.25*). Dissolve 0.100 g in a mixture of 20 mL of *water R* and 1 mL of *1 M hydrochloric acid*, and dilute to 100.0 mL with *water R*. To 10.0 mL add 1 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*. The absorbance at the absorption maximum at 278 nm is 0.59 to 0.63, calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R*.

*Test solution*. Dissolve 0.20 g in 3 mL of *methanol R* and dilute to 10 mL with *methylene chloride R*.

*Reference solution (a).* Dilute 1 mL of the test solution to 100 mL with *methylene chloride R*.

*Reference solution (b).* Dilute 5 mL of reference solution (a) to 10 mL with *methylene chloride R*.

*Reference solution (c).* Dilute 5 mL of reference solution (a) to 20 mL with *methylene chloride R*.

Apply to the plate 10 µL of each solution. Develop over a path corresponding to two-thirds of the plate height using a mixture of 3 volumes of *isopropylamine R*, 3 volumes of *methanol R* and 94 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Heat the plate at 100-105 °C for 15 min, allow to cool, expose to iodine vapour and re-examine under ultraviolet light at 254 nm. By each method of visualisation: any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot obtained with reference solution (a) (1 per cent); not more than 1 such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent); and not more than 4 such spots are more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R*. Add 5 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflection.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.19 mg of $C_{19}H_{28}ClNO$.

STORAGE

Store protected from light.

**01/2008:2000**
**corrected 6.0**

# PENTAZOCINE LACTATE

## Pentazocini lactas

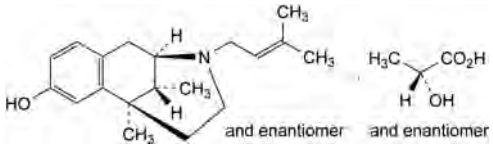


$C_{22}H_{33}NO_4$ $M_r$ 375.5
[17146-95-1]

DEFINITION

(2*RS*,6*RS*,11*RS*)-6,11-Dimethyl-3-(3-methylbut-2-enyl)-1,2,3,4,5,6-hexahydro-2,6-methano-3-benzazocin-8-ol (2*RS*)-2-hydroxypropanoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol, slightly soluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of pentazocine lactate.*

TESTS

**pH** (*2.2.3*): 5.5 to 6.5.

Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Absorbance** (*2.2.25*): 0.50 to 0.54, determined at the absorption maximum at 278 nm.

Dissolve 0.10 g in 10.0 mL of *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 100 mg of the substance to be examined in *acetic anhydride R* and dilute to 5 mL with the same solvent. Heat at 80 °C for 10 min. Dilute 1 mL of the solution to 10 mL with *methanol R*. Mix 1 mL of this solution with 1 mL of the test solution.

*Reference solution (b).* Dilute 1 mL of the test solution to 100 mL with *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *isopropylamine R*, *methanol R*, *methylene chloride R* (3:3:94 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm. Heat at 100-105 °C for 15 min. Allow to cool. Expose to iodine vapour and re-examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (a):

– the chromatogram shows 2 clearly separated spots.

*Limits*: by each method of detection:

– *any impurity*: any spots, apart from the principal spot, are not more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 30 mL of *anhydrous acetic acid R* and add 30 mL of *dioxan R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.55 mg of $C_{22}H_{33}NO_4$.

STORAGE

Protected from light.

**01/2019:0200**

# PENTOBARBITAL

## Pentobarbitalum

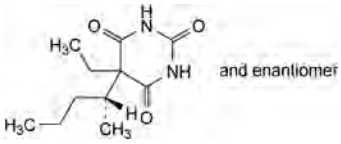


$C_{11}H_{18}N_2O_3$ $M_r$ 226.3

[76-74-4]

DEFINITION

5-Ethyl-5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in anhydrous ethanol. It forms water-soluble compounds with alkali hydroxides and carbonates and with ammonia.

IDENTIFICATION

A. Melting point (*2.2.14*).

*Determination A*: determine the melting point of the substance to be examined.

*Determination B*: mix equal parts of the substance to be examined and *pentobarbital CRS* and determine the melting point of the mixture.

*Results*: the difference between the melting points (which are about 133 °C) is not greater than 2 °C.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *pentobarbital CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Detection*: examine immediately in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 10 mg add about 10 mg of *vanillin R* and 2 mL of *sulfuric acid R*. Mix and heat on a water-bath for 2 min. A reddish-brown colour develops. Cool and add cautiously 5 mL of *anhydrous ethanol R*. The colour becomes violet and then blue.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 6 mL of *water R*.

**Acidity**. Boil 1.0 g with 50 mL of *water R* for 2 min, allow to cool and filter. To 10 mL of the filtrate add 0.15 mL of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to produce a pure yellow colour.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (b) to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.5 mg of *pentobarbital impurity E CRS* in the mobile phase and dilute to 25 mL with the mobile phase. Dilute 1 mL of the solution to 100 mL with the mobile phase. Dissolve 5 mg of the substance to be examined in 5 mL of this solution.

*Reference solution (c).* Dissolve 25.0 mg of *pentobarbital CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of pentobarbital.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to pentobarbital (retention time = about 10 min): impurity E = about 0.92.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity E and pentobarbital.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pentobarbital in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{11}H_{18}N_2O_3$ taking into account the assigned content of *pentobarbital CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

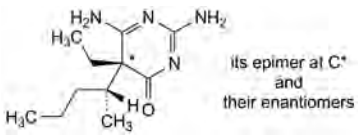


A. mixture of (5*R*)-2,6-diamino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidin-4(5*H*)-one and (5*S*)-2,6-diamino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidin-4(5*H*)-one,

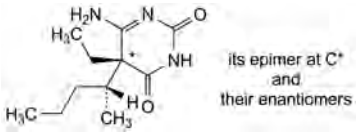


B. mixture of (5*R*)-6-amino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidine-2,4(3*H*,5*H*)-dione and (5*S*)-6-amino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidine-2,4(3*H*,5*H*)-dione,

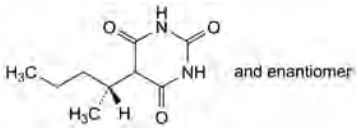


C. 5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione,

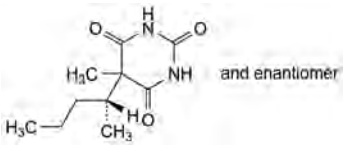


D. 5-methyl-5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione,

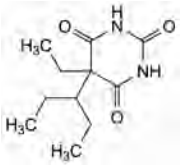

E. 5-ethyl-5-(pentan-3-yl)-1,3-diazinane-2,4,6-trione,

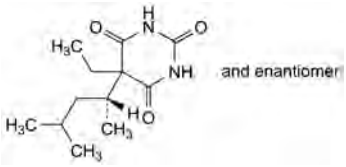


F. 5-ethyl-5-[(2*RS*)-4-methylpentan-2-yl]-1,3-diazinane-2,4,6-trione.

**01/2019:0419**

# PENTOBARBITAL SODIUM

## Pentobarbitalum natricum

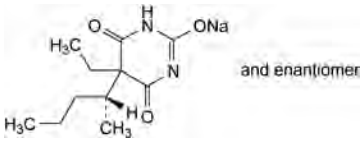


$C_{11}H_{17}N_2NaO_3$ $M_r$ 248.3
[57-33-0]

DEFINITION

Sodium derivative of 5-ethyl-5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: very soluble in water.

IDENTIFICATION

A. Melting point (*2.2.14*).

*Determination A*: dissolve 1 g in 10 mL of *water R* and add 5 mL of *dilute acetic acid R*; a white, crystalline precipitate is formed; filter, wash the precipitate with *water R* and dry at 100-105 °C; determine the melting point of the precipitate.

*Determination B*: mix equal parts of the precipitate and *pentobarbital CRS* and determine the melting point of the mixture.

*Results*: the difference between the melting points (which are about 131 °C) is not greater than 2 °C.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 11 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *pentobarbital CRS* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R, ethanol (96 per cent) R, methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Detection*: examine immediately in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

Monographs d

C. To about 10 mg add about 10 mg of *vanillin R* and 2 mL of *sulfuric acid R*. Mix and heat on a water-bath for 2 min. A reddish-brown colour develops. Cool and add cautiously 5 mL of *anhydrous ethanol R*. The colour becomes violet and then blue.

D. Ignite 1 g. The residue gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 9.6 to 11.0, measured immediately after preparation.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 27.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2.5 mg of *pentobarbital impurity E CRS* in the mobile phase and dilute to 25 mL with the mobile phase. Dilute 1 mL of the solution to 100 mL with the mobile phase. Dissolve 5 mg of the substance to be examined in 5 mL of this solution.

*Reference solution (c)*. Dissolve 25.0 mg of *pentobarbital CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.5 with *dilute phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of pentobarbital.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to pentobarbital (retention time = about 10 min): impurity E = about 0.92.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity E and pentobarbital.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pentobarbital sodium in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Free pentobarbital**: maximum 3.5 per cent.

Dissolve 2.00 g in 75 mL of *dimethylformamide R*, heating gently if necessary. Titrate with *0.1 M sodium methoxide* until the colour changes from olive-green to blue, using 0.25 mL of a 10 g/L solution of *thymol blue R* in *dimethylformamide R* as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium methoxide* is equivalent to 22.63 mg of pentobarbital.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{11}H_{17}N_2NaO_3$ taking into account the assigned content of *pentobarbital CRS* and a conversion factor of 1.097.

STORAGE

In an airtight container.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

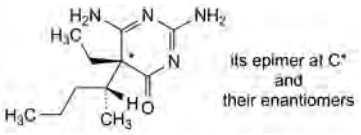


A. mixture of (5*R*)-2,6-diamino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidin-4(5*H*)-one and (5*S*)-2,6-diamino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidin-4(5*H*)-one,

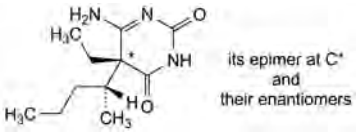


B. mixture of (5*R*)-6-amino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidine-2,4(3*H*,5*H*)-dione and (5*S*)-6-amino-5-ethyl-5-[(2*RS*)-pentan-2-yl]pyrimidine-2,4(3*H*,5*H*)-dione,

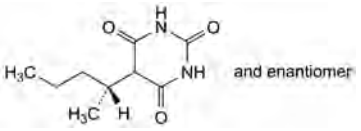


C. 5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione,

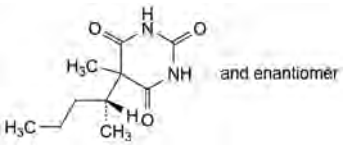


D. 5-methyl-5-[(2*RS*)-pentan-2-yl]-1,3-diazinane-2,4,6-trione,

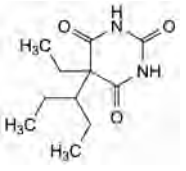

E. 5-ethyl-5-(pentan-3-yl)-1,3-diazinane-2,4,6-trione,

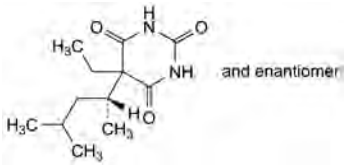


F. 5-ethyl-5-[(2*RS*)-4-methylpentan-2-yl]-1,3-diazinane-2,4,6-trione.

**01/2017:0851**

# PENTOXIFYLLINE

## Pentoxifyllinum

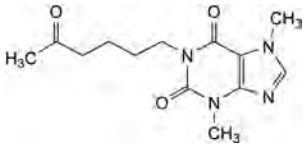


$C_{13}H_{18}N_4O_3$ $M_r$ 278.3
[6493-05-6]

DEFINITION

3,7-Dimethyl-1-(5-oxohexyl)-3,7-dihydro-1*H*-purine-2,6-dione.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 103 °C to 107 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pentoxifylline CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 20 mg of *pentoxifylline CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *methanol R*, *ethyl acetate R* (15:85 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. A 40 per cent *V/V* solution of solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity**. To 8 mL of solution S add 12 mL of *carbon dioxide-free water R* and 0.05 mL of *bromothymol blue solution R1*. The solution is green or yellow. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* A mixture of equal volumes of a 5.44 g/L solution of *potassium dihydrogen phosphate R* and *methanol R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 10.0 mL of reference solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *caffeine R* (impurity F) and 2 mg of *theophylline R* (impurity C) in the solvent mixture, add 1 mL of the test solution and dilute to 10 mL with the solvent mixture.

*Reference solution (d).* Dissolve 5.0 mg of *caffeine R* (impurity F), 5.0 mg of *theobromine R* (impurity A) and 5.0 mg of *theophylline R* (impurity C) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL to 25.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 30 volumes of *methanol R* and 70 volumes of a 5.44 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: mix 30 volumes of a 5.44 g/L solution of *potassium dihydrogen phosphate R* and 70 volumes of *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 85 | 15 |
| 6 - 13 | 85 → 10 | 15 → 90 |
| 13 - 30 | 10 | 90 |
| 30 - 35 | 10 → 85 | 90 → 15 |
| 35 - 45 | 85 | 15 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL.

*Relative retention* with reference to pentoxifylline (retention time = about 12 min): impurity A = about 0.3; impurity C = about 0.4; impurity F = about 0.5; impurity J = about 1.6.

*System suitability*: reference solution (c):
- *retention time*: impurity F = 4 min to 7 min; pentoxifylline = 9 min to 13 min; if necessary adapt the mixing ratio of the mobile phases;
- *resolution*: minimum 4 between the peaks due to impurity C and impurity F.

*Limits*:
- *impurities A, C, F*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent),
- *impurity J*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Place 20 mL of solution S in a separating funnel and shake with 2 quantities, each of 20 mL, of *2-methylpropanol R*. Dilute 10 mL of the aqueous layer to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on 15 mL of solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying over *diphosphorus pentoxide R* at 60 °C at a pressure not exceeding 700 Pa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 5 mL of *anhydrous acetic acid R*. Add 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.83 mg of $C_{13}H_{18}N_4O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, C, F, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, G, H, I, K.*

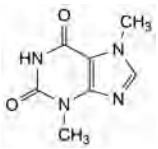

A. theobromine,

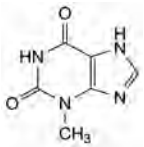

B. 3-methyl-3,7-dihydro-1*H*-purine-2,6-dione,

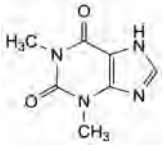

C. theophylline,

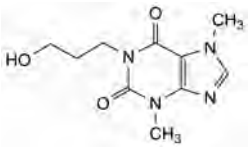

D. 1-(3-hydroxypropyl)-3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione,

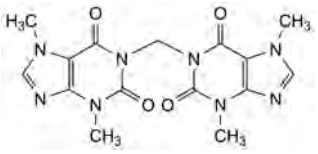

E. 1,1′-methylenebis(3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione),

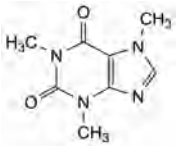

F. caffeine,

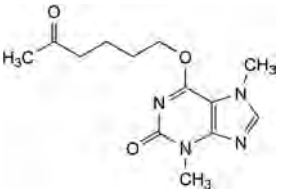

G. 3,7-dimethyl-6-(5-oxohexyloxy)-3,7-dihydro-2*H*-purin-2-one,

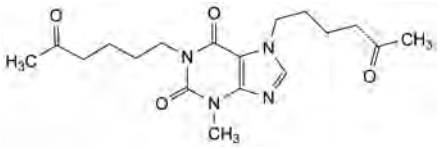

H. 3-methyl-1,7-bis(5-oxohexyl)-3,7-dihydro-1*H*-purine-2,6-dione,

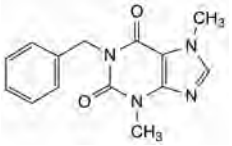

I. 1-benzyl-3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione,

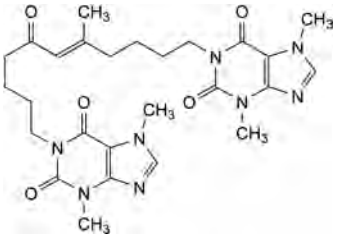

J. 1-[(5*E*)-11-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1*H*-purin-1-yl)-5-methyl-7-oxoundec-5-enyl]-3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione,

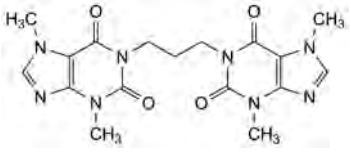

K. 1,1′-(propane-1,3-diyl)bis(3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione).

**01/2008:1621**

# PENTOXYVERINE HYDROGEN CITRATE

## Pentoxyverini hydrogenocitras

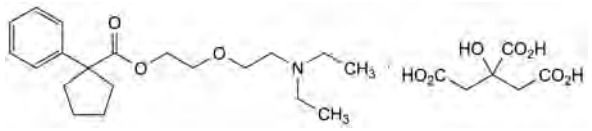

$C_{26}H_{39}NO_{10}$ $M_r$ 525.6
[23142-01-0]

DEFINITION

2-[2-(Diethylamino)ethoxy]ethyl 1-phenylcyclopentanecarboxylate dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very soluble in glacial acetic acid, freely soluble in methanol, soluble in alcohol and in methylene chloride.

mp: about 93 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of pentoxyverine hydrogen citrate*.

B. Dissolve 0.25 g in 5 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.3 to 3.7 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution*. Introduce 5.0 mg of *pentoxyverine impurity A CRS* and 5.0 mg of *pentoxyverine impurity B CRS* in a conical flask, add 5.0 mL of the test solution and dilute to 100.0 mL with the mobile phase. Dilute 3.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm,
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 12 per cent,
- *temperature*: 50 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 1.5 g/L solution of *sodium heptanesulfonate R* adjusted to pH 3.0 with *dilute sulfuric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of pentoxyverine.

*Relative retention* with reference to pentoxyverine (retention time = about 6 min): impurity B = about 0.8; impurity A = about 1.5.

*System suitability*: reference solution:

- *resolution*: minimum of 5.0 between the peaks due to pentoxyverine and to impurity A,
- *signal-to-noise ratio*: minimum of 100 for the peak due to pentoxyverine,
- *symmetry factor*: maximum of 2.0 for the peak due to pentoxyverine.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent),
- *any other impurity*: not more than one-third of the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.1 per cent),
- *total of any other impurity*: not more than the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.3 per cent),
- *disregard limit*: 0.1 times the area of the peak due to pentoxyverine in the chromatogram obtained with the reference solution (0.03 per cent); disregard any peak with a retention time less than or equal to 2.5 min.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 70 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 52.56 mg of $C_{26}H_{39}NO_{10}$.

STORAGE

Protected from light.

IMPURITIES

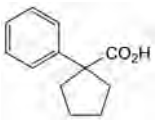

A. 1-phenylcyclopentanecarboxylic acid,

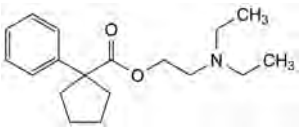

B. 2-(diethylamino)ethyl 1-phenylcyclopentanecarboxylate (caramiphen).

**01/2009:0682**

# PEPSIN POWDER

## Pepsini pulvis

[9001-75-6]

DEFINITION

Powder prepared from the gastric mucosa of pigs, cattle or sheep. It contains gastric proteinases, active in acid medium (pH 1 to 5).

*Activity*: not less than 0.5 Ph. Eur. U./mg (dried substance).

PRODUCTION

The animals from which pepsin powder is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or slightly yellow, crystalline or amorphous powder, hygroscopic.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

The solution in water may be slightly opalescent with a weak acidic reaction.

IDENTIFICATION

In a mortar, pound 30 mg of *fibrin blue R*. Suspend in 20 mL of *dilute hydrochloric acid R2*. Filter the suspension on a filter paper and wash with *dilute hydrochloric acid R2* until a colourless filtrate is obtained. Perforate the filter paper and wash the *fibrin blue R* through it into a conical flask using 20 mL of *dilute hydrochloric acid R2*. Shake before use. Dissolve a quantity of the substance to be examined, equivalent to not less than 20 Ph. Eur. U., in 2 mL of *dilute hydrochloric acid R2* and adjust to pH 1.6 ± 0.1. Add 1 mL of this solution to a test-tube containing 4 mL of the fibrin blue suspension, mix and place in a water-bath at 25 °C with gentle shaking. Prepare a blank solution at the same time and in the same manner using 1 mL of *water R*. After 15 min of incubation the blank solution is colourless and the test solution is blue.

TESTS

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 4 h.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

The activity of pepsin powder is determined by comparing the quantity of peptides, non-precipitable by *trichloroacetic acid solution R* and assayed using the *phosphomolybdotungstic reagent R*, which are released per minute from a substrate of *haemoglobin solution R*, with the quantity of such peptides released by *pepsin powder BRP* from the same substrate in the same conditions.

*For the test solution and the reference solution, prepare the solution and carry out the dilution at 0 °C to 4 °C.*

*Avoid shaking and foaming during preparation of the test and reference solutions.*

*Test solution.* Immediately before use, prepare a solution of the substance to be examined expected to contain 0.5 Ph. Eur. U./mL in *dilute hydrochloric acid R2*; before dilution to volume, adjust to pH 1.6 ± 0.1, if necessary, using *1 M hydrochloric acid*.

*Reference solution.* Less than 15 min before use, prepare a solution of *pepsin powder BRP* containing 0.5 Ph. Eur. U./mL in *dilute hydrochloric acid R2*; before dilution to volume, adjust to pH 1.6 ± 0.1, if necessary, using *1 M hydrochloric acid*.

Designate tubes in duplicate T, $T_b$, $S_1$, $S_{1b}$, $S_2$, $S_{2b}$, $S_3$, $S_{3b}$; designate a tube B.

Add *dilute hydrochloric acid R2* to the tubes as follows:

B: 1.0 mL

$S_1$ and $S_{1b}$: 0.5 mL

$S_2$, $S_{2b}$ and T and $T_b$: 0.25 mL

Add the reference solution to the tubes as follows:

$S_1$ and $S_{1b}$: 0.5 mL

$S_2$ and $S_{2b}$: 0.75 mL

$S_3$ and $S_{3b}$: 1.0 mL

Add 0.75 mL of the test solution to tubes T and $T_b$.

Add 10.0 mL of *trichloroacetic acid solution R* to tubes $S_{1b}$, $S_{2b}$, $S_{3b}$, $T_b$ and B. Mix by shaking.

Place the tubes and *haemoglobin solution R* in a water bath at 25 ± 0.1 °C. When temperature equilibrium is reached, add 5.0 mL of *haemoglobin solution R* to tubes B, $S_{1b}$, $S_{2b}$, $S_{3b}$ and $T_b$. Mix.

At time zero add 5.0 mL of *haemoglobin solution R* successively and at intervals of 30 s to tubes $S_1$, $S_2$, $S_3$ and T.

Mix immediately after each addition.

Table 0682.-1

| | Tubes | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **$S_1$** | **$S_{1b}$** | **$S_2$** | **$S_{2b}$** | **$S_3$** | **$S_{3b}$** | **T** | **$T_b$** | **B** |
| *Dilute hydrochloric acid R2* (mL) | 0.5 | 0.5 | 0.25 | 0.25 | | | 0.25 | 0.25 | 1.0 |
| Reference solution (mL) | 0.5 | 0.5 | 0.75 | 0.75 | 1.0 | 1.0 | | | |
| Test solution (mL) | | | | | | | 0.75 | 0.75 | |
| *Trichloroacetic acid solution R* (mL) | | 10.0 | | 10.0 | | 10.0 | | 10.0 | 10.0 |
| Mix | | + | | + | | + | | + | + |
| Water bath at 25 °C | + | + | + | + | + | + | + | + | + |
| *Haemoglobin solution R* (mL) | | 5.0 | | 5.0 | | 5.0 | | 5.0 | 5.0 |
| Mix | | + | | + | | + | | + | + |
| *Haemoglobin solution R* (mL) | 5.0 | | 5.0 | | 5.0 | | 5.0 | | |
| Mix | + | | + | | + | | + | | |
| Water bath at 25 °C, 10 min | + | + | + | + | + | + | + | + | + |
| *Trichloroacetic acid solution R* (mL) | 10.0 | | 10.0 | | 10.0 | | 10.0 | | |
| Mix | + | | + | | + | | + | | |
| Filter | + | + | + | + | + | + | + | + | + |

Exactly 10 min after adding the *haemoglobin solution R*, stop the reaction by adding, at intervals of 30 s, 10.0 mL of *trichloroacetic acid solution R* to tubes $S_1$, $S_2$, $S_3$ and T (the use of a fast-flowing or blow-out pipette is recommended) and mix.

Filter the contents of each tube (samples and blanks) twice through the same suitable filter paper previously washed with a 50 g/L solution of *trichloroacetic acid R*, then with *water R* and dried. Discard the first 5 mL of filtrate. Place 3.0 mL of each filtrate separately in a tube containing 20 mL of *water R*. Mix.

*A suitable filter paper complies with the following test*: filter 5 mL of a 50 g/L solution of *trichloroacetic acid R* through a 7 cm disc of white filter paper: the absorbance (*2.2.25*) of the filtrate, measured at 275 nm using unfiltered *trichloroacetic acid R* solution as the compensation liquid, is less than 0.04.

Add to each tube 1.0 mL of *sodium hydroxide solution R* and 1.0 mL of *phosphomolybdotungstic reagent R*, beginning with the blanks and then the samples of each set, in a known order.

A schematic presentation of the above operations is shown in Table 0682.-1.

After 15 min measure the absorbance (*2.2.25*) of solutions $S_1$, $S_2$, $S_3$, $S_{1b}$, $S_{2b}$, $S_{3b}$ and T at 540 nm using the filtrate obtained from tube B as the compensation liquid. Correct the average absorbance values for the filtrates obtained from tubes $S_1$, $S_2$ and $S_3$ by subtracting the average values obtained for the filtrates from tubes $S_{1b}$, $S_{2b}$, $S_{3b}$ respectively.

Draw a calibration curve of the corrected values against volume of reference solution used. Determine the activity of the substance to be examined using the corrected absorbance for the test solution ($T - T_b$) together with the calibration curve and taking into account the dilution factors.

STORAGE

Store in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states the activity in European Pharmacopoeia Units per milligram.

**04/2016:1555**

# PERGOLIDE MESILATE

## Pergolidi mesilas

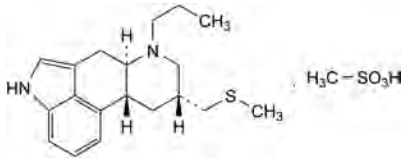

$C_{20}H_{30}N_2O_3S_2$ $M_r$ 410.6
[66104-23-2]

DEFINITION

(6a*R*,9*R*,10a*R*)-9-[(Methylsulfanyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline monomethanesulfonate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in pergolide mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent) and in methylene chloride, very slightly soluble in acetone.

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 23 to – 17 (dried substance).
Dissolve 0.25 g in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *pergolide mesilate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 10 mg of *4,4′-dimethoxybenzophenone R* in *methanol R* and dilute to 10 mL with the same solvent. To 1 mL of the solution add 2 mL of the test solution and dilute to 100 mL with *methanol R*. Dilute 1 mL of this solution to 10 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: mix 5.0 mL of *morpholine for chromatography R* with 995 mL of *water R* and adjust to pH 7.0 with *phosphoric acid R*; use within 24 h;
- *mobile phase B*: *acetonitrile R*, *methanol R*, *tetrahydrofuran R* (1:1:1 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 70 → 0 | 30 → 100 |
| 35 - 40 | 0 → 70 | 100 → 30 |
| 40 - 50 | 70 | 30 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to 4,4′-dimethoxybenzophenone (1st peak) and pergolide (2nd peak).

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solution A.* Dissolve 5.0 mg of *DL-methionine R* in 500 mL of *0.01 M hydrochloric acid.* Add 500 mL of *methanol R* and mix.

*Test solution.* Dissolve 65.0 mg of the substance to be examined in solution A and dilute to 100.0 mL with solution A. Dilute 10.0 mL of this solution to 100.0 mL with solution A.

*Reference solution.* Dissolve 65.0 mg of *pergolide mesilate CRS* in solution A and dilute to 100.0 mL with solution A. Dilute 10.0 mL of this solution to 100.0 mL with solution A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 1 volume of *acetonitrile R*, 1 volume of *methanol R* and 2 volumes of a mixture prepared as follows: dissolve 2.0 g of *sodium octanesulfonate R* in *water R*, add 1.0 mL of *anhydrous acetic acid R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Retention time*: pergolide = about 9 min.

*System suitability*: reference solution:

– *symmetry factor*: maximum 1.5 for the peak due to pergolide.

Calculate the percentage content of $C_{20}H_{30}N_2O_3S_2$ from the assigned content of *pergolide mesilate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

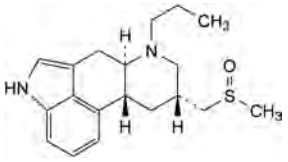

A. (6a*R*,9*R*,10a*R*)-9-[(methylsulfinyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline (pergolide sulfoxide),

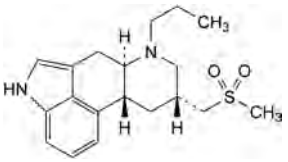

B. (6a*R*,9*R*,10a*R*)-9-[(methylsulfonyl)methyl]-7-propyl-4,6,6a,7,8,9,10,10a-octahydroindolo[4,3-*fg*]quinoline (pergolide sulfone).

**01/2008:2019**

# PERINDOPRIL *tert*-BUTYLAMINE

## *tert*-Butylamini perindoprilum

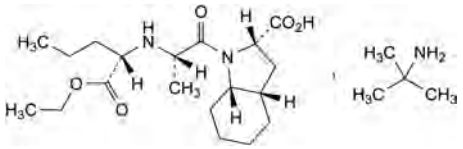

$C_{23}H_{43}N_3O_5$ $M_r$ 441.6

[107133-36-8]

DEFINITION

2-Methylpropan-2-amine (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), soluble or sparingly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 69 to – 66 (anhydrous substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *perindopril tert-butylamine CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

C. Examine the chromatograms obtained in the test for impurity A.

*Results*: in the chromatogram obtained with the test solution a spot is observed with the same $R_F$ as the spot with the higher $R_F$ in the chromatogram obtained with reference solution (c) (*tert*-butylamine).

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *perindopril impurity A CRS* in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 20.0 mL with *methanol R*.

*Reference solution (c).* To 5 mL of reference solution (a) add 5 mL of a 20 g/L solution of *1,1-dimethylethylamine R* in *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *toluene R*, *methanol R* (1:40:60 *V/V/V*).

*Application*: 10 µL of the test solution and reference solutions (b) and (c).

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: expose to iodine vapour for at least 20 h.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

**Stereochemical purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *ethanol (96 per cent) R*. Dilute 1.0 mL of this solution to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *perindopril for stereochemical purity CRS* (containing impurity I) in *ethanol (96 per cent) R* and dilute to 5.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 50 °C for the column and the tubing preceding the column (the method has been developed with a temperature of 50 °C for at least 30 cm of the tubing preceding the column).

*Mobile phase*: mix, in the following order, 21.7 volumes of *acetonitrile R*, 0.3 volumes of *pentanol R*, and 78 volumes of a 1.50 g/L solution of *sodium heptanesulfonate R* previously adjusted to pH 2.0 with a mixture of equal volumes of *perchloric acid R* and *water R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Equilibration*: minimum 4 h.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *perindopril for stereochemical purity CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity I.

*Run time*: 1.5 times the retention time of perindopril.

*Relative retention* with reference to perindopril (retention time = about 100 min): impurity I = about 0.9.

*System suitability*:

– the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *perindopril for stereochemical purity CRS*;
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (a);
– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to perindopril in the chromatogram obtained with reference solution (b).

*Limits*:

– *impurity I*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *disregard limit*: disregard any peak with a relative retention with reference to perindopril of less than 0.6 or more than 1.4.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or maintain them at a temperature below 10 °C.*

*Test solution.* Dissolve 60 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 3 mg of *perindopril for peak identification CRS* (containing impurities B, E, F, H and K) in 1 mL of mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with mobile phase A.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4 mm;
– *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a pore size of 15 nm;
– *temperature*: 60 °C for the column and the tubing preceding the column.

*Mobile phase*:

– *mobile phase A*: *water R* adjusted to pH 2.5 with a mixture of equal volumes of *perchloric acid R* and *water R*;
– *mobile phase B*: 0.03 per cent *V/V* solution of *perchloric acid R* in *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - (5 − *t*) | 95 | 5 |
| (5 − *t*) - (60 − *t*) | 95 → 40 | 5 → 60 |
| (60 − *t*) - (65 − *t*) | 40 → 95 | 60 → 5 |

The isocratic step is described for a chromatographic system with a dwell volume (*D*) of 2 mL. If *D* is different from 2 mL, correct the gradient times with the value *t*, calculated using the following expression:

$$\frac{D - 2}{\text{flow rate}}$$

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *perindopril for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, E, F, H and K.

*Relative retention* with reference to perindopril (retention time = about 25 min): impurity B = about 0.68; impurity K = about 0.72; impurity E = about 1.2; impurity F = about 1.6; impurity H = about 1.8 (impurity H may be eluted as 1 or 2 peaks).

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity K.

*Limits*:

– *impurity E*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
– *impurity B*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);

- *impurities F, H*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.08 mg of $C_{23}H_{43}N_3O_5$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, E, F, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, G, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, AA, BB, CC.*

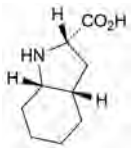

A. (2*S*,3a*S*,7a*S*)-octahydro-1*H*-indole-2-carboxylic acid,

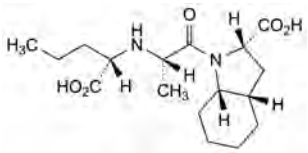

B. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxybutyl]amino]-propanoyl]octahydro-1*H*-indole-2-carboxylic acid (perindoprilat),

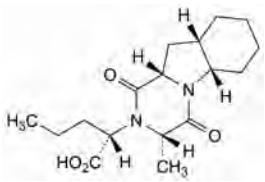

C. (2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*S*)-3-methyl-1,4-dioxodecahydro-pyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,

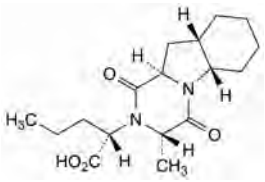

D. (2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*R*)-3-methyl-1,4-dioxodecahydro-pyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoic acid,

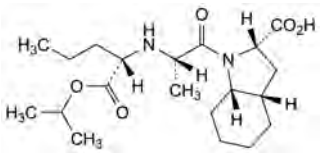

E. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-[(1-methylethoxy)-carbonyl]butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

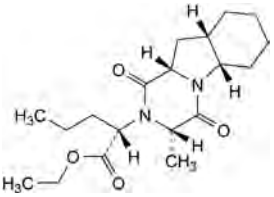

F. ethyl (2*S*)-2-[(3*S*,5a*S*,9a*S*,10a*S*)-3-methyl-1,4-dioxo-decahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]pentanoate,

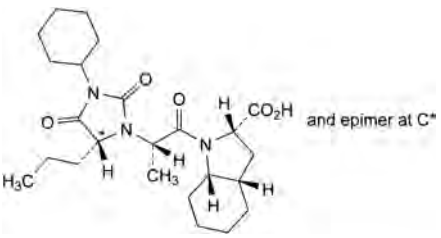


G. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2,4-dioxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

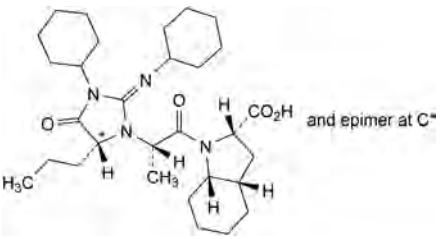


H. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[(5*RS*)-3-cyclohexyl-2-(cyclohexylimino)-4-oxo-5-propylimidazolidin-1-yl]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

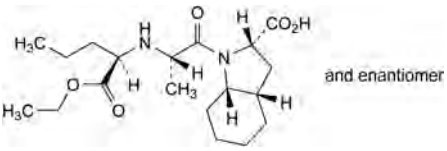


I. (2*RS*,3a*RS*,7a*RS*)-1-[(2*RS*)-2-[[(1*SR*)-1-(ethoxycarbonyl)-butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid ((±)-1″-*epi*-perindopril),

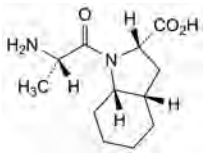

J. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-aminopropanoyl]octahydro-1*H*-indole-2-carboxylic acid,

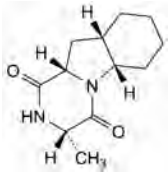

K. (3*S*,5a*S*,9a*S*,10a*S*)-3-methyldecahydropyrazino[1,2-*a*]indole-1,4-dione,

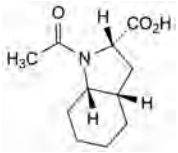

L. (2*S*,3a*S*,7a*S*)-1-acetyloctahydro-1*H*-indole-2-carboxylic acid,

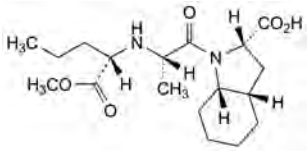

M. (2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-(methoxycarbonyl)-butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

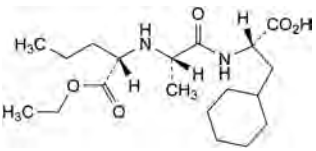

N. (2*S*)-3-cyclohexyl-2-[[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-butyl]amino]propanoyl]amino]propanoic acid,

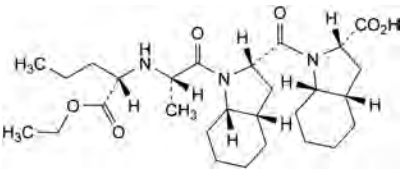

O. (2*S*,3a*S*,7a*S*)-1-[[(2*S*,3a*S*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indol-2-yl]carbonyl]octahydro-1*H*-indole-2-carboxylic acid,

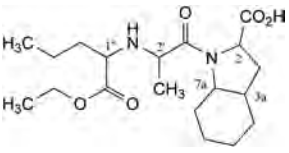

1-[2-[[1-(ethoxycarbonyl)butyl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid,

P. (2*RS*,3a*RS*,7a*RS*)-, (2′*SR*)-, (1″*RS*)-: (±)-2′-*epi*-perindopril,

Q. (2*RS*,3a*RS*,7a*SR*)-, (2′*RS*)-, (1″*RS*)-: (±)-7a-*epi*-perindopril,

R. (2*RS*,3a*SR*,7a*RS*)-, (2′*RS*)-, (1″*RS*)-: (±)-3a-*epi*-perindopril,

S. (2*SR*,3a*RS*,7a*RS*)-, (2′*RS*)-, (1″*RS*)-: (±)-2-*epi*-perindopril,

T. (2*RS*,3a*RS*,7a*RS*)-, (2′*SR*)-, (1″*SR*)-: (±)-1″,2′-di-*epi*-perindopril,

U. (2*RS*,3a*RS*,7a*SR*)-, (2′*RS*)-, (1″*SR*)-: (±)-1″,7a-di-*epi*-perindopril,

V. (2*SR*,3a*SR*,7a*RS*)-, (2′*RS*)-, (1″*RS*)-: (±)-2,3a-di-*epi*-perindopril,

W. (2*SR*,3a*RS*,7a*RS*)-, (2′*RS*)-, (1″*SR*)-: (±)-1″,2-di-*epi*-perindopril,

X. (2*SR*,3a*RS*,7a*SR*)-, (2′*RS*)-, (1″*RS*)-: (±)-2,7a-di-*epi*-perindopril,

Y. (2*SR*,3a*RS*,7a*RS*)-, (2′*SR*)-, (1″*RS*)-: (±)-2,2′-di-*epi*-perindopril,

Z. (2*RS*,3a*SR*,7a*RS*)-, (2′*RS*)-, (1″*SR*)-: (±)-1″,3a-di-*epi*-perindopril,

AA. (2*RS*,3a*SR*,7a*SR*)-, (2′*RS*)-, (1″*RS*)-: (±)-3a,7a-di-*epi*-perindopril,

BB. (2*RS*,3a*SR*,7a*RS*)-, (2′*SR*)-, (1″*RS*)-: (±)-2′,3a-di-*epi*-perindopril,

CC. (2*RS*,3a*RS*,7a*SR*)-, (2′*SR*)-, (1″*RS*)-: (±)-2′,7a-di-*epi*-perindopril.

**01/2019:0862**

# PERITONEAL DIALYSIS, SOLUTIONS FOR

## Solutiones ad peritonealem dialysem

DEFINITION

Preparations for intraperitoneal use containing electrolytes with a concentration close to the electrolytic composition of plasma. They contain glucose in varying concentrations or other suitable osmotic agents.

Solutions for peritoneal dialysis are supplied in:

- rigid or semi-rigid plastic containers;
- flexible plastic containers fitted with a special connecting device; these are generally filled to a volume below their nominal capacity and presented in closed protective envelopes;
- glass containers.

The containers and closures comply with the requirements for containers for preparations for parenteral administration (*3.2*).

Several formulations are used. The concentrations of the components per litre of solution are usually in the following range (see Table 0862.-1).

Table 0862.-1.

| | Concentration (mmol/L) | Concentration (mEq/L) |
|---|---|---|
| Sodium | 125 - 150 | 125 - 150 |
| Potassium | 0 - 4.5 | 0 - 4.5 |
| Calcium | 0 - 2.5 | 0 - 5.0 |
| Magnesium | 0.25 - 1.5 | 0.50 - 3.0 |
| Acetate and/or lactate and/or hydrogen carbonate | 30 - 60 | 30 - 60 |
| Chloride | 90 - 120 | 90 - 120 |
| Glucose | 25 - 250 | |

When hydrogen carbonate is present, the solution of sodium hydrogen carbonate is supplied in a container or a separate compartment and is added to the electrolyte solution immediately before use.

Unless otherwise justified and authorised, antioxidants are not added to the solutions.

IDENTIFICATION

According to the stated composition, the solution to be examined gives the following identification reactions (*2.3.1*):

- potassium: reaction (b);
- calcium: reaction (a);
- sodium: reaction (b);
- chlorides: reaction (a);
- acetates: to 5 mL of the solution to be examined add 1 mL of *hydrochloric acid R* in a test-tube fitted with a stopper and a bent tube, heat and collect a few millilitres of distillate; carry out reaction (b) of acetates on the distillate;
- lactates, hydrogen carbonates: the identification is carried out together with the assay;
- magnesium: to 0.1 mL of *titan yellow solution R* add 10 mL of *water R*, 2 mL of the solution to be examined and 1 mL of *1 M sodium hydroxide*; a pink colour is produced;
- glucose: to 5 mL of the solution to be examined, add 2 mL of *dilute sodium hydroxide solution R* and 0.05 mL of *copper sulfate solution R*; the solution is blue and clear; heat to boiling; an abundant red precipitate is formed.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_4$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 5.0 to 6.5. If the solution contains hydrogen carbonate, the pH is 6.5 to 8.0.

**Hydroxymethylfurfural**. Carry out the test only if glucose is added to the preparation. To a volume of the solution to be examined containing the equivalent of 25 mg of glucose, add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R*, then add 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*) determined at 550 nm after allowing the mixture to stand for 2-3 min is not greater than that of a standard prepared at the same time and in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the solution to be examined (400 ppm expressed with reference to the glucose concentration). If the solution contains hydrogen carbonate, use as the standard a solution containing 20 µg of *hydroxymethylfurfural R* (800 ppm expressed with reference to the glucose concentration).

**Aluminium**: maximum 10 µg/L.

Atomic absorption spectrometry (*2.2.23, Method I or II*). Use a matrix modifier (for example *nitric acid R* and *magnesium nitrate R* in *water R*) in the same quantity for the test solution, the reference solutions and the blank solution.

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions. Method I – direct calibration.*

Prepare the reference solutions by diluting, for example *aluminium standard solution (10 ppm Al) R* with acidified *water R*.

*Reference solutions. Method II – standard additions.*

Prepare at least 3 reference solutions in the test solution, in a range spanning the expected aluminium concentration of the test solution, for example by diluting *aluminium standard solution (10 ppm Al) R* with the test solution.

*Blank solution*: *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: graphite furnace.

**Particulate contamination** (*2.9.19, Method I*). Use 50 mL of the solution to be examined.

**Extractable volume** (*2.9.17*). The solution complies with the test prescribed for parenteral infusions.

**Sterility** (*2.6.1*). The solution complies with the test.

**Bacterial endotoxins** (*2.6.14*): less than 0.05 IU/mL, unless otherwise justified and authorised.

**Pyrogens** (*2.6.8*). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the solution.

ASSAY

**Sodium**: 97.5 per cent to 102.5 per cent of the content of sodium (Na) stated on the label.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*.

*Wavelengths*: 589.0 nm or 589.6 nm (sodium emits as a doublet).

**Potassium**: 95.0 per cent to 105.0 per cent of the content of potassium (K) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used. To 100 mL of the solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Reference solutions.* Prepare the reference solutions using *potassium standard solution (100 ppm K) R*. To 100 mL of each reference solution add 10 mL of a 22 g/L solution of *sodium chloride R*.

*Source*: potassium hollow-cathode lamp.

*Wavelength*: 766.5 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Calcium**: 95.0 per cent to 105.0 per cent of the content of calcium (Ca) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions.* Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Magnesium**: 95.0 per cent to 105.0 per cent of the content of magnesium (Mg) stated on the label.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* If necessary, dilute the solution to be examined with *water R* to a concentration suitable for the instrument to be used.

*Reference solutions.* Prepare the reference solutions using *magnesium standard solution (100 ppm Mg) R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-propane or air-acetylene flame.

**Total chloride**: 95.0 per cent to 105.0 per cent of the content of chloride (Cl) stated on the label.

Dilute an accurately measured volume of the solution to be examined containing the equivalent of about 0.68 mEq of

chloride with an appropriate volume of *water R* in order to immerse the electrode. Carry out a potentiometric titration (*2.2.20*), using *0.1 M silver nitrate*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Acetate**: 95.0 per cent to 105.0 per cent of the content of acetate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of acetate, add 10.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of acetate.

**Lactate**: 95.0 per cent to 105.0 per cent of the content of lactate stated on the label.

To a volume of the solution to be examined, corresponding to about 0.7 mmol of lactate, add 10.0 mL of *0.1 M hydrochloric acid*. Add 50 mL of *acetonitrile R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 0.1 mmol of lactate.

**Sodium hydrogen carbonate**: 95.0 per cent to 105.0 per cent of the content of sodium hydrogen carbonate stated on the label.

Titrate with *0.1 M hydrochloric acid*, a volume of the solution to be examined corresponding to about 0.1 g of sodium hydrogen carbonate, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 8.40 mg of $NaHCO_3$.

**Lactate and hydrogen carbonate**: 95.0 per cent to 105.0 per cent of the content of lactates and hydrogen carbonates stated on the label.

Liquid chromatography (*2.2.29*).

*Test solution*. The solution to be examined.

*Reference solution*. Dissolve in 100 mL of *water for chromatography R* quantities of lactates and hydrogen carbonates, accurately weighed, in order to obtain solutions having concentrations representing about 90 per cent, 100 per cent and 110 per cent of the concentrations stated on the label.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *cation-exchange resin R* (9 µm);
- *temperature*: 60 °C.

*Mobile phase*: *0.005 M sulfuric acid* previously degassed with *helium for chromatography R*.

*Flow rate*: 0.6 mL/min.

*Detection*: differential refractometer.

*Injection*: 20 µL, twice.

*Order of elution*: lactates, hydrogen carbonates.

Determine the concentration of lactate and hydrogen carbonates in the test solution by interpolating the peak area for lactate and the peak height for hydrogen carbonate from the linear regression curve obtained with the reference solutions.

**Reducing sugars** (expressed as glucose): 95.0 per cent to 105.0 per cent of the content of glucose stated on the label.

Transfer a volume of the solution to be examined containing the equivalent of 25 mg of glucose to a 250 mL conical flask with a ground-glass neck and add 25.0 mL of *cupri-citric solution R*. Add a few grains of pumice, fit a reflux condenser, heat so that boiling occurs within 2 min and boil for exactly 10 min. Cool and add 3 g of *potassium iodide R* dissolved in 3 mL of *water R*. Carefully add, in small amounts, 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate* using *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration using 25.0 mL of *water R*.

Calculate the content of reducing sugars expressed as glucose ($C_6H_{12}O_6$), using Table 0862.-2.

Table 0862.-2.

| Volume of *0.1 M sodium thiosulfate* (mL) | Glucose (mg) |
|---|---|
| 8 | 19.8 |
| 9 | 22.4 |
| 10 | 25.0 |
| 11 | 27.6 |
| 12 | 30.3 |
| 13 | 33.0 |
| 14 | 35.7 |
| 15 | 38.5 |
| 16 | 41.3 |

STORAGE

At 4 °C or above.

LABELLING

The label states:

- the formula of the solution for peritoneal dialysis, expressed in grams per litre and in millimoles per litre;
- the calculated osmolarity, expressed in milliosmoles per litre;
- the nominal volume of the solution for peritoneal dialysis in the container;
- that the solution is free from bacterial endotoxins, or where applicable, that it is apyrogenic;
- the storage conditions;
- that the solution is not to be used for intravenous infusion;
- that any unused portion of the solution is to be discarded.

**07/2015:1762**
**corrected 10.0**

# PERMETHRIN (25:75)

## Permethrinum 25:75

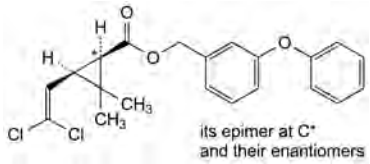


$C_{21}H_{20}Cl_2O_3$ $M_r$ 391.3

Undefined *cis/trans* ratio: [52645-53-1]

DEFINITION

Mixture of the *cis* and *trans* isomers of (3-phenoxyphenyl)-methyl (1*RS*,2*RS*)-2-(2,2-dichloroethenyl)-3,3-dimethylcyclopropane-1-carboxylate.

*Content*:

- *total*: 98.0 per cent to 102.0 per cent (anhydrous substance);
- *cis isomer*: 23.0 per cent to 27.0 per cent (anhydrous substance);
- *trans isomer*: 73.0 per cent to 77.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: colourless or slightly brownish viscous liquid, semi-solid or crystalline solid.

*Solubility*: practically insoluble in water, very soluble in heptane, freely soluble in anhydrous ethanol, sparingly soluble in ethylene glycol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *permethrin (25:75) CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and in *cis/trans* ratio to the 2 principal peaks in the chromatogram obtained with the reference solution (*cis/trans* ratio = about 0.3).

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Warm the substance to be examined at 70-85 °C for 20 min. Dissolve 1.0 g of this substance in *heptane R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50 mg of *permethrin for system suitability CRS* (containing impurities B, C and G) in *heptane R* and dilute to 1.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *heptane R*. Dilute 1.0 mL of this solution to 20.0 mL with *heptane R*.

*Column*:
- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1.5 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 12.0 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 45 |
| | 2 - 26.5 | 45 → 290 |
| | 26.5 - 40.5 | 290 |
| Injection port | | 250 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram supplied with *permethrin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C and G.

*Relative retention* with reference to permethrin (retention time = about 24 min): impurity B = about 0.5; impurity C = about 0.7; impurity G = about 0.9.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurity G and permethrin.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.5;
- *impurities B, C*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent (reference solution (b)).

**Water** (*2.5.32*): maximum 0.25 per cent, determined on 0.100 g using the evaporation technique:
- *temperature*: 110 °C;
- *heating time*: 3 min.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Warm the substance to be examined at 70-85 °C for 20 min. Dissolve 0.100 g of this substance in the mobile phase using sonication and dilute to 50.0 mL with the mobile phase.

*Reference solution*. Warm *permethrin (25:75) CRS* at 70-85 °C for 20 min. Dissolve 0.100 g of this substance in the mobile phase using sonication and dilute to 50.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *dioxan R*, *heptane R* (1.1:98.9 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of *cis*-permethrin.

*Retention time*: *cis*-permethrin = about 6 min.

*Elution order*: *cis*-permethrin, *trans*-permethrin.

*System suitability*: reference solution:
- *resolution*: minimum 2.0 between the peaks due to *cis*-permethrin and *trans*-permethrin.

*Calculation of percentage contents*:
- calculate the percentage content of *cis*-permethrin and *trans*-permethrin by normalisation;
- calculate the percentage content of $C_{21}H_{20}Cl_2O_3$ taking into account the assigned content of *permethrin (25:75) CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F, G, H.*

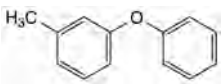


A. 1-methyl-3-phenoxybenzene,

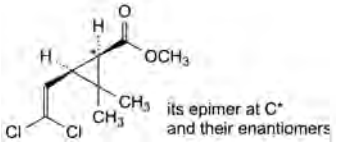


B. methyl (1*RS*,2*RS*)-2-(2,2-dichloroethenyl)-3,3-dimethylcyclopropane-1-carboxylate,

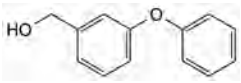

C. (3-phenoxyphenyl)methanol,

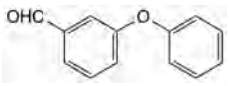

D. 3-phenoxybenzaldehyde,

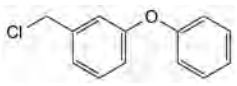

E. 1-(chloromethyl)-3-phenoxybenzene,

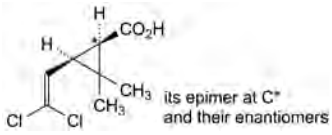


F. (1*RS*,2*RS*)-2-(2,2-dichloroethenyl)-3,3-dimethylcyclopropane-1-carboxylic acid,

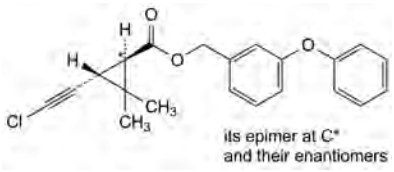


G. 3-(phenoxyphenyl)methyl (1*RS*,2*RS*)-2-(chloroethynyl)-3,3-dimethylcyclopropane-1-carboxylate,

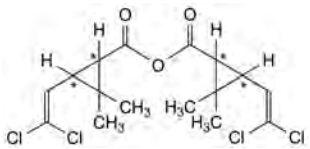

H. 2-(2,2-dichloroethenyl)-3,3-dimethylcyclopropane-1-carboxylic anhydride.

**01/2009:0629**

# PERPHENAZINE

## Perphenazinum

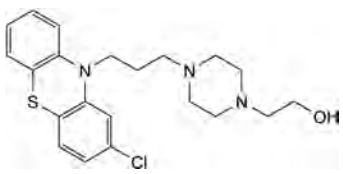

$C_{21}H_{26}ClN_3OS$ $M_r$ 404.0
[58-39-9]

DEFINITION

2-[4-[3-(2-Chloro-10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent). It dissolves in dilute solutions of hydrochloric acid.

IDENTIFICATION

A. Melting point (*2.2.14*): 96 °C to 100 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *perphenazine CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.20 g in 10 mL of *methanol R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use. Carry out the test protected from light.*

*Test solution*. Dissolve 20 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 2 mg of *perphenazine for system suitability CRS* (containing impurities A and B) in 1.0 mL of mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *base-deactivated octylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 35 volumes of *acetonitrile R* and 65 volumes of a 7 g/L solution of *sodium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 10 | 100 → 80 | 0 → 20 |
| 10 - 33 | 80 → 30 | 20 → 70 |
| 33 - 48 | 30 → 100 | 70 → 0 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *perphenazine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to perphenazine (retention time = about 12 min): impurity A = about 0.3; impurity B = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to impurity B and perphenazine.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.6;
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 65 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.20 mg of $C_{21}H_{26}ClN_3OS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B*.

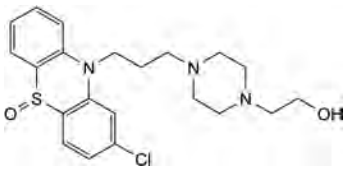

A. 2-[4-[3-(2-chloro-5-oxido-10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol,

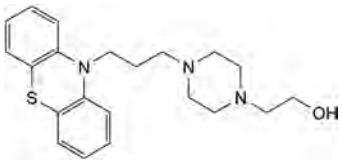

B. 2-[4-[3-(10*H*-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol.

**01/2008:0420**
**corrected 7.0**

# PETHIDINE HYDROCHLORIDE

## Pethidini hydrochloridum

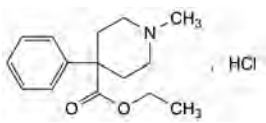

$C_{15}H_{22}ClNO_2$ $M_r$ 283.8
[50-13-5]

DEFINITION

Ethyl 1-methyl-4-phenylpiperidine-4-carboxylate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

If intended for use in the manufacture of parenteral preparations, the manufacturing process is validated to show that the content of impurity B is not more than 0.1 ppm.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in alcohol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 187 °C to 190 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of pethidine hydrochloride.*

C. Dissolve 0.1 g in 10 mL of *ethanol R* and add 10 mL of *picric acid solution R*. A crystalline precipitate is formed which, when washed with *water R* and dried at 100-105 °C, melts (*2.2.14*) at 186 °C to 193 °C. Mix equal quantities of the precipitate and the substance to be examined and determine the melting point of the mixture. The melting point is at least 20 °C lower than that of the precipitate.

D. To 5 mL of solution S (see Tests) add 5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is red.

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 25.0 mL with the same mixture of solvents.

*Test solution (b).* Dissolve 0.125 g of the substance to be examined in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 0.5 mL of test solution (a) to 100.0 mL with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

*Reference solution (b).* Dissolve 10.0 mg of *pethidine impurity A CRS* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 12.5 mg of *1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine R* in a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R* and dilute to 10.0 mL with the same mixture of solvents. Dilute 1.0 mL of the solution to 100.0 mL with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

*Reference solution (d).* Dilute 5.0 mL of reference solution (b) and 1.0 mL of reference solution (c) to 100.0 mL with a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm,

– *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 340 $m^2$/g, a pore size of 10 nm and a carbon loading of 19 per cent.

*Mobile phase*:

– *mobile phase A*: mix equal volumes of a 42.0 g/L solution of *sodium perchlorate R* and of a 11.6 g/L solution of *phosphoric acid R*, adjust to pH 2.0 with *triethylamine R*,

– *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 80 → 75 | 20 → 25 |
| 15 - 31 | 75 → 55 | 25 → 45 |
| 31 - 40 | 55 | 45 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL; inject test solution (b) and reference solution (d).

*Relative retention* with reference to pethidine (retention time = about 24 min): impurity B = about 0.66; impurity A = about 0.68.

*System suitability*: reference solution (d):

- *signal-to-noise ratio*: minimum 10 for the first peak,
- *peak-to-valley ratio*: minimum 4, where $H_p$ = height above the baseline of the peak due to impurity B, and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A.

*Limit*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (10 ppm) if intended for non-parenteral administration.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for impurity B with the following modifications.

*Injection*: 20 µL; inject test solution (a) and reference solution (a).

*Limits*:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.220 g in 50 mL of *alcohol R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.38 mg of $C_{15}H_{22}ClNO_2$.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

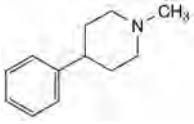

A. 1-methyl-4-phenylpiperidine (MPP),

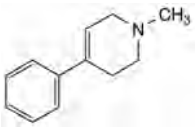

B. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),

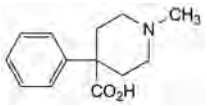

C. 1-methyl-4-phenylpiperidine-4-carboxylic acid,

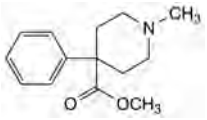

D. methyl 1-methyl-4-phenylpiperidine-4-carboxylate,

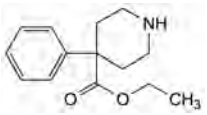

E. ethyl 4-phenylpiperidine-4-carboxylate,

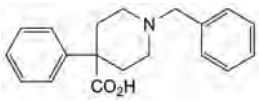

F. 1-benzyl-4-phenylpiperidine-4-carboxylic acid,

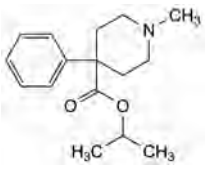

G. 1-methylethyl 1-methyl-4-phenylpiperidine-4-carboxylate,

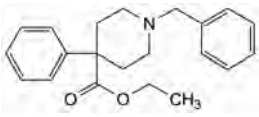

H. ethyl 1-benzyl-4-phenylpiperidine-4-carboxylate,

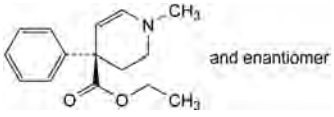


I. ethyl (4*RS*)-1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine-4-carboxylate,

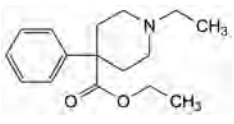

J. ethyl 1-ethyl-4-phenylpiperidine-4-carboxylate.

01/2017:0421

# PHENAZONE

## Phenazonum

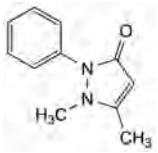

$C_{11}H_{12}N_2O$ $M_r$ 188.2
[60-80-0]

DEFINITION

1,5-Dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *A, B*.

*Second identification*: *A, C, D*.

A. Melting point (*2.2.14*): 109 °C to 113 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *phenazone CRS*.

C. To 1 mL of solution S (see Tests) add 4 mL of *water R* and 0.25 mL of *dilute sulfuric acid R*. Add 1 mL of *sodium nitrite solution R*; a green colour develops.

D. To 1 mL of solution S add 4 mL of *water R* and 0.5 mL of *ferric chloride solution R2*. A red colour develops which is discharged on the addition of *dilute sulfuric acid R*.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*; the solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*; the solution is red. Add 0.25 mL of *methyl red solution R* and 0.4 mL of *0.01 M hydrochloric acid*; the solution is red or yellowish-red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *phenazone impurity A CRS* in the mobile phase, add 10 mL of the test solution and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5.0 mg of *phenazone impurity A CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 6.0 mm;
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 6.8 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. Add 2 mL of *triethylamine R* and adjust to pH 7.0 with *sodium hydroxide solution R*. Add 430 mL of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of phenazone.

*Relative retention* with reference to phenazone (retention time = about 13 min): impurity A = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.0 between the peaks due to impurity A and phenazone.

*Limits*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.05 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dissolve 1.5 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 20 mL of *water R*. Add 2 g of *sodium acetate R*, 1 mL of *dilute acetic acid R* and 25.0 mL of *0.05 M iodine*. Allow to stand protected from light for 30 min. Add 25 mL of *methylene chloride R* and shake until the precipitate dissolves. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 9.41 mg of $C_{11}H_{12}N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

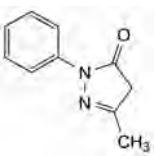

A. 5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one.

**01/2017:1357**

# PHENIRAMINE MALEATE

## Pheniramini maleas

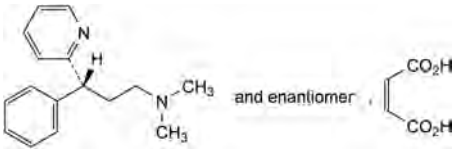


$C_{20}H_{24}N_2O_4$ $M_r$ 356.4
[132-20-7]

DEFINITION

(3*RS*)-*N*,*N*-Dimethyl-3-phenyl-3-(pyridin-2-yl)propan-1-amine (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 106 °C to 109 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 40.0 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of the solution to 50.0 mL with *0.1 M hydrochloric acid.*

*Spectral range*: 220-320 nm.

*Absorption maximum*: at 265 nm.

*Shoulder*: at 261 nm.

*Specific absorbance at the absorption maximum*: 200 to 220.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pheniramine maleate CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 65 mg of *maleic acid R* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 0.10 g of *pheniramine maleate CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *water R*, *anhydrous formic acid R*, *methanol R*, *di-isopropyl ether R* (3:7:20:70 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the chromatogram obtained with the test solution shows 2 clearly separated spots; the upper spot is similar in position and size to the spot in the chromatogram obtained with reference solution (a); the lower spot is similar in position and size to the lower spot in the chromatogram obtained with reference solution (b).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 5.5.

Dissolve 0.20 g in 20 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, mobile phase A (10:90 *V/V*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10.0 mg of *pheniramine impurity A CRS* and 10 mg of *4-benzylpyridine R* (impurity B) in 10.0 mL of the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 10.0 mL with reference solution (a). Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.30 m, Ø = 3.9 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 5.056 g of *sodium heptanesulfonate R* in 900 mL of *water R*, adjust to pH 2.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 37 | 90 → 62 | 10 → 38 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to pheniramine (retention time = about 31 min): maleic acid = about 0.1; impurity A = about 0.9; impurity B = about 0.97.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to impurity B and pheniramine.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: maximum 1.0 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.130 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.82 mg of $C_{20}H_{24}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

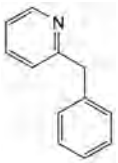

A. 2-benzylpyridine,

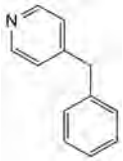

B. 4-benzylpyridine.



**04/2012:0201**

# PHENOBARBITAL

## Phenobarbitalum

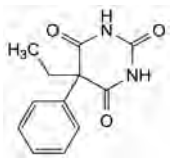

$C_{12}H_{12}N_2O_3$ $M_r$ 232.2

[50-06-6]

DEFINITION

5-Ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent).

It forms water-soluble compounds with alkali hydroxides, carbonates and ammonia.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Determine the melting point (*2.2.14*) of the substance to be examined. Mix equal parts of the substance to be examined and *phenobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 176 °C) is not greater than 2 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenobarbital CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *phenobarbital CRS* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in a mixture of 4 mL of *dilute sodium hydroxide solution R* and 6 mL of *water R*.

**Acidity**. Boil 1.0 g with 50 mL of *water R* for 2 min, allow to cool and filter. To 10 mL of the filtrate add 0.15 mL of *methyl red solution R*. The solution is orange-yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to produce a pure yellow colour.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.125 g of the substance to be examined in 5.0 mL of *methanol R* and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Mix 1.0 mL of the test solution and 20.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase. Mix 1.0 mL of this solution with 2.0 mL of *methanol R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *phenobarbital impurity A CRS* and 5.0 mg of *phenobarbital impurity B CRS* in 2.0 mL of *methanol R* and dilute to 10.0 mL with the mobile phase. Mix 1.0 mL of this solution with 20.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*. Dissolve 6.60 g of *sodium acetate R* in 900 mL of *water R*, add 3 mL of *glacial acetic acid R*, adjust to pH 4.5 with *glacial acetic acid R* and dilute to 1000 mL with *water R*. Mix 60 volumes of this solution with 40 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 2.1 times the retention time of phenobarbital.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to phenobarbital (retention time = about 14 min): impurity A = about 0.2; impurity B = about 0.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities A and B.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *impurity B*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 40 mL of *ethanol (96 per cent) R* and add 20 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 23.22 mg of $C_{12}H_{12}N_2O_3$.

IMPURITIES

*Specified impurities*: *A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

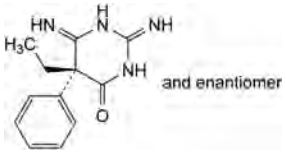


A. (5*RS*)-5-ethyl-2,6-diimino-5-phenyltetrahydropyrimidin-4(1*H*)-one,

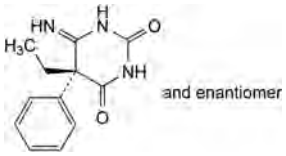


B. (5*RS*)-5-ethyl-6-imino-5-phenyldihydropyrimidine-2,4(1*H*,3*H*)-dione,

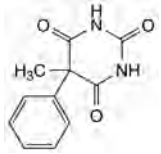


C. 5-methyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione.

**07/2016:0630**

# PHENOBARBITAL SODIUM

## Phenobarbitalum natricum

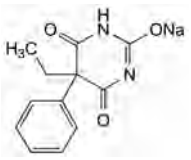


$C_{12}H_{11}N_2NaO_3$ $M_r$ 254.2
[57-30-7]

DEFINITION

Sodium derivative of 5-ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in carbon dioxide-free water (a small fraction may be insoluble), soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Acidify 10 mL of solution S (see Tests) with *dilute hydrochloric acid R* and shake with 20 mL of *ether R*. Separate the ether layer, wash with 10 mL of *water R*, dry over *anhydrous sodium sulfate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100-105 °C (test residue). Determine the melting point (*2.2.14*) of the test residue. Mix equal parts of the test residue and *phenobarbital CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 176 °C) is not greater than 2 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: use the test residue obtained in identification test A.

*Comparison*: *phenobarbital CRS*.

If the spectra obtained in the solid state show differences, dissolve the test residue and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *ethanol (50 per cent V/V) R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 9 mg of *phenobarbital CRS* in *ethanol (50 per cent V/V) R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R, ethanol (96 per cent) R, methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine immediately in ultraviolet light at 254 nm.

Monographs p

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

E. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *ethanol (50 per cent V/V) R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): maximum 10.2.

Dissolve 5.0 g as completely as possible in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 55.0 mg of the substance to be examined in 2.0 mL of *methanol R* and dilute to 10.0 mL with the mobile phase.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in 10.0 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Mix 1.0 mL of test solution (b) and 20.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *phenobarbital impurity A CRS* and 5.0 mg of *phenobarbital impurity B CRS* in 2.0 mL of *methanol R* and dilute to 10.0 mL with the mobile phase. Mix 1.0 mL of the solution and 20.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25.0 mg of *phenobarbital CRS* in 10.0 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Dissolve 6.6 g of *sodium acetate R* in 900 mL of *water R*, add 3 mL of *glacial acetic acid R*, adjust to pH 4.5 with *glacial acetic acid R* and dilute to 1000 mL with *water R*. Mix 60 volumes of this solution and 40 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of phenobarbital.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to phenobarbital (retention time = about 13 min): impurity A = about 0.29; impurity B = about 0.32.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

– for impurity A, use the concentration of impurity A in reference solution (b);

– for impurity B, use the concentration of impurity B in reference solution (b);

– for impurities other than A and B, use the concentration of phenobarbital sodium in reference solution (a).

*Limits*:

– *impurities A, B*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 0.500 g by drying in an oven at 150 °C for 4 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: 10 µL of test solution (b) and reference solution (c).

*Run time*: 1.5 times the retention time of phenobarbital.

Calculate the percentage content of $C_{12}H_{11}N_2NaO_3$ taking into account the assigned content of *phenobarbital CRS* and a conversion factor of 1.095.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E.*

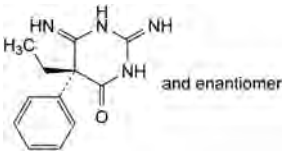


A. (5*RS*)-5-ethyl-2,6-diimino-5-phenyltetrahydropyrimidin-4(1*H*)-one,

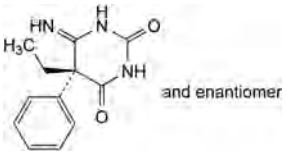


B. (5*RS*)-5-ethyl-6-imino-5-phenyldihydropyrimidine-2,4(1*H*,3*H*)-dione,

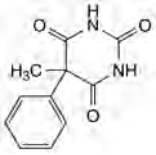


C. 5-methyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione,

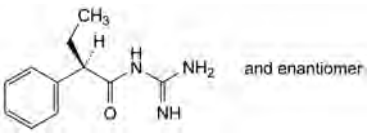


E. (2*RS*)-*N*-carbamimidoyl-2-phenylbutanamide.

**01/2008:0631**
**corrected 6.3**

# PHENOL

## Phenolum

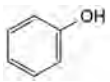

$C_6H_6O$ $M_r$ 94.1
[108-95-2]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: colourless or faintly pink or faintly yellowish, crystals or crystalline masses, deliquescent.

*Solubility*: soluble in water, very soluble in ethanol (96 per cent), in glycerol and in methylene chloride.

IDENTIFICATION

A. Dissolve 0.5 g in 2 mL of *concentrated ammonia R*. The substance dissolves completely. Dilute to about 100 mL with *water R*. To 2 mL of this solution add 0.05 mL of *strong sodium hypochlorite solution R*. A blue colour develops and becomes progressively more intense.

B. To 1 mL of solution S (see Tests) add 10 mL of *water R* and 0.1 mL of *ferric chloride solution R1*. A violet colour is produced which disappears on addition of 5 mL of *2-propanol R*.

C. To 1 mL of solution S add 10 mL of *water R* and 1 mL of *bromine water R*. A white precipitate is formed.

TESTS

**Solution S**. Dissolve 1.0 g in *water R* and dilute to 15 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Acidity**. To 2 mL of solution S add 0.05 mL of *methyl orange solution R*. The solution is yellow.

**Freezing point** (*2.2.18*): minimum 39.5 °C.

**Residue on evaporation**: maximum 0.05 per cent.

Evaporate 5.000 g to dryness on a water-bath and dry at 100-105 °C for 1 h. The residue weighs a maximum of 2.5 mg.

ASSAY

Dissolve 2.000 g in *water R* and dilute to 1000.0 mL with the same solvent. Transfer 25.0 mL of the solution to a ground-glass-stoppered flask and add 50.0 mL of *0.0167 M bromide-bromate* and 5 mL of *hydrochloric acid R*, close the flask, allow to stand with occasional swirling for 30 min. Then allow to stand for 15 min. Add 5 mL of a 200 g/L solution of *potassium iodide R*, shake and titrate with *0.1 M sodium thiosulfate* until a faint yellow colour remains. Add 0.5 mL of *starch solution R* and 10 mL of *chloroform R* and continue the titration with vigorous shaking. Carry out a blank titration.

1 mL of *0.0167 M bromide-bromate* is equivalent to 1.569 mg of $C_6H_6O$.

STORAGE

In an airtight container, protected from light.

**01/2017:1584**

# PHENOLPHTHALEIN

## Phenolphthaleinum

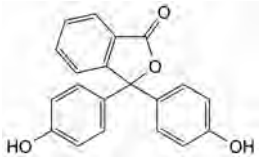

$C_{20}H_{14}O_4$ $M_r$ 318.3
[77-09-8]

DEFINITION

Phenolphthalein contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 3,3-bis(4-hydroxyphenyl)isobenzofuran-1(3*H*)-one, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, practically insoluble in water, soluble in alcohol.

It melts at about 260 °C.

IDENTIFICATION

A. Dissolve 25.0 mg in *alcohol R* and dilute to 100.0 mL with the same solvent (solution A). To 2.0 mL of solution A add 5.0 mL of *1 M hydrochloric acid*and dilute to 50.0 mL with *alcohol R* (solution $A_1$). To 10.0 mL of solution A add 5.0 mL of *1 M hydrochloric acid* and dilute to 50.0 mL with *alcohol R* (solution $A_2$). To 2.0 mL of solution A add 5.0 mL of *1 M sodium hydroxide* and dilute to 50.0 mL with *alcohol R* (solution B). Examined between 220 nm and 250 nm (*2.2.25*), solution $A_1$ shows an absorption maximum at 229 nm. The specific absorbance at the maximum at 229 nm is 922 to 1018. Examined between 250 nm and 300 nm, solution $A_2$ shows an absorption maximum at 276 nm. The specific absorbance at the maximum at 276 nm is 142 to 158. Examined between 230 nm and 270 nm, solution B shows an absorption maximum at 249 nm. The specific absorbance at the maximum at 249 nm is 744 to 822.

B. Dissolve about 10 mg in *alcohol R*. Add 1 mL of *dilute sodium hydroxide solution R*. The solution is red. Add 5 mL of *dilute sulfuric acid R*. The colour disappears.

TESTS

**Solution S**. To 2.0 g add 40 mL of *distilled water R* and heat to boiling. Cool and filter.

**Appearance of solution**. Dissolve 0.20 g in 5 mL of *alcohol R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.15 mL of *bromothymol blue solution R1*. Add 0.05 mL of *0.01 M hydrochloric acid*, the solution is yellow. Add 0.10 mL of *0.01 M sodium hydroxide*, the solution is blue.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R*.

*Test solution*. Dissolve 0.5 g of the substance to be examined in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dilute 1 mL of the test solution to 10 mL with *alcohol R*. Dilute 5 mL of this solution to 100 mL with *alcohol R*.

Monographs

*Reference solution (b).* Dissolve 25 mg of *fluorene R* in *alcohol R*, add 0.5 mL of the test solution and dilute to 10 mL with *alcohol R*.

Apply to the plate 5 µL of the test solution and 5 µL of each of the reference solutions. Develop over a path corresponding to two-thirds of the plate height using a mixture of 50 volumes of *acetone R* and 50 volumes of *methylene chloride R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm and re-examine after exposure to ammonia vapour. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

**Chlorides** (*2.4.4*). Dilute 10 mL of solution S to 15 mL with *water R*. The solution complies with the limit test for chlorides (100 ppm).

**Sulfates** (*2.4.13*). 15 mL of solution S complies with the limit test for sulfates (200 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 5 mL of *dimethylformamide R*. Add 5 mL of *sodium carbonate solution R*, 10 mL of *sodium hydrogen carbonate solution R*, 35 mL of *water R* and 50.0 mL of *0.05 M iodine*. Add 10 mL of *methylene chloride R* and 20 mL of *dilute sulfuric acid R*. Titrate the excess of iodine with *0.1 M sodium thiosulfate*, using 0.3 mL of *starch solution R* added towards the end of the titration, as indicator. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 3.979 mg of $C_{20}H_{14}O_4$.

STORAGE

Store protected from light.

**01/2008:0242**
**corrected 6.0**

# PHENOLSULFONPHTHALEIN

## Phenolsulfonphthaleinum

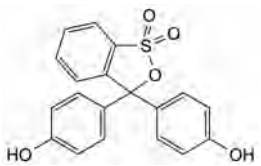

$C_{19}H_{14}O_5S$ $M_r$ 354.4
[143-74-8]

DEFINITION

Phenolsulfonphthalein (phenol red) contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 3,3-bis(4-hydroxyphenyl)-3*H*-2,1-benzoxathiole 1,1-dioxide, calculated with reference to the dried substance.

CHARACTERS

A bright-red to dark-red, crystalline powder, very slightly soluble in water, slightly soluble in alcohol.

IDENTIFICATION

A. Dissolve 10 mg in a 10 g/L solution of *sodium carbonate R* and dilute to 200.0 mL with the sodium carbonate solution. Dilute 5.0 mL of the solution to 100.0 mL with a 10 g/L solution of *sodium carbonate R*. Examined between 400 nm and 630 nm (*2.2.25*), the solution shows an absorption maximum at 558 nm. The specific absorbance at the maximum is 1900 to 2100.

B. Dissolve about 10 mg in 1 mL of *dilute sodium hydroxide solution R* and add 9 mL of *water R*. The solution is deep red. To 5 mL of the solution add a slight excess of *dilute sulfuric acid R*. The colour becomes orange.

C. To 5 mL of the solution prepared for identification test B add 1 mL of *0.0167 M bromide-bromate* and 1 mL of *dilute hydrochloric acid R*, shake and allow to stand for 15 min. Make alkaline with *dilute sodium hydroxide solution R*. An intense violet-blue colour is produced.

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.1 g of the substance to be examined in *0.1 M sodium hydroxide* and dilute to 5 mL with the same solvent.

*Reference solution.* Dilute 0.5 mL of the test solution to 100 mL with *0.1 M sodium hydroxide*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 100 volumes of *tert-pentyl alcohol R*. Allow the plate to dry in air until the solvent has evaporated and expose the plate to the vapour from *concentrated ammonia R*. Examine in ultraviolet light at 254 nm. Not more than one spot, apart from the principal spot, appears in the chromatogram obtained with the test solution and this spot is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Insoluble matter**. To 1.0 g of the finely powdered substance to be examined add 12 mL of *sodium hydrogen carbonate solution R*. Allow to stand for 1 h, shaking frequently. Dilute to 100 mL with *water R* and allow to stand for 15 h. Centrifuge at 2000 *g* to 3000 *g*, for 30 min, decant the supernatant and wash the residue with 25 mL of a 10 g/L solution of *sodium hydrogen carbonate R* and then 25 mL of *water R*. Dry at 100 °C to 105 °C. The residue weighs not more than 5 mg (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.00 g of the powdered substance to be examined by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.2 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.900 g in 15 mL of *1 M sodium hydroxide* and dilute to 250.0 mL with *water R*. To 10.0 mL of the solution in a glass-stoppered flask add 25 mL of *glacial acetic acid R*, 20.0 mL of *0.0167 M potassium bromate*, 5 mL of a 100 g/L solution of *potassium bromide R* and 5 mL of *hydrochloric acid R*. Allow to stand protected from light for 15 min, add 10 mL of a 100 g/L solution of *potassium iodide R* and titrate immediately with *0.1 M sodium thiosulfate*, using 0.1 mL of *starch solution R* as indicator.

1 mL of *0.0167 M potassium bromate* is equivalent to 4.43 mg of $C_{19}H_{14}O_5S$.

**07/2016:0781**

# PHENOXYETHANOL

## Phenoxyethanolum

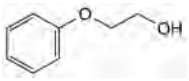

$C_8H_{10}O_2$ $M_r$ 138.2
[122-99-6]

DEFINITION

2-Phenoxyethanol.

*Content*: 99.0 per cent *m/m* to 100.5 per cent *m/m*.

CHARACTERS

*Appearance*: colourless, slightly viscous liquid.

*Solubility*: slightly soluble in water, in arachis oil and in olive oil, miscible with acetone, with ethanol (96 per cent) and with glycerol.

IDENTIFICATION

*First identification*: C.

*Second identification*: A, B, D.

A. Refractive index (*2.2.6*): 1.537 to 1.539.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 80.0 mg in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *water R*.

*Spectral range*: 240-350 nm.

*Absorption maxima*: at 269 nm and 275 nm.

*Specific absorbances at the absorption maxima*:

– at 269 nm: 95 to 105;

– at 275 nm: 75 to 85.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenoxyethanol CRS*.

D. Shake 2 mL with a mixture of 4 g of *potassium permanganate R*, 5.4 g of *sodium carbonate R* and 75 mL of *water R* for 30 min. Add 25 g of *sodium chloride R* and stir continuously for 60 min, filter and acidify with *hydrochloric acid R* to about pH 1.7. The melting point of the precipitate, after recrystallisation from *water R*, is 96 °C to 99 °C (*2.2.14*).

TESTS

**Relative density** (*2.2.5*): 1.105 to 1.110.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution.* Dilute 5.0 g of the substance to be examined to 20.0 mL with *anhydrous ethanol R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *anhydrous ethanol R*.

*Reference solution (b).* Dissolve 25.0 mg of *phenol R* (impurity A) in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (c).* Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) and dilute to 10.0 mL with *anhydrous ethanol R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with *anhydrous ethanol R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.25 mm;

– *stationary phase*: *macrogol 20 000 R* (0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 13 | 90 → 220 |
| | 13 - 23 | 220 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solutions (c) and (d).

*Elution order*: impurity A, phenoxyethanol.

*Retention time*: phenoxyethanol = about 13 min.

*System suitability*: reference solution (c):

– *resolution*: minimum 15 between the peaks due to impurity A and phenoxyethanol.

*Calculation of percentage contents*:

– for each impurity, use the concentration of phenoxyethanol in reference solution (d).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– *reporting threshold*: 0.05 per cent.

ASSAY

To 2.000 g in an acetylation flask fitted with an air condenser, add 10.0 mL of freshly prepared *acetic anhydride solution R1* and heat with frequent shaking in a water-bath for 45 min. Cool and carefully add 10 mL of *water R*. Heat for a further 2 min. Cool, add 10 mL of *butanol R*, shake vigorously and titrate the excess of acetic acid with *1 M sodium hydroxide* using 0.2 mL of *phenolphthalein solution R* as indicator. Repeat the procedure without the substance to be examined. The difference between the volumes used in the titrations represents the amount of acetic anhydride required for the acetylation of the substance to be examined.

1 mL of *1 M sodium hydroxide* is equivalent to 0.1382 g of $C_8H_{10}O_2$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): A.

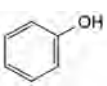

A. phenol.

Monographs

**04/2017:0148**

# PHENOXYMETHYLPENICILLIN

## Phenoxymethylpenicillinum

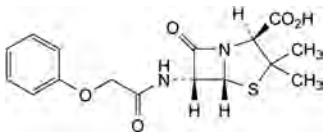

$C_{16}H_{18}N_2O_5S$ $M_r$ 350.4
[87-08-1]

DEFINITION

(2*S*,5*R*,6*R*)-3,3-Dimethyl-7-oxo-6-[(2-phenoxyacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid.

Substance produced by the growth of certain strains of *Penicillium notatum* or related microorganisms.

*Content*: 95.0 per cent to 102.0 per cent for the sum of the percentage contents of phenoxymethylpenicillin and 4-hydroxyphenoxymethylpenicillin (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. pH (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *phenoxymethylpenicillin CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *acetone R.*
*Reference solution (a).* Dissolve 25 mg of *phenoxymethylpenicillin CRS* in 5 mL of *acetone R.*
*Reference solution (b).* Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 mL of *water R.*
*Plate*: *TLC silanised silica gel plate R.*
*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R.*
*Application*: 1 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: expose to iodine vapour until the spots appear and examine in daylight.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R.* Mix the contents of the tube by swirling; the solution is reddish-brown. Place the test-tube on a water-bath for 1 min; a dark reddish-brown colour develops.

TESTS

**pH** (*2.2.3*): 2.4 to 4.0.

Suspend 50 mg in 10 mL of *carbon dioxide-free water R.*

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A.* To 250 mL of *0.2 M potassium dihydrogen phosphate R* add 500 mL of *water R*, adjust to pH 6.5 with an 8.4 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R.*

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Test solution (b). Prepare immediately before use.* Dissolve 80.0 mg of the substance to be examined in solution A and dilute to 20.0 mL with solution A.

*Reference solution (a).* Dissolve 55.0 mg of *phenoxymethylpenicillin potassium CRS* in solution A and dilute to 50.0 mL with solution A.

*Reference solution (b).* Dissolve 9 mg of *phenoxymethylpenicillin for system suitability CRS* (containing impurities B, D, E and F) in 2 mL of solution A.

*Reference solution (c).* Dilute 1.0 mL of test solution (b) to 100.0 mL with solution A.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with solution A.

*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
– *temperature*: 50 °C.

*Mobile phase*:
– *mobile phase A*: *phosphate buffer solution pH 3.4 R*, *methanol R*, *water for chromatography R* (10:30:60 *V/V/V*);
– *mobile phase B*: *phosphate buffer solution pH 3.4 R*, *methanol R*, *water for chromatography R* (5:60:35 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 5 | 85 → 70 | 15 → 30 |
| 5 - 17 | 70 → 0 | 30 → 100 |
| 17 - 22 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *phenoxymethylpenicillin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, D, E and F.

*Relative retention* with reference to phenoxymethylpenicillin (retention time = about 11 min): impurity B = about 0.29; impurity D = about 0.38; impurity E = about 0.55 and 0.61; impurity F = about 0.88 and 0.95.

*System suitability*:
– *resolution*: minimum 3.0 between the peaks due to the epimers of impurity F in the chromatogram obtained with reference solution (b);
– *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:
– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.5; impurity E = 1.3;
– for each impurity, use the concentration of phenoxymethylpenicillin in reference solution (c).

*Limits*:

- *impurity E (sum of isomers), impurity F (sum of epimers)*: for each impurity, maximum 1.0 per cent;
- *impurity B*: maximum 0.2 per cent;
- *any other impurity*: for each impurity, maximum 0.15 per cent;
- *sum of impurities other than D*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurity D (4-hydroxyphenoxymethylpenicillin)**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

*Calculation of percentage content*:

- *correction factor*: multiply the peak area of impurity D by 1.7;
- use the concentration of phenoxymethylpenicillin in reference solution (c).

*Limit*: maximum 1.0 per cent (anhydrous substance).

**Water** (*2.5.12, Method B*): maximum 0.5 per cent, determined on 1.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage content of $C_{16}H_{18}N_2O_5S$ taking into account the assigned content of *phenoxymethylpenicillin potassium CRS* and a conversion factor of 0.902.

Calculate the sum of the percentage contents of phenoxymethylpenicillin and of 4-hydroxyphenoxymethylpenicillin.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: B, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

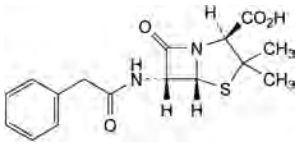

A. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(2-phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (benzylpenicillin),

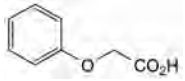

B. phenoxyacetic acid,

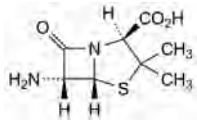

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

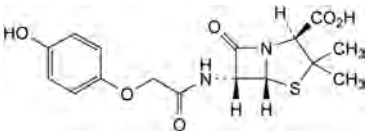

D. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[2-(4-hydroxyphenoxy)acetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (4-hydroxyphenoxymethylpenicillin),

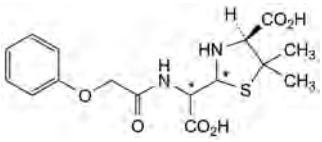

E. (4*S*)-2-[carboxy[(2-phenoxyacetyl)amino]-methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of phenoxymethylpenicillin),

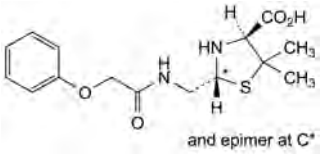


F. (2*RS*,4*S*)-5,5-dimethyl-2-[[(2-phenoxyacetyl)amino]-methyl]-1,3-thiazolidine-4-carboxylic acid (penilloic acids of phenoxymethylpenicillin).

**01/2019:2636**

# PHENOXYMETHYLPENICILLIN (BENZATHINE) TETRAHYDRATE

## Benzathini phenoxymethylpenicillinum tetrahydricum

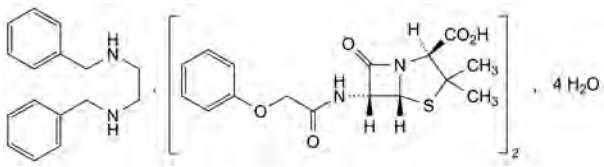

$C_{48}H_{56}N_6O_{10}S_2,4H_2O$ $M_r$ 1013

[2113728-41-7]

DEFINITION

$N^1$,$N^2$-Dibenzylethane-1,2-diamine bis[(2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-(2-phenoxyacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate] tetrahydrate.

Salt of a substance produced by the growth of certain strains of *Penicillium notatum* or related micro-organisms.

*Content*:

- 94.0 per cent to 102.0 per cent for the sum of benzathine phenoxymethylpenicillin and benzathine 4-hydroxyphenoxymethylpenicillin (anhydrous substance) with correction for dispersing or suspending agents;
- *benzathine*: 23.0 per cent to 26.0 per cent (anhydrous substance).

Dispersing or suspending agents (e.g. lecithin) may be added.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *benzathine phenoxymethylpenicillin CRS.*

TESTS

**pH** (*2.2.3*): 4.0 to 6.5.

Suspend 0.600 g in 20 mL of *carbon dioxide-free water R.*

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. To 250 mL of *0.2 M potassium dihydrogen phosphate R* add 500 mL of *water R*, adjust to pH 6.5 with an 8.4 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*.

*Test solution (a).* Dissolve 40.0 mg of the substance to be examined in 10 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Test solution (b). Prepare immediately before use.* Dissolve 80.0 mg of the substance to be examined in 10 mL of *methanol R* and dilute to 20.0 mL with solution A.

*Reference solution (a).* Dissolve 40.0 mg of *benzathine phenoxymethylpenicillin CRS* in 10 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (b).* Dissolve 8 mg of *benzathine phenoxymethylpenicillin for system suitability CRS* (containing impurities B, D, E, F, G, H and I) in 1 mL of *methanol R* and dilute to 2 mL with solution A.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with a mixture of equal volumes of *methanol R* and solution A.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 25.0 mL with a mixture of equal volumes of *methanol R* and solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 3.4 R, methanol R, water for chromatography R* (10:30:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution pH 3.4 R, water for chromatography R, methanol R* (2.5:27.5:70 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 5 | 90 → 75 | 10 → 25 |
| 5 - 17 | 75 → 0 | 25 → 100 |
| 17 - 24 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *benzathine phenoxymethylpenicillin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, D, E, F, G, H and I.

*Relative retention* with reference to phenoxymethylpenicillin (retention time = about 10 min): benzathine = about 0.2; impurity B = about 0.3; impurity D = about 0.4; impurity E = about 0.60 and 0.64; impurity F = about 0.91 and 0.97; impurity H = about 1.36; impurity G = about 1.39; impurity I = about 2.4.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to the epimers of impurity F in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.5; impurity D = 1.7; impurity E = 1.3;
- for each impurity, use the concentration of phenoxymethylpenicillin in reference solution (c).

*Limits*:

- *impurity G*: maximum 2.0 per cent;
- *impurity E (sum of isomers), impurity F (sum of epimers)*: for each impurity, maximum 1.0 per cent;
- *impurities D, H, I*: for each impurity, maximum 0.5 per cent;
- *impurity B*: maximum 0.2 per cent;
- *any other impurity*: for each impurity, maximum 0.15 per cent;
- *sum of impurities other than D*: maximum 4.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to benzathine.

**Water** (*2.5.12, Method B*): 5.0 per cent to 8.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage contents of benzathine ($C_{16}H_{20}N_2$) and benzathine phenoxymethylpenicillin ($C_{48}H_{56}N_6O_{10}S_2$) taking into account the assigned content of *benzathine phenoxymethylpenicillin CRS*.

Calculate the sum of the percentage contents of benzathine phenoxymethylpenicillin and of benzathine 4-hydroxyphenoxymethylpenicillin.

IMPURITIES

*Specified impurities: B, D, E, F, G, H, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

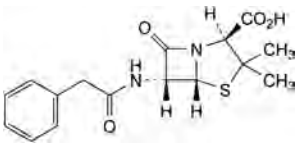

A. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (benzylpenicillin),

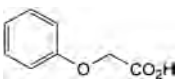

B. phenoxyacetic acid,

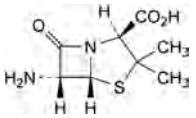

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

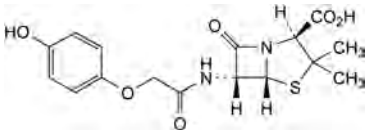

D. (2*S*,5*R*,6*R*)-6-[2-(4-hydroxyphenoxy)acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (4-hydroxyphenoxymethylpenicillin),

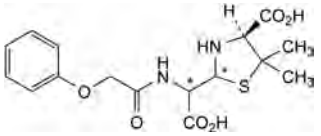

E. (2*Ξ*,4*S*)-2-[(*Ξ*)-carboxy(2-phenoxyacetamido)methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of phenoxymethylpenicillin),

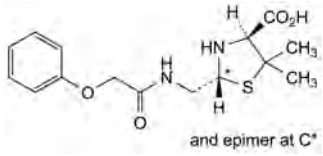


F. (2*RS*,4*S*)-5,5-dimethyl-2-[(2-phenoxyacetamido)methyl]-1,3-thiazolidine-4-carboxylic acid (penilloic acids of phenoxymethylpenicillin),

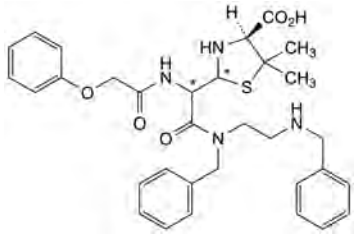

G. (2*Ξ*,4*S*)-2-[(1*Ξ*)-2-[benzyl[2-(benzylamino)ethyl]amino]-2-oxo-1-(2-phenoxyacetamido)ethyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid,

H. unknown structure,

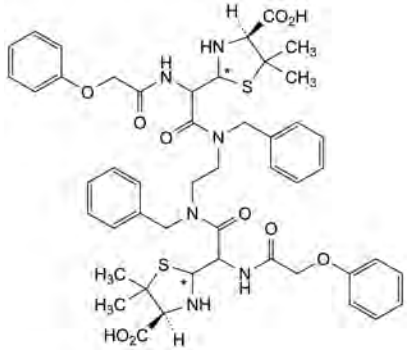

I. (2*Ξ*,2′*Ξ*,4*S*,4′*S*)-2,2′-[ethane-1,2-diylbis[(benzylazanediyl)-[2-oxo-1-(2-phenoxyacetamido)ethane-2,1-diyl]]]-bis(5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid).

**04/2017:0149**

# PHENOXYMETHYLPENICILLIN POTASSIUM

## Phenoxymethylpenicillinum kalicum

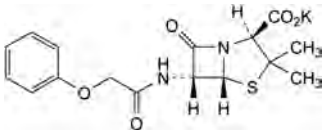

$C_{16}H_{17}KN_2O_5S$ $M_r$ 388.5
[132-98-9]

DEFINITION

Potassium (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(2-phenoxyacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Substance produced by the growth of certain strains of *Penicillium notatum* or related microorganisms.

*Content*: 95.0 per cent to 102.0 per cent for the sum of the percentage contents of phenoxymethylpenicillin potassium and 4-hydroxyphenoxymethylpenicillin potassium (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenoxymethylpenicillin potassium CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *water R*.

*Reference solution (a).* Dissolve 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 mL of *water R*.

*Reference solution (b).* Dissolve 25 mg of *benzylpenicillin potassium CRS* and 25 mg of *phenoxymethylpenicillin potassium CRS* in 5 mL of *water R*.

*Plate*: *TLC silanised silica gel plate R*.

*Mobile phase*: mix 30 volumes of *acetone R* and 70 volumes of a 154 g/L solution of *ammonium acetate R* adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*.

Monographs p

Mix the contents of the tube by swirling; the solution is reddish-brown. Place the test-tube on a water-bath for 1 min; a dark reddish-brown colour develops.

D. It gives reaction (a) of potassium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.5 to 7.5.

Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. To 250 mL of *0.2 M potassium dihydrogen phosphate R* add 500 mL of *water R*, adjust to pH 6.5 with an 8.4 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*.

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Test solution (b). Prepare immediately before use.* Dissolve 80.0 mg of the substance to be examined in solution A and dilute to 20.0 mL with solution A.

*Reference solution (a).* Dissolve 50.0 mg of *phenoxymethylpenicillin potassium CRS* in solution A and dilute to 50.0 mL with solution A.

*Reference solution (b).* Dissolve 8 mg of *phenoxymethylpenicillin for system suitability CRS* (containing impurities B, D, E and F) in 2 mL of solution A.

*Reference solution (c).* Dilute 1.0 mL of test solution (b) to 100.0 mL with solution A.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:

- *mobile phase A*: *phosphate buffer solution pH 3.4 R*, *methanol R*, *water for chromatography R* (10:30:60 *V/V/V*);
- *mobile phase B*: *phosphate buffer solution pH 3.4 R*, *methanol R*, *water for chromatography R* (5:60:35 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 5 | 85 → 70 | 15 → 30 |
| 5 - 17 | 70 → 0 | 30 → 100 |
| 17 - 22 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *phenoxymethylpenicillin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, D, E and F.

*Relative retention* with reference to phenoxymethylpenicillin (retention time = about 11 min): impurity B = about 0.29; impurity D = about 0.38; impurity E = about 0.55 and 0.61; impurity F = about 0.88 and 0.95.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to the epimers of impurity F in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 20 for the principal peak in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.6; impurity E = 1.3;
- for each impurity, use the concentration of phenoxymethylpenicillin potassium in reference solution (c).

*Limits*:

- *impurity E (sum of isomers), impurity F (sum of epimers)*: for each impurity, maximum 1.0 per cent;
- *impurity B*: maximum 0.3 per cent;
- *any other impurity*: for each impurity, maximum 0.15 per cent;
- *sum of impurities other than D*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurity D (4-hydroxyphenoxymethylpenicillin)**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

*Calculation of percentage content*:

- *correction factor*: multiply the peak area of impurity D by 1.7;
- use the concentration of phenoxymethylpenicillin potassium in reference solution (c).

*Limit*: maximum 4.0 per cent (anhydrous substance).

**Water** (*2.5.12, Method B*): maximum 1.0 per cent, determined on 1.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (a) and reference solution (a).

Calculate the percentage content of $C_{16}H_{17}KN_2O_5S$ taking into account the assigned content of *phenoxymethylpenicillin potassium CRS*.

Calculate the sum of the percentage contents of phenoxymethylpenicillin potassium and of 4-hydroxyphenoxymethylpenicillin potassium.

IMPURITIES

*Specified impurities: B, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

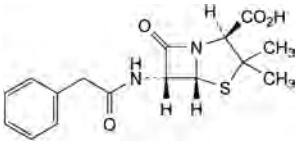

A. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[(2-phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (benzylpenicillin),

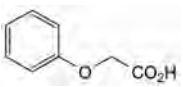

B. phenoxyacetic acid,

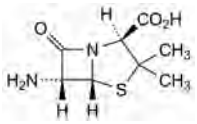

C. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

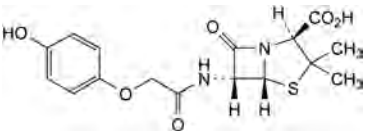

D. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[2-(4-hydroxyphenoxy)acetyl]amino]-4-thia- 1-azabicyclo[3.2.0]heptane-2-carboxylic acid (4-hydroxyphenoxymethylpenicillin),

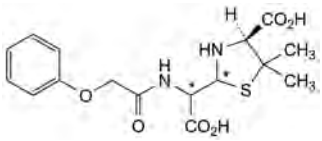

E. (4*S*)-2-[carboxy[(2-phenoxyacetyl)amino]methyl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid (penicilloic acids of phenoxymethylpenicillin),

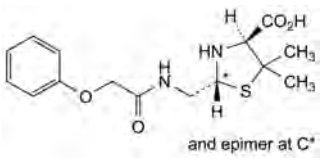


F. (2*RS*,4*S*)-5,5-dimethyl-2-[[(2-phenoxyacetyl)amino]-methyl]-1,3-thiazolidine-4-carboxylic acid (penilloic acids of phenoxymethylpenicillin).

**04/2016:1138**
**corrected 10.0**

# PHENTOLAMINE MESILATE

## Phentolamini mesilas

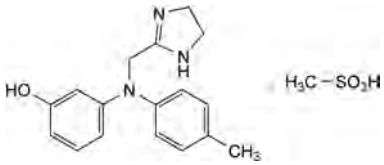

$C_{18}H_{23}N_3O_4S$ $M_r$ 377.5
[65-28-1]

DEFINITION

3-[[(4,5-Dihydro-1*H*-imidazol-2-yl)methyl](4-methylphenyl)amino]phenol methanesulfonate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in phentolamine mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: *C, E.*

*Second identification*: *A, B, D, E.*

A. Melting point (*2.2.14*): 178 °C to 182 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 60.0 mg in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 278 nm.

*Specific absorbance at the absorption maximum*: 220 to 245.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phentolamine mesilate CRS*.

D. Dissolve 0.5 g in a mixture of 5 mL of *ethanol (96 per cent) R* and 5 mL of a 10 g/L solution of *hydrochloric acid R* and add 0.5 mL of a 5 g/L solution of *ammonium vanadate R*. A light green precipitate is produced.

E. Mix 50 mg with 0.2 g of *sodium hydroxide R*, heat to fusion and continue heating for a few seconds. Allow to cool and add 0.5 mL of warm *water R*. Acidify with *dilute hydrochloric acid R* and heat. Sulfur dioxide is evolved, which turns moistened *starch iodate paper R* blue.

TESTS

**Acidity**. Dissolve 0.1 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.1 mL of *methyl red solution R*. If the solution is red, not more than 0.05 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *phentolamine for system suitability CRS* (containing impurities A and C) in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 33 volumes of *acetonitrile for chromatography R* and 67 volumes of a 0.5 g/L solution of *ammonium acetate R* previously adjusted to pH 5.9 with *dilute acetic acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of phentolamine.

*Identification of impurities*: use the chromatogram supplied with *phentolamine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C.

*Relative retention* with reference to phentolamine (retention time = about 15 min): impurity A = about 0.7; impurity C = about 1.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to phentolamine and impurity C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.7;
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 100 mL of *2-propanol R1*. Titrate under a stream of nitrogen with *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Determine the end-point potentiometrically (*2.2.20*), using a glass indicator electrode and a suitable reference electrode. Carry out a blank titration.

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 37.75 mg of $C_{18}H_{23}N_3O_4S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

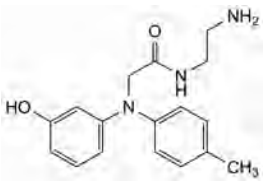


A. *N*-(2-aminoethyl)-2-[(3-hydroxyphenyl)(4-methylphenyl)amino]acetamide,

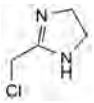


B. 2-(chloromethyl)-4,5-dihydro-1*H*-imidazole,

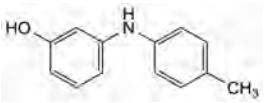


C. 3-[(4-methylphenyl)amino]phenol.

**01/2017:0782**

# PHENYLALANINE

## Phenylalaninum

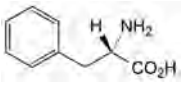


$C_9H_{11}NO_2$ $M_r$ 165.2
[63-91-2]

DEFINITION

(2*S*)-2-Amino-3-phenylpropanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, or shiny, white flakes.

*Solubility*: sparingly soluble in water, very slightly soluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *phenylalanine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 50 mL with the same mixture of solvents.
*Reference solution*. Dissolve 10 mg of *phenylalanine CRS* in a mixture of equal volumes of *glacial acetic acid R* and *water R* and dilute to 50 mL with the same mixture of solvents.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To about 10 mg add 0.5 g of *potassium nitrate R* and 2 mL of *sulfuric acid R*. Heat on a water-bath for 20 min. Allow to cool. Add 5 mL of a 50 g/L solution of *hydroxylamine hydrochloride R* and allow to stand in iced water for 10 min. Add 9 mL of *strong sodium hydroxide solution R*. A violet-red or violet-brown colour develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 10 mL with the same solution.

**Specific optical rotation** (*2.2.7*): – 35.5 to – 33.0 (dried substance).

Dissolve 0.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of phenylalanine in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 3 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution, without further addition of nitric acid, complies with the test.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in a mixture of 5 volumes of *dilute hydrochloric acid R* and 25 volumes of *distilled water R* and dilute to 15 mL with the same mixture of solvents.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.52 mg of $C_9H_{11}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

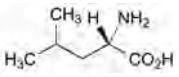


A. (2*S*)-2-amino-4-methylpentanoic acid (leucine),

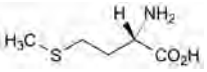


B. (2*S*)-2-amino-4-(methylsulfanyl)butanoic acid (methionine),

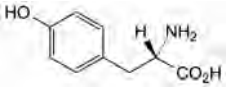


C. (2*S*)-2-amino-3-(4-hydroxyphenyl)propanoic acid (tyrosine),

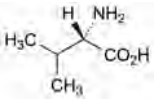


D. (2*S*)-2-amino-3-methylbutanoic acid (valine).

**01/2017:0422**

# PHENYLBUTAZONE

## Phenylbutazonum

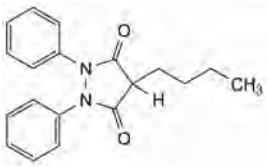

$C_{19}H_{20}N_2O_2$ $M_r$ 308.4
[50-33-9]

DEFINITION

4-Butyl-1,2-diphenylpyrazolidine-3,5-dione.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in alcohol. It dissolves in alkaline solutions.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 104 °C to 107 °C.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 25 mL with the same mixture of solvents.

*Reference solution.* Dissolve 25 mg of *phenylbutazone CRS* in a mixture of equal volumes of *ethanol R* and *methylene chloride R* and dilute to 25 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *acetone R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenylbutazone CRS.*

D. To 0.1 g add 1 mL of *glacial acetic acid R* and 2 mL of *hydrochloric acid R* and heat the mixture under a reflux condenser for 30 min. Cool, add 10 mL of *water R* and filter. To the filtrate add 3 mL of a 7 g/L solution of *sodium nitrite R*. A yellow colour is produced. To 1 mL of the solution add a solution of 10 mg of *β-naphthol R* in 5 mL of *sodium carbonate solution R*. A brownish-red to violet-red precipitate is formed.

TESTS

**Solution S**. Dissolve 1.0 g with shaking in 20 mL of *dilute sodium hydroxide solution R* and maintain the solution at 25 °C for 3 h.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Acidity or alkalinity**. Heat to boiling 1.0 g in 50 mL of *water R*, cool with shaking in a closed flask and filter. To 25 mL of the filtrate add 0.5 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator. Add 0.6 mL of *0.01 M hydrochloric acid* and 0.1 mL of *methyl red solution R*; the solution is red or orange.

**Absorbance** (*2.2.25*): maximum 0.20 for solution S at 420 nm in a 4 cm cell.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 100.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 5 mg of *phenylbutazone impurity B CRS* and 5 mg of *1,2-diphenylhydrazine R* in *acetonitrile R*, add 0.5 mL of the test solution and dilute to 50 mL with *acetonitrile R*. Dilute 2.5 mL to 10 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 1.0 mg of *benzidine R* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 100.0 mL with *acetonitrile R*. Dilute 5.0 mL to 10.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.36 g of *sodium acetate R* in *water R*, adjust to pH 5.2 with a 52.5 g/L solution of *citric acid monohydrate R* and dilute to 1000 mL with *water R*,
- *mobile phase B*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 70 | 30 |
| 10 - 20 | 70 → 40 | 30 → 60 |
| 20 - 35 | 40 | 60 |
| 35 - 40 | 40 → 70 | 60 → 30 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL; inject the test solution and reference solutions (a) and (b).

*Relative retentions* with reference to phenylbutazone (retention time = about 13 min): impurity E = about 0.2; impurity A = about 0.5; impurity B = about 1.2; impurity C = about 1.3; impurity D = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to phenylbutazone and to impurity B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 0.55,
- *impurities A, B*: for each impurity, not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent),
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent); disregard any peak due to impurity E.

**Impurity E**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 280 nm.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

– *signal-to-noise ratio*: minimum 10 for the principal peak.

*Limit*:

– *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (5 ppm).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *acetone R* and add 0.5 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide* until a blue colour is obtained which persists for 15 s. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.84 mg of $C_{19}H_{20}N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

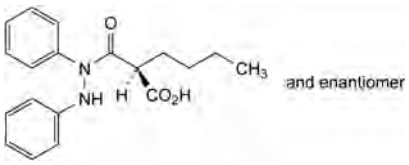


A. (2*RS*)-2-[(1,2-diphenyldiazanyl)carbonyl]hexanoic acid,

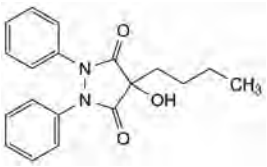


B. 4-butyl-4-hydroxy-1,2-diphenylpyrazolidine-3,5-dione,

C. $C_6H_5$-NH-NH-$C_6H_5$: 1,2-diphenyldiazane (1,2-diphenylhydrazine),

D. $C_6H_5$-N=N-$C_6H_5$: 1,2-diphenyldiazene,

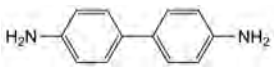


E. biphenyl-4,4′-diamine (benzidine).

**01/2008:1035**
**corrected 7.0**

# PHENYLEPHRINE

## Phenylephrinum

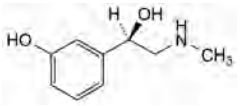


$C_9H_{13}NO_2$ $M_r$ 167.2
[59-42-7]

DEFINITION

(1*R*)-1-(3-Hydroxyphenyl)-2-(methylamino)ethanol.

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

mp: about 174 °C.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenylephrine CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture.* A mixture of equal volumes of *methylene chloride R* and methanolic hydrochloric acid (*hydrochloric acid R* diluted 10-fold with *methanol R*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution.* Dissolve 20 mg of *phenylephrine CRS* in the solvent mixture and dilute to 1 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (0.5:25:70 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of cold air.

*Detection*: examine in ultraviolet light at 254 nm; spray with a 1 g/L solution of *fast red B salt R* in a 50 g/L solution of *sodium carbonate R* and examine in daylight.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 1 mL of *1 M hydrochloric acid*, add 0.05 mL of *copper sulfate solution R* and 1 mL of a 200 g/L solution of *sodium hydroxide R*. A violet colour develops. Add 1 mL of *ether R* and shake. The upper layer remains colourless.

Monographs

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 1 g in *1 M hydrochloric acid* and dilute to 10 mL with the same acid.

**Specific optical rotation** (*2.2.7*): − 53 to − 57 (dried substance).

Dissolve 1.250 g in *1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *dilute hydrochloric acid R*, mobile phase B, mobile phase A (5:200:800 *V/V/V*).

*Buffer solution pH 2.8*. Dissolve 3.25 g of *sodium octanesulfonate monohydrate R* in 1000 mL of *water R* by stirring for 30 min and adjust to pH 2.8 with *dilute phosphoric acid R*.

*Test solution*. Dissolve 41.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *phenylephrine hydrochloride for peak identification CRS* (containing impurities C and E) in 2.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.055 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution pH 2.8 (10:90 *V/V*);
- *mobile phase B*: buffer solution pH 2.8, *acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 93 | 7 |
| 3 - 13 | 93 → 70 | 7 → 30 |
| 13 - 14 | 70 → 93 | 30 → 7 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Relative retention* with reference to phenylephrine (retention time = about 2.8 min): impurity C = about 1.3; impurity E = about 3.6.

*System suitability*:

- *symmetry factor*: maximum 1.9 for the principal peak in the chromatogram obtained with the test solution;
- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to phenylephrine in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.5; impurity E = 0.5;
- *impurities C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.72 mg of $C_9H_{13}NO_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *C, E*.
*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D*.

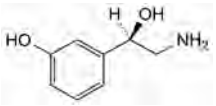

A. (1*R*)-2-amino-1-(3-hydroxyphenyl)ethanol (norphenylephrine),

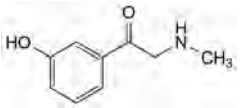

C. 1-(3-hydroxyphenyl)-2-(methylamino)ethanone (phenylephrone),

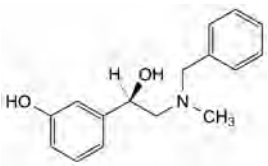

D. (1*R*)-2-(benzylmethylamino)-1-(3-hydroxyphenyl)ethanol (benzylphenylephrine),

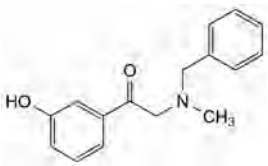

E. 2-(benzylmethylamino)-1-(3-hydroxyphenyl)ethanone (benzylphenylephrone).

**01/2008:0632**
**corrected 7.0**

# PHENYLEPHRINE HYDROCHLORIDE

## Phenylephrini hydrochloridum

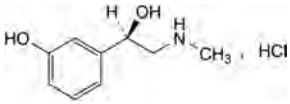

$C_9H_{14}ClNO_2$ $M_r$ 203.7
[61-76-7]

DEFINITION

(1*R*)-1-(3-Hydroxyphenyl)-2-(methylamino)ethanol hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

mp: about 143 °C.

IDENTIFICATION

*First identification: A, C, E.*

*Second identification: A, B, D, E.*

A. Specific optical rotation (see Tests).

B. Melting point (*2.2.14*): 171 °C to 176 °C.
Dissolve 0.3 g in 3 mL of *water R*, add 1 mL of *dilute ammonia R1* and initiate crystallisation by scratching the wall of the tube with a glass rod. Wash the crystals with iced *water R* and dry at 105 °C for 2 h.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *phenylephrine hydrochloride CRS.*

D. Dissolve about 10 mg in 1 mL of *water R* and add 0.05 mL of a 125 g/L solution of *copper sulfate pentahydrate R* and 1 mL of a 200 g/L solution of *sodium hydroxide R*. A violet colour is produced. Add 1 mL of *ether R* and shake; the upper layer remains colourless.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.00 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Specific optical rotation** (*2.2.7*): – 43 to – 47 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (20:80 *V/V*).

*Buffer solution pH 2.8*. Dissolve 3.25 g of *sodium octanesulfonate monohydrate R* in 1000 mL of *water R* by stirring for 30 min and adjust to pH 2.8 with *dilute phosphoric acid R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *phenylephrine hydrochloride for peak identification CRS* (containing impurities C and E) in 2.0 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.055 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, buffer solution pH 2.8 (10:90 *V/V*);
- *mobile phase B*: buffer solution pH 2.8, *acetonitrile R1* (10:90 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 93 | 7 |
| 3 - 13 | 93 → 70 | 7 → 30 |
| 13 - 14 | 70 → 93 | 30 → 7 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Relative retention* with reference to phenylephrine (retention time = about 2.8 min): impurity C = about 1.3; impurity E = about 3.6.

*System suitability*:
- *symmetry factor*: maximum 1.9 for the principal peak in the chromatogram obtained with the test solution;
- *peak-to-valley ratio*: minimum 5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to phenylephrine in the chromatogram obtained with reference solution (b).

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.5; impurity E = 0.5;
- *impurities C, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 500 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 0.5 mL of *0.1 M hydrochloric acid* and 80 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*) using *0.1 M ethanolic sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 20.37 mg of $C_9H_{14}ClNO_2$.

IMPURITIES

*Specified impurities: C, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D.*

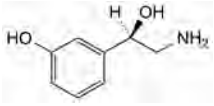

A. (1*R*)-2-amino-1-(3-hydroxyphenyl)ethanol (norphenylephrine),

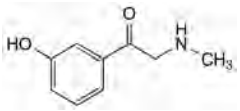

C. 1-(3-hydroxyphenyl)-2-(methylamino)ethanone (phenylephrone),

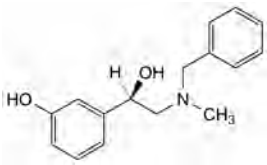

D. (1*R*)-2-(benzylmethylamino)-1-(3-hydroxyphenyl)ethanol (benzylphenylephrine),

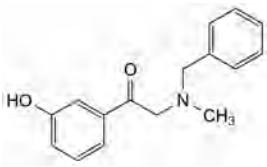

E. 2-(benzylmethylamino)-1-(3-hydroxyphenyl)ethanone (benzylphenylephrone).

**01/2008:2042**

# PHENYLMERCURIC ACETATE

## Phenylhydrargyri acetas

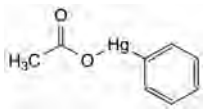

$C_8H_8HgO_2$ $M_r$ 336.7
[62-38-4]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder or small, colourless crystals.

*Solubility*: slightly soluble in water, soluble in acetone and in alcohol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of phenylmercuric acetate.*

B. To 5 mL of solution S (see Tests) add 5 mL of *water R* and 0.1 mL of *sodium sulfide solution R*. A white precipitate is formed that darkens slowly on heating.

C. To 10 mL of solution S add 2 mL of *potassium iodide solution R* and shake vigorously. Filter. The filtrate gives reaction (b) of acetates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in 40 mL of *water R* by heating to boiling. Allow to cool and dilute to 50 mL with *water R*. Prepare the solution immediately before use.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Ionised mercury**: maximum 0.2 per cent.

To 2 mL of solution S add 8 mL of *water R*, 2 mL of *potassium iodide solution R* and 3 mL of *dilute hydrochloric acid R*. Filter. The filtrate is not more coloured than the potassium iodide solution used. Wash the precipitate with 3 mL of *water R*. Combine the filtrate and the washings, add 2 mL of *dilute sodium hydroxide solution R* and dilute to 20 mL with *water R*. 12 mL of this solution complies with test A for heavy metals (*2.4.8*). Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

**Polymercuric benzene compounds**: maximum 1.5 per cent.

Shake 0.2 g with 10 mL of *acetone R*. Filter. Wash the residue twice with 5 mL of *acetone R*. Dry the residue at 105 °C for 1 h. The residue weighs a maximum of 3 mg.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 45 °C for 15 h.

ASSAY

Dissolve with heating 0.300 g in 100 mL of *water R*. Cool and add 3 mL of *nitric acid R*. Titrate with *0.1 M ammonium thiocyanate* using 2 mL of *ferric ammonium sulfate solution R2* as indicator, until a persistent reddish-yellow colour is obtained.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 33.67 mg of phenylmercuric acetate.

STORAGE

Protected from light.

**01/2008:0103**

# PHENYLMERCURIC BORATE

## Phenylhydrargyri boras

DEFINITION

Compound consisting of equimolecular proportions of phenylmercuric orthoborate and phenylmercuric hydroxide ($C_{12}H_{13}BHg_2O_4$; $M_r$ 633) or of the dehydrated form (metaborate, $C_{12}H_{11}BHg_2O_3$; $M_r$ 615) or of a mixture of the 2 compounds.

*Content*:

– *mercury* (Hg; $A_r$ 200.6): 64.5 per cent to 66.0 per cent (dried substance),

– *borates expressed as $H_3BO_3$*: 9.8 per cent to 10.3 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder or colourless, shiny crystals.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *Ph. Eur. reference spectrum of phenylmercuric borate*.

B. To 2 mL of solution S (see Tests) add 8 mL of *water R* and 0.1 mL of *sodium sulfide solution R*. A white precipitate is formed that darkens slowly on heating.

C. Dissolve about 20 mg in 2 mL of *methanol R*. The solution is clear and colourless. Ignite; the solution burns with a green-edged flame.

TESTS

**Solution S**. Dissolve 0.25 g by sprinkling it on the surface of 25 mL of boiling *water R*, cool and dilute to 25 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Ionised mercury**: maximum 0.01 per cent.

To 10 mL of solution S add 2 mL of *potassium iodide solution R* and 3 mL of *dilute hydrochloric acid R*. Filter. The filtrate is colourless. Wash the precipitate with 3 mL of *water R*. Combine the filtrate and the washings, add 2 mL of *dilute sodium hydroxide solution R* and dilute to 20 mL with *water R*. 12 mL of this solution complies with test A for heavy metals (*2.4.8*). Prepare the reference solution using a mixture of 2.5 mL of *lead standard solution (2 ppm Pb) R* and 7.5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 3.5 per cent, determined on 0.50 g by drying in an oven at 45 °C for 15 h ± 30 min.

ASSAY

**Mercury**. Dissolve 0.300 g in 100 mL of *water R* and add 3 mL of *nitric acid R*. Titrate with *0.1 M ammonium thiocyanate*, using 2 mL of *ferric ammonium sulfate solution R2* as indicator, until a persistent reddish-yellow colour is obtained.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 20.06 mg of Hg.

**Borates**. Dissolve 0.600 g with heating in 25 mL of *water R*. Dissolve 10 g of *sorbitol R* in the hot solution and cool. Titrate with *0.1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator, until a persistent pink colour is obtained. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 6.18 mg of $H_3BO_3$.

STORAGE

Protected from light.

**01/2008:0783**

# PHENYLMERCURIC NITRATE

## Phenylhydrargyri nitras

DEFINITION

Mixture of phenylmercuric nitrate ($C_6H_5HgNO_3$; $M_r$ 339.7) and phenylmercuric hydroxide ($C_6H_5HgOH$; $M_r$ 294.7).

*Content*: 62.5 per cent to 64.0 per cent of Hg ($A_r$ 200.6) (dried substance).

CHARACTERS

*Appearance*: white or pale yellow powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent), slightly soluble in hot water. It dissolves in glycerol and in fatty oils.

IDENTIFICATION

A. To 5 mL of solution S (see Tests) add 8 mL of *water R* and 0.1 mL of *sodium sulfide solution R*. A white precipitate is formed that darkens slowly on heating.

B. To 1 mL of a saturated solution of the substance to be examined add 1 mL of *dilute hydrochloric acid R*. A white, flocculent precipitate is formed.

C. To 5 mL of solution S add 1 mL of *dilute hydrochloric acid R*, 2 mL of *methylene chloride R* and 0.2 mL of *dithizone solution R*. Shake. The lower layer is orange-yellow.

D. About 10 mg gives the reaction of nitrates (*2.3.1*).

TESTS

**Solution S**. To 0.1 g add 45 mL of *water R* and heat to boiling with shaking. Cool, filter and dilute to 50 mL with *water R*.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Inorganic mercuric compounds**: maximum 0.1 per cent.

To 10 mL of solution S add 2 mL of *potassium iodide solution R* and 3 mL of *dilute hydrochloric acid R*. Filter. The filtrate is colourless. Wash the precipitate with 2 mL of *water R*. Combine the filtrate and washings, add 2 mL of *dilute sodium hydroxide solution R* and dilute to 20 mL with *water R*. 12 mL of the solution complies with test A for heavy metals (*2.4.8*). Prepare the reference solution using *lead standard solution (1 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* for 24 h.

ASSAY

Dissolve 0.150 g in a mixture of 10 mL of *dilute nitric acid R* and 90 mL of *water R*, heating to boiling. Cool to 15-20 °C. Titrate with *0.1 M ammonium thiocyanate* using 2 mL of *ferric ammonium sulfate solution R2* as indicator, until a persistent reddish-yellow colour is obtained. Carry out a blank titration.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 20.06 mg of Hg.

STORAGE

Protected from light.

**01/2020:0683**

# PHENYLPROPANOLAMINE HYDROCHLORIDE

## Phenylpropanolamini hydrochloridum

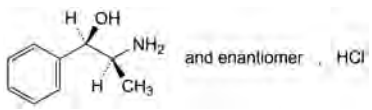


$C_9H_{14}ClNO$ $M_r$ 187.7

[154-41-6]

DEFINITION

(1*RS*,2*SR*)-2-Amino-1-phenylpropan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 194 °C to 197 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenylpropanolamine hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *phenylpropanolamine hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Pretreatment*: spray with a 20 g/L solution of *disodium tetraborate R*, using 8 mL for a plate 100 mm x 200 mm and dry in a stream of cold air for 30 min.

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *butanol R* (6:24:70 *V/V/V*).

*Application*: 10 µL as bands of 10 mm by 3 mm.

*Development*: over 1/2 of the plate.

*Drying*: in a current of warm air.

*Detection*: allow to cool, then spray with a 2 g/L solution of *ninhydrin R* in *ethanol (96 per cent) R* and heat at 110 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide.* The solution is yellow. Add 0.4 mL of *0.01 M hydrochloric acid.* The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 3.4 g of *potassium dihydrogen phosphate R* in 500 mL of *water for chromatography R* and adjust to pH 2.5 with *phosphoric acid R.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *cathine hydrochloride R* (1*S*,2*S*-enantiomer of impurity A) in *methanol R* and dilute to 50 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *methanol R*. Dilute 1 mL of this solution to 10 mL with the test solution.

*Column*:

- *size*: *l* = 0.075 m, Ø = 3.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (1.8 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, buffer solution (2:98 *V/V*);
- *mobile phase B*: *water for chromatography R*, *acetonitrile R1* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 100 | 0 |
| 6 - 14 | 100 → 48 | 0 → 52 |
| 14 - 18 | 48 | 52 |

*Flow rate*: 0.56 mL/min.

*Detection*: spectrophotometer at 206 nm.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to phenylpropanolamine (retention time = about 3 min): impurity A = about 1.2.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to phenylpropanolamine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of phenylpropanolamine hydrochloride in reference solution (a).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.77 mg of $C_9H_{14}ClNO$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

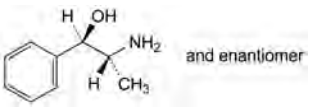


A. (1*RS*,2*RS*)-2-amino-1-phenylpropan-1-ol (racemic cathine, racemic norpseudoephedrine),

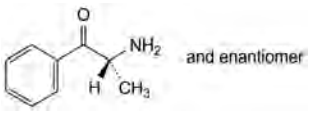

and enantiomer

B. (2*RS*)-2-amino-1-phenylpropan-1-one (racemic cathinone),

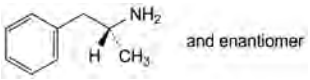

and enantiomer

C. (2*RS*)-1-phenylpropan-2-amine (amfetamine),

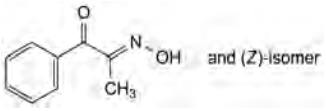

and (*Z*)-isomer

D. (2*EZ*)-2-(hydroxyimino)-1-phenylpropan-1-one (α-isonitrosopropiophenone),

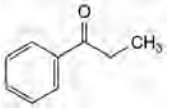


E. 1-phenylpropan-1-one (propiophenone).

**01/2019:1253**

# PHENYTOIN

## Phenytoinum

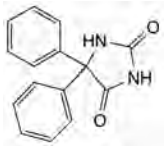


$C_{15}H_{12}N_2O_2$ $M_r$ 252.3
[57-41-0]

DEFINITION

5,5-Diphenylimidazolidine-2,4-dione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *phenytoin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in a mixture of 5 mL of *1 M sodium hydroxide* and 20 mL of *water R*.

**Acidity or alkalinity**. To 1.0 g add 45 mL of *water R* and boil for 2 min. Allow to cool and filter. Wash the filter with *carbon dioxide-free water R* and dilute the combined filtrate and washings to 50 mL with the same solvent. To 10 mL of the solution add 0.15 mL of *methyl red solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red. To 10 mL of the solution add 0.15 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *2,2-diphenylglycine R* (impurity C) in 100 mL of the mobile phase.

*Reference solution (c)*. Dissolve 10 mg of *phenytoin for system suitability CRS* (containing impurities D and E) in the mobile phase, add 1 mL of reference solution (b) and dilute to 10 mL with the mobile phase.

*Reference solution (d)*. Dissolve 50.0 mg of *phenytoin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Mobile phase*: mix 20 volumes of *methanol R2*, 35 volumes of *acetonitrile R1* and 45 volumes of a 5.75 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (c).

*Run time*: 4 times the retention time of phenytoin.

*Identification of impurities*: use the chromatogram supplied with *phenytoin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C, D and E.

*Relative retention* with reference to phenytoin (retention time = about 4 min): impurity C = about 0.5; impurity D = about 0.6; impurity E = about 0.8.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.5 between the peaks due to impurities D and E.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peaks areas of the following impurities by the corresponding correction factor: impurity D = 1.7; impurity E = 1.4;
- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{15}H_{12}N_2O_2$ taking into account the assigned content of *phenytoin CRS*.

IMPURITIES

*Specified impurities: C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, F.*

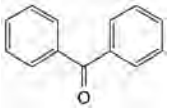

A. diphenylmethanone (benzophenone),

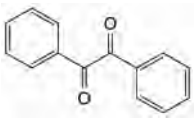

B. diphenylethanedione (benzil),

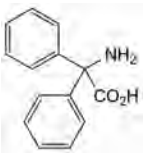

C. amino(diphenyl)acetic acid (2,2-diphenylglycine),

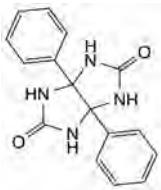

D. 3a,6a-diphenyltetrahydroimidazo[4,5-*d*]imidazole-2,5(1*H*,3*H*)-dione,

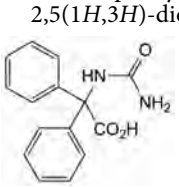

E. (carbamoylamino)diphenylacetic acid,

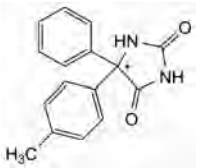

F. (5Ξ)-5-(4-methylphenyl)-5-phenylimidazolidine-2,4-dione.

**01/2019:0521**

# PHENYTOIN SODIUM

## Phenytoinum natricum

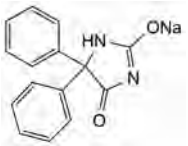

$C_{15}H_{11}N_2NaO_2$ $M_r$ 274.3

[630-93-3]

DEFINITION

Sodium 4-oxo-5,5-diphenyl-4,5-dihydro-1*H*-imidazol-2-olate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: suspend 0.1 g in 20 mL of *water R*. Acidify with *dilute hydrochloric acid R* and shake with 3 quantities, each of 30 mL, of *ethyl acetate R*. Wash the combined ethyl acetate layers with *water R*, evaporate to dryness and dry the residue at 100-105 °C (test residue). Repeat the operations using 0.1 g of *phenytoin sodium CRS* (reference residue). Examine as discs prepared using *potassium bromide R*.

*Comparison*: *phenytoin sodium CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *acetone R*, *methanol R* (50:50 *V/V*).

*Test solution*. Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution*. Dissolve 20 mg of *phenytoin sodium CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *toluene R*, *2-propanol R* (10:40:50 *V/V/V*).

*Application*: 10 µL as bands of 8 mm.

*Development*: over 2/3 of the plate.

*Drying*: at 80 °C for 5 min; allow to cool.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal zone in the chromatogram obtained with the test solution is similar in position and size to the principal zone in the chromatogram obtained with the reference solution.

C. Ignite 1 g and cool. Add 2 mL of *water R* to the residue and neutralise the solution with *hydrochloric acid R*. Filter and dilute the filtrate to 4 mL with *water R*. 0.1 mL of the solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Suspend 1.0 g in 5 mL of *water R* and dilute to 20 mL with *0.1 M sodium hydroxide*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *2,2-diphenylglycine R* (impurity C) in 100 mL of the mobile phase.

*Reference solution (c)*. Dissolve 10 mg of *phenytoin for system suitability CRS* (containing impurities D and E) in the mobile phase, add 1 mL of reference solution (b) and dilute to 10 mL with the mobile phase.

*Reference solution (d)*. Dissolve 50.0 mg of *phenytoin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Mobile phase*: mix 20 volumes of *methanol R2*, 35 volumes of *acetonitrile R1* and 45 volumes of a 5.75 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (c).

*Run time*: 4 times the retention time of phenytoin.

*Identification of impurities*: use the chromatogram supplied with *phenytoin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C, D and E.

*Relative retention* with reference to phenytoin (retention time = about 4 min): impurity C = about 0.5; impurity D = about 0.6; impurity E = about 0.8.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.5 between the peaks due to impurities D and E.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.7; impurity E = 1.4;
- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Free phenytoin**. Dissolve 0.30 g in 10 mL of a mixture of equal volumes of *pyridine R* and *water R*. Add 0.5 mL of *phenolphthalein solution R* and 3 mL of *silver nitrate solution in pyridine R*. Not more than 1.0 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{15}H_{11}N_2NaO_2$ taking into account the assigned content of *phenytoin CRS*, and a conversion factor of 1.09.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, F.*

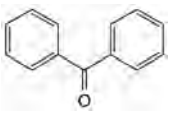

A. diphenylmethanone (benzophenone),

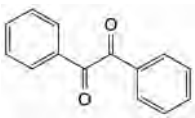

B. diphenylethanedione (benzil),

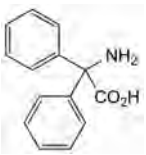


C. amino(diphenyl)acetic acid (2,2-diphenylglycine),

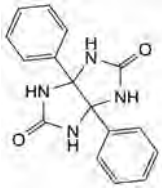


D. 3a,6a-diphenyltetrahydroimidazo[4,5-*d*]imidazole-2,5(1*H*,3*H*)-dione,

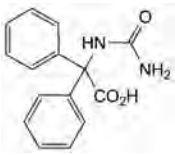

E. (carbamoylamino)diphenylacetic acid,

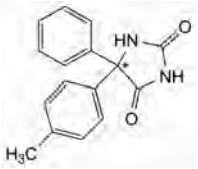

F. (5Ξ)-5-(4-methylphenyl)-5-phenylimidazolidine-2,4-dione.

**01/2017:2301**
**corrected 9.7**

# PHLOROGLUCINOL

## Phloroglucinolum

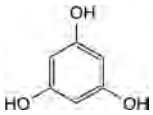

$C_6H_6O_3$ $M_r$ 126.1
[108-73-6]

DEFINITION

Benzene-1,3,5-triol.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anhydrous phloroglucinol CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 0.20 g of *anhydrous phloroglucinol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *hexane R*, *ethyl acetate R* (2:37.5:62.5 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.0.

Dilute 10 mL of solution S to 100 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light*.

*Solvent mixture*: mobile phase B, mobile phase A (10:90 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 6 mg each of *resorcinol R* (impurity B), *phloroglucide R* (impurity D) and *pyrogallol R* (impurity A) in 10 mL of the solvent mixture, add 2 mL of the test solution and dilute to 20 mL with the solvent mixture. Dilute 1 mL of this solution to 50 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 4 mg each of *benzene-1,2,4-triol R* (impurity E), *2,6-dichlorophenol R* (impurity I), *4-chlororesorcinol R* (impurity K) and *3,5-dichloroaniline R* (impurity L) in 10 mL of the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 10 mg of the substance to be examined in 10 mL of the solvent mixture, add 1 mL of reference solution (c) and dilute to 20 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 100 | 0 |
| 9 - 15 | 100 → 50 | 0 → 50 |
| 15 - 25 | 50 → 20 | 50 → 80 |
| 25 - 30 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities E, I, K and L.

*Relative retention* with reference to phloroglucinol (retention time = about 12 min): impurity E = about 0.7; impurity A = about 0.9; impurity D = about 1.3; impurity B = about 1.35; impurity K = about 1.5; impurity I = about 1.8; impurity L = about 2.0.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity A and phloroglucinol; minimum 4.0 between the peaks due to impurities D and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity D = 0.2; impurity E = 0.7; impurity I = 0.6; impurity K = 0.6; impurity L = 0.4;
- *impurities A, D, E, I, K, L*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 3 mL of solution S to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 63.05 mg of $C_6H_6O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E, I, K, L.*

*Other detectable impurities (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also 5.10. Control of impurities in substances for pharmaceutical use): B, O.*

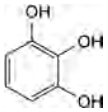

A. benzene-1,2,3-triol (pyrogallol),

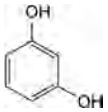

B. benzene-1,3-diol (resorcinol),

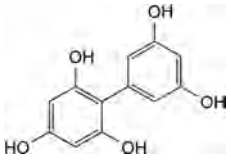

D. 2,3′,4,5′,6-biphenylpentol (phloroglucide),

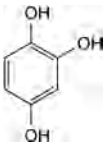

E. benzene-1,2,4-triol,

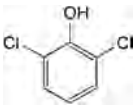

I. 2,6-dichlorophenol,

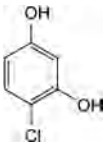

K. 4-chlorobenzene-1,3-diol (4-chlororesorcinol),

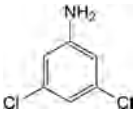

L. 3,5-dichloroaniline,

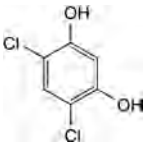

O. 4,6-dichlorobenzene-1,3-diol (4,6-dichlororesorcinol).

**01/2017:2302**
**corrected 9.7**

# PHLOROGLUCINOL DIHYDRATE

## Phloroglucinolum dihydricum

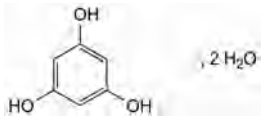

$C_6H_6O_3,2H_2O$ $M_r$ 162.1
[6099-90-7]

DEFINITION

Benzene-1,3,5-triol dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: previously dry the substance to be examined in an oven at 105 °C.

*Comparison*: *anhydrous phloroglucinol CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 0.20 g of *anhydrous phloroglucinol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *hexane R*, *ethyl acetate R* (2:37.5:62.5 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 6.0.

Dilute 10 mL of solution S to 100 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Solvent mixture*: mobile phase B, mobile phase A (10:90 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 6 mg each of *resorcinol R* (impurity B), *phloroglucide R* (impurity D) and *pyrogallol R* (impurity A) in 10 mL of the solvent mixture, add 2 mL of the test solution and dilute to 20 mL with the solvent mixture. Dilute 1 mL of this solution to 50 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 4 mg each of *benzene-1,2,4-triol R* (impurity E), *2,6-dichlorophenol R* (impurity I), *4-chlororesorcinol R* (impurity K) and *3,5-dichloroaniline R* (impurity L) in 10 mL of the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 10 mg of the substance to be examined in 10 mL of the solvent mixture, add 1 mL of reference solution (c) and dilute to 20 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 100 | 0 |
| 9 - 15 | 100 → 50 | 0 → 50 |
| 15 - 25 | 50 → 20 | 50 → 80 |
| 25 - 30 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (b) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities E, I, K and L.

*Relative retention* with reference to phloroglucinol (retention time = about 12 min): impurity E = about 0.7; impurity A = about 0.9; impurity D = about 1.3; impurity B = about 1.35; impurity K = about 1.5; impurity I = about 1.8; impurity L = about 2.0.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to impurity A and phloroglucinol; minimum 4.0 between the peaks due to impurities D and B.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity D = 0.2; impurity E = 0.7; impurity I = 0.6; impurity K = 0.6; impurity L = 0.4;
- *impurities A, D, E, I, K, L*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 3 mL of solution S to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): 20.0 per cent to 23.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.600 g in 50 mL of *water R*. Titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 63.05 mg of $C_6H_6O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E, I, K, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, O.*

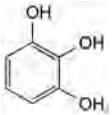


A. benzene-1,2,3-triol (pyrogallol),

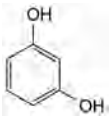

B. benzene-1,3-diol (resorcinol),

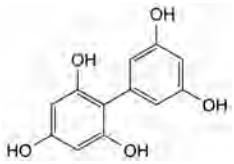

D. 2,3′,4,5′,6-biphenylpentol (phloroglucide),

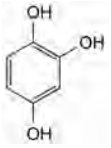

E. benzene-1,2,4-triol,

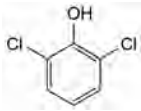

I. 2,6-dichlorophenol,

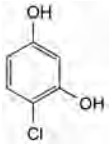

K. 4-chlorobenzene-1,3-diol (4-chlororesorcinol),

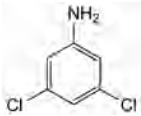

L. 3,5-dichloroaniline,

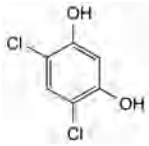

O. 4,6-dichlorobenzene-1,3-diol (4,6-dichlororesorcinol).

**04/2017:0522**

# PHOLCODINE MONOHYDRATE

## Pholcodinum monohydricum

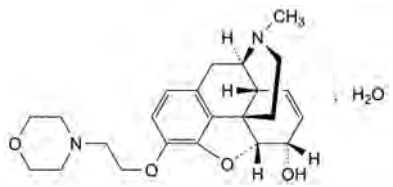

$C_{23}H_{30}N_2O_4,H_2O$ $M_r$ 416.5
[6254-99-5]

DEFINITION

17-Methyl-3-[2-(morpholin-4-yl)ethoxy]-7,8-didehydro-4,5α-epoxymorphinan-6α-ol monohydrate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: sparingly soluble in water, freely soluble in acetone and in ethanol (96 per cent). It dissolves in dilute mineral acids.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pholcodine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): − 98 to − 94 (dried substance).

Dissolve 1.000 g in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*0.02 M phosphate buffer solution*. To 80.0 mL of *0.2 M sodium hydroxide* add 100.0 mL of *0.2 M potassium dihydrogen phosphate R* and dilute to 1.0 L with *water R*.

*Solvent mixture*. Dilute 80 mL of *acetonitrile R* to 1 L with the 0.02 M phosphate buffer solution.

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *codeine R* (impurity B) in the solvent mixture and dilute to 10 mL with the solvent mixture. To 0.5 mL of this solution add 0.5 mL of the test solution and dilute to 50 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5 mg of *pholcodine for peak identification CRS* (containing impurities A, B and D) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 5 mg of *pholcodine for impurity G identification CRS* in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.075 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core phenylhexylsilyl silica gel for chromatography R* (2.6 µm);
- *temperature*: 35 °C.

*Mobile phase*: to 50 mL of *tetrahydrofuran for chromatography R* add 75 mL of *acetonitrile R* and dilute to 1000 mL with the 0.02 M phosphate buffer solution; adjust to pH 7.9 ± 0.05 with *0.2 M sodium hydroxide*; the pH must not exceed 8.0.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of pholcodine.

*Identification of impurities*: use the chromatogram supplied with *pholcodine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and D; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to pholcodine (retention time = about 10 min): impurity A = about 0.4; impurity B = about 0.8; impurity D = about 2.3; impurity G = about 4.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 3.0 between the peaks due to impurity B and pholcodine.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity G by 0.5;

0286

– *impurities A, B, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): 3.9 per cent to 4.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.



ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R*, warming gently. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 19.93 mg of $C_{23}H_{30}N_2O_4$.

IMPURITIES

*Specified impurities*: *A, B, D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F.*

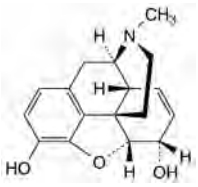

A. 17-methyl-7,8-didehydro-4,5α-epoxymorphinan-3,6α-diol (morphine),

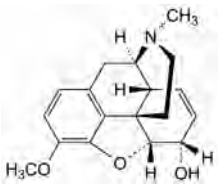

B. 3-methoxy-17-methyl-7,8-didehydro-4,5α-epoxymorphinan-6α-ol (codeine),

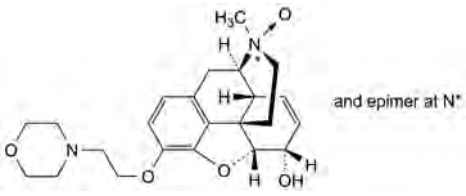


C. (17*RS*)-6α-hydroxy-17-methyl-3-[2-(morpholin-4-yl)ethoxy]-7,8-didehydro-4,5α-epoxymorphinan 17-oxide (pholcodine *N*-oxide),

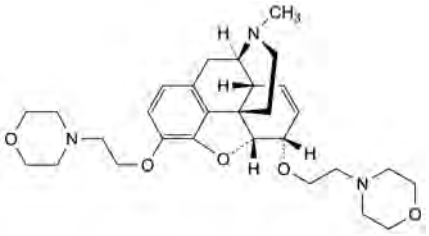

D. 17-methyl-3,6α-bis[2-(morpholin-4-yl)ethoxy]-7,8-didehydro-4,5α-epoxymorphinan,

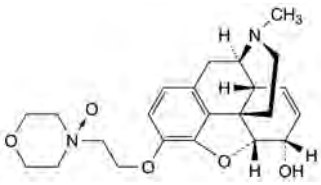

E. 4-[2-[(6α-hydroxy-17-methyl-7,8-didehydro-4,5α-epoxymorphinan-3-yl)oxy]ethyl]morpholine 4-oxide (pholcodine *N*′-oxide),

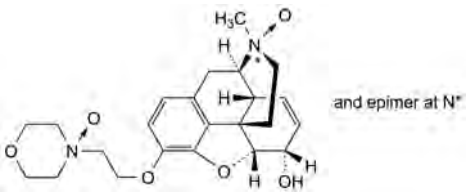


F. (17*RS*)-6α-hydroxy-17-methyl-3-[2-(4-oxidomorpholin-4-ium-4-yl)ethoxy]-7,8-didehydro-4,5α-epoxymorphinan 17-oxide (pholcodine *N*,*N*′-dioxide),

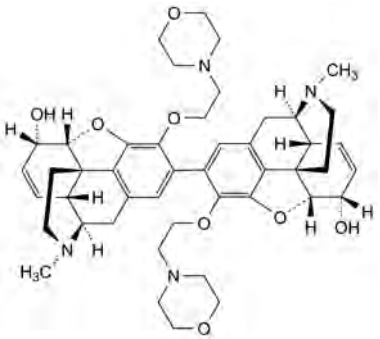

G. 17,17′-dimethyl-3,3′-bis[2-(morpholin-4-yl)ethoxy]-7,7′,8,8′-tetradehydro-4,5α:4′,5′α-diepoxybimorphinan-6α,6′α-diol (pholcodine dimer).

**01/2017:0004**

# PHOSPHORIC ACID, CONCENTRATED

## Acidum phosphoricum concentratum

$H_3PO_4$ $M_r$ 98.0
[7664-38-2]

DEFINITION

*Content*: 84.0 per cent *m/m* to 90.0 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless, syrupy liquid, corrosive.

*Solubility*: miscible with water and with ethanol (96 per cent).

When stored at a low temperature it may solidify into a mass of colourless crystals which do not melt at a temperature below 28 °C.

*Relative density*: about 1.7.

IDENTIFICATION

A. Dilute with *water R*. The solution is strongly acid (*2.2.4*).

B. Solution S (see Tests) neutralised with *dilute sodium hydroxide solution R* gives the reactions of phosphates (*2.3.1*).

TESTS

**Solution S**. Dilute 10.0 g to 150 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Substances precipitated with ammonia**. To 10 mL of solution S add 8 mL of *dilute ammonia R1*. Any opalescence in the solution is not more intense than that in a mixture of 10 mL of solution S and 8 mL of *water R*.

**Hypophosphorous acid and phosphorous acid**. To 5 mL of solution S add 2 mL of *silver nitrate solution R2* and heat on a water-bath for 5 min. The solution shows no change in appearance.

**Chlorides** (*2.4.4*): maximum 50 ppm, determined on solution S.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dilute 1.5 g to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 7.5 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 3 mL of solution S to 10 mL with *water R*.

ASSAY

To 1.000 g add a solution of 10 g of *sodium chloride R* in 30 mL of *water R*. Titrate with *1 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.00 mg of $H_3PO_4$.

STORAGE

In a glass container.

**01/2017:0005**

# PHOSPHORIC ACID, DILUTE

## Acidum phosphoricum dilutum

DEFINITION

*Content*: 9.5 per cent *m/m* to 10.5 per cent *m/m* of $H_3PO_4$ ($M_r$ 98.0).

PREPARATION

To 885 g of *water R* add 115 g of concentrated phosphoric acid and mix.

IDENTIFICATION

A. It is strongly acid (*2.2.4*).

B. Solution S (see Tests), neutralised with *dilute sodium hydroxide solution R*, gives the reactions of phosphates (*2.3.1*).

TESTS

**Solution S**. Dilute 86 g to 150 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Substances precipitated with ammonia**. To 10 mL of solution S add 8 mL of *dilute ammonia R1*. Any opalescence in the solution is not more intense than that in a mixture of 10 mL of solution S and 8 mL of *water R*.

**Hypophosphorous acid and phosphorous acid**. To 5 mL of solution S add 2 mL of *silver nitrate solution R2* and heat on a water-bath for 5 min. The solution shows no change in appearance.

**Chlorides** (*2.4.4*): maximum 6 ppm, determined on solution S.

**Sulfates** (*2.4.13*): maximum 10 ppm, determined on the preparation to be examined.

**Arsenic** (*2.4.2, Method A*): maximum 0.2 ppm, determined on 7.5 mL of solution S.

**Iron** (*2.4.9*): maximum 6 ppm.

Dilute 3 mL of solution S to 10 mL with *water R*.

ASSAY

To 8.60 g add a solution of 10 g of *sodium chloride R* in 30 mL of *water R*. Titrate with *1 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.00 mg of $H_3PO_4$.

**01/2017:0352**

# PHTHALYLSULFATHIAZOLE

## Phthalylsulfathiazolum

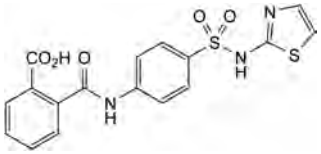

$C_{17}H_{13}N_3O_5S_2$ $M_r$ 403.4
[85-73-4]

DEFINITION

2-[[4-(Thiazol-2-ylsulfamoyl)phenyl]carbamoyl]benzoic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *phthalylsulfathiazole CRS.*

B. To 1 g add 8.5 mL of *dilute sodium hydroxide solution R* and boil under a reflux condenser for 30 min. Cool and add 17.5 mL of *dilute hydrochloric acid R*. Shake vigorously and filter. Neutralise the filtrate with *dilute sodium hydroxide solution R*. Filter, wash the precipitate with *water R*, recrystallise from *water R* and dry the crystals at 100-105 °C. The crystals melt (*2.2.14*) at 200 °C to 203 °C.

C. To 0.1 g in a test-tube add 3 mL of *dilute sulfuric acid R* and 0.5 g of *zinc powder R*. Fumes are evolved which produce a black stain on *lead acetate paper R*.

D. To 0.1 g add 0.5 g of *resorcinol R* and 0.3 mL of *sulfuric acid R* and heat on a water-bath until a homogeneous mixture is obtained. Allow to cool. Add 5 mL of *dilute sodium hydroxide solution R*. Dilute 0.1 mL of this brownish-red mixture to 25 mL with *water R*. An intense green fluorescence appears which disappears on acidification.

E. Dissolve about 10 mg of the crystals obtained in identification test B in 200 mL of *0.1 M hydrochloric acid*. 2 mL of the solution gives the reaction of primary aromatic amines (*2.3.1*) with formation of an orange precipitate.

TESTS

**Appearance of solution** The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *1 M sodium hydroxide* and dilute to 20 mL with the same solvent.

**Acidity**. To 2.0 g add 20 mL of *water R*, shake continuously for 30 min and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Sulfathiazole and other primary aromatic amines**: maximum 2.0 per cent.

Dissolve 5 mg in a mixture of 3.5 mL of *water R*, 6 mL of *dilute hydrochloric acid R* and 25 mL of *ethanol (96 per cent) R*, previously cooled to 15 °C. Place immediately in iced water and add 1 mL of a 2.5 g/L solution of *sodium nitrite R*. Allow to stand for 3 min, add 2.5 mL of a 40 g/L solution of *sulfamic acid R* and allow to stand for 5 min. Add 1 mL of a 4 g/L solution of *naphthylethylenediamine dihydrochloride R* and dilute to 50 mL with *water R*. Measured at 550 nm, the absorbance (*2.2.25*) is not greater than that of a standard prepared at the same time and in the same manner using a mixture of 1 mL of a 100 mL aqueous solution containing 10 mg of *sulfathiazole R* and 0.5 mL of *hydrochloric acid R*; 2.5 mL of *water R*; 6 mL of *dilute hydrochloric acid R*; and 25 mL of *ethanol (96 per cent) R*.

**Loss on drying** (*2.2.32*): maximum 2 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 40 mL of *dimethylformamide R*. Titrate with *0.1 M sodium hydroxide* until the colour becomes blue. using 0.2 mL of *thymolphthalein solution R* as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.17 mg of $C_{17}H_{13}N_3O_5S_2$.

STORAGE

Protected from light.

**01/2008:0286**
**corrected 6.0**

# PHYSOSTIGMINE SALICYLATE

## Physostigmini salicylas

## Eserini salicylas

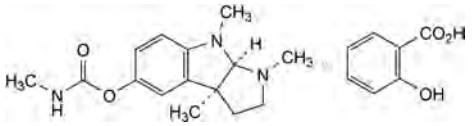

$C_{22}H_{27}N_3O_5$ $M_r$ 413.5
[57-64-7]

DEFINITION

Physostigmine salicylate contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of (3a*S*,8a*R*)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-*b*]indol-5-yl methylcarbamate salicylate, calculated with reference to the dried substance.

CHARACTERS

Colourless or almost colourless crystals, sparingly soluble in water, soluble in alcohol. The crystals gradually become red when exposed to air and light; the colour develops more quickly when the crystals are also exposed to moisture. Aqueous solutions are unstable.

It melts at about 182 °C, with decomposition.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *physostigmine salicylate CRS.*

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Heat about 10 mg in a porcelain dish with a few drops of *dilute ammonia R1*. An orange colour develops. Evaporate the solution to dryness. The residue dissolves in *alcohol R* giving a blue solution. Add 0.1 mL of *glacial acetic acid R*. The colour becomes violet. Dilute with *water R*. An intense red fluorescence appears.

D. Solution S (see Tests) gives reaction (a) of salicylates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.900 g, without heating, in 95 mL of *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Prepare immediately before use.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 5.1 to 5.9.

**Specific optical rotation** (*2.2.7*): – 90 to – 94, determined on solution S and calculated with reference to the dried substance.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution (a).* Dissolve 0.2 g of the substance to be examined in *alcohol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 2.5 mL of test solution (a) to 50 mL with *alcohol R*.

*Reference solution (a).* Dissolve 10 mg of *physostigmine salicylate CRS* in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 2 mL of reference solution (a) to 20 mL with *alcohol R*.

Apply to the plate 20 µL of each solution. Develop over a path of 15 cm using a mixture of 2 volumes of *concentrated ammonia R*, 23 volumes of *2-propanol R* and 100 volumes of *cyclohexane R*. Dry the plate in a current of cold air and carry out a second development in the same direction. Allow the plate to dry in air and spray with freshly prepared *potassium iodobismuthate solution R* and then with *dilute hydrogen peroxide solution R*. Examine the plate within 2 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Eseridine**. To 5 mL of solution S add a few crystals of *potassium iodate R*, 0.05 mL of *dilute hydrochloric acid R* and 2 mL of *chloroform R*. Shake. No violet colour develops in the chloroform layer within 1 min.

**Sulfates** (*2.4.13*). 15 mL of solution S complies with the limit test for sulfates (0.1 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

Dissolve 0.350 g in 50 mL of a mixture of equal volumes of *anhydrous acetic acid R* and *chloroform R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.35 mg of $C_{22}H_{27}N_3O_5$.

STORAGE

Store in an airtight container, protected from light.

**01/2020:3011**

# PHYTOMENADIONE, RACEMIC

## Phytomenadionum racemicum

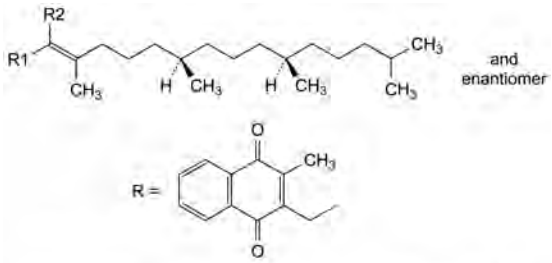


| Phytomenadione | R1 | R2 | Molecular formula | $M_r$ |
|---|---|---|---|---|
| *trans* isomers | R | H | $C_{31}H_{46}O_2$ | 450.7 |
| *cis* isomers | H | R | $C_{31}H_{46}O_2$ | 450.7 |

$C_{31}H_{46}O_2$ $M_r$ 450.7

DEFINITION

Mixture of 2-methyl-3-[(2*E*,7*RS*,11*RS*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]naphthalene-1,4-dione (*trans*-phytomenadione isomers) and 2-methyl-3-[(2*Z*,7*RS*,11*RS*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]naphthalene-1,4-dione (*cis*-phytomenadione isomers).

*Content*:

– *trans*-phytomenadione isomers: minimum 85.0 per cent;
– sum of *trans*-phytomenadione and *cis*-phytomenadione isomers: 97.0 per cent to 103.0 per cent.

CHARACTERS

*Appearance*: clear, intense yellow, viscous, oily liquid.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent), miscible with fatty oils.

It decomposes on exposure to actinic light.

*Refractive index*: about 1.526.

IDENTIFICATION

*Prepare the solutions immediately before use and protect from light.*

A. Optical rotation (see Tests).

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 10.0 mg of the substance to be examined in *trimethylpentane R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 50.0 mL with *trimethylpentane R*.

*Spectral range*: 275-340 nm for test solution (a); 230-280 nm for test solution (b).

*Absorption maxima*: 327 nm for test solution (a); 243 nm, 249 nm, 261 nm and 270 nm for test solution (b).

*Absorption minimum*: 285 nm for test solution (a).

*Specific absorbance at the absorption maximum at 327 nm*: 67 to 73 for test solution (a).

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 2.5 g in *trimethylpentane R* and dilute to 25 mL with the same solvent.

**Optical rotation** (*2.2.7*): – 0.05° to + 0.05°, measured at 25 °C.

Dissolve 0.25 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.00 g.

**Impurity A**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 0.40 g of the substance to be examined in *cyclohexane R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 4.0 mg of *menadione R* (impurity A) in *cyclohexane R* and dilute to 50.0 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *cyclohexane R*, *toluene R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air for 5 min.

*Detection*: examine in ultraviolet light at 254 nm.

*Relative retention* with reference to racemic *trans*-phytomenadione ($R_F$ = about 0.5): impurity A = about 0.4.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 20.0 mg of *phytomenadione CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *trans-epoxyphytomenadione CRS* (impurity B) in 1 mL of the mobile phase. Add 0.4 mL of the solution to 5 mL of reference solution (a) and dilute to 100 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: spherical *silica gel for chromatography R* (5 µm).

*Mobile phase*: *octanol R*, *di-isopropyl ether R*, *heptane R* (1:3.3:1000 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for at least 24 h.

*Injection*: 50 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.6 times the retention time of *trans*-phytomenadione isomers.

*Relative retention* with reference to *trans*-phytomenadione isomers (retention time = about 27 min): impurity B = about 0.6; *cis*-phytomenadione isomers = about 0.65.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity B and *cis*-phytomenadione isomers; minimum 4.0 between the peaks due to *cis*-phytomenadione isomers and *trans*-phytomenadione isomers.

*Calculation of percentage contents*:

– for each impurity, use the concentration of *trans*-phytomenadione isomers in reference solution (c), taking into account their content as determined in the assay.

*Limits*:

– *impurity B*: maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.15 per cent;

– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (d).

*System suitability*: reference solution (d):

– *repeatability*: maximum relative standard deviation of 1.0 per cent for the peak due to *trans*-phytomenadione isomers, determined on 6 injections.

Calculate the percentage contents of *trans*-phytomenadione isomers and *cis*-phytomenadione isomers taking into account the assigned content of *phytomenadione CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B*.

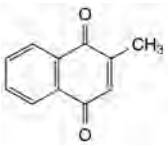


A. 2-methylnaphthalene-1,4-dione (menadione),

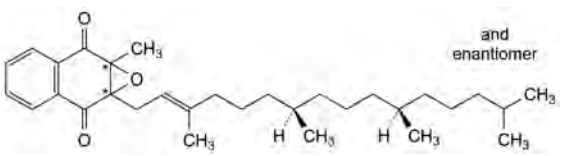


B. (1aΞ,7aΞ)-1a-methyl-7a-[(2*E*,7*RS*,11*RS*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]-1a,7a-dihydronaphtho[2,3-*b*]oxirene-2,7-dione (*trans*-epoxyphytomenadione isomers).

**01/2008:1911**
**corrected 10.0**

# PHYTOSTEROL

## Phytosterolum

DEFINITION

Natural mixture of sterols obtained from plants of the genera *Hypoxis*, *Pinus* and *Picea*.

*Content*: minimum 70.0 per cent of β-sitosterol (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in tetrahydrofuran, sparingly soluble in ethyl acetate.

IDENTIFICATION

A. Mix 1 mL of *acetic anhydride R* with 0.5 mL of solution S (see Tests). After the addition of 0.1 mL of *sulfuric acid R* a red colour is produced, which changes rapidly to violet, then to blue and finally to green.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the peak in the chromatogram obtained with reference solution (b).

TESTS

**Solution S**. Dissolve 1.0 g in *tetrahydrofuran R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Shake 0.20 g with a mixture of 4.0 mL of *ethyl acetate R* and 10.0 mL of *carbon dioxide-free water R* for 3 min. Allow the layers to separate. To the aqueous layer add 0.1 mL of *bromothymol blue solution R1*. If the solution is yellow, not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour to blue. If the solution is blue, not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour to yellow.

**Specific optical rotation** (*2.2.7*): – 28.0 to – 15.0 (dried substance).

Dissolve 0.500 g in *ethyl acetate R* and dilute to 10.0 mL with the same solvent.

**Acid value** (*2.5.1*): maximum 1.0, determined on 2.0 g.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Saponification value** (*2.5.6*): maximum 1.0.

Carry out the test using 2.50 g of the substance to be examined, *0.1 M alcoholic potassium hydroxide*, *0.1 M hydrochloric acid*, and a factor of 5.61 (instead of 28.05).

**Other sterols**. Examine the chromatogram obtained with the test solution in the assay (Figure 1911.-1).

*Composition of the other sterols*:

– *cholesterol*: maximum 0.5 per cent;
– *brassicasterol*: maximum 0.5 per cent;
– *campesterol*: maximum 15.0 per cent;
– *campestanol*: maximum 5.0 per cent;
– *stigmasterol*: maximum 5.0 per cent;
– *sitostanol*: maximum 15.0 per cent;
– *Δ7-stigmastenol*: maximum 5.0 per cent.

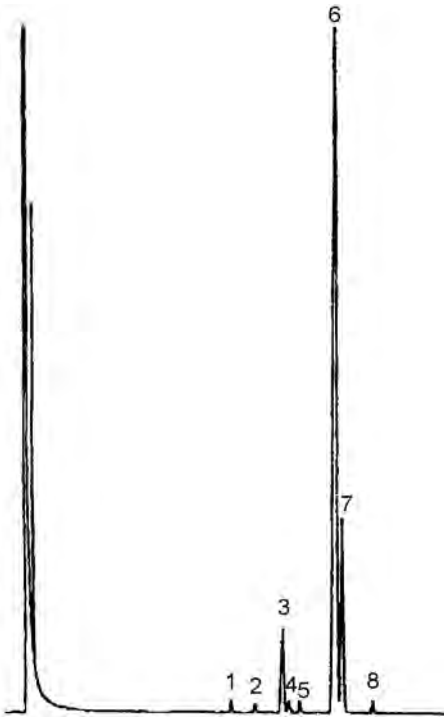


1. cholesterol
2. brassicasterol
3. campesterol
4. campestanol
5. stigmasterol
6. β-sitosterol
7. sitostanol
8. Δ7-stigmastenol

Figure 1911.-1. – *Chromatogram for the assay of phytosterol (trimethylsilyl derivatives)*

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 0.250 g by drying in an oven at 105 °C for 2 h.

**Total ash** (*2.4.16*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 0.100 g in *tetrahydrofuran R* and dilute to 10.0 mL with the same solvent. Introduce 100 µL of this solution into a 3 mL flask and evaporate to dryness under *nitrogen R*. Add 100 µL of a freshly prepared mixture of 50 µL of *1-methylimidazole R* and 1.0 mL of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*, close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

*Reference solution (a).* Dissolve 25 mg of *β-sitosterol R* and 25 mg of *sitostanol R* in *tetrahydrofuran R* and dilute to 10.0 mL with the same solvent. Introduce 100 µL of this solution into a 3 mL flask and evaporate to dryness under *nitrogen R*. Add 100 µL of a freshly prepared mixture of 50 µL of *1-methylimidazole R* and 1.0 mL of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

*Reference solution (b).* Dissolve 0.100 g of *β-sitosterol R* in *tetrahydrofuran R* and dilute to 10.0 mL with the same solvent. Introduce 100 µL of this solution into a 3 mL flask and evaporate to dryness under *nitrogen R*. Add 100 µL of a freshly prepared mixture of 50 µL of *1-methylimidazole R* and 1.0 mL of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat to 100 °C for 15 min. Allow to cool.

*Column*:

– *material*: quartz;
– *size*: *l* = 25 m, Ø = 0.3 mm;

– *stationary phase*: *vinyl(1)phenyl(5)methyl(94)polysiloxane R* (1 µm).

*Carrier gas*: *hydrogen for chromatography R*.

*Flow rate*: 2 mL/min.

*Split ratio*: 1:20.

*Temperature*:

– *column*: 280 °C;

– *injection port and detector*: 300 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retentions* with reference to β-sitosterol (retention time = about 16 min): cholesterol = about 0.7; brassicasterol = about 0.77; campesterol = about 0.84; campestanol = about 0.86; stigmasterol = about 0.9; sitostanol = about 1.02; Δ7-stigmastenol = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.0 between the peaks due to β-sitosterol and sitostanol.

STORAGE

In an airtight container, protected from light.



**01/2017:1358**

# PICOTAMIDE MONOHYDRATE

## Picotamidum monohydricum

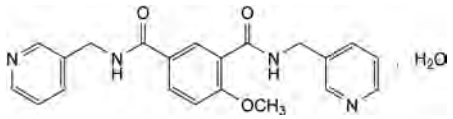

$C_{21}H_{20}N_4O_3,H_2O$ $M_r$ 394.4

DEFINITION

Picotamide monohydrate contains not less than 98.0 per cent and not more than 101.0 per cent of 4-methoxy-*N*,*N*′-bis(pyridin-3-ylmethyl)benzene-1,3-dicarboxamide, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, slightly soluble in water, soluble in ethanol and in methylene chloride. It dissolves in dilute mineral acids.

It shows polymorphism (*5.9*).

IDENTIFICATION

Examine by infrared spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *picotamide monohydrate CRS*. If the spectra obtained in the solid state shows differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. Dissolve 2.5 g in *methanol R* and dilute to 50 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R*.

*Test solution*. Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dilute 1 mL of the test solution to 10 mL with *methanol R*. Dilute 1 mL of the solution to 20 mL with *methanol R*.

*Reference solution (b)*. Dilute 5 mL of reference solution (a) to 10 mL with *methanol R*.

*Reference solution (c)*. Dissolve 0.5 g of the substance to be examined and 5 mg of *picotamide impurity A CRS* in *methanol R* and dilute to 10 mL with the same solvent.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture 0.8 volumes of *glacial acetic acid R*, 1 volume of *water R*, 2.5 volumes of *methanol R* and 8 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the principal spot in the chromatogram obtained with reference solution (a) (0.5 per cent) and not more than one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

**Chlorides** (*2.4.4*). Dissolve 0.25 g in a mixture of 2.5 mL of *dilute nitric acid R* and 12.5 mL of *water R*. The solution complies with the limit test for chlorides (200 ppm).

**Water** (*2.5.12*): 4.5 per cent to 5.0 per cent, determined on 0.300 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in a mixture of 20 mL of *anhydrous acetic acid R* and 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.82 mg of $C_{21}H_{20}N_4O_3$.

IMPURITIES

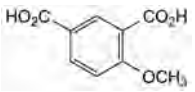

A. 4-methoxybenzene-1,3-dicarboxylic acid,

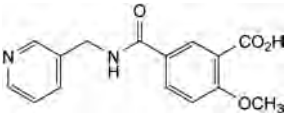

B. 2-methoxy-5-[[(pyridin-3-ylmethyl)amino]carbonyl]-benzoic acid,

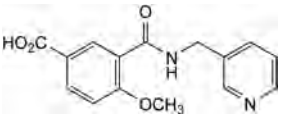

C. 4-methoxy-3-[[(pyridin-3-ylmethyl)amino]carbonyl]-benzoic acid,

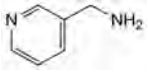

D. (pyridin-3-yl)methanamine.

**04/2016:0633**

# PILOCARPINE HYDROCHLORIDE

## Pilocarpini hydrochloridum

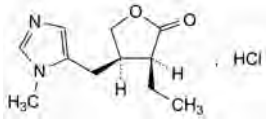

$C_{11}H_{17}ClN_2O_2$ $M_r$ 244.7
[54-71-7]

DEFINITION

(3*S*,4*R*)-3-Ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]-dihydrofuran-2(3*H*)-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: very soluble in water and freely soluble in ethanol (96 per cent).

mp: about 203 °C.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pilocarpine hydrochloride CRS*.

If the substances are examined as discs, prepare them using *potassium chloride R*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 2 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *pilocarpine hydrochloride CRS* in *methanol R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:14:85 *V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 15 cm.

*Drying*: at 100-105 °C for 10 min, then allow to cool.

*Detection*: spray with *dilute potassium iodobismuthate solution R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 4.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 89 to + 93 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 20.0 mL with *water R*.

*Reference solution (b).* Dissolve 5.0 mg of *pilocarpine nitrate for system suitability CRS* (containing impurity A) in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (c).* To 5 mL of the test solution, add 0.1 mL of *ammonia R* and heat the solution on a water-bath for 30 min, cool and dilute to 25 mL with *water R*. Dilute 3 mL of this solution to 25 mL with *water R*. Mainly pilocarpic acid (impurity B) is formed.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.

*Mobile phase*: mix 55 volumes of *methanol R*, 60 volumes of *acetonitrile R* and 885 volumes of a 0.679 g/L solution of *tetrabutylammonium dihydrogen phosphate R* previously adjusted to pH 7.7 with *dilute ammonia R2*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of pilocarpine.

*Elution order*: impurity B, impurity C, impurity A, pilocarpine.

*Retention time*: pilocarpine = about 20 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.6 between the peaks due to impurity A and pilocarpine.

*Limits*:

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
– *sum of impurities A and B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
– *sum of impurities other than A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S. Prepare the standard using 5 mL of *iron standard solution (1 ppm Fe) R* and 5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *ethanol (96 per cent) R* and add 5 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.47 mg of $C_{11}H_{17}ClN_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C*.

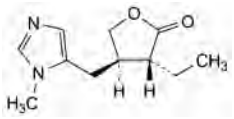

A. (3*R*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)-methyl]dihydrofuran-2(3*H*)-one (isopilocarpine),

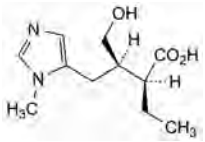

B. (2*S*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (pilocarpic acid),

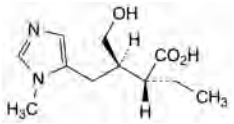

C. (2*R*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (isopilocarpic acid).

**04/2016:0104**

# PILOCARPINE NITRATE

## Pilocarpini nitras

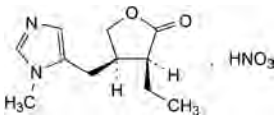

$C_{11}H_{17}N_3O_5$ $M_r$ 271.3
[148-72-1]

DEFINITION

(3*S*,4*R*)-3-Ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]-dihydrofuran-2(3*H*)-one nitrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

It is sensitive to light.

mp: about 174 °C, with decomposition.

IDENTIFICATION

*First identification*: *A, B, D.*

*Second identification*: *C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pilocarpine nitrate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.
*Reference solution.* Dissolve 10 mg of *pilocarpine nitrate CRS* in *water R* and dilute to 10 mL with the same solvent.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:14:85 *V/V/V*).
*Application*: 10 µL.
*Development*: over a path of 15 cm.
*Drying*: at 100-105 °C for 10 min and allow to cool.
*Detection*: spray with *potassium iodobismuthate solution R*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of nitrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent. Prepare immediately before use.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 4.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 80 to + 83 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution.* Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.
*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 20.0 mL with *water R*.
*Reference solution (b).* Dissolve 5.0 mg of *pilocarpine nitrate for system suitability CRS* (containing impurity A) in *water R* and dilute to 50.0 mL with the same solvent.
*Reference solution (c).* To 5 mL of the test solution, add 0.1 mL of *ammonia R* and heat the solution on a water-bath for 30 min, cool and dilute to 25 mL with *water R*. Dilute 3 mL of this solution to 25 mL with *water R*. Mainly pilocarpic acid (impurity B) is formed.
*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R1* (5 µm) with a pore size of 10 nm and a carbon loading of 19 per cent.
*Mobile phase*: mix 55 volumes of *methanol R*, 60 volumes of *acetonitrile R* and 885 volumes of a 0.679 g/L solution of *tetrabutylammonium dihydrogen phosphate R* previously adjusted to pH 7.7 with *dilute ammonia R2*.
*Flow rate*: 1.2 mL/min.
*Detection*: spectrophotometer at 220 nm.
*Injection*: 20 µL.
*Run time*: twice the retention time of pilocarpine.
*Elution order*: impurity B, impurity C, impurity A, pilocarpine.
*Retention time*: pilocarpine = about 20 min.
*System suitability*: reference solution (b):
– *resolution*: minimum 1.6 between the peaks due to impurity A and pilocarpine.
*Limits*:
– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);

- *sum of impurities A and B*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *sum of impurities other than A and B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent); disregard any peak due to the nitrate ion with a relative retention with reference to pilocarpine of about 0.3.

**Chlorides** (*2.4.4*): maximum 70 ppm, determined on solution S.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S. Prepare the standard using 5 mL of *iron standard solution (1 ppm Fe) R* and 5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.13 mg of $C_{11}H_{17}N_3O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

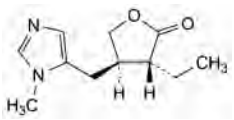

A. (3*R*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl)-methyl]dihydrofuran-2(3*H*)-one (isopilocarpine),

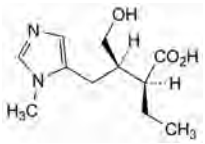

B. (2*S*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (pilocarpic acid),

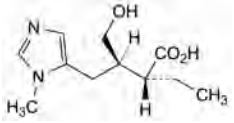

C. (2*R*,3*R*)-2-ethyl-3-(hydroxymethyl)-4-(1-methyl-1*H*-imidazol-5-yl)butanoic acid (isopilocarpic acid).

**07/2018:2179**

# PIMOBENDAN FOR VETERINARY USE

## Pimobendanum ad usum veterinarium

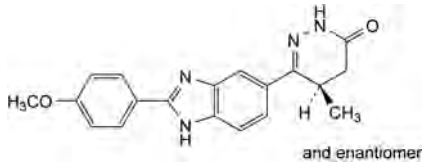


$C_{19}H_{18}N_4O_2$ $M_r$ 334.4
[74150-27-9]

DEFINITION

(5*RS*)-6-[2-(4-Methoxyphenyl)-1*H*-benzimidazol-5-yl]-5-methyl-4,5-dihydropyridazin-3(2*H*)-one.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish, hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in dimethylformamide, slightly soluble in acetone and in methanol.

mp: about 242 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pimobendan CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve the contents of a vial of *pimobendan for system suitability CRS* (impurities A and B) in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.0 g of *potassium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 2.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 85 → 80 | 15 → 20 |
| 6 - 20 | 80 → 20 | 20 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 290 nm.

*Injection*: 10 µL.

*Relative retention* with reference to pimobendan (retention time = about 8.3 min): impurity A = about 1.3; impurity B = about 1.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent).

**Heavy metals** (*2.4.8*): maximum 10 ppm.

2.0 g complies with test F. Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 5 mL of *anhydrous formic acid R*. Add 10 mL of *acetic anhydride R* and 70 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.44 mg of $C_{19}H_{18}N_4O_2$.

STORAGE

In an airtight container.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

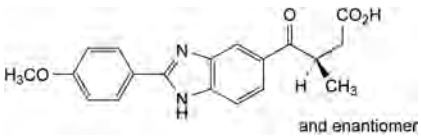


A. (3*RS*)-4-[2-(4-methoxyphenyl)-1*H*-benzimidazol-5-yl]-3-methyl-4-oxobutanoic acid,

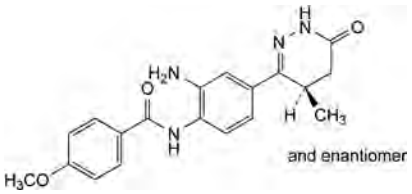


B. (5*RS*)-6-[3-amino-4-(4-methoxybenzamido)phenyl]-5-methyl-4,5-dihydropyridazin-3(2*H*)-one.

**01/2012:1254**

# PIMOZIDE

## Pimozidum

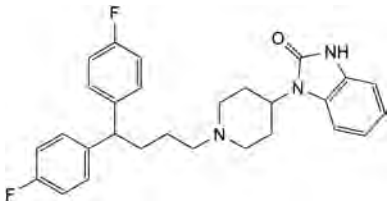


$C_{28}H_{29}F_2N_3O$ $M_r$ 461.6
[2062-78-4]

DEFINITION

1-[1-[4,4-Bis(4-fluorophenyl)butyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 216 °C to 220 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pimozide CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.
*Reference solution (a).* Dissolve 30 mg of *pimozide CRS* in the mobile phase and dilute to 10 mL with the mobile phase.
*Reference solution (b).* Dissolve 30 mg of *pimozide CRS* and 30 mg of *benperidol CRS* in the mobile phase and dilute to 10 mL with the mobile phase.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *acetone R*, *methanol R* (10:90 *V/V*).
*Application*: 10 µL.
*Development*: over 3/4 of the plate.
*Drying*: in a current of warm air for 15 min.
*Detection*: expose to iodine vapour until the spots appear.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, then add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *pimozide CRS* and 2.0 mg of *mebendazole CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: solution containing 2.5 g/L of *ammonium acetate R* and 8.5 g/L of *tetrabutylammonium hydrogen sulfate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 80 → 70 | 20 → 30 |
| 10 - 15 | 70 | 30 |
| 15 - 20 | 80 | 20 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Relative retention* with reference to pimozide (retention time = about 8 min): impurity A = about 0.1; mebendazole = about 0.88; impurity B = about 0.9; impurity C = about 0.95; impurity D = about 1.1; impurity E = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to mebendazole and pimozide.

*Limits*:

- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.75 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 46.16 mg of $C_{28}H_{29}F_2N_3O$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

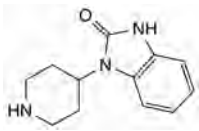

A. 1-(piperidin-4-yl)-1,3-dihydro-2*H*-benzimidazol-2-one,

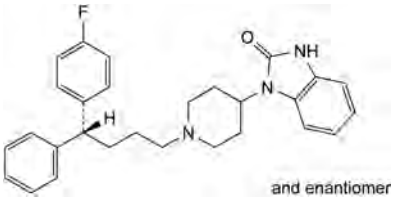


B. 1-[1-[(4*RS*)-4-(4-fluorophenyl)-4-phenylbutyl]piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

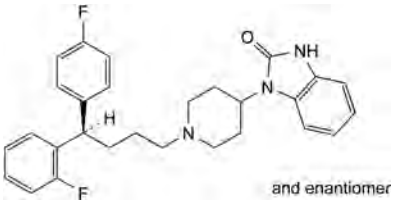


C. 1-[1-[(4*RS*)-4-(2-fluorophenyl)-4-(4-fluorophenyl)butyl]-piperidin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

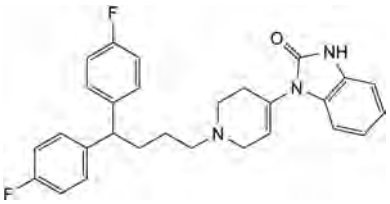

D. 1-[1-[4,4-bis(4-fluorophenyl)butyl]-1,2,3,6-tetrahydropyridin-4-yl]-1,3-dihydro-2*H*-benzimidazol-2-one,

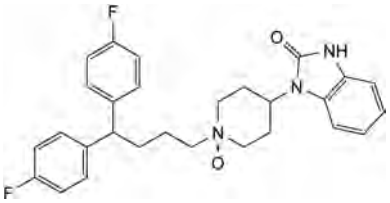

E. 1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl 1-oxide]-1,3-dihydro-2*H*-benzimidazol-2-one.

**01/2017:0634**

# PINDOLOL

## Pindololum

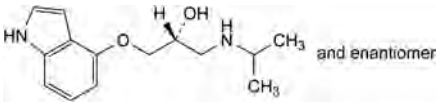


$C_{14}H_{20}N_2O_2$ $M_r$ 248.3

[13523-86-9]

DEFINITION

Pindolol contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (2*RS*)-1-(1*H*-indol-4-yloxy)-3-[(1-methylethyl)amino]propan-2-ol, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in methanol. It dissolves in dilute mineral acids.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 169 °C to 174 °C.

B. Dissolve 20.0 mg in a 0.085 per cent *V/V* solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 mL with the same solution. Dilute 10.0 mL of the solution to 100.0 mL with a 0.085 per cent *V/V* solution of *hydrochloric acid R* in *methanol R*. Examined between 230 nm and 320 nm (*2.2.25*), the solution shows two absorption maxima, at 264 nm and at 287 nm, and a shoulder at 275 nm. The specific absorbance at the maxima are 330 to 350 and 170 to 190, respectively.

C. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *pindolol CRS*.

D. Examine in daylight the chromatograms on plate A obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. Dissolve 0.5 g in *dilute acetic acid R* and dilute to 10 mL with the same acid. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ or $B_5$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance. *Carry out all operations as rapidly as possible, protected from light.*

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents. Prepare immediately before use and apply this solution to the plate last.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *pindolol CRS* in a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.5 mL of reference solution (a) to 50 mL with a mixture of 1 volume of *anhydrous acetic acid R* and 99 volumes of *methanol R*.

A. Apply separately 5 µL of each solution. Develop the plate without delay over a path of 10 cm using a freshly prepared mixture of 4 volumes of *concentrated ammonia R*, 50 volumes of *ethyl acetate R* and 50 volumes of *methanol R*. Dry the plate briefly in a current of cold air. Spray the plate without delay with *dimethylaminobenzaldehyde solution R7* and heat to 50 °C for 20 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent).

B. Apply separately 10 µL of each solution. Develop the plate without delay over a path of 10 cm using a freshly prepared mixture of 4 volumes of *concentrated ammonia R*, 50 volumes of *ethyl acetate R* and 50 volumes of *methanol R*. Dry the plate briefly in a current of cold air. Examine the plate without delay in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot and the spots detected on plate A, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.3 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 80 mL of *methanol R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 24.83 mg of $C_{14}H_{20}N_2O_2$.

STORAGE

Store protected from light.

IMPURITIES

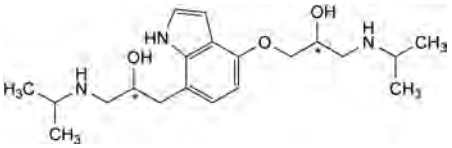

A. 1-[[7-[2-hydroxy-3-[(1-methylethyl)amino]propyl]-1*H*-indol-4-yl]oxy]-3-[(1-methylethyl)amino]propan-2-ol,

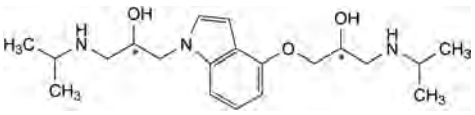

B. 1-[4-[2-hydroxy-3-[(1-methylethyl)amino]propoxy]-1*H*-indol-1-yl]-3-[(1-methylethyl)amino]propan-2-ol,

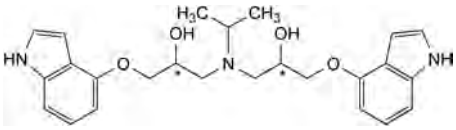

C. 1,1′-[(1-methylethyl)imino]bis[3-(1*H*-indol-4-yloxy)propan-2-ol],

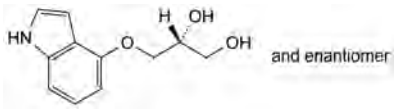


D. (2*RS*)-3-(1*H*-indol-4-yloxy)propane-1,2-diol,

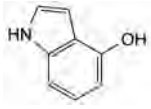

E. 1*H*-indol-4-ol,

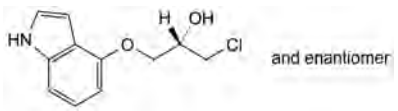


F. (2*RS*)-1-chloro-3-(1*H*-indol-4-yloxy)propan-2-ol.

**01/2017:2601**

# PIOGLITAZONE HYDROCHLORIDE

## Pioglitazoni hydrochloridum

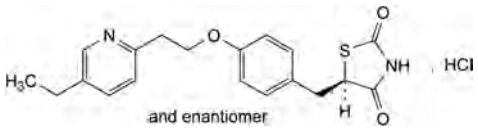


$C_{19}H_{21}ClN_2O_3S$ $M_r$ 392.9
[112529-15-4]

DEFINITION

(5*RS*)-5-[[4-[2-(5-Ethylpyridin-2-yl)ethoxy]phenyl]methyl]thiazolidine-2,4-dione hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white crystals or crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble to soluble in methanol, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pioglitazone hydrochloride CRS*.

B. Dissolve 25 mg in 0.5 mL of *nitric acid R* and add 2 mL of *dilute nitric acid R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): − 0.2 to + 0.2.

Dissolve 2.5 g in *dimethylformamide R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 20 mg of the substance to be examined in 20 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Test solution (b)*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Suspend 5 mg of *pioglitazone for system suitability CRS* (containing impurities B and C) in 5 mL of *methanol R*. Heat at 60 °C for about 30 s, cool to room temperature and dilute to 25.0 mL with the mobile phase. Filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution (c)*. Dissolve 50.0 mg of *pioglitazone hydrochloride CRS* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, 7.71 g/L solution of *ammonium acetate R* (1:25:25 *V/V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 269 nm.

*Injection*: 40 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of pioglitazone.

*Identification of impurities*: use the chromatogram supplied with *pioglitazone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to pioglitazone (retention time = about 7 min): impurity B = about 1.4; impurity C = about 3.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to pioglitazone and impurity B.

*Limits*:

– *impurities B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{19}H_{21}ClN_2O_3S$ taking into account the assigned content of *pioglitazone hydrochloride CRS*.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E.*

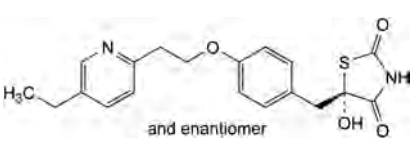


A. (5*RS*)-5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]-phenyl]methyl]-5-hydroxythiazolidine-2,4-dione,

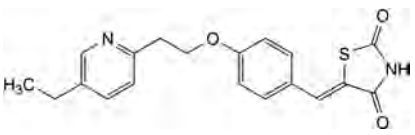


B. (5*Z*)-5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]-phenyl]methylene]thiazolidine-2,4-dione,

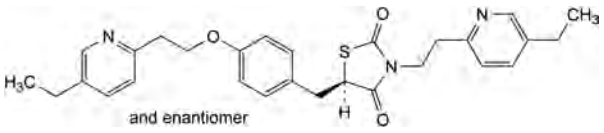


C. (5*RS*)-5-[[4-[2-(5-ethylpyridin-2-yl)ethoxy]-phenyl]methyl]-3-[2-(5-ethylpyridin-2-yl)-ethyl]thiazolidine-2,4-dione,

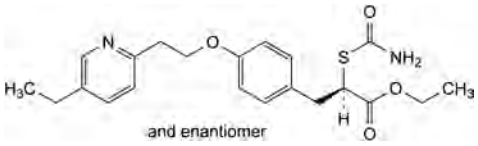


D. ethyl (2*RS*)-2-(carbamoylsulfanyl)-3-[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl]propanoate,

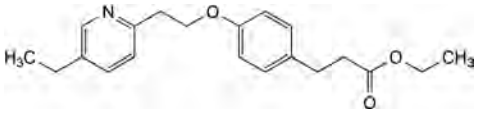

E. ethyl 3-[4-[2-(5-ethylpyridin-2-yl)ethoxy]phenyl]-propanoate.



**01/2017:1743**

# PIPEMIDIC ACID TRIHYDRATE

## Acidum pipemidicum trihydricum

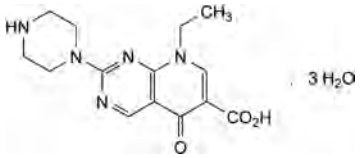

$C_{14}H_{17}N_5O_3,3H_2O$ $M_r$ 357.4
[72571-82-5]

DEFINITION

8-Ethyl-5-oxo-2-(piperazin-1-yl)-5,8-dihydropyrido-[2,3-*d*]pyrimidine-6-carboxylic acid trihydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow or yellow, crystalline powder.

*Solubility*: very slightly soluble in water and in methylene chloride, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of acids and of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pipemidic acid trihydrate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 10 mL of the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 1.0 mg of *ethyl parahydroxybenzoate R* in 2.0 mL of the test solution and dilute to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a solution containing 5.7 g/L of *citric acid monohydrate R* and 1.7 g/L of *sodium decanesulfonate R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of pipemidic acid.

*Relative retention* with reference to pipemidic acid (retention time = about 15 min): ethyl parahydroxybenzoate = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to ethyl parahydroxybenzoate and pipemidic acid.

*Limits*:

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): 14.0 per cent to 16.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.240 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.33 mg of $C_{14}H_{17}N_5O_3$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F.*

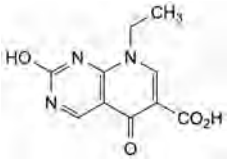

A. 8-ethyl-2-hydroxy-5-oxo-5,8-dihydropyrido-[2,3-*d*]pyrimidine-6-carboxylic acid,

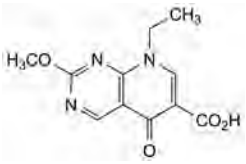

B. 8-ethyl-2-methoxy-5-oxo-5,8-dihydropyrido-[2,3-*d*]pyrimidine-6-carboxylic acid,

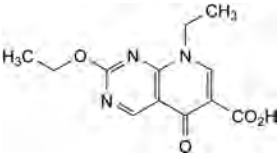

C. 2-ethoxy-8-ethyl-5-oxo-5,8-dihydropyrido-[2,3-*d*]pyrimidine-6-carboxylic acid,

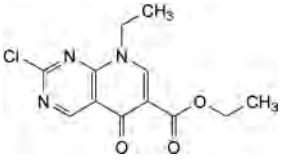

D. ethyl 2-chloro-8-ethyl-5-oxo-5,8-dihydropyrido-[2,3-*d*]pyrimidine-6-carboxylate,

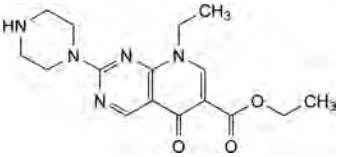

E. ethyl 8-ethyl-5-oxo-2-(piperazin-1-yl)-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylate,

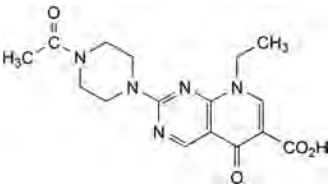

F. 2-(4-acetylpiperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-*d*]pyrimidine-6-carboxylic acid (acetylpipemidic acid).

**01/2017:1169**

# PIPERACILLIN

## Piperacillinum

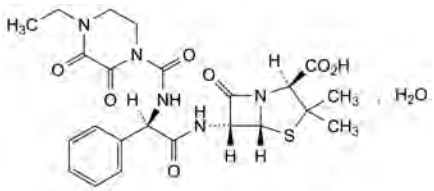

$C_{23}H_{27}N_5O_7S,H_2O$ $M_r$ 535.6
[66258-76-2]

DEFINITION

(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(4-Ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid monohydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, slightly soluble in ethyl acetate.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *piperacillin CRS*.

TESTS

**Solution S**. Dissolve 2.50 g in *sodium carbonate solution R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.10.

**Specific optical rotation** (*2.2.7*): + 168 to + 178 (anhydrous substance).

Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 31.2 g/L solution of *sodium dihydrogen phosphate R* (25:75 *V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. *Prepare the solution immediately before use*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *piperacillin CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10.0 mg of *piperacillin CRS* and 10.0 mg of *anhydrous ampicillin CRS* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 576 mL of *water R*, 200 mL of a 31.2 g/L solution of *sodium dihydrogen phosphate R* and 24 mL of an 80 g/L solution of *tetrabutylammonium hydroxide R*; if necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; add 200 mL of *acetonitrile R*;
- *mobile phase B*: mix 126 mL of *water R*, 200 mL of a 31.2 g/L solution of *sodium dihydrogen phosphate R* and 24 mL of an 80 g/L solution of *tetrabutylammonium hydroxide R*; if necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; add 650 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 88 | 12 |
| $t_R$ - ($t_R$ + 30) | 88 → 0 | 12 → 100 |
| ($t_R$ + 30) - ($t_R$ + 45) | 0 → 88 | 100 → 12 |

$t_R$ = retention time of piperacillin determined with reference solution (b)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of reference solutions (b), (c) and (d) with isocratic elution at the initial mobile phase composition and 20 µL of test solution (b) according to the elution gradient described under Mobile phase.

*System suitability*:

- *resolution*: minimum 10 between the peaks due to impurity A and piperacillin in the chromatogram obtained with reference solution (c); if necessary, adjust the ratio A:B of the mobile phase;
- *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (d);
- *mass distribution ratio*: 2.0 to 3.0 for the peak due to piperacillin in the chromatogram obtained with reference solution (c).

*Limit*:

- *any impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent).

***N*,*N*-Dimethylaniline** (*2.4.26, Method A*): maximum 20 ppm.

**Water** (*2.5.12*): 2.0 per cent to 4.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

IMPURITIES

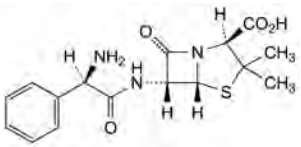

A. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ampicillin),

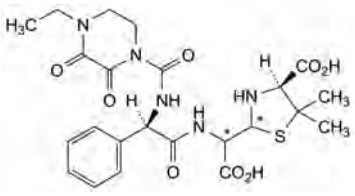

B. (4*S*)-2-[carboxy[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of piperacillin),

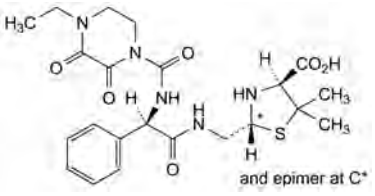


C. (2*RS*,4*S*)-2-[[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of piperacillin),

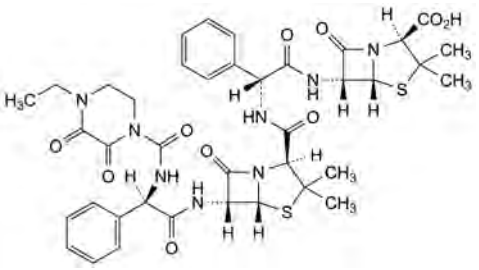

D. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (piperacillinylampicillin),

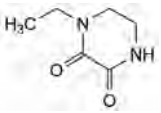

E. 1-ethylpiperazine-2,3-dione,

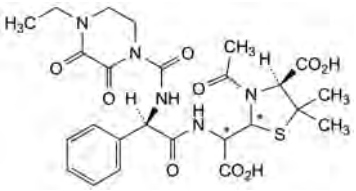

F. (4*S*)-3-acetyl-2-[carboxy[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (acetylated penicilloic acids of piperacillin).

**01/2017:1168**
**corrected 10.0**

# PIPERACILLIN SODIUM

## Piperacillinum natricum

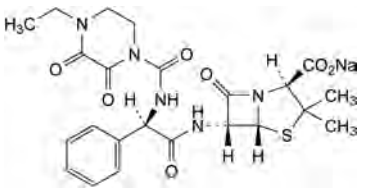

$C_{23}H_{26}N_5NaO_7S$ $M_r$ 539.5
[59703-84-3]

DEFINITION

Sodium (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in ethyl acetate.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.250 g in *water R*, add 0.5 mL of *dilute hydrochloric acid R* and 5 mL of *ethyl acetate R*; stir and allow to stand for 10 min in iced water. Filter the crystals through a small sintered-glass filter (40), applying suction. Wash with 5 mL of *water R* and 5 mL of *ethyl acetate R*, then dry in an oven at 60 °C for 60 min.

*Comparison*: *piperacillin CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 430 nm is not greater than 0.10.

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 175 to + 190 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 31.2 g/L solution of *sodium dihydrogen phosphate R* (25:75 *V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. *Prepare the solution immediately before use*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *piperacillin CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10.0 mg of *piperacillin CRS* and 10.0 mg of *anhydrous ampicillin CRS* (impurity A) in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 576 mL of *water R*, 200 mL of a 31.2 g/L solution of *sodium dihydrogen phosphate R* and 24 mL of an 80 g/L solution of *tetrabutylammonium hydroxide R*; if necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; add 200 mL of *acetonitrile R*;
- *mobile phase B*: mix 126 mL of *water R*, 200 mL of a 31.2 g/L solution of *sodium dihydrogen phosphate R* and 24 mL of an 80 g/L solution of *tetrabutylammonium hydroxide R*; if necessary, adjust to pH 5.5 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*; add 650 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - $t_R$ | 88 | 12 |
| $t_R$ - ($t_R$ + 30) | 88 → 0 | 12 → 100 |
| ($t_R$ + 30) - ($t_R$ + 45) | 0 → 88 | 100 → 12 |

$t_R$ = retention time of piperacillin determined with reference solution (b)

If the mobile phase composition has been adjusted to achieve the required resolution, the adjusted composition will apply at time zero in the gradient and in the assay.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of reference solutions (b), (c) and (d) with isocratic elution at the initial mobile phase composition and 20 µL of test solution (b) according to the elution gradient described under Mobile phase.

*System suitability*:

- *resolution*: minimum 10 between the peaks due to impurity A and piperacillin in the chromatogram obtained with reference solution (c); if necessary, adjust the ratio A:B of the mobile phase;
- *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (d);
- *mass distribution ratio*: 2.0 to 3.0 for the peak due to piperacillin in the chromatogram obtained with reference solution (c).

*Limit*:

- *any impurity*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method A*): maximum 20 ppm.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.07 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: initial composition of the mixture of mobile phases A and B, adjusted where applicable.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of piperacillin sodium, multiplying the result by 1.042.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H*.

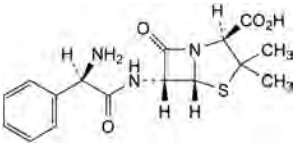

A. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ampicillin),

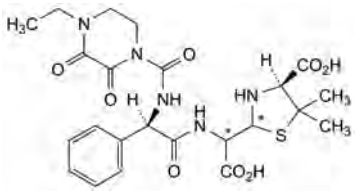

B. (4*S*)-2-[carboxy[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]- 2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of piperacillin),

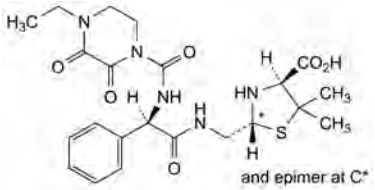


C. (2*RS*,4*S*)-2-[[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]- 2-phenylacetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of piperacillin),

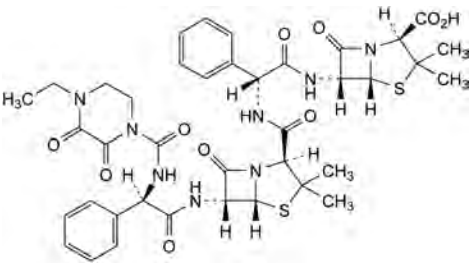

D. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (piperacillinylampicillin),

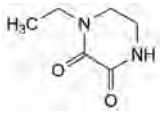

E. 1-ethylpiperazine-2,3-dione,

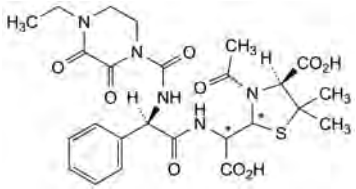

F. (4*S*)-3-acetyl-2-[carboxy[[(2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]amino]- 2-phenylacetyl]-amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (acetylated penicilloic acids of piperacillin),

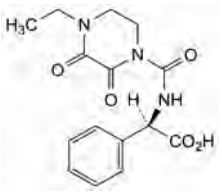

G. (2*R*)-2-[[(4-ethyl-2,3-dioxopiperazin-1-yl)carbonyl]-amino]-2-phenylacetic acid,

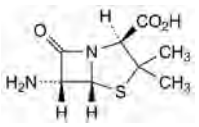

H. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid).

**01/2017:0423**

# PIPERAZINE ADIPATE

## Piperazini adipas

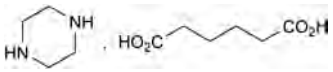

$C_{10}H_{20}N_2O_4$ $M_r$ 232.3
[142-88-1]

DEFINITION

Piperazine adipate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of piperazine hexanedioate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white crystalline powder, soluble in water, practically insoluble in alcohol. It melts at about 250 °C, with decomposition.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *piperazine adipate CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 10 mL of solution S (see Tests) add 5 mL of *hydrochloric acid R* and shake with three quantities, each of 10 mL, of *ether R*. Evaporate the combined ether layers to dryness. The residue, washed with 5 mL of *water R* and dried at 100 °C to 105 °C, melts (*2.2.14*) at 150 °C to 154 °C.

TESTS

**Solution S**. Dissolve 2.5 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in 6 mL of *concentrated ammonia R* and dilute to 10 mL with *ethanol R*.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

*Reference solution (a).* Dissolve 0.1 g of *piperazine adipate CRS* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 25 mg of *ethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 25 mg of *triethylenediamine R* in a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (d).* Dissolve 12.5 mg of *triethylenediamine R* in 5.0 mL of test solution (a) and dilute to 50 mL with a mixture of 2 volumes of *ethanol R* and 3 volumes of *concentrated ammonia R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of *concentrated ammonia R* and 80 volumes of *acetone R*. Dry the plate at 105 °C and spray successively with a 3 g/L solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/L solution of *ninhydrin R* in *ethanol R*. Dry the plate at 105 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray the plate with *0.05 M iodine* and allow to stand for about 10 min. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated spots. Disregard any spots remaining on the line of application.

**Water** (*2.5.12*). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 10 mL of *anhydrous acetic acid R* with gentle heating and dilute to 70 mL with the same acid. Titrate with *0.1 M perchloric acid* using 0.25 mL of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 11.61 mg of $C_{10}H_{20}N_2O_4$.

**01/2017:0424**

# PIPERAZINE CITRATE

## Piperazini citras

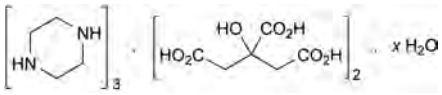

$C_{24}H_{46}N_6O_{14},xH_20$ $M_r$ 643 (anhydrous substance)

DEFINITION

Piperazine citrate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of tripiperazine bis(2-hydroxy-propane-1,2,3-tricarboxylate), calculated with reference to the anhydrous substance. It contains a variable quantity of water.

CHARACTERS

A white or almost white granular powder, freely soluble in water, practically insoluble in ethanol (96 per cent).

After drying at 100 °C to 105 °C, it melts at about 190 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *piperazine citrate CRS*. Dry the substance to be examined and the reference substance at 120 °C for 5 h, powder the substances avoiding uptake of water, prepare discs and record the spectra without delay.

B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.5 g in *water R* and dilute to 5 mL with the same solvent. The solution gives the reaction of citrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a suitable silica gel as the coating substance.

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in 6 mL of *concentrated ammonia R* and dilute to 10 mL with *anhydrous ethanol R*.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 2 volumes of *anhydrous ethanol R* and 3 volumes of *concentrated ammonia R*.

*Reference solution (a).* Dissolve 0.1 g of *piperazine citrate CRS* in a mixture of 2 volumes of *anhydrous ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 25 mg of *ethylenediamine R* in a mixture of 2 volumes of *anhydrous ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 mL with the same mixture of solvents.

Monographs

*Reference solution (c).* Dissolve 25 mg of *triethylenediamine R* in a mixture of 2 volumes of *anhydrous ethanol R* and 3 volumes of *concentrated ammonia R* and dilute to 100 mL with the same mixture of solvents.

*Reference solution (d).* Dissolve 12.5 mg of *triethylenediamine R* in 5.0 mL of test solution (a) and dilute to 50 mL with a mixture of 2 volumes of *anhydrous ethanol R* and 3 volumes of *concentrated ammonia R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a freshly prepared mixture of 20 volumes of *concentrated ammonia R* and 80 volumes of *acetone R*. Dry the plate at 105 °C and spray successively with a 3 g/L solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/L solution of *ninhydrin R* in *anhydrous ethanol R*. Dry the plate at 105 °C for 10 min. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent). Spray the plate with *0.05 M iodine* and allow to stand for about 10 min. Any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows 2 clearly separated spots. Disregard any spots remaining on the line of application.

**Water** (*2.5.12*). 10.0 per cent to 14.0 per cent, determined on 0.300 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 10 mL of *anhydrous acetic acid R* with gentle heating and dilute to 70 mL with the same acid. Titrate with *0.1 M perchloric acid* using 0.25 mL of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 10.71 mg of $C_{24}H_{46}N_6O_{14}$.

**01/2017:0425**
**corrected 10.0**

# PIPERAZINE HYDRATE

## Piperazinum hydricum

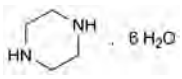

$C_4H_{10}N_2,6H_20$ $M_r$ 194.2
[142-63-2]

DEFINITION

Piperazine hexahydrate.

*Content*: 98.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: colourless, deliquescent crystals.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

mp: about 43 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined and the reference substance *in vacuo* (*2.2.32*) for 48 h, powder the substances avoiding uptake of water, prepare discs and record the spectra without delay.

*Comparison*: *piperazine hydrate CRS*.

B. Examine the chromatograms obtained in the test for related substances after spraying with the ninhydrin solutions.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.5 g in 5 mL of *dilute sodium hydroxide solution R*. Add 0.2 mL of *benzoyl chloride R* and mix. Continue to add *benzoyl chloride R* in portions of 0.2 mL until no further precipitate is formed. Filter and wash the precipitate with a total of 10 mL of *water R* added in small portions. Dissolve the precipitate in 2 mL of hot *ethanol (96 per cent) R* and pour the solution into 5 mL of *water R*. Allow to stand for 4 h, filter, wash the crystals with *water R* and dry at 100-105 °C. The crystals melt (*2.2.14*) at 191 °C to 196 °C.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 10.5 to 12.0 for solution S.

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *anhydrous ethanol R*, *concentrated ammonia R* (40:60 *V/V*).

*Test solution (a).* Dissolve 1.0 g of the substance to be examined in 6 mL of *concentrated ammonia R* and dilute to 10 mL with *anhydrous ethanol R*.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 0.1 g of *piperazine hydrate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 25 mg of *ethylenediamine R* in the solvent mixture and dilute to 100 mL with the solvent mixture.

*Reference solution (c).* Dissolve 25 mg of *triethylenediamine R* in the solvent mixture and dilute to 100 mL with the solvent mixture.

*Reference solution (d).* Dissolve 12.5 mg of *triethylenediamine R* in 5.0 mL of test solution (a) and dilute to 50 mL with the solvent mixture.

*Plate*: suitable silica gel as the coating substance.

*Mobile phase*: *concentrated ammonia R*, *acetone R* (20:80 *V/V*); use a freshly prepared mixture.

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: at 105 °C.

*Detection A*: spray successively with a 3 g/L solution of *ninhydrin R* in a mixture of 3 volumes of *anhydrous acetic acid R* and 100 volumes of *butanol R* and a 1.5 g/L solution of *ninhydrin R* in *anhydrous ethanol R* and dry the plate at 105 °C for 10 min.

*Limits A*: any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

*Detection B*: spray with *0.05 M iodine* and allow to stand for about 10 min.

*Limits B*: any spot corresponding to triethylenediamine in the chromatogram obtained with test solution (a) is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent).

*System suitability*: reference solution (d):

– the chromatogram shows 2 clearly separated spots.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 80.0 mg in 10 mL of *anhydrous acetic acid R* with gentle heating and dilute to 70 mL with the same acid. Titrate with *0.1 M perchloric acid* using 0.25 mL of *naphtholbenzein solution R* as indicator until the colour changes from brownish-yellow to green.

1 mL of *0.1 M perchloric acid* is equivalent to 9.705 mg of $C_4H_{10}N_2,6H_20$.

STORAGE

In an airtight container, protected from light.

**01/2017:1733**

# PIRACETAM

## Piracetamum

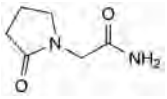

$C_6H_{10}N_2O_2$ $M_r$ 142.2
[7491-74-9]

DEFINITION

2-(2-Oxopyrrolidin-1-yl)acetamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *piracetam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Test solution (b)*. Dilute 10.0 mL of test solution (a) to 50.0 mL with a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R*.

*Reference solution (a)*. Dissolve 5 mg of the substance to be examined and 10 µL of *2-pyrrolidone R* in a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R*. Dilute 5.0 mL of this solution to 50.0 mL with a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R*.

*Reference solution (c)*. Dissolve 50.0 mg of *piracetam CRS* in a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 10.0 mL of this solution to 50.0 mL with a mixture of 10 volumes of *acetonitrile R1* and 90 volumes of *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile R1* and 90 volumes of a 1.0 g/L solution of *dipotassium hydrogen phosphate R*; adjust to pH 6.0 with *dilute phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 8 times the retention time of piracetam.

*Relative retention* with reference to piracetam (retention time = about 4 min): impurity D = about 0.8; impurity A = about 1.15; impurity B = about 2.8; impurity C = about 6.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to piracetam and impurity A,

– *symmetry factor*: maximum 2.0 for the peak due to piracetam.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent),

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_6H_{10}N_2O_2$ from the areas of the peaks and the declared content of *piracetam CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

A. pyrrolidin-2-one (2-pyrrolidone),

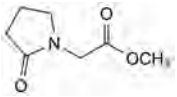

B. methyl (2-oxopyrrolidin-1-yl)acetate,

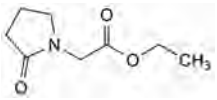

C. ethyl (2-oxopyrrolidin-1-yl)acetate,

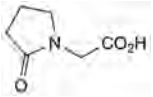

D. (2-oxopyrrolidin-1-yl)acetic acid.



**01/2008:2001**
**corrected 7.0**

# PIRENZEPINE DIHYDROCHLORIDE MONOHYDRATE

## Pirenzepini dihydrochloridum monohydricum

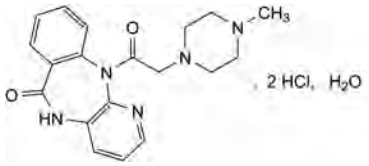

$C_{19}H_{23}Cl_2N_5O_2,H_2O$ $M_r$ 442.3

DEFINITION

11-[(4-Methylpiperazin-1-yl)acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one dihydrochloride monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in methanol, very slightly soluble in ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 30.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *methanol R*. Examined between 240 nm and 360 nm (*2.2.25*), the solution shows an absorption maximum at 283 nm. The specific absorbance at the maximum is 190 to 205 (anhydrous substance).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pirenzepine dihydrochloride monohydrate CRS*.

C. Examine the chromatograms obtained in the test for impurity D.
*Results*: the principal zone obtained in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal zone in the chromatogram obtained with reference solution (d).

D. To 0.2 mL of solution S (see Tests) add 1.8 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 1.0 to 2.0 for solution S.

**Impurity D**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* To 0.10 g add 0.1 mL of *concentrated ammonia R* and dilute to 10 mL with *methanol R*.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* To 0.1 g of *pirenzepine dihydrochloride monohydrate CRS* add 0.1 mL of *concentrated ammonia R* and dilute to 10 mL with *methanol R*.

*Reference solution (b).* Dissolve 25 mg of *methylpiperazine R* in *methanol R* and dilute to 25 mL with the same solvent. Dilute 2.0 mL of the solution to 100 mL with *methanol R*.

*Reference solution (c).* Dilute 5 mL of test solution (a) to 100 mL with *methanol R*. Dilute 4 mL of this solution to 100 mL with *methanol R*. Mix 1 mL with 1 mL of reference solution (b).

*Reference solution (d).* Dilute 1 mL of reference solution (a) to 10 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *ethyl acetate R*, *toluene R* (7:25:28:40 *V/V/V/V*).

*Application*: 20 µL as zones of 20 mm by 2 mm.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour until the zone in the chromatogram obtained with reference solution (b) is clearly visible (at most 60 min).

*System suitability*: the test is not valid unless the chromatogram obtained with reference solution (c) shows 2 clearly separated zones.

*Limit*:

– *impurity D*: any zone corresponding to impurity D in the chromatogram obtained with test solution (a) is not more intense than the zone in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.30 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of the solution add 5 mL of *methanol R* and dilute to 10.0 mL with mobile phase A.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 0.1 g of *1-phenylpiperazine R* in *methanol R* and dilute to 10 mL with the same solvent. Mix 1 mL of the solution with 1 mL of the test solution, add 5 mL of *methanol R* and dilute to 10 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: dissolve 2.0 g of *sodium dodecyl sulfate R* in *water R*, adjust to pH 3.2 with *acetic acid R* and dilute to 1000 mL with *water R*,
- *mobile phase B*: *methanol R*,
- *mobile phase C*: *acetonitrile R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 15 | 55 → 25 | 30 | 15 → 45 |
| 15 - 18 | 25 → 20 | 30 → 0 | 45 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to pirenzepine and 1-phenylpiperazine.

*Limits*:
- *any impurity*: not more than the peak area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.04 per cent).

**Water** (*2.5.12*): 3.5 per cent to 5.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume at the first point of inflection.

1 mL of *0.1 M sodium hydroxide* is equivalent to 42.43 mg of $C_{19}H_{23}Cl_2N_5O_2$.

STORAGE

Protected from light.

IMPURITIES

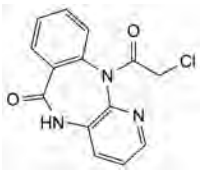

A. 11-(chloroacetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one,

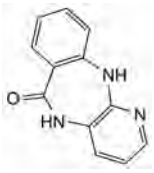

B. 5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one,

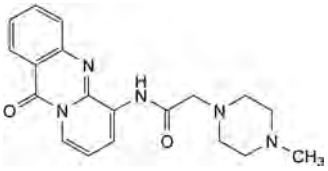

C. 6-[[(4-methylpiperazin-1-yl)acetyl]amino]-11*H*-pyrido[2,1-*b*]quinazolin-11-one,

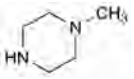

D. 1-methylpiperazine.

**01/2017:1556**

# PIRETANIDE

## Piretanidum

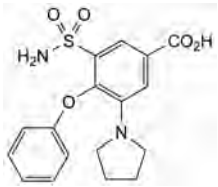

$C_{17}H_{18}N_2O_5S$ $M_r$ 362.4

[55837-27-9]

DEFINITION

4-Phenoxy-3-(pyrrolidin-1-yl)-5-sulfamoylbenzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish-white or yellowish powder.

*Solubility*: very slightly soluble in water, sparingly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *piretanide CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

Dissolve 0.1 g in *methanol R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *anhydrous ethanol R*, *acetonitrile R*, *water R* (10:45:45 *V/V/V*).

*Test solution*. Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *piretanide for system suitability CRS* (containing impurities A, B and C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 0.3 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: a mixture of 35 volumes of *acetonitrile R1* and 65 volumes of a solution prepared as follows: add 1 mL of *trifluoroacetic acid R* to 500 mL of *water for chromatography R*, add 1 mL of *triethylamine R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of piretanide.

*Identification of impurities*: use the chromatogram supplied with *piretanide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to piretanide (retention time = about 10 min): impurity A = about 0.8; impurity B = about 3.1; impurity C = about 4.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 2 between the peaks due to impurity A and piretanide.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than 0.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3.33 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *disregard limit*: 0.17 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.24 mg of $C_{17}H_{18}N_2O_5S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

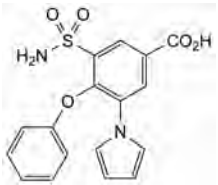

A. 4-phenoxy-3-(1*H*-pyrrol-1-yl)-5-sulfamoylbenzoic acid,

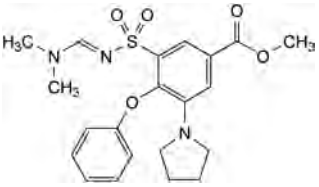

B. methyl-3-[[(dimethylamino)methylidene]sulfamoyl]-4-phenoxy-5-(pyrrolidin-1-yl)benzoate,

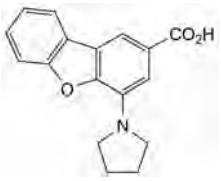

C. 4-(pyrrolidin-1-yl)dibenzo[*b*,*d*]furan-2-carboxylic acid.

**01/2016:2856**

# PIRFENIDONE

## Pirfenidonum

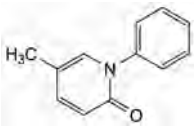

$C_{12}H_{11}NO$ $M_r$ 185.2
[53179-13-8]

DEFINITION

5-Methyl-1-phenylpyridin-2(1*H*)-one.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in ethanol (96 per cent), slightly soluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pirfenidone CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of test solution (b) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *5-methylpyridin-2-amine R* (impurity A) and 5 mg of *5-methylpyridin-2(1H)-one R* (impurity B) in *methanol R* and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 100 mL with the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *pirfenidone CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: add 0.9 mL of *triethylamine R2* to 650 mL of *water for chromatography R* and adjust to pH 3.0 with *phosphoric acid R*. Mix 650 mL of this solution, 130 mL of *methanol R2* and 220 mL of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 15 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of pirfenidone.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to pirfenidone (retention time = about 10 min): impurity A = about 0.35; impurity B = about 0.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pirfenidone in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.05 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.03 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 75 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{12}H_{11}NO$ taking into account the assigned content of *pirfenidone CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

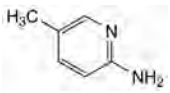

A. 5-methylpyridin-2-amine,

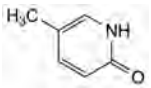

B. 5-methylpyridin-2(1*H*)-one,

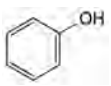

C. phenol,

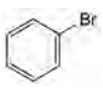

D. bromobenzene.

**01/2017:0944**

# PIROXICAM

## Piroxicamum

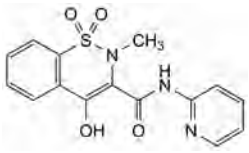

$C_{15}H_{13}N_3O_4S$ $M_r$ 331.4
[36322-90-4]

DEFINITION

4-Hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *piroxicam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness on a water-bath and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 75 mg of the substance to be examined in *acetonitrile R1*, warming slightly if necessary, and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 7 mg of *piroxicam for system suitability CRS* (containing impurities A, B, D, G and J) in *acetonitrile R1* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with *acetonitrile R1*. Dilute 1.0 mL of this solution to 50.0 mL with *acetonitrile R1*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 50 °C.

*Mobile phase*: mix 30 volumes of *acetonitrile R1* and 70 volumes of a 6.81 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of piroxicam.

*Identification of impurities*: use the chromatogram supplied with *piroxicam for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, D, G and J.

*Relative retention* with reference to piroxicam (retention time = about 16 min): impurity A = about 0.1; impurity D = about 0.6; impurity G = about 0.7; impurity B = about 0.8; impurity J = about 1.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurities G and B.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.6;

– *impurities A, B, D, G, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 mL of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.14 mg of $C_{15}H_{13}N_3O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, D, G, J*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, E, F, H, I, K, L*.

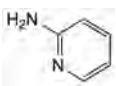

A. pyridin-2-amine,

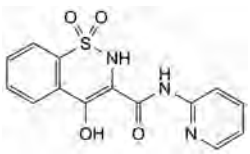

B. 4-hydroxy-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide,

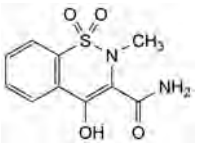

C. 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide,

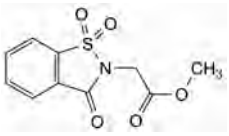

D. methyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3*H*)-yl)acetate,

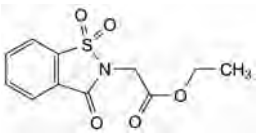

E. ethyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3*H*)-yl)acetate,

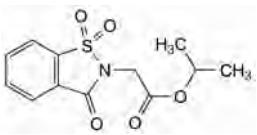

F. 1-methylethyl (1,1-dioxido-3-oxo-1,2-benzisothiazol-2(3*H*)-yl)acetate,

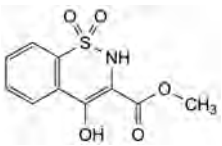

G. methyl 4-hydroxy-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

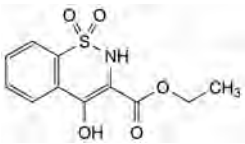

H. ethyl 4-hydroxy-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

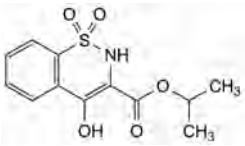

I. 1-methylethyl 4-hydroxy-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

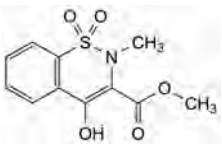

J. methyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

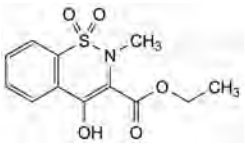

K. ethyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide,

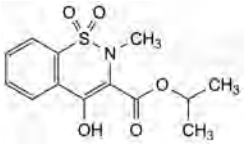

L. 1-methylethyl 4-hydroxy-2-methyl-2*H*-1,2-benzothiazine-3-carboxylate 1,1-dioxide.

**01/2008:0852**
**corrected 6.0**

# PIVAMPICILLIN

## Pivampicillinum

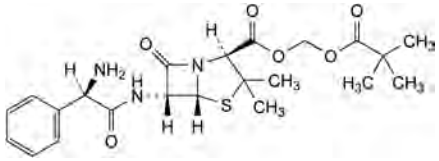

$C_{22}H_{29}N_3O_6S$ $M_r$ 463.6
[33817-20-8]

DEFINITION

Methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]-amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylate 2,2-dimethylpropanoate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methanol, soluble in anhydrous ethanol. It dissolves in dilute acids.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pivampicillin CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of *methanol R*.
*Reference solution (a).* Dissolve 10 mg of *pivampicillin CRS* in 2 mL of *methanol R*.
*Reference solution (b).* Dissolve 10 mg of *bacampicillin hydrochloride CRS*, 10 mg of *pivampicillin CRS* and 10 mg of *talampicillin hydrochloride CRS* in 2 mL of *methanol R*.
*Plate*: *TLC silanised silica gel plate R*.
*Mobile phase*: mix 10 volumes of a 272 g/L solution of *sodium acetate R* adjusted to pH 5.0 with *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *ethanol (96 per cent) R*.
*Application*: 1 µL.
*Development*: over a path of 15 cm.
*Drying*: in a current of warm air.
*Detection*: spray with *ninhydrin solution R1* and heat at 60 °C for 10 min.
*System suitability*: reference solution (b):
– the chromatogram shows 3 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 150 mm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is almost colourless. Place the test-tube in a water-bath for 1 min; a dark yellow colour develops.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method I*).

Dissolve 50 mg in 12 mL of *0.1 M hydrochloric acid*.

**Specific optical rotation** (*2.2.7*): + 208 to + 222 (anhydrous substance).

Dissolve 0.100 g in 5.0 mL of *ethanol (96 per cent) R* and dilute to 10.0 mL with *0.1 M hydrochloric acid*.

**Triethanolamine**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in 1.0 mL of a mixture of 1 volume of *water R* and 9 volumes of *acetonitrile R*.

*Reference solution.* Dissolve 5.0 mg of *triethanolamine R* in a mixture of 1 volume of *water R* and 9 volumes of *acetonitrile R* and dilute to 100 mL with the same mixture of solvents.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methanol R*, *butanol R*, *phosphate buffer solution pH 5.8 R*, *glacial acetic acid R*, *butyl acetate R* (5:15:24:40:80 *V/V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: at 110 °C for 10 min and allow to cool.

*Chlorine treatment*: place at the bottom of a chromatographic tank an evaporating dish containing a mixture of 1 volume of *hydrochloric acid R1*, 1 volume of *water R* and 2 volumes of a 15 g/L solution of *potassium permanganate R*; close the tank and allow to stand for 15 min; place the dried plate in the tank and close the tank; leave the plate in contact with the chlorine vapour in the tank for 15-20 min; withdraw the plate and allow it to stand in air for 2-3 min.

*Detection*: spray with *tetramethyldiaminodiphenylmethane reagent R*.

*Limit*:
– *triethanolamine*: any spot due to triethanolamine is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.05 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 10.0 mL of *acetonitrile R* and dilute to 20 mL with a 1 g/L solution of *phosphoric acid R*.

*Reference solution.* Mix 2.0 mL of the test solution with 9.0 mL of *acetonitrile R* and 9.0 mL of a 1 g/L solution of *phosphoric acid R*.

*Column*:
– *size*: *l* = 0.125 m, Ø = 4 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R*.

*Mobile phase*:

– *mobile phase A*: mix 50 volumes of a 1.32 g/L solution of *ammonium phosphate R*, adjusted to pH 2.5 with a 100 g/L solution of *phosphoric acid R*, and 50 volumes of *acetonitrile R*;

– *mobile phase B*: mix 15 volumes of a 1.32 g/L solution of *ammonium phosphate R*, adjusted to pH 2.5 with a 100 g/L solution of *phosphoric acid R*, and 85 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 12 | 0 | 100 |
| 12 - 17 | 100 | 0 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 50 µL.

*Retention time*: pivampicillin dimer = about 5 min.

*System suitability*: reference solution:

– *ratio of the mass distribution ratio*: minimum 12 for the peak due to pivampicillin dimer to that of the peak due to pivampicillin (principal peak).

*Limits*:

– *total*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with the reference solution (3 per cent);

– *disregard limit*: 0.01 times the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

*Test solution.* To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 10 mL of *0.5 M sulfuric acid*. Heat the tube for 10 min in a water-bath, cool and add 15 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.30 g.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*). *Use the solutions within 2 h of preparation.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *pivampicillin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 25.0 mg of *propyl parahydroxybenzoate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 50.0 mL with the mobile phase. Mix 5.0 mL of this solution with 5.0 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 40 volumes of *acetonitrile R* and 60 volumes of a 2.22 g/L solution of *phosphoric acid R* adjusted to pH 2.5 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to pivampicillin (1st peak) and propyl parahydroxybenzoate (2nd peak) in the chromatogram obtained with reference solution (b);

– *symmetry factor*: maximum 2.0 for the peak due to pivampicillin in the chromatogram obtained with reference solution (b);

– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections of reference solution (a).

Calculate the percentage content of $C_{22}H_{29}N_3O_6S$ from the declared content of *pivampicillin CRS*.

STORAGE

In an airtight container.

IMPURITIES

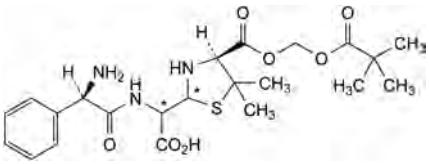

A. 2-[[(2*R*)-2-amino-2-phenylacetyl]amino]-2-[(4*S*)-4-[[[(2,2-dimethylpropanoyl)oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetic acid (penicilloic acids of pivampicillin),

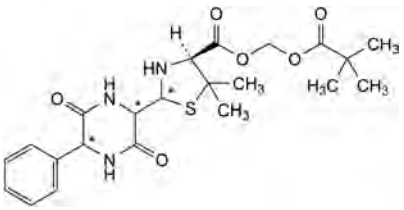

B. methylene (4*S*)-5,5-dimethyl-2-(3,6-dioxo-5-phenylpiperazin-2-yl)thiazolidine-4-carboxylate 2,2-dimethylpropanoate (diketopiperazines of pivampicillin),

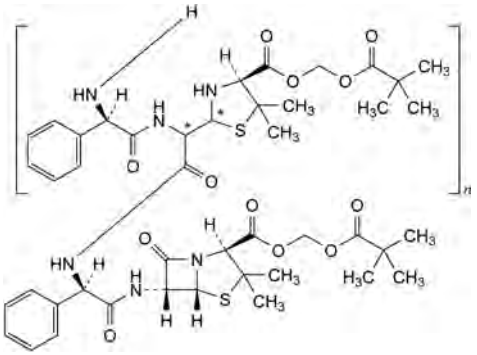

C. co-oligomers of pivampicillin and of penicilloic acids of pivampicillin.

**01/2017:1359**

# PIVMECILLINAM HYDROCHLORIDE

## Pivmecillinami hydrochloridum

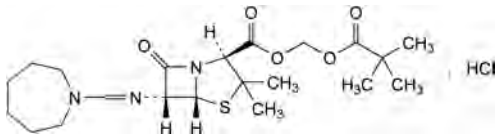

$C_{21}H_{34}ClN_3O_5S$ $M_r$ 476.0
[32887-03-9]

DEFINITION

Methylene 2,2-dimethylpropanoate (2*S*,5*R*,6*R*)-6-[[(hexahydro-1*H*-azepin-1-yl)methylene]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate hydrochloride.
Semi-synthetic product derived from a fermentation product.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, in anhydrous ethanol and in methanol, slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *pivmecillinam hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method I*).
Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 2.8 to 3.8.
Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* To 45 volumes of *acetonitrile R* add 55 volumes of a 13.5 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*.

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *pivmecillinam hydrochloride CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *pivmecillinam hydrochloride CRS* and 5 mg of *pivmecillinam impurity C CRS* in the solvent mixture, and dilute to 50 mL with the solvent mixture.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.55 g of *tetraethylammonium hydrogen sulfate R* and 1.0 g of *tetramethylammonium hydrogen sulfate R* in the solvent mixture and dilute to 1000 mL with the solvent mixture.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b) and (c).

*Run time*: 3 times the retention time of pivmecillinam.

*System suitability*: reference solution (c):
– *resolution*: minimum 3.5 between the peaks due to pivmecillinam (1st peak) and impurity C (2nd peak).

*Limits*:
– *any impurity*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method A*): maximum 20 ppm.

*Test solution.* Prepare as described in the general method but heat at about 27 °C after the addition of *strong sodium hydroxide solution R*, to dissolve the precipitate formed, then add the *trimethylpentane R*.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):
– *repeatability*: maximum relative standard deviation of 1.0 per cent after 6 injections.

Calculate the percentage content of $C_{21}H_{34}ClN_3O_5S$ from the declared content of *pivmecillinam hydrochloride CRS*.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

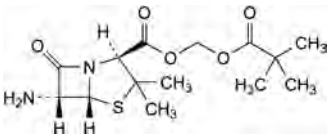

A. methylene (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 2,2-dimethylpropanoate (pivaloyloxymethyl 6-aminopenicillanate),

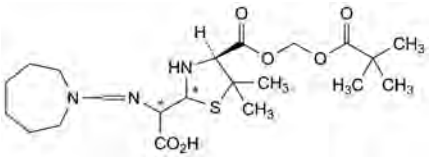

B. 2-[[(hexahydro-1*H*-azepin-1-yl)methylene]amino]-2-[(4*S*)-4-[[[(2,2-dimethylpropanoyl)oxy]methoxy]-carbonyl]-5,5-dimethylthiazolidin-2-yl]acetic acid (penicilloic acids of pivmecillinam),

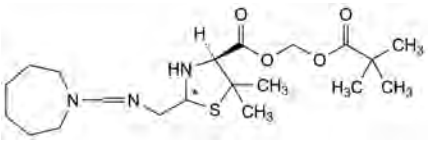

C. methylene 2,2-dimethylpropanoate (2*RS*,4*S*)-2-[[[(hexahydro-1*H*-azepin-1-yl)methylene]amino]methyl]-5,5-dimethylthiazolidin-4-carboxylate,

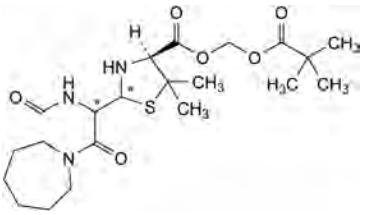

D. methylene 2,2-dimethylpropanoate (4*S*)-2-[1-(formylamino)-2-(hexahydro-1*H*-azepin-1-yl)-2-oxoethyl]-5,5-dimethylthiazolidin-4-carboxylate.

**01/2019:2750**



# PODOPHYLLOTOXIN

## Podophyllotoxinum

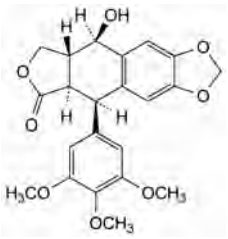

$C_{22}H_{22}O_8$ $M_r$ 414.4
[518-28-5]

DEFINITION

(5*R*,5a*R*,8a*R*,9*R*)-9-Hydroxy-5-(3,4,5-trimethoxyphenyl)-5,8,8a,9-tetrahydrofuro[3′,4′:6,7]naphtho[2,3-*d*][1,3]dioxol-6(5a*H*)-one.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in acetone, soluble in methanol.

It shows polymorphism (*5.9*).

mp: about 184 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *podophyllotoxin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Specific optical rotation** (*2.2.7*): − 112 to − 107 (anhydrous substance).

Dissolve 0.200 g in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 10.0 mL of the mobile phase using sonication for 30 min and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *podophyllotoxin for system suitability CRS* (containing impurities A and C) in 5 mL of the mobile phase.

*Reference solution (c)*. Dissolve 25.0 mg of *podophyllotoxin CRS* in 10.0 mL of the mobile phase using sonication for 30 min and dilute to 25.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: mix 40 volumes of a 1.0 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.5 with *phosphoric acid R*, and 60 volumes of *methanol R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 5 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of podophyllotoxin.

*Identification of impurities*: use the chromatogram supplied with *podophyllotoxin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C.

*Relative retention* with reference to podophyllotoxin (retention time = about 8 min): impurity A = about 0.9; impurity C = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to podophyllotoxin and impurity C.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity C = 0.4;
- for each impurity, use the concentration of podophyllotoxin in reference solution (a).

*Limits*:

- *impurities A, C*: for each impurity, maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{22}H_{22}O_8$ taking into account the assigned content of *podophyllotoxin CRS*.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

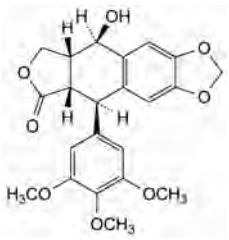

A. (5*R*,5a*S*,8a*R*,9*R*)-9-hydroxy-5-(3,4,5-trimethoxyphenyl)-5,8,8a,9-tetrahydrofuro[3′,4′:6,7]naphtho[2,3-*d*][1,3]-dioxol-6(5a*H*)-one (picropodophyllotoxin),

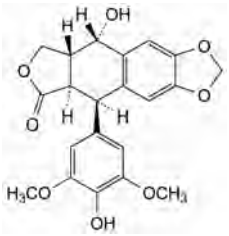

B. (5*R*,5a*R*,8a*R*,9*S*)-9-hydroxy-5-(4-hydroxy-3,5-dimethoxyphenyl)-5,8,8a,9-tetrahydrofuro[3′,4′:6,7]-naphtho[2,3-*d*][1,3]dioxol-6(5a*H*)-one (4′-demethylepipodophyllotoxin),

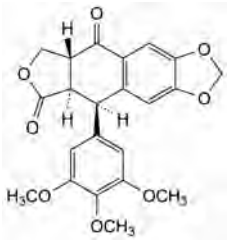

C. (5a*R*,8a*R*,9*R*)-9-(3,4,5-trimethoxyphenyl)-5a,6,8a,9-tetrahydrofuro[3′,4′:6,7]naphtho[2,3-*d*][1,3]dioxol-5,8-dione (podophyllotoxone).

**01/2017:2627**

# POLLENS FOR ALLERGEN PRODUCTS

## Pollines ad producta allergenica

DEFINITION

Pollens for allergen products come primarily from anemophilous plants and secondarily from a few entomophilous plants. They may be processed (e.g. defatted with organic solvents) or unprocessed pollen and they contain soluble substances, including proteins, whose functional activity is unrelated to any antigenic and allergenic properties they may have.

PRODUCTION

Pollens for allergen products are defined by their species and geographic location. Species identification is carried out on the parent plant from which the pollen is collected. The field characteristics and any treatments are specified for cultivated species. Where pollen is collected from wild species, the nature of collection areas is specified. The methods of collection are described and must ensure the origin, quality, consistency and traceability of the pollen. Where applicable, reference is made to good agricultural and collection practice (GACP). The collection, handling and further processing of the pollen are such that a consistent composition of the source material is ensured from batch to batch.

Pollen may be defatted using organic solvents. The content of relevant pesticides, heavy metals and residual solvents is monitored and determined on a number of batches according to a justified sampling plan. Pesticides, heavy metals and residual solvents are limited according to the principles defined in general chapters *2.8.13. Pesticide residues*, *2.4.27. Heavy metals in herbal drugs and herbal drug preparations* and *2.4.24. Identification and control of residual solvents*, respectively.

Where major changes to the production of the pollens for allergen products take place (e.g. when a new process or supplier of pollen source material is introduced), such changes are qualified.

Microbial contamination of the pollen may be unavoidable and should be monitored on a representative number of batches of pollens for allergen products (e.g. defined by collection year and location) according to a justified sampling plan and each time a new supplier and/or a new process for production is introduced; if a determination of microbial contamination is not applicable, this must be justified. Microbial contamination values and potential increases in microbial contamination are monitored during stability studies, in order to assess this aspect along with the pollen characteristics upon storage.

Control methods and acceptance criteria relating to identity and purity of the pollen are established. The acceptance criteria must ensure the consistency of the pollens for allergen products from a qualitative and quantitative point of view. Pollens for allergen products are stored under controlled conditions justified by stability data. The collection and production, as well as the handling of the pollen, are such that consistent quality is ensured from batch to batch.

*POLLENS FOR ALLERGEN PRODUCTS REFERENCE BATCH*

An appropriate reference batch is established for each species. The nature of the reference batch depends on the testing approach to verify batch-to-batch consistency and to establish acceptable quality. The reference batch may be, for example, an internal reference preparation (if available), a source material extract or a sample of a production batch. Its characterisation must be described. The extent of characterisation of the reference batch depends on the pollen, knowledge of the allergenic components and availability of suitable reagents. The reference batch is stored under controlled conditions ensuring its stability.

*BATCH-TO-BATCH CONSISTENCY*

To establish batch-to-batch consistency, one or more of the following tests are performed on each batch. If the microbial contamination test is performed, at least one other test is to be used. The choice of tests must be justified. Variations between different batches are to be expected due to, for example, geographic location of the harvest areas or different climates during the growth period.

**Total protein** (*2.5.33*).

**Protein profile**. Determined by using suitable electrophoresis methods (*2.2.31*, *2.2.54*).

**Allergen profile**. Relevant allergenic components are identified by means of suitable techniques using allergen-specific antibodies.

**Major allergen content**. Determined by using suitable immunochemical methods (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

**Total allergenic activity**. Determined by testing inhibition of the binding capacity of specific immunoglobulin E antibodies or by a suitable equivalent *in vitro* method.

**Microbial contamination** (*2.6.12*).

CHARACTERS

Pollens for allergen products are supplied as coloured powders and consist of grains in a great variety of shapes, densities and sizes depending on the species. Detailed specifications for each type of pollen grain are established.

IDENTIFICATION

Tests on identity are performed on each individual batch of pollen using adequate methods (e.g. microscopy). Specific characteristics, such as colour, size, shape, number and position of apertures, are described and compared with those of a reference batch or reference documents.

Additional tests may be performed as complementary identification determination.

TESTS

**Foreign pollen**. The content of pollen from other species is limited to 1 per cent of total mixed pollens and 0.5 per cent of any individual pollen as determined by a microscopic particle count.

**Foreign matter**. Particles of plant origin (determined by microscopic examination), other than pollen are kept to a minimum but in any case do not exceed 10 per cent unless otherwise justified and authorised. The sum of all non-plant particles (e.g. soil) must not exceed 1 per cent. Detectable mould spores must not exceed 1 per cent.

**Water** (*2.5.12* or *2.5.32*) or **loss on drying** (*2.2.32*). The water content of dried material is determined; specification limits must be supported by batch analysis and stability data.

STORAGE

Pollens for allergen products are stored under controlled conditions justified by stability data.

LABELLING

The label states the species of the pollen.

**04/2019:1464**
**corrected 10.0**

# POLOXAMERS

## Poloxamera

DEFINITION

Synthetic block copolymer of ethylene oxide (oxirane) and propylene oxide (methyloxirane), represented by the following general formula:

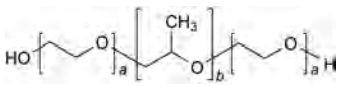


| Poloxamer type | Ethylene oxide units *a* | Propylene oxide units *b* | Content of oxyethylene units (per cent) | Average relative molecular mass |
|---|---|---|---|---|
| 124 | 10 - 15 | 18 - 23 | 44.8 - 48.6 | 2090 - 2360 |
| 188 | 75 - 85 | 25 - 30 | 79.9 - 83.7 | 7680 - 9510 |
| 237 | 60 - 68 | 35 - 40 | 70.5 - 74.3 | 6840 - 8830 |
| 338 | 137 - 146 | 42 - 47 | 81.4 - 84.9 | 12 700 - 17 400 |
| 407 | 95 - 105 | 54 - 60 | 71.5 - 74.9 | 9840 - 14 600 |

A suitable antioxidant may be added.

CHARACTERS

*Appearance*:

- *poloxamer 124*: colourless or almost colourless liquid;
- *poloxamers 188, 237, 338, 407*: white or almost white, waxy powder, microbeads or flakes.

*Solubility*:

- *poloxamers 124, 237, 338, 407*: very soluble in water and in ethanol (96 per cent), practically insoluble in light petroleum (bp: 50-70 °C);
- *poloxamer 188*: soluble in water and in ethanol (96 per cent).

mp: about 50 °C for poloxamers 188, 237, 338 and 407.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: Ph. Eur. chemical reference substance corresponding to the type of poloxamer to be examined.

B. Average relative molecular mass (see Tests).

C. Oxypropylene:oxyethylene ratio (see Tests).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.5 for solution S.

**Ethylene oxide, propylene oxide and dioxan**. Head-space gas chromatography (*2.2.28*).

*Ethylene oxide stock solution.* Introduce 0.5 mL of *ethylene oxide stock solution R2* into a vial and dilute to 50.0 mL with *dimethyl sulfoxide R1*. Mix carefully.

*Ethylene oxide solution.* Dilute 1.0 mL of the ethylene oxide stock solution to 250 mL with *dimethyl sulfoxide R1*.

*Propylene oxide stock solution.* Introduce about 7 mL of *methylene chloride R* into a volumetric flask, add 0.500 g (*m*) of *propylene oxide R* and dilute to 10.0 mL with *methylene chloride R*. Dilute 0.5 mL of this solution to 50.0 mL with *dimethyl sulfoxide R1*. Mix carefully. Calculate the exact concentration of propylene oxide, in milligrams per millilitre, using the following expression:

$$\frac{m \times 1000 \times 0.5}{10 \times 50}$$

*Propylene oxide solution.* Dilute 1.0 mL of the propylene oxide stock solution to 50.0 mL with *dimethyl sulfoxide R1*.

Calculate the exact concentration of propylene oxide, in micrograms per millilitre, using the following expression:

$$\frac{C \times 1000 \times 1}{50}$$

$C$ = concentration of the propylene oxide stock solution, in milligrams per millilitre.

*Dioxan solution.* Introduce 0.100 g (*m*) of *dioxan R* into a flask and dilute to 50.0 mL with *dimethyl sulfoxide R1*. Dilute 2.50 mL of this solution to 100.0 mL with *dimethyl sulfoxide R1*.

Calculate the exact concentration of dioxan, in micrograms per millilitre, using the following expression:

$$\frac{m \times 2.50 \times 1000 \times 1000}{50 \times 100}$$

*Mixture solution.* Dilute a mixture of 6.0 mL of the ethylene oxide solution, 6.0 mL of the propylene oxide solution and 2.5 mL of the dioxan solution to 25.0 mL with *dimethyl sulfoxide R1*.

*Test solution.* To 1.000 g of the substance to be examined in a head-space vial, add 4.0 mL of *dimethyl sulfoxide R1* and close the vial immediately.

*Reference solution.* To 1.000 g of the substance to be examined in a head-space vial, add 2.0 mL of *dimethyl sulfoxide R1* and 2.0 mL of the mixture solution. Close the vial immediately.

*Column*:

- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 5 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.4 mL/min.

*Static head-space conditions*:

- *equilibrium temperature*: 110 °C;
- *equilibration time*: 30 min;
- *transfer-line temperature*: 140 °C;
- *pressurisation time*: 1 min;
- *injection time*: 0.05 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| | 0 - 10 | 70 |
| Column | 10 - 27 | 70 → 240 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: inject a suitable volume of the gaseous phase, for example 1 mL.

*Relative retention* with reference to ethylene oxide (retention time = about 6 min): propylene oxide = about 1.3; methylene chloride = about 1.6; dioxan = about 3.0; dimethyl sulfoxide = about 3.7.

*Limits*:

- *ethylene oxide*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (1 ppm);
- *propylene oxide*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (5 ppm);
- *dioxan*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (10 ppm).

**Average relative molecular mass**. Weigh 15 g (*m*) of the substance to be examined into a 250 mL ground-glass-stoppered flask, add 25.0 mL of *phthalic anhydride solution R* and a few glass beads and swirl to dissolve. Boil gently under a reflux condenser for 1 h, allow to cool and add 2 quantities, each of 10 mL, of *pyridine R*, through the condenser. Add 10 mL of *water R*, mix and allow to stand for 10 min. Add 40.0 mL of *0.5 M sodium hydroxide* and 0.5 mL of a 10 g/L solution of *phenolphthalein R* in *pyridine R*. Titrate with *0.5 M sodium hydroxide* to a light pink endpoint that persists for 15 s and record the volume of sodium hydroxide used (*S*). Prepare a blank. Record the volume of sodium hydroxide used (*B*).

Calculate the average relative molecular mass using the following expression:

$$\frac{4000m}{B-S}$$

**Oxypropylene:oxyethylene ratio**. Nuclear magnetic resonance spectrometry (*2.2.33*).

Use a 100 g/L solution of the substance to be examined in *deuterated chloroform R*. Record the average area of the doublet appearing at about 1.08 ppm due to the methyl groups of the oxypropylene units ($A_1$) and the average area of the composite band from 3.2 ppm to 3.8 ppm due to $CH_2O$ groups of both the oxyethylene and oxypropylene units and the CHO groups of the oxypropylene units ($A_2$) with reference to the internal standard.

Calculate the percentage of oxyethylene, by weight, in the sample being examined using the following expression:

$$\frac{3300\alpha}{33\alpha + 58}$$

where $\alpha = \frac{A_2}{A_1} - 1$

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.000 g.

**Total ash** (*2.4.16*): maximum 0.4 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states the type of poloxamer.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for poloxamers used as dispersing agents or vehicles (type 124).*

**Viscosity** (*2.2.9*): about 400 mPa·s, determined at 25 °C, for the undiluted poloxamer.

*The following characteristics may be relevant for poloxamers used as solubilising agents (types 188 and 237).*

**Viscosity** (*2.2.9*): typically less than 50 mPa·s, determined at 25 °C.

Dissolve 25.0 g in 100.0 mL of *water R*.

**Oxypropylene:oxyethylene ratio** (see Tests).

*The following characteristics may be relevant for poloxamers used as solubilising agents (type 407).*

**Viscosity** (*2.2.9*): typically less than 75 mPa·s, determined at 5 °C.

Dissolve 25.0 g in 100.0 mL of *water R*.

**Oxypropylene:oxyethylene ratio** (see Tests).

*The following characteristics may be relevant for poloxamers used as wetting agents (types 188 and 407).*

**Particle-size distribution** (*2.9.31*).

**Oxypropylene:oxyethylene ratio** (see Tests).

*The following characteristic may be relevant for poloxamers used as viscosity-increasing agents or suspending agents (types 338 and 407).*

**Gel formation**. Dissolve 25.0 g in 100 mL of cold *water R* (5-8 °C) and stir overnight in a refrigerator until dissolution is complete. The viscous solution obtained forms a

thermoreversible gel after heating to 37 °C and does not flow in a capillary tube (*2.2.9*) or flows only very slowly (flow time greater than 10 min).

**01/2017:0733**

# POLYACRYLATE DISPERSION 30 PER CENT

## Polyacrylatis dispersio 30 per centum

DEFINITION

Dispersion in water of a copolymer of ethyl acrylate and methyl methacrylate having a mean relative molecular mass of about 800 000.

*Content*: 28.5 per cent to 31.5 per cent (residue on evaporation).

It may contain a suitable emulsifier.

CHARACTERS

*Appearance*: opaque, white or almost white, slightly viscous liquid.

*Solubility*: miscible with water, soluble in acetone, in anhydrous ethanol and in 2-propanol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of polyacrylate.*

B. To 1 g add 5 mL of *water R* and mix; the mixture remains opaque. Take 3 portions of 1 g and mix separately with 5 g of *anhydrous ethanol R*, 5 g of *acetone R* and 5 g of *2-propanol R*. Transparent solutions are obtained.

C. To 1 g add 10 mL of *0.1 M sodium hydroxide*. The mixture remains opaque.

D. Appearance of a film (see Tests).

E. Dry 4 g in a Petri dish at 60 °C in an oven for 4 h and transfer the resulting clear film to a small test-tube (100 mm × 12 mm). Heat over a flame and collect the fumes that evolve in a 2nd test-tube held over the mouth of the 1st tube. The condensate gives the reaction of esters (*2.3.1*).

TESTS

**Relative density** (*2.2.5*): 1.037 to 1.047.

**Viscosity** (*2.2.10*): maximum 50 mPa·s, determined using a rotating viscometer at 20 °C and a shear rate of 10 $s^{-1}$.

**Appearance of a film**. Pour 1 mL on a glass plate and allow to dry. A clear elastic film is formed.

**Particulate matter**. Filter 100.0 g through a tared stainless steel sieve (90). Rinse with *water R* until a clear filtrate is obtained and dry at 80 °C to constant mass. The residue weighs not more than 0.500 g.

**Residual monomers**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 1.00 g of the substance to be examined in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. To 5.0 mL of a 35 g/L solution of *sodium perchlorate R* add 10.0 mL of the solution dropwise whilst stirring continuously. Centrifuge and filter the clear supernatant. Dilute 5.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution*. Dissolve 10 mg of *ethyl acrylate R* and 10 mg of *methyl methacrylate R* in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *tetrahydrofuran R*. To 10.0 mL of the solution add 5.0 mL of a 35 g/L solution of *sodium perchlorate R* and mix. Dilute 5.0 mL of the mixture to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.12 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: *acetonitrile R1*, *water for chromatography R* (15:85 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: about 50 µL.

*Limit*:

– *residual monomers*: maximum 100 ppm.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dry 1.000 g at 110 °C for 3 h and weigh the residue.

STORAGE

At a temperature of 5 °C to 25 °C, protected from freezing.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for polyacrylate dispersion 30 per cent used as film former or matrix former in prolonged-release dosage forms.*

**Viscosity** (see Tests).

**Appearance of a film** (see Tests).

**Solubility of a film**. Take a piece of the film obtained in the test for appearance of a film and place it in a flask containing a 10.3 g/L solution of *hydrochloric acid R* with stirring. It does not dissolve within 2 h. Take another piece of the film and place it in a flask containing *0.33 M phosphate buffer solution pH 7.5 R* with stirring. It also does not dissolve within 2 h.

**04/2019:0203**
**corrected 10.0**

# POLYMYXIN B SULFATE

## Polymyxini B sulfas

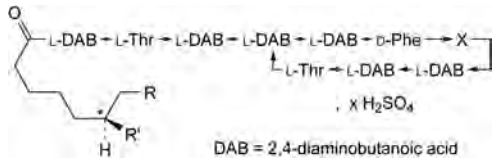


| Polymyxin | R | R′ | X | Molecular formula | $M_r$ |
|---|---|---|---|---|---|
| B1 | $CH_3$ | $CH_3$ | L-Leu | $C_{56}H_{98}N_{16}O_{13}$ | 1203 |
| B2 | H | $CH_3$ | L-Leu | $C_{55}H_{96}N_{16}O_{13}$ | 1189 |
| B3 | $CH_3$ | H | L-Leu | $C_{55}H_{96}N_{16}O_{13}$ | 1189 |
| B1-I | $CH_3$ | $CH_3$ | L-Ile | $C_{56}H_{98}N_{16}O_{13}$ | 1203 |

### DEFINITION

Mixture of the sulfates of polypeptides produced by the growth of certain strains of *Paenibacillus polymyxa*, the main component being polymyxin B1.

*Potency*: minimum 6500 IU/mg (dried substance).

### CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

### IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in 1 mL of a mixture of equal volumes of *hydrochloric acid R* and *water R*. Heat at 135 °C in a sealed tube for 5 h. Evaporate to dryness on a water-bath and continue the heating until the hydrochloric acid has evaporated. Dissolve the residue in 0.5 mL of *water R*.

*Reference solution (a).* Dissolve 20 mg of *leucine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *threonine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (c).* Dissolve 20 mg of *phenylalanine R* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (d).* Dissolve 20 mg of *serine R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Carry out the following procedures protected from light.*

*Mobile phase*: *water R, phenol R* (25:75 *V/V*).

*Application*: 5 µL as bands of 10 mm, then place the plate in the chromatographic tank so that it is not in contact with the mobile phase, and allow it to become impregnated with the vapour of the mobile phase for at least 12 h.

*Development*: over 2/3 of the plate using the same mobile phase.

*Drying*: at 100-105 °C.

*Detection*: spray with *ninhydrin solution R1* and heat at 110 °C for 5 min.

*Results*: the chromatogram obtained with the test solution shows zones corresponding to those in the chromatograms obtained with reference solutions (a), (b) and (c), but shows no zone corresponding to that in the chromatogram obtained with reference solution (d); the chromatogram obtained with the test solution also shows a zone with a very low $R_F$ value (2,4-diaminobutyric acid).

B. Examine the chromatograms obtained in the test for composition.

*Results*: the peaks due to polymyxins B1, B2, B3 and B1-I in the chromatogram obtained with the test solution are similar in retention time to the corresponding peaks in the chromatogram obtained with reference solution (a).

C. Dissolve about 2 mg in 5 mL of *water R* and add 5 mL of a 100 g/L solution of *sodium hydroxide R*. Shake and add dropwise 0.25 mL of a 10 g/L solution of *copper sulfate pentahydrate R*, shaking after each addition. A reddish-violet colour develops.

D. It gives reaction (a) of sulfates (*2.3.1*).

### TESTS

**pH** (*2.2.3*): 5.0 to 7.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: *acetonitrile R*, *water R* (20:80 *V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *polymyxin B sulfate CRS* in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (b).* Dilute 1 mL of reference solution (a) to 100 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 20 volumes of *acetonitrile R1* and 80 volumes of a solution prepared as follows: dissolve 4.46 g of *anhydrous sodium sulfate R* in 900 mL of *water for chromatography R*, adjust to pH 2.3 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 1.4 times the retention time of polymyxin B1.

*Identification of peaks*: use the chromatogram supplied with *polymyxin B sulfate CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to polymyxins B1, B2, B3 and B1-I.

*Relative retention* with reference to polymyxin B1 (retention time = about 35 min): polymyxin B2 = about 0.5; polymyxin B3 = about 0.6; polymyxin B1-I = about 0.8.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to polymyxin B2 and polymyxin B3 in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 20 for the peak due to polymyxin B1 in the chromatogram obtained with reference solution (b).

*Limits*:

- *polymyxin B1-I*: maximum 15.0 per cent;
- *polymyxin B3*: maximum 6.0 per cent;

– *sum of polymyxins B1, B2, B3 and B1-I*: minimum 80.0 per cent;
– *reporting threshold*: 0.40 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition.

*Limits*:
– *any impurity*: for each impurity, maximum 3.0 per cent;
– *total*: maximum 17.0 per cent;
– *reporting threshold*: 0.40 per cent.

**Sulfate**: 15.5 per cent to 17.5 per cent (dried substance).

Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 with *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*, adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continuing the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of $SO_4$.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g by drying at 60 °C by drying *in vacuo* at 60 °C at a pressure not exceeding 0,7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.75 per cent, determined on 1.0 g.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of pyrogens, it complies with the test for pyrogens. Inject, per kilogram of the rabbit's mass, 1 mL of a solution in *water for injections R* containing 1.5 mg of the substance to be examined per millilitre.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**07/2018:2602**
**corrected 10.0**

# POLYOXYPROPYLENE STEARYL ETHER

## Polyoxypropyleni aether stearylicus

DEFINITION

Mixture of ethers of polyoxypropylene with linear alcohols, mainly stearyl alcohol, obtained by the reaction of stearyl alcohol with propylene oxide. It may contain some free polyoxypropylene and various amounts of free stearyl alcohol. The number of moles of propylene oxide reacted per mole of stearyl alcohol is 11 (nominal value). A suitable antioxidant may be added.

CHARACTERS

*Appearance*: colourless or pale yellow, clear or slightly turbid liquid.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent), in mineral oils and in 2-propanol, practically insoluble in propylene glycol and in glycerol.

*Relative density*: about 0.94 at 25 °C.

*Refractive index*: about 1.448 at 25 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *polyoxypropylene stearyl ether CRS*.
B. Hydroxyl value (see Tests).
C. Saponification value (see Tests).
D. Viscosity (*2.2.9*): 83 mPa·s to 95 mPa·s.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

**Hydroxyl value** (*2.5.3, Method A*): 60 to 77.

**Iodine value** (*2.5.4, Method A*): maximum 3.0.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Saponification value** (*2.5.6*): maximum 3.0.

**Propylene oxide**. Head-space gas chromatography (*2.2.28*).

*Propylene oxide stock solution*. Weigh 45 g of cold *macrogol 200 R1* and add 1.000 g (*m*) of *propylene oxide R*. Mix carefully by swirling to ensure a homogeneous solution. Add more *macrogol 200 R1* until the total weight is 50.0 g and mix again. [NOTE: the solution is stable for 1 month if stored at – 20 °C]. Allow to reach room temperature. Dilute 0.50 g of this solution to 100.0 mL with *water R*.

*Propylene oxide standard solution*. Dilute 10.0 mL of propylene oxide stock solution to 100.0 mL with *water R*.

*Propionaldehyde stock solution*. Weigh 0.1 g of *propionaldehyde R* into a volumetric flask and dilute to 100.0 mL with *water R*.

*Test solution (a)*. Weigh 1.00 g of the substance to be examined into a 20 mL head-space vial. Add 0.5 mL of cold *water R* and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap. Mix carefully.

*Test solution (b)*. Weigh 1.00 g of the substance to be examined into a 20 mL head-space vial. Add 0.5 mL of cold propylene oxide standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap. Mix carefully.

*Reference solution*. Introduce 50.0 mL of cold propylene oxide standard solution into a volumetric flask, add 0.5 mL of cold propionaldehyde stock solution and dilute to 100.0 mL with *water R*. Introduce 0.5 mL of this solution into a 20 mL head-space vial.

*Column*:
– *material*: fused silica;
– *size*: *l* = 60 m, Ø = 0.32 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness 5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.6 mL/min.

*Split ratio*: 10:1.

*Static head-space conditions that may be used*:
– *equilibration temperature*: 90 °C;
– *equilibration time*: 45 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 50 |
| | 5 - 31 | 50 → 180 |
| | 31 - 32.7 | 180 → 230 |
| | 32.7 - 37.7 | 230 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: a suitable volume, for example 1.0 mL, of the gaseous phase of test solutions (a) and (b) and of the reference solution.

*Identification of peaks*: use the chromatogram obtained with the reference solution to identify the peaks due to propylene oxide and propionaldehyde.

*Relative retention* with reference to propylene oxide (retention time = about 10.4 min): propionaldehyde = about 0.96.

*System suitability*: reference solution:

- *resolution*: minimum 1.5 between the peaks due to propionaldehyde and propylene oxide;
- *signal-to-noise ratio*: minimum 10 for the peak due to propylene oxide.

Calculate the content of propylene oxide in parts per million using the following expression:

$$\frac{A_1 \times m \times 5}{(A_2 \times M_1) - (A_1 \times M_2)}$$

$A_1$ = area of the peak due to propylene oxide in the chromatogram obtained with test solution (a);

$A_2$ = area of the peak due to propylene oxide in the chromatogram obtained with test solution (b);

$M_1$ = mass of the substance to be examined in test solution (a), in grams;

$M_2$ = mass of the substance to be examined in test solution (b), in grams;

$m$ = mass of propylene oxide used to prepare the propylene oxide stock solution, in grams.

*Limit*:

- *propylene oxide*: maximum 5 ppm.

**Water** (*2.5.12*): maximum 0.7 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent.

Ignite a suitable crucible at 600 ± 25 °C for 30 min, allow to cool in a desiccator over silica gel or other suitable desiccant and weigh. Place 1.0 g of the substance to be examined in the crucible and weigh. Carefully ignite and char the substance using a gas burner in a fume cupboard. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting from "Moisten the substance to be examined…".

STORAGE

In an airtight container.

LABELLING

The label states the number of moles of propylene oxide reacted per mole of stearyl alcohol (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for polyoxypropylene stearyl ether used as solvent or emollient in preparations for cutaneous application.*

**Viscosity** (see Identification).

**01/2017:0426**

# POLYSORBATE 20

## Polysorbatum 20

DEFINITION

Mixture of partial esters of fatty acids, mainly lauric (dodecanoic) acid, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

*Appearance*: oily, yellow or brownish-yellow, clear or slightly opalescent liquid.

*Solubility*: soluble in water, in anhydrous ethanol, in ethyl acetate and in methanol, practically insoluble in fatty oils and in liquid paraffin.

*Relative density*: about 1.10.

*Viscosity*: about 400 mPa·s at 25 °C.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of polysorbate 20.*

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 mL of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

Dissolve 5.0 g in 50 mL of the prescribed solvent mixture.

**Hydroxyl value** (*2.5.3, Method A*): 96 to 108.

**Peroxide value**: maximum 10.0.

Introduce 10.0 g into a 100 mL beaker and dissolve with 20 mL of *glacial acetic acid R*. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration. If the result of the blank determination exceeds 0.1 mL of titration reagent, replace the reagents and repeat the determination.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution, in moles per litre;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): 40 to 50, determined on 4.0 g.

Use 15.0 mL of *0.5 M alcoholic potassium hydroxide* and dilute with 50 mL of *ethanol (96 per cent) R* before carrying out the titration. Heat under reflux for 60 min.

**Composition of fatty acids** (*2.4.22, Method C*). Prepare reference solution (a) as indicated in Table 2.4.22.-2.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 50 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 80 → 220 |
| | 14 - 54 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Composition of the fatty-acid fraction of the substance*:

– *caproic acid*: maximum 1.0 per cent;
– *caprylic acid*: maximum 10.0 per cent;
– *capric acid*: maximum 10.0 per cent;
– *lauric acid*: 40.0 per cent to 60.0 per cent;
– *myristic acid*: 14.0 per cent to 25.0 per cent;
– *palmitic acid*: 7.0 per cent to 15.0 per cent;
– *stearic acid*: maximum 7.0 per cent;
– *oleic acid*: maximum 11.0 per cent;
– *linoleic acid*: maximum 3.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

**01/2017:1914**

# POLYSORBATE 40

## Polysorbatum 40

DEFINITION

Mixture of partial esters of fatty acids, mainly *Palmitic acid (1904)*, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

*Appearance*: oily, viscous, yellowish or brownish-yellow liquid.

*Solubility*: miscible with water, with anhydrous ethanol, with ethyl acetate and with methanol, practically insoluble in fatty oils and in liquid paraffin.

*Relative density*: about 1.10.

*Viscosity*: about 400 mPa·s at 30 °C.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of polysorbate 40.*

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 mL of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

Dissolve 5.0 g in 50 mL of the prescribed solvent mixture.

**Hydroxyl value** (*2.5.3, Method A*): 89 to 105.

**Peroxide value**: maximum 10.0.

Introduce 10.0 g into a 100 mL beaker and dissolve with 20 mL of *glacial acetic acid R*. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration. If the result of the blank determination exceeds 0.1 mL of titration reagent, replace the reagents and repeat the determination.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution, in moles per litre;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): 41 to 52, determined on 4.0 g.

Use 15.0 mL of *0.5 M alcoholic potassium hydroxide* and dilute with 50 mL of *ethanol (96 per cent) R* before carrying out the titration. Heat under reflux for 60 min.

**Composition of fatty acids** (*2.4.22, Method C*). Prepare reference solution (a) as indicated in Table 2.4.22.-1.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 50 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 80 → 220 |
| | 14 - 54 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Composition of the fatty-acid fraction of the substance*:

– *palmitic acid*: minimum 92.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

**01/2017:0427**

# POLYSORBATE 60

## Polysorbatum 60

DEFINITION

Mixture of partial esters of fatty acids, mainly *Stearic acid 50 (1474)*, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

CHARACTERS

*Appearance*: yellowish-brown gelatinous mass which becomes a clear liquid at temperatures above 25 °C.

*Solubility*: soluble in water, in anhydrous ethanol, in ethyl acetate and in methanol, practically insoluble in fatty oils and in liquid paraffin.

*Relative density*: about 1.10.

*Viscosity*: about 400 mPa·s at 30 °C.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of polysorbate 60.*

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

E. Dissolve 0.1 g in 5 mL of *methylene chloride R*. Add 0.1 g of *potassium thiocyanate R* and 0.1 g of *cobalt nitrate R*. Stir with a glass rod. The solution becomes blue.

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

Dissolve 5.0 g in 50 mL of the prescribed solvent mixture.

**Hydroxyl value** (*2.5.3, Method A*): 81 to 96.

**Peroxide value**: maximum 10.0.

Introduce 10.0 g into a 100 mL beaker and dissolve with 20 mL of *glacial acetic acid R*. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration. If the result of the blank determination exceeds 0.1 mL of titration reagent, replace the reagents and repeat the determination.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution, in moles per litre;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): 45 to 55, determined on 4.0 g.

Use 15.0 mL of *0.5 M alcoholic potassium hydroxide* and dilute with 50 mL of *ethanol (96 per cent) R* before carrying out the titration. Heat under reflux for 60 min.

**Composition of fatty acids** (*2.4.22, Method C*). Prepare reference solution (a) as indicated in Table 2.4.22.-1.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: 50 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 80 → 220 |
| | 14 - 54 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Composition of the fatty-acid fraction of the substance*:
- *stearic acid*: 40.0 per cent to 60.0 per cent;
- *sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent.

**Ethylene oxide and dioxan** (*2.4.25, Method A*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

**01/2017:0428**
**corrected 10.0**

# POLYSORBATE 80[(1)]

## Polysorbatum 80

DEFINITION

Mixture of partial esters of fatty acids, mainly *Oleic acid (0799)*, with sorbitol and its anhydrides ethoxylated with approximately 20 moles of ethylene oxide for each mole of sorbitol and sorbitol anhydrides.

♦CHARACTERS

*Appearance*: oily, colourless or brownish-yellow, clear or slightly opalescent liquid.

*Solubility*: dispersible in water, in anhydrous ethanol, in ethyl acetate and in methanol, practically insoluble in fatty oils and in liquid paraffin.

*Relative density*: about 1.10.

*Viscosity*: about 400 mPa·s at 25 °C.♦

IDENTIFICATION

*First identification:* ♦*A*♦*, D.*

◊*Second identification: B, C, D, E.*◊

♦A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of polysorbate 80.*♦

◊B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).◊

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

D. Composition of fatty acids (see Tests).

◊E. Dissolve 0.1 g in 5 mL of *methylene chloride R*. Add 0.1 g of *cobalt nitrate R* and 0.1 g of *potassium thiocyanate R*. Stir with a glass rod. The solution becomes blue.◊

TESTS

**Acid value** (*2.5.1*): maximum 2.0.

Dissolve 5.0 g in 50 mL of the prescribed mixture of solvents.

**Hydroxyl value** (*2.5.3, Method A*): 65 to 80.

**Peroxide value**: maximum 10.0.

Introduce 10.0 g into a 100 mL beaker and dissolve with 20 mL of *glacial acetic acid R*. Add 1 mL of *saturated potassium iodide solution R*, mix and allow to stand for 1 min. Add 50 mL of *carbon dioxide-free water R* and a magnetic stirring bar. Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

Determine the peroxide value using the following expression:

$$\frac{(n_1 - n_2) \times M \times 1000}{m}$$

$n_1$ = volume of *0.01 M sodium thiosulfate* required for the substance to be examined, in millilitres;

$n_2$ = volume of *0.01 M sodium thiosulfate* required for the blank titration, in millilitres;

$M$ = molarity of the sodium thiosulfate solution, in moles per litre;

$m$ = mass of the substance to be examined, in grams.

**Saponification value** (*2.5.6*): 45 to 55, determined on 4.0 g.

Use 30.0 mL of *0.5 M alcoholic potassium hydroxide*, heat under reflux for 60 min and add 50 mL of *anhydrous ethanol R* before carrying out the titration.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 50 cm/s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 14 | 80 → 220 |
| | 14 - 54 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Composition of the fatty-acid fraction of the substance*:

– *myristic acid*: maximum 5.0 per cent;
– *palmitic acid*: maximum 16.0 per cent;
– *palmitoleic acid*: maximum 8.0 per cent;
– *stearic acid*: maximum 6.0 per cent;
– *oleic acid*: minimum 58.0 per cent;
– *linoleic acid*: maximum 18.0 per cent;
– *linolenic acid*: maximum 4.0 per cent.

**Ethylene oxide and dioxan**: maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

Head-space gas chromatography (*2.2.28*).

*Ethylene oxide stock solution*. Dilute 0.5 mL of a commercially available solution of ethylene oxide in methylene chloride (50 mg/mL) to 50.0 mL with *water R*. [NOTE: the solution is stable for 3 months, if stored in vials with polytetrafluoroethylene-coated silicone membrane crimped caps at − 20 °C]. Allow to reach room temperature. Dilute 1.0 mL of this solution to 250.0 mL with *water R*.

*Dioxan stock solution*. Dilute 1.0 mL of *dioxan R* to 200.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Acetaldehyde stock solution*. Weigh about 0.100 g of *acetaldehyde R* into a 100 mL volumetric flask and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Standard solution*. To 6.0 mL of ethylene oxide stock solution add 2.5 mL of dioxan stock solution and dilute to 25.0 mL with *water R*.

*Test solution (a)*. Weigh 1.00 g of the substance to be examined into a 10 mL head-space vial. Add 2.0 mL of *water R*, seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminum cap. Mix carefully.

*Test solution (b)*. Weigh 1.00 g of the substance to be examined into a 10 mL head-space vial. Add 2.0 mL of standard solution, seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminum cap. Mix carefully.

*Reference solution*. Introduce 2.0 mL of acetaldehyde stock solution and 2.0 mL of ethylene oxide stock solution into a 10 mL head-space vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminum cap. Mix carefully.

*Column*:

– *material*: fused silica;
– *size*: *l* = 50 m, Ø = 0.53 mm;
– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4.0 mL/min.

*Split ratio*: 1:3.5.

*Static head-space conditions that may be used*:

– *equilibration temperature*: 80 °C;
– *equilibration time*: 30 min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 18 | 70 → 250 |
| | 18 - 23 | 250 |
| Injection port | | 85 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL of test solutions (a) and (b) and of the reference solution.

*Relative retention* with reference to ethylene oxide (retention time = about 6.5 min): acetaldehyde = about 0.9; dioxan = about 1.9.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to acetaldehyde and ethylene oxide.

Calculate the content of ethylene oxide in ppm using the following expression:

$$\frac{2\ C_{EO} \times A_a}{A_b - A_a}$$

$C_{EO}$ = concentration of added ethylene oxide in test solution (b), in micrograms per millilitre;

$A_a$ = peak area of ethylene oxide in the chromatogram obtained with test solution (a);

$A_b$ = peak area of ethylene oxide in the chromatogram obtained with test solution (b).

Calculate the content of dioxan in ppm using the following expression:

$$\frac{2 \times 1.03 \times C_D \times A_{a'}}{A_{b'} - A_{a'}} \times 1000$$

$C_D$ = concentration of added dioxan in test solution (b), in microlitres per millilitre;

1.03 = density of dioxan, in grams per millilitre;

$A_{a'}$ = peak area of dioxan in the chromatogram obtained with test solution (a);

$A_{b'}$ = peak area of dioxan in the chromatogram obtained with test solution (b).

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.25 per cent, determined on 2.0 g.

STORAGE

In an airtight container, protected from light.

**01/2017:1962**
**corrected 10.0**

# POLY(VINYL ACETATE)

## Poly(vinylis acetas)

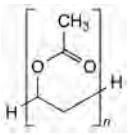

DEFINITION

Poly(vinyl acetate) is a thermoplastic polymer obtained by polymerisation of vinyl acetate using a suitable starter, without solvent or with water or 2-propanol. The vast majority of the acetate moieties are attached to non-neighbouring carbon atoms of the chain.

The index *n* is about 100-17 000. The relative molecular mass lies between 10 000 and 1500 000. The viscosity is 4 mPa·s to 250 mPa·s. The ester value, which characterises the degree of hydrolysis, is 615 to 675.

CHARACTERS

*Appearance*: white or almost white powder or colourless granules or beads.

*Solubility*: practically insoluble in water, freely soluble in ethyl acetate, soluble in ethanol (96 per cent). It is hygroscopic and swells in water.

It softens at temperatures above 40-50 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs prepared as follows: dissolve about 200 mg in 5 mL of *acetone R*, place 100 µL on a *potassium bromide R* disc and dry to evaporate the solvent. Alternatively, the spectrum may be recorded directly by attenuated total reflectance (ATR).

*Comparison*: *poly(vinyl acetate) CRS*.

B. Viscosity (see Tests).

C. Saponify (*2.5.6*) 0.500 g in a mixture of 25.0 mL of *0.5 M alcoholic potassium hydroxide* and 25.0 mL of *water R*. 0.15 mL of the solution obtained gives reaction (b) of acetates (*2.3.1*).

TESTS

**Solution S**. Suspend 50.0 g in 100 mL of *ethyl acetate R* in a borosilicate glass flask with a ground-glass neck. Heat under a reflux condenser with constant stirring for 30 min. Allow to cool. Filter through a sintered-glass filter (16) (*2.1.2*), wash the residue with 50.0 mL of *ethyl acetate R* and pour the filtrate into a 250 mL graduated flask. Dilute to 250 mL with *ethyl acetate R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Viscosity** (*2.2.49, Method A*): 85 per cent to 115 per cent of the value stated on the label.

Determine the viscosity immediately after preparation of solution S at 20 ± 0.1 °C by using a falling ball viscometer.

**Acid value** (*2.5.1*): maximum 2.0, determined on 5.0 g dissolved in 50 mL of *ethanol (96 per cent) R* by shaking for 3 h.

**Ester value** (*2.5.2*): 615 to 675.

Saponify (*2.5.6*) 0.500 g in a mixture of 25.0 mL of *0.5 M alcoholic potassium hydroxide* and 25.0 mL of *water R*.

**Residual peroxides**: maximum 100 ppm, calculated as hydrogen peroxide.

Place 0.85 g in a borosilicate glass flask with a ground-glass neck. Add 10 mL of *ethyl acetate R* and heat under a reflux condenser with constant agitation. Allow to cool. Replace the air in the container with *oxygen-free nitrogen R* and add a solution of 1 mL of *glacial acetic acid R* and 0.5 g of *sodium iodide R* in 40 mL of *water R*. Shake thoroughly and allow to stand protected from light for 20 min. Titrate with *0.005 M sodium thiosulfate* until the yellow colour is discharged. Carry out a blank titration. The difference between the titration volumes is not greater than 1.0 mL.

**Vinyl acetate**. Head-space gas chromatography (*2.2.28*).

*Test solution (a).* Place 0.200 g of the substance to be examined in a 20 mL vial and add 1.0 mL of *dimethylformamide R*. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid.

*Test solution (b).* Place 0.200 g of the substance to be examined in a 20 mL vial and add 1.0 mL of the reference solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid.

*Reference solution.* Place 15.0 mL of *dimethylformamide R* in a 20 mL vial, add 45 µL of *vinyl acetate R* and 50 µL of *butanal R* and dilute to volume with *dimethylformamide R*. Dilute 1.0 mL of the solution to 10.0 mL with *dimethylformamide R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.32 mm;
- *stationary phase*: *vinyl(1)phenyl(5)methyl(94)polysiloxane R* (film thickness 0.32 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 20 mL/min.

*Static head-space conditions that may be used*:

- *equilibration temperature*: 60 °C;
- *equilibration time*: 20 min;
- *transfer-line temperature*: 120 °C;
- *carrier gas*: *nitrogen for chromatography R*.

*Temperature*:

- *column*: 155 °C;
- *injection port*: 120 °C;
- *detector*: 180 °C.

*Detection*: flame ionisation.

*Injection*: 1.6 mL of the gaseous phase of test solutions (a) and (b).

*System suitability*: test solution (b):

- *resolution*: minimum 2.0 between the peaks due to vinyl acetate and butanal;
- *signal-to-noise ratio*: minimum 5 for the peak due to vinyl acetate.

Calculate the percentage content of vinyl acetate using the following expression:

$$\frac{V \times S_1 \times 0.931}{(m_1 S_2 - m_2 S_1) \times 2000}$$

$S_1$ = area (or height) of the peak due to vinyl acetate in the chromatogram obtained with test solution (a);

$S_2$ = area (or height) of the peak due to vinyl acetate in the chromatogram obtained with test solution (b);

$m_1$ = mass of the substance to be examined used to prepare test solution (a), in grams;

$m_2$ = mass of the substance to be examined used to prepare test solution (b), in grams;

0.931 = density of vinyl acetate, in grams per millilitre;

$V$ = volume of vinyl acetate used to prepare the reference solution, in microlitres.

*Limit*:

- *vinyl acetate*: maximum 0.3 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

In an airtight container.

LABELLING

The label states:

- the nominal relative molecular mass;
- the viscosity.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for poly(vinyl acetate) used as matrix former in prolonged-release dosage forms or film former.*

**Viscosity** (see Tests).

**Solubility of a film**. Place 3 mL of solution S on a glass plate and dry. Place the film obtained in 50 mL of *phosphate buffer solution pH 6.8 R* whilst stirring continuously. The film does not dissolve within 30 min.

**01/2009:2152**
**corrected 10.0**

# POLY(VINYL ACETATE) DISPERSION 30 PER CENT

## Poly(vinylis acetas) dispersio 30 per centum

DEFINITION

Dispersion in water of poly(vinyl acetate) having a mean relative molecular mass of about 450 000. It may contain *Povidone (0685)* and a suitable surface-active agent, such as *Sodium laurilsulfate (0098)*, as stabilisers.

*Content*: 25.0 per cent to 30.0 per cent of poly(vinyl acetate).

CHARACTERS

*Appearance*: opaque, white or almost white, slightly viscous liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

It is sensitive to spoilage by microbial contaminants.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry 1 mL *in vacuo*, dissolve the residue in *acetone R*, and spread 1 drop of the solution between 2 *sodium chloride R* plates; remove 1 plate and allow the solvent to evaporate.

*Comparison*: repeat the operation using *poly(vinyl acetate) dispersion 30 per cent CRS*.

B. Place 3 mL on a glass plate and allow to dry. A clear film is formed.

C. 50 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Agglomerates**. Filter 100.0 g through a tared stainless steel sieve (90). Rinse with *water R* until a clear filtrate is obtained and dry to constant mass at 100-105 °C. The mass of the residue is not greater than 0.5 g.

**Vinyl acetate**. Liquid chromatography (*2.2.29*).

*Test solution.* Introduce 0.250 g into a 10 mL volumetric flask and add about 1 mL of *methanol R*. Sonicate. Add about 8 mL of *water R*. Sonicate and dilute to 10.0 mL with *water R*. Centrifuge for about 10 min and filter.

*Reference solution (a).* Dissolve 5.0 mg of *vinyl acetate CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* To 5 mg of *vinyl acetate R* and 5 mg of *1-vinylpyrrolidin-2-one R*, add 10 mL of *methanol R* and sonicate. Dilute to 50 mL with mobile phase A. Dilute 1 mL of the solution to 20 mL with mobile phase A.

A precolumn containing *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) may be used if a matrix effect is observed.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, *methanol R2*, *water for chromatography R* (5:5:90 *V/V/V*);
- *mobile phase B*: *methanol R2*, *acetonitrile R1*, *water for chromatography R* (5:45:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 26 | 100 → 80 | 0 → 20 |
| 26 - 27 | 80 → 0 | 20 → 100 |
| 27 - 30 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to vinyl acetate and 1-vinylpyrrolidin-2-one.

*Limit*:

– *vinyl acetate*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (100 ppm).

**Povidone**: maximum 4.0 per cent.

Carry out the determination of nitrogen by sulfuric acid digestion (*2.5.9*), using 0.25 g. Calculate the percentage content of povidone using the following expression:

$$\frac{N}{0.126}$$

$N$ = percentage content of nitrogen.

**Acetic acid**. Liquid chromatography (*2.2.29*).

*Test solution.* Mix 0.200 g with *water R*. Sonicate for about 10 min and dilute to 10.0 mL with *water R*.

*Reference solution.* Dissolve 30.0 mg of *acetic acid R* and 30 mg of *citric acid monohydrate R* in the mobile phase. Shake gently to dissolve and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: $l$ = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *0.005 M sulfuric acid*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL; after each injection, rinse the column with a mixture of equal volumes of *acetonitrile R1* and *0.005 M sulfuric acid*.

*Retention time*: acetic acid = about 6 min; citric acid = about 8 min.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to acetic acid and citric acid.

*Limit*:

– *acetic acid*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (1.5 per cent).

**Residue on evaporation**: 28.5 per cent to 31.5 per cent, determined on 1.000 g at 110 °C for 5 h.

**Sulfated ash**: maximum 0.5 per cent, determined on 1.0 g.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.00 g of the preparation to be examined in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 ± 25 °C, until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting with "Moisten the substance to be examined...".

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Determine the saponification value (*2.5.6*) on 1.5 g and calculate the percentage content of poly(vinyl acetate) using the following expression:

$$I_s \times 0.1534$$

$I_s$ = saponification value.

STORAGE

At a temperature of 5 °C to 30 °C. Handle the substance so as to minimise microbial contamination.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for poly(vinyl acetate) dispersion 30 per cent used in the manufacture of modified-release dosage forms and to mask taste.*

**Solubility of a film**. Place the film obtained in identification test B in 50 mL of *phosphate buffer solution pH 6.8 R* whilst stirring continuously. The film does not dissolve within 30 min.

**Apparent viscosity** (*2.2.10*): maximum 100 mPa·s, determined using a rotating viscometer at 20 °C and a shear rate of 100 $s^{-1}$.

**01/2017:1961**
**corrected 9.3**

# POLY(VINYL ALCOHOL)

## Poly(alcohol vinylicus)

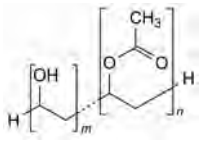


DEFINITION

Poly(vinyl alcohol) is obtained by polymerisation of vinyl acetate, followed by partial or almost complete hydrolysis of poly(vinyl acetate) in the presence of catalytic amounts of alkali or mineral acids.

Poly(vinyl alcohol) polymers comply with the following indices:

$$0 \leq \frac{n}{m} \leq 0.35$$

The mean relative molecular mass lies between 20 000 and 150 000. The viscosity is 3 mPa·s to 70 mPa·s. The ester value, which characterises the degree of hydrolysis, is not greater than 280.

CHARACTERS

*Appearance*: yellowish-white powder or translucent granules.

*Solubility*: soluble in water, slightly soluble in ethanol, practically insoluble in acetone.

Various grades of poly(vinyl alcohol) are available. They differ in their degree of polymerisation and their degree of hydrolysis which determine the physical properties of the different grades. They are characterised by the viscosity and the ester value of the substance.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *poly(vinyl alcohol) CRS*.

The intensities of the absorption bands at about 1720 $cm^{-1}$ and 1260 $cm^{-1}$ are inversely proportional to the degree of hydrolysis.

B. Viscosity (see Tests).

TESTS

**Solution S**. Heat on a water-bath 250 mL of *water R* in a borosilicate round-bottomed flask attached to a reflux condenser with stirrer, add 10.0 g of the substance to be examined (correcting for the loss on drying) and continue heating for 30 min with continuous stirring. Remove the flask from the water-bath and continue stirring until room temperature is reached.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.5 for solution S.

**Viscosity** (*2.2.49, Method A*): 85 per cent to 115 per cent of the value stated on the label.

Determine the viscosity using a falling ball viscometer immediately after preparation of solution S at 20 ± 0.1 °C.

**Acid value**: maximum 3.0.

Add 1 mL of *phenolphthalein solution R* to 50 mL of solution S and titrate with *0.05 M potassium hydroxide* until the pink colour persists for 15 s. Calculate the acid value using the following expression:

$$\frac{2.805V}{2}$$

$V$ = volume of *0.05 M potassium hydroxide* used, in millilitres.

**Ester value** (*2.5.2*): 90 per cent to 110 per cent of the value stated on the label.

Saponify (*2.5.6*) 1.00 g in a mixture of 25.0 mL of *0.5 M alcoholic potassium hydroxide* and 25.0 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

LABELLING

The label states:

- the viscosity for a 40 g/L solution;
- the ester value.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section.*

*Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for poly(vinyl alcohol) used as viscosity-increasing agent, binder or film former.*

**Viscosity** (see Tests).

**Ester value** (see Tests).

**Infrared absorption spectrophotometry** (see Identification A).

**01/2017:1139**

# POTASSIUM ACETATE

## Kalii acetas

$C_2H_3KO_2$ $M_r$ 98.1

[127-08-2]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, deliquescent.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of acetates (*2.3.1*).

B. It gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 9.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Reducing substances**. Dilute 10 mL of solution S to 100 mL with *water R*. Add 5 mL of *dilute sulfuric acid R* and 0.5 mL of a 0.32 g/L solution of *potassium permanganate R*. Mix and boil gently for 5 min. The solution remains pink.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 1 ppm, if intended for use in the manufacture of peritoneal dialysis solutions, haemofiltration solutions or haemodialysis solutions.

*Prescribed solution*. Dissolve 2.0 g in 50 mL of *water R* and add 5 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 1 mL of *aluminium standard solution (2 ppm Al) R*, 5 mL of *acetate buffer solution pH 6.0 R* and 49 mL of *water R*.

*Blank solution*. Mix 5 mL of *acetate buffer solution pH 6.0 R* and 50 mL of *water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Sodium**: maximum 0.5 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution*. Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 80.0 mg in 20 mL of *anhydrous acetic acid R*. Add 0.2 mL of *naphtholbenzein solution R*. Titrate with *0.1 M perchloric acid*. Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 9.81 mg of $C_2H_3KO_2$.

STORAGE

In an airtight container.

**01/2017:0184**

# POTASSIUM BROMIDE

## Kalii bromidum

KBr $M_r$ 119.0

[7758-02-3]

DEFINITION

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water and in glycerol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of bromides (*2.3.1*).

B. Solution S (see Tests) gives the reactions of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromates**. To 10 mL of solution S add 1 mL of *starch solution R*, 0.1 mL of a 100 g/L solution of *potassium iodide R* and 0.25 mL of *0.5 M sulfuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

**Chlorides and sulfates**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.400 g of the substance to be examined in 50 mL of *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dilute 25.0 mL of test solution (a) to 50.0 mL with *water for chromatography R*.

*Reference solution (a)*. To 25.0 mL of test solution (a) add 1.0 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 12.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (b)*. Dilute 10.0 mL of test solution (a) to 100.0 mL with *water for chromatography R*. To 2.0 mL of this solution add 8.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 20.0 mL with *water for chromatography R*.

*Blank solution*: *water for chromatography R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 2 mm;
– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (13 µm).

*Mobile phase*: dissolve 0.600 g of *potassium hydroxide R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent.

*Flow rate*: 0.4 mL/min.

*Detection*: conductivity detector equipped with a suitable ion suppressor.

*Injection*: 50 µL of test solution (b), reference solutions (a) and (b) and the blank solution.

*Run time*: 2.5 times the retention time of bromide.

*Retention time*: chloride = about 5 min; bromide = about 8 min; sulfate = about 16 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 8.0 between the peaks due to chloride and bromide.

*Calculation of percentage contents*:

– for chlorides, use the concentration of chloride in reference solution (a); correct the area of the peak due to chloride in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to chloride in the chromatogram obtained with test solution (b);
– for sulfates, use the concentration of sulfate in reference solution (a); correct the area of the peak due to sulfate in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to sulfate in the chromatogram obtained with test solution (b).

*Limits*:

– *chlorides*: maximum 0.6 per cent;
– *sulfates*: maximum 0.01 per cent.

**Iodides**. To 5 mL of solution S add 0.15 mL of *ferric chloride solution R1* and 2 mL of *methylene chloride R*. Shake and allow to separate. The lower layer is colourless (*2.2.2, Method I*).

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium and alkaline-earth metals** (*2.4.7*): maximum 200 ppm, calculated as Ca.

10.0 g complies with the test for magnesium and alkaline-earth metals. The volume of *0.01 M sodium edetate* used does not exceed 5.0 mL.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 100.0 mg in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 11.90 mg of KBr.

Calculate the percentage content of KBr using the following expression:

$$a - 3.357\ b$$

*a* = percentage content of KBr and KCl obtained in the assay and calculated as KBr;

*b* = percentage content of Cl obtained in the test for chlorides.

**01/2017:1557**

# POTASSIUM CARBONATE

## Kalii carbonas

$K_2CO_3$ $M_r$ 138.2
[584-08-7]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white granular powder, hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in 10 mL of *water R*. The solution is strongly alkaline (*2.2.4*).

B. 2 mL of the solution prepared for identification test A gives the reaction of carbonates and bicarbonates (*2.3.1*).

C. 1 mL of the solution prepared for identification test A gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in 25 mL of *distilled water R*. Slowly add 14 mL of *hydrochloric acid R*. When the effervescence has ceased, boil for a few minutes. Allow to cool and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 0.50 g in 10 mL of *water R*. Carefully add dropwise 1 mL of *nitric acid R*. Boil. Cool, add 5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm.

Dilute 7.50 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 100 ppm.

To 5 mL of solution S add 1 mL of *concentrated ammonia R*. Boil. Cool. Dilute to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 0.300 g by drying in an oven at 120-125 °C for 5 h.

ASSAY

Dissolve 0.500 g in 50 mL of *carbon dioxide-free water R*. Carry out a potentiometric titration (*2.2.20*), using *1 M hydrochloric acid*. Read the volume added at the 2nd point of inflexion.

1 mL of *1 M hydrochloric acid* is equivalent to 69.1 mg of $K_2CO_3$.

STORAGE

In an airtight container.

**01/2017:0185**

# POTASSIUM CHLORIDE

## Kalii chloridum

KCl $M_r$ 74.6
[7447-40-7]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of KCl (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. It gives the reactions of chlorides (*2.3.1*).

B. Solution S (see Tests) gives the reactions of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromides**: maximum 0.1 per cent.

Dilute 1.0 mL of solution S to 50 mL with *water R*. To 5.0 mL of the solution add 2.0 mL of *phenol red solution R2* and 1.0 mL of *chloramine solution R1* and mix immediately. After exactly 2 min add 0.15 mL of *0.1 M sodium thiosulfate*, mix and dilute to 10.0 mL with *water R*. The absorbance (*2.2.25*) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner using 5 mL of a 3.0 mg/L solution of *potassium bromide R*.

**Iodides**. Moisten 5 g by the dropwise addition of a freshly prepared mixture of 0.15 mL of *sodium nitrite solution R*, 2 mL of *0.5 M sulfuric acid*, 25 mL of *iodide-free starch solution R* and 25 mL of *water R*. After 5 min, examine in daylight. The substance shows no blue colour.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 1.0 ppm, if intended for use in the manufacture of haemodialysis solutions.

*Prescribed solution*. Dissolve 4 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Barium**. To 5 mL of solution S add 5 mL of *distilled water R* and 1 mL of *dilute sulfuric acid R*. After 15 min, any opalescence in the solution is not more intense than that in a mixture of 5 mL of solution S and 6 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium and alkaline-earth metals** (*2.4.7*): maximum 200 ppm, calculated as Ca, determined on 10.0 g using 0.15 g of *mordant black 11 triturate R*. The volume of *0.01 M sodium edetate* used does not exceed 5.0 mL.

**Sodium**: maximum 0.1 per cent, if intended for use in the manufacture of parenteral preparations or haemodialysis solutions.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions by diluting as required a solution containing 200 µg of Na per millilitre, prepared as follows: dissolve in *water R* 0.5084 g of *sodium chloride R*, previously dried at 105 °C for 3 h, and dilute to 1000.0 mL with the same solvent.

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 60.0 mg in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 7.46 mg of KCl.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- where applicable, that the substance is suitable for use in the manufacture of haemodialysis solutions.

**01/2017:0400**

# POTASSIUM CITRATE

## Kalii citras

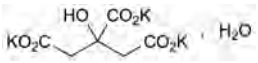

$C_6H_5K_3O_7,H_2O$ $M_r$ 324.4
[6100-05-6]

DEFINITION

Tripotassium 2-hydroxypropane-1,2,3-tricarboxylate monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, granular powder or transparent crystals, hygroscopic.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. To 1 mL of solution S (see Tests) add 4 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

B. 0.5 mL of solution S gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Readily carbonisable substances**. To 0.20 g of the powdered substance to be examined add 10 mL of *sulfuric acid R* and heat in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution $Y_2$ or $GY_2$ (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Oxalates**: maximum 300 ppm.

Dissolve 0.50 g in 4 mL of *water R*, add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 4 mL of a 0.05 g/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

To 10 mL of solution S add 2 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*.

**Sodium**: maximum 0.3 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution.* To 10 mL of solution S add 1 mL of *dilute hydrochloric acid R* and dilute to 100 mL with *distilled water R*.

*Reference solutions.* Prepare the reference solutions using a solution of *sodium chloride R* containing 1 mg of Na per millilitre diluted as necessary with *distilled water R*.

*Wavelength*: 589 nm.

**Water** (*2.5.12*): 4.0 per cent to 7.0 per cent, determined on 0.250 g. Use a mixture of 1 volume of *formamide R* and 2 volumes of *methanol R* as solvent. After adding the substance to be examined, stir for 15 min before titrating.

ASSAY

Dissolve 0.150 g in 20 mL of *anhydrous acetic acid R*, heating to about 50 °C. Allow to cool. Titrate with *0.1 M perchloric acid* using 0.25 mL of *naphtholbenzein solution R* as indicator until a green colour is obtained.

1 mL of *0.1 M perchloric acid* is equivalent to 10.21 mg of $C_6H_5K_3O_7$.

STORAGE

In an airtight container.

**07/2010:1140**
**corrected 10.0**

# POTASSIUM CLAVULANATE

## Kalii clavulanas

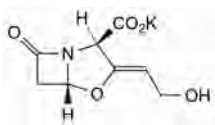

$C_8H_8KNO_5$ $M_r$ 237.3
[61177-45-5]

DEFINITION

Potassium (2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate, the potassium salt of a substance produced by the growth of certain strains of *Streptomyces clavuligerus* or obtained by any other means.

*Content*: 96.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in acetone.

PRODUCTION

The methods of production, extraction and purification are such that clavam-2-carboxylate is eliminated or present at a level not exceeding 0.01 per cent.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of potassium clavulanate*.

B. It gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.400 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**pH** (*2.2.3*): 5.5 to 8.0.

Dilute 5 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 53 to + 63 (anhydrous substance), determined on solution S.

**Polymeric impurities and other impurities absorbing at 278 nm**

Dissolve 50.0 mg in *0.1 M phosphate buffer solution pH 7.0 R* and dilute to 50.0 mL with the same buffer solution. Measure the absorbance immediately.

The absorbance (*2.2.25*) of the solution determined at 278 nm is not greater than 0.40.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.250 g of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10 mg of *lithium clavulanate CRS* and 10 mg of *amoxicillin trihydrate CRS* in mobile phase A and dilute to 100 mL with mobile phase A.

*Reference solution (c).* Dissolve 2 mg of *potassium clavulanate impurity G CRS* in 20 mL of mobile phase A.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: a 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 4.0 with *phosphoric acid R*;
- *mobile phase B*: a mixture of equal volumes of *methanol R* and mobile phase A;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 15 | 100 → 50 | 0 → 50 |
| 15 - 18 | 50 | 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to clavulanate (retention time = about 3 min): impurity E = about 2.3; impurity G = about 3.6.

*System suitability*: reference solution (b):
- *resolution*: minimum 13 between the peaks due to clavulanate (1st peak) and amoxicillin (2nd peak).

*Limits*:
- *impurities E, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Aliphatic amines**. Gas chromatography (*2.2.28*).

The method shown below can be used to determine the following aliphatic amines: 1,1-dimethylethylamine; tetramethylethylenediamine; 1,1,3,3-tetramethylbutylamine; *N*,*N*′-diisopropylethylenediamine; 2,2′-oxybis(*N*,*N*)dimethylethylamine.

*Internal standard solution*: dissolve 50 µL of *3-methylpentan-2-one R* in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Weigh 1.00 g of the substance to be examined into a centrifuge tube. Add 5.0 mL of the internal standard solution, 5.0 mL of *dilute sodium hydroxide solution R*, 10.0 mL of *water R*, 5.0 mL of *2-methylpropanol R* and 5 g of *sodium chloride R*. Shake vigorously for 1 min. Centrifuge to separate the layers.

*Reference solution.* Dissolve 80.0 mg of each of the following amines: *1,1-dimethylethylamine R*; *tetramethylethylenediamine R*; *1,1,3,3-tetramethylbutylamine R*; *N,N′-diisopropylethylenediamine R* and *2,2′-oxybis(N,N-dimethylethylamine) R* in *dilute hydrochloric acid R* and dilute to 200.0 mL with the same acid. Introduce 5.0 mL of this solution into a centrifuge tube. Add 5.0 mL of the internal standard solution, 10.0 mL of *dilute sodium hydroxide solution R*, 5.0 mL of *2-methylpropanol R* and 5 g of *sodium chloride R*. Shake vigorously for 1 min. Centrifuge to separate the layers.

*Column*:
- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 8 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 7 | 35 |
| | 7 - 10.8 | 35 → 150 |
| | 10.8 - 25.8 | 150 |
| Injection port | | 200 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the upper layers obtained from the test solution and the reference solution.

*Relative retention* with reference to 3-methylpentan-2-one (retention time = about 11.4 min): impurity H = about 0.55; impurity J = about 1.07; impurity K = about 1.13; impurity L = about 1.33; impurity M = about 1.57.

*Limit*:

– *total of aliphatic amines*: maximum 0.2 per cent.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.8 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.03 IU/mg if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in a 4.1 g/L solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R*, and dilute to 50.0 mL with the same solution.

*Reference solution (a).* Dissolve 50.0 mg of *lithium clavulanate CRS* in a 4.1 g/L solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R* and dilute to 50.0 mL with the same solution.

*Reference solution (b).* Dissolve 10 mg of *amoxicillin trihydrate CRS* in 10 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.3 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 5 volumes of *methanol R1* and 95 volumes of a 15 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 4.0 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to clavulanate (1st peak) and amoxicillin (2nd peak).

1 mg of clavulanate ($C_8H_9NO_5$) is equivalent to 1.191 mg of $C_8H_8KNO_5$.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

**Test for related substances**: A, B, C, D, E, F, G.

**Test for aliphatic amines**: H, J, K, L, M.

*Specified impurities: E, G, H, J, K, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F.*

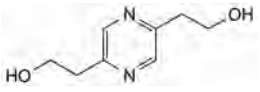

A. 2,2′-(pyrazine-2,5-diyl)diethanol,

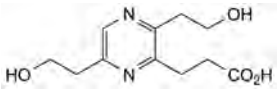

B. 3-[3,6-bis(2-hydroxyethyl)pyrazin-2-yl]propanoic acid,

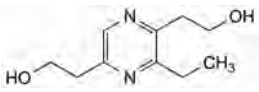

C. 2,2′-(3-ethylpyrazine-2,5-diyl)diethanol,

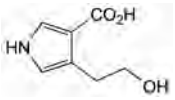

D. 4-(2-hydroxyethyl)-1*H*-pyrrole-3-carboxylic acid,

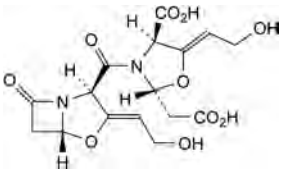

E. (2*R*,4*R*,5*Z*)-2-(carboxymethyl)-5-(2-hydroxyethylidene)-3-[[(2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]oxazolidine-4-carboxylic acid,

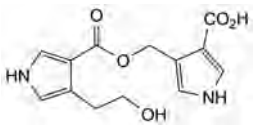

F. 4-[[[[4-(2-hydroxyethyl)-1*H*-pyrrol-3-yl]carbonyl]oxy]methyl]-1*H*-pyrrole-3-carboxylic acid,

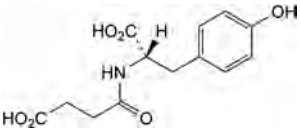

G. 4-[[(1*S*)-1-carboxy-2-(4-hydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (*N*-(hydrogensuccinyl)tyrosine),

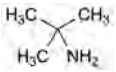

H. 2-methylpropan-2-amine (2-amino-2-methylpropane, *tert*-butylamine, 1,1-dimethylethylamine),

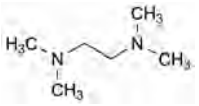

J. *N,N,N′,N′*-tetramethylethane-1,2-diamine (1,2-bis(dimethylamino)ethane, *N,N,N′,N′*-tetramethylethylenediamine),

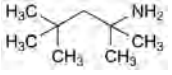

K. 2,4,4-trimethylpentan-2-amine (2-amino-2,4,4-trimethylpentane, 1,1,3,3-tetramethylbutylamine),

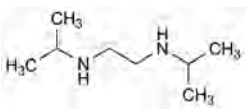

L. *N,N′*-diisopropylethane-1,2-diamine (*N,N′*-bis(1-methylethyl)ethane-1,2-diamine),

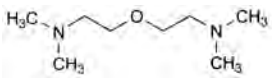

M. 2,2′-oxybis(*N,N*-dimethylethanamine) (bis[2-(dimethylamino)ethyl] ether, *N,N,N′,N′*-tetramethyl(oxydiethylene)diamine).

**07/2010:1653**

# POTASSIUM CLAVULANATE, DILUTED

## Kalii clavulanas dilutus

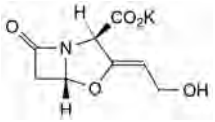

$C_8H_8KNO_5$ $M_r$ 237.3

DEFINITION

Dry mixture of *Potassium clavulanate (1140)* and *Cellulose, microcrystalline (0316)* or *Silica, colloidal anhydrous (0434)* or *Silica, colloidal hydrated (0738)*.

*Content*: 91.2 per cent to 107.1 per cent of the content of potassium clavulanate stated on the label.

CHARACTERS

*Appearance of diluted potassium clavulanate*: white or almost white powder, hygroscopic.

*Solubility of potassium clavulanate*: freely soluble in water, slightly soluble in ethanol (96 per cent), very slightly soluble in acetone.

The solubility of the diluted product depends on the diluent and its concentration.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. It gives reaction (b) of potassium (*2.3.1*).

C. Depending on the diluent used, carry out the corresponding identification test (a) or (b).

(a) A quantity of the substance to be examined, corresponding to 20 mg of cellulose, when placed on a watch-glass and dispersed in 4 mL of *iodinated zinc chloride solution R*, becomes violet-blue.

(b) It gives the reaction of silicates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.8 to 8.0.

Suspend a quantity of the substance to be examined corresponding to 0.200 g of potassium clavulanate in 20 mL of *carbon dioxide-free water R*.

**Polymeric impurities and other impurities absorbing at 278 nm**

Disperse a quantity of the substance to be examined corresponding to 50.0 mg of potassium clavulanate in 10 mL of *0.1 M phosphate buffer solution pH 7.0 R*, dilute to 50.0 mL with the same buffer solution and filter. Measure the absorbance immediately.

The absorbance (*2.2.25*) of the solution determined at 278 nm is not greater than 0.40.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Disperse a quantity of the substance to be examined corresponding to 0.250 g of potassium clavulanate in 5 mL of mobile phase A, dilute to 25.0 mL with mobile phase A and filter.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 10 mg of *amoxicillin trihydrate CRS* in 1 mL of the test solution and dilute to 100 mL with mobile phase A.

*Reference solution (c).* 2 mg of *potassium clavulanate impurity G CRS* in 20 mL of mobile phase A.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: a 7.8 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 4.0 with *dilute phosphoric acid R*;
- *mobile phase B*: a mixture of equal volumes of mobile phase A and *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 15 | 100 → 50 | 0 → 50 |
| 15 - 18 | 50 | 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to clavulanate (retention time = about 3 min): impurity E = about 2.3; impurity G = about 3.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 13 between the peaks due to clavulanate (1st peak) and amoxicillin (2nd peak).

*Limits*:

- *impurities E, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 1.000 g.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Disperse a quantity of the substance to be examined corresponding to 50.0 mg of potassium clavulanate in a 4.1 g/L solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R*, dilute to 50.0 mL with the same solution and filter.

*Reference solution (a).* Dissolve 50.0 mg of *lithium clavulanate CRS* in a 4.1 g/L solution of *sodium acetate R* previously adjusted to pH 6.0 with *glacial acetic acid R* and dilute to 50.0 mL with the same solution.

*Reference solution (b).* Dissolve 10 mg of *amoxicillin trihydrate CRS* in 10 mL of reference solution (a).

*Column*:

– *size*: *l* = 0.3 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (10 µm).

*Mobile phase*: mix 5 volumes of *methanol R1* and 95 volumes of a 15 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 4.0 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to clavulanate (1st peak) and amoxicillin (2nd peak).

1 mg of $C_8H_9NO_5$ is equivalent to 1.191 mg of $C_8H_8KNO_5$.

STORAGE

In an airtight container.

LABELLING

The label states the percentage content of potassium clavulanate and the diluent used to prepare the mixture.

IMPURITIES

*Specified impurities: E, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F.*

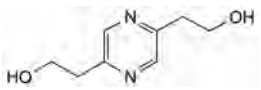

A. 2,2′-(pyrazine-2,5-diyl)diethanol,

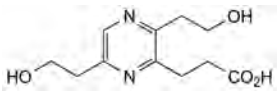

B. 3-[3,6-bis(2-hydroxyethyl)pyrazin-2-yl]propanoic acid,

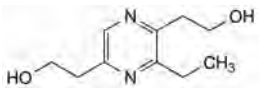

C. 2,2′-(3-ethylpyrazine-2,5-diyl)diethanol,

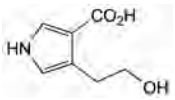

D. 4-(2-hydroxyethyl)-1*H*-pyrrole-3-carboxylic acid,

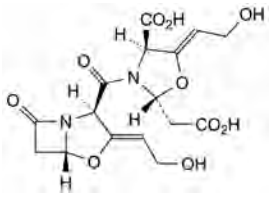

E. (2*R*,4*R*,5*Z*)-2-(carboxymethyl)-5-(2-hydroxyethylidene)-3-[[(2*R*,3*Z*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]oxazolidine-4-carboxylic acid,

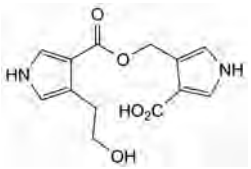

F. 4-[[[[4-(2-hydroxyethyl)-1*H*-pyrrol-3-yl]carbonyl]oxy]-methyl]-1*H*-pyrrole-3-carboxylic acid,

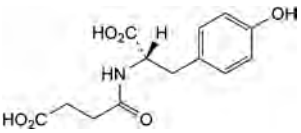

G. 4-[[(1*S*)-1-carboxy-2-(4-hydroxyphenyl)ethyl]amino]-4-oxobutanoic acid (*N*-(hydrogensuccinyl)tyrosine).

**01/2017:0920**
**corrected 10.0**

# POTASSIUM DIHYDROGEN PHOSPHATE

## Kalii dihydrogenophosphas

$KH_2PO_4$ $M_r$ 136.1
[7778-77-0]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly acid (*2.2.4*).

B. Solution S gives reaction (b) of phosphates (*2.3.1*).

C. 0.5 mL of solution S gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.2 to 4.5.

To 5 mL of solution S add 5 mL of *carbon dioxide-free water R*.

**Reducing substances**. To 5 mL of solution S add 5 mL of *dilute sulfuric acid R* and 0.25 mL of *0.02 M potassium permanganate*. Heat on a water-bath for 5 min. The colour of the permanganate is not completely discharged.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 5 mL of solution S add 0.5 mL of *hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Sodium**: maximum 0.1 per cent, if intended for use in the manufacture of parenteral preparations.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using the following solution, diluted as necessary with *water R*: dissolve 0.5084 g of *sodium chloride R*, previously dried at 100-105 °C for 3 h, in *water R* and dilute to 1000.0 mL with the same solvent (200 µg of Na per millilitre).

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 125-130 °C.

ASSAY

Dissolve 1.000 g in 50 mL of *carbon dioxide-free water R*. Titrate with carbonate-free *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 0.1361 g of $KH_2PO_4$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.



**01/2017:2076**

# POTASSIUM HYDROGEN ASPARTATE HEMIHYDRATE

## Kalii hydrogenoaspartas hemihydricus

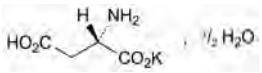


$C_4H_6KNO_4,½H_2O$ $M_r$ 180.2

DEFINITION

Potassium hydrogen (2*S*)-2-aminobutanedioate hemihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, powder or crystalline powder, or colourless crystals.

*Solubility*: very soluble in water, practically insoluble in alcohol and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Examine the chromatograms obtained in the test for ninhydrin-positive substances.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. It gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 7.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 18.0 to + 20.5 (anhydrous substance).

Dissolve 0.50 g in a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 25.0 mL with the same mixture of solvents.

**Ninhydrin-positive substances**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Solution S.

*Test solution (b).* Dilute 1.0 mL of solution S to 10.0 mL with *water R*.

*Reference solution (a).* Dissolve 25 mg of *potassium hydrogen aspartate hemihydrate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (b) to 20.0 mL with *water R*.

*Reference solution (c).* Dissolve 10 mg of *glutamic acid CRS* and 10 mg of the substance to be examined in *water R* and dilute to 25 mL with the same solvent.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated principal spots.

*Limits*: test solution (a):

– *any impurity*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 10 mL of solution S add 5 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 12 mL of solution S add 3 mL of *distilled water R*.

**Ammonium** (*2.4.1, Method B*): maximum 200 ppm, determined on 50 mg.

Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Iron** (*2.4.9*): maximum 30 ppm.

In a separating funnel, dissolve 0.33 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. The aqueous layer complies with the limit test for iron.

**Water** (*2.5.12*): 4.0 per cent to 6.0 per cent, determined on 0.200 g.

Dissolve the substance to be examined in 10 mL of *formamide R1* and add 10 mL of *anhydrous methanol R*.

ASSAY

Dissolve 70.0 mg in 5 mL of *anhydrous formic acid R*, add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 8.56 mg of $C_4H_6KNO_4$.

01/2017:1141

# POTASSIUM HYDROGEN CARBONATE

## Kalii hydrogenocarbonas

$KHCO_3$ $M_r$ 100.1
[298-14-6]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

When heated in the dry state or in solution, it is gradually converted to potassium carbonate.

IDENTIFICATION

A. To 5 mL of solution S (see Tests) add 0.1 mL of *phenolphthalein solution R*. A pale pink colour is produced. Heat; gas is evolved and the colour becomes red.

B. It gives the reaction of carbonates and bicarbonates (*2.3.1*).

C. 1 mL of solution S gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 90 mL of *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Carbonates**. The pH (*2.2.3*) of freshly prepared solution S is not greater than 8.6.

**Chlorides** (*2.4.4*): maximum 150 ppm.

Dilute 7 mL of solution S to 15 mL with *dilute nitric acid R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *acetic acid R*. Prepare the standard using a mixture of 7.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 7.5 mL of *distilled water R*.

**Ammonium** (*2.4.1*): maximum 20 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Calcium** (*2.4.3*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *acetic acid R*. Prepare the standard using a mixture of 5 mL of *calcium standard solution (10 ppm Ca) R* and 10 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm, determined on solution S.

**Sodium**: maximum 0.5 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution*. Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

ASSAY

Dissolve 0.800 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection, or at the point of inflection if only 1 point is detected.

1 mL of *1 M hydrochloric acid* is equivalent to 0.1001 g of $KHCO_3$.

01/2017:1984

# POTASSIUM HYDROGEN TARTRATE

## Kalii hydrogenotartras

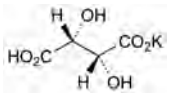


$C_4H_5KO_6$ $M_r$ 188.2
[868-14-4]

DEFINITION

Potassium hydrogen (2*R*,3*R*)-2,3-dihydroxybutane-1,4-dioate.

*Content*: 99.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Suspend 0.5 g in 50 mL of *water R* and boil until dissolution is complete. Allow to cool (solution A). To 5 mL of solution A, add 0.1 mL of *methyl red solution R*. The solution is red.

C. Solution A gives reaction (a) of tartrates (*2.3.1*).

D. Solution A gives reaction (b) of potassium (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 8.0 to + 9.2 (dried substance).

Dissolve 2.50 g in 20 mL of *1 M hydrochloric acid* with heating. Allow to cool. Dilute to 25.0 mL with *water R*.

**Oxalic acid**: maximum 500 ppm.

Dissolve 0.43 g in 4 mL of *water R*. Add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules and boil for 1 min. Allow to stand for 2 min. Collect the liquid in a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of a 50 g/L solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 1 mL of *water R* and 3 mL of a 0.1 g/L solution of *oxalic acid R*.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dissolve 1.0 g with heating in a mixture of 3 mL of *dilute nitric acid R* and 50 mL of *water R*. Dilute to 100 mL with *water R*. Dilute 10 mL of the solution to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Suspend 0.30 g in 3.0 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*. Heat until dissolution is complete.

**Barium**. Suspend 0.50 g in a mixture of 1.5 mL of *dilute hydrochloric acid R* and 8.5 mL of *water R*. Heat until dissolution is complete. Allow to cool. Add 1 mL of *dilute sulfuric acid R*. The solution remains clear (*2.2.1*) on standing for 15 min.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.170 g in 100 mL of *water R* at 100 °C. Titrate the hot solution with *0.1 M sodium hydroxide*, using 0.3 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.82 mg of $C_4H_5KO_6$.

**01/2017:0840**
**corrected 9.4**

# POTASSIUM HYDROXIDE

## Kalii hydroxidum

KOH $M_r$ 56.11
[1310-58-3]

DEFINITION

*Content*: 85.0 per cent to 100.5 per cent of total alkali, calculated as KOH.

CHARACTERS

*Appearance*: white or almost white, crystalline, hard masses, supplied as sticks, pellets or irregularly shaped pieces, deliquescent, absorbing carbon dioxide.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. pH (*2.2.3*): minimum 10.5.
Dissolve 0.1 g in 10 mL of *water R* (solution A used for identification test B). Dilute 1 mL of this solution to 100 mL with *water R*.

B. 1 mL of solution A prepared in identification test A gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S1**. Dissolve 2.5 g in 10 mL of *water R*. Carefully add 2 mL of *nitric acid R* while cooling, and dilute to 25 mL with *dilute nitric acid R*.

**Solution S2**. Dissolve 10 g in 15 mL of *distilled water R*. Carefully add 12 mL of *hydrochloric acid R* while cooling, and dilute to 50 mL with *dilute hydrochloric acid R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Carbonates**: maximum 2.0 per cent, calculated as $K_2CO_3$ as determined in the assay.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S1 to 15 mL with *water R*.

**Phosphates** (*2.4.11*): maximum 100 ppm.

Dilute 1 mL of solution S1 to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 5 mL of solution S2 to 20 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 0.2 ppm, if intended for use in the manufacture of haemodialysis solutions.

*Prescribed solution*. Dissolve 20 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Iron** (*2.4.9*): maximum 10 ppm.

Dilute 5 mL of solution S2 to 10 mL with *water R*.

**Sodium**: maximum 1.0 per cent.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Dissolve 1.00 g in 50 mL of *water R*, add 5 mL of *sulfuric acid R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluting with *water R*.

*Source*: sodium hollow-cathode lamp.

*Wavelength*: 589 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Dissolve 2.000 g in 25 mL of *carbon dioxide-free water R*. Add 25 mL of freshly prepared *barium chloride solution R1* and 0.3 mL of *phenolphthalein solution R*. Add slowly, while shaking, 25.0 mL of *1 M hydrochloric acid* and continue the titration with *1 M hydrochloric acid* until the colour changes from pink to colourless. Add 0.3 mL of *bromophenol blue solution R* and continue the titration with *1 M hydrochloric acid* until the colour changes from violet-blue to yellow.

1 mL of *1 M hydrochloric acid* used in the 2nd part of the titration is equivalent to 69.11 mg of $K_2CO_3$.

1 mL of *1 M hydrochloric acid* used in the combined titrations is equivalent to 56.11 mg of total alkali, calculated as KOH.

STORAGE

In an airtight, non-metallic container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of haemodialysis solutions.

**01/2017:0186**

# POTASSIUM IODIDE

## Kalii iodidum

KI 166.0
[7681-11-0]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in glycerol, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of iodides (*2.3.1*).

B. Solution S gives the reactions of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Alkalinity**. To 12.5 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Iodates**. To 10 mL of solution S add 0.25 mL of *iodide-free starch solution R* and 0.2 mL of *dilute sulfuric acid R* and allow to stand protected from light for 2 min. No blue colour develops.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Thiosulfates**. To 10 mL of solution S add 0.1 mL of *starch solution R* and 0.1 mL of *0.005 M iodine*. A blue colour is produced.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.00 g of previously powdered substance by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 1.500 g in *water R* and dilute to 100.0 mL with the same solvent. To 20.0 mL of the solution add 40 mL of *hydrochloric acid R* and titrate with *0.05 M potassium iodate* until the colour changes from red to yellow. Add 5 mL of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer is decolourised.

1 mL of *0.05 M potassium iodate* is equivalent to 16.60 mg of KI.

STORAGE

Protected from light.

**01/2017:2075**

# POTASSIUM METABISULFITE

## Kalii metabisulfis

$K_2S_2O_5$ $M_r$ 222.3
[16731-55-8]

DEFINITION

Potassium metabisulfite (potassium disulfite).

*Content*: 95.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white powder, or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. pH (see Tests).

B. To 5 mL of solution S (see Tests), add 0.5 mL of *0.05 M iodine*. The mixture is colourless and gives reaction (a) of sulfates (*2.3.1*).

C. Solution S gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**pH** (*2.2.3*): 3.0 to 4.5 for solution S.

**Thiosulfates**. To 2.00 g add 25 mL of a 42.5 g/L solution of *sodium hydroxide R* and 75 mL of *water R*. Shake until dissolved and add 10 mL of *formaldehyde R* and 10 mL of *acetic acid R*. After 5 min, titrate with *0.05 M iodine* using 1 mL of *starch solution R*. Carry out a blank titration. The difference between the volumes consumed in the 2 titrations is not more than 0.15 mL.

**Iron**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dilute 20 mL of solution S to 50 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted as necessary with *water R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Selenium**: maximum 10 ppm.

To 3.0 g add 10 mL of *formaldehyde R*. Carefully add 2 mL of *hydrochloric acid R* in small portions. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a reference solution prepared at the same time in the same manner using 1.0 g of the substance to be examined to which 0.2 mL of *selenium standard solution (100 ppm Se) R* has been added.

**Zinc**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. Dilute 20 mL of solution S to 50 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluted as necessary with *water R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

In a 500 mL conical flask containing 50.0 mL of *0.05 M iodine* introduce 0.150 g and add 5 mL of *hydrochloric acid R*. Titrate the excess of iodine with *0.1 M sodium thiosulfate* using 0.1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.05 M iodine* is equivalent to 5.558 mg of $K_2S_2O_5$.

STORAGE

In an airtight container, protected from light.

**01/2017:1465**

# POTASSIUM NITRATE

## Kalii nitras

$KNO_3$ $M_r$ 101.1
[7757-79-1]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, very soluble in boiling water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives the reaction of nitrates (*2.3.1*).

B. Solution S (see Tests) gives the reactions of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Reducible substances**. To 10 mL of solution S, add 0.5 mL of *dilute sulfuric acid R* and 2 mL of *zinc iodide and starch solution R*. The solution does not become blue within 2 min.

**Chlorides** (*2.4.4*): maximum 20 ppm, if intended for ophthalmic use.

Dissolve 2.5 g in *water R* and dilute to 15 mL with the same solvent.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium** (*2.4.1*): maximum 100 ppm, determined in 1 mL of solution S; maximum 50 ppm if intended for ophthalmic use.

**Calcium** (*2.4.3*): maximum 100 ppm; maximum 50 ppm if intended for ophthalmic use.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm; maximum 10 ppm if intended for ophthalmic use.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Sodium**: maximum 0.1 per cent.

Atomic emission spectrometry (*2.2.22, Method II*).

*Test solution*. Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluting with *water R*.

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Prepare a chromatography column 0.3 m long and 10 mm in internal diameter and filled with 10 g of *strongly acidic ion-exchange resin R* covered with *carbon dioxide-free water R*. Maintain a 1 cm layer of liquid above the resin at all times. Allow 100 mL of *dilute hydrochloric acid R* to run through the column at a flow rate of about 5 mL/min. Wash the column (with the tap completely open) with *carbon dioxide-free water R* until neutral to *blue litmus paper R*. Dissolve 0.200 g of the substance to be examined in 2 mL of *carbon dioxide-free water R* in a beaker and transfer it to the column reservoir, allow the solution to run through the column at a flow rate of about 3 mL/min and collect the eluate. Wash the beaker with 10 mL of *carbon dioxide-free water R* and transfer this solution at the same flow rate to the column before it runs dry. Finally wash the column with 200 mL of *carbon dioxide-free water R* (with the tap completely open) until neutral to *blue litmus paper R*. Titrate the combined eluate and washings with *0.1 M sodium hydroxide*, using 1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 10.11 mg of $KNO_3$.

LABELLING

The label states, where applicable, that the substance is suitable for ophthalmic use.

**01/2017:1987**

# POTASSIUM PERCHLORATE

## Kalii perchloras

$KClO_4$ $M_r$ 138.6
[7778-74-7]

DEFINITION

*Content*: 99.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 0.1 g in 5 mL of *water R*. Add 5 mL of *indigo carmine solution R* and heat to boiling. The colour of the solution does not disappear.

B. Chlorates and chlorides (see Tests).

C. Heat 10 mg over a flame for 2 min. Dissolve the residue in 2 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

D. Dissolve 50 mg with heating in 5 mL of *water R*. Allow to cool to room temperature. The solution gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Suspend 5.0 g in 90 mL of *distilled water R* and heat to boiling. Allow to cool. Filter. Dilute the filtrate to 100 mL with *carbon dioxide-free water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.20 g in *water R* and dilute to 20 mL with the same solvent.

**Acidity or alkalinity**. To 5 mL of solution S add 5 mL of *water R* and 0.1 mL of *phenolphthalein solution R*. Not more than 0.25 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator. To 5 mL of solution S, add 5 mL of *water R* and 0.1 mL of *bromocresol green solution R*. Not more than 0.25 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Chlorates and chlorides** (*2.4.4*): maximum 100 ppm (calculated as chlorides).

To 5 mL of solution S, add 5 mL of *water R* and heat to boiling. Add 1 mL of *nitric acid R* and 0.1 g of *sodium nitrite R*. Allow to cool to room temperature. Dilute to 15 mL with *water R*. The solution complies with the limit test for chlorides. Prepare the standard using 5 mL of *chloride standard solution (5 ppm Cl) R* and 10 mL of *water R*, and adding only 1 mL of *dilute nitric acid R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

Prepare the standard using a mixture of 7.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 7.5 mL of *water R*.

**Calcium** (*2.4.3*): maximum 100 ppm, determined on solution S.

Prepare the standard using a mixture of 7.5 mL of *calcium standard solution (10 ppm Ca) R*, 1 mL of *dilute acetic acid R* and 7.5 mL of *distilled water R*.

ASSAY

Prepare a chromatography column 0.3 m long and 10 mm in internal diameter and filled with 10 g of *strongly acidic ion-exchange resin R* covered with *carbon dioxide-free water R*. Maintain a 1 cm layer of liquid above the resin throughout the determination. Allow 100 mL of *dilute hydrochloric acid R* to run through the column at a flow rate of about 5 mL/min. Wash the column (with the tap completely open) with *carbon dioxide-free water R* until the eluate is neutral to *blue litmus paper R*. Dissolve 0.100 g of the substance to be examined in 10 mL of *carbon dioxide-free water R* in a beaker and transfer it to the column reservoir, allow the solution to run through the column at a flow rate of about 3 mL/min and collect the eluate. Wash the beaker 3 times with 10 mL of *carbon dioxide-free water R* and transfer this solution at the same flow rate to the column before it runs dry. Finally, wash the column with 200 mL of *carbon dioxide-free water R* (with the tap completely open) until the eluate is neutral to *blue litmus paper R*. Titrate the combined eluate and washings with *0.1 M sodium hydroxide*, using 1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 13.86 mg of $KClO_4$.

**01/2008:0121**

# POTASSIUM PERMANGANATE

## Kalii permanganas

$KMnO_4$ $M_r$ 158.0
[7722-64-7]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: dark purple or brownish-black, granular powder or dark purple or almost black crystals, usually having a metallic lustre.

*Solubility*: soluble in cold water, freely soluble in boiling water.

It decomposes on contact with certain organic substances.

IDENTIFICATION

A. Dissolve about 50 mg in 5 mL of *water R* and add 1 mL of *ethanol (96 per cent) R* and 0.3 mL of *dilute sodium hydroxide solution R*. A green colour develops. Heat to boiling. A dark brown precipitate is formed.

B. Filter the mixture obtained in identification test A. The filtrate gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.75 g in 25 mL of *distilled water R*, add 3 mL of *ethanol (96 per cent) R* and boil for 2-3 min. Cool, dilute to 30 mL with *distilled water R* and filter.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Substances insoluble in water**: maximum 1.0 per cent.

Dissolve 0.5 g in 50 mL of *water R* and heat to boiling. Filter through a tared sintered-glass filter (16) (*2.1.2*). Wash with *water R* until the filtrate is colourless and collect the residue on the filter. The residue, dried in an oven at 100-105 °C, weighs a maximum of 5 mg.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 12 mL of solution S to 15 mL with *distilled water R*.

ASSAY

Dissolve 0.300 g in *water R* and dilute to 100.0 mL with the same solvent. To 20.0 mL of the solution add 20 mL of *water R*, 1 g of *potassium iodide R* and 10 mL of *dilute hydrochloric acid R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 3.160 mg of $KMnO_4$.

**01/2017:1986**

# POTASSIUM SODIUM TARTRATE TETRAHYDRATE

## Kalii natrii tartras tetrahydricus

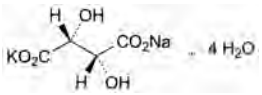


$C_4H_4KNaO_6,4H_2O$ $M_r$ 282.2
[6381-59-5]

DEFINITION

Potassium sodium (+)-(2*R*,3*R*)-2,3-dihydroxybutanedioate tetrahydrate.

*Content*: 98.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless, transparent crystals.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. It gives reaction (b) of tartrates (*2.3.1*).

C. It gives reaction (b) of potassium (*2.3.1*).

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.000 g in *carbon dioxide-free water R*, prepared from *distilled water R*, and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 5 mL of solution S, add 0.1 mL of *phenolphthalein solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Specific optical rotation** (*2.2.7*): + 28.0 to + 30.0 (anhydrous substance), determined on solution S.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 50 ppm.

Dissolve 1.0 g in *distilled water R* and dilute to 15 mL with the same solvent. Prepare the reference solution with a mixture of 5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 10 mL of *distilled water R*.

**Ammonium** (*2.4.1*): maximum 40 ppm, determined on 5 mL of solution S.

**Barium and oxalates**. To 5 mL of solution S, add 3 mL of *calcium sulfate solution R*. Allow to stand for 5 min. Any opalescence in the solution is not more intense than that in a mixture of 3 mL of *calcium sulfate solution R* and 5 mL of *distilled water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): 24.0 per cent to 26.5 per cent, determined on 50.0 mg. Use 50 mL of *anhydrous methanol R*. Titrate slowly.

ASSAY

To 0.100 g of finely powdered substance add 40 mL of *anhydrous acetic acid R* and 20 mL of *acetic anhydride R*. Titrate slowly with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 10.51 mg of $C_4H_4KNaO_6$.

**01/2017:0618**

# POTASSIUM SORBATE

## Kalii sorbas

$C_6H_7KO_2$ $M_r$ 150.2
[590-00-1]

DEFINITION

Potassium (*E,E*)-hexa-2,4-dienoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or granules.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 50.0 mg in *water R* and dilute to 250.0 mL with the same solvent. Dilute 2.0 mL of this solution to 200.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 264 nm.

*Specific absorbance at the absorption maximum*: 1650 to 1900.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *potassium sorbate CRS*.

C. Dissolve 1.0 g in 50 mL of *water R*, add 10 mL of *dilute hydrochloric acid R* and shake. Filter the crystalline precipitate, wash with *water R* and dry *in vacuo* over *sulfuric acid R* for 4 h. The residue obtained melts (*2.2.14*) at 132 °C to 136 °C.

D. Dissolve 0.2 g in 2 mL of *water R* and add 2 mL of *dilute acetic acid R*. Filter. The solution gives reaction (b) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 20 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.25 mL of *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Aldehydes**: maximum 0.15 per cent, expressed as $C_2H_4O$.

Dissolve 1.0 g in a mixture of 30 mL of *water R* and 50 mL of *2-propanol R*, adjust to pH 4 with *1 M hydrochloric acid* and dilute to 100 mL with *water R*. To 10 mL of the solution add 1 mL of *decolorised fuchsin solution R* and allow to stand for 30 min. Any colour in the solution is not more intense than that in a standard prepared at the same time by adding 1 mL of *decolorised fuchsin solution R* to a mixture of 1.5 mL of *acetaldehyde standard solution (100 ppm $C_2H_4O$) R*, 4 mL of *2-propanol R* and 4.5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.120 g in 20 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid* using 0.1 mL of *crystal violet solution R* as indicator until the colour changes from violet to bluish-green.

1 mL of *0.1 M perchloric acid* is equivalent to 15.02 mg of $C_6H_7KO_2$.

STORAGE

Protected from light.

**01/2019:1622**

# POTASSIUM SULFATE

## Kalii sulfas

$K_2SO_4$ $M_r$ 174.3
[7778-80-5]

DEFINITION

*Content*: 98.5 per cent to 101.0 per cent of $K_2SO_4$ (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, practically insoluble in ethanol.

IDENTIFICATION

A. It gives reaction (a) of sulfates (*2.3.1*).

B. It gives reaction (a) of potassium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in 90 mL of *carbon dioxide-free water R* prepared from *distilled water R*, heating gently. Allow to cool and dilute to 100 mL with *carbon dioxide-free water R* prepared from *distilled water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 40 ppm.

Dilute 12.5 mL of solution S to 15 mL with *water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on 10 mL of solution S.

**Magnesium**: maximum 20 ppm.

To 5 mL of solution S add 5 mL of *water R*, 1 mL of *glycerol (85 per cent) R*, 0.15 mL of *titan yellow solution R*, 0.25 mL of *ammonium oxalate solution R* and 5 mL of *dilute sodium hydroxide solution R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time and in the same manner using a mixture of 1 mL of *magnesium standard solution (10 ppm Mg) R* and 9 mL of *water R*.

**Sodium**: maximum 0.10 per cent.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions.* Dissolve in *water R* 0.50 g of *sodium chloride R*, previously dried at 100-105 °C for 3 h, and dilute to 1000.0 mL with the same solvent (200 µg of Na per millilitre). Dilute as required.

*Wavelength*: 589 nm.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 4 h.

ASSAY

Pack 20 g of *strongly acidic ion-exchange resin R* into a glass column, at least 20 cm long and 20 mm in internal diameter, and cover it with *water R*. After 5 min, wash the resin with *water R* until the pH of the eluate is about 6 to 7 using a *pH indicator strip R*. Keep the resin covered with *water R*. Dissolve 0.600 g of the substance to be examined in 10 mL of *water R* in a beaker. Load the solution onto the column and pass through the resin at a flow rate of about 4 mL/min until the resin is just covered with the solution. Using about 200 mL of *water R*, rinse the beaker and pass the rinsings through the column at the same flow rate. Check that the pH of the last eluate is about 6 to 7 using a *pH indicator strip R*.

Titrate the eluate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 87.15 mg of $K_2SO_4$.

**01/2014:0355**

# POTATO STARCH(2)

## Solani amylum

DEFINITION

Potato starch is obtained from the tuber of *Solanum tuberosum* L.

♦CHARACTERS

*Appearance*: very fine, white or almost white powder which creaks when pressed between the fingers.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

Potato starch does not contain starch grains of any other origin. It may contain a minute quantity, if any, of tissue fragments of the original plant.♦

IDENTIFICATION

A. Microscopic examination (*2.8.23*) using a 50 per cent *V/V* solution of *glycerol R*. It presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30-100 µm in size but occasionally exceeding 100 µm, or rounded, 10-35 µm in size. There are occasional compound granules having 2-4 components (Figure 0355.-1). The ovoid and pear-shaped granules have an eccentric hilum and the rounded granules acentric or slightly eccentric hilum. All granules show clearly visible concentric striations. Between orthogonally orientated polarising plates or prisms, the granules show a distinct black cross intersecting at the hilum.

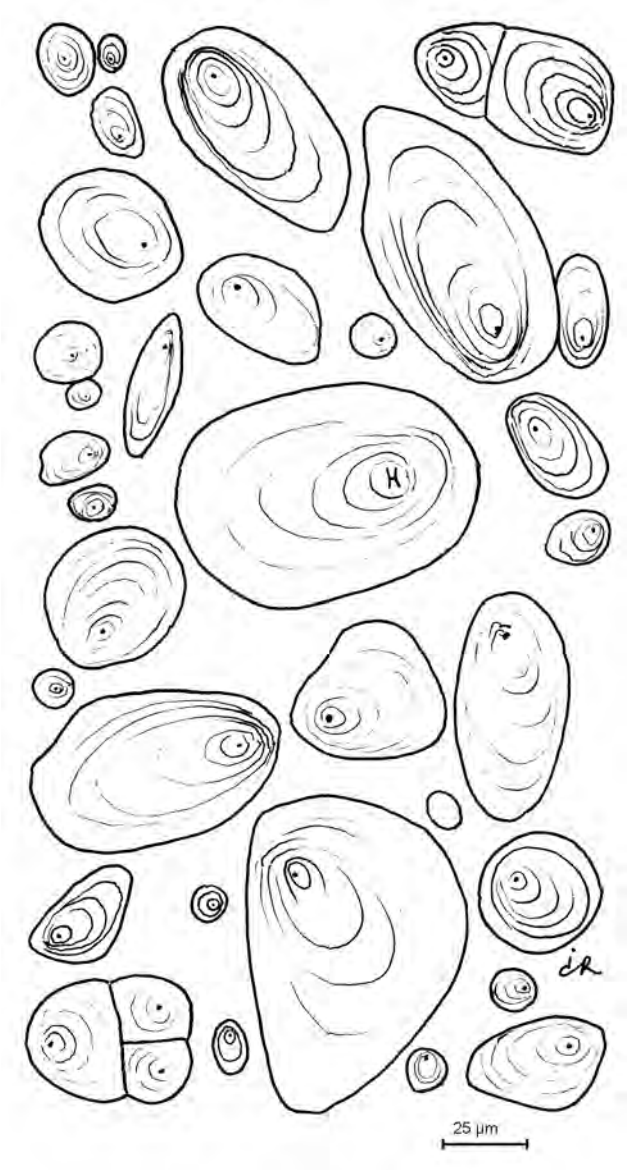


Figure 0355.-1. – *Illustration for identification test A of potato starch*

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A thick, opalescent mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B, add 0.05 mL of *iodine solution R1*. An orange-red to dark blue colour is produced which disappears on heating.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

Shake 5.0 g with 25.0 mL of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

◊**Foreign matter**. Examined under a microscope using a 50 per cent *V/V* solution of *glycerol R*, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.◊

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 10 ppm.

Shake 1.5 g with 15 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the limit test for iron.

**Loss on drying** (*2.2.32*): maximum 20.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

◊Absence of *Salmonella* (*2.6.13*).◊

**07/2017:0685**

# POVIDONE(3)

## Povidonum

◊ 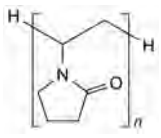 ◊

◊$C_{6n}H_{9n+2}N_nO_n$◊
[9003-39-8]

DEFINITION

α-Hydro-ω-hydropoly[1-(2-oxopyrrolidin-1-yl)ethylene]. It consists of linear polymers of 1-ethenylpyrrolidin-2-one.

*Content*: 11.5 per cent to 12.8 per cent of nitrogen (N; $A_r$ 14.01) (anhydrous substance).

The different types of povidone are characterised by their viscosity in solution expressed as a nominal *K*-value. The nominal *K*-value is 10 to 120.

♦CHARACTERS

*Appearance*: white or yellowish-white, hygroscopic powder or flakes.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methanol, very slightly soluble in acetone.♦

IDENTIFICATION

*First identification: A, D.*

◊*Second identification: B, C, D.*◊

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: dry the substances beforehand at 105 °C for 6 h.
*Comparison*: *povidone CRS*.

◊B. To 1 mL of solution S1 (see Tests) add 0.2 mL of *dimethylaminobenzaldehyde solution R1* and 0.1 mL of *sulfuric acid R*. A pink colour is produced.

C. To 0.1 mL of solution S1 add 5 mL of *water R* and 0.2 mL of *0.05 M iodine*. A red colour is produced.◊

D. To 0.5 g add 10 mL of *water R* and shake. The substance dissolves.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent. Add the substance to be examined to the water in small portions, stirring using a magnetic stirrer.

**Solution S1**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent. Add the substance to be examined to the water in small portions, stirring using a magnetic stirrer.

♦**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$, $BY_6$ or $R_6$ (*2.2.2, Method II*).♦

**pH** (*2.2.3*): 3.0 to 5.0 for solution S, for povidone having a nominal *K*-value of not more than 30; 4.0 to 7.0 for solution S, for povidone having a nominal *K*-value of more than 30.

**Viscosity, expressed as *K*-value**. For povidone having a nominal value of 18 or less, use a 50.0 g/L solution. For povidone having a nominal value of more than 18 and not more than 95, use a 10.0 g/L solution. For povidone having a nominal value of more than 95, use a 1.0 g/L solution. Allow to stand for 1 h and determine the viscosity (*2.2.9*) of the solution at 25 °C, ◊using a size no. 1 viscometer with a minimum flow time of 100 s◊. Calculate the *K*-value using the following expression:

$$\frac{1.5 \log \nu_{rel} - 1}{0.15 + 0.003c} + \frac{\sqrt{300c \log \nu_{rel} + (c + 1.5c \log \nu_{rel})^2}}{0.15c + 0.003c^2}$$

$c$ = concentration of the substance to be examined (anhydrous substance), in grams per 100 mL;

$\nu_{rel}$ = kinematic viscosity of the solution relative to that of *water R*.

The *K*-value of povidone having a nominal *K*-value of 15 or less is 85.0 per cent to 115.0 per cent of the nominal value.

The *K*-value of povidone having a nominal *K*-value of more than 15 is 90.0 per cent to 108.0 per cent of the nominal value.

**Aldehydes**: maximum 500 ppm, expressed as acetaldehyde.

*Test solution*. Dissolve a quantity of the substance to be examined equivalent to 1.0 g of anhydrous substance in *phosphate buffer solution pH 9.0 R* and dilute to 100.0 mL with the same solvent. Stopper the flask tightly and heat at 60 °C for 1 h. Allow to cool to room temperature.

*Reference solution*. Dissolve 0.140 g of *acetaldehyde ammonia trimer trihydrate R* in *water R* and dilute to 200.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *phosphate buffer solution pH 9.0 R*.

Into 3 identical spectrophotometric cells with a path length of 1 cm, introduce separately 0.5 mL of the test solution, 0.5 mL of the reference solution and 0.5 mL of *water R* (blank). To each cell add 2.5 mL of *phosphate buffer solution pH 9.0 R* and 0.2 mL of *nicotinamide-adenine dinucleotide solution R*. Mix and stopper tightly. Allow to stand at 22 ± 2 °C for 2-3 min and measure the absorbance (*2.2.25*) of each solution at 340 nm, using *water R* as the compensation liquid. To each cell add 0.05 mL of *aldehyde dehydrogenase solution R*, mix and stopper tightly. Allow to stand at 22 ± 2 °C for 5 min. Measure the absorbance of each solution at 340 nm using *water R* as the compensation liquid.

Calculate the content of aldehydes using the following expression:

$$\frac{(A_{t2} - A_{t1}) - (A_{b2} - A_{b1})}{(A_{s2} - A_{s1}) - (A_{b2} - A_{b1})} \times \frac{100\,000 \times C}{m}$$

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

$A_{t1}$ = absorbance of the test solution before the addition of aldehyde dehydrogenase;

$A_{t2}$ = absorbance of the test solution after the addition of aldehyde dehydrogenase;

$A_{s1}$ = absorbance of the reference solution before the addition of aldehyde dehydrogenase;

$A_{s2}$ = absorbance of the reference solution after the addition of aldehyde dehydrogenase;

$A_{b1}$ = absorbance of the blank before the addition of aldehyde dehydrogenase;

$A_{b2}$ = absorbance of the blank after the addition of aldehyde dehydrogenase;

$m$ = mass of the substance to be examined (anhydrous substance) in the test solution, in grams;

$C$ = concentration of acetaldehyde in the reference solution, calculated from the mass of *acetaldehyde ammonia trimer trihydrate R* with the factor 0.72, in milligrams per millilitre.

**Peroxides**: maximum 400 ppm, expressed as $H_2O_2$.

Dissolve a quantity of the substance to be examined equivalent to 4.0 g of the anhydrous substance in *water R* and dilute to 100.0 mL with the same solvent (stock solution). To 25.0 mL of the stock solution add 2.0 mL of *titanium trichloride-sulfuric acid reagent R*. Allow to stand for 30 min. The absorbance (*2.2.25*) of the solution, measured at 405 nm using a mixture of 25.0 mL of the stock solution and 2.0 mL of a 13 per cent *V/V* solution of *sulfuric acid R* as the compensation liquid, is not greater than 0.35.

**Formic acid**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve a quantity of the substance to be examined equivalent to 2.0 g of the anhydrous substance in *water R* and dilute to 100.0 mL with the same solvent (stock solution). Transfer a suspension of *strongly acidic ion-exchange resin R* for column chromatography in *water R* to a column of about 0.8 cm in internal diameter to give a packing of about 20 mm in length and keep the strongly acidic ion-exchange resin layer constantly immersed in *water R*. Pour 5 mL of *water R* and adjust the flow rate to about 1 mL/min. When the level of the water comes down to near the top of the strongly acidic ion-exchange resin layer, introduce the stock solution into the column. Discard the first 2 mL of the eluate, then collect 1.5 mL of the solution and use this solution as the test solution.

*Reference solution.* Dissolve 0.100 g of *anhydrous formic acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;

– *stationary phase*: *strongly acidic ion-exchange resin R* for column chromatography (9 µm);

– *temperature*: 35 °C.

*Mobile phase*: dilute 1 mL of *perchloric acid R* to 700 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Retention time*: formic acid = about 8 min.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections;

– *number of theoretical plates*: minimum 1000;

– *symmetry factor*: 0.5 to 1.5.

Calculate the percentage content of formic acid using the following expression:

$$\frac{A_1}{A_2} \times \frac{M}{m}$$

$A_1$ = area of the peak due to formic acid in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to formic acid in the chromatogram obtained with the reference solution;

$m$ = mass of the substance to be examined (anhydrous substance) in the test solution, in grams;

$M$ = mass of *anhydrous formic acid R* in the reference solution, in grams.

*Limit*:

– *formic acid*: maximum 0.5 per cent.

**Hydrazine**. Thin-layer chromatography (*2.2.27*). *Use freshly prepared solutions.*

*Test solution.* Dissolve a quantity of the substance to be examined equivalent to 2.5 g of the anhydrous substance in 25 mL of *water R*. Add 0.5 mL of a 50 g/L solution of *salicylaldehyde R* in *methanol R*, mix and heat in a water-bath at 60 °C for 15 min. Allow to cool, add 2.0 mL of *toluene R*, shake vigorously for 2 min and centrifuge. Use the upper layer.

*Reference solution.* Dissolve 90 mg of *salicylaldehyde azine R* in *toluene R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *toluene R*.

*Plate*: *TLC silanised silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *methanol R* (1:2 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 365 nm.

*Retardation factor*: salicylaldehyde azine = about 0.3.

*Limit*:

– *hydrazine*: any spot due to salicylaldehyde azine is not more intense than the spot in the chromatogram obtained with the reference solution (1 ppm).

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve a quantity of the substance to be examined equivalent to 0.250 g of the anhydrous substance in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *1-vinylpyrrolidin-2-one R* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *1-vinylpyrrolidin-2-one R* (impurity A) and 0.5 g of *vinyl acetate R* in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Precolumn*:

– *size*: *l* = 0.010 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: *acetonitrile for chromatography R*, *water for chromatography R* (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL; ◊after each injection of the test solution, wait for about 2 min and wash the precolumn by passing the mobile phase through the column backwards for 30 min at the same flow rate as applied in the test.

*Relative retention* with reference to vinyl acetate (retention time = about 14 min): impurity A = about 0.6.◊

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity A and vinyl acetate in the chromatogram obtained with reference solution (b);
- *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections of reference solution (a).

Calculate the content of impurity A in parts per million using the following expression:

$$\frac{A_1}{A_2} \times \frac{2.5}{m}$$

$A_1$ = area of the peak due to impurity A in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to impurity A in the chromatogram obtained with reference solution (a);

$m$ = mass of the substance to be examined (anhydrous substance) in the test solution, in grams.

*Limit*:

- *impurity A*: maximum 10 ppm.

**Impurity B**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve a quantity of the substance to be examined equivalent to 0.500 g of the anhydrous substance in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution.* Dissolve 0.150 g of *2-pyrrolidone R* (impurity B) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of the solution to 100.0 mL with the mobile phase.

*Precolumn*:

- *size*: *l* = 0.010 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *methanol R2*, *water for chromatography R* (5:95 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 50 µL; ◊after each injection of the test solution, wait for about 2 min and wash the precolumn by passing the mobile phase through the column backwards for about 30 min at the same flow rate as applied in the test.◊

*Retention time*: impurity B = about 7 min.

*System suitability*: reference solution:

- *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 6 injections;
- *number of theoretical plates*: minimum 5000.

Calculate the percentage content of impurity B using the following expression:

$$\frac{A_1}{A_2} \times \frac{0.3}{m}$$

$A_1$ = area of the peak due to impurity B in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to impurity B in the chromatogram obtained with the reference solution;

$m$ = mass of the substance to be examined (anhydrous substance) in the test solution, in grams.

*Limit*:

- *impurity B*: maximum 3.0 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Place 0.100 g of the substance to be examined (*m* mg) in a combustion flask and add 5 g of a mixture of 1 g of *copper sulfate pentahydrate R*, 1 g of *titanium dioxide R* and 33 g of *dipotassium sulfate R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with a small quantity of *water R*. Add 7 mL of *sulfuric acid R*, allowing it to run down the insides of the flask. Heat the flask gradually until the solution has a clear, yellowish-green colour, and the inside wall of the flask is free from any carbonised material, and then heat for a further 45 min. After cooling, add cautiously 20 mL of *water R*, and connect the flask to the distillation apparatus, which has been previously washed by passing steam through it. To the absorption flask add 30 mL of a 40 g/L solution of *boric acid R*, 3 drops of *bromocresol green-methyl red solution R* and sufficient water to immerse the lower end of the condenser tube. Add 30 mL of *strong sodium hydroxide solution R* through the funnel, rinse the funnel cautiously with 10 mL of *water R*, immediately close the clamp on the rubber tube, then start distillation with steam to obtain 80-100 mL of distillate. Remove the absorption flask from the lower end of the condenser tube, rinsing the end part with a small quantity of *water R*, and titrate the distillate with *0.025 M sulfuric acid* until the colour of the solution changes from green through pale greyish blue to pale greyish reddish-purple. Carry out a blank determination.

1 mL of *0.025 M sulfuric acid* is equivalent to 0.700 mg of N.

♦STORAGE

In an airtight container.♦

LABELLING

The label states the nominal *K*-value.

IMPURITIES

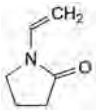


A. 1-ethenylpyrrolidin-2-one (1-vinylpyrrolidin-2-one),

B. pyrrolidin-2-one (2-pyrrolidone).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality*

*of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for povidone used as solubiliser and stabiliser in liquid dosage forms.*

**Viscosity** (*2.2.9*). Determine the dynamic viscosity using a capillary viscometer on a 10 per cent solution (dried substance) at 25 °C. Typical values are shown in Table 0685.-1.

**Molecular mass** (see Viscosity, expressed as *K*-value). Typical values are shown in Table 0685.-1.

*The following characteristic may be relevant for povidone used as binder in tablets and granules.*

**Molecular mass** (see Viscosity, expressed as *K*-value). Typical values are shown in Table 0685.-1.

Table 0685.-1. – *Typical viscosity ranges and ranges for viscosity, expressed as K-value*

| | Viscosity range (mPa·s) | Molecular mass: viscosity, expressed as *K*-value |
|---|---|---|
| Povidone K 12 | 1.3-2.3 | 11-14 |
| Povidone K 17 | 1.5-3.5 | 16-18 |
| Povidone K 25 | 3.5-5.5 | 24-27 |
| Povidone K 30 | 5.5-8.5 | 28-32 |
| Povidone K 90 | 300-700 | 85-95 |

**01/2016:1142**

# POVIDONE, IODINATED

## Povidonum iodinatum

DEFINITION

Complex of iodine and povidone.

*Content*: 9.0 per cent to 12.0 per cent of available iodine (dried substance).

PRODUCTION

It is produced using povidone that complies with the monograph on *Povidone (0685)*, except that the povidone used may contain not more than 2.0 per cent of formic acid and not more than 8.0 per cent of water.

CHARACTERS

*Appearance*: yellowish-brown or reddish-brown, amorphous powder.

*Solubility*: soluble in water and in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *iodinated povidone CRS*.

B. Dissolve 10 mg in 10 mL of *water R* and add 1 mL of *starch solution R*. An intense blue colour is produced.

TESTS

**pH** (*2.2.3*): 1.5 to 5.0.

Dissolve 1.0 g in 10 mL of *carbon dioxide-free water R*.

**Iodide**: maximum 6.0 per cent (dried substance).

Dissolve 0.500 g in 100 mL of *water R*. Add *sodium metabisulfite R* until the colour of the iodine has disappeared. Add 25.0 mL of *0.1 M silver nitrate*, 10 mL of *nitric acid R* and 5 mL of *ferric ammonium sulfate solution R2*. Titrate with *0.1 M ammonium thiocyanate*. Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 12.69 mg of total iodine. From the percentage of total iodine, calculated with reference to the dried substance, subtract the percentage of available iodine as determined in the assay to obtain the percentage of iodide.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Transfer 1.000 g into a ground-glass-stoppered flask containing 150 mL of *water R* and stir for 1 h. Add 0.1 mL of *dilute acetic acid R* and titrate with *0.1 M sodium thiosulfate* using *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 12.69 mg of available iodine.

STORAGE

Protected from light.

**01/2017:2416**
**corrected 10.0**

# PRAMIPEXOLE DIHYDROCHLORIDE MONOHYDRATE

## Pramipexoli dihydrochloridum monohydricum

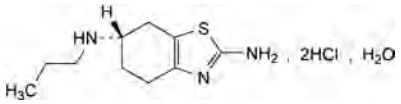


$C_{10}H_{19}Cl_2N_3S,H_2O$ $M_r$ 302.3
[191217-81-9]

DEFINITION

(6*S*)-6-*N*-Propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine dihydrochloride monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in methanol, sparingly soluble or slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

Carry out either tests B, C, D or tests A, B, D.

A. Specific optical rotation (*2.2.7*): – 69.5 to – 67.0 (anhydrous substance).
Dissolve 0.250 g in *methanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pramipexole dihydrochloride monohydrate CRS*.

C. Enantiomeric purity (see Tests).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.1 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 2.8 to 3.4.

Dissolve 0.4 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 5 g of *sodium octanesulfonate monohydrate R* and 9.1 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Solvent mixture*: *acetonitrile R*, buffer solution (20:80 *V/V*).

*Test solution.* Dissolve 75 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 7.5 mg of *pramipexole for system suitability CRS* (containing impurities A, B and C) in 5 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: buffer solution;
- *mobile phase B*: *acetonitrile R*, buffer solution (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 60 → 20 | 40 → 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 264 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *pramipexole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to pramipexole (retention time = about 6 min): impurity A = about 0.7; impurity B = about 1.5; impurity C = about 1.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 6.0 between the peaks due to impurity A and pramipexole.

*Limits*:

- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 6 mg of the substance to be examined in 5 mL of *anhydrous ethanol R* and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *pramipexole impurity D CRS* in the mobile phase and dilute to 10 mL with the mobile phase. To 1 mL of this solution add 1 mL of the test solution and dilute to 20 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R*.

*Mobile phase*: *diethylamine R*, *anhydrous ethanol R*, *hexane R* (0.1:15:85 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 75 µL.

*Run time*: 1.5 times the retention time of pramipexole.

*Relative retention* with reference to pramipexole (retention time = about 11 min): impurity D = about 0.5.

*System suitability*:

- *resolution*: minimum 5.0 between the peaks due to impurity D and pramipexole in the chromatogram obtained with reference solution (a);
- *symmetry factor*: maximum 2.4 for the peak due to pramipexole in the chromatogram obtained with reference solution (b).

*Limit*:

- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Water** (*2.5.12*): 5.0 per cent to 7.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 150 mL of *water R*. Add 10 mL of *dilute nitric acid R2* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 14.213 mg of $C_{10}H_{19}Cl_2N_3S$.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E.*

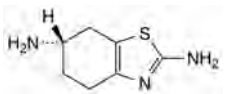

A. (6*S*)-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine,

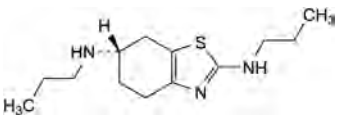

B. (6*S*)-*N*,*N*′-dipropyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine,

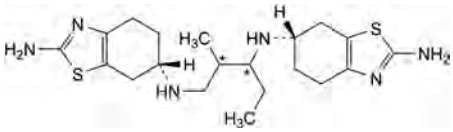

C. mixture of diastereoisomers of (6*S*)-6-*N*-[3-[[(6*S*)-2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl]amino]-1-ethyl-2-methylpropyl]-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine,

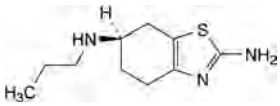

D. (6*R*)-6-*N*-propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine,

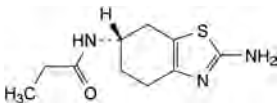

E. *N*-[(6*S*)-2-amino-4,5,6,7-tetrahydro-1,3-benzothiazol-6-yl]propanamide.

**01/2020:3040**

# PRASUGREL HYDROCHLORIDE

## Prasugreli hydrochloridum

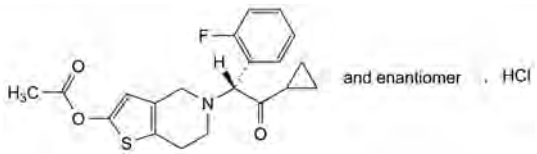


$C_{20}H_{21}ClFNO_3S$ $M_r$ 409.9
[389574-19-0]

DEFINITION

5-[(1*RS*)-2-Cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate hydrochloride.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: slightly soluble in water, freely soluble in methanol, slightly soluble in acetonitrile, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *prasugrel hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (30:70 *V/V*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b).* Dilute 2.0 mL of test solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 25.0 mg of *prasugrel hydrochloride CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 2.0 mL of the solution to 25.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2.5 mg of *prasugrel for system suitability CRS* (containing impurities B and E) in the solvent mixture and dilute to 2 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.8 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 5 °C.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 3.6 times the retention time of prasugrel.

*Identification of impurities*: use the chromatogram supplied with *prasugrel for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B and E.

*Relative retention* with reference to prasugrel (retention time = about 11 min): impurity B = about 1.1; impurity E = about 2.3.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to prasugrel and impurity B.

*Calculation of percentage contents*:
- for each impurity, use the concentration of prasugrel hydrochloride in reference solution (b).

*Limits*:
- *impurity B*: maximum 0.3 per cent;
- *impurity E*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.5 per cent, determined on 0.100 g by direct sample introduction.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 260 nm.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{20}H_{21}ClFNO_3S$ taking into account the assigned content of *prasugrel hydrochloride CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, F, G.*

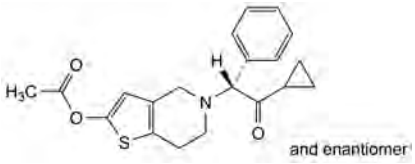


A. 5-[(1*RS*)-2-cyclopropyl-2-oxo-1-phenylethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate,

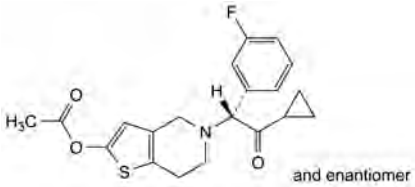


B. 5-[(1*RS*)-2-cyclopropyl-1-(3-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate,

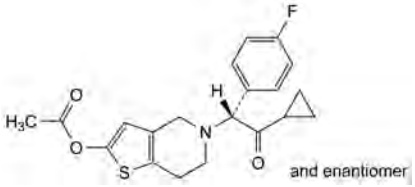


C. 5-[(1*RS*)-2-cyclopropyl-1-(4-fluorophenyl)-2-oxoethyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate,

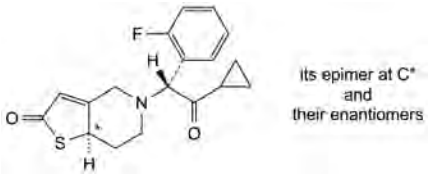


D. mixture of (7a*R*)-5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-5,6,7,7a-tetrahydrothieno[3,2-*c*]pyridin-2(4*H*)-one and (7a*S*)-5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-5,6,7,7a-tetrahydrothieno[3,2-*c*]pyridin-2(4*H*)-one,

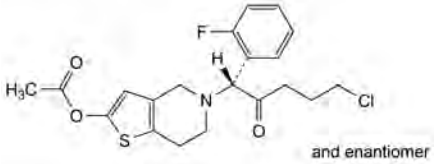


E. 5-[(1*RS*)-5-chloro-1-(2-fluorophenyl)-2-oxopentyl]-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridin-2-yl acetate,

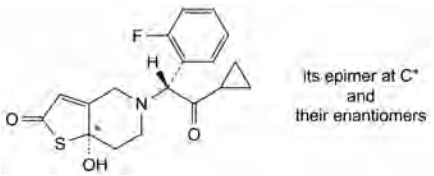


F. mixture of (7a*R*)-5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-7a-hydroxy-5,6,7,7a-tetrahydrothieno[3,2-*c*]pyridin-2(4*H*)-one and (7a*S*)-5-[(1*RS*)-2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-7a-hydroxy-5,6,7,7a-tetrahydrothieno[3,2-*c*]pyridin-2(4*H*)-one,

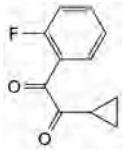

G. 1-cyclopropyl-2-(2-fluorophenyl)ethane-1,2-dione.

**01/2017:2059**

# PRAVASTATIN SODIUM

## Pravastatinum natricum

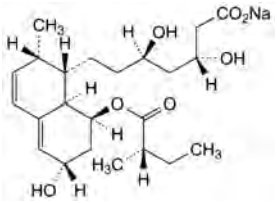

$C_{23}H_{35}NaO_7$ $M_r$ 446.5
[81131-70-6]

DEFINITION

Sodium (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder or crystalline powder, hygroscopic.

*Solubility*: freely soluble in water and in methanol, soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of pravastatin sodium*.

C. 1 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dilute 2.0 mL of solution S to 10.0 mL with *water R*.

**pH** (*2.2.3*): 7.2 to 9.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 153 to + 159 (anhydrous substance).

Dilute 2.0 mL of solution S to 20.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (9:11 *V/V*).

*Test solution (a)*. Dissolve 0.1000 g of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 10.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve the contents of a vial of *pravastatin impurity A CRS* in 1.0 mL of test solution (b).

*Reference solution (b).* Dilute 2.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 12.4 mg of *pravastatin 1,1,3,3-tetramethylbutylamine CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: *glacial acetic acid R, triethylamine R, methanol R, water R* (1:1:450:550 *V/V/V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 238 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 2.5 times the retention time of pravastatin.

*Relative retention* with reference to pravastatin (retention time = about 21 min): impurity F = about 0.1; impurity B = about 0.2; impurity E = about 0.3; impurity G = about 0.4; impurity A = about 0.6; impurity D = about 1.9; impurity C = about 2.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 7.0 between the peaks due to impurity A and pravastatin.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities F, G*: for each impurity, not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Ethanol** (*2.4.24, System A*): maximum 3.0 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{23}H_{35}NaO_7$ using the chromatogram obtained with reference solution (c) and the declared content of pravastatin in *pravastatin 1,1,3,3-tetramethylbutylamine CRS*.

1 mg of pravastatin is equivalent to 1.052 mg of pravastatin sodium.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

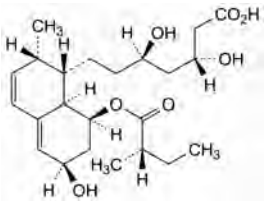

A. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methylbutanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoic acid (6′-epipravastatin),

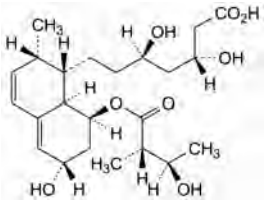

B. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-8-[[(2*S*,3*R*)3-hydroxy-2-methylbutanoyl]oxy]-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoic acid (3″-(*R*)-hydroxypravastatin),

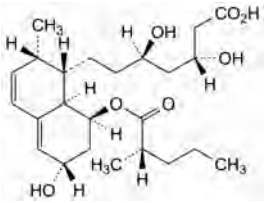

C. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[[(2*S*)-2-methylpentanoyl]oxy]-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoic acid,

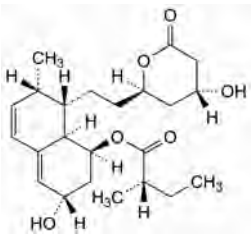

D. (1*S*,3*S*,7*S*,8*S*,8a*R*)-3-hydroxy-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-7-methyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (pravastatin lactone),

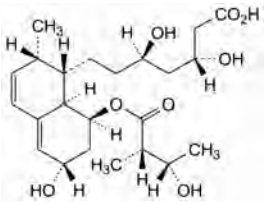

E. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-8-[[(2*S*,3*S*)3-hydroxy-2-methylbutanoyl]oxy]-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]heptanoic acid (3″-(*S*)-hydroxypravastatin),

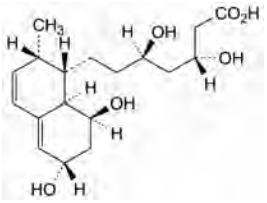

F. (3*R*,5*R*)-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6,8-dihydroxy-2-methyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid,

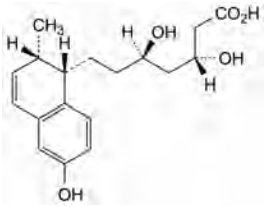

G. (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*)-6-hydroxy-2-methyl-1,2-dihydronaphthalen-1-yl]heptanoic acid.



**01/2017:1466**

# PRAZEPAM

## Prazepamum

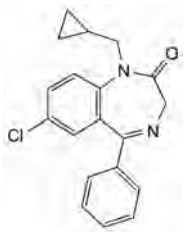

$C_{19}H_{17}ClN_2O$ $M_r$ 324.8
[2955-38-6]

DEFINITION

7-Chloro-1-(cyclopropylmethyl)-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in anhydrous ethanol.

mp: about 145 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prazepam CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water for chromatography R* (50:50 *V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 25 mL of *acetonitrile R1* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (a)*. Dissolve 5 mg of *aminochlorobenzophenone R* (impurity C) in *acetonitrile R1* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (1.8 µm);
- *temperature*: 55 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R1*, *water for chromatography R* (5:95 *V/V*);
- *mobile phase B*: *methanol R1*, *acetonitrile R1* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 55 | 45 |
| 0.5 - 6.5 | 55 → 15 | 45 → 85 |
| 6.5 - 8.5 | 15 | 85 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 2 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to prazepam (retention time = about 3 min): impurity C = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity C and prazepam.

*Calculation of percentage contents*:

- for each impurity, use the concentration of prazepam in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.48 mg of $C_{19}H_{17}ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*, *B*, *C*, *D*.

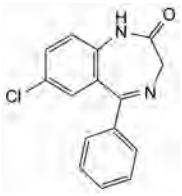

A. 7-chloro-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (nordazepam),

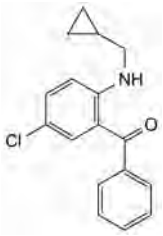

B. [5-chloro-2-[(cyclopropylmethyl)amino]phenyl]phenylmethanone,

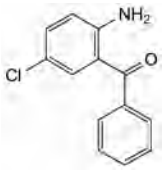

C. (2-amino-5-chlorophenyl)phenylmethanone (aminochlorobenzophenone),

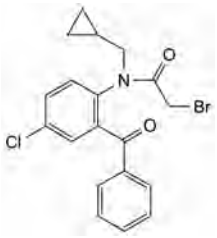

D. *N*-(2-benzoyl-4-chlorophenyl)-2-bromo-*N*-(cyclopropylmethyl)acetamide.

**01/2017:0855**
**corrected 10.0**

# PRAZIQUANTEL

## Praziquantelum

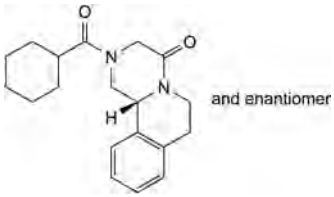


$C_{19}H_{24}N_2O_2$ $M_r$ 312.4
[55268-74-1]

DEFINITION

(11b*RS*)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *praziquantel CRS*.

If the spectra obtained show differences, dissolve 50 mg of the substance to be examined and 50 mg of the reference substance separately in 2 mL of *methanol R*. Evaporate and dry the residue at 60 °C at a pressure not exceeding 0.7 kPa. Record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 40.0 mg of *praziquantel CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 2 mg of *praziquantel for system suitability CRS* (containing impurities A and B) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, *water for chromatography R* (45:55 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Run time*: 4 times the retention time of praziquantel.

*Identification of impurities*: use the chromatogram supplied with *praziquantel for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to praziquantel (retention time = about 10 min): impurity A = about 0.6; impurity B = about 2.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity A and praziquantel.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.4;
- *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C at a pressure not exceeding 0.7 kPa for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{19}H_{24}N_2O_2$ taking into account the assigned content of *praziquantel CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

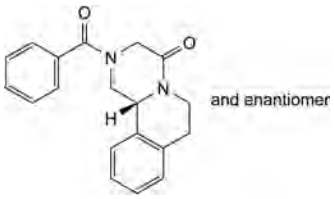


A. (11b*RS*)-2-benzoyl-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one,

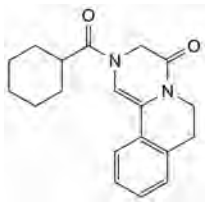

B. 2-(cyclohexylcarbonyl)-2,3,6,7-tetrahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one,

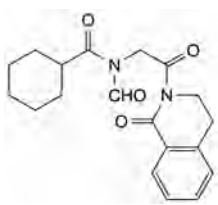

C. *N*-formyl-*N*-[2-oxo-2-(1-oxo-3,4-dihydroisoquinolin-2(1*H*)-yl)ethyl]cyclohexanecarboxamide.

**07/2019:0856**

# PRAZOSIN HYDROCHLORIDE

## Prazosini hydrochloridum

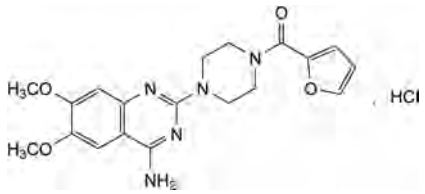


$C_{19}H_{22}ClN_5O_4$ $M_r$ 419.9
[19237-84-4]

DEFINITION

[4-(4-Amino-6,7-dimethoxyquinazolin-2-yl)piperazin-1-yl](furan-2-yl)methanone hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent) and in methanol, practically insoluble in acetone.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 50.0 mg in a 0.1 per cent *V/V* solution of *hydrochloric acid R* in *methanol R* and dilute to 100.0 mL with the same acid solution. Dilute separately 1.0 mL and 5.0 mL of this solution to 100.0 mL with a 0.1 per cent *V/V* solution of *hydrochloric acid R* in *methanol R* (solution A and solution B, respectively). Examined between 220 nm and 280 nm (*2.2.25*), solution A shows an absorption maximum at 247 nm. The specific absorbance at the maximum is 1320 to 1400. Examined between 280 nm and 400 nm, solution B shows 2 absorption maxima, at 330 nm and 343 nm. The specific absorbances at the maxima are 260 to 280 and 240 to 265, respectively.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *prazosin hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *diethylamine R*, 10 volumes of *methanol R* and 10 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Reference solution.* Dissolve 10 mg of *prazosin hydrochloride CRS* in a mixture of 1 volume of *diethylamine R*, 10 volumes of *methanol R* and 10 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Plate*: *TLC silica gel $GF_{254}$ plate R.*
*Mobile phase*: *diethylamine R*, *ethyl acetate R* (5:95 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of warm air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 2 mg in 2 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Use 1.0 mL of this solution to dissolve the contents of a vial of *prazosin impurity A CRS*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 50 volumes of *methanol R* and 50 volumes of a solution containing 3.5 g/L of *sodium pentanesulfonate R* and 3.6 g/L of *tetramethylammonium hydroxide R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of prazosin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to prazosin (retention time = about 8 min): impurity A = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity A and prazosin.

*Calculation of percentage contents*:

- for each impurity, use the concentration of prazosin hydrochloride in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Iron**: maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution*. To 1.0 g add dropwise about 1.5 mL of *nitric acid R*. After fuming has subsided, evaporate on a water-bath and ignite by gradually raising the temperature from 150 °C to 1000 ± 50 °C, maintaining the final temperature for 1 h. Cool, dissolve the residue in 20 mL of *dilute hydrochloric acid R*, evaporate to about 5 mL and dilute to 25.0 mL with *dilute hydrochloric acid R*.

*Reference solutions*. Prepare the reference solutions using *iron standard solution (8 ppm Fe) R*, diluted as necessary with *water R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248 nm.

*Flame*: air-acetylene.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g using a mixture of equal volumes of *methanol R* and *methylene chloride R* as solvent.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout the titration and stop the titration immediately after the end-point has been reached.*

Dissolve 0.350 g in a mixture of 20 mL of *anhydrous formic acid R* and 30 mL of *acetic anhydride R*. Titrate quickly with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.99 mg of $C_{19}H_{22}ClN_5O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

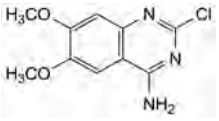

A. 2-chloro-6,7-dimethoxyquinazolin-4-amine,

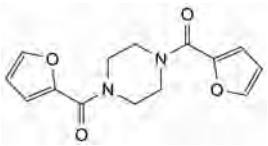

B. (piperazin-1,4-diyl)bis[(furan-2-yl)methanone],

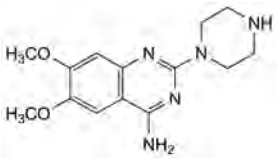

C. 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine,

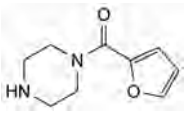

D. (furan-2-yl)(piperazin-1-yl)methanone,

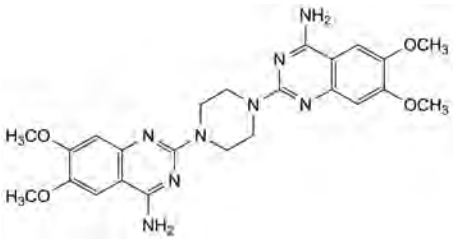

E. 2,2′-(piperazin-1,4-diyl)bis(6,7-dimethoxyquinazolin-4-amine).

**04/2014:1467**

# PREDNICARBATE

## Prednicarbatum

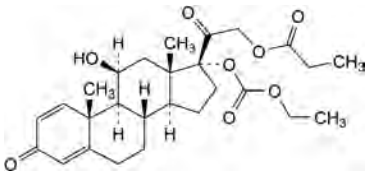

$C_{27}H_{36}O_8$ $M_r$ 488.6
[73771-04-7]

DEFINITION

11β-Hydroxy-3,20-dioxopregna-1,4-diene-17,21-diyl 17-(ethylcarbonate) 21-propanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent), sparingly soluble in propylene glycol.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prednicarbate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *prednicarbate CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *prednisolone acetate CRS* in 5.0 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*; heat at 120 °C for 10 min or until the spots appear and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

TESTS

**Specific optical rotation** (*2.2.7*): + 60 to + 66 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3 mg of *prednicarbate for system suitability A CRS* (containing impurities B, C, D, E and F) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 30.0 mg of *prednicarbate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (50:60 *V/V*).

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 243 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of prednicarbate.

*Identification of impurities*: use the chromatogram supplied with *prednicarbate for system suitability A CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, C, D, E and F.

*Relative retention* with reference to prednicarbate (retention time = about 20 min): impurity B = about 0.25; impurity C = about 0.35; impurity D = about 0.39; impurity E = about 0.6; impurity F = about 1.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to prednicarbate and impurity F; minimum 1.5 between the peaks due to impurities C and D.

*Calculation of percentage contents*:

– for each impurity, use the concentration of prednicarbate in reference solution (b).

*Limits*:

– *impurity F*: maximum 0.8 per cent;
– *impurity C*: maximum 0.5 per cent;
– *impurity E*: maximum 0.3 per cent;
– *impurities B, D*: for each impurity, maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 2.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{27}H_{36}O_8$ taking into account the assigned content of *prednicarbate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, F.*

*Other detectable impurities (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph Substances for pharmaceutical use (2034). It is therefore not necessary to identify these impurities for demonstration of compliance. See also 5.10. Control of impurities in substances for pharmaceutical use): A.*

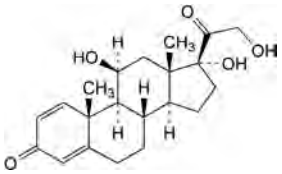

A. 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone),

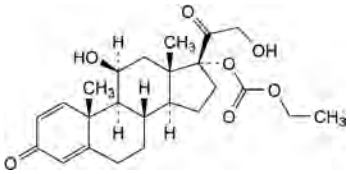

B. 11β,21-dihydroxy-3,20-dioxopregna-1,4-dien-17-yl ethyl carbonate (prednisolone 17-ethylcarbonate),

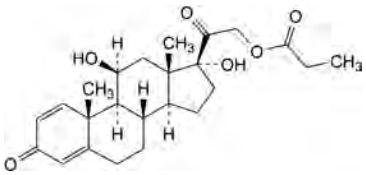

C. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl propanoate (prednisolone 21-propanoate),

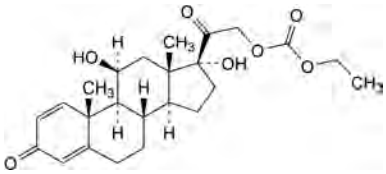

D. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl ethyl carbonate (prednisolone 21-ethylcarbonate),

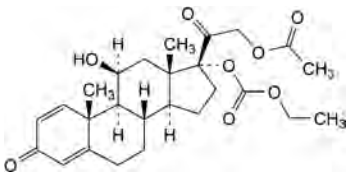

E. 11β-hydroxy-3,20-dioxopregna-1,4-dien-17,21-diyl 21-acetate 17-(ethylcarbonate) (prednisolone 21-acetate 17-ethylcarbonate),

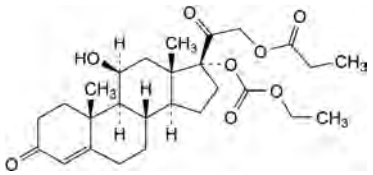

F. 11β-hydroxy-3,20-dioxopregn-4-ene-17,21-diyl 17-(ethylcarbonate) 21-propanoate (1,2-dihydroprednicarbate).

**07/2011:0353**

# PREDNISOLONE

## Prednisolonum

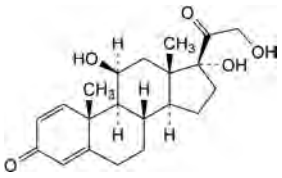

$C_{21}H_{28}O_5$ $M_r$ 360.4
[50-24-8]

DEFINITION

11β,17,21-Trihydroxypregna-1,4-diene-3,20-dione.

*Content*: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, hygroscopic powder.

*Solubility*: very slightly soluble in water, soluble in ethanol (96 per cent) and in methanol, sparingly soluble in acetone, slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prednisolone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**Specific optical rotation** (*2.2.7*): + 113 to + 119 (dried substance).

Dissolve 0.250 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution (a).* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *prednisolone for system suitability CRS* (containing impurities A, B and C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *prednisolone for peak identification CRS* (containing impurities F and J) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *prednisolone CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*, *methanol R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 60 | 40 |
| 14 - 20 | 60 → 20 | 40 → 80 |
| 20 - 25 | 20 | 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *prednisolone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C; use the chromatogram supplied with *prednisolone for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities F and J.

*Relative retention* with reference to prednisolone (retention time = about 12 min): impurity F = about 0.7; impurity B = about 0.9; impurity A = about 1.05; impurity J = about 1.5; impurity C = about 1.7.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to prednisolone.

*Limits*:

- *impurity A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *impurity F*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurities B, C, J*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{21}H_{28}O_5$ from the declared content of *prednisolone CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, F, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, G, H, I.*

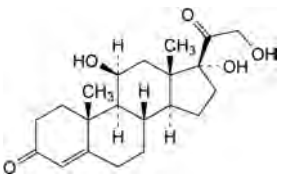

A. 11β,17,21-trihydroxypregn-4-ene-3,20-dione (hydrocortisone),

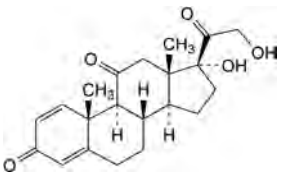

B. 17,21-dihydroxypregna-1,4-diene-3,11,20-trione (prednisone),

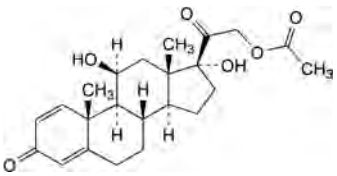

C. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (prednisolone acetate),

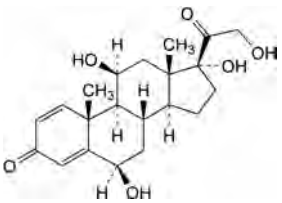

D. 6β,11β,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (6β-hydroxyprednisolone),

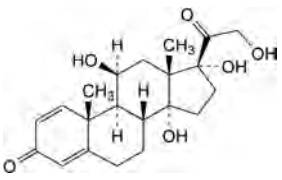

E. 11β,14α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (14α-hydroxyprednisolone),

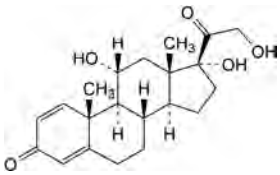

F. 11α,17,21-trihydroxypregna-1,4-diene-3,20-dione (11-*epi*-prednisolone),

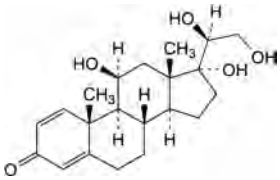

G. 11β,17,20β,21-tetrahydroxypregna-1,4-dien-3-one (20β-hydroxyprednisolone),

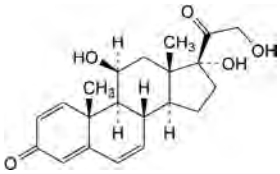

H. 11β,17,21-trihydroxypregna-1,4,6-triene-3,20-dione ($\Delta^6$-prednisolone),

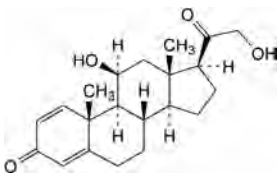

I. 11β,21-dihydroxypregna-1,4-diene-3,20-dione (17-deoxyprednisolone),

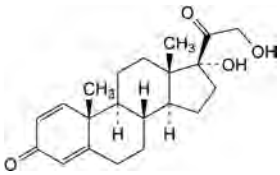

J. 17,21-dihydroxypregna-1,4-diene-3,20-dione (11-deoxyprednisolone).

**01/2008:0734**
**corrected 6.0**

# PREDNISOLONE ACETATE

## Prednisoloni acetas

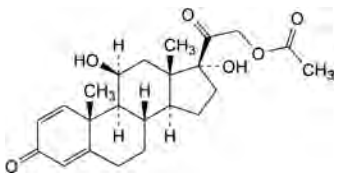

$C_{23}H_{30}O_6$ $M_r$ 402.5
[52-21-1]

DEFINITION

11β,17-Dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *prednisolone acetate CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Reference solution (a).* Dissolve 20 mg of *prednisolone acetate CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 20 mL with the same mixture of solvents.
*Reference solution (b).* Dissolve 10 mg of *prednisolone pivalate CRS* in reference solution (a) and dilute to 10 mL with the same solution.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*
*Application*: 5 µL.
*Development*: over a path of 15 cm.
*Drying*: in air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
*Detection B*: spray with *alcoholic solution of sulfuric acid R.* Heat at 105 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.
*System suitability*: reference solution (b):
– the chromatogram obtained shows 2 clearly separated spots.
*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add the solution to 10 mL of *water R* and mix. The colour fades and there is greenish-yellow fluorescence in ultraviolet light at 365 nm.

D. About 10 mg gives the reaction of acetyl (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 128 to + 137 (dried substance).

Dissolve 70.0 mg in *methanol R2* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Buffer solution pH 4.* Mix 1 volume of *dilute hydrochloric acid R*, 5 volumes of a 68.1 g/L solution of *sodium acetate R*, 15 volumes of a 37.3 g/L solution of *potassium chloride R* and 79 volumes of *water R.*

*Solvent mixture.* Mix equal volumes of *acetonitrile R* and buffer solution pH 4.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2 mg of *prednisolone acetate CRS* and 2 mg of *hydrocortisone acetate CRS* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *prednisolone acetate for peak identification CRS* (containing impurities A, B and C) in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *water R* (350:650 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of prednisolone acetate.

*Identification of impurities*: use the chromatogram supplied with *prednisolone acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to prednisolone acetate (retention time = about 17 min): impurity B = about 0.4; impurity A = about 1.1; impurity C = about 2.0.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to prednisolone acetate and impurity A.

*Limits*:

- *impurities A, B*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity C*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 243 nm.

Calculate the content of $C_{23}H_{30}O_6$ taking the specific absorbance to be 370.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

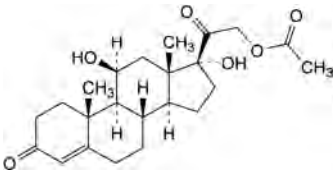

A. 11β,17-dihydroxy-3,20-dioxopregn-4-en-21-yl acetate (hydrocortisone acetate),

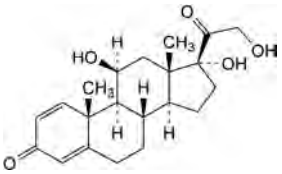

B. 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone),

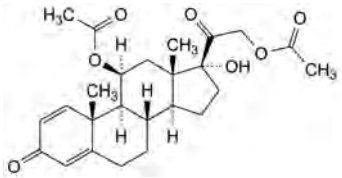

C. 17-hydroxy-3,20-dioxopregna-1,4-diene-11β,21-diyl diacetate (prednisolone 11,21-diacetate),

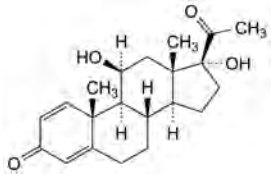

D. 11β,17-dihydroxypregna-1,4-diene3,20-dione,

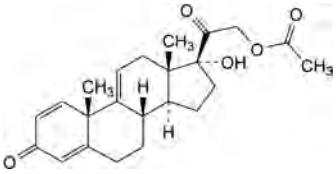

E. 17-hydroxy-3,20-dioxopregna-1,4,9(11)-trien-21-yl acetate.

**01/2008:0736**
**corrected 6.0**

# PREDNISOLONE PIVALATE

## Prednisoloni pivalas

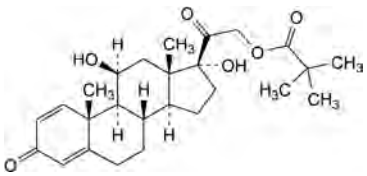

$C_{26}H_{36}O_6$ $M_r$ 444.6
[1107-99-9]

DEFINITION

11β,17-Dihydroxy-3,20-dioxopregna-1,4-dien-21-yl 2,2-dimethylpropanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent), soluble in methylene chloride.

mp: about 229 °C, with decomposition.

IDENTIFICATION

*First identification*: B, C.

*Second identification*: A, C, D.

A. Dissolve 10.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) at the absorption maximum at 415 nm is 0.20 to 0.30.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prednisolone pivalate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution*. Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 10 mg of *prednisolone pivalate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *prednisolone acetate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture. Dilute 5 mL of this solution to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*, heat at 120 °C for 10 min or until the spots appear, and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

D. To 2 mL of *sulfuric acid R*, add about 2 mg and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add this solution to 10 mL of *water R* and mix. The colour fades and there is greenish-yellow fluorescence in ultraviolet light at 365 nm.

TESTS

**Specific optical rotation** (*2.2.7*): + 104 to + 112 (dried substance).

Dissolve 0.250 g in *dioxan R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 62.5 mg of the substance to be examined in 2 mL of a mixture of 1 volume of *water R* and 4 volumes of *tetrahydrofuran R* and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 25 mg of *prednisolone acetate CRS*, 25 mg of *cortisone acetate CRS* and 25 mg of *prednisolone pivalate CRS* in 2 mL of a mixture of 1 volume of *water R* and 4 volumes of *tetrahydrofuran R* and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 25.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: carefully mix 19 mL of *butyl acetate R1* with 37 mL of *tetrahydrofuran R* and 213 mL of *ethylene glycol monomethyl ether R*, then add with 231 mL of *water R*; mix, allow to equilibrate for 1 h and filter through a 0.45 µm filter.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of prednisolone pivalate.

*Retention time*: prednisolone acetate = about 3.5 min; cortisone acetate = about 4.5 min; prednisolone pivalate = about 13 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to prednisolone acetate and cortisone acetate; if necessary, adjust the concentration of water in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent), and not more than one such peak has an area greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *total*: not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (2.5 per cent);
– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 250.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 243 nm.

Calculate the content of $C_{26}H_{36}O_6$ taking the specific absorbance to be 337.

STORAGE

Protected from light.

**01/2008:0735**



# PREDNISOLONE SODIUM PHOSPHATE

## Prednisoloni natrii phosphas

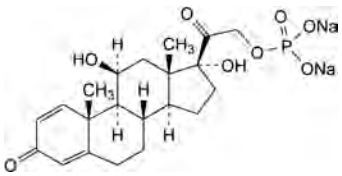

$C_{21}H_{27}Na_2O_8P$ $M_r$ 484.4
[125-02-0]

DEFINITION

11β,17-Dihydroxy-3,20-dioxopregna-1,4-dien-21-yl disodium phosphate.

*Content*: 96.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D, E.*

A. Dissolve 10.0 mg in 5 mL of *water R* and dilute to 100.0 mL with *anhydrous ethanol R*. Place 2.0 mL of this solution in a ground-glass-stoppered tube, add 10.0 mL of *phenylhydrazine-sulfuric acid solution R*, mix and heat in a water-bath at 60 °C for 20 min. Cool immediately. The absorbance (*2.2.25*) at the absorption maximum at 415 nm is 0.10 to 0.20.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prednisolone sodium phosphate CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *ethanol (96 per cent) R*, evaporate to dryness on a water-bath and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *prednisolone sodium phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *dexamethasone sodium phosphate CRS* in *methanol R* and dilute to 10 mL with the same solvent. Dilute 5 mL of this solution to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*, heat at 120 °C for 10 min or until the spots appear, and allow to cool; examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):
– the chromatogram shows 2 spots which may, however, not be completely separated.

D. To 2 mL of *sulfuric acid R* add about 2 mg and shake to dissolve. Within 5 min, an intense red colour develops. When examined in ultraviolet light at 365 nm, a reddish-brown fluorescence is seen. Add this solution to 10 mL of *water R* and mix. The colour fades and there is a greenish-yellow fluorescence in ultraviolet light at 365 nm.

E. To about 40 mg add 2 mL of *sulfuric acid R* and heat gently until white fumes are evolved. Add *nitric acid R* dropwise, continue the heating until the solution is almost colourless, and cool. Add 2 mL of *water R*, heat until white fumes are again evolved, cool, add 10 mL of *water R* and neutralise to *red litmus paper R* with *dilute ammonia R1*. The solution gives reaction (a) of sodium (*2.3.1*) and reaction (b) of phosphates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 9.0 for solution S.

**Specific optical rotation** (*2.2.7*): + 94 to + 100 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25 mg of *prednisolone sodium phosphate CRS* and 25 mg of *prednisolone CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 25.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: into a 250 mL conical flask weigh 1.360 g of *potassium dihydrogen phosphate R* and 0.600 g of *hexylamine R*, mix, allow to stand for 10 min, then dissolve in 185 mL of *water R*; add 65 mL of *acetonitrile R*, mix, and filter through a 0.45 µm filter.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Equilibration*: with the mobile phase for about 30 min.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of prednisolone sodium phosphate.

*Retention time*: prednisolone sodium phosphate = about 6.5 min; prednisolone = about 8.5 min.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.5 between the peaks due to prednisolone sodium phosphate and prednisolone; if necessary, increase the concentration of *acetonitrile R* or *water R* in the mobile phase.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent), and not more than 1 such peak has an area greater than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);

– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent);

– *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Inorganic phosphate**: maximum 1 per cent.

Dissolve 50 mg in *water R* and dilute to 100 mL with the same solvent. To 10 mL of this solution add 5 mL of *molybdovanadic reagent R*, mix, and allow to stand for 5 min. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner using 10 mL of *phosphate standard solution (5 ppm $PO_4$) R*.

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 250.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 247 nm.

Calculate the content of $C_{21}H_{27}Na_2O_8P$ taking the specific absorbance to be 312.

STORAGE

Protected from light.

**01/2018:0354**

# PREDNISONE

## Prednisonum

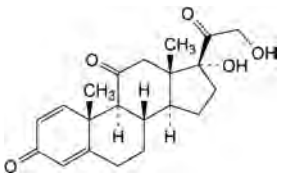

$C_{21}H_{26}O_5$ $M_r$ 358.4
[53-03-2]

DEFINITION

17,21-Dihydroxypregna-1,4-diene-3,11,20-trione.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prednisone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness on a water-bath and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (1:9 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20 mg of *prednisone CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *betamethasone CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R.*

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*Detection B*: spray with *alcoholic solution of sulfuric acid R*. Heat at 120 °C for 10 min or until the spots appear. Allow to cool. Examine in daylight and in ultraviolet light at 365 nm.

*Results B*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour in daylight, fluorescence in ultraviolet light at 365 nm and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

D. Add about 2 mg to 2 mL of *sulfuric acid R* and shake to dissolve. Within 5 min, a yellow colour develops with a blue fluorescence in ultraviolet light at 365 nm. Add this solution to 10 mL of *water R* and mix. The colour fades but the blue fluorescence in ultraviolet light does not disappear.

TESTS

**Specific optical rotation** (*2.2.7*): + 183 to + 191 (dried substance).

Dissolve 0.125 g in *ethanol (96 per cent) R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile for chromatography R, water for chromatography R* (50:50 *V/V*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *prednisone impurity B CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 4 mg of *prednisone for peak identification CRS* (containing impurities A, D and E) in the solvent mixture, add 1.0 mL of reference solution (a) and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 20.0 mg of *prednisone CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 20.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;
– *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (2.5 µm);
– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: 0.68 g/L solution of *potassium dihydrogen phosphate R*, adjusted to pH 2.0 with *phosphoric acid R*;
– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 83 | 17 |
| 2 - 25 | 83 → 80 | 17 → 20 |
| 25 - 28 | 80 → 65 | 20 → 35 |
| 28 - 33 | 65 | 35 |
| 33 - 43 | 65 → 20 | 35 → 80 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 244 nm.

*Injection*: 5 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: Use the chromatogram supplied with *prednisone for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, D and E; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to prednisone (retention time = about 18 min): impurity B = about 1.06; impurity A = about 1.12; impurity D = about 1.6; impurity E = about 1.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to prednisone.

*Calculation of percentage contents*:

– for each impurity, use the concentration of prednisone in reference solution (c).

*Limits*:

– *impurity A*: maximum 0.5 per cent;
– *impurity E*: maximum 0.2 per cent;
– *impurity D*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

ASSAY

*Carry out the test protected from light. Prepare the solutions immediately before use.*

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{21}H_{26}O_5$ taking into account the assigned content of *prednisone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F, G, J, K, L.*

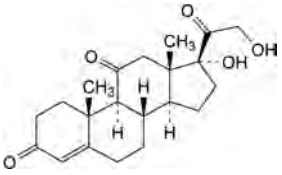


A. 17,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone),

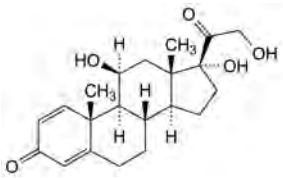

B. 11β,17,21-trihydroxypregna-1,4-diene-3,20-dione (prednisolone),

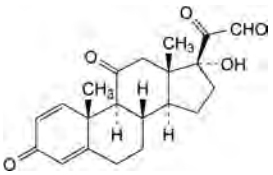

C. 17-hydroxy-3,11,20-trioxopregna-1,4-dien-21-al (prednisone-21-aldehyde),

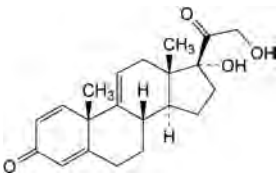

D. 17,21-dihydroxypregna-1,4,9(11)-triene-3,20-dione (deltacortinene),

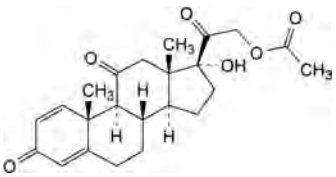

E. 17-hydroxy-3,11,20-trioxopregna-1,4-dien-21-yl acetate (prednisone acetate),

F. unknown structure,

G. unknown structure,

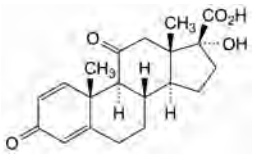

J. 17α-hydroxy-3,11-dioxoandrosta-1,4-diene-17β-carboxylic acid,

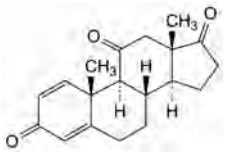

K. androsta-1,4-diene-3,11,17-trione,

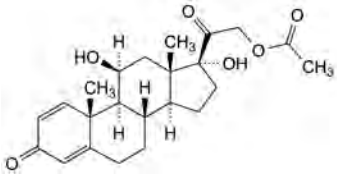

L. 11β,17-dihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (prednisolone acetate).

**07/2017:2777**

# PREGABALIN

## Pregabalinum

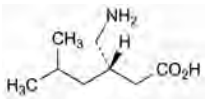

$C_8H_{17}NO_2$ $M_r$ 159.2
[148553-50-8]

DEFINITION

(3*S*)-3-(Aminomethyl)-5-methylhexanoic acid.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: sparingly soluble in water, very slightly soluble in methanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pregabalin CRS*.

B. Examine the chromatograms obtained in the test for enantiomeric purity.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent. Derivatise the solution as described under Derivatisation.

*Reference solution.* Dissolve 2 mg of *pregabalin impurity B CRS* in *water R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*. To 20 mg of *pregabalin CRS*, add 1.0 mL of this solution and dilute to 10.0 mL with *water R*. Derivatise this solution as described under Derivatisation.

*Derivatisation.* Transfer 500 µL of the solution to a reaction vial. Add 500 µL of a 5 g/L solution of *1-fluoro-2,4-dinitrophenyl-5-L-alaninamide R* in *acetonitrile R*. Add 50 µL of an 84 g/L solution of *sodium hydrogen carbonate R*. Seal the vial, mix and derivatise by maintaining the vial at 40 °C for 1 h in a heating/stirring module. Stop the reaction by adding about 50 µL of a 103 g/L solution of *hydrochloric acid R*. Mix thoroughly. To 200 µL of the derivatised solution add 800 µL of the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R*, 1 per cent *V/V* solution of *triethylamine R* previously adjusted to pH 3.0 with *phosphoric acid R* (38:62 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 340 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of the pregabalin derivative.

*Relative retention* with reference to the pregabalin derivative (retention time = about 10 min): impurity B derivative = about 1.3.

*System suitability*: reference solution:

– *resolution*: minimum 4.4 between the peaks due to the pregabalin derivative and impurity B derivative.

*Limit*:

– *impurity B*: maximum 0.15 per cent.

**Related substances**

A. Polar impurities eluting before pregabalin. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 0.100 g of *pregabalin CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 5 mg of *mandelic acid R* (impurity C) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 0.5 mL of the solution to 5.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*: *methanol R2*, 3.40 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 6.3 with *concentrated ammonia R* (15:85 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 1.3 times the retention time of pregabalin.

*Relative retention* with reference to pregabalin (retention time = about 10 min): impurity C = about 0.6.

*System suitability*: reference solution (c):

– *resolution*: minimum 5.0 between the peaks due to impurity C and pregabalin.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pregabalin in reference solution (b).

*Limits*:

– *unspecified impurities eluting before pregabalin*: for each impurity, maximum 0.10 per cent;

– *reporting threshold*: 0.05 per cent.

B. Non-polar impurities eluting after pregabalin. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2.5 mg of *pregabalin impurity D CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase (solution A). Dissolve the contents of a vial of *pregabalin impurity A CRS* in the mobile phase, add 1.0 mL of solution A and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);

– *temperature*: 30 °C.

*Mobile phase*: 3.40 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 6.3 with *concentrated ammonia R*, *methanol R2* (45:55 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of pregabalin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and D.

*Relative retention* with reference to pregabalin (retention time = about 4 min): impurity A = about 2.4; impurity D = about 3.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to impurities A and D.

*Calculation of percentage contents:*

– for impurities A and D, use the concentration of each impurity in reference solution (b);

– for impurities other than A and D, use the concentration of pregabalin in reference solution (a).

*Limits*:

– *impurity A*: maximum 0.15 per cent;

– *unspecified impurities eluting after pregabalin*: for each impurity, maximum 0.10 per cent;

– *reporting threshold*: 0.05 per cent.

*Limit*:

– *total for tests A and B*: maximum 0.5 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.130 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_8H_{17}NO_2$ taking into account the assigned content of *pregabalin CRS*.

IMPURITIES

**Test A for related substances**: C.

**Test B for related substances**: A, D.

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

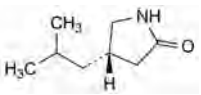


A. (4*S*)-4-(2-methylpropyl)pyrrolidin-2-one,

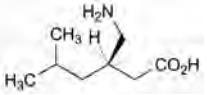


B. (3*R*)-3-(aminomethyl)-5-methylhexanoic acid (pregabalin enantiomer),

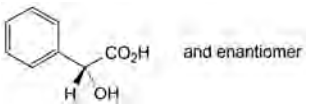


C. (2*RS*)-2-hydroxy-2-phenylacetic acid (mandelic acid),

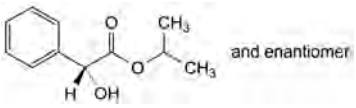


D. 1-methylethyl (2*RS*)-2-hydroxy-2-phenylacetate.

**01/2017:1362**
**corrected 9.4**

# PRILOCAINE

## Prilocainum

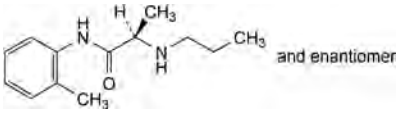


$C_{13}H_{20}N_2O$ $M_r$ 220.3
[721-50-6]

DEFINITION

(2*RS*)-*N*-(2-Methylphenyl)-2-(propylamino)propanamide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, very soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: prepare a film between 2 plates of *sodium chloride R* by heating at 40-45 °C until the substance has melted.

*Comparison*: *prilocaine CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 2.50 g in 15 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.5 mg of the substance to be examined and 3 mg of *prilocaine impurity E CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 33.5 mg of *prilocaine impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 15 mg of *prilocaine for peak identification CRS* (containing impurity G) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 26 volumes of *acetonitrile for chromatography R* and 74 volumes of a solution prepared as follows: dissolve 0.180 g of *sodium dihydrogen phosphate monohydrate R* and 2.89 g of *disodium hydrogen phosphate dihydrate R* in 1000 mL of *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of prilocaine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to prilocaine (retention time = about 25 min): impurity B = about 0.3; impurity G = about 0.8; impurity E = about 1.2.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.0 between the peaks due to prilocaine and impurity E.

*Limits*:
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (100 ppm);
- *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.03 mg of $C_{13}H_{20}N_2O$.

IMPURITIES

*Specified impurities*: *B, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F.*

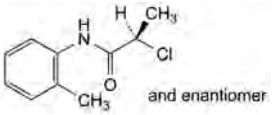


A. (2*RS*)-2-chloro-*N*-(2-methylphenyl)propanamide,

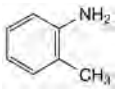

B. 2-methylbenzenamine (*o*-toluidine),

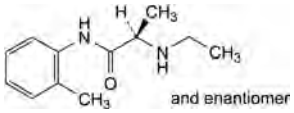


C. (2*RS*)-2-(ethylamino)-*N*-(2-methylphenyl)propanamide,

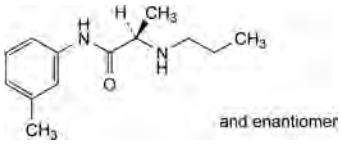


D. (2*RS*)-*N*-(3-methylphenyl)-2-(propylamino)propanamide,

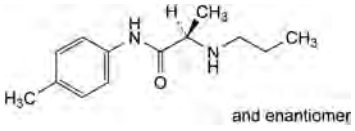


E. (2*RS*)-*N*-(4-methylphenyl)-2-(propylamino)propanamide,

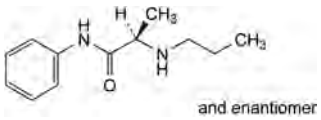


F. (2*RS*)-*N*-phenyl-2-(propylamino)propanamide,

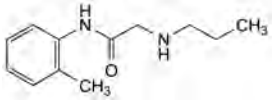

G. *N*-(2-methylphenyl)-2-(propylamino)acetamide.

**01/2017:1363**
**corrected 9.4**

# PRILOCAINE HYDROCHLORIDE

## Prilocaini hydrochloridum

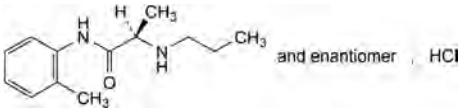


$C_{13}H_{21}ClN_2O$ $M_r$ 256.8
[1786-81-8]

DEFINITION

(2*RS*)-*N*-(2-Methylphenyl)-2-(propylamino)propanamide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water and in ethanol (96 per cent), very slightly soluble in acetone.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 168 °C to 171 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prilocaine hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 20.0 mg of *prilocaine hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 20.0 mg of *lidocaine hydrochloride CRS* and 20.0 mg of *prilocaine hydrochloride CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *1,1-dimethylethyl methyl ether R* (1:5:100 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 12 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 4 mL of solution S to 10 mL with *carbon dioxide-free water R*. Add 0.1 mL of *bromocresol green solution R* and 0.40 mL of *0.01 M sodium hydroxide*; the solution is blue. Add 0.80 mL of *0.01 M hydrochloric acid*; the solution is yellow.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 30 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3 mg of the substance to be examined and 3 mg of *prilocaine impurity E CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 30.0 mg of *prilocaine impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 15 mg of *prilocaine for peak identification CRS* (containing impurity G) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 26 volumes of *acetonitrile for chromatography R* and 74 volumes of a solution prepared as follows: dissolve 0.180 g of *sodium dihydrogen phosphate monohydrate R* and 2.89 g of *disodium hydrogen phosphate dihydrate R* in 1000 mL of *water for chromatography R.*

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of prilocaine.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity G.

*Relative retention* with reference to prilocaine (retention time = about 25 min): impurity B = about 0.3; impurity G = about 0.8; impurity E = about 1.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to prilocaine and impurity E.

*Limits*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (100 ppm);
– *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 25.68 mg of $C_{13}H_{21}ClN_2O$.

IMPURITIES

*Specified impurities: B, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F.*

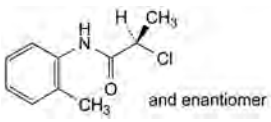


A. (2*RS*)-2-chloro-*N*-(2-methylphenyl)propanamide,

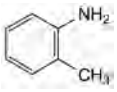

B. 2-methylbenzenamine (*o*-toluidine),

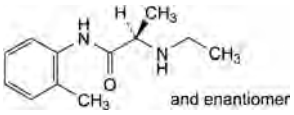


C. (2*RS*)-2-(ethylamino)-*N*-(2-methylphenyl)propanamide,

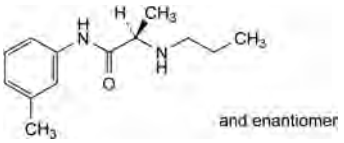


D. (2*RS*)-*N*-(3-methylphenyl)-2-(propylamino)propanamide,

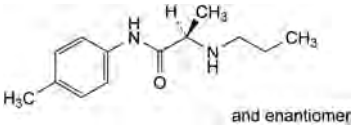


E. (2*RS*)-*N*-(4-methylphenyl)-2-(propylamino)propanamide,

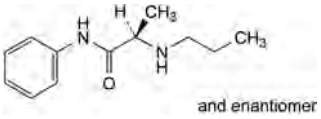


F. (2*RS*)-*N*-phenyl-2-(propylamino)propanamide,

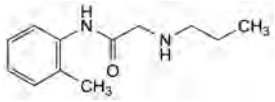

G. *N*-(2-methylphenyl)-2-(propylamino)acetamide.

**01/2019:0635**

# PRIMAQUINE DIPHOSPHATE

## Primaquini diphosphas

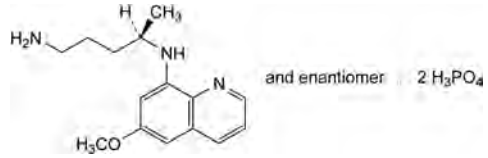


$C_{15}H_{27}N_3O_9P_2$ $M_r$ 455.3
[63-45-6]

DEFINITION

(4*RS*)-$N^4$-(6-Methoxyquinolin-8-yl)pentane-1,4-diamine bis(dihydrogen phosphate).

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: orange, crystalline powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

mp: about 200 °C, with decomposition.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 15 mg in a 1.03 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with a 1.03 g/L solution of *hydrochloric acid R*.

*Spectral range*: 310-450 nm for test solution (a); 215-310 nm for test solution (b).

*Absorption maxima*: at 332 nm and 415 nm for test solution (a); at 225 nm, 265 nm and 282 nm for test solution (b).

*Specific absorbance at the absorption maxima*:

- at 332 nm: 45 to 52 for test solution (a);
- at 415 nm: 27 to 35 for test solution (a);
- at 225 nm: 495 to 515 for test solution (b);
- at 265 nm: 335 to 350 for test solution (b);
- at 282 nm: 330 to 345 for test solution (b).

B. Infrared absorption spectrophotometry (*2.2.24*).

Examine the substances in discs prepared as follows: dissolve separately 0.1 g of the substance to be examined and 0.1 g of the reference substance in 5 mL of *water R*, add 2 mL of *dilute ammonia R2* and 5 mL of *methylene chloride R*, then shake. Dry the methylene chloride layer over 0.5 g of *anhydrous sodium sulfate R*. Prepare a blank disc using about 0.3 g of *potassium bromide R*. Apply dropwise to the disc 0.1 mL of the methylene chloride layer, allowing the methylene chloride to evaporate between applications. Dry the disc at 50 °C for 2 min.

*Comparison*: *primaquine diphosphate CRS*.

C. Thin-layer chromatography (*2.2.27*). *Carry out all operations as rapidly as possible, protected from light. Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.20 g of the substance to be examined in 5 mL of *water R* and dilute to 10 mL with *methanol R*. Dilute 1 mL of this solution to 10 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Reference solution.* Dissolve 20 mg of *primaquine diphosphate CRS* in 5 mL of *water R* and dilute to 10 mL with *methanol R*.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Pretreatment*: wash the plate with the mobile phase and allow to dry in air.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:40:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 50 mg in 5 mL of *water R*. Add 2 mL of *dilute sodium hydroxide solution R* and shake with 2 quantities, each of 5 mL, of *methylene chloride R*. The aqueous layer, acidified by addition of *nitric acid R*, gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution. Use a freshly prepared solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase A, using sonication if necessary, and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 5 mg of *primaquine for system suitability CRS* (containing impurity A) in mobile phase A and dilute to 10 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *trifluoroacetic acid R*, *tetrahydrofuran R*, *acetonitrile R*, *water for chromatography R* (0.1/1/9/90 *V/V/V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 40 | 100 → 50 | 0 → 50 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 25 µL.

*Relative retention* with reference to primaquine (retention time = about 13 min): impurity A = about 0.86.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity A and primaquine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of primaquine diphosphate in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.200 g in 40 mL of *anhydrous acetic acid R*, heating gently. Allow to cool and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.77 mg of $C_{15}H_{27}N_3O_9P_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

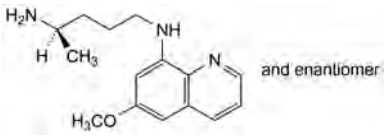


A. (4*RS*)-$N^1$-(6-methoxyquinolin-8-yl)pentane-1,4-diamine (quinocide),

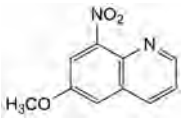


B. 6-methoxy-8-nitroquinoline,

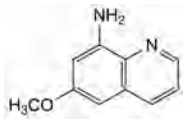

C. 6-methoxyquinolin-8-amine,

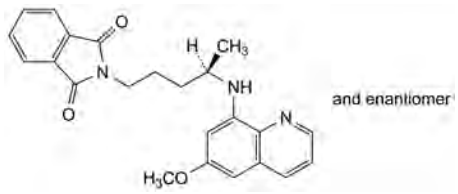


D. 2-[(4*RS*)-4-[(6-methoxyquinolin-8-yl)amino]pentyl]-1*H*-isoindole-1,3(2*H*)-dione.

**01/2017:0584**
**corrected 9.3**

# PRIMIDONE

## Primidonum

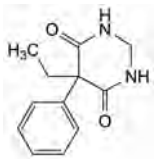

$C_{12}H_{14}N_2O_2$ $M_r$ 218.3
[125-33-7]

DEFINITION

5-Ethyl-5-phenyldihydropyrimidine-4,6(1*H*,5*H*)-dione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in alkaline solutions.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Use the solution prescribed for the assay. Examined between 240 nm and 300 nm (*2.2.25*), the solution shows 3 absorption maxima, at 252 nm, 257 nm and 264 nm, and 2 absorption minima, at 254 nm and 261 nm. The ratio of the absorbance measured at the absorption maximum at 257 nm to that measured at the absorption minimum at 261 nm is 2.00 to 2.20. The identification is valid if, in the test for resolution (*2.2.25*), the ratio of the absorbances is not less than 2.0.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *primidone CRS*.

C. Dissolve 0.1 g in 5 mL of a 5 g/L solution of *chromotropic acid, sodium salt R* in a mixture of 4 volumes of *water R* and 9 volumes of *sulfuric acid R*. A pinkish-blue colour develops on heating.

D. Mix 0.2 g and 0.2 g of *anhydrous sodium carbonate R*. Heat until the mixture melts. Ammonia is evolved which is detectable by its alkaline reaction (*2.2.4*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R1* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R1*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1*.

*Reference solution (b)*. Dissolve 5 mg of *primidone for peak identification CRS* (containing impurities A, B, C, D, E and F) in *methanol R1* and dilute to 5 mL with the same solvent.

*Column*:
- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*.

*Mobile phase*:
- *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 75 | 25 |
| 1 - 6 | 75 → 40 | 25 → 60 |
| 6 - 8 | 40 | 60 |
| 8 - 8.5 | 40 → 75 | 60 → 25 |
| 8.5 - 10 | 75 | 25 |

*Flow rate*: 3.2 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *primidone for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks.

*Relative retention* with reference to primidone (retention time = about 2.2 min): impurity A = about 0.5; impurity B = about 1.4; impurity C = about 1.6; impurity D = about 1.75; impurity E = about 2.0; impurity F = about 2.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.5 between the peaks due to impurity B and impurity C.

*Limits*:
- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.5; impurity C = 1.5; impurity D = 1.4; impurity E = 1.3;
- *impurity F*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 60.0 mg with heating in 70 mL of *ethanol (96 per cent) R*, cool and dilute to 100.0 mL with the same solvent. Prepare a reference solution in the same manner using 60.0 mg of *primidone CRS*. Measure the absorbance (*2.2.25*) of the 2 solutions at the absorption maximum at 257 nm.

Calculate the content of $C_{12}H_{14}N_2O_2$ from the absorbances measured and the concentrations of the solutions.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

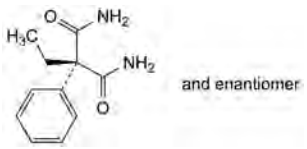


A. 2-ethyl-2-phenylpropanediamide (ethylphenylmalonamide),

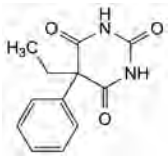


B. 5-ethyl-5-phenylpyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione (phenobarbital),

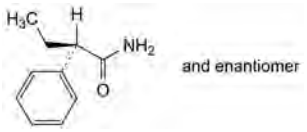


C. (2*RS*)-2-phenylbutanamide,

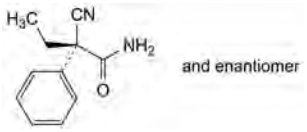


D. (2*RS*)-2-cyano-2-phenylbutanamide,

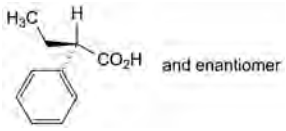


E. (2*RS*)-2-phenylbutanoic acid,

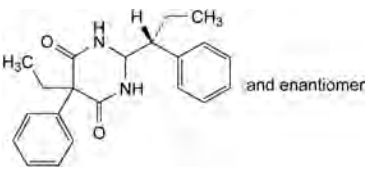


F. 5-ethyl-5-phenyl-2-[(1*RS*)-1-phenylpropyl]dihydropyrimidine-4,6(1*H*,5*H*)-dione.

**01/2017:0243**

# PROBENECID

## Probenecidum

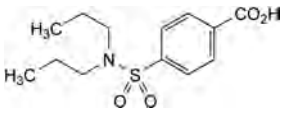


$C_{13}H_{19}NO_4S$ $M_r$ 285.4
[57-66-9]

DEFINITION

4-(Dipropylsulfamoyl)benzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small crystals.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 197 °C to 202 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20 mg in a mixture of 1 volume of *0.1 M hydrochloric acid* and 9 volumes of *ethanol (96 per cent) R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 5.0 mL of the solution to 100.0 mL with a mixture of 1 volume of *0.1 M hydrochloric acid* and 9 volumes of *ethanol (96 per cent) R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 223 nm and 248 nm.

*Specific absorbance at the absorption maximum at 248 nm*: 310 to 350.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *probenecid CRS*.

D. Dissolve 0.2 g in the smallest necessary quantity of *dilute ammonia R2* (about 0.6 mL). Add 3 mL of *silver nitrate solution R2*. A white precipitate is formed which dissolves in an excess of ammonia.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in *1 M sodium hydroxide* and dilute to 10 mL with the same solvent.

**Acidity**. To 2.0 g add 100 mL of *water R* and heat on a water-bath for 30 min. Make up to the original volume with *water R*, allow to cool to room temperature and filter. To 50 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. Not more than 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Mix 1 volume of *glacial acetic acid R* and 100 volumes of *acetonitrile R*.

*Solution B*. Mix 1 volume of *glacial acetic acid R* and 100 volumes of a 6.9 g/L solution of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R* and adjust to pH 3.0 with *dilute phosphoric acid R*.

*Test solution.* Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *potassium 4-sulfobenzoate R* (impurity A) in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped phenylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: solution A, solution B (50:50 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of probenecid.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to probenecid (retention time = about 7 min): impurity A = about 0.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to impurity A and probenecid.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity A by 0.4;
- for each impurity, use the concentration of probenecid in reference solution (a).

*Limits*:
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R*, shaking and heating slightly if necessary. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.54 mg of $C_{13}H_{19}NO_4S$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

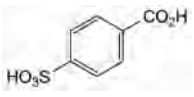

A. 4-sulfobenzoic acid,

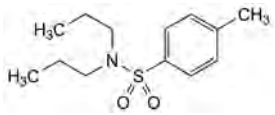

B. 4-methyl-*N*,*N*-dipropylbenzenesulfonamide,

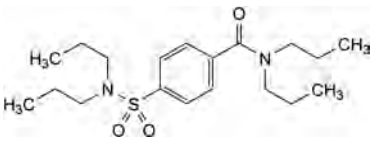

C. 4-(dipropylsulfamoyl)-*N*,*N*-dipropylbenzamide,

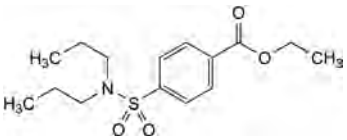

D. ethyl 4-(dipropylsulfamoyl)benzoate.

**01/2017:0567**

# PROCAINAMIDE HYDROCHLORIDE

## Procainamidi hydrochloridum

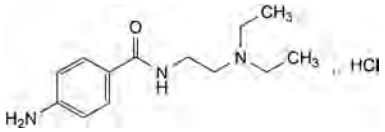

$C_{13}H_{22}ClN_3O$ $M_r$ 271.8
[614-39-1]

DEFINITION

Procainamide hydrochloride contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-[2-(diethylamino)ethyl]benzamide hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or very slightly yellow, crystalline powder, hygroscopic, very soluble in water, freely soluble in alcohol, slightly soluble in acetone.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 166 °C to 170 °C.

B. Dissolve 10.0 mg in *0.1 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *0.1 M sodium hydroxide*. Examined between 220 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 273 nm. The specific absorbance at the maximum is 580 to 610.

C. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *procainamide hydrochloride CRS*.

D. Dilute 1 mL of solution S to 5 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

E. Dilute 1 mL of solution S (see Tests) to 2 mL with *water R*. 1 mL of this solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 5.6 to 6.3.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 0.10 g of the substance to be examined in *alcohol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dilute 1 mL of the test solution to 200 mL with *alcohol R*.

Apply to the plate 5 µL of each solution. Develop over a path of 12 cm using a mixture of 15 volumes of *glacial acetic acid R*, 30 volumes of *water R* and 60 volumes of *butanol R*. Place the plate in a stream of cold air until the plate appears dry. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in 50 mL of *dilute hydrochloric acid R*. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*).

1 mL of *0.1 M sodium nitrite* is equivalent to 27.18 mg of $C_{13}H_{22}ClN_3O$.

STORAGE

Store in an airtight container, protected from light.

**01/2017:0050**

# PROCAINE HYDROCHLORIDE

## Procaini hydrochloridum

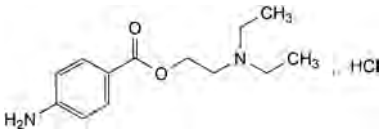

$C_{13}H_{21}ClN_2O_2$ $M_r$ 272.8
[51-05-8]

DEFINITION

Procaine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 2-(diethylamino)ethyl 4-aminobenzoate hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder or colourless crystals, very soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E, F.*

A. Melting point (*2.2.14*): 154 °C to 158 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *procaine hydrochloride CRS*.

C. To about 5 mg add 0.5 mL of *fuming nitric acid R*. Evaporate to dryness on a water-bath, allow to cool and dissolve the residue in 5 mL of *acetone R*. Add 1 mL of *0.1 M alcoholic potassium hydroxide*. Only a brownish-red colour develops.

D. To 0.2 mL of solution S (see Tests) add 2 mL of *water R* and 0.5 mL of *dilute sulfuric acid R* and shake. Add 1 mL of a 1 g/L solution of *potassium permanganate R*. The colour is immediately discharged.

E. It gives reaction (a) of chlorides (*2.3.1*).

F. Dilute 1 mL of solution S to 100 mL with *water R*. 2 mL of this solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). Dilute 4 mL of solution S to 10 mL with *carbon dioxide-free water R*. The pH of the solution is 5.0 to 6.5.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 50 mg of *4-aminobenzoic acid R* in *water R* and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with *water R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 10 cm using a mixture of 4 volumes of *glacial acetic acid R*, 16 volumes of *hexane R* and 80 volumes of *dibutyl ether R*. Dry the plate at 100 °C to 105 °C for 10 min and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.05 per cent). The principal spot in the chromatogram obtained with the test solution remains on the point of application.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.00 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *dilute hydrochloric acid R*. Carry out the determination of primary aromatic amino nitrogen (*2.5.8*).

1 mL of *0.1 M sodium nitrite* is equivalent to 27.28 mg of $C_{13}H_{21}ClN_2O_2$.

STORAGE

Store protected from light.

**07/2019:0244**

# PROCHLORPERAZINE MALEATE

## Prochlorperazini maleas

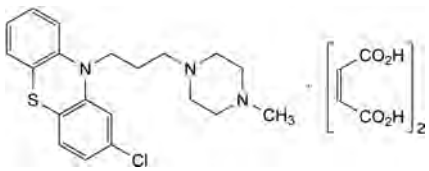

$C_{28}H_{32}ClN_3O_8S$ $M_r$ 606
[84-02-6]

DEFINITION

2-Chloro-10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine bis[hydrogen (2*Z*)-but-2-enedioate].

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale-yellow, crystalline powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: B.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *prochlorperazine maleate CRS*.

B. Identification of phenothiazines by thin-layer chromatography (*2.3.3*) with the following modifications.

*Test solution*. Dissolve 20 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R*, and dilute to 20 mL with the same mixture of solvents.

*Reference solution*. Dissolve 20 mg of *prochlorperazine maleate CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R*, and dilute to 20 mL with the same mixture of solvents.

*Application*: 4 µL.

TESTS

**pH** (*2.2.3*): 3.0 to 4.0 for a freshly prepared saturated solution in *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Buffer solution*. Dissolve 0.96 g of *ammonium carbonate R* in about 800 mL of *water for chromatography R*. Adjust to pH 10.0 with *concentrated ammonia R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *prochlorperazine for system suitability CRS* (containing impurities B and D) in the mobile phase and dilute to 25 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped propyl-2-phenylsilyl amorphous organosilica polymer R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *tetrahydrofuran R*, *methanol R*, buffer solution (15:33:52 *V/V/V*).

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 252 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of prochlorperazine.

*Identification of impurities*: use the chromatogram supplied with *prochlorperazine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and D.

*Relative retention* with reference to prochlorperazine (retention time = about 24 min): maleic acid = about 0.06; impurity B = about 0.56; impurity D = about 1.11.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to prochlorperazine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of prochlorperazine maleate in reference solution (a).

*Limits*:

- *impurity B*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g of the powdered substance to be examined in 50 mL of *anhydrous acetic acid R*, warming on a water-bath. Allow to cool to room temperature. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid*, is equivalent to 30.31 mg of $C_{28}H_{32}ClN_3O_8S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D.*

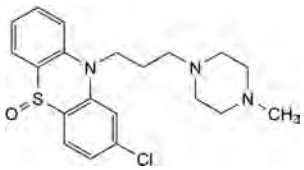

A. 2-chloro-10-[3-(4-methylpiperazin-1-yl)propyl]-5λ⁴-phenothiazin-5(10*H*)-one,

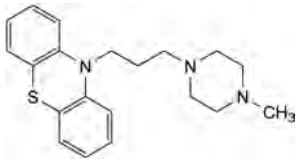

B. 10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine,

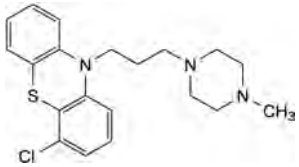

C. 4-chloro-10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine,

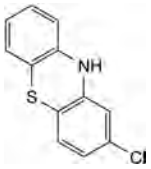

D. 2-chloro-10*H*-phenothiazine.

**07/2015:0429**

# PROGESTERONE

## Progesteronum

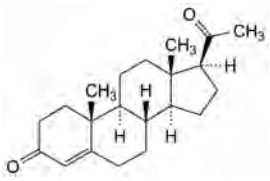

$C_{21}H_{30}O_2$ $M_r$ 314.5
[57-83-0]

DEFINITION

Pregn-4-ene-3,20-dione.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, sparingly soluble in acetone and in fatty oils.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *progesterone CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution*. Dissolve 10 mg of *progesterone CRS* in a mixture of 1 volume of *methanol R* and 9 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ethyl acetate R*, *methylene chloride R* (33:66 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Specific optical rotation** (*2.2.7*): + 186 to + 194 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in mobile phase B and dilute to 50.0 mL with mobile phase B.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (b)*. Dissolve 2 mg of *progesterone for system suitability CRS* (containing impurities B, C, G, I and M) in mobile phase B and dilute to 5.0 mL with mobile phase B.

*Reference solution (c)*. Dissolve 2 mg of *progesterone for peak identification CRS* (containing impurities D, E, J, K and L) in mobile phase B and dilute to 5.0 mL with mobile phase B.

*Reference solution (d)*. Dissolve 10.0 mg of *progesterone for impurity H identification CRS* (with an assigned content of impurity H) in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Reference solution (e)*. Dissolve 20.0 mg of *progesterone CRS* in mobile phase B and dilute to 50.0 mL with mobile phase B.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, *water R* (50:50 *V/V*);
– *mobile phase B*: *water R*, *acetonitrile R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 27 | 100 → 0 | 0 → 100 |
| 27 - 45 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 241 nm and, for impurity H, at 286 nm.

*Injection*: 10 µL of the test solution and reference solutions (a), (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *progesterone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, G, I and M; use the chromatogram supplied with *progesterone for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D + E, J, K and L; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity H.

*Relative retention* with reference to progesterone (retention time = about 14 min): impurity B = about 0.60; impurity J = about 0.65; impurity H = about 0.82; impurity K = about 0.85; impurity C = about 0.93; impurity M = about 1.1; impurity L = about 1.90; impurity I = about 1.95; impurities D and E = about 2.05; impurity G = about 2.65.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 4.0, where $H_p$ = height above the baseline of the peak due to impurity M and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to progesterone.

*Limits*:

- *impurity I (sum of the 2 epimers)*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *sum of impurities D and E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurities G, J, K, L, M*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity H at 286 nm*: maximum 0.15 per cent, calculated from the area of the corresponding peak in the chromatogram obtained with reference solution (d) and taking into account the assigned content of impurity H in *progesterone for impurity H identification CRS*;
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than H*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 2 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (e).

Calculate the percentage content of $C_{21}H_{30}O_2$ taking into account the assigned content of *progesterone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, C, D, E, G, H, I, J, K, L, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

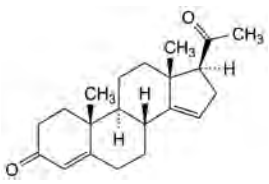

A. pregna-4,14-diene-3,20-dione,

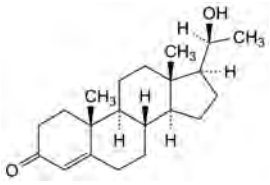

B. (20*S*)-20-hydroxypregn-4-en-3-one,

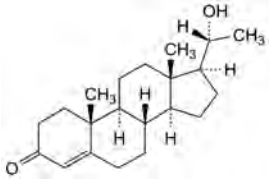

C. (20*R*)-20-hydroxypregn-4-en-3-one,

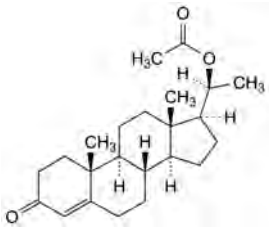

D. (20*S*)-3-oxopregn-4-en-20-yl acetate,

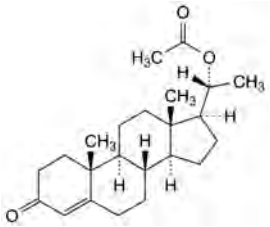

E. (20*R*)-3-oxopregn-4-en-20-yl acetate,

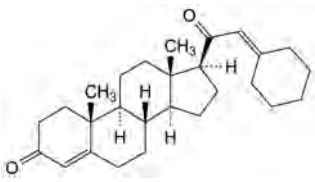

G. 21-(cyclohexylidene)pregn-4-ene-3,20-dione,

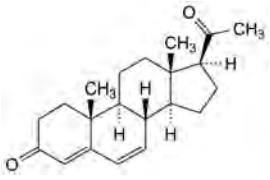

H. pregna-4,6-diene-3,20-dione (Δ6-progesterone),

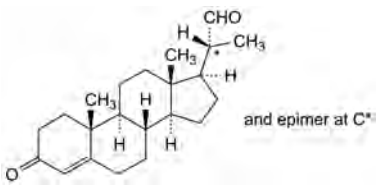


I. (20*RS*)-20-methyl-3-oxopregn-4-en-21-al,

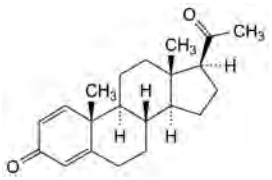

J. pregna-1,4-diene-3,20-dione,

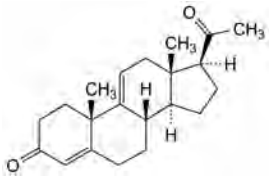

K. pregna-4,9(11)-diene-3,20-dione,

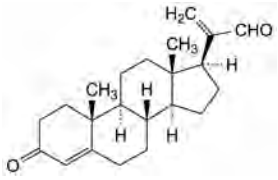

L. 20-methylidene-3-oxopregn-4-en-21-al,

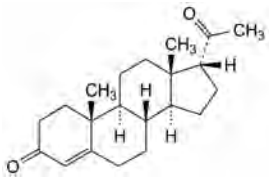

M. (17α)-pregn-4-ene-3,20-dione.



**04/2019:2002**

# PROGUANIL HYDROCHLORIDE

## Proguanili hydrochloridum

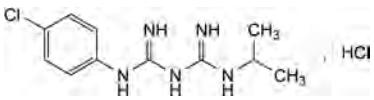

$C_{11}H_{17}Cl_2N_5$ $M_r$ 290.2
[637-32-1]

DEFINITION

$N^1$-(4-Chlorophenyl)-$N^3$-(propan-2-yl)imidodicarbonimidic diamide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *proguanil hydrochloride CRS*.

B. Dissolve 0.4 g in 50 mL of *water R* (solution A). To 15 mL of solution A add 2 mL of *dilute sodium hydroxide solution R*. Extract with 20 mL of *ethyl acetate R*. Wash the organic layer with *water R*, evaporate to dryness and dry at 105 °C. The melting point (*2.2.14*) of the residue is 130 °C to 133 °C.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Acidity or alkalinity**. To 35 mL of *water R* maintained at 60-65 °C, add 0.2 mL of *methyl red mixed solution R*. Neutralise to a grey colour with either *0.01 M sodium hydroxide* or *0.01 M hydrochloric acid*. Add 0.4 g of the substance to be examined and stir until completely dissolved. The solution is grey or green. Not more than 0.2 mL of *0.01 M hydrochloric acid* is required to change the colour of the solution to reddish-violet.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *methanol R*, *water R* (20:20:60 *V/V/V*).

*Test solution (a)*. Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Test solution (b)*. Dissolve 20 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *proguanil for system suitability CRS* (containing impurity G) in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 4.0 mg of *proguanil impurity B CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.075 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 0.65 g of *sodium pentanesulfonate R* and 7.0 g of *sodium perchlorate R* in *water for chromatography R* and dilute to 1000 mL with the same solvent; adjust to pH 3.0 with a 1 per cent *V/V* solution of *trifluoroacetic acid R*;
- *mobile phase B*: dissolve 0.65 g of *sodium pentanesulfonate R* and 7.0 g of *sodium perchlorate R* in a mixture of 20 volumes of *water for chromatography R*, 40 volumes of *acetonitrile for chromatography R* and 40 volumes of *methanol R1* and dilute to 1000 mL with the same solvent mixture; add 4 mL of a 1 per cent *V/V* solution of *trifluoroacetic acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 23 | 75 → 40 | 25 → 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *proguanil for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity G.

*Relative retention* with reference to proguanil (retention time = about 16 min): impurity G = about 1.05.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to proguanil.

*Calculation of percentage contents*:

– for each impurity, use the concentration of proguanil hydrochloride in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.2 per cent;
– *reporting threshold*: 0.05 per cent, except for impurity B.

**Impurity B**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*Relative retention* with reference to proguanil (retention time = about 16 min): impurity B = about 0.2.

*System suitability*: reference solution (c):

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity B.

*Limit*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (200 ppm).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Suspend 0.100 g in 20 mL of *anhydrous acetic acid R*, shake and heat at 50 °C for 5 min. Cool to room temperature and add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 14.51 mg of $C_{11}H_{17}Cl_2N_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, F, G.*

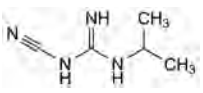

A. *N*-cyano-*N*′-(propan-2-yl)guanidine,

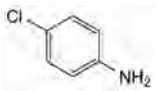

B. 4-chloroaniline,

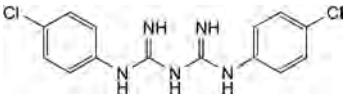

C. $N^1$,$N^3$-bis(4-chlorophenyl)imidodicarbonimidic diamide,

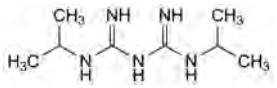

D. $N^1$,$N^3$-bis(propan-2-yl)imidodicarbonimidic diamide,

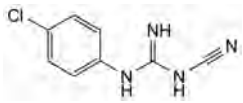

E. *N*-(4-chlorophenyl)-*N*′-cyanoguanidine,

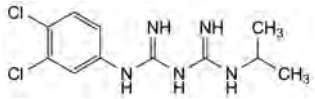

F. $N^1$-(3,4-dichlorophenyl)-$N^3$-(propan-2-yl)imidodicarbonimidic diamide,

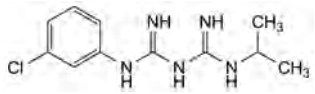

G. $N^1$-(3-chlorophenyl)-$N^3$-(propan-2-yl)imidodicarbonimidic diamide.

**01/2017:0785**

# PROLINE

## Prolinum

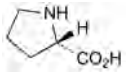

$C_5H_9NO_2$ $M_r$ 115.1
[147-85-3]

DEFINITION

(2*S*)-Pyrrolidine-2-carboxylic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *proline CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in a 1 per cent *V*/*V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Reference solution*. Dissolve 10 mg of *proline CRS* in a 1 per cent *V*/*V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V*/*V*/*V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): − 86.0 to − 84.0 (dried substance).

Dissolve 1.00 g in *water R* and dilute to 25.0 mL with the same solvent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dissolve 30.0 mg of *alanine R* (impurity A) in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of impurity A in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 11.51 mg of $C_5H_9NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*, *B*.

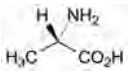


A. (2*S*)-2-aminopropanoic acid (alanine),

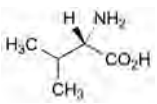


B. (2*S*)-2-amino-3-methylbutanoic acid (valine).

**01/2008:1365**
**corrected 6.0**

# PROMAZINE HYDROCHLORIDE

## Promazini hydrochloridum

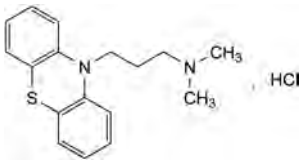

$C_{17}H_{21}ClN_2S$ $M_r$ 320.9
[53-60-1]

DEFINITION

Promazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3-(10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine hydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, slightly hygroscopic, very soluble in water, in alcohol and in methylene chloride.

It melts at about 179 °C.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *promazine hydrochloride CRS.*

B. It complies with the identification test for phenothiazines by thin-layer chromatography (*2.3.3*). Use *promazine hydrochloride CRS* to prepare the reference solution.

C. Dissolve about 5 mg in 2 mL of *sulfuric acid R* and allow to stand for 5 min. An orange colour is produced.

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*). Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. The pH of the freshly prepared solution is 4.2 to 5.2.

**Related substances**. *Carry out the test protected from bright light. Prepare the solutions immediately before use.*

Examine by thin layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R.*

*Test solution.* Dissolve 0.10 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 1 mL of the test solution to 200 mL with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R.*

*Reference solution (b).* Dissolve 10 mg of *chlorprothixene hydrochloride CRS* in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*, add 1 mL of the test solution and dilute to 10 mL with the same mixture of solvents.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R.* Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (a) (0.5 per cent). Disregard any spot at the point of application. The test is not valid unless the chromatogram obtained with reference solution (b) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *alcohol R.* Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide.* Read the volume added between the two points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.09 mg of $C_{17}H_{21}ClN_2S$.

STORAGE

Store protected from light.

IMPURITIES

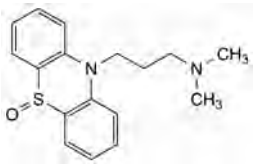

A. 3-(10*H*-phenothiazin-10-yl)-*N*,*N*-dimethylpropan-1-amine *S*-oxide (promazine sulfoxide).

**01/2017:0524**

# PROMETHAZINE HYDROCHLORIDE

## Promethazini hydrochloridum

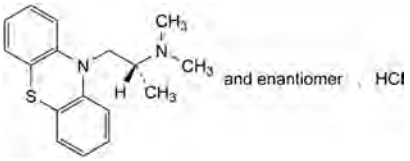

$C_{17}H_{21}ClN_2S$ $M_r$ 320.9
[58-33-3]

DEFINITION

(2*RS*)-*N*,*N*-Dimethyl-1-(10*H*-phenothiazin-10-yl)propan-2-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or faintly yellowish, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 222 °C, with decomposition.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *promethazine hydrochloride CRS.*

B. It complies with the identification test for phenothiazines by thin-layer chromatography (*2.3.3*): use *promethazine hydrochloride CRS* to prepare the reference solution.

C. Dissolve 0.1 g in 3 mL of *water R*. Add dropwise 1 mL of *nitric acid R*. A precipitate is formed which rapidly dissolves to give a red solution, becoming orange and then yellow. Heat to boiling. The solution becomes orange and an orange-red precipitate is formed.

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 4.0 to 5.0, measured immediately after preparation.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and use freshly prepared solutions.*

*Solvent mixture*: *triethylamine R*, *methanol R* (1:1000 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2.5 mg of *promethazine for peak identification CRS* (containing impurities A, B and C) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5.0 mg of *promethazine impurity D CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1 mL of this solution to 100 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm).

*Mobile phase*: mix 20 volumes of *methanol R*, 30 volumes of *acetonitrile R* and 50 volumes of a 3.4 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.0 with *potassium hydroxide R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of promethazine.

*Identification of impurities*: use the chromatogram supplied with *promethazine for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to promethazine (retention time = about 18 min): impurity D = about 0.2; impurity C = about 0.5; impurity B = about 1.4; impurity A = about 1.8.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurities B and A in the chromatogram obtained with reference solution (a);
- the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *promethazine for peak identification CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.5;
- *impurity B*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.8 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 12 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.09 mg of $C_{17}H_{21}ClN_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

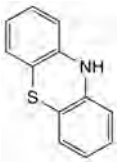


A. phenothiazine,

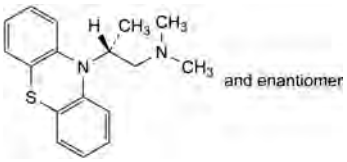


B. (2*RS*)-*N*,*N*-dimethyl-2-(10*H*-phenothiazin-10-yl)propan-1-amine (isopromethazine),

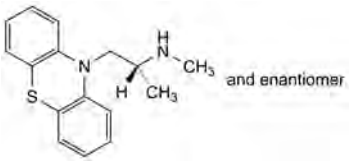


C. (2*RS*)-*N*-methyl-1-(10*H*-phenothiazin-10-yl)propan-2-amine,

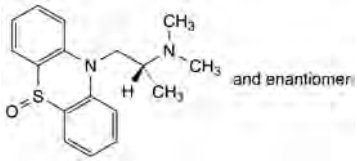


D. (2*RS*)-*N*,*N*-dimethyl-1-(10*H*-phenothiazin-10-yl)propan-2-amine *S*-oxide.

**01/2017:1366**

# PROPACETAMOL HYDROCHLORIDE

## Propacetamoli hydrochloridum

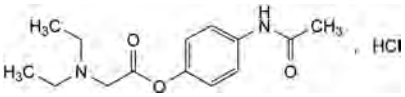

$C_{14}H_{21}ClN_2O_3$ $M_r$ 300.8
[66532-86-3]

DEFINITION

4-(Acetylamino)phenyl (diethylamino)acetate hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of propacetamol hydrochloride*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. *Prepare the solution immediately before use.* Dissolve 1.75 g in *water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*).

**Absorbance** (*2.2.25*): maximum 0.05, determined at 390 nm on solution S.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 4.00 g of the substance to be examined in 8 mL of *acetonitrile R*. Shake for 30 min and filter. Dilute to 10 mL with *acetonitrile R*.

*Reference solution (a).* Dissolve 25 mg of *4-aminophenol R* (impurity B) in *acetonitrile R* and dilute to 50 mL with the same solvent. Dilute 10 mL of this solution to 50 mL with *acetonitrile R*.

*Reference solution (b).* Dilute 5 mL of reference solution (a) to 50 mL with *acetonitrile R*.

*Reference solution (c).* Dilute 0.2 mL of reference solution (a) to 5 mL with the test solution.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *methylene chloride R* (3:4:30:64 *V/V/V/V*).

*Application*: 50 µL of the test solution and of reference solutions (b) and (c).

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm. Spray with a 10 g/L solution of *dimethylaminobenzaldehyde R* in *ethanol (96 per cent) R*.

*Identification of spots*: reference solution (c) shows 2 spots, one visible in ultraviolet light due to propacetamol and the other one yellow, visible after spraying due to impurity B. An additional spot may appear in ultraviolet light and corresponds to impurity A.

*System suitability*: reference solution (c):
– the chromatogram shows 2 clearly separated spots.

*Limit*:
– *impurity B*: any yellow spot due to impurity B not visible in ultraviolet light is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (25 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 2.16 g of *sodium octanesulfonate R* in 900 mL of *water R* and dilute to 1000 mL with the same solvent. Adjust to pH 3.0 with *acetic acid R*.

*Test solution.* Suspend 1.00 g of the substance to be examined in 10.0 mL of *acetonitrile R*. Shake for 10 min. Allow to stand. Take 3.0 mL of the supernatant solution and dilute to 10.0 mL with solution A. Inject immediately.

*Reference solution (a).* Dissolve 50 mg of *paracetamol R* (impurity A) in *acetonitrile R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *acetonitrile R*. Dilute 3.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 10 mg of *paracetamol R* (impurity A) and 0.100 g of *4-aminophenol R* (impurity B) in *acetonitrile R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *acetonitrile R*. Dilute 3.0 mL of this solution to 10.0 mL with solution A.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, solution A (30:70 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 246 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of propacetamol.

*Identification of impurities*: the chromatogram obtained with reference solution (b) shows a peak due to impurity A (1st peak) and a peak due to impurity B (2nd peak).

*Relative retention* with reference to impurity A: impurity B = about 1.6.

*Limits*:
– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (200 ppm);
– *unspecified impurities*: for each impurity, not more than 3.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent taking into account the response factor of paracetamol of 1.6);
– *total*: not more than 6.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent taking into account the relative response factor of paracetamol of 1.6);
– *disregard limit*: 0.01 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3 ppm taking into account the relative response factor of paracetamol of 1.6).

**Methanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 2.0 mL of *propanol R* to 20.0 mL with *water R*. Dilute 1.0 mL of the solution to 25.0 mL with *water R*. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

*Test solution.* Dissolve 2.00 g of the substance to be examined in *water R*, add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Reference solution.* Dilute 0.8 mL of *methanol R* to 50.0 mL with *water R*. Dilute 1.0 mL of the solution to 25.0 mL with *water R*. To 2.0 mL of this solution, add 2.0 mL of the internal standard solution and dilute to 10.0 mL with *water R*.

*Column*:

– *material*: glass;

– *size*: *l* = 2 m, Ø = 2 mm;

– *stationary phase*: carbon molecular sieve impregnated with 0.2 per cent of macrogol 1500.

*Carrier gas*: *nitrogen for chromatography R*.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1.5 | 60 |
| | 1.5 - 5.5 | 60 → 80 |
| | 5.5 - 15.5 | 80 |
| Injection port | | 170 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Limit*:

– *methanol*: calculate the ratio (*R*) of the area of the peak due to methanol to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to methanol to the area of the peak due to the internal standard: this ratio is not greater than *R* (500 ppm).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 25 mL of *anhydrous acetic acid R* and 25 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.08 mg of $C_{14}H_{21}ClN_2O_3$.

STORAGE

Protected from humidity.

IMPURITIES

*Specified impurities: A, B.*

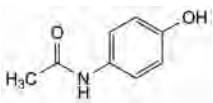

A. *N*-(4-hydroxyphenyl)acetamide (paracetamol),

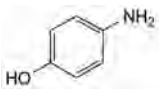

B. 4-aminophenol.

**01/2017:2103**

# PROPAFENONE HYDROCHLORIDE

## Propafenoni hydrochloridum

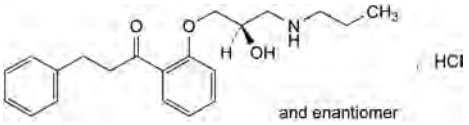


$C_{21}H_{28}ClNO_3$ $M_r$ 377.9

[34183-22-7]

DEFINITION

1-[2-[(2*RS*)-2-Hydroxy-3-(propylamino)propoxy]phenyl]-3-phenylpropan-1-one hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless crystals or white or almost white powder.

*Solubility*: slightly soluble in cold water, soluble in methanol and in hot water, practically insoluble in ethanol (96 per cent).

mp: about 173 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *propafenone hydrochloride CRS*.

B. To 5.0 mL of solution S (see Tests) add 2 drops of *dilute nitric acid R*. A precipitate is formed. After 10 min, filter. 2.0 mL of the clear filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. To 0.500 g in a 100 mL volumetric flask add 50 mL of *water R*, and heat to boiling for 5 min. Allow to cool to room temperature and dilute to 100.0 mL with *carbon dioxide-free water R*.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.2 for solution S.

**Optical rotation** (*2.2.7*): − 0.05° to + 0.05°.

Dissolve 1.00 g in *methanol R* and dilute to 100.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (35:65 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5.0 mg of the substance to be examined and 5.0 mg of *propafenone impurity B CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm,

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 320-350 $m^2/g$ and a pore size of 12-13 nm,

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: 3.42 g/L solution of *dipotassium hydrogen phosphate trihydrate R* adjusted to pH 2.5 with *phosphoric acid R*,

– *mobile phase B*: *acetonitrile for chromatography R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 65 | 35 |
| 8 - 20 | 65 → 30 | 35 → 70 |
| 20 - 30 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Equilibration*: 60 min with the mobile phase at the initial composition, before each series of injections.

*Injection*: 20 µL of the test solution, reference solutions (a), (b) and (c) and of the solvent mixture as a blank.

*Relative retention* with reference to propafenone (retention time = about 5 min): impurity B = about 0.8; impurity D = about 2.3; impurity G = about 3.6; impurity C = about 4.1; impurity F = about 5.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to impurity B and propafenone.

*Limits*:

– *impurities B, C, D, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),

– *disregard limit*: 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.300 g in 2 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 37.79 mg of $C_{21}H_{28}ClNO_3$.

IMPURITIES

*Specified impurities*: *B, C, D, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, E, H.*

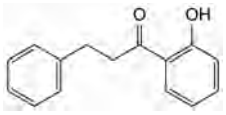

A. 1-(2-hydroxyphenyl)-3-phenylpropan-1-one,

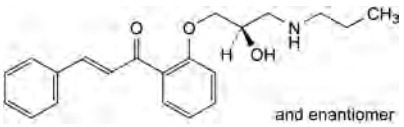


B. (2*E*)-1-[2-[(2*RS*)-2-hydroxy-3-(propylamino)-propoxy]phenyl]-3-phenylprop-2-en-1-one,

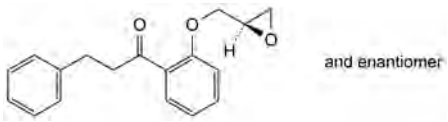


C. 1-[2-[[(2*RS*)-oxiranyl]methoxy]phenyl]-3-phenylpropan-1-one,

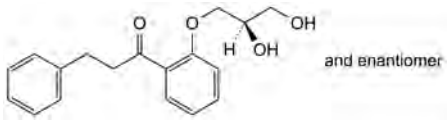


D. 1-[2-[(2*RS*)-2,3-dihydroxypropoxy]phenyl]-3-phenylpropan-1-one,

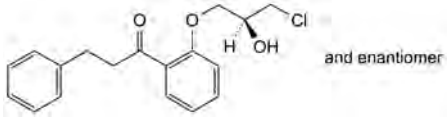


E. 1-[2-[(2*RS*)-3-chloro-2-hydroxypropoxy]phenyl]-3-phenylpropan-1-one,

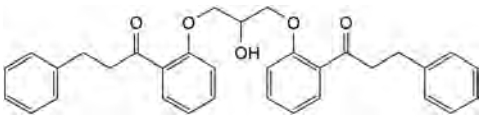

F. 1,1′-[2-hydroxypropane-1,3-diylbis(oxy-2,1-phenylene)]bis(3-phenylpropan-1-one),

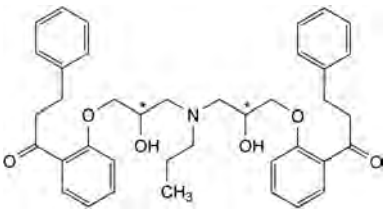

G. 1,1′-[propyliminobis[(2-hydroxypropane-3,1-diyl)oxy-2,1-phenylene]]bis(3-phenylpropan-1-one),

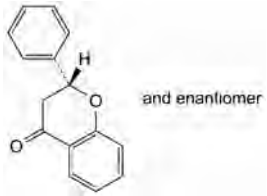


H. (2*RS*)-2-phenyl-2,3-dihydro-4*H*-1-benzopyran-4-one.

**01/2008:2036**
**corrected 10.0**

# PROPANOL

## Propanolum

$C_3H_8O$ $M_r$ 60.1
[71-23-8]

DEFINITION

Propan-1-ol.

CHARACTERS

*Appearance*: clear, colourless liquid.

*Solubility*: miscible with water and with ethanol.

IDENTIFICATION

*First identification: C, B.*

*Second identification: A, B, D.*

A. Refractive index (*2.2.6*): 1.384 to 1.387.

B. Boiling point (*2.2.12*): 96 °C to 98 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of propanol.*

D. To 1.0 mL add 0.10 g of *dinitrobenzoyl chloride R* and 0.05 mL of *sulfuric acid R*. Boil under reflux for 30 min. Evaporate until the excess of propanol is removed, add 5 mL of *heptane R* to the residue and heat to boiling. Filter the hot solution. Wash the crystals formed on cooling with *heptane R* and dry in vacuum (2 kPa, at room temperature for 24 h). The small, colourless, shiny plates melt (*2.2.14*) between 71 °C and 74 °C.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*). Dilute 2 mL to 10 mL with *water R*. After 5 min, the solution is clear (*2.2.1*).

**Acidity or alkalinity**. To 10.0 mL of *carbon dioxide-free water R* add 0.1 mL of *phenolphthalein solution R* and *0.01 M sodium hydroxide* until the solution becomes pale pink. After addition of 5.0 mL of the substance to be examined the colour of the solution does not become more intense. If the colour fades, add 0.2 mL of *0.01 M sodium hydroxide*. The solution is pink.

**Absorbance** (*2.2.25*). Measure the absorbance between 230 nm and 310 nm using *water R* as the compensation liquid. The absorbance *A* is not greater than the following values.

| Wavelength (nm) | Absorbance *A* |
|---|---|
| 230 | 0.300 |
| 250 | 0.100 |
| 270 | 0.030 |
| 290 | 0.020 |
| 310 | 0.010 |

The absorption curve does not show any peaks.

**Reducing substances**. Place 10.0 mL in a test tube of about 20 mm in diameter in a water bath at 20 °C. Keep protected from actinic light and add 1.0 mL of a freshly prepared 0.16 g/L solution of *potassium permanganate R*. The mixture, maintained at 20 °C, slowly changes its colour from violet to red. After 30 min, the test solution is not less intensely coloured (*2.2.2, Method II*) than 10.0 mL of a reference solution prepared as follows: to 5.5 mL of primary solution yellow, add 13.0 mL of primary solution red and dilute to 100.0 mL with *water R*.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution.* The substance to be examined.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *heptane R*. Dilute 1.0 mL of this solution to 10.0 mL with *heptane R*.

*Reference solution (b).* Mix 0.1 mL of *acetone R* and 0.1 mL of *2-propanol R* and dilute to 100 mL with the test solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.4 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: 25 cm/s.

*Split ratio*: 1:200.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 40 |
| | 12 - 28 | 40 → 200 |
| | 28 - 38 | 200 |
| Injection port | | 240 |
| Detector | | 240 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurities D and E.

*Limits*:
- *any impurity*: not more than the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 3 times the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.1 times the area of the peak due to propanol in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Non-volatile matter**: maximum 0.004 per cent.

Evaporate 50 mL of the substance to be examined to dryness at 100 °C and dry the residue in an oven at 100-105 °C to constant mass. The residue weighs a maximum of 2 mg.

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 10 g.

STORAGE

Protected from light.

IMPURITIES

A. methanol,

B. ethanol,

C. propanal,

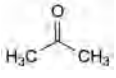

D. propanone (acetone),

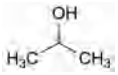

E. isopropyl alcohol (2-propanol),

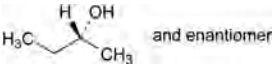


F. butan-2-ol (*sec*-butanol),

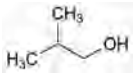

G. 2-methylpropan-1-ol (isobutanol),

H. butan-1-ol (*n*-butanol),

I. pentan-1-ol (*n*-pentanol),

J. hexan-1-ol (*n*-hexanol).

**01/2008:0857**
**corrected 6.0**

# PROPANTHELINE BROMIDE

## Propanthelini bromidum

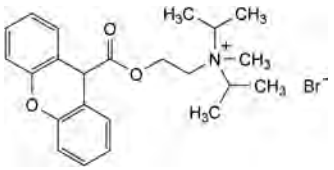

$C_{23}H_{30}BrNO_3$ $M_r$ 448.4
[50-34-0]

DEFINITION

*N*-Methyl-*N*,*N*-bis(1-methylethyl)-2-[(9*H*-xanthen-9-ylcarbonyl)oxy]ethanaminium bromide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, slightly hygroscopic powder.

*Solubility*: very soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 60 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 246 nm and 282 nm.

*Specific absorbance at the absorption maxima*:

– at 246 nm: 115 to 125;

– at 282 nm: 57 to 63.

B. Dissolve 0.2 g in 15 mL of *water R* and add 1 mL of *strong sodium hydroxide solution R*. Boil for 2 min and cool slightly. Add 7.5 mL of *dilute hydrochloric acid R* and filter. Wash the residue with *water R* and recrystallise from *ethanol (50 per cent V/V) R*. Dry at 100-105 °C for 1 h. Dissolve about 10 mg of the residue in 5 mL of *sulfuric acid R*. The solution has an intense yellow colour and shows an intense yellowish-green fluorescence when examined in ultraviolet light at 365 nm.

C. Dissolve 50 mg in 0.1 mL of *water R* in a 25 mL flask and add 1 mL of a saturated solution of *potassium permanganate R*. Attach a fractionating column and a condenser, with the end of the delivery tube immersed in 1 mL of *water R* in a test-tube placed in a bath of iced water. Distil fairly vigorously and continue heating for 1 min after a dry residue has been obtained in the flask. Prepare a blank by introducing into an identical test-tube a volume of *water R* equal to that of the distillate. Place the tubes in a bath of iced water. To each tube, add 0.5 mL of a 20 per cent *V/V* solution of *morpholine R* and 0.5 mL of a freshly prepared 50 g/L solution of *sodium nitroprusside R*. Mix and allow to stand at 0 °C for 5 min, and then at room temperature for 3 min. No blue colour develops in either tube. Add 1 g of *ammonium sulfate R*, mix and allow to stand for 15 min. A stable, intense pink colour develops in the test solution. A brownish-yellow colour develops in the blank.

D. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.6 g in *water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution (a)*. Dissolve 6 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Test solution (b)*. Dissolve 6 mg of the substance to be examined in 30 mL of the solvent mixture. Add 5 mL of reference solution (b) and dilute to 50 mL with the solvent mixture.

*Test solution (c)*. Dissolve 6 mg of *xanthydrol R1* and 6 mg of the substance to be examined in the solvent mixture, then dilute to 50 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 6 mg of *xanthydrol R1* in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5 mL of reference solution (a) to 50 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mixture of equal volumes of *acetonitrile R* and of a solution containing 28 g/L of *sodium perchlorate R* and 11 g/L of *phosphoric acid R*, adjusted to pH 3.8 with *strong sodium hydroxide solution R* and then with *0.1 M sodium hydroxide*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 206 nm.

*Injection*: 20 µL of test solutions (a), (b), (c) and reference solution (a).

*Run time*: twice the retention time of propantheline.

*System suitability*: test solution (c):

– in the chromatogram obtained with test solution (a), there is no peak corresponding to the principal peak in the chromatogram obtained with reference solution (a);

– *resolution*: minimum 8.0 between the peaks due to propantheline and xanthydrol.

*Limits*: test solution (b):

- *any impurity*: for each impurity, not more than the area of the peak due to xanthydrol (1.0 per cent), and not more than one such peak has an area greater than or equal to 0.5 times the area of the peak due to xanthydrol (0.5 per cent);
- *disregard limit*: disregard any peak with a retention time relative to propantheline of less than 0.2 (bromide); disregard the peak due to xanthydrol.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* corresponds to 44.84 mg of $C_{23}H_{30}BrNO_3$.

STORAGE

In an airtight container.

**01/2008:1558**
**corrected 10.0**

# PROPOFOL

## Propofolum

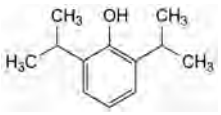

$C_{12}H_{18}O$ $M_r$ 178.3
[2078-54-8]

DEFINITION

2,6-Bis(1-methylethyl)phenol.

*Content*: 98.0 per cent to 102.0 per cent.

This monograph applies to propofol prepared using distillation for purification.

CHARACTERS

*Appearance*: colourless or very light yellow, clear liquid.

*Solubility*: very slightly soluble in water, miscible with hexane and with methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *propofol CRS*.

TESTS

**Refractive index** (*2.2.6*): 1.5125 to 1.5145.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 1.00 g of the substance to be examined in *hexane R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dissolve 0.240 g of the substance to be examined in *hexane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5 µL of the substance to be examined and 15 µL of *propofol impurity J CRS* in *hexane R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b).* Dilute 0.1 mL of *propofol for peak identification CRS* (containing impurities E and G) to 1.0 mL with *hexane R*.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *hexane R*. Dilute 1.0 mL of this solution to 10.0 mL with *hexane R*.

*Reference solution (d).* Dissolve 0.240 g of *propofol CRS* in *hexane R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.20 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *anhydrous ethanol R*, *acetonitrile R*, *hexane R* (1.0:7.5:990 *V/V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: 7 times the retention time of propofol.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities G and E.

*Relative retention* with reference to propofol (retention time = about 3 min): impurity G = about 0.5; impurity I = about 0.6; impurity B = about 0.7; impurity N = about 2.3; impurity D = about 2.5; impurity P = about 2.9; impurity A = about 3.0; impurity C = about 3.4; impurity E = about 4.0; impurity F = about 5.8; impurity H = about 6.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to impurity J and propofol.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity E = 0.25; impurity G = 5.0;
- *impurity G*: not more than twice the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity E*: not more than 0.1 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.01 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.05 per cent);
- *total*: not more than 3 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *disregard limit*: 0.3 times the area of the peak due to propofol in the chromatogram obtained with reference solution (c) (0.03 per cent), except for impurity E.

**Impurities J, K, L and O**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *methylene chloride R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 10.0 mL with *methylene chloride R*.

*Reference solution (b).* Dissolve 5 µL of *propofol impurity J CRS* (corresponding to 5 mg) in *methylene chloride R* and dilute to 100 mL with the same solvent. Dilute 1.0 mL of this solution to 25 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve 4 mg of *propofol CRS* in reference solution (b) and dilute to 1 mL with the same solution.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1.7 mL/min.

*Split ratio*: 1:5.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 80 |
| | 3 - 25 | 80 → 210 |
| | 25 - 40 | 210 |
| Injection port | | 100 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solutions (a) and (c).

*Relative retention* with reference to propofol (retention time = about 17 min): impurity K = about 0.76; impurity L = about 0.81; impurity J = about 1.01; impurity O = about 1.03.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity J, and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to propofol.

*Limits*:

– *impurities J, K, L, O*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{12}H_{18}O$ using the declared content of *propofol CRS*.

STORAGE

Protected from light under an inert gas.

IMPURITIES

*Specified impurities: E, G, J, K, L, O.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, H, I, N, P.*

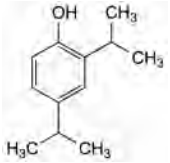

A. 2,4-bis(1-methylethyl)phenol,

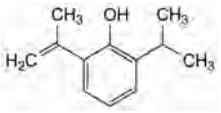

B. 2-(1-methylethenyl)-6-(1-methylethyl)phenol,

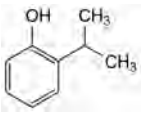

C. 2-(1-methylethyl)phenol,

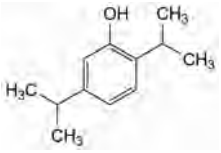

D. 2,5-bis(1-methylethyl)phenol,

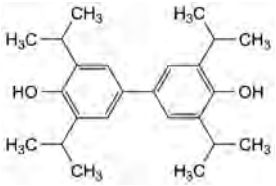

E. 3,3′,5,5′-tetrakis(1-methylethyl)biphenyl-4,4′-diol,

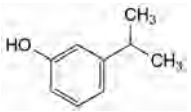

F. 3-(1-methylethyl)phenol,

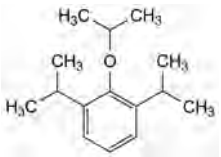

G. 2-(1-methylethoxy)-1,3-bis(1-methylethyl)benzene,

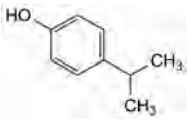

H. 4-(1-methylethyl)phenol,

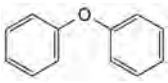

I. oxydibenzene,

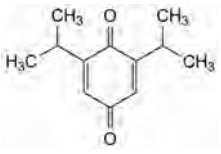

J. 2,6-bis(1-methylethyl)benzene-1,4-dione,

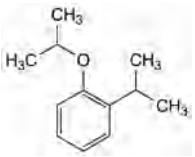

K. 1-(1-methylethoxy)-2-(1-methylethyl)benzene,

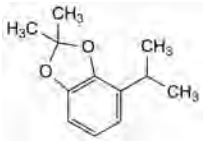

L. 2,2-dimethyl-4-(1-methylethyl)-1,3-benzodioxole,

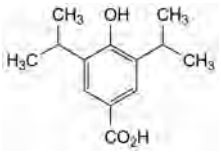

N. 4-hydroxy-3,5-bis(1-methylethyl)benzoic acid,

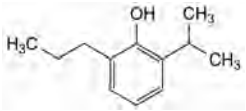

O. 2-(1-methylethyl)-6-propylphenol,

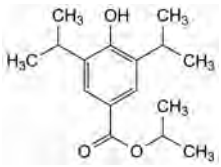

P. 1-methylethyl 4-hydroxy-3,5-bis(1-methylethyl)benzoate.

**01/2020:0568**

# PROPRANOLOL HYDROCHLORIDE

## Propranololi hydrochloridum

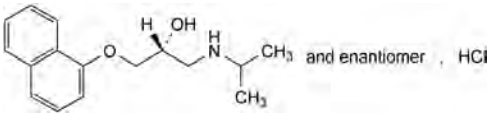


$C_{16}H_{22}ClNO_2$ $M_r$ 295.8
[318-98-9]

DEFINITION

(2*RS*)-1-[(Propan-2-yl)amino]-3-[(naphthalen-1-yl)oxy]propan-2-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water and in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

*First identification*: B, D.

*Second identification*: A, C, D.

A. Melting point (*2.2.14*): 163 °C to 166 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *propranolol hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in 1 mL of *methanol R*.
*Reference solution*. Dissolve 10 mg of *propranolol hydrochloride CRS* in 1 mL of *methanol R*.
*Plate*: *TLC silica gel G plate R*.
*Mobile phase*: *concentrated ammonia R1*, *methanol R* (1:99 *V/V*).
*Application*: 10 µL.
*Development*: over 3/4 of the plate.
*Drying*: at 100-105 °C.
*Detection*: spray with *anisaldehyde solution R* and heat at 100-105 °C until the colour of the spots reaches maximum intensity (10-15 min).
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 2.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent. Add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*; the solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*; the solution is yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10 mg of *propranolol for system suitability CRS* (containing impurity A) in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m; Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 0.31 g of *tetrabutylammonium dihydrogen phosphate R* and 1.6 g of *sodium laurilsulfate R* in a mixture of 1 mL of *sulfuric acid R*, 450 mL of *water for chromatography R* and 550 mL of *acetonitrile R*; adjust to pH 3.3 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 292 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of propranolol.

*Identification of impurities*: use the chromatogram supplied with *propranolol for system suitability CRS* and the chromatogram obtained with reference solution (a)to identify the peak due to impurity A.

*Relative retention* with reference to propranolol (retention time = about 3 min): impurity A = about 0.6.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity A and propranolol.

*Calculation of percentage contents*:
- for each impurity, use the concentration of propranolol hydrochloride in reference solution (b).

*Limits*:
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 29.58 mg of $C_{16}H_{22}ClNO_2$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

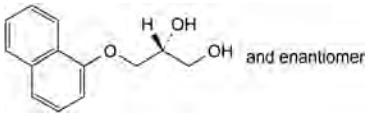


A. (2*RS*)-3-[(naphthalen-1-yl)oxy]propane-1,2-diol,

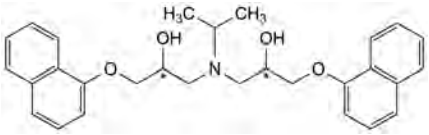


B. 1,1′-[(propan-2-yl)azanediyl]bis[(2Ξ)-3-[(naphthalen-1-yl)oxy]propan-2-ol,

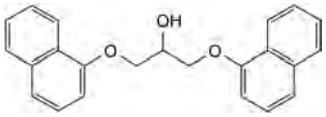


C. 1,3-bis[(naphthalen-1-yl)oxy]propan-2-ol.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 148 °C to 151 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *propyl gallate for ID and assay CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 5 mg of *propyl gallate for ID and assay CRS* in *acetone R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *anhydrous formic acid R*, *ethyl formate R*, *toluene R* (10:40:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air for 10 min.

*Detection*: spray with a mixture of 1 volume of *ferric chloride solution R1* and 9 volumes of *ethanol (96 per cent) R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 10 mL of *water R* by heating to about 70 °C. Cool and add 1 mL of *bismuth subnitrate solution R*. A bright yellow precipitate is formed.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**01/2020:1039**

# PROPYL GALLATE

## Propylis gallas

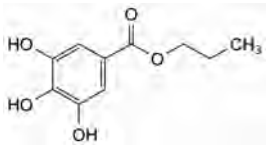


$C_{10}H_{12}O_5$ $M_r$ 212.2

[121-79-9]

DEFINITION

Propyl 3,4,5-trihydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dilute 2.0 mL of test solution (a) to 20.0 mL with *methanol R*.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 25.0 mg of *propyl gallate for ID and assay CRS* in *methanol R* and dilute to 25.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve 25 mg of *methyl parahydroxybenzoate R* in *methanol R* and dilute to 25 mL with the same solvent. Mix 1 mL of the solution and 1 mL of test solution (a), and dilute to 100 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with embedded polar groups R* (3.5 µm);
– *temperature*: 28 °C.

*Mobile phase*:

– *mobile phase A*: 6.8 g/L solution of *potassium dihydrogen phosphate R*;
– *mobile phase B*: *acetonitrile R*;

Monographs p

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 95 | 5 |
| 6 - 25 | 95 → 45 | 5 → 55 |
| 25 - 40 | 45 | 55 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (c).

*Identification of peaks*: use the chromatogram obtained with reference solution (c) to identify the peak due to methyl parahydroxybenzoate.

*Relative retention* with reference to propyl gallate (retention time = about 20 min): methyl parahydroxybenzoate = about 1.05.

*System suitability*: reference solution (c):

– *resolution*: minimum 4.0 between the peaks due to propyl gallate and methyl parahydroxybenzoate.

*Calculation of percentage contents*:

– for each impurity, use the concentration of propyl gallate in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.15 per cent;
– *reporting threshold*: 0.05 per cent.

**Total chlorine**: maximum 200 ppm.

Mix 0.5 g with 2 g of *calcium carbonate R1*. Dry and ignite at 700 ± 50 °C. Take up the residue with 20 mL of *dilute nitric acid R* and dilute to 30 mL with *water R*. 15 mL of the solution, without further addition of *dilute nitric acid R*, complies with the test for chlorides (*2.4.4*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

To 1.65 g add 50 mL of *water R*. Shake for 5 min. Filter.

**Zinc**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* To 2.5 mL of the test solution prepared as described in general chapter *2.4.8* (method C) using 2.0 g of the substance to be examined, add 2.5 mL of *water R*.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *water R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_{10}H_{12}O_5$ taking into account the assigned content of *propyl gallate for ID and assay CRS*.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

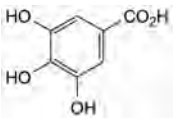


A. 3,4,5-trihydroxybenzoic acid (gallic acid).

**07/2010:0431**

# PROPYL PARAHYDROXYBENZOATE

## Propylis parahydroxybenzoas

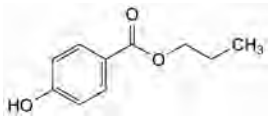


$C_{10}H_{12}O_3$ $M_r$ 180.2
[94-13-3]

DEFINITION

Propyl 4-hydroxybenzoate.

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 96 °C to 99 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *propyl parahydroxybenzoate CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.
*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.
*Reference solution (a).* Dissolve 10 mg of *propyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).
*Application*: 2 µL of test solution (b) and reference solutions (a) and (b).
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. To 2 mL of solution S add 3 mL of *ethanol (96 per cent) R*, 5 mL of *carbon dioxide-free water R* and 0.1 mL of *bromocresol green solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A), 5 mg of *ethyl parahydroxybenzoate R* (impurity C) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50.0 mg of *propyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 2.5 times the retention time of propyl parahydroxybenzoate.

*Relative retention* with reference to propyl parahydroxybenzoate (retention time = about 4.5 min): impurity A = about 0.3; impurity C = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity C and propyl parahydroxybenzoate.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{10}H_{12}O_3$ from the declared content of *propyl parahydroxybenzoate CRS*.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D*.

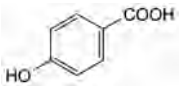


A. 4-hydroxybenzoic acid,

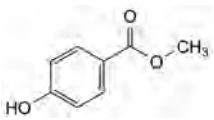


B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

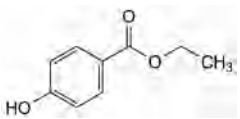


C. ethyl 4-hydroxybenzoate (ethyl parahydroxybenzoate),

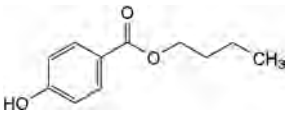


D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

**01/2017:0430**

# PROPYLENE GLYCOL

## Propylenglycolum

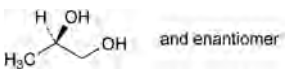


$C_3H_8O_2$ $M_r$ 76.1

[57-55-6]

DEFINITION

Propylene glycol is (*RS*)-propane-1,2-diol.

CHARACTERS

A viscous, clear, colourless, hygroscopic liquid, miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. Relative density (see Tests).

B. Refractive index (see Tests).

C. Boiling point (*2.2.12*): 184 °C to 189 °C.

D. To 0.5 mL add 5 mL of *pyridine R* and 2 g of finely ground *nitrobenzoyl chloride R*. Boil for 1 min and pour into 15 mL of cold *water R* with shaking. Filter, wash the precipitate with 20 mL of a saturated solution of *sodium hydrogen carbonate R* and then with *water R* and dry. Dissolve in boiling *ethanol (80 per cent V/V) R* and filter the hot solution. On cooling, crystals are formed which, after drying at 100-105 °C, melt (*2.2.14*) at 121 °C to 128 °C.

TESTS

**Appearance**. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Relative density** (*2.2.5*): 1.035 to 1.040.

**Refractive index** (*2.2.6*): 1.431 to 1.433.

**Acidity**. To 10 mL add 40 mL of *water R* and 0.1 mL of *bromothymol blue solution R1*. The solution is greenish-yellow. Not more than 0.05 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Oxidising substances**. To 10 mL add 5 mL of *water R*, 2 mL of *potassium iodide solution R* and 2 mL of *dilute sulfuric acid R* and allow to stand in a ground-glass-stoppered flask protected from light for 15 min. Titrate with *0.05 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator. Not more than 0.2 mL of *0.05 M sodium thiosulfate* is required.

**Reducing substances**. To 1 mL add 1 mL of *dilute ammonia R1* and heat in a water-bath at 60 °C for 5 min. The solution is not yellow. Immediately add 0.15 mL of *0.1 M silver nitrate* and allow to stand for 5 min. The solution does not change its appearance.

**Water** (*2.5.12*). Not more than 0.2 per cent, determined on 5.00 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Heat 50 g until it burns and ignite. Allow to cool. Moisten the residue with *sulfuric acid R* and ignite; repeat the operations. The residue weighs not more than 5 mg (0.01 per cent).

STORAGE

Store in an airtight container.

**01/2016:2122**

# PROPYLENE GLYCOL DICAPRYLOCAPRATE

## Propylenglycoli dicaprylocapras

DEFINITION

Propylene glycol diesters of saturated fatty acids, mainly caprylic (octanoic) acid and capric (decanoic) acid, of vegetable origin.

CHARACTERS

*Appearance*: almost colourless or light yellow, oily liquid.

*Solubility*: practically insoluble in water, soluble in fatty oils and in light petroleum, slightly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, C, E.*

A. Refractive index (*2.2.6*): 1.439 to 1.442.

B. Relative density (*2.2.5*): 0.910 to 0.930.

C. Viscosity (*2.2.9*): 9 mPa·s to 13 mPa·s.

D. Composition of fatty acids (see Tests).

E. Saponification value (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acid value** (*2.5.1*): maximum 0.2.

**Hydroxyl value** (*2.5.3, Method A*): maximum 10.

**Iodine value** (*2.5.4*): maximum 1.0.

**Peroxide value** (*2.5.5, Method A*): maximum 1.0.

**Saponification value** (*2.5.6*): 315 to 340.

**Alkaline impurities**. Dissolve 2.00 g of the substance to be examined in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3.0 mL of *ether R*. Add 0.05 mL of *bromophenol blue solution R*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*). Prepare reference solution (a) as indicated in Table 2.4.22.-2.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 70 |
| | 1 - 35 | 70 → 240 |
| | 35 - 50 | 240 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Composition of the fatty-acid fraction of the substance*:

– *caproic acid*: maximum 2.0 per cent;

– *caprylic acid*: 50.0 per cent to 80.0 per cent;

– *capric acid*: 20.0 per cent to 50.0 per cent;

– *lauric acid*: maximum 3.0 per cent;

– *myristic acid*: maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 0.1 per cent, determined on 5.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

Protected from light.

**07/2017:2087**

# PROPYLENE GLYCOL DILAURATE

## Propylenglycoli dilauras

DEFINITION

Mixture of propylene glycol ((2*RS*)-propane-1,2-diol) mono- and diesters of lauric (dodecanoic) acid.

*Content*: minimum 70.0 per cent of diesters and maximum 30.0 per cent of monoesters.

CHARACTERS

*Appearance*: clear, oily liquid at 20 °C, colourless or slightly yellow.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in *methylene chloride R* and dilute to 2 mL with the same solvent.

*Reference solution.* Dissolve 0.1 g of *propylene glycol dilaurate CRS* in *methylene chloride R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine 6 G R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of diesters).

TESTS

**Acid value** (*2.5.1*): maximum 4.0, determined on 5.00 g.

**Iodine value** (*2.5.4, Method A*): maximum 1.0.

**Saponification value** (*2.5.6*): 230 to 250.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-2.

*Composition of the fatty acid fraction of the substance:*

- *caprylic acid*: maximum 0.5 per cent;
- *capric acid*: maximum 2.0 per cent;
- *lauric acid*: minimum 95.0 per cent;
- *myristic acid*: maximum 3.0 per cent;
- *palmitic acid*: maximum 1.0 per cent.

**Free propylene glycol**: maximum 2.0 per cent, determined as prescribed under Assay.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Stock solution.* Introduce 0.100 g of *propylene glycol R* into a flask and dilute to 25.0 mL with *tetrahydrofuran R*.

*Test solution.* In a 15 mL flask, weigh 0.200 g (*m*). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* Into four 15 mL flasks, introduce respectively 0.25 mL, 0.5 mL, 1.0 mL and 2.5 mL of stock solution and add 5.0 mL of *tetrahydrofuran R*. Weigh each flask and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7 mm,
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to propylene glycol: diesters = about 0.85; monoesters = about 0.90.

*Calculations*:

- *free propylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration of propylene glycol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free propylene glycol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 200}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

200 = rounded molar mass of lauric acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y}\ (100 - A - B)\right] - D$$

$A$ = percentage content of free propylene glycol;

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters.

- *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{Y}{X+Y}\ (100 - A - B)$$

STORAGE

Protected from moisture.

**01/2017:1915**

# PROPYLENE GLYCOL MONOLAURATE

## Propylenglycoli monolauras

DEFINITION

Mixture of propylene glycol ((2*RS*)-propane-1,2-diol) mono- and diesters of lauric (dodecanoic) acid.

*Content*:

- *propylene glycol monolaurate (type I)*: 45.0 per cent to 70.0 per cent of monoesters and 30.0 per cent to 55.0 per cent of diesters,
- *propylene glycol monolaurate (type II)*: minimum 90.0 per cent of monoesters and maximum 10.0 per cent of diesters.

CHARACTERS

*Appearance*: clear, oily liquid at 20 °C, colourless or slightly yellow.

*Solubility*: practically insoluble in water, very soluble in ethanol (96 per cent), in methanol and in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in *methylene chloride R* and dilute to 2 mL with the same solvent.

*Reference solution.* Dissolve 0.1 g of *propylene glycol monolaurate CRS* in *methylene chloride R* and dilute to 2 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine 6 G R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the spots in the chromatogram obtained with the test solution are similar in position to those in the chromatogram obtained with the reference solution.

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of monoesters).

TESTS

**Acid value** (*2.5.1*): maximum 4.0, determined on 5.00 g.

**Iodine value** (*2.5.4, Method A*): maximum 1.0.

**Saponification value** (*2.5.6*): 210 to 245 for propylene glycol monolaurate (type I) and 200 to 230 for propylene glycol monolaurate (type II).

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-2.

*Composition of the fatty acid fraction of the substance:*

- *caprylic acid*: maximum 0.5 per cent;
- *capric acid*: maximum 2.0 per cent;
- *lauric acid*: minimum 95.0 per cent;
- *myristic acid*: maximum 3.0 per cent;
- *palmitic acid*: maximum 1.0 per cent.

**Free propylene glycol**: maximum 5.0 per cent for propylene glycol monolaurate (type I) and maximum 1.0 per cent for propylene glycol monolaurate (type II), determined as prescribed under Assay.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Stock solution.* Introduce 0.100 g of *propylene glycol R* into a vial and dilute to 25.0 mL with *tetrahydrofuran R*.

*Test solution.* In a 15 mL flask, weigh 0.200 g ($m$). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Reweigh the flask and calculate the total mass of solvent and substance ($M$).

*Reference solutions.* Into four 15 mL flasks, introduce respectively 0.25 mL, 0.5 mL, 1.0 mL and 2.5 mL of stock solution and add 5.0 mL of *tetrahydrofuran R*. In a fifth 15 mL flask, introduce 5.0 mL of stock solution. Weigh each flask and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

*Column*:

- *size*: $l$ = 0.6 m, Ø = 7 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to propylene glycol: diesters = about 0.85; monoesters = about 0.90.

*Calculations*:

- *free propylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration of propylene glycol ($C$) in milligrams per gram in the test solution and calculate the percentage content of free propylene glycol ($A$) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

- *free fatty acids*: calculate the percentage content of free fatty acids ($D$) using the following expression:

$$\frac{I_A \times 200}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

200 = rounded molar mass of lauric acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y}\,(100 - A - B)\right] - D$$

$A$ = percentage content of free propylene glycol;

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters.

- *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{Y}{X+Y}\,(100 - A - B)$$

STORAGE

Protected from moisture.

LABELLING

The label states the type of propylene glycol monolaurate (type I or type II).

**01/2020:1469**

# PROPYLENE GLYCOL MONOPALMITOSTEARATE

## Propylenglycoli monopalmitostearas

DEFINITION

Mixture of propylene glycol ((2*RS*)-propane-1,2-diol) mono- and diesters of palmitic (hexadecanoic) and stearic (octadecanoic) acids, produced by the condensation of propylene glycol and stearic acid 50 of vegetable or animal origin (see *Stearic acid (1474)*).

*Content*: minimum 50.0 per cent of monoesters.

CHARACTERS

*Appearance*: white or almost white, waxy solid.

*Solubility*: practically insoluble in water, soluble in acetone and in hot ethanol (96 per cent).

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

C. It complies with the assay (content of monoesters).

TESTS

**Melting point** (*2.2.15*): 33 °C to 40 °C.

**Acid value** (*2.5.1*): maximum 4.0.

**Iodine value** (*2.5.4*): maximum 3.0.

**Saponification value** (*2.5.6*): 170 to 185, determined on 2.0 g.

**Composition of fatty acids** (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

– *stearic acid*: 40.0 per cent to 60.0 per cent;

– *sum of contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

**Free propylene glycol**: maximum 5.0 per cent, determined as prescribed under Assay.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of equal volumes of *methanol R* and *methylene chloride R* as the solvent.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*).

*Test solution.* In a 15 mL flask, weigh 0.200 g (*m*). Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Heat gently, if necessary. Reweigh the flask and calculate the total mass of solvent and substance (*M*).

*Reference solutions.* In four 15 mL flasks, weigh respectively 2.5 mg, 5.0 mg, 10.0 mg and 20.0 mg of *propylene glycol R*. Add 5.0 mL of *tetrahydrofuran R* and shake to dissolve. Weigh the flasks again and calculate the concentration of propylene glycol in milligrams per gram for each reference solution.

*Column*:

– *size*: *l* = 0.6 m, Ø = 7.5 mm;

– *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 40 µL.

*Relative retention* with reference to propylene glycol: diesters = about 0.78; monoesters = about 0.84.

*Calculations*:

– *free propylene glycol*: from the calibration curve obtained with the reference solutions, determine the concentration of propylene glycol (*C*) in milligrams per gram in the test solution and calculate the percentage content of free propylene glycol (*A*) in the substance to be examined using the following expression:

$$\frac{C \times M}{m \times 10}$$

– *free fatty acids*: calculate the percentage content of free fatty acids (*D*) using the following expression:

$$\frac{I_A \times 270}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

270 = average rounded molar mass of palmitic acid and stearic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

– *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y}(100 - A - B)\right] - D$$

$A$ = percentage content of free propylene glycol;

$B$ = percentage content of water (see Tests);

$D$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters.

STORAGE

Protected from light.

**01/2017:0525**

# PROPYLTHIOURACIL

## Propylthiouracilum

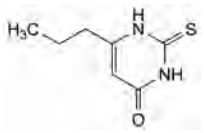


$C_7H_{10}N_2OS$ $M_r$ 170.2

[51-52-5]

DEFINITION

Propylthiouracil contains not less than 98.0 per cent and not more than the equivalent of 100.5 per cent of 2,3-dihydro-6-propyl-2-thioxopyrimidin-4(1*H*)-one, calculated with reference to the dried substance.

CHARACTERS

White or almost white, crystalline powder or crystals, very slightly soluble in water, sparingly soluble in alcohol. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 217 °C to 221 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *propylthiouracil CRS*. Examine as discs prepared using 1 mg of substance and 0.3 g of *potassium bromide R*.

C. Examine the chromatograms obtained in the test for impurity A and related substances in ultraviolet light at 254 nm before exposure of the plate to iodine vapour. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 20 mg add 8 mL of *bromine water R* and shake for a few minutes. Boil until the mixture is decolourised, allow to cool and filter. To the filtrate add 2 mL of *barium chloride solution R1*. A white precipitate is formed whose colour does not become violet on the addition of 5 mL of *dilute sodium hydroxide solution R*.

TESTS

**Impurity A and related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $GF_{254}$ plate R*.

*Test solution (a)*. Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *propylthiouracil CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 50 mg of *thiourea R* in *methanol R* and dilute to 100 mL with the same solvent. Dilute 1 mL of this solution to 100 mL with *methanol R*.

*Reference solution (c)*. Dilute 1 mL of test solution (a) to 100 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 0.1 volumes of *glacial acetic acid R*, 6 volumes of *2-propanol R* and 50 volumes of *chloroform R*. Allow the plate to dry in air. Examine in ultraviolet light at 254 nm. Expose the plate to iodine vapour for 10 min. In the chromatogram obtained with test solution (a), any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.05 per cent) and any spot apart from the principal spot and any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (c) (1.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.300 g add 30 mL of *water R* and 30.0 mL of *0.1 M sodium hydroxide*. Boil and shake until dissolution is complete. Add 50 mL of *0.1 M silver nitrate* while stirring, boil gently for 5 min and cool. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). The volume of *0.1 M sodium hydroxide* used is equal to the sum of the volume added initially and the volume used in the final titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 8.511 mg of $C_7H_{10}N_2OS$.

STORAGE

Store protected from light.

IMPURITIES

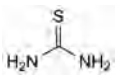

A. thiourea.

**01/2017:0636**

# PROPYPHENAZONE

## Propyphenazonum

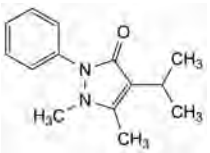

$C_{14}H_{18}N_2O$ $M_r$ 230.3
[479-92-5]

DEFINITION

1,5-Dimethyl-4-(1-methylethyl)-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 102 °C to 106 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *propyphenazone CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 80 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution*. Dissolve 80 mg of *propyphenazone CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *butanol R*, *cyclohexane R*, *ethyl acetate R* (10:45:45 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of hot air for 15 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 1 mL of solution S (see Tests) add 0.1 mL of *ferric chloride solution R1*. A brownish-red colour appears which becomes yellow on addition of 1 mL of *dilute hydrochloric acid R*.

TESTS

**Solution S**. Dissolve 2 g in a mixture of equal volumes of *carbon dioxide-free water R* and *ethanol (96 per cent) R* and dilute to 50 mL with the same mixture of solvents.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*; the solution becomes pink. Add 0.4 mL of *0.01 M hydrochloric acid*; the solution becomes colourless. Add 0.2 mL of *methyl red solution R*. The solution becomes orange or red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 1 mg of the substance to be examined and 1 mg of *phenazone R* (impurity A) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Dissolve 13.7 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 5.2 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water R*. Mix 60 volumes of the solution and 40 volumes of *acetonitrile R1*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of propyphenazone.

*Relative retention* with reference to propyphenazone (retention time = about 7 min): impurity A = about 0.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to impurity A and propyphenazone.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

Dissolve 0.200 g in 10 mL of *anhydrous acetic acid R* and add 75 mL of *ethylene chloride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.03 mg of $C_{14}H_{18}N_2O$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

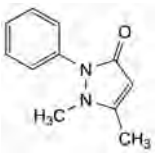

A. 1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one (phenazone),

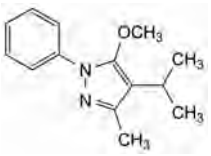

B. 5-methoxy-3-methyl-4-(1-methylethyl)-1-phenyl-1*H*-pyrazole,

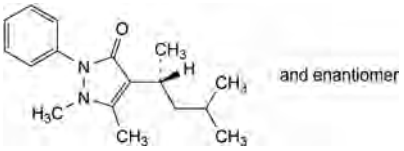


C. 4-[(1*RS*)-1,3-dimethylbutyl]-1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one.

**01/2020:0569**

# PROTAMINE SULFATE

## Protamini sulfas

[9009-65-8]

DEFINITION

Sulfated forms of basic peptides, comprising 4 major chemical species, extracted from the sperm or roe of *Salmonidae*. It binds with heparin in solution, inhibiting its anticoagulant activity; under the conditions of the potency assay this binding gives rise to a precipitate.

*Content*: 90.0 per cent to 110.0 per cent (dried, sulfuric acid-free substance).

*Potency*: 1 mg of protamine sulfate precipitates not less than 100 IU of heparin (dried substance).

PRODUCTION

The animals from which protamine sulfate is derived must fulfil the requirements for the health of animals suitable for human consumption.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances and calculate individually the relative amounts of protamine peptides A, B, C and D.

*Results*:
- the principal peaks in the chromatogram obtained with the test solution (protamine peptides A to D) are similar in retention time to the principal peaks in the chromatogram obtained with reference solution (a);
- protamine peptide A content: 13-18 per cent;
- protamine peptide B content: 21-28 per cent;
- protamine peptide C content: 31-38 per cent;
- protamine peptide D content: 19-24 per cent.

B. It gives the appropriate response when examined as described in the potency assay.

C. It complies with the test for sulfate (see Tests).

TESTS

**Solution S**. Dissolve 0.20 g in *water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ or $Y_6$ (*2.2.2, Method II*).

To 2.5 mL of solution S add 7.5 mL of *water R*.

**Absorbance** (*2.2.25*): maximum 0.1 between wavelengths of 260 nm and 280 nm.

Dilute 2.5 mL of solution S to 5.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 30 mg of the substance to be examined in 400 µL of a 618 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with a 1.03 g/L solution of *hydrochloric acid R*.

*Reference solution (a)*. Dissolve the contents of a vial of *protamine sulfate CRS* in 40 µL of a 618 g/L solution of *hydrochloric acid R* and dilute to 10.0 mL with a 1.03 g/L solution of *hydrochloric acid R*.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with a 1.03 g/L solution of *hydrochloric acid R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped solid core octadecylsilyl silica gel for chromatography R* (3.6 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: *trifluoroacetic acid R*, *acetonitrile R1*, *water for chromatography R* (0.1:5:95 *V/V/V*); degas;
- *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R1*, *water for chromatography R* (0.1:50:50 *V/V/V*); degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 | 5 |
| 2 - 17 | 95 → 80 | 5 → 20 |
| 17 - 17.2 | 80 → 20 | 20 → 80 |
| 17.2 - 20 | 20 | 80 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 5 °C.

*Injection*: 30 µL.

*Relative retention* with reference to protamine peptide A (retention time = about 9-13 min): protamine peptide B = about 1.05; protamine peptide C = about 1.10; protamine peptide D = about 1.15.

*System suitability*:
- the 4 principal peaks (protamine peptides A to D) are eluted between 8 min and 16.5 min in the chromatogram obtained with reference solution (a);
- *resolution*: minimum 2.0 between each of the adjacent principal peaks in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peak due to protamine peptide A in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.8 for the peak due to protamine peptide A, maximum 1.5 for the peak due to protamine peptide B, maximum 2.5 for the peak due to protamine peptide C and maximum 2.2 for the peak due to protamine peptide D in the chromatogram obtained with reference solution (a).

Calculate the percentage content of related substances using the following expression (use the vertical drop approach for integration):

$$100 - \left( \frac{r_A + r_B + r_C + r_D}{r_T} \times 100 \right)$$

$r_{A\text{-}D}$ = areas of the peaks due to protamine peptides A to D;

$r_T$ = sum of the areas of all peaks in the chromatogram.

*Limit*:
- *total*: maximum 8.0 per cent;
- *reporting threshold*: 0.5 per cent.

**Sulfate**: 16 per cent to 24 per cent (dried substance).

Dissolve 0.150 g in 15 mL of *distilled water R* in a beaker. Add 5 mL of *dilute hydrochloric acid R*. Heat to boiling and slowly add to the boiling solution 10 mL of a 100 g/L solution of *barium chloride R*. Cover the beaker and heat on a water-bath for 1 h. Filter. Wash the precipitate several times with small quantities of hot *water R*. Dry and ignite the residue at 600 ± 50 °C to constant mass.

1.0 g of residue is equivalent to 0.4117 g of $SO_4$.

**Iron** (*2.4.9*): maximum 10 ppm.

Dissolve 1.0 g with heating in *water R* and dilute to 10 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

**Protein**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):
- *repeatability*: maximum relative standard deviation of 2.0 per cent for the total area of the peaks above the reporting threshold with a relative retention with reference to protamine peptide A of 0.80-1.30, determined on 6 injections.

Calculate the percentage content of protamine from the areas of all peaks above the reporting threshold with a relative retention with reference to protamine peptide A of 0.80-1.30, taking into account the assigned content of *protamine sulfate CRS*.

**Potency**.

*Test solution (a)*. Dissolve 15.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dilute 2.0 mL of test solution (a) to 3.0 mL with *water R*.

*Test solution (c)*. Dilute 1.0 mL of test solution (a) to 3.0 mL with *water R*.

Use as titrant a 6-fold dilution of *heparin sodium BRP* in *water R* (for example, 1.7 mL diluted to 10.0 mL with *water R*). Titrate each test solution in duplicate as follows: introduce an

accurately measured volume of the solution to be titrated, for example 1.5 mL, into the cell of a suitable colorimeter and set the apparatus for measurement at a suitable wavelength (none is critical) in the visible range. Add the titrant in small volumes until there is a sharp increase in the absorbance and note the volume of titrant added.

Carry out 3 independent assays. For each individual titration, calculate the number of International Units of heparin in the volume of titrant added at the end-point per milligram of the substance to be examined. Calculate the potency of the substance as the average of the 18 values. Test the linearity of the response by the usual statistical methods (for example, *5.3*). Calculate the 3 standard deviations for the results obtained with each of the 3 test solutions. Calculate the 3 standard deviations for the results obtained with each of the 3 independent assays. The assay is not valid unless each of the 6 standard deviations is less than 5 per cent of the average result.

STORAGE

In an airtight, tamper-evident container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

**01/2008:1144**
**corrected 10.0**

# PROTIRELIN

## Protirelinum

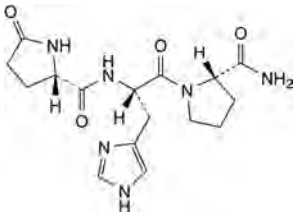

$C_{16}H_{22}N_6O_4$ $M_r$ 362.4
[24305-27-9]

DEFINITION

5-Oxo-L-prolyl-L-histidyl-L-prolinamide.

Synthetic tripeptide with the same sequence of amino acids as the natural hypothalamic neurohormone, which stimulates the release and synthesis of thyrotropin.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or yellowish-white powder, hygroscopic.

*Solubility*: very soluble in water, freely soluble in methanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *protirelin CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Appearance of solution**. A 10 g/L solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): – 62 to – 70 (anhydrous and acetic acid-free substance).

Dissolve 10 mg in 1.0 mL of *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 5.0 mg of the substance to be examined in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve the contents of a vial of *D-His-protirelin CRS* in an appropriate volume of mobile phase A to obtain a concentration of 1 mg/mL. Mix equal volumes of this solution and the test solution.

*Reference solution (b)*. Dilute 0.2 mL of the test solution to 10.0 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 12 nm.

*Mobile phase*:
- *mobile phase A*: a mixture of 100 mL of *acetonitrile for chromatography R*, 1900 mL of *water R* and 2.0 g of *sodium octanesulfonate R*, containing 2.5 mL/L of *tetraethylammonium hydroxide solution R*; adjust to pH 3.5 with *phosphoric acid R*,
- *mobile phase B*: a mixture of 300 mL of *acetonitrile for chromatography R*, 1700 mL of *water R* and 2.0 g of *sodium octanesulfonate R*, containing 2.5 mL/L of *tetraethylammonium hydroxide solution R*; adjust to pH 3.5 with *phosphoric acid R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 74 → 41 | 26 → 59 |
| 30 - 35 | 41 → 74 | 59 → 26 |
| 35 - 50 | 74 | 26 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Relative retention* with reference to protirelin (retention time = about 18 min): impurity C = about 0.2; impurity D = about 0.68; impurity A = about 0.91; impurity B = about 0.95; impurity E = about 1.08.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.5 between the peaks due to impurity A and protirelin,
- *symmetry factor*: 0.9 to 1.2 for the peak due to protirelin.

*Limits*:
- *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Acetic acid** (*2.5.34*): maximum 2.0 per cent.

*Test solution*. Dissolve 40.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of solvents.

**Water** (*2.5.12*): maximum 7.0 per cent, determined on 0.200 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.7 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Reference solution.* Dissolve the contents of a vial of *protirelin CRS* in an appropriate volume of mobile phase A to obtain a concentration of 1.0 mg/mL.

Calculate the content of protirelin ($C_{16}H_{22}N_6O_4$) using the peak areas of the chromatograms obtained with the test solution and the reference solution and the declared content of $C_{16}H_{22}N_6O_4$ in *protirelin CRS*.

STORAGE

In an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the mass of peptide in the container.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

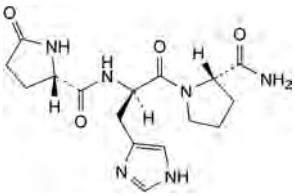

A. 5-oxo-L-prolyl-D-histidyl-L-prolinamide,

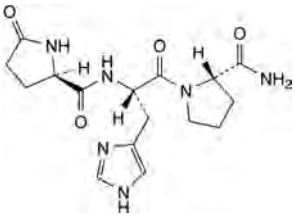

B. 5-oxo-D-prolyl-L-histidyl-L-prolinamide,

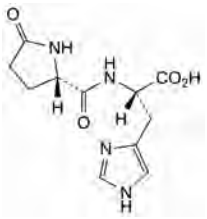

C. 5-oxo-L-prolyl-L-histidine,

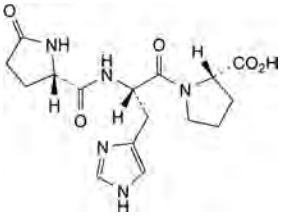

D. 5-oxo-L-prolyl-L-histidyl-L-proline,

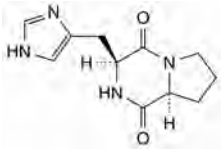

E. (3*S*,8a*S*)-3-(1*H*-imidazol-4-ylmethyl)hexahydropyrrolo-[1,2-*a*]pyrazine-1,4-dione (cyclo(-L-histidyl-L-prolyl-)).

**01/2017:0526**

# PROXYPHYLLINE

## Proxyphyllinum

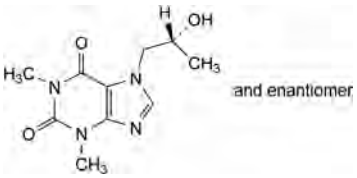


$C_{10}H_{14}N_4O_3$ $M_r$ 238.2
[603-00-9]

DEFINITION

Proxyphylline contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of 7-[(2*RS*)-2-hydroxypropyl]-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, very soluble in water, soluble in alcohol.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 134 °C to 136 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *proxyphylline CRS*. Examine the substances as discs prepared using 0.5 mg to 1 mg of the substance to be examined in 0.3 g of *potassium bromide R*.

C. Dissolve 1 g in 5 mL of *acetic anhydride R* and boil under a reflux condenser for 15 min. Allow to cool and add 100 mL of a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*. Cool in iced water for at least 20 min, shaking from time to time. Filter, wash the precipitate with a mixture of 20 volumes of *ether R* and 80 volumes of *light petroleum R*, recrystallise from *alcohol R* and dry *in vacuo*. The crystals melt (*2.2.14*) at 87 °C to 92 °C.

D. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.25 mL of *bromothymol blue solution R1*. The solution is yellow or green. Not more than 0.4 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $HF_{254}$ *R* as the coating substance.

*Test solution.* Dissolve 0.3 g of the substance to be examined in a mixture of 20 volumes of *water R* and 30 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents. Prepare immediately before use.

*Reference solution (a).* Dilute 1 mL of the test solution to 100 mL with *methanol R*.

*Reference solution (b).* Dilute 0.2 mL of the test solution to 100 mL with *methanol R*.

*Reference solution (c).* Dissolve 10 mg of *theophylline R* in *methanol R*, add 0.3 mL of the test solution and dilute to 10 mL with *methanol R*.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 10 volumes of *ethanol R* and 90 volumes of *chloroform R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (1 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated spots.

**Chlorides** (*2.4.4*). Dilute 2.5 mL of solution S to 15 mL with *water R*. The solution complies with the limit test for chlorides (400 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.200 g in 3.0 mL of *anhydrous formic acid R* and add 50.0 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.82 mg of $C_{10}H_{14}N_4O_3$.

STORAGE

Store protected from light.

**07/2008:1367**

# PSEUDOEPHEDRINE HYDROCHLORIDE

## Pseudoephedrini hydrochloridum

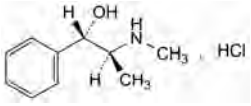


$C_{10}H_{16}ClNO$ $M_r$ 201.7
[345-78-8]

DEFINITION

(1*S*,2*S*)-2-(Methylamino)-1-phenylpropan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water and in ethanol (96 per cent), sparingly soluble in methylene chloride.

mp: about 184 °C.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pseudoephedrine hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (a).* Dissolve 20 mg of *pseudoephedrine hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *ephedrine hydrochloride CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *methylene chloride R*, *concentrated ammonia R*, *2-propanol R* (5:15:80 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 110 °C for 5 min.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dilute 2 mL of solution S to 10 mL with *carbon dioxide-free water R*. Add 0.1 mL of *methyl red solution R* and 0.1 mL of *0.01 M sodium hydroxide*; the solution is yellow. Add 0.2 mL of *0.01 M hydrochloric acid*; the solution is red.

**Specific optical rotation** (*2.2.7*): + 61.0 to + 62.5 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).
*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.
*Reference solution (a).* Dissolve 20.0 mg of *ephedrine hydrochloride CRS* (impurity A) in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.
*Reference solution (b).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.
*Reference solution (c).* Dissolve 10 mg of *ephedrine hydrochloride CRS* (impurity A) in 5 mL of the test solution and dilute to 100 mL with the mobile phase.
*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm).
*Mobile phase*: mix 6 volumes of *methanol R* and 94 volumes of an 11.6 g/L solution of *ammonium acetate R* previously adjusted to pH 4.0 with *glacial acetic acid R*.
*Flow rate*: 1 mL/min.
*Detection*: spectrophotometer at 257 nm.
*Injection*: 20 µL.
*Run time*: 1.5 times the retention time of pseudoephedrine.
*Relative retention* with reference to pseudoephedrine (retention time = about 18 min): impurity A = about 0.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the peaks due to impurity A and pseudoephedrine; if necessary, reduce the content of methanol in the mobile phase.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.170 g in 30 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.17 mg of $C_{10}H_{16}ClNO$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A*.

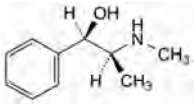

A. (1*R*,2*S*)-2-(methylamino)-1-phenylpropan-1-ol (ephedrine).

**01/2015:2603**

# PULLULAN

## Pullulanum

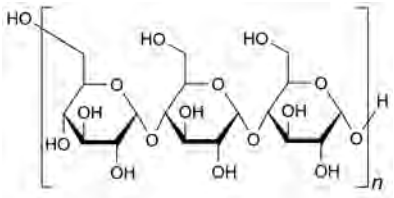

$C_{18n}H_{30n+2}O_{15n+1}$
[9057-02-7]

DEFINITION

Neutral polysaccharide produced by the growth of *Aureobasidium pullulans*. It has a chain structure consisting of α-(1→6)-linked maltotriose units, each of which is composed of three α-(1→4)-linked D-glucopyranose units. It may contain some maltotetraose units.

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Dissolve 10 g in 100 mL of *water R*, adding the substance to be examined in small quantities while stirring. A viscous solution is produced.
B. Mix 10 mL of the solution obtained in identification test A with 0.1 mL of a 10 units/mL solution of *pullulanase R*. Incubate at 25 °C for about 20 min. A substantial decrease in viscosity is observed.
C. Dissolve 2 g in 100 mL of *water R*. To 10 mL of the solution, add 2 mL of *macrogol 600 R*. A white precipitate is formed immediately.

TESTS

**pH** (*2.2.3*): 4.5 to 6.5.

Dissolve 1.0 g in 10 mL of *carbon dioxide-free water R*.

**Viscosity** (*2.2.9*): 100 $mm^2 \cdot s^{-1}$ to 180 $mm^2 \cdot s^{-1}$.

Dissolve 10.0 g (dried substance) in *water R* to make 100 g of solution, adding the substance to be examined in small quantities while stirring. Carry out the test at 30 ± 0.1 °C.

**Mono-, di- and oligosaccharides**: maximum 10.0 per cent for the sum of mono-, di- and oligosaccharides.

*Stock solution*. Dissolve 0.80 g (dried substance) in *water R*, adding the substance to be examined in small quantities while stirring, and dilute to 100.0 mL with the same solvent.

*Test solution*. To 1.0 mL of the stock solution, add 0.1 mL of a saturated solution of *potassium chloride R* (2 g in 5 mL). Mix vigorously with 3 mL of *methanol R*. Centrifuge at 12 000-16 000 *g* for 10 min maintaining at 4 °C. Use the supernatant.

*Reference solution*. Dilute 1.0 mL of the stock solution to 50.0 mL with *water R*.

*Blank solution*: *water R*.

Transfer 0.2 mL of the test solution, the reference solution and the blank solution to separate test tubes, placed in a bath of iced water with each containing 5 mL of a 2 g/L solution of *anthrone R* in *sulfuric acid R1*. Stir immediately, then heat at 90 °C for 10 min. Cool the tubes under running water.

Measure the absorbance (*2.2.25*) of the test solution, the reference solution and the blank solution at 620 nm, using *water R* as the compensation liquid.

Calculate the percentage content of mono-, di- and oligosaccharides using the following expression:

$$100 \times \frac{4.1}{50} \times \frac{A_1 - A_2}{A_3 - A_2}$$

4.1 = dilution factor for the test solution;

50 = dilution factor for the reference solution;

$A_1$ = absorbance of the test solution;

$A_2$ = absorbance of the blank solution;

$A_3$ = absorbance of the reference solution.

**Loss on drying** (*2.2.32*): maximum 6.0 per cent, determined on 1.000 g by drying *in vacuo* at 90 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 2.0 g.

**Nitrogen** (*2.5.9*): maximum 0.05 per cent (dried substance), determined on 4.0 g.

Carry out the determination of nitrogen using 40 mL of *sulfuric acid R* and heating until a green colour is obtained (about 1.5 h) to complete the decomposition and add *strong*

*sodium hydroxide solution R* until a dark brown colour is obtained (about 90 mL).

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

STORAGE

In an airtight container.

**01/2017:1680**
**corrected 10.0**

# PYRANTEL EMBONATE

## Pyranteli embonas

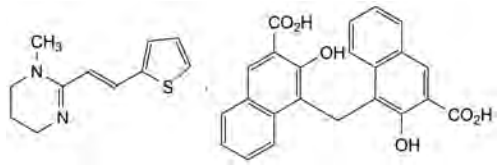

$C_{34}H_{30}N_2O_6S$ $M_r$ 594.7
[22204-24-6]

DEFINITION

1-Methyl-2-[(*E*)-2-(thiophen-2-yl)eth-1-en-1-yl]-1,4,5,6-tetrahydropyrimidine hydrogen 4,4′-methylenebis(3-hydroxynaphthalene-2-carboxylate).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: pale yellow or yellow powder.

*Solubility*: practically insoluble in water, soluble in dimethyl sulfoxide, practically insoluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pyrantel embonate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Solvent mixture*. Mix 5 volumes of *glacial acetic acid R* and 5 volumes of *water for chromatography R*, then add 2 volumes of *diethylamine R* with cooling.

*Test solution (a)*. Dissolve 0.800 g of the substance to be examined in 7 mL of the solvent mixture and dilute to 100.0 mL with *acetonitrile R*.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 10 mg of *pyrantel impurity A CRS* in the solvent mixture, add 2.5 mL of test solution (b) and dilute to 50 mL with the solvent mixture. Dilute 2 mL of this solution to 100 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (b) to 200.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 8.0 mg of *pyrantel impurity D CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *acetonitrile R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: solvent mixture, *acetonitrile for chromatography R* (72:928 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 288 nm and, for impurity D, at 238 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (a) and (b); for impurity D, 50 µL of test solution (a) and reference solution (c).

*Run time*: 4 times the retention time of pyrantel.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to pyrantel (retention time = about 11 min): embonic acid = about 0.5; impurity A = about 1.3; impurity D = about 2.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to pyrantel and impurity A.

*Calculation of percentage contents*:

– for impurity D, use the concentration of impurity D in reference solution (c);
– for impurities other than D, use the concentration of pyrantel embonate in reference solution (b).

*Limits*:

– *impurity D*: maximum 0.2 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *sum of impurities other than D (excluding embonic acid)*: maximum 0.3 per cent;
– *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 360 ppm.

To 0.46 g add 10 mL of *dilute nitric acid R* and 30 mL of *water R*. Heat on a water-bath for 5 min. Cool, dilute to 50 mL with *water R*, mix well and filter.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

To 0.50 g add 2.5 mL of *dilute nitric acid R* and dilute to 50 mL with *distilled water R*. Heat on a water-bath for 5 min, shake for 2 min, cool and filter.

**Iron** (*2.4.9*): maximum 75 ppm.

Ignite 0.66 g at 800 ± 50 °C for 2 h. Dissolve the residue in 2.5 mL of *dilute hydrochloric acid R* with gentle heating for 10 min. Cool and dilute to 50 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

To 0.450 g add 10 mL of *acetic anhydride R* and 50 mL of *glacial acetic acid R*, heat at 50 °C and stir for 10 min. Allow to cool (a clear solution is not obtained). Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 59.47 mg of $C_{34}H_{30}N_2O_6S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use*

*(2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

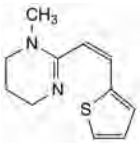

A. 1-methyl-2-[(*Z*)-2-(thiophen-2-yl)eth-1-en-1-yl]-1,4,5,6-tetrahydropyrimidine,

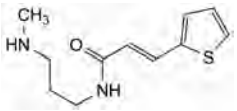

B. (*E*)-*N*-[3-(methylamino)propyl]-3-(thiophen-2-yl)prop-2-enamide,

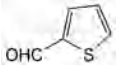

C. thiophene-2-carbaldehyde,

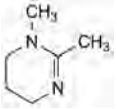

D. 1,2-dimethyl-1,4,5,6-tetrahydropyrimidine.

**01/2017:0859**

# PYRAZINAMIDE

## Pyrazinamidum

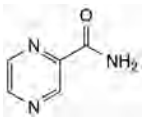

$C_5H_5N_3O$ $M_r$ 123.1
[98-96-4]

DEFINITION

Pyrazine-2-carboxamide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *C.*

*Second identification*: *A, B, D.*

A. Melting point (*2.2.14*): 188 °C to 191 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 50.0 mg in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *water R*.

*Test solution (c).* Dilute 2.0 mL of test solution (a) to 100.0 mL with *water R*.

*Spectral range*: 290-350 nm for test solution (b); 230-290 nm for test solution (c).

*Absorption maxima*: at 310 nm for test solution (b); at 268 nm for test solution (c).

*Specific absorbance at the absorption maximum at 268 nm*: 640 to 680 for test solution (c).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pyrazinamide CRS.*

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *ethanol (96 per cent) R*, evaporate to dryness and record new spectra using the residues.

D. Dissolve 0.1 g in 5 mL of *water R*. Add 1 mL of *ferrous sulfate solution R2.* The solution becomes orange. Add 1 mL of *dilute sodium hydroxide solution R*. The solution becomes dark blue.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 25 mL of solution S add 0.05 mL of *phenolphthalein solution R1* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 1.0 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.15 mL of *methyl red solution R*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent. Dilute 5.0 mL of the solution to 25.0 mL with *water R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 10 mg of *pyrazine-2-carbonitrile R* (impurity B) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *water R*. To 5.0 mL of this solution add 5.0 mL of the test solution and dilute to 25.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: dissolve 6.80 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, add 1.84 g of *sodium hydroxide R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*; add 10.0 mL of *acetonitrile R* and 1.0 mL of *tetrahydrofuran R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 40 µL.

*Run time*: 4 times the retention time of pyrazinamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to pyrazinamide (retention time = about 5 min): impurity B = about 1.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to pyrazinamide and impurity B.

*Limits*:

– *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 12.31 mg of $C_5H_5N_3O$.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

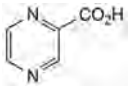

A. pyrazine-2-carboxylic acid,

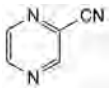

B. pyrazine-2-carbonitrile.

**01/2017:1255**
**corrected 10.0**

# PYRIDOSTIGMINE BROMIDE

## Pyridostigmini bromidum

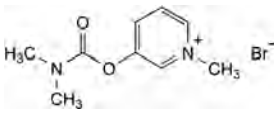

$C_9H_{13}BrN_2O_2$ $M_r$ 261.1
[101-26-8]

DEFINITION

3-[(Dimethylcarbamoyl)oxy]-1-methylpyridinium bromide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, deliquescent powder.

*Solubility*: very soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pyridostigmine bromide CRS.*

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 40 mL of solution S add a few drops of *methyl red solution R*. To 20 mL of this solution add 0.2 mL of *0.02 M sodium hydroxide*. The solution is yellow. To the other 20 mL add 0.2 mL of *0.02 M hydrochloric acid*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase at about 40 °C. Allow to cool and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 4 mg of *pyridostigmine bromide CRS*, 4 mg of *pyridostigmine impurity A CRS* and 4 mg of *pyridostigmine impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 20.0 mL with the mobile phase.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: mix 30 volumes of *acetonitrile R* and 70 volumes of a 4.33 g/L solution of *sodium dodecyl sulfate R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of pyridostigmine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B.

*Relative retention* with reference to pyridostigmine (retention time = about 32 min): impurity B = about 0.7; impurity A = about 0.9.

*System suitability*: reference solution (a):
– *resolution*: minimum 1.5 between the peaks due to impurity A and pyridostigmine.

*Limits*:
– *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent); at most one such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard the peak due to the bromide ion.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.230 g in 10 mL of *anhydrous acetic acid R*. Add 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 26.11 mg of $C_9H_{13}BrN_2O_2$.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

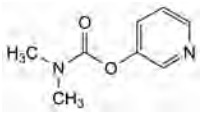

A. pyridin-3-yl dimethylcarbamate,

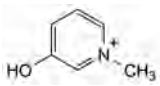

B. 3-hydroxy-1-methylpyridinium.

**01/2020:0245**

# PYRIDOXINE HYDROCHLORIDE

## Pyridoxini hydrochloridum

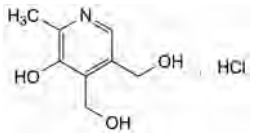

$C_8H_{12}ClNO_3$ $M_r$ 205.6

[58-56-0]

DEFINITION

(5-Hydroxy-6-methylpyridine-3,4-diyl)dimethanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 205 °C, with decomposition.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Solution A*. Dilute 1.0 mL of solution S (see Tests) to 50.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Solution B*. Dilute 1.0 mL of solution A to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Solution C*. Dilute 1.0 mL of solution A to 100.0 mL with the potassium dihydrogen phosphate 0.025 M + disodium hydrogen phosphate 0.025 M solution described in general chapter *2.2.3*.

*Spectral ranges*: 250-350 nm for solution B; 220-350 nm for solution C.

*Absorption maxima*: 288-296 nm for solution B; 248-256 nm and 320-327 nm for solution C.

*Specific absorbances at the absorption maxima*:

- 425 to 445 for solution B at 288-296 nm;
- 175 to 195 for solution C at 248-256 nm;
- 345 to 365 for solution C at 320-327 nm.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pyridoxine hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1.0 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *water R*.

*Reference solution*. Dissolve 0.10 g of *pyridoxine hydrochloride CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *methylene chloride R*, *tetrahydrofuran R*, *acetone R* (9:13:13:65 *V/V/V/V*).

*Application*: 2 µL.

*Development*: in an unsaturated tank, over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 50 g/L solution of *sodium carbonate R* in a mixture of 30 volumes of *ethanol (96 per cent) R* and 70 volumes of *water R*; dry in a current of air, spray with a 1 g/L solution of *dichloroquinonechlorimide R* in *ethanol (96 per cent) R* and examine immediately.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Solution S gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 2.4 to 3.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b)*. Dissolve 2.5 mg of *pyridoxine impurity A CRS* and 2.5 mg of *4-deoxypyridoxine hydrochloride R* (impurity B) in *water R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2.72 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 2.5 times the retention time of pyridoxine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to pyridoxine (retention time = about 12 min): impurity A = about 1.7; impurity B = about 1.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

– for each impurity, use the concentration of pyridoxine hydrochloride in reference solution (a).

*Limits*:

– *impurity B*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating in the reaction medium, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.150 g in 5 mL of *anhydrous formic acid R*. Add 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 20.56 mg of $C_8H_{12}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

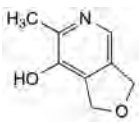

A. 6-methyl-1,3-dihydrofuro[3,4-*c*]pyridin-7-ol,

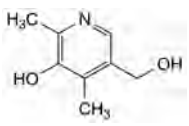

B. 5-(hydroxymethyl)-2,4-dimethylpyridin-3-ol.

**04/2019:0288**

# PYRIMETHAMINE

## Pyrimethaminum

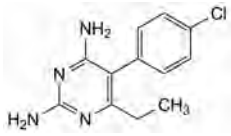

$C_{12}H_{13}ClN_4$ $M_r$ 248.7
[58-14-0]

DEFINITION

5-(4-Chlorophenyl)-6-ethylpyrimidine-2,4-diamine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 239 °C to 243 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.14 g in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*. Dilute 10.0 mL of this solution to 100.0 mL with a 10.3 g/L solution of *hydrochloric acid R*.

*Spectral range*: 250-300 nm.

*Absorption maximum*: 272 nm.

*Absorption minimum*: 261 nm.

*Specific absorption at the absorption maximum*: 310 to 330.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *pyrimethamine CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in the solvent mixture and dilute to 100 mL with the solvent mixture.

*Reference solution.* Dissolve 0.1 g of *pyrimethamine CRS* in the solvent mixture and dilute to 100 mL with the solvent mixture.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *methylene chloride R*, *propanol R*, *glacial acetic acid R*, *toluene R* (4:8:12:76 *V/V/V/V*).

*Application*: 20 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Shake 1.0 g with 50 mL of *carbon dioxide-free water R* for 2 min and filter.

**Appearance of solution**. *Prepare the solution immediately before use.* Dissolve 0.25 g in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink. Add 0.4 mL of *0.01 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The solution is red or orange.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 4 mL of the solvent mixture using sonication and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of the substance to be examined and 10 mg of *pyrimethamine impurity B CRS* in 50 mL of the solvent mixture using sonication and dilute to 100 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.72 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R*, adjust to pH 8.0 with *ammonia R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 65 | 35 |
| 6 - 20 | 65 → 40 | 35 → 60 |
| 20 - 35 | 40 → 55 | 60 → 45 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to pyrimethamine (retention time = about 15 min): impurity B = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurity B and pyrimethamine.

*Calculation of percentage contents*:

- for each impurity, use the concentration of pyrimethamine in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 80 ppm, determined on solution S. Prepare the standard using a mixture of 2.5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 12.5 mL of *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 mL of *anhydrous acetic acid R* with gentle heating and allow to cool. Titrate with *0.1 M perchloric acid* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.87 mg of $C_{12}H_{13}ClN_4$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

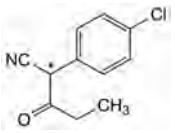


A. (2Ξ)-2-(4-chlorophenyl)-3-oxopentanenitrile,

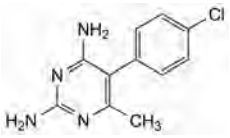


B. 5-(4-chlorophenyl)-6-methylpyrimidine-2,4-diamine,

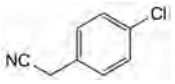


C. (4-chlorophenyl)acetonitrile,

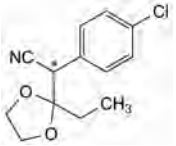


D. (Ξ)-(4-chlorophenyl)(2-ethyl-1,3-dioxolan-2-yl)acetonitrile.

**01/2017:2180**
**corrected 10.0**

# PYRROLIDONE

## Pyrrolidonum

$C_4H_7NO$ $M_r$ 85.1
[616-45-5]

DEFINITION

Pyrrolidin-2-one.

CHARACTERS

*Appearance*: clear, colourless or slightly greyish liquid, or white or almost white crystals, or colourless crystal needles.

*Solubility*: miscible with water, with ethanol (96 per cent) and with most common organic solvents.

mp: about 25 °C; the molten substance remains liquid at temperatures below the melting point.

bp: about 245 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *pyrrolidone CRS.*

B. Relative density (*2.2.5*): 1.112 to 1.115.

C. Refractive index (*2.2.6*): 1.487 to 1.490.

TESTS

*Use the molten substance for all tests.*

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than intensity 7 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Alkalinity**. To 100 mL of *water R* add 1.0 mL of *bromothymol blue solution R1* and adjust to a green colour with *0.02 M potassium hydroxide* or *0.02 M hydrochloric acid*. To 50 mL of this solution add 20 mL of the substance to be examined and titrate with *0.02 M hydrochloric acid* to the initial colour. Not more than 8.0 mL of *0.02 M hydrochloric acid* is required.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution (a)*. Dissolve 1 mL of the substance to be examined and 1 mL of *N-methylpyrrolidone R* (impurity C) in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 1.1 g of the substance to be examined in *methylene chloride R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 20.0 mL with *methylene chloride R*.

*Reference solution (c)*. Dissolve 1 mL of *butyrolactone R* (impurity B) and 1 mL of *butane-1,4-diol R* (impurity A) in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 5 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Split ratio*: 1:80.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 18.75 | 100 → 250 |
| | 18.75 - 30 | 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 0.1 µL.

*Relative retention* with reference to pyrrolidone (retention time = about 13 min): impurity B = about 0.73; impurity A = about 0.76; impurity C = about 0.97.

Use the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurity C and pyrrolidone.

*Limits*:
- *impurity B*: maximum 0.5 per cent;
- *impurities A, C*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.7 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

A. butane-1,4-diol,

B. dihydrofuran-2(3*H*)-one (γ-butyrolactone),

C. 1-methylpyrrolidin-2-one (*N*-methylpyrrolidone).

# Q

Quetiapine fumarate..... 3683
Quinapril hydrochloride..... 3685
Quinidine sulfate..... 3687
Quinine hydrochloride..... 3689
Quinine sulfate..... 3690

**01/2017:2541 corrected 9.6**

# QUETIAPINE FUMARATE

## Quetiapini fumaras

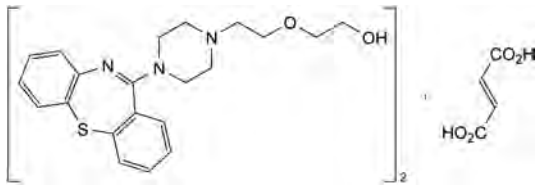

$C_{46}H_{54}N_6O_8S_2$ $M_r$ 883
[111974-72-2]

DEFINITION

Bis[2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethanol] (2*E*)-but-2-enedioate.

*Content*:

– *quetiapine fumarate* ($C_{46}H_{54}N_6O_8S_2$; $M_r$ 883): 99.0 per cent to 101.0 per cent (dried substance);

– *fumaric acid* ($C_4H_4O_4$; $M_r$ 116.1): 12.5 per cent to 13.8 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, in anhydrous ethanol and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *quetiapine fumarate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve the contents of a vial of *quetiapine for system suitability CRS* (containing impurities G and N) in 1.0 mL of the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2.1 mm;

– *stationary phase*: *end-capped, charged surface, ethylene-bridged phenylhexylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 10 volumes of *methanol R1* and 90 volumes of a 3.85 g/L solution of *ammonium acetate R*, previously adjusted to pH 9.0 with *ammonia R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 80 | 20 |
| 8 - 14.50 | 80 → 60 | 20 → 40 |
| 14.50 - 22.60 | 60 → 50 | 40 → 50 |
| 22.60 - 26 | 50 → 30 | 50 → 70 |
| 26 - 29 | 30 → 10 | 70 → 90 |
| 29 - 30 | 10 | 90 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 3.0 µL.

*Identification of impurities*: use the chromatogram supplied with *quetiapine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities G and N.

*Relative retention* with reference to quetiapine (retention time = about 13 min): fumaric acid = about 0.05; impurity G = about 0.5; impurity N = about 1.04.

*System suitability*:

– *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (b);

– *peak-to-valley ratio*: minimum 5.0, where $H_p$ = height above the baseline of the peak due to impurity N and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to quetiapine in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 0.5; impurity N = 2.0;

– for each impurity, use the concentration of quetiapine fumarate in reference solution (b).

*Limits*:

– *impurities G, N*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak due to fumaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**Quetiapine fumarate**. Dissolve 0.170 g in 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.08 mg of $C_{46}H_{54}N_6O_8S_2$.

**Fumaric acid**. Dissolve 0.350 g in 70 mL of a mixture of equal volumes of *methanol R* and *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 5.804 mg of $C_4H_4O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *G, N*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, H, I, J, K, L, O, P, Q, S, T, U, V, W*.

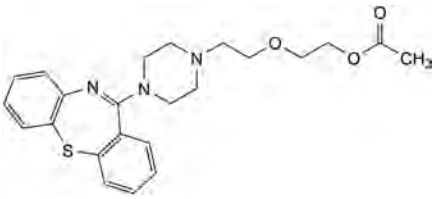

A. 2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethyl acetate,

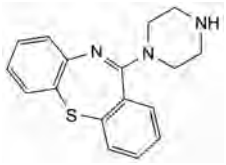

B. 11-(piperazin-1-yl)dibenzo[*b*,*f*][1,4]thiazepine,

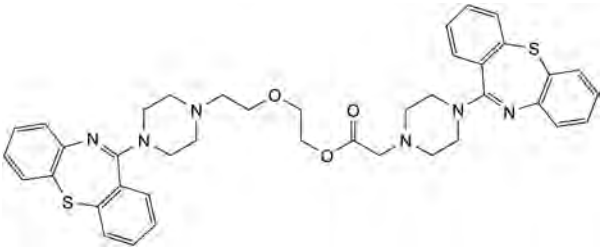

C. 2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethyl 2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]acetate,

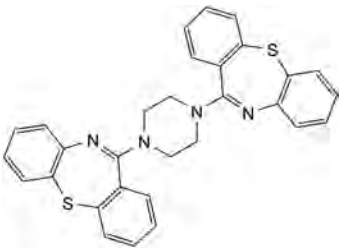

D. 11,11′-(piperazine-1,4-diyl)bis(dibenzo-[*b*,*f*][1,4]thiazepine),

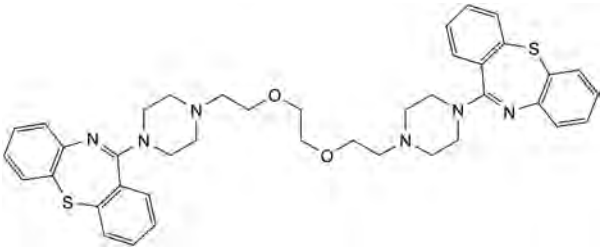

E. 11,11′-[ethylenebis(oxyethylenepiperazine-4,1-diyl)]bis(dibenzo[*b*,*f*][1,4]thiazepine),

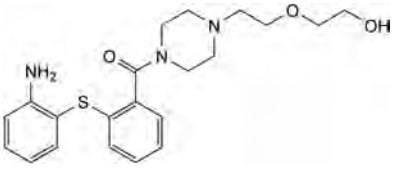

F. [2-[(2-aminophenyl)sulfanyl]phenyl][4-[2-(2-hydroxyethoxy)ethyl]piperazin-1-yl]methanone,

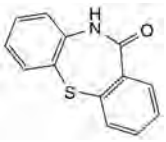

G. dibenzo[*b*,*f*][1,4]thiazepin-11(10*H*)-one,

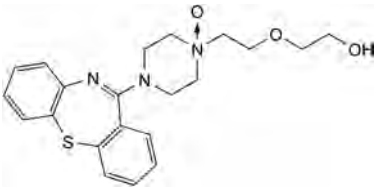

H. 2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)-1-oxidopiperazin-1-yl]ethoxy]ethanol,

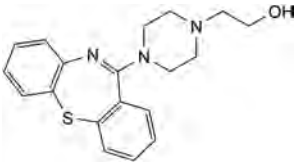

I. 2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethanol,

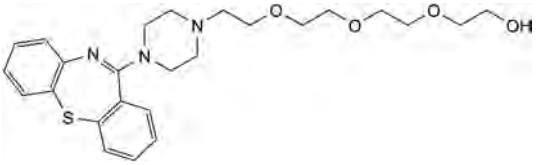

J. 2-[2-[2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethoxy]ethoxy]ethanol,

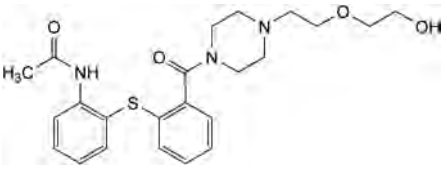

K. *N*-[2-[[2-[[4-[2-(2-hydroxyethoxy)ethyl]piperazin-1-yl]carbonyl]phenyl]sulfanyl]phenyl]acetamide,

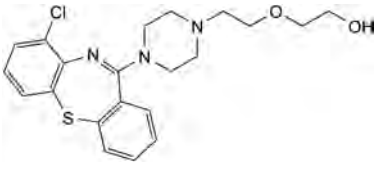

L. 2-[2-[4-(9-chlorodibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethanol,

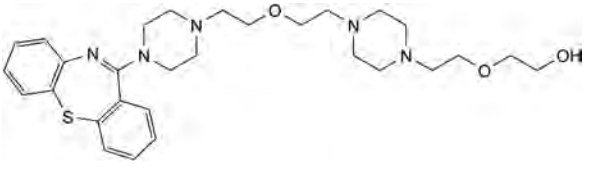

N. 2-[2-[4-[2-[2-[4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethyl]piperazin-1-yl]ethoxy]ethanol,

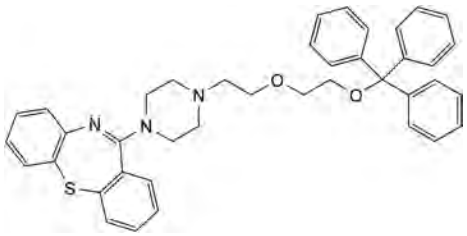

O. 11-[4-[2-[2-(triphenylmethoxy)ethoxy]ethyl]piperazin-1-yl]dibenzo[*b*,*f*][1,4]thiazepine,

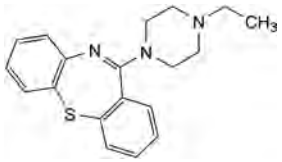

P. 11-(4-ethylpiperazin-1-yl)dibenzo[*b*,*f*][1,4]thiazepine,

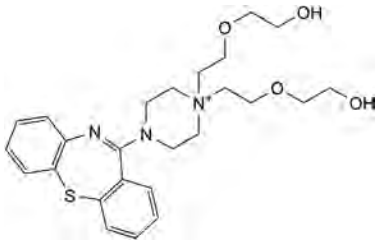

Q. 4-(dibenzo[*b*,*f*][1,4]thiazepin-11-yl)-1,1-bis[2-(2-hydroxyethoxy)ethyl]piperazin-1-ium,

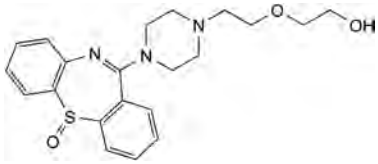

S. 2-[2-[4-(5-oxidodibenzo[*b*,*f*][1,4]thiazepin-11-yl)piperazin-1-yl]ethoxy]ethanol,

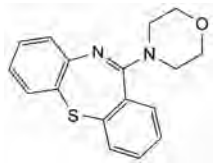

T. 11-(morpholin-4-yl)dibenzo[*b*,*f*][1,4]thiazepine,

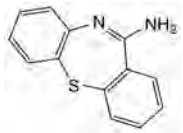

U. dibenzo[*b*,*f*][1,4]thiazepin-11-amine,

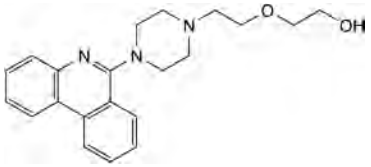

V. 2-[2-[4-(phenanthridin-6-yl)piperazin-1-yl]ethoxy]ethanol,

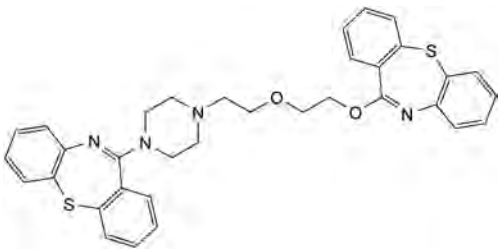

W. 11-(4-[2-[2-(dibenzo[*b*,*f*][1,4]thiazepin-11-yloxy)ethoxy]ethyl]piperazin-1-yl)dibenzo-[*b*,*f*][1,4]thiazepine.

**01/2017:1763**

# QUINAPRIL HYDROCHLORIDE

## Quinaprili hydrochloridum

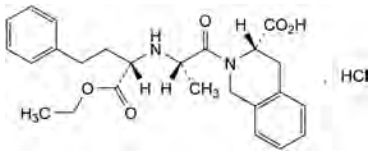

$C_{25}H_{31}ClN_2O_5$ $M_r$ 475.0
[82586-55-8]

DEFINITION

(3*S*)-2-[(2*S*)-2-[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]-amino]propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or slightly pink, hygroscopic powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), very slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *quinapril hydrochloride CRS*.

B. Specific optical rotation (see Tests).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): + 14.4 to + 16.6 (anhydrous substance).

Dissolve 0.500 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Diastereoisomers**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Adjust 500 mL of the mobile phase to pH 6.5 with *concentrated ammonia R*.

*Test solution.* Dissolve 100 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *quinapril for peak identification CRS* (containing impurities G, H and I) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: mix 260 mL of *tetrahydrofuran R* (non-stabilised) with 740 mL of a freshly prepared solution containing 1.08 g/L of *sodium octanesulfonate R* and 2.88 g/L of *ammonium dihydrogen phosphate R*, previously adjusted to pH 4.5 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of quinapril.

*Identification of impurities*: use the chromatogram supplied with *quinapril for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities G, H and I.

*Relative retention* with reference to quinapril (retention time = about 18 min): impurity G = about 0.9; impurity H = about 1.2; impurity I = about 1.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurity G and quinapril;
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity H and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to quinapril.

*Limits*:
- *impurities G, H, I*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 40 volumes of *acetonitrile R1* and 60 volumes of a 2.88 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 6.5 with *dilute ammonia R1*.

*Test solution.* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *quinapril for system suitability CRS* (containing impurities A, C, D, E and G) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* In order to prepare impurity M *in situ*, dissolve 250 mg of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent. Expose this solution to a source of ultraviolet light for 2.5 h and evaporate the solvent. Dissolve 40 mg of the remaining substance in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Column:*
- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Temperature:*
- *column*: 30 °C;
- *autosampler*: 5 °C.

*Mobile phase: acetonitrile R1*, 5.77 g/L solution of *sodium dodecyl sulfate R* adjusted to pH 2.2 with *phosphoric acid R* (48:52 *V/V*).

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of quinapril.

*Identification of impurities*: use the chromatogram supplied with *quinapril for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C, D, E and G; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity M.

*Relative retention* with reference to quinapril (retention time = about 12 min): impurity A = about 0.1; impurity C = about 0.3; impurity D = about 0.4; impurity M = about 0.7; impurities G + H = about 0.9; impurity E = about 2.3.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities C and D; minimum 1.5 between the peaks due to impurity G and quinapril.

*Limits*:
- *correction factor*: for the calculation of content, multiply the peak area of impurity E by 1.5;
- *impurities C, D*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities E, M*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to impurities G + H.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 23.75 mg of $C_{25}H_{31}ClN_2O_5$.

STORAGE

In an airtight container at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities*: *A, C, D, E, G, H, I, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, J.*

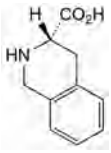


A. (3*S*)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

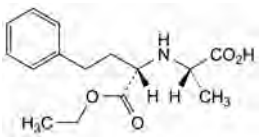

B. (2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-propanoic acid,

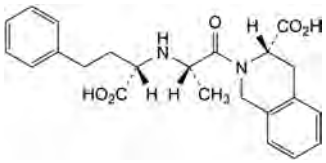

C. (3*S*)-2-[(2*S*)-2-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]-propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

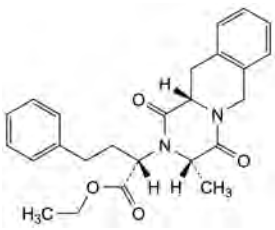

D. ethyl (2*S*)-2-[(3*S*,11a*S*)-3-methyl-1,4-dioxo-1,3,4,6,11,11a-hexahydro-2*H*-pyrazino[1,2-*b*]isoquinolin-2-yl]-4-phenylbutanoate,

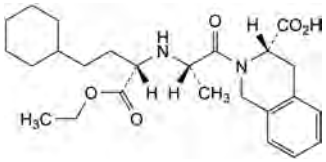

E. (3*S*)-2-[(2*S*)-2-[[(1*S*)-3-cyclohexyl-1-(ethoxycarbonyl)-propyl]amino]propanoyl]-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid,

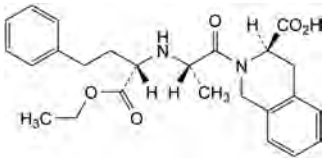

G. (3*R*)-2-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

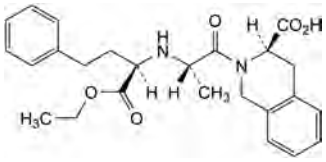

H. (3*R*)-2-[(2*S*)-2-[[(1*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

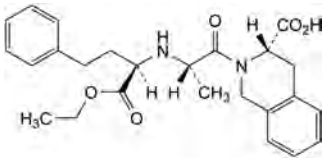

I. (3*S*)-2-[(2*S*)-2-[[(1*R*)-1-(ethoxycarbonyl)-3-phenylpropyl]-amino]propanoyl]-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

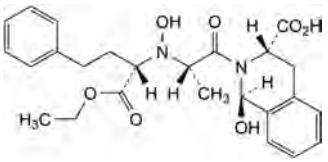

J. (1*R*,3*S*)-2-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl](hydroxy)amino]propanoyl]-1-hydroxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid,

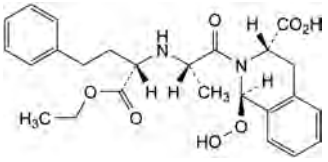

M. (1*R*,3*S*)-2-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1-hydroperoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

**01/2016:0017**

# QUINIDINE SULFATE

## Chinidini sulfas

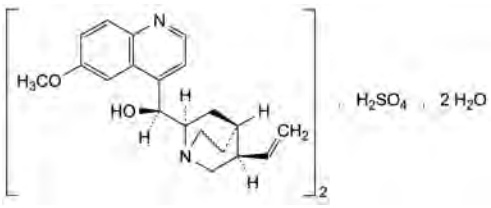

$C_{40}H_{50}N_4O_8S,2H_2O$ $M_r$ 783

[6591-63-5]

DEFINITION

Alkaloid monosulfates, expressed as bis[(*S*)-[(2*R*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol] sulfate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or silky, colourless needles.

*Solubility*: slightly soluble in water, soluble in boiling water and in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.10 g of *quinidine sulfate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *diethylamine R*, *ether R*, *toluene R* (10:24:40 *V/V/V*).

*Application*: 5 µL.

*Development*: twice over a path of 15 cm; dry in a current of air for 15 min between the 2 developments.

*Drying*: at 105 °C for 30 min and allow to cool.

*Detection*: spray with *iodoplatinate reagent R.*

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve about 5 mg in 5 mL of *water R*. Add 0.2 mL of *bromine water R* and 1 mL of *dilute ammonia R2*. A green colour develops.

C. Dissolve 0.1 g in 3 mL of *dilute sulfuric acid R* and dilute to 100 mL with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on addition of 1 mL of *hydrochloric acid R*.

D. Dissolve about 50 mg in 5 mL of hot *water R*, cool, add 1 mL of *silver nitrate solution R1* and stir with a glass rod. After a few minutes, a white precipitate is formed that dissolves on the addition of *dilute nitric acid R*.

E. It gives reaction (a) of sulfates (*2.3.1*).

F. pH (see Tests).

TESTS

**Solution S**. Dissolve 0.500 g in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 6.8.

Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 275 to + 290 (dried substance), determined on solution S.

**Other cinchona alkaloids**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20 mg of the substance to be examined in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (a).* Dissolve 20 mg of *quinine sulfate CRS* (impurity A) in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dissolve 20 mg of *quinidine sulfate CRS* in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (c).* To 1 mL of reference solution (a) add 1 mL of reference solution (b).

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15-0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 mL of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 250 nm for reference solution (e) and at 316 nm for the other solutions.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of quinidine.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurity A and dihydroquinine; use the chromatogram obtained with reference solution (b) to identify the peaks due to quinidine and impurity C; the chromatogram obtained with reference solution (c) shows 4 peaks due to quinidine, impurity A, impurity C and dihydroquinine which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

*Relative retention* with reference to impurity A: dihydroquinine = about 1.4.

*Relative retention* with reference to quinidine: impurity C = about 1.5.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to impurity A and quinidine and minimum 2.0 between the peaks due to impurities C and A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 4 for the principal peak in the chromatogram obtained with reference solution (d);
- *mass distribution ratio*: 3.5 to 4.5 for the peak due to quinidine in the chromatogram obtained with reference solution (b), $t_{R'}$ being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurity C*: maximum 15 per cent;
- *any impurity eluted before quinidine*: for each impurity, maximum 5 per cent;
- *any other impurity*: for each impurity, maximum 2.5 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent).

**Boron**: maximum 5 ppm. *Avoid where possible the use of glassware.*

*Test solution.* Dissolve 1.00 g in a mixture of 0.5 mL of *hydrochloric acid R* and 4.0 mL of *water R*.

*Reference solution.* Dissolve 0.572 g of *boric acid R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. To 1.0 mL of this solution add 3.0 mL of *water R* and 0.5 mL of *hydrochloric acid R*.

*Blank solution.* Add 0.5 mL of *hydrochloric acid R* to 4.0 mL of *water R*.

Add 3.0 mL of a 100 g/L solution of *2-ethylhexane-1,3-diol R* in *methylene chloride R* to the test solution, to the reference solution and to the blank solution, then shake for 1 min. Allow to stand for 6 min. To 1.0 mL of the lower layer, add 2.0 mL of a 3.75 g/L solution of *curcumin R* in *anhydrous acetic acid R* and 0.3 mL of *sulfuric acid R*. Mix and after 20 min add 25.0 mL of *ethanol (96 per cent) R*. Mix. The blank solution is yellow. Any red colour in the test solution is not more intense than that in the reference solution.

**Loss on drying** (*2.2.32*): 3.0 per cent to 5.0 per cent, determined on 1.000 g by drying in an oven at 130 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, using 0.15 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 24.90 mg of $C_{40}H_{50}N_4O_8S$.

STORAGE

Protected from light.

IMPURITIES

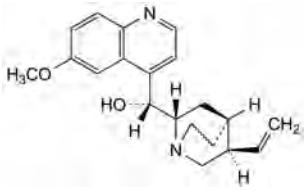

A. (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (quinine),

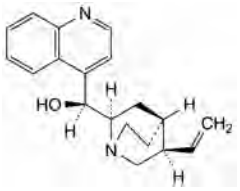

B. (*S*)-[(2*R*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonine),

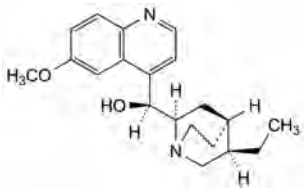

C. (*S*)-[(2*R*,4*S*,5*R*)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinidine).

**01/2016:0018**

# QUININE HYDROCHLORIDE

## Chinini hydrochloridum

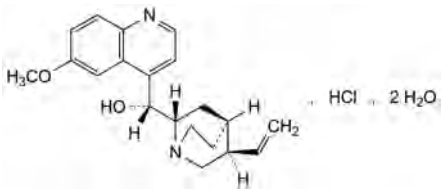

$C_{20}H_{25}ClN_2O_2,2H_2O$ $M_r$ 396.9
[6119-47-7]

DEFINITION

Alkaloid monohydrochlorides, expressed as (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol hydrochloride dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white or colourless, fine, silky needles, often in clusters.

*Solubility*: soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.10 g of *quinine sulfate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *diethylamine R*, *ether R*, *toluene R* (10:24:40 *V/V/V*).

*Application*: 5 µL.

*Development*: twice over a path of 15 cm; dry in a current of air for 15 min between the 2 developments.

*Drying*: at 105 °C for 30 min and allow to cool.

*Detection*: spray with *iodoplatinate reagent R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve about 10 mg in *water R* and dilute to 10 mL with the same solvent. To 5 mL of this solution add 0.2 mL of *bromine water R* and 1 mL of *dilute ammonia R2*. A green colour develops.

C. Dissolve 0.1 g in 3 mL of *dilute sulfuric acid R* and dilute to 100 mL with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on the addition of 1 mL of *hydrochloric acid R*.

D. It gives the reactions of chlorides (*2.3.1*).

E. pH (see Tests).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 6.8.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 245 to − 258 (dried substance).

Dissolve 0.500 g in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Other cinchona alkaloids**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20 mg of the substance to be examined in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (a).* Dissolve 20 mg of *quinine sulfate CRS* in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dissolve 20 mg of *quinidine sulfate CRS* (impurity A) in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (c).* To 1 mL of reference solution (a) add 1 mL of reference solution (b).

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15-0.25 m, Ø = 4.6 mm;
- *stationary phase = octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 mL of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 250 nm for reference solution (e) and at 316 nm for the other solutions.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of quinine.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to quinine and impurity C; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurity A and dihydroquinidine; the chromatogram obtained with reference solution (c) shows 4 peaks due to impurity A, quinine, dihydroquinidine and impurity C, which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

*Relative retention* with reference to quinine: impurity C = about 1.4.

*Relative retention* with reference to impurity A: dihydroquinidine = about 1.5.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to quinine and impurity A and minimum 2.0 between the peaks due to dihydroquinidine and quinine in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 4 for the principal peak in the chromatogram obtained with reference solution (d);
- *mass distribution ratio*: 3.5 to 4.5 for the peak due to impurity A in the chromatogram obtained with reference solution (b), $t_{R'}$ being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurity C*: maximum 10 per cent;
- *any impurity eluted before quinine*: for each impurity, maximum 5 per cent;
- *any other impurity*: for each impurity, maximum 2.5 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent).

**Sulfates** (*2.4.13*): maximum 500 ppm, determined on solution S.

**Barium**. To 15 mL of solution S add 1 mL of *dilute sulfuric acid R*. Allow to stand for 15 min. Any opalescence in the solution is not more intense than that in a mixture of 15 mL of solution S and 1 mL of *distilled water R*.

**Loss on drying** (*2.2.32*): 6.0 per cent to 10.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 inflexion points.

1 mL of *0.1 M sodium hydroxide* is equivalent to 36.09 mg of $C_{20}H_{25}ClN_2O_2$

STORAGE

Protected from light.

IMPURITIES

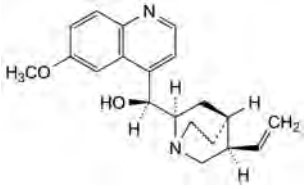

A. (*S*)-[(2*R*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (quinidine),

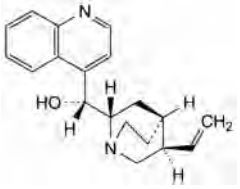

B. (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonidine),

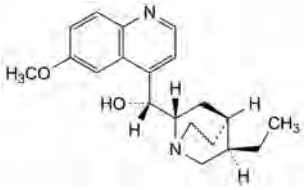

C. (*R*)-[(2*S*,4*S*,5*R*)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinine).

**01/2016:0019**

# QUININE SULFATE

## Chinini sulfas

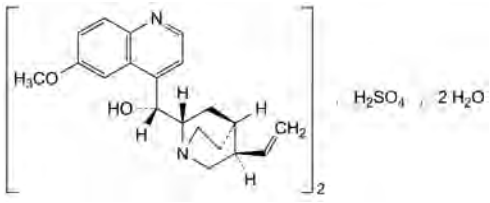

$C_{40}H_{50}N_4O_8S,2H_2O$ $M_r$ 783

[6119-70-6]

DEFINITION

Alkaloid monosulfates, expressed as bis[(*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol] sulfate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or fine, colourless needles.

*Solubility*: slightly soluble in water, sparingly soluble in boiling water and in ethanol (96 per cent).

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.10 g of *quinine sulfate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *diethylamine R*, *ether R*, *toluene R* (10:24:40 *V/V/V*).

*Application*: 5 µL.

*Development*: twice over a path of 15 cm; dry in a current of air for 15 min between the 2 developments.

*Drying*: at 105 °C for 30 min and allow to cool.

*Detection*: spray with *iodoplatinate reagent R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve about 5 mg in 5 mL of *water R*. Add 0.2 mL of *bromine water R* and 1 mL of *dilute ammonia R2*. A green colour develops.

C. Dissolve 0.1 g in 3 mL of *dilute sulfuric acid R* and dilute to 100 mL with *water R*. When examined in ultraviolet light at 366 nm, an intense blue fluorescence appears which disappears almost completely on the addition of 1 mL of *hydrochloric acid R*.

D. Dissolve about 45 mg in 5 mL of *dilute hydrochloric acid R*. The solution gives reaction (a) of sulfates (*2.3.1*).

E. pH (see Tests).

TESTS

**Solution S**. Dissolve 0.500 g in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.7 to 6.6 for a 10 g/L suspension in *water R*.

**Specific optical rotation** (*2.2.7*): − 237 to − 245 (dried substance), determined on solution S.

**Other cinchona alkaloids**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 20 mg of the substance to be examined in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (a).* Dissolve 20 mg of *quinine sulfate CRS* in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dissolve 20 mg of *quinidine sulfate CRS* (impurity A) in 5 mL of the mobile phase, with gentle heating if necessary, and dilute to 10 mL with the mobile phase.

*Reference solution (c).* To 1 mL of reference solution (a) add 1 mL of reference solution (b).

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (e).* Dissolve 10 mg of *thiourea R* in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15-0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5-10 µm).

*Mobile phase*: dissolve 6.8 g of *potassium dihydrogen phosphate R* and 3.0 g of *hexylamine R* in 700 mL of *water R*, adjust to pH 2.8 with *dilute phosphoric acid R*, add 60 mL of *acetonitrile R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 250 nm for reference solution (e) and at 316 nm for the other solutions.

*Injection*: 10 µL.

*Run time*: 2.5 times the retention time of quinine.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to quinine and impurity C; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurity A and dihydroquinidine; the chromatogram obtained with reference solution (c) shows 4 peaks due to impurity A, quinine, dihydroquinidine and impurity C which are identified by comparison of their retention times with those of the corresponding peaks in the chromatograms obtained with reference solutions (a) and (b).

*Relative retention* with reference to quinine: impurity C = about 1.4.

*Relative retention* with reference to impurity A: dihydroquinidine = about 1.5.

*System suitability*:

– *resolution*: minimum 3.0 between the peaks due to quinine and impurity A and minimum 2.0 between the peaks due to dihydroquinidine and quinine in the chromatogram obtained with reference solution (c);

– *signal-to-noise ratio*: minimum 4 for the principal peak in the chromatogram obtained with reference solution (d);

– *mass distribution ratio*: 3.5 to 4.5 for the peak due to impurity A in the chromatogram obtained with reference solution (b), $t_{R'}$ being calculated from the peak due to thiourea in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

– *impurity C*: maximum 10 per cent;

– *any impurity eluted before quinine*: for each impurity, maximum 5 per cent;

– *any other impurity*: for each impurity, maximum 2.5 per cent;

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.2 per cent).

**Loss on drying** (*2.2.32*): 3.0 per cent to 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *chloroform R* and 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.90 mg of $C_{40}H_{50}N_4O_8S$.

STORAGE

Protected from light.

IMPURITIES

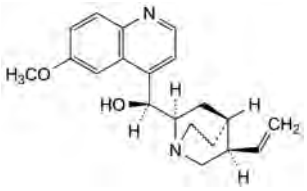


A. (*S*)-[(2*R*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (quinidine),

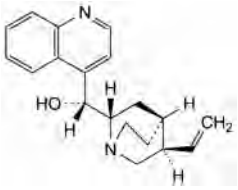


B. (*R*)-[(2*S*,4*S*,5*R*)-5-ethenyl-1-azabicyclo[2.2.2]oct-2-yl](quinolin-4-yl)methanol (cinchonidine),

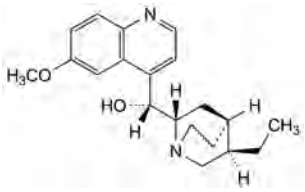


C. (*R*)-[(2*S*,4*S*,5*R*)-5-ethyl-1-azabicyclo[2.2.2]oct-2-yl](6-methoxyquinolin-4-yl)methanol (dihydroquinine).

# R

Rabeprazole sodium.. 3695
Rabeprazole sodium hydrate.. 3696
Racecadotril.. 3698
Raloxifene hydrochloride.. 3699
Raltegravir potassium.. 3701
Raltegravir chewable tablets.. 3702
Raltegravir tablets.. 3704
Ramipril.. 3705
Ranitidine hydrochloride.. 3708
Rapeseed oil, refined.. 3709
Regorafenib monohydrate.. 3710
Remifentanil hydrochloride.. 3711
Repaglinide.. 3714
Reserpine.. 3715
Resorcinol.. 3716
Ribavirin.. 3717
Riboflavin.. 3718
Riboflavin sodium phosphate.. 3720
Rice starch.. 3721
Rifabutin.. 3722
Rifampicin.. 3724
Rifamycin sodium.. 3725
Rifaximin.. 3726
Rilmenidine dihydrogen phosphate.. 3728
Risedronate sodium 2.5-hydrate.. 3729
Risperidone.. 3730
Ritonavir.. 3732
Rivastigmine.. 3736
Rivastigmine hydrogen tartrate.. 3737
Rizatriptan benzoate.. 3739
Rocuronium bromide.. 3740
Ropinirole hydrochloride.. 3742
Ropivacaine hydrochloride monohydrate.. 3744
Rosuvastatin calcium.. 3746
Rotigotine.. 3748
Roxithromycin.. 3750
Rupatadine fumarate.. 3752
Rutoside trihydrate.. 3753

**04/2016:2868**
**corrected 10.0**

# RABEPRAZOLE SODIUM

## Rabeprazolum natricum

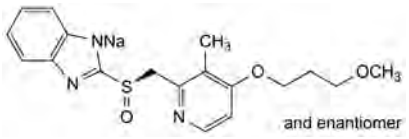


$C_{18}H_{20}N_3NaO_3S$ $M_r$ 381.4
[117976-90-6]

DEFINITION

Sodium 2-[(*RS*)-[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methyl]sulfinyl]benzimidazol-1-ide.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish-white, hygroscopic powder.

*Solubility*: very soluble to freely soluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve the substance to be examined in *methanol R*, evaporate to dryness and record the spectrum using the residue.

*Comparison*: repeat the operations using *rabeprazole sodium hydrate CRS*.

B. Loss on drying (see Tests).

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 9.5 to 11.5.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *methanol R*, *0.1 M phosphate buffer solution pH 11.3 R* (20:80 *V/V*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *rabeprazole for system suitability CRS* (containing impurities A and H) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20.0 mg of *rabeprazole sodium hydrate CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *acetonitrile R* and 95 volumes of a 4.35 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 7.0 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*;
- *mobile phase C*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 2 | 100 | 0 | 0 |
| 2 - 7 | 100 → 85 | 0 | 0 → 15 |
| 7 - 27 | 85 → 30 | 0 → 40 | 15 → 30 |
| 27 - 32 | 30 → 15 | 40 → 55 | 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Autosampler*: set at 6 °C.

*Injection*: 5 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *rabeprazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and H.

*Relative retention* with reference to rabeprazole (retention time = about 19 min): impurity A = about 0.9; impurity H = about 0.98.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity H and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rabeprazole.

*Calculation of percentage contents*:

- for each impurity, use the concentration of rabeprazole sodium in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.8 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{20}N_3NaO_3S$ taking into account the assigned content of *rabeprazole sodium hydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, K*.

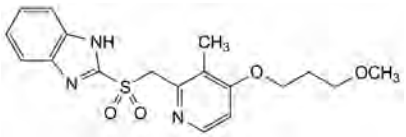

A. 2-[[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]-methyl]sulfonyl]-1*H*-benzimidazole,

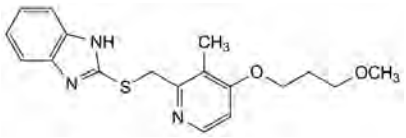

B. 2-[[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]-methyl]sulfanyl]-1*H*-benzimidazole,

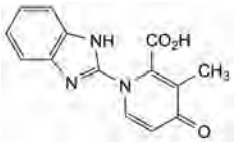

C. 1-(1*H*-benzimidazol-2-yl)-3-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid,

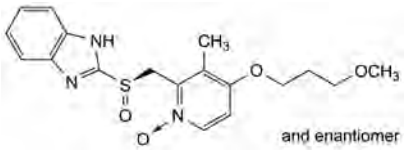


D. 2-[[(*RS*)-(1*H*-benzimidazol-2-yl)sulfinyl]methyl]-4-(3-methoxypropoxy)-3-methylpyridine 1-oxide,

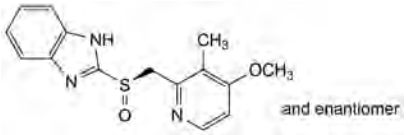


E. 2-[(*RS*)-[(4-methoxy-3-methylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole,

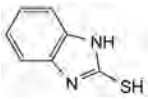

F. 1*H*-benzimidazole-2-thiol,

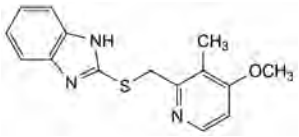

G. 2-[[(4-methoxy-3-methylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole,

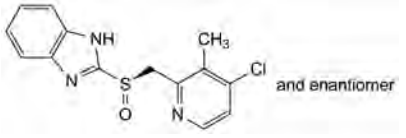


H. 2-[(*RS*)-[(4-chloro-3-methylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole,

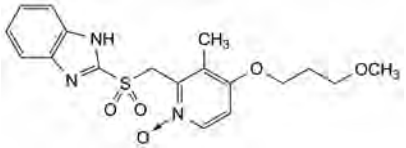

I. 2-[[(1*H*-benzimidazol-2-yl)sulfonyl]methyl]-4-(3-methoxypropoxy)-3-methylpyridine 1-oxide,

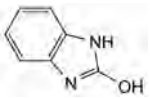

K. 1*H*-benzimidazol-2-ol.

**04/2016:2331**
**corrected 10.0**

# RABEPRAZOLE SODIUM HYDRATE

## Rabeprazolum natricum hydricum

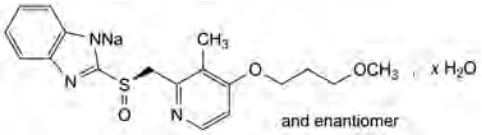


$C_{18}H_{20}N_3NaO_3S,xH_2O$ $M_r$ 381.4 (anhydrous substance)
Anhydrous rabeprazole sodium: [117976-90-6]

DEFINITION

Sodium 2-[(*RS*)-[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]methyl]sulfinyl]benzimidazol-1-ide hydrate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or slightly yellowish-white, hygroscopic, amorphous or crystalline powder.

*Solubility*: very soluble to freely soluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rabeprazole sodium hydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Water (see Tests).

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 9.5 to 11.5.

Dissolve 0.100 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *methanol R*, *0.1 M phosphate buffer solution pH 11.3 R* (20:80 *V/V*).

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *rabeprazole for system suitability CRS* (containing impurities A and H) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 20.0 mg of *rabeprazole sodium hydrate CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: mix 5 volumes of *acetonitrile R* and 95 volumes of a 4.35 g/L solution of *dipotassium hydrogen phosphate R* previously adjusted to pH 7.0 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*;
- *mobile phase C*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 2 | 100 | 0 | 0 |
| 2 - 7 | 100 → 85 | 0 | 0 → 15 |
| 7 - 27 | 85 → 30 | 0 → 40 | 15 → 30 |
| 27 - 32 | 30 → 15 | 40 → 55 | 30 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Autosampler*: set at 6 °C.

*Injection*: 5 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *rabeprazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and H.

*Relative retention* with reference to rabeprazole (retention time = about 19 min): impurity A = about 0.9; impurity H = about 0.98.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity H and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rabeprazole.

*Calculation of percentage contents*:
- for each impurity, use the concentration of rabeprazole sodium hydrate in reference solution (a).

*Limits*:
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 1.5 per cent to 7.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{20}N_3NaO_3S$ taking into account the assigned content of *rabeprazole sodium hydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, K.*

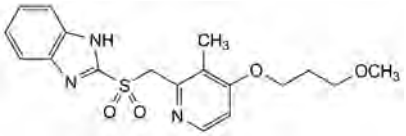

A. 2-[[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]-methyl]sulfonyl]-1*H*-benzimidazole,

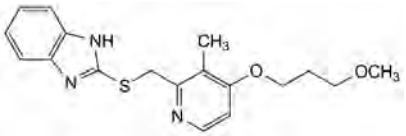

B. 2-[[[4-(3-methoxypropoxy)-3-methylpyridin-2-yl]-methyl]sulfanyl]-1*H*-benzimidazole,

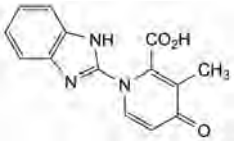

C. 1-(1*H*-benzimidazol-2-yl)-3-methyl-4-oxo-1,4-dihydropyridine-2-carboxylic acid,

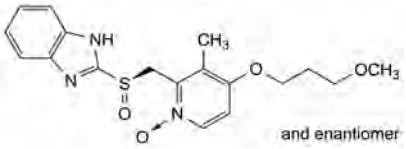


D. 2-[[(*RS*)-(1*H*-benzimidazol-2-yl)sulfinyl]methyl]-4-(3-methoxypropoxy)-3-methylpyridine 1-oxide,

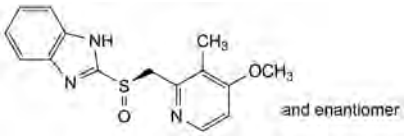


E. 2-[(*RS*)-[(4-methoxy-3-methylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole,

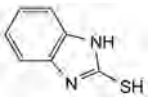

F. 1*H*-benzimidazole-2-thiol,

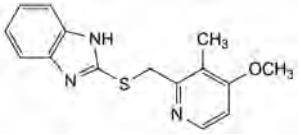

G. 2-[[(4-methoxy-3-methylpyridin-2-yl)methyl]sulfanyl]-1*H*-benzimidazole,

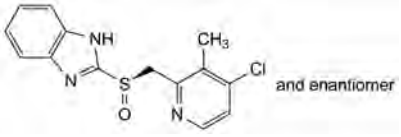


H. 2-[(*RS*)-[(4-chloro-3-methylpyridin-2-yl)methyl]sulfinyl]-1*H*-benzimidazole,

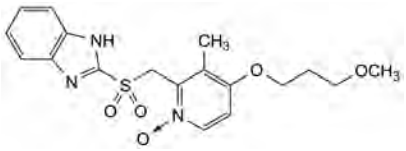

I. 2-[[(1*H*-benzimidazol-2-yl)sulfonyl]methyl]-4-(3-methoxypropoxy)-3-methylpyridine 1-oxide,

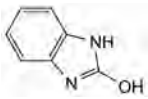

K. 1*H*-benzimidazol-2-ol.

**07/2008:2171**
**corrected 8.4**

# RACECADOTRIL

## Racecadotrilum

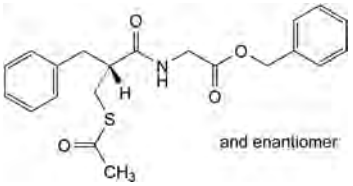


$C_{21}H_{23}NO_4S$ $M_r$ 385.5
[81110-73-8]

DEFINITION

Benzyl [[(2*RS*)-2-[(acetylsulfanyl)methyl]-3-phenylpropanoyl]amino]acetate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *racecadotril CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 5.0 g in 10 mL of *acetone R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b). Prepare immediately before use.* Dilute 500 µL of *racecadotril impurity A CRS* in *acetonitrile R* and dilute to 250.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *racecadotril impurity G CRS* in the solvent mixture and dilute to 50 mL with the solvent mixture. To 5 mL of this solution add 1 mL of test solution (b) and dilute to 100 mL with the solvent mixture.

*Reference solution (d).* Dissolve 50.0 mg of *racecadotril CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 25.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve 2 mg of *racecadotril for peak identification CRS* (containing impurities C, E and F) in 1.0 mL of the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *potassium dihydrogen phosphate R* in *water R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 60 | 40 |
| 5 - 25 | 60 → 20 | 40 → 80 |
| 25 - 35 | 20 | 80 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the solvent mixture, test solution (a) and reference solutions (a), (b), (c) and (e).

*Identification of impurities*: use the chromatogram supplied with *racecadotril for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities C, E and F.

*Relative retention* with reference to racecadotril (retention time = about 16 min): impurity A = about 0.2; impurity C = about 0.3; impurity E = about 0.5; impurity F = about 0.9.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity G and racecadotril.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.4; impurity E = 0.6; impurity F = 0.7;
- *impurities C, E, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{21}H_{23}NO_4S$ from the declared content of *racecadotril CRS*.

IMPURITIES

*Specified impurities: A, C, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, G, H.*

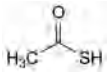

A. ethanethioic acid (thioacetic acid),

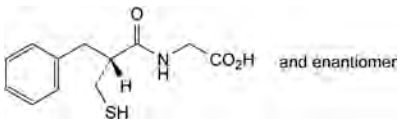


B. [[(2*RS*)-2-benzyl-3-sulfanylpropanoyl]amino]acetic acid,

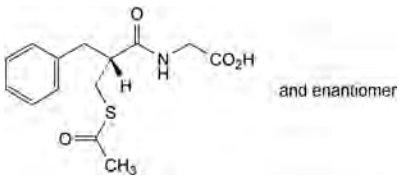


C. [[(2*RS*)-2-[(acetylsulfanyl)methyl]-3-phenylpropanoyl]-amino]acetic acid,

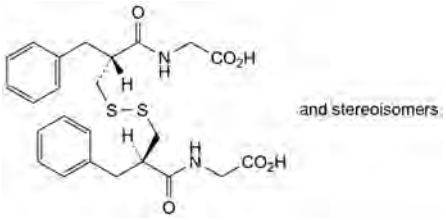


D. 5,10-dibenzyl-4,11-dioxo-7,8-dithia-3,12-diazatetradecanedioic acid,

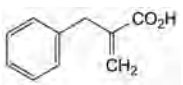

E. 2-benzylprop-2-enoic acid (2-benzylacrylic acid),

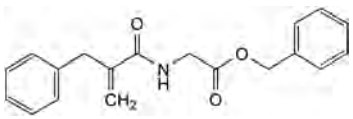

F. benzyl [(2-benzylprop-2-enoyl)amino]acetate,

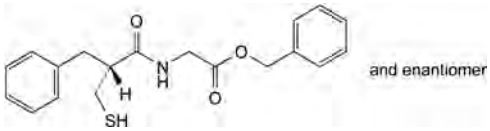


G. benzyl [[(2*RS*)-2-benzyl-3-sulfanylpropanoyl]amino]-acetate,

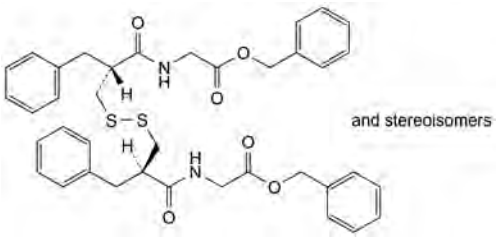


H. dibenzyl 5,10-dibenzyl-4,11-dioxo-7,8-dithia-3,12-diazatetradecanedioate.

**01/2017:2375**

# RALOXIFENE HYDROCHLORIDE

## Raloxifeni hydrochloridum

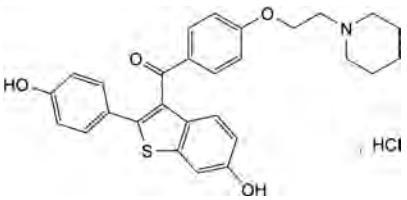


$C_{28}H_{28}ClNO_4S$ $M_r$ 510.0
[82640-04-8]

DEFINITION

[6-Hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl][4-[2-(piperidin-1-yl)ethoxy]phenyl]methanone hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or pale-yellow powder.

*Solubility*: very slightly soluble or practically insoluble in water and in acetone, slightly soluble in ethanol (96 per cent *V/V*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *raloxifene hydrochloride CRS*.

B. Dissolve 20 mg of the substance to be examined in 2 mL of *methanol R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, mobile phase A (30:70 *V/V*).

*Test solution*. Dissolve 30 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. In order to produce impurity C *in situ*, to 6 mg of the substance to be examined add 15 mL of *acetonitrile R*, 3 mL of *water R* and 5 mL of stabilised *strong hydrogen peroxide solution R*. Store at 30 °C for at least 6 h then dilute to 50.0 mL with mobile phase A. To 1.0 mL of this solution add 3 mg of the substance to be examined dissolved in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 3 mg of *raloxifene for peak identification CRS* (containing impurity A) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: 9.0 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 9 | 75 | 25 |
| 9 - 40 | 75 → 50 | 25 → 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Identification of impurity A*: use the chromatogram supplied with *raloxifene for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to raloxifene (retention time = about 18 min): impurity A = about 0.7; impurity C = about 1.2.

*System suitability*:
- *resolution*: minimum 3.0 between the peaks due to raloxifene and impurity C in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.8 for the principal peak in the chromatogram obtained with reference solution (a).

*Limits*:
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer solution pH 2.5*. 7.2 g/L Solution of *potassium dihydrogen phosphate R* adjusted to pH 2.5 with *phosphoric acid R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 50.0 mg of *raloxifene hydrochloride CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. In order to produce impurity C *in situ*, to 6 mg of the substance to be examined add 15 mL of *acetonitrile R*, 3 mL of *water R* and 5 mL of stabilised *strong hydrogen peroxide solution R*. Store at 30 °C for at least 6 h, then dilute to 50.0 mL with buffer solution pH 2.5.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *acetonitrile R*, buffer solution pH 2.5 (33:67 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of raloxifene.

*Relative retention* with reference to raloxifene (retention time = about 3 min): impurity C = about 1.2.

*System suitability*:
- *resolution*: minimum 2.0 between the peaks due to raloxifene and impurity C in the chromatogram obtained with reference solution (b); if necessary, adjust the concentration of acetonitrile in the mobile phase;
- *symmetry factor*: maximum 1.8 for the principal peak in the chromatogram obtained with reference solution (a).

Calculate the percentage content of $C_{28}H_{28}ClNO_4S$ from the declared content of *raloxifene hydrochloride CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

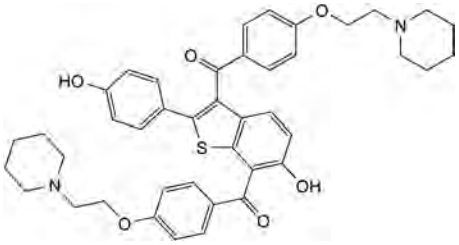


A. [6-hydroxy-2-(4-hydroxyphenyl)-7-[4-[2-(piperidin-1-yl)ethoxy]benzoyl]-1-benzothiophen-3-yl][4-[2-(piperidin-1-yl)ethoxy]phenyl]methanone,

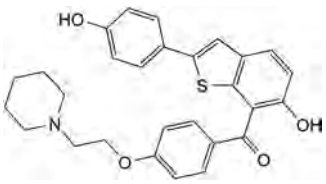


B. [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-7-yl][4-[2-(piperidin-1-yl)ethoxy]phenyl]methanone,

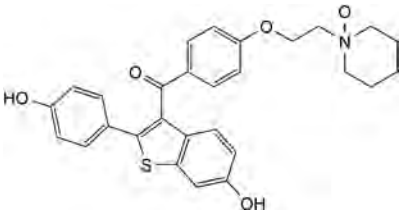


C. [6-hydroxy-2-(4-hydroxyphenyl)-1-benzothiophen-3-yl][4-[2-(piperidin-1-yl)ethoxy]phenyl]methanone *N*-oxide.

**04/2018:2887**
**corrected 10.0**

# RALTEGRAVIR POTASSIUM

## Raltegravirum kalicum

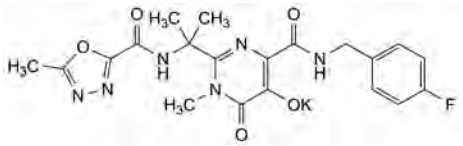

$C_{20}H_{20}FKN_6O_5$ $M_r$ 482.5
[871038-72-1]

DEFINITION

Potassium 4-[[(4-fluorophenyl)methyl]carbamoyl]-1-methyl-2-[2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl]-6-oxo-1,6-dihydropyrimidin-5-olate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *raltegravir potassium CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (b) of potassium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (25:75 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 100 mL of the solvent mixture using sonication for 5 min. Add about 140 mL of the solvent mixture then dilute to 250.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *raltegravir potassium CRS* in 100 mL of the solvent mixture using sonication for 5 min. Add about 140 mL of the solvent mixture then dilute to 250.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *raltegravir impurity E CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 50.0 mL with reference solution (a).

*Reference solution (d)*. In order to prepare impurity C *in situ*, dissolve 20 mg of the substance to be examined in a 40 g/L solution of *sodium hydroxide R* and dilute to 10 mL with the same solvent. Stir the solution for 30 min. To 5 mL of the solution add 5 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the solvent mixture.

*Reference solution (e)*. Dissolve 5 mg of *raltegravir for peak identification CRS* (containing impurities F and G) in 20 mL of the solvent mixture using sonication for 5 min. Add about 25 mL of the solvent mixture then dilute to 50 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 15 °C.

*Mobile phase*:
- *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 75 | 25 |
| 2 - 5 | 75 → 60 | 25 → 40 |
| 5 - 10 | 60 → 55 | 40 → 45 |
| 10 - 19 | 55 → 10 | 45 → 90 |
| 19 - 22 | 10 → 5 | 90 → 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E; use the chromatogram supplied with *raltegravir for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities F and G.

*Relative retention* with reference to raltegravir (retention time = about 10 min): impurity C = about 0.7; impurity E = about 0.95; impurity G = about 1.1; impurity F = about 1.15.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to impurity E and raltegravir.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity C by 1.6;
- for each impurity, use the concentration of raltegravir potassium in reference solution (b).

*Limits*:
- *impurity C*: maximum 0.3 per cent;
- *impurities E, F, G*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.6 per cent, determined on 0.500 g. Use as the solvent a mixture of equal volumes of *methanol R* and *formamide R*.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{20}H_{20}FKN_6O_5$ taking into account the assigned content of *raltegravir potassium CRS*.

IMPURITIES

*Specified impurities: C, E, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, H.*

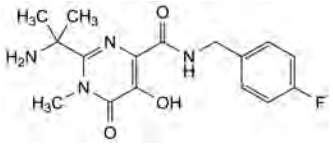

A. 2-(2-aminopropan-2-yl)-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

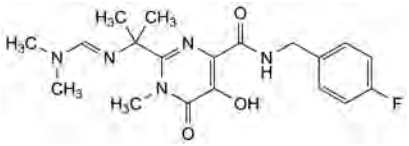

B. 2-[2-[(*E*)-[(dimethylamino)methylidene]amino]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

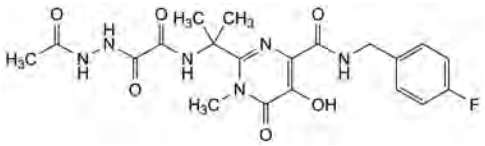

C. 2-[2-[2-(2-acetylhydrazin-1-yl)-2-oxoacetamido]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

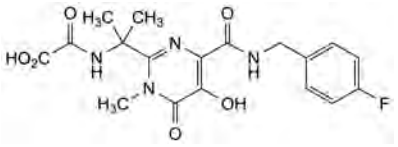

D. *N*-[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamic acid,

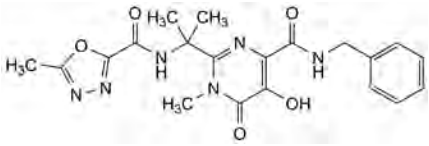

E. *N*-benzyl-5-hydroxy-1-methyl-2-[2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl]-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

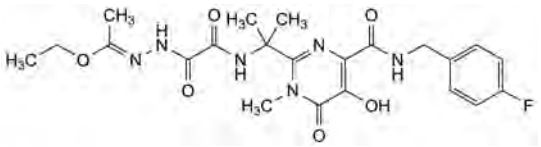

F. ethyl (1*E*)-*N*-[[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamoyl]ethanehydrazonate,

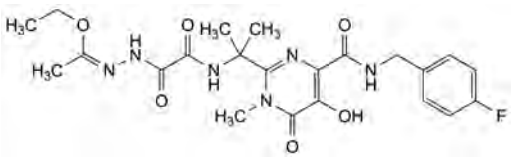

G. ethyl (1*Z*)-*N*-[[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamoyl]ethanehydrazonate,

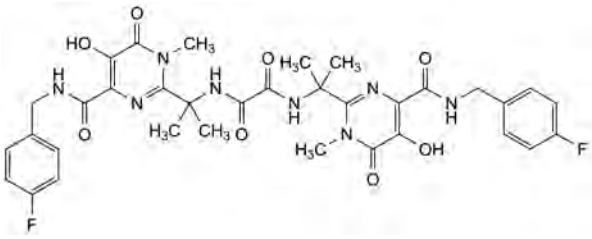

H. *N*,*N*′-bis[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamide.

**07/2018:2939**

# RALTEGRAVIR CHEWABLE TABLETS

## Raltegraviri compressi masticabiles

DEFINITION

Raltegravir chewable tablets contain *Raltegravir potassium (2887)*.

*The tablets comply with the monograph Tablets (0478) and with the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of raltegravir ($C_{20}H_{21}FN_6O_5$) stated on the label.

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay with a diode array detector in the range of 190-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: crush a tablet to a powder and homogenise.

*Comparison*: *raltegravir potassium CRS*.

*Results*: the spectrum obtained shows absorption maxima at about 1633 $cm^{-1}$, 1515 $cm^{-1}$, 1188 $cm^{-1}$, 810 $cm^{-1}$ and 728 $cm^{-1}$, similar to the spectrum obtained with *raltegravir potassium CRS*.

Other absorption maxima may be present in the spectra.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (30:70 *V/V*).

*Test solution*. Place 10 tablets in an appropriate volumetric flask to obtain a concentration of 1 mg/mL of raltegravir and make up to volume with the solvent mixture. Stir vigorously for 1 h. Centrifuge a portion of the solution and dilute 20.0 mL of the clear supernatant to 200.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 22.0 mg of *raltegravir potassium CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 2 mg of *raltegravir impurity E CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the test solution.

*Reference solution (d).* In order to prepare impurities C and D *in situ*, dissolve 20 mg of *raltegravir potassium R* in a 40 g/L solution of *sodium hydroxide R* and dilute to 10 mL with the same solvent. Stir the solution for 2 h at room temperature. To 5 mL of the solution add 5 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of *acetonitrile for chromatography R* and 80 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 27 | 100 → 50 | 0 → 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 15 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C and D; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to raltegravir (retention time = about 22 min): impurity D = about 0.7; impurity C = about 0.8; impurity E = about 0.96.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity E and raltegravir.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.6; impurity D = 1.4;
- for each impurity, use the concentration of raltegravir in reference solution (b).

*Limits*:

- *impurity C*: maximum 0.3 per cent;
- *impurity D*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 0.8 per cent;
- *reporting threshold*: 0.1 per cent.

**Dissolution** (*2.9.3*, *Apparatus 2*). The tablets comply with the test, unless otherwise justified and authorised.

*Dissolution medium*: *water R*, 900 mL.

*Rotation speed*: 50 r/min.

*Time*: 15 min.

*Analysis.* Liquid chromatography (*2.2.29*).

*Test solutions.* Solutions from the dissolution test.

*Reference solution.* Using sonication if necessary, dissolve a suitable quantity of *raltegravir potassium CRS* in a suitable quantity of a mixture of 30 volumes of *acetonitrile R* and 70 volumes of *water R* to obtain a concentration of raltegravir corresponding to the theoretical concentration of raltegravir in the test solution, based on the labelled content of the tablets.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*;
- *temperature*: 40 °C.

*Mobile phase*: mix 38 volumes of *acetonitrile R* and 62 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 5.0 mL/min.

*Detection*: spectrophotometer at 303 nm.

*Injection*: 30 µL.

*Run time*: 1 min.

*System suitability*: reference solution:

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the amount of dissolved raltegravir, expressed as a percentage of the content of raltegravir ($C_{20}H_{21}FN_6O_5$) stated on the label, taking into account the assigned content of *raltegravir potassium CRS* and a conversion factor of 0.9210.

*Acceptance criterion*:

- *Q* = 85 per cent after 15 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

- *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of raltegravir ($C_{20}H_{21}FN_6O_5$) taking into account the assigned content of *raltegravir potassium CRS* and a conversion factor of 0.9210.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph)*: A, B, E.*

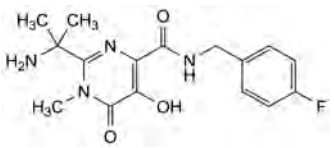

A. 2-(2-aminopropan-2-yl)-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

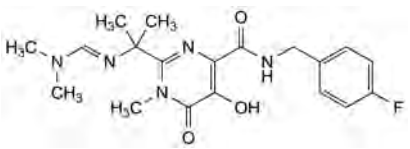

B. 2-[2-[(*E*)-[(dimethylamino)methylidene]amino]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

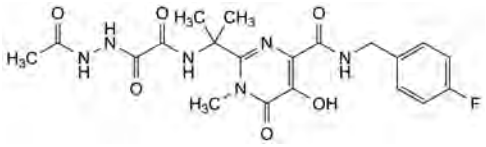

C. 2-[2-[2-(2-acetylhydrazin-1-yl)-2-oxoacetamido]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

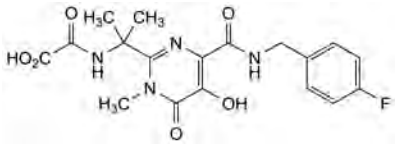

D. *N*-[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamic acid,

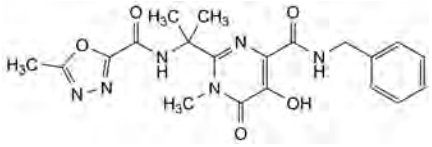

E. *N*-benzyl-5-hydroxy-1-methyl-2-[2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl]-6-oxo-1,6-dihydropyrimidine-4-carboxamide.

**07/2018:2938**



# RALTEGRAVIR TABLETS

## Raltegraviri compressi

DEFINITION

Raltegravir tablets contain *Raltegravir potassium (2887)*.

*The tablets comply with the monograph Tablets (0478) and with the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of raltegravir ($C_{20}H_{21}FN_6O_5$) stated on the label.

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay with a diode array detector in the range of 190-400 nm.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: crush a tablet to a powder and homogenise.

*Comparison*: *raltegravir potassium CRS*.

*Results*: the spectrum obtained shows absorption maxima at about 1633 $cm^{-1}$, 1515 $cm^{-1}$, 1188 $cm^{-1}$, 810 $cm^{-1}$ and 728 $cm^{-1}$, similar to the spectrum obtained with *raltegravir potassium CRS*.

Other absorption maxima may be present in the spectra.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (30:70 *V/V*).

*Test solution*. Place 10 tablets in an appropriate volumetric flask to obtain a concentration of 8 mg/mL of raltegravir and make up to volume with the solvent mixture. Stir vigorously for 1 h. Centrifuge a portion of the solution and dilute 5.0 mL of the clear supernatant to 100.0 mL with the solvent mixture. Dilute 50.0 mL of this solution to 200.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 22.0 mg of *raltegravir potassium CRS* in the solvent mixture and dilute to 200.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *raltegravir impurity E CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the test solution.

*Reference solution (d)*. In order to prepare impurities C and D *in situ,* dissolve 20 mg of *raltegravir potassium R* in a 40 g/L solution of *sodium hydroxide R* and dilute to 10 mL with the same solvent. Stir the solution for 2 h at room temperature. To 5 mL of the solution add 5 mL of a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of *acetonitrile for chromatography R* and 80 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 27 | 100 → 50 | 0 → 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 15 µL of the test solution and reference solutions (b), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C and D; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to raltegravir (retention time = about 22 min): impurity D = about 0.7; impurity C = about 0.8; impurity E = about 0.96.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.5 between the peaks due to impurity E and raltegravir.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 1.6; impurity D = 1.4;
- for each impurity, use the concentration of raltegravir in reference solution (b).

*Limits*:

- *impurity C*: maximum 0.5 per cent;
- *impurity D*: maximum 0.3 per cent;

– *unspecified impurities*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.8 per cent;

– *reporting threshold*: 0.1 per cent.

**Dissolution** (*2.9.3, Apparatus 2*). The tablets comply with the test, unless otherwise justified and authorised. Use sinker devices.

*Dissolution medium*: *water R*, 900 mL.

*Rotation speed*: 100 r/min.

*Time*: 15 min and 60 min.

*Analysis.* Liquid chromatography (*2.2.29*).

*Test solutions.* Solutions from the dissolution test.

*Reference solution.* Using sonication, dissolve a suitable quantity of *raltegravir potassium CRS* in a suitable quantity of a mixture of 30 volumes of *acetonitrile R* and 70 volumes of *water R* to obtain a concentration of raltegravir corresponding to the theoretical concentration of raltegravir in the test solution, based on the labelled content of the tablets.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped monolithic octadecylsilyl silica gel for chromatography R*;

– *temperature*: 40 °C.

*Mobile phase*: mix 38 volumes of *acetonitrile R* and 62 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 5.0 mL/min.

*Detection*: spectrophotometer at 303 nm.

*Injection*: 10 µL.

*Run time*: 1 min.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the amount of dissolved raltegravir, expressed as a percentage of the content of raltegravir ($C_{20}H_{21}FN_6O_5$) stated on the label, taking into account the assigned content of *raltegravir potassium CRS* and a conversion factor of 0.9210.

*Acceptance criteria*:

– 15-45 per cent after 15 min;

– *Q* = 70 per cent after 60 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of raltegravir ($C_{20}H_{21}FN_6O_5$) taking into account the assigned content of *raltegravir potassium CRS* and a conversion factor of 0.9210.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph)*: A, B, E.*

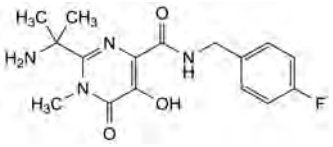

A. 2-(2-aminopropan-2-yl)-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

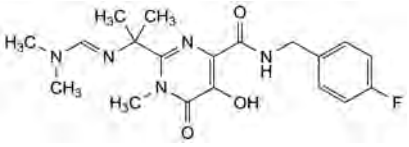

B. 2-[2-[(*E*)-[(dimethylamino)methylidene]amino]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

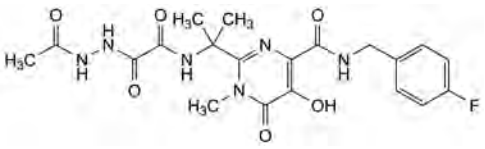

C. 2-[2-[2-(2-acetylhydrazin-1-yl)-2-oxoacetamido]propan-2-yl]-*N*-[(4-fluorophenyl)methyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidine-4-carboxamide,

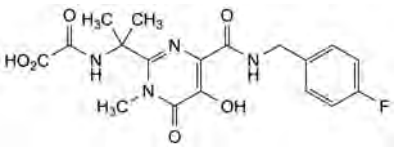

D. *N*-[2-[4-[[(4-fluorophenyl)methyl]carbamoyl]-5-hydroxy-1-methyl-6-oxo-1,6-dihydropyrimidin-2-yl]propan-2-yl]oxamic acid,

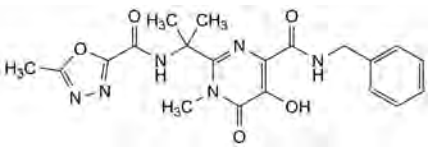

E. *N*-benzyl-5-hydroxy-1-methyl-2-[2-[(5-methyl-1,3,4-oxadiazol-2-yl)formamido]propan-2-yl]-6-oxo-1,6-dihydropyrimidine-4-carboxamide.

**07/2019:1368**

# RAMIPRIL

## Ramiprilum

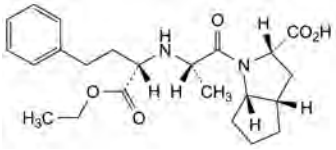

$C_{23}H_{32}N_2O_5$ $M_r$ 416.5

[87333-19-5]

DEFINITION

(2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(2*S*)-1-Ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, freely soluble in methanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ramipril CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.1 g in *methanol R* and dilute to 10.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 32.0 to + 38.0 (dried substance).

Dissolve 0.250 g in a mixture of 14 volumes of *hydrochloric acid R1* and 86 volumes of *methanol R* and dilute to 25.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 2 mg of *ramipril impurity A CRS*, 2 mg of *ramipril impurity B CRS*, 2 mg of *ramipril impurity C CRS* and 2 mg of *ramipril impurity D CRS* in mobile phase A and dilute to 25.0 mL with mobile phase A. To 1.0 mL of this solution, add 5.0 mL of the test solution and dilute to 10.0 mL with mobile phase B.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase B.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with mobile phase B.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 65 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.0 g of *sodium perchlorate R* in a mixture of 0.5 mL of *triethylamine R* and 800 mL of *water for chromatography R*; adjust to pH 3.6 with *phosphoric acid R* and add 200 mL of *acetonitrile R1*;
- *mobile phase B*: dissolve 2.0 g of *sodium perchlorate R* in a mixture of 0.5 mL of *triethylamine R* and 300 mL of *water for chromatography R*; adjust to pH 2.6 with *phosphoric acid R* and add 700 mL of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 90 | 10 |
| 6 - 7 | 90 → 75 | 10 → 25 |
| 7 - 20 | 75 → 65 | 25 → 35 |
| 20 - 30 | 65 → 25 | 35 → 75 |
| 30 - 50 | 25 | 75 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Equilibration*: with the mobile phase at the initial composition for at least 35 min; if a suitable baseline cannot be obtained, use another grade of triethylamine.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to ramipril (retention time = about 18 min): impurity A = about 0.8; impurity B = about 1.3; impurity C = about 1.5; impurity D = about 1.7.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to impurity A and ramipril in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (c);
- *symmetry factor*: 0.8 to 2.0 for the peak due to ramipril in the chromatogram obtained with the test solution.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2.4;
- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying in an oven under high vacuum at 60 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 25 mL of *methanol R* and add 25 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 41.65 mg of $C_{23}H_{32}N_2O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G, H, I, J, K, L, M, N, O.*

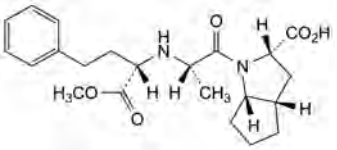


A. (2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(2*S*)-1-methoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramipril methyl ester),

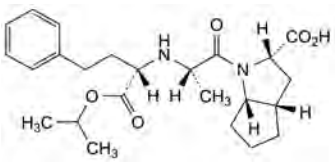

B. (2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(2*S*)-1-oxo-4-phenyl-1-[(propan-2-yl)oxy]butan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramipril isopropyl ester),

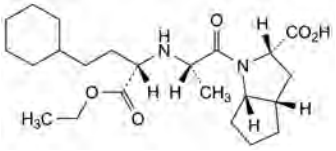

C. (2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(2*S*)-4-cyclohexyl-1-ethoxy-1-oxobutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]-pyrrole-2-carboxylic acid (hexahydroramipril),

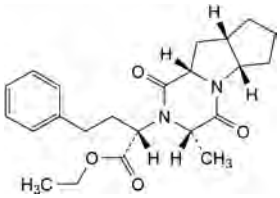

D. ethyl (2*S*)-2-[(3*S*,5a*S*,8a*S*,9a*S*)-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]pyrazin-2-yl]-4-phenylbutanoate (ramipril diketopiperazine),

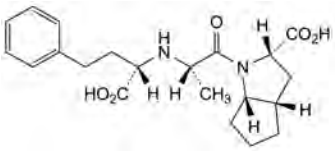

E. (2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxy-3-phenylpropyl]-amino]propanoyl]octahydrocyclopenta[*b*]pyrrole-2-carboxylic acid (ramiprilat),

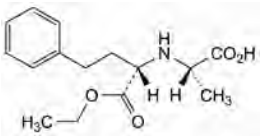

F. (2*S*)-2-[[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]-propanoic acid,

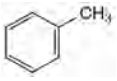

G. methylbenzene (toluene),

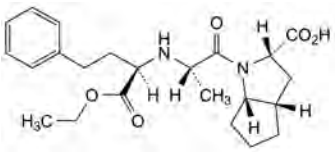

H. (2*S*,3a*S*,6a*S*)-1-[(2*S*)-2-[[(2*R*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]-pyrrole-2-carboxylic acid ((*R*,*S*,*S*,*S*,*S*)-epimer of ramipril),

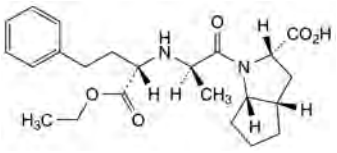

I. (2*S*,3a*S*,6a*S*)-1-[(2*R*)-2-[[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]-pyrrole-2-carboxylic acid ((*S*,*R*,*S*,*S*,*S*)-epimer of ramipril),

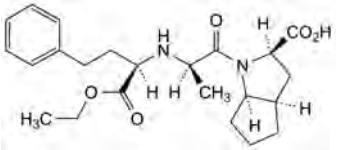

J. (2*R*,3a*R*,6a*R*)-1-[(2*R*)-2-[[(2*R*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]-pyrrole-2-carboxylic acid (enantiomer of ramipril),

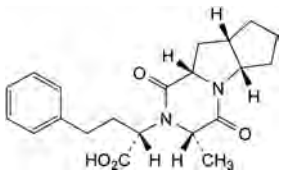

K. (2*S*)-2-[(3*S*,5a*S*,8a*S*,9a*S*)-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]pyrazin-2-yl]-4-phenylbutanoic acid (ramiprilate diketopiperazine),

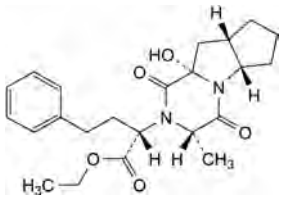

L. ethyl (2*S*)-2-[(3*S*,5a*S*,8a*S*,9a*S*)-9a-hydroxy-3-methyl-1,4-dioxodecahydro-2*H*-cyclopenta[4,5]pyrrolo[1,2-*a*]-pyrazin-2-yl]-4-phenylbutanoate (ramipril hydroxydiketopiperazine),

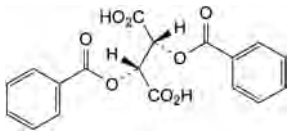

M. (2*R*,3*R*)-2,3-bis(benzoyloxy)butanedioic acid (dibenzoyl tartric acid),

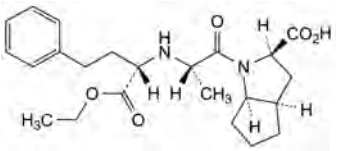

N. (2*R*,3a*R*,6a*R*)-1-[(2*S*)-2-[[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydrocyclopenta[*b*]-pyrrole-2-carboxylic acid ((*S*,*S*,*R*,*R*,*R*)-isomer of ramipril),

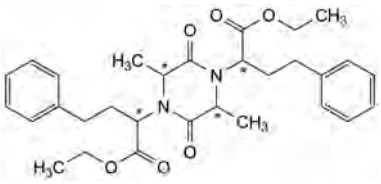

O. diethyl (2*Ξ*,2′*Ξ*)-2,2′-[(2*Ξ*,5*Ξ*)-2,5-dimethyl-3,6-dioxopiperazine-1,4-diyl]bis(4-phenylbutanoate).

07/2017:0946
corrected 10.0

# RANITIDINE HYDROCHLORIDE

## Ranitidini hydrochloridum

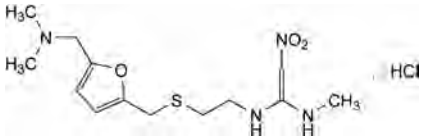

$C_{13}H_{23}ClN_4O_3S$ $M_r$ 350.9
[66357-59-3]

DEFINITION

*N*-[2-[[[5-[(Dimethylamino)methyl]furan-2-yl]methyl]-sulfanyl]ethyl]-*N'*-methyl-2-nitroeth-1-ene-1,1-diamine hydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale yellow, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble or slightly soluble in anhydrous ethanol, very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ranitidine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve 10 mg of the substance to be examined and 10 mg of the reference substance separately in 0.5 mL of *methanol R* in an agate mortar. Evaporate to dryness under a stream of *nitrogen R*. Dry the residues under vacuum for 30 min. Add 3 drops of *liquid paraffin R* to the residues and triturate until the mull shows a milky appearance. Compress the mulls between 2 plates transparent to infrared radiation and record new spectra.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 6.8 g of *potassium dihydrogen phosphate R* in 950 mL of *water for chromatography R*. Adjust to pH 7.1 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 13 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 6.5 mg of *ranitidine for impurity A identification CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve the contents of a vial of *ranitidine impurity J CRS* in 1.0 mL of the test solution.

*Blank solution*: mobile phase A.

*Column*:
- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile for chromatography R*, buffer solution (2:98 *V/V*);
- *mobile phase B*: *acetonitrile for chromatography R*, buffer solution (22:78 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 → 0 | 0 → 100 |
| 10 - 15 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *ranitidine for impurity A identification CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity J.

*Relative retention* with reference to ranitidine (retention time = about 7 min): impurity J = about 0.9; impurity A = about 1.7.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to impurity J and ranitidine in the chromatogram obtained with reference solution (c);
- the chromatogram obtained with the blank solution does not show any peak with the same relative retention as the peak due to impurity A in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity J by 2.0;
- for each impurity, use the concentration of ranitidine hydrochlorhyde in reference solution (b).

*Limits*:
- *impurity A*: maximum 0.3 per cent;
- *impurity J*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.75 per cent, determined on 1.000 g by drying under high vacuum at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.280 g in 35 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.09 mg of $C_{13}H_{23}ClN_4O_3S$.

STORAGE

In airtight container, protected from light.

IMPURITIES

*Specified impurities: A, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, K*.

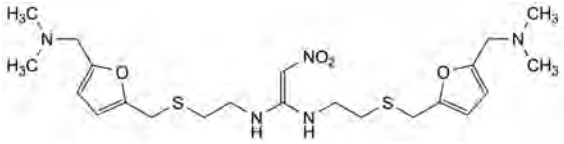

A. *N*,*N*′-bis[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl]ethyl]-2-nitroeth-1-ene-1,1-diamine,

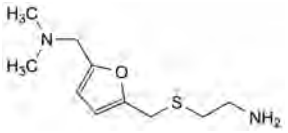

B. 2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl]ethan-1-amine,

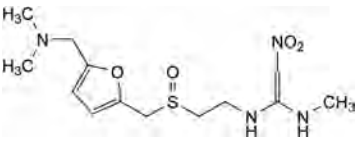

C. *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfinyl]ethyl]-*N*′-methyl-2-nitroeth-1-ene-1,1-diamine,

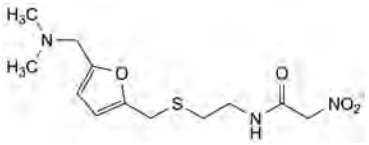

D. *N*-[2-[[[5-[(dimethylamino)methyl]furan-2-yl]methyl]sulfanyl]ethyl]-2-nitroacetamide,

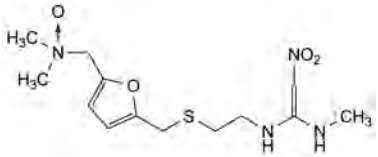

E. [5-[[[2-[[1-(methylamino)-2-nitroeth-1-en-1-yl]amino]ethyl]sulfanyl]methyl]furan-2-yl]-*N*,*N*-dimethylmethanamine *N*-oxide,

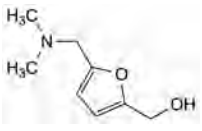

F. [5-[(dimethylamino)methyl]furan-2-yl]methanol,

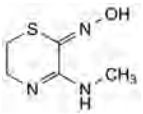

G. [3-(methylamino)-5,6-dihydro-2*H*-1,4-thiazin-2-ylidene]hydroxylamine,

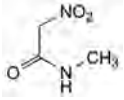

H. *N*-methyl-2-nitroacetamide,

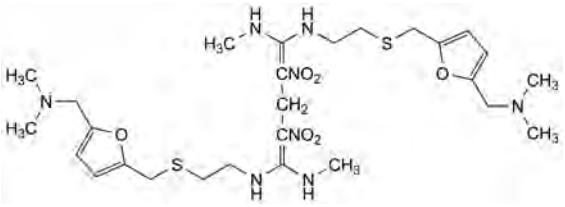

I. 2,2′-methylenebis[*N*-[2-[[[5-[(dimethylamino)methyl]-furan-2-yl]methyl]sulfanyl]ethyl]-*N*′-methyl-2-nitro-eth-1-ene-1,1-diamine],

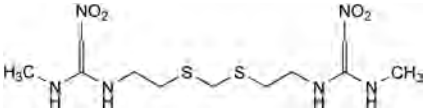

J. *N*,*N*″-[methylenebis(sulfanediylethan-2,1-diyl)]bis(*N*′-methyl-2-nitroeth-1-ene-1,1-diamine),

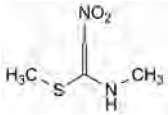

K. *N*-methyl-1-(methylsulfanyl)-2-nitroeth-1-en-1-amine.

**01/2010:1369**

# RAPESEED OIL, REFINED

## Rapae oleum raffinatum

DEFINITION

Fatty oil obtained from the seeds of *Brassica napus* L. and *Brassica campestris* L. by mechanical expression or by extraction. It is then refined. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, light yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

*Relative density*: about 0.917.

*Refractive index*: about 1.473.

IDENTIFICATION

Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

TESTS

**Acid value** (*2.5.1*): maximum 0.5, determined on 10.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *palmitic acid*: 2.5 per cent to 6.0 per cent,
- *stearic acid*: maximum 3.0 per cent,
- *oleic acid*: 50.0 per cent to 67.0 per cent,
- *linoleic acid*: 16.0 per cent to 30.0 per cent,
- *linolenic acid*: 6.0 per cent to 14.0 per cent,
- *eicosenoic acid*: maximum 5.0 per cent,
- *erucic acid*: maximum 2.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states whether the oil is obtained by mechanical expression or by extraction.

**07/2019:3012**

# REGORAFENIB MONOHYDRATE

## Regorafenibum monohydricum

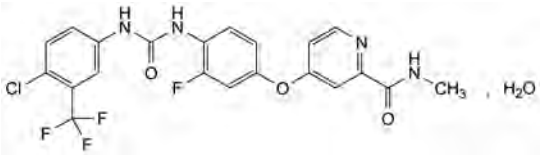

$C_{21}H_{15}ClF_4N_4O_3,H_2O$ $M_r$ 500.8

[1019206-88-2]

DEFINITION

4-[4-[[[4-Chloro-3-(trifluoromethyl)phenyl]carbamoyl]-amino]-3-fluorophenoxy]-*N*-methylpyridine-2-carboxamide monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white or pinkish or brownish powder.

*Solubility*: practically insoluble in water, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent) and in methanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *regorafenib monohydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

B. Water (see Tests).

TESTS

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and store the solutions at 2-8 °C.*

*Test solution.* Dissolve 0.250 g of the substance to be examined in *tetrahydrofuran R* and dilute to 5.0 mL with the same solvent.

*Reference solution.* Dissolve 5.0 mg of *regorafenib impurity A CRS* in *tetrahydrofuran R* and dilute to 50.0 mL with the same solvent. Dilute 2.5 mL of the solution to 50.0 mL with *tetrahydrofuran R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;

– *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 8 volumes of *acetonitrile for chromatography R* and 92 volumes of a solution containing 0.5 g/L of *dipotassium hydrogen phosphate R* and 1.5 g/L of *potassium dihydrogen phosphate R*;

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 17 | 100 → 22 | 0 → 78 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 228 nm.

*Autosampler*: set at 8 °C.

*Injection*: 3.0 µL.

*Relative retention* with reference to regorafenib (retention time = about 14 min): impurity A = about 0.6.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 10.0 per cent determined on 6 injections.

*Calculation of content*:

– for impurity A, use the concentration of impurity A in the reference solution.

*Limit*:

– *impurity A*: maximum 100 ppm.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and store the solutions at 2-8 °C.*

*Test solution (a).* Dissolve 30.0 mg of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent.

*Test solution (b).* Dilute 15.0 mL of test solution (a) to 50.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 30.0 mg of *regorafenib monohydrate CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 15.0 mL of the solution to 50.0 mL with *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 2 mg of *sorafenib CRS* in *methanol R* and dilute to 50 mL with the same solvent. Dilute 0.2 mL of the solution to 2 mL with test solution (a).

*Reference solution (d).* Dissolve 3 mg of *regorafenib impurity C CRS* in *methanol R* and dilute to 100 mL with the same solvent.

*Reference solution (e).* Dissolve 3 mg of *regorafenib impurity D CRS* in *dimethyl sulfoxide R* and dilute to 100 mL with the same solvent.

*Reference solution (f).* Mix 1 mL of reference solution (d) and 1 mL of reference solution (e) and dilute to 20 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 2.1 mm;

– *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (3.5 µm);

– *temperature*: 63 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile for chromatography R*, 0.1 per cent *V/V* solution of *trifluoroacetic acid R* (3:97 *V/V*);

– *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 15 | 100 → 78 | 0 → 22 |
| 15 - 25 | 78 → 60 | 22 → 40 |
| 25 - 33 | 60 → 36 | 40 → 64 |
| 33 - 37 | 36 | 64 |

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Autosampler*: set at 8 °C.

*Injection*: 5 µL of test solution (a) and reference solutions (b), (c) and (f).

*Identification of peaks*: use the chromatogram obtained with reference solution (f) to identify the peaks due to impurities C and D; use the chromatogram obtained with reference solution (c) to identify the peak due to sorafenib.

*Relative retention* with reference to regorafenib (retention time = about 25 min): impurity C = about 0.4; impurity D = about 0.8; sorafenib = about 0.95.

*System suitability*: reference solution (c):

– *resolution*: minimum 3.0 between the peaks due to sorafenib and regorafenib.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.5; impurity D = 0.6;
– for each impurity, use the concentration of regorafenib monohydrate in reference solution (b).

*Limits*:

– *impurity D*: maximum 0.2 per cent;
– *impurity C*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): 3.2 per cent to 4.0 per cent, determined on 50.0 mg using the evaporation technique at 150 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{21}H_{15}ClF_4N_4O_3$ taking into account the assigned content of *regorafenib monohydrate CRS*.

IMPURITIES

*Specified impurities: A, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, E.*

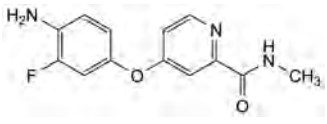

A. 4-(4-amino-3-fluorophenoxy)-*N*-methylpyridine-2-carboxamide,

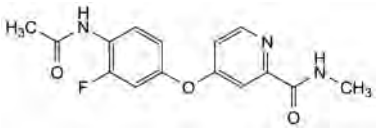

B. 4-(4-acetamido-3-fluorophenoxy)-*N*-methylpyridine-2-carboxamide,

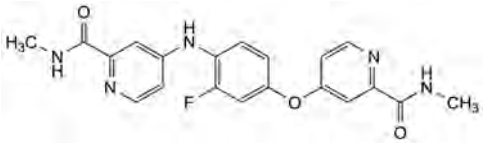

C. 4-[3-fluoro-4-[[2-(methylcarbamoyl)pyridin-4-yl]amino]phenoxy]-*N*-methylpyridine-2-carboxamide,

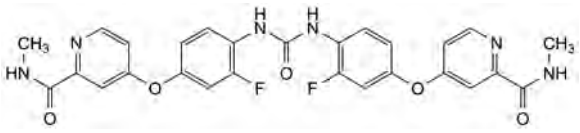

D. $3^3$,$7^2$-difluoro-*N*,*N*′-dimethyl-5-oxo-2,8-dioxa-4,6-diaza-1(4),9(4)-dipyridina-3(1,4),7(1,4)-dibenzenanonaphane-$1^2$,$9^2$-dicarboxamide,

E. $9^4$-chloro-$3^4$-[[[4-chloro-3-(trifluoromethyl)phenyl]-carbamoyl]amino]-$5^3$-fluoro-*N*-methyl-7-oxo-$9^3$-(trifluoromethyl)-2,4-dioxa-6,8-diaza-1(4)-pyridina-3(1,3),5(1,4),9(1)-tribenzenanonaphane-$1^2$-carboxamide.

**07/2017:2644**
**corrected 10.0**

# REMIFENTANIL HYDROCHLORIDE

## Remifentanili hydrochloridum

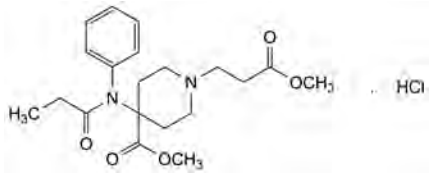

$C_{20}H_{29}ClN_2O_5$ $M_r$ 412.9
[132539-07-2]

DEFINITION

Methyl 1-(3-methoxy-3-oxopropyl)-4-[phenyl(propanoyl)-amino]piperidine-4-carboxylate hydrochloride.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in acetonitrile and in methanol, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *remifentanil hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and keep them at not more than 5 °C.*

*Buffer solution.* Dissolve 6.0 g of *anhydrous sodium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution (a).* Dissolve 30.0 mg of the substance to be examined in mobile phase B and dilute to 20.0 mL with mobile phase B.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20.0 mL with mobile phase B.

*Reference solution (a).* Dissolve 3 mg of *remifentanil impurity mixture CRS* (containing impurities A, B, C, E, L, N and O) in mobile phase B and dilute to 2 mL with mobile phase B.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (c).* Dissolve 30.0 mg of *remifentanil hydrochloride CRS* in mobile phase B and dilute to 20.0 mL with mobile phase B. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase B.

*Column*:
- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped octadecylphenylsilyl silica gel for chromatography R* (2 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: *acetonitrile R1*, *methanol R2*, buffer solution (1:9:90 *V/V/V*);
- *mobile phase B*: *acetonitrile R1*, buffer solution, *methanol R2* (12:40:48 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 85 | 15 |
| 8 - 40 | 85 → 45 | 15 → 55 |

*Flow rate*: 0.65 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5.0 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *remifentanil impurity mixture CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, E, L, N and O.

*Relative retention* with reference to remifentanil (retention time = about 10 min): impurity L = about 0.2; impurity O = about 0.40; impurity B = about 0.44; impurity A = about 0.7; impurity C = about 0.80; impurity N = about 0.85; impurity E = about 1.4.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurities O and B; minimum 2.0 between the peaks due to impurities C and N.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity L by 1.5;
- for each impurity, use the concentration of remifentanil hydrochloride in reference solution (b).

*Limits*:
- *impurity C*: maximum 0.5 per cent;
- *impurity A*: maximum 0.2 per cent;
- *impurities B, E, L*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Methyl acrylate**. Head-space gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 25.0 µL of *propanol R* to 100.0 mL with *water for chromatography R*. Dilute 50.0 mL of the solution to 1000.0 mL with *water for chromatography R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 5.0 mL with the internal standard solution.

*Reference solution.* Dilute 75.0 µL of *methyl acrylate R* to 100.0 mL with the internal standard solution. Dilute 10.0 mL of the solution to 100.0 mL with the internal standard solution. Dilute 7.0 mL of this solution to 100.0 mL with the internal standard solution.

*Precolumn*:
- *material*: deactivated fused silica;
- *size*: *l* = 5 m, Ø = 0.25 mm.

*Column*:
- *material*: fused silica;
- *size*: *l* = 60 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 8.3 mL/min.

*Pressure*: 68.9 kPa.

*Split ratio*: 1:10.

*Static head-space conditions that may be used*:
- *equilibration temperature*: 90 °C;
- *equilibration time*: 5 min;
- *transfer-line temperature*: 180 °C;
- *pressurisation time*: 2 min;
- *injection*: 6 s or 1 mL;
- *withdrawal time*: 12 s;
- *shaker*: on.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 35 | 35 |
| | 35 - 40 | 35 → 210 |
| | 40 - 50 | 210 |
| Injection port | | 180 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Retention time*: propanol = about 25 min; methyl acrylate = about 27 min.

*System suitability*: reference solution:
- *resolution*: minimum 5.0 between the peaks due to propanol and methyl acrylate.

Calculate the content of methyl acrylate, taking its relative density to be 0.955 at 20 °C.

*Limit*:
- *methyl acrylate*: maximum 250 ppm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 85 | 15 |
| 8 - 12 | 85 → 83 | 15 → 17 |
| 12 - 15 | 83 → 15 | 17 → 85 |
| 15 - 25 | 15 | 85 |

*Injection*: test solution (b) and reference solution (c).

*Retention time*: remifentanil = about 11 min.

*System suitability*: reference solution (c):

– *symmetry factor*: maximum 2.2 for the peak due to remifentanil.

Calculate the percentage content of $C_{20}H_{29}ClN_2O_5$ taking into account the assigned content of *remifentanil hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, C, E, L.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, G, H, I, J, K, M, N, O.*

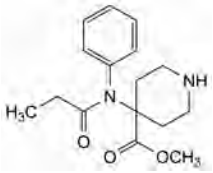

A. methyl 4-[phenyl(propanoyl)amino]piperidine-4-carboxylate,

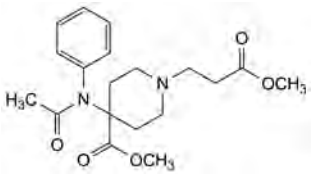

B. methyl 4-[acetyl(phenyl)amino]-1-(3-methoxy-3-oxopropyl)piperidine-4-carboxylate,

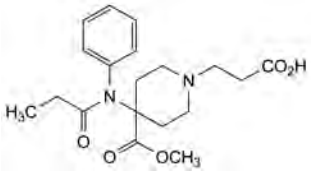

C. 3-[4-(methoxycarbonyl)-4-[phenyl(propanoyl)amino]-piperidin-1-yl]propanoic acid,

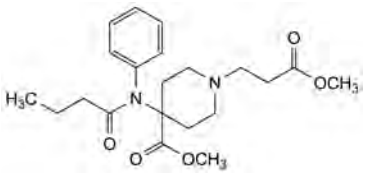

D. methyl 4-[butanoyl(phenyl)amino]-1-(3-methoxy-3-oxopropyl)piperidine-4-carboxylate,

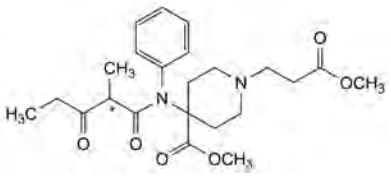

E. methyl 1-(3-methoxy-3-oxopropyl)-4-[(2-methyl-3-oxopentanoyl)(phenyl)amino]piperidine-4-carboxylate,

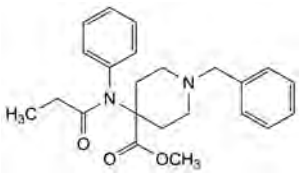

G. methyl 1-benzyl-4-[phenyl(propanoyl)amino]piperidine-4-carboxylate,

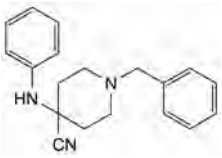

H. 1-benzyl-4-(phenylamino)piperidine-4-carbonitrile,

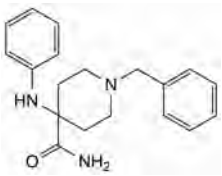

I. 1-benzyl-4-(phenylamino)piperidine-4-carboxamide,

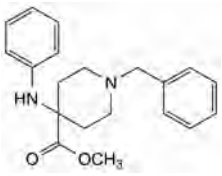

J. methyl 1-benzyl-4-(phenylamino)piperidine-4-carboxylate,

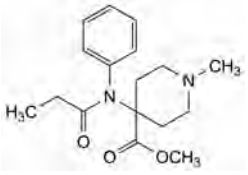

K. methyl 1-methyl-4-[phenyl(propanoyl)amino]piperidine-4-carboxylate,

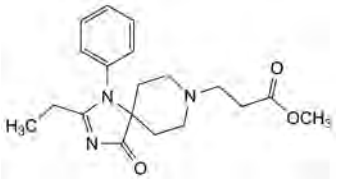

L. methyl 3-(2-ethyl-4-oxo-1-phenyl-1,3,8-triazaspiro[4.5]-dec-2-en-8-yl)propanoate,

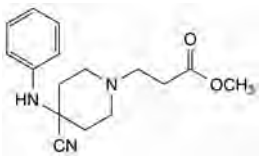

M. methyl 3-[4-cyano-4-(phenylamino)piperidin-1-yl]propanoate,

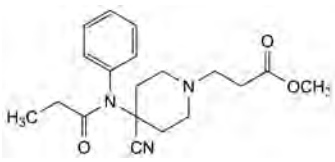

N. methyl 3-[4-cyano-4-[phenyl(propanoyl)amino]piperidin-1-yl]propanoate,

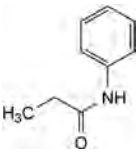

O. *N*-phenylpropanamide.

**01/2008:2135**
**corrected 6.0**

# REPAGLINIDE

## Repaglinidum

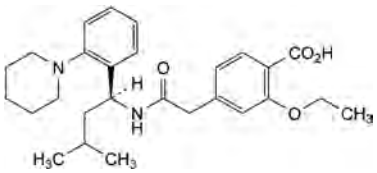

$C_{27}H_{36}N_2O_4$ $M_r$ 452.6
[135062-02-1]

DEFINITION

2-Ethoxy-4-[2-[[(1*S*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]-butyl]amino]-2-oxoethyl]benzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): + 6.3 to + 7.7.

Dissolve 1.00 g in *methanol R* and dilute to 20.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *repaglinide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).
*Prepare the solutions in amber flasks and vials.*

*Test solution*. Dissolve 10.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5.0 mg of *repaglinide impurity E CRS* in *methanol R* and dilute to 50.0 mL with the same of solvent.

*Reference solution (b)*. Dilute 2.0 mL of reference solution (a) to 100.0 mL with *methanol R*.

*Reference solution (c)*. Mix 1.0 mL of the test solution and 10 mL of reference solution (a) and dilute to 50.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.1 m, Ø = 4.0 mm,
- *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1.0 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 4.7 with *dilute sodium hydroxide solution R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 80 → 60 | 20 → 40 |
| 4 - 6 | 60 | 40 |

*Equilibration after installation of the column for use*: using *water R*, slowly increase the flow rate from 0.2 mL/min to 0.5 mL/min. Maintain the flow rate at 0.5 mL/min for 5 min. The column must be washed for 1 h at a flow rate of 1 mL/min with *water R* and for 1 h with the mobile phase at the initial composition prior to the 1st analysis.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Retention time*: repaglinide = about 3.3 min; impurity E = about 5.0 min.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to repaglinide and impurity E.

*Limit*:
- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 2.0 mL of this solution to 100.0 mL with *acetonitrile R*.

*Reference solution (b)*. With the aid of an ultrasonic bath, dissolve the contents of 1 vial of *repaglinide for system suitability CRS* in 2.0 mL of *acetonitrile R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *silica gel for chromatography, alkyl-bonded for use with highly aqueous mobile phases R* (5 µm),
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: 4.0 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.2 with *dilute phosphoric acid R*;

– *mobile phase B*: mobile phase A, *acetonitrile R* (300:700 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 50 → 7 | 50 → 93 |
| 20 - 30 | 7 | 93 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Relative retention* with reference to repaglinide (retention time = about 10 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.4; impurity D = about 1.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurity B and impurity C,
– the chromatogram obtained is similar to the chromatogram supplied with *repaglinide for system suitability CRS*.

*Limits*:

– *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity B = 0.7; impurity C = 3.1;
– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.320 g in 10 mL *methanol R* and add 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 45.26 mg of $C_{27}H_{36}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

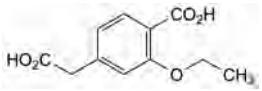

A. 4-(carboxymethyl)-2-ethoxybenzoic acid,

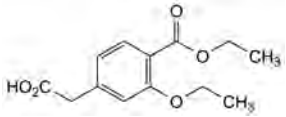

B. [3-ethoxy-4-(ethoxycarbonyl)phenyl]acetic acid,

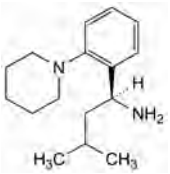

C. (1*S*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butan-1-amine,

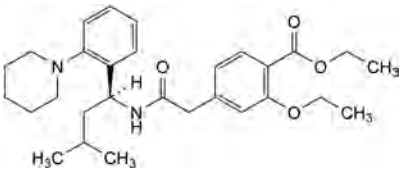

D. ethyl 2-ethoxy-4-[2-[[(1*S*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butyl]amino]-2-oxoethyl]benzoate,

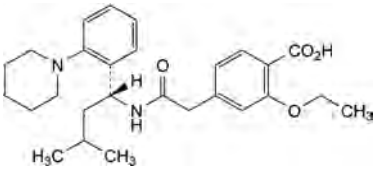

E. 2-ethoxy-4-[2-[[(1*R*)-3-methyl-1-[2-(piperidin-1-yl)phenyl]butyl]amino]-2-oxoethyl]benzoic acid.

**01/2008:0528**
**corrected 10.0**

# RESERPINE

## Reserpinum

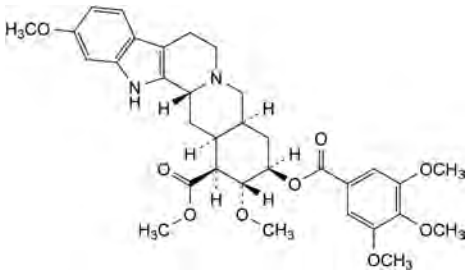

$C_{33}H_{40}N_2O_9$ $M_r$ 609
[50-55-5]

DEFINITION

Methyl 11,17α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimban-16β-carboxylate.

*Content*:

– *reserpine*: 98.0 per cent to 102.0 per cent (dried substance),
– *total alkaloids*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, small crystals or crystalline powder, darkening slowly on exposure to light.

*Solubility*: practically insoluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 20.0 mg in *chloroform R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *ethanol (96 per cent) R*. Examine immediately.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 268 nm.

*Specific absorbance at the absorption maximum*: 265 to 285.

Over the range 288-295 nm, the curve shows a slight absorption minimum followed by a shoulder or a slight absorption maximum; over this range, the specific absorbance is about 170.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *reserpine CRS*.

C. To about 1 mg add 0.1 mL of a 1 g/L solution of *sodium molybdate R* in *sulfuric acid R*. A yellow colour is produced which becomes blue within 2 min.

D. To about 1 mg add 0.2 mL of a freshly prepared 10 g/L solution of *vanillin R* in *hydrochloric acid R*. A pink colour develops within 2 min.

E. Mix about 0.5 mg with 5 mg of *dimethylaminobenzaldehyde R* and 0.2 mL of *glacial acetic acid R* and add 0.2 mL of *sulfuric acid R*. A green colour is produced. Add 1 mL of *glacial acetic acid R*. The colour becomes red.

TESTS

**Specific optical rotation** (*2.2.7*): − 116 to − 128 (dried substance).

Dissolve 0.250 g in *chloroform R* and dilute to 25.0 mL with the same solvent. Examine immediately.

**Oxidation products**. Dissolve 20 mg in *glacial acetic acid R* and dilute to 100.0 mL with the same acid. The absorbance (*2.2.25*) measured immediately at the absorption maximum at 388 nm is not greater than 0.10.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 0.500 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 0.5 g.

ASSAY

**Total alkaloids**. Dissolve 0.500 g in a mixture of 6 mL of *acetic anhydride R* and 40 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 60.9 mg of total alkaloids.

**Reserpine**. *Protect the solutions from light.* Moisten 25.0 mg with 2 mL of *ethanol (96 per cent) R*, add 2 mL of *0.25 M sulfuric acid* and 10 mL of *ethanol (96 per cent) R*, and warm gently to dissolve. Cool and dilute to 100.0 mL with *ethanol (96 per cent) R*. Dilute 5.0 mL of this solution to 50.0 mL with *ethanol (96 per cent) R*. Prepare a reference solution in the same manner using 25.0 mg of *reserpine CRS*. Place 10.0 mL of each solution separately in 2 boiling-tubes, add 2.0 mL of *0.25 M sulfuric acid* and 2.0 mL of a freshly prepared 3 g/L solution of *sodium nitrite R*. Mix and heat in a water-bath at 55 °C for 35 min. Cool, add 1.0 mL of a freshly prepared 50 g/L solution of *sulfamic acid R* and dilute to 25.0 mL with *ethanol (96 per cent) R*. Measure the absorbance (*2.2.25*) of each solution at the absorption maximum at 388 nm, using as the compensation liquid 10.0 mL of the same solution prepared at the same time in the same manner, but omitting the sodium nitrite.

Calculate the content of $C_{33}H_{40}N_2O_9$ from the absorbances measured and the concentrations of the solutions.

STORAGE

Protected from light.

**01/2008:0290**

# RESORCINOL

## Resorcinolum

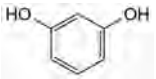


$C_6H_6O_2$ $M_r$ 110.1

[108-46-3]

DEFINITION

Resorcinol contains not less than 98.5 per cent and not more than the equivalent of 101.0 per cent of benzene-1,3-diol, calculated with reference to the dried substance.

CHARACTERS

A colourless or slightly pinkish-grey, crystalline powder or crystals, turning red on exposure to light and air, very soluble in water and in alcohol.

IDENTIFICATION

A. Melting point (*2.2.14*): 109 °C to 112 °C.

B. Dissolve 0.1 g in 1 mL of *water R*, add 1 mL of *strong sodium hydroxide solution R* and 0.1 mL of *chloroform R*, heat and allow to cool. An intense, deep-red colour develops which becomes pale yellow on the addition of a slight excess of *hydrochloric acid R*.

C. Thoroughly mix about 10 mg with about 10 mg of *potassium hydrogen phthalate R*, both finely powdered. Heat over a naked flame until an orange-yellow colour is obtained. Cool and add 1 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R* and shake to dissolve. The solution shows an intense green fluorescence.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$ or $R_5$ (*2.2.2, Method II*) and remains so when heated in a water-bath for 5 min.

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *bromophenol blue solution R2*. Not more than 0.05 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution*. Dissolve 0.5 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dilute 0.1 mL of the test solution to 20 mL with *methanol R*.

Apply separately to the plate 2 µL of each solution. Develop over a path of 15 cm using a mixture of 40 volumes of *ethyl acetate R* and 60 volumes of *hexane R*. Allow the plate to dry in air for 15 min and expose it to iodine vapour. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Pyrocatechol**. To 2 mL of solution S add 1 mL of *ammonium molybdate solution R2* and mix. Any yellow colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 2 mL of a 0.1 g/L solution of *pyrocatechol R*.

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.00 g of powdered substance by drying in a desiccator for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in *water R* and dilute to 250.0 mL with the same solvent. To 25.0 mL of the solution in a ground-glass-stoppered flask add 1.0 g of *potassium bromide R*, 50.0 mL of *0.0167 M potassium bromate*, 15 mL of *chloroform R* and 15.0 mL of *hydrochloric acid R1*. Stopper the flask, shake and allow to stand in the dark for 15 min, shaking occasionally. Add 10 mL of a 100 g/L solution of *potassium iodide R*, shake thoroughly, allow to stand for 5 min and titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.0167 M potassium bromate* is equivalent to 1.835 mg of $C_6H_6O_2$.

STORAGE

Store protected from light.

**01/2017:2109**

# RIBAVIRIN

## Ribavirinum

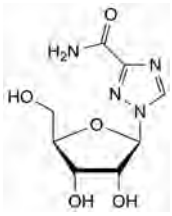

$C_8H_{12}N_4O_5$ $M_r$ 244.2
[36791-04-5]

DEFINITION

1-β-D-Ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), slightly soluble or very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ribavirin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**pH** (*2.2.3*): 4.0 to 6.5.

Dissolve 0.200 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 33 to − 37 (dried substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent. Determine the specific optical rotation within 10 min of preparing the solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. In order to produce impurity A *in situ*, mix 5.0 mL of the test solution and 5.0 mL of a 42 g/L solution of *sodium hydroxide R* and allow to stand for 90 min. Neutralise with 5.0 mL of a 103 g/L solution of *hydrochloric acid R* and mix well.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water for chromatography R*. Dilute 1.0 mL of this solution to 10.0 mL with *water for chromatography R*.

*Reference solution (c)*. Dissolve 50.0 mg of *ribavirin CRS* in *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (3 µm) suitable for use with highly aqueous mobile phases ;
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *anhydrous sodium sulfate R* in 950 mL of *water for chromatography R*, add 2.0 mL of a 5 per cent *V/V* solution of *phosphoric acid R*, adjust to pH 2.8 with a 5 per cent *V/V* solution of *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*, mobile phase A (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 25 | 100 → 0 | 0 → 100 |
| 25 - 35 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 5 µL of the test solution and reference solutions (a) and (b).

*Relative retention* with reference to ribavirin (retention time = about 6 min): impurity A = about 0.8.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to impurity A and ribavirin.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 2.3;
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 5 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_8H_{12}N_4O_5$ from the declared content of *ribavirin CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G.*

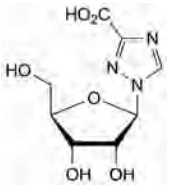

A. 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxylic acid,

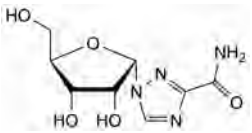

B. 1-α-D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide (anomer),

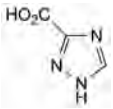

C. 1*H*-1,2,4-triazole-3-carboxylic acid,

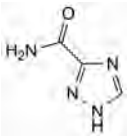

D. 1*H*-1,2,4-triazole-3-carboxamide,

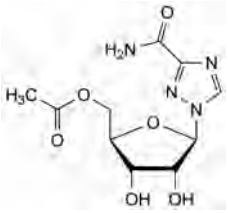

F. 1-(5-*O*-acetyl-β-D-ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide (5′-*O*-acetylribavirin),

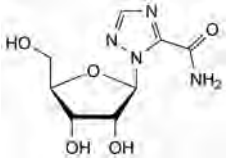

G. 1-β-D-ribofuranosyl-1*H*-1,2,4-triazole-5-carboxamide (*N*-isomer).

**01/2008:0292**

# RIBOFLAVIN

## Riboflavinum

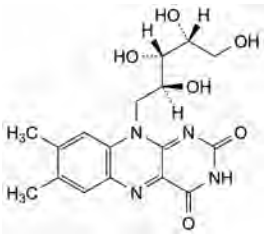

$C_{17}H_{20}N_4O_6$ $M_r$ 376.4

[83-88-5]

DEFINITION

7,8-Dimethyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]-benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione.

This monograph applies to riboflavin produced by fermentation.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or orange-yellow, crystalline powder.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent).

Solutions deteriorate on exposure to light, especially in the presence of alkali.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Suspend 25 mg of the substance to be examined in 10 mL of *water R*, shake for 5 min and filter the suspension to remove the undissolved material.

*Reference solution.* Suspend 25 mg of *riboflavin CRS* in 10 mL of *water R*, shake for 5 min and filter the suspension to remove the undissolved material.

*Plate*: *TLC silica gel plate R* (2-10 μm).

*Mobile phase*: *water R*.

*Application*: as follows, drying in a current of cold air after each individual application:

- *1st application*: 2 μL of *methylene chloride R* then 2 μL of the test solution;
- *2nd application*: 2 μL of *methylene chloride R* then 2 μL of the reference solution.

*Development*: over a path of 6 cm.

*Drying*: in a current of cold air.

*Detection*: examine in ultraviolet light at 365 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 1 mg in 100 mL of *water R*. The solution has, by transmitted light, a pale greenish-yellow colour, and, by reflected light, an intense yellowish-green fluorescence which disappears on the addition of mineral acids or alkalis.

TESTS

**Specific optical rotation** (*2.2.7*): – 115 to – 135 (dried substance).

Dissolve 50.0 mg in *0.05 M sodium hydroxide* free from carbonate and dilute to 10.0 mL with the same alkaline solution. Measure the optical rotation within 30 min of dissolution.

**Absorbance** (*2.2.25*).

*Test solution.* Dilute the final solution prepared for the assay with an equal volume of *water R*.

*Absorption maxima*: at 223 nm, 267 nm, 373 nm and 444 nm.

*Absorbance ratios*:

- $A_{373}/A_{267}$ = 0.31 to 0.33;
- $A_{444}/A_{267}$ = 0.36 to 0.39.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Solution A*: 13.6 g/L solution of *sodium acetate R*.

*Test solution.* With the aid of ultrasound, dissolve 0.120 g of the substance to be examined in 10 mL of *0.1 M sodium hydroxide* and dilute to 100 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (b).* With the aid of ultrasound, dissolve the contents of a vial of *riboflavin for peak identification CRS* (containing impurities C and D) in 1.0 mL of a mixture of 1 volume of mobile phase B and 9 volumes of mobile phase A.

*Reference solution (c).* In order to prepare *in situ* impurities A and B, dissolve 10 mg of the substance to be examined in 1 mL of *0.5 M sodium hydroxide*. Expose to daylight for 1.5 h. Add 0.5 mL of *acetic acid R* and dilute to 100 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: *phosphoric acid R, water R* (1:1000 *V/V*);
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 20 | 90 → 80 | 10 → 20 |
| 20 - 25 | 80 | 20 |
| 25 - 35 | 80 → 50 | 20 → 50 |
| 35 - 45 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 267 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *riboflavin for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to riboflavin (retention time = about 16 min): impurity C = about 0.2; impurity D = about 0.5; impurity A = about 1.4; impurity B = about 1.9.

*System suitability*:

- *resolution*: minimum 5 between the peaks due to impurities A and B in the chromatogram obtained with reference solution (c);
- the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *riboflavin for peak identification CRS*.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity B = 1.4; impurity C = 2.3; impurity D = 1.4;
- *impurity A*: not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent);
- *impurities B, C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit for peaks other than those due to impurity A*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on the residue obtained in the test for loss on drying.

ASSAY

*Carry out the assay protected from light.*

In a brown-glass 500 mL volumetric flask, suspend 65.0 mg in 5 mL of *water R* ensuring that it is completely wetted and dissolve in 5 mL of *dilute sodium hydroxide solution R*. As soon as dissolution is complete, add 100 mL of *water R* and 2.5 mL of *glacial acetic acid R* and dilute to 500.0 mL with *water R*. Place 20.0 mL of this solution in a 200 mL brown-glass volumetric flask, add 3.5 mL of a 14 g/L solution of *sodium acetate R* and dilute to 200.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 444 nm. Calculate the content of $C_{17}H_{20}N_4O_6$ taking the specific absorbance to be 328.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D.*

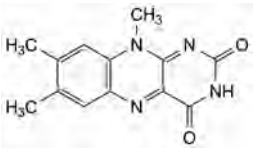

A. 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (lumiflavine),

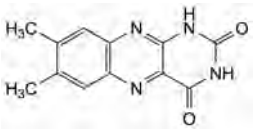

B. 7,8-dimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione,

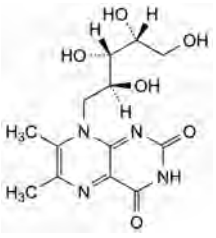

C. 6,7-dimethyl-8-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]-pteridine-2,4(3*H*,8*H*)-dione,

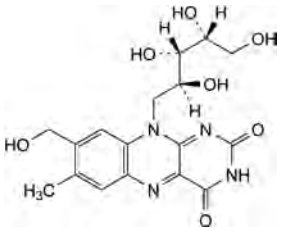

D. 8-(hydroxymethyl)-7-methyl-10-[(2*S*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentyl]benzo[*g*]pteridine-2,4(3*H*,10*H*)-dione.

**01/2017:0786**

# RIBOFLAVIN SODIUM PHOSPHATE

## Riboflavini natrii phosphas

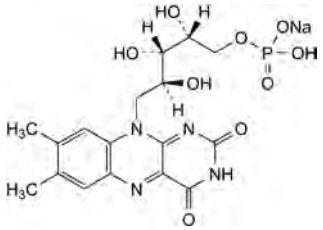

$C_{17}H_{20}N_4NaO_9P$ $M_r$ 478.3
[130-40-5]

DEFINITION

Mixture containing riboflavin 5′-(sodium hydrogen phosphate) as the main component and other riboflavin sodium monophosphates.

*Content*: 73.0 per cent to 79.0 per cent of riboflavin ($C_{17}H_{20}N_4O_6$; $M_r$ 376.4) (dried substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: yellow or orange-yellow, crystalline, hygroscopic powder.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 50.0 mg in *phosphate buffer solution pH 7.0 R* and dilute to 100.0 mL with the same buffer solution. Dilute 2.0 mL of this solution to 100.0 mL with *phosphate buffer solution pH 7.0 R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 266 nm.

*Specific absorbance at the absorption maximum*: 580 to 640.

B. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in position and approximate size to the principal peak in the chromatogram obtained with reference solution (b).

C. Dissolve about 10 mg in *dilute sodium hydroxide solution R* and dilute to 100 mL with the same solution. Expose 1 mL of this solution to ultraviolet light at 254 nm for 5 min, add sufficient *acetic acid R* to make the solution acidic to *blue litmus paper R* and shake with 2 mL of *methylene chloride R*. The lower layer shows yellow fluorescence.

D. To 0.5 g add 10 mL of *nitric acid R* and evaporate the mixture to dryness on a water-bath. Ignite the residue until it becomes white, dissolve the residue in 5 mL of *water R* and filter. The filtrate gives reaction (a) of sodium and reaction (b) of phosphates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.0 to 6.5.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 38.0 to + 43.0 (dried substance).

Dissolve 0.300 g in 18.2 mL of *hydrochloric acid R1* and dilute to 25.0 mL with *water R*.

**Impurity E**. To about 35 mg add 10 mL of *methylene chloride R*, shake for 5 min and filter. The filtrate is not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from actinic light.*

*Test solution*. Dissolve 0.100 g of the substance to be examined in 50 mL of *water R* and dilute to 100.0 mL with the mobile phase. Dilute 8.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 60 mg of *riboflavin CRS* (impurity D) in 1 mL of *hydrochloric acid R* and dilute to 250.0 mL with *water R*. Dilute 4.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 0.100 g of *riboflavin sodium phosphate CRS* in 50 mL of *water R* and dilute to 100.0 mL with the mobile phase. Dilute 8.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, 7.35 g/L solution of *potassium dihydrogen phosphate R* (150:850 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 100 µL.

*Run time*: until the peak due to riboflavin can be clearly evaluated.

*Relative retention* with reference to riboflavin 5′-monophosphate (retention time = about 20 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.5; riboflavin 3′-monophosphate = about 0.7; riboflavin 4′-monophosphate = about 0.9; impurity D = about 2.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to riboflavin 4′-monophosphate and riboflavin 5′-monophosphate.

Calculate the percentage content of free riboflavin (impurity D) and of riboflavin in the form of the diphosphates of riboflavin (impurities A, B, C) from the areas of the peaks in the chromatogram obtained with the test solution and the amount of free riboflavin in reference solution (a).

*Limits*:

- *impurity D*: maximum 6.0 per cent (dried substance);
- *sum of impurities A, B and C*: maximum 6.0 per cent (dried substance).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Inorganic phosphate**: maximum 1.5 per cent.

Dissolve 0.10 g in *water R* and dilute to 100 mL with the same solvent. To 5 mL of this solution, add 10 mL of *water R*,

5 mL of *buffered copper sulfate solution pH 4.0 R*, 2 mL of a 30 g/L solution of *ammonium molybdate R*, 1 mL of a freshly prepared solution containing 20 g/L of *4-methylaminophenol sulfate R* and 50 g/L of *sodium metabisulfite R*, and 1 mL of a 3 per cent *V/V* solution of *perchloric acid R*. Dilute to 25.0 mL with *water R* and measure, within 15 min of its preparation, the absorbance (*2.2.25*) of the solution at 800 nm, using as the compensation liquid a solution prepared in the same manner but without the substance to be examined. The absorbance is not greater than that of a solution prepared as follows: to 15 mL of *phosphate standard solution (5 ppm $PO_4$) R*, add 5 mL of *buffered copper sulfate solution pH 4.0 R*, 2 mL of a 30 g/L solution of *ammonium molybdate R*, 1 mL of a freshly prepared solution containing 20 g/L of *4-methylaminophenol sulfate R* and 50 g/L of *sodium metabisulfite R*, and 1 mL of a 3 per cent *V/V* solution of *perchloric acid R*; dilute to 25.0 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa for 5 h.

ASSAY

*Carry out the assay protected from light.*

Dissolve 0.100 g in 150 mL of *water R*, add 2 mL of *glacial acetic acid R* and dilute to 1000.0 mL with *water R*. To 10.0 mL of this solution add 3.5 mL of a 14 g/L solution of *sodium acetate R* and dilute to 50.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 444 nm.

Calculate the content of $C_{17}H_{20}N_4O_6$ taking the specific absorbance to be 328.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

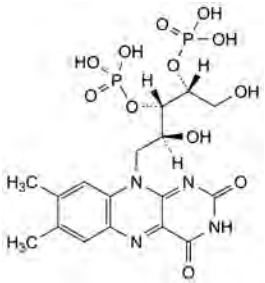

A. riboflavin 3′,4′-diphosphate,

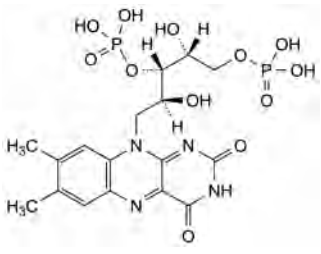

B. riboflavin 3′,5′-diphosphate,

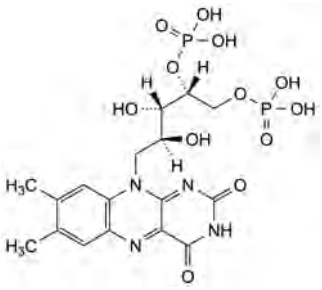

C. riboflavin 4′,5′-diphosphate,

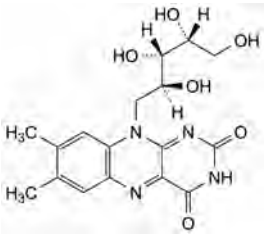

D. riboflavin,

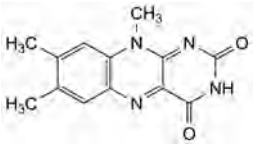

E. 7,8,10-trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (lumiflavin).

**04/2015:0349**

# RICE STARCH[(1)]

## Oryzae amylum

DEFINITION

Rice starch is obtained from the caryopsis of *Oryza sativa* L.

♦CHARACTERS

*Appearance*: very fine, white or almost white powder, which creaks when pressed between the fingers.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

Rice starch does not contain starch grains of any other origin. It may contain traces of, if any, fragments of the endosperm tissue of the fruit.♦

IDENTIFICATION

A. Microscopic examination (*2.8.23*) using a 50 per cent *V/V* solution of *glycerol R*. It presents polyhedral, simple grains 1-10 µm (mostly 4-6 µm) in size (Figure 0349.-1). These simple grains often gather in ellipsoidal, compound grains 50-100 µm in diameter. The grains have a poorly visible central hilum and there are no concentric striations. Between orthogonally orientated polarising plates or prisms, the starch grains show a distinct black cross intersecting at the hilum.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

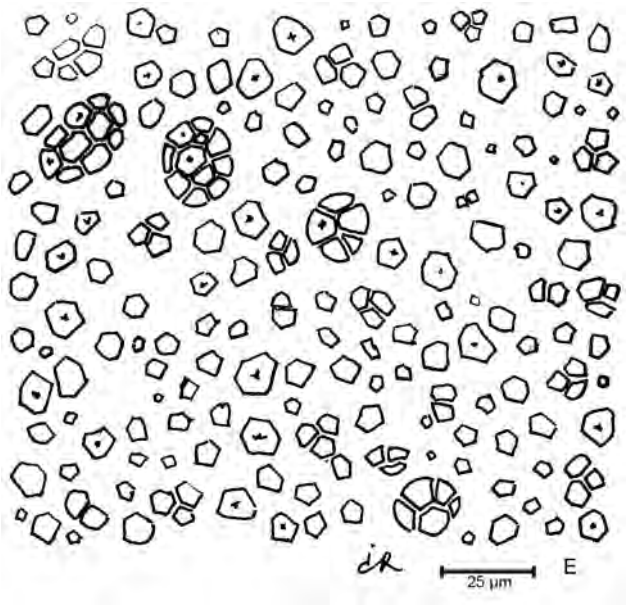


Figure 0349.-1. – *Illustration for identification test A of rice starch*

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B add 0.05 mL of *iodine solution R1*. An orange-red to dark blue colour is produced, which disappears on heating.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

Shake 5.0 g with 25.0 mL of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

♦**Foreign matter**. Examined under a microscope using a 50 per cent *V/V* solution of *glycerol R*, not more than traces of matter other than starch granules are present. No starch grains of any other origin are present.♦

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 10 ppm.

Shake 1.5 g with 15 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.00 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

◊Absence of *Salmonella* (*2.6.13*).◊

**01/2008:1657**
**corrected 6.0**

# RIFABUTIN

## Rifabutinum

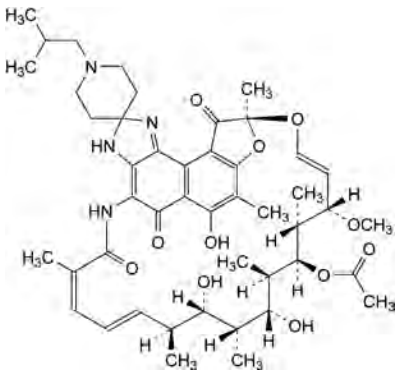

$C_{46}H_{62}N_4O_{11}$ [72559-06-9] $M_r$ 847

DEFINITION

(9*S*,12*E*,14*S*,15*R*,16*S*,17*R*,18*R*,19*R*,20*S*,21*S*,22*E*,24*Z*)-6,18,20-Trihydroxy-14-methoxy-7,9,15,17,19,21,25-heptamethyl-1′-(2-methylpropyl)-5,10,26-trioxo-3,5,9,10-tetrahydrospiro[9,4-(epoxypentadeca[1,11,13]trienimino)-2*H*-furo[2′,3′:7,8]naphtho[1,2-*d*]imidazole-2,4′-piperidine]-16-yl acetate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: reddish-violet amorphous powder.

*Solubility*: slightly soluble in water, soluble in methanol, slightly soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *rifabutin CRS*.

B. Examine the chromatograms obtained in the test for related substances.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution*. Dissolve 10 mg of *rifabutin impurity A CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 10 mL with the same mixture of solvents. Dilute 3 mL of the solution to 100 mL with a mixture of equal volumes of *methanol R* and *methylene chloride R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *acetone R*, *light petroleum R* (23:77 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour for about 5 min, then spray with *potassium iodide and starch solution R* and allow to stand for 5 min.

*Limit*:

– *impurity A*: any spot corresponding to impurity A is not more intense than the spot in the chromatogram obtained with the reference solution (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *rifabutin CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dissolve about 10 mg of *rifabutin CRS* in 2 mL of *methanol R*, add 1 mL of *dilute sodium hydroxide solution R* and allow to stand for about 4 min. Add 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.110 m, Ø = 4.6 mm,

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix equal volumes of *acetonitrile R* and a 13.6 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 6.5 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of rifabutin.

*Relative retention* with reference to rifabutin (retention time = about 9 min): impurity E = about 0.5; impurity B = about 0.6; impurity D = about 0.9; impurity C = about 1.3.

*System suitability*: reference solution (c):

– *resolution*: minimum 2.0 between the second peak of the 3 peaks due to degradation products and the peak due to rifabutin.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent); not more than 1 such peak has an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of ribabutin.

IMPURITIES

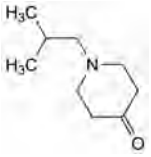

A. 1-(2-methylpropyl)piperidin-4-one,

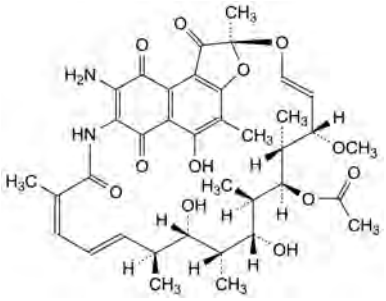

B. 3-aminorifamycin S,

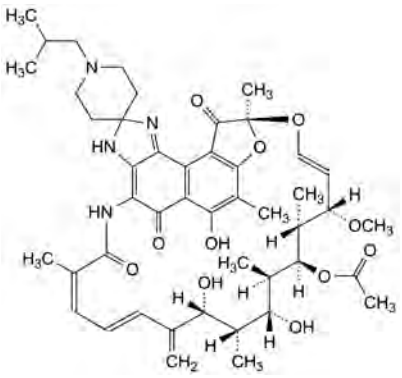

C. 21,31-didehydrorifabutin,

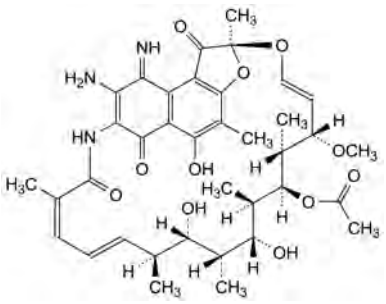

D. 3-amino-4-imidorifamycin S,

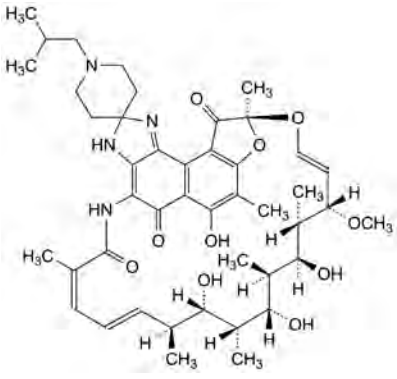

E. 16-deacetylrifabutin.

**01/2017:0052**

# RIFAMPICIN

## Rifampicinum

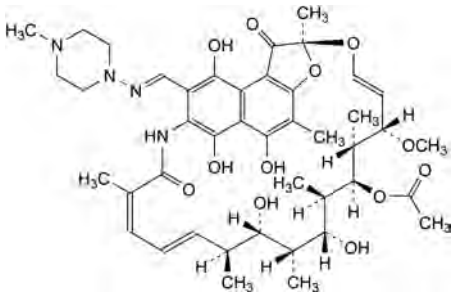

$C_{43}H_{58}N_4O_{12}$ $M_r$ 823

[13292-46-1]

DEFINITION

(2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-5,6,9,17,19-Pentahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-8-[[(4-methylpiperazin-1-yl)imino]methyl]-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]-trienimino)naphto[2,1-*b*]furan-21-yl acetate.

Semi-synthetic antibiotic obtained from rifamycin SV.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: reddish-brown or brownish-red, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol, slightly soluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in 50 mL of *methanol R*. Dilute 1 mL of this solution to 50 mL with *phosphate buffer solution pH 7.4 R*.

*Spectral range*: 220-500 nm.

*Absorption maxima*: at 237 nm, 254 nm, 334 nm and 475 nm.

*Absorbance ratio*: $A_{334}/A_{475}$ = about 1.75.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: mulls in *liquid paraffin R*.

*Comparison*: *rifampicin CRS*.

C. Suspend about 25 mg in 25 mL of *water R*, shake for 5 min and filter. To 5 mL of the filtrate add 1 mL of a 100 g/L solution of *ammonium persulfate R* in *phosphate buffer solution pH 7.4 R* and shake for a few minutes. The colour changes from orange-yellow to violet-red and no precipitate is formed.

TESTS

**pH** (*2.2.3*): 4.5 to 6.5 for a 10 g/L suspension in *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the test solution and the reference solution immediately before use.*

*Solvent mixture.* To 10 volumes of a 210.1 g/L solution of *citric acid monohydrate R* add 23 volumes of a 136.1 g/L solution of *potassium dihydrogen phosphate R*, 77 volumes of a 174.2 g/L solution of *dipotassium hydrogen phosphate R*, 250 volumes of *acetonitrile R* and 640 volumes of *water R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixure.

*Reference solution.* Dissolve 20.0 mg of *rifampicin quinone CRS* (impurity A) in *acetonitrile R* and dilute to 100.0 mL with the same solvent. To 1.0 mL of this solution add 1.0 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.12 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R* and 65 volumes of a solution containing 0.1 per cent *V/V* of *phosphoric acid R*, 1.9 g/L of *sodium perchlorate R*, 5.9 g/L of *citric acid monohydrate R* and 20.9 g/L of *potassium dihydrogen phosphate R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of rifampicin.

*System suitability*: reference solution:

- *resolution*: minimum 4.0 between the peaks due to rifampicin and impurity A; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (1.5 per cent);
- *any other impurity*: for each impurity, not more than the area of the peak due to rifampicin in the chromatogram obtained with the reference solution (1.0 per cent);
- *sum of impurities other than A*: not more than 3.5 times the area of the peak due to rifampicin in the chromatogram obtained with the reference solution (3.5 per cent);
- *disregard limit*: 0.05 times the area of the peak due to rifampicin in the chromatogram obtained with the reference solution (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying at 80 °C at a pressure not exceeding 0.67 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.100 g in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with *phosphate buffer solution pH 7.4 R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 475 nm, using *phosphate buffer solution pH 7.4 R* as the compensation liquid.

Calculate the content of $C_{43}H_{58}N_4O_{12}$, taking the specific absorbance to be 187.

STORAGE

Under nitrogen in an airtight container, protected from light, at a temperature not exceeding 25 °C.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

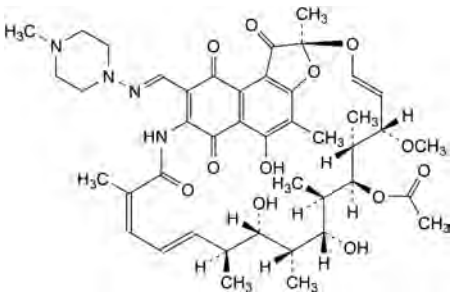

A. rifampicin quinone,

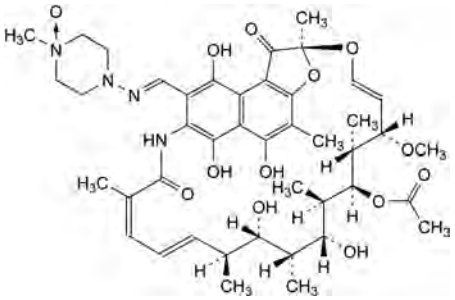

B. rifampicin *N*-oxide.

**01/2019:0432**
**corrected 10.0**

# RIFAMYCIN SODIUM

## Rifamycinum natricum

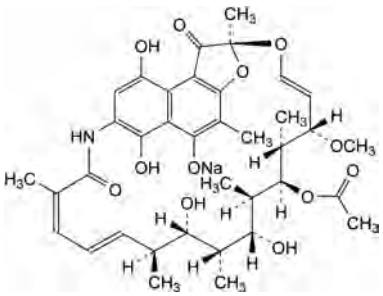

$C_{37}H_{46}NNaO_{12}$ $M_r$ 720
[14897-39-3]

DEFINITION

Sodium (2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-21-(acetyloxy)-6,9,17,19-tetrahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienazano)naphtho[2,1-*b*]furan-5-olate.

Monosodium salt of rifamycin SV, obtained by chemical transformation of rifamycin B, which is produced during the growth of certain strains of *Amycolatopsis mediterranei*. Rifamycin SV may also be obtained directly from certain *A. mediterranei* mutants.

*Potency*: minimum 900 IU/mg (anhydrous substance).

PRODUCTION

It is produced by methods of manufacture designed to minimise or eliminate substances lowering blood pressure.

CHARACTERS

*Appearance*: fine or slightly granular, red powder.

*Solubility*: soluble in water, freely soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs of *potassium bromide R*.
*Comparison*: *rifamycin sodium CRS*.

B. Suspend 70 mg of the substance to be examined in 0.5 mL of *water R*. Add 1.5 mL of *methoxyphenylacetic reagent R* to obtain a clear red solution. Cool in ice-water for 30 min. A precipitate is formed. Place in water at 20 °C and stir for 5 min. The precipitate does not disappear. Add 1 mL of *dilute ammonia R1*. The precipitate dissolves completely. Add 1 mL of *ammonium carbonate solution R*. No precipitate is formed.

TESTS

**pH** (*2.2.3*): 6.5 to 8.0.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Absorbance** (*2.2.25*). Dissolve 20.0 mg in 5 mL of *methanol R* and dilute to 100.0 mL with freshly prepared *phosphate buffer solution pH 7.0 R1* to which 1 g/L of *ascorbic acid R* has been added immediately before use. Dilute 5.0 mL of this solution to 50.0 mL with the same phosphate buffer solution containing ascorbic acid. Allow to stand for 30 min. The solution shows an absorption maximum at 445 nm. The specific absorbance at this absorption maximum is 190 to 210 (anhydrous substance).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Mix 50 volumes of *acetonitrile R* and 50 volumes of a 3.9 g/L solution of *sodium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10.0 mg of *rifamycin B CRS* (impurity A) and 40.0 mg of *rifamycin S CRS* (impurity B) in the solvent mixture and dilute to 200.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 25 mg of the substance to be examined and 8 mg of *rifamycin S CRS* in the solvent mixture and dilute to 250.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: mix 10 volumes of *acetonitrile R* and 90 volumes of a 3.9 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 7.5 with *dilute sodium hydroxide solution R*;
- *mobile phase B*: mix 30 volumes of a 3.9 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 7.5 with *dilute sodium hydroxide solution R* and 70 volumes of *acetonitrile R*;
- *temperature*: minimum 20 °C;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 80 → 20 | 20 → 80 |
| 40 - 45 | 20 | 80 |
| 45 - 47 | 20 → 80 | 80 → 20 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Elution order*: impurity A, rifamycin SV, impurity B.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to rifamycin SV and impurity B.

*Limits*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (2 per cent);

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *sum of impurities other than A and B*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (2 per cent);

– *disregard limit*: 0.05 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Water** (*2.5.12*): 12.0 per cent to 17.0 per cent, determined on 0.200 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.50 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

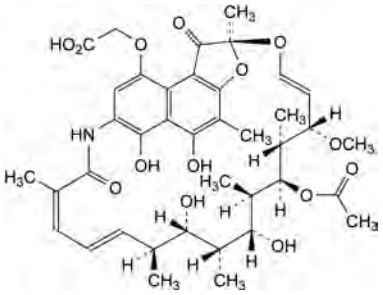

A. [(2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-21-(acetyloxy)-5,6,17,19-tetrahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-1,11-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienazano)naphtho[2,1-*b*]furan-9-yl]acetic acid (rifamycin B),

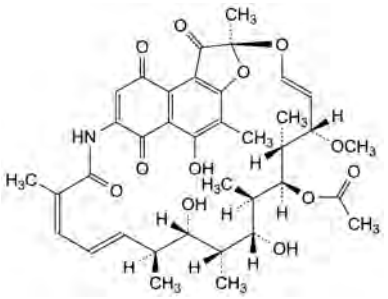

B. [(2*S*,12*Z*,14*E*,16*S*,17*S*,18*R*,19*R*,20*R*,21*S*,22*R*,23*S*,24*E*)-5,17,19-trihydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-1,6,9,11-tetraoxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienazano)naphtho[2,1-*b*]furan-21-yl acetate (rifamycin S),

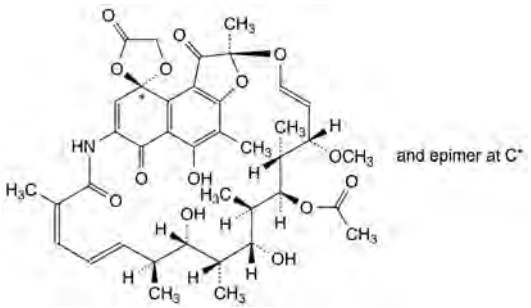


C. [(2*RS*,2′*S*,12′*Z*,14′*E*,16′*S*,17′*S*,18′*R*,19′*R*,20′*R*,21′*S*,22′*R*,-23′*S*,24′*E*)-5′,17′,19′-trihydroxy-23′-methoxy-2′,4′,12′,16′,18′,20′,22′-heptamethyl-1′,4,6′,11′-tetraoxo-1′,2′-dihydrospiro[1,3-dioxolane-2,9′(6′*H*)-[2,7](epoxypentadeca[1,11,13]trienazano)naphtho[2,1-*b*]furan]-21′-yl acetate (rifamycin O).

**01/2017:2362**

# RIFAXIMIN

## Rifaximinum

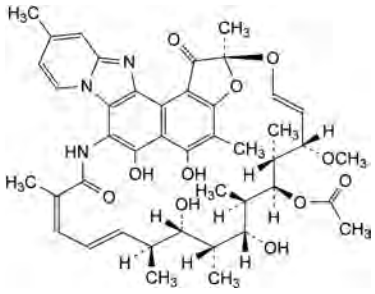

$C_{43}H_{51}N_3O_{11}$ $M_r$ 786

[80621-81-4]

DEFINITION

(2*S*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*R*,27*S*,28*E*)5,6,21,23-Tetrahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octamethyl-1,15-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]-trienoimino)[1]benzofuro[4,5-*e*]pyrido[1,2-*a*]benzimidazol-25-yl acetate.

Semi-synthetic product derived from a fermentation product.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: red-orange, hygroscopic, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rifaximin CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *water R* (40:60 *V/V*).

*Test solution (a)*. Dissolve 0.100 g of the substance to be examined in 8 mL of *acetonitrile R* and dilute to 20 mL with *water R*.

*Test solution (b)*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *rifaximin for system suitability CRS* (containing impurity H) in 4 mL of the solvent mixture.

*Reference solution (c)*. Dissolve 40.0 mg of *rifaximin CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 37 volumes of a 3.16 g/L solution of *ammonium formate R* adjusted to pH 7.2 with *dilute ammonia R1* and 63 volumes of a mixture of equal volumes of *acetonitrile R* and *methanol R*.

*Flow rate*: 1.4 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3 times the retention time of rifaximin.

*Relative retention* with reference to rifaximin (retention time = about 12 min): impurities D and H = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurities D + H and rifaximin.

*Limits*:

- *sum of impurities D and H*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 4.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{43}H_{51}N_3O_{11}$ using the chromatogram obtained with reference solution (c) and the declared content of *rifaximin CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F, G.*

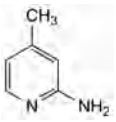

A. 4-methylpyridin-2-amine,

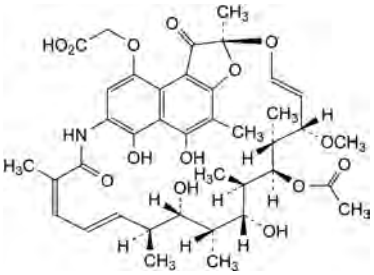

B. rifamycin B,

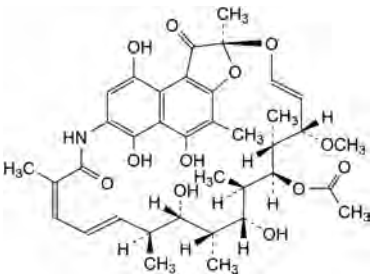

C. rifamycin SV,

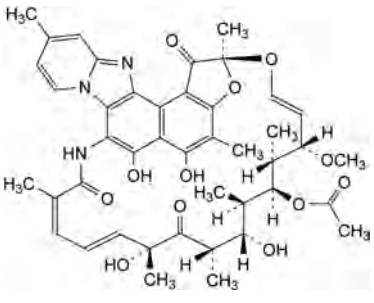

D. rifaximin Y,

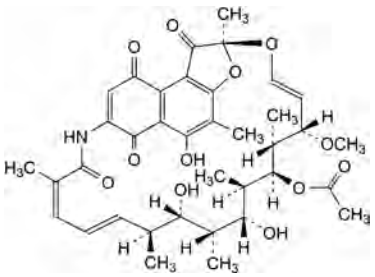

E. rifamycin S,

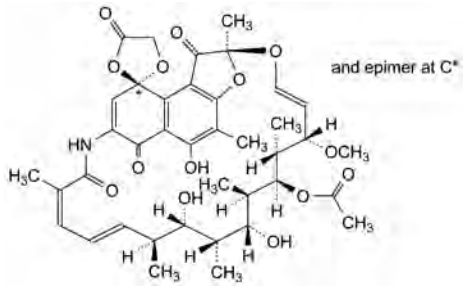


F. rifamycin O,

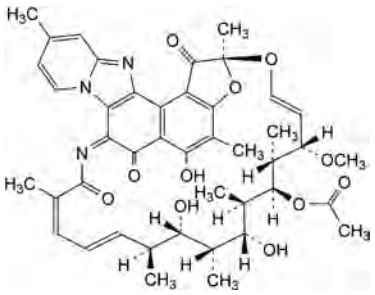

G. (2*S*,7*Z*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*R*,27*S*,28*E*)-5,21,23-trihydroxy-27-methoxy-2,4,11,16,20,22,24,26-octamethyl-1,6,15-trioxo-1,2,6,7-tetrahydro-2,7-(epoxypentadeca[1,11,13]trienonitrilo)[1]benzofuro-[4,5-*e*]pyrido[1,2-*a*]benzimidazol-25-yl acetate (6-*O*,14-didehydrorifaximin),

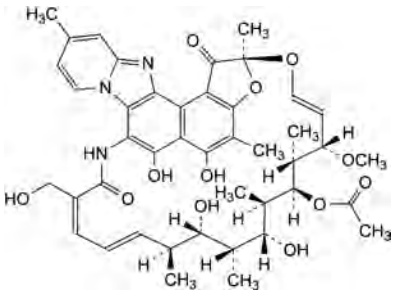

H. (2*S*,16*Z*,18*E*,20*S*,21*S*,22*R*,23*R*,24*R*,25*S*,26*R*,27*S*,28*E*)-5,6,21,23-tetrahydroxy-16-(hydroxymethyl)-27-methoxy-2,4,11,20,22,24,26-heptamethyl-1,15-dioxo-1,2-dihydro-2,7-(epoxypentadeca[1,11,13]trienoimino)[1]benzofuro-[4,5-*e*]pyrido[1,2-*a*]benzimidazol-25-yl acetate (16-desmethyl-16-(hydroxymethyl)rifaximin).

**01/2008:2020**
**corrected 10.0**

# RILMENIDINE DIHYDROGEN PHOSPHATE

## Rilmenidini dihydrogenophosphas

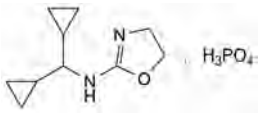

$C_{10}H_{19}N_2O_5P$ $M_r$ 278.2
[85409-38-7]

DEFINITION

*N*-(Dicyclopropylmethyl)-4,5-dihydro-oxazol-2-amine dihydrogen phosphate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of rilmenidine dihydrogen phosphate*.

B. Dissolve 10 mg in *water R* and dilute to 1 mL with the same solvent. The solution gives reaction (b) of phosphates (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 60.0 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R* and dilute 10.0 mL of this solution to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 20.0 mL with *water R*.

*Reference solution (c)*. Dissolve 15.0 mg of *rilmenidine for system suitability CRS* in *water R* and dilute to 5.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm and a carbon loading of 25 per cent,
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3 g of *sodium heptanesulfonate R* in *water R* and dilute to 860 mL with the same solvent; add 130 mL of *methanol R2*, 10 mL of *tetrahydrofuran for chromatography R* and 1.0 mL of *phosphoric acid R*,
- *mobile phase B*: dissolve 3 g of *sodium heptanesulfonate R* in *water R* and dilute to 600 mL with the same solvent; add 350 mL of *acetonitrile for chromatography R*, 50 mL of *tetrahydrofuran for chromatography R* and 1.0 mL of *phosphoric acid R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 100 → 0 | 0 → 100 |
| 14 - 15 | 0 → 100 | 100 → 0 |
| 15 - 30 | 100 | 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rilmenidine (retention time = about 13 min): impurity A = about 0.6; impurity B = about 0.9; impurity C = about 1.4.

With these conditions the inflexion of the baseline, corresponding to the beginning of the gradient, appears on the recorder after a minimum time *t* of 5 min. If this is not the case (*t* < 5 min) modify the chromatographic sequence by adding an isocratic elution with 100 per cent of mobile phase A for a time corresponding to (5-*t*) min before the linear gradient.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rilmenidine.

*Limits*:

– *any impurity*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent),

– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 50 °C for 2 h.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.82 mg of $C_{10}H_{19}N_2O_5P$.

IMPURITIES

*Specified impurities: A, B, C.*

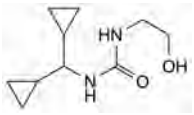

A. 1-(dicyclopropylmethyl)-3-(2-hydroxyethyl)urea,

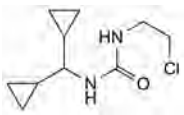

B. 1-(2-chloroethyl)-3-(dicyclopropylmethyl)urea,

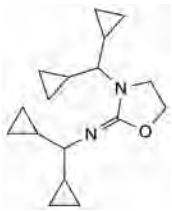

C. *N*,3-bis(dicyclopropylmethyl)oxazolidin-2-imine.

**01/2017:2572**

# RISEDRONATE SODIUM 2.5-HYDRATE

## Natrii risedronas 2.5-hydricus

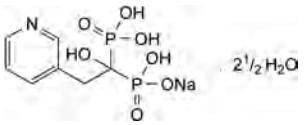

$C_7H_{10}NNaO_7P_2,2½H_2O$ $M_r$ 350.1
[329003-65-8]

DEFINITION

Sodium hydrogen [1-hydroxy-1-phosphono-2-(pyridin-3-yl)ethyl]phosphonate hemipentahydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in methanol. It dissolves in dilute solutions of alkali hydroxides and mineral acids.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *risedronate sodium 2.5-hydrate CRS*.

B. It gives reaction (a) of sodium (*2.3.1*). Dissolution of the substance to be examined is achieved after the addition of the 150 g/L solution of *potassium carbonate R*.

C. Water (see Tests).

TESTS

**pH** (*2.2.3*): 4.0 to 5.0.

Dissolve 0.10 g in *carbon dioxide-free water R* with the aid of an ultrasonic bath and dilute to 10 mL with the same solvent.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Buffer solution.* Dissolve 0.410 g of *sodium edetate R*, 1.7 g of *dipotassium hydrogen phosphate R* and 1.7 g of *tetrabutylammonium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.5 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*.

*Solution A*. Dissolve 100 mg of *sodium chloride R* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase by gentle swirling and heating for 5-10 min and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* To 2.0 mL of the test solution add 5 mg of *risedronate impurity E CRS* and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, buffer solution (10:90 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 263 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of risedronate.

*Relative retention* with reference to risedronate (retention time = about 17 min): impurity E = about 0.95.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity E and risedronate.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to solution A.

B. Liquid chromatography (*2.2.29*) as described in test A for related substances, with the following modifications.

*Reference solution (a).* Dissolve 5.0 mg of the substance to be examined in 50.0 mL of the mobile phase by gentle swirling and heating for 5-10 min, using an ultrasonic bath if necessary.

*Reference solution (b).* Dissolve 5.0 mg of *risedronate impurity A CRS* in the mobile phase by gentle swirling and heating for 5-10 min, using an ultrasonic bath if necessary, and dilute to 50.0 mL with the same solvent.

*Reference solution (c).* Dilute 0.5 mL of reference solution (b) to 20.0 mL with the mobile phase.

*Reference solution (d).* Mix 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) and dilute to 20.0 mL with the mobile phase.

*Mobile phase*: *acetonitrile R*, buffer solution (25:75 *V/V*).

*Injection*: 10 µL of the test solution, reference solutions (b), (c) and (d) and solution A.

*Run time*: 8 times the retention time of risedronate.

*Identification*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to risedronate (retention time = about 4 min): impurity A = about 2.2.

*System suitability*: reference solution (d):

– *resolution*: minimum 10.0 between the peaks due to risedronate and impurity A.

*Limits*:

– *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to solution A and any peak eluting before the peak due to risedronate.

**Water** (*2.5.12*): 11.9 per cent to 13.9 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.125 g in 50 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.26 mg of $C_7H_{10}NNaO_7P_2$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

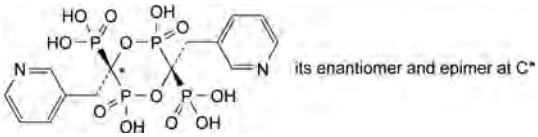


A. [(3*RS*,6*RS* and 3*R*,6*S*-*meso*)-2,5-dihydroxy-2,5-dioxo-3,6-bis[(pyridin-3-yl)methyl]-1,4,2λ$^5$,5λ$^5$-dioxadiphosphinane-3,6-diyl]bis(phosphonic acid),

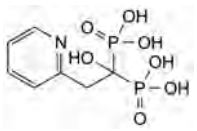

B. [1-hydroxy-2-(pyridin-2-yl)ethylidene]bis(phosphonic acid),

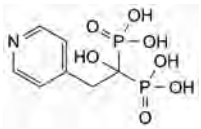

C. [1-hydroxy-2-(pyridin-4-yl)ethylidene]bis(phosphonic acid),

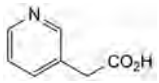

D. 2-(pyridin-3-yl)acetic acid,

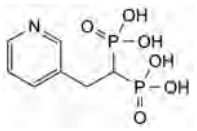

E. [2-(pyridin-3-yl)ethylidene]bis(phosphonic acid).

**01/2011:1559**
**corrected 7.4**

# RISPERIDONE

## Risperidonum

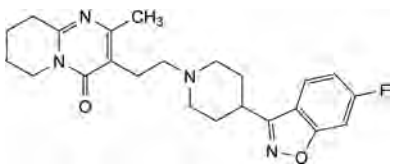

$C_{23}H_{27}FN_4O_2$ $M_r$ 410.5
[106266-06-2]

DEFINITION

3-[2-[4-(6-Fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent). It dissolves in dilute acid solutions.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *risperidone CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.1 g in a 7.5 g/L solution of *tartaric acid R* and dilute to 100 mL with the same acid solution.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *risperidone for system suitability CRS* (containing impurities A, B, C, D and E) in 1.0 mL of *methanol R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 25.0 mL with *methanol R*.

*Reference solution (c)*. Dissolve the contents of a vial of *risperidone impurity K CRS* in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: 5 g/L solution of *ammonium acetate R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 70 | 30 |
| 2 - 17 | 70 → 30 | 30 → 70 |
| 17 - 22 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *risperidone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D and E; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity K.

*Relative retention* with reference to risperidone (retention time = about 12 min): impurity A = about 0.7; impurity B = about 0.75; impurity C = about 0.8; impurity K = about 0.9; impurity D = about 0.94; impurity E = about 1.1.

*System suitability*: reference solution (a):

- the chromatogram obtained is similar to the chromatogram supplied with *risperidone for system suitability CRS*;
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to risperidone.

*Limits*:

- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity K*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.160 g in 70 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with *0.1 M perchloric acid*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.53 mg of $C_{23}H_{27}FN_4O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, H, I, J, L, M.*

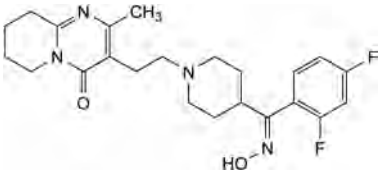

A. 3-[2-[4-[(*E*)-(2,4-difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

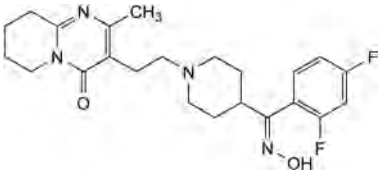

B. 3-[2-[4-[(*Z*)-(2,4-difluorophenyl)(hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

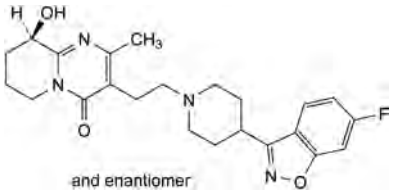


C. (9*RS*)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-9-hydroxy-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

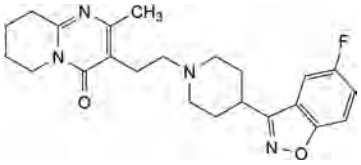

D. 3-[2-[4-(5-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

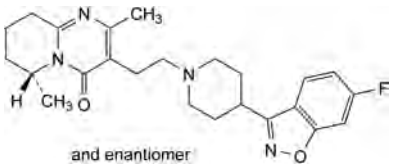


E. (6*RS*)-3-[2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2,6-dimethyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

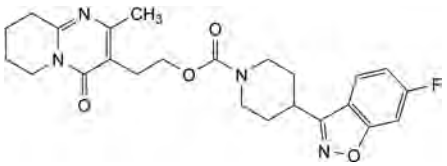

F. 2-[2-methyl-4-oxo-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-3-yl]ethyl 4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-carboxylate,

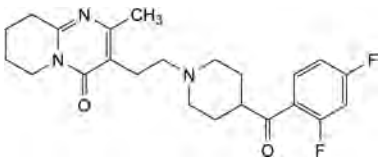

H. 3-[2-[4-(2,4-difluorobenzoyl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

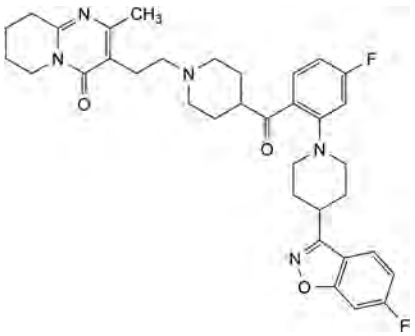

I. 3-[2-[4-[4-fluoro-2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]benzoyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

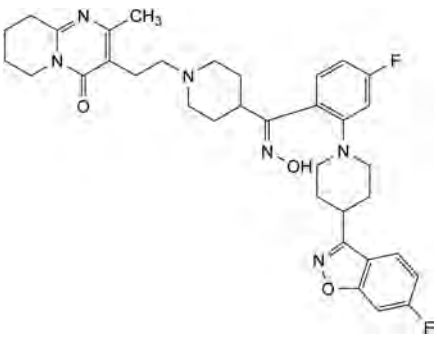

J. 3-[2-[4-[(*Z*)-[4-fluoro-2-[4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidin-1-yl]phenyl](hydroxyimino)methyl]piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one,

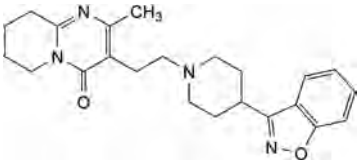

K. 3-[2-[4-(1,2-benzisoxazol-3-yl)piperidin-1-yl]ethyl]-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (desfluoro risperidone),

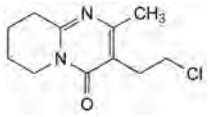

L. 3-(2-chloroethyl)-2-methyl-6,7,8,9-tetrahydro-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (piperidopyrimidinone intermediate),

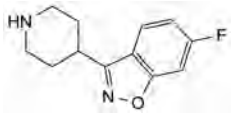

M. 6-fluoro-3-(piperidin-4-yl)-1,2-benzisoxazole.

**01/2017:2136**

# RITONAVIR

## Ritonavirum

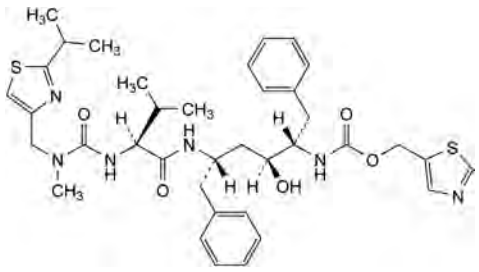

$C_{37}H_{48}N_6O_5S_2$ $M_r$ 721

[155213-67-5]

DEFINITION

Thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]carbamate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

The production method is validated to demonstrate suitable enantiomeric purity of the final product.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methanol and in methylene chloride, very slightly soluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ritonavir CRS*.

If the spectra obtained in the solid state show differences dissolve the substance to be examined and the reference substance separately in *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Mix equal volumes of *acetonitrile R* and a 4.1 g/L solution of *potassium dihydrogen phosphate R*.

*Test solution (a)*. Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Sonicate if necessary.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5.0 mg of *ritonavir for peak identification CRS* (containing impurities E, F, L, O and T) in the solvent mixture and dilute to 5.0 mL with the solvent mixture. Sonicate if necessary.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10.0 mg of *ritonavir CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Sonicate if necessary. Dilute 5.0 mL of this solution to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *butanol R*, 8 volumes of *tetrahydrofuran R*, 18 volumes of *acetonitrile R* and 69 volumes of a 4.1 g/L solution of *potassium dihydrogen phosphate R* filtered through a 0.45 µm nylon membrane;
- *mobile phase B*: mix 5 volumes of *butanol R*, 8 volumes of *tetrahydrofuran R*, 40 volumes of a 4.1 g/L solution of *potassium dihydrogen phosphate R* filtered through a 0.45 µm nylon membrane and 47 volumes of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 60 | 100 | 0 |
| 60 - 120 | 100 → 0 | 0 → 100 |
| 120 - 120.1 | 0 → 100 | 100 → 0 |
| 120.1 - 155 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 50 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *ritonavir for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities E, F, L, O and T.

*Relative retention* with reference to ritonavir (retention time = about 34 min): impurity E = about 0.39; impurity F = about 0.40; impurity L = about 0.8; impurity O = about 1.1; impurity T = about 2.6.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity F.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity F = 1.4; impurity L = 1.9; impurity T = 1.4;
- *impurities E, O*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity T*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities F, L*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{37}H_{48}N_6O_5S_2$ from the declared content of *ritonavir CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: E, F, L, O, T.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, G, H, I, J, K, M, N, P, Q, R, S, U.*

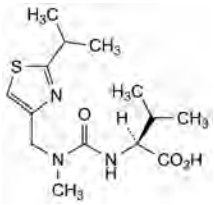

A. (2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoic acid,

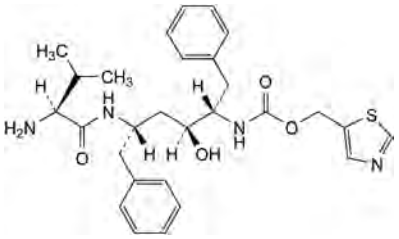

B. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-4-[[(2*S*)-2-amino-3-methylbutanoyl]amino]-1-benzyl-2-hydroxy-5-phenylpentyl]carbamate,

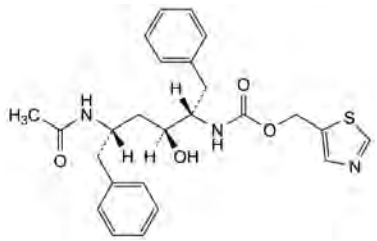

C. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-4-(acetylamino)-1-benzyl-2-hydroxy-5-phenylpentyl]carbamate,

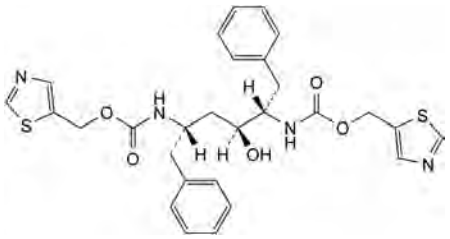

D. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-5-phenyl-4-[[(thiazol-5-ylmethoxy)carbonyl]amino]pentyl]-carbamate,

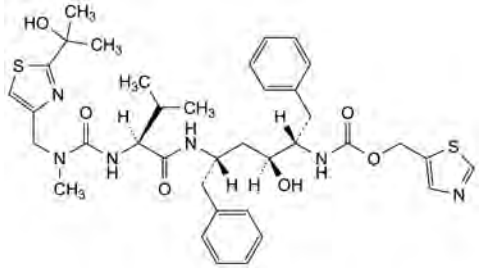

E. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-2-[[[[2-(1-hydroxy-1-methylethyl)thiazol-4-yl]methyl]methylcarbamoyl]amino]-3-methylbutanoyl]-amino]-5-phenylpentyl]carbamate,

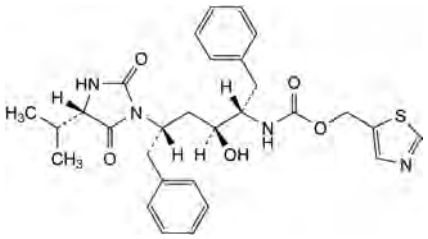

F. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-4-[[(2*S*)-1-benzyl-2-hydroxy-4-[(4*S*)-4-(1-methylethyl)-2,5-dioxoimidazolidin-1-yl]-5-phenylpentyl]carbamate,

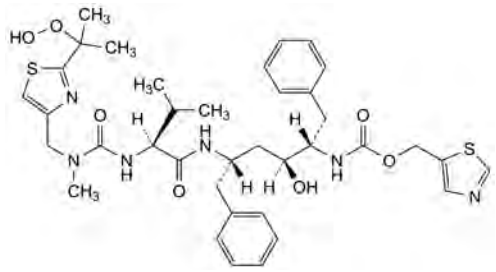

G. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-4-[[(2*S*)-2-[[[[2-(1-hydroperoxy-1-methylethyl)thiazol-4-yl]methyl]methylcarbamoyl]amino]-3-methylbutanoyl]-amino]-2-hydroxy-5-phenylpentyl]carbamate,

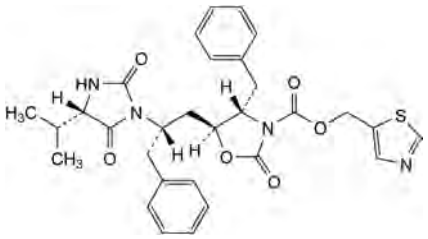

H. thiazol-5-ylmethyl (4*S*,5*S*)-4-benzyl-5-[(2*S*)-2-[(4*S*)-4-(1-methylethyl)-2,5-dioxoimidazolidin-1-yl]-3-phenylpropyl]-2-oxooxazolidine-3-carboxylate,

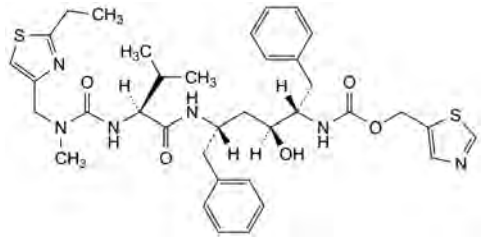

I. thiazol-5-ylmethyl [((1*S*,2*S*,4*S*)-1-benzyl-4-[[(2*S*)-2-[[[[2-ethylthiazol-4-yl]methyl]methylcarbamoyl]-amino]-3-methylbutanoyl]amino]-2-hydroxy-5-phenyl-pentyl]carbamate,

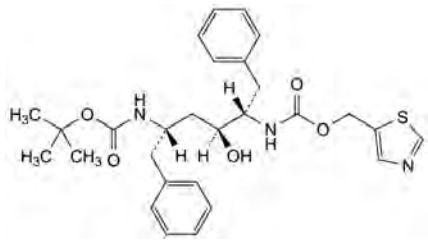

J. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-4-[[(1,1-dimethylethoxy)carbonyl]amino]-2-hydroxy-5-phenylpentyl]carbamate,

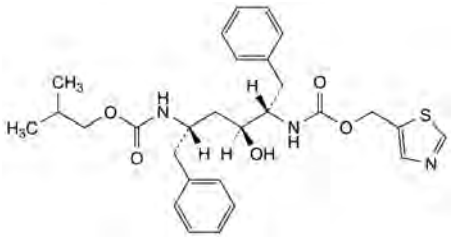

K. thiazol-5-ylmethyl (1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2-methylpropoxy)carbonyl]amino]-5-phenylpentyl]carbamate,

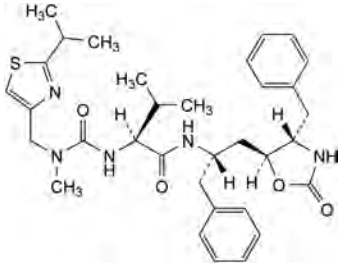

L. (4*S*,5*S*)-4-benzyl-5-[(2*S*)-2-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]-butanoyl]amino]-3-phenylpropyl]oxazolidin-2-one,

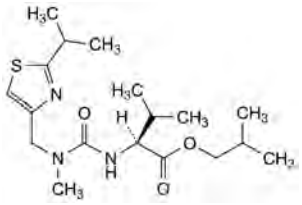

M. 2-methylpropyl (2*S*)-3-methyl-2-[[methyl[[2-(1-methyl-ethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoate,

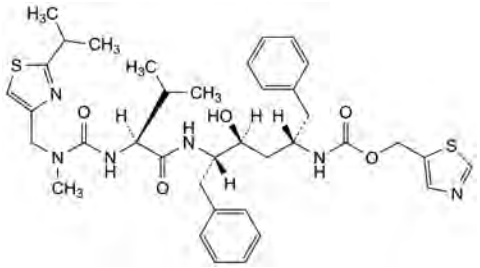

N. thiazol-5-ylmethyl [(1*S*,3*S*,4*S*)-1-benzyl-3-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]carbamate,

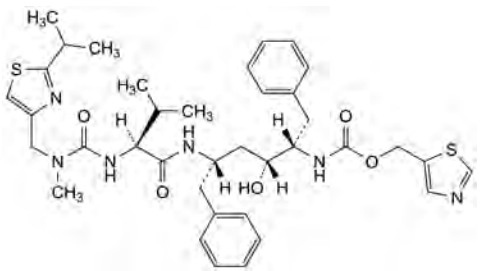

O. thiazol-5-ylmethyl [(1*S*,2*R*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]carbamate,

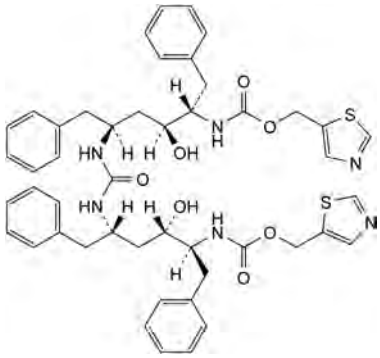

P. bis(thiazol-5-ylmethyl) [carbonylbis[imino[(2*S*,3*S*,5*S*)-3-hydroxy-1,6-diphenylhexane-5,2-diyl]]]dicarbamate,

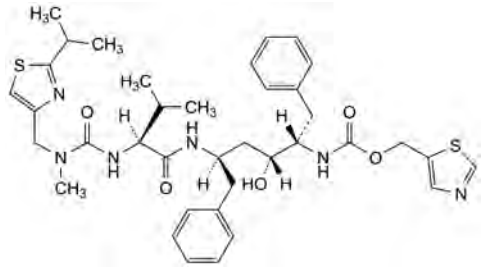

Q. thiazol-5-ylmethyl [(1*S*,2*R*,4*R*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)-thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]carbamate,

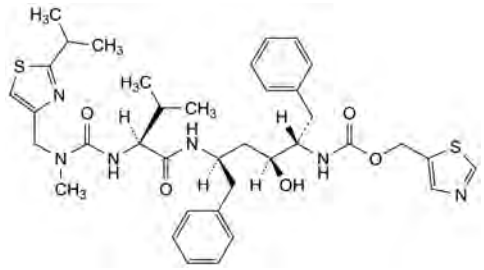

R. thiazol-5-ylmethyl [(1*S*,2*S*,4*R*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]carbamate,

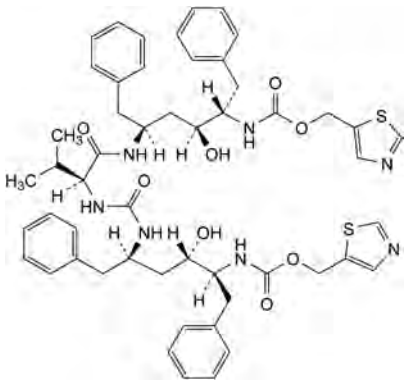

S. thiazol-5-ylmethyl [(1*S*,2*S*,4*S*)-1-benzyl-4-[[(2*S*)-2-[[[(1*S*,3*S*,4*S*)-1-benzyl-3-hydroxy-5-phenyl-4-[[(thiazol-5-ylmethoxy)carbonyl]amino]pentyl]carbamoyl]amino]-3-methylbutanoyl]amino]-2-hydroxy-5-phenylpentyl]-carbamate,

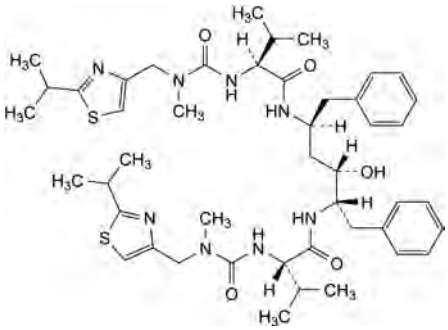

T. (2*S*)-*N*-[(1*S*,2*S*,4*S*)-1-benzyl-2-hydroxy-4-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-5-phenylpentyl]-3-methyl-2-[[methyl[[2-(1-methylethyl)-thiazol-4-yl]methyl]carbamoyl]amino]butanamide,

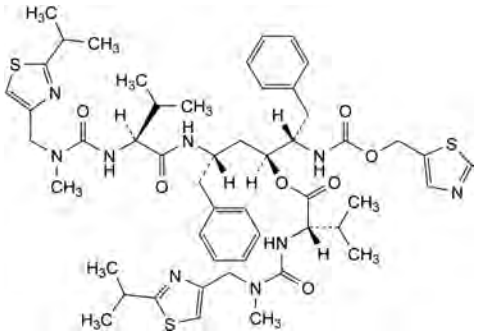

U. (1*S*,3*S*)-3-[[(2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)-thiazol-4-yl]methyl]carbamoyl]amino]butanoyl]amino]-4-phenyl-1-[(1*S*)-2-phenyl-1-[[(thiazol-5-ylmethoxy)-carbonyl]amino]ethyl]butyl (2*S*)-3-methyl-2-[[methyl[[2-(1-methylethyl)thiazol-4-yl]methyl]carbamoyl]amino]-butanoate.

**01/2017:2629**

# RIVASTIGMINE

## Rivastigminum

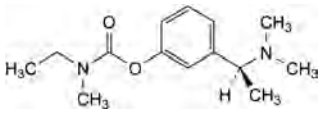

$C_{14}H_{22}N_2O_2$ $M_r$ 250.3
[123441-03-2]

DEFINITION

3-[(1*S*)-1-(Dimethylamino)ethyl]phenyl ethyl(methyl)-carbamate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: viscous, clear, colourless or yellow or very slightly brown, hygroscopic liquid.

*Solubility*: sparingly soluble in water, very soluble in anhydrous ethanol and in heptane.

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): – 44.0 to – 38.0 (anhydrous substance). *Prepare the solution immediately before use.*
Dissolve 0.300 g in *ethyl acetate R* and dilute to 50.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: film.
*Comparison*: *rivastigmine hydrogen tartrate CRS*, treated as follows: dissolve 0.100 g in 30 mL of *buffer solution pH 11 R*, then add 30 mL of *1,1-dimethylethyl methyl ether R* and shake vigorously for 2 min. Allow the layers to separate. Filter the upper organic layer through *anhydrous sodium sulfate R*. Evaporate the filtrate under reduced pressure at a temperature not exceeding 60 °C to obtain a residue. Record the reference spectrum using this residue.

C. Enantiomeric purity (see Tests).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solution A*. Solution containing 1.78 g/L of *disodium hydrogen phosphate dihydrate R* and 1.38 g/L of *sodium dihydrogen phosphate monohydrate R*. Adjust to pH 6.0 with *phosphoric acid R*.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *rivastigmine impurity D CRS* in the mobile phase and dilute to 200.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 1 mg of *rivastigmine hydrogen tartrate CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Column*:
– *size*: *l* = 0.10 m, Ø = 4.0 mm;
– *stationary phase*: *silica gel AGP for chiral chromatography R* (5 µm).

*Mobile phase*: mix 205 µL of *N,N-dimethyloctylamine R* and 20.0 mL of *acetonitrile R1* and dilute to 1000 mL with solution A.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of rivastigmine.

*Relative retention* with reference to rivastigmine (retention time = about 9 min): impurity D = about 0.8.

*System suitability*: reference solution (b):
– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rivastigmine.

*Calculation of percentage content*:
– use the concentration of impurity D in reference solution (a).

*Limit*:
– *impurity D*: maximum 0.3 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 62.5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *rivastigmine for system suitability CRS* (containing impurities A, B and C) in 1.0 mL of the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *rivastigmine hydrogen tartrate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 42 volumes of an 8.9 g/L solution of *disodium hydrogen phosphate dihydrate R* previously adjusted to pH 7.0 with *phosphoric acid R* and 58 volumes of *methanol R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of rivastigmine.

*Identification of impurities*: use the chromatogram supplied with *rivastigmine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to rivastigmine (retention time = about 10 min): impurity A = about 0.4; impurity C = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities C and B.

*Calculation of percentage contents*:

– for each impurity, use the concentration of rivastigmine in reference solution (a).

*Limits*:

– *impurity A*: maximum 0.3 per cent;

– *impurity B*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

Change the solvent after standardisation of the titrant and after every 3rd sample.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

– *symmetry factor*: maximum 2.5 for the peak due to rivastigmine.

Calculate the percentage content of $C_{14}H_{22}N_2O_2$ taking into account the assigned content of *rivastigmine hydrogen tartrate CRS* and a conversion factor of 0.625.

STORAGE

Under an inert gas, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

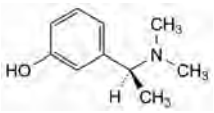

A. 3-[(1*S*)-1-(dimethylamino)ethyl]phenol (dimetol),

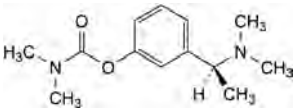

B. 3-[(1*S*)-1-(dimethylamino)ethyl]phenyl dimethylcarbamate,

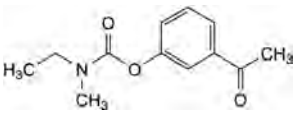

C. 3-acetylphenyl ethyl(methyl)carbamate,

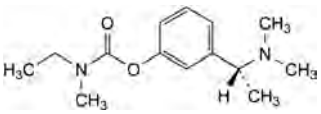

D. 3-[(1*R*)-1-(dimethylamino)ethyl]phenyl ethyl(methyl)carbamate ((*R*)-enantiomer).

**01/2017:2630**
**corrected 10.0**

# RIVASTIGMINE HYDROGEN TARTRATE

## Rivastigmini hydrogenotartras

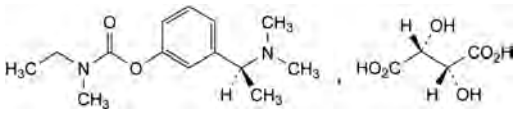

$C_{18}H_{28}N_2O_8$ $M_r$ 400.4

[129101-54-8]

DEFINITION

3-[(1*S*)-1-(Dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methylcarbamate hydrogen (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, very hygroscopic, crystalline or fine crystalline powder.

*Solubility*: very soluble in water, soluble in methanol, very slightly soluble in ethyl acetate.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B or tests B, C.

A. Specific optical rotation (*2.2.7*): + 4.2 to + 5.1.
Dissolve 0.600 g in *methanol R* and dilute to 20.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *rivastigmine hydrogen tartrate CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

C. Enantiomeric purity (see Tests).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solution A.* Solution containing 1.78 g/L of *disodium hydrogen phosphate dihydrate R* and 1.38 g/L of *sodium dihydrogen phosphate monohydrate R*, adjusted to pH 6.0 with *phosphoric acid R*.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *rivastigmine impurity D CRS* in the mobile phase and dilute to 200.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 1 mg of *rivastigmine hydrogen tartrate CRS* in reference solution (a) and dilute to 10.0 mL with the same solution.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *α1-acid-glycoprotein silica gel for chiral separation R* (5 µm).

*Mobile phase*: mix 205 µL of *N,N-dimethyloctylamine R* and 20.0 mL of *acetonitrile R1* and dilute to 1000 mL with solution A.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of rivastigmine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity D.

*Relative retention* with reference to rivastigmine (retention time = about 9 min): impurity D = about 0.8.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rivastigmine.

*Calculation of percentage content*:

- for impurity D, use the concentration of impurity D in reference solution (a).

*Limit*:

- *impurity D*: maximum 0.3 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *rivastigmine for system suitability CRS* (containing impurities A, B and C) in 1.0 mL of the mobile phase.

*Reference solution (c).* Dissolve 50.0 mg of *rivastigmine hydrogen tartrate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 42 volumes of an 8.9 g/L solution of *disodium hydrogen phosphate dihydrate R* previously adjusted to pH 7.0 with *phosphoric acid R* and 58 volumes of *methanol R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of rivastigmine.

*Identification of impurities*: use the chromatogram supplied with *rivastigmine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to rivastigmine (retention time = about 10 min): impurity A = about 0.4; impurity C = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities C and B.

*Calculation of percentage contents*:

- for each impurity, use the concentration of rivastigmine hydrogen tartrate in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to tartaric acid.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (c).

*System suitability*: reference solution (c):

- *symmetry factor*: maximum 2.5 for the peak due to rivastigmine.

Calculate the percentage content of $C_{18}H_{28}N_2O_8$ taking into account the assigned content of *rivastigmine hydrogen tartrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, E, F, G, H.*

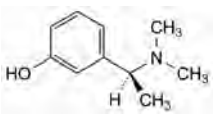

A. 3-[(1*S*)-1-(dimethylamino)ethyl]phenol,

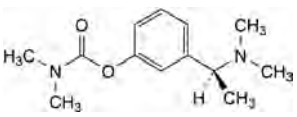

B. 3-[(1*S*)-1-(dimethylamino)ethyl]phenyl *N*,*N*-dimethylcarbamate,

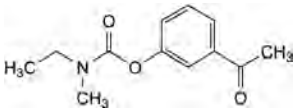

C. 3-acetylphenyl *N*-ethyl-*N*-methylcarbamate,

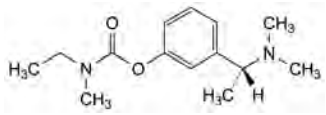

D. 3-[(1*R*)-1-(dimethylamino)ethyl]phenyl *N*-ethyl-*N*-methylcarbamate,

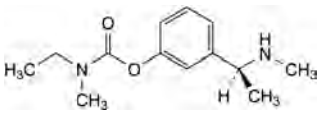

E. 3-[(1*S*)-1-(methylamino)ethyl]phenyl *N*-ethyl-*N*-methylcarbamate,

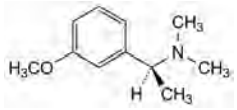

F. (1*S*)-1-(3-methoxyphenyl)-*N*,*N*-dimethylethanamine,

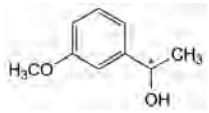

G. 1-(3-methoxyphenyl)ethan-1-ol,

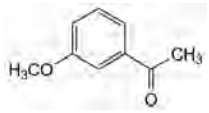

H. 1-(3-methoxyphenyl)ethan-1-one.

**01/2017:2585**

# RIZATRIPTAN BENZOATE

## Rizatriptani benzoas

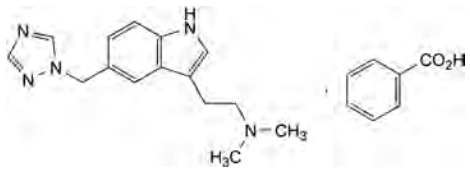

$C_{22}H_{25}N_5O_2$ $M_r$ 391.5
[145202-66-0]

DEFINITION

*N*,*N*-Dimethyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanamine benzoate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder or crystals.

*Solubility*: soluble in water, sparingly soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rizatriptan benzoate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Use silanised glass autosampler vials and freshly prepared solutions.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 50.0 mg of *rizatriptan benzoate CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5 mg of *rizatriptan for system suitability CRS* (containing impurity C) in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *phenylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 160 mL of *acetonitrile R* and 840 mL of *water R*, add 1.0 mL of *trifluoroacetic acid R* and mix;
- *mobile phase B*: to 1000 mL of *acetonitrile R* add 1.0 mL of *trifluoroacetic acid R* and mix;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 100 | 0 |
| 8 - 17 | 100 → 70 | 0 → 30 |
| 17 - 20 | 70 | 30 |
| 20 - 20.1 | 70 → 100 | 30 → 0 |
| 20.1 - 23 | 100 | 0 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *rizatriptan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to rizatriptan (retention time = about 5 min): impurity C = about 1.3; benzoic acid = about 2.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to rizatriptan and impurity C.

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *disregard limit*: the area of the peak due to rizatriptan in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to benzoic acid.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 3.5 for the peak due to rizatriptan.

Calculate the percentage content of $C_{22}H_{25}N_5O_2$ from the declared content of *rizatriptan benzoate CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I.*

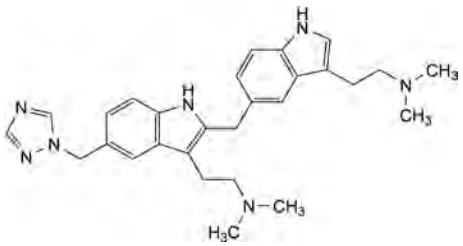

A. 2-[2-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]-*N*,*N*-dimethylethanamine,

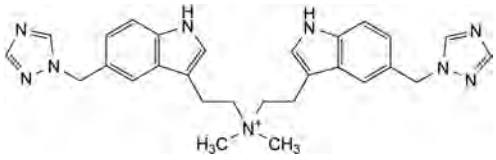

B. *N*,*N*-dimethyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]-*N*-[2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethyl]ethanaminium,

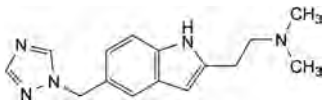

C. *N*,*N*-dimethyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-2-yl]ethanamine,

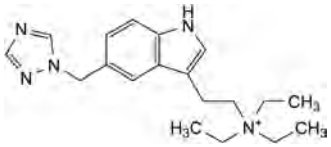

D. *N*,*N*,*N*-triethyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanaminium,

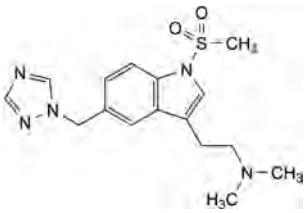

E. *N*,*N*-dimethyl-2-[1-(methylsulfonyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanamine,

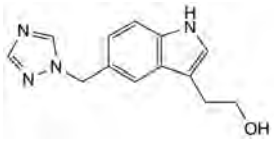

F. 2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanol,

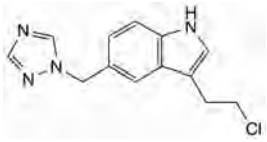

G. 3-(2-chloroethyl)-5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indole,

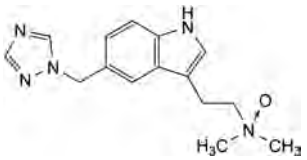

H. *N*,*N*-dimethyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanamine *N*-oxide,

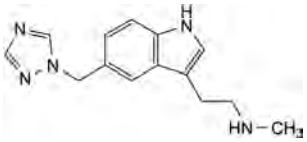

I. *N*-methyl-2-[5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indol-3-yl]ethanamine.

**01/2017:1764**

# ROCURONIUM BROMIDE

## Rocuronii bromidum

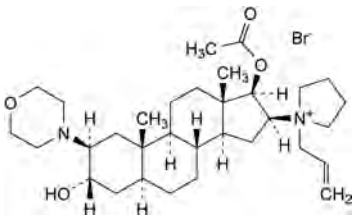

$C_{32}H_{53}BrN_2O_4$ $M_r$ 610

[119302-91-9]

DEFINITION

1-[17β-Acetoxy-3α-hydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium bromide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: almost white or pale yellow, slightly hygroscopic powder.

*Solubility*: freely soluble in water, very soluble in methylene chloride, freely soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *rocuronium bromide CRS*.

B. Solution S (see Tests) gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 28.5 to + 32.0 (anhydrous substance).

Dissolve 0.250 g in a 5.15 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solution.

**pH** (*2.2.3*): 8.9 to 9.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R1* (10:90 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *rocuronium for peak identification CRS* (containing impurities A, B, C, F, G and H) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 10 volumes of a 4.53 g/L solution of *tetramethylammonium hydroxide R* adjusted to pH 7.4 with *phosphoric acid R* and 90 volumes of *acetonitrile R1*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 2.5 times the retention time of rocuronium.

*Identification of impurities*: use the chromatogram supplied with *rocuronium for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, F, G and H.

*Relative retention* with reference to rocuronium (retention time = about 9 min): impurity A = about 0.2; impurity G = about 0.4; impurity F = about 0.75; impurity B = about 0.80; impurity H = about 0.95; impurity C = about 1.2.

*System suitability*: reference solution (b):
- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity H and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to rocuronium.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.5; impurity F = 1.3; impurity G = 0.4; impurity H = 0.4;
- *impurities A, B, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurities F, G, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak eluting before impurity A.

**Chlorides**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.644 g of *sodium bromide R* and 0.824 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (b)*. Dissolve 0.824 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *water R*. Dilute 2.0 mL of this solution to 50.0 mL with *water R*.

*Blank solution*: *water R*.

*Precolumn*:
- *size*: *l* = 0.05 m, Ø = 4.0 mm;
- *stationary phase*: *anion-exchange resin R* (13 µm).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *anion-exchange resin R* (13 µm).

*Mobile phase*: a solution containing 0.063 g/L of *sodium hydrogen carbonate R* and 0.212 g/L of *anhydrous sodium carbonate R*.

*Flow rate*: 2.0 mL/min.

*Detection*: conductivity detector set at 100 µS/V and maintained at 30 °C.

Use a self-regenerating anion suppressor.

*Injection*: 25 µL.

*Retention times*: chloride = about 1.7 min; bromide = about 2.8 min.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.5 between the peaks due to chloride and bromide.

*Limit*:
- *chlorides*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**2-Propanol** (*2.4.24, System A*): maximum 1.0 per cent.

**Water** (*2.5.12*): maximum 4.5 per cent, determined on 0.400 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 40 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 60.97 mg of $C_{32}H_{53}BrN_2O_4$.

STORAGE

In an airtight container, protected from light, at a temperature below – 15 °C.

IMPURITIES

*Specified impurities: A, B, C, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

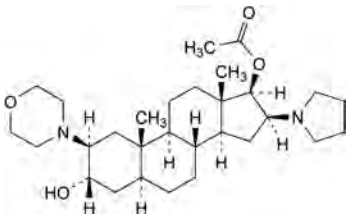

A. 3α-hydroxy-2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstan-17β-yl acetate,

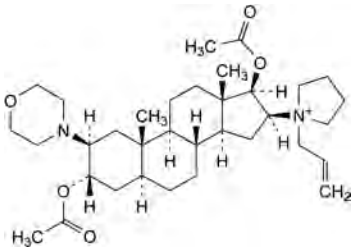

B. 1-[3α,17β-diacetoxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

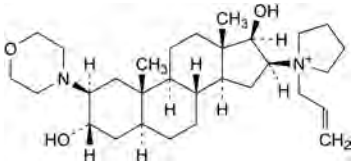

C. 1-[3α,17β-dihydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

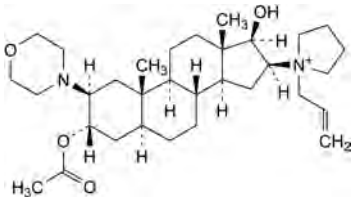

D. 1-[3α-acetoxy-17β-hydroxy-2β-(morpholin-4-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

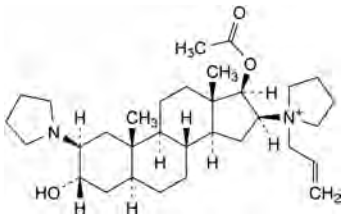

E. 1-[17β-acetoxy-3α-hydroxy-2β-(pyrrolidin-1-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

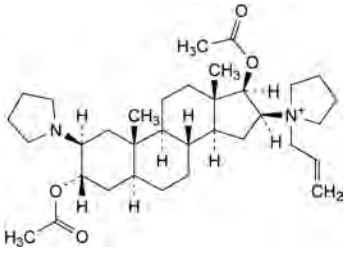

F. 1-[3α,17β-acetoxy-2β-(pyrrolidin-1-yl)-5α-androstan-16β-yl]-1-(prop-2-enyl)pyrrolidinium,

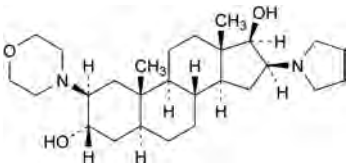

G. 2β-(morpholin-4-yl)-16β-(pyrrolidin-1-yl)-5α-androstane-3α,17β-diol,

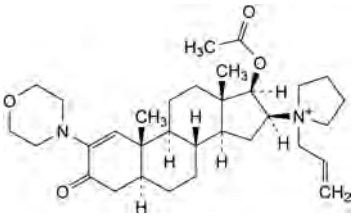

H. 1-[17β-acetoxy-2-(morpholin-4-yl)-3-oxo-5α-androst-1-en-16β-yl]-1-(prop-2-enyl)pyrrolidinium.

**01/2019:2604**

# ROPINIROLE HYDROCHLORIDE

## Ropiniroli hydrochloridum

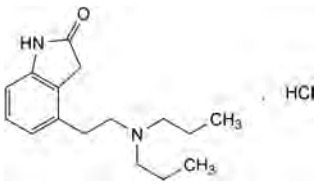

$C_{16}H_{25}ClN_2O$ $M_r$ 296.8
[91374-20-8]

DEFINITION

4-[2-(Dipropylamino)ethyl]-1,3-dihydro-2*H*-indol-2-one hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder.

*Solubility*: freely soluble in water and in methanol, slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ropinirole hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve, using sonication, 50.0 mg of the substance to be examined in 4 mL of mobile phase B and dilute to 25.0 mL with mobile phase A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve, using sonication, 7.5 mg of *ropinirole impurity A CRS* and 5.0 mg of the substance to be examined in 16 mL of mobile phase B and dilute to 100.0 mL with mobile phase A.

*Reference solution (b)*. To 1.0 mL of reference solution (a) add 4 mL of mobile phase B and dilute to 25.0 mL with mobile phase A.

*Reference solution (c).* Dissolve, using sonication, 50.0 mg of *ropinirole hydrochloride CRS* in 4 mL of mobile phase B and dilute to 25.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 20.0 mL with mobile phase A.

*Reference solution (d).* Dissolve 5.0 mg of *ropinirole for peak identification 1 CRS* (containing impurity D) in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Reference solution (e).* Dissolve 5.0 mg of *ropinirole for peak identification 2 CRS* (containing impurities G and H) in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Reference solution (f).* Dissolve 5.0 mg of *ropinirole for peak identification 3 CRS* (containing impurity E) in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.85 g of *ammonium acetate R* in 950 mL of *water for chromatography R*, adjust to pH 2.5 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R*, *acetonitrile R1* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 92 | 8 |
| 15 - 30 | 92 → 80 | 8 → 20 |
| 30 - 50 | 80 → 60 | 20 → 40 |
| 50 - 75 | 60 | 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b), (d), (e) and (f).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *ropinirole for peak identification 1 CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity D; use the chromatogram supplied with *ropinirole for peak identification 2 CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities G and H; use the chromatogram supplied with *ropinirole for peak identification 3 CRS* and the chromatogram obtained with reference solution (f) to identify the peak due to impurity E.

*Relative retention* with reference to ropinirole (retention time = about 30 min): impurity D = about 0.5; impurity A = about 0.9; impurity E = about 1.1; impurity H = about 1.2; impurity G = about 1.4.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to impurity A and ropinirole in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 60 for the peak due to ropinirole in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity G = 2.7; impurity H = 0.5;
- for impurity A, use the concentration of impurity A in reference solution (b);
- for impurities other than A, use the concentration of ropinirole hydrochloride in reference solution (b).

*Limits*:

- *impurities A, D, E, G, H*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase B, mobile phase A (19:81 *V/V*).

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{16}H_{25}ClN_2O$ taking into account the assigned content of *ropinirole hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, D, E, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F.*

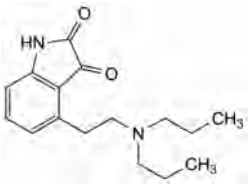

A. 4-[2-(dipropylamino)ethyl]-1*H*-indole-2,3-dione,

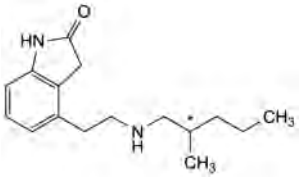

B. 4-[2-[(2-methylpentyl)amino]ethyl]-1,3-dihydro-2*H*-indol-2-one,

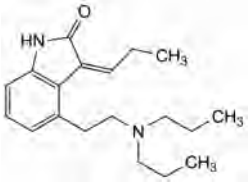

C. (3*Z*)-4-[2-(dipropylamino)ethyl]-3-propylidene-1,3-dihydro-2*H*-indol-2-one,

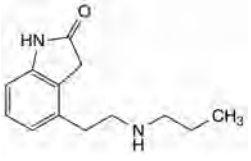

D. 4-[2-(propylamino)ethyl]-1,3-dihydro-2*H*-indol-2-one,

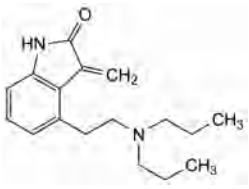

E. 4-[2-(dipropylamino)ethyl]-3-methylidene-1,3-dihydro-2*H*-indol-2-one,

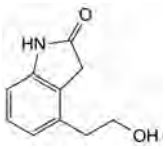

F. 4-(2-hydroxyethyl)-1,3-dihydro-2*H*-indol-2-one,

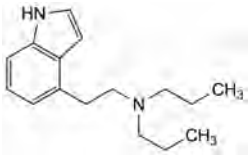

G. 4-[2-(dipropylamino)ethyl]-1*H*-indole,

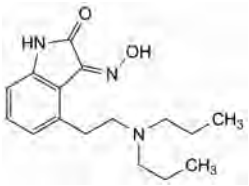

H. (3*Z*)-4-[2-(dipropylamino)ethyl]-3-(hydroxyimino)-1,3-dihydro-2*H*-indol-2-one.

**01/2017:2335**

# ROPIVACAINE HYDROCHLORIDE MONOHYDRATE

## Ropivacaini hydrochloridum monohydricum

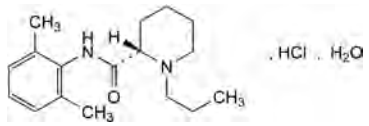

$C_{17}H_{27}ClN_2O,H_2O$ $M_r$ 328.9
[132112-35-7]

DEFINITION

(−)-(2*S*)-*N*-(2,6-Dimethylphenyl)-1-propylpiperidine-2-carboxamide hydrochloride monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent), slightly soluble in methylene chloride.

IDENTIFICATION

Carry out either tests A, C, D or tests A, B, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ropivacaine hydrochloride monohydrate CRS*.

B. Specific optical rotation (*2.2.7*): − 74.0 to − 64.0 (anhydrous substance).
Mix 2 mL of a 200 g/L solution of *sodium hydroxide R* and 30 mL of *water R* and dilute to 100.0 mL with *ethanol (96 per cent) R* (solution A). Dissolve 0.500 g of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

C. It gives reaction (a) of chlorides (*2.3.1*).

D. Enantiomeric purity (see Tests).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Absorbance** (*2.2.25*): maximum 0.030 at 405 nm and maximum 0.025 at 436 nm, determined on solution S prepared immediately before use, with a path length of 5 cm and using *water R* as the compensation liquid.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 55 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of the substance to be examined and 5 mg of *bupivacaine hydrochloride CRS* (impurity A) in the mobile phase and dilute to 5 mL with the mobile phase. Dilute 1 mL of this solution to 100 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*: mix 1.3 mL of a 138 g/L solution of *sodium dihydrogen phosphate R* and 32.5 mL of an 89 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and dilute to 1000 mL with *water R*; mix equal volumes of this solution (pH 8.0) and *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Injection*: 20 µL.

*Detection*: spectrophotometer at 240 nm.

*Run time*: 2.5 times the retention time of ropivacaine.

*Relative retention* with reference to ropivacaine (retention time = about 6 min): impurity A = about 1.6.

*System suitability*: reference solution (b):
- *resolution*: minimum 6.0 between the peaks due to ropivacaine and impurity A.

*Limits*:
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Impurity H**. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution*. Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution.* Dissolve 13.0 mg of *2,6-dimethylaniline hydrochloride R* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Retention time*: impurity H = about 2-3 min.

*Limit*:

– *impurity H*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (10 ppm).

**Enantiomeric purity**. Capillary electrophoresis (*2.2.47*): use the normalisation procedure.

*Test solution.* Dissolve 50 mg of the substance to be examined in *water R* and dilute to 25 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with *water R*.

*Reference solution (b).* Dissolve 1.5 mg of the substance to be examined and 1.5 mg of *ropivacaine impurity G CRS* in *water R* and dilute to 100 mL with the same solvent.

*Capillary*:

– *material*: fused silica;

– *size*: effective length = about 72 cm, total length = 80 cm, Ø = 50 µm.

*Temperature*: 30 °C.

*CZE buffer*: prepare a 13.3 g/L solution of *dimethyl-β-cyclodextrin R* in an 11.5 g/L solution of *phosphoric acid R* previously adjusted to pH 3.0 with *triethanolamine R*. The CZE buffer is prepared and filtered through a membrane filter (nominal pore size 0.45 µm) immediately before use.

*Detection*: spectrophotometer at 206 nm.

*Preconditioning of the capillary*: rinse the capillary at 100 kPa with *water R* for 1 min, with *0.1 M sodium hydroxide* for 10 min and with *water R* for 3 min. If the capillary is new or dry, increase the sodium hydroxide rinse to 30 min.

*Between-run rinsing*: rinse the capillary at 100 kPa with *water R* for 1 min, with *0.1 M sodium hydroxide* for 4 min, with *water R* for 1 min and with the CZE buffer for 4 min.

*Injection*: under pressure (5 kPa) for 5 s.

*Migration*: apply a field strength of 375 V/cm with an initial ramp of 500 V/s and a positive polarity corresponding to a current of 40-45 µA.

*Run time*: 30 min.

*System suitability*:

– *resolution*: minimum 3.7 between the peaks due to impurity G (1st peak) and (*S*)-ropivacaine in the electropherogram obtained with reference solution (b); if necessary, increase the dimethyl-β-cyclodextrin concentration in the CZE buffer or vary the pH between 2.9 and 3.1 or lower the temperature;

– *signal-to-noise ratio*: minimum 10 for the principal peak in the electropherogram obtained with reference solution (a).

*Limit*:

– *impurity G*: maximum 0.5 per cent.

**Water** (*2.5.12*): 5.0 per cent to 6.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.09 mg of $C_{17}H_{27}ClN_2O$.

IMPURITIES

*Specified impurities: A, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F.*

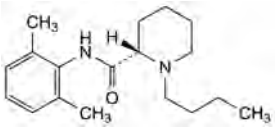

A. (*S*)-bupivacaine,

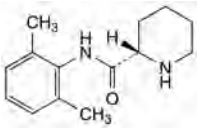

B. (−)-(2*S*)-*N*-(2,6-dimethylphenyl)piperidine-2-carboxamide,

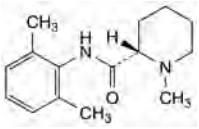

C. (−)-(2*S*)-*N*-(2,6-dimethylphenyl)-1-methylpiperidine-2-carboxamide ((*S*)-mepivacaine),

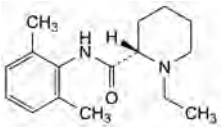

D. (−)-(2*S*)-*N*-(2,6-dimethylphenyl)-1-ethylpiperidine-2-carboxamide,

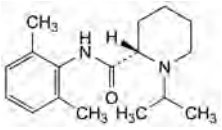

E. (−)-(2*S*)-*N*-(2,6-dimethylphenyl)-1(1-methylethyl)-piperidine-2-carboxamide,

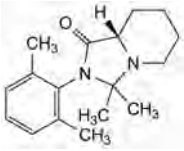

F. (8a*S*)-2-(2,6-dimethylphenyl)-3,3-dimethylhexahydro-imidazo[1,5-*a*]pyridin-1(5*H*)-one (acetone adduct),

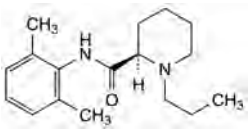

G. (+)-(2*R*)-*N*-(2,6-dimethylphenyl)-1-propylpiperidine-2-carboxamide ((*R*)-ropivacaine),

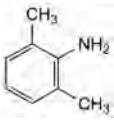

H. 2,6-dimethylaniline.

01/2017:2631

# ROSUVASTATIN CALCIUM

## Rosuvastatinum calcicum

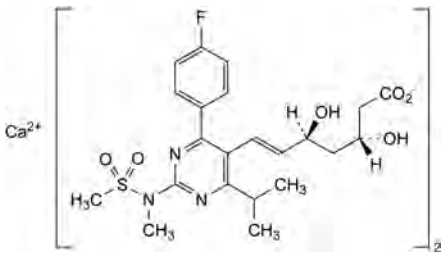

$C_{44}H_{54}CaF_2N_6O_{12}S_2$ $M_r$ 1001
[147098-20-2]

DEFINITION

Calcium bis[(3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoate].

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: slightly soluble in water, freely soluble in methylene chloride, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *rosuvastatin calcium CRS*.

B. Enantiomeric purity (see Tests).

C. It gives reaction (b) of calcium (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile for chromatography R*, *water for chromatography R* (25:75 *V/V*).

*Test solution.* Dissolve 35.0 mg of the substance to be examined in 12 mL of *acetonitrile for chromatography R* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (a).* Dissolve 35.0 mg of *rosuvastatin calcium CRS* in 12 mL of *acetonitrile for chromatography R* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 7 mg of *rosuvastatin for system suitability CRS* (containing impurities A, B and C) in 2.5 mL of *acetonitrile for chromatography R* and dilute to 10 mL with *water for chromatography R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: 1 per cent *V/V* solution of *trifluoroacetic acid R*, *acetonitrile for chromatography R*, *water for chromatography R* (1:29:70 *V/V/V*);
- *mobile phase B*: 1 per cent *V/V* solution of *trifluoroacetic acid R*, *water for chromatography R*, *acetonitrile for chromatography R* (1:24:75 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 | 0 |
| 30 - 50 | 100 → 60 | 0 → 40 |
| 50 - 60 | 60 → 0 | 40 → 100 |
| 60 - 70 | 0 | 100 |

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *rosuvastatin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to rosuvastatin (retention time = about 25 min): impurity A = about 0.9; impurity B = about 1.1; impurity C = about 1.5.

*System suitability*: reference solution (c):
- *resolution*: minimum 2.0 between the peaks due to rosuvastatin and impurity B.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity C by 1.4;
- for each impurity, use the concentration of rosuvastatin in reference solution (b).

*Limits*:
- *impurity C*: maximum 0.8 per cent;
- *impurity B*: maximum 0.5 per cent;
- *impurity A*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture*: *acetonitrile for chromatography R*, *water for chromatography R* (25:75 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 6 mL of *acetonitrile for chromatography R* and dilute to 25.0 mL with *water for chromatography R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *rosuvastatin impurity G CRS* in 1.0 mL of the test solution.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *acetonitrile for chromatography R*, 0.1 per cent *V/V* solution of *trifluoroacetic acid R* (25:75 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL.

*Run time*: 2.6 times the retention time of rosuvastatin.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity G.

*Relative retention* with reference to rosuvastatin (retention time = about 29 min): impurity G = about 0.9.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurity G and rosuvastatin.

*Calculation of percentage content*:

– use the concentration of rosuvastatin in reference solution (a).

*Limit*:

– *impurity G*: maximum 0.1 per cent.

**Water** (*2.5.12*): maximum 6.1 per cent, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{44}H_{54}CaF_2N_6O_{12}S_2$ taking into account the assigned content of *rosuvastatin calcium CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, J.*

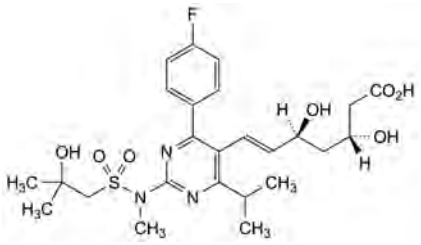

A. (3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-2-[[(2-hydroxy-2-methylpropyl)sulfonyl](methyl)amino]-6-(1-methylethyl)-pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid,

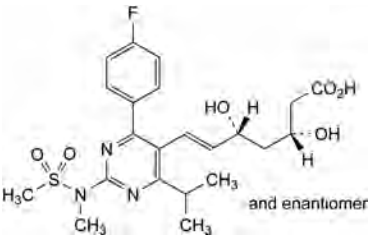


B. (3*RS*,5*RS*,6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid,

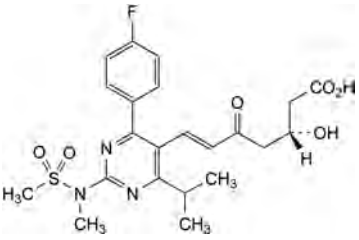

C. (3*R*,6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-3-hydroxy-5-oxohept-6-enoic acid,

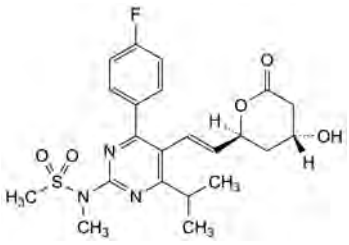

D. *N*-[4-(4-fluorophenyl)-5-[(*E*)-2-[(2*S*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethenyl]-6-(1-methylethyl)-pyrimidin-2-yl]-*N*-methylmethanesulfonamide,

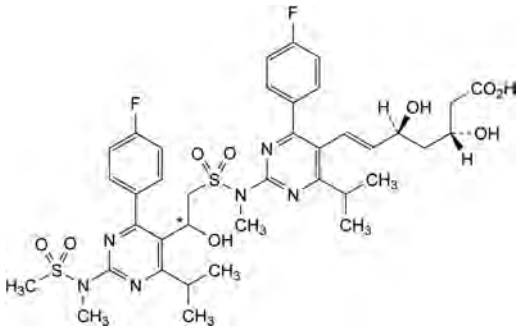

E. (3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-2-[[[2-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-2-hydroxyethyl]sulfonyl](methyl)amino]-6-(1-methylethyl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid,

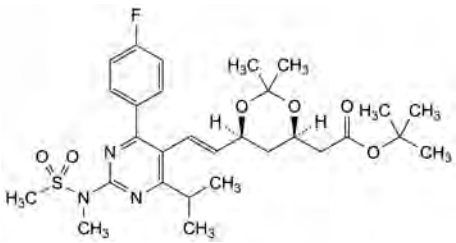

F. 1,1-dimethylethyl [(4*R*,6*S*)-6-[(*E*)-2-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]-pyrimidin-5-yl]ethenyl]-2,2-dimethyl-1,3-dioxan-4-yl]-acetate,

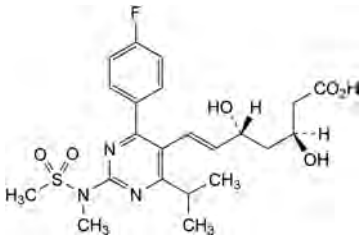

G. (3*S*,5*R*,6*E*)-7-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid,

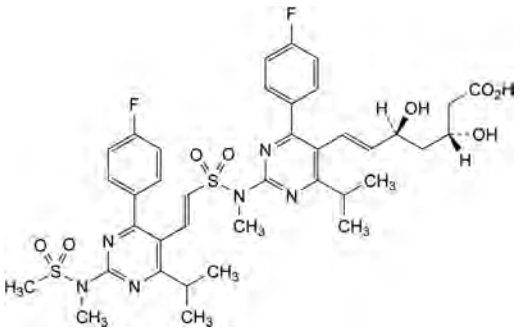

J. (3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-2-[[[(*E*)-2-[4-(4-fluorophenyl)-6-(1-methylethyl)-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl]ethenyl]sulfonyl](methyl)amino]-6-(1-methylethyl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoic acid.

**01/2019:3014**

# ROTIGOTINE

## Rotigotinum

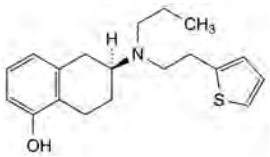

$C_{19}H_{25}NOS$ $M_r$ 315.5

[99755-59-6]

DEFINITION

(6*S*)-6-[Propyl[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-ol.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or light brown powder.

*Solubility*: practically insoluble in water, soluble in anhydrous ethanol, very slightly soluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rotigotine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

B. Enantiomeric purity (see Tests).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solution A*: *methanol R1*, *2-propanol R*, *anhydrous ethanol R* (5:5:90 *V/V/V*).

*Solution B*: 0.1 per cent *V/V* solution of *diethylamine R* in solution A.

*Test solution*. Dissolve 10 mg of the substance to be examined in solution B and dilute to 10 mL with solution B.

*Reference solution*. Dissolve 2 mg of *racemic rotigotine CRS* in 1 mL of solution B.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (10 µm).

*Mobile phase*: *diethylamine R*, solution A, *heptane R* (0.1:2:98 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of rotigotine.

*Relative retention* with reference to rotigotine (retention time = about 22 min): impurity A = about 0.9.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to impurity A and rotigotine.

*Limit*:

– *impurity A*: maximum 0.15 per cent, calculate the ratio of the area of the peak due to impurity A to the sum of the areas of the peaks due to rotigotine and impurity A.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R*.

*Solution B*: 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in *acetonitrile R*.

*Test solution (a)*. Suspend 30.0 mg of the substance to be examined in 2.5 mL of solution B and add 20 mL of solution A. Sonicate for at least 20 min, shaking vigorously at 5 min intervals, until dissolution is complete, then dilute to 50.0 mL with solution A.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with solution A.

*Reference solution (a)*. Suspend 33.0 mg of *rotigotine hydrochloride CRS* in 2.5 mL of solution B and add 20 mL of solution A. Sonicate for at least 20 min, shaking vigorously at 5 min intervals, until dissolution is complete, then dilute to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Suspend 3 mg of *rotigotine impurity B CRS*, 5 mg of *rotigotine impurity C CRS*, 5 mg of *rotigotine impurity G CRS* and 3 mg of *rotigotine impurity H CRS* in 5 mL of solution B and add 40 mL of solution A. Sonicate for at least 20 min, shaking vigorously at 5 min intervals, until dissolution is complete, then dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 50.0 mL with solution A.

*Reference solution (c)*. To 1.0 mL of test solution (a) add 5 mL of solution B and dilute to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped extra-dense bonded octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water for chromatography R* (0.3:1000 *V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile for chromatography R* (0.2:1000 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 82 | 18 |
| 2 - 24 | 82 → 50 | 18 → 50 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, G and H.

*Relative retention* with reference to rotigotine (retention time = about 10 min): impurity B = about 0.2; impurity C = about 0.6; impurity G = about 1.6; impurity H = about 1.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities G and H.

*Calculation of percentage contents*:

– for each impurity, use the concentration of rotigotine in reference solution (c).

*Limits*:

– *impurities C, G*: for each impurity, maximum 0.2 per cent;
– *impurity B*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.6 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.20 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 2.2 for the principal peak.

Calculate the percentage content of $C_{19}H_{25}NOS$ taking into account the assigned content of *rotigotine hydrochloride CRS* and a conversion factor of 0.8964.

IMPURITIES

*Specified impurities: A, B, C, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, H, I, J, K.*

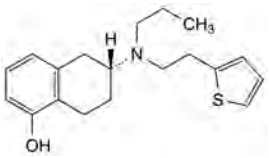

A. (6*R*)-6-[propyl[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-ol ((*R*)-rotigotine),

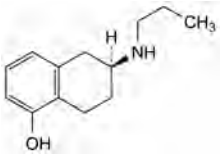

B. (6*S*)-6-(propylamino)-5,6,7,8-tetrahydronaphthalen-1-ol (desthienylethyl rotigotine),

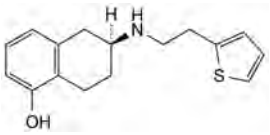

C. (6*S*)-6-[[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-ol (despropyl rotigotine),

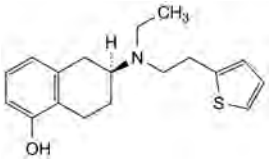

D. (6*S*)-6-[ethyl[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-ol (ethyl rotigotine),

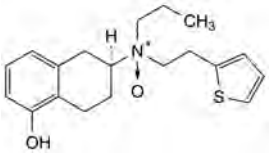

E. (2*S*)-5-hydroxy-*N*-propyl-*N*-[2-(thiophen-2-yl)ethyl]-1,2,3,4-tetrahydronaphthalen-2-amine *N*-oxide (rotigotine *N*-oxide),

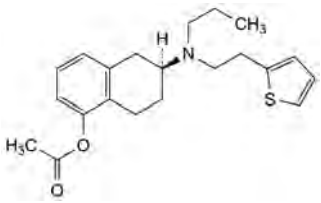

F. (6*S*)-6-[propyl[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-yl acetate (acetyl rotigotine),

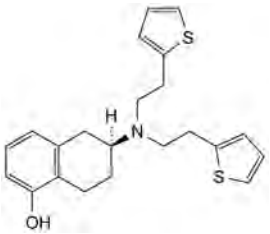

G. (6*S*)-6-[*N*,*N*-bis[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-ol,

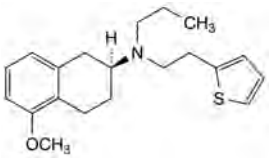

H. (2*S*)-5-methoxy-*N*-propyl-*N*-[2-(thiophen-2-yl)ethyl]-1,2,3,4-tetrahydronaphthalen-2-amine (methoxy rotigotine),

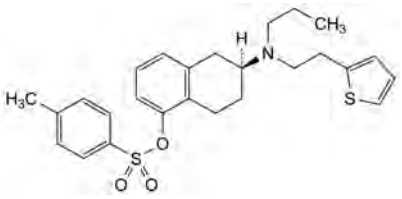

I. (6*S*)-6-[propyl[2-(thiophen-2-yl)ethyl]amino]-5,6,7,8-tetrahydronaphthalen-1-yl 4-methylbenzenesulfonate (rotigotine toluene sulfonic acid ester),

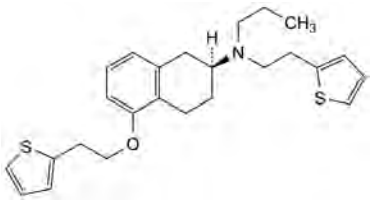

J. (2*S*)-*N*-propyl-5-[2-(thiophen-2-yl)ethoxy]-*N*-[2-(thiophen-2-yl)ethyl]-1,2,3,4-tetrahydronaphthalen-2-amine (rotigotine thienylethyl ether),

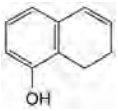

K. 7,8-dihydronaphthalen-1-ol.

**01/2017:1146**

# ROXITHROMYCIN

## Roxithromycinum

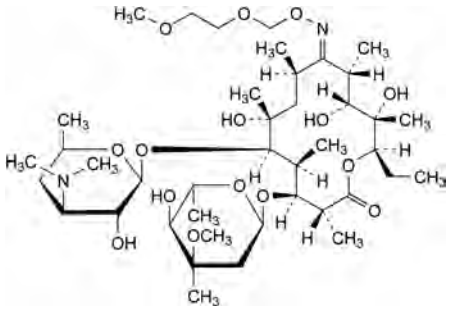

$C_{41}H_{76}N_2O_{15}$ $M_r$ 837
[80214-83-1]

DEFINITION

(3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*S*,12*R*,13*S*,14*R*)-4-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-10-[(*E*)-[(2-methoxyethoxy)methoxy]imino]-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecan-2-one (erythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime]).

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone, in alcohol and in methylene chloride. It is slightly soluble in dilute hydrochloric acid.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *roxithromycin CRS*.

If the spectra obtained shows differences, prepare further spectra using 90 g/L solutions in *methylene chloride R*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 93 to – 96 (anhydrous substance).

Dissolve 0.500 g in *acetone R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Mix 30 volumes of *acetonitrile R* and 70 volumes of a 48.6 g/L solution of *ammonium dihydrogen phosphate R*, adjusted to pH 5.3 with *dilute sodium hydroxide solution R*.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 25.0 mL with solution A.

*Reference solution (a)*. Dissolve 50.0 mg of *roxithromycin CRS* in solution A and dilute to 25.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with solution A.

*Reference solution (c)*. Dissolve 2.0 mg of *roxithromycin for system suitability CRS* in solution A and dilute to 1.0 mL with solution A.

*Reference solution (d)*. Dilute 1.0 mL of *toluene R* to 100.0 mL with *acetonitrile R*. Dilute 0.2 mL of this solution to 200.0 mL with solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a 10 nm pore size and a carbon loading of about 19 per cent,
- *temperature*: 15 °C.

*Mobile phase*:

- *mobile phase A*: mix 26 volumes of *acetonitrile R* and 74 volumes of a 59.7 g/L solution of *ammonium dihydrogen phosphate R*, adjusted to pH 4.3 with *dilute sodium hydroxide solution R*,
- *mobile phase B*: *water R*, *acetonitrile R* (30:70 *V/V*),

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 100 | 0 |
| 50 - 51 | 100 → 90 | 0 → 10 |
| 51 - 80 | 90 | 10 |
| 80 - 81 | 90 → 100 | 10 → 0 |
| 81 - 100 | 100 | 0 |

*Flow rate*: 1.1 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL, using an injector maintained at 8 °C, of the test solution and reference solutions (b), (c) and (d).

*Relative retention* with reference to roxithromycin (retention time = about 22 min): impurity A = about 0.28; impurity B = about 0.31; impurity C = about 0.33; impurity D = about 0.62; impurity E = about 0.67; impurity F = about 0.83; impurity G = about 1.15; impurity K = about 1.7; impurity H = about 1.85; impurity J = about 2.65; impurity I = about 3.1.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

*Limits*:

- *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),

– *impurities A, B, C, D, E, F, H, I, J*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent). Disregard any peak due to toluene (use reference solution (d) to identify it).

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances, with the following modifications.

*Column*:

– *size*: *l* = 0.25 m.

*Mobile phase*: mix 307 volumes of *acetonitrile R* and 693 volumes of a 49.1 g/L solution of *ammonium dihydrogen phosphate R* adjusted to pH 5.3 with *dilute sodium hydroxide solution R*.

*Flow rate*: 1.5 mL/min.

*Injection*: test solution and reference solutions (a) and (c).

*Retention time*: roxithromycin = about 12 min.

*System suitability*: reference solution (c):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to roxithromycin.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *K.*

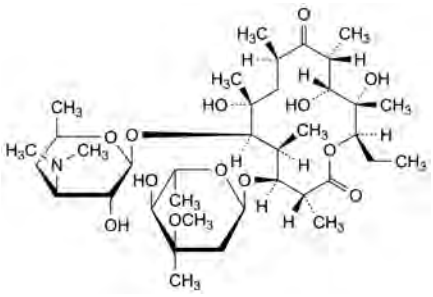

A. (3*R*,4*S*,5*S*,6*R*,7*R*,9*R*,11*R*,12*R*,13*S*,14*R*)-4-[(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-14-ethyl-7,12,13-trihydroxy-3,5,7,9,11,13-hexamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-*xylo*-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione (erythromycin A),

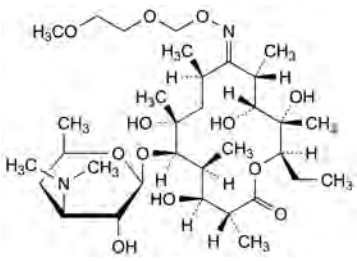

B. 3-*O*-de(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-*ribo*-hexopyranosyl)erythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime],

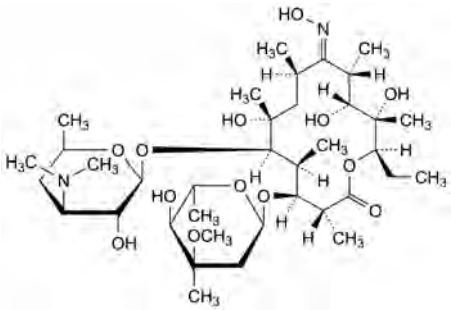

C. erythromycin 9-(*E*)-oxime,

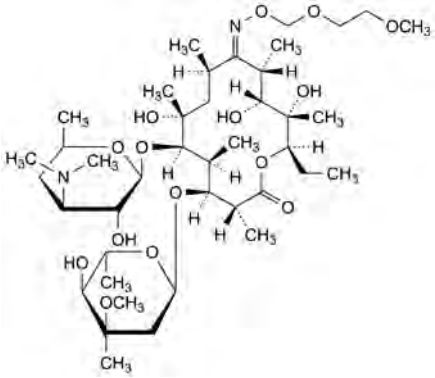

D. erythromycin 9-(*Z*)-[*O*-[(2-methoxyethoxy)methyl]-oxime],

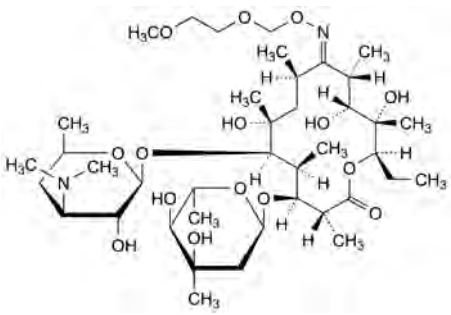

E. 3″-*O*-demethylerythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime],

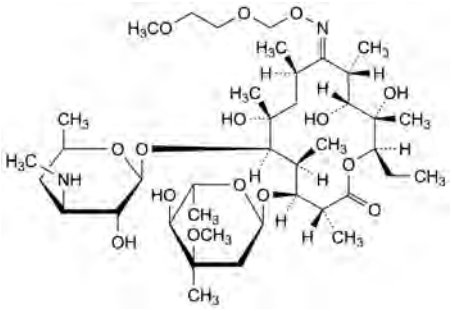

F. 3′-*N*-demethylerythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime],

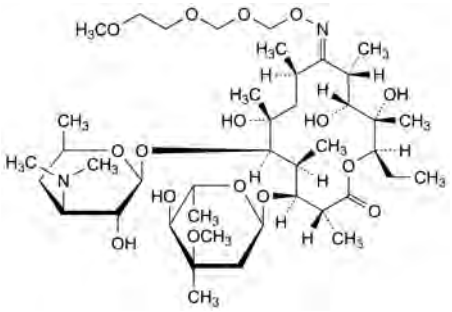

G. erythromycin 9-(*E*)-[*O*-[[(2-methoxyethoxy)methoxy]-methyl]oxime],

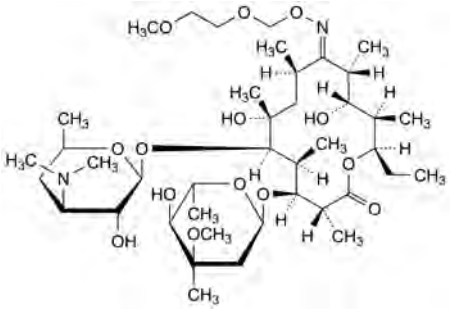

H. 12-deoxyerythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)-methyl]oxime],

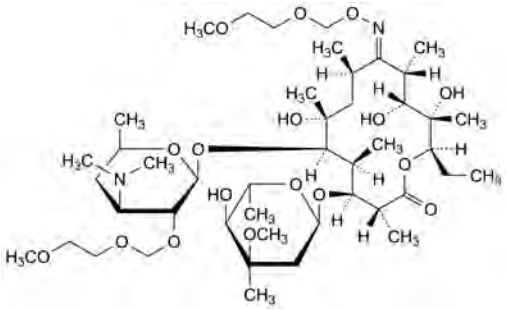

I. 2′-*O*-[(2-methoxyethoxy)methyl]erythromycin 9-(*E*)-[*O*-[(2-methoxyethoxy)methyl]oxime],

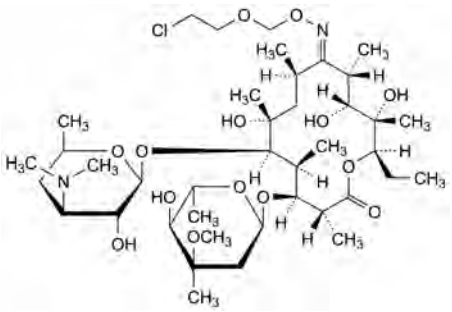

J. erythromycin 9-(*E*)-[*O*-[(2-chloroethoxy)methyl]oxime],

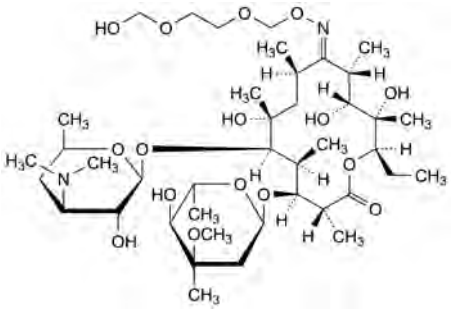

K. erythromycin 9-(*E*)-[*O*-[[2-(hydroxymethoxy)ethoxy]-methyl]oxime].

**01/2018:2888**
**corrected 10.0**

# RUPATADINE FUMARATE

## Rupatadini fumaras

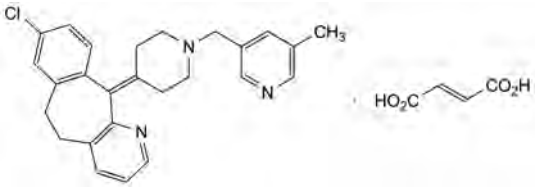

$C_{30}H_{30}ClN_3O_4$ $M_r$ 532.0
[182349-12-8]

DEFINITION

8-Chloro-11-[1-[(5-methylpyridin-3-yl)methyl]piperidin-4-ylidene]-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine (2*E*)-but-2-enedioate.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly pinkish powder.

*Solubility*: very slightly soluble in water, slightly soluble in anhydrous ethanol, very slightly soluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rupatadine fumarate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, mobile phase A (20:80 *V/V*).

*Test solution*. Dissolve 32.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 3 mg of *rupatadine for system suitability CRS* (containing impurities A and B) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: 7.0 g/L solution of *sodium dihydrogen phosphate monohydrate R* in *water for chromatography R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 27 | 80 → 50 | 20 → 50 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 50 µL.

*Identification of impurities*: use the chromatogram supplied with *rupatadine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to rupatadine (retention time = about 17 min): fumaric acid = about 0.1; impurity A = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity A by 1.3;

– for each impurity, use the concentration of rupatadine fumarate in reference solution (a).

*Limits*:

– *impurity B*: maximum 0.5 per cent;

– *impurity A*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.7 per cent;

– *reporting threshold*: 0.05 per cent; disregard the peak due to fumaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* in an oven at 80 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.73 mg of $C_{30}H_{30}ClN_3O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

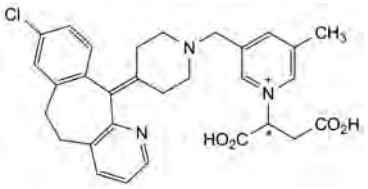

A. 3-[[4-(8-chloro-5,6-dihydro-11*H*-benzo[5,6]-cyclohepta[1,2-*b*]pyridin-11-ylidene)piperidin-1-yl]methyl]-1-(1,2-dicarboxyethyl)-5-methylpyridin-1-ium,

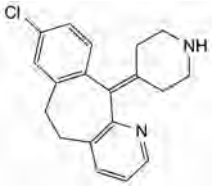

B. 8-chloro-11-(piperidin-4-ylidene)-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine,

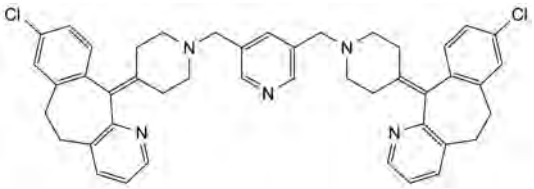

C. 11,11′-[pyridine-3,5-diylbis(methylenepiperidin-1-yl-4-ylidene)]bis(8-chloro-6,11-dihydro-5*H*-benzo[5,6]cyclohepta[1,2-*b*]pyridine).

**01/2013:1795**

# RUTOSIDE TRIHYDRATE

## Rutosidum trihydricum

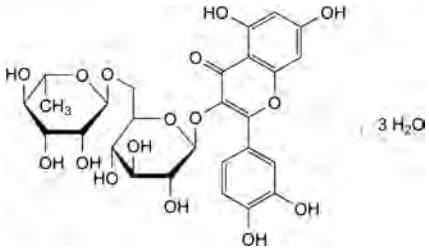

$C_{27}H_{30}O_{16},3H_2O$ $M_r$ 665

[250249-75-3]

DEFINITION

3-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one trihydrate.

*Content*: 95.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow or greenish-yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 50.0 mg in *methanol R*, dilute to 250.0 mL with the same solvent and filter if necessary. Dilute 5.0 mL of the solution to 50.0 mL with *methanol R*.

*Spectral range*: 210-450 nm.

*Absorption maxima*: at 257 nm and 358 nm.

*Specific absorbance at the absorption maximum at 358 nm*: 305 to 330 (anhydrous substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *rutoside trihydrate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution*. Dissolve 25 mg of *rutoside trihydrate CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *butanol R, anhydrous acetic acid R, water R, methyl ethyl ketone R, ethyl acetate R* (5:10:10:30:50 *V/V/V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: spray with a mixture of 2.5 mL of *ferric chloride solution R1* and 7.5 mL of a 10 g/L solution of *potassium ferricyanide R* and examine for 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve 10 mg in 5 mL of *ethanol (96 per cent) R*, add 1 g of *zinc R* and 2 mL of *hydrochloric acid R1*. A red colour develops.

TESTS

**Light-absorbing impurities**. The absorbance (*2.2.25*) is not greater than 0.10 at wavelengths between 450 nm and 800 nm.

Dissolve 0.200 g in 40 mL of *2-propanol R*. Stir for 15 min, dilute to 50.0 mL with *2-propanol R* and filter.

**Substances insoluble in methanol**: maximum 3 per cent.

Shake 2.5 g of the substance to be examined for 15 min in 50 mL of *methanol R* at 20-25 °C. Filter under reduced pressure through a sintered-glass filter (1.6) (*2.1.2*) previously dried for 15 min at 100-105 °C, allowed to cool in a desiccator and tared. Wash the filter 3 times with 20 mL of *methanol R*. Dry the filter for 30 min at 100-105 °C. Allow to cool and weigh. The residue weighs a maximum of 75 mg.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in 20 mL of *methanol R* and dilute to 100.0 mL with mobile phase B.

*Reference solution (a)*. Dissolve 10.0 mg of *rutoside trihydrate CRS* in 2.0 mL of *methanol R* and dilute to 10.0 mL with mobile phase B.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with mobile phase B.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 5 volumes of *tetrahydrofuran R* and 95 volumes of a 15.6 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: mix 40 volumes of *tetrahydrofuran R* and 60 volumes of a 15.6 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 50 → 0 | 50 → 100 |
| 10 - 15 | 0 | 100 |
| 15 - 16 | 0 → 50 | 100 → 50 |
| 16 - 20 | 50 | 50 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Relative retention* with reference to rutoside (retention time = about 7 min): impurity B = about 1.1; impurity A = about 1.2; impurity C = about 2.5.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to rutoside and impurity B.

*Limits*: locate the impurities by comparison with the chromatogram provided with *rutoside trihydrate CRS*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.8; impurity C = 0.5;
- *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Water** (*2.5.12*): 7.5 per cent to 9.5 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 20 mL of *dimethylformamide R*. Titrate with *0.1 M tetrabutylammonium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 30.53 mg of $C_{27}H_{30}O_{16}$.

STORAGE

Protected from light.

IMPURITIES

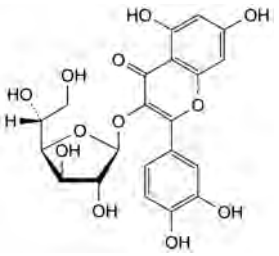


A. 2-(3,4-dihydroxyphenyl)-3-(β-D-glucofuranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one (isoquercitroside),

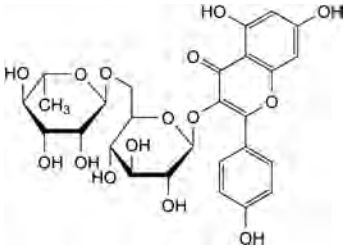

B. 3-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one (kaempferol 3-rutinoside),

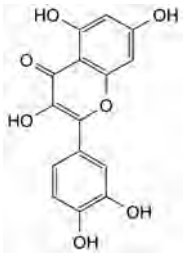

C. 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one (quercetin).

# S


Saccharin.... 3759
Saccharin sodium.. .... 3760
Safflower oil, refined.... 3761
Salbutamol.. .... 3761
Salbutamol sulfate.... 3764
Salicylic acid.. .... 3766
Salmeterol xinafoate.. .... 3767
Salmon oil, farmed.. .... 3769
Saquinavir mesilate.... 3771
Selamectin for veterinary use.... 3773
Selegiline hydrochloride.. .... 3775
Selenium disulfide.. .... 3776
Serine.... 3777
Sertaconazole nitrate.. .... 3778
Sertraline hydrochloride.. .... 3779
Sesame oil, refined.. .... 3781
Sevoflurane.. .... 3783
Shellac .. .... 3785
Sildenafil citrate.. .... 3786
Silica, colloidal anhydrous.. .... 3787
Silica, colloidal hydrated.. .... 3788
Silica, dental type.. .... 3788
Silica, hydrophobic colloidal.. .... 3789
Silver, colloidal, for external use.. .... 3790
Silver nitrate.. .... 3790
Simeticone.. .... 3791
Simvastatin.. .... 3792
Sitagliptin phosphate monohydrate.. .... 3795
Sitagliptin tablets.. .... 3796
Sodium acetate trihydrate.... 3797
Sodium alendronate trihydrate.. .... 3798
Sodium alginate.. .... 3799
Sodium amidotrizoate.... 3800
Sodium aminosalicylate dihydrate.. .... 3801
Sodium ascorbate.... 3802
Sodium aurothiomalate.. .... 3804
Sodium benzoate.. .... 3805
Sodium bromide.. .... 3806
Sodium calcium edetate.... 3807
Sodium caprylate.. .... 3808
Sodium carbonate.... 3809
Sodium carbonate decahydrate.... 3809
Sodium carbonate monohydrate.. .... 3810
Sodium cetostearyl sulfate.... 3810
Sodium chloride.... 3812
Sodium citrate.. .... 3813
Sodium cromoglicate.. .... 3813
Sodium cyclamate.... 3814
Sodium dihydrogen phosphate dihydrate.. .... 3816
Sodium ethyl parahydroxybenzoate.... 3816
Sodium fluoride.. .... 3818
Sodium fusidate.. .... 3818
Sodium glycerophosphate, hydrated.. .... 3821
Sodium hyaluronate.. .... 3821
Sodium hydrogen carbonate.. .... 3824
Sodium hydroxide.. .... 3824
Sodium iodide.. .... 3825
Sodium lactate solution.. .... 3825
Sodium (*S*)-lactate solution.. .... 3826
Sodium laurilsulfate.. .... 3827
Sodium lauroylsarcosinate for external use.... 3828
Sodium metabisulfite.... 3829
Sodium methyl parahydroxybenzoate.. .... 3830
Sodium molybdate dihydrate.. .... 3831
Sodium nitrite.. .... 3832
Sodium nitroprusside.... 3832
Sodium perborate, hydrated.... 3833
Sodium phenylbutyrate.... 3833
Sodium picosulfate.. .... 3835
Sodium polystyrene sulfonate.. .... 3836
Sodium propionate.. .... 3837
Sodium propyl parahydroxybenzoate.. .... 3838
Sodium salicylate.. .... 3839
Sodium selenite.. .... 3840
Sodium selenite pentahydrate.. .... 3840
Sodium starch glycolate (type A).... 3841
Sodium starch glycolate (type B).. .... 3842
Sodium starch glycolate (type C).... 3843
Sodium stearate.... 3844
Sodium stearyl fumarate.... 3845
Sodium sulfate, anhydrous.. .... 3846
Sodium sulfate decahydrate.... 3847
Sodium sulfite.. .... 3847
Sodium sulfite heptahydrate.... 3848
Sodium thiosulfate.... 3848
Sodium valproate.. .... 3849
Solifenacin succinate.. .... 3850
Solutions for organ preservation.. .... 3852
Somatostatin.... 3853
Somatropin.. .... 3854
Somatropin concentrated solution.. .... 3856
Somatropin for injection.. .... 3858
Somatropin solution for injection.. .... 3860
Sorbic acid.. .... 3861
Sorbitan laurate.. .... 3862
Sorbitan oleate.... 3862
Sorbitan palmitate.. .... 3863
Sorbitan sesquioleate.. .... 3863
Sorbitan stearate.. .... 3864
Sorbitan trioleate.. .... 3865
Sorbitol.. .... 3865
Sorbitol, liquid (crystallising).. .... 3867
Sorbitol, liquid (non-crystallising).. .... 3867
Sorbitol, liquid, partially dehydrated.. .... 3868
Sotalol hydrochloride.. .... 3869
Soya phospholipids for injection.. .... 3870
Soya-bean oil, hydrogenated.. .... 3872
Soya-bean oil, refined.... 3873
Spectinomycin dihydrochloride pentahydrate.... 3873
Spectinomycin sulfate tetrahydrate for veterinary use.. .... 3875
Spiramycin.. .... 3877
Spirapril hydrochloride monohydrate.... 3880
Spironolactone.. .... 3881
Squalane.. .... 3883
Squalene.. .... 3886
Stannous chloride dihydrate.... 3886
Stanozolol.. .... 3887
Starch, hydroxypropyl.. .... 3888
Starch, hydroxypropyl, pregelatinised.. .... 3890
Starch, pregelatinised.. .... 3891
Starches, hydroxyethyl.. .... 3892
Stavudine.. .... 3896
Stearic acid.... 3898
Stearoyl macrogolglycerides.. .... 3899
Stearyl alcohol.. .... 3900
Streptokinase concentrated solution.. .... 3901
Streptomycin sulfate.. .... 3902
Sucralfate.. .... 3904
Sucralose.. .... 3905
Sucrose.. .... 3906
Sucrose, liquid.. .... 3908
Sucrose monopalmitate.... 3909
Sucrose stearate.. .... 3911
Sufentanil.. .... 3912
Sufentanil citrate.. .... 3913
Sugar spheres.... 3915

Sulbactam sodium.. 3915
Sulfacetamide sodium.. 3917
Sulfadiazine.. 3918
Sulfadimethoxine.. 3920
Sulfadimethoxine sodium for veterinary use.. 3921
Sulfadimidine.. 3922
Sulfadoxine.. 3924
Sulfafurazole.. 3925
Sulfaguanidine 3926
Sulfamerazine.. 3926
Sulfamethizole.. 3927
Sulfamethoxazole.. 3928
Sulfamethoxypyridazine for veterinary use 3929
Sulfanilamide 3930
Sulfasalazine.. 3931
Sulfathiazole.. 3933
Sulfinpyrazone 3934
Sulfobutylbetadex sodium.. 3935
Sulfur for external use.. 3938
Sulfuric acid 3938
Sulindac 3939
Sulpiride.. 3940
Sultamicillin.. 3941
Sultamicillin tosilate dihydrate.. 3944
Sumatriptan succinate.. 3946
Sunflower oil, refined.. 3948
Suxamethonium chloride.. 3948
Suxibuzone.. 3949

**04/2019:0947**
**corrected 10.0**

# SACCHARIN

## Saccharinum

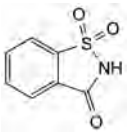

$C_7H_5NO_3S$ $M_r$ 183.2
[81-07-2]

DEFINITION

1,2-Benzisothiazol-3(2*H*)-one 1,1-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: sparingly soluble in boiling water and in ethanol (96 per cent), slightly soluble in cold water. It dissolves in dilute solutions of alkali hydroxides and carbonates.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D, E.*

A. A saturated solution, prepared without heating, turns *blue litmus paper R* red.

B. Melting point (*2.2.14*): 226 °C to 230 °C.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *saccharin CRS*.

D. Mix about 10 mg with about 10 mg of *resorcinol R*, add 0.25 mL of *sulfuric acid R* and carefully heat the mixture over a naked flame until a dark green colour is produced. Allow to cool, add 10 mL of *water R* and *dilute sodium hydroxide solution R* until an alkaline reaction is produced. An intense green fluorescence develops.

E. To 0.2 g add 1.5 mL of *dilute sodium hydroxide solution R*, evaporate to dryness and heat the residue carefully until it melts, avoiding carbonisation. Allow to cool, dissolve the mass in about 5 mL of *water R*, add *dilute hydrochloric acid R* until a weak acid reaction is produced and filter, if necessary. To the filtrate add 0.2 mL of *ferric chloride solution R2*. A violet colour develops.

TESTS

**Solution S**. Dissolve 5.0 g in 20 mL of a 200 g/L solution of *sodium acetate R* and dilute to 25 mL with the same solution.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

***o*- and *p*-Toluenesulfonamide**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 25 mg of *caffeine R* in *methylene chloride R* and dilute to 100 mL with the same solvent.

*Test solution*. Suspend 10.0 g of the substance to be examined in 20 mL of *water R* and dissolve using 5-6 mL of *strong sodium hydroxide solution R*. If necessary adjust the solution to pH 7-8 with *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 50 mL with *water R*. Shake the solution with 4 quantities, each of 50 mL, of *methylene chloride R*. Combine the lower layers, dry over *anhydrous sodium sulfate R* and filter. Wash the filter and the sodium sulfate with 10 mL of *methylene chloride R*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40 °C. Using a small quantity of *methylene chloride R*, quantitatively transfer the residue into a suitable 10 mL tube, evaporate to dryness in a current of nitrogen and dissolve the residue in 1.0 mL of the internal standard solution.

*Blank solution*. Evaporate 200 mL of *methylene chloride R* to dryness in a water-bath at a temperature not exceeding 40 °C. Dissolve the residue in 1 mL of *methylene chloride R*.

*Reference solution*. Dissolve 20.0 mg of *o-toluenesulfonamide R* and 20.0 mg of *toluenesulfonamide R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *methylene chloride R*. Evaporate 5.0 mL of the final solution to dryness in a current of nitrogen. Dissolve the residue in 1.0 mL of the internal standard solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 10 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 2 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 10 mL/min.

*Split ratio*: 1:2.

*Temperature*:
- *column*: 180 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Order of elution*: *o*-toluenesulfonamide, *p*-toluenesulfonamide, caffeine.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to *o*-toluenesulfonamide and *p*-toluenesulfonamide in the chromatogram obtained with the reference solution;
- the chromatogram obtained with the blank solution does not show any peak with the same retention times as the internal standard, *o*-toluenesulfonamide and *p*-toluenesulfonamide.

*Limits*:
- *o-toluenesulfonamide*: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm);
- *p-toluenesulfonamide*: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

**Readily carbonisable substances**. Dissolve 0.20 g in 5 mL of *sulfuric acid R* and keep at 48-50 °C for 10 min. When viewed against a white background, the solution is not more intensely coloured than a solution prepared by mixing 0.1 mL of red primary solution, 0.1 mL of blue primary solution and 0.4 mL of yellow primary solution (*2.2.2*) with 4.4 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.500 g in 40 mL of *ethanol (96 per cent) R*. Add 40 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, using a 10 g/L solution of *phenolphthalein R* in *ethanol (96 per cent) R* as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.32 mg of $C_7H_5NO_3S$.

04/2019:0787
corrected 10.0

# SACCHARIN SODIUM

## Saccharinum natricum

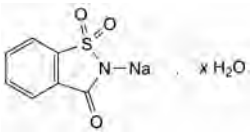

$C_7H_4NNaO_3S,xH_2O$ $M_r$ 205.2 (anhydrous substance)
Anhydrous saccharin sodium: [128-44-9]

DEFINITION

2-Sodio-1,2-benzisothiazol-3(2*H*)-one 1,1-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

It may be anhydrous or contain a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, efflorescent in dry air.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *B, E.*

*Second identification*: *A, C, D, E.*

A. Melting point (*2.2.14*): 226 °C to 230 °C.

To 5 mL of solution S (see Tests) add 3 mL of *dilute hydrochloric acid R*. A white precipitate is formed. Filter and wash with *water R*. Dry the precipitate at 100-105 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs; dry the substances to constant mass at 105 °C before use.

*Comparison*: *saccharin sodium CRS*.

C. Mix about 10 mg with about 10 mg of *resorcinol R*, add 0.25 mL of *sulfuric acid R* and carefully heat the mixture over a naked flame until a dark green colour is produced. Allow to cool, add 10 mL of *water R* and *dilute sodium hydroxide solution R* until an alkaline reaction is produced. An intense green fluorescence develops.

D. To 0.2 g add 1.5 mL of *dilute sodium hydroxide solution R*, evaporate to dryness and heat the residue carefully until it melts, avoiding carbonisation. Allow to cool, dissolve the mass in about 5 mL of *water R*, add *dilute hydrochloric acid R* until a weak acid reaction is produced and filter, if necessary. To the filtrate add 0.2 mL of *ferric chloride solution R2*. A violet colour develops.

E. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 2.0 g in 10 mL of *water R*.

**Acidity or alkalinity**. To 10 mL of solution S add about 0.05 mL of a 10 g/L solution of *phenolphthalein R* in *ethanol (96 per cent) R*. The solution is neither pink nor red. Add 0.05 mL of *0.1 M sodium hydroxide*. The solution becomes pink or red.

***o*- and *p*-Toluenesulfonamide**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 25 mg of *caffeine R* in *methylene chloride R* and dilute to 100 mL with the same solvent.

*Test solution*. Dissolve 10.0 g of the substance to be examined in 50 mL of *water R*. If necessary adjust the solution to pH 7-8 by addition of *1 M sodium hydroxide* or *1 M hydrochloric acid*. Shake the solution with 4 quantities, each of 50 mL, of *methylene chloride R*. Combine the lower layers, dry over *anhydrous sodium sulfate R* and filter. Wash the filter and the sodium sulfate with 10 mL of *methylene chloride R*. Combine the solution and the washings and evaporate almost to dryness in a water-bath at a temperature not exceeding 40 °C. Using a small quantity of *methylene chloride R*, quantitatively transfer the residue into a suitable 10 mL tube, evaporate to dryness in a current of *nitrogen R* and add 1.0 mL of the internal standard solution.

*Blank solution*. Evaporate 200 mL of *methylene chloride R* to dryness in a water-bath at a temperature not exceeding 40 °C. Dissolve the residue in 1 mL of *methylene chloride R*.

*Reference solution*. Dissolve 20.0 mg of *o-toluenesulfonamide R* and 20.0 mg of *toluenesulfonamide R* in *methylene chloride R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *methylene chloride R*. Evaporate 5.0 mL of the final solution to dryness in a current of *nitrogen R*. Take up the residue using 1.0 mL of the internal standard solution.

*Column*:
- *material*: fused silica;
- *size*: *l* = 10 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 2 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 10 mL/min.

*Split ratio*: 1:2.

*Temperature*:
- *column*: 180 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: *o*-toluenesulfonamide, *p*-toluenesulfonamide, caffeine.

*System suitability*:
- *resolution*: minimum 1.5 between the peaks due to *o*-toluenesulfonamide and *p*-toluenesulfonamide in the chromatogram obtained with the reference solution;
- the chromatogram obtained with the blank solution does not show any peak with the same retention times as the internal standard, *o*-toluenesulfonamide and *p*-toluenesulfonamide.

*Limits*:
- *o-toluenesulfonamide*: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm);
- *p-toluenesulfonamide*: the ratio of its area to that of the internal standard is not greater than the corresponding ratio in the chromatogram obtained with the reference solution (10 ppm).

**Readily carbonisable substances**. Dissolve 0.200 g in 5 mL of *sulfuric acid R* and keep at 48-50 °C for 10 min. When viewed against a white background, the solution is not more intensely coloured than a solution prepared by mixing 0.1 mL of red primary solution, 0.1 mL of blue primary solution and 0.4 mL of yellow primary solution (*2.2.2*) with 4.4 mL of *water R*.

**Water** (*2.5.12*): maximum 15.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*, with slight heating if necessary. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 20.52 mg of $C_7H_4NNaO_3S$.

STORAGE

In an airtight container.

**01/2020:2088**

# SAFFLOWER OIL, REFINED

## Carthami oleum raffinatum

DEFINITION

Fatty oil obtained from seeds of *Carthamus tinctorius* L. (type I) or from seeds of hybrids of *Carthamus tinctorius* L. (type II), by expression and/or extraction followed by refining. Type II refined safflower oil is rich in oleic ((9*Z*)-octadec-9-enoic) acid. A suitable antioxidant may be added.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, viscous, yellow or pale yellow liquid.

*Solubility*: practically insoluble in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

| | Type I refined safflower oil | Type II refined safflower oil |
|---|---|---|
| Relative density | about 0.922 | about 0.914 |
| Refractive index | about 1.476 | about 1.472 |

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram for type I or type II shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of type I refined safflower oil:*

- *saturated fatty acids of chain length less than C14*: maximum 0.2 per cent;
- *myristic acid*: maximum 0.2 per cent;
- *palmitic acid*: 4.0 per cent to 10.0 per cent;
- *stearic acid*: 1.0 per cent to 5.0 per cent;
- *oleic acid*: 8.0 per cent to 21.0 per cent;
- *linoleic acid*: 68.0 per cent to 83.0 per cent;
- *linolenic acid*: maximum 0.5 per cent;
- *arachidic acid*: maximum 0.5 per cent;
- *eicosenoic acid*: maximum 0.5 per cent;
- *behenic acid*: maximum 1.0 per cent.

*Composition of the fatty-acid fraction of type II refined safflower oil:*

- *saturated fatty acids of chain length less than C14*: maximum 0.2 per cent;
- *myristic acid*: maximum 0.2 per cent;
- *palmitic acid*: 3.6 per cent to 6.0 per cent;
- *stearic acid*: 1.0 per cent to 5.0 per cent;
- *oleic acid*: 70.0 per cent to 84.0 per cent;
- *linoleic acid*: 7.0 per cent to 23.0 per cent;
- *linolenic acid*: maximum 0.5 per cent;
- *arachidic acid*: maximum 1.0 per cent;
- *eicosenoic acid*: maximum 1.0 per cent;
- *behenic acid*: maximum 1.2 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled, airtight container, protected from light.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- the type of oil (type I or type II).

**01/2011:0529**

# SALBUTAMOL

## Salbutamolum

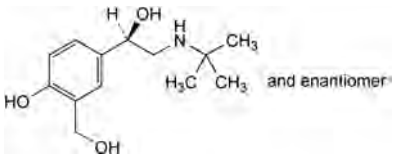


$C_{13}H_{21}NO_3$ $M_r$ 239.3

[18559-94-9]

DEFINITION

(1*RS*)-2-[(1,1-Dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, soluble in ethanol (96 per cent).

mp: about 155 °C, with decomposition.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 80.0 mg in a 10 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with a 10 g/L solution of *hydrochloric acid R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 276 nm.

*Specific absorbance at the absorption maximum*: 66 to 75.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *salbutamol CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 50 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *salbutamol CRS* in *methanol R* and dilute to 50 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethyl acetate R*, *2-propanol R*, *methyl isobutyl ketone R* (3:18:35:45:50 *V/V/V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with a 1 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* in a 90 per cent *V/V* solution of *methanol R*, followed by a 20 g/L solution of *potassium ferricyanide R* in a mixture of 1 volume of *concentrated ammonia R1* and 3 volumes of *water R*, followed by a further spraying with a 1 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* in a 90 per cent *V/V* solution of *methanol R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 50 mL of a 20 g/L solution of *disodium tetraborate R*. Add 1 mL of a 30 g/L solution of *aminopyrazolone R*, 10 mL of *methylene chloride R* and 10 mL of a 20 g/L solution of *potassium ferricyanide R*. Shake and allow to separate. An orange-red colour develops in the methylene chloride layer.

TESTS

**Solution S**. Dissolve 0.50 g in *methanol R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2.0 mg of *salbutamol CRS*, 2 mg of *salbutamol impurity B CRS*, 3.0 mg of *salbutamol impurity D CRS*, 3.0 mg of *salbutamol impurity F CRS* and 3.0 mg of *salbutamol impurity G CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *salbutamol impurity I CRS* in 1.0 mL of the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;
– *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 335 $m^2$/g, a pore size of 10 nm and a carbon loading of 11.7 per cent.

*Mobile phase*: mix 22 volumes of *acetonitrile R1* and 78 volumes of a solution containing 2.87 g/L of *sodium heptanesulfonate R* and 2.5 g/L of *potassium dihydrogen phosphate R* previously ajusted to pH 3.65 with *dilute phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 25 times the retention time of salbutamol.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B, D, F and G; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity I.

*Relative retention* with reference to salbutamol (retention time = about 2 min): impurity B = about 1.3; impurity A = about 1.7; impurity C = about 2.0; impurity D = about 2.7; impurity H = about 3.0; impurity E = about 3.1; impurity G = about 4.1; impurity F = about 6.2; impurity I = about 23.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to salbutamol and impurity B.

*Limits*:

– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *impurity F*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *impurity G*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *impurities A, B, C, E, H, I*: for each impurity, not more than 1.5 times the area of the peak due to salbutamol in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to salbutamol in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: maximum 1.0 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Impurity J**: maximum 0.2 per cent.

Dissolve 50.0 mg in a 1 g/L solution of *hydrochloric acid R* and dilute to 25.0 mL with the same solvent. The absorbance (*2.2.25*) of the solution measured at 310 nm is not greater than 0.10.

**Boron**: maximum 50 ppm.

*Test solution.* To 50 mg of the substance to be examined add 5 mL of a solution containing 13 g/L of *anhydrous sodium carbonate R* and 17 g/L of *potassium carbonate R*. Evaporate to dryness on a water-bath and dry at 120 °C. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool and add 0.5 mL of *water R* and 3.0 mL of a freshly prepared 1.25 g/L solution of *curcumin R* in *glacial acetic acid R*. Warm gently to effect solution, allow to cool and add 3.0 mL of a mixture prepared by adding 5 mL of *sulfuric acid R*, slowly and with stirring, to 5 mL of *glacial acetic acid R*. Mix and allow to stand for 30 min. Dilute to 100.0 mL with *ethanol (96 per cent) R*, filter and use the filtrate.

*Reference solution*. Dissolve 0.572 g of *boric acid R* in 1000.0 mL of *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. To 2.5 mL of this solution add 5 mL of a solution containing 13 g/L of *anhydrous sodium carbonate R* and 17 g/L of *potassium carbonate R*, and treat this mixture in the same manner as the test solution.

Measure the absorbance (*2.2.25*) of the test solution and of the reference solution at the absorption maximum at about 555 nm. The absorbance of the test solution is not greater than that of the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 23.93 mg of $C_{13}H_{21}NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

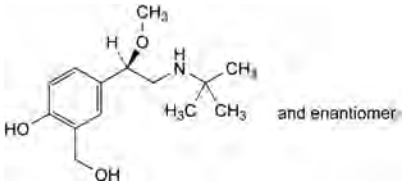


A. 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-methoxyethyl]-2-hydroxyphenyl]methanol,

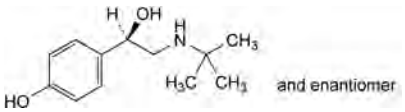


B. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxyphenyl)ethanol,

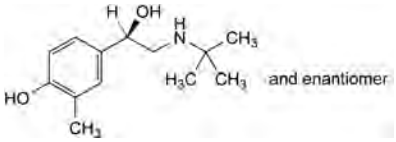


C. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxy-3-methylphenyl)ethanol,

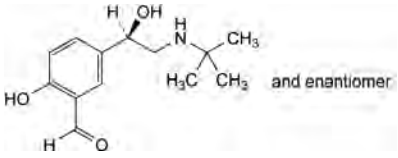


D. 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxybenzaldehyde,

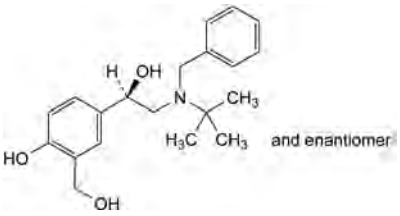


E. (1*RS*)-2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol,

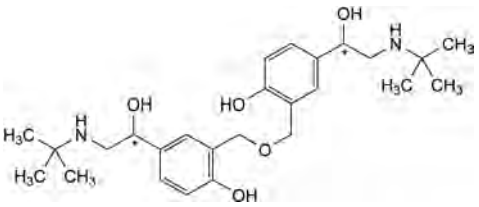


F. 1,1′-[oxybis[methylene(4-hydroxy-1,3-phenylene)]]bis[2-[(1,1-dimethylethyl)amino]ethanol],

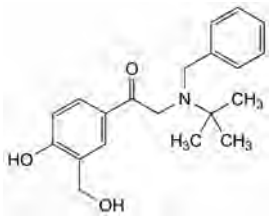


G. 2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone,

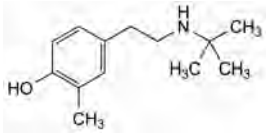


H. 4-[2-[(1,1-dimethylethyl)amino]ethyl]-2-methylphenol,

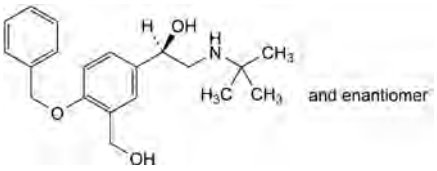


I. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-(benzyloxy)-3-(hydroxymethyl)phenyl]ethanol,

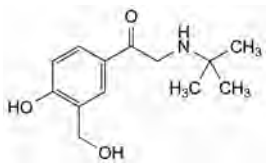


J. 2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone (salbutamone).

Monographs S

07/2011:0687

# SALBUTAMOL SULFATE

## Salbutamoli sulfas

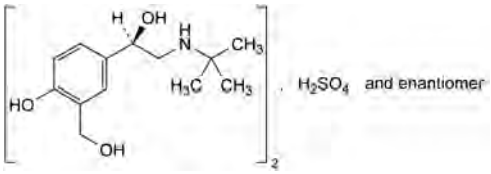


$C_{26}H_{44}N_2O_{10}S$ $M_r$ 576.7
[51022-70-9]

DEFINITION

Bis[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol] sulfate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, practically insoluble or very slightly soluble in ethanol (96 per cent) and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: B, E.*

*Second identification: A, C, D, E.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 80.0 mg in a 10 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same acid. Dilute 10.0 mL of the solution to 100.0 mL with a 10 g/L solution of *hydrochloric acid R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 276 nm.

*Specific absorbance at the absorption maximum*: 55 to 64.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *salbutamol sulfate CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*. Dry the residues and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 12 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 12 mg of *salbutamol sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethyl acetate R*, *2-propanol R*, *methyl isobutyl ketone R* (3:18:35:45:50 *V/V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 1 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* in a 90 per cent *V/V* solution of *methanol R*, followed by a 20 g/L solution of *potassium ferricyanide R* in a mixture of 1 volume of *concentrated ammonia R1* and 3 volumes of *water R*, followed by a further spraying with a 1 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R* in a 90 per cent *V/V* solution of *methanol R*.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 10 mg in 50 mL of a 20 g/L solution of *disodium tetraborate R*. Add 1 mL of a 30 g/L solution of *aminopyrazolone R*, 10 mL of *methylene chloride R* and 10 mL of a 20 g/L solution of *potassium ferricyanide R*. Shake and allow to separate. An orange-red colour develops in the methylene chloride layer.

E. It gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.250 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Acidity or alkalinity**. To 10 mL of solution S add 0.15 mL of *methyl red solution R* and 0.2 mL of *0.01 M sodium hydroxide*. The solution is yellow. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in mobile phase A and dilute to 50.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 3.0 mg of *salbutamol impurity D CRS* and 3.0 mg of *salbutamol impurity F CRS* in mobile phase A and dilute to 50.0 mL with mobile phase A. Dilute 2.0 mL of the solution to 100.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c).* With the aid of ultrasound, dissolve the contents of a vial of *salbutamol impurity J CRS* in 1.0 mL of the test solution.

*Reference solution (d).* Dissolve 1 mg of *salbutamol impurity D CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (e).* Dissolve 4 mg of *salbutamol sulfate for system suitability CRS* (containing impurities C, F, N and O) in mobile phase A, add 0.4 mL of reference solution (d) and dilute to 10.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 3.45 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of a 0.05 per cent *V/V* solution of *triethylamine R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with a 0.05 per cent *V/V* solution of *triethylamine R*;
- *mobile phase B*: *methanol R*, *acetonitrile R* (35:65 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 95 | 5 |
| 5 - 30 | 95 → 10 | 5 → 90 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µl of the test solution and reference solutions (a), (b), (c) and (e).

*Relative retention* with reference to salbutamol (retention time = about 7 min): impurity J = about 0.9; impurity C = about 1.6; impurity N = about 1.67; impurity D = about 1.68; impurity F = about 1.77; impurity O = about 1.82.

*Identification of impurities*: use the chromatogram supplied with *salbutamol sulfate for system suitability CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities C, D, F, N and O; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity J.

*System suitability*:

- *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity N and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity D in the chromatogram obtained with reference solution (e); minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity J and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to salbutamol in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurities D, F*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities C, N, O*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: maximum 0.9 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Boron**: maximum 50 ppm.

*Test solution.* To 50 mg of the substance to be examined add 5 mL of a solution containing 13 g/L of *anhydrous sodium carbonate R* and 17 g/L of *potassium carbonate R*. Evaporate to dryness on a water-bath and dry at 120 °C. Ignite the residue rapidly until the organic matter has been destroyed, allow to cool and add 0.5 mL of *water R* and 3.0 mL of a freshly prepared 1.25 g/L solution of *curcumin R* in *glacial acetic acid R*. Warm gently to effect solution, allow to cool and add 3.0 mL of a mixture prepared by adding 5 mL of *sulfuric acid R*, slowly and with stirring, to 5 mL of *glacial acetic acid R*. Mix and allow to stand for 30 min. Dilute to 100.0 mL with *ethanol (96 per cent) R*, filter and use the filtrate.

*Reference solution.* Dissolve 0.572 g of *boric acid R* in 1000.0 mL of *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*. To 2.5 mL of this solution add 5 mL of a solution containing 13 g/L of *anhydrous sodium carbonate R* and 17 g/L of *potassium carbonate R*, and treat this mixture in the same manner as the test solution.

Measure the absorbance (*2.2.25*) of the test solution and of the reference solution at the absorption maximum at about 555 nm. The absorbance of the test solution is not greater than that of the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 5 mL of *anhydrous formic acid R* and add 35 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 57.67 mg of $C_{26}H_{44}N_2O_{10}S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *C, D, F, N, O*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, G, I, J, K, L, M*.

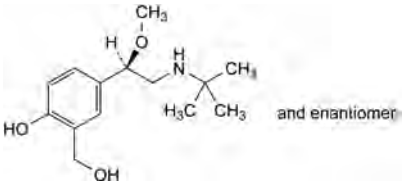


A. [5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-methoxyethyl]-2-hydroxyphenyl]methanol,

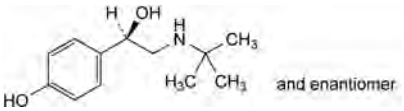


B. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxyphenyl)-ethanol,

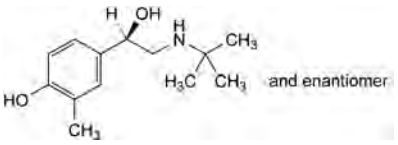


C. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-(4-hydroxy-3-methylphenyl)ethanol,

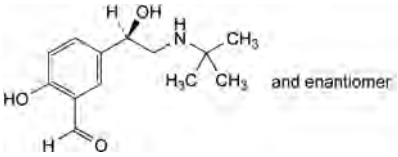


D. 5-[(1*RS*)-2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxybenzaldehyde,

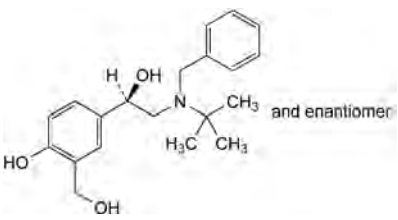


E. (1*RS*)-2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol,

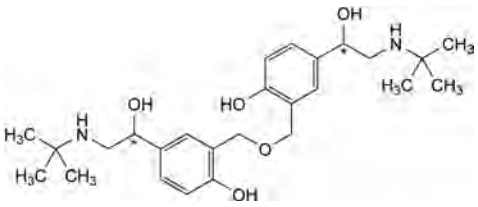

F. 1,1′-[oxybis[methylene(4-hydroxy-1,3-phenylene)]]bis[2-[(1,1-dimethylethyl)amino]ethanol],

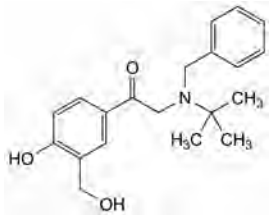

G. 2-[benzyl(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone,

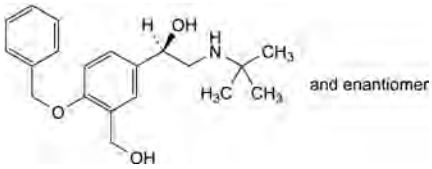


I. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-(benzyloxy)-3-(hydroxymethyl)phenyl]ethanol,

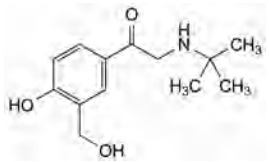

J. 2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanone (salbutamone),

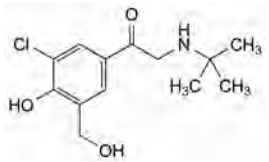

K. 2-[(1,1-dimethylethyl)amino]-1-[3-chloro-4-hydroxy-5-(hydroxymethyl)phenyl]ethanone,

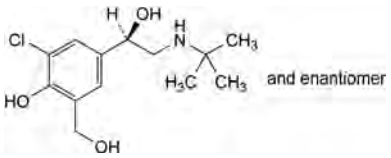


L. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[3-chloro-4-hydroxy-5-(hydroxymethyl)phenyl]ethanol,

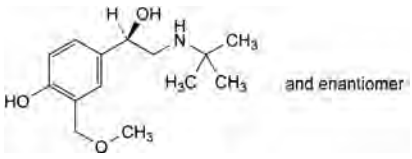


M. (1*RS*)-2-[(1,1-dimethylethyl)amino]-1-[4-hydroxy-3-(methoxymethyl)phenyl]ethanol,

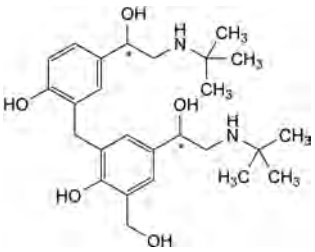

N. 2-[(1,1-dimethylethyl)amino]-1-[3-[[5-[2-[(1,1-dimethylethyl)amino]-1-hydroxyethyl]-2-hydroxyphenyl]methyl]-4-hydroxy-5-(hydroxymethyl)phenyl]-ethanol,

O. unknown structure.

**01/2017:0366**

# SALICYLIC ACID

## Acidum salicylicum

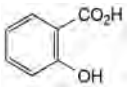

$C_7H_6O_3$ $M_r$ 138.1
[69-72-7]

DEFINITION

2-Hydroxybenzenecarboxylic acid.

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or white or colourless, acicular crystals.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent), sparingly soluble in methylene chloride.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Melting point (*2.2.14*): 158 °C to 161 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *salicylic acid CRS.*

C. Dissolve about 30 mg in 5 mL of *0.05 M sodium hydroxide*, neutralise if necessary and dilute to 20 mL with *water R*. 1 mL of the solution gives reaction (a) of salicylates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in 50 mL of boiling *distilled water R*, cool and filter.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1 g in 10 mL of *ethanol (96 per cent) R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.50 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10 mg of *phenol R* (impurity C) in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *salicylic acid impurity B CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 50 mg of *4-hydroxybenzoic acid R* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (e).* Dilute a mixture of 1.0 mL of each of reference solutions (a), (b) and (c) to 10.0 mL with the mobile phase.

*Reference solution (f).* Dilute a mixture of 0.1 mL of each of reference solutions (a), (b) and (c) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *methanol R*, *water R* (1:40:60 *V/V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 10 µL of the test solution and reference solutions (d), (e) and (f).

*Identification of impurities*: use the chromatogram obtained with reference solution (e) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to impurity C (retention time = about 9.5 min): impurity A = about 0.6; impurity B = about 0.8.

*System suitability*: reference solution (e):

- the 3rd peak in the chromatogram corresponds to the peak due to impurity C in the chromatogram obtained with reference solution (d);
- *resolution*: minimum 1.0 between the peaks due to impurities B and C; if necessary, adjust the quantity of acetic acid in the mobile phase.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.1 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.05 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.02 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (f) (0.05 per cent);
- *total*: not more than twice the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.2 per cent);
- *disregard limit*: 0.3 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (0.03 per cent). Do not disregard the peak due to impurity C.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates**: maximum 200 ppm.

Dissolve 1.0 g in 5 mL of *dimethylformamide R* and add 4 mL of *water R*. Mix thoroughly. Add 0.2 mL of *dilute hydrochloric acid R* and 0.5 mL of a 25 per cent *m/m* solution of *barium chloride R*. After 15 min any opalescence in the solution is not more intense than that in a standard prepared as follows: to 2 mL of *sulfate standard solution (100 ppm $SO_4$) R* add 0.2 mL of *dilute hydrochloric acid R*, 0.5 mL of a 25 per cent *m/m* solution of *barium chloride R*, 3 mL of *water R* and 5 mL of *dimethylformamide R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.120 g in 30 mL of *ethanol (96 per cent) R* and add 20 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenol red solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 13.81 mg of $C_7H_6O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

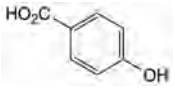

A. 4-hydroxybenzoic acid,

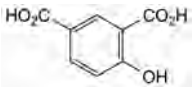

B. 4-hydroxyisophthalic acid,

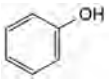

C. phenol.

**04/2014:1765**
**corrected 10.0**

# SALMETEROL XINAFOATE

## Salmeteroli xinafoas

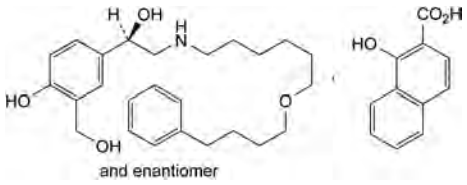


$C_{36}H_{45}NO_7$ $M_r$ 604
[94749-08-3]

DEFINITION

(1*RS*)-1-[4-Hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(4-phenylbutoxy)hexyl]amino]ethanol 1-hydroxynaphthalene-2-carboxylate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in methanol, slightly soluble in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *salmeterol xinafoate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 11 mg of *salmeterol xinafoate for system suitability CRS* (containing impurities E and G) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *salmeterol xinafoate for peak identification CRS* (containing impurity D) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 25.0 mg of *salmeterol xinafoate CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (e).* Dilute 1.0 mL of reference solution (a) to 20.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 24 volumes of a 7.71 g/L solution of *ammonium acetate R* and 24 volumes of a 28.84 g/L solution of *sodium dodecyl sulfate R* and adjust to pH 2.7 with *glacial acetic acid R*; mix with 52 volumes of *acetonitrile R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 16 | 100 | 0 |
| 16 - 36 | 100 → 30 | 0 → 70 |
| 36 - 45 | 30 | 70 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *salmeterol xinafoate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities E and G; use the chromatogram supplied with *salmeterol xinafoate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to salmeterol (retention time = about 13 min): xinafoic acid = about 0.2; impurity D = about 0.8; impurity E = about 0.9; impurity G = about 2.7.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to salmeterol.

*Calculation of percentage contents*:

- for each impurity, use the concentration of salmeterol xinafoate in reference solution (b).

*Limits*:

- *impurities D, G*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to xinafoic acid.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A.

*Injection*: 20 µL of test solution (b) and reference solutions (d) and (e).

*Run time*: until complete elution of the peak due to salmeterol (about 16 min).

*System suitability*: reference solution (e):

- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to salmeterol.

Calculate the percentage content of $C_{36}H_{45}NO_7$ using the chromatogram obtained with reference solution (d) and taking into account the assigned content of *salmeterol xinafoate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: D, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F.*

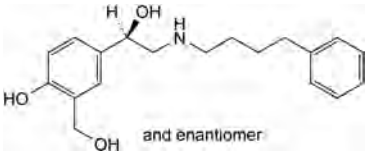


A. (1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[(4-phenylbutyl)amino]ethanol,

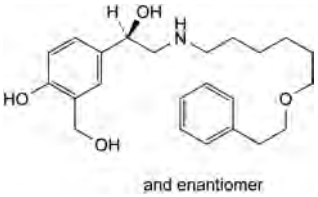


B. (1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(2-phenylethoxy)hexyl]amino]ethanol,

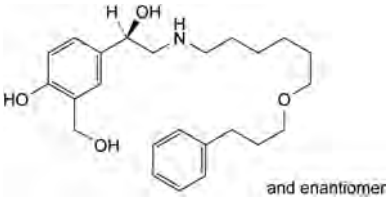


C. (1*RS*)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(3-phenylpropoxy)hexyl]amino]ethanol,

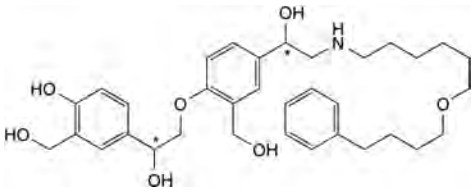

D. 1-[4-[2-hydroxy-2-[4-hydroxy-3-(hydroxymethyl)-phenyl]ethoxy]-3-(hydroxymethyl)phenyl]-2-[[6-(4-phenylbutoxy)hexyl]amino]ethanol,

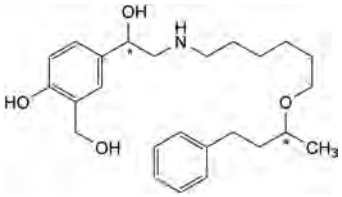

E. 1-[4-hydroxy-3-(hydroxymethyl)phenyl]-2-[[6-(1-methyl-3-phenylpropoxy)hexyl]amino]ethanol,

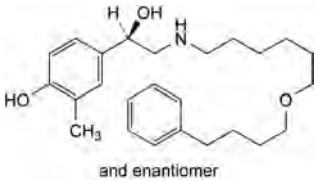


F. (1*RS*)-1-(4-hydroxy-3-methylphenyl)-2-[[6-(4-phenylbutoxy)hexyl]amino]ethanol,

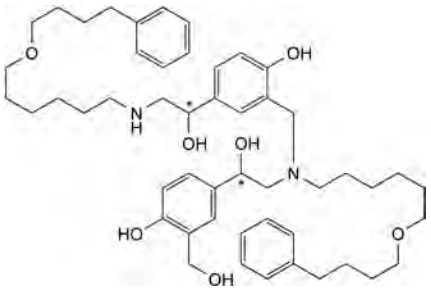

G. 1-[4-hydroxy-3-[[[2-hydroxy-2-[4-hydroxy-3-(hydroxymethyl)phenyl]ethyl][6-(4-phenylbutoxy)hexyl]amino]-methyl]phenyl]-2-[[6-(4-phenylbutoxy)hexyl]amino]-ethanol.

**07/2012:1910**

# SALMON OIL, FARMED

## Salmonis domestici oleum

DEFINITION

Purified fatty oil obtained from fresh farmed *Salmo salar*. The positional distribution (β(2)-acyl) is 60-70 per cent for cervonic (docosahexaenoic) acid (C22:6 n-3; DHA), 25-35 per cent for timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA) and 40-55 per cent for moroctic acid (C18:4 n-3).

*Content*:

– *sum of the contents of EPA and DHA (expressed as triglycerides)*: 10.0 per cent to 28.0 per cent.

A suitable antioxidant may be added.

PRODUCTION

The fish shall only be given feed with a composition that is in accordance with the relevant European Union or other applicable regulations.

The content of dioxins and dioxin-like PCBs (polychlorinated biphenyls) is controlled using methods and limits in accordance with the requirements set in the European Union or other applicable regulations.

The oil is produced by mechanical expression of fresh raw materials, either from the whole fish, or fish where the fillets have been removed, at a temperature not exceeding 100 °C, and without using solvents. After centrifugation, solid substances may be removed from the oil by cooling and filtering (winterisation).

CHARACTERS

*Appearance*: pale pink liquid.

*Solubility*: practically insoluble in water, very soluble in acetone and in heptane, slightly soluble in anhydrous ethanol.

IDENTIFICATION

Examine the $^{13}C$ NMR spectra obtained in the assay for positional distribution (β(2)-acyl) of fatty acids. The spectra contain peaks between 172 ppm and 173 ppm with shifts similar to those in the type spectrum (Figure 1910.-2). The oil to be examined complies with the limits of this assay.

TESTS

**Absorbance** (*2.2.25*): minimum 0.10, measured at the absorption maximum between 470 nm and 480 nm.

Dissolve 5.0 mL in 5.0 mL of *trimethylpentane R*.

**Acid value** (*2.5.1*): maximum 2.0.

**Anisidine value** (*2.5.36*): maximum 10.0.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Linoleic acid** (*2.4.29*): maximum 11.0 per cent.

Identify the peak due to linoleic acid using the chromatogram in Figure 1910.-1. Determine the percentage content by normalisation.

ASSAY

**Positional distribution (β(2)-acyl) of fatty acids**. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Apparatus*: high resolution FT-NMR spectrometer operating at minimum 300 MHz.

*Test solution*. Dissolve 190-210 mg of fresh salmon oil in 500 µL of *deuterated chloroform R*. Prepare at least 3 samples and examine within 3 days.

*Acquisition of $^{13}C$ NMR spectra.* The following parameters may be used:

– *sweep width*: 200 ppm (– 5 to 195 ppm);
– *irradiation frequency offset*: 95 ppm;
– *time domain*: 64 K;
– *pulse delay*: 2 s;
– *pulse program*: zgig 30 (inverse gated, 30° excitation pulse);
– *dummy scans*: 4;
– *number of scans*: 4096.

*Processing and plotting.* The following parameters may be used:

– *size*: 64 K (zero-filling);
– *window multiplication*: exponential;
– *Lorentzian broadening factor*: 0.2 Hz.

Use the $CDCl_3$ signal for shift referencing. The shift of the central peak of the 1:1:1 triplet is set to 77.16 ppm.

Plot the spectral region δ 171.5-173.5 ppm. Compare the spectrum with the reference spectrum in Figure 1910.-2. The shift values lie within the ranges given in Table 1910.-1.

Table 1910.-1. – *Shift values*

| Signal | Shift range (ppm) |
|---|---|
| β DHA | 172.05 - 172.09 |
| α DHA | 172.43 - 172.47 |
| β EPA | 172.52 - 172.56 |
| α EPA | 172.90 - 172.94 |
| β C18:4 | 172.56 - 172.60 |
| α C18:4 | 172.95 - 172.99 |

*System suitability*:

– *signal-to-noise ratio*: minimum 5 for the smallest relevant peak corresponding to α C18:4 signal (in the range δ 172.95-172.99 ppm);

– *peak width at half-height* maximum 0.02 ppm for the central $CDCl_3$ signal (at δ 77.16 ppm).

*Calculation of positional distribution (β(2)-acyl)*: use the following expression:

$$\frac{\beta}{\alpha + \beta} \times 100$$

$\alpha$ = peak area of the corresponding α-carbonyl peak;

$\beta$ = peak area of β-carbonyl peak from C22:6 n-3, C20:5 n-3 or C18:4 n-3, respectively.

*Limits*:

– *cervonic (docosahexaenoic) acid (C22:6 n-3; DHA)*: 60 per cent to 70 per cent.

– *timnodonic (eicosapentaenoic) acid (C20:5 n-3; EPA)*: 25 per cent to 35 per cent;

– *moroctic acid (C18:4 n-3)*: 40 per cent to 55 per cent.

**EPA and DHA** (*2.4.29*). See Figure 1910.-1.

STORAGE

In an airtight, well-filled container, protected from light, under inert gas.

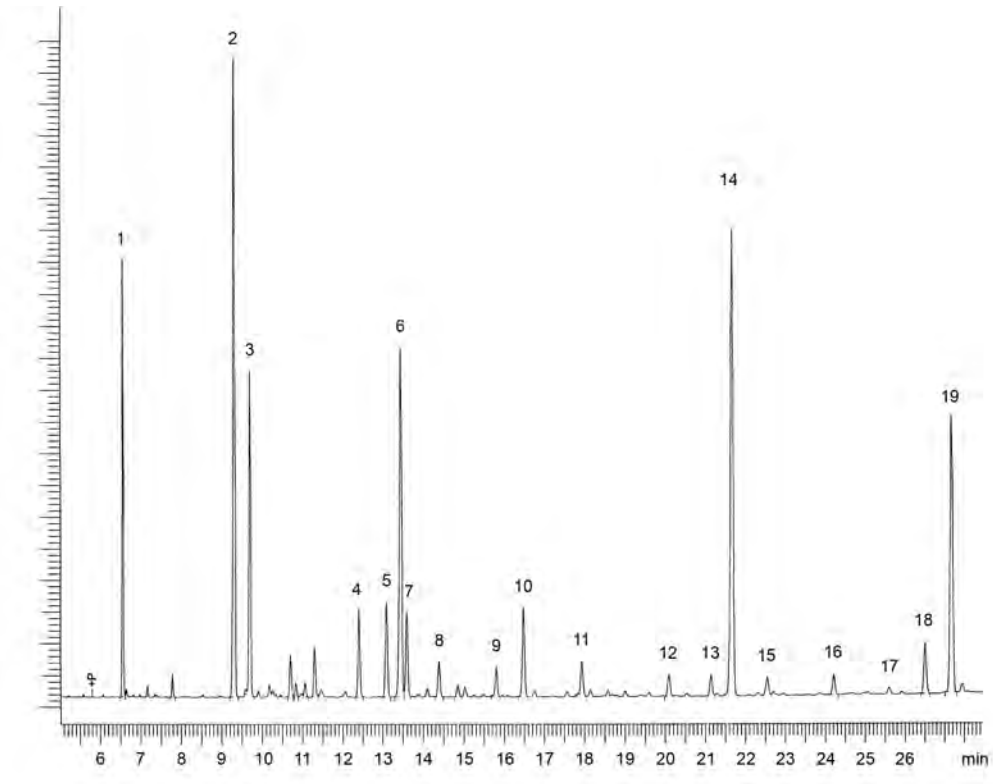


| | | | | |
|---|---|---|---|---|
| 1. C14:0 | 5. C18:0 | 9. C18:3 n-3 | 13. C20:4 n-3 | 17. C22:5 n-6 |
| 2. C16:0 | 6. C18:1 n-9 | 10. C18:4 n-3 | 14. EPA | 18. C22:5 n-3 |
| 3. C16:1 n-7 | 7. C18:1 n-7 | 11. C20:1 n-9 | 15. C22:1 n-11 | 19. DHA |
| 4. C16:4 n-1 | 8. C18:2 n-6 | 12. C20:4 n-6 | 16. C21:5 n-3 | |

Figure 1910.-1. – *Chromatogram for the composition of fatty acids in farmed salmon oil*

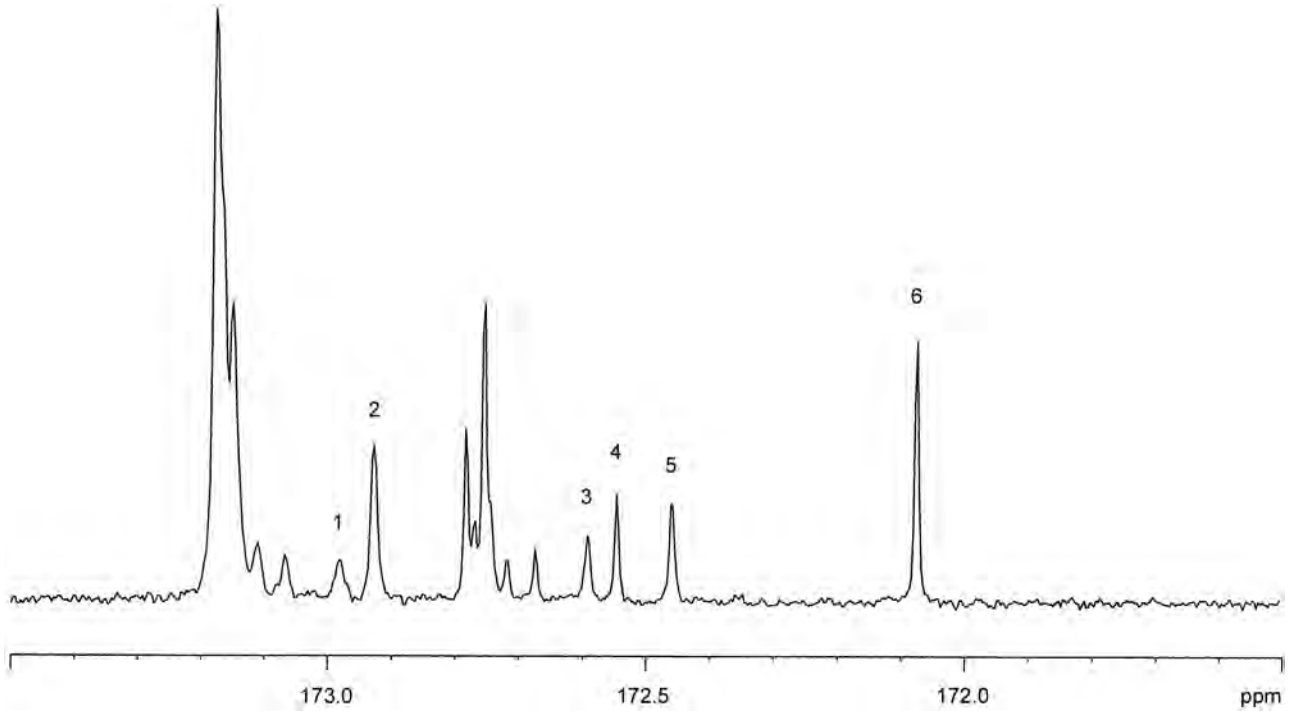


1. α C18:4 2. α EPA 3. β C18:4 4. β EPA 5. α DHA 6. β DHA

Figure 1910.-2. – *$^{13}$C NMR spectrum: carbonyl region of farmed salmon oil*

**01/2017:2267**

# SAQUINAVIR MESILATE

## Saquinaviri mesilas

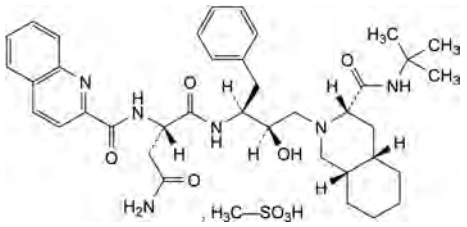

$C_{39}H_{54}N_6O_8S$ $M_r$ 767
[149845-06-7]

DEFINITION

(2*S*)-*N*$^1$-[(1*S*,2*R*)-1-Benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]-2-[(quinolin-2-ylcarbonyl)amino]-butanediamide methanesulfonate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in saquinavir mesilate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *saquinavir mesilate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): – 42.0 to – 35.0 (anhydrous substance).

Dissolve 0.25 g in *anhydrous methanol R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *acetonitrile R1* (47:53 *V/V*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the solvent mixture, using sonication, and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve the contents of a vial of *saquinavir for system suitability CRS* (containing impurities A, B, C and D) in 1.0 mL of the solvent mixture and sonicate for 2 min.

*Reference solution (c)*. Dissolve 30.0 mg of *saquinavir mesilate CRS* in the solvent mixture, using sonication, and dilute to 100.0 mL with the same solvent.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

– *mobile phase A*: to 2.5 mL of *strong sodium hydroxide solution R* add 900 mL of *water for chromatography R*, adjust to pH 1.8 with *perchloric acid R* and dilute to 1000 mL with *water for chromatography R*;

– *mobile phase B*: mobile phase A, *acetonitrile R1* (38:62 *V/V*);

| Time (min) | Mobile phase A (per cent) | Mobile phase B (per cent) |
|---|---|---|
| 0 - 1 | 50 | 50 |
| 1 - 31 | 50 → 0 | 50 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *saquinavir for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to saquinavir (retention time = about 17 min): impurity A = about 0.2; impurity B = about 0.3; impurity C = about 0.5; impurity D = about 0.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to saquinavir.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.5; impurity B = 0.5; impurity C = 2.5;

– *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.03 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (c).

Calculate the percentage content of saquinavir mesilate from the assigned content of *saquinavir mesilate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G, H.*

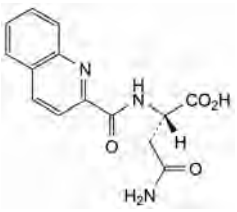

A. (2*S*)-4-amino-4-oxo-2-[(quinolin-2-ylcarbonyl)amino]-butanoic acid,

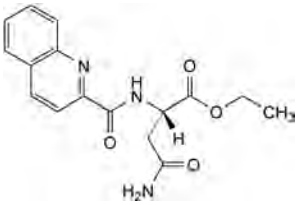

B. ethyl (2*S*)-4-amino-4-oxo-2-[(quinolin-2-ylcarbonyl)-amino]butanoate,

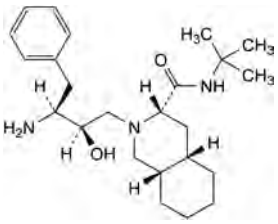

C. (3*S*,4a*S*,8a*S*)-2-[(2*R*,3*S*)-3-amino-2-hydroxy-4-phenylbutyl]-*N*-(1,1-dimethylethyl)decahydroisoquinoline-3-carboxamide,

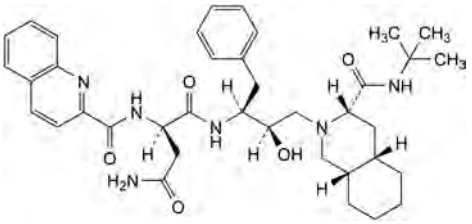

D. (2*R*)-*N*$^1$-[(1*S*,2*R*)-1-benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]-2-[(quinolin-2-ylcarbonyl)amino]-butanediamide (2-*epi*-saquinavir),

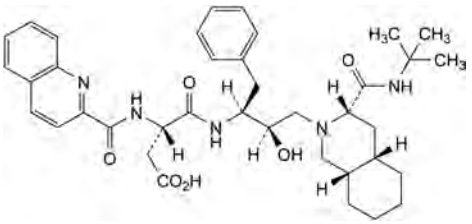

E. (3*S*)-4-[[(1*S*,2*R*)-1-benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]amino]-4-oxo-3-[(quinolin-2-ylcarbonyl)amino]butanoic acid,

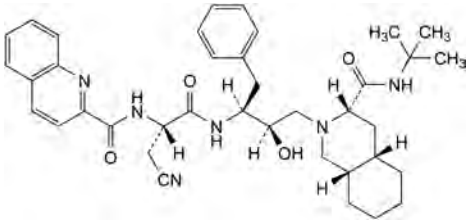

F. *N*-[(1*S*)-2-[[(1*S*,2*R*)-1-benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]amino]-1-(cyanomethyl)-2-oxoethyl]quinoline-2-carboxamide,

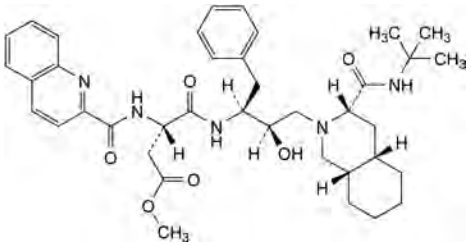

G. methyl (3*S*)-4-[[(1*S*,2*R*)-1-benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]amino]-4-oxo-3-[(quinolin-2-ylcarbonyl)amino]butanoate,

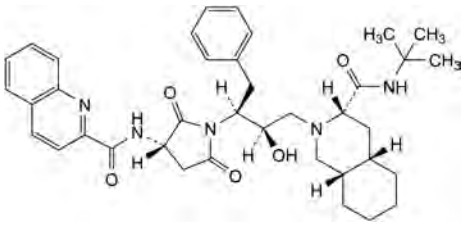

H. *N*-[(3*S*)-1-[(1*S*,2*R*)-1-benzyl-3-[(3*S*,4a*S*,8a*S*)-3-[(1,1-dimethylethyl)carbamoyl]octahydroisoquinolin-2(1*H*)-yl]-2-hydroxypropyl]-2,5-dioxopyrrolidin-3-yl]quinoline-2-carboxamide.

**07/2015:2268**
**corrected 9.0**

# SELAMECTIN FOR VETERINARY USE

## Selamectinum ad usum veterinarium

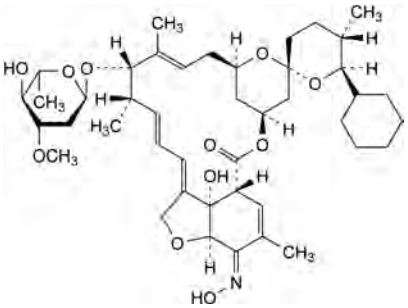

$C_{43}H_{63}NO_{11}$ $M_r$ 770
[220119-17-5]

DEFINITION

(2a*E*,2′*R*,4*E*,5′*S*,6*S*,6′*S*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*,20b*S*)-6′-Cyclohexyl-7-[(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)oxy]-20b-hydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((5*Z*,21*R*,25*S*)-25-cyclohexyl-4′-*O*-de(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-5-demethoxy-25-de(1-methylpropyl)-22,23-dihydro-5-(hydroxyimino)-avermectin $A_{1a}$).

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in isopropyl alcohol, soluble in acetone and in methylene chloride, sparingly soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *selamectin CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 2.5 mg of *selamectin for peak identification CRS* (containing impurities A, C and D) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *selamectin impurity B CRS* in 1.0 mL of reference solution (b).

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 28 | 40 | 60 |
| 28 - 45 | 40 → 20 | 60 → 80 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 243 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *selamectin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to selamectin (retention time = about 22 min): impurity A = about 0.2; impurity B = about 0.4; impurity C = about 0.5; impurity D = about 1.7.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.5 between the peaks due to impurities B and C.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity D by 1.5;
- *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *impurities C, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);

- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Heavy metals** (*2.4.8*): maximum 20 ppm.

Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 20.0 mL with the same solvent. 12 mL of the solution complies with test B. Prepare the reference solution using lead standard solution (2 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with *ethanol (96 per cent) R*. Filter the solution through a membrane filter (nominal pore size 0.45 µm). Compare the spots on the filters obtained with the different solutions. Any brownish-black colour in the spot from the test solution is not more intense than that in the spot from the reference solution.

**Water** (*2.5.12, Method A*): maximum 7.0 per cent, determined on 0.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Reference solution.* Dissolve 50.0 mg of *selamectin CRS* in the mobile phase and dilute to 250.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 30 °C.

*Mobile phase*: *water R*, *acetonitrile R* (20:80 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 243 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of selamectin.

*Retention time*: selamectin = about 9 min.

Calculate the percentage content of $C_{43}H_{63}NO_{11}$ taking into account the assigned content of *selamectin CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D.*

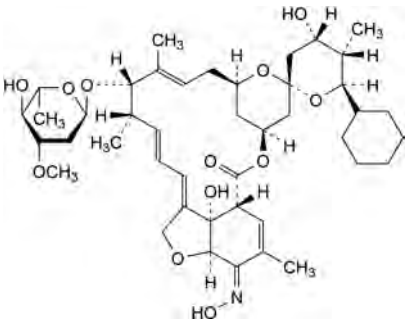

A. (2a*E*,2′*R*,4*E*,4′*S*,5′*S*,6*S*,6′*R*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*Z*,-20a*R*,20b*S*)-6′-cyclohexyl-7-[(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)oxy]-4′,20b-dihydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((5*Z*,21*R*,23*S*,25*R*)-25-cyclohexyl-4′-*O*-de(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-5-demethoxy-25-de(1-methylpropyl)-22,23-dihydro-23-hydroxy-5-(hydroxyimino)avermectin $A_{1a}$),

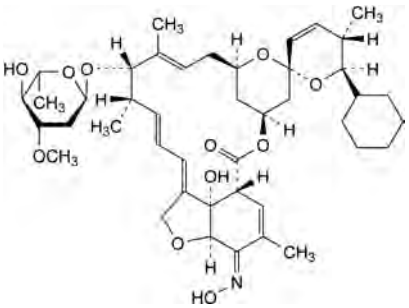

B. (2a*E*,2′*S*,4*E*,5′*S*,6*S*,6′*R*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*,20b*S*)-6′-cyclohexyl-7-[(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)oxy]-20b-hydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-5′,6,6′,7,10,11,14,15,17a,20,20a,20b-dodecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((5*Z*,25*R*)-25-cyclohexyl-4′-*O*-de(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-5-demethoxy-25-de(1-methylpropyl)-5-(hydroxyimino)avermectin $A_{1a}$),

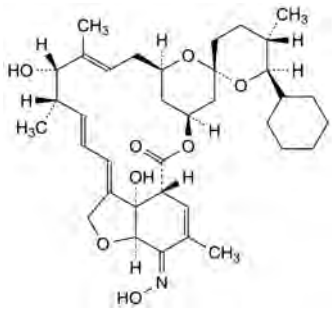

C. (2a*E*,2′*R*,4*E*,5′*S*,6*S*,6′*S*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*,20b*S*)-6′-cyclohexyl-7,20b-dihydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-3′,4′,5′,6,6′,7,10,11,14,15,-17a,20,20a,20b-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((5*Z*,21*R*,25*S*)-25-cyclohexyl-13-*O*-de[2,6-dideoxy-4-*O*-(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)-3-*O*-methyl-α-L-*arabino*-hexopyranosyl]-5-demethoxy-25-de(1-methylpropyl)-22,23-dihydro-5-(hydroxyimino)avermectin $A_{1a}$),

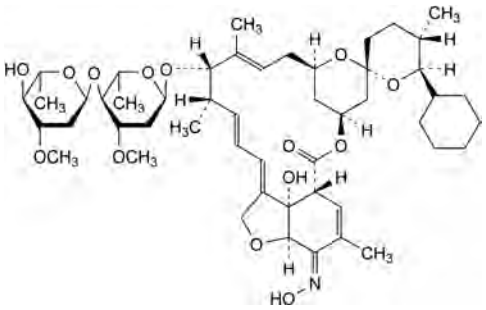

D. (2a*E*,2′*R*,4*E*,5′*S*,6*S*,6′*S*,7*S*,8*E*,11*R*,15*S*,17a*R*,20*Z*,20a*R*,20b*S*)-6′-cyclohexyl-7-[(2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl-(1→4)-2,6-dideoxy-3-*O*-methyl-α-L-*arabino*-hexopyranosyl)oxy]-20b-hydroxy-20-(hydroxyimino)-5′,6,8,19-tetramethyl-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradecahydrospiro[2*H*,17*H*-11,15-methanofuro[4,3,2-*pq*][2,6]benzodioxacyclooctadecine-13,2′-pyran]-17-one ((5*Z*,21*R*,25*S*)-25-cyclohexyl-5-demethoxy-25-de(1-methylpropyl)-22,23-dihydro-5-(hydroxyimino)avermectin $A_{1a}$).

**01/2017:1260**

# SELEGILINE HYDROCHLORIDE

## Selegilini hydrochloridum

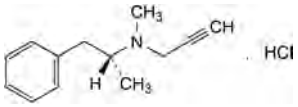

$C_{13}H_{18}ClN$ $M_r$ 223.7
[14611-52-0]

DEFINITION

*N*-Methyl-*N*-[(1*R*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, slightly soluble in acetone and in ethyl acetate.

mp: about 143 °C.

IDENTIFICATION

Carry out either tests A, B, D or tests B, C, D.

A. Specific optical rotation (*2.2.7*): − 12.0 to − 10.0 (dried substance).
Dissolve 2.00 g in *carbon dioxide-free water R* and dilute to 20.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *selegiline hydrochloride CRS*.

C. Enantiomeric purity (see Tests).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 3.5 to 4.5.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in a mixture of 10 µL of *butylamine R* and 1 mL of *2-propanol R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 8.0 mg of *(RS)-selegiline hydrochloride CRS* in a mixture of 10 µL of *butylamine R* and 1 mL of *2-propanol R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 0.5 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R*.

*Mobile phase*: *2-propanol R*, *cyclohexane R* (0.2:99.8 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Relative retention* with reference to (*R*)-selegiline (retention time = about 6 min): impurity E = about 0.9.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity E and (*R*)-selegiline; if necessary, adjust the concentration of 2-propanol in the mobile phase.

*Limit*:
- *impurity E*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Butylammonium acetate buffer solution.* Dilute 4 mL of *butylamine R* in 900 mL of *water R*, adjust to pH 6.5 with *acetic acid R* and dilute to 1000.0 mL with *water R*.

*Test solution.* Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50 mg of the substance to be examined and 10 mg of *butyl parahydroxybenzoate R* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, butylammonium acetate buffer solution (50:50 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 1.7 times the retention time of selegiline.

*Relative retention* with reference to selegiline (retention time = about 14 min): butyl parahydroxybenzoate = about 0.8.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.0 between the peaks due to butyl parahydroxybenzoate and selegiline.

*Limits*:
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.5 kPa for 3 h.

Monographs S

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.37 mg of $C_{13}H_{18}ClN$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, G.*

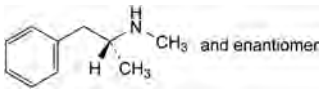


A. (2*RS*)-*N*-methyl-1-phenylpropan-2-amine ((*RS*)-metamfetamine),

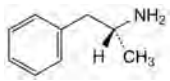


B. (2*R*)-1-phenylpropan-2-amine (amfetamine),

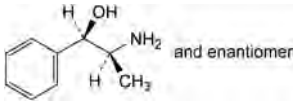


C. (1*RS*,2*SR*)-2-amino-1-phenylpropan-1-ol (phenylpropanolamine),

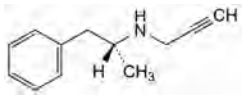


D. *N*-[(1*R*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine (desmethylselegiline),

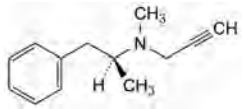


E. *N*-methyl-*N*-[(1*S*)-1-methyl-2-phenylethyl]prop-2-yn-1-amine,

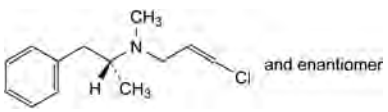


G. (2*EZ*)-3-chloro-*N*-methyl-*N*-[(1*RS*)-1-methyl-2-phenylethyl]prop-2-en-1-amine.

**01/2008:1147**

# SELENIUM DISULFIDE

## Selenii disulfidum

$SeS_2$ $M_r$ 143.1
[7488-56-4]

DEFINITION

*Content*: 52.0 per cent to 55.5 per cent of Se.

CHARACTERS

*Appearance*: bright orange or reddish-brown powder.

*Solubility*: practically insoluble in water.

IDENTIFICATION

A. Gently boil about 50 mg with 5 mL of *nitric acid R* for 30 min. Dilute to 50 mL with *water R* and filter. To 5 mL of the filtrate add 10 mL of *water R* and 5 g of *urea R*. Heat to boiling, cool and add 1.5 mL of *potassium iodide solution R*. A yellow or orange colour is produced which darkens rapidly on standing. This solution is used in identification test B.

B. Allow the coloured solution obtained under identification A to stand for 10 min and filter through *kieselguhr for chromatography R*. 5 mL of the filtrate gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Soluble selenium compounds**: maximum 5 ppm, calculated as Se.

To 10 g add 100 mL of *water R*, mix well, allow to stand for 1 h with frequent shaking and filter. To 10 mL of the filtrate add 2 mL of a 115 g/L solution of *anhydrous formic acid R*, dilute to 50 mL with *water R* and adjust to pH 2.0-3.0 with an 115 g/L solution of *anhydrous formic acid R*. Add 2 mL of a 5 g/L solution of *3,3′-diaminobenzidine tetrahydrochloride R*. Allow to stand for 45 min and then adjust to pH 6.0-7.0 with *dilute ammonia R1*. Shake the solution for 1 min with 10 mL of *toluene R* and allow the phases to separate. The absorbance (*2.2.25*) of the upper layer measured at 420 nm is not greater than that of a standard prepared at the same time and in the same manner beginning at the words "add 2 mL of an 115 g/L solution of *anhydrous formic acid R*" and using 5 mL of *selenium standard solution (1 ppm Se) R* instead of 10 mL of the filtrate.

ASSAY

To 0.100 g add 25 mL of *fuming nitric acid R* and heat on a water-bath for 1 h; a small insoluble residue may remain. Cool and dilute to 100.0 mL with *water R*. To 25.0 mL of this solution add 50 mL of *water R* and 5 g of *urea R* and heat to boiling. Cool, add 7 mL of *potassium iodide solution R* and 3 mL of *starch solution R*. Titrate immediately with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 1.974 mg of Se.

**01/2017:0788**

# SERINE

## Serinum

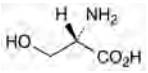

$C_3H_7NO_3$ $M_r$ 105.1
[56-45-1]

DEFINITION

(2*S*)-2-Amino-3-hydroxypropanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *serine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a 1 per cent *V/V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Reference solution.* Dissolve 10 mg of *serine CRS* in a 1 per cent *V/V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To 1 mL of a 10 g/L solution of the substance to be examined in a test tube, add 5 mL of a 20 g/L solution of *sodium periodate R*. Heat on a water-bath and collect the vapour on glass wool moistened with *water R* and inserted in the opening of the test tube. After heating for 5 min, transfer the glass wool to a test tube containing 1 mL of a 15 g/L solution of *chromotropic acid, sodium salt R* and 3 mL of *sulfuric acid R*. Heat on a water-bath for 10 min. A violet-red colour is produced.

TESTS

**Solution S**. Dissolve 2.5 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 14.0 to + 16.0 (dried substance).

Dissolve 2.50 g in *dilute hydrochloric acid R* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):
– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:
– for any ninhydrin-positive substance detected at 570 nm, use the concentration of serine in reference solution (a);
– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:
– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:
– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 10.51 mg of $C_3H_7NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

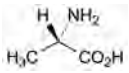

A. (2*S*)-2-aminopropanoic acid (alanine),

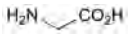

B. 2-aminoacetic acid (glycine),

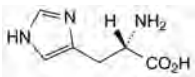

C. (2*S*)-2-amino-3-(imidazol-4-yl)propanoic acid (histidine).

**01/2008:1148**
**corrected 6.1**

# SERTACONAZOLE NITRATE

## Sertaconazoli nitras

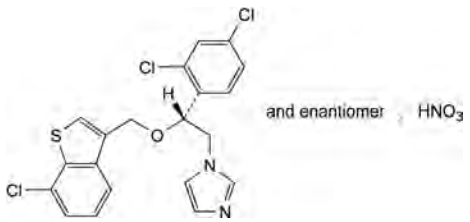


$C_{20}H_{16}Cl_3N_3O_4S$ $M_r$ 500.8
[99592-39-9]

DEFINITION

(*RS*)-1-[2-[(7-Chloro-1-benzothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole nitrate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in methanol, sparingly soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 156 °C to 161 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.1 g in *methanol R* and dilute to 100 mL with the same solvent. Dilute 10 mL of this solution to 100 mL with *methanol R*.

*Spectral range*: 240-320 nm.

*Absorption maxima*: at 260 nm, 293 nm and 302 nm.

*Absorbance ratio*: $A_{302}/A_{293}$ = 1.16 to 1.28.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substances at 100-105 °C for 2 h and examine as discs of *potassium bromide R*.

*Comparison*: *sertaconazole nitrate CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *concentrated ammonia R*, *methanol R* (10:90 *V/V*).

*Test solution.* Dissolve 40 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 40 mg of *sertaconazole nitrate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 20 mg of *miconazole nitrate CRS* in reference solution (a) and dilute to 5 mL with reference solution (a).

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *concentrated ammonia R*, *toluene R*, *dioxan R* (1:40:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air for 15 min.

*Detection*: expose to iodine vapour for 30 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

E. About 1 mg gives the reaction of nitrates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *sertaconazole nitrate CRS* and 5.0 mg of *miconazole nitrate CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *nitrile silica gel for chromatography R1* (10 µm).

*Mobile phase*: *acetonitrile R1*, 1.5 g/L solution of *sodium dihydrogen phosphate R* (37:63 *V/V*).

*Flow rate*: 1.6 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 1.3 times the retention time of sertaconazole.

*Retention time*: nitrate ion = about 1 min; miconazole = about 17 min; sertaconazole = about 19 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to miconazole and sertaconazole.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.25 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard the peak due to the nitrate ion.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of a mixture of equal volumes of *anhydrous acetic acid R* and *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 50.08 mg of $C_{20}H_{16}Cl_3N_3O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

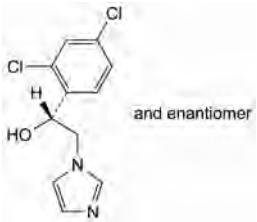


A. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol,

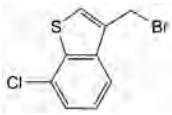


B. 3-(bromomethyl)-7-chloro-1-benzothiophen,

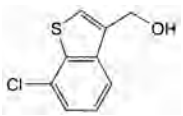


C. (7-chloro-1-benzothiophen-3-yl)methanol.

**04/2019:1705**
**corrected 10.0**

# SERTRALINE HYDROCHLORIDE

## Sertralini hydrochloridum

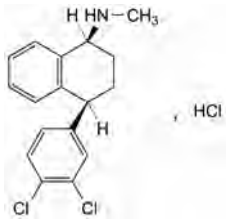


$C_{17}H_{18}Cl_3N$ $M_r$ 342.7
[79559-97-0]

DEFINITION

(1*S*,4*S*)-4-(3,4-Dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine hydrochloride.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble or slightly soluble in anhydrous ethanol, slightly soluble in acetone and in 2-propanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B, C or tests B, C, D.

A. Specific optical rotation (*2.2.7*): + 38.8 to + 43.0 (anhydrous substance), measured at 25 °C.

*Solvent mixture.* Dilute 1 volume of a 103 g/L solution of *hydrochloric acid R* to 20 volumes with *methanol R*.

Dissolve 0.250 g in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sertraline hydrochloride CRS*.

If the spectra obtained in the solid state show differences, record new spectra using 10 g/L solutions in *methylene chloride R*.

C. Dissolve 10 mg in 5 mL of *anhydrous ethanol R* and add 5 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

D. Enantiomeric purity (see Tests).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Prepare the test solution immediately before use.*

*Solvent mixture*: *diethylamine R*, *hexane R*, *2-propanol R* (1:40:60 *V/V/V*).

*Test solution.* Dissolve 60.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *sertraline for system suitability CRS* (containing impurity G) in 1 mL of the solvent mixture.

*Reference solution (b).* Dilute 0.5 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm).

Monographs S

*Mobile phase*: mix 30 volumes of *hexane R* and 70 volumes of a mixture of 1 volume of *diethylamine R*, 25 volumes of *2-propanol R* and 975 volumes of *hexane R*.

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of sertraline.

*Identification of impurities*: use the chromatogram supplied with *sertraline for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity G.

*Relative retention* with reference to sertraline (retention time = about 15 min): impurity G = about 1.3.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to sertraline and impurity G in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peak due to sertraline in the chromatogram obtained with reference solution (b).

*Limit*:

- *impurity G*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent).

**Impurity E**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase A, mobile phase B (50:50 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5.0 mg of *sertraline impurity E CRS* (mandelic acid) in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10 mg of *benzoic acid R* and 20 mg of *mandelic acid R* (impurity E) in the solvent mixture and dilute to 50 mL with the solvent mixture. Dilute 1 mL of the solution to 50 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (3 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 1.0 g of *sodium laurilsulfate R* in 800 mL of *water for chromatography R* and add 200 mL of *acetonitrile for chromatography R*; add 1.0 mL of *phosphoric acid R* and mix;
- *mobile phase B*: dissolve 1.0 g of *sodium laurilsulfate R* in 100 mL of *water for chromatography R* and add 900 mL of *acetonitrile for chromatography R*; add 1.0 mL of *phosphoric acid R* and mix;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 8 | 60 | 40 |
| 8 - 9 | 60 → 10 | 40 → 90 |
| 9 - 16 | 10 | 90 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (b) to identify the peak due to benzoic acid.

*Relative retention* with reference to sertraline (retention time = about 18 min): impurity E = about 0.2; benzoic acid = about 0.3.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity E and benzoic acid.

*Limit*:

- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Introduce 0.250 g of the substance to be examined into a 15 mL stoppered centrifuge tube, add 2.0 mL of *methanol R* and 0.20 mL of a 25 per cent solution of *potassium carbonate R* and mix in a vortex mixer for 30 s. Add 8.0 mL of *methylene chloride R*, stopper the tube and mix in a vortex mixer for 60 s. Add 1 g of *anhydrous sodium sulfate R*, mix well and then centrifuge for about 5 min.

*Reference solution (a)*. Dissolve the contents of a vial of *sertraline for peak identification CRS* (containing impurities A, B, C and F) in 0.2 mL of *methylene chloride R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 20.0 mL with *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *phenyl(50)methyl(50)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 9 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 200 |
| | 1 - 31 | 200 → 260 |
| | 31 - 39 | 260 |
| Injection port | | 250 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of impurities*: use the chromatogram supplied with *sertraline for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and F.

*Relative retention* with reference to sertraline (retention time = about 24 min): impurity B = about 0.5; impurities C and D = about 0.7; impurity A = about 1.05; impurity F = about 1.1.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 15, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to sertraline.

*Limits*:

- *sum of impurities C and D*: maximum 0.8 per cent;
- *impurities A, B, F*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.05 per cent (reference solution (b)).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Buffer solution.* To 28.6 mL of *glacial acetic acid R* slowly add, while stirring and cooling, 34.8 mL of *triethylamine R*, and dilute to 100 mL with *water for chromatography R*. Dilute 10 mL of this solution to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 55.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution.* Dissolve 55.0 mg of *sertraline hydrochloride CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);

– *temperature*: 30 °C.

*Mobile phase*: *methanol R*, buffer solution, *acetonitrile R* (15:40:45 *V/V/V*).

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of sertraline.

*Retention time*: sertraline = about 1.9 min.

Calculate the percentage content of $C_{17}H_{18}Cl_3N$ taking into account the assigned content of *sertraline hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

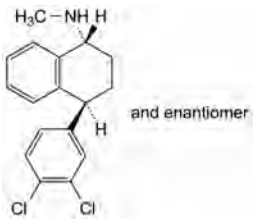


A. (1*RS*,4*SR*)-4-(3,4-dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

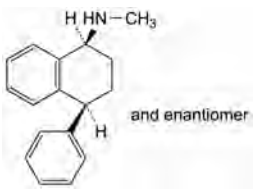


B. (1*RS*,4*RS*)-*N*-methyl-4-phenyl-1,2,3,4-tetrahydronaphthalen-1-amine,

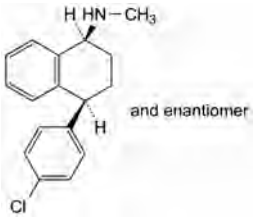


C. (1*RS*,4*RS*)-4-(4-chlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

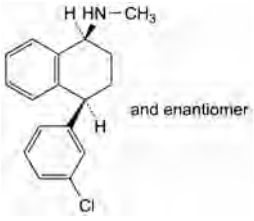


D. (1*RS*,4*RS*)-4-(3-chlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine,

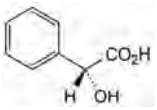


E. (2*R*)-hydroxyphenylacetic acid ((*R*)-mandelic acid),

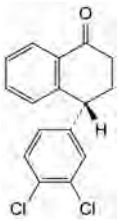


F. (4*R*)-4-(3,4-dichlorophenyl)-3,4-dihydronaphthalen-1(2*H*)-one,

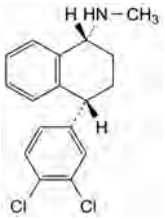


G. (1*R*,4*R*)-4-(3,4-dichlorophenyl)-*N*-methyl-1,2,3,4-tetrahydronaphthalen-1-amine (sertraline enantiomer).

**04/2017:0433**

# SESAME OIL, REFINED

## Sesami oleum raffinatum

DEFINITION

Fatty oil obtained from the ripe seeds of *Sesamum indicum* L. by expression or extraction. It is then refined. Improved colour and odour may be obtained by further refining. It may contain a suitable antioxidant.

CHARACTERS

*Appearance*: clear, light yellow liquid, almost colourless.

*Solubility*: practically insoluble in ethanol (96 per cent), miscible with light petroleum.

*Relative density*: about 0.919.

*Refractive index*: about 1.473.

It solidifies to a butter-like mass at about – 4 °C.

## IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B*.

A. Composition of triglycerides (see Tests).

B. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2-1.

## TESTS

**Acid value** (*2.5.1*): maximum 0.5, determined on 10.0 g; maximum 0.3 if intended for use in the manufacture of parenteral preparations.

**Peroxide value** (*2.5.5*): maximum 10.0; maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 2.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test for alkaline impurities in fatty oils.

**Cottonseed oil**. Mix 5 mL in a test-tube with 5 mL of a mixture of equal volumes of *pentanol R* and a 10 g/L solution of *sulfur R* in *carbon disulfide R*. Warm the mixture carefully until the carbon disulfide is expelled, and immerse the tube to 1/3 of its depth in boiling *saturated sodium chloride solution R*. No reddish colour develops within 15 min.

**Composition of triglycerides**. Liquid chromatography (*2.2.29*).

*Test solution*. Dilute 50.0 mg of the substance to be examined to 10.0 mL with a mixture of equal volumes of *acetone R* and *methylene chloride R*.

*Reference solutions*. Dissolve 80.0 mg of *triolein R* in a mixture of equal volumes of *acetone R* and *methylene chloride R* and dilute to 50.0 mL with the same mixture of solvents. Prepare 5 reference solutions by dilution of this solution so as to cover concentrations ranging from the disregard limit (0.5 per cent) to the upper limit for OLL (30.0 per cent).

Plot the logarithm of the area of the peak due to triolein against the logarithm of the concentration of triolein in the reference solution.

*Column*: 2 columns coupled in series:

- *size of each column*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: *acetone R*, *methylene chloride R*, *acetonitrile R* (5:15:80 *V/V/V*);
- *mobile phase B*: *acetone R*, *acetonitrile R*, *methylene chloride R* (20:20:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 → 75 | 0 → 25 |
| 15 - 25 | 75 | 25 |
| 25 - 70 | 75 → 0 | 25 → 100 |
| 70 - 75 | 0 → 100 | 100 → 0 |
| 75 - 80 | 100 | 0 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 0.7 L/min;
- *evaporator temperature*: 85 °C;
- *nebuliser temperature*: 45 °C.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatograms obtained with the reference solutions to identify the peak due to triolein; identify the other peaks using the chromatogram shown in Figure 0433.-1. The fatty acids are designated as linolenic (Ln), linoleic (L), oleic (O), palmitic (P) and stearic (S).

*System suitability*: test solution:

- *resolution*: minimum 1.5 between the peaks due to OOO (triolein) and SOL.

Using the calibration curve obtained with the reference solutions, determine the percentage content of each peak with an area greater than that of the peak corresponding to the disregard limit (0.5 per cent). Assuming that the sum of these percentage contents is 100 per cent, normalise the percentage content of each of the 8 triglycerides specified below.

*Composition of triglycerides*:

- *LLL*: 7.0 per cent to 19.0 per cent;
- *OLL*: 13.0 per cent to 30.0 per cent;
- *PLL*: 5.0 per cent to 9.0 per cent;
- *OOL*: 12.0 per cent to 23.0 per cent;
- *POL*: 6.0 per cent to 14.0 per cent;
- *OOO*: 5.0 per cent to 16.0 per cent;
- *SOL*: 2.0 per cent to 8.0 per cent;
- *POO*: 2.0 per cent to 10.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

## STORAGE

In an airtight, well-filled container, protected from light; if intended for use in the manufacture of parenteral preparations store under an inert gas in an airtight container.

When the container has been opened, its contents are to be used as soon as possible. Any part of the contents not used at once is protected by an atmosphere of an inert gas.

## LABELLING

The label states:

- whether the oil is obtained by expression or extraction;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- where applicable, the name of the inert gas used.

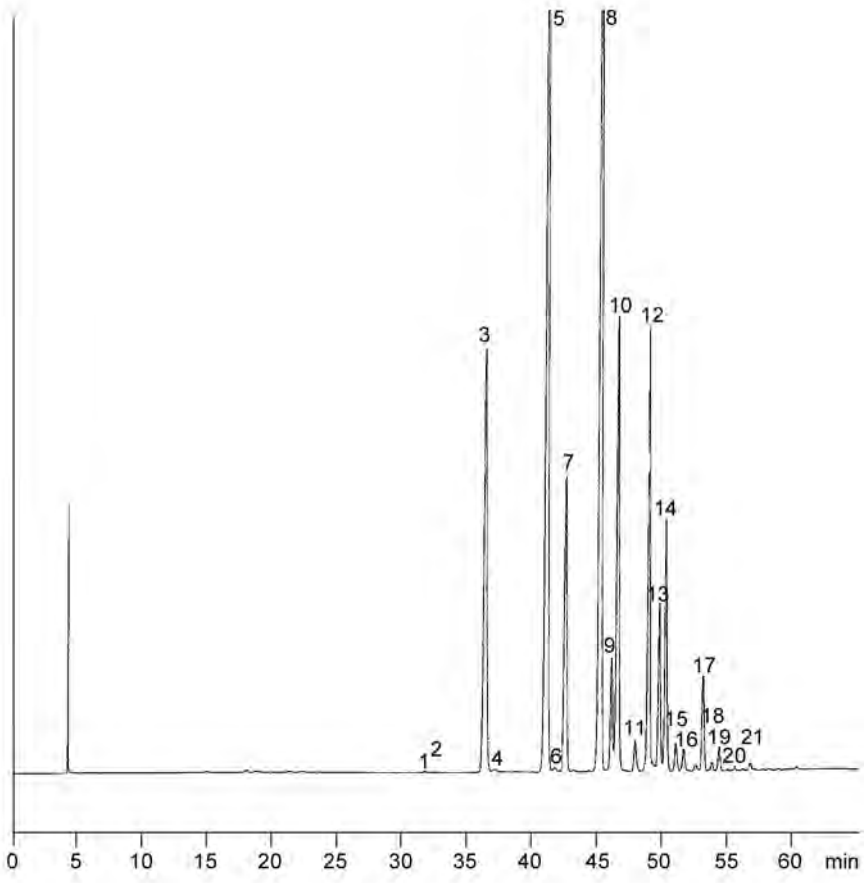


| | | | | | | |
|---|---|---|---|---|---|---|
| 1. LLLn | 4. OLLn | 7. PLL | 10. POL | 13. SOL | 16. PPO | 19. SSL |
| 2. OLnLn | 5. OLL | 8. OOL | 11. PPL | 14. POO | 17. SOO | 20. PPS |
| 3. LLL | 6. OOLn | 9. SLL | 12. OOO | 15. PSL | 18. PSO | 21. SSO |

Figure 0433.-1. – *Chromatogram for the composition of triglycerides in refined sesame oil*

**07/2018:2269**
**corrected 10.0**

# SEVOFLURANE

## Sevofluranum

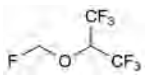


$C_4H_3F_7O$ $M_r$ 200.1
[28523-86-6]

DEFINITION

1,1,1,3,3,3-Hexafluoro-2-(fluoromethoxy)propane.

CHARACTERS

*Appearance*: clear, colourless, volatile liquid.

*Solubility*: slightly soluble in water, miscible with ethanol (96 per cent).

*Relative density*: about 1.52.

bp: about 59 °C.

It is non-flammable.

It decomposes in the presence of Lewis acids; this decomposition is inhibited by water in sufficient quantity.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: examine the substance in the gaseous state or in the liquid state.

*Comparison*: *sevoflurane CRS*.

TESTS

**Acidity or alkalinity**. Introduce 20.0 mL of the substance to be examined and 20 mL of *carbon dioxide-free water R* into a separating funnel, shake for 3 min and allow to stand. Collect the aqueous upper layer and add 0.2 mL of *bromocresol purple solution R*. Not more than 0.10 mL of *0.01 M sodium hydroxide* or 0.60 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Refractive index** (*2.2.6*): 1.2745 to 1.2760.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard*: *methylal R*.

*Test solution*. Introduce 20.0 mL of the substance to be examined into a vial and seal with a cap and septum. Using a microsyringe, add 5 µL of the internal standard and mix thoroughly.

*Reference solution (a)*. Introduce 2.0 mL of *ethylene chloride R* into a screw-cap vial and immediately seal with a cap and septum. Using a microsyringe, add about 20 µL of the substance to be examined. Record the quantity added, in milligrams, of the substance to be examined ($M_2$). Then, using a microsyringe, add about 20 µL of the internal standard. Record the quantity added, in milligrams, of the internal standard ($M_1$).

*Reference solution (b)*: *sevoflurane for system suitability CRS* (containing impurities A and B).

*Reference solution (c).* Introduce 20.0 mL of *ethylene chloride R* into a vial and seal with a cap and septum. Using a microsyringe, add 20 µL of the substance to be examined and mix thoroughly. Dilute 0.5 mL of this solution to 100.0 mL with *ethylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 40 |
| | 10 - 26 | 40 → 200 |
| | 26 - 40 | 200 |
| Injection port | | 200 |
| Detector | | 225 |

*Detection*: flame ionisation.

*Injection*: 2 µL; rinse the syringe with a solution containing *ethylene chloride R* before injection of the reference solutions; rinse the syringe with the substance to be examined before injection of the test solution.

*Identification of impurities*: use the chromatogram supplied with *sevoflurane for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to sevoflurane (retention time = about 6.6 min): impurity A = about 0.78; impurity B = about 0.83; internal standard = about 1.35.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

Calculate the relative response factor ($F_1$) for reference solution (a), using the following expression:

$$\frac{M_1 \times R}{M_2}$$

$M_1$ = mass of the internal standard in reference solution (a), in milligrams;

$M_2$ = mass of the substance to be examined in reference solution (a), in milligrams;

$R$ = ratio of the area of the peak due to sevoflurane to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (a).

Calculate the content of each impurity in the substance to be examined, in parts per million, using the following expression:

$$\frac{0.859 \times R_1 \times 250}{1.52 \times F_1}$$

0.859 = relative density of the internal standard;

1.52 = relative density of sevoflurane;

$R_1$ = ratio of the area of the peak due to the impurity to the area of the peak due to the internal standard from the chromatogram obtained with the test solution;

$F_1$ = relative response factor for reference solution (a).

*Limits*:

- *impurity A*: maximum 25 ppm;
- *impurity B*: maximum 100 ppm;
- *unspecified impurities*: for each impurity, maximum 100 ppm;
- *total*: maximum 300 ppm;
- *disregard limit*: the area of the peak due to sevoflurane in the chromatogram obtained with reference solution (c) (5 ppm).

**Fluorides**: maximum 2 µg/mL.

Potentiometry (*2.2.36, Method I*). *Use plastic utensils throughout this test.*

*Buffer solution.* Dissolve 0.5 g of *sodium citrate R* and 55 g of *sodium chloride R* in 350 mL of *water R*. Carefully add 75 g of *sodium hydroxide R* and shake to dissolve. Cool to room temperature and carefully add 225 mL of *glacial acetic acid R* while stirring. Cool and add 300 mL of *2-propanol R*. Dilute with *water R* to 1000.0 mL. The apparent pH of this solution is between 5.0 and 5.5.

*Test solution.* Introduce 50.0 mL of the substance to be examined and 50.0 mL of *water R* into a separating funnel, shake vigorously for 3 min and allow the layers to separate completely. Dilute 25.0 mL of the upper aqueous layer to 50.0 mL with the buffer solution.

*Fluoride standard solution (1000 ppm F).* Dissolve 221.0 mg of *sodium fluoride R*, previously dried at 150 °C for 4 h, in *water R*. Add 1.0 mL of *0.01 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

*Reference stock solutions.* Dilute the fluoride standard solution (1000 ppm F) with *water R* to obtain solutions having known concentrations of about 5 µg, 2 µg, 0.5 µg, and 0.2 µg of fluoride per millilitre.

*Reference solutions.* Dilute 25.0 mL of each reference stock solution to 50.0 mL with the buffer solution.

*Indicator electrode*: fluoride-selective.

*Reference electrode*: silver-silver chloride.

*Apparatus*: voltmeter capable of a minimum reproducibility of ± 0.2 mV.

Carry out the measurements on the reference solutions and test solution. To take measurements, transfer the solution under test to a 100 mL beaker containing a polytetrafluoroethylene-coated magnetic stirring bar, and immerse the electrodes. Allow to stir on a magnetic stirrer with an insulated top until equilibrium is attained (about 2-3 min), and record the potential. Rinse the electrodes with the buffer solution and dry, taking care to avoid damaging the crystal of the specific-ion electrode.

Calculate the concentration of fluorides using the calibration curve.

**Non-volatile residue**: maximum 100 mg/L.

Transfer 10.0 mL to a tared evaporating dish, evaporate to dryness on a water-bath and dry the residue at 105 °C for 2 h. The residue weighs a maximum of 1.0 mg.

**Water** (*2.5.12*): maximum 0.050 per cent *m/m*, determined on 10.0 mL.

STORAGE

In an airtight, stainless-steel container, protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

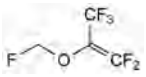

A. 1,1,3,3,3-pentafluoro-2-(fluoromethoxy)prop-1-ene,

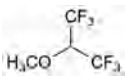

B. 1,1,1,3,3,3-hexafluoro-2-methoxypropane,

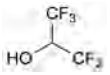

C. 1,1,1,3,3,3-hexafluoropropan-2-ol.

**01/2017:1149**

# SHELLAC

## Lacca

DEFINITION

Purified material obtained from the resinous secretion of the female insect *Kerria lacca* (Kerr) Lindinger (*Laccifer lacca* Kerr). There are 4 types of shellac depending on the nature of the treatment of crude secretion (seedlac): wax-containing shellac, bleached shellac, dewaxed shellac and bleached, dewaxed shellac.

Wax-containing shellac is obtained from seedlac: it is purified by filtration of the molten substance and/or by hot extraction using a suitable solvent.

Bleached shellac is obtained from seedlac by treatment with sodium hypochlorite after dissolution in a suitable alkaline solution, precipitation by dilute acid and drying.

Dewaxed shellac is obtained from wax-containing shellac or seedlac by treatment with a suitable solvent and removal of the insoluble wax by filtering.

Bleached, dewaxed shellac is obtained from wax-containing shellac or seedlac by treatment with sodium hypochlorite after dissolution in a suitable alkaline solution; the insoluble wax is removed by filtration. It is precipitated by dilute acid and dried.

CHARACTERS

*Appearance*: brownish-orange or yellow, shining, translucent, hard or brittle, more or less thin flakes (wax-containing shellac and dewaxed shellac), or a creamy white or brownish-yellow powder (bleached shellac and bleached, dewaxed shellac).

*Solubility*: practically insoluble in water, gives a more or less opalescent solution (wax containing shellac and bleached shellac) or a clear solution (dewaxed shellac and bleached, dewaxed shellac) in anhydrous ethanol. When warmed it is sparingly soluble or soluble in alkaline solutions.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Heat 0.25 g of the powdered substance (500) (*2.9.12*) on a water-bath with 2 mL of *dilute sodium hydroxide solution R* for 5 min. Cool, add 5 mL of *ethyl acetate R* and slowly, with stirring, 2 mL of *dilute acetic acid R*. Shake and filter the upper layer through *anhydrous sodium sulfate R*.

*Reference solution.* Dissolve 6.0 mg of *aleuritic acid R* in 1.0 mL of *methanol R*, heating slightly if necessary.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetic acid R*, *methanol R*, *methylene chloride R*, *ethyl acetate R* (1:8:32:60 *V/V/V/V*).

*Application*: 10 µL, as bands.

*Development*: twice over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the chromatogram obtained with the test solution shows several coloured zones, one of which is similar in position and colour to the zone in the chromatogram obtained with the reference solution. Above this zone the chromatogram obtained with the test solution shows a pink zone and below it several violet zones. Below the zone due to aleuritic acid, there is a light blue zone (shellolic acid) accompanied by zones of the same colour but of lower intensity. Other faint grey and violet zones may be visible.

B. Examine the chromatograms obtained in the test for colophony.

*Results*: for wax-containing shellac, in the chromatogram obtained with the test solution, a more or less strong bluish-grey zone is visible, just above the zone due to thymolphthalein in the chromatogram obtained with the reference solution; for dewaxed shellac, no such zone is visible just above the zone due to thymolphthalein in the chromatogram obtained with the reference solution.

TESTS

**Acid value** (*2.5.1*): 65 to 95 (dried substance).

Examine 1.00 g of the coarsely ground substance. Determine the end-point potentiometrically (*2.2.20*).

**Colophony**. Thin-layer chromatography (*2.2.27*) as described under identification test A with the following modifications.

*Test solution.* Dissolve 50 mg of the powdered substance (500) (*2.9.12*), with heating, in a mixture of 0.5 mL of *methylene chloride R* and 0.5 mL of *methanol R*.

*Reference solution.* Dissolve 2.0 mg of *thymolphthalein R* in 1.0 mL of *methanol R*.

*Detection*: examine in ultraviolet light at 254 nm; mark the quenching zones in the chromatogram obtained with the test solution that have similar $R_F$ values to that of the quenching zone due to thymolphthalein in the chromatogram obtained with the reference solution; spray with *anisaldehyde solution R*, heat at 100-105 °C for 5-10 min and examine in daylight.

*Results*: the chromatogram obtained with the reference solution shows a principal zone with a reddish-violet colour (thymolphthalein). None of the quenching zones in the chromatogram obtained with the test solution that have an $R_F$ value similar to the zone due to thymolphthalein in the reference solution show a more or less strong violet or brownish colour (colophony). Disregard any faint violet zone at this level that does not show quenching before spraying and heating.

**Arsenic** (*2.4.2, Method A*): maximum 3 ppm.

Introduce 0.33 g of the substance to be examined and 5 mL of *sulfuric acid R* into a combustion flask. Carefully add a few millilitres of *strong hydrogen peroxide solution R* and heat to boiling until a clear, colourless solution is obtained. Continue heating to eliminate the water and as much sulfuric acid as possible and dilute to 25 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent for unbleached shellac and maximum 6.0 per cent for bleached shellac, determined on 1.000 g of the powdered substance (500) (*2.9.12*) by drying in an oven at 40-45 °C for 24 h.

STORAGE

Protected from light. Store bleached shellac and bleached, dewaxed shellac at a temperature not exceeding 15 °C.

LABELLING

The label indicates the type of shellac.

**07/2017:2270**
**corrected 10.0**

# SILDENAFIL CITRATE

## Sildenafili citras

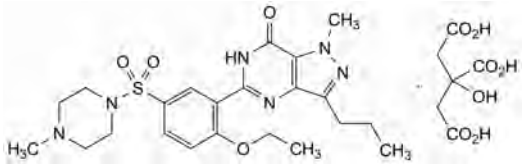

$C_{28}H_{38}N_6O_{11}S$ $M_r$ 667
[171599-83-0]

DEFINITION

5-[2-Ethoxy-5-[(4-methylpiperazin-1-yl)sulfonyl]phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water and in methanol, practically insoluble in hexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sildenafil citrate CRS*.

TESTS

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *concentrated ammonia R*, *water R*, *methanol R* (5:25:75 *V/V/V*).

*Test solution*. Dissolve 35.0 mg of the substance to be examined in 2.0 mL of the solvent mixture, with the aid of ultrasound if necessary.

*Reference solution (a)*. Dissolve 7.0 mg of *imidazole CRS* (impurity E) in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Mix 1 mL of the test solution and 1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *ethyl acetate R*, *methylene chloride R* (1:20:30:50 *V/V/V/V*).

*Application*: 10 µL of the test solution and reference solutions (b) and (c) as bands of 6 mm.

*Development*: over 2/3 of the plate.

*Drying*: at 100 °C for about 15 min.

*Detection*: expose to iodine vapour until the plate is light brown and examine under ultraviolet light at 254 nm.

*Retardation factors*: citrate = about 0; impurity E = about 0.25; sildenafil = about 0.4.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated zones.

*Limit*:

– *impurity E*: any zone due to impurity E is not more intense than the principal zone in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 900 mL of *water for chromatography R* and adjust to pH 6.5 with a 120 g/L solution of *potassium hydroxide R* in *water for chromatography R*. Dilute to 1.0 L with *water for chromatography R*.

*Solvent mixture*: *acetonitrile for chromatography R*, mobile phase A (10:90 *V/V*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 2.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *sildenafil impurity A CRS* in test solution (a) and dilute to 10.0 mL with test solution (a). Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *sildenafil for peak identification CRS* (containing impurity D) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 25.0 mg of *sildenafil citrate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 2.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *acetonitrile for chromatography R*, buffer solution (20:80 *V/V*);
– *mobile phase B*: buffer solution, *methanol R1*, *acetonitrile for chromatography R* (20:20:60 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 75 | 25 |
| 3 - 26 | 75 → 30 | 25 → 70 |
| 26 - 38 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *sildenafil for peak identification CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to sildenafil (retention time = about 16 min): impurity D = about 0.15; impurity A = about 1.25.

*System suitability*: reference solution (a):
- *resolution*: minimum 5.0 between the peaks due to sildenafil and impurity A.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity D by 0.7;
- for each impurity, use the concentration of sildenafil citrate in reference solution (c).

*Limits*:
- *impurities A, D*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to citrate.

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.00 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{28}H_{38}N_6O_{11}S$ taking into account the assigned content of *sildenafil citrate CRS*.

STORAGE

In airtight container.

IMPURITIES

*Specified impurities: A, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F, G.*

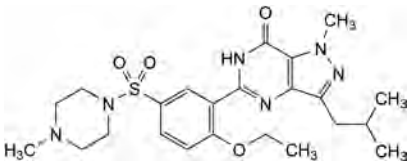

A. 5-[2-ethoxy-5-[(4-methylpiperazin-1-yl)sulfonyl]phenyl]-1-methyl-3-(2-methylpropyl)-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one,

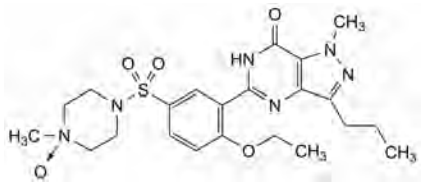

B. 1-methyl-4-[[4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)phenyl]sulfonyl]piperazine 1-oxide,

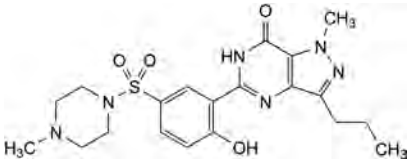

C. 5-[2-hydroxy-5-[(4-methylpiperazin-1-yl)sulfonyl]-phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo-[4,3-*d*]pyrimidin-7-one,

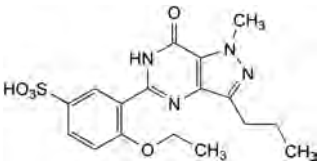

D. 4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6,7-dihydro-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)benzenesulfonic acid,

E. 1*H*-imidazole,

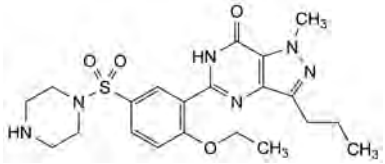

F. 5-[2-ethoxy-5-(piperazin-1-ylsulfonyl)phenyl]-1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one,

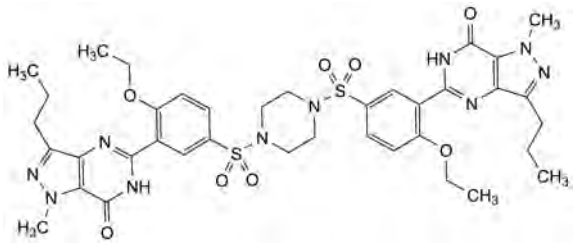

G. 5,5′-[piperazin-1,4-diylbis[dioxo-λ⁶-sulfanediyl(2-ethoxy-5,1-phenylene)]]bis(1-methyl-3-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]pyrimidin-7-one).

**01/2017:0434**

# SILICA, COLLOIDAL ANHYDROUS

## Silica colloidalis anhydrica

$SiO_2$ $M_r$ 60.1

[7631-86-9]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent of $SiO_2$ (ignited substance).

CHARACTERS

*Appearance*: white or almost white, light, fine, amorphous powder, with a particle size of about 15 nm.

*Solubility*: practically insoluble in water and in mineral acids except hydrofluoric acid. It dissolves in hot solutions of alkali hydroxides.

IDENTIFICATION

About 20 mg gives the reaction of silicates (*2.3.1*).

TESTS

**pH** (*2.2.3*): 3.5 to 5.5.

Shake 1.0 g with 30 mL of *carbon dioxide-free water R*.

**Chlorides** (*2.4.4*): maximum 250 ppm.

To 1.0 g add a mixture of 20 mL of *dilute nitric acid R* and 30 mL of *water R* and heat on a water-bath for 15 min, shaking frequently. Dilute to 50 mL with *water R* if necessary, filter and cool. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Loss on ignition**: maximum 5.0 per cent, determined on 0.200 g by ignition in a platinum crucible at 900 ± 50 °C for 2 h. Allow to cool in a desiccator before weighing.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 mL of *sulfuric acid R* and sufficient *ethanol (96 per cent) R* to moisten the residue completely. Add 6 mL of *hydrofluoric acid R* and evaporate to dryness on a hot-plate at 95-105 °C, taking care to avoid loss from sputtering. Wash down the sides of the dish with 6 mL of *hydrofluoric acid R* and evaporate to dryness. Ignite at 900 ± 50 °C, allow to cool in a desiccator and weigh.

The difference between the mass of the final residue and the mass of the residue obtained in the test for loss on ignition gives the amount of $SiO_2$ in the quantity of the substance to be examined used.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for colloidal anhydrous silica used as glidant in tablets and capsules.*

**Specific surface area** (*2.9.26, Method I*). Determine the specific surface area in the $P/P_0$ range of 0.05 to 0.30.

*Sample outgassing*: 20 min at 160 °C.

**01/2017:0738**

# SILICA, COLLOIDAL HYDRATED

## Silica colloidalis hydrica

[63231-67-4]

DEFINITION

Colloidal hydrated silica produced by precipitation or gelation process.

*Content*: 98.0 per cent to 100.5 per cent of $SiO_2$ ($M_r$ 60.1) (ignited substance).

CHARACTERS

*Appearance*: white or almost white, light, fine, amorphous powder.

*Solubility*: practically insoluble in water and in mineral acids, with the exception of hydrofluoric acid. It dissolves in hot solutions of alkali hydroxides.

IDENTIFICATION

About 20 mg gives the reaction of silicates (*2.3.1*).

TESTS

**Solution S**. To 2.5 g add 50 mL of *hydrochloric acid R* and mix. Heat on a water-bath for 30 min, stirring from time to time. Maintain the original volume by adding *dilute hydrochloric acid R*. Evaporate to dryness. Add to the residue a mixture of 8 mL of *dilute hydrochloric acid R* and 24 mL of *water R*. Heat to boiling and filter under reduced pressure through a sintered-glass filter (16) (*2.1.2*). Wash the residue on the filter with a hot mixture of 3 mL of *dilute hydrochloric acid R* and 9 mL of *water R*. Wash with small quantities of *water R*, combine the filtrate and washings and dilute to 50 mL with *water R*.

**pH** (*2.2.3*): 4.0 to 7.0.

Suspend 1.0 g in 30 mL of a 75 g/L solution of *potassium chloride R*.

**Water-absorption capacity**. In a mortar, triturate 5 g with 5 mL of *water R*, added drop by drop. The mixture remains powdery.

**Substances soluble in hydrochloric acid**: maximum 2.0 per cent.

In a platinum dish, evaporate to dryness 10.0 mL of solution S and dry to constant mass at 100-105 °C. The mass of the residue is not more than 10 mg.

**Chlorides** (*2.4.4*): maximum 0.1 per cent.

Heat 0.5 g with 50 mL of *water R* on a water-bath for 15 min. Dilute to 100 mL with *water R* and centrifuge at 1500 *g* for 5 min. Dilute 10 mL of the supernatant to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 1 per cent.

Dilute 2 mL of solution S to 100 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 300 ppm.

To 2 mL of solution S add 28 mL of *water R*.

**Loss on ignition**: maximum 20.0 per cent, determined on 0.200 g in a platinum crucible by heating at 100-105 °C for 1 h and then at 900 ± 50 °C for 2 h.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 mL of *sulfuric acid R* and a quantity of *ethanol (96 per cent) R* sufficient to moisten the residue completely. Add 6 mL of *hydrofluoric acid R* and evaporate to dryness at 95-105 °C, taking care to avoid loss from sputtering. Wash the inside of the dish with 6 mL of *hydrofluoric acid R* and evaporate to dryness again. Ignite at 900 ± 50 °C, allow to cool in a desiccator and weigh. The difference between the mass of the final residue and that of the mass obtained in the test for loss on ignition corresponds to the mass of $SiO_2$ in the test sample.

**01/2017:1562**

# SILICA, DENTAL TYPE

## Silica ad usum dentalem

DEFINITION

Amorphous silica (precipitated, gel or obtained by flame hydrolysis).

*Content*: 94.0 per cent to 100.5 per cent of $SiO_2$ (ignited substance).

CHARACTERS

*Appearance*: white or almost white, light, fine, amorphous powder.

*Solubility*: practically insoluble in water and in mineral acids. It dissolves in hydrofluoric acid and hot solutions of alkali hydroxides.

IDENTIFICATION

About 20 mg gives the reaction of silicates (*2.3.1*).

TESTS

**Solution S**. To 2.5 g add 50 mL of *hydrochloric acid R* and mix. Heat on a water-bath for 30 min, stirring from time to time. Evaporate to dryness. Add to the residue a mixture of 8 mL of *dilute hydrochloric acid R* and 24 mL of *water R*. Heat to boiling and filter under reduced pressure through a sintered-glass filter (16) (*2.1.2*). Wash the residue on the filter with a hot mixture of 3 mL of *dilute hydrochloric acid R* and 9 mL of *water R*. Wash with small quantities of *water R*, combine the washings and the filtrate, and dilute to 50 mL with *water R*.

**pH** (*2.2.3*): 3.2 to 8.9.

Suspend 5 g in a mixture of 5 mL of a 7.46 g/L solution of *potassium chloride R* and 90 mL of *carbon dioxide-free water R*.

**Chlorides**. Liquid chromatography (*2.2.29*) as described in the test for sulfates.

*Retention time*: chlorides = about 4 min.

*Limit*:

– *chlorides*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.3 per cent).

**Sulfates**. Liquid chromatography (*2.2.29*).

*Test solution*. To 0.625 g of the substance to be examined add 30 mL of *water R* and boil for 2 h. Allow to cool and quantitatively transfer to a 50 mL graduated flask. Dilute to 50.0 mL with *water R*. Dilute 5.0 mL of the supernatant to 50.0 mL with *water R* and filter through a membrane filter (nominal pore size 0.45 µm).

*Reference solution*. Dissolve 0.50 g of *anhydrous sodium sulfate R* and 0.062 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with *water R*. Dilute 5.0 mL of the solution to 50.0 mL with *water R*.

*Column*:

– *material*: non-metallic;

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: suitable anion-exchange resin (30-50 µm).

*Mobile phase*: dissolve 0.508 g of *sodium carbonate R* and 0.05 g of *sodium hydrogen carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

*Flow rate*: 1.2 mL/min.

*Detection*: conductivity detector.

*Injection*: 25 µL.

*Retention time*: sulfates = about 8 min.

*Limit*:

– *sulfates*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (4.0 per cent, expressed as sodium sulfate).

**Iron** (*2.4.9*): maximum 400 ppm.

Dilute 2 mL of solution S to 40 mL with *water R*.

**Loss on ignition**: maximum 25.0 per cent, determined on 0.200 g by heating in a platinum crucible at 100-105 °C for 1 h and then at 1000 ± 50 °C for 2 h.

ASSAY

To the residue obtained in the test for loss on ignition add 0.2 mL of *sulfuric acid R* and a quantity of *ethanol (96 per cent) R* sufficient to moisten the residue completely. Add 6 mL of *hydrofluoric acid R* and evaporate to dryness at 95-105 °C, taking care to avoid loss from sputtering. Wash the inside of the crucible with 6 mL of *hydrofluoric acid R* and evaporate to dryness again. Ignite at 900 ± 50 °C, allow to cool in a desiccator and weigh. The difference between the mass of the final residue and that of the mass obtained in the test for loss on ignition corresponds to the mass of $SiO_2$ in the test sample.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for dental type silica used as abrasive.*

**Specific surface area** (*2.9.26, Method I*). Determine the specific surface area in the $P/P_0$ range of 0.05 to 0.30.

*Sample outgasing*: 60 min at 160 °C.

**01/2017:2208**

# SILICA, HYDROPHOBIC COLLOIDAL

## Silica hydrophobica colloidalis

DEFINITION

Colloidal silicon dioxide partly alkylated for hydrophobation.

*Content*: 99.0 per cent to 101.0 per cent $SiO_2$ (ignited substance).

CHARACTERS

*Appearance*: white or almost white, light, fine, amorphous powder, not wettable by water.

*Solubility*: practically insoluble in water and mineral acids except hydrofluoric acid. It dissolves slowly in hot solutions of alkali hydroxides.

IDENTIFICATION

A. About 25 mg ignited in a platinum crucible at 900 ± 50 °C for 2 h gives the reaction of silicates (*2.3.1*).

B. Water-dispersible fraction (see Tests).

TESTS

**Chlorides** (*2.4.4*): maximum 250 ppm.

To 1.0 g add 30 mL of *methanol R* and 20 mL of *dilute nitric acid R*. Heat on a water-bath for 15 min stirring frequently. Cool, dilute to 50 mL with *water R* and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Water-dispersible fraction**: maximum 3.0 per cent.

Place 0.400 g in a 500 mL separating funnel, add 100 mL of *water R* and shake for 1 min. Allow to stand for 1 h. Allow 90 mL of the aqueous phase to run out dropwise without filtration into a suitable dish dried at 140 °C and cooled in a desiccator. Evaporate to dryness at 140 °C, starting at a low temperature to avoid splashing. Cool in a desiccator. The residue weighs a maximum of 12 mg.

**Loss on ignition**: maximum 6.0 per cent, determined on 0.200 g by ignition in a platinum crucible at 900 ± 50 °C for 2 h. It is advisable to place the crucible in a cold oven and then to heat up the oven. Allow to cool in a desiccator before weighing.

ASSAY

To the residue obtained in the test for loss on ignition add sufficient *ethanol (96 per cent) R* to moisten the residue completely and 0.2 mL of *sulfuric acid R*. Add 6 mL of *hydrofluoric acid R* and evaporate to dryness on a hot-plate at about 100 °C, taking care to avoid loss from sputtering. Wash down the sides of the platinum crucible with 6 mL of *hydrofluoric acid R* and evaporate to dryness. Ignite at 900 ± 50 °C, allow to cool in a desiccator and weigh.

The difference between the mass of the residue obtained in the test for loss on ignition and the mass of the final residue gives the amount of $SiO_2$ in the quantity of the substance to be examined.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for hydrophobic colloidal silica used as glidant in tablets and capsules.*

**Specific surface area** (*2.9.26, Method I*). Determine the specific surface area in the $P/P_0$ range of 0.05 to 0.30.

*Sample outgassing*: 20 min at 160 °C.

**01/2008:2281**
**corrected 6.0**

# SILVER, COLLOIDAL, FOR EXTERNAL USE

## Argentum colloidale ad usum externum

DEFINITION

Colloidal metallic silver containing protein.

*Content*: 70.0 per cent to 80.0 per cent of Ag (dried substance).

CHARACTERS

*Appearance*: green or bluish-black metallic shiny flakes or powder, hygroscopic.

*Solubility*: freely soluble or soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. To 5 mL of the filtrate obtained in the test for alkalinity (see Tests) add 0.05 mL of *copper sulfate solution R* and 1 mL of *dilute sodium hydroxide solution R*. Shake. A violet colour appears within 15 min.

B. To 1 mL of solution S (see Tests), add 2 mL of *sodium chloride solution R*. A precipitate is formed which dissolves in an exces of water.

C. Ignite 0.05 g of the substance to be examined. Dissolve the residue in 10 mL of *nitric acid R*. The filtrate gives the reaction of silver (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g of the substance to be examined in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent. Allow to stand for 5 min then shake vigorously. Filter through a tared sintered-glass filter (16) (*2.1.2*) after 30 min.

**Alkalinity**. To 40.0 mL of solution S add 10.0 mL of *0.05 M sulfuric acid* and 2.0 g of *anhydrous sodium sulfate R*. Shake and filter several times if necessary. To 25.0 mL of the clear and colourless solution add 0.1 mL of *phenolphthalein solution R*. Not less than 1.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Silver ions**. To 0.50 g of the substance to be examined add 5 mL of *anhydrous ethanol R*. Shake for 1 min, filter and add 2 mL of *hydrochloric acid R* to the filtrate. No precipitate is formed.

**Sensitivity to electrolytes**. Dissolve 0.1 g of the substance to be examined in 100 mL of *water R*. Transfer a part of the solution into a test tube. When examined viewing horizontally the solution appears clear and reddish-brown. When examined viewing vertically, the solution appears turbid with a greenish-brown fluorescence. To 5 mL of the solution add 5 mL of a solution of 0.50 g/L *sodium chloride R* and mix by shaking for 1 minute. When examined viewing horizontally the solution remains clear and reddish-brown.

**Water insoluble substances**: maximum 1.0 per cent.

Wash the residue obtained on the filter during preparation of solution S 5 times with 10 mL of *water R*. Dry the filter to constant mass at 100-105 °C. The residue weighs a maximum of 12.5 mg.

**Loss on drying** (*2.2.32*): maximum 8.0 per cent, determined on 0.500 g by drying in an oven at 80 °C.

ASSAY

Ignite 0.200 g of the substance to be examined at 650 ± 50 °C until the residue is white. Allow to cool, add 10 mL of a mixture of equal volumes of *nitric acid R* and *water R* and boil for 1 min. Transfer the contents of the crucible into a flask and titrate with *0.1 M ammonium thiocyanate* using 50 mg *ferric sulfate R* as indicator, until a reddish-brown colour appears.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 10.79 mg of Ag.

STORAGE

In an airtight container.

**01/2008:0009**
**corrected 6.0**

# SILVER NITRATE

## Argenti nitras

$AgNO_3$ $M_r$ 169.9
[7761-88-8]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or transparent, colourless crystals.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. 10 mg gives the reaction of nitrates (*2.3.1*).

B. 10 mg gives the reaction of silver (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 2 mL of solution S add 0.1 mL of *bromocresol green solution R*. The solution is blue. To 2 mL of solution S add 0.1 mL of *phenol red solution R*. The solution is yellow.

**Foreign salts**: maximum 0.3 per cent.

To 30 mL of solution S, add 7.5 mL of *dilute hydrochloric acid R*, shake vigorously, heat for 5 min on a water-bath and filter. Evaporate 20 mL of the filtrate to dryness on a water-bath and dry at 100-105 °C. The residue weighs a maximum of 2 mg.

**Aluminium, lead, copper and bismuth**. Dissolve 1.0 g in a mixture of 4 mL of *concentrated ammonia R* and 6 mL of *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

ASSAY

Dissolve 0.300 g in 50 mL of *water R*, add 2 mL of *dilute nitric acid R* and 2 mL of *ferric ammonium sulfate solution R2*. Titrate with *0.1 M ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 16.99 mg of $AgNO_3$.

STORAGE

In a non-metallic container, protected from light.

**01/2017:1470**

# SIMETICONE

## Simeticonum

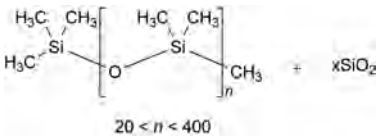


[8050-81-5]

DEFINITION

Mixture of α-trimethylsilyl-ω-methylpoly[oxy(dimethylsilanediyl)] and silicon dioxide.

Simeticone is prepared by incorporation of 4 per cent to 7 per cent silica into poly(dimethylsiloxane) with a degree of polymerisation between 20 and 400.

*Content*: 90.5 per cent to 99.0 per cent of poly(dimethylsiloxane).

PRODUCTION

Poly(dimethylsiloxane) is obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane and the silica is modified at the surface by incorporation of methylsilyl groups.

CHARACTERS

*Appearance*: viscous, greyish-white, opalescent liquid.

*Solubility*: practically insoluble in water, very slightly soluble or practically insoluble in anhydrous ethanol, practically insoluble in methanol, partly miscible with ethyl acetate, with methylene chloride, with methyl ethyl ketone and with toluene.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: thin films between plates of *sodium chloride R*.
*Absorption maxima*: at 2964 $cm^{-1}$, 2905 $cm^{-1}$, 1412 $cm^{-1}$, 1260 $cm^{-1}$ and 1020 $cm^{-1}$.

B. Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

C. The residue obtained in the test for silica under Assay gives the reaction of silicates (*2.3.1*).

TESTS

**Acidity**. To 2.0 g add 25 mL of a mixture of equal volumes of *anhydrous ethanol R* and *ether R*, previously neutralised to 0.2 mL of *bromothymol blue solution R1*, and shake. Not more than 3.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the solution to blue.

**Defoaming activity**

*Foaming solution*. Dissolve 5.0 g of *docusate sodium R* in 1 L of *water R*, warm to 50 °C if necessary.

*Defoaming solution*. To 50 mL of *methyl ethyl ketone R* add 0.250 g of the substance to be examined, warm to not more than 50 °C with shaking.

Into a 250 mL cylindrical tube about 5 cm in diameter introduce 100 mL of foaming solution and 1 mL of defoaming solution. Close tightly and fix the tube on a suitable oscillating shaker that complies with the following conditions:

– 250-300 oscillations per minute;
– angle of oscillation of about 10°;
– oscillation radius of about 10 cm.

Shake for 10 s and record the time between the end of the shaking and the instant the 1st portion of foam-free liquid surface appears.

This duration is not longer than 15 s.

**Mineral oils**. Place 2.0 g in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

**Phenylated compounds**: the corrected absorbance (*2.2.25*) is not greater than 0.2.

*Test solution*. Dissolve 5.0 g with shaking in 10.0 mL of *cyclohexane R*.

*Spectral range*: 200-350 nm.

Calculate the corrected absorbance using the following expression:

$$B - C$$

*B* = absorbance at the absorption maximum between 250 nm and 270 nm;

*C* = absorbance at 300 nm.

**Volatile matter**: maximum 1.0 per cent, determined on 1.00 g by heating in an oven at 150 °C for 2 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

ASSAY

**Silica**. Heat not less than 20.0 mg to 800 °C increasing the temperature by 20 °C/min under a current of *nitrogen R* at a flow rate of 200 mL/min and weigh the residue (silica).

**Poly(dimethylsiloxane)**. Infrared absorption spectrophotometry (*2.2.24*).

*Test solution*. Place about 50 mg (*E*) in a screw-capped 125 mL cylindrical tube, add 25.0 mL of *toluene R*, swirl manually to disperse and add 50 mL of *dilute hydrochloric acid R*, close the tube and place on a vortex mixer; shake for 5 min. Transfer the contents of the tube to a separating funnel, allow to settle and transfer 5 mL of the upper layer to a screw-capped test-tube containing 0.5 g of *anhydrous sodium sulfate R*. Cap and shake vigorously manually. Centrifuge to obtain a clear solution.

*Reference solution*. Introduce about 0.20 g of *dimeticone CRS* (poly(dimethylsiloxane)) into 100.0 mL of *toluene R*. Prepare the reference solution in the same way as for the test solution, using 25.0 mL of the dimeticone solution obtained above.

*Blank solution*. Shake 10 mL of *toluene R* with 1 g of *anhydrous sodium sulfate R*. Centrifuge the resulting suspension.

Record the infrared absorption spectra for the test solution and the reference solution in 0.5 mm cells, from 1330 $cm^{-1}$ to 1180 $cm^{-1}$. Determine the absorbance of the band at 1260 $cm^{-1}$.

Calculate the percentage content of poly(dimethylsiloxane) using the following expression:

$$\frac{25 \times C \times A_M \times 100}{A_E \times E}$$

$A_M$ = absorbance of the test solution;

$A_E$ = absorbance of the reference solution;

$C$ = concentration of the reference solution, in milligrams per millilitre;

$E$ = mass of the substance to be examined, in milligrams.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for simeticone used as defoaming agent.*

**Defoaming activity** (see Tests).

**04/2019:1563**

# SIMVASTATIN

## Simvastatinum

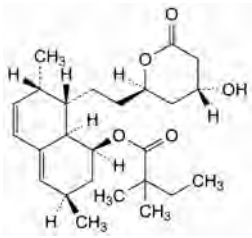

$C_{25}H_{38}O_5$ $M_r$ 418.6

[79902-63-9]

DEFINITION

(1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-Hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

A suitable antioxidant may be added.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *simvastatin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 0.2 g in *methanol R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 285 to + 300 (dried substance).

Dissolve 0.125 g in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 10.0 mg of *simvastatin CRS* in the solvent mixture and dilute to 5.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *simvastatin for system suitability CRS* (containing impurities A, B, C, D, E, F, G, I and J) in 1 mL of the solvent mixture.

*Reference solution (d).* Dissolve 4.0 mg of *simvastatin impurity K CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 5.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped extra-dense bonded octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R1*, 0.1 per cent *V/V* solution of *phosphoric acid R* (40:60 *V/V*);
- *mobile phase B*: 0.1 per cent *V/V* solution of *phosphoric acid R*, *acetonitrile R1* (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 5 | 100 → 80 | 0 → 20 |
| 5 - 33 | 80 → 60 | 20 → 40 |
| 33 - 34 | 60 → 0 | 40 → 100 |
| 34 - 45 | 0 | 100 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 238 nm and, for impurity K, at 200 nm.

*Autosampler*: set at 8 °C.

*Injection*: 5 µL of test solution (a) and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *simvastatin for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D, E, F, G, I and J; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity K.

*Relative retention* with reference to simvastatin (retention time = about 19 min): impurity I = about 0.67; impurity A = about 0.69; impurity E = about 0.81; impurity F = about 0.83; impurity G = about 0.9; impurity K = about 1.2; impurity B = about 1.69; impurity J = about 1.74; impurity C = about 1.76; impurity D = about 2.3.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity E; minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity J.

*Calculation of percentage contents*:

- for impurity K, use the concentration of impurity K in reference solution (d) and the peak areas recorded at 200 nm;
- for impurities other than K, use the concentration of simvastatin in reference solution (a) and the peak areas recorded at 238 nm.

*Limits*:

- *impurities E, F*: for each impurity, maximum 0.5 per cent;
- *sum of impurities A and I*: maximum 0.4 per cent;
- *impurity D*: maximum 0.4 per cent;
- *impurity K at 200 nm*: maximum 0.4 per cent;
- *impurities B, C*: for each impurity, maximum 0.3 per cent;
- *impurities G, J*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 3.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator under high vacuum at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*:

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 70 | 30 |
| 14 - 15 | 70 → 0 | 30 → 100 |
| 15 - 20 | 0 | 100 |

*Injection*: test solution (b) and reference solution (b).

Calculate the percentage content of $C_{25}H_{38}O_5$ taking into account the assigned content of *simvastatin CRS*.

STORAGE

Protected from light. If no antioxidant is present, store under nitrogen, in an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, I, J, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *H, M, N.*

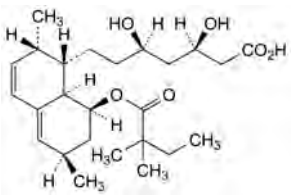

A. (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl)-oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoic acid (tenivastatin),

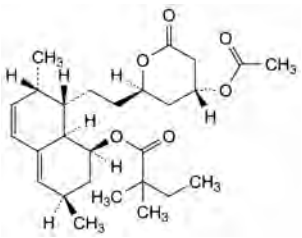

B. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-(acetyloxy)-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate,

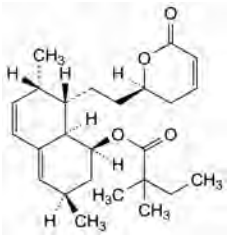

C. (1*S*,3*R*,7*S*,8*S*,8a*R*)-3,7-dimethyl-8-[2-[(2*R*)-6-oxo-3,6-dihydro-2*H*-pyran-2-yl]ethyl]-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate,

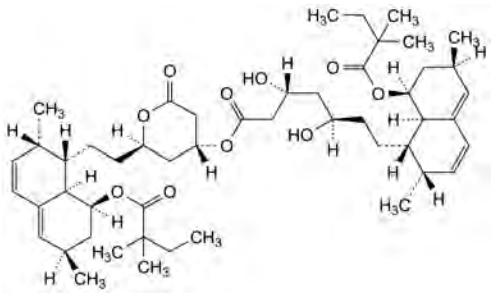

D. (2*R*,4*R*)-2-[2-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]ethyl]-6-oxooxan-4-yl (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoate,

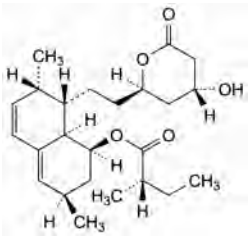

E. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*S*)-2-methylbutanoate (lovastatin),

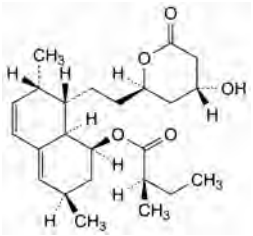

F. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2*R*)-2-methylbutanoate (epilovastatin),

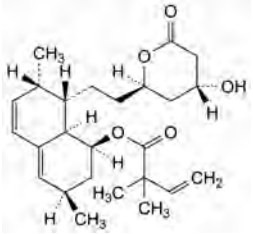

G. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbut-3-enoate,

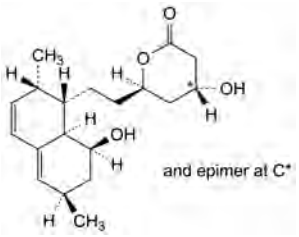


H. (4*RS*,6*R*)-4-hydroxy-6-[2-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-hydroxy-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]ethyl]oxan-2-one,

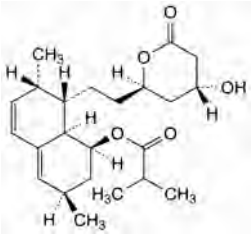

I. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2-methylpropanoate,

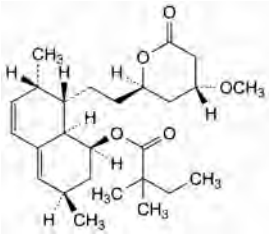

J. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*)-4-methoxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate,

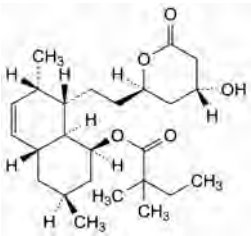

K. (1*S*,3*S*,4a*R*,7*S*,8*S*,8a*S*)-8-[2-[(2*R*,4*R*)-4-hydroxy-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,4,4a,7,8,8a-octahydronaphthalen-1-yl 2,2-dimethylbutanoate,

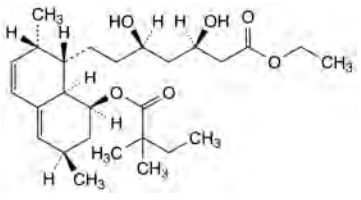

M. ethyl (3*R*,5*R*)-7-[(1*S*,2*S*,6*R*,8*S*,8a*R*)-8-[(2,2-dimethylbutanoyl)oxy]-2,6-dimethyl-1,2,6,7,8,8a-hexahydronaphthalen-1-yl]-3,5-dihydroxyheptanoate,

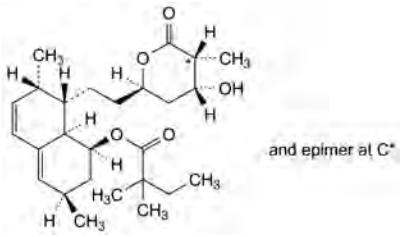


N. (1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4*R*,5*RS*)-4-hydroxy-5-methyl-6-oxooxan-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate.

**04/2017:2778**
**corrected 10.0**

# SITAGLIPTIN PHOSPHATE MONOHYDRATE

## Sitagliptini phosphas monohydricus

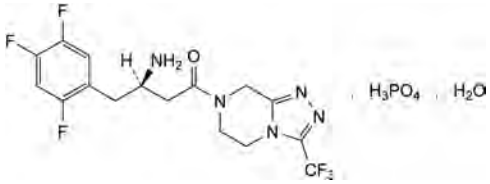

$C_{16}H_{18}F_6N_5O_5P,H_2O$ $M_r$ 523.3
[654671-77-9]

DEFINITION

(3*R*)-3-Amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-4-(2,4,5-trifluorophenyl)-butan-1-one phosphate monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sitagliptin phosphate monohydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Enantiomeric purity (see Tests).

C. Dissolve 0.200 g in *water R* and dilute to 5.0 mL with the same solvent. The solution gives reaction (a) of phosphates (*2.3.1*).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: *water R*, *methanol R* (10:90 *V/V*).

*Test solution*. Dissolve 80 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 8 mg of *sitagliptin containing impurity A CRS* in 1 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm);
– *temperature*: 35 °C.

*Mobile phase*: *water for chromatography R*, *diethylamine R*, *heptane R*, *anhydrous ethanol R* (1:1:400:600 *V/V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 268 nm.

*Injection*: 10 µL.

*Run time*: 1.6 times the retention time of sitagliptin.

*Identification of impurities*: use the chromatogram supplied with *sitagliptin containing impurity A CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to sitagliptin (retention time = about 15 min): impurity A = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity A and sitagliptin.

*Limit*:

– *impurity A*: maximum 0.5 per cent;
– *reporting threshold*: 0.1 per cent (reference solution (a)).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 0.1 per cent *V/V* solution of *phosphoric acid R* (5:95 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 250.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *sitagliptin phosphate monohydrate CRS* in the solvent mixture and dilute to 250.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. In order to prepare the fumarate adduct *in situ*, heat 1 mL of *water R*, 1 mg of *sodium stearyl fumarate R* and 10 mg of the substance to be examined in a tightly closed vial at 80 °C for about 30 h. Dilute to 100 mL with the solvent mixture and stir for 1 h. Centrifuge a portion of the solution and use the clear supernatant.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped cyanosilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: mix 15 volumes of *acetonitrile R1* and 85 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 5.5 times the retention time of sitagliptin.

*Relative retention* with reference to sitagliptin (retention time = about 5.5 min): fumarate adduct = about 1.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to sitagliptin and the fumarate adduct.

*Calculation of percentage contents*:

– for each impurity, use the concentration of sitagliptin phosphate monohydrate in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 3.3 per cent to 3.7 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*Run time*: twice the retention time of sitagliptin.

Calculate the percentage content of $C_{16}H_{18}F_6N_5O_5P$ taking into account the assigned content of *sitagliptin phosphate monohydrate CRS*.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

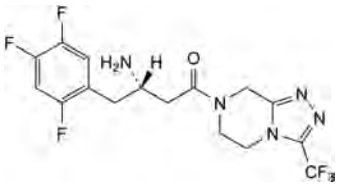

A. (3*S*)-3-amino-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-4-(2,4,5-trifluorophenyl)butan-1-one,

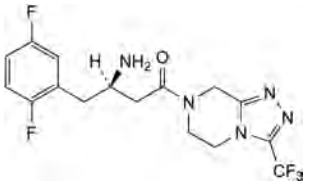

B. (3*R*)-3-amino-4-(2,5-difluorophenyl)-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]butan-1-one,

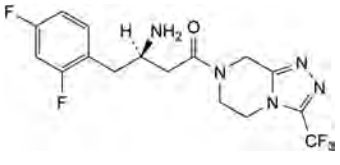

C. (3*R*)-3-amino-4-(2,4-difluorophenyl)-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]butan-1-one.

**04/2016:2927**
**corrected 10.0**

# SITAGLIPTIN TABLETS

## Sitagliptini compressi

DEFINITION

Tablets containing *Sitagliptin phosphate monohydrate (2778)*.

*They comply with the monograph Tablets (0478) and the following additional requirements.*

*Content*: 95.0 per cent to 105.0 per cent of the content of sitagliptin ($C_{16}H_{15}F_6N_5O$) stated on the label.

IDENTIFICATION

*Carry out either tests A, B or tests B, C.*

A. Record the UV spectrum of the principal peak in the chromatograms obtained with the solutions used in the assay with a diode array detector.

*Results*: the UV spectrum of the principal peak in the chromatogram obtained with the test solution is similar to the UV spectrum of the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: crush a tablet to a powder and homogenise.

*Comparison*: *sitagliptin phosphate monohydrate CRS*.

*Results*: the spectrum obtained shows absorption maxima at about 1669 $cm^{-1}$, 1513 $cm^{-1}$, 1425 $cm^{-1}$, 1207 $cm^{-1}$, 880 $cm^{-1}$ and 844 $cm^{-1}$, similar to the spectrum obtained with *sitagliptin phosphate monohydrate CRS*. Other absorption maxima may be present in the spectra.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, 0.1 per cent *V/V* solution of *phosphoric acid R* (5:95 *V/V*).

*Test solution.* To 10 tablets, add a suitable volume of the solvent mixture to obtain a concentration of sitagliptin of 1 mg/mL. Stir vigorously for 1 h. Dilute 2.0 mL of the solution to 25.0 mL with the solvent mixture. Centrifuge a portion of the solution and use the clear supernatant.

*Reference solution (a).* Dissolve 25.0 mg of *sitagliptin phosphate monohydrate CRS* in the solvent mixture and dilute to 250.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* In order to prepare impurity FP-A (fumarate adduct) *in situ*, heat 1 mL of *water R* and either 1 sitagliptin tablet containing sodium stearyl fumarate as an excipient or 1 mg of *sodium stearyl fumarate R* and 10 mg of *sitagliptin phosphate monohydrate CRS* in a tightly closed vial at 80 °C for about 30 h. Dilute to 100 mL with the solvent mixture and stir for 1 h. Centrifuge a portion of the solution and use the clear supernatant.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped cyanosilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 15 volumes of *acetonitrile R1* and 85 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.0 with *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 7 times the retention time of sitagliptin.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity FP-A.

*Relative retention* with reference to sitagliptin (retention time = about 5.5 min): impurity FP-A = about 1.2.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to sitagliptin and impurity FP-A.

*Calculation of percentage contents*:

– for each impurity, use the concentration of sitagliptin in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.1 per cent.

**Disintegration** (*2.9.1*). The tablets comply with the test, unless otherwise justified or authorised.

*Medium*: *water R*.

*Time*: 5 min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

*Run time*: twice the retention time of sitagliptin.

*System suitability*: reference solution (a):

– *repeatability*: maximum relative standard deviation of 1.5 per cent determined on 6 injections.

Calculate the percentage content of sitagliptin ($C_{16}H_{15}F_6N_5O$) taking into account the assigned content of *sitagliptin phosphate monohydrate CRS*.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph): *FP-A, FP-B, FP-C, FP-D, FP-E.*

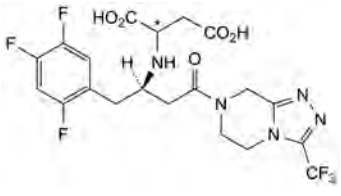

FP-A. 2-[[(2*R*)-4-oxo-4-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-1-(2,4,5-trifluorophenyl)butan-2-yl]amino]butanedioic acid,

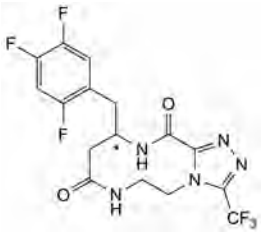

FP-B. 3-(trifluoromethyl)-10-[(2,4,5-trifluorophenyl)methyl]-6,7,10,11-tetrahydro[1,2,4]triazolo[3,4-*c*][1,4,7]triazecine-8,12(5*H*,9*H*)-dione,

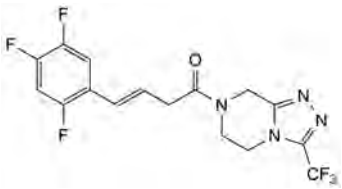

FP-C. (3*E*)-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]-triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-4-(2,4,5-trifluoro-phenyl)but-3-en-1-one,

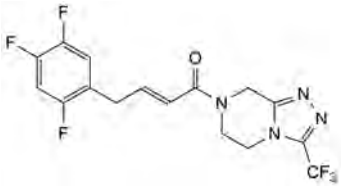

FP-D. (2*E*)-1-[3-(trifluoromethyl)-5,6-dihydro[1,2,4]-triazolo[4,3-*a*]pyrazin-7(8*H*)-yl]-4-(2,4,5-trifluoro-phenyl)but-2-en-1-one,

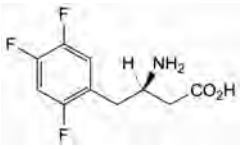

FP-E. (3*R*)-3-amino-4-(2,4,5-trifluorophenyl)butanoic acid.

**01/2017:0411**

# SODIUM ACETATE TRIHYDRATE

## Natrii acetas trihydricus

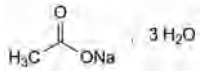

$C_2H_3NaO_2,3H_2O$ $M_r$ 136.1
[6131-90-4]

DEFINITION

Sodium ethanoate trihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. 1 mL of solution S (see Tests) gives reaction (b) of acetates (*2.3.1*).

B. 1 mL of solution S gives reaction (a) of sodium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.5 to 9.0.

Dilute 5 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Reducing substances**. Dissolve 5.0 g in 50 mL of *water R*, then add 5 mL of *dilute sulfuric acid R* and 0.5 mL of *0.002 M potassium permanganate*. The pink colour persists for at least 1 h. Prepare a blank in the same manner but without the substance to be examined.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 0.2 ppm, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution*. Dissolve 20 g in 100 mL of *water R* and adjust to pH 6.0 by the addition of *1 M hydrochloric acid* (about 10 mL).

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Calcium and magnesium**: maximum 50 ppm, calculated as Ca.

To 200 mL of *water R* add 10 mL of *ammonium chloride buffer solution pH 10.0 R*, 0.1 g of *mordant black 11 triturate R*, 2.0 mL of *0.05 M zinc chloride* and, dropwise, *0.02 M sodium edetate* until the colour changes from violet to blue. Add to the solution 10.0 g of the substance to be examined and shake to dissolve. Titrate with *0.02 M sodium edetate* until the blue colour is restored. Not more than 0.65 mL of *0.02 M sodium edetate* is required.

**Iron** (*2.4.9*): maximum 10 ppm, determined on 10 mL of solution S.

**Loss on drying** (*2.2.32*): 39.0 per cent to 40.5 per cent, determined on 1.000 g by drying in an oven at 130 °C. Introduce the substance to be examined into the oven while the latter is cold.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*, add 5 mL of *acetic anhydride R*, mix and allow to stand for 30 min. Using 0.3 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until a green colour is obtained.

1 mL of *0.1 M perchloric acid* is equivalent to 8.20 mg of $C_2H_3NaO_2$.

STORAGE

In an airtight container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

**01/2017:1564**

# SODIUM ALENDRONATE TRIHYDRATE

## Natrii alendronas trihydricus

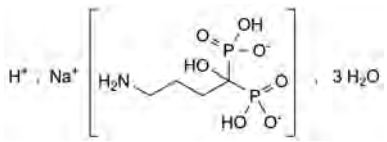

$C_4H_{12}NNaO_7P_2,3H_2O$ $M_r$ 325.1
[121268-17-5]

DEFINITION

Monosodium trihydrogen (4-amino-1-hydroxybutylidene)bisphosphonate trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium alendronate trihydrate CRS*.

B. Loss on drying (see Tests).

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**pH** (*2.2.3*): 4.0 to 5.0 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solution A*. Dissolve 29.4 g of *sodium citrate R* in *water R* and dilute to 1.0 L with the same solvent.

*Solution B*. Dissolve 19.1 g of *disodium tetraborate R* in *water R* and dilute to 1.0 L with the same solvent.

*Solution C. Prepare immediately before use.* Dissolve 0.200 g of *(9-fluorenyl)methyl chloroformate R* in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Buffer solution*. Dissolve 2.84 g of *anhydrous disodium hydrogen phosphate R* and 5.88 g of *sodium citrate R* in 1.9 L of *water R*, adjust to pH 8.0 with either *phosphoric acid R* or a 42 g/L solution of *sodium hydroxide R* and dilute to 2.0 L with *water R*.

*Test solution*. Dissolve 30 mg of the substance to be examined in solution A and dilute to 50 mL with solution A. Transfer 5 mL of the solution to a 50 mL polypropylene screw-cap centrifuge tube containing 5 mL of solution B. Add 5 mL of *acetonitrile R* and 5 mL of solution C. Shake for 45 s and allow to stand at room temperature for 30 min. Add 20 mL of *methylene chloride R* and shake vigorously for 1 min. Centrifuge for 5-10 min and use the clear upper layer.

*Reference solution (a)*. Dissolve 15 mg of *4-aminobutanoic acid R* (impurity A) in solution A and dilute to 100 mL with solution A. Dilute 10 mL of the solution to 50 mL with solution A. Take 5 mL of this solution and proceed as described for the test solution, starting from 'to a 50 mL polypropylene screw-cap centrifuge tube'.

*Reference solution (b).* Dissolve 3 mg of *sodium alendronate for system suitability CRS* (containing impurity D) in solution A and dilute to 5 mL with solution A. Take this solution and proceed as described for the test solution, starting from 'to a 50 mL polypropylene screw-cap centrifuge tube'.

*Blank solution.* Take 5 mL of solution A and proceed as described for the test solution, starting from 'to a 50 mL polypropylene screw-cap centrifuge tube'.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.1 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (10 µm);
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, buffer solution (15:85 *V/V*);
- *mobile phase B*: buffer solution, *acetonitrile R* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 18 | 100 → 50 | 0 → 50 |
| 18 - 28 | 50 → 0 | 50 → 100 |

*Flow rate*: 1.8 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 20 µL of the test solution, reference solutions (a), (b) and the blank solution.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *sodium alendronate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to alendronate (retention time = about 7 min): impurity D = about 1.4; impurity A = about 1.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to alendronate and impurity D.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.4;
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurities B and C**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 50.0 mg of *sodium alendronate trihydrate CRS* in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dissolve 3.0 g of *phosphoric acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 2.5 g of *phosphorous acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Reference solution (d).* Mix 2.0 mL of reference solution (b) with 2.0 mL of reference solution (c) and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *anion-exchange resin R1* (7 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 0.2 mL of *anhydrous formic acid R* with 1 L of *water R*; adjust to pH 3.5 with *2 M sodium hydroxide R*.

*Flow rate*: 1.2 mL/min.

*Detection*: differential refractometer.

*Injection*: 100 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of alendronate.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity C.

*Relative retention* with reference to alendronate (retention time = about 16 min): impurity B = about 1.3; impurity C = about 1.6.

*Limits*:

- *impurities B, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.5 per cent).

**Loss on drying** (*2.2.32*): 16.1 per cent to 17.1 per cent, determined on 1.000 g by drying in an oven at 140-145 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for impurities B and C with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_4H_{12}NNaO_7P_2$ taking into account the assigned content of *sodium alendronate trihydrate CRS*.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

A. 4-aminobutanoic acid (γ-aminobutyric acid),

B. phosphate,

C. phosphite,

D. unknown structure.

**01/2017:0625**

# SODIUM ALGINATE

## Natrii alginas

DEFINITION

Sodium alginate consists mainly of the sodium salt of alginic acid, which is a mixture of polyuronic acids $[(C_6H_8O_6)_n]$ composed of units of D-mannuronic acid and L-guluronic acid. Sodium alginate is obtained mainly from algae belonging to the Phaeophyceae.

CHARACTERS

*Appearance*: white or pale yellowish-brown powder.

*Solubility*: slowly soluble in water forming a viscous, colloidal solution, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 0.2 g with shaking in 20 mL of *water R*. To 5 mL of this solution add 1 mL of *calcium chloride solution R*. A voluminous gelatinous mass is formed.

B. To 10 mL of the solution prepared in identification test A add 1 mL of *dilute sulfuric acid R*. A gelatinous mass is formed.

C. To 5 mg add 5 mL of *water R*, 1 mL of a freshly prepared 10 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and 5 mL of *hydrochloric acid R*. Boil for 3 min, cool, add 5 mL of *water R*, and shake with 15 mL of *di-isopropyl ether R*. Carry out a blank test. The upper layer obtained with the substance to be examined exhibits a deeper bluish-red colour than that obtained with the blank.

D. Sulfated ash (see Tests). The residue obtained, dissolved in 2 mL of *water R*, gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.10 g in *water R* with constant stirring, dilute to 30 mL with the same solvent and allow to stand for 1 h.

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dilute 1 mL of solution S to 10 mL with *water R*.

**Chlorides**: maximum 1.0 per cent.

To 2.50 g add 50 mL of *dilute nitric acid R*, shake for 1 h and dilute to 100.0 mL with *dilute nitric acid R*. Filter. To 50.0 mL of the filtrate add 10.0 mL of *0.1 M silver nitrate* and 5 mL of *toluene R*. Titrate with *0.1 M ammonium thiocyanate*, using 2 mL of *ferric ammonium sulfate solution R2* as indicator and shaking vigorously towards the end point.

1 mL of *0.1 M silver nitrate* is equivalent to 3.545 mg of Cl.

**Calcium**: maximum 1.5 per cent.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 0.10 g in 50 mL of *dilute ammonia R2*, heating on a water-bath. Allow to cool and dilute to 100.0 mL with *distilled water R* (solution (a)). Dilute 3.0 mL of solution (a) to 100.0 mL with *distilled water R*.

*Reference solutions.* Prepare 3 reference solutions in the same manner as the test solution but add 0.75 mL, 1.0 mL and 1.5 mL respectively of *calcium standard solution (100 ppm Ca) R* to the 3.0 mL of solution (a).

Set the zero of the instrument using a mixture of 1.5 volumes of *dilute ammonia R2* and 98.5 volumes of *distilled water R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 0.1000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): 30.0 per cent to 36.0 per cent (dried substance), determined on 0.1000 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for sodium alginate used as viscosity-increasing agent or binder.*

**Apparent viscosity** (*2.2.10*). Carry out the test on a 10 g/L solution (dried substance). Determine the dynamic viscosity at 20 °C using a rotating viscometer at 20 r/min.

**01/2017:1150**

# SODIUM AMIDOTRIZOATE

## Natrii amidotrizoas

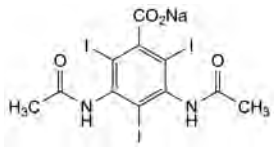

$C_{11}H_8I_3N_2NaO_4$ $M_r$ 636

[737-31-5]

DEFINITION

Sodium amidotrizoate contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of sodium 3,5-bis(acetylamino)-2,4,6-tri-iodobenzoate, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white powder, freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

It melts at about 261 °C with decomposition.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sodium amidotrizoate CRS*. Dry both the substance to be examined and the reference substance at 100 °C to 105 °C for 3 h.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (b).

C. Heat 50 mg gently in a small porcelain dish over a naked flame. Violet vapour is evolved.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Dilute 1 mL of solution S to 10 mL with *water R*. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of solution S is 7.5 to 9.5.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $GF_{254}$ plate R. Prepare the solutions in subdued light and develop the chromatograms protected from light*.

*Test solution (a).* Dissolve 0.50 g of the substance to be examined in a 3 per cent *V/V* solution of *ammonia R* in *methanol R* and dilute to 10 mL with the same solution.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a 3 per cent *V/V* solution of *ammonia R* in *methanol R*.

*Reference solution (a).* Dilute 1 mL of test solution (b) to 50 mL with a 3 per cent *V/V* solution of *ammonia R* in *methanol R*.

*Reference solution (b).* Dissolve 50 mg of *sodium amidotrizoate CRS* in a 3 per cent *V/V* solution of *ammonia R* in *methanol R* and dilute to 10 mL with the same solution.

Apply separately to the plate 2 µL of each solution. Develop over a path of 15 cm using a mixture of 20 volumes of *anhydrous formic acid R*, 25 volumes of *methyl ethyl ketone R* and 60 volumes of *toluene R*. Allow the plate to dry until the solvents have evaporated and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Free aromatic amines**. *Maintain the solutions and reagents in iced water, protected from light*. To 0.50 g in a 50 mL volumetric flask add 15 mL of *water R*. Shake and add 1 mL of *dilute sodium hydroxide solution R*. Cool in iced water, add 5 mL of a freshly prepared 5 g/L solution of *sodium nitrite R* and 12 mL of *dilute hydrochloric acid R*. Shake gently and allow to stand for exactly 2 min after adding the hydrochloric acid. Add 10 mL of a 20 g/L solution of *ammonium sulfamate R*. Allow to stand for 5 min, shaking frequently, and add 0.15 mL of a 100 g/L solution of *α-naphthol R* in *ethanol (96 per cent) R*. Shake and allow to stand for 5 min. Add 3.5 mL of *buffer solution pH 10.9 R*, mix and dilute to 50.0 mL with *water R*. The absorbance (*2.2.25*), measured within 20 min at 485 nm using as the compensation liquid a solution prepared at the same time and in the same manner but omitting the substance to be examined, is not greater than 0.30.

**Free iodine and iodides**. Not more than 50 ppm. Dissolve 1.0 g in *distilled water R* and dilute to 10 mL with the same solvent. Add dropwise *dilute nitric acid R* until the precipitation is complete, then add 3 mL of *dilute nitric acid R*. Filter and wash the precipitate with 5 mL of *water R*. Collect the filtrate and washings. Add 1 mL of *strong hydrogen peroxide solution R* and 1 mL of *methylene chloride R*. Shake. The lower layer is not more intensely coloured than a reference solution prepared simultaneously and in the same manner, using a mixture of 5 mL of *iodide standard solution (10 ppm I) R*, 3 mL of *dilute nitric acid R* and 15 mL of *water R*.

**Water** (*2.5.12*). Not more than 11.0 per cent, determined on 0.400 g by the semi-micro determination of water.

ASSAY

To 0.150 g in a 250 mL round-bottomed flask add 5 mL of *strong sodium hydroxide solution R*, 20 mL of *water R*, 1 g of *zinc powder R* and a few glass beads. Boil under a reflux condenser for 30 min. Allow to cool and rinse the condenser with 20 mL of *water R*, adding the rinsings to the flask. Filter through a sintered-glass filter (*2.1.2*) and wash the filter with several quantities of *water R*. Collect the filtrate and washings. Add 40 mL of *dilute sulfuric acid R* and titrate immediately with *0.1 M silver nitrate*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 21.20 mg of $C_{11}H_8I_3N_2NaO_4$.

STORAGE

Store protected from light.

IMPURITIES

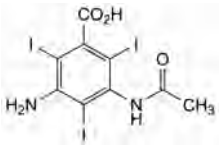

A. 3-acetylamino-5-amino-2,4,6-tri-iodobenzoic acid,

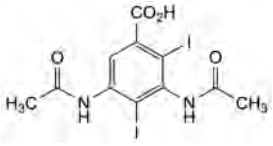

B. 3,5-bis(acetylamino)-2,4-di-iodobenzoic acid.

**01/2017:1993**
**corrected 10.0**

# SODIUM AMINOSALICYLATE DIHYDRATE

## Natrii aminosalicylas dihydricus

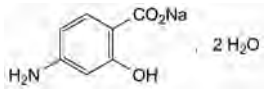

$C_7H_6NNaO_3,2H_2O$ $M_r$ 211.2
[6018-19-5]

DEFINITION

Sodium 4-amino-2-hydroxybenzoate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder or crystals, slightly hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium aminosalicylate dihydrate CRS*.

B. Introduce 0.3 g into a porcelain crucible. Cautiously heat on a small flame until vapour is evolved. Cover the crucible with a watch glass and collect the white sublimate. The melting point (*2.2.14*) of the sublimate is 120 °C to 124 °C.

C. To 0.1 mL of solution S (see Tests) add 5 mL of *water R* and 0.1 mL of *ferric chloride solution R1*. A reddish-brown colour develops.

D. 2 mL of solution S gives the reaction of primary aromatic amines (*2.3.1*).

E. 0.5 mL of solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. The freshly prepared solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_5$ (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 25 mL with the same solvent.

**pH** (*2.2.3*): 6.5 to 8.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Use freshly prepared solutions and mobile phases.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *3-aminophenol R* (impurity A) in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *mesalazine CRS* (impurity B) in *water R* and dilute to 100.0 mL with the same solvent. To 10.0 mL of this solution add 1.0 mL of reference solution (a) and dilute to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: spherical *base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: dissolve 2.2 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *water R* and dilute to 1000.0 mL with the same solvent,
- *mobile phase B*: dissolve 1.7 g of *perchloric acid R* and 1.0 g of *phosphoric acid R* in *acetonitrile R* and dilute to 1000.0 mL with the same solvent,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 30 | 100 → 40 | 0 → 60 |

*Flow rate*: 1.25 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL of the test solution and reference solution (b).

*Relative retention* with reference to 4-aminosalicylate (retention time = about 12 min): impurity A = about 0.30; impurity B = about 0.37.

*System suitability*: reference solution (b):

- *resolution*: minimum 4.0 between the peaks due to impurity A and impurity B.

*Limits*:

- *impurity A*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent),
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.10 per cent),
- *total*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (1.0 per cent),
- *disregard limit*: 0.05 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): 16.0 per cent to 17.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.150 g in 20 mL of *water R*. Add 10 mL of a 500 g/L solution of *sodium bromide R* and 25 mL of *glacial acetic acid R*. Add 5 mL of *0.1 M sodium nitrite* rapidly and continue the titration with the same titrant, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium nitrite* is equivalent to 17.52 mg of $C_7H_6NNaO_3$.

STORAGE

In an airtight container, protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A, B.*

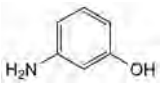

A. 3-aminophenol,

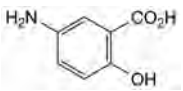

B. 5-amino-2-hydroxybenzoic acid (mesalazine).

**07/2019:1791**

# SODIUM ASCORBATE

## Natrii ascorbas

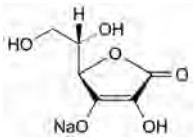

$C_6H_7NaO_6$ $M_r$ 198.1
[134-03-2]

DEFINITION

Sodium (2*R*)-2-[(1*S*)-1,2-dihydroxyethyl]-4-hydroxy-5-oxo-2,5-dihydrofuran-3-olate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish, crystalline powder or crystals.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium ascorbate CRS*.

C. To 1 mL of solution S (see Tests) add 0.2 mL of *dilute nitric acid R* and 0.2 mL of *silver nitrate solution R2*. A grey precipitate is formed.

D. 1 mL of solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*); examine the colour immediately after preparation of the solution.

**pH** (*2.2.3*): 7.0 to 8.0 determined on freshly prepared solution S.

**Specific optical rotation** (*2.2.7*): + 103 to + 108 (dried substance), determined on freshly prepared solution S.

**Impurity E**: maximum 0.2 per cent.

*Test solution.* Dissolve 0.25 g in 5 mL of *water R*. Add 1 mL of *dilute acetic acid R* and 0.5 mL of *calcium chloride solution R*.

*Reference solution.* Dissolve 70 mg of *oxalic acid R* (dihydrate of impurity E) in *water R* and dilute to 500 mL with the same solvent; to 5 mL of the solution add 1 mL of *dilute acetic acid R* and 0.5 mL of *calcium chloride solution R*.

Allow the solutions to stand for 1 h. Any opalescence in the test solution is not more intense than that in the reference solution.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Phosphate buffer solution.* Dissolve 6.8 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to about 175 mL with the same solvent. Filter through a membrane filter (nominal pore size 0.45 µm) and dilute to 1000 mL with *water for chromatography R*.

*Test solution.* Dissolve 0.500 g of the substance to be examined in the phosphate buffer solution and dilute to 10.0 mL with the phosphate buffer solution.

*Reference solution (a).* Dissolve 10.0 mg of *ascorbic acid impurity C CRS* (impurity C) in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *ascorbic acid impurity D CRS* (impurity D) and 5.0 mg of *ascorbic acid CRS* in the mobile phase, add 2.5 mL of reference solution (a) and dilute to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1 mL of the test solution to 200 mL with the mobile phase. Mix 1 mL of this solution and 1 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*: phosphate buffer solution, *acetonitrile R1* (25:75 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of ascorbic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to ascorbic acid (retention time = about 11 min): impurity D = about 0.4; impurity C = about 1.7.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to ascorbic acid and impurity C in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 20 for the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Limits*:

- *impurities C, D*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than C and D*: not more than twice the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the peak due to ascorbic acid in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 150 ppm.

To 10 mL of solution S add 2 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*.

**Copper**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.0 g in *0.1 M nitric acid* and dilute to 25.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) using *copper standard solution (10 ppm Cu) R*, diluting with *0.1 M nitric acid*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Atomisation device*: air-acetylene flame.

**Iron**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 5.0 g in *0.1 M nitric acid* and dilute to 25.0 mL with the same acid.

*Reference solutions.* Prepare the reference solutions (0.2 ppm, 0.4 ppm and 0.6 ppm) using *iron standard solution (20 ppm Fe) R*, diluting with *0.1 M nitric acid*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 0.25 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 80 mg in a mixture of 10 mL of *dilute sulfuric acid R* and 80 mL of *carbon dioxide-free water R*. Add 1 mL of *starch solution R*. Titrate with *0.05 M iodine* until a persistent violet-blue colour is obtained.

1 mL of *0.05 M iodine* is equivalent to 9.91 mg of $C_6H_7NaO_6$.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

*Specified impurities*: *C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, F, G, H.*

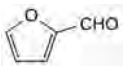


A. furan-2-carbaldehyde,

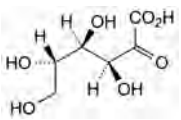


C. L-*xylo*-hex-2-ulosonic acid (L-sorbosonic acid),

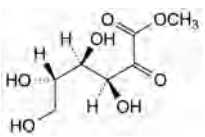


D. methyl L-*xylo*-hex-2-ulosonate (methyl L-sorbosonate),

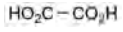

E. oxalic acid,

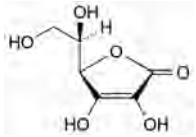

F. (5*R*)-5-[(1*R*)-1,2-dihydroxyethyl]-3,4-dihydroxyfuran-2(5*H*)-one,

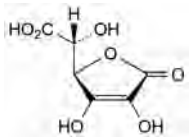

G. (*R*)-[(2*R*)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]hydroxyacetic acid,

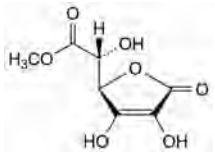

H. methyl (*R*)-[(2*R*)-3,4-dihydroxy-5-oxo-2,5-dihydrofuran-2-yl]hydroxyacetate.

**01/2008:1994**
**corrected 10.0**

# SODIUM AUROTHIOMALATE

## Natrii aurothiomalas

DEFINITION

Mixture of monosodium and disodium salts of (2*RS*)-2-(aurosulfanyl)butanedioic acid.

*Content*:

- *gold* (Au; $A_r$ 197.0): 44.5 per cent to 46.0 per cent (dried substance);
- *sodium* (Na; $A_r$ 22.99): 10.8 per cent to 11.8 per cent (dried substance).

CHARACTERS

*Appearance*: fine, pale yellow, hygroscopic powder.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Dissolve 20 mg in *water R* and dilute to 2 mL with the same solvent. Add 2 mL of *strong hydrogen peroxide solution R* and 1 mL of *sodium hydroxide solution R*. Carefully heat to boiling and boil for 30 s. A precipitate is produced that appears brownish-black by reflected light and bluish-green by transmitted light.

B. To 20 mg add 2 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

C. Ignite 100 mg, dissolve the residue in *hydrochloric acid R* and dilute to 10 mL with the same acid. Allow to stand. 5 mL of the clear supernatant gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent. Filter, seal in an ampoule and heat at 100 °C for 1 h. Cool and dilute the contents of the ampoule to 100 mL with *water R*. The solution remains clear and is not more intensely coloured than a 0.100 g/L solution of *potassium ferricyanide R*.

**pH** (*2.2.3*): 6.0 to 7.0.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *fumaric acid R* and 100.0 mg of *thiomalic acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

*Reference solution (b).* Dissolve 12.0 mg of *thiomalic acid R* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dissolve 10.0 mg of *maleic acid R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 90 volumes of a 10.5 g/L solution of *phosphoric acid R*, 100 volumes of *methanol R2* and 810 volumes of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of impurity C.

*Relative retention* with reference to impurity C (retention time = about 8 min): impurity A = about 0.4; impurity B = about 0.6. Aurothiomalate does not elute under the chromatographic conditions described.

*System suitability*: reference solution (a):

- *resolution*: minimum 5.0 between the peaks due to impurities B and C.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent).

**Glycerol**: maximum 8.0 per cent.

*Test solution.* Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dilute 0.80 g of *glycerol R* to 100.0 mL with *water R*.

*Reference solution (b).* To 2.5 mL of reference solution (a) add 7.5 mL of *water R*.

*Reference solution (c).* To 5.0 mL of reference solution (a) add 5.0 mL of *water R*.

*Reference solution (d).* To 7.5 mL of reference solution (a) add 2.5 mL of *water R*.

*Blank solution.* 10 mL of *water R*.

To the test solution, reference solutions (b), (c) and (d) and the blank solution, add 2.5 mL of a freshly prepared 235 g/L solution of *sodium hydroxide R* and mix. Add dropwise in 0.2 mL increments a 38.0 g/L solution of *cupric chloride R*, shaking vigorously after each addition, until the solutions become slightly turbid. Then add 0.2 mL of the 38.0 g/L solution of *cupric chloride R*. Stopper and shake vigorously for

1 min. Dilute to 25.0 mL with *water R* and mix. Centrifuge for 2 min. Measure the absorbance (*2.2.25*) of the supernatant solution of a 1 cm layer at 635 nm. Use the solution prepared from the blank solution as the compensation liquid. Draw a calibration curve and calculate the content of glycerol in the sample.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

ASSAY

**Gold**. Heat 0.2 g with 10 mL of *sulfuric acid R* and continue to boil gently until a clear, pale yellow liquid is produced. Cool, add about 1 mL of *nitric acid R* dropwise and boil for 1 h. Cool, dilute to 70 mL with *water R*, boil for 5 min and filter. Wash the residue of gold with *water R* at 60 °C. Dry and ignite at a temperature of at least 600 °C for 3 h. Weigh the residue and calculate the percentage content of Au.

**Sodium**. Evaporate to dryness the filtrate and washings obtained in the assay for gold, moisten with *sulfuric acid R* and ignite at 600 ± 50 °C for 3 h.

1.000 g of residue is equivalent to 0.324 g of Na.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C.*

A. (*Z*)-butenedioic acid (maleic acid),

B. (*E*)-butenedioic acid (fumaric acid),

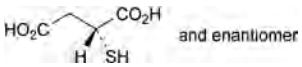


C. (2*RS*)-2-sulfanylbutanedioic acid (thiomalic acid).

**01/2017:0123**

# SODIUM BENZOATE

## Natrii benzoas

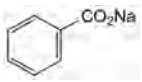

$C_7H_5NaO_2$ $M_r$ 144.1
[532-32-1]

DEFINITION

Sodium benzenecarboxylate.

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or granular powder or flakes, slightly hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (90 per cent *V/V*).

IDENTIFICATION

A. It gives reactions (b) and (c) of benzoates (*2.3.1*).

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 10 mL of *carbon dioxide-free water R* and 0.2 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M sodium hydroxide* or *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Halogenated compounds**: maximum 200 ppm for ionised chlorine and maximum 300 ppm for total chlorine.

*All glassware used must be chloride-free and may be prepared by soaking overnight in a 500 g/L solution of nitric acid R, rinsed with water R and stored full of water R. It is recommended that glassware be reserved exclusively for this test.*

*Test solution*. To 20.0 mL of solution S add 5 mL of *water R* and dilute to 50.0 mL with *ethanol (96 per cent) R*.

*Determination of ionised chlorine*

In three 25 mL volumetric flasks, prepare the following solutions.

*Solution (a)*. To 4.0 mL of the test solution add 3 mL of *dilute sodium hydroxide solution R* and 3 mL of *ethanol (96 per cent) R*. This solution is used to prepare solution A.

*Solution (b)*. To 3 mL of *dilute sodium hydroxide solution R* add 2 mL of *water R* and 5 mL of *ethanol (96 per cent) R*. This solution is used to prepare solution B.

*Solution (c)*. To 4.0 mL of *chloride standard solution (8 ppm Cl) R* add 6.0 mL of *water R*. This solution is used to prepare solution C.

In a fourth 25 mL volumetric flask, place 10 mL of *water R*. To each flask add 5 mL of *ferric ammonium sulfate solution R5*, mix and add dropwise and with swirling 2 mL of *nitric acid R* and 5 mL of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 mL with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm in a 2 cm cell the absorbance (*2.2.25*) of solution A using solution B as the compensation liquid, and the absorbance of solution C using the solution obtained with 10 mL of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C.

*Determination of total chlorine*

*Solution (a)*. To 10.0 mL of the test solution add 7.5 mL of *dilute sodium hydroxide solution R* and 0.125 g of *nickel-aluminium alloy R* and heat on a water-bath for 10 min. Allow to cool to room temperature, filter into a 25 mL volumetric flask and wash the filter with 3 quantities, each of 2 mL, of *ethanol (96 per cent) R* (a slight precipitate may form that disappears on acidification). Dilute the filtrate and washings to 25.0 mL with *water R*. This solution is used to prepare solution A.

*Solution (b)*. In the same manner, prepare a similar solution replacing the test solution by a mixture of 5 mL of *ethanol (96 per cent) R* and 5 mL of *water R*. This solution is used to prepare solution B.

*Solution (c)*. To 6.0 mL of *chloride standard solution (8 ppm Cl) R* add 4.0 mL of *water R*. This solution is used to prepare solution C.

In four 25 mL volumetric flasks, place separately 10 mL of solution (a), 10 mL of solution (b), 10 mL of solution (c) and 10 mL of *water R*. To each flask add 5 mL of *ferric ammonium sulfate solution R5*, mix and add dropwise and with swirling 2 mL of *nitric acid R* and 5 mL of *mercuric thiocyanate solution R*. Shake. Dilute the contents of each flask to 25.0 mL with *water R* and allow the solutions to stand in a water-bath at 20 °C for 15 min. Measure at 460 nm in a 2 cm cell the absorbance (*2.2.25*) of solution A

using solution B as the compensation liquid, and the absorbance of solution C using the solution obtained with 10 mL of *water R* as the compensation liquid. The absorbance of solution A is not greater than that of solution C.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.00 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.250 g in 20 mL of *anhydrous acetic acid R*, heating to 50 °C if necessary. Cool. Using 0.05 mL of *naphtholbenzein solution R* as indicator, titrate with *0.1 M perchloric acid* until a green colour is obtained.

1 mL of *0.1 M perchloric acid* is equivalent to 14.41 mg of $C_7H_5NaO_2$.

**01/2017:0190**

# SODIUM BROMIDE

## Natrii bromidum

NaBr $M_r$ 102.9
[7647-15-6]

DEFINITION

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, granular powder or small, colourless, transparent or opaque crystals, slightly hygroscopic.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of bromides (*2.3.1*).

B. Solution S (see Tests) gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromates**. To 10 mL of solution S add 1 mL of *starch solution R*, 0.1 mL of a 100 g/L solution of *potassium iodide R* and 0.25 mL of *0.5 M sulfuric acid* and allow to stand protected from light for 5 min. No blue or violet colour develops.

**Chlorides and sulfates**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 0.400 g of the substance to be examined in 50 mL of *water for chromatography R* and dilute to 100.0 mL with the same solvent.

*Test solution (b)*. Dilute 25.0 mL of test solution (a) to 50.0 mL with *water for chromatography R*.

*Reference solution (a)*. To 25.0 mL of test solution (a) add 1.0 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 12.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (b)*. Dilute 10.0 mL of test solution (a) to 100.0 mL with *water for chromatography R*. To 2.0 mL of this solution add 8.0 mL of *chloride standard solution (50 ppm Cl) R* and dilute to 20.0 mL with *water for chromatography R*.

*Blank solution*: *water for chromatography R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 2 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (13 µm).

*Mobile phase*: dissolve 0.600 g of *potassium hydroxide R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent.

*Flow rate*: 0.4 mL/min.

*Detection*: conductivity detector equipped with a suitable ion suppressor.

*Injection*: 50 µL of test solution (b), reference solutions (a) and (b) and the blank solution.

*Run time*: 2.5 times the retention time of bromide.

*Retention time*: chloride = about 5 min; bromide = about 8 min; sulfate = about 16 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 8.0 between the peaks due to chloride and bromide.

*Calculation of percentage contents*:

- for chlorides, use the concentration of chloride in reference solution (a); correct the area of the peak due to chloride in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to chloride in the chromatogram obtained with test solution (b);
- for sulfates, use the concentration of sulfate in reference solution (a); correct the area of the peak due to sulfate in the chromatogram obtained with reference solution (a) by subtracting the area of the peak due to sulfate in the chromatogram obtained with test solution (b).

*Limits*:

- *chlorides*: maximum 0.6 per cent;
- *sulfates*: maximum 0.01 per cent.

**Iodides**. To 5 mL of solution S add 0.15 mL of *ferric chloride solution R1* and 2 mL of *methylene chloride R*. Shake and allow to separate. The lower layer is colourless (*2.2.2, Method I*).

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium and alkaline-earth metals** (*2.4.7*): maximum 200 ppm, calculated as Ca.

10.0 g complies with the test for magnesium and alkaline-earth metals. The volume of *0.01 M sodium edetate* used does not exceed 5.0 mL.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 85.0 mg in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 10.29 mg of NaBr.

Calculate the percentage content of NaBr using the following expression:

$$a - 2.902\,b$$

*a* = percentage content of NaBr and NaCl obtained in the assay and calculated as NaBr;

*b* = percentage content of Cl obtained in the test for chlorides.

STORAGE

In an airtight container.

**01/2017:0231**

# SODIUM CALCIUM EDETATE

## Natrii calcii edetas

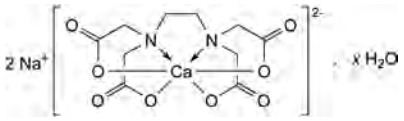

$C_{10}H_{12}CaN_2Na_2O_8,xH_2O$ $M_r$ 374.3 (anhydrous substance)
[62-33-9]

DEFINITION

Disodium [(ethylenedinitrilo)tetraacetato]calciate(2−).

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water of crystallisation.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *sodium calcium edetate CRS*.

B. Dissolve 2 g in 10 mL of *water R*, add 6 mL of *lead nitrate solution R*, shake and add 3 mL of *potassium iodide solution R*. No yellow precipitate is formed. Make alkaline to *red litmus paper R* by the addition of *dilute ammonia R2* and add 3 mL of *ammonium oxalate solution R*. A white precipitate is formed.

C. Ignite. The residue gives reaction (b) of calcium (*2.3.1*).

D. Dissolve 0.5 g in 10 mL of *water R* and add 10 mL of *potassium pyroantimonate solution R*. A white, crystalline precipitate is formed. The formation of the precipitate is accelerated by rubbing the wall of the tube with a glass rod.

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 8.0.

Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Impurity A**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture.* Dissolve 10.0 g of *ferric sulfate pentahydrate R* in 20 mL of *0.5 M sulfuric acid* and add 780 mL of *water R*. Adjust to pH 2.0 with *1 M sodium hydroxide* and dilute to 1000 mL with *water R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution.* Dissolve 40.0 mg of *nitrilotriacetic acid R* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. To 1.0 mL of this solution add 0.1 mL of the test solution and dilute to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: spherical *graphitised carbon for chromatography R1* (5 µm) with a specific surface area of 120 $m^2$/g and a pore size of 25 nm.

*Mobile phase*: dissolve 50.0 mg of *ferric sulfate pentahydrate R* in 50 mL of *0.5 M sulfuric acid* and add 750 mL of *water R*; adjust to pH 1.5 with *0.5 M sulfuric acid* or *1 M sodium hydroxide*, add 20 mL of *ethylene glycol R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 273 nm.

*Injection*: 20 µL; filter the solutions and inject immediately.

*Run time*: 4 times the retention time of the iron complex of impurity A.

*Retention time*: iron complex of impurity A = about 5 min; iron complex of edetic acid = about 10 min.

*System suitability*: reference solution:

– *resolution*: minimum 7 between the peaks due to the iron complex of impurity A and the iron complex of edetic acid;
– *signal-to-noise ratio*: minimum 50 for the peak due to impurity A.

*Limit*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Disodium edetate**: maximum 1.0 per cent.

Dissolve 5.0 g in 250 mL of *water R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Not more than 1.5 mL of *0.1 M magnesium chloride* is required to change the colour of the indicator to violet.

**Chlorides**: maximum 0.1 per cent.

Dissolve 0.7 g in *water R* and dilute to 20 mL with the same solvent. Add 30 mL of *dilute nitric acid R*, allow to stand for 30 min and filter. Dilute 10 mL of the filtrate to 50 mL with *water R*. Use this solution as the test solution. Prepare the reference solution using 0.40 mL of *0.01 M hydrochloric acid*, add 6 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Filter both solutions if necessary. Add 1 mL of *silver nitrate solution R2* to the test solution and to the reference solution and mix. After standing for 5 min protected from light, any opalescence in the test solution is not more intense than that in the reference solution.

**Iron** (*2.4.9*): maximum 80 ppm.

Dilute 2.5 mL of solution S to 10 mL with *water R*. Add 0.25 g of *calcium chloride R* to the test solution and the standard before the addition of the *thioglycollic acid R*.

**Water** (*2.5.12*): 5.0 per cent to 13.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.500 g in *water R* and dilute to 200 mL with the same solvent. To 20.0 mL of this solution, add 80 mL of *water R* and adjust to pH 2 with *dilute nitric acid R*. Titrate with *0.01 M bismuth nitrate*, using 0.1 mL of a 1 g/L solution of *xylenol orange R* as indicator. The colour of the solution changes from yellow to red.

1 mL of *0.01 M bismuth nitrate* is equivalent to 3.74 mg of $C_{10}H_{12}CaN_2Na_2O_8$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

Monographs S

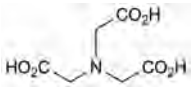

A. nitrilotriacetic acid.

**01/2017:1471**

# SODIUM CAPRYLATE

## Natrii caprylas

$C_8H_{15}NaO_2$ $M_r$ 166.2
[1984-06-1]

DEFINITION

Sodium octanoate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble or freely soluble in water, freely soluble in acetic acid, sparingly soluble in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. To 0.2 mL of solution S (see Tests) add 0.3 mL of *water R*. The solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 8.0 to 10.5 for solution S.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 0.116 g in *water R* and dilute to 5 mL with the same solvent. Add 1 mL of a 2.8 per cent *V/V* solution of *sulfuric acid R* and shake with 10 mL of *ethyl acetate R*. Separate the organic layer and dry over *anhydrous sodium sulfate R*.

*Reference solution (a).* Dissolve 0.10 g of *caprylic acid CRS* in *ethyl acetate R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of the test solution to 100 mL with *ethyl acetate R*. Dilute 5 mL of this solution to 50 mL with *ethyl acetate R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 100 |
| | 1 - 25 | 100 → 220 |
| | 25 - 35 | 220 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 5 for the principal peak.

*Limits*:

- *any impurity*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 1.000 g.

ASSAY

Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.62 mg of $C_8H_{15}NaO_2$.

IMPURITIES

A. hexanoic acid,

B. heptanoic acid,

C. nonanoic acid,

D. decanoic acid,

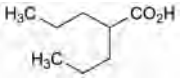

E. 2-propylpentanoic acid (valproic acid),

F. methyl octanoate,

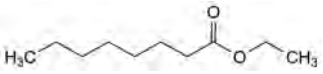

G. ethyl octanoate,

H. methyl decanoate,

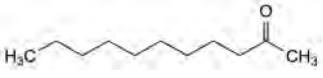

I. undecan-2-one,

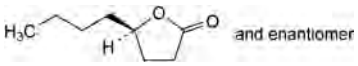


J. (*RS*)-5-butyltetrahydrofuran-2-one (γ-hydroxyoctanoic acid lactone).

**01/2017:0773**

# SODIUM CARBONATE

## Natrii carbonas

$Na_2CO_3$ $M_r$ 106.0
[497-19-8]

DEFINITION

*Content*: 99.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, slightly granular powder, hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in *water R* and dilute to 10 mL with the same solvent. The solution is strongly alkaline (*2.2.4*).

B. The solution prepared for identification test A gives the reaction of carbonates (*2.3.1*).

C. The solution prepared for identification test A gives reaction (a) of sodium (*2.3.1*).

D. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.0 g in portions in a mixture of 5 mL of *hydrochloric acid R* and 25 mL of *distilled water R*. Heat to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

Dissolve 2.0 g in 10 mL of *water R*.

**Alkali hydroxides and bicarbonates**. Dissolve 0.4 g in 20 mL of *water R*. Add 20 mL of *barium chloride solution R1* and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. The solution does not become red. Boil the remainder of the filtrate for 2 min. The solution remains clear (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 125 ppm.

Dissolve 0.4 g in *water R*, add 4 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 250 ppm, determined on solution S.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying at 300 ± 15 °C.

ASSAY

Dissolve 0.400 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection.

1 mL of *1 M hydrochloric acid* is equivalent to 52.99 mg of $Na_2CO_3$.

STORAGE

In an airtight container.

**01/2017:0191**

# SODIUM CARBONATE DECAHYDRATE

## Natrii carbonas decahydricus

$Na_2CO_3,10H_2O$ $M_r$ 286.1
[6132-02-1]

DEFINITION

*Content*: 36.7 per cent to 40.0 per cent of $Na_2CO_3$.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless, transparent crystals, efflorescent.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in *water R* and dilute to 10 mL with the same solvent. The solution is strongly alkaline (*2.2.4*).

B. The solution prepared for identification test A gives the reaction of carbonates (*2.3.1*).

C. The solution prepared for identification test A gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in portions in a mixture of 5 mL of *hydrochloric acid R* and 25 mL of *distilled water R*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

Dissolve 4.0 g in 10 mL of *water R*.

**Alkali hydroxides and bicarbonates**. Dissolve 1.0 g in 20 mL of *water R*, add 20 mL of *barium chloride solution R1* and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. The solution does not become red. Heat the remainder of the filtrate to boiling for 2 min. The solution remains clear (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dissolve 1.0 g in *water R*, add 4 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

ASSAY

Dissolve 1.000 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection.

1 mL of *1 M hydrochloric acid* is equivalent to 52.99 mg of $Na_2CO_3$.

STORAGE

In an airtight container.

**01/2017:0192**

# SODIUM CARBONATE MONOHYDRATE

## Natrii carbonas monohydricus

$Na_2CO_3,H_2O$ $M_r$ 124.0

[5968-11-6]

DEFINITION

*Content*: 83.0 per cent to 87.5 per cent of $Na_2CO_3$.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 1 g in *water R* and dilute to 10 mL with the same solvent. The solution is strongly alkaline (*2.2.4*).

B. The solution prepared for identification test A gives the reaction of carbonates (*2.3.1*).

C. The solution prepared for identification test A gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in portions in a mixture of 5 mL of *hydrochloric acid R* and 25 mL of *distilled water R*. Heat the solution to boiling and cool. Add *dilute sodium hydroxide solution R* until the solution is neutral and dilute to 50 mL with *distilled water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

Dissolve 2.0 g in 10 mL of *water R*.

**Alkali hydroxides and bicarbonates**. Dissolve 0.4 g in 20 mL of *water R*, add 20 mL of *barium chloride solution R1* and filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. The solution does not become red. Heat the remainder of the filtrate to boiling for 2 min. The solution remains clear (*2.2.1*).

**Chlorides** (*2.4.4*): maximum 125 ppm.

Dissolve 0.4 g in *water R*, add 4 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 250 ppm, determined on solution S.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm, determined on 5 mL of solution S.

**Iron** (*2.4.9*): maximum 50 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

ASSAY

Dissolve 0.500 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection.

1 mL of *1 M hydrochloric acid* is equivalent to 52.99 mg of $Na_2CO_3$.

STORAGE

In an airtight container.

**04/2018:0847**
**corrected 10.0**

# SODIUM CETOSTEARYL SULFATE

## Natrii cetylo- et stearylosulfas

DEFINITION

Mixture of sodium cetyl sulfate (sodium hexadecyl sulfate, $C_{16}H_{33}NaO_4S$; $M_r$ 344.5) and sodium stearyl sulfate (sodium octadecyl sulfate, $C_{18}H_{37}NaO_4S$; $M_r$ 372.5). A suitable buffer may be added.

*Content*:

– *sodium cetostearyl sulfate*: minimum 90.0 per cent (anhydrous substance);

– *sodium cetyl sulfate*: minimum 40.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or pale yellow, amorphous or crystalline powder.

*Solubility*: soluble in hot water giving an opalescent solution, practically insoluble in cold water, partly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, D, F.*

*Second identification: A, C, D, E, F.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 50 mg of the substance to be examined in 10 mL of *ethanol (70 per cent V/V) R*, heating on a water-bath.

*Reference solution*. Dissolve 50 mg of *sodium cetostearyl sulfate CRS* in 10 mL of *ethanol (70 per cent V/V) R*, heating on a water-bath.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *acetone R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 50 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*; heat at 120 °C until spots appear (about 5 min).

*Results*: the principal spots in the chromatogram obtained with the test solution are similar in position and colour to the principal spots in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with test solution (b) are similar in retention time to the 2 principal peaks in the chromatograms obtained with reference solutions (a) and (b).

C. Dissolve 0.1 g in 10 mL of *water R* and shake. A foam is formed.

D. It gives a yellow colour to a non-luminous flame.

E. To 0.1 mL of the solution prepared for identification test C add 0.1 mL of a 1 g/L solution of *methylene blue R* and 2 mL of *dilute sulfuric acid R*. Add 2 mL of *methylene chloride R* and shake. The methylene chloride layer has an intense blue colour.

F. Mix about 10 mg with 10 mL of *anhydrous ethanol R*. Heat to boiling on a water-bath, shaking frequently. Filter immediately and evaporate to dryness. Dissolve the residue in 7 mL of *water R*, add 3 mL of *dilute hydrochloric acid R*

and evaporate the solution to half its volume. Allow to cool. Filter. To the filtrate add 1 mL of *barium chloride solution R1*. A white, crystalline precipitate is formed.

TESTS

**Acidity or alkalinity**. Dissolve 0.5 g with heating in a mixture of 10 mL of *water R* and 15 mL of *ethanol (90 per cent V/V) R*. Add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Add 0.1 mL of *0.1 M sodium hydroxide*. The solution is red.

**Sodium chloride and sodium sulfate**: maximum 8.0 per cent for the sum of the percentage contents.

*Sodium chloride*. Dissolve 5.00 g in 50 mL of *water R*, add *dilute nitric acid R* dropwise until the solution is neutral to *blue litmus paper R*. Titrate with *0.1 M silver nitrate* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

*Sodium sulfate*. Dissolve 0.500 g in 20 mL of *water R*, warming gently if necessary, and add 1 mL of a 0.5 g/L solution of *dithizone R* in *acetone R*. If the solution is red, add *1 M nitric acid* dropwise until a bluish-green colour is obtained. Add 2.0 mL of *dichloroacetic acid solution R* and 80 mL of *acetone R*. Titrate with *0.01 M lead nitrate* until a persistent orange-red colour is obtained.

1 mL of *0.01 M lead nitrate* is equivalent to 1.420 mg of $Na_2SO_4$.

**Free cetostearyl alcohol**: maximum 4.0 per cent.

From the chromatogram obtained with test solution (a) in the assay, calculate the percentage content of free cetostearyl alcohol in the substance to be examined using the following expression and taking into account the assigned content of the chemical reference substances:

$$\frac{A_1}{A_2} \times \frac{m_2}{m_1} \times \frac{2}{100} \times 100$$

$A_1$ = sum of the areas of the peaks due to cetyl alcohol and stearyl alcohol in the chromatogram obtained with test solution (a);

$A_2$ = area of the peak due to the internal standard in the chromatogram obtained with test solution (a);

$m_1$ = mass of the substance to be examined in test solution (a), in milligrams;

$m_2$ = mass of the internal standard in the internal standard solution, in milligrams.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 0.200 g of *1-nonadecanol CRS* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Test solution (a)*. Dissolve 0.300 g of the substance to be examined in 50.0 mL of *anhydrous ethanol R* and add 2.0 mL of the internal standard solution and 48.0 mL of *water R*. Shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers. Reserve the lower layer for the preparation of test solution (b). Wash the combined upper layers with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Test solution (b)*. Transfer 25.0 mL of the lower layer obtained in the preparation of test solution (a) to a 200 mL flask that can be fitted with a reflux condenser. Add 10.0 mL of the internal standard solution and 20 mL of *hydrochloric acid R*. Boil under a reflux condenser for 2 h. Allow to cool and shake with 4 quantities, each of 20 mL, of *pentane R*. Combine the upper layers, wash with 2 quantities, each of 20 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (a)*. Dissolve 0.100 g of *cetyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Reference solution (b)*. Dissolve 0.100 g of *stearyl alcohol CRS* in 25.0 mL of the internal standard solution. Add 25 mL of *water R* and shake with 4 quantities, each of 25 mL, of *pentane R*, adding *sodium chloride R*, if necessary, to facilitate the separation of the layers. Combine the upper layers, wash with 2 quantities, each of 30 mL, of *water R*, dry over *anhydrous sodium sulfate R* and filter.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Elution order*: cetyl alcohol, stearyl alcohol, 1-nonadecanol.

Calculate the percentage content of sodium cetyl sulfate in the substance to be examined using the following expression and taking into account the assigned content of *cetyl alcohol CRS*:

$$A_x \times \frac{A_2}{A_1} \times \frac{m_{x,y}}{A_{x,y}} \times \frac{1}{m} \times 100 \times 4 \times \frac{1}{2.5} \times F_x$$

$A_x$ = area of the peak due to cetyl alcohol in the chromatogram obtained with test solution (b);

$A_{x,y}$ = area of the peak due to *cetyl alcohol CRS* in the chromatogram obtained with reference solution (a);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with test solution (b);

$A_2$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a);

$F_x$ = conversion factor from cetyl alcohol to sodium cetyl sulfate (1.421);

$m$ = mass of the substance to be examined in test solution (a), in milligrams;

$m_{x,y}$ = mass of *cetyl alcohol CRS* in reference solution (a), in milligrams.

Calculate the percentage content of sodium stearyl sulfate in the substance to be examined using the following expression and taking into account the assigned content of *stearyl alcohol CRS*:

$$A_z \times \frac{A_3}{A_1} \times \frac{m_{z,y}}{A_{z,y}} \times \frac{1}{m} \times 100 \times 4 \times \frac{1}{2.5} \times F_z$$

$A_z$ = area of the peak due to stearyl alcohol in the chromatogram obtained with test solution (b);

$A_{z,y}$ = area of the peak due to *stearyl alcohol CRS* in the chromatogram obtained with reference solution (b);

$A_1$ = area of the peak due to the internal standard in the chromatogram obtained with test solution (b);

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (b);

$F_z$ = conversion factor from stearyl alcohol to sodium stearyl sulfate (1.377);

$m$ = mass of the substance to be examined in test solution (a), in milligrams;

$m_{z,y}$ = mass of *stearyl alcohol CRS* in reference solution (b), in milligrams.

The percentage content of sodium cetostearyl sulfate corresponds to the sum of the percentage contents of sodium cetyl sulfate and sodium stearyl sulfate.

LABELLING

The label states, where appropriate, the name and concentration of any added buffer.

**01/2017:0193**

# SODIUM CHLORIDE[(1)]

## Natrii chloridum

NaCl $M_r$ 58.44
[7647-14-5]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals or white or almost white pearls.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol.♦

IDENTIFICATION

A. It gives reaction (a) of chlorides (*2.3.1*).

B. It gives the reactions of sodium (*2.3.1*).

TESTS

◊*If the substance is in the form of pearls, crush before use.*◊

**Solution S**. Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

♦**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).♦

**Acidity or alkalinity**. To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Bromides**: maximum 100 ppm.

To 0.5 mL of solution S add 4.0 mL of *water R*, 2.0 mL of *phenol red solution R2* and 1.0 mL of a 0.1 g/L solution of *chloramine R* and mix immediately. After exactly 2 min, add 0.15 mL of *0.1 M sodium thiosulfate*, mix and dilute to 10.0 mL with *water R*. The absorbance (*2.2.25*) of the solution measured at 590 nm, using *water R* as the compensation liquid, is not greater than that of a standard prepared at the same time and in the same manner, using 5.0 mL of a 3.0 mg/L solution of *potassium bromide R*.

**Ferrocyanides**. Dissolve 2.0 g in 6 mL of *water R*. Add 0.5 mL of a mixture of 5 mL of a 10 g/L solution of *ferric ammonium sulfate R* in a 2.5 g/L solution of *sulfuric acid R* and 95 mL of a 10 g/L solution of *ferrous sulfate R*. No blue colour develops within 10 min.

**Iodides**. Moisten 5 g by the dropwise addition of a freshly prepared mixture of 0.15 mL of *sodium nitrite solution R*, 2 mL of *0.5 M sulfuric acid*, 25 mL of *iodide-free starch solution R* and 25 mL of *water R*. After 5 min, examine in daylight. The mixture shows no blue colour.

**Nitrites**. To 10 mL of solution S add 10 mL of *water R*. The absorbance (*2.2.25*) is not greater than 0.01 at 354 nm.

**Phosphates** (*2.4.11*): maximum 25 ppm.

Dilute 2 mL of solution S to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 30 mL with *distilled water R*.

**Aluminium** (*2.4.17*): maximum 0.2 ppm, if intended for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions.

*Prescribed solution*. Dissolve 20.0 g in 100 mL of *water R* and add 10 mL of *acetate buffer solution pH 6.0 R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *water R*.

♦**Arsenic** (*2.4.2, Method A*): maximum 1 ppm, determined on 5 mL of solution S.♦

**Barium**. To 5 mL of solution S add 5 mL of *distilled water R* and 2 mL of *dilute sulfuric acid R*. After 2 h, any opalescence in the solution is not more intense than that in a mixture of 5 mL of solution S and 7 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 2 ppm, determined on solution S.

Prepare the standard using a mixture of 4 mL of *iron standard solution (1 ppm Fe) R* and 6 mL of *water R*.

**Magnesium and alkaline-earth metals** (*2.4.7*): maximum 100 ppm, calculated as Ca and determined on 10.0 g.

Use 0.150 g of *mordant black 11 triturate R*. The volume of *0.01 M sodium edetate* used is not more than 2.5 mL.

**Potassium**: maximum 500 ppm, if intended for use in the manufacture of parenteral preparations or haemodialysis, haemofiltration or peritoneal dialysis solutions.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. Dissolve 1.00 g in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solutions*. Dissolve 1.144 g of *potassium chloride R*, previously dried at 100-105 °C for 3 h, in *water R* and dilute to 1000.0 mL with the same solvent (600 µg of K per millilitre). Dilute as required.

*Wavelength*: 766.5 nm.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

♦**Bacterial endotoxins** (*2.6.14*): less than 5 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.♦

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

ASSAY

Dissolve 50.0 mg in *water R* and dilute to 50 mL with the same solvent. Titrate with *0.1 M silver nitrate* determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

♦LABELLING

The label states:

– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;

– where applicable, that the substance is suitable for use in the manufacture of peritoneal dialysis solutions, haemodialysis solutions or haemofiltration solutions. ♦

**01/2017:0412**

# SODIUM CITRATE

## Natrii citras

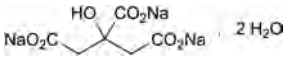

$C_6H_5Na_3O_7,2H_2O$ $M_r$ 294.1
[6132-04-3]

DEFINITION

Trisodium 2-hydroxypropane-1,2,3-tricarboxylate dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or white or almost white, granular crystals, slightly deliquescent in moist air.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. To 1 mL of solution S (see Tests) add 4 mL of *water R*. The solution gives the reaction of citrates (*2.3.1*).

B. 1 mL of solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. Not more than 0.2 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Readily carbonisable substances**. To 0.20 g of the powdered substance to be examined add 10 mL of *sulfuric acid R* and heat in a water-bath at 90 ± 1 °C for 60 min. Cool rapidly. The solution is not more intensely coloured than reference solution $Y_2$ or $GY_2$ (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Oxalates**: maximum 300 ppm.

Dissolve 0.50 g in 4 mL of *water R*, add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules and heat on a water-bath for 1 min. Allow to stand for 2 min, decant the liquid into a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of *potassium ferricyanide solution R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 mL of a 50 mg/L solution of *oxalic acid R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

To 10 mL of solution S add 2 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*.

**Water** (*2.5.12*): 11.0 per cent to 13.0 per cent, determined on 0.300 g. Use as the solvent a mixture of 20 mL of *methanol R*, 30 mL of *formamide R* and 5 g of *salicylic acid R*.

**Pyrogens** (*2.6.8*). If intended for use in the manufacture of large-volume parenteral preparations, the competent authority may require that it comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of a freshly prepared solution in *water for injections R* containing per millilitre 10.0 mg of the substance to be examined and 7.5 mg of pyrogen-free *calcium chloride R*.

ASSAY

Dissolve 0.150 g in 20 mL of *anhydrous acetic acid R*, heating to about 50 °C. Allow to cool. Titrate with *0.1 M perchloric acid*, using 0.25 mL of *naphtholbenzein solution R* as indicator until a green colour is obtained.

1 mL of *0.1 M perchloric acid* is equivalent to 8.602 mg of $C_6H_5Na_3O_7$.

STORAGE

In an airtight container.

**01/2017:0562**

# SODIUM CROMOGLICATE

## Natrii cromoglicas

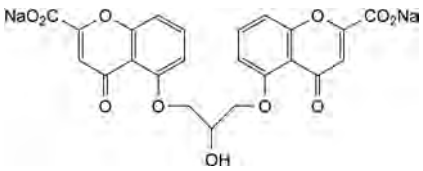

$C_{23}H_{14}Na_2O_{11}$ $M_r$ 512.3
[15826-37-6]

DEFINITION

Disodium 5,5′-[(2-hydroxypropane-1,3-diyl)dioxy]bis(4-oxo-4*H*-1-benzopyran-2-carboxylate).

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in *phosphate buffer solution pH 7.4 R* and dilute to 100.0 mL with the same buffer solution. Dilute 10.0 mL of this solution to 100.0 mL with *phosphate buffer solution pH 7.4 R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 239 nm and 327 nm.

*Absorbance ratio*: $A_{327}/A_{239}$ = 0.25 to 0.30.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium cromoglicate CRS*.

C. Dissolve about 5 mg in 0.5 mL of *methanol R*. Add 3 mL of a 5 g/L solution of *aminopyrazolone R* in *methanol R* containing 1 per cent *V/V* of *hydrochloric acid R*. Allow to stand for 5 min. An intense yellow colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.4 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.25 mL of *methyl red solution R*. The solution is red.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water R*, *acetonitrile R* (40:60 *V/V*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 7 mg of *sodium cromoglicate for system suitability CRS* (containing impurity C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, 10 g/L solution of *tetrabutylammonium hydrogen sulfate R* (5:95 *V/V*);
- *mobile phase B*: *acetonitrile R*, 10 g/L solution of *tetrabutylammonium hydrogen sulfate R* (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 → 0 | 0 → 100 |
| 15 - 20 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 330 nm.

*Injection*: 10 µL.

*Relative retention* with reference to sodium cromoglicate (retention time = about 11 min): impurity C = about 1.1.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to sodium cromoglicate and impurity C.

*Limits*:

- *impurity C*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Oxalates**: maximum 0.35 per cent.

Dissolve 0.10 g in 20 mL of *water R*, add 5.0 mL of *iron salicylate solution R* and dilute to 50.0 mL with *water R*. Measure the absorbance (*2.2.25*) at 480 nm. The absorbance is not less than that of a reference solution prepared in the same manner using 0.35 mg of *oxalic acid R* instead of the substance to be examined.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying at 105 °C and at a pressure of 0.3-0.6 kPa.

ASSAY

Dissolve 0.200 g with heating in a mixture of 5 mL of *2-propanol R* and 25 mL of *ethylene glycol R*. Cool and add a mixture of 6 mL of *tetrahydrofuran R* and 24 mL of *acetonitrile R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.62 mg of $C_{23}H_{14}Na_2O_{11}$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *C*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B*.

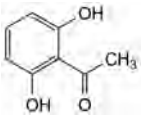

A. 1-(2,6-dihydroxyphenyl)ethanone,

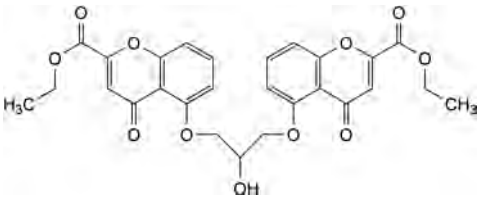

B. diethyl 5,5′-[(2-hydroxypropane-1,3-diyl)dioxy]bis(4-oxo-4*H*-1-benzopyran-2-carboxylate),

C. unknown structure.

**01/2017:0774**
**corrected 10.0**

# SODIUM CYCLAMATE

## Natrii cyclamas

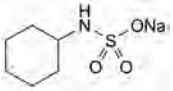

$C_6H_{12}NNaO_3S$ $M_r$ 201.2
[139-05-9]

DEFINITION

Sodium *N*-cyclohexylsulfamate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sodium cyclamate CRS*.

B. Examine the chromatograms obtained in the test for impurity A.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 1 mL of solution S (see Tests), add 1 mL of *water R* and 2 mL of *silver nitrate solution R1*, then shake. A white, crystalline precipitate is formed.

D. To 1 mL of solution S add 5 mL of *water R*, 2 mL of *dilute hydrochloric acid R* and 4 mL of *barium chloride solution R1* and mix. The solution is clear. Add 2 mL of *sodium nitrite solution R*. A voluminous white precipitate is formed and gas is given off.

E. A mixture of 1 mL of solution S and 1 mL of *water R* gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 7.5 for solution S.

**Absorbance** (*2.2.25*): maximum 0.10, determined at 270 nm on solution S.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Solution S.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *water R*.

*Reference solution (a).* Dissolve 0.10 g of *sodium cyclamate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *sulfamic acid R* (impurity A) in *water R* and dilute to 100 mL with the same solvent.

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: *concentrated ammonia R*, *water R*, *ethyl acetate R*, *propanol R* (10:10:20:70 *V/V/V/V*).

*Application*: 2 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of warm air, then heat at 105 °C for 5 min.

*Detection*: spray the hot plate with *strong sodium hypochlorite solution R* diluted to a concentration of 5 g/L of active chlorine. Place in a current of cold air until an area of coating below the points of application gives at most a faint blue colour with a drop of *potassium iodide and starch solution R*; avoid prolonged exposure to cold air. Spray with *potassium iodide and starch solution R* and examine the chromatograms within 5 min.

*Limit*: test solution (a):

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Impurities B, C and D**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 2 µL of *tetradecane R* in *methylene chloride R* and dilute to 100 mL with the same solvent.

*Test solution.* Dissolve 2.00 g of the substance to be examined in 20 mL of *water R*, add 0.5 mL of *strong sodium hydroxide solution R* and shake with 30 mL of *toluene R*. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of *dilute acetic acid R* and *water R*. Separate the lower layer, add 0.5 mL of *strong sodium hydroxide solution R* and 0.5 mL of the internal standard solution and shake. Use the lower layer immediately after separation.

*Reference solution.* Dissolve 10.0 mg (about 12 µL) of *cyclohexylamine R* (impurity C), 1.0 mg (about 1.1 µL) of *dicyclohexylamine R* (impurity D) and 1.0 mg (about 1 µL) of *aniline R* (impurity B) in *water R*, then dilute to 1000 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *water R* (solution A). To 20.0 mL of solution A, add 0.5 mL of *strong sodium hydroxide solution R* and extract with 30 mL of *toluene R*. Shake 20 mL of the upper layer with 4 mL of a mixture of equal volumes of *dilute acetic acid R* and *water R*. Separate the lower layer, add 0.5 mL of *strong sodium hydroxide solution R* and 0.5 mL of the internal standard solution and shake. Use the lower layer immediately after separation.

*Column*:

– *material*: fused silica;
– *size*: *l* = 25 m, Ø = 0.32 mm;
– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.51 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.8 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 85 |
| | 1 - 9 | 85 → 150 |
| | 9 - 13 | 150 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1.5 µL; use a split vent at a flow rate of 20 mL/min.

*Relative retention* with reference to impurity C (retention time = about 2.3 min): impurity B = about 1.4; tetradecane = about 4.3; impurity D = about 4.5.

*Limits*:

– *impurity C*: maximum 10 ppm;
– *impurities B, D*: for each impurity, maximum 1 ppm.

**Sulfates** (*2.4.13*): maximum 0.1 per cent.

Dilute 1.5 mL of solution S to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

ASSAY

Dissolve without heating 0.150 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.12 mg of $C_6H_{12}NNaO_3S$.

IMPURITIES

*Specified impurities: A, B, C, D.*

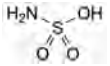

A. sulfamic acid,

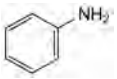

B. aniline (phenylamine),

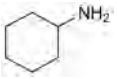

C. cyclohexanamine,

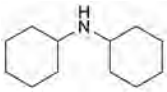

D. *N*-cyclohexylcyclohexanamine.

**01/2017:0194**

# SODIUM DIHYDROGEN PHOSPHATE DIHYDRATE

## Natrii dihydrogenophosphas dihydricus

$NaH_2PO_4,2H_2O$ $M_r$ 156.0
[13472-35-0]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: very soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly acid (*2.2.4*).

B. Solution S gives the reactions of phosphates (*2.3.1*).

C. Solution S previously neutralised using a 100 g/L solution of *potassium hydroxide R* gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.2 to 4.5.

To 5 mL of solution S add 5 mL of *carbon dioxide-free water R*.

**Reducing substances**. To 5 mL of solution S add 0.25 mL of *0.02 M potassium permanganate* and 5 mL of *dilute sulfuric acid R* and heat in a water-bath for 5 min. The colour of the permanganate is not completely discharged.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 5 mL of solution S add 0.5 mL of *hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): 21.5 per cent to 24.0 per cent, determined on 0.50 g by drying in an oven at 130 °C.

ASSAY

Dissolve 2.500 g in 40 mL of *water R*. Titrate with carbonate-free *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 0.120 g of $NaH_2PO_4$.

**01/2017:2134**

# SODIUM ETHYL PARAHYDROXYBENZOATE

## Ethylis parahydroxybenzoas natricus

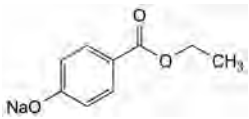

$C_9H_9NaO_3$ $M_r$ 188.2
[35285-68-8]

DEFINITION

Sodium 4-(ethoxycarbonyl)phenolate.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, soluble in anhydrous ethanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.5 g in 50 mL of *water R*. Immediately add 5 mL of *hydrochloric acid R1*. Filter and wash the precipitate with *water R*. Dry *in vacuo* at 80 °C for 2 h. It melts (*2.2.14*) at 115 °C to 118 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: the precipitate obtained in identification A.
*Comparison*: *ethyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 0.100 g of the substance to be examined in 10 mL of *water R*. Immediately add 2 mL of *hydrochloric acid R* and shake with 50 mL of *methylene chloride R*. Evaporate the lower layer to dryness and take up the residue with 10 mL of *acetone R*.
*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.
*Reference solution (a).* Dissolve 5 mg of *ethyl parahydroxybenzoate CRS* in *acetone R* and dilute to 5 mL with the same solvent.
*Reference solution (b).* Dissolve 5 mg of *methyl parahydroxybenzoate CRS* (impurity B) in 0.5 mL of test solution (a) and dilute to 5 mL with *acetone R*.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: *glacial acetic acid R, water R, methanol R* (1:30:70 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 mL of solution S (see Tests) add 1 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S, examined immediately after preparation, is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.5 to 10.5.

Dilute 1 mL of solution S to 100 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A), 5 mg of *methyl parahydroxybenzoate R* (impurity B) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 50.0 mg of *ethyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 4 times the retention time of ethyl parahydroxybenzoate.

*Relative retention* with reference to ethyl parahydroxybenzoate (retention time = about 3 min): impurity A = about 0.5; impurity B = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity B and ethyl parahydroxybenzoate.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;

– *impurity A*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

– *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 350 ppm.

To 10 mL of solution S add 30 mL of *water R* and 1 mL of *nitric acid R* and dilute to 50 mL with *water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*. Prepare the standard using a mixture of 1 mL of *water R* and 14 mL of *chloride standard solution (5 ppm Cl) R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 25 mL of solution S add 5 mL of *distilled water R* and 10 mL of *hydrochloric acid R* and dilute to 50 mL with *distilled water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_9H_9NaO_3$ from the declared content of *ethyl parahydroxybenzoate CRS*, multiplied by a correction factor of 1.132.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

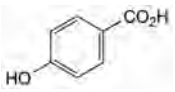


A. 4-hydroxybenzoic acid,

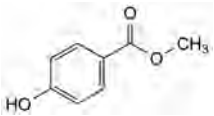


B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

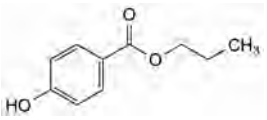


C. propyl 4-hydroxybenzoate (propyl parahydroxybenzoate),

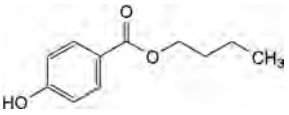


D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

Monographs S

07/2016:0514

# SODIUM FLUORIDE

## Natrii fluoridum

NaF $M_r$ 41.99
[7681-49-4]

DEFINITION

*Content*: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. To 2 mL of solution S (see Tests) add 0.5 mL of *calcium chloride solution R*. A gelatinous white precipitate is formed that dissolves on adding 5 mL of *ferric chloride solution R1*.

B. To about 4 mg add a mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R* and mix. The colour changes from red to yellow.

C. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* without heating and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Dissolve 2.5 g of *potassium nitrate R* in 40 mL of solution S and dilute to 50 mL with *carbon dioxide-free water R*. Cool to 0 °C and add 0.2 mL of *phenolphthalein solution R*. If the solution is colourless, not more than 1.0 mL of *0.1 M sodium hydroxide* is required to produce a red colour that persists for at least 15 s. If the solution is red, not more than 0.25 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Fluorosilicates**. Heat to boiling the neutralised solution obtained in the test for acidity or alkalinity and titrate whilst hot. Not more than 0.75 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to red.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dissolve 0.25 g in 10 mL of a 223 g/L solution of *aluminium nitrate R*. Add 5 mL of *distilled water R* and 0.6 mL of *hydrochloric acid R1*. Prepare the standard by mixing 0.6 mL of *hydrochloric acid R1*, 5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 10 mL of a 223 g/L solution of *aluminium nitrate R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 130 °C for 3 h.

ASSAY

Dissolve 0.100 g in *water R* and dilute to 60 mL with the same solvent. Titrate with *0.1 M lanthanum nitrate*, determining the end-point potentiometrically (*2.2.20*) using a fluoride-selective indicator electrode and a silver-silver chloride reference electrode.

1 mL of *0.1 M lanthanum nitrate* is equivalent to 12.60 mg of NaF.

01/2012:0848

# SODIUM FUSIDATE

## Natrii fusidas

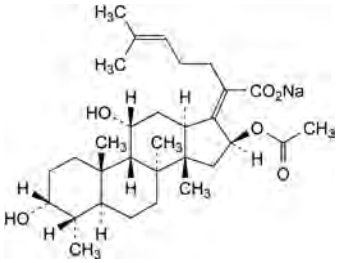

$C_{31}H_{47}NaO_6$ $M_r$ 538.7
[751-94-0]

DEFINITION

Sodium *ent*-(17*Z*)-16α-(acetyloxy)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oate.

Antimicrobial substance produced by fermentation of certain strains of *Fusidium coccineum* or by any other means.

*Content*: 97.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium fusidate CRS*.

B. Ignite 1 g. The residue gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

Dissolve 1.5 g in 10 mL of *water R*.

**pH** (*2.2.3*): 7.5 to 9.0.

Dissolve 0.125 g in 10 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *methanol R*, 5 g/L solution of *phosphoric acid R*, *acetonitrile R* (10:40:50 *V/V/V*).

*Test solution.* Dissolve 25 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 2 mg of *fusidic acid for peak identification CRS* (containing impurities A, B, C, D, F, G, H and N) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve the contents of a vial of *fusidic acid impurity mixture CRS* (containing impurities I, K, L and M) in 1.0 mL of the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *methanol R*, *acetonitrile R*, 5 g/L solution of *phosphoric acid R* (20:40:40 *V/V/V*);

– *mobile phase B*: 5 g/L solution of *phosphoric acid R*, *methanol R*, *acetonitrile R* (10:20:70 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 100 | 0 |
| 3 - 28 | 100 → 0 | 0 → 100 |
| 28 - 33 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *fusidic acid for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, F, G, H and N; use the chromatogram supplied with *fusidic acid impurity mixture CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities I, K, L and M.

*Relative retention* with reference to fusidic acid (retention time = about 18 min): impurity A = about 0.4; impurity B = about 0.5; impurity C = about 0.6; impurity D = about 0.63; impurity N = about 0.65; impurity F = about 0.7; impurity G = about 0.82; impurity H = about 0.85; impurity I = about 0.96; impurity K = about 1.18; impurity L = about 1.23; impurity M = about 1.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurities G and H.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity D = 0.7; impurity F = 0.3; impurity I = 0.6; impurity K = 0.6;

– *impurity M*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *impurity G*: not more than 0.7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);

– *impurity L*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *impurity B*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.4 per cent);

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);

– *impurities C, D, F, I, K, N*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.400 g in 30 mL of *water R* and add 40 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 53.87 mg of $C_{31}H_{47}NaO_6$.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, F, G, I, K, L, M, N.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, H, J, O.*

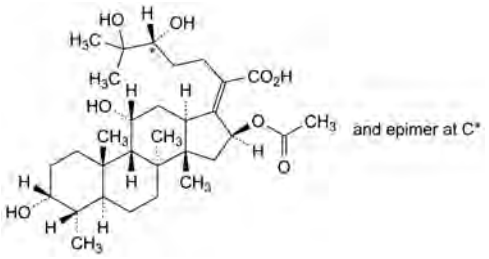


A. *ent*-(24*SR*,17*Z*)-16α-(acetyloxy)-3β,11β,24,25-tetrahydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholest-17(20)-en-21-oic acid (24,25-dihydro-24,25-dihydroxyfusidic acid),

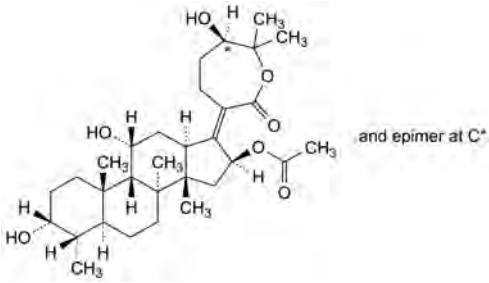


B. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*SR*)-6-hydroxy-7,7-dimethyl-2-oxooxepan-3-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate (24,25-dihydro-24,25-dihydroxyfusidic acid 21,25-lactone),

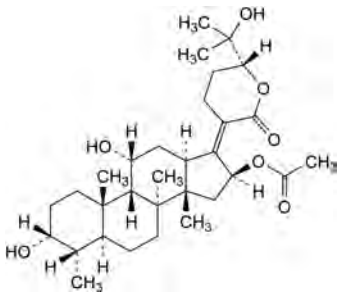

C. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*S*)-6-(1-hydroxy-1-methylethyl)-2-oxodihydro-2*H*-pyran-3(4*H*)-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate ((24*R*)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone),

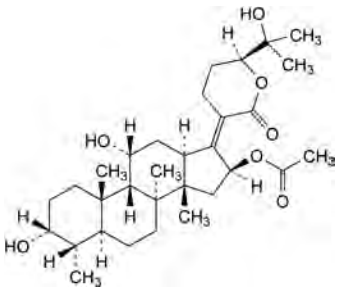

D. *ent*-(17*Z*)-3β,11β-dihydroxy-17-[(6*R*)-6-(1-hydroxy-1-methylethyl)-2-oxodihydro-2*H*-pyran-3(4*H*)-ylidene]-4β,8,14-trimethyl-18-nor-5β,10α-androstan-16α-yl acetate ((24*S*)-24,25-dihydro-24,25-dihydroxyfusidic acid 21,24-lactone),

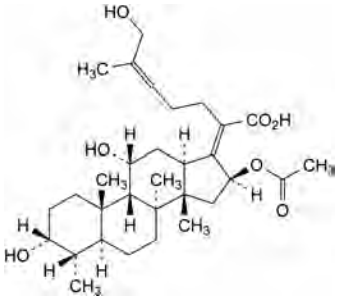

E. *ent*-(17*Z*,24*EZ*)-16α-(acetyloxy)-3β,11β,26-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (26-hydroxyfusidic acid),

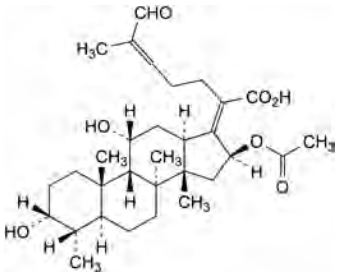

F. *ent*-(17*Z*,24*EZ*)-16α-(acetyloxy)-3β,11β-dihydroxy-4β,8,14-trimethyl-26-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (26-oxofusidic acid),

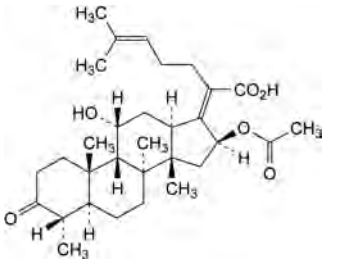

G. *ent*-(17*Z*)-16α-(acetyloxy)-11β-hydroxy-4β,8,14-trimethyl-3-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (3-didehydrofusidic acid),

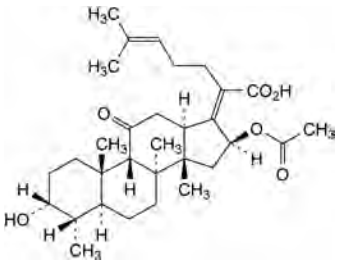

H. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-11-oxo-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (11-didehydrofusidic acid),

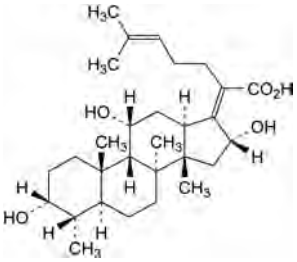

I. *ent*-(17*Z*)-3β,11β,16β-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (16-*epi*-deacetylfusidic acid),

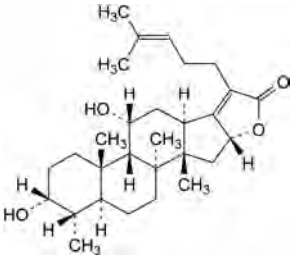

J. *ent*-(17*Z*)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dieno-21(16β)-lactone (16-*epi*-deacetylfusidic acid 21,16-lactone),

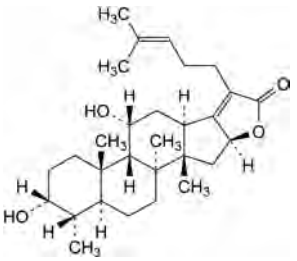

K. *ent*-(17*Z*)-3β,11β-dihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dieno-21(16α)-lactone (deacetylfusidic acid 21,16-lactone),

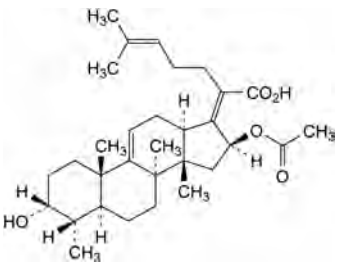

L. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-9(11),17(20),24-trien-21-oic acid (9,11-anhydrofusidic acid),

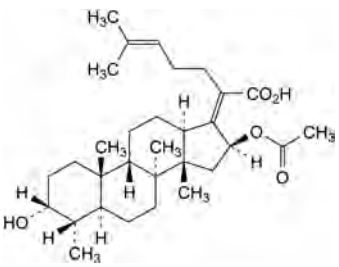

M. *ent*-(17*Z*)-16α-(acetyloxy)-3β-hydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (11-deoxyfusidic acid),

N. unknown structure,

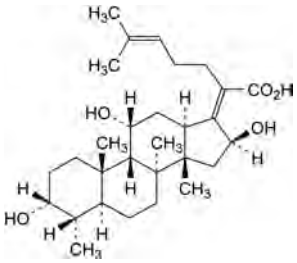

O. *ent*-(17*Z*)-3β,11β,16α-trihydroxy-4β,8,14-trimethyl-18-nor-5β,10α-cholesta-17(20),24-dien-21-oic acid (deacetylfusidic acid).

**01/2017:1995**

# SODIUM GLYCEROPHOSPHATE, HYDRATED

## Natrii glycerophosphas hydricus

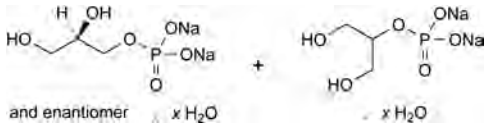

$C_3H_7Na_2O_6P,xH_2O$ $M_r$ 216.0 (anhydrous substance)

DEFINITION

Mixture of variable proportions of hydrated disodium (2*RS*)-2,3-dihydroxypropyl phosphate and hydrated disodium 2-hydroxy-1-(hydroxymethyl)ethyl phosphate. The mixture may contain various amounts of other glycerophosphate esters.

*Content*: 98.0 per cent to 105.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or crystals.

*Solubility*: freely soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

B. To 0.1 g add 5 mL of *dilute nitric acid R*. Heat to boiling and boil for 1 min. Cool. The solution gives reaction (b) of phosphates (*2.3.1*).

C. In a test-tube fitted with a glass tube, mix 0.1 g with 5 g of *potassium hydrogen sulfate R*. Heat strongly and direct the white vapour into 5 mL of *decolorised fuchsin solution R*. A violet-red colour develops which becomes violet upon heating for 30 min on a water-bath.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Alkalinity**. To 10 mL of solution S add 0.2 mL of *phenolphthalein solution R*. Not more than 1.0 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator ($n_2$).

**Glycerol and ethanol (96 per cent)-soluble substances**: maximum 1.0 per cent.

Shake 1.000 g with 25 mL of *ethanol (96 per cent) R* for 10 min. Filter. Evaporate the filtrate on a water-bath and dry the residue at 70 °C for 1 h. The residue weighs not more than 10 mg.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 2.5 mL of solution S to 15 mL with *water R*.

**Phosphates** (*2.4.11*): maximum 0.1 per cent.

Dilute 1 mL of solution S to 10 mL with *water R*. Dilute 1 mL of this solution to 100 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dilute 3 mL of solution S to 15 mL with *water R*.

**Calcium**: maximum 100 ppm, if intended for use in the manufacture of parenteral preparations.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

*Test solution.* Dissolve 0.50 g in a 1 per cent *V/V* solution of *nitric acid R* and dilute to 50.0 mL with the same solution.

*Reference solutions.* Prepare the reference solutions using *calcium standard solution (100 ppm Ca) R*, diluting with a 1 per cent *V/V* solution of *nitric acid R*.

*Wavelength*: 396.8 nm.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Water** (*2.5.12*): 25.0 per cent to 35.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.250 g in 30 mL of *water R*. Titrate with *0.05 M sulfuric acid*, determining the end-point potentiometrically (*2.2.20*), ($n_1$).

Calculate the percentage content of sodium glycerophosphate (anhydrous substance) using the following expression:

$$\frac{216.0\left(n_1 - \frac{n_2}{4}\right)}{m(100 - a)}$$

$a$ = percentage content of water;

$n_1$ = volume of *0.05 M sulfuric acid* used in the assay, in millilitres;

$n_2$ = volume of *0.1 M hydrochloric acid* used in the test for alkalinity, in millilitres;

$m$ = mass of the substance to be examined, in grams.

**01/2017:1472**
**corrected 10.0**

# SODIUM HYALURONATE

## Natrii hyaluronas

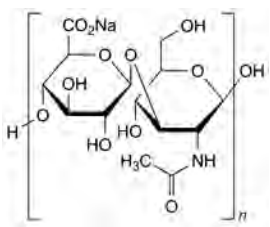

$(C_{14}H_{20}NNaO_{11})_n$
[9067-32-7]

DEFINITION

Sodium salt of hyaluronic acid, a glycosaminoglycan consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine disaccharide units. It is extracted from cocks' combs or obtained by fermentation from *Streptococci*, Lancefield Groups A and C.

*Content*: 95.0 per cent to 105.0 per cent (dried substance).

*Intrinsic viscosity*: 90 per cent to 120 per cent of the value stated on the label.

PRODUCTION

Where applicable, the animals from which sodium hyaluronate is derived must fulfil the requirements for the health of animals suitable for human consumption.

When produced by fermentation of gram-positive bacteria, the process must be shown to reduce or eliminate pyrogenic or inflammatory components of the cell wall.

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder or fibrous aggregate.

*Solubility*: sparingly soluble or soluble in water, practically insoluble in acetone and in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of sodium hyaluronate*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Weigh a quantity of the substance to be examined equivalent to 0.10 g of the dried substance and add 30.0 mL of a 9 g/L solution of *sodium chloride R*. Mix gently on a shaker until dissolved (about 12 h).

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 600 nm is not greater than 0.01.

**pH** (*2.2.3*): 5.0 to 8.5.

Dissolve the substance to be examined in *carbon dioxide-free water R* to obtain a solution containing a quantity equivalent to 5 mg of the dried substance per millilitre.

**Intrinsic viscosity**. *Sodium hyaluronate is very hygroscopic and must be protected from moisture during weighing.*

*Buffer solution (0.15 M sodium chloride in 0.01 M phosphate buffer solution pH 7.0)*. Dissolve 0.78 g of *sodium dihydrogen phosphate R* and 4.50 g of *sodium chloride R* in *water R* and dilute to 500.0 mL with the same solvent (solution A). Dissolve 1.79 g of *disodium hydrogen phosphate dodecahydrate R* and 4.50 g of *sodium chloride R* in *water R* and dilute to 500.0 mL with the same solvent (solution B). Mix solutions A and B until a pH of 7.0 is reached. Filter through a sintered-glass filter (4) (*2.1.2*).

*Test solution (a)*. Weigh 0.200 g ($m_{0p}$) *(NOTE: this value is only indicative and should be adjusted after an initial measurement of the viscosity of test solution (a))* of the substance to be examined and dilute with 50.0 g ($m_{0s}$) of buffer solution at 4 °C. Mix the solution by shaking at 4 °C during 24 h. Weigh 5.00 g ($m_{1p}$) of the solution and dilute with 100.0 g ($m_{1s}$) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) (*2.1.2*), and discard the first 10 mL.

*Test solution (b)*. Weigh 30.0 g ($m_{2p}$) of test solution (a) and dilute with 10.0 g ($m_{2s}$) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) (*2.1.2*) and discard the first 10 mL.

*Test solution (c)*. Weigh 20.0 g ($m_{3p}$) of test solution (a) and dilute with 20.0 g ($m_{3s}$) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) (*2.1.2*) and discard the first 10 mL.

*Test solution (d)*. Weigh 10.0 g ($m_{4p}$) of test solution (a) and dilute with 30.0 g ($m_{4s}$) of buffer solution at 25 °C. Mix this solution by shaking for 20 min. Filter the solution through a sintered-glass filter (100) (*2.1.2*) and discard the first 10 mL.

Determine the flow-times (*2.2.9*) for the buffer solution ($t_0$) and for the 4 test solutions ($t_1$, $t_2$, $t_3$ and $t_4$), at 25.00 ± 0.03 °C. Use an appropriate suspended level viscometer (specifications: viscometer constant about 0.005 mm²/s², kinematic viscosity of 1-5 mm²/s, internal diameter of tube *R* 0.53 mm, volume of bulb *C* 5.6 mL, internal diameter of tube *N* 2.8-3.2 mm) with a funnel-shaped lower capillary end. Use the same viscometer for all measurements; measure all outflow times in triplicate. The test is not valid unless the results do not differ by more than 0.35 per cent from the mean and if the flow time $t_1$ is not less than 1.6 and not more than 1.8 times $t_0$. If this is not the case, adjust the value of $m_{0p}$ and repeat the procedure.

*Calculation of the relative viscosities*

Since the densities of the sodium hyaluronate solutions and of the solvent are almost equal, the relative viscosities $\eta_{ri}$ (being $\eta_{r1}$, $\eta_{r2}$, $\eta_{r3}$ and $\eta_{r4}$) can be calculated from the ratio of the flow times for the respective solutions $t_i$ (being $t_1$, $t_2$, $t_3$and $t_4$) to the flow time of the solvent $t_0$, but taking into account the kinetic energy correction factor for the capillary ($B$ = 30 800 s³), using the following expression:

$$\frac{t_i - \frac{B}{t_i^2}}{t_0 - \frac{B}{t_0^2}}$$

*Calculation of the concentrations*

Calculate the concentration $c_1$ (expressed in kg/m³) of sodium hyaluronate in test solution (a) using the following expression:

$$\frac{m_{0p} \times x \times (100 - h) \times m_{1p} \times \rho_{25}}{100 \times 100 \times \left(m_{0p} + m_{0s}\right) \times \left(m_{1p} + m_{1s}\right)}$$

$x$ = percentage content of sodium hyaluronate as determined under Assay;

$h$ = percentage loss on drying;

$\rho_{25}$ = 1005 kg/m³ (density of the test solution at 25 °C).

Calculate the concentration $c_2$ (expressed in kg/m³) of sodium hyaluronate in test solution (b) using the following expression:

$$c_1 \times \frac{m_{2p}}{m_{2s} + m_{2p}}$$

Calculate the concentration $c_3$ (expressed in kg/m³) of sodium hyaluronate in test solution (c) using the following expression:

$$c_1 \times \frac{m_{3p}}{m_{3s} + m_{3p}}$$

Calculate the concentration $c_4$ (expressed in kg/m³) of sodium hyaluronate in test solution (d) using the following expression:

$$c_1 \times \frac{m_{4p}}{m_{4s} + m_{4p}}$$

*Calculation of the intrinsic viscosity*

Calculate the intrinsic viscosity [$\eta$] by linear least-squares regression analysis using the Martin equation:

$$\log_{10}\left(\frac{\eta_r - 1}{c}\right) = \log_{10}[\eta] + k[\eta]c$$

The decimal antilogarithm of the intercept is the intrinsic viscosity expressed in m³/kg.

**Sulfated glycosaminoglycans**: maximum 1 per cent, if the product is extracted from cocks' combs.

*Appropriate safety precautions are to be taken when handling perchloric acid at elevated temperature.*

*Test solution.* Introduce a quantity of the substance to be examined equivalent to 50.0 mg of the dried substance into a test-tube 150 mm long and 16 mm in internal diameter and dissolve in 1.0 mL of *perchloric acid R*.

*Reference solution.* Dissolve 0.149 g of *anhydrous sodium sulfate R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of this solution to 100.0 mL with *water R*. Evaporate 1.0 mL in a test-tube 150 mm long and 16 mm in internal diameter in a heating block at 90-95 °C, and dissolve the residue in 1.0 mL of *perchloric acid R*.

Plug each test-tube with a piece of glass wool. Place the test-tubes in a heating block or a silicone oil bath maintained at 180 °C and heat until clear, colourless solutions are obtained (about 12 h). Remove the test-tubes and cool to room temperature. Add to each test-tube 3.0 mL of a 33.3 g/L solution of *barium chloride R*, cap and shake vigorously. Allow the test-tubes to stand for 30 min. Shake each test-tube once again, and determine the absorbance (*2.2.25*) at 660 nm, using *water R* as a blank.

The absorbance obtained with the test solution is not greater than the absorbance obtained with the reference solution.

**Nucleic acids**. The absorbance (*2.2.25*) of solution S at 260 nm is maximum 0.5.

**Protein**: maximum 0.3 per cent; maximum 0.1 per cent, if intended for use in the manufacture of parenteral preparations.

*Test solution (a).* Dissolve the substance to be examined in *water R* to obtain a solution containing a quantity equivalent to about 10 mg of the dried substance per millilitre.

*Test solution (b).* Mix equal volumes of test solution (a) and *water R*.

*Reference solutions.* Prepare a 0.5 mg/mL stock solution of *bovine albumin R* in *water R*. Prepare 5 dilutions of the stock solution containing between 5 µg/mL and 50 µg/mL of *bovine albumin R*.

Add 2.5 mL of freshly prepared *cupri-tartaric solution R3* to test-tubes containing 2.5 mL of *water R* (blank), 2.5 mL of the test solutions (a) or (b) or 2.5 mL of the reference solutions. Mix after each addition. After about 10 min, add to each test-tube 0.50 mL of a mixture of equal volumes of *phosphomolybdotungstic reagent R* and *water R* prepared immediately before use. Mix after each addition. After 30 min, measure the absorbance (*2.2.25*) of each solution at 750 nm against the blank. From the calibration curve obtained with the 5 reference solutions determine the content of protein in the test solutions.

**Chlorides** (*2.4.4*): maximum 0.5 per cent.

Dissolve 67 mg in 100 mL of *water R*.

**Iron**: maximum 80 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve a quantity of the substance to be examined equivalent to 0.25 g of the dried substance in 1 mL of *nitric acid R* by heating on a water-bath. Cool and dilute to 10.0 mL with *water R*.

*Reference solutions.* Prepare 2 reference solutions in the same manner as the test solution, adding 1.0 mL and 2.0 mL respectively of *iron standard solution (10 ppm Fe) R* to the dissolved substance to be examined.

*Source*: iron hollow-cathode lamp using a transmission band of 0.2 nm.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 20.0 per cent, determined on 0.500 g by drying at 100-110 °C over *diphosphorus pentoxide R* for 6 h.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*). Use 1 g of the substance to be examined.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins; less than 0.05 IU/mg, if intended for use in the manufacture of intra-ocular preparations or intra-articular preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Determine the glucuronic acid content by reaction with carbazole as described below.

*Reagent A.* Dissolve 0.95 g of *disodium tetraborate R* in 100.0 mL of *sulfuric acid R*.

*Reagent B.* Dissolve 0.125 g of *carbazole R* in 100.0 mL of *anhydrous ethanol R*.

*Test solution.* Prepare this solution in triplicate. Dissolve 0.170 g of the substance to be examined in *water R* and dilute to 100.0 g with the same solvent. Dilute 10.0 g of this solution to 200.0 g with *water R*.

*Reference stock solution.* Dissolve 0.100 g of *D-glucuronic acid R*, previously dried to constant mass in vacuum over *diphosphorus pentoxide R* (*2.2.32*), in *water R* and dilute to 100.0 g with the same solvent.

*Reference solutions.* Prepare 5 dilutions of the reference stock solution containing between 6.5 µg/g and 65 µg/g of *D-glucuronic acid R*.

Place 25 test-tubes, numbered 1 to 25, in iced water. Add 1.0 mL of the 5 reference solutions in triplicate to the test-tubes 1 to 15 (reference tubes), 1.0 mL of the 3 test solutions in triplicate to the test-tubes 16 to 24 (sample tubes), and 1.0 mL of *water R* to test-tube 25 (blank). Add to each test-tube 5.0 mL of freshly prepared reagent A, previously cooled in iced water. Tightly close the test-tubes with plastic caps, shake the contents, and place on a water bath for exactly 15 min. Cool in iced water, and add to each test tube 0.20 mL of reagent B. Recap the tubes, shake, and put them again on a water-bath for exactly 15 min. Cool to room temperature and measure the absorbance (*2.2.25*) of the solutions at 530 nm, against the blank.

From the calibration curve obtained with the mean absorbances read for each reference solution, determine the mean concentrations of D-glucuronic acid in the test solutions.

Calculate the percentage content of sodium hyaluronate using the following expression:

$$\frac{c_g}{c_s} \times Z \times \frac{100}{100 - h} \times \frac{401.3}{194.1}$$

$c_g$ = mean of concentrations of D-glucuronic acid in the test solutions, in milligrams per gram;

$c_s$ = mean of concentrations of the substance to be examined in the test solutions, in milligrams per gram;

$Z$ = determined percentage content of $C_6H_{10}O_7$ in *D-glucuronic acid R*;

$h$ = percentage loss on drying;

401.3 = relative molecular mass of the disaccharide fragment;

194.1 = relative molecular mass of glucuronic acid.

STORAGE

In an airtight container, protected from light and humidity. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

– the intrinsic viscosity;

– the origin of the substance;

- the intended use of the substance;
- where applicable, that the substance is suitable for parenteral administration other than intra-articular administration;
- where applicable, that the substance is suitable for parenteral administration, including intra-articular administration;
- where applicable that the material is suitable for intra-ocular use.

**01/2017:0195**

# SODIUM HYDROGEN CARBONATE

## Natrii hydrogenocarbonas

$NaHCO_3$ $M_r$ 84.0
[144-55-8]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

When heated in the dry state or in solution, it gradually changes into sodium carbonate.

IDENTIFICATION

A. To 5 mL of solution S (see Tests) add 0.1 mL of *phenolphthalein solution R*. A pale pink colour is produced. Heat; gas is evolved and the solution becomes red.

B. It gives the reaction of carbonates and bicarbonates (*2.3.1*).

C. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in 90 mL of *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Carbonates**. The pH (*2.2.3*) of freshly prepared solution S is not greater than 8.6.

**Chlorides** (*2.4.4*): maximum 150 ppm.

To 7 mL of solution S add 2 mL of *nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

To a suspension of 1.0 g in 10 mL of *distilled water R* add *hydrochloric acid R* until neutral and about 1 mL in excess. Dilute to 15 mL with *distilled water R*.

**Ammonium** (*2.4.1*): maximum 20 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*. Prepare the standard using a mixture of 5 mL of *water R* and 10 mL of *ammonium standard solution (1 ppm $NH_4$) R*.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Calcium** (*2.4.3*): maximum 100 ppm.

To a suspension of 1.0 g in 10 mL of *distilled water R* add *hydrochloric acid R* until neutral and dilute to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dissolve 0.5 g in 5 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*.

ASSAY

Dissolve 0.750 g in 50 mL of *carbon dioxide-free water R*. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 2nd point of inflection, or at the point of inflection if only 1 point is detected.

1 mL of *1 M hydrochloric acid* is equivalent to 84.0 mg of $NaHCO_3$.

**01/2017:0677**

# SODIUM HYDROXIDE

## Natrii hydroxidum

NaOH $M_r$ 40.00
[1310-73-2]

DEFINITION

*Content*: 97.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline masses, supplied as pellets, sticks or slabs, deliquescent, readily absorbing carbon dioxide.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. pH (*2.2.3*): minimum 11.0.
Dissolve 0.1 g in 10 mL of *water R*. Dilute 1 mL of the solution to 100 mL with *water R*.

B. 2 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. *Carry out the procedure described below with caution.* Dissolve 5.0 g in 12 mL of *water R*. Add 17 mL of *hydrochloric acid R1*, adjust to pH 7 with a 103 g/L solution of *hydrochloric acid R* and dilute to 50 mL with *water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in 10 mL of *water R*.

**Carbonates**: maximum 2.0 per cent, calculated as $Na_2CO_3$ as determined in the assay.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 5 mL of *water R*, acidify the solution with about 4 mL of *nitric acid R* and dilute to 15 mL with *water R*. The solution, without addition of *dilute nitric acid R*, complies with the test.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dissolve 0.75 g in 6 mL of *distilled water R*, adjust to pH 7 with *hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

ASSAY

Dissolve 2.000 g in about 80 mL of *carbon dioxide-free water R*. Add 0.3 mL of *phenolphthalein solution R* and titrate with *1 M hydrochloric acid*. Add 0.3 mL of *methyl orange solution R* and continue the titration with *1 M hydrochloric acid*.

1 mL of *1 M hydrochloric acid* used in the 2nd part of the titration is equivalent to 0.1060 g of $Na_2CO_3$.

1 mL of *1 M hydrochloric acid* used in the combined titrations is equivalent to 40.00 mg of total alkali, calculated as NaOH.

STORAGE

In an airtight, non-metallic container.

**01/2017:0196**

# SODIUM IODIDE

## Natrii iodidum

NaI $M_r$ 149.9
[7681-82-5]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, hygroscopic.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of iodides (*2.3.1*).

B. Solution S gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Alkalinity**. To 12.5 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.7 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator.

**Iodates**. To 10 mL of solution S add 0.25 mL of *iodide-free starch solution R* and 0.2 mL of *dilute sulfuric acid R* and allow to stand protected from light for 2 min. No blue colour develops.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Thiosulfates**. To 10 mL of solution S add 0.1 mL of *starch solution R* and 0.1 mL of *0.005 M iodine*. A blue colour is produced.

**Iron** (*2.4.9*): maximum 20 ppm.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 1.00 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 1.300 g in *water R* and dilute to 100.0 mL with the same solvent. To 20.0 mL of the solution add 40 mL of *hydrochloric acid R* and titrate with *0.05 M potassium iodate* until the colour changes from red to yellow. Add 5 mL of *chloroform R* and continue the titration, shaking vigorously, until the chloroform layer is decolorised.

1 mL of *0.05 M potassium iodate* is equivalent to 14.99 mg of NaI.

STORAGE

Protected from light.

**01/2017:1151**

# SODIUM LACTATE SOLUTION

## Natrii lactatis solutio

DEFINITION

Solution of a mixture of the enantiomers of sodium 2-hydroxypropanoate in approximately equal proportions.

*Content*: minimum declared content 50 per cent *m/m* of sodium 2-hydroxypropanoate ($C_3H_5NaO_3$; $M_r$ 112.1); 96.0 per cent to 104.0 per cent of the content of sodium lactate stated on the label.

CHARACTERS

*Appearance*: clear, colourless, slightly syrupy liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. To 0.1 mL add 10 mL of *water R*. 5 mL of the solution gives the reaction of lactates (*2.3.1*).

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dilute a quantity of the substance to be examined corresponding to 40.0 g of sodium lactate to 200 mL with *distilled water R*.

**Appearance of solution**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 9.0 for the substance to be examined.

**Reducing sugars and sucrose**. To 5 mL of the substance to be examined add 0.2 mL of *copper sulfate solution R* and 2 mL of *dilute sodium hydroxide solution R*. The solution is clear and blue and remains so on boiling. Add to the hot solution 4 mL of *hydrochloric acid R*. Boil for 1 min. Add 6 mL of *strong sodium hydroxide solution R* and heat to boiling again. The solution is clear and blue.

**Methanol**. Gas chromatography (*2.4.24*).

*Limit*:

– *methanol*: maximum 50 ppm, calculated with reference to sodium lactate, if intended for use in the manufacture of parenteral preparations, dialysis, haemodialysis or haemofiltration solutions.

**Chlorides** (*2.4.4*): maximum 50 ppm calculated with reference to sodium lactate.

Dilute 5 mL of solution S to 15 mL with *water R*. The solution complies with the test for chlorides.

**Oxalates and phosphates**. To 1 mL of the substance to be examined add 15 mL of *ethanol (96 per cent) R* and 2 mL of *calcium chloride solution R*. Heat at 75 °C for 5 min. Any opalescence in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 1 mL of the substance to be examined, 15 mL of *ethanol (96 per cent) R* and 2 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm calculated with reference to sodium lactate.

To 7.5 mL of solution S, add 1.9 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*. The solution complies with the test for sulfates without addition of 0.5 mL of *acetic acid R*. Acidify the standard solution with 0.05 mL of *hydrochloric acid R1* instead of 0.5 mL of *acetic acid R*.

Monographs S

**Aluminium**: maximum 0.1 ppm, if intended for use in the manufacture of parenteral preparations, dialysis, haemodialysis or haemofiltration solutions.

Atomic absorption spectrometry (*2.2.23, Method I*). For the preparation of the solutions, use equipment that is aluminium-free or that will not release aluminium under the conditions of use (glass, polyethylene, etc).

*Modifier solution.* Dissolve 100.0 g of *ammonium nitrate R* in a mixture of 4 mL of *nitric acid R* and 50 mL of *water R* and dilute to 200 mL with *water R*.

*Blank solution.* Dilute to 2.0 mL of the modifier solution to 25.0 mL with *water R*.

*Test solution.* To 5.0 g add 2.0 mL of the modifier solution and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions immediately before use (0.010 ppm to 0.050 ppm of aluminium) using *aluminium standard solution (200 ppm Al) R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: a graphite furnace.

*Carrier gas*: *argon R*.

*Conditions*: the device is equipped with a non-specific absorption correction system. Heat the oven to 120 °C for as many seconds as there are microlitres of solution introduced into the apparatus, then heat at 1000 °C for 30 s and finally at 2700 °C for 6 s.

**Barium**. To 10 mL of solution S add 10 mL of *calcium sulfate solution R*. Allow to stand for 30 min. Any opalescence (*2.2.1*) in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 10 mL of solution S and 10 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm calculated with reference to sodium lactate.

Dilute 5 mL of solution S to 10 mL with *water R*. The solution complies with the test for iron.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/g, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve a quantity of the substance to be examined corresponding to 75.0 mg of sodium lactate in a mixture of 10 mL of *glacial acetic acid R* and 20 mL of *acetic anhydride R*. Allow to stand for 15 min. Add 1 mL of *naphtholbenzein solution R* and titrate with *0.1 M perchloric acid*.

1 mL of *0.1 M perchloric acid* is equivalent to 11.21 mg of $C_3H_5NaO_3$.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of dialysis, haemodialysis and haemofiltration solutions,
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations,
- the declared content of sodium lactate.

**01/2017:2033**
**corrected 10.0**

# SODIUM (*S*)-LACTATE SOLUTION

## Natrii (*S*)-lactatis solutio

DEFINITION

*Content*: minimum 50.0 per cent *m/m* of sodium (*S*)-2-hydroxypropanoate ($C_3H_5NaO_3$; $M_r$ 112.1); 96.0 per cent to 104.0 per cent of the content of sodium lactate stated on the label, not less than 95.0 per cent of which is the (*S*)-enantiomer.

CHARACTERS

*Appearance*: clear, colourless, slightly syrupy liquid.

*Solubility*: miscible with water and with ethanol (96 per cent).

IDENTIFICATION

A. To 0.1 mL add 10 mL of *water R*. 5 mL of the solution gives the reaction of lactates (*2.3.1*).

B. It gives reaction (a) of sodium (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dilute a quantity of the substance to be examined corresponding to 40.0 g of sodium lactate to 200 mL with *distilled water R*.

**Appearance of solution**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.5 to 9.0 for the substance to be examined.

**Reducing sugars and sucrose**. To 5 mL of the substance to be examined add 2 mL of *dilute sodium hydroxide solution R* and 0.2 mL of *copper sulfate solution R*. The solution is clear and blue and remains so on boiling. Add to the hot solution 4 mL of *hydrochloric acid R*. Boil for 1 min. Add 6 mL of *strong sodium hydroxide solution R* and heat to boiling again. The solution is clear and blue.

**Methanol**. Gas chromatography (*2.4.24*).

*Limit*:

- *methanol*: maximum 50 ppm, calculated with reference to sodium lactate, if intended for use in the manufacture of parenteral preparations, dialysis, haemodialysis or haemofiltration solutions.

**Chlorides** (*2.4.4*): maximum 50 ppm calculated with reference to sodium lactate.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Oxalates and phosphates**. To 1 mL of the substance to be examined add 15 mL of *ethanol (96 per cent) R* and 2 mL of *calcium chloride solution R*. Heat at 75 °C for 5 min. Any opalescence in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 1 mL of the substance to be examined, 15 mL of *ethanol (96 per cent) R* and 2 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm calculated with reference to sodium lactate.

To 7.5 mL of solution S, add 1.9 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*. The solution complies with the limit test for sulfates without addition of 0.5 mL of *acetic acid R*. Acidify the standard solution with 0.05 mL of *hydrochloric acid R1* instead of 0.5 mL of *acetic acid R*.

**Aluminium**: maximum 0.1 ppm, if intended for use in the manufacture of parenteral preparations, dialysis, haemodialysis or haemofiltration solutions.

Atomic absorption spectrometry (*2.2.23, Method I*). For the preparation of the solutions, use equipment that is aluminium-free or that will not release aluminium under the conditions of use (glass, polyethylene, etc).

*Modifier solution.* Dissolve 100.0 g of *ammonium nitrate R* in a mixture of 50 mL of *water R* and 4 mL of *nitric acid R* and dilute to 200 mL with *water R*.

*Blank solution.* Dilute 2.0 mL of the modifier solution to 25.0 mL with *water R*.

*Test solution.* To 5.0 g add 2.0 mL of the modifier solution and dilute to 25.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions immediately before use (0.010 ppm to 0.050 ppm of aluminium) using *aluminium standard solution (200 ppm Al) R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: a graphite furnace.

*Carrier gas*: *argon R*.

*Conditions*: the device is equipped with a non-specific absorption correction system. Heat the oven to 120 °C for as many seconds as there are microlitres of solution introduced into the apparatus, then heat at 1000 °C for 30 s and finally at 2700 °C for 6 s.

**Barium**. To 10 mL of solution S add 10 mL of *calcium sulfate solution R*. Allow to stand for 30 min. Any opalescence (*2.2.1*) in the solution is not more intense than that of a standard prepared at the same time and in the same manner using a mixture of 10 mL of solution S and 10 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 10 ppm calculated with reference to sodium lactate.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/g if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve a quantity of the substance to be examined corresponding to 75.0 mg of sodium lactate in a mixture of 10 mL of *glacial acetic acid R* and 20 mL of *acetic anhydride R*. Allow to stand for 15 min. Add 1 mL of *naphtholbenzein solution R* and titrate with *0.1 M perchloric acid*.

1 mL of *0.1 M perchloric acid* is equivalent to 11.21 mg of $C_3H_5NaO_3$.

**(*S*)-enantiomer**. Transfer a quantity of the substance to be examined corresponding to 2.50 g of sodium lactate into a 50 mL volumetric flask, dilute with about 30 mL of *water R* and add 5.0 g of *ammonium molybdate R*. Dissolve and dilute with *water R* to 50.0 mL. Measure the angle of optical rotation (*2.2.7*). Calculate the percentage content of (*S*)-enantiomer using the expression:

$$50 + \left(24.04 \times \alpha \times \frac{5.0}{m} \times \frac{50}{c}\right)$$

$\alpha$ = angle of optical rotation (absolute value);

$m$ = mass of the substance to be examined, in grams;

$c$ = percentage content of $C_3H_5NaO_3$ in the substance to be examined.

The complex of sodium (*S*)-lactate formed under these test conditions is laevorotatory.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of dialysis, haemodialysis and haemofiltration solutions;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- the declared content of sodium lactate.

**04/2016:0098**
**corrected 9.1**

# SODIUM LAURILSULFATE(2)

## Natrii laurilsulfas

Sodium dodecyl sulfate: [151-21-3]

DEFINITION

Mixture of sodium alkyl sulfates consisting mainly of sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$; $M_r$ 288.4).

*Content*:

- *sodium alkyl sulfates*: minimum 85.0 per cent, expressed as $C_{12}H_{25}NaO_4S$.

♦CHARACTERS

*Appearance*: white or pale yellow, powder or crystals.

*Solubility*: freely soluble in water giving an opalescent solution, partly soluble in ethanol (96 per cent). ♦

IDENTIFICATION

A. Dissolve 0.1 g in 10 mL of *water R* and shake. A copious foam is formed.

B. To 0.1 mL of the solution prepared for identification test A, add 0.1 mL of a 1 g/L solution of *methylene blue R* and 2 mL of *dilute sulfuric acid R*. Add 2 mL of *methylene chloride R* and shake. An intense blue colour develops in the methylene chloride layer.

C. Place 2.5 g in a silica or platinum crucible and add 2 mL of a 500 g/L solution of *sulfuric acid R*. Heat on a water-bath, then cautiously raise the temperature progressively over an open flame. Ignite, preferably in a muffle furnace, at 600 ± 25 °C. Continue heating until all black particles have disappeared. Cool, add a few drops of a 100 g/L solution of *sulfuric acid R*, and heat and ignite as above. Add a few drops of *ammonium carbonate solution R*, evaporate to dryness, and ignite as above. Cool, dissolve the residue in 50 mL of *water R* and mix. To 2 mL of this solution add 4 mL of *potassium pyroantimonate solution R1*. If necessary, rub the inside of the test-tube with a glass rod. A white, crystalline precipitate is formed.

D. Prepare a 100 g/L solution. After acidification with *hydrochloric acid R* and boiling for 20 min, no precipitate is formed. Add a 120 g/L solution of *barium chloride R*; a white precipitate is produced.

TESTS

**Alkalinity**. Dissolve 1.0 g in 100 mL of *carbon dioxide-free water R* and add 0.1 mL of *phenol red solution R*. Not more than 0.5 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

(2) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Non-esterified alcohols**: maximum 4.0 per cent.

Dissolve 10.0 g in 100 mL of *water R*, add 100 mL of *ethanol (96 per cent) R* and shake the solution with 3 quantities, each of 50 mL, of *light petroleum R*, adding *sodium chloride R*, if necessary, to promote separation of the 2 layers. Wash the combined organic layers with 3 quantities, each of 50 mL, of *water R*, dry over *anhydrous sodium sulfate R*, filter and evaporate on a water-bath until the solvent has evaporated. Heat the residue at 105 °C for 30 min and cool. The residue weighs a maximum of 0.4 g.

**Sodium chloride and sodium sulfate**: maximum 8.0 per cent for the total percentage content.

*Sodium chloride*. Dissolve 5.00 g in 50 mL of *water R*, add *dilute nitric acid R* dropwise, if necessary, until the solution is neutral, and add 5.0 mL of a 5.84 g/L solution of *sodium chloride R*. Titrate with *0.1 M silver nitrate* using 0.1 mL of a 2 g/L solution of *sodium fluoresceinate R* as indicator, to the first appearance of turbidity with solution colour change from yellowish-green to orange through to yellow. Perform a blank determination.

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

*Sodium sulfate*. Dissolve 1.00 g in 10 mL of *water R*, add 100 mL of *ethanol (96 per cent) R* and heat at a temperature just below the boiling point for 2 h. Filter while hot through a glass filter with a porosity of 5-10 µm and wash with 100 mL of boiling *ethanol (96 per cent) R*. Dissolve the precipitate by washing with 150 mL of *water R*, collecting the washings in a beaker. Add 10 mL of *dilute hydrochloric acid R*, heat to boiling, add 25 mL of a 120 g/L solution of *barium chloride R* and allow to stand overnight. Collect the precipitate and wash with *water R* until the last washing shows no opalescence with *0.1 M silver nitrate*. Dry the precipitate, ignite to constant mass at 500-600 °C by raising the temperature gradually, and weigh as barium sulfate.

1 mg of barium sulfate is equivalent to 0.609 mg of $Na_2SO_4$.

ASSAY

Dissolve 1.50 g in *water R*, warming if necessary, and dilute to 1.0 L with the same solvent. To 10.0 mL of the solution add 15 mL of *methylene chloride R*, 20 mL of *water R* and 25 mL of *methylene blue solution R*. Titrate with *0.004 M benzethonium chloride*, shaking vigorously and allowing the layers to separate before each addition, until almost the same blue colour is obtained in the 2 layers.

1 mL of *0.004 M benzethonium chloride* is equivalent to 1.154 mg of sodium alkyl sulfates, expressed as $C_{12}H_{25}NaO_4S$.

**04/2016:2542**

# SODIUM LAUROYLSARCOSINATE FOR EXTERNAL USE

## Natrii lauroylsarcosinas ad usum externum

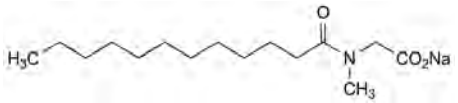


$C_{15}H_{28}NNaO_3$ $M_r$ 293.4
[137-16-6]

DEFINITION

Mixture of sodium *N*-acyl-*N*-methylglycinates, mainly consisting of sodium *N*-lauroyl-*N*-methylglycinate (sodium [dodecanoyl(methyl)amino]acetate).

A suitable chelating agent (e.g. edetic acid (EDTA)) may be added.

PRODUCTION

Sodium lauroylsarcosinate is typically obtained using the reaction of lauroyl chloride with sodium sarcosinate (sodium *N*-methylglycinate) under aqueous alkaline conditions.

The production method is validated to ensure a limit of maximum 0.1 per cent of free sodium sarcosinate.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water giving a clear or slightly turbid solution, sparingly soluble in ethanol 96 per cent.

mp: about 145 °C.

IDENTIFICATION

A. Dissolve 0.1 g in 10 mL of *water R* and shake. Copious foam is formed.

B. Examine the chromatograms obtained in the test for composition of fatty acids.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

C. Place 0.5 g in a silica crucible and ignite in a muffle furnace at 600 ± 25 °C for 1 h. Allow to cool to room temperature and dissolve the residue in 10 mL of *water R*. 0.5 mL of the solution obtained gives reaction (b) of sodium (*2.3.1*). Rub the inside of the tube to initiate the precipitation.

TESTS

**pH** (*2.2.3*): 7.5 to 8.5.

Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Composition of fatty acids**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution (a)*. To 2.5 g in a 150 mL round-bottomed flask add 30.0 mL of *boron trifluoride-methanol solution R*, fit a reflux condenser and boil for 20 min. Add 120 mL of hot *water R* so that the upper layer is brought into the neck of the flask in order to recover the methyl esters. Dissolve 0.50 g of the methyl esters in 10.0 mL of *heptane R*.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *heptane R*.

*Reference solution (a)*. Prepare 0.50 g of the mixture of calibrating substances with the composition described in Table 2542.-1, dissolve in *heptane R* and dilute to 50.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *heptane R*. Dilute 1.0 mL of this solution to 10.0 mL with *heptane R*.

*Reference solution (c)*. To 0.5 g of *sodium lauroylsarcosinate CRS* in a 25 mL round-bottomed flask, add 6.0 mL of *boron trifluoride-methanol solution R*, fit a reflux condenser and boil for 20 min. Add 20.0 mL of hot *water R* so that the upper layer is brought into the neck of the flask in order to recover the methyl esters. Dissolve 0.25 g of the methyl esters in 50.0 mL of *heptane R*.

Table 2542.-1. – *Mixture of calibrating substances*

| Mixture of the following substances | Composition (per cent *m/m*) |
|---|---|
| *Methyl caproate R* | 10 |
| *Methyl caprylate R* | 10 |
| *Methyl decanoate R* | 20 |
| *Methyl laurate R* | 20 |
| *Methyl myristate R* | 40 |

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:5.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 3 | 100 |
| | 3 - 13 | 100 → 200 |
| | 13 - 19 | 200 → 230 |
| | 19 - 29 | 230 |
| | 29 - 31 | 230 → 250 |
| | 31 - 40 | 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to methyl caprylate, methyl decanoate, methyl laurate and methyl myristate.

*System suitability*:
– *resolution*: minimum 4.0 between the peaks due to methyl caprylate and methyl decanoate in the chromatogram obtained with reference solution (a);
– *signal-to-noise ratio*: minimum 20 for the peak due to methyl laurate in the chromatogram obtained with reference solution (b).

Disregard any peak eluting after the peak due to methyl myristate, as well as any peak with an area less than 0.05 per cent of the total area of the peaks used for quantification.

*Composition of the fatty-acid fraction of the substance*: test solution (a):
– *capric acid*: maximum 0.5 per cent;
– *lauric acid*: minimum 98.0 per cent;
– *myristic acid*: maximum 1.5 per cent.

**Sodium laurate**. Proceed as described in the test for composition of fatty acids with the following modifications.

*Internal standard*: *undecanoic acid R*.

*Test solution*. To 2.5 g in a 150 mL round-bottomed flask add 0.100 g of the internal standard. Add 30.0 mL of *boron trifluoride-methanol solution R*, fit a reflux condenser and boil for 20 min. Add 120 mL of hot *water R* so that the upper layer is brought into the neck of the flask in order to recover the methyl esters. Dissolve 0.50 g of the methyl esters in 10.0 mL of *heptane R*.

Calculate the percentage content of sodium laurate using the following expression:

$$\frac{A_1 \times m_2 \times 1.110 \times 100}{A_2 \times m_1}$$

$A_1$ = area of the peak due to methyl laurate in the chromatogram obtained with the test solution;

$A_2$ = area of the peak due to methyl undecanoate in the chromatogram obtained with the test solution;

$m_1$ = mass of the substance to be examined in the test solution, in grams;

$m_2$ = mass of the internal standard in the test solution, in grams;

1.110 = ratio of the molecular mass of sodium laurate to that of lauric acid (222/200).

*Limit*:
– *sodium laurate*: maximum 5.0 per cent.

**Sodium chloride**: maximum 0.5 per cent.

Dissolve 0.500 g in 50 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *water R*. Add 10 mL of *nitric acid R* and titrate with *0.005 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

Calculate the percentage content of sodium chloride using the following expression:

$$\frac{M \times V \times 58.44}{m \times 10}$$

$M$ = molarity of the silver nitrate solution, in moles per litre;

$V$ = volume of *0.005 M silver nitrate* used, in mL;

$m$ = mass of the substance to be examined, in grams.

**Sodium sulfate**: maximum 1.5 per cent.

*Test solution*. In a 150 mL borosilicate-glass beaker, dissolve 0.3 g in 20 mL of *deionised distilled water R*. Add 0.3 mL of *naphtharson solution R1*, 1.0 mL of *perchloric acid R* and 70.0 mL of *2-propanol R*.

Degas the solution obtained under vacuum for 30 s or using an ultrasonic bath for 2 min, then titrate with *0.005 M barium perchlorate*.

Determine the end-point using a suitable autotitrator equipped with a photometric sensor at 523 nm and at 25 °C. Adjust the stirrer speed to avoid the formation of bubbles.

1 mL of *0.005 M barium perchlorate* is equivalent to 0.7102 mg of $Na_2SO_4$.

**01/2017:0849**

# SODIUM METABISULFITE

## Natrii metabisulfis

$Na_2S_2O_5$ $M_r$ 190.1
[7681-57-4]

DEFINITION

Sodium metabisulfite also called sodium disulfite.

*Content*: 95.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. pH (see Tests).
B. To 0.4 mL of *iodinated potassium iodide solution R* add 8 mL of *distilled water R* and 1 mL of solution S diluted 1 to 10 in *distilled water R*. The solution is colourless and gives reaction (a) of sulfates (*2.3.1*).
C. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.5 to 5.0 for solution S.

**Thiosulfates**. To 5 mL of solution S add 5 mL of *dilute hydrochloric acid R*. The solution remains clear (*2.2.1*) for at least 15 min.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm.

Mix 0.20 g with 2 mL of *water R* in a dish. Add, drop by drop, 1.5 mL of *nitric acid R*. Evaporate the mixture to dryness on a water-bath. Heat over a flame until no more vapour is evolved. Take up the residue in 25 mL of *water R*.

**Iron** (*2.4.9*): maximum 20 ppm, determined on solution S.

ASSAY

Dissolve 0.200 g in 50.0 mL of *0.05 M iodine* and add 5 mL of *dilute hydrochloric acid R*. Titrate the excess of iodine with *0.1 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.05 M iodine* is equivalent to 4.753 mg of $Na_2S_2O_5$.

STORAGE

Protected from light.

**01/2017:1262**

# SODIUM METHYL PARAHYDROXYBENZOATE

## Methylis parahydroxybenzoas natricus

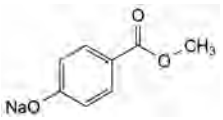


$C_8H_7NaO_3$ $M_r$ 174.1
[5026-62-0]

DEFINITION

Sodium 4-(methoxycarbonyl)phenolate.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.5 g in 50 mL of *water R*. Immediately add 5 mL of *hydrochloric acid R1*. Filter and wash the precipitate with *water R*. Dry *in vacuo* at 80 °C for 2 h. The precipitate melts (*2.2.14*) at 125 °C to 128 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: precipitate obtained in identification test A.
*Comparison*: *methyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 10 mL of *water R*. Immediately add 2 mL of *hydrochloric acid R* and shake with 50 mL of *1,1-dimethylethyl methyl ether R*. Evaporate the upper layer to dryness and take up the residue with 10 mL of *acetone R*.
*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.
*Reference solution (a).* Dissolve 10 mg of *methyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.
*Reference solution (b).* Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).
*Application*: 5 µL of test solution (b) and reference solutions (a) and (b).
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– the chromatogram shows 2 clearly separated principal spots.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 mL of solution S (see Tests) add 1 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S examined immediately after preparation is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*). Dilute 1 mL of solution S to 100 mL with *carbon dioxide-free water R*. The pH of the solution is 9.5 to 10.5.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *4-hydroxybenzoic acid R* (impurity A) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 50.0 mg of *methyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 5 times the retention time of methyl parahydroxybenzoate.

*Relative retention* with reference to methyl parahydroxybenzoate (retention time = about 2.3 min): impurity A = about 0.6.

*System suitability*: reference solution (a):
– *resolution*: minimum 2.0 between the peaks due to impurity A and methyl parahydroxybenzoate.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
- *impurity A*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 350 ppm.

To 10 mL of solution S, add 30 mL of *water R* and 1 mL of *nitric acid R* and dilute to 50 mL with *water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*. Prepare the standard using 14 mL of *chloride standard solution (5 ppm Cl) R* to which 1 mL of *water R* has been added.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 25 mL of solution S, add 5 mL of *distilled water R* and 10 mL of *hydrochloric acid R* and dilute to 50 mL with *distilled water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_8H_7NaO_3$ using the declared content of *methyl parahydroxybenzoate CRS* and multiplying by a correction factor of 1.145.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

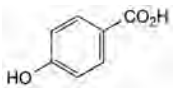

A. 4-hydroxybenzoic acid,

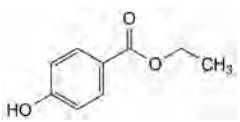

B. ethyl 4-hydroxybenzoate (ethyl parahydroxybenzoate),

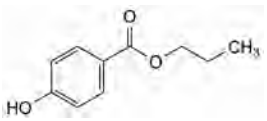

C. propyl 4-hydroxybenzoate (propyl parahydroxybenzoate),

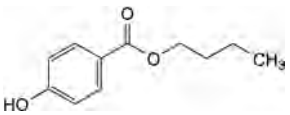

D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

**04/2019:1565**

# SODIUM MOLYBDATE DIHYDRATE

## Natrii molybdas dihydricus

$MoNa_2O_4,2H_2O$ $M_r$ 241.9
[10102-40-6]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. Loss on drying (see Tests).

B. Dissolve 0.2 g in 5 mL of a mixture of equal volumes of *nitric acid R* and *water R* and add 0.1 g of *ammonium chloride R*. Add 0.3 mL of *disodium hydrogen phosphate solution R* and heat slowly at 50-60 °C. A yellow precipitate is formed.

C. Dissolve 0.15 g in 2 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Chlorides**: maximum 50 ppm.

To 10 mL of a mixture of equal volumes of *nitric acid R* and *water R* add 10 mL of solution S with shaking. Add 1 mL of *0.1 M silver nitrate*. Any opalescence in the solution is not more intense after 5 min than that of a standard solution prepared at the same time in the same manner with 10 mL of *chloride standard solution (5 ppm Cl) R*.

**Phosphates**: maximum 200 ppm.

Dissolve 2.0 g by heating in 13 mL of *water R*. In the still-hot solution, dissolve 8.0 g of *ammonium nitrate R1*. Add this solution to 27 mL of a mixture of equal volumes of *nitric acid R* and *water R*. Any yellow colour or opalescence in the solution is not more intense within 3 h than that in a standard solution prepared at the same time in the same manner as follows: dissolve 1.0 g in 12 mL of *water R* and add 1 mL of *phosphate standard solution (200 ppm $PO_4$) R*.

**Ammonium** (*2.4.1, Method B*): maximum 10 ppm, determined on 0.10 g.

Prepare the standard using 1 mL of *ammonium standard solution (1 ppm $NH_4$) R*.

**Loss on drying** (*2.2.32*): 14.0 per cent to 16.0 per cent, determined on 1.000 g by drying in an oven at 140 °C for 3 h.

ASSAY

Dissolve 0.100 g in 30 mL of *water R*, add 0.5 g of *hexamethylenetetramine R* and 0.1 mL of a 250 g/L solution of *nitric acid R*. Heat to 60 °C. Titrate with *0.05 M lead nitrate* using *4-(2-pyridylazo)resorcinol monosodium salt R* as indicator.

1 mL of *0.05 M lead nitrate* is equivalent to 10.30 mg of $MoNa_2O_4$.

**01/2017:1996**

# SODIUM NITRITE

## Natrii nitris

$NaNO_2$ $M_r$ 69.0
[7632-00-0]

DEFINITION

*Content*: 98.5 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: colourless crystals or mass or yellowish rods, hygroscopic.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Dilute 1 mL of solution S1 (see Tests) to 25 mL with *water R*. To 0.1 mL of the solution add 1 mL of *sulfanilic acid solution R1*. Allow to stand for 2-3 min. Add 1 mL of *β-naphthol solution R* and 1 mL of *dilute sodium hydroxide solution R*. An intense red colour develops.

B. To 1 mL of the solution prepared for identification test A add 3 mL of a 20 g/L solution of *phenazone R* and 0.4 mL of *dilute sulfuric acid R*. An intense green colour develops.

C. To 0.15 mL of solution S1, add 0.35 mL of *water R*. The solution gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S1**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Solution S2**. Dissolve 3 g in *distilled water R*. Cautiously add 10 mL of *nitric acid R* and evaporate to dryness. Dissolve the residue in 10 mL of *distilled water R*, neutralise with *dilute sodium hydroxide solution R* and dilute to 30 mL with *distilled water R*.

**Appearance of solution**. Solution S1 is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S1, add 0.05 mL of *phenol red solution R*. Add 0.1 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is yellow.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 10 mL of solution S2 to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S2 to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying *in vacuo*.

ASSAY

Dissolve 0.400 g in 100.0 mL of *water R*. Introduce 20.0 mL of the solution, while stirring continuously and keeping the tip of the pipette below the surface of the liquid, into a conical flask containing 30.0 mL of *0.1 M cerium sulfate*. Immediately stopper the flask and allow to stand for 2 min. Add 10 mL of a 200 g/L solution of *potassium iodide R* and 2 mL of *starch solution R*.

While stirring continuously, titrate with *0.1 M sodium thiosulfate* until the blue colour disappears. Carry out a blank titration.

1 mL of *0.1 M cerium sulfate* is equivalent to 3.45 mg of $NaNO_2$.

STORAGE

In an airtight container.

**01/2016:0565**

# SODIUM NITROPRUSSIDE

## Natrii nitroprussias

$Na_2[Fe(CN)_5(NO)],2H_2O$ $M_r$ 298.0
[13755-38-9]

DEFINITION

Sodium pentacyanonitrosylferrate (III) dihydrate.

*Content*: 99.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: reddish-brown powder or crystals.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 0.700 g in *water R* and dilute to 100.0 mL with the same solvent. Examine the solution immediately after preparation.

*Spectral range*: 350-600 nm.

*Absorption maximum*: at 395 nm.

*Shoulder*: at about 510 nm.

*Absorption minimum*: at 370 nm.

*Specific absorbance at the absorption maximum*: 0.65 to 0.80.

B. Dissolve about 20 mg in 2 mL of *water R* and add 0.1 mL of *sodium sulfide solution R*. A deep violet-red colour is produced.

C. Dissolve 50 mg in 1 mL of *water R* and acidify the solution by the addition of *hydrochloric acid R*. Place a drop of the solution in an oxidising flame. A persistent yellow colour is produced.

TESTS

**Insoluble matter**: maximum 100 ppm.

Dissolve 10 g without heating in 50 mL of *water R*. Allow to stand for 30 min and filter through a sintered-glass filter (16) (*2.1.2*). Wash the filter with cold *water R* until the filtrate is colourless. Dry the residue on the filter at 105 °C. The residue weighs a maximum of 1 mg.

**Chlorides** (*2.4.4*): maximum 200 ppm.

In a metallic crucible (nickel) mix 1.0 g with 8 mL of a 200 g/L solution of *sodium hydroxide R*. Heat slowly and evaporate carefully to dryness over a small flame, then heat to a dull red colour for 30 min. Allow to cool and transfer the solid residue with 3 quantities, each of 8 mL, of *dilute sulfuric acid R*. Filter the sulfuric acid extracts on a filter paper and collect the filtrates. Render the filtrate acid to *blue litmus paper R* by adding, if necessary, a few drops of *dilute sulfuric acid R*. Wash the crucible and the filter paper with 3 quantities, each

of 10 mL, of *water R*, add the washings to the main sulfuric acid solution and dilute to 60 mL with *water R*. Mix.

**Ferricyanides**: maximum 200 ppm.

Dissolve 1.25 g in *acetate buffer solution pH 4.6 R* and dilute to 50.0 mL with the same buffer solution. Use three 50 mL volumetric flasks (*A, B, C*). To flask *B* add 1.0 mL of *ferricyanide standard solution (50 ppm $Fe(CN)_6$) R*. To flasks *A* and *B* add 1 mL of a 5 g/L solution of *ferrous ammonium sulfate R*. To the 3 flasks add 10.0 mL of the solution of the substance to be examined. Dilute the contents of each flask to 50.0 mL with *water R*. Allow to stand for 30 min. The absorbance (*2.2.25*) of the solution in flask *A* measured at 720 nm using the solution in flask *C* as the compensation liquid is not greater than the absorbance of the solution in flask *B* measured at 720 nm using the solution in flask *A* as the compensation liquid.

**Ferrocyanides**: maximum 200 ppm.

Dissolve 4.0 g in *water R* and dilute to 100.0 mL with the same solvent. Use three 50 mL volumetric flasks (*A*, *B*, *C*). To flask *B* add 2.0 mL of *ferrocyanide standard solution (100 ppm $Fe(CN)_6$) R*. To flasks *A* and *B* add 1 mL of *ferric chloride solution R2*. To the 3 flasks add 25.0 mL of the solution of the substance to be examined. Dilute the contents of each flask to 50.0 mL with *water R*. Allow to stand for 30 min. The absorbance (*2.2.25*) of the solution in flask *A* measured at 695 nm using the solution in flask *C* as the compensation liquid is not greater than the absorbance of the solution in flask *B* measured at 695 nm using the solution in flask *A* as the compensation liquid.

**Sulfates**: maximum 100 ppm.

*Test solution*. Dissolve 3.6 g in 120 mL of *distilled water R*, add with mixing 4 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 20 mL of a 250 g/L solution of *cupric chloride R* and dilute to 150.0 mL with *distilled water R*. Allow to stand for 16 h and filter or centrifuge until a clear light-blue solution is obtained.

*Reference solution*. To 40 mL of *sulfate standard solution (10 ppm $SO_4$) R* add 80 mL of *distilled water R* and 12-13 mL of a 250 g/L solution of *cupric chloride R*. Dilute to 150.0 mL with *distilled water R*. The volume of cupric chloride solution added is such that the colour of the final solution matches that of the test solution.

Allow the solutions to stand. Filter both solutions, discarding the first 25 mL of filtrate. To 100 mL of each filtrate, add 0.5 mL of *acetic acid R*. Mix and add 2 mL of a 250 g/L solution of *barium chloride R* and mix again. The test solution is not more opalescent than the reference solution.

**Water** (*2.5.12*): 9.0 per cent to 15.0 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.250 g in 100 mL of *water R* and add 0.1 mL of *dilute sulfuric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 13.10 mg of $Na_2[Fe(CN)_5(NO)]$.

STORAGE

Protected from light.

**01/2017:1997**

# SODIUM PERBORATE, HYDRATED

## Natrii perboras hydricus

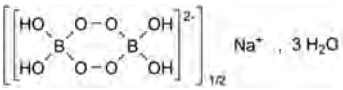


$NaBO_3,4H_2O$ or $NaBO_2,H_2O_2,3H_2O$ $M_r$ 153.9

DEFINITION

*Content*: 96.0 per cent to 103.0 per cent.

CHARACTERS

*Appearance*: colourless, prismatic crystals or white or almost white powder, stable in the crystalline form.

*Solubility*: sparingly soluble in water, with slow decomposition. It dissolves in dilute mineral acids.

IDENTIFICATION

A. Dissolve 20 mg in a mixture of 1 mL of *dilute sulfuric acid R* and 1 mL of *water R*. Add 1 mL of *potassium iodide solution R*. A reddish-brown colour appears.

B. The mixture obtained by treating about 100 mg with 0.1 mL of *sulfuric acid R* and 5 mL of *methanol R* burns with a greenish flame when ignited.

C. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dissolve 0.15 g in 15 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 1.2 per cent.

Dissolve 0.13 g in 150 mL of *distilled water R*.

**Iron** (*2.4.9*): maximum 20 ppm.

Dissolve 2.5 g in 10 mL of *dilute hydrochloric acid R* with heating, evaporate to dryness, with stirring, and dissolve the residue in 25 mL of hot *water R*. Dilute 5 mL of the obtained solution to 10 mL with *water R*.

ASSAY

Dissolve 0.300 g in 50.0 mL of *water R*. Dilute 10.0 mL of the solution to 50 mL with *water R* and add 10 mL of *dilute sulfuric acid R*. Titrate with *0.02 M potassium permanganate*.

1 mL of *0.02 M potassium permanganate* is equivalent to 7.693 mg of $NaH_8BO_7$.

STORAGE

In an airtight container.

**01/2017:2183**
**corrected 10.0**

# SODIUM PHENYLBUTYRATE

## Natrii phenylbutyras

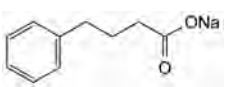


$C_{10}H_{11}NaO_2$ $M_r$ 186.2
[1716-12-7]

DEFINITION

Sodium 4-phenylbutanoate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium phenylbutyrate CRS*.

B. Dissolve 0.15 g in 2 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**pH** (*2.2.3*): 6.5 to 7.5.

Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Impurity C**. Gas chromatography (*2.2.28*).

*Silylation solution*. To 2 mL of *N,O-bis(trimethylsilyl)trifluoroacetamide R* add 0.04 mL of *chlorotrimethylsilane R* and mix.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 3 mL of *water R* and add 0.5 mL of *hydrochloric acid R*. Extract with 2 quantities, each of 5 mL, of *methylene chloride R*. Evaporate the combined methylene chloride extracts to dryness in a vial with a screw cap and add 0.5 mL of the silylation solution. Seal the vial and heat at 70 ± 5 °C for 20 min.

*Reference solution (a)*. Dissolve 5.0 mg of *sodium phenylbutyrate impurity C CRS* in *methylene chloride R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with *methylene chloride R*. Place 1.0 mL of this solution in a vial with a screw cap, evaporate to dryness and add 0.5 mL of the silylation solution. Seal the vial and heat at 70 ± 5 °C for 20 min.

*Reference solution (c)*. Dissolve 10 mg of the substance to be examined in 25 mL of *water R*. To 3 mL of this solution add 0.1 mL of *hydrochloric acid R*. Extract with 2 quantities, each of 5 mL, of *methylene chloride R*. Combine the methylene chloride extracts in a vial with a screw cap and add 2 mL of reference solution (a). Evaporate to dryness and add 0.5 mL of the silylation solution. Seal the vial and heat at 70 ± 5 °C for 20 min.

*Column*:
- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.9 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 50 |
| | 5 - 27 | 50 → 270 |
| | 27 - 32 | 270 |
| Injection port | | 270 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to phenylbutyrate (retention time = about 20 min): impurity C = about 0.98.

*System suitability*: reference solution (c):
- *resolution*: minimum 3.0 between the peaks due to impurity C and phenylbutyrate.

*Limit*:
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.20 g of the substance to be examined in 10 mL of *methanol R* and dilute to 50.0 mL with *water R*.

*Reference solution (a)*. Dissolve 4.0 mg of *α-tetralone R* (impurity B) in 10 mL of *methanol R* and dilute to 200.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 0.20 g of the substance to be examined in 10 mL of *methanol R*, add 1 mL of reference solution (a) and dilute to 50 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 50.0 mL with *water R*.

*Reference solution (d)*. Dissolve 5.0 mg of *3-benzoylpropionic acid R* (impurity A) in 2.5 mL of *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *methanol R*, *water R* (1:49:50 *V/V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of phenylbutyrate.

*Relative retention* with reference to phenylbutyrate (retention time = about 17 min): impurity A = about 0.3; impurity B = about 0.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 6 between the peaks due to impurity B and phenylbutyrate.

*Limits*:
- *impurity A*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.01 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (0.05 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent);
- *disregard limit of impurities other than B*: 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (d) (0.03 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

ASSAY

Disperse 0.150 g in 50 mL of *anhydrous acetic acid R*. The opalescence of the solution disappears during the titration. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.62 mg of $C_{10}H_{11}NaO_2$.

IMPURITIES

*Specified impurities: A, B, C.*

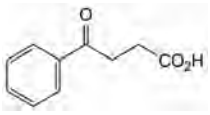

A. 4-oxo-4-phenylbutanoic acid (3-benzoylpropionic acid),

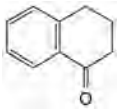

B. 3,4-dihydronaphthalen-1(2*H*)-one (α-tetralone),

C. 4-cyclohexylbutanoic acid.

**01/2013:1031**

# SODIUM PICOSULFATE

## Natrii picosulfas

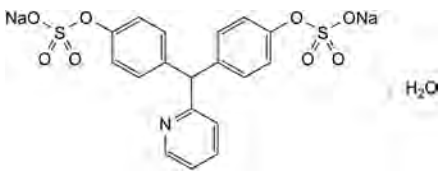

$C_{18}H_{13}NNa_2O_8S_2,H_2O$ $M_r$ 499.4

DEFINITION

4,4′-[(Pyridin-2-yl)methylene]diphenyl bis(sodium sulfate) monohydrate.

*Content*: 98.5 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium picosulfate CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution.* Dissolve 20 mg of *sodium picosulfate CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.
*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *ethyl acetate R* (2.5:12.5:25:60 *V/V/V/V*).
*Application*: 5 µL.
*Development*: over 1/2 of the plate.
*Drying*: in a current of warm air for 15 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To 5 mL of solution S (see Tests) add 1 mL of *dilute hydrochloric acid R* and heat to boiling. Add 1 mL of *barium chloride solution R1*. A white precipitate is formed.

D. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.05 mL of *phenolphthalein solution R*. The solution is colourless. Not more than 0.25 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *picosulfate for system suitability CRS* (containing impurities A and B) in 1.0 mL of the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R1* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: dissolve 2.3 g of *disodium hydrogen phosphate dihydrate R* in 800 mL of *water for chromatography R*, add 0.2 g of *cetyltrimethylammonium bromide R*, adjust to pH 7.5 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*; mix 550 mL of this solution and 450 mL of *acetonitrile R* (if necessary vary the buffer/acetonitrile proportion in 10 mL increments in order to fulfil the resolution requirement).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 263 nm.

*Injection*: 40 µL.

*Run time*: twice the retention time of picosulfate.

*Identification of impurities*: use the chromatogram supplied with *picosulfate for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to picosulfate (retention time = about 7.4 min): impurity B = about 0.5; impurity A = about 0.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to impurities B and A.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity B = 0.5;
- *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

Monographs S

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): 3.0 per cent to 5.0 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.400 g in 80 mL of *methanol R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 48.14 mg of $C_{18}H_{13}NNa_2O_8S_2$.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

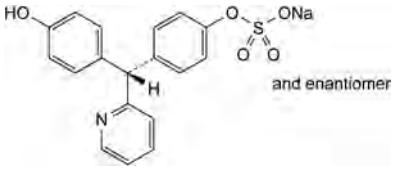


A. 4-[(*RS*)-(4-hydroxyphenyl)(pyridin-2-yl)methyl]phenyl sodium sulfate,

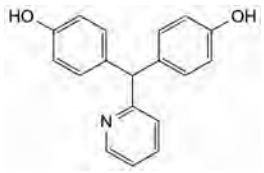


B. 4,4′-[(pyridin-2-yl)methylene]diphenol,

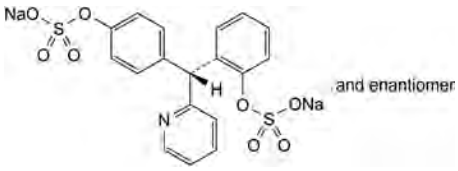


C. 2-[(*RS*)-(pyridin-2-yl)[4-(sulfonatooxy)phenyl]methyl]-phenyl disodium sulfate.

**01/2017:1909**

# SODIUM POLYSTYRENE SULFONATE

## Natrii polystyrenesulfonas

DEFINITION

Polystyrene sulfonate resin prepared in the sodium form.

*Exchange capacity*: 2.8 mmol to 3.4 mmol of potassium per gram (dried substance).

*Content*: 9.4 per cent to 11.0 per cent of Na (dried substance).

CHARACTERS

*Appearance*: almost white or light brown powder.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs using finely ground substance.

*Comparison*: *Ph. Eur. reference spectrum of sodium polystyrene sulfonate*.

B. Suspend 0.1 g in *water R*, add 2 mL of a 150 g/L solution of *potassium carbonate R*, and heat to boiling. Allow to cool and filter. To the filtrate add 4 mL of *potassium pyroantimonate solution R* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

TESTS

**Styrene**. Liquid chromatography (*2.2.29*).

*Test solution*. Shake 10.0 g of the substance to be examined with 10 mL of *acetone R* for 30 min, centrifuge and use the supernatant.

*Reference solution*. Dissolve 10 mg of *styrene R* in *acetone R* and dilute to 100 mL with the same solvent. Dilute 1 mL of this solution to 100 mL with *acetone R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (1:1 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Limit*:

– *styrene*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (1 ppm).

**Calcium**: maximum 0.10 per cent.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. To 1.10 g add 5 mL of *hydrochloric acid R*, heat to boiling, cool and add 10 mL of *water R*. Filter, wash the filter and residue with *water R* and dilute the filtrate and washing to 25.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *calcium standard solution (400 ppm Ca) R*, diluted as necessary with *water R*.

*Wavelength*: 422.7 nm.

**Potassium**: maximum 0.10 per cent.

Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution*. To 1.10 g add 5 mL of *hydrochloric acid R*, heat to boiling, cool and add 10 mL of *water R*. Filter, wash the filter and residue with *water R* and dilute the filtrate and washings to 25.0 mL with *water R*.

*Reference solutions*. Prepare the reference solutions using *potassium standard solution (100 ppm K) R*, diluted as necessary with *water R*.

*Wavelength*: 766.5 nm.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Microbial contamination** (*2.6.13*)

Bile-tolerant gram-negative bacteria: acceptance criterion less than $10^2$ CFU/g.

ASSAY

**Sodium**. Atomic emission spectrometry (*2.2.22, Method I*).

*Test solution.* In a platinum crucible moisten 0.90 g with a few drops of *sulfuric acid R*, ignite very gently and allow to cool. Moisten with a few drops of *sulfuric acid R* again, ignite at 800 ± 50 °C until a carbon-free ash is obtained and allow to cool.

Add 20 mL of *water R* to the crucible, warm gently on a water-bath until dissolution, cool, transfer quantitatively to a 100 mL graduated flask and dilute to 100.0 mL with *water R*. Dilute 5 mL of this solution to 1000.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *sodium standard solution (200 ppm Na) R*, diluted as necessary with *water R*.

*Wavelength*: 589 nm.

**Exchange capacity**. Atomic emission spectrometry (*2.2.22, Method I*).

*Solution A*. 9.533 g/L solution of *potassium chloride R*.

*Test solution.* To 1.6 g of the substance to be examined in a dry 250 mL ground-glass-stoppered flask add 100 mL of solution A, stopper and shake for 15 min. Filter, discard the first 20 mL of the filtrate and dilute 4 mL of the filtrate to 1000 mL with *water R*.

*Reference solutions.* Prepare the reference solutions by diluting 0, 1, 2, 3 and 4 mL of solution A respectively and 4, 3, 2, 1 and 0 mL of a 7.63 g/L solution of *sodium chloride R* to 1000 mL with *water R*.

*Wavelength*: 766.5 nm.

Prepare a calibration curve using the reference solutions and calculate the potassium exchange capacity of the substance to be examined in millimoles per gram taking the concentration of potassium in solution A as 128 mmoles of K per litre.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

A. styrene.

**01/2017:2041**
**corrected 10.0**

# SODIUM PROPIONATE

## Natrii propionas

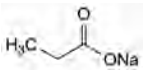


$C_3H_5NaO_2$ $M_r$ 96.1
[137-40-6]

DEFINITION

Sodium propanoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless crystals or, white or almost white powder, slightly hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of sodium propionate.*

B. Dissolve 0.1 g in a mixture of 2 mL of *copper sulfate solution R* and 2 mL of *methylene chloride R*. Shake vigorously and allow to stand. Both the upper and the lower layer show a blue colour.

C. To 5 mL of solution S (see Tests) add 2 mL of *0.1 M silver nitrate*. A white precipitate is formed.

D. Solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 7.8 to 9.2.

Dilute 1 mL of solution S to 5 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of the substance to be examined and 10 mg of *sodium acetate R* in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dilute 1 mL of *phosphoric acid R* to 1000 mL with *water for chromatography R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (a):
- *resolution*: minimum 5.0 between the peaks due to sodium acetate and sodium propionate.

*Limits*:
- *any impurity*: not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *total*: not more than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Readily oxidisable substances**. In a ground-glass-stoppered conical flask introduce 10 g of the substance to be examined. Add 100 mL of *water R* and stir to dissolve. Add 25 mL of *sodium hypobromite solution R* and 10 mL of a 200 g/L solution of *sodium acetate R*, stopper the flask and allow to stand for 15 min. Add 10 mL of *potassium iodide solution R* and 20 mL of *hydrochloric acid R* while cooling. Titrate with *0.1 M sodium thiosulfate*, adding 2 mL of *starch solution R* towards the end of the titration. Carry out a blank titration. The difference between the volumes used in the 2 titrations is not greater than 2.2 mL.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by heating in an oven at 105 °C for 3 h.

ASSAY

Dissolve 80.0 mg in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 9.61 mg of $C_3H_5NaO_2$.

STORAGE

In an airtight container.

**01/2017:1263**

# SODIUM PROPYL PARAHYDROXYBENZOATE

## Propylis parahydroxybenzoas natricus

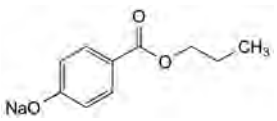


$C_{10}H_{11}NaO_3$ $M_r$ 202.2
[35285-69-9]

DEFINITION

Sodium 4-(propoxycarbonyl)phenolate.

*Content*: 94.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Dissolve 0.5 g in 50 mL of *water R*. Immediately add 5 mL of *hydrochloric acid R1*. Filter and wash the precipitate with *water R*. Dry *in vacuo* at 80 °C for 2 h. The precipitate melts (*2.2.14*) at 96 °C to 99 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: precipitate obtained in identification test A.

*Comparison*: *propyl parahydroxybenzoate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 10 mL of *water R*. Immediately add 2 mL of *hydrochloric acid R* and shake with 50 mL of *1,1-dimethylethyl methyl ether R*. Evaporate the upper layer to dryness and take up the residue with 10 mL of *acetone R*.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.

*Reference solution (a).* Dissolve 10 mg of *propyl parahydroxybenzoate CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *ethyl parahydroxybenzoate CRS* in 1 mL of test solution (a) and dilute to 10 mL with *acetone R*.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:30:70 *V/V/V*).

*Application*: 5 µL of test solution (b) and reference solutions (a) and (b).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 1 mL of solution S (see Tests) add 1 mL of *water R*. The solution gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R*, and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S, examined immediately after preparation, is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.5 to 10.5.

Dilute 1 mL of solution S to 100 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *ethyl parahydroxybenzoate CRS* (impurity C), 5 mg of *4-hydroxybenzoic acid R* (impurity A) and 5 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 50.0 mg of *propyl parahydroxybenzoate CRS* in 2.5 mL of *methanol R* and dilute to 50.0 mL with the mobile phase. Dilute 10.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 20.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 6.8 g/L solution of *potassium dihydrogen phosphate R*, *methanol R* (35:65 *V/V*).

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 2.5 times the retention time of propyl parahydroxybenzoate.

*Relative retention* with reference to propyl parahydroxybenzoate (retention time = about 4.5 min): impurity A = about 0.3; impurity C = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to impurity C and propyl parahydroxybenzoate.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 1.4;
– *impurity A*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (c) (4.0 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);

- *sum of impurities other than A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 350 ppm.

To 10 mL of solution S, add 1 mL of *nitric acid R* and 30 mL of *water R* and dilute to 50 mL with *water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*. Prepare the standard using 14 mL of *chloride standard solution (5 ppm Cl) R* to which 1 mL of *water R* has been added.

**Sulfates** (*2.4.13*): maximum 300 ppm.

To 25 mL of solution S, add 5 mL of *distilled water R* and 10 mL of *hydrochloric acid R* and dilute to 50 mL with *distilled water R*. Shake and filter. Dilute 10 mL of the filtrate to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{10}H_{11}NaO_3$ from the declared content of *propyl parahydroxybenzoate CRS*, multiplied by a correction factor of 1.122.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

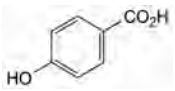


A. 4-hydroxybenzoic acid,

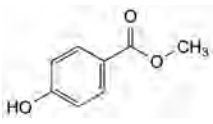


B. methyl 4-hydroxybenzoate (methyl parahydroxybenzoate),

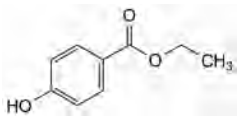


C. ethyl 4-hydroxybenzoate (ethyl parahydroxybenzoate),

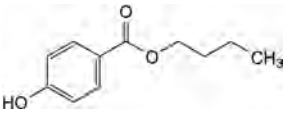


D. butyl 4-hydroxybenzoate (butyl parahydroxybenzoate).

**01/2017:0413**

# SODIUM SALICYLATE

## Natrii salicylas

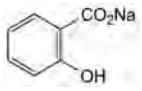


$C_7H_5NaO_3$ $M_r$ 160.1
[54-21-7]

DEFINITION

Sodium 2-hydroxybenzenecarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small, colourless crystals or shiny flakes.

*Solubility*: freely soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sodium salicylate CRS*.

B. Solution S (see Tests) gives the reactions of salicylates (*2.3.1*).

C. It gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. To 20 mL of solution S add 0.1 mL of *phenol red solution R*. The solution is yellow. Not more than 2.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to violet-red.

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 5 mL of solution S add 5 mL of *water R* and 10 mL of *dilute nitric acid R* and filter. Dilute 10 mL of the filtrate to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 600 ppm.

Dilute 2.5 mL of solution S to 15 mL with *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.00 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.130 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.01 mg of $C_7H_5NaO_3$.

STORAGE

In an airtight container, protected from light.

01/2016:2740

# SODIUM SELENITE

## Natrii selenis

$Na_2SeO_3$ $M_r$ 172.9
[10102-18-8]

DEFINITION

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline deliquescent powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 50 mg in 5 mL of a mixture of equal volumes of *dilute hydrochloric acid R* and *water R* and heat to boiling. Add 50 mg of *ascorbic acid R*; a red precipitate is formed which may become black.

B. Dissolve 50 mg in a mixture of 1 mL of *dilute hydrochloric acid R* and 5 mL of *water R*. Add 1 mL of *barium chloride solution R1*; the solution remains clear.

C. It gives reaction (a) of sodium (*2.3.1*).

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.8 to 10.8 for solution S.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dissolve 1.0 g in 5 mL of *water R*, add 9 mL of *nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates and selenates** (*2.4.13*): maximum 300 ppm (determined as sulfates).

Dissolve 0.5 g in 10 mL of *distilled water R*, add 0.5 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*.

**Iron**: maximum 50 ppm.

To 2 mL of solution S add 2 mL of a 200 g/L solution of *sulfosalicylic acid R* and 5 mL of *concentrated ammonia R*, and dilute to 10 mL with *water R*. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 1 mL of *iron standard solution (10 ppm Fe) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 130 °C for 2 h.

ASSAY

Dissolve 80.0 mg in 50 mL of *water R*. Add 1.0 mL of *anhydrous formic acid R*, 25.0 mL of *0.1 M sodium thiosulfate* and 0.5 g of *potassium iodide R*. Titrate immediately with *0.05 M iodine* using *starch solution R* as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 4.323 mg of $Na_2SeO_3$.

STORAGE

In an airtight container.

01/2016:1677

# SODIUM SELENITE PENTAHYDRATE

## Natrii selenis pentahydricus

$Na_2SeO_3,5H_2O$ $M_r$ 263.0
[26970-82-1]

DEFINITION

*Content*: 98.5 per cent to 101.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Dissolve 50 mg in 5 mL of a mixture of equal volumes of *dilute hydrochloric acid R* and *water R* and heat to boiling. Add 50 mg of *ascorbic acid R*; a red precipitate is formed which may become black.

B. Dissolve 50 mg in a mixture of 1 mL of *dilute hydrochloric acid R* and 5 mL of *water R*. Add 1 mL of *barium chloride solution R1*; the solution remains clear.

C. It gives reaction (a) of sodium (*2.3.1*).

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 9.8 to 10.8 for solution S.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dissolve 1.0 g in 5 mL of *water R*, add 9 mL of *nitric acid R* and dilute to 15 mL with *water R*.

**Sulfates and selenates** (*2.4.13*): maximum 300 ppm (determined as sulfates).

Dissolve 0.5 g in 10 mL of *distilled water R*, add 0.5 mL of *hydrochloric acid R1* and dilute to 15 mL with *distilled water R*.

**Iron**: maximum 50 ppm.

To 2 mL of solution S add 2 mL of a 200 g/L solution of *sulfosalicylic acid R*, 5 mL of *concentrated ammonia R* and dilute to 10 mL with *water R*. The solution is not more intensely coloured than a reference solution prepared at the same time and in the same manner using 1 mL of *iron standard solution (10 ppm Fe) R*.

ASSAY

Dissolve 0.120 g in 50 mL of *water R*. Add 1.0 mL of *anhydrous formic acid R*, 25.0 mL of *0.1 M sodium thiosulfate* and 0.5 g of *potassium iodide R*. Titrate immediately with *0.05 M iodine* using *starch solution R* as indicator. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 6.575 mg of $Na_2SeO_3,5H_2O$.

STORAGE

In an airtight container.

**01/2020:0983**

# SODIUM STARCH GLYCOLATE (TYPE A)[3]

## Carboxymethylamylum natricum A

DEFINITION

Sodium salt of a cross-linked partly *O*-carboxymethylated starch.

*Content*: 2.8 per cent to 4.2 per cent of Na ($A_r$ 22.99) (substance washed with ethanol (80 per cent *V/V*) and dried).

♦ CHARACTERS

*Appearance*: white or almost white, fine, free-flowing powder, very hygroscopic.

*Solubility*: practically insoluble in methylene chloride. It gives a translucent suspension in water. ♦

IDENTIFICATION

◊ A. pH (see Tests).

B. Prepare with shaking and without heating a mixture of 4.0 g of the substance to be examined and 20 mL of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 mL of *carbon dioxide-free water R* and shake. A suspension forms that settles after standing. ◊

C. To an acidified solution, add *iodinated potassium iodide solution R1*. The solution becomes blue or violet.

D. Solution S2 (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S1**. Centrifuge the suspension obtained in identification test B at 2500 *g* for 10 min. Collect carefully the supernatant.

**Solution S2**. Place 2.5 g in a silica or platinum crucible and add 2 mL of a 500 g/L solution of *sulfuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulfuric acid R*, heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 mL of *water R*.

◊ **Appearance of solution S1**. Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*). ◊

**pH** (*2.2.3*): 5.5 to 7.5.

Disperse 1.0 g in 30 mL of *carbon dioxide-free water R*.

**Sodium glycolate**: maximum 2.0 per cent. *Carry out the test protected from light.*

*Test solution.* Place 0.20 g in a beaker. Add 5 mL of *acetic acid R* and 5 mL of *water R*. Stir until dissolution is complete (about 10 min). Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 mL with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant.

*Reference solution.* Dissolve 0.310 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*) at room temperature overnight, in *water R* and dilute to 500.0 mL with the same solvent. To 5.0 mL of this solution add 5 mL of *acetic acid R* and allow to stand for about 30 min. Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 mL with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant.

Heat 2.0 mL of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 mL of *2,7-dihydroxynaphthalene solution R*. Shake and heat in a water-bath for 20 min. Cool under running water, transfer to a volumetric flask and dilute to 25.0 mL with *sulfuric acid R*, maintaining the flask under running water. Within 10 min, measure the absorbance at 540 nm (*2.2.25*) using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 mL of the reference solution.

**Sodium chloride**: maximum 7.0 per cent.

Place 0.500 g in a beaker and suspend in 100 mL of *water R*. Add 1 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*), using a silver-based indicator electrode and a double-junction reference electrode containing a 100 g/L solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket.

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

**Iron** (*2.4.9*): maximum 20 ppm, determined on 10 mL of solution S2.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 1.5 h.

**Microbial contamination**. It complies with the test for *Escherichia coli* and *Salmonella* (*2.6.13*).

ASSAY

Shake about 1 g with 20 mL of *ethanol (80 per cent V/V) R*, stir for 10 min and filter. Repeat the operation until chloride has been completely extracted and verify the absence of chloride using *silver nitrate solution R2*. Dry the residue at 105 °C to constant mass. To 0.700 g of the dried residue, add 80 mL of *glacial acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 2.299 mg of Na.

♦ STORAGE

In an airtight container, protected from light. ♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sodium starch glycolate (type A) used as disintegrant in tablets.*

**Settling volume**. Introduce 70 mL of *water R* in a flask, add 1.0 g of the substance to be examined (dried substance) and stir with a glass rod so that no lumps remain. Transfer the

(3) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

suspension to a 100 mL graduated cylinder, rinse the walls of the flask with *water R* and dilute to 100 mL with the same solvent. Stir again until the substance is evenly distributed. Allow to stand for 2 h. The volume of the sediment is typically less than 45 mL.

**Degree of substitution**. Place 1.000 g (dried substance) in a 500 mL conical flask, add 300 mL of a 100 g/L solution of *sodium chloride R* and 25.0 mL of *0.1 M sodium hydroxide*, stopper the flask and allow to stand for 5 min, shaking occasionally. Add 0.05 mL of *m-cresol purple solution R* and about 15 mL of *0.1 M hydrochloric acid* from a burette. Insert the stopper and shake. If the solution is violet, add *0.1 M hydrochloric acid* in 1 mL portions until the solution becomes yellow, shaking after each addition. Titrate with *0.1 M sodium hydroxide* until the colour turns to violet. Calculate the number of milliequivalents (*M*) of base required to neutralise the equivalent of 1 g of dried substance.

Calculate the degree of acid carboxymethyl substitution (*A*) using the following expression:

$$A = \frac{1150M}{(7102 - 412M - 80C)}$$

*C* = sulfated ash as a percentage, determined as follows.

Introduce 1.3 g of the substance to be examined in a flask and add 20 mL of *ethanol (80 per cent V/V) R*. Shake for 10 min and filter on a sintered-glass filter (*2.1.2*). Repeat this operation 4 times. Add to the residue 10 mL of *ethanol (96 per cent) R* and filter. Collect the residue and place it in an oven at 105 °C for 4 h. Place the dried residue in a mortar and crush. Determine the sulfated ash content (*C*) (*2.4.14*) on 1.000 g of the powder obtained.

Calculate the degree of sodium carboxymethyl substitution (*S*) using the following expression:

$$S = \frac{(162 + 58A)C}{(7102 - 80C)}$$

The degree of substitution is the sum of *A* and *S*.

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**01/2020:0984**

# SODIUM STARCH GLYCOLATE (TYPE B)[4]

## Carboxymethylamylum natricum B

DEFINITION

Sodium salt of a cross-linked partly *O*-carboxymethylated starch.

*Content*: 2.0 per cent to 3.4 per cent of Na ($A_r$ 22.99) (substance washed with ethanol (80 per cent *V/V*) and dried).

♦ CHARACTERS

*Appearance*: white or almost white, fine, free-flowing powder, very hygroscopic.

*Solubility*: practically insoluble in methylene chloride. It gives a translucent suspension in water. ♦

IDENTIFICATION

◊ A. pH (see Tests).

B. Prepare with shaking and without heating a mixture of 4.0 g of the substance to be examined and 20 mL of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 mL of *carbon dioxide-free water R* and shake. A suspension forms that settles after standing. ◊

C. To an acidified solution, add *iodinated potassium iodide solution R1*. The solution becomes blue or violet.

D. Solution S2 (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S1**. Centrifuge the suspension obtained in identification test B at 2500 *g* for 10 min. Collect carefully the supernatant.

**Solution S2**. Place 2.5 g in a silica or platinum crucible and add 2 mL of a 500 g/L solution of *sulfuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, and then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *dilute sulfuric acid R* and heat and incinerate as above. Allow to cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 mL of *water R*.

◊ **Appearance of solution S1**. Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*). ◊

**pH** (*2.2.3*): 3.0 to 5.0.

Disperse 1.0 g in 30 mL of *carbon dioxide-free water R*.

**Sodium glycolate**: maximum 2.0 per cent. *Carry out the test protected from light.*

*Test solution*. Place 0.20 g in a beaker. Add 5 mL of *acetic acid R* and 5 mL of *water R*. Stir until dissolution is complete (about 10 min). Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 mL with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant.

*Reference solution*. Dissolve 0.310 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*) at room temperature overnight, in *water R* and dilute to 500.0 mL with the same solvent. To 5.0 mL of this solution add 5 mL of *acetic acid R* and allow to stand for about 30 min. Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 mL with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant.

Heat 2.0 mL of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 mL of *2,7-dihydroxynaphthalene solution R*. Shake and heat in a water-bath for 20 min. Cool under running water, transfer quantitatively to a volumetric flask and dilute to 25.0 mL with *sulfuric acid R*, maintaining the flasks under running water. Within 10 min, measure the absorbance at 540 nm (*2.2.25*) using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 mL of the reference solution.

**Sodium chloride**: maximum 7.0 per cent.

Place 0.500 g in a beaker and suspend in 100 mL of *water R*. Add 1 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*), using a silver-based indicator electrode and a double-junction reference electrode containing a 100 g/L solution of *potassium nitrate R* in the outerjacket and a standard filling solution in the inner jacket.

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

(4) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

**Iron** (*2.4.9*): maximum 20 ppm, determined on 10 mL of solution S2.

**Loss on drying** (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 1.5 h.

**Microbial contamination**. It complies with the test for *Escherichia coli* and *Salmonella* (*2.6.13*).

ASSAY

Shake about 1 g with 20 mL of *ethanol (80 per cent V/V) R*, stir for 10 min and filter. Repeat the operation until chloride has been completely extracted and verify the absence of chloride using *silver nitrate solution R2*. Dry the residue at 105 °C to constant mass. To 0.700 g of the dried residue, add 80 mL of *glacial acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 2.299 mg of Na.

♦ STORAGE

In an airtight container, protected from light. ♦

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sodium starch glycolate (type B) used as disintegrant in tablets.*

**Settling volume**. Introduce 70 mL of *water R* in a flask, add 1.0 g of the substance to be examined (dried substance) and stir with a glass rod so that no lumps remain. Transfer the suspension to a 100 mL graduated cylinder, rinse the walls of the flask with *water R* and dilute to 100 mL with the same solvent. Stir again until the substance is evenly distributed. Allow to stand for 2 h. The volume of the sediment is typically less than 45 mL.

**Degree of substitution**. Place 1.000 g (dried substance) in a 500 mL conical flask, add 300 mL of a 100 g/L solution of *sodium chloride R* and 25.0 mL of *0.1 M sodium hydroxide*, stopper the flask and allow to stand for 5 min, shaking occasionally. Add 0.05 mL of *m-cresol purple solution R* and about 15 mL of *0.1 M hydrochloric acid* from a burette. Insert the stopper and shake. If the solution is violet, add *0.1 M hydrochloric acid* in 1 mL portions until the solution becomes yellow, shaking after each addition. Titrate with *0.1 M sodium hydroxide* until the colour turns to violet. Calculate the number of milliequivalents (*M*) of base required to neutralise the equivalent of 1 g of dried substance.

Calculate the degree of acid carboxymethyl substitution (*A*) using the following expression:

$$A = \frac{1150M}{(7102 - 412M - 80C)}$$

*C* = sulfated ash as a percentage, determined as follows.

Introduce 1.3 g of the substance to be examined in a flask and add 20 mL of *ethanol (80 per cent V/V) R*. Shake for 10 min and filter on a sintered-glass filter (*2.1.2*). Repeat this operation 4 times. Add to the residue 10 mL of *ethanol (96 per cent) R* and filter. Collect the residue and place it in an oven at 105 °C for 4 h. Place the dried residue in a mortar and crush. Determine the sulfated ash content (*C*) (*2.4.14*) on 1.000 g of the powder obtained.

Calculate the degree of sodium carboxymethyl substitution (*S*) using the following expression:

$$S = \frac{(162 + 58A)C}{(7102 - 80C)}$$

The degree of substitution is the sum of *A* and *S*.

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**01/2020:1566**

# SODIUM STARCH GLYCOLATE (TYPE C)(5)

## Carboxymethylamylum natricum C

DEFINITION

Sodium salt of a partly *O*-carboxymethylated starch, cross-linked by physical dehydration.

*Content*: 2.8 per cent to 5.0 per cent of Na ($A_r$ 22.99) (substance washed with ethanol (80 per cent *V/V*) and dried).

CHARACTERS

*Appearance*: white or almost white, fine, free-flowing powder, very hygroscopic.

*Microscopic examination*: it is seen to consist of granules, irregularly shaped, ovoid or pear-shaped, 30-100 µm in size, or rounded, 10-35 µm in size; compound granules consisting of 2-4 components occur occasionally; the granules have an eccentric hilum and clearly visible concentric striations; between crossed nicol prisms, the granules show a distinct black cross intersecting at the hilum; small crystals are visible at the surface of the granules. The granules show considerable swelling in contact with water.

*Solubility*: soluble in water, practically insoluble in methylene chloride. It gives a translucent gel-like product in water.

IDENTIFICATION

A. pH (see Tests).

B. Prepare with shaking and without heating a mixture of 4.0 g of the substance to be examined and 20 mL of *carbon dioxide-free water R*. The mixture has the appearance of a gel. Add 100 mL of *carbon dioxide-free water R* and shake: the gel remains stable (difference from types A and B). Keep the gel for the tests for appearance of gel and pH.

C. To 5 mL of the gel obtained in identification test B add 0.05 mL of *iodine solution R1*. A dark blue colour is produced.

D. Solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Place 2.5 g in a silica or platinum crucible and add 2 mL of a 500 g/L solution of *sulfuric acid R*. Heat on a water-bath, then cautiously over a naked flame, raising the temperature progressively, and then incinerate in a muffle furnace at 600 ± 25 °C. Continue heating until all black particles have disappeared. Allow to cool, add a few drops of *sulfuric acid R*, heat and incinerate as above. Allow to

(5) In contrast to sodium starch glycolate type A and type B, this monograph has NOT undergone pharmacopoeial harmonisation.

cool, add a few drops of *ammonium carbonate solution R*, evaporate to dryness and incinerate cautiously. Allow to cool and dissolve the residue in 50 mL of *water R*.

**Appearance of gel**. The gel obtained in identification test B is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 7.5 for the gel obtained in identification test B.

**Sodium glycolate**: maximum 2.0 per cent. *Carry out the test protected from light.*

*Test solution.* Place 0.20 g in a beaker. Add 5 mL of *acetic acid R* and 5 mL of *water R*. Stir until dissolution is complete (about 10 min). Add 50 mL of *acetone R* and 1 g of *sodium chloride R*. Filter through a fast filter paper impregnated with *acetone R*, rinse the beaker and filter with *acetone R*. Combine the filtrate and washings and dilute to 100.0 mL with *acetone R*. Allow to stand for 24 h without shaking. Use the clear supernatant.

*Reference solution.* Dissolve 0.310 g of *glycolic acid R*, previously dried in a desiccator (*2.2.32*), in *water R* and dilute to 500.0 mL with the same solvent. To 5.0 mL of this solution, add 5 mL of *acetic acid R* and allow to stand for about 30 min. Add 50 mL of *acetone R* and 1 g of *sodium chloride R* and dilute to 100.0 mL with *acetone R*.

Heat 2.0 mL of the test solution on a water-bath for 20 min. Cool to room temperature and add 20.0 mL of *2,7-dihydroxynaphthalene solution R*. Shake and heat on a water-bath for 20 min. Cool under running water, transfer to a volumetric flask and dilute to 25.0 mL with *sulfuric acid R*, maintaining the flask under running water. Within 10 min, measure the absorbance (*2.2.25*) at 540 nm using *water R* as the compensation liquid. The absorbance of the solution prepared with the test solution is not greater than that of a solution prepared at the same time and in the same manner with 2.0 mL of the reference solution.

**Sodium chloride**: maximum 1 per cent.

Shake 1.00 g with 20 mL of *ethanol (80 per cent V/V) R* for 10 min and filter. Repeat the operation 4 times. Dry the residue to constant mass at 100 °C and set aside for the assay. Combine the filtrates. Evaporate to dryness, take up the residue with *water R* and dilute to 25.0 mL with the same solvent. To 10.0 mL of the solution add 30 mL of *water R* and 5 mL of *dilute nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*), using a silver-based indicator electrode and a double-junction reference electrode containing a 100 g/L solution of *potassium nitrate R* in the outer jacket and a standard filling solution in the inner jacket.

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

**Iron** (*2.4.9*): maximum 20 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 7.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Microbial contamination**. It complies with the test for *Escherichia coli* and *Salmonella* (*2.6.13*).

ASSAY

To 0.500 g of the dried and crushed residue obtained in the test for sodium chloride add 80 mL of *anhydrous acetic acid R* and heat under a reflux condenser for 2 h. Cool the solution to room temperature. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank test.

1 mL of *0.1 M perchloric acid* is equivalent to 2.299 mg of Na.

STORAGE

In an airtight container, protected from light.

**01/2009:2058**
**corrected 7.3**

# SODIUM STEARATE

## Natrii stearas

DEFINITION

Mixture of sodium salts of different fatty acids consisting mainly of stearic (octadecanoic) acid [$C_{17}H_{35}COONa$; $M_r$ 306.5] and palmitic (hexadecanoic) acid [$C_{15}H_{31}COONa$; $M_r$ 278.4].

*Content*:

- *sodium*: 7.4 per cent to 8.5 per cent ($A_r$ 22.99) (dried substance);
- *stearic acid in the fatty acid fraction*: minimum 40 per cent;
- *sum of stearic acid and palmitic acid in the fatty acid fraction*: minimum 90 per cent.

CHARACTERS

*Appearance*: white or yellowish, fine powder, greasy to the touch.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Freezing point (*2.2.18*): minimum 53 °C for the residue obtained in the preparation of solution S (see Tests).

B. Acid value (*2.5.1*): 195 to 210, determined on 0.200 g of the residue obtained in the preparation of solution S dissolved in 25 mL of the prescribed mixture of solvents.

C. Examine the chromatograms obtained in the assay of stearic acid and palmitic acid.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the 2 principal peaks in the chromatogram obtained with the reference solution.

D. Solution S gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. To 10.0 g add 100 mL of *peroxide-free ether R* and 80 mL of *acetic acid R*. Boil under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 8 mL, of *acetic acid R*. Combine the aqueous layers, wash with 30 mL of *peroxide-free ether R* and dilute to 100 mL with *distilled water R* (solution S). Evaporate the ether layers to dryness on a water-bath and dry the residue at 100-105 °C.

**Acidity or alkalinity**. Suspend 2.0 g in 50 mL of previously neutralised *ethanol (96 per cent) R*. Heat under reflux to dissolve and add 3 drops of *phenolphthalein solution R*; the solution is colourless. Not less than 0.60 mL and not more than 0.85 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 0.2 per cent.

Dilute 0.25 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.3 per cent.

Dilute 0.5 mL of solution S to 15 mL with *distilled water R*.

**Nickel**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Place 50.0 mg of the substance to be examined in a polytetrafluoroethylene digestion flask and add 0.5 mL of a mixture of 1 volume of *heavy metal-free hydrochloric acid R* and 5 volumes of *heavy metal-free nitric acid R*. Allow to digest at 170 °C for 5 h. Allow to cool. Dissolve the residue in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *nickel standard solution (10 ppm Ni) R*, diluting as necessary with *water R*.

*Source*: nickel hollow-cathode lamp.

*Wavelength*: 232.0 nm.

*Atomisation device*: furnace.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent.

In a weighing glass introduce 1.0 g of previously washed *sand R*, dry at 105 °C and weigh. Add 0.500 g of the substance to be examined and 10 mL of *ethanol (96 per cent) R*. Evaporate at 80 °C and dry the residue at 105 °C for 4 h.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

**Sodium**. Dissolve 0.250 g with gentle heating in a mixture of 5 mL of *acetic anhydride R* and 20 mL of *anhydrous acetic acid R*. Cool and add 20 mL of *dioxan R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 2.299 mg of Na.

**Stearic acid and palmitic acid**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* In a conical flask fitted with a reflux condenser, dissolve 0.10 g of the substance to be examined in 5 mL of *boron trifluoride-methanol solution R*. Boil under a reflux condenser for 10 min. Add 4 mL of *heptane R* through the condenser and boil again under a reflux condenser for 10 min. Allow to cool. Add 20 mL of *saturated sodium chloride solution R*. Shake and allow the layers to separate. Remove about 2 mL of the organic layer and dry over 0.2 g of *anhydrous sodium sulfate R*. Dilute 1.0 mL of the solution to 100.0 mL with *heptane R*.

*Reference solution.* Prepare the reference solution in the same manner as the test solution using 50.0 mg of *palmitic acid CRS* and 50.0 mg of *stearic acid CRS* instead of the substance to be examined.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 70 |
| | 2 - 36 | 70 → 240 |
| | 36 - 41 | 240 |
| Injection port | | 220 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to methyl stearate (retention time = about 40 min): methyl palmitate = about 0.88.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to methyl palmitate and methyl stearate.

Calculate the content of stearic acid and palmitic acid.

STORAGE

In an airtight container, protected from light.

**07/2010:1567**
**corrected 10.0**

# SODIUM STEARYL FUMARATE

## Natrii stearylis fumaras

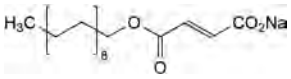


$C_{22}H_{39}NaO_4$ $M_r$ 390.5
[4070-80-8]

DEFINITION

Sodium octadecyl (*E*)-butenedioate.

*Content*: 99.0 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, fine powder with agglomerates of flat, circular particles.

*Solubility*: practically insoluble in water, slightly soluble in methanol, practically insoluble in acetone and in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sodium stearyl fumarate CRS*.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Silylation solution.* To 2 mL of *N,O-bis(trimethylsilyl)trifluoroacetamide R* add 0.02 mL of *chlorotrimethylsilane R* and mix.

*Test solution.* Introduce 15.0 mg of the substance to be examined in a vial with a screw cap and add 1 mL of the silylation solution. Seal the vial and heat at about 70 °C for 1 h. After the reaction a precipitate remains in the vial; filter the solution through a nylon filter (pore size 0.45 µm).

*Reference solution.* Introduce 1.0 mg of *sodium stearyl maleate CRS* and 1.0 mg of *sodium stearyl fumarate CRS* into a vial with a screw cap and add 1 mL of the silylation solution. Seal the vial and heat at about 70 °C for 1 h.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.53 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.15 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2 mL/min.

*Split ratio*: 1:25.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 180 |
| | 1 - 21 | 180 → 320 |
| | 21 - 26 | 320 |
| Injection port | | 250 |
| Detector | | 320 |

*Detection*: flame ionisation.

*Injection*: 2 µL.

*Relative retention* with reference to stearyl trimethylsilyl fumarate (retention time = about 9 min): stearyl alcohol = 0.30; stearyl trimethylsilyl ether = 0.35; palmityl trimethylsilyl fumarate = 0.80; heptadecyl trimethylsilyl fumarate = 0.85; stearyl trimethylsilyl maleate = 0.90; nonadecyl trimethylsilyl fumarate = 1.05; eicos-11-enyl trimethylsilyl fumarate = 1.15; distearyl fumarate = 2.25.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks in the chromatogram obtained with the reference solution.

*Limits*:

– *any impurity*: maximum 0.5 per cent;
– *total*: maximum 5.0 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.250 g, accurately weighed, in 10 mL of *methylene chloride R* and add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 39.05 mg of $C_{22}H_{39}NaO_4$.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sodium stearyl fumarate used as a lubricant in tablets and capsules.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26, Method I*).

**01/2019:0099**

# SODIUM SULFATE, ANHYDROUS

## Natrii sulfas anhydricus

$Na_2SO_4$ $M_r$ 142.0
[7757-82-6]

DEFINITION

Anhydrous disodium sulfate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water.

IDENTIFICATION

A. It gives reaction (a) of sulfates (*2.3.1*).

B. It gives reaction (a) of sodium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 2.2 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 450 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Calcium** (*2.4.3*): maximum 450 ppm, if intended for use in the manufacture of parenteral preparations.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 90 ppm, if intended for use in the manufacture of parenteral preparations.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium**: maximum 200 ppm, if intended for use in the manufacture of parenteral preparations.

To 10 mL of solution S add 1 mL of *glycerol (85 per cent) R*, 0.15 mL of *titan yellow solution R*, 0.25 mL of *ammonium oxalate solution R* and 5 mL of *dilute sodium hydroxide solution R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 5 mL of *magnesium standard solution (10 ppm Mg) R* and 5 mL of *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 130 °C.

ASSAY

Pack 20 g of *strongly acidic ion-exchange resin R* into a glass column, at least 20 cm long and 20 mm in internal diameter, and cover it with *water R*. After 5 min, wash the resin with *water R* until the pH of the eluate is about 6 to 7 using a *pH indicator strip R*. Keep the resin covered with *water R*. Dissolve 0.500 g of the substance to be examined in 10 mL of *water R* in a beaker. Load the solution onto the column and pass through the resin at a flow rate of about 4 mL/min until the resin is just covered with the solution. Using about 200 mL of *water R*, rinse the beaker and pass the rinsings through the column at the same flow rate. Check that the pH of the last eluate is about 6 to 7 using a *pH indicator strip R*.

Titrate the eluate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 71.00 mg of $Na_2SO_4$.

STORAGE

Store in an airtight container.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2019:0100**

# SODIUM SULFATE DECAHYDRATE

## Natrii sulfas decahydricus

$Na_2SO_4,10H_2O$ $M_r$ 322.2
[7727-73-3]

DEFINITION

Disodium sulfate decahydrate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless, transparent crystals.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent). It partly dissolves in its own water of crystallisation at about 33 °C.

IDENTIFICATION

A. It gives reaction (a) of sulfates (*2.3.1*).

B. It gives reaction (a) of sodium (*2.3.1*).

C. Loss on drying (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Calcium** (*2.4.3*): maximum 200 ppm, if intended for use in the manufacture of parenteral preparations.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Iron** (*2.4.9*): maximum 40 ppm, if intended for use in the manufacture of parenteral preparations.

Dilute 5 mL of solution S to 10 mL with *water R*.

**Magnesium**: maximum 100 ppm, if intended for use in the manufacture of parenteral preparations.

To 10 mL of solution S add 1 mL of *glycerol (85 per cent) R*, 0.15 mL of *titan yellow solution R*, 0.25 mL of *ammonium oxalate solution R* and 5 mL of *dilute sodium hydroxide solution R* and shake. Any pink colour in the test solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 5 mL of *magnesium standard solution (10 ppm Mg) R* and 5 mL of *water R*.

**Loss on drying** (*2.2.32*): 52.0 per cent to 57.0 per cent, determined on 1.000 g by drying at 30 °C for 1 h, then at 130 °C.

ASSAY

Pack 20 g of *strongly acidic ion-exchange resin R* into a glass column, at least 20 cm long and 20 mm in internal diameter, and cover it with *water R*. After 5 min, wash the resin with *water R* until the pH of the eluate is about 6 to 7 using a *pH indicator strip R*. Keep the resin covered with *water R*. Dissolve 1.100 g of the substance to be examined in 10 mL of *water R* in a beaker. Load the solution onto the column and pass through the resin at a flow rate of about 4 mL/min until the resin is just covered with the solution. Using about 200 mL of *water R*, rinse the beaker and pass the rinsings through the column at the same flow rate. Check that the pH of the last eluate is about 6 to 7 using a *pH indicator strip R*.

Titrate the eluate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 0.161 g of $Na_2SO_4,10H_2O$.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

**01/2017:0775**

# SODIUM SULFITE

## Natrii sulfis

$Na_2SO_3$ $M_r$ 126.0
[7757-83-7]

DEFINITION

*Content*: 95.0 per cent to 100.5 per cent of $Na_2SO_3$.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. To 5 mL of solution S add 0.5 mL of *0.05 M iodine*. The solution is colourless and gives reaction (a) of sulfates (*2.3.1*).

C. Solution S gives reaction (a) of sodium (*2.3.1*).

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 5 g in *water R* and dilute to 100 mL with the same solvent.

**Solution S1**. To 10.0 g add 25 mL of *water R*. Shake until mostly dissolved, carefully and progressively add 15 mL of *hydrochloric acid R*. Heat to boiling. Cool and dilute to 100.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Thiosulfates**: maximum 0.1 per cent.

To 2.00 g add 100 mL of *water R*. Shake, add 10 mL of *formaldehyde solution R* and 10 mL of *acetic acid R*. Allow to stand for 5 min. Add 0.5 mL of *starch solution R* and titrate with *0.05 M iodine*. Carry out a blank titration. The difference between the volumes used in the titrations is not more than 0.15 mL.

**Iron** (*2.4.9*): maximum 10 ppm, determined on solution S1.

**Selenium**: maximum 10 ppm.

To 3.0 g add 10 mL of *formaldehyde solution R*, carefully and progressively add 2 mL of *hydrochloric acid R*. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 1.0 g of the substance to be examined to which 0.2 mL of *selenium standard solution (100 ppm Se) R* has been added.

**Zinc**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 2.0 mL of solution S1 to 10.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *water R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Introduce 0.250 g into a 500 mL conical flask containing 50.0 mL of *0.05 M iodine*. Shake until completely dissolved. Add 1 mL of *starch solution R* and titrate the excess of iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 6.30 mg of $Na_2SO_3$.

**01/2017:0776**

# SODIUM SULFITE HEPTAHYDRATE

## Natrii sulfis heptahydricus

$Na_2SO_3,7H_2O$ $M_r$ 252.2
[10102-15-5]

DEFINITION

*Content*: 48.0 per cent to 52.5 per cent of $Na_2SO_3$.

CHARACTERS

*Appearance*: colourless crystals.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is slightly alkaline (*2.2.4*).

B. To 5 mL of solution S add 0.5 mL of *0.05 M iodine*. The solution is colourless and gives reaction (a) of sulfates (*2.3.1*).

C. Solution S gives reaction (a) of sodium (*2.3.1*).

D. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 10 g in *water R* and dilute to 100 mL with the same solvent.

**Solution S1**. To 20.0 g add 25 mL of *water R*. Shake until mostly dissolved, and carefully and progressively, add 15 mL of *hydrochloric acid R*. Heat to boiling. Cool and dilute to 100.0 mL with *water R*.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method I*).

**Thiosulfates**: maximum 0.05 per cent.

To 4.00 g add 100 mL of *water R*. Shake to dissolve, add 10 mL of *formaldehyde solution R* and 10 mL of *acetic acid R*. Allow to stand for 5 min, then add 0.5 mL of *starch solution R* and titrate with *0.05 M iodine*. Carry out a blank titration. The difference between the volumes used in the titrations is not more than 0.15 mL.

**Iron** (*2.4.9*): maximum 5 ppm, determined on solution S1.

**Selenium**: maximum 5 ppm.

To 6.0 g add 10 mL of *formaldehyde solution R*, carefully and progressively add 2 mL of *hydrochloric acid R*. Heat on a water-bath for 20 min. Any pink colour in the solution is not more intense than that of a standard prepared at the same time and in the same manner using 2.0 g of the substance to be examined to which 0.2 mL of *selenium standard solution (100 ppm Se) R* has been added.

**Zinc**: maximum 12 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 2.0 mL of solution S1 to 10.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *water R*.

*Source*: zinc hollow-cathode lamp.

*Wavelength*: 213.9 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Introduce 0.500 g into a 500 mL conical flask containing 50.0 mL of *0.05 M iodine*. Shake until completely dissolved. Add 1 mL of *starch solution R* and titrate the excess of iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 6.30 mg of $Na_2SO_3$.

**01/2017:0414**

# SODIUM THIOSULFATE

## Natrii thiosulfas

$Na_2S_2O_3,5H_2O$ $M_r$ 248.2
[10102-17-7]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of $Na_2S_2O_3,5H_2O$.

CHARACTERS

*Appearance*: transparent, colourless crystals, efflorescent in dry air.

*Solubility*: very soluble in water, practically insoluble in ethanol 96 per cent. It dissolves in its water of crystallisation at about 49 °C.

IDENTIFICATION

A. It decolourises *iodinated potassium iodide solution R*.

B. To 0.5 mL of solution S (see Tests) add 0.5 mL of *water R* and 2 mL of *silver nitrate solution R2*. A white precipitate is formed which rapidly becomes yellowish and then black.

C. To 2.5 mL of solution S add 2.5 mL of *water R* and 1 mL of *hydrochloric acid R*. A precipitate of sulfur is formed and gas is evolved which gives a blue colour to *starch iodate paper R*.

D. 1 mL of solution S gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. The freshly prepared solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 10.0 g in 50 mL of *distilled water R*, add 1 mL of *0.1 M sodium hydroxide* and dilute to 100 mL with the same solvent.

**pH** (*2.2.3*): 6.0 to 8.4 for the freshly prepared solution S.

**Sulfates and sulfites** (*2.4.13*): maximum 0.2 per cent.

Dilute 2.5 mL of freshly prepared solution S to 10 mL with *distilled water R*. To 3 mL of this solution first add 2 mL of *iodinated potassium iodide solution R* and continue the addition dropwise until a very faint persistent yellow colour appears. Dilute to 15 mL with *distilled water R*.

**Sulfides**. To 10 mL of solution S add 0.05 mL of a freshly prepared 50 g/L solution of *sodium nitroprusside R*. The solution does not become violet.

ASSAY

Dissolve 0.500 g in 20 mL of *water R* and titrate with *0.05 M iodine*, using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.05 M iodine* is equivalent to 24.82 mg of $Na_2S_2O_3,5H_2O$.

STORAGE

In an airtight container.

**01/2017:0678**

# SODIUM VALPROATE

## Natrii valproas

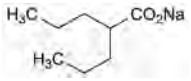


$C_8H_{15}NaO_2$ $M_r$ 166.2

[1069-66-5]

DEFINITION

Sodium 2-propylpentanoate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline, hygroscopic powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sodium valproate CRS*.

If the spectra obtained in the solid state show differences, record new spectra using discs prepared by placing 50 µL of a 100 g/L solution in *methanol R* on a disc of *potassium bromide R* and evaporating the solvent *in vacuo*. Examine immediately.

B. 2 mL of solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in 20 mL of *distilled water R* in a separating funnel, add 5 mL of *dilute nitric acid R* and shake. Allow the mixture to stand for 12 h. Use the aqueous lower layer.

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *water R* and dilute to 10 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 1.0 g in 10 mL of *water R*. Add 0.1 mL of *phenolphthalein solution R*. Not more than 0.75 mL of *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in 10 mL of *water R*. Add 5 mL of *dilute sulfuric acid R* and shake with 3 quantities, each of 20 mL, of *heptane R*. Dilute the combined upper layers to 100.0 mL with *heptane R*.

*Reference solution (a)*. Dissolve 5 mg of *valproic acid for system suitability CRS* (containing impurity K) in 1.0 mL of *heptane R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *heptane R*.

*Column*:

- *material*: wide-bore fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 8 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 80 |
| | 5 - 15 | 80 → 150 |
| | 15 - 28.3 | 150 → 190 |
| | 28.3 - 30 | 190 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to valproic acid (retention time = about 17 min): impurity K = about 0.97.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity K and valproic acid.

*Limits*:

- *impurity K*: not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *total*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.03 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Chlorides** (*2.4.4*): maximum 200 ppm.

To 5 mL of solution S add 10 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm, determined on solution S.

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.150 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 16.62 mg of $C_8H_{15}NaO_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *K*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these

Monographs S

impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, F, G, I, J, L.*

A. pentanoic acid (valeric acid),

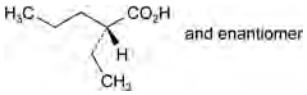

B. (2*RS*)-2-ethylpentanoic acid,

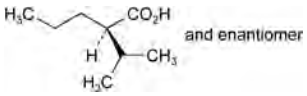

C. (2*RS*)-2-(1-methylethyl)pentanoic acid,

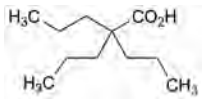

D. 2,2-dipropylpentanoic acid,

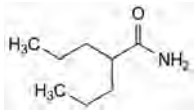

F. 2-propylpentanamide,

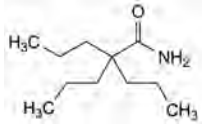

G. 2,2-dipropylpentanamide,

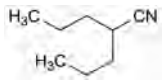

I. 2-propylpentanenitrile,

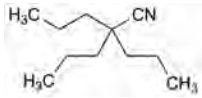

J. 2,2-dipropylpentanenitrile,

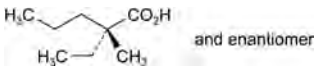

K. (2*RS*)-2-ethyl-2-methylpentanoic acid,

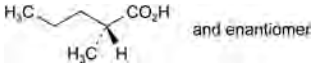

L. (2*RS*)-2-methylpentanoic acid.

**01/2016:2779**
**corrected 10.0**

# SOLIFENACIN SUCCINATE

## Solifenacini succinas

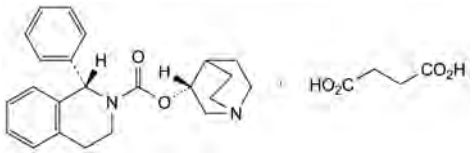

$C_{27}H_{32}N_2O_6$ $M_r$ 480.6
[242478-38-2]

DEFINITION

(3*R*)-1-Azabicyclo[2.2.2]octan-3-yl (1*S*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate hydrogen butanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or light yellow powder.

*Solubility*: very soluble or freely soluble in water, soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *solifenacin succinate CRS*.

B. Isomeric purity (see Tests).

TESTS

**Isomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve the contents of a vial of *solifenacin for system suitability CRS* (containing impurities F, G and H) in 1.0 mL of the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *diethylamine R*, *anhydrous ethanol R*, *heptane R* (0.1:200:800 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of solifenacin.

*Identification of impurities*: use the chromatogram supplied with *solifenacin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities F, G and H.

*Relative retention* with reference to solifenacin (retention time = about 17 min): impurity F = about 0.7; impurity H = about 0.76; impurity G = about 0.84.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurities F and H;

– *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity H.

*Calculation of percentage contents*:

– for each impurity, use the concentration of solifenacin succinate in reference solution (b).

*Limits*:

– *impurities F, G, H*: for each impurity, maximum 0.15 per cent.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution*. Dissolve 5.0 mg of *3-quinuclidinol R* (impurity E) in *methanol R* and dilute to 50.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *concentrated ammonia R*, *anhydrous ethanol R*, *toluene R* (6:31:63 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 1 h.

*Detection*: expose to iodine vapour for at least 2 h.

*Relative retention* with reference to solifenacin ($R_F$ = about 0.6): impurity E = about 0.2.

*Limit*:

– *impurity E*: any spot due to impurity E is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Dissolve 0.6 g of *ammonium phosphate R* in 1 L of *water R*, add 1.0 mL of *diethylamine R* and adjust to pH 11 with *concentrated ammonia R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 100.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 5 mg of *1-phenyl-1,2,3,4-tetrahydroisoquinoline R* (impurity A) in the test solution and dilute to 25.0 mL with the test solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2.1 mm;

– *stationary phase*: *end-capped ethylene-bridged polar-embedded octadecylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);

– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R1*, solvent mixture (45:55 *V/V*);

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 90 | 10 |
| 0.5 - 10.5 | 90 → 68 | 10 → 32 |
| 10.5 - 12.5 | 68 | 32 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 2.5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to solifenacin (retention time = about 2 min): succinate = about 0.2; impurity A = about 0.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to impurity A and solifenacin.

*Calculation of percentage contents*:

– for each impurity, use the concentration of solifenacin succinate in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.3 per cent;

– *reporting threshold*: 0.05 per cent; disregard the peak due to succinate.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 48.06 mg of $C_{27}H_{32}N_2O_6$.

IMPURITIES

*Specified impurities: E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, I.*

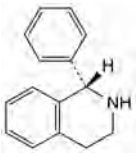


A. (1*S*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline,

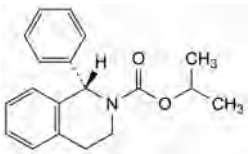


B. propan-2-yl (1*S*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate,

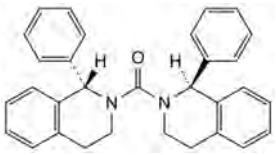


C. bis[(1*S*)-1-phenyl-3,4-dihydroisoquinolin-2(1*H*)-yl]methanone,

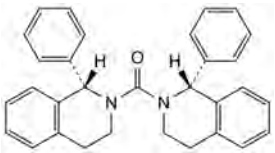

D. [(1*R*)-1-phenyl-3,4-dihydroisoquinolin-2(1*H*)-yl][(1*S*)-1-phenyl-3,4-dihydroisoquinolin-2(1*H*)-yl]methanone,

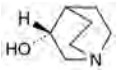

E. (3*R*)-1-azabicyclo[2.2.2]octan-3-ol (3-quinuclidinol),

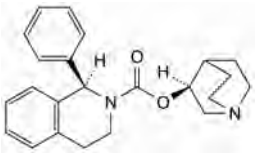

F. (3*S*)-1-azabicyclo[2.2.2]octan-3-yl (1*R*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate,

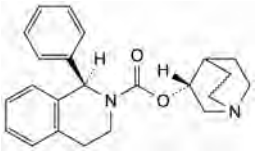

G. (3*R*)-1-azabicyclo[2.2.2]octan-3-yl (1*R*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate,

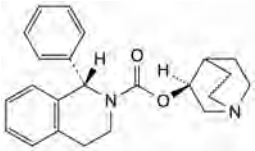

H. (3*S*)-1-azabicyclo[2.2.2]octan-3-yl (1*S*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carboxylate,

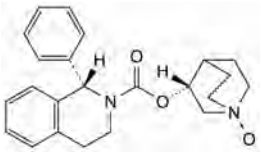

I. (3*R*)-3-[[(1*S*)-1-phenyl-3,4-dihydroisoquinoline-2(1*H*)-carbonyl]oxy]-1-azabicyclo[2.2.2]octane 1-oxide (solifenacin *N*-oxide).

**01/2020:1264**

# SOLUTIONS FOR ORGAN PRESERVATION

## Solutiones ad conservationem partium corporis

DEFINITION

Solutions for organ preservation are sterile, aqueous preparations, intended for storage, protection and/or perfusion of mammalian body organs that are in particular destined for transplantation.

They contain electrolytes that are typically at a concentration close to the intracellular electrolyte composition.

They may contain carbohydrates (such as glucose or mannitol), amino acids, calcium-complexing agents (such as citrate or phosphate), hydrocolloids (such as starch or gelatin derivatives) and other excipients, for example to make the preparation isotonic with blood, to adjust or buffer the pH, to prevent deterioration of the ingredients, but not to adversely affect the intended action of the preparation or, at the concentration used, to cause toxicity or undue local irritation. Solutions for organ preservation may also contain active substances or these may be added immediately before use.

Solutions for organ preservation, examined under suitable conditions of visibility, are clear and practically free from particles.

Solutions for organ preservation may also be presented as concentrated solutions. They are diluted to the prescribed volume with a prescribed liquid immediately before use. After dilution, they comply with the requirements for solutions for organ preservation.

Before use, the solutions for organ preservation are cooled below room temperature, typically to 2 °C to 6 °C, to reduce the temperature of the body organ and its metabolism.

Where applicable, the containers for solutions for organ preservation comply with the requirements for *Materials used for the manufacture of containers* (*3.1* and subsections) and *Containers* (*3.2* and subsections). Solutions for organ preservation are supplied in glass containers (*3.2.1*) or in other containers such as plastic containers (*3.2.2* and *3.3.8*). The tightness of the container is ensured by suitable means. Closures ensure a good seal, prevent the access of micro-organisms and other contaminants and usually permit the withdrawal of a part or the whole of the contents without removal of the closure. The plastic materials or elastomers of which the closure is composed are sufficiently firm and elastic to allow the passage of a needle with the least possible shedding of particles.

PRODUCTION

Solutions for organ preservation are prepared using materials and methods designed to ensure their sterility and to avoid the introduction of contaminants and the growth of micro-organisms; recommendations on this aspect are provided in the text on *Methods of preparation of sterile products* (*5.1.1*).

Unless otherwise justified and authorised, solutions for organ preservation are prepared from *water for injections R* and do not contain antimicrobial preservatives.

TESTS

**pH** (*2.2.3*): 5.0 to 8.0.

**Osmolality** (*2.2.35*): 250 mosmol/kg to 380 mosmol/kg.

**Hydroxymethylfurfural**. If the solution contains glucose, it complies with the following test: to a volume of the preparation to be examined containing the equivalent of 25 mg of glucose, add 5.0 mL of a 100 g/L solution of *p-toluidine R* in *2-propanol R* containing 10 per cent *V/V* of *glacial acetic acid R* and 1.0 mL of a 5 g/L solution of *barbituric acid R*. The absorbance (*2.2.25*), determined at 550 nm after allowing the mixture to stand for 2 min to 3 min, is not greater than that of a standard prepared at the same time in the same manner using a solution containing 10 µg of *hydroxymethylfurfural R* in the same volume as the preparation to be examined.

**Particulate contamination**. Carry out the test for sub-visible particles (*2.9.19*) using 50 mL of preparation to be examined. The solution contains not more than 50 particles per millilitre larger than 10 µm and not more than 5 particles per millilitre larger than 25 µm.

Products for which the label states that the product is to be used with a final filter are exempted from these requirements.

**Sterility** (*2.6.1*). The solution complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mL.

**Pyrogens** (*2.6.8*). Solutions for which a validated test for bacterial endotoxins cannot be carried out comply with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the solution, unless otherwise justified and authorised.

LABELLING

The label states:

- that the solution is not to be used for injection;
- the formula of the solution for organ preservation expressed in grams per litre and in millimoles per litre;
- the nominal volume of the solution for organ preservation in the container;
- the osmolality, expressed in milliosmoles per kilogram;
- that any unused portion of the ready-to-use solution, of the concentrated solution or of the diluted solution must be discarded;
- the storage conditions;
- if applicable, that the solution is to be used in conjunction with a final filter.

In addition, for concentrated solutions the label states that the solution must be diluted with a suitable liquid immediately before use.

**04/2014:0949**
**corrected 10.0**

# SOMATOSTATIN

## Somatostatinum

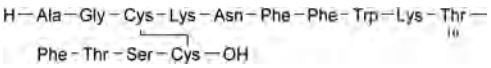


$C_{76}H_{104}N_{18}O_{19}S_2$ $M_r$ 1638
[38916-34-6]

DEFINITION

L-Alanylglycyl-L-cysteinyl-L-lysyl-L-asparaginyl-L-phenylalanyl-L-phenylalanyl-L-tryptophyl-L-lysyl-L-threonyl-L-phenylalanyl-L-threonyl-L-seryl-L-cysteine cyclic (3→14)-disulfide.

Synthetic cyclic tetradecapeptide having the structure of the hypothalamic hormone that inhibits the release of human growth hormone. It contains a variable quantity of acetic acid. It is available in the freeze-dried form.

*Content*: 95.0 per cent to 104.0 per cent (anhydrous and acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, amorphous powder.

*Solubility*: freely soluble in water and in acetic acid, practically insoluble in methylene chloride.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids taking 1/8 of the sum of the number of moles of aspartic acid, alanine, lysine, glycine and phenylalanine as equal to 1. The values fall within the following limits: aspartic acid: 0.90 to 1.10; glycine: 0.90 to 1.10; alanine: 0.90 to 1.10; phenylalanine: 2.7 to 3.3; serine: 0.7 to 1.05; threonine: 1.4 to 2.1; half-cystine: 1.4 to 2.1; lysine: 1.8 to 2.2. Not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): – 47 to – 37 (anhydrous and acetic acid-free substance).

Dissolve 2.0 mg in 1.0 mL of a 1 per cent *V/V* solution of *glacial acetic acid R*.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *somatostatin CRS* in *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (b)*. Dissolve the contents of a vial of *somatostatin CRS* in a 1 mg/mL solution of *dithioerythritol R* to obtain a concentration of 1.0 mg/mL. Allow to stand at room temperature for about 1 h. Mix 1 volume of this solution and 1 volume of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm.

*Mobile phase*:

- *mobile phase A*: *trifluoroacetic acid R*, *acetonitrile R*, *water R* (0.1:20:80 *V/V/V*);
- *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile R*, *water R* (0.1:45:55 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 → 0 | 0 → 100 |
| 25 - 30 | 0 | 100 |
| 30 - 32 | 0 → 100 | 100 → 0 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 15 µL of the test solution and reference solution (b).

*Relative retention* with reference to somatostatin (retention time = about 12 min): reduced somatostatin = about 1.09.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.5 between the peak due to reduced somatostatin and the principal peak;
- *number of theoretical plates*: minimum 15 000, calculated for the principal peak;
- *symmetry factor*: maximum 2.7 for the principal peak.

*Limits*:

- *any impurity*: maximum 1 per cent;
- *total*: maximum 2 per cent;
- *disregard limit*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 3.0 per cent to 15.0 per cent.

*Test solution*. Dissolve 7.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A, then dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 8.0 per cent, determined on 10.0 mg.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of the test solution and reference solution (a).

Calculate the content of somatostatin ($C_{76}H_{104}N_{18}O_{19}S_2$) taking into account the assigned content of $C_{76}H_{104}N_{18}O_{19}S_2$ in *somatostatin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

**01/2008:0951**
**corrected 10.0**

# SOMATROPIN

## Somatropinum

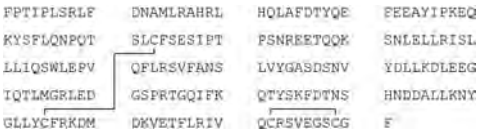


$C_{990}H_{1528}N_{262}O_{300}S_7$ $M_r$ 22 125

DEFINITION

Protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary.

*Content*: 91.0 per cent to 105.0 per cent (anhydrous substance).

By convention, for the purpose of labelling somatropin preparations, 1 mg of anhydrous somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is equivalent to 3.0 IU of biological activity.

PRODUCTION

Somatropin is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority.

Somatropin complies with the following additional requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: white or almost white powder.

IDENTIFICATION

A. Capillary electrophoresis (*2.2.47*) as described in the test for charged variants with the following modifications.

*Injection*: test solution (b); under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

*Results*: in the electropherogram obtained, only 1 principal peak, corresponding to somatropin, is detected: no doubling of this peak is observed.

B. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

C. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Prepare a solution of the substance to be examined in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* to obtain a solution containing 2.0 mg/mL of somatropin and transfer about 1.0 mL to a tube made from a suitable material such as polypropylene. Prepare a 1 mg/mL solution of *trypsin for peptide mapping R* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* and add 30 µL to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain at 2-8 °C.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution, but using *somatropin CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION.* Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5-10 µm) with a pore size of 30 nm;
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: to 100 mL of *water for chromatography R*, add 1 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 80 | 0 → 20 |
| 20 - 40 | 80 → 75 | 20 → 25 |
| 40 - 65 | 75 → 50 | 25 → 50 |
| 65 - 70 | 50 → 20 | 50 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Maintain the solutions at 2-8 °C and use within 24 h. If an automatic injector is used, maintain it at 2-8 °C.*

*Test solution.* Prepare a solution of the substance to be examined in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, containing 2.0 mg/mL of somatropin.

*Reference solution.* Prepare a solution of *somatropin CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, containing 2.0 mg/mL of somatropin.

*Resolution solution.* Dissolve the contents of a vial of *somatropin/desamidosomatropin resolution mixture CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* to obtain a concentration of 2 mg/mL of somatropin.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm; a silica saturation column is placed between the pump and the injector valve;
- *temperature*: 45 °C.

*Mobile phase*: *propanol R*, *0.05 M tris-hydrochloride buffer solution pH 7.5 R* (29:71 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Preconditioning of the column*: rinse with 200-500 mL of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in a 50 per cent *V/V* solution of *acetonitrile R*; repeat as necessary, to improve column performance.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin (retention time = about 33 min; if necessary adjust the concentration of *propanol R* in the mobile phase): desamidosomatropin = about 0.85.

*System suitability*: resolution solution:

- *resolution*: minimum 1.0 between the peaks due to desamidosomatropin and somatropin;
- *symmetry factor*: 0.9 to 1.8 for the peak due to somatropin.

*Limit*:

- *total*: maximum 6.0 per cent.

**Dimer and related substances of higher molecular mass**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Prepare a solution of the substance to be examined in *0.025 M phosphate buffer solution pH 7.0 R*, containing 1.0 mg/mL of somatropin.

*Reference solution.* Dissolve the contents of a vial of *somatropin CRS* in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Resolution solution.* Place 1 vial of *somatropin CRS* in an oven at 50 °C for a period sufficient to generate 1-2 per cent of dimer (typically 12-24 h). Dissolve its contents in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000.

*Mobile phase*: *2-propanol R*, *0.063 M phosphate buffer solution pH 7.0 R* (3:97 *V/V*); filter and degas.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin monomer (retention time = 12 min to 17 min): related substances of higher molecular mass = about 0.65; somatropin dimer = about 0.9.

*System suitability*: resolution solution:

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limit*:

- *sum of the peaks with retention times less than that of the principal peak*: maximum 4.0 per cent.

**Charged variants**. Capillary electrophoresis (*2.2.47*).

*Test solution (a).* Prepare a solution of the substance to be examined containing 1 mg/mL of somatropin.

*Test solution (b).* Mix equal volumes of test solution (a) and the reference solution.

*Reference solution.* Dissolve the contents of a vial of *somatropin CRS* in *water R* and dilute with the same solvent to obtain a concentration of 1 mg/mL.

*Capillary*:

- *material*: uncoated fused silica;
- *size*: effective length = at least 70 cm, Ø = 50 µm.

*Temperature*: 30 °C.

*CZE buffer*: 13.2 g/L solution of *ammonium phosphate R* adjusted to pH 6.0 with *phosphoric acid R* and filtered.

*Detection*: spectrophotometer at 200 nm.

*Set the autosampler to store the samples at 4 °C during analysis.*

*Preconditioning of the capillary*: rinse with *1 M sodium hydroxide* for 20 min, with *water R* for 10 min and with the CZE buffer for 20 min.

*Between-run rinsing*: rinse with *0.1 M sodium hydroxide* for 2 min and with the CZE buffer for 6 min.

*Note: rinsing times may be adapted according to the length of the capillary and the equipment used.*

*Injection*: test solution (a) and the reference solution; under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

The injection time and pressure may be adapted in order to meet the system suitability criteria.

*Migration*: apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 min, using the CZE buffer as the electrolyte in both buffer reservoirs.

*Relative migration* with reference to somatropin: deamidated forms = 1.02 to 1.11.

*System suitability*: reference solution:

- the electropherogram obtained is similar to the electropherogram of somatropin supplied with *somatropin CRS*; 2 peaks ($I_1$, $I_2$) eluting prior to the principal peak and at least 2 peaks ($I_3$, $I_4$) eluting after the principal peak are clearly visible.

  *Note: peak $I_2$ corresponds to the cleaved form and peak $I_4$ corresponds to the deamidated forms, eluting as a doublet.*

*Limits*:

- *deamidated forms*: maximum 5.0 per cent;
- *any other impurity*: for each impurity, maximum 2.0 per cent;
- *total*: maximum 10.0 per cent.

**Water** (*2.5.32*): maximum 10.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Size-exclusion chromatography (*2.2.30*) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) from the declared content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin CRS*.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

**01/2008:0950**
**corrected 10.0**

# SOMATROPIN CONCENTRATED SOLUTION

## Somatropini solutio concentrata

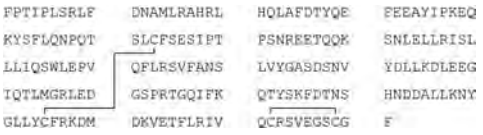


$C_{990}H_{1528}N_{262}O_{300}S_7$ $M_r$ 22 125

DEFINITION

Solution containing a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary. It may contain buffer salts and other auxiliary substances.

*Content*: 91.0 per cent to 105.0 per cent of the amount of somatropin stated on the label.

By convention, for the purpose of labelling somatropin preparations, 1 mg of anhydrous somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is equivalent to 3.0 IU of biological activity.

PRODUCTION

Somatropin concentrated solution is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of at least 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority.

Somatropin concentrated solution complies with the following additional requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: clear or slightly turbid, colourless solution.

IDENTIFICATION

A. Capillary electrophoresis (*2.2.47*) as described in the test for charged variants with the following modifications.

*Injection*: test solution (b); under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

*Results*: in the electropherogram obtained, only 1 principal peak, corresponding to somatropin, is detected: no doubling of this peak is observed.

B. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

C. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Dilute the solution to be examined with *0.05 M tris-hydrochloride buffer solution pH 7.5 R* so that it contains 2.0 mg/mL of somatropin and transfer about 1.0 mL to a tube made from a suitable material such as polypropylene. Prepare a 1 mg/mL solution of *trypsin for peptide mapping R* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* and add 30 µL to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain at 2-8 °C.

*Note: If a 2 mg/mL somatropin concentration is not obtainable, a similar digest relationship (micrograms of trypsin per milligram of somatropin) may be used.*

*Reference solution.* Prepare at the same time and in the same manner as for the test solution, but using *somatropin CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5-10 µm) with a pore size of 30 nm;
– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
– *mobile phase B*: to 100 mL of *water for chromatography R*, add 1 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 80 | 0 → 20 |
| 20 - 40 | 80 → 75 | 20 → 25 |
| 40 - 65 | 75 → 50 | 25 → 50 |
| 65 - 70 | 50 → 20 | 50 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Maintain the solutions at 2-8 °C and use within 24 h. If an automatic injector is used, maintain it at 2-8 °C.*

*Test solution.* Dilute the solution to be examined in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, so as to contain 2.0 mg/mL of somatropin. A weaker solution may be prepared, in which case the injection volume is adjusted accordingly.

*Reference solution.* Prepare a solution of *somatropin CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, containing 2.0 mg/mL of somatropin.

*Resolution solution.* Dissolve the contents of a vial of *somatropin/desamidosomatropin resolution mixture CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* to obtain a concentration of 2 mg/mL of somatropin.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm; a silica saturation column is placed between the pump and the injector valve;
– *temperature*: 45 °C.

*Mobile phase*: *propanol R*, *0.05 M tris-hydrochloride buffer solution pH 7.5 R* (29:71 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Preconditioning of the column*: rinse with 200-500 mL of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in a 50 per cent *V/V* solution of *acetonitrile R*; repeat as necessary, to improve column performance.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin (retention time = about 33 min; if necessary adjust the concentration of *propanol R* in the mobile phase): desamidosomatropin = about 0.85.

*System suitability*: resolution solution:
– *resolution*: minimum 1.0 between the peaks due to desamidosomatropin and somatropin;
– *symmetry factor*: 0.9 to 1.8 for the peak due to somatropin.

*Limit*:
– *total*: maximum 6.0 per cent.

**Dimer and related substances of higher molecular mass**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Dilute the solution to be examined in *0.025 M phosphate buffer solution pH 7.0 R*, so as to contain 1.0 mg/mL of somatropin.

*Reference solution*. Dissolve the contents of a vial of *somatropin CRS* in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Resolution solution*. Place 1 vial of *somatropin CRS* in an oven at 50 °C for a period sufficient to generate 1-2 per cent of dimer (typically 12-24 h). Dissolve its contents in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Column*:
– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000.

*Mobile phase*: *2-propanol R*, *0.063 M phosphate buffer solution pH 7.0 R* (3:97 *V/V*); filter and degas.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin monomer (retention time = 12 min to 17 min): related substances of higher molecular mass = about 0.65; somatropin dimer = about 0.9.

*System suitability*: resolution solution:
– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limit*:
– *sum of the peaks with retention times less than that of the principal peak*: maximum 4.0 per cent.

**Charged variants**. Capillary electrophoresis (*2.2.47*).

*Test solution (a)*. Dilute the solution to be examined so as to obtain a concentration of 1 mg/mL of somatropin.

*Test solution (b)*. Mix equal volumes of test solution (a) and the reference solution.

*Reference solution*. Dissolve the contents of a vial of *somatropin CRS* in *water R* and dilute with the same solvent to obtain a concentration of 1 mg/mL.

*Capillary*:
– *material*: uncoated fused silica;
– *size*: effective length = at least 70 cm, Ø = 50 µm.

*Temperature*: 30 °C.

*CZE buffer*: 13.2 g/L solution of *ammonium phosphate R* adjusted to pH 6.0 with *phosphoric acid R* and filtered.

*Detection*: spectrophotometer at 200 nm.

*Set the autosampler to store the samples at 4 °C during analysis.*

*Preconditioning of the capillary*: rinse with *1 M sodium hydroxide* for 20 min, with *water R* for 10 min and with the CZE buffer for 20 min.

*Between-run rinsing*: rinse with *0.1 M sodium hydroxide* for 2 min and with the CZE buffer for 6 min.

*Note: rinsing times may be adapted according to the length of the capillary and the equipment used.*

*Injection*: test solution (a) and the reference solution; under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s. The injection time and pressure may be adapted in order to meet the system suitability criteria.

*Migration*: apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 min, using the CZE buffer as the electrolyte in both buffer reservoirs.

*Relative migration* with reference to somatropin: deamidated forms = 1.02 to 1.11.

*System suitability*: reference solution:
– the electropherogram obtained is similar to the electropherogram of somatropin supplied with *somatropin CRS*; 2 peaks ($I_1$, $I_2$) eluting prior to the principal peak and at least 2 peaks ($I_3$, $I_4$) eluting after the principal peak are clearly visible.

*Note: peak $I_2$ corresponds to the cleaved form and peak $I_4$ corresponds to the deamidated forms, eluting as a doublet.*

*Limits*:
– *deamidated forms*: maximum 5.0 per cent;
– *any other impurity*: for each impurity, maximum 2.0 per cent;
– *total*: maximum 10.0 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU in the volume that contains 1 mg of somatropin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Size-exclusion chromatography (*2.2.30*) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) from the declared content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin CRS*.

STORAGE

In an airtight container at a temperature of – 20 °C. Avoid repeated freezing and thawing. If the solution is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:
– the content of somatropin in milligrams per millilitre;
– the name and concentration of any auxiliary substance.

**01/2008:0952**
**corrected 10.0**

# SOMATROPIN FOR INJECTION

## Somatropinum iniectabile

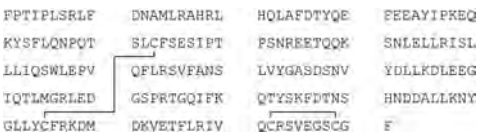


$C_{990}H_{1528}N_{262}O_{300}S_7$ $M_r$ 22 125

DEFINITION

Freeze-dried, sterile preparation of a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary.

*Content*: 89.0 per cent to 105.0 per cent of the amount of somatropin stated on the label.

By convention, for the purpose of labelling somatropin preparations, 1 mg of anhydrous somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is equivalent to 3.0 IU of biological activity.

Somatropin for injection complies with the requirements of the monograph *Parenteral preparations (0520)*.

PRODUCTION

Somatropin for injection is prepared either from *Somatropin (0951)* or from *Somatropin concentrated solution (0950)*, or by a method based on recombinant DNA (rDNA) technology in which the injectable preparation is produced without the isolation of an intermediate solid or liquid bulk. In the latter case, during the course of product development, it must be demonstrated that the manufacturing process produces a product having a biological activity of not less than 2.5 IU/mg, using a validated bioassay based on growth promotion and approved by the competent authority. The purified preparation, to which buffers and stabilisers may be added, is filtered through a bacteria-retentive filter, aseptically distributed in sterile containers of glass type I (*3.2.1*) and freeze-dried. The containers are immediately sealed so as to exclude microbial contamination and moisture.

Somatropin for injection complies with the following additional requirements.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

*Where somatropin for injection is prepared from Somatropin (0951) or from Somatropin concentrated solution (0950), compliance with the requirements for host-cell-derived proteins, host-cell- and vector-derived DNA, identification test A, identification test C and charged variants need not be reconfirmed by the manufacturer during subsequent production of somatropin for injection.*

CHARACTERS

*Appearance*: white or almost white powder.

IDENTIFICATION

A. Capillary electrophoresis (*2.2.47*) as described in the test for charged variants with the following modifications.

*Injection*: test solution (b); under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

*Results*: in the electropherogram obtained, only 1 principal peak, corresponding to somatropin, is detected: no doubling of this peak is observed.

B. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

C. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Test solution.* Prepare a solution of the substance to be examined in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* to obtain a solution containing 2.0 mg/mL of somatropin and transfer about 1.0 mL to a tube made from a suitable material such as polypropylene. Prepare a 1 mg/mL solution of *trypsin for peptide mapping R* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* and add 30 µL to the solution of the substance to be examined. Cap the tube and place in a water-bath at 37 °C for 4 h. Remove from the water-bath and stop the reaction immediately, for example by freezing. If analysed immediately using an automatic injector, maintain at 2-8 °C.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution, but using *somatropin CRS* instead of the substance to be examined.

*CHROMATOGRAPHIC SEPARATION*. Liquid chromatography (*2.2.29*).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5-10 µm) with a pore size of 30 nm;
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dilute 1 mL of *trifluoroacetic acid R* to 1000 mL with *water for chromatography R*;
- *mobile phase B*: to 100 mL of *water for chromatography R* add 1 mL of *trifluoroacetic acid R* and dilute to 1000 mL with *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 → 80 | 0 → 20 |
| 20 - 40 | 80 → 75 | 20 → 25 |
| 40 - 65 | 75 → 50 | 25 → 50 |
| 65 - 70 | 50 → 20 | 50 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 100 µL.

*System suitability*: the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of somatropin digest supplied with *somatropin CRS*.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

D. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Maintain the solutions at 2-8 °C and use within 24 h. If an automatic injector is used, maintain at 2-8 °C.*

*Test solution.* Prepare a solution of the substance to be examined in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, containing 2.0 mg/mL of somatropin.

*Reference solution.* Prepare a solution of *somatropin CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R*, containing 2.0 mg/mL of somatropin.

*Resolution solution.* Dissolve the contents of a vial of *somatropin/desamidosomatropin resolution mixture CRS* in *0.05 M tris-hydrochloride buffer solution pH 7.5 R* to obtain a concentration of 2 mg/mL of somatropin.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped butylsilyl silica gel for chromatography R* (5 µm) with a pore size of 30 nm; a silica saturation column is placed between the pump and the injector valve;
– *temperature*: 45 °C.

*Mobile phase*: *propanol R*, *0.05 M tris-hydrochloride buffer solution pH 7.5 R* (29:71 *V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Preconditioning of the column*: rinse with 200-500 mL of a 0.1 per cent *V/V* solution of *trifluoroacetic acid R* in a 50 per cent *V/V* solution of *acetonitrile R*; repeat as necessary, to improve column performance.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin (retention time = about 33 min; if necessary adjust the concentration of *propanol R* in the mobile phase): desamidosomatropin = about 0.85.

*System suitability*: resolution solution:

– *resolution*: minimum 1.0 between the peaks due to desamidosomatropin and somatropin;
– *symmetry factor*: 0.9 to 1.8 for the peak due to somatropin.

*Limit*:

– *total*: maximum 13.0 per cent.

**Dimer and related substances of higher molecular mass**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Prepare a solution of the substance to be examined in *0.025 M phosphate buffer solution pH 7.0 R*, containing 1.0 mg/mL of somatropin.

*Reference solution.* Dissolve the contents of a vial of *somatropin CRS* in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Resolution solution.* Place 1 vial of *somatropin CRS* in an oven at 50 °C for a period sufficient to generate 1-2 per cent of dimer (typically 12-24 h). Dissolve its contents in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000.

*Mobile phase*: *2-propanol R*, *0.063 M phosphate buffer solution pH 7.0 R* (3:97 *V/V*); filter and degas.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin monomer (retention time = 12 min to 17 min): related substances of higher molecular mass = about 0.65; somatropin dimer = about 0.9.

*System suitability*: resolution solution:

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limit*:

– *sum of the peaks with retention times less than that of the principal peak*: maximum 6.0 per cent.

**Charged variants**. Capillary electrophoresis (*2.2.47*).

*Test solution (a).* Prepare a solution of the substance to be examined containing 1 mg/mL of somatropin.

*Test solution (b).* Mix equal volumes of test solution (a) and the reference solution.

*Reference solution.* Dissolve the contents of a vial of *somatropin CRS* in *water R* and dilute with the same solvent to obtain a concentration of 1 mg/mL.

*Capillary*:

– *material*: uncoated fused silica;
– *size*: effective length = at least 70 cm, Ø = 50 µm.

*Temperature*: 30 °C.

*CZE buffer*: 13.2 g/L solution of *ammonium phosphate R* adjusted to pH 6.0 with *phosphoric acid R* and filtered.

*Detection*: spectrophotometer at 200 nm.

*Set the autosampler to store the samples at 4 °C during analysis.*

*Preconditioning of the capillary*: rinse with *1 M sodium hydroxide* for 20 min, with *water R* for 10 min and with the CZE buffer for 20 min.

*Between-run rinsing*: rinse with *0.1 M sodium hydroxide* for 2 min and with the CZE buffer for 6 min.

*Note: rinsing times may be adapted according to the length of the capillary and the equipment used.*

*Injection*: test solution (a) and the reference solution; under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

The injection time and pressure may be adapted in order to meet the system suitability criteria.

*Migration*: apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 min, using CZE buffer as the electrolyte in both buffer reservoirs.

*Relative migration* with reference to somatropin: deamidated forms = 1.02 to 1.11.

*System suitability*: reference solution:

– the electropherogram obtained is similar to the electropherogram of somatropin supplied with *somatropin CRS*; 2 peaks ($I_1$, $I_2$) eluting prior to the principal peak and at least 2 peaks ($I_3$, $I_4$) eluting after the principal peak are clearly visible.

*Note: peak $I_2$ corresponds to the cleaved form and peak $I_4$ corresponds to the deamidated forms, eluting as a doublet.*

*Limits*:

– *deamidated forms*: maximum 6.5 per cent;
– *any other impurity*: for each impurity, maximum 2.0 per cent;
– *total*: maximum 11.5 per cent.

**Water** (*2.5.32*): maximum 3.0 per cent, unless otherwise justified and authorised.

**Bacterial endotoxins** (*2.6.14*): less than 5 IU/mg.

ASSAY

Size-exclusion chromatography (*2.2.30*) as described in the test for dimer and related substances of higher molecular mass.

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) from the declared content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin CRS*.

STORAGE

In a sterile, airtight, tamper-evident container, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the content of somatropin in the container, in milligrams;
- the composition and volume of the liquid to be added for reconstitution;
- the time within which the reconstituted solution shall be used and the storage conditions during this period;
- the name and quantity of any excipient;
- the storage temperature;
- that the preparation shall not be shaken during reconstitution.

**01/2016:2370**
**corrected 10.0**

# SOMATROPIN SOLUTION FOR INJECTION

## Somatropini solutio iniectabilis

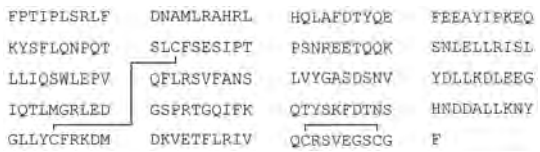


$C_{990}H_{1528}N_{262}O_{300}S_7$ $M_r$ 22 125

DEFINITION

Sterile preparation of a protein having the structure (191 amino-acid residues) of the major component of growth hormone produced by the human pituitary.

*Content*: 90.0 per cent to 110.0 per cent of the amount of somatropin stated on the label.

By convention, for the purpose of labelling somatropin preparations, 1 mg of anhydrous somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is equivalent to 3.0 IU of biological activity.

PRODUCTION

Somatropin solution for injection is prepared either from *Somatropin (0951)* or from *Somatropin concentrated solution (0950)*.

CHARACTERS

*Appearance*: colourless solution.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related proteins.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related proteins** (excluding deamidated forms). Liquid chromatography (*2.2.29*): use the normalisation procedure. *Maintain the solutions at 2-8 °C and use within 24 h.*

*Solution A*. Dilute 5 µL of *strong hydrogen peroxide solution R* to 100 mL with *water for chromatography R*.

*Test solution*. Dilute the preparation to be examined with an appropriate buffer, for example *0.025 M phosphate buffer solution pH 7.0 R*, to obtain a concentration of 1.6 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *somatropin CRS* in *water for chromatography R* to obtain a concentration of 1.6 mg/mL.

*Reference solution (b)*. Dissolve the contents of a vial of *somatropin CRS* in solution A to obtain a concentration of 1.6 mg/mL. Allow to stand for 1-7 days to obtain a sufficient amount of sulfoxide forms.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm) with a pore size of 100 nm;
- *temperature*: 45 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 82.6 g of *ammonium sulfate R* and 34.5 g of *sodium dihydrogen phosphate monohydrate R* in 2950 mL of *water for chromatography R*; add 25.0 mL of *perchloric acid R*, 2000 mL of *acetonitrile for chromatography R* and dilute to 5000 mL with *water for chromatography R*;
- *mobile phase B*: to 4000 mL of *acetonitrile for chromatography R* add 1000 mL of *water for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 – 60 | 77 → 72 | 23 → 28 |
| 60 – 61 | 72 → 77 | 28 → 23 |
| 61 – 72 | 77 | 23 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Relative retention* with reference to somatropin (retention time = about 40 min): [MetO$^{125}$]somatropin (double peak) = about 0.40; [β-Asp$^{107}$]somatropin = about 0.78; [MetO$^{14}$]somatropin = about 0.91; 130,131-anhydrosomatropin = about 1.27; [seco-130/131]somatropin = about 1.31.

If necessary, adjust the percentage of mobile phase B in the gradient so that the main peak elutes at about 40 min.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to [MetO$^{14}$]somatropin and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to somatropin.

*Limit*:

- *total*: maximum 10 per cent.

**Dimer and related substances of higher molecular mass**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution*. Dilute the preparation to be examined with *0.025 M phosphate buffer solution pH 7.0 R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (a)*. Dissolve the contents of a vial of *somatropin CRS* in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Reference solution (b).* Place a vial of *somatropin CRS* in an oven at 50 °C for a period sufficient to generate 1-2 per cent of dimer (typically 12-24 h). Dissolve its contents in *0.025 M phosphate buffer solution pH 7.0 R* and dilute with the same solution to obtain a concentration of 1.0 mg/mL.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;

– *stationary phase*: *hydrophilic silica gel for chromatography R* of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 5000 to 150 000.

*Mobile phase*: *2-propanol R*, *0.063 M phosphate buffer solution pH 7.0 R* (3:97 *V/V*); filter and degas.

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Injection*: 20 µL of the test solution and reference solution (b).

*Relative retention* with reference to somatropin monomer (retention time = 12 min to 17 min): related substances of higher molecular mass = about 0.65; somatropin dimer = about 0.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to the dimer and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to the monomer.

*Limit*:

– *sum of the areas of the peaks with retention times less than that of the principal peak*: maximum 4.0 per cent.

**Deamidated forms**. Capillary electrophoresis (*2.2.47*).

*Test solution.* Dilute the preparation to be examined to obtain a concentration of 1 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *somatropin CRS* in *water for chromatography R* and dilute with the same solvent to obtain a concentration of 1 mg/mL.

*Capillary*:

– *material*: uncoated fused silica;

– *size*: effective length = at least 70 cm, Ø = 50 µm.

*Temperature*: 30 °C.

*CZE buffer*: 13.2 g/L solution of *ammonium phosphate R* adjusted to pH 6.0 with *phosphoric acid R* and filtered.

*Detection*: spectrophotometer at 200 nm.

*Autosampler*: set at 8 °C.

*Preconditioning of the capillary*: rinse with *1 M sodium hydroxide* for 20 min, with *water for chromatography R* for 10 min and with the CZE buffer for 20 min.

*Between-run rinsing*: rinse with *0.1 M sodium hydroxide* for 2 min and with the CZE buffer for 6 min.

*NOTE: rinsing times may be adapted according to the length of the capillary and the equipment used.*

*Injection*: test solution and reference solution; under pressure or vacuum, using the following sequence: sample injection for at least 3 s then CZE buffer injection for 1 s.

The injection time and pressure may be adapted in order to meet the system suitability criteria.

*Migration*: apply a field strength of 217 V/cm (20 kV for capillaries of 92 cm total length) for 80 min, using CZE buffer as the electrolyte in both buffer reservoirs.

*Relative migration* with reference to somatropin: deamidated forms = 1.02 to 1.11.

*System suitability*: reference solution:

– the electropherogram obtained is similar to the electropherogram supplied with *somatropin CRS*; 2 peaks ($I_1$, $I_2$) eluting prior to the principal peak and at least 2 peaks ($I_3$, $I_4$) eluting after the principal peak are clearly visible.

*NOTE: peak $I_2$ corresponds to the cleaved form and peak $I_4$ corresponds to the deamidated forms, eluted as a doublet.*

*Limit*:

– *deamidated forms*: maximum 20 per cent of the total area of the peaks.

ASSAY

Size-exclusion chromatography (*2.2.30*) as described in the test for dimer and related substances of higher molecular mass with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the content of somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) taking into account the assigned content of $C_{990}H_{1528}N_{262}O_{300}S_7$ in *somatropin CRS*.

STORAGE

In a sterile, airtight, tamper-evident container, protected from light, at a temperature of 2 °C to 8 °C.

**01/2017:0592**

# SORBIC ACID

## Acidum sorbicum

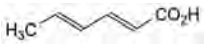


$C_6H_8O_2$ $M_r$ 112.1

[110-44-1]

DEFINITION

(*E,E*)-Hexa-2,4-dienoic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 132 °C to 136 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *water R* and dilute to 250.0 mL with the same solvent. Dilute 2.0 mL of this solution to 200.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 264 nm.

*Specific absorbance at the absorption maximum*: 2150 to 2550.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sorbic acid CRS*.

D. Dissolve 0.2 g in 2 mL of *ethanol (96 per cent) R* and add 0.2 mL of *bromine water R*. The solution is decolorised.

TESTS

**Solution S**. Dissolve 1.25 g in *ethanol (96 per cent) R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Aldehydes**: maximum 0.15 per cent, calculated as $C_2H_4O$.

Dissolve 1.0 g in a mixture of 30 mL of *water R* and 50 mL of *2-propanol R*, adjust to pH 4 with *0.1 M hydrochloric acid* or *0.1 M sodium hydroxide* and dilute to 100 mL with *water R*. To 10 mL of this solution add 1 mL of *decolorised fuchsin solution R* and allow to stand for 30 min. Any colour in the

solution is not more intense than that in a standard prepared at the same time by adding 1 mL of *decolorised fuchsin solution R* to a mixture of 1.5 mL of *acetaldehyde standard solution (100 ppm $C_2H_4O$) R*, 4 mL of *2-propanol R* and 4.5 mL of *water R*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 2.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.1000 g in 20 mL of *ethanol (96 per cent) R*. Using 0.2 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 11.21 mg of $C_6H_8O_2$.

STORAGE

Protected from light.

**04/2017:1040**

## SORBITAN LAURATE

### Sorbitani lauras

DEFINITION

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with lauric (dodecanoic) acid.

CHARACTERS

*Appearance*: brownish-yellow, viscous liquid.

*Solubility*: practically insoluble but dispersible in water, miscible with ethanol (96 per cent).

*Relative density*: about 0.98.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 7.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 330 to 358.

**Iodine value** (*2.5.4*): maximum 10.

**Peroxide value** (*2.5.5*): maximum 5.0.

**Saponification value** (*2.5.6*): 158 to 170.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

Prepare reference solution (a) as indicated in tables 2.4.22.-1 and 2.4.22.-2.

*Composition of the fatty acid fraction of the substance*:

- *caproic acid*: maximum 1.0 per cent;
- *caprylic acid*: maximum 10.0 per cent;
- *capric acid*: maximum 10.0 per cent;
- *lauric acid*: 40.0 per cent to 60.0 per cent;
- *myristic acid*: 14.0 per cent to 25.0 per cent;
- *palmitic acid*: 7.0 per cent to 15.0 per cent;
- *stearic acid*: maximum 7.0 per cent;
- *oleic acid*: maximum 11.0 per cent;
- *linoleic acid*: maximum 3.0 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent.

STORAGE

Protected from light.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan laurate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**04/2017:1041**

## SORBITAN OLEATE

### Sorbitani oleas

DEFINITION

Mixture usually obtained by esterification of 1 mole of sorbitol and its mono-and di-anhydrides per mole of oleic ((9*Z*)-octadec-9-enoic) acid. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: brownish-yellow, viscous liquid.

*Solubility*: practically insoluble but dispersible in water, soluble in fatty oils producing a hazy solution, miscible with ethanol (96 per cent).

*Relative density*: about 0.99.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Composition of fatty acids (see Tests).

*Margaric acid*: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

**Acid value** (*2.5.1*): maximum 8.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 190 to 210.

**Iodine value** (*2.5.4*): 62 to 76.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Saponification value** (*2.5.6*): 145 to 160.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance*:

- *myristic acid*: maximum 5.0 per cent;
- *palmitic acid*: maximum 16.0 per cent;

– *palmitoleic acid*: maximum 8.0 per cent;
– *stearic acid*: maximum 6.0 per cent;
– *oleic acid*: 65.0 per cent to 88.0 per cent;
– *linoleic acid*: maximum 18.0 per cent;
– *linolenic acid*: maximum 4.0 per cent;
– *fatty acids with chain length greater than $C_{18}$*: maximum 4.0 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

The label states the origin of the oleic acid used (animal or vegetable).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan oleate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**04/2017:1042**

# SORBITAN PALMITATE

## Sorbitani palmitas

DEFINITION

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with palmitic (hexadecanoic) acid.

CHARACTERS

*Appearance*: yellow or yellowish powder, waxy flakes or hard masses.

*Solubility*: practically insoluble in water, soluble in fatty oils, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.15*): 44 °C to 51 °C.
   Introduce the melted substance into the glass capillary tubes and allow to stand at a temperature below 10 °C for 24 h.
B. Hydroxyl value (see Tests).
C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 8.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 270 to 305.

**Peroxide value** (*2.5.5*): maximum 5.0.

**Saponification value** (*2.5.6*): 140 to 155.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance*:
– *palmitic acid*: minimum 92.0 per cent;
– *stearic acid*: maximum 6.0 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent.

STORAGE

Protected from light.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan palmitate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**04/2017:1916**

# SORBITAN SESQUIOLEATE

## Sorbitani sesquioleas

DEFINITION

Mixture usually obtained by esterification of 2 moles of sorbitol and its mono- and di-anhydrides per 3 moles of oleic ((9*Z*)-octadec-9-enoic) acid. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: pale yellow or slightly brownish-yellow paste, which becomes a viscous, oily, brownish-yellow liquid at about 25 °C.

*Solubility*: dispersible in water, soluble in fatty oils, slightly soluble in ethanol (96 per cent).

*Relative density*: about 0.99.

IDENTIFICATION

A. Hydroxyl value (see Tests).
B. Iodine value (see Tests).
C. Composition of fatty acids (see Tests).
   *Margaric acid*: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

**Acid value** (*2.5.1*): maximum 16.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 180 to 215.

**Iodine value** (*2.5.4*): 70 to 95.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Saponification value** (*2.5.6*): 145 to 166.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance*:

- *myristic acid*: maximum 5.0 per cent;
- *palmitic acid*: maximum 16.0 per cent;
- *palmitoleic acid*: maximum 8.0 per cent;
- *stearic acid*: maximum 6.0 per cent;
- *oleic acid*: 65.0 per cent to 88.0 per cent;
- *linoleic acid*: maximum 18.0 per cent;
- *linolenic acid*: maximum 4.0 per cent;
- *fatty acids with chain length greater than* $C_{18}$: maximum 4.0 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

The label states the origin of the oleic acid used (animal or vegetable).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan sesquioleate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**04/2017:1043**

# SORBITAN STEARATE

## Sorbitani stearas

DEFINITION

Mixture usually obtained by partial esterification of sorbitol and its mono- and di-anhydrides with *Stearic acid 50 (1474)* or *Stearic acid 70 (1474)*.

CHARACTERS

*Appearance*: pale yellow, waxy solid.

*Solubility*: practically insoluble but dispersible in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.15*): 50 °C to 60 °C.

Introduce the melted substance into the capillary tubes and allow to stand at a temperature below 10 °C for 24 h.

B. Hydroxyl value (see Tests).

C. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 10.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 235 to 260.

**Peroxide value** (*2.5.5*): maximum 5.0.

**Saponification value** (*2.5.6*): 147 to 157.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance:*

| | Type of fatty acid used | Composition of fatty acids |
|---|---|---|
| Sorbitan stearate (type I) | Stearic acid 50 | *Stearic acid*: 40.0 per cent to 60.0 per cent,<br>*Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent. |
| Sorbitan stearate (type II) | Stearic acid 70 | *Stearic acid*: 60.0 per cent to 80.0 per cent,<br>*Sum of the contents of palmitic and stearic acids*: minimum 90.0 per cent. |

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent.

STORAGE

Protected from light.

LABELLING

The label states the type of sorbitan stearate.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan stearate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**04/2017:1044**

# SORBITAN TRIOLEATE

## Sorbitani trioleas

DEFINITION

Mixture usually obtained by esterification of 1 mole of sorbitol and its mono-anhydride per 3 moles of oleic ((9*Z*)-octadec-9-enoic) acid. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: pale yellow, light yellowish or brown solid, which becomes a viscous, oily, brownish-yellow liquid at about 25 °C.

*Solubility*: practically insoluble but dispersible in water, soluble in fatty oils, slightly soluble in ethanol (96 per cent).

*Relative density*: about 0.98.

IDENTIFICATION

A. Hydroxyl value (see Tests).

B. Iodine value (see Tests).

C. Composition of fatty acids (see Tests).

*Margaric acid*: maximum 0.2 per cent for oleic acid of vegetable origin and maximum 4.0 per cent for oleic acid of animal origin.

TESTS

**Acid value** (*2.5.1*): maximum 16.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 55 to 75.

**Iodine value** (*2.5.4*): 76 to 90.

**Peroxide value** (*2.5.5*): maximum 10.0.

**Saponification value** (*2.5.6*): 170 to 190.

Carry out the saponification for 1 h.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*).

*Composition of the fatty acid fraction of the substance:*

- *myristic acid*: maximum 5.0 per cent;
- *palmitic acid*: maximum 16.0 per cent;
- *palmitoleic acid*: maximum 8.0 per cent;
- *stearic acid*: maximum 6.0 per cent;
- *oleic acid*: 65.0 per cent to 88.0 per cent;
- *linoleic acid*: maximum 18.0 per cent;
- *linolenic acid*: maximum 4.0 per cent;
- *fatty acids with chain length greater than* $C_{18}$: maximum 4.0 per cent.

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g.

**Total ash** (*2.4.16*): maximum 0.5 per cent, determined on 1.5 g.

STORAGE

Protected from light.

LABELLING

The label states the origin of the oleic acid used (animal or vegetable).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitan trioleate used as emulsifier and co-solubiliser in creams.*

**Composition of fatty acids** (see Tests).

**Hydroxyl value** (see Tests).

**07/2019:0435**

# SORBITOL

## Sorbitolum

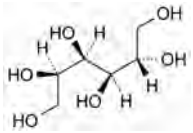

$C_6H_{14}O_6$ $M_r$ 182.2

[50-70-4]

DEFINITION

D-Glucitol (D-sorbitol).

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Dissolve 0.5 g with heating in a mixture of 0.5 mL of *pyridine R* and 5 mL of *acetic anhydride R*. After 10 min, pour the solution into 25 mL of *water R* and allow to stand in iced water for 2 h. The precipitate, recrystallised from a small volume of *ethanol (96 per cent) R* and dried *in vacuo*, melts (*2.2.14*) at 98 °C to 104 °C.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 25 mg of *sorbitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 25 mg of *mannitol CRS* and 25 mg of *sorbitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *ethyl acetate R*, *propanol R* (10:20:70 *V/V/V*).

*Application*: 2 µL.

*Development*: over 2/3 of the plate.

Monographs S

*Drying*: in air.

*Detection*: spray with *4-aminobenzoic acid solution R*; dry in a current of cold air until the acetone is removed; heat at 100 °C for 15 min; allow to cool and spray with a 2 g/L solution of *sodium periodate R*; dry in a current of cold air; heat at 100 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Specific optical rotation (*2.2.7*): + 4.0 to + 7.0 (anhydrous substance).

Dissolve 5.00 g of the substance to be examined and 6.4 g of *disodium tetraborate R* in 40 mL of *water R*. Allow to stand for 1 h, shaking occasionally, and dilute to 50.0 mL with *water R*. Filter if necessary.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 5 g in *water R* and dilute to 50 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 20 $\mu S \cdot cm^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent, expressed as glucose equivalent.

Dissolve 5.0 g in 6 mL of *water R* with the aid of gentle heat. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 5.0 g of the substance to be examined in 20 mL of *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.50 g of *sorbitol CRS* in 2 mL of *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 100.0 mL with *water R*.

*Reference solution (d).* Dissolve 0.5 g of *sorbitol R* and 0.5 g of *mannitol R* (impurity A) in 5 mL of *water R* and dilute to 10 mL with the same solvent.

*Column*:

– *size*: *l* = 0.3 m, Ø = 7.8 mm;
– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
– *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of sorbitol.

*Relative retention* with reference to sorbitol (retention time = about 27 min): impurity C = about 0.6; impurity A = about 0.8; impurity B = about 1.1.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to impurity A and sorbitol.

*Limits*:

– *any impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
– *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 1.00 g. Use a mixture of 1 volume of *formamide R1* and 2 volumes of *anhydrous methanol R* as solvent.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
– TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
– absence of *Escherichia coli* (*2.6.13*);
– absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins:

– less than 4 IU/g for parenteral preparations having a concentration of less than 100 g/L of sorbitol;
– less than 2.5 IU/g for parenteral preparations having a concentration of 100 g/L or more of sorbitol.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_6H_{14}O_6$ taking into account the assigned content of *sorbitol CRS*.

LABELLING

The label states:

– where applicable, the maximum concentration of bacterial endotoxins;
– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

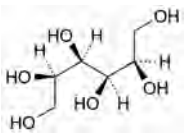

A. D-mannitol,

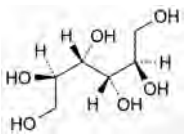

B. D-iditol,

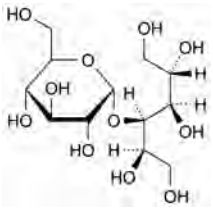

C. 4-*O*-α-D-glucopyranosyl-D-glucitol (D-maltitol).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for sorbitol used as filler and binder in tablets.*

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**07/2019:0436**

# SORBITOL, LIQUID (CRYSTALLISING)

## Sorbitolum liquidum cristallisabile

DEFINITION

Aqueous solution of a hydrogenated, partly hydrolysed starch.

*Content*:

- anhydrous substance: 68.0 per cent *m/m* to 72.0 per cent *m/m*,
- D-glucitol (D-sorbitol, $C_6H_{14}O_6$): 92.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless, syrupy liquid, miscible with water.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g add 40 mL of *water R* and 6.4 g of *disodium tetraborate R*, allow to stand for 1 h, shaking occasionally, and dilute to 50.0 mL with *water R*. Filter if necessary. The angle of rotation (*2.2.7*) is 0° to + 1.5°.

C. It is a clear, syrupy liquid at a temperature of 25 °C.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 7.0 g to 50 mL with *water R*.

**Conductivity** (*2.2.38*): maximum 10 $\mu S \cdot cm^{-1}$ measured on the undiluted liquid sorbitol (crystallising) while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent calculated as glucose equivalent.

To 5.0 g add 6 mL of *water R*, 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): 28.0 per cent to 32.0 per cent *m/m*, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Mix 1.00 g of the substance to be examined with 20 mL of *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 65.0 mg of *sorbitol CRS* in 2 mL of *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dissolve 65 mg of *mannitol R* and 65 mg of *sorbitol R* in 2 mL of *water R* and dilute to 5 mL with the same solvent.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm,
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm),
- *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL.

*Run time*: twice the retention time of sorbitol.

*Relative retention* with reference to sorbitol (retention time = about 27 min): mannitol = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to mannitol and to sorbitol.

Calculate the percentage content of $C_6H_{14}O_6$ taking into account the assigned content of *sorbitol CRS*.

**07/2019:0437**

# SORBITOL, LIQUID (NON-CRYSTALLISING)

## Sorbitolum liquidum non cristallisabile

DEFINITION

Aqueous solution of a hydrogenated, partly hydrolysed starch.

*Content*:

- anhydrous substance: 68.0 per cent *m/m* to 72.0 per cent *m/m*,
- D-glucitol (D-sorbitol, $C_6H_{14}O_6$): 72.0 per cent to 92.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless, syrupy liquid, miscible with water.

IDENTIFICATION

A. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. To 7.0 g add 40 mL of *water R* and 6.4 g of *disodium tetraborate R*. Allow to stand for 1 h, shaking occasionally, and dilute to 50.0 mL with *water R*. Filter if necessary. The angle of rotation (*2.2.7*) is + 1.5° to + 3.5°.

C. It is a clear, syrupy liquid at 25 °C.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 7.0 g to 50 mL with *water R*.

**Conductivity** (*2.2.38*): maximum 10 µS·cm$^{-1}$ measured on the undiluted liquid sorbitol (non crystallising) while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent calculated as glucose equivalent.

To 5.0 g add 6 mL of *water R*, 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Reducing sugars after hydrolysis**: maximum 9.3 per cent calculated as glucose equivalent.

To 6.0 g add 35 mL of *water R*, 40 mL of *1 M hydrochloric acid* and a few glass beads. Boil under a reflux condenser for 4 h. Cool and neutralise with *dilute sodium hydroxide solution R* using 0.2 mL of *bromothymol blue solution R1* as indicator. Cool and dilute to 100.0 mL with *water R*. To 3.0 mL of the solution add 5 mL of *water R*, 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R*. When the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 8.0 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): 28.0 per cent to 32.0 per cent *m/m*, determined on 0.100 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Mix 1.00 g of the substance to be examined with 20 mL of *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 55.0 mg of *sorbitol CRS* in 2 mL of *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b).* Dissolve 55 mg of *mannitol R* and 55 mg of *sorbitol R* in 2 mL of *water R* and dilute to 5 mL with the same solvent.

*Column*:
- *size*: *l* = 0.3 m, Ø = 7.8 mm,
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm),
- *temperature*: 85 ± 1 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 35 °C).

*Injection*: 20 µL.

*Run time*: twice the retention time of sorbitol.

*Relative retention* with reference to sorbitol (retention time = about 27 min): mannitol = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to mannitol and to sorbitol.

Calculate the percentage content of $C_6H_{14}O_6$ taking into account the assigned content of *sorbitol CRS*.

**07/2019:2048**

# SORBITOL, LIQUID, PARTIALLY DEHYDRATED

## Sorbitolum liquidum partim deshydricum

DEFINITION

Partially dehydrated liquid sorbitol is obtained by acid-catalysed partial internal dehydration of liquid sorbitol. It contains not less than 68.0 per cent *m/m* and not more than 85.0 per cent *m/m* of anhydrous substances, composed of a mixture of mainly D-sorbitol and 1,4-sorbitan, with mannitol, hydrogenated oligo- and disaccharides, and sorbitans.

*Content* (nominal value):
- 1,4-sorbitan ($C_6H_{12}O_5$): minimum 15.0 per cent (anhydrous substance);
- D-sorbitol ($C_6H_{14}O_6$): minimum 25.0 per cent (anhydrous substance).

The contents of 1,4-sorbitan and D-sorbitol are within 95.0 per cent to 105.0 per cent of the nominal values.

CHARACTERS

*Appearance*: clear, colourless, syrupy liquid.

*Solubility*: miscible with water, practically insoluble in mineral oils and vegetable oils.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

*Results*: the 2 principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the peaks in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dilute the substance to be examined with *carbon dioxide-free water R* prepared from *distilled water R* to obtain a solution containing 50.0 per cent *m/m* of anhydrous substance.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Measure the conductivity of solution S, while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.3 per cent, calculated as glucose (anhydrous substance).

To an amount of the substance to be examined equivalent to 3.3 g of anhydrous substance, add 3 mL of *water R*, 20.0 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min. Maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous

shaking, add 25 mL of a mixture of 6 mL of *hydrochloric acid R* and 94 mL of *water R*. When the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 2 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Water** (*2.5.12*): 15.0 per cent to 32.0 per cent, determined on 0.100 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.400 g of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50.0 mg of *sorbitol CRS* and 20.0 mg of *1,4-sorbitan CRS* in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 0.100 g of *mannitol R* and 0.100 g of *sorbitol R* in *water R* and dilute to 10 mL with the same solvent.

*Column*:

- *size*: *l* = 0.3 m, Ø = 7.8 mm;
- *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);
- *temperature*: 80 ± 5 °C.

*Mobile phase*: degassed *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (e.g. 30-35 °C).

*Injection*: 40 µL.

*Relative retention* with reference to D-sorbitol (retention time = about 25 min): 1,4-sorbitan = about 0.5; mannitol = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to mannitol and D-sorbitol.

Calculate the percentage contents of 1,4-sorbitan ($C_6H_{12}O_5$) and D-sorbitol ($C_6H_{14}O_6$) taking into account the assigned contents of *1,4-sorbitan CRS* and *sorbitol CRS*.

LABELLING

The label states the content of D-sorbitol and the content of 1,4-sorbitan (= nominal values).

**01/2017:2004**

# SOTALOL HYDROCHLORIDE

## Sotaloli hydrochloridum

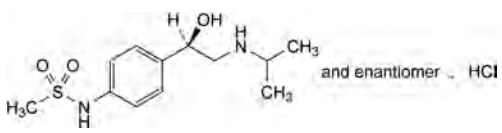


$C_{12}H_{21}ClN_2O_3S$ $M_r$ 308.8

[959-24-0]

DEFINITION

*N*-[4-[(1*RS*)-1-Hydroxy-2-[(1-methylethyl)amino]ethyl]-phenyl]methanesulfonamide hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, soluble in alcohol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sotalol hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.0.

Dilute 5.0 mL of solution S to 10.0 mL with *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dilute 25.0 mL of solution S to 50.0 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 3.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 8.0 mg of *sotalol impurity B CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Reference solution (d)*. Dilute 1.5 mL of reference solution (b) to 100 mL with the mobile phase. To 1 mL of this solution add 1 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 2 g of *sodium octanesulfonate R* in 790 mL of *water R*. Adjust to pH 3.0 with *phosphoric acid R* and add 210 mL of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 228 nm.

*Injection*: 10 µL; inject the test solution and reference solutions (a), (c) and (d).

*Run time*: 2.5 times the retention time of sotalol.

*System suitability*: reference solution (d):

- *resolution*: minimum 4.0 between the peaks due to sotalol and to impurity B.

*Limits*:

- *impurity B*: not more than 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent), and not more than 1 such peak has an area greater than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total of other impurities*: not more than 1.65 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.17 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Palladium**: maximum 0.5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 1.00 g in a mixture of 0.25 volumes of *nitric acid R*, 0.75 volumes of *hydrochloric acid R* and 99.0 volumes of *water R* and dilute to 20.0 mL with the same mixture of solvents.

*Reference solutions.* Use solutions containing 0.02 µg, 0.03 µg and 0.05 µg of palladium per millilitre, freshly prepared by dilution of *palladium standard solution (0.5 ppm Pd) R* with a mixture of 0.25 volumes of *nitric acid R*, 0.75 volumes of *hydrochloric acid R* and 99.0 volumes of *water R*.

*Source*: palladium hollow-cathode lamp.

*Wavelength*: 247.6 nm.

Use a graphite tube.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

*In order to avoid overheating during the titration, mix thoroughly throughout and stop the titration immediately after the end-point has been reached.*

Dissolve 0.250 g in 10 mL of *anhydrous formic acid R*, if necessary with the aid of ultrasound. Add 40 mL of *acetic anhydride R* and titrate immediately with *0.1 M perchloric acid*. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.88 mg of $C_{12}H_{21}ClN_2O_3S$.

STORAGE

Protected from light.

IMPURITIES

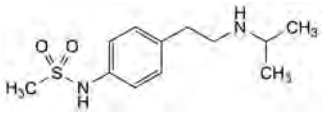

A. *N*-[4-[2-[(1-methylethyl)amino]ethyl]phenyl]-methanesulfonamide,

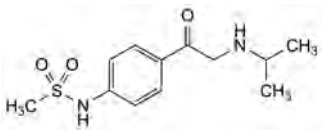

B. *N*-[4-[[(1-methylethyl)amino]acetyl]phenyl]-methanesulfonamide,

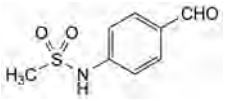

C. *N*-(4-formylphenyl)methanesulfonamide,

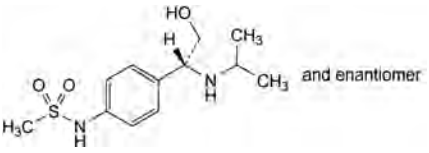


D. *N*-[4-[(1*RS*)-2-hydroxy-1-[(1-methylethyl)amino]ethyl]-phenyl]methanesulfonamide.

**07/2019:2316**

# SOYA PHOSPHOLIPIDS FOR INJECTION

## Phospholipida ex soia ad iniectabile

Soya lecithins: [8030-76-0]
Soya phosphatidylcholines: [97281-47-5]

DEFINITION

Mixture of natural phospholipids of soya, and fractions thereof, for injection. A suitable stabiliser such as α-tocopherol may be added.

*Content*:

– *phosphatidylcholine*: minimum 60.0 per cent (anhydrous substance);
– *phosphatidylethanolamine*: maximum 12.0 per cent (anhydrous substance);
– *lysophosphatidylcholine*: maximum 4.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: light yellow or brownish, porous agglomerates or waxy solid mass.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent) and in heptane.

IDENTIFICATION

*Carry out either test A or test B. If N-acyl-phosphatidylethanolamine is not detected with test A or B, carry out test C.*

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *phosphatidylethanolamine from soya bean CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 20 mg of *lysophosphatidylcholine from soya bean CRS* and 20 mg of *N-acyl-phosphatidylethanolamine from soya bean CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (c).* Dissolve 15 mg of *phosphatidylcholine from soya bean CRS* in *methanol R*, add 3 mL of reference solution (a) and 1 mL of reference solution (b) and dilute to 50.0 mL with *methanol R*.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: *water R*, *methanol R*, *chloroform R* (4:25:65 *V/V/V*).

*Application*: 4 µL of the test solution and reference solution (c), as slim bands.

*Development*: over 2/3 of the plate.

*Detection*: spray with *copper sulfate solution R1* and heat at 170 °C for 5-15 min; the phospholipids appear as brownish-grey bands.

*Retardation factors*: lysophosphatidylcholine = about 0.15; phosphatidylcholine = about 0.3; phosphatidylethanolamine = about 0.6; *N*-acyl-phosphatidylethanolamine = about 0.8.

*System suitability*: reference solution (c):

– the chromatogram shows 4 clearly separated bands.

*Results*:

- the principal band in the chromatogram obtained with the test solution is similar in position and colour to the band due to phosphatidylcholine in the chromatogram obtained with reference solution (c);
- the band due to *N*-acyl-phosphatidylethanolamine is visible in the chromatogram obtained with the test solution.

B. Liquid chromatography (*2.2.29*) as described in the assay with the following modifications.

*Injection*: test solution and reference solution (h).

*Identification of peaks*: use the chromatogram obtained with reference solution (h) to identify the peak due to *N*-acyl-phosphatidylethanolamine.

*Relative retention* with reference to phosphatidylcholine (retention time = about 9 min): *N*-acyl-phosphatidyl-ethanolamine = about 0.2 (may be eluted as 1 or 2 peaks).

*Results*:

- the principal peak in the chromatogram obtained with the test solution is due to phosphatidylcholine;
- the peak due to *N*-acyl-phosphatidylethanolamine in the chromatogram obtained with the test solution has a signal-to-noise ratio not less than 3.

C. Composition of fatty acids (*2.4.22, Method B*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the substance*:

- *linoleic acid (C18:2)*: 59 per cent to 71 per cent;
- *palmitic acid (C16:0)*: 10 per cent to 17 per cent;
- *oleic acid (C18:1)*: 6 per cent to 15 per cent;
- *α-linolenic acid (C18:3)*: 4 per cent to 9 per cent;
- *stearic acid (C18:0)*: 2 per cent to 5 per cent.

TESTS

**Acid value** (*2.5.1*): maximum 12.

Dissolve 2.00 g in 50 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *ether R*, previously neutralised with *0.1 M potassium hydroxide*, using 0.5 mL of *phenolphthalein solution R1* as indicator.

**Peroxide value** (*2.5.5, Method A*): maximum 5.

Titrate with *0.01 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*).

**Iodine value** (*2.5.4, Method A*): minimum 83.

**Free fatty acids**. Thin-layer chromatography (*2.2.27*). *Carry out the test at room temperature.*

*Solvent mixture*: *water R*, *heptane R*, *2-propanol R* (8:40:50 *V/V/V*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution.* Dissolve 10.0 mg of *oleic acid R* and 10 mg of *refined soya-bean oil R* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *acetic acid R*, *ether R*, *heptane R* (1:30:70 *V/V/V*).

*Application*: 20 µL.

*Development*: over 2/3 of the plate.

*Detection*: spray with *copper sulfate solution R1* and heat at 170 °C for 5-15 min.

*Retardation factors*: oleic acid = about 0.3; soya-bean oil = about 0.5.

*System suitability*: reference solution:

- the chromatogram shows 2 clearly separated spots.

*Limit*: any spot due to oleic acid is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500-1.00 g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14, Method A*): less than 6 IU/g. The suitability of the method must be verified and a risk assessment must be carried out according to general chapter *5.1.10. Guidelines for using the test for bacterial endotoxins* since the phospholipids can interfere with the test.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 8 volumes of *water R* and 46 volumes of *2-propanol R*, then add 46 volumes of *hexane R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*NOTE: if saturation of the peak due to phosphatidylcholine is observed, the test solution may be diluted with the solvent mixture to quantify this compound.*

*Reference solution ($a_1$).* Dissolve 60 mg of *phosphatidylcholine from soya bean CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_1$), ($c_1$), ($d_1$), ($e_1$), ($f_1$).* Dilute reference solution ($a_1$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of phosphatidylcholine in the test solution.

*Reference solution ($a_2$).* Dissolve 40 mg of *phosphatidylethanolamine from soya bean CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_2$), ($c_2$), ($d_2$), ($e_2$), ($f_2$).* Dilute reference solution ($a_2$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of phosphatidylethanolamine in the test solution.

*Reference solution ($a_3$).* Dissolve 40 mg of *lysophosphatidylcholine from soya bean CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solutions ($b_3$), ($c_3$), ($d_3$), ($e_3$), ($f_3$).* Dilute reference solution ($a_3$) with the solvent mixture to obtain 5 reference solutions, the concentrations of which span 60 per cent to 140 per cent of the expected content of lysophosphatidylcholine in the test solution.

*Reference solution (g).* Mix 5 mL of reference solution ($a_1$) and 5 mL of reference solution ($a_2$).

*Reference solution (h).* Dissolve 5 mg of *N-acyl-phosphatidylethanolamine from soya bean CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *diol silica gel for chromatography R* (5 µm);
- *temperature*: 55 °C.

*Mobile phase*:

- *mobile phase A*: mix 0.08 volumes of *triethylamine R*, 1.5 volumes of *glacial acetic acid R*, 17 volumes of *2-propanol R* and 81.42 volumes of *hexane R*;
- *mobile phase B*: mix 0.08 volumes of *triethylamine R*, 1.5 volumes of *glacial acetic acid R*, 14 volumes of *water for chromatography R* and 84.42 volumes of *2-propanol R*;

Monographs S

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.0 | 95 | 5 |
| 2.0 - 7.0 | 95 → 80 | 5 → 20 |
| 7.0 - 10.5 | 80 → 60 | 20 → 40 |
| 10.5 - 17.0 | 60 → 0 | 40 → 100 |
| 17.0 - 19.5 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 2.5 mL/min;
- *evaporation temperature*: 50 °C.

*Injection*: 20 µL of the test solution and reference solutions ($b_{1,2,3}$), ($c_{1,2,3}$), ($d_{1,2,3}$), ($e_{1,2,3}$), ($f_{1,2,3}$) and (g).

*Identification of peaks*: use the chromatograms obtained with reference solutions ($f_1$), ($f_2$) and ($f_3$) to identify the peaks due to phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine.

*Relative retention* with reference to phosphatidylcholine (retention time = about 9 min): phosphatidylethanolamine = about 0.8; lysophosphatidylcholine = about 1.2.

Establish calibration curves with the logarithm of the concentration of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine in reference solutions ($b_{1,2,3}$), ($c_{1,2,3}$), ($d_{1,2,3}$), ($e_{1,2,3}$) and ($f_{1,2,3}$) as the abscissa and the logarithm of the corresponding peak areas as the ordinate.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to phosphatidylethanolamine and phosphatidylcholine in the chromatogram obtained with reference solution (g);
- *phosphatidylcholine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_1$);
  - *correlation coefficient*: minimum 0.995;
- *phosphatidylethanolamine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_2$);
  - *correlation coefficient*: minimum 0.995;
- *lysophosphatidylcholine*:
  - *repeatability*: maximum relative standard deviation of 5.0 per cent determined on 6 injections of reference solution ($f_3$);
  - *correlation coefficient*: minimum 0.99.

Calculate the percentage contents of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine using the calibration curves.

STORAGE

Under nitrogen, protected from light, at − 15 °C or below.

LABELLING

The label states:

- the specified content range of phosphatidylcholine;
- the specified content range of phosphatidylethanolamine.

01/2020:1265

# SOYA-BEAN OIL, HYDROGENATED

## Soiae oleum hydrogenatum

DEFINITION

Product obtained by refining, bleaching, hydrogenation and deodorisation of oil obtained from seeds of *Glycine max* (L.) Merr. (*G. hispida* (Moench) Maxim.). The product consists mainly of triglycerides of palmitic and stearic acids.

CHARACTERS

*Appearance*: white or almost white mass or powder which melts to a clear, pale yellow liquid when heated.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, in light petroleum (bp: 65-70 °C) after heating and in toluene, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (see Tests).

B. Composition of fatty acids (see Tests).

TESTS

**Melting point** (*2.2.15*): 66 °C to 72 °C.

**Acid value** (*2.5.1*): maximum 0.5.

Dissolve 10.0 g in 50 mL of a hot mixture of equal volumes of *ethanol (96 per cent) R* and *toluene R*, previously neutralised with *0.1 M potassium hydroxide* using 0.5 mL of *phenolphthalein solution R1* as indicator. Titrate the solution immediately while still hot.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). Dissolve 2.0 g with gentle heating in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3 mL of *toluene R*. Add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*. Not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour to yellow.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*) with the following modifications. Use the mixture of calibrating substances in Table 2.4.22.-3.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 0.65 mL/min.

*Split ratio*: 1:100.

*Temperature*:

- *column*: 180 °C for 20 min;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Composition of the fatty-acid fraction of the substance*:

- *saturated fatty acids of chain length less than $C_{14}$*: maximum 0.1 per cent;
- *myristic acid*: maximum 0.5 per cent;
- *palmitic acid*: 9.0 per cent to 16.0 per cent;
- *stearic acid*: 79.0 per cent to 89.0 per cent;
- *oleic acid and isomers*: maximum 4.0 per cent;
- *linoleic acid and isomers*: maximum 1.0 per cent;

– *linolenic acid and isomers*: maximum 0.2 per cent;
– *arachidic acid*: maximum 1.0 per cent;
– *behenic acid*: maximum 1.0 per cent.

STORAGE

Protected from light.

**01/2020:1473**

# SOYA-BEAN OIL, REFINED

## Soiae oleum raffinatum

DEFINITION

Fatty oil obtained from seeds of *Glycine max* (L.) Merr. (*Glycine hispida* (Moench) Maxim.) by extraction and subsequent refining. It may contain a suitable antioxidant.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, pale yellow liquid.

*Solubility*: practically insoluble in ethanol (96 per cent), miscible with light petroleum (bp: 50-70 °C).

*Relative density*: about 0.922.

*Refractive index*: about 1.475.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).
*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.5.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:
– *saturated fatty acids of chain length less than $C_{14}$*: maximum 0.1 per cent;
– *myristic acid*: maximum 0.2 per cent;
– *palmitic acid*: 9.0 per cent to 13.0 per cent;
– *palmitoleic acid*: maximum 0.3 per cent;
– *stearic acid*: 2.5 per cent to 5.0 per cent;
– *oleic acid and isomer*: 17.0 per cent to 30.0 per cent;
– *linoleic acid*: 48.0 per cent to 58.0 per cent;
– *linolenic acid*: 5.0 per cent to 11.0 per cent;
– *arachidic acid*: maximum 1.0 per cent;
– *eicosenoic acid*: maximum 1.0 per cent;
– *behenic acid*: maximum 1.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In a well-filled container, protected from light, at a temperature not exceeding 25 °C. If intended for use in the manufacture of parenteral preparations, store under an inert gas.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations and the name of the inert gas.

**01/2008:1152**
**corrected 10.0**

# SPECTINOMYCIN DIHYDROCHLORIDE PENTAHYDRATE

## Spectinomycini dihydrochloridum pentahydricum

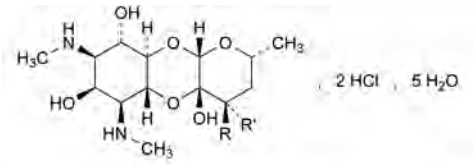


| Compound | R | R' | Molec. Formula | $M_r$ |
|---|---|---|---|---|
| spectinomycin | R + R' = O | | $C_{14}H_{26}Cl_2N_2O_7,5H_2O$ | 495.4 |
| (4*R*)-dihydro-spectinomycin | OH | H | $C_{14}H_{28}Cl_2N_2O_7,5H_2O$ | 497.4 |

DEFINITION

Mixture of (2*R*,4a*R*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-4a,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one dihydrochloride pentahydrate (spectinomycin dihydrochloride pentahydrate) and of (2*R*,4*R*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]-benzodioxine-4,4a,7,9-tetrol dihydrochloride pentahydrate ((4*R*)-dihydrospectinomycin dihydrochloride pentahydrate).

It is produced by *Streptomyces spectabilis* or by any other means.

*Content*:
– *(4R)-dihydrospectinomycin dihydrochloride*: maximum 9.0 per cent (anhydrous substance);
– *sum of the contents of spectinomycin dihydrochloride and (4R)-dihydrospectinomycin dihydrochloride*: 93.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *spectinomycin hydrochloride CRS*.

B. Dilute 1.0 mL of solution S (see Tests) to 10 mL with *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 2.0 mL of solution S to 20.0 mL with *water R*.

**pH** (*2.2.3*): 3.8 to 5.6 for solution S.

**Specific optical rotation** (*2.2.7*): + 15.0 to + 21.0 (anhydrous substance), determined on solution S within 20 min of preparation.

**Related substances**. Liquid chromatography (*2.2.29*). *In order to avoid formation of anomers, prepare the solutions immediately before use.*

*Test solution.* Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3 mg of *spectinomycin for system suitability CRS* in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 3.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: ambient and constant.

*Mobile phase*: dissolve 4.2 g of *oxalic acid R* and 2.0 mL of *heptafluorobutyric acid R* in *water R* and dilute to 1000 mL with *water R*; adjust to pH 3.2 with *sodium hydroxide solution R*, add 105 mL of *acetonitrile R* and mix; filter through a 0.45 µm filter and degas with *helium for chromatography R* for 10 min.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted with *carbon dioxide-free water R* to obtain a final concentration of NaOH of 21 g/L. Degas the solution with *helium for chromatography R* for 10 min before use. Add it pulse-less to the column effluent using a 375 µL polymeric mixing coil.

*Post-column flow rate*: 0.5 mL/min.

*Detection*: pulsed amperometric detection or equivalent with a gold indicator electrode having preferably a diameter of 1.4 mm or greater, a suitable reference electrode and a stainless steel counter electrode, held at + 0.12 V detection, + 0.70 V oxidation and – 0.60 V reduction potentials respectively, with pulse durations according to the instrument used. Keep the detection cell at ambient and constant temperature. Clean the gold indicator electrode with an eraser and damp precision wipe prior to start-up of the system to enhance the detector sensitivity and increase the signal-to-noise ratio.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of spectinomycin.

*Identification of impurities*: use the chromatogram supplied with *spectinomycin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, D and E.

*Relative retention* with reference to spectinomycin (retention time = 11 min to 20 min): impurity A = about 0.5; impurity F = about 0.53; impurity G = about 0.6; impurity D = about 0.7; impurity E = about 0.9; (4*R*)-dihydrospectinomycin = about 1.3; impurity C = about 1.4.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity E and spectinomycin.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.4;
- *impurities A, C, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurities D, E*: for each impurity, not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent); disregard the peak due to (4*R*)-dihydrospectinomycin.

**Water** (*2.5.12*): 16.0 per cent to 20.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.09 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Prepare the solutions using a 0.42 per cent *m/m* solution of *sodium hydrogen carbonate R*.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Allow to stand for not less than 15 h and not more than 72 h (formation of anomers). Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution.* Dissolve 40.0 mg of *spectinomycin hydrochloride CRS* (containing (4*R*)-dihydrospectinomycin) in *water R* and dilute to 50.0 mL with the same solvent. Allow to stand for the same period of time as the test solution (formation of anomers). Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*System suitability*:

- *repeatability*: maximum relative standard deviation of 3.0 per cent for the principal peak after 6 injections of the reference solution.

Calculate the sum of the percentage contents of spectinomycin dihydrochloride and (4*R*)-dihydrospectinomycin dihydrochloride from the declared contents of $C_{14}H_{26}Cl_2N_2O_7$ and $C_{14}H_{28}Cl_2N_2O_7$ in *spectinomycin hydrochloride CRS*.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A, C, D, E, F, G*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B*.

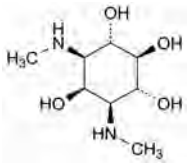

A. 1,3-dideoxy-1,3-bis(methylamino)-*myo*-inositol (actinamine),

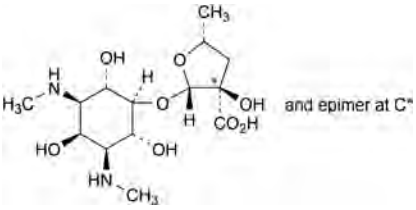


B. (2*S*,3*RS*,5*R*)-3-hydroxy-5-methyl-2-[[(1*r*,2*R*,3*S*,4*r*,5*R*,6*S*)-2,4,6-trihydroxy-3,5-bis(methylamino)cyclohexyl]oxy]-tetrahydrofuran-3-carboxylic acid (actinospectinoic acid),

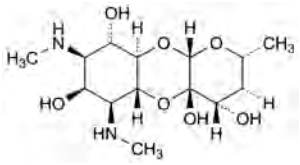

C. (2*R*,4*S*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]benzodioxine-4,4a,7,9-tetrol ((4*S*)-dihydrospectinomycin),

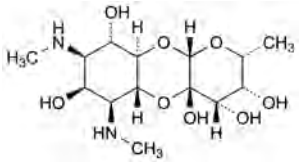

D. (2*R*,3*R*,4*S*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]benzodioxine-3,4,4a,7,9-pentol (dihydroxyspectinomycin),

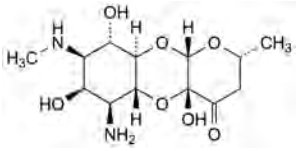

E. (2*R*,4a*R*,5a*R*,6*S*,7*R*,8*R*,9*S*,9a*R*,10a*S*)-6-amino-4a,7,9-trihydroxy-2-methyl-8-(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one (*N*-desmethylspectinomycin),

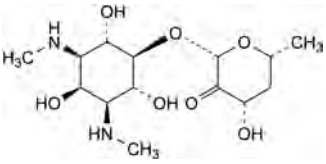

F. (2*S*,4*S*,6*R*)-4-hydroxy-6-methyl-2-[[(1*r*,2*R*,3*S*,4*r*,5*R*,6*S*)-2,4,6-trihydroxy-3,5-bis(methylamino)cyclohexyl]oxy]-dihydro-2*H*-pyran-3(4*H*)-one (triol spectinomycin),

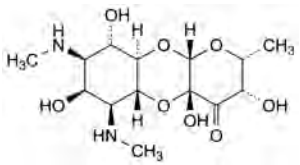

G. (2*R*,3*S*,4a*R*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-3,4a,7,9-tetrahydroxy-2-methyl-6,8-bis(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one (tetrahydroxyspectinomycin).

**01/2008:1658**
**corrected 10.0**

# SPECTINOMYCIN SULFATE TETRAHYDRATE FOR VETERINARY USE

## Spectinomycini sulfas tetrahydricus ad usum veterinarium

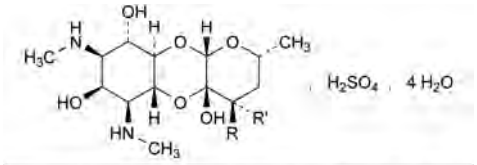


| Compound | R | R' | Molec. Formula | $M_r$ |
|---|---|---|---|---|
| spectinomycin | R + R' = O | | $C_{14}H_{26}N_2O_{11}S,4H_2O$ | 502.5 |
| (4*R*)-dihydro-spectinomycin | OH | H | $C_{14}H_{28}N_2O_{11}S,4H_2O$ | 504.5 |

DEFINITION

Mixture of (2*R*,4a*R*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-4a,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one sulfate tetrahydrate (spectinomycin sulfate tetrahydrate) and (2*R*,4*R*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]-benzodioxine-4,4a,7,9-tetrol sulfate tetrahydrate ((4*R*)-dihydrospectinomycin sulfate tetrahydrate).

It is produced by *Streptomyces spectabilis* or by any other means.

*Content*:

– *(4R)-dihydrospectinomycin sulfate*: maximum 2.0 per cent (anhydrous substance);
– *sum of the contents of spectinomycin sulfate and (4R)-dihydrospectinomycin sulfate*: 93.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *spectinomycin sulfate tetrahydrate CRS*.

B. Dilute 1.0 mL of solution S (see Tests) to 10 mL with *water R*. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.8 to 5.6 for solution S.

**Specific optical rotation** (*2.2.7*): + 10.0 to + 14.0 (anhydrous substance).

Dissolve 2.50 g in an 8 mL/L solution of *concentrated ammonia R1* and dilute to 25.0 mL with the same solvent. Allow the solution to stand at room temperature for not less than 30 min and not more than 2 h prior to determination.

**Related substances**. Liquid chromatography (*2.2.29*). *In order to avoid the formation of anomers, prepare the solutions immediately before use.*

*Test solution.* Dissolve 15.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3 mg of *spectinomycin for system suitability CRS* in the mobile phase and dilute to 20 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 3.0 mL of reference solution (b) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: ambient and constant.

*Mobile phase*: dissolve 4.2 g of *oxalic acid R* and 2.0 mL of *heptafluorobutyric acid R* in *water R* and dilute to 1000 mL with *water R*; adjust to pH 3.2 with *sodium hydroxide solution R*; add 105 mL of *acetonitrile R* and mix; filter through a 0.45 µm filter and degas with *helium for chromatography R* for 10 min.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted with *carbon dioxide-free water R* to obtain a final concentration of NaOH of 21 g/L. Degas the solution with *helium for chromatography R* for 10 min before use. Add it pulse-less to the column effluent using a 375 µL polymeric mixing coil.

*Post-column flow rate*: 0.5 mL/min.

*Detection*: pulsed amperometric detection or equivalent with a gold indicator electrode having preferably a diameter of 1.4 mm or greater, a suitable reference electrode and a stainless steel counter electrode, held at + 0.12 V detection, + 0.70 V oxidation and – 0.60 V reduction potentials respectively, with pulse durations according to the instrument used. Keep the detection cell at ambient and constant temperature. Clean the gold indicator electrode with an eraser and damp precision wipe prior to start-up of the system to enhance the detector sensitivity and increase the signal-to-noise ratio.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of spectinomycin.

*Identification of impurities*: use the chromatogram supplied with *spectinomycin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, D and E.

*Relative retention* with reference to spectinomycin (retention time = 11 min to 20 min): impurity A = about 0.5; impurity D = about 0.7; impurity E = about 0.9; (4*R*)-dihydrospectinomycin = about 1.3.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity E and spectinomycin.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.4;
- *impurities A, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity D*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (4.0 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (6.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent); disregard the peak due to (4*R*)-dihydrospectinomycin.

**Water** (*2.5.12*): 12.0 per cent to 16.5 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins. Prepare the solutions using a 0.42 per cent *m/m* solution of *sodium hydrogen carbonate R*.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Test solution.* Dissolve 40.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Allow to stand for not less than 15 h and not more than 72 h (formation of anomers). Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution.* Dissolve 40.0 mg of *spectinomycin hydrochloride CRS* (containing (4*R*)-dihydrospectinomycin) in *water R* and dilute to 50.0 mL with the same solvent. Allow to stand for the same period of time as the test solution (formation of anomers). Dilute 5.0 mL of this solution to 50.0 mL with the mobile phase.

*System suitability*:

- *repeatability*: maximum relative standard deviation of 3.0 per cent for the principal peak after 6 injections of the reference solution.

Calculate the sum of the percentage contents of spectinomycin sulfate and (4*R*)-dihydrospectinomycin sulfate from the declared contents of $C_{14}H_{26}Cl_2N_2O_7$ and $C_{14}H_{28}Cl_2N_2O_7$ in *spectinomycin hydrochloride CRS*, applying a correction factor of 1.062.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, F, G.*

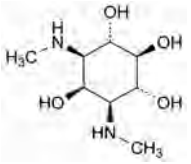


A. 1,3-dideoxy-1,3-bis(methylamino)-*myo*-inositol (actinamine),

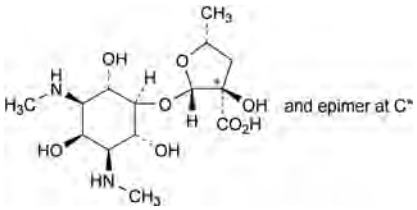


B. (2*S*,3*RS*,5*R*)-3-hydroxy-5-methyl-2-[[(1*r*,2*R*,3*S*,4*r*,5*R*,6*S*)-2,4,6-trihydroxy-3,5-bis(methylamino)cyclohexyl]oxy]-tetrahydrofuran-3-carboxylic acid (actinospectinoic acid),

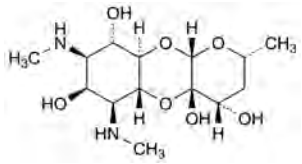

C. (2*R*,4*S*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]-benzodioxine-4,4a,7,9-tetrol ((4*S*)-dihydrospectinomycin),

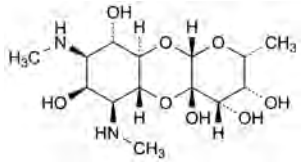

D. (2*R*,3*R*,4*S*,4a*S*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-2-methyl-6,8-bis(methylamino)decahydro-2*H*-pyrano[2,3-*b*][1,4]benzo-dioxine-3,4,4a,7,9-pentol (dihydroxyspectinomycin),

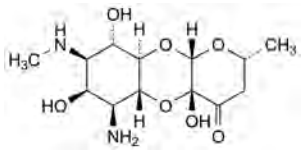

E. (2*R*,4a*R*,5a*R*,6*S*,7*R*,8*R*,9*S*,9a*R*,10a*S*)-6-amino-4a,7,9-trihydroxy-2-methyl-8-(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one (*N*-desmethylspectinomycin),

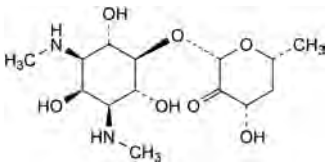

F. (2*S*,4*S*,6*R*)-4-hydroxy-6-methyl-2-[[(1*r*,2*R*,3*S*,4*r*,5*R*,6*S*)-2,4,6-trihydroxy-3,5-bis(methylamino)cyclohexyl]oxy]-dihydro-2*H*-pyran-3(4*H*)-one (triol spectinomycin),

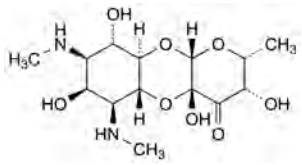

G. (2*R*,3*S*,4a*R*,5a*R*,6*S*,7*S*,8*R*,9*S*,9a*R*,10a*S*)-3,4a,7,9-tetra-hydroxy-2-methyl-6,8-bis(methylamino)decahydro-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one (tetrahydroxyspectinomycin).

**01/2017:0293**
**corrected 10.0**

# SPIRAMYCIN

## Spiramycinum

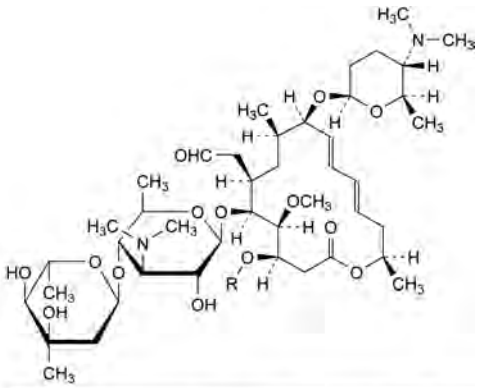

| Compound | R | Molec Formula | $M_r$ |
|---|---|---|---|
| Spiramycin I | H | $C_{43}H_{74}N_2O_{14}$ | 843.1 |
| Spiramycin II | CO-$CH_3$ | $C_{45}H_{76}N_2O_{15}$ | 885.1 |
| Spiramycin III | CO-$CH_2$-$CH_3$ | $C_{46}H_{78}N_2O_{15}$ | 899.1 |

DEFINITION

Macrolide antibiotic produced by the growth of certain strains of *Streptomyces ambofaciens* or obtained by any other means. The main component is (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (spiramycin I; $M_r$ 843). Spiramycin II (4-*O*-acetylspiramycin I) and spiramycin III (4-*O*-propanoylspiramycin I) are also present.

*Potency*: minimum 4100 IU/mg (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder, slightly hygroscopic.

*Solubility*: slightly soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methanol.

IDENTIFICATION

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 232 nm.

*Specific absorbance at the absorption maximum*: about 340.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 40 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 40 mg of *spiramycin CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 40 mg of *erythromycin A CRS* in *methanol R* and dilute to 10 mL with the same solvent.

Monographs S

*Plate*: *TLC silica gel G plate R*.

*Mobile phase*: the upper layer of a mixture of 4 volumes of *2-propanol R*, 8 volumes of a 150 g/L solution of *ammonium acetate R* previously adjusted to pH 9.6 with *strong sodium hydroxide solution R*, and 9 volumes of *ethyl acetate R*.

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *anisaldehyde solution R1* and heat at 110 °C for 5 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). If in the chromatogram obtained with the test solution 1 or 2 spots occur with $R_F$ values slightly higher than that of the principal spot, these spots are similar in position and colour to the secondary spots in the chromatogram obtained with reference solution (a) and differ from the spots in the chromatogram obtained with reference solution (b).

C. Dissolve 0.5 g in 10 mL of *dilute sulfuric acid R1* and add 25 mL of *water R*. Adjust to about pH 8 with *0.1 M sodium hydroxide* and dilute to 50 mL with *water R*. To 5 mL of this solution add 2 mL of a mixture of 1 volume of *water R* and 2 volumes of *sulfuric acid R*. A brown colour develops.

TESTS

**pH** (*2.2.3*): 8.5 to 10.5.

Dissolve 0.5 g in 5 mL of *methanol R* and dilute to 100 mL with *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 80 to − 85 (dried substance).

Dissolve 1.00 g in a 10 per cent *V/V* solution of *dilute acetic acid R* and dilute to 50.0 mL with the same acid solution.

**Composition**. Liquid chromatography (*2.2.29*) as described in the test for related substances.

*Injection*: test solution and reference solution (a).

Calculate the percentage content using the declared content of spiramycins I, II and III in *spiramycin CRS*.

*Limits* (dried substance):

- *spiramycin I*: minimum 80.0 per cent;
- *spiramycin II*: maximum 5.0 per cent;
- *spiramycin III*: maximum 10.0 per cent;
- *sum of spiramycins I, II and III*: minimum 90.0 per cent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use*.

*Solvent mixture*: *methanol R*, *water R* (30:70 *V/V*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *spiramycin CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 2.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 5 mg of *spiramycin CRS* in 15 mL of *buffer solution pH 2.2 R* and dilute to 25 mL with *water R*, then heat in a water-bath at 60 °C for 5 min and cool under cold water.

*Blank solution*. The solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm) (polar-embedded octadecylsilyl methylsilica gel), with a pore size of 12.5 nm and a carbon loading of 15 per cent;
- *temperature*: 70 °C.

*Mobile phase*: mix 5 volumes of a 34.8 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 6.5 with a 27.2 g/L solution of *potassium dihydrogen phosphate R*, 40 volumes of *acetonitrile R* and 55 volumes of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 20 µL of the blank solution, the test solution and reference solutions (b) and (c).

*Run time*: 3 times the retention time of spiramycin I.

*Identification of spiramycins*: use the chromatogram supplied with *spiramycin CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to spiramycins I, II and III.

*Relative retention* with reference to spiramycin I (retention time = 20 min to 30 min): impurity F = about 0.41; impurity A = about 0.45; impurity D = about 0.50; impurity G = about 0.66; impurity B = about 0.73; impurity H = about 0.87; spiramycin II = about 1.4; spiramycin III = about 2.0; impurity E = about 2.5.

If necessary adjust the composition of the mobile phase by changing the amount of acetonitrile.

*System suitability*: reference solution (c):

- *resolution*: minimum 10.0 between the peaks due to impurity A and spiramycin I.

*Limits*:

- *impurities A, B, D, E, F, G, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (10.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); disregard the peaks due to spiramycins I, II and III.

**Loss on drying** (*2.2.32*): maximum 3.5 per cent, determined on 0.500 g by drying *in vacuo* at 80 °C at a pressure not exceeding 0.7 kPa for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, D, E, F, G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

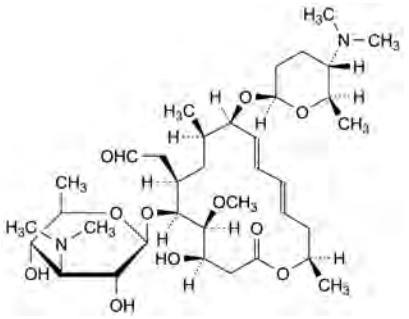

A. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (neospiramycin I),

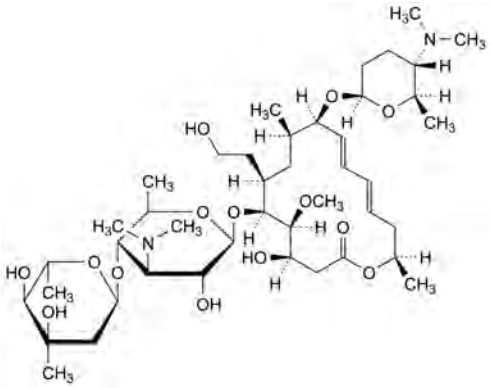

B. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-4-hydroxy-7-(2-hydroxyethyl)-5-methoxy-9,16-dimethyl-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (spiramycin IV),

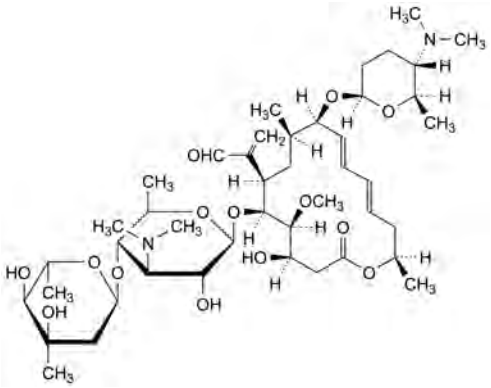

C. (4*R*,5*S*,6*S*,7*S*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-7-(1-formylethenyl)-4-hydroxy-5-methoxy-9,16-dimethyl-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (17-methylenespiramycin I),

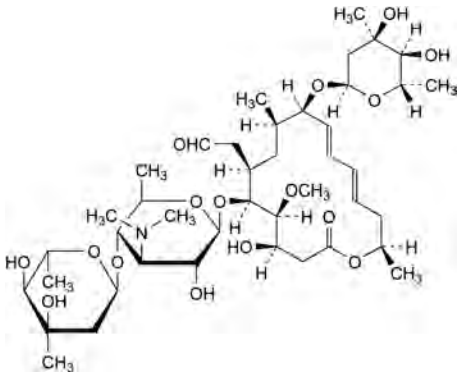

D. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-10-[(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)oxy]-4-hydroxy-5-methoxy-9,16-dimethyl-7-(2-oxoethyl)-oxacyclohexadeca-11,13-dien-2-one (spiramycin V),

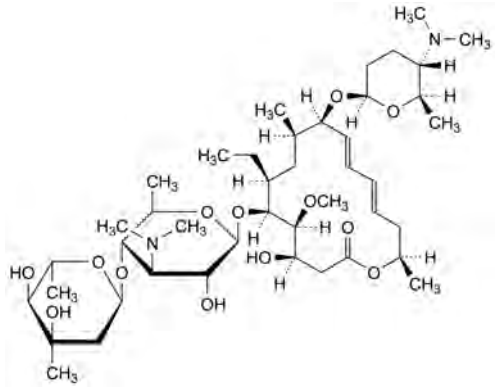

E. (4*R*,5*S*,6*S*,7*S*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-7-ethyl-4-hydroxy-5-methoxy-9,16-dimethyl-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-2-one (18-deoxy-18-dihydrospiramycin I or DSPM),

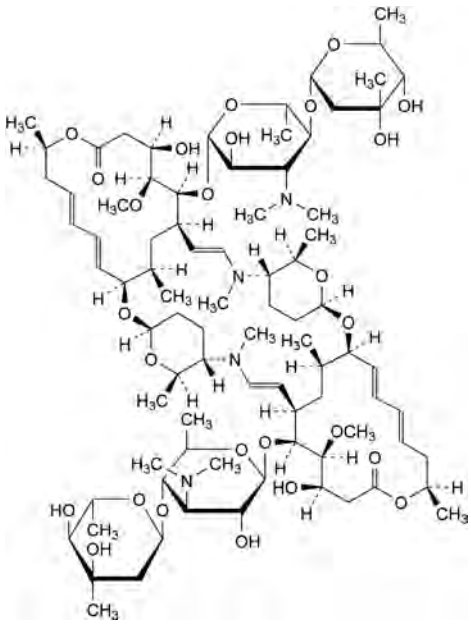

F. spiramycin dimer,

Monographs S

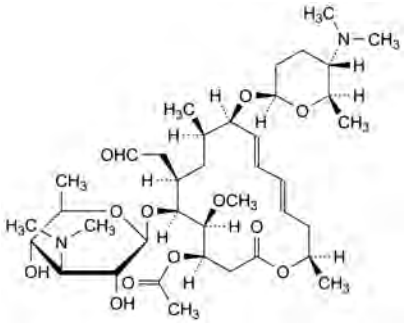

G. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-2-oxo-7-(2-oxoethyl)-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-4-yl acetate (neospiramycin II),

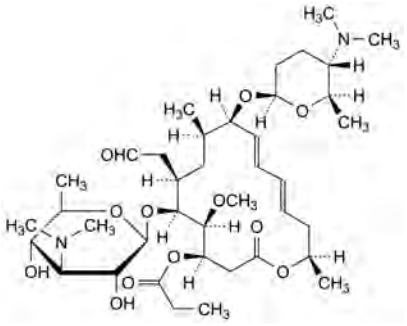

H. (4*R*,5*S*,6*S*,7*R*,9*R*,10*R*,11*E*,13*E*,16*R*)-6-[[3,6-dideoxy-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-5-methoxy-9,16-dimethyl-2-oxo-7-(2-oxoethyl)-10-[[2,3,4,6-tetradeoxy-4-(dimethylamino)-β-D-*erythro*-hexopyranosyl]oxy]oxacyclohexadeca-11,13-dien-4-yl propanoatate (neospiramycin III).

**01/2016:1766**

# SPIRAPRIL HYDROCHLORIDE MONOHYDRATE

## Spiraprili hydrochloridum monohydricum

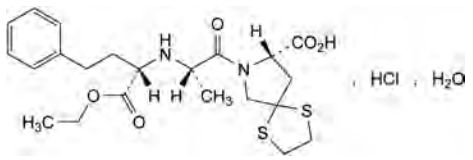

$C_{22}H_{31}ClN_2O_5S_2,H_2O$ $M_r$ 521.1

DEFINITION

(8*S*)-7-[(2*S*)-2-[[(1*S*)-1-(Ethoxycarbonyl)-3-phenylpropyl]-amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid hydrochloride monohydrate.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, fine crystalline powder.

*Solubility*: very slightly soluble in water, soluble in methanol, slightly soluble in acetonitrile, practically insoluble in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *spirapril hydrochloride monohydrate CRS*.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Specific optical rotation** (*2.2.7*): – 13.0 to – 11.0 (anhydrous substance).

Dissolve 0.200 g in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 6 mg of *spirapril for system suitability CRS* (containing impurities B and D) in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 70 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 4.5 g of *tetramethylammonium hydroxide R* in 900 mL of *water R*, add 100 mL of *acetonitrile R1* and adjust to pH 2.2 with *phosphoric acid R*;
- *mobile phase B*: dissolve 4.5 g of *tetramethylammonium hydroxide R* in 400 mL of *water R*, add 600 mL of *acetonitrile R1* and adjust to pH 2.2 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 90 | 10 |
| 4 - 14 | 90 → 10 | 10 → 90 |
| 14 - 20 | 10 | 90 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Relative retention* with reference to spirapril (retention time = about 10 min): impurity C = about 0.6; impurity B = about 0.7; impurity A = about 1.26; impurity D = about 1.38.

*System suitability*: reference solution (a):
- *resolution*: minimum 3.5 between the peaks due to impurity B and spirapril, and minimum 5.5 between the peaks due to spirapril and impurity D.

*Limits*:
- *impurity D*: not more than 0.8 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity B*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurities A, C*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): 3.0 per cent to 4.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix equal volumes of *acetonitrile R1* and *water R*.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *spirapril hydrochloride monohydrate CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 6.0 mg of *spirapril for system suitability CRS* (spirapril spiked with impurity B and impurity D) in a mixture of 2 volumes of *acetonitrile R* and 8 volumes of *water R* and dilute to 20 mL with the same mixture of solvents.

*Solution A.* Dissolve 4.5 g of *tetramethylammonium hydroxide R* in 900 mL of *water R*, adjust to pH 1.75 with *phosphoric acid R* and add 100 mL of *acetonitrile R1*.

*Solution B.* Dissolve 4.5 g of *tetramethylammonium hydroxide R* in 400 mL of *water R*, adjust to pH 1.75 with *phosphoric acid R* and add 600 mL of *acetonitrile R1*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 70 °C.

*Mobile phase*: solution A, solution B (45:55 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Retention time*: spirapril = 1.6 min to 2.9 min; impurity D = about 13 min. Adjust the proportion of solution B in the mobile phase if necessary.

*System suitability*: reference solution (b):

– *resolution*: minimum 15 between the peaks due to spirapril and impurity D.

Calculate the percentage content of $C_{22}H_{31}ClN_2O_5S_2$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *spirapril hydrochloride monohydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

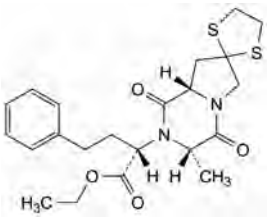

A. ethyl (2*S*)-2-[(3′*S*,8′a*S*)-3′-methyl-1′,4′-dioxohexahydrospiro[1,3-dithiolane-2,7′(6′*H*)-pyrrolo[1,2-*a*]pyrazin]-2′-yl]-4-phenylbutanoate,

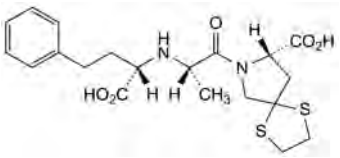

B. (8*S*)-7-[(2*S*)-2-[[(1*S*)-1-carboxy-3-phenylpropyl]amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylic acid (spiraprilat),

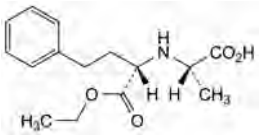

C. (2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoic acid,

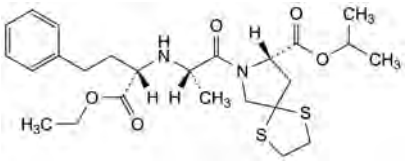

D. 1-methylethyl (8*S*)-7-[(2*S*)-2-[[(1*S*)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]propanoyl]-1,4-dithia-7-azaspiro[4.4]nonane-8-carboxylate.

**07/2019:0688**

# SPIRONOLACTONE

## Spironolactonum

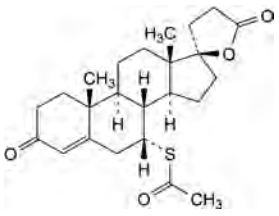

$C_{24}H_{32}O_4S$ $M_r$ 416.6

[52-01-7]

DEFINITION

*S*-[(2′*R*)-3,5′-Dioxo-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-7α-yl] ethanethioate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B, C*.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *spironolactone CRS*.

If the spectra obtained in the solid state shows differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 20 mg of *spironolactone CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *water R*, *cyclohexane R*, *ethyl acetate R* (1:24:75 *V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. To about 10 mg add 2 mL of a 50 per cent *V/V* solution of *sulfuric acid R* and shake. An orange solution with an intense yellowish-green fluorescence is produced. Heat the solution gently; the colour becomes deep red and hydrogen sulfide, which blackens *lead acetate paper R*, is evolved. Add the solution to 10 mL of *water R*; a greenish-yellow solution is produced, showing opalescence or a precipitate.

TESTS

**Specific optical rotation** (*2.2.7*): − 46 to − 41 (dried substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in 2.5 mL of *tetrahydrofuran R* and dilute to 25.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (b) to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve with the aid of ultrasound the contents of a vial of *spironolactone for system suitability CRS* (containing impurities A, C, D, E and I) in 1.0 mL of the solvent mixture.

*Reference solution (c)*. Dissolve 50.0 mg of *spironolactone CRS* in 2.5 mL of *tetrahydrofuran R* and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 5.0 mg of *canrenone CRS* (impurity F) in 2.5 mL of *tetrahydrofuran R* and dilute to 25.0 mL with the solvent mixture. Dilute 3.0 mL of this solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase*: *acetonitrile R*, *tetrahydrofuran R*, *methanol R1*, *water for chromatography R* (15:20:425:540 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (d).

*Run time*: 2.5 times the retention time of spironolactone.

*Identification of impurities*: use the chromatogram supplied with *spironolactone for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C, D, E and I; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity F.

*Relative retention* with reference to spironolactone (retention time = about 26 min): impurity A = about 0.95; impurity F = about 1.2; impurity C = about 1.5; impurity D = about 1.6; impurity E = about 1.7; impurity I = about 1.9.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to spironolactone.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 2.3;
- *impurity I*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurities E, F*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurities A, C*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *impurity D*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Free thiol compounds**. To 2.0 g add 20 mL of *water R*, shake for 1 min and filter. To 10 mL of the filtrate add 0.05 mL of *0.01 M iodine* and 0.1 mL of *starch solution R* and mix. A blue colour develops.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{24}H_{32}O_4S$ from the assigned content of *spironolactone CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, C, D, E, F, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, G, H.*

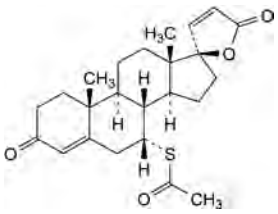

A. *S*-[(2′*R*)-3,5′-dioxo-5′*H*-spiro[androst-4-ene-17,2′-furan]-7α-yl] ethanethioate (Δ20-spironolactone),

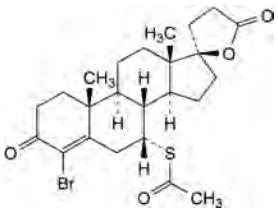

B. *S*-[(2′*R*)-4-bromo-3,5′-dioxo-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-7α-yl] ethanethioate (4-bromospironolactone),

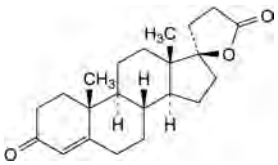

C. (2′*R*)-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-3,5′-dione (aldone),

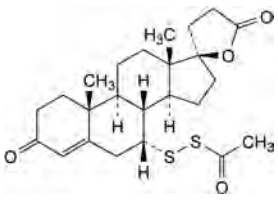

D. *S*-[(2′*R*)-3,5′-dioxo-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-7α-yl] ethane(dithioperoxate) (disulfanyl-spironolactone),

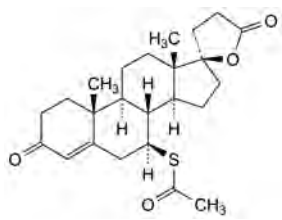

E. *S*-[(2′*R*)-3,5′-dioxo-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-7β-yl] ethanethioate (7β-spironolactone),

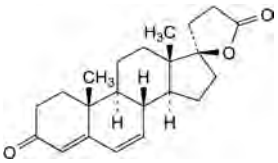

F. (2′*R*)-3′,4′-dihydro-5′*H*-spiro[androst-4,6-diene-17,2′-furan]-3,5′-dione (canrenone),

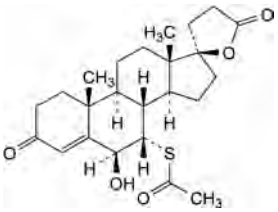

G. *S*-[(2′*R*)-6β-hydroxy-3,5′-dioxo-3′,4′-dihydro-5′*H*-spiro[androst-4-ene-17,2′-furan]-7α-yl] ethanethioate (6β-hydroxy-spironolactone),

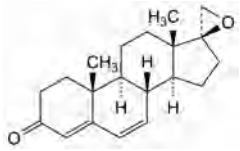

H. (2′*S*)-spiro[androst-4,6-diene-17,2′-oxiran]-3-one,

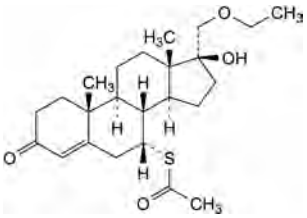

I. *S*-[17α-(ethoxymethyl)-17β-hydroxy-3-oxoandrost-4-en-7α-yl] ethanethioate.

**01/2020:1630**

# SQUALANE

## Squalanum

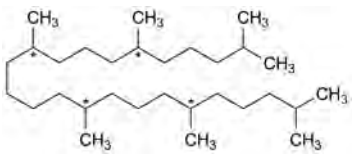

$C_{30}H_{62}$ $M_r$ 422.8

[111-01-3]

DEFINITION

(6*E*,10*E*,15*E*,19*E*)-2,6,10,15,19,23-Hexamethyltetracosane (perhydrosqualene). It may be of vegetable (unsaponifiable matter of olive oil), animal (shark liver oil) or synthetic origin.

*Content*: 96.0 per cent to 103.0 per cent.

CHARACTERS

*Appearance*: clear, colourless, oily liquid.

*Solubility*: practically insoluble in water, miscible with most fats and oils, freely soluble in acetone and in cyclohexane, practically insoluble in ethanol (96 per cent).

*Relative density*: about 0.815.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *squalane CRS*.

B. Refractive index (see Tests).

C. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).
The chromatogram obtained with squalane of animal origin does not show a peak due to cyclosqualane and does not show a peak with a relative retention of 1.1 with reference to squalane (Figure 1630.-1).
The chromatogram obtained with squalane of vegetable origin shows a peak due to cyclosqualane (Figure 1630.-2).
The chromatogram obtained with squalane of synthetic origin shows a peak with a relative retention of 1.1 with reference to squalane (Figure 1630.-3).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Refractive index** (*2.2.6*): 1.450 to 1.454.

**Acid value** (*2.5.1*): maximum 0.2.

**Iodine value** (*2.5.4, Method A*): maximum 4.0.

**Saponification value** (*2.5.6*): maximum 3.0.

**Total ash** (*2.4.16*): maximum 0.5 per cent, determined on 1.000 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* To 1.0 mL of *dimethylacetamide R* (DMA) add 100.0 mL of *heptane R*.

*Test solution.* Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 25.0 mL with the same solution.

*Reference solution (a).* Dissolve 0.100 g of *squalane CRS* in the internal standard solution and dilute to 25.0 mL with the same solution.

*Reference solution (b).* To 0.1 mL of *methyl erucate R* add 0.100 g of the substance to be examined, dissolve in the internal standard solution and dilute to 25.0 mL with the same solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.7 mL/min.

*Split ratio*: 1:12.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 39 | 60 - 290 |
| | 39 - 50 | 290 |
| Injection port | | 275 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to squalane (retention time = about 41 min): internal standard = about 0.2; methyl erucate = about 0.9; cyclosqualane = 1.05.

*System suitability*: reference solution (b):

- *resolution*: minimum 5 between the peaks due to methyl erucate and squalane.

Calculate the percentage content of $C_{30}H_{62}$ taking into account the assigned content of *squalane CRS*.

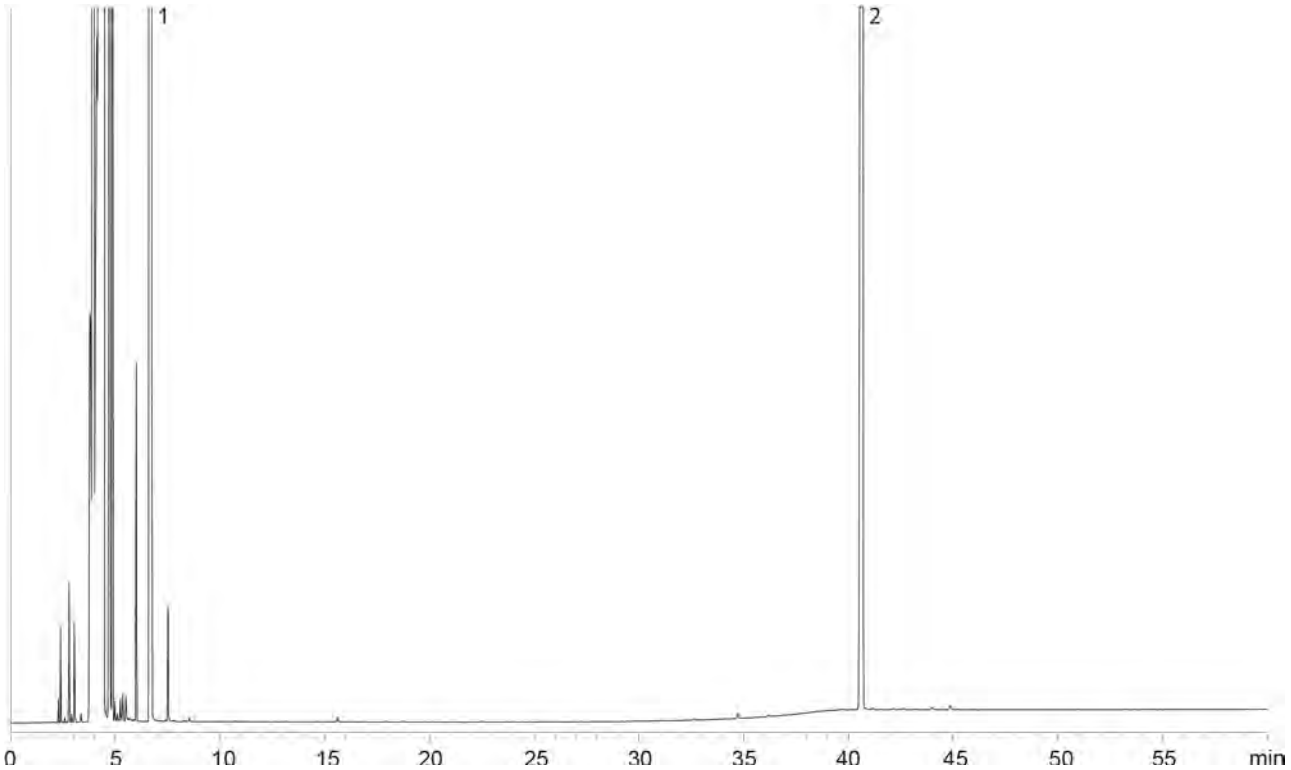


1. DMA 2. squalane

Figure 1630.-1. – *Chromatogram of squalane of animal origin*

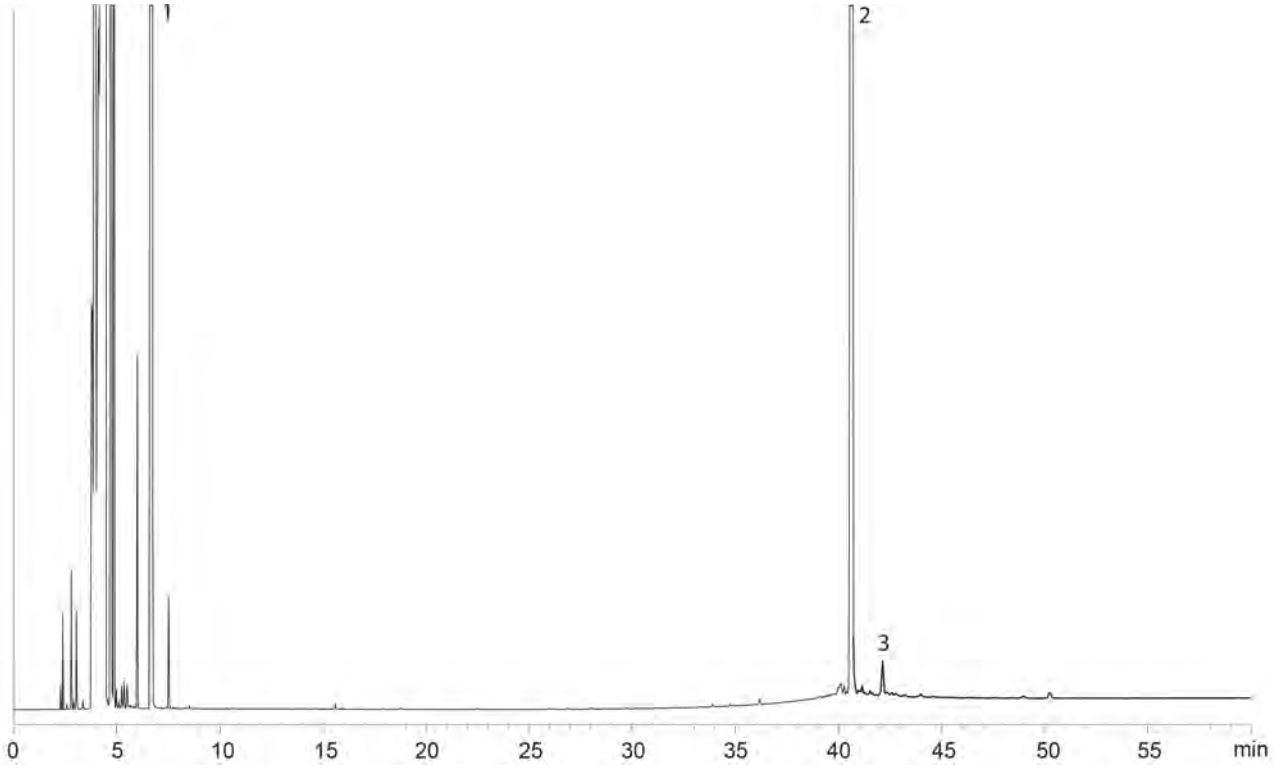


1. DMA 2. squalane 3. cyclosqualane

Figure 1630.-2. – *Chromatogram of squalane of vegetable origin*

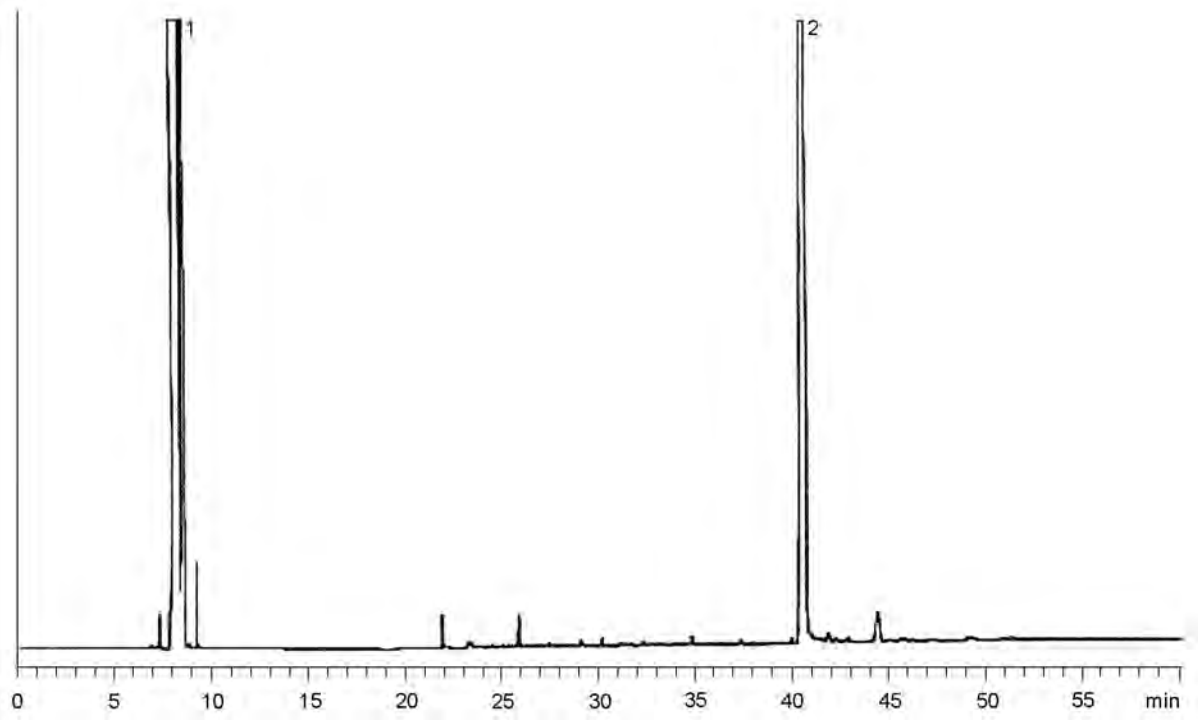


1. DMA 2. squalane

Figure 1630.-3. – *Chromatogram of squalane of synthetic origin*

LABELLING

The label states the origin of squalane (vegetable, animal or synthetic).

01/2020:2805

# SQUALENE

## Squalenum

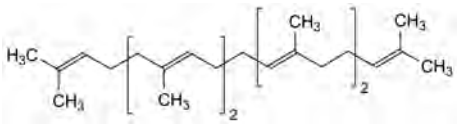

$C_{30}H_{50}$ $M_r$ 410.7

[111-02-4]

DEFINITION

(6*E*,10*E*,14*E*,18*E*)-2,6,10,15,19,23-Hexamethyltetracosa-2,6,10,14,18,22-hexaene. It may be of vegetable or animal origin.

*Content*: 97.0 per cent to 103.0 per cent (anhydrous substance).

This monograph applies to squalene used as an adjuvant in vaccines.

CHARACTERS

*Appearance*: clear, colourless or light yellow, oily liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone and in cyclohexane, soluble in ethanol (96 per cent).

*Relative density*: about 0.86.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *squalene CRS*.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Refractive index** (*2.2.6*): 1.491 to 1.499.

**Acid value** (*2.5.1*): maximum 1.0.

**Iodine value** (*2.5.4, Method B*): 350 to 450, determined on 0.06 g.

**Peroxide value** (*2.5.5, Method A*): maximum 5.0.

**Saponification value** (*2.5.6*): maximum 5.0.

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 2.00 g.

ASSAY

Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 0.100 g of the substance to be examined in *heptane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.100 g of *squalene CRS* in *heptane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 0.100 g of the substance to be examined and 0.100 g of *methyl lignocerate R* in *heptane R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.7 mL/min.

*Split ratio*: 1:12.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 12 | 290 |
| Injection port | | 275 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to squalene (retention time = about 8.1 min): methyl lignocerate = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to methyl lignocerate and squalene.

Calculate the percentage content of $C_{30}H_{50}$ taking into account the assigned content of *squalene CRS*.

STORAGE

In an airtight container, protected from light.

LABELLING

The label states the origin of squalene (vegetable or animal).

01/2017:1266

# STANNOUS CHLORIDE DIHYDRATE

## Stannosi chloridum dihydricum

$SnCl_2,2H_2O$ $M_r$ 225.6
[10025-69-1]

DEFINITION

*Content*: 98.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals, efflorescent in air.

*Solubility*: freely soluble in water (the solution becomes cloudy after standing or on dilution), freely soluble in ethanol (96 per cent). It dissolves in dilute hydrochloric acid.

IDENTIFICATION

A. To 1 mL of solution S1 (see Tests) add 5 mL of *water R* and 0.05 mL of *mercuric chloride solution R*. A blackish-grey precipitate is formed.

B. Dissolve 1.0 g in 3.0 mL of *water R*. Add 0.5 mL of *dilute sodium hydroxide solution R* to the cloudy solution; a yellowish flocculent precipitate is formed. Add 6.5 mL of *water R*. To 1.0 mL of the previously shaken suspension add 1.0 mL of *strong sodium hydroxide solution R*; the precipitate dissolves and the resulting solution is clear and colourless.

C. Dissolve 10 mg in 2 mL of *dilute nitric acid R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S1**. To 0.40 g add 1 mL of *dilute hydrochloric acid R* and dilute to 20 mL with *distilled water R*.

**Solution S2**. Dissolve 1.0 g in *dilute hydrochloric acid R* and dilute to 30 mL with the same acid. Heat to boiling. Add 30 mL of *thioacetamide solution R* and boil for 15 min (solution A). Take 5 mL, filter and heat the filtrate to boiling. Add 5 mL of *thioacetamide solution R* and boil for 15 min. If a precipitate is formed, add the remainder of solution A (solution A′) to the mixture. Add 10 mL of *thioacetamide solution R* and boil. Repeat the series of operations from "Take

5 mL" until a precipitate is no longer formed on addition of *thioacetamide solution R* to the filtrate obtained from the 5 mL of solution A (solution A′, solution A″, etc. respectively). If no precipitate is formed or if no more precipitate is formed combine the solution obtained with the remainder of solution A (solution A′, solution A″, etc. respectively), filter and wash the precipitate with 10 mL of *water R*. Heat the filtrate until the resulting vapour no longer turns a moistened piece of *lead acetate paper R* blackish-grey. Allow to cool and dilute to 50 mL with *water R*.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 10.0 g in *dilute hydrochloric acid R* and dilute to 20 mL with the same acid.

**Substances not precipitated by thioacetamide**: maximum 0.2 per cent.

Evaporate 25 mL of solution S2 to dryness and ignite at 600 ± 50 °C. The residue weighs a maximum of 1 mg.

**Sulfates** (*2.4.13*): maximum 500 ppm, determined on solution S1.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 5 mL of solution S2 to 10 mL with *water R*.

ASSAY

Dissolve 0.100 g in 50 mL of *water R*, freed from oxygen by purging with carbon dioxide or nitrogen for 15 min. Add 1.5 mL of *hydrochloric acid R1*, 5 g of *sodium potassium tartrate R*, 10 g of *sodium hydrogen carbonate R* and 1 mL of *starch solution R*. Titrate immediately with *0.05 M iodine*. Carry out a blank titration.

1 mL of *0.05 M iodine* is equivalent to 11.28 mg of $SnCl_2,2H_2O$.

STORAGE

In an airtight container.

**04/2012:1568**

# STANOZOLOL

## Stanozololum

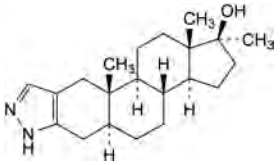

$C_{21}H_{32}N_2O$ $M_r$ 328.5
[10418-03-8]

DEFINITION

17-Methyl-2′*H*-5α-androst-2-eno[3,2-*c*]pyrazol-17β-ol.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, slightly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *stanozolol CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness at room temperature under an air-stream and record new spectra using the residues.

B. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Specific optical rotation** (*2.2.7*): + 37 to + 41 (dried substance).

Dissolve 60.0 mg in *methanol R* and dilute to 20.0 mL with the same solvent.

**Impurities A and B**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *methanol R1*, *methylene chloride R* (10:90 *V/V*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1.0 mL of the solvent mixture.

*Reference solution*. Dissolve 2 mg of *stanozolol CRS*, 2.0 mg of *stanozolol impurity A CRS* and 2.0 mg of *stanozolol impurity B CRS* in 1.0 mL of the solvent mixture. Dilute 0.1 mL of the solution to 2.0 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *ethyl acetate R*, *cyclohexane R* (2:48:50 *V/V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *vanillin reagent R* and heat at 120 °C.

*System suitability*: reference solution:

– the chromatogram shows 3 clearly separated spots, due to stanozolol, impurity A and impurity B, in order of increasing $R_F$ value.

*Limits*:

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent);
– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 15.0 mg of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 1 mg of *stanozolol CRS* and 1 mg of *stanozolol impurity B CRS* in *methanol R* and dilute to 20.0 mL with the same solvent.

*Reference solution (c)*. Dissolve 15.0 mg of *stanozolol CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 1 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*, *methanol R* (30:70 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 228 nm.

Monographs S

*Injection*: 25 µL of the test solution and reference solutions (a) and (b).

*Run time*: 3 times the retention time of stanozolol.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to stanozolol (retention time = about 12 min): impurity B = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to stanozolol and impurity B.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying at 105 °C at a pressure not exceeding 0.7 kPa.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.85 mg of $C_{21}H_{32}N_2O$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B.*

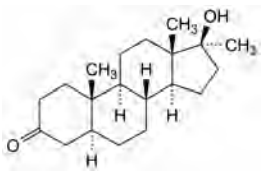

A. 17β-hydroxy-17-methyl-5α-androstan-3-one (mestanolone),

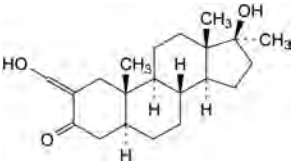

B. 17β-hydroxy-2-(hydroxymethylene)-17-methyl-5α-androstan-3-one (oxymetholone).

**01/2013:2165**
**corrected 10.0**

# STARCH, HYDROXYPROPYL

## Amylum hydroxypropylum

[9049-76-7]

DEFINITION

Hydroxypropyl starch is a partially substituted 2-hydroxypropylether of *Maize starch (0344)*, *Potato starch (0355)*, cassava starch, *Rice starch (0349)* or *Pea starch (2403)* chemically modified by etherification with the reagent propylene oxide. In addition, this starch may be partially hydrolysed using acids or enzymes to obtain 'thinned starch' with reduced viscosity.

*Content*:

– *hydroxypropyl groups*: 0.5 per cent to 7.0 per cent.

PRODUCTION

The production of hydroxypropyl starch shall be in compliance with the requirements of the European legislation for food additives.

Mixing of starches from different botanical sources prior to chemical modification is not allowed.

CHARACTERS

*Appearance*: white or slightly yellowish powder.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

IDENTIFICATION

A. Examined under a microscope, using not less than 20 × magnification and using a mixture of equal volumes of *glycerol R* and *water R*, it appears as follows according to the botanical source stated on the label.
   – *Maize-based hydroxypropyl starch*: it presents either angular polyhedral granules of irregular sizes with diameters of about 2-23 µm or rounded or spheroidal granules of irregular sizes with diameters of about 25-35 µm; the central hilum consists of a distinct cavity or 2-to-5-rayed cleft and there are no concentric striations; between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
   – *Potato-based hydroxypropyl starch*: it presents granules, either irregularly shaped, ovoid or pear-shaped, usually 30-100 µm in size but occasionally exceeding 100 µm, or rounded, 10-35 µm in size; there are occasional compound granules having 2-4 components; the ovoid and pear-shaped granules have an eccentric hilum and the rounded granules a centric or slightly eccentric hilum; all granules show clearly visible concentric striations; between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
   – *Cassava-based hydroxypropyl starch*: it presents spherical granules with one truncated side, typically 5-35 µm in diameter and having a circular or several-rayed central cleft; some granules may also be egg-shaped or cap-shaped; the hilum is centric, sometimes slightly fissured; between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
   – *Rice-based hydroxypropyl starch*: it presents polyhedral, simple granules 1-10 µm, mostly 4-6 µm, in size; these simple granules often gather in ellipsoidal, compound granules 50-100 µm in diameter; the granules have a poorly visible central hilum and there are no concentric striations; between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross intersecting at the hilum.
   – *Pea-based hydroxypropyl starch*: it presents a majority of large elliptical granules, 25-45 µm in size, sometimes irregular or reniform; it also presents a minority of small rounded granules, 5-8 µm in size; granules can present cracks or irregularities; sometimes, granules show barely visible concentric striations; exceptionally,

granules show a slit along the main axis; between orthogonally orientated polarising plates or prisms, the starch granules show a distinct black cross.

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A translucent or clear mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B add 0.05 mL of *iodine solution R1*. An orange-red or dark blue colour is produced, which disappears on heating.

D. Introduce 0.1 g into a 100 mL volumetric flask and add 12.5 mL of *dilute sulfuric acid R*. Place the flask in a water-bath and heat until the sample is dissolved. Cool and dilute to 100 mL with *water R*. Introduce 1 mL of this solution into a 25 mL graduated test-tube with glass stopper and, with the tube immersed in cold water, add dropwise 8 mL of *sulfuric acid R*. Mix well and place the tube in a water-bath for exactly 3 min. Immediately transfer the tube to an ice-bath until the solution is chilled. Add 0.6 mL of *ninhydrin solution R2*, carefully allowing the reagent to run down the walls of the test-tube. Immediately shake well, and place the tube in a water-bath at 25 °C for 100 min. Dilute to 25 mL with *sulfuric acid R* and mix by inverting the tube several times. Do not shake. A violet colour develops within 5 min.

TESTS

**pH** (*2.2.3*): 4.5 to 8.0.

Shake 5.0 g with 25.0 mL of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

**Foreign matter**. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, not more than traces of matter other than starch granules are present.

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*)

– For hydroxypropyl starch obtained from maize, potato, cassava or rice: maximum 20 ppm.

  Shake 1.0 g with 20 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test for iron.

– For hydroxypropyl starch obtained from pea: maximum 50 ppm.

  Shake 1.0 g with 50 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test for iron.

**Loss on drying** (*2.2.32*) determined on 1.000 g by drying in an oven at 130 °C for 90 min:

– maximum 15.0 per cent for hydroxypropyl starch obtained from maize, cassava, rice or pea;

– maximum 20.0 per cent for hydroxypropyl starch obtained from potato.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

Nuclear magnetic resonance spectrometry (*2.2.33*).

*Internal standard solution*. Disperse 50.0 mg of *3-trimethylsilyl-1-propanesulfonic acid sodium salt CRS* in about 5 g of *deuterium oxide R1*, weighed to the nearest 0.1 mg. Store in a sealed bottle.

*Test solution*. Disperse 20 g of the substance to be examined in 200.0 mL of *carbon dioxide-free water R* at room temperature. Agitate for 15 min and filter. Repeat the operation twice.

If problems of poor dispersibility or slow filtration are encountered, use cooled *carbon dioxide-free water R* for the washing operation. Dry the washed starch for at least 4 h in an oven *in vacuo* at 30 ± 5 °C. Determine the moisture content ($W$) on 5 g of this washed and dried sample using the test for loss on drying. Weigh 12.0 mg (dried substance) of the washed and dried sample in a 5 mm NMR tube. Add 0.1 mL of *deuterium chloride solution R* and 0.75 mL of *deuterium oxide R1*. Cap the tube, mix, and place it in a water-bath until a clear solution is obtained (3 min to 1 h maximum). When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube and weigh to the nearest 0.1 mg. Add 0.05 mL of the internal standard solution and weigh to the nearest 0.1 mg. Determine the mass of the internal standard solution introduced. Mix thoroughly.

*Apparatus*: FT-NMR spectrometer at minimum 300 MHz.

*Acquisition of $^1H$ NMR spectra*. The following parameters may be used:

– *sweep width*: 8 ppm (– 1.0 to + 7 ppm);

– *irradiation frequency offset*: none;

– *time domain*: 64 K at least;

– *pulse width*: 90°;

– *pulse delay*: 10 s;

– *dummy scans*: 0;

– *number of scans*: 8.

Use the $CH_3$ signal of the internal standard for shift referencing. The shift of the singlet is set to 0 ppm.

Record the FID signal.

Call the integration sub-routine after phase corrections and baseline correction between – 0.5 ppm and + 6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at + 1.2 ppm ($A_2$), and of the methyl groups at 0 ppm of the internal standard ($A_1$) without $^{13}C$-satellites.

*Results*: measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function; calculate the hydroxypropyl groups content as a percentage *m/m* (dried substance) using the following expression:

$$\frac{3A_2}{A_1} \times \frac{P}{100} \times \frac{W_1 \times m_1}{218} \times 59 \times \frac{100}{m} \times \frac{100}{100 - W}$$

3 = numerical value representing the 3 methyl groups in the internal standard;

$A_1$ = area of the methyl groups in the internal standard;

$A_2$ = area of the methyl groups of hydroxypropyl;

$P$ = percentage content of *3-trimethylsilyl-1-propanesulfonic acid sodium salt CRS*;

$W_1$ = mass fraction of the internal standard in the internal standard solution, in milligrams per gram;

$m_1$ = mass of the internal standard solution in the NMR tube, in grams;

218 = molar mass of the internal standard, in grams per mole;

59 = molar mass of the hydroxypropyl group, in grams per mole;

$m$ = mass of the washed and dried sample in the NMR tube, in milligrams;

$W$ = moisture content, as a percentage *m/m*.

LABELLING

The label states the botanical source of the starch and the type of modification.

07/2013:2645
corrected 10.0

# STARCH, HYDROXYPROPYL, PREGELATINISED

## Amylum hydroxypropylum pregelificatum

DEFINITION

Pregelatinised hydroxypropyl starch is prepared from *Starch, hydroxypropyl (2165)* by mechanical processing in the presence of water, with or without heat, to rupture all or part of the starch granules, and subsequent drying.

*Content*:

– *hydroxypropyl groups*: 0.5 per cent to 7.0 per cent.

PRODUCTION

The production of pregelatinised hydroxypropyl starch shall be in compliance with the requirements of the European legislation for food additives.

CHARACTERS

*Appearance*: white or slightly yellowish powder.

IDENTIFICATION

A. It swells in cold water.

B. Disperse 0.5 g in 2 mL of *water R*, without heating, and add 0.05 mL of *iodine solution R1*. A reddish-violet or greyish-blue colour is produced.

C. To 0.1 g add 12.5 mL of *dilute sulfuric acid R*. Heat in a water-bath until the sample is dissolved. Cool and dilute to 100 mL with *water R*. Introduce 1 mL of the solution into a 25 mL graduated test-tube with a ground-glass stopper and, with the tube immersed in cold water, add dropwise 8 mL of *sulfuric acid R*. Mix well and place the tube in a water-bath for exactly 3 min. Immediately transfer the tube to an ice-bath until the solution is chilled. Add 0.6 mL of *ninhydrin solution R2*, carefully allowing the reagent to run down the walls of the test-tube. Immediately shake well, and place the tube in a water-bath at 25 °C for 100 min. Dilute to 25 mL with *sulfuric acid R* and mix by inverting the tube several times. Do not shake. A violet colour develops within 5 min.

TESTS

**pH** (*2.2.3*): 4.5 to 8.0.

Progressively add 3.0 g to 100.0 mL of *carbon dioxide-free water R*, stirring continuously. Determine the pH when a homogeneous solution is obtained.

**Impurity A**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Mix 50.0 mg of *propane-1,3-diol R* with *anhydrous pyridine R* and dilute to 100.0 mL with the same solvent.

*Test solution.* To 0.200 g of the substance to be examined add 1.0 mL of the internal standard solution and 9.0 mL of *anhydrous pyridine R*. Heat under a reflux condenser for 20 min. Allow to cool. Transfer 1.0 mL of this solution to a 2 mL vial with a screw cap fitted with a septum. Add 0.1 mL of *chlorotrimethylsilane R* and 0.2 mL of *hexamethyldisilazane R*. Close and mix. Allow to stand for 15 min.

*Reference solution.* Mix 50.0 mg of *propane-1,3-diol R* and 50.0 mg of *propylene glycol CRS* (impurity A) with *anhydrous pyridine R* and dilute to 100.0 mL with the same solvent. Transfer 0.1 mL of the solution to a 2 mL vial with a screw cap fitted with a septum. Add 0.1 mL of *chlorotrimethylsilane R*, 0.2 mL of *hexamethyldisilazane R* and 0.9 mL of *anhydrous pyridine R*. Close and mix. Allow to stand for 15 min.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*NOTE: the column must be desorbed regularly. Conditions: temperature program of 70 °C to 300 °C at a rate of 7 °C/min. Maintain for 10 min at 300 °C.*

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3 mL/min.

*Split ratio*: 1:30.

*Temperature*:

– *column*: 70 °C;

– *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to the trimethylsilyl derivative of propane-1,3-diol (retention time = about 8.5 min): trimethylsilyl derivative of propylene glycol = about 0.7.

*System suitability*: reference solution:

– *resolution*: minimum 5.0 between the peaks due to the trimethylsilyl derivative of propylene glycol and the trimethylsilyl derivative of propane-1,3-diol.

*Calculation of percentage contents*: use the internal standard method.

*Limit*:

– *impurity A*: maximum 0.1 per cent.

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

Use a mixture of equal volumes of *methanol R* and *water R* as solvent.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*)

– For pregelatinised hydroxypropyl starch obtained from maize, potato, cassava or rice: maximum 20 ppm.

  Dissolve the residue obtained in the test for sulfated ash in 20 mL of *dilute hydrochloric acid R* and filter. The filtrate complies with the test for iron.

– For pregelatinised hydroxypropyl starch obtained from pea: maximum 50 ppm.

  Dissolve the residue obtained in the test for sulfated ash in 50 mL of *dilute hydrochloric acid R* and filter. The filtrate complies with the test for iron.

**Loss on drying** (*2.2.32*) determined on 1.000 g by drying in an oven at 130 °C for 90 min:

– maximum 15.0 per cent for pregelatinised hydroxypropyl starch obtained from maize, cassava, rice or pea;

– maximum 20.0 per cent for pregelatinised hydroxypropyl starch obtained from potato.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

Nuclear magnetic resonance spectrometry (*2.2.33*).

*Internal standard solution.* Disperse 50.0 mg of *3-trimethylsilyl-1-propanesulfonic acid sodium salt CRS* in about 5 g of *deuterium oxide R1*, weighed to the nearest 0.1 mg. Store in a sealed bottle.

*Test solution.* Dry 5.000 g of the substance to be examined at 130 °C for 90 min. Weigh 12.0 mg of the dried substance in a 5 mm NMR tube. Add 0.1 mL of *deuterium chloride solution R* and 0.75 mL of *deuterium oxide R1*. Cap the tube, mix, and place it in a water-bath until a clear solution is obtained (3 min to maximum 1 h). When a clear solution is obtained, allow to cool to room temperature. Dry the exterior of the tube and weigh to the nearest 0.1 mg. Add 0.05 mL of the internal standard solution and weigh to the nearest 0.1 mg. Determine the mass of the internal standard solution introduced. Mix thoroughly.

*Apparatus*: FT-NMR spectrometer operating at minimum 300 MHz.

*Acquisition of $^1$H NMR spectra.* The following parameters may be used:

- *sweep width*: 8 ppm (– 1.0 to + 7 ppm);
- *irradiation frequency offset*: none;
- *time domain*: at least 64 K;
- *pulse width*: 90°;
- *pulse delay*: 10 s;
- *dummy scans*: 0;
- *number of scans*: 8.

Use the $CH_3$ signal of the internal standard for shift referencing. The shift of the singlet is set to 0 ppm.

Record the FID signal.

Call the integration sub-routine after phase corrections and baseline correction between – 0.5 ppm and + 6 ppm.

Measure the peak areas of the doublet from the methyl groups of the hydroxypropyl function at + 1.2 ppm ($A_2$), and of the methyl groups at 0 ppm of the internal standard ($A_1$) without $^{13}$C-satellites.

*Results*: measure the signal coming from the 3 protons of the methyl group in the hydroxypropyl function; calculate the percentage content of hydroxypropyl groups using the following expression:

$$\frac{3A_2}{A_1} \times \frac{P}{100} \times \frac{W_1 \times m_1}{218} \times 59 \times \frac{100}{m}$$

| | | |
|---|---|---|
| 3 | = | numerical value representing the 3 methyl groups in the internal standard; |
| $A_1$ | = | area of the methyl groups in the internal standard; |
| $A_2$ | = | area of the methyl groups of hydroxypropyl; |
| $P$ | = | percentage content of *3-trimethylsilyl-1-propanesulfonic acid sodium salt CRS*; |
| $W_1$ | = | mass fraction of the internal standard in the internal standard solution, in milligrams per gram; |
| $m_1$ | = | mass of the internal standard solution in the NMR tube, in grams; |
| 218 | = | molar mass of the internal standard, in grams per mole; |
| 59 | = | molar mass of the hydroxypropyl group, in grams per mole; |
| $m$ | = | mass of the substance to be examined in the NMR tube, in milligrams. |

LABELLING

The label states the botanical source of the starch and the type of modification.

IMPURITIES

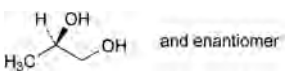


A. (2*RS*)-propane-1,2-diol (propylene glycol).

**01/2010:1267**
**corrected 9.0**

# STARCH, PREGELATINISED

## Amylum pregelificatum

DEFINITION

Pregelatinised starch is prepared from *Maize starch (0344)*, *Potato starch (0355)* or *Rice starch (0349)* by mechanical processing in the presence of water, with or without heat, to rupture all or part of the starch granules, and subsequent drying. It contains no added substances but it may be modified to render it compressible and to improve its flow characteristics.

CHARACTERS

*Appearance*: white or yellowish-white powder.

It swells in cold water.

IDENTIFICATION

A. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R* it presents irregular, translucent, white or yellowish-white flakes or pieces with an uneven surface. Under polarised light (between crossed nicol prisms), starch granules with a distinct black cross intersecting at the hilum may be seen.

B. Disperse 0.5 g in 2 mL of *water R* without heating and add 0.05 mL of *iodine solution R1*. A reddish-violet or blue colour is produced.

TESTS

**pH** (*2.2.3*): 4.5 to 7.0.

Progressively add 3.0 g to 100.0 mL of *carbon dioxide-free water R*, stirring continuously. Determine the pH when a homogeneous solution is obtained.

**Oxidising substances** (*2.5.30*). It complies with the test for oxidising substances. Use a mixture of equal volumes of *methanol R* and *water R* as solvent.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 20 ppm.

Dissolve the residue obtained in the test for sulfated ash in 20 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test.

**Foreign matter**. Examined under a microscope using a mixture of equal volumes of *glycerol R* and *water R*, not more than traces of matter other than starch granules are present.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

LABELLING

The label states the type of starch used as starting material.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be*

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*present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for pregelatinised starch used as filler, binder or disintegrant in tablets and in hard capsules.*

**Cold-water-soluble matter**. Transfer 100 mL of *water R* at 25 ± 1 °C into a beaker and add 1.000-3.000 g of the substance to be examined while stirring. Continue to stir for 10 min. Transfer 35 mL of the dispersion to a centrifuge tube and centrifuge at 3000 *g* for 15 min. Transfer 25 mL of the supernatant to a crucible that has previously been dried in an oven at 120 ± 2 °C for 4 h and weighed to the nearest 0.1 mg. Evaporate to dryness on a water-bath, then place the crucible in an oven at 120 ± 2 °C for 4 h. Allow to cool in a desiccator. Weigh the crucible to the nearest 0.1 mg again.

Determine the percentage of cold-water-soluble matter using the following expression:

$$\frac{(B - A) \times \frac{100}{25} \times 100}{S \times \frac{100 - C}{100}}$$

*A* = initial crucible mass, in grams;

*B* = final crucible mass, in grams;

*C* = loss on drying, in per cent;

*S* = sample mass, in grams.

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**01/2019:1785**
**corrected 10.0**

# STARCHES, HYDROXYETHYL

## Amyla hydroxyethyla

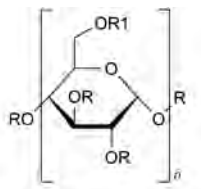


R = -[$CH_2CH_2O$]$_{n'}$H (*n'* = 0, 1, 2...)

R1 = -[$CH_2CH_2O$]$_{n''}$H (*n''* = 0 or 1) or glucose

[$C_6H_{10}O_5(C_2H_4O)_x$]$_n$ with *x* = molar substitution
[9005-27-0]

DEFINITION

Hydroxyethyl starches are partially substituted poly(2-hydroxyethyl)ethers of waxy maize starch or potato starch, which primarily consist of amylopectine. The type of hydroxyethyl starch is defined by 2 numbers: the mean molecular weight (*Mw*) and the number of hydroxyethyl groups per anhydroglucose unit expressed as the molar substitution (*MS*). Hydroxyethyl starch is also characterised by the number of hydroxyethyl groups located at the C2 group over the number of hydroxyethyl groups located at C6, expressed as the C2/C6 ratio. The parameters *Mw*, *MS* and C2/C6 ratio are determined by the reaction conditions of the production.

PRODUCTION

Hydroxyethyl starches are produced from waxy maize starch or potato starch by acidic hydrolysis and reaction with ethylene oxide and purified by ultrafiltration.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in dimethyl sulfoxide, practically insoluble in anhydrous ethanol.

Hydroxyethyl starches are hygroscopic until they reach a water content of about 12 per cent to 15 per cent.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *medium Mw hydroxyethyl starch CRS.*
*Results*: the spectrum obtained shows the same absorption bands as the spectrum obtained with *medium Mw hydroxyethyl starch CRS*. Due to the difference in the substitution of the substance, the intensity of some absorption bands can vary.

B. To 5 mL of solution S (see Tests), add 0.1 mL of *0.05 M iodine*. A reddish-brown or blue-violet colour appears.

C. Molecular weight (see Tests).

TESTS

**Solution S**. Dissolve 5.0 g of the substance to be examined (dried substance) in *carbon dioxide-free water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*).

**pH** (*2.2.3*): 4.5 to 7.0.
To 25 mL of solution S, add 0.2 mL of a saturated solution of *potassium chloride R*.

**Absorbance** (*2.2.25*): maximum 0.025, determined at 400 nm on solution S filtered through a 0.2 µm filter.

**Molecular weight (*Mw*) and molecular weight distribution**. Size-exclusion chromatography (*2.2.30*).

*Buffer solution*. Dissolve 54.34 g of *sodium acetate R* in *water R*, add 100.0 mL of *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

*Test stock solution*. Dissolve 2.0 g of the substance to be examined (dried substance) in *water R* and dilute to 50 mL with the same solvent. Add 10.0 mL of the buffer solution and dilute to 100.0 mL with *water R*.

*Reference solution (a)*. To prepare reference solution (a):
- if the nominal *Mw* of the substance to be examined is below 300 000, use *medium Mw hydroxyethyl starch CRS*;
- if the nominal *Mw* of the substance to be examined is above 300 000, use *high Mw hydroxyethyl starch CRS*.

Dissolve 0.4 g of *medium Mw hydroxyethyl starch CRS* or *high Mw hydroxyethyl starch CRS* in 10 mL of *water R*. Add 2.0 mL of the buffer solution and dilute to 20.0 mL with *water R*.

*Reference solution (b)*. Dilute 10.0 mL of reference solution (a) to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dilute 10.0 mL of reference solution (b) to 20.0 mL with the mobile phase.

*Reference solution (d)*. Dilute 10.0 mL of reference solution (c) to 20.0 mL with the mobile phase.

*Column*:
- *stationary phase*: *hydroxylated polymethacrylate gel R*;

– 4 columns to be connected in series:

| Length in m | Internal diameter in mm | Particle size in µm | Pore size in nm |
|---|---|---|---|
| 0.30 | 7.5 | 17 | > 100 |
| 0.30 | 7.5 | 17 | 100 |
| 0.30 | 7.5 | 10 | 20 |
| 0.30 | 7.5 | 10 | 12.5 |

*Mobile phase*. Dilute 100.0 mL of the buffer solution to 1000 mL with *water for chromatography R*.

*Flow rate*: 0.5-1.0 mL/min.

*Detection*: multiple-angle light scattering (MALS) detector and refractometer maintained at a constant temperature, connected in series.

*Injection volume*: 50 µL.

Determine the suitable working solution as follows: inject reference solutions (a) and (b), the mean *Mw* determined with reference solution (b) does not deviate by more than 3 per cent from the mean *Mw* determined with reference solution (a). If the deviation meets the requirement, use reference solution (a) to check the system suitability criterion.

If the deviation is higher, inject reference solution (c) and determine the mean *Mw*. The mean *Mw* determined with reference solution (c) does not deviate by more than 3 per cent from the mean *Mw* determined with reference solution (b). If the deviation meets the requirement, use reference solution (b) to check the system suitability criterion.

If the deviation is higher, inject reference solution (d) and determine the mean *Mw*. The mean *Mw* determined with reference solution (d) does not deviate by more than 3 per cent from the mean *Mw* determined with reference solution (c). If the deviation meets the requirement, use reference solution (c) to check the system suitability criterion.

*System suitability*:

– *mean Mw*: within 5 per cent of the value assigned to the *medium Mw hydroxyethyl starch CRS* or *high Mw hydroxyethyl starch CRS*.

If necessary, dilute the test stock solution in order to have the same concentration as that of the reference solution used to check the system suitability.

*Results*: use a suitable integrator to determine the mean *Mw* and the *Mw* of the lowest and highest 10 per cent mass fraction.

| Low *Mw* 2000 - 100 000 | Medium *Mw* 100 000 - 300 000 | High *Mw* 300 000 - 900 000 |
|---|---|---|
| Determined *Mw* = nominal *Mw* ± 15 per cent | | |
| *Mw* at 10 per cent lowest fraction > 10 per cent of nominal *Mw* | *Mw* at 10 per cent lowest fraction > 15 000 | *Mw* at 10 per cent lowest fraction > 15 000 |
| *Mw* at 10 per cent highest fraction < 300 per cent of nominal *Mw* | *Mw* at 10 per cent highest fraction < 300 per cent of nominal *Mw* | *Mw* at 10 per cent highest fraction < 500 per cent of nominal *Mw* |

**C2/C6 ratio**. Gas chromatography (*2.2.28*).

*Solution A*. Mix equal volumes of *dilute sulfuric acid R* and *water R*.

*Test solution*. Introduce 0.18 g of the substance to be examined into a 5 mL vial. Add 3.0 mL of solution A, cap, seal the vial and shake until dissolution. Heat the vials for 4 h in a heating block already preheated to 100 °C, shaking them from time to time. Cool to room temperature. Open the vial and carefully add 0.9 g of *barium carbonate R*. Shake carefully and then centrifuge at about 9000 *g* for about 15 min. Test the clear supernatant for neutral pH with pH paper. If the solution is still acid, add more *barium carbonate R* in portions of 0.2 g until the solution is neutral. Filter the clear supernatant (pore size 0.45 µm). Introduce 0.5 mL of the filtrate into a autosampler vial and evaporate to dryness at 40 °C (several hours are usually needed). Take up the residue with 0.50 mL of *pyridine R*, 0.25 mL of *N,O-bis(trimethylsilyl)acetamide R* and 25 µL of *chlorotrimethylsilane R*. Seal the vial and heat to 40 °C for 1 h shaking from time to time. Cool to room temperature. Place the vial into the autosampler and perform 3 injections from each vial. Prepare in duplicate.

*Reference solution*. Prepare as prescribed for the test solution but using *medium Mw hydroxyethyl starch CRS* instead of the substance to be examined.

*Column*:

– *size*: *l* = 15 m, Ø = 0.32 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R* at a constant pressure of 69 kPa.

*Split ratio*: 1:20.

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 150 |
| | 1 - 25 | 150 → 270 |
| | 25 - 28 | 270 |
| Injection port | | 250 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Identification of peaks*: use the chromatogram supplied with *medium Mw hydroxyethyl starch CRS* and the chromatogram obtained with the reference solution to identify the peaks due to derivatised product 1, derivatised product 2, derivatised product 3, 2-*O*-hydroxyethyl-α-D-glucose, 6-*O*-hydroxyethyl-α-D-glucose, 2-*O*-hydroxyethyl-β-D-glucose and 6-*O*-hydroxyethyl-β-D-glucose.

*System suitability*: reference solution:

– *resolution*: minimum 1.5 between the peaks due to 2-*O*-hydroxyethyl-β-D-glucose and 6-*O*-hydroxyethyl-β-D-glucose;
– *symmetry factor*: 0.6 to 1.5 for the peak due to derivatised product 1;
– *repeatability*: maximum relative standard deviation of 5.0 per cent for derivatised product 1 after 3 injections.

Calculate the C2/C6 ratio using the following expression:

$$\frac{A_1 + A_2 + A_3 + A_4 + A_5}{A_6 + A_7}$$

$A_1$ = area of the peak due to derivatised product 1;

$A_2$ = area of the peak due to derivatised product 2;

$A_3$ = area of the peak due to derivatised product 3;

$A_4$ = area of the peak due to 2-*O*-hydroxyethyl-α-D-glucose;

$A_5$ = area of the peak due to 2-*O*-hydroxyethyl-β-D-glucose;

$A_6$ = area of the peak due to 6-*O*-hydroxyethyl-α-D-glucose;

$A_7$ = area of the peak due to 6-*O*-hydroxyethyl-β-D-glucose.

Calculate the mean C2/C6 ratio from the values obtained with the 2 test solutions.

The test is not valid unless the difference of the 2 values is not more than 5 per cent.

*Limit*: within 20.0 per cent of the nominal value.

**Molar substitution (*MS*)**. Gas chromatography (*2.2.28*).

The content of hydroxyethyl groups is determined after hydrolysis with hydriodic acid as iodoethane.

*Internal standard solution*. Dilute 1.0 mL of *toluene R* to 200.0 mL with *xylene R*.

*Test solution*. Introduce 50.0 mg of the substance to be examined and about 0.10-0.15 g of *adipic acid R* in a 5 mL vial. Add 1.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R*. Tightly seal and cap the vial with a septum and an aluminium, centre tear-off seal. Prepare the test solution 5 times.

*Reference solutions*. In each of 7 vials of 5 mL, introduce about 0.10-0.15 g of *adipic acid R*. To each vial add 1.0 mL of the internal standard solution and 2.0 mL of *hydriodic acid R*. Tightly seal and cap the vials with a septum and an aluminium, centre tear-off seal. Weigh the vials with an accuracy of 0.01 mg. Introduce respectively 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 60 mg and 70 mg of *iodoethane R* with a 100 µL syringe piercing the septa carefully. Weigh the vials again with an accuracy of 0.01 mg and calculate the exact amount of *iodoethane R* added.

Determine the mass of the vials to the nearest 1 mg. Place the vials for 10 h into a heating block already preheated to 150 °C. After cooling to room temperature, determine the mass of each vial to the nearest 1 mg. Disregard any vial with a loss in mass of more than 5 mg. From 4 vials of the test solution and 5 of the reference solutions, take-up 100 µL of the upper layer. Introduce in an autosampler vial and dilute with 1.0 mL of *xylene R*. Seal immediately the vials and shortly shake.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 8 mL/min.

*Split ratio*: 1:20.

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 50 |
| | 4 - 16 | 50 → 230 |
| | 16 - 20 | 230 |
| Injection port | | 200 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection volume*: 1 µL; inject each solution twice.

*Relative retention* with reference to toluene (retention time = about 7.5 min): iodoethane = about 0.5.

*System suitability*: reference solutions:

- *resolution*: minimum 1.5 between the peaks due to iodoethane and toluene;
- calculate the ratio of the area of the peak due to *iodoethane R* to the area of the peak due to the internal standard for each chromatogram. Calculate the linear regression curve plotting the ratios calculated for the reference solutions against the quantity of *iodoethane R* added (in milligrams). The coefficient of determination $R^2$ is not less than 0.990.

*Results*: calculate the quantity (*T*) of iodoethane in milligrams present in the test solution using the following expression:

$$\frac{A - B}{M}$$

$A$ = ratio of the area of the peak due to iodoethane to the area of the peak due to the internal standard in the chromatogram obtained with the test solution;

$B$ = y-intercept of the curve;

$M$ = slope of the curve.

Then calculate the percentage content of ethylene oxide (*C*) using the following expression:

$$\frac{44.05 \times T \times 100}{155.97 \times m}$$

$m$ = mass of the substance to be examined, in milligrams;

44.05 = molecular mass of ethylene oxide;

155.97 = molecular mass of iodoethane.

Then calculate the *MS* using the following expression:

$$\frac{C \times 162.14}{(100 - C) \times 44.05}$$

162.14 = molecular mass of anhydroglucose;

44.05 = molecular mass of ethylene oxide.

Calculate the mean *MS* from the values obtained with the 4 test solutions.

*Limit*: 0.05 to 2.4, and within 8.0 per cent of the nominal value.

**Ethylene glycol**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 1.0 g of the substance to be examined (dried substance) in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution*. Dissolve 0.800 g of *ethylene glycol R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 200.0 mL with *water R*. Dilute 2.0 mL of this solution to 200.0 mL with *water R*.

*Precolumn*:

- *size*: *l* = 0.01 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography compatible with 100 per cent aqueous mobile phases R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Post-column solution*. Dilute 750 mL of *2 M sodium hydroxide R* to 1000 mL with *water for chromatography R*.

*Flow rate of post-column solution*: 0.2 mL/min.

*Detection*: pulsed amperometric detector.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of ethylene glycol.

*Retention time*: ethylene glycol = about 4 min.

*System suitability*: reference solution:

- *signal-to-noise ratio*: minimum 10 for the principal peak;
- *repeatability*: maximum relative standard deviation of 10.0 per cent determined on 6 injections.

After a maximum of 8 sample injections, wash the column using the following program.

*Rinsing solution*: *acetonitrile for chromatography R*, *water for chromatography R* (20:80 *V/V*).

| Time (min) | Mobile phase (per cent *V/V*) | Rinsing solution (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 75 | 25 |
| 15 - 20 | 75 → 0 | 25 → 100 |
| 20 - 25 | 0 | 100 |
| 25 - 30 | 0 → 100 | 100 → 0 |
| 30 - 100 | 100 | 0 |

*Limit*:

– *ethylene glycol*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (40 ppm).

**2-Chloroethanol**. Gas chromatography (*2.2.28*).

*Solvent mixture*: *methanol R*, *acetonitrile R* (25:75 *V/V*).

*Internal standard solution.* Dissolve 0.250 g of *2,6-dimethylaniline R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 0.5 mL of the solution to 50.0 mL with the solvent mixture.

*Test solution.* Introduce 1.0 g of the substance to be examined into a 20 mL vial. Add 10.0 mL of the solvent mixture. Close tightly. Treat in an ultrasonic bath for 3.5 h. Allow to cool to room temperature. To 1.0 mL of this solution add 0.8 mL of the internal standard solution.

*Reference solution.* Dissolve 0.250 g of *2-chloroethanol R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture. To 1.0 mL of this solution add 0.8 mL of the internal standard solution.

*Precolumn*:

– *material*: fused silica;
– *size*: *l* = 10 m, Ø = 0.53 mm;
– *stationary phase*: *polar-deactivated macrogol R*.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

*Carrier gas*: *hydrogen for chromatography R*.

*Flow rate*: 2.9 mL/min.

*Split program*:

| Time (min) | Split state | Split ratio |
|---|---|---|
| initial | on | 1:20 |
| 0.01 | off | 1:20 |
| 0.50 | on | 1:20 |

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 4 | 45 |
| | 4 - 23.5 | 45 → 240 |
| | 23.5 - 28.5 | 240 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution:

– *signal-to-noise ratio*: minimum 10 for the peak due to 2-chloroethanol;
– *repeatability*: maximum relative standard deviation of 10.0 per cent after 6 injections.

*Limit*:

– *2-chloroethanol*: calculate the ratio (*R*) of the area of the peak due to 2-chloroethanol to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; calculate the ratio of the area of the peak due to 2-chloroethanol to the area of the peak due to the internal standard from the chromatogram obtained with the test solution: this ratio is not greater than *R* (5 ppm).

**Ethylene oxide**. Head-space gas chromatography (*2.2.28*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in 1.0 mL of *water R*. Close the vial tightly. Prepare in duplicate.

*Reference stock solution.* Introduce 80 mL of *water R* in a 100 mL volumetric flask. Cool at about 4 °C for at least 30 min. Place the flask on an analytical balance and slowly introduce 1.0 g of *ethylene oxide R*. Determine the precise quantity of ethylene oxide by differential weighing. Dilute to 100.0 mL with *water R*. Store in the refrigerator and use within 4 weeks.

*Reference solution (a).* Dilute 1.0 mL of the reference stock solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Use within 24 h.

*Reference solution (b).* Dissolve 1.0 g of the substance to be examined in 1.0 mL of reference solution (a). Close the vial tightly. Prepare in duplicate.

*Column*:

– *material*: quartz;
– *size*: *l* = 30 m, Ø = 0.32 mm;
– *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.5 µm).

*Carrier gas*: *helium for chromatography R* at a pressure of 110.3 kPa.

*Split ratio*: 1:35.

*Static head-space conditions which may be used*:

– *equilibration temperature*: 80 °C;
– *equilibration time*: 40 min;
– *transfer-line temperature*: 150 °C;
– *pressurisation time*: 2.0 min;
– *injection time*: 3 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 40 |
| | 20 - 30 | 40 → 240 |
| | 30 - 40 | 240 |
| Injection port | | 140 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: inject a suitable volume of the gaseous phase of the test solution and reference solution (b).

*System suitability*:

– *signal-to-noise ratio*: minimum 10 for the peak due to ethylene oxide in the chromatogram obtained with reference solution (b).

*Limit*:

– *ethylene oxide*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (1 ppm).

**Sodium chloride**: maximum 0.1 per cent.

*Test solution.* In a 250 mL conical flask, dissolve 10.0 g of the substance to be examined in 100 mL of *water R*. Add 2 mL of *dilute nitric acid R* and 5.0 mL of a 9 g/L solution of *sodium chloride R*.

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*Reference solution.* In a 250 mL conical flask, dilute 5.0 mL of a 9 g/L solution of *sodium chloride R* with 100 mL of *water R*. Add 2 mL of *dilute nitric acid R*.

Carry out a potentiometric titration (*2.2.20*) with *0.1 M silver nitrate*. Calculate the percentage content of sodium chloride using the following expression:

$$\frac{(n_1 - n_2) \times 5.844 \times 100}{m}$$

$n_1$ = volume of *0.1 M silver nitrate* used for the test solution, in millilitres;

$n_2$ = volume of *0.1 M silver nitrate* used for the reference solution, in millilitres;

$m$ = mass of the substance to be examined in the test solution, in milligrams.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying at 105 °C.

**Bacterial endotoxins** (*2.6.14*): less than 2.5 IU/g.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

LABELLING

The label states the mean molecular weight, molar substitution and C2/C6 ratio (nominal values).

**01/2015:2130**

# STAVUDINE

## Stavudinum

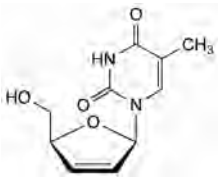

$C_{10}H_{12}N_2O_4$ $M_r$ 224.2

[3056-17-5]

DEFINITION

1-(2,3-Dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, sparingly soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (*2.2.7*): – 45.9 to – 39.5 (anhydrous substance).

Dissolve 0.100 g in *water R* and dilute to 10.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *stavudine CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C until use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dilute 0.5 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b).* Dilute 20.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 5 mg of *stavudine for system suitability CRS* (containing impurities A, B, C, E and G) in *water R* and dilute to 10.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 35 volumes of *acetonitrile for chromatography R* and 965 volumes of a 0.77 g/L solution of *ammonium acetate R*;
- *mobile phase B*: mix 250 volumes of *acetonitrile for chromatography R* and 750 volumes of a 0.77 g/L solution of *ammonium acetate R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 20 | 100 → 0 | 0 → 100 |
| 20 - 30 | 0 | 100 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *stavudine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, E and G.

*Relative retention* with reference to stavudine (retention time = about 10 min): impurity A = about 0.3; impurity B = about 0.50; impurity C = about 0.53; impurity E = about 1.1; impurity G = about 1.9.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B; minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to stavudine.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity A by 0.7;
- for impurity A, use the concentration of stavudine in reference solution (a);
- for impurities other than A, use the concentration of stavudine in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *impurity G*: maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Impurity I**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C until use.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 2 mg of *stavudine impurity I CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *acetonitrile for chromatography R* and 70 volumes of a 1.15 g/L solution of *ammonium dihydrogen phosphate R* previously adjusted to pH 6.8 with *triethylamine R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 266 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of stavudine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity I.

*Relative retention* with reference to stavudine (retention time = about 3 min): impurity I = about 6.0.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 40 for the principal peak.

*Calculation of percentage content*:

- *correction factor*: multiply the peak area of impurity I by 1.7;
- for impurity I, use the concentration of stavudine in reference solution (b).

*Limit*:

- *impurity I*: maximum 0.15 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C until use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 10.0 mg of *stavudine CRS* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (b).* Dissolve 5 mg of *thymine R* (impurity A) and 7.5 mg of *thymidine R* (impurity C) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.033 m, Ø = 4.0 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: mix 5 volumes of *acetonitrile for chromatography R* and 95 volumes of a 0.77 g/L solution of *ammonium acetate R*.

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 25 µL.

*Run time*: twice the retention time of stavudine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C.

*Relative retention* with reference to stavudine (retention time = about 4 min): impurity A = about 0.4; impurity C = about 0.6.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to impurities A and C in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.6 for the peak due to stavudine in the chromatogram obtained with reference solution (a).

Calculate the percentage content of $C_{10}H_{12}N_2O_4$ using the chromatograms obtained with the test solution and reference solution (a) and taking into account the assigned content of *stavudine CRS*.

STORAGE

Protected from light and humidity.

IMPURITIES

*Specified impurities: A, G, I.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, H.*

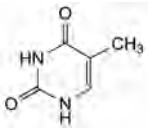

A. 5-methylpyrimidine-2,4(1*H*,3*H*)-dione (thymine),

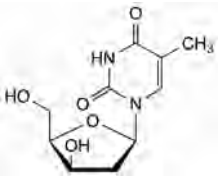

B. 1-(2-deoxy-β-D-*threo*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (3′-epithymidine),

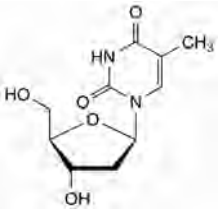

C. 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (thymidine),

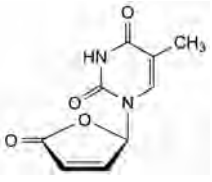

D. 1-[(2*R*)-5-oxo-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

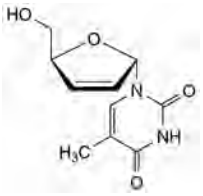

E. 1-(2,3-dideoxy-α-D-*glycero*-pent-2-enofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (stavudine anomer α),

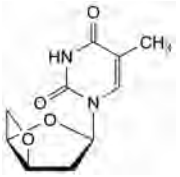

F. 1-(3,5-anhydro-2-deoxy-β-D-*threo*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

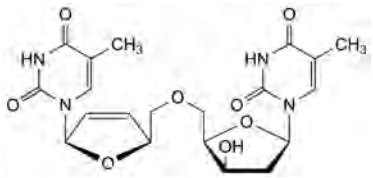

G. 1-[2-deoxy-5-*O*-[[(2*S*,5*R*)-5-[5-methyl-2,4-dioxo-3,4-dihydropyrimidine-1(2*H*)-yl]-2,5-dihydrofuran-2-yl]methyl]-β-D-*threo*-pentofuranosyl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

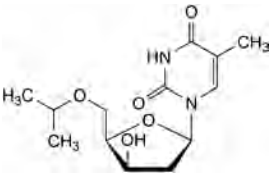

H. 1-[2-deoxy-5-*O*-(1-methylethyl)-β-D-*threo*-pentofuranosyl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

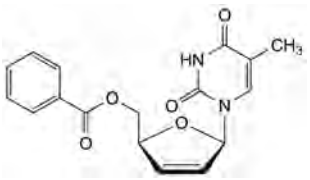

I. 1-(5-*O*-benzoyl-2,3-dideoxy-β-D-*glycero*-pent-2-enofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione.

**04/2015:1474**
**corrected 9.0**

# STEARIC ACID[(6)]

## Acidum stearicum

DEFINITION

Mixture consisting mainly of stearic (octadecanoic) acid ($C_{18}H_{36}O_2$; $M_r$ 284.5) and palmitic (hexadecanoic) acid ($C_{16}H_{32}O_2$; $M_r$ 256.4) obtained from fats or oils of vegetable or animal origin.

*Content*:

| | |
|---|---|
| Stearic acid 50 | *Stearic acid*: 40.0 per cent to 60.0 per cent.<br>*Sum of the contents of stearic and palmitic acids*: minimum 90.0 per cent. |
| Stearic acid 70 | *Stearic acid*: 60.0 per cent to 80.0 per cent.<br>*Sum of the contents of stearic and palmitic acids*: minimum 90.0 per cent. |
| Stearic acid 95 | *Stearic acid*: minimum 90.0 per cent.<br>*Sum of the contents of stearic and palmitic acids*: minimum 96.0 per cent. |

♦CHARACTERS

*Appearance*: white or almost white, waxy, flaky crystals, white or almost white hard masses, or white or yellowish-white powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in light petroleum (bp: 50-70 °C).♦

IDENTIFICATION

A. Freezing point (see Tests).

B. Acid value (*2.5.1*): 194 to 212, determined on 0.5 g.

C. Examine the chromatograms obtained in the assay.
*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time to those in the chromatogram obtained with the reference solution.

TESTS

◊**Appearance**. Heat the substance to be examined to about 75 °C. The resulting liquid is not more intensely coloured than reference solution $Y_7$ or $BY_7$ (*2.2.2, Method I*).◊

**Acidity**. Melt 5.0 g, shake for 2 min with 10 mL of hot *carbon dioxide-free water R*, cool slowly and filter. To the filtrate add 0.05 mL of *methyl orange solution R*. No red colour develops.

**Iodine value** (*2.5.4*). See Table 1474.-1.

**Freezing point** (*2.2.18*). See Table 1474.-1.

Table 1474.-1.

| Type | Iodine value | Freezing point (°C) |
|---|---|---|
| Stearic acid 50 | maximum 4.0 | 53 - 59 |
| Stearic acid 70 | maximum 4.0 | 57 - 64 |
| Stearic acid 95 | maximum 1.5 | 64 - 69 |

♦**Nickel** (*2.4.31*): maximum 1 ppm.♦

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. In a conical flask fitted with a reflux condenser, dissolve 0.100 g of the substance to be examined in 5 mL of *boron trifluoride-methanol solution R*. Boil under reflux for 10 min. Add 4.0 mL of *heptane R* through the condenser and boil again under reflux for 10 min. Allow to cool. Add 20 mL of a saturated solution of *sodium chloride R*. Shake and allow the layers to separate. Remove about 2 mL of the organic layer and dry it over 0.2 g of *anhydrous sodium sulfate R*. Dilute 1.0 mL of this solution to 10.0 mL with *heptane R*.

*Reference solution*. Prepare the reference solution in the same manner as the test solution using 50 mg of *palmitic acid CRS* and 50 mg of *stearic acid CRS* instead of the substance to be examined.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

(6) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 70 |
| | 2 - 36 | 70 → 240 |
| | 36 - 41 | 240 |
| Injection port | | 220 |
| Detector | | 260 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to methyl stearate: methyl palmitate = about 0.9.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to methyl palmitate and methyl stearate;
- *repeatability*: maximum relative standard deviation of 3.0 per cent for the areas of the peaks due to methyl palmitate and methyl stearate, determined on 6 injections; maximum 1.0 per cent for the ratio of the areas of the peaks due to methyl palmitate to the areas of the peaks due to methyl stearate, determined on 6 injections.

LABELLING

The label states the type of stearic acid (50, 70, 95).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for stearic acid used as a lubricant in tablets and capsules.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26, Method I*).

**04/2017:1268**

# STEAROYL MACROGOLGLYCERIDES

## Macrogolglyceridorum stearates

DEFINITION

Mixtures of monoesters, diesters and triesters of glycerol and monoesters and diesters of macrogols with a mean relative molecular mass between 300 and 4000.

They are obtained by partial alcoholysis of saturated oils containing mainly triglycerides of stearic (octadecanoic) acid, using macrogol, or by esterification of glycerol and macrogol with saturated fatty acids, or by mixing glycerol esters and condensates of ethylene oxide with the fatty acids of these hydrogenated oils.

The hydroxyl value is within 15 units of the nominal value. The saponification value is within 10 units of the nominal value.

CHARACTERS

*Appearance*: pale yellow waxy solid.

*Solubility*: dispersible in warm water and in warm liquid paraffin, freely soluble in methylene chloride, soluble in warm anhydrous ethanol.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 1.0 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *hexane R*, *ether R* (30:70 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: spray with a 0.1 g/L solution of *rhodamine B R* in *ethanol (96 per cent) R* and examine in ultraviolet light at 365 nm.

*Results*: the chromatogram shows a spot due to triglycerides with an $R_F$ value of about 0.9 ($R_{st}$ 1) and spots due to 1,3-diglycerides ($R_{st}$ 0.7), to 1,2-diglycerides ($R_{st}$ 0.6), to monoglycerides ($R_{st}$ 0.1) and to esters of macrogol ($R_{st}$ 0).

B. Hydroxyl value (see Tests).

C. Saponification value (see Tests).

D. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Hydroxyl value** (*2.5.3, Method A*): within 15 units of the nominal value, determined on 1.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 6.0, determined on 2.0 g.

**Saponification value** (*2.5.6*): within 10 units of the nominal value, determined on 2.0 g.

**Alkaline impurities**. Introduce 5.0 g into a test-tube and carefully add a mixture, neutralised if necessary with *0.01 M hydrochloric acid* or with *0.01 M sodium hydroxide*, of 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *ethanol (96 per cent) R*, 0.3 mL of *water R* and 10 mL of *ethanol (96 per cent) R*. Shake and allow to stand. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

**Free glycerol**: maximum 3.0 per cent.

Dissolve 1.20 g in 25.0 mL of *methylene chloride R*. Heat if necessary. After cooling, add 100 mL of *water R*. Shake and add 25.0 mL of *periodic acetic acid solution R*. Shake and allow to stand for 30 min. Add 40 mL of a 75 g/L solution of *potassium iodide R*. Allow to stand for 1 min. Add 1 mL of *starch solution R*. Titrate the iodine with *0.1 M sodium thiosulfate*. Carry out a blank titration.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.3 mg of glycerol.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method A*).

*Composition of the fatty-acid fraction of the substance*:

- *lauric acid*: maximum 5.0 per cent;
- *myristic acid*: maximum 5.0 per cent;
- *stearic acid and palmitic acid*: different nominal amounts and minimum 90.0 per cent for the sum of $C_{18}H_{36}O_2$ and $C_{16}H_{32}O_2$.

**Ethylene oxide and dioxan** (*2.4.25*): maximum 1 ppm of ethylene oxide and maximum 10 ppm of dioxan.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g. Use a mixture of 30 volumes of *anhydrous methanol R* and 70 volumes of *methylene chloride R* as solvent.

**Total ash** (*2.4.16*): maximum 0.2 per cent.

LABELLING

The label states:

- the nominal hydroxyl value;
- the nominal saponification value;
- the type of the macrogol used (mean relative molecular mass) or the number of moles of ethylene oxide reacted per mole of substance (nominal value).

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for stearoyl macrogolglycerides used as self-emulsifying agents, solubilisers, modified-release agents and wetting agents.*

**Hydroxyl value** (see Tests).

**Saponification value** (see Tests).

**Composition of fatty acids** (see Tests).



**01/2008:0753**
**corrected 10.0**

# STEARYL ALCOHOL

## Alcohol stearylicus

DEFINITION

Mixture of solid alcohols, mainly octadecan-1-ol ($C_{18}H_{38}O$; $M_r$ 270.5), of animal or vegetable origin.

*Content*: minimum 95.0 per cent of $C_{18}H_{38}O$.

CHARACTERS

*Appearance*: white or almost white, unctuous flakes, granules or mass.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent). When melted, it is miscible with fatty oils, with liquid paraffin and with melted wool fat.

IDENTIFICATION

Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (b).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

Dissolve 0.50 g in 20 mL of boiling *ethanol (96 per cent) R*. Allow to cool.

**Melting point** (*2.2.14*): 57 °C to 60 °C.

**Acid value** (*2.5.1*): maximum 1.0.

**Hydroxyl value** (*2.5.3, Method A*): 197 to 217.

**Iodine value** (*2.5.4, Method A*): maximum 2.0.

Dissolve 2.00 g in *methylene chloride R*, warming if necessary and dilute to 25 mL with the same solvent.

**Saponification value** (*2.5.6*): maximum 2.0.

ASSAY

Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. Dissolve 0.100 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 50 mg of *cetyl alcohol R* in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dissolve 50 mg of *stearyl alcohol CRS* in *ethanol (96 per cent) R* and dilute to 5 mL with the same solvent.

*Reference solution (c)*. Mix 1 mL of reference solution (a) and 1 mL of reference solution (b) and dilute to 10 mL with *ethanol (96 per cent) R*.

*Column*:

- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *methylpolysiloxane R* (1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 20 | 150 → 250 |
| | 20 - 40 | 250 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL of the test solution and reference solutions (b) and (c).

*System suitability*: reference solution (c):

- *resolution*: minimum 5.0 between the peaks due to cetyl alcohol and stearyl alcohol.

Calculate the percentage content of $C_{18}H_{38}O$.

**01/2019:0356**
**corrected 10.0**

# STREPTOKINASE CONCENTRATED SOLUTION

## Streptokinasi solutio concentrata

DEFINITION

Streptokinase concentrated solution is a preparation of a protein obtained from culture filtrates of certain strains of haemolytic *Streptococcus* group C; it has the property of combining with human plasminogen to form plasminogen activator. It may contain buffer salts and other excipients. The potency is not less than 510 IU per microgram of nitrogen.

CHARACTERS

*Appearance*: clear, colourless liquid.

IDENTIFICATION

A. Place 0.5 mL of citrated human plasma in a polystyrene tube maintained in a water-bath at 37 °C. Add 0.1 mL of a dilution of the preparation to be examined containing 10 000 IU of streptokinase activity per millilitre in *phosphate buffer solution pH 7.2 R* and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL in *phosphate buffer solution pH 7.2 R*. Mix immediately. A clot forms and lyses within 30 min. Repeat the procedure using citrated bovine plasma. The clot does not lyse within 60 min.

B. Perform an immunochemical test using double immunodiffusion techniques (*2.7.1*). Place in the central cavity about 80 µL of goat or rabbit antistreptokinase serum containing about 10 000 units of antistreptokinase activity per millilitre; place in each of the surrounding cavities about 80 µL of a dilution of the preparation to be examined containing 125 000 IU of streptokinase activity per millilitre. Allow the plates to stand in a humidified tank for 24 h. Only one precipitation arc appears and it is well defined.

TESTS

**pH** (*2.2.3*): 6.8 to 7.5.

Dilute the preparation to be examined in *carbon dioxide-free water R* to obtain a solution containing at least 1000 000 IU of streptokinase activity per millilitre.

**Streptodornase**: maximum 10 IU of streptodornase activity per 100 000 IU of streptokinase activity.

*Test solution.* Dilute the preparation to be examined in *imidazole buffer solution pH 6.5 R* to obtain a solution containing 150 000 IU of streptokinase activity per millilitre.

*Reference solution.* Dissolve in *imidazole buffer solution pH 6.5 R* a reference preparation of streptodornase, calibrated in International Units against the International Standard of streptodornase, to obtain a solution containing 20 IU of streptodornase activity per millilitre. The equivalence in International Units of the International Standard is stated by the World Health Organization.

To each of 8 numbered centrifuge tubes, add 0.5 mL of a 1 g/L solution of *sodium deoxyribonucleate R* in *imidazole buffer solution pH 6.5 R*. To tube number 1 and tube number 2 add 0.25 mL of *imidazole buffer solution pH 6.5 R*, 0.25 mL of the test solution and, immediately, 3.0 mL of perchloric acid (25 g/L $HClO_4$). Mix, centrifuge at about 3000 *g* for 5 min and measure the absorbances (*2.2.25*) of the supernatant liquids at 260 nm, using as the compensation liquid a mixture of 1.0 mL of *imidazole buffer solution pH 6.5 R* and 3.0 mL of perchloric acid (25 g/L $HClO_4$) (absorbances $A_1$ and $A_2$). To the other 6 tubes (numbers 3 to 8) add 0.25 mL, 0.25 mL, 0.125 mL, 0.125 mL, 0 mL and 0 mL respectively of *imidazole buffer solution pH 6.5 R*; add to each tube 0.25 mL of the test solution and 0 mL, 0 mL, 0.125 mL, 0.125 mL, 0.25 mL and 0.25 mL respectively of the reference solution. Mix the contents of each tube and heat at 37 °C for 15 min. To each tube add 3.0 mL of perchloric acid (25 g/L $HClO_4$), mix and centrifuge. Measure the absorbances (*2.2.25*) of the supernatant liquids at 260 nm using the compensation liquid described above (absorbances $A_3$ to $A_8$). The absorbances comply with the following requirement:

$$(A_3 + A_4) - (A_1 + A_2) < \frac{(A_5 + A_6 + A_7 + A_8)}{2} - (A_3 + A_4)$$

**Streptolysin**. In a polystyrene tube, use a quantity of the preparation to be examined equivalent to 500 000 IU of streptokinase activity and dilute to 0.5 mL with a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*. Add 0.4 mL of a 23 g/L solution of *sodium thioglycollate R*. Heat in a water-bath at 37 °C for 10 min. Add 0.1 mL of a solution of a reference preparation of human antistreptolysin O containing 5 IU/mL. Heat at 37 °C for 5 min. Add 1 mL of *rabbit erythrocyte suspension R*. Heat at 37 °C for 30 min. Centrifuge at about 1000 *g*. In the same manner, prepare a polystyrene tube in which the solution of the preparation to be examined has been replaced by 0.5 mL of a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*. Measure the absorbances (*2.2.25*) of the supernatant liquids at 550 nm. The absorbance of the test solution is not more than 50 per cent greater than that of the reference solution.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dilute the preparation to be examined with *water R* to obtain a concentration of about 0.5-1 g/L, depending on the chromatographic system used.

*Reference solution.* Dilute 1 volume of *streptokinase for system suitability CRS* with 49 volumes of *water R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (10 µm) with a pore size of 200 nm;
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *trifluoroacetic acid R*, *water for chromatography R* (1:1000 *V/V*); degas;
- *mobile phase B*: *trifluoroacetic acid R*, *acetonitrile for chromatography R* (1:1000 *V/V*); degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 1 | 68 | 32 |
| 1 - 4 | 68 → 52 | 32 → 48 |
| 4 - 5 | 52 | 48 |
| 5 - 7 | 0 | 100 |
| 7 - 10 | 68 | 32 |

The above conditions may be modified to improve the separation efficiency of the chromatographic system.

*Flow rate*: 5 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Retention time*: streptokinase = 2.3 min to 2.8 min.

*System suitability*: reference solution:

- *symmetry factor*: maximum 1.9 for the peak due to streptokinase;

– *peak-to-valley ratio*: minimum 2, where $H_p$ = height above the baseline of the 1st peak eluting after the principal peak and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the 2nd peak eluting after the principal peak;
– the chromatogram obtained with the reference solution is similar to the chromatogram supplied with *streptokinase for system suitability CRS*.

*Limit*:

– *total*: maximum 5 per cent.

**Bacterial endotoxins** (*2.6.14*): less than 0.02 IU per 100 IU of streptokinase activity, if intended for use without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

**Nitrogen** (*2.5.9*).

**Potency**

The potency of streptokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of streptokinase calibrated in International Units; the formation of plasmin is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for streptokinase. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*Reference and test solutions*

Prepare 2 independent series of at least 3 dilutions of each of the preparation to be examined and of the reference preparation of streptokinase in *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1*, in the linear range of the assay (a range of 0.5-4.0 IU/mL has been found suitable). Prepare and maintain all solutions at 37 °C.

*Substrate solution*

Mix 1.0 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 7.4 R* with 1.0 mL of *chromogenic substrate R3*. Add 5 µL of a 100 g/L solution of *polysorbate 20 R*. Keep at 37 °C in a water-bath. Immediately before commencing the activation assay, add 45 µL of a 1 mg/mL solution of *human plasminogen R*.

*Method*

Analyse each streptokinase dilution, maintained at 37 °C, in duplicate. Initiate the activation reaction by adding 60 µL of each dilution to 40 µL of substrate solution. For blank wells, use 60 µL of *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R1* instead of the reference and test solutions. Allow the reaction to proceed at 37 °C for 20 min and read the absorbance (*2.2.25*) at 405 nm. If a suitable thermostatted plate reader is available, this may be used to monitor the reaction. Alternatively, it may be necessary to stop the reaction after 20 min using 50 µL of a 50 per cent *V/V* solution of *glacial acetic acid R*. Best results are obtained when the absorbance for the highest streptokinase concentration is between 0.1 and 0.2 (after blank subtraction). If necessary, adjust the time of incubation in order to reach this range of absorbances.

Calculate the regression of the absorbance on log concentrations of the solutions of the preparation to be examined and of the reference preparation of streptokinase and calculate the potency of the preparation to be examined using a suitable statistical method, for example the parallel-line assay (*5.3*).

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 80 per cent and not more than 125 per cent of the estimated potency.

STORAGE

In an airtight container, protected from light and at a temperature of – 20 °C. If the preparation is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

– the number of International Units of streptokinase activity per milligram, calculated with reference to the dried preparation;
– that the preparation is suitable for use in the manufacture of parenteral preparations.

**01/2019:0053**
**corrected 10.0**

# STREPTOMYCIN SULFATE

## Streptomycini sulfas

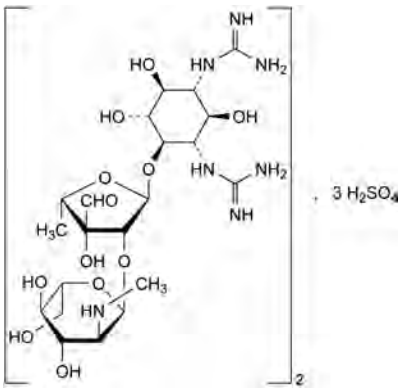


$C_{42}H_{84}N_{14}O_{36}S_3$ $M_r$ 1457
[3810-74-0]

DEFINITION

Bis[$N^1$,$N^3$-dicarbamimidoyl-4-*O*-[5-deoxy-2-*O*-[2-deoxy-2-(methylamino)-α-L-glucopyranosyl]-3-*C*-formyl-α-L-lyxofuranosyl]-D-streptamine] trisulfate, a substance produced by the growth of certain strains of *Streptomyces griseus* or obtained by any other means. Stabilisers may be added. The potency is not less than 720 IU/mg, calculated with reference to the dried substance.

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure.

CHARACTERS

A white or almost white powder, hygroscopic, very soluble in water, practically insoluble in anhydrous ethanol.

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using a plate coated with a 0.75 mm layer of the following mixture: mix 0.3 g of *carbomer R* with 240 mL of *water R* and allow to stand, with moderate shaking, for 1 h; adjust to pH 7 by the gradual addition, with continuous shaking, of *dilute sodium hydroxide solution R* and add 30 g of *silica gel H R*.

Heat the plate at 110 °C for 1 h, allow to cool and use immediately.

*Test solution*. Dissolve 10 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *streptomycin sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *kanamycin monosulfate CRS*, 10 mg of *neomycin sulfate CRS* and 10 mg of *streptomycin sulfate CRS* in *water R* and dilute to 10 mL with the same solvent.

Apply separately to the plate 10 µL of each solution. Develop over a path of 12 cm using a 70 g/L solution of *potassium dihydrogen phosphate R*. Dry the plate in a current of warm air, and spray with a mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 460 g/L solution of *sulfuric acid R*. Heat at 150 °C for 5 min to 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows three clearly separated spots.

B. Dissolve 5 mg to 10 mg in 4 mL of *water R* and add 1 mL of *1 M sodium hydroxide*. Heat in a water-bath for 4 min. Add a slight excess of *dilute hydrochloric acid R* and 0.1 mL of *ferric chloride solution R1*. A violet colour develops.

C. Dissolve 0.1 g in 2 mL of *water R*, add 1 mL of *α-naphthol solution R* and 2 mL of a mixture of equal volumes of *strong sodium hypochlorite solution R* and *water R*. A red colour develops.

D. Dissolve about 10 mg in 5 mL of *water R* and add 1 mL of *1 M hydrochloric acid*. Heat in a water-bath for 2 min. Add 2 mL of a 5 g/L solution of *α-naphthol R* in *1 M sodium hydroxide* and heat in a water-bath for 1 min. A faint yellow colour develops.

E. It gives the reactions of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is not more intensely coloured than intensity 3 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*). Allow to stand protected from light, at a temperature of about 20 °C for 24 h. Solution S is not more opalescent than reference suspension II (*2.2.1*).

**pH** (*2.2.3*). The pH of solution S is 4.5 to 7.0.

**Methanol**. Examine by gas chromatography (*2.2.28*).

*Test solution.* Dissolve 1.00 g of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution.* Dilute 12.0 mg of *methanol R* to 100 mL with *water R*.

The chromatographic procedure may be carried out using:

– a column 1.5 m to 2.0 m long and 2 mm to 4 mm in internal diameter, packed with *ethylvinylbenzene-divinylbenzene copolymer R* (150 µm to 180 µm),

– *nitrogen for chromatography R* as the carrier gas at a constant flow rate of 30 mL to 40 mL per minute,

– a flame-ionisation detector.

Maintain the column at a constant temperature between 120 °C and 140 °C and the injection port and the detector at a temperature at least 50 °C higher than that of the column. Inject the test solution and the reference solution. The area of the peak due to methanol in the chromatogram obtained with the test solution is not greater than the area of the peak in the chromatogram obtained with the reference solution (0.3 per cent).

**Streptomycin B**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance.

*Test solution.* Dissolve 0.2 g of the substance to be examined in a freshly prepared mixture of 3 volumes of *sulfuric acid R* and 97 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents. Heat under a reflux condenser for 1 h, cool, rinse the condenser with *methanol R* and dilute to 20 mL with the same solvent (10 g/L solution).

*Reference solution.* Dissolve 36 mg of *mannose R* in a freshly prepared mixture of 3 volumes of *sulfuric acid R* and 97 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents. Heat under a reflux condenser for 1 h, cool, rinse the condenser with *methanol R* and dilute to 50 mL with the same solvent. Dilute 5 mL of the solution to 50 mL with *methanol R* (0.3 g/L solution expressed as strepto-mycin B; 1 mg of *mannose R* is equivalent to 4.13 mg of streptomycin B).

Apply separately to the plate 10 µL of each solution. Develop over a path of 13 cm to 15 cm using a mixture of 25 volumes of *glacial acetic acid R*, 25 volumes of *methanol R* and 50 volumes of *toluene R*. Allow the plate to dry in air and spray with a freshly prepared mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 20 per cent *V/V* solution of *sulfuric acid R* and heat at 110 °C for 5 min. Any spot corresponding to streptomycin B in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (3.0 per cent).

**Loss on drying** (*2.2.32*). Not more than 7.0 per cent, determined on 1.000 g by drying at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 24 h.

**Sulfated ash** (*2.4.14*). Not more than 1.0 per cent, determined on 1.000 g.

**Sulfate**. 18.0 per cent to 21.5 per cent of sulfate ($SO_4$), calculated with reference to the dried substance. Dissolve 0.250 g in 100 mL of *water R* and adjust the solution to pH 11 using *concentrated ammonia R*. Add 10.0 mL of *0.1 M barium chloride* and about 0.5 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate* adding 50 mL of *ethanol (96 per cent) R* when the colour of the solution begins to change and continue the titration until the violet-blue colour disappears.

1 mL of *0.1 M barium chloride* is equivalent to 9.606 mg of sulfate ($SO_4$).

**Colorimetric test**. Dry the substance to be examined and *streptomycin sulfate CRS* at 60 °C over *diphosphorus pentoxide R* at a pressure not exceeding 0.1 kPa for 24 h. Dissolve 0.100 g of the dried substance to be examined in *water R* and dilute to 100.0 mL with the same solvent. Prepare a reference solution in the same manner using 0.100 g of the dried *streptomycin sulfate CRS*. Place 5.0 mL of each solution separately in two volumetric flasks and in a third flask place 5 mL of *water R*. To each flask add 5.0 mL of *0.2 M sodium hydroxide* and heat for exactly 10 min in a water-bath. Cool in ice for exactly 5 min, add 3 mL of a 15 g/L solution of *ferric ammonium sulfate R* in *0.5 M sulfuric acid*, dilute to 25.0 mL with *water R* and mix. Exactly 20 min after the addition of the ferric ammonium sulfate solution measure the absorbance (*2.2.25*) of the test solution and the reference solution in a 2 cm cell at the maximum at 525 nm, using as compensation liquid the solution prepared from 5 mL of *water R*. The absorbance of the test solution is not less than 90.0 per cent of that of the reference solution.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for removal of bacterial endotoxins.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

Store in an airtight container. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

01/2020:1796

# SUCRALFATE

## Sucralfatum

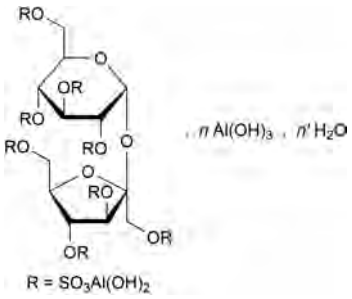


$C_{12}H_{30}Al_8O_{51}S_8[Al(OH)_3]_n[H_2O]_{n'}$

in which $n$ = 8 to 10 and $n'$ = 22 to 31

DEFINITION

β-D-Fructofuranosyl-α-D-glucopyranoside octakis(dihydroxyaluminium sulfate) with 8-10 molecules of aluminium hydroxide and 22-31 molecules of water.

*Content*:

- *β-D-fructofuranosyl-α-D-glucopyranoside octakis(sulfate)* (*sucrose octasulfate*) ($C_{12}H_{14}O_{35}S_8^{8-}$; $M_r$ 975): 30.0 per cent to 38.0 per cent;
- *aluminium* (Al; $A_r$ 26.98): 15.5 per cent to 18.5 per cent.

CHARACTERS

*Appearance*: white or almost white, amorphous powder.

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sucralfate CRS*.

B. To 2 g add 10 mL of a 10.3 g/L solution of *hydrochloric acid R* and boil. Cool and neutralise with a 4 g/L solution of *sodium hydroxide R*. To 5 mL of the solution add 0.15 mL of freshly prepared *copper sulfate solution R* and 2 mL of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 mL of *dilute hydrochloric acid R* and boil for 1 min. Add 4 mL of *dilute sodium hydroxide solution R*; an orange precipitate is formed immediately.

C. Dissolve about 15 mg in a mixture of 0.5 mL of *dilute hydrochloric acid R* and 2 mL of *water R*. The solution gives the reaction of aluminium (*2.3.1*).

TESTS

**Impurity A**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 450.0 mg of the substance to be examined in a mixture of equal volumes of an 88 g/L solution of *sodium hydroxide R* and a 196.2 g/L solution of *sulfuric acid R* and dilute to 10.0 mL with the same mixture of solvents. Without delay, while shaking at a moderate rate, add a volume (*V*), accurately measured in millilitres, of a 4 g/L solution of *sodium hydroxide R* to adjust the solution to approximately pH 2.3. Dilute the solution with (15.0 − *V*) mL of *water R*. Shake for 1 min. If the pH is not between 2.3 and 3.5, repeat the test using a different volume of a 4 g/L solution of *sodium hydroxide R*.

*Reference solution (a).* Dissolve 40.0 mg of *potassium sucrose octasulfate CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 70 g/L solution of *ammonium sulfate R*, adjusted to pH 3.5 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: differential refractometer.

*Injection*: 50 µL of the test solution and reference solution (b).

*Relative retention* with reference to sucrose octasulfate (retention time = about 6 min): impurity A = about 0.6.

*System suitability*: reference solution (b):

- *number of theoretical plates*: minimum 400;
- *symmetry factor*: maximum 4.0.

*Limit*:

- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (5.0 per cent).

**Neutralising capacity**. Disperse 0.25 g in 100.0 mL of *0.1 M hydrochloric acid*, previously heated at 37 °C, stir continuously for 1 h in a water-bath at 37 °C and cool. Titrate 20.0 mL of this solution with *0.1 M sodium hydroxide* to pH 3.5; not more than 14.0 mL of *0.1 M sodium hydroxide* is required.

**Chlorides** (*2.4.4*): maximum 0.50 per cent.

Dissolve 0.10 g in 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Dilute 5 mL of this solution to 15 mL with *water R*.

ASSAY

**Aluminium**. Disperse 1.0 g in 10 mL of *6 M hydrochloric acid R*. Heat with continuous stirring in a water-bath at 70 °C for 5 min. Cool to room temperature, transfer quantitatively to a volumetric flask, dilute to 250.0 mL with *water R*, and mix. Filter the solution, discarding the 1st portion of the filtrate. To 10.0 mL of the solution, add 10.0 mL of *0.1 M sodium edetate* and 30 mL of a mixture of equal volumes of *ammonium acetate solution R* and *dilute acetic acid R*. Heat in a water-bath at 70 °C for 5 min, then cool. Add 25 mL of *ethanol (96 per cent) R* and 1 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *ethanol (96 per cent) R*. Titrate the excess of sodium edetate with *0.1 M zinc sulfate* until the colour changes to pink.

1 mL of *0.1 M sodium edetate* is equivalent to 2.698 mg of Al.

**Sucrose octasulfate**. Liquid chromatography (*2.2.29*) as described in the test for impurity A with the following modifications.

*Mobile phase*: 132 g/L solution of *ammonium sulfate R*, adjusted to pH 3.5 with *phosphoric acid R*.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{14}O_{35}S_8$ taking into account the assigned content of *potassium sucrose octasulfate CRS* and by multiplying the potassium sucrose octasulfate content by 0.757.

IMPURITIES

*Specified impurities: A.*

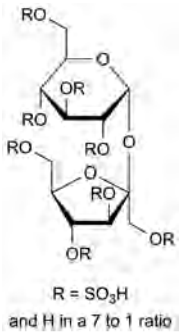


A. β-D-fructofuranosyl-α-D-glucopyranoside heptakis(hydrogen sulfate).

**07/2017:2368**

# SUCRALOSE

## Sucralosum

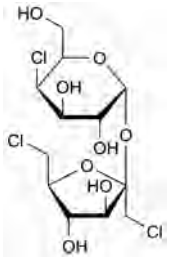


$C_{12}H_{19}Cl_3O_8$ $M_r$ 397.6
[56038-13-2]

DEFINITION

1,6-Dichloro-1,6-dideoxy-β-D-fructofuranosyl 4-chloro-4-deoxy-α-D-galactopyranoside.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in anhydrous ethanol, slightly soluble in ethyl acetate.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sucralose CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 84.0 to + 87.5 (anhydrous substance).

Dissolve 2.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Impurities H and I**

*Test solution*. Dissolve 2.5 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 1.0 g of *mannitol R* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 1.0 g of *mannitol R* and 4.0 mg of *fructose R* in *water R* and dilute to 10.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Application*: 5 µL by applying the solution slowly in 1 µL aliquots and allowing the plate to dry between applications; the 3 spots must be of a similar size.

*Detection*: spray with a solution prepared as follows: dissolve 1.23 g of *p-anisidine R* and 1.66 g of *phthalic acid R* in 100 mL of *methanol R*; store the solution in darkness and in a refrigerator to prevent it becoming discoloured; discard if the solution becomes discoloured; heat the plate at 100 ± 2 °C for 15 min and examine immediately against a dark background.

*System suitability*: the spot due to mannitol obtained with reference solution (a) is colourless; darkening of the mannitol spot indicates that the plate has been held for too long in the oven and a 2nd plate has to be prepared.

*Limit*:

– *sum of impurities H and I*: any spot is not more intense than the spot due to fructose obtained with reference solution (b) (0.1 per cent).

**Related substances**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 1.0 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 0.5 mL of the test solution to 100.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *sucralose impurity B CRS* in 1.0 mL of the test solution.

*Plate*: *TLC octadecylsilyl silica gel plate R*.

*Mobile phase*: *acetonitrile R*, 50 g/L solution of *sodium chloride R* (30:70 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with a 15 per cent *V/V* solution of *sulfuric acid R* in *methanol R* and heat at 125 °C for 10 min.

*Retardation factors*: impurity A = about 0.3; impurity B = about 0.35; sucralose = about 0.45; impurity F = about 0.67; impurity G = about 0.70; impurity E = about 0.72; impurity D = about 0.8.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots due to impurity B and sucralose.

*Limits*:

– *impurities A, B, D, E, F, G*: any spot, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.7 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.25 g of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution*. Dissolve 0.25 g of *sucralose CRS* in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, *water R* (15:85 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: refractometer maintained at a constant temperature.

*Injection*: 20 µL.

*Retention time*: sucralose = about 3 min.

*System suitability*: reference solution:

– *symmetry factor*: maximum 2.0 for the peak due to sucralose.

Calculate the percentage content of $C_{12}H_{19}Cl_3O_8$ taking into account the assigned content of *sucralose CRS*.

IMPURITIES

*Specified impurities*: *A, B, D, E, F, G, H, I.*

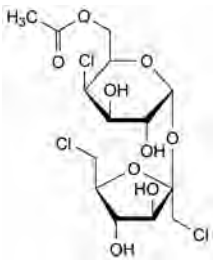

A. 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl 6-*O*-acetyl-4-chloro-4-deoxy-α-D-galactopyranoside (6-*O*-acetylsucralose),

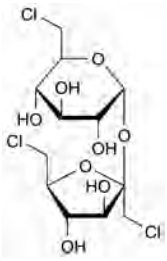

B. 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl 6-chloro-6-deoxy-α-D-glucopyranoside (1′,6,6′-trichloro-1′,6,6′-trideoxysucrose),

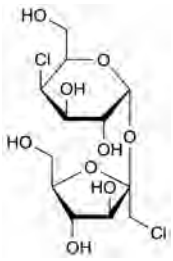

D. 1-chloro-1-deoxy-β-D-fructofuranosyl 4-chloro-4-deoxy-α-D-galactopyranoside (1′,4-dichloro-1′,4-dideoxygalactosucrose),

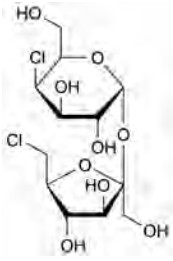

E. 6-chloro-6-deoxy-β-D-fructofuranosyl 4-chloro-4-deoxy-α-D-galactopyranoside (4,6′-dichloro-4,6′-dideoxygalactosucrose),

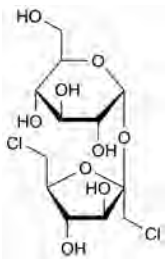

F. 1,6-dichloro-1,6-dideoxy-β-D-fructofuranosyl α-D-glucopyranoside (1′,6′-dichloro-1′,6′-dideoxysucrose),

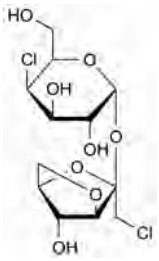

G. 3,6-anhydro-1-chloro-1-deoxy-β-D-fructofuranosyl 4-chloro-4-deoxy-α-D-galactopyranoside (3′,6′-anhydro-1′,4-dichloro-1′,4-dideoxygalactosucrose),

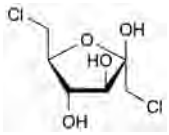

H. 1,6-dichloro-1,6-dideoxy-β-D-fructofuranose,

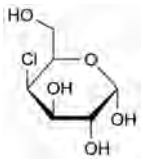

I. 4-chloro-4-deoxy-α-D-galactopyranose.

**01/2016:0204**

# SUCROSE[7]

## Saccharum

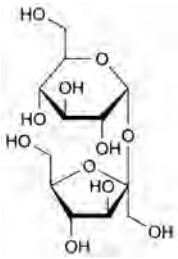

$C_{12}H_{22}O_{11}$ $M_r$ 342.3

[57-50-1]

DEFINITION

β-D-Fructofuranosyl α-D-glucopyranoside.

It contains no additives.

♦CHARACTERS

*Appearance*: white or almost white, crystalline powder, or lustrous, colourless or white or almost white crystals.

*Solubility*: very soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sucrose CRS.*

B. Thin-layer chromatography (*2.2.27*).

(7) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation.*

*Test solution.* Dissolve 10 mg of the substance to be examined in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 10 mg of *sucrose CRS* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 10 mg of *fructose R*, 10 mg of *glucose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in a mixture of 2 volumes of *water R* and 3 volumes of *methanol R* and dilute to 20 mL with the same mixture of solvents.

*Plate*: *TLC silica gel G plate R.*

*Mobile phase*: *cold saturated boric acid solution R*, 60 per cent *V/V* solution of *glacial acetic acid R*, *anhydrous ethanol R*, *acetone R*, *ethyl acetate R* (10:15:20:60:60 *V/V/V/V/V*).

*Application*: 2 µL.

*Development*: in an unsaturated tank, over 3/4 of the plate.

*Drying*: in a current of warm air.

*Detection*: spray with a solution of 0.5 g of *thymol R* in a mixture of 5 mL of *sulfuric acid R* and 95 mL of *ethanol (96 per cent) R*. Heat the plate at 130 °C for 10 min.

*System suitability*: the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dilute 1 mL of solution S (see Tests) to 100 mL with *water R*. To 5 mL of the solution add 0.15 mL of freshly prepared *copper sulfate solution R* and 2 mL of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 mL of *dilute hydrochloric acid R* and boil for 1 min. Add 4 mL of *dilute sodium hydroxide solution R*. An orange precipitate is formed immediately. ♦

TESTS

**Solution S**. Dissolve 50.0 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*).

**Conductivity** (*2.2.38*): maximum 35 µS·cm$^{-1}$ at 20 °C.

Dissolve 31.3 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent. Measure the conductivity of the solution ($C_1$), while gently stirring with a magnetic stirrer, and that of the water used for preparing the solution ($C_2$). The readings must be stable within 1 per cent over a period of 30 s. Calculate the conductivity of the solution of the substance to be examined using the following expression:

$$C_1 - 0.35\,C_2$$

**Specific optical rotation** (*2.2.7*): + 66.3 to + 67.0.

Dissolve 26.0 g in *water R* and dilute to 100.0 mL with the same solvent.

♦**Colour value**: maximum 45.

Dissolve 50.0 g in 50.0 mL of *water R*. Mix, filter (diameter of pores 0.45 µm) and degas. Measure the absorbance (*2.2.25*) at 420 nm, using a minimum path length of 4 cm (a path length of 10 cm or more is preferred).

Calculate the colour value using the following expression:

$$\frac{A \times 1000}{b \times c}$$

$A$ = absorbance measured at 420 nm;

$b$ = path length in centimetres;

$c$ = concentration of the solution, in grams per millilitre, calculated from the refractive index (*2.2.6*) of the solution; use Table 0204.-1 and interpolate the values if necessary.

Table 0204.-1

| $n_D^{20}$ | $c$ (g/mL) |
|---|---|
| 1.4138 | 0.570 |
| 1.4159 | 0.585 |
| 1.4179 | 0.600 |
| 1.4200 | 0.615 |
| 1.4221 | 0.630 |
| 1.4243 | 0.645 |
| 1.4264 | 0.661 |

*System suitability*:

– *repeatability*: the absolute difference between 2 results is not greater than 3. ♦

**Dextrins**. If intended for use in the manufacture of large-volume parenteral preparations, it complies with the test for dextrins. To 2 mL of solution S add 8 mL of *water R*, 0.05 mL of *dilute hydrochloric acid R* and 0.05 mL of *0.05 M iodine*. The solution remains yellow.

**Reducing sugars**. To 5 mL of solution S in a test-tube about 150 mm long and 16 mm in diameter add 5 mL of *water R*, 1.0 mL of *1 M sodium hydroxide* and 1.0 mL of a 1 g/L solution of *methylene blue R*. Mix and place in a water-bath. After exactly 2 min, take the tube out of the bath and examine the solution immediately. The blue colour does not disappear completely. Ignore any blue colour at the air/solution interface.

**Sulfites**: maximum 10 ppm, calculated as $SO_2$.

Determine the sulfites content by a suitable enzymatic method based on the following reactions. Sulfite is oxidised by sulfite oxidase to sulfate and hydrogen peroxide which in turn is reduced by nicotinamide-adenine dinucleotide-peroxidase in the presence of reduced nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidised is proportional to the amount of sulfite.

*Test solution.* Dissolve 4.0 g of the substance to be examined in freshly prepared *distilled water R* and dilute to 10.0 mL with the same solvent.

*Reference solution.* Dissolve 4.0 g of the substance to be examined in freshly prepared *distilled water R*, add 0.5 mL of *sulfite standard solution (80 ppm $SO_2$) R* and dilute to 10.0 mL with freshly prepared *distilled water R*.

*Blank solution.* Freshly prepared *distilled water R*.

Separately introduce 2.0 mL each of the test solution, the reference solution and the blank in 10 mm cuvettes and add the reagents as described in the instructions in the kit for sulfite determination. Measure the absorbance (*2.2.25*) at the absorption maximum at about 340 nm before and at the end of the reaction time and subtract the value obtained with the blank.

The absorbance difference of the test solution is not greater than half the absorbance difference of the reference solution.

**Loss on drying** (*2.2.32*): maximum 0.1 per cent, determined on 2.000 g by drying in an oven at 105 °C for 3 h.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mg, if intended for use in the manufacture of large-volume parenteral preparations.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of large-volume parenteral preparations.

**04/2018:2797**

# SUCROSE, LIQUID

## Saccharum liquidum

DEFINITION

Aqueous solution of sucrose.

*Content*:

– *dry matter*: 66.0 per cent *m/m* to 67.5 per cent *m/m*.

CHARACTERS

*Appearance*: clear, colourless or pale yellow, viscous liquid.

*Solubility*: miscible with glycerol.

IDENTIFICATION

A. Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

B. Dilute 75 g to 100 mL with *water R*. Dilute 1 mL of the solution to 100 mL with *water R*. To 5 mL of this solution add 0.15 mL of freshly prepared *copper sulfate solution R* and 2 mL of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 mL of *dilute hydrochloric acid R* and boil for 1 min. Add 4 mL of *dilute sodium hydroxide solution R*. An orange precipitate is formed immediately.

TESTS

**Colour value**: maximum 30.

Dilute a quantity of the substance to be examined corresponding to 50.0 g of dry matter (see Tests) to 100.0 g with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm) and allow to stand for about 15 min. Measure the absorbance (*2.2.25*) at 420 nm, using a minimum path length of 5 cm (a path length of 10 cm or more is preferred).

Calculate the colour value using the following expression:

$$\frac{A \times 1000}{b \times c}$$

$A$ = absorbance measured at 420 nm;

$b$ = path length, in centimetres;

$c$ = concentration of the solution obtained, in grams per millilitre, calculated from the refractive index (*2.2.6*) of the solution; use Table 2797.-1 and interpolate the values if necessary.

Table 2797.-1

| $n_D^{20}$ | $c$ (g/mL) |
|---|---|
| 1.4138 | 0.570 |
| 1.4159 | 0.585 |
| 1.4179 | 0.600 |
| 1.4200 | 0.615 |
| 1.4221 | 0.630 |
| 1.4243 | 0.645 |
| 1.4264 | 0.661 |

*System suitability*:

– *repeatability*: the absolute difference between 2 results is not greater than 3.

**Conductivity** (*2.2.38*): maximum 35 µS·cm$^{-1}$ at 20 °C.

Dilute a quantity of the substance to be examined corresponding to 31.3 ± 0.1 g of dry matter (see Tests) to 100.0 mL with *carbon dioxide-free water R*. Measure the conductivity of the solution ($C_1$), while gently stirring with a magnetic stirrer, and that of the water used for preparing the solution ($C_2$). The readings must be stable within 1 per cent over a period of 30 s. Calculate the conductivity of the solution of the substance to be examined using the following expression:

$$C_1 - 0.35\ C_2$$

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dilute 0.200 g of the substance to be examined to 10.0 mL with *water R*.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 20.0 mL with *water R*.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 200.0 mL with *water R*.

*Reference solution (b)*. Dissolve 10.0 mg of *raffinose pentahydrate R* (impurity A) and 14.0 mg of *glucose R* (impurity C) in *water R* and dilute to 50.0 mL with the same solvent. To 1.0 mL of the solution add 26.0 mg of *sucrose CRS* and dilute to 2.0 mL with *water R*.

*Reference solution (c)*. Dissolve 33.0 mg of *sucrose CRS* in *water R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Precolumn*:

– *size*: *l* = 0.03 m, Ø = 8.0 mm;

– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm).

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;

– *stationary phase*: *strong cation-exchange resin (calcium form) R* (9 µm);

– *temperature*: 80 ± 1 °C.

*Mobile phase*: *water for chromatography R*.

*Flow rate*: 0.5 mL/min.

*Detection*: differential refractometer maintained at a constant temperature (about 40 °C).

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 3 times the retention time of sucrose.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A (or B) and C.

Impurity A is present in sucrose obtained from sugar beet and impurity B is present in sucrose obtained from sugar cane.

*Relative retention* with reference to sucrose (retention time = about 10 min): impurities A and B = about 0.9; impurity C = about 1.2.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to sucrose; minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to sucrose.

*Calculation of percentage contents*:

– for each impurity, use the concentration of sucrose in reference solution (a).

*Limits*:

– *impurity C*: maximum 0.7 per cent;

– *unspecified impurities*: for each impurity, maximum 0.5 per cent;

– *total*: maximum 2.0 per cent;

– *reporting threshold*: 0.05 per cent.

**Sulfites**: maximum 10 ppm, calculated as $SO_2$.

Determine the sulfites content by a suitable enzymatic method based on the following reactions. Sulfite is oxidised by sulfite oxidase to sulfate and hydrogen peroxide which in turn is reduced by nicotinamide-adenine dinucleotide-peroxidase in the presence of reduced nicotinamide-adenine dinucleotide (NADH). The amount of NADH oxidised is proportional to the amount of sulfite.

*Test solution*. Dilute 4.0 g of the substance to be examined to 10.0 mL with freshly prepared *distilled water R*.

*Reference solution*. To 4.0 g of the substance to be examined add 0.5 mL of *sulfite standard solution (80 ppm $SO_2$) R* and dilute to 10.0 mL with freshly prepared *distilled water R*.

*Blank solution*: freshly prepared *distilled water R*.

Separately introduce 2.0 mL each of the test solution, the reference solution and the blank solution into 10 mm cuvettes and add the reagents as described in the instructions in the kit for sulfite determination. Measure the absorbance (*2.2.25*) at the absorption maximum at about 340 nm before and at the end of the reaction time and subtract the value obtained with the blank.

The absorbance difference of the test solution is not greater than half the absorbance difference of the reference solution.

**Dry matter**. Determine the refractive index (*2.2.6*). Use Table 2797.-2 and interpolate the values if necessary.

Table 2797.-2

| Dry matter (per cent *m/m*) | $n_D^{20}$ |
|---|---|
| 65 | 1.453478 |
| 66 | 1.455839 |
| 67 | 1.458217 |
| 68 | 1.460613 |
| 69 | 1.463026 |

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D.*

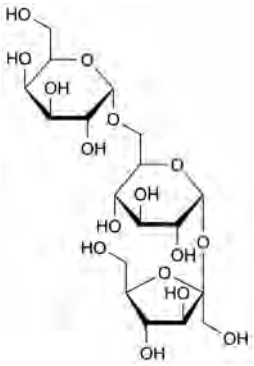

A. β-D-fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside (raffinose),

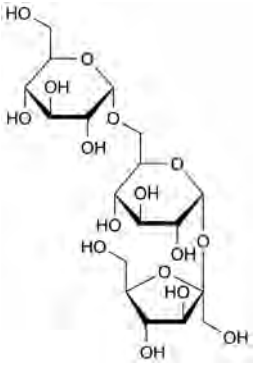

B. β-D-fructofuranosyl α-D-glucopyranosyl-(1→6)-α-D-glucopyranoside (theanderose),

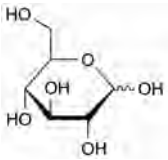

C. D-glucopyranose (glucose),

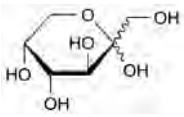

D. D-*arabino*-hex-2-ulopyranose (fructose).

**01/2017:2319**

# SUCROSE MONOPALMITATE

## Sacchari monopalmitas

DEFINITION

Mixture of sucrose monoesters, mainly sucrose monopalmitate, obtained by transesterification of palmitic (hexadecanoic) acid methyl esters of vegetable origin with *Sucrose (0204)*. The manufacture of the fatty acid methyl esters includes a distillation step.

It contains variable quantities of mono-, di-, tri- and polyesters.

*Content*:

– *monoesters*: minimum 55.0 per cent;

– *diesters*: maximum 40.0 per cent;

– *sum of triesters and polyesters*: maximum 20.0 per cent.

CHARACTERS

*Appearance*: white or almost white, unctuous powder.

*Solubility*: very slightly soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Composition of fatty acids (see Tests).

B. It complies with the limits of the assay.

TESTS

**Acid value** (*2.5.1*): maximum 6.0, determined on 3.00 g.

Use a freshly neutralised mixture of 1 volume of *water R* and 2 volumes of *2-propanol R* as solvent and heat gently.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance:*

- *lauric acid*: maximum 3.0 per cent;
- *myristic acid*: maximum 3.0 per cent;
- *palmitic acid*: 70.0 per cent to 85.0 per cent;
- *stearic acid*: 10.0 per cent to 25.0 per cent;
- *sum of the contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

**Free sucrose**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *tetrahydrofuran for chromatography R* (12.5:87.5 *V/V*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 4.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *sucrose CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* In 4 volumetric flasks, introduce respectively 5.0 mg, 10.0 mg, 20.0 mg and 25.0 mg of *sucrose CRS*, dissolve in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *aminopropylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: 0.01 g/L solution of *ammonium acetate R* in *acetonitrile for chromatography R*;
- *mobile phase B*: 0.01 g/L solution of *ammonium acetate R* in a mixture of 10 volumes of *water for chromatography R* and 90 volumes of *tetrahydrofuran for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 1 | 100 | 0 | 1.0 |
| 1 - 9 | 100 → 0 | 0 → 100 | 1.0 |
| 9 - 16 | 0 | 100 | 1.0 |
| 16 - 16.01 | 0 | 100 | 1.0 → 2.5 |
| 16.01 - 32 | 0 | 100 | 2.5 |

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 1.0 mL/min;
- *evaporator temperature*: 45 °C;
- *nebuliser temperature*: 40 °C.

*Injection*: 20 µL.

*Retention time*: about 26 min.

*System suitability*: reference solution (a):

- *signal-to-noise ratio*: minimum 10.

*Limit*: maximum 4.0 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.20 g.

**Total ash** (*2.4.16*): maximum 1.5 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Dissolve 60.0 mg of the substance to be examined in *tetrahydrofuran R* and dilute to 4.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1.2 mL/min.

*Detection*: differential refractometer.

*Injection*: 20 µL.

*Relative retention* with reference to monoesters (retention time = about 13 min): diesters = about 0.93; triesters and polyesters = about 0.91.

*Calculations*:

- *disregard limit*: disregard the peaks having a signal-to-noise ratio less than 10;
- *free fatty acids*: calculate the percentage content of free fatty acids (*C*), using the following expression:

$$\frac{I_A \times 256}{56.11 \times 10}$$

$I_A$ = acid value (see Tests);

256 = rounded molar mass of palmitic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

- *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y+Z}\,(100-A-B)\right]-C$$

$A$ = percentage content of free sucrose (see Tests);

$B$ = percentage content of water (see Tests);

$C$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters;

$Z$ = area of the peak due to triesters and polyesters.

- *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{Y}{X+Y+Z}\,(100-A-B)$$

- *sum of triesters and polyesters*: calculate the sum of the percentage contents of triesters and polyesters using the following expression:

$$\frac{Z}{X+Y+Z}\,(100-A-B)$$

STORAGE

Protected from humidity.

**01/2017:2318**

# SUCROSE STEARATE

## Sacchari stearas

DEFINITION

Mixture of sucrose esters, mainly sucrose stearate, obtained by transesterification of stearic (octadecanoic) acid methyl esters of vegetable origin with *sucrose (0204)*. The manufacture of the fatty acid methyl esters includes a distillation step.

It contains variable quantities of mono-, di-, tri- and polyesters.

*Content*:

*Sucrose stearate type I*:

- *monoesters*: minimum 50.0 per cent;
- *diesters*: maximum 40.0 per cent;
- *sum of triesters and polyesters*: maximum 25.0 per cent;

*Sucrose stearate type II*:

- *monoesters*: 20.0 per cent to 45.0 per cent;
- *diesters*: 30.0 per cent to 40.0 per cent;
- *sum of triesters and polyesters*: maximum 30.0 per cent;

*Sucrose stearate type III*:

- *monoesters*: 15.0 per cent to 25.0 per cent;
- *diesters*: 30.0 per cent to 45.0 per cent;
- *sum of triesters and polyesters*: 35.0 per cent to 50.0 per cent.

CHARACTERS

*Appearance*: white or almost white, unctuous powder.

*Solubility*: very slightly soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

A. Composition of fatty acids (see Tests).

B. It complies with the limits of the assay.

TESTS

**Acid value** (*2.5.1*): maximum 6.0, determined on 3.00 g.

Use a freshly neutralised mixture of 1 volume of *water R* and 2 volumes of *2-propanol R* as solvent and heat gently.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance:*

- *lauric acid*: maximum 3.0 per cent;
- *myristic acid*: maximum 3.0 per cent;
- *palmitic acid*: 25.0 per cent to 40.0 per cent;
- *stearic acid*: 55.0 per cent to 75.0 per cent;
- *sum of the contents of palmitic acid and stearic acid*: minimum 90.0 per cent.

**Free sucrose**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *water for chromatography R*, *tetrahydrofuran for chromatography R* (12.5:87.5 *V/V*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 4.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 20.0 mg of *sucrose CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* In 4 volumetric flasks, introduce respectively 5.0 mg, 10.0 mg, 20.0 mg and 25.0 mg of *sucrose CRS*, dissolve in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *aminopropylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*:

- *mobile phase A*: 0.01 g/L solution of *ammonium acetate R* in *acetonitrile for chromatography R*;
- *mobile phase B*: 0.01 g/L solution of *ammonium acetate R* in a mixture of 10 volumes of *water for chromatography R* and 90 volumes of *tetrahydrofuran for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Flow rate (mL/min) |
|---|---|---|---|
| 0 - 1 | 100 | 0 | 1.0 |
| 1 - 9 | 100 → 0 | 0 → 100 | 1.0 |
| 9 - 16 | 0 | 100 | 1.0 |
| 16 - 16.01 | 0 | 100 | 1.0 → 2.5 |
| 16.01 - 32 | 0 | 100 | 2.5 |

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criterion:

- *carrier gas*: *nitrogen R*;
- *flow rate*: 1.0 mL/min;
- *evaporator temperature*: 45 °C;
- *nebuliser temperature*: 40 °C.

*Injection*: 20 µL.

*Retention time*: about 26 min.

*System suitability*: reference solution (a):

- *signal-to-noise ratio*: minimum 10.

*Limit*:

- *sucrose*: maximum 4.0 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.20 g.

**Total ash** (*2.4.16*): maximum 1.5 per cent.

ASSAY

Size-exclusion chromatography (*2.2.30*): use the normalisation procedure.

*Test solution.* Dissolve 60.0 mg of the substance to be examined in *tetrahydrofuran R* and dilute to 4.0 mL with the same solvent.

*Column*:

- *size*: *l* = 0.6 m, Ø = 7 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: *tetrahydrofuran R*.

*Flow rate*: 1.2 mL/min.

*Detection*: differential refractometer.

*Injection*: 20 µL.

*Relative retention* with reference to monoesters (retention time = about 13 min): diesters = about 0.94; triesters and polyesters = about 0.92.

*Calculations*:

- *disregard limit*: disregard the peaks having a signal-to-noise ratio less than 10;
- *free fatty acids*: calculate the percentage content of free fatty acids (*C*) using the following expression:

$$\frac{I_A \times 284}{56.11 \times 10}$$

Monographs S

$I_A$ = acid value (see Tests);

284 = rounded molar mass of stearic acid, in grams per mole;

56.11 = molar mass of potassium hydroxide, in grams per mole.

– *monoesters*: calculate the percentage content of monoesters using the following expression:

$$\left[\frac{X}{X+Y+Z}\ (100-A-B)\right]-C$$

$A$ = percentage content of free sucrose (see Tests);

$B$ = percentage content of water (see Tests);

$C$ = percentage content of free fatty acids;

$X$ = area of the peak due to monoesters and free fatty acids;

$Y$ = area of the peak due to diesters;

$Z$ = area of the peak due to triesters and polyesters.

– *diesters*: calculate the percentage content of diesters using the following expression:

$$\frac{Y}{X+Y+Z}\ (100-A-B)$$

– *sum of triesters and polyesters*: calculate the sum of the percentage contents of triesters and polyesters using the following expression:

$$\frac{Z}{X+Y+Z}\ (100-A-B)$$

LABELLING

The label states the type of sucrose stearate (type I, II or III).

STORAGE

Protected from humidity.

**01/2012:1569**

# SUFENTANIL

## Sufentanilum

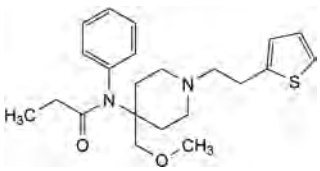

$C_{22}H_{30}N_2O_2S$ $M_r$ 386.6

[56030-54-7]

DEFINITION

*N*-[4-(Methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in methanol.

mp: about 98 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of sufentanil.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.10 g in *methanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* In order to produce impurity E *in situ*, dissolve 10 mg of the substance to be examined in 10.0 mL of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Add 10.0 mL of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 mL of *methanol R*. Filter.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 20.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 5 g/L solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*;

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 40 | 10 → 60 |
| 15 - 20 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to sufentanil (retention time = about 13 min): impurity D = about 0.85; impurity E = about 0.9; impurity F = about 0.95; impurity H = about 1.1.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to impurity E and sufentanil.

*Limits*:

– *impurities D, F, H*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 2 h.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 38.66 mg of $C_{22}H_{30}N_2O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *D, F, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, G, I.*

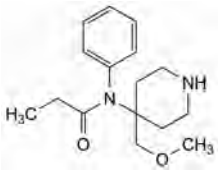

A. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,

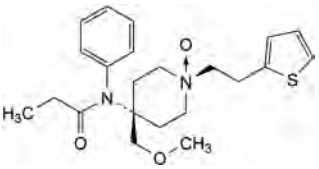

B. *cis*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide,

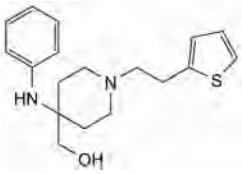

C. [4-(phenylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methanol,

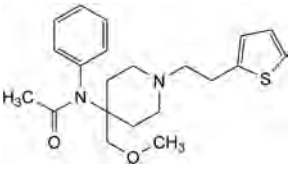

D. *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylacetamide,

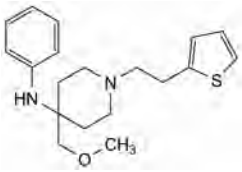

E. 4-(methoxymethyl)-*N*-phenyl-1-[2-(thiophen-2-yl)ethyl]piperidin-4-amine,

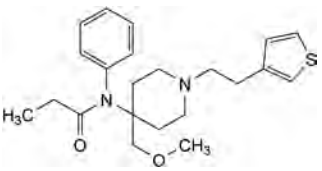

F. *N*-[4-(methoxymethyl)-1-[2-(thiophen-3-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide,

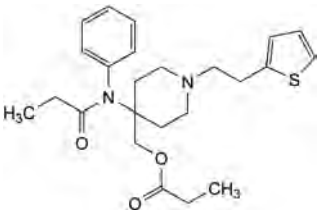

G. [4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methyl propanoate,

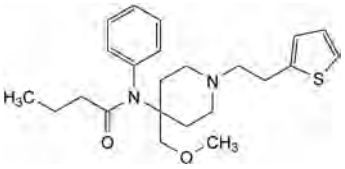

H. *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylbutanamide,

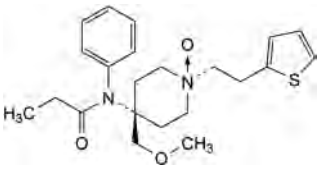

I. *trans*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide.

**01/2012:1269**
**corrected 10.0**

# SUFENTANIL CITRATE

## Sufentanili citras

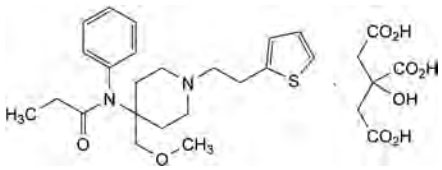

$C_{28}H_{38}N_2O_9S$ $M_r$ 578.7
[60561-17-3]

DEFINITION

*N*-[4-(Methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide citrate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent).

mp: about 140 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of sufentanil citrate.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g in *water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. In order to produce impurity E *in situ*, dissolve 10 mg of the substance to be examined in 10.0 mL of *dilute hydrochloric acid R*. Heat on a water-bath under a reflux condenser for 4 h. Add 10.0 mL of *dilute sodium hydroxide solution R*. Evaporate to dryness on a water-bath. Cool and take up the residue in 10 mL of *methanol R*. Filter.

*Reference solution (b)*. Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.1 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: 5 g/L solution of *ammonium carbonate R* in a mixture of 10 volumes of *tetrahydrofuran R* and 90 volumes of *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 90 → 40 | 10 → 60 |
| 15 - 20 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to sufentanil (retention time = about 13 min): impurity A = about 0.3; impurity B = about 0.4; impurity I = about 0.45; impurity C = about 0.7; impurity D = about 0.85; impurity E = about 0.9; impurity F = about 0.95; impurity G = about 1.05; impurity H = about 1.1.

*System suitability*: reference solution (a):

- *resolution*: minimum 4.0 between the peaks due to impurity E and sufentanil.

*Limits*:

- *impurities A, B, C, D, E, F, G, H, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak with a relative retention with reference to sufentanil of 0.05 or less.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C.

ASSAY

Dissolve 0.400 g in 50 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R* and titrate with *0.1 M perchloric acid*, using 0.2 mL of *naphtholbenzein solution R* as indicator.

1 mL of *0.1 M perchloric acid* is equivalent to 57.87 mg of $C_{28}H_{38}N_2O_9S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I.*

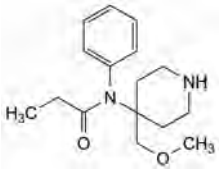

A. *N*-[4-(methoxymethyl)piperidin-4-yl]-*N*-phenylpropanamide,

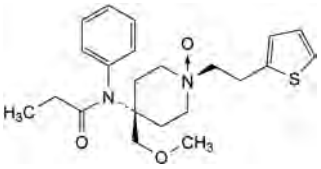

B. *cis*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide,

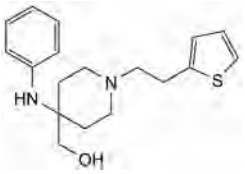

C. [4-(phenylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methanol,

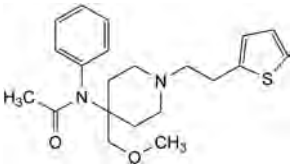

D. *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylacetamide,

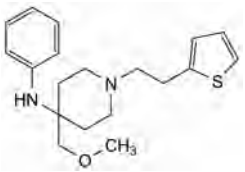

E. 4-(methoxymethyl)-*N*-phenyl-1-[2-(thiophen-2-yl)ethyl]piperidin-4-amine,

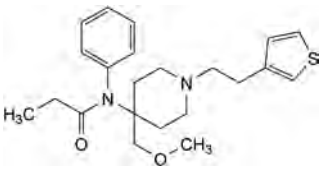

F. *N*-[4-(methoxymethyl)-1-[2-(thiophen-3-yl)ethyl]piperidin-4-yl]-*N*-phenylpropanamide,

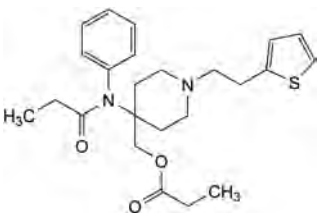

G. [4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]methyl propanoate,

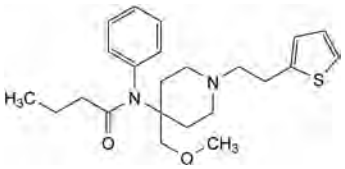

H. *N*-[4-(methoxymethyl)-1-[2-(thiophen-2-yl)ethyl]piperidin-4-yl]-*N*-phenylbutanamide,

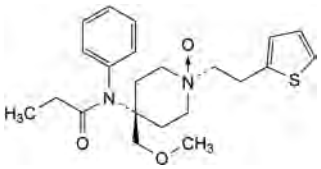

I. *trans*-4-(methoxymethyl)-4-(phenylpropanoylamino)-1-[2-(thiophen-2-yl)ethyl]piperidine 1-oxide.

**01/2017:1570**

# SUGAR SPHERES

## Sacchari sphaerae

DEFINITION

Sugar spheres contain not more than 92 per cent of sucrose, calculated on the dried basis. The remainder consists of maize starch and may also contain starch hydrolysates and colour additives. The diameter of sugar spheres varies usually from 200 µm to 2000 µm and the upper and lower limits of the size of the sugar spheres are stated on the label.

IDENTIFICATION

A. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel G plate R*.

*Solvent mixture*: *water R*, *methanol R* (2:3 *V/V*).

*Test solution.* Mix 2 mL of solution S (see Tests) with 3 mL of *methanol R* and dilute to 20 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *sucrose CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *fructose R*, 10 mg of *glucose R*, 10 mg of *lactose monohydrate R* and 10 mg of *sucrose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

Apply to the plate 2 µL of each solution and thoroughly dry the points of application. Develop over a path of 15 cm using a mixture of 10 volumes of *water R*, 15 volumes of *methanol R*, 25 volumes of *anhydrous acetic acid R* and 50 volumes of *ethylene chloride R*, measured accurately as a slight excess of water causes cloudiness of the solution. Dry the plate in a current of warm air. Repeat the development immediately after renewing the mobile phase. Dry the plate in a current of warm air and spray evenly with a 5 g/L solution of *thymol R* in a mixture of 5 volumes of *sulfuric acid R* and 95 volumes of *ethanol (96 per cent) R*. Heat at 130 °C for 10 min. The principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a). The test is not valid unless the chromatogram obtained with reference solution (b) shows 4 clearly separated spots.

B. To a water slurry of the insoluble portion obtained in the assay, add 0.05 mL of *iodine solution R1*. A dark-blue colour is produced, which disappears on heating.

C. To 5 mL of solution S add 0.15 mL of freshly prepared *copper sulfate solution R* and 2 mL of freshly prepared *dilute sodium hydroxide solution R*. The solution is blue and clear and remains so after boiling. To the hot solution add 4 mL of *dilute hydrochloric acid R* and boil for 1 min. Add 4 mL of *dilute sodium hydroxide solution R*. An orange precipitate is formed immediately.

TESTS

**Solution S**. To 0.5 g in a 100 mL volumetric flask add 80 mL of *water R* and shake until the sucrose is dissolved. Dilute to 100.0 mL with *water R*. Filter under vacuum to obtain a clear solution.

**Fineness** (*2.9.35*): not less than 90 per cent *m/m* of the sugar spheres are between the lower and the upper limits of the size of the sugar spheres stated on the label.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 2 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

**Sucrose content**

Weigh 10.000 g of ground sugar spheres in a 100 mL flask and make up to 100.0 mL with *water R*. Stir and decant. Filter under vacuum to obtain a clear solution (the insoluble portion is used for identification test B). Measure the angle of optical rotation (*2.2.7*) and calculate the sucrose percentage content using the following expression:

$$\frac{10^6 \times \alpha}{66.5 \times l \times m \times (100 - H)}$$

$\alpha$ = angle of rotation;

$l$ = length of the polarimeter tube, in decimetres;

$m$ = exact mass of the sample, in grams;

$H$ = loss on drying.

LABELLING

The label states the upper and the lower limits of the size of the sugar spheres.

**01/2017:2209**
**corrected 10.0**

# SULBACTAM SODIUM

## Sulbactamum natricum

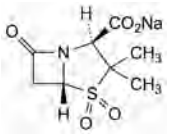

$C_8H_{10}NNaO_5S$ $M_r$ 255.2

[69388-84-7]

DEFINITION

Sodium (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate 4,4-dioxide.

Semi-synthetic product derived from a fermentation product.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble in ethyl acetate, very slightly soluble in ethanol (96 per cent). It is freely soluble in dilute acids.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sulbactam sodium CRS*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 2.0 g in *water R* and dilute to 20 mL with the same solvent.

**Absorbance** (*2.2.25*): maximum 0.10 at 430 nm.

Dissolve 1.0 g in *water R* and dilute to 100.0 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 7.2; if the substance is sterile: 5.2 to 7.2.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 219 to + 233 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. 2.72 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 4.0 with *dilute phosphoric acid R*.

*Solution B*. Dilute 2 mL of *acetonitrile R1* to 100.0 mL with solution A.

*Test solution*. Suspend 77.0 mg of the substance to be examined in 2 mL of *acetonitrile R1*, sonicate for about 5 min and dilute to 100.0 mL with solution A.

*Reference solution (a)*. Suspend 70.0 mg of *sulbactam CRS* in 2 mL of *acetonitrile R1*, sonicate for about 5 min and dilute to 100.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with solution B. Dilute 1.0 mL of this solution to 10.0 mL with solution B.

*Reference solution (c)*. Dissolve 15.0 mg of *6-aminopenicillanic acid R* (impurity B) in solution A and dilute to 50.0 mL with solution A.

*Reference solution (d)*. Mix 1 mL of reference solution (a) and 1 mL of reference solution (c) and dilute to 25.0 mL with solution B.

*Reference solution (e)*. Dissolve 8 mg of *sulbactam for peak identification CRS* (containing impurities A, C, D, E and F) in 1 mL of *acetonitrile R1*, sonicate for about 5 min and dilute to 10 mL with solution B.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.0 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 5.44 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 4.0 with *dilute phosphoric acid R*;
- *mobile phase B*: mobile phase A, *acetonitrile R1* (40:60 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2.0 | 98 | 2 |
| 2.0 - 9.5 | 98 → 50 | 2 → 50 |
| 9.5 - 12.0 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of the test solution, solution B and reference solutions (b), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *sulbactam for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peaks due to impurities A, C, D, E and F; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity B.

*Relative retention* with reference to sulbactam (retention time = about 3 min): impurity A = about 0.4; impurity B = about 0.7; impurity C = about 2.3; impurity D = about 3.1; impurity E = about 3.3; impurity F = about 3.9.

*System suitability*: reference solution (d):

- *resolution*: minimum 5.0 between the peaks due to impurity B and sulbactam.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.6; impurity B = 0.5; impurity D = 0.5; impurity F = 0.6;
- for each impurity, use the concentration of sulbactam in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *impurities C, E*: for each impurity, maximum 0.2 per cent;
- *impurities B, D, F*: for each impurity, maximum 0.1 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent *m/m*.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Bacterial endotoxins** (*2.6.14, Method A*): less than 0.17 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_8H_{10}NNaO_5S$ taking into account the assigned content of *sulbactam CRS* and a conversion factor of 1.094.

STORAGE

In an airtight container. If the substance is sterile, store in a sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical*

*use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

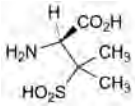

A. (2*S*)-2-amino-3-methyl-3-sulfinobutanoic acid,

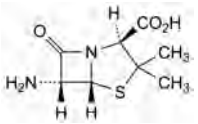

B. (2*S*,5*R*,6*R*)-6-amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-aminopenicillanic acid),

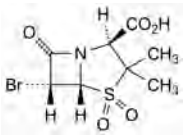

C. (2*S*,5*R*,6*R*)-6-bromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (6-bromopenicillanic acid sulfone),

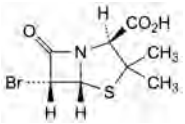

D. (2*S*,5*R*,6*R*)-6-bromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6-bromopenicillanic acid),

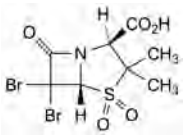

E. (2*S*,5*R*)-6,6-dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (6,6-dibromopenicillanic acid sulfone),

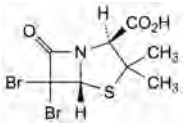

F. (2*S*,5*R*)-6,6-dibromo-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (6,6-dibromopenicillanic acid),

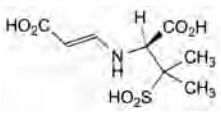

G. (2*S*)-2-[[(2*E*)-2-carboxyethenyl]amino]-3-methyl-3-sulfinobutanoic acid.

**01/2017:0107**

# SULFACETAMIDE SODIUM

## Sulfacetamidum natricum

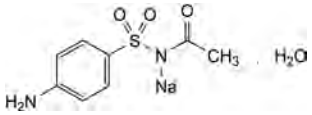

$C_8H_9N_2NaO_3S,H_2O$ $M_r$ 254.2

DEFINITION

Sodium acetyl[(4-aminophenyl)sulfonyl]azanide monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: B, F.*

*Second identification: A, C, E, F.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 0.1 g in *phosphate buffer solution pH 7.0 R* and dilute to 100.0 mL with the same buffer solution. Dilute 1.0 mL of this solution to 100.0 mL with *phosphate buffer solution pH 7.0 R*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 255 nm.

*Specific absorbance at the absorption maximum*: 660 to 720 (anhydrous substance).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sulfacetamide sodium CRS*.

C. Melting point (*2.2.14*): 181 °C to 185 °C.

Dissolve 1 g in 10 mL of *water R*, add 6 mL of *dilute acetic acid R* and filter. Wash the precipitate with a small quantity of *water R* and dry at 100-105 °C for 4 h.

E. Dissolve about 1 mg of the precipitate obtained in identification C, with heating, in 1 mL of *water R*. The solution gives the reaction of primary aromatic amines (*2.3.1*) with formation of an orange-red precipitate.

F. Solution S (see Tests) gives the reactions of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 8.0 to 9.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and carry out the test protected from light.*

*Test solution*. Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *sulfacetamide sodium CRS* and 5 mg of *sulfanilamide R* (impurity A) in 1.0 mL of the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *methanol R*, *water for chromatography R* (1:10:89 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Run time*: 7 times the retention time of sulfacetamide.

*Relative retention* with reference to sulfacetamide (retention time = about 5 min): impurity A = about 0.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to impurity A and sulfacetamide.

*Limits*:

– *correction factor*: for the calculation of the content, multiply the peak area of impurity A by 0.5;

– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dissolve 2.5 g in *distilled water R* and dilute to 25 mL with the same solvent. Add 25 mL of *dilute acetic acid R*, shake for 30 min and filter. 15 mL of the filtrate complies with the limit test for sulfates.

**Water** (*2.5.12*). 6.0 per cent to 8.0 per cent, determined on 0.200 g.

ASSAY

Dissolve 0.500 g in a mixture of 50 mL of *water R* and 20 mL of *dilute hydrochloric acid R*. Cool the solution in a bath of iced water and carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 23.62 mg of $C_8H_9N_2NaO_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

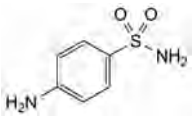

A. 4-aminobenzenesulfonamide (sulfanilamide),

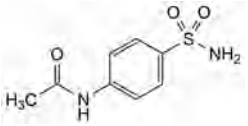

B. *N*-(4-sulfamoylphenyl)acetamide,

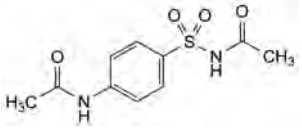

C. *N*-[[4-(acetylamino)phenyl]sulfonyl]acetamide,

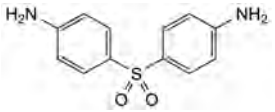

D. 4,4′-sulfonyldianiline (dapsone).

**01/2017:0294**

# SULFADIAZINE

## Sulfadiazinum

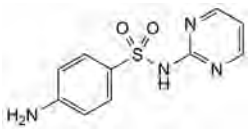

$C_{10}H_{10}N_4O_2S$ $M_r$ 250.3

[68-35-9]

DEFINITION

4-Amino-*N*-(pyrimidin-2-yl)benzenesulfonamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white, yellowish-white or pinkish-white, crystalline powder or crystals.

*Solubility*: practically insoluble in water, slightly soluble in acetone, very slightly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sulfadiazine CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *concentrated ammonia R*, *methanol R* (4:96 *V/V*)
*Test solution.* Dissolve 20 mg of the substance to be examined in 3 mL of the solvent mixture and dilute to 5.0 mL with the solvent mixture.
*Reference solution.* Dissolve 20 mg of *sulfadiazine CRS* in 3 mL of the solvent mixture and dilute to 5.0 mL with the solvent mixture.
*Plate*: *TLC silica gel* $F_{254}$ *plate R.*

*Mobile phase*: *dilute ammonia R1*, *water R*, *nitromethane R*, *dioxan R* (3:5:40:50 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 105 °C.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Place 3 g in a dry tube. Immerse the lower part of the tube, inclined at 45°, in a silicone oil bath and heat to about 270 °C. The substance to be examined decomposes and a white or yellowish-white sublimate is formed, which, after recrystallisation from *toluene R* and drying at 100 °C, melts (*2.2.14*) at 123 °C to 127 °C.

D. Dissolve about 5 mg in 10 mL of a 103 g/L solution of *hydrochloric acid R*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_5$, $BY_5$ or $GY_5$ (*2.2.2, Method II*).

Dissolve 0.8 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*.

**Acidity**. To 1.25 g, finely powdered, add 25 mL of *carbon dioxide-free water R*. Heat at about 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: 40 g/L solution of *sodium hydroxide R*, *acetonitrile R*, *water R* (2:20:60 *V/V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with *water R*.

*Reference solution (a)*. Dissolve 5.0 mg of *sulfadiazine impurity A CRS* and 5.0 mg of *sulfanilic acid RV* (impurity B) in the solvent mixture and dilute to 10.0 mL with *water R*. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 3.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve the contents of a vial of *acetylsulfadiazine CRS* (impurity E) in 1 mL of the mobile phase.

*Reference solution (d)*. Dissolve 5 mg of *sulfadiazine for identification of impurity F CRS* in the solvent mixture and dilute to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R*, 2.8 g/L solution of *phosphoric acid R* (10:90 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: 7 times the retention time of sulfadiazine.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity F.

*Relative retention* with reference to sulfadiazine (retention time = about 8.5 min): impurity A = about 0.26; impurity B = about 0.30; impurity E = about 2.1; impurity F = about 6.0.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity E by 0.7;
- *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity E*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity F*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in a mixture of 20 mL of *dilute hydrochloric acid R* and 50 mL of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 25.03 mg of $C_{10}H_{10}N_4O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

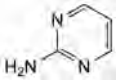

A. pyrimidin-2-amine,

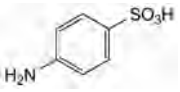

B. 4-aminobenzenesulfonic acid (sulfanilic acid),

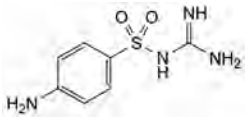

C. [(4-aminophenyl)sulfonyl]guanidine (sulfaguanidine),

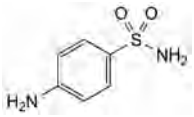

D. 4-aminobenzenesulfonamide (sulfanilamide),

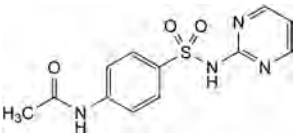

E. *N*-[4-(pyrimidin-2-ylsulfamoyl)phenyl]acetamide (acetylsulfadiazine),

F. unknown structure.

**01/2017:2741**
**corrected 9.4**

# SULFADIMETHOXINE

## Sulfadimethoxinum

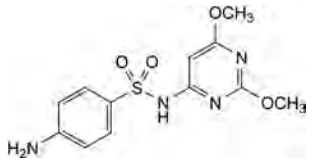

$C_{12}H_{14}N_4O_4S$ $M_r$ 310.3
[122-11-2]

DEFINITION

4-Amino-*N*-(2,6-dimethoxypyrimidin-4-yl)benzene-1-sulfonamide.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in methylene chloride, very slightly soluble in ethanol (96 per cent).

It is freely soluble in dilute sodium hydroxide and sparingly soluble in dilute hydrochloric acid.

mp: 197 °C to 202 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sulfadimethoxine CRS*.

TESTS

**Acidity**. Suspend 0.5 g in 25 mL of *carbon dioxide-free water R*. Heat the suspension at 70 °C for 5 min, cool quickly to room temperature and filter. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to reach pH 7.0.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 6.0 g of *sodium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 7.0 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 20.0 mg of the substance to be examined in 25 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (a)*. Dissolve 20.0 mg of *sulfadimethoxine CRS* in 25 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 4 mg of *sulfadimethoxine for peak identification CRS* (containing impurities A and F) in 5 mL of *methanol R* and dilute to 20 mL with solution A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, solution A (25:75 *V/V*);
- *mobile phase B*: *methanol R*, *acetonitrile R*, solution A (25:35:40 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 30 | 100 → 0 | 0 → 100 |
| 30 - 35 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *sulfadimethoxine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and F.

*Relative retention* with reference to sulfadimethoxine (retention time = about 11 min): impurity F = about 0.4; impurity A = about 1.2.

*System suitability*:

- *resolution*: minimum 2.5 between the peaks due to sulfadimethoxine and impurity A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity F = 1.7;
- for each impurity, use the concentration of sulfadimethoxine in reference solution (b).

*Limits*:

- *impurities A, F*: for each impurity, maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{14}N_4O_4S$ taking into account the assigned content of *sulfadimethoxine CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

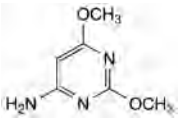

A. 2,6-dimethoxypyrimidin-4-amine,

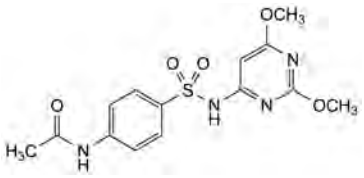

B. *N*-[4-[(2,6-dimethoxypyrimidin-4-yl)sulfamoyl]phenyl]-acetamide,

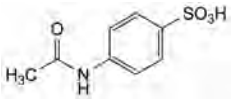

C. 4-(acetylamino)benzene-1-sulfonic acid,

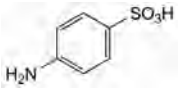

D. 4-aminobenzene-1-sulfonic acid (sulfanilic acid),

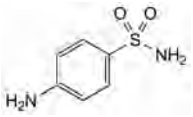

E. 4-aminobenzene-1-sulfonamide (sulfanilamide),

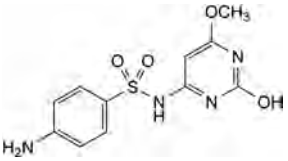

F. 4-amino-*N*-(2-hydroxy-6-methoxypyrimidin-4-yl)benzene-1-sulfonamide.

**01/2015:2745**
**corrected 10.0**

# SULFADIMETHOXINE SODIUM FOR VETERINARY USE

## Sulfadimethoxinum natricum ad usum veterinarium

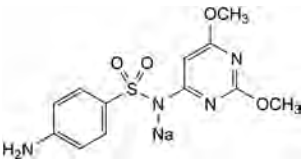

$C_{12}H_{13}N_4NaO_4S$ $M_r$ 332.3
[1037-50-9]

DEFINITION

Sodium [(4-aminophenyl)sulfonyl](2,6-dimethoxypyrimidin-4-yl)azanide.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 1 g in 20 mL of *water R*; add 0.20 mL of *glacial acetic acid R*; filter the precipitate, wash with 1 mL of *water R* and dry at 105 °C for 2 h.

*Comparison*: *sulfadimethoxine CRS*.

B. 2 mL of the filtrate obtained in identification test A gives reaction (a) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent.

**pH** (*2.2.3*): 8.5 to 10.0.

Dissolve 0.2 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 6.0 g of *sodium dihydrogen phosphate R* in 950 mL of *water for chromatography R*, adjust to pH 7.0 with *dilute sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 22.0 mg of the substance to be examined in 76 mL of solution A and dilute to 100.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 20.0 mg of *sulfadimethoxine CRS* in 25 mL of *methanol R* and dilute to 100.0 mL with solution A.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 4 mg of *sulfadimethoxine for peak identification CRS* (containing impurities A and F) in 5 mL of *methanol R* and dilute to 20 mL with solution A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: *methanol R*, solution A (25:75 *V/V*);
- *mobile phase B*: *methanol R*, *acetonitrile R*, solution A (25:35:40 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 30 | 100 → 0 | 0 → 100 |
| 30 - 35 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *sulfadimethoxine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and F.

*Relative retention* with reference to sulfadimethoxine (retention time = about 11 min): impurity F = about 0.4; impurity A = about 1.2.

*System suitability*:
- *resolution*: minimum 2.5 between the peaks due to sulfadimethoxine and impurity A in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 40 for the principal peak in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:
- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.4; impurity F = 1.7;
- for each impurity, use the concentration of sulfadimethoxine sodium in reference solution (b).

*Limits*:
- *impurities A, F*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.10 per cent.

**Heavy metals** (*2.4.8*): maximum 20 ppm.

*Solvent*: *water R*.

0.5 g complies with test H. Prepare the reference solution using 1 mL of *lead standard solution (10 ppm Pb) R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.200 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{12}H_{13}N_4NaO_4S$ taking into account the assigned content of *sulfadimethoxine CRS* and a conversion factor of 1.071.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

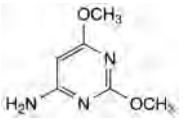

A. 2,6-dimethoxypyrimidin-4-amine,

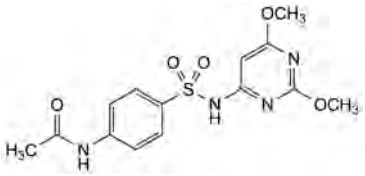

B. *N*-[4-[(2,6-dimethoxypyrimidin-4-yl)sulfamoyl]phenyl]-acetamide,

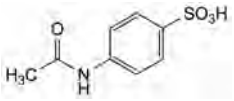

C. 4-(acetylamino)benzene-1-sulfonic acid,

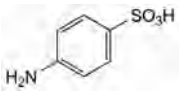

D. 4-aminobenzene-1-sulfonic acid (sulfanilic acid),

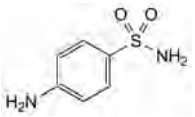

E. 4-aminobenzene-1-sulfonamide (sulfanilamide),

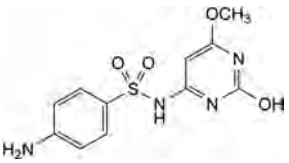

F. 4-amino-*N*-(2-hydroxy-6-methoxypyrimidin-4-yl)benzene-1-sulfonamide.

**01/2017:0295**

# SULFADIMIDINE

## Sulfadimidinum

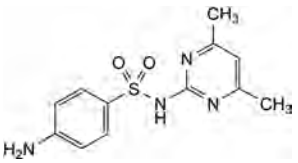

$C_{12}H_{14}N_4O_2S$ $M_r$ 278.3

[57-68-1]

DEFINITION

4-Amino-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or crystals.

*Solubility*: very slightly soluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sulfadimidine CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *concentrated ammonia R*, *methanol R* (4:96 *V/V*).
*Test solution.* Dissolve 20 mg of the substance to be examined in 3 mL of the solvent mixture and dilute to 5.0 mL with the solvent mixture.
*Reference solution.* Dissolve 20 mg of *sulfadimidine CRS* in 3 mL of the solvent mixture and dilute to 5.0 mL with the solvent mixture.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.
*Mobile phase*: *dilute ammonia R1*, *water R*, *nitromethane R*, *dioxan R* (3:5:40:50 *V/V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: at 100-105 °C for 30 min.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Place 3 g in a dry tube. Immerse the lower part of the tube, inclined at 45°, in a silicone-oil bath and heat to about 270 °C. The substance to be examined decomposes and a white or yellowish-white sublimate is formed which, after recrystallisation from *toluene R* and drying at 100 °C, melts (*2.2.14*) at 150 °C to 154 °C.

D. Dissolve about 5 mg in 10 mL of a 103 g/L solution of *hydrochloric acid R*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_5$, $BY_5$ or $GY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*.

**Acidity**. To 1.25 g of the finely powdered substance to be examined, add 25 mL of *carbon dioxide-free water R*. Heat at about 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: 40 g/L solution of *sodium hydroxide R*, *acetonitrile R*, *water R* (2.5:25:75 *V/V/V*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 41 mL of the solvent mixture and dilute to 50.0 mL with *water R*.

*Reference solution (a).* Dissolve 5 mg of *sulfacetamide sodium CRS* (impurity E) and 5 mg of *sulfaguanidine CRS* (impurity C) in 41 mL of the solvent mixture and dilute to 100.0 mL with *water R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (c).* Dissolve 20 mg of *sulfadimidine for peak identification CRS* (containing impurity G) in 16.4 mL of the solvent mixture and dilute to 20.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: mix 10 volumes of *acetonitrile R* and 90 volumes of a 0.6 per cent *V/V* solution of *acetic acid R* previously adjusted to pH 6.5 with a 250 g/L solution of *ammonia R*;
- *mobile phase B*: mix equal volumes of *acetonitrile R* and a 0.6 per cent *V/V* solution of *acetic acid R* previously adjusted to pH 6.5 with a 250 g/L solution of *ammonia R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 35 | 100 → 0 | 0 → 100 |
| 35 - 45 | 0 | 100 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 241 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *sulfadimidine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity G.

*Relative retention* with reference to sulfadimidine (retention time = about 20 min): impurity E = about 0.13; impurity C = about 0.15; impurity D = about 0.2; impurity G = about 1.7.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurities E and C.

*Limits*:
- *impurities C, D, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 mL of *dilute hydrochloric acid R* and 50 mL of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 27.83 mg of $C_{12}H_{14}N_4O_2S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D, G.*

Monographs S

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F.*

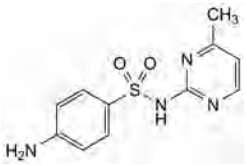

A. 4-amino-*N*-(4-methylpyrimidin-2-yl)benzenesulfonamide (sulfamerazine),

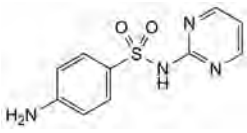

B. 4-amino-*N*-pyrimidin-2-ylbenzenesulfonamide (sulfadiazine),

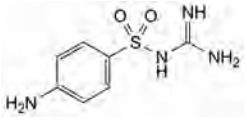

C. (4-aminophenylsulfonyl)guanidine (sulfaguanidine),

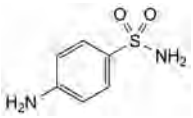

D. 4-aminobenzenesulfonamide (sulfanilamide),

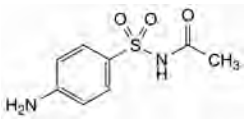

E. *N*-[(4-aminophenyl)sulfonyl]acetamide (sulfacetamide),

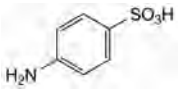

F. 4-aminobenzenesulfonic acid (sulfanilic acid),

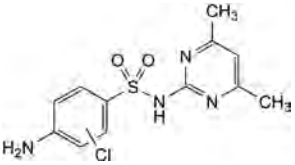

G. 4-amino-2-chloro-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide or 4-amino-3-chloro-*N*-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide.

**01/2017:0740**

# SULFADOXINE

## Sulfadoxinum

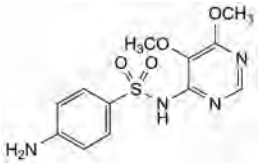

$C_{12}H_{14}N_4O_4S$ $M_r$ 310.3
[2447-57-6]

DEFINITION

Sulfadoxine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(5,6-dimethoxypyrimidin-4-yl)benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystalline powder or crystals, very slightly soluble in water, slightly soluble in alcohol and in methanol. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 198 °C, with decomposition.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfadoxine CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.5 g in 1 mL of a 40 per cent *V/V* solution of *sulfuric acid R*, heating gently. Continue heating until a crystalline precipitate appears (about 2 min). Allow to cool and add 10 mL of *dilute sodium hydroxide solution R*. Cool again. Add 25 mL of *ether R* and shake for 5 min. Separate the ether layer, dry over *anhydrous sodium sulfate R* and filter. Evaporate the solvent by heating in a water-bath. The residue melts (*2.2.14*) at 80 °C to 82 °C or at 90 °C to 92 °C.

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*. The solution is not more intensely coloured than reference solution $Y_5$, $BY_5$ or $GY_5$ (*2.2.2, Method II*).

**Acidity**. To 1.25 g, finely powdered, add 25 mL of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in a bath of iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 3 mL of a mixture of 2 volumes of *concentrated*

*ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 5 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *sulfadoxine CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 2.5 mL of test solution (b) to 100 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using 0.250 g and determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 31.03 mg of $C_{12}H_{14}N_4O_4S$.

STORAGE

Store protected from light.

**01/2017:0741**

# SULFAFURAZOLE

## Sulfafurazolum

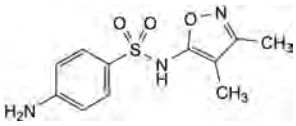

$C_{11}H_{13}N_3O_3S$ $M_r$ 267.3
[127-69-5]

DEFINITION

Sulfafurazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(3,4-dimethylisoxazol-5-yl)benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white, crystalline powder or crystals, practically insoluble in water, sparingly soluble in alcohol, slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 197 °C, with decomposition.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfafurazole CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 0.5 g add 1 mL of a 40 per cent *V/V* solution of *sulfuric acid R* and heat over a low flame to dissolve. Continue heating until a crystalline precipitate appears (about 2 min). Allow to cool and add 10 mL of *dilute sodium hydroxide solution R*. Cool. Shake the solution for 5 min with 25 mL of *ether R*. Separate the ether layer, dry over *anhydrous sodium sulfate R* and filter. Evaporate the solvent by heating on a water-bath. The residue melts (*2.2.14*) at 119 °C to 123 °C.

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 0.4 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*, with gently warming if necessary. The solution is not more intensely coloured than reference solution $Y_6$, $BY_6$ or $GY_6$ (*2.2.2, Method II*).

**Acidity**. To 1.25 g, finely powdered, add 25 mL of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 5 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *sulfafurazole CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.25 mL of test solution (b) to 50 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *concentrated ammonia R*, 25 volumes of *methanol R* and 75 volumes of *methylene chloride R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *acetone R*. Titrate with *0.1 M tetrabutylammonium hydroxide* using a 4 g/L solution of *thymol blue R* in *methanol R* as indicator.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 26.73 mg of $C_{11}H_{13}N_3O_3S$.

STORAGE

Store protected from light.

**01/2017:1476**
**corrected 10.0**

# SULFAGUANIDINE

## Sulfaguanidinum

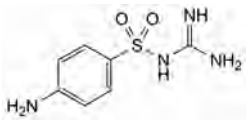

$C_7H_{10}N_4O_2S$ $M_r$ 214.3
[57-67-0]

DEFINITION

Sulfaguanidine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of (4-aminophenylsulfonyl)guanidine, calculated with reference to the dried substance.

CHARACTERS

A white or almost white, fine crystalline powder, very slightly soluble in water, slightly soluble in acetone, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute solutions of mineral acids.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 189 °C to 193 °C, determined on the dried substance.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfaguanidine CRS*.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

E. Suspend 0.1 g in 2 mL of *water R*, add 1 mL of *α-naphthol solution R* and 2 mL of a mixture of equal volumes of *water R* and *strong sodium hypochlorite solution R*. A red colour develops.

TESTS

**Solution S**. To 2.5 g, add 40 mL of *carbon dioxide-free water R*. Heat at about 70 °C for 5 min. Cool while stirring in iced water for about 15 min, filter and dilute to 50 mL with *carbon dioxide-free water R*.

**Acidity**. To 20 mL of solution S, add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin layer chromatography (*2.2.27*), using a *TLC silica gel $GF_{254}$ plate R*.

*Test solution (a).* Dissolve 50 mg of the substance to be examined in *acetone R* and dilute to 5 mL with the same solvent.

*Test solution (b).* Dilute 2 mL of test solution (a) to 10 mL with *acetone R*.

*Reference solution (a).* Dissolve 10 mg of *sulfaguanidine CRS* in *acetone R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dilute 5 mL of test solution (b) to 200 mL with *acetone R*.

*Reference solution (c).* Dilute 5 mL of reference solution (b) to 10 mL with *acetone R*.

*Reference solution (d).* Dissolve 10 mg of *sulfanilamide R* in test solution (b) and dilute to 5 mL with the same solution.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *anhydrous formic acid R*, 20 volumes of *methanol R* and 70 volumes of *methylene chloride R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.25 per cent). The test is not valid unless the chromatogram obtained with reference solution (d) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*): not more than 8.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.175 g in 50 mL of *dilute hydrochloric acid R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 21.42 mg of $C_7H_{10}N_4O_2S$.

STORAGE

Store protected from light.

IMPURITIES

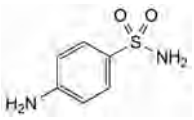

A. 4-aminobenzenesulfonamide (sulfanilamide),

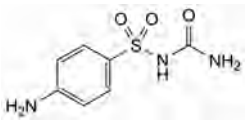

B. *N*-[(4-aminophenyl)sulfonyl]urea (sulfacarbamide).

**01/2017:0358**
**corrected 10.0**

# SULFAMERAZINE

## Sulfamerazinum

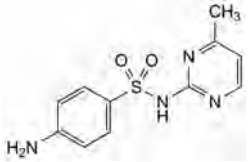

$C_{11}H_{12}N_4O_2S$ $M_r$ 264.3
[127-79-7]

DEFINITION

Sulfamerazine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(4-methyl-2-pyrimidinyl)benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

White, yellowish-white or pinkish-white, crystalline powder or crystals, very slightly soluble in water, sparingly soluble in acetone, slightly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 235 °C, with decomposition.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfamerazine CRS*. Examine the substances as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place 3 g in a dry tube. Incline the tube by about 45°, immerse the bottom of the tube in a silicone-oil bath and heat to about 270 °C. The substance decomposes, producing a white or yellowish-white sublimate which, after recrystallisation from *toluene R* and drying at 100 °C, melts (*2.2.14*) at 157 °C to 161 °C.

D. Dissolve about 20 mg in 0.5 mL of *dilute hydrochloric acid R* and add 1 mL of *water R*. The solution gives, without further addition of acid, the identification reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 0.8 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*. The solution is not more intensely coloured than reference solution $Y_4$, $BY_4$ or $GY_4$ (*2.2.2, Method II*).

**Acidity**. To 1.25 g, finely powdered, add 40 mL of *carbon dioxide-free water R* and heat at about 70 °C for 5 min. Cool for about 15 min in iced water and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*) using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

*Reference solution (a).* Dissolve 10 mg of *sulfamerazine CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 2.5 mL of test solution (b) to 50 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

Apply to the plate 5 µL of each solution. Develop over a path of 15 cm with a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2500 g in a mixture of 20 mL of *dilute hydrochloric acid R* and 50 mL of *water R*. Cool the solution in iced water. Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 26.43 mg of $C_{11}H_{12}N_4O_2S$.

STORAGE

Store protected from light.

**01/2017:0637**

# SULFAMETHIZOLE

## Sulfamethizolum

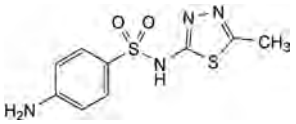

$C_9H_{10}N_4O_2S_2$ $M_r$ 270.3

[144-82-1]

DEFINITION

Sulfamethizole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(5-methyl-1,3,4-thiadiazol-2-yl)benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystalline powder or crystals, very slightly soluble in water, soluble in acetone, sparingly soluble in alcohol. It dissolves in dilute solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 210 °C.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfamethizole CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 50 mg in 4 mL of *methanol R* and add 0.2 mL of a 40 g/L solution of *copper acetate R*. A flocculent, yellowish-green precipitate is formed, changing to dark green.

D. Dissolve about 5 mg in *1 M hydrochloric acid* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*. The solution is not more intensely coloured than reference solution $Y_5$, $BY_5$ or $GY_5$ (*2.2.2, Method II*).

**Acidity**. To 1.25 g add 25 mL of *carbon dioxide-free water R* and heat at 70 °C for 5 min. Cool for about 15 min in iced water and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a)*. Dissolve 0.30 g of the substance to be examined in *acetone R* and dilute to 10 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.

*Reference solution (a)*. Dissolve 30 mg of *sulfamethizole CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dilute 1 mL of test solution (b) to 20 mL with *acetone R*.

Apply to the plate 2 µL of each solution. Develop over a path of 15 cm using a mixture of 15 volumes of *methanol R* and 80 volumes of *chloroform R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using 0.2500 g and determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 27.03 mg of $C_9H_{10}N_4O_2S_2$.

STORAGE

Store protected from light.

**01/2017:0108**

# SULFAMETHOXAZOLE

## Sulfamethoxazolum

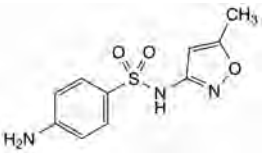

$C_{10}H_{11}N_3O_3S$ $M_r$ 253.3
[723-46-6]

DEFINITION

4-Amino-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, sparingly soluble in ethanol (96 per cent). It dissolves in dilute solutions of sodium hydroxide and in dilute acids.

IDENTIFICATION

*First identification*: *A, B*.

*Second identification*: *A, C, D*.

A. Melting point (*2.2.14*): 169 °C to 172 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sulfamethoxazole CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution*. Dissolve 20 mg of *sulfamethoxazole CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Plate*: *TLC silica gel* $F_{254}$ *plate R*.

*Mobile phase*: *dilute ammonia R1*, *water R*, *nitromethane R*, *dioxan R* (3:5:41:51 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$, $BY_5$ or $GY_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in a mixture of 5 mL of *dilute sodium hydroxide solution R* and 5 mL of *water R*.

**Acidity**. To 1.25 g, finely powdered, add 25 mL of *water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 45 mL of the mobile phase, sonicate at about 45 °C for 10 min, cool and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 1 mg of the substance to be examined and 1 mg of *sulfamethoxazole impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 1.0 mg of *sulfamethoxazole impurity F CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm,
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*: mix 35 volumes of *methanol R2* and 65 volumes of a 13.6 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 5.3 with a 20 g/L solution of *potassium hydroxide R*.

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of sulfamethoxazole.

*Relative retention* with reference to sulfamethoxazole (retention time = about 10 min): impurity D = about 0.3; impurity E = about 0.35; impurity F = about 0.45; impurity C = about 0.5; impurity A = about 1.2; impurity B = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to sulfamethoxazole and impurity A.

*Limits*:

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay of primary aromatic amino-nitrogen (*2.5.8*), using 0.200 g and determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 25.33 mg of $C_{10}H_{11}N_3O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

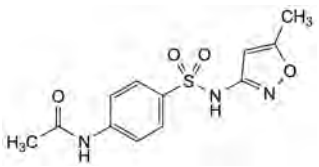

A. *N*-[4-[(5-methylisoxazol-3-yl)sulfamoyl]phenyl]acetamide,

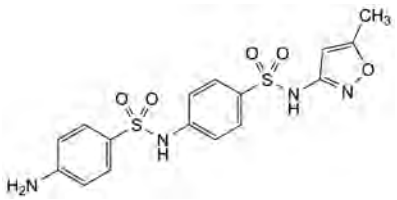

B. 4-[[(4-aminophenyl)sulfonyl]amino]-*N*-(5-methylisoxazol-3-yl)benzenesulfonamide,

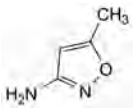

C. 5-methylisoxazol-3-amine,

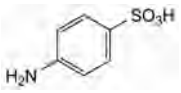

D. 4-aminobenzenesulfonic acid (sulfanilic acid),

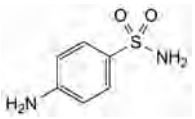

E. 4-aminobenzenesulfonamide (sulfanilamide),

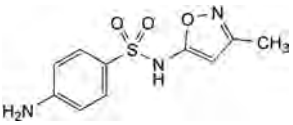

F. 4-amino-*N*-(3-methylisoxazol-5-yl)benzenesulfonamide.

**01/2008:0638**
**corrected 6.0**

# SULFAMETHOXYPYRIDAZINE FOR VETERINARY USE

## Sulfamethoxypyridazinum ad usum veterinarium

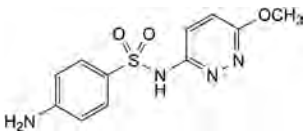

$C_{11}H_{12}N_4O_3S$ $M_r$ 280.3
[80-35-3]

DEFINITION

Sulfamethoxypyridazine for veterinary use contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(6-methoxypyridazin-3-yl)-benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, colouring slowly on exposure to light, practically insoluble in water, sparingly soluble in acetone, slightly soluble in alcohol, very slightly soluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

It melts at about 180 °C, with decomposition.

IDENTIFICATION

*First identification: A, B.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfamethoxypyridazine CRS*. Examine the substances prepared as discs.

B. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.5 g in 1 mL of a 40 per cent *V/V* solution of *sulfuric acid R*, heating gently. Continue heating until a crystalline precipitate appears (about 2 min). Cool and add 10 mL of *dilute sodium hydroxide solution R*. Cool again, add 25 mL of *ether R* and shake the solution for 5 min. Separate the ether layer, dry over *anhydrous sodium sulfate R* and filter. Evaporate the ether by heating in a water-bath. An oily residue is obtained which becomes crystalline on cooling; if necessary, scratch the wall of the container with a glass rod. The residue melts (*2.2.14*) at 102 °C to 106 °C.

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in a mixture of 10 mL of *1 M sodium hydroxide* and 15 mL of *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_4$ or $BY_4$ (*2.2.2, Method II*).

**Acidity**. To 1.25 g, finely powdered, add 25 mL of *carbon dioxide-free water R*. Heat at 70 °C for 5 min. Cool in iced water for about 15 min and filter. To 20 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.5 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin layer chromatography (*2.2.27*), using *TLC silica gel $GF_{254}$ plate R*.

*Test solution (a)*. Dissolve 0.10 g of the substance to be examined in *acetone R* and dilute to 5 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *acetone R*.

*Reference solution (a)*. Dissolve 20 mg of *sulfamethoxypyridazine CRS* in *acetone R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dilute 2.5 mL of test solution (b) to 50 mL with *acetone R*.

Apply separately to the plate 5 µL of each solution. Develop over a path of 15 cm using a mixture of 1 volume of *dilute ammonia R1*, 9 volumes of *water R*, 30 volumes of *2-propanol R* and 50 volumes of *ethyl acetate R*. Dry the plate at 100-105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Heavy metals** (*2.4.8*). 1.0 g complies with test D for heavy metals (20 ppm). Prepare the reference solution using 2 mL of *lead standard solution (10 ppm Pb) R*.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the assay of primary aromatic amino-nitrogen (*2.5.8*), using 0.2500 g, determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 28.03 mg of $C_{11}H_{12}N_4O_3S$.

STORAGE

Protected from light.

**01/2017:1571**

# SULFANILAMIDE

## Sulfanilamidum

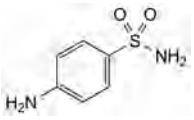

$C_6H_8N_2O_2S$ $M_r$ 172.2
[63-74-1]

DEFINITION

Sulfanilamide contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-aminobenzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

White or yellowish-white crystals or fine powder, slightly soluble in water, freely soluble in acetone, sparingly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 164.5 °C to 166.0 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfanilamide CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further acidification, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Solution S**. To 2.5 g add 50 mL of *carbon dioxide-free water R*. Heat at about 70 °C for about 5 min. Cool in iced water for about 15 min and filter.

**Acidity**. To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $F_{254}$ plate R*.

*Test solution (a)*. Dissolve 20 mg of the substance to be examined in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Test solution (b)*. Dissolve 0.10 g of the substance to be examined in 0.5 mL of *concentrated ammonia R* and dilute to 5 mL with *methanol R*. If the solution is not clear, heat gently until dissolution is complete.

*Reference solution (a).* Dissolve 20 mg of *sulfanilamide CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.25 mL of test solution (a) to 50 mL with a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R*.

*Reference solution (c).* Dissolve 20 mg of the substance to be examined and 20 mg of *sulfamerazine CRS* in 3 mL of a mixture of 2 volumes of *concentrated ammonia R* and 48 volumes of *methanol R* and dilute to 5 mL with the same mixture of solvents.

Apply to the plate 5 µL of each solution. Develop over a path corresponding to two-thirds of the plate height using a mixture of 3 volumes of *dilute ammonia R1*, 5 volumes of *water R*, 40 volumes of *nitromethane R* and 50 volumes of *dioxan R*. Dry the plate at 100 °C to 105 °C and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (b), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). The test is not valid unless the chromatogram obtained with reference solution (c) shows two clearly separated principal spots.

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using 0.140 g and determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 17.22 mg of $C_6H_8N_2O_2S$.

STORAGE

Store protected from light.

**01/2017:0863**
**corrected 9.2**

# SULFASALAZINE

## Sulfasalazinum

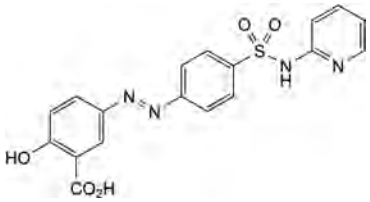

$C_{18}H_{14}N_4O_5S$ $M_r$ 398.4
[599-79-1]

DEFINITION

2-Hydroxy-5-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]-benzoic acid.

*Content*: 97.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: bright yellow or brownish-yellow, fine powder.

*Solubility*: practically insoluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *sulfasalazine CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *dilute ammonia R3* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *dilute ammonia R3*.

*Reference solution (b).* Dissolve 1.0 mg of *sulfasalazine derivative for resolution CRS* in 10.0 mL of reference solution (a). Dilute 1.0 mL of this solution to 10.0 mL with reference solution (a).

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: in a 1000 mL volumetric flask dissolve 1.13 g of *sodium dihydrogen phosphate R* and 2.5 g of *sodium acetate R* in 900 mL of *water R*; adjust to pH 4.8 with *glacial acetic acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: mobile phase A, *methanol R* (10:40 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 60 → 45 | 40 → 55 |
| 15 - 25 | 45 | 55 |
| 25 - 60 | 45 → 0 | 55 → 100 |
| 60 - 65 | 0 | 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 320 nm.

*Injection*: 20 µL.

*Relative retention* with reference to sulfasalazine: impurity H = about 0.16; impurity I = about 0.28; impurity C = about 0.80; impurity F = about 0.85; impurity G = about 1.39; impurity E = about 1.63; impurity B = about 1.85; impurity D = about 1.90; impurity A = about 2.00.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to sulfasalazine and sulfasalazine derivative for resolution.

*Limits*:

- *impurities A, B, C, D, E, F, G, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak with a retention time less than 6 min (due to impurities H and J).

**Impurities H and J**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *dilute ammonia R3* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *salicylic acid R* (impurity H) and 5.0 mg of *sulfapyridine CRS* (impurity J) in *dilute ammonia R3* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 2.0 mL of reference solution (a) to 100.0 mL with *dilute ammonia R3*.

*Column*:

– *size*: *l* = 0.25, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mobile phase B (described in the test for related substances), mobile phase A (described in the test for related substances) (30:70 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 20 µL of the test solution and reference solution (b).

*Run time*: 10 min.

*Retention time*: impurity H = about 6 min; impurity J = about 7 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 2 between the peaks due to impurities H and J.

*Limits*:

– *impurities H, J*: for each impurity, not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 140 ppm.

To 1.25 g add 50 mL of *distilled water R*. Heat at about 70 °C for 5 min. Cool and filter. To 20 mL of the filtrate add 1 mL of *nitric acid R*, allow to stand for 5 min and filter to obtain a clear solution.

**Sulfates** (*2.4.13*): maximum 400 ppm.

To 20 mL of the filtrate prepared for the test for chlorides add 1 mL of *dilute hydrochloric acid R*, allow to stand for 5 min and filter.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in *0.1 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Transfer 5.0 mL of this solution to a 1000 mL volumetric flask containing about 750 mL of *water R*. Add 20.0 mL of a 6 g/L solution of *glacial acetic acid R* and dilute to 1000.0 mL with *water R*. Prepare a standard solution at the same time and in the same manner using 0.150 g of *sulfasalazine CRS*. Measure the absorbance (*2.2.25*) of the 2 solutions at the absorption maximum at 359 nm.

Calculate the content of $C_{18}H_{14}N_4O_5S$ from the absorbances measured and the concentration of the solutions.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F, G, H, I, J.*

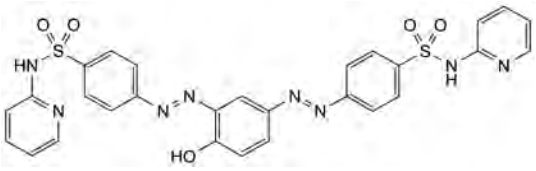

A. 4,4′-[(4-hydroxy-1,3-phenylene)bis(diazenediyl)]bis[*N*-(pyridin-2-yl)benzenesulfonamide],

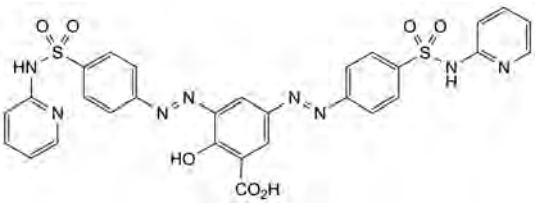

B. 2-hydroxy-3,5-bis[2-[4-(pyridin-2-ylsulfamoyl)phenyl]-diazenyl]benzoic acid,

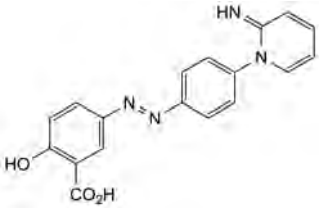

C. 2-hydroxy-5-[2-[4-(2-iminopyridin-1(2*H*)-yl)phenyl]-diazenyl]benzoic acid,

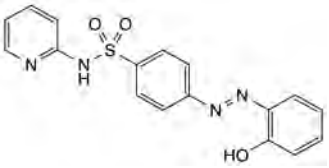

D. 4-[2-(2-hydroxyphenyl)diazenyl]-*N*-(pyridin-2-yl)benzenesulfonamide,

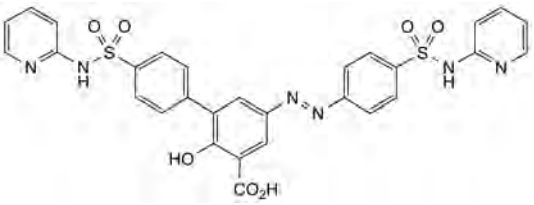

E. 2-hydroxy-4′-(pyridin-2-ylsulfamoyl)-5-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]diazenyl]biphenyl-3-carboxylic acid,

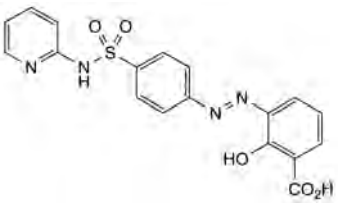

F. 2-hydroxy-3-[2-[4-(pyridin-2-ylsulfamoyl)phenyl]-diazenyl]benzoic acid,

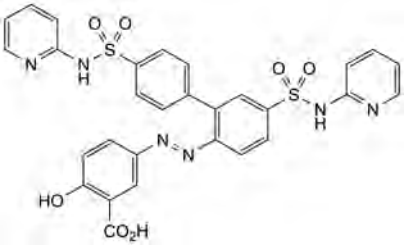

G. 5-[2-[4′,5-bis(pyridin-2-ylsulfamoyl)biphenyl-2-yl]diazenyl]-2-hydroxybenzoic acid,

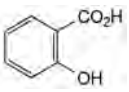

H. 2-hydroxybenzenecarboxylic acid (salicylic acid),

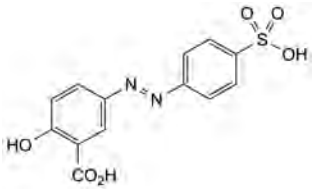

I. 2-hydroxy-5-[2-(4-sulfophenyl)diazenyl]benzoic acid,

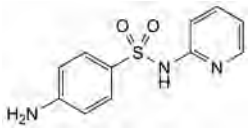

J. 4-amino-*N*-(pyridin-2-yl)benzenesulfonamide (sulfapyridine).

**01/2017:0742**

# SULFATHIAZOLE

## Sulfathiazolum

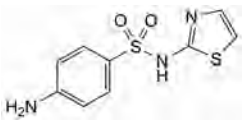

$C_9H_9N_3O_2S_2$ $M_r$ 255.3
[72-14-0]

DEFINITION

Sulfathiazole contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-amino-*N*-(thiazol-2-yl)benzenesulfonamide, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, practically insoluble in water, slightly soluble in alcohol, practically insoluble in methylene chloride. It dissolves in dilute solutions of alkali hydroxides and in dilute mineral acids.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 200 °C to 203 °C. Melting may occur at about 175 °C, followed by solidification and a second melting between 200 °C and 203 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *sulfathiazole CRS*. Examine the substances prepared as discs. If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *alcohol R*, evaporate to dryness *in vacuo* and record the spectra again using the residues.

C. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 10 mg in a mixture of 10 mL of *water R* and 2 mL of *0.1 M sodium hydroxide* and add 0.5 mL of *copper sulfate solution R*. A greyish-blue or purple precipitate is formed.

E. Dissolve about 5 mg in 10 mL of *1 M hydrochloric acid*. Dilute 1 mL of the solution to 10 mL with *water R*. The solution, without further addition of acid, gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. Dissolve 1.0 g in 10 mL of *1 M sodium hydroxide*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_4$ (*2.2.2, Method II*).

**Acidity**. To 1.0 g add 50 mL of *carbon dioxide-free water R*. Heat to 70 °C for 5 min. Cool rapidly to 20 °C and filter. To 25 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel H R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 10 mL with the same mixture of solvents.

*Test solution (b).* Dilute 1 mL of test solution (a) to 5 mL with a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R*.

*Reference solution (a).* Dissolve 20 mg of *sulfathiazole CRS* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (b).* Dissolve 50 mg of *sulfanilamide R* in a mixture of 1 volume of *concentrated ammonia R* and 9 volumes of *alcohol R* and dilute to 100 mL with the same mixture of solvents. Dilute 1 mL of this solution to 10 mL with the same mixture of solvents.

Apply to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 18 volumes of *ammonia R* and 90 volumes of *butanol R*. Dry the plate at 100 °C to 105 °C for 10 min and spray with a 1 g/L solution of *dimethylaminobenzaldehyde R* in *alcohol R* containing 1 per cent *V/V* of *hydrochloric acid R*. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using 0.200 g, determining the end-point electrometrically.

1 mL of *0.1 M sodium nitrite* is equivalent to 25.53 mg of $C_9H_9N_3O_2S_2$.

STORAGE

Store protected from light.

**01/2017:0790**

# SULFINPYRAZONE

## Sulfinpyrazonum

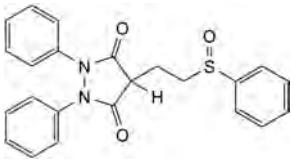

$C_{23}H_{20}N_2O_3S$ $M_r$ 404.5
[57-96-5]

DEFINITION

1,2-Diphenyl-4-[2-(phenylsulfinyl)ethyl]pyrazolidine-3,5-dione.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, sparingly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.16*): 131 °C to 135 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 30.0 mg in *0.01 M sodium hydroxide* and dilute to 100.0 mL with the same alkaline solution. Dilute 1.0 mL of this solution to 20.0 mL with *0.01 M sodium hydroxide*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 260 nm.

*Specific absorbance at the absorption maximum*: 530 to 580.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sulfinpyrazone CRS*.

D. Dissolve about 10 mg in 3 mL of *acetone R* and add a mixture of 0.2 mL of *ferric chloride solution R2* and 3 mL of *water R*. A red to violet colour develops.

TESTS

**Appearance of solution in acetone**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm using a path length of 4 cm is not greater than 0.10.

Dissolve 1.25 g in *acetone R* and dilute to 25 mL with the same solvent.

**Appearance of solution in 1 M sodium hydroxide**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm using a path length of 4 cm is not greater than 0.15.

Dissolve 1.25 g, heating gently if necessary, in 25 mL of *1 M sodium hydroxide*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (10:40 *V/V*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5.0 mg of *sulfinpyrazone impurity A CRS* and 5.0 mg of *sulfinpyrazone impurity B CRS* in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve the contents of a vial of *sulfinpyrazone for system suitability CRS* (containing impurity C) in 1.0 mL of the solvent mixture.

*Reference solution (e).* Dilute 1.0 mL of reference solution (a) to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *tetrahydrofuran for chromatography R*, *acetonitrile R*, 0.3 per cent *V/V* solution of *phosphoric acid R* (7:35:58 *V/V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 235 nm.

*Injection*: 20 µl.

*Run time*: 7 times the retention time of sulfinpyrazone.

*Identification of impurities*: use the chromatogram supplied with *sulfinpyrazone for system suitability CRS* and the chromatogram obtained with reference solution (d) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and B.

*Relative retention* with reference to sulfinpyrazone (retention time = about 3.5 min): impurity C = about 0.8; impurity A = about 1.6; impurity B = about 4.8.

*System suitability*: reference solution (d):

– *resolution*: minimum 2.0 between the peaks due to impurity C and sulfinpyrazone.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (e) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (e) (0.10 per cent);
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (e) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 25 mL of *acetone R*. Add 0.5 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide* until the colour changes from yellow to blue.

1 mL of *0.1 M sodium hydroxide* is equivalent to 40.45 mg of $C_{23}H_{20}N_2O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

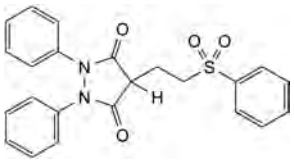

A. 1,2-diphenyl-4-[2-(phenylsulfonyl)ethyl]pyrazolidine-3,5-dione,

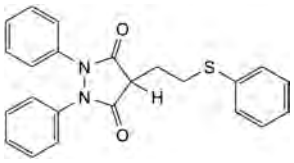

B. 1,2-diphenyl-4-[2-(phenylsulfanyl)ethyl]pyrazolidine-3,5-dione,

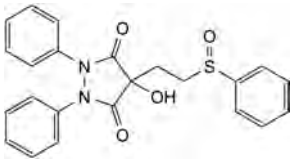

C. 4-hydroxy-1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-pyrazolidine-3,5-dione.

**07/2019:2804**
**corrected 10.0**

# SULFOBUTYLBETADEX SODIUM

## Sulfobutylbetadexum natricum

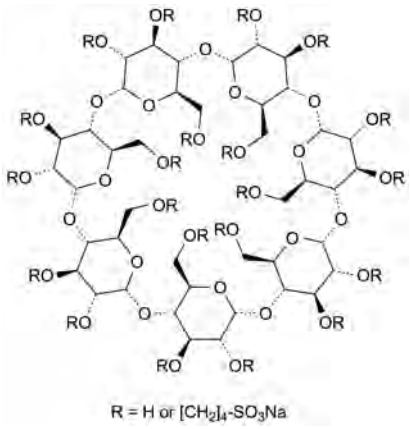


$C_{42}H_{70-n}O_{35}(C_4H_8SO_3Na)_n$ $M_r$ 2163 when $n$ = 6.5
[182410-00-0]

DEFINITION

Sulfobutylbetadex sodium (β-cyclodextrin, sodium 4-sulfonatobutyl ether) is the sodium salt of a partially substituted poly(sulfobutyl) ether of betadex.

*Content*:

– *sulfobutylbetadex sodium*: 95.0 per cent to 105.0 per cent (anhydrous substance);
– *average number of sulfobutyl groups per cyclodextrin ring, expressed as average degree of substitution (DS)*: 5.9 to 6.6.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sulfobutylbetadex sodium CRS*.

*Results*: the spectrum obtained with the substance to be examined shows the same absorption bands as the spectrum obtained with *sulfobutylbetadex sodium CRS*. Due to differences in the degree of substitution of the substance, the relative intensity of some absorption bands may vary.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 7.50 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.5 for solution S.

Use intensive nitrogen purging to avoid carbon dioxide absorption during measurement.

**Reducing sugars**: maximum 0.05 per cent.

*Reagent solution.* Dissolve 1.0 g of *triphenyltetrazolium chloride R* in 100 mL of *aldehyde-free methanol R*.

*Test solution.* Dissolve 0.45 g of the substance to be examined in 2.0 mL of *dimethyl sulfoxide R* in a test tube and add 0.5 mL of a 40 g/L solution of *sodium hydroxide R* and 7.5 mL of the reagent solution. Mix and store at room temperature.

*Reference solution.* Prepare at the same time and in the same manner as for the test solution, using 0.10 mL of a 2.25 g/L solution of *glucose R* instead of the substance to be examined.

After 1 h, measure the absorbance (*2.2.25*) of the solutions at the absorption maximum at 482 nm. The absorbance of the test solution is not greater than that of the reference solution.

**Impurities A, C and D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.300 g of the substance to be examined in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 15.0 mg of *betadex CRS* (impurity A) in *water R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 50.0 mL with *water R*.

*Reference solution (b).* Dissolve 15.0 mg of *sulfobutylbetadex impurity C CRS* in *water R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Reference solution (c).* Dissolve 15.0 mg of *sulfobutylbetadex impurity D CRS* in *water R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*.

*Reference solution (d).* Dissolve 0.300 g of *sulfobutylbetadex sodium CRS* in 1.0 mL of reference solution (b) and 0.5 mL of reference solution (c), and dilute to 5.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.0 mm;
– *stationary phase*: *4-dimethylaminobenzylcarbamidesilyl silica gel for chromatography R* (3 µm);
– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: to 4.0 mL of *triethylamine R* add 900 mL of *water for chromatography R* and adjust to pH 4.5 with *anhydrous formic acid R*; dilute to 1000 mL with *water for chromatography R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 8 | 90 → 60 | 10 → 40 |
| 8 - 14 | 60 → 40 | 40 → 60 |
| 14 - 20 | 40 | 60 |

*Flow rate*: 1.1 mL/min.

*Detection*: evaporative light-scattering detector; the following settings have been found to be suitable; if the detector has different setting parameters, adjust the detector settings so as to comply with the system suitability criteria.

– *carrier gas*: *nitrogen R*;

– *flow rate*: 1.5 L/min;

– *evaporator temperature*: 50 °C.

*Equilibration*: at initial isocratic conditions for 10 min.

*Injection*: 10 µL of the test solution and reference solutions (a) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C and D.

*Relative retention* with reference to impurity A (retention time = about 2.9 min): impurity C = about 0.7; impurity D = about 2.0; sulfobutylbetadex elutes as several peaks after impurity D unless heart-cutting is applied.

*System suitability*:

– *resolution*: minimum 2.0 between the peaks due to impurities C and A in the chromatogram obtained with reference solution (d);

– *repeatability*: maximum relative standard deviation of 3.0 per cent for the area of the peak due to impurity A determined on 5 injections of reference solution (a), using the initial isocratic conditions for 4 min.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.1 per cent);

– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.05 per cent);

– disregard the peak due to the sodium counter-ion (corresponding to the dead volume of the column) and any peak eluting after impurity D (due to sulfobutylbetadex).

**Impurity B**. Gas chromatography (*2.2.28*). *Prepare the solutions immediately before use.*

*Internal standard solution.* Dissolve 25.0 mg of *diethyl sulfone R* in 10.0 mL of *dimethylformamide R* and dilute to 1000.0 mL with *water R*. Dilute 1.0 mL of the solution to 100.0 mL with *water R*.

*Test solutions (a), (b), (c) and (d).* In 4 test tubes with screw caps, prepare 4 solutions as indicated in Table 2804.-1. Mix each test tube on a vortex mixer for 30 s and allow to stand for at least 5 min or until complete separation of the phases. Decant the organic phase into a vial and seal.

*Reference solution (a).* Dissolve 50.0 mg of *sulfobutylbetadex impurity B CRS* in 5.0 mL of *dimethylformamide R* and dilute with *water R* to obtain a concentration of 2.0 µg/mL.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 2.0 mL with *water R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 4.0 mL with *water R*.

*Blank solution.* Prepare as described for test solution (d), but omitting the substance to be examined.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 2.4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 100 → 200 |
| | 10 - 11.5 | 200 → 250 |
| | 11.5 - 16.5 | 250 |
| Injection port | | 250 |
| Detector | | 270 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to the internal standard (retention time = about 6.5 min): impurity B = about 1.4.

*System suitability*:

– *repeatability*: maximum relative standard deviation of 10.0 per cent for the corrected ratios (as described under Calculation of content) determined on 5 injections of test solution (b).

*Calculation of content*: calculate the ratio of the area of the peak due to impurity B to the area of the peak due to the internal standard in the chromatograms obtained with test solutions (a), (b), (c) and (d) respectively. Subtract from these values the ratio of the area of the peak due to impurity B to the area of the peak due to the internal standard in the chromatogram obtained with the blank solution (diethyl sulfone may contain trace amounts of impurity B). Plot the

Table 2804.-1.

| | Substance to be examined (g) | Internal standard solution (mL) | Solvent/solution (mL) | *Methylene chloride R* (mL) | Final content of *sulfobutylbetadex impurity B CRS* (µg) |
|---|---|---|---|---|---|
| *Blank solution* | 0 | 4.0 | *Water R*, 1.0 | 1.0 | 0 |
| *Test solution (a)* | 1.000 | 4.0 | Reference solution (a), 1.0 | 1.0 | 2.0 |
| *Test solution (b)* | 1.000 | 4.0 | Reference solution (b), 1.0 | 1.0 | 1.0 |
| *Test solution (c)* | 1.000 | 4.0 | Reference solution (c), 1.0 | 1.0 | 0.5 |
| *Test solution (d)* | 1.000 | 4.0 | *Water R*, 1.0 | 1.0 | 0 |

added quantity of impurity B (in micrograms) as the abscissa and the corrected ratios as the ordinates. Extrapolate the line joining the points on the graph until it meets the *x*-axis. The distance between this point and the origin corresponds to the content of impurity B in micrograms per gram (ppm) of the substance to be examined.

*Limit*:

– *impurity B*: maximum 0.5 ppm.

**Chlorides** (*2.4.4*): maximum 0.12 per cent (corresponding to 0.20 per cent expressed as sodium chloride).

Dissolve a quantity of the substance to be examined corresponding to 42 mg of anhydrous substance in 15.0 mL of *water R*.

**Water** (*2.5.12*): maximum 10.0 per cent, determined on 0.200 g.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

– TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
– TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
– absence of *Escherichia coli* (*2.6.13*);
– absence of *Salmonella* (*2.6.13*).

ASSAY

**Sulfobutylbetadex sodium**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution.* Dissolve 0.100 g of *sulfobutylbetadex sodium CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.30 m, Ø = 7.8 mm;
– *stationary phase*: *polymethacrylate gel R*.

*Mobile phase*: dissolve 10.11 g of *potassium nitrate R* in a mixture of 1 volume of *acetonitrile for chromatography R* and 4 volumes of *water for chromatography R* and dilute to 1000 mL with the same mixture of solvents.

*Flow rate*: 1.0 mL/min.

*Detection*: differential refractometer maintained at 35 ± 2 °C.

*Injection*: 20 µL.

*Between-run rinsing*: rinse the column with a mixture of 1 volume of *acetonitrile for chromatography R* and 9 volumes of *water for chromatography R*.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 2.0 per cent for the area of the peak due to sulfobutylbetadex, determined on 5 injections.

Calculate the percentage content of sulfobutylbetadex sodium taking into account the assigned content of *sulfobutylbetadex sodium CRS*.

**Average degree of substitution**. Nuclear magnetic resonance spectrometry (*2.2.33*).

The average degree of substitution (*DS*) is calculated from the ratio between the signals of the inner-chain protons of the sulfobutyl ether groups (-O-$CH_2$-**$CH_2$**-**$CH_2$**-$CH_2$-$SO_3H$) and the signals of the glycosidic protons attached to the C1 carbon of the anhydroglucose units.

*Test solution.* Introduce not less than 10.0 mg of the substance to be examined, previously dried for at least 1 day in a desiccator, into a 5 mm NMR tube. Add about 0.70 mL of *deuterium oxide R1*. Cap the tube and mix thoroughly.

*Apparatus*: FT-NMR spectrometer operating at minimum 250 MHz and suitable for recording at 25 °C.

*Acquisition of $^1H$ NMR spectra.* The following parameters may be used:

– *sweep width*: 10 ppm (0 to + 10.0 ppm);
– *irradiation frequency offset*: none;
– *time domain*: 32 K;
– *pulse width*: 90°;
– *pulse delay*: 15 s;
– *dummy scans*: 2;
– *number of scans*: 16.

Call the integration sub-routine after phase corrections and baseline correction between − 1 ppm and + 9 ppm.

The signal of exchangeable protons (solvent) at + 4.8 ppm is used as reference.

Make a zero filling at least twice in size relative to the acquisition data file and transform the FID to the spectrum without any correction of the Gaussian broadening factor (GB = 0) and with a line broadening factor not greater than 0.3 Hz (LB ≤ 0.3). Adjust integral level and tilting.

Measure the peak areas of the signals between 1.43 ppm and 2.12 ppm ($A_1$, -O-$CH_2$-**$CH_2$**-**$CH_2$**-$CH_2$-$SO_3H$), and the signals of the glycosidic protons between 4.95 ppm and 5.41 ppm ($A_2$, -O-**CH**-O-).

Calculate the average degree of substitution (*DS*) using the following expression:

$$\frac{7 \times A_1}{4 \times A_2}$$

$A_1$ = area of the peaks due to the inner methylene protons (indicated in bold) of the sulfobutyl ether functional group;

$A_2$ = area of the peaks due to the glycosidic protons (indicated in bold).

STORAGE

In an airtight container.

LABELLING

The label states:

– the average degree of substitution (*DS*);
– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C, D.*

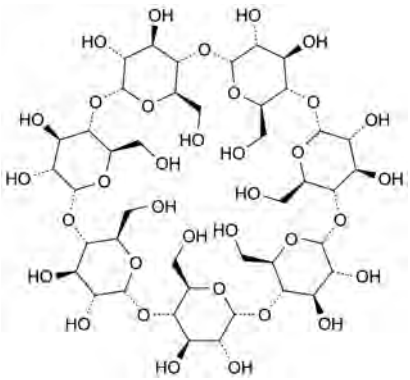

A. cycloheptakis-(1→4)-(α-D-glucopyranosyl) (betadex or cyclomaltoheptaose or β-cyclodextrin),

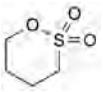

B. 1,2λ⁶-oxathiane-2,2-dione,

C. 4-hydroxybutane-1-sulfonic acid,

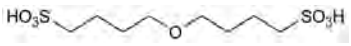

D. 4,4′-oxydi(butane-1-sulfonic acid).

**01/2008:0953**

# SULFUR FOR EXTERNAL USE

## Sulfur ad usum externum

S $A_r$ 32.07
[7704-34-9]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: yellow powder.

*Solubility*: practically insoluble in water, soluble in carbon disulfide, slightly soluble in vegetable oils.

mp: about 120 °C.

The size of most of the particles is not greater than 20 µm and that of almost all the particles is not greater than 40 µm.

IDENTIFICATION

A. Heated in the presence of air, it burns with a blue flame, emitting sulfur dioxide which changes the colour of moistened *blue litmus paper R* to red.

B. Heat 0.1 g with 0.5 mL of *bromine water R* until decolourised. Add 5 mL of *water R* and filter. The solution gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. To 5 g add 50 mL of *carbon dioxide-free water R* prepared from *distilled water R*. Allow to stand for 30 min with frequent shaking and filter.

**Appearance of solution**. Solution S is colourless (*2.2.2, Method II*).

**Odour** (*2.3.4*). It has no perceptible odour of hydrogen sulfide.

**Acidity or alkalinity**. To 5 mL of solution S add 0.1 mL of *phenolphthalein solution R1*. The solution is colourless. Add 0.2 mL of *0.01 M sodium hydroxide*. The solution is red. Add 0.3 mL of *0.01 M hydrochloric acid*. The solution is colourless. Add 0.15 mL of *methyl red solution R*. The solution is orange-red.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Sulfides**. To 10 mL of solution S add 2 mL of *buffer solution pH 3.5 R* and 1 mL of a freshly prepared 1.6 g/L solution of *lead nitrate R* in *carbon dioxide-free water R*. Shake. After 1 min any colour in the solution is not more intense than that in a reference solution prepared at the same time using 1 mL of *lead standard solution (10 ppm Pb) R*, 9 mL of *carbon dioxide-free water R*, 2 mL of *buffer solution pH 3.5 R* and 1.2 mL of *thioacetamide reagent R*.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Carry out the oxygen-flask method (*2.5.10*), using 60.0 mg in a 1000 mL combustion flask. Absorb the combustion products in a mixture of 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of *water R*. Heat to boiling, boil gently for 2 min and cool. Using 0.2 mL of *phenolphthalein solution R* as indicator, titrate with *0.1 M sodium hydroxide* until the colour changes from colourless to red. Carry out a blank titration under the same conditions.

1 mL of *0.1 M sodium hydroxide* is equivalent to 1.603 mg of S.

STORAGE

Protected from light.

**01/2017:1572**

# SULFURIC ACID

## Acidum sulfuricum

$H_2SO_4$ $M_r$ 98.1
[7664-93-9]

DEFINITION

*Content*: 95.0 per cent *m/m* to 100.5 per cent *m/m*.

CHARACTERS

*Appearance*: colourless, oily liquid, very hygroscopic.

*Solubility*: miscible with water and with ethanol (96 per cent) producing intense heat.

*Relative density*: about 1.84.

IDENTIFICATION

A. Carefully add 1 mL to 100 mL of *water R*. The solution is strongly acid (*2.2.4*).

B. The solution obtained in identification test A gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Carefully pour, while cooling, 5 mL into 30 mL of *water R* and dilute to 50 mL with the same solvent.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Mix carefully, while cooling, 3.3 g with 30 mL of *water R*. Neutralise with *ammonia R* and dilute to 50 mL with *water R*.

**Nitrates**. Add 5 mL to 5 mL of *water R*. Cool to room temperature and add 0.5 mL of *indigo carmine solution R*. The blue colour persists for at least 1 min.

**Arsenic** (*2.4.2, Method A*): maximum 1 ppm.

Mix, while cooling, 1 g with 20 mL of *water R* and dilute to 25 mL with the same solvent.

**Iron** (*2.4.9*): maximum 25 ppm.

Cautiously evaporate 10.0 g and ignite to dull redness. Dissolve the ignition residue in 1 mL of *dilute hydrochloric acid R* with gentle heating and dilute to 25 mL with *water R*. Dilute 1 mL of this solution to 10 mL with *water R*.

ASSAY

Weigh accurately a ground-glass-stoppered flask containing 30 mL of *water R*. Introduce 0.2 mL, cool and weigh again. Titrate with *1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M sodium hydroxide* is equivalent to 49.04 mg of $H_2SO_4$.

STORAGE

In an airtight container.

**01/2017:0864**

# SULINDAC

## Sulindacum

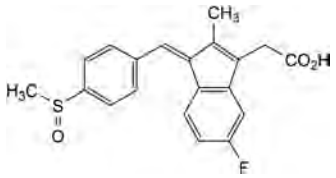


$C_{20}H_{17}FO_3S$ $M_r$ 356.4

[38194-50-2]

DEFINITION

(*Z*)-[5-Fluoro-2-methyl-1-[4-(methylsulfinyl)benzylidene]-1*H*-inden-3-yl]acetic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in methylene chloride, sparingly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 182 °C to 186 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in a 0.3 per cent *V/V* solution of *hydrochloric acid R* in *methanol R* and dilute to 100 mL with the same acid solution. Dilute 2 mL of this solution to 50 mL with a 0.3 per cent *V/V* solution of *hydrochloric acid R* in *methanol R*.

*Spectral range*: 230-350 nm.

*Absorption maxima*: at 284 nm and 327 nm.

*Shoulder*: at about 258 nm.

*Absorbance ratio*: $A_{284}/A_{327}$ = 1.10 to 1.20.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *sulindac CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of hot *methanol R*, evaporate to dryness and record new spectra using the residues.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *sulindac CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *diflunisal CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 2 mL with reference solution (a).

*Plate*: *TLC silica gel $GF_{254}$ plate R.*

*Mobile phase*: *glacial acetic acid R, methylene chloride R, acetone R* (1:49:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

E. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. Add 1.0 mL of the filtrate to a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*. Mix, allow to stand for 5 min and compare the colour of the solution with that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 20.0 mg of *sulindac CRS* (which has an assigned content of (*E*)-isomer) in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (10 µm).

*Mobile phase*: *glacial acetic acid R, ethanol (96 per cent) R, ethyl acetate R, ethanol-free chloroform R* (1:4:100:400 *V/V/V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of sulindac.

*Identification of peaks*: the chromatogram obtained with reference solution (b) shows a principal peak due to sulindac and a peak due to the (*E*)-isomer.

*Relative retention* with reference to sulindac: (*E*)-isomer = about 1.75.

From the chromatograms obtained with the test solution and reference solution (b), determine the percentage content of (*E*)-isomer, taking into account the assigned content of this isomer in *sulindac CRS*.

*Limits*:

– (*E*)-isomer: maximum 0.5 per cent;

– *impurities A, B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *methanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 35.64 mg of $C_{20}H_{17}FO_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C.*

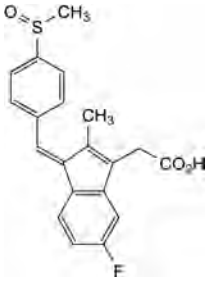

A. (*E*)-[5-fluoro-2-methyl-1-[4-(methylsulfinyl)benzylidene]-1*H*-inden-3-yl]acetic acid,

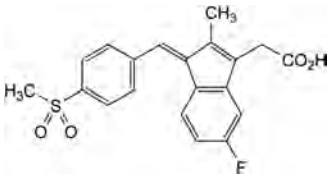

B. (*Z*)-[5-fluoro-2-methyl-1-[4-(methylsulfonyl)-benzylidene]-1*H*-inden-3-yl]acetic acid,

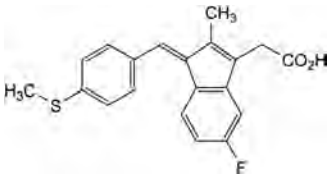

C. (*Z*)-[5-fluoro-2-methyl-1-[4-methylsulfanyl)-benzylidene]-1*H*-inden-3-yl]acetic acid.

**01/2017:1045**

# SULPIRIDE

## Sulpiridum

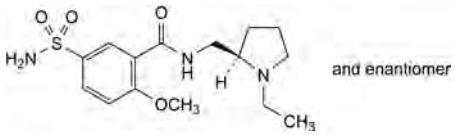


$C_{15}H_{23}N_3O_4S$ $M_r$ 341.4

[15676-16-1]

DEFINITION

*N*-[[(2*RS*)-1-Ethylpyrrolidin-2-yl)]methyl]-2-methoxy-5-sulfamoylbenzamide.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent) and in methylene chloride. It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 177 °C to 181 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *sulpiride CRS*.

C. Examine the chromatograms obtained in the test for impurity A.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To about 1 mg in a porcelain dish, add 0.5 mL of *sulfuric acid R* and 0.05 mL of *formaldehyde solution R*. Examined in ultraviolet light at 365 nm, the solution shows blue fluorescence.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method I*).

Dissolve 1.0 g in *dilute acetic acid R* and dilute to 10 mL with the same acid.

**Impurity A.** Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.20 g of the substance to be examined in *methanol R*, sonicate until dissolution is complete and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *sulpiride CRS* in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 5.0 mg of *sulpiride impurity A CRS* in *methanol R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *dioxan R*, *methanol R*, *methylene chloride R* (2:10:14:90 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 1/2 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm for identification test C and then spray with *ninhydrin solution R*; heat at 100-105 °C for 15 min and examine in daylight.

*Limit*: test solution (a):

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined and 5 mg of *sulpiride impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of *acetonitrile R*, 10 volumes of *methanol R* and 80 volumes of a solution containing 6.8 g/L of *potassium dihydrogen phosphate R* and 1 g/L of *sodium octanesulfonate R*, previously adjusted to pH 3.3 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of sulpiride.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to sulpiride (retention time = about 15 min): impurity B = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurity B and sulpiride.

*Calculation of percentage contents*:

– for each impurity, use the concentration of sulpiride in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.3 per cent;
– *reporting threshold*: 0.05 per cent.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Shake 1.0 g with 20 mL of *water R*. Filter through a sintered-glass filter (40) (*2.1.2*). To 10 mL of the filtrate add 5 mL of *water R*.

**Iron** (*2.4.9*): maximum 10 ppm.

Ignite 1.0 g in a silica crucible. To the residue add 1 mL of *1 M hydrochloric acid*, 3 mL of *water R* and 0.1 mL of *nitric acid R*. Heat on a water-bath for about 5 min. Place the solution in a test-tube. Rinse the crucible with 4 mL of *water R*. Collect the rinsings in the test-tube and dilute to 10 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 80 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.14 mg of $C_{15}H_{23}N_3O_4S$.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G.*

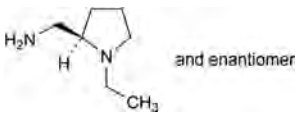


A. [(2*RS*)-1-ethylpyrrolidin-2-yl]methanamine,

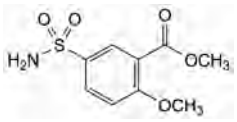


B. methyl 2-methoxy-5-sulfamoylbenzoate,

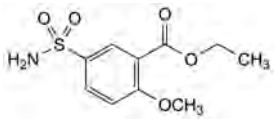


C. ethyl 2-methoxy-5-sulfamoylbenzoate,

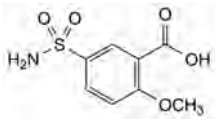


D. 2-methoxy-5-sulfamoylbenzoic acid,

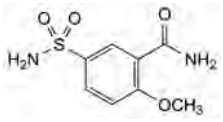


E. 2-methoxy-5-sulfamoylbenzamide,

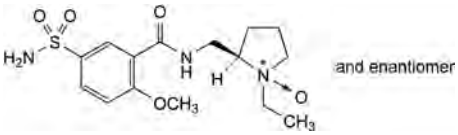


F. *N*-[[(2*RS*)-1-ethyl-1-oxidopyrrolidin-2-yl]methyl]-2-methoxy-5-sulfamoylbenzamide,

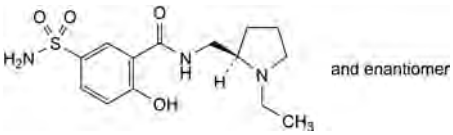


G. *N*-[[(2*RS*)-1-éthylpyrrolidin-2-yl]méthyl]-2-hydroxy-5-sulfamoylbenzamide.

**01/2017:2211**
**corrected 10.0**

# SULTAMICILLIN

## Sultamicillinum

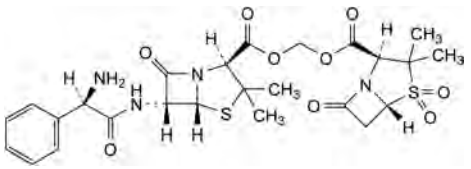


$C_{25}H_{30}N_4O_9S_2$ $M_r$ 594.7
[76497-13-7]

DEFINITION

Methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-aminophenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: practically insoluble in water, very slightly soluble in methanol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sultamicillin CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 190 to + 210 (anhydrous substance).

Dissolve 0.500 g in *dimethylformamide R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or keep at 2-8 °C for not more than 6 h.*

*Solution A*: *methanol R1*, *acetonitrile R1* (20:80 *V/V*).

*Solution B*. Dissolve 1.56 g of *sodium dihydrogen phosphate R* in 900 mL of *water R*. Add 7.0 mL of *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Blank solution*: solution B, solution A (30:70 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 35 mL of solution A and sonicate for about 1 min. Add 13 mL of solution B, mix and sonicate for about 1 min. Dilute to 50.0 mL with solution B and mix.

*Reference solution (a)*. Dissolve 70.0 mg of *sultamicillin tosilate CRS* in 35 mL of solution A and sonicate for about 1 min. Add 13 mL of solution B, mix and sonicate for about 1 min. Dilute to 50.0 mL with solution B and mix.

*Reference solution (b)*. Suspend 15 mg of *sultamicillin tosilate CRS* in 20 mL of a 0.4 g/L solution of *sodium hydroxide R* and sonicate in an ultrasonic bath for about 5 min. Add 20 mL of a 0.36 g/L solution of *hydrochloric acid R* and dilute to 100 mL with *water R*.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (a) to 100.0 mL with the blank solution.

*Reference solution (d)*. Dissolve 17.3 mg of *ampicillin trihydrate CRS* (impurity C) and 15.0 mg of *sulbactam CRS* (impurity A) in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (e)*. Dissolve 5 mg of *sultamicillin for peak identification CRS* (containing impurity G) in 7.0 mL of solution A and sonicate for about 1 min. Dilute to 10.0 mL with solution B, mix and sonicate for about 1 min.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 4.68 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 95 → 30 | 5 → 70 |
| 15 - 16 | 30 | 70 |
| 16 - 16.5 | 30 → 95 | 70 → 5 |
| 16.5 - 20 | 95 | 5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the blank solution, the test solution and reference solutions (b), (c), (d) and (e).

*Identification of impurities*: use the chromatogram supplied with *sultamicillin for peak identification CRS* and the chromatogram obtained with reference solution (e) to identify the peak due to impurity G.

*Relative retention* with reference to sultamicillin (retention time = about 9.3 min): impurity A = about 0.41; ampicillin penicilloic acid = about 0.47; impurity B = about 0.50; impurity C = about 0.55; impurity D = about 0.94; impurity E = about 1.09; impurity F = about 1.26; impurity G = about 1.42.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to ampicillin penicilloic acid and impurity B and minimum 2.5 between the peaks due to impurities B and C.

*Limits*:

- *impurity G*: not more than the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.3 per cent);
- *impurities D, E, F*: for each impurity, not more than 0.3 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *any other impurity*: for each impurity, not more than 0.3 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *total*: not more than 3 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (3.0 per cent);
- *disregard limit*: 0.1 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Ethyl acetate**. Head-space gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.200 g in 7.0 mL of a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R*.

*Reference solution*. Dissolve 0.200 g of *ethyl acetate R* in 240 mL of a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R* and dilute to 250.0 mL with the same mixture of solvents. Dilute 5.0 mL of this solution to 7.0 mL with a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R*.

*Close the vials immediately with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous solution.*

*Column*:

- *material*: fused silica;
- *size*: *l* = 50 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness: 1.8 µm or 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:5.

*Static head-space conditions that may be used:*

- *equilibration temperature*: 105 °C;
- *equilibration time*: 45 min;

– *transfer-line temperature*: 110 °C;

– *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 70 |
| | 6 - 16 | 70 → 220 |
| | 16 - 18 | 220 |
| Injection port | | 140 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 mL.

*Relative retention* with reference to dimethylformamide (retention time = about 14 min): ethyl acetate = about 0.7.

*Limit*:

– *ethyl acetate*: maximum 2.5 per cent.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of sultamicillin ($C_{25}H_{30}N_4O_9S_2$) from the declared content of $C_{25}H_{30}N_4O_9S_2$ in *sultamicillin tosilate CRS* and by multiplying the sultamicillin tosilate content by 0.7752.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

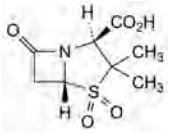

A. (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid 4,4-dioxide (sulbactam),

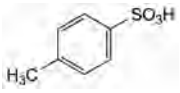

B. 4-methylbenzenesulfonic acid (*p*-toluenesulfonic acid),

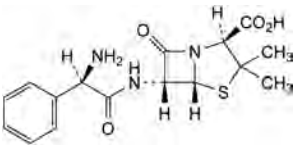

C. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ampicillin),

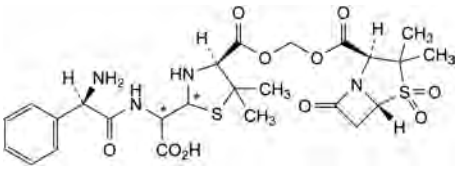

D. [[(2*R*)-aminophenylacetyl]amino][(4*S*)-4-[[[[[(2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]-hept-2-yl]carbonyl]oxy]methoxy]carbonyl]-5,5-dimethyl-thiazolidin-2-yl]acetic acid (penicilloic acids of sultamicillin),

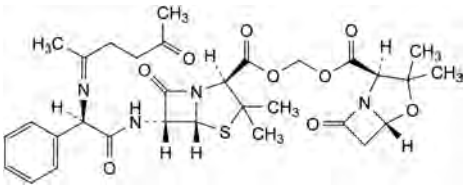

E. methylene (2*S*,5*R*,6*R*)-3,3-dimethyl-6-[[(2*R*)-[(1-methyl-4-oxopentylidene)amino]phenylacetyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate,

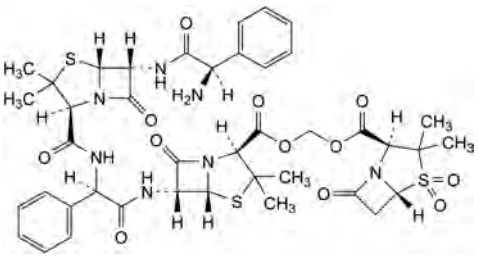

F. methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-aminophenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylate (ampicillin sultamicillin amide),

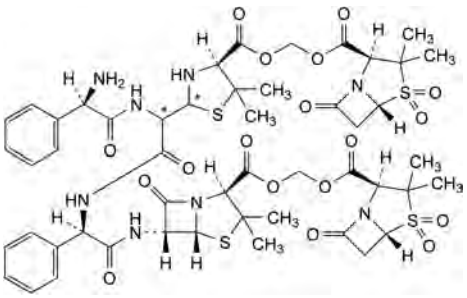

G. methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-[[[[(2*R*)-aminophenyl-acetyl]amino][(4*S*)-4-[[[[[(2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]-oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetyl]amino]phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo-[3.2.0]heptane-2-carboxylate (sultamicillin dimer).

01/2017:2212
corrected 10.0

# SULTAMICILLIN TOSILATE DIHYDRATE

## Sultamicillini tosilas dihydricus

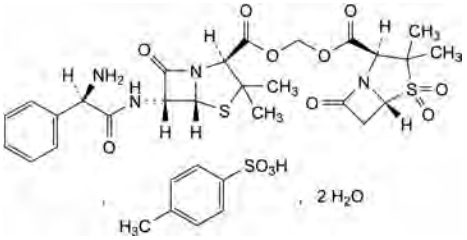

$C_{32}H_{38}N_4O_{12}S_3,2H_2O$ $M_r$ 803

DEFINITION

4-Methylbenzenesulfonate of methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-aminophenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ$^6$-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate dihydrate.

Semi-synthetic product derived from a fermentation product.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl toluenesulfonate esters are genotoxic and are potential impurities in sultamicillin tosilate dihydrate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general method *2.5.40. Methyl, ethyl and isopropyl toluenesulfonate in active substances* is available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sultamicillin tosilate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 178 to + 195 (anhydrous substance).

Dissolve 1.000 g in *dimethylformamide R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or keep at 2-8 °C for not more than 6 h.*

*Solution A*: *methanol R1*, *acetonitrile R1* (20:80 *V/V*).

*Solution B*. Dissolve 1.56 g of *sodium dihydrogen phosphate R* in 900 mL of *water R*. Add 7.0 mL of *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Blank solution*: solution B, solution A (30:70 *V/V*).

*Test solution*. Dissolve 70.0 mg of the substance to be examined in 35 mL of solution A and sonicate for about 1 min. Add 13 mL of solution B, mix and sonicate for about 1 min. Dilute to 50.0 mL with solution B and mix.

*Reference solution (a)*. Dissolve 70.0 mg of *sultamicillin tosilate CRS* in 35 mL of solution A and sonicate for about 1 min. Add 13 mL of solution B, mix and sonicate for about 1 min. Dilute to 50.0 mL with solution B and mix.

*Reference solution (b)*. Suspend 15 mg of the substance to be examined in 20 mL of a 0.4 g/L solution of *sodium hydroxide R* and sonicate in an ultrasonic bath for about 5 min. Add 20 mL of a 0.36 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

*Reference solution (c)*. Dissolve 0.200 g of the substance to be examined in 70.0 mL of solution A and sonicate for about 1 min. Add 25.0 mL of solution B, mix and sonicate for about 1 min. Dilute to 100.0 mL with solution B and mix. Dilute 1.0 mL of this solution to 100.0 mL with the blank solution.

*Reference solution (d)*. Dissolve 32.3 mg of *ampicillin trihydrate CRS* (impurity B) and 7.0 mg of *sulbactam CRS* (impurity A) in *water R* and dilute to 1000 mL with the same solvent.

*Column*:

- *size*: *l* = 0.10 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: 4.68 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 95 → 30 | 5 → 70 |
| 15 - 16 | 30 | 70 |
| 16 - 16.5 | 30 → 95 | 70 → 5 |
| 16.5 - 20 | 95 | 5 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL of the blank solution, the test solution and reference solutions (b), (c) and (d).

*Relative retention* with reference to sultamicillin (retention time = about 9.3 min): impurity A = about 0.41; ampicillin penicilloic acid = about 0.47; tosilate = about 0.50; impurity B = about 0.55; impurity C = about 0.94; impurity D = about 1.09; impurity F = about 1.23; impurity E = about 1.26; impurity G = about 1.42.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.5 between the peaks due to ampicillin penicilloic acid and tosilate and minimum 2.5 between the peaks due to tosilate and impurity B.

*Limits*:

- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (2.0 per cent);
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.5 per cent);
- *impurities C, D, E, F, G*: for each impurity, not more than 0.5 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *any other impurity*: for each impurity, not more than 0.5 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *total*: not more than 4 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (4.0 per cent);

– *disregard limit*: 0.1 times the area of the peak due to sultamicillin in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Ethyl acetate**. Head-space gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.200 g in 7.0 mL of a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R*.

*Reference solution*. Dissolve 0.200 g of *ethyl acetate R* in 240 mL of a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R* and dilute to 250.0 mL with the same mixture of solvents. Dilute 5.0 mL of this solution to 7.0 mL with a mixture of 1 volume of *water R* and 99 volumes of *dimethylformamide R*.

*Immediately close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimped cap. Shake to obtain a homogeneous solution.*

*Column*:

– *material*: fused silica;
– *size*: *l* = 50 m, Ø = 0.32 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness: 1.8 µm or 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 35 cm/s.

*Split ratio*: 1:5.

*Static head-space conditions that may be used:*

– *equilibration temperature*: 105 °C;
– *equilibration time*: 45 min;
– *transfer-line temperature*: 110 °C;
– *pressurisation time*: 30 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 6 | 70 |
| | 6 - 16 | 70 → 220 |
| | 16 - 18 | 220 |
| Injection port | | 140 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 mL.

*Relative retention* with reference to dimethylformamide (retention time = about 14 min): ethyl acetate = about 0.7.

*Limit*:

– *ethyl acetate*: maximum 2.0 per cent.

**Water** (*2.5.12*): 4.0 per cent to 6.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of sultamicillin tosilate ($C_{32}H_{38}N_4O_{12}S_3$) from the declared content of *sultamicillin tosilate CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

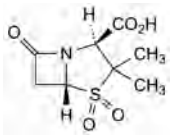

A. (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid 4,4-dioxide (sulbactam),

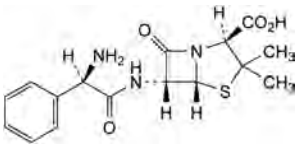

B. (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (ampicillin),

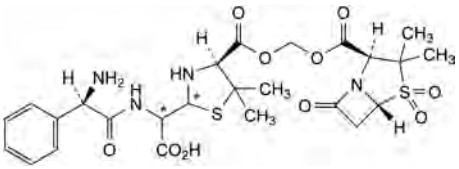

C. [[(2*R*)-aminophenylacetyl]amino][(4*S*)-4-[[[[[(2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]-hept-2-yl]carbonyl]oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetic acid (penicilloic acids of sultamicillin),

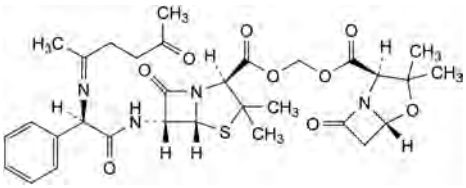

D. methylene (2*S*,5*R*,6*R*)-3,3-dimethyl-6-[[(2*R*)-[(1-methyl-4-oxopentylidene)amino]phenylacetyl]amino]-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylate,

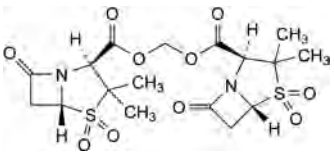

E. methylene bis[(2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate] (sulbactam methylene ester),

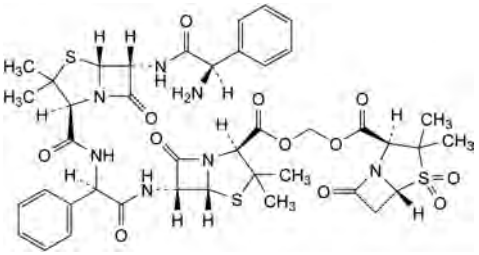

F. methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-[[[(2*S*,5*R*,6*R*)-6-[[(2*R*)-aminophenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]hept-2-yl]carbonyl]amino]-phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (ampicillin sultamicillin amide),

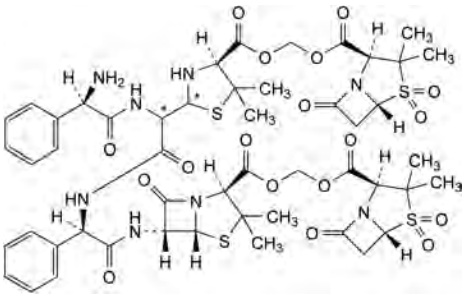

G. methylene (2*S*,5*R*,6*R*)-6-[[(2*R*)-[[[[(2*R*)-amino-phenylacetyl]amino][(4*S*)-4-[[[[[(2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-azabicyclo[3.2.0]hept-2-yl]-carbonyl]oxy]methoxy]carbonyl]-5,5-dimethylthiazolidin-2-yl]acetyl]amino]phenylacetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate (2*S*,5*R*)-3,3-dimethyl-4,4,7-trioxo-4λ⁶-thia-1-aza-bicyclo[3.2.0]heptane-2-carboxylate (sultamicillin dimer).

**01/2017:1573**
**corrected 10.0**

# SUMATRIPTAN SUCCINATE

## Sumatriptani succinas

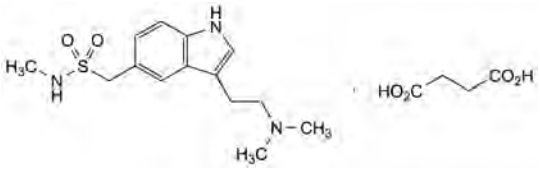

$C_{18}H_{27}N_3O_6S$ $M_r$ 413.5
[103628-48-4]

DEFINITION

1-[3-[2-(Dimethylamino)ethyl]-1*H*-indol-5-yl]-*N*-methylmethanesulfonamide hydrogen butanedioate.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, sparingly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *sumatriptan succinate CRS*.

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 4.5 to 5.3.

Dilute 2.5 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Absorbance** (*2.2.25*): maximum 0.10, determined at 440 nm on solution S.

**Impurities A and H**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 3 mg of *sumatriptan for system suitability CRS* (containing impurities A and H) in the mobile phase and dilute to 1.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 10 volumes of a 771 g/L solution of *ammonium acetate R* and 90 volumes of *methanol R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of sumatriptan.

*Identification of impurities*: use the chromatogram supplied with *sumatriptan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and H.

*Relative retention* with reference to sumatriptan (retention time = about 2 min): impurity A = about 1.9; impurity H = about 2.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurities A and H.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.6;
– *impurity A*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
– *impurity H*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 2.925 g of *sodium dihydrogen phosphate R* in 600 mL of *water R*, adjust to pH 6.5 with *strong sodium hydroxide solution R*, dilute to 750 mL with *water R*, add 250 mL of *acetonitrile R* and mix.

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Test solution (b)*. Dissolve 15.0 mg of the substance to be examined in solution A and dilute to 100.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *sumatriptan impurity mixture CRS* (containing impurities B, C, D and E) in the mobile phase and dilute to 1 mL with the mobile phase.

*Reference solution (c)*. Dissolve 15.0 mg of *sumatriptan succinate CRS* in solution A and dilute to 100.0 mL with solution A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 25 volumes of *acetonitrile R* with 75 volumes of a solution prepared as follows: dissolve 0.970 g of *dibutylamine R*, 0.735 g of *phosphoric acid R* and 2.93 g of *sodium dihydrogen phosphate R* in 750 mL of *water R*, adjust to pH 6.5 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 282 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: 4 times the retention time of sumatriptan.

*Identification of impurities*: use the chromatogram supplied with *sumatriptan impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D and E.

*Relative retention* with reference to sumatriptan (retention time = about 7 min): impurity E = about 0.5; impurity B = about 0.6; impurity D = about 0.7; impurity C = about 0.8.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity C and sumatriptan;
- the chromatogram shows 5 clearly separated peaks.

*Limits*:

- *impurities B, C, D*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity E*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{18}H_{27}N_3O_6S$ taking into account the assigned content of *sumatriptan succinate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F, G.*

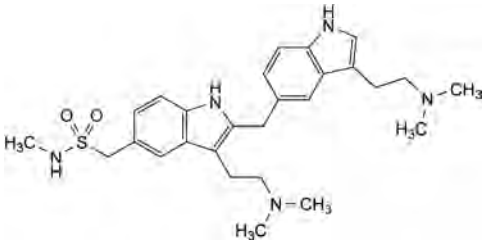

A. 1-[3-[2-(dimethylamino)ethyl]-2-[[3-[2-(dimethylamino)-ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methyl-methanesulfonamide,

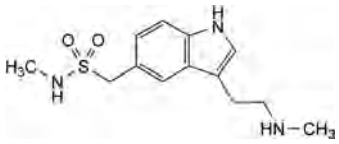

B. *N*-methyl-1-[3-[2-(methylamino)ethyl]-1*H*-indol-5-yl]-methanesulfonamide,

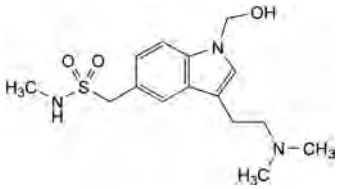

C. 1-[3-[2-(dimethylamino)ethyl]-1-(hydroxymethyl)-1*H*-indol-5-yl]-*N*-methylmethanesulfonamide,

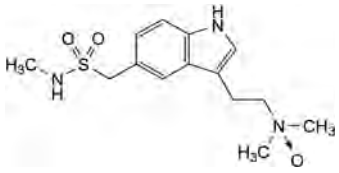

D. *N*,*N*-dimethyl-2-[5-[(methylsulfamoyl)methyl]-1*H*-indol-3-yl]ethan-1-amine *N*-oxide,

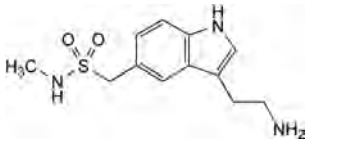

E. 1-[3-(2-aminoethyl)-1*H*-indol-5-yl]-*N*-methylmethane-sulfonamide,

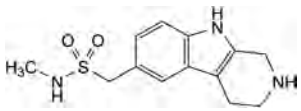

F. *N*-methyl-1-(2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-6-yl)methanesulfonamide,

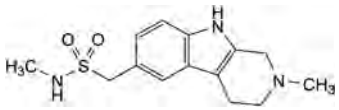

G. *N*-methyl-1-(2-methyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indol-6-yl)methanesulfonamide,

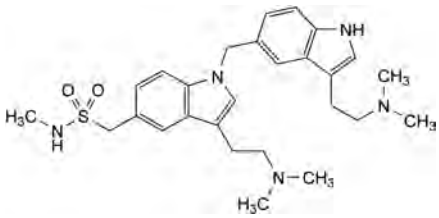

H. 1-[3-[2-(dimethylamino)ethyl]-1-[[3-[2-(dimethylamino)-ethyl]-1*H*-indol-5-yl]methyl]-1*H*-indol-5-yl]-*N*-methyl-methanesulfon amide.

01/2010:1371

# SUNFLOWER OIL, REFINED

## Helianthi annui oleum raffinatum

DEFINITION

Fatty oil obtained from the seeds of *Helianthus annuus* L. by mechanical expression or by extraction. It is then refined. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: clear, light yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

*Relative density*: about 0.921.

*Refractive index*: about 1.474.

IDENTIFICATION

Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

TESTS

**Acid value** (*2.5.1*): maximum 0.5, determined on 10.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Unsaponifiable matter** (*2.5.7*): maximum 1.5 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method A*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil:*

- *palmitic acid*: 4.0 per cent to 9.0 per cent,
- *stearic acid*: 1.0 per cent to 7.0 per cent,
- *oleic acid*: 14.0 per cent to 40.0 per cent,
- *linoleic acid*: 48.0 per cent to 74.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states whether the oil is obtained by mechanical expression or by extraction.

01/2008:0248

# SUXAMETHONIUM CHLORIDE

## Suxamethonii chloridum

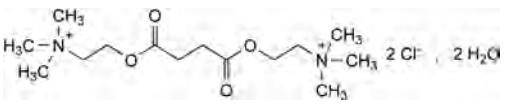

$C_{14}H_{30}Cl_2N_2O_4,2H_2O$ $M_r$ 397.3

[6101-15-1]

DEFINITION

Suxamethonium chloride contains not less than 98.0 per cent and not more than the equivalent of 102.0 per cent of 2,2′-[butanedioylbis(oxy)]bis(*N,N,N*-trimethylethanaminium) dichloride, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, hygroscopic, freely soluble in water, slightly soluble in alcohol.

It melts at about 160 °C, determined without previous drying.

IDENTIFICATION

*First identification: A, D.*

*Second identification: B, C, D.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *suxamethonium chloride CRS*. Examine the substances prepared as discs.

B. To 1 mL of solution S (see Tests) add 9 mL of *water R*, 10 mL of *dilute sulfuric acid R* and 30 mL of *ammonium reineckate solution R*. A pink precipitate is formed. Allow to stand for 30 min, filter, wash with *water R*, with *alcohol R* and then with *ether R* and dry at 80 °C. The melting point (*2.2.14*) of the precipitate is 180 °C to 185 °C.

C. Dissolve about 25 mg in 1 mL of *water R* and add 0.1 mL of a 10 g/L solution of *cobalt chloride R* and 0.1 mL of *potassium ferrocyanide solution R*. A green colour is produced.

D. About 20 mg gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*). Dilute 4 mL of solution S to 10 mL with *water R*. The solution is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*. The pH of the solution is 4.0 to 5.0.

**Choline chloride**. Examine by thin-layer chromatography (*2.2.27*), using *cellulose for chromatography R1* as the coating substance.

*Test solution.* Dissolve 0.4 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.4 g of *suxamethonium chloride CRS* and 2 mg of *choline chloride R* in *methanol R* and dilute to 10 mL with the same solvent.

Apply to the plate 5 µL of each solution. Prepare the mobile phase as follows: shake together for 10 min, 10 volumes of *anhydrous formic acid R*, 40 volumes of *water R* and 50 volumes of *butanol R*; allow to stand and use the upper layer. Develop over a path of 15 cm. Dry the plate in a current of air and spray with *potassium iodobismuthate solution R*. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot corresponding to choline chloride in the chromatogram obtained with the reference solution (0.5 per cent). The test is not valid unless the chromatogram obtained with the reference solution shows two clearly separated spots.

**Water** (*2.5.12*). 8.0 per cent to 10.0 per cent, determined on 0.30 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.07 mg of $C_{14}H_{30}Cl_2N_2O_4$.

STORAGE

Store in an airtight container, protected from light.

**01/2017:1574**

# SUXIBUZONE

## Suxibuzonum

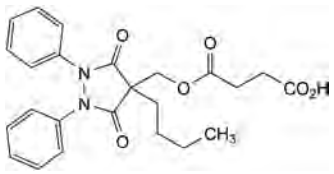

$C_{24}H_{26}N_2O_6$ $M_r$ 438.5
[27470-51-5]

DEFINITION

4-[(4-Butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl)methoxy]-4-oxobutanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent), practically insoluble in cyclohexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *suxibuzone CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1 g in *anhydrous ethanol R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.10 g of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.8 mg of *phenylbutazone CRS* (impurity A), 2.8 mg of *suxibuzone impurity B CRS* and 2.8 mg of *suxibuzone impurity C CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 4 mg of *phenylbutazone CRS* (impurity A) in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 10 mg of *phenylbutazone CRS* (impurity A) in *acetonitrile R* and dilute to 25.0 mL with the same solvent. Mix 10.0 mL of this solution with 1.0 mL of the test solution and dilute the mixture to 25.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 44 volumes of *acetonitrile R* and 56 volumes of a solution prepared as follows: dissolve 6.7 g of *citric acid monohydrate R* and 2.4 g of *tris(hydroxymethyl)aminomethane R* in 950 mL of *water R*, adjust to pH 3.0 with *citric acid monohydrate R* and dilute to 1000 mL with *water R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 10 µL.

*Relative retention* with reference to suxibuzone (retention time = about 7 min): impurity C = 0.7; impurity A = 1.4; impurity B = 3.3.

*System suitability*: reference solution (c):

- *resolution*: minimum of 2.0 between the peaks due to suxibuzone and impurity A.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.7 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.07 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven *in vacuo* at 60 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in previously neutralised *anhydrous ethanol R* and dilute to 10 mL with the same solvent. Carry out a potentiometric titration (*2.2.20*) using *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 43.85 mg of $C_{24}H_{26}N_2O_6$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C*.

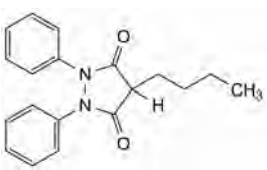

A. 4-butyl-1,2-diphenylpyrazolidine-3,5-dione (phenylbutazone),

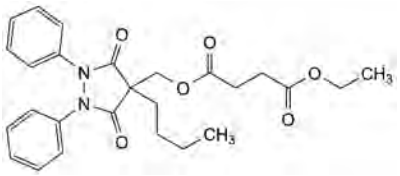

B. (4-butyl-3,5-dioxo-1,2-diphenylpyrazolidin-4-yl)methyl ethyl butanedioate,

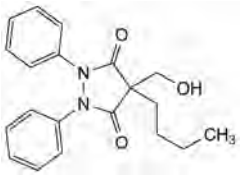

C. 4-butyl-4-(hydroxymethyl)-1,2-diphenyl-1,2-dihydro-4*H*-pyrazole-3,5-dione.

# T


Tacalcitol monohydrate.... 3953
Tacrolimus monohydrate.... 3954
Tadalafil.... 3957
Talc.... 3959
Tamoxifen citrate.... 3961
Tamsulosin hydrochloride.... 3963
Tannic acid.... 3964
Tapentadol hydrochloride.... 3965
Tartaric acid.... 3966
Teicoplanin.... 3967
Telmisartan.... 3970
Temazepam.... 3972
Temozolomide.... 3973
Tenoxicam.... 3974
Terazosin hydrochloride dihydrate.... 3976
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Terbutaline sulfate.... 3979
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Terfenadine.... 3982
Teriparatide.... 3983
Terlipressin.... 3986
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Testosterone enantate.... 3992
Testosterone isocaproate.... 3994
Testosterone propionate.... 3995
Tetracaine.... 3997
Tetracaine hydrochloride.... 3998
Tetracosactide.... 3999
Tetracycline.... 4000
Tetracycline hydrochloride.... 4001
Tetrazepam.... 4003
Tetryzoline hydrochloride.... 4004
Theobromine.... 4005
Theophylline.... 4005
Theophylline monohydrate.... 4007
Theophylline-ethylenediamine.... 4008
Theophylline-ethylenediamine hydrate.... 4009
Thiamazole.... 4011
Thiamine hydrochloride.... 4012
Thiamine nitrate.... 4014
Thiamphenicol.... 4015
Thiocolchicoside crystallised from ethanol.... 4016
Thiocolchicoside hydrate.... 4018
Thioctic acid.... 4020
Thiomersal.... 4021
Thiopental sodium and sodium carbonate.... 4022
Thioridazine.... 4024
Thioridazine hydrochloride.... 4025
Threonine.... 4027
Thymol.... 4028
Tiabendazole.... 4029
Tiamulin for veterinary use.... 4030
Tiamulin hydrogen fumarate for veterinary use.... 4032
Tianeptine sodium.... 4035
Tiapride hydrochloride.... 4036
Tiaprofenic acid.... 4037
Tibolone.... 4039
Ticarcillin sodium.... 4040
Ticlopidine hydrochloride.... 4041
Tigecycline.... 4043
Tilidine hydrochloride hemihydrate.... 4045
Timolol maleate.... 4046
Tinidazole.... 4048
Tinzaparin sodium.... 4049
Tioconazole.... 4050
Tiotropium bromide monohydrate.... 4051
Titanium dioxide.... 4052
Tizanidine hydrochloride.... 4053
Tobramycin.... 4055
all-*rac*-α-Tocopherol.... 4057
*RRR*-α-Tocopherol.... 4058
all-*rac*-α-Tocopheryl acetate.... 4059
*RRR*-α-Tocopheryl acetate.... 4060
α-Tocopheryl acetate concentrate (powder form).... 4062
DL-α-Tocopheryl hydrogen succinate.... 4062
*RRR*-α-Tocopheryl hydrogen succinate.... 4064
Tolbutamide.... 4066
Tolfenamic acid.... 4067
Tolnaftate.... 4068
Tolterodine tartrate.... 4070
Topiramate.... 4071
Torasemide.... 4073
Tosylchloramide sodium.... 4074
Tramadol hydrochloride.... 4074
Tramazoline hydrochloride monohydrate.... 4076
Trandolapril.... 4077
Tranexamic acid.... 4078
Trapidil.... 4079
Trehalose dihydrate.... 4080
Tretinoin.... 4082
Triacetin.... 4083
Triamcinolone.... 4083
Triamcinolone acetonide.... 4085
Triamcinolone hexacetonide.... 4086
Triamterene.... 4088
Tribenoside.... 4089
Tributyl acetylcitrate.... 4090
Tri-*n*-butyl phosphate.... 4091
Trichloroacetic acid.... 4092
Triclabendazole for veterinary use.... 4093
Triethyl citrate.... 4094
Trifluoperazine hydrochloride.... 4095
Triflusal.... 4095
Triglycerides, medium-chain.... 4096
Triglycerol diisostearate.... 4097
Trihexyphenidyl hydrochloride.... 4098
Trimebutine maleate.... 4099
Trimetazidine dihydrochloride.... 4100
Trimethadione.... 4101
Trimethoprim.... 4102
Trimipramine maleate.... 4104
Trolamine.... 4106
Trometamol.... 4107
Tropicamide.... 4108
Tropisetron hydrochloride.... 4110
Trospium chloride.... 4111
Troxerutin.... 4112
Trypsin.... 4114
Tryptophan.... 4115
Tuberculin for human use, old.... 4117
Tuberculin purified protein derivative, avian.... 4119
Tuberculin purified protein derivative, bovine.... 4120
Tuberculin purified protein derivative for human use.... 4120
Tylosin for veterinary use.... 4122
Tylosin phosphate bulk solution for veterinary use.... 4127
Tylosin phosphate for veterinary use.... 4131
Tylosin tartrate for veterinary use.... 4136
Tyrosine.... 4140
Tyrothricin.... 4142

**01/2017:2272**
**corrected 10.0**

# TACALCITOL MONOHYDRATE

## Tacalcitolum monohydricum

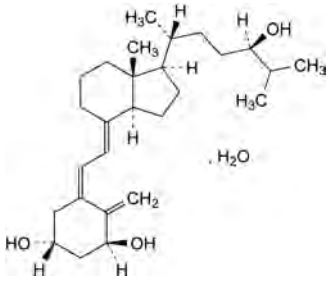

$C_{27}H_{44}O_3,H_2O$ $M_r$ 434.7
[93129-94-3]

DEFINITION

(5*Z*,7*E*)-(24*R*)-9,10-Secocholesta-5,7,10(19)-triene-1α,3β,24-triol.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

A reversible isomerisation to pre-tacalcitol takes place in solution, depending on temperature and time. The activity is due to both compounds.

It is sensitive to air, heat and light.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol and in ethyl acetate.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of tacalcitol monohydrate*.

B. It complies with test B for related substances (see Tests).

C. Water (see Tests).

TESTS

**Related substances**.

A. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use avoiding exposure to light and air.*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *tacalcitol monohydrate CRS* in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Heat 3 mL of the test solution at 80 °C for 30 min. Cool the solution to room temperature.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (d).* Dissolve the contents of a vial of *tacalcitol impurity A CRS* in 1 mL of the test solution.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 μm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R* (40:60 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 50 μL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 2.5 times the retention time of tacalcitol.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A; use the chromatogram obtained with reference solution (b) to identify the peak due to pre-tacalcitol.

*Relative retention* with reference to tacalcitol (retention time = about 11 min): pre-tacalcitol = about 0.8; impurity A = about 0.9.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to impurity A and tacalcitol.

*Calculation of percentage contents*:
- for each impurity, use the concentration of tacalcitol monohydrate in reference solution (c).

*Limits*:
- *impurity A*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to pre-tacalcitol.

B. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 2.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dissolve the contents of a vial of *tacalcitol for system suitability CRS* (containing impurity B) in 0.5 mL of the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (10 μm).

*Mobile phase*: *anhydrous ethanol R*, *heptane R* (14:86 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 50 μL.

*Run time*: 2.5 times the retention time of tacalcitol.

*Identification of impurities*: use the chromatogram supplied with *tacalcitol for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to tacalcitol (retention time = about 12 min): impurity A = about 0.7; impurity B = about 0.85.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity B and tacalcitol.

*Calculation of percentage contents*:
- for each impurity, use the concentration of tacalcitol monohydrate in reference solution (b).

*Limits*:
- *impurity B*: maximum 1.0 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- disregard any peak with a relative retention with reference to tacalcitol of about 0.7 (impurity A).

**Water** (*2.5.32*): 3.8 per cent to 4.8 per cent, determined on 10.0 mg by direct sample introduction.

Monographs T

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution and reference solutions (a) and (b).

Calculate the percentage content of $C_{27}H_{44}O_3$ taking into account the assigned content of *tacalcitol monohydrate CRS* and, if present, the peak due to pre-tacalcitol.

STORAGE

In an airtight container, under an inert gas, protected from light at a temperature not exceeding − 15 °C.

IMPURITIES

**Test A for related substances:** A.

**Test B for related substances:** B, C.

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

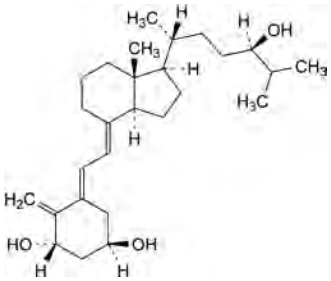

A. (5*E*,7*E*)-(24*R*)-9,10-secocholesta-5,7,10(19)-triene-1α,3β,24-triol (*trans*-tacalcitol),

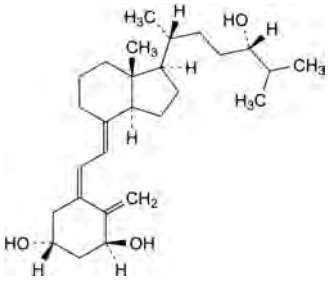

B. (5*Z*,7*E*)-(24*S*)-9,10-secocholesta-5,7,10(19)-triene-1α,3β,24-triol ((24*S*)-tacalcitol),

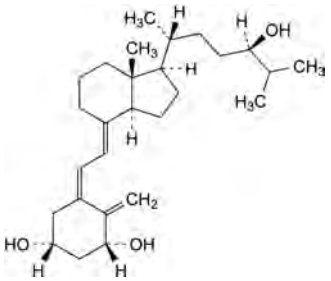

C. (5*Z*,7*E*)-(24*R*)-9,10-secocholesta-5,7,10(19)-triene-1β,3β,24-triol (1β-tacalcitol).

**01/2018:2244**
**corrected 10.0**

# TACROLIMUS MONOHYDRATE

## Tacrolimusum monohydricum

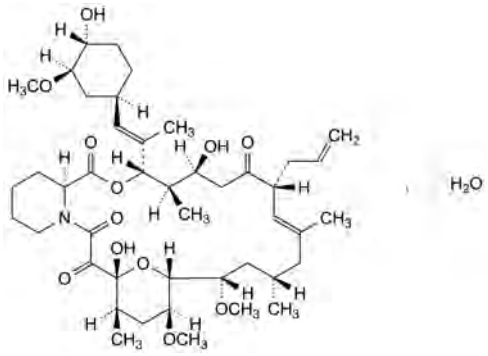

$C_{44}H_{69}NO_{12},H_2O$ $M_r$ 822

[109581-93-3]

DEFINITION

(3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(prop-2-en-1-yl)-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7H-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone monohydrate.

Substance produced by the growth of certain strains of *Streptomyces tsukubaensis*.

*Content*: 97.0 per cent to 102.0 per cent for the sum of tacrolimus, tacrolimus compound I and tacrolimus compound II (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tacrolimus monohydrate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_7$ (*2.2.2, Method II*).

Dissolve 0.20 g in *ethanol (96 per cent) R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Allow the test and reference solutions to stand for 3 h at room temperature before use.*

*Solvent mixture*: *water R*, *acetonitrile R* (30:70 *V/V*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 30.0 mg of *tacrolimus monohydrate CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 3.0 mg of *tacrolimus for system suitability CRS* (containing impurity A) in the solvent mixture and dilute to 1.0 mL with the solvent mixture.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: mix 20 volumes of a mixture of 19 volumes of *1,1-dimethylethyl methyl ether R* and 81 volumes of *acetonitrile for chromatography R* with 80 volumes of a 0.04 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: mix 20 volumes of a 0.04 per cent *V/V* solution of *phosphoric acid R* with 80 volumes of a mixture of 19 volumes of *1,1-dimethylethyl methyl ether R* and 81 volumes of *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 72 | 28 |
| 30 - 53 | 72 → 15 | 28 → 85 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Autosampler*: set at 4 °C.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *tacrolimus for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to tacrolimus (retention time = about 24 min): tacrolimus compound I = broad peak at about 0.5; impurity B = sharp peak at about 0.5; tacrolimus compound II = about 0.6; impurity A = about 0.9.

*System suitability*: reference solution (b):

- *resolution*: minimum 3.0 between the peaks due to impurity A and tacrolimus.

*Integration*: the peak due to impurity B, which co-elutes with tacrolimus compound I, is a rider peak that is to be integrated using tangential skimming. It is necessary that the integration area of the peak due to tacrolimus compound I preserves the Gaussian peak shape.

*Calculation of percentage contents*:

- for each impurity, use the concentration of tacrolimus monohydrate in reference solution (c).

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.15 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peaks due to tacrolimus compound I and tacrolimus compound II.

**Water** (*2.5.12*): 1.5 per cent to 4.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of the sum of tacrolimus, tacrolimus compound I and tacrolimus compound II taking into account the assigned content of *tacrolimus monohydrate CRS*.

STORAGE

In an airtight container. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, H, I.*

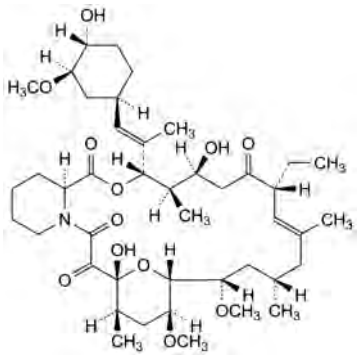

A. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-8-ethyl-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone,

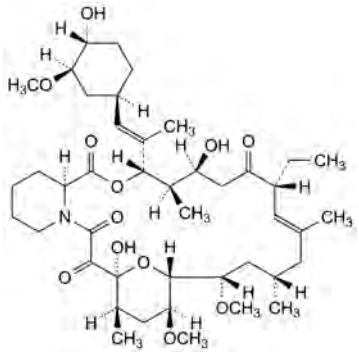

B. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*S*,26a*S*)-8-ethyl-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone,

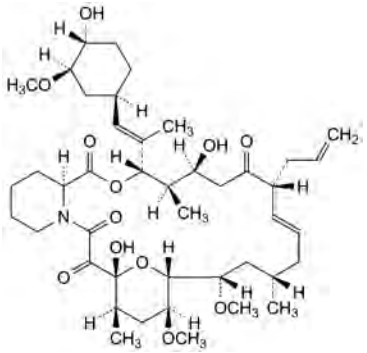

C. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,12,18-trimethyl-8-(prop-2-en-1-yl)-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone,

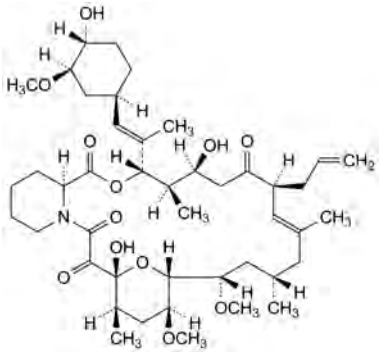

D. (3*S*,4*R*,5*S*,8*S*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(prop-2-en-1-yl)-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone,

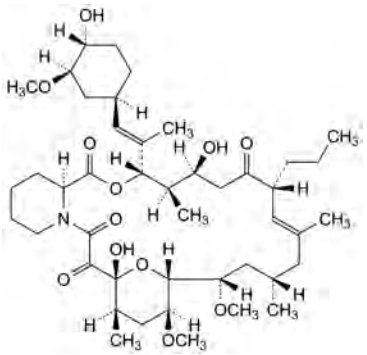

E. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-propyl-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone,

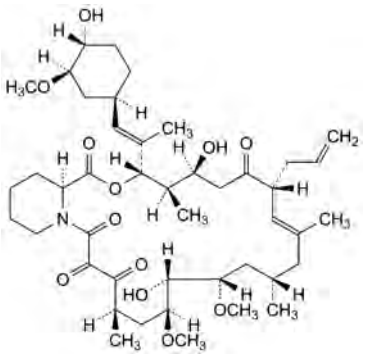

F. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-5,15-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(prop-2-en-1-yl)-3,4,5,6,11,12,13,14,15,16,17,18,24,25,26,26a-hexadecahydro-7*H*-pyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,19,20,21(8*H*,23*H*)-pentone (tacrolimus compound I),

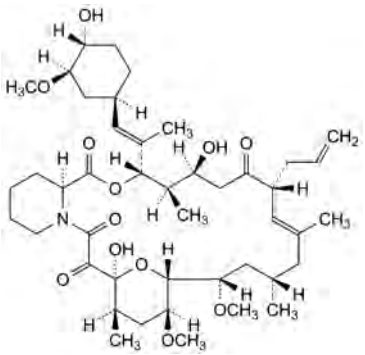

G. (3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*S*,26a*S*)-5,19-dihydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(prop-2-en-1-yl)-3,4,5,6,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-octadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone (tacrolimus compound II),

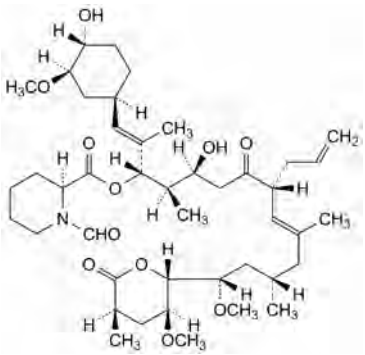

H. (1*E*,3*S*,4*R*,5*S*,8*R*,9*E*,12*S*,14*S*)-5-hydroxy-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]-14-methoxy-14-[(2*R*,3*S*,5*R*)-3-methoxy-5-methyl-6-oxooxan-2-yl]-2,4,10,12-tetramethyl-7-oxo-8-(prop-2-en-1-yl)tetradeca-1,9-dien-3-yl (2*S*)-1-formylpiperidine-2-carboxylate,

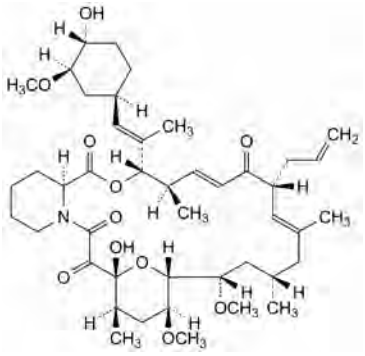

I. (3*S*,4*R*,5*E*,8*R*,9*E*,12*S*,14*S*,15*R*,16*S*,18*R*,19*R*,26a*S*)-19-hydroxy-3-[(1*E*)-1-[(1*R*,3*R*,4*R*)-4-hydroxy-3-methoxycyclohexyl]prop-1-en-2-yl]-14,16-dimethoxy-4,10,12,18-tetramethyl-8-(prop-2-en-1-yl)-3,4,8,11,12,13,14,15,16,17,18,19,24,25,26,26a-hexadecahydro-7*H*-15,19-epoxypyrido[2,1-*c*][1,4]oxaazacyclotricosine-1,7,20,21(23*H*)-tetrone.

**04/2012:2606**
**corrected 10.0**

# TADALAFIL

## Tadalafilum

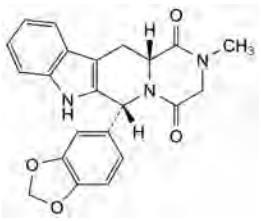

$C_{22}H_{19}N_3O_4$ $M_r$ 389.4
[171596-29-5]

DEFINITION

(6*R*,12a*R*)-6-(1,3-Benzodioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]-pyrido[3,4-*b*]indole-1,4-dione.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in dimethyl sulfoxide, slightly soluble in methylene chloride.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tadalafil CRS*.

B. Liquid chromatography (*2.2.29*) as described in the test for impurities A, B and C with the following modification.
*Injection*: test solution and reference solution (a).
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

C. Specific optical rotation (*2.2.7*): + 78.0 to + 84.0 (dried substance).
Dissolve 0.250 g in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent.

TESTS

**Impurities A, B and C**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R*, *hexane R*, *2-propanol R* (20:40:40 *V/V/V*).

*Solution A*. Dissolve 27 g of *tetrabutylammonium hydroxide R* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 25.0 mg of *tadalafil CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. In order to prepare impurity A *in situ*, dissolve 25 mg of the substance to be examined in 40 mL of the solvent mixture. Add 1 mL of solution A, mix well and allow to stand for 20 min. Add 1 mL of *trifluoroacetic acid R* and dilute to 100 mL with the solvent mixture.

*Reference solution (d)*. To 1 mL of the test solution add 1 mL of reference solution (c) and dilute to 50 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (10 µm);
- *temperature*: 30 °C.

*Mobile phase*: *hexane R*, *2-propanol R1* (50:50 *V/V*).

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 222 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (d).

*Run time*: 2.2 times the retention time of tadalafil.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peak due to impurity A.

*Relative retention* with reference to tadalafil (retention time = about 11 min): impurity A = about 0.8.

*System suitability*: reference solution (d)
- *resolution*: minimum 2.0 between the peaks due to impurity A and tadalafil.

*Limits*:
- *impurity A*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Do not use sonication during the preparation of the solutions.*

*Solvent mixture*: *acetonitrile R*, *2-propanol R* (50:50 *V/V*).

*Solution A*. Dissolve 27 g of *tetrabutylammonium hydroxide R* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Test solution (a)*. Dissolve 40 mg of the substance to be examined in 50 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A.

*Test solution (b)*. Dissolve 50.0 mg of the substance to be examined in 50 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A. To 10.0 mL of this solution add 25.0 mL of *acetonitrile R* and dilute to 50.0 mL with mobile phase A.

*Reference solution (a)*. To 1.0 mL of test solution (a) add 50 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A. To 1.0 mL of this solution add 5 mL of *acetonitrile R* and dilute to 10.0 mL with mobile phase A.

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*Reference solution (b).* In order to prepare impurity A *in situ*, dissolve 4 mg of the substance to be examined in 50 mL of the solvent mixture. Add 1 mL of solution A, mix, and allow to stand for 40 min. Add 1 mL of *trifluoroacetic acid R* and dilute to 100 mL with the solvent mixture.

*Reference solution (c).* Dilute 1 mL of reference solution (b) to 50 mL with test solution (a).

*Reference solution (d).* Dissolve 50.0 mg of *tadalafil CRS* in 50 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A. To 10.0 mL of this solution add 25.0 mL of *acetonitrile R* and dilute to 50.0 mL with mobile phase A.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 1.0 mL of *trifluoroacetic acid R* with *water for chromatography R* and dilute to 1000 mL with the same solvent;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 85 | 15 |
| 3 - 30 | 85 → 5 | 15 → 95 |
| 30 - 33 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurities A + C.

*Relative retention* with reference to tadalafil (retention time = about 16 min): impurities A and C = about 1.03.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 3.3, where $H_p$ = height above the baseline of the peak due to impurities A + C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to tadalafil.

*Limits*:

- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to impurity A and/or C.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: *acetonitrile R*, mobile phase A (45:55 *V/V*).

*Flow rate*: 1.5 mL/min.

*Injection*: test solution (b) and reference solution (d).

*Run time*: twice the retention time of tadalafil (retention time = about 4.5 min).

Calculate the percentage content of $C_{22}H_{19}N_3O_4$ from the declared content of *tadalafil CRS*.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I*.

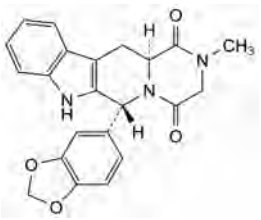

A. (6*R*,12a*S*)-6-(1,3-benzodioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-1,4-dione,

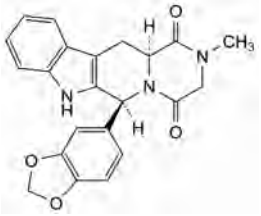

B. (6*S*,12a*S*)-6-(1,3-benzodioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-1,4-dione,

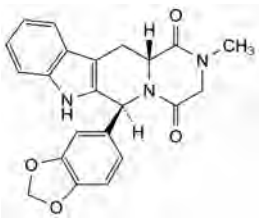

C. (6*S*,12a*R*)-6-(1,3-benzodioxol-5-yl)-2-methyl-2,3,6,7,12,12a-hexahydropyrazino[1′,2′:1,6]pyrido[3,4-*b*]indole-1,4-dione,

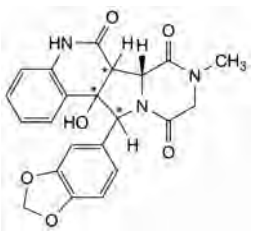

D. (6b*R*)-12-(1,3-benzodioxol-5-yl)-12a-hydroxy-8-methyl-6a,6b,8,9,12,12a-hexahydropyrazino[1′,2′:1,2]-pyrrolo[3,4-*c*]quinoline-6,7,10(5*H*)-trione,

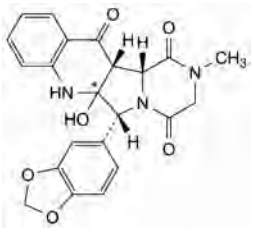

E. (6*R*,12a*R*,12b*R*)-6-(1,3-benzodioxol-5-yl)-6a-hydroxy-2-methyl-2,3,6a,7,12a,12b-hexahydropyrazino[1′,2′:1,5]-pyrrolo[3,4-*b*]quinoline-1,4,12(6*H*)-trione,

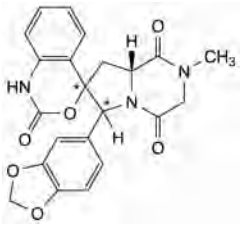

F. (8a′*R*)-6′-(1,3-benzodioxol-5-yl)-2′-methyl-2′,3′,8′,8a′-tetrahydro-6′*H*-spiro[3,1-benzoxazine-4,7′-pyrrolo[1,2-*a*]pyrazine]-1′,2,4′(1*H*)-trione,

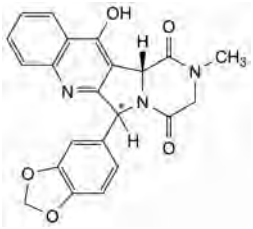

G. (12b*R*)-6-(1,3-benzodioxol-5-yl)-12-hydroxy-2-methyl-2,3,6,12b-tetrahydropyrazino[1′,2′:1,5]pyrrolo-[3,4-*b*]quinoline-1,4-dione,

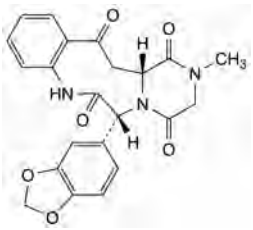

H. (6*R*,14a*R*)-6-(1,3-benzodioxol-5-yl)-2-methyl-2,3,14,14a-tetrahydropyrazino[1,2-*d*][1,4]benzodiazonine-1,4,7,13-(6*H*,8*H*)-tetrone,

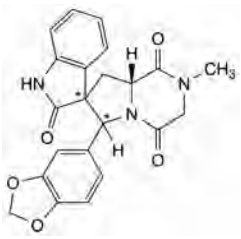

I. (8a′*R*)-6′-(1,3-benzodioxol-5-yl)-2′-methyl-2′,3′,8′,8a′-tetrahydro-6′*H*-spiro[indole-3,7′-pyrrolo[1,2-*a*]pyrazine]-1′,2,4′(1*H*)-trione.

**04/2012:0438**

# TALC

## Talcum

[14807-96-6]

DEFINITION

Powdered, selected, natural, hydrated magnesium silicate. Pure talc has the formula $Mg_3Si_4O_{10}(OH)_2$ ($M_r$ 379.3). It may contain variable amounts of associated minerals among which chlorites (hydrated aluminium and magnesium silicates), magnesite (magnesium carbonate), calcite (calcium carbonate) and dolomite (calcium and magnesium carbonate) are predominant.

PRODUCTION

Talc derived from deposits that are known to contain associated asbestos is not suitable for pharmaceutical use. The manufacturer is responsible for demonstrating by the test for amphiboles and serpentines that the product is free from asbestos. The presence of amphiboles and of serpentines is revealed by X-ray diffraction or by infrared spectrophotometry (see A and B). If detected, the specific morphological criteria of asbestos are investigated by a suitable method of optical microscopy to determine whether tremolite asbestos or chrysotile is present, as described below.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium bromide R*.

In the range 740 $cm^{-1}$ to 760 $cm^{-1}$ using scale expansion, any absorption band at 758 ± 1 $cm^{-1}$ may indicate the presence of tremolite or of chlorite. If the absorption band remains after ignition of the substance to be examined at 850 ± 50 °C for at least 30 min, it indicates the presence of the tremolite. In the range 600 $cm^{-1}$ to 650 $cm^{-1}$ using scale expansion, any absorption band or shoulder may indicate the presence of serpentines.

B. X-ray diffraction.

*Preparation*: place the sample on the sample holder; pack and smooth its surface with a polished glass microscope slide.

*Radiation*: Cu Kα monochromatic, 40 kV, 24-30 mA.

*Incident slit*: 1°.

*Detection slit*: 0.2°.

*Goniometer speed*: 1/10° 2θ/min.

*Scanning range*: 10-13° 2θ and 24-26° 2θ.

*Sample*: not oriented.

*Results*: the presence of amphiboles is detected by a diffraction peak at 10.5 ± 0.1° 2θ, the presence of serpentines is detected by diffraction peaks at 24.3 ± 0.1° 2θ and at 12.1 ± 0.1° 2θ.

*If, by one of the 2 methods, amphiboles and/or serpentine are detected, examine by a suitable method of optical microscopy to determine the asbestos character.*

The presence of asbestos is shown if the following 2 criteria are met:

– a range of length to width ratios of 20:1 to 100:1, or higher for fibres longer than 5 μm;
– capability of splitting into very thin fibrils;

and if at least 2 of the following 4 criteria are met:

– parallel fibres occurring in bundles;
– fibre bundles displaying frayed ends;
– fibres in the form of thin needles;

Monographs T

– matted masses of individual fibres and/or fibres showing curvature.

CHARACTERS

*Appearance*: light, homogeneous, white or almost white powder, greasy to the touch (non abrasive).

*Solubility*: practically insoluble in water, in ethanol (96 per cent) and in dilute solutions of acids and alkali hydroxides.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs of *potassium bromide R.*

*Absorption bands*: at 3677 ± 2 $cm^{-1}$, 1018 ± 2 $cm^{-1}$ and 669 ± 2 $cm^{-1}$.

B. In a platinum crucible, melt a mixture of 0.2 g of *anhydrous sodium carbonate R* and 2.0 g of *potassium carbonate R*. To the melted mass add 0.1 g of the substance to be examined and heat until the mixture is completely melted. Allow to cool and transfer the melted mass into an evaporating dish with 50 mL of hot *water R*. Add *hydrochloric acid R* until effervescence ceases. Add 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Allow to cool. Add 20 mL of *water R*, heat to boiling and filter (the residue is used for identification test C). To 5 mL of the filtrate add 1 mL of *ammonia R* and 1 mL of *ammonium chloride solution R* and filter. To the filtrate add 1 mL of *disodium hydrogen phosphate solution R*. A white, crystalline precipitate is formed.

C. The residue obtained in identification test B gives the reaction of silicates (*2.3.1*).

TESTS

**Solution S1**. Weigh 10.0 g into a conical flask fitted with a reflux condenser, gradually add 50 mL of *0.5 M hydrochloric acid* while stirring and heat on a water-bath for 30 min. Allow to cool. Transfer the mixture to a beaker and allow the undissolved material to settle. Filter the supernatant through medium-speed filter paper into a 100 mL volumetric flask, retaining as much as possible of the insoluble material in the beaker. Wash the residue and the beaker with 3 quantities, each of 10 mL, of hot *water R*. Wash the filter with 15 mL of hot *water R*, allow the filtrate to cool and dilute to 100.0 mL with the same solvent.

**Solution S2**. *Perchlorates mixed with heavy metals are known to be explosive. Take proper precautions while performing this procedure.* Weigh 0.5 g in a 100 mL polytetrafluoroethylene dish, add 5 mL of *hydrochloric acid R*, 5 mL of *lead-free nitric acid R* and 5 mL of *perchloric acid R*. Stir gently then add 35 mL of *hydrofluoric acid R* and evaporate slowly to dryness on a hot plate. To the residue, add 5 mL of *hydrochloric acid R*, cover with a watch-glass, heat to boiling and allow to cool. Rinse the watch-glass and the dish with *water R*. Transfer into a volumetric flask, rinse the dish with *water R* and dilute to 50.0 mL with the same solvent.

**Acidity or alkalinity**. Boil 2.5 g with 50 mL of *carbon dioxide-free water R* under reflux. Filter *in vacuo*. To 10 mL of the filtrate add 0.1 mL of *bromothymol blue solution R1*; not more than 0.4 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to green. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R1*; not more than 0.3 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Water-soluble substances**: maximum 0.2 per cent.

To 10.0 g add 50 mL of *carbon dioxide-free water R*, heat to boiling and maintain boiling under a reflux condenser for 30 min. Allow to cool, filter through a medium-speed filter paper and dilute to 50.0 mL with *carbon dioxide-free water R*. Take 25.0 mL of the filtrate, evaporate to dryness and heat at 105 °C for 1 h. The residue weighs a maximum of 10 mg.

**Aluminium**: maximum 2.0 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* To 5.0 mL of solution S2 add 10 mL of a 25.34 g/L solution of *caesium chloride R*, 10.0 mL of *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks, each containing 10.0 mL of *hydrochloric acid R* and 10 mL of a 25.34 g/L solution of *caesium chloride R*, introduce respectively 5.0 mL, 10.0 mL, 15.0 mL and 20.0 mL of *aluminium standard solution (100 ppm Al) R* and dilute to 100.0 mL with *water R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: nitrous oxide-acetylene flame.

**Calcium**: maximum 0.9 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* To 5.0 mL of solution S2 add 10.0 mL of *hydrochloric acid R*, 10 mL of *lanthanum chloride solution R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks, each containing 10.0 mL of *hydrochloric acid R* and 10 mL of *lanthanum chloride solution R*, introduce respectively 1.0 mL, 2.0 mL, 3.0 mL and 5.0 mL of *calcium standard solution (100 ppm Ca) R1* and dilute to 100.0 mL with *water R*.

*Source*: calcium hollow-cathode lamp.

*Wavelength*: 422.7 nm.

*Atomisation device*: nitrous oxide-acetylene flame.

**Iron**: maximum 0.25 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* To 2.5 mL of solution S1, add 50.0 mL of *0.5 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks, each containing 50.0 mL of *0.5 M hydrochloric acid*, introduce respectively 2.0 mL, 2.5 mL, 3.0 mL and 4.0 mL of *iron standard solution (250 ppm Fe) R* and dilute to 100.0 mL with *water R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

*Correction*: deuterium lamp.

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use solution S1.

*Reference solutions.* Into 4 identical volumetric flasks, each containing 50.0 mL of *0.5 M hydrochloric acid*, introduce respectively 5.0 mL, 7.5 mL, 10.0 mL and 12.5 mL of *lead standard solution (10 ppm Pb) R1* and dilute to 100.0 mL with *water R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 217.0 nm.

*Atomisation device*: air-acetylene flame.

**Magnesium**: 17.0 per cent to 19.5 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dilute 0.5 mL of solution S2 to 100.0 mL with *water R*. To 4.0 mL of the solution, add 10.0 mL of *hydrochloric acid R*, 10 mL of *lanthanum chloride solution R* and dilute to 100.0 mL with *water R*.

*Reference solutions.* Into 4 identical volumetric flasks, each containing 10.0 mL of *hydrochloric acid R* and 10 mL of *lanthanum chloride solution R*, introduce respectively 2.5 mL, 3.0 mL, 4.0 mL and 5.0 mL of *magnesium standard solution (10 ppm Mg) R1* and dilute to 100.0 mL with *water R*.

*Source*: magnesium hollow-cathode lamp.

*Wavelength*: 285.2 nm.

*Atomisation device*: air-acetylene flame.

**Loss on ignition**: maximum 7.0 per cent, determined on 1.00 g by ignition to constant weight at 1050-1100 °C.

**Microbial contamination**

If intended for cutaneous administration:

– TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If intended for oral administration:

– TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);

– TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

LABELLING

The label states, where applicable, that the substance is suitable for oral or cutaneous administration.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for talc used as antisticking agent or glidant in tablets and capsules or as antiadhesive in coated and film-coated tablets.*

**Particle-size distribution** (*2.9.31*).

**Specific surface area** (*2.9.26*).

**04/2016:1046**

# TAMOXIFEN CITRATE

## Tamoxifeni citras

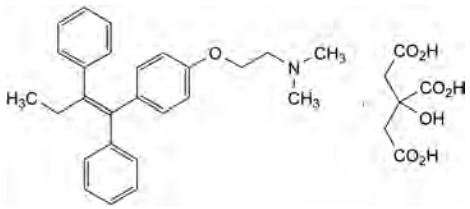

$C_{32}H_{37}NO_8$ $M_r$ 563.6

[54965-24-1]

DEFINITION

2-[4-[(*Z*)-1,2-Diphenylbut-1-en-1-yl]phenoxy]-*N*,*N*-dimethylethan-1-amine dihydrogen 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in methanol, slightly soluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification*: *B*.

*Second identification*: *A, C*.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20 mg in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maxima*: at 237 nm and 275 nm.

*Absorbance ratio*: $A_{237}/A_{275}$ = 1.45 to 1.65.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tamoxifen citrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *acetone R*, evaporate to dryness and record new spectra using the residues.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *tamoxifen citrate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *clomifene citrate CRS* and 10 mg of *tamoxifen citrate CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *triethylamine R*, *toluene R* (10:90 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution.* Dissolve 15 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 3 mg of *tamoxifen citrate for performance test CRS* (containing impurities A and F) in the mobile phase and dilute to 2.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 40 volumes of *acetonitrile R* and 60 volumes of *water R* containing 0.9 g/L of *sodium dihydrogen phosphate R* and 4.8 g/L of *N,N-dimethyloctylamine R*; adjust to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of tamoxifen.

*Identification of impurities*: use the chromatogram supplied with *tamoxifen citrate for performance test CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and F.

*Relative retention* with reference to tamoxifen (retention time = about 20 min): impurity A = about 0.8; impurity F = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurities A and F; baseline separation between the peaks due to impurity F and tamoxifen.

*Limits*:

– *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *impurity F*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak due to the citrate.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 65 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 75 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 56.36 mg of $C_{32}H_{37}NO_8$.

IMPURITIES

*Specified impurities*: *A, F*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, G, H*.

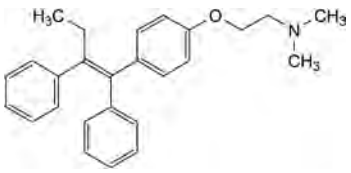

A. 2-[4-[(*E*)-1,2-diphenylbut-1-en-1-yl]phenoxy]-*N*,*N*-dimethylethan-1-amine ((*E*)-isomer),

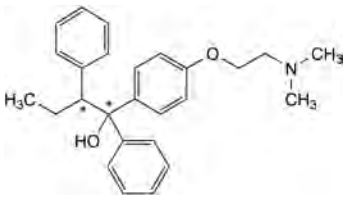

B. 1-[4-[2-(dimethylamino)ethoxy]phenyl]-1,2-diphenylbutan-1-ol,

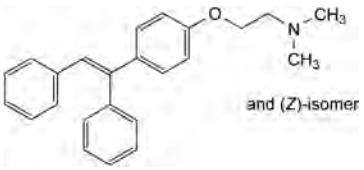


C. 2-[4-[(*EZ*)-1,2-diphenyleth-1-en-1-yl]phenoxy]-*N*,*N*-dimethylethan-1-amine,

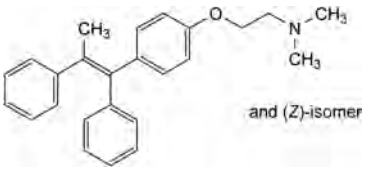


D. 2-[4-[(*EZ*)-1,2-diphenylprop-1-en-1-yl]phenoxy]-*N*,*N*-dimethylethan-1-amine,

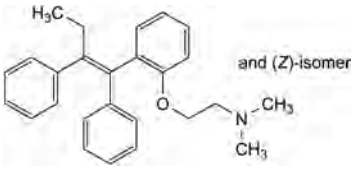


E. 2-[2-[(*EZ*)-1,2-diphenylbut-1-en-1-yl]phenoxy]-*N*,*N*-dimethylethan-1-amine,

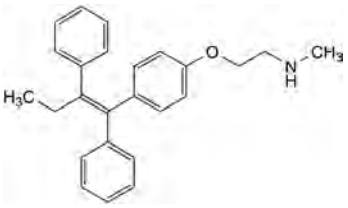

F. 2-[4-[(*Z*)-1,2-diphenylbut-1-en-1-yl]phenoxy]-*N*-methylethan-1-amine,

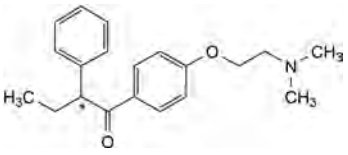

G. 1-[4-[2-(dimethylamino)ethoxy]phenyl]-2-phenylbutan-1-one,

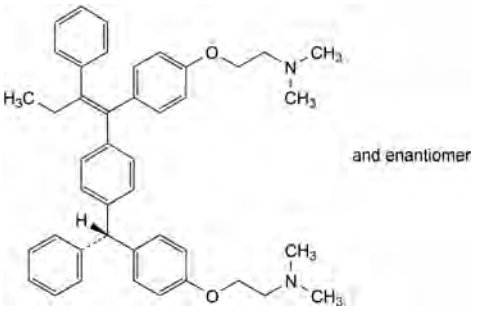


H. 2-[4-[(*RS*)-[4-[(*Z*)-1-[4-[2-(dimethylamino)-ethoxy]phenyl]-2-phenylbut-1-en-1-yl]phenyl]-(phenyl)methyl]phenoxy]-*N*,*N*-dimethylethan-1-amine.

**01/2017:2131**
**corrected 10.0**

# TAMSULOSIN HYDROCHLORIDE

## Tamsulosini hydrochloridum

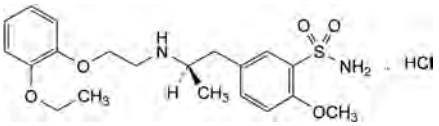

$C_{20}H_{29}ClN_2O_5S$ $M_r$ 445.0
[106463-17-6]

DEFINITION

5-[(2*R*)-2-[[2-(2-Ethoxyphenoxy)ethyl]amino]propyl]-2-methoxybenzenesulfonamide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble in water, freely soluble in formic acid, slightly soluble in anhydrous ethanol.

mp: about 230 °C.

IDENTIFICATION

Carry out either tests A, C, D or tests A, B, D.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tamsulosin hydrochloride CRS*.

B. Specific optical rotation (*2.2.7*): – 20.5 to – 17.5 (dried substance).
Dissolve with heating 0.15 g in *water R* and dilute to 20.0 mL with the same solvent.

C. Enantiomeric purity (see Tests).

D. Dissolve with heating 0.75 g in *water R* and dilute to 100.0 mL with the same solvent. Take 5 mL of the solution and cool in an ice-bath. Add 3 mL of *dilute nitric acid R* and shake. Allow to stand at room temperature for 30 min and filter. The filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**

A. Impurities eluting before tamsulosin. Liquid chromatography (*2.2.29*).
*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.
*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.
*Reference solution (b)*. Dissolve 4 mg of *tamsulosin impurity D CRS* and 4 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase. Dilute 2 mL of the solution to 20 mL with the mobile phase.
*Reference solution (c)*. Dissolve 4 mg of *tamsulosin impurity H CRS* and 4 mg of the substance to be examined in the mobile phase and dilute to 20 mL with the mobile phase. Dilute 2 mL of the solution to 20 mL with the mobile phase.
*Column*:
– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.
*Mobile phase*: dissolve 3.0 g of *sodium hydroxide R* in a mixture of 8.7 mL of *perchloric acid R* and 1900 mL of *water for chromatography R*; adjust to pH 2.0 with *0.5 M sodium hydroxide* and dilute to 2000 mL with *water for chromatography R*; to 1400 mL of this solution add 600 mL of *acetonitrile for chromatography R*.
*Flow rate*: 1.3 mL/min.
*Detection*: spectrophotometer at 225 nm.
*Injection*: 10 µL of the test solution and reference solutions (a) and (b).
*Run time*: 1.5 times the retention time of tamsulosin (retention time = about 6 min).
*System suitability*: reference solution (b):
– *resolution*: minimum 6.0 between the peaks due to impurity D and tamsulosin.
*Limits*:
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

B. Impurities eluting after tamsulosin. Liquid chromatography (*2.2.29*) as described in test A with the following modifications.
*Mobile phase*: dissolve 3.0 g of *sodium hydroxide R* in a mixture of 8.7 mL of *perchloric acid R* and 1900 mL of *water for chromatography R*; adjust to pH 2.0 with *0.5 M sodium hydroxide* and dilute to 2000 mL with *water for chromatography R*; add 2000 mL of *acetonitrile for chromatography R*.
*Flow rate*: 1.0 mL/min.
*Injection*: 10 µL of the test solution and reference solutions (a) and (c).
*Run time*: 5 times the retention time of tamsulosin (retention time = about 2.5 min).
*System suitability*: reference solution (c):
– *resolution*: minimum 2.0 between the peaks due to tamsulosin and impurity H.
*Limits*:
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities eluting before tamsulosin in test A and after tamsulosin in test B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).
*Test solution*. Dissolve 50.0 mg of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.
*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.
*Reference solution (b)*. Dissolve 5 mg of *tamsulosin racemate CRS* in *methanol R* and dilute to 25 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *methanol R*.
*Column*:
– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *amylose derivative of silica gel for chiral separation R*;
– *temperature*: 40 °C.
*Mobile phase*: *diethylamine R*, *methanol R1*, *anhydrous ethanol R*, *hexane R* (1:150:200:650 *V/V/V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Relative retention* with reference to tamsulosin (retention time = about 14 min): impurity G = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity G and tamsulosin.

*Limit*:

– *impurity G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 5.0 mL of *anhydrous formic acid R*, add 75 mL of a mixture of 2 volumes of *acetic anhydride R* and 3 volumes of *glacial acetic acid R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 44.50 mg of $C_{20}H_{29}ClN_2O_5S$.

IMPURITIES

*Specified impurities: G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, H, I.*

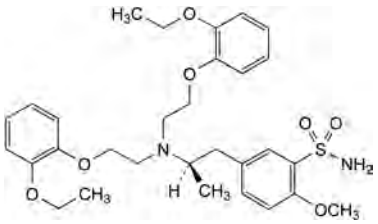

A. 5-[(2*R*)-2-[bis[2-(2-ethoxyphenoxy)ethyl]amino]propyl]-2-methoxybenzenesulfonamide,

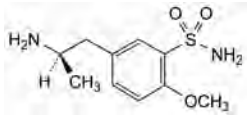

B. 5-[(2*R*)-2-aminopropyl]-2-methoxybenzenesulfonamide,

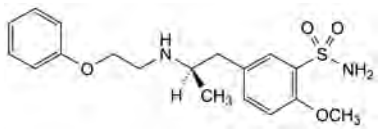

C. 2-methoxy-5-[(2*R*)-2-[(2-phenoxyethyl)amino]propyl]-benzenesulfonamide,

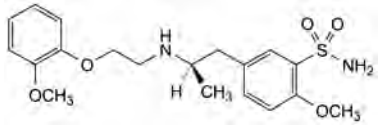

D. 2-methoxy-5-[(2*R*)-2-[[2-(2-methoxyphenoxy)ethyl]-amino]propyl]benzenesulfonamide,

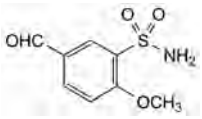

E. 5-formyl-2-methoxybenzenesulfonamide,

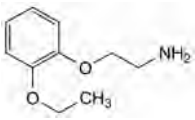

F. 2-(2-ethoxyphenoxy)ethanamine,

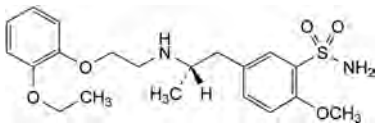

G. 5-[(2*S*)-2-[[2-(2-ethoxyphenoxy)ethyl]amino]propyl]-2-methoxybenzenesulfonamide,

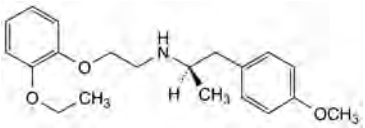

H. (2*R*)-*N*-[2-(2-ethoxyphenoxy)ethyl]-1-(4-methoxyphenyl)-propan-2-amine,

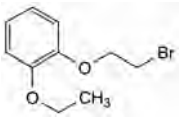

I. 1-(2-bromoethoxy)-2-ethoxybenzene.

**01/2008:1477**
**corrected 6.0**

# TANNIC ACID

## Tanninum

DEFINITION

Mixture of esters of glucose with gallic acid and 3-galloylgallic acid.

CHARACTERS

*Appearance*: yellowish-white or slightly brown amorphous light powder or shiny plates.

*Solubility*: very soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in glycerol (85 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Dilute 0.1 mL of solution S (see Tests) to 5 mL with *water R*. Add 0.1 mL of *ferric chloride solution R1*. A blackish-blue colour is produced which becomes green on the addition of 1 mL of *dilute sulfuric acid R*.

B. To 1 mL of solution S, add 3 mL of a 1 g/L solution of *gelatin R*. The mixture becomes turbid and a flocculent precipitate is formed.

C. Dilute 0.1 mL of solution S to 5 mL with *water R*. Add 0.3 mL of *barium hydroxide solution R*. A greenish-blue precipitate is formed.

TESTS

**Solution S**. Dissolve 4.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*).

**Dextrins, gum, salts, sugars**. To 2 mL of solution S, add 2 mL of *ethanol (96 per cent) R*. The solution is clear. Add 1 mL of *ether R*. The solution remains clear for at least 10 min.

**Resins**. To 5 mL of solution S, add 5 mL of *water R*. The mixture remains clear (*2.2.1*) for at least 15 min.

**Loss on drying** (*2.2.32*): maximum 12.0 per cent, determined on 0.200 g by drying at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

Protected from light.

**01/2020:3035**

# TAPENTADOL HYDROCHLORIDE

## Tapentadoli hydrochloridum

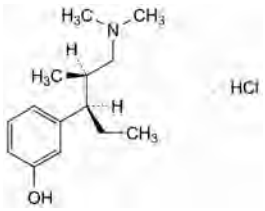


$C_{14}H_{24}ClNO$ $M_r$ 257.8
[175591-09-0]

DEFINITION

3-[(2*R*,3*R*)-1-(Dimethylamino)-2-methylpentan-3-yl]phenol hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in tapentadol hydrochloride. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in methanol, soluble in anhydrous ethanol, very slightly soluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tapentadol hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

C. Enantiomeric purity (see Tests).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *diethylamine R*, *2-propanol R*, *heptane R* (0.2:10:89.8 *V/V/V*).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in a mixture of 40 µL of *diethylamine R* and 2 mL of *2-propanol R* using sonication and dilute to 20.0 mL with *heptane R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *tapentadol impurity A CRS* and 5 mg of the substance to be examined in a mixture of 0.2 mL of *diethylamine R* and 10 mL of *2-propanol R* using sonication and dilute to 100 mL with *heptane R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm).

*Mobile phase*: *diethylamine R*, *2-propanol R*, *heptane R* (0.1:2:98 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of tapentadol.

*Relative retention* with reference to tapentadol (retention time = about 13 min): impurity A = about 1.2.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to tapentadol and impurity A.

*Limit*:
- *impurity A*: maximum 1.0 per cent, calculated as the ratio of the area of the peak due to impurity A to the sum of the areas of the peaks due to tapentadol and impurity A;
- *reporting threshold*: 0.10 per cent (reference solution (a)).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *phosphoric acid R*, *methanol R*, *water R* (0.1:20:80 *V/V/V*).

*Test solution (a)*. Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 30.0 mg of *tapentadol hydrochloride CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 3 mg of *tapentadol impurity C CRS* in the solvent mixture and dilute to 5 mL with the solvent mixture. Add 0.5 mL of the solution to 30 mg of the substance to be examined and dilute to 50 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*:
- *mobile phase A*: *phosphoric acid R*, *methanol R2*, *water for chromatography R* (0.1:10:90 *V/V/V*);
- *mobile phase B*: *phosphoric acid R*, *water for chromatography R*, *methanol R2* (0.1:10:90 *V/V/V*);

Monographs T

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 100 | 0 |
| 2 - 44 | 100 → 61 | 0 → 39 |
| 44 - 44.5 | 61 → 0 | 39 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Relative retention* with reference to tapentadol (retention time = about 15 min): impurity C = about 0.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity C and tapentadol.

*Calculation of percentage contents*:

– for each impurity, use the concentration of tapentadol hydrochloride in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.4 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{14}H_{24}ClNO$ taking into account the assigned content of *tapentadol hydrochloride CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

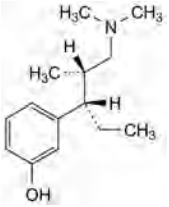

A. 3-[(2*S*,3*S*)-1-(dimethylamino)-2-methylpentan-3-yl]phenol,

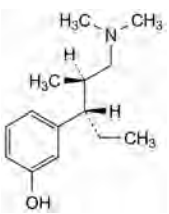

B. 3-[(2*R*,3*S*)-1-(dimethylamino)-2-methylpentan-3-yl]phenol,

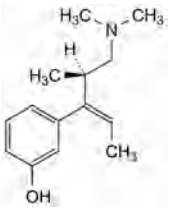

C. 3-[(2*Z*,4*R*)-5-(dimethylamino)-4-methylpent-2-en-3-yl]phenol,

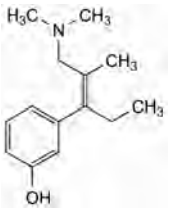

D. 3-[(2*Z*)-1-(dimethylamino)-2-methylpent-2-en-3-yl]phenol,

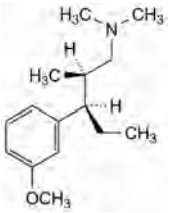

E. (2*R*,3*R*)-3-(3-methoxyphenyl)-*N*,*N*,2-trimethylpentan-1-amine.

**01/2017:0460**

# TARTARIC ACID

## Acidum tartaricum

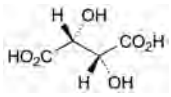

$C_4H_6O_6$ $M_r$ 150.1

[87-69-4]

DEFINITION

(2*R*,3*R*)-2,3-Dihydroxybutanedioic acid.

The substance is of natural origin, obtained by extraction of lees during winemaking.

*Content*: 99.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) is strongly acid (*2.2.4*).

B. It gives the reactions of tartrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *distilled water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 12.0 to + 12.8 (dried substance).

Dissolve 5.00 g in *water R* and dilute to 25.0 mL with the same solvent.

**Oxalic acid**: maximum 360 ppm, calculated as anhydrous oxalic acid.

Dissolve 0.80 g in 4 mL of *water R*. Add 3 mL of *hydrochloric acid R* and 1 g of *zinc R* in granules and boil for 1 min. Allow to stand for 2 min. Collect the liquid in a test-tube containing 0.25 mL of a 10 g/L solution of *phenylhydrazine hydrochloride R* and heat to boiling. Cool rapidly, transfer to a graduated cylinder and add an equal volume of *hydrochloric acid R* and 0.25 mL of a 50 g/L solution of *potassium ferricyanide R*. Shake and allow to stand for 30 min. Any pink colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 4 mL of a 0.1 g/L solution of *oxalic acid R*.

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 150 ppm.

Dilute 10 mL of solution S to 15 mL with *distilled water R*.

**Calcium** (*2.4.3*): maximum 200 ppm.

To 5 mL of solution S add 10 mL of a 50 g/L solution of *sodium acetate R* in *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.650 g in 25 mL of *water R*. Titrate with *1 M sodium hydroxide* using 0.5 mL of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 mL of *1 M sodium hydroxide* is equivalent to 75.05 mg of $C_4H_6O_6$.

**07/2019:2358**

# TEICOPLANIN

## Teicoplaninum

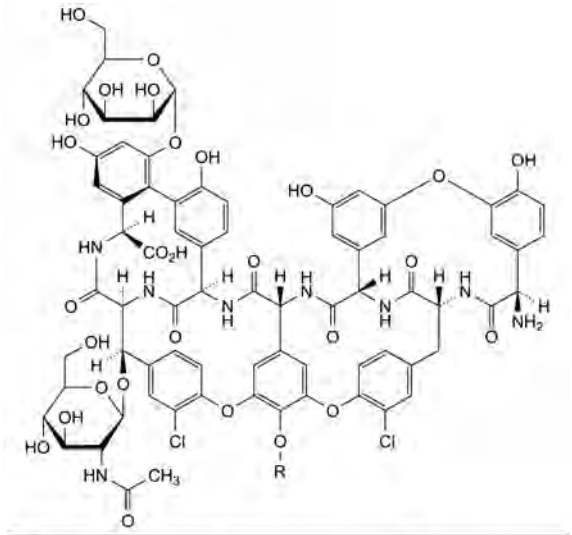

| teicoplanin | R | R' |
|---|---|---|
| $A_{2\text{-}1}$ $C_{88}H_{95}Cl_2N_9O_{33}$ $M_r$ 1878 | | |
| $A_{2\text{-}2}$ $C_{88}H_{97}Cl_2N_9O_{33}$ $M_r$ 1880 | | |
| $A_{2\text{-}3}$ $C_{88}H_{97}Cl_2N_9O_{33}$ $M_r$ 1880 | | |
| $A_{2\text{-}4}$ $C_{89}H_{99}Cl_2N_9O_{33}$ $M_r$ 1894 | | |
| $A_{2\text{-}5}$ $C_{89}H_{99}Cl_2N_9O_{33}$ $M_r$ 1894 | | |
| $A_{3\text{-}1}$ $C_{72}H_{68}Cl_2N_8O_{28}$ $M_r$ 1564 | H | |
| $A_{2\text{-}1a}$ $C_{87}H_{95}Cl_2N_9O_{33}$ $M_r$ 1866 | | |
| $A_{2\text{-}1b}$ $C_{87}H_{95}Cl_2N_9O_{33}$ $M_r$ 1866 | | |

[61036-62-2]

DEFINITION

Mixture of glycopeptides produced by certain strains of *Actinoplanes teichomyceticus sp.*; the 6 principal components of the mixture are teicoplanins $A_{2\text{-}1}$ to $A_{2\text{-}5}$ and teicoplanin $A_{3\text{-}1}$, and 2 minor components are teicoplanins $A_{2\text{-}1a}$ and $A_{2\text{-}1b}$.

Fermentation product.

*Potency*: minimum 900 IU/mg (anhydrous and sodium chloride-free substance).

CHARACTERS

*Appearance*: white or yellowish, amorphous powder.

*Solubility*: freely soluble in water, sparingly soluble in dimethylformamide, practically insoluble in ethanol (96 per cent).

Monographs T

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *teicoplanin for identification CRS*.

B. Examine the chromatograms obtained in the test for composition.

*Results*: the principal peaks (teicoplanins $A_{3\text{-}1}$, $A_{2\text{-}1}$, $A_{2\text{-}2}$, $A_{2\text{-}3}$, $A_{2\text{-}4}$ and $A_{2\text{-}5}$) and the 2 minor peaks (teicoplanins $A_{2\text{-}1a}$ and $A_{2\text{-}1b}$) in the chromatogram obtained with the test solution are similar in retention time and size to the principal peaks in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_3$ or $B_4$ (*2.2.2, Method I*).

Dissolve 0.8 g in 10 mL of *water R*.

**pH** (*2.2.3*): 6.5 to 7.5.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *teicoplanin for identification CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of reference solution (a) to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

*Reference solution (c)*. Dissolve 50.0 mg of *mesityl oxide CRS* (impurity A) in *water R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 900 mL of a 3.0 g/L solution of *anhydrous sodium dihydrogen phosphate R*, adjusted to pH 6.0 with a 40 g/L solution of *sodium hydroxide R*, and 100 mL of *acetonitrile R*;
- *mobile phase B*: mix 300 mL of a 3.0 g/L solution of *anhydrous sodium dihydrogen phosphate R*, adjusted to pH 6.0 with a 40 g/L solution of *sodium hydroxide R*, and 700 mL of *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 50 | 0 → 50 |
| 30 - 31 | 50 → 10 | 50 → 90 |
| 31 - 35 | 10 | 90 |

*Flow rate*: 2.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Identification*: use the chromatogram supplied with *teicoplanin for identification CRS* and the chromatogram obtained with reference solution (a) to identify the groups and components.

*Relative retention* with reference to teicoplanin $A_{2\text{-}2}$ (retention time = about 18 min):

- teicoplanin $A_3$ group ≤ 0.70:
  - teicoplanin $A_{3\text{-}1}$ = about 0.43.
- teicoplanin $A_2$ group > 0.70 including:
  - teicoplanin $A_{2\text{-}1}$ group > 0.70 and < 1.00:
    - teicoplanin $A_{2\text{-}1a}$ = about 0.85;
    - teicoplanin $A_{2\text{-}1b}$ = about 0.88;
    - teicoplanin $A_{2\text{-}1}$ = about 0.93;
  - teicoplanin $A_{2\text{-}2}$ = 1.00;
  - teicoplanin $A_{2\text{-}3}$ group > 1.00 and < 1.12:
    - teicoplanin $A_{2\text{-}3}$ = about 1.03;
  - teicoplanin $A_{2\text{-}4}$ = about 1.12;
  - teicoplanin $A_{2\text{-}5}$ group > 1.12 and < 1.25:
    - teicoplanin $A_{2\text{-}5}$ = about 1.15;
  - teicoplanin $A_{2\text{-}6}$ group ≥ 1.25:
    - teicoplanin-like related substance RS $A_{2\text{-}6a}$ = about 1.25;
    - teicoplanin-like related substance RS $A_{2\text{-}6b}$ = about 1.30;
    - teicoplanin-like related substance RS $A_{2\text{-}6c}$ = about 1.38.

*System suitability*:

- the chromatogram obtained with reference solution (a) is similar to the chromatogram supplied with *teicoplanin for identification CRS*;
- *resolution*: minimum 1.0 between the peaks due to teicoplanin $A_{2\text{-}4}$ and teicoplanin $A_{2\text{-}5}$ in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 40 for the peak due to teicoplanin $A_{2\text{-}2}$ in the chromatogram obtained with reference solution (b).

Calculate the percentage contents using the following equations:

$$\text{teicoplanin } A_3 \text{ group} = \frac{0.83 \times S_3}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_2 \text{ group} = \frac{S_2}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}1} \text{ group} = \frac{S_{2-1}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}1a} = \frac{A_{2-1a}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}1b} = \frac{A_{2-1b}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}1} = \frac{A_{2-1}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}2} = \frac{A_{2-2}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}3} \text{ group} = \frac{S_{2-3}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}3} = \frac{A_{2-3}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}4} = \frac{A_{2-4}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}5} \text{ group} = \frac{S_{2-5}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}5} = \frac{A_{2-5}}{S_2 + 0.83 \times S_3} \times 100$$

$$\text{teicoplanin } A_{2\text{-}6} \text{ group} = \frac{S_{2-6}}{S_2 + 0.83 \times S_3} \times 100$$

$S_2$ = sum of the areas of the peaks due to teicoplanin $A_2$ group in the chromatogram obtained with the test solution;

$S_3$ = sum of the areas of the peaks due to teicoplanin $A_3$ group in the chromatogram obtained with the test solution; disregard any peak due to impurity A;

$S_{2\text{-}6}$ = sum of the areas of the peaks with a relative retention greater than or equal to 1.25 in the chromatogram obtained with the test solution;

$S_{2\text{-}1}$ = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}1}$ group in the chromatogram obtained with the test solution;

$A_{2\text{-}1a}$ = area of the peak due to teicoplanin $A_{2\text{-}1a}$ in the chromatogram obtained with the test solution;

$A_{2\text{-}1b}$ = area of the peak due to teicoplanin $A_{2\text{-}1b}$ in the chromatogram obtained with the test solution;

$A_{2\text{-}1}$ = area of the peak due to teicoplanin $A_{2\text{-}1}$ in the chromatogram obtained with the test solution;

$A_{2\text{-}2}$ = area of the peak due to teicoplanin $A_{2\text{-}2}$ in the chromatogram obtained with the test solution;

$S_{2\text{-}3}$ = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}3}$ group in the chromatogram obtained with the test solution;

$A_{2\text{-}3}$ = area of the peak due to teicoplanin $A_{2\text{-}3}$ in the chromatogram obtained with the test solution;

$A_{2\text{-}4}$ = area of the peak due to teicoplanin $A_{2\text{-}4}$ in the chromatogram obtained with the test solution;

$S_{2\text{-}5}$ = sum of the areas of the peaks due to teicoplanin $A_{2\text{-}5}$ group in the chromatogram obtained with the test solution;

$A_{2\text{-}5}$ = area of the peak due to teicoplanin $A_{2\text{-}5}$ in the chromatogram obtained with the test solution;

*Limits*:

- *teicoplanin $A_2$ group*: 84.0 per cent to 98.0 per cent;
- *teicoplanin $A_{2\text{-}2}$*: 37.0 per cent to 50.0 per cent;
- *teicoplanin $A_{2\text{-}1}$ group*: 10.0 per cent to 19.0 per cent;
- *teicoplanin $A_{2\text{-}5}$ group*: 7.0 per cent to 17.0 per cent;
- *teicoplanin $A_{2\text{-}4}$*: 7.0 per cent to 15.0 per cent;
- *teicoplanin $A_{2\text{-}5}$*: 7.0 per cent to 15.0 per cent;
- *teicoplanin $A_{2\text{-}3}$ group*: 5.0 per cent to 11.0 per cent;
- *teicoplanin $A_3$ group*: 4.0 per cent to 12.0 per cent;
- *teicoplanin $A_{2\text{-}3}$*: 4.0 per cent to 8.5 per cent;
- *teicoplanin $A_{2\text{-}1}$*: 2.0 per cent to 7.0 per cent;
- *teicoplanin $A_{2\text{-}1a}$*: 0.5 per cent to 5.5 per cent;
- *teicoplanin $A_{2\text{-}1b}$*: 0.5 per cent to 4.0 per cent;
- *teicoplanin $A_{2\text{-}6}$ group*: maximum 5.0 per cent;
- *disregard limit*: 0.25 per cent.

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition. Use the normalisation procedure.

Use the chromatogram obtained with reference solution (a) to identify all peaks present above the disregard limit as teicoplanin-like related substances. Any peak present in any part of the chromatogram obtained with the test solution that cannot be correlated to a peak above the disregard limit in reference solution (a) should be considered as a non-teicoplanin-like impurity, unless it is characterized by other means.

A teicoplanin-like related substance is defined as a substance that shares the same glycopeptide core structure of the parent molecule, composed of a linear heptapeptide aglycone, an α-D-mannose and an acetyl-β-D-glucosamine.

The R′ side chains in the teicoplanin-like related substances RS $A_{2\text{-}6a}$, RS $A_{2\text{-}6b}$ and RS $A_{2\text{-}6c}$ are unknown.

Calculate the percentage contents using the following equations:

$$\text{teicoplanin-like related substance (x)} = \frac{A_{RSTLx}}{(S2 + 0.83 \times S_3)} \times 100$$

$A_{RSTLx}$ = area of the peak due to the teicoplanin-like related substance (x) in the chromatogram obtained with the test solution;

$$\text{non-teicoplanin-like impurity (x)} = \frac{A_{Ix}}{(S2 + 0.83 \times S_3)} \times 100$$

$A_{Ix}$ = area of the peak due to the non-teicoplanin-like impurity (x) in the chromatogram obtained with the test solution.

*Limits*:

- *teicoplanin-like related substance RS $A_{2\text{-}6c}$*: maximum 2.5 per cent;
- *teicoplanin-like related substance RS $A_{2\text{-}6a}$*: maximum 1.5 per cent;
- *teicoplanin-like related substance RS $A_{2\text{-}6b}$*: maximum 1.5 per cent;
- *any non-teicoplanin-like impurity other than impurity A*: maximum 0.5 per cent;
- *total non-teicoplanin-like impurities other than impurity A*: maximum 1.5 per cent.

**Impurity A**. Liquid chromatography (*2.2.29*) as described in the test for composition with the following modifications.

*Injection*: 20 µL of the test solution and reference solution (c).

*Relative retention* with reference to teicoplanin $A_{2\text{-}2}$ (retention time = about 18 min): impurity A = about 0.6.

*Calculation of percentage content*:

- for impurity A, use the concentration of impurity A in reference solution (c).

*Limit*:

- *impurity A*: maximum 0.2 per cent.

**Chlorides**: maximum 5.0 per cent, expressed as sodium chloride (anhydrous substance).

Dissolve 1.000 g in 300 mL of *water R*, stir and acidify with 2 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

**Water** (*2.5.12*): maximum 15.0 per cent, determined on 0.300 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*), using the diffusion method. Use *teicoplanin CRS* as the reference substance.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A.*

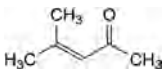

A. 4-methylpent-3-en-2-one (mesityl oxide).

**04/2019:2154**

# TELMISARTAN

## Telmisartanum

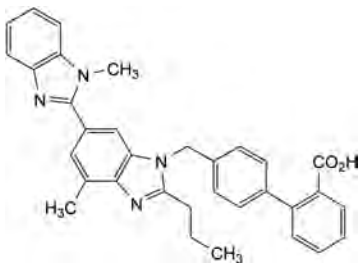

$C_{33}H_{30}N_4O_2$ $M_r$ 514.6

[144701-48-4]

DEFINITION

4′-[[4-Methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl][1,1′-biphenyl]-2-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in methanol, sparingly soluble in methylene chloride. It dissolves in a 40 g/L solution of *sodium hydroxide R*.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *telmisartan CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in hot *anhydrous ethanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_4$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 40 g/L solution of *sodium hydroxide R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. To 25 mg of the substance to be examined add about 5 mL of *methanol R* and 100 µL of a 40 g/L solution of *sodium hydroxide R*. Dissolve using sonication and dilute to 50.0 mL with *methanol R*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *telmisartan for system suitability CRS* (containing impurities A, B, C, E and F) in 2 mL of *methanol R*.

*Reference solution (c)*. To 5 mg of *telmisartan for peak identification CRS* (containing impurity D) add about 5 mL of *methanol R* and 100 µL of a 40 g/L solution of *sodium hydroxide R*. Dissolve using sonication and dilute to 10 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm;
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 2.0 g of *potassium dihydrogen phosphate R* and 3.8 g of *sodium pentanesulfonate monohydrate R1* in 900 mL of *water for chromatography R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *methanol R1*, *acetonitrile for chromatography R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 70 | 30 |
| 3 - 28 | 70 → 20 | 30 → 80 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *telmisartan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, E and F; use the chromatogram supplied with *telmisartan for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to telmisartan (retention time = about 15 min): impurity A = about 0.2; impurity E = about 0.6; impurity F = about 0.7; impurity B = about 0.9; impurity C = about 1.5; impurity D = about 1.6.

*System suitability*: reference solution (b):

- the chromatogram obtained with reference solution (b) is similar to the chromatogram supplied with *telmisartan for system suitability CRS*;
- *resolution*: minimum 3.0 between the peaks due to impurity B and telmisartan.

*Limits*:

- *impurities C, D*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *impurities A, B*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.190 g in 5 mL of *anhydrous formic acid R*. Add 75 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.73 mg of $C_{33}H_{30}N_4O_2$.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G, H, I, J.*

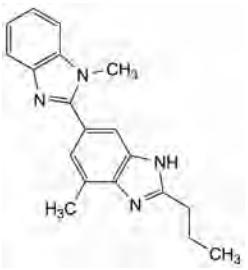

A. 4-methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazole,

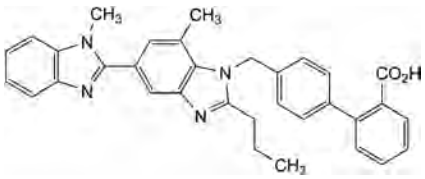

B. 4′-[[7-methyl-5-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl][1,1′-biphenyl]-2-carboxylic acid,

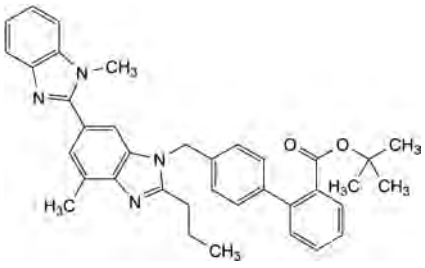

C. *tert*-butyl 4′-[[4-methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl][1,1′-biphenyl]-2-carboxylate,

D. unknown structure,

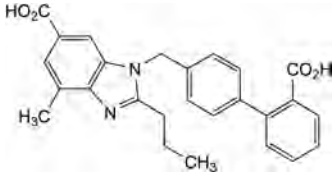

E. 1-[(2′-carboxy[1,1′-biphenyl]-4-yl)methyl]-4-methyl-2-propyl-1*H*-benzimidazole-6-carboxylic acid,

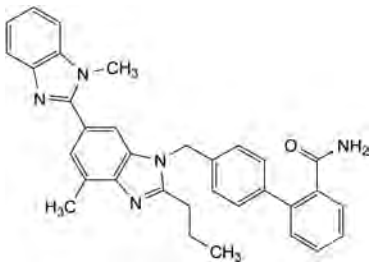

F. 4′-[[4-methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl][1,1′-biphenyl]-2-carboxamide,

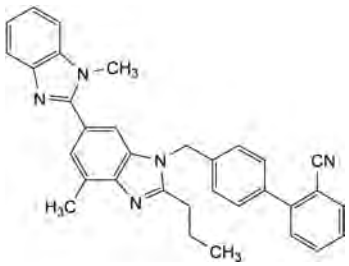

G. 4′-[[4-methyl-6-(1-methyl-1*H*-benzimidazol-2-yl)-2-propyl-1*H*-benzimidazol-1-yl]methyl][1,1′-biphenyl]-2-carbonitrile,

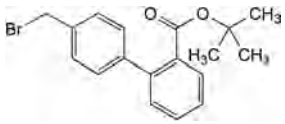

H. *tert*-butyl 4′-(bromomethyl)[1,1′-biphenyl]-2-carboxylate,

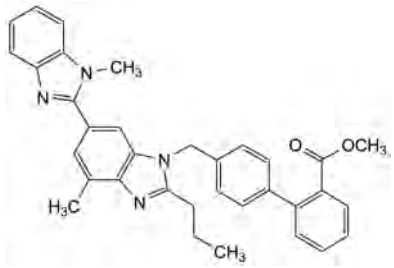

I. methyl 4′-[(1,7′-dimethyl-2′-propyl-1*H*,3′*H*-[2,5′-bibenzimidazol]-3′-yl)methyl][1,1′-biphenyl]-2-carboxylate,

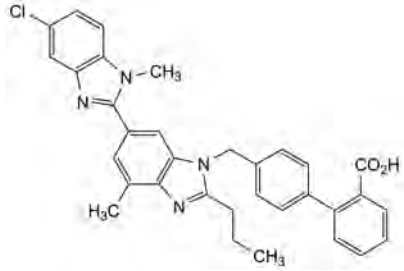

J. 4′-[(5-chloro-1,7′-dimethyl-2′-propyl-1*H*,3′*H*-[2,5′-bibenzimidazol]-3′-yl)methyl][1,1′-biphenyl]-2-carboxylic acid.

**01/2008:0954**
**corrected 6.0**

# TEMAZEPAM

## Temazepamum

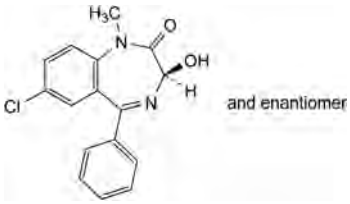


$C_{16}H_{13}ClN_2O_2$ $M_r$ 300.7
[846-50-4]

DEFINITION

(3*RS*)-7-Chloro-3-hydroxy-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *temazepam CRS*.

TESTS

**Impurity A**: maximum 0.05 per cent.

Dissolve 0.400 g in *methylene chloride R* and dilute to 20.0 mL with the same solvent. The absorbance (*2.2.25*) is not greater than 0.30 at 409 nm.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with a mixture of 1 volume of *water R* and 9 volumes of *methanol R*.

*Reference solution (b).* Dissolve 1 mg of *oxazepam R*, 1 mg of *temazepam impurity F CRS* and 1 mg of *temazepam impurity G CRS* in a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 25 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 1 mg of *temazepam impurity C CRS* and 1 mg of *temazepam impurity D CRS* with a mixture of 1 volume of *water R* and 9 volumes of *methanol R* and dilute to 25 mL with the same mixture of solvents.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm).

*Mobile phase*:

- *mobile phase A*: solution containing 4.9 g/L of *sodium dihydrogen phosphate R* and 0.63 g/L of *disodium hydrogen phosphate dodecahydrate R* (pH 5.6);
- *mobile phase B*: *methanol R*;
- *mobile phase C*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 18 | 54 | 39 | 7 |
| 18 - 25 | 54 → 22 | 39 → 63 | 7 → 15 |
| 25 - 31 | 22 | 63 | 15 |
| 31 - 37 | 22 → 54 | 63 → 39 | 15 → 7 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Relative retention* with reference to temazepam (retention time = about 16 min): impurity E = about 0.55; impurity F = about 0.67; impurity G = about 0.73; impurity B = about 0.8; impurity D = about 1.2; impurity C = about 1.3; impurity A = about 1.5.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity F and impurity G;
- *peak-to-valley ratio*: minimum 1.7, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity F = 3.2; impurity G = 3.1;
- *impurities B, C, D, E, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *nitroethane R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.07 mg of $C_{16}H_{13}ClN_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

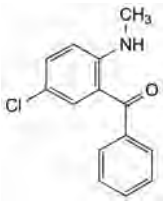


A. [5-chloro-2-(methylamino)phenyl]phenylmethanone,

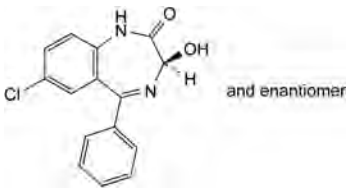


B. (3*RS*)-7-chloro-3-hydroxy-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one (oxazepam),

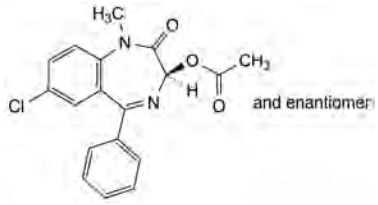


C. (3*RS*)-7-chloro-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepin-3-yl acetate,

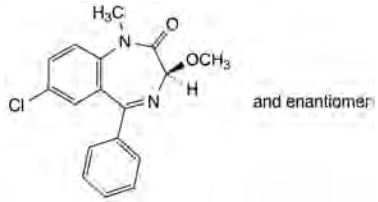


D. (3*RS*)-7-chloro-3-methoxy-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

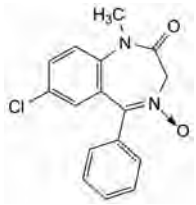


E. 7-chloro-1-methyl-5-phenyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one 4-oxide,

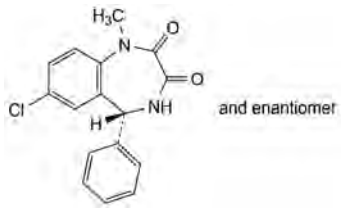


F. (5*RS*)-7-chloro-1-methyl-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione,

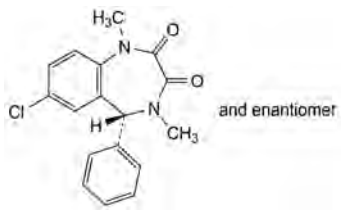


G. (5*RS*)-7-chloro-1,4-dimethyl-5-phenyl-4,5-dihydro-1*H*-1,4-benzodiazepine-2,3-dione.

**04/2019:2780**

# TEMOZOLOMIDE

## Temozolomidum

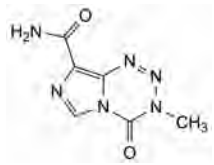


$C_6H_6N_6O_2$ $M_r$ 194.2
[85622-93-1]

DEFINITION

3-Methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]tetrazine-8-carboxamide.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly brown or slightly pink powder.

*Solubility*: sparingly soluble in water, soluble in dimethyl sulfoxide, very slightly soluble in ethanol (96 per cent), practically insoluble in toluene.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *temozolomide CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (d).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 25.0 mg of the substance to be examined in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 10.0 mL with *dimethyl sulfoxide R*.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with *dimethyl sulfoxide R*. Dilute 1.0 mL of this solution to 10.0 mL with *dimethyl sulfoxide R*.

*Reference solution (b)*. In order to prepare impurities A, B and E *in situ*, mix 5 mL of a 10.3 g/L solution of *hydrochloric acid R* and 5 mL of test solution (a). Heat the mixture in a water-bath for 1 h.

*Reference solution (c)*. Dissolve 2 mg of *temozolomide for peak identification CRS* (containing impurity D) in 2 mL of *dimethyl sulfoxide R*.

*Reference solution (d)*. Dissolve 25.0 mg of *temozolomide CRS* in *dimethyl sulfoxide R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *dimethyl sulfoxide R*.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 0.94 g/L solution of *sodium hexanesulfonate R* in a mixture of 4 volumes of *methanol R* and 96 volumes of a 0.5 per cent *V/V* solution of *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

Monographs T

*Injection*: 10 µL of test solution (a) and reference solutions (a), (b) and (c).

*Run time*: 3 times the retention time of temozolomide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and E; use the chromatogram supplied with *temozolomide for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to temozolomide (retention time = about 11 min): impurity E = about 0.4; impurity D = about 0.5; impurity B = about 0.9; impurity A = about 1.7. The peak due to impurity A in the chromatogram obtained with test solution (a) may be split.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity B and temozolomide.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.4; impurity E = 0.6;
– for each impurity, use the concentration of temozolomide in reference solution (a).

*Limits*:

– *impurity D*: maximum 0.5 per cent;
– *impurity A* (*sum of the peaks*): maximum 0.15 per cent;
– *impurities B, E*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.8 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 0.4 per cent, determined on 50.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_6H_6N_6O_2$ taking into account the assigned content of *temozolomide CRS*.

IMPURITIES

*Specified impurities: A, B, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

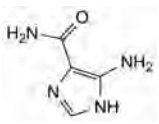

A. 5-amino-1*H*-imidazole-4-carboxamide,

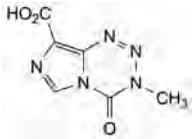

B. 3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*][1,2,3,5]-tetrazine-8-carboxylic acid,

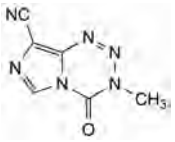

C. 3-methyl-4-oxo-3,4-dihydroimidazo[5,1-*d*]-[1,2,3,5]tetrazine-8-carbonitrile,

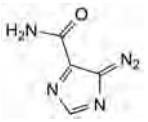

D. 4-diazo-4*H*-imidazole-5-carboxamide,

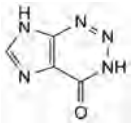

E. 3,7-dihydro-4*H*-imidazo[4,5-*d*][1,2,3]triazin-4-one (2-azahypoxanthine).

**01/2017:1156**
**corrected 9.4**

# TENOXICAM

## Tenoxicamum

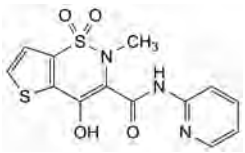

$C_{13}H_{11}N_3O_4S_2$ $M_r$ 337.4
[59804-37-4]

DEFINITION

4-Hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxamide 1,1-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, very slightly soluble in anhydrous ethanol. It dissolves in solutions of acids and alkalis.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tenoxicam CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methylene chloride R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.10 g in *methylene chloride R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture.* Mix equal volumes of *acetonitrile R* and *water R*. Adjust to apparent pH 3.2 with *dilute phosphoric acid R1*.

*Test solution.* Dissolve 35 mg of the substance to be examined in the solvent mixture, sonicate and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 7 mg of *pyridin-2-amine R* (impurity A) in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *tenoxicam impurity mixture CRS* (impurities B, G and H) in 1.0 mL of the test solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *diisopropylcyanosilyl silica gel for chromatography R* (3.5 µm);
- *temperature*: 35 °C.

*Mobile phase*:

- *mobile phase A*: mix 25 volumes of *methanol R2* and 75 volumes of *water for chromatography R* and adjust to apparent pH 3.2 with *dilute phosphoric acid R1*;
- *mobile phase B*: mix 25 volumes of *water for chromatography R* and 75 volumes of *methanol R2* and adjust to apparent pH 3.2 with *dilute phosphoric acid R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 96 | 4 |
| 5 - 16 | 96 → 76 | 4 → 24 |
| 16 - 25 | 76 | 24 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Identification of impurities*:

- use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A;
- use the chromatogram supplied with *tenoxicam impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B, G and H; for identification of impurities G and H, which may be inverted in the elution order, take into account the heights of the corresponding peaks in the chromatogram supplied with *tenoxicam impurity mixture CRS*.

*Relative retention* with reference to tenoxicam (retention time = about 12 min): impurity A = about 0.1; impurity G = about 0.85; impurity H = about 0.9; impurity B = about 1.3.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.3 between the peaks due to impurity H (or G if peaks are inverted) and tenoxicam, and between the peaks due to impurities G and H; if necessary, optimise the apparent pH of the mobile phases within the range 3.0-3.4.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.2; impurity B = 2.0;
- *impurities A, B*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 5 mL of *anhydrous formic acid R*. Add 70 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 33.74 mg of $C_{13}H_{11}N_3O_4S_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D, E, F, G, H.*

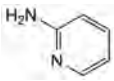

A. pyridin-2-amine,

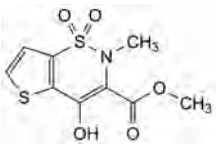

B. methyl 4-hydroxy-2-methyl-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxylate 1,1-dioxide,

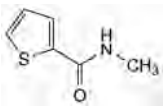

C. *N*-methylthiophene-2-carboxamide,

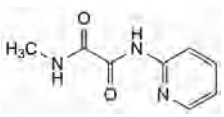

D. *N*-methyl-*N'*-(pyridin-2-yl)-ethanediamide,

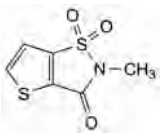

E. 2-methylthieno[2,3-*d*]isothiazol-3(2*H*)-one 1,1-dioxide,

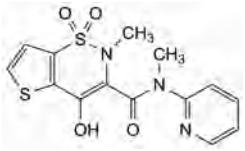

F. 4-hydroxy-*N*,2-dimethyl-*N*-(pyridin-2-yl)-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxamide 1,1-dioxide,

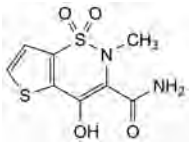

G. 4-hydroxy-2-methyl-2*H*-thieno[2,3-*e*]1,2-thiazine-3-carboxamide 1,1-dioxide,

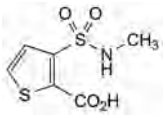

H. 3-[(methylamino)sulfonyl]thiophene-2-carboxylic acid.

**01/2017:2021**

# TERAZOSIN HYDROCHLORIDE DIHYDRATE

## Terazosini hydrochloridum dihydricum

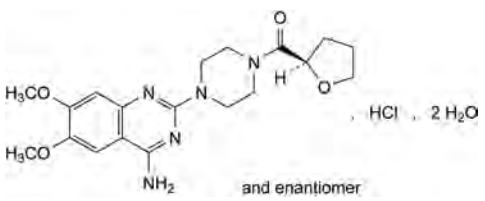

$C_{19}H_{26}ClN_5O_4,2H_2O$ $M_r$ 459.9
[70024-40-7]

DEFINITION

1-(4-Amino-6,7-dimethoxyquinazolin-2-yl)-4-[[(2*RS*)-tetrahydrofuran-2-yl]carbonyl]piperazine hydrochloride dihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in methanol, very slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *terazosin hydrochloride dihydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.00 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dilute 10 mL of solution S to 20 mL with *water R*.

**pH** (*2.2.3*): 3.0 to 5.0 for solution S.

**Impurities N and O**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 5 mg of *terazosin impurity A CRS* and 5.0 mg of *terazosin impurity N CRS* in *acetonitrile R1* using sonication, add 5.0 mL of the test solution and dilute to 50.0 mL with *acetonitrile R1*. Dilute 10.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 10.0 mL of reference solution (a) to 100.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*: dissolve 2.80 g of *sodium laurilsulfate R* in 1000.0 mL of *water R* and add 11.0 mL of a solution containing 202.4 g/L of *triethylamine R* and 230.0 g/L of *phosphoric acid R*; adjust to pH 2.5 with *phosphoric acid R*; mix 600 volumes of this solution with 400 volumes of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of terazosin.

*Relative retention* with reference to terazosin (retention time = about 10 min): impurity O = about 0.2; impurity N = about 0.3; impurity A = about 0.4.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurities A and N.

*Limits*:
- *impurity N*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *impurity O*: not more than the area of the peak due to terazosin in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *terazosin for system suitability CRS* (containing impurities A, B, C, J, K and M) in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (c)*. Dissolve 5.0 mg of *terazosin impurity L CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (d)*. To 5 mg of *terazosin impurity E CRS*, add 70 mL of *methanol R* and 30 mL of *water R*. Allow to stand for at least 1 h to dissolve the substance. Use sonication if necessary.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 2 volumes of *triethylamine R*, 350 volumes of *acetonitrile R*, and 1650 volumes of a solution containing 6 g/L of *sodium citrate R* and 14.25 g/L of *anhydrous citric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 245 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of terazosin.

*Identification of impurities*: use the chromatogram supplied with *terazosin for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, J, K and M; use the chromatograms obtained with reference solutions (c) and (d) to identify the peaks due to impurities L and E respectively.

*Retention time*: terazosin = about 11 min.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurities B and J; if necessary, adjust the proportion of the aqueous component in the mobile phase (an increase in the proportion of the aqueous component increases the retention times);
- the chromatogram obtained is similar to the chromatogram supplied with *terazosin for system suitability CRS*; in case of insufficient separation of the impurities, reduce the amount of triethylamine in the mobile phase.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity C = 0.7; impurity M = 1.6;
- *impurities A, C, E, K*: for each impurity, not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity L*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.1 per cent);
- *impurities B, J, M*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 7.0 per cent to 8.6 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *methanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 42.39 mg of $C_{19}H_{26}ClN_5O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, E, J, K, L, M, N, O.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, F, G, H, I.*

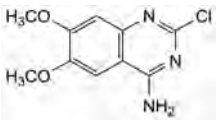

A. 2-chloro-6,7-dimethoxyquinazolin-4-amine,

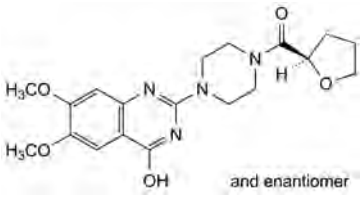


B. 1-(4-hydroxy-6,7-dimethoxyquinazolin-2-yl)-4-[[(2*RS*)-tetrahydrofuran-2-yl]carbonyl]piperazine,

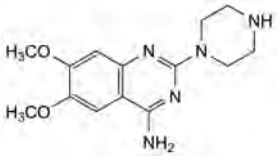

C. 6,7-dimethoxy-2-(piperazin-1-yl)quinazolin-4-amine,

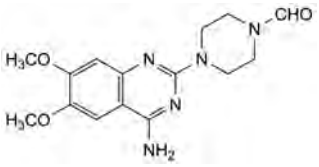

D. 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-formylpiperazine,

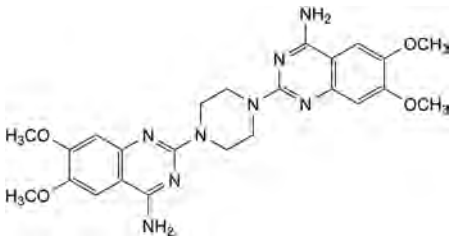

E. 2,2′-(piperazine-1,4-diyl)bis(6,7-dimethoxyquinazolin-4-amine),

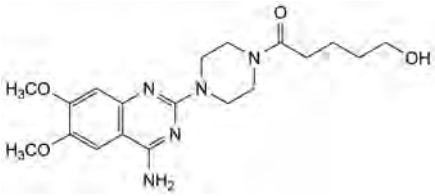

F. 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-(5-hydroxypentanoyl)piperazine,

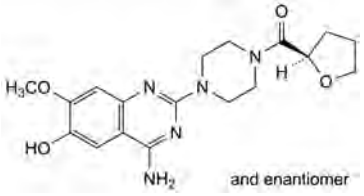


G. 1-(4-amino-6-hydroxy-7-methoxyquinazolin-2-yl)-4-[[(2*RS*)-tetrahydrofuran-2-yl]carbonyl]piperazine,

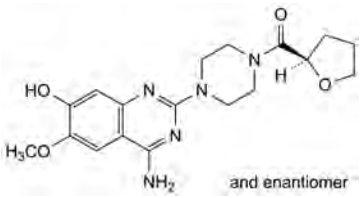


H. 1-(4-amino-7-hydroxy-6-methoxyquinazolin-2-yl)-4-[[(2*RS*)-tetrahydrofuran-2-yl]carbonyl]piperazine,

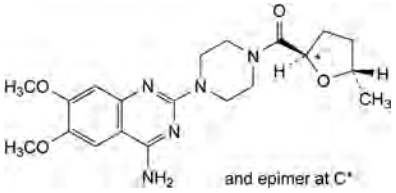


I. 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-[[(2*RS*,5*S*)-5-methyltetrahydrofuran-2-yl]carbonyl]piperazine,

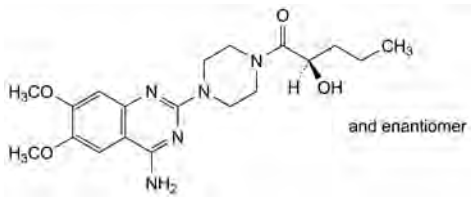


J. 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-[(2*RS*)-2-hydroxypentanoyl]piperazine,

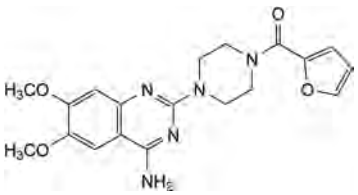


K. 1-(4-amino-6,7-dimethoxyquinazolin-2-yl)-4-(furan-2-ylcarbonyl)piperazine (prazosin),

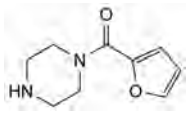


L. 1-(furan-2-ylcarbonyl)piperazine,

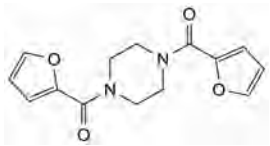


M. 1,4-bis(furan-2-ylcarbonyl)piperazine,

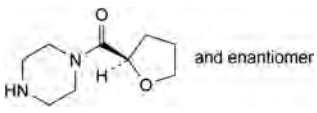


N. 1-[[(2*RS*)-tetrahydrofuran-2-yl]carbonyl]piperazine,

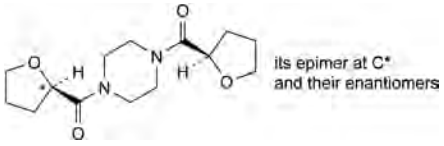


O. 1,4-bis[(tetrahydrofuran-2-yl)carbonyl]piperazine.

**01/2010:1734**

# TERBINAFINE HYDROCHLORIDE

## Terbinafini hydrochloridum

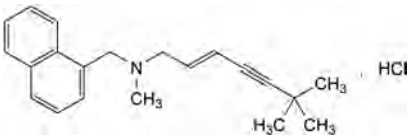


$C_{21}H_{26}ClN$ $M_r$ 327.9
[78628-80-5]

DEFINITION

(2*E*)-*N*,6,6-Trimethyl-*N*-(naphthalen-1-ylmethyl)hept-2-en-4-yn-1-amine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly or slightly soluble in water, freely soluble in anhydrous ethanol and in methanol, slightly soluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *terbinafine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*) using *anhydrous ethanol R* as solvent.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Solvent mixture A*: *acetonitrile R*, *water R* (50:50 *V/V*).

*Solvent mixture B*: *acetonitrile R*, *methanol R* (40:60 *V/V*).

*Buffer solution*. Dilute 2.0 mL of *triethylamine R1* to 950 mL with *water R*. Adjust to pH 7.5 with a mixture of 5 volumes of *glacial acetic acid R* and 95 volumes of *water R* and dilute to 1000.0 mL with *water R*.

*Test solution*. Dissolve 25 mg of the substance to be examined in solvent mixture A and dilute to 50.0 mL with solvent mixture A.

*Reference solution (a)*. Dissolve 5 mg of *terbinafine for system suitability CRS* (containing impurities B and E) in 10.0 mL of solvent mixture A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solvent mixture A. Dilute 1.0 mL of this solution to 10.0 mL with solvent mixture A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: buffer solution, solvent mixture B (30:70 *V/V*);
- *mobile phase B*: buffer solution, solvent mixture B (5:95 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 25 | 100 → 0 | 0 → 100 |
| 25 - 30 | 0 | 100 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *terbinafine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and E.

*Relative retention* with reference to terbinafine (retention time = about 15 min): impurity B = about 0.9; impurity E = about 1.7.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurity B and terbinafine.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity E by 0.5;
– *impurity B*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *impurity E*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R*, add 5 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide* determining the end-point potentiometrically (*2.2.20*). Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 32.79 mg of $C_{21}H_{26}ClN$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, F.*

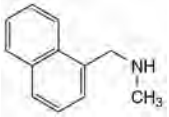

A. *N*-methyl-*C*-(naphthalen-1-yl)methanamine,

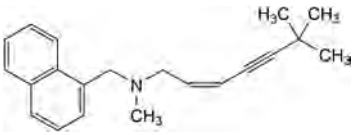

B. (2*Z*)-*N*,6,6-trimethyl-*N*-(naphthalen-1-ylmethyl)hept-2-en-4-yn-1-amine (*cis*-terbinafine),

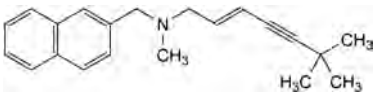

C. (2*E*)-*N*,6,6-trimethyl-*N*-(naphthalen-2-ylmethyl)hept-2-en-4-yn-1-amine (*trans*-isoterbinafine),

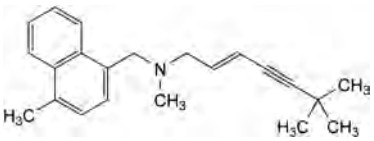

D. (2*E*)-*N*,6,6-trimethyl-*N*-[(4-methylnaphthalen-1-yl)methyl]hept-2-en-4-yn-1-amine (4-methylterbinafine),

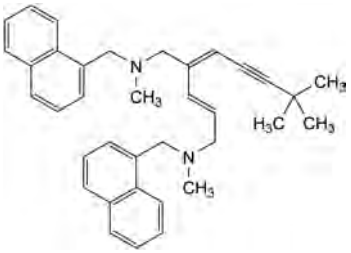

E. (2*E*,4*E*)-4-(4,4-dimethylpent-2-yn-1-ylidene)-*N*,*N*′-dimethyl-*N*,*N*′-bis(naphthalen-1-ylmethyl)pent-2-ene-1,5-diamine,

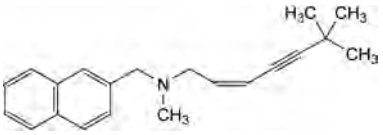

F. (2*Z*)-*N*,6,6-trimethyl-*N*-(naphthalen-2-ylmethyl)hept-2-en-4-yn-1-amine (*cis*-isoterbinafine).

**01/2020:0690**

# TERBUTALINE SULFATE

## Terbutalini sulfas

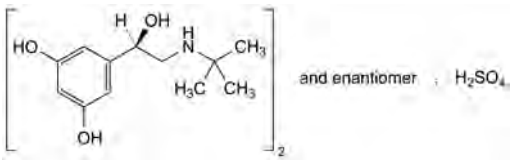

$C_{24}H_{40}N_2O_{10}S$ $M_r$ 548.6
[23031-32-5]

DEFINITION

Bis[5-[(1*RS*)-2-(*tert*-butylamino)-1-hydroxyethyl]benzene-1,3-diol] sulfate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *terbutaline sulfate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *aldehyde-free methanol R*, evaporate to dryness and record new spectra using the residues.

B. 5 mL of solution S (see Tests) gives reaction (a) of sulfates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 400 nm in a 2 cm cell is not greater than 0.11.

**Acidity**. To 10 mL of solution S add 0.05 mL of *methyl red solution R*. Not more than 1.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 75.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 7.5 mg of *terbutaline impurity C CRS* and 22.5 mg of *terbutaline sulfate CRS* in the mobile phase and dilute to 50 mL with the mobile phase. Dilute 1 mL of the solution to 100 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: prepare a 0.050 M ammonium formate solution as follows: dissolve 3.15 g of *ammonium formate R* in about 980 mL of *water for chromatography R*, adjust to pH 3.0 by adding about 8 mL of *anhydrous formic acid R* and dilute to 1000 mL with *water for chromatography R*. Dissolve 4.23 g of *sodium hexanesulfonate R* in 770 mL of the 0.050 M ammonium formate solution, then add 230 mL of *methanol R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 276 nm.

*Injection*: 20 µL.

*Run time*: 6 times the retention time of terbutaline.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity C.

*Relative retention* with reference to terbutaline (retention time = about 10 min): impurity C = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity C and terbutaline.

*Calculation of percentage contents*:

- for each impurity, use the concentration of terbutaline sulfate in reference solution (b).

*Limits*:

- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

ASSAY

Dissolve 0.400 g in 70 mL of *anhydrous acetic acid R* with heating. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 54.86 mg of $C_{24}H_{40}N_2O_{10}S$.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

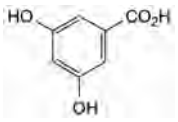

A. 3,5-dihydroxybenzoic acid (α-resorcylic acid),

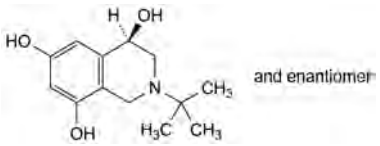


B. (4*RS*)-2-*tert*-butyl-1,2,3,4-tetrahydroisoquinoline-4,6,8-triol,

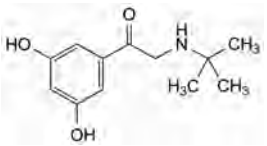

C. 2-(*tert*-butylamino)-1-(3,5-dihydroxyphenyl)ethan-1-one,

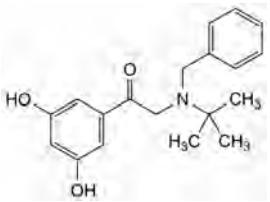

D. 2-[benzyl(*tert*-butyl)amino]-1-(3,5-dihydroxyphenyl)ethan-1-one.

**01/2012:1270**

# TERCONAZOLE

## Terconazolum

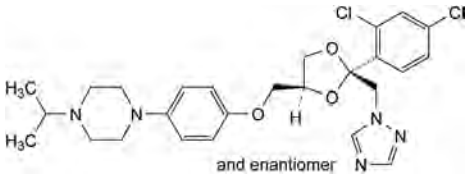


$C_{26}H_{31}Cl_2N_5O_3$ $M_r$ 532.5

[67915-31-5]

DEFINITION

1-[4-[[(2*RS*,4*SR*)-2-(2,4-Dichlorophenyl)-2-[(1*H*-1,2,4-triazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetone, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *terconazole CRS.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *acetone R*, evaporate to dryness in a current of air and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 30 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 30 mg of *terconazole CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 30 mg of *ketoconazole CRS* and 30 mg of *terconazole CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel plate R.*

*Mobile phase*: *ammonium acetate solution R*, *dioxan R*, *methanol R* (20:40:40 *V/V/V*).

*Application*: 5 µL.

*Development*: in an unsaturated tank over half of the plate.

*Drying*: in a current of warm air for 15 min.

*Detection*: expose to iodine vapour until the spots appear and examine in daylight.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. To 30 mg in a porcelain crucible add 0.3 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 1.0 g in *methylene chloride R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2.0 mg of *ketoconazole CRS* and 2.5 mg of *terconazole CRS* in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 5.0 mL of this solution to 20.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.1 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

– *mobile phase A*: 3.4 g/L solution of *tetrabutylammonium hydrogen sulfate R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 95 → 50 | 5 → 50 |
| 10 - 15 | 50 | 50 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to terconazole (retention time = about 7.5 min): ketoconazole = about 0.8; impurity A = about 0.85; impurity B = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 13 between the peaks due to ketoconazole and terconazole.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent);

– *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);

– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 70 mL of a mixture of 1 volume of *anhydrous acetic acid R* and 7 volumes of *methyl ethyl ketone R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically at the 2nd point of inflexion (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 17.75 mg of $C_{26}H_{31}Cl_2N_5O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

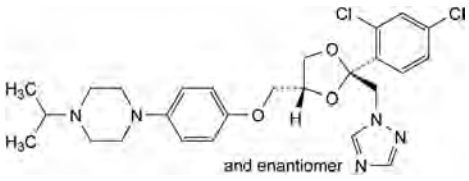


A. 1-[4-[[(2*RS*,4*RS*)-2-(2,4-dichlorophenyl)-2-[(1*H*-1,2,4-triazol-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine,

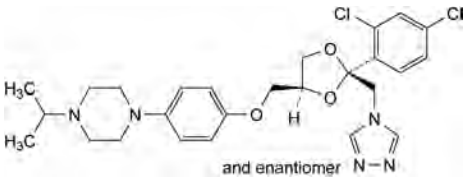


B. 1-[4-[[(2*RS*,4*SR*)-2-(2,4-dichlorophenyl)-2-[(4*H*-1,2,4-triazol-4-yl)methyl]-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)piperazine.

**01/2008:0955**
**corrected 6.1**

# TERFENADINE

## Terfenadinum

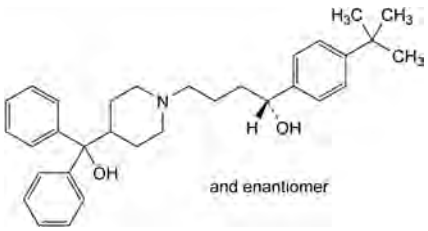


$C_{32}H_{41}NO_2$ $M_r$ 471.7
[50679-08-8]

DEFINITION

(1*RS*)-1-[4-(1,1-Dimethylethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butan-1-ol.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, soluble in methanol. It is very slightly soluble in dilute hydrochloric acid.

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 146 °C to 152 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 259 nm.

*Shoulders*: at 253 nm and 270 nm.

*Specific absorbance at the absorption maximum*: 13.5 to 14.9.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *terfenadine CRS.*

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 50 mg of *terfenadine CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *methanol R*, *methylene chloride R* (10:90 *V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 15 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 15 mg of *terfenadine impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase. To 5.0 mL of this solution, add 5.0 mL of the test solution and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 10.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 0.1 g of *potassium iodide R* in the mobile phase and dilute to 100 mL with the mobile phase. Dilute 1 mL of this solution to 100 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dilute 600 mL of *acetonitrile R1* to 1 L with *diethylammonium phosphate buffer solution pH 6.0 R.*

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 217 nm.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of terfenadine.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to terfenadine and impurity A;
- *mass distribution ratio*: minimum 2.0 for the peak due to terfenadine; use *potassium iodide R* as the unretained compound (reference solution (d)).

*Limits*:

- *impurities A, B, C, D, E, F, G, H, I, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.025 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.005 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.5 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.17 mg of $C_{32}H_{41}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

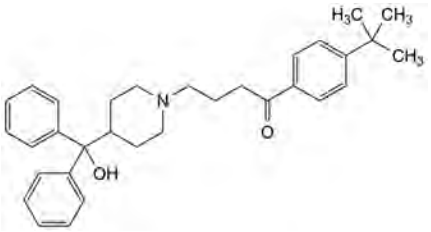

A. 1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(hydroxydiphenylmethyl)piperidin-1-yl]butan-1-one,

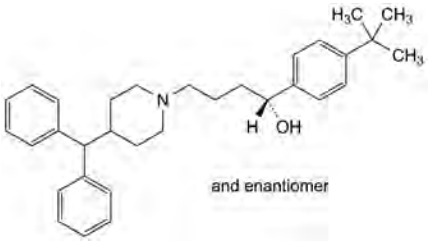


B. (1*RS*)-1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(diphenylmethyl)piperidin-1-yl]butan-1-ol,

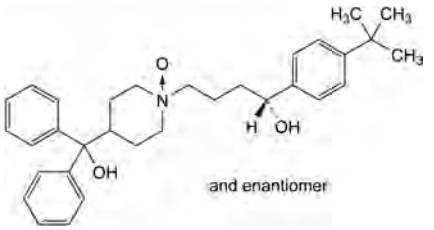


C. 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]-4-(hydroxydiphenylmethyl)piperidine 1-oxide,

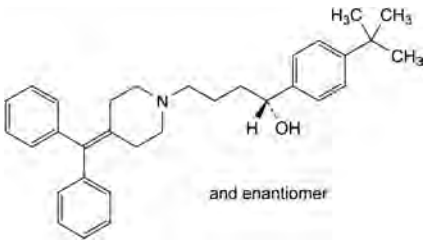


D. (1*RS*)-1-[4-(1,1-dimethylethyl)phenyl]-4-[4-(diphenylmethylene)piperidin-1-yl]butan-1-ol,

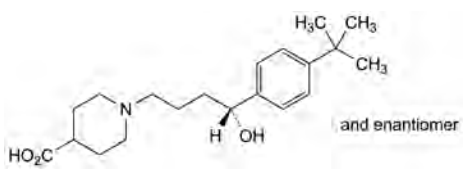


E. 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]piperidine-4-carboxylic acid,

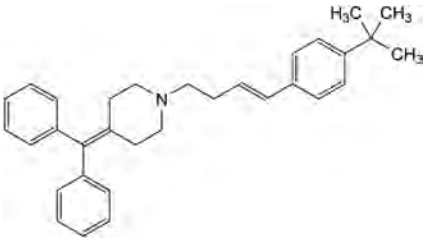

F. 1-[4-[4-(1,1-dimethylethyl)phenyl]but-3-enyl]-4-(diphenylmethylene)piperidine,

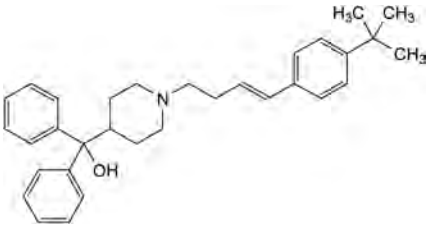

G. [1-[4-[4-(1,1-dimethylethyl)phenyl]but-3-enyl]piperidin-4-yl]diphenylmethanol,

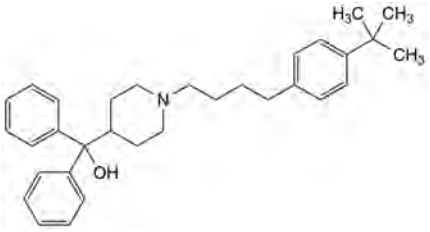

H. [1-[4-[4-(1,1-dimethylethyl)phenyl]butyl]-piperidin-4-yl]diphenylmethanol,

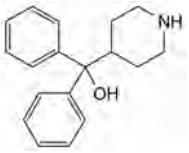

I. diphenyl(piperidin-4-yl)methanol,

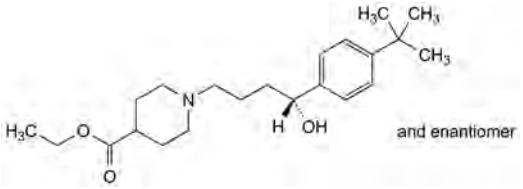


J. ethyl 1-[(4*RS*)-4-[4-(1,1-dimethylethyl)phenyl]-4-hydroxybutyl]piperidine-4-carboxylate.

**01/2017:2829**
**corrected 10.0**

# TERIPARATIDE

## Teriparatidum

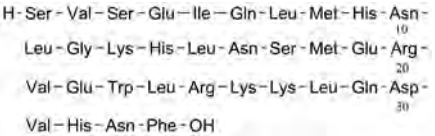


$C_{181}H_{291}N_{55}O_{51}S_2$ $M_r$ 4118

[52232-67-4]

DEFINITION

Tetratriacontapeptide in which the sequence of amino acids is the same as that of the 1-34 *N*-terminal fragment of endogeneous human parathyroid hormone (rhPTH).

*Content*: 95.0 per cent to 105.0 per cent (anhydrous, acetic acid- and chloride-free substance).

PRODUCTION

Teriparatide is produced by a method based on recombinant DNA (rDNA) technology. During the course of product development it must be demonstrated that the manufacturing process produces a biologically active protein using a suitable bioassay as approved by the competent authority.

Prior to release, the following tests are carried out on each batch of teriparatide, unless exemption has been granted by the competent authority.

**Host-cell-derived proteins**. The limit is approved by the competent authority.

**Host-cell- and vector-derived DNA**. The limit is approved by the competent authority.

CHARACTERS

*Appearance*: white or almost white, very hygroscopic powder.

*Solubility*: freely soluble in water and in methanol, practically insoluble in acetonitrile.

IDENTIFICATION

A. Peptide mapping (*2.2.55*).

*SELECTIVE CLEAVAGE OF THE PEPTIDE BONDS*

*Solution A*. Dissolve 230 mg of *anhydrous disodium hydrogen phosphate R* and 60 mg of *sodium dihydrogen phosphate monohydrate R* in 100 mL of *water R* and adjust to pH 7.8 with *sodium hydroxide solution R*.

*Test solution*. Dissolve the substance to be examined in solution A to obtain a concentration of 1.5 mg/mL and transfer 150 µL of the solution to a clean tube. Add 90 µL of a 0.25 mg/mL solution of *glutamyl endopeptidase for peptide mapping R* in solution A. Mix and incubate at 37 °C for 18-24 h. Stop the reaction by adding 660 µL of mobile phase A to reach a final digested protein concentration of about 0.25 mg/mL.

*NOTE: if a teriparatide concentration of 1.5 mg/mL is not obtainable, a similar ratio of micrograms of endopeptidase per milligram of teriparatide may be used.*

*Reference solution*. Prepare at the same time and in the same manner as for the test solution but using *teriparatide CRS* instead of the substance to be examined.

*Blank solution*. Prepare at the same time and in the same manner as for the test solution but omitting the substance to be examined.

*CHROMATOGRAPHIC SEPARATION*

Liquid chromatography (*2.2.29*). *Store the solutions at 2-8 °C and use them within 72 h.*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm) with a pore size of 30 nm;
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 1 mL of *trifluoroacetic acid R* and 1000 mL of *water R*; filter and degas;
- *mobile phase B*: mix 1 mL of *trifluoroacetic acid R*, 400 mL of *water R* and 600 mL of *acetonitrile for chromatography R*; filter and degas;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 6 | 96 | 4 |
| 6 - 20 | 96 → 45 | 4 → 55 |
| 20 - 25 | 45 → 0 | 55 → 100 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 20 µL.

*System suitability*:

- the chromatogram obtained with the reference solution is qualitatively similar to the chromatogram of teriparatide digest supplied with *teriparatide CRS*;
- in the chromatogram obtained with the reference solution, identify the peaks due to digest fragments I, II, III, IV and V:

  *symmetry factor*: maximum 2.3 for the peak due to fragment IV;

  *resolution*: minimum 1.5 between the peaks due to fragments I and III.

*Results*: the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with the reference solution.

TESTS

**Impurities with molecular masses greater than that of teriparatide**. Size-exclusion chromatography (*2.2.30*): use the normalisation procedure. *Store the solutions at 2-8 °C and use them within 72 h.*

*Test solution*. Dissolve the substance to be examined in *water R* to obtain a concentration of 1 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *teriparatide CRS* in *water R* to obtain a concentration of 1 mg/mL.

*Blank solution*: *water R*.

*Resolution solution*. Incubate a vial of *teriparatide CRS* at 75 °C for 16-24 h. After incubation, dissolve the contents of the vial in *water R* to obtain a concentration of 1 mg/mL of degraded teriparatide.

*Column*:

- *size*: *l* = 0.30 m, Ø = 7.8 mm;
- *stationary phase*: *hydrophilic silica gel for chromatography R* (5-10 µm) with a pore size of 12.5 nm, of a grade suitable for fractionation of globular proteins of relative molecular mass up to 150 000.

*Mobile phase*: add 1 mL of *trifluoroacetic acid R* to 750 mL of *water R*, mix with 250 mL of *acetonitrile for chromatography R* and degas.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of teriparatide monomer.

*Retention time*: teriparatide monomer = about 17 min.

*System suitability*:

- the chromatogram obtained with the reference solution is similar to the chromatogram supplied with *teriparatide CRS*;

– *resolution*: minimum 2.0 between the peaks due to teriparatide dimer and monomer in the chromatogram obtained with the resolution solution.

*Limit*:

– *sum of the peaks eluted before the principal peak*: maximum 0.3 per cent; disregard any peak with a retention time greater than that of the peak due to teriparatide monomer.

**Related proteins**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Store the solutions at 2-8 °C and use them within 48 h.*

*Buffer solution.* Dissolve 28.4 g of *anhydrous sodium sulfate R* in 900 mL of *water R* and adjust to pH 2.3 with *phosphoric acid R*. Dilute to 1000 mL with *water for chromatography R* and filter.

*Test solution.* Dissolve the substance to be examined in mobile phase A to obtain a concentration of 0.7 mg/mL.

*Reference solution.* Dissolve the contents of a vial of *teriparatide CRS* in mobile phase A to obtain a concentration of 0.7 mg/mL.

*Blank solution*: mobile phase A.

*Resolution solution.* Dissolve the contents of a vial of *teriparatide for system suitability CRS* in mobile phase A to obtain a concentration of 1 mg/mL.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3.5 µm) with a pore size of 30 nm;
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 10 volumes of *acetonitrile for chromatography R* and 90 volumes of the buffer solution and degas; apply mild heating at 20-25 °C while stirring continuously during analysis;
– *mobile phase B*: mix equal volumes of *acetonitrile for chromatography R* and the buffer solution and degas; apply mild heating at 20-25 °C while stirring continuously during analysis;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 → 65 | 0 → 35 |
| 5 - 35 | 65 → 60 | 35 → 40 |
| 35 - 45 | 60 → 0 | 40 → 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 214 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 20 µL.

*Relative retention* with reference to teriparatide (retention time = 20-25 min): related protein A ([MetO$^{8}$,MetO$^{18}$]teriparatide) = about 0.40; related protein B ([MetO$^{8}$]teriparatide) = about 0.49; related protein C ([MetO$^{18}$]teriparatide) = about 0.57; related protein D = about 1.06; related protein E = about 1.14.

*System suitability*: resolution solution:

– the chromatogram obtained is similar to the chromatogram supplied with *teriparatide for system suitability CRS*;
– *resolution*: minimum 1.5 between the peaks due to teriparatide and related protein D;
– *symmetry factor*: 0.8 to 2.0 for the peak due to teriparatide;

*Results*:

– the profile of the chromatogram obtained with the test solution corresponds to that of the chromatogram obtained with the reference solution.

*Limits*:

– *sum of related proteins A, B and C*: maximum 0.5 per cent;
– *any other related protein*: maximum 0.5 per cent;
– *total*: maximum 2.0 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 7.0 per cent, determined on 10 mg using the evaporation technique:

– *temperature*: 100 °C;
– *heating time*: 8 min.

**Bacterial endotoxins** (*2.6.14*): less than 50 IU/mg.

ASSAY

**Acetate**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Store the solutions at 2-8 °C and use them within 72 h.*

*Test solution.* Dissolve the substance to be examined in the mobile phase to obtain a concentration of 5 mg/mL.

*Reference solutions.* Dissolve separately 100 mg, 200 mg and 300 mg of *anhydrous sodium acetate R* in the mobile phase and dilute to 100 mL with the mobile phase. Further dilute 1.0 mL of each solution to 10.0 mL with the mobile phase to prepare a standard curve with acetate concentrations in the range of 0.072-0.216 mg/mL.

Plot peak areas versus injected acetate content and perform linear regression to create a standard curve.

*Column*:

– *size*: *l* = 0.25 m, Ø = 9.0 mm;
– *stationary phase*: *ion-exclusion resin for chromatography R* (7.5 µm).

*Mobile phase*: 0.5 per cent *V/V* solution of *dilute sulfuric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 2-8 °C.

*Injection*: 100 µL.

*Run time*: 1.5 times the retention time of acetate.

*Retention time*: acetate = about 10 min.

*System suitability*:

– *repeatability*: maximum relative standard deviation of 1.25 per cent for the area of the principal peak, determined on 3 injections of the middle reference solution;
– the correlation coefficient (*r*) calculated for the standard curve is not less than 0.999.

Calculate the acetate content using the standard curve and the area of the peak due to acetate in the chromatogram obtained with the test solution.

**Chloride**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Use the solutions within 72 h.*

*Test solution.* Dissolve the substance to be examined in *water R* to obtain a concentration of 1 mg/mL.

*Reference solution (a).* Dissolve 165.9 mg of *sodium chloride R*, previously dried at 105 °C for 30 min, in *water R* and dilute to 100 mL with the same solvent.

*Reference solution (b).* Dissolve 150 mg of *sodium nitrite R* in *water R* and dilute to 100 mL with the same solvent. Mix 1.0 mL of the solution and 2.5 mL of reference solution (a) and dilute to 100 mL with *water R*.

*Reference solutions.* Dilute reference solution (a) with *water R* to prepare a standard curve with at least 4 concentrations in the range of 10-40 µg/mL.

Plot peak areas versus injected chloride content and perform linear regression to create a standard curve.

*Precolumn*:

– *size*: *l* = 0.05 m, Ø = 4.0 mm;
– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (15 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (15 µm).

*Mobile phase*: dissolve 285.7 mg of *sodium hydrogen carbonate R* and 381.6 mg of *anhydrous sodium carbonate R* in *water R* and dilute to 2000 mL with the same solvent.

*Flow rate*: 2.0 mL/min.

*Detection*: conductivity detector; use a self-regenerating anion suppressor at 100 mA.

*Injection*: 50 µL.

*Run time*: 6 times the retention time of chloride.

*Retention time*: chloride = about 1.6 min; nitrite = about 1.8 min.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to chloride and nitrite;
– *symmetry factor*: maximum 2.0 for the peaks due to chloride and nitrite;
– *repeatability*: maximum relative standard deviation of 2.0 per cent for the areas of the peaks due to chloride and nitrite, determined on 5 injections;
– the correlation coefficient (*r*) calculated for the standard curve is not less than 0.999.

Calculate the chloride content using the standard curve and the area of the peak due to chloride in the chromatogram obtained with the test solution.

**Content**. Liquid chromatography (*2.2.29*) as described in the test for related proteins with the following modifications.

*Test solution*. Dissolve the substance to be examined in the mobile phase to obtain a concentration of 0.25 mg/mL.

*Reference solution*. Dissolve the contents of a vial of *teriparatide CRS* in the mobile phase to obtain a concentration of 0.25 mg/mL.

*Mobile phase*: mobile phase A, mobile phase B (63:37 *V/V*); apply mild heating at 20-25 °C while stirring continuously during analysis.

*Run time*: 1.5 times the retention time of teriparatide.

*Retention time*: teriparatide = about 10 min.

*System suitability*: reference solution:

– *symmetry factor*: 0.8 to 1.5 for the peak due to teriparatide;
– *repeatability*: maximum relative standard deviation of 1.25 per cent for the area of the peak due to teriparatide, determined on 3 injections.

Calculate the percentage content of teriparatide ($C_{181}H_{291}N_{55}O_{51}S_2$) taking into account the assigned content of *teriparatide CRS*.

STORAGE

In an airtight container, protected from light, at − 10 °C or below.

**07/2017:2646**

# TERLIPRESSIN

## Terlipressinum

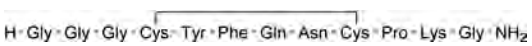


$C_{52}H_{74}N_{16}O_{15}S_2$ $M_r$ 1227
[14636-12-5]

DEFINITION

Glycyl-glycyl-glycyl-L-cysteinyl-L-tyrosyl-L-phenylalanyl-L-glutaminyl-L-asparaginyl-L-cysteinyl-L-prolyl-L-lysylglycinamide cyclic (4→9)-disulfide.

Synthetic dodecapeptide analogue of the natural hormone vasopressin. It is available as an acetate.

*Content*: 95.0 per cent to 105.0 per cent (anhydrous, acetic acid-free substance).

CHARACTERS

*Appearance*: white or almost white fluffy powder, hygroscopic.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

B. Nuclear magnetic resonance spectrometry (*2.2.64*).

*Preparation*: 2.9 mg/mL solution in *deuterium oxide R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R*, adjusted to pH 4 with *deuterated acetic acid R*.

*Comparison*: dissolve the contents of a vial of *terlipressin for NMR identification CRS* in *deuterium oxide R* containing 20 µg/mL of *deuterated sodium trimethylsilylpropionate R*, adjusted to pH 4 with *deuterated acetic acid R*, to obtain a concentration of 2.9 mg/mL.

*Operating conditions*:

– *field strength*: minimum 300 MHz;
– *temperature*: 23 °C.

*Results*: examine the $^1$H NMR spectrum from 0 to 9 ppm. The $^1$H NMR spectrum obtained is qualitatively similar to the $^1$H NMR spectrum obtained with *terlipressin for NMR identification CRS*.

C. Amino acid analysis (*2.2.56*). Method 1 for hydrolysis and method 1 for analysis are suitable.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking 1/9 of the sum of the number of moles of aspartic acid, glutamic acid, proline, glycine, phenylalanine and lysine as equal to 1. The values fall within the following limits: glycine 3.6 to 4.4; half-cystine 1.4 to 2.2; tyrosine 0.7 to 1.1; phenylalanine, glutamic acid, aspartic acid, proline and lysine 0.9 to 1.1. Not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): − 108 to − 88 (anhydrous and acetic acid-free substance).

Dissolve 125.0 mg in 25.0 mL of a 1 per cent *V/V* solution of *glacial acetic acid R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Buffer solution*. Dissolve 3.30 g of *ammonium sulfate R* in *water for chromatography R* and dilute to 5000 mL with the same solvent. Add 1.0 mL of *sulfuric acid R* and mix well; filter through a membrane filter (nominal pore size 0.45 µm).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in 50.0 mL of a 9 g/L solution of *sodium chloride R*.

*Solution A*. Dissolve the contents of a vial of *terlipressin impurity mixture CRS* (containing impurities A, D and L) in 1.0 mL of a 9 g/L solution of *sodium chloride R*.

*Solution B*. Dissolve the contents of a vial of *terlipressin CRS* in a 9 g/L solution of *sodium chloride R* and dilute to 2.5 mL with the same solution.

*Reference solution (a)*. Dilute 1.0 mL of solution B to 2.0 mL with a 9 g/L solution of *sodium chloride R*.

*Reference solution (b)*. Mix 20 µL of solution A and 500 µL of solution B, and dilute to 1.0 mL with a 9 g/L solution of *sodium chloride R*.

*Reference solution (c).* Mix 100 µL of solution A and 500 µL of solution B, and dilute to 1.0 mL with a 9 g/L solution of *sodium chloride R*.

*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with a 9 g/L solution of *sodium chloride R*. Dilute 1.0 mL of this solution to 10.0 mL with a 9 g/L solution of *sodium chloride R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: *methanol R*, buffer solution (18.5:81.5 *V/V*);
- *mobile phase B*: *methanol R*, buffer solution (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 100 | 0 |
| 35 - 55 | 100 → 0 | 0 → 100 |
| 55 - 65 | 0 | 100 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 200 µL of the test solution and reference solutions (a), (b), (c) and (d).

*Identification of impurities*: use the chromatogram supplied with *terlipressin impurity mixture CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, D and L.

*Relative retention* with reference to terlipressin (retention time = about 21 min): impurity E = about 0.4; impurity C = about 0.5; impurity B = about 0.7; impurity A = about 0.85; impurity G = about 1.2; impurity H = about 1.4; impurities I and F = about 2.0; impurity L = about 2.2; impurity D = about 2.3; impurity K = about 2.5; impurity J = about 2.8.

*System suitability*:

- *resolution*: minimum 1.4 between the peak due to impurity A and the principal peak in the chromatogram obtained with reference solution (c); the peaks due to impurities L and D are separated as shown in the chromatogram supplied with *terlipressin impurity mixture CRS*.
- *signal-to-noise ratio*: minimum 50 for the peak due to impurity A in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 2.0 for the peak due to terlipressin in the chromatogram obtained with reference solution (a);
- *repeatability*: maximum relative standard deviation of 2.0 per cent determined on 5 injections of reference solution (a).

*Calculation of percentage contents*:

- for each impurity, use the concentration of terlipressin in reference solution (d).

*Limits*:

- *impurity D*: maximum 0.6 per cent;
- *unspecified impurities*: for each impurity, maximum 0.5 per cent;
- *total*: maximum 1.5 per cent;
- *reporting threshold*: 0.1 per cent.

**Acetic acid** (*2.5.34*): 8.0 per cent to 14.0 per cent.

*Test solution.* Dissolve 20.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A, and dilute to 20.0 mL with the same mixture of solvents.

**Water** (*2.5.32*): maximum 10.0 per cent, determined on 10.0 mg using the evaporation technique at 120 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 200 µL of the test solution and reference solution (a).

Calculate the percentage content of terlipressin ($C_{52}H_{74}N_{16}O_{15}S_2$) taking into account the assigned content of $C_{52}H_{74}N_{16}O_{15}S_2$ in *terlipressin CRS*.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states the terlipressin content ($C_{52}H_{74}N_{16}O_{15}S_2$).

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, F, G, H, I, J, K, L.*

A. des-1-glycine-terlipressin,

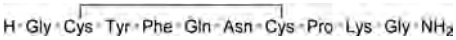


B. des-1,2-diglycine-terlipressin,

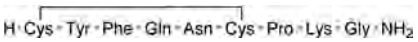


C. des-(1-3)-terlipressin,

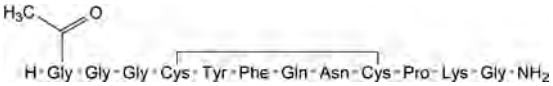


D. $N^1$-acetylterlipressin,

E. [6-D-phenylalanine]terlipressin,

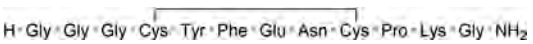


F. [7-L-glutamic acid]terlipressin,

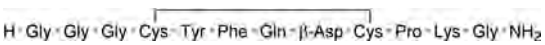


G. [8-L-β-aspartic acid]terlipressin,

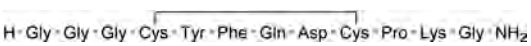


H. [8-L-α-aspartic acid]terlipressin,

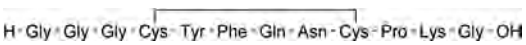


I. [12-glycine]terlipressin,

J. [12-glycine]terlipressin ethyl ester,

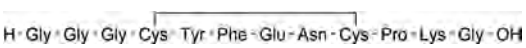


K. [7-L-glutamic acid,12-glycine]terlipressin,

L. [8-L-aspartic acid,12-glycine]terlipressin.

04/2019:2940
corrected 10.0

# TERPIN MONOHYDRATE

## Terpinum monohydricum

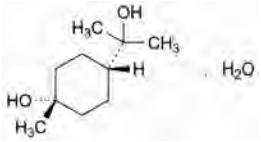


$C_{10}H_{20}O_2,H_2O$ $M_r$ 190.3
[2451-01-6]

DEFINITION

(1*s*,4*s*)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexan-1-ol monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in ethanol (96 per cent), slightly soluble in methylene chloride.

mp: about 117 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *terpin monohydrate CRS*.

B. Water (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *ethanol (96 per cent) R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.02 M sodium hydroxide* or *0.02 M hydrochloric acid* is required to change the colour of the indicator.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution*. Dissolve 0.500 g of *biphenyl R* in 1 mL of *methylene chloride R* and dilute to 25.0 mL with *methanol R*.

*Test solution (a)*. Dissolve 0.375 g of the substance to be examined in *methanol R* and dilute to 25.0 mL with the same solvent.

*Test solution (b)*. Dissolve 85.0 mg of the substance to be examined in *methanol R*, add 2.0 mL of the internal standard solution and dilute to 100.0 mL with *methanol R*.

*Reference solution (a)*. Dissolve 15.0 mg of *trans-terpin R* (impurity D) in test solution (a) and dilute to 10.0 mL with test solution (a). Dilute 1.0 mL of the solution to 10.0 mL with test solution (a).

*Reference solution (b)*. Dissolve 85.0 mg of *terpin monohydrate CRS* in *methanol R*, add 2.0 mL of the internal standard solution and dilute to 100.0 mL with *methanol R*.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1.0 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 11 | 180 |
| | 11 - 14 | 180 → 270 |
| | 14 - 15 | 270 |
| Injection port | | 220 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (a) and reference solution (a).

*Relative retention* with reference to terpin (retention time = about 6.3 min): impurity D = about 1.05; internal standard = about 1.2.

*System suitability*: reference solution (a):
- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to terpin.

*Limits*:
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 8.0 per cent to 10.0 per cent, determined on 0.200 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modification.

*Injection*: 0.5 µL of test solution (b) and reference solution (b).

Calculate the percentage content of terpin using the following expression:

$$\frac{A_1 \times m_2 \times A_4 \times 100}{A_2 \times m_1 \times A_3}$$

$A_1$ = area of the peak due to terpin in the chromatogram obtained with test solution (b);

$A_2$ = area of the peak due to terpin in the chromatogram obtained with reference solution (b);

$A_3$ = area of the peak due to the internal standard in the chromatogram obtained with test solution (b);

$A_4$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (b);

$m_1$ = mass of the substance to be examined used to prepare test solution (b), in milligrams;

$m_2$ = mass of *terpin monohydrate CRS* used to prepare reference solution (b), in milligrams.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

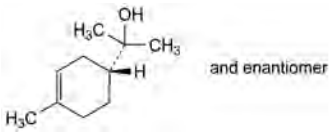


A. 2-[(1*RS*)-4-methylcyclohex-3-en-1-yl]propan-2-ol (α-terpineol),

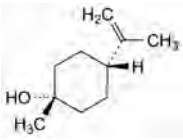


B. (1*s*,4*s*)-1-methyl-4-(prop-1-en-2-yl)cyclohexan-1-ol (*cis*-β-terpineol),

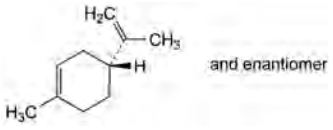


C. (4*RS*)-1-methyl-4-(prop-1-en-2-yl)cyclohex-1-ene (limonene),

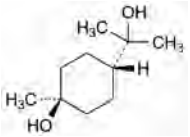


D. (1*r*,4*r*)-4-(2-hydroxypropan-2-yl)-1-methylcyclohexan-1-ol (*trans*-terpin).

**01/2008:1373**
**corrected 7.0**

# TESTOSTERONE

## Testosteronum

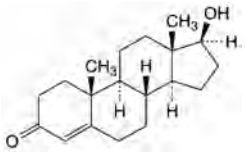


$C_{19}H_{28}O_2$ $M_r$ 288.4
[58-22-0]

DEFINITION

17β-Hydroxyandrost-4-en-3-one.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white crystalline powder, or colourless or yellowish-white crystals.

*Solubility*: practically insoluble in water, freely soluble in alcohol and in methylene chloride, practically insoluble in fatty oils.

mp: about 155 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *testosterone CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 106 to + 114 (dried substance).

Dissolve 0.250 g in *ethanol R* and dilute to 25.0 mL with the same solvent.

**Impurities D and F**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 1 mg of *stanolone R* in *methanol R* and dilute to 10 mL with the same solvent. In 1 mL of this solution, dissolve 10 mg of *testosterone for impurity D identification CRS* (testosterone spiked with about 1 per cent of impurity D).

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (b) to 10.0 mL with *methanol R*.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (b) to 10.0 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (6-8 μm).

*Preconditioning* (in the dark): add about 5 g of powdered *silver nitrate R* to 100 mL of *methanol R*. Stir the suspension for 30 min. Filter or decant the suspension and immerse the plate in the silver nitrate solution for at least 30 min. Dry at 75 °C for 30 min.

A pre-conditioned plate can be stored in the dark for 5-7 days.

*Mobile phase*: *acetic acid R*, *ethanol R*, *dioxan R*, *methylene chloride R* (1:2:10:90 *V/V/V/V*).

*Application*: 2 μL.

*Development*: in a saturated tank over 3/4 of the plate.

*Drying*: allow to stand at room temperature and protected from light for 30 min.

*Detection*: spray with a 200 g/L solution of *toluenesulfonic acid R* in *ethanol R* and heat at 105 °C for 10 min. Examine in ultraviolet light at 365 nm.

*System suitability*: the chromatogram obtained with reference solution (a) shows 3 clearly separated spots; impurity D $R_F$ = about 0.5; testosterone $R_F$ = about 0.65; impurity F $R_F$ = about 0.7.

*Limits*:

- *impurity D*: any spot due to impurity D is not more intense than the spot in the chromatogram obtained with reference solution (c) (0.2 per cent),
- *impurity F*: any spot due to impurity F is not more intense than the spot in the chromatogram obtained with reference solution (d) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 10 mg of *testosterone for system suitability CRS* (containing impurities C and I) in 1 mL of *methanol R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 20.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (b) to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 μm) with a pore size of 15 nm,
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *water for chromatography R*, *methanol R* (45:55 *V/V*),
- *mobile phase B*: *methanol R*,

Monographs T

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 4 | 100 | 0 |
| 4 - 24 | 100 → 60 | 0 → 40 |
| 24 - 53 | 60 → 0 | 40 → 100 |
| 53 - 55 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL.

*Relative retention* with reference to testosterone (retention time = about 18 min): impurity G = about 0.6; impurity H = about 0.8; impurity A = about 0.9; impurity I = about 0.95; impurity C = about 1.2; impurity E = about 1.7; impurity J = about 2.1; impurity B = about 2.5.

*System suitability*: reference solution (a):

– *resolution*: minimum baseline separation between the peaks due to impurity I and testosterone.

*Limits*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and I:

– *correction factor*: for the calculation of content, multiply the peak area of impurity I by 2.9,
– *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
– *impurity I*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent),
– *impurities A, B, E, G, H, J*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent),
– *total*: not more than 1.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 0.500 g by drying in an oven at 105 °C for 2 h.

ASSAY

Dissolve 50.0 mg in *alcohol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL to 100.0 mL with *alcohol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241 nm.

Calculate the content of $C_{19}H_{28}O_2$ taking the specific absorbance to be 569.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J.*

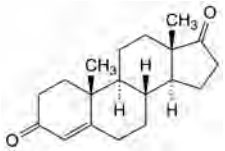

A. androst-4-ene-3,17-dione (androstenedione),

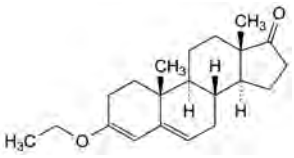

B. 3-ethoxyandrosta-3,5-dien-17-one (androstenedione ethylenolether),

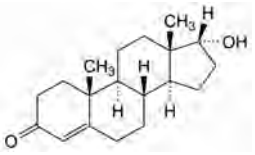

C. 17α-hydroxyandrost-4-en-3-one (epitestosterone),

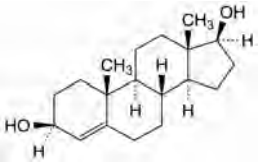

D. androst-4-ene-3β,17β-diol (Δ4-androstenediol),

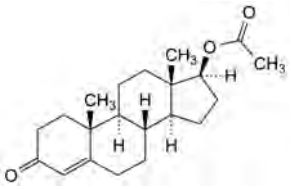

E. 3-oxoandrost-4-en-17β-yl acetate (testosterone acetate),

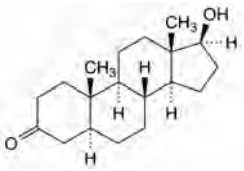

F. 17β-hydroxy-5α-androstan-3-one (androstanolone, stanolone),

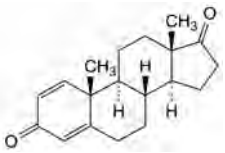

G. androsta-1,4-diene-3,17-dione (androstadienedione).

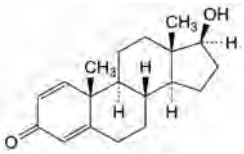

H. 17β-hydroxyandrosta-1,4-dien-3-one (boldenone),

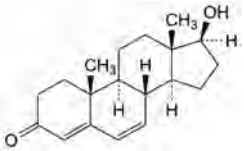

I. 17β-hydroxyandrosta-4,6-dien-3-one (Δ6-testosterone),

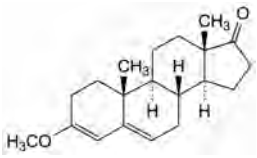

J. 3-methoxyandrosta-3,5-dien-17-one (androstenedione methylenolether).

**01/2008:1736**
**corrected 10.0**

# TESTOSTERONE DECANOATE

## Testosteroni decanoas

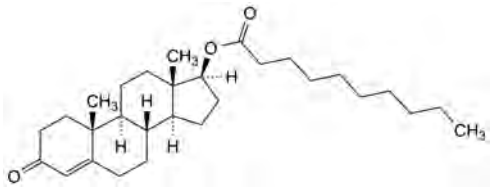

$C_{29}H_{46}O_3$ $M_r$ 442.7

DEFINITION

3-Oxoandrost-4-en-17β-yl decanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in acetone, in methylene chloride and in anhydrous ethanol, freely soluble in fatty oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *testosterone decanoate CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in 20 mL of *methanol R*.

**Specific optical rotation** (*2.2.7*): + 75.0 to + 80.0 (dried substance).

Dissolve 0.200 g in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve the contents of a vial of *testosterone decanoate for system suitability CRS* (containing impurities A, B, C, D, E and F) in 1 mL of the mobile phase.

*Reference solution (b)*. Dilute 10.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 20.0 mg of *testosterone decanoate CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *water R*, *acetonitrile R* (5:95 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of testosterone decanoate.

*Identification of impurities*: use the chromatogram supplied with *testosterone decanoate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E and F.

*Relative retention* with reference to testosterone decanoate (retention time = about 20 min): impurity A = about 0.2; impurity B = about 0.6; impurities C and G = about 0.79; impurity D = about 0.83; impurity E = about 1.3; impurity F = about 1.7.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurities C and D.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak area of impurity A by 0.7;
- *impurities A, B, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *sum of impurities C and G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Free acid**. Dissolve 0.65 g in 10 mL of *ethanol (96 per cent) R*, previously neutralised to *bromothymol blue solution R3*, and titrate immediately with *0.01 M sodium hydroxide*, using 0.1 mL of *bromothymol blue solution R3* as indicator. Not more than 0.6 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at a pressure not exceeding 0.7 kPa.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of the test solution and reference solution (c).

Calculate the percentage content of $C_{29}H_{46}O_3$ from the declared content of *testosterone decanoate CRS*.

STORAGE

At a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

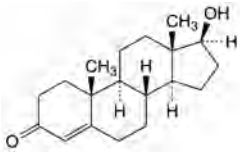

A. testosterone,

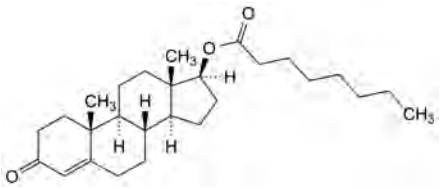

B. 3-oxoandrost-4-en-17β-yl octanoate (testosterone octanoate),

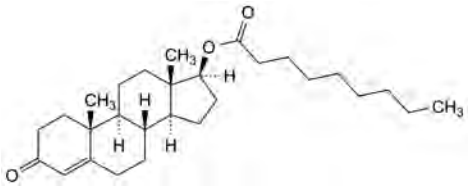

C. 3-oxoandrost-4-en-17β-yl nonanoate (testosterone nonanoate),

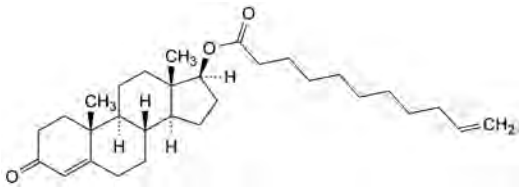

D. 3-oxoandrost-4-en-17β-yl undec-10-enoate (testosterone undecylenate),

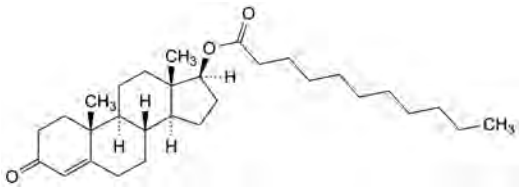

E. 3-oxoandrost-4-en-17β-yl undecanoate (testosterone undecanoate),

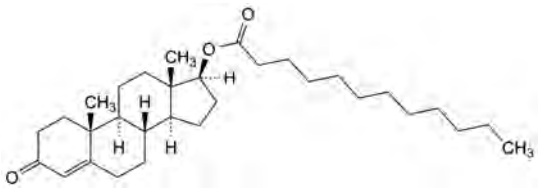

F. 3-oxoandrost-4-en-17β-yl dodecanoate (testosterone laurate),

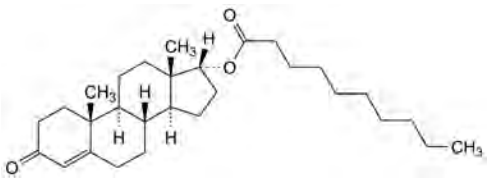

G. 3-oxoandrost-4-en-17α-yl decanoate (epitestosterone decanoate).

**04/2011:1048**
**corrected 10.0**

# TESTOSTERONE ENANTATE

## Testosteroni enantas

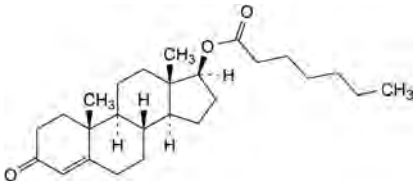

$C_{26}H_{40}O_3$ $M_r$ 400.6
[315-37-7]

DEFINITION

3-Oxoandrost-4-en-17β-yl heptanoate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in anhydrous ethanol, freely soluble in fatty oils.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Melting point (*2.2.14*): 34 °C to 39 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *testosterone enantate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *methanol R*, *methylene chloride R* (10:90 *V/V*).
*Test solution.* Dissolve 5 mg of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Reference solution (a).* Dissolve 5 mg of *testosterone enantate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Reference solution (b).* Dissolve 5 mg of *testosterone enantate CRS*, 5 mg of *testosterone decanoate CRS* and 5 mg of *testosterone isocaproate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R*.
*Mobile phase*: *water R*, *acetonitrile R*, *2-propanol R* (20:40:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 3/4 of the plate.
*Drying*: in air, then at 100 °C for 10 min; allow to cool.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).
*Detection B*: spray with *alcoholic solution of sulfuric acid R*; heat at 120 °C for 10 min; allow to cool and examine in daylight.
*Results B*: the principal spot in the chromatogram obtained with the test solution is green and is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

*System suitability*: reference solution (b):

- the chromatogram shows 3 clearly separated principal spots by each method of visualisation.

D. To about 25 mg add 2 mL of a 10 g/L solution of *potassium hydroxide R* in *methanol R* and boil under a reflux condenser for 1 h. Cool. Add 10 mL of *water R*. Acidify with *dilute hydrochloric acid R* until *blue litmus paper R* turns red. Filter and wash the precipitate with a small quantity of *water R*. The residue, after drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h, melts (*2.2.14*) at 150 °C to 153 °C.

TESTS

**Specific optical rotation** (*2.2.7*): + 81 to + 86 (dried substance).

Dissolve 0.100 g in *anhydrous ethanol R* and dilute to 10.0 mL with the same solvent.

**Impurity A**: maximum 0.16 per cent.

Dissolve 0.50 g in 10 mL of *ethanol (96 per cent) R* previously neutralised to *bromothymol blue solution R3*. Titrate immediately with *0.01 M sodium hydroxide* using 0.1 mL of *bromothymol blue solution R3* as indicator. Not more than 0.6 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Impurity H**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in 1.0 mL of *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 3.0 mg of *testosterone enantate impurity H CRS* in 20.0 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ethyl acetate R*, *cyclohexane R1* (40:60 *V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a 200 g/L solution of *toluenesulfonic acid R* in *ethanol (96 per cent) R* and heat at 120 °C for 10 min; examine in ultraviolet light at 366 nm.

*System suitability*: reference solution:

- the chromatogram shows a clearly visible spot due to impurity H.

*Limit*:

- *impurity H*: any spot due to impurity H is not more intense than the principal spot in the chromatogram obtained with the reference solution (0.15 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve with the aid of ultrasound the contents of a vial of *testosterone enantate for system suitability CRS* (containing impurities F and G) in the mobile phase and dilute to 1.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 2 mg of *testosterone enantate for peak identification CRS* (containing impurity E) in 1.0 mL of the mobile phase.

*Reference solution (d)*. Dissolve 2 mg of *testosterone caproate CRS* (impurity B) and 2 mg of *testosterone CRS* (impurity D) in the mobile phase and dilute to 5.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped dodecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*: *water R*, *acetonitrile R* (30:70 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of testosterone enantate.

*Identification of impurities*: use the chromatogram supplied with *testosterone enantate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities F and G; use the chromatogram supplied with *testosterone enantate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity E; use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and D.

*Relative retention* with reference to testosterone enantate (retention time = about 22 min): impurity D = about 0.1; impurity B = about 0.7; impurity E = about 0.8; impurity F = about 0.85; impurity G = about 0.9.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.3 between the peaks due to impurities F and G.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 6.3;
- *impurity D*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurities E, F*: for each impurity, not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity G*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at a pressure not exceeding 0.7 kPa.

ASSAY

Dissolve 50.0 mg in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *anhydrous ethanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 241 nm.

Calculate the content of $C_{26}H_{40}O_3$ taking the specific absorbance to be 422.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, B, D, E, F, G, H.*

A. heptanoic acid,

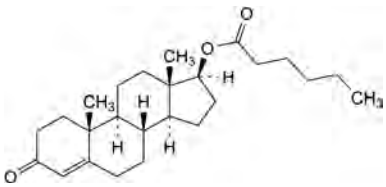

B. 3-oxoandrost-4-en-17β-yl hexanoate (testosterone caproate),

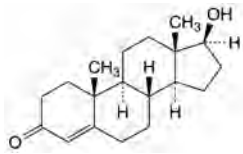

D. 17β-hydroxyandrost-4-en-3-one (testosterone),

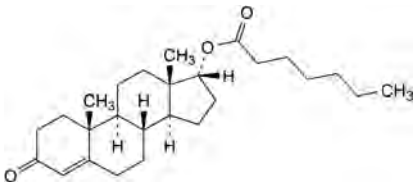

E. 3-oxoandrost-4-en-17α-yl heptanoate (17α-testosterone enantate),

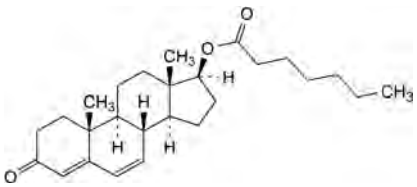

F. 3-oxoandrosta-4,6-dien-17β-yl heptanoate (Δ6-testosterone enantate),

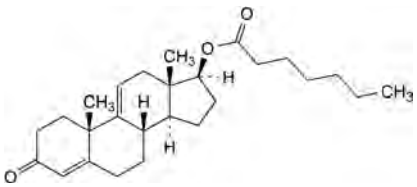

G. 3-oxoandrosta-4,9(11)-dien-17β-yl heptanoate (Δ9(11)-testosterone enantate),

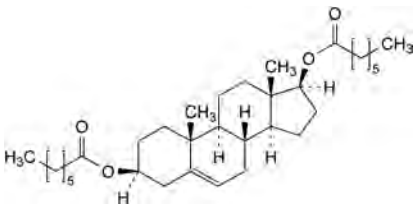

H. androst-5-ene-3β,17β-diyl diheptanoate.

**01/2008:1737**
**corrected 10.0**

# TESTOSTERONE ISOCAPROATE

## Testosteroni isocaproas

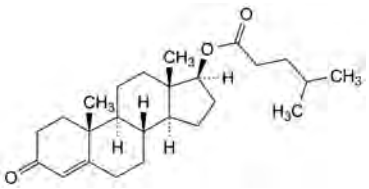

$C_{25}H_{38}O_3$ $M_r$ 386.6

DEFINITION

3-Oxoandrost-4-en-17β-yl 4-methylpentanoate.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in acetone and in methylene chloride, freely soluble in fatty oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *testosterone isocaproate CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.20 g in 20 mL of *methanol R*.

**Specific optical rotation** (*2.2.7*): + 82.0 to + 88.0 (dried substance).

Dissolve 0.200 g in *anhydrous ethanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2 mg of *testosterone isocaproate for system suitability CRS* (containing impurities A, B, C, D, E, F and G) in 10 mL of the mobile phase.

*Reference solution (b)*. Dilute 10.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 20.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve 20.0 mg of *testosterone isocaproate CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: *water R*, *acetonitrile R* (15:85 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of testosterone isocaproate.

*Identification of impurities*: use the chromatogram supplied with *testosterone isocaproate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, E, F and G.

*Relative retention* with reference to testosterone isocaproate (retention time = about 14 min): impurity A = about 0.2; impurity B = about 0.4; impurity C = about 0.5; impurity D = about 0.7; impurity G = about 0.8; impurity E = about 1.1; impurity F = about 1.4.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to testosterone isocaproate.

*Limits*:

- *impurities A, B, C, D, E, F, G*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Free acid**. Dissolve 0.44 g in 10 mL of *ethanol (96 per cent) R*, previously neutralised to *bromothymol blue solution R3*, and titrate immediately with *0.01 M sodium hydroxide*, using 0.1 mL of *bromothymol blue solution R3* as indicator. Not more than 0.6 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at a pressure not exceeding 0.7 kPa.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 20 µL of the test solution and reference solution (c).

Calculate the percentage content of $C_{25}H_{38}O_3$ from the declared content of *testosterone isocaproate CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G.*

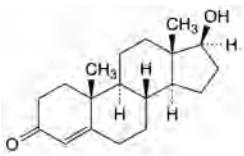

A. testosterone,

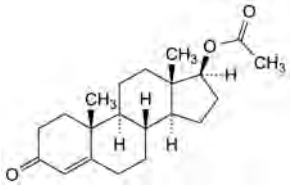

B. 3-oxoandrost-4-en-17β-yl acetate (testosterone acetate),

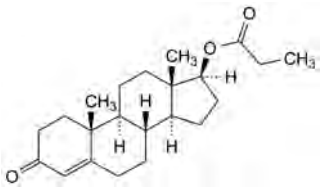

C. testosterone propionate,

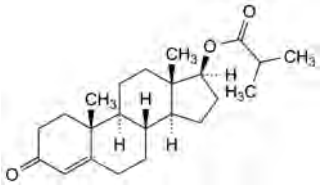

D. 3-oxoandrost-4-en-17β-yl 2-methylpropanoate (testosterone isobutyrate),

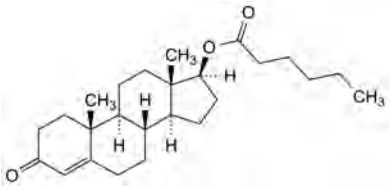

E. 3-oxoandrost-4-en-17β-yl hexanoate (testosterone caproate),

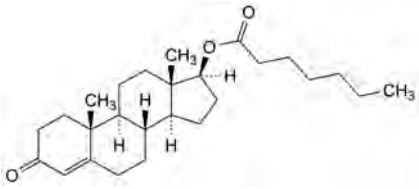

F. testosterone enantate,

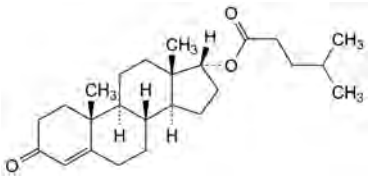

G. 3-oxoandrost-4-en-17α-yl 4-methylpentanoate (epitestosterone isocaproate).

**04/2012:0297**

# TESTOSTERONE PROPIONATE

## Testosteroni propionas

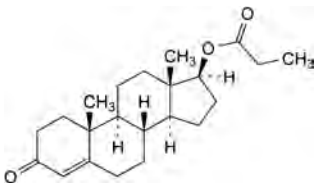

$C_{22}H_{32}O_3$ $M_r$ 344.5
[57-85-2]

DEFINITION

3-Oxoandrost-4-en-17β-yl propanoate.

*Content*: 97.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder or colourless crystals.

*Solubility*: practically insoluble in water, freely soluble in acetone and in ethanol (96 per cent), soluble in fatty oils.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *testosterone propionate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 84 to + 90 (dried substance).

Dissolve 0.250 g in *ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 2 mg of *testosterone propionate for system suitability CRS* (containing impurities A, B and C) in 5.0 mL of *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (c).* Dissolve 20.0 mg of *testosterone propionate CRS* in 50.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *methanol R* (20:80 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Run time*: twice the retention time of testosterone propionate.

*Identification of impurities*: use the chromatogram supplied with *testosterone propionate for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to testosterone propionate (retention time = about 8 min): impurity C = about 0.4; impurity A = about 0.7; impurity B = about 1.4.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to testosterone propionate.

*Limits*:

- *impurity A*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 7 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.7 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_{22}H_{32}O_3$ taking into account the assigned content of *testosterone propionate CRS*.

IMPURITIES

*Specified impurities*: *A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E.*

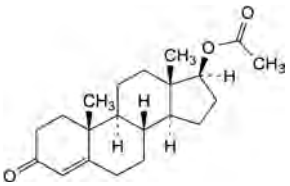

A. 3-oxoandrost-4-en-17β-yl acetate (testosterone acetate),

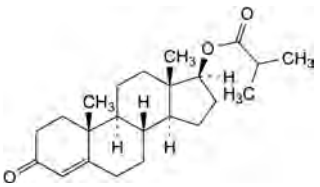

B. 3-oxoandrost-4-en-17β-yl 2-methylpropanoate (testosterone isobutyrate),

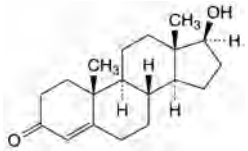

C. 17β-hydroxyandrost-4-en-3-one (testosterone),

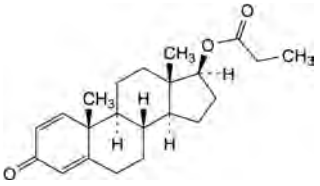

D. 3-oxoandrosta-1,4-dien-17β-yl propanoate,

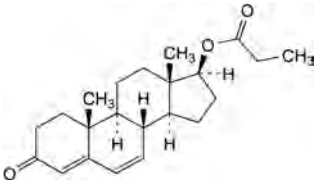

E. 3-oxoandrosta-4,6-dien-17β-yl propanoate.

**01/2020:2909**

# TETRACAINE

## Tetracainum

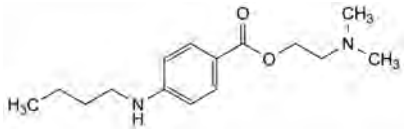

$C_{15}H_{24}N_2O_2$ $M_r$ 264.4
[94-24-6]

DEFINITION

2-(Dimethylamino)ethyl 4-(butylamino)benzoate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, freely soluble in methylene chloride, soluble in ethanol (96 per cent).

mp: about 44 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tetracaine CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *acetonitrile R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b)*. Dissolve 10 mg of *4-aminobenzoic acid R* (impurity A), 10 mg of *4-(butylamino)benzoic acid R* (impurity B) and 10 mg of *methyl 4-(butylamino)benzoate R* (impurity C) in *acetonitrile R* and dilute to 50 mL with the same solvent. Dilute 1 mL of the solution to 10 mL with the test solution.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl amorphous organosilica polymer for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 1.36 g of *potassium dihydrogen phosphate R* in *water for chromatography R*, add 0.5 mL of *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 80 | 20 |
| 3 - 18 | 80 → 40 | 20 → 60 |
| 18 - 23 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to tetracaine (retention time = about 7 min): impurity A = about 0.4; impurity B = about 1.6; impurity C = about 2.2.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to tetracaine and impurity B.

*Calculation of percentage contents*:

- *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.7; impurity C = 0.7;
- for each impurity, use the concentration of tetracaine in reference solution (a).

*Limits*:

- *impurities B, C*: for each impurity, maximum 0.15 per cent;
- *impurity A*: maximum 0.05 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent, except for impurity A.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 30 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of 25 volumes of *water R* and 75 volumes of *ethanol (96 per cent) R*. Titrate with *0.1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M hydrochloric acid* is equivalent to 26.44 mg of $C_{15}H_{24}N_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

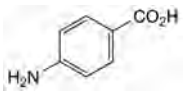

A. 4-aminobenzoic acid,

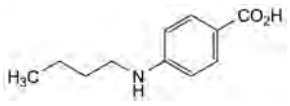

B. 4-(butylamino)benzoic acid,

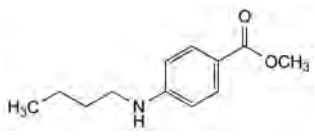

C. methyl 4-(butylamino)benzoate.

01/2017:0057

# TETRACAINE HYDROCHLORIDE

## Tetracaini hydrochloridum

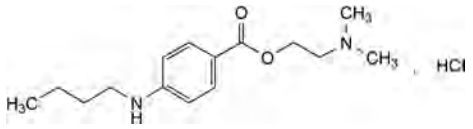

$C_{15}H_{25}ClN_2O_2$ $M_r$ 300.8
[136-47-0]

DEFINITION

2-(Dimethylamino)ethyl 4-(butylamino)benzoate hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

It melts at about 148 °C or it may occur in either of 2 other crystalline forms which melt respectively at about 134 °C and 139 °C. Mixtures of these forms melt within the range 134 °C to 147 °C.

IDENTIFICATION

*First identification: A, B, D.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tetracaine hydrochloride CRS.*

B. To 10 mL of solution S (see Tests) add 1 mL of *ammonium thiocyanate solution R*. A white, crystalline precipitate is formed which, after recrystallisation from *water R* and drying at 80 °C for 2 h, melts (*2.2.14*) at about 131 °C.

C. To about 5 mg add 0.5 mL of *fuming nitric acid R*. Evaporate to dryness on a water-bath, allow to cool and dissolve the residue in 5 mL of *acetone R*. Add 1 mL of *0.1 M alcoholic potassium hydroxide*. A violet colour develops.

D. Solution S gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dilute 2 mL of solution S to 10 mL with *water R*.

**pH** (*2.2.3*): 4.5 to 6.5.

Dilute 1 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use or store them at 2-8 °C.*

*Solvent mixture*: *acetonitrile R*, *water R* (20:80 *V/V*).

*Test solution.* Dissolve 50 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *tetracaine for system suitability CRS* (containing impurities A, B and C) in 2 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 1.36 g of *potassium dihydrogen phosphate R* in *water R*, add 0.5 mL of *phosphoric acid R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 80 | 20 |
| 3 - 18 | 80 → 40 | 20 → 60 |
| 18 - 23 | 40 | 60 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 300 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *tetracaine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to tetracaine (retention time = about 8 min): impurity A = about 0.3; impurity B = about 1.7; impurity C = about 2.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 5.0 between the peaks due to tetracaine and impurity B.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.6; impurity C = 0.7;
- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *impurities B, C*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 30.08 mg of $C_{15}H_{25}ClN_2O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

Monographs

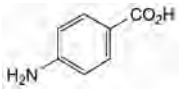

A. 4-aminobenzoic acid,

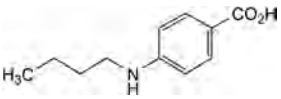

B. 4-(butylamino)benzoic acid,

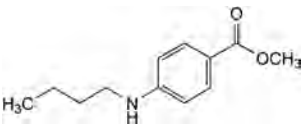

C. methyl 4-(butylamino)benzoate.

**04/2010:0644**

# TETRACOSACTIDE

## Tetracosactidum

H - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Trp - Gly - Lys - Pro - Val - Gly - Lys - Lys - Arg - Arg - Pro - Val - Lys - Val - Tyr - Pro - OH

$C_{136}H_{210}N_{40}O_{31}S$ $M_r$ 2933
[16960-16-0]

DEFINITION

Synthetic tetracosapeptide, in which the sequence of amino acids is the same as that of the first 24 residues of human corticotropin. It increases the rate at which corticoid hormones are secreted by the adrenal glands. It is available as an acetate.

*Content*: 90 per cent to 102 per cent (anhydrous and acetic acid-free substance). By convention, 1 µg of tetracosactide is equivalent to 1 IU of tetracosactide.

CHARACTERS

*Appearance*: white or yellow, amorphous powder.

*Solubility*: sparingly soluble in water.

IDENTIFICATION

A. Examine the chromatograms obtained in the test for related peptides.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with the reference solution.

B. Amino acid analysis (*2.2.56*). For hydrolysis use Method 1 and for analysis use Method 1.

Express the content of each amino acid in moles. Calculate the relative proportions of the amino acids, taking that of valine to be equivalent to 3. The values fall within the following limits: lysine 3.5 to 4.7; histidine 0.9 to 1.1; arginine 2.7 to 3.3; serine 1.1 to 2.2; glutamic acid 0.9 to 1.1; proline 2.5 to 3.5; glycine 1.8 to 2.2; methionine 0.9 to 1.1; tyrosine 1.7 to 2.2; phenylalanine 0.9 to 1.1. Not more than traces of other amino acids are present.

TESTS

**Specific optical rotation** (*2.2.7*): − 99 to − 109 (anhydrous and acetic acid-free substance).

Dissolve 10.0 mg in 1.0 mL of a mixture of 1 volume of *glacial acetic acid R* and 99 volumes of *water R*.

**Absorbance** (*2.2.25*): 0.51 to 0.61 (anhydrous and acetic acid-free substance), determined at the absorption maximum between 240 nm and 280 nm, at 276 nm. The ratio of the absorbance at the maximum at 276 nm to the absorbance at 248 nm is 2.4 to 2.9.

Dissolve 1.0 mg in *0.1 M hydrochloric acid* and dilute to 5.0 mL with the same acid.

**Related peptides**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve an accurately weighed quantity of the substance to be examined in *water R* to obtain the same concentration as in reference solution (a).

*Reference solution (a).* Dissolve the contents of a vial of *tetracosactide CRS* in *water R* to obtain a concentration of about 1 mg/mL, as indicated in the leaflet provided with the reference standard.

*Reference solution (b).* In order to prepare impurity A *in situ*, dissolve 1.0 mg of the substance to be examined in 1 mL of a 1 per cent *V/V* solution of *glacial acetic acid R*, add 50 µL of a mixture of 1 volume of *strong hydrogen peroxide solution R* and 999 volumes of *water R*, and allow to stand for 2 h.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 25 °C.

*Mobile phase*:

- *mobile phase A*: mix 5.0 mL of *glacial acetic acid R*, 60 mL of *acetonitrile R* and 5.0 g of *ammonium sulfate R* and dilute to 1000 mL with *water R*;
- *mobile phase B*: mix 5.0 mL of *glacial acetic acid R*, 310 mL of *acetonitrile R* and 5.0 g of *ammonium sulfate R* and dilute to 1000 mL with *water R*;
- *mobile phase C*: *acetonitrile R*.

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) | Mobile phase C (per cent *V/V*) |
|---|---|---|---|
| 0 - 50 | 55 → 40 | 45 → 60 | 0 |
| 50 - 50.1 | 40 → 0 | 60 → 15 | 0 → 85 |
| 50.1 - 55 | 0 | 15 | 85 |
| 55 - 55.1 | 0 → 55 | 15 → 45 | 85 → 0 |
| 55.1 - 60 | 55 | 45 | 0 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *tetracosactide CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to tetracosactide (retention time = about 26 min): impurity A = about 0.3; impurity B = about 0.95.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity B and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to tetracosactide.

*Limits*:

- *impurity A*: maximum 3 per cent;
- *impurity B*: maximum 4 per cent;
- *unspecified impurities*: for each impurity, maximum 2.5 per cent;
- *sum of impurities other than A*: maximum 9 per cent.

**Acetic acid** (*2.5.34*): 8.0 per cent to 13.0 per cent.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A and dilute to 10.0 mL with the same mixture of mobile phases.

**Water** (*2.5.32*): maximum 14.0 per cent, determined on 20.0-50.0 mg.

**Bacterial endotoxins** (*2.6.14*): less than 10 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related peptides.

Calculate the content of $C_{136}H_{210}N_{40}O_{31}S$ using the declared content of *tetracosactide CRS*.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the mass of peptide in the container;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B.*

A. tetracosactide sulfoxide,

B. unknown structure.

**01/2017:0211**

# TETRACYCLINE

## Tetracyclinum

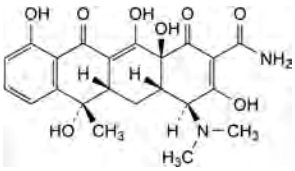

$C_{22}H_{24}N_2O_8$ $M_r$ 444.4

[60-54-8]

DEFINITION

(4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(Dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide.

Substance produced by certain strains of *Streptomyces aerofaciens* or obtained by any other means.

*Content*: 88.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: very slightly soluble in water, soluble in ethanol (96 per cent) and in methanol, sparingly soluble in acetone. It dissolves in dilute acid and alkaline solutions.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *tetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *oxytetracycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R*.

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A violet-red colour develops. Add the solution to 2.5 mL of *water R*. The colour becomes yellow.

C. Dissolve about 10 mg in a mixture of 1 mL of *dilute nitric acid R* and 5 mL of *water R*. Shake and add 1 mL of *silver nitrate solution R2*. Any opalescence in the solution is not more intense than that in a mixture of 1 mL of *dilute nitric acid R*, 5 mL of *water R* and 1 mL of *silver nitrate solution R2*.

TESTS

**pH** (*2.2.3*): 3.5 to 6.0.

Suspend 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 260 to − 280 (dried substance).

Dissolve 0.250 g in *0.1 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (a).* Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (b).* Dissolve 12.5 mg of *4-epitetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (c).* Dissolve 10.0 mg of *anhydrotetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

*Reference solution (d).* Dissolve 10.0 mg of *4-epianhydrotetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (e).* Mix 1.0 mL of reference solution (a), 2.0 mL of reference solution (b) and 5.0 mL of reference solution (d) and dilute to 25.0 mL with *0.01 M hydrochloric acid*.

*Reference solution (f).* Mix 40.0 mL of reference solution (b), 20.0 mL of reference solution (c) and 5.0 mL of reference solution (d) and dilute to 200.0 mL with *0.01 M hydrochloric acid*.

*Reference solution (g).* Dilute 1.0 mL of reference solution (c) to 50.0 mL with *0.01 M hydrochloric acid*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm);
– *temperature*: 60 °C.

*Mobile phase*: weigh 80.0 g of *2-methyl-2-propanol R* and transfer to a 1000 mL volumetric flask with the aid of 200 mL of *water R*; add 100 mL of a 35 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 with *dilute phosphoric acid R*, 200 mL of a 10 g/L solution of *tetrabutylammonium hydrogen sulfate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R* and 10 mL of a 40 g/L solution of *sodium edetate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R*; dilute to 1000.0 mL with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL; inject the test solution and reference solutions (e), (f) and (g).

*System suitability*:
– *resolution*: minimum 2.5 between the peaks due to impurity A (1st peak) and tetracycline (2nd peak) and minimum 8.0 between the peaks due to tetracycline and impurity D (3rd peak) in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase;
– *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (g);
– *symmetry factor*: maximum 1.25 for the peak due to tetracycline in the chromatogram obtained with reference solution (e).

*Limits*:
– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (5.0 per cent);
– *impurity B* (eluting on the tail of the principal peak): not more than 0.4 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (2.0 per cent);
– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (1.0 per cent);
– *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 13.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{24}N_2O_8$.

STORAGE

Protected from light.

IMPURITIES

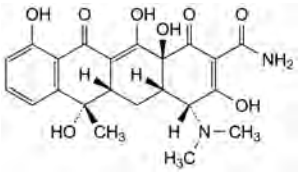

A. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epitetracycline),

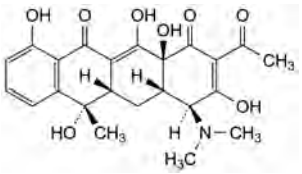

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyltetracycline),

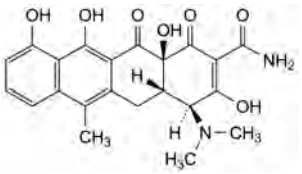

C. (4*S*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

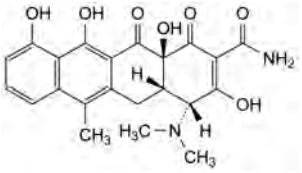

D. (4*R*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline).

**01/2017:0210**
**corrected 10.0**

# TETRACYCLINE HYDROCHLORIDE

## Tetracyclini hydrochloridum

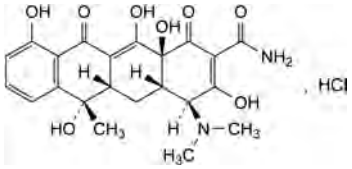

$C_{22}H_{25}ClN_2O_8$ $M_r$ 480.9
[64-75-5]

DEFINITION

(4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-(Dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide hydrochloride.

Substance produced by certain strains of *Streptomyces aerofaciens* or obtained by any other means.

*Content*: 95.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone. It dissolves in solutions of alkali hydroxides and carbonates. Solutions in water become turbid on standing, owing to the precipitation of tetracycline.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

Monographs T

*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 5 mg of *tetracycline hydrochloride CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *tetracycline hydrochloride CRS*, 5 mg of *demeclocycline hydrochloride R* and 5 mg of *oxytetracycline hydrochloride R* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC octadecylsilyl silica gel $F_{254}$ plate R.*

*Mobile phase*: mix 20 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 60 volumes of a 63 g/L solution of *oxalic acid R* previously adjusted to pH 2 with *concentrated ammonia R.*

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

B. To about 2 mg add 5 mL of *sulfuric acid R*. A violet-red colour develops. Add the solution to 2.5 mL of *water R*. The colour becomes yellow.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 1.8 to 2.8.

Dissolve 0.1 g in 10 mL of *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): − 240 to − 255 (dried substance).

Dissolve 0.250 g in *0.1 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (a).* Dissolve 25.0 mg of *tetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

*Reference solution (b).* Dissolve 15.0 mg of *4-epitetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (c).* Dissolve 10.0 mg of *anhydrotetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

*Reference solution (d).* Dissolve 10.0 mg of *4-epianhydrotetracycline hydrochloride CRS* in *0.01 M hydrochloric acid* and dilute to 50.0 mL with the same acid.

*Reference solution (e).* Mix 1.0 mL of reference solution (a), 2.0 mL of reference solution (b) and 5.0 mL of reference solution (d) and dilute to 25.0 mL with *0.01 M hydrochloric acid.*

*Reference solution (f).* Mix 20.0 mL of reference solution (b), 10.0 mL of reference solution (c) and 5.0 mL of reference solution (d) and dilute to 200.0 mL using *0.01 M hydrochloric acid.*

*Reference solution (g).* Dilute 1.0 mL of reference solution (c) to 50.0 mL with *0.01 M hydrochloric acid.*

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm);
- *temperature*: 60 °C.

*Mobile phase*: weigh 80.0 g of *2-methyl-2-propanol R* and transfer to a 1000 mL volumetric flask with the aid of 200 mL of *water R*; add 100 mL of a 35 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 9.0 with *dilute phosphoric acid R*, 200 mL of a 10 g/L solution of *tetrabutylammonium hydrogen sulfate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R* and 10 mL of a 40 g/L solution of *sodium edetate R* adjusted to pH 9.0 with *dilute sodium hydroxide solution R*; dilute to 1000.0 mL with *water R.*

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL; inject the test solution and reference solutions (e), (f) and (g).

*System suitability*:

- *resolution*: minimum 2.5 between the peaks due to impurity A (1st peak) and tetracycline (2nd peak) and minimum 8.0 between the peaks due to tetracycline and impurity D (3rd peak) in the chromatogram obtained with reference solution (e); if necessary, adjust the concentration of 2-methyl-2-propanol in the mobile phase;
- *signal-to-noise ratio*: minimum 3 for the principal peak in the chromatogram obtained with reference solution (g);
- *symmetry factor*: maximum 1.25 for the peak due to tetracycline in the chromatogram obtained with reference solution (e).

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (3.0 per cent);
- *impurity B* (eluting on the tail of the principal peak): not more than 0.5 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (f) (1.5 per cent);
- *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent);
- *impurity D*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.5 per cent).

**Loss on drying** (*2.2.32*): maximum 2.0 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.5 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{22}H_{25}ClN_2O_8$.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, tamper-evident container.

IMPURITIES

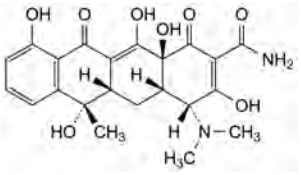

A. (4*R*,4a*S*,5a*S*,6*S*,12a*S*)-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (4-epitetracycline),

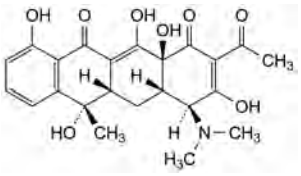

B. (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-2-acetyl-4-(dimethylamino)-3,6,10,12,12a-pentahydroxy-6-methyl-4a,5a,6,12a-tetrahydrotetracene-1,11(4*H*,5*H*)-dione (2-acetyl-2-decarbamoyltetracycline),

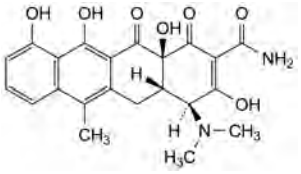

C. (4*S*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (anhydrotetracycline),

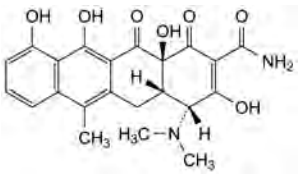

D. (4*R*,4a*S*,12a*S*)-4-(dimethylamino)-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-1,4,4a,5,12,12a-hexahydrotetracene-2-carboxamide (4-epianhydrotetracycline).

**01/2008:1738**
**corrected 7.0**

# TETRAZEPAM

## Tetrazepamum

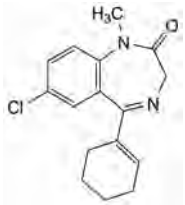

$C_{16}H_{17}ClN_2O$ $M_r$ 288.8
[10379-14-3]

DEFINITION

7-Chloro-5-(cyclohex-1-enyl)-1-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: light yellow or yellow crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, soluble in acetonitrile.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of tetrazepam*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of the substance to be examined and 5.0 mg of *tetrazepam impurity C CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *acetonitrile R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 50.0 mL with *acetonitrile R*. Dilute 1.0 mL of this solution to 10.0 mL with *acetonitrile R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

- *mobile phase A*: mix 40 volumes of *acetonitrile R* and 60 volumes of a 3.4 g/L solution of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 100 | 0 |
| 35 - 40 | 100 → 55 | 0 → 45 |
| 40 - 50 | 55 | 45 |

*Flow rate*: 1.5 mL/min.

*Detection*: a spectrophotometer at 229 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to tetrazepam and to impurity C.

*Limits*:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 0.750 g in 10 mL of *methylene chloride R* and add 15 mL of *water R*. Shake and separate the 2 layers. Dilute 10 mL of the aqueous layer to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.230 g in 50.0 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.88 mg of $C_{16}H_{17}ClN_2O$.

STORAGE

Protected from light.

IMPURITIES

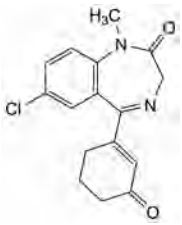

A. 7-chloro-1-methyl-5-(3-oxocyclohex-1-enyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

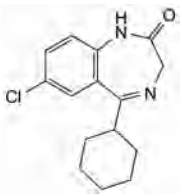

B. 7-chloro-5-cyclohexyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

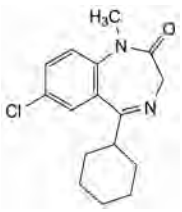

C. 7-chloro-5-cyclohexyl-1-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

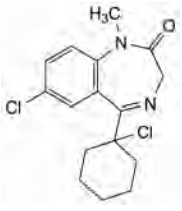

D. 7-chloro-5-(1-chlorocyclohexyl)-1-methyl-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one,

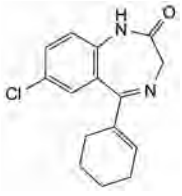

E. 7-chloro-5-(cyclohex-1-enyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one.

**01/2008:2101**
**corrected 10.0**

# TETRYZOLINE HYDROCHLORIDE

## Tetryzolini hydrochloridum

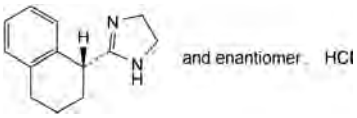


$C_{13}H_{17}ClN_2$ $M_r$ 236.7
[522-48-5]

DEFINITION

2-[(1*RS*)-1,2,3,4-Tetrahydronaphthalen-1-yl]-4,5-dihydro-1*H*-imidazole hydrochloride.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, in anhydrous ethanol and in ethanol (96 per cent), practically insoluble in acetone.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tetryzoline hydrochloride CRS*.

B. Dissolve 50 mg in 10 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 1.0 g of the substance to be examined in a mixture of 25 volumes of *1 M sodium hydroxide* and 75 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution*. Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of 25 volumes of *1 M sodium hydroxide* and 75 volumes of *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with a mixture of 25 volumes of *1 M sodium hydroxide* and 75 volumes of *methanol R*.

*Column*:
- *material*: fused silica,
- *size*: *l* = 25 m, Ø = 0.32 mm,
- *stationary phase*: *methylpolysiloxane R* (1 µm).

*Carrier gas*: *helium for chromatography R*.

*Split ratio*: 1:40.

*Flow rate*: 2.5 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 8 | 160 |
| | 8 - 11 | 160 → 220 |
| | 11 - 15 | 220 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to tetryzoline (retention time = about 12 min): impurity A = about 0.5.

*System suitability*: reference solution:

– *signal-to-noise ratio*: minimum 50 for the principal peak.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with the reference solution (0.1 per cent),
– *total*: not more than twice the area of the principal peak in the chromatogram obtained with the reference solution (0.2 per cent),
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with the reference solution (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 100 mL of a mixture of 3 volumes of *anhydrous acetic acid R* and 7 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 23.67 mg of $C_{13}H_{17}ClN_2$.

IMPURITIES

*Specified impurities: A.*

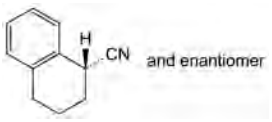


A. (1*RS*)-1,2,3,4-tetrahydronaphthalene-1-carbonitrile (α-cyanotetraline).

**01/2017:0298**

# THEOBROMINE

## Theobrominum

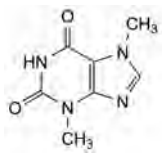


$C_7H_8N_4O_2$ $M_r$ 180.2
[83-67-0]

DEFINITION

Theobromine contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione, calculated with reference to the dried substance.

CHARACTERS

A white or almost white powder, very slightly soluble in water and in anhydrous ethanol, slightly soluble in ammonia. It dissolves in dilute solutions of alkali hydroxides and in mineral acids.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *theobromine CRS*.

B. Dissolve about 20 mg in 2 mL of *dilute ammonia R1*, warming slightly, and cool. Add 2 mL of *silver nitrate solution R2*. The solution remains clear. Boil the solution for a few minutes. A white, crystalline precipitate is formed.

C. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Acidity**. To 0.4 g add 20 mL of boiling *water R* and boil for 1 min. Allow to cool and filter. Add 0.05 mL of *bromothymol blue solution R1*. The solution is yellow or yellowish-green. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution*. To 0.2 g of the finely powdered substance to be examined add 10 mL of a mixture of 4 volumes of *methanol R* and 6 volumes of *chloroform R*. Heat under a reflux condenser on a water-bath for 15 min, shaking occasionally. Cool and filter.

*Reference solution*. Dissolve 5 mg of *theobromine CRS* in a mixture of 4 volumes of *methanol R* and 6 volumes of *chloroform R* and dilute to 50 mL with the same mixture of solvents.

Apply separately to the plate 10 µL of each solution. Develop over a path of 15 cm using a mixture of 10 volumes of *concentrated ammonia R*, 30 volumes of *acetone R*, 30 volumes of *chloroform R* and 40 volumes of *butanol R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve, with stirring, 0.150 g in 125 mL of boiling *water R*, cool to 50-60 °C and add 25 mL of *0.1 M silver nitrate*. Using 1 mL of *phenolphthalein solution R* as indicator, titrate dropwise with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

**01/2017:0299**

# THEOPHYLLINE

## Theophyllinum

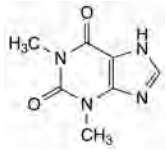


$C_7H_8N_4O_2$ $M_r$ 180.2
[58-55-9]

DEFINITION

1,3-Dimethyl-3,7-dihydro-1*H*-purine-2,6-dione.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides, in ammonia and in mineral acids.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 270 °C to 274 °C, determined after drying at 100-105 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of theophylline.*

C. Heat 10 mg with 1.0 mL of a 360 g/L solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 mL of *diazotised sulfanilic acid solution R*. A red colour slowly develops. Carry out a blank test.

D. Loss on drying (see Tests).

E. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g with heating in *carbon dioxide-free water R*, cool and dilute to 75 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 50 mL of solution S add 0.1 mL of *methyl red solution R*. The solution is red. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *theobromine R* in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase. Dilute 5 mL of this solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 7 volumes of *acetonitrile for chromatography R* and 93 volumes of a 1.36 g/L solution of *sodium acetate R* containing 5.0 mL/L of *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of theophylline.

*Relative retention* with reference to theophylline (retention time = about 6 min): impurity C = about 0.3; impurity B = about 0.4; impurity D = about 0.5; impurity A = about 2.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to theobromine and theophylline.

*Limits*:

– *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 100 mL of *water R*, add 20 mL of *0.1 M silver nitrate* and shake. Add 1 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

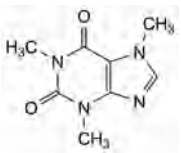

A. 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (caffeine),

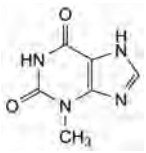

B. 3-methyl-3,7-dihydro-1*H*-purine-2,6-dione,

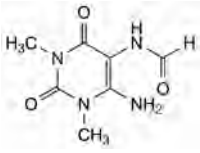

C. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

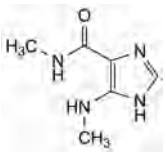

D. *N*-methyl-5-(methylamino)-1*H*-imidazole-4-carboxamide (theophyllidine),

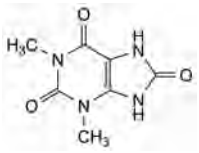

E. 1,3-dimethyl-7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione,

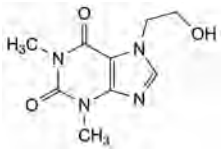

F. 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (etofylline).

**01/2017:0302**

# THEOPHYLLINE MONOHYDRATE

## Theophyllinum monohydricum

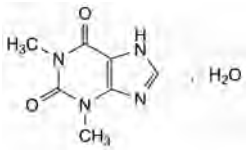

$C_7H_8N_4O_2,H_2O$ $M_r$ 198.2
[5967-84-0]

DEFINITION

1,3-Dimethyl-3,7-dihydro-1*H*-purine-2,6-dione monohydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides, in ammonia and in mineral acids.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D, E.*

A. Melting point (*2.2.14*): 270 °C to 274 °C, determined after drying at 100-105 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: dry the substance to be examined at 100-105 °C before use.
*Comparison*: *Ph. Eur. reference spectrum of theophylline.*

C. Heat 10 mg with 1.0 mL of a 360 g/L solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 mL of *diazotised sulfanilic acid solution R*. A red colour slowly develops. Carry out a blank test.

D. Water (see Tests).

E. It gives the reaction of xanthines (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g with heating in *carbon dioxide-free water R*, cool and dilute to 75 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 50 mL of solution S add 0.1 mL of *methyl red solution R*. The solution is red. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *theobromine R* in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase. Dilute 5 mL of this solution to 50 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 7 volumes of *acetonitrile for chromatography R* and 93 volumes of a 1.36 g/L solution of *sodium acetate R* containing 5.0 mL/L of *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of theophylline.

*Relative retention* with reference to theophylline (retention time = about 6 min): impurity C = about 0.3; impurity B = about 0.4; impurity D = about 0.5; impurity A = about 2.5.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to theobromine and theophylline.

*Limits*:
- *impurities A, B, C, D*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 8.0 per cent to 9.5 per cent, determined on 0.20 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.160 g in 100 mL of *water R*, add 20 mL of *0.1 M silver nitrate* and shake. Add 1 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

IMPURITIES

*Specified impurities: A, B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F.*

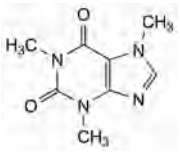

A. 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (caffeine),

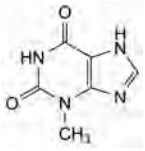

B. 3-methyl-3,7-dihydro-1*H*-purine-2,6-dione,

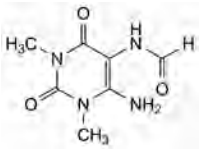

C. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

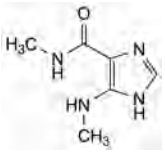

D. *N*-methyl-5-(methylamino)-1*H*-imidazole-4-carboxamide (theophyllidine),

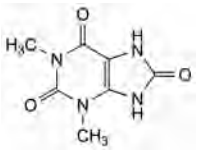

E. 1,3-dimethyl-7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione,

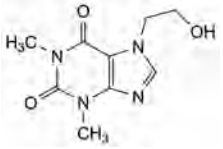

F. 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (etofylline).

**01/2017:0300**

# THEOPHYLLINE-ETHYLENEDIAMINE

## Theophyllinum et ethylenediaminum

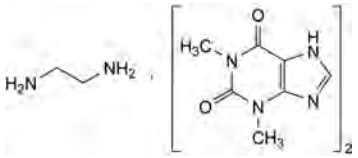

$C_{16}H_{24}N_{10}O_4$ $M_r$ 420.4
[317-34-0]

DEFINITION

*Content*:

– *theophylline* ($C_7H_8N_4O_2$; $M_r$ 180.2): 84.0 per cent to 87.4 per cent (anhydrous substance);
– *ethylenediamine* ($C_2H_8N_2$; $M_r$ 60.1): 13.5 per cent to 15.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellowish powder, sometimes granular, hygroscopic.

*Solubility*: freely soluble in water (the solution becomes cloudy through absorption of carbon dioxide), practically insoluble in anhydrous ethanol.

IDENTIFICATION

*First identification*: *B, C, E.*

*Second identification*: *A, C, D, E, F.*

Dissolve 1.0 g in 10 mL of *water R* and add 2 mL of *dilute hydrochloric acid R* dropwise with shaking. Filter. Use the precipitate for identification tests A, B, D and F and the filtrate for identification test C.

A. Melting point (*2.2.14*): 270 °C to 274 °C, determined after washing the precipitate with *water R* and drying at 105 °C.
B. Infrared absorption spectrophotometry (*2.2.24*).
   *Preparation*: precipitate, washed with *water R* and dried at 105 °C.
   *Comparison*: *theophylline CRS*.
C. To the filtrate add 0.2 mL of *benzoyl chloride R*, make alkaline with *dilute sodium hydroxide solution R* and shake vigorously. Filter the precipitate, wash with 10 mL of *water R*, dissolve in 5 mL of hot *ethanol (96 per cent) R* and add 5 mL of *water R*. A precipitate is formed, which, when washed and dried at 105 °C, melts (*2.2.14*) at 248 °C to 252 °C.
D. Heat about 10 mg of the precipitate with 1.0 mL of a 360 g/L solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 mL of *diazotised sulfanilic acid solution R*. A red colour slowly develops. Carry out a blank test.
E. Water (see Tests).
F. The precipitate gives the reaction of xanthines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g with gentle warming in 10 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 47 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *theobromine R* (impurity G) in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase. Dilute 5 mL of this solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 7 volumes of *acetonitrile for chromatography R* and 93 volumes of a 1.36 g/L solution of *sodium acetate R* containing 0.50 per cent *V/V* of *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of theophylline.

*Relative retention* with reference to theophylline (retention time = about 6 min): impurity G = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity G and theophylline.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.5 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**Ethylenediamine**. Dissolve 0.250 g in 30 mL of *water R*. Add 0.1 mL of *bromocresol green solution R*. Titrate with *0.1 M hydrochloric acid* until a green colour is obtained.

1 mL of *0.1 M hydrochloric acid* is equivalent to 3.005 mg of $C_2H_8N_2$.

**Theophylline**. Heat 0.200 g to constant mass in an oven at 135 °C. Dissolve the residue with heating in 100 mL of *water R*, allow to cool, add 20 mL of *0.1 M silver nitrate* and shake. Add 1 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

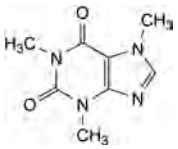

A. 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (caffeine),

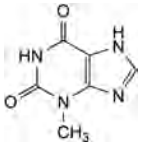

B. 3-methyl-3,7-dihydro-1*H*-purine-2,6-dione,

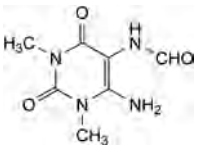

C. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

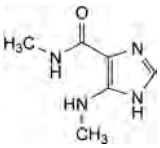

D. *N*-methyl-5-(methylamino)-1*H*-imidazole-4-carboxamide,

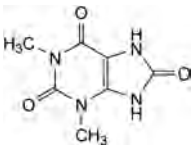

E. 1,3-dimethyl-7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione,

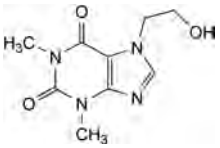

F. 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (etofylline),

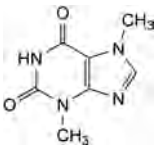

G. 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theobromine).

**01/2017:0301**

# THEOPHYLLINE-ETHYLENEDIAMINE HYDRATE

## Theophyllinum et ethylenediaminum hydricum

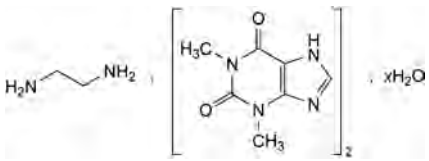

$C_{16}H_{24}N_{10}O_4,xH_2O$ $M_r$ 420.4 (anhydrous substance)
[72487-55-9]

DEFINITION

*Content*:

– *theophylline* ($C_7H_8N_4O_2$; $M_r$ 180.2): 84.0 per cent to 87.4 per cent (anhydrous substance);
– *ethylenediamine* ($C_2H_8N_2$; $M_r$ 60.1): 13.5 per cent to 15.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or slightly yellowish powder, sometimes granular.

*Solubility*: freely soluble in water (the solution becomes cloudy through absorption of carbon dioxide), practically insoluble in anhydrous ethanol.

IDENTIFICATION

*First identification: B, C, E.*

*Second identification: A, C, D, E, F.*

Dissolve 1.0 g in 10 mL of *water R* and add 2 mL of *dilute hydrochloric acid R* dropwise with shaking. Filter. Use the precipitate for identification tests A, B, D and F and the filtrate for identification test C.

A. Melting point (*2.2.14*): 270 °C to 274 °C, determined after washing the precipitate with *water R* and drying at 105 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: precipitate, washed with *water R* and dried at 105 °C.

*Comparison*: *theophylline CRS*.

C. To the filtrate add 0.2 mL of *benzoyl chloride R*, make alkaline with *dilute sodium hydroxide solution R* and shake vigorously. Filter the precipitate, wash with 10 mL of *water R*, dissolve in 5 mL of hot *ethanol (96 per cent) R* and add 5 mL of *water R*. A precipitate is formed which, when washed and dried at 105 °C, melts (*2.2.14*) at 248 °C to 252 °C.

D. Heat about 10 mg of the precipitate with 1.0 mL of a 360 g/L solution of *potassium hydroxide R* in a water-bath at 90 °C for 3 min, then add 1.0 mL of *diazotised sulfanilic acid solution R*. A red colour slowly develops. Carry out a blank test.

E. Water (see Tests).

F. The precipitate gives the reaction of xanthines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $GY_6$ (*2.2.2, Method II*).

Dissolve 0.5 g with gentle warming in 10 mL of *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 10 mg of *theobromine R* (impurity G) in the mobile phase, add 5 mL of the test solution and dilute to 100 mL with the mobile phase. Dilute 5 mL of this solution to 50 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (7 µm).

*Mobile phase*: mix 7 volumes of *acetonitrile for chromatography R* and 93 volumes of a 1.36 g/L solution of *sodium acetate R* containing 0.50 per cent *V/V* of *glacial acetic acid R*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of theophylline.

*Relative retention* with reference to theophylline (retention time = about 6 min): impurity G = about 0.6.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity G and theophylline.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 3.0 per cent to 8.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

**Ethylenediamine**. Dissolve 0.250 g in 30 mL of *water R*. Add 0.1 mL of *bromocresol green solution R*. Titrate with *0.1 M hydrochloric acid* until a green colour is obtained.

1 mL of *0.1 M hydrochloric acid* is equivalent to 3.005 mg of $C_2H_8N_2$.

**Theophylline**. Heat 0.200 g to constant mass in an oven at 135 °C. Dissolve the residue with heating in 100 mL of *water R*, allow to cool, add 20 mL of *0.1 M silver nitrate* and shake. Add 1 mL of *bromothymol blue solution R1*. Titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 18.02 mg of $C_7H_8N_4O_2$.

STORAGE

In a well-filled, airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G.*

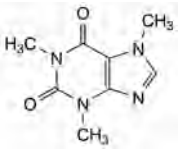

A. 1,3,7-trimethyl-3,7-dihydro-1*H*-purine-2,6-dione (caffeine),

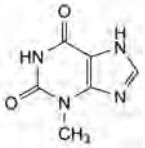

B. 3-methyl-3,7-dihydro-1*H*-purine-2,6-dione,

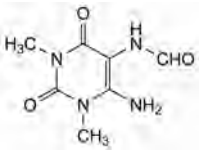

C. *N*-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)formamide,

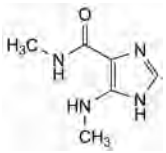

D. *N*-methyl-5-(methylamino)-1*H*-imidazole-4-carboxamide,

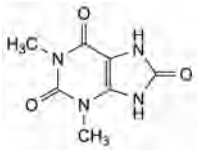

E. 1,3-dimethyl-7,9-dihydro-1*H*-purine-2,6,8(3*H*)-trione,

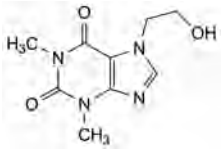

F. 7-(2-hydroxyethyl)-1,3-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (etofylline),

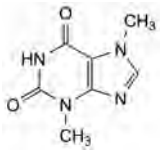

G. 3,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione (theobromine).

**01/2017:1706**
**corrected 10.0**

# THIAMAZOLE

## Thiamazolum

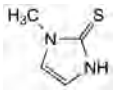

$C_4H_6N_2S$ $M_r$ 114.2
[60-56-0]

DEFINITION

1-Methyl-1,3-dihydro-2*H*-imidazole-2-thione.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or pale brown, crystalline powder.

*Solubility*: freely soluble in water, freely soluble in methylene chloride, freely soluble or soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: A, C.

*Second identification*: A, B, D.

A. Melting point (*2.2.14*): 143 °C to 146 °C.

B. Dissolve 25 mg in 10 mL of a 0.28 per cent *V/V* solution of *sulfuric acid R* and dilute to 50.0 mL with the same solution. Dilute 1.0 mL of this solution to 100.0 mL with a 0.28 per cent *V/V* solution of *sulfuric acid R*. Examined between 200 nm and 300 nm (*2.2.25*), the solution shows 2 absorption maxima, at 211 nm and 251 nm. The ratio of the absorbance measured at the absorption maximum at 251 nm to that measured at the absorption maximum at 211 nm is 2.5 to 2.7.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *thiamazole CRS*.

D. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 5.0 mg of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.
*Reference solution (a)*. Dissolve 5.0 mg of *thiamazole CRS* in *methanol R* and dilute to 5.0 mL with the same solvent.
*Reference solution (b)*. Dissolve 5.0 mg of *2-methylimidazole R* in *methanol R* and dilute to 5.0 mL with the same solvent. Dilute 1.0 mL of this solution to 2.0 mL with the test solution.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *concentrated ammonia R1*, *2-propanol R*, *toluene R* (1:24:75 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: examine in ultraviolet light at 254 nm.
*System suitability*: reference solution (b):
– expose the plate to iodine vapour for 30 min; the chromatogram shows 2 clearly separated spots.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 2.0 g in *water R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Related substances**. Gas chromatography (*2.2.28*).
*Test solution*. Dissolve 0.100 g of the substance to be examined in *chloroform R* and dilute to 10.0 mL with the same solvent.
*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *chloroform R*. Dilute 1.0 mL of this solution to 10.0 mL with *chloroform R*.
*Reference solution (b)*. Dissolve 5.0 mg of *thiamazole impurity A CRS*, 5.0 mg of *1-methylimidazole R1* and 5.0 mg of *thiamazole impurity C CRS* in *chloroform R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *chloroform R*.
*Column*:
– *material*: fused silica,
– *size*: *l* = 30.0 m, Ø = 0.25 mm,
– *stationary phase*: *base-deactivated phenyl(5)methyl(95)polysiloxane R* (film thickness 0.5 µm).
*Carrier gas*: *helium for chromatography R*.
*Flow rate*: 1.5 mL/min.
*Split ratio*: 3:20.
*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 100 |
| | 2 - 7 | 100 → 250 |
| | 7 - 22 | 250 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.
*Injection*: 1 µL.

*Relative retention* with reference to thiamazole (retention time = about 6.5 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurity A and impurity B.

*Limits*:

– *impurities A, B, C*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
– *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent),
– *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 75 mL of *water R*. Add 15.0 mL of *0.1 M sodium hydroxide*, mix and add with stirring, about 30 mL of *0.1 M silver nitrate*. Continue the titration with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 11.42 mg of $C_4H_6N_2S$.

IMPURITIES

*Specified impurities: A, B, C.*

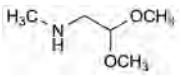

A. 2,2-dimethoxy-*N*-methylethanamine,

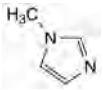

B. 1-methyl-1*H*-imidazole,

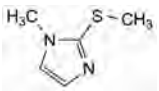

C. 1-methyl-2-(methylsulfanyl)-1*H*-imidazole.

**01/2017:0303**
**corrected 10.0**

# THIAMINE HYDROCHLORIDE

## Thiamini hydrochloridum

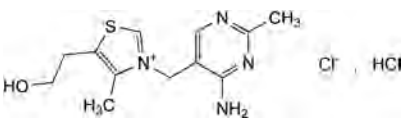

$C_{12}H_{18}Cl_2N_4OS$ $M_r$ 337.3
[67-03-8]

DEFINITION

3-[(4-Amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium chloride hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: freely soluble in water, soluble in glycerol, slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *thiamine hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *water R*, evaporate to dryness and record new spectra using the residues.

B. Dissolve about 20 mg in 10 mL of *water R*, add 1 mL of *dilute acetic acid R* and 1.6 mL of *1 M sodium hydroxide*, heat on a water-bath for 30 min and allow to cool. Add 5 mL of *dilute sodium hydroxide solution R*, 10 mL of *potassium ferricyanide solution R* and 10 mL of *butanol R* and shake vigorously for 2 min. The upper alcoholic layer shows an intense light-blue fluorescence, especially in ultraviolet light at 365 nm. Repeat the test using 0.9 mL of *1 M sodium hydroxide* and 0.1 g of *anhydrous sodium sulfite R* instead of 1.6 mL of *1 M sodium hydroxide*. Practically no fluorescence is seen.

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ or $GY_7$ (*2.2.2, Method II*).

Dilute 2.5 mL of solution S to 5 mL with *water R*.

**pH** (*2.2.3*): 2.7 to 3.3.

Dilute 2.5 mL of solution S to 10 mL with *water R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.35 g of the substance to be examined in 15.0 mL of a 5 per cent *V/V* solution of *glacial acetic acid R* and dilute to 100.0 mL with *water R*.

*Reference solution (a)*. Dissolve the contents of a vial of *thiamine for system suitability CRS* (containing impurities A, B and C) in 1 mL of a 0.75 per cent *V/V* solution of *glacial acetic acid R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 45 °C.

*Mobile phase*:

– *mobile phase A*: 3.764 g/L solution of *sodium hexanesulfonate R* adjusted to pH 3.1 with *phosphoric acid R*;
– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 27 | 90 → 70 | 10 → 30 |
| 27 - 35 | 70 → 50 | 30 → 50 |
| 35 - 42 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *thiamine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to thiamine (retention time = about 30 min): impurity A = about 0.3; impurity B = about 0.9; impurity C = about 1.2.

*System suitability*: reference solution (a):

– *resolution*: minimum 3.0 between the peaks due to impurity B and thiamine; minimum 2.0 between the peaks due to thiamine and impurity C.

*Calculation of percentage contents*:

– for each impurity, use the concentration of thiamine hydrochloride in reference solution (b).

*Limits*:

– *impurity B*: maximum 0.3 per cent;

– *impurities A, C*: for each impurity, maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.400 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

## ASSAY

Dissolve 0.110 g in 5 mL of *anhydrous formic acid R* and add 50 mL of *acetic anhydride R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*) and carrying out the titration within 2 min. Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 16.86 mg of $C_{12}H_{18}Cl_2N_4OS$.

## STORAGE

In a non-metallic container, protected from light.

## IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G, H.*

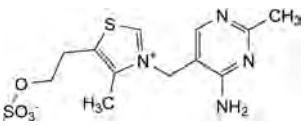

A. 2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethyl sulfate (thiamine sulfate ester),

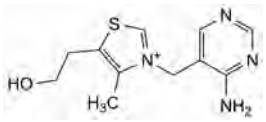

B. 3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium (desmethylthiamine),

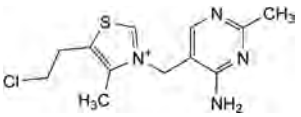

C. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-chloroethyl)-4-methyl-1,3-thiazol-3-ium (chlorothiamine),

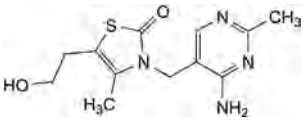

D. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2(3*H*)-one (oxothiamine),

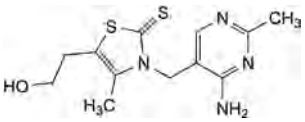

E. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2(3*H*)-thione (thioxothiamine),

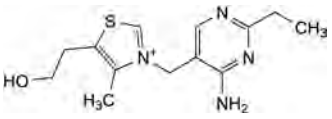

F. 3-[(4-amino-2-ethylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium (ethylthiamine),

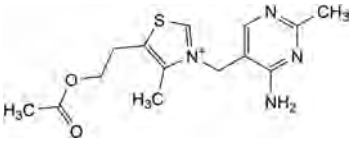

G. 5-[2-(acetyloxy)ethyl]-3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium (acetylthiamine),

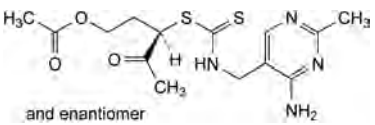


H. (3*RS*)-3-[[[(4-amino-2-methylpyrimidin-5-yl)methyl]thiocarbamoyl]sulfanyl]-4-oxopentyl acetate (ketodithiocarbamate).

01/2017:0531
corrected 10.0

# THIAMINE NITRATE

## Thiamini nitras

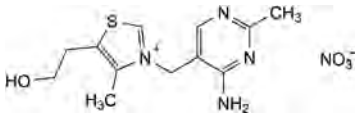

$C_{12}H_{17}N_5O_4S$ $M_r$ 327.4
[532-43-4]

DEFINITION

3-[(4-Amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium nitrate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or small, colourless crystals.

*Solubility*: sparingly soluble in water, freely soluble in boiling water, slightly soluble in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *thiamine nitrate CRS*.

B. Dissolve about 20 mg in 10 mL of *water R*, add 1 mL of *dilute acetic acid R* and 1.6 mL of *1 M sodium hydroxide*, heat on a water-bath for 30 min and allow to cool. Add 5 mL of *dilute sodium hydroxide solution R*, 10 mL of *potassium ferricyanide solution R* and 10 mL of *butanol R* and shake vigorously for 2 min. The upper alcoholic layer shows an intense light-blue fluorescence, especially in ultraviolet light at 365 nm. Repeat the test using 0.9 mL of *1 M sodium hydroxide* and 0.2 g of *sodium sulfite heptahydrate R* instead of 1.6 mL of *1 M sodium hydroxide*. Practically no fluorescence is produced.

C. About 5 mg gives the reaction of nitrates (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.8 to 7.6 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.35 g of the substance to be examined in 15.0 mL of a 5 per cent *V/V* solution of *glacial acetic acid R* and dilute to 100.0 mL with *water R*.

*Reference solution (a)*. Dissolve the contents of a vial of *thiamine for system suitability CRS* (containing impurities A, B and C) in 1 mL of a 0.75 per cent *V/V* solution of *glacial acetic acid R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:
– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 45 °C.

*Mobile phase*:
– *mobile phase A*: 3.764 g/L solution of *sodium hexanesulfonate R* adjusted to pH 3.1 with *phosphoric acid R*;
– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 90 | 10 |
| 2 - 27 | 90 → 70 | 10 → 30 |
| 27 - 35 | 70 → 50 | 30 → 50 |
| 35 - 42 | 50 | 50 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *thiamine for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to thiamine (retention time = about 30 min): impurity A = about 0.3; impurity B = about 0.9; impurity C = about 1.2.

*System suitability*: reference solution (a):
– *resolution*: minimum 3.0 between the peaks due to impurity B and thiamine; minimum 2.0 between the peaks due to thiamine and impurity C.

*Calculation of percentage contents*:
– for each impurity, use the concentration of thiamine nitrate in reference solution (b).

*Limits*:
– *impurity B*: maximum 0.6 per cent;
– *impurity C*: maximum 0.4 per cent;
– *impurity A*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 1.0 per cent;
– *reporting threshold*: 0.05 per cent; disregard the peak due to the nitrate ion at the beginning of the chromatogram.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 5 mL of *anhydrous formic acid R* and add 50 mL of *acetic anhydride R*. Titrate immediately with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*) and carrying out the titration within 2 min. Carry out a blank titration.

1.0 mL of *0.1 M perchloric acid* is equivalent to 16.37 mg of $C_{12}H_{17}N_5O_4S$.

STORAGE

In a non-metallic container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use*

*(2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, F, G, H.*

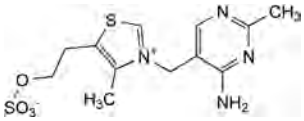

A. 2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethyl sulfate (thiamine sulfate ester),

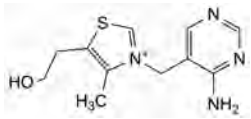

B. 3-[(4-aminopyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium (desmethylthiamine),

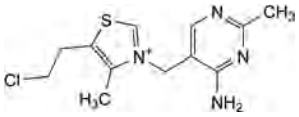

C. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-chloroethyl)-4-methyl-1,3-thiazol-3-ium (chlorothiamine),

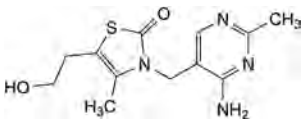

D. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2(3*H*)-one (oxothiamine),

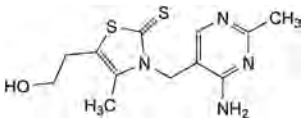

E. 3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-2(3*H*)-thione (thioxothiamine),

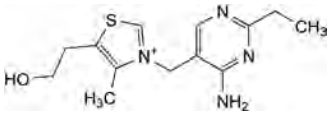

F. 3-[(4-amino-2-ethylpyrimidin-5-yl)methyl]-5-(2-hydroxyethyl)-4-methyl-1,3-thiazol-3-ium (ethylthiamine),

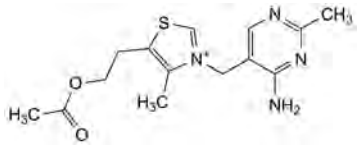

G. 5-[2-(acetyloxy)ethyl]-3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium (acetylthiamine),

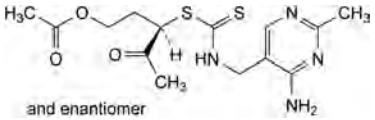


H. (3*RS*)-3-[[[(4-amino-2-methylpyrimidin-5-yl)methyl]thiocarbamoyl]sulfanyl]-4-oxopentyl acetate (ketodithiocarbamate).

**01/2017:0109**

# THIAMPHENICOL

## Thiamphenicolum

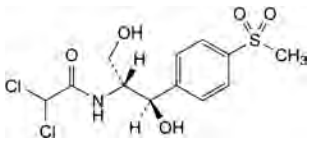

$C_{12}H_{15}Cl_2NO_5S$ $M_r$ 356.2
[15318-45-3]

DEFINITION

2,2-Dichloro-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)-2-[4-(methylsulfonyl)phenyl]ethyl]acetamide.

*Content*: 98.0 per cent to 100.5 per cent (dried substance).

CHARACTERS

*Appearance*: fine, white or yellowish-white, crystalline powder or crystals.

*Solubility*: slightly soluble in water, very soluble in dimethylacetamide, freely soluble in acetonitrile and in dimethylformamide, soluble in methanol, sparingly soluble in acetone and in anhydrous ethanol, slightly soluble in ethyl acetate.

A solution in anhydrous ethanol is dextrorotatory and a solution in dimethylformamide is laevorotatory.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dry the substance to be examined and the reference substance at 100-105 °C for 2 h; examine as discs of *potassium bromide R*.

*Comparison*: *thiamphenicol CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 0.1 g of *thiamphenicol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *silica gel* $GF_{254}$ *R* as the coating substance.

*Mobile phase*: *methanol R*, *ethyl acetate R* (3:97 *V/V*).

*Application*: 5 µL.

*Development*: over a path of 10 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the spot in the chromatogram obtained with the reference solution.

C. To 50 mg in a porcelain crucible add 0.5 g of *anhydrous sodium carbonate R*. Heat over an open flame for 10 min. Allow to cool. Take up the residue with 5 mL of *dilute nitric acid R* and filter. To 1 mL of the filtrate add 1 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Acidity or alkalinity**. Shake 0.1 g with 20 mL of *carbon dioxide-free water R* and add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.1 mL of *0.02 M hydrochloric acid* or *0.02 M sodium hydroxide* is required to change the colour of the indicator.

Monographs T

**Specific optical rotation** (*2.2.7*): – 24 to – 21 (dried substance).

Dissolve 1.25 g in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

**Melting point** (*2.2.14*): 163 °C to 167 °C.

**Absorbance** (*2.2.25*).

*Test solution (a).* Dissolve 20 mg in *water R*, heating to about 40 °C, and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 2.5 mL of test solution (a) to 50.0 mL with *water R*.

*Spectral range*: 240-300 nm for test solution (a); 200-240 nm for test solution (b).

*Absorption maxima*: at 266 nm and 273 nm for test solution (a); at 224 nm for test solution (b).

*Specific absorbances at the absorption maxima*:

– at 266 nm: 25 to 28 for test solution (a);
– at 273 nm: 21.5 to 23.5 for test solution (a);
– at 224 nm: 370 to 400 for test solution (b).

**Chlorides** (*2.4.4*): maximum 200 ppm.

Shake 0.5 g with 30 mL of *water R* for 5 min and filter.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.300 g in 30 mL of *ethanol (96 per cent) R*, add 20 mL of a 500 g/L solution of *potassium hydroxide R*, mix and heat under a reflux condenser for 4 h. Cool, add 100 mL of *water R*, neutralise with *dilute nitric acid R* and add 5 mL of the same acid in excess. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank test.

1 mL of *0.1 M silver nitrate* is equivalent to 17.81 mg of $C_{12}H_{15}Cl_2NO_5S$.

STORAGE

In an airtight container, protected from light.

**04/2016:2896**
**corrected 10.0**

# THIOCOLCHICOSIDE CRYSTALLISED FROM ETHANOL

## Thiocolchicosidum ex ethanolo cristallisatum

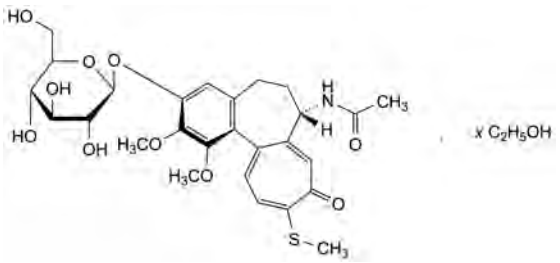


$C_{27}H_{33}NO_{10}S,xC_2H_6O$ $M_r$ 563.6 (ethanol-free substance)
Anhydrous thiocolchicoside: [602-41-5]

DEFINITION

*N*-[(7*S*,12a*R*$_a$)-3-(β-D-Glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of ethanol.

CHARACTERS

*Appearance*: yellow crystalline powder, hygroscopic.

*Solubility*: sparingly soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

mp: about 208 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *thiocolchicoside crystallised from ethanol CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.100 g in *methanol R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 600 to – 570 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 100.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *methanol R*.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *thiocolchicoside for system suitability CRS* (containing impurities D, E, G, H, K and L) in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (c).* Dissolve 20.0 mg of *thiocolchicoside hydrate CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2.1 mm;
– *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 0.22 g of *ammonium formate R* in 350 mL of *water for chromatography R* and mix with 25 mL of *tetrahydrofuran R*;
– *mobile phase B*: dilute 45 µL of *anhydrous formic acid R* in 900 mL of *acetonitrile R* and mix with 100 mL of *tetrahydrofuran R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 99 | 1 |
| 2 - 6 | 99 → 92 | 1 → 8 |
| 6 - 7.5 | 92 → 70 | 8 → 30 |
| 7.5 - 8.8 | 70 → 50 | 30 → 50 |
| 8.8 - 9.2 | 50 → 2 | 50 → 98 |
| 9.2 - 10 | 2 | 98 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 370 nm.

*Injection*: 1.0 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *thiocolchicoside for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities D, E, H and L.

*Relative retention* with reference to thiocolchicoside (retention time = about 6 min): impurity D = about 0.2; impurity H = about 0.4; impurity E = about 1.05; impurity L = about 1.11.

*System suitability*:

- *signal-to-noise ratio*: minimum 50 for the principal peak in the chromatogram obtained with reference solution (a);
- *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to thiocolchicoside in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

- *correction factor*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.7; impurity H = 2.8;
- for each impurity, use the concentration of the substance to be examined in reference solution (a).

*Limits*:

- *impurity D*: maximum 0.4 per cent;
- *impurities E, H*: for each impurity, maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent; disregard the peak due to impurity L.

**Ethanol** (*2.4.24*): maximum 1.5 per cent.

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{33}NO_{10}S$ taking into account the assigned content of *thiocolchicoside hydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, E, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, G, J, K, L.*

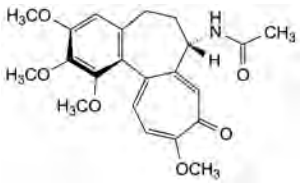

A. *N*-[(7*S*,12a$R_a$)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicine),

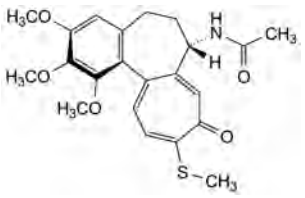

B. *N*-[(7*S*,12a$R_a$)-1,2,3-trimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (thiocolchicine),

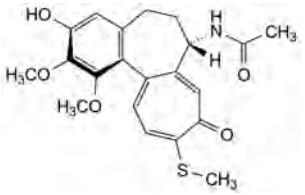

C. *N*-[(7*S*,12a$R_a$)-3-hydroxy-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (3-*O*-demethylthiocolchicine),

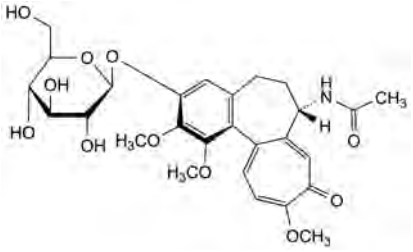

D. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicoside),

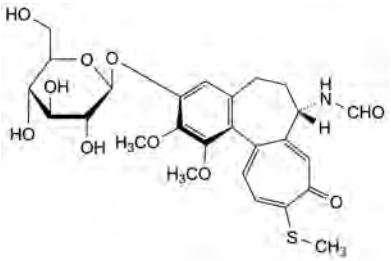

E. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]formamide (*N*-deacetyl-*N*-formylthiocolchicoside),

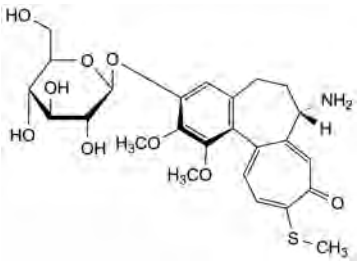

G. (7*S*,12a$R_a$)-7-amino-3(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-6,7-dihydrobenzo-[*a*]heptalen-9(5*H*)-one (*N*-deacetylthiocolchicoside),

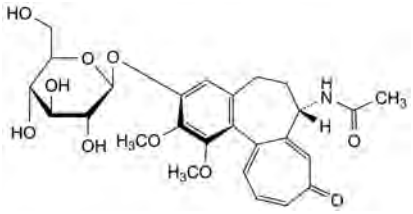

H. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (10-demethoxycolchicoside),

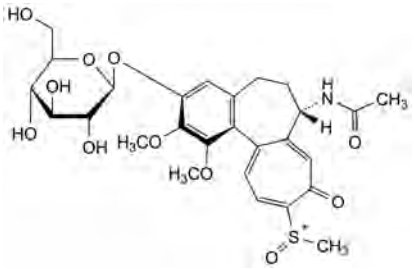

J. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfinyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (thiocolchicoside *S*-oxide),

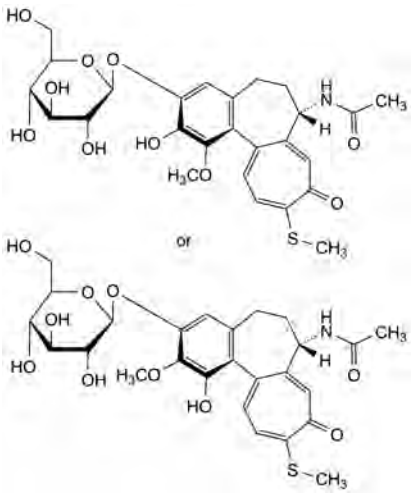

K. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-2-hydroxy-1-methoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide or *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1-hydroxy-2-methoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide,

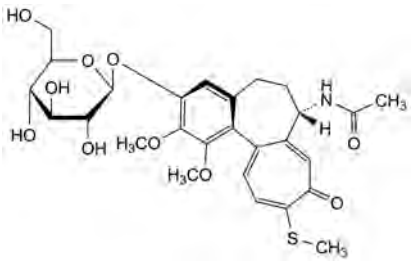

L. *N*-[(7*S*,12a$S_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (12a$S_a$-thiocolchicoside).

**04/2016:2814**
**corrected 10.0**

# THIOCOLCHICOSIDE HYDRATE

## Thiocolchicosidum hydricum

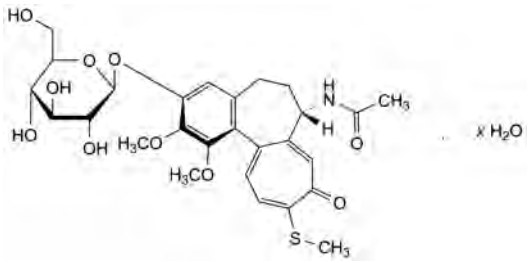

$C_{27}H_{33}NO_{10}S,xH_2O$ $M_r$ 563.6 (anhydrous substance)
Anhydrous thiocolchicoside: [602-41-5]

DEFINITION

*N*-[(7*S*,12a$R_a$)-3-(β-D-Glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: yellow crystalline powder, slightly hygroscopic.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: about 272 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *thiocolchicoside hydrate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.100 g in *methanol R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): − 600 to − 570 (anhydrous substance).

Dissolve 0.500 g in *water R* and dilute to 100.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution (a).* Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 20.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *methanol R*.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *thiocolchicoside for system suitability CRS* (containing impurities D, E, G, H, K and L) in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (c).* Dissolve 20.0 mg of *thiocolchicoside hydrate CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.10 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped ethylene-bridged phenylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);

– *temperature*: 25 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 0.22 g of *ammonium formate R* in 350 mL of *water for chromatography R* and mix with 25 mL of *tetrahydrofuran R*;

– *mobile phase B*: dilute 45 µL of *anhydrous formic acid R* in 900 mL of *acetonitrile R* and mix with 100 mL of *tetrahydrofuran R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 99 | 1 |
| 2 - 6 | 99 → 92 | 1 → 8 |
| 6 - 7.5 | 92 → 70 | 8 → 30 |
| 7.5 - 8.8 | 70 → 50 | 30 → 50 |
| 8.8 - 9.2 | 50 → 2 | 50 → 98 |
| 9.2 - 10 | 2 | 98 |

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 370 nm.

*Injection*: 1.0 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *thiocolchicoside for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities D, E, G, H, K and L.

*Relative retention* with reference to thiocolchicoside (retention time = about 6 min): impurity D = about 0.2; impurity H = about 0.4; impurity G = about 0.7; impurity K = about 0.8; impurity E = about 1.05; impurity L = about 1.11.

*System suitability*:

– *signal-to-noise ratio*: minimum 50 for the principal peak in the chromatogram obtained with reference solution (a);

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to thiocolchicoside in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.7; impurity H = 2.8;

– for each impurity, use the concentration of thiocolchicoside hydrate in reference solution (a).

*Limits*:

– *impurity E*: maximum 1.0 per cent;

– *impurity H*: maximum 0.7 per cent;

– *impurity K*: maximum 0.3 per cent;

– *impurities D, G*: for each impurity, maximum 0.2 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 2.5 per cent;

– *reporting threshold*: 0.05 per cent; disregard the peak due to impurity L.

**Water** (*2.5.12*): maximum 2.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (c).

Calculate the percentage content of $C_{27}H_{33}NO_{10}S$ taking into account the assigned content of *thiocolchicoside hydrate CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, E, G, H, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, J, L.*

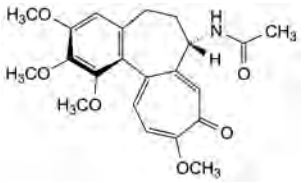

A. *N*-[(7*S*,12a$R_a$)-1,2,3,10-tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicine),

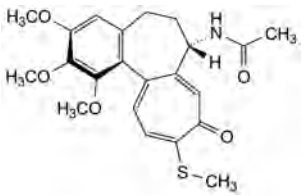

B. *N*-[(7*S*,12a$R_a$)-1,2,3-trimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (thiocolchicine),

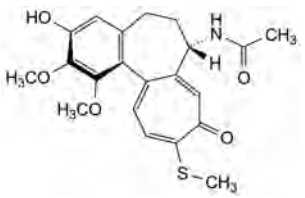

C. *N*-[(7*S*,12a$R_a$)-3-hydroxy-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (3-*O*-demethylthiocolchicine),

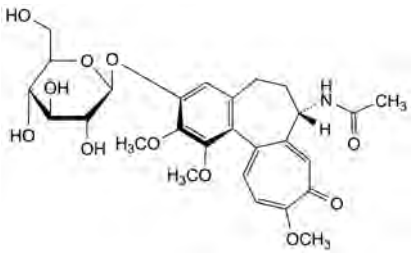

D. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2,10-trimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (colchicoside),

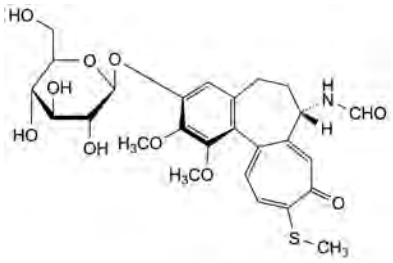

E. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]formamide (*N*-deacetyl-*N*-formylthiocolchicoside),

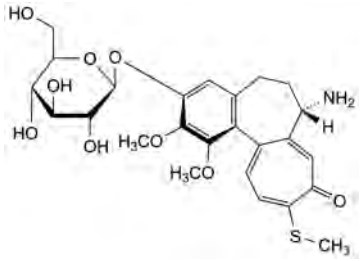

G. (7*S*,12a$R_a$)-7-amino-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-6,7-dihydrobenzo[*a*]-heptalen-9(5*H*)-one (*N*-deacetylthiocolchicoside),

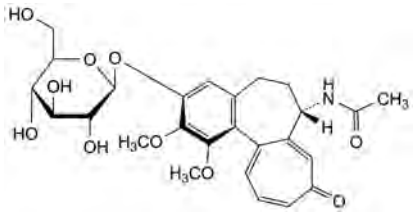

H. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (10-demethoxycolchicoside),

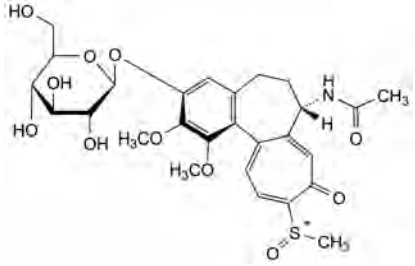

J. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfinyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (thiocolchicoside *S*-oxide),

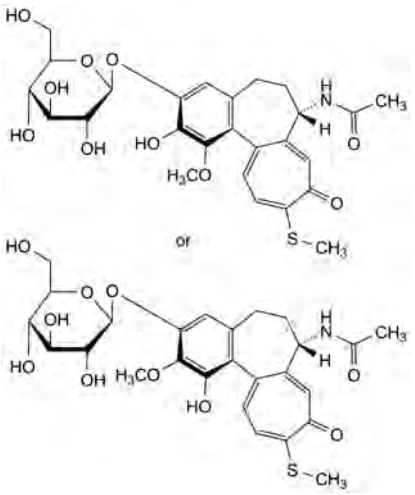

K. *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-2-hydroxy-1-methoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide or *N*-[(7*S*,12a$R_a$)-3-(β-D-glucopyranosyloxy)-1-hydroxy-2-methoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide,

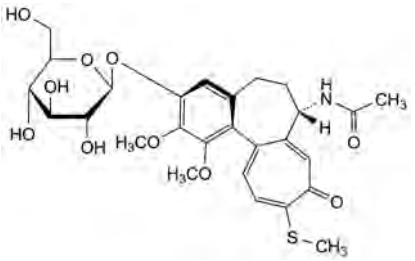

L. *N*-[(7*S*,12a$S_a$)-3-(β-D-glucopyranosyloxy)-1,2-dimethoxy-10-(methylsulfanyl)-9-oxo-5,6,7,9-tetrahydrobenzo[*a*]heptalen-7-yl]acetamide (12a$S_a$-thiocolchicoside).

**01/2008:1648**

# THIOCTIC ACID

## Acidum thiocticum

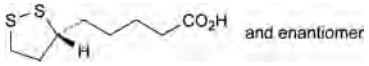

$C_8H_{14}O_2S_2$ $M_r$ 206.3
[1077-28-7]

DEFINITION

5-[(3*RS*)-1,2-Dithiolan-3-yl]pentanoic acid.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: very slightly soluble in water, very soluble in dimethylformamide, freely soluble in methanol.

mp: about 61 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *thioctic acid CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Dissolve 0.50 g in a 20 g/L solution of *sodium hydroxide R* and dilute to 10 mL with the same solution.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in *dimethylformamide R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *thioctic acid containing impurity B CRS* in *dimethylformamide R* and dilute to 1.0 mL with the same solvent (1.0 per cent impurity B solution).

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: 25 per cent *V/V* solution of *ammonia R*, *water R*, *ethyl acetate R*, *propanol R* (5:10:40:40 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 50 °C for 20 min.

*Detection*: expose to iodine vapour for 30 min or until the spots appear.

*System suitability*: reference solution:

– the chromatogram shows 2 clearly separated principal spots due to impurity B ($R_F$ = 0.0) and thioctic acid ($R_F$ = about 0.3).

*Limit*:

– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (1.0 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Solvent mixture*: a mixture of equal volumes of *acetonitrile R1* and a 0.7 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.6 with *phosphoric acid R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *thioctic acid for system suitability CRS* (containing impurity A) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 50.0 mg of *thioctic acid CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Blank solution.* Solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 35 °C.

*Mobile phase*: mix 8 volumes of *acetonitrile R1*, 41 volumes of a 0.7 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*, and 51 volumes of *methanol R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of thioctic acid.

*Relative retention* with reference to thioctic acid (retention time = about 6 min): impurity A = about 2.2.

*System suitability*:

– *resolution*: minimum 6.0 between the peaks due to thioctic acid and impurity A in the chromatogram obtained with reference solution (a);
– *symmetry factor*: maximum 2.0 for the peak due to thioctic acid in the chromatogram obtained with reference solution (c).

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.6;
– *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.2 per cent, determined on 1.000 g by drying *in vacuo* at 40 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_8H_{14}O_2S_2$ from the peak areas and the declared content of *thioctic acid CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

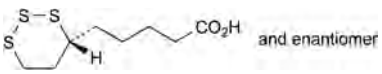


A. 5-[(4*RS*)-1,2,3-trithian-4-yl]pentanoic acid,

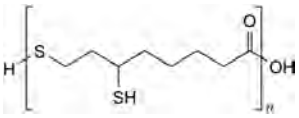


B. α-hydro-ω-hydroxypoly[sulfanediyl(3-sulfanyl-8-oxooctane-1,8-diyl)] (mixture of thioctic acid polymers).

**01/2008:1625**
**corrected 10.0**

# THIOMERSAL

## Thiomersalum

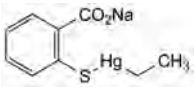


$C_9H_9HgNaO_2S$ $M_r$ 404.8

[54-64-8]

DEFINITION

Sodium ethyl[2-sulfanylbenzoato(2-)-*O*,*S*]mercurate(1-).

*Content*: 97.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, sparingly soluble or soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 103 °C to 115 °C.
Dissolve 0.5 g in *water R* and dilute to 10 mL with the same solvent. Add 2 mL of *dilute hydrochloric acid R*. A white precipitate is formed. Wash the precipitate with *water R* and dry *in vacuo* (*2.2.32*) at a pressure not exceeding 0.7 kPa.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *thiomersal CRS*.

C. Treat 50 mg by the oxygen-flask method (*2.5.10*). Use a mixture of 1 mL of *strong hydrogen peroxide solution R* and 50 mL of *water R* to absorb the combustion products. To the solution add 5 mL of *dilute nitric acid R*. 0.1 mL of this solution gives reaction (a) of mercury (*2.3.1*). To the remaining part of the solution add 10 mL of *dilute hydrochloric acid R* and filter. 5 mL of the filtrate, without further addition of acid, gives reaction (a) of sulfates (*2.3.1*).

D. Solution S (see Tests) gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 6.0 to 8.0.

Dilute 5 mL of solution S to 50 mL with *carbon dioxide-free water R*.

**Inorganic mercury compounds**: maximum 0.70 per cent.

*Protect the solutions from light throughout the procedure.*

*Test solution.* Dissolve 25 mg of the substance to be examined in *water R* and dilute to 25.0 mL with the same solvent.

*Reference solution.* Dissolve 95.0 mg of *mercuric chloride R* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

*Test, reference and blank preparations.* Label five 10 mL volumetric flasks A, B, C, D and E. Place 5 mL of the test solution in flasks A, B, C and D. To each of the flasks C and D add 0.5 mL of the reference solution. Dilute the contents of flasks A and C to 10 mL with *water R* (blank preparations A and C). Dilute the contents of flasks B and D to 10 mL with a freshly prepared 332 g/L solution of *potassium iodide R* (test preparation B and reference preparation D). Place 5 mL of a 332 g/L solution of *potassium iodide R* in flask E. Dilute to 10 mL with *water R* (blank preparation E).

Measure the absorbance (*2.2.25*) of each solution ($A_a$, $A_b$, $A_c$, $A_d$ and $A_e$) at 323 nm using *water R* as the compensation liquid. Calculate the content of inorganic mercury compounds, expressed as Hg from the expression:

$$\frac{(A_b - A_a - A_e) \times m_R \times 0.1847}{(A_d - A_c - A_b + A_a) \times m_T}$$

$m_R$ = mass of mercuric chloride in the reference solution in milligrams,

$m_T$ = mass of the substance to be examined in milligrams.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in a desiccator at a pressure not exceeding 0.7 kPa for 24 h.

ASSAY

Place 0.5 g in a 100 mL long-necked combustion flask, add 5 mL of *sulfuric acid R* and heat gently until charring occurs, continue to heat and add dropwise *strong hydrogen peroxide solution R* until the mixture is colourless. Dilute with *water R*, evaporate until slight fuming occurs, dilute to 10 mL with *water R*, cool down and titrate with *0.1 M ammonium thiocyanate* using *ferric ammonium sulfate solution R2* as indicator.

1 mL of *0.1 M ammonium thiocyanate* is equivalent to 20.24 mg of $C_9H_9HgNaO_2S$.

STORAGE

Protected from light.

**07/2012:0212**

# THIOPENTAL SODIUM AND SODIUM CARBONATE

## Thiopentalum natricum et natrii carbonas

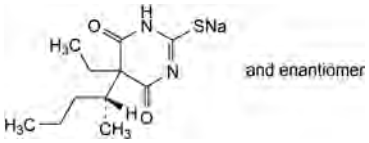


$C_{11}H_{17}N_2NaO_2S$ $M_r$ 264.3

DEFINITION

Mixture of sodium 5-ethyl-5-[(1*RS*)-1-methylbutyl]-4,6-dioxo-1,4,5,6-tetrahydropyrimidine-2-thiolate and anhydrous sodium carbonate.

*Content*:

– *thiopental*: 84.0 per cent to 87.0 per cent (dried substance);
– *sodium*: 10.2 per cent to 11.2 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish-white, hygroscopic powder.

*Solubility*: freely soluble in water, partly soluble in anhydrous ethanol.

IDENTIFICATION

*First identification: A, B, E.*

*Second identification: A, C, D, E.*

A. Acidify 10 mL of solution S (see Tests) with *dilute hydrochloric acid R*. An effervescence is produced. Shake with 20 mL of *1,1-dimethylethyl methyl ether R*. Separate the upper layer, wash with 10 mL of *water R*, dry over *anhydrous sodium sulfate R* and filter. Evaporate the filtrate to dryness and dry the residue at 100-105 °C. Determine the melting point (*2.2.14*) of the residue. Mix equal parts of the residue and *thiopental CRS* and determine the melting point of the mixture. The difference between the melting points (which are about 160 °C) is not greater than 2 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: use the residue obtained in Identification test A.
*Comparison*: *thiopental CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 0.1 g of the substance to be examined in *water R* and dilute to 100 mL with the same solvent.
*Reference solution.* Dissolve 85 mg of *thiopental CRS* in 10 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*.
*Plate*: *TLC silica gel $GF_{254}$ plate R*.
*Mobile phase*: *concentrated ammonia R*, *ethanol (96 per cent) R*, *methylene chloride R* (5:15:80 *V/V/V*); use the lower layer.
*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Detection*: examine immediately in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives the reaction of non-nitrogen substituted barbiturates (*2.3.1*).

E. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $GY_3$ (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 5.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 2 mg of *thiopental for system suitability CRS* (containing impurities A, B, C and D) in the mobile phase and dilute to 2.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, 1 g/L solution of *phosphoric acid R* (35:65 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of thiopental.

*Identification of impurities*: use the chromatogram supplied with *thiopental for system suitability CRS* (containing impurities A, B, C and D) and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and D.

*Relative retention* with reference to thiopental (retention time = about 20 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.9; impurity D = about 1.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities A and B; minimum 1.5 between the peaks due to impurity C and thiopental.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 1.5;

– *impurity C*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent);

– *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);

– *impurity D*: not more than 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (5.0 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 330 ppm.

To 5 mL of solution S add 35 mL of *water R* and 10 mL of *dilute nitric acid R*. Shake with 3 quantities, each of 25 mL, of *1,1-dimethylethyl methyl ether R* and discard the upper layer. Eliminate the organic solvent from the lower layer by heating on a water-bath. 15 mL of the solution complies with the test for chlorides.

**Loss on drying** (*2.2.32*): maximum 2.5 per cent, determined on 0.500 g by drying *in vacuo* at 100 °C for 4 h.

ASSAY

**Sodium**. Dissolve 0.400 g in 30 mL of *water R*. Add 0.1 mL of *methyl red solution R* and titrate with *0.1 M hydrochloric acid* until a red colour is obtained. Boil gently for 2 min. Allow to cool and, if necessary, continue the titration with *0.1 M hydrochloric acid* until the red colour is again obtained.

1 mL of *0.1 M hydrochloric acid* is equivalent to 2.299 mg of Na.

**Thiopental**. Dissolve 0.150 g in 5 mL of *water R*. Add 2 mL of *dilute sulfuric acid R* and shake with 4 quantities, each of 10 mL, of *chloroform R*. Combine the chloroform layers, filter and evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 30 mL of previously neutralised *dimethylformamide R* and add 0.1 mL of a 2 g/L solution of *thymol blue R* in *methanol R*. Titrate immediately with *0.1 M lithium methoxide* until a blue colour is obtained. Protect the solution from atmospheric carbon dioxide during the titration.

1 mL of *0.1 M lithium methoxide* is equivalent to 24.23 mg of $C_{11}H_{18}N_2O_2S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *B, C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

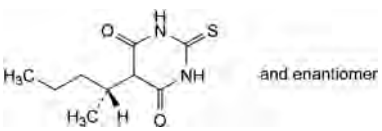


A. 5-[(1*RS*)-1-methylbutyl]-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

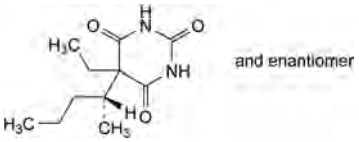


B. 5-ethyl-5-[(1*RS*)-1-methylbutyl]pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione,

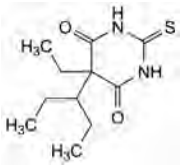

C. 5-ethyl-5-(1-ethylpropyl)-2-thioxo-2,3-dihydropyrimidine-4,6(1*H*,5*H*)-dione,

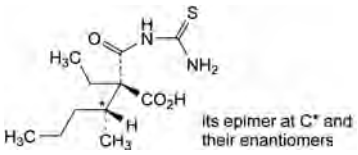


D. mixture of (2*RS*,3*RS*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid and (2*RS*,3*SR*)-2-(carbamothioylcarbamoyl)-2-ethyl-3-methylhexanoic acid.

**01/2017:2005**

# THIORIDAZINE

## Thioridazinum

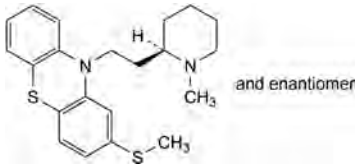


$C_{21}H_{26}N_2S_2$ $M_r$ 370.6
[50-52-2]

DEFINITION

10-[2-[(2*RS*)-1-Methylpiperidin-2-yl]ethyl]-2-(methylsulfanyl)-10*H*-phenothiazine

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, freely soluble in methanol, soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *thioridazine CRS*.

TESTS

**Solution S**. Dissolve 1.25 g in *methanol R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test as quickly as possible and protected from light.*

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 100 mL with the same solvent.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b).* Dissolve the contents of a vial of *thioridazine for system suitability CRS* (containing impurities A, B, C, D and E) in 1.0 mL of *methanol R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* resistant to bases up to pH 11.

*Mobile phase*:
- *mobile phase A*: *triethylamine R1*, *acetonitrile R*, *water R* (2:400:600 *V/V/V*);
- *mobile phase B*: *triethylamine R1*, *acetonitrile R* (2:1000 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 35 | 100 → 5 | 0 → 95 |
| 35 - 40 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *thioridazine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to thioridazine (retention time = about 30 min): impurity D = about 0.1; impurity A = about 0.3; impurity C = about 0.4; impurity B = about 0.5; impurity E = about 0.6.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.5 between the peaks due to impurities C and B.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity B = 2.4; impurity C = 0.5; impurity D = 1.5;
- *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g *in vacuo* at 50 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 37.06 mg of $C_{21}H_{26}N_2S_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or

by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F.*

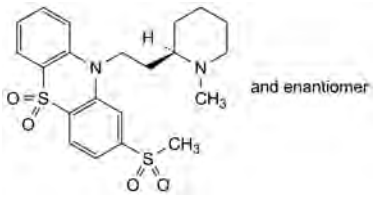


A. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfonyl)-10*H*-phenothiazine 5,5-dioxide,

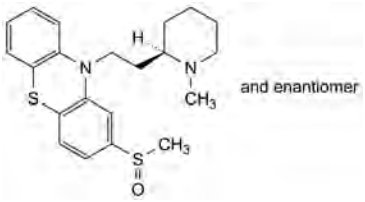


B. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10*H*-phenothiazine (mesoridazine),

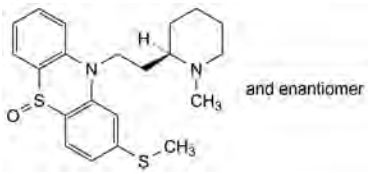


C. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfanyl)-10*H*-phenothiazine 5-oxide,

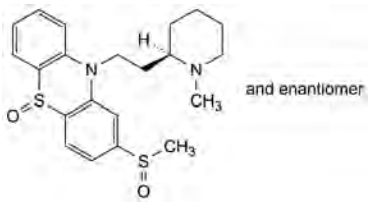


D. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10*H*-phenothiazine 5-oxide,

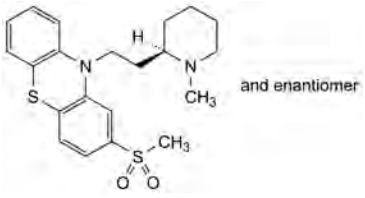


E. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfonyl)-10*H*-phenothiazine (sulforidazine),

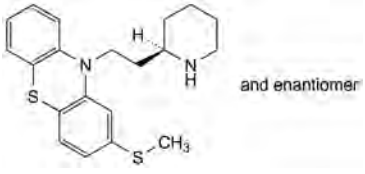


F. 2-(methylsulfanyl)-10-[2-[(2*RS*)-piperidin-2-yl]ethyl]-10*H*-phenothiazine (northioridazine).

**01/2017:0586**

# THIORIDAZINE HYDROCHLORIDE

## Thioridazini hydrochloridum

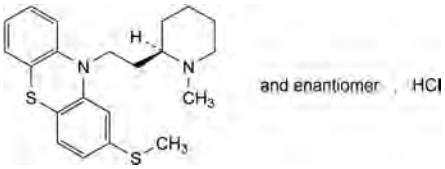


$C_{21}H_{27}ClN_2S_2$ $M_r$ 407.0
[130-61-0]

DEFINITION

10-[2-[(2*RS*)-1-Methylpiperidin-2-yl]ethyl]-2-(methylsulfanyl)-10*H*-phenothiazine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, soluble in ethanol 96 per cent.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *thioridazine hydrochloride CRS*.

B. 0.2 g gives reaction (b) of chlorides (*2.3.1*).

TESTS

*Carry out all operations protected from light.*

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 6 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 20 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°.

Dissolve 1.0 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test as quickly as possible and protected from light.*

*Test solution*. Dissolve 20.0 mg of the substance to be examined in *methanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dilute 5.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 2.0 mL of this solution to 100.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *thioridazine for system suitability CRS* (containing impurities A, B, C, D and E) in 1.0 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) resistant to bases up to pH 11.

*Mobile phase*:

- *mobile phase A*: *triethylamine R1*, *acetonitrile R*, *water R* (2:400:600 *V/V/V*);
- *mobile phase B*: *triethylamine R1*, *acetonitrile R* (2:1000 *V/V*);

Monographs T

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 100 | 0 |
| 5 - 35 | 100 → 5 | 0 → 95 |
| 35 - 40 | 5 | 95 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 25 µL.

*Identification of impurities*: use the chromatogram supplied with *thioridazine for system suitability CRS* to identify the peaks due to impurities A, B, C, D and E .

*Relative retention* with reference to thioridazine (retention time = about 30 min): impurity D = about 0.1; impurity A = about 0.3; impurity C = about 0.4; impurity B = about 0.5; impurity E = about 0.6; impurity F = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to impurities C and B.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity B = 2.4; impurity C = 0.5; impurity D = 1.5;

– *impurities A, B, C, D, E*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *anhydrous acetic acid R* and 60 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 40.70 mg of $C_{21}H_{27}ClN_2S_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *F*.

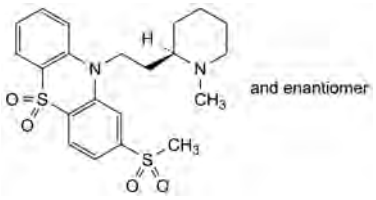


A. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfonyl)-10*H*-phenothiazine 5,5-dioxide,

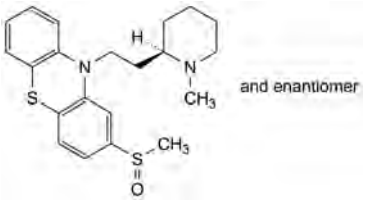


B. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10*H*-phenothiazine,

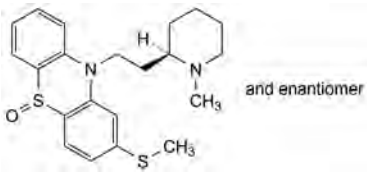


C. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfanyl)-10*H*-phenothiazine 5-oxide,

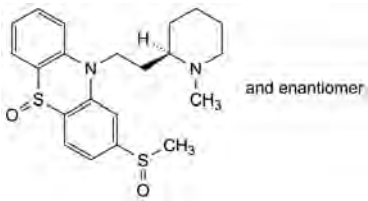


D. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfinyl)-10*H*-phenothiazine 5-oxide,

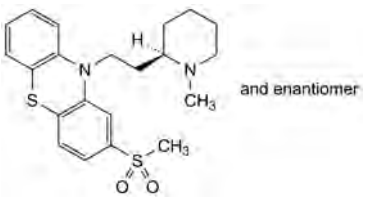


E. 10-[2-[(2*RS*)-1-methylpiperidin-2-yl]ethyl]-2-(methylsulfonyl)-10*H*-phenothiazine,

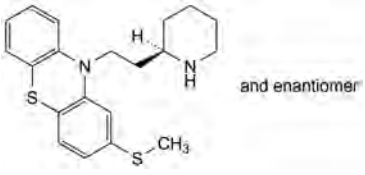


F. 2-(methylsulfanyl)-10-[2-[(2*RS*)-piperidin-2-yl]ethyl]-10*H*-phenothiazine.

**01/2017:1049**

# THREONINE

## Threoninum

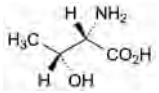

$C_4H_9NO_3$ $M_r$ 119.1
[72-19-5]

DEFINITION

(2*S*,3*R*)-2-Amino-3-hydroxybutanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *threonine CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 10 mg of the substance to be examined in a 1 per cent *V*/*V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Reference solution*. Dissolve 10 mg of *threonine CRS* in a 1 per cent *V*/*V* solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Plate*: *TLC silica gel plate R*.
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V*/*V*/*V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Mix 1 mL of a 2 g/L solution of the substance to be examined and 1 mL of a 20 g/L solution of *sodium periodate R*. Add 0.2 mL of *piperidine R* and 0.1 mL of a 25 g/L solution of *sodium nitroprusside R*. A blue colour develops that changes to yellow after a few minutes.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 6.5 for solution S.

**Specific optical rotation** (*2.2.7*): − 29.0 to − 27.6 (dried substance).

Dissolve 1.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* (impurity D) and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):
- *resolution*: minimum 1.5 between the peaks due to impurity D and leucine.

*Calculation of percentage contents*:
- for any ninhydrin-positive substance detected at 570 nm, use the concentration of threonine in reference solution (a);
- for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:
- *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:
- *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of

*methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 5 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 11.91 mg of $C_4H_9NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E.*

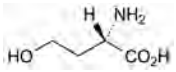

A. (2*S*)-2-amino-4-hydroxybutanoic acid (homoserine),

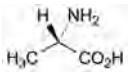

B. (2*S*)-2-aminopropanoic acid (alanine),

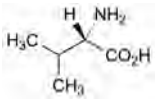

C. (2*S*)-2-amino-3-methylbutanoic acid (valine),

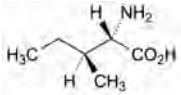

D. (2*S*,3*S*)-2-amino-3-methylpentanoic acid (isoleucine),

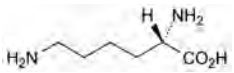

E. (2*S*)-2,6-diaminohexanoic acid (lysine).

**01/2008:0791**

# THYMOL

## Thymolum

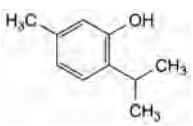

$C_{10}H_{14}O$ $M_r$ 150.2

[89-83-8]

DEFINITION

5-Methyl-2-(methylethyl)phenol.

CHARACTERS

*Appearance*: colourless crystals.

*Solubility*: very slightly soluble in water, very soluble in ethanol (96 per cent), freely soluble in essential oils and in fatty oils, sparingly soluble in glycerol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: *B.*

*Second identification*: *A, C, D.*

A. Melting point (*2.2.14*): 48 °C to 52 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *thymol CRS.*

C. Dissolve 0.2 g with heating in 2 mL of *dilute sodium hydroxide solution R* and add 0.2 mL of *chloroform R*. Heat on a water-bath. A violet colour develops.

D. Dissolve about 2 mg in 1 mL of *anhydrous acetic acid R*. Add 0.15 mL of *sulfuric acid R* and 0.05 mL of *nitric acid R*. A bluish-green colour develops.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension IV (*2.2.1*) and not more intensely coloured than reference solution $R_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in 10 mL of *dilute sodium hydroxide solution R*.

**Acidity**. To 1.0 g in a 100 mL glass-stoppered conical flask add 20 mL of *water R*. Boil until dissolution is complete, cool and stopper the flask. Shake vigorously for 1 min. Add a few crystals of the substance to be examined to initiate crystallisation. Shake vigorously for 1 min and filter. To 5 mL of the filtrate add 0.05 mL of *methyl red solution R* and 0.05 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1 mL of the test solution to 100 mL with *ethanol (96 per cent) R*.

*Reference solution (b)*. Dilute 1 mL of reference solution (a) to 10 mL with *ethanol (96 per cent) R*.

*Reference solution (c)*. Dilute 5 mL of reference solution (b) to 10 mL with *ethanol (96 per cent) R*.

*Column*:

- *material*: glass or steel;
- *size*: *l* = 4 m, Ø = 2 mm;
- *stationary phase*: *diatomaceous earth for gas chromatography R*, impregnated with a mixture suitable for the separation of free fatty acids.

*Carrier gas*: *nitrogen for chromatography R.*

*Flow rate*: 30 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 2 | 80 |
| | 2 - 22 | 80 → 240 |
| | 22 - 37 | 240 |
| Injection port | | 250 |
| Detector | | 300 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*System suitability*: reference solution (b):

- *signal-to-noise ratio*: minimum 5 for the principal peak.

*Limits*:

- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Residue on evaporation**: maximum 0.05 per cent.

Evaporate 2.00 g on a water-bath and heat in an oven at 100-105 °C for 1 h. The residue weighs not more than 1.0 mg.

STORAGE

Protected from light.

**01/2017:0866**

# TIABENDAZOLE

## Tiabendazolum

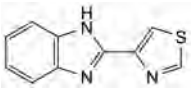

$C_{10}H_7N_3S$ $M_r$ 201.2
[148-79-8]

DEFINITION

Tiabendazole contains not less than 98.0 per cent and not more than the equivalent of 101.0 per cent of 2-(thiazol-4-yl)-1*H*-benzimidazole, calculated with reference to the anhydrous substance.

CHARACTERS

A white or almost white, crystalline powder, practically insoluble in water, slightly soluble in alcohol and in methylene chloride. It dissolves in dilute mineral acids. It melts at about 300 °C.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Dissolve 25 mg in *0.1 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 2.0 mL of the solution to 100.0 mL with *0.1 M hydrochloric acid*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows two absorption maxima, at 243 nm and 302 nm. The ratio of the absorbance measured at the maximum at 302 nm to that measured at the maximum at 243 nm is 1.8 to 2.1.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *tiabendazole CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. Dissolve about 5 mg in *0.1 M hydrochloric acid* and dilute to 5 mL with the same acid. Add 3 mg of *p-phenylenediamine dihydrochloride R* and shake until dissolved. Add 0.1 g of *zinc powder R*, mix, allow to stand for 2 min and add 5 mL of *ferric ammonium sulfate solution R2*. A bluish-violet colour develops.

TESTS

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $HF_{254}$ *R* as the coating substance.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Test solution (b).* Dilute 2 mL of test solution (a) to 20 mL with *methanol R*.

*Reference solution (a).* Dissolve 20 mg of *tiabendazole CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (b).* Dilute 1 mL of test solution (b) to 10 mL with *methanol R*.

*Reference solution (c).* Dilute 1 mL of test solution (b) to 25 mL with *methanol R*.

Apply separately to the plate 20 µL of each solution. Develop over a path of 15 cm using a mixture of 2.5 volumes of *water R*, 10 volumes of *acetone R*, 25 volumes of *glacial acetic acid R* and 62.5 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent) and at most one such spot is more intense than the spot in the chromatogram obtained with reference solution (c) (0.4 per cent).

***o*-Phenylenediamine**. To 5.0 g in a flask fitted with a ground-glass stopper, add 25 mL of a mixture of 1 volume of *methanol R* and 2 volumes of *water R*. Shake for 3 min. Filter through a sintered-glass filter (16) (*2.1.2*) under reduced pressure. To 10 mL of the filtrate add 0.5 mL of *hydrochloric acid R* and 0.5 mL of *acetylacetone R* and shake until the solution is clear. The solution is not more intensely coloured than reference solution $R_7$ (*2.2.2, Method I*) (10 ppm).

**Water** (*2.5.12*). Not more than 0.5 per cent, determined on 1.00 g by the semi-micro determination of water.

**Sulfated ash** (*2.4.14*). Not more than 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.12 mg of $C_{10}H_7N_3S$.

STORAGE

Store protected from light.

IMPURITIES

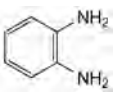

A. benzene-1,2-diamine.

Monographs T

01/2008:1660
corrected 6.5

# TIAMULIN FOR VETERINARY USE

## Tiamulinum ad usum veterinarium

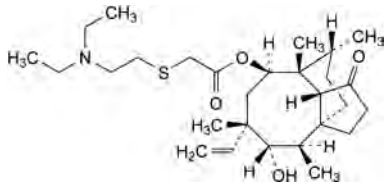

$C_{28}H_{47}NO_4S$ $M_r$ 493.8
[55297-95-5]

DEFINITION

(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-Ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl] sulfanyl]acetate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: sticky, translucent yellowish mass, slightly hygroscopic.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, freely soluble in anhydrous ethanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of tiamulin*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm is not greater than 0.050.

Dissolve 2.5 g in 50 mL of *methanol R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Ammonium carbonate buffer solution pH 10.0*. Dissolve 10.0 g of *ammonium carbonate R* in *water R*, add 22 mL of *perchloric acid solution R* and dilute to 1000.0 mL with *water R*. Adjust to pH 10.0 with *concentrated ammonia R1*.

*Solvent mixture*: *acetonitrile R1*, ammonium carbonate buffer solution pH 10.0 (50:50 *V/V*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 0.250 g of *tiamulin hydrogen fumarate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dilute 0.1 mL of *toluene R* to 100 mL with *acetonitrile R*. Dilute 0.1 mL of this solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, ammonium carbonate buffer solution pH 10.0, *methanol R1* (21:30:49 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 212 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of tiamulin.

*Relative retention* with reference to tiamulin (retention time = about 18 min): impurity A = about 0.22; impurity B = about 0.5; impurity C = about 0.66; impurity D = about 1.1; impurity F = about 1.6; impurity E = about 2.4.

*System suitability*: reference solution (a):

- baseline separation between the peaks due to tiamulin and impurity D.

*Limits*:

- *impurities A, B, C, D, E, F*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); disregard any peak present in the chromatogram obtained with reference solution (c).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 80 °C.

**Bacterial endotoxins** (*2.6.14, Method D*): less than 0.4 IU/mg, determined in a 1 mg/mL solution in *anhydrous ethanol R* (endotoxin free) diluted 1:40 with water for bacterial endotoxins test.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{28}H_{47}NO_4S$, from the declared content of *tiamulin hydrogen fumarate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, J, K, L, M, N, O, P, Q, R.*

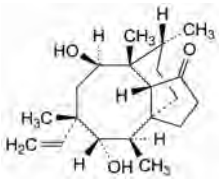

A. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5,8-dihydroxy-4,6,9,10-tetramethyloctahydro-3a,9-propano-3a*H*-cyclopentacycloocten-1(4*H*)-one (mutilin),

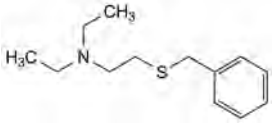

B. 2-(benzylsulfanyl)-*N*,*N*-diethylethanamine,

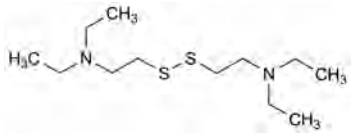

C. 2,2′-(disulfane-1,2-diyl)bis(*N*,*N*-diethylethanamine),

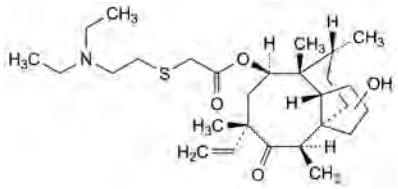

D. (3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenylhydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]-sulfanyl]acetate,

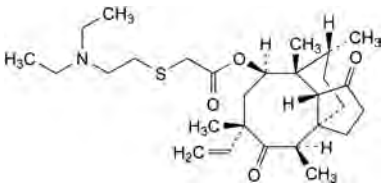

E. (3a*S*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1,5-dioxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino) ethyl]sulfanyl]acetate (11-oxotiamulin),

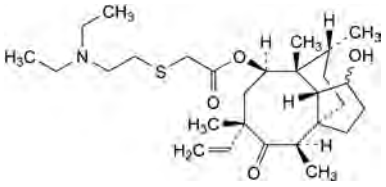

F. (1*RS*,3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-1-hydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]acetate (1-hydroxy-11-oxotiamulin),

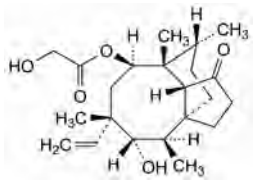

G. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl hydroxyacetate (pleuromutilin),

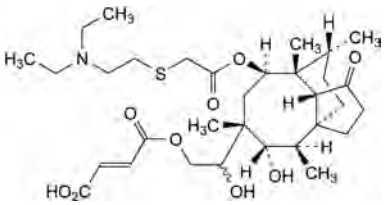

H. (2*E*)-4-[(2*RS*)-2-[(3a*S*,4*R*,5*S*,6*R*,8*R*,9*R*,9a*R*,10*R*)-8-[[[[2-(diethylamino)ethyl]sulfanyl]acetyl]oxy]-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-6-yl]-2-hydroxyethoxy]-4-oxobut-2-enoic acid (19,20-dihydroxytiamulin 20-fumarate),

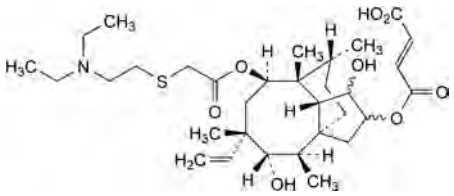

I. (2*E*)-4-[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-8-[[[[2-(diethylamino)ethyl]sulfanyl]acetyl]oxy]-6-ethenyl-1,5-dihydroxy-4,6,9,10-tetramethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-2-yl]oxy]-4-oxobut-2-enoic acid (2,3-dihydroxytiamulin 2-fumarate),

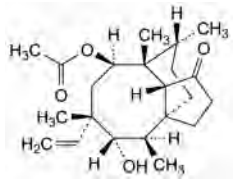

J. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl acetate (mutilin 14-acetate),

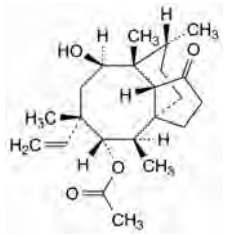

K. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-8-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5-yl acetate (mutilin 11-acetate),

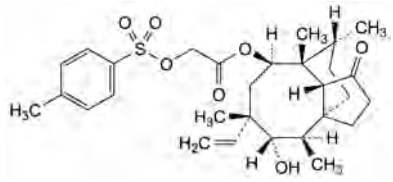

L. (3aS,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[(4-methylphenyl)sulfonyl]-oxy]acetate (pleuromutilin 22-tosylate),

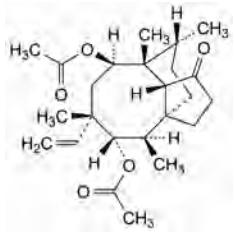

M. (3aS,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5,8-diyl diacetate (mutilin 11,14-diacetate),

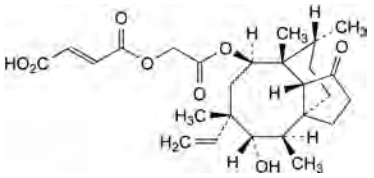

N. (2*E*)-4-[2-[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl]oxy]-2-oxoethoxy]-4-oxobut-2-enoic acid (pleuromutilin 22-fumarate),

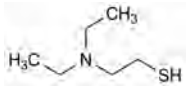

O. 2-(diethylamino)ethanethiol,

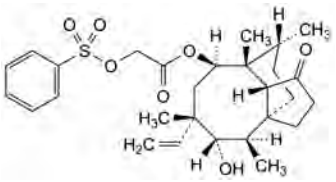

P. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [(phenylsulfonyl)oxy]acetate,

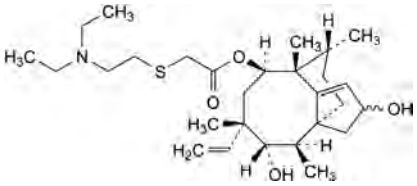

Q. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,10*R*)-6-ethenyl-2,5-dihydroxy-4,6,9,10-tetramethyl-2,3,4,5,6,7,8,9-octahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]acetate (3,4-didehydro-2-hydroxytiamulin),

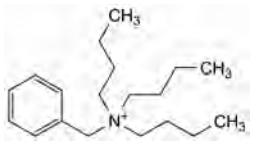

R. *N*-benzyl-*N*,*N*-dibutylbutan-1-aminium.

**01/2008:1659**
**corrected 6.0**

# TIAMULIN HYDROGEN FUMARATE FOR VETERINARY USE

## Tiamulini hydrogenofumaras ad usum veterinarium

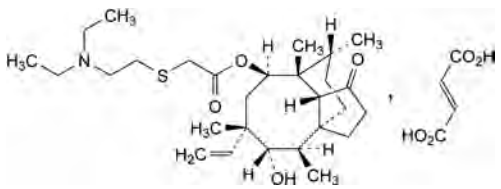

$C_{32}H_{51}NO_8S$ $M_r$ 610
[55297-96-6]

DEFINITION

(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-Ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]acetate hydrogen (*E*)-butenedioate.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.5 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or light yellow, crystalline powder.

*Solubility*: soluble in water, freely soluble in anhydrous ethanol and soluble in methanol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tiamulin hydrogen fumarate CRS*.

TESTS

**pH** (*2.2.3*): 3.1 to 4.1.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Ammonium carbonate buffer solution pH 10.0*. Dissolve 10.0 g of *ammonium carbonate R* in *water R*, add 22 mL of *perchloric acid solution R* and dilute to 1000.0 mL with *water R*. Adjust to pH 10.0 with *concentrated ammonia R1*.

*Solvent mixture*: ammonium carbonate buffer solution pH 10.0, *acetonitrile R1* (50:50 *V/V*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 0.200 g of *tiamulin hydrogen fumarate CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 40.0 mg of *fumaric acid R* in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (d)*. Dissolve 4 mg of *tiamulin for peak identification CRS* (tiamulin hydrogen fumarate containing impurities B, C, D, F, H and I) in the solvent mixture and dilute to 1 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm),
- *temperature*: 30 °C.

*Mobile phase*: *acetonitrile R1*, ammonium carbonate buffer solution pH 10.0, *methanol R1* (21:30:49 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 212 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of tiamulin.

*Identification of impurities*: use the chromatogram supplied with *tiamulin for peak identification CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities B and H.

*Relative retention* with reference to tiamulin (retention time = about 18 min): impurity G = about 0.2; impurity A = about 0.22; impurity H = about 0.23; impurity I = about 0.3; impurity J = about 0.4; impurity K = about 0.45; impurity B = about 0.5; impurity L = about 0.65; impurity C = about 0.66; impurity F = about 0.8; impurity M = about 0.85; impurity D = about 1.1; impurity S = about 1.4; impurity T = about 1.6; impurity E = 2.4.

*System suitability*: reference solution (a):

– baseline separation between the peaks due to tiamulin and impurity D.

*Limits*:

– *impurities B, H*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent),
– *impurities A, C, D, E, F, G, I, J, K, L, M, S, T*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent),
– *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent),
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (3.0 per cent),
– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent); disregard any peak present in reference solution (c).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{32}H_{51}NO_8S$ from the declared content of *tiamulin hydrogen fumarate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K, L, M, S, T.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *N, O, P, Q, R.*

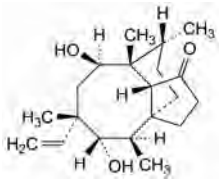

A. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5,8-dihydroxy-4,6,9,10-tetramethyloctahydro-3a,9-propano-3a*H*-cyclopentacycloocten-1(4*H*)-one (mutilin),

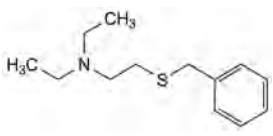

B. 2-(benzylsulfanyl)-*N,N*-diethylethanamine,

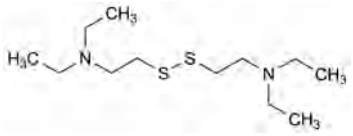

C. 2,2'-(disulfane-1,2-diyl)bis(*N,N*-diethylethanamine),

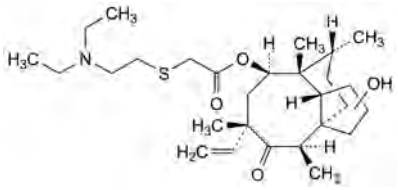

D. (3a*R*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenylhydroxy-4,6,9,10-tetramethyl-5-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]-sulfanyl]acetate,

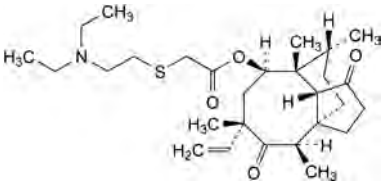

E. (3a*S*,4*R*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1,5-dioxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]-acetate (11-oxotiamulin),

F. impurity of unknown structure with a relative retention of about 0.8,

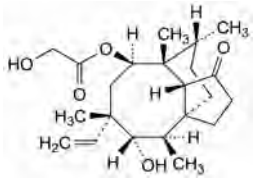

G. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl hydroxyacetate (pleuromutilin),

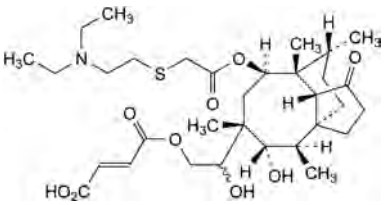

H. (2*E*)-4-[(2*RS*)-2-[(3a*S*,4*R*,5*S*,6*R*,8*R*,9*R*,9a*R*,10*R*)-8-[[[[2-(diethylamino)ethyl]sulfanyl]acetyl]oxy]-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-6-yl]-2-hydroxyethoxy]-4-oxobut-2-enoic acid (19,20-dihydroxytiamulin 20-fumarate),

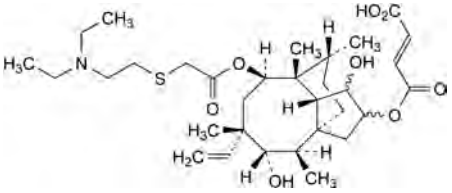

I. (2*E*)-4-[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-8-[[[[2-(diethylamino)ethyl]sulfanyl]acetyl]oxy]-6-ethenyl-1,5-dihydroxy-4,6,9,10-tetramethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-2-yl]oxy]-4-oxobut-2-enoic acid (2,3-dihydroxytiamulin 2-fumarate),

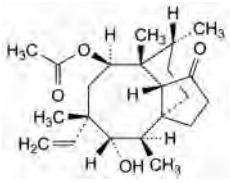

J. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl acetate (mutilin 14-acetate),

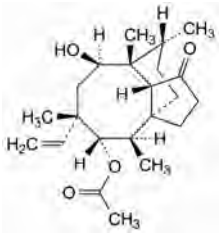

K. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-8-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5-yl acetate (mutilin 11-acetate),

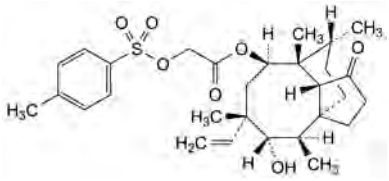

L. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[(4-methylphenyl)-sulfonyl]oxy]acetate (pleuromutilin 22-tosylate),

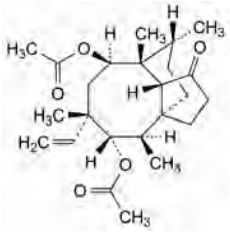

M. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-5,8-diyl diacetate (mutilin 11,14-diacetate),

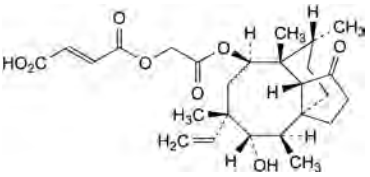

N. (2*E*)-4-[2-[[(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl]oxy]-2-oxoethoxy]-4-oxobut-2-enoic acid (pleuromutilin 22-fumarate),

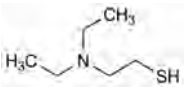

O. 2-(diethylamino)ethanethiol,

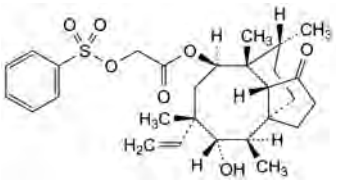

P. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [(phenylsulfonyl)oxy]acetate,

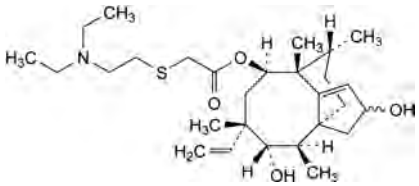

Q. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,10*R*)-6-ethenyl-2,5-dihydroxy-4,6,9,10-tetramethyl-2,3,4,5,6,7,8,9-octahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]acetate (3,4-didehydro-2-hydroxytiamulin),

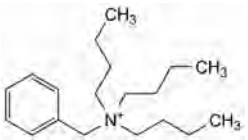

R. *N*-benzyl-*N*,*N*-dibutylbutan-1-aminium,

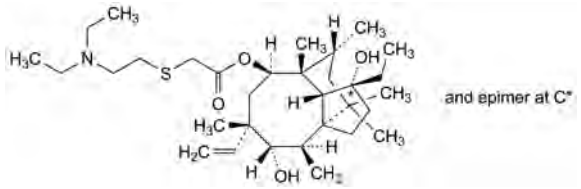


S. (1*RS*,3a*R*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-1-ethyl-1,5-dihydroxy-4,6,9,10,12,12-hexamethyldecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-(diethylamino)ethyl]sulfanyl]acetate,

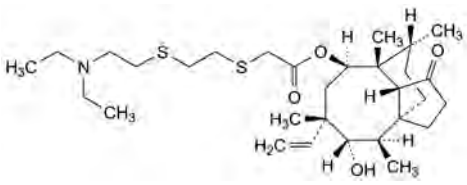

T. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopentacycloocten-8-yl [[2-[[2-(diethylamino)-ethyl]sulfanyl]ethyl]sulfanyl]acetate.

**01/2008:2022**
**corrected 10.0**

# TIANEPTINE SODIUM

## Tianeptinum natricum

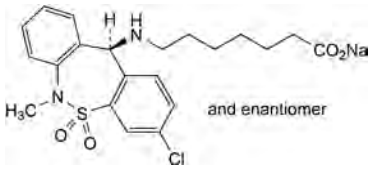


$C_{21}H_{24}ClN_2NaO_4S$ $M_r$ 458.9
[30123-17-2]

DEFINITION

Sodium 7-[[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo-[*c*,*f*][1,2]thiazepin-11-yl]amino]heptanoate *S*,*S*-dioxide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish powder, very hygroscopic.

*Solubility*: freely soluble in water, in methanol and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of tianeptine sodium*.

B. It gives reaction (a) of sodium (*2.3.1*).

TESTS

**Impurity A**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 1 mL of *ethyl 5-bromovalerate R* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of the solution to 250.0 mL with *anhydrous ethanol R*.

*Test solution.* Dissolve 0.1000 g of the substance to be examined in the internal standard solution and dilute to 2.0 mL with the same solution.

*Reference solution.* Dissolve 10.0 mg of *tianeptine impurity A CRS* in the internal standard solution and dilute to 200.0 mL with the same solution.

*Column*:
- *material*: fused silica,
- *size*: *l* = 25 m, Ø = 0.25 mm,
- *stationary phase*: *cyanopropylpolysiloxane R* (film thickness 0.2 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 26 cm/s.

*Split ratio*: 1:100.

*Temperature*:
- *column*: 150 °C,
- *injection port and detector*: 210 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: twice the retention time of ethyl 5-bromovalerate.

*System suitability*: reference solution:
- *elution order*: ethanol, ethyl 5-bromovalerate, impurity A,
- *resolution*: minimum 10 between the peaks due to ethyl 5-bromovalerate and impurity A,
- *signal-to-noise ratio*: minimum 20 for the peak due to impurity A.

*Limit*:
- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Mix 50 volumes of *methanol R* and 50 volumes of *water R*.

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 20 mg of *sodium tianeptine for system suitability CRS* in the solvent mixture and dilute to 200 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm) with a pore size of 0.01 µm,
- *temperature*: 30 °C.

*Mobile phase*:
- *mobile phase A*: mix 21 volumes of *methanol R1*, 31.5 volumes of *acetonitrile R1* and 47.5 volumes of a 2 g/L solution of *sodium laurilsulfate R*, adjusted to pH 2.5 with *phosphoric acid R*,
- *mobile phase B*: mix 20 volumes of *methanol R1*, 20 volumes of a 2 g/L solution of *sodium laurilsulfate R*, adjusted to pH 2.5 with *phosphoric acid R* and 60 volumes of *acetonitrile R1*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 35 | 100 | 0 |
| 35 - 45 | 100 → 40 | 0 → 60 |
| 45 - 60 | 40 | 60 |
| 60 - 70 | 40 → 100 | 60 → 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to tianeptine (retention time = about 30 min): impurity C = about 0.4; impurity D1 = about 0.6; impurity D2 = about 0.8; impurity E = about 1.1; impurity B = about 1.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.5 between the peaks due to tianeptine and impurity E.

*Limits*:
- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 8 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent),
- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.165 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 22.95 mg of $C_{21}H_{24}ClN_2NaO_4S$.

STORAGE

In an airtight container.

IMPURITIES

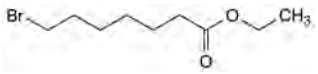

A. ethyl 7-bromoheptanoate,

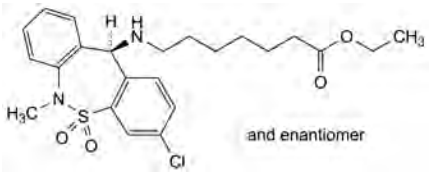


B. ethyl 7-[[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo-[*c*,*f*][1,2]thiazepin-11-yl]amino]heptanoate *S*,*S*-dioxide,

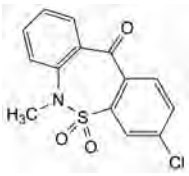

C. 3-chloro-6-methyldibenzo[*c*,*f*][1,2]thiazepin-11(6*H*)-one *S*,*S*-dioxide,

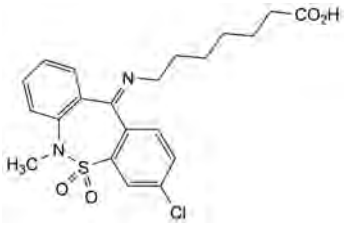

D. 7-[[(11*RS*)-3-chloro-6-methyldibenzo[*c*,*f*][1,2]thiazepin-11(6*H*)-ylidene]amino]heptanoic acid *S*,*S*-dioxide,

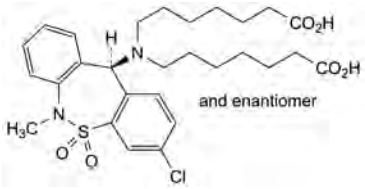


E. 7,7′-[[(11*RS*)-3-chloro-6-methyl-6,11-dihydrodibenzo-[*c*,*f*][1,2]thiazepin-11-yl]imino]diheptanoic acid *S*,*S*-dioxide.

**01/2017:1575**
**corrected 10.0**

# TIAPRIDE HYDROCHLORIDE

## Tiapridi hydrochloridum

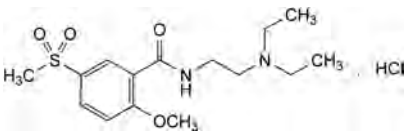

$C_{15}H_{25}ClN_2O_4S$ $M_r$ 364.9
[51012-33-0]

DEFINITION

*N*-[2-(Diethylamino)ethyl]-2-methoxy-5-(methylsulfonyl)-benzamide hydrochloride.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, soluble in methanol, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tiapride hydrochloride CRS*.

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 450 nm is not greater than 0.030.

**pH** (*2.2.3*): 4.0 to 6.0 for solution S.

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.400 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20.0 mg of *metoclopramide impurity E CRS* (impurity C) in *methanol R* and dilute to 50 mL with the same solvent. Dilute 2.0 mL of the solution to 20 mL with *methanol R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *dioxan R*, *methanol R*, *methylene chloride R* (2:10:14:90 *V/V/V/V*).

*Application*: 10 µL.

*Development*: over 4/5 of the plate.

*Drying*: in air.

*Detection*: spray with a 2 g/L solution of *ninhydrin R* in *butanol R* and heat at 100 °C for 15 min.

*Retardation factors*: impurity C = about 0.1; tiapride = about 0.6.

*Limit*:

– *impurity C*: any spot due to impurity C is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *tiapride hydrochloride CRS* and 5.0 mg of *tiapride N-oxide CRS* (impurity D) in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: dissolve 5.44 g of *potassium dihydrogen phosphate R* and 0.08 g of *sodium octanesulfonate R* in 780 mL of *water R*, adjust to pH 2.7 with *phosphoric acid R* and dilute to 800 mL with *water R*; add 150 mL of *methanol R* and 50 mL of *acetonitrile R* and mix.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of tiapride.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to tiapride (retention time = about 7 min): impurity D = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 4.0 between the peaks due to tiapride and impurity D.

*Calculation of percentage contents*:

– for each impurity, use the concentration of tiapride hydrochloride in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 20 mL of *anhydrous acetic acid R*. Add 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 36.49 mg of $C_{15}H_{25}ClN_2O_4S$.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D.*

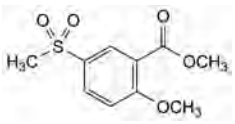

A. methyl 2-methoxy-5-(methylsulfonyl)benzoate,

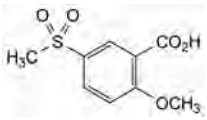

B. 2-methoxy-5-(methylsulfonyl)benzoic acid,

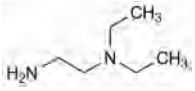

C. *N*,*N*-diethylethane-1,2-diamine,

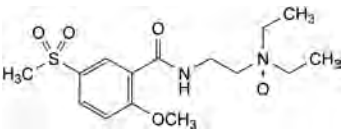

D. *N*-[2-(diethyloxidoaminol)ethyl]-2-methoxy-5-(methylsulfonyl)benzamide (tiapride *N*-oxide).

**01/2017:1157**

# TIAPROFENIC ACID

## Acidum tiaprofenicum

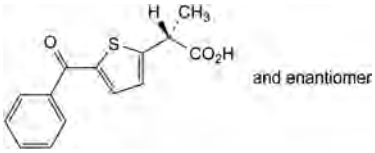


$C_{14}H_{12}O_3S$ $M_r$ 260.3

[33005-95-7]

DEFINITION

(2*RS*)-2-(5-Benzoylthiophen-2-yl)propanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification: C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 95 °C to 99 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 25.0 mg in *ethanolic hydrochloric acid R* and dilute to 50.0 mL with the same solvent. Dilute 1.0 mL of this solution to 50.0 mL with *ethanolic hydrochloric acid R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 305 nm.

*Shoulder*: at 262 nm.

*Specific absorbance at the absorption maximum*: 550 to 590.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tiaprofenic acid CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 10 mg of *tiaprofenic acid CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 10 mg of *ketoprofen CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 2 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *acetic acid R*, *methylene chloride R*, *acetone R* (1:20:80 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 2.0 g in *ethanol (96 per cent) R* and dilute to 20 mL with the same solvent.

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 0.50 g in *ethyl acetate R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 10.0 mg of *tiaprofenic acid impurity C CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution (d).* Dilute 1.0 mL of reference solution (a) to 2.0 mL with reference solution (c).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *glacial acetic acid R*, *hexane R*, *methylene chloride R* (0.25:20:500:500 *V/V/V/V*); add the water to the acetic acid, then hexane and methylene chloride; sonicate the mixture for 2 min. Do not degas with helium during analysis.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 250 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of tiaprofenic acid.

*Relative retention* with reference to tiaprofenic acid: impurity A = about 0.19; impurity B = about 0.43; impurity C = about 0.86.

*System suitability*: reference solution (d):

– *resolution*: minimum 3.0 between the peaks due to impurity C and tiaprofenic acid.

*Limits*:

– *impurity C*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *sum of impurities other than C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 25 mL of *ethanol (96 per cent) R*. Add 25 mL of *water R* and 0.5 mL of *phenolphthalein solution R*. Titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 26.03 mg of $C_{14}H_{12}O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, D, E, F.*

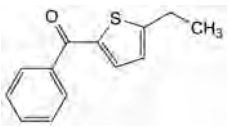


A. (5-ethylthiophen-2-yl)phenylmethanone,

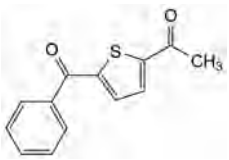


B. 1-(5-benzoylthiophen-2-yl)ethanone,

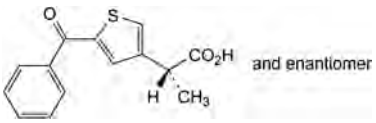


C. (2*RS*)-2-(5-benzoylthiophen-3-yl)propanoic acid,

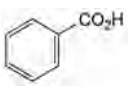


D. benzoic acid,

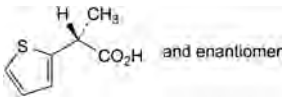


E. (2*RS*)-2-(thiophen-2-yl)propanoic acid,

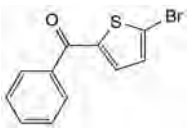


F. (5-bromothiophen-2-yl)phenylmethanone.

**01/2008:1739**
**corrected 6.0**

# TIBOLONE

## Tibolonum

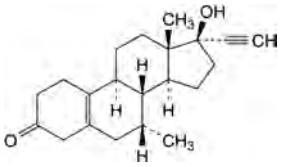

$C_{21}H_{28}O_2$ $M_r$ 312.5

DEFINITION

17-Hydroxy-7α-methyl-19-nor-17α-pregn-5(10)-en-20-yn-3-one.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or crystals.

*Solubility*: practically insoluble in water, soluble in acetone and in methanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of tibolone.*

If the spectra obtained in the solid state show differences, dissolve the substance to be examined in the minimum volume of *anhydrous ethanol R*, evaporate to dryness on a water-bath and record a new spectrum using the residue.

TESTS

**Specific optical rotation** (*2.2.7*): + 100 to + 106 (dried substance).

Dissolve 0.250 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Select a brand of acetonitrile such that the formation of possible artefact peaks, eluting after impurity C at relative retentions 0.6 to 0.8, is avoided.*

*Solvent mixture*: *water R*, *acetonitrile R1* (25:75 *V/V*).

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 4 mg of *tibolone for system suitability CRS* (containing impurities A, B, C, D and E) in 1 mL of the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *methanol R*, *acetonitrile R1*, *water R* (8:40:52 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 5 µL.

*Run time*: 3 times the retention time of tibolone.

*Identification of impurities*: use the chromatogram supplied with *tibolone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D and E.

*Relative retention* with reference to tibolone (retention time = about 14 min): impurity A = about 0.22; impurity B = about 0.24; impurity C = about 0.58; impurity D = about 1.12; impurity E = about 2.24.

*System suitability*: reference solution (a):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to tibolone.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.7; impurity B = 1.5; impurity C = 2.1;
- *impurities A, E*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *impurity B*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity C*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurity D*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 60 mL of *tetrahydrofuran R*. Add 25 mL of a 100 g/L solution of *silver nitrate R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.25 mg of $C_{21}H_{28}O_2$.

STORAGE

At a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

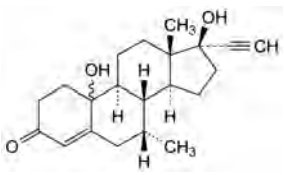

A. 10,17-dihydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one,

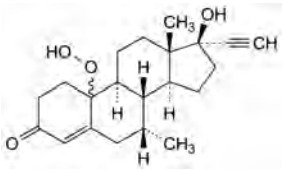

B. 10-hydroperoxy-17-hydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one,

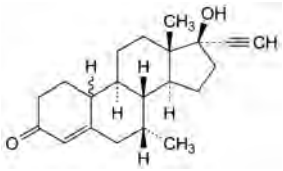

C. 17-hydroxy-7α-methyl-19-nor-10ξ,17α-pregn-4-en-20-yn-3-one,

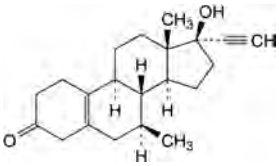

D. 17-hydroxy-7β-methyl-19-nor-17α-pregn-5(10)-en-20-yn-3-one,

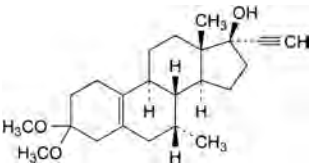

E. 3,3-dimethoxy-7α-methyl-19-nor-17α-pregn-5(10)-en-20-yn-17-ol.

**01/2008:0956**
**corrected 10.0**

# TICARCILLIN SODIUM

## Ticarcillinum natricum

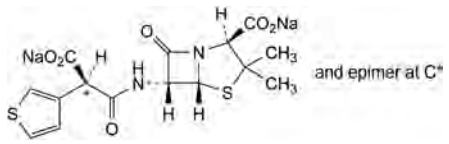


$C_{15}H_{14}N_2Na_2O_6S_2$ $M_r$ 428.4
[4697-14-7]

DEFINITION

Disodium (2*S*,5*R*,6*R*)-6-[[(2*RS*)-2-carboxylato-2-(thiophen-3-yl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Semi-synthetic product derived from a fermentation product.

*Content*: 89.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly yellow, hygroscopic powder.

*Solubility*: freely soluble in water, soluble in methanol.

IDENTIFICATION

*First identification: A, D, E.*

*Second identification: B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 50 mg of the substance to be examined in 1 mL of *water R*, add 0.1 mL of *hydrochloric acid R1*, swirl and allow to stand in iced water for 10 min. Filter the precipitate and rinse with 2 mL of *water R*. Dissolve in a mixture of 1 volume of *water R* and 9 volumes of *acetone R*. Evaporate the solvent almost to dryness, then dry in an oven at 60 °C for 30 min.

*Comparison*: repeat the operations using *ticarcillin monosodium CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *ticarcillin monosodium CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 25 mg of *carbenicillin sodium CRS* and 25 mg of *ticarcillin monosodium CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Plate*: *TLC silanised silica gel plate R.*

*Mobile phase*: mix 10 volumes of *acetone R* and 90 volumes of a 154 g/L solution of *ammonium acetate R*, adjusted to pH 5.0 with *glacial acetic acid R*.

*Application*: 1 µL.

*Development*: over a path of 12 cm.

*Drying*: in a current of hot air.

*Detection*: expose to iodine vapour.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Place about 2 mg in a test-tube about 15 cm long and 15 mm in diameter. Moisten with 0.05 mL of *water R* and add 2 mL of *sulfuric acid-formaldehyde reagent R*. Mix the contents of the tube by swirling; the solution is brown. Place the test-tube in a water-bath for 1 min; a dark reddish-brown colour develops.

D. It gives reaction (a) of sodium (*2.3.1*).

E. Specific optical rotation (see Tests).

TESTS

**Solution S**. Dissolve 2.50 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.5 to 7.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 172 to + 187 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase A and dilute to 25.0 mL with mobile phase A.

*Reference solution (a).* Dissolve 20.0 mg of *ticarcillin impurity A CRS* in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 5.0 mL of this solution to 50.0 mL with mobile phase A.

*Reference solution (b).* Dilute 1 mL of the test solution to 50 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1.3 g/L solution of *ammonium phosphate R* adjusted to pH 7.0 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*, mobile phase A (50:50 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 30 | 100 → 30 | 0 → 70 |
| 30 - 40 | 30 | 70 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the 2 principal peaks (diastereoisomers).

*Limits*:
- *impurity A*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (4 per cent);
- *any other impurity*: for each impurity, not more than 1.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (2.5 per cent).

***N,N*-Dimethylaniline** (*2.4.26, Method B*): maximum 20 ppm.

**2-Ethylhexanoic acid** (*2.4.28*): maximum 0.5 per cent *m/m*.

**Water** (*2.5.12*): maximum 5.5 per cent, determined on 0.150 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.05 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Reference solution.* Dissolve 50.0 mg of *ticarcillin monosodium CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 10.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *methanol R* and 80 volumes of a 1.3 g/L solution of *ammonium phosphate R* adjusted to pH 7.0 with *phosphoric acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*System suitability*: reference solution:
- *resolution*: minimum 2.5 between the 2 principal peaks;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the 2 peaks due to ticarcillin after 6 injections.

Calculate the percentage content of ticarcillin sodium as the sum of the areas of the 2 peaks, multiplying the content of ticarcillin monosodium by 1.054.

STORAGE

In an airtight container, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

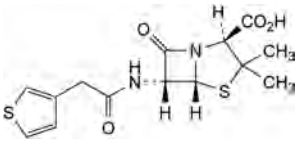

A. (2*S*,5*R*,6*R*)-3,3-dimethyl-7-oxo-6-[[(thiophen-3-yl)acetyl]amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (decarboxyticarcillin),

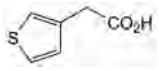

B. (thiophen-3-yl)acetic acid,

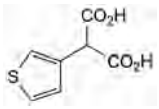

C. 2-(thiophen-3-yl)propanedioic acid (3-thienylmalonic acid),

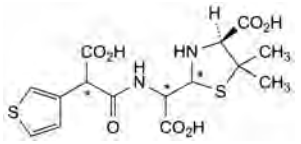

D. (4*S*)-2-[carboxy[[2-carboxy-2-(thiophen-3-yl)acetyl]amino]methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penicilloic acids of ticarcillin),

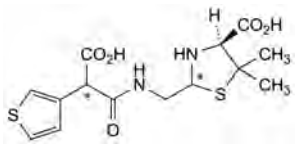

E. (4*S*)-2-[[[2-carboxy-2-(thiophen-3-yl)acetyl]amino]-methyl]-5,5-dimethylthiazolidine-4-carboxylic acid (penilloic acids of ticarcillin).

**01/2017:1050**

# TICLOPIDINE HYDROCHLORIDE

## Ticlopidini hydrochloridum

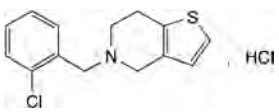

$C_{14}H_{15}Cl_2NS$ $M_r$ 300.2

[53885-35-1]

DEFINITION

5-(2-Chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water and in anhydrous ethanol, very slightly soluble in ethyl acetate.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 40 mg in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with *water R*.

*Spectral range*: 250-350 nm for test solution (a); 200-350 nm for test solution (b).

*Absorption maxima*: at 268 nm and 275 nm for test solution (a); at 214 nm and 232 nm for test solution (b).

*Absorption ratio*: $A_{268}/A_{275}$ = 1.1 to 1.2.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ticlopidine hydrochloride CRS*.

C. Mix about 6 mg of *citric acid monohydrate R* and 0.3 mL of *acetic anhydride R*. Add about 5 mg of the substance to be examined and heat in a water-bath at 80 °C. A red colour develops.

D. About 20 mg gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in a 1 per cent *V/V* solution of *hydrochloric acid R* and dilute to 20 mL with the same solution.

**pH** (*2.2.3*): 3.5 to 4.0.

Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (20:80 *V/V*).

*Test solution.* Dissolve 0.250 g of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution.* Dissolve 5.0 mg of *ticlopidine impurity F CRS* in the solvent mixture. Add 1.00 mL of the test solution and dilute to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: 0.95 g/L solution of *sodium pentanesulfonate monohydrate R*, adjusted to pH 3.4 with a 50 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 45 | 80 → 20 | 20 → 80 |
| 45 - 50 | 20 | 80 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL; inject the solvent mixture as a blank.

*Identification of impurities*: use the chromatogram obtained with the reference solution to identify the peak due to impurity F.

*Retention time*: ticlopidine = about 15 min.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peaks due to ticlopidine and impurity F; if necessary, adjust the pH of mobile phase A;
- *signal-to-noise ratio*: minimum 50 for the peak due to ticlopidine.

*Limits*:

- *impurity F*: not more than 0.5 times the area of the corresponding peak in the chromatogram obtained with the reference solution (0.05 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to ticlopidine in the chromatogram obtained with the reference solution (0.10 per cent);
- *total*: not more than the area of the peak due to ticlopidine in the chromatogram obtained with the reference solution (0.10 per cent);
- *disregard limit*: 0.5 times the area of the peak due to ticlopidine in the chromatogram obtained with the reference solution (0.05 per cent). Do not disregard the peak due to impurity F.

**Formaldehyde**: maximum 20 ppm.

Dissolve 0.200 g in 4.0 mL of *water R*. Add 0.4 mL of *dilute sodium hydroxide solution R*. Centrifuge, filter the supernatant through cotton previously impregnated with *water R* and dilute to 5.0 mL with *water R*. Transfer to a test-tube. Add 5.0 mL of *acetylacetone reagent R1*. Place the test-tube in a water-bath at 40 °C for 40 min. The test solution is not more intensely coloured than a standard prepared at the same time and in the same manner using 5.0 mL of a 0.8 ppm solution of formaldehyde ($CH_2O$), obtained by dilution of *formaldehyde standard solution (5 ppm $CH_2O$) R* with *water R*. Examine the tubes down their vertical axis.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 15 mL of *anhydrous acetic acid R*. Add 35 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.02 mg of $C_{14}H_{15}Cl_2NS$.

IMPURITIES

*Specified impurities: F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, G, H, I, J, K, L.*

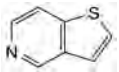


A. thieno[3,2-*c*]pyridine,

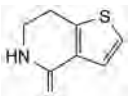


B. 6,7-dihydrothieno[3,2-*c*]pyridin-4(5*H*)-one,

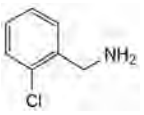

C. (2-chlorophenyl)methanamine,

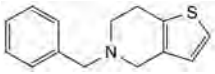

D. 5-benzyl-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine,

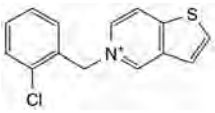

E. 5-(2-chlorobenzyl)thieno[3,2-*c*]pyridinium,

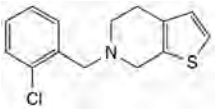

F. 6-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine,

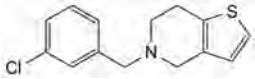

G. 5-(3-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine,

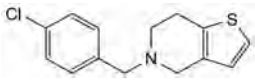

H. 5-(4-chlorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-*c*]pyridine,

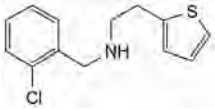

I. *N*-(2-chlorobenzyl)-2-(thiophen-2-yl)ethanamine,

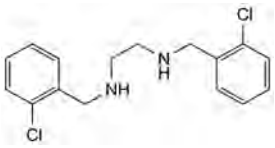

J. *N*,*N*′-bis(2-chlorobenzyl)ethane-1,2-diamine,

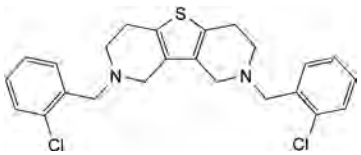

K. 2,8-bis(2-chlorobenzyl)-1,2,3,4,6,7,8,9-octahydrothieno[3,2-*c*:4,5-*c*′]dipyridine (bis-ticlopidine),

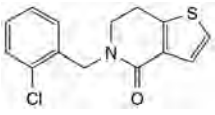

L. 5-(2-chlorobenzyl)-6,7-dihydrothieno[3,2-*c*]pyridin-4(5*H*)-one.

**01/2019:2825**
**corrected 10.0**

# TIGECYCLINE

## Tigecyclinum

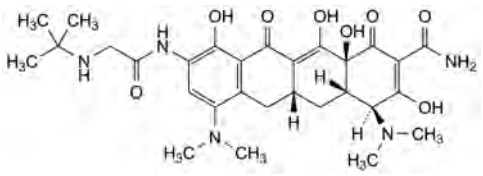

$C_{29}H_{39}N_5O_8$ $M_r$ 585.7
[220620-09-7]

DEFINITION

(4*S*,4a*S*,5a*R*,12a*S*)-9-[2-(*tert*-Butylamino)acetamido]-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide.

Semi-synthetic product derived from a fermentation product.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: orange, hygroscopic, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in anhydrous ethanol and in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tigecycline CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**pH** (*2.2.3*): 7.7 to 8.2.

Dissolve 0.500 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light. Store the solutions at 2-8 °C and use them within 12 h.*

*Solution A*. Solution containing 4.35 g/L of *dipotassium hydrogen phosphate R* and 0.5 g/L of *sodium hydrogensulfite R* adjusted to pH 8.0 with a 56.1 g/L solution of *potassium hydroxide R*.

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in solution A and dilute to 100.0 mL with solution A.

*Test solution (b)*. Dilute 10.0 mL of test solution (a) to 50.0 mL with solution A.

*Reference solution (a)*. Dissolve 50.0 mg of *tigecycline CRS* in solution A and dilute to 100.0 mL with solution A. Dilute 10.0 mL of this solution to 50.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (c)*. Dissolve 2 mg of *tigecycline impurity B CRS* and 2 mg of *minocycline hydrochloride R* (impurity C) in solution A and dilute to 25 mL with solution A. Dilute 3 mL of the solution to 20 mL with solution A. Dissolve 2.5 mg of *tigecycline for system suitability CRS* (containing impurity A) in 1 mL of this solution and dilute to 5 mL with solution A.

*Reference solution (d).* Dissolve 2.5 mg of *tigecycline for system suitability CRS* (containing impurity A) in solution A and dilute to 5 mL with solution A.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: dissolve 4.35 g of *dipotassium hydrogen phosphate R* and 0.93 g of *sodium edetate R* in 940 mL of *water for chromatography R*, and adjust to pH 6.4 with *phosphoric acid R*; make up to 950 mL with *water for chromatography R* and dilute to 1000 mL with *acetonitrile for chromatography R*;
- *mobile phase B*: dissolve 4.35 g of *dipotassium hydrogen phosphate R* and 0.93 g of *sodium edetate R* in 490 mL of *water for chromatography R*, and adjust to pH 6.4 with *phosphoric acid R*; make up to 500 mL with *water for chromatography R* and dilute to 1000 mL with *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 85 | 15 |
| 2 - 42 | 85 → 57 | 15 → 43 |
| 42 - 57 | 57 → 0 | 43 → 100 |
| 57 - 60 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 25 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *tigecycline for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to tigecycline (retention time = about 20 min): impurity B = about 0.6; impurity A = about 0.7; impurity C = about 1.6.

*System suitability*: reference solution (c):

- *resolution*: minimum 1.6 between the peaks due to impurities B and A.

*Calculation of percentage contents*:

- for each impurity, use the concentration of tigecycline in reference solution (b).

*Limits*:

- *impurity A*: maximum 0.6 per cent;
- *impurity C*: maximum 0.2 per cent;
- *impurity B*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.32*): maximum 2.5 per cent.

Inject 1.0 mL of a 50 mg/mL solution of the substance to be examined in *dimethyl sulfoxide R2*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Column*:

- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 14 volumes of *acetonitrile for chromatography R* and 86 volumes of a solution containing 4.35 g/L of *dipotassium hydrogen phosphate R* and 0.93 g/L of *sodium edetate R* previously adjusted to pH 6.2 with *phosphoric acid R*.

*Injection*: 20 µL of test solution (b) and reference solutions (a) and (d).

*Run time*: 4.5 times the retention time of tigecycline.

*Relative retention* with reference to tigecycline (retention time = about 12 min): impurity A = about 0.6.

*System suitability*: reference solution (d):

- *resolution*: minimum 3.0 between the peaks due to impurity A and tigecycline.

Calculate the percentage content of $C_{29}H_{39}N_5O_8$ using the chromatogram obtained with reference solution (a) and taking into account the assigned content of *tigecycline CRS*.

STORAGE

Under nitrogen, in an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

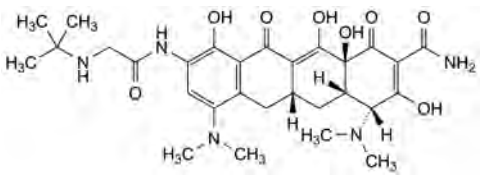

A. (4*R*,4a*S*,5a*R*,12a*S*)-9-[2-(*tert*-butylamino)acetamido]-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide,

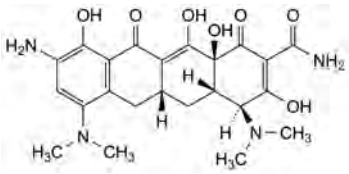

B. (4*S*,4a*S*,5a*R*,12a*S*)-9-amino-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (9-aminominocycline),

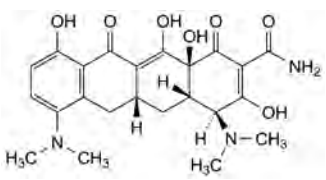

C. (4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-3,10,12,12a-tetrahydroxy-1,11-dioxo-1,4,4a,5,5a,6,11,12a-octahydrotetracene-2-carboxamide (minocycline),

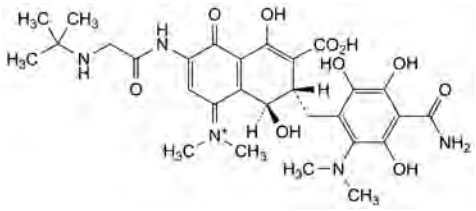

D. (7*S*,8*R*)-3-[2-(*tert*-butylamino)acetamido]-7-[[4-carbamoyl-2-(dimethylamino)-3,5,6-trihydroxyphenyl]methyl]-6-carboxy-5,8-dihydroxy-*N*,*N*-dimethyl-4-oxo-7,8-dihydronaphthalen-1(4*H*)-iminium.

**01/2020:1767**

# TILIDINE HYDROCHLORIDE HEMIHYDRATE

## Tilidini hydrochloridum hemihydricum

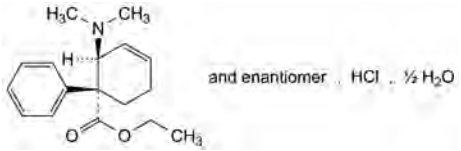


$C_{17}H_{24}ClNO_2,½H_2O$ $M_r$ 318.8
[255733-17-6]

DEFINITION

Ethyl (1*RS*,6*SR*)-6-(dimethylamino)-3,6-dihydro[1,1′-biphenyl]-1(2*H*)-carboxylate hydrochloride hemihydrate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

A suitable antioxidant may be added.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tilidine hydrochloride hemihydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

**Acidity or alkalinity**. To 20.0 mL of solution S add 0.2 mL of *0.01 M sodium hydroxide*. The pH is not less than 4.1. Add 0.4 mL of *0.01 M hydrochloric acid*. The pH is not more than 4.3.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *tilidine for system suitability CRS* (containing impurities D and E) in the mobile phase and dilute to 5 mL with the mobile phase.

*Precolumn*:
- *size*: *l* = 4 mm, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix equal volumes of *acetonitrile for chromatography R* and a 0.98 g/L solution of *ammonium carbonate R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of tilidine.

*Identification of impurities*: use the chromatogram supplied with *tilidine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities D and E; impurities D and E may be inverted in the elution order, therefore take into account the heights of the corresponding peaks in the chromatogram supplied with *tilidine for system suitabity CRS*.

*Relative retention* with reference to tilidine (retention time = about 15 min): impurity E = about 0.4; impurity D = about 0.5 (D and E may be inverted).

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurities E and D.

*Calculation of percentage contents*:
- *correction factor*: multiply the peak area of impurity E by 0.5;
- for each impurity, use the concentration of tilidine hydrochloride hemihydrate in reference solution (a).

*Limits*:
- *impurity E*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): 2.5 per cent to 3.1 per cent, determined on 0.300 g.

ASSAY

Dissolve 0.250 g in a mixture of 10 mL of *anhydrous acetic acid R* and 50 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 30.98 mg of $C_{17}H_{24}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *E*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D*.

Monographs T

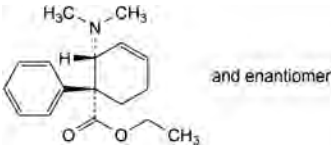


A. ethyl (1*RS*,6*RS*)-6-(dimethylamino)-3,6-dihydro[1,1′-biphenyl]-1(2*H*)-carboxylate,

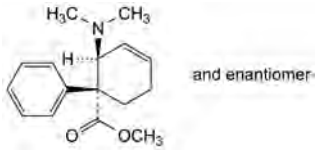


B. methyl (1*RS*,6*SR*)-6-(dimethylamino)-3,6-dihydro[1,1′-biphenyl]-1(2*H*)-carboxylate,

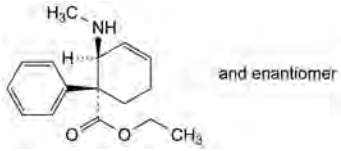


C. ethyl (1*RS*,6*SR*)-6-(methylamino)-3,6-dihydro[1,1′-biphenyl]-1(2*H*)-carboxylate,

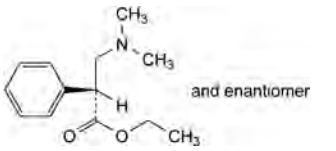


D. ethyl (2*RS*)-3-(dimethylamino)-2-phenylpropanoate,

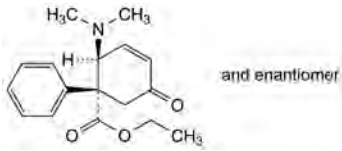


E. ethyl (1*RS*,6*SR*)-6-(dimethylamino)-3-oxo-3,6-dihydro[1,1′-biphenyl]-1(2*H*)-carboxylate.

**01/2017:0572**

# TIMOLOL MALEATE

## Timololi maleas

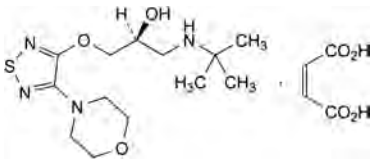

$C_{17}H_{28}N_4O_7S$ $M_r$ 432.5
[26921-17-5]

DEFINITION

(2*S*)-1-[(1,1-Dimethylethyl)amino]-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol (*Z*)-butenedioate.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water and in ethanol (96 per cent).

mp: about 199 °C, with decomposition.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (*2.2.7*): − 6.2 to − 5.7.
Dissolve 1.000 g in *1 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *timolol maleate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 5 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution.* Dissolve 5 mg of *timolol maleate CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.
*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (1:20:80 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: expose to iodine vapour for 2 h.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Triturate 0.1 g with a mixture of 1 mL of *dilute sodium hydroxide solution R* and 3 mL of *water R*. Shake with 3 quantities, each of 5 mL, of *ether R*. To 0.1 mL of the aqueous layer add a solution containing 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min; no violet-red colour develops. Neutralise the remainder of the aqueous layer with *dilute sulfuric acid R* and add 1 mL of *bromine water R*. Heat on a water-bath for 15 min, then heat to boiling and cool. To 0.2 mL of this solution add a solution containing 10 mg of *resorcinol R* in 3 mL of *sulfuric acid R*. Heat on a water-bath for 15 min; a violet-red colour develops. Add 0.2 mL of a 100 g/L solution of *potassium bromide R* and heat for 5 min on a water-bath; the colour becomes violet-blue.

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_8$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 3.8 to 4.3 for solution S.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*). *Carry out the test protected from actinic light.*
*Solvent mixture*: *methylene chloride R*, *2-propanol R* (10:30 *V/V*).
*Test solution.* Dissolve 30.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.
*Reference solution (a).* Dissolve 30 mg of *timolol maleate CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.
*Reference solution (b).* Dissolve 3 mg of *(R)-timolol CRS* (impurity A) in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 10.0 mL with the solvent mixture.
*Reference solution (c).* Dilute 1 mL of reference solution (a) to 100 mL with the solvent mixture. Mix 1 mL of this solution with 1 mL of reference solution (b).
*Reference solution (d).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (5 µm).

*Mobile phase*: *diethylamine R*, *2-propanol R*, *hexane R* (2:40:960 *V/V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 297 nm.

*Injection*: 5 µL.

*Elution order*: impurity A is eluted first.

*System suitability*:

– *resolution*: minimum 4.0 between the peaks due to impurity A and the (*S*)-enantiomer in the chromatogram obtained with reference solution (c);

– the retention times of the principal peaks due to the (*S*)-enantiomer in the chromatograms obtained with the test solution and reference solution (a) are identical.

*Limit*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (d) (1.0 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 20 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 3 mg of *timolol impurity F CRS* in *methanol R* and dilute to 20.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with mobile phase A (solution A). Dissolve the contents of a vial of *timolol for system suitability CRS* (containing impurities B, C and D) in 1.0 mL of solution A.

*Reference solution (c)*. Dissolve 2 mg of the substance to be examined and 20 mg of *maleic acid R* in 10 mL of *acetonitrile R*. Evaporate 1 mL of the solution to dryness under a stream of *nitrogen R* in an amber glass vial. Heat the open vial at 105 °C for 1 h. Reconstitute the residue with 1.0 mL of mobile phase A (*in situ* preparation of impurity E).

*Column*:

– *size*: *l* = 0.150 m, Ø = 3.9 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mixture of equal volumes of *methanol R* and a 4.32 g/L solution of *sodium octanesulfonate R* previously adjusted to pH 3.0 with *glacial acetic acid R*;

– *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 97.5 | 2.5 |
| 10 - 11 | 97.5 → 70 | 2.5 → 30 |
| 11 - 20 | 70 | 30 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 295 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram supplied with *timolol for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, C, D and F; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to timolol (retention time = about 7.5 min): maleic acid = about 0.1; impurity D = about 0.3; impurity E = about 0.4; impurity B = about 0.7; impurity F = about 0.8; impurity C = about 2.1.

*System suitability*: reference solution (b):

– *resolution*: minimum 1.5 between the peaks due to impurities B and F.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity D by 0.6;

– *impurities B, C, D, E, F*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 60 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 43.25 mg of $C_{17}H_{28}N_4O_7S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G, H, I, J.*

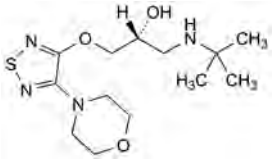

A. (2*R*)-1-[(1,1-dimethylethyl)amino]-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol ((*R*)-timolol),

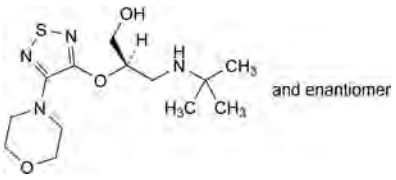


B. (2*RS*)-3-[(1,1-dimethylethyl)amino]-2-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-1-ol,

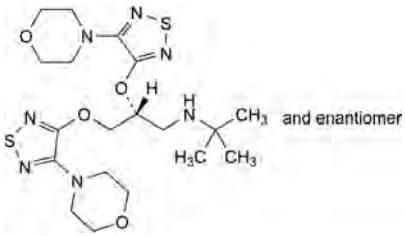

C. (2*RS*)-*N*-(1,1-dimethylethyl)-2,3-bis[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-1-amine,

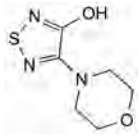

D. 4-(morpholin-4-yl)-1,2,5-thiadiazol-3-ol,

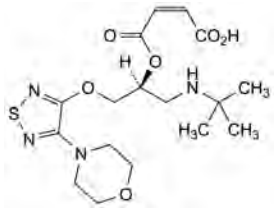

E. (2*Z*)-4-[(1*S*)-1-[[(1,1-dimethylethyl)amino]methyl]-2-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]ethoxy]-4-oxobut-2-enoic acid,

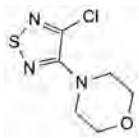

F. 4-(4-chloro-1,2,5-thiadiazol-3-yl)morpholine,

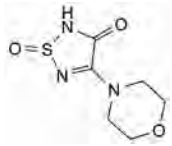

G. 4-(morpholin-4-yl)-1,2,5-thiadiazol-3(2*H*)-one 1-oxide,

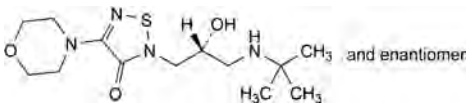

H. 2-[(2*RS*)-3-[(1,1-dimethylethyl)amino]-2-hydroxypropyl]-4-(morpholin-4-yl)-1,2,5-thiadiazol-3(2*H*)-one,

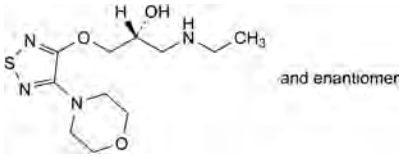

I. (2*RS*)-1-(ethylamino)-3-[[4-(morpholin-4-yl)-1,2,5-thiadiazol-3-yl]oxy]propan-2-ol,

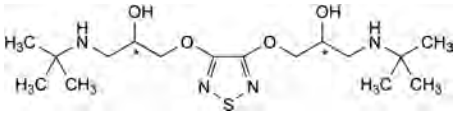

J. 1,1′-[1,2,5-thiadiazol-3,4-diylbis(oxy)]bis[3-[(1,1-dimethylethyl)amino]propan-2-ol].

**01/2017:1051**

# TINIDAZOLE

## Tinidazolum

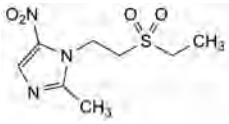

$C_8H_{13}N_3O_4S$ $M_r$ 247.3
[19387-91-8]

DEFINITION

1-[2-(Ethylsulfonyl)ethyl]-2-methyl-5-nitro-1*H*-imidazole.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: almost white or pale yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in methylene chloride, sparingly soluble in methanol.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D, E.*

A. Melting point (*2.2.14*): 125 °C to 128 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 10.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 310 nm.

*Specific absorbance at the absorption maximum*: 340 to 360.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tinidazole CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 20 mg of *tinidazole CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Pretreatment*: heat at 110 °C for 1 h and allow to cool.

*Mobile phase*: *butanol R*, *ethyl acetate R* (25:75 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

E. To about 10 mg add about 10 mg of *zinc powder R*, 0.3 mL of *hydrochloric acid R* and 1 mL of *water R*. Heat in a water-bath for 5 min and cool. The solution gives the reaction of primary aromatic amines (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.0 g in *acetone R* and dilute to 20 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Protect solutions from light.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in 10.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *tinidazole impurity A CRS* and 5.0 mg of *tinidazole impurity B CRS* in 10.0 mL of *methanol R* and dilute to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 3.0 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

Regular column conditioning by subsequent flushing with 50 mL of *water R*, 100 mL of *methanol R*, 25 mL of *water R* and 100 mL of the mobile phase is recommended.

*Mobile phase*: *acetonitrile R*, *methanol R*, *water R* (10:20:70 *V/V/V*).

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 320 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of tinidazole.

*Relative retention* with reference to tinidazole (retention time = about 6 min): impurity A = about 0.6; impurity B = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurities A and B.

*Limits*:

– *impurities A, B*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 25 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 24.73 mg of $C_8H_{13}N_3O_4S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

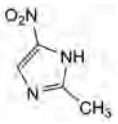

A. 2-methyl-5-nitro-1*H*-imidazole,

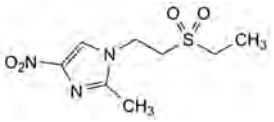

B. 1-[2-(ethylsulfonyl)ethyl]-2-methyl-4-nitro-1*H*-imidazole.

**01/2008:1271**

# TINZAPARIN SODIUM

## Tinzaparinum natricum

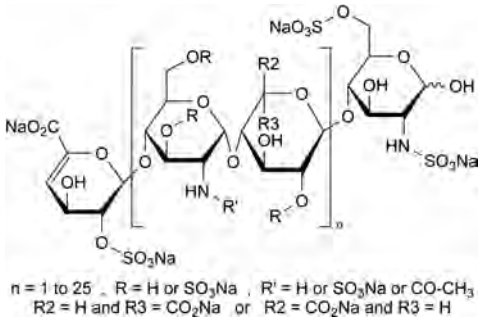


DEFINITION

Tinzaparin sodium is the sodium salt of a low-molecular-mass heparin that is obtained by controlled enzymatic depolymerisation of heparin from porcine intestinal mucosa using heparinase from *Flavobacterium heparinum*. The majority of the components have a 2-*O*-sulfo-4-enepyranosuronic acid structure at the non-reducing end and a 2-*N*,6-*O*-disulfo-D-glucosamine structure at the reducing end of their chain.

*Tinzaparin sodium complies with the monograph on Low-molecular-mass heparins (0828) with the modifications and additional requirements below.*

The mass-average relative molecular mass ranges between 5500 and 7500 with a characteristic value of about 6500.

The degree of sulfatation is 1.8 to 2.5 per disaccharide unit.

The potency is not less than 70 IU and not more than 120 IU of anti-factor Xa activity per milligram calculated with reference to the dried substance. The ratio of the anti-factor Xa activity to anti-factor IIa activity is between 1.5 and 2.5.

IDENTIFICATION

Carry out identification test A as described in the monograph *Low-molecular-mass heparins (0828)* using *tinzaparin sodium CRS*.

Carry out identification test C as described in the monograph *Low-molecular-mass heparins (0828)*. The following requirements apply.

The mass-average relative molecular mass ranges between 5500 and 7500. The mass percentage of chains lower than 2000 is not more than 10.0 per cent. The mass percentage of chains between 2000 and 8000 ranges between 60.0 and 72.0 per cent. The mass percentage of chains above 8000 ranges between 22.0 and 36.0 per cent.

Monographs T

TESTS

**Appearance of solution**. Dissolve 1.0 g in 10 mL of *water R*. The solution is clear (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*)

**Absorbance** (*2.2.25*). Dissolve 50.0 mg in 100 mL of *0.01 M hydrochloric acid*. The specific absorbance, measured at 231 nm and calculated with reference to the dried substance, is 8.0 to 12.5.

**01/2008:2074**

# TIOCONAZOLE

## Tioconazolum

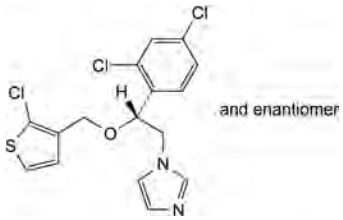


$C_{16}H_{13}Cl_3N_2OS$ $M_r$ 387.7
[65899-73-2]

DEFINITION

1-[(2*RS*)-2-[(2-Chlorothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very slightly soluble in water, very soluble in methylene chloride, freely soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of tioconazole*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 2.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *tioconazole for system suitability CRS* in the mobile phase and dilute to 2.5 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm) with a specific surface area of 170 $m^2$/g, a pore size of 12 nm and a carbon loading of 10 per cent.

*Mobile phase*: mix 1 volume of a 1.7 g/L solution of *tetrabutylammonium dihydrogen phosphate R* previously adjusted to pH 7.4 with *dilute ammonia R2* and 3 volumes of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 218 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of tioconazole.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.0 between the peaks due to impurity B and impurity C (locate impurities A, B and C by comparison with the chromatogram provided with *tioconazole for system suitability CRS*).

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.7; impurity C = 1.7.
- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent),
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent),
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 38.77 mg of $C_{16}H_{13}Cl_3N_2OS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D.*

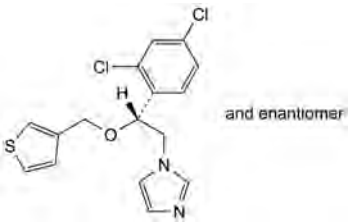


A. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[(thiophen-3-yl)methoxy]ethyl]-1*H*-imidazole,

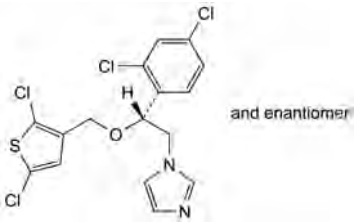


B. 1-[(2*RS*)-2-(2,4-dichlorophenyl)-2-[(2,5-dichlorothiophen-3-yl)methoxy]ethyl]-1*H*-imidazole,

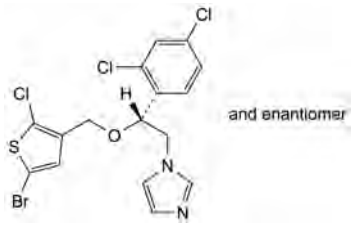


C. 1-[(2*RS*)-2-[(5-bromo-2-chlorothiophen-3-yl)methoxy]-2-(2,4-dichlorophenyl)ethyl]-1*H*-imidazole,

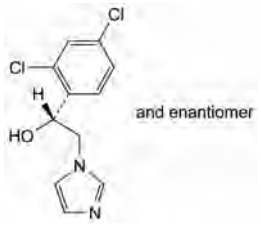


D. (1*RS*)-1-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-yl)ethanol.

**01/2018:2420**

# TIOTROPIUM BROMIDE MONOHYDRATE

## Tiotropii bromidum monohydricum

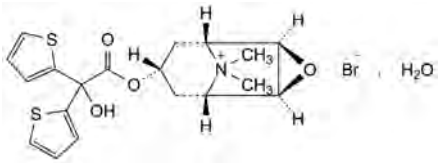


$C_{19}H_{22}BrNO_4S_2,H_2O$ $M_r$ 490.4
[411207-31-3]

DEFINITION

(1*R*,2*R*,4*S*,5*S*,7*s*)-7-[[Hydroxy[di(thiophen-2-yl)]acetyl]oxy]-9,9-dimethyl-3-oxa-9-azatricyclo[3.3.1.$0^{2,4}$]nonan-9-ium bromide monohydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish-white powder or crystals.

*Solubility*: sparingly soluble in water, soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tiotropium bromide monohydrate CRS*.

B. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 0.2 g in *water R* and dilute to 20 mL with the same solvent.

**Impurities G and H**. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use.*

*Solvent mixture.* Dilute 1 volume of a 103 g/L solution of *hydrochloric acid R* to 100 volumes with *methanol R*.

*Test solution.* Dissolve 0.40 g of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *tiotropium impurity mixture CRS* (containing impurities G and H) in 1.0 mL of the solvent mixture.

*Reference solution (b).* Mix 0.1 mL of the test solution with 0.1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Mobile phase*: *water R*, *anhydrous formic acid R*, *acetonitrile R*, *methylene chloride R* (10:15:35:50 *V/V/V/V*).

*Application*: 10 µL of the test solution and reference solution (a), and 20 µL of reference solution (b).

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: expose to iodine vapour until the spots are clearly visible (about 15 min); remove the plate and examine immediately.

*Retardation factors*: impurity G = about 0.33; impurity H = about 0.38; tiotropium = about 0.64.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Limits*:

– *impurity G*: any spot due to impurity G is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.1 per cent);
– *impurity H*: any spot due to impurity H is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (b).* Dissolve 4 mg of *tiotropium for system suitability 1 CRS* (containing impurity C) in 2.0 mL of mobile phase B.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.0 mm;
– *stationary phase*: *propylsilyl silica gel for chromatography R* (3.5 µm);
– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: dissolve 1.0 g of *sodium methanesulfonate R* and 5.0 g of *potassium dihydrogen phosphate R* in about 980 mL of *water R*, adjust to pH 3.0 with *dilute phosphoric acid R* and dilute to 1000 mL with *water R*;
– *mobile phase B*: *methanol R1*, *acetonitrile for chromatography R*, mobile phase A (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 90 | 10 |
| 3 - 17 | 90 → 80 | 10 → 20 |
| 17 - 28 | 80 → 25 | 20 → 75 |
| 28 - 30 | 25 | 75 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Injection*: 5 µL.

Monographs T

*Identification of impurities*: use the chromatogram supplied with *tiotropium for system suitability 1 CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to tiotropium (retention time = about 15 min): impurity C = about 1.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.4 between the peaks due to tiotropium and impurity C.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): 2.5 per cent to 4.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.35 g in 100 mL of *water R*. Add 10 mL of *dilute nitric acid R2*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 47.24 mg of $C_{19}H_{22}BrNO_4S_2$.

IMPURITIES

*Specified impurities: G, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E, F.*

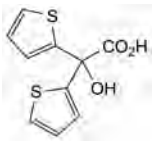

A. hydroxy[di(thiophen-2-yl)]acetic acid,

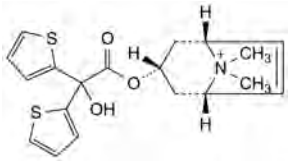

C. (1*R*,3*s*,5*S*)-3-[[hydroxy[di(thiophen-2-yl)]acetyl]oxy]-8,8-dimethyl-8-azabicyclo[3.2.1]oct-6-en-8-ium,

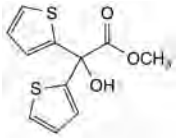

E. methyl hydroxy[di(thiophen-2-yl)]acetate,

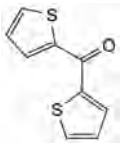

F. di(thiophen-2-yl)methanone,

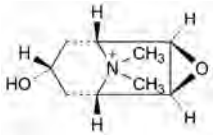

G. (1*R*,2*R*,4*S*,5*S*,7*s*)-7-hydroxy-9,9-dimethyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]nonan-9-ium,

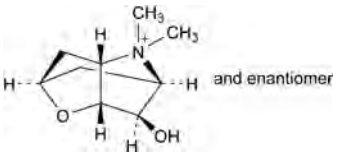


H. (1*s*,3*RS*,4*RS*,5*RS*,7*SR*)-4-hydroxy-6,6-dimethyl-2-oxa-6-azatricyclo[3.3.1.0$^{3,7}$]nonan-6-ium.

**01/2017:0150**

# TITANIUM DIOXIDE

## Titanii dioxidum

$TiO_2$ $M_r$ 79.9
[13463-67-7]

DEFINITION

*Content*: 98.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water. It does not dissolve in dilute mineral acids but dissolves slowly in hot concentrated sulfuric acid.

IDENTIFICATION

A. When strongly heated, it becomes pale yellow; the colour disappears on cooling.

B. To 5 mL of solution S2 (see Tests) add 0.1 mL of *strong hydrogen peroxide solution R*. An orange-red colour appears.

C. To 5 mL of solution S2 add 0.5 g of *zinc R* in granules. After 45 min, the mixture has a violet-blue colour.

TESTS

**Solution S1**. Shake 20.0 g with 30 mL of *hydrochloric acid R* for 1 min. Add 100 mL of *distilled water R* and heat the mixture to boiling. Filter the hot mixture through a hardened filter paper until a clear filtrate is obtained. Wash the filter with 60 mL of *distilled water R* and dilute the combined filtrate and washings to 200 mL with *distilled water R*.

**Solution S2**. Mix 0.500 g (*m* g) with 5 g of *anhydrous sodium sulfate R* in a 300 mL long-necked combustion flask. Add 10 mL of *water R* and mix. Add 10 mL of *sulfuric acid R* and boil vigorously, with the usual precautions, until a clear solution is obtained. Cool, add slowly a cooled mixture of 30 mL of *water R* and 10 mL of *sulfuric acid R*, cool again and dilute to 100.0 mL with *water R*.

**Appearance of solution**. Solution S2 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. Shake 5.0 g with 50 mL of *carbon dioxide-free water R* for 5 min. Centrifuge or filter until a clear solution is obtained. To 10 mL of the solution add 0.1 mL of *bromothymol blue solution R1*. Not more than 1.0 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Water-soluble substances**: maximum 0.5 per cent.

To 10.0 g add a solution of 0.5 g of *ammonium sulfate R* in 150 mL of *water R* and boil for 5 min. Cool, dilute to 200 mL with *water R* and filter until a clear solution is obtained. Evaporate 100 mL of the solution to dryness in a tared evaporating dish and ignite. The residue weighs a maximum of 25 mg.

**Antimony**: maximum 100 ppm.

To 10 mL of solution S2 add 10 mL of *hydrochloric acid R* and 10 mL of *water R*. Cool to 20 °C, if necessary, and add 0.15 mL of *sodium nitrite solution R*. After 5 min, add 5 mL of a 10 g/L solution of *hydroxylamine hydrochloride R* and 10 mL of a freshly prepared 0.1 g/L solution of *rhodamine B R*. Mix thoroughly after each addition. Shake vigorously with 10.0 mL of *toluene R* for 1 min. Allow to separate and centrifuge for 2 min if necessary. Any pink colour in the toluene phase is not more intense than that in the toluene phase of a standard prepared at the same time in the same manner using a mixture of 5.0 mL of *antimony standard solution (1 ppm Sb) R*, 10 mL of *hydrochloric acid R* and 15 mL of a solution containing 0.5 g of *anhydrous sodium sulfate R* and 2 mL of *sulfuric acid R* instead of the mixture of 10 mL of solution S2, 10 mL of *hydrochloric acid R* and 10 mL of *water R*.

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm.

Place 0.50 g in a 250 mL round-bottomed flask, fitted with a thermometer, a funnel with stopcock and a vapour-outlet tube connected to a flask containing 30 mL of *water R*. Add 50 mL of *water R*, 0.5 g of *hydrazine sulfate R*, 0.5 g of *potassium bromide R* and 20 g of *sodium chloride R*. Through the funnel, add dropwise 25 mL of *sulfuric acid R*, heat and maintain the temperature of the liquid at 110-115 °C for 20 min. Collect the vapour in the flask containing 30 mL of *water R*. Dilute to 50 mL with *water R*. 20 mL of the solution complies with the test.

**Barium**. To 10 mL of solution S1 add 1 mL of *dilute sulfuric acid R*. After 30 min, any opalescence in the solution is not more intense than that in a mixture of 10 mL of solution S1 and 1 mL of *distilled water R*.

**Iron**: maximum 200 ppm.

To 8 mL of solution S2 add 4 mL of *water R*. Mix and add 0.05 mL of *bromine water R*. Allow to stand for 5 min and remove the excess of bromine with a current of air. Add 3 mL of *potassium thiocyanate solution R*. Any colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 4 mL of *iron standard solution (2 ppm Fe) R* and 8 mL of a 200 g/L solution of *sulfuric acid R*.

ASSAY

To 300 g of *zinc R* in granules (710) add 300 mL of a 20 g/L solution of *mercuric nitrate R* and 2 mL of *nitric acid R*, shake for 10 min and wash with *water R*. Pack the amalgamated zinc into a glass tube about 400 mm long and about 20 mm in diameter fitted with a tap and a filter plate. Pass through the column 100 mL of *dilute sulfuric acid R* followed by 100 mL of *water R*, making sure that the amalgam is always covered with liquid. Pass slowly at a rate of about 3 mL/min through the column a mixture of 100 mL of *dilute sulfuric acid R* and 100 mL of *water R* followed by 100 mL of *water R*. Collect the eluate in a 500 mL conical flask containing 50.0 mL of a 150 g/L solution of *ferric ammonium sulfate R* in a mixture of 1 volume of *sulfuric acid R* and 3 volumes of *water R*. Add 0.1 mL of *ferroin R* and titrate immediately with *0.1 M ammonium and cerium nitrate* until a greenish colour is obtained ($n_1$ mL). Pass slowly at a rate of about 3 mL/min through the column a mixture of 50 mL of *dilute sulfuric acid R* and 50 mL of *water R*, followed by 20.0 mL of solution S2, a mixture of 50 mL of *dilute sulfuric acid R* and 50 mL of *water R* and finally 100 mL of *water R*. Collect the eluate in a 500 mL conical flask containing 50.0 mL of a 150 g/L solution of *ferric ammonium sulfate R* in a mixture of 1 volume of *sulfuric acid R* and 3 volumes of *water R*. Rinse the lower end of the column with *water R*, add 0.1 mL of *ferroin R* and titrate immediately with *0.1 M ammonium and cerium nitrate* until a greenish colour is obtained ($n_2$ mL).

Calculate the percentage content of $TiO_2$ using the following expression:

$$\frac{3.99 \times (n_2 - n_1)}{m}$$

$m$ = mass of the substance to be examined used for the preparation of solution S2, in grams.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristic may be relevant for titanium dioxide used as opacifier in solid oral dosage forms and in preparations for cutaneous application.*

**Particle-size distribution** (*2.9.31*).

**01/2017:2578**
**corrected 10.0**

# TIZANIDINE HYDROCHLORIDE

## Tizanidini hydrochloridum

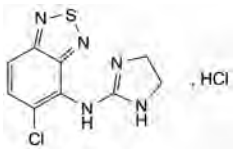


$C_9H_9Cl_2N_5S$ $M_r$ 290.2
[64461-82-1]

DEFINITION

5-Chloro-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-2,1,3-benzothiadiazol-4-amine hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tizanidine hydrochloride CRS*.

B. Solution S (see Tests) gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**pH** (*2.2.3*): 3.7 to 5.0 for solution S.

**Impurity H**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *methanol R* and dilute to 5.0 mL with the same solvent.

*Reference solution*. Dissolve 8.0 mg of *ethylenediamine R* (impurity H) in *methanol R* and dilute to 100.0 mL with the same solvent.

*Plate*: *TLC cellulose plate R*.

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (1:25:75 *V/V/V*).

*Application*: 5 µL.

*Development*: over 1/2 of the plate.

*Drying*: in a current of cold air.

*Detection*: spray with a mixture of 15 volumes of a 20 g/L solution of *ninhydrin R* in *glacial acetic acid R* and 85 volumes of *butanol R*, then heat at 100 °C for 5 min.

*Relative retention* with reference to tizanidine ($R_F$ = about 0.8): impurity H = about 0.3.

*Limit*:

– *impurity H*: any spot due to impurity H is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 30.0 mg of the substance to be examined in mobile phase A, using sonication if necessary, and dilute to 25.0 mL with mobile phase A. Dilute 5.0 mL of the solution to 50.0 mL with mobile phase A.

*Reference solution (a)*. Dissolve 3 mg of *tizanidine impurity B CRS* in mobile phase A and dilute to 25.0 mL with mobile phase A. Dilute 1.0 mL of the solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with the test solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (c)*. Dissolve 30.0 mg of *tizanidine hydrochloride CRS* in mobile phase A, using sonication if necessary, and dilute to 25.0 mL with mobile phase A. Dilute 5.0 mL of the solution to 50.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3.5 µm);

– *temperature*: 50 °C.

*Mobile phase*:

– *mobile phase A*: mix 18 volumes of *acetonitrile R1* and 82 volumes of a 3.86 g/L solution of *sodium pentanesulfonate monohydrate R* previously adjusted to pH 3.0 with *phosphoric acid R*;

– *mobile phase B*: mix 20 volumes of a 3.86 g/L solution of *sodium pentanesulfonate monohydrate R* previously adjusted to pH 3.0 with *phosphoric acid R* and 80 volumes of *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 100 | 0 |
| 15 - 25 | 100 → 0 | 0 → 100 |
| 25 - 27 | 0 | 100 |

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity B.

*Relative retention* with reference to tizanidine (retention time = about 6 min): impurity B = about 1.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to tizanidine and impurity B.

*Calculation of percentage contents*:

– for each impurity, use the concentration of tizanidine hydrochloride in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (c).

Calculate the percentage content of $C_9H_9Cl_2N_5S$ taking into account the assigned content of *tizanidine hydrochloride CRS*.

IMPURITIES

*Specified impurities*: *H*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, I*.

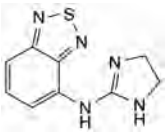

A. *N*-(4,5-dihydro-1*H*-imidazol-2-yl)-2,1,3-benzothiadiazol-4-amine,

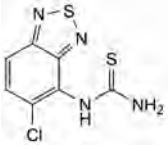

B. *N*-(5-chloro-2,1,3-benzothiadiazol-4-yl)thiourea,

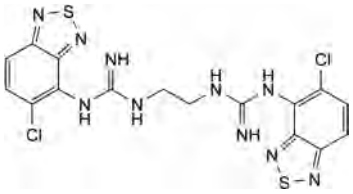

C. 1,1′-ethane-1,2-diylbis[3-(5-chloro-2,1,3-benzothiadiazol-4-yl)guanidine],

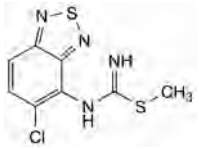

D. methyl *N*-(5-chloro-2,1,3-benzothiadiazol-4-yl)-carbamimidothioate,

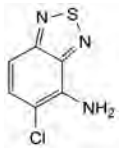

E. 5-chloro-2,1,3-benzothiadiazol-4-amine,

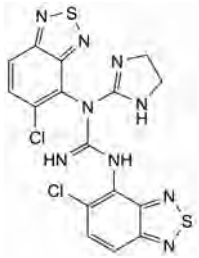

F. 1,3-bis(5-chloro-2,1,3-benzothiadiazol-4-yl)-1-(4,5-dihydro-1*H*-imidazol-2-yl)guanidine,

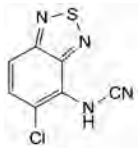

G. (5-chloro-2,1,3-benzothiadiazol-4-yl)cyanamide,

H. ethane-1,2-diamine (ethylenediamine),

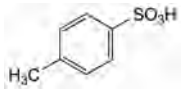

I. 4-methylbenzenesulfonic acid (*p*-toluenesulfonic acid).

**01/2008:0645**
**corrected 10.0**

# TOBRAMYCIN

## Tobramycinum

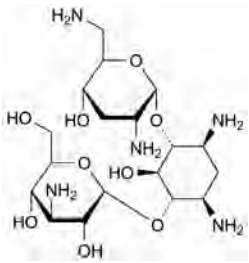

$C_{18}H_{37}N_5O_9$ $M_r$ 467.5
[32986-56-4]

DEFINITION

4-*O*-(3-Amino-3-deoxy-α-D-glucopyranosyl)-2-deoxy-6-*O*-(2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl)-L-streptamine.

Substance produced by *Streptomyces tenebrarius* or obtained by any other means.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is produced by methods of manufacture designed to eliminate or minimise substances lowering blood pressure.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: A.

*Second identification*: B, C.

A. Nuclear magnetic resonance spectrometry (*2.2.33*).

*Preparation*: 100 g/L solution in *deuterium oxide R*.

*Comparison*: 100 g/L solution of *tobramycin CRS* in *deuterium oxide R*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in *water R* and dilute to 5 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *tobramycin CRS* in *water R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dissolve 4 mg of *neomycin sulfate CRS* and 4 mg of *kanamycin monosulfate CRS* in 1 mL of reference solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *methylene chloride R*, *concentrated ammonia R*, *methanol R* (17:33:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection*: spray with a mixture of equal volumes of a 2 g/L solution of *1,3-dihydroxynaphthalene R* in *ethanol (96 per cent) R* and a 460 g/L solution of *sulfuric acid R*; heat at 105 °C for 5-10 min.

*System suitability*: the chromatogram obtained with reference solution (b) shows 3 major spots which are clearly separated.

Monographs T

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 5 mg in 5 mL of *water R*. Add 5 mL of a 1 g/L solution of *ninhydrin R* in *ethanol (96 per cent) R* and heat in a water-bath for 3 min. A violet-blue colour develops.

TESTS

**pH** (*2.2.3*): 9.0 to 11.0.

Dissolve 1.0 g in 10 mL of *carbon dioxide-free water R*.

**Specific optical rotation** (*2.2.7*): + 138 to + 148 (anhydrous substance).

Dissolve 1.00 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *tobramycin CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Reference solution (d).* Dissolve 10.0 mg of *kanamycin B sulfate CRS* in 20.0 mL of the mobile phase. To 1.0 mL of this solution, add 2.0 mL of reference solution (a) and dilute to 10.0 mL with the mobile phase.

*Reference solution (e).* Dilute 10.0 mL of reference solution (a) to 25.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (8 µm) with a pore size of 100 nm;
- *temperature*: 55 °C.

*Mobile phase*: mixture prepared with *carbon dioxide-free water R* containing 52 g/L of *anhydrous sodium sulfate R*, 1.5 g/L of *sodium octanesulfonate R*, 3 mL/L of *tetrahydrofuran R* stabilised with *butylhydroxytoluene R*, and 50 mL/L of *0.2 M potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *dilute phosphoric acid R*. Degas.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: *carbonate-free sodium hydroxide solution R* diluted 25-fold with *carbon dioxide-free water R*, which is added pulselessly to the column effluent using a 375 µL polymeric mixing coil.

*Flow rate*: 0.3 mL/min.

*Detection*: pulsed amperometric detector or equivalent with a gold working electrode, a silver-silver chloride reference electrode and a stainless steel auxiliary electrode which is the cell body, held at respectively + 0.05 V detection, + 0.75 V oxidation and − 0.15 V reduction potentials, with pulse durations according to the instrument used. The temperature of the detector is set at 35 °C.

*NOTE: to prevent problems due to salt precipitation, the electrochemical cell can be flushed with water R overnight.*

*Injection*: 20 µL using a refrigerated injector (4-8 °C); inject test solution (a) and reference solutions (b), (c) and (d).

*Run time*: 1.5 times the retention time of tobramycin.

*Relative retention* with reference to tobramycin (retention time = about 18 min): impurity C = about 0.35; impurity B = about 0.40, impurity A = about 0.70.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to impurity A and to tobramycin in the chromatogram obtained with reference solution (d); if necessary, adjust the concentration of sodium octanesulfonate in the mobile phase;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *any impurity*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent) and not more than 1 such peak has an area greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (c) (1.5 per cent);
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (b) (0.25 per cent).

**2-Methyl-1-propanol** (*2.4.24, System B*): maximum 1.0 per cent *m/m*.

**Water** (*2.5.12*): maximum 8.0 per cent, determined on 0.30 g.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 2.0 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (e).

Calculate the percentage content of tobramycin.

STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

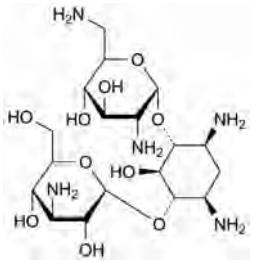

A. 4-*O*-(3-amino-3-deoxy-α-D-glucopyranosyl)-2-deoxy-6-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-L-streptamine (kanamycin B),

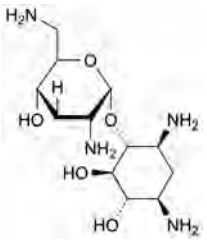

B. 2-deoxy-4-*O*-(2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl)-D-streptamine (nebramine),

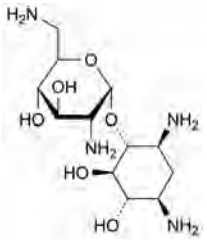

C. 2-deoxy-4-*O*-(2,6-diamino-2,6-dideoxy-α-D-glucopyranosyl)-D-streptamine (neamine).

**07/2011:0692**
**corrected 10.0**

# all-*rac*-α-TOCOPHEROL

## int-*rac*-α-Tocopherolum

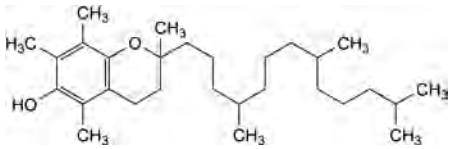

$C_{29}H_{50}O_2$ $M_r$ 430.7
[10191-41-0]

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-ol.

*Content*: 96.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or yellowish-brown, viscous, oily liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

*First identification*: *A, B.*

*Second identification*: *A, C.*

A. Optical rotation (*2.2.7*): − 0.01° to + 0.01°.

Dissolve 2.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *α-tocopherol CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of *cyclohexane R.*

*Reference solution.* Dissolve 10 mg of *α-tocopherol CRS* in 2 mL of *cyclohexane R.*

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of the internal standard solution.

*Test solution (b).* Dissolve 0.100 g of the substance to be examined in 10 mL of *cyclohexane R.*

*Reference solution (a).* Dissolve 0.100 g of *α-tocopherol CRS* in 10.0 mL of the internal standard solution.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined and 10 mg of *α-tocopheryl acetate R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dissolve 10 mg of *all-rac-α-tocopherol for peak identification CRS* (containing impurities A and B) in *cyclohexane R* and dilute to 1 mL with the same solvent.

*Reference solution (d).* Dilute 1.0 mL of test solution (b) to 100.0 mL with *cyclohexane R*. Dilute 1.0 mL of this solution to 10.0 mL with *cyclohexane R.*

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

- *column*: 280 °C;
- *injection port and detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (b) and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of all-*rac*-α-tocopherol.

*Identification of impurities*: use the chromatogram supplied with *all-rac-α-tocopherol for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to all-*rac*-α-tocopherol (retention time = about 9 min): squalane = about 0.5; impurity A = about 0.7; impurity B = about 0.8; impurities C and D = about 1.05 (eluting immediately after the all-*rac*-α-tocopherol peak).

*System suitability*: reference solution (b):

- *resolution*: minimum 3.5 between the peaks due to all-*rac*-α-tocopherol and α-tocopheryl acetate.

*Limits*:

- *impurity A*: maximum 0.5 per cent;
- *impurity B*: maximum 1.5 per cent;
- *sum of impurities C and D*: maximum 1.0 per cent;
- *any other impurity*: for each impurity, maximum 0.25 per cent;
- *total*: maximum 2.5 per cent;
- *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: minimum 0.6 for the principal peak.

Calculate the percentage content of $C_{29}H_{50}O_2$ from the declared content of *α-tocopherol CRS*.

STORAGE

Under an inert gas, protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

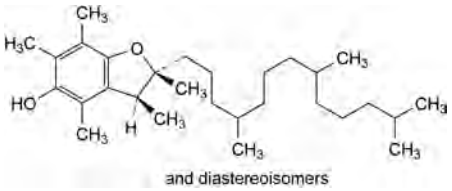


A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol,

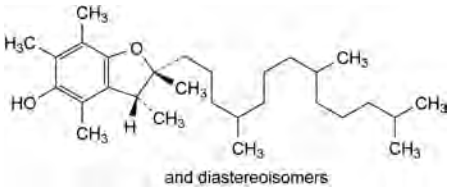


B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-ol,

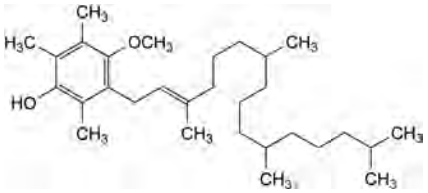

C. 4-methoxy-2,3,6-trimethyl-5-[(all-*RS*,*E*)-3,7,11,15-tetramethylhexadec-2-enyl]phenol,

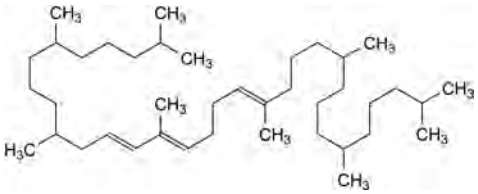

D. (all-*RS*,all-*E*)-2,6,10,14,19,23,27,31-octamethyldotriaconta-12,14,18-triene.

**04/2013:1256**
**corrected 10.0**

# *RRR*-α-TOCOPHEROL

## *RRR*-α-Tocopherolum

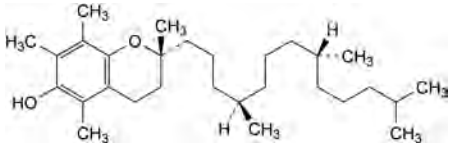

$C_{29}H_{50}O_2$ $M_r$ 430.7

[59-02-9]

DEFINITION

(2*R*)-2,5,7,8-Tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol.

*Content*: 94.5 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or yellowish-brown, viscous, oily liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol, in methylene chloride and in fatty oils.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Optical rotation (*2.2.7*): + 0.05° to + 0.10°.
Dissolve 2.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *α-tocopherol CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 2 mL of *cyclohexane R*.
*Reference solution.* Dissolve 10 mg of *α-tocopherol CRS* in 2 mL of *cyclohexane R*.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of the internal standard solution.

*Test solution (b).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of *cyclohexane R*.

*Reference solution (a).* Dissolve 0.100 g of *α-tocopherol CRS* in 10.0 mL of the internal standard solution.

*Reference solution (b).* Dissolve 10 mg of *α-tocopherol R* and 10 mg of *α-tocopheryl acetate R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Column*:

– *material*: fused silica;
– *size*: *l* = 30 m, Ø = 0.25 mm;
– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 280 |
| Injection port | | 290 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (b) and reference solution (b).

*System suitability*: reference solution (b):

– *resolution*: minimum 3.5 between the peaks due to α-tocopherol and α-tocopheryl acetate.

*Limits*:

– *total*: maximum 4.0 per cent;

– *disregard limit*: 0.1 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: minimum 0.6 for the principal peak.

Calculate the percentage content of $C_{29}H_{50}O_2$ taking into account the assigned content of *α-tocopherol CRS*.

STORAGE

Under an inert gas, protected from light.

IMPURITIES

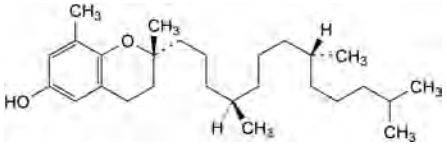

A. (2*R*)-2,8-dimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol (*RRR*-δ-tocopherol),

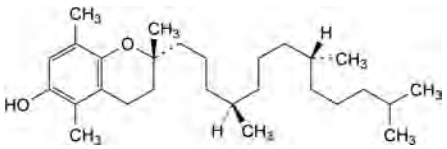

B. (2*R*)-2,5,8-trimethyl-2-[(4*R*,8*R*)4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol (*RRR*-β-tocopherol),

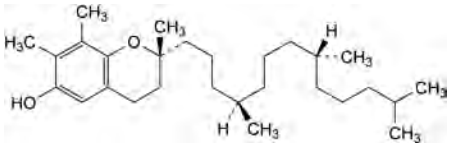

C. (2*R*)-2,7,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol (*RRR*-γ-tocopherol).

**07/2011:0439**
**corrected 10.0**

# all-*rac*-α-TOCOPHERYL ACETATE

## int-*rac*-α-Tocopherylis acetas

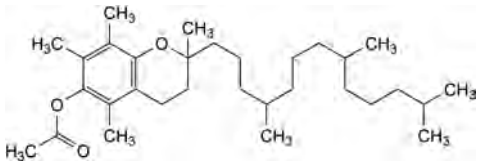

$C_{31}H_{52}O_3$ $M_r$ 472.7

[7695-91-2]

DEFINITION

all-*rac*-2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

*Content*: 96.5 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or slightly greenish-yellow, viscous, oily liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol and in fatty oils.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Optical rotation (*2.2.7*): – 0.01° to + 0.01°.
Dissolve 2.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *α-tocopheryl acetate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve about 10 mg of the substance to be examined in 2 mL of *cyclohexane R*.
*Reference solution.* Dissolve about 10 mg of *α-tocopheryl acetate CRS* in 2 mL of *cyclohexane R*.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of the internal standard solution.

*Test solution (b).* Dissolve 0.100 g of the substance to be examined in 10 mL of *cyclohexane R*.

*Reference solution (a).* Dissolve 0.100 g of *α-tocopheryl acetate CRS* in 10.0 mL of the internal standard solution.

*Reference solution (b).* Dissolve 10 mg of the substance to be examined and 10 mg of *α-tocopherol R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c).* Dissolve 10 mg of *all-rac-α-tocopheryl acetate for peak identification CRS* (containing impurities A and B) in *cyclohexane R* and dilute to 1 mL with the same solvent.

*Reference solution (d).* Dilute 1.0 mL of test solution (b) to 100.0 mL with *cyclohexane R*. Dilute 1.0 mL of this solution to 10.0 mL with *cyclohexane R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.25 mm;

– *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

– *column*: 280 °C;

– *injection port and detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (b) and reference solutions (a), (b), (c) and (d); inject directly onto the column or via a sufficiently inert, glass-lined injection port using an automatic injection device or other reproducible injection method.

*Run time*: twice the retention time of all-*rac*-α-tocopheryl acetate.

*Identification of impurities*: use the chromatogram supplied with *all-rac-α-tocopheryl acetate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A and B.

*Relative retention* with reference to all-*rac*-α-tocopheryl acetate (retention time = about 15 min): squalane = about 0.4; impurity A = about 0.7; impurity B = about 0.8; impurity C = about 0.9; impurities D and E = about 1.05 (eluting immediately after the all-*rac*-α-tocopheryl acetate peak).

*System suitability*:

– *resolution*: minimum 3.5 between the peaks due to impurity C and all-*rac*-α-tocopheryl acetate in the chromatogram obtained with reference solution (b);

– in the chromatogram obtained with reference solution (a), the area of the peak due to impurity C is not greater than 0.2 per cent of the area of the peak due to all-*rac*-α-tocopheryl acetate.

*Limits*:

– *impurities A, C*: for each impurity, maximum 0.5 per cent;

– *impurity B*: maximum 1.5 per cent;

– *sum of impurities D and E*: maximum 1.0 per cent;

– *any other impurity*: for each impurity, maximum 0.25 per cent;

– *total*: maximum 2.5 per cent;

– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: minimum 0.6 for the principal peak.

Calculate the percentage content of $C_{31}H_{52}O_3$ from the declared content of *α-tocopheryl acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

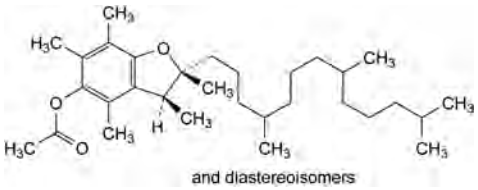


A. all-*rac-trans*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,

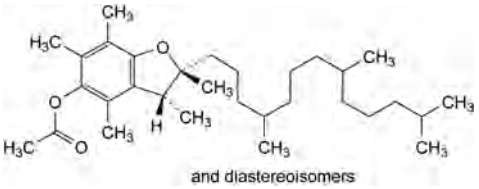


B. all-*rac-cis*-2,3,4,6,7-pentamethyl-2-(4,8,12-trimethyltridecyl)-2,3-dihydrobenzofuran-5-yl acetate,

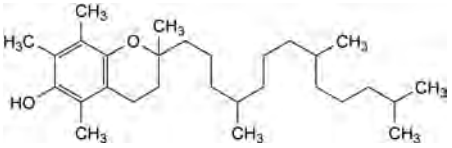

C. all-*rac*-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-3,4-dihydro-2*H*-1-benzopyran-6-ol (all-*rac*-α-tocopherol),

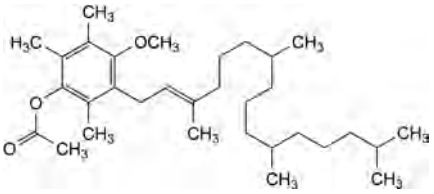

D. 4-methoxy-2,3,6-trimethyl-5-[(all-*RS*,*E*)-3,7,11,15-tetramethylhexadec-2-enyl]phenyl acetate,

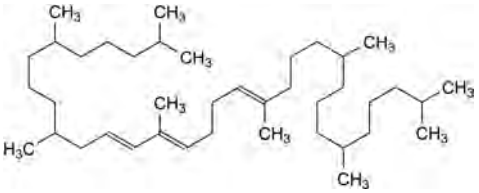

E. (all-*RS*,all-*E*)-2,6,10,14,19,23,27,31-octamethyldotriaconta-12,14,18-triene.

**04/2013:1257**
**corrected 10.0**

# *RRR*-α-TOCOPHERYL ACETATE

## *RRR*-α-Tocopherylis acetas

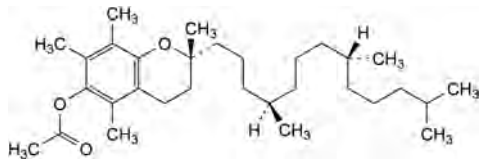

$C_{31}H_{52}O_3$ $M_r$ 472.7

DEFINITION

(2*R*)-2,5,7,8-Tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate.

*Content*: 95.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: clear, colourless or slightly greenish-yellow, viscous, oily liquid.

*Solubility*: practically insoluble in water, freely soluble in acetone, in anhydrous ethanol and in fatty oils, soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification*: *A, C.*

A. Optical rotation (*2.2.7*): + 0.25° to + 0.35°.
Dissolve 2.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *α-tocopheryl acetate CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 10 mg of the substance to be examined in 2 mL of *cyclohexane R*.
*Test solution (b).* In a ground-glass stoppered tube, dissolve about 10 mg of the substance to be examined in 2 mL of *2.5 M alcoholic sulfuric acid R*. Heat on a water-bath for 5 min. Cool and add 2 mL of *water R* and 2 mL of *cyclohexane R*. Shake for 1 min. Use the upper layer.
*Reference solution (a).* Dissolve 10 mg of *α-tocopheryl acetate CRS* in 2 mL of *cyclohexane R*.
*Reference solution (b).* Prepare as described for test solution (b), using *α-tocopheryl acetate CRS* instead of the substance to be examined.
*Plate*: *TLC silica gel $F_{254}$ plate R*.
*Mobile phase*: *ether R, cyclohexane R* (20:80 *V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of air.
*Detection*: examine in ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there may be 2 spots depending on the degree of hydrolysis: the spot with the higher $R_F$ value is due to α-tocopheryl acetate and corresponds to the spot in the chromatogram obtained with reference solution (a); the spot with the lower $R_F$ value is due to α-tocopherol.

TESTS

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Internal standard solution.* Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Test solution (a).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of the internal standard solution.

*Test solution (b).* Dissolve 0.100 g of the substance to be examined in 10.0 mL of *cyclohexane R*.

*Reference solution (a).* Dissolve 0.100 g of *α-tocopheryl acetate CRS* in 10.0 mL of the internal standard solution.

*Reference solution (b).* Dissolve 10 mg of *α-tocopherol R* and 10 mg of *α-tocopheryl acetate R* in *cyclohexane R* and dilute to 100.0 mL with the same solvent.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 15 | 280 |
| Injection port | | 290 |
| Detector | | 290 |

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (b) and reference solutions (a) and (b); inject directly onto the column or via a sufficiently inert, glass-lined injection port using an automatic injection device or other reproducible injection method.

*System suitability*:
- *resolution*: minimum 3.5 between the peaks due to α-tocopherol and α-tocopheryl acetate in the chromatogram obtained with reference solution (b);
- in the chromatogram obtained with reference solution (a), the area of the peak due to α-tocopherol is not greater than 0.2 per cent of the area of the peak due to α-tocopheryl acetate.

*Limits*:
- *total*: maximum 4.0 per cent;
- *disregard limit*: 0.1 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modifications.

*Injection*: test solution (a) and reference solution (a).

*System suitability*: reference solution (a):
- *symmetry factor*: minimum 0.6 for the principal peak.

Calculate the percentage content of $C_{31}H_{52}O_3$ taking into account the assigned content of *α-tocopheryl acetate CRS*.

STORAGE

Protected from light.

IMPURITIES

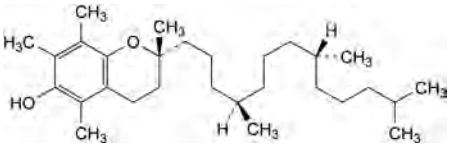

A. (2*R*)-2,5,7,8-tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-ol (*RRR*-α-tocopherol),

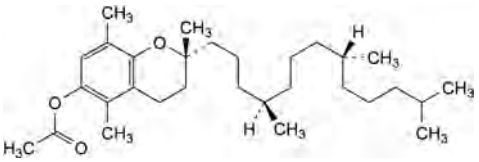

B. (2*R*)-2,5,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate (*RRR*-β-tocopheryl acetate),

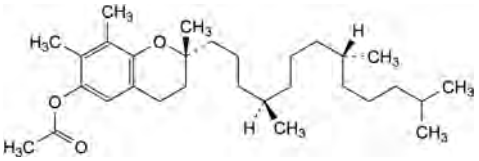

C. (2*R*)-2,7,8-trimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate (*RRR*-γ-tocopheryl acetate),

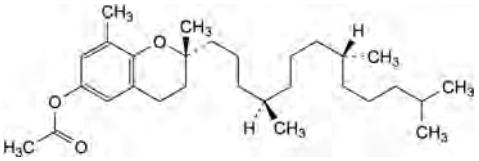

D. (2*R*)-2,8-dimethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl acetate (*RRR*-δ-tocopheryl acetate).

Monographs T

**01/2011:0691**
**corrected 10.0**

# α-TOCOPHERYL ACETATE CONCENTRATE (POWDER FORM)

## α-Tocopherylis acetatis pulvis

DEFINITION

Preparation obtained either by finely dispersing *all-rac-α-Tocopheryl acetate (0439)* in a suitable carrier of suitable quality (for example gelatin, acacia, carbohydrates, lactoproteins or a mixture thereof) or by adsorbing *all-rac-α-Tocopheryl acetate (0439)* on silicic acid of suitable quality.

*Content*: 90.0 per cent to 115.0 per cent of the α-tocopheryl acetate content stated on the label, which is not less than 25 g per 100 g of concentrate.

CHARACTERS

*Appearance*: almost white, yellowish or light brown, small particles.

*Solubility*: practically insoluble or swells or forms a dispersion in water, depending on the formulation.

IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* To a quantity of the preparation to be examined corresponding to 50 mg of α-tocopheryl acetate add 5 mL of *0.01 M hydrochloric acid* and treat with ultrasound at 60 °C. Add 5 mL of *anhydrous ethanol R* and 10 mL of *cyclohexane R*, shake for 1 min and centrifuge for 5 min. Use the upper layer.

*Reference solution.* Dissolve 50 mg of *α-tocopheryl acetate CRS* in *cyclohexane R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: 3/4 of the plate.

*Drying*: in a current of air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

B. Examine the chromatograms obtained in the assay.

*Results*:

- the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a);
- in the chromatogram obtained with reference solution (c) no additional principal peak is observed when compared with the chromatogram obtained with the test solution.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 1.0 g of *squalane R* in *cyclohexane R* and dilute to 500.0 mL with the same solvent.

*Test solution.* Weigh accurately a quantity of the preparation to be examined corresponding to about 0.100 g of α-tocopheryl acetate into a 250 mL conical flask. Add 20 mL of *1 M hydrochloric acid* and sonicate at 70 °C for 20 min. Add 50 mL of *anhydrous ethanol R* and 50.0 mL of the internal standard solution. Mix thoroughly for 30 min using a magnetic stirrer. Allow the 2 layers to separate and use the upper layer.

*Reference solution (a).* Dissolve 0.100 g of *α-tocopheryl acetate CRS* in 50.0 mL of the internal standard solution.

*Reference solution (b).* Dissolve 10 mg of *α-tocopherol R* and 10 mg of *α-tocopheryl acetate CRS* in 5.0 mL of *cyclohexane R*.

*Reference solution (c).* Mix 1.0 mL of the test solution and 1.0 mL of reference solution (a).

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

*Split ratio*: 1:100.

*Temperature*:

- *column*: 280 °C;
- *injection port and detector*: 290 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL; inject directly onto the column or via a sufficiently inert, glass-lined injection port.

*Run time*: 1.1 times the retention time of α-tocopheryl acetate.

*Relative retention* with reference to α-tocopheryl acetate (retention time = about 12 min): squalane = about 0.5; α-tocopherol = about 0.9.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to α-tocopherol and α-tocopheryl acetate in the chromatogram obtained with reference solution (b);
- in the chromatogram obtained with reference solution (a), the area of the peak due to α-tocopherol is not greater than 0.002 times the area of the peak due to α-tocopheryl acetate (0.2 per cent).

Calculate the percentage content of $C_{31}H_{52}O_3$ from the declared content of *α-tocopheryl acetate CRS*.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states the content of α-tocopheryl acetate, expressed in grams per 100 g of concentrate.

**01/2017:1258**
**corrected 10.0**

# DL-α-TOCOPHERYL HYDROGEN SUCCINATE

## DL-α-Tocopherylis hydrogenosuccinas

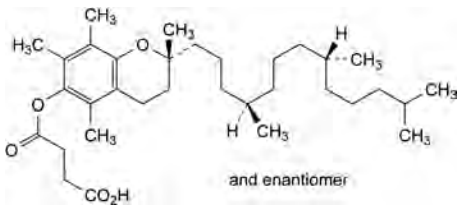


$C_{33}H_{54}O_5$ $M_r$ 530.8

DEFINITION

(2*RS*)-2,5,7,8-Tetramethyl-2-[(4*RS*,8*RS*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl hydrogen succinate.

*Content*: 96.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, soluble in acetone and in anhydrous ethanol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Absorbance (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *RRR-α-tocopheryl hydrogen succinate CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 10 mg of the substance to be examined in 2 mL of *cyclohexane R*.

*Test solution (b).* In a ground-glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 mL of *2.5 M alcoholic sulfuric acid R*. Heat on a water-bath for 5 min. Cool and add 2 mL of *water R* and 2 mL of *cyclohexane R*. Shake for 1 min. Use the upper layer.

*Reference solution (a).* Dissolve 10 mg of *RRR-α-tocopheryl hydrogen succinate CRS* in 2 mL of *cyclohexane R*.

*Reference solution (b).* Prepare as described for test solution (b), using *RRR-α-tocopheryl hydrogen succinate CRS* instead of the substance to be examined.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *ether R*, *cyclohexane R* (0.2:20:80 *V/V/V*).

*Application*: 10 µL.

*Development*: over a path of 15 cm.

*Drying*: in a current of air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are 2 spots: the spot with the higher $R_F$ value is due to α-tocopherol, the spot with the lower $R_F$ value is due to DL-α-tocopheryl hydrogen succinate and corresponds to the spot obtained with reference solution (a). Depending on the degree of hydrolysis, the lower spot may be weak or even absent.

*Detection B*: spray with a mixture of 10 volumes of *hydrochloric acid R*, 40 volumes of a 2.5 g/L solution of *ferric chloride R* in *ethanol (96 per cent) R* and 40 volumes of a 10 g/L solution of *phenanthroline hydrochloride R* in *ethanol (96 per cent) R*.

*Results B*: in the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α-tocopherol is orange.

D. Optical rotation (see Tests).

TESTS

**Optical rotation** (*2.2.7*): − 0.01° to + 0.01°.

Dissolve 2.50 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Absorbance** (*2.2.25*).

*Solution A*. Dissolve 0.150 g in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

*Test solution (a).* Dilute 10.0 mL of solution A to 100.0 mL with *anhydrous ethanol R*.

*Test solution (b).* Dilute 20.0 mL of solution A to 50.0 mL with *anhydrous ethanol R*.

*Absorption maximum*: at 284 nm for test solution (a).

*Absorption minimum*: at 254 nm for test solution (b).

*Specific absorbance at the absorption maximum*: 35 to 38 for test solution (a).

*Specific absorbance at the absorption minimum*: 6.0 to 8.0 for test solution (b).

**Acid value** (*2.5.1*): 101 to 108, determined on 1.00 g.

**Free tocopherol**: maximum 1.0 per cent.

Dissolve 0.500 g in 100 mL of *0.25 M alcoholic sulfuric acid R*. Add 20 mL of *water R* and 0.1 mL of a 2.5 g/L solution of *diphenylamine R* in *sulfuric acid R*. Titrate with *0.01 M ammonium and cerium sulfate* until a blue colour is obtained that persists for at least 5 s. Carry out a blank titration.

1 mL of *0.01 M ammonium and cerium sulfate* is equivalent to 2.154 mg of free tocopherol.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.300 g of *dotriacontane R* in *hexane R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Weigh 30.0 mg of the substance to be examined into a 20 mL vial. Add 2.0 mL of *methanol R*, 1.0 mL of *dimethoxypropane R* and 0.1 mL of *hydrochloric acid R*. Cap tightly and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 10.0 mL of the internal standard solution. Vortex into solution.

*Reference solution.* Weigh 30.0 mg of *RRR-α-tocopheryl hydrogen succinate CRS* into a 20 mL vial. Add 2.0 mL of *methanol R*, 1.0 mL of *dimethoxypropane R* and 0.1 mL of *hydrochloric acid R*. Cap tightly and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 10.0 mL of the internal standard solution. Vortex into solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3-6 mL/min.

*Split ratio*: 1:10 to 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 200 → 250 |
| | 10 - 20 | 250 |
| Injection port | | 300 |
| Detector | | 330 |

*Detection*: flame ionisation.

*Injection*: 1 µL; inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method.

*System suitability*: reference solution:

- *resolution*: minimum 12.0 between the peaks due to dotriacontane and DL-α-tocopheryl hydrogen succinate.

Monographs T

*Interference test.* Dissolve 0.100 g of the substance to be examined in *hexane R* and dilute to 50.0 mL with the same solvent. Inject 1 µL of the solution and record the chromatogram. If a peak is detected with the same retention time as that of the peak due to dotriacontane, calculate the area of this peak relative to the peak area of the substance to be examined. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(corr)}$ for the final calculation.

$$S'_{D(corr)} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

$S'_D$ = area of the peak due to dotriacontane in the chromatogram obtained with the test solution;

$S_I$ = area of the peak with the same retention time as that of the peak due to dotriacontane in the chromatogram obtained in the interference test;

$S'_T$ = area of the peak due to DL-α-tocopheryl hydrogen succinate in the chromatogram obtained with the test solution;

$S_{TI}$ = area of the peak due to DL-α-tocopheryl hydrogen succinate in the chromatogram obtained in the interference test.

Measure the areas of the peaks due to *RRR-α-tocopheryl hydrogen succinate CRS* ($S_T$) and dotriacontane ($S_D$) in the chromatogram obtained with the reference solution and the areas of the peaks due to DL-α-tocopheryl hydrogen succinate ($S'_T$) and dotriacontane ($S'_D$) in the chromatogram obtained with the test solution.

Determine the response factor (RF) for DL-α-tocopheryl hydrogen succinate from the areas of the peaks due to *RRR-α-tocopheryl hydrogen succinate CRS* and dotriacontane in the chromatogram obtained with the reference solution, using the following expression:

$$\frac{S_D \times m_T}{S_T \times m_D}$$

Calculate the percentage content of DL-α-tocopheryl hydrogen succinate using the following expression:

$$\frac{100 \times S'_T \times m_D \times RF}{S'_{D(corr)} \times m}$$

$S_D$ = area of the peak due to dotriacontane in the chromatogram obtained with the reference solution;

$S'_{D(corr)}$ = corrected area of the peak due to dotriacontane in the chromatogram obtained with the test solution;

$S_T$ = area of the peak due to *RRR-α-tocopheryl hydrogen succinate CRS* in the chromatogram obtained with the reference solution;

$S'_T$ = area of the peak due to DL-α-tocopheryl hydrogen succinate in the chromatogram obtained with the test solution;

$m_D$ = mass of dotriacontane in the test solution and in the reference solution, in milligrams;

$m_T$ = mass of *RRR-α-tocopheryl hydrogen succinate CRS* in the reference solution, in milligrams;

$m$ = mass of the substance to be examined in the test solution, in milligrams.

STORAGE

Protected from light.

**01/2017:1259**
**corrected 10.0**

# *RRR*-α-TOCOPHERYL HYDROGEN SUCCINATE

## *RRR*-α-Tocopherylis hydrogenosuccinas

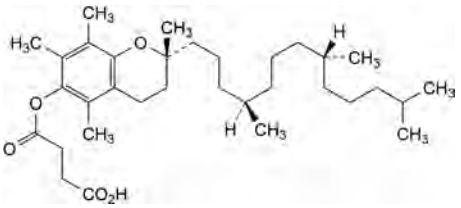

$C_{33}H_{54}O_5$ $M_r$ 530.8
[4345-03-3]

DEFINITION

(2*R*)-2,5,7,8-Tetramethyl-2-[(4*R*,8*R*)-4,8,12-trimethyltridecyl]-3,4-dihydro-2*H*-1-benzopyran-6-yl hydrogen succinate.

*Content*: 96.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in methylene chloride, soluble in acetone and in anhydrous ethanol.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Absorbance (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *RRR-α-tocopheryl hydrogen succinate CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution (a).* Dissolve 10 mg of the substance to be examined in 2 mL of *cyclohexane R*.
*Test solution (b).* In a ground-glass-stoppered tube, dissolve 10 mg of the substance to be examined in 2 mL of *2.5 M alcoholic sulfuric acid R*. Heat on a water-bath for 5 min. Cool and add 2 mL of *water R* and 2 mL of *cyclohexane R*. Shake for 1 min. Use the upper layer.
*Reference solution (a).* Dissolve 10 mg of *RRR-α-tocopheryl hydrogen succinate CRS* in 2 mL of *cyclohexane R*.
*Reference solution (b).* Prepare as described for test solution (b), using *RRR-α-tocopheryl hydrogen succinate CRS* instead of the substance to be examined.
*Plate*: *TLC silica gel $F_{254}$ plate R.*
*Mobile phase*: *glacial acetic acid R, ether R, cyclohexane R* (0.2:20:80 *V/V/V*).
*Application*: 10 µL.
*Development*: over a path of 15 cm.
*Drying*: in a current of air.
*Detection A*: examine in ultraviolet light at 254 nm.
*Results A*: the principal spot in the chromatogram obtained with test solution (a) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a). In the chromatograms obtained with test solution (b) and reference solution (b), there are 2 spots: the spot with the higher $R_F$ value is due to α-tocopherol, the spot with the lower $R_F$ value is due to *RRR*-α-tocopheryl

Monographs T

hydrogen succinate and corresponds to the spot obtained with reference solution (a). Depending on the degree of hydrolysis, the lower spot may be weak or even absent.

*Detection B*: spray with a mixture of 10 volumes of *hydrochloric acid R*, 40 volumes of a 2.5 g/L solution of *ferric chloride R* in *ethanol (96 per cent) R* and 40 volumes of a 10 g/L solution of *phenanthroline hydrochloride R* in *ethanol (96 per cent) R*.

*Results B*: in the chromatograms obtained with test solution (b) and reference solution (b), the spot due to α-tocopherol is orange.

D. After saponification, the resulting *RRR*-α-tocopherol is dextrorotatory (*2.2.7*). The specific optical rotation after oxidation to the quinone form is not less than + 24.

*Carry out the test avoiding exposure to actinic light.* Transfer 1.0 g to a round bottomed, ground-glass-stoppered, 250 mL flask, dissolve in 30 mL of *anhydrous ethanol R* and heat under reflux for 3 min. While the solution is boiling, add, through the condenser, 20 mL of *2 M alcoholic potassium hydroxide R*. Continue heating under reflux for 20 min and, without cooling, add 4.0 mL of *hydrochloric acid R* dropwise through the condenser. Cool, rinse the condenser with 10 mL of *anhydrous ethanol R*, transfer the contents of the flask to a 500 mL separating funnel, and rinse the flask with 4 quantities, each of 25 mL, of *water R* and 4 quantities, each of 25 mL, of *ether R*. Add the rinsings to the separating funnel. Shake vigorously for 2 min, allow the layers to separate and collect each of the 2 layers in individual separating funnels. Shake the aqueous layer with 2 quantities, each of 50 mL, of *ether R* and add these extracts to the main ether extract. Wash the combined ether extracts with 4 quantities, each of 100 mL, of *water R* and discard the washings.

To the ether solution add 40 mL of a 100 g/L solution of *potassium ferricyanide R* in an 8 g/L solution of *sodium hydroxide R* and shake for 3 min. Wash the ether solution with 4 quantities, each of 50 mL, of *water R*, discard the washings and dry the ether layer over *anhydrous sodium sulfate R*. Evaporate the ether on a water-bath under reduced pressure or in an atmosphere of nitrogen until a few millilitres remain, then complete the evaporation removing the last traces of ether without the application of heat. Immediately dissolve the residue in 25.0 mL of *trimethylpentane R* and determine the optical rotation.

Calculate the specific optical rotation of the substance in the test solution using as *c* the number of grams equivalent to α-tocopherol (factor 0.811) in 1000 mL.

TESTS

**Absorbance** (*2.2.25*).

*Solution A*. Dissolve 0.150 g in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

*Test solution (a).* Dilute 10.0 mL of solution A to 100.0 mL with *anhydrous ethanol R*.

*Test solution (b).* Dilute 20.0 mL of solution A to 50.0 mL with *anhydrous ethanol R*.

*Absorption maximum*: at 284 nm for test solution (a).

*Absorption minimum*: at 254 nm for test solution (b).

*Specific absorbance at the absorption maximum*: 35 to 38 for test solution (a).

*Specific absorbance at the absorption minimum*: 6.0 to 8.0 for test solution (b).

**Acid value** (*2.5.1*): 101 to 108, determined on 1.00 g.

**Free tocopherol**: maximum 1.0 per cent.

Dissolve 0.500 g in 100 mL of *0.25 M alcoholic sulfuric acid R*. Add 20 mL of *water R* and 0.1 mL of a 2.5 g/L solution of *diphenylamine R* in *sulfuric acid R*. Titrate with *0.01 M ammonium and cerium sulfate* until a blue colour is obtained that persists for at least 5 s. Carry out a blank titration.

1 mL of *0.01 M ammonium and cerium sulfate* is equivalent to 2.154 mg of free tocopherol.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 0.300 g of *dotriacontane R* in *hexane R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Weigh 30.0 mg of the substance to be examined into a 20 mL vial. Add 2.0 mL of *methanol R*, 1.0 mL of *dimethoxypropane R* and 0.1 mL of *hydrochloric acid R*. Cap tightly and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 10.0 mL of the internal standard solution. Vortex into solution.

*Reference solution.* Weigh 30.0 mg of *RRR-α-tocopheryl hydrogen succinate CRS* into a 20 mL vial. Add 2.0 mL of *methanol R*, 1.0 mL of *dimethoxypropane R* and 0.1 mL of *hydrochloric acid R*. Cap tightly and sonicate. Allow to stand in the dark for 1 h ± 5 min. Remove from the dark, uncap and evaporate just to dryness on a steam bath with the aid of a stream of nitrogen. Add 10.0 mL of the internal standard solution. Vortex into solution.

*Column*:

- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 3-6 mL/min.

*Split ratio*: 1:10 to 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 10 | 200 → 250 |
| | 10 - 20 | 250 |
| Injection port | | 300 |
| Detector | | 330 |

*Detection*: flame ionisation.

*Injection*: 1 µL; inject directly onto the column or via a glass-lined injection port using an automatic injection device or some other reproducible injection method.

*System suitability*: reference solution:

- *resolution*: minimum 12.0 between the peaks due to dotriacontane and *RRR*-α-tocopheryl hydrogen succinate.

*Interference test.* Dissolve 0.100 g of the substance to be examined in *hexane R* and dilute to 50.0 mL with the same solvent. Inject 1 µL of the solution and record the chromatogram. If a peak is detected with the same retention time as that of the peak due to dotriacontane, calculate the area of this peak relative to the peak area of the substance to be examined. If the relative peak area is greater than 0.5 per cent, use the corrected peak area $S'_{D(corr)}$ for the final calculation.

$$S'_{D(corr)} = S'_D - \frac{S_I \times S'_T}{S_{TI}}$$

$S'_D$ = area of the peak due to dotriacontane in the chromatogram obtained with the test solution;

$S_I$ = area of the peak with the same retention time as that of the peak due to dotriacontane in the chromatogram obtained in the interference test;

$S'_T$ = area of the peak due to *RRR*-α-tocopheryl hydrogen succinate in the chromatogram obtained with the test solution;

$S_{TI}$ = area of the peak due to *RRR*-α-tocopheryl hydrogen succinate in the chromatogram obtained in the interference test.

Measure the areas of the peaks due to *RRR-α-tocopheryl hydrogen succinate CRS* ($S_T$) and dotriacontane ($S_D$) in the chromatogram obtained with the reference solution and the areas of the peaks due to *RRR*-α-tocopheryl hydrogen succinate ($S'_T$) and dotriacontane ($S'_D$) in the chromatogram obtained with the test solution.

Determine the response factor (RF) for *RRR*-α-tocopheryl hydrogen succinate from the areas of the peaks due to *RRR-α-tocopheryl hydrogen succinate CRS* and dotriacontane in the chromatogram obtained with the reference solution, using the following expression:

$$\frac{S_D \times m_T}{S_T \times m_D}$$

Calculate the percentage content of *RRR*-α-tocopheryl hydrogen succinate using the following expression:

$$\frac{100 \times S'_T \times m_D \times RF}{S'_{D(corr)} \times m}$$

$S_D$ = area of the peak due to dotriacontane in the chromatogram obtained with the reference solution;

$S'_{D(corr)}$ = corrected area of the peak due to dotriacontane in the chromatogram obtained with the test solution;

$S_T$ = area of the peak due to *RRR-α-tocopheryl hydrogen succinate CRS* in the chromatogram obtained with the reference solution;

$S'_T$ = area of the peak due to *RRR*-α-tocopheryl hydrogen succinate in the chromatogram obtained with the test solution;

$m_D$ = mass of dotriacontane in the test solution and in the reference solution, in milligrams;

$m_T$ = mass of *RRR-α-tocopheryl hydrogen succinate CRS* in the reference solution, in milligrams;

$m$ = mass of the substance to be examined in the test solution, in milligrams.

STORAGE

Protected from light.

**01/2017:0304**

# TOLBUTAMIDE

## Tolbutamidum

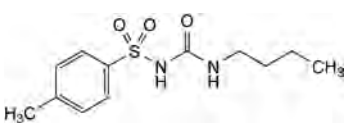

$C_{12}H_{18}N_2O_3S$ $M_r$ 270.3

[64-77-7]

DEFINITION

1-Butyl-3-[(4-methylphenyl)sulfonyl]urea.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, C.*

*Second identification: A, B, D.*

A. Melting point (*2.2.14*): 126 °C to 130 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution (a).* Dissolve 25.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 10.0 mL of test solution (a) to 250.0 mL with *methanol R*.

*Spectral range*: 245-300 nm for test solution (a); 220-235 nm for test solution (b).

*Absorption maxima*: at 258 nm, 263 nm and 275 nm for test solution (a); at 228 nm for test solution (b).

*Shoulder*: at 268 nm for test solution (a).

*Specific absorbance at the absorption maximum*: 480 to 520 for test solution (b).

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tolbutamide CRS*.

D. To 0.2 g add 8 mL of a 500 g/L solution of *sulfuric acid R* and heat under a reflux condenser for 30 min. Allow to cool. Crystals are formed which, after recrystallisation from hot *water R* and drying at 105 °C, melt (*2.2.14*) at 135 °C to 140 °C.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.2 g in 5 mL of *dilute sodium hydroxide solution R* and add 5 mL of *water R*.

**pH** (*2.2.3*): 4.5 to 5.5.

To 2.0 g add 50 mL of *carbon dioxide-free water R* and heat at 70 °C for 5 min. Cool rapidly and filter.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10 mg of *toluenesulfonamide R* (impurity A) and 10 mg of *toluenesulfonylurea R* (impurity B) in the mobile phase and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 35 volumes of *acetonitrile R1* and 65 volumes of a 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.5 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of tolbutamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurities A and B.

*Relative retention* with reference to tolbutamide (retention time = about 18 min): impurity B = about 0.2; impurity A = about 0.3.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impuritites A and B.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 20 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with *0.1 M sodium hydroxide*, using 1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M sodium hydroxide* is equivalent to 27.03 mg of $C_{12}H_{18}N_2O_3S$.

STORAGE

In an airtight container.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C.*

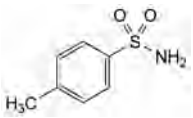

A. (4-methylphenyl)sulfonamide (toluenesulfonamide),

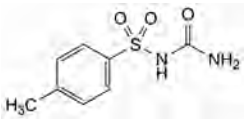

B. 1-[(4-methylphenyl)sulfonyl]urea (toluenesulfonylurea),

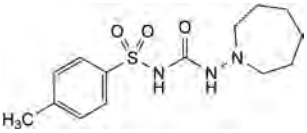

C. 1-azepan-1-yl-3-[(4-methylphenyl)sulfonyl]urea (tolazamide).

**07/2019:2039**

# TOLFENAMIC ACID

## Acidum tolfenamicum

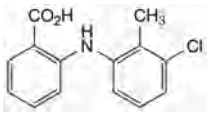

$C_{14}H_{12}ClNO_2$ $M_r$ 261.7
[13710-19-5]

DEFINITION

2-(3-Chloro-2-methylanilino)benzoic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in dimethylformamide, sparingly soluble in anhydrous ethanol and in methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

mp: about 213 °C.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Dissolve 20 mg in a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol R* and dilute to 100 mL with the same mixture of solvents. Dilute 5.0 mL of the solution to 50 mL with a mixture of 1 volume of *1 M hydrochloric acid* and 99 volumes of *methanol R*. Examined between 250 nm and 380 nm (*2.2.25*), the solution shows 2 absorption maxima, at 286 nm and 345 nm. The ratio of the absorbance measured at the maximum at 286 nm to that measured at the maximum at 345 nm is 1.2 to 1.4.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tolfenamic acid CRS*.

C. Thin-layer chromatography (*2.2.27*).
*Test solution*. Dissolve 25 mg of the substance to be examined in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Reference solution*. Dissolve 25 mg of *tolfenamic acid CRS* in a mixture of 1 volume of *methanol R* and 3 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.
*Mobile phase*: *glacial acetic acid R*, *dioxan R*, *toluene R* (1:25:90 *V/V/V*).
*Application*: 10 µL.
*Development*: over 2/3 of the plate.
*Drying*: in a current of warm air.
*Detection*: ultraviolet light at 254 nm.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in 5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the mobile phase.

Monographs T

*Reference solution (a).* Dissolve 25.0 mg of *2-chlorobenzoic acid R* (impurity A) and 25.0 mg of *3-chloro-2-methylaniline R* (impurity B) in 5 mL of *anhydrous ethanol R* and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *water for chromatography R*, *anhydrous ethanol R* (0.2:35:65 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 232 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of tolfenamic acid.

*Relative retention* with reference to tolfenamic acid (retention time = about 15 min): impurity A = about 0.25; impurity B = about 0.34.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.5 between the peaks due to impurity A and to impurity B.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurity B*: not more than half the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.05 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.01 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g with the aid of ultrasound in 100 mL of *anhydrous ethanol R*. Add 0.1 mL of *phenol red solution R* and titrate with *0.1 M sodium hydroxide*.

1 mL of *0.1 M sodium hydroxide* is equivalent to 26.17 mg of $C_{14}H_{12}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C.*

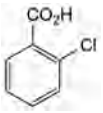

A. 2-chlorobenzoic acid,

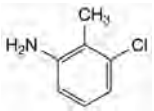

B. 3-chloro-2-methylaniline,

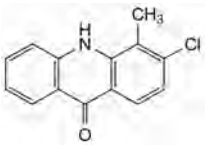

C. 3-chloro-4-methylacridin-9(10*H*)-one.

**04/2011:1158**

# TOLNAFTATE

## Tolnaftatum

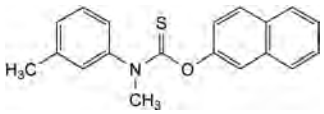

$C_{19}H_{17}NOS$ $M_r$ 307.4
[2398-96-1]

DEFINITION

*O*-Naphthalen-2-yl methyl(3-methylphenyl)carbamothioate.

*Content*: 97.0 per cent to 103.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone and in methylene chloride, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tolnaftate CRS*.

TESTS

**Impurity D**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.400 g of the substance to be examined in 2 mL of *methylene chloride R*. Extract with 3 quantities, each of 3 mL, of *0.01 M hydrochloric acid*. Combine the aqueous phases and dilute to 10.0 mL with *0.01 M hydrochloric acid*.

*Reference solution (a).* Dissolve 20.0 mg of *N-methyl-m-toluidine R* (impurity D) in 50.0 mL of *methylene chloride R*.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *methylene chloride R*. Take 2.0 mL of this solution and extract with 3 quantities, each of 3 mL, of *0.01 M hydrochloric acid*. Combine the aqueous phases and dilute to 10.0 mL with *0.01 M hydrochloric acid*.

*Reference solution (c).* Dissolve 10 mg of the substance to be examined in 25 mL of *methanol R*. Add 2 mL of this solution to 2 mL of reference solution (a) and dilute to 25 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *methanol R*, *water R* (0.1:10:90 *V/V/V*);

– *mobile phase B*: *trifluoroacetic acid R*, *water R*, *methanol R* (0.1:10:90 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 70 | 30 |
| 3 - 8 | 70 → 0 | 30 → 100 |
| 8 - 20 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 100 µL of the test solution and reference solution (b); 10 µL of reference solution (c).

*Relative retention* with reference to tolnaftate (retention time = about 15 min): impurity D = about 0.25.

*System suitability*:

– *resolution*: minimum 5.0 between the peaks due to impurity D and tolnaftate in the chromatogram obtained with reference solution (c);

– *symmetry factor*: maximum 1.9 for the peak due to impurity D in the chromatogram obtained with reference solution (b).

*Limit*:

– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (20 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25 mg of the substance to be examined in 5 mL of *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b).* Dissolve 5 mg of *tolnaftate for system suitability CRS* (containing resolution component A) in 5.0 mL of *methanol R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water R*, *methanol R* (0.1:30:70 *V/V/V*);

– *mobile phase B*: *trifluoroacetic acid R*, *water R*, *methanol R* (0.1:10:90 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 100 | 0 |
| 12 - 30 | 100 → 0 | 0 → 100 |
| 30 - 33 | 0 | 100 |

*Flow rate*: 1.0 mL/ min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Relative retention* with reference to tolnaftate (retention time = about 18 min): resolution component A = about 0.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 5.0 between the peaks due to resolution component A and tolnaftate.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 50.0 mg in *methanol R* and dilute to 250.0 mL with the same solvent. Dilute 2.0 mL of this solution to 50.0 mL with *methanol R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 257 nm.

Calculate the content of $C_{19}H_{17}NOS$ taking the specific absorbance to be 720.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B.*

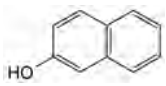


A. naphthalen-2-ol (β-naphthol),

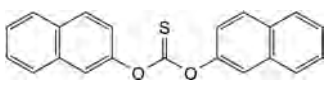


B. *O*,*O*-dinaphthalen-2-yl carbonothioate,

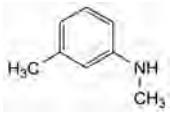


D. *N*,3-dimethylaniline (*N*-methyl-*m*-toluidine).

04/2015:2781
corrected 10.0

# TOLTERODINE TARTRATE

## Tolterodini tartras

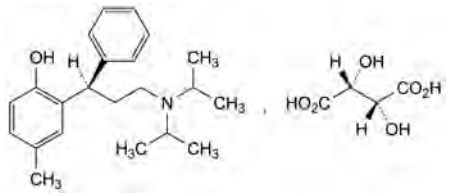

$C_{26}H_{37}NO_7$ $M_r$ 475.6
[124937-52-6]

DEFINITION

2-[(1*R*)-3-[Bis(1-methylethyl)amino]-1-phenylpropyl]-4-methylphenol (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: sparingly soluble in water, slightly soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tolterodine tartrate CRS*.

B. Enantiomeric purity (see Tests).

C. It gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 0.29 g of *sodium dihydrogen phosphate monohydrate R* and 0.47 g of *disodium hydrogen phosphate dihydrate R* in 1 L of *water R*. Dissolve 1.04 g of *tetrabutylammonium bromide R* in this solution.

*Test solution*. Dissolve 4.0 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2 mg of *tolterodine for system suitability CRS* (containing impurity F) in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dilute 0.5 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.10 m, Ø = 2 mm;
– *stationary phase*: *α1-acid-glycoprotein silica gel for chiral separation R* (5 µm).

*Mobile phase*: *2-methylpropanol R*, solution A (7:93 *V/V*).

*Flow rate*: 0.2 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of tolterodine.

*Relative retention* with reference to tolterodine (retention time = about 23 min): impurity F = about 0.9.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity F and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to tolterodine.

*Calculation of percentage content*:

– for impurity F, use the concentration of tolterodine tartrate in reference solution (b).

*Limit*:

– *impurity F*: maximum 0.6 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in 10 mL of *methanol R1*, sonicate until dissolution is complete and dilute to 25.0 mL with *methanol R1*.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with *methanol R1*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R1*.

*Reference solution (b)*. Dissolve 2 mg of *tolterodine impurity E CRS* in *methanol R1* and dilute to 10.0 mL with *methanol R1*. Dilute 1.0 mL of the solution to 20.0 mL with *methanol R1*.

*Reference solution (c)*. Dissolve 25 mg of the substance to be examined in 10 mL of *methanol R1*. Sonicate until dissolution is complete. Add 5 mL of reference solution (b) and dilute to 25.0 mL with *methanol R1*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: mix 5 volumes of *triethylamine R2* and 450 volumes of a 2.88 g/L solution of *ammonium dihydrogen phosphate R*; adjust to pH 5.9 with a 50 per cent *V/V* solution of *phosphoric acid R*; add 550 volumes of *methanol R1*;
– *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 45 | 100 → 80 | 0 → 20 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to tolterodine (retention time = about 7 min): tartaric acid = about 0.2; impurity E = about 0.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 1.5 between the peaks due to impurity E and tolterodine.

*Calculation of percentage contents*:

– for each impurity, use the concentration of tolterodine tartrate in reference solution (a).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.3 per cent;
– *reporting threshold*: 0.05 per cent; disregard the peak due to tartaric acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 30.0 mL of *anhydrous acetic acid R* and sonicate until dissolution is complete. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 47.56 mg of $C_{26}H_{37}NO_7$.

IMPURITIES

*Specified impurities: F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D, E, G.*

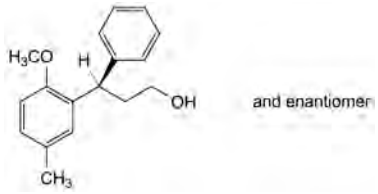


A. (3*RS*)-3-(2-methoxy-5-methylphenyl)-3-phenylpropan-1-ol,

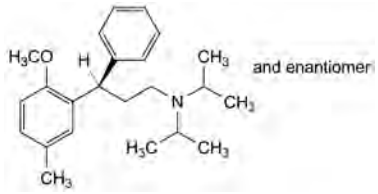


C. (3*RS*)-3-(2-methoxy-5-methylphenyl)-*N*,*N*-bis(1-methylethyl)-3-phenylpropan-1-amine,

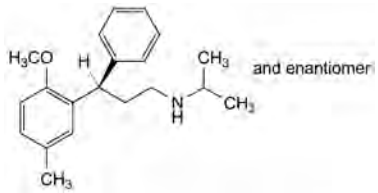


D. (3*RS*)-3-(2-methoxy-5-methylphenyl)-*N*-(1-methylethyl)-3-phenylpropan-1-amine,

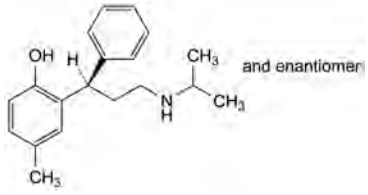


E. 4-methyl-2-[(1*RS*)-3-[(1-methylethyl)amino]-1-phenylpropyl]phenol,

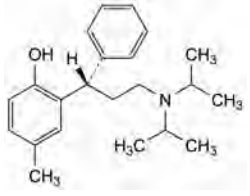


F. 2-[(1*S*)-3-[bis(1-methylethyl)amino]-1-phenylpropyl]-4-methylphenol,

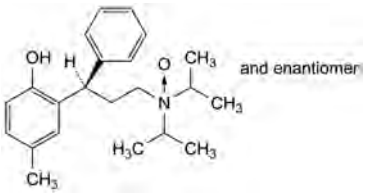


G. 2-[(1*R*)-3-[bis(1-methylethyl)amino]-1-phenylpropyl]-4-methylphenol *N*-oxide.

**01/2020:2616**

# TOPIRAMATE

## Topiramatum

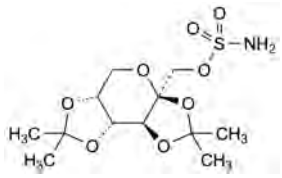


$C_{12}H_{21}NO_8S$ $M_r$ 339.4

[97240-79-4]

DEFINITION

2,3:4,5-Bis-*O*-(propan-2,2-diyl)-β-D-fructopyranose 1-sulfamate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *topiramate CRS.*

TESTS

**Specific optical rotation** (*2.2.7*): − 32 to − 29 (anhydrous substance).

Dissolve 0.200 g in *methanol R* and dilute to 50.0 mL with the same solvent.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in 1.0 mL of *methanol R*.

*Reference solution (a)*. Dissolve 10.0 mg of *topiramate impurity A CRS* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 5.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve 40 mg of the substance to be examined in 1 mL of reference solution (a).

*Plate*: *TLC silanised silica gel plate R*.

*Pretreatment*: wash the plate with *methanol R* and allow to dry in air.

*Mobile phase*: *methanol R*, *acetonitrile R*, 29 g/L solution of *sodium chloride R* (15:35:50 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: spray with a solution prepared by dissolving 3 g of *phenol R* in 95 mL of *ethanol (96 per cent) R* and carefully adding 5 mL of *sulfuric acid R*; allow to dry in air, then heat at 125 °C for 10 min. Use transmittance mode.

Monographs T

*Retardation factors*: topiramate = about 0.43; impurity A = about 0.55.

*System suitability*:

– the chromatogram obtained with reference solution (b) shows 2 clearly separated spots due to topiramate and impurity A.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the corresponding spot in the chromatogram obtained with reference solution (a) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *acetonitrile R1*, mobile phase A (20:80 *V/V*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 20.0 mL with the solvent mixture. Dilute 5.0 mL of this solution to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 15.0 mg of *topiramate impurity E CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 200.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of the substance to be examined in 1 mL of a mixture of 2 volumes of *acetonitrile R1*, 4 volumes of *water for chromatography R* and 4 volumes of *anhydrous formic acid R*. Incubate at 60 °C for 30 min (*in situ* preparation of impurity C). Dilute 20 µL of the solution to 1 mL with a 25 mg/L solution of *fructose R* (impurity E).

*Reference solution (d).* Dissolve 25.0 mg of *topiramate CRS* in 10.0 mL of the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 5.0 mL of the solution to 50.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped solid core pentafluorophenylpropylsilyl silica gel for chromatography R* (2.6 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: 1.93 g/L solution of *ammonium acetate R* adjusted to pH 3.5 with *glacial acetic acid R*;
– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 80 | 20 |
| 5 - 15 | 80 → 50 | 20 → 50 |
| 15 - 15.1 | 50 → 80 | 50 → 20 |

*Flow rate*: 1.0 mL/min.

*Detection*: charged aerosol detector:

– *range*: 100 pA;
– *filter*: none.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity E.

*Relative retention* with reference to topiramate (retention time = about 5.6 min): impurity E = about 0.18; impurity C = about 0.25.

*System suitability*: reference solution (c):

– *resolution*: minimum 4.5 between the peaks due to impurities E and C.

*Calculation of percentage contents*:

– for impurity E, use the concentration of impurity E in reference solution (b) and the height of the peak due to impurity E;
– for impurities other than E, use the concentration of topiramate in reference solution (a).

*Limits*:

– *impurity E*: maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.2 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{12}H_{21}NO_8S$ taking into account the assigned content of *topiramate CRS*.

IMPURITIES

*Specified impurities: A, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

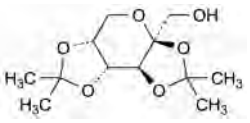

A. 2,3:4,5-bis-*O*-(propan-2,2-diyl)-β-D-fructopyranose,

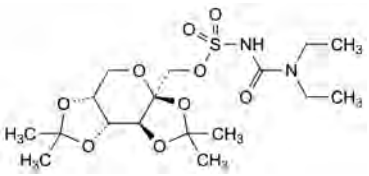

B. 2,3:4,5-bis-*O*-(propan-2,2-diyl)-β-D-fructopyranose 1-[*N*-(diethylcarbamoyl)sulfamate],

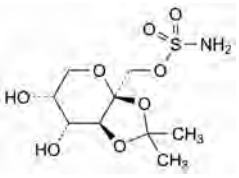

C. 2,3-*O*-(propan-2,2-diyl)-β-D-fructopyranose 1-sulfamate,

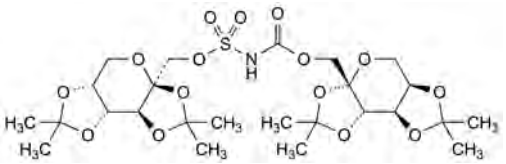

D. 2,3:4,5-bis-*O*-(propan-2,2-diyl)-β-D-fructopyranose 1-[*N*-[[[2,3:4,5-bis-*O*-(propan-2,2-diyl)-β-D-fructopyranosyl]oxy]carbamoyl]sulfamate],

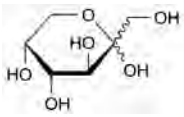

E. D-fructopyranose (fructose).

**01/2017:2132**

# TORASEMIDE

## Torasemidum

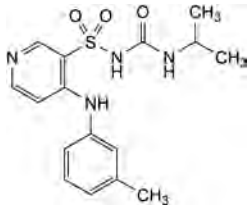

$C_{16}H_{20}N_4O_3S$ $M_r$ 348.4
[56211-40-6]

DEFINITION

1-(1-Methylethyl)-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl]urea.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, slightly soluble in ethanol (96 per cent). It is sparingly soluble in dilute solutions of alkali hydroxides and slightly soluble in dilute acids.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anhydrous torasemide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 2.7 g of *potassium dihydrogen phosphate R* in 950 mL of *water R*, adjust to pH 3.5 with *phosphoric acid R* and dilute to 1000 mL with *water R*.

*Test solution*. Dissolve 20.0 mg of the substance to be examined in 15 mL of *methanol R* and sonicate for 15 min. Add 22.5 mL of solution A, cool to room temperature and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2.0 mg of *torasemide for system suitability CRS* (containing impurities A, B, C and D) in 2.5 mL of *methanol R* and dilute to 5.0 mL with solution A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve the contents of a vial of *torasemide impurity E CRS* in 0.5 mL of *methanol R*. Add 0.5 mL of solution A.

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *methanol R*, solution A (40:60 *V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of torasemide.

*Identification of impurities*: use the chromatogram supplied with *torasemide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C and D; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity E.

*Relative retention* with reference to torasemide (retention time = about 10 min): impurity A = about 0.3; impurity B = about 0.4; impurity C = about 0.5; impurity E = about 0.7; impurity D = about 2.3.

*System suitability*:

- *resolution*: minimum 3.0 between the peaks due to impurities B and C in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 100 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 5.1; impurity B = 0.76;
- *impurity B*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *impurities A, C, D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 34.84 mg of $C_{16}H_{20}N_4O_3S$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

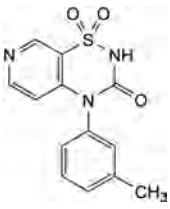

A. 4-(3-methylphenyl)-2*H*-pyrido[4,3-*e*]-1,2,4-thiadiazin-3(4*H*)-one 1,1-dioxide,

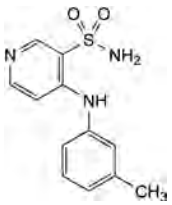

B. 4-[(3-methylphenyl)amino]pyridine-3-sulfonamide,

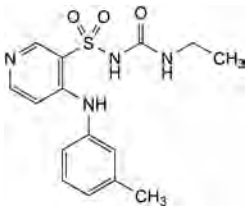

C. 1-ethyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl]urea,

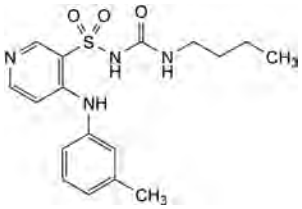

D. 1-butyl-3-[[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl]urea,

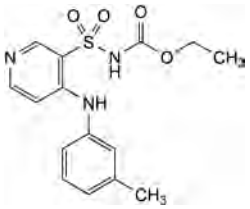

E. ethyl [[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl]carbamate.

**01/2008:0381**
**corrected 10.0**

# TOSYLCHLORAMIDE SODIUM

## Tosylchloramidum natricum

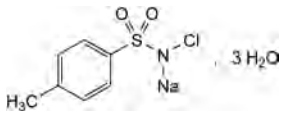

$C_7H_7ClNNaO_2S,3H_2O$ $M_r$ 281.7

DEFINITION

Sodium *N*-chloro-4-methylbenzene-sulfonimidate trihydrate.

*Content*: 98.0 per cent to 103.0 per cent of $C_7H_7ClNNaO_2S,3H_2O$.

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) turns *red litmus paper R* blue and then bleaches it.

B. To 10 mL of solution S add 10 mL of *dilute hydrogen peroxide solution R*. A white precipitate is formed which dissolves on heating. Filter the hot solution and allow to cool. White crystals are formed which, when washed and dried at 100-105 °C, melt (*2.2.14*) at 137 °C to 140 °C.

C. Ignite cautiously 1 g, because of the risk of deflagration. Dissolve the residue in 10 mL of *water R*. The solution gives reaction (a) of chlorides (*2.3.1*).

D. The solution prepared for identification test C gives reaction (a) of sulfates (*2.3.1*).

E. The solution prepared for identification test C gives reaction (b) of sodium (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 8.0 to 10.0 for solution S.

**Ortho compound**. To 2 g add 10 mL of *water R*, mix, add 1 g of *sodium metabisulfite R* and heat to boiling. Cool to 0 °C, filter rapidly and wash with 3 quantities, each of 5 mL, of iced *water R*. The precipitate, dried *in vacuo* (*2.2.32*) at a pressure not exceeding 0.6 kPa, melts (*2.2.14*) at a minimum of 134 °C.

**Residue insoluble in anhydrous ethanol**: maximum 2 per cent.

Shake 1.00 g with 20 mL of *anhydrous ethanol R* for 30 min, filter on a tared filter, wash any residue with 5 mL of *anhydrous ethanol R* and dry at 100-105 °C. The residue weighs a maximum of 20 mg.

ASSAY

Dissolve 0.125 g in 100 mL of *water R* in a ground-glass-stoppered flask. Add 1 g of *potassium iodide R* and 5 mL of *dilute sulfuric acid R*. Allow to stand for 3 min. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 14.08 mg of $C_7H_7ClNNaO_2S,3H_2O$.

STORAGE

In an airtight container, protected from light.

**01/2017:1681**

# TRAMADOL HYDROCHLORIDE

## Tramadoli hydrochloridum

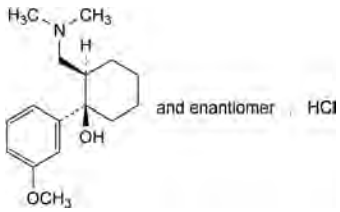

$C_{16}H_{26}ClNO_2$ $M_r$ 299.8
[36282-47-0]

DEFINITION

(1*RS*,2*RS*)-2-[(Dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in methanol, very slightly soluble in acetone.

IDENTIFICATION

*First identification: B, D.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 180 °C to 184 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tramadol hydrochloride CRS*.

C. Chromatograms obtained in the test for impurity E.

*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity**. To 10 mL of solution S, add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Not more than 0.4 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Optical rotation** (*2.2.7*): − 0.10° to + 0.10°, determined on solution S.

**Impurity E**. Thin-layer chromatography (*2.2.27*).

*Test solution (a).* Dissolve 0.10 g in *methanol R* and dilute to 2 mL with the same solvent.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 25 mg of *tramadol hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *tramadol impurity E CRS* in 5 mL of *methanol R*. Dilute 1 mL of the solution to 10 mL with *methanol R*.

*Reference solution (c).* Dissolve 5 mg of *tramadol impurity A CRS* in 1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*, prewashed with *methanol R*.

*Mobile phase*: *concentrated ammonia R*, *2-propanol R*, *toluene R* (1:19:80 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate. Saturate the plate for 20 min with *concentrated ammonia R*. For this, add *concentrated ammonia R* to one trough of a twin trough tank. Just before developing, add the mobile phase to the other trough. Place the plate in the chromatographic tank, ensuring that the layer of silica gel is orientated towards the middle of the tank.

*Drying*: in air.

*Detection*: expose the plate to iodine vapour for 1 h, examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (c) shows 2 clearly separated spots.

*Limit*: test solution (a):

– *impurity E*: any spot corresponding to impurity E is not more intense and not greater than the spot in the chromatogram obtained with reference solution (b) (0.2 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.15 g of the substance to be examined in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *tramadol impurity A CRS* in 4.0 mL of the test solution and dilute to 100 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *end-capped base-deactivated octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 295 volumes of *acetonitrile R* and 705 volumes of a mixture of 0.2 mL of *trifluoroacetic acid R* and 100 mL of *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*Run time*: 4 times the retention time of tramadol.

*Relative retention* with reference to tramadol (retention time = about 5 min): impurity A = about 0.85.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and tramadol.

*Limits*:

– *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 25 mL of *anhydrous acetic acid R* and add 10 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.98 mg of $C_{16}H_{26}ClNO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

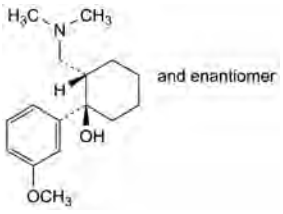


A. (1*RS*,2*SR*)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol,

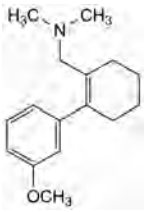

B. [2-(3-methoxyphenyl)cyclohex-1-enyl]-*N*,*N*-dimethylmethanamine,

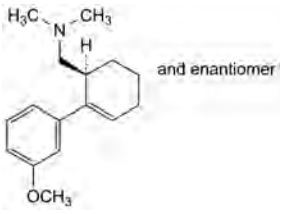


C. (1*RS*)-[2-(3-methoxyphenyl)cyclohex-2-enyl]-*N*,*N*-dimethylmethanamine,

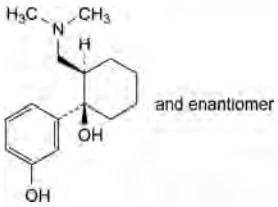


D. (1*RS*,2*RS*)-2-[(dimethylamino)methyl]-1-(3-hydroxyphenyl)cyclohexanol,

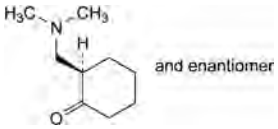


E. (2*RS*)-2-[(dimethylamino)methyl]cyclohexanone.

**01/2008:1597**

# TRAMAZOLINE HYDROCHLORIDE MONOHYDRATE

## Tramazolini hydrochloridum monohydricum

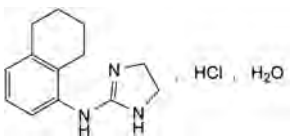

$C_{13}H_{18}ClN_3,H_2O$ $M_r$ 269.8
[74195-73-6]

DEFINITION

*N*-(5,6,7,8-Tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazol-2-amine hydrochloride monohydrate.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tramazoline hydrochloride monohydrate CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.9 to 6.3 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 5.0 mg of *tramazoline impurity A CRS* and 5.0 mg of *tramazoline impurity B CRS* in 5 mL of a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R* and add 5 mL of the test solution.

*Reference solution (b)*. Dilute 0.2 mL of reference solution (a) to 100 mL with a mixture of 50 volumes of *acetonitrile R* and 50 volumes of *water R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 2.0 g/L solution of *sodium dodecyl sulfate R* in a mixture of 6 volumes of *2-propanol R*, 42 volumes of *acetonitrile R* and 52 volumes of *water R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 5 µL.

*Run time*: 3 times the retention time of tramazoline.

*Relative retention* with reference to tramazoline (retention time = about 6.5 min): impurity A = about 0.71; impurity B = about 0.86.

*System suitability*: reference solution (a):
- the chromatogram obtained shows 3 clearly separated peaks,
- *resolution*: minimum 1.5 between tramazoline and impurity B.

*Limits*:
- *impurity A*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *impurity B*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent),
- *any other impurity*: not more than the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *sum of other impurities*: not more than twice the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.2 per cent),

– *disregard limit*: 0.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.02 per cent).

**Water** (*2.5.12*): 6.2 per cent to 7.2 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 2.000 g in a mixture of 5 mL of *0.1 M hydrochloric acid* and 75 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*) using *1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *1 M sodium hydroxide* is equivalent to 251.8 mg of $C_{13}H_{18}ClN_3$.

IMPURITIES

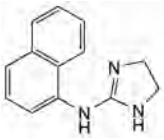

A. *N*-(naphthalen-1-yl)-4,5-dihydro-1*H*-imidazol-2-amine,

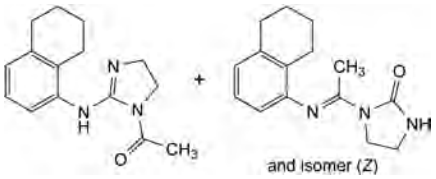


B. mixture of 1-acetyl-*N*-(5,6,7,8-tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazol-2-amine and 1-[(*EZ*)-1-[(5,6,7,8-tetrahydronaphthalen-1-yl)imino]ethyl]imidazolidin-2-one.

**07/2019:2245**

# TRANDOLAPRIL

## Trandolaprilum

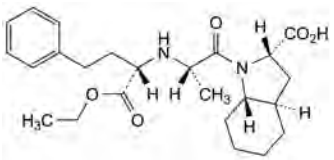

$C_{24}H_{34}N_2O_5$ $M_r$ 430.5
[87679-37-6]

DEFINITION

(2*S*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(2*S*)-1-Ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *trandolapril CRS*.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 1.0 g in *methanol R* and dilute to 10 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): – 18.5 to – 16.5 (anhydrous substance).

Dissolve 1.0 g in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in mobile phase A and dilute to 10.0 mL with mobile phase A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Reference solution (b)*. Dissolve 5 mg of *trandolapril impurity C CRS* and 5 mg of *trandolapril impurity D CRS* in mobile phase A and dilute to 5 mL with mobile phase A. Dilute 1 mL of the solution to 20 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (3.5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: mix 25 volumes of *acetonitrile R1* and 75 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.5 ± 0.1 with *phosphoric acid R*;
– *mobile phase B*: mix equal volumes of *acetonitrile R1* and a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.2 ± 0.1 with *phosphoric acid R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 95 | 5 |
| 20 - 35 | 95 → 5 | 5 → 95 |
| 35 - 45 | 5 | 95 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C and D.

*Relative retention* with reference to trandolapril (retention time = about 14.5 min): impurity C = about 2.1; impurity D = about 2.5.

*System suitability*: reference solution (b):

– *resolution*: minimum 4 between the peaks due to impurities C and D.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity C by 2.2;
– *impurity D*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *sum of impurities other than D*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g in a porcelain or quartz crucible.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 43.05 mg of $C_{24}H_{34}N_2O_5$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: C, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, E, F.*

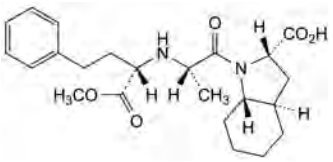

A. (2*S*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(2*S*)-1-methoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid (methyl ester derivative),

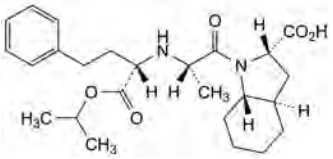

B. (2*S*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(2*S*)-1-oxo-4-phenyl-1-[(propan-2-yl)oxy]butan-2-yl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid (isopropyl ester derivative),

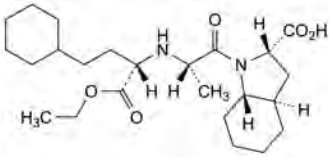

C. (2*S*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(2*S*)-4-cyclohexyl-1-ethoxy-1-oxobutan-2-yl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid (hexahydrotrandolapril),

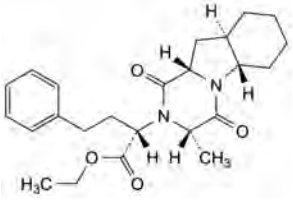

D. ethyl (2*S*)-2-[(3*S*,5a*S*,9a*R*,10a*S*)-3-methyl-1,4-dioxodecahydropyrazino[1,2-*a*]indol-2(1*H*)-yl]-4-phenylbutanoate (trandolapril diketopiperazine),

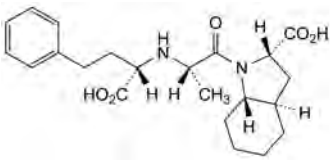

E. (2*S*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(1*S*)-1-carboxy-3-phenylpropyl]-amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid (trandolaprilate),

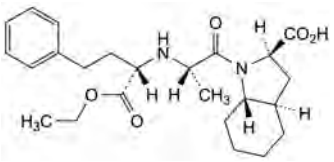

F. (2*R*,3a*R*,7a*S*)-1-[(2*S*)-2-[[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino]propanoyl]octahydro-1*H*-indole-2-carboxylic acid.

**01/2017:0875**

# TRANEXAMIC ACID

## Acidum tranexamicum

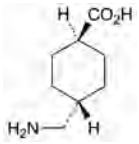

$C_8H_{15}NO_2$ $M_r$ 157.2

[1197-18-8]

DEFINITION

*trans*-4-(Aminomethyl)cyclohexanecarboxylic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water and in glacial acetic acid, practically insoluble in acetone and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *tranexamic acid CRS*.

TESTS

**pH** (*2.2.3*): 7.0 to 8.0.

Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 20 mg of *tranexamic acid CRS* (containing impurity C) in *water R* and dilute to 2 mL with the same solvent.

*Reference solution (c).* Dissolve 12 mg of *4-aminomethylbenzoic acid R* (impurity D) in *water R* and dilute to 100 mL with the same solvent. Dilute 1 mL of the solution to 50 mL with *water R*. Dilute 5 mL of this solution to 200 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm or *l* = 0.25 m, Ø = 6.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: dissolve 11.0 g of *anhydrous sodium dihydrogen phosphate R* in 500 mL of *water R* and add 5 mL of *triethylamine R* and 1.4 g of *sodium laurilsulfate R*. Adjust to pH 2.5 with *dilute phosphoric acid R* and dilute to 600 mL with *water R*. Add 400 mL of *methanol R* and mix.

*Flow rate*: 0.9 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of tranexamic acid.

*Identification of impurities*: use the chromatogram supplied with *tranexamic acid CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retention* with reference to tranexamic acid (retention time = about 13 min): impurity C = about 1.1; impurity D = about 1.3; impurity B = about 1.5; impurity A = about 2.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to tranexamic acid and impurity C.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.2; impurity C = 0.005; impurity D = 0.006;
- *impurity A*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *impurity B*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of unspecified impurities*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.025 per cent).

**Halides expressed as chlorides** (*2.4.4*): maximum 140 ppm.

Dissolve 1.2 g in *water R* and dilute to 50 mL with the same solvent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.140 g in 20 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 15.72 mg of $C_8H_{15}NO_2$.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

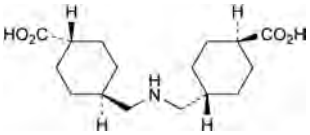

A. *trans*,*trans*-4,4′-(iminodimethylene)di(cyclohexanecarboxylic) acid,

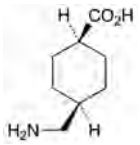

B. *cis*-4-(aminomethyl)cyclohexanecarboxylic acid,

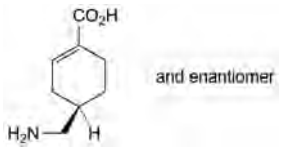


C. (*RS*)-4-(aminomethyl)cyclohex-1-enecarboxylic acid,

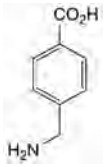

D. 4-aminomethylbenzoic acid.

**01/2017:1576**

# TRAPIDIL

## Trapidilum

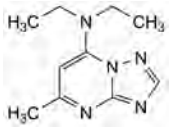

$C_{10}H_{15}N_5$ $M_r$ 205.3

[15421-84-8]

DEFINITION

*N*,*N*-Diethyl-5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-amine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, soluble in ethanol and in methylene chloride.

mp: about 102 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *trapidil CRS*.

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.2 mL of *methyl red solution R* and 0.2 mL of *0.01 M hydrochloric acid*. The solution is red. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *trapidil impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *trapidil impurity B CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (c).* Mix equal volumes of reference solution (a) and reference solution (b).

*Column*:

- *size*: *l* = 0.125 m, Ø = 4.0 mm,
- *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm),

*Mobile phase*: 50 mL of *methanol R*, 75 mL of *acetonitrile R* and 800 mL of a 1.7 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 2.45 with *phosphoric acid R*; dilute to 1000 mL with *water R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 205 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of trapidil.

*System suitability*:

- *resolution*: minimum of 4.0 between the peaks due to impurity A and impurity B in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent),
- *any other impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent),
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent).
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.01 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 0.25 g in 10 mL of *water R* and dilute to 15 mL with *water R*. Prepare the standard using 5 mL of *chloride standard solution (5 ppm Cl) R*.

**Ammonium** (*2.4.1*): maximum 20 ppm.

0.50 g complies with limit test A. Prepare the standard using 0.1 mL of *ammonium standard solution (100 ppm $NH_4$) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.180 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.53 mg of $C_{10}H_{15}N_5$.

STORAGE

Protected from light.

IMPURITIES

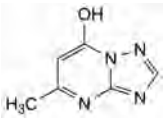

A. 5-methyl-[1,2,4]triazolo[1,5-*a*]pyrimidin-7-ol,

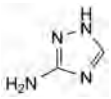

B. 1,2,4-triazol-3-amine.

**01/2017:2297**

# TREHALOSE DIHYDRATE

## Trehalosum dihydricum

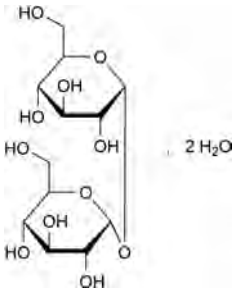

$C_{12}H_{22}O_{11},2H_2O$ $M_r$ 378.3

[6138-23-4]

DEFINITION

α-D-Glucopyranosyl α-D-glucopyranoside dihydrate (α,α-trehalose dihydrate). It is obtained by enzymatic modification of starch.

*Content*: 97.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, slightly soluble in methanol, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *trehalose dihydrate CRS*.

B. Dissolve 2 g in 5 mL of *water R*. To 1 mL of this solution add 0.4 mL of a 50 g/L solution of *α-naphthol R* in *ethanol (96 per cent) R* and mix thoroughly. Carefully add 2 mL of *sulfuric acid R*. A violet colour develops at the interface.

C. Dissolve 1 g in 25 mL of *water R*. To 2 mL of this solution add 1 mL of *dilute hydrochloric acid R* and mix. Keep the solution for 20 min at room temperature. Add 4 mL of a 40 g/L solution of *sodium hydroxide R* and 2 mL of a 40 g/L solution of *glycine R* and mix. Heat the solution in a water-bath for 10 min. No brown colour develops.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 197 to + 201 (anhydrous substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.100 g of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 0.100 g of *trehalose dihydrate CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dilute 5.0 mL of reference solution (b) to 25.0 mL with *water R*.

*Reference solution (d)*. Dissolve 25 mg of *glucose R* (impurity A) and 25 mg of *maltotriose R* in *water R*, add 2.5 mL of reference solution (a) and dilute to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.3 m, Ø = 8 mm;
- *stationary phase*: *strong cation-exchange resin (sodium form) R* (6 µm);
- *temperature*: 80 °C.

*Mobile phase*: *water R*.

*Flow rate*: 0.4 mL/min.

*Detection*: refractometer maintained at 40 °C.

*Injection*: 20 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: twice the retention time of trehalose.

*Identification of impurities*: use the chromatogram obtained with reference solution (d) to identify the peaks due to impurities A and B; impurity B has the same retention time as maltotriose.

*Relative retention* with reference to trehalose (retention time = about 15 min): impurity B = about 0.9; impurity A = about 1.2.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to maltotriose and trehalose.

*Limits*:

- *impurities A, B*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.1 per cent).

**Chlorides** (*2.4.4*): maximum 125 ppm.

Dilute 4 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 7.5 mL of solution S to 15 mL with *distilled water R*.

**Soluble starch**. Dissolve 1 g in 10 mL of *water R*. Add 0.1 mL of *iodine solution R1*. No blue colour develops.

**Water** (*2.5.12*): 9.0 per cent to 11.0 per cent, determined on 0.10 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Microbial contamination**

If intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

If not intended for use in the manufacture of parenteral preparations:

- TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*);
- TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*);
- absence of *Escherichia coli* (*2.6.13*);
- absence of *Salmonella* (*2.6.13*).

**Bacterial endotoxins** (*2.6.14*). If intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins:

- less than 4 IU/g for parenteral preparations having a concentration of 100 g/L or less of trehalose dihydrate;
- less than 2.5 IU/g for parenteral preparations having a concentration of more than 100 g/L of trehalose dihydrate.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of trehalose from the declared content of *trehalose dihydrate CRS*.

LABELLING

The label states:

- where applicable, the maximum concentration of bacterial endotoxins;
- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B.*

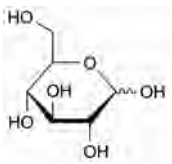

A. D-glucopyranose (glucose),

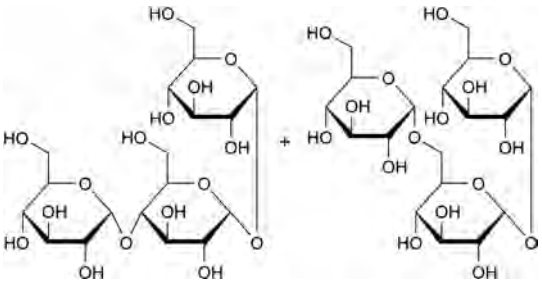

B. oligosaccharides, mainly glucosyltrehalose: mixture of α-D-glucopyranosyl α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (4-*O*-glucosyltrehalose or α-D-maltosyl α-D-glucoside) and α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl α-D-glucopyranoside (6-*O*-glucosyltrehalose or α-D-isomaltosyl α-D-glucoside).

01/2017:0693

# TRETINOIN

## Tretinoinum

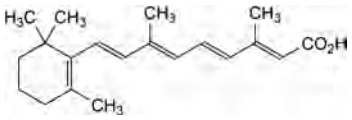

$C_{20}H_{28}O_2$ $M_r$ 300.4
[302-79-4]

DEFINITION

(2*E*,4*E*,6*E*,8*E*)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow or light orange, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in methylene chloride, slightly soluble in ethanol (96 per cent).

mp: about 182 °C, with decomposition.

It is sensitive to air, heat and light, especially in solution.

*Carry out all operations as rapidly as possible and avoid exposure to actinic light; use freshly prepared solutions.*

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tretinoin CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *tretinoin CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $GF_{254}$ plate R*.

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *peroxide-free ether R*, *cyclohexane R* (2:4:40:54 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 5 mg in 2 mL of *antimony trichloride solution R*. An intense red colour develops and later becomes violet.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 10.0 mg of *isotretinoin CRS* (impurity A) in *methanol R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Mix 1.0 mL of reference solution (a) and 0.5 mL of the test solution and dilute to 25.0 mL with *methanol R*.

*Reference solution (c).* Dilute 0.5 mL of the test solution to 100.0 mL with *methanol R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*: *glacial acetic acid R*, *water R*, *methanol R* (5:225:770 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 355 nm.

*Injection*: 10 µL.

*Run time*: 1.2 times the retention time of tretinoin.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to tretinoin (retention time = about 29 min): impurity A = about 0.75.

*System suitability*: reference solution (b):

- *resolution*: minimum 5.0 between the peaks due to impurity A and tretinoin.

*Limits*:

- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for Pharmaceutical use (2034)* do not apply.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* for 16 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 70 mL of *acetone R*. Titrate with *0.1 M tetrabutylammonium hydroxide in 2-propanol*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 30.04 mg of $C_{20}H_{28}O_2$.

STORAGE

Under an inert gas, in an airtight container, protected from light.

It is recommended that the contents of an opened container be used as soon as possible and any unused part be protected by an atmosphere of inert gas.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G*.

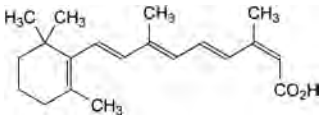

A. (2*Z*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (isotretinoin),

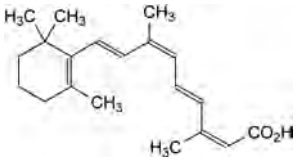

B. (2*Z*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9,13-di-*cis*-retinoic acid),

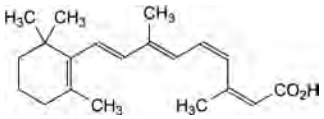

C. (2*Z*,4*Z*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (11,13-di-*cis*-retinoic acid),

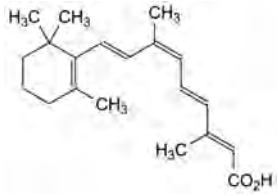

D. (2*E*,4*E*,6*Z*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid (9-*cis*-retinoic acid),

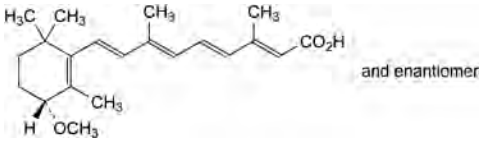


F. (2*E*,4*E*,6*E*,8*E*)-9-[(3*RS*)-3-methoxy-2,6,6-trimethylcyclohex-1-enyl]-3,7-dimethylnona-2,4,6,8-tetraenoic acid (*rac*-4-methoxytretinoin),

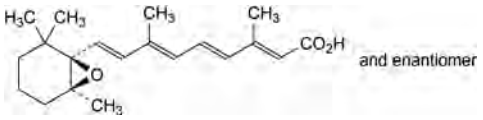


G. (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,2,6-trimethyl-7-oxabicyclo[4.1.0]hept-1-yl)nona-2,4,6,8-tetraenoic acid (*rac*-5,6-epoxytretinoin).

**01/2008:1106**
**corrected 6.0**

# TRIACETIN

## Triacetinum

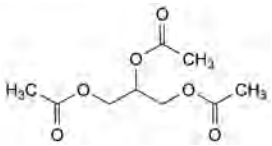

$C_9H_{14}O_6$ $M_r$ 218.2
[102-76-1]

DEFINITION

Propane-1,2,3-triyl triacetate.

*Content*: 97.0 per cent to 100.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, colourless, slightly viscous oily liquid.

*Solubility*: soluble in water, miscible with ethanol (96 per cent) and toluene.

bp: about 260 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of triacetin*.

TESTS

**Appearance**. It is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity**. Dissolve 5.00 g in 25 mL of *anhydrous ethanol R*, previously neutralised to 0.2 mL of *phenolphthalein solution R* and add 0.20 mL of *0.1 M sodium hydroxide*. The pink colour of the mixture persists for 15 s.

**Relative density** (*2.2.5*): 1.159 to 1.164.

**Refractive index** (*2.2.6*): 1.429 to 1.432.

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 5.00 g.

ASSAY

Introduce 0.300 g into a 250 mL borosilicate glass flask fitted with a reflux condenser. Add 25.0 mL of *0.5 M alcoholic potassium hydroxide* and a few glass beads. Attach the condenser and heat under reflux for 30 min. Add 1 mL of *phenolphthalein solution R1* and titrate immediately with *0.5 M hydrochloric acid*. Carry out a blank test under the same conditions. Calculate the content from the difference in consumption of alkali in the main and the blank procedure.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 36.37 mg of $C_9H_{14}O_6$.

STORAGE

In a well-filled container.

**01/2008:1376**

# TRIAMCINOLONE

## Triamcinolonum

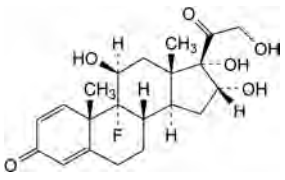

$C_{21}H_{27}FO_6$ $M_r$ 394.4
[124-94-7]

DEFINITION

9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione.

*Content*: 97.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, slightly soluble in methanol, practically insoluble in methylene chloride.

It shows polymorphism (*5.9*).

Monographs T

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triamcinolone CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness, dry the residues at 60 °C at a pressure not exceeding 0.7 kPa and record new spectra using the residues.

B. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use and protect from light. Examine the plate under ultraviolet light immediately after development.*

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *triamcinolone CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *dexamethasone CRS* in reference solution (a) and dilute to 10 mL with the same solution.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: the chromatogram obtained with reference solution (b) shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Specific optical rotation** (*2.2.7*): + 65 to + 72 (anhydrous substance).

Dissolve 0.100 g in *dimethylformamide R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use and protect from light.*

*Test solution*. Dissolve 25.0 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *water R* and dilute to 10.0 mL with the same mixture of solvents.

*Reference solution (a)*. Dissolve 2 mg of *triamcinolone CRS* and 2 mg of *triamcinolone impurity C CRS* in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100.0 mL with the same mixture of solvents.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with a mixture of equal volumes of *methanol R* and *water R*.

*Blank*: *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: a mixture prepared as follows: in a 1000 mL volumetric flask mix 525 mL of *methanol R* with 400 mL of *water R* and allow to equilibrate; adjust the volume to 1000 mL with *water R* and mix again.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer set at 238 nm.

*Injection*: 20 µL.

*Run time*: 4.5 times the retention time of triamcinolone.

*Retention time*: triamcinolone = about 11 min.

*System suitability*: reference solution (a):

- *resolution*: minimum of 1.8 between the peaks due to triamcinolone and to impurity C.

*Limits*:

- *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1 per cent) and not more than 2 such peaks have an area greater than half the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent),
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2 per cent),
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

ASSAY

*Prepare the solutions immediately before use and protect from light.*

Dissolve 50.0 mg in *alcohol R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 100.0 mL with *alcohol R*. Measure the absorbance (*2.2.25*) at the maximum at 238 nm.

Calculate the content of $C_{21}H_{27}FO_6$ taking the specific absorbance to be 389.

STORAGE

Protected from light.

IMPURITIES

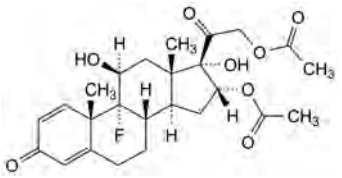

A. 9-fluoro-11β,17-dihydroxy-3,20-dioxopregna-1,4-diene-16α,21-diyl diacetate (triamcinolone 16,21-diacetate),

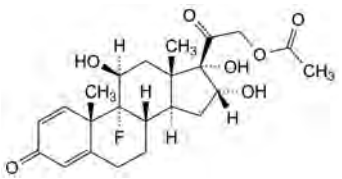

B. 9-fluoro-11β,16α,17-trihydroxy-3,20-dioxopregna-1,4-dien-21-yl acetate (triamcinolone 21-acetate),

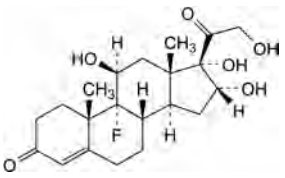

C. 9-fluoro-11β,16α,17,21-tetrahydroxypregn-4-ene-3,20-dione (pretriamcinolone).

**07/2012:0533**

# TRIAMCINOLONE ACETONIDE

## Triamcinoloni acetonidum

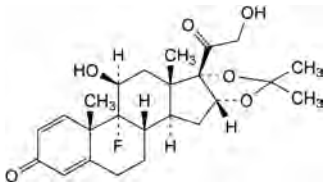

$C_{24}H_{31}FO_6$ $M_r$ 434.5
[76-25-5]

DEFINITION

9-Fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in ethanol (96 per cent).

It shows polymorphism (*5.9*).

IDENTIFICATION

*First identification: A, C.*

*Second identification: B, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triamcinolone acetonide CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *methanol R* and evaporate to dryness. Using the residues, prepare halogen salt discs or mulls in *liquid paraffin R* and record new spectra.

B. Thin-layer chromatography (*2.2.27*). *Prepare the solutions immediately before use and protect from light.*

*Test solution*. Dissolve 10 mg of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (a)*. Dissolve 20 mg of *triamcinolone acetonide CRS* in *methanol R* and dilute to 20 mL with the same solvent.

*Reference solution (b)*. Dissolve 10 mg of *triamcinolone hexacetonide CRS* in reference solution (a) and dilute to 10 mL with reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: add a mixture of 1.2 volumes of *water R* and 8 volumes of *methanol R* to a mixture of 15 volumes of *ether R* and 77 volumes of *methylene chloride R*.

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm, immediately after development.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

D. Mix about 5 mg with 45 mg of *heavy magnesium oxide R* and ignite in a crucible until an almost white residue is obtained (usually less than 5 min). Allow to cool, add 1 mL of *water R*, 0.05 mL of *phenolphthalein solution R1* and about 1 mL of *dilute hydrochloric acid R* to render the solution colourless. Filter. To a freshly prepared mixture of 0.1 mL of *alizarin S solution R* and 0.1 mL of *zirconyl nitrate solution R*, add 1.0 mL of the filtrate. Mix, allow to stand for 5 min and compare the colour of the solution to that of a blank prepared in the same manner. The test solution is yellow and the blank is red.

TESTS

**Specific optical rotation** (*2.2.7*): + 110 to + 117 (anhydrous substance).

Dissolve 0.100 g in *ethanol (96 per cent) R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Reference solution (a).* Dissolve 5 mg of *triamcinolone acetonide for system suitability CRS* (containing impurities B and C) in mobile phase B and dilute to 5.0 mL with mobile phase B.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase B. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase B.

*Reference solution (c).* Dissolve 25.0 mg of *triamcinolone acetonide CRS* in mobile phase B and dilute to 25.0 mL with mobile phase B.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*:

– *mobile phase A*: *acetonitrile R*, *water for chromatography R* (32:68 *V/V*);
– *mobile phase B*: *water for chromatography R*, *acetonitrile R* (35:65 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 40 | 100 → 0 | 0 → 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *triamcinolone acetonide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to triamcinolone acetonide (retention time = about 16 min): impurity C = about 0.7; impurity B = about 0.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.5 between the peaks due to impurities C and B.

Monographs T

*Limits*:

- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity C*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

ASSAY

*Carry out the assay protected from light.*

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Mobile phase*: mobile phase A.

*Injection*: test solution and reference solution (c).

*Run time*: 1.5 times the retention time of triamcinolone acetonide.

*Retention time*: triamcinolone acetonide = about 16 min.

Calculate the percentage content of $C_{24}H_{31}FO_6$ taking into account the assigned content of *triamcinolone acetonide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F.*

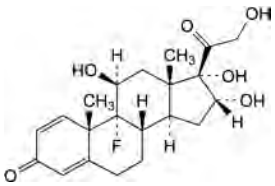

A. 9-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione (triamcinolone),

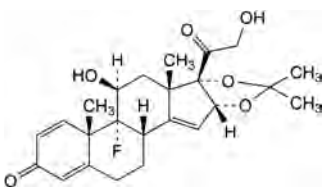

B. 9-fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4,14-triene-3,20-dione (Δ14-triamcinolone acetonide),

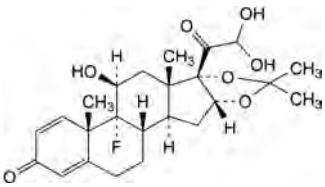

C. 9-fluoro-11β,21,21-trihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione (triamcinolone acetonide 21-aldehyde hydrate),

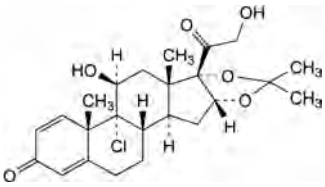

D. 9-chloro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-1,4-diene-3,20-dione (9α-chloro triamcinolone acetonide),

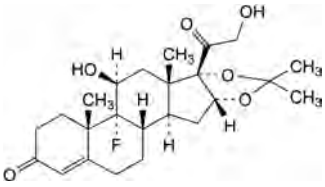

E. 9-fluoro-11β,21-dihydroxy-16α,17-(1-methylethylidenedioxy)pregna-4-ene-3,20-dione (1,2-dihydrotriamcinolone acetonide),

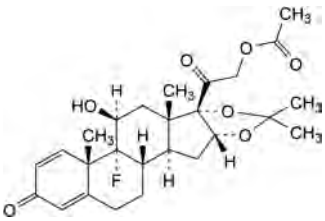

F. 9-fluoro-11β-hydroxy-16α,17-(1-methylethylidenedioxy)-3,20-dioxopregna-1,4-dien-21-yl acetate (21-acetate triamcinolone acetonide).

**01/2017:0867**

# TRIAMCINOLONE HEXACETONIDE

## Triamcinoloni hexacetonidum

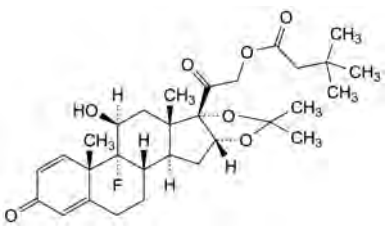

$C_{30}H_{41}FO_7$ $M_r$ 532.6

[5611-51-8]

DEFINITION

9-Fluoro-11β-hydroxy-2′,2′-dimethyl-3,20-dioxo-(16β*H*)-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 3,3-dimethylbutanoate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, sparingly soluble in anhydrous ethanol and in methanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triamcinolone hexacetonide CRS*.

B. Examine the chromatograms obtained in the assay.

*Results*: the principal peak in the chromatogram obtained with test solution (b) is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (c).

TESTS

**Specific optical rotation** (*2.2.7*): + 95 to + 101 (anhydrous substance).

Dissolve 0.100 g in *methylene chloride R* and dilute to 10.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution (a).* Dissolve 25.0 mg of the substance to be examined in *methanol R* and dilute to 10.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 5.0 mL with *methanol R*.

*Reference solution (a).* Dissolve 5 mg of *triamcinolone hexacetonide for system suitability CRS* (containing impurities B and C) in *methanol R* and dilute to 2.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 25.0 mg of *triamcinolone hexacetonide CRS* in *methanol R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 5.0 mL with *methanol R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *methanol R* (25:75 *V/V*).

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a) and (b).

*Run time*: twice the retention time of triamcinolone hexacetonide.

*Identification of impurities*: use the chromatogram supplied with *triamcinolone hexacetonide for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to triamcinolone hexacetonide (retention time = about 8 min): impurity B (epimer 1) = about 0.79; impurity B (epimer 2) = about 0.81; impurity C = about 1.3.

*System suitability*: reference solution (a):

– *resolution*: minimum 4.0 between the peaks due to triamcinolone hexacetonide and impurity C.

*Calculation of percentage contents:*

– for each impurity, use the concentration of triamcinolone hexacetonide in reference solution (b).

*Limits*:

– *impurity B*: for the sum of the areas of the 2 epimer peaks, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *total*: maximum 0.3 per cent;
– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.50 g.

ASSAY

*Carry out the test protected from light.*

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{30}H_{41}FO_7$ taking into account the assigned content of *triamcinolone hexacetonide CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

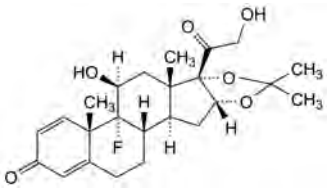

A. 9-fluoro-11β,21-dihydroxy-2′,2′-dimethyl-(16β*H*)-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-diene-3,20-dione (triamcinolone acetonide),

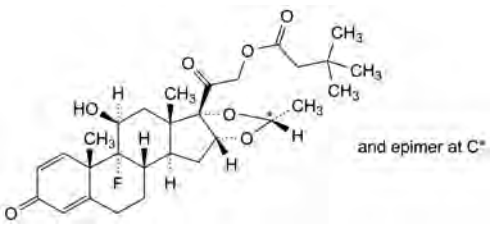


B. (2′*RS*)-9-fluoro-11β-hydroxy-2′-methyl-3,20-dioxo-(16β*H*)-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 3,3-dimethylbutanoate,

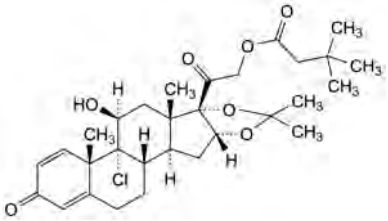

C. 9-chloro-11β-hydroxy-2′,2′-dimethyl-3,20-dioxo-(16β*H*)-[1,3]dioxolo[4′,5′:16,17]pregna-1,4-dien-21-yl 3,3-dimethylbutanoate.

04/2008:0058
corrected 6.3

# TRIAMTERENE

## Triamterenum

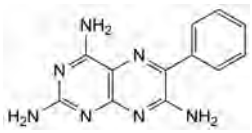

$C_{12}H_{11}N_7$ $M_r$ 253.3
[396-01-0]

DEFINITION

6-Phenylpteridine-2,4,7-triamine.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, crystalline powder.

*Solubility*: very slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triamterene CRS*.

TESTS

**Acidity**. Boil 1.0 g with 20 mL of *water R* for 5 min, cool, filter and wash the filter with 3 quantities, each of 10 mL, of *water R*. Combine the filtrate and washings and add 0.3 mL of *phenolphthalein solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Impurity D**. Gas chromatography (*2.2.28*).

*Internal standard solution*. Dilute 0.1 mL of *nitrobenzene R* to 100 mL with *methanol R*. Dilute 1 mL of this solution to 50 mL with *methanol R*.

*Test solution*. Introduce 0.800 g of the substance to be examined into a suitable vial, add 5 mL of *dimethyl sulfoxide R* and heat until the sample is dissolved (do not heat to boiling). Allow to cool. Add 5 mL of cold *methanol R* to enhance the precipitation of triamterene. Filter and wash the filter with 5 mL of *methanol R*. Combine the filtrate and washing, add 2.0 mL of the internal standard solution and dilute to 20.0 mL with *methanol R*.

*Reference solution*. Dissolve 20.0 mg of *benzyl cyanide R* (impurity D) in *methanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 50.0 mL with *methanol R*. To 2.0 mL of this solution add 2.0 mL of the internal standard solution and 5 mL of *dimethyl sulfoxide R* and dilute to 20.0 mL with *methanol R*.

*Blank solution*. Dilute 5 mL of *dimethyl sulfoxide R* to 20 mL with *methanol R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.5 mL/min.

*Split ratio*: 1:15.

*Temperature*:

- *column*: 170 °C;
- *injection port*: 210 °C;
- *detector*: 230 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: twice the retention time of the internal standard.

*Relative retention* with reference to the internal standard (retention time = about 6 min): impurity D = about 1.6.

*System suitability*: reference solution:

- *resolution*: minimum 2.0 between the peak due to impurity D and the nearest peak due to the solvent (blank solution);
- *signal-to-noise ratio*: minimum 10 for the peak due to impurity D.

*Limit*:

- *impurity D*: calculate the ratio (*R*) of the area of the peak due to impurity D to the area of the peak due to the internal standard from the chromatogram obtained with the reference solution; from the chromatogram obtained with the test solution, calculate the ratio of the area of the peak due to impurity D to the area of the peak due to the internal standard: this ratio is not greater than *R* (50 ppm).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 10.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *nitrosotriaminopyrimidine CRS* (impurity A) in the mobile phase and dilute to 100.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c)*. Dissolve the contents of a vial of *triamterene impurity B CRS* in 200 µL of *dimethyl sulfoxide R*. Add 5.0 mL of the test solution and dilute to 50.0 mL with the mobile phase. Filter the solution through a membrane filter (nominal pore size 0.45 µm) before injection.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: spherical *end-capped octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *butylamine R*, *acetonitrile R*, *methanol R*, *water R* (2:200:200:600 *V/V/V/V*), adjusted to pH 5.3 with *acetic acid R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 320 nm and at 355 nm.

*Injection*: 50 µL.

*Relative retention* with reference to triamterene (retention time = about 5 min): impurity A = about 0.6; impurity B = about 0.8; impurity C = about 1.7.

*System suitability*:

- *resolution*: minimum 1.5 between the peaks due to impurity B and triamterene in the chromatogram obtained with reference solution (c) at 355 nm; if necessary, increase the quantity of *water R* in the mobile phase;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b) at 320 nm.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 1.8; impurity C = 1.5;
- *impurity A at 320 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (50 ppm);

- *impurities B, C at 355 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities at 355 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total at 355 nm*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit at 355 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 mL of *anhydrous formic acid R* and add 100 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 25.33 mg of $C_{12}H_{11}N_7$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

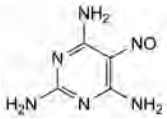

A. 5-nitrosopyrimidine-2,4,6-triamine (nitrosotriamino-pyrimidine),

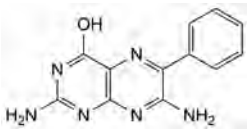

B. 2,7-diamino-6-phenylpteridin-4-ol,

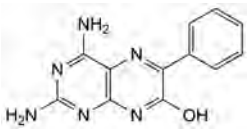

C. 2,4-diamino-6-phenylpteridin-7-ol,

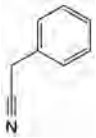

D. phenylacetonitrile (benzyl cyanide).

**01/2017:1740**

# TRIBENOSIDE

## Tribenosidum

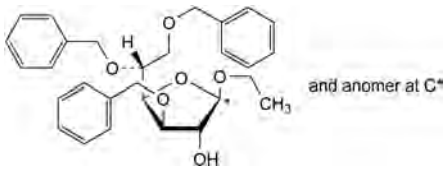


$C_{29}H_{34}O_6$ $M_r$ 478.6
[10310-32-4]

DEFINITION

Mixture of α- and β-anomers of ethyl 3,5,6-tri-*O*-benzyl-D-glucofuranoside.

*Content*: 96.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: yellowish or pale yellow, clear, viscous liquid.

*Solubility*: practically insoluble in water, very soluble in acetone, in methanol and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tribenoside CRS*.

TESTS

**Solution S**. Dissolve 4.00 g in *methanol R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm has a maximum of 0.10.

**Specific optical rotation** (*2.2.7*): − 40.0 to − 31.0.

Dilute 2.0 mL of solution S to 20.0 mL with *methanol R*.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 1.000 g of the substance to be examined in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 25.0 mL with the same mixture of solvents.

*Test solution (b).* Dissolve 50.0 mg of the substance to be examined in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (a).* Dilute 25.0 mg of *benzaldehyde CRS* (impurity C) and 30.0 mg of *tribenoside impurity A CRS* to 100.0 mL with *acetonitrile R*. Introduce 20.0 mL of this solution into a 50 mL volumetric flask, add 2.5 mL of *water R* and dilute to 50.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 50.0 mg of *tribenoside CRS* in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution (c).* Dissolve 12.0 mg of *tribenoside impurity D CRS* in a mixture of 5 volumes of *water R* and 95 volumes of *acetonitrile R* and dilute to 100.0 mL with the same mixture of solvents.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm).

*Mobile phase*:

- *mobile phase A*: 0.1 per cent *V/V* solution of *phosphoric acid R*;

Monographs T

– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 55 → 10 | 45 → 90 |
| 40 - 55 | 10 | 90 |

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (a), (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and C; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity D.

*Relative retentions* with reference to the β-anomer of tribenoside (retention time = about 18 min): α-anomer = about 1.1; impurity C = about 0.2; impurity B = about 0.6; impurity D = about 0.8; impurity A = about 1.4.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to the α-anomer and to the β-anomer of tribenoside.

*Limits*:

– *impurity A*: not more than 1.7 times the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
– *impurity C*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.5 per cent); if the area of the peak due to impurity C in the chromatogram obtained with the test solution is greater than the area of the corresponding peak in the chromatogram obtained with reference solution (a) (0.25 per cent), dilute the test solution to obtain an area equal to or smaller than the area of the peak in the chromatogram obtained with reference solution (a); calculate the content of impurity C taking into account the dilution factor;
– *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
– *any other impurity*: not more than the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *total*: maximum 2.0 per cent;
– *disregard limit*: 0.17 times the area of the peak due to impurity A in the chromatogram obtained with reference solution (a) (0.05 per cent).

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (b).

Calculate the sum of the percentage contents of the α-anomer and the β-anomer of tribenoside taking into account the assigned content of *tribenoside CRS*.

STORAGE

Under nitrogen, in an airtight container.

IMPURITIES

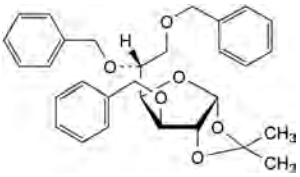

A. 3,5,6-tri-*O*-benzyl-1,2-*O*-(1-methylethylidene)-α-D-glucofuranose,

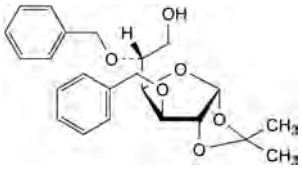

B. 3,5-di-*O*-benzyl-1,2-*O*-(1-methylethylidene)-α-D-glucofuranose,

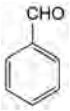

C. benzaldehyde,

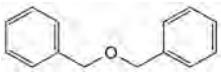

D. dibenzyl ether.

**01/2017:1770**
**corrected 10.0**

# TRIBUTYL ACETYLCITRATE

## Tributylis acetylcitras

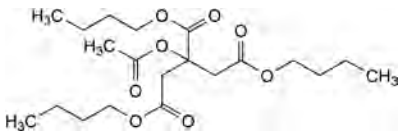

$C_{20}H_{34}O_8$ $M_r$ 402.5
[77-90-7]

DEFINITION

Tributyl 2-(acetyloxy)propane-1,2,3-tricarboxylate.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, oily liquid.

*Solubility*: not miscible with water, miscible with ethanol (96 per cent) and with methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: thin films between 2 sodium chloride plates.

*Comparison*: *tributyl acetylcitrate CRS*.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. Dilute 10 g with 10 mL of previously neutralised *ethanol (96 per cent) R* and add 0.5 mL of *bromothymol blue solution R2*. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Refractive index** (*2.2.6*): 1.442 to 1.445.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.5 g of the substance to be examined in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (a)*. Dissolve 50 mg of the substance to be examined and 50 mg of *tributyl citrate R* (impurity A) in *methylene chloride R* and dilute to 20 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with *methylene chloride R*. Dilute 1.0 mL of this solution to 25.0 mL with *methylene chloride R*.

*Reference solution (c).* Dissolve the contents of a vial of *tributyl acetylcitrate for peak identification CRS* (containing impurities B and C) in 1 mL of *methylene chloride R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *cyanopropyl(25)phenyl(25)methyl(50)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 36 cm/s.

*Split ratio*: 1:20.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 7 | 70 → 210 |
| | 7 - 50 | 210 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL; inject via an inert, glass-lined injection port using an automatic injection device.

*Identification of impurities*: use the chromatogram supplied with *tributyl acetylcitrate for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities B and C; use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to tributyl acetylcitrate (retention time = about 24 min): impurity B = about 0.70; impurity C = about 0.83; impurity A = about 0.87.

*System suitability*:

- *resolution*: minimum 2.0 between the peaks due to impurity A and tributyl acetylcitrate in the chromatogram obtained with reference solution (a);
- *repeatability*: maximum relative standard deviation of 5.0 per cent after 6 injections of reference solution (b).

*Limits*:

- *impurity A*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.6 per cent);
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.4 per cent);
- *impurity B*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.25 per cent, determined on 2.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 1.500 g into a 250 mL borosilicate glass flask. Add 25 mL of *2-propanol R*, 50 mL of *water R*, 25.0 mL of *1 M sodium hydroxide* and a few glass beads. Heat under a reflux condenser for 3 h. Allow to cool. Add 1 mL of *phenolphthalein solution R1* and titrate with *1 M hydrochloric acid*. Carry out a blank titration.

1 mL of *1 M sodium hydroxide* is equivalent to 100.6 mg of $C_{20}H_{34}O_8$.

IMPURITIES

*Specified impurities*: *A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

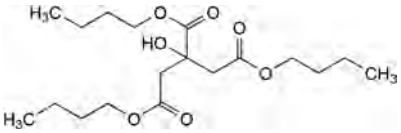

A. tributyl 2-hydroxypropane-1,2,3-tricarboxylate (tributyl citrate),

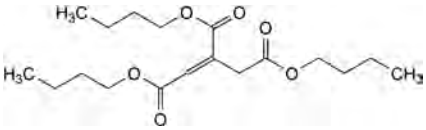

B. tributyl propene-1,2,3-tricarboxylate (tributyl aconitate),

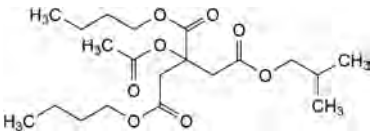

C. 1,2-dibutyl 3-(2-methylpropyl) 2-(acetyloxy)propane-1,2,3-tricarboxylate,

D. butan-1-ol,

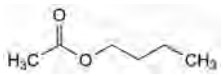

E. butyl acetate.

**01/2017:1682**
**corrected 10.0**

# TRI-*n*-BUTYL PHOSPHATE

## Tri-*n*-butylis phosphas

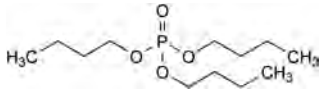

$C_{12}H_{27}O_4P$ $M_r$ 266.3

[126-73-8]

CHARACTERS

*Appearance*: clear, colourless or pale yellow liquid.

*Solubility*: slightly soluble in water, miscible with ethanol (96 per cent).

bp: about 289 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tri-n-butyl phosphate CRS*.

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Acidity**. Dissolve 50 mL in 50 mL of *ethanol (96 per cent) R* previously adjusted with *0.02 M potassium hydroxide* or *0.02 M hydrochloric acid* to a bluish-green colour, using 0.5 mL of *bromothymol blue solution R1* as indicator. Titrate with *0.02 M potassium hydroxide* to the initial bluish-green coloration. Not more than 0.8 mL of *0.02 M potassium hydroxide* is required.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution*. The substance to be examined.

*Reference solution*. Dissolve 10 mg of the substance to be examined and 10 mg of *methyl myristate R* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (5 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 32 cm/s.

*Split ratio*: 65:1.

*Temperature*:

- *column*: 250 °C;
- *injection port and detector*: 250 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Run time*: twice the retention time of tri-*n*-butyl phosphate.

*System suitability*: reference solution:

- *resolution*: minimum 10 between the peaks due to tri-*n*-butyl phosphate and methyl myristate.

*Limits*:

- *any impurity*: for each impurity, maximum 0.3 per cent;
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.01 per cent.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 15 mL of *ethanol (70 per cent V/V) R*. The solution complies with the test. Prepare the reference solution using 10 mL of *chloride standard solution (5 ppm Cl) R* and 5 mL of *anhydrous ethanol R*.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.0 g.

STORAGE

Protected from light.

IMPURITIES

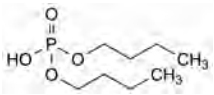

A. dibutyl hydrogen phosphate,

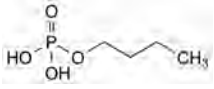

B. butyl dihydrogen phosphate,

C. $H_3C$-$[CH_2]_3$-OH: butan-1-ol,

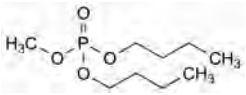

D. dibutyl methyl phosphate,

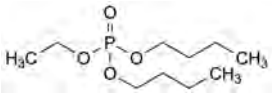

E. dibutyl ethyl phosphate,

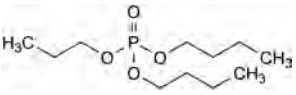

F. dibutyl propyl phosphate,

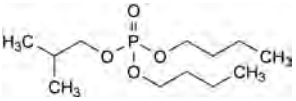

G. dibutyl 2-methylpropyl phosphate,

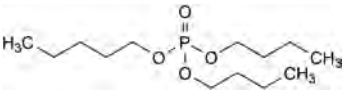

H. dibutyl pentyl phosphate,

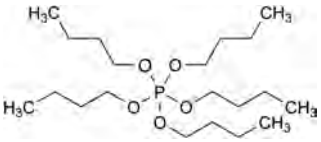

I. pentabutyl phosphate.

**01/2008:1967**
**corrected 6.0**

# TRICHLOROACETIC ACID

## Acidum trichloroaceticum

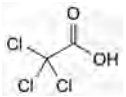

$C_2HCl_3O_2$ $M_r$ 163.4
[76-03-9]

DEFINITION

2,2,2-Trichloroacetic acid.

*Content*: 98.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline mass or colourless crystals, very deliquescent.

*Solubility*: very soluble in water, in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: *A*.

*Second identification*: *B, C*.

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of trichloroacetic acid*.

B. To 0.5 mL of solution S (see Tests) add 2 mL of *pyridine R* and 5 mL of *strong sodium hydroxide solution R*. Shake vigorously and heat in a water-bath at 60-70 °C for 5 min. The upper layer shows an intense red colour.

C. Solution S is strongly acidic (*2.2.4*).

TESTS

**Solution S**. Dissolve 2.5 g in *water R* and dilute to 25 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 20 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 16.34 mg of $C_2HCl_3O_2$.

STORAGE

In an airtight container.

**07/2015:2609**

# TRICLABENDAZOLE FOR VETERINARY USE

## Triclabendazolum ad usum veterinarium

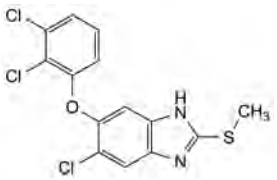

$C_{14}H_9Cl_3N_2OS$ $M_r$ 359.7
[68786-66-3]

DEFINITION

5-Chloro-6-(2,3-dichlorophenoxy)-2-(methylsulfanyl)-1*H*-benzimidazole.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triclabendazole CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 10 mL of *acetonitrile R* and dilute to 25.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *triclabendazole for system suitability CRS* ( impurities A, B and D) in 1.0 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase.* Dissolve 0.77 g of *ammonium acetate R* in 800 mL of *water for chromatography R*, add 1 mL of *triethylamine R* and mix; adjust to pH 4.5 with *glacial acetic acid R* and dilute to 1 L with *water for chromatography R*. Mix 40 volumes of this solution and 60 volumes of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 305 nm.

*Injection*: 20 µL.

*Run time*: 2.5 times the retention time of triclabendazole.

*Identification of impurities*: use the chromatogram supplied with *triclabendazole for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and D.

*Relative retention* with reference to triclabendazole (retention time = about 10 min): impurity A = about 0.6; impurity B = about 0.7; impurity D = about 1.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurities A and B.

*Calculation of percentage contents*:

– *correction factors*: multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 1.9; impurity D = 2.7;

– for each impurity, use the concentration of triclabendazole in reference solution (a).

*Limits*:

– *impurities A, D*: for each impurity, maximum 0.3 per cent;

– *unspecified impurities*: for each impurity, maximum 0.20 per cent;

– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.10 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.280 g in 50 mL of *anhydrous acetic acid R*. Allow to cool and titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.97 mg of $C_{14}H_9Cl_3N_2OS$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

Monographs T

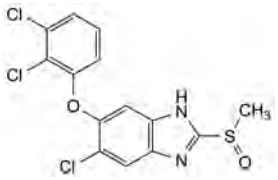

A. 5-chloro-6-(2,3-dichlorophenoxy)-2-(methylsulfinyl)-1*H*-benzimidazole,

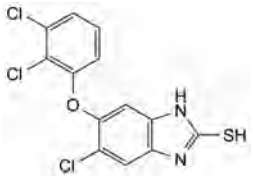

B. 5-chloro-6-(2,3-dichlorophenoxy)-1*H*-benzimidazole-2-thiol,

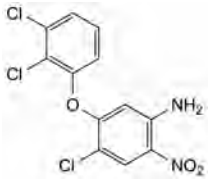

D. 4-chloro-5-(2,3-dichlorophenoxy)-2-nitroaniline.

**01/2017:1479**
**corrected 10.0**

# TRIETHYL CITRATE

## Triethylis citras

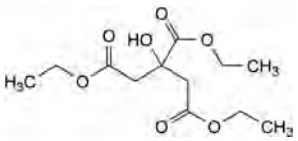

$C_{12}H_{20}O_7$ $M_r$ 276.3
[77-93-0]

DEFINITION

Triethyl 2-hydroxypropane-1,2,3-tricarboxylate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: clear, viscous, colourless or almost colourless, hygroscopic liquid.

*Solubility*: soluble in water, miscible with ethanol (96 per cent), slightly soluble in fatty oils.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Refractive index (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *Ph. Eur. reference spectrum of triethyl citrate.*

C. It gives the reaction of esters (*2.3.1*).

D. To 0.5 mL add 5 mL of *ethanol (96 per cent) R* and 4 mL of *dilute sodium hydroxide solution R*. Boil under reflux for about 10 min. 2 mL of the solution gives the reaction of citrates (*2.3.1*).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Acidity**. Dilute 10 g with 10 mL of previously neutralised *ethanol (96 per cent) R*, add 0.5 mL of *bromothymol blue solution R2*. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to blue.

**Refractive index** (*2.2.6*): 1.440 to 1.446.

**Related substances**. Gas chromatography (*2.2.28*): use the normalisation procedure.

*Test solution.* Dissolve 1.0 mL of the substance to be examined in *methylene chloride R* and dilute to 50.0 mL with the same solvent.

*Reference solution.* Dissolve 1.0 mL of the substance to be examined and 0.5 mL of *methyl tridecanoate R* in *methylene chloride R*, then dilute to 50.0 mL with the same solvent.

*Column*:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (5 µm).

*Carrier gas*: *helium for chromatography R.*

*Linear velocity*: about 26 cm/s.

*Split ratio*: about 1:50.

*Temperature*:
- *column*: 200 °C;
- *injection port and detector*: 220 °C.

*Detection*: flame ionisation.

*Injection*: 1.0 µL.

*Run time*: twice the retention time of triethyl citrate.

*Retention time*: triethyl citrate = about 13.6 min.

*System suitability*: reference solution:
- *resolution*: minimum 1.5 between the peaks due to triethyl citrate and methyl tridecanoate.

*Limits*:
- *any impurity*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 0.5 per cent;
- *disregard limit*: 0.04 per cent.

**Water** (*2.5.12*): maximum 0.25 per cent, determined on 1.000 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Introduce 1.500 g into a 250 mL borosilicate-glass flask fitted with a reflux condenser. Add 25 mL of *2-propanol R*, 50 mL of *water R*, 25.0 mL of *1 M sodium hydroxide* and a few glass beads. Heat under a reflux condenser for 1 h. Allow to cool. Add 1 mL of *phenolphthalein solution R1* and titrate with *1 M hydrochloric acid*. Carry out a blank titration.

1 mL of *1 M sodium hydroxide* is equivalent to 92.1 mg of $C_{12}H_{20}O_7$.

STORAGE

In an airtight container.

IMPURITIES

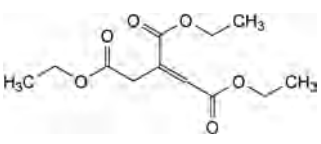

A. triethyl propene-1,2,3-tricarboxylate (triethyl aconitate).

**01/2008:0059**
**corrected 6.0**

# TRIFLUOPERAZINE HYDROCHLORIDE

## Trifluoperazini hydrochloridum

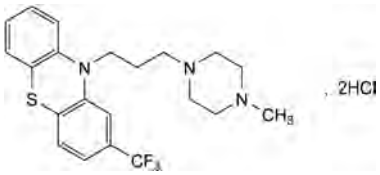

$C_{21}H_{26}Cl_2F_3N_3S$ $M_r$ 480.4
[440-17-5]

DEFINITION

Trifluoperazine hydrochloride contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 10-[3-(4-methylpiperazin-1-yl)propyl]-2-(trifluoromethyl)-10*H*-phenothiazine dihydrochloride, calculated with reference to the dried substance.

CHARACTERS

A white to pale yellow, crystalline powder, hygroscopic, freely soluble in water, soluble in alcohol.

It melts at about 242 °C, with decomposition.

IDENTIFICATION

A. *Protect the solutions from bright light and measure the absorbances immediately.* Dissolve 50 mg in *0.1 M hydrochloric acid* and dilute to 500 mL with the same acid. Examined between 280 nm and 350 nm, the solution shows an absorption maximum (*2.2.25*) at 305 nm. Dilute 5 mL of the solution to 100 mL with *0.1 M hydrochloric acid*. Examined between 230 nm and 280 nm, this solution shows an absorption maximum at 255 nm. The specific absorbance at this maximum is about 650.

B. It complies with the identification test for phenothiazines by thin-layer chromatography (*2.3.3*): use *trifluoperazine hydrochloride CRS* to prepare the reference solution.

C. Place 0.25 g in a 100 mL separating funnel, add 5 mL of *water R* and 2 mL of *dilute sodium hydroxide solution R*. Shake vigorously with 20 mL of *ether R*. Wash the ether layer with 5 mL of *water R*, add 0.15 g of *maleic acid R* and evaporate the ether. The residue, recrystallised from 30 mL of *alcohol R* and dried, melts (*2.2.14*) at about 192 °C.

D. Dissolve about 0.5 mg in 1 mL of *water R*, add 0.1 mL of *bromine water R* and shake for about 1 min. Add dropwise 1 mL of *sulfuric acid R* with constant, vigorous agitation. A red colour develops.

E. Dissolve about 50 mg in 5 mL of *water R* and add 2 mL of *nitric acid R*. A dark-red colour develops which turns to pale yellow. The solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*). Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent. The pH of the solution is 1.6 to 2.5.

**Related substances**. *Carry out the test protected from bright light.*

Examine by thin-layer chromatography (*2.2.27*), using a *TLC silica gel $GF_{254}$ plate R*.

*Test solution.* Dissolve 0.2 g of the substance to be examined in a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents. Prepare immediately before use.

*Reference solution.* Dilute 1 mL of the test solution to 200 mL with a mixture of 5 volumes of *diethylamine R* and 95 volumes of *methanol R*.

Apply to the plate 10 µL of each solution. Develop over a path of 12 cm using a mixture of 10 volumes of *acetone R*, 10 volumes of *diethylamine R* and 80 volumes of *cyclohexane R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with the test solution, apart from the principal spot, is not more intense than the spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Loss on drying** (*2.2.32*). Not more than 1.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *alcohol R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 48.04 mg of $C_{21}H_{26}Cl_2F_3N_3S$.

STORAGE

Store in an airtight container, protected from light.

**01/2017:1377**

# TRIFLUSAL

## Triflusalum

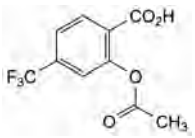

$C_{10}H_7F_3O_4$ $M_r$ 248.2
[322-79-2]

DEFINITION

2-(Acetyloxy)-4-(trifluoromethyl)benzoic acid.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, very soluble in anhydrous ethanol, freely soluble in methylene chloride.

mp: about 118 °C, with decomposition.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *triflusal CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in *acetonitrile R* and dilute to 20.0 mL with the same solvent. *Prepare the solution immediately before use.*

*Reference solution (a).* Dissolve 5.0 mg of *triflusal impurity B CRS* in *acetonitrile R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 25.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve 2.5 mg of the substance to be examined in *acetonitrile R*, add 5 mL of reference solution (a) and dilute to 10.0 mL with *acetonitrile R*. *Prepare the solution immediately before use.*

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4-5 µm).

*Mobile phase*:

- *mobile phase A*: 0.5 per cent *V/V* solution of *phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 80 → 30 | 20 → 70 |
| 20 - 25 | 30 | 70 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 237 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity B.

*Relative retention* with reference to triflusal (retention time = about 11 min): impurity B = about 1.2.

*System suitability*: reference solution (c):

- *resolution*: minimum 3.0 between the peaks due to triflusal and impurity B.

*Limits*:

- *impurity B*: not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *sum of impurities other than B*: not more than 0.5 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *disregard limit*: 0.25 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous ethanol R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 24.82 mg of $C_{10}H_7F_3O_4$.

STORAGE

In an airtight container, at a temperature not exceeding 25 °C.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D.*

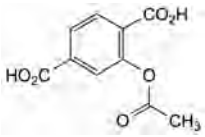

A. 2-(acetyloxy)benzene-1,4-dicarboxylic acid (2-acetoxyterephthalic acid),

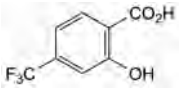

B. 2-hydroxy-4-(trifluoromethyl)benzoic acid (4-(trifluoromethyl)salicylic acid),

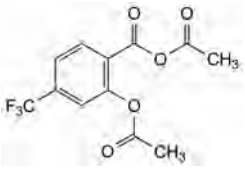

C. acetic 2-(acetyloxy)-4-(trifluoromethyl)benzoic anhydride,

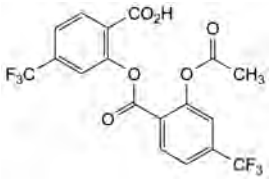

D. 2-[[2-(acetyloxy)-4-(trifluoromethyl)benzoyl]oxy]-4-(trifluoromethyl)benzoic acid.

**01/2020:0868**

# TRIGLYCERIDES, MEDIUM-CHAIN

## Triglycerida media

DEFINITION

Mixture of triglycerides of saturated fatty acids, mainly of caprylic (octanoic) acid and of capric (decanoic) acid. The fatty acids are obtained from the oil extracted from the hard, dried fraction of the endosperm of *Cocos nucifera* L. or from the dried endosperm of *Elaeis guineensis* Jacq.

*Content*: minimum 95.0 per cent of saturated fatty acids with 8 and 10 carbon atoms.

CHARACTERS

*Appearance*: colourless or slightly yellowish, oily liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent), with methylene chloride, with light petroleum and with fatty oils.

*Relative density*: about 0.95.

*Refractive index*: about 1.446.

IDENTIFICATION

*First identification: C, D.*

*Second identification: A, B, D.*

A. Iodine value (*2.5.4*): maximum 1.0.

B. Saponification value (*2.5.6*): 310 to 360.

C. Composition of fatty acids (see Tests).

D. Viscosity (see Tests).

TESTS

**Appearance**. The substance to be examined is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method I*).

**Alkaline impurities**. Dissolve 2.00 g in a mixture of 1.5 mL of *ethanol (96 per cent) R* and 3.0 mL of *ether R*. Add 0.05 mL of *bromophenol blue solution R*. Not more than 0.15 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to yellow.

**Viscosity** (*2.2.9*): 25 mPa·s to 33 mPa·s.

**Acid value** (*2.5.1*): maximum 0.2.

**Hydroxyl value** (*2.5.3, Method A*): maximum 10.

**Peroxide value** (*2.5.5, Method A*): maximum 1.0.

**Composition of fatty acids**. Gas chromatography (*2.4.22, Method C*) with the following modifications. Use the mixture of calibrating substances in Table 2.4.22.-2.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.32 mm;

– *stationary phase*: *macrogol 20 000 R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.3 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 70 |
| | 1 - 35 | 70 → 240 |
| | 35 - 50 | 240 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Split ratio*: 1:100.

*Composition of the fatty-acid fraction of the substance*:

– *caproic acid*: maximum 2.0 per cent;

– *caprylic acid*: 50.0 per cent to 80.0 per cent;

– *capric acid*: 20.0 per cent to 50.0 per cent;

– *lauric acid*: maximum 3.0 per cent;

– *myristic acid*: maximum 1.0 per cent;

– *fatty acids of chain length greater than or equal to* $C_{16}$: maximum 1.0 per cent.

**Water** (*2.5.32*): maximum 0.2 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 2.0 g.

STORAGE

In a well-filled container, protected from light.

LABELLING

The label states, where applicable, that the substance is intended for use in parenteral nutrition.

**04/2012:2032**

# TRIGLYCEROL DIISOSTEARATE

## Triglyceroli diisostearas

DEFINITION

Mixture of polyglycerol diesters of mainly isostearic acid, obtained by esterification of polyglycerol and isostearic acid. The polyglycerol consists mainly of triglycerol.

CHARACTERS

*Appearance*: clear, yellowish, viscous liquid.

*Solubility*: practically insoluble in water, miscible with ethanol (96 per cent) and with fatty oils.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: film between 2 plates of *sodium chloride R*.

*Comparison*: *triglycerol diisostearate CRS*.

B. Composition of fatty acids (see Tests).

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_3$ (*2.2.2, Method I*).

Mix 10 mL with 10 mL of *ethanol (96 per cent) R*.

**Acid value** (*2.5.1*): maximum 3.0, determined on 1.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 180 to 230, determined on 0.25 g.

**Iodine value** (*2.5.4, Method B*): maximum 5.0.

**Peroxide value** (*2.5.5, Method B*): maximum 6.0.

**Saponification value** (*2.5.6*): 128 to 160.

**Composition of fatty acids** (*2.4.22, Method B*). Use the mixture of calibrating substances in Table 2.4.22.-1.

*Composition of the fatty-acid fraction of the substance*:

– *sum of the contents of the fatty acids eluting between palmitic acid and stearic acid*: minimum 60.0 per cent;

– *sum of the contents of myristic acid, palmitic acid and stearic acid*: maximum 11.0 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 2.00 g.

**Sulfated ash**: maximum 0.5 per cent, determined on 1.0 g.

Heat a silica crucible to redness for 30 min, allow to cool in a desiccator and weigh. Evenly distribute 1.00 g of the substance to be examined in the crucible and weigh. Dry at 100-105 °C for 1 h and ignite in a muffle furnace at 600 °C ± 25 °C until the substance is thoroughly charred. Carry out the test for sulfated ash (*2.4.14*) on the residue obtained, starting with "Moisten the substance to be examined...".

STORAGE

In an airtight container, protected from light.

01/2008:1626
corrected 6.0

# TRIHEXYPHENIDYL HYDROCHLORIDE

## Trihexyphenidyli hydrochloridum

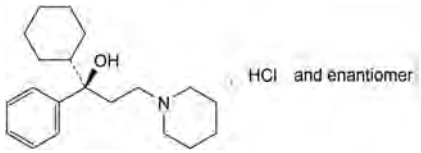


$C_{20}H_{32}ClNO$ $M_r$ 337.9
[52-49-3]

DEFINITION

(1*RS*)-1-Cyclohexyl-1-phenyl-3-(piperidin-1-yl)propan-1-ol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 250 °C, with decomposition.

IDENTIFICATION

*First identification*: *A, D.*

*Second identification*: *B, C, D.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *trihexyphenidyl hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 25 mg of the substance to be examined in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Reference solution.* Dissolve 25 mg of *trihexyphenidyl hydrochloride CRS* in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 10 mL with the same mixture of solvents.
*Plate*: *TLC silica gel G plate R.*
*Mobile phase*: *diethylamine R*, *hexane R* (5:95 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with a 0.1 g/L solution of *chloroplatinic acid R* in *hydrochloric acid R* containing 0.4 per cent *V/V* of *hydriodic acid R*.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve 0.5 g in 5 mL of warm *methanol R* and make just alkaline to *red litmus paper R* with *sodium hydroxide solution R*. A precipitate is formed which, after recrystallisation from *methanol R*, melts (*2.2.14*) at about 113°C to 115 °C.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 5.2 to 6.2.

Dissolve 0.5 g with heating in 25 mL of *carbon dioxide-free water R*. Cool to room temperature and dilute to 50 mL with *carbon dioxide-free water R*.

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°.

Dissolve 1.25 g in a mixture of 20 volumes of *methanol R* and 80 volumes of *methylene chloride R* and dilute to 25.0 mL with the same mixture of solvents.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 20.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase. Dilute 10.0 mL to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 10.0 mg of *trihexyphenidyl impurity A CRS* in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Reference solution (d).* To 1 mL of reference solution (b), add 1 mL of the test solution and dilute to 100 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 200 mL of *water R* with 0.2 mL of *triethylamine R*. Adjust to pH 4.0 with *phosphoric acid R* and add 800 mL of *acetonitrile R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of trihexyphenidyl.

*System suitability*: reference solution (d):
- *resolution*: minimum 4.0 between the peaks due to trihexyphenidyl and to impurity A.

*Limits*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 0.5 per cent;
- *disregard limit*: 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *ethanol (96 per cent) R* and add 5.0 mL of *0.01 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mLof *0.1 M sodium hydroxide* is equivalent to 33.79 mg of $C_{20}H_{32}ClNO$.

IMPURITIES

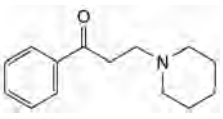


A. 1-phenyl-3-(piperidin-1-yl)propan-1-one.

Monographs T

**01/2017:2182**
**corrected 10.0**

# TRIMEBUTINE MALEATE

## Trimebutini maleas

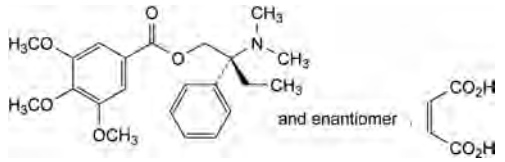


$C_{26}H_{33}NO_9$ $M_r$ 503.5
[34140-59-5]

DEFINITION

(2*RS*)-2-(Dimethylamino)-2-phenylbutyl 3,4,5-trimethoxybenzoate (*Z*)-butenedioate.

*Content*: 99.0 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in acetonitrile, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 133 °C.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *trimebutine maleate CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in *acetone R*, sonicate and dilute to 100 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture.* Dissolve 0.24 g of *anhydrous sodium dihydrogen phosphate R* in 180 mL of *water R* and adjust to pH 2.5 with *dilute phosphoric acid R*; dilute to 200 mL with *water R*. Add 50 mL of *acetonitrile R* and mix.

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *methyl 3,4,5-trimethoxybenzoate R* (impurity C) in 10 mL of the solvent mixture. Dilute 1 mL of the solution to 20 mL with the solvent mixture.

*Reference solution (c).* Dissolve the contents of a vial of *trimebutine for system suitability CRS* (containing impurities D and E) in 1 mL of reference solution (b).

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *polar end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 25 °C.

*Mobile phase*:
- *mobile phase A*: dissolve 3.6 g of *anhydrous sodium dihydrogen phosphate R* in 990 mL of *water for chromatography R* and adjust to pH 3.0 with *phosphoric acid R*; dilute to 1000 mL with *water for chromatography R*;
- *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 78 | 22 |
| 3 - 6.5 | 78 → 65 | 22 → 35 |
| 6.5 - 15 | 65 → 60 | 35 → 40 |
| 15 - 35 | 60 | 40 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL of the test solution and reference solutions (a) and (c).

*Identification of impurities*: use the chromatogram supplied with *trimebutine for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities C, D and E.

*Relative retention* with reference to trimebutine (retention time = about 12 min): maleic acid = about 0.17; impurity E = about 0.9; impurity D = about 1.3; impurity C = about 1.4.

*System suitability*: reference solution (c):
- *resolution*: minimum 1.5 between the peaks due to impurities D and C;
- *peak-to-valley ratio*: minimum 10, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to trimebutine.

*Limits*:
- *impurity E*: not more than 6 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.6 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent); disregard any peak due to maleic acid.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 50.35 mg of $C_{26}H_{33}NO_9$.

IMPURITIES

*Specified impurities*: *E.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D.*

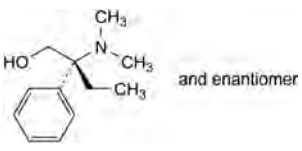


A. (2*RS*)-2-(dimethylamino)-2-phenylbutanol,

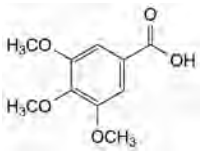

B. 3,4,5-trimethoxybenzoic acid,

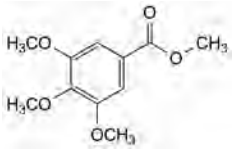

C. methyl 3,4,5-trimethoxybenzoate,

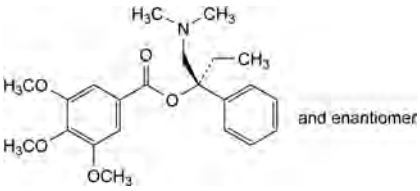


D. (1*RS*)-1-[(dimethylamino)methyl]-1-phenylpropyl 3,4,5-trimethoxybenzoate,

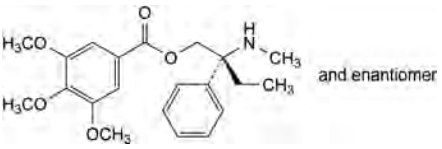


E. (2*RS*)-2-(methylamino)-2-phenylbutyl 3,4,5-trimethoxybenzoate.

**01/2008:1741**
**corrected 10.0**

# TRIMETAZIDINE DIHYDROCHLORIDE

## Trimetazidini dihydrochloridum

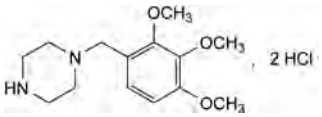


$C_{14}H_{24}Cl_2N_2O_3$ $M_r$ 339.3
[13171-25-0]

DEFINITION

1-(2,3,4-Trimethoxybenzyl)piperazine dihydrochloride.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, slightly hygroscopic.

*Solubility*: freely soluble in water, sparingly soluble in alcohol.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of trimetazidine dihydrochloride*.

B. Dissolve 25 mg in 5 mL of *water R*. 2 mL of the solution gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 20.0 mg of *trimetazidine for system suitability CRS* in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dilute 2.0 mL of the test solution to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (c)*. Dilute 25.0 mL of reference solution (b) to 50.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm,
- *stationary phase*: spherical *octadecylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm,
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 357 volumes of *methanol R* and 643 volumes of a 2.87 g/L solution of *sodium heptanesulfonate R* adjusted to pH 3.0 with *dilute phosphoric acid R*,
- *mobile phase B*: *methanol R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 50 | 95 → 75 | 5 → 25 |
| 50 - 52 | 75 → 95 | 25 → 5 |

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 240 nm.

*Equilibration*: for at least 1 h with the mobile phase at the initial composition.

*Injection*: 10 µL.

*Relative retention* with reference to trimetazidine (retention time = about 25 min): impurity D = about 0.2; impurity C = about 0.4; impurity H = about 0.6; impurities A and I = about 0.9; impurity E = about 0.95; impurity F = about 1.4; impurity B = about 1.8.

*System suitability*:

- *peak-to-valley ratio*: minimum 3, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the principal peak in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *correction factors*: for the calculation of contents, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.55; impurity C = 0.37; impurity F = 0.71;
- *impurities A, B, C, D, E, F, H, I*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *any other impurity*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);

– *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Impurity G**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 22.6 mg of *piperazine hydrate R* in *methanol R* and dilute to 100 mL with the same solvent. Dilute 10 mL of the solution to 100 mL with *methanol R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *concentrated ammonia R*, *alcohol R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 100-105 °C for 30 min.

*Detection*: spray with *iodoplatinate reagent R*.

*Limit*:

– *impurity G*: any spot due to impurity G is not more intense than the spot in the chromatogram obtained with the reference solution (0.1 per cent, expressed as anhydrous piperazine).

**Loss on drying** (*2.2.32*): maximum 2.5 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C at a pressure not exceeding 15 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.120 g in 50.0 mL of *water R*. Add 1 mL of *nitric acid R* and titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 16.96 mg of $C_{14}H_{24}Cl_2N_2O_3$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I.*

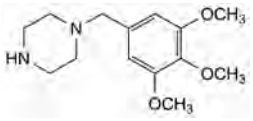

A. 1-(3,4,5-trimethoxybenzyl)piperazine,

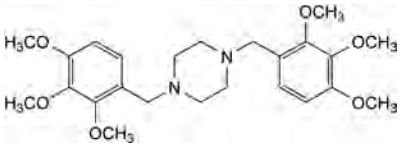

B. 1,4-bis(2,3,4-trimethoxybenzyl)piperazine,

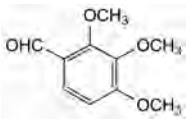

C. 2,3,4-trimethoxybenzaldehyde,

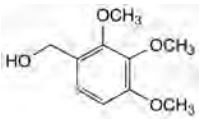

D. (2,3,4-trimethoxyphenyl)methanol,

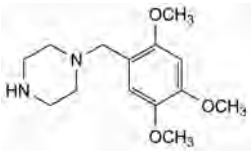

E. 1-(2,4,5-trimethoxybenzyl)piperazine,

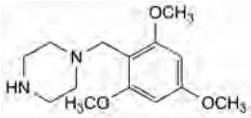

F. 1-(2,4,6-trimethoxybenzyl)piperazine,

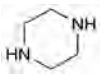

G. piperazine,

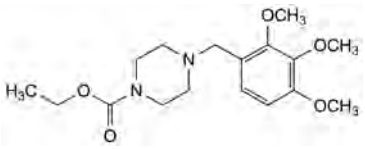

H. ethyl 4-(2,3,4-trimethoxybenzyl)piperazine-1-carboxylate,

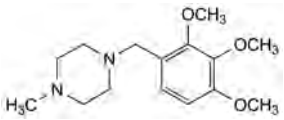

I. 1-methyl-4-(2,3,4-trimethoxybenzyl)piperazine (*N*-methyltrimetazidine).

**01/2017:0440**

# TRIMETHADIONE

## Trimethadionum

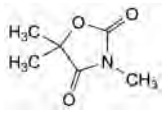

$C_6H_9NO_3$ $M_r$ 143.1
[127-48-0]

DEFINITION

3,5,5-Trimethyloxazolidine-2,4-dione.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: colourless or almost colourless crystals.

*Solubility*: soluble in water, very soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 45 °C to 47 °C, determined without previous drying.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs prepared using 3 mg of substance per 0.4 g of *potassium bromide R*.

*Comparison*: *trimethadione CRS*.

C. To 2 mL of solution S (see Tests) add 1 mL of *barium hydroxide solution R*. A white precipitate is formed, which dissolves on addition of 1 mL of *dilute hydrochloric acid R*.

D. Dissolve 0.3 g in a mixture of 5 mL of *alcoholic potassium hydroxide solution R* and 5 mL of *ethanol (96 per cent) R*. Allow to stand for 10 min. Add 0.05 mL of *phenolphthalein solution R1* and neutralise exactly with *hydrochloric acid R*. Evaporate to dryness on a water-bath and take up the residue with 4 quantities, each of 5 mL, of *ether R*. Filter the combined ether layers and evaporate to dryness. The residue, recrystallised from 5 mL of *toluene R* and dried, melts (*2.2.14*) at about 80 °C.

TESTS

**Solution S**. Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 40 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 10 mL of solution S add 0.1 mL of *methyl red solution R*. Not more than 0.1 mL of *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide* is required to change the colour of the indicator.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.00 g by drying in a desiccator over *anhydrous silica gel R* for 6 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Gas chromatography (*2.2.28*).

*Internal standard solution*. Dissolve 0.125 g of *decanol R* in *anhydrous ethanol R* and dilute to 25 mL with the same solvent.

*Test solution*. Dissolve 0.100 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the same solution.

*Reference solution*. Dissolve 0.100 g of *trimethadione CRS* in the internal standard solution and dilute to 10.0 mL with the same solution.

*Column*:

– *material*: stainless steel,

– *size*: *l* = 0.75 m, Ø = 3 mm,

– *stationary phase*: *styrene-divinylbenzene copolymer R* (125-150 µm).

*Carrier gas*: *nitrogen for chromatography R*.

*Flow rate*: 20 mL/min.

*Temperature*:

– *column*: 210 °C,

– *injection port*: 240 °C,

– *detector*: 270 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

Calculate the content of $C_6H_9NO_3$ from the declared content of *trimethadione CRS*.

STORAGE

Protected from light.

**01/2017:0060**
**corrected 10.0**

# TRIMETHOPRIM

## Trimethoprimum

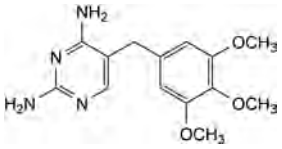

$C_{14}H_{18}N_4O_3$ $M_r$ 290.3
[738-70-5]

DEFINITION

5-(3,4,5-Trimethoxybenzyl)pyrimidine-2,4-diamine.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white powder.

*Solubility*: very slightly soluble in water, slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification*: *C*.

*Second identification*: *A, B, D*.

A. Melting point (*2.2.14*): 199 °C to 203 °C.

B. Dissolve about 20 mg in *0.1 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 10.0 mL with *0.1 M sodium hydroxide*. Examined between 230 nm and 350 nm (*2.2.25*), the solution shows an absorption maximum at 287 nm. The specific absorbance at the absorption maximum is 240 to 250.

C. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *trimethoprim CRS*.

D. Dissolve about 25 mg, heating if necessary, in 5 mL of *0.005 M sulfuric acid* and add 2 mL of a 16 g/L solution of *potassium permanganate R* in *0.1 M sodium hydroxide*. Heat to boiling and add to the hot solution 0.4 mL of *formaldehyde R*. Mix, add 1 mL of *0.5 M sulfuric acid*, mix and heat again to boiling. Cool and filter. To the filtrate, add 2 mL of *methylene chloride R* and shake vigorously. The organic layer, examined in ultraviolet light at 365 nm, shows green fluorescence.

TESTS

**Appearance of solution**. The solution is not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 0.5 g in 10 mL of a mixture of 1 volume of *water R*, 4.5 volumes of *methanol R* and 5 volumes of *methylene chloride R*.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *trimethoprim for system suitability CRS* (containing impurity E) in 1 mL of the mobile phase.

*Column*:

– *size*: *l* = 0.250 m, Ø = 4.0 mm;

– *stationary phase*: *base-deactivated octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 30 volumes of *methanol R* and 70 volumes of a 1.4 g/L solution of *sodium perchlorate R* adjusted to pH 3.6 with *phosphoric acid R*.

*Flow rate*: 1.3 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL loop injector.

*Run time*: 11 times the retention time of trimethoprim.

*Relative retention* with reference to trimethoprim (retention time = about 5 min): impurity C = about 0.8; impurity E = about 0.9; impurity A = about 1.5; impurity D = about 2.0; impurity G = about 2.1; impurity B = about 2.3; impurity J = about 2.7; impurity F = about 4.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurity E and trimethoprim.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.43; impurity E = 0.53; impurity J = 0.66;

– *any impurity*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *total*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak corresponding to impurity H (relative retention = about 10.3).

B. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5.0 mg of *trimethoprim CRS* and 5.0 mg of *trimethoprim impurity B CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *nitrile silica gel for chromatography R* (5 µm) with a specific surface area of 350 $m^2$/g and a pore diameter of 10 nm.

*Mobile phase*: dissolve 1.14 g of *sodium hexanesulfonate R* in 600 mL of a 13.6 g/L solution of *potassium dihydrogen phosphate R*; adjust to pH 3.1 with *phosphoric acid R* and mix with 400 mL of *methanol R*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL loop injector.

*Run time*: 6 times the retention time of trimethoprim.

*Relative retention* with reference to trimethoprim (retention time = about 4 min): impurity H = about 1.8; impurity I = about 4.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to trimethoprim and impurity B.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity H = 0.50; impurity I = 0.28;

– *any impurity*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);

– *total*: not more than 0.4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.04 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.02 per cent); disregard any peak due to impurity B (relative retention = about 1.3).

**Impurity K**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in 35.0 mL of *citrate buffer solution pH 5.0 R*, add 10.0 mL of *1,1-dimethylethyl methyl ether R*, shake thoroughly and centrifuge for 10 min. Use the upper layer.

*Reference solution*. Dilute 5.0 mL of *hydrochloric acid R* to 50.0 mL with *water R*, add 12.5 mg of *aniline R* and shake thoroughly. Add 10.0 µL of this solution and 10.0 mL of *1,1-dimethylethyl methyl ether R* to 35.0 mL of *citrate buffer solution pH 5.0 R*, shake thoroughly and centrifuge for 10 min. Use the upper layer.

*Column*:

– *material*: fused silica;

– *size*: *l* = 30 m, Ø = 0.53 mm;

– *stationary phase*: *methylpolysiloxane R* (film thickness 3 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 12 mL/min.

*Temperature*:

– *column*: 80 °C;

– *injection port*: 230 °C;

– *detector*: 270 °C.

*Detection*: nitrogen-phosphorus detector.

*Injection*: 3 µL.

*Run time*: 15 min.

*System suitability*: reference solution:

– *repeatability*: maximum relative standard deviation of 5.0 per cent after 6 injections.

*Limit*:

– *impurity K*: not more than the area of the corresponding peak in the chromatogram obtained with the reference solution (5 ppm).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 29.03 mg of $C_{14}H_{18}N_4O_3$.

IMPURITIES

**Test A for related substances**: A, B, C, D, E, F, G, H, J.

**Test B for related substances**: B, H, I.

**Test for impurity K**: K.

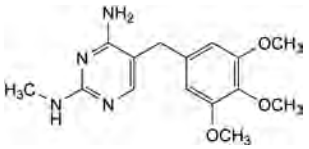

A. $N^2$-methyl-5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine,

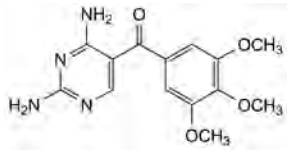

B. (2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl)-methanone,

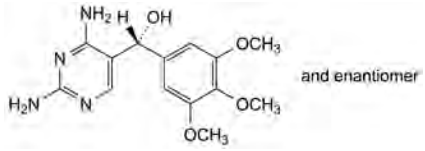


C. (*RS*)-(2,4-diaminopyrimidin-5-yl)(3,4,5-trimethoxyphenyl)methanol,

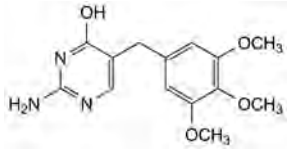

D. 2-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-4-ol,

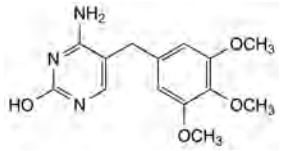

E. 4-amino-5-(3,4,5-trimethoxybenzyl)pyrimidin-2-ol,

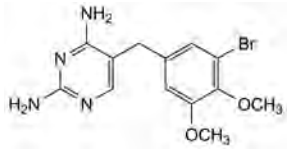

F. 5-(3-bromo-4,5-dimethoxybenzyl)pyrimidine-2,4-diamine,

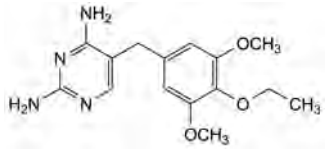

G. 5-(4-ethoxy-3,5-dimethoxybenzyl)pyrimidine-2,4-diamine,

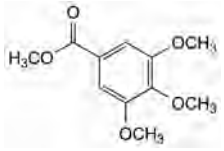

H. methyl 3,4,5-trimethoxybenzoate,

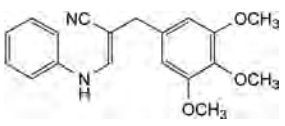

I. 3-(phenylamino)-2-(3,4,5-trimethoxybenzyl)prop-2-enenitrile,

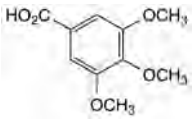

J. 3,4,5-trimethoxybenzoic acid,

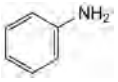

K. aniline.

**01/2017:0534**

# TRIMIPRAMINE MALEATE

## Trimipramini maleas

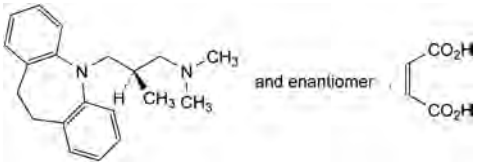


$C_{24}H_{30}N_2O_4$ $M_r$ 410.5
[521-78-8]

DEFINITION

(2*RS*)-3-(10,11-Dihydro-5*H*-dibenzo[*b*,*f*]azepin-5-yl)-*N*,*N*,2-trimethylpropan-1-amine hydrogen (2*Z*)-but-2-enedioate.

*Content*: 98.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water and in ethanol (96 per cent).

IDENTIFICATION

A. Melting point (*2.2.14*): 140 °C to 144 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *trimipramine maleate CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Buffer solution pH 7.7*: 2.64 g/L solution of *ammonium phosphate R* in *water for chromatography R*; adjust to pH 7.7 with *phosphoric acid R*.

*Test solution.* Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of the substance to be examined and 5 mg of *iminodibenzyl R* (impurity F) in the mobile phase and dilute to 10 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (c).* Dissolve the contents of a vial of *trimipramine for peak identification CRS* (containing impurities A, B, C, D and E) in 1 mL of *acetonitrile R1*.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.0 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *acetonitrile R1*, buffer solution pH 7.7 (38:62 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of trimipramine.

*Identification of impurities*: use the chromatogram supplied with *trimipramine for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B, C, D and E; doubling of the peak due to impurity E may be observed.

*Relative retention* with reference to trimipramine (retention time = about 27 min): impurity A = about 0.1; impurity B = about 0.3; impurity C = about 0.4; impurity D = about 0.5; impurity F = about 1.4; impurity E = about 1.5.

*System suitability*:

- *resolution*: minimum 3.5 between the peaks due to trimipramine and impurity F in the chromatogram obtained with reference solution (a);
- the chromatogram obtained with reference solution (c) is similar to the chromatogram supplied with *trimipramine for peak identification CRS*.

*Limits*:

- *correction factor*: for the calculation of content, multiply the peak area of impurity F by 0.5;
- *impurity E*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *impurity F*: not more than 2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurities A, B, C, D*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 41.05 mg of $C_{24}H_{30}N_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, D, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *G.*

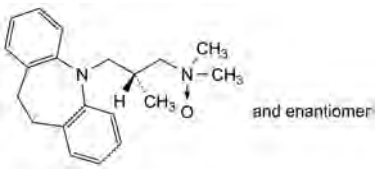


A. (2*RS*)-3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,*N*,2-trimethylpropan-1-amine *N*-oxide,

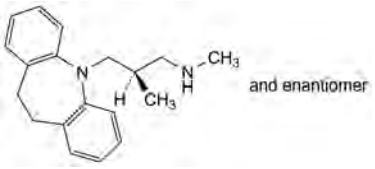


B. (2*RS*)-3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-*N*,2-dimethylpropan-1-amine,

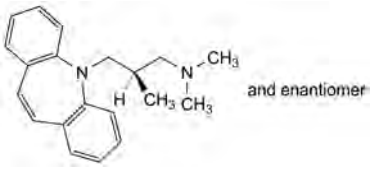


C. (2*RS*)-3-(5*H*-dibenzo[b,f]azepin-5-yl)-*N*,*N*,2-trimethylpropan-1-amine,

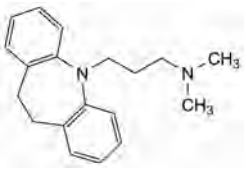


D. imipramine,

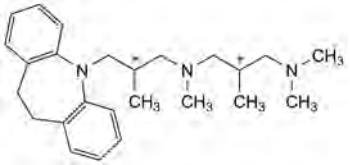


E. mixture of the stereoisomers of *N*-[3-(10,11-dihydro-5*H*-dibenzo[*b,f*]azepin-5-yl)-2-methylpropyl]-*N*,*N*′,*N*′,2-tetramethylpropane-1,3-diamine,

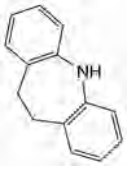


F. 10,11-dihydro-5*H*-dibenzo[*b,f*]azepine,

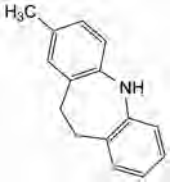


G. 2-methyl-10,11-dihydro-5*H*-dibenzo[*b,f*]azepine.

**01/2017:1577**
**corrected 10.0**

# TROLAMINE

## Trolaminum

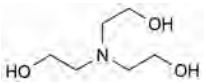

$C_6H_{15}NO_3$ $M_r$ 149.2
[102-71-6]

DEFINITION

2,2′,2″-Nitrilotriethanol.

*Content*: 99.0 per cent *m/m* to 103.0 per cent *m/m* of total bases (anhydrous substance).

CHARACTERS

*Appearance*: clear, viscous, colourless or slightly yellow liquid, very hygroscopic.

*Solubility*: miscible with water and with ethanol (96 per cent), soluble in methylene chloride.

IDENTIFICATION

*First identification: B, C.*

*Second identification: A, B, D.*

A. Relative density (*2.2.5*): 1.120 to 1.130.

B. Refractive index (*2.2.6*): 1.482 to 1.485.

C. Examine the chromatograms obtained in the test for related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

D. To 1 mL add 0.3 mL of *copper sulfate solution R*. A blue colour develops. Add 2.5 mL of *dilute sodium hydroxide solution R* and heat to boiling. The blue colour remains unchanged.

TESTS

**Solution S**. Dissolve 12 g in *water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 5.0 g of *3-aminopropanol R* in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution.* Dissolve 10.0 g of the substance to be examined in *water R*. Add 1.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Reference solution (a).* Dissolve 1.0 g of *trolamine CRS* in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dissolve 0.1 g of *trolamine impurity A CRS*, 0.5 g of *trolamine impurity B CRS* and 0.1 g of *trolamine CRS* in *water R* and dilute to 10.0 mL with the same solvent. To 1.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Column*:

- *material*: fused silica;
- *size*: *l* = 25 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.50 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:35.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 | 60 |
| | 0 - 8.5 | 60 → 230 |
| | 8.5 - 14 | 230 |
| Injection port | | 260 |
| Detector | | 280 |

*Detection*: flame ionisation.

*Injection*: 2 µL; if necessary inject a blank solution.

*Elution order*: impurity A, 3-aminopropanol, impurity B, trolamine.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to 3-aminopropanol and impurity A.

*Limits*:

- *impurity A*: calculate the ratio (*R1*) of the area of the peak due to impurity A to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to impurity A to the area of the peak due to the internal standard: this ratio is not greater than *R1* (0.1 per cent);
- *impurity B*: calculate the ratio (*R2*) of the area of the peak due to impurity B to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the area of any peak due to impurity B to the area of the peak due to the internal standard: this ratio is not greater than *R2* (0.5 per cent);
- *total*: calculate the ratio (*R4*) of the area of the peak due to trolamine to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b); from the chromatogram obtained with the test solution, calculate the ratio of the sum of the areas of any peaks, apart from the principal peak and the peak due to the internal standard, to the area of the peak due to the internal standard: this ratio is not greater than 10 times *R4* (1.0 per cent);
- *disregard limit*: 0.5 times the ratio of the area of the peak due to trolamine to the area of the peak due to the internal standard from the chromatogram obtained with reference solution (b) (0.05 per cent).

**Impurity C**. Gas chromatography (*2.2.28*).

*Solvent mixture*: *acetone R*, *chloroform R* (10:50 *V/V*).

*Preparation of solid phase extraction columns*

*Column A*. Fill a glass chromatography column (*l* = 400 mm; Ø = 20 mm) fitted with a polytetrafluoroethylene stopcock and a sintered-glass filter (160) (*2.1.2*) with 3 g of *anhydrous sodium sulfate R* and cover with a mixture of 17 g of *kieselguhr for chromatography R* and 3 g of *potassium carbonate R*. Settle the column bed by gently tapping the column.

*Column B*. Fill a glass chromatography column (*l* = 400 mm; Ø = 20 mm) fitted with a polytetrafluoroethylene stopcock and a sintered-glass filter (160) (*2.1.2*) with a slurry of 25 g of *silica gel for chromatography R* (0.063 to 0.200 mm) in the solvent mixture. Apply slight pressure to settle the column and cover the column bed with 5 g of *anhydrous sodium sulfate R*.

*Standard solution (a).* Dissolve 50 µL of *N-nitrosodiethanolamine R* (impurity C) in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 100 µL of this solution to 100.0 mL with *methanol R*.

*Standard solution (b).* Dilute 10.0 mL of standard solution (a) to 50.0 mL with *methanol R*.

*Standard solution (c).* Dissolve 50 mg of *N-nitrosodiisopropanolamine R* in *methanol R* and dilute to 50.0 mL with the same solvent. Dilute 100 µL of this solution to 100.0 mL with *methanol R*.

*Test solution.* To 2.000 g of the substance to be examined add 200 µL of *methanol R* and 0.5 g of *sulfamic acid R*. Dissolve in 8 mL of *water for chromatography R* and apply the solution to column A. Rinse the vessel twice with 1.5 mL of *water for chromatography R*, applying the rinsings to the column as well. After 15 min of equilibration time elute the column with 100 mL of *ethyl acetate R*, collecting the eluate in a 250 mL distillation flask. Evaporate the eluate to dryness. Take up the residue in 1 mL of the solvent mixture, apply to column B and let it settle. Rinse the flask twice with 2 mL of the solvent mixture, apply the rinsings to the column and let it settle. Wash the column with 100 mL of the solvent mixture and discard. Elute the column with 120 mL of *acetone R*, collecting the eluate in a 250 mL distillation flask. Evaporate the eluate to dryness. Transfer the residue with the aid of a small volume of *acetone R* into a vial and evaporate again to dryness under a stream of *nitrogen R*. Dissolve the residue in 100 µL of *trimethylpentane for chromatography R*, add 100 µL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat at 70 °C for 1 h.

*Reference solution (a).* To 2.000 g of the substance to be examined add 200 µL of standard solution (b) and 0.5 g of *sulfamic acid R*. Dissolve in 8 mL of *water for chromatography R*, then proceed exactly as described for the test solution.

*Reference solution (b).* To 1.0 mL of standard solution (a) add 4.0 mL of standard solution (c) and mix. Transfer 500 µL of the solution to a vial and evaporate to dryness under a stream of *nitrogen R*. Dissolve the residue in 200 µL of *trimethylpentane for chromatography R*, add 200 µL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat at 70 °C for 1 h.

*Reference solution (c).* In a vial, evaporate 200 µL of standard solution (b) to dryness under a stream of *nitrogen R*. Dissolve the residue in 100 µL of *trimethylpentane for chromatography R*, add 100 µL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat at 70 °C for 1 h.

*Blank solution.* In a gas chromatography vial, evaporate 200 µL of *methanol R* to dryness under a stream of *nitrogen R*. Dissolve the residue in 100 µL of *trimethylpentane for chromatography R* add 100 µL of *N-methyltrimethylsilyl-trifluoroacetamide R* and heat at 70 °C for 1 h.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m; Ø = 0.25 mm;
- *stationary phase*: *base-deactivated phenyl(5)methyl(95)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 2 mL/min.

*Split ratio*: 1:10.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 180 → 280 |
| | 5 - 10 | 280 |
| Injection port | | 220 |

*Detection*: chemoluminescence:

- dual plasma burner in nitrosamine mode;
- burner temperature: 450 °C;
- oxygen flow rate: 4.4-5.0 mL/min.

*Injection*: 4 µL.

*System suitability*:

- *resolution*: minimum 1.3 between the peaks due to impurity C and *N*-nitrosodiisopropanolamine in the chromatogram obtained with reference solution (b);
- *recovery*: minimum 50 per cent. The difference between the area of the peak due to impurity C in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution is not less than 0.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (c).

*Limits*:

- *impurity C*: not more than the difference between the area of the peak due to impurity C in the chromatogram obtained with reference solution (a) and the area of the corresponding peak in the chromatogram obtained with the test solution (24 ppb).

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.000 g.

Open the titration vessel, introduce the substance to be examined directly into the previously titrated solvent. Stopper the flask immediately.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g. Do not carry out the initial heating on a water-bath.

ASSAY

Dissolve 1.200 g in 75 mL of *carbon dioxide-free water R*. Add 0.3 mL of *methyl red solution R*. Titrate with *1 M hydrochloric acid*.

1 mL of *1 M hydrochloric acid* is equivalent to 0.149 g of $C_6H_{15}NO_3$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

A. 2-aminoethanol (ethanolamine),

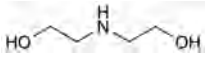

B. 2,2′-iminodiethanol (diethanolamine),

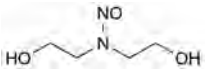

C. 2,2′-(nitrosoimino)diethanol (*N*-nitrosodiethanolamine).

**01/2017:1053**

# TROMETAMOL

## Trometamolum

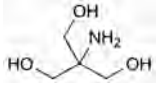

$C_4H_{11}NO_3$ $M_r$ 121.1

[77-86-1]

DEFINITION

Trometamol contains not less than 99.0 per cent and not more than the equivalent of 100.5 per cent of aminomethylidynetri(methanol), calculated with reference to the dried substance.

CHARACTERS

A white or almost white, crystalline powder, or colourless crystals, freely soluble in water, sparingly soluble in alcohol, very slightly soluble in ethyl acetate.

IDENTIFICATION

*First identification*: B, C.

*Second identification*: A, B, D.

A. Solution S (see Tests) is strongly alkaline (*2.2.4*).

B. Melting point (*2.2.14*): 168 °C to 174 °C.

C. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *trometamol CRS*.

D. Examine the chromatograms obtained in the test for related substances. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*). The pH of freshly prepared solution S is 10.0 to 11.5.

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. Wash the plate with *methanol R* before applying the solutions.

*Test solution (a)*. Dissolve 0.20 g in 1 mL of *water R*, with heating, and dilute to 10 mL with *methanol R*.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a)*. Dissolve 20 mg of *trometamol CRS* in *methanol R* and dilute to 10 mL with the same solvent.

*Reference solution (b)*. Dilute 1 mL of test solution (a) to 100 mL with *methanol R*.

Apply to the plate 10 µL of each solution. Develop over a path of 10 cm using a mixture of 10 volumes of *dilute ammonia R1* and 90 volumes of *2-propanol R*. Dry the plate at 100 °C to 105 °C. Spray with a 5 g/L solution of *potassium permanganate R* in a 10 g/L solution of *sodium carbonate R*. After about 10 min examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (1.0 per cent).

**Chlorides** (*2.4.4*). To 10 mL of solution S add 2.5 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution complies with the limit test for chlorides (100 ppm).

**Iron** (*2.4.9*). Dissolve 1.0 g in *water R* and dilute to 10 mL with the same solvent. The solution complies with the limit test for iron (10 ppm).

**Loss on drying** (*2.2.32*). Not more than 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 0.03 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Dissolve 0.100 g in 20 mL of *water R*. Add 0.2 mL of *methyl red solution R*. Titrate with *0.1 M hydrochloric acid* until the colour changes from yellow to red.

1 mL of *0.1 M hydrochloric acid* is equivalent to 12.11 mg of $C_4H_{11}NO_3$.

IMPURITIES

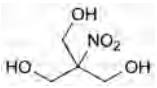


A. nitromethylidynetri(methanol).

**01/2011:1159**

# TROPICAMIDE

## Tropicamidum

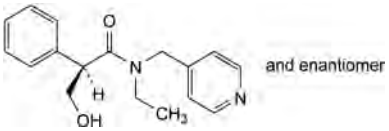


$C_{17}H_{20}N_2O_2$ $M_r$ 284.4

[1508-75-4]

DEFINITION

(2*RS*)-*N*-Ethyl-3-hydroxy-2-phenyl-*N*-(pyridin-4-ylmethyl)propanamide.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

*First identification*: C.

*Second identification*: A, B, D, E.

A. Melting point (*2.2.14*): 95 °C to 98 °C.

B. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution*. Dissolve 20.0 mg in *0.1 M hydrochloric acid* and dilute to 50.0 mL with the same acid. Dilute 2.0 mL of this solution to 20.0 mL with *0.1 M hydrochloric acid*.

*Spectral range*: 230-350 nm.

*Absorption maximum*: at 254 nm.

*Specific absorbance at the absorption maximum*: 170 to 190.

C. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tropicamide CRS*.

D. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution*. Dissolve 10 mg of *tropicamide CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *concentrated ammonia R*, *methanol R*, *methylene chloride R* (0.5:5:95 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the spot in the chromatogram obtained with the reference solution.

Monographs T

E. Dissolve about 5 mg in 3 mL of a mixture of 9 mL of *acetic anhydride R*, 1 mL of *acetic acid R* and 0.1 g of *citric acid monohydrate R*. Heat on a water-bath for 5-10 min. A reddish-yellow colour is produced.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.1 g in *ethanol (96 per cent) R* and dilute to 10 mL with the same solvent.

**Optical rotation** (*2.2.7*): − 0.1° to + 0.1°.

Dissolve 2.5 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 3 mL of *acetonitrile R1* and dilute to 50.0 mL with *water for chromatography R*.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water for chromatography R*. Dilute 1.0 mL of this solution to 10.0 mL with *water for chromatography R*.

*Reference solution (b).* Dissolve 5 mg of *4-[(ethylamino)methyl]pyridine R* (impurity A), 5.0 mg of *tropicamide impurity C CRS* and 5.0 mg of *tropicamide impurity D CRS* in 2 mL of *acetonitrile R1* and dilute to 50.0 mL with *water for chromatography R*. Dilute 1.0 mL of this solution to 10.0 mL with *water for chromatography R*.

*Reference solution (c).* Dissolve 5 mg of *tropicamide for peak identification CRS* (containing impurity B) in 1.0 mL of *acetonitrile R1* and dilute to 10.0 mL with *water for chromatography R*.

*Reference solution (d).* To 1 mL of reference solution (b) add 1 mL of reference solution (c).

*Reference solution (e).* Dilute 1.5 mL of reference solution (b) to 10.0 mL with *water for chromatography R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 40 °C.

*Mobile phase.* Dissolve 0.135 g of *sodium dodecyl sulfate R* and 3.4 mL of *phosphoric acid R* in 950 mL of *water for chromatography R*. Adjust to pH 3.0 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water for chromatography R*. Mix 73 volumes of this solution with 27 volumes of *acetonitrile R1*.

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 210 nm and at 254 nm.

*Injection*: 15 µL.

*Run time*: 3 times the retention time of tropicamide.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, C and D.

*Relative retention* with reference to tropicamide (retention time = about 11 min): impurity C = about 0.4; impurity A = about 0.5; impurity D = about 0.8; impurity B = about 2.3.

*System suitability*: reference solution (d):

- *resolution at 210 nm*: minimum 2.0 between the peaks due to impurities C and A;
- *resolution at 210 nm*: minimum 2.0 between the peaks due to impurities A and D.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.8; impurity B = 0.6;
- *impurity B at 254 nm*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *impurity A at 254 nm*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.15 per cent);
- *impurity C at 210 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.15 per cent);
- *impurity D at 210 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (e) (0.15 per cent);
- *unspecified impurities at 254 nm*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *sum of impurities other than C and D at 254 nm*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *disregard limit at 254 nm*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Chlorides** (*2.4.4*): maximum 100 ppm.

Dissolve 1.0 g with heating in 8 mL of *acetic acid R*, cool and dilute to 10 mL with the same acid. Dilute 5 mL of this solution to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 80 °C at a pressure not exceeding 0.7 kPa for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 50 mL of *anhydrous acetic acid R*. Add 0.2 mL of *naphtholbenzein solution R* and titrate with *0.1 M perchloric acid* until the colour changes from orange to green.

1 mL of *0.1 M perchloric acid* is equivalent to 28.44 mg of $C_{17}H_{20}N_2O_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D.*

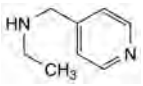


A. *N*-(pyridin-4-ylmethyl)ethanamine,

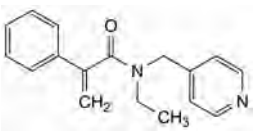


B. *N*-ethyl-2-phenyl-*N*-(pyridin-4-ylmethyl)propenamide,

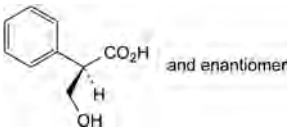


C. (2*RS*)-3-hydroxy-2-phenylpropanoic acid (tropic acid),

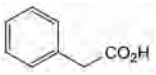

D. phenylacetic acid.

**01/2008:2102**
**corrected 6.0**

# TROPISETRON HYDROCHLORIDE

## Tropisetroni hydrochloridum

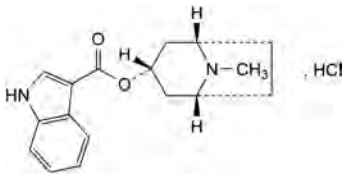

$C_{17}H_{21}ClN_2O_2$ $M_r$ 320.8
[105826-92-4]

DEFINITION

Hydrochloride of (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl 1*H*-indole-3-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble or soluble in water, sparingly soluble in ethanol (96 per cent), very slightly soluble in methylene chloride.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50 mg in *methanol R* and dilute to 25.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *methanol R*.

*Spectral range*: 220-360 nm.

*Absorption maxima*: at 228 nm and 282 nm.

*Absorbance ratio*: $A_{228}/A_{282}$ = 1.3 to 1.4.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tropisetron hydrochloride CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Reference solution.* Dissolve 5 mg of *tropisetron hydrochloride CRS* in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5 mL with the same mixture of solvents.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *anhydrous formic acid R*, *water R*, *methanol R*, *methylene chloride R* (2:2:30:70 *V/V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in cold air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Detection B*: spray first with a solution prepared as follows: dissolve 0.85 g of *bismuth subnitrate R* in a mixture of 10 mL of *acetic acid R* and 40 mL of *water R*; to 5 mL of this solution add 5 mL of a 400 g/L solution of *potassium iodide R* and dilute to 100 mL with *water R*. Then spray with *strong hydrogen peroxide solution R*.

*Results*: for both detection methods, the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

Dissolve 1.00 g in *water R* and dilute to 20 mL with the same solvent.

**Impurity A**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.200 g of the substance to be examined in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 5.0 mL with the same mixture of solvents.

*Reference solution (a).* Dissolve 5.0 mg of *tropine CRS* (impurity A) in a mixture of equal volumes of *methanol R* and *methylene chloride R* and dilute to 25.0 mL with the same mixture of solvents.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with a mixture of equal volumes of *methanol R* and *methylene chloride R*. To 0.1 mL of this solution add 1.0 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ammonia R*, *methanol R*, *methylene chloride R* (5:40:60 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of cold air.

*Detection*: dip the plate in *potassium iodobismuthate solution R1*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity A*: any spot due to impurity A is not more intense than the spot in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.100 g of the substance to be examined in mobile phase A and dilute to 20.0 mL with mobile phase A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 10.0 mL with mobile phase A.

*Reference solution (b).* Dissolve 5.0 mg of *tropisetron impurity B CRS* and 5 mg of *ethyl indole-3-carboxylate CRS* in mobile phase A and dilute to 20.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 50.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *triethylamine R*, *acetonitrile R*, *water R*, *methanol R* (0.3:35:400:565 *V/V/V/V*);

– *mobile phase B*: *triethylamine R*, *acetonitrile R*, *water R*, *methanol R* (0.3:100:100:800 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 14 | 100 | 0 |
| 14 - 32 | 100 → 0 | 0 → 100 |
| 32 - 36 | 0 | 100 |
| 36 - 37 | 0 → 100 | 100 → 0 |
| 37 - 52 | 100 | 0 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Relative retention* with reference to tropisetron (retention time = about 22 min): impurity B = about 0.05; ethyl indole-3-carboxylate = about 0.2.

*System suitability*: reference solution (b):

– *resolution*: minimum 4 between the peaks due to impurity B and ethyl indole-3-carboxylate.

*Limits*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (b) (0.1 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

***N,N*-Dimethylaniline**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution*. Dissolve 250 mg of the substance to be examined in mobile phase A and dilute to 5.0 mL with mobile phase A.

*Reference solution*. Dissolve 10.0 mg of *N,N-dimethylaniline R* in mobile phase A and dilute to 100.0 mL with mobile phase A. Dilute 1.0 mL of this solution to 100.0 mL with mobile phase A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: *triethylamine R, acetonitrile R, water R, methanol R* (0.3:35:400:565 *V/V/V/V*);
– *mobile phase B*: *triethylamine R, acetonitrile R, water R*; *methanol R* (0.3:100:100:800 *V/V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 11 | 100 → 0 | 0 → 100 |
| 11 - 30 | 0 | 100 |
| 30 - 31 | 0 → 100 | 100 → 0 |
| 31 - 50 | 100 | 0 |

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 248 nm.

*Injection*: 20 µL.

*Limit*:

– *N,N-dimethylaniline*: not more than the area of the principal peak in the chromatogram obtained with the reference solution (20 ppm).

**Loss on drying** (*2.2.32*): maximum 0.3 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 10 mL of *anhydrous acetic acid R* and add 70 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 32.08 mg of $C_{17}H_{21}ClN_2O_2$.

IMPURITIES

*Specified impurities: A, B.*

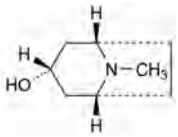

A. (1*R*,3*r*,5*S*)-8-methyl-8-azabicyclo[3.2.1]octan-3-ol (tropine),

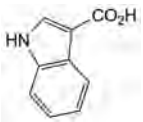

B. 1*H*-indole-3-carboxylic acid.

**01/2008:1798**
**corrected 6.0**

# TROSPIUM CHLORIDE

## Trospii chloridum

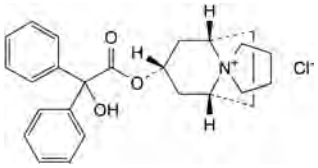

$C_{25}H_{30}ClNO_3$ $M_r$ 428.0

[10405-02-4]

DEFINITION

(1*R*,3*r*,5*S*)-3-[(Hydroxydiphenylacetyl)oxy]spiro[8-azoniabicyclo[3.2.1]octane-8,1′-pyrrolidinium] chloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: very soluble in water, freely soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *trospium chloride CRS.*

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 3.0 g in *carbon dioxide-free water R* and dilute to 30 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.0.

Dilute 5 mL of solution S to 50 mL with *carbon dioxide-free water R*.

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.20 g of the substance to be examined in 2.0 mL of *methanol R*.

*Reference solution (a).* Dissolve 1.0 mg of *trospium impurity C CRS* in 2.0 mL of *methanol R*.

*Reference solution (b).* Dilute 1.0 mL of test solution to 10.0 mL with *methanol R*. To 50 µL of this solution add 1 mL of reference solution (a).

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *glacial acetic acid R*, *hydrochloric acid R*, *acetonitrile R* (1:3.5:45 *V/V/V*).

*Application*: 10 µL as bands.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air until the odour of acetic acid is no longer perceptible.

*Detection*: spray with *potassium iodobismuthate solution R* and subsequently with a 5 g/L solution of *sodium nitrite R*.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly visible and separated zones.

*Limit*:

– *impurity C*: any zone due to impurity C is not more intense than the zone in the chromatogram obtained with reference solution (a) (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 30.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 6.0 mg of *trospium impurity A CRS* in the mobile phase and dilute to 20.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 7.5 mg of *trospium impurity B CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase.

*Reference solution (c).* Dilute a mixture of 0.3 mL of the test solution, 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 1 volume of *triethylamine R* and 3 volumes of *phosphoric acid R* with 700 volumes of *water R* and add 300 volumes of *acetonitrile R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Injection*: 20 µL.

*Run time*: 3 times the retention time of trospium.

*Relative retention* with reference to trospium (retention time = about 10 min): impurity B = about 0.7; impurity A = about 1.9.

*System suitability*: reference solution (c):

– *resolution*: minimum 3 between the peaks due to impurity B and trospium.

*Limits*:

– *impurity B*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
– *impurity A*: not more than 3 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
– *unspecified impurities*: for each impurity, not more than 0.2 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.10 per cent);
– *total*: not more than twice the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (1.0 per cent);
– *disregard limit*: 0.1 times the area of the peak due to impurity B in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of *water R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 42.80 mg of $C_{25}H_{30}ClNO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

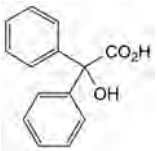


A. hydroxydiphenylacetic acid (benzilic acid),

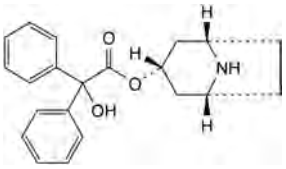


B. (1*R*,3*r*,5*S*)-8-azabicyclo[3.2.1]oct-3-yl hydroxydiphenylacetate,

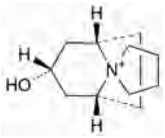


C. (1*R*,3*r*,5*S*)-3-hydroxyspiro[8-azoniabicyclo[3.2.1]octane-8,1′-pyrrolidinium].

**01/2017:2133**
**corrected 10.0**

# TROXERUTIN

## Troxerutinum

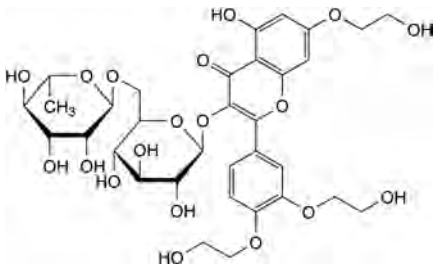


$C_{33}H_{42}O_{19}$ $M_r$ 743

[7085-55-4]

DEFINITION

Mixture of *O*-hydroxyethylated derivatives of rutoside containing minimum 80 per cent of 2-[3,4-bis(2-hydroxyethoxy)phenyl]-3-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-7-(2-hydroxyethoxy)-4*H*-1-benzopyran-4-one (tris(hydroxyethyl)rutin).

*Content*: 95.0 per cent to 105.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellowish-green, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in ethanol (96 per cent) and practically insoluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *troxerutin CRS*.

B. Examine the chromatograms obtained in the test for composition.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in position and size to the principal peak in the chromatogram obtained with the reference solution (a).

TESTS

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution.* Dissolve 10.0 mg of the substance to be examined in the mobile phase, if necessary using an ultrasonic bath and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 10.0 mg of *troxerutin CRS* in the mobile phase, if necessary using an ultrasonic bath and dilute to 10.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1 mL of reference solution (a) to 10 mL with the mobile phase. Dilute 1 mL of this solution to 100 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm,
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 20 volumes of *acetonitrile R* and 80 volumes of a 15.6 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 4.4 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*.

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 350 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of the main compound of troxerutin (tris(hydroxyethyl)rutin).

*Relative retention* with reference to tris(hydroxyethyl)rutin (retention time = about 25 min): tetrakis(hydroxyethyl)-rutin = about 0.5; mono(hydroxyethyl)rutin = about 0.8; bis(hydroxyethyl)rutin = about 1.1.

*System suitability*: reference solution (a):
- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to bis(hydroxyethyl)rutin and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to tris(hydroxyethyl)rutin;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:
- *principal peak*: minimum 80 per cent,
- *any other peak*: for each peak, maximum 5 per cent, except for 1 peak which can be maximum 10 per cent.
- *disregard limit*: area of the principal peak in the chromatogram obtained with reference solution (b).

**Ethylene oxide**. Head-space gas chromatography (*2.2.28*).

*Test solution.* To 1.00 g of the substance to be examined in a vial, add 1.0 mL of *water R*. Mix to obtain a homogeneous solution. Heat at 70 °C for 45 min.

*Reference solution.* To 1.00 g of the substance to be examined in a vial, add 50 µL of *ethylene oxide solution R4* and 950 µL of *water R* and close tightly. Mix to obtain a homogeneous solution. Heat at 70 °C for 45 min.

*Column*:
- *material*: fused silica,
- *size*: *l* = 30 m, Ø = 0.32 mm,
- *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 1 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1.1 mL/min.

*Static head-space conditions which may be used*:
- *equilibration temperature*: 70 °C,
- *equilibration time*: 45 min,
- *transfer line temperature*: 110 °C,
- *pressurisation time*: 2 min,
- *injection time*: 12 s.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 40 |
| | 5 - 18 | 40 → 200 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1.0 mL.

The peak due to ethylene oxide is identified by injecting solutions of ethylene oxide of increasing concentration.

Determine the content of ethylene oxide (ppm) in the substance to be examined using the following expression:

$$\frac{A_1 \times m_1}{(A_2 \times m_2) - (A_1 \times m_3)}$$

$A_1$ = area of the peak due to ethylene oxide in the chromatogram obtained with the test solution,

$A_2$ = area of the peak due to ethylene oxide in the chromatogram obtained with the reference solution,

$m_1$ = mass of ethylene oxide in the reference solution, in micrograms,

$m_2$ = mass of the substance to be examined in the test solution, in grams,

$m_3$ = mass of the substance to be examined in the reference solution, in grams.

*Limit*:
- *ethylene oxide*: maximum 1 ppm.

**Loss on drying** (*2.2.32*): maximum 5.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 4 h.

**Sulfated ash** (*2.4.14*): maximum 0.4 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 100.0 mL of *water R*. Dilute 10.0 mL of this solution to 100.0 mL with *water R*. Dilute 10.0 mL to 100.0 mL with *water R*. Measure the absorbance (*2.2.25*) at the absorption maximum at 350 nm.

Calculate the percentage content of $C_{33}H_{42}O_{19}$ taking the specific absorbance to be 250.

STORAGE

In an airtight container, protected from light.

01/2016:0694

# TRYPSIN

## Trypsinum

[9002-07-7]

DEFINITION

Trypsin is a proteolytic enzyme obtained by the activation of trypsinogen extracted from the pancreas of mammals. It has an activity of not less than 0.5 microkatal per milligram, calculated with reference to the dried substance. In solution, it has maximum enzymic activity at pH 8; the activity is reversibly inhibited at pH 3, the pH at which it is most stable.

PRODUCTION

The animals from which trypsin is derived must fulfil the requirements for the health of animals suitable for human consumption.

The method of manufacture is validated to demonstrate that the product, if tested, would comply with the following test.

**Histamine** (*2.6.10*): not more than 1 µg of histamine base per 0.2 microkatal of trypsin activity. Use a 10 g/L solution of the substance to be examined in *0.0015 M borate buffer solution pH 8.0 R* inactivated by heating on a water-bath for 30 min. Carry out dilutions with a 9 g/L solution of *sodium chloride R*.

CHARACTERS

*Appearance*: white or almost white, crystalline or amorphous powder, hygroscopic if amorphous.

*Solubility*: sparingly soluble in water.

IDENTIFICATION

A. Dilute 1 mL of solution S (see Tests) to 100 mL with *water R*. In a depression in a white spot-plate, mix 0.1 mL of this solution with 0.2 mL of *tosylarginine methyl ester hydrochloride solution R*. A reddish-violet colour develops within 3 min.

B. Dilute 0.5 mL of solution S to 5 mL with *water R*. Add 0.1 mL of a 20 g/L solution of *tosyl-lysyl-chloromethane hydrochloride R*. Adjust to pH 7.0, shake for 2 h and dilute to 50 mL with *water R*. In one of the depressions of a white spot-plate, mix 0.1 mL of this solution with 0.2 mL of *tosylarginine methyl ester hydrochloride solution R*. No reddish-violet colour develops within 3 min.

TESTS

**Solution S**. Dissolve 0.10 g in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension III (*2.2.1*).

**pH** (*2.2.3*): 3.0 to 6.0 for solution S.

**Specific absorbance** (*2.2.25*): 13.5 to 16.5, determined at the absorption maximum at 280 nm; maximum 7.0, determined at the absorption minimum at 250 nm.

Dissolve 30.0 mg in *0.001 M hydrochloric acid* and dilute to 100.0 mL with the same acid.

**Chymotrypsin**.

*Test solution*. To 1.8 mL of *buffer solution pH 8.0 R* add 7.4 mL of *water R* and 0.5 mL of *0.2 M acetyltyrosine ethyl ester R*. While shaking the solution, add 0.3 mL of solution S and start a timer. After exactly 5 min, measure the pH (*2.2.3*).

*Reference solution*. Prepare in the same manner as the test solution, replacing solution S by 0.3 mL of a 0.5 g/L solution of *chymotrypsin BRP*, and measure the pH (*2.2.3*) exactly 5 min after adding the chymotrypsin.

The pH of the test solution is higher than that of the reference solution.

**Loss on drying** (*2.2.32*): not more than 5.0 per cent, determined on 0.500 g by drying at 60 °C at a pressure not exceeding 0.67 kPa for 2 h.

**Microbial contamination**

TAMC: acceptance criterion $10^4$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

Absence of *Salmonella* (*2.6.13*).

ASSAY

The activity of trypsin is determined by comparing the rate at which it hydrolyses *benzoylarginine ethyl ester hydrochloride R* with the rate at which *trypsin BRP* hydrolyses the same substrate in the same conditions.

*Apparatus*. Use a reaction vessel of about 30 mL capacity provided with:

- a device that will maintain a temperature of 25.0 ± 0.1 °C;
- a stirring device (for example, a magnetic stirrer);
- a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents.

An automatic or manual titration device may be used. For the latter, the burette is graduated in 0.005 mL and the pH meter is provided with a wide-range scale and glass-silver-silver chloride or other suitable electrodes.

*Test solution*. Dissolve a suitable quantity of the substance to be examined in *0.001 M hydrochloric acid* and dilute to 25.0 mL with the same acid in order to obtain a solution containing approximately 700 nanokatals per millilitre.

*Reference solution*. Dissolve 25.0 mg of *trypsin BRP* in *0.001 M hydrochloric acid* and dilute to 25.0 mL with the same acid.

Store the solutions at 0-5 °C. Warm 1 mL of each solution to about 25 °C over 15 min and use 50 µL of each solution for each titration. Carry out the titration in an atmosphere of nitrogen. Transfer 10.0 mL of *0.0015 M borate buffer solution pH 8.0 R* to the reaction vessel and, while stirring, add 1.0 mL of a freshly prepared 6.86 g/L solution of *benzoylarginine ethyl ester hydrochloride R*. When the temperature is steady at 25.0 ± 0.1 °C (after about 5 min) adjust to pH 8.0 exactly with *0.1 M sodium hydroxide*. Add 50 µL of the test solution and start a timer. Maintain at pH 8.0 by the addition of *0.1 M sodium hydroxide*, the tip of the microburette being immersed in the solution; note the volume added every 30 s. Follow the reaction for 8 min. Calculate the volume of *0.1 M sodium hydroxide* used per second. Carry out a titration in the same manner using the reference solution and calculate the volume of *0.1 M sodium hydroxide* used per second.

Calculate the activity in microkatals per milligram using the following expression:

$$\frac{m' \times V}{m \times V'} \times A$$

$m$ = mass of the substance to be examined, in milligrams;

$m'$ = mass of *trypsin BRP*, in milligrams;

$V$ = volume of *0.1 M sodium hydroxide* used per second by the test solution;

$V'$ = volume of *0.1 M sodium hydroxide* used per second by the reference solution;

$A$ = activity of *trypsin BRP*, in microkatals per milligram.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C.

LABELLING

The label states:

- the activity in microkatals per milligram;
- for the amorphous substance, that it is hygroscopic.

**01/2017:1272**

# TRYPTOPHAN

## Tryptophanum

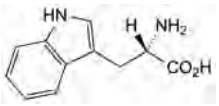

$C_{11}H_{12}N_2O_2$ $M_r$ 204.2

[73-22-3]

DEFINITION

(2*S*)-2-Amino-3-(1*H*-indol-3-yl)propanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline or amorphous powder.

*Solubility*: sparingly soluble in water, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of mineral acids and alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tryptophan CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *glacial acetic acid R*, *water R* (50:50 *V/V*).
*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 50 mL with the solvent mixture.
*Reference solution.* Dissolve 10 mg of *tryptophan CRS* in the solvent mixture and dilute to 50 mL with the solvent mixture.
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. Dissolve about 20 mg in 10 mL of *water R*. Add 5 mL of *dimethylaminobenzaldehyde solution R6* and 2 mL of *hydrochloric acid R1*. Heat on a water-bath. A purple-blue colour develops.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

Dissolve 0.1 g in *1 M hydrochloric acid* and dilute to 10 mL with the same acid.

**Specific optical rotation** (*2.2.7*): − 33.0 to − 30.0 (dried substance).

Dissolve 0.25 g in *water R*, heating on a water-bath if necessary, and dilute to 25.0 mL with the same solvent.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

- *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

- for any ninhydrin-positive substance detected at 570 nm, use the concentration of tryptophan in reference solution (a);
- for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

- *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Impurity A and other related substances**. Liquid chromatography (*2.2.29*). *Prepare the standard, test and reference solutions immediately before use.*

*Buffer solution pH 2.3.* Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 1000 mL of *water R*. Add about 700 mL of a 2.9 g/L solution of *phosphoric acid R* and adjust to pH 2.3 with the same acid solution.

*Solvent mixture*: *acetonitrile R*, *water R* (10:90 *V/V*).

*Standard solution.* Dissolve 10.0 mg of *N-acetyltryptophan R* in the solvent mixture and dilute to 100.0 mL with the solvent mixture. Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Test solution (a).* Dissolve 0.10 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b).* Dissolve 0.10 g of the substance to be examined in the standard solution and dilute to 10.0 mL with the standard solution.

*Reference solution (a).* Dissolve the contents of a vial of *1,1′-ethylidenebistryptophan CRS* (impurity A) in 1.0 mL of the solvent mixture.

*Reference solution (b).* Dissolve the contents of a vial of *1,1′-ethylidenebistryptophan CRS* (impurity A) in 1.0 mL of the standard solution.

*Reference solution (c).* Dilute 0.5 mL of reference solution (a) to 5.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, buffer solution pH 2.3 (115:885 *V/V*);
- *mobile phase B*: *acetonitrile R*, buffer solution pH 2.3 (350:650 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 10 | 100 | 0 |
| 10 - 45 | 100 → 0 | 0 → 100 |
| 45 - 65 | 0 | 100 |

*Flow rate*: 0.7 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 20 µL of test solutions (a) and (b) and reference solutions (b) and (c).

*Retention time*: tryptophan = about 8 min; *N*-acetyltryptophan = about 29 min; impurity A = about 34 min.

*System suitability*:

- *resolution*: minimum 8.0 between the peaks due to *N*-acetyltryptophan and impurity A in the chromatogram obtained with reference solution (b); if necessary, adjust the time programme for the elution gradient (an increase in the duration of elution with mobile phase A produces longer retention times and a better resolution);
- *signal-to-noise ratio*: minimum 15 for the principal peak in the chromatogram obtained with reference solution (c);
- *symmetry factor*: maximum 3.5 for the peak due to impurity A in the chromatogram obtained with reference solution (b);
- in the chromatogram obtained with test solution (a) there is no peak with the same retention time as *N*-acetyltryptophan (in such case correct the area of the *N*-acetyltryptophan peak).

*Limits*: test solution (b):

- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (10 ppm);
- *sum of impurities with a retention time less than that of tryptophan*: not more than 0.6 times the area of the peak due to *N*-acetyltryptophan in the chromatogram obtained with reference solution (b) (100 ppm);
- *sum of impurities with a retention time greater than that of tryptophan and up to 1.8 times the retention time of N-acetyltryptophan*: not more than 1.9 times the area of the peak due to *N*-acetyltryptophan in the chromatogram obtained with reference solution (b) (300 ppm);
- *disregard limit*: 0.02 times the area of the peak due to *N*-acetyltryptophan in the chromatogram obtained with reference solution (b); disregard the peak due to *N*-acetyltryptophan.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 3 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution, without any further addition of nitric acid, complies with the test.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in a mixture of 5 volumes of *dilute hydrochloric acid R* and 25 volumes of *distilled water R*, and dilute to 15 mL with the same mixture of solvents.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

- *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 20 ppm.

In a separating funnel, dissolve 0.50 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Examine the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

## ASSAY

Dissolve 0.150 g in 3 mL of *anhydrous formic acid R*. Add 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 20.42 mg of $C_{11}H_{12}N_2O_2$.

## STORAGE

Protected from light.

## IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F, G, H, I, J, K, L.*

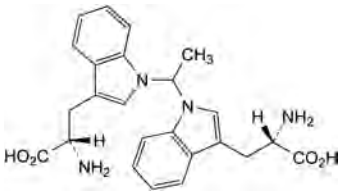

A. 3,3′-[ethane-1,1-diylbis(1*H*-indol-1,3-diyl)]bis[(2*S*)-2-aminopropanoic acid] (ethane-1,1-diylbistryptophan),

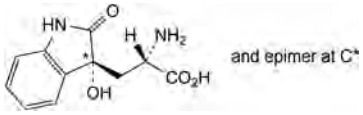


B. (2*S*)-2-amino-3-[(3*RS*)-3-hydroxy-2-oxo-2,3-dihydro-1*H*-indol-3-yl]propanoic acid (dioxyindolylalanine),

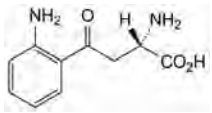

C. (2*S*)-2-amino-4-(2-aminophenyl)-4-oxobutanoic acid (kynurenine),

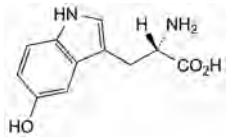

D. (2*S*)-2-amino-3-(5-hydroxy-1*H*-indol-3-yl)propanoic acid (5-hydroxytryptophan),

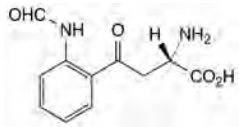

E. (2*S*)-2-amino-4-[2-(formylamino)phenyl]-4-oxobutanoic acid (*N*-formylkynurenine),

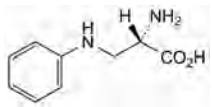

F. (2*S*)-2-amino-3-(phenylamino)propanoic acid (3-phenylaminoalanine),

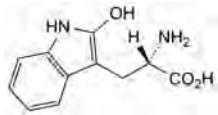

G. (2*S*)-2-amino-3-(2-hydroxy-1*H*-indol-3-yl)propanoic acid (2-hydroxytryptophan),

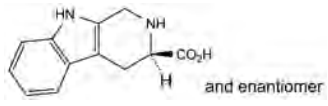


H. (3*RS*)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid,

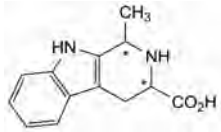

I. 1-methyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid,

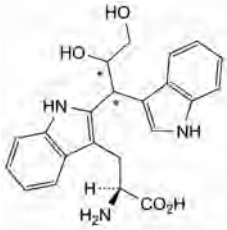

J. (2*S*)-2-amino-3-[2-[2,3-dihydroxy-1-(1*H*-indol-3-yl)propyl]-1*H*-indol-3-yl]propanoic acid,

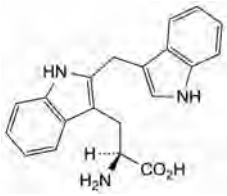

K. (2*S*)-2-amino-3-[2-(1*H*-indol-3-ylmethyl)-1*H*-indol-3-yl]propanoic acid,

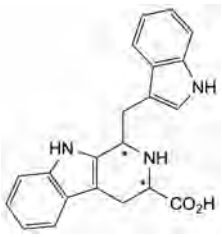

L. 1-(1*H*-indol-3-ylmethyl)-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole-3-carboxylic acid.

**01/2008:0152**

# TUBERCULIN FOR HUMAN USE, OLD

## Tuberculinum pristinum ad usum humanum

DEFINITION

Old tuberculin for human use consists of a filtrate, concentrated by heating, containing the soluble products of the culture and lysis of one or more strains of *Mycobacterium bovis* and/or *Mycobacterium tuberculosis* that is capable of demonstrating a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

Old tuberculin for human use in concentrated form is a transparent, viscous, yellow or brown liquid.

PRODUCTION

*GENERAL PROVISIONS*

The production of old tuberculin is based on a seed-lot system. The production method shall have been shown to yield consistently old tuberculin of adequate potency and safety in man. A batch of old tuberculin, calibrated in International Units by the method described under Assay and for which adequate clinical information is available as to its activity in man, is set aside to serve as a reference preparation.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*SEED LOTS*

The strains of mycobacteria used shall be identified by historical records that include information on their origin and subsequent manipulation.

The working seed lots used to inoculate the media for the production of a concentrated harvest shall not have undergone more than 4 subcultures from the master seed lot.

Only seed lots that comply with the following requirements may be used for propagation.

**Identification**. The species of mycobacterium of the master and working seed lots is identified.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

*PROPAGATION AND HARVEST*

The bacteria are grown in a liquid medium which may be a glycerolated broth or a synthetic medium. Growth must be typical for the strain. The culture is inactivated by a suitable method, such as treatment in an autoclave (121 °C for not less than 30 min) or in flowing steam at a temperature not less than 100 °C for at least 1 h. The culture liquid, from which the micro-organisms may or may not have been separated by filtration, is concentrated by evaporation, usually to one-tenth of its initial volume. The preparation is free from live mycobacteria. The concentrated harvest is shown to comply with the test for mycobacteria (*2.6.2*) before addition of any antimicrobial preservative or other substance that might interfere with the test. Phenol (5 g/L) or another suitable antimicrobial preservative that does not give rise to false positive reactions may be added.

Only a concentrated harvest that complies with the following requirements may be used in the preparation of the final bulk tuberculin.

**pH** (*2.2.3*). The pH of the concentrated harvest is 6.5 to 8.

**Glycerol**. Where applicable, determine the glycerol content of the concentrated harvest. The amount is within the limits approved for the particular product.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not more than 115 per cent of the intended amount. If phenol has been used in the preparation, the concentration is not more than 5 g/L (*2.5.15*).

**Sensitisation**. Carry out the test described under Tests.

**Sterility** (*2.6.1*). The concentrated harvest complies with the test for sterility, carried out using 10 mL for each medium.

**Potency**. Determine the potency as described under Assay.

*FINAL BULK TUBERCULIN*

The concentrated harvest is diluted aseptically.

Only a final bulk tuberculin that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk tuberculin complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

The final bulk tuberculin is distributed aseptically into sterile containers which are then closed so as to prevent contamination.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

The following tests may be omitted on the final lot if they have been carried out at the stages indicated:

- live mycobacteria: concentrated harvest,
- sensitisation: concentrated harvest,
- toxicity: concentrated harvest or final bulk tuberculin,
- antimicrobial preservative: final bulk tuberculin.

IDENTIFICATION

Inject increasing doses of the preparation to be examined intradermally into healthy, white or pale-coloured guinea-pigs, specifically sensitised (for example, as described under Assay). A reaction varying from erythema to necrosis is produced at the site of the injection. Similar injections administered to non-sensitised guinea-pigs do not stimulate a reaction. The assay may also serve as identification.

TESTS

*Old tuberculin for human use in concentrated form (≥ 100 000 IU/mL) complies with each of the tests prescribed below; the diluted product complies with the tests for antimicrobial preservative and sterility.*

**Toxicity**. Inject a quantity equivalent to 50 000 IU subcutaneously into each of two healthy guinea-pigs weighing 250 g to 350 g and which have not been subjected to any treatment likely to interfere with the test. Observe the animals for 7 days. No adverse effect is produced.

**Sensitisation**. Use 3 guinea-pigs that have not been subjected to any treatment likely to interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig about 500 IU of the preparation to be examined in a volume of 0.1 mL. 2 to 3 weeks after the third injection, administer the same dose intradermally to the same animals and to a control group of 3 guinea-pigs of the same mass that have not previously received injections of tuberculin. After 24 h to 72 h, the reactions in the 2 groups of animals are not substantially different.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the amount stated on the label. If phenol has been used in the preparation, the concentration is not more than 5 g/L (*2.5.15*).

**Live mycobacteria** (*2.6.2*). It complies with the test for mycobacteria.

**Sterility** (*2.6.1*). It complies with the test for sterility.

ASSAY

The potency of old tuberculin is determined by comparing the reactions produced by the intradermal injection of increasing doses of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension containing a suitable amount (0.1 mg to 0.4 mg/mL) of heat-inactivated, dried mycobacteria in mineral oil with or without emulsifier; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than 6 pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 mL of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least three injections on each side and not more than a total of 12 injection points per animal. Use at least three different doses of the reference preparation and at least 3 different doses of the preparation to be examined. For both preparations, use doses such that the highest dose is about 10 times the lowest dose. Choose the doses such that when they are injected the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject each dose intradermally in a constant volume of 0.1 mL or 0.2 mL. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical

methods, assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the preparation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store protected from light.

LABELLING

The label states:

- the number of International Units per millilitre,
- the species of mycobacterium used to prepare the product,
- the name and quantity of any antimicrobial preservative or any other excipient,
- the expiry date,
- where applicable, that old tuberculin is not to be injected in its concentrated form but diluted so as to administer not more than 100 IU per dose.

**01/2008:0535**
**corrected 10.0**

# TUBERCULIN PURIFIED PROTEIN DERIVATIVE, AVIAN

## Tuberculini aviarii derivatum proteinosum purificatum

DEFINITION

Avian tuberculin purified protein derivative (avian tuberculin PPD) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium avium* capable of revealing a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

PRODUCTION

It is obtained from the water-soluble fractions prepared by heating in free-flowing steam and subsequently filtering cultures of *M. avium* grown in a liquid synthetic medium. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. An antimicrobial preservative that does not give rise to false positive reactions, such as phenol, may be added. The final sterile preparation, free from mycobacteria, is distributed aseptically into sterile tamper-evident glass containers, which are then closed so as to prevent contamination. The preparation may be freeze-dried.

*The identification, the tests and the determination of potency apply to the liquid form and to the freeze-dried form after reconstitution as stated on the label.*

IDENTIFICATION

Inject a range of graded doses intradermally at different sites into suitably sensitised albino guinea-pigs, each weighing not less than 250 g. After 24-28 h, reactions appear in the form of oedematous swellings with erythema, with or without necrosis, at the points of injection. The size and severity of the reactions vary according to the dose. Unsensitised guinea-pigs show no reactions to similar injections.

TESTS

**pH** (*2.2.3*): 6.5 to 7.5.

**Phenol** (*2.5.15*): maximum 5 g/L, if the preparation to be examined contains phenol.

**Sensitising effect**. Use a group of 3 guinea-pigs that have not been treated with any material that will interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig a dose of the preparation to be examined equivalent to 500 IU in 0.1 mL. 15-21 days after the 3rd injection, inject the same dose (500 IU) intradermally into these animals and into a control group of 3 guinea-pigs of the same mass, which have not previously received injections of tuberculin. 24-28 h after the last injections, the reactions of the 2 groups are not significantly different.

**Toxicity**. Use 2 guinea-pigs, each weighing not less than 250 g, that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each guinea-pig 0.5 mL of the preparation to be examined. Observe the animals for 7 days. No abnormal effects occur during the observation period.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

POTENCY

The potency of avian tuberculin purified protein derivative is determined by comparing the reactions produced in sensitised guinea-pigs by the intradermal injection of a series of dilutions of the preparation to be examined with those produced by known concentrations of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Sensitise not fewer than 8 albino guinea-pigs, each weighing 400-600 g, by the deep intramuscular injection of a suitable dose of inactivated or live *M. avium*. Not less than 4 weeks after the sensitisation of the guinea-pigs, shave their flanks to provide space for not more than 4 injection sites on each side. Prepare dilutions of the preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5-7.5) containing 0.005 g/L of *polysorbate 80 R*. Use not fewer than 3 doses of the reference preparation and not fewer than 3 doses of the preparation to be examined. Choose the doses such that the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. Allocate the dilutions randomly to the sites, for example using a Latin square design. Inject each dose intradermally in a constant volume of 0.1 mL or 0.2 mL. Measure the diameters of the lesions after 24-28 h and calculate the results of the test using the usual statistical methods (for example, *5.3*) and assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the tuberculins.

The test is not valid unless the confidence limits ($P$ = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 75 per cent and not more than 133 per cent of the stated potency. The stated potency is not less than 20 000 IU/mL.

STORAGE

Protected from light, at a temperature of 5 ± 3 °C.

LABELLING

The label states:

- the potency in International Units per millilitre;
- the name and quantity of any excipient;
- for freeze-dried preparations:
  - the name and volume of the reconstituting liquid to be added;
  - that the product is to be used immediately after reconstitution.

**01/2008:0536**
**corrected 10.0**

# TUBERCULIN PURIFIED PROTEIN DERIVATIVE, BOVINE

## Tuberculini bovini derivatum proteinosum purificatum

DEFINITION

Bovine tuberculin purified protein derivative (bovine tuberculin PPD) is a preparation obtained from the heat-treated products of growth and lysis of *Mycobacterium bovis* capable of revealing a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

PRODUCTION

It is obtained from the water-soluble fractions prepared by heating in free-flowing steam and subsequently filtering cultures of *M. bovis* grown in a liquid synthetic medium. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. An antimicrobial preservative that does not give rise to false positive reactions, such as phenol, may be added. The final sterile preparation, free from mycobacteria, is distributed aseptically into sterile, tamper-evident glass containers, which are then closed so as to prevent contamination. The preparation may be freeze-dried.

*The identification, the tests and the determination of potency apply to the liquid form and to the freeze-dried form after reconstitution as stated on the label.*

IDENTIFICATION

Inject a range of graded doses intradermally at different sites into suitably sensitised albino guinea-pigs, each weighing not less than 250 g. After 24-28 h, reactions appear in the form of oedematous swellings with erythema, with or without necrosis, at the points of injection. The size and severity of the reactions vary according to the dose. Unsensitised guinea-pigs show no reactions to similar injections.

TESTS

**pH** (*2.2.3*): 6.5 to 7.5.

**Phenol** (*2.5.15*): maximum 5 g/L, if the preparation to be examined contains phenol.

**Sensitising effect**. Use a group of 3 guinea-pigs that have not been treated with any material that will interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig a dose of the preparation to be examined equivalent to 500 IU in 0.1 mL. 15-21 days after the 3rd injection, inject the same dose (500 IU) intradermally into these animals and into a control group of 3 guinea-pigs of the same mass, which have not previously received injections of tuberculin. 24-28 h after the last injections, the reactions of the 2 groups are not significantly different.

**Toxicity**. Use 2 guinea-pigs, each weighing not less than 250 g, that have not previously been treated with any material that will interfere with the test. Inject subcutaneously into each guinea-pig 0.5 mL of the preparation to be examined. Observe the animals for 7 days. No abnormal effects occur during the observation period.

**Sterility**. It complies with the test for sterility prescribed in the monograph *Vaccines for veterinary use (0062)*.

POTENCY

The potency of bovine tuberculin purified protein derivative is determined by comparing the reactions produced in sensitised guinea-pigs by the intradermal injection of a series of dilutions of the preparation to be examined with those produced by known concentrations of a reference preparation calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Sensitise not fewer than 8 albino guinea-pigs, each weighing 400-600 g, by the deep intramuscular injection of 0.0001 mg of wet mass of living *M. bovis* of strain AN5 suspended in 0.5 mL of a 9 g/L solution of *sodium chloride R*. Not less than 4 weeks after the sensitisation of the guinea-pigs, shave their flanks to provide space for not more than 4 injection sites on each side. Prepare dilutions of the preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5-7.5) containing 0.005 g/L of *polysorbate 80 R*. Use not fewer than 3 doses of the reference preparation and not fewer than 3 doses of the preparation to be examined. Choose the doses such that the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. Allocate the dilutions randomly to the sites, for example using a Latin square design. Inject each dose intradermally in a constant volume of 0.1 mL or 0.2 mL. Measure the diameters of the lesions after 24-28 h and calculate the results of the test using the usual statistical methods (for example, *5.3*) and assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the tuberculins.

The test is not valid unless the confidence limits ($P$ = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency. The estimated potency is not less than 66 per cent and not more than 150 per cent of the stated potency. The stated potency is not less than 20 000 IU/mL.

STORAGE

Protected from light, at a temperature of 5 ± 3 °C.

LABELLING

The label states:
- the potency in International Units per millilitre;
- the name and quantity of any excipient;
- for freeze-dried preparations:
  - the name and volume of the reconstituting liquid to be added;
  - that the product is to be used immediately after reconstitution.

**01/2008:0151**

# TUBERCULIN PURIFIED PROTEIN DERIVATIVE FOR HUMAN USE

## Tuberculini derivatum proteinosum purificatum ad usum humanum

DEFINITION

Tuberculin purified protein derivative (tuberculin PPD) for human use is a preparation obtained by precipitation from the heated products of the culture and lysis of *Mycobacterium bovis* and/or *Mycobacterium tuberculosis* and capable of demonstrating a delayed hypersensitivity in an animal sensitised to micro-organisms of the same species.

Tuberculin PPD is a colourless or pale-yellow liquid; the diluted preparation may be a freeze-dried powder which upon dissolution gives a colourless or pale-yellow liquid.

## PRODUCTION

*GENERAL PROVISIONS*

The production of tuberculin PPD is based on a seed-lot system. The production method shall have been shown to yield consistently tuberculin PPD of adequate potency and safety in man. A batch of tuberculin PPD, calibrated in International Units by method A described under Assay and for which adequate clinical information is available as to its activity in man, is set aside to serve as a reference preparation.

The International Unit is the activity of a stated quantity of the International Standard. The equivalence in International Units of the International Standard is stated by the World Health Organization.

*SEED LOTS*

The strains of mycobacteria used shall be identified by historical records that include information on their origin and subsequent manipulation.

The working seed lots used to inoculate the media for production of a concentrated harvest shall not have undergone more than 4 subcultures from the master seed lot.

Only seed lots that comply with the following requirements may be used for propagation.

**Identification**. The species of mycobacterium of the master and working seed lots is identified.

**Bacterial and fungal contamination**. Carry out the test for sterility (*2.6.1*), using 10 mL for each medium. The working seed lot complies with the test for sterility except for the presence of mycobacteria.

*PROPAGATION AND HARVEST*

The bacteria are grown in a liquid synthetic medium. Growth must be typical for the strain. The culture is inactivated by a suitable method such as treatment in an autoclave (121 °C for not less than 30 min) or in flowing steam at a temperature not less than 100 °C for at least 1 h and filtered. The active fraction of the filtrate, consisting mainly of protein, is isolated by precipitation, washed and re-dissolved. The preparation is free from mycobacteria. The concentrated harvest is shown to comply with the test for mycobacteria (*2.6.2*) before addition of any antimicrobial preservative or other substance that might interfere with the test. Phenol (5 g/L) or another suitable antimicrobial preservative that does not give rise to false positive reactions may be added; a suitable stabiliser intended to prevent adsorption on glass or plastic surfaces may be added. The concentrated harvest may be freeze-dried. Phenol is not added to preparations that are to be freeze-dried.

Only a concentrated harvest that complies with the following requirements may be used in the preparation of the final bulk tuberculin PPD.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than 85 per cent and not more than 115 per cent of the intended amount. If phenol has been used in the preparation, the concentration is not more than 5 g/L (*2.5.15*).

**Sensitisation**. Carry out the test described under Tests.

**Sterility** (*2.6.1*). The concentrated harvest, reconstituted if necessary, complies with the test for sterility, carried out using 10 mL for each medium.

**Potency**. Determine the potency as described under Assay.

*FINAL BULK TUBERCULIN PPD*

The concentrated harvest is diluted aseptically, after reconstitution if necessary.

Only a final bulk tuberculin PPD that complies with the following requirement may be used in the preparation of the final lot.

**Sterility** (*2.6.1*). The final bulk tuberculin PPD complies with the test for sterility, carried out using 10 mL for each medium.

*FINAL LOT*

The final bulk tuberculin PPD is distributed aseptically into sterile containers which are then closed so as to prevent contamination. It may be freeze-dried.

Only a final lot that is satisfactory with respect to each of the requirements given below under Identification, Tests and Assay may be released for use.

The following tests may be omitted on the final lot if they have been carried out at the stages indicated:

- live mycobacteria: concentrated harvest
- sensitisation: concentrated harvest
- toxicity: concentrated harvest or final bulk tuberculin PPD
- antimicrobial preservative: final bulk tuberculin PPD.

## IDENTIFICATION

Inject increasing doses of the preparation to be examined intradermally into healthy, white or pale-coloured guinea-pigs, specifically sensitised (for example as described under Assay). A reaction varying from erythema to necrosis is produced at the site of the injection. Similar injections administered to non-sensitised guinea-pigs do not stimulate a reaction. The assay may also serve as identification.

## TESTS

*Tuberculin purified protein derivative for human use in concentrated form (≥ 100 000 IU/mL) complies with each of the tests prescribed below; the diluted product complies with the tests for pH, antimicrobial preservative and sterility.*

**pH** (*2.2.3*). The pH of the preparation, reconstituted if necessary as stated on the label, is 6.5 to 7.5.

**Toxicity**. Inject subcutaneously 50 000 IU of the preparation to be examined into each of two healthy guinea-pigs weighing 250 g to 350 g and which have not been subjected to any treatment likely to interfere with the test. Observe the animals for 7 days. No adverse effect is produced.

**Sensitisation**. Use 3 guinea-pigs that have not been subjected to any treatment likely to interfere with the test. On 3 occasions at intervals of 5 days, inject intradermally into each guinea-pig about 500 IU of the preparation to be examined in a volume of 0.1 mL. 2 to 3 weeks after the third injection, administer the same dose intradermally to the same animals and to a control group of three guinea-pigs of the same mass that have not previously received injections of tuberculin. After 24 h to 72 h, the reactions in the 2 groups of animals are not substantially different.

**Antimicrobial preservative**. Where applicable, determine the amount of antimicrobial preservative by a suitable chemical or physico-chemical method. The content is not less than the minimum amount shown to be effective and not more than 115 per cent of the amount stated on the label. If phenol has been used in the preparation, the concentration is not more than 5 g/L (*2.5.15*).

**Live mycobacteria** (*2.6.2*). It complies with the test for mycobacteria.

**Sterility** (*2.6.1*). It complies with the test for sterility.

## ASSAY

Use method A or, where the preparation contains 1 IU to 2 IU, use method B.

*METHOD A*

The potency of tuberculin PPD is determined by comparing the reactions produced by the intradermal injection of increasing doses of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension containing a suitable amount (0.1 mg/mL to 0.4 mg/mL) of heat-inactivated, dried mycobacteria in mineral oil with or without emulsifier; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than six pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 mL of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least 3 injections on each side but not more than a total of 12 injection points per animal. Prepare dilutions of the preparation to be examined and of the reference preparation using isotonic phosphate-buffered saline (pH 6.5 to 7.5) containing 50 mg/L of *polysorbate 80 R*. If the preparation to be examined is freeze-dried and does not contain a stabiliser, reconstitute it using the liquid described above. Use at least 3 different doses of the reference preparation and at least 3 different doses of the preparation to be examined. For both preparations, use doses such that the highest dose is about 10 times the lowest dose. Choose the doses such that when they are injected the lesions produced have a diameter of not less than 8 mm and not more than 25 mm. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject each dose intradermally in a constant volume of 0.1 mL or 0.2 mL. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical methods, assuming that the diameters of the lesions are directly proportional to the logarithm of the concentration of the preparation.

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

*METHOD B*

The potency of tuberculin PPD is determined by comparing the reactions produced by the intradermal injection of the preparation to be examined into sensitised guinea-pigs with the reactions produced by known concentrations of the reference preparation.

Prepare a suspension in mineral oil with or without emulsifier and containing a suitable amount (0.1 mg/mL to 0.4 mg/mL) of heat-inactivated, dried mycobacteria; use mycobacteria of a strain of the same species as that used in the preparation to be examined. Sensitise not fewer than 6 pale-coloured guinea-pigs weighing not less than 300 g by injecting intramuscularly or intradermally a total of about 0.5 mL of the suspension, divided between several sites if necessary. Carry out the test after the period of time required for optimal sensitisation which is usually 4 to 8 weeks after sensitisation. Depilate the flanks of the animals so that it is possible to make at least 3 injections on each side but not more than a total of 12 injection points per animal. Prepare dilutions of the reference preparation using isotonic phosphate-buffered saline (pH 6.5 to 7.5) containing 50 mg/L of *polysorbate 80 R*. Use at least 3 different doses of the reference preparation such that the highest dose is about 10 times the lowest dose and the median dose is the same as that of the preparation to be examined. In any given test, the order of the dilutions injected at each point is chosen at random in a Latin square design. Inject the preparation to be examined and each dilution of the reference preparation intradermally in a constant volume of 0.1 mL or 0.2 mL. Measure the diameters of the lesions 24 h to 48 h later and calculate the results of the test by the usual statistical methods, assuming that the areas of the lesions are directly proportional to the logarithm of the concentration of the preparation to be examined. (This dose relationship applies to this assay and not necessarily to other test systems.)

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

Store protected from light.

LABELLING

The label states:

- the number of International Units per container,
- the species of mycobacteria used to prepare the product,
- the name and quantity of any antimicrobial preservative or any other excipient,
- the expiry date,
- for freeze-dried products, a statement that the product is to be reconstituted using the liquid provided by the manufacturer,
- where applicable, that tuberculin PPD is not to be injected in its concentrated form but diluted so as to administer not more than 100 IU per dose.

If the package does not contain a leaflet warning that the inhalation of concentrated tuberculin PPD may produce toxic effects, this warning must be shown on the label on the container together with a statement that the powder must be handled with care.

**01/2018:1273**
**corrected 10.0**

# TYLOSIN FOR VETERINARY USE

## Tylosinum ad usum veterinarium

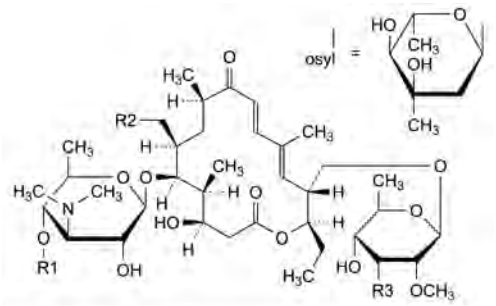


| Tylosin | R1 | R2 | R3 | Mol. Formula | $M_r$ |
|---|---|---|---|---|---|
| A | osyl | CHO | $OCH_3$ | $C_{46}H_{77}NO_{17}$ | 916 |
| B | H | CHO | $OCH_3$ | $C_{39}H_{65}NO_{14}$ | 772 |
| C | osyl | CHO | OH | $C_{45}H_{75}NO_{17}$ | 902 |
| D | osyl | $CH_2OH$ | $OCH_3$ | $C_{46}H_{79}NO_{17}$ | 918 |

[1401-69-0]

DEFINITION

Mixture of macrolide antibiotics produced by certain strains of *Streptomyces fradiae*. The main component is 2-[(4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[(6-deoxy-2,3-di-*O*-methyl-β-D-allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-2,10-dioxo-1-oxacyclohexadeca-

Monographs T

11,13-dien-7-yl]acetaldehyde (tylosin, tylosin A). Tylosin B (desmycosin, 4′-*O*-demycarosyltylosin), tylosin C (macrocin, 3‴-*O*-demethyltylosin) and tylosin D (relomycin, 19-deoxo-19-hydroxytylosin) may also be present.

*Content*: minimum 900 IU/mg (dried substance). Tylosins A, B, C and D contribute to the potency.

CHARACTERS

*Appearance*: almost white or slightly yellow powder.

*Solubility*: very slightly soluble in water, freely soluble in ethanol (96 per cent) and in methylene chloride, practically insoluble in heptane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *tylosin CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 1.00 g in 20 mL of a mixture of equal volumes of *acetonitrile R* and *water R*.

**pH** (*2.2.3*): 8.5 to 10.5.

Suspend 0.25 g in 10 mL of *carbon dioxide-free water R*.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Phosphate buffer pH 5.5*. Dissolve 25.82 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust to pH 5.5 with a 13.2 g/L solution of *dipotassium hydrogen phosphate R* and dilute to 1.0 L with *water R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 5 mL of *acetonitrile R* and dilute to 25.0 mL with *water R*.

*Reference solution (a)*. Dissolve 5 mg of *tylosin for system suitability CRS* (containing tylosins A, B, C and D, and impurities A, E, N, O, R and S) in 1 mL of *acetonitrile R* and dilute to 5.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: phosphate buffer pH 5.5, *acetonitrile R*, *water for chromatography R* (10:27.5:62.5 *V/V/V*);
- *mobile phase B*: phosphate buffer pH 5.5, *water for chromatography R*, *acetonitrile R* (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 45 | 100 → 84 | 0 → 16 |
| 45 - 65 | 84 | 16 |
| 65 - 70 | 84 → 44 | 16 → 56 |
| 70 - 82 | 44 | 56 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *tylosin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to tylosins A, B, C and D, and impurities A, E, N, O, R and S.

*Relative retention* with reference to tylosin A (retention time = about 65 min): impurity E = about 0.23; tylosin B = about 0.31; impurity A = about 0.38; tylosin C = about 0.60; tylosin D = about 0.78; impurity N = about 0.81; impurity O = about 0.85; impurity R = about 1.17; impurity S = about 1.20.

*System suitability*:

- *resolution*: reference solution (a): minimum 2.0 between the peaks due to tylosin B and impurity A; minimum 1.5 between the peaks due to impurities N and O; minimum 1.3 between the peaks due to impurities R and S;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *tylosin A*: minimum 80.0 per cent;
- *sum of tylosins A, B, C and D*: minimum 95.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition. Use the normalisation procedure.

*Limits*:

- *impurity A*: maximum 2.0 per cent;
- *sum of impurities eluting between impurity A and tylosin C*: maximum 2.0 per cent;
- *impurities N, O*: for each impurity, maximum 1.0 per cent;
- *impurities E, R, S*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.50 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Impurity II (tyramine)**: maximum 0.35 per cent, or maximum 0.15 per cent if intended for use in the manufacture of parenteral preparations.

In a 25.0 mL volumetric flask, dissolve 50.0 mg in 5.0 mL of a 3.4 g/L solution of *phosphoric acid R*. Add 1.0 mL of *pyridine R* and 2.0 mL of a saturated solution of *ninhydrin R* (about 40 g/L). Close the flask with aluminium foil and heat in a water-bath at 85 °C for 30 min. Cool the solution rapidly and dilute to 25.0 mL with *water R*. Mix and measure immediately the absorbance (*2.2.25*) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 35 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*. If intended for use in the manufacture of parenteral preparations, the absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 15 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*.

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *tylosin CRS* as the chemical reference substance.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, E, N, O, R, S, II.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, I, J, K, L, M, P, Q, T, U, V, W, X, Y, Z, AA, BB, CC, DD, EE, FF, GG, HH.*

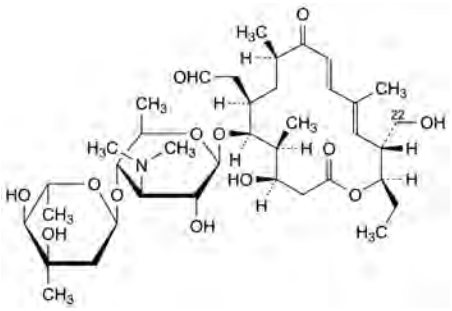

A. 22-*O*-demycinosyltylosin,

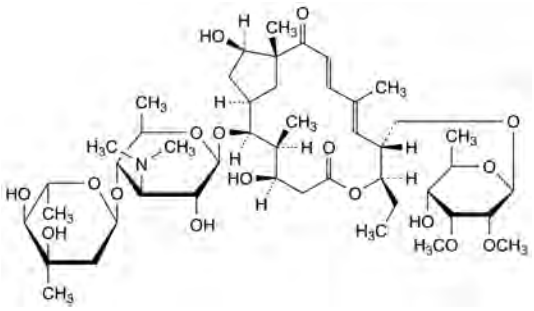

B. tylosin A aldol,

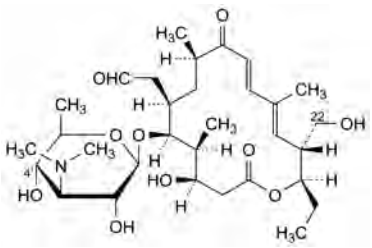

C. 4′-*O*-demycarosyl-22-*O*-demycinosyltylosin (demycinosyldesmycosin),

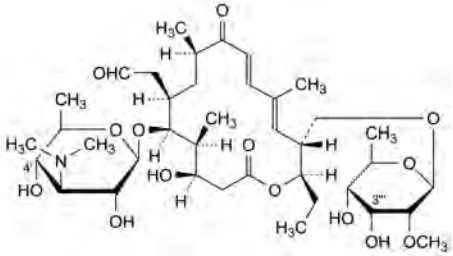

D. 3‴-*O*-demethyl-4′-*O*-demycarosyltylosin (demycarosylmacrocin, lactenocin),

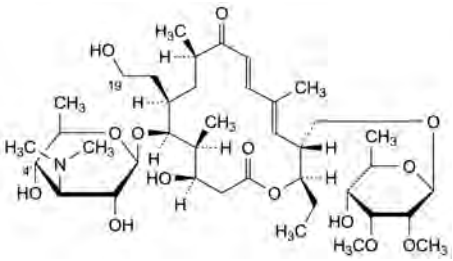

E. 4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin (demycarosylrelomycin),

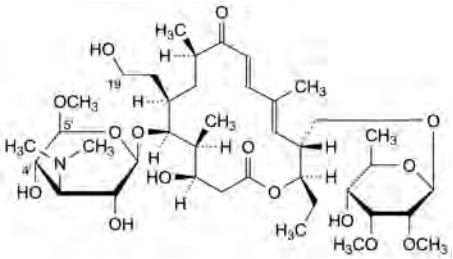

F. 5′-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-5′-methoxytylosin,

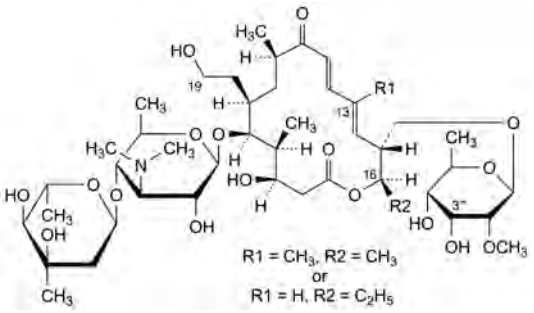


G. 16-deethyl-3‴-*O*-demethyl-19-deoxo-19-hydroxy-16-methyltylosin or 3‴-*O*,13-didemethyl-19-deoxo-19-hydroxytylosin,

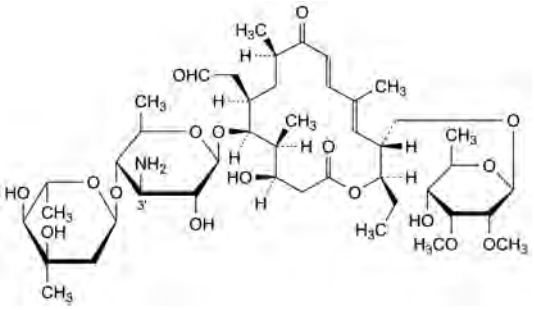

H. 3′-*N*,3′-*N*-didemethyltylosin,

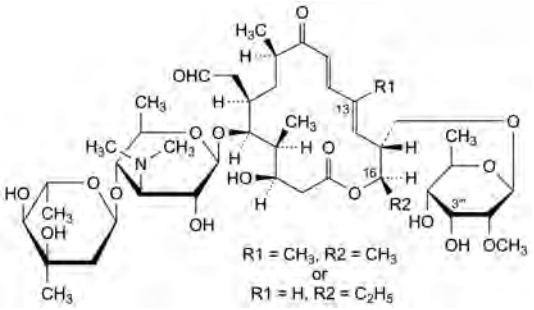


I. 16-deethyl-3‴-*O*-demethyl-16-methyltylosin or 3‴-*O*,13-didemethyltylosin,

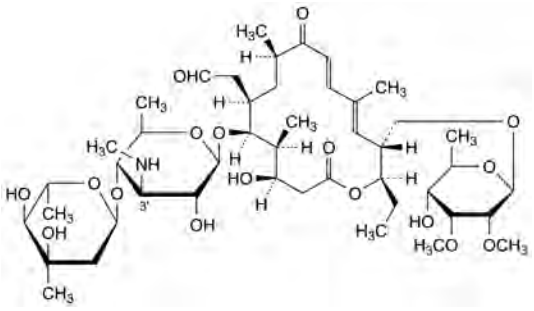

J. 3′-*N*-demethyltylosin,

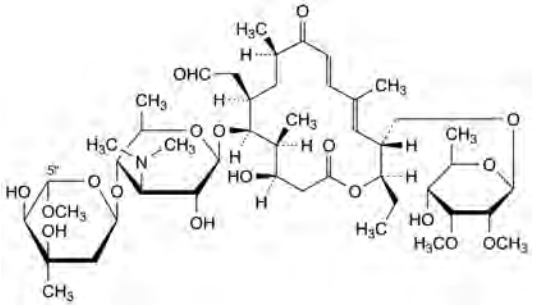

K. 5″-demethyl-5″-methoxytylosin,

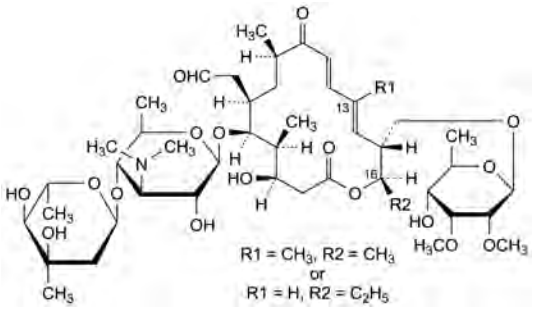


L. 16-deethyl-16-methyltylosin or 13-demethyltylosin,

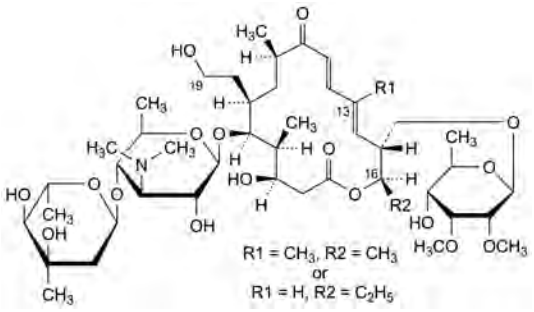


M. 16-deethyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-19-deoxo-19-hydroxytylosin,

N. unknown structure,

O. unknown structure,

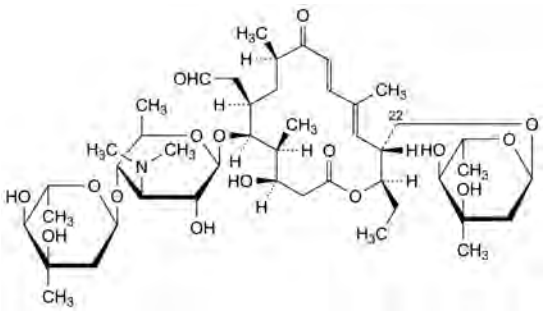

P. 22-*O*-demycinosyl-22-*O*-mycarosyltylosin,

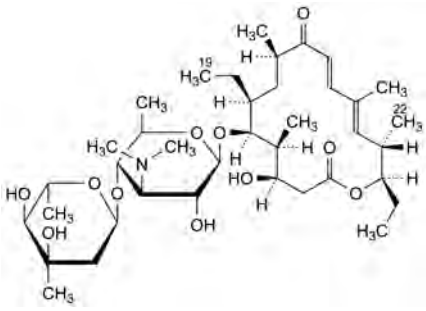

Q. 22-demycinosyloxy-19-deoxotylosin,

R. unknown structure,

S. unknown structure,

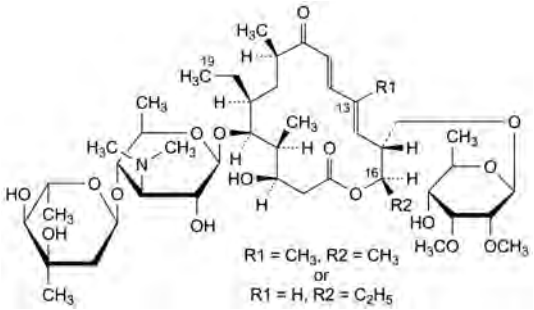


T. 16-deethyl-19-deoxo-16-methyltylosin or 13-demethyl-19-deoxotylosin,

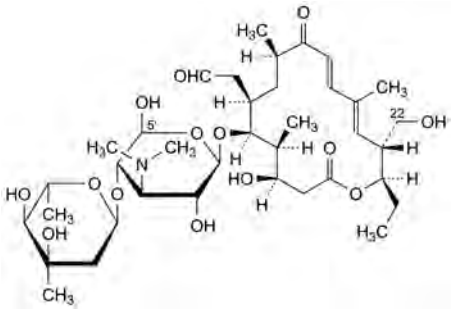

U. 5′-demethyl-22-*O*-demycinosyl-5′-hydroxytylosin,

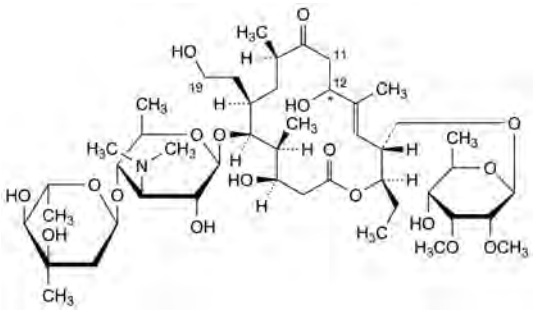

V. 19-deoxo-12,19-dihydroxy-11,12-dihydrotylosin,

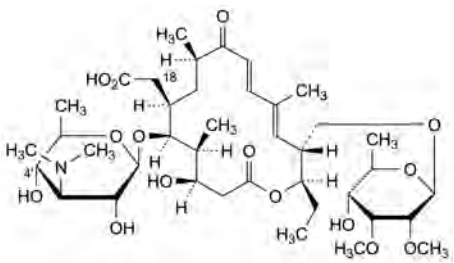

W. 18-carboxy-18-deformyl-4′-*O*-demycarosyltylosin,

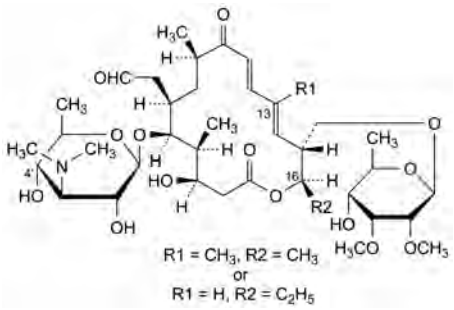


X. 16-deethyl-4′-*O*-demycarosyl-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyltylosin,

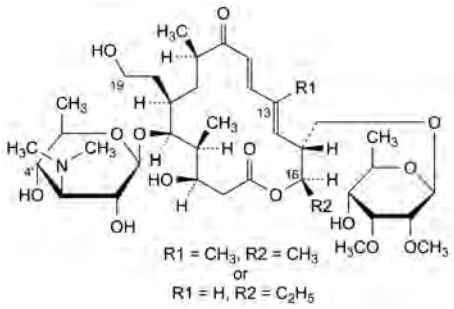


Y. 16-deethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin,

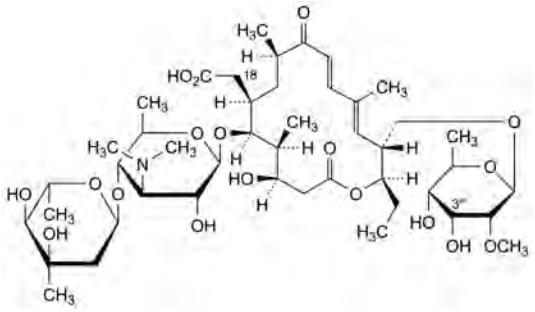

Z. 18-carboxy-18-deformyl-3‴-O-demethyltylosin,

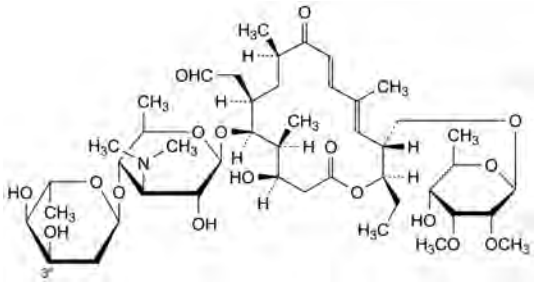

AA. 3″-demethyltylosin,

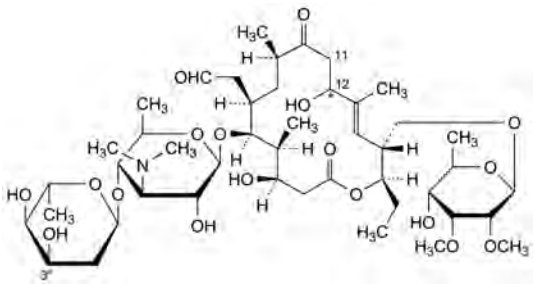

BB. 3″-demethyl-12-hydroxy-11,12-dihydrotylosin,

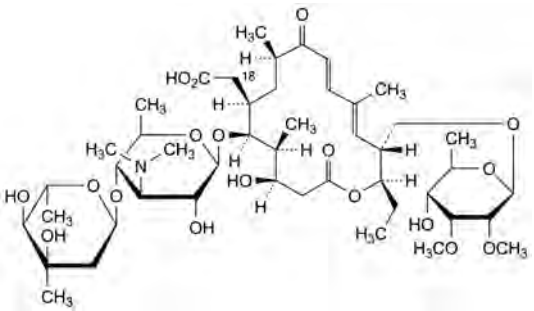

CC. 18-carboxy-18-deformyltylosin,

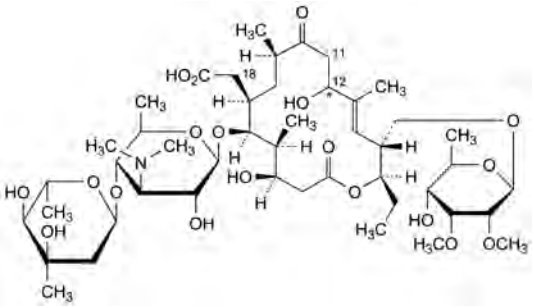

DD. 18-carboxy-18-deformyl-12-hydroxy-11,12-dihydrotylosin,

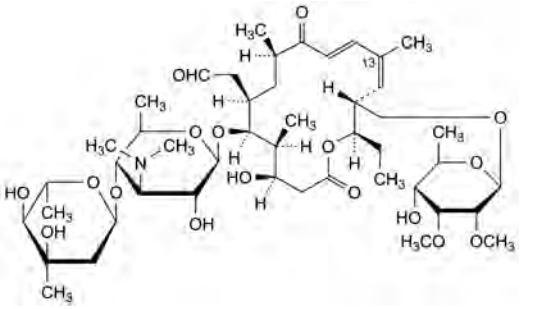

EE. (13*Z*)-tylosin,

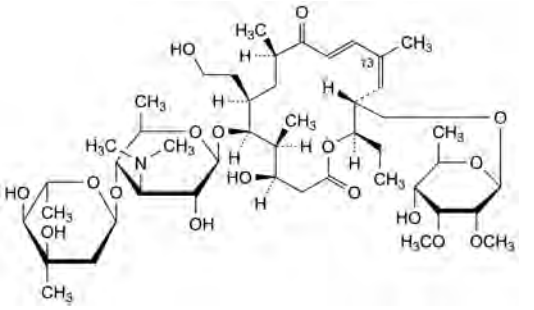

FF. (13*Z*)-19-deoxo-19-hydroxytylosin ((13*Z*)-relomycin),

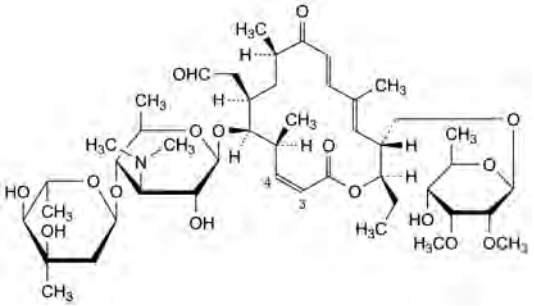

GG. 4-dehydroxy-3,4-didehydrotylosin,

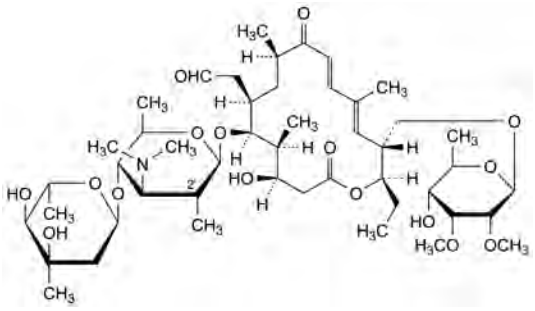

HH. 2′-deoxy-2′-methyltylosin,

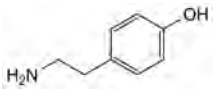

II. 4-(2-aminoethyl)phenol (tyramine).

**01/2018:1661**

# TYLOSIN PHOSPHATE BULK SOLUTION FOR VETERINARY USE

## Tylosini phosphatis solutio ad usum veterinarium

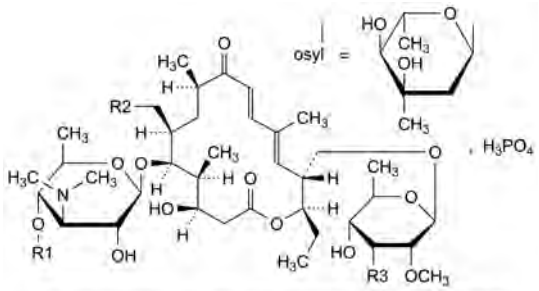

| Tylosin | R1 | R2 | R3 | Mol. Formula | $M_r$ |
|---|---|---|---|---|---|
| A | osyl | CHO | $OCH_3$ | $C_{46}H_{80}NO_{21}P$ | 1014 |
| B | H | CHO | $OCH_3$ | $C_{39}H_{68}NO_{18}P$ | 870 |
| C | osyl | CHO | OH | $C_{45}H_{78}NO_{21}P$ | 1000 |
| D | osyl | $CH_2OH$ | $OCH_3$ | $C_{46}H_{82}NO_{21}P$ | 1016 |

### DEFINITION

Solution of the dihydrogen phosphate of a mixture of macrolide antibiotics produced by certain strains of *Streptomyces fradiae*. The main component is 2-[(4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[(6-deoxy-2,3-di-*O*-methyl-β-D-allopyranosyl)oxy]-methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-gluco-pyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-2,10-dioxo-1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde monophosphate (tylosin monophosphate, tylosin A monophosphate). The monophosphates of tylosin B (desmycosin, 4′-*O*-demycarosyltylosin), tylosin C (macrocin, 3‴-*O*-demethyltylosin) and tylosin D (relomycin, 19-deoxo-19-hydroxytylosin) may also be present. The solution also contains sodium dihydrogen phosphate.

*Content*: minimum 815 IU/mg of dry residue. Tylosins A, B, C and D contribute to the potency.

### CHARACTERS

*Appearance*: yellow or brownish-yellow, viscous liquid.

*Solubility*: miscible with water.

### IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: use the dry residue prepared as described in the assay.

*Comparison*: *tylosin phosphate CRS*.

B. Dilute a quantity of the bulk solution to be examined equivalent to 400 000 IU of tylosin phosphate to 10 mL with *water R*. The solution gives reaction (a) of phosphates (*2.3.1*).

### TESTS

**pH** (*2.2.3*): 5.5 to 6.5.

Dilute 1.0 g of the bulk solution to be examined to 10 mL with *carbon dioxide-free water R*.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Phosphate buffer pH 5.5*. Dissolve 25.82 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust to pH 5.5 with a 13.2 g/L solution of *dipotassium hydrogen phosphate R* and dilute to 1.0 L with *water R*.

*Test solution*. Dilute 1.0 g of the bulk solution to be examined to 50.0 mL with *water R*. Dilute 3.0 mL of this solution to 20.0 mL with *water R* (1.0 mg/mL of dried residue).

*Reference solution (a)*. Dissolve 5 mg of *tylosin for system suitability CRS* (containing tylosins A, B, C and D, and impurities A, E, N, O, R and S) in 1 mL of *acetonitrile R* and dilute to 5.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:

- *mobile phase A*: phosphate buffer pH 5.5, *acetonitrile R*, *water for chromatography R* (10:27.5:62.5 *V/V/V*);
- *mobile phase B*: phosphate buffer pH 5.5, *water for chromatography R*, *acetonitrile R* (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 45 | 100 → 84 | 0 →16 |
| 45 - 65 | 84 | 16 |
| 65 - 70 | 84 → 44 | 16 → 56 |
| 70 - 82 | 44 | 56 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *tylosin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to tylosins A, B, C and D, and impurities A, E, N, O, R and S.

*Relative retention* with reference to tylosin A (retention time = about 65 min): impurity E = about 0.23; tylosin B = about 0.31; impurity A = about 0.38; tylosin C = about 0.60; tylosin D = about 0.78; impurity N = about 0.81; impurity O = about 0.85; impurity R = about 1.17; impurity S = about 1.20.

*System suitability*:

- *resolution*: reference solution (a): minimum 2.0 between the peaks due to tylosin B and impurity A; minimum 1.5 between the peaks due to impurities N and O; minimum 1.3 between the peaks due to impurities R and S;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *tylosin A*: minimum 80.0 per cent;
- *sum of tylosins A, B, C and D*: minimum 95.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition. Use the normalisation procedure.

*Limits*:

- *impurity A*: maximum 2.0 per cent;
- *sum of impurities eluting between impurity A and tylosin C*: maximum 2.0 per cent;
- *impurities N, O*: for each impurity, maximum 1.0 per cent;
- *impurities E, R, S*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.50 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Impurity II (tyramine)**: maximum 0.35 per cent.

In a 25.0 mL volumetric flask, dilute a quantity of the bulk solution to be examined equivalent to 50 000 IU of tylosin phosphate to 5.0 mL with a 3.4 g/L solution of *phosphoric acid R*. Add 1.0 mL of *pyridine R* and 2.0 mL of a saturated solution of *ninhydrin R* (about 40 g/L). Close the flask with aluminium foil and heat in a water-bath at 85 °C for 20-30 min. Cool the solution rapidly and dilute to 25.0 mL with *water R*. Mix and measure immediately the absorbance (*2.2.25*) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 35 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*.

**Phosphate**: 8.5 per cent to 10.0 per cent, calculated with reference to the dry residue (see Assay).

*Test solution*. Dilute a quantity of the bulk solution to be examined equivalent to 200 000 IU of tylosin phosphate to 50 mL with *water R*. Add 5.0 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*. To 2.0 mL of this solution add successively, mixing after each addition, 10.0 mL of *water R*, 5.0 mL of *ammonium molybdate reagent R2*, 1.0 mL of *hydroquinone solution R* and 1.0 mL of a 200 g/L solution of *sodium metabisulfite R*. Allow to stand for at least 20 min and dilute to 50.0 mL with *water R*. Mix thoroughly.

*Reference solution (a)*. To 1.0 mL of a standard solution containing 0.430 g/L of *potassium dihydrogen phosphate R* (corresponds to 300 ppm of $PO_4$) add successively, mixing after each addition, 10.0 mL of *water R*, 5.0 mL of *ammonium molybdate reagent R2*, 1.0 mL of *hydroquinone solution R* and 1.0 mL of a 200 g/L solution of *sodium metabisulfite R*. Allow to stand for at least 20 min and dilute to 50.0 mL with *water R*. Mix thoroughly.

*Reference solution (b)*. Prepare as reference solution (a) but using 2.0 mL of the standard solution.

*Reference solution (c)*. Prepare as reference solution (a) but using 5.0 mL of the standard solution.

*Compensation liquid*. Prepare as reference solution (a) but omitting the standard solution.

Measure the absorbance (*2.2.25*) of the test solution and of the reference solutions at 650 nm. Draw a calibration curve with the absorbances of the 3 reference solutions as a function of the quantity of phosphate in the solutions and read from the curve the quantity of phosphate in the test solution. Determine the percentage content of phosphate, calculated with reference to the dry residue (see Assay).

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

Use *tylosin CRS* as the chemical reference substance. Calculate the content from the mass of the dry residue and the activity of the solution.

*Dry residue*. Dry 3.0 g of the bulk solution to be examined *in vacuo* at 60 °C for 3 h and weigh.

STORAGE

Protected from light, at a temperature of 2 °C to 8 °C.

IMPURITIES

*Specified impurities: A, E, N, O, R, S, II.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, I, J, K, L, M, P, Q, T, U, V, W, X, Y, Z, AA, BB, CC, DD, EE, FF, GG, HH.*

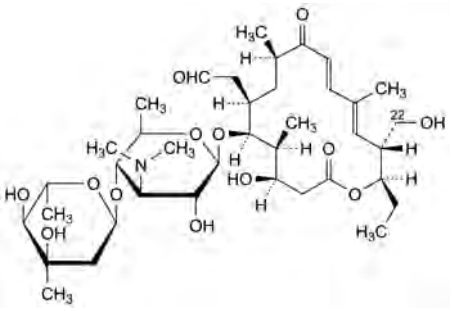

A. 22-*O*-demycinosyltylosin,

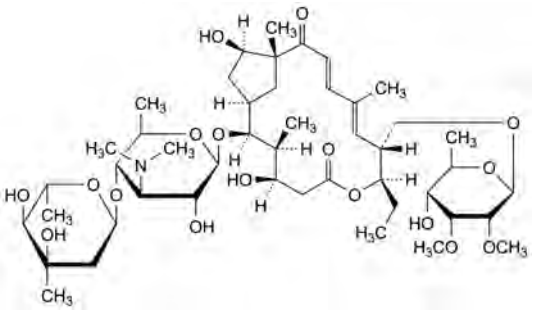

B. tylosin A aldol,

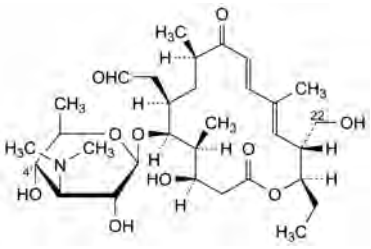

C. 4′-*O*-demycarosyl-22-*O*-demycinosyltylosin (demycinosyldesmycosin),

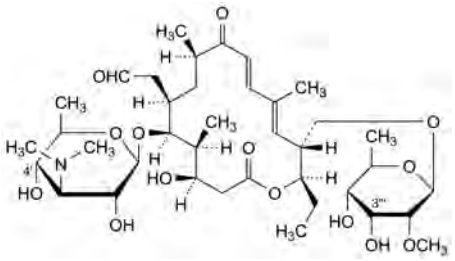

D. 3‴-*O*-demethyl-4′-*O*-demycarosyltylosin (demycarosylmacrocin, lactenocin),

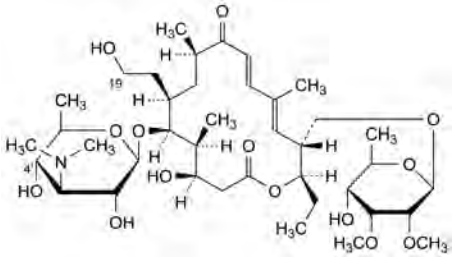

E. 4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin (demycarosylrelomycin),

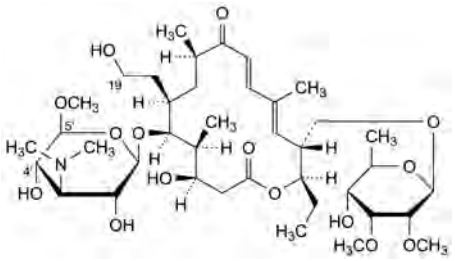

F. 5′-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-5′-methoxytylosin,

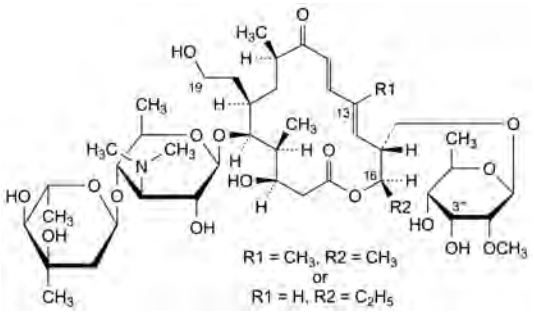


G. 16-deethyl-3‴-*O*-demethyl-19-deoxo-19-hydroxy-16-methyltylosin or 3‴-*O*,13-didemethyl-19-deoxo-19-hydroxytylosin,

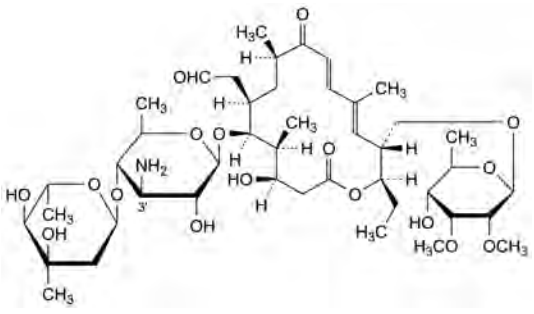

H. 3′-*N*,3′-*N*-didemethyltylosin,

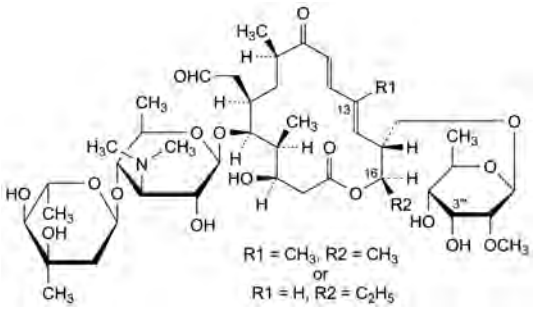


I. 16-deethyl-3‴-*O*-demethyl-16-methyltylosin or 3‴-*O*,13-didemethyltylosin,

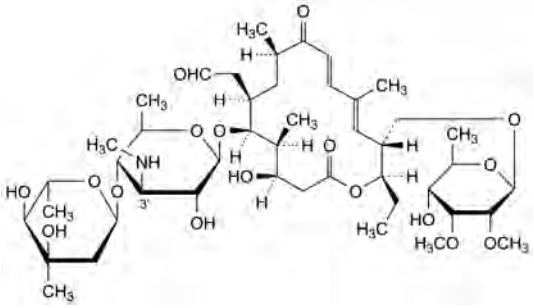

J. 3′-*N*-demethyltylosin,

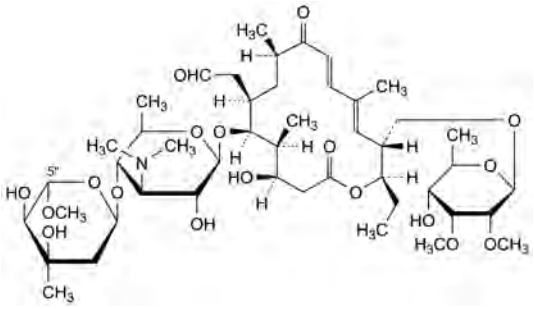

K. 5″-demethyl-5″-methoxytylosin,

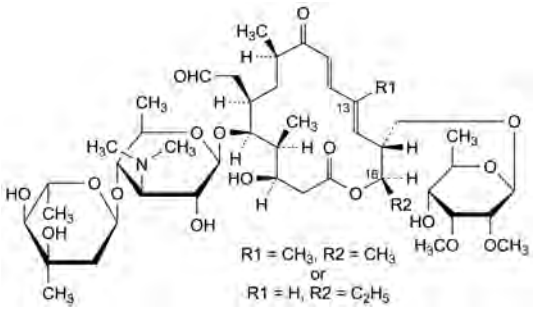


L. 16-deethyl-16-methyltylosin or 13-demethyltylosin,

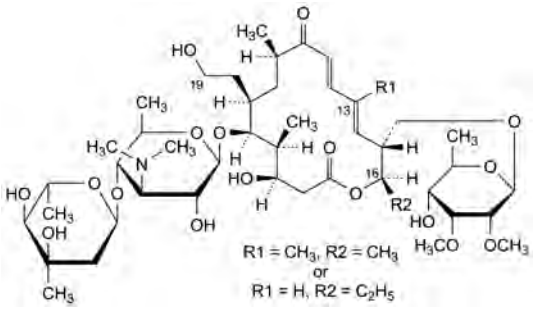


M. 16-deethyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-19-deoxo-19-hydroxytylosin,

N. unknown structure,

O. unknown structure,

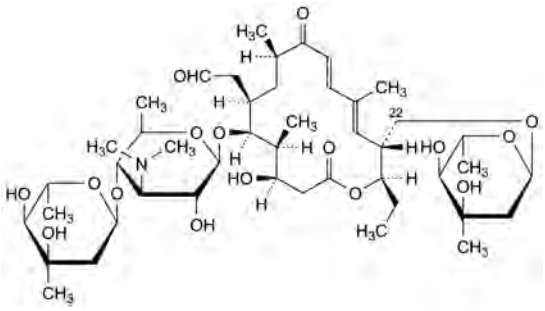

P. 22-*O*-demycinosyl-22-*O*-mycarosyltylosin,

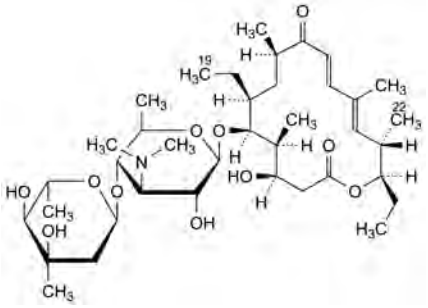

Q. 22-demycinosyloxy-19-deoxotylosin,

R. unknown structure,

S. unknown structure,

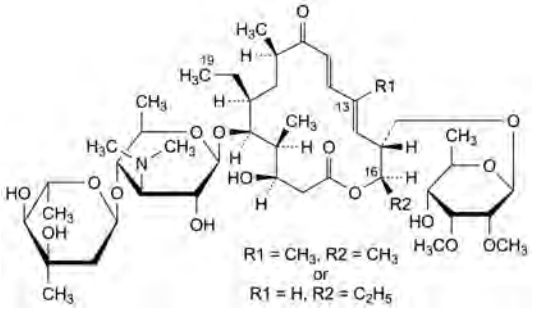


T. 16-deethyl-19-deoxo-16-methyltylosin or 13-demethyl-19-deoxotylosin,

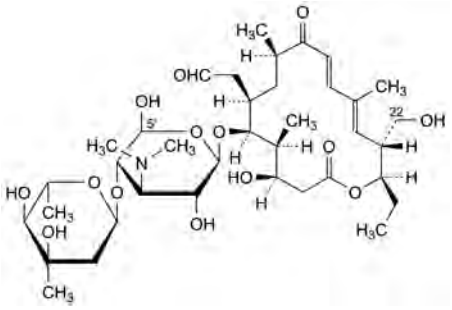

U. 5′-demethyl-22-*O*-demycinosyl-5′-hydroxytylosin,

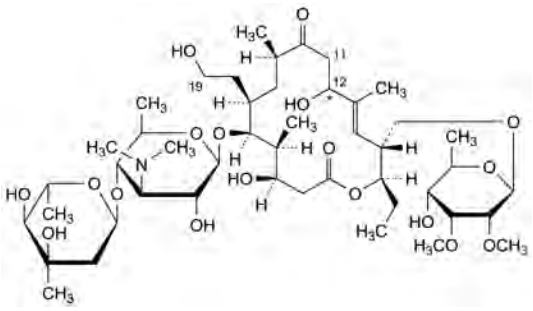

V. 19-deoxo-12,19-dihydroxy-11,12-dihydrotylosin,

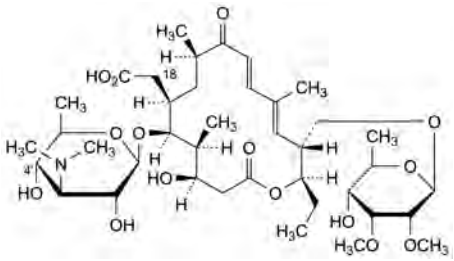

W. 18-carboxy-18-deformyl-4′-*O*-demycarosyltylosin,

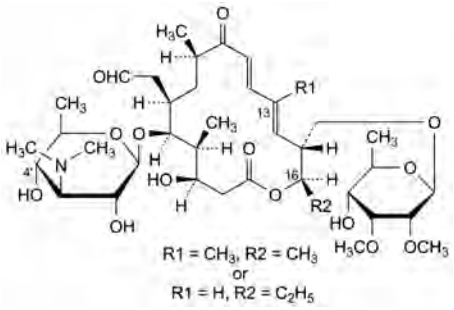


X. 16-deethyl-4′-*O*-demycarosyl-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyltylosin,

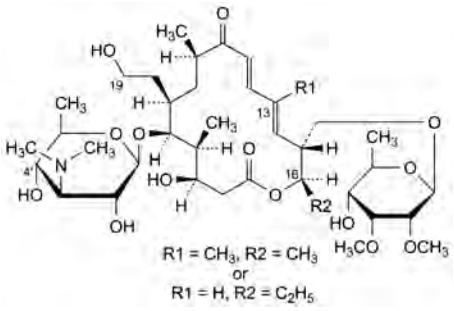


Y. 16-deethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin,

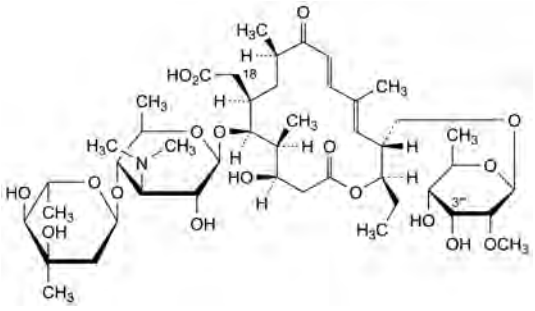

Z. 18-carboxy-18-deformyl-3‴-*O*-demethyltylosin,

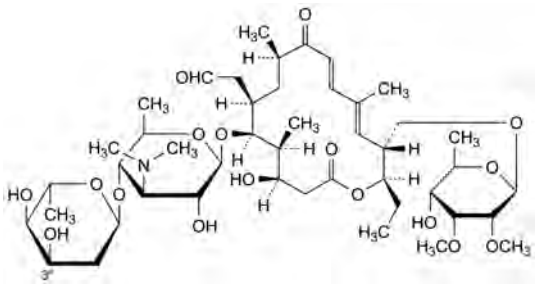

AA. 3″-demethyltylosin,

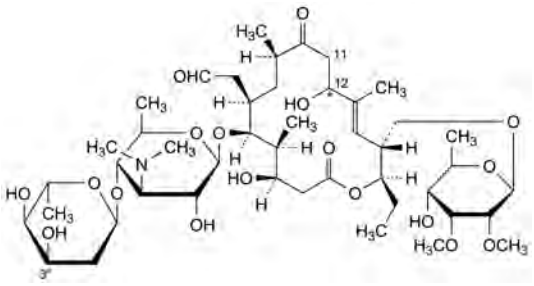

BB. 3″-demethyl-12-hydroxy-11,12-dihydrotylosin,

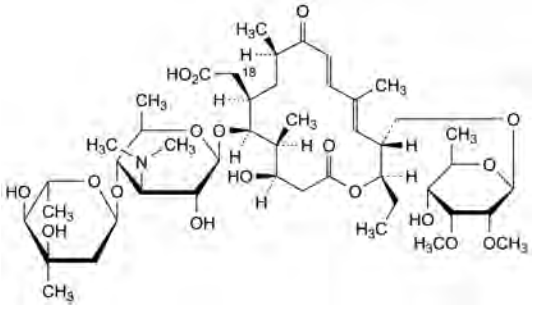

CC. 18-carboxy-18-deformyltylosin,

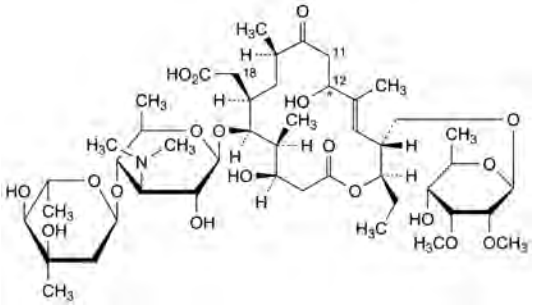

DD. 18-carboxy-18-deformyl-12-hydroxy-11,12-dihydrotylosin,

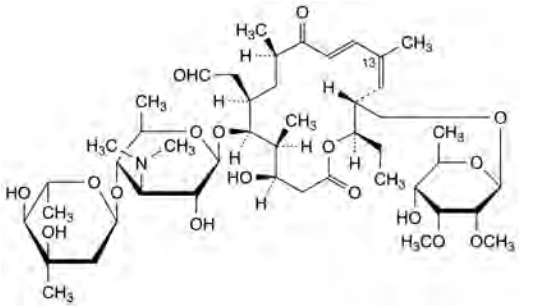

EE. (13*Z*)-tylosin,

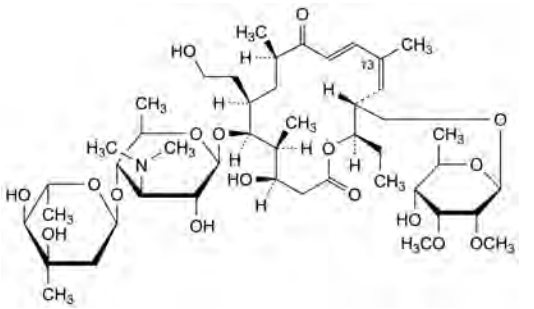

FF. (13*Z*)-19-deoxo-19-hydroxytylosin ((13*Z*)-relomycin),

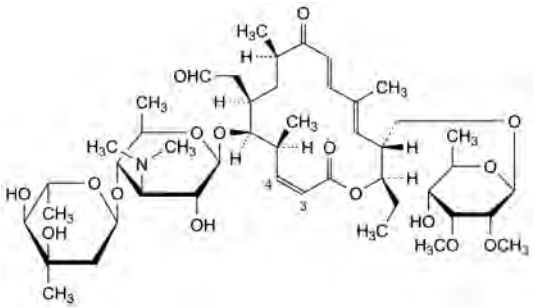

GG. 4-dehydroxy-3,4-didehydrotylosin,

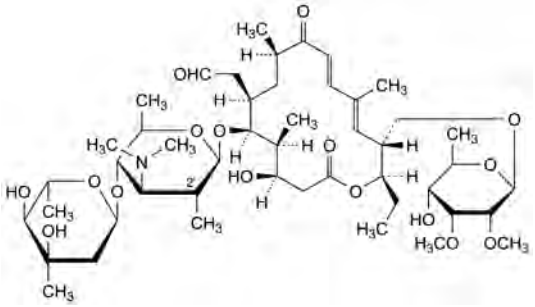

HH. 2′-deoxy-2′-methyltylosin,

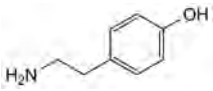

II. 4-(2-aminoethyl)phenol (tyramine).

**01/2018:2802**
**corrected 10.0**

# TYLOSIN PHOSPHATE FOR VETERINARY USE

## Tylosini phosphas ad usum veterinarium

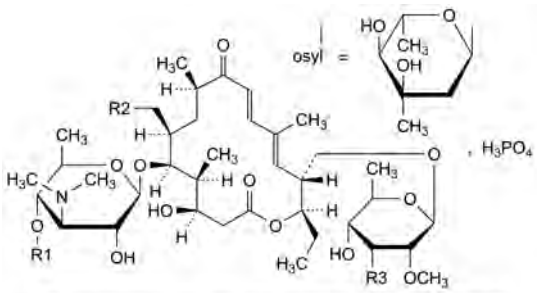

| Tylosin | R1 | R2 | R3 | Mol. Formula | $M_r$ |
|---|---|---|---|---|---|
| A | osyl | CHO | $OCH_3$ | $C_{46}H_{80}NO_{21}P$ | 1014 |
| B | H | CHO | $OCH_3$ | $C_{39}H_{68}NO_{18}P$ | 870 |
| C | osyl | CHO | OH | $C_{45}H_{78}NO_{21}P$ | 1000 |
| D | osyl | $CH_2OH$ | $OCH_3$ | $C_{46}H_{82}NO_{21}P$ | 1016 |

[1405-53-4]

DEFINITION

Dihydrogen phosphate of a mixture of macrolide antibiotics produced by certain strains of *Streptomyces fradiae*. The main component is 2-[(4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[(6-deoxy-2,3-di-*O*-methyl-β-D-allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-2,10-dioxo-

Monographs T

1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde monophosphate (tylosin monophosphate, tylosin A monophosphate). The monophosphates of tylosin B (desmycosin, 4′-*O*-demycarosyltylosin), tylosin C (macrocin, 3‴-*O*-demethyltylosin) and tylosin D (relomycin, 19-deoxo-19-hydroxytylosin) may also be present.

*Content*: minimum 900 IU/mg (dried substance). Tylosins A, B, C and D contribute to the potency.

CHARACTERS

*Appearance*: white or slightly yellow, hygroscopic powder.

*Solubility*: freely soluble in water and in ethanol (96 per cent), practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tylosin phosphate CRS*.

B. Dissolve 50 mg in 10 mL of *water R*. The solution gives reaction (a) of phosphates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method II*).

Dissolve 1.00 g in 20 mL of a mixture of equal volumes of *acetonitrile R* and *water R*.

**pH** (*2.2.3*): 6.0 to 7.2.

Dissolve 0.25 g in 10 mL of *carbon dioxide-free water R*.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Phosphate buffer pH 5.5*. Dissolve 25.82 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust to pH 5.5 with a 13.2 g/L solution of *dipotassium hydrogen phosphate R* and dilute to 1.0 L with *water R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 5 mL of *acetonitrile R* and dilute to 25.0 mL with *water R*.

*Reference solution (a)*. Dissolve 5 mg of *tylosin for system suitability CRS* (containing tylosins A, B, C and D, and impurities A, E, N, O, R and S) in 1 mL of *acetonitrile R* and dilute to 5.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:
- *mobile phase A*: phosphate buffer pH 5.5, *acetonitrile R*, *water for chromatography R* (10:27.5:62.5 *V/V/V*);
- *mobile phase B*: phosphate buffer pH 5.5, *water for chromatography R*, *acetonitrile R* (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 45 | 100 → 84 | 0 → 16 |
| 45 - 65 | 84 | 16 |
| 65 - 70 | 84 → 44 | 16 → 56 |
| 70 - 82 | 44 | 56 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *tylosin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to tylosins A, B, C and D, and impurities A, E, N, O, R and S.

*Relative retention* with reference to tylosin A (retention time = about 65 min): impurity E = about 0.23; tylosin B = about 0.31; impurity A = about 0.38; tylosin C = about 0.60; tylosin D = about 0.78; impurity N = about 0.81; impurity O = about 0.85; impurity R = about 1.17; impurity S = about 1.20.

*System suitability*:
- *resolution*: reference solution (a): minimum 2.0 between the peaks due to tylosin B and impurity A; minimum 1.5 between the peaks due to impurities N and O; minimum 1.3 between the peaks due to impurities R and S;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:
- *tylosin A*: minimum 80.0 per cent;
- *sum of tylosins A, B, C and D*: minimum 95.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition. Use the normalisation procedure.

*Limits*:
- *impurity A*: maximum 2.0 per cent;
- *sum of impurities eluting between impurity A and tylosin C*: maximum 2.0 per cent;
- *impurities N, O*: for each impurity, maximum 1.0 per cent;
- *impurities E, R, S*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.50 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Impurity II (tyramine)**: maximum 0.35 per cent, or maximum 0.15 per cent if intended for use in the manufacture of parenteral preparations.

In a 25.0 mL volumetric flask, dissolve 50.0 mg in 5.0 mL of a 3.4 g/L solution of *phosphoric acid R*. Add 1.0 mL of *pyridine R* and 2.0 mL of a saturated solution of *ninhydrin R* (about 40 g/L). Close the flask with aluminium foil and heat in a water-bath at 85 °C for 20-30 min. Cool the solution rapidly and dilute to 25.0 mL with *water R*. Mix and measure immediately the absorbance (*2.2.25*) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 35 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*. If intended for use in the manufacture of parenteral preparations, the absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 15 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*.

**Loss on drying** (*2.2.32*): maximum 4.5 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 6.0 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *tylosin CRS* as the chemical reference substance.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

Monographs T

IMPURITIES

*Specified impurities: A, E, N, O, R, S, II.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, I, J, K, L, M, P, Q, T, U, V, W, X, Y, Z, AA, BB, CC, DD, EE, FF, GG, HH.*

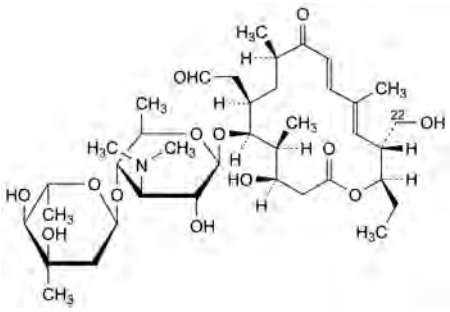

A. 22-*O*-demycinosyltylosin,

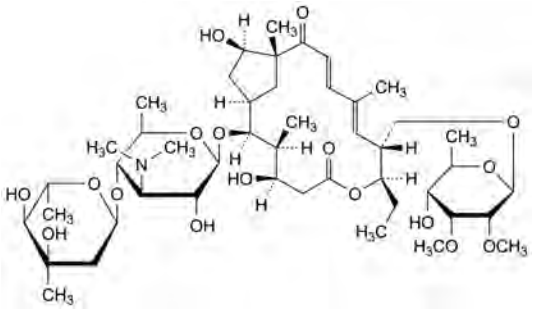

B. tylosin A aldol,

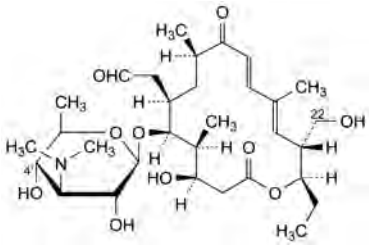

C. 4′-*O*-demycarosyl-22-*O*-demycinosyltylosin (demycinosyldesmycosin),

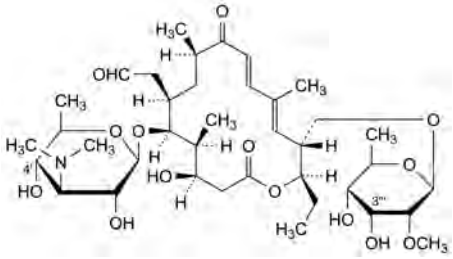

D. 3‴-*O*-demethyl-4′-*O*-demycarosyltylosin (demycarosylmacrocin, lactenocin),

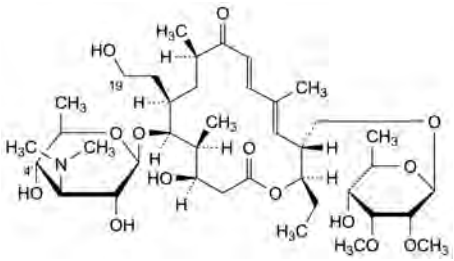

E. 4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin (demycarosylrelomycin),

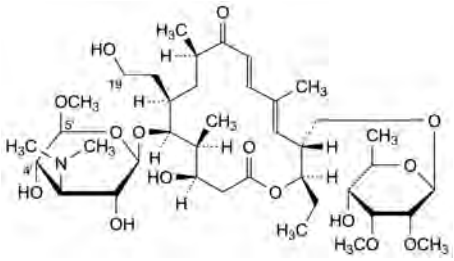

F. 5′-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-5′-methoxytylosin,

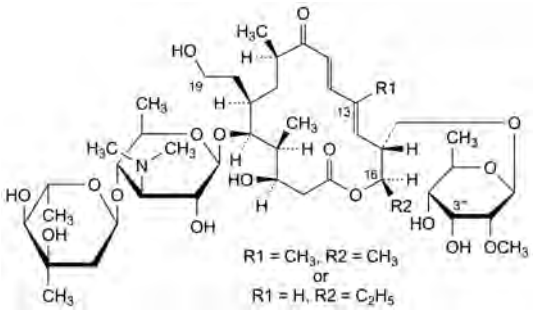


G. 16-deethyl-3‴-*O*-demethyl-19-deoxo-19-hydroxy-16-methyltylosin or 3‴-*O*,13-didemethyl-19-deoxo-19-hydroxytylosin,

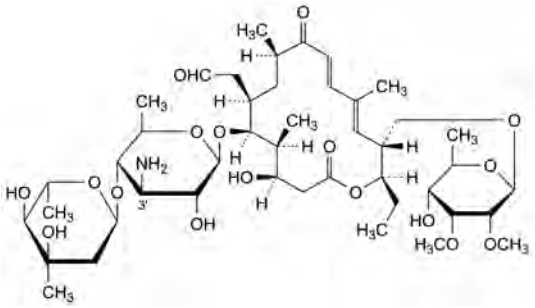

H. 3′-*N*,3′-*N*-didemethyltylosin,

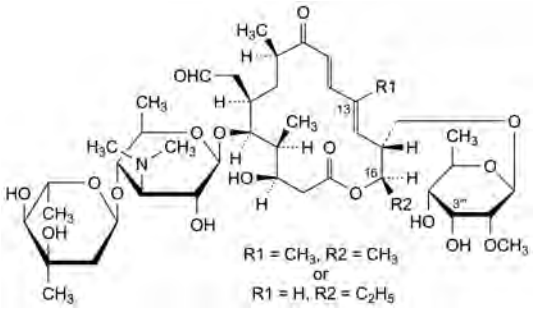


I. 16-deethyl-3‴-*O*-demethyl-16-methyltylosin or 3‴-*O*,13-didemethyltylosin,

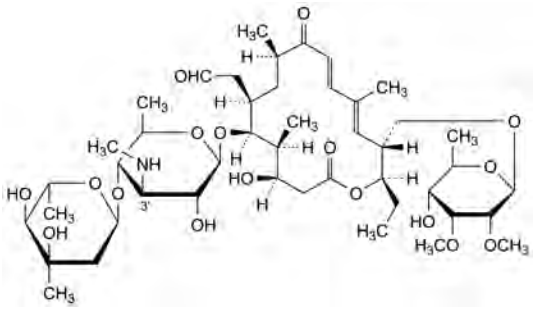

J. 3′-*N*-demethyltylosin,

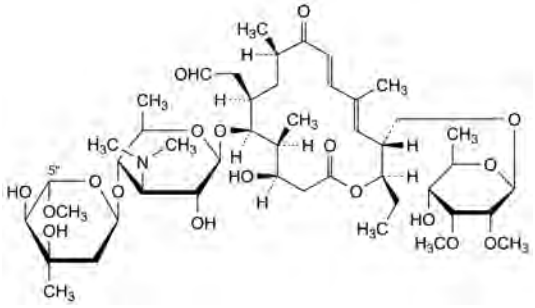

K. 5″-demethyl-5″-methoxytylosin,

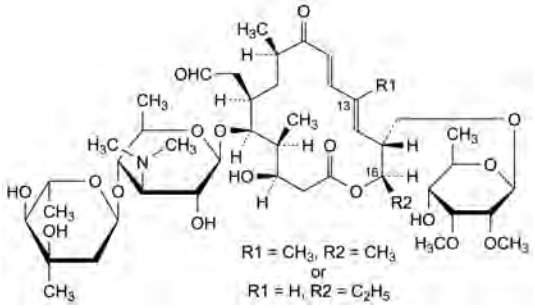


L. 16-deethyl-16-methyltylosin or 13-demethyltylosin,

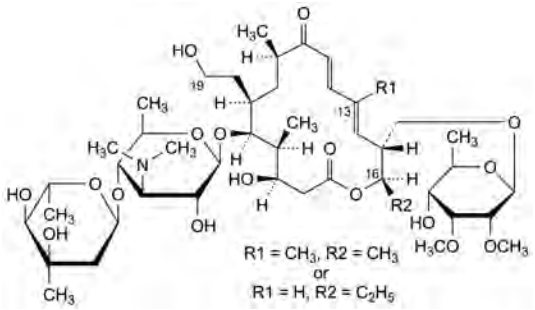


M. 16-deethyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-19-deoxo-19-hydroxytylosin,

N. unknown structure,

O. unknown structure,

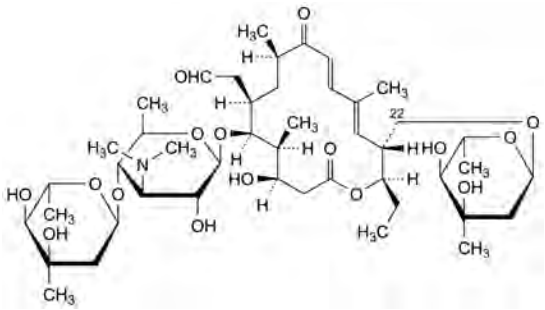

P. 22-*O*-demycinosyl-22-*O*-mycarosyltylosin,

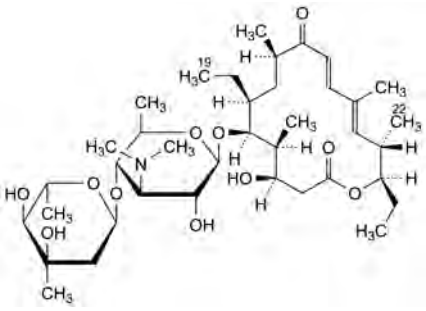

Q. 22-demycinosyloxy-19-deoxotylosin,

R. unknown structure,

S. unknown structure,

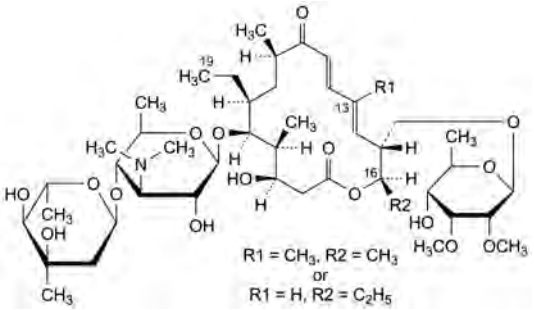


T. 16-deethyl-19-deoxo-16-methyltylosin or 13-demethyl-19-deoxotylosin,

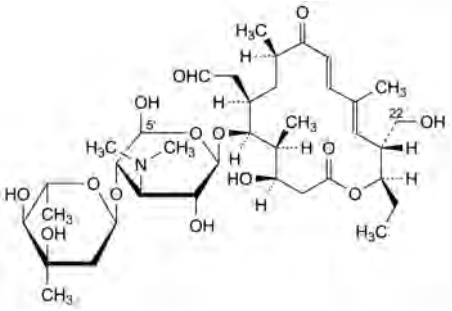

U. 5′-demethyl-22-*O*-demycinosyl-5′-hydroxytylosin,

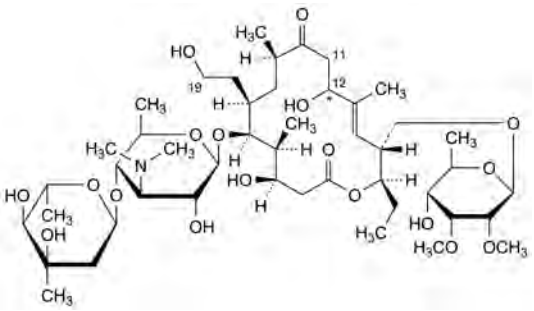

V. 19-deoxo-12,19-dihydroxy-11,12-dihydrotylosin,

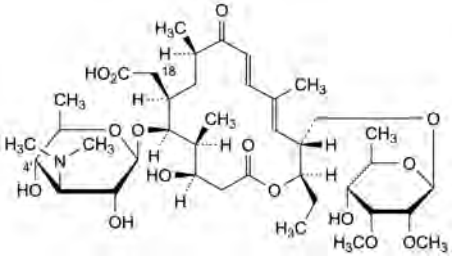

W. 18-carboxy-18-deformyl-4′-*O*-demycarosyltylosin,

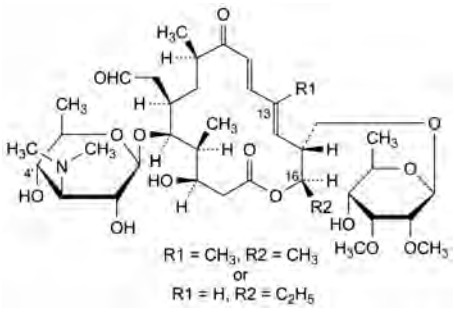


X. 16-deethyl-4′-*O*-demycarosyl-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyltylosin,

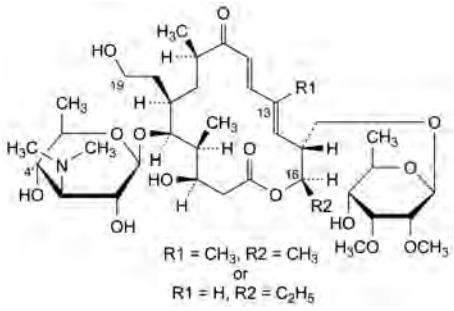


Y. 16-deethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin,

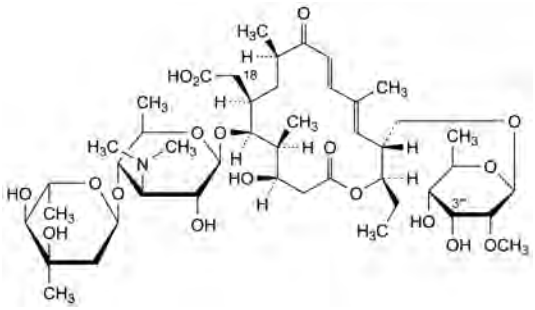

Z. 18-carboxy-18-deformyl-3‴-*O*-demethyltylosin,

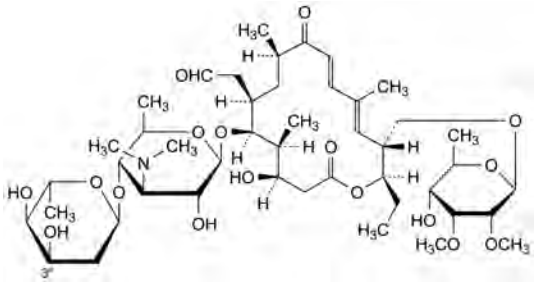

AA. 3″-demethyltylosin,

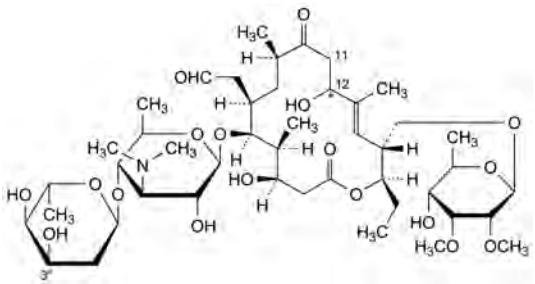

BB. 3″-demethyl-12-hydroxy-11,12-dihydrotylosin,

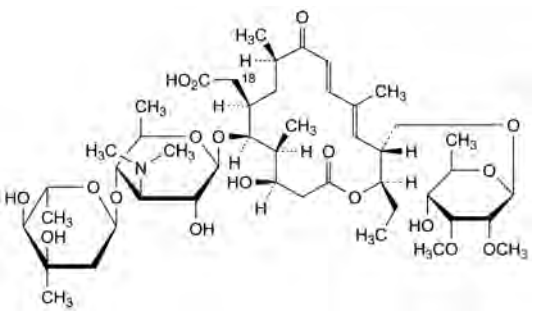

CC. 18-carboxy-18-deformyltylosin,

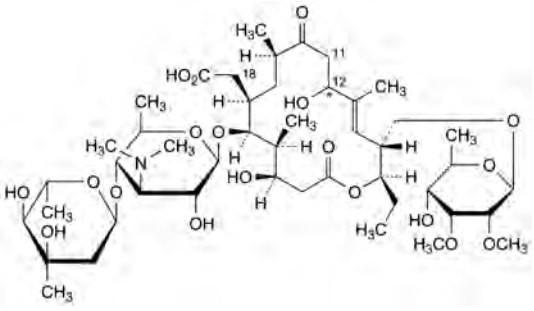

DD. 18-carboxy-18-deformyl-12-hydroxy-11,12-dihydrotylosin,

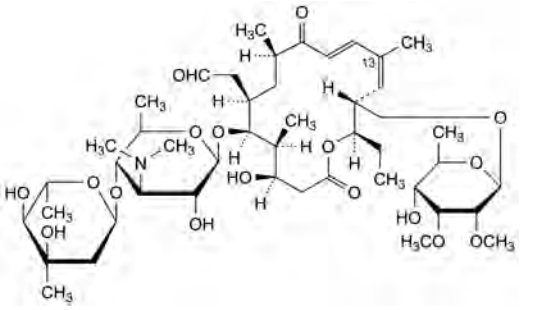

EE. (13*Z*)-tylosin,

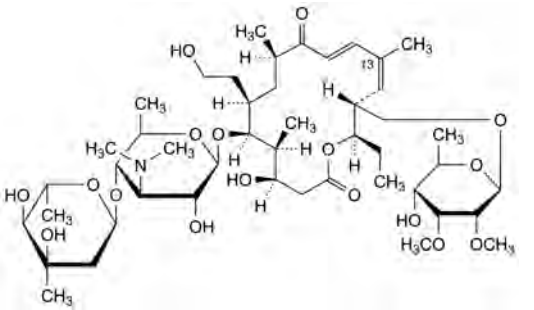

FF. (13*Z*)-19-deoxo-19-hydroxytylosin ((13*Z*)-relomycin),

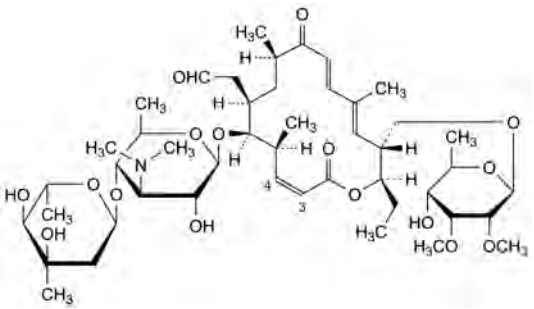

GG. 4-dehydroxy-3,4-didehydrotylosin,

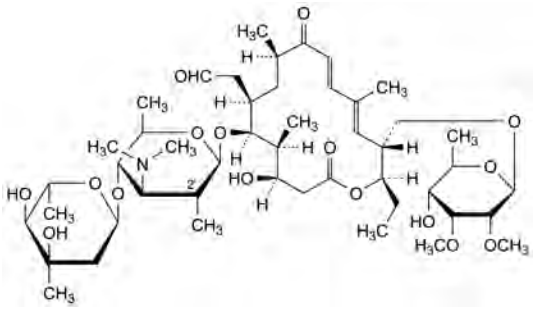

HH. 2′-deoxy-2′-methyltylosin,

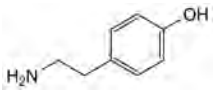

II. 4-(2-aminoethyl)phenol (tyramine).

**01/2018:1274**
**corrected 10.0**

# TYLOSIN TARTRATE FOR VETERINARY USE

## Tylosini tartras ad usum veterinarium

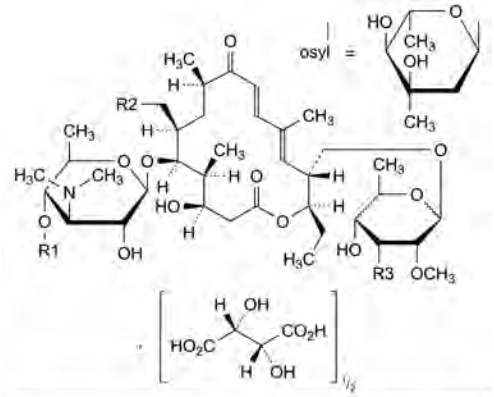

| Tylosin | R1 | R2 | R3 | Mol. Formula | $M_r$ |
|---|---|---|---|---|---|
| A | osyl | CHO | $OCH_3$ | $C_{48}H_{80}NO_{20}$ | 991 |
| B | H | CHO | $OCH_3$ | $C_{41}H_{68}NO_{17}$ | 847 |
| C | osyl | CHO | OH | $C_{47}H_{78}NO_{20}$ | 977 |
| D | osyl | $CH_2OH$ | $OCH_3$ | $C_{48}H_{82}NO_{20}$ | 993 |

[1405-54-5]

DEFINITION

Hemitartrate of a mixture of macrolide antibiotics produced by certain strains of *Streptomyces fradiae*. The main component is 2-[(4*R*,5*S*,6*S*,7*R*,9*R*,11*E*,13*E*,15*R*,16*R*)-15-[[(6-deoxy-2,3-di-*O*-methyl-β-D-allopyranosyl)oxy]methyl]-6-[[3,6-dideoxy-4-*O*-(2,6-dideoxy-3-*C*-methyl-α-L-*ribo*-hexopyranosyl)-3-(dimethylamino)-β-D-glucopyranosyl]oxy]-16-ethyl-4-hydroxy-5,9,13-trimethyl-2,10-dioxo-1-oxacyclohexadeca-11,13-dien-7-yl]acetaldehyde hemi[(2*R*,3*R*)-2,3-dihydroxybutanedioate] (tylosin hemitartrate, tylosin A hemitartrate). The hemitartrates of tylosin B (desmycosin, 4′-*O*-demycarosyltylosin), tylosin C (macrocin, 3‴-*O*-demethyltylosin) and tylosin D (relomycin, 19-deoxo-19-hydroxytylosin) may also be present.

*Content*: minimum 900 IU/mg (dried substance). Tylosins A, B, C and D contribute to the potency.

CHARACTERS

*Appearance*: almost white or slightly yellow, hygroscopic powder.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methylene chloride, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tylosin tartrate CRS*.

B. Dissolve about 30 mg in a mixture of 0.15 mL of *water R*, 2.5 mL of *acetic anhydride R* and 7.5 mL of *pyridine R*. Allow to stand for about 10 min. A green colour is produced.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_3$ (*2.2.2, Method II*).

Dissolve 1.00 g in 20 mL of a mixture of equal volumes of *acetonitrile R* and *water R*.

**pH** (*2.2.3*): 5.0 to 7.2.

Dissolve 0.25 g in 10 mL of *carbon dioxide-free water R*.

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Phosphate buffer pH 5.5*. Dissolve 25.82 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*, adjust to pH 5.5 with a 13.2 g/L solution of *dipotassium hydrogen phosphate R* and dilute to 1.0 L with *water R*.

*Test solution*. Dissolve 25.0 mg of the substance to be examined in 5 mL of *acetonitrile R* and dilute to 25.0 mL with *water R*.

*Reference solution (a)*. Dissolve 5 mg of *tylosin for system suitability CRS* (containing tylosins A, B, C and D, and impurities A, E, N, O, R and S) in 1 mL of *acetonitrile R* and dilute to 5.0 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 60 °C.

*Mobile phase*:
- *mobile phase A*: phosphate buffer pH 5.5, *acetonitrile R*, *water for chromatography R* (10:27.5:62.5 *V/V/V*);
- *mobile phase B*: phosphate buffer pH 5.5, *water for chromatography R*, *acetonitrile R* (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 25 | 100 | 0 |
| 25 - 45 | 100 → 84 | 0 → 16 |
| 45 - 65 | 84 | 16 |
| 65 - 70 | 84 → 44 | 16 → 56 |
| 70 - 82 | 44 | 56 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 20 µL.

*Identification of peaks*: use the chromatogram supplied with *tylosin for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to tylosins A, B, C and D, and impurities A, E, N, O, R and S.

Monographs T

*Relative retention* with reference to tylosin A (retention time = about 65 min): impurity E = about 0.23; tylosin B = about 0.31; impurity A = about 0.38; tylosin C = about 0.60; tylosin D = about 0.78; impurity N = about 0.81; impurity O = about 0.85; impurity R = about 1.17; impurity S = about 1.20.

*System suitability*:

- *resolution*: reference solution (a): minimum 2.0 between the peaks due to tylosin B and impurity A; minimum 1.5 between the peaks due to impurities N and O; minimum 1.3 between the peaks due to impurities R and S;
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (b).

*Limits*:

- *tylosin A*: minimum 80.0 per cent;
- *sum of tylosins A, B, C and D*: minimum 95.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Related substances**. Liquid chromatography (*2.2.29*) as described in the test for composition. Use the normalisation procedure.

*Limits*:

- *impurity A*: maximum 2.0 per cent;
- *sum of impurities eluting between impurity A and tylosin C*: maximum 2.0 per cent per cent;
- *impurities N, O*: for each impurity, maximum 1.0 per cent;
- *impurities E, R, S*: for each impurity, maximum 0.5 per cent;
- *any other impurity*: for each impurity, maximum 0.50 per cent;
- *total*: maximum 5.0 per cent;
- *reporting threshold*: 0.10 per cent (reference solution (b)).

**Impurity II (tyramine)**: maximum 0.35 per cent, or maximum 0.15 per cent if intended for use in the manufacture of parenteral preparations.

In a 25.0 mL volumetric flask, dissolve 50.0 mg in 5.0 mL of a 3.4 g/L solution of *phosphoric acid R*. Add 1.0 mL of *pyridine R* and 2.0 mL of a saturated solution of *ninhydrin R* (about 40 g/L). Close the flask with aluminium foil and heat in a water-bath at 85 °C for 30 min. Cool the solution rapidly and dilute to 25.0 mL with *water R*. Mix and measure immediately the absorbance (*2.2.25*) of the solution at 570 nm using a blank solution as the compensation liquid. The absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 35 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*. If intended for use in the manufacture of parenteral preparations, the absorbance is not greater than that of a standard prepared at the same time and in the same manner using 5.0 mL of a 15 mg/L solution of *tyramine R* (impurity II) in a 3.4 g/L solution of *phosphoric acid R*.

**Loss on drying** (*2.2.32*): maximum 4.5 per cent, determined on 1.000 g by drying at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 2.5 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*). Use *tylosin CRS* as the chemical reference substance.

STORAGE

In an airtight container, protected from light. If the substance is sterile, the container is also sterile and tamper-evident.

IMPURITIES

*Specified impurities: A, E, N, O, R, S, II.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, F, G, H, I, J, K, L, M, P, Q, T, U, V, W, X, Y, Z, AA, BB, CC, DD, EE, FF, GG, HH.*

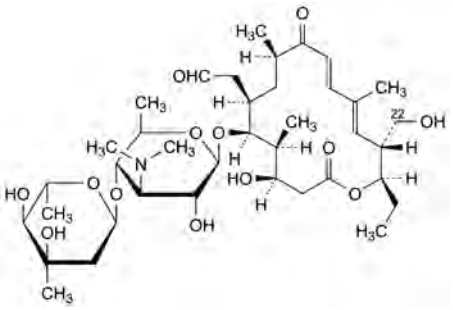

A. 22-*O*-demycinosyltylosin,

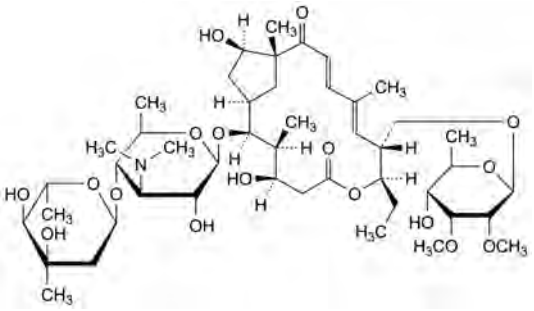

B. tylosin A aldol,

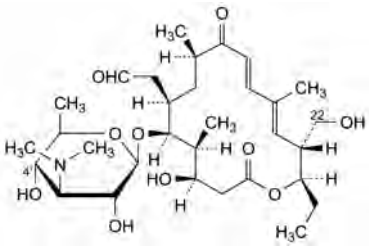

C. 4′-*O*-demycarosyl-22-*O*-demycinosyltylosin (demycinosyldesmycosin),

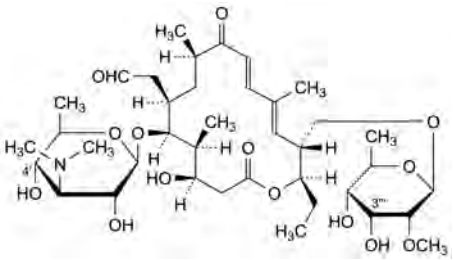

D. 3‴-*O*-demethyl-4′-*O*-demycarosyltylosin (demycarosylmacrocin, lactenocin),

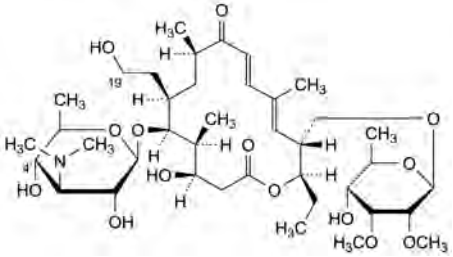

E. 4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin (demycarosylrelomycin),

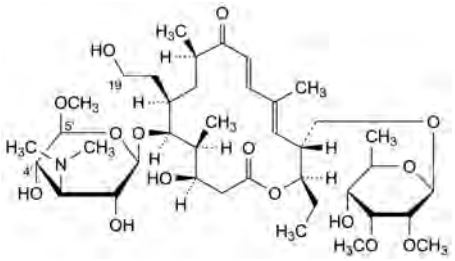

F. 5′-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-5′-methoxytylosin,

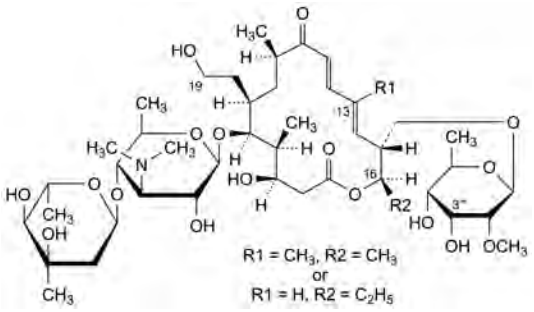


G. 16-deethyl-3‴-*O*-demethyl-19-deoxo-19-hydroxy-16-methyltylosin or 3‴-*O*,13-didemethyl-19-deoxo-19-hydroxytylosin,

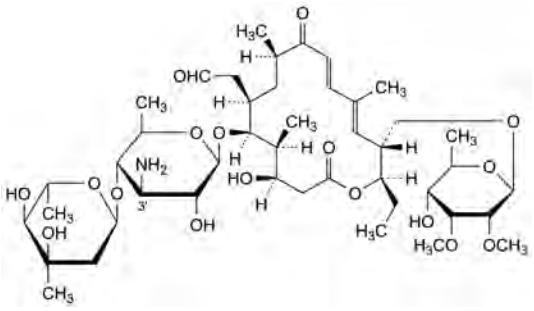

H. 3′-*N*,3′-*N*-didemethyltylosin,

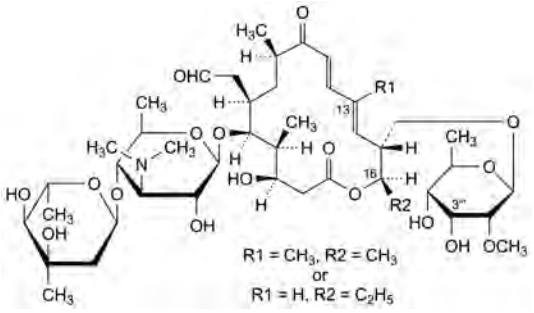


I. 16-deethyl-3‴-*O*-demethyl-16-methyltylosin or 3‴-*O*,13-didemethyltylosin,

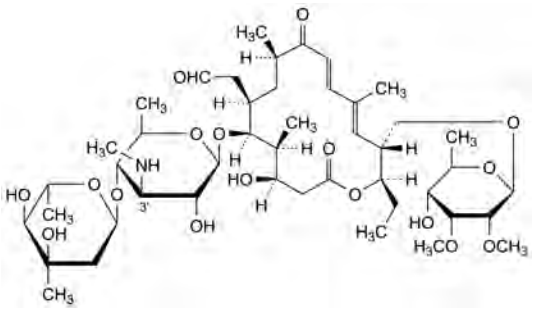

J. 3′-*N*-demethyltylosin,

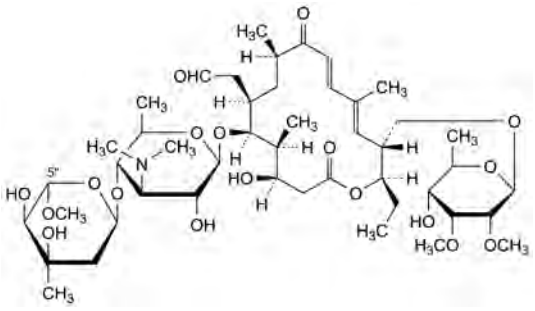

K. 5″-demethyl-5″-methoxytylosin,

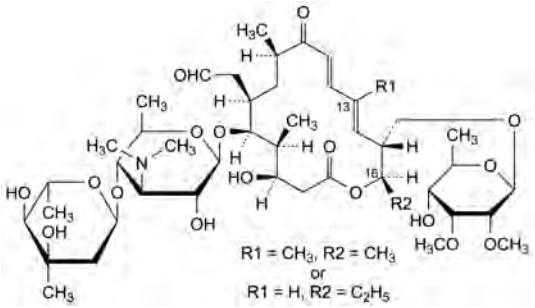


L. 16-deethyl-16-methyltylosin or 13-demethyltylosin,

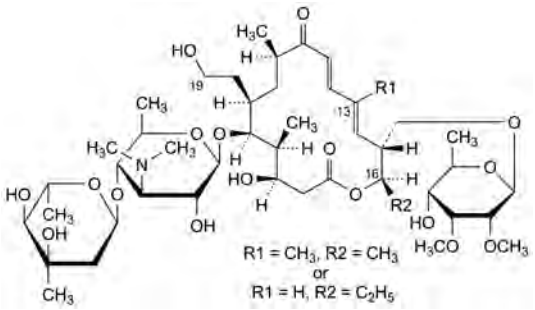


M. 16-deethyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-19-deoxo-19-hydroxytylosin,

N. unknown structure,

O. unknown structure,

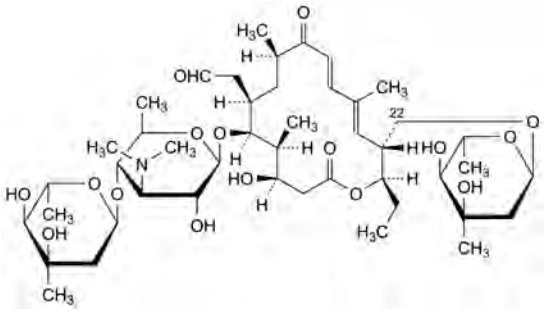

P. 22-*O*-demycinosyl-22-*O*-mycarosyltylosin,

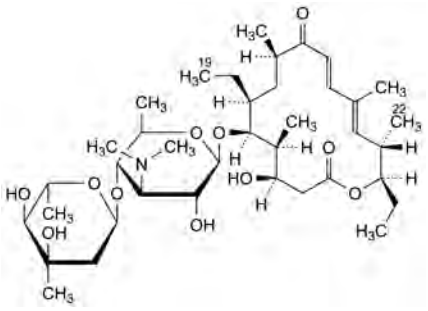

Q. 22-demycinosyloxy-19-deoxotylosin,

R. unknown structure,

S. unknown structure,

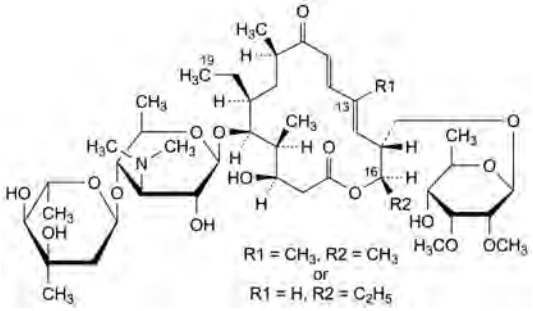


T. 16-deethyl-19-deoxo-16-methyltylosin or 13-demethyl-19-deoxotylosin,

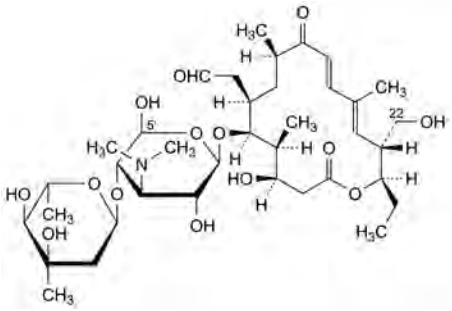

U. 5′-demethyl-22-*O*-demycinosyl-5′-hydroxytylosin,

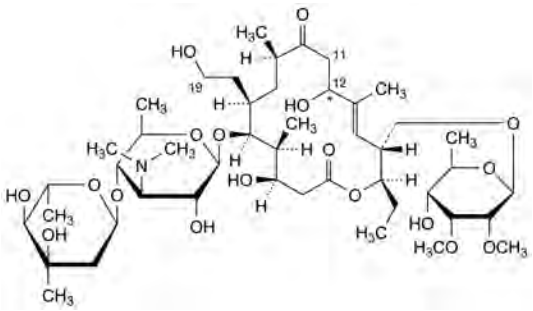

V. 19-deoxo-12,19-dihydroxy-11,12-dihydrotylosin,

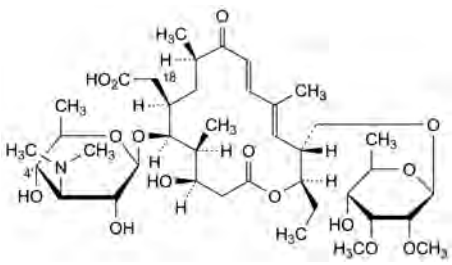

W. 18-carboxy-18-deformyl-4′-*O*-demycarosyltylosin,

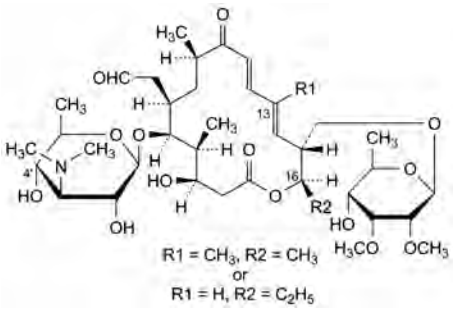


X. 16-deethyl-4′-*O*-demycarosyl-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyltylosin,

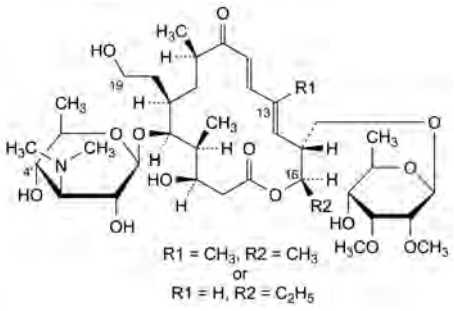


Y. 16-deethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxy-16-methyltylosin or 13-demethyl-4′-*O*-demycarosyl-19-deoxo-19-hydroxytylosin,

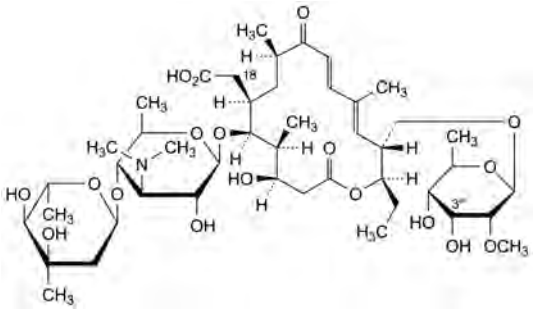

Z. 18-carboxy-18-deformyl-3‴-*O*-demethyltylosin,

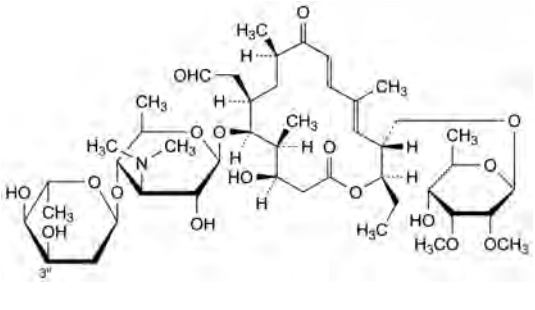

AA. 3″-demethyltylosin,

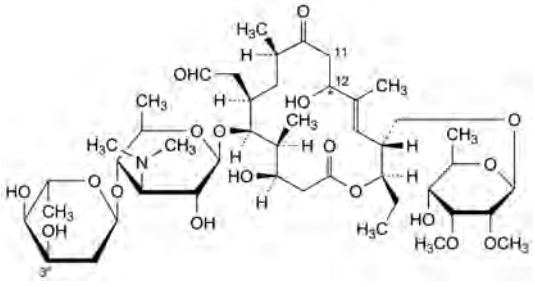

BB. 3″-demethyl-12-hydroxy-11,12-dihydrotylosin,

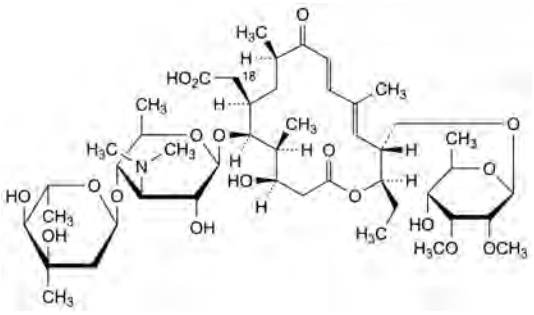

CC. 18-carboxy-18-deformyltylosin,

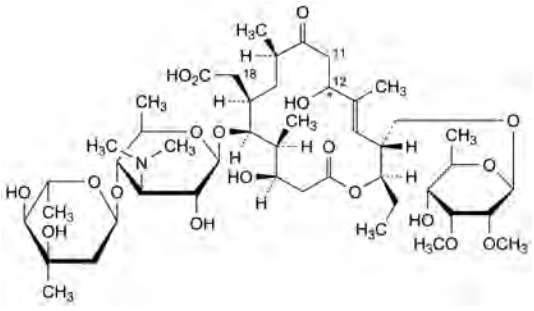

DD. 18-carboxy-18-deformyl-12-hydroxy-11,12-dihydrotylosin,

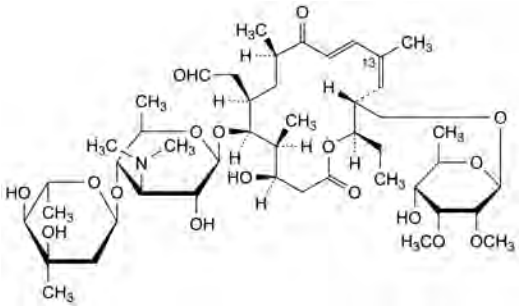

EE. (13*Z*)-tylosin,

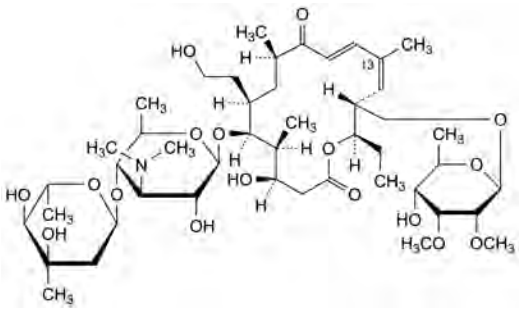

FF. (13*Z*)-19-deoxo-19-hydroxytylosin ((13*Z*)-relomycin),

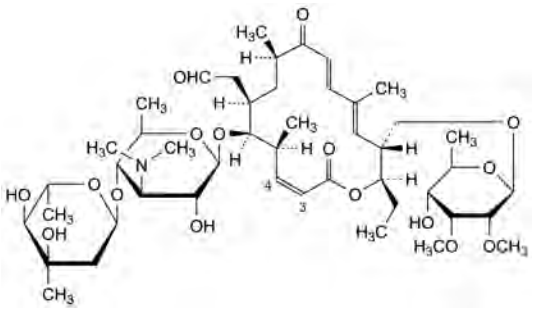

GG. 4-dehydroxy-3,4-didehydrotylosin,

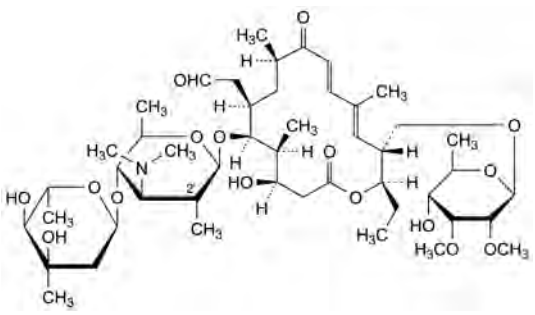

HH. 2′-deoxy-2′-methyltylosin,

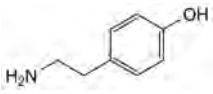

II. 4-(2-aminoethyl)phenol (tyramine).

**01/2017:1161**

# TYROSINE

## Tyrosinum

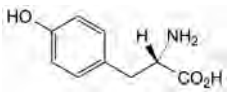

$C_9H_{11}NO_3$ $M_r$ 181.2
[60-18-4]

DEFINITION

(2*S*)-2-Amino-3-(4-hydroxyphenyl)propanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: very slightly soluble in water, practically insoluble in ethanol (96 per cent). It dissolves in dilute mineral acids and in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D, E.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *tyrosine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in 1 mL of *dilute ammonia R2* and dilute to 50 mL with *water R.*
*Reference solution.* Dissolve 10 mg of *tyrosine CRS* in 1 mL of *dilute ammonia R2* and dilute to 50 mL with *water R.*
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *concentrated ammonia R1*, *propanol R* (30:70 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

D. To about 50 mg add 1 mL of *dilute nitric acid R*. A dark red colour is produced within 15 min.

E. Dissolve about 30 mg in 2 mL of *dilute sodium hydroxide solution R*. Add 3 mL of a freshly prepared mixture of equal volumes of a 100 g/L solution of *sodium nitrite R* and a solution of 0.5 g of *sulfanilic acid R* in a mixture of 6 mL of *hydrochloric acid R1* and 94 mL of *water R*. An orange-red colour is produced.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method II*).

Dissolve 0.5 g in *dilute hydrochloric acid R* and dilute to 20 mL with the same acid.

**Specific optical rotation** (*2.2.7*): – 12.3 to – 11.0 (dried substance).

Dissolve 1.25 g in a mixture of equal volumes of *dilute hydrochloric acid R* and *water R* and dilute to 25.0 mL with the same mixture of solvents.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution.* Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b).* Dissolve 30.0 mg of *phenylalanine R* (impurity A) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c).* Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (d).* Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (e).* Dissolve 30 mg of *isoleucine R* and 30 mg of *leucine R* in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (e):

– *resolution*: minimum 1.5 between the peaks due to isoleucine and leucine.

*Calculation of percentage contents*:

– for impurity A, use the concentration of impurity A in reference solution (b);

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of tyrosine in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (c); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *impurity A at 570 nm*: maximum 0.5 per cent;

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 0.6 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dissolve 0.25 g in 3 mL of *dilute nitric acid R* and dilute to 15 mL with *water R*. The solution, without further addition of nitric acid, complies with the test.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve, with gentle heating, 0.5 g in 5 mL of *dilute hydrochloric acid R* and dilute to 15 mL with *distilled water R*.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (d) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (d) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.150 g in 5 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 18.12 mg of $C_9H_{11}NO_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

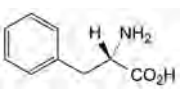


A. (2*S*)-2-amino-3-phenylpropanoic acid (phenylalanine),

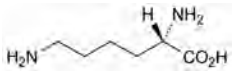

B. (2*S*)-2,6-diaminohexanoic acid (lysine),

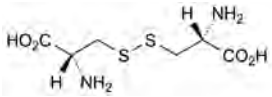

C. 3,3′-disulfanediylbis[(2*R*)-2-aminopropanoic acid] (cystine).

**01/2008:1662**

# TYROTHRICIN

## Tyrothricinum

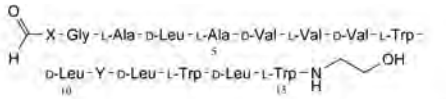

| Gramicidin | Mol. formula | $M_r$ | X | Y |
|---|---|---|---|---|
| A1 | $C_{99}H_{140}N_{20}O_{17}$ | 1882 | L-Val | L-Trp |
| A2 | $C_{100}H_{142}N_{20}O_{17}$ | 1896 | L-Ile | L-Trp |
| C1 | $C_{97}H_{139}N_{19}O_{18}$ | 1859 | L-Val | L-Tyr |
| C2 | $C_{98}H_{141}N_{19}O_{18}$ | 1873 | L-Ile | L-Tyr |

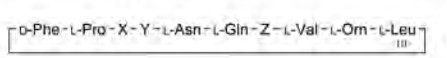

| Tyrocidin | Mol. formula | $M_r$ | X | Y | Z |
|---|---|---|---|---|---|
| A | $C_{66}H_{88}N_{13}O_{13}$ | 1271 | L-Phe | D-Phe | L-Tyr |
| B | $C_{68}H_{89}N_{14}O_{13}$ | 1311 | L-Trp | D-Phe | L-Tyr |
| C | $C_{70}H_{90}N_{15}O_{13}$ | 1350 | L-Trp | D-Trp | L-Tyr |
| D | $C_{72}H_{91}N_{16}O_{12}$ | 1373 | L-Trp | D-Trp | L-Trp |
| E | $C_{66}H_{88}N_{13}O_{12}$ | 1255 | L-Phe | D-Phe | L-Phe |

### DEFINITION

Mixture of antimicrobial linear and cyclic polypeptides, isolated from the fermentation broth of *Brevibacillus brevis* Dubos. It consists mainly of gramicidins and tyrocidins as described above; other related compounds may be present in smaller amounts.

*Potency*: 180 IU/mg to 280 IU/mg (dried substance).

### CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, soluble in ethanol (96 per cent) and in methanol.

### IDENTIFICATION

*First identification*: B.

*Second identification*: A.

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 5 mg of the substance to be examined in 4.0 mL of *ethanol (96 per cent) R*.

*Reference solution.* Dissolve 5 mg of *tyrothricin CRS* in 4.0 mL of *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *methanol R*, *butanol R*, *water R*, *acetic acid R*, *butyl acetate R* (2.5:7.5:12:20:40 *V/V/V/V/V*).

*Application*: 1 µL.

*Development*: over 2/3 of the plate.

*Drying*: in a current of warm air.

*Detection A*: examine in ultraviolet light at 254 nm.

*Results A*: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

*Detection B*: spray with *dimethylaminobenzaldehyde solution R2*. Heat the plate in a current of warm air until the spots appear.

*System suitability*: reference solution:

– the chromatogram shows 2 clearly separated spots or groups of spots.

*Results B*: the principal spots or groups of principal spots in the chromatogram obtained with the test solution are similar in position, colour and size to the principal spots or groups of principal spots in the chromatogram obtained with the reference solution. The upper group corresponds to gramicidins, the lower group to tyrocidins.

B. Composition (see Tests).

### TESTS

**Composition**. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 5 mg of the substance to be examined in 2 mL of *methanol R* and dilute to 5.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *tyrothricin CRS* in 2 mL of *methanol R* and dilute to 5.0 mL with the mobile phase.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm),
– *temperature*: 60 °C.

*Mobile phase*: 0.79 g/L solution of *ammonium sulfate R*, *methanol R* (25:75 *V/V*).

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 25 µL.

*Run time*: 6 times the retention time of gramicidin A1. Use the chromatogram obtained with reference solution (a) and the chromatogram supplied with *tyrothricin CRS* to identify the peaks due to gramicidin A1, gramicidin A2 and the tyrocidins.

*Relative retention* with reference to gramicidin A1 (retention time = about 10 min): gramicidin C1 = about 0.8; gramicidin C2 = about 0.9; gramicidin A2 = about 1.1; tyrocidins = about 1.5 to 6.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 3.0, where $H_p$ = height above the baseline of the peak due to gramicidin A2 and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to gramicidin A1.

*Limits*:

– *sum of gramicidins*: 25 per cent to 50 per cent,
– *sum of tyrocidins*: 50 per cent to 70 per cent,
– *total*: minimum 85 per cent,
– *disregard limit*: the sum of the areas of the peaks due to gramicidins in the chromatogram obtained with reference solution (b).

**Loss on drying** (*2.2.32*): maximum 4.0 per cent, determined on 1.000 g by drying under high vacuum at 60 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 1.5 per cent, determined on 1.0 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*) using the turbidimetric method. Use *gramicidin CRS* as the reference substance.

*Test solution*. Prepare a solution of tyrothricin containing about the same amount of gramicidin as the corresponding solution of *gramicidin CRS* i.e. 5 times more concentrated.

STORAGE

In an airtight container, protected from light.

# U

Ubidecarenone.. ..... 4147
Undecylenic acid..... 4148
Urea.. ..... 4149
Urofollitropin.. ..... 4149
Urokinase.. ..... 4151
Ursodeoxycholic acid .. ..... 4152

**07/2016:1578**

# UBIDECARENONE

## Ubidecarenonum

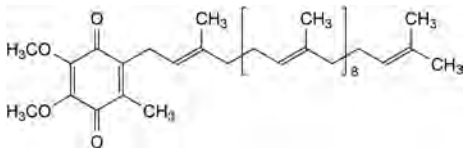

$C_{59}H_{90}O_4$ $M_r$ 863
[303-98-0]

DEFINITION

2-[(*all-E*)-3,7,11,15,19,23,27,31,35,39-Decamethyltetraconta-2,6,10,14,18,22,26,30,34,38-decaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: yellow or orange, hygroscopic, crystalline powder.

*Solubility*: practically insoluble in water, soluble in acetone, very slightly soluble in anhydrous ethanol.

It gradually decomposes and darkens on exposure to light.

mp: about 48 °C.

*Carry out all operations protected from light.*

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ubidecarenone CRS*.

B. Examine the chromatograms obtained in the assay.
*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time and size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 25.0 mL of *anhydrous ethanol R* by heating at about 50 °C for 2 min. Allow to cool.

*Reference solution (a).* Dissolve 25.0 mg of *ubidecarenone CRS* in 25.0 mL of *anhydrous ethanol R* by heating at about 50 °C for 2 min. Allow to cool.

*Reference solution (b).* Dissolve 2 mg of *ubidecarenone impurity D CRS* in 2.0 mL of the test solution by heating at about 50 °C for 2 min. Allow to cool. Dilute 1.0 mL of this solution to 50.0 mL with *anhydrous ethanol R*.

*Reference solution (c).* Dilute 1.0 mL of the test solution to 100.0 mL with *anhydrous ethanol R*. Dilute 5.0 mL of this solution to 10.0 mL with *anhydrous ethanol R*.

*Column*:
- *size*: *l* = 0.2 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *anhydrous ethanol R*, *methanol R2* (20:80 *V/V*).

*Flow rate*: 1.7 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 10 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of ubidecarenone.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to ubidecarenone (retention time = about 14 min): impurity D = about 0.7.

*System suitability*: reference solution (b):
- *resolution*: minimum 6.5 between the peaks due to impurity D and ubidecarenone.

*Calculation of percentage contents*:
- for each impurity, use the concentration of ubidecarenone in reference solution (c).

*Limits*:
- *impurity D*: maximum 0.3 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.6 per cent,
- *reporting threshold*: 0.05 per cent.

**Impurity F**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light.*

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 25.0 mL of *hexane R*.

*Reference solution (a).* Dissolve the contents of a vial of *ubidecarenone for system suitability CRS* (containing impurity F) in 1.0 mL of *hexane R*.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with *hexane R*.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *silica gel for chromatography R* (7 µm).

*Mobile phase*: *ethyl acetate R*, *hexane R* (3:97 *V/V*).

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 275 nm.

*Injection*: 20 µL.

*Run time*: 1.2 times the retention time of ubidecarenone.

*Identification of impurities*: use the chromatogram supplied with *ubidecarenone for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity F.

*Relative retention* with reference to ubidecarenone (retention time = about 10 min): impurity F = about 0.85.

*System suitability*: reference solution (a):
- *resolution*: minimum 1.5 between the peaks due to impurity F and ubidecarenone.

*Limit*:
- *impurity F*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Water** (*2.5.12*): maximum 0.2 per cent, determined on 3.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{59}H_{90}O_4$ taking into account the assigned content of *ubidecarenone CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: D, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, E, G.*

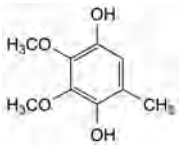

A. 2,3-dimethoxy-5-methylbenzene-1,4-diol,

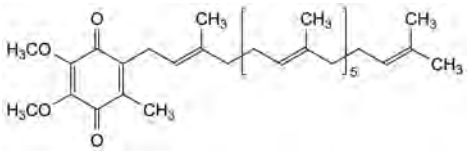

B. 2-[(*all-E*)-3,7,11,15,19,23,27-heptamethyloctadocosa-2,6,10,14,18,22,26-heptaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione (ubiquinone-7),

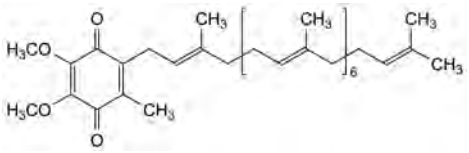

C. 5,6-dimethoxy-3-methyl-2-[(*all-E*)-3,7,11,15,19,23,27,31-octamethyldotriaconta-2,6,10,14,18,22,26,30-octaenyl]-benzene-1,4-dione (ubiquinone-8),

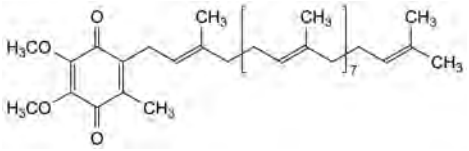

D. 5,6-dimethoxy-3-methyl-2-[(*all-E*)-3,7,11,15,19,23,27,31,35-nonamethylhexatriaconta-2,6,10,14,18,22,26,30,34-nonaenyl]benzene-1,4-dione (ubiquinone-9),

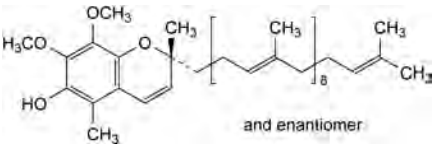


E. (2*RS*)-7,8-dimethoxy-2,5-dimethyl-2-[(*all-E*)-4,8,12,16,20,24,28,32,36-nonamethylheptatriaconta-3,7,11,15,19,23,27,31,35-nonaenyl]-2*H*-1-benzopyran-6-ol (ubicromenol),

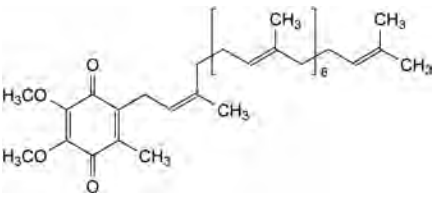

F. 2-[(2*Z*,6*E*,10*E*,14*E*,18*E*,22*E*,26*E*,30*E*,34*E*)-3,7,11,15,19,23,27,31,35,39-decamethyltetraconta-2,6,10,14,18,22,26,30,34,38-decaenyl]-5,6-dimethoxy-3-methylbenzene-1,4-dione (ubidecarenone (*Z*)-isomer),

G. unknown structure.

**01/2008:0461**

# UNDECYLENIC ACID

## Acidum undecylenicum

$C_{11}H_{20}O_2$ $M_r$ 184.3
[112-38-9]

DEFINITION

Undec-10-enoic acid.

*Content*: 97.0 per cent to 102.0 per cent.

CHARACTERS

*Appearance*: white or very pale yellow, crystalline mass or colourless or pale yellow liquid.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent) and in fatty and essential oils.

IDENTIFICATION

A. Refractive index (*2.2.6*): 1.447 to 1.450, determined at 25 ± 0.5 °C.

B. Freezing point (*2.2.18*): 21 °C to 24 °C.

C. To 2.0 g add 2 mL of freshly distilled *aniline R* and boil under a reflux condenser for 10 min. Allow to cool and add 30 mL of *ether R*. Shake with 3 quantities, each of 20 mL, of *dilute hydrochloric acid R* and then with 20 mL of *water R*. Evaporate the organic layer to dryness on a water-bath. After recrystallising twice from *ethanol (70 per cent V/V) R* and drying *in vacuo* for 3 h, the residue melts (*2.2.14*) at 66 °C to 68 °C.

D. Dissolve 0.1 g in a mixture of 2 mL of *dilute sulfuric acid R* and 5 mL of *glacial acetic acid R*. Add dropwise 0.25 mL of *potassium permanganate solution R*. The colour of the potassium permanganate is discharged.

TESTS

**Peroxide value** (*2.5.5, Method A*): maximum 10.

**Fixed and mineral oils**. To 1.0 g add 5 mL of *sodium carbonate solution R* and 25 mL of *water R* and boil for 3 min. The hot solution is not more opalescent than reference suspension II (*2.2.1*).

**Water-soluble acids**. To 1.0 g add 20 mL of *water R* heated to 35-45 °C and shake for 2 min. Cool and filter the aqueous layer through a moistened filter. To 10 mL of the filtrate add 0.1 mL of *phenolphthalein solution R*. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Degree of unsaturation**. Dissolve 85.0 mg in a mixture of 5 mL of *dilute hydrochloric acid R* and 30 mL of *glacial acetic acid R*. Using 0.05 mL of *indigo carmine solution R1* as indicator, added towards the end of the titration, titrate with *0.0167 M bromide-bromate* until the colour changes from blue to yellow. 8.9 mL to 9.4 mL of *0.0167 M bromide-bromate* is required. Carry out a blank titration.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 0.50 g.

ASSAY

Dissolve 0.750 g in 10 mL of *ethanol (96 per cent) R*. Titrate with *0.5 M sodium hydroxide* using 0.1 mL of *phenolphthalein solution R* as indicator, until a pink colour is obtained.

1 mL of *0.5 M sodium hydroxide* is equivalent to 92.14 mg of $C_{11}H_{20}O_2$.

STORAGE

In a non-metallic container, protected from light.

**01/2017:0743**

# UREA

## Ureum

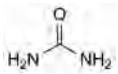

$CH_4N_2O$ $M_r$ 60.1

[57-13-6]

DEFINITION

Carbamide.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or transparent crystals, slightly hygroscopic.

*Solubility*: very soluble in water, soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C, D.*

A. Melting point (*2.2.14*): 132 °C to 135 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *urea CRS*.

C. Dissolve 0.1 g in 1 mL of *water R*. Add 1 mL of *nitric acid R*. A white, crystalline precipitate is formed.

D. Heat 0.5 g in a test tube until it liquefies and the liquid becomes turbid. Cool, dissolve in a mixture of 1 mL of *dilute sodium hydroxide solution R* and 10 mL of *water R* and add 0.05 mL of *copper sulfate solution R*. A reddish-violet colour is produced.

TESTS

**Solution S**. Dissolve 10.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

To 2.5 mL of solution S add 7.5 mL of *water R*.

**Alkalinity**. To 2.5 mL of solution S add 7.5 mL of *water R*, 0.1 mL of *methyl red solution R* and 0.4 mL of *0.01 M hydrochloric acid*. The solution is red to orange.

**Biuret**: maximum 0.1 per cent.

To 10 mL of solution S add 5 mL of *water R*, 0.5 mL of a 5 g/L solution of *copper sulfate pentahydrate R* and 0.5 mL of *strong sodium hydroxide solution R*. Allow to stand for 5 min. Any reddish-violet colour in the solution is not more intense than that in a standard prepared at the same time in the same manner using 10 mL of a 0.2 g/L solution of *biuret R*.

**Ammonium** (*2.4.1*): maximum 500 ppm, determined on 0.1 mL of solution S.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.2000 g in *water R* and dilute to 50.0 mL with the same solvent. Introduce 1.0 mL of the solution into a combustion flask. Add 4 g of a powdered mixture of 100 g of *dipotassium sulfate R*, 5 g of *copper sulfate pentahydrate R* and 2.5 g of *selenium R*, and 3 glass beads. Wash any adhering particles from the neck into the flask with 5 mL of *sulfuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; take precautions to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of *water R*, cool again and place in a steam-distillation apparatus. Add 30 mL of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect the distillate in 15 mL of a 40 g/L solution of *boric acid R* to which has been added 0.2 mL of *methyl red mixed solution R* and enough *water R* to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with *0.01 M sulfuric acid*.

1 mL of *0.01 M sulfuric acid* is equivalent to 0.6006 mg of $CH_4N_2O$.

STORAGE

In an airtight container.

**01/2020:0958**

# UROFOLLITROPIN

## Urofollitropinum

[97048-13-0]

DEFINITION

Dried preparation containing menopausal gonadotrophin obtained from the urine of post-menopausal women. It has follicle-stimulating activity and no or virtually no luteinising activity.

*Potency*: minimum 90 IU of follicle-stimulating hormone (hFSH) per milligram.

*Ratio of the number of units of luteinising hormone (interstitial-cell-stimulating hormone) [hLH(ICSH)] to the number of units of follicle-stimulating hormone:* maximum 1/60.

PRODUCTION

It is prepared by suitable collection and extraction procedures followed by purification steps.

The method of preparation includes steps that have been shown to remove and/or inactivate extraneous agents. In addition, the process is designed to minimise microbial contamination.

CHARACTERS

*Appearance*: almost white or slightly yellowish powder.

*Solubility*: soluble in water.

IDENTIFICATION

When administered to immature female rats as prescribed in the assay, it causes enlargement of the ovaries.

TESTS

**Residual luteinising activity**. The International Units of FSH and LH are the activities contained in stated amounts of the International Standard of human urinary follicle-stimulating

hormone and luteinising hormone (interstitial-cell-stimulating hormone) which consists of a mixture of a freeze-dried extract of urine of post-menopausal women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats approximately 21 days old and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 4 equal groups of at least 6 animals. If sets of 4 litter mates are available, assign one litter mate at random from each set to each group and mark according to litter.

Inject subcutaneously into each rat 50 IU of *serum gonadotrophin R* on the first day and 25 IU of *chorionic gonadotrophin R* on the fourth day, each in 0.5 mL of *phosphate-albumin buffered saline pH 7.2 R*.

Choose 3 doses of the reference preparation such that the smallest dose produces a depletion of the ovarian ascorbic acid content in all the rats and the largest dose does not produce a maximal depletion in all the rats. Use doses in geometric progression; as an initial approximation, total doses of 0.5 IU, 1.0 IU and 2.0 IU may be tried although the dose to be used will depend on the sensitivity of the animals.

Choose a dose of the preparation to be examined expected to contain 60 *X* IU of follicle-stimulating hormone (hFSH), in which *X* = the number of International Units of hLH in the middle dose of the reference preparation.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation in 1.0 mL of *phosphate-albumin buffered saline pH 7.2 R*. Inject into a tail vein to each separate group of rats 6 days after the injection of chorionic gonadotrophin. Exactly 4 h after the injection, euthanise the rats and remove the ovaries from each animal. Remove any extraneous fluid and tissue from the ovaries and weigh the ovaries immediately.

Treat the combined ovaries from each rat separately, as follows. Crush and homogenise within 2 min in a freshly prepared 25 g/L solution of *metaphosphoric acid R* at a temperature of 4 °C and dilute to 7 mL with the same solution. Allow the homogenate to stand for 30 min at 4 °C and centrifuge at 4 °C at approximately 2500 *g*. Filter the supernatant, if necessary, through a 0.22 µm filter.

Prepare a fresh solution consisting of a mixture of 2 mL of a 45.3 g/L solution of *sodium acetate R* adjusted to pH 7 with *acetic acid R*, 3 mL of *water R* and 2 mL of *dichlorophenolindophenol standard solution R*. Mix 2 mL of this solution with 2 mL of the clear supernatant. 30 s after mixing, measure the absorbance (*2.2.25*) of the solution at the maximum at about 520 nm. Use as reference a solution with a known content of *ascorbic acid CRS* in a 25 g/L solution of *metaphosphoric acid R*, treated by the same process.

Calculate the amount of ascorbic acid from the ascorbic acid standard curve obtained and express in milligrams per 0.1 g of ovary to obtain the ascorbic acid content of the ovaries. Calculate the mean and its variance of the ascorbic acid content of the ovaries of the rats treated with the preparation to be examined.

For each dose-group of the reference preparation, plot the mean ascorbic acid content of the ovaries as a function of the logarithm of the dose and analyse the regression of the ascorbic acid content on the logarithm of the dose injected, using standard methods of analysis (the method of least squares).

The test is not valid unless:

- the slope constant *b* is significant at the 0.05 significance level;
- for the groups treated with the reference preparation, the sum of squares due to linear regression is equal to at least 95 per cent of the total sum of squares of the ascorbic acid content;
- the within-group variance of the ascorbic acid content of the group receiving the preparation to be examined is not significantly different at the 0.05 significance level from the within-group variance of the ascorbic acid content of the groups receiving the reference preparation.

The mean ascorbic acid content of the ovaries of the rats treated with the preparation to be examined is not significantly lower than that of the rats treated with the middle dose of the reference preparation (calculated from the regression equation) at the 0.05 significance level.

**Water** (*2.5.32*): maximum 5.0 per cent.

**Bacterial endotoxins** (*2.6.14, Method C*): less than 0.40 IU per International Unit of urofollitropin, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

The follicle-stimulating activity of urofollitropin is estimated by comparing under given conditions its effect in enlarging the ovaries of immature rats treated with chorionic gonadotrophin with the same effect of the International Standard preparation of human urinary follicle-stimulating hormone and luteinising hormone or of a reference preparation calibrated in International Units. The International Units of FSH and LH are the activities contained in stated amounts of the International Standard of human urinary follicle-stimulating hormone and luteinising hormone (interstitial-cell-stimulating hormone) which consists of a mixture of a freeze-dried extract of urine of post-menopausal women with lactose. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Use immature female rats of the same strain, 19-28 days old, differing in age by not more than 3 days and having masses such that the difference between the heaviest and the lightest rat is not more than 10 g. Assign the rats at random to 6 equal groups of at least 5 animals. If sets of 6 litter mates are available, assign one litter mate from each set to each group and mark according to litter.

Choose 3 doses of the reference preparation and 3 doses of the preparation to be examined such that the smallest dose produces a positive response in some of the rats and the largest dose does not produce a maximal response in all the rats. Use doses in geometric progression and as an initial approximation total doses of 1.5 IU, 3.0 IU and 6.0 IU may be tried although the dose will depend on the sensitivity of the animals used, which may vary widely.

Dissolve separately the total quantities of the preparation to be examined and of the reference preparation corresponding to the daily doses to be used in sufficient *phosphate-albumin buffered saline pH 7.2 R* such that the daily dose is administered in a volume of about 0.5 mL. The buffer solution shall contain in the daily dose not less than 14 IU of chorionic gonadotrophin to ensure complete luteinisation. Add a suitable antimicrobial preservative such as 4 g/L of phenol or 0.02 g/L of thiomersal. Store the solutions at 5 ± 3 °C.

Inject subcutaneously into each rat the daily dose allocated to its group. Repeat the injection of each dose 24 h and 48 h after the first injection. About 24 h after the last injection, euthanise the rats and remove the ovaries from each animal. Remove any extraneous fluid and tissue from the ovaries and weigh the 2 combined ovaries of each animal immediately. Calculate the results by the usual statistical methods (for example, *5.3*) using the mass of the 2 combined ovaries as the response (a suitable correction of the organ mass with reference to the mass of the animal from which it was taken may be applied; an analysis of covariance may be used).

The estimated potency is not less than 80 per cent and not more than 125 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 64 per cent and not more than 156 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature of 2 °C to 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states:

- the number of International Units of follicle-stimulating hormone per container;
- the potency, in International Units of follicle-stimulating hormone per milligram.

**01/2020:0695**

# UROKINASE

## Urokinasum

[9039-53-6]

DEFINITION

Enzyme, obtained from human urine, that activates plasminogen. It consists of a mixture of low-molecular-mass (LMM) ($M_r$ 33 000) and high-molecular-mass (HMM) ($M_r$ 54 000) forms, the high-molecular-mass form being predominant.

*Potency*: minimum 70 000 IU per milligram of protein.

PRODUCTION

It is prepared by suitable collection and extraction procedures followed by purification steps.

The method of preparation includes steps that have been shown to remove and/or inactivate extraneous agents. In addition, the process is designed to minimise microbial contamination and vasoactive substances.

CHARACTERS

*Appearance*: white or almost white, amorphous powder.

*Solubility*: soluble in water.

IDENTIFICATION

A. Place separately in 2 haemolysis tubes 0.5 mL of citrated human plasma and 0.5 mL of citrated bovine plasma and maintain in a water-bath at 37 °C. To each tube add 0.1 mL of a solution containing a quantity of the substance to be examined equivalent to 1000 IU/mL in *phosphate buffer solution pH 7.4 R* and 0.1 mL of a solution containing a quantity of *human thrombin R* equivalent to 20 IU/mL in *phosphate buffer solution pH 7.4 R*. Shake immediately. In both tubes, a clot forms and lyses within 30 min.

B. Carry out identification by a suitable immunodiffusion test.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 10 mg in 10 mL of *water R*.

**Thromboplastic contaminants**

*Test solutions.* Dissolve suitable quantities of the substance to be examined in *barbital buffer solution pH 7.4 R* to obtain solutions with activities of 5000 IU/mL, 2500 IU/mL, 1250 IU/mL, 625 IU/mL and 312 IU/mL.

To each of 6 haemolysis tubes 1 cm in internal diameter add 0.1 mL of *citrated rabbit plasma R*. Allocate the test solutions 1 to each of 5 of the tubes; add to each tube 0.1 mL of the solution allocated to it and to the 6th tube add 0.1 mL of *barbital buffer solution pH 7.4 R* (blank). Incubate the tubes at 25 ± 0.5 °C for 5 min and add 0.1 mL of a 3.675 g/L solution of *calcium chloride R*. Measure with a stop-watch the coagulation time for each tube. Plot the shortening of the recalcification time (clotting time of the blank minus clotting time measured) against log concentration in International Units. Extrapolate the best-fitting straight line through the 5 points until it reaches the log-concentration axis. The urokinase activity at the intersection point, which represents the limit concentration for coagulant activity (zero coagulant activity), is not less than 150 IU/mL.

**Molecular fractions**. Size-exclusion chromatography (*2.2.30*).

*Test solution.* Dissolve about 1 mg in 1.0 mL of *0.02 M phosphate buffer solution pH 8.0 R*. Prepare immediately before use.

*Column*:

- *size*: *l* = 0.9 m, Ø = 16 mm;
- *stationary phase*: *cross-linked dextran for chromatography R3*;
- *temperature*: 5 °C.

*Mobile phase*: 17.5 g/L solution of *sodium chloride R* in *0.02 M phosphate buffer solution pH 8.0 R*.

*Flow rate*: 0.1 mL/min.

Apply the test solution to the head of the column rinsing twice with 0.5 mL portions of the buffer and carry out the elution. The eluate may be collected in fractions of 1 mL. Measure the absorbance (*2.2.25*) of the eluate at the maximum at 280 nm and plot the individual values on a graph. Draw perpendicular lines towards the axis of the abscissae from the minima before the HMM peak, between the HMM and the LMM peaks, and after the LMM peak, thus identifying the fractions to be considered in calculating the HMM/LMM activity ratio. Pool the HMM fractions and, separately, the LMM fractions. Determine separately the urokinase activity in International Units of each of the fraction pools by the method prescribed under Assay. The ratio of the urokinase activity in the HMM fraction pool to that in the LMM fraction pool is not less than 2.0.

**Total protein**. Determine the nitrogen content, using 10 mg, by the method of sulfuric acid digestion (*2.5.9*) and calculate the quantity of protein by multiplying by 6.25.

ASSAY

The potency of urokinase is determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of urokinase calibrated in International Units; the formation of plasmin is measured by the determination of the lysis time of a fibrin clot under given conditions.

The International Unit is the activity contained in a stated amount of the International Reference Preparation, which consists of freeze-dried urokinase with lactose. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

Unless otherwise prescribed, use *phosphate buffer solution pH 7.4 R* containing 30 g/L of *bovine albumin R* for the preparation of the solutions and dilutions used in the assay.

*Test solution.* Prepare a solution of the substance to be examined expected to have an activity of 1000 IU/mL.

*Reference solution.* Prepare a solution of a reference preparation having an activity of 1000 IU/mL.

Keep the test solution and the reference solution in iced water and use within 6 h. Prepare 3 serial 1.5-fold dilutions of the reference preparation such that the longest clot-lysis time is less than 20 min and the shortest clot-lysis time is greater than 3 min. Prepare 3 similar dilutions of the test solution. Keep the solutions in iced water and use within 1 h. Use 24 tubes 8 mm in diameter. Label the tubes $T_1$, $T_2$ and $T_3$ for the dilutions of the test solution and $S_1$, $S_2$ and $S_3$ for the dilutions of the reference solution, allocating 4 tubes to each dilution. Place the tubes in iced water. Into each tube, introduce 0.2 mL

of the appropriate dilution, 0.2 mL of *phosphate buffer solution pH 7.4 R* containing 30 g/L of *bovine albumin R* and 0.1 mL of a solution of *human thrombin R* having an activity of not less than 20 IU/mL. Place the tubes in a water-bath at 37 °C and allow to stand for 2 min to attain temperature equilibrium. Using an automatic pipette, introduce into the bottom of the 1st tube 0.5 mL of a 10 g/L solution of *bovine euglobulins R*, ensuring mixing. At intervals of 5 s, introduce successively into the remaining tubes 0.5 mL of a 10 g/L solution of *bovine euglobulins R*. Using a stop-watch, measure for each tube the time in seconds that elapses between the addition of the euglobulins solution and the lysis of the clot. Plot the logarithms of the lysis times for the substance to be examined and for the reference preparation against the logarithms of the concentration and calculate the activity of the substance to be examined using the usual statistical methods.

The estimated potency is not less than 90 per cent and not more than 111 per cent of the stated potency. The confidence limits ($P$ = 0.95) of the estimated potency are not less than 80 per cent and not more than 125 per cent of the stated potency.

STORAGE

In an airtight container, protected from light, at a temperature not exceeding 8 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

LABELLING

The label states the potency in International Units per milligram of protein.

**01/2017:1275**

# URSODEOXYCHOLIC ACID

## Acidum ursodeoxycholicum

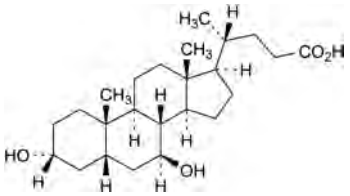

$C_{24}H_{40}O_4$ $M_r$ 392.6
[128-13-2]

DEFINITION

3α,7β-Dihydroxy-5β-cholan-24-oic acid.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in ethanol (96 per cent), slightly soluble in acetone, practically insoluble in methylene chloride.

mp: about 202 °C.

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ursodeoxycholic acid CRS.*

B. Examine the chromatograms obtained in the test for impurity C.
*Results*: the principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 10 mg in 1 mL of *sulfuric acid R*. Add 0.1 mL of *formaldehyde solution R* and allow to stand for 5 min. Add 5 mL of *water R*. The suspension obtained is greenish-blue.

TESTS

**Specific optical rotation** (*2.2.7*): + 58.0 to + 62.0 (dried substance).

Dissolve 0.500 g in *anhydrous ethanol R* and dilute to 25.0 mL with the same solvent.

**Impurity C**. Thin-layer chromatography (*2.2.27*).

*Solvent mixture*: *water R*, *acetone R* (10:90 *V/V*).

*Test solution (a).* Dissolve 0.40 g of the substance to be examined in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Test solution (b).* Dilute 1 mL of test solution (a) to 10 mL with the solvent mixture.

*Reference solution (a).* Dissolve 40 mg of *ursodeoxycholic acid CRS* in the solvent mixture and dilute to 10 mL with the solvent mixture.

*Reference solution (b).* Dissolve 20 mg of *lithocholic acid CRS* (impurity C) in the solvent mixture and dilute to 10.0 mL with the solvent mixture (solution A). Dilute 2.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* To 5 mL of solution A add 10 mg of *chenodeoxycholic acid CRS* (impurity A) and dilute to 50 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *glacial acetic acid R*, *acetone R*, *methylene chloride R* (1:30:60 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: at 120 °C for 10 min.

*Detection*: spray immediately with a 47.6 g/L solution of *phosphomolybdic acid R* in a mixture of 1 volume of *sulfuric acid R* and 20 volumes of *glacial acetic acid R* and heat at 120 °C until blue spots appear on a lighter background.

*System suitability*: reference solution (c):

– the chromatogram shows 2 clearly separated principal spots.

*Limit*: test solution (a):

– *impurity C*: any spot due to impurity C is not more intense than the principal spot in the chromatogram obtained with reference solution (b) (0.1 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, mobile phase (10:90 *V/V*).

*Test solution.* Dissolve 60 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve the contents of a vial of *ursodeoxycholic acid for system suitability CRS* (containing impurities A and H) in 1.0 mL of the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C ± 1 °C.

*Mobile phase*: mix 30 volumes of *acetonitrile R*, 37 volumes of a 0.78 g/L solution of *sodium dihydrogen phosphate R* adjusted to pH 3 with *phosphoric acid R*, and 40 volumes of *methanol R*.

*Flow rate*: 0.8 mL/min.

*Detection*: refractometer at 35 ± 1 °C.

*Injection*: 150 µL.

*Run time*: 4 times the retention time of ursodeoxycholic acid.

*Identification of impurities*: use the chromatogram supplied with *ursodeoxycholic acid for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and H.

*Relative retention* with reference to ursodeoxycholic acid (retention time = about 14 min): impurity H = about 0.9; impurity A = about 2.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity H and ursodeoxycholic acid.

*Limits*:

– *impurity A*: not more than 10 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);

– *total*: not more than 15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (1.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.350 g in 50 mL of *ethanol (96 per cent) R*, previously neutralised to 0.2 mL of *phenolphthalein solution R*. Add 50 mL of *water R* and titrate with *0.1 M sodium hydroxide* until a pink colour is obtained.

1 mL of *0.1 M sodium hydroxide* is equivalent to 39.26 mg of $C_{24}H_{40}O_4$.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, G, H, I.*

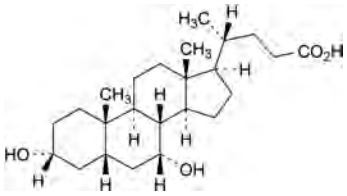

A. 3α,7α-dihydroxy-5β-cholan-24-oic acid (chenodeoxycholic acid),

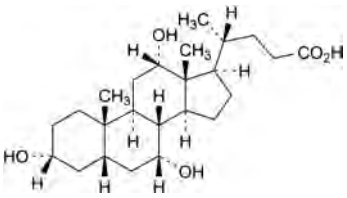

B. 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid (cholic acid),

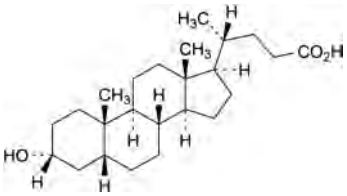

C. 3α-hydroxy-5β-cholan-24-oic acid (lithocholic acid),

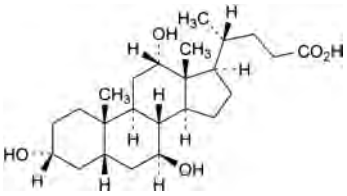

D. 3α,7β,12α-trihydroxy-5β-cholan-24-oic acid (ursocholic acid),

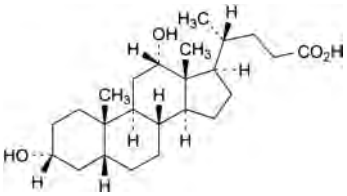

E. 3α,12α-dihydroxy-5β-cholan-24-oic acid (deoxycholic acid),

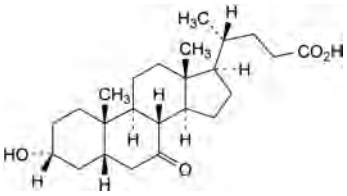

F. 3α-hydroxy-7-oxo-5β-cholan-24-oic acid,

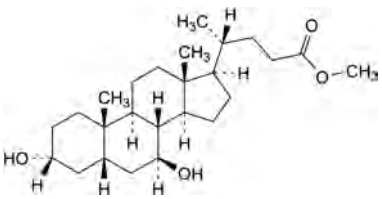

G. methyl 3α,7β-dihydroxy-5β-cholan-24-oate,

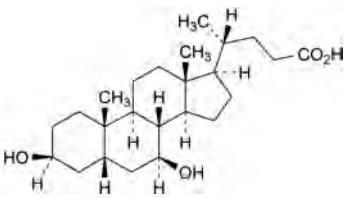

H. 3β,7β-dihydroxy-5β-cholan-24-oic acid,

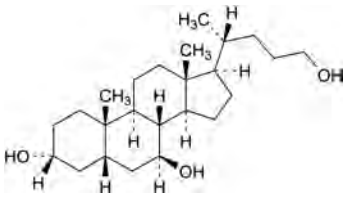


I. 5β-cholane-3α,7β,24-triol.

# V

Valaciclovir hydrochloride.. 4157
Valaciclovir hydrochloride hydrate.. 4160
Valine 4162
Valnemulin hydrochloride for veterinary use 4164
Valproic acid 4165
Valsartan.. 4167
Vancomycin hydrochloride.. 4168
Vanillin 4172
Vardenafil hydrochloride trihydrate 4172
Vecuronium bromide.. 4173
Vedaprofen for veterinary use 4175
Venlafaxine hydrochloride.. 4176
Verapamil hydrochloride.. 4178
Vigabatrin.. 4180
Vinblastine sulfate.. 4181
Vincamine 4182
Vincristine sulfate.. 4183
Vindesine sulfate 4185
Vinorelbine tartrate.. 4186
Vinpocetine.. 4188
Viscose wadding, absorbent.. 4189
Vitamin A.. 4191
Vitamin A concentrate (oily form), synthetic.. 4192
Vitamin A concentrate (powder form), synthetic.. 4193
Vitamin A concentrate (solubilisate/emulsion), synthetic.. 4194
Voriconazole 4196

**07/2019:1768**

# VALACICLOVIR HYDROCHLORIDE

## Valacicloviri hydrochloridum

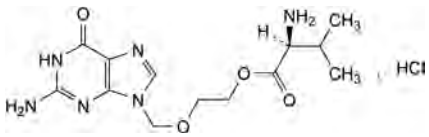

$C_{13}H_{21}ClN_6O_4$ $M_r$ 360.8
[124832-27-5]

DEFINITION

2-[(2-Amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)-methoxy]ethyl L-valinate hydrochloride.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in anhydrous ethanol.

It shows polymorphism (*5.9*).

IDENTIFICATION

Carry out either tests A, B, C, E or tests A, B, D, E.

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *anhydrous valaciclovir hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *anhydrous ethanol R* and evaporate to dryness in a desiccator, under high vacuum, over *diphosphorus pentoxide R*. Record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

C. It complies with the limit for impurity R given in test A for related substances.

D. Optical rotation (*2.2.7*): laevorotatory.

Dissolve 2.50 g in *water R* and dilute to 50.0 mL with the same solvent.

E. Water (see Tests).

TESTS

**Impurities E, F and G**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in 2 mL of *water R* and dilute to 5.0 mL with *ethanol (96 per cent) R*.

*Reference solution (a)*. Dissolve 5 mg of *valaciclovir impurity D CRS*, 5.0 mg of *valaciclovir impurity E CRS*, 5.0 mg of *valaciclovir impurity G CRS* and 8.4 mg of *valaciclovir impurity F para-toluenesulfonate CRS* in a mixture of 2 mL of *water R* and 6 mL of *ethanol (96 per cent) R*, and dilute to 10.0 mL with *ethanol (96 per cent) R*.

*Reference solution (b)*. Dilute 3.0 mL of reference solution (a) to 10.0 mL with *ethanol (96 per cent) R*.

*Reference solution (c)*. Dilute 2.0 mL of reference solution (a) to 10.0 mL with *ethanol (96 per cent) R*.

*Reference solution (d)*. Dilute 0.5 mL of reference solution (a) to 10.0 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Pretreatment*: wash the plate with *methanol R* until the solvent front has migrated over at least 4/5 of the plate; allow the plate to dry.

*Mobile phase*: *concentrated ammonia R*, *tetrahydrofuran R*, *methanol R*, *methylene chloride R* (3:12:34:54 *V/V/V/V*); use freshly prepared mobile phase.

*Application*: 4 µL of the test solution and reference solutions (b), (c) and (d).

*Development*: over 4/5 of the plate.

*Drying*: in a current of air.

*Detection*: examine in ultraviolet light at 254 nm for impurities E and G; spray with a 0.1 g/L solution of *fluorescamine R* in *ethylene chloride R* and examine in ultraviolet light at 365 nm for impurity F.

*Retardation factors*: impurity A = about 0; impurity B = about 0.2; valaciclovir = about 0.3; impurity C = about 0.5; impurity D = about 0.6; impurity E = about 0.7; impurity F = about 0.75; impurity G = about 0.79; impurity C is masked by the leading edge of the spot due to valaciclovir; impurities F and G may co-elute, but this does not adversely affect their quantification because they are visualised differently.

*System suitability*: the chromatograms obtained with reference solutions (b), (c) and (d) each show 3 clearly separated spots when examined under ultraviolet light at 254 nm, due to impurities D, E and G.

*Limits*:

- *impurity E*: any spot due to impurity E is not more intense than the corresponding spot in the chromatogram obtained with reference solution (c) (0.2 per cent);
- *impurity F*: any spot due to impurity F is not more intense than the corresponding spot in the chromatogram obtained with reference solution (b) (0.3 per cent calculated as hydrochloride salt);
- *impurity G*: any spot due to impurity G is not more intense than the corresponding spot in the chromatogram obtained with reference solution (d) (0.05 per cent).

**Related substances**.

A. Impurities A, B, I and R. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution.

*Reference solution (a)*. Dissolve 2.5 mg of *valaciclovir for system suitability CRS* (containing impurities A, B, C, D, H, I, J, M and R) in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 5.0 mL with the same solution.

*Reference solution (b)*. Dissolve 50.0 mg of *anhydrous valaciclovir hydrochloride CRS* in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution.

*Reference solution (c)*. Dilute 3.0 mL of the test solution to 100.0 mL with a 0.5 per cent *V/V* solution of *hydrochloric acid R*. Dilute 1.0 mL of this solution to 100.0 mL with a 0.5 per cent *V/V* solution of *hydrochloric acid R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.0 mm;
- *stationary phase*: *crown-ether silica gel for chiral separation R* (5 µm);
- *temperature*: 10 °C.

*Mobile phase*: *perchloric acid R*, *methanol R*, *water for chromatography R* (0.5:5:95 *V/V/V*).

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 1.5 times the retention time of valaciclovir.

*Identification of impurities*: use the chromatogram supplied with *valaciclovir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A + B, C + R, D, I and M.

*Relative retention* with reference to valaciclovir (retention time = about 21 min): impurities A and B = about 0.2; impurity I = about 0.4; impurities C and R = about 0.6; impurity D = about 0.7; impurity M = about 1.3.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurities C and R.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurities A and B by 0.7;
– *impurity R*: maximum 3.0 per cent; for the calculation, subtract the content of impurity C as determined in test B for related substances from the content of the coeluting impurities C and R as determined in this test;
– *sum of impurities A and B*: maximum 2.0 per cent;
– *impurity I*: maximum 0.2 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.03 per cent); disregard any peaks due to impurities other than A + B, C + R or I.

B. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Use the solutions within 24 h of preparation.*

*Solvent mixture*: *ethanol (96 per cent) R*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 40 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2.5 mg of *valaciclovir for system suitability CRS* (containing impurities A, B, C, D, H, I, J, M and R) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped phenylhexylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 15 °C.

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water for chromatography R* (0.2:100 *V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *methanol R2* (0.2:100 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 35 | 90 → 60 | 10 → 40 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *valaciclovir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, H, I, J and M.

*Relative retention* with reference to valaciclovir (retention time = about 19 min): impurity A = about 0.3; impurity B = about 0.4; impurity H = about 0.5; impurity C = about 1.06; impurity I = about 1.09; impurity D = about 1.2; impurity J = about 1.3; impurity M = about 1.6.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to valaciclovir;
– the chromatogram obtained is similar to the chromatogram supplied with *valaciclovir for system suitability CRS*.

*Limits*:

– *impurity M*: maximum 1.5 per cent;
– *impurity D*: maximum 0.5 per cent;
– *impurity C*: maximum 0.3 per cent;
– *impurities H, J*: for each impurity, maximum 0.10 per cent;
– *unspecified impurities*: for each impurity, maximum 0.05 per cent;
– *disregard limit*: 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent); disregard the peaks due to impurities A, B and I.

*Limit*:

– *total for tests A and B*: maximum 5.0 per cent.

**Chloride**: 9.4 to 9.9 per cent (anhydrous and solvent-free substance).

Dissolve 0.350 g in 100 mL of *water R* and add 0.2 mL of *nitric acid R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M silver nitrate*. Use a silver indicator electrode and a silver-silver chloride reference electrode or a combined silver electrode. Discard the result from the first titration, which is used to condition the electrodes. Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 3.543 mg of Cl.

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{13}H_{21}ClN_6O_4$ taking into account the assigned content of *anhydrous valaciclovir hydrochloride CRS*.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, M, R.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *K, L, N, O, P, Q.*

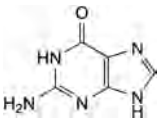


A. 2-amino-1,9-dihydro-6*H*-purin-6-one (guanine),

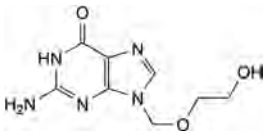

B. 2-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one (aciclovir),

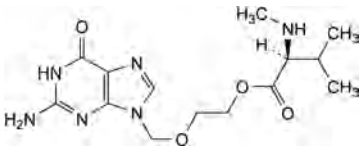

C. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-methyl-L-valinate,

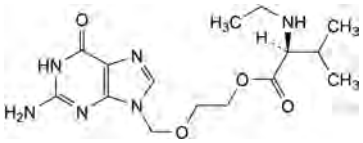

D. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-ethyl-L-valinate,

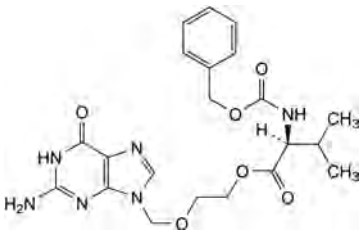

E. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-[(benzyloxy)carbonyl]-L-valinate,

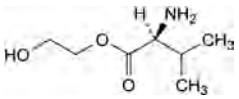

F. 2-hydroxyethyl L-valinate,

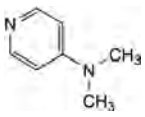

G. *N*,*N*-dimethylpyridin-4-amine,

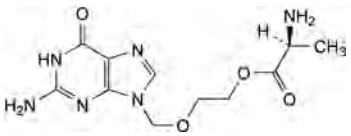

H. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl L-alaninate,

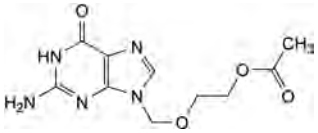

I. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl acetate,

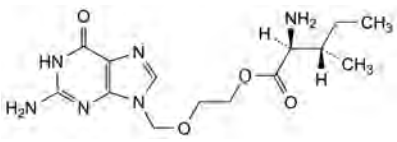

J. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl L-isoleucinate,

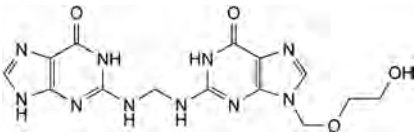

K. 9-[(2-hydroxyethoxy)methyl]-2-[[[(6-oxo-6,9-dihydro-1*H*-purin-2-yl)amino]methyl]amino]-1,9-dihydro-6*H*-purin-6-one,

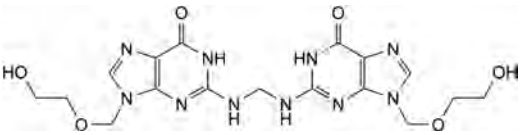

L. 2,2′-(methylenediazanediyl)bis[9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one],

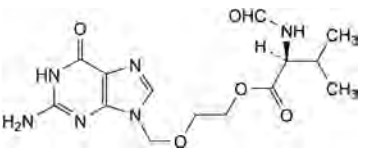

M. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-formyl-L-valinate,

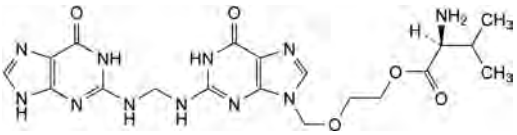

N. 2-[[6-oxo-2-[[[(6-oxo-6,9-dihydro-1*H*-purin-2-yl)amino]methyl]amino]-1,6-dihydro-9*H*-purin-9-yl]methoxy]ethyl L-valinate,

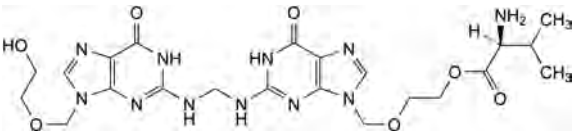

O. 2-[[2-[[[9-[(2-hydroxyethoxy)methyl]-6-oxo-6,9-dihydro-1*H*-purin-2-yl]amino]methyl]amino]-6-oxo-1,6-dihydro-9*H*-purin-9-yl]methoxy]ethyl L-valinate,

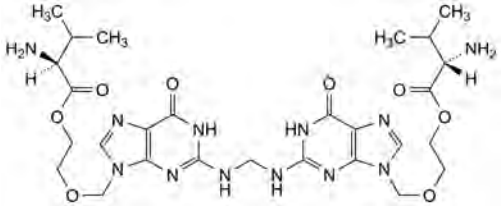

P. [methylenebis[azanediyl(6-oxo-1,6-dihydro-9*H*-purine-2,9-diyl)methyleneoxyethan-2,1-diyl]] di-L-valinate,

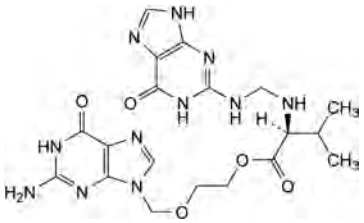

Q. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-[[(6-oxo-6,9-dihydro-1*H*-purin-2-yl)amino]methyl]-L-valinate,

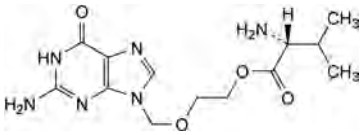

R. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl D-valinate.

**07/2019:2751**

# VALACICLOVIR HYDROCHLORIDE HYDRATE

## Valacicloviri hydrochloridum hydricum

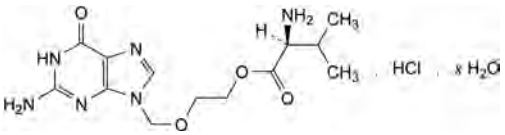

$C_{13}H_{21}ClN_6O_4,xH_2O$ $M_r$ 360.8 (anhydrous substance)
[1218948-84-5]

DEFINITION

2-[(2-Amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]-ethyl L-valinate hydrochloride hydrate.

*Content*: 95.0 per cent to 102.0 per cent (anhydrous substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water, very slightly soluble in anhydrous ethanol, practically insoluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve the substance to be examined in the minimum volume of *water R*, evaporate to dryness at room temperature and record the spectrum using the residue.

*Comparison*: repeat the operations using *anhydrous valaciclovir hydrochloride CRS*.

B. It complies with the limit for impurity R (see test A for related substances).

C. Water (see Tests).

D. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Impurities G and S**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.250 g of the substance to be examined in 2 mL of *water R* and dilute to 5.0 mL with *ethanol (96 per cent) R*.

*Reference solution*. Dissolve 5.0 mg of *valaciclovir impurity G CRS* and 5.0 mg of *valaciclovir impurity S CRS* in a mixture of 2 mL of *water R* and 6 mL of *ethanol (96 per cent) R* and dilute to 10.0 mL with *ethanol (96 per cent) R*. Dilute 0.5 mL of the solution to 10.0 mL with *ethanol (96 per cent) R*.

*Plate*: *TLC silica gel $F_{254}$ plate R* (2-10 µm).

*Pretreatment*: wash the plate with *methanol R* until the solvent front has migrated over at least 4/5 of the plate; allow to dry in air.

*Mobile phase*: *concentrated ammonia R*, *tetrahydrofuran R*, *methanol R*, *methylene chloride R* (3:12:34:54 *V/V/V/V*); use freshly prepared mobile phase.

*Application*: 4 µL.

*Development*: over 4/5 of the plate.

*Drying*: in a current of air.

*Detection*: examine in ultraviolet light at 254 nm.

*Retardation factors*: valaciclovir = about 0.3; impurity S = about 0.7; impurity G = about 0.8.

*System suitability*: the chromatogram obtained with the reference solution shows 2 clearly separated spots due to impurities S and G.

*Limits*:

– *impurity G*: any spot due to impurity G is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.05 per cent);

– *impurity S*: any spot due to impurity S is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.05 per cent).

**Related substances**

A. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Test solution*. Dissolve 50.0 mg of the substance to be examined in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution.

*Reference solution (a)*. Dissolve 2.5 mg of *valaciclovir for system suitability CRS* (containing impurities A, B, C, D, H, M and R) in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 5.0 mL with the same solution.

*Reference solution (b)*. Dissolve 50.0 mg of *anhydrous valaciclovir hydrochloride CRS* in a 0.5 per cent *V/V* solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solution.

*Reference solution (c)*. Dilute 3.0 mL of the test solution to 100.0 mL with a 0.5 per cent *V/V* solution of *hydrochloric acid R*. Dilute 1.0 mL of this solution to 100.0 mL with a 0.5 per cent *V/V* solution of *hydrochloric acid R*.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.0 mm;

– *stationary phase*: *crown-ether silica gel for chiral separation R* (5 µm);

– *temperature*: 10 °C.

*Mobile phase*: *perchloric acid R*, *methanol R*, *water for chromatography R* (0.5:5:95 *V/V/V*).

*Flow rate*: 0.75 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL of the test solution and reference solutions (a) and (c).

*Run time*: 1.5 times the retention time of valaciclovir.

*Identification of impurities*: use the chromatogram supplied with *valaciclovir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A + B, C + R, D and M.

*Relative retention* with reference to valaciclovir (retention time = about 17 min): impurities A and B = about 0.2; impurities C and R = about 0.6; impurity D = about 0.7; impurity M = about 1.3.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity D and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurities C and R.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurities A and B by 0.7;
– *impurity R*: maximum 3.0 per cent; for the calculation, subtract the content of impurity C as determined in test B for related substances from the content of the coeluting impurities C and R as determined in this test;
– *sum of impurities A and B*: maximum 2.0 per cent;
– *disregard limit*: the area of the principal peak in the chromatogram obtained with reference solution (c) (0.03 per cent); disregard any peaks due to impurities other than A + B and C + R.

B. Liquid chromatography (*2.2.29*): use the normalisation procedure. *Use the solutions within 24 h of preparation.*

*Solvent mixture*: *ethanol (96 per cent) R*, *water R* (20:80 *V/V*).

*Test solution*. Dissolve 80 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 1.6 mg of *valaciclovir for system suitability CRS* (containing impurities A, B, C, D, H, M and R) in the solvent mixture and dilute to 2.0 mL with the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 20.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 2 mg of *valaciclovir impurity P CRS* in the solvent mixture and dilute to 25.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped phenylhexylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 15 °C.

*Mobile phase*:

– *mobile phase A*: *trifluoroacetic acid R*, *water for chromatography R* (0.2:100 *V/V*);
– *mobile phase B*: *trifluoroacetic acid R*, *methanol R2* (0.2:100 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 90 | 10 |
| 5 - 35 | 90 → 60 | 10 → 40 |
| 35 - 45 | 60 | 40 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 10 µL.

*Identification of impurities*: use the chromatogram supplied with *valaciclovir for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B, C, D, H and M; use the chromatogram obtained with reference solution (c) to identify the peak due to impurity P.

*Relative retention* with reference to valaciclovir (retention time = about 20 min): impurity A = about 0.3; impurity B = about 0.4; impurity H = about 0.5; impurity C = about 1.06; impurity D = about 1.2; impurity M = about 1.6; impurity P = about 2.0.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to valaciclovir.

*Limits*:

– *impurity M*: maximum 0.6 per cent;
– *impurity D*: maximum 0.3 per cent;
– *impurity C*: maximum 0.2 per cent;
– *impurities H, P*: for each impurity, maximum 0.10 per cent;
– *unspecified impurities*: for each impurity, maximum 0.05 per cent;
– *disregard limit*: 0.6 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent); disregard the peaks due to impurities A and B.

*Limit*:

– *total for tests A and B*: maximum 4.0 per cent.

**Water** (*2.5.12*): 4.5 per cent to 11.0 per cent, determined on 0.100 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution and reference solution (b).

Calculate the percentage content of $C_{13}H_{21}ClN_6O_4$ taking into account the assigned content of *anhydrous valaciclovir hydrochloride CRS*.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, B, C, D, G, H, M, P, R, S.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *I, J, N.*

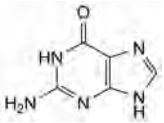

A. 2-amino-1,9-dihydro-6*H*-purin-6-one (guanine),

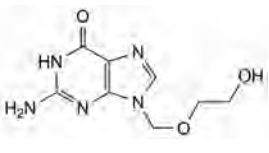

B. 2-amino-9-[(2-hydroxyethoxy)methyl]-1,9-dihydro-6*H*-purin-6-one (aciclovir),

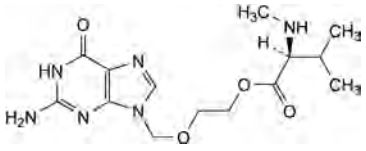

C. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-methyl-L-valinate,

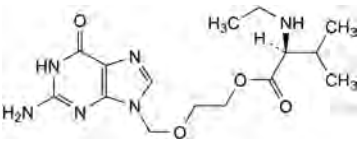

D. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-ethyl-L-valinate,

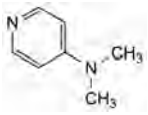

G. *N*,*N*-dimethylpyridin-4-amine,

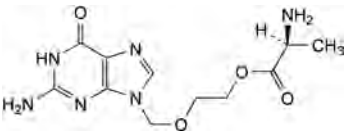

H. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl L-alaninate,

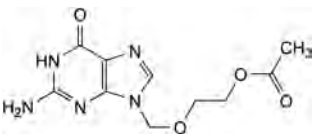

I. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl acetate,

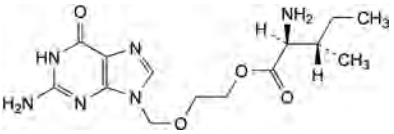

J. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl L-isoleucinate,

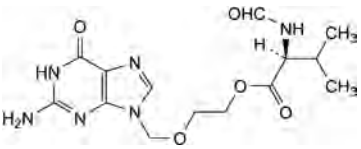

M. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-formyl-L-valinate,

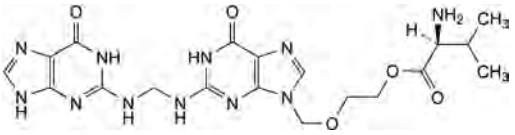

N. 2-[[6-oxo-2-[[[(6-oxo-6,9-dihydro-1*H*-purin-2-yl)amino]methyl]amino]-1,6-dihydro-9*H*-purin-9-yl]methoxy]ethyl L-valinate,

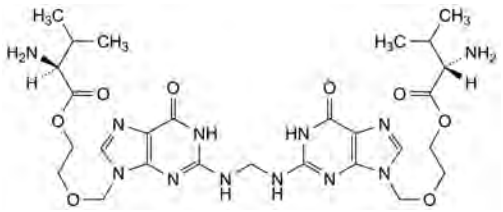

P. [methylenebis[azanediyl(6-oxo-1,6-dihydro-9*H*-purine-2,9-diyl)methyleneoxyethan-2,1-diyl]] di-L-valinate,

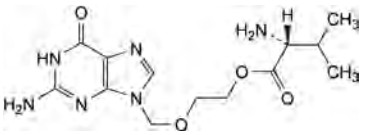

R. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl D-valinate,

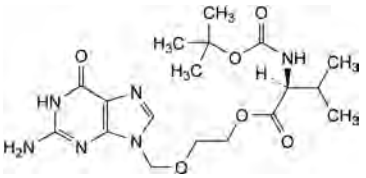

S. 2-[(2-amino-6-oxo-1,6-dihydro-9*H*-purin-9-yl)methoxy]ethyl *N*-(*tert*-butoxycarbonyl)-L-valinate.

**01/2017:0796**

# VALINE

## Valinum

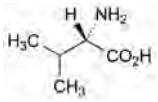

$C_5H_{11}NO_2$ $M_r$ 117.1
[72-18-4]

DEFINITION

(2*S*)-2-Amino-3-methylbutanoic acid.

Product of fermentation or of protein hydrolysis.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless crystals.

*Solubility*: soluble in water, very slightly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: A, B.*

*Second identification: A, C.*

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *valine CRS.*

C. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 10 mg of the substance to be examined in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Reference solution.* Dissolve 10 mg of *valine CRS* in a 10.3 g/L solution of *hydrochloric acid R* and dilute to 50 mL with the same solution.
*Plate*: *TLC silica gel plate R.*
*Mobile phase*: *glacial acetic acid R*, *water R*, *butanol R* (20:20:60 *V/V/V*)
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Detection*: spray with *ninhydrin solution R* and heat at 105 °C for 15 min.
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 2.5 g in *water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_6$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 26.5 to + 29.0 (dried substance).

Dissolve 2.00 g in *hydrochloric acid R1* and dilute to 25.0 mL with the same acid.

**Ninhydrin-positive substances**. Amino acid analysis (*2.2.56*). For analysis, use Method 1.

The concentrations of the test solution and the reference solutions may be adapted according to the sensitivity of the equipment used. The concentrations of all solutions are adjusted so that the system suitability requirements described in general chapter *2.2.46* are fulfilled, keeping the ratios of concentrations between all solutions as described.

*Solution A*: *dilute hydrochloric acid R1* or a sample preparation buffer suitable for the apparatus used.

*Test solution*. Dissolve 30.0 mg of the substance to be examined in solution A and dilute to 50.0 mL with solution A.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 2.0 mL of this solution to 10.0 mL with solution A.

*Reference solution (b)*. Dissolve 30.0 mg of *proline R* in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Reference solution (c)*. Dilute 6.0 mL of *ammonium standard solution (100 ppm $NH_4$) R* to 50.0 mL with solution A. Dilute 1.0 mL of this solution to 100.0 mL with solution A.

*Reference solution (d)*. Dissolve 30 mg of *isoleucine R* (impurity B) and 30 mg of *leucine R* (impurity C) in solution A and dilute to 50.0 mL with solution A. Dilute 1.0 mL of the solution to 200.0 mL with solution A.

*Reference solution (e)*. Dissolve 30.0 mg of *isoleucine R* (impurity B) in solution A and dilute to 100.0 mL with solution A. Dilute 1.0 mL of the solution to 250.0 mL with solution A.

*Blank solution*: solution A.

Inject suitable, equal amounts of the test, blank and reference solutions into the amino acid analyser. Run a program suitable for the determination of physiological amino acids.

*System suitability*: reference solution (d):

– *resolution*: minimum 1.5 between the peaks due to impurities B and C.

*Calculation of percentage contents*:

– for impurity B, use the concentration of impurity B in reference solution (e);

– for any ninhydrin-positive substance detected at 570 nm, use the concentration of valine in reference solution (a);

– for any ninhydrin-positive substance detected at 440 nm, use the concentration of proline in reference solution (b); if a peak is above the reporting threshold at both wavelengths, use the result obtained at 570 nm for quantification.

*Limits*:

– *impurity B at 570 nm*: maximum 0.4 per cent;

– *any ninhydrin-positive substance*: for each impurity, maximum 0.2 per cent;

– *total*: maximum 1.0 per cent;

– *reporting threshold*: 0.05 per cent.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

**Chlorides** (*2.4.4*): maximum 200 ppm.

Dilute 10 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 300 ppm.

Dissolve 0.5 g in *distilled water R* and dilute to 15 mL with the same solvent.

**Ammonium**. Amino acid analysis (*2.2.56*) as described in the test for ninhydrin-positive substances with the following modifications.

*Injection*: test solution, reference solution (c) and blank solution.

*Limit*:

– *ammonium at 570 nm*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.02 per cent), taking into account the peak due to ammonium in the chromatogram obtained with the blank solution.

**Iron** (*2.4.9*): maximum 10 ppm.

In a separating funnel, dissolve 1.0 g in 10 mL of *dilute hydrochloric acid R*. Shake with 3 quantities, each of 10 mL, of *methyl isobutyl ketone R1*, shaking for 3 min each time. To the combined organic layers add 10 mL of *water R* and shake for 3 min. Use the aqueous layer.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 3 mL of *anhydrous formic acid R*. Add 30 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 11.71 mg of $C_5H_{11}NO_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

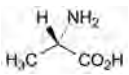

A. (2*S*)-2-aminopropanoic acid (alanine),

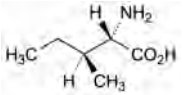

B. (2*S*,3*S*)-2-amino-3-methylpentanoic acid (isoleucine),

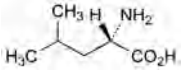

C. (2*S*)-2-amino-4-methylpentanoic acid (leucine).

**01/2008:2137**

# VALNEMULIN HYDROCHLORIDE FOR VETERINARY USE

## Valnemulini hydrochloridum ad usum veterinarium

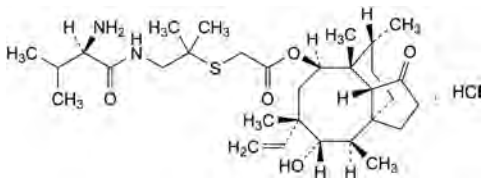

$C_{31}H_{53}ClN_2O_5S$ $M_r$ 601

[133868-46-9]

DEFINITION

(3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-Ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopenta[8]annulen-8-yl [[2-[[(2*R*)-2-amino-3-methylbutanoyl]amino]-1,1-dimethylethyl]sulfanyl]acetate hydrochloride.

Semi-synthetic product derived from a fermentation product.

*Content*: 96.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or yellowish, amorphous powder, hygroscopic.

*Solubility*: freely soluble in water and in anhydrous ethanol, practically insoluble in *tert*-butyl methyl ether.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *valnemulin hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**pH** (*2.2.3*): 3.0 to 6.0.

Dissolve 2.0 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Specific optical rotation** (*2.2.7*): + 15.5 to + 18.0 (anhydrous substance).

Dissolve 0.250 g in *water R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Phosphate buffer solution pH 2.5*. Dissolve 8.0 g of *disodium hydrogen phosphate dodecahydrate R* and 3.0 g of *potassium dihydrogen phosphate R* in *water for chromatography R* and dilute to 1000.0 mL with the same solvent. Adjust to pH 2.5 with *phosphoric acid R*.

*Solvent mixture*. Mix equal volumes of *acetonitrile R1* and *water for chromatography R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *valnemulin impurity E CRS* and 5 mg of the substance to be examined in the solvent mixture and dilute to 25 mL with the solvent mixture.

*Reference solution (c)*. Dissolve the contents of a vial of *valnemulin for peak identification CRS* (containing impurities A, B and C) in 1 mL of the solvent mixture.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);
- *temperature*: 50 °C.

*Mobile phase*:
- *mobile phase A*: phosphate buffer solution pH 2.5, *water R* (25:75 *V/V*);
- *mobile phase B*: phosphate buffer solution pH 2.5, *acetonitrile R1* (25:75 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 95 → 55 | 5 → 45 |
| 2 - 4.5 | 55 → 50 | 45 → 50 |
| 4.5 - 5.5 | 50 → 35 | 50 → 65 |
| 5.5 - 6.85 | 35 | 65 |
| 6.85 - 10 | 35 → 0 | 65 → 100 |
| 10 - 13 | 0 | 100 |
| 13 - 14 | 0 → 95 | 100 → 5 |
| 14 - 20 | 95 | 5 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 200 nm.

*Injection*: 5 µL.

*Identification of impurities*: use the chromatogram supplied with *valnemulin for peak identification CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to valnemulin (retention time = about 7 min): impurity D = about 0.2; impurity A = about 0.7; impurity B = about 0.85; impurity E = about 0.9; impurity C = about 1.1.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to impurity E and valnemulin.

*Limits*:
- *correction factors*: for the calculation of content multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 3.2; impurity E = 4.2;
- *impurity A*: not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *impurity B*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (2.0 per cent);
- *impurity C*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (1.0 per cent);
- *any other impurity*: for each impurity, not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (3.0 per cent);
- *disregard limit*: 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent); disregard the peak due to the chloride ion.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.500 g.

ASSAY

Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 50.0 mL with the same mixture of solvents.

*Reference solution*. Dissolve 50.0 mg of *valnemulin hydrogen tartrate CRS* in a mixture of equal volumes of *acetonitrile R1* and *water R* and dilute to 50.0 mL with the same mixture of solvents.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4.6 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (3 µm);

– *temperature*: 45 °C.

*Mobile phase*: mix 43 volumes of *acetonitrile R1* and 57 volumes of a solution containing 0.94 g/L of *disodium hydrogen phosphate dodecahydrate R* and 8.7 g/L of *potassium dihydrogen phosphate R* previously adjusted to pH 2.5 with *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 5 µL.

*Run time*: 3 times the retention time of valnemulin (retention time = about 2.4 min).

Calculate the percentage content of $C_{31}H_{53}ClN_2O_5S$, using the declared content of *valnemulin hydrogen tartrate CRS* and by multiplying by 0.841.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A, B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E.*

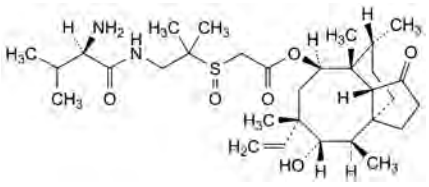

A. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopenta[8]annulen-8-yl [[2-[[(2*R*)-2-amino-3-methylbutanoyl]amino]-1,1-dimethylethyl]sulfinyl]acetate (valnemulin sulfoxide),

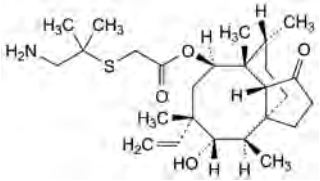

B. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopenta[8]annulen-8-yl [(2-amino-1,1-dimethylethyl)sulfanyl]acetate (dimethyl cysteaminyl pleuromulin),

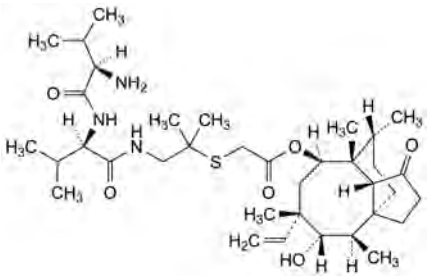

C. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopenta[8]annulen-8-yl [[2-[[(2*R*)-2-[[(2*R*)-2-amino-3-methylbutanoyl]amino]-3-methylbutanoyl]amino]-1,1-dimethylethyl]sulfanyl]acetate (valyl-valneumulin),

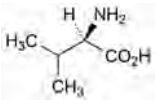

D. (2*R*)-2-amino-3-methylbutanoic acid (D-valine),

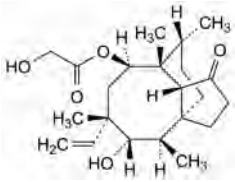

E. (3a*S*,4*R*,5*S*,6*S*,8*R*,9*R*,9a*R*,10*R*)6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecahydro-3a,9-propano-3a*H*-cyclopenta[8]annulen-8-yl 2-hydroxyacetate (pleuromulin).

**01/2017:1378**

# VALPROIC ACID

## Acidum valproicum

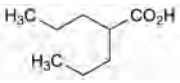

$C_8H_{16}O_2$ $M_r$ 144.2
[99-66-1]

DEFINITION

2-Propylpentanoic acid.

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: colourless or very slightly yellow, clear liquid, slightly viscous.

*Solubility*: very slightly soluble in water, miscible with ethanol (96 per cent) and with methylene chloride. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *valproic acid CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 2.0 g in *dilute sodium hydroxide solution R* and dilute to 10 mL with the same alkaline solution.

**Related substances**. Gas chromatography (*2.2.28*).

*Test solution*. Dissolve 0.500 g of the substance to be examined in *heptane R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of *valproic acid for system suitability CRS* (containing impurity K) in 1.0 mL of *heptane R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with *heptane R*.

*Column*:

- *material*: wide-bore fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *macrogol 20 000 2-nitroterephthalate R* (film thickness 0.5 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 8 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 80 |
| | 5 - 15 | 80 → 150 |
| | 15 - 28.3 | 150 → 190 |
| | 28.3 - 30 | 190 |
| Injection port | | 220 |
| Detector | | 220 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to valproic acid (retention time = about 17 min): impurity K = about 0.97.

*System suitability*: reference solution (a):

- *resolution*: minimum 2.0 between the peaks due to impurity K and valproic acid.

*Limits*:

- *impurity K*: not more than 0.15 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent);
- *total*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *disregard limit*: 0.03 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.03 per cent).

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.100 g in 25 mL of *ethanol (96 per cent) R*. Add 2 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 14.42 mg of $C_8H_{16}O_2$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities*: *K*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, L*.

A. pentanoic acid (valeric acid),

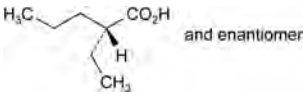


B. (2*RS*)-2-ethylpentanoic acid,

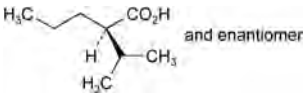


C. (2*RS*)-2-(1-methylethyl)pentanoic acid,

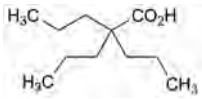

D. 2,2-dipropylpentanoic acid,

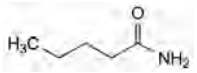

E. pentanamide (valeramide),

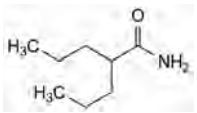

F. 2-propylpentanamide,

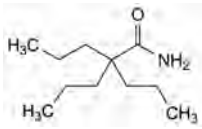

G. 2,2-dipropylpentanamide,

H. pentanenitrile (valeronitrile),

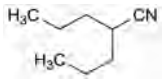

I. 2-propylpentanenitrile,

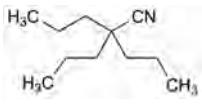

J. 2,2-dipropylpentanenitrile,

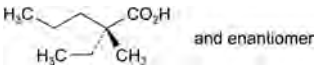


K. (2*RS*)-2-ethyl-2-methylpentanoic acid,

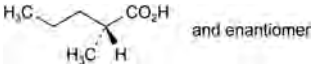


L. (2*RS*)-2-methylpentanoic acid.

**01/2020:2423**

# VALSARTAN

## Valsartanum

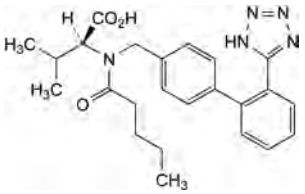

$C_{24}H_{29}N_5O_3$ $M_r$ 435.5
[137862-53-4]

DEFINITION

(2*S*)-3-Methyl-2-[pentanoyl[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]amino]butanoic acid.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

PRODUCTION

As *N*-nitrosodimethylamine (NDMA) and *N*-nitrosodiethylamine (NDEA) are classified as probable human carcinogens, manufacturers must ensure that their manufacturing process does not generate such impurities and develop appropriate control strategies. To allow manufacturers to make the necessary changes to their process, a transition period has been agreed by competent authorities and strict temporary limits on levels of these impurities introduced in the Test section.

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: practically insoluble in water, freely soluble in anhydrous ethanol, sparingly soluble in methylene chloride.

IDENTIFICATION

Carry out either tests A, B or tests A, C.

A. Infrared absorption spectrophotometry (*2.2.24*).
   *Comparison*: *valsartan CRS*.

B. Enantiomeric purity (see Tests).

C. Specific optical rotation (*2.2.7*): – 69.0 to – 64.0 (anhydrous substance).
   Dissolve 0.200 g in *methanol R* and dilute to 20.0 mL with the same solvent.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 5 mg of *valsartan for peak identification CRS* (containing impurity A) in the mobile phase and dilute to 5 mL with the mobile phase.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *cellulose derivative of silica gel for chiral separation R* (5 µm).

*Mobile phase*: *trifluoroacetic acid R*, *2-propanol R*, *hexane R* (0.1:15:85 *V/V/V*).

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 10 µL.

*Run time*: 1.5 times the retention time of valsartan.

*Identification of impurities*: use the chromatogram supplied with *valsartan for peak identification CRS* and the chromatogram obtained with reference solution (a) to identify the peak due to impurity A.

*Relative retention* with reference to valsartan (retention time = about 13 min): impurity A = about 0.6.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurity A and valsartan.

*Limit*:
- *impurity A*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50 mg of the substance to be examined in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve the contents of a vial of *valsartan for system suitability CRS* (containing impurity C) in 1 mL of the mobile phase.

*Column*:
- *size*: *l* = 0.125 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R1*, *water for chromatography R* (1:500:500 *V/V/V*).

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 10 µL.

*Run time*: 6 times the retention time of valsartan.

*Identification of impurities*: use the chromatogram supplied with *valsartan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peak due to impurity C.

*Relative retention* with reference to valsartan (retention time = about 5 min): impurity C = about 0.8.

*System suitability*: reference solution (b):
- *resolution*: minimum 3.0 between the peaks due to impurity C and valsartan.

*Limits*:
- *impurity C*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.12*): maximum 2.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Nitrosamines**. Carry out the test by a suitable method.

The substance to be examined does not contain either NDMA or NDEA above the limits provided below or both impurities at whatever level:
- *N-nitrosodimethylamine (NDMA)*: maximum 0.300 ppm;
- *N-nitrosodiethylamine (NDEA)*: maximum 0.082 ppm.

ASSAY

Dissolve 0.170 g in 70 mL of *2-propanol R*. Titrate with *0.1 M tetrabutylammonium hydroxide in 2-propanol*, determining the endpoint potentiometrically (*2.2.20*). Perform all operations under nitrogen.

1 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* is equivalent to 21.78 mg of $C_{24}H_{29}N_5O_3$.

STORAGE

In an airtight container.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B.*

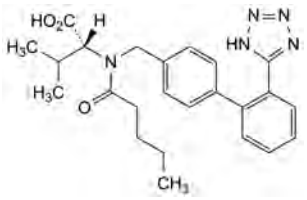

A. (2*R*)-3-methyl-2-[pentanoyl[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]amino]butanoic acid,

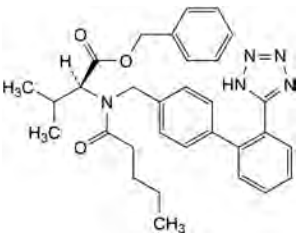

B. benzyl (2*S*)-3-methyl-2-[pentanoyl[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]amino]butanoate,

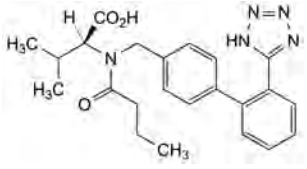

C. (2*S*)-2-[butanoyl[[2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]amino]-3-methylbutanoic acid.

**07/2019:1058**
**corrected 10.0**

# VANCOMYCIN HYDROCHLORIDE

## Vancomycini hydrochloridum

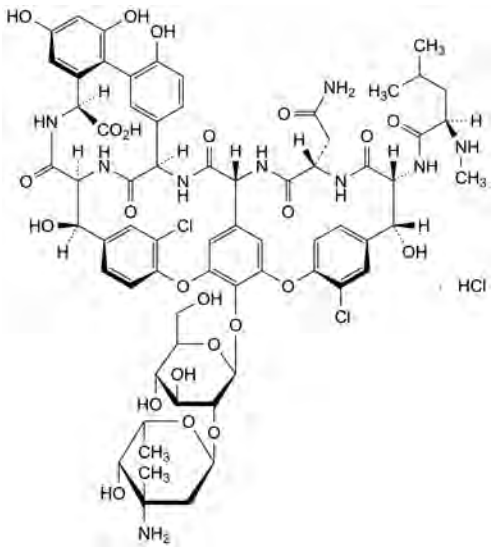

$C_{66}H_{76}Cl_3N_9O_{24}$ $M_r$ 1486

DEFINITION

Monohydrochloride of (3*S*,6*R*,7*R*,8*M*,18*M*,22*R*,23*S*,26*S*,30a*M*,36*R*,38a*R*)-3-(2-amino-2-oxoethyl)-44-[[2-*O*-(3-amino-2,3,6-trideoxy-3-*C*-methyl-α-L-*lyxo*-hexopyranosyl)-β-D-glucopyranosyl]oxy]-10,19-dichloro-7,22,28,30,32-pentahydroxy-6-[(2*R*)-4-methyl-2-(methylamino)pentanamido]-2,5,24,38,39-pentaoxo-2,3,4,5,6,7,23,24,25,26,36,37,38,38a-tetradecahydro-22*H*-23,36-(azanomethano)-8,11:18,21-dietheno-13,16:31,35-dimetheno-1*H*,13*H*-[1,6,9]oxadiazacyclohexadecino[4,5-*m*][10,2,16]benzoxadiazacyclotetracosine-26-carboxylic acid (vancomycin B hydrochloride).

Substance produced by certain strains of *Amycolatopsis orientalis*.

*Potency*: minimum 1050 IU/mg (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic powder.

*Solubility*: freely soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Examine the chromatograms obtained in the test for vancomycin B and related substances.

*Results*: the principal peak in the chromatogram obtained with the test solution is similar in retention time to the principal peak in the chromatogram obtained with reference solution (a).

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and its absorbance (*2.2.25*) at 450 nm is not greater than 0.10, and its absorbance at 370 nm is not greater than 0.65.

Dissolve 2.50 g in *water R* and dilute to 25.0 mL with the same solvent.

**pH** (*2.2.3*): 2.5 to 4.5.

Dissolve 0.50 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Vancomycin B and related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solution A*. Dissolve 7.0 g of *tris(hydroxymethyl)aminomethane R* in approximately 950 mL of *water for chromatography R*. Measure the temperature of the solution and adjust to the target pH indicated in Table 1058.-1 using a 20 per cent *V/V* solution of *glacial acetic acid R* in *water for chromatography R* and dilute to 1000 mL with *water for chromatography R*.

Table 1058.-1

| Temperature of solution A (°C) | Target pH |
|---|---|
| 15 | 8.27 ± 0.05 |
| 16 | 8.25 ± 0.05 |
| 17 | 8.22 ± 0.05 |
| 18 | 8.19 ± 0.05 |
| 19 | 8.16 ± 0.05 |
| 20 | 8.13 ± 0.05 |
| 21 | 8.11 ± 0.05 |
| 22 | 8.08 ± 0.05 |
| 23 | 8.05 ± 0.05 |
| 24 | 8.03 ± 0.05 |
| 25 | 8.00 ± 0.05 |

*Test solution.* Dissolve 20.0 mg of the substance to be examined in *water R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dissolve 4 mg of *vancomycin for system suitability CRS* (containing impurities A, C, F, H, I, J, K and M) in 1.0 mL of *water R*.

*Reference solution (b).* In order to prepare impurities B, D, E, G and L *in situ*, expose 4 mg of *vancomycin for system suitability CRS* (containing impurities A, C, F, H, I, J, K and M) to 80-100 per cent relative humidity at 42 ± 2 °C for at least 7 days. Add 1 mL of *water R* and dissolve the sample completely using sonication.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with a 0.1 per cent *V/V* solution of *acetic acid R*. Dilute 1.0 mL of this solution to 10.0 mL with a 0.1 per cent *V/V* solution of *acetic acid R*.

*Column*:

- *size*: *l* = 0.15 m, Ø = 2.1 mm;
- *stationary phase*: *end-capped, charged surface, ethylene-bridged octadecylsilyl silica gel for chromatography (hybrid material) R* (1.7 µm);
- *temperature*: 40 ± 2 °C.

*Mobile phase*:

- *mobile phase A*: *acetonitrile R*, *methanol R*, solution A (3:4:93 *V/V/V*);
- *mobile phase B*: *acetonitrile R*, *methanol R*, solution A (10:40:50 *V/V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 7 | 88 | 12 |
| 7 - 21 | 88 → 75 | 12 → 25 |
| 21 - 35 | 75 → 25 | 25 → 75 |
| 35 - 37 | 25 | 75 |

*Flow rate*: 0.30 ± 0.02 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Autosampler*: set at 5 °C.

*Injection*: 2 µL.

*Identification of peaks*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, C, F, H, I, J, K and M; use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B, D, E, G and L.

*Relative retention* with reference to vancomycin B (retention time = about 19 min): impurity E = about 0.37; impurity L = about 0.66; impurity B = about 0.70; impurity A = about 0.76; impurity F = about 0.82; impurity G = about 0.90; impurity H = about 0.94; impurity M = about 1.11; impurity I = about 1.14; impurity J = about 1.20; impurity D = about 1.24; impurity K = about 1.50; impurity C = about 1.86.

*System suitability*:

- *resolution*: minimum 1.5 and maximum 4.0 between the peaks due to impurities G and H, and minimum 1.5 and maximum 5.0 between the peaks due to impurities L and B in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the peak due to vancomycin B in the chromatogram obtained with reference solution (c).

*Limits*:

- *vancomycin B*: minimum 91.0 per cent;
- *impurities A, H*: for each impurity, maximum 3.0 per cent;
- *sum of impurities B and E*: maximum 2.0 per cent;
- *impurity J*: maximum 1.6 per cent;
- *impurities D, F, M*: for each impurity, maximum 1.5 per cent;
- *impurities G, I, K*: for each impurity, maximum 1.2 per cent;
- *impurity C*: maximum 1.0 per cent;
- *any other impurity eluting before vancomycin B*: for each impurity, maximum 0.8 per cent, and not more than 5 such impurities exceed 0.30 per cent;
- *any other impurity eluting after vancomycin B*: for each impurity, maximum 0.8 per cent, and not more than 3 such impurities exceed 0.30 per cent;
- *total of impurities*: maximum 9.0 per cent;
- *reporting threshold*: 0.10 per cent.

**Water** (*2.5.12*): maximum 5.0 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 1.0 per cent, determined on 1.00 g.

ASSAY

Carry out the microbiological assay of antibiotics (*2.7.2*).

STORAGE

In an airtight container, protected from light at a temperature of 2 °C to 8 °C. If the substance is sterile, the container is also sterile and tamper-evident.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

*Specified impurities: A, B, C, D, E, F, G, H, I, J, K, M.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *L.*

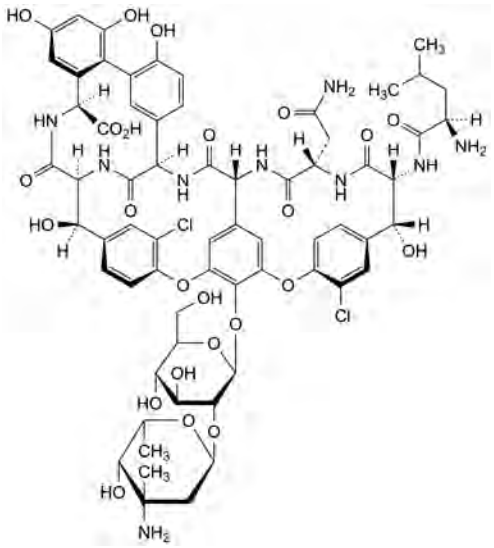

A. $N^{2.1}$-demethylvancomycin B,

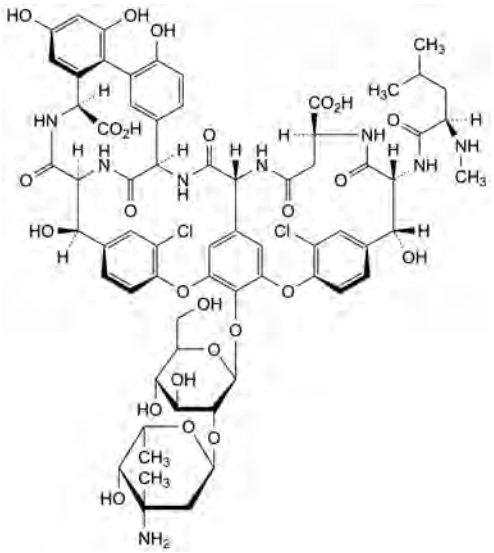

B. (1.2*M*)-[L-β-Asp$^3$]vancomycin B (3.2-*syn*-chloro[L-β-Asp$^3$]vancomycin B) (CDP-1 major),

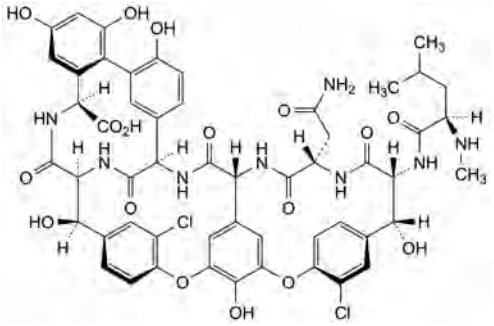

C. $O^{4.4}$-de-[2-*O*-(3-amino-2,3,6-trideoxy-3-*C*-methyl-α-L-*lyxo*-hexopyranosyl)-β-D-glucopyranosyl]vancomycin B (aglucovancomycin B),

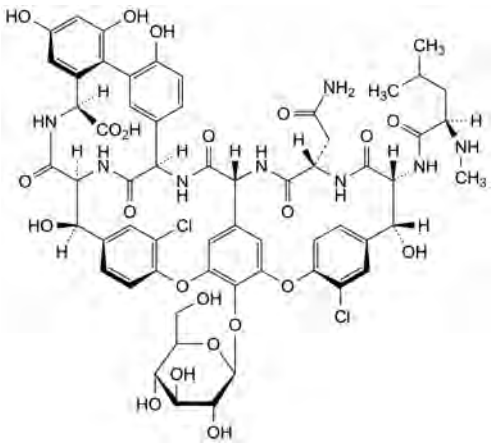

D. $O^{4.4}$-de-[2-*O*-(3-amino-2,3,6-trideoxy-3-*C*-methyl-α-L-*lyxo*-hexopyranosyl)-β-D-glucopyranosyl]-$O^{4.4}$-β-D-glucopyranosyl-vancomycin B (desvancosaminylvancomycin B),

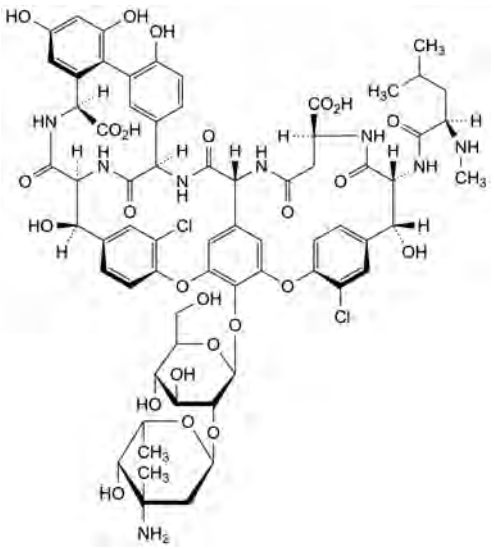

E. [L-β-Asp$^3$]vancomycin B (CDP-1 minor),

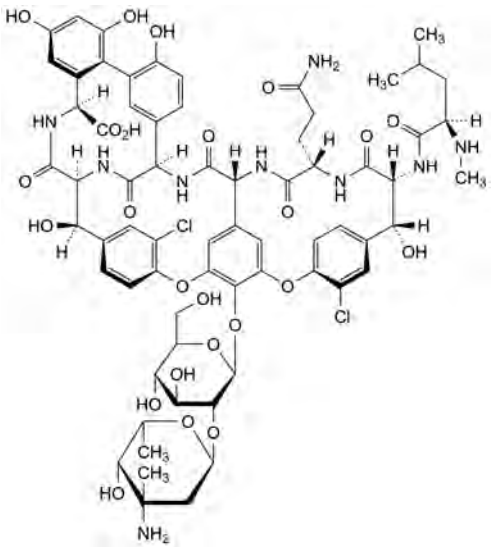

F. [L-α-Gln$^3$]vancomycin B,

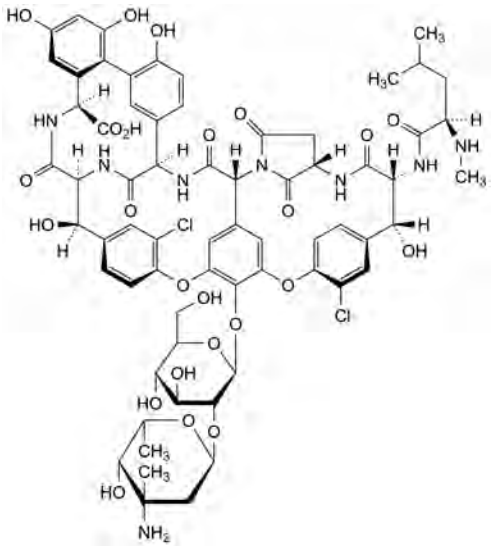

G. 4.3,$N^{\alpha,4}$-anhydro-[L-α-Asp$^3$]vancomycin B,

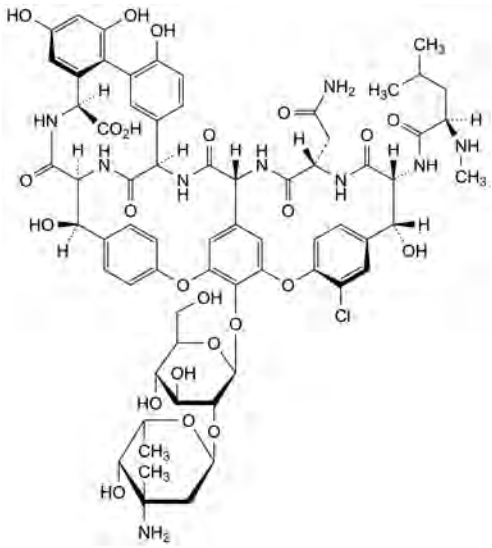

H. 3.6-dechlorovancomycin B (mono-dechloro-6-vancomycin B),

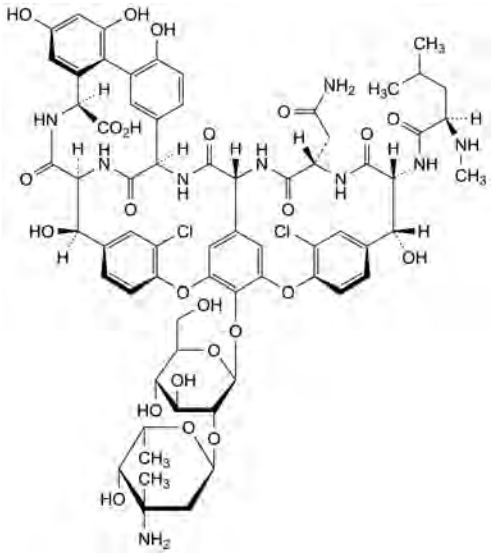

I. (1.2*M*)-vancomycin B (3.2-*syn*-chloro-vancomycin B),

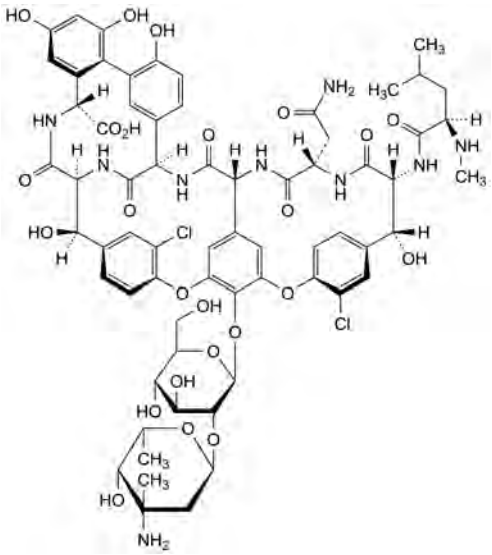

J. ($C^{\alpha,7}R$)-vancomycin B (26-*epi*-vancomycin B),

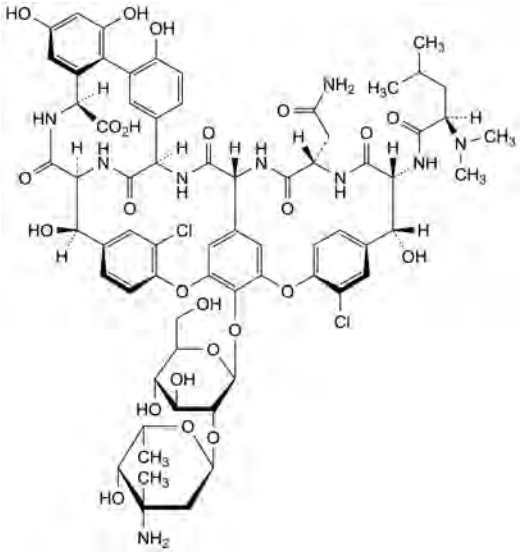

K. [*N*,*N*-dimethyl-D-Leu$^1$]vancomycin B,

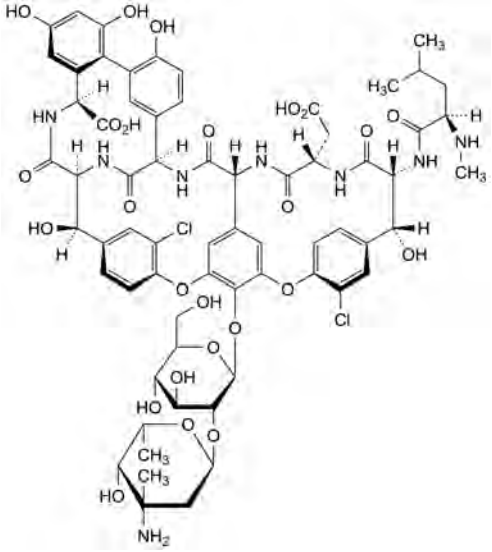

L. [L-α-Asp$^3$]vancomycin B),

M. unknown structure.

01/2008:0747

# VANILLIN

## Vanillinum

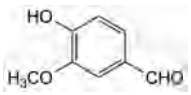

$C_8H_8O_3$ $M_r$ 152.1
[121-33-5]

DEFINITION

Vanillin contains not less than 99.0 per cent and not more than the equivalent of 101.0 per cent of 4-hydroxy-3-methoxybenzaldehyde, calculated with reference to the dried substance.

CHARACTERS

White or slightly yellowish, crystalline powder or needles, slightly soluble in water, freely soluble in alcohol and in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C, D.

A. Melting point (*2.2.14*): 81 °C to 84 °C.

B. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the spectrum obtained with *vanillin CRS*. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances in daylight after spraying. The principal spot in the chromatogram obtained with test solution (b) is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

D. To 5 mL of a saturated solution of the substance to be examined add 0.2 mL of *ferric chloride solution R1*. A blue colour is produced. Heat to 80 °C. The solution becomes brown. On cooling, a white precipitate is formed.

TESTS

**Appearance of solution**. Dissolve 1.0 g in *alcohol R* and dilute to 20 mL with the same solvent. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_6$ (*2.2.2, Method II*).

**Related substances**. Examine by thin-layer chromatography (*2.2.27*), using *silica gel* $GF_{254}$ *R* as the coating substance.

*Test solution (a)*. Dissolve 0.1 g of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.

*Test solution (b)*. Dilute 1 mL of test solution (a) to 10 mL with *methanol R*.

*Reference solution (a)*. Dissolve 10 mg of *vanillin CRS* in *methanol R* and dilute to 5 mL with the same solvent.

*Reference solution (b)*. Dilute 0.5 mL of test solution (a) to 100 mL with *methanol R*.

Apply to the plate 5 µL of each solution. Develop in an unsaturated tank over a path of 10 cm using a mixture of 0.5 volumes of *anhydrous acetic acid R*, 1 volume of *methanol R* and 98.5 volumes of *methylene chloride R*. Dry the plate in a current of cold air. Examine in ultraviolet light at 254 nm. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent). Spray with *dinitrophenylhydrazine-aceto-hydrochloric solution R* and examine in daylight. Any spot in the chromatogram obtained with test solution (a), apart from the principal spot, is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.5 per cent).

**Reaction with sulfuric acid**. Dissolve 50 mg in 5 mL of *sulfuric acid R*. After 5 min, the solution is not more intensely coloured than a mixture of 4.9 mL of yellow primary solution and 0.1 mL of red primary solution or a mixture of 4.9 mL of yellow primary solution and 0.1 mL of blue primary solution (*2.2.2, Method I*).

**Loss on drying** (*2.2.32*). Not more than 1.0 per cent, determined on 1.000 g by drying in a desiccator for 4 h.

**Sulfated ash** (*2.4.14*). Not more than 0.05 per cent, determined on 2.0 g.

ASSAY

Dissolve 0.120 g in 20 mL of *alcohol R* and add 60 mL of *carbon dioxide-free water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 15.21 mg of $C_8H_8O_3$.

STORAGE

Store protected from light.

07/2014:2782
corrected 10.0

# VARDENAFIL HYDROCHLORIDE TRIHYDRATE

## Vardenafili hydrochloridum trihydricum

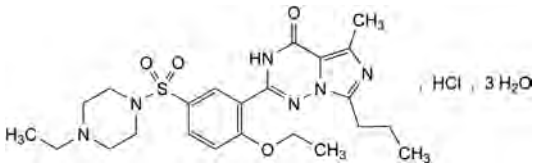

$C_{23}H_{33}ClN_6O_4S,3H_2O$ $M_r$ 579.1
[330808-88-3]

DEFINITION

2-[2-Ethoxy-5-[(4-ethylpiperazin-1-yl)sulfonyl]phenyl]-5-methyl-7-propylimidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one hydrochloride trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly brown or yellow powder.

*Solubility*: slightly soluble in water, freely soluble in anhydrous ethanol, practically insoluble in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *vardenafil hydrochloride CRS*.

B. Water (see Tests).

C. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Protect the solutions from light.*

*Solvent mixture*: *acetonitrile R*, mobile phase A (20:80 *V/V*).

*Test solution (a)*. Dissolve 50.0 mg of the substance to be examined in 20 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A.

Monographs U-V

*Test solution (b).* Dilute 15.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 50.0 mg of *vardenafil hydrochloride CRS* in 20 mL of *acetonitrile R* and dilute to 100.0 mL with mobile phase A. Dilute 15.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 5 mg of *vardenafil for system suitability CRS* (containing impurity A) in 2 mL of *acetonitrile R* and dilute to 10 mL with mobile phase A.

*Column*:
- *size*: *l* = 0.25 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm);
- *temperature*: 45 °C.

*Mobile phase*:
- *mobile phase A*: solution containing 0.7 g/L of *disodium hydrogen phosphate dihydrate R* and 1.3 g/L of *potassium dihydrogen phosphate R*;
- *mobile phase B*: *acetonitrile for chromatography R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 2 | 80 | 20 |
| 2 - 22 | 80 → 25 | 20 → 75 |
| 22 - 27 | 25 | 75 |

*Flow rate*: 0.5 mL/min.

*Detection*: spectrophotometer at 242 nm.

*Injection*: 10 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *vardenafil for system suitability CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to vardenafil (retention time = about 16 min): impurity A = about 0.8.

*System suitability*: reference solution (c):
- *resolution*: minimum 5.0 between the peaks due to impurity A and vardenafil.

*Calculation of percentage contents:*
- for each impurity, use the concentration of vardenafil hydrochloride trihydrate in reference solution (b).

*Limits*:
- *impurity A*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.3 per cent;
- *reporting threshold*: 0.05 per cent.

**Sulfates** (*2.4.13*): maximum 400 ppm.

Suspend 0.5 g in 20 mL of a 5.15 g/L solution of *hydrochloric acid R* and stir for 15 min. Filter if complete dissolution is not obtained.

**Water** (*2.5.12*): 8.8 per cent to 10.5 per cent, determined on 60.0 mg.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: 10 µL of test solution (b) and reference solution (a).

Calculate the percentage content of $C_{23}H_{33}ClN_6O_4S$ taking into account the assigned content of *vardenafil hydrochloride CRS*.

IMPURITIES

*Specified impurities*: *A*.

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C*.

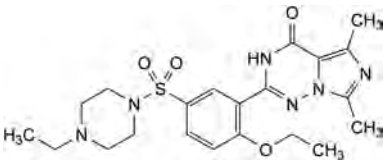

A. 2-[2-ethoxy-5-[(4-ethylpiperazin-1-yl)sulfonyl]phenyl]-5,7-dimethylimidazo[5,1-*f*][1,2,4]triazin-4(3*H*)-one,

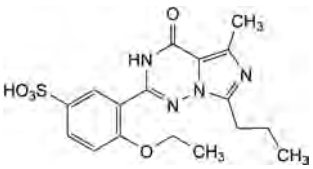

B. 4-ethoxy-3-(5-methyl-4-oxo-7-propyl-3,4-dihydroimidazo[5,1-*f*][1,2,4]triazin-2-yl)benzenesulfonic acid,

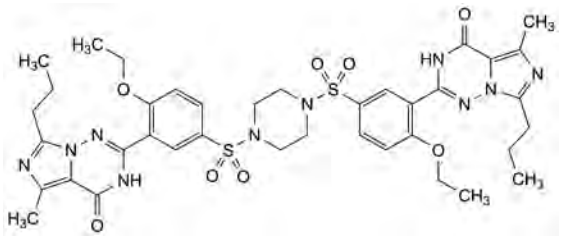

C. 2,2′-[piperazine-1,4-diylbis[(sulfonyl)(4-ethoxybenzene-1,3-diyl)]]bis[5-methyl-7-propylimidazo[5,1-*f*][1,2,4]-triazin-4(3*H*)-one].

**04/2017:1769**
**corrected 10.0**

# VECURONIUM BROMIDE

## Vecuronii bromidum

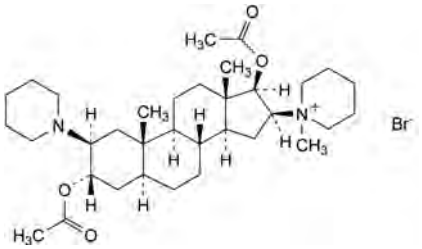

$C_{34}H_{57}BrN_2O_4$ $M_r$ 638

[50700-72-6]

DEFINITION

1-[3α,17β-Bis(acetyloxy)-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidin-1-ium bromide.

*Content*: 99.0 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white crystals or crystalline powder.

*Solubility*: slightly soluble in water, freely soluble in methylene chloride, sparingly soluble in acetonitrile and in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *vecuronium bromide CRS*.

C. It gives reaction (a) of bromides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in a 5.15 g/L solution of *hydrochloric acid R* and dilute to 50.0 mL with the same solution.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

**Specific optical rotation** (*2.2.7*): + 30.5 to + 35.0 (anhydrous substance), determined on solution S.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 0.10 g of the substance to be examined in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (a)*. Dissolve 5 mg of the substance to be examined and 5 mg of *pancuronium bromide CRS* (impurity B) in *methylene chloride R* and dilute to 5.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 5.0 mg of *pancuronium bromide CRS* (impurity B) in *methylene chloride R* and dilute to 100.0 mL with the same solvent.

*Plate*: *TLC silica gel plate R* (2-10 µm).

*Mobile phase*: dissolve 1 g of *sodium bromide R* in 5 mL of *water R*. Add 85 mL of *2-propanol R*, then 10 mL of *acetonitrile R*.

*Application*: 1 µL.

*Development*: in an unsaturated tank, over 2/3 of the plate.

*Drying*: in air for 30 min.

*Detection*: spray with a 2.5 g/L solution of *iodine R* in a mixture of equal volumes of *methanol R* and *methylene chloride R*.

*System suitability*: reference solution (a):

– the chromatogram shows 2 clearly separated spots.

*Limit*:

– *impurity B*: any spot due to impurity B is not more intense than the spot in the chromatogram obtained with reference solution (b) (0.25 per cent).

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solution A*: 0.2 g/L solution of *hydrochloric acid R* in *methanol R2*.

*Test solution*. Dissolve 40 mg of the substance to be examined in solution A and dilute to 20.0 mL with solution A.

*Reference solution (a)*. Dissolve 4 mg of *vecuronium for system suitability CRS* (containing impurities C and E) in solution A and dilute to 2.0 mL with the solution A.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with solution A. Dilute 1.0 mL of this solution to 10.0 mL with solution A.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 40 °C.

*Mobile phase*: mix 135 volumes of an 18.0 g/L solution of *tetramethylammonium hydroxide R* previously adjusted to pH 6.9 with *phosphoric acid R*, 250 volumes of *methanol R2* and 615 volumes of *acetonitrile R1*.

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Autosampler*: set at 4 °C.

*Injection*: 20 µL.

*Run time*: 5 times the retention time of vecuronium.

*Identification of impurities*: use the chromatogram supplied with *vecuronium for system suitability CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities C and E.

*Relative retention* with reference to vecuronium (retention time = about 5 min): impurity C = about 0.8; impurity E = about 1.2.

*System suitability*:

– *signal-to-noise ratio*: minimum 28 for the principal peak in the chromatogram obtained with reference solution (b);

– *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the principal peak in the chromatogram obtained with reference solution (a).

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity C by 1.4;

– for each impurity, use the concentration of vecuronium bromide in reference solution (b).

*Limits*:

– *impurity C*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.2 per cent;

– *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.450 g in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 63.8 mg of $C_{34}H_{57}BrN_2O_4$.

STORAGE

In an airtight container, protected from light and moisture.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, D, E, F.*

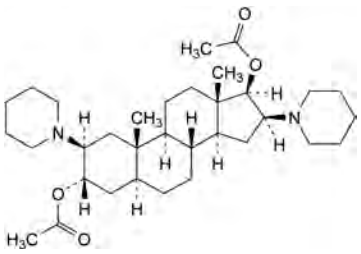

A. 2β,16β-di(piperidin-1-yl)-5α-androstane-3α,17β-diyl diacetate,

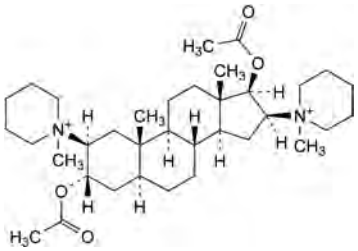

B. 1,1′-[3α,17β-bis(acetyloxy)-5α-androstane-2β,16β-diyl]bis(1-methylpiperidin-1-ium) (pancuronium),

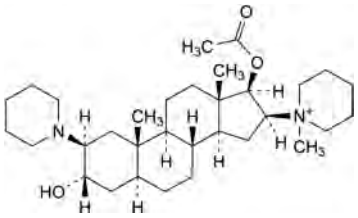

C. 1-[17β-(acetyloxy)-3α-hydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidin-1-ium,

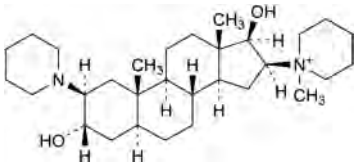

D. 1-[3α,17β-dihydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidin-1-ium,

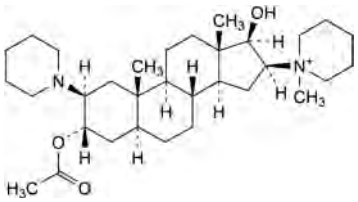

E. 1-[3α-(acetyloxy)-17β-hydroxy-2β-(piperidin-1-yl)-5α-androstan-16β-yl]-1-methylpiperidin-1-ium,

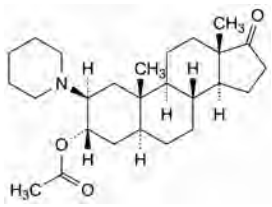

F. 2β-(piperidin-1-yl)-17-oxo-5α-androstan-3α-yl acetate.

**07/2009:2248**

# VEDAPROFEN FOR VETERINARY USE

## Vedaprofenum ad usum veterinarium

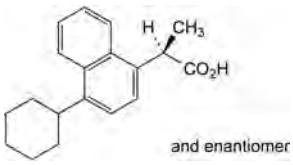


$C_{19}H_{22}O_2$ $M_r$ 282.4
[71109-09-6]

DEFINITION

(2*RS*)-2-(4-Cyclohexyl-1-naphthyl)propanoic acid.

*Content*: 98.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: practically insoluble in water, freely soluble in acetone, soluble in methanol. It dissolves in dilute solutions of alkali hydroxides.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *vedaprofen CRS*.

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

Dissolve 2.0 g in *acetone R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with *methanol R*. Dilute 1.0 mL of this solution to 10.0 mL with *methanol R*.

*Reference solution (b)*. Dissolve the contents of a vial of *vedaprofen impurity mixture CRS* (impurities A, B and C) in 1.0 mL of reference solution (a).

*Column*:

- *size*: *l* = 0.10 m, Ø = 3.0 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: dissolve 1.70 g of *tetrabutylammonium hydrogen sulfate R* in 1000 mL of a mixture of 20 volumes of *water R* and 80 volumes of *methanol R*.

*Flow rate*: 0.4 mL/min.

*Detection*: spectrophotometer at 288 nm.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of vedaprofen.

*Identification of impurities*: use the chromatogram supplied with *vedaprofen impurity mixture CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to vedaprofen (retention time = about 6 min): impurity C = about 0.8; impurity A = about 1.8; impurity B = about 3.7.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity C and vedaprofen.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity B by 0.7;

– *impurities A, B*: for each impurity, not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.4 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.20 per cent);

– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent).

**Heavy metals** (*2.4.8*): maximum 10 ppm.

Dissolve 1.0 g in a mixture of 15 volumes of *water R* and 85 volumes of *acetone R* and dilute to 20 mL with the same mixture of solvents. 12 mL of the solution complies with test B. Prepare the reference solution using lead standard solution (0.5 ppm Pb) obtained by diluting *lead standard solution (100 ppm Pb) R* with a mixture of 15 volumes of *water R* and 85 volumes of *acetone R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.3 per cent, determined on 0.500 g.

ASSAY

Dissolve 0.200 g in 50 mL of *ethanol (96 per cent) R* and add 1.0 mL of *0.1 M hydrochloric acid*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 28.24 mg of $C_{19}H_{22}O_2$.

IMPURITIES

*Specified impurities: A, B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *C, D.*

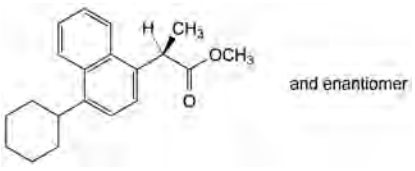


A. methyl (2*RS*)-2-(4-cyclohexyl-1-naphthyl)propanoate,

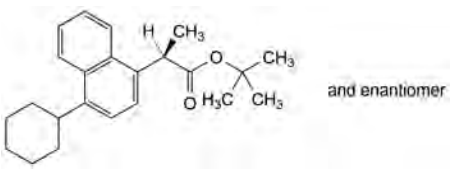


B. 1,1-dimethylethyl (2*RS*)-2-(4-cyclohexyl-1-naphthyl)propanoate,

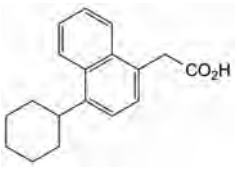


C. (4-cyclohexyl-1-naphthyl)acetic acid,

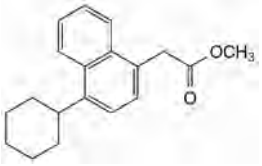


D. methyl (2*RS*)-2-(4-cyclohexyl-1-naphthyl)acetate.

**01/2017:2119**

# VENLAFAXINE HYDROCHLORIDE

## Venlafaxini hydrochloridum

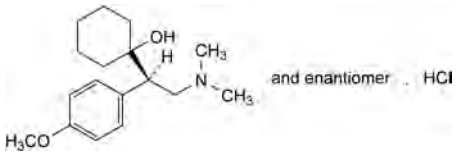


$C_{17}H_{28}ClNO_2$ $M_r$ 313.9
[99300-78-4]

DEFINITION

1-[(1*RS*)-2-(Dimethylamino)-1-(4-methoxyphenyl)ethyl]-cyclohexanol hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water and in methanol, soluble in anhydrous ethanol, slightly soluble or practically insoluble in acetone.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *venlafaxine hydrochloride CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *2-propanol R*, evaporate to dryness and record new spectra using the residues.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Acidity or alkalinity**. Dissolve 0.20 g in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent. Add 0.05 mL of *methyl red solution R* and 0.1 mL of *0.01 M hydrochloric acid*. The solution is pink. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve the contents of a vial of *venlafaxine for system suitability CRS* (containing impurities D and F) in 1.0 mL of the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography R* (5 µm) with a pore size of 10 nm.

*Mobile phase*: mix 510 volumes of *acetonitrile R* and 1490 volumes of a solution prepared as follows: dissolve 17 g of *ammonium dihydrogen phosphate R* in 1490 mL of *water R* and adjust to pH 4.4 using *phosphoric acid R*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 225 nm.

*Injection*: 20 µL.

*Run time*: 10 times the retention time of venlafaxine.

*Relative retention* with reference to venlafaxine (retention time = about 9 min): impurity D = about 0.9; impurity F = about 3.4.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.5 between the peaks due to impurity D and venlafaxine.

*Limits*:

- *impurity F*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.1 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* at 80 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in a mixture of 5.0 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R*. Carry out a potentiometric titration (*2.2.20*), using *0.1 M sodium hydroxide*. Read the volume added between the 2 points of inflexion. Carry out a blank titration.

1 mL of *0.1 M sodium hydroxide* is equivalent to 31.39 mg of $C_{17}H_{28}ClNO_2$.

IMPURITIES

*Specified impurities: F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, G, H.*

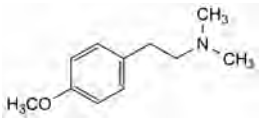

A. 2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine,

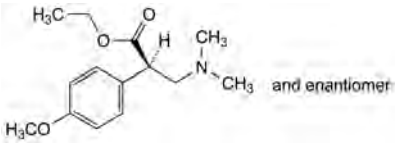


B. ethyl (2*RS*)-3-(dimethylamino)-2-(4-methoxyphenyl)-propanoate,

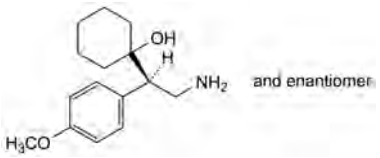


C. 1-[(1*RS*)-2-amino-1-(4-methoxyphenyl)ethyl]-cyclohexanol,

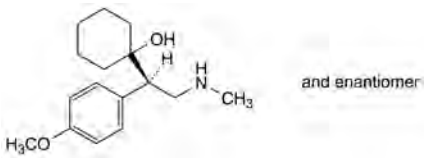


D. 1-[(1*RS*)-1-(4-methoxyphenyl)-2-(methylamino)ethyl]-cyclohexanol,

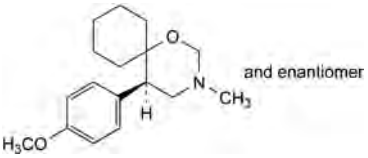


E. (5*RS*)-5-(4-methoxyphenyl)-3-methyl-1-oxa-3-azaspiro[5.5]undecane,

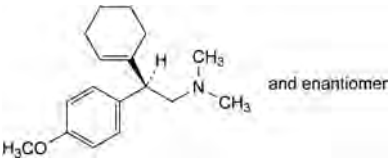


F. (2*RS*)-2-(cyclohex-1-enyl)-2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine,

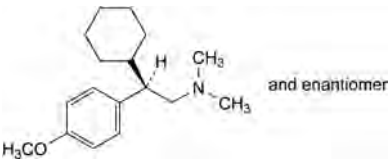


G. (2*RS*)-2-cyclohexyl-2-(4-methoxyphenyl)-*N*,*N*-dimethylethanamine,

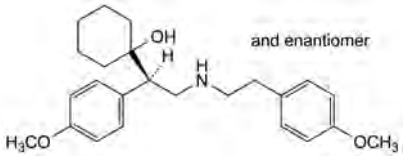


H. 1-[(1*RS*)-1-(4-methoxyphenyl)-2-[[2-(4-methoxyphenyl)-ethyl]amino]ethyl]cyclohexanol.

**01/2017:0573**

# VERAPAMIL HYDROCHLORIDE

## Verapamili hydrochloridum

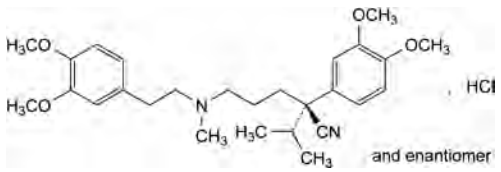


$C_{27}H_{39}ClN_2O_4$ $M_r$ 491.1
[152-11-4]

DEFINITION

(2*RS*)-2-(3,4-Dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]-2-(1-methylethyl)pentanenitrile hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, freely soluble in methanol, sparingly soluble in ethanol (96 per cent).

mp: about 144 °C.

IDENTIFICATION

*First identification*: *B, D.*

*Second identification*: *A, C, D.*

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 20.0 mg in *0.01 M hydrochloric acid* and dilute to 100.0 mL with the same acid. Dilute 5.0 mL of this solution to 50.0 mL with *0.01 M hydrochloric acid*.

*Spectral range*: 210-340 nm.

*Absorption maxima*: at 229 nm and 278 nm.

*Shoulder*: at 282 nm.

*Absorbance ratio*: $A_{278}/A_{229}$ = 0.35 to 0.39.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *verapamil hydrochloride CRS.*

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 5 mL with the same solvent.

*Reference solution (a).* Dissolve 20 mg of *verapamil hydrochloride CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 5 mg of *papaverine hydrochloride CRS* in reference solution (a) and dilute to 5 mL with the same solution.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *diethylamine R, cyclohexane R* (15:85 *V/V*).

*Application*: 5 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated principal spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

D. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 1.0 g in *carbon dioxide-free water R* while gently heating and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.5 to 6.0 for solution S.

**Optical rotation** (*2.2.7*): – 0.10° to + 0.10°, determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (37:63 *V/V*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 5 mg of *verapamil hydrochloride CRS*, 5 mg of *verapamil impurity I CRS* and 5 mg of *verapamil impurity M CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture. Dilute 1 mL of this solution to 10 mL with the solvent mixture.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped polar-embedded octadecylsilyl amorphous organosilica polymer R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 6.97 g/L solution of *dipotassium hydrogen phosphate R* adjusted to pH 7.20 with *phosphoric acid R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 22 | 63 | 37 |
| 22 - 27 | 63 → 35 | 37 → 65 |
| 27 - 35 | 35 | 65 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 278 nm.

*Injection*: 10 µL.

*Relative retention* with reference to verapamil (retention time = about 15 min): impurity I = about 1.3; impurity M = about 2.4.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to verapamil and impurity I;
– impurity M elutes from the column.

*Limits*:

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.400 g in 50 mL of *anhydrous ethanol R* and add 5.0 mL of *0.01 M hydrochloric acid*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Measure the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 49.11 mg of $C_{27}H_{39}ClN_2O_4$.

STORAGE

Protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P.*

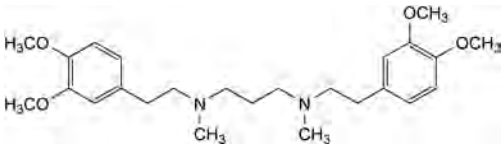

A. *N,N′*-bis[2-(3,4-dimethoxyphenyl)ethyl]-*N,N′*-dimethylpropane-1,3-diamine,

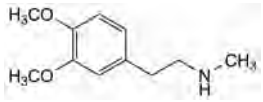

B. 2-(3,4-dimethoxyphenyl)-*N*-methylethanamine,

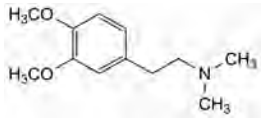

C. 2-(3,4-dimethoxyphenyl)-*N,N*-dimethylethanamine,

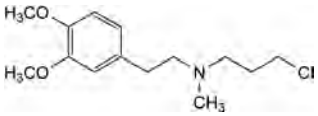

D. 3-chloro-*N*-[2-(3,4-dimethoxyphenyl)ethyl]-*N*-methylpropan-1-amine,

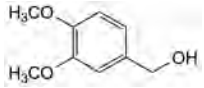

E. (3,4-dimethoxyphenyl)methanol,

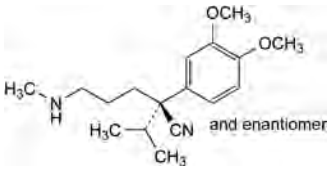

F. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-(methylamino)-2-(1-methylethyl)pentanenitrile,

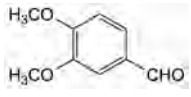

G. 3,4-dimethoxybenzaldehyde,

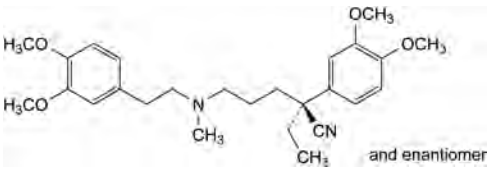

H. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]-2-ethylpentanenitrile,

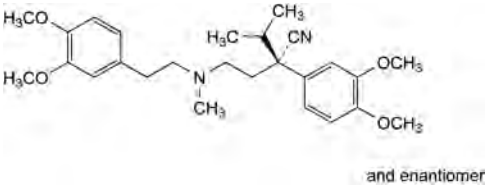

I. (2*RS*)-2-(3,4-dimethoxyphenyl)-2-[2-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]ethyl]-3-methylbutanenitrile,

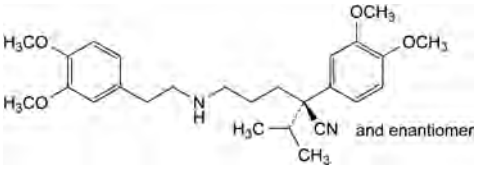

J. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl]amino]-2-(1-methylethyl)pentanenitrile (*N*-norverapamil),

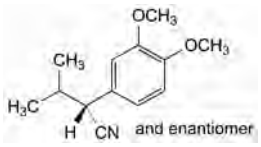

K. (2*RS*)-2-(3,4-dimethoxyphenyl)-3-methylbutanenitrile,

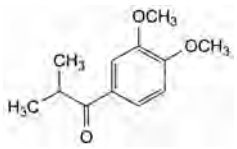

L. 1-(3,4-dimethoxyphenyl)-2-methylpropan-1-one,

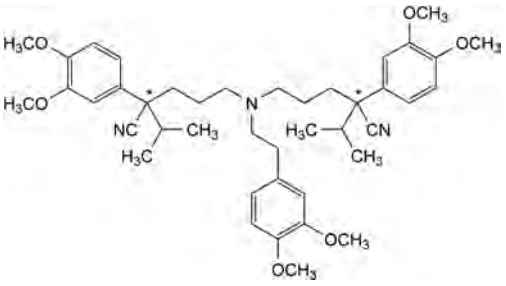

M. 5,5′-[[2-(3,4-dimethoxyphenyl)ethyl]imino]bis[2-(3,4-dimethoxyphenyl)-2-(1-methylethyl)pentanenitrile],

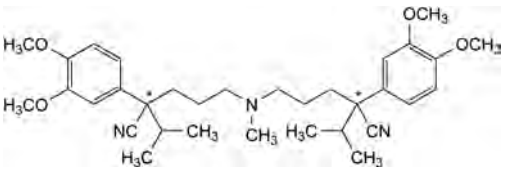

N. 5,5′-(methylimino)bis[2-(3,4-dimethoxyphenyl)-2-(1-methylethyl)pentanenitrile],

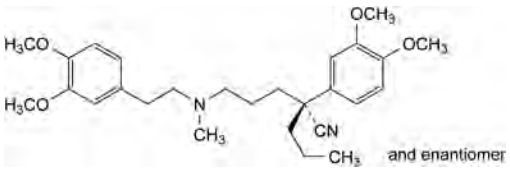


O. (2*RS*)-2-(3,4-dimethoxyphenyl)-5-[[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino]- 2-propylpentanenitrile,

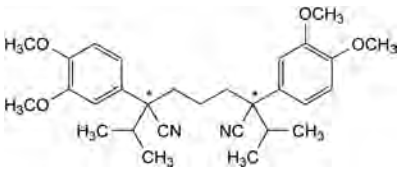


P. 2,6-bis(3,4-dimethoxyphenyl)-2,6-bis(1-methylethyl)-heptane-1,7-dinitrile.

**01/2020:2305**

# VIGABATRIN

## Vigabatrinum

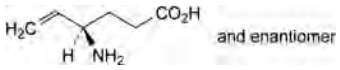


$C_6H_{11}NO_2$ $M_r$ 129.2
[60643-86-9]

DEFINITION

(4*RS*)-4-Aminohex-5-enoic acid.

*Content*: 98.5 per cent to 101.5 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: freely soluble in water, slightly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *vigabatrin CRS*.

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R*, using sonication if necessary, and dilute to 5.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 7.5 mg of *vigabatrin impurity A CRS* and 7.5 mg of *vigabatrin impurity B CRS* in *water R* and dilute to 50.0 mL with the same solvent. Dilute 2.0 mL of the solution to 20.0 mL with *water R*.

*Reference solution (c).* Dissolve 5.0 mg of *vigabatrin impurity E CRS* and 10.0 mg of *vigabatrin impurity D CRS* in *water R* and dilute to 50.0 mL with the same solvent. To 2.0 mL of the solution add 1.0 mL of reference solution (a) and dilute to 20.0 mL with *water R*.

*Reference solution (d).* Dilute 2 mL of reference solution (b) to 10 mL with *water R*.

*Column*:

– *size*: *l* = 0.10 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped solid core phenylhexylsilyl silica gel for chromatography R* (2.7 µm);
– *temperature*: 45 °C.

*Mobile phase*: dissolve 2.1 g of *perfluoroheptanoic acid R* in a mixture of 195 mL of *methanol R2* and 805 mL of *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Post-column solution*: *methanol R2*.

*Post-column flow rate*: 0.8 mL/min.

*Detection*: charged aerosol detector (gas source: nitrogen at 35 psi) and, for impurities A and B, spectrophotometer at 210 nm.

*Autosampler*: set at 15 °C.

*Injection*: 30 µL of the test solution and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of vigabatrin.

*Identification of impurities*: use the chromatogram obtained with the spectrophotometer and with reference solution (b) to identify the peaks due to impurities A and B; use the chromatogram obtained with the charged aerosol detector and with reference solution (c) to identify the peaks due to impurities D and E.

*Relative retention* with reference to vigabatrin (retention time = about 11 min): impurity A = about 0.3; impurity E = about 0.5; impurity D = about 0.6; impurity B = about 2.3.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to impurities E and D in the chromatogram obtained with reference solution (c);
– *signal-to-noise ratio*: minimum 15 for the peak due to vigabatrin in the chromatogram obtained with reference solution (c); minimum 10 for the peak due to impurity B in the chromatogram obtained with reference solution (d);
– *symmetry factor*: minimum 0.6 for the peaks due to impurity D and vigabatrin in the chromatogram obtained with reference solution (c); minimum 0.6 for the peaks due to impurities A and B in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:

– for impurities A and B, use the concentration of each impurity in reference solution (b);
– for impurity D, use the concentration of impurity D in reference solution (c);
– for impurities other than A, B and D, use the concentration of vigabatrin in reference solution (c).

*Limits*:

– *impurity D*: maximum 0.2 per cent;
– *impurities A, B (spectrophotometer at 210 nm)*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.05 per cent;
– *total*: maximum 0.5 per cent;
– *reporting threshold*: 0.03 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.300 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 90 mg in 50 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 12.92 mg of $C_6H_{11}NO_2$.

IMPURITIES

*Specified impurities: A, B, D.*

Monographs U-V

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): E.

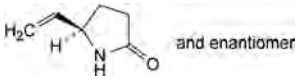


A. (5*RS*)-5-ethenylpyrrolidin-2-one,

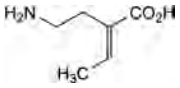

B. (2*E*)-2-(2-aminoethyl)but-2-enoic acid,

D. 4-aminobutanoic acid (GABA),

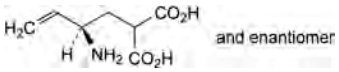


E. 2-[(2*RS*)-2-aminobut-3-en-1-yl]propanedioic acid.

**01/2008:0748**
**corrected 10.0**

# VINBLASTINE SULFATE

## Vinblastini sulfas

$C_{46}H_{60}N_4O_{13}S$ $M_r$ 909
[143-67-9]

DEFINITION

Vinblastine sulfate contains not less than 95.0 per cent and not more than the equivalent of 104.0 per cent of methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulfate, calculated with reference to the dried substance.

CHARACTERS

A white or slightly yellowish, crystalline powder, very hygroscopic, freely soluble in water, practically insoluble in alcohol.

IDENTIFICATION

A. Examine by infrared absorption spectrophotometry (*2.2.24*), comparing with the *Ph. Eur. reference spectrum of vinblastine sulfate*.

B. Examine the chromatograms obtained in the assay. The principal peak in the chromatogram obtained with the test solution is similar in position and approximate size to the principal peak in the chromatogram obtained with reference solution (a).

TESTS

**Solution S**. Dissolve 50.0 mg in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**pH** (*2.2.3*). Dilute 3 mL of solution S to 10 mL with *carbon dioxide-free water R*. The pH of this solution is 3.5 to 5.0.

**Related substances**. Examine the chromatograms obtained in the assay. In the chromatogram obtained with the test solution, the area of any peak apart from the principal peak is not greater than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent) and the sum of the areas of any such peaks is not greater than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent). Disregard any peak with an area less than that of the peak in the chromatogram obtained with reference solution (d).

**Loss on drying**. Not more than 15.0 per cent, determined on 3 mg by thermogravimetry (*2.2.34*). Heat to 200 °C at a rate of 5 °C/min, under a stream of *nitrogen for chromatography R*, at a flow rate of 40 mL/min.

ASSAY

Examine by liquid chromatography (*2.2.29*).

*Keep the solutions in iced water before use.*

*Test solution.* Dilute 1.0 mL of solution S (see Tests) to 5.0 mL with *water R*.

*Reference solution (a).* Dissolve the contents of a vial of *vinblastine sulfate CRS* in 5.0 mL of *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (b).* Dissolve 1.0 mg of *vincristine sulfate CRS* in 1.0 mL of reference solution (a).

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with *water R*.

*Reference solution (d).* Dilute 1.0 mL of reference solution (c) to 20.0 mL with *water R*.

The chromatographic procedure may be carried out using:

- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octylsilyl silica gel for chromatography R* (5 µm). Place between the injector and the column a precolumn packed with suitable silica gel,
- as mobile phase at a flow rate of 1.0 mL/min a mixture of 38 volumes of a 1.5 per cent *V/V* solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R*, 12 volumes of *acetonitrile R* and 50 volumes of *methanol R*,
- as detector a spectrophotometer set at 262 nm,
- a loop injector.

Inject 10 µL of each solution and record the chromatograms for 3 times the retention time of the peak due to vinblastine. The assay is not valid unless: in the chromatogram obtained with reference solution (b) the resolution between the peaks due to vincristine and vinblastine is not less than 4; the peak in the chromatogram obtained with reference solution (d) has a signal-to-noise ratio not less than 5. Calculate the percentage content of $C_{46}H_{60}N_4O_{13}S$ from the area of the principal peak in each of the chromatograms obtained with the test solution and reference solution (a) and from the declared content of *vinblastine sulfate CRS*.

STORAGE

Store in an airtight, glass container, protected from light, at a temperature not exceeding – 20 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident glass container.

**01/2020:1800**

# VINCAMINE

## Vincaminum

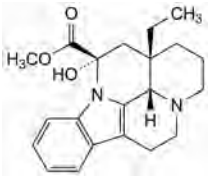

$C_{21}H_{26}N_2O_3$ $M_r$ 354.5
[1617-90-9]

DEFINITION

Methyl 14-hydroxyvincane-14β-carboxylate.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *vincamine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 44.3 to + 49.0 (dried substance).

Dissolve 0.1 g in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use. Dissolve the samples using sonications, while avoiding any overheating.*

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 10 mL of *tetrahydrofuran R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 200.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of *vincamine for system suitability CRS* (containing impurities A, B and C) in the mobile phase and dilute to 10 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with embedded polar groups R* (5 µm).

*Mobile phase*: *tetrahydrofuran R*, *acetonitrile R*, 15.4 g/L solution of *ammonium acetate R* (17:18:65 *V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 272 nm.

*Injection*: 20 µL.

*Run time*: 3.5 times the retention time of vincamine.

*Identification of impurities*: use the chromatogram supplied with *vincamine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to vincamine (retention time = about 10 min): impurity A = about 0.8; impurity B = about 0.9; impurity C = about 1.35.

*System suitability*: reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to impurity B and vincamine.

*Calculation of percentage contents*:
- for each impurity, use the concentration of vincamine in reference solution (a).

*Limits*:
- *impurities A, C*: for each impurity, maximum 0.5 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.0 per cent;
- *reporting threshold*: 0.05 per cent.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.2 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 30 mL of a mixture of 1 volume of *acetic anhydride R* and 5 volumes of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.45 mg of $C_{21}H_{26}N_2O_3$.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D.*

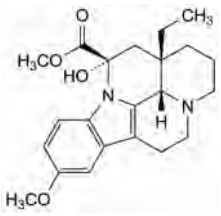

A. methyl 14-hydroxy-10-methoxyvincane-14β-carboxylate (10-methoxyvincamine),

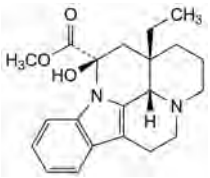

B. methyl 14-hydroxyvincane-14α-carboxylate (14-*epi*-vincamine),

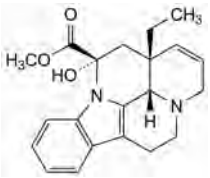

C. methyl 14-hydroxy-17,18-didehydrovincane-14β-carboxylate (17,18-didehydrovincamine),

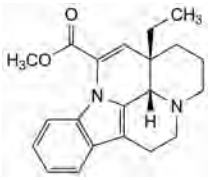

D. methyl 14,15-didehydrovincane-14-carboxylate (apovincamine).

**01/2008:0749**
**corrected 10.0**

# VINCRISTINE SULFATE

## Vincristini sulfas

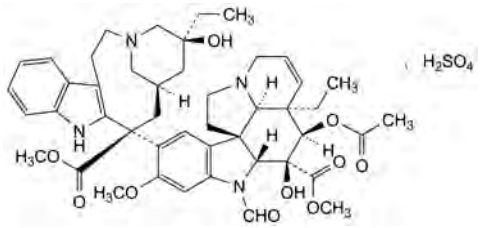

$C_{46}H_{58}N_4O_{14}S$ $M_r$ 923
[2068-78-2]

DEFINITION

Methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate sulfate.

*Content*: 95.0 per cent to 104.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder, very hygroscopic.

*Solubility*: freely soluble in water, slightly soluble in alcohol.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of vincristine sulfate.*

TESTS

**Solution S**. Dissolve 50.0 mg in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent. *Keep the solution in iced water to carry out the test for related substances.*

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 3.5 to 4.5.

Dilute 2 mL of solution S to 10 mL with *carbon dioxide-free water R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Keep the solutions in iced water before use.*

*Test solution*. Dilute 1.0 mL of solution S to 5.0 mL with *water R*.

*Reference solution (a)*. Dissolve the contents of a vial of *vincristine sulfate CRS* in 5.0 mL of *water R* to obtain a concentration of 1.0 mg/mL.

*Reference solution (b)*. Dissolve 1.0 mg of *vinblastine sulfate CRS* in 1.0 mL of reference solution (a).

*Reference solution (c)*. Dilute 1.0 mL of the test solution to 50.0 mL with *water R*.

*Reference solution (d)*. Dilute 1.0 mL of reference solution (c) to 20.0 mL with *water R*.

*Precolumn*:

– *stationary phase*: *octylsilyl silica gel for chromatography R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm,
– *stationary phase*: *octylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 1.5 per cent *V/V* solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R*,
– *mobile phase B*: *methanol R*,

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 12 | 38 | 62 |
| 12 - 27 | 38 → 8 | 62 → 92 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 297 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):

– *resolution*: minimum 4 between the peaks due to vincristine and vinblastine.

*Limits*:

– *any impurity*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (2.0 per cent),
– *total*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (5.0 per cent),
– *disregard limit*: area of the peak in the chromatogram obtained with reference solution (d) (0.1 per cent).

**Loss on drying**: maximum 12.0 per cent, determined on 3 mg by thermogravimetry (*2.2.34*). Heat the substance to be examined to 200 °C increasing the temperature by 5 °C/min, under a current of *nitrogen for chromatography R*, at a flow rate of 40 mL/min.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances, with the following modifications.

*Mobile phase*: mix 30 volumes of a 1.5 per cent *V/V* solution of *diethylamine R* adjusted to pH 7.5 with *phosphoric acid R* and 70 volumes of *methanol R*.

*Flow rate*: 1.0 mL/min.

Calculate the percentage content of $C_{46}H_{58}N_4O_{14}S$ using the chromatogram obtained with reference solution (a) and the declared content of *vincristine sulfate CRS*.

STORAGE

In an airtight, glass container, protected from light, at a temperature not exceeding – 20 °C. If the substance is sterile, store in a sterile, airtight, tamper-evident glass container.

IMPURITIES

*Specified impurities*: *A, B, C, D, H.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *E, F, G.*

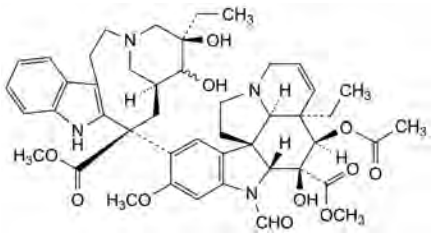

A. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*R*,7*S*,9*S*)-5-ethyl-5,6-dihydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-b]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (3′-hydroxy-VCR),

B. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*R*,7*S*,9*S*)-5-ethyl-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (4′-deoxyvincristine),

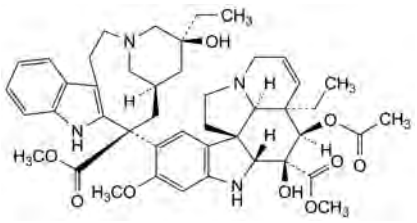

C. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*S*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]indol-9-yl]-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (*N*-desmethylvinblastine),

D. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino-[5,4-*b*]indol-9-yl]-6-formyl-4,5-dihydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino-[8,1-*cd*]carbazole-5-carboxylate (deacetylvincristine),

E. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-3a-ethyl-9-[(5*S*,7*R*,9*S*)-5-ethyl-5-hydroxy-9-(methoxycarbonyl)-1,4,5,6,7,8,9,10-octahydro-2*H*-3,7-methanoazacycloundecino[5,4-*b*]-indol-9-yl]-4,5-dihydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino-[8,1-*cd*]carbazole-5-carboxylate (deacetylvinblastine),

F. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(1a*S*,11*S*,13*S*,13a*R*)-1a-ethyl-11-(methoxycarbonyl)-1a,4,5,10,11,12,13,13a-octahydro-2*H*-3,13-methano-oxireno[9,10]azacycloundecino[5,4-*b*]indol-11-yl]-5-hydroxy-8-methoxy-6-methyl-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (leurosine),

G. methyl (3a*R*,4*R*,5*S*,5a*R*,10b*R*,13a*R*)-4-(acetyloxy)-3a-ethyl-9-[(1a*S*,11*S*,13*S*,13a*R*)-1a-ethyl-11-(methoxycarbonyl)-1a,4,5,10,11,12,13,13a-octahydro-2*H*-3,13-methano-oxireno[9,10]azacycloundecino[5,4-*b*]indol-11-yl]-6-formyl-5-hydroxy-8-methoxy-3a,4,5,5a,6,11,12,13a-octahydro-1*H*-indolizino[8,1-*cd*]carbazole-5-carboxylate (formylleurosine),

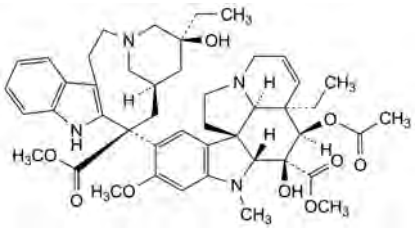

H. vinblastine.

**01/2018:1276**
**corrected 10.0**

# VINDESINE SULFATE

## Vindesini sulfas

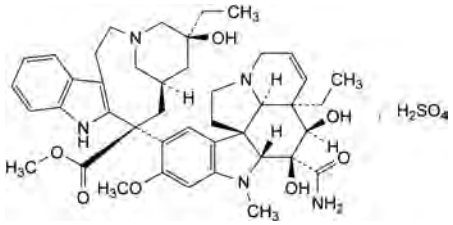

$C_{43}H_{57}N_5O_{11}S$ $M_r$ 852
[59917-39-4]

DEFINITION

3-(Carbamoyl)-$O^4$-deacetyl-3-de(methoxycarbonyl)vincaleukoblastine sulfate.

*Content*: 96.0 per cent to 103.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, amorphous, hygroscopic substance.

*Solubility*: freely soluble in water and in methanol, practically insoluble in cyclohexane.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of vindesine sulfate*.

TESTS

**Solution S**. Dissolve 50 mg in *carbon dioxide-free water R* and dilute to 10 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_7$ (*2.2.2, Method I*).

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Keep the solutions in iced water before use.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

*Reference solution (b).* Dissolve 1.0 mg of *desacetylvinblastine CRS* in *water R*, add 1.0 mL of the test solution and dilute to 50.0 mL with *water R*.

*Reference solution (c).* In order to prepare impurity A *in situ*, dissolve 0.2 g of the substance to be examined in *dilute hydrogen peroxide solution R* and dilute to 20.0 mL with the same solvent. Dilute 2.0 mL of the solution to 10.0 mL with *water R*. Inject the solution within 1 h of preparation.

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:
- *mobile phase A*: 1.5 per cent *V/V* solution of *diethylamine R* adjusted to pH 7.4 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 49 | 51 |
| 40 - 49 | 49 → 30 | 51 → 70 |
| 49 - 60 | 30 | 70 |

*Flow rate*: 2 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 200 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to vindesine (retention time = about 25 min): impurity A = about 0.2.

*System suitability*: reference solution (b):
- the retention time of vindesine is less than 40 min;
- *resolution*: minimum 2.0 between the peaks due to vindesine and desacetylvinblastine;
- *symmetry factor*: maximum 2.0 for the peak due to vindesine.

*Limits*:
- *impurity A*: not more than 2.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);
- *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.8 per cent);
- *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Water** (*2.5.32*): maximum 5.0 per cent, determined on 50.0 mg using the evaporation technique at 150 °C; weigh the sample in an inert atmosphere and carry out a blank test.

ASSAY

Liquid chromatography (*2.2.29*). *Keep the solutions in iced water before use.*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in *water R* and dilute to 10.0 mL with the same solvent.

*Reference solution (a).* Dissolve and dilute the entire contents of a vial of *vindesine sulfate CRS* with *water R* to yield a concentration of approximately 0.50 mg/mL.

*Reference solution (b).* Add 1.0 mg of *desacetylvinblastine CRS* to 2.0 mL of reference solution (a).

*Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 38 volumes of a 1.5 per cent *V/V* solution of *diethylamine R*, previously adjusted to pH 7.4 with *phosphoric acid R*, and 62 volumes of *methanol R*.

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*System suitability*: reference solution (b):
- *resolution*: minimum 1.5 between the peaks due to vindesine and desacetylvinblastine;
- *symmetry factor*: maximum 2.0 for the peak due to vindesine;
- *repeatability*: maximum relative standard deviation of 1.5 per cent for the peak due to vindesine after 5 injections.

Calculate the percentage content of $C_{43}H_{57}N_5O_{11}S$ taking into account the assigned content of *vindesine sulfate CRS*.

STORAGE

In an airtight, high-density polyethylene container with a high-density polyethylene cap, at a temperature of – 50 °C or below. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C.*

A. 3-(carbamoyl)-$O^4$-deacetyl-3-de(methoxycarbonyl)vincaleukoblastine $N^{6'}$-oxide (vindesine $N^3$-oxide),

B. vincaleukoblastine (vinblastine),

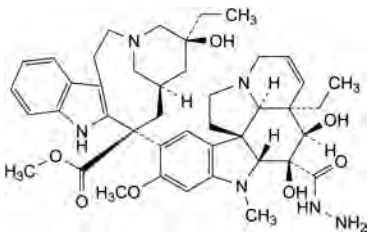

C. $O^4$-deacetyl-23-demethoxy-23-hydrazinylvincaleukoblastine (deacetylvinblastine hydrazide).

**01/2020:2107**

# VINORELBINE TARTRATE

## Vinorelbini tartras

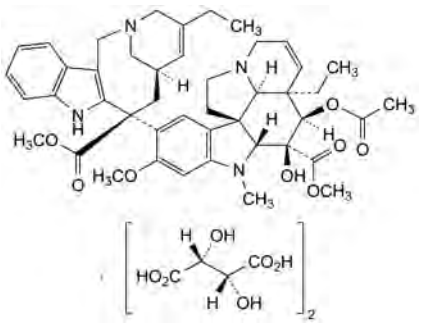

$C_{53}H_{66}N_4O_{20}$ $M_r$ 1079
[125317-39-7]

DEFINITION

4′-Deoxy-3′,4′-didehydro-8′-norvincaleukoblastine dihydrogen bis[(2*R*,3*R*)-2,3-dihydroxybutanedioate].

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder, hygroscopic.

*Solubility*: freely soluble in water and in methanol, practically insoluble in hexane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 10 mg in 5 mL of *water R*. Add 0.5 mL of *sodium hydroxide solution R*. Extract with 5 mL of *methylene chloride R*. Dry the organic layer over *anhydrous sodium sulfate R*, filter and reduce its volume to about 0.5 mL by evaporation and apply to a disc of *potassium bromide R*. Evaporate and record the spectrum.

*Comparison*: *vinorelbine tartrate CRS*, treated as described above.

B. It gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Solution S**. Dissolve a quantity equivalent to 0.140 g of the anhydrous substance in *carbon dioxide-free water R* and dilute to 10.0 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and its absorbance (*2.2.25*) at 420 nm is not greater than 0.030.

**pH** (*2.2.3*): 3.3 to 3.8 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*): use the normalisation procedure.

*Solvent mixture*: mobile phase A, mobile phase B (45:55 *V/V*).

*Test solution.* Dissolve 35.0 mg of the substance to be examined in the solvent mixture and dilute to 25.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 14 mg of *vinorelbine tartrate CRS* in *water R* and dilute to 10 mL with the same solvent. Expose this solution for 1 h to a xenon lamp apparatus at a wavelength of 310-880 nm, supplying a dose of 1600 kJ/m$^2$ at a fluence rate of 500 W/m$^2$ in order to generate impurity A.

*Reference solution (b).* Dilute 1.0 mL of the test solution to 20.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 100.0 mL with the solvent mixture.

*Reference solution (c).* Dissolve 7 mg of *vinorelbine for peak identification A CRS* (containing impurity K) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Reference solution (d).* Dissolve 7 mg of *vinorelbine for peak identification B CRS* (containing impurities C and I) in the solvent mixture and dilute to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*:

- *mobile phase A*: mix 14 mL of *diethylamine R* and 986 mL of *water for chromatography R* and adjust to pH 7.5 with *dilute phosphoric acid R*;
- *mobile phase B*: *acetonitrile R*, *methanol R* (20:80 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 45 | 55 |
| 5 - 45 | 45 → 20 | 55 → 80 |
| 45 - 50 | 20 | 80 |

*Flow rate*: 2.0 mL/min.

*Detection*: spectrophotometer at 267 nm.

*Autosampler*: set at 5 °C.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peak due to impurity A; use the chromatogram supplied with *vinorelbine for peak identification B CRS* and the chromatogram obtained with reference solution (d) to identify the peaks due to impurities C and I; use the chromatogram supplied with *vinorelbine for peak identification A CRS* and the chromatogram obtained with reference solution (c) to identify the peak due to impurity K.

*Relative retention* with reference to vinorelbine (retention time = about 20 min): impurity C = about 0.65; impurity K = about 0.8; impurity A = about 0.9; impurity I = about 1.2.

*System suitability*: reference solution (a):

- *resolution*: minimum 1.5 between the peaks due to impurity A and vinorelbine.

*Limits*:

- *impurity A*: maximum 0.3 per cent;
- *impurities C, I, K*: for each impurity, maximum 0.2 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent (principal peak in the chromatogram obtained with reference solution (b)).

**Boron**: maximum 50 ppm.

*Test solution.* Dissolve 0.10 g of the substance to be examined in 2 mL of *water R*. Slowly add 10.0 mL of *sulfuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 mL of a 0.5 g/L solution of *carminic acid R* in *sulfuric acid R*.

*Reference solution.* Dilute 2.5 mL of a 0.572 g/L solution of *boric acid R* to 100.0 mL with *water R*. To 2.0 mL of this solution slowly add 10.0 mL of *sulfuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 mL of a 0.5 g/L solution of *carminic acid R* in *sulfuric acid R*.

*Blank solution.* To 2.0 mL of *water R* slowly add 10.0 mL of *sulfuric acid R* while cooling in iced water. Stir and allow to warm to room temperature. Add 10.0 mL of a 0.5 g/L solution of *carminic acid R* in *sulfuric acid R*.

After 45 min, measure the absorbance (*2.2.25*) of the test solution and the reference solution, between 560 nm and 650 nm, using the blank solution as compensation liquid. The maximum absorbance value of the test solution is not greater than that of the reference solution.

**Fluorides**: maximum 50 ppm.

Potentiometry (*2.2.36, Method I*) using a fluoride-selective indicator electrode and a silver-silver chloride reference electrode.

*Test solution.* Dissolve 0.19 g of the substance to be examined in 20 mL of *water R*. Add 5.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50 mL with *water R*.

*Reference solutions.* To 0.6 mL, 0.8 mL, 1.0 mL, 1.2 mL and 1.4 mL of *fluoride standard solution (10 ppm F) R*, add 5.0 mL of *total-ionic-strength-adjustment buffer R* and dilute to 50 mL with *water R*.

Introduce the electrodes into the reference solutions and allow to stand for 5 min. Determine the potential difference between the electrodes after 1 min of stabilisation. Using semi-logarithmic paper plot the potential difference obtained for each reference solution as a function of concentration of fluoride. Using exactly the same conditions, determine the potential difference obtained with the test solution and calculate the content of fluoride.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.250 g.

ASSAY

Dissolve 0.350 g in 40 mL of *glacial acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 53.96 mg of $C_{53}H_{66}N_4O_{20}$.

STORAGE

Under an inert gas, protected from light, at a temperature not exceeding – 15 °C.

IMPURITIES

*Specified impurities: A, C, I, K.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, G, H, J.*

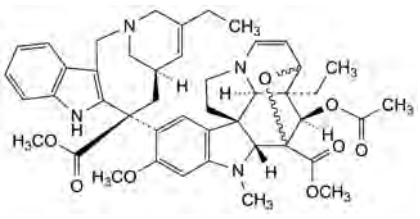

A. 3,4′-dideoxy-3,6ξ-epoxy-3′,4′,7,8-tetradehydro-6,7-dihydro-8′-nor-3ξ-vincaleukoblastine,

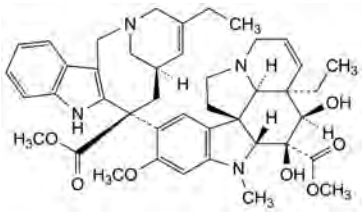

B. $O^4$-deacetyl-4′-deoxy-3′,4′-didehydro-8′-norvincaleukoblastine,

C. unknown structure,

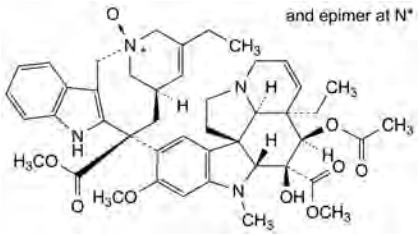


D. (6′*RS*)-4′-deoxy-3′,4′-didehydro-8′-norvincaleukoblastine 6′-oxide,

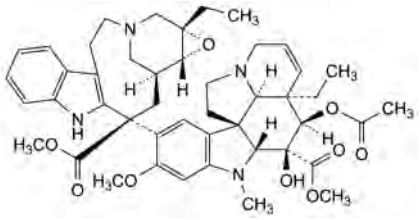

E. 4′-deoxy-3′α,4′α-epoxyvincaleukoblastine (leurosine),

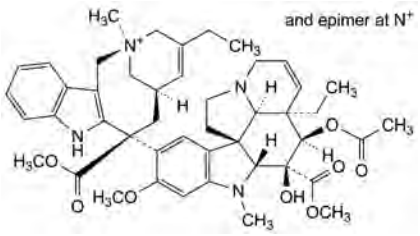


F. (6′*RS*)-4′-deoxy-6′-methyl-3′,4′-didehydro-6′-azonia-8′-norvincaleukoblastine,

G. 4′-deoxy-3′α,4′α-epoxy-8′-norvincaleukoblastine,

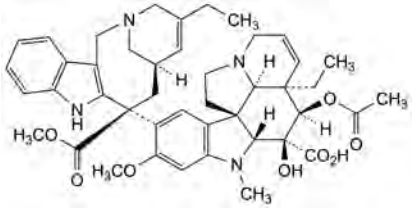

H. 4′-deoxy-3′,4′-didehydro-8′,24-dinorvincaleukoblastine,

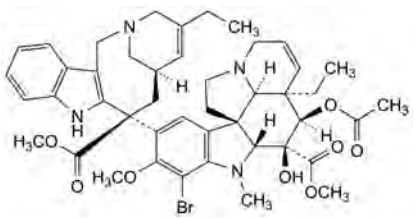

I. 17-bromo-4′-deoxy-3′,4′-didehydro-8′-norvincaleukoblastine,

J. 4′-deoxy-3′,4′-didehydrovincaleukoblastine,

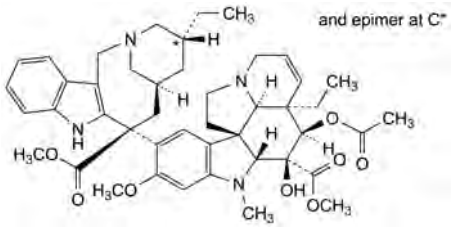


K. 4′-deoxy-8′-nor-4′αβ-vincaleukoblastine.

**01/2008:2139**
**corrected 7.3**

# VINPOCETINE

## Vinpocetinum

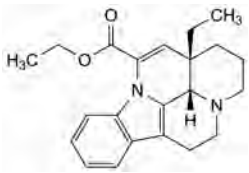

$C_{22}H_{26}N_2O_2$ $M_r$ 350.5
[42971-09-5]

DEFINITION

Ethyl (13a*S*,13b*S*)-13a-ethyl-2,3,5,6,13a,13b-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate.

*Content*: 98.5 per cent to 101.5 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellow, crystalline powder.

*Solubility*: practically insoluble in water, soluble in methylene chloride, slightly soluble in anhydrous ethanol.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *vinpocetine CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 127 to + 134 (dried substance).

Dissolve 0.25 g in *dimethylformamide R* and dilute to 25.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5.0 mg of *vinpocetine impurity B CRS*, 6.0 mg of *vinpocetine impurity A CRS*, 5.0 mg of *vinpocetine impurity C CRS* and 5.0 mg of *vinpocetine impurity D CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) and 1.0 mL of reference solution (b) to 20.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: 15.4 g/L solution of *ammonium acetate R*, *acetonitrile R* (45:55 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 280 nm.

*Injection*: 15 µL.

*Run time*: 3 times the retention time of vinpocetine.

*Relative retention* with reference to vinpocetine (retention time = about 16 min): impurity A = about 0.4; impurity D = about 0.68; impurity B = about 0.75; impurity C = about 0.83.

*System suitability*: reference solution (c):

- *resolution*: minimum 2.0 between the peaks due to impurities D and B.

*Limits*:

- *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.6 per cent);
- *impurities B, D*: for each impurity, not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.5 per cent);
- *impurity C*: not more than 0.6 times the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the peak due to vinpocetine in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than 10 times the area of the peak due to vinpocetine in the chromatogram obtained with reference solution (c) (1.0 per cent);
- *disregard limit*: 0.5 times the area of the peak due to vinpocetine in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying *in vacuo* in an oven at 100 °C for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in 50 mL of a mixture of equal volumes of *acetic anhydride R* and *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 35.05 mg of $C_{22}H_{26}N_2O_2$.

IMPURITIES

*Specified impurities: A, B, C, D.*

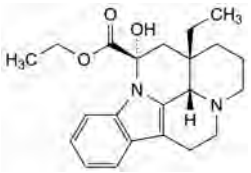

A. ethyl (12*S*,13a*S*,13b*S*)-13a-ethyl-12-hydroxy-2,3,5,6,12,13,13a,13b-octahydro-1*H*-indolo[3,2,1-*de*]-pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (ethyl vincaminate),

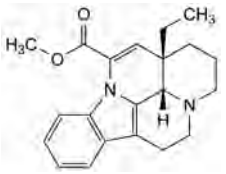

B. methyl (13a*S*,13b*S*)-13a-ethyl-2,3,5,6,13a,13b-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (apovincamine),

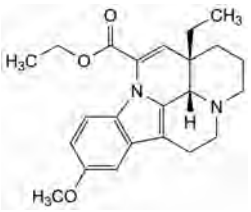

C. ethyl (13a*S*,13b*S*)-13a-ethyl-10-methoxy-2,3,5,6,13a,13b-hexahydro-1*H*-indolo[3,2,1-*de*]pyrido[3,2,1-*ij*][1,5]-naphthyridine-12-carboxylate (methoxyvinpocetine),

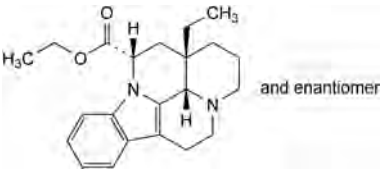


D. ethyl (12*RS*,13a*RS*,13b*RS*)-13a-ethyl-2,3,5,6,12,13,13a,13b-octahydro-1*H*-indolo[3,2,1-*de*]-pyrido[3,2,1-*ij*][1,5]naphthyridine-12-carboxylate (dihydrovinpocetine).

**01/2008:0034**
**corrected 6.0**

# VISCOSE WADDING, ABSORBENT

## Lanugo cellulosi absorbens

DEFINITION

Absorbent viscose wadding consists of bleached, carefully carded, new fibres of regenerated cellulose obtained by the viscose process, with or without the addition of titanium dioxide, of linear density 1.0 dtex to 8.9 dtex (dtex = mass of 10 000 m of fibre, expressed in grams) and cut to a suitable staple length. It does not contain any compensatory colouring matter.

CHARACTERS

It is white or very slightly yellow, has a lustrous or matt appearance, and is soft to the touch.

IDENTIFICATION

A. Viscose rayon fibres may be solid or hollow; hollow fibres may have a continuous lumen or be compartmented. The fibres have an average length of 25 mm to 80 mm and when examined under a microscope in the dry state, or when mounted in *alcohol R* and *water R*, the following characters are observed. They are usually of a more or less uniform width, with many longitudinal parallel lines distributed unequally over the width. The ends are cut more or less straight. Matt fibres contain numerous granular particles of approximately 1 µm average diameter.

*Solid fibres*. In longitudinal view, the surface of the fibres may be uneven or crenate. Fibres having an approximately circular or elliptical cross section have a diameter of about 10 µm to 20 µm and those that are flattened and twisted ribbons vary in width from 15 µm to 20 µm as the twisting of the filament reveals first the major axis and then the minor axis. They are about 4 µm in thickness. Other solid cross sections are Y-shaped and have protruding limbs with the major axis 5 µm to 25 µm in length and the minor axis 2 µm to 8 µm wide.

*Hollow fibres*. Fibres with a continuous, hollow lumen have a diameter of up to about 30 µm; they are thin-walled, with a wall thickness of about 5 µm. When mounted in *alcohol R* and *water R*, the lumen is clearly indicated in many fibres by the presence of many entrapped air bubbles.

*Compartmented fibres*. These fibres may have a diameter of up to 80 µm; they are hollow, having a central lumen which is divided up into several compartments. Individual compartments vary in size but typically may be up to about 60 µm in length and there may be more than one compartment across the width of each fibre. Some compartments show entrapped air bubbles when the fibres are mounted in *alcohol R* and *water R*.

B. When treated with *iodinated zinc chloride solution R*, the fibres become violet.

C. To 0.1 g add 10 mL of *zinc chloride-formic acid solution R*. Heat to 40 °C and allow to stand for 2 h 30 min, shaking occasionally. It dissolves completely except for the matt variety where titanium dioxide particles remain.

D. Dissolve the residue obtained in the test for sulfated ash by warming gently with 5 mL of *sulfuric acid R*. Allow to cool and add 0.2 mL of *dilute hydrogen peroxide solution R*. The solution obtained from the lustrous variety undergoes no change in colour; that from the matt variety shows an orange-yellow colour, the intensity of which depends on the quantity of titanium dioxide present.

TESTS

**Solution S**. Place 15.0 g in a suitable vessel, add 150 mL of *water R*, close the vessel and allow to macerate for 2 h. Decant the solution, squeeze the residual liquid carefully from the sample with a glass rod and mix. Reserve 10 mL of the solution for the test for surface-active substances and filter the remainder.

**Acidity or alkalinity**. To 25 mL of solution S add 0.1 mL of *phenolphthalein solution R* and to another 25 mL add 0.05 mL of *methyl orange solution R*. Neither solution is pink.

**Foreign fibres**. Examined under a microscope, it is seen to consist exclusively of viscose fibres, except that occasionally a few isolated foreign fibres may be present.

**Fluorescence**. Examine a layer about 5 mm in thickness under ultraviolet light at 365 nm. It displays only a slight brownish-violet fluorescence. It shows no intense blue fluorescence, apart from that which may be shown by a few isolated fibres.

**Absorbency**

*Apparatus*. A dry cylindrical copper-wire basket 8.0 cm high and 5.0 cm in diameter. The wire of which the basket is constructed is about 0.4 mm in diameter, the mesh is 1.5 cm to 2.0 cm wide and the mass of the basket is 2.7 ± 0.3 g.

*Sinking time*. Not more than 10 s. Weigh the basket to the nearest centigram ($m_1$). Take a total of 5.00 g in approximately equal quantities from 5 different places in the product to be examined, place loosely in the basket and weigh the filled basket to the nearest centigram ($m_2$). Fill a beaker 11 cm to 12 cm in diameter to a depth of 10 cm with water at about 20 °C. Hold the basket horizontally and drop it from a height of about 10 mm into the water. Measure with a stopwatch the time taken for the basket to sink below the surface of the water. Calculate the result as the average of 3 tests.

*Water-holding capacity*. Not less than 18.0 g of water per gram. After the sinking time has been measured, remove the basket from the water, allow it to drain for exactly 30 s suspended in a horizontal position over the beaker, transfer it to a tared beaker ($m_3$) and weigh to the nearest centigram ($m_4$). Calculate the water-holding capacity per gram of absorbent viscose wadding using the following expression:

$$\frac{m_4 - (m_2 + m_3)}{m_2 - m_1}$$

Calculate the result as the average of 3 tests.

**Ether-soluble substances**. Not more than 0.30 per cent. In an extraction apparatus, extract 5.00 g with *ether R* for 4 h at a rate of at least 4 extractions per hour. Evaporate the ether extract and dry the residue to constant mass at 100 °C to 105 °C.

**Extractable colouring matter**. In a narrow percolator, slowly extract 10.0 g with *alcohol R* until 50 mL of extract is obtained. The liquid obtained is not more intensely coloured (*2.2.2, Method II*) than reference solution $Y_5$, $GY_6$ or a reference solution prepared as follows: to 3.0 mL of blue primary solution add 7.0 mL of hydrochloric acid (10 g/L HCl) and dilute 0.5 mL of this solution to 10.0 mL with hydrochloric acid (10 g/L HCl).

**Surface-active substances**. Introduce the 10 mL portion of solution S reserved before filtration into a 25 mL graduated ground-glass-stoppered cylinder with an external diameter of 20 mm and a wall thickness of not greater than 1.5 mm, previously rinsed 3 times with *sulfuric acid R* and then with *water R*. Shake vigorously 30 times in 10 s, allow to stand for 1 min and repeat the shaking. After 5 min, any foam present does not cover the entire surface of the liquid.

**Water-soluble substances**. Not more than 0.70 per cent. Boil 5.00 g in 500 mL of *water R* for 30 min, stirring frequently. Replace the water lost by evaporation. Decant the liquid, squeeze the residual liquid carefully from the sample with a glass rod and mix. Filter the liquid whilst hot. Evaporate 400 mL of the filtrate (corresponding to 4/5 of the mass of the sample taken) and dry the residue to constant mass at 100 °C to 105 °C.

**Hydrogen sulfide**. To 10 mL of solution S add 1.9 mL of *water R*, 0.15 mL of *dilute acetic acid R* and 1 mL of *lead acetate solution R*. After 2 min, the solution is not more intensely coloured than a reference solution prepared at the same time using 0.15 mL of *dilute acetic acid R*, 1.2 mL of *thioacetamide reagent R*, 1.7 mL of *lead standard solution (10 ppm Pb) R* and 10 mL of solution S.

**Loss on drying** (*2.2.32*). Not more than 13.0 per cent, determined on 5.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*). Not more than 0.45 per cent for the lustrous variety and not more than 1.7 per cent for the matt variety. Introduce 5.00 g into a previously heated and cooled, tared crucible. Heat cautiously over a naked flame and then carefully to dull redness at 600 °C. Allow to cool, add a few drops of *dilute sulfuric acid R*, then heat and incinerate until all the black particles have disappeared. Allow to cool. Add a few drops of *ammonium carbonate solution R*. Evaporate and incinerate carefully, allow to cool and weigh again. Repeat the incineration for periods of 5 min to constant mass.

STORAGE

Store in a dust-proof package in a dry place.

**01/2008:0217**

# VITAMIN A

## Vitaminum A

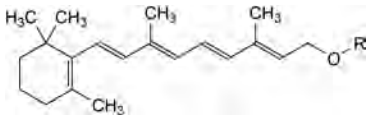

| Substance | R | Molecular formula | $M_r$ |
|---|---|---|---|
| all-(*E*)-retinol | H | $C_{20}H_{30}O$ | 286.5 |
| all-(*E*)-retinol acetate | CO-$CH_3$ | $C_{22}H_{32}O_2$ | 328.5 |
| all-(*E*)-retinol propionate | CO-$C_2H_5$ | $C_{23}H_{34}O_2$ | 342.5 |
| all-(*E*)-retinol palmitate | CO-$C_{15}H_{31}$ | $C_{36}H_{60}O_2$ | 524.9 |

DEFINITION

*Vitamin A* refers to a number of substances of very similar structure (including (*Z*)-isomers) found in animal tissues and possessing similar activity. The principal and biologically most active substance is all-(*E*)-retinol (all-(*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraen-1-ol; $C_{20}H_{30}O$). Vitamin A is generally used in the form of esters such as the acetate, propionate and palmitate.

*Synthetic retinol ester* refers to an ester (acetate, propionate or palmitate) or a mixture of synthetic retinol esters.

The activity of vitamin A is expressed in retinol equivalents (R.E.). 1 mg R.E. corresponds to the activity of 1 mg of all-(*E*)-retinol. The activity of the other retinol esters is calculated stoichiometrically, so that 1 mg R.E. of vitamin A corresponds to the activity of:

- 1.147 mg of all-(*E*)-retinol acetate,
- 1.195 mg of all-(*E*)-retinol propionate,
- 1.832 mg of all-(*E*)-retinol palmitate.

International Units (IU) are also used to express the activity of vitamin A. 1 IU of vitamin A is equivalent to the activity of 0.300 µg of all-(*E*)-retinol. The activity of the other retinol esters is calculated stoichiometrically, so that 1 IU of vitamin A is equivalent to the activity of:

- 0.344 µg of all-(*E*)-retinol acetate,
- 0.359 µg of all-(*E*)-retinol propionate,
- 0.550 µg of all-(*E*)-retinol palmitate,

1 mg of retinol equivalent is equivalent to 3333 IU.

CHARACTERS

*Appearance*:

Retinol acetate: pale-yellow crystals (mp: about 60 °C). Once melted retinol acetate tends to yield a supercooled melt.

Retinol propionate: reddish-brown oily liquid.

Retinol palmitate: a fat-like, light yellow solid or a yellow oily liquid, if melted (mp: about 26 °C).

*Solubility*: all retinol esters are practically insoluble in water, soluble or partly soluble in anhydrous ethanol and miscible with organic solvents.

Vitamin A and its esters are very sensitive to the action of air, oxidising agents, acids, light and heat.

*Carry out the assay and all tests as rapidly as possible, avoiding exposure to actinic light and air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and heat; use freshly prepared solutions.*

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution.* Prepare a solution containing about 3.3 IU of vitamin A per microlitre in *cyclohexane R* containing 1 g/L of *butylhydroxytoluene R*.

*Reference solution.* Prepare a 10 mg/mL solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *cyclohexane R* containing 1 g/L of *butylhydroxytoluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 3 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution:

- the chromatogram shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate.

*Results*: the composition of esters is confirmed by the correspondence of the principal spot or spots of the test solution with those obtained with the reference solution.

B. Related substances (see Tests).

TESTS

**Retinol**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Prepare a solution in *cyclohexane R*, stabilised with a solution containing 1 g/L of *butylhydroxytoluene R*, containing about 330 IU of vitamin A per microlitre.

*Reference solution.* Shake 1 mL of the test solution with 20 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol* for 2 min and dilute to 100 mL with *cyclohexane R*, stabilised with a solution containg 1 g/L of *butylhydroxytoluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 3 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution:

- in the chromatogram obtained no or only traces of the retinol esters are seen.

*Limit*: any spot corresponding to retinol in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with the reference solution (1.0 per cent).

**Related substances**. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* The solution described under Activity.

*Absorption maximum*: at 325 nm to 327 nm.

*Absorbance ratios*:

- $A_{300}/A_{326}$ = maximum 0.60;
- $A_{350}/A_{326}$ = maximum 0.54;
- $A_{370}/A_{326}$ = maximum 0.14.

The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ACTIVITY

The activity of the substance is determined in order to be taken into account for the production of concentrates.

Dissolve 25-100 mg, weighed with an accuracy of 0.1 per cent, in 5 mL of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10 IU/mL to 15 IU/mL. Measure the absorbance (*2.2.25*) at the absorption maximum at 326 nm. Calculate the activity of vitamin A in International Units per gram from the expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

$A_{326}$ = absorbance at 326 nm,

$m$ = mass of the substance to be examined, in grams,

$V$ = total volume to which the substance to be examined is diluted to give 10 IU/mL to 15 IU/mL,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

STORAGE

In an airtight container, protected from light.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram,
- the name of the ester or esters.

IMPURITIES

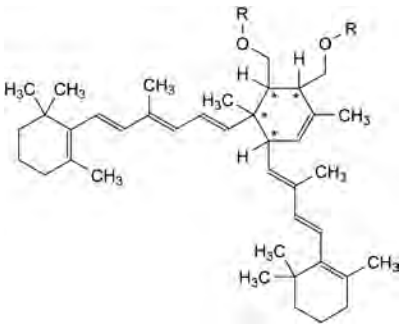

A. R = H, CO-$CH_3$: kitols (Diels-Alder dimers of vitamin A),

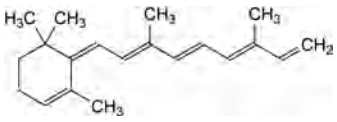

B. (3*E*,5*E*,7*E*)-3,7-dimethyl-9-[(1*Z*)-2,6,6-trimethylcyclohex-2-enylidene]nona-1,3,5,7-tetraene (anhydro-vitamin A),

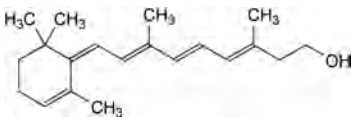

C. (3*E*,5*E*,7*E*)-3,7-dimethyl-9-[(1*Z*)-2,6,6-trimethylcyclohex-2-enylidene]nona-3,5,7-trien-1-ol (*retro*-vitamin A),

D. oxidation products of vitamin A.

**01/2018:0219**

# VITAMIN A CONCENTRATE (OILY FORM), SYNTHETIC

## Vitaminum A syntheticum densatum oleosum

DEFINITION

Oily concentrate prepared from synthetic retinol ester (*0217*) as is or by dilution with a suitable vegetable fatty oil.

*Content*: 95.0 per cent to 110.0 per cent of the vitamin A content stated on the label, which is not less than 500 000 IU/g.

It may contain suitable stabilisers such as antioxidants.

CHARACTERS

*Appearance*: yellow or brownish-yellow, oily liquid.

*Solubility*: practically insoluble in water, soluble or partly soluble in anhydrous ethanol, miscible with organic solvents.

Partial crystallisation may occur in highly concentrated solutions.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Prepare a solution containing about 3.3 IU of vitamin A per microlitre in *cyclohexane R* containing 1 g/L of *butylhydroxytoluene R*.

*Reference solution.* Prepare a 10 mg/mL solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *cyclohexane R* containing 1 g/L of *butylhydroxytoluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 3 µL.

*Development*: immediately, over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution:

- the chromatogram shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate.

*Results*: the composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

TESTS

**Acid value** (*2.5.1*): maximum 2.0, determined on 2.0 g.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0.

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light, air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat; use freshly prepared solutions. If partial crystallisation has occurred, homogenise the material at a temperature of about 65 °C, but avoid prolonged heating.*

*Carry out the assay according to Method A. If the assay is not shown to be valid, use Method B.*

**Method A**. Ultraviolet absorption spectrophotometry (*2.2.25*).

Dissolve 25-100 mg, weighed with an accuracy of 0.1 per cent, in 5 mL of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10-15 IU/mL.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio $A_\lambda/A_{326}$ for each wavelength.

If the ratios do not exceed: 0.60 at 300 nm, 0.54 at 350 nm, 0.14 at 370 nm, calculate the content of vitamin A in International Units per gram using the following expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

$A_{326}$ = absorbance at 326 nm,

$m$ = mass of the preparation to be examined, in grams,

$V$ = total volume to which the preparation to be examined is diluted to give 10-15 IU/mL,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

If one or more of the ratios $A_\lambda/A_{326}$ exceeds the values given, or if the wavelength of the absorption maximum does not lie between 325 nm and 327 nm, use Method B.

**Method B**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Introduce 0.100 g of the preparation to be examined into a 100 mL volumetric flask and dissolve immediately in 5 mL of *pentane R*. Add 40 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl gently and let the mixture react for 10 min at 60-65 °C for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to 100.0 mL with *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*, and homogenise carefully to avoid air bubbles.

*Test solution (b).* Dilute test solution (a) with *2-propanol R* to a final concentration of 100 IU/mL.

*Reference solution (a).* Introduce about 0.100 g of *retinol acetate CRS* into a 100 mL volumetric flask and proceed as described for test solution (a).

*Reference solution (b).* Dilute reference solution (a) with *2-propanol R* to a final concentration of 100 IU/mL. Homogenise carefully to avoid air bubbles.

*Column*:

- *size*: $l$ = 0.125 m, Ø = 4 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *methanol R* (5:95 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL of test solution (b) and reference solution (b).

*Run time*: 1.5 times the retention time of retinol.

*Retention time*: retinol = about 3 min.

Calculate the content of vitamin A in International Units per gram using the following expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to retinol in the chromatogram obtained with test solution (b),

$A_2$ = area of the peak due to retinol in the chromatogram obtained with reference solution (b),

$C$ = concentration of *retinol acetate CRS* in International Units per gram, determined by method A; the absorption ratios $A_\lambda/A_{326}$ must conform,

$m_1$ = mass of the substance to be examined in test solution (a), in milligrams,

$m_2$ = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

STORAGE

In an airtight container, protected from light.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram,
- the name of the ester or esters,
- the name of any added stabilisers,
- the method of restoring the solution if partial crystallisation has occurred.

**01/2018:0218**

# VITAMIN A CONCENTRATE (POWDER FORM), SYNTHETIC

## Vitamini A synthetici densati pulvis

DEFINITION

Powder concentrate obtained by dispersing a synthetic retinol ester (*0217*) in a matrix of *Gelatin (0330)* or *Acacia (0307)* or other suitable material.

*Content*: 95.0 per cent to 115.0 per cent of the vitamin A content stated on the label, which is not less than 250 000 IU/g.

It may contain suitable stabilisers such as antioxidants.

CHARACTERS

*Appearance*: yellowish powder usually in the form of particles of almost uniform size.

*Solubility*: practically insoluble in water, swells or forms an emulsion, depending on the formulation.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution.* Introduce a quantity of the preparation to be examined containing about the equivalent of 17 000 IU of vitamin A into a 20 mL glass-stoppered test tube. Add about 20 mg of *bromelains R*, 2 mL of *water R* and about 150 µL of *2-propanol R*, swirling gently for 2-5 min in a water-bath at 60-65 °C. Cool to below 30 °C and add 5 mL of *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*. Shake vigorously for 1 min, allow to stand for a few minutes and use the supernatant solution.

*Reference solution.* Prepare a 10 mg/mL solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 3 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution:

- the chromatogram shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate.

*Results*: the composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

TESTS

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light, air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat.*

Liquid chromatography (*2.2.29*).

*Test solution (a)*. Introduce 0.200 g of the preparation to be examined into a 100 mL volumetric flask. Add 20-30 mg of *bromelains R*, 5.0 mL of *water R* and 0.15 mL of *2-propanol R*. Heat gently in a water-bath at 60 °C for about 5 min, swirling occasionally. Add 40 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl gently and let the mixture react for 10 min at 60-65 °C for hydrolysis, swirling occasionally. Ensure that all sample material is wetted. Allow to cool to room temperature, dilute to 100.0 mL with *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*, and homogenise carefully to avoid air bubbles. The solution may be turbid.

*Test solution (b)*. Dilute test solution (a) with *2-propanol R* to a final concentration of 100 IU/mL. Filter before injection.

*Reference solution (a)*. Introduce about 0.100 g of *retinol acetate CRS* into a 100 mL volumetric flask and dissolve immediately in 5 mL of *pentane R*. Add 40 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl gently and let the mixture react for 10 min at 60-65 °C for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to 100.0 mL with *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*, and homogenise carefully to avoid air bubbles.

*Reference solution (b)*. Dilute reference solution (a) with *2-propanol R* to a final concentration of 100 IU/mL. Homogenise carefully to avoid air bubbles.

*Column*:

- *size*: $l$ = 0.125 m, Ø = 4 mm,
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *methanol R* (5:95 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL of test solution (b) and reference solution (b).

*Run time*: 1.5 times the retention time of retinol.

*Retention time*: retinol = about 3 min.

Calculate the content of vitamin A using the following expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to retinol in the chromatogram obtained with test solution (b),

$A_2$ = area of the peak due to retinol in the chromatogram obtained with reference solution (b),

$C$ = concentration of *retinol acetate CRS* in International Units per gram, determined by the method below,

$m_1$ = mass of the substance to be examined in test solution (a), in milligrams,

$m_2$ = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

The exact concentration of *retinol acetate CRS* is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dissolve 25-100 mg of *retinol acetate CRS*, weighed with an accuracy of 0.1 per cent, in 5 mL of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10-15 IU/mL.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio $A_\lambda/A_{326}$ for each wavelength.

If the ratios do not exceed: 0.60 at 300 nm, 0.54 at 350 nm, 0.14 at 370 nm, calculate the content of vitamin A in International Units per gram using the following expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

$A_{326}$ = absorbance at 326 nm,

$m$ = mass of *retinol acetate CRS*, in grams,

$V$ = total volume to which the *retinol acetate CRS* is diluted to give 10-15 IU/mL,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

The absorbance ratios $A_\lambda/A_{326}$ must conform.

STORAGE

In an airtight container, protected from light.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

- the number of International Units per gram,
- the name of the ester or esters,
- the name of the principal excipient or excipients used and the name of any added stabilisers.

**01/2018:0220**

# VITAMIN A CONCENTRATE (SOLUBILISATE/EMULSION), SYNTHETIC

## Vitaminum A syntheticum, solubilisatum densatum in aqua dispergibile

DEFINITION

Liquid concentrate (water is generally used as solvent) of a synthetic retinol ester (*0217*) and a suitable solubiliser.

*Content*: 95.0 per cent to 115.0 per cent of the vitamin A content stated on the label, which is not less than 100 000 IU/g.

It may contain suitable stabilisers such as antimicrobial preservatives and antioxidants.

CHARACTERS

*Appearance*: yellow or yellowish liquid of variable opalescence and viscosity. Highly concentrated solutions may become cloudy at low temperature or take the form of a gel.

A mixture of 1 g with 10 mL of *water R* previously warmed to 50 °C gives, after cooling to 20 °C, a uniform, slightly opalescent and slightly yellow dispersion.

IDENTIFICATION

Thin-layer chromatography (*2.2.27*).

*Test solution*. Introduce a quantity of the preparation to be examined containing about the equivalent of 17 000 IU of vitamin A into a 20 mL glass-stoppered test tube. Add 5 mL of *2-propanol R* containing 1 g/L of *butylhydroxytoluene R* and mix thoroughly.

*Reference solution*. Prepare a 10 mg/mL solution of *retinol esters CRS* (i.e. 3.3 IU of each ester per microlitre) in *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*.

*Plate*: *TLC silica gel $F_{254}$ plate R*.

*Mobile phase*: *ether R*, *cyclohexane R* (20:80 *V/V*).

*Application*: 3 µL.

*Development*: over a path of 15 cm.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution:

– the chromatogram shows the individual spots of the corresponding esters. The elution order from bottom to top is: retinol acetate, retinol propionate and retinol palmitate.

*Results*: the composition of the test solution is confirmed by the correspondence of the principal spot or spots with those obtained with the reference solution.

TESTS

**Related substances**. The thresholds indicated under Related substances (Table 2034.-1) in the general monograph *Substances for pharmaceutical use (2034)* do not apply.

ASSAY

*Carry out the assay as rapidly as possible, avoiding exposure to actinic light, air, oxidising agents, oxidation catalysts (e.g. copper, iron), acids and prolonged heat.*

Liquid chromatography (*2.2.29*).

*Test solution (a)*. Introduce 0.200 g of the preparation to be examined into a 100 mL volumetric flask. Add 40 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl into dispersion and let the mixture react for 10 min at 60-65 °C for hydrolysis, swirling occasionally. Ensure that all sample material is wetted. Allow to cool to room temperature, dilute to 100.0 mL with *2-propanol R* containing 1 g/L of *butylhydroxytoluene R* and homogenise carefully to avoid air bubbles. Residue of the matrix may make the solution more or less cloudy.

*Test solution (b)*. Dilute test solution (a) with *2-propanol R* to a final concentration of 100 IU/mL. Filter before injection.

*Reference solution (a)*. Introduce about 0.100 g of *retinol acetate CRS* into a 100 mL volumetric flask and dissolve immediately in 5 mL of *pentane R*. Add 40 mL of *0.1 M tetrabutylammonium hydroxide in 2-propanol*. Swirl gently and let the mixture react for 10 min at 60-65 °C for hydrolysis, swirling occasionally. Allow to cool to room temperature, dilute to 100.0 mL with *2-propanol R* containing 1 g/L of *butylhydroxytoluene R*, and homogenise carefully to avoid air bubbles.

*Reference solution (b)*. Dilute reference solution (a) with *2-propanol R* to a final concentration of 100 IU/mL. Homogenise carefully to avoid air bubbles.

*Column*:

– *size*: *l* = 0.125 m, Ø = 4 mm,

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water R*, *methanol R* (5:95 *V/V*).

*Flow rate*: 1 mL/min.

*Detection*: spectrophotometer at 325 nm.

*Injection*: 10 µL of test solution (b) and reference solution (b).

*Run time*: 1.5 times the retention time of retinol.

*Retention time*: retinol = about 3 min.

Calculate the content of vitamin A using the following expression:

$$\frac{A_1 \times C \times m_2}{A_2 \times m_1}$$

$A_1$ = area of the peak due to retinol in the chromatogram obtained with test solution (b),

$A_2$ = area of the peak due to retinol in the chromatogram obtained with reference solution (b),

$C$ = concentration of *retinol acetate CRS* in International Units per gram, determined by the method below,

$m_1$ = mass of the substance to be examined in test solution (a), in milligrams,

$m_2$ = mass of *retinol acetate CRS* in reference solution (a), in milligrams.

The exact concentration of *retinol acetate CRS* is assessed by ultraviolet absorption spectrophotometry (*2.2.25*). Dissolve 25-100 mg of *retinol acetate CRS*, weighed with an accuracy of 0.1 per cent, in 5 mL of *pentane R* and dilute with *2-propanol R1* to a presumed concentration of 10-15 IU/mL.

Verify that the absorption maximum of the solution lies between 325 nm and 327 nm and measure the absorbances at 300 nm, 326 nm, 350 nm and 370 nm. Repeat the readings at each wavelength and take the mean values. Calculate the ratio $A_\lambda/A_{326}$ for each wavelength.

If the ratios do not exceed: 0.60 at 300 nm, 0.54 at 350 nm, 0.14 at 370 nm, calculate the content of vitamin A in International Units per gram using the following expression:

$$\frac{A_{326} \times V \times 1900}{100 \times m}$$

$A_{326}$ = absorbance at 326 nm,

$m$ = mass of *retinol acetate CRS*, in grams,

$V$ = total volume to which the *retinol acetate CRS* is diluted to give 10-15 IU/mL,

1900 = factor to convert the specific absorbance of esters of retinol into International Units per gram.

The absorbance ratios $A_\lambda/A_{326}$ must conform.

STORAGE

In an airtight container, protected from light, at the temperature stated on the label.

Once the container has been opened, its contents are to be used as soon as possible; any part of the contents not used at once should be protected by an atmosphere of inert gas.

LABELLING

The label states:

– the number of International Units per gram,

– the name of the ester or esters,

– the name of the principal solubiliser or solubilisers used and the name of any added stabilisers,

– the storage temperature.

01/2017:2576
corrected 10.0

# VORICONAZOLE

## Voriconazolum

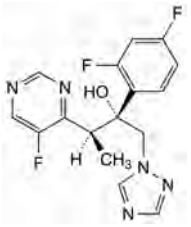

$C_{16}H_{14}F_3N_5O$ $M_r$ 349.3
[137234-62-9]

DEFINITION

(2*R*,3*S*)-2-(2,4-Difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, freely soluble in acetone and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *voriconazole CRS*.

B. Enantiomeric purity (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 0.5 g in a 103 g/L solution of *hydrochloric acid R* and dilute to 20 mL with the same solution.

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 25.0 mg of the substance to be examined in 2 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *voriconazole impurity D CRS* in 2 mL of *acetonitrile R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 25 mg of the substance to be examined in 2 mL of *acetonitrile R*, add 1 mL of reference solution (a) and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *silica gel BC for chiral chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: mix 18 volumes of *acetonitrile R* and 82 volumes of a 0.77 g/L solution of *ammonium acetate R* previously adjusted to pH 5.0 with *glacial acetic acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 256 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 2.5 times the retention time of voriconazole.

*Relative retention* with reference to voriconazole (retention time = about 7 min): impurity D = about 1.5.

*System suitability*: reference solution (b):
- *resolution*: minimum 4.0 between the peaks due to voriconazole and impurity D.

*Limit*:
- *impurity D*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent).

**Impurity E**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in 5.0 mL of *methanol R* and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 25.0 mg of *voriconazole impurity E CRS* in 50 mL of *methanol R* and dilute to 100.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 17 mg of *sodium chloride R* in *water R* and dilute to 200.0 mL with the same solvent. Mix 1 mL of the solution, 1 mL of reference solution (a) and 25 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* To 1.0 mL of reference solution (a) add 25 mL of *methanol R* and dilute to 50.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *strongly basic anion-exchange resin for chromatography R* (8.5 µm);
- *temperature*: 40 °C.

*Mobile phase*: to 1500 mL of *water R* add 500 mL of *methanol R*, mix and degas; add about 175 µL of a 470 g/L solution of *sodium hydroxide R* and mix.

*Flow rate*: 1.0 mL/min.

*Detection*: conductivity detector; use a self-regenerating anion suppressor.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: twice the retention time of impurity E.

*Relative retention* with reference to impurity E (retention time = about 4 min): chloride = about 1.5.

*System suitability*:
- *resolution*: minimum 3.5 between the peaks due to impurity E and chloride in the chromatogram obtained with reference solution (b);
- *symmetry factor*: maximum 1.7 for the peak due to impurity E in the chromatogram obtained with reference solution (c).

*Limit*:
- *impurity E*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 50.0 mg of the substance to be examined in the mobile phase, sonicating if necessary, and dilute to 100.0 mL with the mobile phase. Mix well to ensure complete dissolution.

*Test solution (b).* Dilute 5.0 mL of test solution (a) to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 50.0 mg of *voriconazole CRS* in the mobile phase, sonicating if necessary, and dilute to 100.0 mL with the mobile phase. Mix well to ensure complete dissolution. Dilute 5.0 mL of the solution to 100.0 mL with the mobile phase.

*Reference solution (b).* Suspend 0.100 g of the substance to be examined in 10 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 20 mL with the mobile phase; sonicate if necessary. Allow to stand for 30 min. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase (*in situ* degradation to obtain impurities A and C).

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase and mix. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase and mix well.

*Reference solution (d).* Dissolve 2 mg of *voriconazole impurity B CRS* in the mobile phase and dilute to 200 mL with the mobile phase. Dilute 1.0 mL of the solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 35 °C.

*Mobile phase*: mix 15 volumes of *acetonitrile R*, 30 volumes of *methanol R* and 55 volumes of a 1.90 g/L solution of *ammonium formate R* previously adjusted to pH 4.0 with *anhydrous formic acid R* while stirring continuously.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 256 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (d).

*Run time*: 3 times the retention time of voriconazole.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A and C; use the chromatogram obtained with reference solution (d) to identify the peak due to impurity B.

*Relative retention* with reference to voriconazole (retention time = about 8 min): impurity A = about 0.25; impurity C = about 0.3; impurity B = about 0.6.

*System suitability*: reference solution (b):

- *resolution*: minimum 1.8 between the peaks due to impurities A and C.

*Limits*:

- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity A = 0.7; impurity B = 2.1; impurity C = 0.7;
- *impurities A, B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *sum of impurities A, B, C, D, E and unspecified impurities*: maximum 0.5 per cent;
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Water** (*2.5.12*): maximum 0.4 per cent, determined on 1.00 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g in a platinum crucible.

**Bacterial endotoxins** (*2.6.14*): less than 0.2 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{16}H_{14}F_3N_5O$ from the declared content of *voriconazole CRS*.

STORAGE

If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

*Specified impurities*: *A, B, C, D, E.*

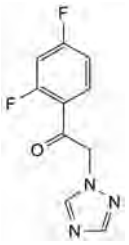

A. 1-(2,4-difluorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanone,

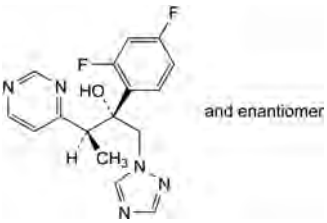


B. (2*RS*,3*SR*)-2-(2,4-difluorophenyl)-3-pyrimidin-4-yl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol,

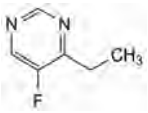

C. 4-ethyl-5-fluoropyrimidine,

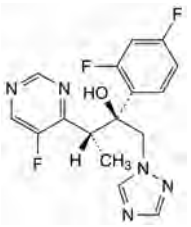

D. (2*S*,3*R*)-2-(2,4-difluorophenyl)-3-(5-fluoropyrimidin-4-yl)-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol (voriconazole enantiomer),

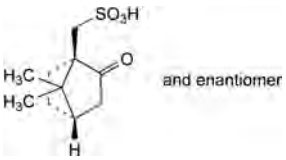


E. [(1*R*,4*S*)-7,7-dimethyl-2-oxobicyclo[2.2.1]hept-1-yl]methanesulfonic acid ((±)-10-camphorsulfonic acid).

# W

Warfarin sodium.... 4201
Warfarin sodium clathrate.... 4202
Water for injections.... 4203
Water for preparation of extracts.... 4206
Water, purified.... 4207
Wheat starch.... 4209
Wheat-germ oil, refined.... 4210
Wheat-germ oil, virgin.... 4210
Wool alcohols.... 4210
Wool fat.... 4211
Wool fat, hydrogenated.... 4215
Wool fat, hydrous.... 4216

**04/2014:0698**

# WARFARIN SODIUM

## Warfarinum natricum

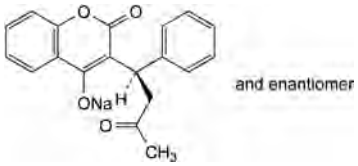


$C_{19}H_{15}NaO_4$ $M_r$ 330.3
[129-06-6]

DEFINITION

Sodium 2-oxo-3-[(1*RS*)-3-oxo-1-phenylbutyl]-2*H*-1-benzopyran-4-olate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, amorphous powder.

*Solubility*: very soluble in water and in ethanol (96 per cent), soluble in acetone, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *warfarin sodium CRS*.

B. Propan-2-ol (see Tests).

C. It gives reaction (b) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 7.6 to 8.6.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (25:75 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *4-hydroxycoumarin R* (impurity B) and 2 mg of *benzalacetone R* (impurity C) in 25 mL of *methanol R* and dilute to 100 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.0 mm;
- *stationary phase*: *cyanosilyl silica gel for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (1:25:75 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of warfarin.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to warfarin (retention time = about 9 min): impurity B = about 0.4; impurity C = about 0.6.

*System suitability*: reference solution (a):
- *resolution*: minimum 2.0 between the peaks due to impurities B and C.

*Limits*:
- *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.5; impurity C = 0.4;
- *impurities B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
- *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Phenolic ketones**. Dissolve 1.25 g in a 20 g/L solution of *sodium hydroxide R* and dilute to 10.0 mL with the same solvent. The absorbance (*2.2.25*) is maximum 0.20 measured at 385 nm within 15 min of preparing the solution.

**Propan-2-ol** (*2.4.24, System A*): maximum 0.5 per cent.

**Water** (*2.5.12*): maximum 4.0 per cent, determined on 0.750 g.

ASSAY

Dissolve 0.100 g in *0.01 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *0.01 M sodium hydroxide*. Dilute 10.0 mL of this solution to 100.0 mL with *0.01 M sodium hydroxide*. Measure the absorbance (*2.2.25*) at the absorption maximum at 308 nm.

Calculate the percentage content of $C_{19}H_{15}NaO_4$ taking the specific absorbance to be 431.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): A.

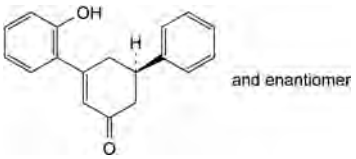


A. (5*RS*)-3-(2-hydroxyphenyl)-5-phenylcyclohex-2-enone,

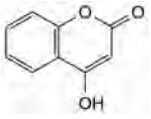


B. 4-hydroxy-2*H*-1-benzopyran-2-one (4-hydroxycoumarin),

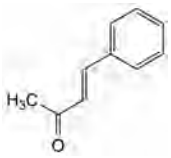

C. (3*E*)-4-phenylbut-3-en-2-one (benzalacetone).

**04/2014:0699**

# WARFARIN SODIUM CLATHRATE

## Warfarinum natricum clathratum

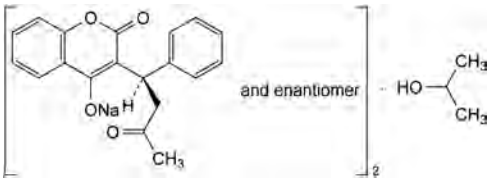

DEFINITION

Mixture, in the form of a clathrate, of warfarin sodium (sodium 2-oxo-3-[(1*RS*)-3-oxo-1-phenylbutyl]-2*H*-1-benzopyran-4-olate) and propan-2-ol in molecular proportions 2:1 (equivalent to about 92 per cent of warfarin sodium).

*Content*:

– *warfarin sodium*: 98.0 per cent to 102.0 per cent (anhydrous and propan-2-ol-free substance);
– *propan-2-ol*: 8.0 per cent to 8.5 per cent.

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent), soluble in acetone, very slightly soluble in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *warfarin sodium clathrate CRS*.

B. Propan-2-ol (see Tests).

C. It gives reaction (b) of sodium (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Dissolve 1.0 g in *water R* and dilute to 20 mL with the same solvent.

**pH** (*2.2.3*): 7.6 to 8.6.

Dissolve 1.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *methanol R*, *water R* (25:75 *V/V*).

*Test solution*. Dissolve 40.0 mg of the substance to be examined in the solvent mixture and dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *4-hydroxycoumarin R* (impurity B) and 2 mg of *benzalacetone R* (impurity C) in 25 mL of *methanol R* and dilute to 100 mL with *water R*.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;
– *stationary phase*: *cyanosilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: *glacial acetic acid R*, *acetonitrile R*, *water R* (1:25:75 *V/V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 260 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of warfarin.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities B and C.

*Relative retention* with reference to warfarin (retention time = about 9 min): impurity B = about 0.4; impurity C = about 0.6.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurities B and C.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity B = 0.5; impurity C = 0.4;
– *impurities B, C*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *total*: not more than 3 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.3 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent).

**Phenolic ketones**. Dissolve 1.25 g in a 20 g/L solution of *sodium hydroxide R* and dilute to 10.0 mL with the same solvent. The absorbance (*2.2.25*) is maximum 0.20 measured at 385 nm within 15 min of preparing the solution.

**Propan-2-ol** (*2.4.24, System A*): 8.0 per cent to 8.5 per cent.

**Water** (*2.5.12*): maximum 0.3 per cent, determined on 2.500 g.

ASSAY

Dissolve 0.100 g in *0.01 M sodium hydroxide* and dilute to 100.0 mL with the same solvent. Dilute 10.0 mL of the solution to 100.0 mL with *0.01 M sodium hydroxide*. Dilute 10.0 mL of this solution to 100.0 mL with *0.01 M sodium hydroxide*. Measure the absorbance (*2.2.25*) at the absorption maximum at 308 nm.

Calculate the percentage content of warfarin sodium ($C_{19}H_{15}NaO_4$) taking the specific absorbance to be 431.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: B, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A*.

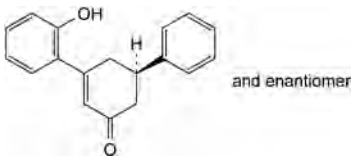


A. (5*RS*)-3-(2-hydroxyphenyl)-5-phenylcyclohex-2-enone,

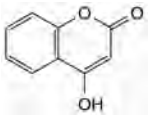


B. 4-hydroxy-2*H*-1-benzopyran-2-one (4-hydroxycoumarin),

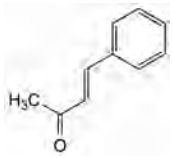


C. (3*E*)-4-phenylbut-3-en-2-one (benzalacetone).

**04/2017:0169**

# WATER FOR INJECTIONS

## Aqua ad iniectabile

$H_2O$ $M_r$ 18.02

DEFINITION

Water for the preparation of medicines for parenteral administration when water is used as vehicle (water for injections in bulk) and for dissolving or diluting substances or preparations for parenteral administration (sterilised water for injections).

## Water for injections in bulk

PRODUCTION

Water for injections in bulk is obtained from water that complies with the regulations on water intended for human consumption laid down by the competent authority or from purified water. It is produced either:

- by distillation in an apparatus of which the parts in contact with the water are of neutral glass, quartz or a suitable metal and which is fitted with an effective device to prevent the entrainment of droplets; or
- by a purification process that is equivalent to distillation. Reverse osmosis, which may be single-pass or double-pass, coupled with other appropriate techniques such as electro-deionisation, ultrafiltration or nanofiltration, is suitable. Notice is given to the supervisory authority of the manufacturer before implementation.

For all methods of production, correct operation monitoring and maintenance of the system are essential. In order to ensure the appropriate quality of the water, validated procedures, in-process monitoring of the electrical conductivity, and regular monitoring of total organic carbon and microbial contamination are applied.

The first portion of water obtained when the system begins to function is discarded.

Water for injections in bulk is stored and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

**Microbiological monitoring**. During production and subsequent storage, appropriate measures are taken to ensure that the microbial count is adequately controlled and monitored. Appropriate alert and action levels are set so as to detect adverse trends. Under normal conditions, an appropriate action level is a microbial count of 10 CFU per 100 mL when determined by filtration through a membrane with a nominal pore size not greater than 0.45 µm, using R2A agar, using at least 200 mL of water for injections in bulk and incubating at 30-35 °C for not less than 5 days. For aseptic processing, stricter alert levels may need to be applied.

*R2A agar*

| | |
|---|---|
| Yeast extract | 0.5 g |
| Proteose peptone | 0.5 g |
| Casein hydrolysate | 0.5 g |
| Glucose | 0.5 g |
| Starch | 0.5 g |
| Dipotassium hydrogen phosphate | 0.3 g |
| Magnesium sulfate, anhydrous | 0.024 g |
| Sodium pyruvate | 0.3 g |
| Agar | 15.0 g |
| Purified water | to 1000 mL |

Adjust the pH so that after sterilisation it is 7.2 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

*Growth promotion of R2A agar*

- *Preparation of test strains.* Use standardised stable suspensions of test strains or prepare them as stated in Table 0169.-1. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial strains separately as described in Table 0169.-1. Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *Bacillus subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

- *Growth promotion.* Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described. Inoculate plates of R2A agar separately with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 0169.-1. Incubate under the conditions described in the table. Growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms must be comparable to that obtained with a previously tested and approved batch of medium.

Table 0169.-1. – *Growth promotion of R2A agar*

| Micro-organism | Preparation of the test strain | Growth promotion |
|---|---|---|
| *Pseudomonas aeruginosa*<br>such as:<br>ATCC 9027<br>NCIMB 8626<br>CIP 82.118<br>NBRC 13275 | Casein soyabean digest agar or casein soyabean digest broth<br>30-35 °C<br>18-24 h | R2A agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |
| *Bacillus subtilis*<br>such as:<br>ATCC 6633<br>NCIMB 8054<br>CIP 52.62<br>NBRC 3134 | Casein soyabean digest agar or casein soyabean digest broth<br>30-35 °C<br>18-24 h | R2A agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |

**Total organic carbon** (*2.2.44*): maximum 0.5 mg/L.

**Conductivity**. Determine the conductivity off-line or in-line under the following conditions.

*EQUIPMENT*

*Conductivity cell*:

– electrodes of a suitable material such as stainless steel;

– cell constant: the cell constant is generally certified by the supplier and is subsequently verified at suitable intervals using a certified reference solution with a conductivity less than 1500 μS·cm$^{-1}$ or by comparison with a cell having a certified cell constant. The cell constant is confirmed if the value found is within 2 per cent of the certified value, otherwise re-calibration must be performed.

*Conductometer*: accuracy of 0.1 μS·cm$^{-1}$ or better at the lowest range.

*System calibration (conductivity cell and conductometer)*:

– against one or more suitable certified reference solutions;

– accuracy: within 3 per cent of the measured conductivity plus 0.1 μS·cm$^{-1}$.

*Conductometer calibration*: calibration is carried out for each range of measurement to be used, after disconnection of the conductivity cell, using certified precision resistors or equivalent devices with an uncertainty not greater than 0.1 per cent of the certified value.

If in-line conductivity cells cannot be dismantled, system calibration may be performed against a calibrated conductivity-measuring instrument with a conductivity cell placed close to the cell to be calibrated in the water flow.

*Temperature measurement*: tolerance ± 2 °C.

*PROCEDURE*

*Stage 1*

1. Measure the conductivity without temperature compensation, recording simultaneously the temperature. Temperature-compensated measurement may be performed after suitable validation.

2. Using Table 0169.-2, find the closest temperature value that is not greater than the measured temperature. The corresponding conductivity value is the limit at that temperature.

3. If the measured conductivity is not greater than the value in Table 0169.-2, the water to be examined meets the requirements of the test for conductivity. If the conductivity is higher than the value in Table 0169.-2, proceed with stage 2.

Table 0169.-2. – *Stage 1*
*Temperature and conductivity requirements (for non-temperature-compensated conductivity measurements)*

| Temperature (°C) | Conductivity (μS·cm$^{-1}$) |
|---|---|
| 0 | 0.6 |
| 5 | 0.8 |
| 10 | 0.9 |
| 15 | 1.0 |
| 20 | 1.1 |
| 25 | 1.3 |
| 30 | 1.4 |
| 35 | 1.5 |
| 40 | 1.7 |
| 45 | 1.8 |
| 50 | 1.9 |
| 55 | 2.1 |
| 60 | 2.2 |
| 65 | 2.4 |
| 70 | 2.5 |
| 75 | 2.7 |
| 80 | 2.7 |
| 85 | 2.7 |
| 90 | 2.7 |
| 95 | 2.9 |
| 100 | 3.1 |

*Stage 2*

4. Transfer a sufficient amount of the water to be examined (100 mL or more) to a suitable container, and stir the test sample. Adjust the temperature, if necessary, and while maintaining it at 25 ± 1 °C, begin vigorously agitating the test sample while periodically observing the conductivity. When the change in conductivity (due to uptake of atmospheric carbon dioxide) is less than 0.1 μS.cm$^{-1}$ per 5 min, note the conductivity.

5. If the conductivity is not greater than 2.1 μS.cm$^{-1}$, the water to be examined meets the requirements of the test for conductivity. If the conductivity is greater than 2.1 μS.cm$^{-1}$, proceed with stage 3.

*Stage 3*

6. Perform this test within approximately 5 min of the conductivity determination in step 5 under stage 2, while maintaining the sample temperature at 25 ± 1 °C. Add a recently prepared saturated solution of *potassium chloride R* to the test sample (0.3 mL per 100 mL of the test sample), and determine the pH (*2.2.3*) to the nearest 0.1 pH unit.

7. Using Table 0169.-3, determine the conductivity limit at the measured pH value in step 6. If the measured conductivity in step 4 under stage 2 is not greater than the conductivity requirements for the pH determined, the water to be examined meets the requirements of the test for conductivity. If either the measured conductivity is greater than this value or the pH is outside the range of 5.0-7.0, the water to be examined does not meet the requirements of the test for conductivity.

Table 0169.-3. – *Stage 3*
*pH and conductivity requirements (for atmosphere- and temperature-equilibrated samples)*

| pH | Conductivity ($\mu S \cdot cm^{-1}$) |
|---|---|
| 5.0 | 4.7 |
| 5.1 | 4.1 |
| 5.2 | 3.6 |
| 5.3 | 3.3 |
| 5.4 | 3.0 |
| 5.5 | 2.8 |
| 5.6 | 2.6 |
| 5.7 | 2.5 |
| 5.8 | 2.4 |
| 5.9 | 2.4 |
| 6.0 | 2.4 |
| 6.1 | 2.4 |
| 6.2 | 2.5 |
| 6.3 | 2.4 |
| 6.4 | 2.3 |
| 6.5 | 2.2 |
| 6.6 | 2.1 |
| 6.7 | 2.6 |
| 6.8 | 3.1 |
| 6.9 | 3.8 |
| 7.0 | 4.6 |

CHARACTERS

*Appearance*: clear and colourless liquid.

TESTS

**Nitrates**: maximum 0.2 ppm.

Place 5 mL in a test-tube immersed in iced water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *nitrogen-free sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 mL of *nitrate-free water R* and 0.5 mL of *nitrate standard solution (2 ppm $NO_3$) R*.

**Aluminium** (*2.4.17*): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution*. To 400 mL of the water to be examined add 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *distilled water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mL.

## Sterilised water for injections

DEFINITION

Water for injections in bulk that has been distributed into suitable containers, closed and sterilised by heat in conditions which ensure that the product still complies with the test for bacterial endotoxins. Sterilised water for injections is free from any added substances.

Examined in suitable conditions of visibility, it is clear and colourless.

Each container contains a sufficient quantity of water for injections to permit the nominal volume to be withdrawn.

TESTS

**Acidity or alkalinity**. To 20 mL add 0.05 mL of *phenol red solution R*. If the solution is yellow, it becomes red on the addition of 0.1 mL of *0.01 M sodium hydroxide*; if red, it becomes yellow on the addition of 0.15 mL of *0.01 M hydrochloric acid*.

**Conductivity**: maximum 25 $\mu S \cdot cm^{-1}$ for containers with a nominal volume of 10 mL or less; maximum 5 $\mu S \cdot cm^{-1}$ for containers with a nominal volume greater than 10 mL.

Use equipment and the calibration procedure as defined under Water for injections in bulk, maintaining the sample temperature at 25 ± 1 °C.

**Oxidisable substances**. For containers with a nominal volume less than 50 mL: heat 100 mL to boiling with 10 mL of *dilute sulfuric acid R*, add 0.4 mL of *0.02 M potassium permanganate* and boil for 5 min; the solution remains faintly pink.

For containers with a nominal volume equal to or greater than 50 mL: heat 100 mL to boiling with 10 mL of *dilute sulfuric acid R*, add 0.2 mL of *0.02 M potassium permanganate* and boil for 5 min; the solution remains faintly pink.

**Chlorides** (*2.4.4*): maximum 0.5 ppm for containers with a nominal volume of 100 mL or less.

15 mL complies with the limit test for chlorides. Prepare the standard using a mixture of 1.5 mL of *chloride standard solution (5 ppm Cl) R* and 13.5 mL of *water R*. Examine the solutions down the vertical axes of the tubes.

For containers with a nominal volume greater than 100 mL, use the following test: to 10 mL add 1 mL of *dilute nitric acid R* and 0.2 mL of *silver nitrate solution R2*. The solution shows no change in appearance for at least 15 min.

**Nitrates**: maximum 0.2 ppm.

Place 5 mL in a test-tube immersed in iced water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *nitrogen-free sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 mL of *nitrate-free water R* and 0.5 mL of *nitrate standard solution (2 ppm $NO_3$) R*.

**Sulfates**. To 10 mL add 0.1 mL of *dilute hydrochloric acid R* and 0.1 mL of *barium chloride solution R1*. The solution shows no change in appearance for at least 1 h.

**Aluminium** (*2.4.17*): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution*. To 400 mL of the water to be examined add 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *distilled water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

**Ammonium**: for containers with a nominal volume less than 50 mL: maximum 0.6 ppm; for containers with a nominal volume equal to or greater than 50 mL: maximum 0.2 ppm.

Containers with a nominal volume less than 50 mL: to 20 mL add 1 mL of *alkaline potassium tetraiodomercurate solution R*; after 5 min, examine the solution down the vertical axis of the tube; the solution is not more intensely coloured than a standard prepared at the same time by adding 1 mL of *alkaline potassium tetraiodomercurate solution R* to a mixture of 4 mL of *ammonium standard solution (3 ppm $NH_4$) R* and 16 mL of *ammonium-free water R*.

Containers with a nominal volume equal to or greater than 50 mL: to 20 mL add 1 mL of *alkaline potassium tetraiodomercurate solution R*; after 5 min, examine the solution down the vertical axis of the tube; the solution is not more intensely coloured than a standard prepared at the same time by adding 1 mL of *alkaline potassium tetraiodomercurate solution R* to a mixture of 4 mL of *ammonium standard solution (1 ppm $NH_4$) R* and 16 mL of *ammonium-free water R*.

**Calcium and magnesium**. To 100 mL add 2 mL of *ammonium chloride buffer solution pH 10.0 R*, 50 mg of *mordant black 11 triturate R* and 0.5 mL of *0.01 M sodium edetate*. A pure blue colour is produced.

**Residue on evaporation**: maximum 4 mg (0.004 per cent) for containers with a nominal volume of 10 mL or less; maximum 3 mg (0.003 per cent) for containers with a nominal volume greater than 10 mL.

Evaporate 100 mL to dryness on a water-bath and dry in an oven at 100-105 °C.

**Particulate contamination: sub-visible particles** (*2.9.19*). It complies with test A or test B, as appropriate.

**Sterility** (*2.6.1*). It complies with the test for sterility.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mL.

**04/2012:2249**

# WATER FOR PREPARATION OF EXTRACTS

## Aqua ad extracta praeparanda

DEFINITION

Water intended for the preparation of *Herbal drug extracts (0765)* complies with the sections Purified water in bulk or Purified water in containers in the monograph *Purified water (0008)*, or is water intended for human consumption of a quality equivalent to that defined in Directive 98/83/EC which is monitored according to the Production section described below.

PRODUCTION

When water intended for human consumption is used as water for preparation of extracts it is a clear, colourless liquid. It is stored (where necessary) and distributed under conditions designed to prevent growth of micro-organisms and to avoid other contamination.

For monitoring purposes, the following tests are carried out at regular intervals to demonstrate consistency in the quality of the water used for the preparation of extracts.

**Conductivity** (*2.2.38*): maximum 2500 $\mu S \cdot cm^{-1}$, measured at 20 °C.

**Nitrate**. Liquid chromatography (*2.2.29*).

*Test solution*. The substance to be examined.

*Reference solution*. Dissolve 0.163 g of *potassium nitrate R* and 0.149 g of *potassium bromide R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4 mm;

– *stationary phase*: *anion-exchange resin R3*.

*Mobile phase*: dissolve 0.265 g of *anhydrous sodium carbonate R* and 0.210 g of *sodium hydrogen carbonate R* in *water R* and dilute to 1000.0 mL with the same solvent.

*Flow rate*: 1.2 mL/min.

*Detection*: conductivity detector, using a self-regenerating anion suppressor.

*Injection*: 25 µL.

*Run time*: twice the retention time of nitrate.

*Relative retention* with reference to nitrate (retention time = about 7 min): bromide = about 0.9.

*System suitability*: reference solution:

– *resolution*: minimum 2.0 between the peaks due to bromide and nitrate.

*Limit*:

– *nitrate*: maximum 50 ppm.

**Microbiological monitoring**. Appropriate measures are taken to ensure that the microbial count is adequately controlled and monitored. Appropriate alert and action levels are set so as to detect adverse trends.

Under normal conditions, an appropriate action level is a microbial count of 100 CFU/mL, determined by filtration through a membrane with a nominal pore size not greater than 0.45 µm, using casein soya bean digest agar and incubating at 30-35 °C for not less than 5 days.

The size of the sample is to be chosen in relation to the expected result.

*Casein soya bean digest agar*

| | |
|---|---|
| Pancreatic digest of casein | 15.0 g |
| Papaic digest of soya bean | 5.0 g |
| Sodium chloride | 5.0 g |
| Agar | 15.0 g |
| Purified water | to 1000 mL |

Adjust the pH so that after sterilisation it is 7.3 ± 0.2. Sterilise in an autoclave using a validated cycle.

*Growth promotion of casein soya bean digest agar*

– *Preparation of test strains*. Use standardised stable suspensions of test strains or prepare them as stated in Table 2249.-1. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial strains separately as described in Table 2249.-1. Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *Bacillus subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

– *Growth promotion*. Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described. Inoculate plates of casein soya bean digest agar separately with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2249.-1. Incubate under the conditions described in this table. Growth obtained must not differ by a factor greater than 2 from the

calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms must be comparable to that obtained with a previously tested and approved batch of medium.

Table 2249.-1. – *Growth promotion of casein soya bean digest agar*

| Micro-organism | Preparation of the test strain | Growth promotion |
|---|---|---|
| *Pseudomonas aeruginosa*<br>such as:<br>ATCC 9027<br>NCIMB 8626<br>CIP 82.118<br>NBRC 13275 | Casein soya bean digest agar or casein soya bean digest broth<br>30-35 °C<br>18-24 h | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |
| *Bacillus subtilis*<br>such as:<br>ATCC 6633<br>NCIMB 8054<br>CIP 52.62<br>NBRC 3134 | Casein soya bean digest agar or casein soya bean digest broth<br>30-35 °C<br>18-24 h | Casein soya bean digest agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |

**04/2018:0008**

# WATER, PURIFIED

## Aqua purificata

$H_2O$ $M_r$ 18.02

DEFINITION

Water for the preparation of medicines other than those that are required to be both sterile and apyrogenic, unless otherwise justified and authorised.

## Purified water in bulk

PRODUCTION

Purified water in bulk is prepared by distillation, by ion exchange, by reverse osmosis or by any other suitable method from water that complies with the regulations on water intended for human consumption laid down by the competent authority.

Purified water in bulk is stored and distributed in conditions designed to prevent growth of micro-organisms and to avoid any other contamination.

**Microbiological monitoring**. During production and subsequent storage, appropriate measures are taken to ensure that the microbial count is adequately controlled and monitored. Appropriate alert and action levels are set so as to detect adverse trends. Under normal conditions, an appropriate action level is a microbial count of 100 CFU/mL, determined by filtration through a membrane with a nominal pore size not greater than 0.45 µm, using R2A agar and incubating at 30-35 °C for not less than 5 days. The size of the sample is to be chosen in relation to the expected result.

*R2A agar*

| | |
|---|---|
| Yeast extract | 0.5 g |
| Proteose peptone | 0.5 g |
| Casein hydrolysate | 0.5 g |
| Glucose | 0.5 g |
| Starch | 0.5 g |
| Dipotassium hydrogen phosphate | 0.3 g |
| Magnesium sulfate, anhydrous | 0.024 g |
| Sodium pyruvate | 0.3 g |
| Agar | 15.0 g |
| Purified water | to 1000 mL |

Adjust the pH so that after sterilisation it is 7.2 ± 0.2. Sterilise by heating in an autoclave at 121 °C for 15 min.

*Growth promotion of R2A agar*

– *Preparation of test strains.* Use standardised stable suspensions of test strains or prepare them as stated in Table 0008.-1. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial strains separately as described in Table 0008.-1. Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *Bacillus subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.
– *Growth promotion.* Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described. Inoculate plates of R2A agar separately with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 0008.-1. Incubate under the conditions described in the table. Growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms must be comparable to that obtained with a previously tested and approved batch of medium.

Table 0008.-1. – *Growth promotion of R2A agar*

| Micro-organism | Preparation of the test strain | Growth promotion |
|---|---|---|
| *Pseudomonas aeruginosa*<br>such as:<br>ATCC 9027<br>NCIMB 8626<br>CIP 82.118<br>NBRC 13275 | Casein soyabean digest agar or casein soyabean digest broth<br>30-35 °C<br>18-24 h | R2A agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |
| *Bacillus subtilis*<br>such as:<br>ATCC 6633<br>NCIMB 8054<br>CIP 52.62<br>NBRC 3134 | Casein soyabean digest agar or casein soyabean digest broth<br>30-35 °C<br>18-24 h | R2A agar<br>≤ 100 CFU<br>30-35 °C<br>≤ 3 days |

**Total organic carbon or oxidisable substances**. Carry out the test for total organic carbon (*2.2.44*) with a limit of 0.5 mg/L or alternatively the following test for oxidisable substances: to 100 mL add 10 mL of *dilute sulfuric acid R* and 0.1 mL of *0.02 M potassium permanganate* and boil for 5 min; the solution remains faintly pink.

**Conductivity**. Determine the conductivity off-line or in-line under the following conditions.

*EQUIPMENT*

*Conductivity cell*:

– electrodes of a suitable material such as stainless steel;
– cell constant: the cell constant is generally certified by the supplier and is subsequently verified at suitable intervals using a certified reference solution with a conductivity less than 1500 µS·cm$^{-1}$ or by comparison with a cell having a certified cell constant; the cell constant is confirmed if the value found is within 2 per cent of the certified value, otherwise re-calibration must be performed.

*Conductometer*: accuracy of 0.1 µS·cm$^{-1}$ or better at the lowest range.

*System calibration (conductivity cell and conductometer)*:

– against one or more suitable certified reference solutions;

– accuracy: within 3 per cent of the measured conductivity plus 0.1 µS·cm$^{-1}$.

*Conductometer calibration*: calibration is carried out for each range of measurement to be used, after disconnection of the conductivity cell, using certified precision resistors or equivalent devices with an uncertainty not greater than 0.1 per cent of the certified value.

If in-line conductivity cells cannot be dismantled, system calibration may be performed against a calibrated conductivity-measuring instrument with a conductivity cell placed close to the cell to be calibrated in the water flow.

*Temperature measurement*: tolerance ± 2 °C.

*PROCEDURE*

Measure the conductivity without temperature compensation, recording simultaneously the temperature. Temperature-compensated measurement may be performed after suitable validation.

The water to be examined meets the requirements if the measured conductivity at the recorded temperature is not greater than the value in Table 0008.-2.

Table 0008.-2. – *Temperature and conductivity requirements*

| Temperature (°C) | Conductivity (µS·cm$^{-1}$) |
|---|---|
| 0 | 2.4 |
| 10 | 3.6 |
| 20 | 4.3 |
| 25 | 5.1 |
| 30 | 5.4 |
| 40 | 6.5 |
| 50 | 7.1 |
| 60 | 8.1 |
| 70 | 9.1 |
| 75 | 9.7 |
| 80 | 9.7 |
| 90 | 9.7 |
| 100 | 10.2 |

For temperatures not listed in Table 0008.-2, calculate the maximal permitted conductivity by interpolation between the next lower and next higher data points in the table.

**Elemental impurities**. If purified water in bulk does not meet the requirement for conductivity prescribed for *Water for injections (0169)* in bulk, a risk assessment according to general chapter *5.20. Elemental impurities* is carried out. The risk assessment should consider the role of water in the manufacturing process, in particular when water is used in a process but is no longer present in the final product.

CHARACTERS

*Appearance*: clear and colourless liquid.

TESTS

**Nitrates**: maximum 0.2 ppm.

Place 5 mL in a test-tube immersed in iced water, add 0.4 mL of a 100 g/L solution of *potassium chloride R*, 0.1 mL of *diphenylamine solution R* and, dropwise with shaking, 5 mL of *nitrogen-free sulfuric acid R*. Transfer the tube to a water-bath at 50 °C. After 15 min, any blue colour in the solution is not more intense than that in a reference solution prepared at the same time in the same manner using a mixture of 4.5 mL of *nitrate-free water R* and 0.5 mL of *nitrate standard solution (2 ppm $NO_3$) R*.

**Aluminium** (*2.4.17*): maximum 10 ppb, if intended for use in the manufacture of dialysis solutions.

*Prescribed solution*. To 400 mL of the water to be examined add 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

*Reference solution*. Mix 2 mL of *aluminium standard solution (2 ppm Al) R*, 10 mL of *acetate buffer solution pH 6.0 R* and 98 mL of *distilled water R*.

*Blank solution*. Mix 10 mL of *acetate buffer solution pH 6.0 R* and 100 mL of *distilled water R*.

**Bacterial endotoxins** (*2.6.14*): less than 0.25 IU/mL, if intended for use in the manufacture of dialysis solutions without a further appropriate procedure for removal of bacterial endotoxins.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

## Purified water in containers

DEFINITION

Purified water in bulk that has been filled and stored in conditions designed to assure the required microbiological quality. It is free from any added substances.

CHARACTERS

*Appearance*: clear and colourless liquid.

TESTS

It complies with the tests prescribed in the section on Purified water in bulk and with the following additional tests.

**Acidity or alkalinity**. To 10 mL, freshly boiled and cooled in a borosilicate glass flask, add 0.05 mL of *methyl red solution R*. The solution is not coloured red.

To 10 mL add 0.1 mL of *bromothymol blue solution R1*. The solution is not coloured blue.

**Oxidisable substances**. To 100 mL add 10 mL of *dilute sulfuric acid R* and 0.1 mL of *0.02 M potassium permanganate* and boil for 5 min. The solution remains faintly pink.

**Chlorides**. To 10 mL add 1 mL of *dilute nitric acid R* and 0.2 mL of *silver nitrate solution R2*. The solution shows no change in appearance for at least 15 min.

**Sulfates**. To 10 mL add 0.1 mL of *dilute hydrochloric acid R* and 0.1 mL of *barium chloride solution R1*. The solution shows no change in appearance for at least 1 h.

**Ammonium**: maximum 0.2 ppm.

To 20 mL add 1 mL of *alkaline potassium tetraiodomercurate solution R*. After 5 min, examine the solution down the vertical axis of the tube. The solution is not more intensely coloured than a standard prepared at the same time by adding 1 mL of *alkaline potassium tetraiodomercurate solution R* to a mixture of 4 mL of *ammonium standard solution (1 ppm $NH_4$) R* and 16 mL of *ammonium-free water R*.

**Calcium and magnesium**. To 100 mL add 2 mL of *ammonium chloride buffer solution pH 10.0 R*, 50 mg of *mordant black 11 triturate R* and 0.5 mL of *0.01 M sodium edetate*. A pure blue colour is produced.

**Residue on evaporation**: maximum 0.001 per cent.

Evaporate 100 mL to dryness on a water-bath and dry in an oven at 100-105 °C. The residue weighs a maximum of 1 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^2$ CFU/mL (*2.6.12*). Use casein soya bean digest agar.

LABELLING

The label states, where applicable, that the substance is suitable for use in the manufacture of dialysis solutions.

**01/2020:0359**

# WHEAT STARCH[(1)]

## Tritici amylum

DEFINITION

Wheat starch is obtained from the caryopsis of *Triticum aestivum* L. (*T. vulgare* Vill.).

♦CHARACTERS

*Appearance*: very fine, white or almost white powder that creaks when pressed between the fingers.

*Solubility*: practically insoluble in cold water and in ethanol (96 per cent).

Wheat starch does not contain starch granules of any other origin. It may contain a minute quantity, if any, of tissue fragments of the original plant.♦

IDENTIFICATION

A. Microscopic examination (*2.8.23*) using a 50 per cent *V/V* solution of *glycerol R*. It shows large and small granules, and, very rarely, intermediate sizes (Figure 0359.-1). The large granules, 10-60 µm in diameter, are discoid or, more rarely, reniform in surface view. The central hilum and striations are invisible or barely visible and the granules sometimes show cracks on the edges. In side view, the granules are elliptical and fusiform and the hilum appears as a slit along the main axis. The small granules, rounded or polyhedral, are 2-10 µm in diameter. Between orthogonally orientated polarising plates or prisms, the granules show a distinct black cross intersecting at the hilum.

Figure 0359.-1. – *Illustration for identification test A of wheat starch*

B. Suspend 1 g in 50 mL of *water R*, boil for 1 min and cool. A thin, cloudy mucilage is formed.

C. To 1 mL of the mucilage obtained in identification test B add 0.05 mL of *iodine solution R1*. A dark blue colour is produced, which disappears on heating.

TESTS

**pH** (*2.2.3*): 4.5 to 7.0.

Shake 5.0 g with 25.0 mL of *carbon dioxide-free water R* for 60 s. Allow to stand for 15 min.

◊**Foreign matter**. Examined under a microscope using a 50 per cent *V/V* solution of *glycerol R*, not more than traces of matter other than starch granules are present. No starch granules of any other origin are present.◊

**Total protein**: maximum 0.3 per cent (corresponding to 0.048 per cent of nitrogen).

Determine the nitrogen content by sulfuric acid digestion as follows, and calculate the quantity of protein by multiplying by 6.25.

Carry out a blank determination by placing 4 g of a powdered mixture of 100 g of *dipotassium sulfate R*, 3 g of *copper sulfate pentahydrate R* and 3 g of *titanium dioxide R*, and 3 glass beads in a combustion flask. Wash any adhering particles from the neck into the flask with 25 mL of *sulfuric acid R*, allowing it to run down the sides of the flask, and swirl to mix the contents. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; take precautions to prevent the upper part of the flask from becoming overheated. Continue heating until a clear solution is obtained. Cool, dissolve the solid material by cautiously adding 25 mL of *water R* to the mixture, cool again and place in a steam-distillation apparatus. Add a suitable volume of *strong sodium hydroxide solution R* to change the colour of the solution from bluish-green to brown or black, and distil immediately by passing steam through the mixture. Collect about 40 mL of distillate in 50.0 mL of *0.01 M hydrochloric acid*, adding enough *water R* if necessary to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with *0.025 M sodium hydroxide* ($n_1$ mL), using *methyl red mixed solution R* as indicator.

Repeat the test adding 3.0 g ($m$ g) of the substance to be examined to the combustion flask, and using the same volume of *strong sodium hydroxide solution R*. Titrate the distillate as described for the blank determination with *0.025 M sodium hydroxide* ($n_2$ mL). Calculate the percentage content of nitrogen using the following expression:

$$\frac{0.03503(n_1 - n_2)}{m}$$

**Oxidising substances** (*2.5.30*): maximum 20 ppm, calculated as $H_2O_2$.

**Sulfur dioxide** (*2.5.29*): maximum 50 ppm.

**Iron** (*2.4.9*): maximum 10 ppm.

Shake 1.5 g with 15 mL of *dilute hydrochloric acid R*. Filter. The filtrate complies with the test.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 130 °C for 90 min.

**Sulfated ash** (*2.4.14*): maximum 0.6 per cent, determined on 1.0 g.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

Absence of *Escherichia coli* (*2.6.13*).

◊Absence of *Salmonella* (*2.6.13*).◊

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter *5.8. Pharmacopoeial harmonisation*.

01/2020:1379

# WHEAT-GERM OIL, REFINED

## Tritici aestivi oleum raffinatum

DEFINITION

Fatty oil obtained from the germ of the grain of *Triticum aestivum* L. by cold expression or by other suitable mechanical means and/or by extraction. It is then refined. A suitable antioxidant may be added.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, light yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

*Relative density*: about 0.925.

*Refractive index*: about 1.475.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 0.9, or maximum 0.3 if intended for use in the manufacture of parenteral preparations.

**Peroxide value** (*2.5.5, Method A*): maximum 10.0, or maximum 5.0 if intended for use in the manufacture of parenteral preparations.

**Unsaponifiable matter** (*2.5.7*): maximum 5.0 per cent, determined on 5.0 g.

**Alkaline impurities** (*2.4.19*). It complies with the test.

**Composition of fatty acids** (*2.4.22, Method C*). Use the mixture of calibrating substances in Table 2.4.22.-3.

*Composition of the fatty-acid fraction of the oil*:

- *palmitic acid*: 14.0 per cent to 19.0 per cent;
- *stearic acid*: maximum 2.0 per cent;
- *oleic acid*: 12.0 per cent to 23.0 per cent;
- *linoleic acid*: 52.0 per cent to 59.0 per cent;
- *linolenic acid*: 3.0 per cent to 10.0 per cent;
- *eicosenoic acid*: maximum 2.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

LABELLING

The label states:

- where applicable, that the substance is suitable for use in the manufacture of parenteral preparations;
- whether the oil is obtained by mechanical means, by extraction or by a combination of the 2.

01/2020:1480

# WHEAT-GERM OIL, VIRGIN

## Tritici aestivi oleum virginale

DEFINITION

Fatty oil obtained from the germ of the grain of *Triticum aestivum* L. by cold expression or other suitable mechanical means.

PRODUCTION

The oil is prepared using materials and methods designed to ensure that the content of brassicasterol (*2.4.23*) in the sterol fraction of the oil is not greater than 0.3 per cent.

CHARACTERS

*Appearance*: clear, light yellow or golden-yellow liquid.

*Solubility*: practically insoluble in water and in ethanol (96 per cent), miscible with light petroleum (bp: 40-60 °C).

*Relative density*: about 0.925.

*Refractive index*: about 1.475.

IDENTIFICATION

*First identification: B.*

*Second identification: A.*

A. Identification of fatty oils by thin-layer chromatography (*2.3.2*).

*Results*: the chromatogram obtained is similar to the corresponding chromatogram shown in Figure 2.3.2.-1.

B. Composition of fatty acids (see Tests).

TESTS

**Acid value** (*2.5.1*): maximum 20.0.

**Peroxide value** (*2.5.5, Method A*): maximum 15.0.

**Unsaponifiable matter** (*2.5.7*): maximum 5.0 per cent, determined on 5.0 g.

**Composition of fatty acids** (*2.4.22, Method C*).

*Composition of the fatty-acid fraction of the oil*:

- *palmitic acid*: 14.0 per cent to 19.0 per cent;
- *stearic acid*: maximum 2.0 per cent;
- *oleic acid*: 12.0 per cent to 23.0 per cent;
- *linoleic acid*: 52.0 per cent to 59.0 per cent;
- *linolenic acid*: 3.0 per cent to 10.0 per cent;
- *eicosenoic acid*: maximum 2.0 per cent.

**Water** (*2.5.32*): maximum 0.1 per cent, determined on 1.00 g.

STORAGE

In an airtight, well-filled container, protected from light.

**04/2012:0593**
**corrected 10.0**

# WOOL ALCOHOLS

## Alcoholes adipis lanae

DEFINITION

Mixture of sterols and higher aliphatic alcohols from wool fat. A suitable antioxidant may be added.

*Content*: minimum 30.0 per cent of cholesterol.

CHARACTERS

*Appearance*: pale-yellow or brownish-yellow, brittle mass becoming plastic on heating.

*Solubility*: practically insoluble in water, soluble in boiling anhydrous ethanol and in methylene chloride, slightly soluble in ethanol (90 per cent *V/V*).

IDENTIFICATION

Dissolve 50 mg in 5 mL of *methylene chloride R* and add 1 mL of *acetic anhydride R* and 0.1 mL of *sulfuric acid R*. Within a few seconds, a green colour develops.

TESTS

**Appearance of solution**. To 1.0 g add 10 mL of *light petroleum R1* and shake while warming in a water-bath. The substance dissolves completely. After cooling, the solution is clear (*2.2.1*).

**Alkalinity**. Dissolve 2.0 g in 25 mL of hot *ethanol (90 per cent V/V) R* and add 0.5 mL of *phenolphthalein solution R1*. No red colour develops.

**Melting point** (*2.2.15*): minimum 56 °C.

Melt the substance to be examined by heating in a water-bath at a temperature which exceeds the expected melting point by not more than 10 °C; introduce the substance to be examined into the capillary tubes and allow to stand on ice for at least 2 h.

**Water-absorption capacity**. Place 0.6 g of the substance to be examined and 9.4 g of *white soft paraffin R* in a mortar and melt on a water-bath. Allow to cool and incorporate 20 mL of *water R*, added in portions. Within 24 h no water is released from the almost white, ointment-like emulsion.

**Acid value** (*2.5.1*): maximum 2.0.

If necessary, heat in a water-bath under a reflux condenser to dissolve the substance to be examined.

**Hydroxyl value** (*2.5.3, Method A*): 120 to 180.

**Peroxide value** (*2.5.5, Method A*): maximum 15.

Take from the substance to be examined wedge-shaped pieces whose base consists of part of the surface. Melt the pieces before carrying out the determination. Before adding 0.5 mL of *saturated potassium iodide solution R*, cool the solution obtained to room temperature.

**Saponification value** (*2.5.6*): maximum 12.0, determined on 10.00 g. Heat under reflux for 4 h.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 2.000 g by drying in an oven at 105 °C.

**Total ash** (*2.4.16*): maximum 0.1 per cent.

ASSAY

Gas chromatography (*2.2.28*). *Homogenise the sample before use.*

*Internal standard solution.* Dissolve 0.125 g of *pregnenolone isobutyrate CRS* in *heptane R* and dilute to 50.0 mL with the same solvent.

*Test solution.* Dissolve 75.0 mg of the substance to be examined in 10.0 mL of the internal standard solution and dilute to 25.0 mL with *heptane R*.

*Reference solution.* Dissolve 25.0 mg of *cholesterol CRS* in 10.0 mL of the internal standard solution and dilute to 25.0 mL with *heptane R*.

*Injection liner*:

- *packing material*: quartz wool;
- *size*: *l* = 78.5 mm, Ø = 4.0 mm.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50.

*Temperature*:

- *column*: 275 °C;
- *injection port*: 285 °C;
- *detector*: 300 °C.

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Relative retention* with reference to pregnenolone isobutyrate (retention time = about 8 min): cholesterol = about 1.2.

*System suitability*: reference solution:

- *resolution*: minimum 5.0 between the peaks due to pregnenolone isobutyrate and cholesterol.

Calculate the percentage content of cholesterol in the substance to be examined taking into account the assigned content of *cholesterol CRS*.

STORAGE

In a well-filled container, protected from light.

**04/2012:0134**
**corrected 10.0**

# WOOL FAT

## Adeps lanae

DEFINITION

Purified, anhydrous, waxy substance obtained from the wool of sheep (*Ovis aries*). A suitable antioxidant may be added.

CHARACTERS

*Appearance*: yellow, unctuous substance. When melted, it is a clear or almost clear, yellow liquid. A solution in light petroleum is opalescent.

*Solubility*: practically insoluble in water, slightly soluble in boiling anhydrous ethanol.

Characteristic odour.

IDENTIFICATION

A. In a test-tube, dissolve 0.5 g in 5 mL of *methylene chloride R* and add 1 mL of *acetic anhydride R* and 0.1 mL of *sulfuric acid R*. A green colour develops.

B. Dissolve 50 mg in 5 mL of *methylene chloride R*, add 5 mL of *sulfuric acid R* and shake. A red colour develops and an intense green fluorescence appears in the lower layer when examined in daylight, with illumination from behind the observer.

TESTS

**Water-soluble acid or alkaline substances**. Melt 5.0 g on a water-bath and shake vigorously for 2 min with 75 mL of *water R* previously heated to 90-95 °C. Allow to cool and filter through filter paper previously rinsed with *water R*. To 60 mL of the filtrate (which may not be clear) add 0.25 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.02 M hydrochloric acid* or 0.15 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Water-absorption capacity**. Place 10 g of molten wool fat in a mortar and allow to cool to room temperature. Weigh the mortar. Add *water R* in portions of 0.2-0.5 mL from a burette, stirring vigorously after each addition to incorporate the *water R*. Instead of a pestle, use a high-density polypropylene cylindrical rod (120 mm long and 10 mm in diameter, for

example). The end-point is reached when visible droplets remain which cannot be incorporated. Weigh the mortar again and determine the amount of water absorbed by weight difference. Not less than 20 mL of *water R* is absorbed.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g dissolved in 25 mL of the prescribed mixture of solvents.

**Peroxide value** (*2.5.5, Method A*): maximum 20.

Before adding 0.5 mL of *saturated potassium iodide solution R*, cool the solution obtained to room temperature.

**Saponification value** (*2.5.6*): 90 to 105, determined on 2.00 g while heating under reflux for 4 h.

**Water-soluble oxidisable substances**. To 10 mL of the filtrate obtained in the test for water-soluble acid or alkaline substances add 1 mL of *dilute sulfuric acid R* and 0.1 mL of *0.02 M potassium permanganate*. After 10 min, the solution is not completely decolourised.

**Paraffins**: maximum 1.0 per cent.

*The tap and cotton plugs used must be free from grease.* Prepare a column of anhydrous aluminium oxide 0.23 m long and 20 mm in diameter by adding a slurry of *anhydrous aluminium oxide R* and *light petroleum R1* to a glass tube fitted with a tap and containing *light petroleum R1* (before use, dehydrate the anhydrous aluminium oxide by heating it in an oven at 600 °C for 3 h). Allow to settle and reduce the depth of the layer of solvent above the column to about 40 mm. Dissolve 3.0 g of the substance to be examined in 50 mL of warm *light petroleum R1*, cool, pass the solution through the column at a flow rate of 3 mL/min and wash with 250 mL of *light petroleum R1*. Concentrate the combined eluate and washings to low bulk by distillation, evaporate to dryness on a water-bath and heat the residue at 105 °C for periods of 10 min until 2 successive weighings do not differ by more than 1 mg. The residue weighs a maximum of 30 mg.

**Pesticide residues**: maximum 0.05 ppm for each organochlorine pesticide, 0.5 ppm for each other pesticide and 1 ppm for the sum of all the pesticides.

*All glassware used is thoroughly washed using phosphate-free detergent as follows. The glassware is immersed in a bath of detergent solution (5 per cent in deionised water) and allowed to soak for 24 h. The detergent is washed off with copious amounts of acetone and hexane for pesticide analysis. It is important to keep glassware specifically for pesticide analyses, it must not be mixed up with glassware used for other applications. The glassware used must be free of chlorinated solvents, plastics and rubber materials, in particular phthalate plasticisers, oxygenated compounds and nitrogenated solvents such as acetonitrile. Use hexane, toluene and acetone for pesticide analysis. Use HPLC grade reagents for ethyl acetate, cyclohexane and water.*

The test consists of the isolation of pesticide residues by size-exclusion chromatography (*2.2.30*) followed by solid phase extraction and identification by gas chromatography coupled with an electron capture detector or a thermionic detector.

*ISOLATION OF THE PESTICIDE RESIDUES.* As detector, use a UV-visible spectrophotometer set at a wavelength of 254 nm to calibrate the chromatographic column for gel permeation.

Calibration is extremely important in gel permeation chromatography (GPC) to check that the pressure, solvent flow rate, solvent ratio, temperature and column conditions remain constant. The gel permeation column is to be calibrated at regular intervals using a standard mixture prepared as follows: into a 1000 mL volumetric flask, introduce 50.00 g of *maize oil R*, 0.20 g of *methoxychlor R* and 50.0 mg of *perylene R*. Dilute to 1000.0 mL with a mixture of equal volumes of *cyclohexane R* and *ethyl acetate R*.

To calibrate the column, set the mobile phase at a flow rate of 5 mL/min with a mixture of equal volumes of *cyclohexane R* and *ethyl acetate R*. Inject 5 mL of the standard mixture and record the resulting chromatogram. The retention times for the analytes must not vary by more than ± 5 per cent between calibrations. If the retention time shift is greater than ± 5 per cent, take corrective action. Excessive retention time shifts may be caused by:

- poor laboratory temperature control;
- the pump containing air; this can be verified by measuring the flow rate: collect 25 mL of column eluate in a volumetric flask and record the time (300 ± 5 s);
- a leak in the system.

Changes in pressure, in mobile phase flow rate or in column temperature conditions, as well as column contamination, can affect pesticide retention times and are to be monitored. If the flow rate or column pressure are outside desired bands the precolumn or column is to be replaced.

*Test solution.* In a volumetric flask, dissolve 1 g of the substance to be examined, accurately weighed, in a mixture of 1 volume of *ethyl acetate R* and 7 volumes of *cyclohexane R*. Add 1 mL of an internal standard (2 ppm, either *isodrin R* or *ditalimphos R*) and dilute to 20 mL. *The internal standard solutions are used to establish that recoveries of the pesticides from the GPC purification stage, evaporation and solid phase extraction stage are at acceptable levels. Recovery levels of the internal standard solutions from the wool fat are determined by comparing the peak areas of the wool fat extracts with peak areas of solutions of the internal standards.*

*Precolumn*:

- *size*: *l* = 0.075 m, Ø = 21.2 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm).

*Gel permeation column*:

- *size*: *l* = 0.3 m, Ø = 21.2 mm;
- *stationary phase*: *styrene-divinylbenzene copolymer R* (5 µm).

*Mobile phase*: *ethyl acetate R*, *cyclohexane R* (10:70 *V/V*).

*Flow rate*: 5 mL/min.

*Detection*: spectrophotometer at 254 nm.

Inject 5 mL of the test solution. Discard the first 95 mL (19 min) of eluate containing the substance to be examined. Collect the next 155 mL of eluate (31 min) containing any pesticide residues in an evaporating vessel.

Place the 155 mL of the eluate collected from the gel permeation chromatography column into an evaporating vessel. Place this vessel in an autoevaporator setting the water-bath temperature at 45 °C and the nitrogen pressure at 55 kPa. Evaporate the eluate down to 0.5 mL.

To prepare the solid phase extraction cartridges take some *magnesium silicate for pesticide residue analysis R* and heat it in a muffle furnace at 700 °C for 4 h to remove moisture and polychlorinated biphenyls. Subsequently allow the magnesium silicate to cool for 2 h and transfer it directly to an oven at 100-105 °C, and allow to stand for 30 min. Transfer the magnesium silicate to a stoppered glass jar and allow to equilibrate for 48 h. This material may be used for up to 2 weeks. After that period the magnesium silicate is to be reactivated, by heating at 600 °C for 2 h in a muffle furnace. Remove the magnesium silicate from the furnace, cool and store in a stoppered glass jar. The magnesium silicate is deactivated by adding 1 per cent of *water R*. After the water has been added, shake the magnesium silicate intermittently over 15 min just prior to use. The deactivated magnesium silicate is suitable for use for up to 1 week. It is essential that only deactivated magnesium silicate is used.

Take a 6 mL empty solid phase extraction cartridge and weigh into the cartridge 1 g of the deactivated magnesium silicate.

At this stage the GPC fraction still contains about 10 per cent of the substance to be examined, so further clean-up is necessary. A separate isolation procedure is carried out a) for organochlorine and synthetic pyrethroid pesticides and

b) for organophosphorus pesticides. Place a preconditioned solid phase extraction cartridge containing l g of deactivated *magnesium silicate for pesticide residue analysis R* onto a vacuum manifold.

Condition the cartridge by adding 10 mL of *toluene R* and allowing the solvent to elute through the cartridge. Place the 0.5 mL of the solvent fraction from the evaporating vessel on the preconditioned cartridge. Elute the pesticide fractions from the cartridges using 20 mL of either of the 2 different solvent systems shown below:

a) for determination of the organochlorine and synthetic pyrethroid pesticides: *toluene R*; a very small amount of the substance to be examined is co-eluted;

b) for determination of the organophosphorus pesticides: a mixture of 2 volumes of *acetone R* and 98 volumes of *toluene R*; this solvent system is used to elute all the pesticides including the more polar organophosphorus pesticides; unfortunately, some of the substance to be examined is co-eluted with this solvent system, which can interfere with the electron capture detector.

Collect the eluate from the extraction cartridges in 25 mL glass vials. Quantitatively transfer the eluate to an evaporating vessel, washing the vial with 3 quantities, each of 10 mL, of *hexane R*.

Place the evaporating vessel on the autoevaporator and evaporate the solid phase extraction fractions down to 0.5 mL. The water-bath temperature is set at 45 °C and the nitrogen pressure is 55 kPa.

Examine the residues by gas chromatography (*2.2.28*) using electron capture and thermionic detectors as described below.

*Recovery*. Calculate the recovery correction factor ($R_{cf}$) of the internal standards (*ditalimphos R* or *isodrin R*) added to the test solution using the following expression:

$$\frac{A_2}{A_1} \times 100$$

$A_1$ = peak area of an internal standard 1 ppm in solution;

$A_2$ = peak area of internal standard extracted from the test solution.

5 mL of the 20 mL test solution containing 1 mL of 2 ppm internal standard concentrated to 0.5 mL is equivalent to 1 ppm of the internal standard in the solution.

If the recovery of the internal standards falls outside the range of 70 per cent to 110 per cent the test is not valid.

*Reference solutions*. Prepare reference solutions of pesticides using the pesticides standards at a concentration of 0.5 ppm (see composition of reference solutions A to D in Table 0134.-1). Commercially available pesticides may be purchased. The individual standards have a concentration of 10 ppm.

Table 0134.-1. – *Composition of the reference solutions*

| | |
|---|---|
| **Reference solution A (0.5 ppm or 0.5 mg/L) (organochlorine and synthetic pyrethroid pesticides)** | **Reference solution B (0.5 ppm or 0.5 mg/L) (organochlorine and synthetic pyrethroid pesticides)** |
| *Cyhalothrin R* | *Aldrin R* |
| *Cypermethrin R* | *o,p'-DDT R* |
| *o,p'-DDE R* | *o,p'-DDD R* |
| *p,p'-DDE R* | *p,p'-DDD R* |
| *p,p'-DDT R* | *Dieldrin R* |
| *Deltamethrin R* | *α-Endosulfan R* |
| *Endrin R* | *β-Endosulfan R* |
| *Heptachlor R* | *Fenvalerate R* |
| *Heptachlor epoxide R* | *α-Hexachlorocyclohexane R* |
| *Hexachlorobenzene R* | *β-Hexachlorocyclohexane R* |
| *Lindane R* | *δ-Hexachlorocyclohexane R* |
| *Tecnazene R* | *Methoxychlor R* |
| | *Permethrin R* |
| **Reference solution C (0.5 ppm or 0.5 mg/L) (organophosphorus pesticides)** | **Reference solution D (0.5 ppm or 0.5 mg/L) (organophosphorus pesticides)** |
| *Bromophos-ethyl R* | *Bromophos R* |
| *Carbophenothion R* | *Chlorpyriphos R* |
| *Chlorfenvinphos R* | *Chlorpyriphos-methyl R* |
| *Diazinon R* | *Coumaphos R* |
| *Dichlofenthion R* | *Phosalone R* |
| *Ethion R* | *Pirimiphos-ethyl R* |
| *Fenchlorphos R* | *Tetrachlorvinphos R* |
| *Malathion R* | |
| *Propetamphos R* | |
| **Reference solution E (electron capture detector calibration mixture)** | **Reference solution F (thermionic detector calibration mixture)** |
| *Aldrin R* (0.01 mg/L) | *Chlorfenvinphos R* (0.05 mg/L) |
| *Cypermethrin R* (0.1 mg/L) | *Diazinon R* (0.05 mg/L) |
| *o,p'-DDD R* (0.01 mg/L) | *Ethion R* (0.05 mg/L) |
| *Deltamethrin R* (0.1 mg/L) | *Fenchlorphos R* (0.05 mg/L) |
| *Endrin R* (0.01 mg/L) | *Propetamphos R* (0.05 mg/L) |
| *β-Hexachlorocyclohexane R* (0.01 mg/L) | |
| **Reference solution G (internal standard organophosphorus pesticide)** | **Reference solution H (internal standard organochlorine pesticide)** |
| *Ditalimphos R* (2 ppm or 2.0 mg/L) | *Isodrin R* (2 ppm or 2.0 mg/L) |
| *Ditalimphos R* (1 ppm or 1.0 mg/L) | *Isodrin R* (1 ppm or 1.0 mg/L) |

At the same time prepare solutions of pesticides equivalent to the limit of detection of the method (see recommended compositions in Table 0134.-1). These reference solutions are used to optimise the electron capture detector and thermionic detector to achieve the detection limits of the method (reference solutions E and F).

To prepare the reference solutions at the different concentrations use a calibrated pipette and volumetric flasks. To prepare the internal standard solutions G and H use a four-place analytical balance, pipette and volumetric flasks.

*IDENTIFICATION AND QUANTIFICATION OF THE PESTICIDE RESIDUES*. To identify any pesticide residues compare the chromatograms obtained with chromatograms obtained with reference solutions A to D.

The identity of the pesticides can be confirmed by spiking samples or overlaying chromatograms using an integration package on a computer. The interpretation of pesticides in trace residue analyses is extremely complex. The detectors, particularly the electron capture detector, are prone to

interference, both from the substance to be examined itself, and from solvents, reagents and apparatus used in the extraction. These peaks can easily be misinterpreted or quoted as a false positive. Confirmation of pesticides can be achieved by running samples and standards on different capillary columns (see chromatographic systems A or B described below). The peaks can be identified by using the information in Table 0134.-2.

A knowledge of the different responses the pesticides have with the 2 detectors is useful in identification of unknown peaks.

Table 0134.-2. – *Elution order of the pesticides on chromatographic systems A and B*

| Chromatographic system A | Chromatographic system B |
|---|---|
| Tecnazene | Tecnazene |
| α-Hexachlorocyclohexane | Hexachlorobenzene |
| Hexachlorobenzene | α-Hexachlorocyclohexane |
| β-Hexachlorocyclohexane | Diazinon |
| Lindane | Lindane |
| Propetamphos | Propetamphos |
| δ-Hexachlorocyclohexane | Heptachlor |
| Diazinon | Dichlofenthion |
| Dichlofenthion | Aldrin |
| Chlorpyriphos-methyl | Chlorpyriphos-methyl |
| Heptachlor | Fenchlorphos |
| Fenchlorphos | β-Hexachlorocyclohexane |
| Aldrin | δ-Hexachlorocyclohexane |
| Malathion | Pirimiphos-ethyl |
| Chlorpyriphos | Chlorpyriphos |
| Bromophos | Bromophos |
| Pirimiphos-ethyl | Malathion |
| Heptachlor epoxide | Heptachlor epoxide |
| Chlorfenvinphos (*E*) | *o,p'*-DDE |
| Chlorfenvinphos (*Z*) | Chlorfenvinphos (*E*) |
| Bromophos-ethyl | α-Endosulfan |
| *o,p'*-DDE | Chlorfenvinphos (*Z*) |
| α-Endosulfan | Bromophos-ethyl |
| Tetrachlorvinphos | *p,p'*-DDE |
| Dieldrin | Dieldrin |
| *p,p'*-DDE | Tetrachlorvinphos |
| *o,p'*-DDT | *o,p'*-DDT |
| Endrin | Endrin |
| β-Endosulfan | *o,p'*-DDD |
| *o,p'*-DDD | *p,p'*-DDD |
| *p,p'*-DDD | β-Endosulfan |
| Ethion | Ethion |
| Carbophenothion | *p,p'*-DDT |
| *p,p'*-DDT | Carbophenothion |
| Methoxychlor | Methoxychlor |
| Phosalone | Cyhalothrin |
| Cyhalothrin (2 isomers) | *cis*-Permethrin |
| *cis*-Permethrin | Phosalone |
| *trans*-Permethrin | *trans*-Permethrin |
| Coumaphos | Cypermethrin (4 isomers) |
| Cypermethrin (4 isomers) | Coumaphos |
| Fenvalerate (2 isomers) | Fenvalerate (2 isomers) |
| Deltamethrin | Deltamethrin |

Once the pesticides have been identified, calculate the content of each pesticide using the following expression:

$$C_p = \frac{P_p \times D \times C_e}{P_e} \times \frac{100}{R_{cf}}$$

$C_p$ = concentration of identified pesticide (ppm);

$P_p$ = peak area of the individual pesticide in the test sample obtained;

$C_e$ = concentration of the individual pesticide in the external standard (ppm);

$P_e$ = peak area of the individual pesticide in the external standard;

$D$ = dilution factor;

$R_{cf}$ = recovery correction factor.

The dilution factor ($D$) can be defined as follows:

$$\frac{V_1}{m \times \frac{V_2}{V_3}}$$

$V_1$ = volume of sample obtained after the 2nd evaporation stage;

$m$ = sample weight;

$V_2$ = GPC injection volume;

$V_3$ = sample volumetric flask volume.

*Chromatographic system A*:

*Precolumn*:

– *material*: deactivated silica;

– *size*: *l* = 4.5 m, Ø = 0.53 mm.

*Column*:

– *material*: fused silica;

– *size*: *l* = 60 m, Ø = 0.25 mm;

– *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 25 cm/s.

*Pressure*: 180 kPa.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 75 |
| | 1 - 5 | 75 → 175 |
| | 5 - 30 | 175 → 275 |
| | 30 - 40 | 275 → 285 |
| | 40 - 55 | 285 |
| Injection port | | 300 |
| Detector | | 350 |

*Detection*: electron capture or thermionic specific detector.

*Injection*: 2 µL.

*Chromatographic system B* which may be used for confirmation analysis:

*Precolumn*:

– *material*: deactivated silica;

– *size*: *l* = 4.5 m, Ø = 0.53 mm.

*Column*:

– *material*: fused silica;

– *size*: *l* = 60 m, Ø = 0.25 mm;

– *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R*.

*Linear velocity*: 25 cm/s.

*Pressure*: 180 kPa.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 75 |
| | 1 - 5 | 75 → 175 |
| | 5 - 30 | 175 → 275 |
| | 30 - 40 | 275 → 285 |
| | 40 - 55 | 285 |
| Injection port | | 300 |
| Detector | | 350 |

*Detection*: electron capture or thermionic specific detector.

*Injection*: 2 µL.

**Chlorides**: maximum 150 ppm.

Boil 1.0 g with 20 mL of *ethanol (90 per cent V/V) R* in a round-bottomed flask fitted with a reflux condenser for 5 min. Cool, add 40 mL of *water R* and 0.5 mL of *nitric acid R* and filter. To the filtrate add 0.15 mL of a 10 g/L solution of *silver nitrate R* in *ethanol (90 per cent V/V) R*. Allow to stand for 5 min protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by adding 0.15 mL of a 10 g/L solution of *silver nitrate R* in *ethanol (90 per cent V/V) R* to a mixture of 0.2 mL of *0.02 M hydrochloric acid*, 20 mL of *ethanol (90 per cent V/V) R*, 40 mL of *water R* and 0.5 mL of *nitric acid R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 1 h.

**Sulfated ash** (*2.4.14*): maximum 0.15 per cent.

Ignite 5.0 g and use the residue to determine the sulfated ash.

STORAGE

At a temperature not exceeding 25 °C.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for wool fat used in water-emulsifying ointments and lipophilic creams.*

**Water-absorption capacity** (see Tests).

**Drop point** (*2.2.17, Method A*). To fill the metal cup, melt the wool fat on a water-bath, cool to about 50 °C, pour into the cup and allow to stand at 15-20 °C for 24 h. The drop point is typically 38 °C to 44 °C.

**01/2017:0969**
**corrected 10.0**

# WOOL FAT, HYDROGENATED

## Adeps lanae hydrogenatus

DEFINITION

Mixture of higher aliphatic alcohols and sterols obtained from the direct, high-pressure, high-temperature hydrogenation of *wool fat (0134)* during which the esters and acids present are reduced to the corresponding alcohols. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: white or pale yellow, unctuous substance.

*Solubility*: practically insoluble in water, soluble in boiling anhydrous ethanol and in light petroleum.

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (see Tests).

B. Examine the chromatograms obtained in the test for fatty alcohols and sterols.

*Results*: the principal peaks in the chromatogram obtained with the test solution are similar in retention time and size to the principal peaks in the chromatogram obtained with reference solution (a).

C. Dissolve 50 mg in 5 mL of *methylene chloride R* and add 1 mL of *acetic anhydride R* and 0.1 mL of *sulfuric acid R*. A green colour is produced.

TESTS

**Melting point** (*2.2.15*): 45 °C to 55 °C. Allow to stand at 20 °C for 16 h.

**Acid value** (*2.5.1*): maximum 1.0, determined on 5.0 g.

**Hydroxyl value** (*2.5.3, Method A*): 140 to 180.

**Saponification value** (*2.5.6*): maximum 8.0. Heat under reflux for 4 h.

**Fatty alcohols and sterols**. Gas chromatography (*2.2.28*).

*Test solution.* Dissolve 0.25 g of the substance to be examined in 60 mL of *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dissolve 0.25 g of *hydrogenated wool fat CRS* in 60 mL of *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dissolve 50 mg of *cetyl alcohol CRS* and 50 mg of *stearyl alcohol CRS* in 60 mL of *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

*Column*:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* or another non-polar phase (film thickness 0.25 µm).

*Carrier gas*: *helium for chromatography R.*

*Flow rate*: 1 mL/min.

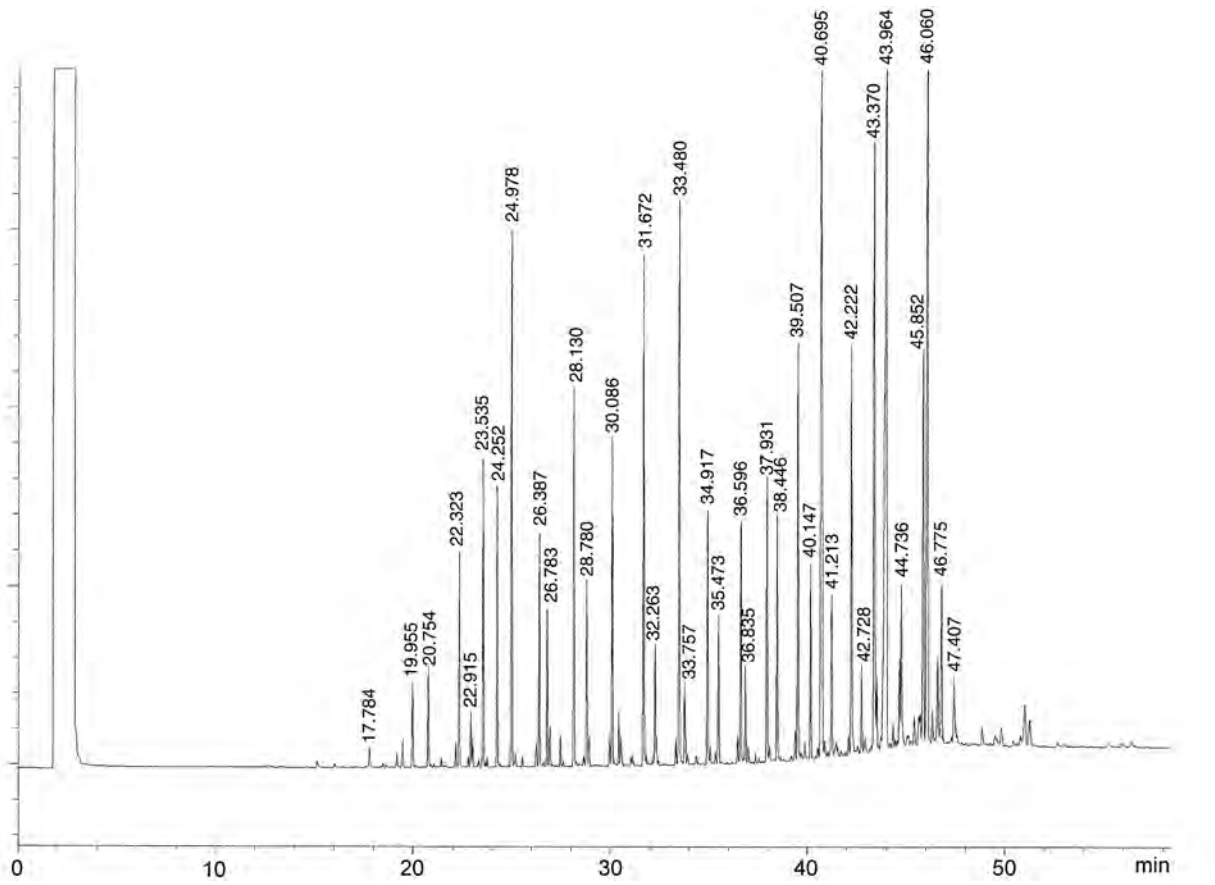


Figure 0969.-1. – *Chromatogram for the test for fatty alcohols and sterols in hydrogenated wool fat: reference solution (a)*

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 100 |
| | 5 - 45 | 100 → 300 |
| | 45 - 60 | 300 |
| Injection port | | 325 |
| Detector | | 350 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

*Results*: the chromatogram obtained with the test solution does not differ significantly from the chromatogram obtained with reference solution (a) (Figure 0969.-1) and it does not show enhanced peaks with retention times corresponding to cetyl alcohol and stearyl alcohol present in the chromatogram obtained with reference solution (b).

**Loss on drying** (*2.2.32*): maximum 3.0 per cent, determined on 2.000 g by drying in an oven at 105 °C for 1 h.

**Total ash** (*2.4.16*): maximum 0.1 per cent, determined on 5.0 g.

STORAGE

In a well-filled container, protected from light.

**04/2012:0135**

# WOOL FAT, HYDROUS

## Adeps lanae cum aqua

DEFINITION

Mixture of 75 per cent *m/m* of wool fat and 25 per cent *m/m* of water. It is obtained by the gradual addition of water to melted wool fat with continuous stirring. A suitable antioxidant may be added.

CHARACTERS

*Appearance*: pale yellow, unctuous substance.

IDENTIFICATION

A. In a test-tube, dissolve 0.5 g in 5 mL of *methylene chloride R* and add 1 mL of *acetic anhydride R* and 0.1 mL of *sulfuric acid R*. A green colour develops.

B. Dissolve 50 mg in 5 mL of *methylene chloride R*, add 5 mL of *sulfuric acid R* and shake. A red colour develops and an intense green fluorescence appears in the lower layer when examined in daylight, with illumination from behind the observer.

TESTS

**Water-soluble acid or alkaline substances**. Melt 6.7 g on a water-bath and shake vigorously for 2 min with 75 mL of *water R* previously heated to 90-95 °C. Allow to cool and filter through filter paper previously rinsed with *water R*. To 60 mL of the filtrate (which may not be clear) add 0.25 mL of *bromothymol blue solution R1*. Not more than 0.2 mL of *0.02 M hydrochloric acid* or 0.15 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator.

**Drop point** (*2.2.17*): 38 °C to 44 °C.

To fill the metal cup, melt the residue obtained in the test for wool-fat content on a water-bath, cool to about 50 °C, pour into the cup and allow to stand at 15-20 °C for 24 h.

**Water-absorption capacity**. Place 10 g of the residue obtained in the test for wool-fat content in a mortar. Add *water R* in portions of 0.2-0.5 mL from a burette, stirring vigorously after each addition to incorporate the *water R*. The end-point is reached when visible droplets remain which cannot be incorporated. Not less than 20 mL of *water R* is absorbed.

**Acid value** (*2.5.1*): maximum 0.8, determined on 5.0 g dissolved in 25 mL of the prescribed mixture of solvents.

**Peroxide value** (*2.5.5, Method A*): maximum 15.

**Saponification value** (*2.5.6*): 67 to 79, determined on 2.00 g while heating under reflux for 4 h.

**Water-soluble oxidisable substances**. To 10 mL of the filtrate obtained in the test for water-soluble acid or alkaline substances add 1 mL of *dilute sulfuric acid R* and 0.1 mL of *0.02 M potassium permanganate*. After 10 min, the solution is not completely decolourised.

**Paraffins**: maximum 1.0 per cent.

*The tap and cotton plugs used must be free from grease.* Prepare a column of anhydrous aluminium oxide 230 mm long and 20 mm in diameter by adding a slurry of *anhydrous aluminium oxide R* and *light petroleum R1* to a glass tube fitted with a tap and containing *light petroleum R1*. Allow to settle and reduce the depth of the layer of solvent above the column to about 40 mm. Dissolve 3.0 g of the residue obtained in the test for wool-fat content in 50 mL of warm *light petroleum R1*, cool, pass the solution through the column at a rate of 3 mL/min and wash with 250 mL of *light petroleum R1*. Concentrate the combined eluate and washings to low bulk by distillation, evaporate to dryness on a water-bath and heat the residue at 105 °C for periods of 10 min until 2 successive weighings do not differ by more than 1 mg. The residue weighs a maximum of 30 mg.

**Chlorides**: maximum 115 ppm.

Boil 1.3 g with 20 mL of *ethanol (90 per cent V/V) R* under a reflux condenser for 5 min. Cool, add 40 mL of *water R* and 0.5 mL of *nitric acid R* and filter. To the filtrate add 0.15 mL of a 10 g/L solution of *silver nitrate R* in *ethanol (90 per cent V/V) R*. Allow to stand for 5 min, protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time by adding 0.15 mL of a 10 g/L solution of *silver nitrate R* in *ethanol (90 per cent V/V) R* to a mixture of 0.2 mL of *0.02 M hydrochloric acid*, 20 mL of *ethanol (90 per cent V/V) R*, 40 mL of *water R* and 0.5 mL of *nitric acid R*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent.

Ignite 5.0 g and use the residue.

**Wool-fat content**: 72.5 per cent to 77.5 per cent.

In a suitable tared dish containing a glass rod, heat 30.0 g to constant mass on a water-bath, stirring continuously. Weigh the residue.

STORAGE

At a temperature not exceeding 25 °C.

# X

Xanthan gum.... 4221
Xylazine hydrochloride for veterinary use.. .... 4222
Xylitol.. .... 4223
Xylometazoline hydrochloride.. .... 4225
Xylose.. .... 4226

**07/2017:1277**

# XANTHAN GUM

## Xanthani gummi

[11138-66-2]

DEFINITION

High-molecular-mass anionic polysaccharide produced by fermentation of carbohydrates with *Xanthomonas campestris*. It consists of a principal chain of β(1→4)-linked D-glucose units with trisaccharide side chains, on alternating anhydroglucose units, consisting of 1 glucuronic acid unit included between 2 mannose units. Most of the terminal units contain a pyruvate moiety and the mannose unit adjacent to the principal chain may be acetylated at C-6.

Xanthan gum has a relative molecular mass of approximately $1 \times 10^6$. It exists as the sodium, potassium or calcium salt.

*Content*: minimum 1.5 per cent of pyruvate acetal groups ($C_3H_3O_2$; $M_r$ 71.1) (dried substance).

CHARACTERS

*Appearance*: white or yellowish-white, free-flowing powder.

*Solubility*: soluble in water giving a highly viscous solution, practically insoluble in organic solvents.

IDENTIFICATION

A. In a flask, suspend 1 g in 15 mL of *0.1 M hydrochloric acid*. Close the flask with a fermentation bulb containing *barium hydroxide solution R* and heat carefully for 5 min. The barium hydroxide solution shows a white turbidity.

B. To 300 mL of *water R*, previously heated to 80 °C and stirred rapidly with a mechanical stirrer in a 400 mL beaker, add, at the point of maximum agitation, a dry blend of 1.5 g of *carob bean gum R* and 1.5 g of the substance to be examined. Stir until the mixture forms a solution, and then continue stirring for 30 min or longer. Do not allow the water temperature to drop below 60 °C during stirring. Discontinue stirring and allow the mixture to stand for at least 2 h. A firm rubbery gel forms after the temperature drops below 40 °C but no such gel forms in a 1 per cent control solution of the sample prepared in the same manner but omitting the carob bean gum.

TESTS

**pH** (*2.2.3*): 6.0 to 8.0 for a 10.0 g/L solution in *carbon dioxide-free water R*.

**2-Propanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 0.50 g of *2-methyl-2-propanol R* to 500 mL with *water R*.

*Test solution.* To 200 mL of *water R* in a 1000 mL round-bottomed flask, add 5.0 g of the substance to be examined and 1 mL of a 10 g/L emulsion of *dimeticone R* in *liquid paraffin R*, stopper the flask and shake for 1 h. Distil about 90.0 mL, mix the distillate with 4.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Reference solution.* Dilute a suitable quantity of *2-propanol R*, accurately weighed, with *water R* to obtain a solution having a known concentration of 2-propanol of about 1 mg/mL. To 4.0 mL of this solution add 4.0 mL of the internal standard solution and dilute to 100.0 mL with *water R*.

*Column*:

– *size*: *l* = 1.8 m, Ø = 4.0 mm;

– *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (149-177 μm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 30 mL/min.

*Temperature*:

– *column*: 165 °C;

– *injection port and detector*: 200 °C.

*Detection*: flame ionisation.

*Injection*: 5 μL.

*Relative retention* with reference to 2-propanol: 2-methyl-2-propanol = about 1.5.

*Limit*:

– *2-propanol*: maximum 750 ppm.

**Loss on drying** (*2.2.32*): maximum 15.0 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2.5 h.

**Total ash** (*2.4.16*): 6.5 per cent to 16.0 per cent.

ASSAY

*Test solution.* Dissolve a quantity of the substance to be examined corresponding to 120.0 mg of the dried substance in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution.* Dissolve 45.0 mg of *pyruvic acid R* in *water R* and dilute to 500.0 mL with the same solvent.

Place 10.0 mL of the test solution in a 50 mL round-bottomed flask, add 20.0 mL of *0.1 M hydrochloric acid* and weigh. Boil on a water-bath under a reflux condenser for 3 h. Weigh and adjust to the initial mass with *water R*. In a separating funnel mix 2.0 mL of the solution with 1.0 mL of *dinitrophenylhydrazine-hydrochloric solution R*. Allow to stand for 5 min and add 5.0 mL of *ethyl acetate R*. Shake and allow the solids to settle. Collect the upper layer and shake with 3 quantities, each of 5.0 mL, of *sodium carbonate solution R*. Combine the aqueous layers and dilute to 50.0 mL with *sodium carbonate solution R*. Mix. Treat 10.0 mL of the reference solution at the same time and in the same manner as for the test solution.

Immediately measure the absorbance (*2.2.25*) of the 2 solutions at 375 nm, using *sodium carbonate solution R* as the compensation liquid.

The absorbance of the test solution is not less than that of the reference solution, which corresponds to a content of pyruvoyl groups of not less than 1.5 per cent.

FUNCTIONALITY-RELATED CHARACTERISTICS

*This section provides information on characteristics that are recognised as being relevant control parameters for one or more functions of the substance when used as an excipient (see chapter 5.15). Some of the characteristics described in the Functionality-related characteristics section may also be present in the mandatory part of the monograph since they also represent mandatory quality criteria. In such cases, a cross-reference to the tests described in the mandatory part is included in the Functionality-related characteristics section. Control of the characteristics can contribute to the quality of a medicinal product by improving the consistency of the manufacturing process and the performance of the medicinal product during use. Where control methods are cited, they are recognised as being suitable for the purpose, but other methods can also be used. Wherever results for a particular characteristic are reported, the control method must be indicated.*

*The following characteristics may be relevant for xanthan gum used as viscosity-increasing agent.*

**Viscosity** (*2.2.10*): typically minimum 600 mPa·s.

Add 3.0 g (dried substance) within 45-90 s into 250 mL of a 12 g/L solution of *potassium chloride R* in a 500 mL beaker stirring with a low-pitch propeller-type stirrer rotating at 800 r/min. When adding the substance take care that agglomerates are destroyed. Add an additional quantity of 44 mL of *water R*, to rinse any adhering residue from the walls of the beaker. Stir the preparation at 800 r/min for 2 h whilst maintaining the temperature at 24 ± 1 °C. Determine the viscosity within 15 min at 24 ± 1 °C using a rotating

viscosimeter set at 60 r/min and equipped with a rotating spindle 1.78 mm high and 12.60 mm in diameter which is attached to a shaft 3.2 mm in diameter. The distance from the top of the cylinder to the lower tip of the shaft should be 25.60 mm and the immersion depth 50.0 mm.

*The following characteristics may be relevant for xanthan gum used as matrix former in prolonged-release tablets.*

**Viscosity**: see test above.

**Particle-size distribution** (*2.9.31* or *2.9.38*).

**Powder flow** (*2.9.36*).

**07/2019:1481**

# XYLAZINE HYDROCHLORIDE FOR VETERINARY USE

## Xylazini hydrochloridum ad usum veterinarium

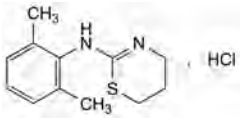

$C_{12}H_{17}ClN_2S$ $M_r$ 256.8
[23076-35-9]

DEFINITION

*N*-(2,6-Dimethylphenyl)-5,6-dihydro-4*H*-1,3-thiazin-2-amine hydrochloride.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder, hygroscopic.

*Solubility*: freely soluble in water, very soluble in methanol, freely soluble in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *xylazine hydrochloride CRS*.

If the spectra obtained show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness at 60 °C at a pressure of 10-20 kPa, and record new spectra using the residues.

B. It gives reaction (b) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 5.0 g in *carbon dioxide-free water R*, heating at 60 °C if necessary; allow to cool and dilute to 50.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.5 for solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Solvent mixture*. Mix 8 volumes of *acetonitrile R*, 30 volumes of *methanol R* and 62 volumes of a 2.72 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.2 with *dilute sodium hydroxide solution R*.

*Test solution*. Dissolve 0.100 g of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 50.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5.0 mg of *bupivacaine impurity F CRS* ( xylazine impurity A) in *acetonitrile R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c)*. Dilute 1.0 mL of reference solution (b) to 100.0 mL with the solvent mixture.

*Reference solution (d)*. Dilute 1 mL of the test solution to 100 mL with the solvent mixture. Mix 10 mL of this solution with 10 mL of reference solution (b). Dilute 1 mL of this solution to 5 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 30 volumes of *methanol R1* and 70 volumes of a 2.72 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 7.2 with *dilute sodium hydroxide solution R*;
- *mobile phase B*: *methanol R1*, *acetonitrile for chromatography R* (30:70 *V/V*);

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 15 | 89 → 28 | 11 → 72 |
| 15 - 21 | 28 | 72 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 230 nm.

*Injection*: 20 µL of the test solution and reference solutions (a), (c) and (d).

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity A.

*Relative retention* with reference to xylazine (retention time = about 8 min): impurity A = about 0.8.

*System suitability*: reference solution (d):

- *resolution*: minimum 4.0 between the peaks due to impurity A and xylazine.

*Calculation of percentage contents*:

- for impurity A, use the concentration of impurity A in reference solution (c);
- for impurities other than A, use the concentration of xylazine hydrochloride in reference solution (a).

*Limits*:

- *impurity A*: maximum 0.01 per cent;
- *unspecified impurities*: for each impurity, maximum 0.20 per cent;
- *total*: maximum 0.2 per cent;
- *reporting threshold*: 0.10 per cent, except for impurity A.

**Heavy metals** (*2.4.8*): maximum 10 ppm.

12 mL of solution S complies with test A. Prepare the reference solution using 10 mL of *lead standard solution (1 ppm Pb) R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C for 2 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 mL of *ethanol (96 per cent) R*. Add 25 mL of *water R*. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M sodium hydroxide* is equivalent to 25.68 mg of $C_{12}H_{17}ClN_2S$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D.*

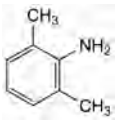

A. 2,6-dimethylaniline (2,6-xylidine),

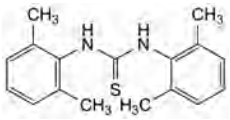

B. *N*,*N*′-bis(2,6-dimethylphenyl)thiourea,

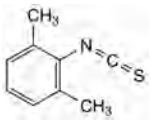

C. 1-isothiocyanato-2,6-dimethylbenzene,

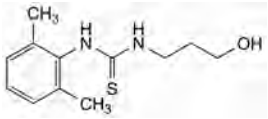

D. *N*-(2,6-dimethylphenyl)-*N*′-(3-hydroxypropyl)thiourea.

**07/2016:1381**
**corrected 10.0**

# XYLITOL

## Xylitolum

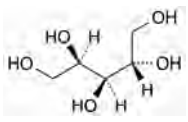

$C_5H_{12}O_5$ $M_r$ 152.1
[87-99-0]

DEFINITION

*Meso*-xylitol.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder or crystals.

*Solubility*: very soluble in water, sparingly soluble in ethanol (96 per cent).

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

A. Melting point (*2.2.14*): 92 °C to 96 °C.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: mulls in *liquid paraffin R*.

*Comparison*: *xylitol CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 25 mg of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (a).* Dissolve 25 mg of *xylitol CRS* in *water R* and dilute to 10 mL with the same solvent.

*Reference solution (b).* Dissolve 25 mg of *mannitol R* and 25 mg of *xylitol R* in *water R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *water R*, *ethyl acetate R*, *propanol R* (10:20:70 *V/V/V*).

*Application*: 2 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air.

*Detection*: spray with *4-aminobenzoic acid solution R*, dry in a current of cold air until the acetone is removed, then heat at 100 °C for 15 min; allow to cool, spray with a 2 g/L solution of *sodium periodate R*, dry in a current of cold air, then heat at 100 °C for 15 min.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension IV (*2.2.1*) and not more intensely coloured than reference solution $BY_7$ (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 50.0 mL with the same solvent.

**Conductivity** (*2.2.38*): maximum 20 µS·cm$^{-1}$.

Dissolve 20.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100.0 mL with the same solvent. Measure the conductivity of the solution while gently stirring with a magnetic stirrer.

**Reducing sugars**: maximum 0.2 per cent, calculated as glucose equivalent.

Dissolve 5.0 g in 6 mL of *water R* with the aid of gentle heat. Cool and add 20 mL of *cupri-citric solution R* and a few glass beads. Heat so that boiling begins after 4 min and maintain boiling for 3 min. Cool rapidly and add 100 mL of a 2.4 per cent *V/V* solution of *glacial acetic acid R* and 20.0 mL of *0.025 M iodine*. With continuous shaking, add 25 mL of a mixture of 6 volumes of *hydrochloric acid R* and 94 volumes of *water R* and, when the precipitate has dissolved, titrate the excess of iodine with *0.05 M sodium thiosulfate* using 1 mL of *starch solution R*, added towards the end of the titration, as indicator. Not less than 12.8 mL of *0.05 M sodium thiosulfate* is required.

**Related substances**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dissolve 5 mg of *erythritol R* in *water R* and dilute to 25.0 mL with the same solvent.

*Test solution (a).* Dissolve 5.000 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* Dilute 1.0 mL of test solution (a) to 10.0 mL with *water R*.

*Reference solution (a).* Dissolve 5.0 mg each of *L-arabinitol CRS* (impurity A), *galactitol CRS* (impurity B), *mannitol CRS* (impurity C) and *sorbitol CRS* (impurity D) in *water R* and dilute to 20.0 mL with the same solvent.

*Reference solution (b).* Dissolve 50.0 mg of *xylitol CRS* in *water R* and dilute to 10.0 mL with the same solvent.

Pipette 1.0 mL of test solutions (a) and (b) and reference solutions (a) and (b) into 4 separate 100 mL round-bottomed flasks. Add 1.0 mL of the internal standard solution to each of the flasks containing test solution (a) or reference solution (a), and 5.0 mL of the internal standard solution to each of the flasks containing test solution (b) or reference solution (b). Evaporate each mixture to dryness in a water-bath at 60 °C by suitable means. Dissolve each dry residue in 1 mL of *anhydrous pyridine R*, add 1 mL of *acetic anhydride R* to each flask and boil each solution under reflux for 1 h to complete acetylation.

*Column*:

– *size*: *l* = 30 m, Ø = 0.25 mm;

– *stationary phase*: *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (0.25 µm).

*Carrier gas*: *nitrogen R*.

*Flow rate*: 1 mL/min.

*Split ratio*: 1:50 to 1:100.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 1 | 170 |
| | 1 - 6 | 170 → 230 |
| | 6 - 30 | 230 |
| Injection port | | 250 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL of test solution (a) and reference solution (a) (solutions obtained after derivatisation).

*Relative retention* with reference to xylitol (retention time = about 15 min): internal standard = about 0.6; impurity A = about 0.9; impurity C = about 1.4; impurity B = about 1.45; impurity D = about 1.5.

*System suitability*: reference solution (a):

– *resolution*: minimum 2.0 between the peaks due to impurities B and D.

Calculate the percentage content of each related substance in the substance to be examined using the following expression:

$$100 \times \frac{m_s}{m_u} \times \frac{R_u}{R_s}$$

$m_s$ = mass of the particular component in 1 mL of reference solution (a), in milligrams;

$m_u$ = mass of the substance to be examined in 1 mL of test solution (a), in milligrams;

$R_s$ = ratio of the area of the peak due to the particular derivatised component to the area of the peak due to the derivatised internal standard in the chromatogram obtained with reference solution (a);

$R_u$ = ratio of the area of the peak due to the particular derivatised component to the area of the peak due to the derivatised internal standard in the chromatogram obtained with test solution (a).

The sum of the percentage contents of the related substances in the chromatogram obtained with test solution (a) is not greater than 2.0 per cent. Disregard any peak with an area corresponding to a percentage content of 0.05 per cent or less.

**Lead** (*2.4.10*): maximum 0.5 ppm.

Dissolve the substance to be examined in 150.0 mL of the prescribed mixture of solvents.

**Nickel** (*2.4.15*): maximum 1 ppm.

Dissolve the substance to be examined in 150.0 mL of the prescribed mixture of solvents.

**Water** (*2.5.12*): maximum 1.0 per cent, determined on 1.00 g.

**Bacterial endotoxins** (*2.6.14*): less than 4 IU/g if the concentration is less than 100 g/L of xylitol and less than 2.5 IU/g if the concentration is 100 g/L or more of xylitol, when intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Gas chromatography (*2.2.28*) as described in the test for related substances with the following modifications.

*Injection*: 1 µL of test solution (b) and reference solution (b) (solutions obtained after derivatisation).

Calculate the percentage content of $C_5H_{12}O_5$ using the following expression:

$$T \times \frac{m_t}{m_v} \times \frac{R_v}{R_t}$$

T = declared percentage content of *xylitol CRS*;

$m_t$ = mass of *xylitol CRS* in 1 mL of reference solution (b), in milligrams;

$m_v$ = mass of the substance to be examined in 1 mL of test solution (b), in milligrams;

$R_t$ = ratio of the area of the peak due to derivatised xylitol to the area of the peak due to the derivatised internal standard in the chromatogram obtained with reference solution (b);

$R_v$ = ratio of the area of the peak due to derivatised xylitol to the area of the peak due to the derivatised internal standard in the chromatogram obtained with test solution (b).

LABELLING

The label states:

– where applicable, the maximum concentration of bacterial endotoxins;

– where applicable, that the substance is suitable for use in the manufacture of parenteral preparations.

IMPURITIES

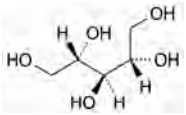

A. L-arabinitol,

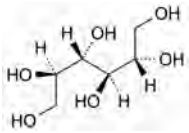

B. *meso*-galactitol,

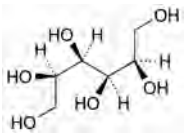

C. D-mannitol,

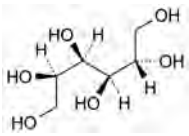

D. D-glucitol (D-sorbitol).

**01/2008:1162**
**corrected 7.0**

# XYLOMETAZOLINE HYDROCHLORIDE

## Xylometazolini hydrochloridum

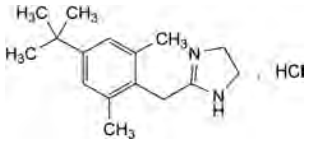

$C_{16}H_{25}ClN_2$ $M_r$ 280.8
[1218-35-5]

DEFINITION

2-[4-(1,1-Dimethylethyl)-2,6-dimethylbenzyl]-4,5-dihydro-1*H*-imidazole hydrochloride.

*Content*: 99.0 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: freely soluble in water, in ethanol (96 per cent) and in methanol.

IDENTIFICATION

*First identification: A, E.*

*Second identification: B, C, D, E.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *xylometazoline hydrochloride CRS.*

B. Thin-layer chromatography (*2.2.27*).
*Test solution.* Dissolve 20 mg of the substance to be examined in *methanol R* and dilute to 5 mL with the same solvent.
*Reference solution.* Dissolve 20 mg of *xylometazoline hydrochloride CRS* in *methanol R* and dilute to 5 mL with the same solvent.
*Plate*: *TLC silica gel G plate R.*
*Mobile phase*: *concentrated ammonia R, methanol R* (5:100 *V/V*).
*Application*: 5 µL.
*Development*: over 2/3 of the plate.
*Drying*: in air.
*Chlorine treatment*: at the bottom of a chromatographic tank place a beaker containing a mixture of 1 volume of *hydrochloric acid R1*, 1 volume of *water R* and 2 volumes of a 15 g/L solution of *potassium permanganate R*. Close the tank and allow to stand for 15 min. Place the dried plate in the tank and reclose the tank. Leave the plate in contact with the chlorine vapour for 5 min. Withdraw the plate and place it in a current of cold air until the excess of chlorine is removed and an area of the coating below the points of application does not give a blue colour with a drop of *potassium iodide and starch solution R.*
*Detection*: spray with *potassium iodide and starch solution R.*
*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

C. Dissolve about 0.5 mg in 1 mL of *methanol R*. Add 0.5 mL of a freshly prepared 50 g/L solution of *sodium nitroprusside R* and 0.5 mL of a 20 g/L solution of *sodium hydroxide R*. Allow to stand for 10 min and add 1 mL of an 80 g/L solution of *sodium hydrogen carbonate R*. A violet colour develops.

D. Dissolve 0.2 g in 1 mL of *water R*, add 2.5 mL of *ethanol (96 per cent) R* and 2 mL of *1 M sodium hydroxide*. Mix thoroughly and examine in ultraviolet light at 365 nm. The solution shows no fluorescence or at most the same fluorescence as a blank solution prepared in the same manner. The identification is not valid unless a solution prepared in the same manner using *naphazoline hydrochloride CRS* instead of the substance to be examined shows a distinct bluish fluorescence.

E. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

Dissolve 2.5 g in *water R* and dilute to 50.0 mL with the same solvent.

**Acidity or alkalinity**. Dissolve 0.25 g in *carbon dioxide-free water R* and dilute to 25 mL with the same solvent. Add 0.1 mL of *methyl red solution R* and 0.1 mL of *0.01 M hydrochloric acid*. The solution is red. Not more than 0.2 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to yellow.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 50.0 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Allow to stand for 1 h before injection.

*Reference solution (a).* Dilute 5.0 mL of the test solution to 100.0 mL with *water R*. Dilute 2.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (b).* Dissolve 5.0 mg of *xylometazoline impurity A CRS* and 5 mg of the substance to be examined in *water R* and dilute to 50.0 mL with the same solvent. Dilute 10.0 mL of this solution to 50.0 mL with *water R*.

*Reference solution (c).* Dilute 5.0 mL of reference solution (b) to 50.0 mL with *water R*.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography with polar incorporated groups R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 1.36 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*;

– *mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 5 | 70 | 30 |
| 5 - 20 | 70 → 15 | 30 → 85 |
| 20 - 35 | 15 | 85 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 220 nm.

*Injection*: 10 µL.

*Relative retention* with reference to xylometazoline (retention time = about 7.2 min): impurity A = about 0.79.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.5 between the peaks due to impurity A and xylometazoline.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);

– *total*: not more than 5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.200 g in 25 mL of *anhydrous acetic acid R* and add 10 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 28.08 mg of $C_{16}H_{25}ClN_2$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E, F.*

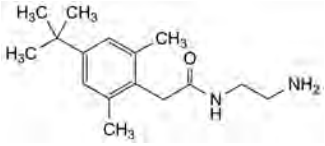

A. *N*-(2-aminoethyl)-2-[4-(1,1-dimethylethyl)-2,6-dimethylphenyl]acetamide,

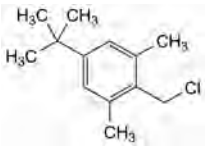

B. 2-(chloromethyl)-5-(1,1-dimethylethyl)-1,3-dimethylbenzene,

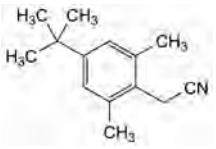

C. [4-(1,1-dimethylethyl)-2,6-dimethylphenyl]acetonitrile,

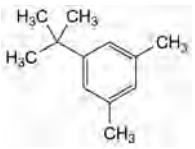

D. 1-(1,1-dimethylethyl)-3,5-dimethylbenzene,

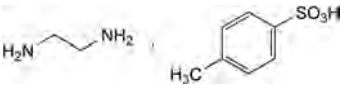

E. ethane-1,2-diamine mono(4-methylbenzenesulfonate),

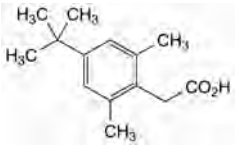

F. [4-(1,1-dimethylethyl)-2,6-dimethylphenyl]acetic acid.

**01/2017:1278**

# XYLOSE

## Xylosum

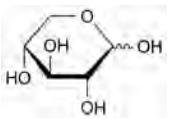

$C_5H_{10}O_5$ $M_r$ 150.1

[58-86-6]

DEFINITION

D-Xylopyranose.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless needles.

*Solubility*: freely soluble in water, soluble in hot ethanol (96 per cent).

IDENTIFICATION

*First identification: A.*

*Second identification: B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).
*Preparation*: discs.
*Comparison*: *xylose CRS*.

B. Thin-layer chromatography (*2.2.27*).
*Solvent mixture*: *water R*, *methanol R* (2:3 *V/V*).

*Test solution.* Dissolve 10 mg of the substance to be examined in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (a).* Dissolve 10 mg of *xylose CRS* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Reference solution (b).* Dissolve 10 mg of *fructose R*, 10 mg of *glucose R* and 10 mg of *xylose R* in the solvent mixture and dilute to 20 mL with the solvent mixture.

*Plate*: *TLC silica gel plate R.*

*Mobile phase*: *water R*, *methanol R*, *anhydrous acetic acid R*, *ethylene chloride R* (10:15:25:50 *V/V/V/V*); measure the volumes accurately since a slight excess of water produces cloudiness.

*Application*: 2 µL; thoroughly dry the points of application.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: spray with a 5 g/L solution of *thymol R* in a mixture of 5 volumes of *sulfuric acid R* and 95 volumes of *ethanol (96 per cent) R*. Heat in an oven at 130 °C for 10 min.

*System suitability*: reference solution (b):

– the chromatogram shows 3 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve 0.1 g in 10 mL of *water R*. Add 3 mL of *cupri-tartaric solution R* and heat. An orange or red precipitate is formed.

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**Acidity or alkalinity**. To 50 mL of solution S add 0.3 mL of *phenolphthalein solution R1*. The solution is colourless. Not more than 0.2 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator to pink.

**Specific optical rotation** (*2.2.7*): + 18.5 to + 19.5 (dried substance).

Dissolve 10.0 g in 80 mL of *water R*, add 1 mL of *dilute ammonia R2* and dilute to 100.0 mL with *water R*. Allow to stand for 30 min.

**Chlorides** (*2.4.4*): maximum 330 ppm.

Dilute 1.5 mL of solution S to 15 mL with *water R*.

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C at a pressure not exceeding 0.7 kPa.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

# Y

Yohimbine hydrochloride.. ..... 4231

**01/2008:2172**

# YOHIMBINE HYDROCHLORIDE

## Yohimbini hydrochloridum

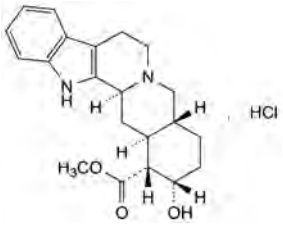

$C_{21}H_{27}ClN_2O_3$ $M_r$ 390.9
[65-19-0]

DEFINITION

Methyl 17α-hydroxyyohimban-16α-carboxylate hydrochloride.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly yellowish, crystalline powder.

*Solubility*: sparingly soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *yohimbine hydrochloride CRS*.

B. It gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.500 g in *carbon dioxide-free water R* with heating, allow to cool to room temperature and dilute to 50.0 mL with the same solvent.

**pH** (*2.2.3*): 3.5 to 5.5 for solution S.

**Specific optical rotation** (*2.2.7*): + 101.0 to + 105.0 (dried substance), determined on solution S.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions protected from light.*

*Test solution.* Dissolve 10.0 mg of the substance to be examined in *methanol R* and dilute to 50.0 mL with the same solvent.

*Reference solution (a).* Dissolve 5.0 mg of *yohimbine hydrochloride CRS* (containing impurities A, F and G) in *methanol R* and dilute to 25.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with *methanol R*.

*Reference solution (c).* Dilute 1.0 mL of reference solution (b) to 10.0 mL with *methanol R*.

*Column*:
- *size*: *l* = 0.125 m, Ø = 4.0 mm;
- *stationary phase*: *octylsilyl silica gel for chromatography R* (4 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 50 mL of a 9.08 g/L solution of *potassium dihydrogen phosphate R*, 100 mL of an 11.88 g/L solution of *disodium hydrogen phosphate dihydrate R*, 285 mL of *acetonitrile R*, 4.0 g of *sodium laurilsulfate R* and 355 mL of *water R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 10 µL.

*Run time*: 3 times the retention time of yohimbine.

*Relative retention* with reference to yohimbine (retention time = about 7 min): impurity F = about 0.65; impurity G = about 0.70; impurity A = about 0.75.

*System suitability*: reference solution (a):
- *peak-to-valley ratio*: minimum 1.3, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity A; and minimum 1.3, where $H_p$ = height above the baseline of the peak due to impurity G and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity F.

*Limits*:
- *sum of impurities A and G*: not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (1.0 per cent);
- *impurity F*: not more than 4 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.4 per cent);
- *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);
- *total*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (2.0 per cent);
- *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution and reference solution (a).

Calculate the percentage content of $C_{21}H_{27}ClN_2O_3$ from the declared content of *yohimbine hydrochloride CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, F, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, C, D, E.*

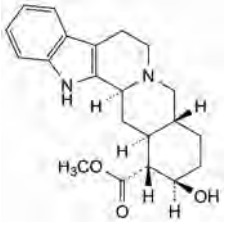

A. methyl 17β-hydroxyyohimban-16α-carboxylate (β-yohimbine),

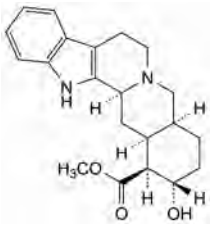

B. methyl 17α-hydroxy-20α-yohimban-16β-carboxylate (α-yohimbine),

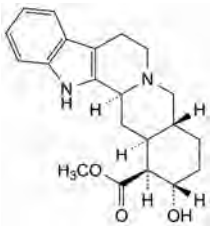

C. methyl 17α-hydroxyyohimban-16β-carboxylate (corynantheine),

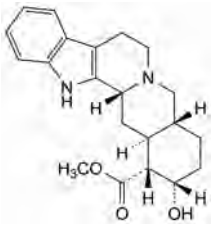

D. methyl 17α-hydroxy-3β-yohimban-16α-carboxylate (pseudo-yohimbine),

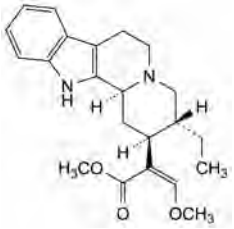

E. methyl (2*Z*)-2-[(2*S*,3*R*,12b*S*)-3-ethyl-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl]-3-methoxyprop-2-enoate,

F. unknown structure,

G. unknown structure.

# Z

Zanamivir hydrate.. 4235
Zidovudine.. 4236
Zinc acetate dihydrate.. 4238
Zinc acexamate.. 4239
Zinc chloride.. 4241
Zinc gluconate.. 4241
Zinc oxide.. 4242
Zinc stearate.. 4242
Zinc sulfate heptahydrate.. 4243
Zinc sulfate hexahydrate.. 4244
Zinc sulfate monohydrate.. 4244
Zinc undecylenate.. 4244
Ziprasidone hydrochloride monohydrate.. 4245
Ziprasidone mesilate trihydrate.. 4247
Zoledronic acid monohydrate.. 4249
Zolmitriptan.. 4251
Zolpidem tartrate.. 4252
Zopiclone.. 4254
Zuclopenthixol decanoate.. 4255

**04/2015:2611**

# ZANAMIVIR HYDRATE

## Zanamivirum hydricum

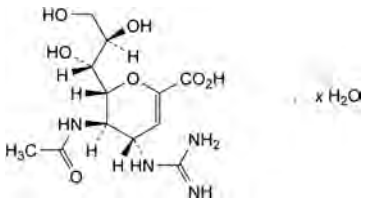

$C_{12}H_{20}N_4O_7,xH_2O$ $M_r$ 332.3 (anhydrous substance)
[551942-41-7]

DEFINITION

(2*R*,3*R*,4*S*)-3-(Acetylamino)-4-carbamimidoylamino-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

It contains a variable quantity of water.

CHARACTERS

*Appearance*: white or almost white, slightly hygroscopic powder.

*Solubility*: slightly soluble in water, practically insoluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *zanamivir hydrate CRS*.

TESTS

**Specific optical rotation** (*2.2.7*): + 36.0 to + 38.5 (dried substance).

Dissolve 0.250 g in 25.0 mL of *water R*; sonicate until dissolution is complete.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a)*. Dissolve 23.0 mg of the substance to be examined in 20 mL of *water R* and dilute to 50.0 mL with *acetonitrile R1*.

*Test solution (b)*. Dilute 5.0 mL of test solution (a) to 50.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 23.0 mg of *zanamivir for assay CRS* in 20 mL of *water R* and dilute to 50.0 mL with *acetonitrile R1*. Dilute 5.0 mL of the solution to 50.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *zanamivir for system suitability CRS* (containing impurities A, B, C and E) in 6 mL of *water R* and dilute to 10.0 mL with *acetonitrile R1*.

*Reference solution (c)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (d)*. Dissolve 3.0 mg of *zanamivir impurity F CRS* in the mobile phase and dilute to 100.0 mL with the mobile phase.

*Reference solution (e)*. Dilute 1.0 mL of reference solution (d) to 100.0 mL with the mobile phase. Dilute 3.0 mL of this solution to 20.0 mL with the mobile phase.

*Column*:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amino alkyl vinyl polymer for chromatography R* (5 µm);
- *temperature*: 30 °C.

*Mobile phase*: 0.7 g/L solution of *sulfuric acid R* previously adjusted to pH 5.5 with *dilute ammonia R3*, *acetonitrile R1* (40:60 *V/V*).

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 234 nm.

*Preconditioning of the column*: prior to first use, rinse with a 0.7 g/L solution of *ammonium sulfate R* at 1.5 mL/min at 30 °C for about 1 h; prior to each use, rinse with the mobile phase for at least 8 h.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (e).

*Run time*: 3 times the retention time of zanamivir.

*Identification of impurities*: use the chromatogram supplied with *zanamivir for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C and E; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity F.

*Relative retention* with reference to zanamivir (retention time = about 9 min): impurity F = about 0.3; impurity B = about 0.6; impurity C = about 0.75; impurity E = about 0.8; impurity A = about 2.6.

*System suitability*:
- *signal-to-noise ratio*: minimum 10 for the principal peak in the chromatogram obtained with reference solution (e);
- *peak-to-valley ratio*: minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity E and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity C in the chromatogram obtained with reference solution (b).

*Calculation of percentage contents*:
- for impurity F, use the concentration of impurity F in reference solution (e);
- for impurities other than F, use the concentration of zanamivir in reference solution (c).

*Limits*:
- *impurity A*: maximum 0.5 per cent;
- *impurity B*: maximum 0.3 per cent;
- *impurity C*: maximum 0.2 per cent;
- *impurity F*: maximum 0.01 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 1.2 per cent;
- *reporting threshold*: 0.05 per cent, except for impurity F.

**Loss on drying** (*2.2.32*): 4.0 per cent to 9.0 per cent, determined on 1.000 g by drying *in vacuo* at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modification.

*Injection*: test solution (b) and reference solution (a).

Calculate the percentage content of $C_{12}H_{20}N_4O_7$ taking into account the assigned content of *zanamivir for assay CRS*.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Specified impurities*: *A, B, C, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general

acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *D, E, H.*

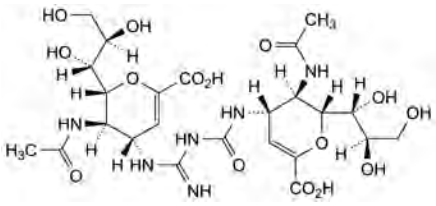

A. (2*R*,3*R*,4*S*)-3-(acetylamino)-4-[[[*N*-[(2*R*,3*R*,4*S*)-3-(acetylamino)-6-carboxy-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-4-yl]carbamimidoyl]-carbamoyl]amino]-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid,

B. unknown structure,

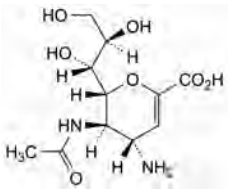

C. (2*R*,3*R*,4*S*)-3-(acetylamino)-4-amino-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid,

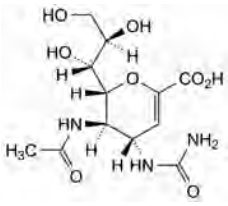

D. (2*R*,3*R*,4*S*)-3-(acetylamino)-4-(carbamoylamino)-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid,

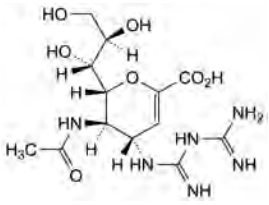

E. (2*R*,3*R*,4*S*)-3-(acetylamino)-4-[(carbamimidoyl-carbamimidoyl)amino]-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid,

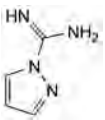

F. 1*H*-pyrazole-1-carboximidamide,

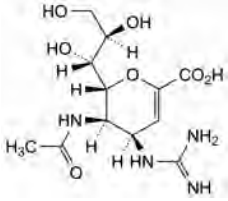

H. (2*R*,3*R*,4*R*)-3-(acetylamino)-4-carbamimidoylamino-2-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]-3,4-dihydro-2*H*-pyran-6-carboxylic acid.

**01/2017:1059**

# ZIDOVUDINE

## Zidovudinum

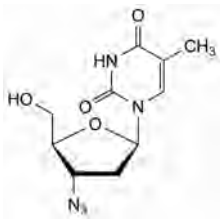

$C_{10}H_{13}N_5O_4$ $M_r$ 267.2
[30516-87-1]

DEFINITION

1-(3-Azido-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione.

*Content*: 97.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or slightly brownish powder.

*Solubility*: sparingly soluble in water, soluble in anhydrous ethanol, practically insoluble in heptane.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Specific optical rotation (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *zidovudine CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in the minimum volume of *water R*, evaporate to dryness in a desiccator, under high vacuum over *diphosphorus pentoxide R* and record new spectra using the residues.

TESTS

**Appearance of solution**. The solution is not more opalescent than reference suspension I (*2.2.1*) and not more intensely coloured than reference solution $BY_5$ (*2.2.2, Method II*).

Dissolve 0.5 g in 50 mL of *water R*, heating if necessary.

**Specific optical rotation** (*2.2.7*): + 60.5 to + 63.0 (dried substance), measured at 25 °C.

Dissolve 0.50 g in *anhydrous ethanol R* and dilute to 50.0 mL with the same solvent.

**Related substances**

A. Liquid chromatography (*2.2.29*).

*Solvent mixture*. Mix 4 volumes of *acetonitrile R*, 20 volumes of *methanol R* and 76 volumes of a 2 g/L solution of *ammonium acetate R* previously adjusted to pH 6.8 with *dilute acetic acid R*.

*Test solution (a)*. Dissolve 20.0 mg of the substance to be examined in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 10.0 mL of test solution (a) to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve 2 mg of *thymine R* (impurity C) and 2 mg of *zidovudine impurity B CRS* in the solvent mixture and dilute to 50.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 5 mg of *zidovudine for system suitability CRS* (containing impurities A, G and H) in reference solution (a) and dilute to 5.0 mL with reference solution (a).

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (d).* Dissolve 20.0 mg of *zidovudine CRS* in the solvent mixture and dilute to 20.0 mL with the solvent mixture. Dilute 10.0 mL of the solution to 50.0 mL with the solvent mixture.

*Reference solution (e).* Dissolve 1 mg of *zidovudine impurity D CRS* in a mixture of 4 volumes of *acetonitrile R*, 40 volumes of *methanol R* and 56 volumes of a 2 g/L solution of *ammonium acetate R* previously adjusted to pH 6.8 with *dilute acetic acid R* and dilute to 50.0 mL with the same mixture of solvents. Dilute 5.0 mL of the solution to 10.0 mL with the same mixture of solvents.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*:

– *mobile phase A*: 2 g/L solution of *ammonium acetate R* previously adjusted to pH 6.8 with *dilute acetic acid R*;
– *mobile phase B*: *acetonitrile R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 3 | 95 | 5 |
| 3 - 18 | 95 → 85 | 5 → 15 |
| 18 - 28 | 85 → 30 | 15 → 70 |
| 28 - 43 | 30 | 70 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 265 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b), (c) and (e).

*Identification of impurities*: use the chromatogram supplied with *zidovudine for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities A, B, C, G and H; use the chromatogram obtained with reference solution (e) to identify the peak due to impurity D.

*Relative retention* with reference to zidovudine (retention time = about 16 min): impurity C = about 0.2; impurity A = about 0.5; impurity H = about 0.95; impurity B = about 1.05; impurity G = about 1.5; impurity D = about 2.0.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity H and zidovudine; minimum 2.0 between the peaks due to zidovudine and impurity B.

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity C by 0.6;
– for each impurity, use the concentration of zidovudine in reference solution (c).

*Limits*:

– *impurity G*: maximum 0.5 per cent;
– *impurities A and C*: for each impurity, maximum 0.15 per cent;
– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *reporting threshold*: 0.05 per cent; disregard any peak due to impurity D and any peak eluted after this impurity.

B. Liquid chromatography (*2.2.29*).

*Test solution.* Dissolve 0.5 g of the substance to be examined in 10 mL of *acetonitrile R1* and dilute to 100.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5.0 mg of *zidovudine impurity D CRS* in *acetonitrile R1* and dilute to 10.0 mL with the same solvent.

*Reference solution (b).* Dilute 1.0 mL of reference solution (a) to 100.0 mL with the mobile phase.

*Reference solution (c).* Dilute 1.0 mL of reference solution (a) to 50.0 mL with the test solution.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: *base-deactivated end-capped octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: *water for chromatography R*, *acetonitrile R1* (30:70 *V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Run time*: 10 times the retention time of zidovudine.

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity D.

*Relative retention* with reference to zidovudine (retention time = about 1.5 min): impurity D = about 2.5.

*System suitability*: reference solution (c):

– *resolution*: minimum 5.0 between the peaks due to zidovudine and impurity D.

*Calculation of percentage contents*:

– for each impurity, use the concentration of impurity D in reference solution (b).

*Limits*:

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;
– *reporting threshold*: 0.05 per cent; disregard any peak eluted before impurity D.

*Limit*:

– *total for tests A and B*: maximum 1.0 per cent.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modification.

*Injection*: test solution (b) and reference solution (d).

Calculate the percentage content of $C_{10}H_{13}N_5O_4$ taking into account the assigned content of *zidovudine CRS*.

STORAGE

Protected from light.

IMPURITIES

**Test A for related substances**: A, B, C, E, F, G, H.

**Test B for related substances**: D, J, K.

*Specified impurities: A, C, G.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034).* It is therefore not necessary to identify these

impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, H, J, K.*

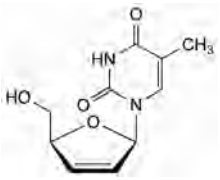

A. 1-[(2*R*,5*S*)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

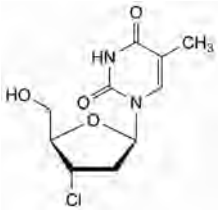

B. 1-(3-chloro-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

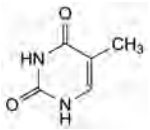

C. 5-methylpyrimidine-2,4(1*H*,3*H*)-dione (thymine),

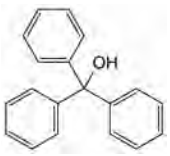

D. triphenylmethanol,

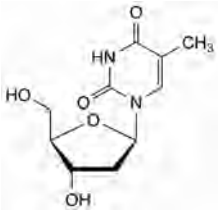

E. 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (thymidine),

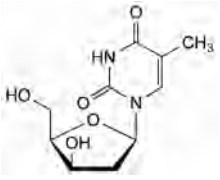

F. 1-(2-deoxy-β-D-*threo*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

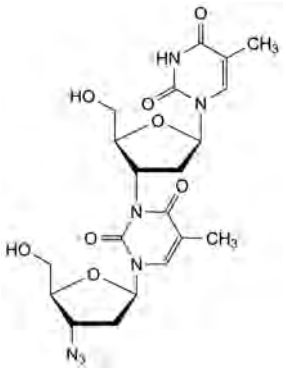

G. 1-[(2*R*,4*S*,5*S*)-4-azido-5-(hydroxymethyl)tetrahydrofuran-2-yl]-3-[(2*S*,3*S*,5*R*)-2-(hydroxymethyl)-5-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2*H*)-yl)tetrahydrofuran-3-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione,

H. unknown structure,

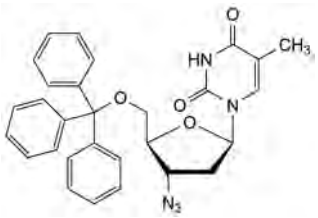

J. 1-[3-azido-2,3-dideoxy-5-*O*-(triphenylmethyl)-β-D-*erythro*-pentofuranosyl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione (trityl zidovudine),

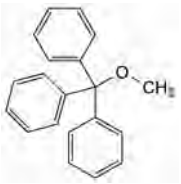

K. 1,1′,1″-(methoxymethanetriyl)tribenzene (methyl trityl ether).

**01/2008:1482**
**corrected 7.0**

# ZINC ACETATE DIHYDRATE

## Zinci acetas dihydricus

$C_4H_6O_4Zn,2H_2O$ $M_r$ 219.5
[5970-45-6]

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent of $C_4H_6O_4Zn,2H_2O$.

CHARACTERS

*Appearance*: white or almost white crystalline powder or flakes.

*Solubility*: freely soluble in water, soluble in ethanol (96 per cent).

IDENTIFICATION

A. It gives reaction (a) of acetates (*2.3.1*).

B. It gives the reaction of zinc (*2.3.1*).

TESTS

**Solution S**. Dissolve 10.0 g in *carbon dioxide-free water R* prepared from *distilled water R* and dilute to 100 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.8 to 7.0.

Dilute 10 mL of solution S to 20 mL with *carbon dioxide-free water R*.

**Reducing substances**. Boil for 5 min a mixture of 10 mL of solution S, 90 mL of *water R*, 5 mL of *dilute sulfuric acid R* and 1.5 mL of a 0.3 g/L solution of *potassium permanganate R*. The pink colour of the solution remains.

**Chlorides** (*2.4.4*): maximum 50 ppm.

Dilute 10 mL of solution S with 15 mL of *water R*.

**Sulfates** (*2.4.13*): maximum 100 ppm, determined on solution S.

**Aluminium**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.50 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluted with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: aluminium hollow-cathode lamp.

*Wavelength*: 309.3 nm.

*Atomisation device*: air-acetylene or acetylene-nitrous oxide flame.

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Cadmium**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use the solution described in the test for aluminium.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluted with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

**Copper**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Use the solution described in the test for iron.

*Reference solutions.* Prepare the reference solutions using *copper standard solution (10 ppm Cu) R*, diluted with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: copper hollow-cathode lamp.

*Wavelength*: 324.8 nm.

*Atomisation device*: air-acetylene flame.

**Iron**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 1.25 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluted with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 5.00 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R*, diluting with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Atomisation device*: air-acetylene flame.

ASSAY

Dissolve 0.200 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 21.95 mg of $C_4H_6O_4Zn,2H_2O$.

STORAGE

In a non-metallic container.

**07/2010:1279**
**corrected 7.0**

# ZINC ACEXAMATE

## Zinci acexamas

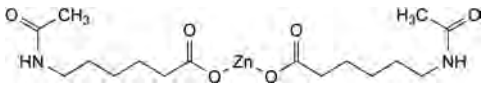

$C_{16}H_{28}N_2O_6Zn$ $M_r$ 409.8
[70020-71-2]

DEFINITION

Zinc 6-(acetylamino)hexanoate.

*Content*: 97.5 per cent to 101.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: soluble in water, practically insoluble in acetone and in ethanol (96 per cent). It dissolves in dilute nitric acid.

mp: about 198 °C.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *zinc acexamate CRS*.

B. 5 mL of solution S (see Tests) gives the reaction of zinc (*2.3.1*).

TESTS

**Solution S**. Dissolve 0.5 g in *carbon dioxide-free water R* and dilute to 20 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension IV (*2.2.1*) and is colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 5.0 to 7.0 for solution S.

**Impurity B**. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 0.30 g of the substance to be examined in *water R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 15 mg of *6-aminohexanoic acid R* (impurity B) in *water R* and dilute to 10 mL with the same solvent. Dilute 1 mL of this solution to 10 mL with *water R*.

*Plate*: *TLC silica gel plate R*.

*Mobile phase*: *ammonia R*, *water R*, *ethanol (96 per cent) R* (2:30:68 *V/V/V*).

*Application*: 5 µL; allow to dry in air.

*Development*: over a path of 15 cm.

*Drying*: in a current of warm air.

*Detection*: spray with *ninhydrin solution R* and heat at 100-105 °C for 15 min.

*Limit*:

– *impurity B*: any spot due to impurity B is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (0.5 per cent).

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution (a).* Dissolve 0.50 g of the substance to be examined in *water R* and dilute to 100.0 mL with the same solvent.

*Test solution (b).* To 20.0 mL of test solution (a), add 20 mL of the mobile phase and 0.4 mL of a 100 g/L solution of *phosphoric acid R*, then dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 40 mg of *N-acetyl-ε-caprolactam R* (impurity C) in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b).* Dilute 5.0 mL of reference solution (a) to 100.0 mL with *water R*.

*Reference solution (c).* Dissolve 20 mg of *zinc acexamate impurity A CRS* in *water R* and dilute to 50.0 mL with the same solvent.

*Reference solution (d).* Dissolve 40 mg of *ε-caprolactam R* (impurity D) in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R*.

*Reference solution (e).* To 20.0 mL of test solution (a), add 5.0 mL of reference solution (b), 5.0 mL of reference solution (c), 5.0 mL of reference solution (d) and 0.4 mL of a 100 g/L solution of *phosphoric acid R*, then dilute to 50.0 mL with the mobile phase.

*Reference solution (f).* To 5.0 mL of reference solution (c), add 5.0 mL of reference solution (b), 5.0 mL of reference solution (d) and 0.4 mL of a 100 g/L solution of *phosphoric acid R*, then dilute to 50.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

*Mobile phase*: mix 0.2 volumes of *phosphoric acid R*, 8 volumes of *acetonitrile R* and 92 volumes of *water R*, then adjust to pH 4.5 with *dilute ammonia R1*.

*Flow rate*: 1.2 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b), (e) and (f).

*Run time*: 8 times the retention time of zinc acexamate.

*Elution order*: zinc acexamate, impurity D, impurity A, impurity C.

*System suitability*: reference solution (e):

– *resolution*: minimum 3.0 between the peaks due to zinc acexamate and impurity D; if necessary, adjust the mobile phase to pH 4.7 with *dilute ammonia R1*.

*Limits*:

– *impurity A*: not more than the area of the corresponding peak in the chromatogram obtained with reference solution (f) (2 per cent);

– *impurities C, D*: for each impurity, not more than 1.5 times the area of the corresponding peak in the chromatogram obtained with reference solution (f) (0.15 per cent);

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (f) (0.05 per cent);

– *sum of impurities other than A*: not more than 5 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (f) (0.5 per cent);

– *disregard limit*: 0.5 times the area of the peak due to impurity C in the chromatogram obtained with reference solution (f) (0.05 per cent).

**Arsenic** (*2.4.2, Method A*): maximum 2 ppm, determined on 0.5 g.

**Cadmium**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 2.50 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

**Iron**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 1.25 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *iron standard solution (20 ppm Fe) R*, diluting with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: iron hollow-cathode lamp.

*Wavelength*: 248.3 nm.

*Atomisation device*: air-acetylene flame.

**Lead**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

*Test solution.* Dissolve 5.00 g in 20 mL of a 200 g/L solution of *cadmium- and lead-free nitric acid R* and dilute to 25.0 mL with the same acid solution.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R*, diluting with a 200 g/L solution of *cadmium- and lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm.

*Atomisation device*: air-acetylene flame.

**Loss on drying** (*2.2.32*): maximum 1.0 per cent, determined on 1.000 g by drying in an oven at 105 °C.

ASSAY

Dissolve 0.400 g in 10 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 40.98 mg of $C_{16}H_{28}N_2O_6Zn$.

STORAGE

In a non-metallic container.

IMPURITIES

*Specified impurities: A, B, C, D.*

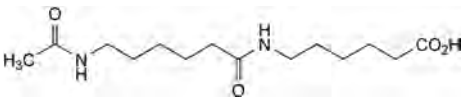

A. 6-[[6-(acetylamino)hexanoyl]amino]hexanoic acid,

B. 6-aminohexanoic acid (6-aminocaproic acid),

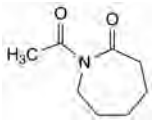

C. 1-acetylhexahydro-2*H*-azepin-2-one (*N*-acetyl-ε-caprolactam),

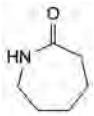

D. hexahydro-2*H*-azepin-2-one (ε–caprolactam).

**01/2008:0110 corrected 9.0**

# ZINC CHLORIDE

## Zinci chloridum

$ZnCl_2$ $M_r$ 136.3
[7646-85-7]

DEFINITION

*Content*: 95.0 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or cast in white or almost white sticks, deliquescent.

*Solubility*: very soluble in water, freely soluble in ethanol (96 per cent) and in glycerol.

IDENTIFICATION

A. Dissolve 0.5 g in *dilute nitric acid R* and dilute to 10 mL with the same acid. The solution gives reaction (a) of chlorides (*2.3.1*).

B. 5 mL of solution S (see Tests) gives the reaction of zinc (*2.3.1*).

TESTS

**Solution S**. To 2.0 g add 38 mL of *carbon dioxide-free water R* prepared from *distilled water R* and add *dilute hydrochloric acid R* dropwise until dissolution is complete. Dilute to 40 mL with *carbon dioxide-free water R* prepared from *distilled water R*.

**pH** (*2.2.3*): 4.6 to 5.5.

Dissolve 1.0 g in 9 mL of *carbon dioxide-free water R*, ignoring any slight turbidity.

**Oxychlorides**. Dissolve 10.0 g in 10 mL of *carbon dioxide-free water R*. The solution is not more opalescent than reference suspension II (*2.2.1*). To 1.5 mL of the solution add 7.5 mL of *ethanol (96 per cent) R*. The solution may become cloudy within 10 min. Any cloudiness disappears on the addition of 0.2 mL of *dilute hydrochloric acid R*.

**Sulfates** (*2.4.13*): maximum 200 ppm.

Dilute 5 mL of solution S to 15 mL with *distilled water R*. Prepare the standard using a mixture of 5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 10 mL of *distilled water R*.

**Aluminium, calcium, iron, magnesium**. To 8 mL of solution S add 2 mL of *concentrated ammonia R* and shake. The solution is clear (*2.2.1*) and colourless (*2.2.2, Method II*). Add 1 mL of *disodium hydrogen phosphate solution R*. The solution remains clear for at least 5 min. Add 0.2 mL of *sodium sulfide solution R*. A white precipitate is formed and the supernatant remains colourless.

**Ammonium** (*2.4.1*): maximum 400 ppm.

Dilute 0.5 mL of solution S to 15 mL with *water R*.

ASSAY

Dissolve 0.250 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 13.63 mg of $ZnCl_2$.

STORAGE

In a non-metallic container.

**01/2017:2164**

# ZINC GLUCONATE

## Zinci gluconas

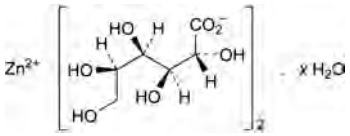

$C_{12}H_{22}ZnO_{14},xH_2O$ $M_r$ 455.7 (anhydrous substance)

DEFINITION

Anhydrous or hydrated zinc D-gluconate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: soluble in water, practically insoluble in anhydrous ethanol and in methylene chloride.

IDENTIFICATION

A. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 20 mg of the substance to be examined in 1 mL of *water R*.

*Reference solution*. Dissolve 20 mg of *calcium gluconate CRS* in 1 mL of *water R*, heating if necessary in a water-bath at 60 °C.

*Plate*: *TLC silica gel plate R* (5-40 µm) [or *TLC silica gel plate R* (2-10 µm)].

*Mobile phase*: *concentrated ammonia R*, *ethyl acetate R*, *water R*, *ethanol (96 per cent) R* (10:10:30:50 *V/V/V/V*).

*Application*: 1 µL.

*Development*: over 3/4 of the plate.

*Drying*: at 100-105 °C for 20 min, then allow to cool to room temperature.

*Detection*: spray with a solution containing 25 g/L of *ammonium molybdate R* and 10 g/L of *cerium sulfate R* in *dilute sulfuric acid R*, and heat at 100-105 °C for about 10 min.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position, colour and size to the principal spot in the chromatogram obtained with the reference solution.

B. Dissolve 0.1 g in 5 mL of *water R*. Add 0.5 mL of *potassium ferrocyanide solution R*. A white precipitate is formed that does not dissolve upon the addition of 5 mL of *hydrochloric acid R*.

TESTS

**Solution S**. Dissolve 1.0 g in *water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Sucrose and reducing sugars**. Dissolve 0.5 g in a mixture of 2 mL of *hydrochloric acid R1* and 10 mL of *water R*. Boil for 5 min, allow to cool, add 10 mL of *sodium carbonate solution R* and allow to stand for 10 min. Dilute to 25 mL with *water R* and filter. To 5 mL of the filtrate add 2 mL of *cupri-tartaric solution R* and boil for 1 min. Allow to stand for 2 min. No red precipitate is formed.

**Chlorides** (*2.4.4*): maximum 500 ppm.

Dilute 5 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 500 ppm.

Dissolve 2.0 g in a mixture of 10 mL of *acetic acid R* and 90 mL of *distilled water R*.

**Cadmium**: maximum 2 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 5.00 g in 20 mL of *deionised distilled water R* with the aid of ultrasound and dilute to 25.0 mL with the same solvent.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with *deionised distilled water R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene flame.

**Water** (*2.5.32*): maximum 12.0 per cent, determined on 80.0 mg.

**Microbial contamination**

TAMC: acceptance criterion $10^3$ CFU/g (*2.6.12*).

TYMC: acceptance criterion $10^2$ CFU/g (*2.6.12*).

ASSAY

Dissolve 0.400 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 45.57 mg of $C_{12}H_{22}ZnO_{14}$.

STORAGE

In a non-metallic, airtight container.

**01/2008:0252**
**corrected 7.0**

# ZINC OXIDE

## Zinci oxidum

ZnO $M_r$ 81.4
[1314-13-2]

DEFINITION

*Content*: 99.0 per cent to 100.5 per cent (ignited substance).

CHARACTERS

*Appearance*: soft, white or faintly yellowish-white, amorphous powder, free from gritty particles.

*Solubility*: practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute mineral acids.

IDENTIFICATION

A. It becomes yellow when strongly heated; the yellow colour disappears on cooling.

B. Dissolve 0.1 g in 1.5 mL of *dilute hydrochloric acid R* and dilute to 5 mL with *water R*. The solution gives the reaction of zinc (*2.3.1*).

TESTS

**Alkalinity**. Shake 1.0 g with 10 mL of boiling *water R*. Add 0.1 mL of *phenolphthalein solution R* and filter. If the filtrate is red, not more than 0.3 mL of *0.1 M hydrochloric acid* is required to change the colour of the indicator.

**Carbonates and substances insoluble in acids**. Dissolve 1.0 g in 15 mL of *dilute hydrochloric acid R*. It dissolves without effervescence and the solution is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

**Arsenic** (*2.4.2, Method A*): maximum 5 ppm, determined on 0.2 g.

**Cadmium**: maximum 10 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 2.0 g in 14 mL of a mixture of equal volumes of *water R* and *cadmium- and lead-free nitric acid R*, boil for 1 min, cool and dilute to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a 3.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene or air-propane flame.

**Iron** (*2.4.9*): maximum 200 ppm.

Dissolve 50 mg in 1 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*. Use in this test 0.5 mL of *thioglycollic acid R*.

**Lead**: maximum 50 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution.* Dissolve 5.0 g in 24 mL of a mixture of equal volumes of *water R* and *cadmium- and lead-free nitric acid R*, boil for 1 min, cool and dilute to 100.0 mL with *water R*.

*Reference solutions.* Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R* and diluting with a 3.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm; 217.0 nm may be used depending on the apparatus.

*Atomisation device*: air-acetylene flame.

**Loss on ignition**: maximum 1.0 per cent, determined on 1.00 g by ignition to constant mass at 500 ± 50 °C.

ASSAY

Dissolve 0.150 g in 10 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 8.14 mg of ZnO.

**01/2008:0306**
**corrected 7.0**

# ZINC STEARATE

## Zinci stearas

[557-05-1]

DEFINITION

Zinc stearate [$(C_{17}H_{35}COO)_2Zn$; $M_r$ 632] may contain varying proportions of zinc palmitate [$(C_{15}H_{31}COO)_2Zn$; $M_r$ 576.2] and zinc oleate [$(C_{17}H_{33}COO)_2Zn$; $M_r$ 628].

*Content*: 10.0 per cent to 12.0 per cent of Zn.

CHARACTERS

*Appearance*: light, white or almost white, amorphous powder, free from gritty particles.

*Solubility*: practically insoluble in water and in anhydrous ethanol.

IDENTIFICATION

A. Freezing point (*2.2.18*): minimum 53 °C, determined on the residue obtained in the preparation of solution S (see Tests).

B. Neutralise 5 mL of solution S to *red litmus paper R* with *strong sodium hydroxide solution R*. The solution gives the reaction of zinc (*2.3.1*).

TESTS

**Solution S**. To 5.0 g add 50 mL of *ether R* and 40 mL of a 7.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R* in *distilled water R*. Heat under a reflux condenser until dissolution is complete. Allow to cool. In a separating funnel, separate the aqueous layer and shake the ether layer with 2 quantities, each of 4 mL, of *distilled water R*. Combine the aqueous layers, wash with 15 mL of *ether R* and heat on a water-bath until ether is completely eliminated. Allow to cool and dilute to 50.0 mL with *distilled water R* (solution S). Evaporate the ether layer to dryness and dry the residue at 105 °C.

**Appearance of solution**. Solution S is not more intensely coloured than reference solution $Y_6$ (*2.2.2, Method II*).

**Appearance of solution of fatty acids**. Dissolve 0.5 g of the residue obtained in the preparation of solution S in 10 mL of *chloroform R*. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_5$ (*2.2.2, Method II*).

**Acidity or alkalinity**. Shake 1.0 g with 5 mL of *ethanol (96 per cent) R* and add 20 mL of *carbon dioxide-free water R* and 0.1 mL of *phenol red solution R*. Not more than 0.3 mL of *0.1 M hydrochloric acid* or 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour of the indicator.

**Acid value of the fatty acids** (*2.5.1*): 195 to 210.

Dissolve 0.20 g of the residue obtained in the preparation of solution S in 25 mL of the prescribed mixture of solvents.

**Chlorides** (*2.4.4*): maximum 250 ppm.

Dilute 2 mL of solution S to 15 mL with *water R*.

**Sulfates** (*2.4.13*): maximum 0.6 per cent.

Dilute 1 mL of solution S to 50 mL with *distilled water R*. Dilute 12.5 mL of this solution to 15 mL with *distilled water R*.

**Cadmium**: maximum 5 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Dilute 20.0 mL of solution S to 50.0 mL with a 3.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R*.

*Reference solutions*. Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R* and diluting with a 3.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R*.

*Source*: cadmium hollow-cathode lamp.

*Wavelength*: 228.8 nm.

*Atomisation device*: air-acetylene or an air-propane flame.

**Lead**: maximum 25 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

*Test solution*. Solution S.

*Reference solutions*. Prepare the reference solutions using *lead standard solution (0.1 per cent Pb) R* and diluting with a 3.5 per cent *V/V* solution of *cadmium- and lead-free nitric acid R*.

*Source*: lead hollow-cathode lamp.

*Wavelength*: 283.3 nm. Depending on the apparatus the line at 217.0 nm may be used.

*Atomisation device*: air-acetylene flame.

ASSAY

To 1.000 g add 50 mL of *dilute acetic acid R* and boil for at least 10 min or until the layer of fatty acids is clear, adding more *water R* as necessary to maintain the original volume. Cool and filter. Wash the filter and the flask with *water R* until the washings are no longer acid to *blue litmus paper R*. Combine the filtrate and washings. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 6.54 mg of Zn.

**01/2008:0111**
**corrected 6.0**

# ZINC SULFATE HEPTAHYDRATE

## Zinci sulfas heptahydricus

$ZnSO_4,7H_2O$ $M_r$ 287.5
[7446-20-0]

DEFINITION

*Content*: 99.0 per cent to 104.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless, transparent crystals, efflorescent.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of sulfates (*2.3.1*).

B. Solution S gives the reaction of zinc (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.4 to 5.6 for solution S.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dilute 3.3 mL of solution S to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*. Use in this test 0.5 mL of *thioglycollic acid R*.

ASSAY

Dissolve 0.200 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 28.75 mg of $ZnSO_4,7H_20$.

STORAGE

In a non-metallic, airtight container.

**01/2008:1683 corrected 6.0**

# ZINC SULFATE HEXAHYDRATE

## Zinci sulfas hexahydricus

$ZnSO_4,6H_2O$ $M_r$ 269.5
[13986-24-8]

DEFINITION

*Content*: 99.0 per cent to 104.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder or colourless transparent crystals, efflorescent.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of sulfates (*2.3.1*).

B. Solution S gives the reaction of zinc (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.4 to 5.6 for solution S.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dilute 3.3 mL of solution S to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*. Use in this test 0.5 mL of *thioglycollic acid R*.

ASSAY

Dissolve 0.200 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 26.95 mg of $ZnSO_4,6H_20$.

STORAGE

In a non-metallic, airtight container.

**01/2010:2159**

# ZINC SULFATE MONOHYDRATE

## Zinci sulfas monohydricus

$ZnSO_4,H_2O$ $M_r$ 179.5

DEFINITION

*Content*: 99.0 per cent to 101.0 per cent.

CHARACTERS

*Appearance*: white or almost white, crystalline powder, or colourless, transparent crystals.

*Solubility*: very soluble in water, practically insoluble in ethanol (96 per cent).

IDENTIFICATION

A. Solution S (see Tests) gives the reactions of sulfates (*2.3.1*).

B. Solution S gives the reaction of zinc (*2.3.1*).

C. It complies with the limits of the assay.

TESTS

**Solution S**. Dissolve 2.5 g in *carbon dioxide-free water R* and dilute to 50 mL with the same solvent.

**Appearance of solution**. Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

**pH** (*2.2.3*): 4.0 to 5.6 for solution S.

**Chlorides** (*2.4.4*): maximum 300 ppm.

Dilute 3.3 mL of solution S to 15 mL with *water R*.

**Iron** (*2.4.9*): maximum 100 ppm.

Dilute 2 mL of solution S to 10 mL with *water R*. Use 0.5 mL of *thioglycollic acid R* in this test.

ASSAY

Dissolve 0.160 g in 5 mL of *dilute acetic acid R*. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 17.95 mg of $ZnSO_4,H_2O$.

STORAGE

In a non-metallic container.

**01/2008:0539 corrected 6.0**

# ZINC UNDECYLENATE

## Zinci undecylenas

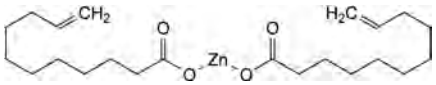


$C_{22}H_{38}O_4Zn$ $M_r$ 431.9
[557-08-4]

DEFINITION

Zinc di(undec-10-enoate).

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: white or almost white, fine powder.

*Solubility*: practically insoluble in water and in ethanol (96 per cent).

mp: 116 °C to 121 °C, it may leave a slight solid residue.

IDENTIFICATION

A. To 2.5 g add 10 mL of *water R* and 10 mL of *dilute sulfuric acid R*. Shake with 2 quantities, each of 10 mL, of *ether R*. Reserve the aqueous layer for identification test C. Wash the combined ether layers with *water R* and evaporate to dryness. To the residue add 2 mL of freshly distilled *aniline R* and boil under a reflux condenser for 10 min. Allow to cool and add 30 mL of *ether R*. Shake with 3 quantities, each of 20 mL, of *dilute hydrochloric acid R* and then with 20 mL of *water R*. Evaporate the organic layer to dryness on a water-bath. The residue, after recrystallisation twice from *ethanol (70 per cent V/V) R* and drying *in vacuo* for 3 h, melts (*2.2.14*) at 66 °C to 68 °C.

B. Dissolve 0.1 g in a mixture of 2 mL of *dilute sulfuric acid R* and 5 mL of *glacial acetic acid R*. Add dropwise 0.25 mL of *potassium permanganate solution R*. The colour of the potassium permanganate solution is discharged.

C. A mixture of 1 mL of the aqueous layer obtained in identification test A and 4 mL of *water R* gives the reaction of zinc (*2.3.1*).

TESTS

**Alkalinity**. Mix 1.0 g with 5 mL of *ethanol (96 per cent) R* and 0.5 mL of *phenol red solution R*. Add 50 mL of *carbon dioxide-free water R* and examine immediately. No reddish colour appears.

**Alkali and alkaline-earth metals**: maximum 2.0 per cent.

To 1.0 g add 25 mL of *water R* and 5 mL of *hydrochloric acid R* and heat to boiling. Filter whilst hot. Wash the filter and the residue with 25 mL of hot *water R*. Combine the filtrate and washings and add *concentrated ammonia R* until alkaline. Add 7.5 mL of *thioacetamide solution R* and heat on a water-bath for 30 min. Filter and wash the precipitate with 2 quantities, each of 10 mL, of *water R*. Combine the filtrate and washings, evaporate to dryness on a water-bath and ignite. The residue weighs a maximum of 20 mg.

**Sulfates** (*2.4.13*): maximum 500 ppm.

To 0.1 g add a mixture of 2 mL of *dilute hydrochloric acid R* and 10 mL of *distilled water R* and heat to boiling. Cool, filter and dilute to 15 mL with *distilled water R*. Prepare the standard using 5 mL of *sulfate standard solution (10 ppm $SO_4$) R* and 10 mL of *distilled water R*.

**Loss on drying** (*2.2.32*): maximum 1.5 per cent, determined on 0.500 g by drying in an oven at 105 °C.

**Degree of unsaturation**. Dissolve 0.100 g in a mixture of 5 mL of *dilute hydrochloric acid R* and 30 mL of *glacial acetic acid R*. Using 0.05 mL *indigo carmine solution R1*, added towards the end of the titration as indicator. Titrate with *0.0167 M bromide-bromate* until the colour changes from blue to yellow. 9.1 mL to 9.4 mL of *0.0167 M bromide-bromate* is required. Carry out a blank titration.

ASSAY

To 0.350 g add 25 mL of *dilute acetic acid R* and heat to boiling. Carry out the complexometric titration of zinc (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 43.19 mg of $C_{22}H_{38}O_4Zn$.

STORAGE

Protected from light.

**01/2017:2421**

# ZIPRASIDONE HYDROCHLORIDE MONOHYDRATE

## Ziprasidoni hydrochloridum monohydricum

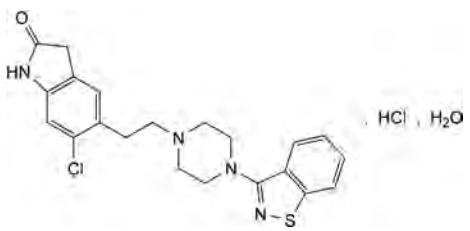


$C_{21}H_{22}Cl_2N_4OS,H_2O$ $M_r$ 467.4
[138982-67-9]

DEFINITION

5-[2-[4-(1,2-Benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one hydrochloride monohydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or slightly pink powder.

*Solubility*: practically insoluble in water, slightly soluble in methanol and in methylene chloride.

It shows polymorphism (*5.9*).

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).
*Comparison*: *ziprasidone hydrochloride monohydrate CRS*.
If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

B. Suspend 30 mg in 2 mL of *water R*, acidify with 0.15 mL of *dilute nitric acid R* and filter. The clear filtrate gives reaction (a) of chlorides (*2.3.1*).

TESTS

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solvent mixture A*: *water R*, *methanol R* (40:60 *V/V*).

*Solvent mixture B*: *hydrochloric acid R*, *water R*, *methanol R* (0.04:20:80 *V/V/V*).

*Test solution (a)*. Dissolve 23 mg of the substance to be examined in solvent mixture A and dilute to 100.0 mL with solvent mixture A.

*Test solution (b)*. Dissolve 23 mg of the substance to be examined in solvent mixture B and dilute to 50.0 mL with solvent mixture B.

*Reference solution (a)*. Dissolve 2.5 mg of *ziprasidone for system suitability 1 CRS* (containing impurities A, B and C) in solvent mixture A and dilute to 10.0 mL with solvent mixture A.

*Reference solution (b)*. Dilute 1.0 mL of test solution (b) to 100.0 mL with solvent mixture B. Dilute 1.0 mL of this solution to 10.0 mL with solvent mixture B.

*Reference solution (c)*. Dissolve the contents of a vial of *ziprasidone for system suitability 2 CRS* (containing impurities D and E) in 1.0 mL of solvent mixture B.

A. *Column*:
- *size*: *l* = 0.15 m, Ø = 4.6 mm;
- *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:
- *mobile phase A*: mix 40 volumes of *methanol R* and 60 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 2.5 with *phosphoric acid R*;
- *mobile phase B*: *methanol R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 20 | 100 | 0 |
| 20 - 21 | 100 → 0 | 0 → 100 |
| 21 - 24 | 0 | 100 |

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 20 µL of test solutions (a) and (b) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *ziprasidone for system suitability 1 CRS* and the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to ziprasidone (retention time = about 7 min): impurity A = about 0.4; impurity B = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (a):

– *peak-to-valley ratio*: minimum 1.2, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B.

*Limits*:

– *correction factor*: for the calculation of content, multiply the peak area of impurity A by 0.7;
– *impurity B in test solution (b)*: not more than twice the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
– *impurity A in test solution (b)*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *impurity C in test solution (a)*: not more than 0.75 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *unspecified impurities in test solution (b)*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *disregard limit in test solution (b)*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard the peak due to impurity C and any peak with a retention time greater than 20 min.

B. *Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 35 °C.

*Mobile phase*: mix 5 volumes of *methanol R*, 40 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 6.0 with a 280 g/L solution of *potassium hydroxide R*, and 55 volumes of *acetonitrile R1*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 20 µL of test solution (b) and reference solutions (b) and (c).

*Run time*: 11 times the retention time of ziprasidone.

*Identification of impurities*: use the chromatogram supplied with *ziprasidone for system suitability 2 CRS* and the chromatogram obtained with reference solution (c) to identify the peaks due to impurities D and E.

*Relative retention* with reference to ziprasidone (retention time = about 4.5 min): impurity D = about 2.0; impurity E = about 3.0.

*System suitability*: reference solution (c):

– *resolution*: minimum 6.0 between the peaks due to ziprasidone and impurity D.

*Limits*:

– *correction factors*: for the calculation of content, multiply the peak areas of the following impurities by the corresponding correction factor: impurity D = 1.4; impurity E = 0.5;
– *impurities D, E*: for each impurity, not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.15 per cent);
– *unspecified impurities*: for each impurity, not more than the area of the principal peak in the chromatogram obtained with reference solution (b) (0.10 per cent);
– *disregard limit*: 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.05 per cent); disregard any peak eluting before the peak due to ziprasidone.

*Limit*:

– *total for tests A and B*: maximum 0.5 per cent.

**Water** (*2.5.12*): 3.7 per cent to 5.0 per cent, determined on 0.250 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solvent mixture*: *water R*, *methanol R* (40:60 *V/V*).

*Test solution.* Dissolve 23.0 mg of the substance to be examined in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Reference solution.* Dissolve 23.0 mg of *ziprasidone hydrochloride monohydrate CRS* in the solvent mixture and dilute to 100.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 4.6 mm;
– *stationary phase*: spherical *octylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 40 °C.

*Mobile phase*: mix 40 volumes of *methanol R* and 60 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* adjusted to pH 3.0 with *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of ziprasidone.

*Retention time*: ziprasidone = about 7 min.

*System suitability*: reference solution:

– *symmetry factor*: maximum 2.0 for the peak due to ziprasidone.

Calculate the percentage content of $C_{21}H_{22}Cl_2N_4OS$ from the declared content of *ziprasidone hydrochloride monohydrate CRS*.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: A, B, C, D, E.*

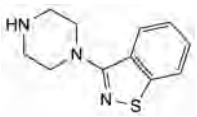

A. 3-piperazin-1-yl-1,2-benzisothiazole,

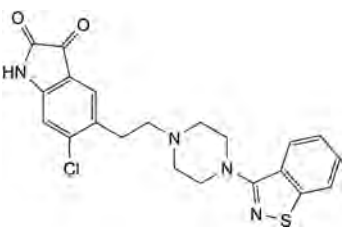

B. 5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1*H*-indole-2,3-dione,

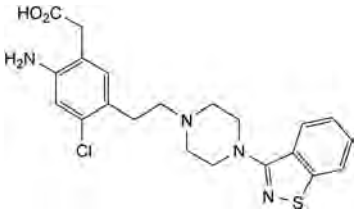

C. 2-[2-amino-5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-4-chlorophenyl]acetic acid,

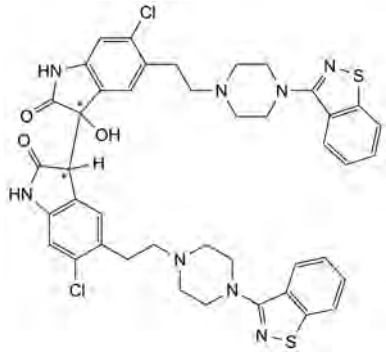

D. 5,5′-bis[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]-ethyl]-6,6′-dichloro-3-hydroxy-1,1′,3,3′-tetrahydro-2*H*,2′*H*-3,3′-biindole-2,2′-dione,

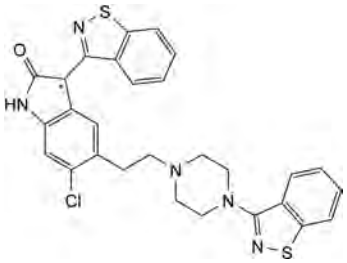

E. 3-(1,2-benzisothiazol-3-yl)-5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one.

**04/2016:2649**
**corrected 10.0**

# ZIPRASIDONE MESILATE TRIHYDRATE

## Ziprasidoni mesilas trihydricus

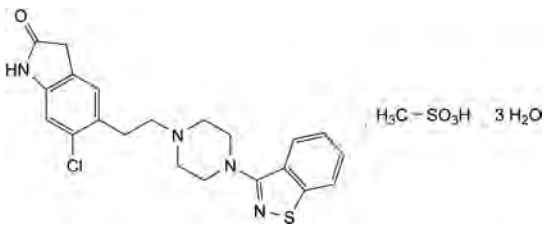

$C_{22}H_{25}ClN_4O_4S_2,3H_2O$ $M_r$ 563.1
[199191-69-0]

DEFINITION

5-[2-[4-(1,2-Benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one methanesulfonate trihydrate.

*Content*: 98.0 per cent to 102.0 per cent (anhydrous substance).

PRODUCTION

It is considered that alkyl methanesulfonate esters are genotoxic and are potential impurities in ziprasidone mesilate trihydrate. The manufacturing process should be developed taking into consideration the principles of quality risk management, together with considerations of the quality of starting materials, process capability and validation. The general methods *2.5.37. Methyl, ethyl and isopropyl methanesulfonate in methanesulfonic acid*, *2.5.38. Methyl, ethyl and isopropyl methanesulfonate in active substances* and *2.5.39. Methanesulfonyl chloride in methanesulfonic acid* are available to assist manufacturers.

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: very slightly soluble in water, slightly soluble in methanol, practically insoluble in acetonitrile.

It shows polymorphism (*5.9*).

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *ziprasidone mesilate trihydrate CRS*.

If the spectra obtained in the solid state show differences, dissolve the substance to be examined and the reference substance separately in *methanol R*, evaporate to dryness and record new spectra using the residues.

TESTS

**Related substances**

*Carry out the tests protected from light and prepare the solutions immediately before use.*

A. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *hydrochloric acid R*, *water R*, *methanol R* (0.04:40:60 *V/V/V*).

*Test solution (a).* Dissolve 27.0 mg of the substance to be examined in 35 mL of the solvent mixture, sonicate for about 2 min and shake. Dilute to 50.0 mL with the solvent mixture.

*Test solution (b).* Dilute 2.0 mL of test solution (a) to 20.0 mL with the solvent mixture.

*Reference solution (a).* Dissolve 27.0 mg of *ziprasidone hydrochloride monohydrate CRS* in 35 mL of the solvent mixture, sonicate for about 2 min and shake. Dilute to 50.0 mL with the solvent mixture. Dilute 2.0 mL of the solution to 20.0 mL with the solvent mixture.

*Reference solution (b).* Dissolve 2.5 mg of *ziprasidone for system suitability 1 CRS* (containing impurities B and C) in a mixture of 40 volumes of *water R* and 60 volumes of *methanol R* and dilute to 10 mL with the same mixture of solvents.

*Reference solution (c).* Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.15 m, Ø = 3.9 mm;
- *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*:

- *mobile phase A*: mix 40 volumes of *methanol R1* and 60 volumes of a 6.8 g/L solution of *potassium dihydrogen phosphate R* previously adjusted to pH 3.0 with *phosphoric acid R*;
- *mobile phase B*: *methanol R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 40 | 100 | 0 |
| 40 - 41 | 100 → 0 | 0 → 100 |
| 41 - 50 | 0 | 100 |

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 20 µL of test solution (a) and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram supplied with *ziprasidone for system suitability 1 CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities B and C.

*Relative retention* with reference to ziprasidone (retention time = about 6 min): impurity B = about 0.8; impurity C = about 0.9.

*System suitability*: reference solution (b):

– *peak-to-valley ratio*: minimum 1.5, where $H_p$ = height above the baseline for the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B; minimum 2.0, where $H_p$ = height above the baseline for the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to ziprasidone.

*Calculation of percentage contents*:

– for each impurity, use the concentration of ziprasidone mesilate trihydrate in reference solution (c).

*Limits*:

– *impurity B*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak with a retention time greater than 40 min.

B. Liquid chromatography (*2.2.29*).

*Solvent mixture*: *hydrochloric acid R*, *water R*, *methanol R* (0.04:20:80 *V/V/V*).

*Test solution*. Dissolve 27 mg of the substance to be examined in 35 mL of the solvent mixture, sonicate for about 2 min and shake. Dilute to 50.0 mL with the solvent mixture.

*Reference solution (a)*. Dissolve the contents of a vial of *ziprasidone for system suitability 2 CRS* (containing impurities D and E) in 1 mL of the solvent mixture.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;

– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);

– *temperature*: 35 °C.

*Mobile phase*: mix 8 volumes of *methanol R1*, 42 volumes of *acetonitrile for chromatography R* and 50 volumes of a solution prepared as follows: dissolve 3.4 g of *potassium dihydrogen phosphate R* and 21.6 g of *sodium octanesulfonate R* in 900 mL of *water for chromatography R*, adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 229 nm.

*Injection*: 20 µL.

*Run time*: 15 times the retention time of ziprasidone.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities D and E.

*Relative retention* with reference to ziprasidone (retention time = about 3 min): impurity E = about 2.6; impurity D = about 7.8.

*System suitability*: reference solution (a):

– *resolution*: minimum 5.0 between the peaks due to impurities E and D.

*Calculation of percentage contents*:

– for each impurity, use the concentration of ziprasidone mesilate trihydrate in reference solution (b).

*Limits*:

– *impurity D*: maximum 0.15 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *reporting threshold*: 0.05 per cent; disregard any peak eluting before the peak due to ziprasidone.

*Limit*:

– *total for tests A and B*: maximum 0.2 per cent.

**Water** (*2.5.12*): 8.5 per cent to 10.1 per cent, determined on 0.050 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

**Bacterial endotoxins** (*2.6.14*): less than 17.5 IU/mg, if intended for use in the manufacture of parenteral preparations without a further appropriate procedure for the removal of bacterial endotoxins.

ASSAY

Liquid chromatography (*2.2.29*) as described in test A for related substances with the following modifications.

*Injection*: 20 µL of test solution (b) and reference solution (a).

*System suitability*: reference solution (a):

– *symmetry factor*: maximum 1.7 for the peak due to ziprasidone.

Calculate the percentage content of $C_{22}H_{25}ClN_4O_4S_2$ taking into account the assigned content of *ziprasidone hydrochloride monohydrate CRS* and a conversion factor of 1.133.

STORAGE

Protected from light. If the substance is sterile, store in a sterile, airtight, tamper-evident container.

IMPURITIES

**Test A for related substances**: A, B, C.

**Test B for related substances**: D, E.

*Specified impurities: B, D.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, E.*

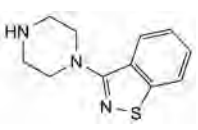


A. 3-piperazin-1-yl-1,2-benzisothiazole,

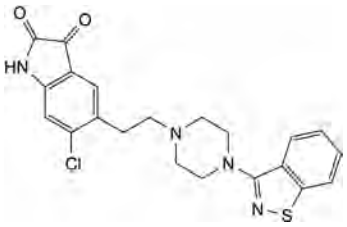

B. 5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1*H*-indole-2,3-dione,

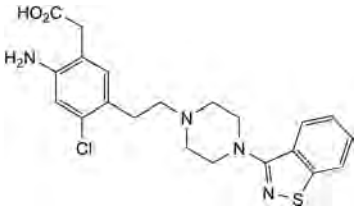

C. 2-[2-amino-5-[2-[4-(1,2-benzisothiazol-3-yl)-piperazin-1-yl]ethyl]-4-chlorophenyl]acetic acid,

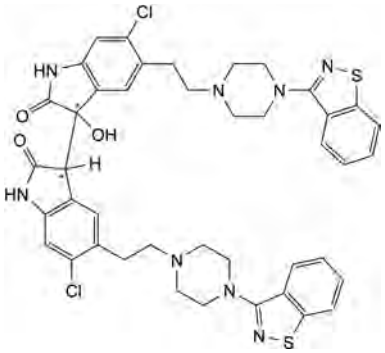

D. 5,5′-bis[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]-ethyl]-6,6′-dichloro-3-hydroxy-1,1′,3,3′-tetrahydro-2*H*,2′*H*-3,3′-biindole-2,2′-dione,

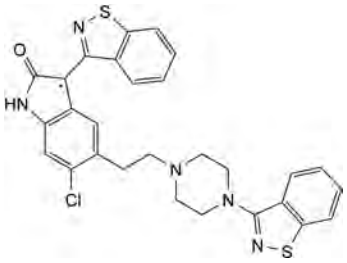

E. 3-(1,2-benzisothiazol-3-yl)-5-[2-[4-(1,2-benzisothiazol-3-yl)piperazin-1-yl]ethyl]-6-chloro-1,3-dihydro-2*H*-indol-2-one.

**01/2019:2743**

# ZOLEDRONIC ACID MONOHYDRATE

## Acidum zoledronicum monohydricum

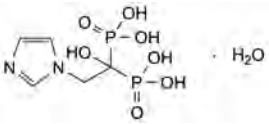

$C_5H_{10}N_2O_7P_2,H_2O$ $M_r$ 290.1
[165800-06-6]

DEFINITION

[1-Hydroxy-2-(1*H*-imidazol-1-yl)ethane-1,1-diyl]bis-(phosphonic acid) monohydrate.

*Content*: 99.0 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, crystalline powder.

*Solubility*: slightly soluble in water, practically insoluble in anhydrous ethanol and in heptane.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *zoledronic acid monohydrate CRS*.

B. Water (see Tests).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $B_7$ or $BY_7$ (*2.2.2, Method II*).

Dissolve 0.5 g in a 4 g/L solution of *sodium hydroxide R* and dilute to 50.0 mL with the same solution.

**pH** (*2.2.3*): 1.8 to 2.8.

Dissolve 0.150 g in *carbon dioxide-free water R* and dilute to 50.0 mL with the same solvent. Sonicate for 10 min, if necessary.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solution A*. Dissolve 10.8 g of *sodium octanesulfonate R* and 37 mg of *sodium edetate R* in *water for chromatography R*, add 10 mL of *perchloric acid R* and 2 mL of *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

*Test solution*. Dissolve 40 mg of the substance to be examined in the mobile phase, sonicate for 30 min, and dilute to 20.0 mL with the mobile phase.

*Reference solution (a)*. Dissolve 2 mg of *zoledronic acid impurity A CRS*, 5 mg of *zoledronic acid impurity B CRS* and 2 mg of *sodium nitrate R* in the mobile phase and dilute to 50.0 mL with the mobile phase. To 1.0 mL of the solution add 7.0 mL of the mobile phase and dilute to 20.0 mL with the test solution.

*Reference solution (b)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *end-capped phenylhexylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 20 °C.

*Mobile phase*: *acetonitrile R1*, solution A (4:96 *V/V*).

*Flow rate*: 0.6 mL/min.

*Detection*: spectrophotometer at 215 nm.

*Preconditioning*: precondition the instrument and the column once before each series of injections as follows:

- *instrument*: rinse the instrument without a column with a 25 per cent *V/V* solution of *acetic acid R* at 5 mL/min for about 20 min. Then, rinse with *water for chromatography R* at 5 mL/min for about 2 h;
- *column*: rinse the column with the mobile phase at 0.6 mL/min for about 1 h. During the rinsing, inject the test solution 15 times, applying a run time of about 3 min for each injection.

*Injection*: 10 µL.

*Run time*: 5 times the retention time of zoledronic acid.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities A and B and the nitrate ion.

*Relative retention* with reference to zoledronic acid (retention time = about 6 min): nitrate = about 0.6; impurity B = about 0.7; impurity A = about 0.9.

*System suitability*: reference solution (a):

– *resolution*: minimum 1.5 between the peaks due to impurity A and zoledronic acid; minimum 1.5 between the peaks due to the nitrate ion and impurity B.

*Calculation of percentage contents*:

– *correction factor*: multiply the peak area of impurity B by 1.9;

– for each impurity, use the concentration of zoledronic acid monohydrate in reference solution (b).

*Limits*:

– *impurity B*: maximum 0.5 per cent;

– *unspecified impurities*: for each impurity, maximum 0.10 per cent;

– *total*: maximum 0.5 per cent;

– *reporting threshold*: 0.05 per cent, disregard the peak due to the nitrate ion.

**Impurities E and F**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 50.0 mg of the substance to be examined in mobile phase B, sonicate if necessary, and dilute to 10.0 mL with mobile phase B.

*Reference solution (a)*. Dissolve 95.0 mg of *anhydrous sodium dihydrogen phosphate R* and 79.0 mg of *phosphorous acid R* (impurity E) in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (b)*. Dissolve 34.0 mg of *sodium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

*Reference solution (c)*. To 1.0 mL of reference solution (a) add 0.5 mL of reference solution (b) and dilute to 100.0 mL with mobile phase B.

*Reference solution (d)*. Dilute 0.1 mL of reference solution (a) to 100.0 mL with mobile phase B.

*Precolumn*:

– *size*: *l* = 0.05 m, Ø = 4.0 mm;

– *stationary phase*: *anion-exchange resin R* (13 µm).

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.0 mm;

– *stationary phase*: *anion-exchange resin R* (9 µm);

– *temperature*: 30 °C.

*Mobile phase*:

– *mobile phase A*: *carbon dioxide-free water R*;

– *mobile phase B*: 4.0 g/L solution of *sodium hydroxide R* in *carbon dioxide-free water R*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 13 | 80 → 70 | 20 → 30 |
| 13 - 17 | 70 → 60 | 30 → 40 |
| 17 - 29 | 60 | 40 |

*Flow rate*: 1.0 mL/min.

*Detection*: conductivity detector.

*Injection*: 20 µL.

*Identification of impurities*: use the chromatogram obtained with reference solution (a) to identify the peaks due to impurities E and F; use the chromatogram obtained with reference solution (b) to identify the peak due to the chloride ion.

*Relative retention* with reference to chloride (retention time = about 5 min): impurity E = about 1.2; impurity F = about 3.4.

*System suitability*:

– *resolution*: minimum 1.5 between the peaks due to the chloride ion and impurity E in the chromatogram obtained with reference solution (c);

– *signal-to-noise ratio*: minimum 10 for the peak due to impurity F in the chromatogram obtained with reference solution (d).

*Calculation of percentage contents*:

– for each impurity, use the concentration of the corresponding impurity in reference solution (c).

*Limits*:

– *impurities E, F*: for each impurity, maximum 0.15 per cent.

**Water** (*2.5.12*): 5.0 per cent to 7.5 per cent, determined on 0.100 g.

ASSAY

Dissolve 0.150 g in 50 mL of *carbon dioxide-free water R*. Sonicate for 10 min, if necessary. Titrate with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Read the volume added at the 3rd inflexion point.

1 mL of *0.1 M sodium hydroxide* is equivalent to 9.07 mg of $C_5H_{10}N_2O_7P_2$.

IMPURITIES

*Specified impurities: B, E, F.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C, D.*

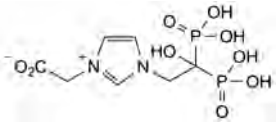

A. [1-(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium-3-yl]acetate,

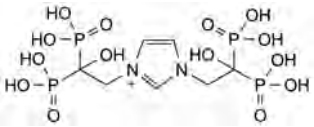

B. 1,3-bis(2-hydroxy-2,2-diphosphonoethyl)-1*H*-imidazol-3-ium,

C. 1*H*-imidazole,

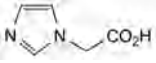

D. (1*H*-imidazol-1-yl)acetic acid,

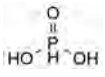

E. phosphonic acid (phosphorous acid),

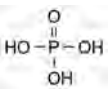

F. phosphoric acid.

**07/2018:2737**

# ZOLMITRIPTAN

## Zolmitriptanum

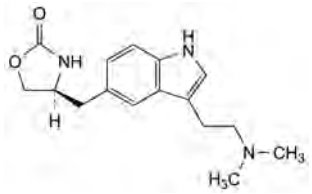

$C_{16}H_{21}N_3O_2$ $M_r$ 287.4
[139264-17-8]

DEFINITION

(4*S*)-4-[[3-[2-(Dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1,3-oxazolidin-2-one.

*Content*: 97.5 per cent to 102.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white powder.

*Solubility*: slightly soluble or very slightly soluble in water, freely soluble in methanol, sparingly soluble in acetone.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *zolmitriptan CRS*.

TESTS

**Enantiomeric purity**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25.0 mg of the substance to be examined in the mobile phase and dilute to 25.0 mL with the mobile phase.

*Reference solution (a)*. Dilute 1.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b)*. Dissolve 5 mg of *zolmitriptan impurity A CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase. To 1.0 mL of the solution add 1.0 mL of the test solution and dilute to 10.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 50.0 mL with the mobile phase.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *amylose derivative of silica gel for chiral separation R* (5 µm);
- *temperature*: 35 °C.

*Mobile phase*: *diethylamine R*, *2-propanol R*, *methanol R*, *heptane R* (0.1:10:15:75 *V/V/V/V*).

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 285 nm.

*Injection*: 10 µL.

*Run time*: twice the retention time of zolmitriptan.

*Relative retention* with reference to zolmitriptan (retention time = about 7 min): impurity A = 0.7.

*System suitability*: reference solution (b):

- *resolution*: minimum 2.0 between the peaks due to impurity A and zolmitriptan.

*Calculation of percentage content*:

- for impurity A, use the concentration of zolmitriptan in reference solution (a).

*Limit*:

- *impurity A*: maximum 0.10 per cent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Solvent mixture*: mobile phase B, mobile phase A (10:90 *V/V*).

*Test solution (a)*. Dissolve 10.0 mg of the substance to be examined in the solvent mixture and dilute to 10.0 mL with the solvent mixture.

*Test solution (b)*. Dilute 1.0 mL of test solution (a) to 5.0 mL with the solvent mixture.

*Reference solution (a)*. Dilute 1.0 mL of test solution (a) to 100.0 mL with the solvent mixture. Dilute 1.0 mL of this solution to 10.0 mL with the solvent mixture.

*Reference solution (b)*. Dissolve 5 mg of *zolmitriptan for system suitability CRS* (containing impurities C, H and I) in the solvent mixture and dilute to 5.0 mL with the solvent mixture.

*Reference solution (c)*. Dissolve 10.0 mg of *zolmitriptan CRS* in the solvent mixture and dilute to 10.0 mL with the solvent mixture. Dilute 1.0 mL of the solution to 5.0 mL with the solvent mixture.

*Column*:

- *size*: *l* = 0.10 m, Ø = 3.0 mm;
- *stationary phase*: *end-capped solid core phenylhexylsilyl silica gel for chromatography R* (2.7 µm);
- *temperature*: 20 °C.

*Mobile phase A*: dissolve 2.72 g of *potassium dihydrogen phosphate R* and 0.94 g of *sodium hexanesulfonate R* in *water for chromatography R*, adjust to pH 2.0 with *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*;

*Mobile phase B*: *acetonitrile R1*;

| Time (min) | Mobile phase A (per cent *V/V*) | Mobile phase B (per cent *V/V*) |
|---|---|---|
| 0 - 0.5 | 90 | 10 |
| 0.5 - 4 | 90 → 85 | 10 → 15 |
| 4 - 8 | 85 | 15 |
| 8 - 9 | 85 → 80 | 15 → 20 |
| 9 - 10 | 80 | 20 |
| 10 - 12 | 80 → 70 | 20 → 30 |
| 12 - 13 | 70 | 30 |

*Flow rate*: 0.8 mL/min.

*Detection*: spectrophotometer at 210 nm.

*Injection*: 2 µL of test solution (a) and reference solutions (a) and (b).

*Identification of impurities*: use the chromatogram supplied with *zolmitriptan for system suitability CRS* and the chromatogram obtained with reference solution (b) to identify the peaks due to impurities C, H and I.

*Relative retention* with reference to zolmitriptan (retention time = about 5 min): impurity H = about 0.97; impurity I = about 1.1; impurity C = about 2.0.

*System suitability*: reference solution (b):

- *peak-to-valley ratio*: minimum 8, where $H_p$ = height above the baseline of the peak due to impurity H and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to zolmitriptan; minimum 1.5, where $H_p$ = height above the baseline of the peak due to impurity I and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to zolmitriptan.

*Calculation of percentage contents*:

- *correction factor*: multiply the peak area of impurity C by 2.0;
- for each impurity, use the concentration of zolmitriptan in reference solution (a).

*Limits*:

- *impurity C*: maximum 0.15 per cent;
- *unspecified impurities*: for each impurity, maximum 0.10 per cent;
- *total*: maximum 0.5 per cent;
- *reporting threshold*: 0.05 per cent.

**Water** (*2.5.12*): maximum 0.5 per cent, determined on 0.500 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Liquid chromatography (*2.2.29*) as described in the test for related substances with the following modifications.

*Detection*: spectrophotometer at 283 nm.

*Injection*: 2 µL of test solution (b) and reference solution (c).

Calculate the percentage content of $C_{16}H_{21}N_3O_2$ taking into account the assigned content of *zolmitriptan CRS*.

IMPURITIES

*Specified impurities: A, C.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *B, D, E, F, G, H, I.*

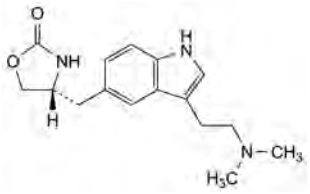

A. (4*R*)-4-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]-1,3-oxazolidin-2-one,

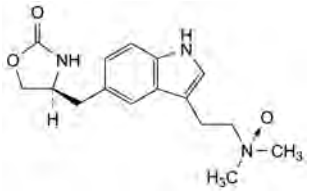

B. *N*,*N*-dimethyl-2-[5-[[(4*S*)-2-oxo-1,3-oxazolidin-4-yl]methyl]-1*H*-indol-3-yl]ethan-1-amine *N*-oxide,

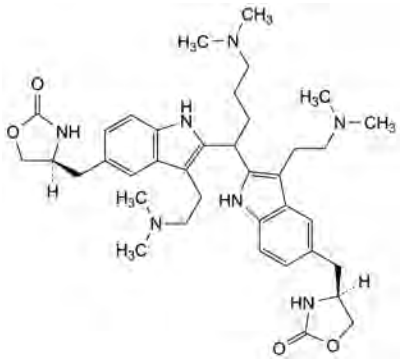

C. (4*S*,4′*S*)-4,4′-[[4-(dimethylamino)butane-1,1-diyl]bis[[3-[2-(dimethylamino)ethyl]-1*H*-indole-2,5-diyl]methylene]]bis(1,3-oxazolidin-2-one),

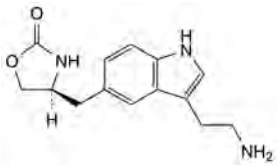

D. (4*S*)-4-[[3-(2-aminoethyl)-1*H*-indol-5-yl]methyl]-1,3-oxazolidin-2-one,

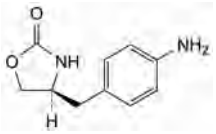

E. (4*S*)-4-[(4-aminophenyl)methyl]-1,3-oxazolidin-2-one,

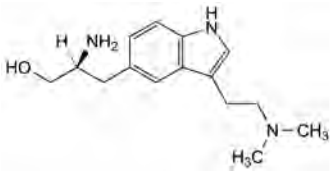

F. (2*S*)-2-amino-3-[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]propan-1-ol,

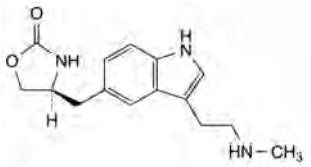

G. (4*S*)-4-[[3-[2-(methylamino)ethyl]-1*H*-indol-5-yl]methyl]-1,3-oxazolidin-2-one,

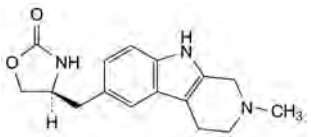

H. (4*S*)-4-[(2-methyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indol-6-yl)methyl]-1,3-oxazolidin-2-one,

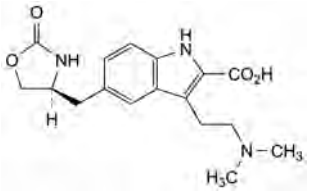

I. 3-[2-(dimethylamino)ethyl]-5-[[(4*S*)-2-oxo-1,3-oxazolidin-4-yl]methyl]-1*H*-indole-2-carboxylic acid.

**01/2011:1280**

# ZOLPIDEM TARTRATE

## Zolpidemi tartras

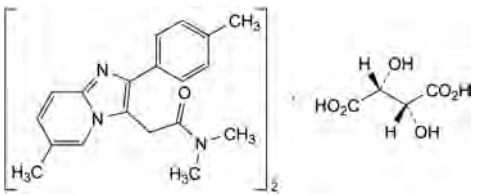

$C_{42}H_{48}N_6O_8$ $M_r$ 765

[99294-93-6]

DEFINITION

Bis[*N*,*N*-dimethyl-2-[6-methyl-2-(4-methylphenyl)-imidazo[1,2-*a*]pyridin-3-yl]acetamide] (2*R*,3*R*)-2,3-dihydroxybutanedioate.

*Content*: 98.5 per cent to 101.0 per cent (anhydrous substance).

CHARACTERS

*Appearance*: white or almost white, hygroscopic, crystalline powder.

*Solubility*: slightly soluble in water, sparingly soluble in methanol, practically insoluble in methylene chloride.

IDENTIFICATION

*First identification*: *A, C.*

*Second identification*: *B, C.*

A. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: dissolve 0.10 g in 10 mL of *0.1 M hydrochloric acid*. Add 10 mL of *water R*. Add dropwise with stirring 1 mL of *dilute ammonia R2*. Filter and collect the resulting precipitate. Wash the precipitate with *water R* and then dry at 105 °C for 2 h. Examine the precipitate as a disc.

*Comparison*: repeat the operations using 0.10 g of *zolpidem tartrate CRS*.

B. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 50 mg of the substance to be examined in 5 mL of *methanol R*, add 0.1 mL of *diethylamine R* and dilute to 10 mL with *methanol R*.

*Reference solution (a).* Dissolve 50 mg of *zolpidem tartrate CRS* in 5 mL of *methanol R*, add 0.1 mL of *diethylamine R* and dilute to 10 mL with *methanol R*.

*Reference solution (b).* Dissolve 50 mg of *flunitrazepam CRS* in 5 mL of *methylene chloride R* and dilute to 10 mL with the same solvent. Mix 1 mL of this solution with 1 mL of reference solution (a).

*Plate*: *TLC silica gel $F_{254}$ plate R.*

*Mobile phase*: *diethylamine R*, *cyclohexane R*, *ethyl acetate R* (10:45:45 *V/V/V*).

*Application*: 5 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*System suitability*: reference solution (b):

– the chromatogram shows 2 clearly separated spots.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

C. Dissolve about 0.1 g in 1 mL of *methanol R* heating gently. 0.1 mL of this solution gives reaction (b) of tartrates (*2.3.1*).

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*) and not more intensely coloured than reference solution $Y_6$ or $BY_6$ (*2.2.2, Method II*). *Prepare the solutions protected from light and carry out the test as rapidly as possible.*

Triturate 0.25 g with 0.125 g of *tartaric acid R*. Dissolve the mixture in 20 mL of *water R* and dilute to 25 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*).

*Test solution*. Dissolve 25 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (a).* Dissolve 5 mg of *zolpidem impurity A CRS* in the mobile phase and dilute to 50.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 5 mg of the substance to be examined in the mobile phase and dilute to 50.0 mL with the mobile phase. To 10 mL of this solution, add 10 mL of reference solution (a).

*Reference solution (c).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.15 m, Ø = 3.9 mm;

– *stationary phase*: *octadecylsilyl silica gel for chromatography R* (4 µm).

*Mobile phase*: mix 18 volumes of *acetonitrile R*, 23 volumes of *methanol R* and 59 volumes of a 5.6 g/L solution of *phosphoric acid R* adjusted to pH 5.5 with *triethylamine R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 254 nm.

*Injection*: 20 µL of the test solution and reference solutions (b) and (c).

*Identification of impurities*: use the chromatogram obtained with reference solution (b) to identify the peak due to impurity A.

*Relative retention* with reference to zolpidem (retention time = about 10 min): tartaric acid = about 0.16; impurity A = about 0.8.

*System suitability*: reference solution (b):

– *resolution*: minimum 2.0 between the peaks due to impurity A and zolpidem.

*Limits*:

– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.10 per cent);

– *total*: not more than the area of the principal peak in the chromatogram obtained with reference solution (c) (0.2 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (c) (0.05 per cent); disregard any peak due to tartaric acid.

**Water** (*2.5.12*): maximum 3.0 per cent, determined on 0.50 g.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 20 mL of *anhydrous acetic acid R* and 20 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 38.24 mg of $C_{42}H_{48}N_6O_8$.

STORAGE

In an airtight container, protected from light.

IMPURITIES

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A.*

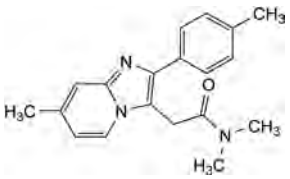

A. *N*,*N*-dimethyl-2-[7-methyl-2-(4-methylphenyl)-imidazo[1,2-*a*]pyridin-3-yl]acetamide.

**01/2017:1060**

# ZOPICLONE

## Zopiclonum

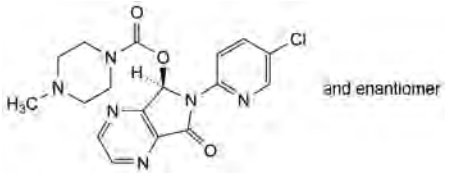


$C_{17}H_{17}ClN_6O_3$ $M_r$ 388.8
[43200-80-2]

DEFINITION

(5*RS*)-6-(5-Chloropyridin-2-yl)-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-yl 4-methylpiperazine-1-carboxylate.

*Content*: 98.5 per cent to 100.5 per cent.

CHARACTERS

*Appearance*: white or slightly yellowish powder.

*Solubility*: practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, practically insoluble in ethanol (96 per cent). It dissolves in dilute mineral acids.

IDENTIFICATION

*First identification*: B.

*Second identification*: A, C.

A. Ultraviolet and visible absorption spectrophotometry (*2.2.25*).

*Test solution.* Dissolve 50.0 mg in a 3.5 g/L solution of *hydrochloric acid R* and dilute to 100.0 mL with the same solvent. Dilute 2.0 mL of this solution to 100.0 mL with a 3.5 g/L solution of *hydrochloric acid R*.

*Spectral range*: 220-350 nm.

*Absorption maximum*: at 303 nm.

*Specific absorbance at the absorption maximum*: 340 to 380.

B. Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *zopiclone CRS*.

C. Thin-layer chromatography (*2.2.27*).

*Test solution.* Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reference solution.* Dissolve 10 mg of *zopiclone CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel* $GF_{254}$ *plate R*.

*Mobile phase*: *triethylamine R*, *acetone R*, *ethyl acetate R* (2:50:50 *V/V/V*).

*Application*: 10 µL.

*Development*: over 2/3 of the plate.

*Drying*: in air.

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

TESTS

**Solution S**. Dissolve 1.0 g in *dimethylformamide R* and dilute to 20.0 mL with the same solvent.

**Appearance of solution**. Solution S is not more opalescent than reference suspension II (*2.2.1*) and not more intensely coloured than intensity 5 of the range of reference solutions of the most appropriate colour (*2.2.2, Method II*).

**Optical rotation** (*2.2.7*): – 0.05° to + 0.05°.

Dilute 10.0 mL of solution S to 50.0 mL with *dimethylformamide R*.

**Related substances**. Liquid chromatography (*2.2.29*). *Prepare the solutions immediately before use.*

*Test solution.* Dissolve 40.0 mg of the substance to be examined in the mobile phase and dilute to 10.0 mL with the mobile phase.

*Reference solution (a).* Dilute 2.0 mL of the test solution to 100.0 mL with the mobile phase. Dilute 1.0 mL of this solution to 10.0 mL with the mobile phase.

*Reference solution (b).* Dissolve 4.0 mg of *zopiclone oxide CRS* (impurity A) in the mobile phase and dilute to 10.0 mL with the mobile phase. To 5.0 mL of the solution add 0.5 mL of the test solution and dilute to 50.0 mL with the mobile phase.

*Reference solution (c).* Dissolve 2.0 mg of *zopiclone impurity B CRS* in the mobile phase and dilute to 5.0 mL with the mobile phase. Dilute 1.0 mL of the solution to 100.0 mL with the mobile phase.

*Column*:

– *size*: *l* = 0.25 m, Ø = 4.6 mm;
– *stationary phase*: *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
– *temperature*: 30 °C.

*Mobile phase*: mix 38 volumes of *acetonitrile R* and 62 volumes of a solution containing 8.1 g/L of *sodium laurilsulfate R* and 1.6 g/L of *sodium dihydrogen phosphate R*, previously adjusted to an apparent pH of 3.5 with a 10 per cent *V/V* solution of *phosphoric acid R*.

*Flow rate*: 1.5 mL/min.

*Detection*: spectrophotometer at 303 nm.

*Injection*: 20 µL.

*Run time*: 1.5 times the retention time of zopiclone.

*Identification of impurities*: use the chromatogram obtained with reference solution (c) to identify the peak due to impurity B.

*Retention time*: zopiclone = 27 min to 31 min; if necessary, adjust the concentration of acetonitrile in the mobile phase.

*Relative retention* with reference to zopiclone: impurity B = about 0.1; impurity A = about 0.9.

*System suitability*: reference solution (b):

– *resolution*: minimum 3.0 between the peaks due to impurity A and zopiclone; if necessary, adjust the mobile phase to pH 4.0 with a 10 per cent *V/V* solution of *phosphoric acid R*.

*Limits*:

– *impurity B*: not more than twice the area of the corresponding peak in the chromatogram obtained with reference solution (c) (0.2 per cent);
– *unspecified impurities*: for each impurity, not more than 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
– *total (excluding impurity B)*: not more than the area of the principal peak in the chromatogram obtained with reference solution (a) (0.2 per cent);

– *disregard limit*: 0.25 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**2-Propanol**. Gas chromatography (*2.2.28*).

*Internal standard solution.* Dilute 5 mL of *ethanol R1* to 100 mL with *ethylene chloride R*. Dilute 1 mL of the solution to 10 mL with *ethylene chloride R*.

*Test solution.* Dissolve 0.25 g of the substance to be examined in *ethylene chloride R*, add 0.5 mL of the internal standard solution and dilute to 5.0 mL with *ethylene chloride R*.

*Reference solution.* Dilute 4.5 mL of *2-propanol R* to 100.0 mL with *ethylene chloride R*. To 1.0 mL of the solution add 10.0 mL of the internal standard solution and dilute to 100.0 mL with *ethylene chloride R*.

*Column*:

– *material*: fused silica;

– *size*: *l* = 10 m, Ø = about 0.53 mm;

– *stationary phase*: *styrene-divinylbenzene copolymer R* (film thickness 20 µm).

*Carrier gas*: *helium for chromatography R*.

*Flow rate*: 4 mL/min.

*Temperature*:

| | Time (min) | Temperature (°C) |
|---|---|---|
| Column | 0 - 5 | 50 |
| | 5 - 10 | 50 → 70 |
| | 10 - 14 | 70 |
| | 14 - 20.5 | 70 → 200 |
| | 20.5 - 27.5 | 200 |
| Injection port | | 150 |
| Detector | | 250 |

*Detection*: flame ionisation.

*Injection*: 1 µL.

Calculate the percentage content *m/m* of 2-propanol taking its density to be 0.785 g/mL at 20 °C.

*Limit*:

– *2-propanol*: maximum 0.7 per cent *m/m*.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.300 g in a mixture of 10 mL of *anhydrous acetic acid R* and 40 mL of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 38.88 mg of $C_{17}H_{17}ClN_6O_3$.

STORAGE

Protected from light.

IMPURITIES

*Specified impurities: B.*

*Other detectable impurities* (the following substances would, if present at a sufficient level, be detected by one or other of the tests in the monograph. They are limited by the general acceptance criterion for other/unspecified impurities and/or by the general monograph *Substances for pharmaceutical use (2034)*. It is therefore not necessary to identify these impurities for demonstration of compliance. See also *5.10. Control of impurities in substances for pharmaceutical use*): *A, C.*

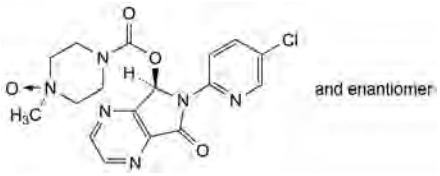


A. (5*RS*)-6-(5-chloropyridin-2-yl)-7-oxo-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-yl 4-methylpiperazine-1-carboxylate 4-oxide (zopiclone oxide),

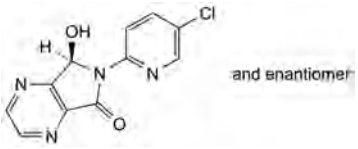


B. (7*RS*)-6-(5-chloropyridin-2-yl)-7-hydroxy-6,7-dihydro-5*H*-pyrrolo[3,4-*b*]pyrazin-5-one,

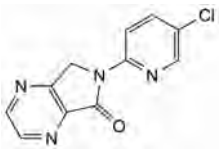

C. 6-(5-chloropyridin-2-yl)-6,7-dihydro-5*H*-pyrrolo-[3,4-*b*]pyrazin-5-one.

**01/2017:1707**

# ZUCLOPENTHIXOL DECANOATE

## Zuclopenthixoli decanoas

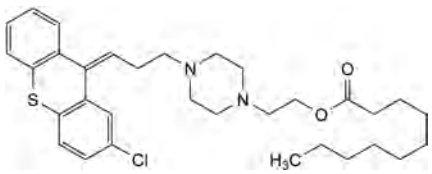

$C_{32}H_{43}ClN_2O_2S$ $M_r$ 555.2

[64053-00-5]

DEFINITION

2-[4-[3-[(9*Z*)-2-Chloro-9*H*-thioxanthen-9-ylidene]propyl]-piperazin-1-yl]ethyl decanoate.

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

CHARACTERS

*Appearance*: yellow, viscous, oily liquid.

*Solubility*: very slightly soluble in water, very soluble in ethanol (96 per cent) and in methylene chloride.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

*Comparison*: *Ph. Eur. reference spectrum of zuclopenthixol decanoate.*

TESTS

**Appearance of solution**. The solution is clear (*2.2.1*).

Using an ultrasonic bath, dissolve 1.0 g in *ethanol (96 per cent) R* and dilute to 20.0 mL with the same solvent.

**Related substances**. Liquid chromatography (*2.2.29*). *Carry out the test protected from light and prepare the solutions immediately before use.*

*Solution A*. Dissolve 8.89 g of *docusate sodium R* in *water R*, stirring for about 6-8 h, and dilute to 1000 mL with the same solvent.

*Test solution.* Dissolve 25.0 mg of the substance to be examined in *acetonitrile R* and dilute to 100.0 mL with the same solvent.

*Reference solution (a).* Dilute 1.0 mL of the test solution to 100.0 mL with *acetonitrile R*.

*Reference solution (b).* Dissolve 5.0 mg of *zuclopenthixol impurity B CRS* in *acetonitrile R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *acetonitrile R*.

*Reference solution (c).* Dissolve the contents of a vial of *zuclopenthixol for system suitability CRS* (zuclopenthixol decanoate containing impurities A, B and C) in 1 mL of *methanol R*.

*Column*:

- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: spherical *end-capped octadecylsilyl silica gel for chromatography R* (5 µm);
- *temperature*: 40 °C.

*Mobile phase*: mix 25 volumes of solution A and 75 volumes of *anhydrous ethanol R*, then add 0.1 volumes of *phosphoric acid R*.

*Flow rate*: 1.0 mL/min.

*Detection*: spectrophotometer at 270 nm.

*Injection*: 20 µL.

*Run time*: twice the retention time of zuclopenthixol decanoate.

*Identification of impurities*: use the chromatogram supplied with *zuclopenthixol for system suitability CRS* and the chromatograms obtained with reference solutions (b) and (c) to identify the peaks due to impurities A, B and C.

*Relative retention* with reference to zuclopenthixol decanoate (retention time = about 12 min): impurity C = about 0.4; impurity B = about 0.5; impurity A = about 1.1.

*System suitability*: reference solution (c):

- *peak-to-valley ratio*: minimum 2.0, where $H_p$ = height above the baseline of the peak due to impurity C and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to impurity B; and minimum 2.5, where $H_p$ = height above the baseline of the peak due to impurity A and $H_v$ = height above the baseline of the lowest point of the curve separating this peak from the peak due to zuclopenthixol decanoate.

*Limits*:

- *impurity A*: not more than 1.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.3 per cent);
- *impurity B*: not more than 0.2 times the area of the principal peak in the chromatogram obtained with reference solution (b) (0.2 per cent);
- *impurity C*: not more than 0.3 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.3 per cent);
- *unspecified impurities*: for each impurity, not more than 0.1 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.10 per cent);
- *total*: not more than 1.5 times the area of the principal peak in the chromatogram obtained with reference solution (a) (1.5 per cent);
- *disregard limit*: 0.05 times the area of the principal peak in the chromatogram obtained with reference solution (a) (0.05 per cent).

**Loss on drying** (*2.2.32*): maximum 0.5 per cent, determined on 1.000 g by drying in an oven at 60 °C at a pressure not exceeding 0.7 kPa for 3 h.

**Sulfated ash** (*2.4.14*): maximum 0.1 per cent, determined on 1.0 g.

ASSAY

Dissolve 0.250 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 27.76 mg of $C_{32}H_{43}ClN_2O_2S$.

STORAGE

Under an inert gas in an airtight container, protected from light, at a temperature not exceeding – 20 °C.

IMPURITIES

*Specified impurities: A, B, C.*

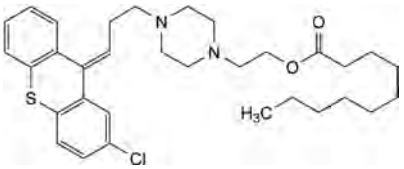


A. 2-[4-[3-[(9*E*)-2-chloro-9*H*-thioxanthen-9-ylidene]propyl]-piperazin-1-yl]ethyl decanoate,

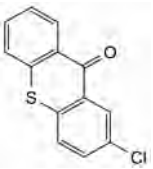


B. 2-chloro-9*H*-thioxanthen-9-one,

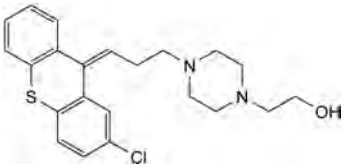


C. 2-[4-[3-[(9*Z*)-2-chloro-9*H*-thioxanthen-9-ylidene]propyl]-piperazin-1-yl]ethanol.

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## How to contact us

*Information and orders* **Internet: www.edqm.eu**

**European Directorate for the Quality of Medicines & HealthCare (EDQM)**
Council of Europe - 7 allée Kastner
CS 30026, F-67081 STRASBOURG, FRANCE
**Tel:** +33 (0)3 88 41 30 30
**Fax:** +33 (0)3 88 41 27 71

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# EUROPEAN PHARMACOPOEIA – 10th EDITION

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CombiStats is a computer program for the statistical analysis of data from biological assays in agreement with general chapter 5.3. *Statistical analysis of results of biological assays and tests* of the 10th Edition of the European Pharmacopoeia.

# KEY TO MONOGRAPHS



*Version date of the text*

**01/2012:0884**
**corrected 10.0**

*Text reference number*

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## CARBIMAZOLE(1)

Carbimazolum

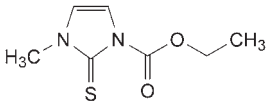

$C_7H_{10}N_2O_2S$

[22232-54-8] $M_r$ 186.2

*CAS number*

DEFINITION

Ethyl 3-methyl-2-thioxo-2,3-dihydro-1*H*-imidazole-1-carboxylate.

*Chemical name in accordance with IUPAC nomenclature rules*

*Content*: 98.0 per cent to 102.0 per cent (dried substance).

*For the meaning of black and white diamonds see chapter 5.8. Pharmacopoeial harmonisation*

♦CHARACTERS

*Appearance*: white or yellowish-white, crystalline powder.

*Solubility*: slightly soluble in water, soluble in acetone and in ethanol (96 per cent). ♦

IDENTIFICATION

*First identification: B.*

*Second identification: A, C.*

*Application of the first and second identification is defined in the General Notices (chapter 1)*

◊A. Melting point (*2.2.14*): 122 °C to 125 °C.◊

B. Infrared absorption spectrophotometry (*2.2.24*).

*Preparation*: discs.

*Comparison*: *carbimazole CRS*.

*Reference standard available from the EDQM (see http://crs.edqm.eu)*

C. Thin-layer chromatography (*2.2.27*).

*Test solution*. Dissolve 10 mg of the substance to be examined in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Reagent described in chapter 4*

*Reference solution*. Dissolve 10 mg of *carbimazole CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

*Plate*: *TLC silica gel GF254 plate R*.

*Further information on certain reagents available in the Knowledge database (http://go.edqm.eu/knowledge)*

*Mobile phase*: *acetone R*, *methylene chloride R* (20:80 *V/V*).

*Application*: 10 µL.

*Development*: over 3/4 of the plate.

*Drying*: in air for 30 min.

*Vertical line in the margin indicating where the text has been modified*

*Detection*: examine in ultraviolet light at 254 nm.

*Results*: the principal spot in the chromatogram obtained with the test solution is similar in position and size to the principal spot in the chromatogram obtained with the reference solution.

*Horizontal line in the margin indicating where part of the text has been deleted*

TESTS

**Related substances**. Liquid chromatography (*2.2.29*).

*Reference to a general chapter*

*Test solution.* Dissolve 5.0 mg of the substance to be examined in 10.0 mL of a mixture of 20 volumes of *acetonitrile R* and 80 volumes of *water R*. Use this solution within 5 min of preparation.

(1) This monograph has undergone pharmacopoeial harmonisation. See chapter 5.8. *Pharmacopoeial harmonisation*.

SPECIMEN

*IMPORTANT NOTICE*

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The European Pharmacopoeia contains a number of general monographs covering classes of products. These general monographs give requirements that are applicable to all products in the given class or, in some cases, to any product in the given class for which there is a specific monograph in the Pharmacopoeia (see *1. General Notices*, General monographs). Where no restriction on scope of a general monograph is given in a preamble, it is applicable to all products in the class defined, irrespective of whether there is an individual monograph for the product in the Pharmacopoeia.

Whenever a monograph is used, it is essential to ascertain whether there is a general monograph applicable to the product in question. The general monographs listed below are published in the General Monographs section (unless otherwise stated). This list is updated where necessary and republished in each supplement.

Allergen products (1063)

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Herbal drugs (1433)

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*(published in the Homoeopathic Preparations section)*

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*(published in the Homoeopathic Preparations section)*

Immunosera for human use, animal (0084)

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Live biotherapeutic products for human use (3053)

Methods of preparation of homoeopathic stocks and potentisation (2371)
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Monoclonal antibodies for human use (2031)

Mother tinctures for homoeopathic preparations (2029)
*(published in the Homoeopathic Preparations section)*

Pharmaceutical preparations (2619)

Products of fermentation (1468)

Products with risk of transmitting agents of animal spongiform encephalopathies (1483)

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Recombinant DNA technology, products of (0784)

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Vaccines for veterinary use (0062)

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